



Enhancing the Glucose Flux of an Engineered EP-Bifido Pathway for High Poly(Hydroxybutyrate) Yield Production

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Background: As the greenhouse effect becomes more serious and carbon dioxide emissions continue rise, the application prospects of carbon sequestration or carbon-saving pathways increase. Previously, we constructed an EP-bifido pathway in *Escherichia coli* by combining Embden-Meyerhof-Parnas pathway, pentose phosphate pathway and “bifid shunt” for high acetyl-CoA production. There is much room for improvement in the EP-bifido pathway, including in production of target compounds such as poly(hydroxybutyrate) (PHB).

Result: To optimize the EP-bifido pathway and obtain higher PHB yields, we knocked out the specific phosphoenolpyruvate phosphate transferase system (PTS) component II Gcg, encoded by *ptsG*. This severely inhibited the growth and sugar consumption of the bacterial cells. Subsequently, we used multiple automated genome engineering (MAGE) to optimize the ribosome binding site (RBS) sequences of *galP* (galactose: H (+) symporter) and *glk* (glucokinase gene bank: NC_017262.1), encoding galactose permease and glucokinase, respectively. Growth and glucose uptake were partially restored in the bacteria. Finally, we introduced the *glf* (UDP-galactopyranose) from *Zymomonas mobilis* mutase sugar transport vector into the host strain genome.

Conclusion: After optimizing RBS of *galP*, the resulting strain L-6 obtained a PHB yield of 71.9% (mol/mol) and a 76 wt% PHB content using glucose as the carbon source. Then when *glf* was integrated into the genome strain L-6, the resulting strain M-6 reached a 5.81 g/L PHB titer and 85.1 wt% PHB content.

Keywords: poly(hydroxybutyrate) yield, glucose flux, EP-bifido pathway, MAGE, *Escherichia coli*

INTRODUCTION

In 2018, global carbon dioxide emissions increased 1.7% over the previous year, hitting a record high of 33.143 billion tons. Accelerating the adoption of renewable energy and improving energy efficiency in response to global warming are urgent priorities (International Energy Agency, 2019). Bio-manufacturing, which uses food crops as raw materials, has wide application prospects.

Through biological manufacturing, biomass resources can be converted to ethanol, polylactic acid, 1, 3-propanediol, and other bulk chemicals (Ragauskas et al., 2006; Lee et al., 2012). A key barrier to this process is the CO₂ emissions that occur during natural aerobic fermentation. Many carbon dioxide fixation pathways have been exploited using the six carbon fixation pathways discovered in nature (Gong et al., 2016). However, complex reaction steps and enzyme requirements limit the broad application of these carbon sequestration pathways (Erb et al., 2007; Schwander et al., 2016).

In natural microorganism fermentation, glucose can be transformed through the Embden-Meyerhof-Parnas (EMP) pathway to pyruvate. Pyruvate metabolism leads to the production of two molecules of acetyl-CoA (AcCoA), the key precursor of ethanol, butanol, fatty acids, amino acids, and pharmaceuticals. This process produces two molecules of CO₂ from one mol of glucose, making it an uneconomical way to biosynthesize products using AcCoA as precursor. Other glycol metabolism pathways, such as the phosphoketolase pathway, employ a pentose phosphate specific transketolase to produce a mixture of ethanol, lactic acid, and CO₂. Additionally, the bifido bacteria exclusive bifid shunt pathway can generate 1 mol of lactic acid and 1.5 mol of acetate from 1 mol of glucose (Meile et al., 2001; Posthuma et al., 2002). However, all of these glucose metabolic pathways lose carbon in the form of CO₂ during the decarboxylation process. Therefore, several carbon-saving pathways, including the non-oxidative glycolytic (NOG) and EP-bifido pathways, have been engineered. The NOG pathway can transform all six carbon atoms of glucose into three AcCoA molecules without CO₂ loss (Bogorad et al., 2013). However, it cannot provide the reducing power NADPH that is needed for PHB and other chemicals biosynthesis. The EP-bifido pathway employs EMP, pentose phosphate pathway (PPP) and the Bifido shunt for high-yield of AcCoA generation. As a reducing power sponsor, the oxidation part of the PPP consumes 1 mol of glucose, and provides 2 mol of NADPH and 1 mol of xylulose-5-phosphate (X5P). The enzyme encoded by the *fxpk* gene of the EP-bifido pathway has both fructose-6-phosphate (F6P) and X5P activity. It is able to catalyze X5P to form acetyl phosphate (AcP) and glyceraldehyde 3-phosphate (G3P) or split F6P to form erythrose 4-phosphate and AcP. The former G3P can generate AcP through carbon rearrangement, each of these processes releases only 1 mol of CO₂, thus saving the carbon source to a certain extent (Figure 1). This carbon-saving pathway has been applied to the production of several compounds that use AcCoA as the precursor (Wang et al., 2019). Previously, we achieved a relatively high level of production and yield, but there remains room to improve carbon conversion in our system.

In *Escherichia coli*, glucose is transported through the phosphate transferase system (PTS). This system is involved in phosphoenolpyruvate (PEP)-dependent sugar transport and its activity has an important impact on carbon flux redistribution in the PEP and pyruvate nodes (Gosset, 2005). Glucose was phosphorylated to G6P by the phosphoryl generated from PEP, which was dephosphorylated to form pyruvate. Then pyruvate further decarboxylated to AcCoA and released 1 mol of CO₂, leading to the loss of the carbon source. In addition, PEP is

a key intermediate metabolite of the EMP pathway. Therefore, an increase in the EMP pathway reduces the carbon conversion efficiency of the EP-bifido pathway.

Poly(hydroxybutyrate) (PHB) is the most common poly(hydroxyalkanoate) (PHA). PHB can be synthesized and accumulated by more than 300 microorganisms as both an energy and carbon store (Lee and Choi, 2001; van der Walle et al., 2001). The *in vivo* biosynthesis of PHB requires three steps using AcCoA as the precursor, and PHB production has been intensively studied (Lee et al., 1994; Wang Q. et al., 2009). Many strategies have been applied to engineer *E. coli* to improve PHB production, however, the yield still has much room to progress. By overexpressing NAD kinase, recombinant *E. coli* produced 14 g/L PHB and the yield based on glucose reached 0.31 mol/mol (0.15 g PHB/g glucose) (Li et al., 2009). By applying fed-batch strategy, *E. coli* could accumulate 125 g/L PHB, but the yield based on glucose was only 0.46 mol/mol (0.22 g PHB/g glucose) (Mozumder et al., 2014). Previously, we have achieved relatively high level of PHB content and PHB yield (68.4 wt% and 63.7% mol/mol, respectively) (Wang et al., 2019). In this study, we optimized the EP-bifido pathway for improved PHB production in *E. coli*. The non-PTS glucose transport pathway genes *glk*, *galP* and heterogeneous *glf* were enhanced and introduced into EP-bifido strains through multiple automated genome engineering (MAGE) and conditional-replication, integration, excision, and retrieval (CRIM) plasmids. The improved PHB production indicates that our modification increased the efficiency of artificial carbon-saving pathways for high carbon conversion rate from glucose.

MATERIALS AND METHODS

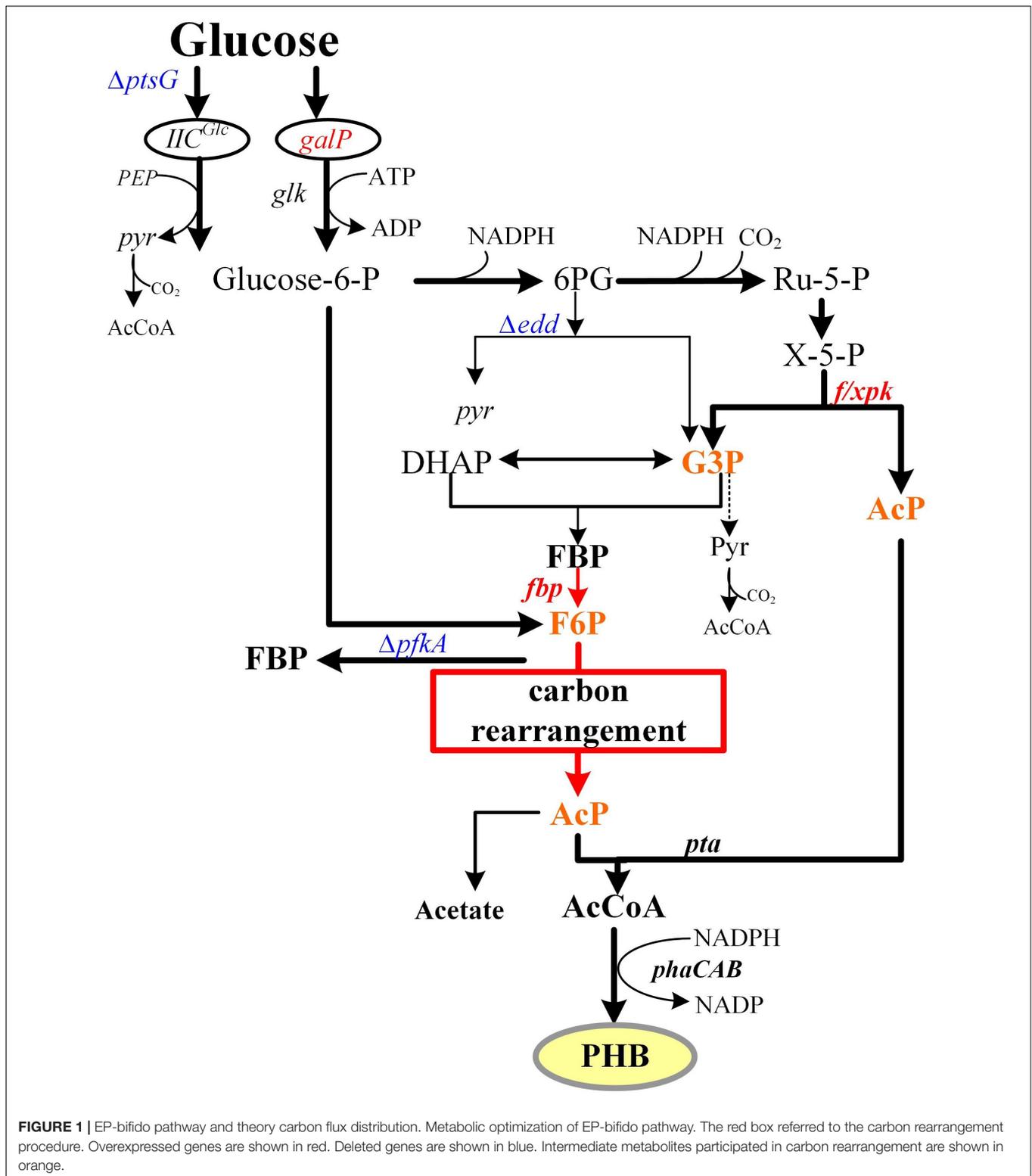
Culture Media and Conditions

For plasmid preparation, *E. coli* strains were cultured at 37°C on a rotary shaker (220 rpm) in test. For plasmid preparation, *E. coli* strains were cultured at 37°C on a rotary shaker (220 rpm) in test tubes containing 5 mL Lysogeny broth (LB) medium. For PHB biosynthesis, 50-mL shake flask cultures were started by 2% inoculation from the 5-mL LB culture. The 50-mL cultures contained M9 minimal medium with 0.2% yeast extract containing 20 g/L glucose and shaken at 37°C in a rotary shaker (120 rpm) for 48 h. Overnight cultures were shaken at 37°C in a rotary shaker (220 rpm). Antibiotics were added as follows: ampicillin (Amp) 100 µg/mL, spectinomycin (Spc) 50 µg/mL, and chloramycetin (Cm) 25 µg/mL. For MAGE procedure, strains were cultivated in SOB medium.

Lysogeny broth medium contains (g/L): tryptone (10), yeast extract (5), and NaCl (10). M9 medium contains (g/L): Na₂HPO₄·12H₂O (15.138), KH₂PO₄ (3), NaCl (0.5), and NH₄Cl (1). SOB medium contains (g/L): tryptone (20), yeast extract (5), and NaCl (5).

Strains and Plasmids

All *E. coli* strains and plasmids used are listed in Table 1. DH-EP was used as the starting strain for further genetic



manipulation. All primers used for molecular manipulations are listed in **Table 2**.

The Red homologous recombination method was employed for gene deletion. The pTKRED complementary plasmid was

transformed into the target strain. Deletion fragments of *ptsG* gene were amplified from the JW1087-2 single-gene knockout mutant (Baba et al., 2006) (bought from Coli Genetic Stock Center, CGSC) using primers Q-ptsG-F/Q-ptsG-R.

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain and plasmids	Relevant properties	Sources
Strains		
JW1087-2	JW25113 derivative, $\Delta ptsG::kan$	Baba et al. (2006)
DH-EP	DH5 α derivative, $\Delta edd \Delta pfkA$	Wang et al. (2019)
DH-EPP	DH5 α derivative, $\Delta edd \Delta pfkA \Delta ptsG$	This study
L-6	DH-EPP derivative, $\Delta edd \Delta pfkA \Delta ptsG galP$ RBS:TGAAAGGGAAA	This study
M-6	L-6 derivative, $\Delta edd \Delta pfkA \Delta ptsG::trc-rbs-glf_{zm}$	This study
Plasmids		
pCAB	pBluescriptII SK, phbC and phbAB gene from <i>Ralstonia eutropha</i>	Wang et al. (2019)
pCDFtrc	Cloning vector, Spe ^R	Wang et al. (2019)
pFF	pCDFtrc, <i>fxpK</i> gene from <i>B. adolescentis</i> and <i>fbp</i> gene from <i>E. coli</i>	Wang et al. (2019)
pKD3	Template plasmid with Cm ^R gene and FLP recognition target	Datsenko and Wanner (2000)
pTKRED	P _{BAD} promoter containing plasmid, Spe ^R	Kuhlman and Cox (2010)
pCP20	Helper plasmid expressing FLP recombinase, ts-rep, Amp ^R , Cm ^R	Datsenko and Wanner (2000)
pAH69	Helper plasmid expressing HK022 integrase, Amp ^R	Haldimann and Wanner (2001)

TABLE 2 | Key oligonucleotide primers used in this study for DNA manipulation.

Primers	Sequence (5'-3')
Q-ptsG-F	5'-GGCTGTGTTGAAAGGTGTTGC-3'
Q-ptsG-R	5'-AACGCGCTATATTGCAGAGG-3'
Glf-F	5'-GGTCGGTAAATCGCTGCTTGACAATTAATCATCCGGC TCGTATAATGTCTAGAGAAAGAGGAGAAATACTAGATGAG TTCTGAAAGTAGTCAGGGTC-3'
Glf-R	5'-GCCTACCCGGATATTATCGTGAGGATGCGAATTGTG TAGGCTGGAGCTGCTTC-3'
R6K-F	5'-TCGCATCCTCACGATAATATCCGGGTAGGC-3'
R6K-R	5'-TTGTCAAGCAGCATCAGCGATTACCGACCGATCC GGCCACGATGCGTCC-3'

Measurement of Extracellular Metabolites

A spectrophotometer was used to measure the optical density at 600 nm (OD₆₀₀) of the bacterial culture. PHB was quantified using gas chromatography (GC). Cells were harvested by centrifugation at 6,000 × g for 10 min, 4°C. The cell pellets were washed twice with distilled water and lyophilized for 7 h. Before GC analysis, 1 mL chloroform, 850 μL methanol, and 150 μL sulfuric acid (98%, w/w) were added to the weighed cells in vials. The vials were incubated at 100°C for 1 h. Then, 1 mL water was added for stratification and cooling vials. After standing for 1 h, the mixture separated into layers and the heavier chloroform phase was transferred to new vial for GC analysis. The GC detection process was performed using a Shimadzu GC2010 gas chromatograph (Kyoto, Japan) equipped with an AOC-20i auto injector and a Restek Rtx-5 column. PHB standard samples of methyl-(R)-3-hydroxybutyrate (Sigma-Aldrich) were dissolved

in chloroform and analyzed by GC. The temperature program used was: 80°C for 1 min, ramped to 120°C at 10°C/min, then ramped to 160°C at 45°C/min for 5 min, and the total time was 10.89 min.

For extracellular metabolite analysis, 1 mL of culture was centrifuged at 12,000 × g for 2 min. The supernatant was filtered through a 0.22-μm syringe filter for high-performance liquid chromatography analysis. Glucose, acetate, and pyruvate were measured on an ion exchange column (HPX-87H; Bio-Rad Labs) with a differential refractive index detector (Shimadzu RID-10A). A 0.5-mL/min mobile phase using a 5-mM H₂SO₄ solution was applied to the column. The column was operated at 65°C.

MAGE Procedure

The ribosome binding sites (RBSs) designed for the modulation of *GalP* and *glk* transcription rates were 5'-GTCGTACTC ACCTATCTTAATTCACAATAAAAAATAACCADRRRRRRD DDDATCATGCCTGACGCTAAAAAACAGGGGCGGTCAAA CAAG-3' (D = A, G, T; and R = A, G) and 5'-GCCGCCACA TCACCGACTAATGCATACTTTGTCAATTCTHHHHYYYYYH HGCTAAAGTCAAATAATTCTTTCTCACACTGTAAATAC CT-3' (H = T, C, A; and Y = T, C), respectively, with four phosphorothioated bases at the 5' terminus. The initiation of MAGE requires that pTKRED was transformed into the target strain. The MAGE cycles were performed by growing DH-EPP in 5 mL SOB medium at 30°C and shaking at 220 rpm for 12 h. For the first MAGE round, 5-mL shake flask cultures using SOB broth were started with a 1% inoculation from the overnight culture. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce λ-prophage (*bet*, *gam*, and *exo*) gene expression. Cells were then incubated at 30°C and shaking at 220 rpm until reaching an OD₆₀₀ of 0.5 to 0.6. Cells were collected (2 mL), pelleted, and washed three times with cold sterile water to make them electrocompetent. ssDNA mixture (1 μM) was added to electrocompetent cells and electroporated at 2.5 kV. To start the second MAGE round, cells were recovered in 5 mL SOC with IPTG until their OD₆₀₀ reached 0.5 to 0.6, after which cells underwent pelleting, washing, and electroporation. Three to four MAGE rounds were performed per day and 16 cycles were performed in total. The resulting pool of variants were then characterized using the Nile red assay.

Screening of PHB Competent Cells by Nile Red Assay

When PHB is combined with Nile red dye a red color is produced. We transformed the pCAB plasmid into these variants and added 100 μL Amp, 50 μL IPTG, 20 g/L glucose, and 200 μL Nile red dye to the solid M9 medium supplemented with 0.2% yeast extract. The MAGE variants were diluted 200-fold and spread onto several plates. After incubation at 37°C for 16 h, the plates were placed at 4°C for 3 days to allow the color reaction to develop. Based on the color difference, we picked single red colonies for sequencing. For all the sequenced colonies with mutations identified, the pTKRED plasmid was removed and the strains were transformed with the pFF and pCAB plasmids for further verification of the PHB competent cells.

Integration of glf

For *glf* integration, the *trc-rbs-glf* module was amplified from *Zymomonas mobilis* genomic DNA by PCR using primers GLF-F and GLF-R. The PCR product (*trc-rbs-glf*) was cloned into a vector that carries R6K replicon and phage attachment sites (*attP*). This plasmid was named R6K-*glf* and was confirmed by DNA sequencing. The pAH69 helper plasmid was transformed into the L-6 strain. The target strain carrying pAH69 was incubated overnight at 30°C and transferred to 37°C for 1 h before transfection. Then the pR6K-*glf* plasmid was introduced into L-6 by electroporation. The centrifuged bacteria were plated onto plates containing 25 µg/mL kanamycin for overnight incubation at 37°C. R6K-*glf* positive transformants were selected by their kan^R phenotype and were verified by PCR.

Determination of CO₂ Emissions

CO₂ emission was determined using a thermostatic oscillation incubator with a CO₂ detector (BCP-CO₂, Bluesens, Germany) that monitored CO₂ volume every 20 s and transmitted the data to a computer. Cultures were grown at 37°C with shaking at 150 rpm.

RESULTS

Inhibiting PTS to Reduce PEP Consumption

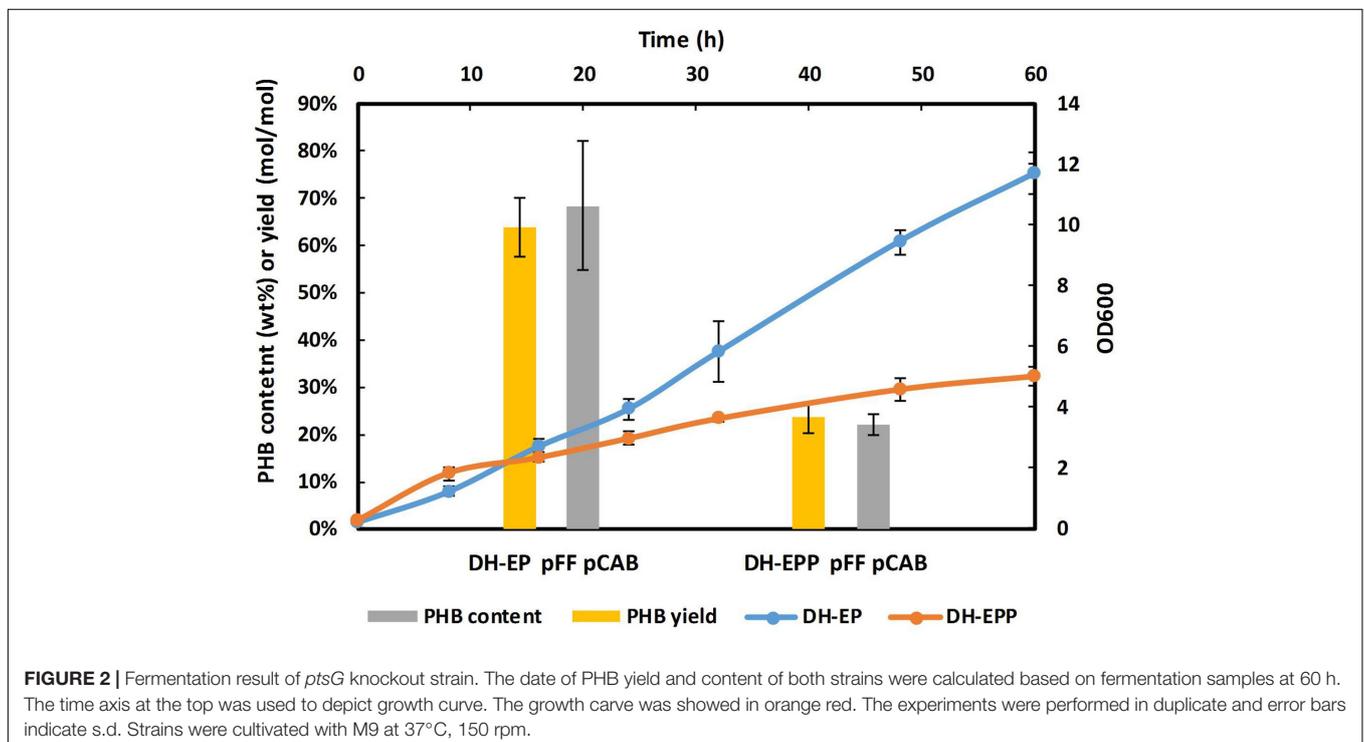
In *E. coli*, glucose is transported through the PTS system. This system is involved in PEP-dependent sugar transport and its activity has an important impact on carbon flux redistribution

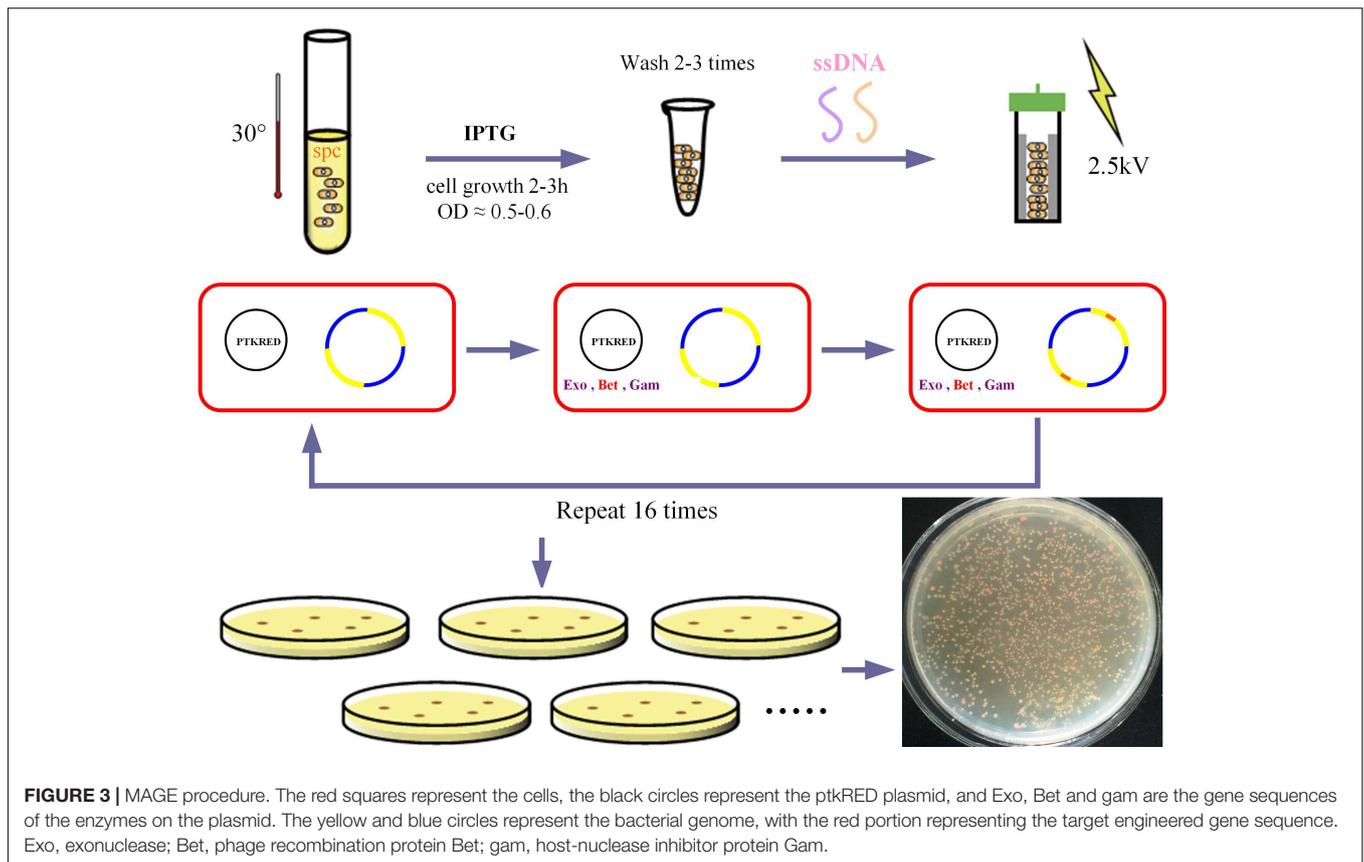
in the PEP and pyruvate nodes (Gosset, 2005). Glucose transport into the cytoplasm by EIICB^{Glc} (encoded by *ptsG*) is coupled to its phosphorylation. The phosphate group is derived from PEP and is transferred via a cascade of proteins, enzyme I (EI), HPr, EIIA, and EIIB. This procedure consumes almost half of the PEP (Valle et al., 1996; Wang et al., 2012). Glucose was phosphorylated to G6P by phosphoryl generated from PEP dephosphorylated to pyruvate, the formed pyruvate is further decarboxylated to AcCoA and releases 1 mol CO₂, leading to carbon source loss. While PEP is not the precursor in our study, PEP consumption would convert carbon flux to the EMP pathway, which is not desirable in our EP-bifido pathway. Therefore, modulation of the PEP-independent uptake and phosphorylation system is required. Knocking out *ptsG* and replacing it with other glucose transport pathways is a common method used in the production of PEP-precursor products (Gosset, 2005; Lee et al., 2005; Li et al., 2013; Kyselova et al., 2018). Therefore, we knocked out *ptsG* in strain DH-EP and named the strain DH-EPP. But found that *ptsG* deletion severely impaired the growth capacity of the resulting strain. Compared with DH-EP (pFFpCAB) strain, PHB yield of DH-EPP strain decreased from 63.7 to 26.3% (mol/mol) (Figure 2).

For glucose uptake recovery, replacing PTS with an alternative PEP-independent uptake and phosphorylation system could be an efficient solution to this problem.

Improving Glucose Flux Through Non-PTS Pathway

When *E. coli* strains lack PTS, the low affinity galactose: H⁺ symporter, GalP, encoded by *galP*, is induced. Glucose





internalized by GalP must be phosphorylated by glucokinase (encoded by *glk*), which catalyzes the ATP-dependent phosphorylation of glucose to generate G6P in the cytoplasm without CO₂ emission, thus saving the carbon source (Gosset, 2005). Therefore, we modified *galP* and *glk* expression levels to improve the glucose utilization rate. While increasing *galP* and *glk* transcriptional levels through plasmid overexpression is a pervasive strategy (Hernandez-Montalvo et al., 2003; Wang et al., 2006), it has several disadvantages, including metabolic burden and unexpected lateral effects. Modulation of *galP* and *glk* expression levels through plasmid overexpression or high strength promoter substitution cannot provide multiple combination of expression intensity for screening. Discovering a way to effectively modulate the transcription of the two genes to an optimal strength in combination is a pressing problem. We adopted MAGE to simultaneously regulate *galP* and *glk* expression levels (Wang H.H. et al., 2009). Using this approach, colonies with high PHB yields can be identified using Nile red dye staining.

After 16 rounds of MAGE modulation (Figure 3), recombinant strains were screened by Nile red staining. Recombinant strains with higher PHB production showed redder color. Screening and sequencing results are shown in Table 3. Using single colony color screening, we found that the RBS of both *glk* and *galP* genes were changed, and the amount of ssDNA (single string DNA) recombination of *glk* exceeded that of *galP*. This may be because the location of the *glk* gene is more

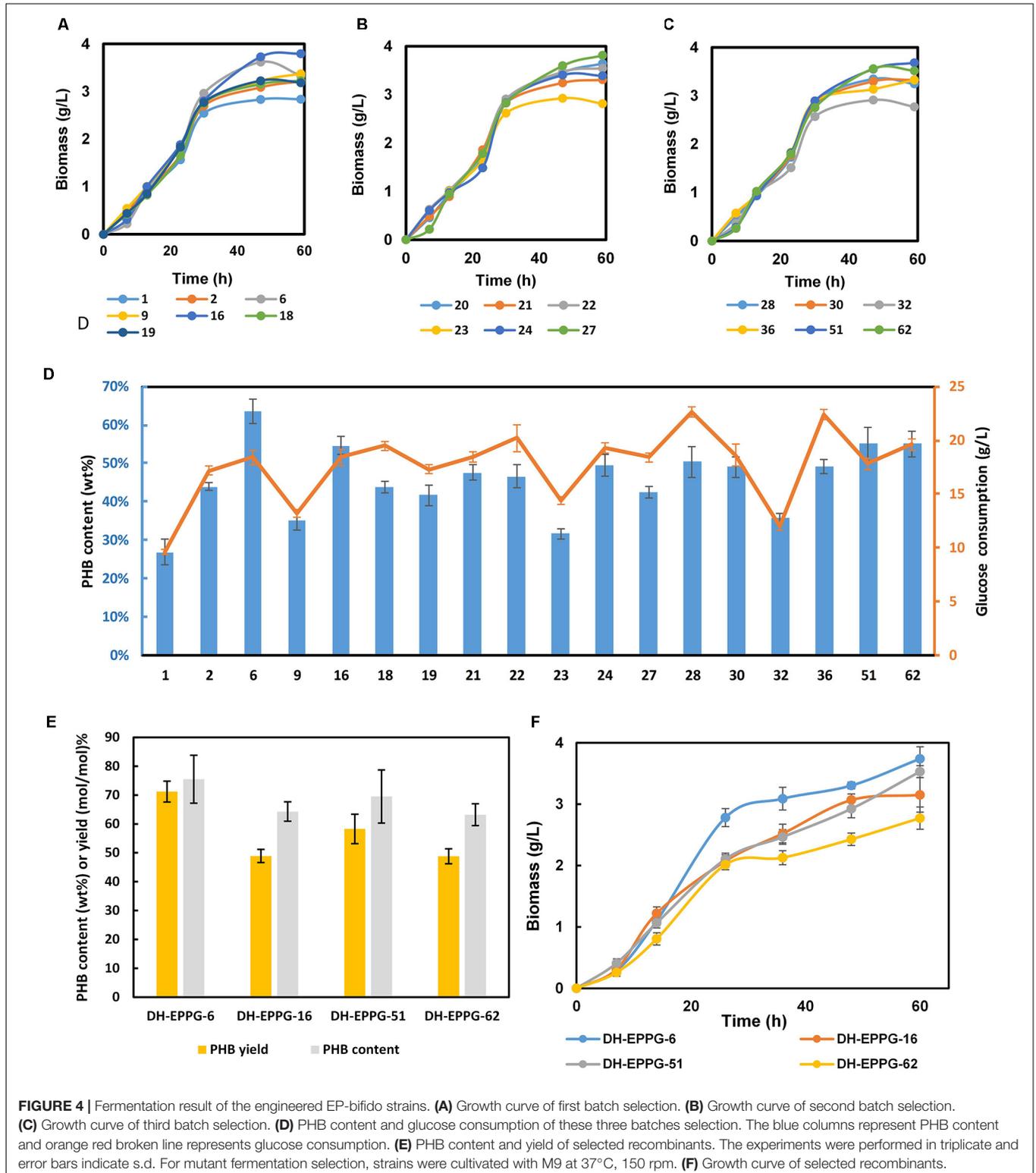
susceptible to ssDNA recombination during genome replication. However, no recombinant was screened out in which the two genes were simultaneously mutated.

TABLE 3 | Screening result of MAGE recombination strains.

Strain number	Gene	Origin RBS sequence	Mutated RBS sequence
1	<i>glk</i>	GGAGCAGTTGA	GAAGGGAGAGG
2	<i>glk</i>	GGAGCAGTTGA	GAAGAAATGA
6	<i>galP</i>	TATTGGAGGGC	TGAAAGGGAAA
9	<i>glk</i>	GGAGCAGTTGA	GGAGGGATGGA
16	<i>glk</i>	GGAGCAGTTGA	TAGGAGGAGTT
18	<i>glk</i>	GGAGCAGTTGA	AAAAGGGTTA
19	<i>glk</i>	GGAGCAGTTGA	GAAGGAGGGGT
20	<i>galP</i>	TATTGGAGGGC	GGAGAGGGTTA
21	<i>glk</i>	GGAGCAGTTGA	TGGGGGAGGG
22	<i>glk</i>	GGAGCAGTTGA	AAAGGGTTTG
23	<i>glk</i>	GGAGCAGTTGA	GAAGGGTTTG
24	<i>glk</i>	GGAGCAGTTGA	AGAGGAAGAGA
27	<i>glk</i>	GGAGCAGTTGA	AAAAGGGATAG
28	<i>glk</i>	GGAGCAGTTGA	TTGGAAGATAT
30	<i>glk</i>	GGAGCAGTTGA	TGAGGAATGAA
32	<i>glk</i>	GGAGCAGTTGA	GTGAAATAGA
36	<i>glk</i>	GGAGCAGTTGA	TTAGGGGGAGT
51	<i>glk</i>	GGAGCAGTTGA	TTAAGGGATAT
62	<i>glk</i>	GGAGCAGTTGA	GGAAGGAGAAT

Nineteen mutant strains were selected and transformed with pFF and pCAB, subsequently. Three batches fermentation of these 19 strains lead to the selection of strains in consideration of glucose consumption and PHB content, named EPPG-6,

EPPG-16, EPPG-51, and EPPG-62 (Figure 4D). Then, we repeated fermentation using the four selected strains (Figure 4E). Fermentation results showed that after glucose transport system modulation, the DH-EPPG-6 strain had a recovered growth rate.



The PHB yield reached 71.2% (mol/mol), which was 63.7% higher than that of the strain DH-EP. Then DH-EPPG-6 strain was renamed L-6, in which the wild type RBS sequence of *galP* was mutated to TGAAAGGGAAA.

Expressing Heterogeneous Sugar Transporter to Reinforce Glucose Uptake

To further enhance the consumption of glucose, we compared the kinetic parameters of several transporters with their glucose transport capacity and energy consumption during glucose internalization and phosphorylation (Gosset, 2005). Because the

transmembrane proton potential is a form of energy, the energy consumption of the *glf* sugar transporter from *Zymomonas mobilis* is comparable to that of the *E. coli* glucose-specific PTS (PTS^{Glc}). Compared with *galP*, the *glf_{zm}* transporter used less energy to produce a higher maximum velocity. Therefore, the high-rate, low-energy sugar transporter *glf_{zm}* was chosen to improve the glucose absorption capacity of engineered bacteria. Then *glf_{zm}* was inserted at the attP genomic site of strain L-6 using the CRIM plasmid system (Haldimann and Wanner, 2001), resulting the strain M-6. We deduced that *glf* integration was conducive to growth recovery in later growth stages. In **Figure 5B**, M-6 (pFFpCAB) showed a better growth

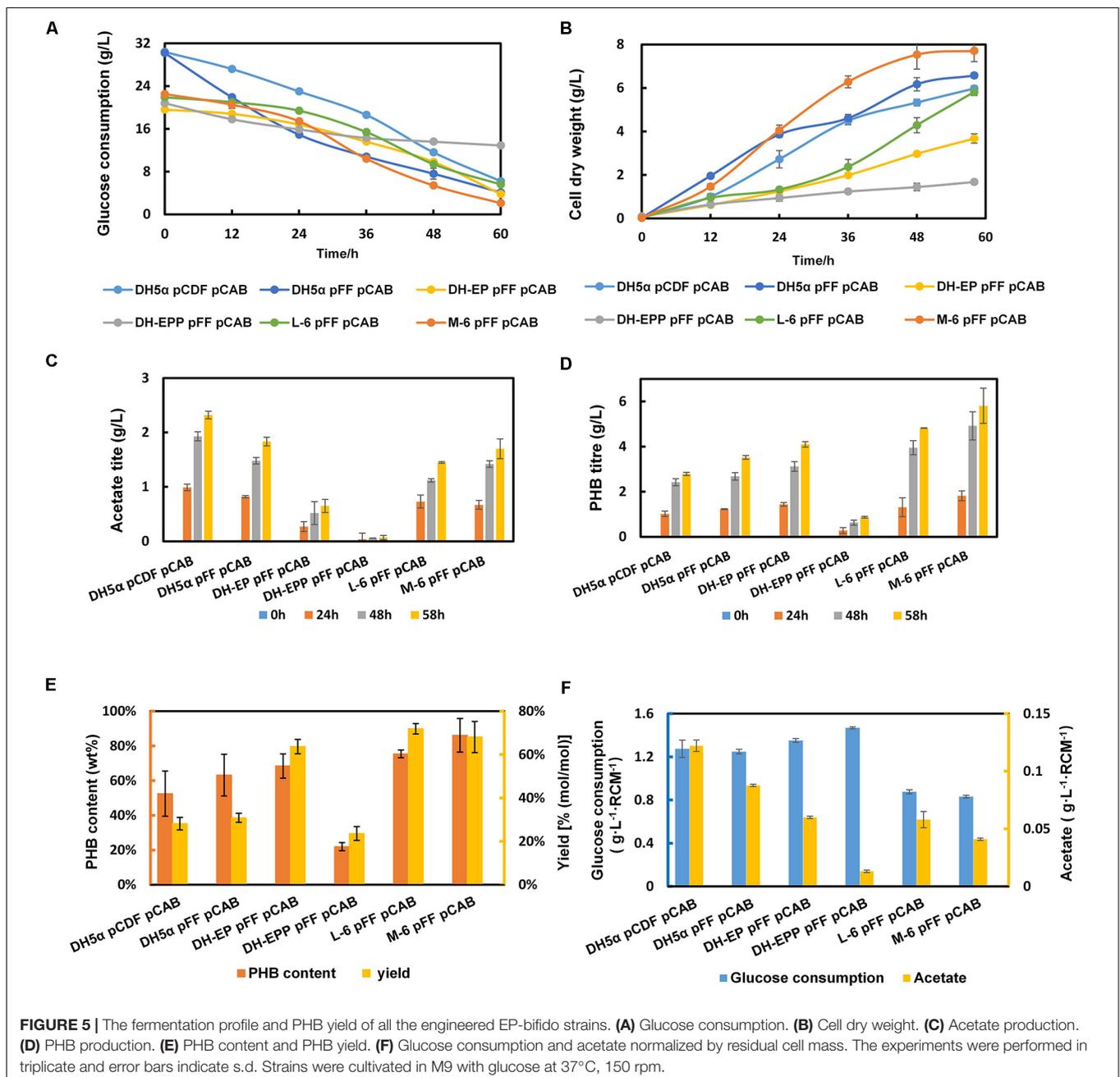


TABLE 4 | Specific growth rates and glucose utilization rates of DH5 α , DH-EP (pFFpCAB), DH-EPP (pFFpCAB), L-6 (pFFpCAB), and M-6 (pFFpCAB).

Strains	Specific growth rate (h ⁻¹)	Specific glucose utilization rate (g·L ⁻¹ ·h ⁻¹)
DH5 α (pFFpCAB)	0.35 ± 0.011	0.78 ± 0.02
DH-EP (pFFpCAB)	0.20 ± 0.020	0.41 ± 0.03
DH-EPP (pFFpCAB)	0.09 ± 0.002	0.23 ± 0.01
L-6 (pFFpCAB)	0.28 ± 0.018	0.52 ± 0.01
M-6 (pFFpCAB)	0.50 ± 0.026	0.71 ± 0.03

Specific growth rates and glucose utilization rates were calculated based on the 0–48 h data.

TABLE 5 | PHB productivity of the engineered PHB-producing strains.

Strains	PHB titer (g·L ⁻¹ ·h ⁻¹)
DH5 α (pCDF pCAB)	0.048 ± 0.07
DH5 α (pFF pCAB)	0.061 ± 0.08
DH-EP (pFF pCAB)	0.071 ± 0.012
DH-EPP (pFF pCAB)	0.015 ± 0.04
L-6 (pFF pCAB)	0.083 ± 0.015
M-6 (pFF pCAB)	0.100 ± 0.078

curve than the control DH5 α (pCDFpCAB). Simultaneously, as we expected, gross glucose consumption improved compared with L-6, increased from 16.2 to 20.4 g/L (Figure 5A). The glucose consumption of all engineered strains changed obviously after every modification step. PHB titer of M-6 improved significantly, from 4.82 to 5.81 g/L in comparison with that of L-6 (Figure 5D), and PHB content in M-6 strain reached 85.1 wt% (Figure 5E). The only drawback was that PHB yield of M-6 reached 68.1% (mol/mol) slightly decreased compared with L-6 (Figure 5E). In general, compared with parental strain DH-EP (pFFpCAB), the PHB titer and content improved 41.71 and 24.41% in M-6, respectively. And compared with the control DH5 α (pCDFpCAB), the PHB content and yield of M-6 improved 61.9 and 141.7% in M-6, respectively (Figure 5). All the engineered strains produced some amount of acetate, M-6 and L-6 produced less acetate than DH-EP strain. We also calculated

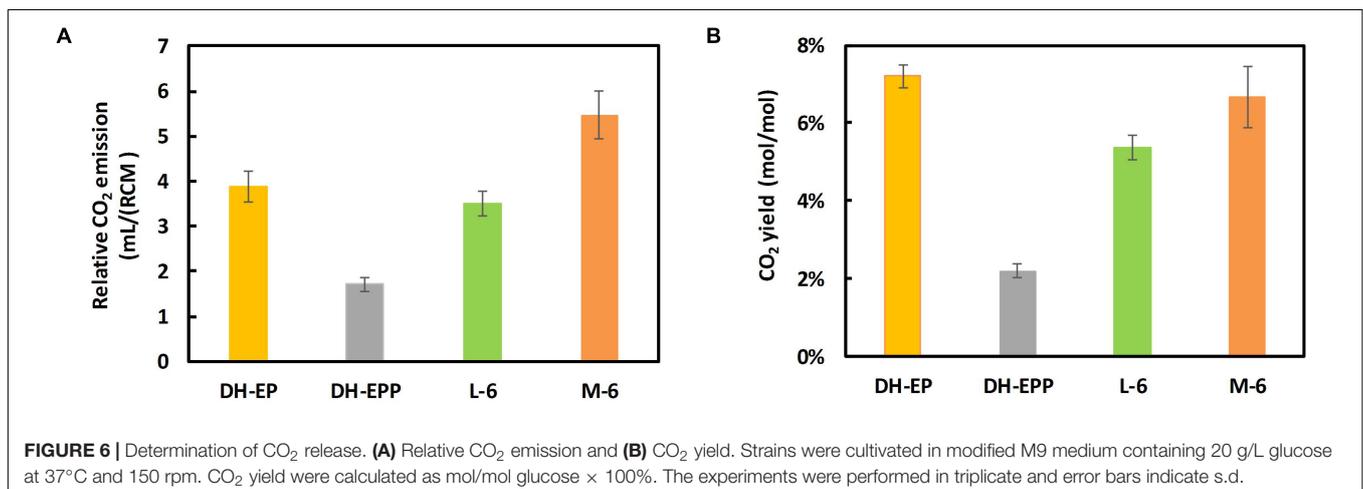
the acetate formation and glucose consumption normalized by residual cell mass (RCM).

In order to confirm that the glucose consumption rates in our engineered strains are indeed improved, we calculated the specific growth rate and glucose utilization rates of DH-EP (pFFpCAB), DH5 α -EPP (pFFpCAB), L-6 (pFFpCAB), and M-6 (pFFpCAB). After *ptsG* gene was deleted, the specific growth rate of DH-EPP decreased from 0.20 to 0.09 h⁻¹, and the glucose utilization rate decreased from 0.78 to 0.41 g L⁻¹ h⁻¹ (Table 4). After *glf_{zm}* integration based on L-6, the specific growth rate of M-6 (pFFpCAB) increased obviously from 0.28 to 0.50 h⁻¹, improved 42.8% compared to that of DH5 α (pFFpCAB). The growth consumption rate recovered to 0.71 g L⁻¹ h⁻¹, 73% higher than DH-EP (pFFpCAB). M-6 and L-6 showed lower normalized glucose consumption. Thus M-6 and L-6 had improved PHB biosynthesis and PHB productivity since they produced less by-product acetate and consumed less glucose per residual cell mass (Figure 5F and Table 5).

We further examined the CO₂ release from the constructed EP-bifido strains. The total CO₂ release of DH5 α -EPP (pFFpCAB) decreased to 32.5% compared to DH-EP (pFFpCAB) (Figure 6A). And the CO₂ emission of L-6 (pFFpCAB), M-6 (pFFpCAB) improved 177 and 332% compared to that of DH5 α -EPP (pFFpCAB). We believe that the restoring growth contributed to the increased CO₂ release. The CO₂ yield of DH5 α -EPP (pFFpCAB), L-6 (pFFpCAB), and M-6 (pFFpCAB) decreased to 16.9% (mol/mol), 74.4% (mol/mol) and 92.4% (mol/mol) compared to their controls, respectively (Figure 6B). The above data confirmed the recovery of growth after PTS system deficiency and the decreased CO₂ emission from the L-6 (pFFpCAB) and M-6 (pFFpCAB) strains.

DISCUSSION

As environmental problems intensify, carbon saving or carbon sequestration pathways have become a new focus for bio-manufacturing. Previously, we successfully constructed an efficient carbon-saving pathway in *E. coli* called the EP-bifido



pathway. This pathway has been applied to the production of several compounds that use acetyl-CoA as a precursor. As a degradable material, PHB has great application prospects. While we believe that there is potential for further optimization of PHB production by the EP-bifido pathway. In this study, we knocked out the *ptsG* gene, a key glucose transporter of the PTS system that employs PEP as phosphate donor. The glucose consumption rate and cell growth were significantly reduced in the *ptsG* mutant under aerobic fermentation conditions. The deficiency in the PTS system dramatically impairs glucose uptake and causes growth restriction. It is speculated that the reason for growth restriction is not insufficient glucose uptake, but the subsequent decrease in glucose phosphorylation efficiency due to limited glucokinase activity (Steinsiek and Bettenbrock, 2012).

To overcome this growth hindrance, we optimized the RBS sequences of *galP* and *glk* genes, encoding glucose permease and glucokinase, respectively. Subsequently, we introduced the *Z. mobilis* glucose transporter, *glf_{zm}*, into the L-6 high-yield strain, and observed cell growth recovery. After optimization, PHB yield reached 71.9% (mol/mol) in L-6 strain. In the resulting strain M-6, the intracellular PHB content reached 85.1 wt%, and the titer reached 5.81 g/L. Previously, most studies have compensated for PTS knockout-induced inhibition of glucose uptake by overexpression of *glk*, and *galP* or by heterologous expression of *glf* (Snoep et al., 1994; Gosset, 2005; Lin et al., 2018). Instead, we applied MAGE technology to directly alter genomic *glk* and *galP* to optimize their expression (Gallagher et al., 2014). Meanwhile, the RBS library constructed using MAGE provided rich genotypes for subsequent screening of high-yield PHB strains. After *glf_{zm}* integration, recovered growth rate and glucose consumption was evident in strain M-6. Compared with parent strain DH-EP (pFFpCAB), the glucose consumption of M-6 increased 4.6 g/L, 29.1% higher than that of parent strain in **Figure 5A**. The PHB content and yield of strain M-6 improved compared with that of DH-EP(pFFpCAB). M-6 and L-6 showed lower normalized glucose consumption. Thus M-6 and L-6 had improved PHB production since they produced less by-product acetate and consumed less glucose per residual cell mass. We inferred that increased glucose uptake enhanced flux through

EMP, which is supported by increased cell growth. The growth of DH-EP strain improved with decreased acetate formation in M-6 strain. The specific growth rate and CO₂ release data of constructed strains further confirmed our inference. Our study provided an efficient way for improving glucose absorption and total carbon conversion rate in artificial carbon-saving pathways by replacing PTS with other glucose transporters. It also describes an efficient screening strategy for MAGE ssDNA recombineering technology. The efficient utilization of carbon sources has been one of the determinant for high productivity in microbial fermentation. In the future, the effective allocation of carbon resources and the construction of effective strategies for balancing cell growth and product biosynthesis will still be the direction of metabolic engineering.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

QW and QQ designed the work. YLi, YLu, and JX performed the experiments. ZS and YLi analyzed the ¹³C-MFA data. QQ and QL encouraged this project. YLi and QW wrote the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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