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Construction of a constitutively expressed homo-fermentative pathway in *Lactobacillus brevis*

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Abstract *Lactobacillus brevis* is a promising lactic acid producing strain that simultaneously utilizes glucose and xylose from lignocellulosic hydrolysate without carbon catabolic repression and inhibition. The production of by-products acetic acid and ethanol has been the major drawback of this strain. Two genes, *pfk*A (fructose-6-phosphate kinase [PFK]) and *fba*A (fructose-1,6-biphosphate aldolase [FBA]), that encode the key enzymes of the EMP/glycolytic pathway from *Lactobacillus rhamnosus*, were fused to the downstream of the strong promoter P32 and expressed in *L. brevis* s3f4 as a strategy to minimize the formation of by-products. By expressing the two enzymes, a homo-fermentative pathway for lactic acid production was constructed. The lactic acid yields achieved from glucose in the transformants were 1.12 and 1.16 mol/mol, which is higher than that of the native strain (0.74 mol/mol). However, the lactic acid yield from xylose in the transformants stayed the same as that of the native strain. Enzyme assay indicated that the activity of the foreign protein FBA in the transformants was much higher than that of the native strains, but was ten times lower than that in *L. rhamnosus*. This result was consistent with the metabolic flux analysis, which indicated that the conversion efficiency of the expressed PFK and FBA was somewhat low. Less than 20 % of the carbons accumulated in the form of fructose-6-phosphate were converted into glyceraldehyde-3-phosphate (GAP) by the expressed PFK and FBA. Metabolic flux analysis also indicated that the enzyme phosphoketolase (XPK) played an important role in splitting the carbon flow from the pentose phosphate pathway to the phosphoketolase pathway. This study suggested that the lactic acid yield of *L. brevis* could be improved by constructing a homo-fermentative pathway.

Keywords *Lactobacillus brevis* · Homo-fermentative · Lactic acid · Fructose-6-phosphate kinase · Fructose-1,6-biphosphate aldolase

Introduction

Lactic acid can be commercially produced from renewable feedstock, such as plant derived starch. Raw material is one of the major contributors to lactic acid's production cost, in addition to cost of purification. It was estimated that feedstock cost accounts for about 30 to 40 % of the total lactic acid production cost (Akerberg and Zacchi 2000; Tejayadi and Cheryan 1995; Oh et al. 2005). Moreover, non-food renewable biomass is preferred for large-scale lactic acid production. Therefore, a cheaper and sustainable feedstock that can substitute the crop-derived starch is needed for biochemical production of lactic acid.

Lignocellulose is one of the most abundant biomass resources and therefore could be used as a sustainable non-food feedstock for the production of biofuels and chemicals such as lactic acid. The process of utilizing lignocellulosic biomass as the carbon source for fermentation include pretreatment and hydrolysis to degrade plant cell wall and release sugar. However, most industrial strains of lignocellulose are unable to ferment the hydrolysate efficiently. One of the major problems for its utilization is the fact that sugars derived from the biomass are a mixture of glucose and xylose. Xylose is not fermentable by most lactic acid bacteria.

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A large number of lactic acid producing strains have been screened or developed in the past decades for pentose utilization (Picataggio et al. 1998; Patel et al. 2006; Bai et al. 2008; Ilmen et al. 2007; Wang et al. 2011). However, in most cases, the pentose catabolism was suppressed by the glucose also present in the mixture (Zhang et al. 2007; Dien et al. 2001; Iyer et al. 2000). The selective and sequential utilization of mixed sugars by most microbes not only makes the fermentation process complex, it also often reduces the yield and productivity (Kim et al. 2010a). Moreover, a number of toxic compounds are often formed during the pretreatment process of lignocellulosic materials, besides the sugars. These compounds inhibit the growth of most microbes. In fact, many excellent lactic acid producing strains, such as *Escherichia coli* (Shoemaker 2004) and *Bacillus coaglulans* (Gorichem 2007), were sensitive to the inhibitors.

Lactobacillus brevis was found to be a promising strain for use in lactic acid production in a lignocellulosic biorefinery, because of its excellent performance in simultaneous utilization of xylose/glucose, and its strong ability to resist toxic compounds generated by the pretreatment of lignocelluloses (Guo et al. 2010; Kim et al. 2010b; Winkler and Kao 2011). Different from most xylose utilizing strains that ferment mixed carbohydrate sequentially, *L. brevis* exhibited a relaxed control of sugar utilization. It simultaneously consumed numerous sugars, such as xylose, glucose, and arabinose without carbon catabolite repression (Guo et al. 2010; Kim et al. 2009). However, the theoretical yield of lactic acid from pentose or hexose was only 1.0 (mol/mol) in *L. brevis*, as the consumed carbons were partly lost in the form of acetic acid and ethanol through the hetero-fermentative pathway.

It was reported that there were two different pathways for xylose metabolism existing in lactic acid bacterium *Lactococcus lactis* IO-1 (Tanaka et al. 2002; Shinkawa et al. 2011); one was phosphoketolase (PK) pathway (hetero-fermentative pathway) and another was pentose phosphate (PP)/glycolytic pathway (homo-fermentative pathway). The PK pathway catalyzes the cleavage of D-xylulose-5-phosphate to equal-molar amounts of glyceraldehyde 3-phosphate and acetylphosphate; hence, the yield coefficients of lactate from xylose or glucose in strains that possess only this pathway do not exceed 1.0 mol/mol. The PP/glycolytic pathway converts xylose or glucose to lactate as sole product, and the theoretical yield coefficient of lactate from xylose or glucose in strains that possess only PP/glycolytic pathway will be 1.67 or 2.00 mol/mol. But in strains that possess the two pathways, such as *L. lactis* IO-1, the yield coefficient of lactate from pentose will be between 1.0 and 1.67 mol/mol. Hence, the yield coefficient of lactate from xylose or glucose is dependent on the proportion of carbon flux distribution between the two pathways. Higher proportion of carbon flux to the PP/glycolytic pathway will result in higher lactate yield.

It was determined through analyzing the genetic and metabolic information of *L. brevis* in the KEGG database that *L. brevis* possess all the enzymes needed for homo-fermentative metabolism of lactic acid, except the fructose-6-phosphate kinase (PFK) and fructose-1,6-biphosphate aldolase (FBA) enzymes. Thus, it is logical to assume that constitutively expressing the *pfk* and *fba* genes in *L. brevis* may introduce the PP/glycolytic pathway in *L. brevis* (Fig. 1). If successful, the carbon flux in *L. brevis* may change from the PK pathway to the created homo-fermentative pathway, and hence increase lactate yield.

In this study, a constitutively expressed homo-fermentative pathway in *L. brevis* was constructed through expressing the two key enzymes of the glycolytic pathway PFK and FBA from *L. rhamnosus*. As a result the lactic acid yield from glucose was enhanced in the recombinant strains in comparison with the native strain. The metabolic flux of the transformant with glucose and xylose as carbon sources were analyzed. Metabolic flux analysis results indicated that with glucose as the carbon source, the carbon flux was redirected into the created glycolytic (homo-fermentative) pathway from the PK pathway in the engineered strains.

Materials and methods

Strains and plasmids

Lactobacillus rhamonosus CGMCC 1.2134 was used for cloning the *pfk* and *fba* genes. *L. brevis* s3f4 was used as the host for metabolic engineering. *E. coli* DH5α was used for gene cloning and manipulation. The strains, plasmids, and primers used in this work are described in Table S1.

Medium and culture conditions

E. coli was grown in Luria–Bertani medium; 100 μg/ml of ampicillin was added to the medium when required. *Lactobacillus* strains were routinely cultivated at 30°C in MRS medium containing 10 g/l peptone, 8 g/l beef extract, 4 g/l yeast extract, 1 ml/l Tween 80, 2 g/l K_2HPO_4, 5 g/l sodium acetate·$3H_2O$, 2 g/l triammonium citrate, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $MnSO_4$, and suitable carbon sources were added according to specific purposes. For selection of the transformants, 20 g/l of glucose was added to the medium; for testing the fermentation ability of the transformants, 60 g/l of glucose or 60 g/l of xylose were added to the medium, respectively. For selection and cultivation of transformants, 10 μg/ml of erythromycin were added to the medium.

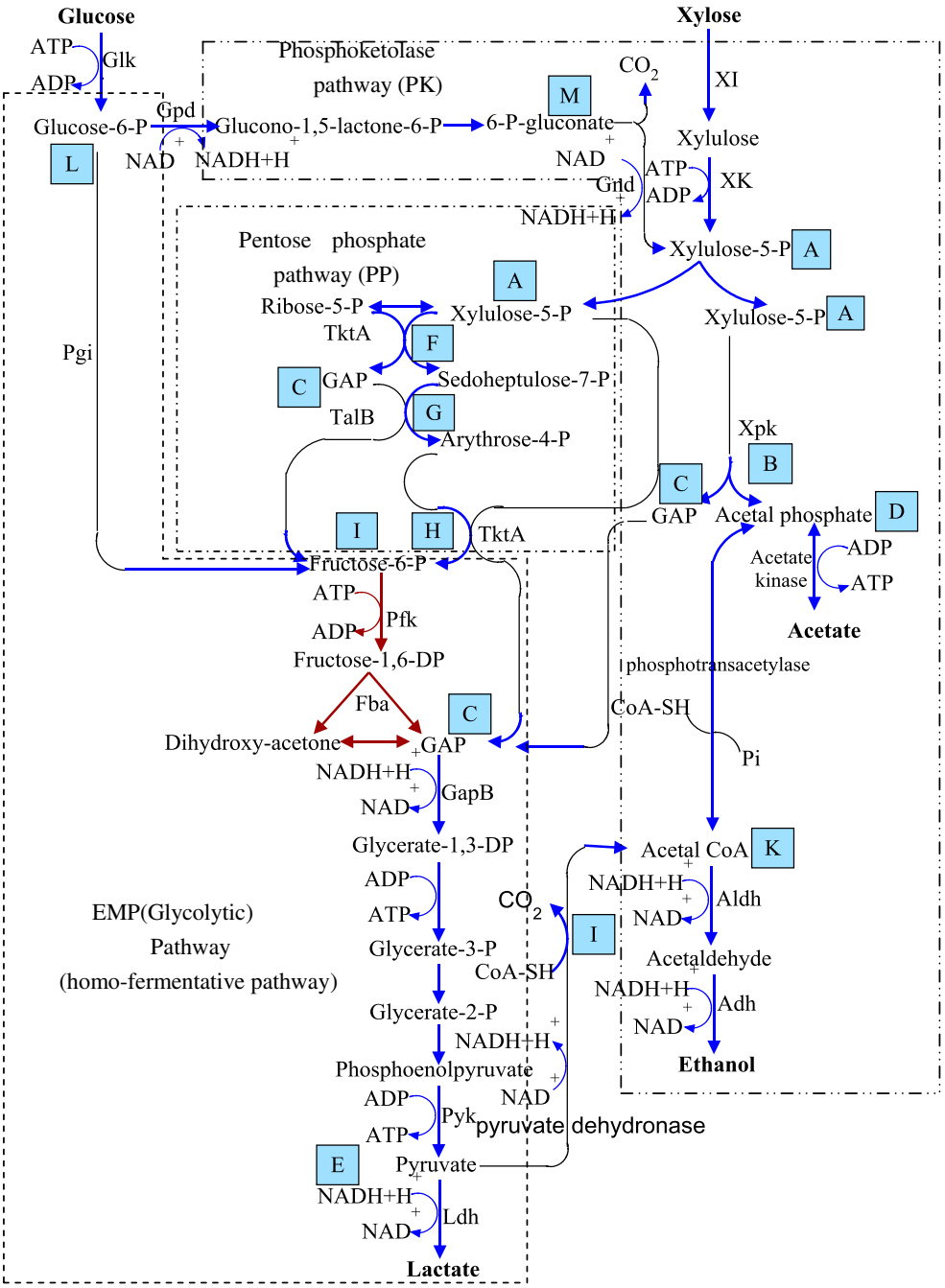

Fig. 1 Proposed xylose and glucose metabolism in engineered *L. brevis*

Molecular biology

By using standard cloning techniques, the structural genes of *pfk*A and *fba*A were amplified from the genome extract of *L. rhamnosus* CGMCC 1.2134 by PCR procedure, respectively. As the genome *L. rhamnosus* CGMCC 1.2134 was not sequenced, primers were designed according to the nucleotide sequence of the two genes in *L. rhamnosus* GG with assigned GenBank accession numbers 8422059 and 8420539. Promoter P32 was amplified from the plasmid extract of pMG36e (Van De Guchte et al. 1989). Recombinant genes of P32*pfk* and P32*fba* with a fusion of the P32 promoter were constructed by overlap extension PCR. Cloning vectors pGEMT-P_{32}pfk and pGEMT-P32fba were constructed by inserting the PCR product into the T-vector pGEMT. Constructed plasmids were submitted for sequencing, and the sequencing results (data not shown) indicated that cloned *pfk*A and *fba*A structural genes of *L. rhamnosus* CGMCC 1.2134 were identical to that of *L. rhamnosus* GG. Correctly constructed plasmids were applied for the following work. Expression plasmid pMG36e-P_{32}fba was constructed by inserting the recombinant gene P32fba (*Eco*RI/*Xba*I digested fragment from pGEMT-P_{32}fba) into the

shuttle vector pMG36e in the *Eco*RI/*Xba*I sites. Expression plasmid pMG36e-P32pfk-P32fba was generated by inserting the P32-pfk (*Eco*RI/*Xma*I) fragment into the recombinant plasmid pMG36e-P32fba in the *Eco*RI/*Xma*I sites.

The plasmid maps of the constructed expression plasmids were shown in Fig. S1.

Transformation of the constructed expression plasmid into *L. brevis* s3f4 was conducted according to the procedure described by Aukrust et al. (1995). Colonies formed on the MRS selective plate were selected and inoculated on MRS plates with xylose as carbon source containing 10 μg/ml erythromycin. Then, strains were inoculated in 10 ml of MRS liquid culture with xylose as carbon source with 10 μg/ml erythromycin at 30°C for 17 to 20 h. Next, the cultures were centrifuged and the strain pellets were collected for plasmid extraction using the plasmid extraction kit. Successful introduction of expression cassettes into *L. brevis* s3f4 was confirmed by PCR using the plasmid extracts of the transformants as template with specific primers.

To identify whether the constructed plasmids were introduced into *L. brevis* s3f4 successfully, three pairs of primers were designed for identification PCR. Primers Upmg and Lpmg were designed based on the nucleotide sequence of the plasmid vector pMG36e. Primers fbalrhlap/fbalrhend were designed according to the structural gene of *fba*A, and primers P32xma2/fbalrhend were designed according to the recombinant gene P32*fba*A. PCR with primers Upmg/Lpmg and fbalrhlap/fbalrhend verified the successful transformation of s3f4 with plasmid pMG36e-P32pfk-P32fba encoding genes P32pfkA and P32fbaA. PCR with primers Upmg/Lpmg and P32xma2/fbalrhend verified the successful transformation of the native strain with plasmids pMG36e-P32fba encoding the recombinant gene P32fbaA. PCR gel to verify the successful transformation of *L. brevis* s3f4 with the constructed expression plasmids was shown in Fig. S1.

Fermentation conditions

Batch fermentation of the wild-type strains and transformants were conducted at 30°C in a 50-ml flask with 30 ml (5 % [v/v]) inoculums of working volume at 150 rpm. The fermentation was conducted in semi-anaerobic matter by sealing the flask with brown packing papers. Fermentation samples were taken at 0, 4, 24, 30 and 36 h for further analysis, and experiments were carried out in duplicate.

Enzymatic assays

The native strain *L. brevis* s3f4 was grown in MRS liquid medium supplemented with xylose as carbon source, and 10 μg/ml of erythromycin was added to the culture medium for cultivation of the transformants. Static cultures were grown overnight with 100 ml volume at 30°C in 250-ml Erlenmeyer flasks. Cells were harvested in early stationary phase, and washed twice with 0.9 % physiological saline, and once with 10 mM Tris–HCL buffer (pH7.0) supplemented with 1 mM dithiothreitol, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, and resuspended in 5 ml of Tris–HCl buffer for ultrasonic oscillation. Cell debris was removed by centrifugation at 10,000×*g* at 4°C for 10 min, and the collected supernatant (crude extracts) was used for further analysis. Procedures described by Baumann and Baumann (1975) were followed to assay the fructose-6-phosphate kinase and the fructose1,6-diphosphate aldolase activity of the crude cell extracts.

Analysis

Glucose, xylose, lactic acid, formic acid, ethanol and acetic acid were measured via HPLC using a refractive index detector and an IC-Pak Ion Exclusion column 7.8×300 mm (Waters, Milford, MA, USA). Samples were eluted using 2 mM H_2SO_4 at a flow rate of 0.6 ml/min at 50 °C.

Results

Enzyme assay of the native strain and transformants

To examine whether the introduced genes P_{32}*pfk*A and P_{32}*fba*A were identified and expressed properly by the host strain *L. brevis* s3f4, crude enzyme extracts of the transformants and native strain were subjected to enzyme assay (Table 1). The results showed that both PFK and FBA activity was detected in the transformants, which indicated that exogenous *pfk*A and *fba*A genes might be expressed properly by the transformants. However, PFK activity was also detected in the native strain *L. brevis* s3f4 (lower than the transformants), although genes encoding PFK were not found in the published genomic information of *L. brevis* ATCC 367. The FBA enzyme activity in the native strain was also detected at a very low level. With the control of the strong promoter P32, the *fba*A gene was found expressed in transformants with enzyme activity achieving 0.22 and 0.45 U/ml, which was about 20 and 45 times higher than that of the native strain, respectively. However, the activity of the expressed foreign protein FBA in the transformants was about ten times lower than in the strain *L. rhamnosus* CGMCC 1.2134

Fermentation of glucose and xylose by the native strain and the transformants

To verify whether the homo-fermentative pathway was constructed successfully in the transformants by expressing the PFK and FBA enzyme, performance of the native strain and

Table 1 Enzyme activity of transformants and native strains

Samples		Native strain	Transformants		Original strain for gene cloning
		s3f4	pf	pp	*L. rhamnosus*
Enzyme activity (U/mg protein)	pfk	2.88±0.56	5.08±0.92	4.88±1.02	6.97±0.65
	fba	0.015±0.005	0.12±0.03	0.35±0.10	3.80±0.16

the transformants with glucose or xylose as carbon source was investigated (Fig. 2).

Fermentation profiles of L. *brevis* s3f4 and the transformants were investigated and compared (Fig. 2). According to Fig. 2, cells of *L. brevis* s3f4 and the transformants grew well with both glucose and xylose as carbon sources. The transformants kept the same fermentation pattern in comparison with the native strain. The highest lactic acid concentration was achieved at 30 h with both glucose and xylose as carbon sources by both the native and the transformants strains. The total carbon yield of the lactic acid, acetic acid, and ethanol relative to the amount of consumed glucose by s3f4 was 0.86 mol/mol at 30 h, which indicated that carbons might be lost in the form of carbon dioxide. However, the total carbon yields of the lactic acid, acetic acid, and ethanol relative to the amount of substrate by the transformants were 0.98 and 0.99 mol/mol at 30 h. The results indicated that all the consumed glucose might be metabolized into lactic acid, acetic acid, and ethanol by the transformants. With xylose as carbon source, the total carbon yields of the lactic acid, acetic acid, and ethanol relative to the amount of substrate by the original and the transformants *L. brevis* pf and *L. brevis* pp were1.20, 1.28 and 1.35 (mol/mol at 30 h. It means besides xylose, small amount of carbon source (introduced by the nitrogen source) existed in the culture medium might be utilized by the strain. In the late fermentation phase (about 36 h) the lactic acid concentration of the native and the transformants was decreased, which means that when glucose or xylose were consumed completely by the native strain and transformants, lactic acid was metabolized by the strains as carbon source. The fermentation data of the native strain and the transformants at 30 h were applied to calculate the metabolic flux, and detailed data were shown in Tables 2 and 3.

When 56 g/l of glucose was consumed, the lactic acid yield of the transformants was higher than that of the native strains. More glucose consumed was converted into lactic acid by the transformants, which means that a constitutive homo-fermentative pathway was constructed by expressing the *pfk*A and *fba*A genes in *L. brevis*. Transformants of *L. brevis* pf with the foreign gene *fba*A exhibited the same lactic acid yield from glucose with transformants *L. brevis* pp possessing both the *pfk*A and *fba*A genes. This can be explained by the enzyme assay results, as PFK enzyme activity was detected in the native strain and the transformants *L. brevis* pf without the foreign gene *pfk*A. Ethanol and acetate were the two major carbon by-products in both the native and the transformant strains with glucose as carbon source. Yields of ethanol of the transformants were lower than that of the native strain, whereas yields of acetic acid of the transformants were higher than that of the native strain.

With 60 g/l xylose as carbon source, the lactic acid yields of the transformants were almost the same with the native strain. Yields of the two carbon by-product of the transformants were equal to that of the native strain. It means that the proposed homo-fermentative pathway established by expressing *pfk*A and *fba*A genes might have failed to function with xylose as a carbon source.

Metabolic flux analysis

To investigate the detailed carbon metabolic fluxes, the fermentation results at 30 h of the transformants and the native strains were used to calculate the metabolic flux based on carbon balance.

The metabolic pathways of the transformants for glucose and xylose utilization are shown in Fig. 1. For analyzing the metabolic flux of the host and the transformants using xylose as carbon source, methods developed by Ohara et al. (2007) were used in this work. For analyzing the metabolic flux of the strains with glucose and xylose as carbon sources, the junctions A–M were set; the same letters were organized in a circuit diagram and each pathway was named f_1–f_{26} (Fig. 3). The circuit was expressed in an augmented matrix (Fig. 4), and the calculated results were listed in Tables 4 and 5.

With glucose as the carbon source, carbons contained in the measured products were almost equal to that of glucose consumed in the transformants. But about 13 % of carbons was lost when 312 mmol glucose were consumed by the native strain *L. brevis* s3f4. As the FBA enzyme activity of the native strain was very low, we presumed that the value of f_{17} of the native strain was 0, with glucose as the carbon source. With xylose as the carbon source, carbons contained in the measured products were higher than those contained in the xylose consumed. Hence, we presumed that all the products were produced through the central metabolic pathway (PK pathway, and the created glycolytic pathway/PP pathway), carbon dioxide lost at the nod of J (Fig. 3) was negligible, and the value of f_{21} in the augmented matrix was set to zero when

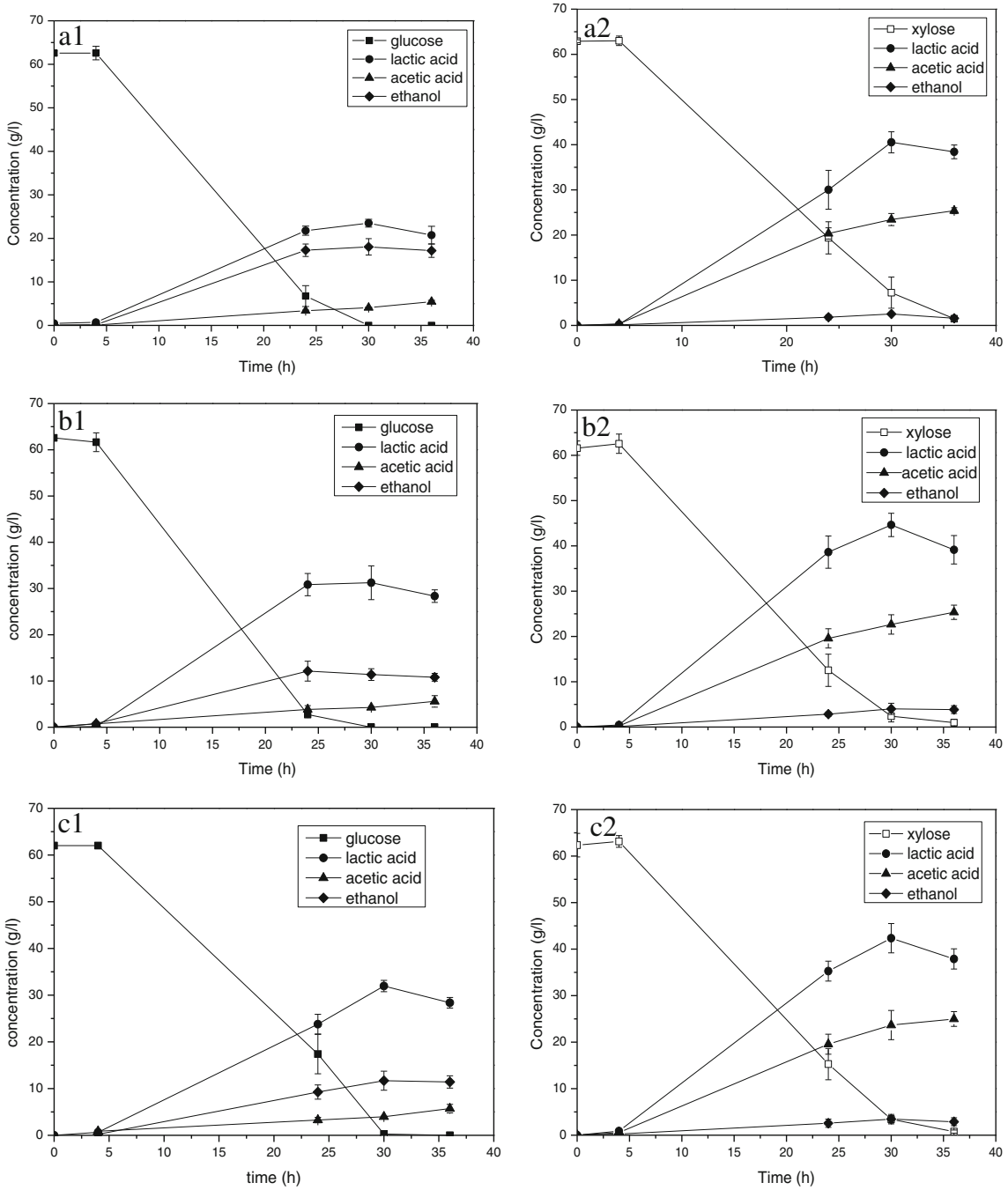

Fig. 2 Fermentation profile of *L. brevis* s3f4 (**a1**, **a2**), transformants pf (**b1**, **b2**), and transformants pp (**c1**, **c2**) with xylose or glucose as carbon source

Table 2 Fermentation results of the transformants and native strains with glucose as carbon source

Strains	Time (h)	Consumed glucose (g/l)	Lactic acid (g/l)	Acetic acid (g/l)	Ethanol (g/l)	$Y_{lac/s}$ (mol/mol)
L. brevis s3f4	30	56.21±0.12	20.94±0.58	2.57±0.54	19.37±3.13	0.74±0.02
L. brevis pf	30	56.36±0.07	31.36±0.79	5.39±0.48	14.23±1.54	1.12±0.03
L. *brevis* pp	30	56.33±0.16	32.71±0.97	6.04±0.40	12.87±0.52	1.16±0.03

$Y_{lac/s}$ yield of lactic acid from substrate

Table 3 Fermentation results of the transformants and native strains with xylose as carbon source

Strains	Time (h)	Consumed xylose (g/l)	Lactic acid (g/l)	Acetic acid (g/l)	Ethanol (g/l)	$Y_{lac/s}$ (mol/mol)
L. brevis s3f4	30	56.06±3.45	40.53±2.34	23.43±1.34	2.53±0.18	1.20±0.05
L. brevis pf	30	46.51±4.20	36.37±2.75	23.84±1.49	2.18±0.21	1.30±0.02
L. brevis pp	30	52.38±4.44	40.52±2.81	22.45±1.58	3.30±0.72	1.29±0.02

$Y_{lac/s}$ yield of lactic acid from substrate

metabolic fluxes were calculated with xylose as the carbon source. The calculated f_1–f_{26} results are listed in Tables 3 and 5.

The calculated results (Table 4) indicated that almost all the glucose consumed flowed into the EMP pathway at the node G-6-P of the metabolic network in the transformants, as the value of f_{24} was almost equal to f_{23}. The positive value of f_{17} indicated that the homo-fermentative pathway was constructed successfully by expressing *fba* or *pfk*+*fba* genes in *L. brevis* s3f4. However, efficiency of the constructed pathway was somewhat low; when 1735 or 1773 mmol carbons were accumulated in the junction I (fructose-6-phosphate), only 277 or 357 mmol carbons were converted into GAP by the expressed PFK and FBA enzymes. About 1,600 mmol excessive carbons in fructose-6-phosphate were re-entered into the PK pathway through carbon chain rearrangement.

With xylose as the carbon source, the lactic acid yield (mol/mol) of the transformants from xylose was the same as that of the native strain. Metabolic flux analysis results listed in Table 5 revealed that, with xylose as the carbon source, f_1 was equal to f_2 in both the native strain and the transformants, which means that by expressing *pfk*A and *fba*A genes in the transformants, the majority of carbons still flowed into the PK pathway instead of the PP pathway at the node of xylulose-5-p. It suggests that PK played an important role in carbon distribution between the PK pathway and the created glycolytic pathway, as it seized most carbons at the node of xylulose-5-p.

Discussion

In this study, a homo-fermentative pathway was constructed in *L. brevis* s3f4 by expressing *pfk* and *fba* genes. The lactic acid yield from glucose of the transformants was enhanced in comparison with the native strains. With about 60 g/l glucose as the carbon source, the lactic acid yield of the transformants *L. brevis* pp and *L. brevis* pf reached 1.16 and 1.12 mol/mol, respectively, higher than that of the native strain. The lactic acid yield of the transformants with plasmid pMG36e-P32fba was almost equal to that of the transformants with plasmid pMG36e-P32pfk-P32fba, which means that only expressing the *fba*A gene might be enough for construction of the homo-fermentative pathway in *L. brevis* s3f4. This result was identical with the enzyme assay result of the native strain, as the PFK activity was detected in the native strain. Saier et al. (1996) reported that PFK and FBA in *L. brevis* were inducible, and function exclusively in fructose utilization. In this study, PFK enzyme activity was detectable with xylose as carbon

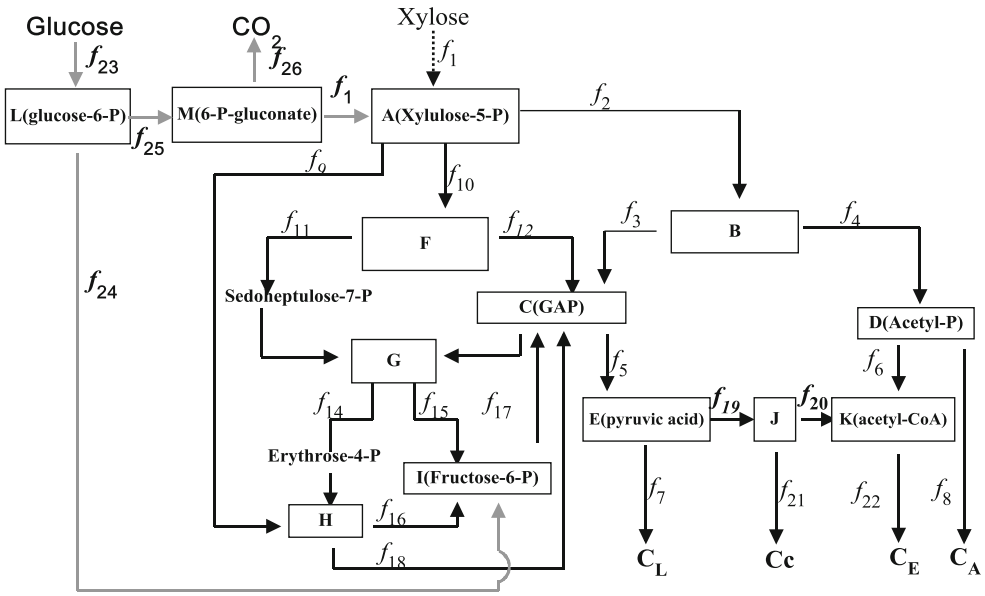

Fig. 3 Circuit of modified metabolic pathways in transformants

Fig. 4 Matrix derived from carbon balance and stoichiometry of carbon flow

	f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	f_{15}	f_{16}	f_{17}	f_{18}	f_{19}	f_{20}	f_{21}	f_{22}	f_{23}	f_{24}	f_{25}	f_{26}	
A	1	−1	0	0	0	0	0	0	−1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	1	−1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	1	0	−1	0	0	0	0	0	0	1	−1	0	0	0	1	1	0	0	0	0	0	0	0	0	0
D	0	0	0	1	0	−1	0	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	1	0	−1	0	0	0	0	0	0	0	0	0	0	0	−1	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	1	−1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	1	0	1	−1	−1	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	−1	0	−1	0	0	0	0	0	0	0	0	0
I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	−1	0	0	0	0	0	0	1	0	0	0
J	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	−1	0	0	0	0	0	0
K	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	−1	0	0	0	0	0
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	−1	0	0	0
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	0	0
Lactic acid	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C_L	
Carbon dioxic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	C_C	
Ethanol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	C_E	
Acetic acid	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C_A	
Glucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	C_G	
B_{out}	0	0	2	−3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
F_{out}	0	0	0	0	0	0	0	0	0	0	3	−7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
G_{in}	0	0	0	0	0	0	0	0	0	0	3	0	−7	0	0	0	0	0	0	0	0	0	0	0	0	0	
G_{out}	0	0	0	0	0	0	0	0	0	0	0	0	0	6	−4	0	0	0	0	0	0	0	0	0	0	0	
H_{in}	0	0	0	0	0	0	0	0	4	0	0	0	0	−5	0	0	0	0	0	0	0	0	0	0	0	0	
H_{out}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	−6	0	0	0	0	0	0	0	0	
J_{out}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−2	0	0	0	0	0	
M_{out}	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−5	0

source under semi-anaerobically environment. Therefore, *L. brevis* s3f4 may possess an inducible homo-fermentative pathway for lactic acid production.

Although, the lactic acid yield of the transformant from glucose was enhanced to 1.19 mol/mol, the yield is much lower than the theory yield of the homo-fermentation pathway 2.0 mol/mol. This result can be explained considering two aspects: one, the low activity of the expressed foreign genes; and two, the carbon flux distribution between the PK pathway and the created glycolytic pathway in the transformants. With the expressed PFK and FBA enzymes, the PFK activity of the transformants was equivalent to that of the homo-fermentative *L. rhamnosus* CGMCC 1.2134, but the FBA activity of the transformants was much lower than that in *L. rhamnosus* CGMCC 1.2134. The low activity of the expressed foreign FBA enzyme might lead to low conversion efficiency of 6-phosphate fructose to GAP, which consequently resulted in accumulation of carbons in the form of 6-phosphate fructose. Metabolic flux analysis results of the transformants with glucose as the carbon source were consistent with the enzyme assay results. Although the majority of carbons flowed into the glycolytic pathway at the upstream of the pathway by expressing the *pfk*A and *fba*A genes, only about 15 % or 20 % of the carbons (Table 4) were converted into GAP (which were further converted into lactic acid) by the expressed PFK and FBA enzymes in the transformants. Excessive carbons in the form of fructose-6-phosphate were redirected into the PK pathway through carbon chain rearrangement by the PP pathway, which means that the constructed homo-fermentative pathway functioned, but its efficiency was low. As a result, the key enzyme of the PK pathway XPK re-seized the excessive carbons, and converted the carbons into lactic acid and the two carbon by-products, either acetic acid or ethanol.

The lactic acid yield of the transformants was improved with glucose as the carbon source. But the lactic acid yield of the transformants with xylose as the carbon source had no change in comparison with the native strain. Xylose metabolic analysis results also indicated that almost 100 % of the carbons were distributed into the PK pathway at the nod of XPK instead of the PP pathway. This explained why the constructed homo-fermentative pathway failed to function in the transformants with xylose as the carbon source, as all carbon fluxes had flowed into the PK pathway at the upstream of the created pathway.

The lactic acid yield of *L. brevis* from glucose was improved by expressing PFK and FBA enzymes; however, its lactic acid yield was still lower than the reported homo-fermentative lactic acid bacteria, such as *B. coagulace* (Patel et al. 2006), for which the lactic acid yield achieved 1.52 mol/mol with xylose as the carbon source. The lactic acid yield of some metabolically engineered bacteria such as *Lactobacillus* MNOT 4 (Picataggio et al. 1998) was reported to achieve 1.57 mol/mol with xylose as carbon source. All reported strains with high lactic acid yield possessed only a homo-fermentative pathway for producing lactic acid. In this study, the modified transformants possessed both the homo-fermentative and hetero-fermentative pathways. As the hetero-fermentative pathway distributed more carbons from the constructed homo-fermentative pathway, the lactic acid yield was destined to be lower than the obligate homo-fermentative lactic acid producing strains. According to the enzyme assay and metabolic flux analysis results, efforts should be made to improve the activity of the FBA enzyme and redirect the carbon flow from the PK pathway to the created Glycolytic pathway to improve the lactic acid yield. Okano et al. (2009) and Yoshida et al. (2011) reported that, by knocking out the *xpk* genes and introducing the *tkt* gene in

Table 4 Calculation results for metabolic fluxes of the strains with glucose as carbon source (mM carbon)

Strains		f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	f_{15}	f_{16}	f_{17}	f_{18}	f_{19}	f_{20}	f_{21}	f_{22}	f_{23}	f_{24}	f_{25}	f_{26}
Native strain	*L. brevis* s3f4	840	1,272	504	769	937	683	698	86	2,449	−2,881	−2,017	−864	−864	−1,153	−1,729	864	0	432	239	159	80	842	1,874	864	1,009	168
Transformants	*L. brevis* pf	119	1,942	1,165	777	1,078	597	1,045	180	−608	−1,215	−850	−365	−365	−486	−729	−729	277	−364	33	22	11	619	1,879	1,735	144	24
	L. brevis pp	86	1,856	1,113	743	1,117	542	1,090	201	−590	−1,180	−826	−354	−354	−472	−708	−708	357	−354	27	18	9	560	1,878	1,773	105	17

Table 5 Calculation results for metabolic fluxes of the strains with xylose as carbon source (mM carbon)

Strains		f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	f_{15}	f_{16}	f_{17}	f_{18}	f_{19}	f_{20}	f_{21}	f_{22}
Native strain	*L. brevis* s3f4	2,242	2,228	1,337	891	1,351	110	1,351	781	5	10	7	3	3	4	6	6	12	3	0	0	0	110
Transformants	*L. brevis* pf	2,102	2,224	1,334	889	1,212	95	1,212	795	−41	−81	−57	−24	−24	−32	−49	−49	−97	−24	0	0	0	95
	L. brevis pp	2,242	2,230	1,338	892	1,351	143	1,351	748	4	9	6	3	3	3	5	5	10	3	0	0	0	143

L. plantrum or *Lactococcus lactis*, the carbon flow could be redirected from the PK pathway to the PP pathway, and the lactic acid yield was enhanced as a result. This strategy can also be applied to improve the fermentation ability of *L. brevis* s3f4 transformants. As a constitutively expressed homo-fermentative pathway has been constructed by expressing PFK and *FBA* in *L. brevis* s3f4, the PK pathway would be cut off by deleting the XPK gene, and all the carbons might be redirected into the constructed homo-fermentative pathway. By further enhancing the expression level of the *fba*A gene in the transformants, one can improve the efficiency of the constructed homo-fermentative pathway, and enhance the lactic acid yield to a level equivalent to the theoretical yield of the homo-fermentative pathway.

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