Received: 24 November 2021 | Revised: 28 January 2022 | Accepted: 12 February 2022

DOI: 10.1002/bit.28065

ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING WILEY

Engineering *Corynebacterium glutamicum* for synthesis of poly (3-hydroxybutyrate) from lignocellulose biomass

Ci Jin | Jing Li | Zhen Huang | Xushen Han | Jie Bao

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

Correspondence
Jie Bao, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Rd, Shanghai 200237, China.
Email: jbao@ecust.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 21978083, 31961133006

Abstract

Lignocellulose is the only feasible carbohydrates feedstock for commercial scale and carbon neutral production of poly(3-hydroxybutyrate) (PHB) biopolymer by its great abundance and availability. Microbial cell factories for fermentative PHB synthesis are highly restricted by the growth suppression of inhibitors from lignocellulose pretreatment. This study targeted a potential PHB-producing cell factory *Corynebacterium glutamicum* owing to its strong inhibitors tolerance. A systematic metabolic engineering was conducted starting with the stable PHB synthesis pathway construction from glucose and xylose, followed by the enhancement of PHB synthesis on PHA synthase activity and stability, cell morphology modification, and growth factors regulation. The relocation of the PHA synthase on the cell membrane guided by secrete signal peptides and cell membrane display motifs increased the PHB content by 2.4 folds. Excessive nitrogen preferentially promoted the PHB synthesis capacity and resulted in the PHB content increased by 13.3 folds. Modification of the genes responsible for cell division changed the cell morphology but the cell size was not enlarged to a PHB accumulation favorable environment. The metabolic engineering of *C. glutamicum* resulted in a high fermentative production of PHB using wheat straw as feedstock. This study provided an important microbial cell factory choice for PHB production using lignocellulose feedstock.

KEYWORDS
ammonia, *Corynebacterium glutamicum*, lignocellulose, PHA synthase, poly(3-hydroxybutyrate) (PHB)

1 | INTRODUCTION

Poly(3-hydroxybutyrate) (PHB) is an important carbon neutral biopolymer to replace petroleum-derived plastics due to its excellent thermochemical property, biocompatibility, and biodegradability (Arrieta et al., 2017; Bugnicourt, 2014; Chen & Wu, 2005; Raza et al., 2018). Fermentative production of PHB requires carbohydrates feedstock with sustainable and stable supply. Lignocellulose biomass is the only feasible carbohydrates option to meet this requirement (Wyman & Dale, 2015). Microbial strains used for PHB fermentation from lignocellulose feedstock had been investigated in previous studies, including *Burkholderia cepacia* (Pan et al., 2012), *Ralstonia eutropha* (Kim et al., 2016), *Bacillus megaterium* (de Souza et al., 2020; Y. Zhang et al., 2013), and *Paracoccus* sp. (Sawant et al., 2015). However, the cell viability of these microbes was generally poor in lignocellulose hydrolysate and the consequent PHB fermentation performance was far below those using glucose as feedstock (Gowda &

Ci Jin and Jing Li are equally contributed to this study.

Shivakumar, 2014; Kourilova et al., 2021; Silva et al., 2004; Yu & Stahl, 2008).

Corynebacterium glutamicum is a potential microbial cell factory for PHB fermentative production in high-solids content lignocellulose hydrolysate by its strong inhibitors-tolerance, well cell growth, and excellent metabolism performance (Han et al., 2019; Jin et al., 2020; Shin et al., 2016; X. Wang et al., 2018; Wen & Bao, 2019; Wen et al., 2018; Zhou et al., 2019). Jo et al. (2006) expressed PHB synthetic gene cluster (*phaCAB*) by plasmid to achieve PHB accumulation in *C. glutamicum* 13869; Matsumoto et al. (2011) expressed *phaCB* and the acetoacetyl-coenzyme synthase (AACS) instead of the *phaA* by plasmid in *C. glutamicum* 13869 to change PHB synthesis pathway; Song et al. (2013) expressed *phaCAB* and the gene HPA (fusion of α-amylase and anchor protein) by plasmid in *C. glutamicum* 13032 to utilize starch; Liu et al. (2007) expressed *phaCAB* in glutamic acid producing strain *C. glutamicum* 14067 and *C. glutamicum* S9114 to simultaneously produce PHB and glutamic acid. However, the PHB titer and yield obtained by these studies are away from practical application, thus further metabolic engineering needs to be conducted. The obstacles ahead for effective PHB production from lignocellulose by *C. glutamicum* include: (i) lack of xylose assimilation pathway results in the yield loss up to 30%; (ii) unstable PHB synthesis pathway (in plasmids) leads to a low PHB accumulation and unstable synthesis activity; (iii) cell morphology unable to match the high PHB accumulation; and (iv) improper growth and substrate factors on PHB synthesis.

Here, we show systematic metabolic engineering of *C. glutamicum* to overcome these obstacles. Successive integration of PHB synthesis genes (*phaA*, *phaB*, and *phaC*) into the genome of xylose-utilizing *C. glutamicum* to obtain a stable PHB-producing strain. Rate-limiting PHA synthase (*phaC*) was strengthened by relocation to cell membrane. Cell morphology was modified to a favorable PHB accumulating environment. The crucial growth factors were regulated to preferentially stimulate the PHB synthesis. The finally obtained engineered *C. glutamicum* strain reached the high PHB production from the practical lignocellulose. This study provided an important microbial cell factory choice for PHB production from lignocellulose feedstock.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

The strains used in this study were listed in Table 1. *Escherichia coli* was cultured in the Luria-Bertani (LB) medium with 50 μg/ml of kanamycin if needed at 37°C and 200 rpm shaking rate.

C. glutamicum S9114 is storage B460 of the Shanghai Industrial Institute of Microorganism (SIIM, http://www.gsysiim.com/). Flask culture of *C. glutamicum* was conducted at 30°C, 200 rpm, and pH 7.0. The seed medium contained 25.0 g/L of glucose, 1.0 g/L of KH_2PO_4, 3.0 g/L of urea, 0.6 g/L of $MgSO_4$, 5.0 g/L of yeast extract, and 10.0 g/L of peptone. The yeast-peptone (YP) medium was used for PHB fermentation in shaking flask, containing 1.0 g/L of KH_2PO_4, 3.0 g/L of urea, 0.6 g/L of $MgSO_4$, 5.0 g/L of yeast extract, 10.0 g/L of peptone, and 60.0 g/L of glucose or xylose.

Fermentation of *C. glutamicum* was conducted in 3 L fermentor in the CGXII medium containing 120.0 g/L of glucose, 5.0 g/L of urea, 1.0 g/L of K_2HPO_4, 1.0 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4$, 0.01 g/L of $CaCl_2$, 0.01 g/L of $FeSO_4{\cdot}7H_2O$, 0.01 g/L of $MnSO_4{\cdot}H_20$, 1.0 mg/L of $ZnSO_4{\cdot}7H_2O$, 0.2 mg/L of $CuSO_4$, 0.02 mg/L of $NiCl_2{\cdot}6H_2O$, 0.1 mg/L of biotin.

Biodetoxification strain *Amorphotheca resinae* ZN1 was isolated in our previous study and stored at the Chinese General Microbial Collections (CGMCC) with the storage number (CGMCC 7452; J. Zhang et al., 2010). *A. resinae* ZN1 were cultured in the potato dextrose agar (PDA) medium containing 200.0 g/L of potato extract, 20.0 g/L of glucose, and 15.0 g/L of agar.

2.2 | Enzymes and reagents

Cellulase CTec 2.0 was purchased from Novozymes China. The cellulase activity, cellobiase activity, and protein concentration were 256.0 FPU/ml, 4653.3 CBU/ml, and 86.3 mg/ml, respectively, according to the NREL protocols (Adney & Baker, 1996) and the Bradford method (Bradford, 1976).

Restriction endonuclease was purchased from Thermo Fisher Scientific. DNA polymerase and DNA ligase were purchased from Takara. The Seamless Cloning Kit was purchased from Hanheng Biotechnology. The Plasmid Extraction Kit, PCR Product Purification Recovery Kit, and Gel Recovery Kit were purchased from Generay Biotechnology.

Commercial PHB sample was purchased from Sigma-Aldrich (purity >99%) as standard. Other chemical reagents were all analytical pure and purchased from local suppliers.

2.3 | Plasmids and recombinants construction

The plasmids used and constructed in this study were listed in Table 1. The primers used were listed in Table S1. The PHB synthesis gene cluster *phaCAB* (H16_RS07135) was obtained from *R. eutropha* H16 and synthesized by Shanghai Generay Biotech.

The C-terminal sequences of the secretion protein Ncgl1289 (assigned as *Ncgl*) and CGR_RS04950 (assigned as *CGR*), and the sequence of porin CGS9114_RS00645 (assigned as *porC*) and CGS9114_RS09485 (assigned as *porB*) were used as cell membrane anchoring motifs according to Yim et al. (2016), L. Zhang et al. (2015), and Tateno et al. (2009).

The expression plasmids and integration plasmids used in this study were constructed according to our previous study (Jin et al., 2020).

TABLE 1 Strains and plasmids used

Strains	Characteristics	Sources
Escherichia coli DH5α	Host for plasmid construction	Lab stock
E. coli BL21	Genes *PntAB*, *udhA*, and *araE* source	Lab stock
Gluconobacter oxydans DSM2003	Gene *SGDH* source	DSMZ*
Amorphotheca resinae ZN1 (CGMCC 7542)	Biodetoxification fungus	Zhang et al. (2010)
C. glutamicum S9114	Glutamic acid production strain	SIIM‡
C. glutamicum-Δ*ldhA1*::*xylAB* (GJ01)	*C. glutamicum* S9114 with xylose utilization ability	Jin et al. (2020)
C. glutamicum GJ01-p*PH36-phaCAB*	*C. glutamicum* S9114 harboring the plasmid p*PH36-phaCAB*	This study
C. glutamicum GJ01-p*Peftu-phaCAB*	*C. glutamicum* S9114 harboring the plasmid p*Peftu-phaCAB*	This study
C. glutamicum-Δ*ldhA1*::*xylAB*-Δ*pdh*::*phaA*-Δ*MscCG*::*phaB*-Δ*ldhA2*::*phaC* (JH01)	*Pdh*, *MscCG* and *ldhA2* knockout and integration of the expression cassette P*eftu_phaA*, P*eftu_phaB* and P*eftu_phaC* in *C. glutamicum* GJ01	This study
JH01-p*Peftumob*	*C. glutamicum* JH01 harboring the plasmid p*Peftumob* as control	This study
JH01-p*Peftu-phaA*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-phaA*	This study
JH01-p*Peftu-phaB*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-phaB*	This study
JH01-p*Peftu-phaC*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-phaC*	This study
JH01-p*Peftu-PntAB*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-PntAB*	This study
JH01-p*Peftu-udhA*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-udhA*	This study
JH01-p*Peftu-SGDH*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-SGDH*	This study
JH01-p*Peftu-fasR*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-fasR*	This study
JH01-p*Peftu-aceE*	*C. glutamicum* JH01 harboring the plasmid p*Peftu-aceE*	This study
JH01-*odhA*_RBS0.1	RBS with 0.1 a.u. substitution of *odhA* in *C. glutamicum* JH01	This study
JH01-Δ*ack*::*araE* (JH02)	*Ack* knockout and the integration of the expression cassette P*H36_araE* in JH01	This study
JH02-p*Peftumob*	*C. glutamicum* JH02 harboring the plasmid p*Peftumob* as control	This study
JH02-p*Peftu-FtsZ*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-FtsZ*	This study
JH02-p*Peftu-pknA*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-pknA*	This study
JH02-p*Peftu-pknB*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-pknB*	This study
JH02-p*Peftu-DivIVA*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-DivIVA*	This study
JH02-p*Peftu-RodA*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-RodA*	This study
JH02-p*Peftu-WhcD*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-WhcD*	This study
JH02-*pknA*_RBS0.1	RBS with 0.1 a.u. substitution of *pknA* in *C. glutamicum* JH02	This study
JH02- *pknB*_RBS0.1	RBS with 0.1 a.u. substitution of *pknB* in *C. glutamicum* JH02	This study
JH02- *DivIVA*_RBS0.1	RBS with 0.1 a.u. substitution of *DivIVA* in *C. glutamicum* JH02	This study
JH02- *FtsZ*_RBS100	RBS with 100 a.u. substitution of *FtsZ* in *C. glutamicum* JH02	This study
JH02-p*Peftu-*(*Ncgl*)*phaC*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-*(*Ncgl*)*phaC*	This study
JH02-p*Peftu-*(*CGR*)*phaC*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-*(*CGR*)*phaC*	This study
JH02-p*Peftu-*(*Ncgl*)*phaC*(*gfp*)	*C. glutamicum* JH02 harboring the plasmid p*Peftu-*(*Ncgl*)*phaC*(*gfp*)	This study
JH02-p*Peftu-*(*porB*)*phaC*	*C. glutamicum* JH02 harboring the plasmid p*Peftu-*(*porB*)*phaC*	This study

(Continues)

TABLE 1 (Continued)

Strains	Characteristics	Sources
JH02-pP*eftu*-(*porC*)*phaC*	*C. glutamicum* JH02 harboring the plasmid pP*eftu*-(*porC*)*phaC*	This study
JH02-pP*eftu*-(*porB*)*phaC*(*gfp*)	*C. glutamicum* JH02 harboring the plasmid pP*eftu*-(*porB*)*phaC*(*gfp*)	This study
pP*H36*mob	Expression vector for *C. glutamicum*, P*H36* promoter, kanamycin resistance (Km^R)	Jin and Bao (2021)
pP*eftu*mob	Expression vector for *C. glutamicum*, P*eftu* promoter, Km^R	Jin and Bao (2021)
pK18mobsacB	Mobilizable vector for selection of double crossover in *C. glutamicum*, Km^R	Y. Wang et al. (2018)
pP*H36-phaCAB*	Vector for expression of *phaCAB* by P*H36* promoter	This study
pP*eftu-phaCAB*	Vector for expression of *phaCAB* by P*eftu* promoter	This study
pP*eftu-phaA*	Vector for expression of *phaA* by P*eftu* promoter	This study
pP*eftu-phaB*	Vector for expression of *phaB* by P*eftu* promoter	This study
pP*eftu-phaC*	Vector for expression of *phaC* by P*eftu* promoter	This study
pP*eftu-PntAB*	Vector for expression of *PntAB* by P*eftu* promoter	This study
pP*eftu-udhA*	Vector for expression of *udhA* by P*eftu* promoter	This study
pP*eftu-SGDH*	Vector for expression of *SGDH* by P*eftu* promoter	This study
pP*eftu-fasR*	Vector for expression of *fasR* by P*eftu* promoter	This study
pP*eftu-aceE*	Vector for expression of *aceE* by P*eftu* promoter	This study
pP*eftu-FtsZ*	Vector for expression of *FtsZ* by P*eftu* promoter	This study
pP*eftu-pknA*	Vector for expression of *pknA* by P*eftu* promoter	This study
pP*eftu-pknB*	Vector for expression of *pknB* by P*eftu* promoter	This study
pP*eftu-DivIVA*	Vector for expression of *DivIVA* by P*eftu* promoter	This study
pP*eftu-RodA*	Vector for expression of *RodA* by P*eftu* promoter	This study
pP*eftu-WhcD*	Vector for expression of *WhcD* by P*eftu* promoter	This study
pP*eftu*-(*Ncgl*)*phaC*	Vector for expression of (*Ncgl*)*phaC* by P*eftu* promoter	This study
pP*eftu*-(*CGR*)*phaC*	Vector for expression of (*CGR*)*phaC* by P*eftu* promoter	This study
pP*eftu*-(*Ncgl*)*phaC*(*gfp*)	Vector for expression of (*Ncgl*)*phaC*(*gfp*) by P*eftu* promoter	This study
pP*eftu*-(*porB*)*phaC*	*C. glutamicum* JH02 harboring the plasmid pP*eftu*-(*porB*)*phaC*	This study
pP*eftu*-(*porC*)*phaC*	*C. glutamicum* JH02 harboring the plasmid pP*eftu*-(*porC*)*phaC*	This study
pP*eftu*-(*porB*)*phaC*(*gfp*)	*C. glutamicum* JH02 harboring the plasmid pP*eftu*-(*porB*)*phaC*(*gfp*)	This study
pK18-Δ*pdh*::*phaA*	Vector for replacement of *pdh* by integrating the P*eftu_phaA* into the genome	This study
pK18-Δ*MscCG*::*phaB*	Vector for replacement of *MscCG* by integrating the P*eftu_phaB* into the genome	This study
pK18-Δ*ldhA2*::*phaC*	Vector for replacement of *ldhA2* by integrating the P*eftu_phaC* into the genome	This study
pK18-*odhA*_RBS0.1	Vector for RBS with 0.1 a.u. substitution of *odhA* in the genome	Wen and Bao (2019)
pK18-Δ*ack*::*araE*	Vector for replacement of *ack* by integrating the P*H36_araE* into the genome	Jin et al. (2020)
pK18-*FtsZ*_RBS100	Vector for RBS with 100 a.u. substitution of *FtsZ* in the genome	This study
pK18-*pknA*_RBS0.1	Vector for RBS with 0.1 a.u. substitution of *pknA* in the genome	This study
pK18-*pknB*_RBS0.1	Vector for RBS with 0.1 a.u. substitution of *pknB* in the genome	This study
pK18-*DivIVA*_RBS0.1	Vector for RBS with 0.1 a.u. substitution of *DivIVA* in the genome	This study

Note: DSMZ* indicates the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.
SIIM‡ indicates the collection center of Shanghai Industrial Institute of Microorganism, Shanghai, China.

2.4 | Lignocellulose feedstock and biorefinery processing

Wheat straw was collected from Nanyang, Henan, China, in spring 2021, then air-dried and milled before use. The wheat straw contained 30.5% of cellulose, 24.9% of xylan, 21.6% of Klason lignin, and 9.7% of ash according to the NREL protocols (Sluiter et al., 2008, 2012). The rest components included arabinan, mannan, galactan, acid-soluble lignin, water-soluble carbohydrates, crude protein, and some undetermined components (Han et al., 2018; Niu et al., 2016). Dry acid pretreatment was conducted according to our previous works (Han & Bao, 2018; He et al., 2014; J. Zhang et al., 2011). $Ca(OH)_2$ slurry (20%, w/w) was used to neutralize the sulfuric acid catalyst in the pretreated wheat straw to pH 5.5. *A. resinae* ZN1 was subsequently inoculated for biodetoxification according to our previous work (He et al., 2016).

The pretreated and detoxified wheat straw was hydrolyzed with the cellulase dosage of 4 mg protein/g dry matter (DM) at 50°C, 150 rpm, and pH 4.8 for 48 h (Han et al., 2019). The wheat straw hydrolysate slurry was centrifuged to remove the lignin solids to obtain the wheat straw hydrolysate with 101.7 g/L of glucose, 37.3 g/L of xylose, 0.9 g/L of acetic acid, 0.003 g/L of furfural, and 0.019 g/L of 5-hydroxymethylfurfural (HMF). The nutrient salts of 1.0 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 0.6 g/L $MgSO_4$, and 20.0 g/L $(NH_4)_2SO_4$ were added to the hydrolysate before fermentation.

2.5 | PHB fermentation

C. glutamicum was stretched on LB agar at 30°C for 48 h. A single colony was inoculated in a 10-ml seed medium and cultured at 30°C, 200 rpm for 16 h. In flask fermentation, the seed was transferred into 30 ml YP medium at inoculum size of 10% (v/v), and cultured at 30°C and 200 rpm. The pH was maintained at 7.0 by adding 5 M NaOH every 12 h. In fermentors, the seed was transferred into an 80-ml seed medium at inoculum size of 10% (v/v), and cultured at 30°C and 200 rpm for 12 h. The seed was subsequently inoculated to the 3 L fermentor (Biotech-3BG-4, Baoxing Biotech Co.) with an 800-ml fermentation medium at 30°C, 1.4 vvm, and 600 rpm stirring rate. The pH was maintained at 7.0 by automatically adding 5 M NaOH or 25% (w/w) aqueous ammonium solution. 200 ml of glucose liquor (500 g/L) or wheat straw hydrolysate was added every 24 h during fed-batch fermentation. In pure sugar fermentation, the initial medium contained 20 g/L of ammonia sulfate and 5 g/L urea, then 4 g ammonia sulfate was added every 36 h. The aqueous ammonia (20%, w/w) was added for maintaining the pH at 7.0. In wheat straw hydrolysate fermentation, the initial medium contained 10 g/L of ammonia sulfate, and the aqueous ammonia (20%, w/w) was added as pH regulator. The total ammonium concentration was calculated based on the initial 800 ml of the medium. The actual ammonia concentration was below the total ammonia concentration, because of the increased medium volume by the addition of aqueous ammonia solution and the growth consumption of the microbes.

2.6 | PHB extraction, GC-MS identification, and analysis

10 ml of fermentation broth was centrifuged to remove supernatant, and the cells were washed two times with deionized water and freeze-dried for 24 h.

20 mg of the dried bacteria or pure PHB was mixed with 2 ml of 97% (v/v) methanol (containing 3% sulfuric acid) and 2 ml of chloroform in the esterification tube at 100°C for 6 h. 1 ml of deionized water was added to the esterification tube and shaken for 5 min. The sample was absorbed from the lower organic phase and detected by Agilent 6890 GC-MS.

20 mg of the dried cells were mixed with 1 ml of sulfuric acid and reacted at 100°C for 40 min, then diluted 10 times and filtered. The samples were detected by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu) with HPX-87H column (Bio-Rad).

2.7 | Analytical methods

The HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu) with HPX-87H column (Bio-Rad) was used for detecting the content of D-glucose, D-xylose, acetic acid, furfural, and HMF. The column temperature was maintained at 65°C and the mobile phase was 5 mM H_2SO_4 with the flow rate of 0.6 ml/min.

2.8 | PHB content and yield calculation

PHB content was defined as the ratio of the recovered PHB weight to the dry cell weight (DCW)

$$\text{PHB content} = \frac{PHB}{DCW} \times 100\% \quad (1)$$

where *PHB* represents the concentration of PHB and *DCW* represents the concentration of dry cell weight.

The yield of PHB was defined as the ratio of the increased PHB concentration to the consumed glucose and xylose

$$\text{PHB yield} = \frac{[PHB] \times V - [PHB]_0 \times V_0}{[X+G]_0 \times V_0 - [X+G] \times V} \times 100\% \quad (2)$$

where $[PHB]$ and $[PHB]_0$ represent the final and initial concentrations of PHB, respectively, $[X+G]$ and $[X+G]_0$ represent the final and initial concentrations of xylose and glucose, respectively, and V and V_0 represent the final and initial volumes of the fermentation broth, respectively.

2.9 | Cell collection, RNA extraction, and qRT-PCR

The ammonia addition of 2 and 45 g/L was set as control and experimental group, respectively. 80 ml of seed was inoculated to 800 ml of fermentation medium in a 3-L fermentor at 30°C, 600 rpm, and pH 7.0. 20 ml of fermentation broth was taken at

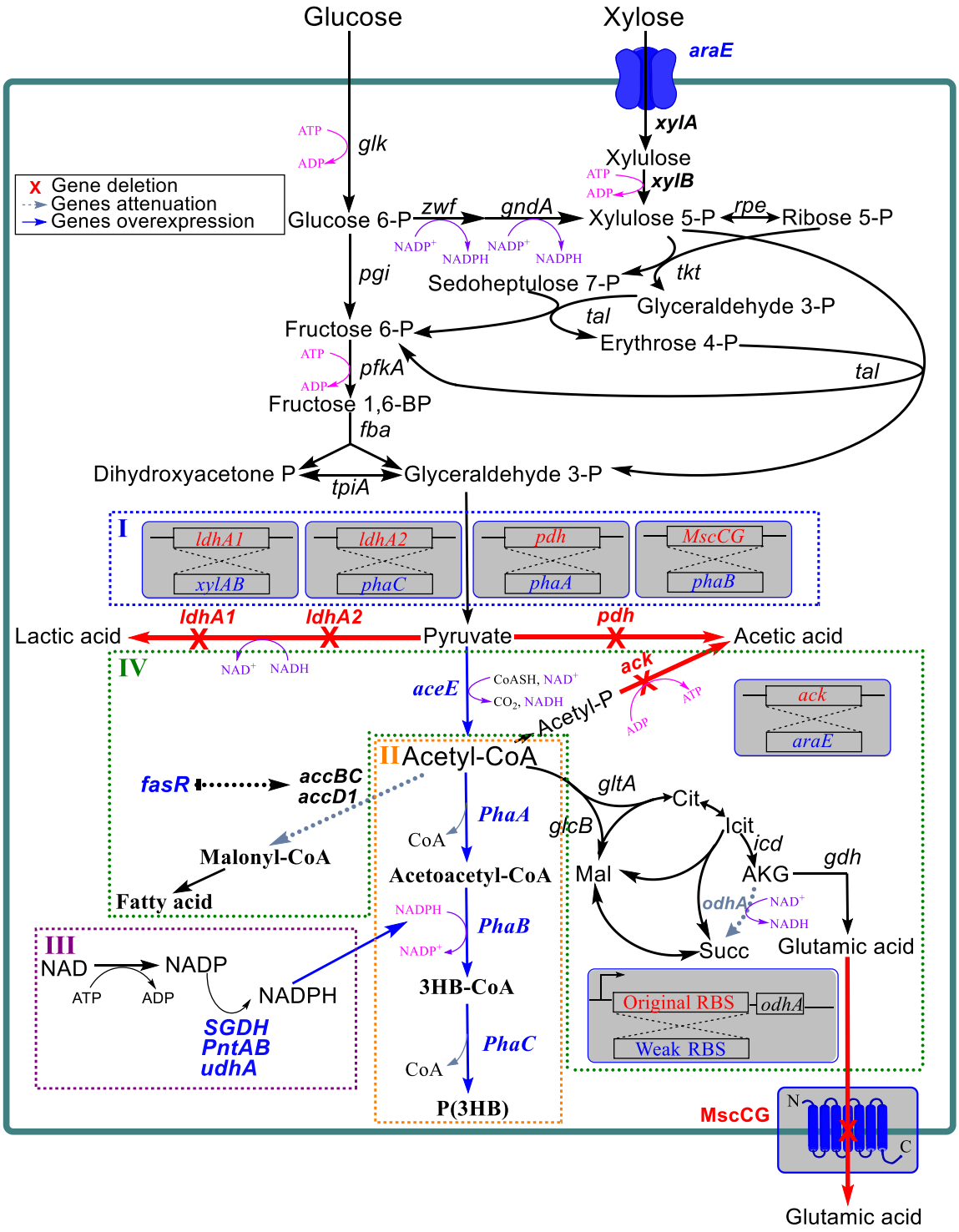


FIGURE 1 Continued

6, 8, 10, and 12 h, and the cell samples after centrifuge were stored at −80°C refrigerator. The primers used in the qRT-PCR experiments were designed based on the whole genome of *C. glutamicum* using Primer Premier 5, which are listed in Table S2. The 16s rRNA of *C. glutamicum* was used as an internal control to normalize the differences in total RNA quantity. The relative expression of genes was quantified using the formula $2^{-\Delta\Delta C_t}$.

2.10 | Transmission electron microscope

One cubic millimeter of wet cells was mixed with 1.0 ml of 2.5% glutaraldehyde solution and suspended in a centrifuge tube for more than 6 h. Then, the cells were washed with 0.1 M phosphate-buffered solution (PBS) for three times and fixed with 4% paraformaldehyde fixative for 2 h. After fixation, the cells were washed

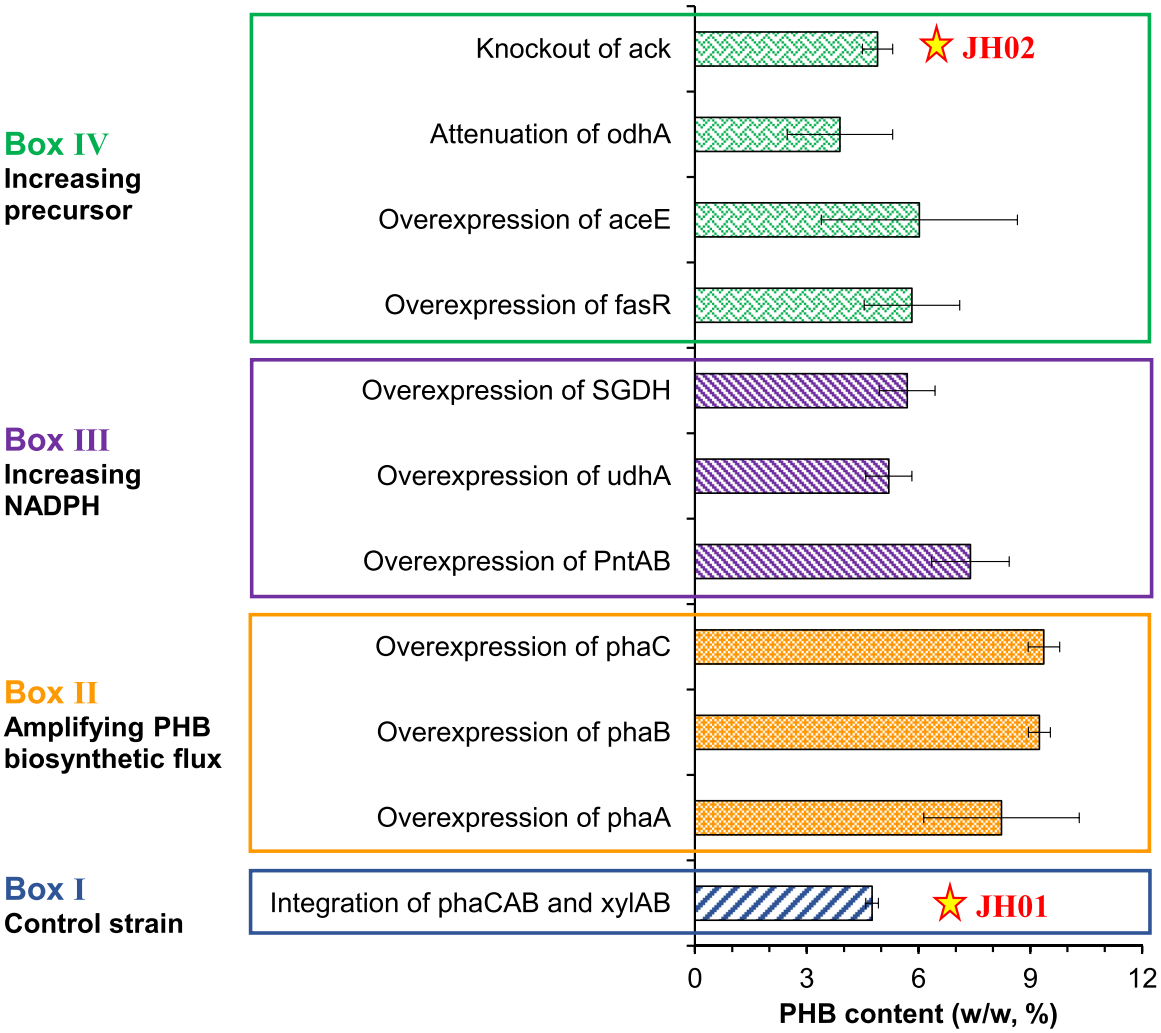


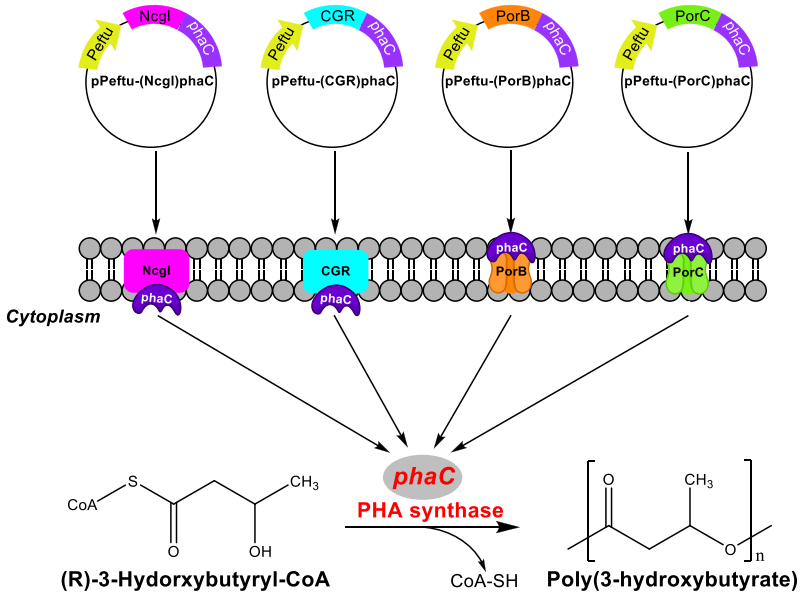
FIGURE 1 Increasing PHB production of *Corynebacterium glutamicum* by metabolic engineering of PHB synthesis-related pathways. The recombinant JH01 as control strain for integration of *phaCAB* and *xylAB* in *C. glutamicum* S9114; the recombinant JH01-odhA_RBS0.1 for attenuation of *odhA* in JH01; the recombinant JH01-Δack::*araE* assigned as JH02 for knockout of *ack* in JH01; other genes overexpression were conducted by plasmid in JH01. The fermentation was conducted in 250 ml flasks containing 30 ml yeast-peptone (YP) medium at 30°C and 200 rpm. Red crosses, gene knockout; blue arrow, genome integration of genes; gray dotted arrow, attenuation of genes. Box I, amplifying PHB synthetic pathway flux; Box II, enhancing NADPH regeneration; Box III, increasing acetyl-CoA accumulation. *araE*, encoding pentose transporter; *xylA*, encoding xylose isomerase; *xylB*, encoding xylulose kinase; *glk*, encoding glucokinase; *zwf*, encoding glucose-6-phosphate dehydrogenase; *gndA*, encoding 6-phosphogluconolactonase; *pgi*, encoding glucose-6-phosphate isomerase; *pfkA*, encoding 6-phosphofructokinase; *fba*, encoding fructose-bisphosphate aldolase; *tpiA*, encoding triosephosphate isomerase; *aceE*, encoding pyruvate dehydrogenase E1 component; *ldhA1*, encoding lactate dehydrogenase 1; *ldhA2*, encoding lactate dehydrogenase 2; *pdh*, encoding pyruvate dehydrogenase; *ack*, encoding acetate kinase; *gltA*, encoding citrate synthase; *icd*, encoding isocitrate dehydrogenase; *odhA*, encoding 2-oxoglutarate dehydrogenase E1 component; *glcB*, encoding malate synthase G; *gdh*, encoding glutamate dehydrogenase; *tkt*, encoding transketolase; *tal*, encoding transaldolase; *accBC*, encoding acetyl-CoA carboxylase BC component; *accD1*, encoding acetyl-CoA carboxylase D1 component; *fasR*, encoding fatty acid biosynthesis transcriptional regulator; *SGDH*, *PntAB*, and *udhA*, encoding transhydrogenase; *phaA*, encoding β-ketothiolase; *phaB*, encoding acetylacetyl-CoA reductase; *phaC*, encoding PHA synthase; CIT, citrate; ICIT, isocitrate; AKG, α-oxoglutarate; SUCC, succinate; Mal, malate; MscCG, glutamate exporter; 3HB-CoA, 3-hydroxybutyryl-CoA; P(3HB), poly(3-hydroxybutyrate)

with 0.1 M PBS for three times again, and it was dehydrated with gradient ethanol before freeze-drying. The prepared cell samples were characterized by transmission electron microscopy (FEI Tecnai Spirit G2 BioTWIN, FEI) and field emission electron microscopy (GeminiSEM 500, ZEISS).

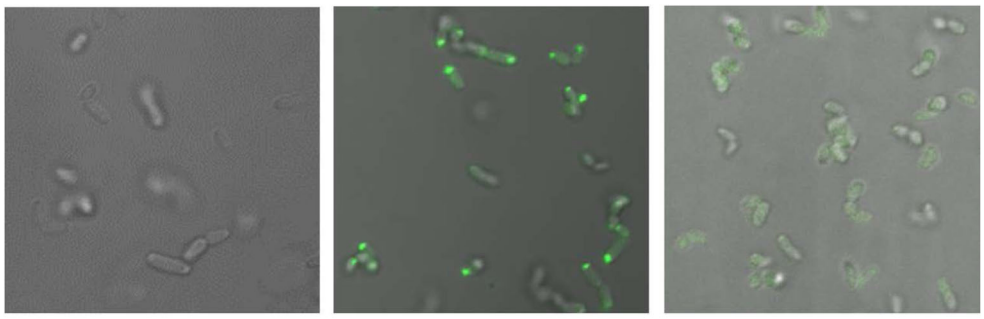
2.11 | Laser confocal microscope observations

After centrifuge, the cells samples were washed with PBS for 3 times and diluted for 10 times, which were characterized by the laser confocal microscope (A1R, Nikon).

(a) Diagram of membrane-located expression of PHA synthase



(b) Fluorescence microscopy of membrane-located expression of PHA synthase



(c) PHB synthesis by membrane-located expression of PHA synthase

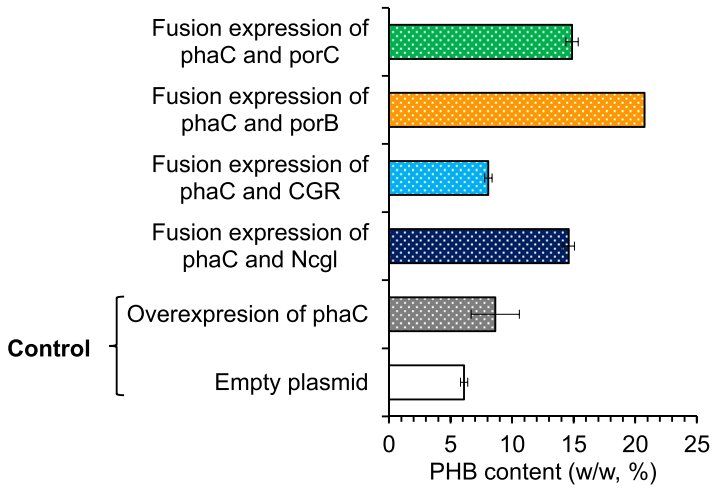


FIGURE 2 PHB synthesis by the membrane-located expression of PHA synthase. (a) Diagram of membrane-located expression of PHA synthase; (b) Fluorescence microscopy of membrane-located expression of PHA synthase; (c) PHB generation by membrane-located expression of PHA synthase. All genes overexpression were conducted by plasmid in JH02. The fermentation was conducted in 250 ml flasks containing 30 ml yeast-peptone (YP) medium at 30°C and 200 rpm. All the data were collected after 48 h fermentation. *Ncgl* and *CGR*, encoding secret signal peptides; *porB* and *porC*, encoding cell membrane display motifs; *phaC*, encoding PHA synthase; *gfp*, encoding green fluorescent protein

3 | RESULTS AND DISCUSSION

3.1 | Constructing PHB synthesis pathway in *C. glutamicum*

The metabolic engineering of *C. glutamicum* for PHB synthesis started with the construction of PHB synthesis and xylose assimilating pathways into the genome of *C. glutamicum* S9114 for stable PHB production (Figure 1, Box I).

Xylose utilization is an essential step when lignocellulose is used. The xylose assimilating gene cluster (*xylAB*) was induced and homogeneously integrated into the genome of *C. glutamicum* S9114 to obtain a xylose assimilating recombinant *C. glutamicum* GJ01 (Jin et al., 2020, Figure 1, Box I).

The P*eftu* promoter was selected for effectively expressing PHB synthesis genes (*phaCAB*) in *C. glutamicum* (Table S3). The genes of the PHB synthesis cluster *phaCAB* were independently integrated into the different locus of the genome of *C. glutamicum* GJ01. The gene *phaC* (PHA synthase) was firstly integrated into the genome by substituting the original *ldhA2* (lactate dehydrogenase). The gene *phaB* (acetylacetyl-CoA reductase) was subsequently integrated into the genome by substituting the original *MscCG* (glutamate secretion channel protein). Finally, the gene *phaA* (β-ketothiolase) was integrated into the genome by substituting the original *pdh* (pyruvate dehydrogenase; Figure 1, Box I). The obtained *phaCAB* genes integrating strain was assigned as *C. glutamicum* JH01. Figure S1 shows the transmission electron microscope (TEM) images of *C. glutamicum* JH01, and the generated PHB was extracted, esterified, and analyzed by GC-MS compared with PHB standard. However, only 3.9% (w/w) and 2.2% (w/w) of PHB were obtained by the *C. glutamicum* JH01 from glucose and xylose, respectively (Table S4). Further metabolic engineering is needed for improving PHB production.

The systematic metabolic engineering on *C. glutamicum* for improving PHB production was illustrated in Figure 1 (Box II, III, IV).

Box II, amplifying PHB synthesis pathway flux. The genes *phaA*, *phaB*, and *phaC* responsible for PHB synthesis from acetyl-CoA were overexpressed in *C. glutamicum* JH01. The PHB content was increased by 1.7, 1.9, and 2.0 folds, respectively, compared to the control strain JH01-pP*eftu*mob.

Box III, enhancing NADPH regeneration to supply cofactor for PHB synthesis. The *PntAB*, *udhA*, and *SGDH* (transhydrogenase) responsible for conversion of $NADP^+$ to NADPH were expressed. The results showed that the overexpression of *PntAB*, *udhA*, and *SGDH* increased the PHB content by 1.6, 0.1, and 0.2 folds, respectively, compared to the control strain JH01-P*eftu*mob.

Box IV, increasing acetyl-CoA (crucial precursor of PHB synthesis) accumulation. The *aceE* (phosphotransacetylase) and *fasR* (fatty acid biosynthesis transcriptional regulator) genes were overexpressed to promote the flux of pyruvate to acetyl-CoA and reduce the flux of acetyl-CoA to fatty acid, respectively. The *ack* (acetate kinase) was substituted by *araE* (pentose transporter) to reduce the flux of acetyl-CoA to acetic acid and increase xylose transmembrane rate. The ribosomal binding site (RBS) of *odhA* (α-oxoglutarate dehydrogenase) was attenuated to reduce the flux of acetyl-CoA to TCA cycle. The fermentation results showed that increasing precursor supply had little affection on PHB synthesis.

The individual effect of engineering results shows that overexpression of *phaCAB*, overexpression of *pntAB*, and deletion of *ack* are beneficial for PHB synthesis (Figure 1). However, both *phaCAB* and *pntAB* were overexpressed by plasmids, and the multiple plasmids expression system is unstable. The *ack* deletion was conducted in the genome, and other genes could be expressed by plasmids for further engineering, thus the *ack* deletion strain was assigned as *C. glutamicum* JH02 as the base strain.

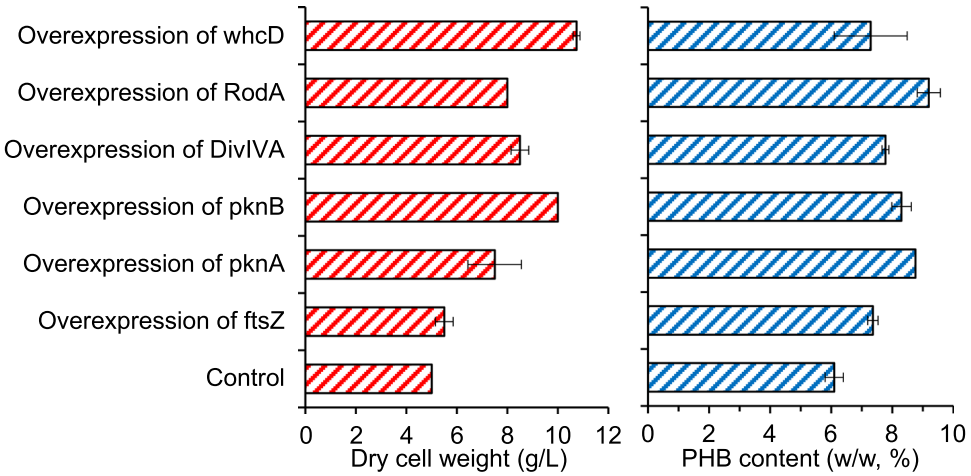
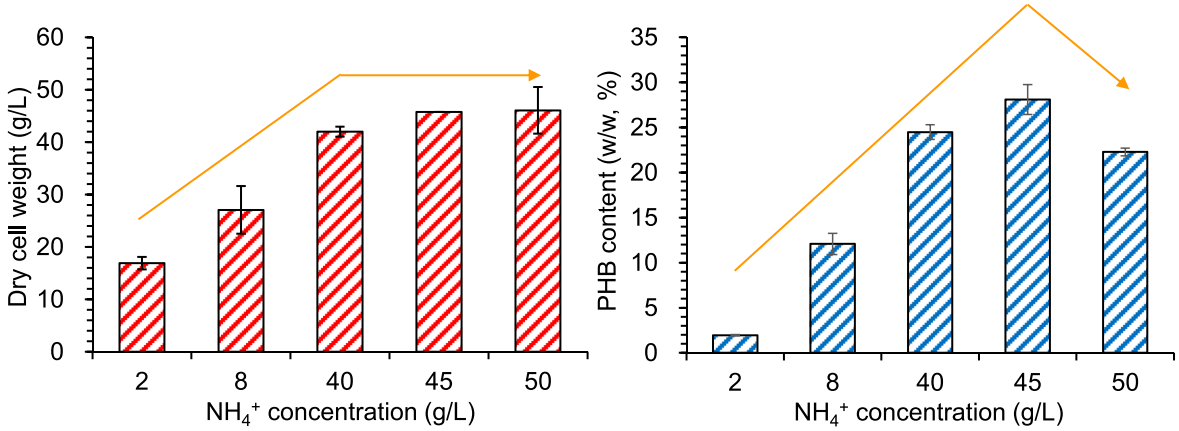


FIGURE 3 Increasing PHB production of *Corynebacterium glutamicum* by changing the cell morphology. All genes overexpression were conducted by plasmid in JH02. The fermentation was conducted in 250 ml flasks containing 30 ml yeast-peptone (YP) medium at 30°C and 200 rpm. All the data were collected after 48 h fermentation

(a) PHB production of *C. glutamicum* by varying ammonia uptake



(b) Transcription analysis of *C. glutamicum* varying ammonia uptake level

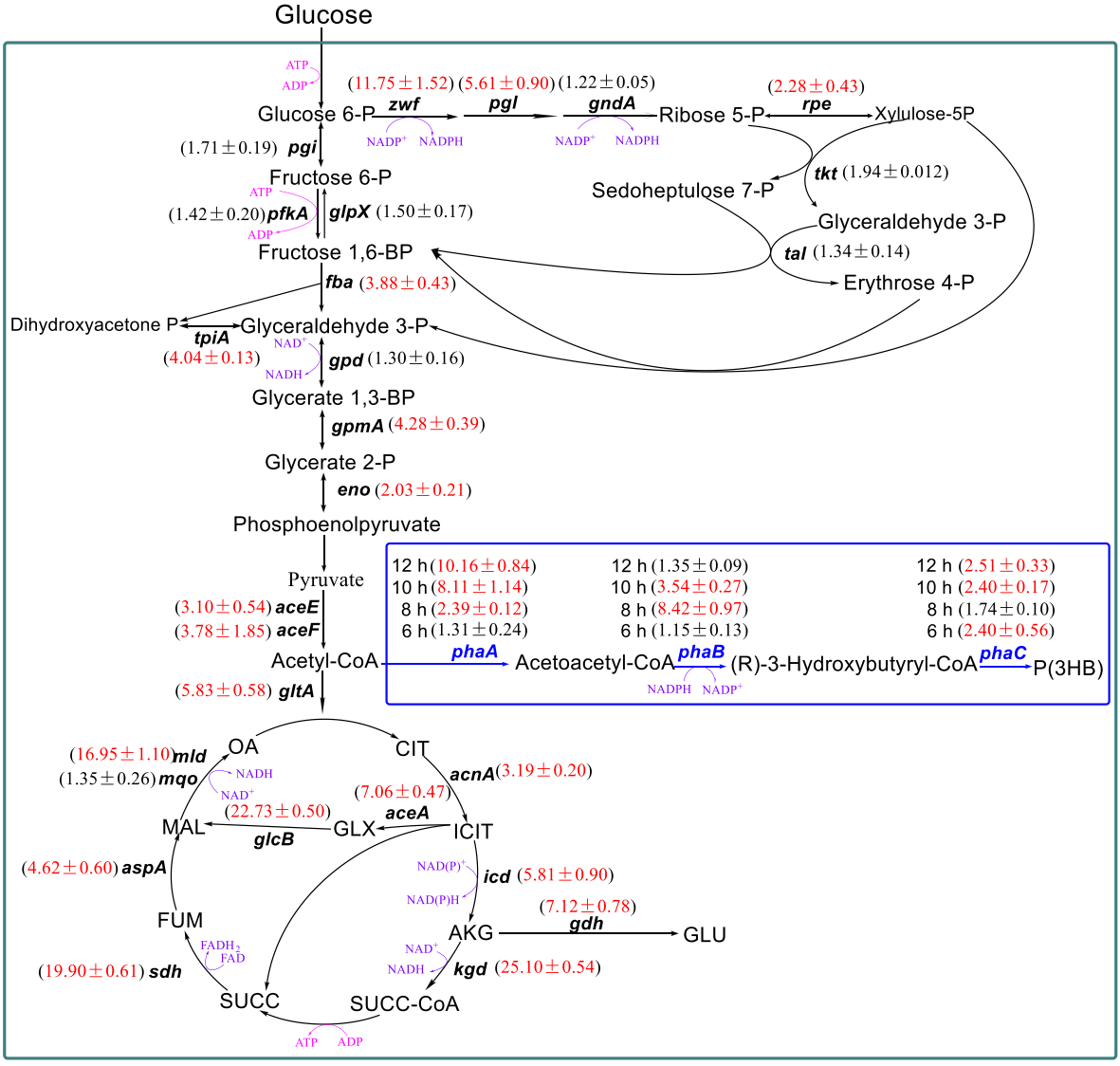


FIGURE 4 (See caption on next page)

3.2 | Increasing PHB production by membrane-located expression of PHA synthase

The enhancement of PHA synthase (PhaC) activity and stability is the key step to overcome the rate-limiting step of PHB synthesis in the engineered *C. glutamicum*. Localization of enzymes on cell membrane is a general method used for increasing the protein's activity and stability. In *Bacillus thuringiensis*, chitinase was re-located on the cell membrane with anchoring motifs and resulted in increased chitinase activity and stability (Tang et al., 2017). In *E. coli*, xylanase activity was increased by the re-location on the cell membrane with anchoring motifs (Chen et al., 2012). This study re-located PhaC on the cell membrane of *C. glutamicum* to increase the activity and stability of PHA synthase. The re-localization of PhaC provided a micro-environment by embodying PhaC in the cell membrane as carrier with less effect from the cytoplasm components, similar to entrapment immobilization of enzymes. The localization of PhaC also lessened the effect of protein dilution by fixation into the cell membrane and the stability of PhaC might also be increased.

The secrete signal peptides (*Ncgl* and *CGR*, Yim et al., 2016; L. Zhang et al., 2015) and cell membrane display motifs (*porB* and *porC*, Tateno et al., 2009) were used as membrane anchoring. The N-terminal of *phaC* was fused at the C-terminal of *Ncgl*, *CGR*, *porB*, and *porC*, respectively, to achieve membrane-located expression of PHA synthase (Figure 2a). Figure 2b showed that the green fluorescence (*gfp*) was fused to the C-terminal of (*Ncgl*)*phaC* and (*porB*)*phaC* was focused on the cell poles when the secrete signal peptide *Ncgl* was used, or equably distributed in the cell when the cell membrane display motif *porB* was used. Figure 2c shows that the fusion expression of *Ncgl*, *CGR*, *porB*, and *porC* with *phaC* increased the PHB content by 0.7, 0, 1.4, and 0.7 folds compared to the control strain JH02-P*eftu*-*phaC*, respectively.

3.3 | Increasing PHB production by modifying cell size

PHB is an intracellular accumulating polymer with occupation of large cell space. Reports showed that the PHB production increased with enlarging cell size (Y. Wang et al., 2014; Wu et al., 2016). Here, we regulated the cell growth and division cycles to change the *C. glutamicum* cells into a favorable PHB accumulating environment. Several genes related to the cell growth and division cycle of *C. glutamicum* were selected including *DivIVA*, *RodA*, *ftsZ*, *pknA*, *pknB*, and *whcD*. Among the selected regulators, *DivIVA* and *RodA* are responsible for cell polar growth (Letek et al., 2008; Sieger et al., 2013). FtsZ is responsible for recruiting other proteins to cause invagination of plasma membrane resulting in cell division (Ramos et al., 2005), which process was also controlled by some transcriptional regulators including PknA, PknB, and WhcD (Fiuza et al., 2008; Lee et al., 2017).

Both amplification and attenuation of the genes were conducted to oversee the change of cell morphology. For amplification, the *DivIVA*, *RodA*, *ftsZ*, *pknA*, *pknB*, and *whcD* genes were overexpressed in *C. glutamicum* JH02, respectively. Figure 3 showed that over-expression of *pknB* and *WhcD* genes increased the cell mass growth by 1.0 and 1.3 folds, respectively, compared to the control strain JH02-P*eftu*mob. However, no variation of cell size was observed (Figure S2), indicating that overexpression of these genes promoted PHB production did not lead to the change of cell size, although the cell growth was increased. For attenuation, *DivIVA*, *ftsZ*, *pknA*, and *pknB* were attenuated by substitution of ribosomal binding site (RBS) sequence in *C. glutamicum* JH02, respectively. The attenuation of *RodA* and *WhcD* were failed to achieve due to the harsh inhibition of cell growth. The attenuation of DivIVA changed the cell morphology from a rod-like shape to a spherical shape, however, the PHB content was decreased (Figure S2).

Several genes related to the cell growth and division cycle were overexpressed and attenuated to change the *C. glutamicum* cells into a favorable PHB accumulating environment. However, the cell size change of *C. glutamicum* was not achieved by rational engineering, although the cell mass was increased. Therefore, the PHB content of these recombinant strains is similar to the control strain JH02-pP*eftu*mob (~8% PHB content), but less than the strain JH02-pP*eftu*-(*porB*)phaC (~20% PHB content).

3.4 | Promotion of PHB production by excessive nitrogen addition

Nitrogen deprivation is generally regarded as an important factor for PHB production by restriction of its cell growth (Cabello

FIGURE 4 Increasing PHB production of *Corynebacterium glutamicum* JH02 by varying ammonia uptake. (a) PHB production of *C. glutamicum* by varying ammonia uptake; (b) transcription analysis of *C. glutamicum* varying ammonia uptake level. The fed-batch fermentation was conducted in the 3 L fermentor for 96 h at 30°C, 600 rpm, 1.4 vvm, and pH 7.0. The ammonia sulfate was used to adjust ammonia concentration of CGXII medium. The ammonia concentration of the control group and experimental group for transcription analysis is 2 and 40 g/L, respectively. *zwf*, encoding glucose-6-phosphate dehydrogenase; *pgl*, encoding 6-phosphogluconolactonase; *rpe*, ribulose-5-phosphate-epimerase; *gndA*, encoding 6-phosphogluconolactonase; *pgi*, encoding glucose-6-phosphate isomerase; *pfkA*, encoding 6-phosphofructokinase; *glpX*, fructose 1,6-bisphosphatase; *fba*, encoding fructose-bisphosphate aldolase; *tpiA*, encoding triosephosphate isomerase; *gpd*, encoding glyceraldehyde-3-phosphate dehydrogenase; *gpmA*, encoding phosphoglycerate mutase; *eno*, encoding phosphopyruvate hydratase; *aceE*, encoding pyruvate dehydrogenase E1 component; *aceF*, encoding dihydrolipoamide acetyltransferase; *gltA*, encoding citrate synthase; *acnA*, encoding aconitate hydratase; *icd*, encoding isocitrate dehydrogenase; *glcB*, encoding malate synthase G; *gdh*, encoding glutamate dehydrogenase; *tkt*, encoding transketolase; *tal*, encoding transaldolase; *aceA*, encoding isocitrate lyase; *kgd*, encoding α-ketoglutarate decarboxylase; *sdh*, encoding succinate dehydrogenase; *aspA*, encoding fumarate hydratase; *mqo*, encoding malate:quinone oxidoreductase; *mld*, encoding malate dehydrogenase; *phaA*, encoding β-ketothiolase; *phaB*, encoding acetylacetyl-CoA reductase; *phaC*, encoding PHA synthase; CIT, citrate; ICIT, isocitrate; AKG, α-oxoglutarate; GlU, glutamic acid; SUCC-CoA, Succinyl-CoA; SUCC, succinate; FUM, fumarate; Mal, malate; OA, Oxaloacetate; P(3HB), poly(3-hydroxybutyrate)

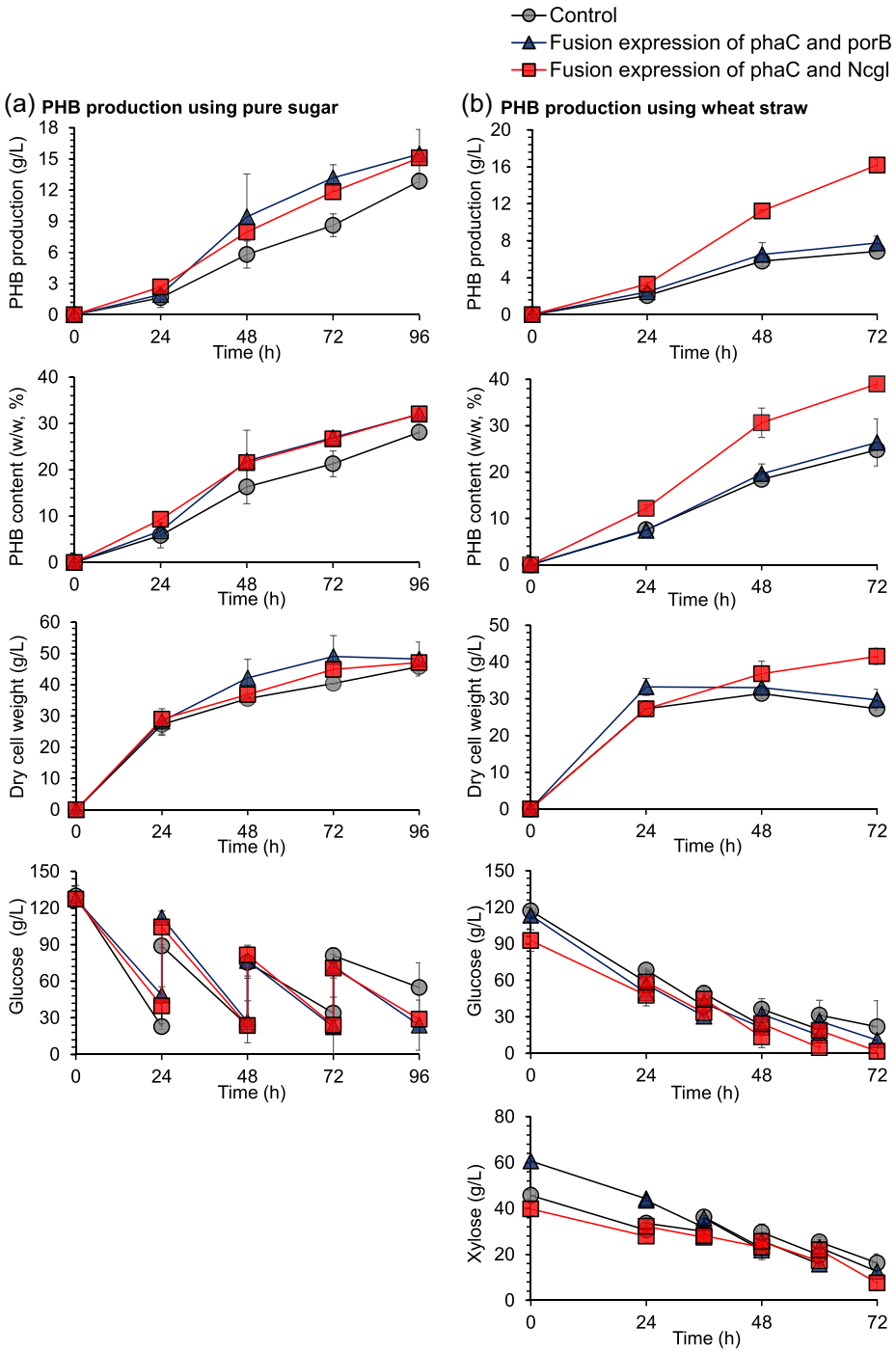


FIGURE 5 Fed-batch fermentation of *Corynebacterium glutamicum* recombinants for PHB production using pure sugars and wheat straw feedstocks. (a) PHB production using pure sugar; (b) PHB production using wheat straw hydrolysate. JH02-p*Eftu*mob as the control strain with empty plasmid p*Eftu*mob in JH02; JH02-p*Eftu*-(*Ncgl*)*phaC* for fusion expression of *phaC* and *Ncgl* in JH02; JH02-p*Eftu*-(*porB*)*phaC* for fusion expression of *phaC* and *CGR* in JH02. The fed-batch fermentation was conducted in the 3 L fermentor at 30°C, 600 rpm, 1.4 vvm, and pH 7.0. 200 ml of 500 g/L glucose or wheat straw hydrolysate was added every 24 h

et al., 2016; Zhu et al., 2013). This study tested the ammonia concentration of the fermentation medium and found that nitrogen surplus, instead of deprivation, was crucially important for PHB synthesis. The ammonia addition was respectively set as 2, 8, 40, 45, and 50 g/L to increase PHB production of *C. glutamicum*. Figure 4a shows that the dry cell weight (DCW) and PHB content were increased by 1.7 and 13.3 folds, respectively, with the ammonia addition from 2 to 45 g/L, then declined with further increase of ammonia addition from 45 to 50 g/L. The results indicate that excessive nitrogen in a specific range not only

increased cell growth, but also promoted PHB synthesis of *C. glutamicum*.

qRT-PCR was conducted under varying ammonia addition to study the nitrogen surplus effects on PHB synthesis by *C. glutamicum* (Figure 4b). The ammonia addition of control and experimental group was set as 2 and 45 g/L, respectively. Figure 4b shows that nitrogen surplus promoted PHB synthesis of *C. glutamicum* in three aspects as follows: (i) PHB synthesis pathway was enhanced by the significant upregulation expression of PHB synthesis genes (*phaA*, *phaB*, and *phaC*); (ii) NADPH regeneration was increased to supply coenzyme for PHB synthesis by the upregulation expression of glucose oxidation genes (*zwf* and *pgl*); (iii) substance and energy metabolism were enhanced to improve cell growth and cell viability by the upregulation expression of genes involved in the glycolytic pathway and tricarboxylic acid cycle (TCA cycle). It is speculated that the high ammonia concentration activated the environmental transcription factors of the constitutive promotor eftu, instead of specific inducers, for the upregulation of *phaCAB* genes. The confirmation of the transcription factors related to ammonia activation is under investigation.

3.5 | PHB fermentation in synthetic medium and wheat straw hydrolysate

The engineered *C. glutamicum* recombinants, JH02-pP*eftu*-(*Ncgl*)*phaC* and JH02-pP*eftu*-(*porB*)*phaC*, were used in fed-batch fermentation using pure sugars and wheat straw feedstock (Figure 5).

In pure sugars, Figure 5a shows that the highest PHB production by JH02-pP*eftu*-(*porB*)*phaC* reached 15.5 g/L, which was approximately 20% greater than that of control strain JH02-pPeftumob (12.9 g/L). The highest PHB content by JH02-pP*eftu*-(*Ncgl*)*phaC* reached 32.1% (w/w), which was approximately 14% greater than that of control strain JH02-pPeftumob (28.1% w/w).

In wheat straw hydrolysate, Figure 5b shows that the highest PHB production and content by JH02-pP*eftu*-(*Ncgl*)*phaC* reached 16.2 g/L and 39.0% (w/w), which was approximately 135% and 57% greater than that of control strain JH02-pPeftumob (6.9 g/L and 24.8% w/w). However, the PHB production of JH02-pP*eftu*-(*porB*)*phaC* using wheat straw hydrolysate (7.8 g/L and 26.4 wt%) was far lower than that using pure sugars (15.5 g/L and 32.0% w/w). We speculated that the cell surface display motif PorB used in JH02-pP*eftu*-(*porB*)*phaC* exposed PHA synthase to the severe environment of wheat straw hydrolysate leading to the reduction of PHA synthase activity. While the Ncgl motif used in JH02-pP*eftu*-(*Ncgl*)*phaC* located the PHA synthase in the intracellular membrane to make it keep from extracellular severe environment. The above results indicated that the JH02-P*eftu*-(*Ncgl*)*phaC* was the most suitable strain for PHB production in the wheat straw hydrolysate.

Table 2 shows that the *C. glutamicum* JH02-P*eftu*-(*Ncgl*)*phaC* produced the maximum PHB (41.6 g/L of cell mass, 39.0% PHB content, 16.2 g/L of PHB) using typical lignocellulose feedstock compared with the previous reports. The integration of *phaCAB* into the genome provided a microbial cell factory for further engineering, and membrane-located expression, morphology engineering on PHB synthesis. The unique biodetoxification approach by *A. resinae* ZN1 provided the wheat straw hydrolysate with the high fermentable sugars (101.7 g/L of glucose and 37.3 g/L of xylose) and the least inhibitors (0.9 g/L of acetic acid, 0.003 g/L of furfural, and 0.019 g/L of 5-hydroxymethylfurfural) for PHB fermentation. The xylose utilization capacity of the engineered *C. glutamicum*, as well as the proper ammonia supplementation (35 g/L of the total

TABLE 2 Summary of the reported PHB productions by *Corynebacterium glutamicum*

Strategies	Feedstock	Titer (g/L)	Content (w/w, %)	Yield (g/100 g sugars)	Sources
Expression of *phaCAB* by plasmid in *C. glutamicum* 13869	Glucose	2.8	22.5	4.7	Jo et al. (2006)
Expression of *phaCB* and the acetoacetyl-coenzyme synthase (AACS) instead of the *phaA* by plasmid in *C. glutamicum* 13869	Glucose	1.7	19.0	2.8	Matsumoto et al. (2011)
Expression of *phaCAB* and the gene HPA (fusion of α-amylase and anchor protein) by plasmid in *C. glutamicum* 13032	Starch	0.4	6.4	0.9	Song et al. (2013)
Expression of *phaCAB* by plasmid in *C. glutamicum* 14067; simultaneous production of PHB and glutamic acid	Glucose	0.2	2.2	0.3	Liu et al. (2007)
Expression of *phaCAB* by plasmid in *C. glutamicum* S9114; simultaneous production of PHB and glutamic acid	Glucose	1.0	12.1	1.3	Liu et al. (2007)
Integration of *phaCAB* into genome and membrane-located expression of *phaC* in *C. glutamicum* GJ01; nitrogen surplus	Glucose	15.5	32.0	6.4	This study
	Wheat straw (glucose and xylose)	16.2	39.0	13.2	This study

TABLE 3 Summary of the reported results of PHB production from lignocellulose biomass

Producing strain	Carbon source	Pretreatment and detoxification	CDW (g/L)	Content (wt%)	Titer (g/L)	Sources
Azotobacter beijerinickii	Coir pitch (Glucose)	Autoclave	5.0	48.0	2.4	Prabu and Murugesan (2010)
Burkholderia cepacia	Wood hydrolysate (Xylose)	Acid pretreatment; membrane filtration	17.0	51.4	8.7	Pan et al. (2012)
Ralstonia eutropha	Bagasse hydrolysate (Glucose)	Acid pretreatment;	6.0	65.0	3.9	Yu and Stahl (2008)
Bacillus megaterium	Oil palm empty fruit bunch (Glucose and xylose)	Alkaline pretreatment	24.2	51.6	12.5	Zhang et al. (2013)
B. cepacia	Bagasse hydrolysate (Glucose and xylose)	Acid pretreatment; overliming and activated carbon	4.4	53.0	2.3	Silva et al. (2004)
Burkholderia sacchari	Bagasse hydrolysate (Glucose and xylose)	Acid pretreatment; Overliming and activated carbon	4.4	62.0	2.7	Silva et al. (2004)
Bacillus thuringiensis	Bagasse hydrolysate (Glucose)	Acid pretreatment; overliming	10.6	39.6	4.2	Gowda and Shivakumar (2014)
R. eutropha	Sunflower stalk hydrolysate (Glucose and xylose)	Hydrothermal pretreatment	11.0	72.5	7.9	Kim et al. (2016)
B. megaterium	Corn husk hydrolysate (Glucose and xylose)	Biologically pretreatment	1.7	57.8	1.0	de Souza et al. (2020)
Paracoccus sp.	Corn stover (Glucose and xylose)	Alkaline pretreatment	14.8	72.0	9.7	Sawant et al. (2015)
Corynebacterium glutamicum	Wheat straw hydrolysate (Glucose and xylose)	Acid pretreatment; bio-detoxification	41.6	39.0	16.2	This study

ammonia supplementation) also played the important role in PHB production.

When different strains were used on PHB production from lignocellulose feedstock, only low cell mass below 10.0 g/L and PHB around 4.0 g/L were obtained due to the inhibitors suppression (Table 3; Gowda & Shivakumar, 2014; Kourilova et al., 2021; Prabu & Murugesan, 2010; Silva et al., 2004; Yu & Stahl, 2008). Although the detoxification led to increased cell growth (below 20.0 g/L) and PHB generation (below 15.0 g/L; de Souza et al., 2020; Kim et al., 2016; Pan et al., 2012; Prabu & Murugesan, 2010; Sawant et al., 2015), these detoxification approaches (evaporation, membrane filtration, and activated carbon) are highly costly with heavy wastes generation and sugar loss, thus not feasible for any practical applications (Jönsson & Martín, 2016). The selection of the robust *C. glutamicum* showed the obvious advantage when lignocellulose was used as the carbohydrate feedstock.

4 | CONCLUSION

Lignocellulose is an important feedstock for industrial PHB production, but the inhibitors generated in pretreatment suppress cell growth of traditional PHB-producing strains. In this study, *C. glutamicum* was used as starting strain with strong inhibitors tolerance, and PHB synthesis and xylose utilization pathway were constructed to achieve PHB production from lignocellulose-derived sugars. Some strategies were successfully applied on *C. glutamicum* for increasing PHB production for the first time, including membrane-located expression of PHA synthase, alteration of cell growth and division, and addition of excessive nitrogen. The final strain produced 16.2 g/L of PHB with 39.0% (w/w) from wheat straw hydrolysate without high-cost detoxification. This study provided an important microbe option to produce PHB using lignocellulose feedstock.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China (21978083, 31961133006).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All supporting data are available in Supporting Information or from the corresponding author on request.

ORCID

Jie Bao https://orcid.org/0000-0001-6521-3099

REFERENCES

Adney, B., & Baker, J. (1996). *Measurement of cellulase activities. NREL/AP-006*. National Renewable Energy Laboratory.

Arrieta, M. P., Samper, M. D., Aldas, M., & Lopez, J. (2017). On the use of PLA-PHB blends for sustainable food packaging applications. *Materials*, *10*, 1008. https://doi.org/10.3390/ma10091008

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bugnicourt, E. (2014). Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging. *eXPRESS Polymer Letters*, *8*, 791–808. https://doi.org/10.3144/expresspolymlett.2014.82

Cabello, A. T., Rspi, J., & Ruiz, B. (2016). Influence of the C/N ratio on synthesis of polyhydroxybutyrate (PHB) by *Ralstonia eutropha* using juice manufacturing wastewater as carbon source. *New Biotechnology*, *33*, S153. https://doi.org/10.1016/j.nbt.2016.06.1251

Chen, G. Q., & Wu, Q. (2005). Microbial production and applications of chiral hydroxyalkanoates. *Applied Microbiology and Biotechnology*, *67*, 592–599. https://doi.org/10.1007/s00253-005-1917-2

Chen, Y. P., Hwang, I. E., Lin, C. J., Wang, H. J., & Tseng, C. P. (2012). Enhancing the stability of xylanase from *Cellulomonas fimi* by cell-surface display on *Escherichia coli*. *Journal of Applied Microbiology*, *112*, 455–463. https://doi.org/10.1111/j.1365-2672.2012.05232.x

Fiuza, M., Canova, M. J., Zanella-Cleon, I., Becchi, M., Cozzone, A. J., Mateos, L. M., Kremer, L., Gil, J. A., & Molle, V. (2008). From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division. *Journal of Biological Chemistry*, *283*, 18099–18112. https://doi.org/10.1074/jbc.A802615200

Gowda, V., & Shivakumar, S. (2014). Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of *Bacillus thuringiensis* IAM 12077. *Brazilian Archives of Biology and Technology*, *57*, 55–61. https://doi.org/10.1590/S1516-89132014000100009

Han, X., & Bao, J. (2018). General method to correct the fluctuation of acid based pretreatment efficiency of lignocellulose for highly efficient bioconversion. *ACS Sustainable Chemistry and Engineering*, *6*, 4212–4219. https://doi.org/10.1021/acssuschemeng.7b04601

Han, X., Hong, F., Liu, G., & Bao, J. (2018). An approach of utilizing water-soluble carbohydrates in lignocellulose feedstock for promotion of cellulosic l -lactic acid production. *ACS Sustainable Chemistry & Engineering*, *6*, 4212–4219. https://doi.org/10.1021/acs.jafc.8b03592

Han, X., Li, L., & Bao, J. (2019). Microbial extraction of biotin from lignocellulose biomass and its application on glutamic acid production. *Bioresource Technology*, *288*, 121523. https://doi.org/10.1016/j.biortech.2019.121523

He, Y., Zhang, J., & Bao, J. (2014). Dry dilute acid pretreatment by co-currently feeding of corn stover feedstock and dilute acid solution without impregnation. *Bioresource Technology*, *158*, 360–364. https://doi.org/10.1016/j.biortech.2014.02.074

He, Y., Zhang, J., & Bao, J. (2016). Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation. *Biotechnology for Biofuels*, *9*, 19. https://doi.org/10.1186/s13068-016-0438-9

Jin, C., & Bao, J. (2021). Lysine production by dry biorefining of wheat straw and cofermentation of *Corynebacterium glutamicum*. *Journal of Agricultural and Food Chemistry*, *69*, 1900–1906. https://doi.org/10.1021/acs.jafc.0c07902

Jin, C., Huang, Z., & Bao, J. (2020). High-titer glutamic acid production from lignocellulose using an engineered *Corynebacterium glutamicum* with simultaneous co-utilization of xylose and glucose. *ACS Sustainable Chemistry & Engineering*, *8*, 6315–6322. https://doi.org/10.1021/acssuschemeng.9b07839

Jo, S. J., Maeda, M., Ooi, T., & Taguchi, S. (2006). Production system for biodegradable polyester polyhydroxybutyrate by *Corynebacterium glutamicum*. *Journal of Bioscience and Bioengineering*, *102*, 233–236. https://doi.org/10.1263/jbb.102.233

Jönsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, *199*, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Kim, H. S., Oh, Y. H., Jang, Y. -A., Kang, K. H., David, Y., & Yu, J. H. (2016). Recombinant *Ralstonia eutropha* engineered to utilize xylose and its use for the production of poly(3-hydroxybutyrate) from sunflower stalk hydrolysate solution. *Microbial Cell Factories*, *15*, 95. https://doi.org/10.1186/s12934-016-0495-6

Kourilova, X., Novackova, I., Koller, M., & Obruca, S. (2021). Evaluation of mesophilic *Burkholderia sacchari*, thermophilic *Schlegelella thermodepolymerans* and halophilic *Halomonas halophila* for polyhydroxyalkanoates production on model media mimicking lignocellulose hydrolysates. *Bioresource Technology*, *325*, 124704. https://doi.org/10.1016/j.biortech.2021.124704

Lee, D. S., Kim, Y., & Lee, H. S. (2017). The whcD gene of *Corynebacterium glutamicum* plays roles in cell division and envelope formation. *Microbiology*, *163*, 131–143. https://doi.org/10.1099/mic.0.000399

Letek, M., Ordonez, E., Vaquera, J., Margolin, W., Flardh, K., Mateos, L. M., & Gil, J. A. (2008). Diviva is required for polar growth in the mreb-lacking rod-shaped actinomycete *Corynebacterium glutamicum*. *Journal of Bacteriology*, *190*, 3283–3292. https://doi.org/10.1128/JB.01934-07

Liu, Q., Ouyang, S. -p, Kim, J., & Chen, G. Q. (2007). The impact of PHB accumulation on l-glutamate production by recombinant *Corynebacterium glutamicum*. *Journal of Biotechnology*, *132*, 273–279. https://doi.org/10.1016/j.jbiotec.2007.03.014

Matsumoto, K., Yamada, M., Leong, C. R., Jo, S. J., Kuzuyama, T., & Taguchi, S. (2011). A new pathway for poly(3-hydroxybutyrate) production in *Escherichia coli* and *Corynebacterium glutamicum* by functional expression of a new acetoacetyl-coenzyme a synthase. *Bioscience, Biotechnology, and Biochemistry*, *75*, 364–366. https://doi.org/10.1271/bbb.100682

Niu, W., Han, L., Liu, X., Huang, G., Chen, L., Xiao, W., & Yang, Z. (2016). Twenty-two compositional characterization s and theoretical energy potentials of extensively diversified China's crop residues. *Energy*, *100*, 238–250. https://doi.org/10.1016/j.energy.2016.01.093

Pan, W., Nomura, C. T., & Nakas, J. P. (2012). Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology. *Bioresource Technology*, *125*, 275–282. https://doi.org/10.1016/j.biortech.2012.08.107

Prabu, C. S., & Murugesan, A. G. (2010). Effective utilization and management of coir industrial waste for the production of poly-beat-hydroxybutyrate (PHB) using the bacterium azotobacter beijerinickii. *International Journal of Environmental Research*, *4*, 519–524.

Ramos, A., Letek, M., Campelo, A. B., Vaquera, J., Mateos, L. M., & Gil, J. A. (2005). Altered morphology produced by *ftsZ* expression in *Corynebacterium glutamicum* ATCC 13869. *Microbiology*, *151*, 2563–2572. https://doi.org/10.1099/mic.0.28036-0

Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration & Biodegradation*, *126*, 45–56. https://doi.org/10.1016/j.ibiod.2017.10.001

Sawant, S. S., Salunke, B. K., & Kim, B. E. (2015). Degradation of corn stover by fungal cellulase cocktail for production of polyhydroxyalkanoates by moderate halophile *Paracoccus* sp. *Bioresource Technology*, *194*, 245–255. https://doi.org/10.1016/j.biortech.2015.07.019

Shin, K. C., Sim, D. H., Seo, M. J., & Oh, D. K. (2016). Increased production of food-grade d-tagatose from d-galactose by permeabilized and

immobilized cells of *Corynebacterium glutamicum*, a GRAS host, expressing d-galactose isomerase from geobacillus thermodenitrificans. *Journal of Agricultural and Food Chemistry*, *64*, 8146–8153. https://doi.org/10.1021/acs.jafc.6b03588

Sieger, B., Schubert, K., Donovan, C., & Bramkamp, M. (2013). The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*. *Molecular Microbiology*, *90*, 966–982. https://doi.org/10.1111/mmi.12411

Silva, L. F., Taciro, M. K., Ramos, M. E. M., Carter, J. M., Pradella, J. G., & Gomez, J. G. (2004). Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. *Journal of Industrial Microbiology and Biotechnology*, *31*, 245–254. https://doi.org/10.1007/s10295-004-0136-7

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of sugars, byproducts, and degradation products in liquid fraction process samples. NREL/TP-510-42623*. National Renewable Energy Laboratory.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2012). *Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618*. National Renewable Energy Laboratory.

Song, Y., Matsumoto, K., Tanaka, T., Kondo, A., & Taguchi, S. (2013). Single-step production of polyhydroxybutyrate from starch by using α-amylase cell-surface displaying system of *Corynebacterium glutamicum*. *Journal of Bioscience and Bioengineering*, *115*, 12–14. https://doi.org/10.1016/j.jbiosc.2012.08.004

de Souza, L., Manasa, Y., & Shivakumar, S. (2020). Bioconversion of lignocellulosic substrates for the production of polyhydroxyalkanoates. *Biocatalysis and Agricultural Biotechnology*, *28*, 101754. https://doi.org/10.1016/j.bcab.2020.101754

Tang, M., Sun, X., Zhang, S., Wan, J., Li, L., & Ni, H. (2017). Improved catalytic and antifungal activities of *Bacillus thuringiensis* cells with surface display of Chi9602 Delta SP. *Journal of Applied Microbiology*, *122*, 106–118. https://doi.org/10.1111/jam.13333

Tateno, T., Hatada, K., Tanaka, T., Fukuda, H., & Kondo, A. (2009). Development of novel cell surface display in *Corynebacterium glutamicum* using porin. *Applied Genetics and Molecular Biotechnology*, *84*, 733–739. https://doi.org/10.1007/s00253-009-2021-9

Wang, X., Khushk, I., Xiao, Y., Gao, Q., & Bao, J. (2018). Tolerance improvement of *Corynebacterium glutamicum* on lignocellulose derived inhibitors by adaptive evolution. *Applied Microbiology and Biotechnology*, *102*, 377–388. https://doi.org/10.1007/s00253-017-8627-4

Wang, Y., Cao, G., Xu, D., Fan, L., Wu, X., Ni, X., Zhao, S., Zheng, P., Sun, J., & Ma, Y. (2018). A Novel *Corynebacterium glutamicum* L-Glutamate Exporter. *Applied and Environmental Microbiology*, *84*, e02691–17. https://doi.org/10.1128/AEM.02691-17

Wang, Y., Wu, H., Jiang, X., & Chen, G. Q. (2014). Engineering *Escherichia coli* for enhanced production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in larger cellular space. *Metabolic Engineering*, *25*, 183–193. https://doi.org/10.1016/j.ymben.2014.07.010

Wen, J., & Bao, J. (2019). Engineering *Corynebacterium glutamicum* triggers glutamic acid accumulation in biotin-rich corn stover hydrolysate. *Biotechnology for Biofuels*, *12*, 86. https://doi.org/10.1186/s13068-019-1428-5

Wen, J., Xiao, Y., Liu, T., Gao, Q., & Bao, J. (2018). Rich biotin content in lignocellulose biomass plays the key role in determining cellulosic glutamic acid accumulation by *Corynebacterium glutamicum*. *Biotechnology for Biofuels*, *11*, 132. https://doi.org/10.1186/s13068-018-1132-x

Wu, H., Fan, Z., Jiang, X., Chen, J., & Chen, G. Q. (2016). Enhanced production of polyhydroxybutyrate by multiple dividing *E. coli*. *Microbial Cell Factories*, *15*, 128. https://doi.org/10.1186/s12934-016-0531-6

Wyman, C. E., & Dale, B. E. (2015). Producing biofuels via the sugar platform. *Chemical Engineering Progress*, *111*, 45.

Yim, S. S., Choi, J. W., Lee, R. J., Lee, Y. J., Lee, S. H., Kim, S. Y., & Jeong, K. J. (2016). Development of a new platform for secretory production of recombinant proteins in *Corynebacterium glutamicum*. *Biotechnology and Bioengineering*, *113*, 163–172. https://doi.org/10.1002/bit.25692

Yu, J., & Stahl, H. (2008). Microbial utilization and biopolyester synthesis of bagasse hydrolysates. *Bioresource Technology*, *99*, 8042–8048. https://doi.org/10.1016/j.biortech.2008.03.071

Zhang, J., Wang, X., Chu, D., He, Y., & Bao, J. (2011). Dry pretreatment of lignocellulose with extremely low steam and water usage for bioethanol production. *Bioresource Technology*, *102*(6), 4480–4488. https://doi.org/10.1016/j.biortech.2011.01.005

Zhang, J., Zhu, Z., Wang, X., Wang, N., Wang, W., & Bao, J. (2010). Biodetoxification of toxins generated from lignocellulose pretreatment using a newly isolated fungus, *Amorphotheca resinae* ZN1, and the consequent ethanol fermentation. *Biotechnology for Biofuels*, *3*, 26. https://doi.org/10.1186/1754-6834-3-26

Zhang, L., Jia, H., & Xu, D. (2015). Construction of a novel twin-arginine translocation (Tat)-dependant type expression vector for secretory production of heterologous proteins in *Corynebacterium glutamicum*. *Plasmid*, *82*, 50–55. https://doi.org/10.1016/j.plasmid.2015.10.004

Zhang, Y., Sun, W., Wang, H., & Geng, A. (2013). Polyhydroxybutyrate production from oil palm empty fruit bunch using *Bacillus megaterium*. *Bioresource Technology*, *147*, 307–314. https://doi.org/10.1016/j.biortech.2013.08.029

Zhou, P. P., Khushk, I., Gao, Q. Q., & Bao, J. (2019). Tolerance and transcriptional analysis of *Corynebacterium glutamicum* on biotransformation of toxic furaldehyde and benzaldehyde inhibitory compounds. *Journal of Industrial Microbiology and Biotechnology*, *46*, 951–963. https://doi.org/10.1007/s10295-019-02171-9

Zhu, R. L., Wang, S. Y., Li, J., Wang, K., Miao, L., Ma, B., Gong, L. X., & Peng, Y. Z. (2013). Effect of influent C/N ratio on nitrogen removal using PHB as electron donor in a post-denitritation SBR. *Journal of Chemical Technology and Biotechnology*, *88*, 1834–1905. https://doi.org/10.1002/jctb.4047

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Jin, C., Li, J., Huang, Z., Han, X., & Bao, J. (2022). Engineering *Corynebacterium glutamicum* for synthesis of poly(3-hydroxybutyrate) from lignocellulose biomass. *Biotechnology and Bioengineering*, 1–16. https://doi.org/10.1002/bit.28065