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METABOLIC PATHWAY MODIFICATION FOR PRODUCTION OF XYLITOL FROM GLUCOSE IN *ESCHERICHIA COLI*

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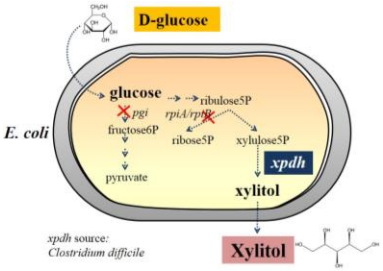
Graphical abstract



Abstract

Glucose is a cheap and readily available substrate for production of large-scale chemicals. Synthesis of xylitol, a high demand chemical in global market is currently done by using xylose, which contributes to its high operational cost. Studies on production of xylitol from glucose have explored several approaches, from sequential fermentation to multiple and single gene expression. Xylitol-5-phosphate dehydrogenase (XPDH), is an enzyme that enables conversion of glucose to xylitol in a single step fermentation. This study explores conversion of xylitol from glucose in *E. coli* by the expression of *xpdh* from *Clostridium difficile* with modifications in metabolic pathways to enhance xylitol production. The *xpdh* gene was carried by pACYC-Duet-1 expression vector and induced by the addition of IPTG. Initial screening of *E. coli* expressing *xpdh* (NA116) was done by shake-flask fermentation for 24 hours and its metabolites were analyzed by HPLC. NA116 was able to produce 0.273 g/L xylitol from 4.33 g/L consumed glucose in 24 hours. Further metabolic pathway modification to eliminate competing pathways yielded four mutants, NA207 (Δ*rpiA*), NA208 (Δ*rpiB*), NA209 (Δ*pgi*) and NA211 (Δ*rpi*Δ*pgi*). Screening of mutants for xylitol production showed that highest xylitol production from glucose was achieved by NA211 with almost double the amount of the original strain, 0.585 g/L. This showed successful xylitol conversion from glucose in a single fermentation in *E. coli* with improved yield through metabolic pathway modification.

Keywords: Xylitol, metabolic engineering, glucose, *Escherichia coli*, single gene

Abstrak

Glukosa ialah substrat yang murah dan mudah didapati bagi penghasilan bahan kimia berskala besar. Xilitol, satu bahan kimia yang mempunyai permintaan tinggi di pasaran global disintesis menggunakan xilosa, yang menyumbang kepada kos operasi yang tinggi. Kajian tentang penghasilan xilitol daripada glukosa telah meneroka beberapa pendekatan, daripada penapaian berurutan kepada pengekspresani beberapa atau satu gen. Xilitol-5-fosfat dehidrogenase (XPDH), ialah satu enzim yang membenarkan penukaran glukosa kepada xilitol dalam satu langkah penapaian. Kajian ini meneroka penukaran xilitol daripada glukosa dalam *E. coli* melalui pengekspresan *xpdh* daripada *Clostridium difficile* dan melakukan pengubahsuaian dalam laluan metabolik *E. coli* bagi meningkatkan pengeluaran xyiitol. Gen *xpdh* dibawa oleh vektor pengekspresan pACYC-Duet-1 dan diaruh dengan penambahan IPTG. Pemeriksaan awal *E. coli* yang mengekspreskan *xpdh* (NA116) dijalankan melalui penapaian di dalam kelalang kon selama 24 jam dan

metabolitnya dianalisa menggunakan HPLC. NA116 mampu menghasilkan 0.273 g/L xilitol daripada 4.33 g/L glukosa yang diambil oleh *E. coli* dalam tempoh 24 jam. Pengubahsuaian laluan metabolik selanjutnya dilakukan untuk menghapuskan laluan bersaing telah berjaya menghasilkan empat mutan, NA207 (Δ*rpiA*), NA208 (Δ*rpiB*), NA209 (Δ*pgi*) dan NA211 (Δ*rpiA*Δ*pgi*). Saringan mutan bagi pengeluaran xilitol menunjukkan bahawa pengeluaran xilitol tertinggi daripada glukosa dicapai oleh NA211 dengan hampir dua kali ganda jumlah mikrob asal (NA116), 0.585 g/L. Ini menunjukkan bahawa xyiitol dapat dihasilkan daripada glukosa dalam hanya satu proses penapaian di dalam *E. coli*, dan juga berlaku peningkatan dalam jumlah xilitol melalui pengubahsuaian jalan metabolik.

Kata kunci: xilitol, kejuruteraan metabolik, glukosa, *Escherichia coli*, gen tunggal

1.0 INTRODUCTION

Xylitol is a five-carbon sugar alcohol that has gained high demand in recent years due to its wide applications in various industries. It has been identified as one of the top ten value-added chemicals from biomass by the Department of Energy, United States [1]. In 2016, global market of xylitol was estimated to have reached 190.9 thousand metric tons and valued at USD725.9 million, where 70% of the demand comes from chewing gums and confectionery manufacturing [2]. Demands for xylitol has increased over 40 times over four decades, with production of 6000 tons in 1978 to 190.0 thousand tons in 2016. It is estimated to reach 266.5 thousand metric tons and valued at USD 1 billion in 2022 [3, 4].

Xylitol is a naturally occurring polyol, which presents in a minute quantity of about 1% in fruits and vegetables such as strawberry, raspberry, spinach and cauliflower [5]. It is the sweetest of the polyols, with a sweetness level similar to sucrose but only has about half caloric value of sucrose (2.4 cal/g to 4.0 cal/g) [6]. Industrial applications of xylitol involve food, pharmaceuticals, and odontological due to its advantages of being anti-cariogenic, anti-diabetic, has low-glycemic index, aids in tooth rehardening, and prevents otitis, ear and upper respiratory infections [7]. Metabolism of xylitol in the body is independent of insulin which makes it a suitable sugar alternative for diabetic patients and post-surgical patients [8, 9]. It is also a natural prebiotic by decreasing fecal pH and accommodates the growth of gram-positive gut bacteria [10, 11].

Current global production of xylitol is done through chemical hydrolysis of pure xylose using Raney nickel catalyst. The process involves the extraction and purification of xylose from lignocellulosic biomass and then hydrogenation of xylose to xylitol [12]. Although this process yields high xylitol with 98% conversion from pure xylose, the catalytic hydrogenation process is hindered by two main factors. Firstly, the process requires the use of pure xylose which involves multiple purification steps of hardwood hydrolysate, and secondly, the reduction process is energy consuming where it is carried out at high temperature and high pressure (80-140 °C; ~50 atm) [13]. These factors contribute to high operational costs and thus expensive xylitol. Therefore, development of a microbial system for production of xylitol is highly desirable as it could utilize impure carbon sources and operate at mild conditions.

Studies on microbial systems for xylitol production have explored various microorganisms including fungi, yeast and bacteria [14–16]. Most researches have focused on the conversion of xylitol from xylose by the expression of xylose reductase gene (XR). However, xylose is an expensive raw material with an approximate of USD 2500 per ton [17]. In recent years, several molecular approaches have been applied to also enable conversion of xylitol from other lignocellulosic sugars such as glucose and arabinose [18–22]. The use of glucose as a substrate for xylitol conversion has attracted attention due to it being readily available and cheap for large scale applications. The first report on glucose to xylitol conversion was done in three sequential fermentation steps, which involved three different microbes for conversion of glucose to arabitol, then to xylulose and then xylitol [23]. The process was further developed in a mixed-culture fermentation by *Gluconobacter oxydans* and *Escherichia coli* expressing xylitol dehydrogenase (*xdh*) in which allowed conversion of xylitol from xylulose and arabitol, two intermediates in glucose metabolism [24]. A single fermentation process using the same technique was reported in *Pichia pastosis* which expressed D-arabitol dehydrogenase and xylitol dehydrogenase [25]. Meanwhile, a more simple manipulation of xylitol conversion from glucose was observed in *Bacillus subtilis* by the expression of a single gene, *xpdh* which converts xylulose-5-phosphate to xylitol [26].

E. coli is a well-known industrial microbe, which has been applied for industrial production of succinic acid, lactic acid, 1,3-propanediol and other value added chemicals [27]. As a host, it has several advantages compared to other industrial microbes for the ability to use various carbon sources in both aerobic and anaerobic growth, has high growth and metabolic rates, well established genetic, metabolic

and physiological background, and has numerous genetics tools for metabolic engineering [28].

Xylitol conversion in *E. coli* has been studied, with most focus on using xylose or D-arabitol as substrate [29–34]. As to date, there has yet a report for a single fermentation step for glucose to xylitol conversion in *E. coli*. In this study, we constructed a recombinant *E. coli* expressing *xpdh* enzyme from *Clostridium difficile* and subjected the strain under metabolic pathways modification to enhance conversion of xylitol from glucose.

2.0 METHODOLOGY

2.1 Microbial Strains and Culture Conditions

E. coli BL21 (DE3) (Novagen) was used as cloning and expression host. Molecular manipulation of the host through DNA recombination and PCR was conducted as described in [35]. The *E. coli* strain was grown on Luria-Bertani (LB) medium or on LB agar plate, incubated at 37 °C for ~17 hours. For production analysis, the cells were grown in LB broth, supplemented with 0.1 mM IPTG, 34 µg/mL chloramphenicol, 50 µg/mL kanamycin and 10g/L of sugar (glucose, arabinose or xylose). The culture was grown at 37 °C while shaking at 200 rpm. All strains used are listed in Table 1.

2.2 Plasmid Construction

Xylitol phosphate dehydrogenase (*xpdh*) from *Clostridium difficile*, as reported by [26] was codon optimized for *E. coli* and synthesized by GenScript (New Jersey, USA). The plasmid containing xpdh gene (pUC19-xpdh) was digested with *Nde*I and *Xho*I restriction enzymes and ligated into the expression vector pACYC-Duet-1 digested with the same enzymes yielding pACYC-xpdh. The resulting plasmid was transformed into electrocompetent *E. coli* BL21 through electroporation yielding BL21-xpdh (Eppendorf, Germany).

2.3 Expression and SDS-PAGE Analysis

BL21-xpdh was inoculated into LB medium supplemented with 34 µg/mL chloramphenicol. The expression of xpdh was induced by adding 0.1 mM IPTG at cell density OD_{600} ~0.6 and incubated for 24 hours. Cells were harvested at 11337 ×*g* for 5 minutes and resuspended in 400 µL BugBuster protein extraction reagent supplemented with 0.1% v/v benzonase, 0.2 mg/ml lysozyme and 1mM phenylmethylsulfonyl fluoride (PMSF) from Novagen (Darmstadt, Germany). Twenty microliters of the total lysate of soluble fraction was run on 12% acrylamide gel, and the proteins were stained with Coomassie Brilliant Blue after electrophoresis.

2.4 Strain Construction

Disruptions of *E. coli* chromosomal genes were done as described by Datsenko and Wanner, 2000. A gene cassette carrying kanamycin resistant gene flanked by FRT (FLP recognition target) at both sides was PCR-amplified from plasmid template, pKD4 by using primers containing 35-nt extensions homologous to the flanking sequence of the targeted gene. The gene cassette was electroporated into competent *E. coli* BL21 carrying λ-red recombinase plasmid, pKD46. The transformed cells were recovered by incubation in LB medium at 37 °C while shaking at 200 rpm for an hour and then plated on selective LB agar containing 50 µg/mL kanamycin. The kanamycin marker in the resulting *E. coli* mutants were removed by pCP20 helper plasmid expressing FLP recombinase and cultured at 42 °C. The gene disruption was verified by PCR of the flanking sequence of the targeted genes. The primers used in this study are listed in Table S1.

Table 1 List of *E. coli* mutants and plasmid vectors used in this study

Strain/plasmid	Genotype or phenotype	Source/ Reference
Plasmid		
pACYC-Duet-1	Cm^R; *E. coli* shuttle vector for regulated dual genes expression; (P_7, *lacI*, P15A *ori*)	Novagen
pACYC-xpdh	pACYC-Duet-1 containing xylitol-phosphate dehydrogenase from *Clostridium difficile*	[26]
pkD46	Amp^R; λ red recombinase vector; (araC-P_{araB}, *β γ exo*, repA 101 *ori*)	[36]
pKD4	Amp^R; FRT-flanked kanamycin resistance gene; (P_{R6K}, R6Kγ *ori*)	[36]
pCP20	Amp^R; expresses yeast FLP recombinase gene; ([cI857] (lambda) (ts), ts-rep)	[36]
***E. coli* strain**		
BL21 (DE3)	*E. coli* str. B F^- *ompT gal dcm lon* $hsdS_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S)	Novagen
NA116	*E. coli* BL21 (DE3) pACYC-*xpdh*	This study
NA207	*E. coli* BL21 Δ*rpiA*::FRT-kan-FRT::pACYC-*xpdh*	This study
NA208	*E. coli* BL21 Δ*rpiB*::FRT-kan-FRT::pACYC-*xpdh*	This study
NA209	*E. coli* BL21 Δ*pgi*::FRT-kan-FRT::pACYC-*xpdh*	This study
NA211	*E. coli* BL21 Δ*rpiA*Δ*pgi*::FRT-kan-FRT::pACYC-*xpdh*	This study

2.5 Xylitol Conversion Fermentation

The seed culture of *E. coli* strain carrying pACYC-xpdh was prepared by inoculating a single colony in 10 mL LB broth supplemented with 34 µg/mL chloramphenicol and 50 µg/mL kanamycin at 37 °C and 200 rpm for ~17 hours. Xylitol production experiments were conducted in 250-mL shake-flasks, containing 50 mL of LB medium supplemented with the same antibiotics as the seed culture. The medium was inoculated with 1% of the seed culture and incubated at 37 °C and 200 rpm. The cells were induced by adding 0.1 mM IPTG and 10 g/L sugar D-glucose when the optical density of the culture OD_{600} reached ~0.6. The culture was sampled periodically by withdrawing 2mL each time to determine OD_{600} and the concentration of residual substrates and products. The removed culture was centrifuged at 11337 ×*g* for 10 minutes and the supernatant was transferred to a fresh tube and stored at -20 °C for subsequent HPLC analysis. Each experiment was performed in triplicate and the values of the data are reported in mean and standard error.

2.6 Analysis of Sugars and Metabolites

Concentrations of sugars and cultural metabolites were measured by using high performance liquid chromatography (HPLC) system, equipped with 1525 binary pump, 2414 refractive index detector (RID) and 2707 autosampler (Waters, USA). Glucose and xylitol were separated by using Rezex RPM-monosaccharide Pb^{+} column (length; 300 x 7.8 mm, Phenomenex, California, USA). The system was operated using filtered pure water as its mobile phase, running at flow rate 0.6 mL/min with 75 °C oven and 40 °C detector temperatures. Meanwhile, ethanol and acetate were separated by using Rezex ROA-organic acid H+ (length; 250 x 4.6 mm, Phenomenex, California, USA) with 0.005N sulfuric acid as mobile phase, with 0.4 mL/min flow rate and oven and detector temperatures of 50 °C and 40 °C respectively.

3.0 RESULTS AND DISCUSSION

3.1 Construction and Expression of XPDH in *E. coli*

To study the conversion of xylitol from glucose in *E. coli*, xylitol-5-phosphate dehydrogenase (*xpdh*) from *C. difficile* [26] was cloned into pACYC-Duet-1 expression vector and expressed in *E. coli* BL21 (DE3), denoted as NA116 strain. The nucleotide sequence corresponding to the *xpdh* protein sequence was codon-optimized for *E. coli* and synthesized (GenScript, USA), PCR amplified and cloned into pACYC-Duet-1 expression vector. The gene size was 1054 kb long and its expression was controlled under the T7 promoter. The expression of the gene was induced by the addition of IPTG which yielded a protein size of ~38 kDa on SDS-PAGE, which coincides with the predicted molecular weight of *xpdh (*Figure 1).

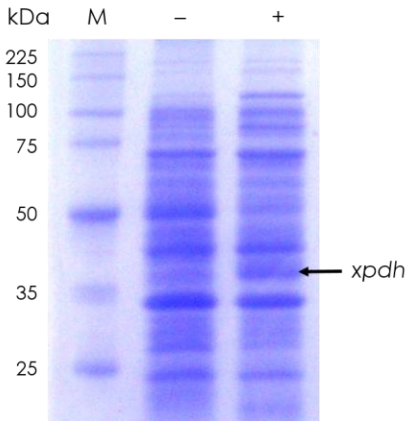


Figure 1 Expression of recombinant enzyme, xylitol phosphate dehydrogenase (xpdh) in *E. coli*. Soluble cell extracts were prepared from *E. coli* BL21(DE3) wild type (-) and *E. coli* BL21(DE3) carrying pACYC-xpdh (NA116) (+) induced for 24-hours at 37°C. The soluble fraction of the cell lysate was analyzed on SDS-PAGE using a 12% acrylamide gel. The arrow indicates *xpdh*. Lane M is the protein marker.

The enzyme activity for *xpdh* was not conducted due to the absence of commercial xylulose-5-phosphate. Thus, the functionality of the *xpdh* in *E. coli* was shown by xylitol production screening. Initial screening in fermentation medium added with 10 g/L glucose showed that NA116 successfully produced 0.273 g/L xylitol after 24 hours with 4.33 g/L glucose consumed, which gives net xylitol conversion of 0.063 g/g (Table 1). Compared to the original study in *B. subtilis*, the yield was much lower due to the use of different expression host and the original study also has modified pathways for xylitol conversion [26].

Table 1 Production of xylitol using xpdh gene

Substrate consumed (g/L)	Xylitol produced (g/L)	Percent conversion (%)	Organism	Ref
4.33	0.273	6.3	*E. coli*	This study
10	2.3	23	*B. subtilis*	[26]

In previous reports on direct glucose conversion to xylitol, two key enzymes were used; arabitol dehydrogenase (*ardh*) which converts arabitol to xylulose, and xylulose dehydrogenase (*xdh*) which converts xylulose to xylitol [25, 37]. The system was applied in *Pichia pastoris* to convert glucose to xylitol in a single fermentation, and it also observed low xylitol yield, at 0.079 g/g conversion [25]. The report stated that the main reason for low xylitol yield was due to low flux of glucose to pentose phosphate pathway. In relation to this study, the conversion of

xylitol by *xpdh* expression also occurred in the pentose phosphate pathway which has competing flux with glycolysis. Moreover, as direct conversion of glucose to xylitol by *xpdh* involved a series of reactions, it is possible to have certain reactions becoming limiting steps for the xylitol conversion.

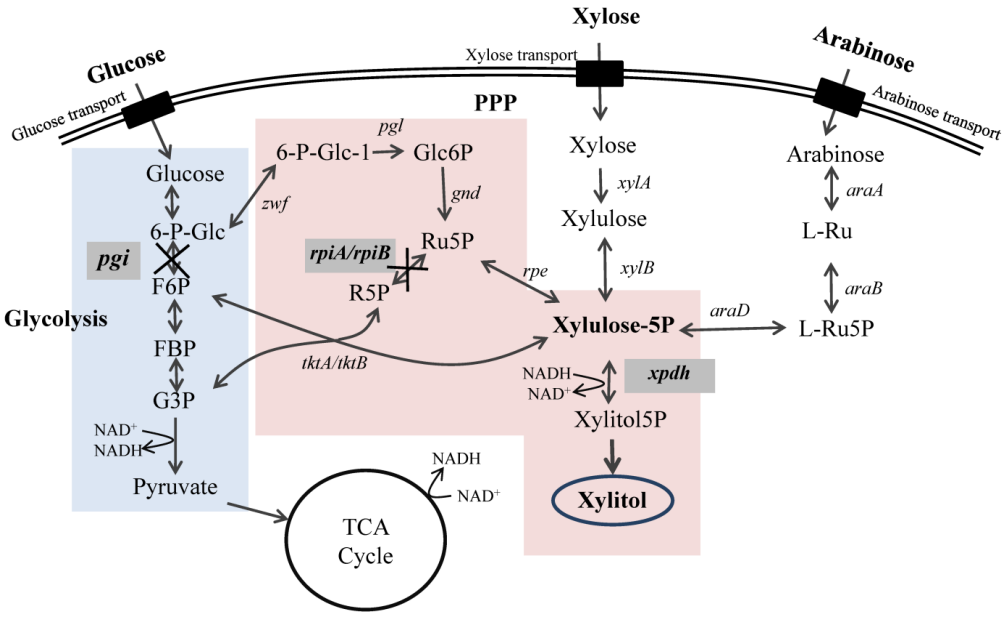


Figure 2 Schematic representation of *E. coli* metabolic pathway manipulations for xylitol conversion from glucose, xylose and arabinose. Recombinant gene, *xpdh* from *Clostridium difficile* converts xylulose-5-phosphate to xylitol-5-phophate which is further dephosphorylated to xylitol. Two metabolic pathways encoded by *pgi* and *rpiA/rpiB* were deleted to divert the flux towards *xpdh* pathway. PPP, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; 6-P-Glc, glucose-6-phosphate; 6-P-Glc-1, 6-phosphoglucono-δ-lactone; Glc6P, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; Xylulose-5P, xylulose-5-phosphate; xylitol5P, xylitol-5-phosphate; R5P, ribose-5-phosphate; L-Ru, L-ribulose; L-Ru5P, L-ribulose-5-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-phosphate; G3P, glyceraldehyde-3-phosphate; *pgi*, phosphoglucose isomerase; *zwf*, glucose-6-phosphate dehydrogenase; *pgl*, 6-phosphogluconolactonase; *gnd*, 6-phosphogluconate dehydrogenase; *rpiA*, ribose-5-phosphate isomerase A; *rpiB*, ribose-5-phosphate isomerase B; *rpe*, ribulose-5-phosphate-3-epimerase; *tktA*, transketolase A; *tktB*, transketolase B; *xylA*, xylose isomerase; *xylB*, xylulokinase; *araA*, L-arabinose isomerase; *araB*, L-ribulokinase; *araD*, L-ribulose-5-phosphate-4-epimerase.

The glucose metabolic pathway consists of two reactions that divert its carbon from forming xylulose-5-phosphate, encoded by *pgi* and *rpiA/rpiB* thus explained its lower conversion (Figure 2). Therefore, metabolic modifications were done targeting these reactions.

Several studies in xylitol conversion from glucose also reported that the introduced *ardh* and *xdh* became the limiting step because the enzymes required the use of NADH cofactor [21, 38, 39]. As *xpdh* reaction also required NADH as cofactor, the low yield of xylitol could also be attributed to cofactor imbalance in the system.

3.3 Optimization of Metabolic Pathway for Xylitol Conversion

Metabolic pathway modifications involving *pgi*, *rpiA* and *rpiB* showed significant improvement on xylitol production in *E. coli*. Manipulation of the genes generated four *E. coli* mutants carrying single and double gene deletions identified as NA207 (Δ*rpiA*), NA208 (Δ*rpiB*), NA209 (Δ*pgi*) and NA211 (Δ*rpiA*Δ*pgi*) (Table 1).

Screening on glucose as substrate, all mutants showed improvement in xylitol production compared to the original recombinant strain, NA116 (BL21-*xpdh*) with significant difference ($p<0.05$). Highest xylitol production from glucose was achieved by NA211 (Δ*rpiA*Δ*pgi*) with 0.584 g/L at 72 h fermentation, followed by NA208 (Δ*rpiB*) with 0.435 g/L, NA207 (Δ*rpiA*) with 0.380g/L, NA209 (Δ*pgi*) with 0.320 g/L and the parent strain, NA116 (BL21-xpdh) with 0.284 g/L (Table 3). Production of xylitol showed exponential increase within 24 h in all strains, and entered stationary phase afterwards (Figure 3).

Through the 72 h fermentation, all strains showed highest performance for xylitol conversion during the first 24 h. However, the specific productivity of the *E. coli* strains during the 24 h fermentation showed a slight different trend where only NA211 (Δ*rpiA*Δ*pgi*) and NA207 (Δ*rpiA*) recorded significant xylitol conversion compared to the parent strain, NA116 (Table 3). Highest xylitol productivity was achieved at 24 h fermentation for most strains while NA207 (Δ*rpiA*)

and NA211 (Δ*rpiA*Δ*pgi*) reached productivity peak at 12 h (Table 2). It could be related that the best xylitol conversion was maintained in the 24 h when the cells were actively growing and consuming most substrates during the batch fermentation. Highest performance was achieved by NA211 (Δ*rpiA*Δ*pgi*) which showed highest xylitol productivity with 0.087 g/g xylitol conversion at 12 hour fermentation followed by NA207 (Δ*rpiA*) at 0.072 g/g.

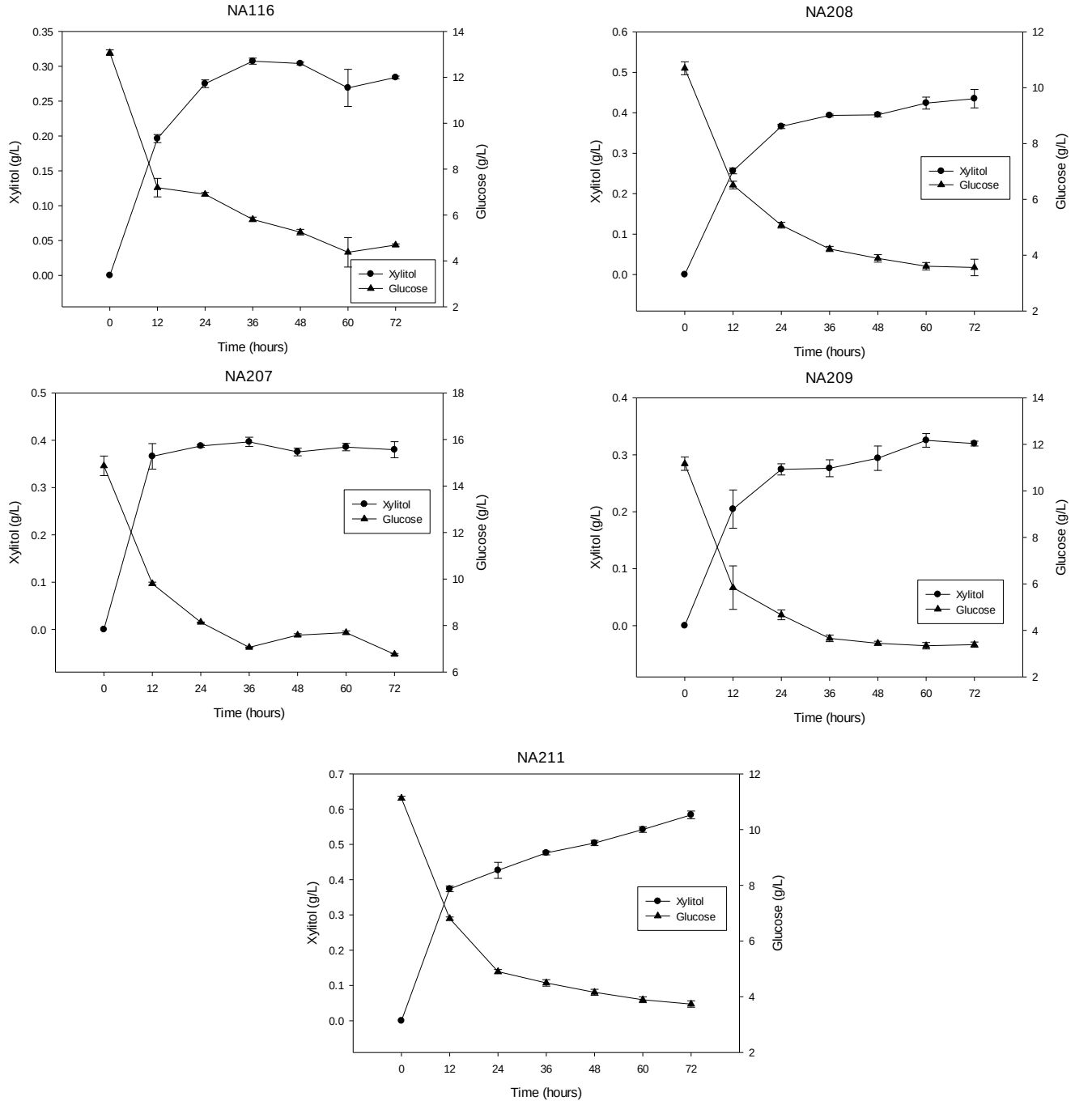


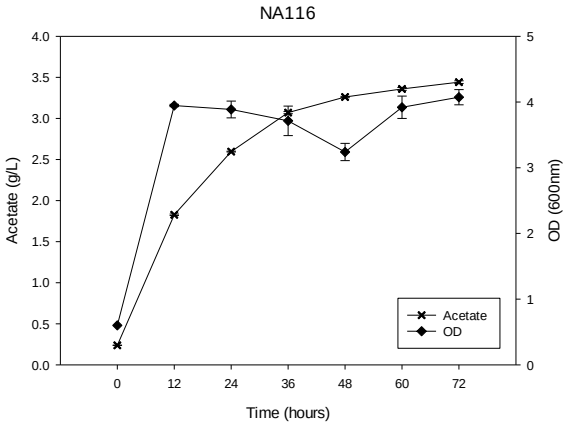
Figure 3 Production of xylitol from glucose by *E. coli* mutants, NA116 (BL21-*xpdh*), NA207 (Δ*rpiA*), NA208 (Δ*rpiB*), NA209 (Δ*pgi*) and NA211 (Δ*rpiA*Δ*pgi*). Each data represents the mean of triplicate cultures with error bars indicating standard error

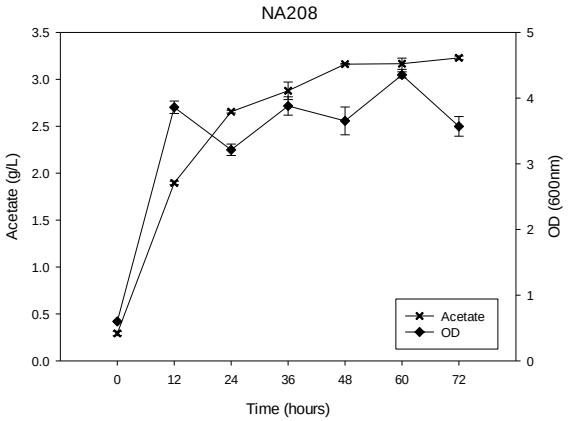
Table 2 Specific production of xylitol from consumed glucose at different fermentation time.

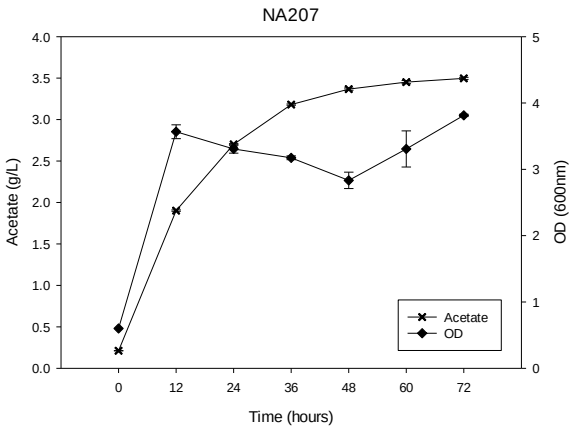
Strain	Specific xylitol production from glucose, $Y_{P/S}$ (g/g)					
	12-hr	24-hr	36-hr	48-hr	60-hr	72-hr
NA116	0.048	**0.063**	0.056	0.051	0.039	0.043
NA207	**0.072**	0.058	0.051	0.052	0.054	0.047
NA208	0.061	**0.065**	0.061	0.058	0.060	0.061
NA209	0.038	**0.042**	0.037	0.038	0.042	0.041
NA211	**0.087**	0.069	0.072	0.071	0.075	0.078

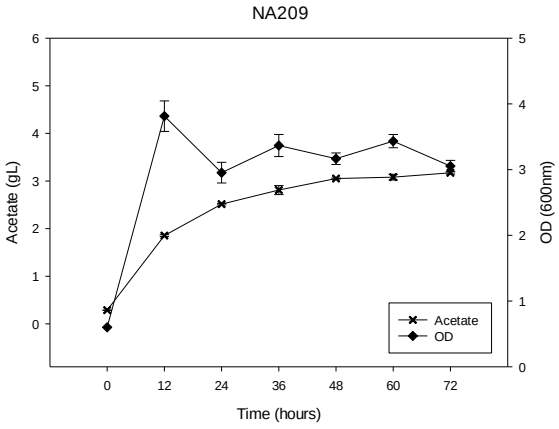
Compared to all mutants, NA211 (Δ*rpiA*Δ*pgi*) showed highest conversion of xylitol with up to two times (0.584 g/L) compared to that of the original strain, NA116 (0.284 g/L) ($p < 0.05$) and highest overall specific productivity, 0.078 g/g (Table 3). This was caused by the increased formation of X5P due to the disruption of competing pathways, *pgi* and *rpiA*, which increased the glucose carbon flux towards pentose phosphate pathway [40, 41]. It is also interesting to note that the disruption of the Rpi reaction (encoded by *rpiA* and *rpiB*) caused reverse reactions in the non-oxidative branch of pentose phosphate pathway, particularly Tkt and Tal genes, to generate ribulose-5-phosphate (R5P), an essential metabolite in *E. coli* [42]. Since reverse reaction for generation of R5P would also yield X5P, it was assumed that Rpi pathway disruption also increased the flux for *xpdh* reactions, thus resulting in higher xylitol. However, it was also noted that the attempt for complete disruption of the Rpi pathway through double deletions of *rpiA* and *rpiB* was not achieved in this study. In another report, *rpiA* was suggested to be an essential pathway in *E. coli* [40], as it is the major enzyme for the ribose isomerase reaction, whereas *rpiB* is only induced when *rpiA* is disrupted [43]. As we were unable to construct the double deletions of *rpiA* and *rpiB*, it correlates with the previous assumption that the reaction is essential. It is noted that xylitol production in most strains reached saturation after 24 hours of fermentation (Figure 3).

Growth of *E. coli* on fermentable sugars such as glucose would generate acetate through the oxidation of pyruvate via acetyl-coA. There are several factors that caused acetate accumulation, which include the imbalance of glucose channeled towards product formation and cell growth [44].









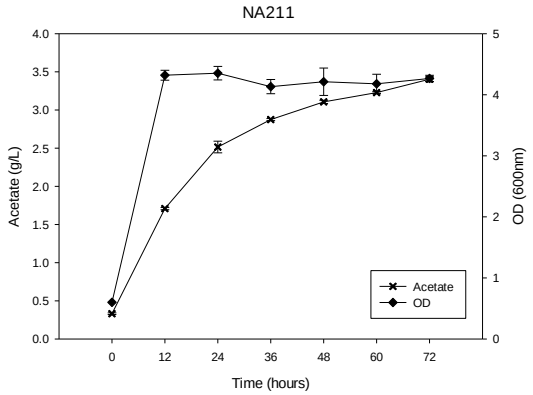


Figure 4 Acetate and growth profile of *E. coli* mutants. NA116 (BL21-*xpdh*), NA207 (Δ*rpiA*), NA208 (Δ*rpiB*), NA209 (Δ*pgi*) and NA211 (Δ*rpiA*Δ*pgi*). Each data represents the mean of triplicate cultures with error bars indicating standard error

Table 3 Summary of xylitol production from *E. coli* mutants. All metabolites reported were at 72-hour fermentation

Strain	Sugar consumed (g/L)	Final Xylitol (g/L)	Xylitol productivities ($gL^{-1}h^{-1}$)	Specific production $Y_{p/s}$	Final acetate (g/L)	Final ethanol (g/L)	Final OD_{600}	Final pH
NA116	6.56	0.284	0.004	0.043	3.442	0.835	4.073	4.43
NA207	8.107	0.380	0.005	0.047	3.497	0.955	3.813	4.37
NA208	7.137	0.435	0.006	0.061	3.229	0.843	3.570	4.42
NA209	7.781	0.320	0.004	0.041	3.172	0.818	3.053	4.46
NA211	7.485	0.584	0.008	0.078	3.403	0.934	4.27	4.34

It is observed in this study that fermentation of the *E. coli* strains on glucose observed high acetate, with over 2 g/L after 24 hours (Figure 4). Generally, acetate concentration of over 2 g/L could cause adverse effects on *E. coli* growth and synthesis of recombinant protein [45]. All mutants in this study showed high acetate concentration of more than 3 g/L at the end of fermentation, 72 h, with highest concentration at 3.497 g/L by NA207, while lowest at 3.172 g/L by NA209. Consequently, this high level of acetate was seen to have affected the mutants' growth and xylitol production, where all mutants entered stationary phase after only 12 hours despite the presence of glucose remaining in the culture (Figure 3 and Figure 4).

Similar observation was also reported in the production of xylitol in *E. coli* using xylose-glucose mixture, where high acetate secretion caused lower xylitol production due to imbalance in glucose channeling in biomass and product formation [46]. The study also reported that increasing the flux towards xylitol formation by eliminating all competing pathways in xylose conversion to xylitol reduced acetate accumulation and thus improved xylitol formation. In application to this study, complete removal of competing pathways for xylitol formation from glucose was not feasible as Rpi reaction was essential to the cell [43], and Tkt (*tktA*/*tktB*) mutants were also reported to have truncated growth, thus require the pyridoxine and aromatic amino acids for growth [47]. Carbon diversion towards acetate formation is highly undesirable, which caused carbon loss in chemical conversion. However, further manipulation in fermentation conditions and also metabolic modifications in acetate pathway in future study can help to reduce acetate flux thus improving product formation.

Comparing to other studies, conversion of xylitol from glucose in *E.coli* has mainly focused on the glucose → arabitol → xylulose → xylitol route using two key enzymes which are arabitol dehydrogenase (*ardh*) and xylitol dehydrogenase (*xdh*) [21, 48, 49]. In a study using a mix culture of *E. coli* expressing *xdh* and *ardh* separately, high xylitol yield was reported with 0.87 g/g xylitol from arabitol [49]. Another strategy of using a mix culture of *E. coli* expressing *xdh* and alcohol dehydrogenase (*adh*) for cofactor regeneration, and *Gluconobac-ter thailandicus* observed 0.89 g/g xylitol conversion from arabitol [48]. Highest xylitol conversion from glucose in this study was observed at 0.078 g/g by NA211 (Δ*rpiA*Δ*pgi*) (Table 3) which is considerably low compared to the studies above. However, this is expected as arabitol conversion to xylitol only requires two steps conversion by *ardh* (arabitol to xylulose) and *xdh* (xylulose to xylitol). Meanwhile, when compared to

the studies using glucose as initial substrate, *Zygosaccharomyces rouxii* expressing both *ardh* and *xdh* produced 0.038 g/g xylitol from glucose [50]. Improved yield was also reported by glucose conversion to xylitol in *P. pastoris* with 0.078 g/g xylitol from glucose [25]. As this is the first report of xylitol conversion from glucose in *E. coli*, our yield at 0.078 g/g xylitol to glucose by NA211 is comparable to these studies. This also showed that production of xylitol using only *xpdh* could also give a high yield as to when expressing two enzymes, *ardh* and *xdh*.

4.0 CONCLUSION

In this study, we were able to show that the expression of a single gene, *xpdh* allowed xylitol conversion from glucose in a single fermentation in *E. coli*. The ability of the strain to convert xylitol from glucose could open many opportunities in the study of microbial conversion of xylitol. Metabolic modification of *E. coli* showed significant improvement of xylitol yield from glucose with twice the amount of xylitol compared to the original strain. Further manipulation to improve the system could be done to disrupt Tkt pathway to eliminate X5P from being used for cell growth and thus concentrated the flux towards xylitol production. Process optimization should also be applied to reduce acetate formation and thus increase cell viability for xylitol production.

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References

[1] Bozell, J. J., and Petersen, G. R. 2010. Technology Development for the Production of Biobased Products from Biorefinery Carbohydrates—the US Department of Energy's "Top 10" Revisited. *Green Chem*. 12(4): 539-554. https://doi.org/ 10.1039/B922014C.

[2] Industry Experts. 2014. Xylitol, A Global Market Overview. *Industry Experts*. Retrieved January 7, 2018, from https://www.marketresearch.com/ product/sample-8164119.pdf.

[3] Delgado Arcaño, Y., Valmaña García, O. D., Mandelli, D., Carvalho, W. A., and Magalhães Pontes, L. A. 2020. Xylitol: A Review on the Progress and Challenges of Its Production by Chemical Route. *Catalysis Today*. 344: 2-14. https://doi.org/ 10.1016/j.cattod.2018.07.060.

[4] Hyvönen, L., Koivistoinen, P., and Voirol, F. 1982. Food Technological Evaluation of Xylitol. *Advances in Food Research*. Cambridge: Academic Press. 28: 373-403.

[5] Ur-Rehman, S., Mushtaq, Z., Zahoor, T., Jamil, A., and Murtaza, M. A. 2015. Xylitol: A Review on Bioproduction, Application, Health Benefits, and Related Safety Issues. *Critical Reviews in Food Science and Nutrition*. 55(11): 1514-1528. https://doi.org/10.1080/10408398.2012.702288.

[6] Grembecka, M. 2015. Sugar Alcohols—Their Role in the Modern World of Sweeteners: A Review. *European Food Research and Technology*. 241(1): 1-14. https://doi.org/10.1007/s00217-015-2437-7.

[7] Prakasham, R. S., Rao, R. S., and Hobbs, P. J. 2009. Current Trends in Biotechnological Production of Xylitol and Future Prospects. *Current Trends in Biotechnology and Pharmacy*. 3(1): 8-36.

[8] Su, B., Wu, M., Lin, J., and Yang, L. 2013. Metabolic Engineering Strategies for improving Xylitol Production from Hemicellulosic Sugars. *Biotechnology Letters*. 35(11): 1781-1789. https://doi.org/10.1007/s10529-013-1279-2.

[9] Parajó, J. C., Domínguez, H., and Domínguez, J. M. 1998. Biotechnological Production of Xylitol. Part 1: Interest of Xylitol and Fundamentals of Its Biosynthesis. *Bioresource Technology*. 65(3): 191-201. https://doi.org/10.1016/S0960-8524(98)00038-8.

[10] O'Donnell, K., and Kearsley, M. W. 2012. *Sweeteners and Sugar Alternatives in Food Technology*. Second Edition. Oxford: Wiley Blackwell. https://doi.org/ 10.1002/9781118373941

[11] Gasmi Benahmed, A., Gasmi, A., Arshad, M., Shanaida, M., Lysiuk, R., Peana, M., ... Bjørklund, G. 2020. Health Benefits of Xylitol. *Applied Microbiology and Biotechnology*. 104(17): 7225-7237. https://doi.org/10.1007/s00253-020-10708-7.

[12] Rafiqul, I. S. M., and Sakinah, A. M. M. 2013. Processes for the Production of Xylitol-A Review. *Food Reviews International*. 29(2): 127-156. https://doi.org/10.1080/87559129.2012.714434.

[13] Albuquerque, T. L. De, Da Silva, I. J., De MacEdo, G. R., and Rocha, M. V. P. 2014. Biotechnological Production of Xylitol from Lignocellulosic Wastes: A Review. *Process Biochemistry*. 49(11): 1779-1789. https://doi.org/10.1016/j.procbio.2014.07.010.

[14] Chen, X., Jiang, Z. H., Chen, S., and Qin, W. 2010. Microbial and Bioconversion Production of D-xylitol and Its Detection and Application. *International Journal of Biological Sciences*. 6(7): 834-844. https://doi.org/10.7150/ijbs.6.834.

[15] Pal, S., Mondal, A. K., and Sahoo, D. K. 2016. Molecular Strategies for Enhancing Microbial Production of Xylitol. *Process Biochemistry*. 51(7): 809-819. https://doi.org/10.1016/j.procbio.2016.03. 017.

[16] Da Silva, S. S., and Chandel, A. K. 2012. *D-Xylitol.)D-Xylitol: Fermentative Production, Application and Commercialization*. Heidelberg: Springer https://doi.org/10.1007/978-3-642-31887-0.

[17] Xu, Y., Chi, P., Bilal, M., and Cheng, H. 2019. Biosynthetic Strategies to Produce Xylitol: An Economical Venture. *Applied Microbiology and Biotechnology*. 103(13): 5143-5160. https://doi.org/ 10.1007/s00253-019-09881-1.

[18] Dhar, K. S., Wendisch, V. F., and Nampoothiri, K. M. 2016. Engineering of Corynebacterium Glutamicum for Xylitol Production from Lignocellulosic Pentose Sugars. *Journal of Biotechnology*. 230: 63-71. https://doi.org/10.1016/j.jbiotec.2016.05.011.

[19] Qi, Y., Zhang, H., Yun, J., Yang, M., Zhang, G., Xue, Y., ... Ran, L. 2017. Biosynthesis of Xylitol from Glucose: Microorganism, Key Enzymes and Genetically Engineered Strains. *American Journal of Bioscience and Bioengineering*. 5(5): 109-112. https://doi.org/10.11648/j.bio.20170505.13.

[20] Sakakibara, Y., Saha, B. C., and Taylor, P. 2009. Microbial Production of Xylitol from l-arabinose by Metabolically Engineered Escherichia coli. *Journal of Bioscience and Bioengineering*. 107(5): 506-511. https://doi.org/10.1016/j.jbiosc.2008.12.017.

[21] Zhou, P., Li, S., Xu, H., Feng, X., and Ouyang, P. 2012. Construction and Co-expression of Plasmid Encoding Xylitol Dehydrogenase and a Cofactor Regeneration

Enzyme for the Production of Xylitol from d-arabitol. *Enzyme and Microbial Technology*. 51(2): 119-124. https://doi.org/10.1016/j.enzmictec. 2012.05.002.

[22] Yoon, B. H., Jeon, W. Y., Shim, W. Y., and Kim, J. H. 2011. L-Arabinose Pathway Engineering for Arabitol-free xylitol Production in Candida Tropicalis. *Biotechnology Letters*. 33(4): 747-753. https:// doi.org/10.1007/s10529-010-0487-2.

[23] Onishi, H., and Suzuki, T. 1969. Microbial Production of Xylitol from Glucose. *Applied Microbiology*. 18(6): 1031-1035. https://doi.org/10.1016/j.biortech. 2010.10.074.

[24] Qi, X. H., Zhu, J. F., Yun, J. H., Lin, J., Qi, Y. L., Guo, Q., and Xu, H. 2016. Enhanced Xylitol Production: Expression of Xylitol Dehydrogenase from Gluconobacter oxydans and Mixed Culture of Resting Cell. *Journal of Bioscience and Bioengineering*. 122(3): 257-262. https://doi.org/ 10.1016/j.jbiosc.2016.02.009.

2014. Genetically Engineered Pichia Pastoris Yeast for Conversion of Glucose to Xylitol by a Single-fermentation Process. *Applied Microbiology and Biotechnology*. 98(8): 3539-3552. https://doi.org/10.1007/s00253-013-5501-x.

[26] Povelainen, M., and Miasnikov, A. N. 2007. Production of Xylitol by Metabolically Engineered Strains of Bacillus Subtilis. *Journal of Biotechnology*. 128(1): 24-31. https://doi.org/10.1016/j.jbiotec.2006. 09.008.

[27] Yu, C., Cao, Y., Zou, H., and Xian, M. 2011. Metabolic Engineering of Escherichia coli for Biotechnological Production of High-value Organic Acids and Alcohols. *Applied Microbiology and Biotechnology*. 89(3): 573-583. https://doi.org/ 10.1007/s00253-010-2970-z.

[28] Wang, C., Pfleger, B. F., and Kim, S. W. 2017. Reassessing Escherichia coli as a Cell Factory for Biofuel Production. *Current Opinion in Biotechnology*. 45: 92-103. https://doi.org/10.1016/ j.copbio.2017.02.010.

[29] Su, B., Wu, M., Zhang, Z., Lin, J., and Yang, L. 2015. Efficient Production of Xylitol from Hemicellulosic Hydrolysate Using Engineered Escherichia coli. *Metabolic Engineering*. 31: 112-122. https://doi.org/ 10.1016/j.ymben.2015.07.003.

[30] Li, S., Ye, Z., Moreb, E. A., Hennigan, J. N., Castellanos, D. B., Yang, T., and Lynch, M. D. 2021. Dynamic Control Over Feedback Regulatory Mechanisms Improves NADPH Flux and Xylitol Biosynthesis in Engineered E. coli. *Metabolic Engineering*. 64(January): 26-40. https://doi.org/ 10.1016/j.ymben.2021.01.005.

[31] Abd Rahman, N. H., Md. Jahim, J., Abdul Munaim, M. S., A. Rahman, R., Fuzi, S. F. Z., and Md. Illias, R. 2020. Immobilization of Recombinant Escherichia coli on Multi-walled Carbon Nanotubes for Xylitol Production. *Enzyme and Microbial Technology*. 135(August 2019): 109495. https://doi.org/ 10.1016/j.enzmictec.2019.109495.

[32] Jin, L. Q., Xu, W., Yang, B., Liu, Z. Q., and Zheng, Y. G. 2019. Efficient Biosynthesis of Xylitol from Xylose by Coexpression of Xylose Reductase and Glucose Dehydrogenase in Escherichia coli. *Applied Biochemistry and Biotechnology*. 187(4): 1143-1157. https://doi.org/10.1007/s12010-018-2878-0.

[33] Chin, J. W., and Cirino, P. C. 2011. Improved NADPH Supply for Xylitol Production by Engineered Escherichia coli with Glycolytic Mutations. *Biotechnology Progress*. 27(2): 333-341. https://doi.org/10.1002/btpr.559.

[34] Kim, S. M., Choi, B. Y., Ryu, Y. S., Jung, S. H., Park, J. M., Kim, G. H., and Lee, S. K. 2015. Simultaneous Utilization of Glucose and Xylose via Novel Mechanisms in Engineered Escherichia coli. *Metabolic Engineering*. 30: 141-148. https://doi.org/ 10.1016/j.ymben.2015.05.002.

[35] Yamada, Y., Miura, K., Kumagai, T., Hayakawa, C., Miyazaki, S., Matsumoto, A., … Wakamatsu, N. 2001. Molecular Analysis of Japanese Patients with Rett Syndrome: Identification of Five Novel Mutations and Genotype-phenotype Correlation Communicated by Mark H. Paalman Online Citation: Human Mutation, Mutation in Brief #443 (2001) Online http://journals.wil. *Human Mutation*. 18(3): 253. https://doi.org/10.1002/humu.1186.abs.

[36] Datsenko, K. A., and Wanner, B. L. 2000. One-step Inactivation of chromosomal Genes in Escherichia coli K-12 using PCR Products. *Proceedings of the National Academy of Sciences*. 97(12): 6640-6645. https://doi.org/10.1073/pnas.120163297.

[37] Yahashi, Y., Horitsu, H., Kawai, K., Suzuki, T., and Takamizawa, K. 1996. Production of Xylitol from D-xylose by Candida Tropicalis: The Effect of D-glucose Feeding. *Journal of Fermentation and Bioengineering*. 81(2): 148-152. https://doi.org/ 10.1016/0922-338X(96)87593-3.

[38] Li, S., Zhang, J., Xu, H., and Feng, X. 2016. Improved Xylitol Production from d-arabitol by Enhancing the Coenzyme Regeneration Efficiency of the Pentose Phosphate Pathway in Gluconobacter Oxydans. *Journal of Agricultural and Food Chemistry*. 64(5): 1144-1150. https://doi.org/10.1021/acs.jafc.5b05509.

[39] Zhang, J., Li, S., Xu, H., Zhou, P., Zhang, L., and Ouyang, P. 2013. Purification of Xylitol Dehydrogenase and Improved Production of Xylitol by Increasing XDH Activity and NADH Supply in Gluconobacter Oxydans. *Journal of Agricultural and Food Chemistry*. 61(11): 2861-2867. https://doi.org/ 10.1021/jf304983d.

[40] Long, C. P., Gonzalez, J. E., Sandoval, N. R., and Antoniewicz, M. R. 2016. Characterization of Physiological Responses to 22 Gene Knockouts in Escherichia coli Central Carbon Metabolism. *Metabolic Engineering*. 37: 102-113. https://doi.org/ 10.1016/j.ymben.2016.05.006.

[41] Hua, Q., Yang, C., Baba, T., Mori, H., and Shimizu, K. 2003. Responses of the Central Metabolism in Escherichia coli to Phosphoglucose Isomerase and Glucose-6-Phosphate Dehydrogenase Knockouts. *Journal of Bacteriology*. 185(24): 7053-7067. https://doi.org/10.1128/JB.185.24.7053-7067.2003.

[42] Nakahigashi, K., Toya, Y., Ishii, N., Soga, T., Hasegawa, M., Watanabe, H., … Tomita, M. 2009. Systematic Phenome Analysis of Escherichia Coli Multiple-knockout Mutants Reveals Hidden Reactions in Central Carbon Metabolism. *Molecular Systems Biology*. 5(306): 1-14. https://doi.org/10.1038/ msb.2009.65.

[43] Ishii, N., Nakahigashi, K., Baba, T., Robert, M., Soga, T., Kanai, A., … Tomita, M. 2007. Multiple High-throughput Analyses Monitor the Response of E. coli to Perturbations. *Science*. 316(5824): 593-597. https://doi.org/10.1126/science.1132067.

[25] Cheng, H., Lv, J., Wang, H., Wang, B., Li, Z., and Deng, Z.

[44] Bernal, V., Castaño-Cerezo, S., and Cánovas, M. 2016. Acetate Metabolism Regulation in Escherichia coli: Carbon Overflow, Pathogenicity, and Beyond. *Applied Microbiology and Biotechnology*. 100(21): 8985-9001. https://doi.org/10.1007/s00253-016-7832-x.

[45] Shiloach, J., and Fass, R. 2005. Growing E. coli to high cell density - A Historical Perspective on Method Development. *Biotechnology Advances*. 23(5): 345-357. https://doi.org/10.1016/ j.biotechadv.2005.04.004.

[46] Cirino, P. C., Chin, J. W., and Ingram, L. O. 2006. Engineering Escherichia coli for Xylitol Production from Glucose-xylose Mixtures. *Biotechnology and Bioengineering*. 95(6): 1167-1176. https://doi.org/ 10.1002/bit.21082.

[47] Zhao, G., and Winkler, M. E. 1994. An Escherichia coli K-12 tktA tktB Mutant Deficient in Transketolase Activity Requires Pyridoxine (Vitamin B6) as well as the Aromatic Amino Acids and Vitamins for Growth. *Journal of Bacteriology*. 176(19): 6134-6138. https://doi.org/10.1128/jb.176.19.6134-6138.1994.

[48] Zhang, H., Yun, J., Zabed, H., Yang, M., Zhang, G., Qi, Y., … Qi, X. 2018. Production of Xylitol by Expressing Xylitol Dehydrogenase and Alcohol Dehydrogenase from Gluconobacter Thailandicus and Co-biotransformation of Whole Cells. *Bioresource Technology*. 257(January): 223-228. https://doi.org/10.1016/j.biortech.2018.02.095.

[49] Qi, X., Zhang, H., Magocha, T. A., An, Y., Yun, J., Yang, M., … Cao, Z. 2017. Improved Xylitol Production by Expressing a Novel D-arabitol Dehydrogenase from Isolated Gluconobacter sp. JX-05 and Co-biotransformation of

Whole Cells. *Bioresource Technology*. 235(March): 50-58. https://doi.org/10.1016/j.biortech.2017.03.107.
[50] Harkki, A. M., Andrey Novomirovich, M., Juha Heikki Antero, A., and Ossi Antero, P. 2004. Manufacture of xylitol Using Recombinant Microbial Hosts. United States Patent No. 6,723,540 (B1). Retrieved from https://www.google.com.my/patents/US6723540.

Appendix

Table S1 Primers used in the study

Gene	Primer	Length	Sequence (5'-sequence-3')
rpiA	rpiA_del_F	56	TGCGTGTGAAATTTCATACCACAGGCGAAACGATCGTGTAGGCTGGAGCTGCTTCG
	rpiA_del_R	54	AGAATTTTTTTAACGGGGGAGGTTCCCCCGTCAGACATATGAATATCCTCCTTA
	rpiA_ver_F	23	TTCATACCACAGGCGAAACGATC
	rpiA_ver_R	23	TAGAGCAATCGACAATAGCCAGG
rpiB	rpiB_del_F	56	TTGATTGTGAAGTTTTGCACGGACGGGGAAGATGAGTGTAGGCTGGAGCTGCTTCG
	rpiB_del_R	54	AATCATTACTCATCCATGCAAGTAGTGGATGAATCCATATGAATATCCTCCTTA
	rpiB_ver_F	23	GATCAGGAAGGCGTAATTCATCG
	rpiB_ver_R	21	CATGCAAGTAGTGGATGAATC
pgi	pgi_del_F	71	CGCTACAATCTTCCAAAGTCACAATTCTCAAAATCAGAAGAGTATTGCTAGTGTAGGCTGGAGCTGCTTCG
	pgi_del_R	69	AATCGTAAAGCCCATTTTTCCAGCGAAGCCGCTGGAAAAAATAATGGAACCATATGAATATCCTCCTTA
	pgi_ver_F	22	CTGTGACTGGCGCTACAATCTT
	pgi_ver_R	22	TATGTAGGCCGGATAAGGCGTT
ptsG	ptsG_del_F	71	CACGCGTGAGAACGTAAAAAAAGCACCCATACTCAGGAGCACTCTCAATTGTGTAGGCTGGAGCTGCTTCG
	ptsG_del_R	69	GTAAAAAAGGCAGCCATCTGGCTGCCTTAGTCTCCCCAACGTCTTACAGACATATGAATATCCTCCTTA
	ptsG_ver_F	20	AAGCACCCATACTCAGGAGC
	ptsG_ver_R	20	CATCTGGCTGCCTTAGTCTC
pKD4 verify	k2		CGGTGCCCTGAATGAACTGC
	kt		CGGCCACAGTCGATGAATCC