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Improvement of glycerol catabolism in *Bacillus licheniformis* for production of poly-γ-glutamic acid

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Abstract *Bacillus licheniformis* WX-02 is a well-studied strain to produce poly-γ-glutamic acid (γ-PGA) with numerous applications. This study is to improve WX-02 strain's capability of assimilating glycerol, a major byproduct of biofuels industries, through metabolic manipulation. Through gene knockout, the GlpK pathway was identified as the sole functional glycerol catabolism pathway, while the DhaK pathway was inactive for this strain under either aerobic or anaerobic conditions. The enhancement of glycerol utilization was attempted by substituting the native *glpFK* promoter with the constitutive promoter (P43), *ytzE* promoter (PytzE), and *bacABC* operon promoter (PbacA), respectively. The glycerol consumptions of the corresponding mutant strains WX02-P43glpFK, WX02-PytzEglpFK, and WX02-PbacAglpFK were 30.9, 26.42, and 18.8% higher than that of the WX-02 strain, respectively. The γ-PGA concentrations produced by the three mutant strains were 33.71, 23.39, and 30.05% higher than that of WX-02 strain, respectively. When biodiesel-derived crude glycerol was used as the carbon source, the mutant WX02-P43glpFK produced 16.63 g L^{-1} of γ-PGA, with a productivity of 0.35 g L^{-1} h^{-1}. Collectively, this study demonstrated that glycerol can be used as an effective substrate for producing γ-PGA by metabolic engineering *B. licheniformis* strains.

Keywords *Bacillus licheniformis* · Crude glycerol · Poly-γ-glutamic acid · Promoter replacement

Yangyang Zhan and Chengjun Zhu have contributed equally to this work.

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Introduction

Poly-γ-glutamic acid (γ-PGA) is a naturally occurring biopolymer, consisting of the D- and L-glutamate monomers, which are connected through amide linkages between α-amino group and γ-carboxylic acid group (Luo et al. 2016; Ogunleye et al. 2015). The production of γ-PGA can be based on chemical synthesis, enzymatic synthesis, and microbial fermentation (Sanda et al. 2001). Among those methods, microbial fermentation is most cost-effective with numerous advantages such as the utilization of inexpensive raw materials, high purity of final product, mild reaction condition, and environment friendly (Luo et al. 2016). Due to its water soluble, biodegradable, non-toxic, and edible characteristics, γ-PGA has been used in various areas, such as thickener in food industry, moisturizer in cosmetics, drug carrier and tissue engineering in the medical area, flocculants and heavy metal absorbent for the wastewater treatment, and fertilizer in agriculture (Bajaj and Singhal 2011; Shih et al. 2002; Wang et al. 2008).

Fermentative production of γ-PGA is commonly achieved through *Bacillus* species such as *Bacillus anthracis*, *Bacillus subtilis*, *B. licheniformis*, *Bacillus megaterium*, *Bacillus*

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pumilis, and *Bacillus amyloliquefaciens* (Bajaj and Singhal 2011). Among those species, *B. licheniformis* is a promising γ-PGA producer as it is a generally regarded as safe (GRAS) strain. The *B. licheniformis* WX-02 strain has been extensively studied as an efficient producer of γ-PGA (Li et al. 2014; Tian et al. 2014; Wang et al. 2016; Wei et al. 2010). In addition to γ-PGA production, *B. licheniformis* can also be used as an expression platform for enzymes, amino acids, antibiotics, biofuels, and other secondary metabolites (Qiu et al. 2016)

Microbial production of γ-PGA commonly uses glucose as the fermentation substrate (Luo et al. 2016). The use of glycerol as a substrate for microbial γ-PGA production can be a promising option, particularly when glycerol can be obtained as a byproduct from the biodiesel industries (Mattam et al. 2013). Biodiesel-derived crude glycerol has been regarded as a low value or even waste material with a disposal challenge (Dobson et al. 2012). As it is not economical to refine the crude glycerol into high purity products, using crude glycerol as a substrate for fermentative γ-PGA production provides an opportunity to utilize this large quantity of byproduct (Dobson et al. 2012). Du et al. (2005) reported that glycerol can affect the biosynthesis of cell membrane phospholipids and their ester-linked fatty acids, which resulted in better membrane permeability for enhanced secretion of γ-PGA. As a matter of fact, glycerol has been used as a co-substrate with glucose for cell growth and γ-PGA production of *B. licheniformis* ATCC 9945a, *B. subtilis* NX-2, and *B. subtilis* CGMCC0833 (Ko and Gross 1998; Wu et al. 2008, 2010).

Several *B. licheniformis* strains have been reported capable of growing and synthesizing γ-PGA when using glycerol as the carbon source (Cromwick et al. 1996; Du et al. 2005; Shih et al. 2002). It is widely known that glycerol transport facilitator (GlpF) facilitates glycerol uptake through the cell membrane in microorganisms such as *Escherichia coli* (Murarka et al. 2008), *Pseudomonas putida* (Nikel et al. 2014), *Enterococcus faecalis* (Bizzini et al. 2010), and *B. subtilis* (Darbon et al. 2002). Two pathways are commonly involved in glycerol catabolism. One is the GlpK pathway (aerobic or respiration pathway) occurred in *E. coli* (Murarka et al. 2008). This pathway initiates with ATP-dependent glycerol kinase (GlpK) converting glycerol to glycerol-3-phosphate (glycerol-3-P), which is oxidized to dihydroxyacetone phosphate (DHAP) (Fig. 1). The other is the DhaK pathway (anaerobic or fermentation pathway) occurred in *Citrobactor freundii* (Yazdani and Gonzalez 2007). This pathway starts with NAD^+-dependent glycerol dehydrogenase (GldA) for catalysis of glycerol to dihydroxyacetone, which is then phosphorylated to DHAP by DHA kinase (DhaK) (Bizzini et al. 2010; Nikel et al. 2014) (Fig. 1). For *B. licheniformis*, however, glycerol catabolism pathway is still unknown. The appealing of using crude glycerol in *B. licheniformis* requires a detailed investigation aboout the glycerol metabolism in this species, which would then provide a guidance of using metabolic engineering approach to enhance the glycerol uptake and γ-PGA production by *B. licheniformis*. The aim of this study is to identify the genes responsible for the glycerol assimilation of *B. licheniformis* WX-02 strain and develop metabolic engineering approaches to improve its γ-PGA production by this strain.

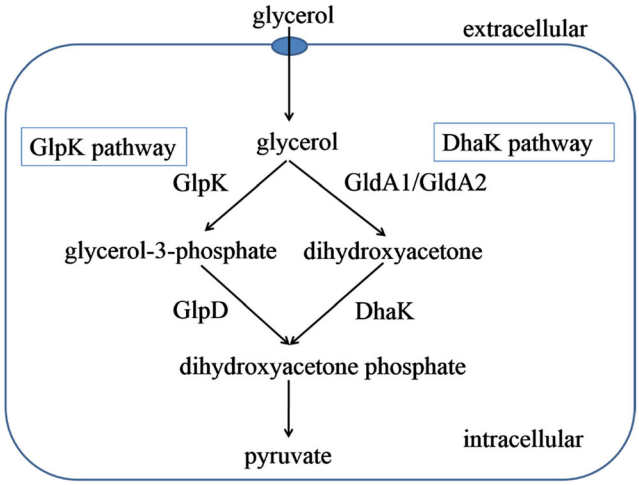

Fig. 1 Two catabolism pathways for glycerol utilization. GlpK, glycerol kinase; GlpD, glycerol-3-phosphate dehydrogenase; GldA1/2, glycerol dehydrogenase; DhaK, dihydroxyacetone kinase

Materials and methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are listed in Table S1. *Escherichia coli* DH5α was used for cloning; *B. licheniformis* WX-02 strain (CCTCC M208065) was used as the initial strain (Wei et al. 2010). *E. coli* DH5α and *B. licheniformis* strains were grown at 37 °C in Luria Bertani (LB) medium. The solid media contained 18 g L^{-1} agar. When necessary, the *E. coli* and *B. licheniformis* cultures were added with 20 mg mL^{-1} kanamycin, 100 mg mL^{-1} ampicillin, and 20 mg mL^{-1} tetracycline.

B. licheniformis seed were grown in 250-mL flasks containing 50-mL LB medium. The flasks were maintained at 37 °C in a rotary shaker with 180 rpm. Cooper's minimal medium was used to growth experiments with minor modifications (Qiu et al. 2014). The medium consists of (per liter) 5 g NH_4NO_3, 60 mmol KH_2PO_4, 80 mmol Na_2HPO_4, 0.2 mmol $MnSO_4{\cdot}H_2O$, 0.8 mmol $MgSO_4{\cdot}7H_2O$, 7 μmol $CaCl_2$, and 4 μmol Na_2-EDTA. The medium was supplemented with 2% (wt/vol) glycerol as the sole carbon source. Experiments under aerobic conditions were performed with 250-mL Erlenmeyer flasks, and the cultures were incubated at 37 °C with vigorous agitation (180 rpm). While the anaerobic culture was performed in the 30-mL glass tubes sealed with rubber stoppers under the static condition (Bizzini et al. 2010).

Both culture systems were inoculated with 2% (*v*/*v*) seed culture and contained 30 mL of Cooper's medium.

The medium for γ-PGA production contained (per liter) 50 g glycerol, 12 g citric acid, 30 g sodium glutamate, 8 g ammonium chloride, 1 g $K_2HPO_4{\cdot}3H_2O$, 1 g $MgSO_4{\cdot}7H_2O$, 1 g $ZnSO_4{\cdot}7H_2O$, 1 g $CaCl_2$, and 0.15 g $MnSO_4{\cdot}H_2O$. The medium was adjusted to pH 7.2 before autoclaved at 115 °C. The medium was then inoculated with 3% (*v*/*v*) seed culture in 250-mL Erlenmeyer flasks (50-mL working volume) and incubated at 37 °C in a rotary shaker with at 180 rpm for 48 h.

Biodiesel-derived crude glycerol was obtained from Hebei Longhai Biofuels Co., Ltd., China. The crude glycerol stream (containing 82% glycerol, *v*/*v*) was diluted with deionized water with a 1:4 ratio (*v*/*v*), and then treated with activated carbon (1.5 g activated carbon per 100 mL diluted solution). The mixture was agitated for 30 min at 60 °C, and centrifuged at 12,000 rpm for 5 min. The supernatant was then filtered through a paper filter to remove any remaining particles. The resulting filtrates were analyzed for glycerol concentration.

Deletion of chromosomal genes and the promoter replacements

The primers used for chromosomal manipulation are listed in Table S2. Deletion of *glpK*, *glpD*, *gldA*1, *gldA*2, and *dhaK* genes, and the replacements of the native promoter of *glpFK* operon with P43, PytzE, or PbacA were performed following our protocols reported previously (Qiu et al. 2014, 2016). The T2(2)-ori vector was used for the gene deletions and promoter replacement (Qiu et al. 2014). The P43, PytzE, and PbacA promoters were amplified from *B. subtilis* 168, *B. licheniformis* WX-02, and *B. licheniformis* DW2 (CCTCC M2011344), respectively. The recombinant fragments were constructed through splicing overlap extension PCR (SOE-PCR). The fusing fragments were transformed into the T2(2)-ori vector, generating recombinant plasmids which were transformed into *B. licheniformis* WX-02 strain via electroporation (Xue et al. 1999). The positive transformant was incubated in LB medium containing kanamycin at 45 °C for 8 h and then streaked onto LB agar containing kanamycin for 8 h incubation to obtain single-crossover recombinants. The selected single-cross clones were grown in LB medium at 37 °C with serial subcultures to promote homologous recombination. The kanamycin sensitive colonies resulting from a double crossover event were verified by PCR using the corresponding primers (Table S2) and the gene-deleted clones and promoter-replaced clones were verified by DNA-sequencing.

Complementary experiments for the WX-02Δ*glpK* and WX-02Δ*glpD* strains

To complement the mutation strain WX-02Δ*glpK* and WX-02Δ*glpD*, *glpK* and *glpD* were expressed in the shuttle vector pHY300PLK, respectively (Qiu et al. 2016). The P43 promoter was amplified from *B. subtilis* 168 genomic DNA, while *glpK*, *glpD*, and the terminator of *amyL* gene were amplified from *B. licheniformis* WX-02 genomic DNA. The P43 promoter, gene fragment, and terminator were fused by SOE-PCR, resulting in the fusing fragments P43-*glpK*-TamyL and P43-*glpD*-TamyL, which were then respectively inserted into *Eco*RI/*Xba*I-cut pHY300PLK to form the plasmid pHY-*glpK* and pHY-*glpD*. The expression vector pHY-*glpK* and pHY-*glpD* were transformed into the corresponding mutants. Positive transformants with tetracycline resistance were verified by PCR and DNA-sequencing.

Transcription analysis

Transcription analysis was performed according to the method reported previously (Wang et al. 2016). The biomass of the strains WX-02, WX02-P43glpFK, WX02-PytzEglpFK and WX02-PbacAglpFK were harvested after being cultured in flasks for 24 h, and subject to RNA extraction with TRIzol® Reagent (Invitrogen, USA). DNase I enzyme (TaKaRa, Japan) was used to digest DNA following the manufacturer's instructions, and RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) was used to amplify the first stand of cDNA. The real-time PCR was performed with SYBR® Select Master Mix (ABI, USA) with the manufacturer's instructions. The primers listed in Table S3 were used for amplifying the genes, and 16S rRNA from *B. licheniformis* WX-02 strain was used as the reference gene to normalize the data. The expression levels for the recombinant strains were compared to the wild-type strain after normalization. All assays were performed in triplicates.

Analytical procedures

For determination of cell growth, the fermentation broth was centrifuged at 12,000 rpm for 10 min, the separated cells were resuspended in distilled water, then were determined by the absorbance at 600 nm with a spectrophotometer (Bio-Rad, USA). Glutamic acid concentration was determined using a SBA-40C bioanalyzer (Academy of Sciences, Shandong, China) (Li et al. 2014). The concentrations of γ-PGA were determined using a gel permeation chromatography (GPC) system according to previous methods (Wang et al. 2016). In brief, a TSK Gel G6000 PWXL gel permeation chromatogram column (7.8 mm × 300 mm, Tosoh, Tokyo, Japan) was used, the samples were eluted with a mixture of 25 mmol L^{-1} sodium sulfate solution: acetonitrile (8:1) at a flow rate of 0.5 mL min^{-1} and detected at 220 nm. The γ-PGA was quantified by the external standards.

To determine glycerol concentration, citric acid concentration, and other metabolites contents, cultures were appropriately diluted with ethanol and the mixture was vortex mixed, then

centrifuged at 12,000 rpm for 10 min to remove cells and γ-PGA. The supernatant was then filtered through a Millipore membrane with the pore size of 0.22 μm. The resulting filtrate containing acetoin and 2,3-butanediol (2,3-BD) was transferred to GC vials for further GC analysis (Agilent, USA). Acetoin and 2,3-BD concentrations were determined by gas chromatography method described previously (Qi et al. 2014). Organic acids (citric acid, acetic acid, and lactic acid) concentrations were determined by a high-performance liquid chromatography (HPLC) method reported previously (Qiu et al. 2016). Glycerol in the filtrate was quantified by a HPLC system (Agilent, USA) with an evaporative light-scattering detector (ELSD, Agilent, USA) with Inertsil NH_2 column (250 mm × 4.6 mm, 5 μm; GL Science Tokyo, Japan). The column was eluted with acetonitrile-water (4:1, *v*/*v*) at 30 °C with 1 mL min^{-1} flow rate. The ELSD were set as the drift tube temperature of 50 °C and gas flow of nebulizer of 1.6 L min^{-1}. The glycerol was determined by the external standards.

Statistical analysis

All the experiments were performed in three replicates. The data were represented as the mean value ± standard deviation. The level of significance of the differences was evaluated by means of ANOVA, with $\alpha = 0.05$.

Results

Determination of glycerol metabolic pathway in *B. licheniformis* WX-02

In silico analysis of the genome sequence of *B. licheniformis* WX-02 (Yangtse et al. 2012) revealed that the putative genes involving in the GlpK pathway were clustered in the *glp* loci and the putative genes encoding enzymes of the DhaK pathway were scattered into three gene structures encoding for putative glycerol dehydrogenase (GldA1 and GldA2), and putative dihydroxyacetone kinase (DhaK), respectively (data not shown). Several mutant strains were constructed to investigate its glycerol catabolism pathways (Table S1). The growth of those mutants under aerobic or anaerobic conditions was tested using glycerol as the sole carbon source.

Under aerobic condition, the *B. licheniformis* WX-02 strain proved to use glycerol as the carbon source (Fig. 2). However, neither Δ*glpK* nor Δ*glpD* mutant could use glycerol as the carbon source (Fig. 2), indicating the essentiality of both *glpK* and *glpD* in the glycerol utilization in *B. licheniformis* WX-02. Figure 2 also shows that the growth of the mutant strains with deletion of either *gldA*1/*gldA*2 or *dhaK* was almost the same as that of the wild-type strain, indicating that the defect of DhaK pathway had little influence on glycerol utilization.

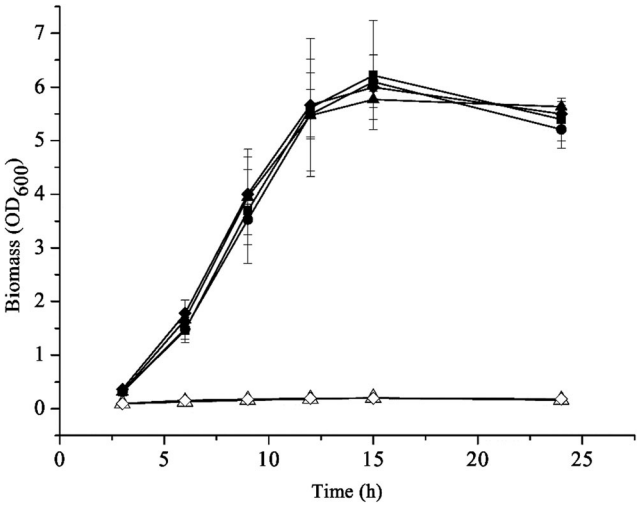

Fig. 2 Growth of the wild-type and the knockout strains of *B. licheniformis* under aerobic condition. Glycerol (20 g L^{-1}) was used as the carbon source. Symbols for different strains: wild-type strain WX-02 (■), mutant Δ*glpK* (△), mutant Δ*glpD* (◇), mutant Δ*gldA*1 (▲), mutant Δ*gldA*2 (◆), and mutant Δ*dhaK* (●). Data are represented as the means of three replicates and bars represent the standard deviations

Under anaerobic condition, *B. licheniformis* requires an electron acceptor, e.g., nitrate. Therefore, *B. licheniformis* WX-02 was able to grow in glycerol-containing medium with the addition of ammonium nitrate (Fig. 3). However, neither the mutants WX-02Δ*glpK* nor WX-02Δ*glpD* could grow on glycerol-containing medium (Fig. 3). The growth of WX-02Δ*gld*A1, WX-02Δ*gld*A2, and WX-02Δ*dhaK* were similar to the wild-type strain (Fig. 3). These results indicated that the GlpK pathway was functional and the DhaK pathway was inactive under the anaerobic condition.

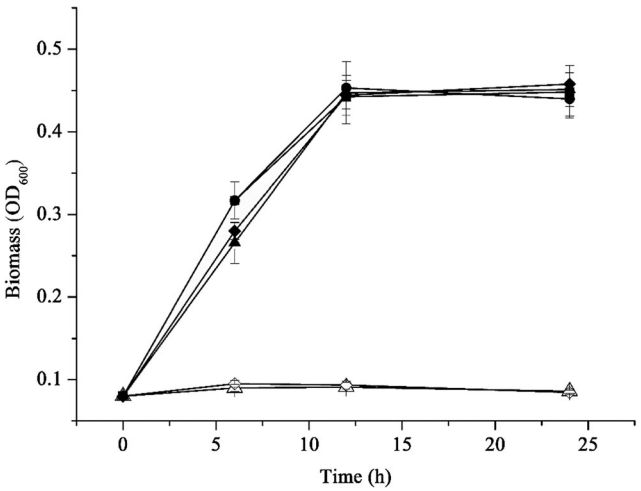

Fig. 3 Growth of the wild-type and the knockout strains of *B. licheniformis* under anaerobic condition. Glycerol (20 g L^{-1}) was used as the carbon source. Symbols for different strains: wild-type strain WX-02 (■), mutant Δ*glpK* (△), mutant Δ*glpD* (◇), mutant Δ*gldA*1 (▲), mutant Δ*gldA*2 (◆), and mutant Δ*dhaK* (●). Data are represented as the means of three replicates and bars represent the standard deviations

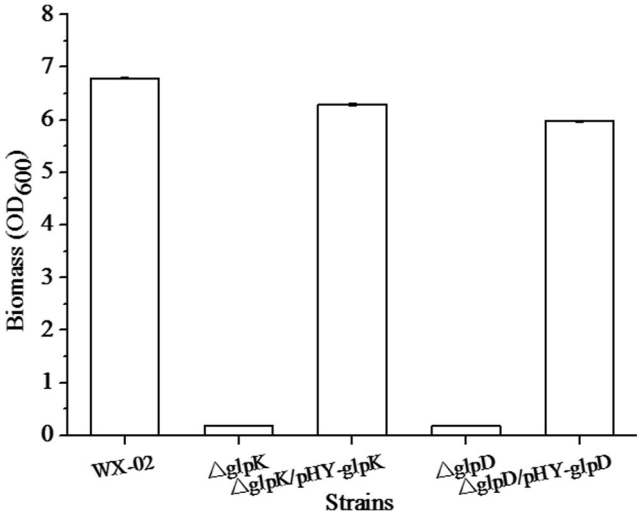

Fig. 4 Cell growth of the control strain (WX-02), knockout strains (WX-02Δ*glpK* and WX-02Δ*glpD*), and their corresponding complementary strains. The cells were grown in minimal medium containing 20 g L^{-1} glycerol for 14 h. Data are represented as the means of three replicates and bars represent the standard deviations

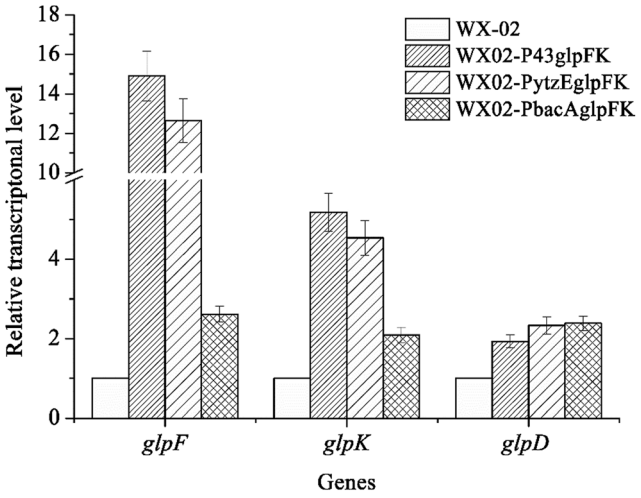

Fig. 5 The transcription levels of selected genes in different *B. licheniformis* strains (WX02-P43glpFK, WX02-PytzEglpFK, WX02-PbacAglpFK, and the wild-type strain WX-02)

Collectively, above results indicated in either aerobic or anaerobic conditions, DhaK pathway was inactive in glycerol metabolism and the GlpK pathway was the major pathway involving in glycerol metabolism in *B. licheniformis*. To further confirm the glycerol metabolism in *B. licheniformis*, the complementary strains of *glpK* (WX-02Δ*glpK*/pHY-*glpK*) and *glpD* (WX-02Δ*glpD*/pHY-*glpD*) were constructed, respectively. The two complementary strains successfully restored their abilities of utilizing glycerol for the growth, with comparable biomass to that of the wild-type (Fig. 4). These results further confirmed that glycerol was metabolized through the GlpK pathway in *B. licheniformis* WX-02.

Enhancement of glycerol consumption and γ-PGA production by replacing *glpFK* promoter

Since *B. licheniformis* WX-02 utilize glycerol through the GlpK pathway, and GlpF is widely known as the glycerol uptake facilitator with GlpK being the rate-limiting enzyme (EC. 1976), it is reasonable to speculate that the enhanced GlpFK activity facilitates glycerol utilization. Therefore, replacing *glpFK* promoter by a stronger promoter (P43, PbacA, or PytzE) was attempted; the resultant mutants were denoted as WX02-P43glpFK, WX02-PbacAglpFK, and WX02-PytzEglpFK, respectively. The glycerol consumption and γ-PGA production were then evaluated for the wild-type strain (WX-02) and the three mutant strains. As shown in Table 1, the glycerol consumption of the mutant WX02-P43glpFK was higher (30.09%) than that of the WX-02 strain. This mutant strain also produced 17.65 g L^{-1} γ-PGA, 33.71% higher than that of the WX-02 strain. The mutants WX02-PytzEglpFK and WX02-PbacAglpFK produced 16.29 and 17.17 g L^{-1} γ-PGA, which were 23.39 and 30.05% higher than that of the wild-type strain, respectively. The glycerol consumption of WX02-PytzEglpFK and WX02-PbacAglpFK were 26.42 and 18.8% higher than that of the wild-type strain (Table 1).

To further explore the effect of replacing *glpFK* promoter on glycerol consumption, the transcript levels of *glpF*, *glpK*, and *glpD* were measured in the wild-type strain and the mutants. As shown in Fig. 5, replacing the *glpFK* promoter with stronger promoters resulted in the upregulation of the

Table 1 Comparison of cell growth, glycerol consumption, and γ-PGA production by different *B. licheniformis* strains

B. licheniformis strains	Biomass (OD_{600})	Glycerol consumption (g L^{-1})	Glycerol consumption rate (g L^{-1} h^{-1})	γ-PGA yield (g L^{-1})	γ-PGA productivity (g L^{-1} h^{-1})
WX-02	10.58 ± 0.54	22.35 ± 1.79	0.47	13.2 ± 0.53	0.28
WX02-P43glpFK	13.51 ± 0.89	29.08 ± 1.98	0.61	17.65 ± 1.8	0.37
WX02-PytzEglpFK	13.17 ± 1.12	28.26 ± 1.06	0.59	16.29 ± 0.69	0.34
WX02-PbacAglpFK	12.80 ± 0.36	26.56 ± 1.27	0.55	17.17 ± 0.31	0.36

Strains were grown in 250-mL flasks containing 50-mL medium and incubated in a rotary shaker with 180 rpm at 37 °C for 48 h. The initial glycerol concentration was 50 g L^{-1}. Data are presented as mean ± SDs of three replicates

transcriptions of the *glpF*, *glpK*, and *glpD*, with the expression levels of the three genes displayed a similar trend to the glycerol consumption. Collectively, the above results show the mutant WX02-P43glpFK is the best strain that leads to the highest glycerol consumption and γ-PGA production.

Substrate consumption, cell growth, and metabolites production of the mutant WX02-P43glpFK

The kinetics of cell growth, glycerol consumption, citric acid consumption, glutamic acid consumption, production of γ-PGA, and the major metabolites (2,3-BD, acetoin, acetic acid, and lactic acid) of the mutant WX02-P43glpFK were evaluated to provide an insight on the effects of strengthening glycerol catabolism pathway in *B. licheniformis* (Fig. 6). The results of the wild-type strain WX-02 was also presented as the comparison baseline. As shown in Fig. 6a, the growth of WX02-P43glpFK strain was similar to the WX-02 strain in the first 32 h, but had a higher biomass afterwards. The mutant also accumulated higher γ-PGA than the WX-02 strain (Fig.6a). The WX02-P43glpFK strain also exhibited higher utilizations of glycerol, citric acid, and glutamic acid than that of the WX-02 strain (Fig. 6b). Acetoin and 2,3-BD as important overflow metabolites in the γ-PGA production were also monitored during the fermentation period. Acetoin was accumulated by both the mutant and the wild-type strain with comparable levels; while, 2,3-BD yield in the WX02-P43glpFK was much higher than that of the WX-02 (Fig. 6c). Figure 6d shows the accumulation of the acetic acid and lactic acid, another two overflow metabolites in the γ-PGA production; the WX02-P43glpFK strain produced less acetic acid and lactic acid than the WX-02 strain (Fig. 6d).

Use of biodiesel-derived glycerol for γ-PGA production by the WX02-P43glpFK strain

Above results demonstrated that the mutant WX02-P43glpFK produced the highest γ-PGA with the glycerol utilization capacity, therefore, biodiesel-derived crude glycerol was further used to grow this strain. As shown in Table 2, when 65 g L^{-1} crude glycerol (the equivalent to 50 g L^{-1} pure glycerol) was added as the carbon source, the WX02-P43glpFK strain produced 16.63 g L^{-1} γ-PGA, corresponding a productivity of 0.35 g L^{-1} h^{-1}, higher than that from the wild-type WX-02

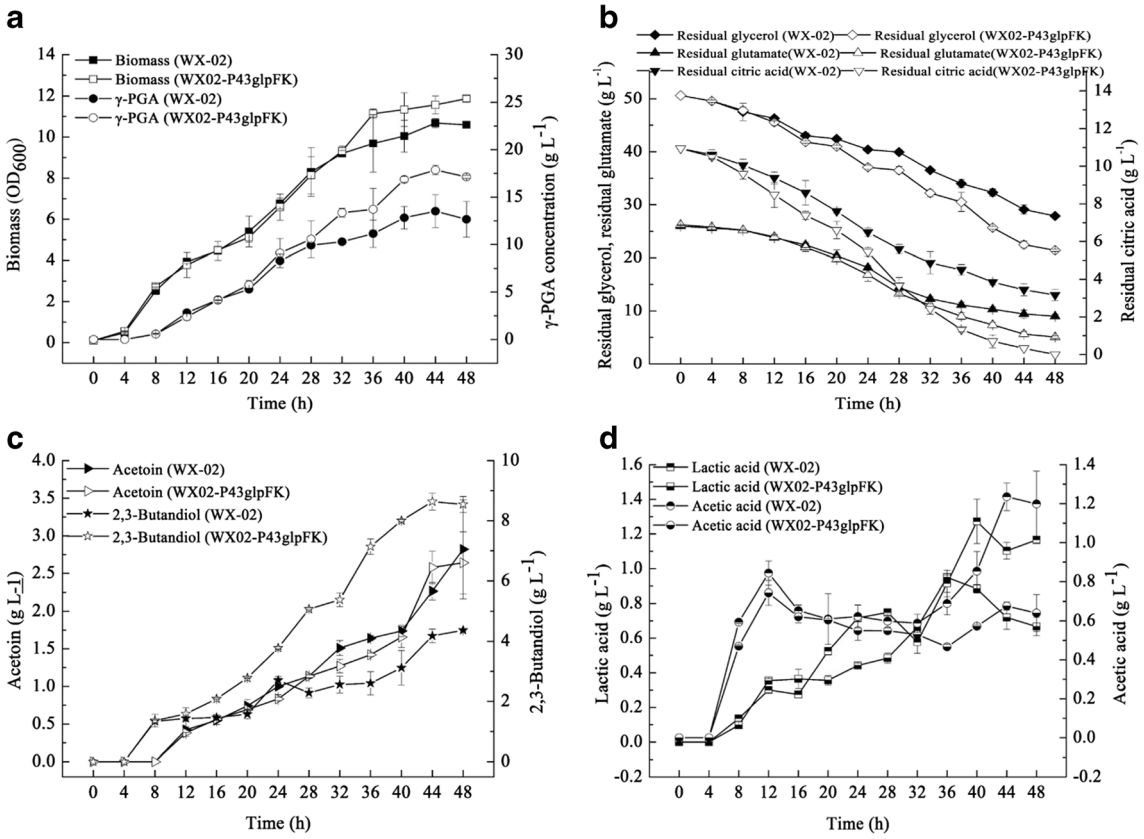

Fig. 6 Fermentation profile of *B. licheniformis* WX-02 and WX02-P43glpFK strain in the flask-shaking experiments for γ-PGA production. **a** Biomass density and γ-PGA yield. **b** Residual glycerol, residual glutamate, and residual citric acid. **c** Acetoin and 2,3-BD concentrations, **e** Lactic acid and acetic acid concentrations. Data are represented as the means of three replicates and bars represent the standard deviations

Table 2 Comparison of γ-PGA production by various *Bacillus* strains using glycerol as a carbon source

Strains	Main nutrients (g L^{-1})	Culture time (h)	γ-PGA yield (g L^{-1})	γ-PGA Productivity (g L^{-1} h^{-1})	Reference
B. licheniformis ATCC 9945a	Glycerol 80, citric acid 12, glutamic acid 20	96	17–23	0.18–0.24	(Cromwick et al. 1996)
B. licheniformis CCRC 12826	Glycerol 157.11, citric acid 24.5, glutamic acid 57.3	96	19.62	0.2	(Shih et al. 2002)
B. subtilis RKY3	Glycerol 17.6, glutamic acid 59.6	24	48.7	2.03	(Jeong et al. 2010)
B. subtilis IFO 3335	Glycerol 20, citric acid 20, glutamic acid 30	96	10–20	0.1–0.21	(Goto and Kunioka 1992)
B. subtilis C1	Glycerol 170, citric acid 22,	144	21.4	0.15	(Shih et al. 2005)
B. licheniformis WBL-3	Glycerol 70, citric acid 10, glutamic acid 20	96	19.3	0.2	(Du et al. 2005)
B. subtilis BL53	Glycerol 80, citric acid 12, glutamic acid 20	72	10.4	0.14	(da Silva et al. 2009)
B. licheniformis WX-02	Glycerol 50, citric acid 12, sodium glutamate 30	48	13.2	0.28	This study
B. licheniformis WX02-P43glpFK	Glycerol 50, citric acid 12, sodium glutamate 30	48	17.65	0.37	This study
B. licheniformis WX02-P43glpFK	Crude glycerol 65, citric acid 12, sodium glutamate 30	48	16.63	0.35	This study

strain (0.28 g L^{-1} h^{-1}). These results clearly show the potential of using biodiesel-derived glycerol for high-level production of γ-PGA with metabolic manipulation of *B. licheniformis*.

Discussion

Glycerol catabolism pathways have been researched in many organisms (Bizzini et al. 2010; Holmberg et al. 1990; Murarka et al. 2008; Nikel et al. 2014; Oren 2017). The glycerol catabolism pathways in *B. licheniformis* has been unknown. In this study, it was revealed that orthologues of GlpK and GlpD belong to the GlpK pathway in the *B. subtilis* genome. The genes encoding putative glycerol dehydrogenase (the *gldA*1 and *gldA*2) are present in two loci of *B. licheniformis* WX-02 chromosome. The gene *dhaK* encoding the dihydroxyacetone kinase has been identified in *Citrobacter* (ATP dependent) (Daniel et al. 1995) and *E.coli* (PEP dependent) (Gutknecht et al. 2001). This work identified an ATP-dependent putative dihydroxyacetone kinase encoding gene (*dhaK*) in *B. licheniformis* WX-02 strain.

Two distinct glycerol catabolism pathways exist in *E. faecalis* MMH594 and *Pseudomonas putida* KT2440 (Bizzini et al. 2010; Nikel et al. 2014). In this work, in silico analysis of the genome sequence of *B. licheniformis* WX-02 strain revealed the genes encoding enzymes in the GlpK pathway and the DhaK pathway exist in this strain. Previous studies indicated that glycerol might be metabolized through the GlpK pathway under the aerobic growth condition or through the DhaK pathway under the anaerobic condition (Bizzini et al. 2010; Jacobs and VanDemark 1960). In this work, it shows that glycerol catabolism of *B. licheniformis* was different from the previous reports (Jacobs and VanDemark 1960). The Δ*glpK* or Δ*glpD* mutants failed to grow on glycerol under either aerobic or anaerobic condition (Figs. 2 and 3). The results indicated that GlpK pathway is essential for glycerol catabolism in WX-02 in either aerobic or anaerobic condition. Furthermore, the complementary strains *glpK* and *glpD* restored glycerol catabolism, with comparable growth to that of the wild-type strain. These complementation results further confirmed that the involvement of GlpK pathway in *B. licheniformis* for its glycerol catabolism. The growth of WX-02Δ*gld*A1, WX-02Δ*gld*A2, and WX-02Δ*dhaK* mutants were similar to that of the wild-type strain, indicating little effect of DhaK pathway on the growth of WX-02 strain. The GldA1 from WX-02 strain has been investigated for its catalytic activities on various substrates (Qiu et al. 2016). It exhibited the high activity on D-2,3-BD and low activity on glycerol (Qiu et al. 2016). Thus, the DhaK pathway of *B. licheniformis* WX-02 was regarded as an inactive or a silent glycerol catabolism pathway. Alternatively, the enzymes in the DhaK pathway may have other functions such as catalysis of D-AC to D-2,3-BD by GldA1 (Qiu et al. 2016). All these results indicated that *B. licheniformis* WX-02 uses GlpK pathway to use glycerol.

Compared to other carbon sources such as glucose, glycerol has more reducing equivalents, and thus is capable of producing high-yield fuels and chemicals such as γ-PGA production (Chen and Liu 2016). However, the glycerol consumption rate in *B. licheniformis* was generally lower than that of glucose, leading to the reduced γ-PGA production (Yao et al. 2015). Since *glpFK* forms an operon, and *glpK* is the

rate-limiting step in GlpK pathway (Clomburg and Gonzalez 2013), overexpression of *glpF* and *glpK* in *B. licheniformis* may increase glycerol flux. Use of stronger promoters has been reported to generate higher transcription for the lichenysin production in *B. licheniformis* (Qiu et al. 2014). In this study, the replacement of the native promoter of *glpFK* with stronger promoters (P43, PbacA and PytzE) also promotes the glycerol consumption and γ-PGA production (Table 1). Among these three promoters, P43 is most effective for glycerol catabolism (Table 1 and Fig. 5). The mutant WX02-P43glpFK strain exhibited the highest glycerol consumption rate.

Our study shows that overexpression of the *glpFK* operon genes affected the whole cellular metabolism (Fig. 6). Compared to the wild-type strain, the mutant WX02-P43glpFK increased its flux through the 2, 3-BD biosynthesis pathways, resulting in lower accumulation of acetic acid and lactic acid. The elevated glycerol consumption led to an NADH surplus and a high intracellular NADH/NAD^+ ratio; the 2,3-BD production was mainly attributed to this excess reducing equivalents (Wang et al. 2017). Previous research reported that biosynthesis of acetoin and 2,3-BD helped to maintain intracellular redox and pH homeostasis (Tsau et al. 1992). The mutant WX02-P43glpFK had a higher utilization rate of citric acid, an important intermediate in tricarboxylic acid cycle (TCA cycle) providing cellular energy and carbon skeleton (Ruffing and Chen 2011) and regulating the metabolic fluxes in glycolysis, pentose phosphate pathway, and TCA cycle (Chen et al. 2010). The transcriptional profiles of key genes were measured by qRT-PCR (Fig. S1). The expression levels of *icd*, *citZ*, and *citB* genes involved in the TCA cycle were upregulated by 1.12-fold, 1.29-fold, and 1.6-fold, respectively, while the transcription levels of the regulator genes *ccpC* and *codY* were downregulated (Fig. S1). The regulator CcpC can repress *citZ* and *citB* genes, CodY also can repress *citB* gene, and active the acetate and lactate biosynthesis pathways (Sonenshein 2007). All these results indicate that the relative flux distribution through the TCA cycle was increased, resulting in more energy, NADH and glutamate, and affecting the cellular metabolism. The metabolic flux of the mutant WX02-P43glpFK should be analyzed to elucidate the glycerol metabolism.

For *B. licheniformis* WX-02, γ-PGA can be produced without L-glutamate, although external addition of glutamate could increase γ-PGA production. In this paper, the utilization glutamic acid increased (Fig. 6b) and the expression of *gltT* encoding glutamate transport protein of the mutant WX02-P43glpFK was 1.99 times higher than the WX-02 (Fig. S1). The upregulation of *gltT* gene supported the higher glutamate consumption rate of the WX02-P43glpFK. The ^{13}C labeling metabolic flux analysis may be used to elucidate the metabolic flux of γ-PGA in the future study (Yao et al. 2010).

Biodiesel-derived glycerol has been considered as a waste in biofuel industry (Mattam et al. 2013). Microbial conversion of glycerol into value-added products can be an ideal option to utilize this waste material (Yang et al. 2012). The use of glycerol for microbial production of γ-PGA not only reduces the cost of γ-PGA production, but also improves the economic viability of the biodiesel industry. This study elucidated the GlpK pathway as the sole functional glycerol catabolism pathway in *B. licheniformis*. Through the promoter replacement, the mutant WX02-P43glpFK was successfully constructed as the ideal strain for enhancing glycerol utilization and γ-PGA production, with 16.63 g L^{-1} γ-PGA titer and 0.35 g L^{-1} h^{-1} γ-PGA productivity when using biodiesel-derived crude glycerol as the carbon source (Table 2). The establishment of this mutant can be a substantial progress towards industrial γ-PGA production.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

Bajaj I, Singhal R (2011) Poly (glutamic acid)—an emerging biopolymer of commercial interest. Bioresour Technol 102:5551–5561. doi:10.1016/j.biortech.2011.02.047

Bizzini A, Zhao C, Budin-Verneuil A, Sauvageot N, Giard J, Auffray Y, Hartke A (2010) Glycerol is metabolized in a complex and strain-dependent manner in *Enterococcus faecalis*. J Bacteriol 192:779–785. doi:10.1128/JB.00959-09

Chen Y, Li S, Xiong J, Li Z, Bai J, Zhang L, Ye Q, Ouyang P, Ying H (2010) The mechanisms of citrate on regulating the distribution of carbon flux in the biosynthesis of uridine 5′-monophosphate by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 86:75–81. doi:10.1007/s00253-009-2287-y

Chen Z, Liu D, (2016) Toward glycerol biorefinery: metabolic engineering for the production of biofuels and chemicals from glycerol. Biotechnol Biofuels 9:205

Clomburg JM, Gonzalez R (2013) Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. Trends Biotechnol 31:20–28. doi:10.1016/j.tibtech.2012.10.006

Cromwick AM, Birrer GA, Gross RA (1996) Effects of pH and aeration on gamma-poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. Biotechnol Bioeng 50:222–

227. doi:10.1002/(SICI)1097-0290(19960420)50:2<222::AID-BIT10>3.0.CO;2-P

da Silva GP, Mack M, Contiero J (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27:30–39. doi:10.1016/j.biotechadv.2008.07.006

Daniel R, Stuertz K, Gottschalk G (1995) Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii*. J Bacteriol 177:4392–4401. doi:10.1128/jb.177.15.4392-4401.1995

Darbon E, Servant P, Poncet S, Deutscher J (2002) Antitermination by GlpP, catabolite repression via CcpA and inducer exclusion triggered by P-GlpK dephosphorylation control *Bacillus subtilis glpFK* expression. Mol Microbiol 43:1039–1052. doi:10.1046/j.1365-2958.2002.02800.x

Dobson R, Gray V, Rumbold K (2012) Microbial utilization of crude glycerol for the production of value-added products. J Ind Microbiol Biotechnol 39:217–226. doi:10.1007/s10295-011-1038-0

Du G, Yang G, Qu Y, Chen J, Lun S (2005) Effects of glycerol on the production of poly(γ-glutamic acid) by *Bacillus licheniformis*. Process Biochem 40:2143–2147. doi:10.1016/j.procbio.2004.08.005

EC L (1976) Glycerol dissimilation and its regulation in bacteria. Annu Rev Microbiol 30:535–578. doi:10.1146/annurev.mi.30.100176.002535

Goto A, Kunioka M (1992) Biosynthesis and hydrolysis of poly(gamma-glutamic acid) from *Bacillus subtilis* IF03335. Biosci Biotechnol Biochem 56:1031–1035. doi:10.1271/bbb.56.1031

Gutknecht R, Beutler R, Garcia-Alle LF, Baumann U, Erni B (2001) The dihydroxyacetone kinase of *Escherichia coli* utilizes a phosphoprotein instead of ATP as phosphoryl donor. The EMBO J 20:2480–2486. doi:10.1093/emboj/20.10.2480

Holmberg C, Beijer L, Rutberg B, Rutberg L (1990) Glycerol catabolism in *Bacillus subtilis*: nucleotide sequence of the genes encoding glycerol kinase (*glpK*) and glycerol-3-phosphate dehydrogenase (*glpD*). J Gen Microbiol 136:2367–2375. doi:10.1099/00221287-136-12-2367

Jacobs NJ, VanDemark PJ (1960) Comparison of the mechanism of glycerol oxidation in aerobically and anaerobically grown *Streptococcus faecalis*. J Bacteriol 79:532–538

Jeong JH, Kim JN, Wee YJ, Ryu HW (2010) The statistically optimized production of poly(gamma-glutamic acid) by batch fermentation of a newly isolated *Bacillus subtilis* RKY3. Bioresour Technol 101:4533–45339. doi:10.1016/j.biortech.2010.01.080

Ko YH, Gross RA (1998) Effects of glucose and glycerol on gamma-poly(glutamic acid) formation by *Bacillus licheniformis* ATCC 9945a. Biotechnol Bioeng 57:430–437. doi:10.1002/(SICI)1097-0290(19980220)57:4<430::AID-BIT6>3.0.CO;2-N

Li X, Gou X, Long D, Ji Z, Hu L, Xu D, Liu J, Chen S (2014) Physiological and metabolic analysis of nitrate reduction on poly-gamma-glutamic acid synthesis in *Bacillus licheniformis* WX-02. Arch Microbiol 196:791–799. doi:10.1007/s00203-014-1014-y

Luo Z, Guo Y, Liu J, Qiu H, Zhao M, Zou W, Li S (2016) Microbial synthesis of poly-gamma-glutamic acid: current progress, challenges, and future perspectives. Biotechnol Biofuels 9:134. doi:10.1186/s13068-016-0537-7

Mattam AJ, Clomburg JM, Gonzalez R, Yazdani SS (2013) Fermentation of glycerol and production of valuable chemical and biofuel molecules. Biotechnol Lett 35:831–842. doi:10.1007/s10529-013-1240-4

Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R (2008) Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 74:1124–1135. doi:10.1128/AEM.02192-07

Nikel PI, Kim J, de Lorenzo V (2014) Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440. Environ Microbiol 16:239–254. doi:10.1111/1462-2920.12224

Ogunleye A, Bhat A, Irorere VU, Hill D, Williams C, Radecka I (2015) Poly-gamma-glutamic acid: production, properties and applications. Microbiology 161:1–17. doi:10.1099/mic.0.081448-0

Oren A (2017) Glycerol metabolism in hypersaline environments. Environ Microbiol 19:851–863. doi:10.1111/1462-2920.13493

Qi G, Kang Y, Li L, Xiao A, Zhang S, Wen Z, Xu D, Chen S (2014) Deletion of meso-2,3-butanediol dehydrogenase gene *budC* for enhanced D-2,3-butanediol production in *Bacillus licheniformis*. Biotechnol Biofuels 7:16. doi:10.1186/1754-6834-7-16

Qiu Y, Xiao F, Wei X, Wen Z, Chen S (2014) Improvement of lichenysin production in *Bacillus licheniformis* by replacement of native promoter of lichenysin biosynthesis operon and medium optimization. Appl Microbiol Biotechnol 98:8895–8903. doi:10.1007/s00253-014-5978-y

Qiu Y, Zhang J, Li L, Wen Z, Nomura CT, Wu S, Chen S (2016) Engineering *Bacillus licheniformis* for the production of meso-2,3-butanediol. Biotechnol Biofuels 9:117. doi:10.1186/s13068-016-0522-1

Ruffing AM, Chen RR (2011) Citrate stimulates oligosaccharide synthesis in metabolically engineered *Agrobacterium* sp. Appl Biochem Biotechnol 164:851–866. doi:10.1007/s12010-011-9179-1

Sanda F, Fujiyama T, Endo T (2001) Chemical synthesis of poly-γ-glutamic acid by polycondensation of γ-glutamic acid dimer: synthesis and reaction of poly-γ-glutamic acid methyl ester. J Polym Sci Pol Chem 39:732–741. doi:10.1002/1099-0518(20010301)39:5<732::AID-POLA1045>3.0.CO;2-P

Shih IL, Van YT, Chang YN (2002) Application of statistical experimental methods to optimize production of poly(γ-glutamic acid) by *Bacillus licheniformis* CCRC 12826. Enzym Microb Technol 31:213–220. doi:10.1016/S0141-0229(02)00103-5

Shih IL, Wu PJ, Shieh CJ (2005) Microbial production of a poly(γ-glutamic acid) derivative by *Bacillus subtilis*. Process Biochem 40:2827–2832. doi:10.1016/j.procbio.2004.12.009

Sonenshein AL (2007) Control of key metabolic intersections in *Bacillus subtilis*. Nat Rev Microbiol 5:917–927. doi:10.1038/nrmicro1772

Tian G, Fu J, Wei X, Ji Z, Ma X, Qi G, Chen S (2014) Enhanced expression of *pgdS* gene for high production of poly-γ-glutamic aicd with lower molecular weight in *Bacillus licheniformis* WX-02. J Chem Technol Biotech 89:1825–1832. doi:10.1002/jctb.4261

Tsau JL, Guffanti AA, Montville TJ (1992) Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. Appl Environ Microbiol 5:891–894

Wang Q, Chen S, Zhang J, Sun M, Liu Z, Yu Z (2008) Co-producing lipopeptides and poly-gamma-glutamic acid by solid-state fermentation of *Bacillus subtilis* using soybean and sweet potato residues and its biocontrol and fertilizer synergistic effects. Bioresour Technol 99:3318–3323. doi:10.1016/j.biortech.2007.05.052

Wang J, Yuan H, Wei X, Chen J, Chen S (2016) Enhancement of poly-γ-glutamic acid production by alkaline pH stress treatment in *Bacillus licheniformis* WX-02. J Chem Technol Biotech 91:2399–2403. doi:10.1002/jctb.4822

Wang Y, Tao F, Xin B, Liu H, Gao Y, Zhou NY, Xu P (2017) Switch of metabolic status: redirecting metabolic flux for acetoin production from glycerol by activating a silent glycerol catabolism pathway. Metab Eng 39:90–101. doi:10.1016/j.ymben.2016.10.020

Wei X, Ji Z, Chen S (2010) Isolation of halotolerant *Bacillus licheniformis* WX-02 and regulatory effects of sodium chloride on yield and molecular sizes of poly-gamma-glutamic acid. Appl Biochem Biotechnol 160:1332–1340. doi:10.1007/s12010-009-8681-1

Wu Q, Xu H, Shi N, Yao J, Li S, Ouyang P (2008) Improvement of poly(gamma-glutamic acid) biosynthesis and redistribution of metabolic flux with the presence of different additives in *Bacillus*

subtilis CGMCC 0833. Appl Biochem Biotechnol 79:527–535. doi:10.1007/s00253-008-1462-x

Wu Q, Xu H, Liang J, Yao J (2010) Contribution of glycerol on production of poly(gamma-glutamic acid) in *Bacillus subtilis* NX-2. Appl Biochem Biotechnol 160:386–392. doi:10.1007/s12010-008-8320-2

Xue GP, Johnson JS, Dalrymple BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Meth 34: 183–191

Yang F, Hanna M, Sun R (2012) Value-added uses for crude glycerol—a byproduct of biodiesel production. Biotechnol Biofuels 5:13. doi: 10.1186/1754-6834-5-13

Yangtse W, Zhou Y, Lei Y, Qiu Y, Wei X, Ji Z, Qi G, Yong Y, Chen L, Chen S (2012) Genome sequence of *Bacillus licheniformis* WX-02. J Bacteriol 194:3561–3562. doi:10.1128/JB.00572-12

Yao J, Xu H, Shi N, Cao X, Feng X, Li S, Ouyang P (2010) Analysis of carbon metabolism and improvement of gamma-polyglutamic acid production from *Bacillus subtilis* NX-2. Appl Biochem Biotechnol 160:2332–2341. doi:10.1007/s12010-009-8798-2

Yao R, Liu Q, Hu H, Wood TK, Zhang X (2015) Metabolic engineering of *Escherichia coli* to enhance acetol production from glycerol. Appl Microbiol Biotechnol 99:7945–7952. doi:10.1007/s00253-015-6732-9

Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219. doi:10.1016/j.copbio.2007.05.002