공학석사 학위논문

Modulation of sugar transport for improved production of 3-hydroxypropionic acid (3-HP) in engineered *Escherichia coli*

당운반체 조절을 통한 **3-**히드록시프로피온산의 생산성 향상

2015년 8월

서울대학교 대학원

협동과정 바이오엔지니어링 전공

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이 논문을 공학석사학위논문으로 제출함

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Abstract

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Since xylose is the second abundunt sugar next to glucose in lignocellulosic biomass, production of various chemicals by using xylose as a carbon source is important. 3-Hydroxypropionic acid (3-HP) was selected as a target material which is a precursor for various chemicals including acrylic acid, methyl acrylate and acrylamide. However, production of 3-HP from xylose showed glycerol accumulation during cultivation. Because the glycerol accumulation is known for inhibiting glycerol dehydratase which is a key enzyme in biosynthesis of 3-HP, alleviating glycerol accumulation is necessary to increase 3-HP production. The *crr* and *ptsHI* genes are known for encoding the PTS system. The phosphorylated CRR protein inhibits glycerol uptake when xylose remained in media by activating transcription of catabolite-repressed genes, and the phosphorylated HPR protein encoded by the *ptsHI* genes inhibits glycerol uptake by combining with a glycerol facilitator. Therefore, deletion of the *crr* and *ptsHI* genes involved in inhibition of glycerol uptake are necessary.

Although deletion of the *crr* and *ptsHI* genes decreased glycerol accumulation by 30% relative to the control strain, this strategy also reduced xylose uptake rate and 3-HP concentration. As another way to resolve the glycerol accumulation problem, the genes for sugar transporters, *galP*, *glpF* and *xylE*, were expressed constitutively under the endogenous *E. coli* promoter. The *galP* gene encoding galactose permease and the *glpF* gene encoding glycerol facilitator are overexpressed when the glycerol is used as a sole carbon source. The *xylE* gene encoding xylose symporter is expected to enhance xylose uptake rate. As a result, the strain overexpressing the *galP* gene decreased glycerol accumulation by 55% compare with the control strain without reducing 3-HP concentration. The strain overexpressing the *glpF* and *xylE* increased glycerol accumulation by 25% and 58% relative to the control strain, respectively. To clarify this result, fermentation was carried out in R/5 medium containing 20 g/L xylose. The batch fermentation resulted in 4.01 g/L of 3-HP and 5.71 g/L of DCW without accumulation of glycerol. Expression of the *galP* gene which was identified by Western blotting seemed to redirect the carbon flux from glycerol to cell mass. To produce high concentration of 3-HP, a fed-batch fermentation using the *galP* overexpressed strain was carried out by feeding xylose continuously resulted in 39.1 g/L of 3-HP concentration and 0.40 g/L·h of 3-HP productivity without

accumulation of glycerol.

Keywords : Metabolic engineering, 3-Hydroxypropionic acid, *Escherichia coli*, xylose, glycerol accumulation, *galP*, 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. 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 opening for 3-HP has been estimated at 3.63 million tons per year (Raj *et al.* 2008).

The 3-HP production via microbial fermentation can be devided into two major approaches based on the utilized carbon sources, glucose

and glycerol (Choi *et al.* 2015). The 3-HP production from glucose has been studied by Cargill, Codexis. Systems for glucose conversion to 3-HP, lactic pathway and β-alanine pathway. However, little is known about a measure of 3-HP production by the aforementioned two systems (US DOE, 2005). The first report to date is 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 our previous report, new aldehyde dehydrogenase from Pseudomonas aeruginosa were identifed and glycerol kinase(*glpK*) and propanediol oxidoreductase (*yqhD*) were disrupted. 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 recent study, *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). The 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 Research of 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) *ΔglpKΔgldA*	*Δglpk, gldA* *aldH, dhaBR* *GPD1,GPP2*	22.2	0.21	0.10	Fed-batch **(xylose)**	Lee thesis, (2012)

1.2 Hemicellulose and xylose

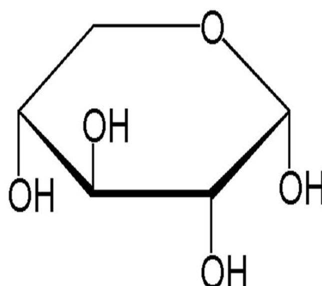
Hemicellulose, the second major constituent of lignocellusose (Table 1.3) is a highly branched and complex heteropolymer that contains hexoses (D-glucose, D-galactose, D-mannose, L-rhamnose, L-fructose), pentoses (D-xylose an L-arabinose) and uronic acids (D-glucuronic acid and D-galacturonic acid). 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 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, 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

Figure 1.1. Structure of xylose



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

E. coli is able to metabolize xylose via the pentose phosphate pathway (David & Weismeyer 1970). Through pentose phosphate pathway, xylose is converted to dihydroxyacetone phosphate (DHAP), and then DHAP is converted to glycerol 3-phosphate catalyzed 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; glycerol dehydrogenase (GPD), glycerol 3-phosphatese (GPP), glycerol dehydratase (DHAB), and aldehyde dehydrogenase (ALDH) contribute to the ability to convert xylose to 3-HP in *E. coli* (Figure 1.2).

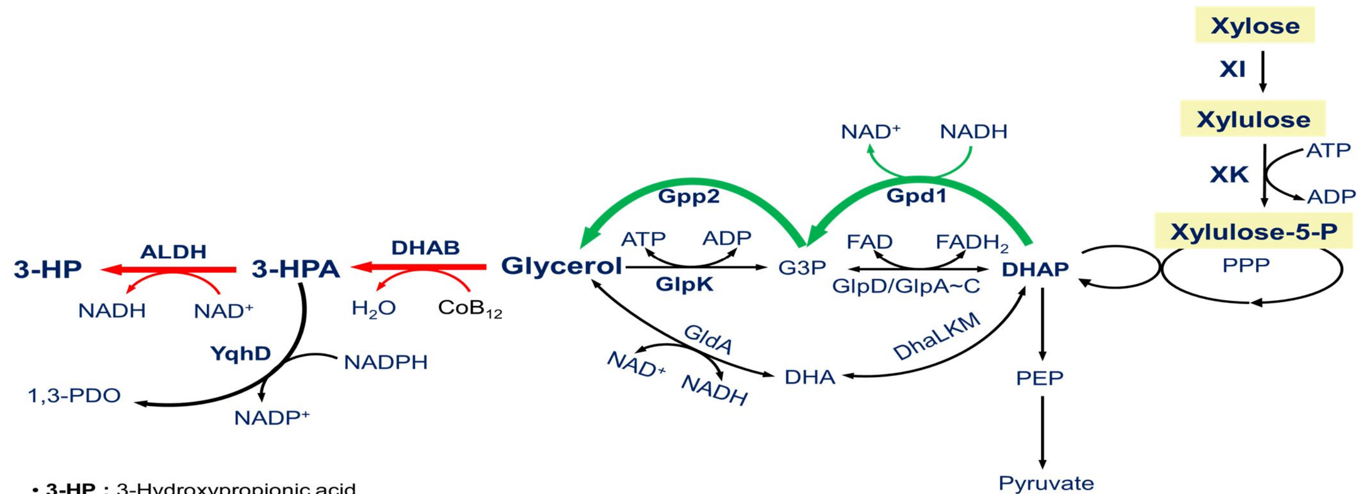


Figure 1.2. Biosynthetic pathway to 3-hydroxypropionic acid from xylose in recombinant *E. coli*

1.4 Phosphoenolpyruvate-dependent phosphotransferase system (PTS system)

In an environment of mixed available carbons, *E. coli* generally has one preferred carbon source. *E. coli* primarily chooses glucose when exposed to a carbon mixture. Since then, glucose has been regarded as the classical “preferred” carbon source and has been studied for decades in order to unravel the molecular mechanisms of carbon source-transport and its regulation. The major glucose transport system of *E. coli* was first described in 1966 when a glucose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) was identified (Kundig *et al.* 1964).

PTS of *E. coli* K-12 consists of a range of more than 20 different carbohydrate specific Enzymes II (EIIs), which catalyze concomitant transport and phosphorylation of the carbohydrate (Gabor *et al.* 2011). The use of PEP, an intermediate of the glycolysis, as a phosphoryl group donor couples carbohydrate transport and metabolism tightly. In the case of the glucose–PTS the phosphate group is subsequently transferred from EI~P to HPr, from HPr~P to the soluble $EIIA^{Glc}$ (sometimes also called $EIIA^{Crr}$, gene *crr*, part of the *ptsHIcrr* operon), and finally from $EIIA^{Glc}$~P to the glucose-specific membrane protein $EIICB^{Glc}$ (gene *ptsG*), which mediates uptake and

phosphorylation of glucose (Jahreis *et al.* 2008). Cells use the information about the phosphorylation levels of the various PTS-proteins to control the cellular carbon flux (Gabor *et al.* 2011).

1.5 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). Transporter are integral transmembrane proteins. That is, 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)

1.6 Research objectives

This study was focused on the enhancement of 3-HP production by minimizing glycerol accumulation in metabolically engineered *E. coli*. To achieve this goal, the *E. coli* PTS system was modulated and *E. coli* transporter protein was expressed constitutively.

The specific objectives of this research were described as follows :

1. Modulation of the PTS system by knock-out of *crr* and *ptsHI* genes encoding the PTS system.
2. Overexpression of *E. coli galP*, *glpF* and *xylE* genes.
3. Enhancement of 3-HP production in metabolically engineered *E. coli* by 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 BL21star(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* BL21star(DE3) was used as a host strain for 3-HP production. *GlpK*, *yqhD*, *ptsG* gene were disrupted in *E. coli* BL21star(DE3) in previous research (Joung thesis, 2013).

Plasmids pELDRR haboring *Lactobacillus brevis* glycerol dehydratase (DhaB1, DhaB2, DhaB3) and glycerol dehydratase reactivase (DhaR1, DhaR2) genes under T7 promoter (Kwak *et al.* 2013). Second plasmid used in this study pCPaGGRmut haboring 4 genes under T7 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 synthetic promoter.

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(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) *ΔglpKΔyqhDΔptsG*	Joung thesis (2013)
Δgyp-crr	BL21(DE3) *ΔglpKΔyqhDΔptsGΔcrr*	In this study
Δgyp-ptsHI	BL21(DE3) *ΔglpKΔyqhDΔptsGΔptsHI*	In this study
Plasmids		
pCPaGGRmut	Sm^R, pCDFduet-1 based plasmid containing *aldH*, GPD1, GPP2 and xylR fragment with natural P_R promoter under the T_7 promoter	Joung thesis (2013)
pCPaGGRmutGlpF	Sm^R, *glpF* gene with natural promoter cloned in pCPaGGRmut	In this study
pCPaGGRmutGalP	Sm^R, *galP* gene with natural promoter cloned in pCPaGGRmut	In this study
pCPaGGRmutxylE	Sm^R, *xylE* gene with natural promoter cloned in pCPaGGRmut	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 DNA Manipulation

2.2.1 Gene deletion

E. coli BL21 star (DE3) was used as a host strain for 3-HP production. For the cloning of kanamycin resistance cassette to delete *E. coli* choromosomal gene encoding phosphoenolpyruvate-dependent phosphotransferase system, plasmid pKD13 used as the template of the polymerase chain reaction (PCR). Primers, containing sequences designed for homologous recombination with the *crr, ptsHI* genes, listed in Table 2.2 were used to amplify. To express λ recombinase, plasmid pKD46 was introduced to *E. coli* BL21 star (DE3). Elimination of kanamycin resistance gene was performed by the aid of a helper plasmid pCP20 which expressed FLP recombinase by thermal induction and then the helper plasmid was cured. Disruption of chromosomal genes was confirmed by colony PCR using PCR-primers F-chk and R-chk (Table 2.2). 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-crr	**TCCACGAGATGCGGCCCAATTTACTGCTTAGGAGAAGATC**GTGTAGGCTGGAGCTGCTTC	crr
F-ptsHI	**TTAGTTCCACAACACTAAACCTATAAGTTGGGGAAATACA**GTGTAGGCTGGAGCTGCTTC	ptsHI
R-crr/ptsHI	**AAATGGCGCCGATGGGCGCCATTTTTCACTGCGGCAAGAA**ATTCCGGGGATCCGTCGACC	crr / ptsHI
F-chk	ACGGACGAGTTAATGACGCTGG	
R-chk	TTGCTGAAAGGGAAGGCGTTAAC	
F-galP	*TATGCGACTCCTGCA*ATTACACTGATGTGATTTGCTTCACATCT	galP
R-galP	*TATTAATTTCCTAAT*ATTAATCGTGAGCGCCTATTTCGCG	
R-galP-his	*TATTAATTTCCTAAT*ATTAGTGGTGGTGGTGGTGGTGATCGTGAGCGCCTATTTCGCG	
F-glpF	*TATGCGACTCCTGCA*GGCACACACATTTTAAGTTCGATATTTCTC	glpF
R-glpF	*TATTAATTTCCTAAT*ATTACAGCGAAGCTTTTTGTTCTGAAGGAG	
F-xylE	*TATGCGACTCCTGCA*CAATTTTGGATAATTATCACAATTAAGATCACAG	xlyE
R-xylE	*TATTAATTTCCTAAT*ATTACAGCGTAGCAGTTTGTTGTGTTTTC	

* Bolded sequences are homologous regions with *crr, ptsHI* genes in the *E. coli* choromosomes

* Italic sequences are homologous regions with pCPaGGRmut EcoN1 site

* Underlined sequences are six histidine sequence to western blotting

2.2.2 *E. coli* transporter expressing plasmid vector construction

For the cloning of *E. coli* transporter, chromosomal DNA of *E. coli* K-12 was used as the template of the polymerase chain reaction (PCR). Primers listed in Table 2.2 were used to amplify. The expression vectors for *E. coli* transporter is derived from pCDFDeut-1 (Novagen, Germany).

2.3 Gene deletion progress

2.3.1 Preparation of kanamycin resistance cassette

PCRs were performed with Applied Biosystems Veriti 96well Thermal Cycler (Lincoln, CA, USA) and prime STAR HS Premix from TAKARA (Otsu, Japan). PCRs for cloning of Kanamycin resistance cassettes were performed in 25 µL of DNA polymerase premix from TAKARA(Otsu, Japan) containing 20 pM each F-crr, F-ptsHI and R-crr/ptsHI primers (Table 2.2), and 1 µL pKD13 which is template of cloning respectively. 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 appropriate time for length of target gene (1 min per 1 kb DNA) at 72°C, followed by 7 min at 72°C during the last cycle.

2.3.2 Expression of λ red recombinase in host strain

Plasmid pKD46 introduced to each expression strain as using $CaCl_2$ method (Dagert & Ehrlich 1979). Strain which is harboring pKD46 cultured in 100ml LB medium containing L-arabinose (10 mM), ampicilin (5 mg/mL) at 30°C until O.D. at 600 nm reached 0.5~0.8.

2.3.3 Kanamycin resistance cassette insert to expression strain

All cells harvested by centrifugation at 3000 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 is 3 times. The cells are concentrated in 200 μL ,using 10% glycerol. Then prepared kanamycin resistance cassette 16 μL put on the 100 μL cell by electroporation method as using Gene-Pulser EC2 program (Bio-RAD, USA).

2.3.4 Recombination and adaptation

The cell which is harboring kanamycin resistance cassette, shaking incubation in 1 ml LB medium about 1 hr and that do overnight at room temperature. Spreading the cells to LBK plate at 37°C. Colonies, having the kanamycin resistance, are selected. Kanamycin resistance cassette insertion is verified by the PCR method using check primers (Table 2.2)

2.3.5 Elimination of kanamycin resistance cassette

Plasmid pCP20 introduced to each kanamycin resistance strain as using $CaCl_2$ method (Dagert & Ehrlich 1979). Spreading the cells 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)

2.4 *E. coli* transporter DNA manipulation and transformation

2.4.1 Preparation of DNA

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

2.4.2 Polymerase chain reaction

PCRs were performed with Applied Biosystems Veriti 96 well Thermal Cycler (Lincoln, CA, USA). PCRs for cloning of transporter gene which is *galp*, *glpF*, *xylE* from *E. coli* K-12 was 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 *E. coli* K-12 genomic DNA which is template of cloning, respectively. 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 last cycle.

2.4.3 Digestion and ligation of DNA

Restriction enzymes *EcoN1* and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). pCPaGGRmut vector was digested with *EcoN1*. In-Fusion HD Cloning Kit obtained from Takara (Otsu, Japan) was used for ligation PCR products and the plasmid vector pCPaGGRmut.

2.4.4 Transformation

Plasmid pCPaGGRmutgalP, pCPaGGRmutglpF, pCPaGGRmutxylE introduced to *E. coli* TOP10 which is cloning vector as using CaCl2 method (Dagert & Ehrlich 1979). After plasmid were preparation, transformed to expression strain, *E. coli* BL21star(DE3)

2.5 Culture conditions

2.5.1 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 glycerol and 3-HP. Riesenberg medium consisting 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.

2.5.2 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 shaking incubator (Vision, Buchon, Korea). To produce glycerol and 3-HP, flask culture was performed at 500 mL baffled flask (NALGENE, USA) with 100 mL R/5 medium containing 10 g/L xylose. The 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, respectively) and coenzyme B_{12} (final concentration 20 μM,

respectively) were added as the inducer and cofactor of glycerol dehydratase when O.D.$_{600}$ reached to 3. And at the same time, temperature changed to 25℃ and the flask was covered with aluminum foil.

2.5.3 Batch fermentation in a bioreactor

Batch fermentation was performed in a bioreactor of 3 L jar (Fermentec, Seoul, Korea) with 1 L initial working volume of a R/5 medium containing 20 g/L xylose and antibiotics of the same concentration as flask culture. The 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 pre-culture was transferring to bioreactor. Main culture was carried out at 37℃, 1200 rpm. Aeration rate and agitation speed were in 1,200 rpm and 2 vvm of air supply. When the cell O.D.600 reached to 10, IPTG and coenzyme B_{12} were added and temperature shift was performed, same as flask culture. Aeration rate were shifted at 1 vvm and agitation speed was shifted to 1,200 rpm to 600 rpm after induction.

2.5.4 Fed-batch fermentation in a bioreactor

Fed-batch fermentation was carried out in a 2.5 L jar fermentor (KObiotech, Incheon, Korea) with a 1 L working volume of R/5

medium. Transferring the 100 mL of pre-culture and main culture was the same with the batch fermentation. When the cell $O.D._{600}$ reached to 8, IPTG and coenzyme B_{12} were added and temperature shifted to 25℃ without changing aeration rate and agitation speed. Feeding solutions were conversed to organic acids by the metabolic processes of cells. The feeding solution was composed of 500 g/L xylose and 14 g/L $MgSO_4 \cdot 7H_2O$. After 10 h, feeding solution was fed on continuously. The pH was automatically controlled at 6.78 to 6.82 by addition of 28% ammonia water.

2.6 Analytical methods

2.6.1 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:

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

2.6.2 High performance liquid chromatography analysis

The concentrations of 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.

2.6.3 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.

2.6.4 Western blot

Native proteins were seperated by gel electrophoresis on sodium dodecyl sulfate polyacrylamide gel. Then, the proteins were transferred to a PVDF membrane. Transferred membrane were blocked with 5% non-fat dry milk several hours and next His-probe antibodies were treated. After several hours to probing, adding the appropriate substrate to the membrane, target protein produced a fluorescence. That fluorescence was detected on the film at the dark room. All buffers used in western blot are listed in Table 2.4.

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

Table 2.4 Composition of buffers used in western blot

10X transfer buffer		**Washing buffer (TBST)**		**Blocking buffer**
Tris	30.3 g/L	Tris	6 g/L	5% skim milk in TBST
Glycine	144 g/L			
Dillute to 1X		NaCl	29.22 g/L	
DDW	700 mL			
Methnol	200 mL	0.1 % Tween 20	1 ml/L	
10X Transbuffer	100 mL			

Chapter 3. Results and discussions

3.1 Production of 3-HP from xylose

3.1.1 Problem of glycerol accumulation during culture

In previous research, strain Δgyp was transformed with plasmid pCPaGGRmut and pELDRR to product 3-HP. To measure 3-HP producing capacity , the strain Δgyp/pELDRR/pCPaGGRmut was cultured in R/5 medium containing 10 g/L xylose. When cell mass reached an $O.D._{600}$ of 3, IPTG and coenzyme B_{12} were added to final concentrations of 0.1 mM and 20 μM respectively.

As the result of flask culture, the consumption rate of xylose was 0.67 g/L·h and final concentration of 3-HP was 1.0 g/L (Figure 3.1). However, glycerol accumulated at 1.7 g/L in initial 15h and final concentration of glycerol was 1.0 g/L. Conversion of glycerol to 3-HP was initiated only after xylose was depleted completely.

Although glycerol was precursor of 3-HPA, high concentration of glycerol inactivates glycerol dehydratase which convert glycerol to 3-HPA (Knietsch *et al.* 2003). It is a result of 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).

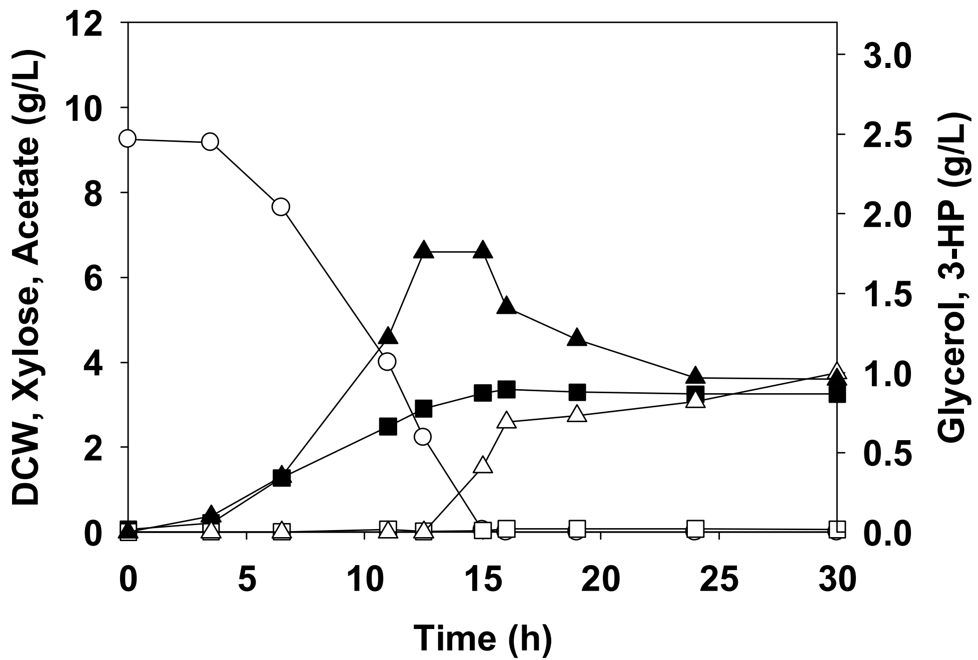


Figure 3.1. 100 mL flask culture of Δgyp/pELDRR/pCPaGGRmut in R/5 medium containing 10 g/L xylose.

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

3.2 Catabolite derepression

3.2.1 Deletion of *crr* and *ptsHI* genes

Δgyp/pELDRR/pCPaGGRmut cannot consume xylose and glycerol simultaneously. Therefore *crr* and *ptsHI* genes which are known for inhibiting *glpF* gene encoding glycerol facilitator were targeted (Darbon *et al.* 2002).

3.2.2 Sequences of *crr* and *ptsHI* genes

crr gene sequence is as follow.

ATGGGTTTGTTCGATAAACTGAAATCTCTGGTTTCCGACGACAAGAAGGAT

ACCGGAACTATTGAGATCATTGCTCCGCTCTCTGGCGAGATCGTCAATATCG

AAGACGTGCCGGATGTCGTTTTTGCGGAAAAAATCGTTGGTGATGGTATTG

CTATCAAACCAACGGGTAACAAAATGGTCGCGCCAGTAGACGGCACCATTG

GTAAAATCTTTGAAACCAACCACGCATTCTCTATCGAATCTGATAGCGGCGT

TGAACTGTTCGTCCACTTCGGTATCGACACCGTTGAACTGAAAGGCGAAGG

CTTCAAGCGTATTGCTGAAGAAGGTCAGCGCGTGAAAGTTGGCGATACTGT

CATTGAATTTGATCTGCCGCTGCTGGAAGAGAAAGCCAAGTCTACCCTGAC

TCCGGTTGTTATCTCCAACATGGACGAAATCAAAGAACTGATCAAACTGTCC

GGTAGCGTAACCGTGGGTGAAACCCCGG

TTATCCGCATCAAGAAGTAA

ptsHI genes sequence is as follow

ATGTTCCAGCAAGAAGTTACCATTACCGCTCCGAACGGTCTGCACACCCGC

CCTGCTGCCCAGTTTGTAAAAGAAGCTAAGGGCTTCACTTCTGAAATTACTG

TGACTTCCAACGGCAAAAGCGCCAGCGCGAAAAGCCTGTTTAAACTGCAG

ACTCTGGGCCTGACTCAAGGTACCGTTGTGACTATCTCCGCAGAAGGCGAA

GACGAGCAGAAAGCGGTTGAACATCTGGTTAAACTGATGGCGGAACTCGA

GTAATTTCCCGGGTTCTTTTAAAAATCAGTCACAAGTAAGGTAGGGTTATGA

TTTCAGGCATTTTAGCATCCCCGGGTATCGCTTTCGGTAAAGCTCTGCTTCT

GAAAGAAGACGAAATTGTCATTGACCGGAAAAAAATTTCTGCCGACCAGG

TTGATCAGGAAGTTGAACGTTTTCTGAGCGGTCGTGCCAAGGCATCAGCCC

AGCTGGAAACGATCAAAACGAAAGCTGGTGAAACGTTCGGTGAAGAAAA

GAAGCCATCTTTGAAGGGCATATTATGCTGCTCGAAGATGAGGAGCTGGAG

CAGGAAATCATAGCCCTGATTAAAGATAAGCACATGACAGCTGACGCAGCT

GCTCATGAAGTTATCGAAGGTCAGGCTTCTGCCCTGGAAGAGCTGGATGAT

GAATACCTGAAAGAACGTGCGGCTGACGTACGTGATATCGGTAAGCGCCTG

CTGCGCAACATCCTGGGCCTGAAGATTATCGACCTGAGCGCCATTCAGGAT

GAAGTCATTCTGGTTGCCGCTGACCTGACGCCGTCCGAAACCGCACAGCTG

AACCTGAAGAAGGTGCTGGGTTTCATCACCGACGCGGGTGGCCGTACTTCC

CACACCTCTATCATGGCGCGTTCTCTGGAACTACCTGCTATCGTGGGTACCG

GTAGCGTCACCTCTCAGGTGAAAAATGACGACTATCTGATTCTGGATGCCGT

AAATAATCAGGTTTACGTCAATCCAACCAACGAAGTTATTGATAAAATGCGC

GCTGTTCAGGAGCAAGTGGCTTCTGAAAAAGCAGAGCTTGCTAAACTGAA

AGATCTGCCAGCTATTACGCTGGACGGTCACCAGGTAGAAGTATGCGCTAA

CATTGGTACGGTTCGTGACGTTGAAGGTGCAGAGCGTAACGGCGCTGAAG

GCGTTGGTCTGTATCGTACTGAGTTCCTGTTCATGGACCGCGACGCACTGCC

CACTGAAGAAGAACAGTTTGCTGCTTACAAAGCAGTGGCTGAAGCGTGTG

GCTCGCAAGCGGTTATCGTTCGTACCATGGACATCGGCGGCGACAAAGAGC

TGCCATACATGAACTTCCCGAAAGAAGAGAACCCGTTCCTCGGCTGGCGCG

CTATCCGTATCGCGATGGATCGTAAAGAGATCCTGCGCGATCAGCTCCGCGC

TATCCTGCGTGCCTCGGCTTTCGGTAAATTGCGCATTATGTTCCCGATGATCA

TCTCTGTTGAAGAAGTGCGTGCACTGCGCAAAGAGATCGAAATCTACAAAC

AGGAACTGCGCGACGAAGGTAAAGCGTTTGACGAGTCAATTGAAATCGGC

GTAATGGTGGAAACACCGGCTGCCGCAACAATTGCACGTCATTTAGCCAAA

GAAGTTGATTTCTTTAGTATCGGCACCAATGATTTAACGCAGTACACTCTGG

CAGTTGACCGTGGTAATGATATGATTTCACACCTTTACCAGCCAATGTCACC

GTCCGTGCTGAACTTGATCAAGCAAGTTATTGATGCTTCTCATGCTGAAGGC

AAATGGACTGGCATGTGTGGTGAGCTTGCTGGCGATGAACGTGCTACACTT

CTGTTGCTGGGGATGGGTCTGGACGAATTCTCTATGAGCGCCATTTCTATCC

CGCGCATTAAGAAGATTATCCGTAACACGAACTTCGAAGATGCGAAGGTGT

TAGCAGAGCAGGCTCTTGCTCAACCGACAACGGACGAGTTAATGACGCTG

GTTAACAAGTTCATTGAAGAAAAAACAATCTGCTAATCCACGAGATGCGGC

CCAATTTACTGCTTAGGAGAAGATCATGGGTTTGTTCGATAAACTGAAATCT

CTGGTTTCCGACGACAAGAAGGATACCGGAACTATTGAGATCATTGCTCCG

CTCTCTGGCGAGATCGTCAATATCGAAGACGTGCCGGATGTCGTTTTTGCG

GAAAAAATCGTTGGTGATGGTATTGCTATCAAACCAACGGGTAACAAAATG

GTCGCGCCAGTAGACGGCACCATTGGTAAAATCTTTGAAACCAACCACGCA

TTCTCTATCGAATCTGATAGCGGCGTTGAACTGTTCGTCCACTTCGGTATCGA

CACCGTTGAACTGAAAGGCGAAGGCTTCAAGCGTATTGCTGAAGAAGGTC

AGCGCGTGAAAGTTGGCGATACTGTCATTGAATTTGATCTGCCGCTGCTGG

AAGAGAAAGCCAAGTCTACCCTGACTCCGGTTGTTATCTCCAACATGGACG

AAATCAAAGAACTGATCAAACTGTCCGGTAGCGTAACCGTGGGTGAAACCC

CGGTTATCCGCATCAAGAAGTAA

3.2.3 Confirmation of gene deletion

Disruption of genes was confirmed by colony PCR using check primers (Table 2.2). The results of deletion are shown in (Figure3.2)

3.2.4 Flask culture of PTS system deleted strains

To evaluate the effects of deletion of *crr* and *ptsHI* genes, comparison experiments were carried out. The plasmid pCPaGGRmut and pELDRR which were necessary to product 3-HP were transformed into strain Δgyp, Δgyp-crr and Δgyp-ptsHI. Strain Δgyp/pCPaGGRmut/pELDRR, Δgyp-crr/pCPaGGRmut/pELDRR, Δgyp-ptsHI/pCPaGGRmut/pELDRR cultured in R/5 medium containing 10 g/L of xylose.

During 30 h of cultivation, Δgyp-crr/pCPaGGRmut/pELDRR showed 1.2 g/L of glycerol accumulation and another strain Δgyp-ptsHI/pCPaGGRmut/pELDRR showed 1.35 g/L of glycerol

accumulation (Figure 3.3). Catabolite derepression between xylose and glycerol resulted in 30% decreased glycerol accumulation than that of the control strain. Although glycerol accumulation was alleviated, xylose uptake was also decreased. That resulted low 3-HP concentration also. Results are summarized in Table 3.1.

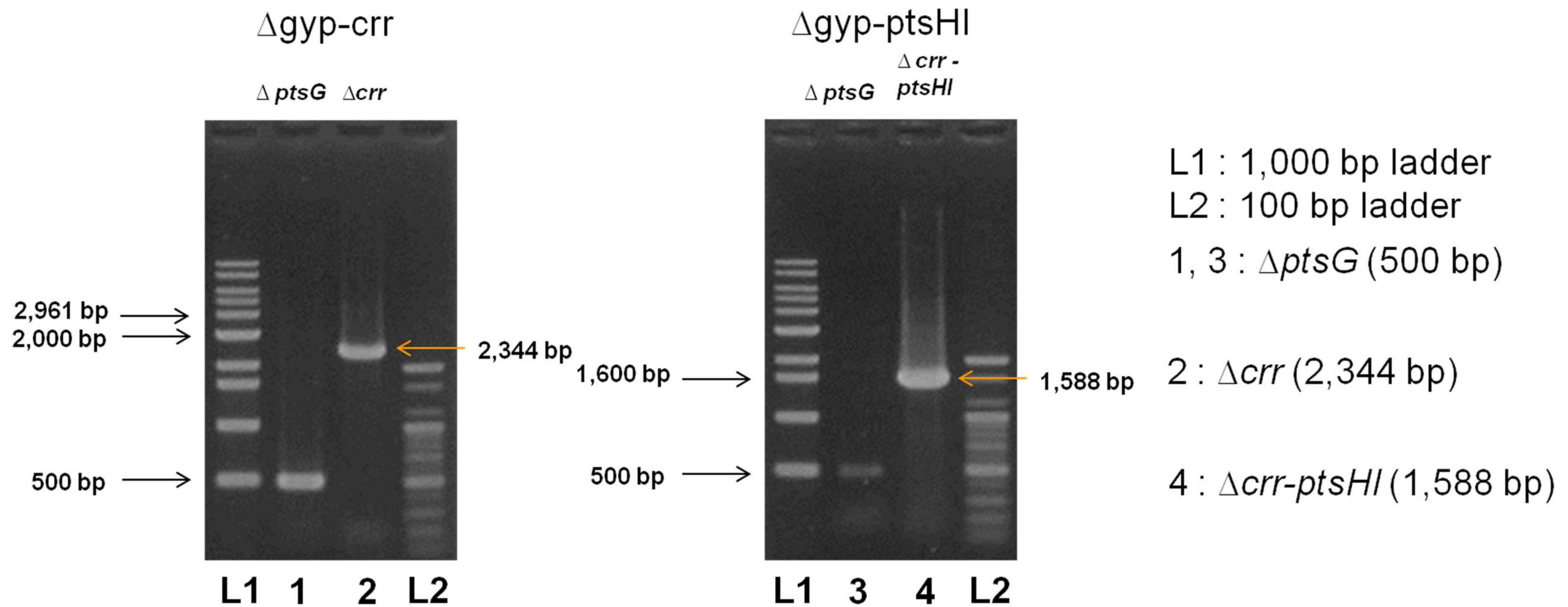


Figure 3.2. Confirmation of crr and ptsHI gene disruption

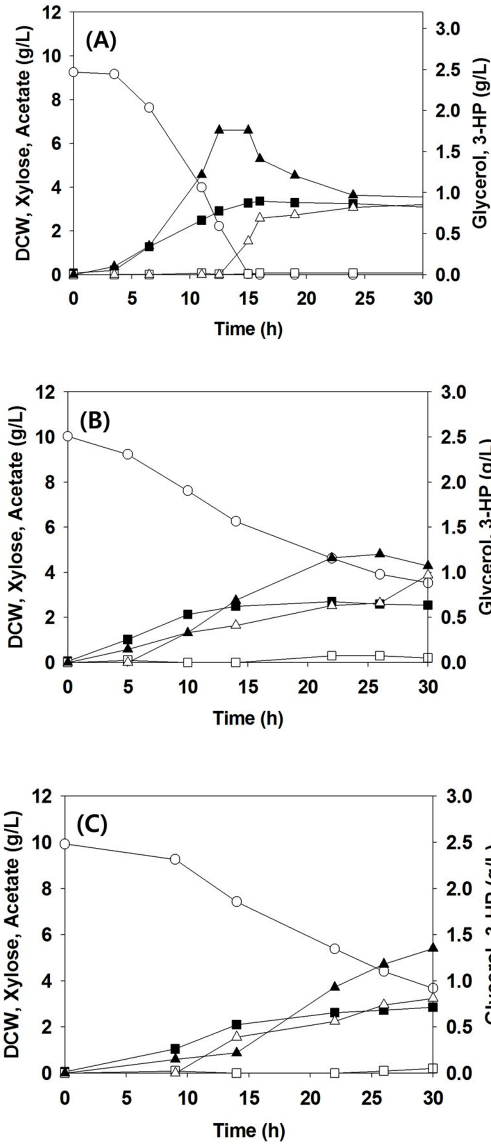


Figure 3.3. Results of flask culture PTS deletion strain in xylose medium (A) ∆gyp/pCPaGGRmut/pELDRR (B) ∆gyp-crr/pCPaGGRmut (C) ∆gyp-ptsHI/pCPaGGRmut/pELDRR

Symbols : ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol

Table 3.1. Results of PTS deletion strain in flask culture

Strains	DCW (g/L)	Glycerol accumulation (g/L)	3-HP concentration (g/L)	Xylose uptake rate (g/L·h)
Δgyp pELDRR/pCPaGGRmut	3.00	1.72	1.05	0.67
Δgyp-crr pELDRR/pCPaGGRmut	2.69	1.20	0.84	0.21
Δgyp-ptsHI pELDRR/pCPaGGRmut	2.85	1.35	0.81	0.20

3.3 Expression of *E. coli* transporters

Since previous approaches which deleted PTS involved genes resulted in decreased xylose uptake rates and final 3-HP concentration, second approaches were attempted that *E. coli* endogenous transporters were overexpressed. Transporter was known for proteins which uptakes metabolites into the *E. coli* through membrane (Lemieux *et al.* 2004).

In *E. coli* BL21star(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 protein than the translocation protein can be processed. Saturation of translocation protein 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.

3.3.1 Sequences of *galP*, *glpF* and *xylE*

galP gene sequence is as follow

ATTACACTGATGTGATTTGCTTCACATCTTTTTACGTCGTACTCACCTATCTTA

ATTCACAATAAAAAATAACCATATTGGAGGGCATC**ATGCCTGACGCTAAAAA**

ACAGGGGCGGTCAAACAAGGCAATGACGTTTTTCGTCTGCTTCCTTGCCG

CTCTGGCGGGATTACTCTTTGGCCTGGATATCGGTGTAATTGCTGGCGCAC

TGCCGTTTATTGCAGATGAATTCCAGATTACTTCGCACACGCAAGAATGGG

TCGTAAGCTCCATGATGTTCGGTGCGGCAGTCGGTGCGGTGGGCAGCGGC

TGGCTCTCCTTTAAACTCGGGCGCAAAAAGAGCCTGATGATCGGCGCAAT

TTTGTTTGTTGCCGGTTCGCTGTTCTCTGCGGCTGCGCCAAACGTTGAAGT

ACTGATTCTTTCCCGCGTTCTACTGGGGCTGGCGGTGGGTGTGGCCTCTTA

TACCGCACCGCTGTACCTCTCTGAAATTGCGCCGGAAAAAATTCGTGGCA

GTATGATCTCGATGTATCAGTTGATGATCACTATCGGGATCCTCGGTGCTTAT

CTTTCTGATACCGCCTTCAGCTACACCGGTGCATGGCGCTGGATGCTGGGT

GTGATTATCATCCCGGCAATTTTGCTGCTGATTGGTGTCTTCTTCCTGCCAG

ACAGCCCACGTTGGTTTGCCGCCAAACGCCGTTTTGTTGATGCCGAACGC

GTGCTGCTACGCCTGCGTGACACCAGCGCGGAAGCGAAACGCGAACTGG

ATGAAATCCGTGAAAGTTTGCAGGTTAAACAGAGTGGCTGGGCGCTGTTT

AAAGAGAACAGCAACTTCCGCCGCGCGGTGTTCCTTGGCGTACTGTTGCA

GGTAATGCAGCAATTCACCGGGATGAACGTCATCATGTATTACGCGCCGAA

AATCTTCGAACTGGCGGGTTATACCAACACTACCGAGCAAATGTGGGGGA

CCGTGATTGTCGGCCTGACCAACGTACTTGCCACCTTTATCGCAATCGGCCT

TGTTGACCGCTGGGGACGTAAACCAACGCTAACGCTGGGCTTCCTGGTGA

TGGCTGCTGGCATGGGCGTACTCGGTACAATGATGCATATCGGTATTCACTC

TCCGTCGGCGCAGTATTTCGCCATCGCCATGCTGCTGATGTTTATTGTCGGT

TTTGCCATGAGTGCCGGTCCGCTGATTTGGGTACTGTGCTCCGAAATTCAG

CCGCTGAAAGGCCGCGATTTTGGCATCACCTGCTCCACTGCCACCAACTG

GATTGCCAACATGATCGTTGGCGCAACGTTCCTGACCATGCTCAACACGCT

GGGTAACGCCAACACCTTCTGGGTGTATGCGGCTCTGAACGTACTGTTTAT

CCTGCTGACATTGTGGCTGGTACCGGAAACCAAACACGTTTCGCTGGAAC

ATATTGAACGTAATCTGATGAAAGGTCGTAAACTGCGCGAAATAGGCGCTC

ACGATCACCACCACCACCACCACTAATATTAGGAAATTAATA

glpF gene sequence is as follow

GGCACACACATTTTAAGTTCGATATTTCTCGTTTTTGCTCGTTAACGATAAGT

TTACAGCATGCCTACAAGCATCGTGGAGGTCCGTGACTTTCACGCATACAAC

AAACATTAACTCTTCAGGATCCGATT**ATGAGTCAAACATCAACCTTGAAAG**

GCCAGTGCATTGCTGAATTCCTCGGTACCGGGTTGTTGATTTTCTTCGGTG

TGGGTTGCGTTGCAGCACTAAAAGTCGCTGGTGCGTCTTTTGGTCAGTGG

GAAATCAGTGTCATTTGGGGACTGGGGGTGGCAATGGCCATCTACCTGAC

CGCAGGGGTTTCCGGCGCGCATCTTAATCCCGCTGTTACCATTGCATTGTG

GCTGTTTGCCTGTTTCGACAAGCGCAAAGTTATTCCTTTTATCGTTTCACAA

GTTGCCGGCGCTTTCTGTGCTGCGGCTTTAGTTTACGGGCTTTACTACAAT

TTATTTTTCGACTTCGAGCAGACTCATCACATTGTTCGCGGCAGCGTTGAA

AGTGTTGATCTGGCTGGCACTTTCTCTACTTACCCTAATCCTCATATCAATTT

TGTGCAGGCTTTCGCAGTTGAGATGGTGATTACCGCTATTCTGATGGGGCT

GATCCTGGCGTTAACGGACGATGGCAACGGTGTACCACGCGGCCCTTTGG

CTCCCTTGCTGATTGGTCTACTGATTGCGGTCATTGGCGCATCTATGGGCCC

ATTGACAGGTTTTGCCATGAACCCAGCGCGTGACTTCGGTCCGAAAGTCT

TTGCCTGGCTGGCGGGCTGGGGCAATGTCGCCTTTACCGGCGGCAGAGA

CATTCCTTACTTCCTGGTGCCGCTTTTCGGCCCTATCGTTGGCGCGATTGTA

GGTGCATTTGCCTACCGCAAACTGATTGGTCGCCATTTGCCTTGCGATATCT

GTGTTGTGGAAGAAAAGGAAACCACAACTCCTTCAGAACAAAAAGCTTC

GCTGTAA

xylE gene sequence is as follow

CAATTTTGGATAATTATCACAATTAAGATCACAGAAAAGACATTACGTAAAC

GCATTGTAAAAAATGATAATTGCCTTAACTGCCTGACAATTCCAACATCAATG

CACTGATAAAAGATCAGAATGGTCTAAGGCAGGTCTGA**ATGAATACCCAGT**

ATAATTCCAGTTATATATTTTCGATTACCTTAGTCGCTACATTAGGTGGTTTAT

TATTTGGCTACGACACCGCCGTTATTTCCGGTACTGTTGAGTCACTCAATAC

CGTCTTTGTTGCTCCACAAAACTTAAGTGAATCCGCTGCCAACTCCCTGTTA

GGGTTTTGCGTGGCCAGCGCTCTGATTGGTTGCATCATCGGCGGTGCCCTC

GGTGGTTATTGCAGTAACCGCTTCGGTCGTCGTGATTCACTTAAGATTGCT

GCTGTCCTGTTTTTTATTTCTGGTGTAGGTTCTGCCTGGCCAGAACTTGGTT

TTACCTCTATAAACCCGGACAACACTGTGCCTGTTTATCTGGCAGGTTATGT

CCCGGAATTTGTTATTTATCGCATTATTGGCGGTATTGGCGTTGGTTTAGCC

TCAATGCTCTCGCCAATGTATATTGCGGAACTGGCTCCAGCTCATATTCGCG

GGAAACTGGTCTCTTTTAACCAGTTTGCGATTATTTTCGGGCAACTTTTAG

TTTACTGCGTAAACTATTTTATTGCCCGTTCCGGTGATGCCAGCTGGCTGAA

TACTGACGGCTGGCGTTATATGTTTGCCTCGGAATGTATCCCTGCACTGCTG

TTCTTAATGCTGCTGTATACCGTGCCAGAAAGTCCTCGCTGGCTGATGTCGC

GCGGCAAGCAAGAACAGGCGGAAGGTATCCTGCGCAAAATTATGGGCAA

CACGCTTGCAACTCAGGCAGTACAGGAAATTAAACACTCCCTGGATCATG

GCCGCAAAACCGGTGGTCGTCTGCTGATGTTTGGCGTGGGCGTGATTGTA

ATCGGCGTAATGCTCTCCATCTTCCAGCAATTTGTCGGCATCAATGTGGTGC

TGTACTACGCGCCGGAAGTGTTCAAAACGCTGGGGGCCAGCACGGATATC

GCGCTGTTGCAGACCATTATTGTCGGAGTTATCAACCTCACCTTCACCGTTC

TGGCAATTATGACGGTGGATAAATTTGGTCGTAAGCCACTGCAAATTATCG

GCGCACTCGGAATGGCAATCGGTATGTTTAGCCTCGGTACCGCGTTTTACA

CTCAGGCACCGGGTATTGTGGCGCTACTGTCGATGCTGTTCTATGTTGCCG

CCTTTGCCATGTCCTGGGGTCCGGTATGCTGGGTACTGCTGTCGGAAATCT

TCCCGAATGCTATTCGTGGTAAAGCGCTGGCAATCGCGGTGGCGGCCCAG

TGGCTGGCGAACTACTTCGTCTCCTGGACCTTCCCGATGATGGACAAAAAC

TCCTGGCTGGTGGCCCATTTCCACAACGGTTTCTCCTACTGGATTTACGGTT

GTATGGGCGTTCTGGCAGCACTGTTTATGTGGAAATTTGTCCCGGAAACC

AAAGGTAAAACCCTTGAGGAGCTGGAAGCGCTCTGGGAACCGGAAACG

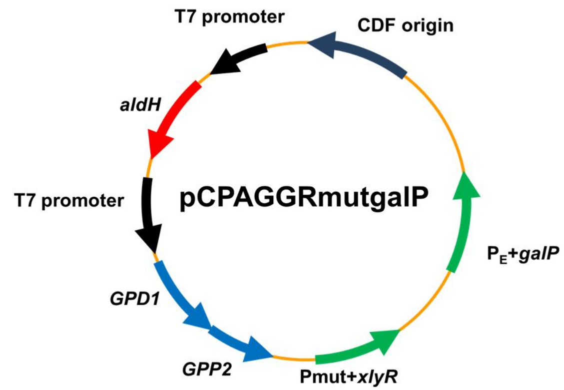
AAGAAAACACAACAAACTGCTACGCTGTAA

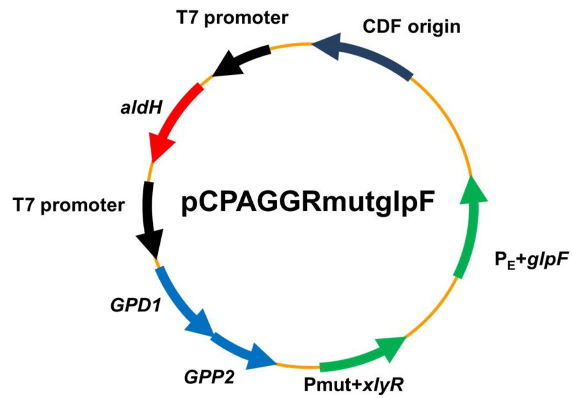
* The underlined sequences correspond to promoter region

* The bolded sequences correspond to ORF region

3.3.2 Construction of *E. coli* transporter expression strain

In previous study, plasmid pCPaGGRmut 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 expression of the *E. coli* transporter constitutively under the control of native *E. coli* promoter, three types of transporter, *galP*, *glpF* and *xylE*, were introducing in pCPaGGRmut plasmid (Figure 3.4).





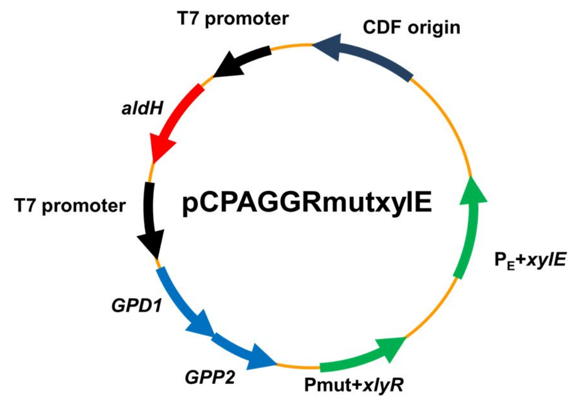


Figure 3.4. Genetic maps of pCPaGGRmutgalP, pCPaGGRmutglpF and pCPaGGRmutxylE

3.3.3 Flask culture of transporter overexpression strain

To investigate 3-HP production and glycerol accumulation, flask culutres of strain ∆gyp/pELDRR/pCPaGGRmutgalP, ∆gyp/pELDRR/ pCPaGGRmutglpF and ∆gyp/pELDRR/pCPaGGRmutxylE in R/5 medium containing 10 g/L xylose were carried out. During 30 h of cultivation, only ∆gyp/pELDRR/pCPaGGRmutgalP strain shown lower glycerol accumulation of 0.82 g/L, and that is lower by 55% than that of control strain. Also, it showed the same level of final concentration of 3-HP and xylose uptake rates (Figure 3.5). Results are summarized in Table 3.2.

3.3.4 Batch fermentation

In order to confirm these results, batch fermentation which contained higher concentration of carbon source more than 10 g/L of xylose was carried out.

During 12h of cultivation, control strain, ∆gyp/pELDRR/pCPaGGRmut, and ∆gyp/pELDRR/pCPaGGRmutgalP were consumed 20 g/L of xylose. Batch culture of ∆gyp/pELDRR/pCPaGGR resulted in 5.35 g/L DCW, 0.24 g/L 3-HP concentration and 2.48 g/L glycerol concentration, and batch culture of ∆gyp/pELDRR/pCPaGGRmutgalP resulted in 5.71 g/L DCW, 4.01 g/L 3-HP concentration and 0 g/L glycerol concentration (Figure 3.6, Table 3.3).

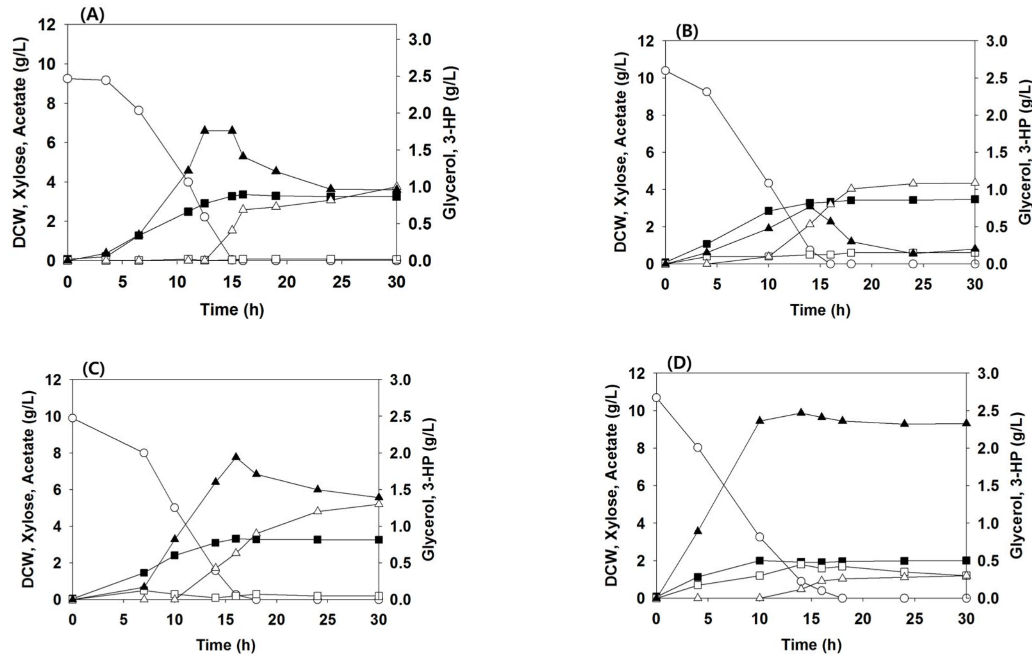


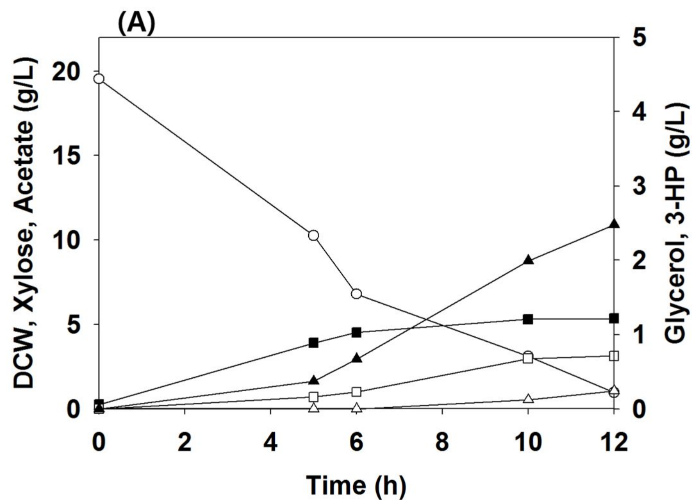
Figure 3.5. Results of flask culture transporter expression strain in xylose medium

(A) ∆gyp/pELDRR/pCPaGGRmut, (B) ∆gyp/pELDRR/pCPaGGRmutgalP, (C) ∆gyp/pELDRR/pCPaGGRmutglpF, (D) ∆gyp/pELDRR/pCPaGGRmut

Symbols : ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol

Table 3.2 Results of transporter expression strain in flask culture.

Strains	DCW (g/L)	Glycerol accumulation (g/L)	3-HP concentration (g/L)	Xylose uptake rate (g/L·h)
Δgyp pELDRR/pCPaGGRmut	3.00	1.72	1.05	0.67
Δgyp pELDRR/pCPaGGRmutgalP	3.47	0.82	1.12	0.67
Δgyp-crr pELDRR/pCPaGGRmutglpF	3.31	2.16	1.15	0.56
Δgyp-ptsHI pELDRR/pCPaGGRmutxylE	2.01	2.73	0.34	0.56



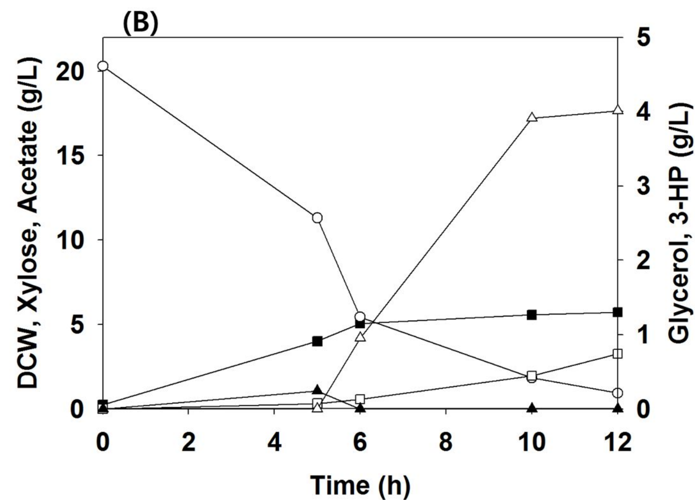


Figure 3.6. Results of batch culture in xylose medium

(A) ∆gyp/pELDRR/pCPaGGRmut, (B) ∆gyp/pELDRR/pCPaGGRmutgalP

Symbols : ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol

Table 3.3 Results of batch culture in fermentor

Strains	DCW (g/L)	Glycerol accumulation (g/L)	3-HP concentration (g/L)
Δgyp pELDRR/pCPaGGRmut	5.35	2.48	0.24
Δgyp pELDRR/pCPaGGRmutgalP	5.71	0	4.01

3.4 Confirmation of *galP* expression

3.4.1 SDS-PAGE & Western blot

In the result of batch culture of ∆gyp/pELDRR/pCPaGGRmutgalP, difference of concentration level of 3-HP and glycerol had been shown, significantly. It is assumed that expression of *galP* gene changed the results. For the confirmation of expression of the *galP* gene, plasmid pELDRR and pCPaGGRmutgalP were transformed into competent ∆gyp. Although it was confirmed that the proteins, DHAB1, DHAB2, DHAB3, DHAR1, DHAR2, ALDH, GPD1 and GPP2, were expressed solubly in ∆gyp by IPTG induction (Final concentration 0.1 mM), *galP* which introduced in the plasmid was not shown in SDS-PAGE gel (FIgure 3.7).

For confirmation of *galP* gene expression, Western blotting was carried out. Because antigen is necessary for western blotting, his tag which consists of six repeats of histidine amino acid was introduced at 3` of *galP* gene (Figure 3.8).

Western blotting analysis showed *galP* gene (51kDa) band (FIgure 3.9).

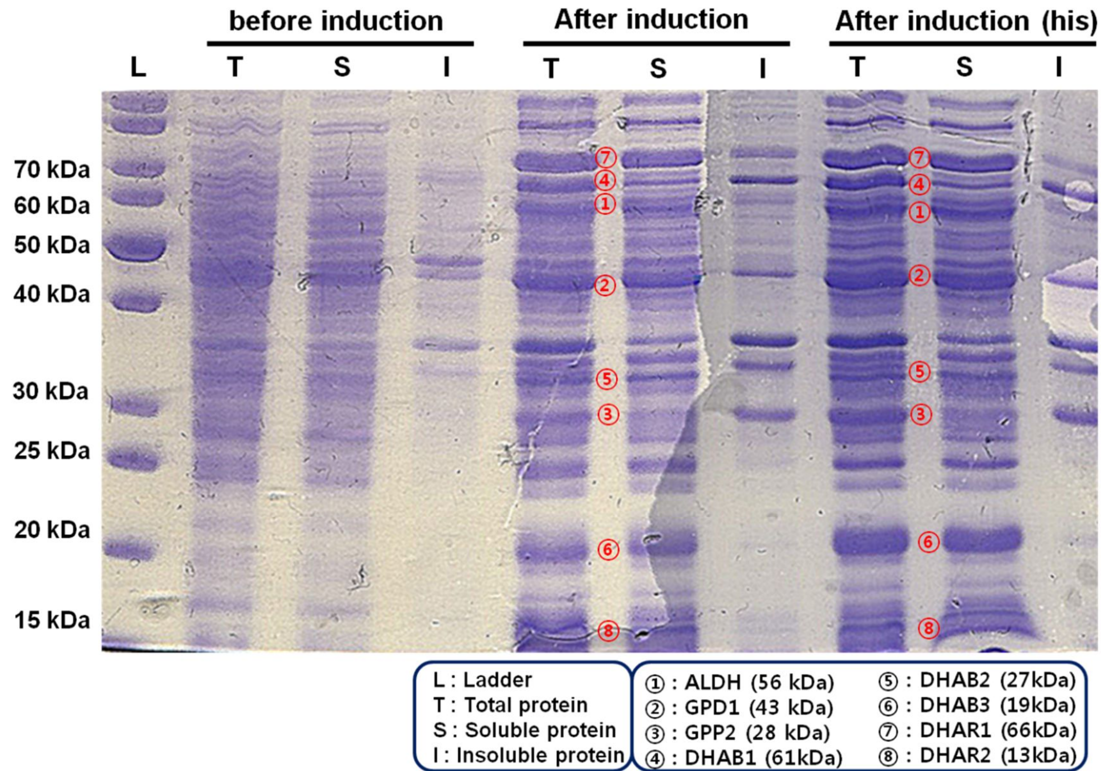


Figure 3.7. SDS-PAGE analysis of pCPaGGRmut and pELDRR plamid expression

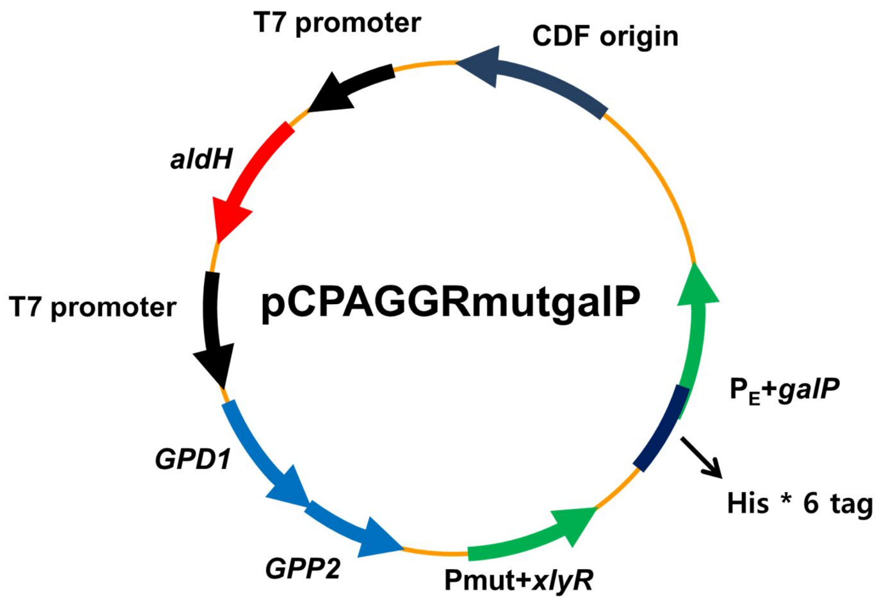


Figure 3.8. Genetic map of pCPaGGRmutgalP introduced his tag

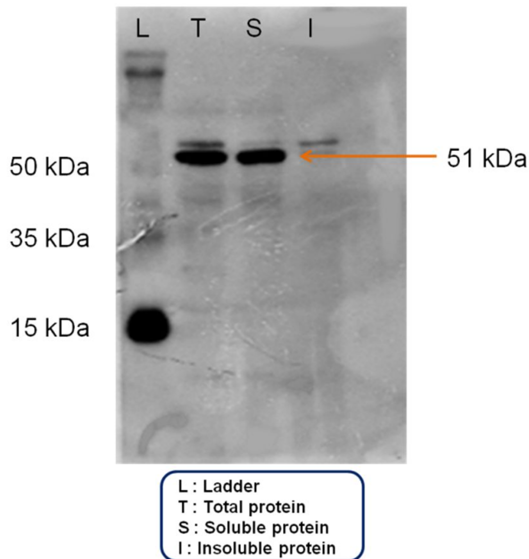


Figure 3.9. Western blot analysis of *galP* expression

3.4.2 Effect of *galP* expression on glycerol metabolism

Since Δgyp/pELDRR/pCPaGGRmutgalP exhibited low accumulation of glycerol, experiments to identify *galP* effects on glycerol metabolism were carried out.

To identify *galP* expression on glycerol metabolism, Δgyp/pCPaGGRmut and Δgyp/pCPaGGRmutgalP which was not contained pELDRR was constructed. As pELDRR was not carried in this strains, conversion glycerol to 3-HPA was not happened.

The strain Δgyp/pCPaGGRmut and Δgyp/pCPaGGRmutgalP were cultured in R/5 medium containing 10 g/L xylose. Although Δgyp/pCPaGGRmut produced 4.94 g/L of glycerol and 2.28 g/L of DCW, Δgyp/pCPaGGRmutgalP produced 2.43 g/L of glycerol and 3.13 g/L of DCW. As a result of *galP* constitutive expression, carbon flux moved from glycerol to central metabolism than that of the control strain (Figure 3.10). This phenomenon explained in Zhang's article. In this article, *galP* expression enhanced TCA cycle and resultingly, pyruvate concentration was higher than that of control strain (Zhang *et al.* 2009).

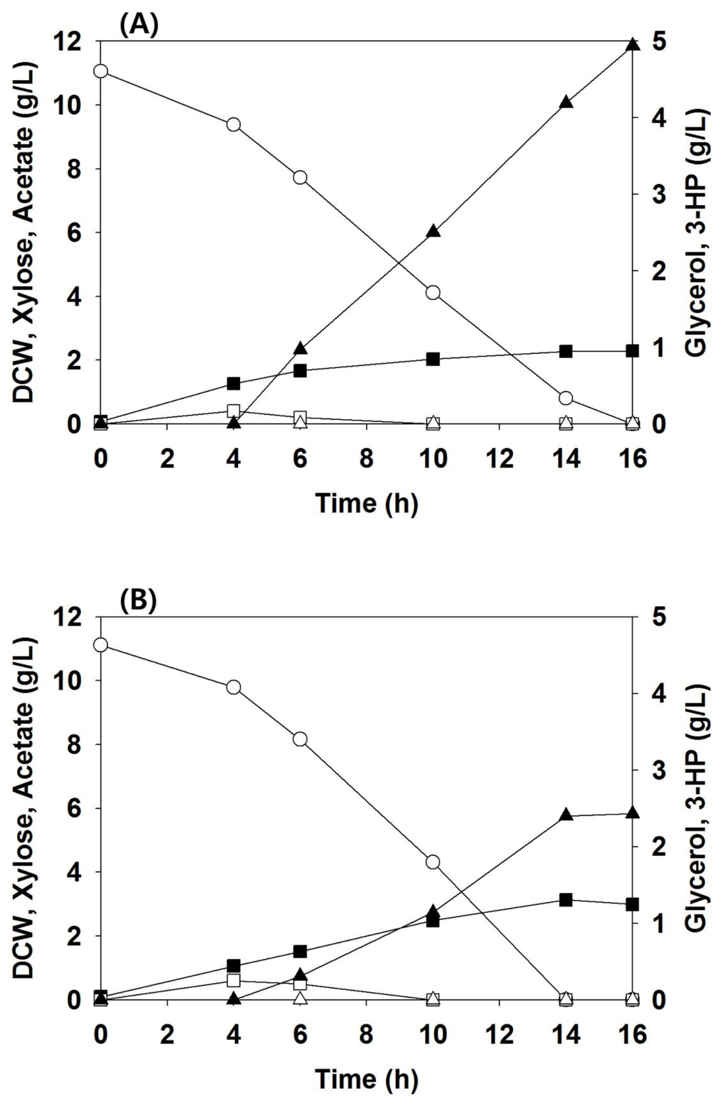


Figure 3.10. Results of flask culture (A) ∆gyp/pCPaGGRmut and (B) ∆gyp/pCPaGGRmutgalp in R/5 medium containing 10 g/L xylose.

Symbols : ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol

3.5 Production of 3-HP from xylose in *galP* expression strain

3.5.1 Fed-batch fermentation using xylose feeding strategy

In flask culture and batch culture, ∆gyp/pELDRR/pCPaGGRmutgalP showed lower glycerol accumulation and higher 3-HP concentration than that of control strain, ∆gyp/pELDRR/pCPaGGRmut.

To achieve high concentration of 3-HP, fed-batch fermentations were carried out. To keep the cell growth and to maximize 3-HP production, xylose continuous condition was carried out. After 94 h of cultivation, 39.1 g/L of 3-HP was produced, and productivity of 3-HP was 0.40 g/L·h respectively. The productivity of 3-HP in fed-batch was increased by 20% than that of batch result.

In this study, reduced of accumulation of glycerol which inhibit glycerol dehydratase gene was drived through *galP* expression constitutively. The results suggest that *galP* gene overexpression would apply to other chemicals which require to use glycerol dehydratase gene.

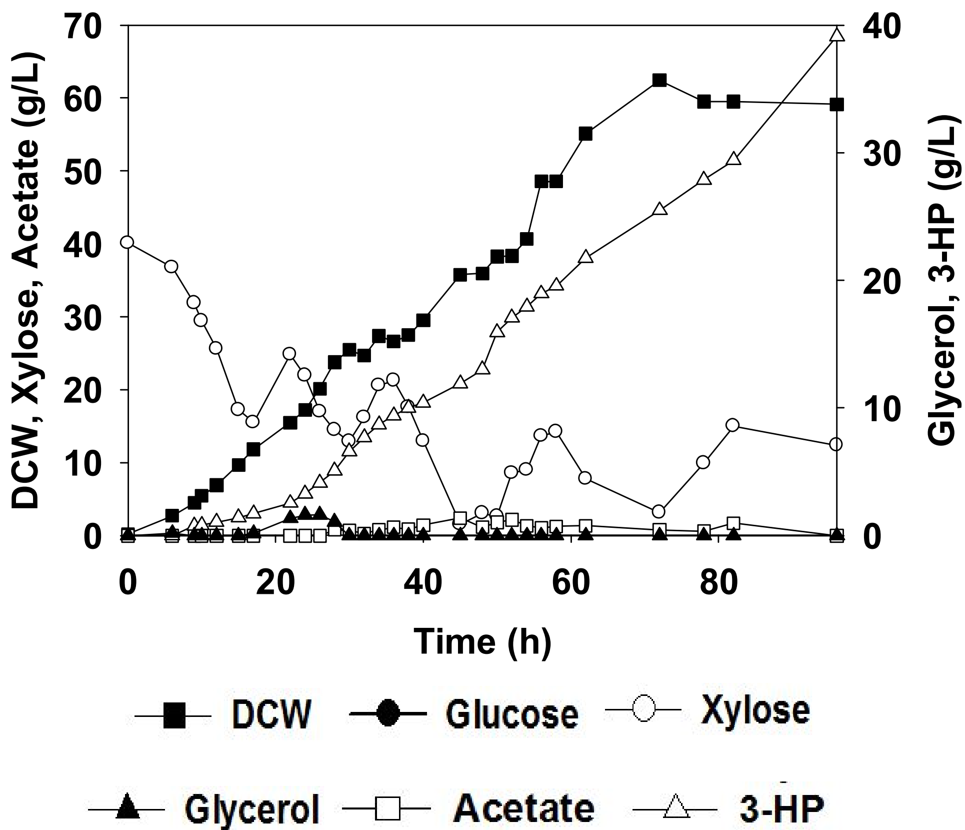


Figure 3.11. Profile of fed-batch fermentation of Δgyp/pELDRR/pCPaGGRmutgalP

Table 3.4. Results of fed-batch fermentation of Δgyp/pELRDRR/ pCPaGGRmutgalP

Strain	DCW (g/L)	3-HP (g/L)	3-HP productivity (g/L·h)	Glycerol (g/L)
Δgyp / pELDRR pCPaGGRmutgalP	59.1	39.1	0.40	0

Chapter 4. Conclusions

This thesis can draw the following conclusions :

(1) Although deletion of the *crr* and *ptsHI* genes encoding the PTS-system decreased glycerol accumulation by 30% relative to the control strain, this strategy also decreased xylose uptake rate by 70%.

(2) Constitutive expression of the *galP* gene under the control of the native *E. coli* promoter decreased glycerol accumulation by 55% and increased cell mass by 37% relative to the control strain by redirecting carbon flux from glycerol to cell growth.

(3) In xylose limited fed-batch fermentation using engineered *E. coli* overexpressing the *galP* gene, 3-HP titer of 39.1 g/L and 3-HP productivity of 0.40 g/L·h were obtained without glycerol accumulation.

Chapter 5. References

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국 문 초 록

석유 분포의 불균등성과 의존성으로 인해 석유를 기반으로 하는 화학 산업에서 바이오매스를 이용하는 화학 산업이라는 새로운 패러다임이 제시되고 있다. 바이오 화학 산업의 적용을 위한 생산 목적 물질로는 미국 에너지성에서 선정한 바이오매스로부터 생산할 가치가 있는 바이오 화학 소재 중 다양한 화학 물질의 전구체로 사용되는 3-히드록시프로피온 산을 선정하였다. 하지만 2 세대 바이오매스로부터 추출되는 목당을 이용하여 3-히드록시프로피온산을 생산함에 있어서 발효 도중에 글리세롤이 축적 되는 문제를 보였고, 이는 생산 경로 상 중요한 유전자인 글리세롤 탈수효소의 역가를 저해시킨다는 연구가 알려져 있다. 이러한 문제점을 해결하기 위해서 글리세롤의 축적을 줄일 필요가 있었고, 그에 대한 접근 방식으로 글리세롤의 유입을 저해함을 매개하는 *crr* 과 *ptsHI* 유전자의 파쇄하는 방법과 운반 단백질을 발현하는 방법을 선택하였다. *crr* 유전자와 *ptsHI* 유전자의 파쇄는 글리세롤의 축적을 약 30% 줄일 수 있었지만 자일로스의 대사

속도를 약 70% 낮추는 단점을 보였고, 그에 따라 3-히드록시프로피온산의 생산성이 낮아지는 단점을 보였다. 그 다음 접근 방식을 적용하기 위해서 선정한 운반 단백질들의 발현을 위한 벡터를 구축하였고, 플라스크 배양 결과 구축한 벡터 중 pCPaGGRmutgalP 만이 3-히드록시프로피온산의 생산 저해 없이 글리세롤이 약 55%정도 줄어드는 결과를 확인할 수 있었다. 이러한 결과를 확인하기 위해서 20 g/L 의 자일로스가 포함된 R/5 배지에서 발효를 진행하였고, 그 결과 글리세롤이 쌓이지 않으면서, 4.01 g/L 의 3-HP 의 생산을 확인할 수 있었다. 도입해준 *galP* 의 발현은 웨스턴블롯팅을 통해 확인하였고, *galP* 의 발현은 탄소 흐름을 글리세롤에서 DCW 로 변화시키는 것으로 예상된다. 이렇게 선정된 Δgyp/pELDRR/pCPaGGRmut 를 이용하여 고 농도의 3-히드록시 프로피온산을 생산하기 위해 목당을 꾸준히 주입해주는 유가식 배양을 진행하였고, 그 결과 글리세롤이 쌓이지 않으면서 약 39.1 g/L 의 3-히드록시프로피온산을 생산할 수 있었다. 이러한

접근 방식은 글리세롤 탈수효소를 이용하는 대사공학에 널리 쓰일 수 있을 것이라고 예상할 수 있다.

주요어 : 대사공학, 3-히드록시프로피온산, 대장균, 목당,

글리세롤 축적, *galP*, 유가식 배양

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