Journal of Bioscience and Bioengineering
VOL. 127 No. 3, 288–293, 2019

www.elsevier.com/locate/jbiosc

Enhanced production of D-lactate from mixed sugars in *Corynebacterium glutamicum* by overexpression of glycolytic genes encoding phosphofructokinase and triosephosphate isomerase

Yota Tsuge,[1,2] Naoto Kato,[1] Shogo Yamamoto,[1] Masako Suda,[1] and Masayuki Inui[1,3,*]

Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan,[1] Institute for Frontier Science Initiative, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan,[2] and Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan[3]

Received 27 February 2018; accepted 5 August 2018
Available online 6 September 2018

The use of mixed sugars containing glucose and xylose in lignocellulosic biomass is desirable for the microbial production of chemicals and fuels. We investigated the effect of individual or simultaneous overexpression of glycolytic genes on D-lactate production from a mixture of glucose and xylose by a recombinant xylose-assimilating *Corynebacterium glutamicum* strain. The individual overexpression of genes encoding phosphofructokinase (PFK) and triosephosphate isomerase (TPI) increased D-lactate production rate by 71% and 34%, respectively, with corresponding increases (2.4- and 1.8-fold) in the glucose consumption; however, the amount of xylose consumed not altered. D-Lactate yield was also increased by 5.5%, but only in the strain overexpressing the gene encoding PFK. In the parent strain and the strains overexpressing the genes encoding PFK or TPI, a reduction in D-lactate production occurred at approximately 900 mM after 32 h. However, the strain that simultaneously overexpressed the genes encoding PFK and TPI continued to produce D-lactate after 32 h, with the eventual production of 1326 mM after production for 80 h in mineral salts medium. Our findings contribute to the cost-effective, large-scale production of D-lactate from mixed sugars.

[**Key words:** *Corynebacterium glutamicum*; Oxygen deprivation; D-Lactate; Lactic acid; Metabolic engineering; Glycolytic enzyme; Xylose; Mixed sugars; Lignocellulose]

Corynebacterium glutamicum is well known as an amino acid producer such as glutamate and lysine (1). We have recently found that glucose consumption was elevated in oxygen deprived conditions compared with aerobic growth conditions (2). Enzymes in the glycolytic pathway and reductive branch of the tricarboxylic acid cycle, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), phosphoenolpyruvate carboxylase (PEPC), and malate dehydrogenase (MDH), were upregulated under oxygen deprivation (2). To exploit this feature, a bioprocess was developed by using a high-cell density culture of *C. glutamicum* under oxygen deprivation (3). This bioprocess has enabled the bulk production of useful chemicals and fuels such as lactate, succinate, valine, alanine, ethanol, and isobutanol (3–10). Recently, overexpression of the glycolytic genes has been shown to improve the productivity of the target chemicals under oxygen deprived conditions, but the effective genes varied depending on the targets (6,8,9).

Lignocellulosic materials are the most abundant renewable organic resource on earth. In the near future, the use of renewable resources for the production of chemicals and fuels is expected to increase, because they provide a cost-effective sugar source. Lignocellulose contains various type of sugars including glucose, xylose, and arabinose. The ratio of contained sugars varies depending on the materials (11). Several types of lignocellulosic materials such as corn stover, sugarcane bagasse, and rice straw have been utilized for microbial production of chemicals and fuels after hydrolysis (12,13). Besides fermentable sugars, several chemicals such as furfural and 3-hydroxymethl furfural are produced in the process of hydrolysis, which inhibit bacterial growth and fermentation (14). Although it is still difficult for complete detoxification of the fermentation inhibitors, several approaches such as adaptive evolution or genetic engineering have been applied to circumvent the toxicity (14).

Lactic acid is currently used in the food, cosmetic, and pharmaceutical industries. Owing to increased concerns about climate change, lactic acid provides an attractive source for non-fuel-derived plastic from lignocellulosic biomass. To circumvent the low melting temperature of poly-L-lactic acid (PLLA), a stereocomplex of poly lactic acid (scPLA), a mixture of the polymerized L- and D-lactic acid, is expected to be highly successful (15,16). To synthesize high quality scPLA, highly optically pure stereoisomers of both D- and L-lactic acid are necessary. Microbial production is ideal because it produces either isomer, depending on the chiral specific lactate dehydrogenase (LDH) (17). Although the microbial production of optically pure L-lactic acid from mixture of glucose and xylose has been demonstrated by using several bacteria and

* Corresponding author at: Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan. Tel.: +81 774 75 2308; fax: +81 774 72 2321.
E-mail address: mmg-lab@rite.or.jp (M. Inui).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.08.002

yeast, the report on D-lactate production from the mixed sugars is only limited by *Escherichia coli* and *Lactobacillus* species (18–23).

In this study, we examined the effect of individual or simultaneous overexpression of ten glycolytic genes on D-lactate production from mixed sugars (glucose and xylose) in *C. glutamicum* under oxygen deprived conditions. We elucidated the optimal combinations of gene overexpression to achieve a high production of D-lactate from mixed sugars.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and plasmids All plasmids and strains used in this study are listed in Table S1. *E. coli* and *C. glutamicum* strains were grown at 37 °C in Luria–Bertani (LB) medium and nutrient-rich A medium at 30 °C, respectively, as described previously (24). Kanamycin (50 μg/mL) and/or 1.5% agar were supplemented when necessary.

DNA manipulation and sequencing Transformation of *E. coli and C. glutamicum* was performed as described previously (25). Sequencing was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Waltham, MA, USA), the GENETYX program (GENETYX Corporation, Tokyo, Japan) was used for analysis of the DNA sequence data.

Chromosomal integration of glycolytic genes Ten glycolytic genes were integrated into the chromosome as a second copy via a markerless system by using pCRA725 suicide vector. Each glycolytic gene was integrated into the specific genomic regions, identified as dispensable for cell growth (26). The integration vectors are listed in Table S1, which was constructed in a previous study (24). The vectors were introduced into the genome by electroporation. Single crossover mutants were selected on a plate supplemented with kanamycin. The mutants were cultivated for 16 h in BT-medium [composition (per liter): 7 g ammonium sulfate, 2 g urea, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 6 mg $Fe_2SO_4{\cdot}7H_2O$, 4.2 mg $Mn_2SO_4{\cdot}H_2O$, 0.2 mg biotin, and 0.2 mg thiamine] containing 10% sucrose and the cells were spread on a BT-medium agar plate containing 10% sucrose. The deletion strains were selected through screening for sucrose resistance and kanamycin sensitivity.

Bioprocess forD-lactate production under oxygen deprived conditions For D-lactate production, two 500 mL cultures were harvested by centrifugation (5000 × *g*, 4 °C, 10 min) after production for 15 h at 33 °C. The cell precipitate was subsequently washed once with BT medium without urea. Six grams of cells were resuspended in 54 mL of BT medium without urea to yield 10% of cells in a reaction volume of 60 mL. At the beginning of the reaction, 253 mM of glucose and 177 mM of xylose were added as a model sugar mixture based on the previous reports (11,27,28). When if depletion of either occurred during the reaction, both glucose and xylose were added with the same ratio. The cell suspensions were constantly agitated at 33 °C without aeration. The pH of the bioprocess was maintained at 7.0 throughout the reaction by the addition of 5.0 N ammonia solution using a pH controller (DT-1023, ABLE Corporation, Tokyo, Japan).

Analytical procedures Glucose, xylose, D-lactate, succinate, acetate, dihydroxyacetone (DHA), and glycerol were analyzed by a high performance liquid chromatography (HPLC) as described previously (24) The optical purity of D-lactate was analyzed by using an HPLC system (8020, Tosoh Corporation, Tokyo, Japan), equipped with an UV detector, and a Chiralpak MA (+) column (Daicel CPI Company, Japan) operated at 25 °C with a mobile phase of 2 mM $CuSO_4$ and a flow rate of 0.5 mL/min. Cell growth was monitored by the measurement of the absorbance at 610 nm using a spectrophotometer (DU800, Beckman Coulter, Brea, CA, USA).

Enzyme assay One milliliter of reaction solution at 2 h was collected and centrifuged at 10,000 × *g*, 4 °C for 5 min. Cell pellets were washed with sonication buffer (100 mM Tris–HCl buffer, pH 7.5, 1 mM $MgCl_2$, 2 mM dithiothreitol [DTT]). The cells were resuspended in 1 mL of the same buffer, and sonicated for 45 min (pulse, 5 s; interval, 10 s) with ultrasonic homogenizer (UCD-200, Cosmo Bio, Tokyo, Japan) in an ice water bath with 0.5-g glass beads (150–212 μm, Sigma-Aldrich, St. Louis, MO, USA). The enzyme assay for glycolytic enzymes was performed as described previously (24) by measuring the shift at 340 nm for NAD(P)H/NAD(P).

RESULTS

Xylose assimilation decreased D-lactate yield and increased by-product yields under oxygen deprivation conditions In a previous study, the *E. coli xylA* and *xylB* genes encoding xylose isomerase and xylulokinase, respectively, were integrated into the non-essential genomic regions distributed throughout the *C. glutamicum* R chromosome to enable the assimilation of xylose (29). Xylose is therefore metabolized through the pentose phosphate pathway, which leads to glyceraldehyde-3-phosphate and fructose-6-phosphate in the Embden–Meyerhof pathway (Fig. 1). In addition, the introduction of *araE* enhanced xylose consumption under oxygen deprived conditions (29). The resultant strain, ACX-araE, can be used as a parent strain for the production of chemicals and fuels from xylose. To switch off L-lactate production, the native *L-ldhA* gene was eliminated and a plasmid pCRB215 harboring the *D-ldhA* gene from *Lactobacillus delbrueckii* under the control of the *gapA* promoter was introduced to produce optically pure D-lactate (24). Furthermore, the *ppc* gene encoding phosphoenolpyruvate carboxylase (PEPC), which was mainly responsible for succinate production under oxygen deprived conditions (3), was deleted. The resultant strain named CRZ5/pCRB215 was used as the parent strain in this study to investigate the effect of the overexpression of glycolytic genes on D-lactate production from mixture of glucose and xylose under oxygen deprived conditions.

First, CRZ5/pCRB215 was cultivated to produce D-lactate from the mixed sugars. After production for 10 h, 302 ± 39 mM D-lactate was produced (Fig. 2). Interestingly, the consumption of xylose (159 ± 2 mM) was greater than that of glucose (88 ± 25 mM) (Table 1). The yield of D-lactate was dramatically decreased to 68.6 ± 4.8% in comparison with that determined in a previous study on D-lactate production from the sole carbon source of glucose (87.0 ± 1.7%) (24). Instead, the by-products of DHA and glycerol were markedly produced at yields of 11.3 ± 1.1% and 6.4 ± 0.2%, respectively (Table 1). These results indicated that the simultaneous utilization of glucose and xylose in a D-lactate producing strain caused an overflow of the glycolytic flux upstream of glyceraldehydes-3-phosphate, which resulted in the accumulation of DHA and glycerol. The accumulation of DHA and glycerol in the medium was also observed on D-lactate production from glucose with yield of 6.0 ± 1.0% and 5.8 ± 0.3%, respectively, when the gene encoding GLK was overexpressed, most likely because of a similar overflow metabolism (24).

Individual overexpression of genes encoding PFK or TPI improved D-lactate production rate from a mixture of glucose and xylose via increased the amount of glucose consumed The above result led us to overexpress the gene encoding GAPDH in CRZ5/pCRB215 to release the overflow metabolism potentially caused by the accumulation of glyceraldehyde-3-phosphate and increase the D-lactate yield. Indeed, in a previous study, the additional overexpression of the gene encoding GAPDH in a strain overexpressing the gene encoding GLK encoding gene released the overflow metabolism, which decreased the yields of DHA and glycerol and increased the D-lactate yield from the mixture of glucose and xylose (8). Therefore, we first overexpressed the gene encoding GAPDH in CRZ5/pCRB215 through the integration of a second copy of the gene into the chromosome under the control of the constitutive *tac* promoter. The resultant strain (LPglc4/pCRB215) was subjected to the bioprocess under oxygen deprived conditions to produce D-lactate from glucose and xylose. Unexpectedly, LPglc4/pCRB215 (overexpressing the gene encoding GAPDH; hereinafter, only the name of overproduced enzyme is shown) resulted in a 37% decrease in D-lactate compared with that of the parent strain (190 ± 27 mM; Fig. 2). Furthermore, the amount of glucose consumed decreased by 47% and the amount of xylose consumed decreased by 11%–47 ± 9 mM and 141 ± 8 mM, respectively, compared with the parent strain (Table 1). D-Lactate yield was also decreased by 16.1%–57.6 ± 6.5%. The yields of the by-products were not dramatically altered. These results showed that overexpression of the gene encoding GAPDH was not

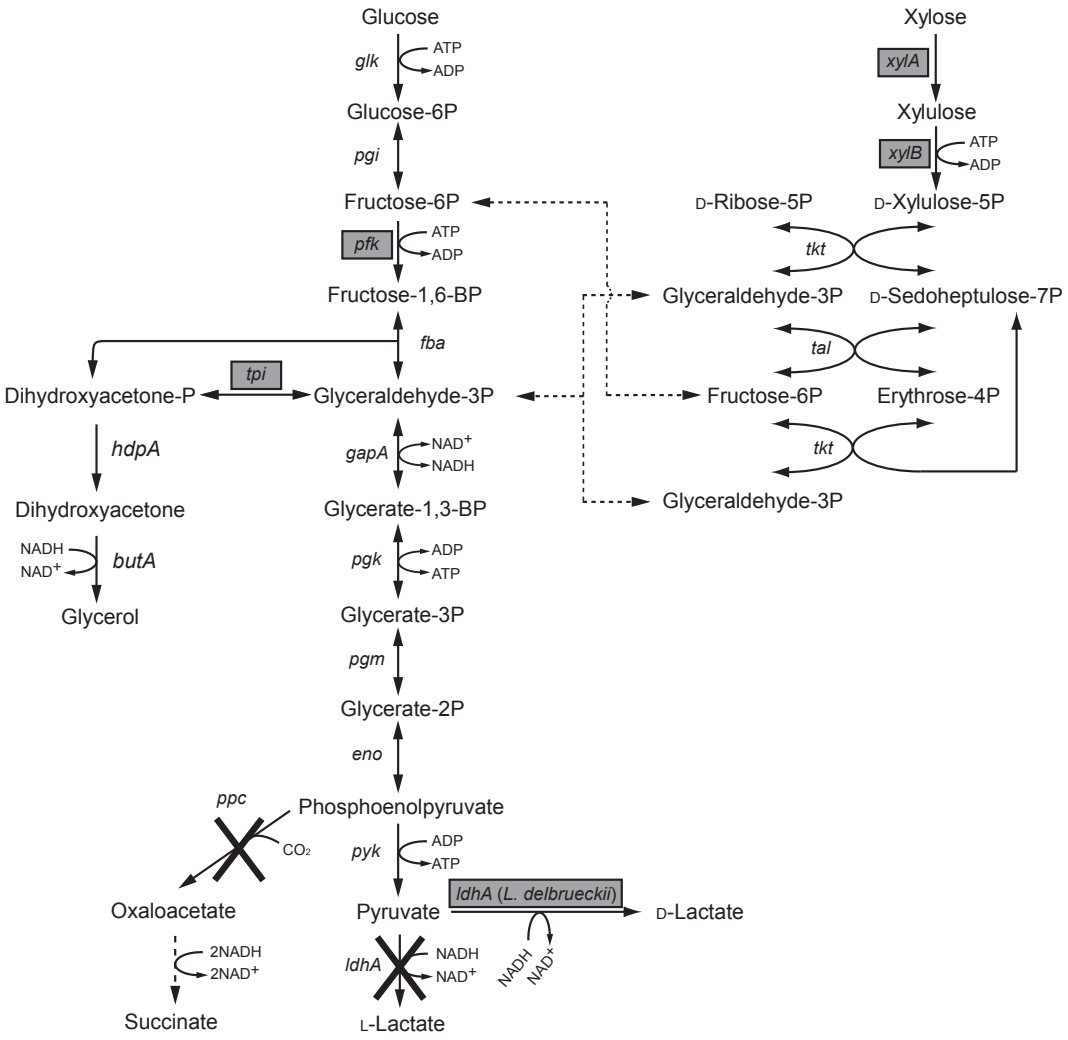

FIG. 1. Biosynthetic pathway of D-lactate. The endogenous glycolytic genes were chromosomally overexpressed. The gray boxes represent the genes for which overexpression elevated D-lactate production or genes introduced into *Corynebacterium glutamicum* cells. The native *L-ldhA* gene and the *ppc* gene, marked with a cross, were deleted from the chromosome.

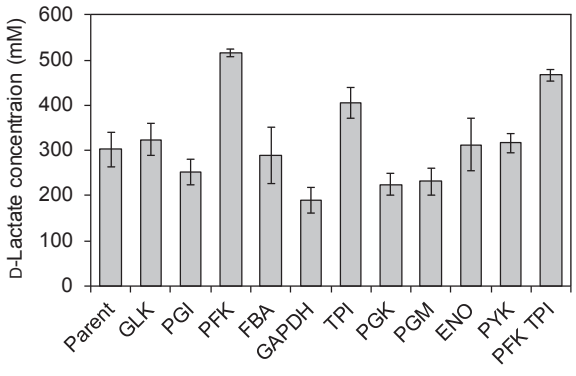

FIG. 2. Short-term D-lactate production over 10 h with strains overexpressing the genes encoding glycolytic enzymes under oxygen deprivation conditions. The overexpressed glycolytic genes are indicated on the x-axis. The strain names correspond to the overproduced enzymes as follows: GLK, LPglc255; PGI, LPglc263; PFK, LPglc257; FBA, LPglc259; GAPDH, LPglc4; TPI, LPglc258; PGK, LPglc262; PGM, LPglc261; ENO, LPglc260; PYK, LPglc18; PFK TPI, LPglc283. The parent strain is CRZ5. The bars indicate the standard deviations calculated from triplicate measurements.

effective and negatively impacted D-lactate production from a mixture of glucose and xylose.

The above results indicated that it was highly possible that the effect of the overexpression of the other glycolytic genes on D-lactate production from mixed sugars could be very different to effects of glucose as a sole carbon source. Therefore, we individually overexpressed the other nine glycolytic genes in the parent strain. The overexpression was induced through the integration of a second copy of each gene into the chromosome under the control of the constitutive *tac* promoter. All enzyme activities were successfully increased by the overexpression of each gene; the increases varied from a 1.4-fold increase in the strain overexpressing the gene encoding ENO to a 9.5-fold increase in the strain overexpressing the gene encoding PYK (Table 2). All strains were cultivated for 10 h under oxygen deprived conditions to produce D-lactate from a mixture of glucose and xylose. Two strains, overexpressing the genes encoding PFK (LPglc257/pCRB215) and TPI (LPglc258/pCRB215), showed increased D-lactate production. LPglc257/pCRB215 and LPglc258/pCRB215 produced 515 ± 8 and 405 ± 33 mM D-lactate, respectively, which was 71% and 34% higher than that of the parental strain (Fig. 2). Unexpectedly, both strains consumed more glucose than the parent strain (2.4-fold for LPglc257/pCRB215, and 1.8-fold for LPglc258/pCRB215; Table 1),

TABLE 1. Comparison of D-lactate producing strains overexpressing glycolytic genes.[a]

Strain	LDH activity (U/mg protein)	Glucose consumption rate (mM/h)	Xylose consumption rate (mM/h)	Yield (%)[b]				
				D-Lactate	Succinate	Acetate	DHA	Glycerol
CRZ5/pCRB215	59 ± 1	8.8 ± 2.5	15.9 ± 0.2	68.6 ± 4.8	1.6 ± 0.1	1.7 ± 0.3	11.3 ± 1.1	6.4 ± 0.2
LPglc255/pCRB215	71 ± 11	9.7 ± 1.0	16.2 ± 1.1	69.7 ± 3.5	1.6 ± 0.3	1.7 ± 0.3	11.1 ± 1.0	5.3 ± 0.5
LPglc263/pCRB215	66 ± 3	6.4 ± 0.8	15.4 ± 1.1	65.4 ± 2.7	1.7 ± 0.1	1.6 ± 0.2	10.9 ± 0.1	4.7 ± 0.1
LPglc257/pCRB215	66 ± 6	21.3 ± 1.3	16.0 ± 0.5	74.4 ± 1.2	2.4 ± 0.1	2.2 ± 0.1	11.4 ± 1.0	6.0 ± 0.2
LPglc259/pCRB215	64 ± 4	9.3 ± 1.8	16.2 ± 0.1	67.8 ± 5.0	1.4 ± 0.3	1.8 ± 0.3	11.3 ± 0.7	5.7 ± 0.3
LPglc4/pCRB215	57 ± 3	4.7 ± 0.9	14.1 ± 0.8	57.6 ± 6.5	1.3 ± 0.1	1.4 ± 0.1	10.0 ± 1.5	4.8 ± 0.1
LPglc258/pCRB215	60 ± 7	15.7 ± 1.6	16.5 ± 0.3	68.6 ± 3.3	2.1 ± 0.1	1.6 ± 0.3	10.3 ± 0.3	7.0 ± 2.1
LPglc262/pCRB215	58 ± 3	5.3 ± 1.4	13.9 ± 2.0	66.8 ± 3.3	1.1 ± 0.1	1.6 ± 0.5	9.4 ± 1.2	5.7 ± 0.7
LPglc261/pCRB215	55 ± 4	6.7 ± 1.6	14.4 ± 1.2	61.7 ± 1.3	1.5 ± 0.2	1.9 ± 0.1	10.5 ± 0.1	5.7 ± 0.1
LPglc260/pCRB215	46 ± 5	10.7 ± 2.5	14.5 ± 1.5	68.6 ± 5.1	2.0 ± 0.1	1.8 ± 0.3	10.0 ± 0.1	6.2 ± 0.3
LPglc18/pCRB215	42 ± 3	8.9 ± 1.3	16.2 ± 0.2	70.7 ± 2.6	1.9 ± 0.2	2.0 ± 0.4	10.2 ± 0.3	5.3 ± 0.4
LPglc283/pCRB215	42 ± 3	19.0 ± 1.2	10.3 ± 0.3	84.5 ± 1.1	0.8 ± 0.1	1.6 ± 0.3	7.0 ± 1.2	4.8 ± 0.2

The bioprocess reaction was performed for 10 h.
[a] The values represent average data with standard deviations from three independent experiments.
[b] Yields are based on mol of fermentation products produced from mol of glucose consumption (100% means 2 mol of D-lactate per 1 mol of glucose).

but the amount of xylose consumed was comparable in all strains tested.

Simultaneous overexpression of genes encoding PFK and TPI increased D-lactate concentration in long-term production Finally, we overexpressed the gene encoding TPI in LPglc257/pCRB215 (PFK) to simultaneously overexpress genes encoding PFK and TPI and determine if D-lactate production could be improved further. However, the strain (LPglc283/pCRB215) resulted in slightly decreased D-lactate production of 466 ± 12 mM after production for 10 h, which was 9.5% lower than that observed in the LPglc257/pCRB215 (PFK) strain (Fig. 2). Interestingly, the amount of xylose consumed markedly decreased by 36% (to 103 ± 3 mM) in comparison with that of the LPglc257/pCRB215 (PFK) strain (Table 1). The yields of D-lactate and by-products were also altered: D-Lactate yield markedly increased to 84.5 ± 1.1%; in contrast, the yields of DHA and glycerol were decreased to 7.0 ± 1.2% and 4.8 ± 0.2%, respectively, compared with that of LPglc257/pCRB215 (PFK) (Table 1). These results showed that D-lactate yield decreased inversely to the amount of xylose consumed when mixed sugars were used.

TABLE 2. Enzyme activity of glycolytic enzymes in the parental strain and overexpressing strains of glycolytic genes.

Enzyme	Enzyme activities (U/mg protein)		
	CRZ5/pCRB215	Overexpressing strains	LPglc283/pCRB215
GLK	0.0024 ± 0.0006	0.0088 ± 0.027	N.D.
PGI	0.14 ± 0.03	0.45 ± 0.09	N.D.
PFK	0.013 ± 0.004	0.02 ± 0.003	0.1 ± 0.001
FBA	0.15 ± 0.02	0.22 ± 0.005	N.D.
GAPDH	1.0 ± 0.05	5.0 ± 0.04	N.D.
TPI	3.9 ± 0.5	24 ± 1	14 ± 1
PGK	2.2 ± 0.04	20 ± 0.2	N.D.
PGM	0.27 ± 0.008	2.5 ± 0.08	N.D.
ENO	0.36 ± 0.01	0.58 ± 0.03	N.D.
PYK	0.22 ± 0.04	2.1 ± 0.09	N.D.

The strains of overexpressing glycolytic genes used for enzyme assay are as follows: GLK, LPglc255/pCRB215; GPI, LPglc263/pCRB215; PFK, LPglc257/pCRB215; FBA, LPglc259/pCRB215; GAPDH, LPglc4/pCRB215; TPI, LPglc258/pCRB215; PGK, LPglc262/pCRB215; PGM, LPglc261/pCRB215; ENO, LPglc260/pCRB215; PYK, LPglc18/pCRB215. Cells were collected at 2 h in the bioprocess reaction.

Next, we conducted the long-term production of CRZ5/pCRB215, LPglc257/pCRB215 (PFK), LPglc258/pCRB215 (TPI), and LPglc283/pCRB215 (PFK, TPI) strain. Interestingly, glucose consumption was accelerated by LPglc283/pCRB215 (540 ± 57 mM) than by LPglc257/pCRB215 (425 ± 23 mM) (Table 3). In contrast, the amount of xylose consumed by LPglc283/pCRB215 (PFK, TPI) decreased to 252 ± 6 mM from 375 ± 8 mM by LPglc257/pCRB215 (PFK). Although the initial D-lactate production rate in the first 10 h was varied among CRZ5/pCRB215, LPglc257/pCRB215 and LPglc258/pCRB215, the three strains slowed the production of D-lactate after 32 h, and reached similar D-lactate concentration after production for 80 h (Fig. 3). The D-lactate production rate after 32 h in CRZ5/pCRB215, LPglc257/pCRB215 (PFK) and LPglc258/pCRB215 (TPI) was 5.6, 3.3 and 5.6 mM/h, respectively. In contrast, LPglc283/pCRB215 (PFK, TPI), which had an initial D-lactate production rate 9.5% lower than that of LPglc257/pCRB215 (PFK), continued to produce D-lactate, with more than 1000 mM after 32 h, and 1326 ± 65 mM after production for 80 h (Fig. 3). The D-lactate production rate after 32 h in LPglc283/pCRB215 (PFK, TPI) was 8.7 mM/h, which was 2.6-fold higher than that of LPglc257/pCRB215 (PFK).

DISCUSSION

In this study, we investigated the effect of overexpression of glycolytic genes on D-lactate production from mixture of glucose and xylose in *C. glutamicum*. We showed that simultaneous overexpression of genes encoding PFK and TPI cooperatively enhances D-lactate production from the mixed sugars.

The overexpression of the gene encoding PFK might contribute to an increase in the amount of glucose consumed through the increased concentration of fructose-1,6-bisphosphate, which activates LDH (30,31). Moreover, continuous supply of fructose-1,6-bisphosphate by overexpression of the gene encoding PFK might be critical for high production of D-lactate from the mixed sugars without reducing the production rate throughout the production. In contrast, the yields of D-lactate and by-products were not altered in LPglc258/pCRB215 (TPI), which indicated that the distribution of carbon flux was not largely altered in this strain. Although the LDH activity varied

TABLE 3. Comparison of D-lactate producing strains overexpressing glycolytic genes for long term bioprocess reaction under oxygen deprivation.

Strain	D-Lactate concentration (mM)	Glucose consumed (mM)	Xylose consumed (mM)	Yield (%)				
				D-Lactate	Succinate	Acetate	DHA	Glycerol
CRZ5/pCRB215	992 ± 68	425 ± 23	375 ± 8	60.3 ± 3.1	2.8 ± 0.2	1.3 ± 0.1	8.4 ± 1.9	6.0 ± 0.3
LPglc283/pCRB215	1326 ± 65	540 ± 57	252 ± 6	74.0 ± 2.2	0.7 ± 0.2	1.0 ± 0.1	2.9 ± 0.9	4.6 ± 0.5

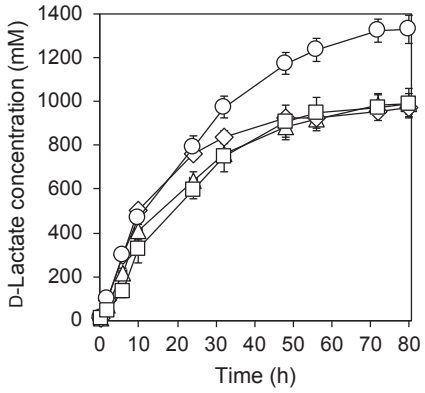

FIG. 3. Long-term D-lactate production by CRZ5/pCRB215 (squares), LPglc257/pCRB215 (diamonds), LPglc258/pCRB215 (triangles), and LPglc283/pCRB215 (circles) under oxygen deprivation conditions. The data points represent averages calculated from triplicate measurements. The standard deviations are indicated by bars or are within each symbol.

from 42 ± 3 U/mg protein in the strain overexpressing the gene encoding PYK to 71 ± 11 U/mg protein in the strain overexpressing the gene encoding GLK, a clear correlation between LDH activity and D-lactate production rate was not observed (Table 2). The effect of overexpression of glycolytic genes varied depending on the target products and sugar sources. Overexpression of the gene encoding GAPDH showed no apparent effect for enhancing D-lactate production from mixture of glucose and xylose, which was in contrast to a previous study on D-lactate production from glucose as sole carbon source, which reported an increase in D-lactate production rate of 39% (24). However, from the perspective of by-products, the additional overexpression of the gene encoding GAPDH in the strain overexpressing the gene encoding GLK released the accumulation of DHA and glycerol and decreased their yield by 74% and 24%, respectively (24). Although the cause of the disadvantages of the overexpression of the gene encoding GAPDH in this study was unclear, it may be related to the potential increase of the NADH/NAD^+ ratio in the mixed culture in comparison with the culture with only glucose, which inhibited GAPDH activity (32,33).

The ratio of the amount of glucose and xylose consumed is important for the complete utilization of the sugars in lignocellulosic biomass, which are varied depending on the materials. Overexpression of the gene encoding PFK and TPI decreased xylose consumption, but the ratio of the amount of glucose and xylose consumed in LPglc283/pCRB215 (PFK, TPI) (2.14) was similar to the ratio of the content of glucose and xylose in wheat straw (1.91) (11). In addition to the decreased yield of all by-products (succinate, acetate, DHA, and glycerol) in LPglc283/pCRB215 (PFK, TPI), the simultaneous overexpression of genes encoding PFK and TPI fine-tuned the metabolic flux in the glycolytic pathway to enable D-lactate production from a mixture of glucose and xylose. In the current study using the described bioprocess under oxygen deprived conditions, higher D-lactate concentration (974 mM; 87.7 g/L) was produced with yield of 0.81 g/g of consumed sugar after 32 h; eventually, D-lactate was produced up to 1326 mM (119.3 g/L) with a yield of 0.79 g/g of consumed sugar after 80 h (Table 3).

In conclusion, we identified two genes encoding PFK and TPI that were able to enhance D-lactate production from mixed sugars containing glucose and xylose when overexpressed in xylose-assimilating *C. glutamicum*. Furthermore, the simultaneous overexpression of genes encoding PFK and TPI improved D-lactate production and achieved a concentration of 1326 mM (119.3 g/L) after the long-term production (80 h), which is the highest titer reported to date from the mixed sugars (Table). We have identified the two genes responsible for the conversion of DHAP to glycerol via DHA (34,35). The introduction of additional genetic modifications into LPglc283/pCRB215 may allow further improvements in the production of D-lactate from the mixed sugars in *C. glutamicum*.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.08.002.

ACKNOWLEDGMENTS

The authors declare no conflict of interest. This work was supported by a grant from New Energy and Industrial Technology Development Organization (NEDO).

References

1. **Kinoshita, S.:** Glutamic acid bacteria, pp. 115–146, in: Demain, A. L. and Solomon, N. A. (Eds.), Biology of industrial microorganisms. Cummings, London (1985).
2. **Inui, M., Suda, M., Okino, S., Nonaka, H., Puskas, L. G., Vertes, A. A., and Yukawa, H.:** Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions, Microbiology, **153**, 2491–2504 (2007).
3. **Inui, M., Kawaguchi, H., Murakami, S., Vertes, A. A., and Yukawa, H.:** Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions, J. Mol. Microbiol. Biotechnol., **8**, 243–254 (2004).
4. **Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J. W., Wendisch, V. F., and Eikmanns, B. J.:** *Corynebacterium glutamicum* tailored for efficient isobutanol production, Appl. Environ. Microbiol., **77**, 3300–3310 (2011).
5. **Hasegawa, S., Suda, M., Uematsu, K., Natsuma, Y., Hiraga, K., Jojima, T., Inui, M., and Yukawa, H.:** Engineering of *Corynebacterium glutamicum* for high-yield L-valine production under oxygen deprivation conditions, Appl. Environ. Microbiol., **79**, 1250–1257 (2013).
6. **Litsanov, B., Brocker, M., and Bott, M.:** Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate, Appl. Environ. Microbiol., **78**, 3325–3337 (2012).
7. **Okino, S., Noburyu, R., Suda, M., Jojima, T., Inui, M., and Yukawa, H.:** An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain, Appl. Microbiol. Biotechnol., **81**, 459–464 (2008).
8. **Tsuge, Y., Yamamoto, S., Kato, N., Suda, M., Vertès, A. A., Yukawa, H., and Inui, M.:** Overexpression of the phosphofructokinase encoding gene is crucial for achieving high production of D-lactate in *Corynebacterium glutamicum* under oxygen deprivation, Appl. Microbiol. Biotechnol., **99**, 4679–4689 (2015).
9. **Yamamoto, S., Gunji, W., Suzuki, H., Toda, H., Suda, M., Jojima, T., Inui, M., and Yukawa, H.:** Overexpression of genes encoding glycolytic enzymes in *Corynebacterium glutamicum* enhances glucose metabolism and alanine production under oxygen deprivation conditions, Appl. Environ. Microbiol., **78**, 4447–4457 (2012).
10. **Yamamoto, S., Suda, M., Niimi, S., Inui, M., and Yukawa, H.:** Strain optimization for efficient isobutanol production using *Corynebacterium glutamicum* under oxygen deprivation, Biotechnol. Bioeng., **110**, 2938–2948 (2013).
11. **Aristidou, A. and Penttila, M.:** Metabolic engineering applications to renewable resource utilization, Curr. Opin. Biotechnol., **11**, 187–198 (2000).
12. **Kawaguchi, H., Hasunuma, T., Ogino, C., and Kondo, A.:** Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks, Curr. Opin. Biotechnol., **42**, 30–39 (2016).
13. **Paulova, L., Patakova, P., Branska, B., Rychtera, M., and Melzoch, K.:** Lignocellulosic ethanol: Technology design and its impact on process efficiency, Biotechnol. Adv., **33**, 1091–1107 (2015).
14. **Jönsson, L. J. and Martín, C.:** Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects, Bioresour. Technol., **199**, 103–112 (2016).
15. **Fukushima, K., Chang, Y. H., and Kimura, Y.:** Enhanced stereocomplex formation of poly(L-lactic acid) and poly(D-lactic acid) in the presence of stereoblock poly(lactic acid), Macromol. Biosci., **7**, 829–835 (2007).
16. **Ikada, Y., Jamshidi, K., Tsuji, H., and Hyon, S.:** Stereocomplex formation between enantiomeric poly(lactides), Macromolecules, **20**, 904–906 (1987).
17. **Tsuge, Y., Kawaguchi, H., Sasaki, K., and Kondo, A.:** Engineering cell factories for producing building block chemicals for bio-polymer synthesis, Microb. Cell Fact., **15**, 19 (2016).
18. **Hama, S., Mizuno, S., Kihara, M., Tanaka, T., Ogino, C., Noda, H., and Kondo, A.:** Production of D-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*, Bioresour. Technol., **187**, 167–172 (2015).

19. **Lu, H., Zhao, X., Wang, Y., Ding, X., Wang, J., Garza, E., Manow, R., Iverson, A., and Zhou, S.:** Enhancement of D-lactic acid production from a mixed glucose and xylose substrate by the *Escherichia coli* strain JH15 devoid of the glucose effect, BMC Biotechnol., **19**, 19 (2016).
20. **Tsuge, Y., Kawaguchi, H., Sasaki, K., Tanaka, T., and Kondo, A.:** Two-step production of D-lactate from mixed sugars by growing and resting cells of metabolically engineered *Lactobacillus plantarum*, Appl. Microbiol. Biotechnol., **98**, 4911–4918 (2014).
21. **Utrilla, J., Vargas-Tah, A., Trujillo-Martínez, B., Gosset, G., and Martinez, A.:** Production of D-lactate from sugarcane bagasse and corn stover hydrolysates using metabolic engineered *Escherichia coli* strains, Bioresour. Technol., **220**, 208–214 (2016).
22. **Yi, X., Zhang, P., Sun, J., Tu, Y., Gao, Q., Zhang, J., and Bao, J.:** Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock, J. Biotechnol., **217**, 112–121 (2016).
23. **Yoshida, S., Okano, K., Tanaka, T., Ogino, C., and Kondo, A.:** Homo-D-lactic acid production from mixed sugars using xylose-assimilating operon-integrated *Lactobacillus plantarum*, Appl. Microbiol. Biotechnol., **92**, 67–76 (2011).
24. **Tsuge, Y., Yamamoto, S., Suda, M., Inui, M., and Yukawa, H.:** Reactions upstream of glycerate-1,3-bisphosphate drive *Corynebacterium glutamicum* D-lactate productivity under oxygen deprivation, Appl. Microbiol. Biotechnol., **97**, 6693–6703 (2013).
25. **Tsuge, Y., Ogino, H., Teramoto, H., Inui, M., and Yukawa, H.:** Deletion of *cgR_1596* and *cgR_2070*, encoding NlpC/P60 proteins, causes a defect in cell separation in *Corynebacterium glutamicum* R, J. Bacteriol., **190**, 8204–8214 (2008).
26. **Suzuki, N., Okayama, S., Nonaka, H., Tsuge, Y., Inui, M., and Yukawa, H.:** Large-scale engineering of the *Corynebacterium glutamicum* genome, Appl. Environ. Microbiol., **71**, 3369–3372 (2005).
27. **Kaparaju, P., Serrano, M., Thomsen, A. B., Kongjan, P., and Angelidaki, I.:** Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept, Bioresour. Technol., **100**, 2562–2568 (2009).
28. **Persson, T., Ren, J. L., Joelsson, E., and Jönsson, A. S.:** Fractionation of wheat and barley straw to access high-molecular-mass hemicelluloses prior to ethanol production, Bioresour. Technol., **100**, 3906–3913 (2009).
29. **Sasaki, M., Jojima, T., Kawaguchi, H., Inui, M., and Yukawa, H.:** Engineering of pentose transport in *Corynebacterium glutamicum* to improve simultaneous utilization of mixed sugars, Appl. Microbiol. Biotechnol., **85**, 105–115 (2009).
30. **Arai, K., Hishida, A., Ishiyama, M., Kamata, T., Uchikoba, H., Fushinobu, S., Matsuzawa, H., and Taguchi, H.:** An absolute requirement of fructose 1,6-bisphosphate for the *Lactobacillus casei* L-lactate dehydrogenase activity induced by a single amino acid substitution, Protein Eng., **15**, 35–41 (2002).
31. **Gaspar, P., Neves, A. R., Shearman, C. A., Gasson, M. J., Baptista, A. M., Turner, D. L., Soares, C. M., and Santos, H.:** The lactate dehydrogenases encoded by the *ldh* and *ldhB* genes in *Lactococcus lactis* exhibit distinct regulation and catalytic properties-comparative modeling to probe the molecular basis, FEBS J., **274**, 5924–5936 (2007).
32. **Dominguez, H., Rollin, C., Guyonvarch, A., Guerquin-Kern, J. L., Cocaign-Bousquet, M., and Lindley, N. D.:** Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose, Eur. J. Biochem., **254**, 96–102 (1998).
33. **Tsuge, Y., Uematsu, K., Yamamoto, S., Suda, M., Yukawa, H., and Inui, M.:** Glucose consumption rate critically depends on redox state in *Corynebacterium glutamicum* under oxygen deprivation, Appl. Microbiol. Biotechnol., **99**, 5573–5582 (2015).
34. **Jojima, T., Igari, T., Gunji, W., Suda, M., Inui, M., and Yukawa, H.:** Identification of a HAD superfamily phosphatase, HdpA, involved in 1,3-dihydroxyacetone production during sugar catabolism in *Corynebacterium glutamicum*, FEBS Lett., **586**, 4228–4232 (2012).
35. **Jojima, T., Igari, T., Moteki, Y., Suda, M., Yukawa, H., and Inui, M.:** Promiscuous activity of (*S,S*)-butanediol dehydrogenase is responsible for glycerol production from 1,3-dihydroxyacetone in *Corynebacterium glutamicum* under oxygen-deprived conditions, Appl. Microbiol. Biotechnol., **99**, 1427–1433 (2015).