A Thesis for the Degree of Master of Science

Modulation of glycerol metabolism for enhanced production of 3-hydroxypropionic acid from glucose and xylose in engineered *Escherichia coli*

재조합 대장균에서 포도당과 목당 혼합당으로부터 3-히드록시프로피온산 생산을 위한 글리세롤 대사과정 조절에 관한 연구

By

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School of Agricultural Biotechnology
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農學碩士學位論文

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Abstract

Replacement of conventional petro-based chemicals with biomass-based substances is a central paradigm in the chemical industry. Hroxypropionic acid (3-HP) was selected as a target product, which is a precursor for various chemicals including acrylic acid, methyl acrylate and acrylamide.

In previous research, accumulation of glycerol was observed during 3-HP producing culture from a mixture of glucose and xylose, which is supposed to be a result of imbalance between glycerol synthesis and consumption. It is known that accumulated glycerol inhibits glycerol dehydratase which is a key enzyme in biosynthesis of 3-HP. Therefore, it is necessary to alleviate glycerol accumulation for improving 3-HP production.

For this purpose, the *gpsA* gene encoding glycerol-3-phosphate dehydrogenase derived from *Escherichia coli* K-12 which has lower activity than GPD1 from *Saccharomyces cerevisiae* was introduced to *E. coli* BL21 star (DE3). During 35 h of cultivation, the Δgyp/pELDRR/pCPaGAR strain showed glycerol accumulation of 1.24 g/L and 3-HP concentration of 1.51 g/L, which are lower by 46% and higher by 48% than those of the Δgyp/pELDRR/pCPaGGR strain, respectively.

To achieve high concentration of 3-HP, fed-batch fermentation was carried out with feeding a mixture of glucose and xylose. After 71 h of cultivation, accumulated glycerol was transformed to 3-HP completely by using the *gpsA* gene with a reduced level of glycerol synthesis. Finally, 37.6 g/L of 3-HP which is 33% higher than that of the control strain was produced. Productivity of 0.63 g/L·h and yield of 0.17 $g_{3\text{-}HP}/g_{sugar}$ were also obtained.

In this study, glycerol accumulation which inhibits glycerol dehydratase was reduced by replacing the *GPD1* gene by the *gpsA* gene. It is supposed to be a result of reduction of a carbon flux from dihydroxyacetone phosphate to glycerol-3-phosphate by GpsA which has lower activity than GPD1. As a result, enhanced production of 3-HP was possible by minimizing inactivation of glycerol dehydratase by glycerol. The result suggests that the *gpsA* gene overexpression would be applied to production of other chemicals which require using the glycerol dehydratase gene.

In the previous research, the Δgyp/pELDRR/pCPaGGR strain showed that xylose uptake rate decreased with culture time in glucose and xylose limited fed-batch fermentation, which resulted in a reduction of the titer and productivity of 3-HP. Therefore, xylose uptake rate needs to be increased for improving 3-HP production.

A xylose transporter was introduced in order to enhance xylose uptake rate in a mixture of glucose and xylose. The 376th amino acid residue, asparagine, was changed to phenyalanine in the *E. coli* galactose transporter GalP for simultaneous consumption of glucose and xylose. During 16 h of cultivation, the Δgyp/pACYCDuet-1_galPm showed an enhancement in xylose uptake rate by 34% and a decrease in glucose uptake rate by 22% compared to the control strain. Insufficient xylose uptake rate of 3-HP producing strains was improved by introducing the GalPm protein which has a high affinity with xylose.

Furthermore, the *xylFGH* genes were deleted to increase ATP availability and to secure more space for the GalPm in the cell membrane. During 16 h of cultivation, the Δgypx/pACYCDuet-1_galPm strain showed improved uptake rate of glucose by 20% and of xylose by 16%.

To investigate 3-HP production, flask culture of the Δgypx/pELDRR/pCPaGGRgalPm in R/5 medium containing 5 g/L glucose and 3 g/L xylose was carried out. During 30 h of cultivation, the Δgypx/pELDRR/pCPaGGRgalPm strain showed xylose uptake rate of 0.45 g/L·h and glucose uptake rate of 0.25 g/L·h, which is higher by 61% and lower by 36% than those of the control strain, respectively. Also, it showed 3-HP concentration of 1.10 g/L, and that

is higher by 8% than that of control strain. Further, this effect was maximized by deletion of the *xylFGH* genes to increase ATP availability and to secure membrane space for GalPm in the cell membrane. Improving sugar uptake rate and increasing ATP availability seem to contribute to enhanced cell growth and 3-HP production.

The result suggests that the GalPm protein with point mutation and deletion of the *xylFGH* genes would be applied to production of other chemicals from cellulosic biomass.

Keywords: Metabolic engineering, 3-hydroxypropionic acid, *Escherichia coli*, glucose and xylose metabolism, fed-batch fermentation

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Chapter 1. Introduction

1.1 3-Hydroxypropionic acid

3-Hydroxypropionic acid (3-HP, $C_3H_6O_3$—MW 90.08) holds the third most important place on the current list of the US DOE's top 12 platform chemicals among renewable biomass products (Bozell and Peterson, 2010). The 3-HP molecule contains two functional groups with different properties that make it a suitable precursor for synthesizing many optically active substances. The applications of 3-HP in chemical industries are diverse. It is used as a crosslinking agent for polymer coatings, metal lubricants, and antistatic agents for textiles. In addition, 3-HP can serve as a precursor for several key compounds, such as 1,3-propanediol ($C_3H_8O_2$—MW 76.09), acrylic acid ($C_3H_4O_2$—MW 72.06), methyl acrylate ($C_4H_6O_2$—MW 86.09), acrylamide (C_3H_5NO—MW 71.08), ethyl 3-HP ($C_5H_{10}O_3$—MW 118.13), malonic acid ($C_3H_4O_4$—MW 104.06) and acrylonitrile (C_3H_3N—MW 53.06) (Table 1.1). For these reasons, the global market size for 3-HP has been estimated at 3.63 million tons per year (Raj et al., 2008).

3-HP production via microbial fermentation can be devided into two major approaches based on the carbon sources utilized, glucose and glycerol (Choi et al., 2015). 3-HP production from glucose has been

studied by Cargill, Codexis. There are the lactic pathway and β-alanine pathway for conversion of glucose to 3-HP. However, little is known about a measure of 3-HP production by the aforementioned two systems (US DOE, 2005). The first report to date was announced by Suthers and Cameron. Two bacterial pathways, glycerol dehydratase from *Klebsiella pneumpniae* and aldehyde dehydrogenase from *Escherichia coli*, *Saccharomyces cerevisiae* and human were introduced into recombinant *E. coli*.

In a previous report, new aldehyde dehydrogenase from *Pseudomonas aeruginosa* which has higher activity than that of *E. coli* was identified. Glycerol kinase (*glpK*) and propanediol oxidoreductase (*yqhD*) were disrupted to reduce amount of by-products. Fed-batch fermentation using this strain resulted in 57.3 g/L 3-HP concentration, 1.59 g/L·h productivity and 0.88 g/g yield (Kim et al., 2014). Recently, it was reported that engineered *E. coli* expressing glycerol dehydratase from *K. pneumonia* and aldehyde dehydrogenase from *Cupriavidus necator* produced 3-HP at a maximum level of 71.9 g/L (Chu et al., 2015). In another study, the *E. coli* W strain was reported to be significantly better for 3-HP production due to its higher tolerance to 3-HP compared with *E. coli* K-12 (Sankaranarayanan et al., 2014). Previous studies on 3-HP production are summarized in Table 1.2.

Table 1.1 3-HP based product opportunities

(Industrial Bioproducts: Today and Tomorrow, DOE, U.S.A., 2003)

Compound	Application	Market size (M lb)	Price ($/lb)
Acrylic acid	Acrylates(coatings, adhesives) comonomer, superabsorbent polymers detergent polymers	2,000	0.48
Acrylonitrile	Acrylic fibers (carpets, clothing) acrylonitrile-butadiene-styrene and styrene-acrylonitrile (pipes and fittings, automobiles) nitrile rubber copolymers, adiponitrile, acrylamide	3,130	0.31 ~0.37
Acrylamide	Polyacrylamide comonomer (styrene-butadiene latex, acrylic resins, many others)	206	1.76 ~1.86
1,3-Propanediol	Polyethylene terephthalate, polybutylene terephthalate, nylon applications	small	0.30 ~0.50
Malonic acid	Blowing agent (formed plastic), silver plating brightening agent, tanning auxiliary	<1	high

Table 1.2 Recent researches about 3-HP production

Host	Amplified and deleted genes	3-HP titer (g/L)	Yield (g/g)	Productivity (g/L·H)	Cultivation (carbon source)	Reference
K. pneumoniae DSMZ 2026	*ΔdhaT, yqhD dhaB, puuC*	28.0	0.39	0.58	Fed-batch **(glycerol)**	(Ashok et al., 2013)
K. pneumoniae *ΔdhaT, yqhD*	*ΔdhaT, yqhD aldH, prpE, phaC*	2.03	-	0.04	Batch **(glycerol)**	(Xinjun et al., 2015)
E. coli BL21_mcr_acc_pntAB	*mcr, msr pntAB, acc*	0.20	0.03	0.01	Batch **(Glucose)**	(Rathnasingh et al., 2012)
E. coli BX3_0240	*ΔfabD mcr, acc*	49.0	0.23	0.71	Fed-batch **(Glucose)**	(Lynch et al., 2014)
E. coli SH501	*gabD4*	71.9	-	1.80	Fed-batch **(glycerol)**	(Chu et al., 2015)
E. coli W3110	dhaB123, gdrAB KGSADH	41.5	0.31	0.86	Fed-batch **(glycerol)**	(Sankaranarayanan et al., 2014)
E. coli BL21(DE3) *ΔglpKΔyqhD*	*Δglpk, yqhD aldH, dhaBR*	57.3	0.93	1.43	Fed-batch **(glycerol)**	(Kim et al., 2014)
E. coli BL21(DE3) *ΔglpKΔyqhDΔptsG*	*Δglpk, yqhD, ptsG aldH, dhaBR GPD1,GPP2, xylR*	29.7	0.36	0.54	Fed-batch **(glucose, xylose)**	Joung thesis, (2014)

1.2 Hemicellulose and xylose

Hemicellulose, the second major constituent of lignocellusose (Table 1.3) is a highly branched and complex heteropolymer that contains hexoses (D-glucose, D-galactose, D-mannose, L-rhamnose, L-fructose), pentoses (D-xylose an L-arabinose) and uronic acids (D-glucuronic acid and D-galacturonic acid). Hemicellulose composition is strongly dependent on the plant sources (Aristidou & Penttilä 2000). Hemicelluloses are the world's second most abundant family of polymers after cellulose and thus represent an enormous renewable resource for the chemical industry. Annually, 60 billion tons of hemicelluloses are produced on the earth and remain almost completely unused (Xu et al., 2006).

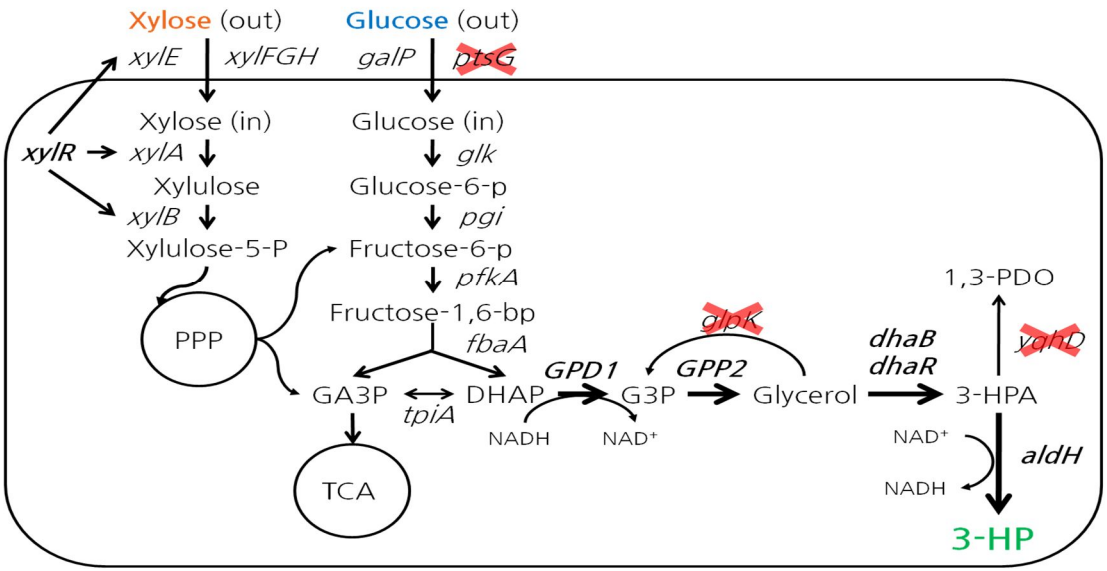
Hemicellulosic sugars, especially D-xylose, are relatively abundant in agricultural residues and plants wastes. Moreover, due to the random, amorphous structure with little strength of hemicelluloses their recovery by acid hydrolysis is easier and more efficient than the recovery of D-glucose from cellulose which has a crystalline, strong structure that, is resistant to hydrolysis. These advantages make hemicellulosic sugars a favorable feedstock in the biotechnology industry, especially for production of biofuels and other biochemicals (Jeffries 1983).

Table 1.3 Polymer composition of lignocellulose (IEA, 2003)

Fraction	Content in lignocellulose	Major monomers
Cellulose	33-51%	Glucose
Hemicellulose	19-34%	Glucose, mannose, galactose, xylose and arabinose
Lignin	20-30%	Aromatic alcohols
Pectin	1-20%	Galacturonic acid and rhamnose

1.3 Metabolic pathway from glucose and xylose to 3-HP in *E. coli*

During the uptake of glucose, one unit of ATP is used to convert the glucose to D-glucose-6-phosphate. The transport of glucose into the cell occurs through the phosphotransferase system (PTS). D-glucose-6-phosphate is then converted to fructose-6-phosphate and finally to pyruvate through the glycolysis pathway (Keseler, I.M., et al., 2005). *E. coli* is also able to metabolize xylose via the pentose phosphate pathway (David & Weismeyer 1970). Intracellular glucose and xylose are converted to dihydroxyacetone phosphate (DHAP), and then DHAP is converted to glycerol 3-phosphate by glycerol dehydrogenase (GPD) and glycerol 3-phosphate is converted to glycerol catalyzed by glycerol 3-phosphatase (GPP) (Meynial Salles et al., 2007). Glycerol is further converted into 3-HP through 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (DHAB) and aldehyde dehydrogenase (ALDH) (Kwak et al., 2013). Therefore, four enzymes including glycerol dehydrogenase (GPD), glycerol 3-phosphatese (GPP), glycerol dehydratase (DHAB), and aldehyde dehydrogenase (ALDH) contribute to the ability to convert glucose and xylose to 3-HP in *E. coli* (Figure 1.1).



G3P: glycerol-3-phosphate
GA3P: glyceraldehyde 3-phosphate
DHAP: dihydroxyacetone phosphate
3-HPA: 3-hydroxypropionaldehyde
1,3-PDO: 1,3-propanediol
3-HP: 3-hydroxypropionic acid

GPD: glycerol-3-phosphate dehydrogenase
GPP: glycerol-3-phosphatase from *Saccharomyces cerevisiae*
dhaB: glycerol dehydratase
dhaR: glycerol dehydratase reactivase from *Lactobacillus brevis* KCTC 33069

aldH: semialdehyde dehydrogenase from *Pseudomonas aeruginosa*
yqhD: 1,3-propandiol oxidoreductase
glpK: glycerol kinase

Figure 1.1 Biosynthesis pathway to 3-hydroxypropionic acid from glucose and xylose in engineered *E. coli*

1.4 Transporter

A membrane transport protein (or simply transporter) is a membrane protein involved in the movement of ions, small molecules or macromolecules across a biological membrane (Dahl et al., 2004). Transporters are integral transmembrane proteins. They exist permanently within and span the membrane across which they transport substances. The proteins may assist in the movement of substances by facilitated diffusion or active transport (H Lodish, 2000)

The *E. coli* takes up metabolites through the membrane by transporters. (Lemieux et al., 2004). In the *E. coli* BL21 star (DE3) strain, the strong *lacUV5* promoter is used for T7RNAP expression (Studier & Moffatt 1986). However, for most membrane proteins, this strong overexpression leads to the production of more proteins than the translocation protein which can be processed. Saturation of translocation proteins makes most overexpressed membrane proteins impossible to insert into the membrane. Membrane proteins that cannot insert into the membrane end up aggregating in the cytoplasm (Wagner et al., 2008). In this reason, targeted transporter encoding genes were expressed under the endogenous *E. coli* promoter.

1.5 Research objectives

This study was focused on the enhancement of 3-HP production by minimizing glycerol accumulation and improving xylose uptake rate in metabolically engineered *E. coli*. First, the glycerol-3-phosphate dehydrogenase encoding gene would be replaced by *gpsA* gene from *E. coli* K-12 to minimize glycerol accumulation. Secondly, *E. coli* transporter protein would be modulated and expressed constitutively for improving xylose uptake rate. Also, expression of the effective transporter would be optimized with more space in the cell membrane by deletion of genes encoding unnecessary proteins for xylose uptake in a mixture of glucose and xylose.

The specific objectives of this research were described as follows:

1. Minimization of glycerol accumulation by changing glycerol-3-phosphate dehydrogenase encoding gene from *GPD1* of *S. cerevisiae* to *gpsA* of *E. coli* K-12
2. Improvement of xylose uptake rate by point mutation of the *E. coli galP* gene and deletion of the *xylFGH* genes
3. Enhancement of 3-HP production in metabolically engineered *E. coli* by glucose and xylose limited fed-batch fermentation

Chapter 2. Materials and methods

2.1 Strains and Plasmids

For plasmid construction and 3-HP production, *E. coli* TOP10 and BL21 star (DE3) (Invitrogen Co., Carlsbad, CA, USA) were used (Table 2.1). *E. coli* TOP10 was used for the transformation, plasmid preparation and DNA manipulation, and *E. coli* BL21 star (DE3) was used as a host strain for 3-HP production. The *glpK*, *yqhD* and *ptsG* genes were disrupted in *E. coli* BL21 star (DE3) in previous research (Joung thesis, 2013).

Plasmid pELDRR is haboring *Lactobacillus brevis* glycerol dehydratase (DhaB1, DhaB2, DhaB3) and glycerol dehydratase reactivase (DhaR1, DhaR2) genes under the T_7 promoter (Kwak et al., 2013). Plasmid pCPaGGR is haboring 4 genes under the T_7 promoter which were aldehyde dehydrogenase derived from *Pseudomonas aeruginosa*, glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase derived from *Saccharomyces cerevisiae* and *xylR* gene derived from *E. coli* K-12 strain with the synthetic promoter (Jung et al., 2015).

All strains and plasmids used in this study are listed in Table 2.1.

Table 2.1 List of strains and plasmids used in this study

Strains / Plasmids	Main characteristics	Reference / Source
Strains		
E. coli TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen
E. coli BL21 star (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Invitrogen
Δgyp	BL21(DE3) Δ*glpK*Δ*yqhD*Δ*ptsG*	Jung et al., (2015)
Δgypx	BL21(DE3) Δ*glpK*Δ*yqhD*Δ*ptsG*Δ*xylFGH*	In this study
Plasmids		
pCPaGGR	Sm^R, pCDFduet-1 based plasmid containing *aldH*, *GPD1*, *GPP2* under the T_7 promoter and *xylR* with natural P_R promoter	Jung et al., (2015)
pCPaGppR	Sm^R, pCDFduet-1 based plasmid containing *aldH*, *GPP2* under the T_7 promoter and *xylR* with natural P_R promoter	In this study
pCPaGAR	Sm^R, *gpsA* gene with T_7 promoter cloned in pCPaGppR	In this study
pCPaGGRgalP	Sm^R, *galP* gene with natural promoter cloned in pCPaGGR	Kim thesis (2015)
pCPaGGRgalPm	Sm^R, *galPm* gene with natural promoter cloned in pCPaGGR	In this study

(be continued)

Strains / Plasmids	Main characteristics	Reference / Source
pACYCDuet-1	Cm^R; T_7 promoter; P15A ori; two sets of MCS; MCS I, His•tag; MCS II, S•tag; *lac*I	Novagen
pACYCDuet-1_galP	Cm^R, *galP* gene with natural promoter cloned in pACYCDuet-1	In this study
pACYCDuet-1_galPm	Cm^R, *galPm* gene with natural promoter cloned in pACYCDuet-1	In this study
pELDRR	KanR, pET-29b(+) based plasmid containing *L. brevis dhaB*, *dhaR* cluster under the T_7 promoter	Kwak et al., (2012)

2.2 Gene deletion progress

E. coli BL21 star (DE3) was used as a host strain for 3-HP production. For the cloning of the kanamycin resistance cassette to delete the *E. coli* choromosomal gene, plasmid pKD13 was used as the template of the polymerase chain reaction (PCR). Primers which are designed for homologous recombination with the *xylFGH* genes were used to amplify the kanamycin resistance cassette. To express λ recombinase, plasmid pKD46 was introduced to *E. coli* BL21 star (DE3). Elimination of the kanamycin resistance gene was performed by using a helper plasmid pCP20 which is able to express FLP recombinase by thermal induction and then the helper plasmid was cured. Disruption of the chromosomal genes was confirmed by colony PCR using PCR-primers F_xylFGH_chk and R_xylFGH_chk. All primers used in this study are listed in Table 2.2.

Table 2.2 List of primers used in this study

Name	Necleotide sequence (5` to 3`)	Target gene
F_gpsA_XhoI	CCGCTCGAGATGAACCAACGTAATGCTTCAATGACT	*gpsA*
R_gpsA_PacI	CCTTAATTAATTAGTGGCTGCTGCGCTCGT	
F_xylFGH_del	**CTTATTAAAACTGTCCTCTAACTACAGAAGGCCCTACACC**GTGTAGGCTGGAGCTGCTTC	*xylFGH*
R_xylFGH_del	**CCGGTAATACGTAACCGGCTTTGAGAAAATTTTTATCAAA**ATTCCGGGGATCCGTCGACC	
F_xylFGH_chk	GTTACGTTTATCGCGGTGATTGTTA	
R_xylFGH_chk	GCTGAATCATGCAAAAACTCAAAACC	
F_galP_mut	GGCCTGACCTTCGTACTTGC	*galP*
R_galP_mut	GCAAGTACGAAGGTCAGGCC	
F_galP_inf	*TATGCGACTCCTGCA*ATTACACTGATGTGATTTGCTTCACATCT	
R_galP_inf	*TATTAATTTCCTAAT*ATTAATCGTGAGCGCCTATTTCGC	
F_galP_EcoNI	GGCCTGCATTAGGATTACACTGATGTGATTTGCTTCACAT	
R_galP_XhoI	CCGCTCGAGATTAATCGTGAGCGCCTATTTCG	

* Bolded sequences are homologous regions with *xylFGH* genes in the *E. coli* BL21 star (DE3) chromosomes

* Italic sequences are homologous regions with pCPaGGR EcoN1 site

* The underlined sequences correspond to restriction enzyme site

2.2.1 Preparation of kanamycin resistance cassette

PCRs were performed with an Applied Biosystems Veriti 96well Thermal Cycler (Lincoln, CA, USA) and prime STAR HS Premix from TAKARA (Otsu, Japan). PCRs for cloning of the kanamycin resistance cassettes were performed in 25 μL of DNA polymerase premix from TAKARA(Otsu, Japan) containing 20 pM each F_xylFGH_del, R_xylFGH_del primers (Table 2.2), and 1 μL pKD13 which is a template of cloning. After heating the reaction tubes for 5 min at 95°C, 30 cycles of PCR amplification were performed as follows: 10 sec at 98°C, 5 sec at 55°C, and 1 min per 1 kb DNA at 72°C, followed by 7 min at 72°C during the last cycle.

2.2.2 Expression of λ red recombinase in host strain

Plasmid pKD46 was introduced to each expression strain by using $CaCl_2$ method (Dagert & Ehrlich 1979). Δgyp with pKD46 was cultured in 100ml LB medium containing L-arabinose (10 mM) and ampicilin (5 mg/mL) at 30°C until $O.D._{600\ nm}$ reached 0.5~0.8.

2.2.3 Kanamycin resistance cassette insert to expression strain

All cells harvested by centrifugation at 3,000 rpm for 15 min at 4°C were carefully resuspended in 30 mL of ice-cold 10% (v/v) glycerol solution about 30 min on ice. This procedure was repeated three times.

The cells were concentrated in 200 μL using 10% glycerol. Then 16 μL of the prepared kanamycin resistance cassette was introduced to the 100 μL cell by electroporation method by using the Gene-Pulser EC2 program (Bio-RAD, USA).

2.2.4 Recombination and adaptation

The cells harboring the kanamycin resistance cassette were incubated in 1 ml LB medium about 1 hr. The cells were spread to LBK plate and incubated at 37°C about 18 hrs. Colonies with the kanamycin resistance were selected. Kanamycin resistance cassette insertion was verified by the PCR method using check primers (Table 2.2)

2.2.5 Elimination of kanamycin resistance cassette

Plasmid pCP20 was introduced to each kanamycin resistance strain by using the $CaCl_2$ method (Dagert & Ehrlich 1979). The cells were spread to LBA plate at 30°C about 24 hrs, then incubation at 43 °C to cure. Gene deletion is verified by the PCR method using check primers (Table 2.2)

2.3 *E. coli* DNA manipulation and transformation

2.3.1 Preparation of DNA

Mini-scale preparation of plasmid DNA was carried out by using Plasmid Miniprep Kit from Takara (Otsu, Japan). Preparation of the *E. coli* K-12 chromosomal DNA for PCR template was carried out by using the DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany). PCR amplified or enzyme treated DNA was purified by using the Hiyield™Gel/PCR DNA Extraction Kit from Real Biotech Corporation (Taipei, Taiwan).

2.3.2 Polymerase Chain Reaction (PCR)

PCRs were performed with an Applied Biosystems Veriti 96 well Thermal Cycler (Lincoln, CA, USA). PCRs for cloning of genes from *E. coli* K-12 were performed in 50 μL of PrimeStar™ dyemix solution from Takara (Otsu, Japan) containing 20 pM each of forward and reverse primers (Table 2.2), and 1 μL of the *E. coli* K-12 genomic DNA which is a template of cloning. After heating the reaction tubes for 5 min at 95℃, 30 cycles of PCR amplification were performed as follows: 10 sec at 98℃, 5 sec at 55℃ and 2 min at 72℃, followed by 7 min at 72℃ during the last cycle.

2.3. 3 Point mutation by overlap extension PCR

The 376th amino acid residue of GalP, asparagine was replaced by phenylalanine using overlap extension PCR with four primers, F_galP_mut, R_galP_mut, F_galP_EcoNI and R_galP_XhoI. This procedure is composed of 3-steps. First, the fragments for overlap extension PCR were amplified by PCR with two primer sets; F_galP_EcoNI and R_galP_mut; F_galP_mut and R_galP_XhoI. After heating the reaction tubes for 5 min at 95℃, 30 cycles of PCR amplification were performed as follows: 10 sec at 98℃, 5 sec at 55℃ and 1 min at 72℃, followed by 7 min at 72℃ during the last cycle. After PCR, two PCR solutions were purified with the DNA-spin^{TM} Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea). The purified PCR products were used as a template for overlap extension PCR with F_galP_EcoNI and R_galP_XhoI. After heating the reaction tubes for 5 min at 95℃, 30 cycles of PCR amplification were performed as follows: 10 sec at 98℃, 5 sec at 55℃ and 2 min at 72℃, followed by 7 min at 72℃ during the last cycle.

2.3.4 Digestion and ligation of DNA

Restriction enzymes *EcoNI, XhoI* and *PacI* and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). Plasmid pCPaGppR was digested with *XhoI*

and *PacI*. Plasmid pCPaGGR was digested with *EcoNI*. Plasmid pACYCDuet-1 was digested with *EcoNI* and *XhoI*. The In-Fusion HD Cloning Kit obtained from Takara (Otsu, Japan) was used for ligation of PCR products and plasmid pCPaGGR.

2.3.5 Transformation

Plasmids pCPaGGR, pCPaGppR, pCPaGAR, pCPaGGRgalPm, pACYCDuet-1_galPm and pELDRR were transformed to *E. coli* BL21 star (DE3) by using $CaCl_2$ method (Dagert & Ehrlich 1979).

2.4 Culture conditions

2.4.1 Growth media

A chemically defined Riesenberg medium (Kim et al., 2014) supplemented with 5 g/L of yeast extract (R/5 medium) was used for production of 3-HP. Riesenberg medium consists of (per liter) 1.7 g citric acid, 13.5 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$ and a trace element solution 10 mL (contains per liter of 5 M HCl : 10 g $FeSO_4 \cdot 7H_2O$, 2.25 g $ZnSO4 \cdot 7H_2O$, 1 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 2 g $CaCl_2$, 0.1 g $(NH_4)_6Mo_7O_{24}$, 0.23 g $Na_2B_4O_7 \cdot 10H_2O$). Medium pH was adjusted at 6.8 with 5 N NaOH.

2.4.2 Flask culture

The cell stock was transferred to a test tube containing 5 mL of LB medium (1% yeast extract, 2% bacto-tryptone and 1% NaCl) with appropriate antibiotics and incubated overnight at 37℃ and 250 rpm in a shaking incubator (Vision, Buchon, Korea). To produce 3-HP, flask culture was performed in a 500 mL baffled flask (NALGENE, USA) with 100 mL R/5 medium containing 5 g/L glucose and 3 g/L xylose. 5 mL seed culture was inoculated in R/5 medium with suitable antibiotics and cultivated at 37℃ and 250 rpm. IPTG (final concentration 0.1 mM) and coenzyme B_{12} (final concentration 20 μM) were added as the inducer and cofactor of glycerol dehydratase when $O.D._{600nm}$ reached to 3. At the same time, temperature was changed to 25℃ and the flask was covered with aluminum foil for absence of light considering vulnerability of coenzyme B_{12} about light.

2.4.3 Fed-batch fermentation in a bioreactor

Fed-batch fermentation was carried out in a 2.5 L jar fermentor (Kobiotech, Incheon, Korea) with 1 L initial working volume of R/5 medium containing 13 g/L glucose and 7 g/L xylose and the same concentration of antibiotics as the flask culture. 100 mL pre-culture was prepared in a 500 mL flask and grown in a shaking incubator at 37℃ and 250 rpm for 12 h. After 12 h, 100 mL of the pre-culture was

transferred to a bioreactor. Main culture was carried out at 37℃ with aeration rate of 2 vvm and agitation speed of 1,200 rpm. When the cell $O.D._{600nm}$ reached 30, IPTG and coenzyme B_{12} were added and temperature was shifted to 25℃ without changing aeration rate and agitation speed. The feeding solution is composed of 500 g/L glucose and 250 g/L xylose with 20 g/L $MgSO_4 \cdot 7H_2O$. After induction, the feeding solution was fed continuously. The media pH was automatically controlled between 6.78 and 6.82 by addition of 28% ammonia water.

2.5 Analytical methods

2.5.1 Dry cell weight

Cell growth was monitored by measuring the optical density of culture broth at 600 nm using a spectrophotometer (OPTIZEN POP, MECASYS, Korea). Optical density was converted into dry cell mass by using the following conversion equation:

$$\text{Dry cell mass (g/L)} = 0.365 \times O.D._{600nm}$$

2.5.2 High performance liquid chromatography analysis

The concentrations of glucose, xylose, glycerol, 3-HP and acetate

were measured by a high performance liquid chromatography (1200 series, Agilent, Santa Clara, CA, USA) with a Aminex HPX-87H Ion Exclusion Column (BIO-RAD, Richmond, CA, USA) heated at 60°C. A mobile phase of 5 mM H_2SO_4 was used at a flow rate of 0.5 mL/min (Sluiter et al., 2006). Detection was made with a reflective index detector and an UV detector at 210 nm.

2.5.3 SDS-PAGE analysis

The crude extract samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 14% polyacrylamide gel) (Table 2.3) and detected by staining the gels with Coomassie brilliant blue.

Table 2.3 Composition of 14% polyacrylamide gel for SDS-PAGE

Separating gel buffer(pH 8.8)		Stacking gel buffer(pH 6.8)	
Tris-Cl	1.5 M	Tris-Cl	0.5 M
SDS	0.40%	SDS	0.40%
Separating gel		Stacking gel	
Acrylamide	4000 μL	Acrylamide	800 μL
4X Separating buffer	3100 μL	4X Separating buffer	1600 μL
Deionized water	2800 μL	Deionized water	2500 μL
10% SDS	120 μL	10% SDS	60 μL
TEMED	10 μL	TEMED	5 μL
Ammonium persulfate	100 μL	Ammonium persulfate	100 μL

Chapter 3. Results and discussions

3.1 Production of 3-HP from glucose and xylose

3.1.1 Problem of glycerol accumulation during culture

In previous research, the strain Δgyp was transformed with plasmids pCPaGGR and pELDRR to produce 3-HP. To measure 3-HP producing capacity, the strain Δgyp/pELDRR/pCPaGGR (Jung et al., 2015) was cultured in R/5 medium containing 5 g/L glucose and 3 g/L xylose. When cell mass reached $O.D._{600}$ of 3, IPTG and coenzyme B_{12} were added at final concentrations of 0.1 mM and 20 μM, respectively.

As a result of flask culture, final concentration of 3-HP was 1.02 g/L (Figure 3.1). However, glycerol accumulated at 2.31 g/L in initial 14h and final concentration of glycerol was 1.59 g/L. Conversion of glycerol to 3-HP was initiated only after glucose and xylose were depleted completely.

Although glycerol was a precursor of 3-HPA, high concentrations of glycerol inactivate glycerol dehydratase which catalyze conversion of glycerol to 3-HPA (Knietsch et al., 2003). It seems to be due to the fact that glycerol deactivates glycerol dehydratase by the homolysis of the Co-C bond of coenzyme b12 binding to the apoenzyme and for protection of radical intermediates from undesired side reactions during catalysis (Toraya 2000).

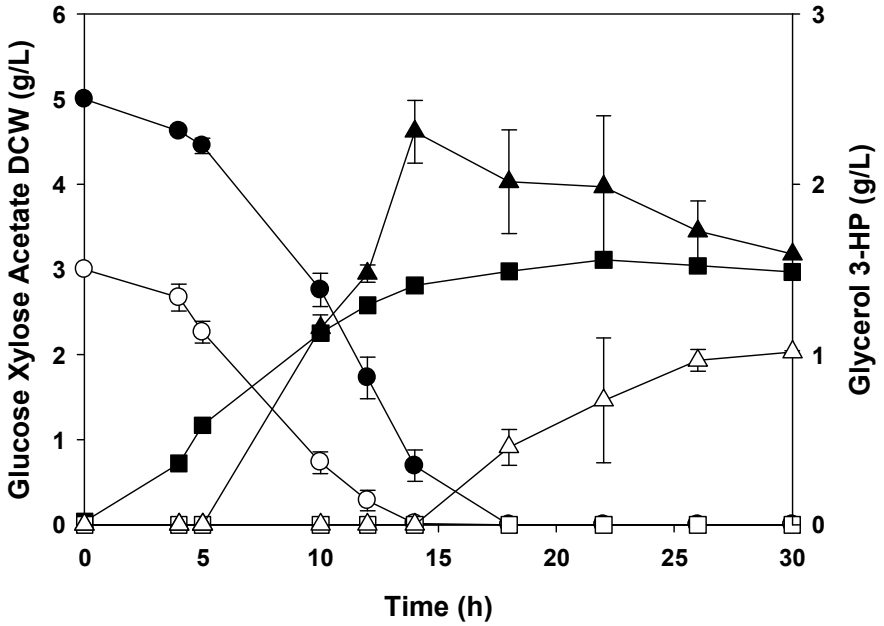


Figure 3.1. Profile of batch fermentation of the Δgyp/pELDRR/pCPaGGR in R/5 medium containing 5 g/L glucose and 3 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

3.2 Manipulation of glycerol synthesis pathway

3.2.1 Construction of the strain without *GPD1* gene

E. coli BL21 star (DE3) has the endogenous glycerol-3-phosphate dehydrogenase encoding gene, *gpsA*. To reduce the amount of glycerol produced during fermentation, plasmid without the *GPD1* gene, pCPaGppR was constructed for expression of *aldH* derived from *P. aeruginosa*, *GPP2* derived from *S. cerevisiae* and *xylR* derived from *E. coli* with engineered promoter (Figure 3.2). Therefore pELDRR and pCPaGppR were transformed into Δgyp to alleviate accumulation of glycerol during 3-HP production.

3.2.2 Flask culture of the strain without *GPD1* gene

To investigate 3-HP production and glycerol accumulation, flask culture of the strain Δgyp/pELDRR/pCPaGppR in R/5 medium containing 5 g/L glucose and 3 g/L xylose was carried out. During 30 h of cultivation, Δgyp/pELDRR/pCPaGppR strain showed glycerol accumulation of 0.10 g/L, which is much lower than that of Δgyp/pELDRR/pCPaGGR strain. Also, it showed the similar level of final concentration of 3-HP (Figure 3.3).

Reduction of a carbon flux from dihydroxyacetone phosphate to glycerol-3-phosphate by absence of the *GPD1* gene led to an alleviated glycerol accumulation. Judging from this result, a problem

of glycerol accumulation during 3-HP producing fermentation was supposed to be a result by imbalance between glycerol synthesis and consumption. Results are summarized in Table 3.1

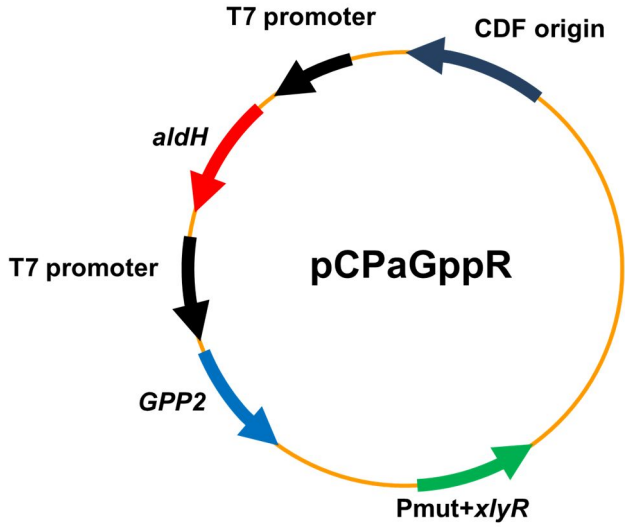


Figure 3.2. Genetic map of plasmid pCPaGppR

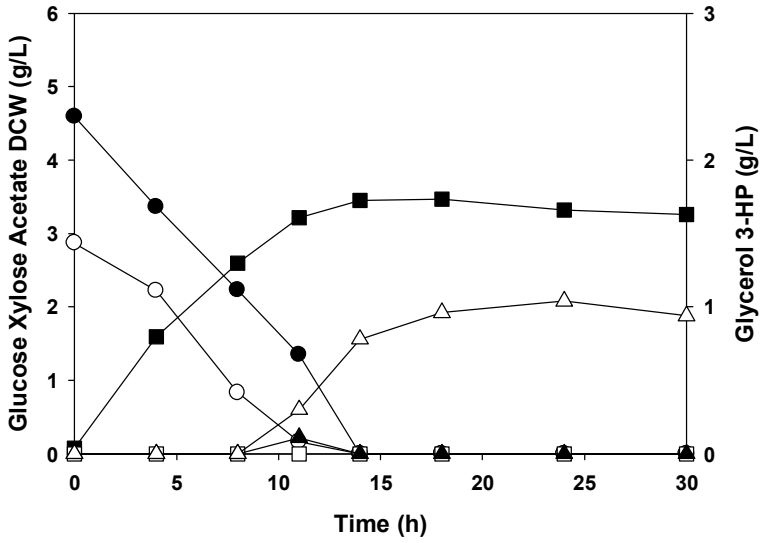


Figure 3.3. Profile of batch fermentation of the Δgyp/pELDRR/pCPaGppR in R/5 medium containing 5 g/L glucose and 3 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.1 Results of the strain without *GPD1* gene in flask culture

Strains	DCW (g/L)	Glycerol accumulation (g/L)	Final glycerol concentration (g/L)	3-HP concentration (g/L)
Δgyp pELDRR/pCPaGGR	3.09	2.31	1.59	1.02
Δgyp pELDRR/pCPaGppR	3.48	0.10	0	0.94

3.3 Expression of the glycerol-3-phosphate dehydrogenase derived from *E. coli* K-12

Judging from the aforementioned results, a problem of glycerol accumulation during 3-HP producing fermentation was supposed to be a result by imbalance between glycerol synthesis and consumption. Therefore, introduction of an alternative gene encoding glycerol-3-phosphate dehydrogenase which has lower activity than that of GPD1 from *S. cerevisiae* is necessary to alleviate glycerol accumulation for producing high concentration of 3-HP.

For this purpose, the *gpsA* gene encoding glycerol-3-phosphate dehydrogenase derived from *E. coli* K-12 was overexpressed under the T_7 promoter.

3.3.1 Sequences of the *gpsA* gene

The *gpsA* gene sequences are as follows.

ATGAACCAACGTAATGCTTCAATGACTGTGATCGGTGCCGGC
TCGTACGGCACCGCTCTTGCCATCACCCTGGCAAGAAATGGC
CACGAGGTTGTCCTCTGGGGCCATGACCCTGAACATATCGCA
ACGCTTGAACGCGACCGCTGTAACGCCGCGTTTCTCCCCGAT
GTGCCTTTTCCCGATACGCTCCATCTTGAAAGCGATCTCGCC
ACTGCGCTGGCAGCCAGCCGTAATATTCTCGTCGTCGTACCC
AGCCATGTCTTTGGTGAAGTGCTGCGCCAGATTAAACCACTG

ATGCGTCCTGATGCGCGTCTGGTGTGGGCGACCAAAGGGCT

GGAAGCGGAAACCGGACGTCTGTTACAGGACGTGGCGCGT

GAGGCCTTAGGCGATCAAATTCCGCTGGCGGTTATCTCTGGC

CCAACGTTTGCGAAAGAACTGGCGGCAGGTTTACCGACAGC

TATTTCGCTGGCCTCGACCGATCAGACCTTTGCCGATGATCTC

CAGCAGCTGCTGCACTGCGGCAAAAGTTTCCGCGTTTACAG

CAATCCGGATTTCATTGGCGTGCAGCTTGGCGGCGCGGTGAA

AAACGTTATTGCCATTGGTGCGGGGATGTCCGACGGTATCGG

TTTTGGTGCGAATGCGCGTACGGCGCTGATCACCCGTGGGCT

GGCTGAAATGTCGCGTCTTGGTGCGGCGCTGGGTGCCGACC

CTGCCACCTTTATGGGCATGGCGGGGCTTGGCGATCTGGTGC

TTACCTGTACCGACAACCAGTCGCGTAACCGCCGTTTTGGCA

TGATGCTCGGTCAGGGCATGGATGTACAAAGCGCGCAGGAG

AAGATTGGTCAGGTGGTGGAAGGCTACCGCAATACGAAAGA

AGTCCGCGAACTGGCGCATCGCTTCGGCGTTGAAATGCCAAT

AACCGAGGAAATTTATCAAGTATTATATTGCGGAAAAAACGC

GCGCGAGGCAGCATTGACTTTACTAGGTCGTGCACGCAAGG

ACGAGCGCAGCAGCCACTAA

3.3.2 Construction of the strain expressing *gpsA* gene

Plasmid pCPaGAR was constructed for expression of *aldH* derived from *P. aeruginosa*, *GPP2* derived from *S. cerevisiae*, *gpsA* derived

from *E. coli* with the T_7 promoter and *xylR* derived from *E. coli* with the engineered promoter (Figure 3.5).

For the confirmation of expression of the *gpsA* gene along with other genes which are necessary for 3-HP production, plasmids pELDRR and pCPaGAR were transformed into competent Δgyp. Expression of the proteins was confirmed with SDS-PAGE analysis (Figure 3.6) The proteins DHAB1, DHAB2, DHAB3, DHAR1, DHAR2, ALDH, GPP2 and GpsA were expressed solubly in Δgyp by IPTG induction (0.1 mM of final concentration).

3.3.3 Flask culture of the strain expressing *gpsA* gene

To investigate 3-HP production and glycerol accumulation, flask cultures of the strains Δgyp/pELDRR/pCPaGGR and Δgyp/pELDRR/ pCPaGAR in R/5 medium containing 5 g/L glucose and 3 g/L xylose were carried out. During 35 h of cultivation, then Δgyp/pELDRR/pCPaGAR strain showed glycerol concentration of 1.24 g/L, and that is lower by 46% than that of the control strain. Also, it showed 3-HP concentration of 1.51 g/L, and that is higher by 48% than that of the control strain (Figure 3.4).

Replacing the *GPD1* gene by the *gpsA* gene led to an alleviated accumulation of glycerol with reduction of a carbon flux from dihydroxyacetone phosphate to glycerol-3-phosphate. As a result,

efficient conversion of 3-HPA to 3-HP was possible with an alleviated inactivation of glycerol dehydratase. Results are summarized in Table 3.2.

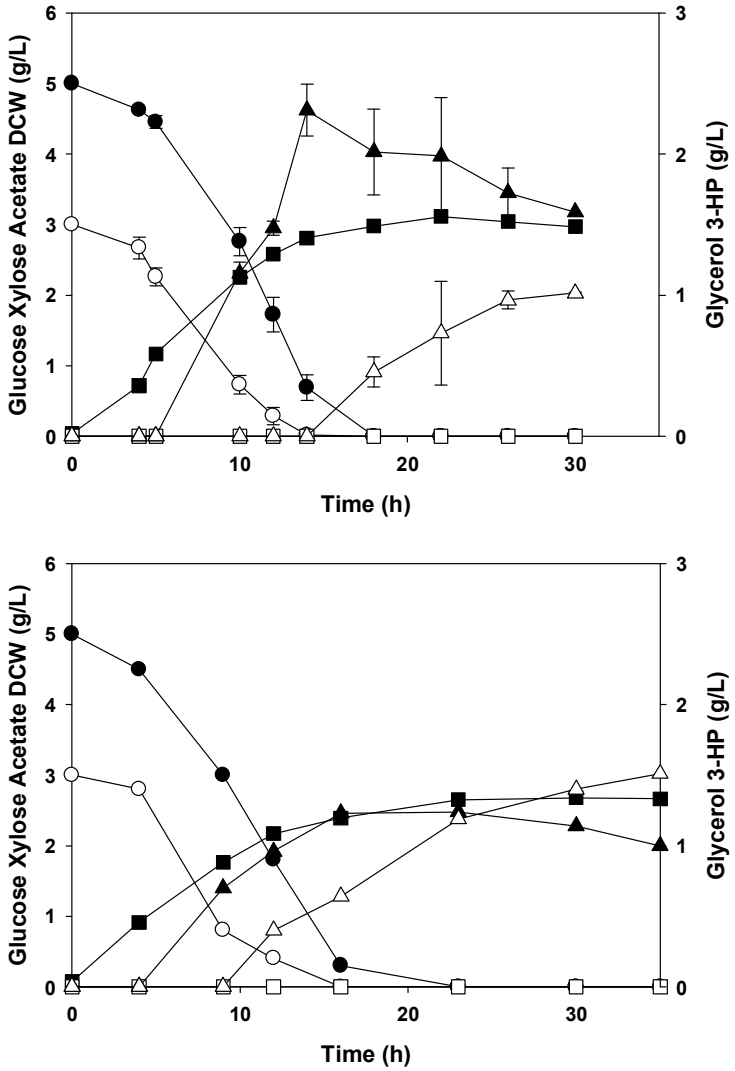


Figure 3.4. Profiles of batch fermentation of (A) ∆gyp/pELDRR/pCPaGGR and (B) ∆gyp/pELDRR/pCPaGAR in R/5 medium containing 5 g/L glucose and 3 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.2 Results of the *gpsA* gene expression strain in flask culture

Strains	DCW (g/L)	Glycerol accumulation (g/L)	Final glycerol concentration (g/L)	3-HP concentration (g/L)
Δgyp pELDRR/pCPaGGR	3.09	2.31	1.59	1.02
Δgyp pELDRR/pCPaGAR	2.69	1.24	1.00	1.51

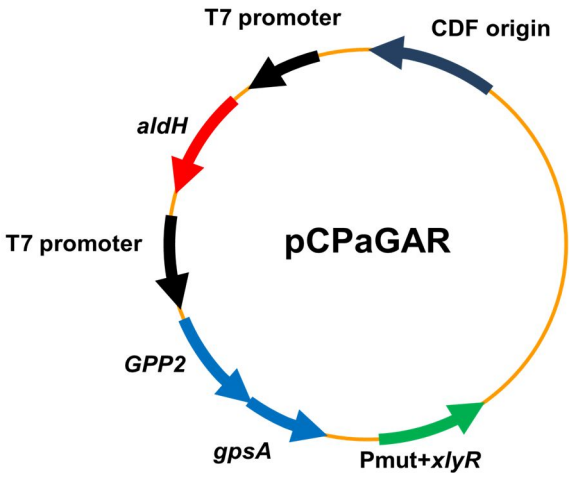
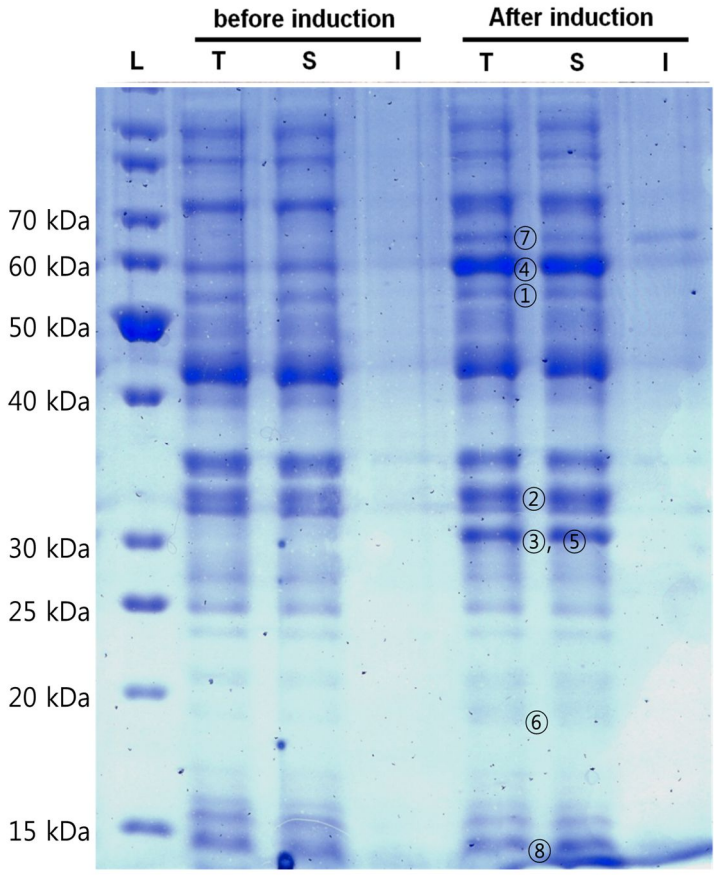


Figure 3.5. Genetic map of plasmid pCPaGAR



L: Ladder T: Total protein S:Soluble protein I: Insoluble protein	①: ALDH (56 kDa) ⑤: DHAB2 (27 kDa) ②: GPSA (36 kDa) ⑥: DHAB3 (19 kDa) ③: GPP2 (28 kDa) ⑦: DHAR1 (66 kDa) ④: DHAB1 (61 kDa) ⑧: DHAR2 (13 kDa)

Figure 3.6. SDS-PAGE analysis of pCPaGAR and pELDRR plasmid expression

3.3.4 Fed-batch fermentation using constant feeding strategy

In the flask culture, Δgyp/pELDRR/pCPaGAR showed lower glycerol accumulation and higher 3-HP concentration than the control strain, Δgyp/pELDRR/pCPaGGR.

To achieve high concentration of 3-HP, glucose and xylose limited fed-batch fermentation were carried out. After 71 h of cultivation, accumulated glycerol was transformed to 3-HP completely. Finally, 37.6 g/L of 3-HP which is 33% higher than that of control strain was produced. Productivity and yield of 3-HP were 0.63 g/L·h and 0.17 $g_{3\text{-HP}}/g_{sugar}$ respectively (Figure 3.7). Results are summarized in Table 3.3.

In this study, glycerol accumulation which inhibits glycerol dehydratase was reduced by replacing the *GPD1* gene by the *gpsA* gene. It is supposed to be a result of reduction of a carbon flux from dihydroxyacetone phosphate to glycerol-3-phosphate by GpsA which has lower activity than GPD1. As a result, enhanced production of 3-HP was possible with alleviated inactivation of glycerol dehydratase by reducing glycerol concentration. The result suggests that the *gpsA* gene overexpression would be applied to production of other chemicals which require using glycerol dehydratase gene.

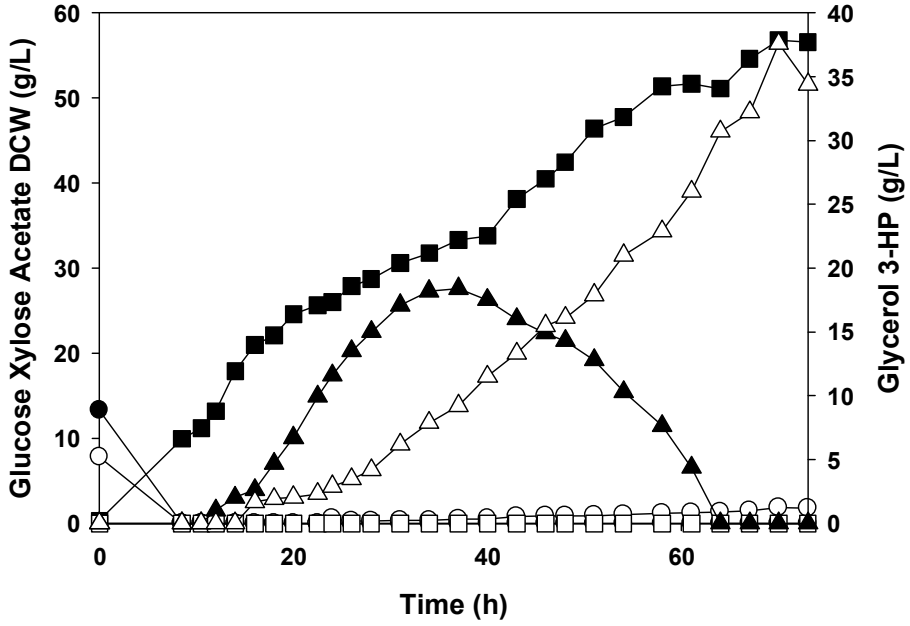


Figure 3.7. Profile of fed-batch fermentation of the Δgyp pELDRR/pCPaGAR

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.3 Result of fed-batch fermentation of Δgyp pELDRR/pCPaGAR

Strain	DCW (g/L)	Glycerol (g/L)	3-HP (g/L)	3-HP yield (g 3-HP/g sugar)	3-HP productivity (g/L·h)
Δgyp pELDRR/pCPaGAR	56.8	0	37.6	0.17	0.63

3.4 Expression of the mutant *galP* gene

In the previous research, the Δgyp/pELDRR/pCPaGGR strain showed that xylose uptake rate decreased with long culture time in glucose and xylose limited fed-batch fermentation. It resulted in a reduction of the titer and productivity of 3-HP. Therefore, xylose uptake rate needs to be increased for improving 3-HP production.

Introduction of xylose transporter was aimed at improving xylose uptake rate in a mixture of glucose and xylose. However, every natural xylose transporter was entirely inhibited by glucose. Thus construction of mutant transporters which show high affinity for xylose in a mixture of glucose and xylose was necessary.

The 376^{th} amino acid residue, asparagine, was exchanged to phenyalanine in the yeast hexose transporter GAL2 for simultaneous consumption of glucose and xylose. From this point mutation, mutant GAL2 showed the higher affinity for xylose than wild GAL2. The conserved asparagine residue is found in the same region of the *E. coli* galactose transporter GalP and is positioned to the extracellular side of the central sugar-binding pocket (Alexander et al., 2014).

Therefore, that conserved region of the galactose transporter derived from *E. coli* K-12, which is essential for affinity with xylose, is

engineered to make mutant galactose transporter encoding gene, *galPm*.

3.4.1 Sequences of the *galPm* gene

The *galPm* gene sequences are as follows.

ATTACACTGATGTGATTTGCTTCACATCTTTTTACGTCGTACT

CACCTATCTTAATTCACAATAAAAAATAACCATATTGGAGGG

CATC**ATGCCTGACGCTAAAAAACAGGGGCGGTCAAACAA**

GGCAATGACGTTTTTCGTCTGCTTCCTTGCCGCTCTGGC

GGGATTACTCTTTGGCCTGGATATCGGTGTAATTGCTGGC

GCACTGCCGTTTATTGCAGATGAATTCCAGATTACTTCGC

ACACGCAAGAATGGGTCGTAAGCTCCATGATGTTCGGTG

CGGCAGTCGGTGCGGTGGGCAGCGGCTGGCTCTCCTTT

AAACTCGGGCGCAAAAAGAGCCTGATGATCGGCGCAATT

TTGTTTGTTGCCGGTTCGCTGTTCTCTGCGGCTGCGCCA

AACGTTGAAGTACTGATTCTTTCCCGCGTTCTACTGGGG

CTGGCGGTGGGTGTGGCCTCTTATACCGCACCGCTGTAC

CTCTCTGAAATTGCGCCGGAAAAAATTCGTGGCAGTATG

ATCTCGATGTATCAGTTGATGATCACTATCGGGATCCTCG

GTGCTTATCTTTCTGATACCGCCTTCAGCTACACCGGTGC

ATGGCGCTGGATGCTGGGTGTGATTATCATCCCGGCAAT

TTTGCTGCTGATTGGTGTCTTCTTCCTGCCAGACAGCCC

ACGTTGGTTTGCCGCCAAACGCCGTTTTGTTGATGCCGA

ACGCGTGCTGCTACGCCTGCGTGACACCAGCGCGGAAG

CGAAACGCGAACTGGATGAAATCCGTGAAAGTTTGCAGG

TTAAACAGAGTGGCTGGGCGCTGTTTAAAGAGAACAGCA

ACTTCCGCCGCGCGGTGTTCCTTGGCGTACTGTTGCAGG

TAATGCAGCAATTCACCGGGATGAACGTCATCATGTATTA

CGCGCCGAAAATCTTCGAACTGGCGGGTTATACCAACAC

TACCGAGCAAATGTGGGGGACCGTGATTGTCGGCCTGAC

CTT**CGTACTTGCCACCTTTATCGCAATCGGCCTTGTTGA**

CCGCTGGGGACGTAAACCAACGCTAACGCTGGGCTTCCT

GGTGATGGCTGCTGGCATGGGCGTACTCGGTACAATGAT

GCATATCGGTATTCACTCTCCGTCGGCGCAGTATTTCGCC

ATCGCCATGCTGCTGATGTTTATTGTCGGTTTTGCCATGA

GTGCCGGTCCGCTGATTTGGGTACTGTGCTCCGAAATTC

AGCCGCTGAAAGGCCGCGATTTTGGCATCACCTGCTCCA

CTGCCACCAACTGGATTGCCAACATGATCGTTGGCGCAA

CGTTCCTGACCATGCTCAACACGCTGGGTAACGCCAACA

CCTTCTGGGTGTATGCGGCTCTGAACGTACTGTTTATCCT

GCTGACATTGTGGCTGGTACCGGAAACCAAACACGTTTC

GCTGGAACATATTGAACGTAATCTGATGAAAGGTCGTAA

ACTGCGCGAAATAGGCGCTCACGATCACCACCACCACCA

CCACTAATATTAGGAAATTAATA

* The underlined sequences correspond to the promoter region

* The bolded sequences correspond to the ORF region

* The bigger letters correspond to the mutated region

3.4.2 Construction of the strain expressing *galPm* gene

In previous study, plasmid pCPaGGR was constructed for expression of *aldH* derived from *P. aeruginosa*, *GPD1* and *GPP2* derived from *S. cerevisiae* and *xylR* derived from *E. coli* with engineered promoter. To express the mutant galactose transporter constitutively under the control of the native *E. coli* promoter, the *galPm* gene was introduced to plasmids pACYCDuet-1 and pCPaGGR (Figure 3.8).

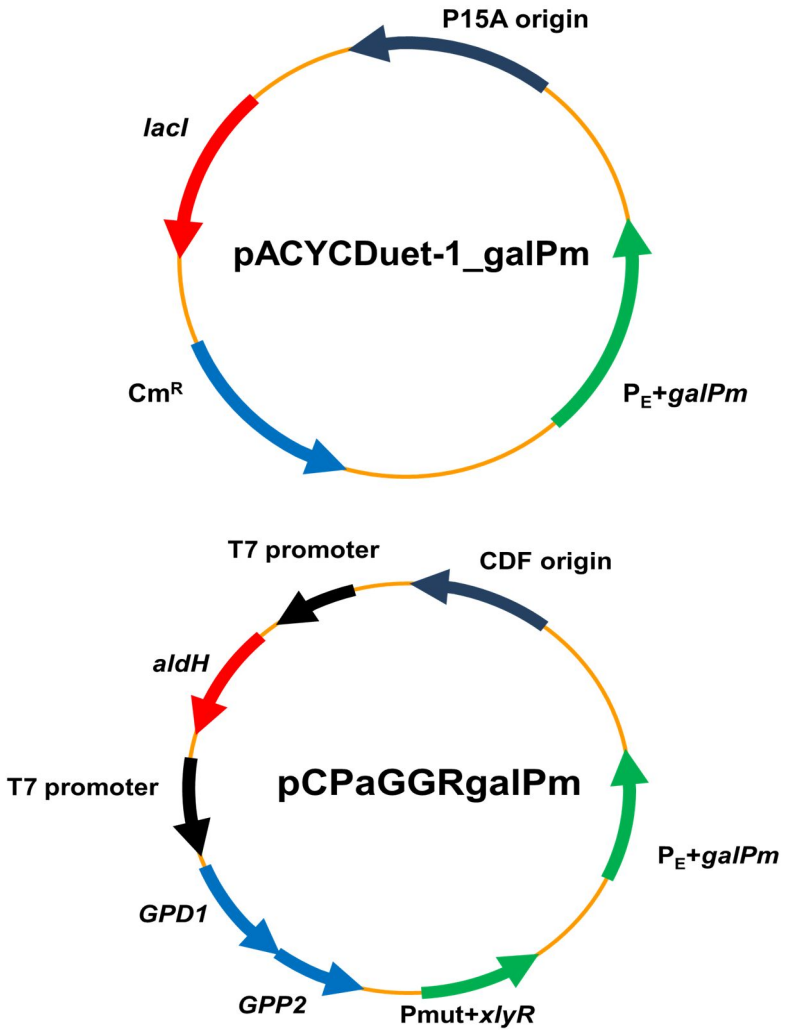


Figure 3.8. Genetic maps of plasmids pACYCDuet-1_galPm and pCPaGGRgalPm

3.4.3 Flask culture of the strain expressing *galPm* gene

To investigate xylose uptake rate, flask culutres of the strains Δgyp/pACYCDuet-1, Δgyp/pACYCDuet-1_galP and Δgyp/pACYCDuet-1_galPm were carried out in R/5 medium containing 10 g/L glucose and 6 g/L xylose. During 16 h of cultivation, the Δgyp/pACYCDuet-1_galP strain showed a 52% increase in glucose uptake rate and a 72% decrease in xylose uptake rate, compared to the control strain. On the other hand, the Δgyp/pACYCDuet-1_galPm showed an enhancement in xylose uptake rate by 34% and a decrease in glucose uptake rate by 22%, compared to the control strain (Figure 3.9.).

In other words, introduction of the GalPm protein with a point mutation in the 376th amino acid residue from asparagines to phenylalanine led to an improvement of xylose uptake rate in a mixture of glucose and xylose. Results are summarized in Table 3.4.

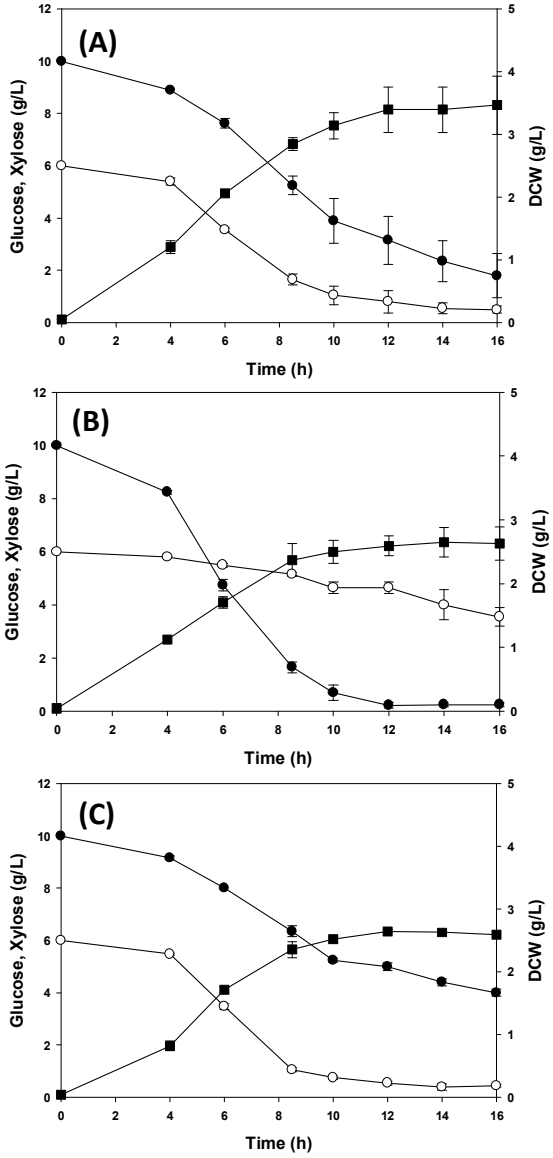


Figure 3.9. Profiles of batch fermentation of (A) Δgyp/pACYCDuet-1, (B) Δgyp/pACYCDuet-1_galP and (C) Δgyp/pACYCDuet-1_galPm in R/5 medium containing 10 g/L glucose and 6 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.4 Results of the *galPm* gene expression strain in flask culture

Strains	Volumetric total sugar uptake rate[1] (g/L·h)	Volumetric glucose uptake rate[2] (g/L·h)	Volumetric xylose uptake rate[3] (g/L·h)
Δgyp/pACYCDuet-1	1.56	0.83	0.73
Δgyp/pACYCDuet-1galP	1.46	1.26	0.20
Δgyp/pACYCDuet-1_galPm	1.63	0.65	0.98

[1]Total sugar uptake rate was calculated by division of total sugar concentration of glucose and xylose by the hours sugar was depleted.

[2]Glucose uptake rate was calculated by division of glucose concentration by the hours glucose was depleted

[3]Xylose uptake rate was calculated by division of xylose concentration by the hours xylose was depleted.

3.5 Deletion of the *xylFGH* genes

There are two major systems for xylose transport in *E. coli*, the proton/xylose symporter coded by *xylE* and the ABC transporter encoded by the *xylF, xylG* and *xylH* genes (Sumiya et al., 1995). Because ATP is consumed to internalize xylose and to phosphorylate xylulose, low ATP/xylose molar yields (0.67) and slow growth are observed in homolactic strains (Hasona et al., 2004). Furthermore, the *xylF, xylG* and *xylH* genes deleted strain showed a 50% increase in growth rate and similar specific xylose consumption rate (J. Utrilla et al., 2012). Therefore, in this study, the *xylFGH* genes were deleted to increase ATP availability and to secure more space for GalPm protein in the cell membrane.

3.5.1 Sequences of the *xylFGH* genes

The *xylF* gene sequences are as follows.

ATGAAAATAAAGAACATTCTACTCACCCTTTGCACCTCACTC
CTGCTTACCAACGTTGCTGCACACGCCAAAGAAGTCAAAAT
AGGTATGGCGATTGATGATCTCCGTCTTGAACGCTGGCAAAA
AGATCGAGATATCTTTGTGAAAAAGGCAGAATCTCTCGGCGC
GAAAGTATTTGTACAGTCTGCAAATGGCAATGAAGAAACAC
AAATGTCGCAGATTGAAAACATGATAAACCGGGGTGTCGAT

GTTCTTGTCATTATTCCGTATAACGGTCAGGTATTAAGTAACG

TTGTAAAAGAAGCCAAACAAGAAGGCATTAAAGTATTAGCTT

ACGACCGTATGATTAACGATGCGGATATCGATTTTTATATTTCT

TTCGATAACGAAAAAGTCGGTGAACTGCAGGCAAAAGCCCT

GGTCGATATTGTTCCGCAAGGTAATTACTTCCTGATGGGCGG

CTCGCCGGTAGATAACAACGCCAAGCTGTTCCGCGCCGGAC

AAATGAAAGTGTTAAAACCTTACGTTGATTCCGGAAAAATTA

AAGTCGTTGGTGACCAATGGGTTGATGGCTGGTTACCGGAA

AACGCATTGAAAATTATGGAAAACGCGCTAACCGCCAATAAT

AACAAAATTGATGCTGTAGTTGCCTCAAACGATGCCACCGCA

GGTGGGGCAATTCAGGCATTAAGCGCGCAAGGTTTATCAGG

GAAAGTAGCAATCTCCGGCCAGGATGCGGATCTCGCAGGTAT

TAAACGTATTGCTGCCGGTACGCAAACTATGACGGTGTATAA

ACCTATTACGTTGTTGGCAAATACTGCCGCAGAAATTGCCGT

TGAATTGGGCAATGGTCAGGAGCCAAAAGCAGATACCTCAC

TGAATAATGGCCTGAAAGATGTCCCCTCCCGCCTCCTGACAC

CGATCGATGTGAATAAAAACAACATCAAAGATACGGTAATTA

AAGACGGATTCCACAAAGAGAGCGAGCTGTAA

The *xylG* gene sequences are as follows.

ATGCCTTATCTACTTGAAATGAAGAACATTACCAAAACCTTC

GGCAGTGTGAAGGCGATTGATAACGTCTGCTTGCGGTTGAAT

GCTGGCGAAATCGTCTCACTTTGTGGGGAAAATGGGTCTGGT

AAATCAACGCTGATGAAAGTGCTGTGTGGTATTTATCCCCAT

GGCTCCTACGAAGGCGAAATTATTTTTGCGGGAGAAGAGATT

CAGGCGAGTCACATCCGCGATACCGAACGCAAAGGTATCGC

CATCATTCATCAGGAATTGGCCCTGGTGAAAGAATTGACCGT

GCTGGAAAATATCTTCCTGGGTAACGAAATAACCCACAATGG

CATTATGGATTATGACCTGATGACGCTACGCTGTCAGAAGCT

GCTCGCACAGGTCAGTTTATCCATTTCACCTGATACCCGCGT

TGGCGATTTAGGGCTTGGGCAACAACAACTGGTTGAAATTG

CCAAGGCACTTAATAAACAGGTGCGCTTGTTAATTCTCGATG

AACCGACAGCCTCATTAACTGAGCAGGAAACGTCGGTTTTA

CTGGATATTATTCGCGATCTACAACAGCACGGTATCGCCTGTA

TTTATATTTCGCACAAACTCAACGAAGTCAAAGCGATTTCCG

ATACGATTTGCGTTATTCGCGACGGACAGCACATTGGTACGC

GTGATGCTGCCGGAATGAGTGAAGACGATATTATCACCATGA

TGGTCGGGCGAGAGTTAACCGCGCTTTACCCTAATGAACCAC

ATACCACCGGAGATGAAATATTACGTATTGAACATCTGACGG

CATGGCATCCGGTCAATCGTCATATTAAACGAGTTAATGATGT

CTCGTTTTCCCTGAAACGTGGCGAAATACTGGGTATTGCCGG

ACTCGTTGGTGCCGGACGTACCGAGACCATTCAGTGCCTGTT

TGGCGTGTGGCCCGGACAATGGGAAGGAAAAATTTATATTGA

TGGCAAACAGGTAGATATTCGTAACTGTCAGCAAGCCATCGC

CCAGGGGATTGCGATGGTACCCGAAGACAGAAAGCGCGACG

GCATCGTTCCGGTAATGGCGGTTGGTAAAAATATTACCCTCG

CCGACTCAATAAATTTACCGGTGGCATTAGCCAGCTTGATGA

CGCGGCAGAGCAAAAATGTATTCTGGAATCAATCCAGCAAC

TCAAAGTTAAAACGTCGTCCCCCGACCTTGCTATTGGACGTT

TGAGCGGCGGCAATCAGCAAAAAGCGATCCTCGCTCGCTGT

CTGTTACTTAACCCGCGCATTCTCATTCTTGATGAACCCACCA

GGGGTATCGATATTGGCGCGAAATACGAGATCTACAAATTAAT

TAACCAACTCGTCCAGCAGGGTATTGCCGTTATTGTCATCTCT

TCCGAATTACCTGAAGTGCTCGGCCTTAGCGATCGTGTACTG

GTGATGCATGAAGGGAAACTAAAAGCCAACCTGATAAATCAT

AACCTGACTCAGGAGCAGGTGATGGAAGCCGCATTGAGGAG

CGAACATCATGTCGAAAAGCAATCCGTCTGA

The *xylH* gene sequences are as follows.

ATGTCGAAAAGCAATCCGTCTGAAGTGAAATTGGCCGTACC

GACATCCGGTAGCTTCTCCGGGCTGAAATCACTGAATTTGCA

GGTCTTCGTGATGATTGCAGCTATCATCGCAATCATGCTGTTC

TTTACCTGGACCACCGATGGTGCCTACTTAAGCGCCCGTAAC

GTCTCCAACCTGTTACGCCAGACCGCGATTACCGGCATCCTC

GCGGTAGGAATGGTGTTCGTCATAATTTCTGCTGAAATCGAC

CTTTCCGTCGGCTCAATGATGGGGCTATTAGGTGGCGTCGCG

GCGATTTGTGACGTCTGGTTAGGCTGGCCTTTGCCACTTACC

ATCATTGTGACGTTGGTTCTGGGACTGCTTCTCGGTGCCTGG

AACGGATGGTGGGTCGCGTACCGCAAAGTCCCTTCATTTATT

GTCACCCTCGCGGGCATGTTGGCATTTCGCGGCATACTCATT

GGCATCACCAACGGCACGACTGTTTCCCCCACCAGCGCCGC

GATGTCACAAATTGGGCAAAGCTATCTACCTGCCAGCACTGG

CTTCATCATTGGCGCGCTTGGCTTAATGGCTTTTGTTGGTTGG

CAATGGCGCGGAAGAATGCGCCGTCAGGCTTTGGGTTTGCA

GTCTCCGGCCTCTACCGCAGTAGTCGGTCGCCAGGCTTTAAC

CGCTATCATCGTATTAGGCGCAATCTGGCTGTTGAATGATTAC

CGTGGCGTTCCCACTCCTGTTCTGCTGCTGACGTTGCTGTTA

CTCGGCGGAATGTTTATGGAAACGCGGACGGCATTTGGACG

ACGCATTTATGCCATCGGCGGCAATCTGGAAGCAGCACGTCT

CTCCGGGATTAACGTTGAACGCACCAAACTTGCCGTGTTCGC

GATTAACGGATTAATGGTAGCCATCGCCGGATTAATCCTTAGT

TCTCGACTTGGCGCTGGTTCACCTTCTGCGGGAAATATCGCC

GAACTGGACGCAATTGCAGCATGCGTGATTGGCGGCACCAG

CCTGGCTGGCGGTGTGGGAAGCGTTGCCGGAGCAGTAATGG

GGGCATTTATCATGGCTTCACTGGATAACGGCATGAGTATGAT

GGATGTACCGACCTTCTGGCAGTATATCGTTAAAGGTGCGAT

TCTGTTGCTGGCAGTATGGATGGACTCCGCAACCAAACGCC

GTTCTTGA

3.5.2 Confirmation of gene deletion

Disruption of the genes was confirmed by colony PCR using check primers (Table 2.2). The length of PCR product amplified with check primers was 3767 bp for the *xylFGH* gens in BL21 (DE3) strain and 180 bp for Δ*xylFGH* in the Δgypx strain. The result of deletion is shown in (Figure 3.10).

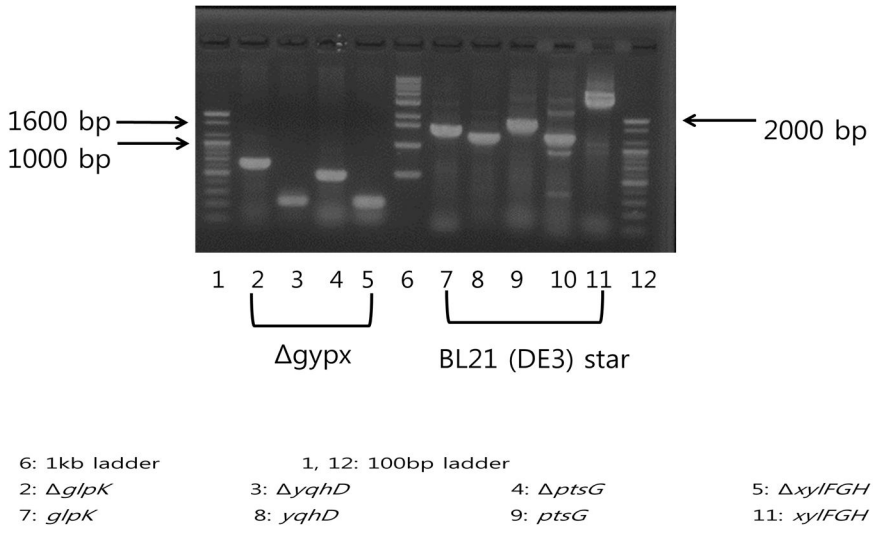


Figure 3.10. Confirmation of deletion of the *xylFGH* genes

3.5.3 Flask culture of the *xylFGH* genes deleted strain

To evaluate the effect of deletion of the *xylFGH* genes, comparison experiments were carried out. Plasmid pACYCDuet-1 and pACYCDuet-1_galPm were transformed into the strains Δgyp and Δgypx. The strains Δgyp/pACYCDuet-1, Δgyp/pACYCDuet-1_galPm and Δgypx/pACYCDuet-1_galPm were cultured in R/5 medium containing 10 g/L of glucose and 6 g/L xylose.

During 16 h of cultivation, the Δgypx/pACYCDuet-1_galPm strain showed improved uptake rate in glucose and xylose by 20% and 16%, respectively, compared to the Δgyp/pACYCDuet-1_galPm strain (Figure 3.9.).

Deletion of the *xylFGH* genes led to a proper expression of the GalPm protein, which was a result of an additional enhanced xylose uptake rate. For improved 3-HP production, these genetic manipulation should be introduced to the stains which produce 3-HP. Results are summarized in Table 3.5.

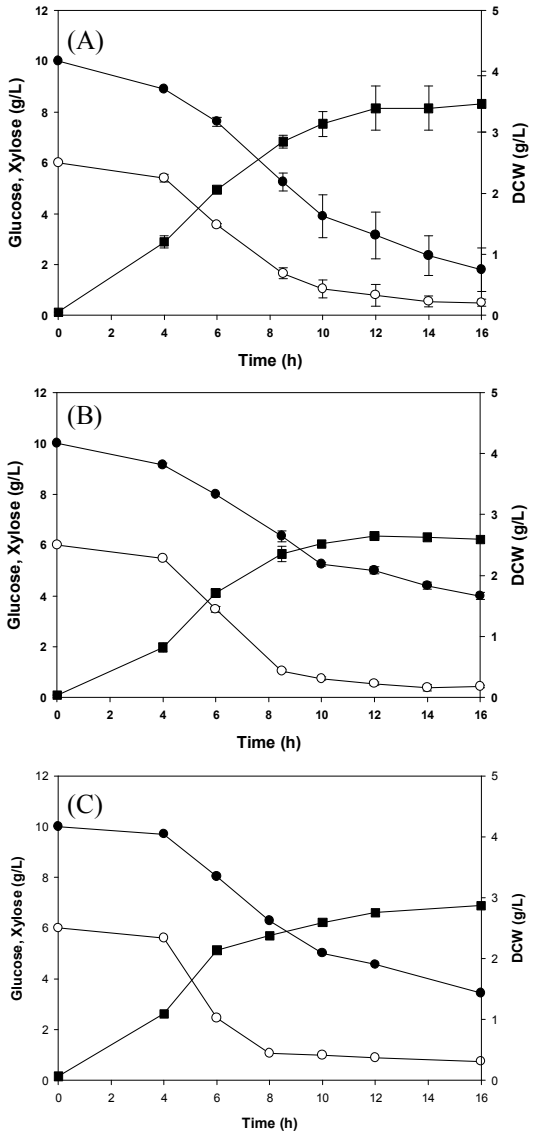


Figure 3.11. Profiles of batch fermentation of (A) Δgyp/pACYCDuet-1, (B) Δgyp/pACYCDuet-1_galPm and (C)

Δgypx/pACYCDuet-1_galPm in R/5 medium containing 10 g/L glucose and 6 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.5 Results of the *xylFGH* genes deleted strain in flask culture

Strains	Volumetric total sugar uptake rate[1] (g/L·h)	Volumetric glucose uptake rate[2] (g/L·h)	Volumetric xylose uptake rate[3] (g/L·h)
Δgyp/pACYCDuet-1	1.56	0.83	0.73
Δgyp/pACYCDuet-1_galPm	1.63	0.65	0.98
Δgypx/pACYCDuet-1_galPm	1.92	0.78	1.14

[1]Total sugar uptake rate was calculated by division of total sugar concentration of glucose and xylose by the hours sugar was depleted.

[2]Glucose uptake rate was calculated by division of glucose concentration by the hours glucose was depleted

[3]Xylose uptake rate was calculated by division of xylose concentration by the hours xylose was depleted.

3.5.4 Flask culture for 3-HP production with enhanced xylose uptake rate

To investigate 3-HP production, the strains Δgyp and Δgypx are transformed with plasmid pELDRR and pCPaGGR or pCPaGGRgalPm to produce 3-HP. Flask cultures of the strains Δgyp/pELDRR/pCPaGGR and Δgypx/pELDRR/ pCPaGGRgalPm in R/5 medium containing 5 g/L glucose and 3 g/L xylose were carried out. During 30 h of cultivation, the Δgypx/pELDRR/pCPaGGRgalPm strain showed xylose uptake rate of 0.45 g/L·h and glucose uptake rate of 0.25 g/L·h, and that is higher by 61% and lower by 36% than that of control strain, respectively. Also, it showed 3-HP concentration of 1.10 g/L, and that is higher by 8% than that of control strain (Figure 3.12). Results are summarized in Table 3.6.

In this study, GalPm protein, which has a high affinity with xylose, was introduced. As a result, insufficient xylose uptake rate of 3-HP producing strains was improved. Further, deletion of the *xylFGH* genes was carried out to increase ATP availability and secure membrane space for GalPm protein in the cell membrane. Finally, proper expression of the efficient protein for xylose uptake rate, GalPm protein, and increased ATP availability led to enhanced xylose uptake rate, cell growth and 3-HP production. The result suggests that

GalPm protein with point mutation and deletion of *xylFGH* genes would be applied to production of other chemicals which use a mixture of glucose and xylose.

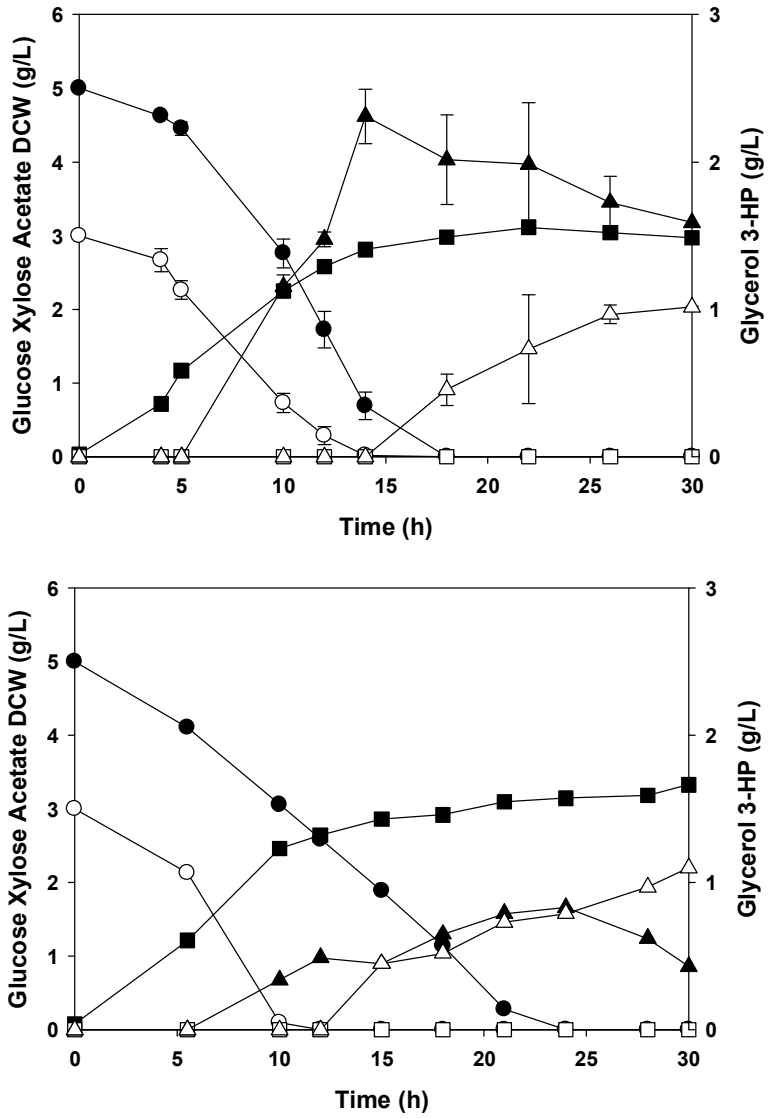


Figure 3.12. Profiles of batch fermentation of (A) Δgyp/pELDRR/pCPaGGR and (B) Δgypx/pELDRR/pCPaGGRgalPm in R/5 medium containing 5 g/L glucose and 3 g/L xylose.

Symbols: ●, Glucose; ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol; △, 3-HP.

Table 3.6 Results of the 3-HP producing strains in flask culture

Strains	DCW (g/L)	Total sugar uptake rate (g/L·h)	Glucose uptake rate (g/L·h)	Xylose uptake rate (g/L·h)	Glycerol (g/L)	3-HP (g/L)
Δgyp/pELDRR/pCPaGGR	3.09	0.67	0.39	0.28	1.59	1.02
Δgypx/pELDRR/pCPaGGRgalPm	3.33	0.70	0.25	0.45	0.43	1.10

Chapter 4. Conclusions

This thesis can draw the following conclusions:

(1) Overexpression of the *gpsA* gene from *E. coli* K-12 MG1655 under the control of the T_7 promoter decreased glycerol accumulation by 42% and increased 3-HP concentration by 48% in batch culture compared to the strains expressing the *GPD1* gene from *S. cerevisiae*

(2) Fed-batch fermentation of Δgyp/pELDRR/pCPaGAR using a limited glucose and xylose feeding strategy resulted in 37.6 g/L of 3-HP concentration, 0.63 g/L·h of productivity and 0.13 $g_{3\text{-HP}}/g_{sugar}$ of yield.

(3) Constitutive expression of the *galPm* gene and deletion of the *xylFGH* genes in the strain Δgypx/pELDRR/pCPaGGRgalPm improved xylose uptake rate by 47% and 3-HP of concentration by 8%, compared to the strain Δgyp/pELDRR/pCPaGGR.

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국 문 초 록

석유 분포의 불균등성과 가격의 불안정성 및 환경정인 문제가 대두되고 있는 시점에서, 석유를 기반으로 하는 화학 산업에서 바이오매스를 이용하는 화학 산업이라는 새로운 패러다임이 제시되고 있다. 이러한 바이오 화학 산업의 적용을 위한 생산 목적 물질로는 미국 에너지성에서 선정한 바이오매스로부터 생산할 가치가 있는 바이오 화학 소재로 선정되었으며, 다양한 화학 물질의 전구체로 사용되는 3-히드록시프로피온산을 선정하였다.

선행 연구에서는, 2 세대 바이오매스로부터 추출되는 포도당과 목당을 이용하여 3-히드록시프로피온산을 생산함에 있어서 발효 도중에 글리세롤이 축적되는 문제를 보였고, 이는 생산 경로의 중요한 유전자인 글리세롤 탈수효소의 역가를 저해시킨다는 연구가 알려져 있다. 이러한 문제점을 해결하기 위해서 글리세롤의 축적을 줄일 필요가 있었고, 글리세롤 축적의 원인이 글리세롤 생산과 소모의 불균형에 있다는 것을 확인하였다.

따라서 포도당과 목당으로부터 글리세롤의 생산에 관여하는 글리세롤-3-포스페이트 디하이드로제나아제(glycerol-3-phosphate dehydrogenase)의 역가를 줄이기 위하여 기존의 *Saccharomyces cerevisiae* 유래의 것(GPD1)을 *Escherichia*

coli 유래의 것(GpsA)으로 교체하였다. 결과적으로 5 g/L 의 포도당과 3 g/L 의 목당이 포함된 R/5 배지에서의 회분식 발효를 통해 기존보다 글리세롤 축적이 46% 감소하고 3-히드록시프로피온산의 생산은 48% 더 이루어졌다. 또한 구축된 Δgyp/pELDRR/pCPaGAR 를 이용하여 고농도의 3-히드록시프로피온산을 생산하기 위해 포도당과 목당을 기저 수준으로 유지해주는 유가식 배양을 진행하였다. 그 결과, 생산된 글리세롤이 모두 3-히드록시프로피온산으로 전환되면서 약 37.6 g/L 의 3-히드록시프로피온산을 생산할 수 있었다. 이러한 접근 방식은 글리세롤 탈수효소를 이용하는 대사공학에 널리 쓰일 수 있을 것이라고 예상할 수 있다.

이와 더불어 포도당과 목당을 통한 유가식 배양 시에 목당의 소모속도가 느려져 목당이 축적되고 결과적으로 3-히드록시프로피온산에 대한 생산성이 낮아지는 문제점을 보였다. 이러한 문제점을 해결하기 위해서 *S. cerevisiae* 의 육탄당 운반 단백질인 GAL2 단백질에 점 돌연변이를 가한 경우, 포도당과 목당 혼합당에서 목당에 대한 특이성이 증가해 목당 소모속도가 증가하는 현상을 보고한 연구를 참고하였다. 이를 토대로 *E. coli* 의 GalP 단백질에 대해 점 돌연변이를 실시하였고 결과적으로 포도당과 목당

혼합당에서 목당의 소모속도가 34% 증가하는 것을 확인할 수 있었다.

또한 변형된 GalPm 단백질의 세포 표면에서의 부분을 늘려 이 효과를 극대화하기 위하여, 효율적이지 못한 다른 막 단백질의 발현을 막고자 하였다. 이를 위해 효과가 미미하다고 알려진 목당 운반 단백질인 XylFGH의 발현을 막고자 *xylFGH* 유전자를 파쇄하는 실험을 진행하였다. 그 결과 변형된 GalPm가 보다 효율적으로 세포 표면에 발현되어, 이에 따라 포도당과 목당의 소모속도가 각각 20%와 16%만큼 증가하였다. 이러한 유전적 변형을 3-히드록시프로피온산 생산 균주에 도입한 결과, 목당 소모속도가 61% 향상되고 3-히드록시프로피온산의 생산량 또한 8% 증가하였다.

이러한 접근방식과 기술은 섬유소 유래의 바이오매스로부터 대사공학적으로 화학소재를 생산할 때 범용적으로 쓰일 수 있을 것으로 보인다.

주요어: 대사공학, 3-히드록시프로피온산, 대장균, 글리세롤 축적, 포도당과 목당 대사, 유가식 배양

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