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Simultaneous conversion of glucose and xylose to 3-hydroxypropionic acid in engineered *Escherichia coli* by modulation of sugar transport and glycerol synthesis

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HIGHLIGHTS

- Glycerol synthetic genes were expressed for 3-HP production from glucose and xylose.
- *PtsG* deletion and *xylR* overexpression facilitated efficient consumption of sugars.
- The strain JHS01300 harboring *GPD1* and *GPP2* produced glycerol with 48% yield.
- A fed-batch culture using mixed sugars resulted in 29.7 g/L 3-HP and 0.36 g/g yield.

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ABSTRACT

Escherichia coli expressing the *Lactobacillus brevis* $dhaB_1B_2B_3$ and $dhaR_1R_2$ clusters and *Pseudomonas aeruginosa aldhH* was engineered to produce 3-HP from glucose and xylose via the glycerol biosynthetic pathway. Glycerol, a key precursor for 3-HP biosynthesis was produced by overexpression of the *GPD1* and *GPP2* genes from *Saccharomyces cerevisiae*. For relief of carbon catabolite repression, deletion of the chromosomal *ptsG* gene and overexpression of the endogenous *xylR* gene rendered engineered *E. coli* JHS01300/pCPaGGRm to utilize glucose and xylose simultaneously and to produce glycerol at 0.48 g/g yield and 0.35 g/L-h productivity. Finally, engineered *E. coli* JHS01300/pELDRR + pCPaGGRm produced 29.4 g/L of 3-HP with 0.54 g/L-h productivity and 0.36 g/g yield in a sugar-limited fed-batch fermentation. It was concluded that dual modulation of sugar transport and glycerol biosynthesis is a promising strategy for efficient conversion of glucose and xylose to 3-HP.

1. Introduction

3-Hydroxypropionic acid (3-HP) is a three-carbon carboxylic acid containing a β-hydroxyl group and can be converted to various commodity chemicals such as acrylic acid, acrylonitrile, methyl acrylate, and 1,3-propanediol (Kumar et al., 2013). It was also selected as a top value-added bio-chemical from cellulosic biomass by the US Department of Energy (Bozell and Petersen, 2010). For microbial production of 3-HP from carbohydrates, various microorganisms have been engineered such as *Lactobacillus*, *Escherichia*, *Klebsiella* species. Glycerol, a major by-product in biodiesel manufacturing is used as a carbon source for 3-HP production in many cases. From glycerol to 3-HP, two enzymes are involved such as glycerol dehydratase and aldehyde dehydrogenase (Fig. 1). By expression of the two enzymes, recombinant *Klebsiella pneumoniae* WM3/pUC18*kan-aldHec* strains produced 48.9 g/L 3-HP with 0.41 mol/mol yield in a fed-batch culture using glycerol only (Huang et al., 2013). Our group constructed an engineered *Escherichia coli* system expressing the *Lactobacillus brevis dhaB* and *dhaR* gene cluster and *E. coli aldH* gene and produced 14.3 g/L 3-HP from glycerol by a two-step feeding strategy in the fed-batch mode of operation (Kwak et al., 2013). By expression of a strong aldehyde dehydrogenase from *Pseudomonas aeruginosa* and deletion of glycerol kinase gene (*glpK*) and alcohol dehydrogenase gene (*yqhD*), engineered *E. coli* △*glpK*△*yqhD*/pELDRR + pCPa72 produced

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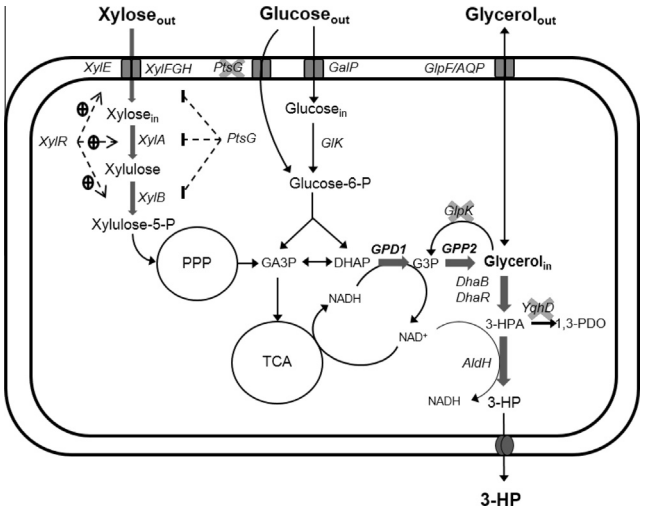

Fig. 1. Metabolic pathway for 3-HP production in recombinant *E. coli* JHS01300 strain harboring plasmids pELDRR and pCPaGGRm. The name of metabolites are abbreviated as follows: 3-HP, 3-hydroxypropionic acid; 3-HPA, 3-hydroxypropionaldehyde; 1,3-PDO, 1,3-propanediol; GA3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate. Italicized letters indicate metabolic enzymes or regulatory proteins: *DhaB*, glycerol dehydratase; *DhaR*, glycerol dehydratase reactivating factor; *AldH*, aldehyde dehydrogenase; *GPD1*, glycerol-3-phosphate dehydrogenase; *GPP2*, glycerol-3-phosphatase; *GlpK*, glycerol kinase; *YqhD*, alcohol dehydrogenase; *PtsG*, glucose transporter $IICB_{Glc}$; *GlpF*, glycerol transporter; *AQP*, aquaporins; *GalP*, galactose permease; *Glk*, glucose kinase; *XylFGH*, xylose ABC transporter; *XylA*, xylose isomerase; *XylB*, xylulokinase; *XylR*, xylose DNA-binding transcriptional activator.

57.3 g/L 3-HP with 0.88 g/g yield and 1.59 g/L-h productivity by the same fed-batch culture strategy (Kim et al., 2014). Meanwhile, glucose is also used as the substrate for 3-HP production. In most cases, glucose is metabolized into pyruvate in the glycolysis and the pyruvate is converted into 3-HP via different intermediates such as malonyl-CoA, acrylogyl-CoA, ß-alanine and so on (Henry et al., 2010; Jiang et al., 2009; Kumar et al., 2013). Among various pathways, the malonyl-CoA mediated strategy conferred recombinant *E. coli* BX3_0240 to be able to produce almost 50 g/L 3-HP from glucose (Lynch et al., 2014).

Cellulosic biomass is the second-generation biomass and composed of various carbohydrates. Glucose and xylose are two major monosugars in cellulosic biomass and should be metabolized by cells efficiently and simultaneously for development of bioprocess with cost-competitiveness. In general, microbial cells including *E. coli* have the carbon catabolite repression (CCR) (or glucose repression) mechanism. Glucose inhibits the utilization of other sugars such as xylose before the glucose depletion, so glucose and xylose in culture medium are metabolized sequentially in a row (Kim et al., 2010). By relief of CCR, many research efforts have been made to develop *E. coli* mutant strains able to consume glucose and xylose simultaneously. For example, the disruption of the phosphoenolpyruate:sugar-transferring system (PTS) resulted in an improvement of pentose consumption rate in the presence of glucose (Nichols et al., 2001), but xylose consumption was still partially repressed by glucose in a PTS-negative *E. coli* strain (Hernandez-Montalvo et al., 2001). Replacement of the native cyclic AMP receptor protein gene (*crp*) with its engineered one and deletion of the *mgs* gene responsible for activation of the glycolysis shunt pathway also relieved CCR in some degree (Cirino et al., 2006; Yomano et al., 2009). An evolved mutant of *E. coli* cultured on xylose and arabinose for a long time was able to consume glucose and xylose simultaneously (Kim et al., 2010).

Even though 3-HP was successfully produced from glycerol or glucose, there has been no report for 3-HP production by using both glucose and xylose. In this study, we constructed the metabolic pathway from glucose and xylose to 3-HP via the glycerol biosynthetic pathway (Fig. 1). For glycerol production, two genes coding for glycerol-3-phosphate dehydrogenase (GPD1) and glycerol-3-phosphatase (GPP2) were overexpressed in *E. coli*. For efficient utilization of glucose and xylose, CCR was relieved by deletion of PtsG (enzyme IIBC in PTS) and overexpression of XylR (transcriptional activator of the *xyl* operon). Finally, the expression of 3-HP-producing enzymes (Kim et al., 2014) and genetic modifications for simultaneous consumption of glucose and xylose enabled engineered *E. coli* to produce high amount of 3-HP via a sugar-limited fed-batch fermentation.

2. Methods

2.1. Strains and plasmids

E. coli TOP10 (Invitrogen Co., Carlsbad, CA, USA) and BL21 Star (DE3) Δ*glpK*Δ*yqhD* (Kim et al., 2014) named as JHS01169 were used for plasmid construction and 3-HP production, respectively. Plasmids pELDRR harboring the glycerol dehydratase genes ($dhaB_1$-B_2B_3) and its reactivase genes ($dhaR_1R_2$) from *L. brevis* (Kwak et al., 2013) and pCPa72 containing the *P. aeruginosa aldH* gene coding for aldehyde dehydrogenase (Kim et al., 2014) were used for production of 3-HP. Plasmids pKD13, pKD46 and pCP20 were used for deletion of the *ptsG* gene in the *E. coli* chromosome (Datsenko and Wanner, 2000). *E. coli* strains and plasmids used in this study are listed in Table 1.

2.2. Genetic manipulation

The *GPD1* and *GPP2* genes were amplified by polymerase chain reaction (PCR) from the *Saccharomyces cerevisiae* D452-2 chromosome with the corresponding PCR primers (Table 2). Each PCR product was digested with appropriate restriction enzymes: *Nde*I and *Xho*I for *GPD1*; *Xho*I and *Pac*I for *GPP2*. They were ligated subsequently into plasmid pCPa72, yielding plasmid pCPaGG. For expression of the *xylR* gene, the *xylR* gene fragment with the promoter sequence (Supplement Fig. 2) was PCR-amplified from the chromosome of *E. coli* BL21 Star (DE3) using the corresponding primer sets: F-*xylR*-*Nhe*I, F-*xylR*m-*Nhe*I and R-*xylR*-*Nhe*I (Table 2). The PCR products of the *xylR* fragment were digested with *Nhe*I, and then were combined with linearized pCPaGG with the same restriction enzyme, resulting in construction of plasmids pCPaGGR and pCPaGGRm. Deletion of the *ptsG* gene in the *E. coli* chromosome followed the previous report with minor modification (Datsenko and Wanner, 2000). Briefly, the *ptsG*-deletion cassette with the kanamycin resistant gene was PCR-amplified from the template of plasmid pKD13 with the corresponding DNA oligomers of F-*ptsG* and R-*ptsG* (Table 2). The PCR amplicon was introduced into *E. coli* JHS01169 harboring plasmid pKD46. After selection of the *ptsG*-deleted transformants alive on LB medium with kanamycin and ampicillin, plasmid pCP20 was transformed into the selected clone. To cure plasmid pCP20, the selected transformant against ampicillin was incubated at 43 °C. The final engineered *E. coli* BL21 Star (DE3) strains without the chromosomal *ptsG* gene as well as *glpK* and *yqhD* was named JHS01300.

2.3. Culture conditions

Luria–Bertani (LB) medium (1% yeast extract, 2% bacto-tryptone and 1% NaCl) with appropriate antibiotics was used for genetic manipulation and pre-cultivation. Batch culture was performed

Table 1
Strains and plasmids used in this study.

Name	Description	Sources
Strain		
E. coli TOP10	F– mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (araleu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
JHS01169	*E. coli* BL21 Star (DE3) Δ*glpK* Δ*yqhD*	Kim et al. (2014)
JHS01300	*E. coli* BL21 Star (DE3) Δ*glpK* Δ*yqhD*Δ*ptsG*	This study
Plasmid		
pKD13	Amp^R, Kan^R, oriR6K	Datsenko and Wanner (2000)
pKD46	Phage λ red recombinase, temperature sensitive replicon	
pCP20	Amp^R, Chl^R, Yeast Flp recombinase, temperature sensitive replicon	
pCPa72	Sm^R, pCDFduet-1 based plasmid containing *Pseudomonas aeruginosa* semialdehyde dehydrogenase gene under the *T7* promoter	Kim et al. (2014)
pCPaGG	Sm^R, *GPD1* and *GPP2* cloned in *Nde*I, and *Pac*I sites of pCPa72	This study
pCPaGGR	Sm^R, *xylR* fragment with endogenous P_R promoter cloned in *Nhe*I site of pCPaGG	This study
pCPaGGRm	Sm^R, *xylR* fragment with synthetic promoter *Pmut* cloned in *Nhe*I site of pCPaGG	This study
pELDRR	Kan^R, pET-29b(+)-based plasmid containing *L. brevis dhaB*, *dhaR* cluster under the *T7* promoter	Kwak et al. (2013)

using a 500 mL-scale baffled flask (Nalgene Co., Rochester, NY, USA) containing 100 mL of R/5 medium (Reisenberg medium with 5 g/L yeast extract) with glucose and/or xylose at 37 °C, 250 rpm and pH 6.8 (Cho et al., 2015). When optical density (OD_{600}) of cultured broth reached 3, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and coenzyme B_{12} (Sigma–Aldrich Co., USA) were added at the final concentrations of 0.2 mM and 20 μM, respectively, and cultivation temperature was shifted down to 25 °C to promote soluble expressions (Kwak et al., 2013). Sugar-limited fed-batch fermentations were carried out in a 2.5 L-scale jar fermentor (Kobiotech, Incheon, Korea) with 1 L working volume of R/5 medium containing 13 g/L glucose and 7 g/L xylose initially. To maintain a dissolved oxygen level above 10%, agitation speed and aeration rate were set at 1,300 rpm and 1 vvm of air supply, respectively. After depletion of glucose and xylose added initially, a feeding solution composed of 500 g/L glucose and 250 g/L xylose was constantly fed into the fermentor at 1 g/h. When the cell mass reached an OD_{600} of 20, addition of IPTG and coenzyme B_{12}, and temperature shift to 25 °C were carried out as the same as the batch culture, and agitation speed was changed from 1300 to 600 rpm.

2.4. Determination of cell and metabolites concentration

The concentrations of glucose, xylose, glycerol, 3-HP and acetate were measured by high performance liquid chromatography (1200 series, Agilent Technologies Inc., Santa Clara, CA, USA) with a Rezex™ ROA-Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) heated at 60 °C. 5 mM H_2SO_4 was flowed at 0.6 mL/min (Park et al., 2011). A reflective index detector and an UV detector at 210 nm were used for detection. To determine cell concentration, an optical density (OD_{600}) of the cells was measured using a spectrophotometer (UV-1601, Shimadzu Co., Tokyo, Japan) at 600 nM. Dry cell mass was calculated using a pre-determined conversion factor, 0.365 g/L/OD_{600}.

2.5. Enzyme activity assay

Determination of xylulokinase activity followed a previous report with some modifications (Park et al., 2012). To prepare the crude enzyme extract, the *E. coli* cells were cultured in R/5 medium containing 5 g/L glucose and 3 g/L xylose. After the IPTG induction in the mid-exponential phase and 4 h of more incubation, the cells were collected by centrifugation at 6000*g* for 15 m at 4 °C and the cell pellets were suspended in 100 mM potassium phosphate buffer (pH 7.0). After cell disruption and centrifugation, the supernatant was used as crude enzyme solution. The enzyme reaction mixture (20 mM glycine, 5 mM $MgSO_4$, 1.1 mM ATP, 2.3 mM phosphoenolpyruvate, 0.4 mM NADH, 2U pyruvate kinase and 2U lactate dehydrogenase at pH 7.0) was pre-warmed at 25 °C. After addition of 8.5 mM xylulose and the crude enzyme solution, reduction of NADH was monitored at 340 nm. One unit of xylulokinase was defined as the amount of the enzyme able to reduce 1 μmol NADH per minute. Protein concentration was determined by a pro-

Table 2
List of primers used in this study.

Name	Nucleotide sequence (5′ to 3′)	Target gene
F-*GPD1*-*Nde*I	GGAATTCCATATGATGTCTGCTGCTGCTGATAGATTAA	*GPD1*
R-*GPD1*-*Xho*I	CCGCTCGAGCTAATCTTCATGTAGATCTAATTCTTCAATC	
F-*GPP2*-*Xho*I	CCGCTCGAGAGGAGGAAATAAAATGGGATTGACTACTAAACCTCTATCT	*GPP2*
R-*GPP2*-*Pac*I	CCTTAATTAATTACCATTTCAACAGATCGTCCTTAG	
F-*ptsG*	ACGTAAAAAAAGCACCCATACTCAGGAGCACTCTCAATTGTGTAGGCTGGAGCTGCTTC	*ptsG*
R-*ptsG*	AGCCATCTGGCTGCCTTAGTCTCCCCAACGTCTTACAGAATTCCGGGGATCCGTCGACC	
F-*ptsG* check	GCTCTCCCCCCTTGCC	
R-*ptsG* check	CCGGCACGTATCAATTCTGAATAA	
F-*xylR*-*Nhe*I	CTAGCTAGCTGGACTCCGCAACCAAACGCCGTACTTTATTTTGATAAAAATTTTCTC	*xylR*
F-*xylRm*-*Nhe*I	CTAGCTAGCTGGACTCCGCAACCAAACGCCGTACTTTATTTTGATAAAAATTTTCTC	
R-*xylR*-*Nhe*I	CTAGCTAGCCATCGCTACAACATGACCTCGCTAT	
F-*rrsA*	AGGCCTTCGGGTTGTAAAGT	*rrsA*
R-*rrsA*	ATTCCGATTAACGCTTGCAC	
F-*xylA*	GAGGGCGCGTCGTTAAAAG	*xylA*
R-*xylA*	CGCTGTAACAACTTGCGTTG	
F-*xylB*	GGTATTGCCGGCCAGATG	*xylB*
R-*xylB*	GTAAATCCGGGCATCATCAGG	
F-*xylF*	GGTGACCAATGGGTTGATGG	*xylF*
R-*xylF*	ATCCGCATCCTGGCCGG	
F-*xylR*	CGACGTCCCCATTGTTGGG	*xylR*
R-*xylR*	GAAATCGATATTCGCGCTCAGT	

The underlined sequences indicate the recognition site of each restriction enzyme, of which name is described beside the primer name.

tein assay kit (Bio-Rad Co., Richmond, CA, USA) using bovine serum albumin (BSA) as a standard protein. Specific xylulokinase activity (U/mg protein) was calculated by division of enzyme activity with protein concentration of the crude enzyme solution. The assay was repeated independently in triplicate.

2.6. Quantitative real-time PCR

To analyze the transcription levels of *xylA*, *xylB*, *xylF* and *xylR* genes located in the *xyl* operon, total RNA was isolated from *E. coli* cells using the RNeasy Mini Kit (Qiagen Co., Valencia, CA, USA) and used for cDNA synthesis using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, Marietta, OH, USA). MiniOpticon Real-Time PCR system (Bio-Rad, Hercules, CA, USA) with a SYBR premix Ex Taq (Takara, Otsu, Shiga, Japan) was used for quantitative real-time PCR. DNA oligomers specific for *xylA*, *xylB*, *xylF*, and *xylR* are listed in Table 2. The *rrsA* gene encoding 16S rRNA served as the internal standard to normalize the differences in RNA quantity among the samples (Kobayashi et al., 2006).

3. Results and discussion

3.1. Construction of glycerol synthetic pathway in E. coli

To produce 3-HP from glucose and xylose, the glycerol biosynthetic pathway from dihydroxyacetone phosphate to glycerol was newly introduced into recombinant *E. coli* JHS01169 because *E. coli* generally does not have essential enzymes for glycerol production (Yu et al., 2011). Since *S. cerevisiae* is a high glycerol producer and its osmo-tolerant strain produced 130 g/L glycerol with 32 g/L-d of productivity (Wang et al., 2001), we amplified two essential genes coding for glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol 3-phosphatase (*GPP2*), and subcloned into plasmid pCPa72, resulting in construction of plasmid pCPaGG. Co-expressions of *GDP1* and *GPP2* were confirmed in the strain JHS01300/pELDRR + pCPaGG using SDS–PAGE (Supplement Fig. 4). To evaluate a glycerol-producing performance, engineered *E. coli* JHS01169 containing plasmid pCPaGG or pCPa72 was cultured batchwise in R/5 medium with 8 g/L glucose or 8 g/L xylose. As shown in Table 3 and Supplement Fig. 1, introduction of both *GPD1* and *GPP2* genes allowed JHS01169/pCPaGG to produce 3.1–3.7 g/L glycerol from glucose or xylose with 0.39–0.46 g/g yield, whereas the control strain of JHS01169/pCPa72 did not synthesize glycerol at all. Glycerol accumulating in culture medium was not consumed in aerobic condition even after the depletion of glucose or xylose (Supplement Fig. 1), since the *glpK* gene coding for glycerol kinase was deleted in JHS01169 and hence the *glpK* deletion minimized glycerol dissimilation as reported elsewhere (Yu et al., 2011). This result is consistent with previous study that the *GlpK-GlpD*/*GlpABC* pathway is the major glycerol utilization pathway while the *GldA-DhaLKM* pathway exerts negligible impact on glycerol metabolism in aerobic condition (Kim et al., 2014). Finally, glycerol production from glucose and xylose was successfully achieved by expression of both *GPD1* and *GPP2* in recombinant *E. coli* JHS01169 without glycerol reutilization, of which system was used for further engineering of *E. coli*.

3.2. Effect of ptsG deletion on conversion of glucose and xylose to glycerol

In *E. coli*, the *ptsG* gene encoding the glucose-specific transporter (enzyme EIIBC) of the phosphotransferase system is known to play a central role in CCR and the inducer exclusion mechanism (Gosset, 2005). In order to relieve CCR and to utilize glucose and xylose simultaneously, the chromosomal *ptsG* gene was disrupted in the JHS01169 strain, resulting in the construction of the JHS01300 strain losing the *glpK*, *yqhD* and *ptsG* genes. To investigate the effect of the *ptsG* deletion, JHS01169/pCPaGG and JHS01300/pCPaGG were cultivated in R/5 medium with 5 g/L glucose and 3 g/L xylose (Fig. 2A and B). While JHS01300/pCPaGG consumed 2.1 g/L of xylose (70% of initial xylose concentration), JHS01169/pCPaGG utilized only 0.6 g/L of xylose in 15 h. The final cell mass (3.8 g/L) and glycerol concentration (3.5 g/L) of JHS01300/pCPaGG were 15% and 46% higher than those of JHS01169/pCPaGG, respectively (Table 3). Especially, deletion of *ptsG* considerably increased the xylose consumption rate of JHS01300/pCPaGG by a 3.5-fold, relative to that of JHS01169/pCPaGG. Deletion of *ptsG* certainly increased xylose consumption and glycerol production by the relief of CCR, and reduced glucose consumption rate, of which phenomena were coincided with the previous report (Hernandez-Montalvo et al., 2001). Meanwhile, 30% of xylose initially added still remained when glucose was depleted at 15 h (Fig. 2B), indicating that modulation of CCR was not enough for the recombinant *E. coli* strain to utilize glucose and xylose in the same consumption rate. Thus, an additional strategy for more efficient utilization of xylose during glucose consumption should be devised.

3.3. Synergistic effect of ptsG deletion and xylR overexpression on glycerol production from glucose and xylose

The *xyl* operon of *E. coli* is composed of six genes (*xylABFGHR*), in which the *xylA* and *xylB* genes encode two metabolic enzymes of xylose isomerase and xylulokinase, respectively, and the *xylF*, *xylG* and *xylH* genes code for xylose transport components (Groff et al., 2012). XylR encoded by the *xylR* gene is known to activate the *xyl* operon as a transcriptional enhancer (Ni et al., 2013) and its overexpression enabled *E. coli* to utilize l-arabinose together with xylose (Groff et al., 2012). To investigate the effect of XylR overexpression on xylose utilization, the *E. coli xylR* gene was introduced into plasmid pCPaGG under the endogenous *xylR* promoter (P_R) or a

Table 3
Summarized results of batch cultures of recombinant *E. coli* strains producing glycerol from glucose and/or xylose.

Strain	Plasmid	Carbon source	Dry cell mass (g/L)	Glucose consumption rate (g/L-h)	Xylose consumption rate (g/L-h)	Glycerol concentration (g/L)	Glycerol yield (g/g total sugar)	Glycerol productivity (g/L-h)
JHS01169	pCPa72	Glc (8)[a]	5.2 ± 0.15	0.67 ± 0.00	–	N.D.[b]	–	–
	pCPaGG	Glc (8)[a]	4.4 ± 0.47	0.52 ± 0.03	–	3.7 ± 0.06	0.46 ± 0.03	0.23 ± 0.02
		Xyl (8)	3.3 ± 0.21	–	0.40 ± 0.02	3.1 ± 0.17	0.39 ± 0.01	0.19 ± 0.02
		Glc (5) + Xyl (3)	3.3 ± 0.36	0.42 ± 0.00	0.04 ± 0.01	2.4 ± 0.25	0.43 ± 0.05	0.22 ± 0.01
JHS01300	pCPaGG	Glc (5) + Xyl (3)	3.8 ± 0.24	0.36 ± 0.05	0.14 ± 0.02	3.5 ± 0.23	0.49 ± 0.03	0.32 ± 0.02
	pCPaGGRm	Glc (5) + Xyl (3)	4.0 ± 0.11	0.33 ± 0.01	0.20 ± 0.00	3.8 ± 0.06	0.48 ± 0.01	0.35 ± 0.01

[a] Numbers in the parenthesis indicate the concentrations (g/L) of glucose (Glc) and xylose (Xyl).
[b] N.D., not detected.

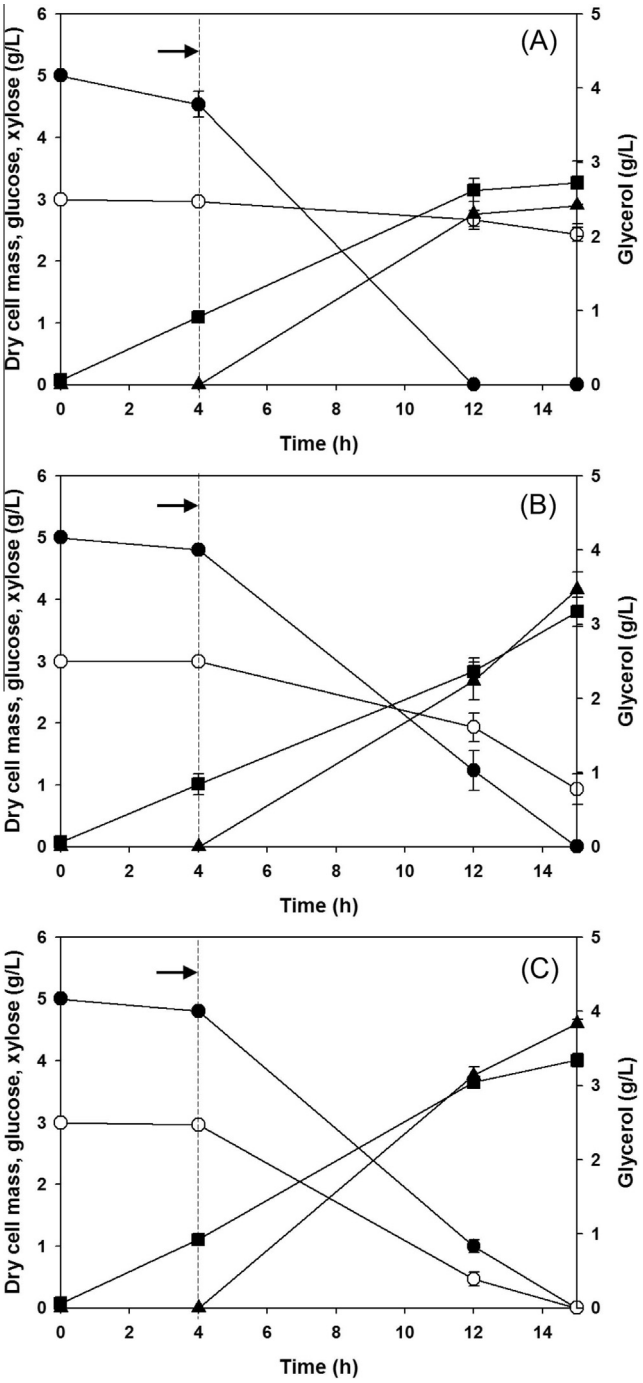

Fig. 2. Batch cultures of JHS01169/pCPaGG (A) and JHS01300 harboring pCPaGG (B) or pCPaGGRm (C) in R/5 medium with 5 g/L glucose and 3 g/L xylose. ■, dry cell mass; ●, glucose; ○, xylose; ▲, glycerol; arrow, IPTG induction, coenzyme B_{12} addition and temperature shift from 37 °C to 25 °C. The experiment was conducted in triplicate.

synthetic promoter (*Pmut*), resulting in construction of plasmids pCPaGGR and pCPaGGRm, respectively. The *Pmut* promoter was highly homologous to the consensus promoter sequence (Supplement Fig. 2A). JHS01300 strains transformed with plasmid pCPaGG, pCPaGGR or pCPaGGRm were cultured in a mixture of 2 g/L glucose and 1.2 g/L of xylose without IPTG induction (Supplement Fig. 2B). The JHS01300/pCPaGGRm strain demonstrated the highest specific xylose consumption rate of 0.12 g xylose/g dry cell mass-h, which was 28% and 49% higher than those for JHS01300 strains harboring plasmid pCPaGGR or pCPaGG, respectively. To assess the synergistic effect of the constitutive expression of *xylR* and deletion of *ptsG* on glycerol production from glucose and xylose, the JHS01300/pCPaGGRm was cultured using 5 g/L glucose and 3 g/L xylose (Fig. 2C). When comparing to the culture profile of JHS01300/pCPaGG (Fig. 2B), *xylR* overexpression in JHS01300/pCPaGGRm led to simultaneous consumption of glucose and xylose in 15 h without a notable influence on cell growth and glucose consumption. Finally, xylose consumption rate and maximum glycerol productivity were increased by 5.3-fold and 1.37-fold via the overexpression of *xylR* and deletion of *ptsG*, respectively, (Table 3). The *xylR* modulation did not affect the yield of glycerol to total sugars utilized. The transcription of the *xyl* operon in *E. coli* is regulated by two independent regulators of XylR and the cyclic AMP-CRP complex (cAMP-CRP) (Song and Park, 1997). The full transcriptional activation of the *xyl* operon is known to require the attachment of both XylR and cAMP-CRP onto their corresponding binding sites located between the open reading frames of *xylA* and *xylF*. Many research efforts have been made to increase CRP level in *E. coli* to enhance xylose consumption rate in the presence of glucose by genetic modification of the PTS-related genes (Gosset, 2005; Yao et al., 2011). However, an increment of CRP level by the *ptsG* deletion did not fully relieve CCR on xylose utilization. Meanwhile, expression of XylR in *E. coli* without any genetic modification was also ineffective to activate the transcription of the *xyl* operon (Song and Park, 1997). Considering the simultaneous consumption of glucose and xylose, and increases in xylose consumption rate and glycerol production rate for JHS01300/pCPaGGRm, it is certain that a combinatorial modification of *xylR* overexpression and chromosomal *ptsG* deletion is an effective way to produce 3-HP from glucose and xylose via the glycerol biosynthetic pathway.

3.4. Transcriptional and translational analysis of the xyl operon

To assess the effect of *xylR* overexpression and *ptsG* deletion on transcription levels of the *xyl* operon, PT-PCR was carried out to quantify transcripts of *xylA*, *xylB*, *xylF* and *xylR* genes in the strains JHS01169/pCPaGG, JHS01300/pCaGG and JHS01300/pCaGGRm. As shown in the culture profiles of the three strains (Fig. 2), the cells were collected at the mid-exponential growth phase (8 h) where both glucose and xylose were still present in the medium. As shown in Table 4, the *ptsG* deletion in JHS01300/pCPaGG triggered the mRNA levels of the four *xyl* genes considerably by over 12 times, relative to JHS01169/pCPaGG. Furthermore, JHS01300/pCPaGGRm with dual modification of *ptsG* deletion and *xylR* overexpression showed 1.4–6.4 times increases in mRNA levels of the *xyl* genes, compared to JHS01300/pCPaGG without *ptsG* only (Table 4). For the analysis of the *xyl* operon in translation level, xylulokinase encoded by *xylB* was chosen as the indicator enzyme since it is known as rate-limiting enzyme in the xylose metabolism (Park et al., 2012). Stepwise increases in its specific activity were observed according to disruption of *ptsG* (2.2 times) and overexpression of *xylR* (1.7 times) (Table 4). Taken together, a strategy of *ptsG* disruption and *xylR* overexpression was proven to synergistically enhance both the transcriptional and translational levels of the genes involved in the *xyl* operon. Finally, it was concluded that JHS01300/pCPaGGRm was an effective glycerol producer for further 3-HP production from glucose and xylose.

3.5. Batch production of 3-HP from glucose and xylose

To produce 3-HP from glucose and xylose, plasmid pELDRR harboring the *dhaB*$_1$*B*$_2$*B*$_3$ and *dhaR*$_1$*R*$_2$ gene cluster from *L. brevis* (Kwak et al., 2013) was introduced into JHS01169/pCPaGG, JHS01300/pCPaGG and JHS01300/pCPaGGRm, respectively. The three *E. coli* strains able to produce 3-HP were cultured batchwise using 5 g/L glucose and 3 g/L xylose, of which culture profiles and results are

Table 4
Quantitative analysis of gene and protein expression involved in the *xyl* operon.

Strain	Plasmid	Relative transcriptional level				Specific xylulokinase activity (U/mg)
		xylA	*xylB*	*xylF*	*xylR*	
JHS01169	pCPaGG	1.0 ± 0.06	1.0 ± 0.06	1.0 ± 0.06	1.0 ± 0.20	0.013 ± 0.003
JHS01300	pCPaGG	77.7 ± 11.9	30.7 ± 2.02	64.7 ± 2.92	12.6 ± 0.61	0.029 ± 0.001
JHS01300	pCPaGGRm	132.4 ± 3.67	51.6 ± 3.68	88.6 ± 15.0	81.2 ± 1.59	0.050 ± 0.002

The recombinant cells were cultured in R/5 medium containing 5 g/L glucose and 3 g/L xylose and the samples were collected in the mid-exponential phase at 8 h (Fig. 2).

displayed in Fig. 3 and Table 5. The JHS01169/pELDRR + pCPaGG strain used glucose completely and produced 0.54 g/L glycerol (Fig. 3A). The synthesized glycerol was converted completely to 0.36 g/L 3-HP. Most xylose was not metabolized in 26 h of the culture. Meanwhile, a *ptsG*-deleted strain of JHS01300/pELDRR + pCPaGG metabolized glucose and xylose simultaneously at a similar rate of 0.22 g/L-h and consumed completely in 22 h (Fig. 3B). After IPTG induction, glycerol was produced gradually to 2.57 g/L with 0.1 g/L-h and 3-HP accumulated at 0.8 g/L in 26 h of batch culture. A 2.2-fold increase in 3-HP concentration by the *ptsG* deletion in JHS01300/pELDRR + pCPaGG was owing to 10 times faster consumption of xylose and 4.8-fold higher production of glycerol than JHS01169/pELDRR + pCPaGG. Finally, the *ptsG*-deleted and *xylR*-overexpressing strain of JHS01300/pELDRR + pCPaGGRm was cultured under the same condition (Fig. 3C). The batch culture resulted in a 24% increase in total sugar consumption rate and a 20% increase in 3-HP titer, relative to those for JHS01300/pELDRR + pCPaGG.

3.6. Effect of sugar presence on 3-HP production from glycerol

Although JHS01300/pELDRR + pCPaGG and JHS01300/pELDRR + pCPaGGRm were able to successfully produce 3-HP from glucose and xylose in a batch culture, a considerable amount of glycerol accumulated in the media (Fig. 3). In the batch culture of JHS01300/pELDRR + pCPaGGRm, especially, conversion of glycerol to 3-HP started after the depletion of glucose and xylose. In a previous report, over 1 g/L glucose in culture medium allowed accumulation of glycerol and prevention of 1,3-propanediol production (Jin and Lee, 2008), suggesting that glucose and/or xylose might hinder the conversion of glycerol to 3-HP in our systems. To verify this inhibitory effect, engineered *E. coli* JHS01300/pELDRR + pCPa72 was cultured using 1 g/L glycerol and 3 g/L glucose or 3 g/L xylose (Supplement Fig. 3). When the strain was grown using glycerol only, 0.83 g/L 3-HP accumulated in the medium, while less than 0.3 g/L 3-HP was produced in the presence of glycerol and glucose or xylose, indicating that glycerol conversion to 3-HP was inhibited by glucose and xylose. For production of 3-HP from glycerol, the JHS01300/pELDRR + pCPa72 cells should take up extracellular glycerol because it does not have the glycerol biosynthetic pathway from glucose and xylose. GlpF is the main glycerol transporter with the facilitated diffusion mechanism and allows both export and import of glycerol (Lu et al., 2003). Since expression of GlpF under the control of the *glpFK* promoter is activated by cAMP-CRP (Weissenborn et al., 1992), the CCR-negative strain of JHS01300 (Δ*glpK*Δ*yqhD*Δ*ptsG*) can express GlpF protein in some extent. Despite the existence of GlpF, the reduction of the glycerol conversion might be owing to the inhibition of GlpF-mediated glycerol transport by glucose and xylose. Even though an exact mechanism of this inhibition is not unveiled, but glucose is generally known to inhibit the transport of the second sugars such as xylose (Lee et al., 2002).

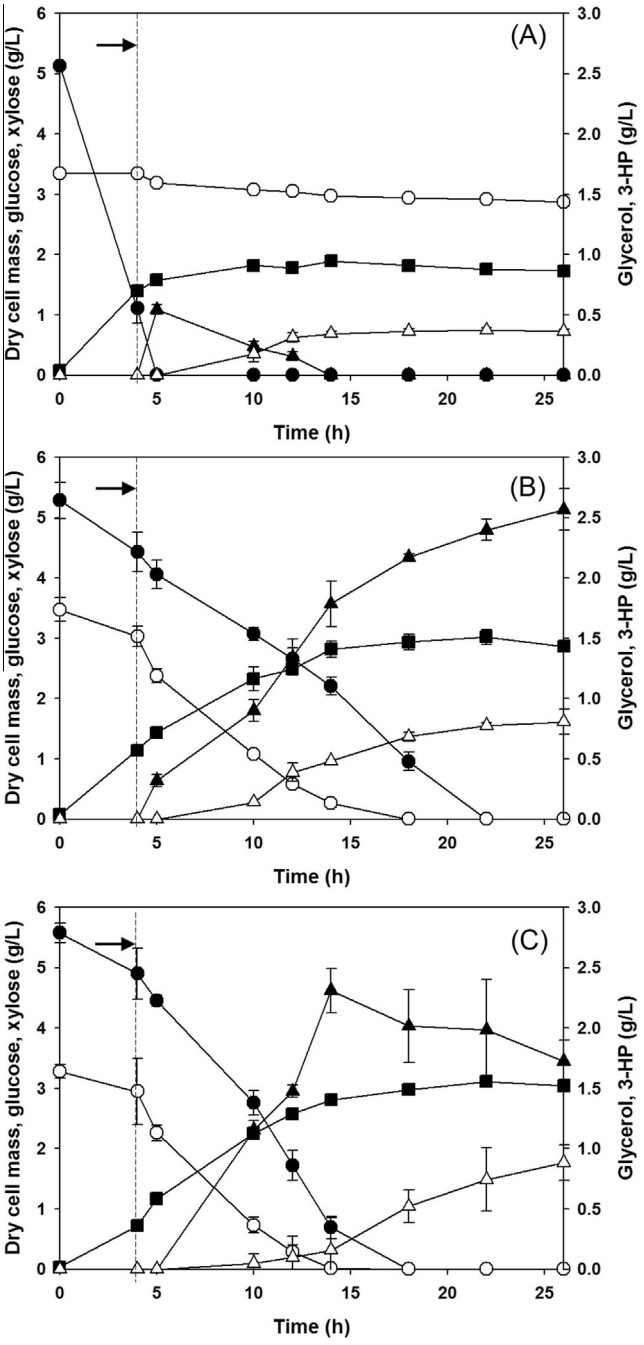

Fig. 3. Batch cultures of JHS01169/pELDRR + pCPaGG (A), JHS01300/pELDRR + pCPaGG (B) or pELDRR + pCPaGGRm (C) in R/5 medium with 5 g/L glucose and 3 g/L xylose. ■, dry cell mass; ●, glucose; ○, xylose; ▲, glycerol; △, 3-HP; arrow, IPTG induction, coenzyme B_{12} addition and temperature shift from 37 °C to 25 °C. The experiment was conducted in triplicate.

Table 5
Summarized results of batch and fed-batch cultures of recombinant *E. coli* strains producing 3-HP from glucose and xylose.

Strain	Plasmid	Culture type	Dry cell mass (g/L)	Glucose consumption rate (g/L-h)	Xylose consumption rate (g/L-h)	3-HP concentration (g/L)	3-HP productivity (g/L-h)[a]	3-HP yield (g/g total sugars)
JHS01169	pELDRR + pCPaGG	Batch	1.72 ± 0.13	1.03 ± 0.01	0.02 ± 0.00	0.36 ± 0.02	0.014 ± 0.001	0.04 ± 0.00
JHS01300	pELDRR + pCPaGG	Batch	2.86 ± 0.02	0.24 ± 0.01	0.20 ± 0.01	0.81 ± 0.10	0.033 ± 0.004	0.09 ± 0.01
	pELDRR + pCPaGGRm	Batch	3.00 ± 0.03	0.35 ± 0.02	0.27 ± 0.01	0.97 ± 0.06	0.037 ± 0.002	0.11 ± 0.01
	pELDRR + pCPaGGRm	Fed-batch	13.3	0.74	0.32	29.7	0.54	0.36

[a] 3-HP productivity was calculated in the period of glycerol production stage after IPTG induction.

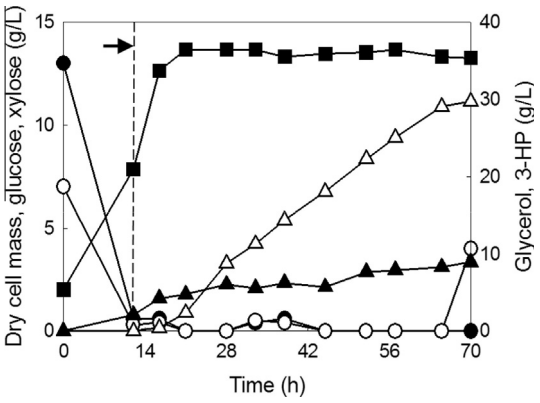

Fig. 4. Sugar-limited fed-batch fermentation of JHS01300/pELDRR + pCPaGGRm in R/5 medium containing 13 g/L glucose and 7 g/L xylose. After depletion of initial glucose and xylose, feeding of a mixture with 500 g/L glucose and 250 g/L xylose was started at 1 g/h of feeding rate (arrow). ■, dry cell mass; ●, glucose; ○, xylose; ▲, glycerol; △, 3-HP; arrow, IPTG induction, coenzyme B_{12} addition, temperature shift from 37 °C to 25 °C and agitation speed shift from 1300 rpm to 600 rpm.

3.7. Sugar-limited fed-batch culture for production of 3-HP from glucose and xylose

As mentioned above, the presence of glucose and xylose in culture medium prevented the efficient conversion of glycerol to 3-HP. To solve this problem, we designed a sugar-limited fed-batch fermentation to maintain basal levels of glucose and xylose in culture broth during fed-batch mode of operation. By considering that the ratio of consumption rate of glucose to xylose of JHS01300/pELDRR + pCPaGGRm was about 1.7:1, the concentrated feeding solution of 500 g/L glucose and 250 g/L xylose was formulated. After batch culture using initial glucose and xylose for 12 h, IPTG and coenzyme B_{12} were added and a concentrated feeding solution was fed at a constant rate (Fig. 4). By the sugar-limited fed-batch fermentation, JHS01300/pELDRR + pCPaGGRm finally produced 29.7 g/L of 3-HP with a yield of 0.36 g/g total sugar (equivalent to 0.67 mol/mol) and a productivity of 0.54 g/L-h (Table 5). A previous research for 3-HP production from glucose via the malonyl-CoA pathway reported that a fed-batch culture of recombinant *E. coli* BX3_0240 resulted in 49.0 g/L 3-HP concentration, 0.46 mol/mol of 3-HP yield and 0.71 g/L-h of productivity (Lynch et al., 2014). In spite of low titer and productivity, our strategy for 3-HP production from glucose and xylose via the glycerol pathway showed 45% higher 3-HP yield than the other method using the malonyl-CoA pathway. As shown in Fig. 4 and 95 g/L glycerol was still present at the end of the fermentation, which was probably ascribed to mismatch of carbon fluxes between glycerol biosynthesis and 3-HP production. To solve this problem and enhance all fermentation parameters for 3-HP production, concerted expression of genes involved in the biosynthesis of glycerol seems to be necessary to enhance the glucose and xylose consumption rate by modulation of the *xyl* operon.

4. Conclusion

To produce 3-HP from glucose and xylose, *E. coli* was engineered by introducing the glycerol biosynthetic enzymes encoded by *S. cerevisiae GPD1* and *GPP2* and deleting the chromosomal *ptsG* and overexpressing the endogenous *xylR*. The resulting strain, *E. coli* JHS01300/pELDRR + pCPaGGRm metabolized glucose and xylose simultaneously to glycerol and hence produced 3-HP ultimately. The sugar-limited fed-batch fermentation using the glucose and xylose mixture resulted in a remarkable enhancement of 3-HP production via the glycerol pathway. Dual modulation of CCR relief and glycerol biosynthesis is a promising strategy for efficient production of 3-HP from a mixture of glucose and xylose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.09.079.

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