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

당문반체 조절을 통한 3-히드록시프로피온산의 생산성 향상

지도교수 서 진호

이 논문을 공학석사학위논문으로 제출함

2015년 8월

서울대학교 대학원

협동과정 바이오엔지니어링 전공

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김 준희의 석사학위논문을 인준함

2015년 8월

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Abstract

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Since xylose is the second abundant 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 ptsHl 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

Student Number: 2013-23190
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Chapter 1. Introduction

1.1 3-Hydroxypropionic acid

3-Hydroxypropionic acid (3-HP, C$_3$H$_6$O$_3$—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$_3$H$_6$O$_2$—MW 76.09), acrylic acid (C$_3$H$_4$O$_2$—MW 72.06), methyl acrylate (C$_4$H$_6$O$_2$—MW 86.09), acrylamide (C$_3$H$_5$NO—MW 71.08), ethyl 3-HP (C$_5$H$_{10}$O$_3$—MW 118.13), malonic acid (C$_3$H$_4$O$_4$—MW 104.06) and acrylonitrile (C$_3$H$_3$N—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 \textit{Escherichia coli}, Saccharomyces cerevisiae and human were introduced into recombinant \textit{E. coli}. In our previous report, new aldehyde dehydrogenase from Pseudomonas aeruginosa were identified and glycerol kinase(\textit{glpK}) and propanediol oxidoreductase (\textit{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 \textit{E. coli} expressing glycerol dehydratase from \textit{K. pneumonia} and aldehyde dehydrogenase from \textit{Cupriavidus necator} produced 3-HP at a maximum level of 71.9 g/L (Chu et al. 2015). In another recent study, \textit{E. coli} W strain was reported to be significantly better for 3-HP production due to its higher tolerance to 3-HP compared with \textit{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


<table>
<thead>
<tr>
<th>Compound</th>
<th>Application</th>
<th>Market size (M lb)</th>
<th>Price ($/lb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylic acid</td>
<td>Acrylates (coatings, adhesives) comonomer, superabsorbent polymers detergent polymers</td>
<td>2,000</td>
<td>0.48</td>
</tr>
<tr>
<td>Acrylonitrile</td>
<td>Acrylic fibers (carpets, clothing) acrylonitrile-butadiene-styrene and styrene-acrylonitrile (pipes and fittings, automobiles) nitrile rubber copolymers, adiponitrile, acrylamide</td>
<td>3,130</td>
<td>0.31 ~0.37</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Polyacrylamide comonomer (styrene-butadiene latex, acrylic resins, many others)</td>
<td>206</td>
<td>1.76 ~1.86</td>
</tr>
<tr>
<td>1,3-Propanediol</td>
<td>Polyethylene terephthalate, polybutylene terephthalate, nylon applications</td>
<td>small</td>
<td>0.30 ~0.50</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>Blowing agent (formed plastic), silver plating brightening agent, tanning auxiliary</td>
<td>&lt;1</td>
<td>high</td>
</tr>
</tbody>
</table>
Table 1.2 Research of 3-HP production

<table>
<thead>
<tr>
<th>Host</th>
<th>Amplified and deleted genes</th>
<th>3-HP titer (g/L)</th>
<th>Yield (g/g)</th>
<th>Productivity (g/L·H)</th>
<th>Cultivation (carbon source)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>ΔdhaT, yqhD, dhaB, puuC</td>
<td>28.0</td>
<td>0.39</td>
<td>0.58</td>
<td>Fed-batch (glycerol)</td>
<td>(Ashok et al. 2013)</td>
</tr>
<tr>
<td>DSMZ 2026</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>ΔdhaT, yqhD, aldH, prpE, phaC</td>
<td>2.03</td>
<td>-</td>
<td>0.04</td>
<td>Batch (glycerol)</td>
<td>(Xinjun et al. 2015)</td>
</tr>
<tr>
<td>ΔdhaT, yqhD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>mcr, msr, pntAB, acc</td>
<td>0.20</td>
<td>0.03</td>
<td>0.01</td>
<td>Batch (Glucose)</td>
<td>(Rathnasingh et al. 2012)</td>
</tr>
<tr>
<td>BL21_mcr_acc_pntAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ΔfabD, mcr, acc</td>
<td>49.0</td>
<td>0.23</td>
<td>0.71</td>
<td>Fed-batch (Glucose)</td>
<td>(Lynch et al. 2014)</td>
</tr>
<tr>
<td>BX3_0240</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>gabD4</td>
<td>71.9</td>
<td>-</td>
<td>1.80</td>
<td>Fed-batch (glycerol)</td>
<td>(Chu et al. 2015)</td>
</tr>
<tr>
<td>SH501</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>dhaB123, gdrAB, KGSADH</td>
<td>41.5</td>
<td>0.31</td>
<td>0.86</td>
<td>Fed-batch (glycerol)</td>
<td>(Sankaranarayanan et al. 2014)</td>
</tr>
<tr>
<td>W3110</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ΔglpKΔyqhD, aldH, dhaBR</td>
<td>57.3</td>
<td>0.93</td>
<td>1.43</td>
<td>Fed-batch (glycerol)</td>
<td>(Kim et al. 2014)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔglpKΔglpk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔglpKΔgldA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Δglpk, gldA, aldH, dhaBR</td>
<td>22.2</td>
<td>0.21</td>
<td>0.10</td>
<td>Fed-batch (xylose)</td>
<td>Lee thesis, (2012)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔglpKΔgldA</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.2 Hemicellulose and xylose

Hemicellulose, the second major constituent of lignocellulose (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)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Content in lignocellulose</th>
<th>Major monomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>33-51%</td>
<td>Glucose</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>19-34%</td>
<td>Glucose, mannose, galactose, xylose and arabinose</td>
</tr>
<tr>
<td>Lignin</td>
<td>20-30%</td>
<td>Aromatic alcohols</td>
</tr>
<tr>
<td>Pectin</td>
<td>1-20%</td>
<td>Galacturonic acid and rhamnose</td>
</tr>
</tbody>
</table>

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-phosphatase (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\textsuperscript{Glc} (sometimes also called EIIA\textsuperscript{Crr}, gene *crr*, part of the *ptsHlcrr* operon), and finally from EIIA\textsuperscript{Glc}∼P to the glucose-specific membrane protein EIICB\textsuperscript{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 harboring *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 harboring 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

<table>
<thead>
<tr>
<th>Strains / Plasmids</th>
<th>Main characteristics</th>
<th>Reference / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ^-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>F- ompT gal dcm lon hsdS6 (rB^- mB^-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Δgyp</td>
<td>BL21(DE3) ΔglpKΔyqhDΔptsG</td>
<td>Joung thesis (2013)</td>
</tr>
<tr>
<td>Δgyp-crr</td>
<td>BL21(DE3) ΔglpKΔyqhDΔptsGΔcrr</td>
<td>In this study</td>
</tr>
<tr>
<td>Δgyp-ptsHI</td>
<td>BL21(DE3) ΔglpKΔyqhDΔptsGΔptsHI</td>
<td>In this study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPaGGRmut</td>
<td>Sm^R, pCDFduet-1 based plasmid containing <em>aldH</em>, GPD1, GPP2 and <em>xylR</em> fragment with natural P_r promoter under the T_7 promoter</td>
<td>Joung thesis (2013)</td>
</tr>
<tr>
<td>pCPaGGRmutGlpF</td>
<td>Sm^R, <em>glpF</em> gene with natural promoter cloned in pCPaGGRmut</td>
<td>In this study</td>
</tr>
<tr>
<td>pCPaGGRmutGalP</td>
<td>Sm^R, <em>galP</em> gene with natural promoter cloned in pCPaGGRmut</td>
<td>In this study</td>
</tr>
<tr>
<td>pCPaGGRmutxylE</td>
<td>Sm^R, <em>xylE</em> gene with natural promoter cloned in pCPaGGRmut</td>
<td>In this study</td>
</tr>
<tr>
<td>pELDRR</td>
<td>KanR, pET-29b(+) based plasmid containing <em>L. brevis dhaB, dhaR</em> cluster under the T_7 promoter</td>
<td>Kwak <em>et al.</em> (2012)</td>
</tr>
</tbody>
</table>
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* chromosomal 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, ptsH1* 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>
<thead>
<tr>
<th><strong>Name</strong></th>
<th><strong>Nucleotide sequence (5′ to 3′)</strong></th>
<th><strong>Target gene</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F-crr</strong></td>
<td><code>TCCACGAGATGCAGGCGGCGGCTGCTTATTTTAGGAGAGAGATC</code> <code>GTGTAGGTGGCTGAAGCTTGCTTCC</code></td>
<td>crr</td>
</tr>
<tr>
<td><strong>F-ptsHI</strong></td>
<td><code>TTAGTTCCACAAACAACATACAAACTAAGTTGGGAAATACAC</code> <code>GTGTAGGTGGCTGAAGCTTGCTTCC</code></td>
<td>ptsHI</td>
</tr>
<tr>
<td><strong>R-crr/ptsHI</strong></td>
<td><code>AAATGGCGGCGATGCGGCGGCGCGCCATTTTTTCATCGGCGCAAGAAA</code> <code>ATTCCGGGATCGCGTGCACC</code></td>
<td>crr / ptsHI</td>
</tr>
<tr>
<td><strong>F-chk</strong></td>
<td><code>ACCGACGAGTTAATGACGCTTGG</code></td>
<td></td>
</tr>
<tr>
<td><strong>R-chk</strong></td>
<td><code>TTGCTGAAAGGGAAGCCGTAAAAC</code></td>
<td></td>
</tr>
<tr>
<td><strong>F-galP</strong></td>
<td><code>TATGCAGCTCCTGCAATTACACTGATGATTTGGCTTCATCATC</code></td>
<td>galP</td>
</tr>
<tr>
<td><strong>R-galP</strong></td>
<td><code>TATTAATTTCCCTAAATATATCTGATGCGGCTATTTCCGG</code></td>
<td></td>
</tr>
<tr>
<td><strong>R-galP-his</strong></td>
<td><code>TATTAATTTCCCTAAATATATCTGATGCGGCTATTTCCGG</code></td>
<td></td>
</tr>
<tr>
<td><strong>F-glpF</strong></td>
<td><code>TATGCAGCTCCTGCGAGGAGCACCACACTTTTAAATTTTCGATATTTCAC</code></td>
<td>glpF</td>
</tr>
<tr>
<td><strong>R-glpF</strong></td>
<td><code>TATTAATTTCCCTAAATATATCTGATGCGGCTATTTCCGG</code></td>
<td></td>
</tr>
<tr>
<td><strong>F-xyLE</strong></td>
<td><code>TATGCAGCTCCTGCAATTATTTGGAATTATACAAATGATGATCACAG</code></td>
<td>xyle</td>
</tr>
<tr>
<td><strong>R-xyLE</strong></td>
<td><code>TATTAATTTCCCTAAATATATCTGATGCGGCTATTTTCGATTTTC</code></td>
<td></td>
</tr>
</tbody>
</table>
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 \(\lambda\) red recombinase in host strain

Plasmid pKD46 introduced to each expression strain as using \(\text{CaCl}_2\) 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 purified 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°C, 30 cycles of PCR amplification were performed as follows: 10 sec at 98°C, 5 sec at 55°C and 2 min at 72°C, followed by 7 min at 72°C 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$_2$PO$_4$, 4 g (NH$_4$)$_2$HPO$_4$ and a trace element solution 10 mL (contains per liter of 5 M HCl : 10 g FeSO$_4$·7H$_2$O, 2.25 g ZnSO$_4$·7H$_2$O, 1 g CuSO$_4$·5H$_2$O, 0.5 g MnSO$_4$·4H$_2$O, 2 g CaCl$_2$, 0.1 g (NH$_4$)$_6$Mo$_7$O$_{24}$, 0.23 g Na$_2$B$_4$O$_7$·10H$_2$O). 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°C 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°C 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.\textsubscript{600} reached to 3. And at the same time, temperature changed to 25°C 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°C 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°C, 1200 rpm. Aeration rate and agitation speed were in 1,200 rpm and 2 vvm of air supply. When the cell O.D.\textsubscript{600} reached to 10, IPTG and coenzyme B\textsubscript{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.\textsubscript{600} reached to 8, IPTG and coenzyme B\textsubscript{12} were added and temperature shifted to 25°C without changing aeration rate and agitation speed. Feeding solutions were converted to organic acids by the metabolic processes of cells. The feeding solution was composed of 500 g/L xylose and 14 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O. 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:

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

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$_2$SO$_4$ 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**

<table>
<thead>
<tr>
<th></th>
<th>Separating gel buffer (pH 8.8)</th>
<th>Stacking gel buffer (pH 6.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl</td>
<td>1.5 M</td>
<td>Tris-Cl</td>
</tr>
<tr>
<td>SDS</td>
<td>0.40%</td>
<td>SDS</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4000 μL</td>
<td>Acrylamide</td>
</tr>
<tr>
<td>4X Separating buffer</td>
<td>3100 μL</td>
<td>4X Separating buffer</td>
</tr>
<tr>
<td>Deionized water</td>
<td>2800 μL</td>
<td>Deionized water</td>
</tr>
<tr>
<td>10% SDS</td>
<td>120 μL</td>
<td>10% SDS</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td>TEMED</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>100 μL</td>
<td>Ammonium persulfate</td>
</tr>
</tbody>
</table>

**Table 2.4 Composition of buffers used in western blot**

<table>
<thead>
<tr>
<th></th>
<th>10X transfer buffer</th>
<th>Washing buffer (TBST)</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>30.3 g/L</td>
<td>Tris</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>144 g/L</td>
<td>6 g/L</td>
<td></td>
</tr>
<tr>
<td><strong>Dilute to 1X</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>700 mL</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>200 mL</td>
<td>29.22 g/L</td>
<td></td>
</tr>
<tr>
<td>10X Transbuffer</td>
<td>100 mL</td>
<td>0.1 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween 20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 ml/L</td>
<td>5% skim milk in TBST</td>
</tr>
</tbody>
</table>
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.\textsubscript{600} of 3, IPTG and coenzyme B\textsubscript{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\textperiodcentered 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 \textit{et al.} 2003). It is a result of that glycerol deactivates glycerol dehydratase by the homolysis of the Co-C bond of coenzyme b\textsubscript{12} 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 follows.

`ATGGGGTTTGTCCGATAAACTGAAATCTCTTGTTTCCGACGACAAGGAT
ACCGGAACAGATCTTGCTCCGCTCTCTGGCGAGATCGTCAATATCG
AAGACGTGCGGATGTCGTTTTTGCGGAAAAATCGTTGGTGATGGTATTG
CTATCAAACCAACCGGTAACAAAAATGGTCGCGCCAGTAGACGGACCCATTG
GTAAAATCTTTGAAAAACCACCAGCATTTCTCTATCGAATCTGATAGCGGCGT
TGAACTGTTCTCCACTTCGATGATCGACACCCTGTAACGAGGAGCCGAGG
CTTCAAGCGGTATGGCTGAAGAAGGTCGCGCCGTAAGATGGTGGAGACTGT
CATTGAATGGGTATCTCCGTGCAGTGGAAGAAAGCAAGTCTACCGTACGC
TCGGGTGTATCTCCGATATGGAGAATCAAGAATGTGCTAAGACCTGTC
GGTAGCGTAACCGTGTCGAAACCCCGG
TTATCCGCATCAAGAAGTAA`

ptsHI genes sequence is as follow:

`ATGGGGTTTGTCCGATAAACTGAAATCTCTTGTTTCCGACGACAAGGAT
ACCGGAACAGATCTTGCTCCGCTCTCTGGCGAGATCGTCAATATCG
AAGACGTGCGGATGTCGTTTTTGCGGAAAAATCGTTGGTGATGGTATTG
CTATCAAACCAACCGGTAACAAAAATGGTCGCGCCAGTAGACGGACCCATTG
GTAAAATCTTTGAAAAACCACCAGCATTTCTCTATCGAATCTGATAGCGGCGT
TGAACTGTTCTCCACTTCGATGATCGACACCCTGTAACGAGGAGCCGAGG
CTTCAAGCGGTATGGCTGAAGAAGGTCGCGCCGTAAGATGGTGGAGACTGT
CATTGAATGGGTATCTCCGTGCAGTGGAAGAAAGCAAGTCTACCGTACGC
TCGGGTGTATCTCCGATATGGAGAATCAAGAATGTGCTAAGACCTGTC
GGTAGCGTAACCGTGTCGAAACCCCGG
TTATCCGCATCAAGAAGTAA`
CATTTGGTACGGTTCTGGAACGTTGAAGGAGGCAGACGTAACGGGCGCTGAAG
GCGTTGTCTGTATCGTACTGAGTTTAGCTGTGGGCTGACGTGAGCTGTTGC
CACTGAAAGAACAGTTTCTGCTTAACAAAGCACGTGGGTAGGCTGAGCG
TGCTCGAACGGCCAAGGGAAAAGAGGAAACCCCTTTCTGGGCTGGCGAG
CTATCCTGCTACGAGATCCTGCGAGACGCAGCTCAGCTGCCGAC
TATCCTGCCTCGGCTTTCCGGTAATTTGCGCATTATGTTCCTCCGATGATC
TCTCTGGGAAGAAGTGCGCGACTGCGAAAGAGATCGAATCTAACAAAC
AGGAACTGCGACGACAGGAAGGTAAAGCGTTTGACGAGTCAATTGAAATCGGC
GTAATGGTGAAAACACCGGCTGCCAACAATTGACGTCATTAGCCAAA
GAAAGTTTAGTTCTTAGTATCGGCACCCAAATGATTAACGCACTACACTGG
CAGTTGACCCTGTTGAAATGATATGATTTTACCACACCTTACCATGCGATGGGC
GTCCCGTGCTGAACTTGATCAAGCAAGTTATTGATGCTTCTCATGCTGAAGGC
AAATGGACTGCAATGGGTGAGCTTGCGAGCTGACGCTGACACTTT
CTTTGGCTGGGGATGGGTCTGGACGAATTCTCTATGAGCGCCATTTCTATCC
CGGCATTAAAGAAGATTATCCGTAACACGAACTTCGGAAGATCGGAAGGTT
TAGCAGAGCGGGTCTTTGCTCAACCGAACAACGGAGCAGTTAAATGACGCTG
GTAAACAAAGTTCTATTGAAAGAAACAAATCTGCTAAATCCAGAAGATCGG
CCAATTATGCTGGGTAGGCTGATCCCGTTGTCTCGATAAAAATGAAATCT
CTGGTTTCCGACGAAAAGGATACCCGGAACTATTGAGATCATGCTTGCTCCG
CTCTCTGGCGAGATCGTCAATATCGAAGACGTGCCGGATGTCGTTTTTGCG
GAAAAATCGTTGGTGATGGTATTGCTTACAAACCAACGGGTAACAAAATG
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

- Δgyp-crr
- ΔptsG  Δcrr

- Δgyp-ptsHI
- Δcrr  ΔptsG  ptsHI

L1: 1,000 bp ladder
L2: 100 bp ladder
1, 3: ΔptsG (500 bp)
2: Δcrr (2,344 bp)
4: Δcrr-ptsHI (1,588 bp)
Figure 3.3. Results of flask culture PTS deletion strain in xylose medium (A) \(\Delta gyp/pCPaGGRmut/pELDRR\) (B) \(\Delta gyp-crr/pCPaGGRmut\) (C) \(\Delta gyp-ptsHI/pCPaGGRmut/pELDRR\)

Symbols: ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol
<table>
<thead>
<tr>
<th>Strains</th>
<th>DCW (g/L)</th>
<th>Glycerol accumulation (g/L)</th>
<th>3-HP concentration (g/L)</th>
<th>Xylose uptake rate (g/L·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆gyp pELDRR/pCPaGGRmut</td>
<td>3.00</td>
<td>1.72</td>
<td>1.05</td>
<td>0.67</td>
</tr>
<tr>
<td>∆gyp-crr pELDRR/pCPaGGRmut</td>
<td>2.69</td>
<td>1.20</td>
<td>0.84</td>
<td>0.21</td>
</tr>
<tr>
<td>∆gyp-ptsHI pELDRR/pCPaGGRmut</td>
<td>2.85</td>
<td>1.35</td>
<td>0.81</td>
<td>0.20</td>
</tr>
</tbody>
</table>
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 uptake 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
ATTACACTGATGATTTGGCTTCACATCTTTTTACGTGACTACCTATCTTA
ATTCAACAATAAAAATAAACCATATTGGAGGCGATCATGCCTGACGCTAAAA
ACAGGGGGCGGTAACAAAGGCAATGACGTTTTCGTCTGCTTTCTTGCCG
CTCTGGCGGGGATTACTCTTTGGCCGATATCGGTGTAATTGCTGGCGCAC
TGCCCTTATTGCAGATGAATTCCAGATTCTTCGACACGCAAGATGGG
TCGTAAGCTCATGATTTGCAGCGAGTCTGGTGCGGTGGGTGGCTCTTA
TACGCCACCGCTGTACCTCTCTGGAATTTGCAGCGGCTACAAATCTG
GTATGATTCGATGTATCAGTTGATGATCCTACCTCTGTGGATCGGCAAT
TTTCTGATACCCGCCCTTCAGCTACACCGGTGCTGCGCTGCGGGATAC
GTGATTATCATCCCCCGGAATTTTGCTGTGATTGGGTCTTCTTCTTCAG
ACAGCCCGTTGGGTTCGCCCGGCGGCGGCGGTGTCACCCGACGAGACGGA
GTGCTGTACGCGCCGTGATACCAAGCGCGGAAAGCGGAAACTGG
ATGAAATCCGTGAAAGTTTGCAGTTAAACAGAGTGGCTGGGCGCTT
AAAGAGAAGACGAACTTCGCCGCGGCGGCGGTGTCTTCTGGCGTACCTGT
GGTAATGCAACGCGAACACTTCGCCGCGGCGGCGGTGTTCTTCTGCGTACCGA
AATCTTGAAACTGGGCGGTTATACACACGACAGCAATGCGGGGTGGGGA
CCGTGATTGTGGGCTGACCAACGTACTTGCCACCTTTATCGCAATCGCCCT
TGTTGACCGCTGGGACGCTAAACACCGCTACGCGCTTCTGCTGGTGAC
TGGCTGCTGGCATGGGCGTGACTCGGTACAATGATGCATATCGGTATTCTC
TCCGTGGCGGCAGTATTTTCGCCATCGCCATGCTGCTGATGTTTATTGTGGGT
TTTGCCATGAGTGCGCGGCCGCTCGTATTGGGTACTGTGCTGCCAAATTACAG
CCGCTGAAGGCGCGGATTATTGGGCCATCACCTGCTCCAATCGCCACCAACTG
GATTGCAACATGATCGTGTTGGCGCGCTCTGACGCAGTACTGTTTAT
GGTAACGCCAACACCTTCGTTGGGTATGCGGCTCTGAAACGTACTGTTTAT
CCTGCTGAGACTTGCTTGCTGCCGAAACAAACCAGTCTTGCGTGGAAAC
ATATGAACGTAATCTGATGAAAGGTCGTAAACCTGCGAAATAGGCGCTC
ACGATCACCACCCACCACCACCACACTATATTAGGAAAAATTA

glpF gene sequence is as follow
GGCACACACATTTTAAGTTTCGATAATTTCCTCGTTTTTGCTCTGTTAAACGATAAGTTTACAGCATGCTACAAAGCATCGTGAGGTCCGTGACTTTCACGCATACAAC
AAACATTAACCTCTTCCAGGATCCGATTGAGTCAAACATCAACCTTGAAAG
GCCAGTCATTGCTGAATTCTCCGCTGCGTTGGTTTCTTTTCGCGTGG
TGGGTGCCTGCAACTAAAAGTCGCTGCTGTGCTTTTGCTGCAGTG
GAAATCACTGTATTTGGGGGACTGGGGGTGGCAATGGCCATTACCTCT
CGCAGGGGTCTCCGCGGCGCAGCTTTATCCCAGCTGATTACCTGCTGTGG
GCTGTGTCGCTGCTGTTTTTGCTGTTTTTGCTGTTTTACACAA
GGTGCCGCGCCTTCTGTCGTCCGCGTCTTACTGTTACCGTTACAAAT
TTTTCTCGACAGCAGACTCATCAGATTGTCGCTCTCTTCTACGCTGCTGCTGCTGCT
AGTGTGCCTTCGAGACGCAGACTCATCAGATTGTCGCTCTCTTCTACGCTGCTGCTGCTGCT
TGTCAGCGCCTGAGATGTTGATTACCGCTATTTCGATGGGGCT
GATCCTGGCGTTAACGGACGATGGCAACGGTGTACCACGCGGCCCTTTGG
CTCCCTTGGCTGATTGGTCTACTGATTGCGGTCATTGGCGCATCTATGGGCCC
ATTGACAGGTTTTGGCCATGAACCCAGCGCGTGACTTCGGTCCGAAAGTCT
TTGCCTGGCTGGCGGGCTGGGGCAATGTCGCCTTTACCGGCGGCAGAGA
CATTCCTTACTTCCTGTTGCCGCTTTTCGGCCCTATCGTTGGCGCGATTGTA
GGTGCATTGCTACCCGAAACCTGATTGGTCGACCTTTGCTTGCGATATCT
GTGTTGGGAAGAAAGGGAAACCACAACCTCCTTCAGAAAGAAGCTTC
GCTGTAA

*xylE* gene sequence is as follow

CAATTTTGGATAATTATCACAATTAAGATCACAAGAAAAAGACATTACGTAAAC
GCATTTGAAAAATGAATATTGCTTTAACTGCTGACAATTCCAACATCAATG
CACTGATAAAAAAGATCGAATGGCTAAGGCAGGTCTGAA
ATGAAATCCAGT
ATAATTCAGTTATATTTTCGATTACCTTAGTCGACATTAGGTGGTTTAT
TATTTTGCTACGACACCGCGTTATATCCGGTACTGTTGAGTCACTCAATAC
CGTCTTTTGCTCCACAACAAAATTAAGTGAATCCGCTGCCAACCTCCGTGA
GGGTTTTCGCTGGCCAGCGCTCTGATGTGGTTGCTCATCGGCCTGCTGGCC
GGTGTTTAGTGCAAGCCGCTTGCGTCTGAGTTCACTAAGATTGCT
GCTGTCCCTGGTTTTTTATTTCTGGGTAGTTCTGCCTGCTGTTCTTAT
TTACCTCTATAAACCCGGACAACACTGTGCTCTGTTATTATCGCAGGGTTATGT
CCCAGAATTGTTATTGATATGGCGATATTGGCGCTGTTGTTAGCC
TCAATGCTCTCAGCAATGTATATTGCCGAAACTGGCTCCAGCTCATATTTCGCG
GGAAACTGGTCTCTTTAACCAGTTTTGCGATTATTTTCCGGGCAACTTTTAG
TTTACTGCGTAAAACTATTTTTATTGGCCCGTACCGGATGCCAGCTGGCTGAA
TACTGACGGCTGGCGTTATATGATTGTTCCTCGGCAGCTGGGCTGATGTGCG
TTCTTAATGCTGCTGATACCGGTGCTCCGGTGATGCCAGCTGGCTGAA
GCGGCAAGCAAGACAGAGGCGGGAAGGTATCCTCGCTGGGCTGATGGTC
CACGCTTGCAACTCAGGGAAGTTAAAACACTCCCTCGGATGTGAC
GCCGCAAAACCCGTTGGGTCTGTCTGCTGATGTTTTGCGTGGGCGTGATTGTA
ATCGGCGTAAATGCTCTCCCCATCTCAAAATTTGCGCAATCAATGTGGTC
TGTACTACGCCGCGGAAAGTTTCAAAACGCTGGGCGGCGACACGGATATC
GCGCTGTTCAGACCACTATTGTGCGAGTTATAACCTACCTATCCAGTTTC
TGGCAATTATGAGGTTGAAAAATTTTGGTCGTAAGCCACTGCAAAATTATG
GCGCAGTCGGAAATGGCAATCGGTATGTTTAGCCCTCGGTACCAGCGTTTTACA
CTCAGGCACCGGTATTGTCGGCTACTTCGAGTCTGTTCTATGTGGCC
CCTTTGCCATGTCCGTTGGGTACCGATGCTGCTGCTGAAACCT
TCCCGAATGCTATTGTGGTAAGCGCTGGCAATCGCGGTGCGGCAGTTACG
TGGCTGGCGGAAACTATTCGTCTCCTGGGACCTTCCGGATGAGGCAAAAACC
TCCTGCTGGTGCGCCATTTCACCAAGGTTTCTCCTACTCGGATTACGGTT
GTATGGGCGTTCTGGCAGGACTGTTATGTGAAATTTTTGGTCGGAACACCC
AAAGGTAAAAACCCCTGAGGAGCTGGAAGCCTCGGGAAACGCGCTTG
AAGAAAAACCAACAAACTGCTACGCTGTAA

* 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 cultures of strain Δgyp/pELDRR/pCPaGGRmutgalP, Δgyp/pELDRR/pCPaGGRmutglpF and Δgyp/pELDRR/pCPaGGRmutxylE in R/5 medium containing 10 g/L xylose were carried out. During 30 h of cultivation, only Δgyp/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, Δgyp/pELDRR/pCPaGGRmut, and Δgyp/pELDRR/pCPaGGRmutgalP were consumed 20 g/L of xylose. Batch culture of Δgyp/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 Δgyp/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) $\Delta$gyp/pELDRR/pCPaGGRmut, (B) $\Delta$gyp/pELDRR/pCPaGGRmutgalP, (C) $\Delta$gyp/pELDRR/pCPaGGRmutglpF, (D) $\Delta$gyp/pELDRR/pCPaGGRmut

Symbols: ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol
Table 3.2 Results of transporter expression strain in flask culture.

<table>
<thead>
<tr>
<th>Strains</th>
<th>DCW (g/L)</th>
<th>Glycerol accumulation (g/L)</th>
<th>3-HP concentration (g/L)</th>
<th>Xylose uptake rate (g/L·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δgyp pELDRR/pCPaGGRmut</td>
<td>3.00</td>
<td>1.72</td>
<td>1.05</td>
<td>0.67</td>
</tr>
<tr>
<td>Δgyp pELDRR/pCPaGGRmutgalP</td>
<td>3.47</td>
<td>0.82</td>
<td>1.12</td>
<td>0.67</td>
</tr>
<tr>
<td>Δgyp-crr pELDRR/pCPaGGRmutglpF</td>
<td>3.31</td>
<td>2.16</td>
<td>1.15</td>
<td>0.56</td>
</tr>
<tr>
<td>Δgyp-ptsHI pELDRR/pCPaGGRmutxyIe</td>
<td>2.01</td>
<td>2.73</td>
<td>0.34</td>
<td>0.56</td>
</tr>
</tbody>
</table>
Figure 3.6. Results of batch culture in xylose medium

(A) $\Delta gyp/pELDRR/pCPaGGRmut$, (B) $\Delta gyp/pELDRR/pCPaGGRmutgalP$

Symbols: ○, Xylose; ■, DCW; □, Acetate; ▲, Glycerol
<table>
<thead>
<tr>
<th>Strains</th>
<th>DCW (g/L)</th>
<th>Glycerol accumulation (g/L)</th>
<th>3-HP concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆gyp</td>
<td>5.35</td>
<td>2.48</td>
<td>0.24</td>
</tr>
<tr>
<td>pELDRR/pCPaGGRmut</td>
<td>5.71</td>
<td>0</td>
<td>4.01</td>
</tr>
</tbody>
</table>
3.4 Confirmation of \textit{galP} expression

3.4.1 SDS-PAGE & Western blot

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

For confirmation of \textit{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 \textit{galP} gene (Figure 3.8).

Western blotting analysis showed \textit{galP} gene (51kDa) band (Figure 3.9).
Figure 3.7. SDS-PAGE analysis of pCPaGGRmut and pELDRR plasmid 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 \textit{galP} expression on glycerol metabolism

Since $\Delta gyp/pELDRR/pCPaGGRmutgalP$ exhibited low accumulation of glycerol, experiments to identify \textit{galP} effects on glycerol metabolism were carried out.

To identify \textit{galP} expression on glycerol metabolism, $\Delta gyp/pCPaGGRmut$ and $\Delta gyp/pCPaGGRmutgalP$ 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 $\Delta gyp/pCPaGGRmut$ and $\Delta gyp/pCPaGGRmutgalP$ were cultured in R/5 medium containing 10 g/L xylose. Although $\Delta gyp/pCPaGGRmut$ produced 4.94 g/L of glycerol and 2.28 g/L of DCW, $\Delta gyp/pCPaGGRmutgalP$ produced 2.43 g/L of glycerol and 3.13 g/L of DCW. As a result of \textit{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, \textit{galP} expression enhanced TCA cycle and resultingly, pyruvate concentration was higher than that of control strain (Zhang \textit{et al.} 2009).
Figure 3.10. Results of flask culture (A) Δgyp/pCPaGGRmut and (B) Δgyp/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, Δgyp/pELDRR/pCPaGGRmutgalP showed lower glycerol accumulation and higher 3-HP concentration than that of control strain, Δgyp/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 $\Delta gyp/pELDRR/pCPaGGRmutgalP$

Table 3.4. Results of fed-batch fermentation of $\Delta gyp/pELDRR/pCPaGGRmutgalP$

<table>
<thead>
<tr>
<th>Strain</th>
<th>DCW (g/L)</th>
<th>3-HP (g/L)</th>
<th>3-HP productivity (g/L-h)</th>
<th>Glycerol (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta gyp / pELDRR$</td>
<td>59.1</td>
<td>39.1</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td>$pCPaGGRmutgalP$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4. Conclusions

This thesis can draw the following conclusions:

(1) Although deletion of the crr and ptsH1 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


and Molecular Life Sciences 57: 106-127.


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 선평한 바이오매스로부터 생산할 가치가 있는 바이오 화학 소재 중 다양한 화학 물질의 전구체로 사용되는 3-히드록시프로피온산을 선정하였다. 하지만 2세대 바이오매스로부터 추출되는 목당을 이용하여 3-히드록시프로피온산을 생산함에 있어서 발효 도중에 글리세롤이 축적되는 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 $ptsHl$ 유전자의 파쇄하는 방법과 운반 단백질을 발현하는 방법을 선택하였다. $crr$ 유전자와 $ptsHl$ 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/L의 3-HP의 생산을 확인할 수 있었다. 도입해준 galP 의 발현은 웨스턴블로팅을 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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