JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Systematic Approach To Engineer *Escherichia coli* Pathways for Co-utilization of a Glucose−Xylose Mixture

Chung-Jen Chiang,[†] Hong Min Lee,[§] Hong Jhih Guo,[§] Zei Wen Wang,[§] Li-Jen Lin,[#] and Yun-Peng Chao*[,§,⊥]

[†]Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan 40402

[§]Department of Chemical Engineering, Feng Chia University, 100 Wenhwa Road, Taichung, Taiwan 40724

[#]School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan 40402

[⊥]Department of Biotechnology, Asia University, Taichung, Taiwan 41354

ABSTRACT: Glucose and xylose are two major sugars of lignocellulosic hydrolysate. The regulatory program of catabolite repression in *Escherichia coli* dictates the preferred utilization of glucose over xylose, which handicaps the development of the lignocellulose-based fermentation process. To co-utilize a glucose−xylose mixture, the *E. coli* strain was manipulated by pathway engineering in a systematic way. The approach included (1) blocking catabolite repression, (2) enhancing glucose transport, (3) increasing the activity of the pentose phosphate pathway, and (4) eliminating undesirable pathways. Moreover, the ethanol synthetic pathway from *Zymomonas mobilis* was introduced into the engineered strain. As a consequence, the resulting strain was able to simultaneously metabolize glucose and xylose and consume all sugars (30 g/L each) in 16 h, leading to 97% of the theoretical ethanol yield. Overall, this indicates that this approach is effective and straightforward to engineer *E. coli* for the desired trait.

KEYWORDS: *mixed sugars, metabolic engineering, biofuel, substituted chemicals*

INTRODUCTION

The enormous emission of greenhouse gas from the growing consumption of fossil fuels in recent decades has worsened the global climate. This results in global warming that exerts an adverse impact on the environment and human health. The insecure supply of fossil fuels and the drastic climate change have encouraged the search for alternative energy sources.[1] One good example of applying alternative energy is the current use of bioethanol for transportation. Bioethanol on the market has been produced from sugar cane and corn starch by a microbial fermentation process. However, these sugars are commonly plagued with the food-and-feed debate, and their reduction effect on greenhouse gas emission is not appreciated.[2] Apparently, these concerns have overshadowed sustainability of the edible feedstock-based microbial process for production of biofuels and substitutes for petroleum-based chemicals.

As recognized, lignocellulose is the most abundant resource in nature. Mainly from plant cell walls, this biomaterial is renewable and sustainably available. In addition to low costs and less environmental impact, production of biofuels and platform chemicals from lignocellulose is attractive because the process is not geographically restricted and can ease the ethical conflict between food and feed.[3] Recently, considerable research efforts have been put into developing an integrated production process of lignocellulosic fuels and value-added chemicals by microbes.[4] Cellulose and hemicellulose are two major components of lignocellulose. After hydrolysis, lignocellulosic biomass is decomposed to two dominant sugars, glucose and xylose. However, most naturally occurring microorganisms are unable to co-utilize these two sugars in an effective manner.[5] This unequivocally presents the first barrier to the development of a microbial fermentation process for the production of lignocellulosic fuels and chemicals.

Escherichia coli has been recognized as a bioprocess-friendly strain. This bacterial strain grows rapidly and can be easily cultured in a simple medium. A wealth of knowledge on the genetics and physiology of *E. coli* is also available, which greatly facilitates genetic manipulation of the strain. In particular, this bacterium is able to metabolize an array of monosaccharides.[6] However, *E. coli* prefers glucose to other sugars. It is recognized that glucose enters *E. coli* with the aid of glucose-specific permease $EIICB^{glc}$ (encoded by *ptsG*) in the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) (Figure 1). During glucose transport, the level of cyclic AMP (cAMP) is lowered by dephosphorylated $EIIA^{Glc}$ (encoded by *crr*), which in turn limits the availability of the cAMP and catabolite activator protein (cAMP−CAP) complex. The expression of genes that are involved in the catabolism of sugars other than glucose generally requires the cAMP−CAP complex and, consequently, is repressed. This type of a regulatory mechanism is known as catabolite repression.[6] Accordingly, a diauxic fermentation pattern occurs in *E. coli* when both glucose and xylose are present. Xylose is utilized only after complete depletion of glucose. Such a carbohydrate-utilizing pattern prolongs the fermentation process, which reduces the productivity and even makes xylose metabolism ineffective.[7]

In this study, we attempted to alter the physiological trait of *E. coli* for co-utilization of glucose and xylose using a molecular

Received: March 21, 2013
Revised: July 9, 2013
Accepted: July 14, 2013
Published: July 15, 2013

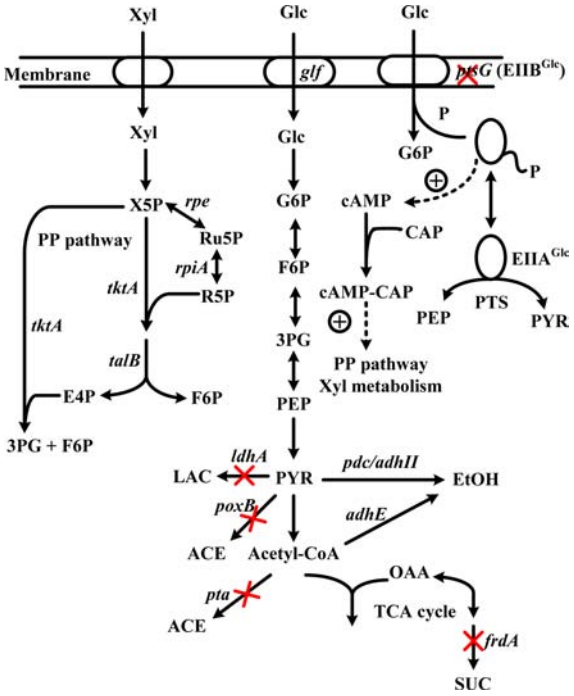

Figure 1. Central metabolic pathway leading to ethanol in *E. coli*. Shown are the pathways related to the synthesis of ethanol. The deleted genes are marked by "X". The gene encoding the enzyme that catalyzes the pathway reaction is indicated as follows: *adhII*, alcohol dehydrogenase; *frdA*, fumarate reductase; *glf*, glucose facilitator; *ldhA*, lactate dehydrogenase; *pdc*, pyruvate decarboxylase; *poxB*, pyruvate oxidase; *pta*, phosphate acetyltransferase; *ptsG*, glucose-specific PTS permease; *rpe*, ribulose-5-phosphate 3-epimerase; *rpiA*, ribose-5-phosphate isomerase A; *talB*, transaldolase B; *tktA*, transketolase A. Abbreviations: ACE, acetate; E4P, erythrose 4-phosphate; EtOH, ethanol; F6P, fructose 6-phosphate; Glc, glucose; G6P, glucose 6-phosphate; LAC, lactate; OAA, oxaloacetate; 3PG, 3-phosphoglycer-aldehyde; PEP, phosphoenol pyruvate; PYR, pyruvate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; SUC, succinate; TCA, tricarboxylic acid; Xyl, xylose; X5P, xylulose 5-phosphate.

approach. This was systematically carried out by (1) decoupling catabolite repression of glucose, (2) enhancing glucose transport, (3) increasing the activity of the pentose phosphate (PP) pathway, and (4) eliminating undesirable pathways that lead to waste products. To illustrate the bacterial phenotype, a heterologous synthesis pathway for ethanol was introduced into the engineered *E. coli* strain. Consequently, the resulting strain was able to coferment glucose and xylose and produced ethanol in an effective way.

MATERIALS AND METHODS

Bacterial Strains and Culturing Condition. *E. coli* strains DH5α and BW25142 were employed as intermediate cells for DNA cloning. Meanwhile, *E. coli* strain BL21 was engineered to produce ethanol. Unless stated otherwise, bacterial strains were grown on Luria−Bertani (LB) medium,[8] and the bacterial growth was monitored by turbidimetrical measurement at a wavelength of 550 nm. To produce ethanol, engineered strains were first cultured overnight. The overnight cultures were then inoculated into 125 mL Erlenmeyer flasks containing 25 mL of culture medium to have the initial cell density reach 0.33 mg dry cell weight (DCW)/mL. The culture medium consisted of LB medium with ampicillin (50 μg/mL) plus 30 g/L glucose and 30 g/L xylose. Alternatively, bacteria were grown in NBS minimal medium[9] augmented with 30 g/L glucose and 30 g/L xylose. The oxygen-limited fermentation was carried out by growing bacterial cultures in flasks that were sealed by parafilm and incubated in an orbital shaker at 37 °C and 150 rpm. Samples were collected for determination of sugars, ethanol, and organic compounds during the time course.

Gene Cloning. Table 1 lists the plasmids and primers applied in this study, and the genes subjected to manipulation are depicted in Figure 1. The *pdc* and *adhII* genes from *Zymomonas mobilis* were cloned by polymerase chain reaction (PCR) with primers Pdc1-Pdc2 and AdhE1-AdhE2, respectively. The resulting PCR DNAs containing the *pdc* and *adhII* genes were digested by either *Nde*I−*Bam*HI or *Bam*HI−*Xho*I. The two DNA fragments were purified and subsequently incorporated into the *Nde*I and *Xho*I sites of plasmid pND707[10] to produce plasmid pND-pet. As a result, plasmid pND-pet contains the *pdc* and *adhII* genes under the control of the $\lambda P_R P_L$ promoter ($\lambda P_R P_L$-*pdc-adhII*). Moreover, the DNA fragment containing $\lambda P_R P_L$-*pdc-adhII* was amplified from plasmid pND-pet by PCR with primers LaP1-LaP2. The resulting PCR DNA was then digested with *Bam*HI and spliced into integration plasmid pP21-Km,[11] which was cleaved with *Bam*HI−*Sma*I to obtain plasmid pP21-pet.

The *glf* gene of *Z. mobilis* was amplified by PCR with primers Glf1−Glf2. After the *Xba*I−*Xho*I digestion, the resulting PCR DNA was spliced into plasmid pND707 to give plasmid pND-glf. The DNA fragment containing the *glf* gene fused to the $\lambda P_R P_L$ promoter was then amplified by PCR with primers LaP1−LaP2 from plasmid pND-glf. Followed by the *Bam*HI digestion, the PCR DNA was incorporated into integration plasmid pHK-Km[11] that was restricted with *Bam*HI−*Sma*I to obtain plasmid pHK-glf.

The *rpe* and *tktA* genes were amplified by PCR from strain BL21 using primers Rpe1−Rpe2 and TktA1−TktA2, respectively. By either *Nde*I−-*Eco*RI or *Eco*RI−*Xho*I digestion, the two PCR DNAs were spliced into the *Nde*I and *Eco*RI sites of plasmid pND707 to produce plasmid pND-rTA. Moreover, the DNA fragment comprising the $\lambda P_R P_L$ promoter-driven *rpe* and *tktA* genes was amplified from plasmid pND-rTA by PCR with primers LaP1−LaP3. Cleaved with *Bam*HI, the resulting PCR DNA was incorporated into the *Bam*HI and *Sma*I sites of integration plasmid pPhi80-Km[11] to generate plasmid pPhi80-rTA

Similarly, the *rpiA* and *talB* genes of *E. coli* were amplified by PCR using primers RpiA1−RpiA2 and TalB1−TalB2, respectively. After cleavage of either *Nde*I−*Eco*RI or *Eco*RI−*Xho*I, the PCR DNAs were spliced into the *Nde*I and *Xho*I sites of plasmid pND707 to obtain plasmid pND-rTB. The DNA fragment involving the *rpiA* and *talB* genes driven by the $\lambda P_R P_L$ promoter was amplified from plasmid pND-rTB by PCR with primers LaP4-LaP5. The PCR DNA was cut by *Sal*I and then incorporated into the *Sal*I and *Sma*I sites of integration plasmid pLambda-Km,[11] consequently resulting in plasmid pLam-rTB.

Gene Insertion. The passenger genes carried by integration plasmids were integrated into *E. coli* genome essentially following the method as previously reported.[11] In brief, plasmid pHK-glf was transformed into the *ptsG* gene-deficient strain (i.e., BL-G) that harbored helper plasmid pAH69.[12] Plasmid pAH69 carries the HK022 integrase gene (*int*HK022) regulated by the λP_R promoter. The cells were then cultured in LB medium at 37 °C for 1 h, followed by exposure to 39 °C for another 3 h. The cells were harvested by centrifugation and spread onto LB agar plates plus kanamycin (25 μg/mL) at 39 °C overnight. The kanamycin-resistant integrants were further examined for sensitivity to ampicillin as an indicator of curing of helper plasmid. To remove the inserted marker and replicon, integrants were transformed with helper plasmid pCP20.[12] Plasmid pCP20 carries the Flp gene that is induced thermally. The plasmid-bearing integrants were cultured in a routine way at 30 °C. Upon exposure to 40 °C for 30 min, integrants were collected and spread on LB agar plates at 39 °C overnight. Integrants that showed sensitivity to kanamycin (indicating elimination of marker and replicon) and ampicillin (indicating loss of the helper plasmid) were verified for the inserted *glf* by in situ PCR. One resulting integrant was selected and designated BL-Gf.

In a similar manner, genomic insertion of the DNA containing the $\lambda P_R P_L$ promoter-driven *rpe* and *tktA* genes was carried out by transformation of plasmid pPhi80-rTA into strain BL-Gf bearing helper plasmid pAH123.[12] Plasmid pAH123 carries the Φ80 integrase gene (*int*Φ80). Subsequently, the redundant plasmid DNA backbone

Table 1. Strains, Plasmids, and Primers Applied in This Study[a]

		main characteristics	source
E. coli strain			
	BL21	F$^-$ *dcm gal ompT hsdS*(r_B^- m_B^-)	lab collection
	BL-G	as BL21Δ*ptsG*	this study
	BL-Gf	as BL-G HK022::λP_RP_L-*glf*	this study
	BL21e	as BL-Gf Φ80:: λP_RP_L-*rpe-tktA*	this study
	BL21e-RB	as BL21e λ:: λP_RP_L-*rpiA-talB*	this study
	BL-A1	as BL21e-RBΔ*poxB*::FRT-*kan*-FRT	this study
	BL-A2	as BL-A1Δ*pta*::FRT-*kan*-FRT	this study
	BL-A3	as BL-A2Δ*ldhA*::FRT-*kan*-FRT	this study
	BL-A4	as BL-A3Δ*frdA*::FRT-*kan*-FRT	this study
	BL21p	as BL-A4 P21:: λP_RP_L-*pdc-adhII*	this study
	BW25142	*endA1 recA1 hsdR514 rph1 galU95 uidA* (*MluI*)::*pir116*	12
	CGSC9031	Δ*ptsG*::FRT-*kan*-FRT	14
	CGSC9216	Δ*ldhA*::FRT-*kan*-FRT	14
	CGSC9216	Δ*frdA*::FRT-*kan*-FRT	14
	DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	lab collection
plasmid			
	pAH69	λP_R-*int*HK022 *bla*$^+$	12
	pAH123	λP_R-*int*Φ80 *bla*$^+$	12
	pAH121	λP_R-*int*P21 *bla*$^+$	12
	pCP20	λP_R-*flp bla*$^+$	12
	pHK-Km	HK*attP* FRT-*kan*-R6K *ori*-FRT	11
	pHK-glf	as pHK-Km but *glf*$^+$	this study
	pINT-ts	λP_R-*intλ bla*$^+$	12
	pKD13	FRT-*kan*-FRT	13
	pKD46	P*araBAD*-*λRed bla*$^+$	13
	pLambda-Km	*λattP* FRT-*kan*-R6K *ori*-FRT	11
	pLam-rTB	as pLambda-Km but *rpiA*$^+$ and *talB*$^+$	this study
	pMC-poxKm	Δ*poxB*::FRT-*kan*-FRT	this study
	pMC-ptaKm	Δ*pta*::FRT-*kan*-FRT	this study
	pND707	λP_RP_L *bla*$^+$	10
	pND-pet	as pND707 but *pdc*$^+$ and *adhII*$^+$	this study
	pND-glf	as pND707 but *glf*$^+$	this study
	pND-rTA	as pND707 but *rpe*$^+$ and *tktA*$^+$	this study
	pND-rTB	as pND707 but *rpiA*$^+$ and *talB*$^+$	this study
	pPhi80-Km	*ϕ80attP* FRT-*kan*-R6K *ori*-FRT	11
	pPhi80-rTA	as pPhi80-Km but *rpe*$^+$ and *tktA*$^+$	this study
	pP21-Km	P21*attP* FRT-*kan*-R6K *ori*-FRT	11
	pP21-pet	as pP21-Km but *pdc*$^+$ and *adhEII*$^+$	this study

		main characteristics source
primer		
	Glf1	TGTCTCTAGAAGCATGCAGGAGGAATCG
	Glf2	AGCAACTCGAGTTACTTCTGGGAGCGCCAC
	LaP1	AAGGGGGATCCATCTAACACCGTGCGTGTTG
	LaP2	AGCAACTCGAGTTACTTCTGGGAGCGCCAC
	Rpe1	TATACATATGAAACAGTATTTGATTGC
	Rpe2	CTGAATTCAAACTTATTCATGACTTACC
	TktA1	ACGGGAATTCAGGAGGAGTCAAAATG
	TktA2	GGGCCTCGAGTTACAGCAGTTCTTTTC
	Rpe3	AGGGGGATCCATCTAACACCGTGCGTGTTG
	LaP3	GGGCCTCGAGTTACAGCAGTTCTTTTC
	RpiA1	AATGCCATATGAATTTCATACCACAGGC-GAAAC
	RpiA2	TGGAGGAATTCCCGTCAGATCATTTCACAATG
	TalB1	TTTGAATTCAGGAGGATACTATCATGACG
	TalB2	CTAACTCGAGGTCGACGTTACAGCA GATCGCCGATC
	LaP4	AAGGGGGATCCATCTAACACCGTGCGTGTTG
	LaP5	CTAACTCGAGGTCGACGTTACAG CAGATCGCCGATC
	PtsG1	TGGGTGAAACCGGGCTGG
	PtsG2	AGCCGTCTGACCACCACG
	PoxB1	TTAGAAGCTTGCAGGGGTGAAACGCATCTG
	PoxB2	ATTAGACTAGTGGCTGGGTTGATATCAATC
	PoxB3	ATTAGGAATTCGTGATTGCGGTGGCAATC
	PoxB4	ATTAGGTCGACGGTACCAAACTG GCGCAACTGCTG
	KD1	TTAGGAATTCGTGTAGGCTGGAGCTGCTTC
	KD2	ATTCCGGGGATCCGTCGACC
	Pta1	TGTCCAAGCTTATTATGCTGATCCCTACC
	Pta2	GTTCGACTAGTTTAGAAATGCGCGCGTC
	Pta3	ACGATGAATTCCATCAGCACATCTTTCTG
	Pta4	ACCGTGTCGACGGTACCTGATCGC-GACTCGTGC
	LdhA1	TCTTATGAAACTCGCCGTTTATAG
	LdhA2	TTAAACCAGTTCGTTCGGGCAG
	FrdA1	GAAAGTCGACGAATCCCGCCCAGG
	FrdA2	CAAGAAAGCTTGTTGATAAGAAAGG
	Pdc1	TATACATATGAGTTATACTGTCGGTAC
	Pdc2	CCATGGATCCTTATCCTCCTCCGAGGAGCTTG
	AdhE1	ATGTGGATCCAGGATATAGCTATGGCTTCTT-CAACTTTTTATATTC
	AdhE2	AGGACTCGAGTTAGAAAGCGCTCAGGAAGAG

[a]Restriction sites included in primers are underlined. Abbreviations: *attP*, prophage attachment site; *bla*, ampicillin-resistant determinant; *kan*, kanamycin-resistant determinant; P*araBAD*, *araBAD* promoter; λP_RP_L, λP_RP_L promoter; λP_R, λP_R promoter; *ori*, plasmid replication origin.

flanked by FRT sites in the bacterial chromosome was removed by the act of Flp that was expressed from helper plasmid pCP20. The resulting integrant was renamed BL21e. Moreover, plasmid pLam-rTB was transformed into strain BL21e with helper plasmid pINT-ts[12] that expresses the λ integrase gene (*intλ*). The integrants with insertion of *rpiA* and *talB* genes were further manipulated for elimination of integrated marker and replicon by using plasmid pCP20. One resulting strain was chosen and designated BL21e-RB.

Finally, strain BL21p was obtained by integration of λP_RP_L-*pdc-adhII* on plasmid pP21-pet into strain BL-A4 (see below) with the aid of helper plasmid pAH121,[12] which contains the P21 integrase gene (*int*P21).

Gene Deletion. The *ptsG* gene of strain BL21 was deleted according to a previous study.[13] *E. coli* strain CGSC9031[14] carries the *ptsG* gene that is interrupted by insertion of a DNA cassette containing a kanamycin-resistant gene (*kan*) flanked by FRT sites (FRT-*kan*-FRT). The DNA bearing the *ptsG* gene inserted with FRT-*kan*-FRT along two homologous extensions was then amplified from strain CGSC9031 by PCR with primers PtsG1−PtsG2. The PCR DNA was electroporated into strain BL21 harboring helper plasmid pKD46,[13] which carries the λRed gene under the control of the arabinose promoter (P*araBAD*). After the λRed-mediated homologous recombination, integrants exhibiting resistance to kanamycin but susceptibility to ampicillin (loss of plasmid pKD46) were selected and proceeded to remove the integrated marker by using plasmid pCP20 as described. One resulting strain with the deletion of the *ptsG* gene was designated BL-G.

The DNA cassettes involving the *poxB* and *pta* genes inserted with FRT-*kan*-FRT were constructed in several steps. First, the *poxB* and *pta* genes were amplified by PCR with primers PoxB1−-PoxB2 and Pta1−Pta2, respectively. After digestion with *Hin*dIII−*Spe*I, the PCR DNAs were incorporated into plasmid pMCS-5 to obtain plasmids pMC-pox and pMC-pta. PCR was utilized to create the internal *Eco*RI and *Sal*I sites within the structural *poxB* and *pta* genes from plasmids pMC-pox and pMC-pta with primers PoxB3−PoxB4 and Pta3−Pta4. Meanwhile, FRT-*kan*-FRT tagged with *Eco*RI and *Sal*I sites was

dx.doi.org/10.1021/jf401230r | J. Agric. Food Chem. 2013, 61, 7583−7590

generated by PCR from plasmid pKD13[13] with primers KD1−KD2. Finally, the amplified FRT-*kan*-FRT was incorporated into the PCR-created *Eco*RI and *Sal*I sites of plasmids pMC-pox and pMC-pta to give plasmid pMC-poxKm and pMC-ptaKm. To delete the *poxB* gene, the DNA cassette in plasmid pMC-poxKm was amplified by PCR with primers PoxB1−PoxB2 and electroporated into strain BL21e-RB with helper plasmid pKD46. Subsequent removal of the inserted marker was conducted with plasmid pCP20, resulting in strain BL-A1. The *pta* gene of strain BL-A1 was eliminated exactly in the same way by using the PCR amplification of the DNA cassette from plasmid pMC-ptaKm with primers Pta1−Pta2. Later elimination of the inserted marker gave rise to strain BL-A2.

In a similar way, the DNA fragment comprising the *ldhA* gene interrupted with FRT-*kan*-FRT was amplified from strain CGSC9216[14] by PCR with primers LdhA1−LdhA2. This DNA fragment was used to introduce the deletion of the *ldhA* gene in strain BL-A2. After removal of the inserted marker, it resulted in strain BL-A3. Moreover, deletion of the *frdA* gene in strain BL-A3 was performed by PCR amplification of the DNA fragment that contains the FRT-*kan*-FRT-inserted *frdA* gene from strain CGSC10964[14] with primers FrdA1−FrdA2. The resulting strain without the marker was designated BL-A4.

Analytical Methods. Glucose and xylose were determined by high-performance liquid chromatography (HPLC) equipped with an ICSep ICE-ION-300 column (Transgenomic, USA). The mobile phase composed of 0.0085 N sulfuric acid was pumped at 0.4 mL/min, and the eluate was analyzed with the refractive index (RID-10A, Shimadzu, Japan) at 40 °C. With the same HPLC apparatus, organic acids were analyzed using the UV detector at 60 °C. Meanwhile, ethanol was measured by gas chromatography (GBPI, Korea) loaded with a Porapak Q 80/100 column (Supelco, USA). The carrier gas consisting of air, nitrogen, and hydrogen was provided at the predetermined gauge pressure following the manufacturer's instruction.

RESULTS

Co-utilization of Glucose and Xylose. The strategies for pathway engineering of *E. coli* are outlined in Figure 1. As reported previously, the expression of the *lacZ* gene that is subject to catabolite repression could be induced in a *ptsG*-deficient *E. coli* strain irrespective of glucose.[15] This result implies that a null mutation of *ptsG* decouples the glucose-mediated control circuit. Strain BL-G was first constructed by deletion of the *ptsG* gene in strain BL21. In this study, production of ethanol was taken as an example for illustration. Therefore, plasmid pND-pet carrying the *pdc* and *adhII* genes of *Z. mobilis* was then transformed into strains BL21 and BL-G, thus resulting in recombinant BL21/pND-pet and BL-G/pND-pet strains. The fermentation of the recombinant strains was then carried out in the presence of glucose and xylose. As shown in Figure 2A, strain BL21/pND-pet consumed all of the glucose but barely metabolized xylose. In contrast, strain BL-G/pND-pet was able to co-utilize glucose and xylose while it consumed all of the glucose and 60% of xylose at the end of the fermentation. However, the consumption rate of glucose for the BL-G/pND-pet strain was slower than that for the BL21/pND-pet strain. This indicates that the deficient *ptsG* impairs glucose utilization of *E. coli*. Finally, strains BL21/pND-pet and BL-G/pND-pet produced ethanol with yields of 14.3 and 22 g/L (Figure 2B), respectively.

Enhancement of the Glucose-Utilizing Rate. The *ptsG*-defective BL-G strain was able to co-metabolize glucose and xylose but at the expense of the glucose utilization rate. It has been reported that the *glf* gene encoding the glucose facilitator of *Z. mobilis* is functional in *E. coli*.[16] Therefore, the *glf* gene was introduced into strain BL-G to give strain BL-Gf. Strain BL-Gf with plasmid pND-pet (i.e., BL-Gf/pND-pet) was then cultured in a similar way and examined for its fermentative

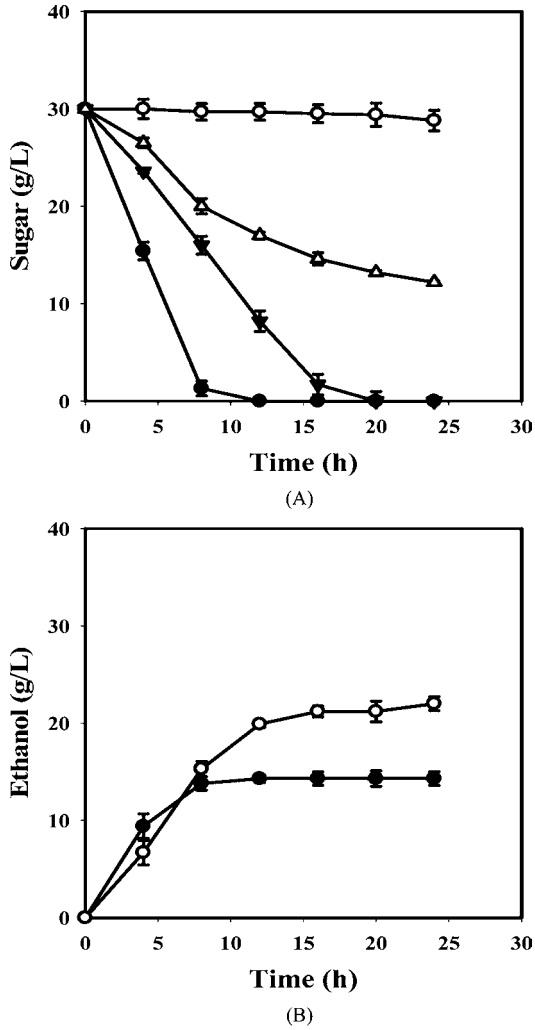

Figure 2. Time profiles of bacterial fermentation: (A) sugar consumption curve (●, glucose for strain BL21/pND-pet; ○, xylose for strain BL21/pND-pet; ▼, glucose for strain strain BL-G/pND-pet; △, xylose for strain BL-G/pND-pet); (B) ethanol production curve (●, strain BL21/pND-pet; ○, strain BL-G/pND-pet). Recombinant strains BL21/pND-pet and BL-G/pND-pet were grown in shake flasks containing LB medium with glucose and xylose. Sugars and ethanol concentrations in the culture broth were determined during the time course. The experiments were conducted in triplicate.

performance during the time course. Consequently, the glucose-utilizing rate of strain BL-Gf/pND-pet increased, whereas the xylose-utilizing rate of the strain was not improved (Figure 3 and Table 2). This suggests that the functional *glf* recovers the capability of glucose transport in the *ptsG*-lacking strain. Finally, the BL-Gf/pND-pet strain produced ethanol of 23 g/L at the end of fermentation.

Enhancement of the PP Pathway. The poor xylose catabolism in strain BL-Gf/pND-pet implies the presence of rate-limiting steps in the xylose-utilizing pathway. Accordingly, the PP pathway of the strain was enhanced (Figure 1). An extra copy of the *rpiA*, *tktA*, *rpe*, and *talB* genes under the control of a heterologous promoter was integrated into strain BL-Gf, consequently generating strain BL21e-RB. The inserted gene clusters of *rpe*−*tktA* and *rpiA*−*talB* in the BL21e-RB strain were further verified by in situ PCR using primers LaP1−LaP3 and LaP4−LaP5, respectively. Under a similar culturing condition, strain BL21e-RB with plasmid pND-pet (i.e., BL21e-RB/pND-pet)

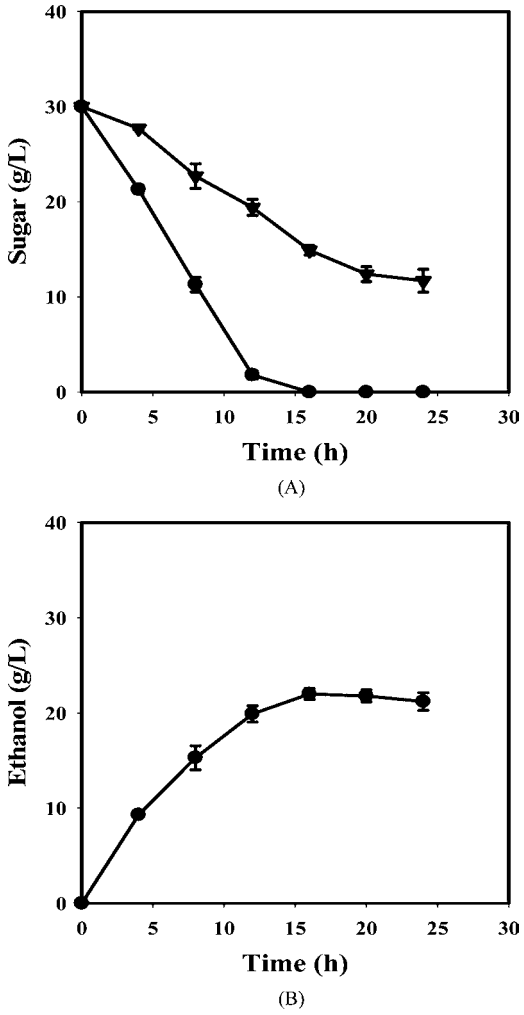

Figure 3. Fermentation profiles for strain BL-Gf/pND-pet: (A) sugar consumption curve (●, glucose; ▼, xylose); (B) ethanol production curve. The shake flask culture was performed in LB medium with glucose and xylose. The experiments were conducted in triplicate.

Table 2. Summary of Fermentation Kinetics for *E. coli* Strains[a]

strain[b]	medium	μG (g/L-h)	μX (g/L-h)	YE (g/L-h)
BL21	LB	3.0 ± 0.2	0.03 ± 0.00	1.7 ± 0.2
BL-G	LB	1.8 ± 0.1	1.1 ± 0.1	1.7 ± 0.1
BL-Gf	LB	2.3 ± 0.1	0.9 ± 0.0	1.6 ± 0.1
BL21e-RB	LB	2.4 ± 0.1	1.3 ± 0.1	2.0 ± 0.1
BL-A4	LB	2.8 ± 0.2	2.4 ± 0.2	2.0 ± 0.1
BL21p	NBS	2.1 ± 0.1	1.8 ± 0.1	1.6 ± 0.1

[a]Each strain exhibited a comparable growth and biomass production. The data were taken from the linear range of experiments shown in Figures 2−6. The concentration of glucose and xylose in the indicated medium was 30 g/L for each. μG, volumetric consumption rate of glucose; μX, volumetric consumption rate of xylose; YE, volumetric productivity of ethanol. [b]Except strain BL21p, all *E. coli* strains harbor plasmid pND-pet.

was grown in a mixture containing glucose and xylose. As compared to the parent BL-G/pND-pet strain, strain BL21e-RB/pND-pet exhibited a higher consumption rate of xylose and a comparable utilization rate of glucose (Figure 4 and Table 2). Nevertheless, strain BL21e-RB/pND-pet was still unable to consume all of the xylose and produced ethanol of 26 g/L at the end.

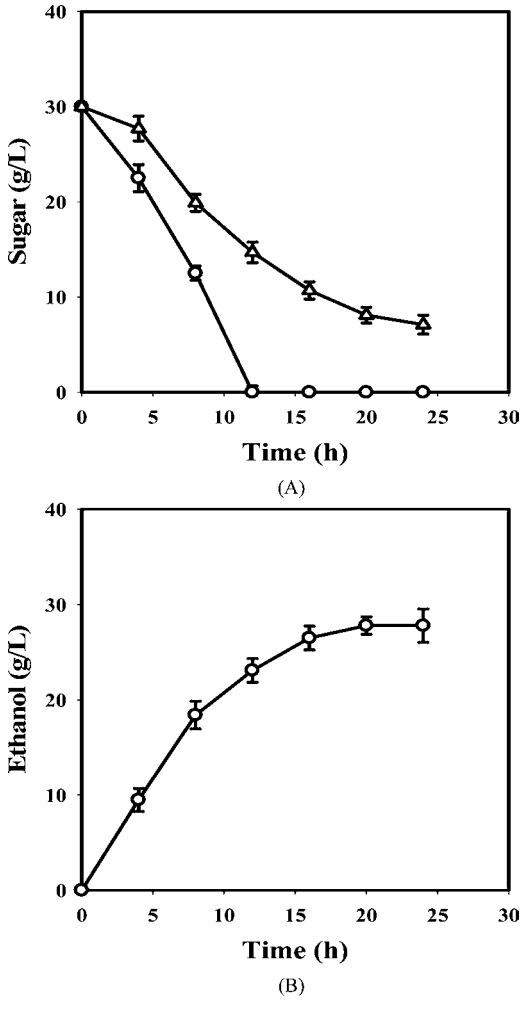

Figure 4. Fermentation profiles for strain BL21e-RB/pND-pet: (A) sugar consumption curve (○, glucose; △, xylose); (B) ethanol production curve. The bacterial culture in shake flasks was carried out in LB medium with glucose and xylose. The experiments were conducted in triplicate.

Elimination of Competing Pathways. *E. coli* is known to undergo mixed-acid fermentation. As analyzed, ethanol was a major product for strain BL21e-RB/pND-pet, whereas the strain also produced small amounts of organic acids, including lactate, succinate, and acetate. In the wild-type BL21 strain, consumption of xylose was severely retarded even after glucose was exhausted (Figure 2). This is likely due to a combined inhibitory effect of mixed acids, which results in the perturbed flux distribution in cells. This fact probably also holds true for strain BL21e-RB. Elimination of undesired pathways leading to these waste products is expected to be favorable for the strain's performance. Accordingly, the *ldhA*, *poxB*, *pta*, and *frdA* genes of strain BL21e-RB were all deleted (Figure 1), resulting in strain BL-A4. Strain BL-A4 carrying plasmid pND-pet (i.e., BL-A4/pND-pet) was cultured in a similar way. As a consequence, glucose and xylose were simultaneously consumed and all sugars were depleted around 16 h (Figure 5). In addition, strain BL-A4/pND-pet produced ethanol with a yield reaching 29 g/L at the end of fermentation. This ethanol yield accounts for 97% of the theoretical yield.

Finally, it was intriguing to see how the developed strain performed in a cost-effective medium. Therefore, the *pdc* and *adhII* genes were integrated into strain BL-A4 to give strain

dx.doi.org/10.1021/jf401230r | J. Agric. Food Chem. 2013, 61, 7583−7590

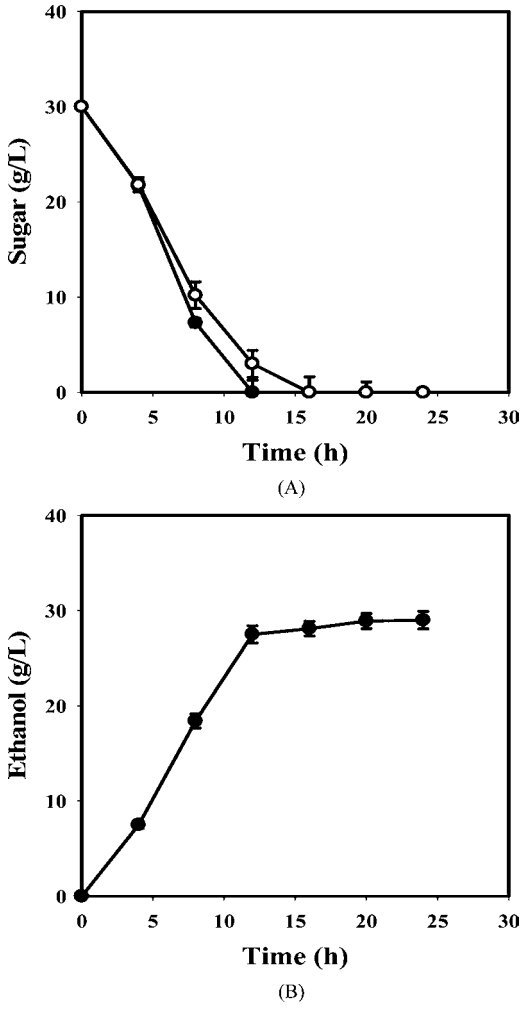

Figure 5. Fermentation profiles for strain BL-A4/pND-pet: (A) sugar consumption curve (●, glucose; ○, xylose); (B) ethanol production curve. The shake flask culture was carried out in LB medium with glucose and xylose. The experiments were conducted in triplicate.

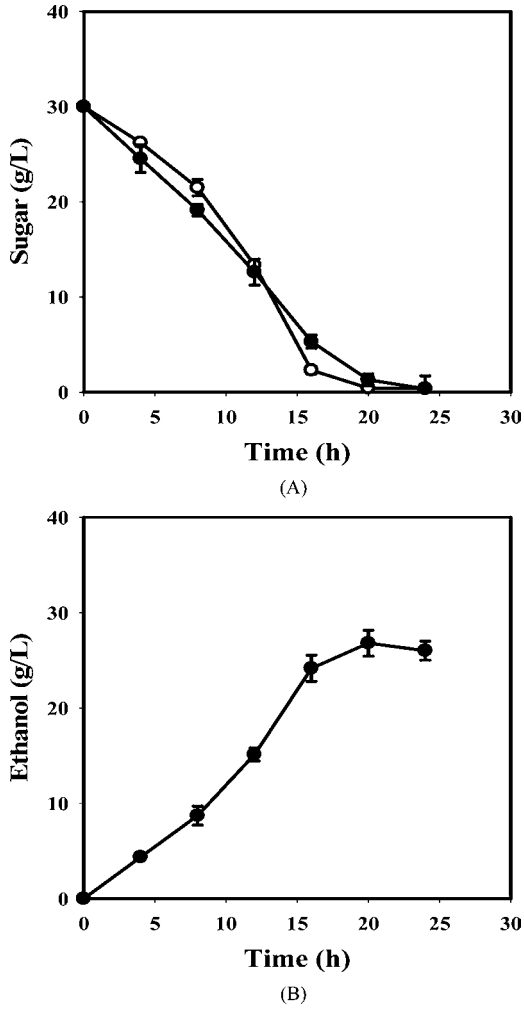

Figure 6. Fermentation profiles for strain BL21p: (A) sugar consumption curve (○, glucose; ●, xylose); (B) ethanol production curve. The shake flask culture was grown in NBS minimal medium with glucose and xylose. The experiments were conducted in triplicate.

BL21p. The plasmid-free strain was then grown in a minimal medium containing the sugar mixture. As a result, the BL21p strain was able to co-metabolize glucose and xylose and consumed all of these two sugars roughly at the same time (Figure 6). At the end, the strain produced around 27 g/L ethanol.

■ DISCUSSION

Economic production of fuels or chemicals from lignocellulosic hydrolysates cannot be realized until microbes are able to efficiently utilize sugar mixtures.[5] However, catabolite repression is a prevailing phenomenon that programs the sequential utilization of sugars by microbes.[6] Other sugars are not metabolized until glucose is used up, which makes utilization of sugar mixtures ineffective. In this study, the *ptsG* gene was inactivated to deregulate the catabolite repression in *E. coli*. In agreement with a previous study,[17] the *ptsG*-negative strain (i.e., BL-G) with plasmid pND-pet enabled the co-metabolization of glucose and xylose but at the expense of the glucose utilization rate (Table 2). In *E. coli*, three alternative systems are known for transport of glucose, including *manXYZ*, *mglABC*, and *galP*.[7] The expression of *mglABC* is repressed by Mlc, and Mlc can be sequestered by the dephosphorylated PtsG.[18] In the absence of *ptsG*, Mlc becomes free and exerts its repression on *manXYZ*. Therefore, MglABC and/or GalP are likely involved in glucose transport in strain BL-G but appear to be less effective than PtsG.

To improve the bacterial glucose utilization capability, the *glf* gene of *Z. mobilis* was introduced into strain BL-G. The resulting BL-Gf strain with plasmid pND-pet exhibited a 30% increase in the volumetric consumption rate of glucose compared with strain BL-G carrying plasmid pND-pet (Table 2). Glf in *E. coli* is reported to exhibit a xylose-transporting activity, which is subject to inhibition by glucose.[19] Nevertheless, the contribution of Glf to xylose transport in strain BL-Gf that lacks *ptsG* is unclear. Moreover, strain BL21e-RB was constructed with enhancement of all key enzymes in the nonoxidative branch of the PP pathway in strain BL-Gf. Consequently, strain BL21e-RB with plasmid pND-pet exhibited 45 and 25% increases in the volumetric consumption rate of xylose and the ethanol productivity relative to strain BL-Gf carrying plasmid pND-pet (Table 2). This result suggests that xylose catabolism of the *E. coli* strain is limited in the nonoxidative branch of the PP pathway.

E. coli is known to undergo mixed-acid fermentation. As illustrated in the wild-type BL21 strain with plasmid pND-pet, consumption of xylose was severely retarded even after glucose

was exhausted (Figure 2). This is likely due to a combined inhibitory effect of mixed acids that results in perturbed flux distribution in cells. This fact probably also holds true for strain BL21e-RB. After deletion of the gene for the synthesis of organic acids, the resulting BL-A4 strain with plasmid pND-pet exhibited an improved volumetric consumption rate of glucose and of xylose (Table 2). In particular, the xylose utilization rate of the strain was increased by >75% and to a level comparable to the glucose assimilation rate. Nevertheless, ethanol productivity for strain BL-A4 remained unimproved. This suggests that the ethanol production rate is likely saturated in the strain under the experimental condition. Finally, an ethanol-producing strain free of plasmids (i.e., BL21p) was constructed from strain BL-A4. Strain BL21p was still able to co-utilize the glucose−xylose mixture and to exhibit a high efficiency of ethanol production in minimal medium (Figure 6).

Several alternative strategies have been proposed to achieve co-utilization of the glucose−xylose mixture in *E. coli*. In one case, the PTS-negative mutant with a glucose-negative phenotype is created by deletion of *ptsHI* and *crr* genes. After the evolutional selection, this mutant strain restores its growth on glucose (denoted PTS^- Glu^+) resulting from high cellular activity of GalP and Glk.[20] Furthermore, the PTS^- Glu^+ strain is subject to adaptive evolution to achieve co-utilization of glucose and xylose.[21] In another example, an *E. coli* LY160 strain is obtained from a lactate-overproducing strain that is randomly inserted with the ethanol-synthesized genes of *Z. moblilis* and selected in a minimal medium with xylose.[22] After deletion of the *mgsA* gene, a derivate of strain LY160 is found to increase the ability of co-utilizing sugar mixtures.[23] As reported, the level of glucose-6-phosphate (G6P) is high in the *mgsA*-deficient strain.[24] A high level of G6P renders the *ptsG* mRNA unstable, which in turn lowers the uptake rate of glucose.[25] In addition, a CRP* mutant strain that does not require cAMP to activate expression of the CRP−cAMP complex-controlled genes is applied to xylitol production from the glucose−xylose mixture.[26] However, the CRP*-mediated effect seems to be strain-dependent and is unable to completely abolish catabolite repression. Recently, one interesting study has reported the construction of a minimal strain for efficient production of ethanol simply by reducing the functional space of the central metabolism.[27] As a consequence, the approach leads the engineered strain to co-utilization of glucose and xylose. However, this minimal strain shows a slow utilization rate of glucose relative to its parent strain.

In summary, our proposed method of pathway engineering is feasible and straightforward to manipulate *E. coli* for efficient co-utilization of glucose and xylose in either complex or minimal medium (Figure 6). In particular, this method does not involve any adaptive evolution and selection, which is in contrast to most previously reported approaches. The usefulness of the sugar mixture-utilizing strain BL-A4 is not limited and can also be applied to the production of value-added chemicals, such as lactic acid.[28]

AUTHOR INFORMATION

Corresponding Author

*(Y.-P.C.) E-mail: ypchao@fcu.edu.tw. Phone: 886-4-24517250, ext. 3677. Fax: 886-4-24510890.

Funding

This work is supported by the National Science Council of Taiwan (NSC 98-3114-E-035-001-CC2; NSC 101-2221-E-035-057-MY3) and the Ministry of Economic Affairs (101-EC-17-A-10-S1-156).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Schubert, C. Can biofuels finally take center stage? *Nat. Biotechnol.* **2006**, *24*, 777−784.

(2) Farrell, A. E.; Plevin, R. J.; Turner, B. T.; Jones, A. D.; O'Hare, M.; Kammen, D. M. Ethanol can contribute to energy and environmental goals. *Science* **2006**, *311*, 506−508.

(3) Himmel, M. E.; Ding, S. Y.; Johnson, D. K.; Adney, W. S.; Nimlos, M. R.; Brady, J. W.; Foust, T. D. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **2007**, *315*, 804−807.

(4) Geddes, C. C.; Nieves, I. U.; Ingram, L. O. Advances in ethanol production. *Curr. Opin. Biotechnol.* **2011**, *22*, 312−319.

(5) Kim, J. H.; Block, D. E.; Mills, D. A. Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1077−1085.

(6) Deutscher, J.; Francke, C.; Postma, P. W. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev* **2006**, *70*, 939−1031.

(7) Gosset, G. Improvement of *Escherichia coli* production strains by modification of the phosphoenolpyruvate:sugar phosphotransferase system. *Microb. Cell Fact.* **2005**, *4*, 14.

(8) Miller, J. H. *Experiments in Molecular Genetics*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1972.

(9) Martinez, A.; Grabar, T. B.; Shanmugam, K. T.; Yomano, L. P.; York, S. W.; Ingram, L. O. Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. *Biotechnol. Lett.* **2007**, *29*, 397−404.

(10) Love, C. A.; Lilley, P. E.; Dixon, N. E. Stable high-copy-number bacteriophage λ promoter vectors for overproduction of proteins in *Escherichia coli*. *Gene* **1996**, *176*, 49−53.

(11) Chiang, C. J.; Chen, P. T.; Chao, Y. P. Replicon-free and markerless methods for genomic insertion of DNAs in phage attachment sites and controlled expression of chromosomal genes in *Escherichia coli*. *Biotechnol. Bioeng.* **2008**, *101*, 985−995.

(12) Haldimann, A.; Wanner, B. L. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. *J. Bacteriol.* **2001**, *183*, 6384−6393.

(13) Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640−6645.

(14) Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2006**, *2*, 2006.0008.

(15) Chatterjee, R.; Millard, C. S.; Champion, K.; Clark, D. P.; Donnelly, M. I. Mutation of the *ptsG* gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*. *Appl. Environ. Microbiol.* **2001**, *67*, 148−154.

(16) Parker, C.; Barnell, W. O.; Snoep, J. L.; Ingram, L. O.; Conway, T. Characterization of the *Zymomonas mobilis* glucose facilitator gene product (*glf*) in recombinant *Escherichia coli*: examination of transport mechanism, kinetics and the role of glucokinase in glucose transport. *Mol. Microbiol.* **1995**, *15*, 796−802.

(17) Nichols, N. N.; Dien, B. S.; Bothast, R. J. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 120−125.

(18) Plumbridge, J. Regulation of gene expression in the PTS in *Escherichia coli*: the role and interactions of Mlc. *Curr. Opin. Microbiol.* **2002**, *5*, 187−193.

(19) Ren, C.; Chen, T.; Zhang, J.; Liang, L.; Lin, Z. An evolved xylose transporter from *Zymomonas mobilis* enhances sugar transport in *Escherichia coli*. *Microb. Cell Fact.* **2009**, *8*, 66.
(20) Hernández-Montalvo, V.; Martinez, A.; Hernández-Chavez, G.; Bolivar, F.; Valle, F.; Gosset, G. Expression of *galP* and *glk* in a *Escherichia coli* PTS mutant restores glucose transport and increases glycolytic flux to fermentation products. *Biotechnol. Bioeng.* **2003**, *83*, 687−694.
(21) Balderas-Hernández, V. E.; Hernández-Montalvo, V.; Bolívar, F.; Gosset, G.; Martínez, A. Adaptive evolution of *Escherichia coli* inactivated in the phosphotransferase system operon improves co-utilization of xylose and glucose under anaerobic conditions. *Appl. Biochem. Biotechnol.* **2011**, *163*, 485−496.
(22) Yomano, L. P.; York, S. W.; Zhou, S.; Shanmugam, K. T.; Ingram, L. O. Re-engineering *Escherichia coli* for ethanol production. *Biotechnol. Lett.* **2008**, *30*, 2097−2103.
(23) Yomano, L. P.; York, S. W.; Shanmugam, K. T.; Ingram, L. O. Deletion of methylglyoxal synthase gene (*mgsA*) increased sugar co-metabolism in ethanol-producing *Escherichia coli*. *Biotechnol. Lett.* **2009**, *31*, 1389−1398.
(24) Yao, R.; Hirose, Y.; Sarkar, D.; Nakahigashi, K.; Ye, Q.; Shimizu, K. Catabolic regulation analysis of *Escherichia coli* and its *crp*, *mlc*, *mgsA*, *pgi* and *ptsG* mutants. *Microb. Cell Fact.* **2011**, *10*, 67.
(25) Morita, T.; El-Kazzaz, W.; Tanaka, Y.; Inada, T.; Aiba, H. Accumulation of glucose 6-phosphate or fructose 6-phosphate is responsible for destabilization of glucose transporter mRNA in *Escherichia coli*. *J. Biol. Chem.* **2003**, *278*, 15608−15614.
(26) Khankal, R.; Luziatelli, F.; Chin, J. W.; Frei, C. S.; Cirino, P. C. Comparison between *Escherichia coli* K-12 strains W3110 and MG1655 and wild-type *E. coli* B as platforms for xylitol production. *Biotechnol. Lett.* **2008**, *30*, 1645−1653.
(27) Trinh, C. T.; Unrean, P.; Srienc, F. Minimal *Escherichia coli* cell for the most efficient production of ethanol from hexoses and pentoses. *Appl. Environ. Microbiol.* **2008**, *74*, 3634−3643.
(28) Chao, Y. P.; Chiang, C. J.; Lee, H. M.; Wang, Z. W.; Chen, P. T. Method for simultaneous fermentation of pentose and hexose. U.S. patent pending, 2012.