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The main byproducts and metabolic flux profiling of γ-PGA-producing strain *B. subtilis* ZJU-7 under different pH values

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is an extracellular anionic polymer with many potential applications. Although microbial fermentation is the common approach to produce γ-PGA, the broth at the latter stage usually becomes very viscous and severely influences the metabolites producing pattern of target microbe. In this study, acetoin and 2,3-butanediol were confirmed to be the main byproducts of γ-PGA-producing strain *B. subtilis* ZJU-7 (*B. subtilis* CGMCC1250), and their effects on the cell growth and γ-PGA biosynthesis were further investigated in shake flasks. The outcome indicated that both acetoin and 2,3-butanediol showed clear impairment on γ-PGA production of *B. subtilis* ZJU-7. Moreover, the extracellular metabolites profiles of fermentation under three different pH values were acquired and the metabolic flux redistribution of pathways related to γ-PGA biosynthesis was calculated based on the collected data. As a result, the metabolic flux favored to distribute toward glycolytic pathway at pH 6.5, in which the ingestion rate of extracellular glutamic acid was higher and the subsequent γ-PGA biosynthesis was enhanced. The present work provided us a deep insight into the metabolic flux control of γ-PGA biosynthesis, which will stimulate some novel metabolic engineering strategies to improve the productivity of γ-PGA in future.

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1. Introduction

Poly (γ-glutamic acid) (γ-PGA) is composed of D- and L-glutamic acid units via amide linkages between α-amino and γ-carboxylic acid groups. Several members of *Bacilli* species are able to synthesize γ-PGA in vivo, such as *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* (Birrer et al., 1994; Goto and Kunioka, 1992; Liu et al., 2011). Possessing several beneficial characteristics such as water soluble, anionic, non-toxic, biocompatible, biodegradable and edible, γ-PGA has presented great potential in its industrial applications (Bajaj and Singhal, 2011). For fermentative production of γ-PGA, *B. licheniformis* and *B. subtilis* have been normally chosen because of their easy cultivation and general biosafety (GRAS). Intensive studies of these strains regarding enzymatic synthesis regulations, nutrient requirements, and cultivation conditions were carried out to improve cell growth, γ-PGA production and desirable ratio in chain D/L-repeat unit composition (Ashiuchi et al., 2004; Shih and Van, 2001). The metabolic pathways related to the γ-PGA biosynthesis were also studied (Wu et al., 2008). According to the origin of basal unit glutamate, γ-PGA producing strains are divided into two groups: one needs the addition of external L-glutamate and the other can self-synthesize γ-PGA without supply of L-glutamate (Kunioka, 1997; Zhang et al., 2012).

The fermentation of γ-PGA biosynthesis is generally an aerobic process, but the high viscosity of broth at the latter stage of fermentation seriously hampers the transfer of dissolved oxygen and thus forms the anaerobic-like environment affecting the metabolites producing pattern of microbes. It was reported that *B. subtilis* could grow under the anaerobic fermentation condition by respiration with nitrate as the terminal electron acceptor

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; T3P, triose-3-phosphate pool; Xyl5P, xylulose-5-phosphate; Rib5p, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; PG3, 3-phosphoglycerate; C1, methyl group bound to tetrahydrofolate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; αKG, α-ketoglutaric acid; FUM, fumarate; MAL, maleic acid; Glu, intracellular glutamic acid; exGlu, extracellular glutamic acid; PGAex, extracellular poly γ-glutamic acid; 3H2B, intracellular acetoin; 3H2Bex, extracellular acetoin; 23BDOex, extracellular 2,3-butanediol.

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(Hartig et al., 2006). The nuclear magnetic resonance scan analysis of in vivo metabolites indicated that *B. subtilis* had performed mixed acid-butanediol fermentation without oxygen, and hardly with any formate detected (Earl et al., 2008). Further metabolome analysis implied that pyruvate was probably not metabolized by pyruvate formate lyase (PFL) to generate acetyl coenzyme A (CoA) and formate, but oxidatively decarboxylated by pyruvate dehydrogenase (PDH) to produce butanediol. The fermentation metabolites produced by wild-type *B. subtilis* include acetate, ethanol, lactate, 2,3-butanediol (2,3-BDO), and a small portion of acetoin (Nakano et al., 1998; Ramos et al., 2000). In addition, acetate and 2,3-BDO were the major byproduct identified during the γ-PGA production by *B. licheniformis* 9455a (Birrer et al., 1994).

Over the last decade, genome scale metabolic models have been widely established and are available for more than 50 organisms. Among them, metabolic flux balancing is a valuable tool to gain insight into the complex responses and capabilities of whole cellular metabolism (Dauner et al., 2001). The model of center carbon metabolism pathways in *B. subtilis* CGMCC0833 using glucose and glutamate has been proposed (Wu et al., 2008). However, the effects of such byproducts on the fermentation process have not been investigated yet. In our previous work, *B. subtilis* strain ZJU-7 (*B. subtilis* CGMCC1250) showed great capacity to produce γ-PGA as high as 80 g/l, with yeast extract as an alternative nitrogen source to reduce the cost of γ-PGA production (Chen et al., 2010). In the present work, we focused on the measurement of main metabolic byproducts in *B. subtilis* ZJU-7 and tried to evaluate the effects of metabolic byproducts and pH control strategies on cell growth and PGA production. Especially, the metabolic flux redistribution under different pH values was adopted to explore the strategy to improve γ-PGA productivity.

2. Material and methods

2.1. Cultivation of B. subtilis ZJU-7 in shake flask

B. subtilis ZJU-7 was isolated from fermented bean curd and reserved in our lab (Shi et al., 2006a). It was deposited in CGMCC (China General Microbiological Culture Collection Center) and named as *B. subtilis* CGMCC1250. The strain was cultured in 250 ml flask containing 20 ml seed medium (per liter: 10 g tryptone, 5 g beef extract, and 5 g NaCl) and incubated at 37 °C and 200 rpm for overnight. Three milliliters of such seed culture was then inoculated into 30 ml production medium (initial pH 6.5) that comprised of 80 g/l L-glutamic acid, 10 g/l NaCl, 1 g/l $CaCl_2$, 1 g/l $MgSO_4$, 80 g/l glucose, and 40 g/l yeast extract. *B. subtilis* ZJU-7 was cultivated at 37 °C and 200 rpm for 48 hours to produce γ-PGA (Shi et al., 2006b).

To investigate the effects of main byproducts on cell growth of *B. subtilis* ZJU-7 and γ-PGA production, different concentrations of acetoin (0, 2, 5, 15 g/l, respectively) and 2,3-BDO (0, 10, 20, 30 g/l, respectively) were added into the medium at the beginning of the cultivation and the beginning of stationary phase, respectively. After 6 hours growth, samples were taken every 4 hours until the exhaustion of carbon source.

2.2. Fed-batch fermentation of B. subtilis ZJU-7

For fed-batch fermentation in the fermentors, *B. subtilis* ZJU-7 was first cultured in 250 ml flask containing 30 ml seed medium and incubated at 37 °C and 200 rpm for overnight. Such culture was then inoculated into 500 ml production medium and grew at 37 °C and 200 rpm for 36 h to obtain the mature seed broth. The resulted 500 ml seed broth was transferred into a 10-l fermentor (Shanghai Baoxing Bioengineering Equipment Co., Shanghai, China) containing 5-l production medium (initial concentrations of glucose was 20 g/l, L-glutamate was 40 g/l) for fermentation. The temperature was kept at 37 °C. The pH was automatically controlled at a certain value (5.7, 6.5 or 7.3) with the addition of 25% (v/v) NH_4OH or 1 M HCl. The aeration rate was set at 1 vvm, with agitation rate ranging from 350 to 650 rpm to maintain enough dissolved oxygen. The pulse feeding of 750 g/l glucose solution using a peristaltic pump at the rate of $2\,g\,l^{-1}\,h^{-1}$ was started when the concentration of glucose in the broth was lower than 5 g/l. The pulse interval was manually adjusted to maintain the concentration of glucose in the range of 3–8 g/l. The fermentation process was lasted for about 48–72 h (Huang et al., 2011).

2.3. Analysis of fermentation samples

The concentrations of CO_2 and O_2 in the exhaust gas were measured online using gas analyzer (MultiRAE IR Gas Monitor, RAE Systems). The DO and pH values of broth were measured online using related electrodes (Mettler Toledo).

The cell density of broth was measured at 600 nm by a 752 s UV–Vis spectrophotometer. For determination of cell dry weight, 10 ml culture was centrifuged at 3030g and 4 °C for 20 min using Eppendorf 5810 R Centrifuge. The resulted cell pellets were washed three times with 0.15 M NaCl and resuspended in 20 ml of 0.15 M NaCl. Such mixture was dried at 55 °C to a constant weight; 20 ml of 0.15 M NaCl was also dried to a constant weight as the control to get the final cell dry weight.

For GC analysis, the cell pellet from centrifugation was washed once with 20 mM Tris–HCl (pH 7.6) and resuspended in 5 ml dd H_2O, 4-fold volume of methanol was added to precipitate γ-PGA and other polysaccharides. After centrifugation, the collected supernatant was subjected to GC analysis. An Agilent 6850 GC equipped with split/splittless injector was applied. Helium with a constant flow rate of 1 ml/min was used as carrier gas. The splitless injection was set at 250 °C, and the total run time was 6.5 min. The initial temperature of column oven was set at 60 °C and maintained for 2 min, and then the temperature was raised to 300 °C with the rate of 75 °C/min. The final ramping at 300 °C was kept for 2 min. The authentic samples of ethanol, acetate, lactate, propionate, acetoin and 2,3-BDO were also analyzed by GC to provide standard references for metabolites characterization.

The concentrations of glucose and glutamate were measured enzymatically using a bioanalyzer (SBA-40 C, Shandong Academy of Sciences). The yield of γ-PGA was measured offline by gel permeation chromatography (GPC) system following the previously reported method (Wu et al., 2008) using an Agilent 1100 HPLC system equipped with TSK G4000PXWL column (30 cm × 7.8 mm × 10 μm), RID detector and 0.01 M $NaNO_3$ as the mobile phase. Every experiment was repeated at least three times, and the experimental errors were less than 4%.

2.4. Metabolic flux profiling

The previously described biochemical reaction network of *B. subtilis* (Wu et al., 2008) was augmented with the proposed pathway and shunts involved in the biosynthesis of acetoin and 2,3-BDO (shown in Appendix A). The model constituted reactions from the intermediary metabolisms (including glycolysis, pentose–phosphate pathway, tricarboxylic acid (TCA) cycle), cell mass formation, and biosynthesis of main by-products (acetoin and 2,3-BDO) detected in our experiments. From this network, a stoichiometric matrix containing 30 unknown fluxes and 30 metabolite balances (including 8 balances of the measured substrates and products: biomass, glucose, L-glutamate, γ-PGA, acetoin, 2,3-BDO, CO_2, and O_2) was constructed. Some linear reactions in the model were lumped together for simplification. The cell mass term in the model was constructed by considering all required metabolites,

cofactors, and energy from the central metabolic pathways for the synthesis of the cell mass components including proteins, nucleic acids, lipids, and carbohydrates (Dauner et al., 2001). The cell mass term presented here had reconnected the cell composition to eight precursors (3-phosphoglycerate, phosphoenolpyruvate, ribose-5-phosphate, erythose-4-phosphate, pyruvate, acetyl-CoA, α-ketoglutarate, and methyl-tetrahydrofolate) along with ATP, NAD/NADH, and NADP/NADPH. The cell mass term was estimated on the basis of experimental cell dry weight reported in the previous literatures of *B. subtilis* (Wu et al., 2008).

Briefly, the balances of all metabolites represented in the model were calculated starting from a randomly chosen flux distribution. After 32 h cultivation when the strain was at the stationary stage, the reaction rates of glucose uptake, glutamate uptake, O_2 uptake, cell growth, γ-PGA synthesis, byproducts accumulation, and CO_2 evolution were measured to calculate the metabolic fluxes at the predetermined period. The quality of the fit is judged by the X^2 (error) criterion. Through an iterative process of flux estimation and signal fitting, a flux solution is sought that corresponds to a minimal X^2 value. This optimal solution represents the maximum-likelihood flux distribution in the investigated metabolic system that reflects both the physiological data and the GC/LC analysis data. To determine the biochemical energy content of carbon substrates, the steady-state flux balance was formulated as an optimization problem in which the production of a particular product or quotients of the production of pairs of products were maximized, subject to

$$S \cdot v \geq b \quad (1)$$

where S is the stoichiometric matrix, v is the vector of fluxes, and b is the rate vector of metabolite production (Dauner et al., 2002). For this purpose, either a variant of Mehrotra's predictor-corrector algorithm, a primal-dual interior point method, or a sequential quadratic programming method was used, as implemented in the linprog and fmincon functions of the MATLAB Optimization Toolbox (The MathWorks, Inc.). Calculations were carried out on a personal computer using Matlab 7.0.

3. Results

3.1. Fed-batch fermentation and byproducts analysis

In the previous work, *B. subtilis* ZJU-7 was shown to produce approximately 80 g/l γ-PGA in the broth (Huang et al., 2011). In order to evaluate the possible effects of highly viscous fermentation broth on the glucose catabolism, fed-batch cultivations of *B. subtilis* ZJU-7 were conducted in a 10-l bench-top fermentor. Sample aliquots were collected to monitor the viscosity, cell density, residual substrate and γ-PGA concentration in broth. As shown in Fig. 1, the dissolved oxygen (DO) dropped dramatically to less than 10% at the exponential phase. The biomass reached its peak at 26 h, and the viscosity of broth was continuously increasing to over 4000 mPa·s, which was consistent with the accumulation of γ-PGA biopolymer. During the entire fermentation, the DO of broth was kept at a low level (less than 20%), indicating that *B. subtilis* cells were not undergoing fully aerobic environment.

To establish the extracellular byproduct profiles of *B. subtilis* ZJU-7, three different stages of fed-batch fermentation including the exponential phase (10 h), the stable phase (26 h) and the decline phase (38 h) were monitored by GC analyses to examine the accumulation of main byproducts. The obtained data suggested that acetoin and 2,3-BDO were the major byproducts during the γ-PGA biosynthesis process (Fig. 2). No remarkable amount of acetate, ethanol, lactate and propionate were detected in the broth samples after the removal of γ-PGA and other polysaccharides via methanol precipitation.

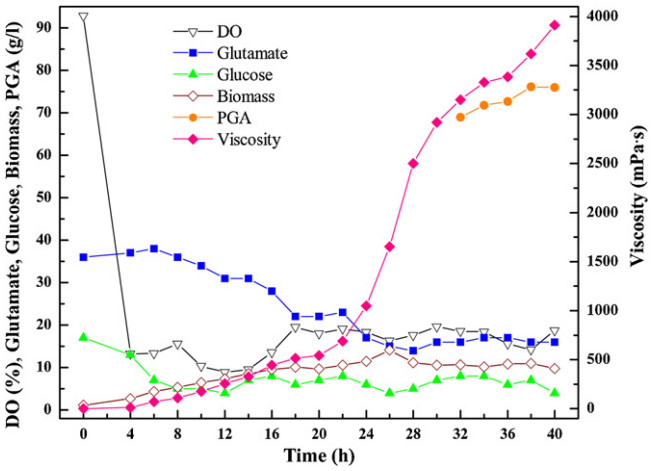

Fig. 1. Profiles of fed-batch production of γ-PGA in 10-l fermentors at 37 °C. The initial concentration of glucose was 20 g/l, and the pH was modulated at 6.5. The pulse feeding interval of 750 g/l glucose solution was manually adjusted to maintain the concentration of glucose in the range of 3–8 g/l in the broth. The pH was controlled with automatic adjustment of 25% (v/v) NH_4OH or 1 M HCl.

3.2. Effects of acetoin and 2,3-BDO on fermentation of B. subtilis ZJU-7

After the identification of two primary byproducts, their effects on the fermentation of *B. subtilis* ZJU-7, especially for the cell growth and γ-PGA biosynthesis, were further examined in shake flasks. Different concentrations of acetoin were first added into the medium at the beginning of cultivation, respectively. The result indicated that the cell growth of *B. subtilis ZJU*-7 was inhibited seriously by the addition of acetoin (Fig. 3A). The supplement of low concentration acetoin (2 g/l) greatly reduced the cell growth of *B. subtilis* ZJU-7 to as low as 3 g/l cell mass, which was only about 25% of that from *B. subtilis* cultivation without acetoin addition. Further increase of acetoin concentration up to 5 g/l could almost totally inhibit the cell growth of *B. subtilis* ZJU-7. On the other hand, the addition of 2,3-BDO had no evident negative effect on cell growth (Fig. 3B). The final pH values of the broth were around 6.5–7.0. Moreover, with the addition of suitable concentration (lower than 30 g/l) of 2,3-BDO, the cell density at the lag phase of

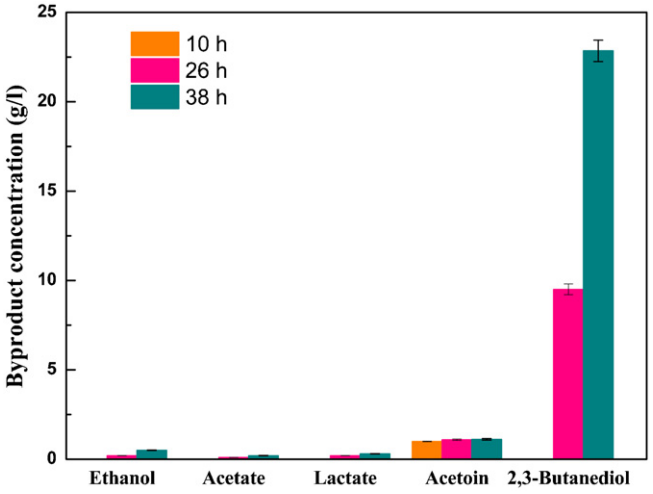

Fig. 2. The measurements of byproducts during the fed-batch fermentation of *B. subtilis* ZJU-7 in 10-l fermentors at 10 h, 26 h and 38 h, respectively. The cultivation conditions of fed-batch process were the same as those in Fig. 1. After removing γ-PGA and other polysaccharides via methanol precipitation, the broth samples were analyzed by GC.

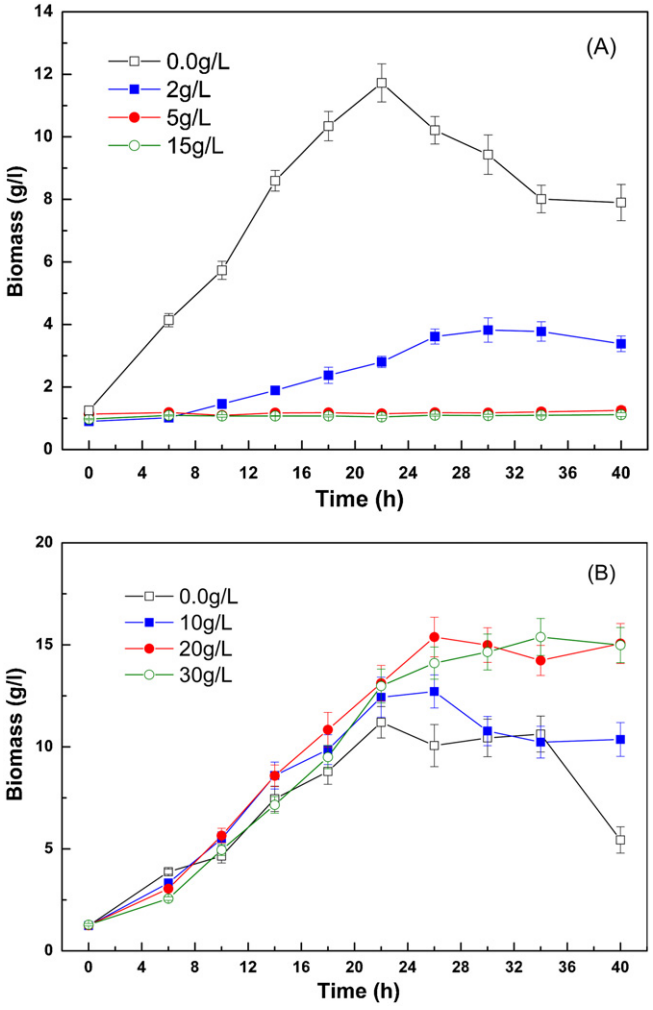

Fig. 3. Effects of initial addition of acetin (A) and 2,3 butanediol (B) on cell growth of *B. subtilis* ZJU-7. The experiments were carried out triplicately in 250 ml shake flasks containing 30 ml production medium at 37 °C.

fermentation was even slightly boosted (Fig. 3B). Considering the possible exhaustion of glucose at that stage, 2,3-BDO may be served as an alternative carbon source for the growth of *B. subtilis* ZJU-7.

For better simulation of fermentation process, various concentrations of acetoin and 2,3-BDO were also respectively added into the shake flask culture during the stationary phase, which was the period to present highest synthetic efficiency in both γ-PGA and byproducts. After cultivating in shaking flasks for 36 h, the biomass, yield of γ-PGA and residual sugar in the medium were monitored (Table 1). Examination with microscopy showed that the total number of living cells decreased in the presence of acetoin. Therefore, the addition of acetoin was obviously toxic to the cells and thus limited the production of γ-PGA. On the contrary, the supplement of low concentration of 2,3-BDO such as 10 g/l or 20 g/l had no influence on cell growth, but the production of γ-PGA was partially impaired, while the concentration of 30 g/l had exhibited negative effect on production of γ-PGA (15.36 ± 0.62 g/l) compared with the result of control samples (29.06 ± 1.27 g/l).

3.3. The byproduct profiles of fed-batch fermentation under different pH values

Considering the importance of pH during the fermentation, the profiles of acetoin and 2,3-BDO during the fed-batch fermentation were investigated in the 10-l bench-top fermentor under three

Table 1
The effects of acetoin and 2,3-butanediol on γ-PGA synthesis and cell growth in the shake flasks.

Byproducts (g/l)		Biomass	Residual sugar	γ-PGA
Acetoin	2,3-BDO	(g/l)	(g/l)	(g/l)
0	0	12.57 ± 0.33	0.0 ± 0.01	29.06 ± 1.27
2	0	9.27 ± 0.21	31.0 ± 1.02	17.10 ± 0.64
5	0	6.39 ± 0.12	46.0 ± 1.67	15.64 ± 0.71
15	0	5.80 ± 0.07	44.0 ± 1.58	14.98 ± 0.54
0	10	11.51 ± 0.35	6.0 ± 0.31	19.68 ± 0.83
0	20	12.12 ± 0.38	7.0 ± 0.48	19.46 ± 0.92
0	30	9.16 ± 0.23	21.0 ± 0.83	15.36 ± 0.62

All the experiments were carried out triplicately in 250 ml shake flasks containing 30 ml production media. A certain quantity of acetoin or 2,3-butanediol was added into the broth at 26 h, respectively. The biomass, residual sugar and γ-PGA were measured after 40 h fermentation.

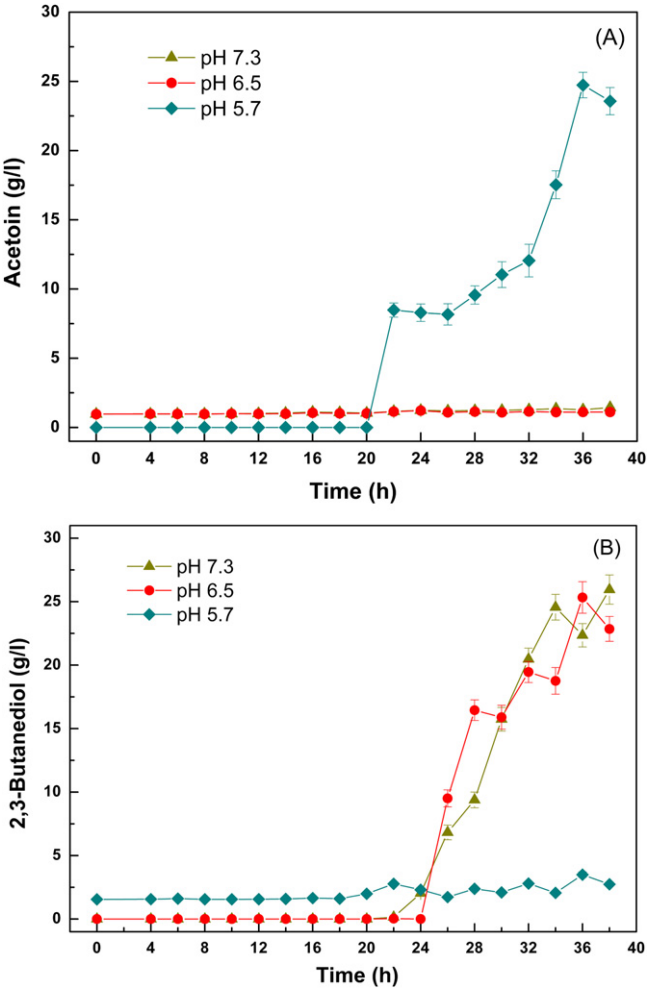

Fig. 4. The accumulation of acetoin (A) and 2,3-butanediol (B) during the fed-batch fermentation at pH 5.7, pH 6.5 and pH 7.3, respectively. Different pH controls were carried out by automatically adding 25% (v/v) NH_4OH or 1 M HCl. Other cultivation conditions of these fed-batch processes were the same as those in Fig. 1.

different pH values (5.7, 6.5 and 7.3), respectively. The results suggested that although the biosynthesis of main product γ-PGA was not evidently affected by pH, the pattern and production of byproducts were significantly changed (Fig. 4). At pH 5.7, acetoin was the major byproduct while the synthesis of 2,3-BDO was almost eliminated. The production of acetoin was detected at 20 h after inoculation and reached its highest concentration of 24.7 ± 0.92 g/l at 38 h (Fig. 4A). In contrast, 2,3-BDO was the primary byproduct at pH 6.5 and pH 7.3, while the production of acetoin was inhibited.

Table 2
List of the metabolic reactions involved in γ-PGA biosynthesis by *B. subtilis* ZJU-7.

R1	1 Glc + 1 ATP ↔ 1 G6P + 1 ADP
R2	1 G6P ↔ 1 F6P
R3	1 F6P + 1 ATP ↔ 2 T3P + 1 ADP
R4	1 T3P + 1 ADP + 1 NAD ↔ 1 PG3 + 1 ATP + 1 NADH
R5	1 PG3 ↔ 1 PEP
R6	1 PEP + 1 ADP ↔ 1 Pyr + 1 ATP
R7	1 Pyr + 1 NAD ↔ 1 AcCoA + 1 NADH + 1 CO_2
R8	1 G6P +1 NADP ↔ 1 R5P + 2 NADPH + 1 CO_2
R9	1 R5P ↔ 1 Xyl5P
R10	1 R5P ↔ 1 Rib5P
R11	1 Xyl5P + 1 Rib5P ↔ 1 T3P + 1 S7P
R12	1 Xyl5P + 1 E4P ↔ 1 F6P + 1 T3P
R13	1 T3P + 1 S7P ↔ 1 F6P + 1 E4P
R14	1 PG3 + 1 NADPH ↔ 1 NADH + 1 CO_2 + 2 C1
R15	1 AcCoA + 1 OAA + 1 NADP ↔ 1 αKG + 1 NADPH + 1 CO_2
R16	1 αKG + 1 FAD + 1 NAD ↔ 1 Fum + 1 FADH2 + 1 NADH + 1 CO_2
R17	1 Fum ↔ 1 Mal
R18	1 Mal + 1 NAD ↔ 1 OAA + 1 NADH
R19	1 αKG +1 NADPH ↔ 1 Glu + 1 NADP
R20	1 Glu + 1 ATP ↔ 1 PGA + 1 ADP
R21	1 exGlu + 1 ATP ↔ 1 Glu + 1 ADP
R22	1 Pyr + 1 CO_2 ↔ 1 OAA
R23	1 Mal + 1 NAD ↔ 1 Pyr + 2 CO_2 + 1 NADH
R24	1 FADH2+ 0.5 O_2 ↔ 0.8667 ATP + 1 FAD + 1 H_2O
R25	1 NADPH + 0.5 O_2 ↔ 1.3 ATP + 1 NADP + 1 H_2O
R26	1 NADH + 0.5 O_2 ↔ 1.3 ATP + 1 NAD + 1 H_2O
R27[a]	0.0014 PG3 + 0.0007 PEP + 0.0025 Pyr + 0.0021 AcCoA + 0.0386 ATP + 0.0008 RIB5P + 0.0163 NADPH + 0.0003 E4P + 0.0011 αKG + 0.0019 OAA + 0.0002 C1 → Biomass + 0.0036 NADH + 0.0022 CO_2
R28	1 3H2B + 1 ATP ↔ 1 3H2Bex + 1 ADP
R29	1 3H2B + 1 NADH ↔ 1 23BDOex + 1 NAD
R30	1 3H2B + 1 NAD ↔ 1 AcCoA + 1 NADH

[a] The biomass composition of *B. subtilis* was cited from the published data (Dauner et al., 2001).

The profiles of 2,3-BDO under pH 6.5 and pH 7.3 were very similar; its accumulation was started around 24 h and approached to the highest concentration of about 25 g/l around 36 h (Fig. 4B).

3.4. Metabolic network model modification and fluxes profiling under different pH values

Metabolic flux balance analysis is a tool to study the alteration of intracellular carbon flux responding to environmental changes. Thus, the possible central carbon source metabolism network could be complemented with the estimation of metabolic fluxes. Considering about the quasi-steady-state assumption, the fed-batch fermentation period in 10-l fermentor between 32 h and 34 h was chosen to calculate the metabolic fluxes under three different pH values (5.7, 6.5 and 7.3). According to the previous literature (Dauner et al., 2001; Wu et al., 2008) and verification of byproduct in the present work, the metabolic network of γ-PGA biosynthesis in *B. subtilis* ZJU-7 was presumably composed of 30 biochemical reactions (Table 2). The mean reaction rates of eight parameters (glucose, glutamate, O_2, biomass, γ-PGA, CO_2, acetoin and 2,3-BDO) were measured and exhibited in Table 3, which were employed as the primary data to calculate the corresponding metabolic fluxes. To calculate the flux distribution in the whole metabolic network, other 22 unknown fluxes were assigned as zero and the vector v_n of 30 fluxes for each of the 30 metabolite balances was used as a starting for an iterative process of flux estimation and signal fitting. A flux solution of the 30 metabolite balances was sought that corresponded to a minimal X^2 value which presented the maximum-likelihood flux distribution in the central carbon metabolism network during the chosen period. The resulted network of center carbon metabolism, γ-PGA synthetic pathways and byproducts accumulation were schemed out (Fig. 5). The fluxes of different pathways related to oxalacetic acid (OAA),

Table 3
Determination of mean reaction rates of eight parameters involved in γ-PGA biosynthesis by *B. subtilis* ZJU-7 under three pH control strategies. All reaction rates were normalized with respect to the glucose uptake rate.

	Mean reaction rate (mmol g_{DCW}^{-1} h^{-1})		
	pH 5.7	pH 6.5	pH 7.3
Glucose	−100.0	−100.0	−100.0
Glutamate	−34.0	−45.9	−17.7
O_2	−107.1	−118.6	−122.3
Biomass	3.3	4.2	4.1
γ-PGA	80.0	121.0	53.1
Acetoin	46.1	10.1	5.4
2,3-BDO	15.7	42.3	36.8
CO_2	193.8	170.6	91.2

which was the main precursor of α-ketoglutaric acid (α-KG) in the tricarboxylic acid (TCA) cycle, were varied significantly at three different pH values (data not shown). At pH 6.5, the pathways from pyruvic acid (PYR) and maleic acid (MAL) to OAA presented best performance, thus the metabolic flux was focused on the biosynthesis of α-KG, which could be subsequently converted into endogenous glutamic acid for production of γ-PGA. At pH 5.7, the metabolic fluxes branched into glycolytic pathway and pentose-phosphate pathway were very similar, while under other two pH values, glycolytic pathway presented higher enzyme-catalyzed reaction activity. As shown in Table 4, the total accumulation of redox cofactors (FAD, NADP, NAD) at pH 6.5 was higher than those at pH 5.7 or 7.3, while no major difference in biomass was observed under different pH controls. This suggested that at the stationary phase different pH values did not seriously disturb the cell growth but apparently influenced the cellular energy metabolism.

Normally, the productivity of a biosynthesis process could be raised by the increasing of the substrate uptake and conversion. In our modeling of metabolic network, the highest rates of extracellular glutamate ingestion and endogenous glutamate synthesis were 45.9 and 78.0 mmol g_{DCW}^{-1} h^{-1}, respectively, both at pH 6.5 (Fig. 5B), which resulted in a high concentration of γ-PGA (76.12 g/l) and low concentration of byproducts (1.17 g/l acetoin and 7.52 g/l 2,3-BDO). When the broth pH was switched to 5.7, the rates of extracellular glutamate ingestion, endogenous glutamate synthesis, and γ-PGA formation were reduced to 34.0, 46.0 and 80.0 mmol g_{DCW}^{-1} h^{-1}, respectively. Obviously, as shown in Fig. 5A, the metabolic flux from glucose shunted partially into acetoin related pathways, leading to lower γ-PGA synthesis rate and higher acetoin accumulation (17.53 g/l) at pH 5.7. This similar phenomenon of metabolic flux redistribution appeared at pH 7.3, while 2,3-BDO was the main byproduct with the rate of 36.8 mmol g_{DCW}^{-1} h^{-1} (Fig. 5C). From 32 h to 34 h, the production of γ-PGA at pH 5.7, 6.5 and 7.3 were 2.9 g/l, 4 g/l, and 3 g/l, respectively. The above results indicated that low pH and the lack of sufficient partial pressure of oxygen could activate byproduct synthetic pathways and deviate the metabolic flux from glycolysis, therefore, reducing the found productivity of γ-PGA from 4.5 g l^{-1} h^{-1} (at pH 6.5) to 3 g l^{-1} h^{-1} (at pH 5.7). High pH (7.3) would also decrease γ-PGA production to 68.32 g/l and boost the formation of 2,3-BDO

Table 4
Calculated metabolic fluxes related to cell growth and energy metabolism in γ-PGA biosynthesis at different pH control strategies.

Metabolic reactions	Metabolic fluxes		
	pH 5.7	pH 6.5	pH 7.3
R24	3.70	3.50	1.10
R25	0.00	0.00	0.00
R26	210.50	249.20	243.60
R27	3.28	4.12	4.10

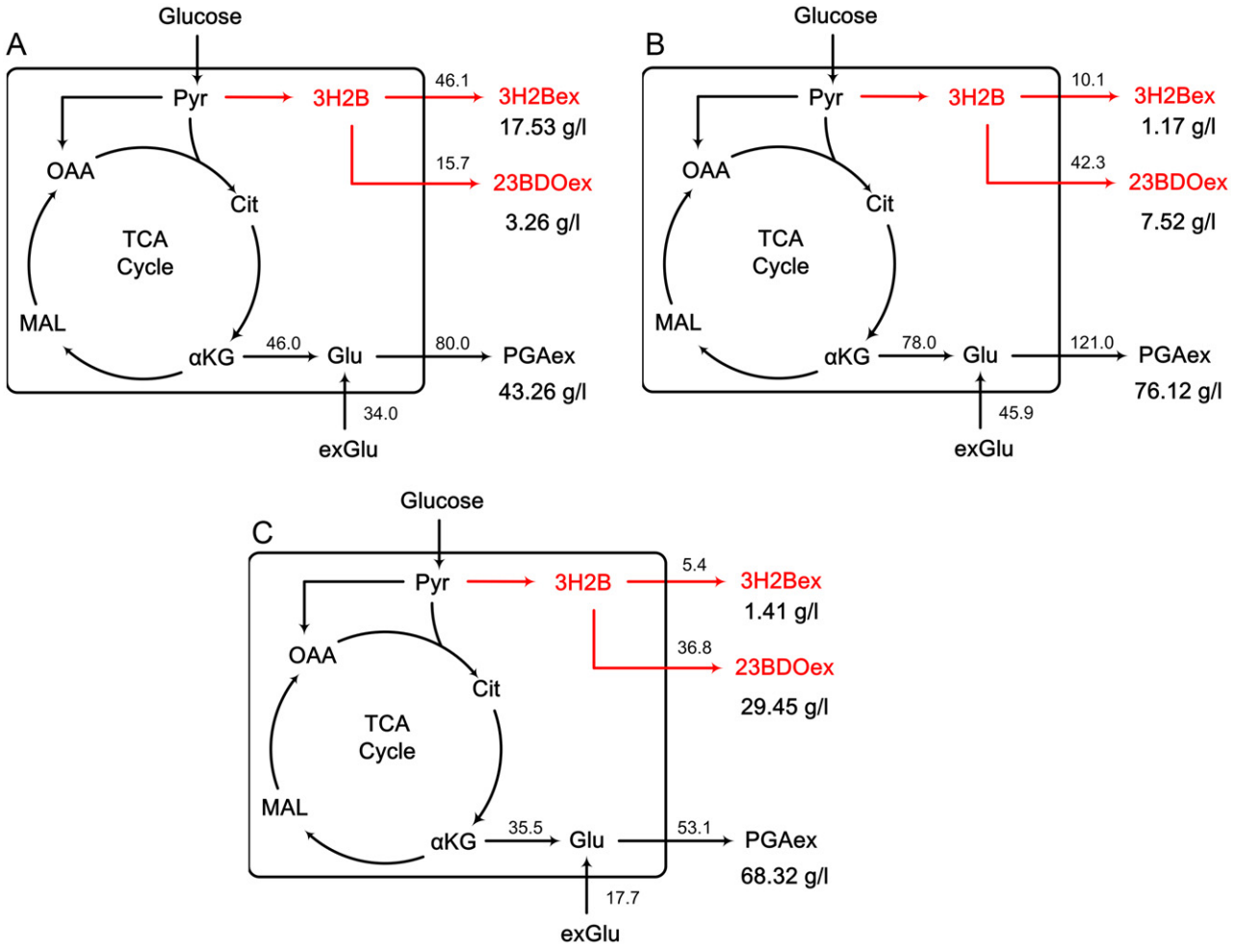

Fig. 5. Calculated carbon flux redistribution of *B. subtilis* ZJU-7 in 10-l fermentors under different pH values: (A) pH 5.7; (B) pH 6.5; (C) pH 7.3. The metabolic pathways in red color were proposed in the present work. The metabolic fluxes were given in units of mmol g_{DCW}^{-1} h^{-1}.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(29.45 g/l). These data were coincident with our previous report that pH 6.5 was favorable for γ-PGA production by *B. subtilis* ZJU-7 (Chen et al., 2010). Thus, the modeling and calculation process of metabolic network could be revised by inviting metabolic reactions of byproducts synthesis, and better simulating the realistic situation of γ-PGA fermentation. Providing the detailed information about the changes of the cellular metabolic phenotype, our results enriched the understanding of γ-PGA production in bacteria grown on glucose and glutamate.

4. Discussion

The high molecular weight and cross-linked structure of γ-PGA had caused high viscosity of fermentation broth, which led to the poor efficiency of oxygen transfer and became a bottleneck to limit the improvement of γ-PGA production. Moreover, the lack of oxygen may induce the anaerobic behavior of *B. subtilis* and further affect the cell growth and the biosynthesis of γ-PGA. In this work, the analysis of main byproducts from *B. subtilis* ZJU-7 fermentation and the evaluation of their possible effects were executed. It was well-known that a variety of end-products (acetate, ethanol, lactate, propionate, etc.) were often observed in the broth, when different bacteria were cultivated with glucose as the sole carbon source (Papagianni, 2012). Although a variety of micro-organisms were reported to produce γ-PGA with different productivities (Birrer et al., 1994; Cao et al., 2011; Goto and Kunioka, 1992), the investigations of their byproducts are often neglected. In the present work, acetoin and 2,3-BDO were confirmed to be two primary byproducts during the cultivation process with *B. subtilis* ZJU-7. Especially, the accumulated acetoin was very toxic to the growth of *B. subtilis* ZJU-7 whenever it was added into the medium. Further fed-batch experiments in the shake flasks indicated that both two byproducts exerted negative effects on the biosynthesis of γ-PGA. The possible negative effect of acetoin may ascribe to its toxicity toward cell growth, which further impaired the production of γ-PGA, while the limiting influence of 2,3-BDO was probably due to the competition among different pathways in the central carbon metabolism network. The investigation of acetoin and 2,3-BDO on cell growth and γ-PGA synthesis provided us valuable information about the viscous fermentation process of *B. subtilis* ZJU-7 and suggested an alternative strategy to improve the production of γ-PGA, by reducing or eliminating the production of acetoin and 2,3-BDO. One possible way to reduce the formation of such byproducts is to improve the dissolved oxygen (DO) level in the broth, either by optimizing the fermentation operations or supplementing the air stream with pure oxygen. However, considering about the high viscosity cultivation process, the above engineering methodology may not be practical to enhance the oxygen transfer during fermentation. Thus, another rational strategy is to eliminate the biosynthesis of acetoin and 2,3-BDO in the genetic level, via knocking out their necessary biosynthetic genes in *B. subtilis*.

The biosynthesis of anaerobic byproducts was obviously triggered by the shortage of dissolved oxygen. Our acquired byproducts profiles under different conditions had revealed that pH was also an important factor to affect the pattern and yield of anaerobic byproducts. When fermentation broth became viscous, the DO became insufficient, and the production of acetoin was boosted at lower pH to inhibit cell growth, thus impaired the production of other metabolites, while at the suitable pH, the pathway of acetoin was dormant, the central carbon metabolic flux was switched from biosynthesis of γ-PGA to the production of 2,3-BDO. These results confirmed that under viscous environment, *B. subtilis* ZJU-7 would employ the nitrate respiration route and synthesize byproducts. In addition, the concentration of 2,3-BDO was fluctuated in the range of 15–25 g/l during the late phase of fermentation at pH 6.5 and 7.3. The possible hypothesis was that 2,3-BDO could serve as an alternative carbon source for *B. subtilis* ZJU-7. Thus, the biosynthesis and consumption of 2,3-BDO were coexisted during the cultivation, especially when its total concentration was more than 15 g/l, leading to the variation of its apparent concentration.

Although endogenous or exogenous glutamate was the direct source of the carbon backbone in the γ-PGA biosynthesis, other carbon sources such as glucose, fructose, sucrose, and glycerol were iteratively reported to be indispensable in large amounts (Bajaj and Singhal, 2011; Shih and Van, 2001). Those substrates were mainly consumed in glycolysis, pentose–phosphate pathway, and tricarboxylic acid (TCA) cycle, providing energy for cell respiration and growth. However, in the stationary phase of fed-batch fermentation when cell growth was minimal, the rates of extracellular glutamate uptake and CO_2 release were observed to be dropping down slowly (Huang et al., 2011). It suggests that the glucose uptake was not totally converted into endogenous glutamate or degraded into CO_2. In this work, the measurement of main byproducts and investigation of their effects on cell growth and γ-PGA biosynthesis provided an explanation to where and how the surplus carbon source was gone. In addition, the effects of different pH values on byproduct production behavior were examined, elucidating a possible reason for the varied γ-PGA productivity at different pH values. The original consideration of different byproducts for metabolic network construction and fluxes prediction could provide us insight into the γ-PGA biosynthetic network and guide us developing novel strategies for byproducts elimination to enhance the production of γ-PGA.

5. Conclusions

Acetoin and 2,3-BDO have been confirmed as the major byproducts during the fermentative production of γ-PGA in *B. subtilis* ZJU-7. The negative effects of these byproducts on cell growth and γ-PGA biosynthesis were further evaluated at three different pH values, and the corresponding metabolic fluxes profiling was performed to scheme the central carbon metabolism of *B. subtilis* ZJU-7 involved in the production of γ-PGA. The comparison indicated that the biosynthesis of acetoin was induced at low pH and insufficient DO in the broth, which directly inhibited the cell growth and subsequent γ-PGA biosynthesis. With the suitable pH at 6.5, the lack of oxygen could deviate the metabolic flux from γ-PGA production to the biosynthesis of 2,3-BDO. The present work not only made a deep insight for byproducts formation but also promised one metabolic engineering strategy to further improve γ-PGA productivity in *Bacillus subtillis.*

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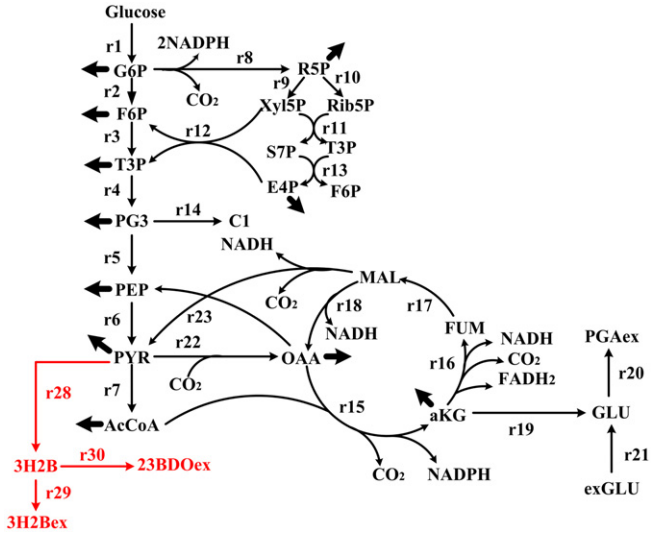
Appendix A.

Metabolic network of *B. subtilis* ZJU-7 related to γ-PGA biosynthesis grown on glucose and glutamate. The metabolic pathways in red color were proposed in the present work. (For interpretation of the references to color in the figure in Appendix, the reader is referred to the web version of the article.)



References

Ashiuchi, M., Shimanouchi, K., Nakamura, H., Kamei, T., Soda, K., Park, C., Sung, M.H., Misono, H., 2004. Enzymatic synthesis of high-molecular-mass poly-γ-glutamate and regulation of its stereochemistry. Applied and Environmental Microbiology 70, 4249–4255.

Bajaj, I., Singhal, R., 2011. Poly(glutamic acid) – an emerging biopolymer of commercial interest. Bioresource Technology 102, 5551–5561.

Birrer, G.A., Cromwick, A.M., Gross, R.A., 1994. γ-Poly(glutamic acid) formation by *Bacillus licheniformis* 9945: physiological and biochemical studies. International Journal of Biological Macromolecules 16, 265–275.

Cao, M.F., Geng, W.T., Liu, L., Song, C.J., Xie, H., Guoa, W.B., Jin, Y.H., Wang, S.F., 2011. Glutamic acid independent production of poly-γ-glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of pgsBCA genes. Bioresource Technology 102, 4251–4257.

Chen, J., Shi, F., Zhang, B., Zhu, F., Cao, W.F., Xu, Z.N., Xu, G.H., Cen, P.L., 2010. Effects of cultivation conditions on the production of γ-PGA with *Bacillus subtilis* ZJU-7. Applied Biochemistry and Biotechnology 160, 370–377.

Dauner, M., Bailey, J.E., Sauer, U., 2001. Metabolic flux analysis with a comprehensive isotopomer model in *Bacillus subtilis*. Biotechnology and Bioengineering 76, 144–156.

Dauner, M., Sonderegger, M., Hochuli, M., Szyperski, T., Wüthrich, K., Hohmann, H.P., Sauer, U., Bailey, J.E., 2002. Intracellular carbon fluxes in riboflavin-producing *Bacillus subtilis* during growth on two-carbon substrate mixtures. Applied and Environmental Microbiology 68, 1760–1771.

Earl, A.M., Losick, R., Kolter, R., 2008. Ecology and genomics of *Bacillus subtilis*. Trends in Microbiology 16, 269–275.

Goto, A., Kunioka, M., 1992. Biosynthesis and hydrolysis of poly(γ-glutamic acid) from *Bacillus subtilis* IFO3335. Bioscience Biotechnology and Biochemistry 56, 1031–1035.

Hartig, E., Hartmann, A., Schatzle, M., Albertini, A.M., Jahn, D., 2006. The *Bacillus subtilis* nrdEF genes, encoding a class Ib ribonucleotide reductase, are essential for aerobic and anaerobic growth. Applied and Environment Microbiology 72, 5260–5265.

Huang, J., Du, Y.M., Xu, G.H., Zhang, H.L., Zhu, F., Huang, L., Xu, Z.N., 2011. High yield and cost-effective production of poly(γ-glutamic acid) with *Bacillus subtilis*. Engineering in Life Sciences 4, 1–7.

Kunioka, M., 1997. Biosynthesis and chemical reactions of poly(amino acid)s from microorganisms. Applied Microbiology and Biotechnology 47, 469–475.

Liu, J., Ma, X., Wang, Y., Liu, F., Qiao, J., Li, X.Z., Liu, Qiao, J., Li, X.Z., Gao, X., Zhou, T., 2011. Depressed biofilm production in *Bacillus amyloliquefaciens* C06 causes γ-polyglutamic acid (γ-PGA) overproduction. Current Microbiology 62, 235–241.

Nakano, M.M., Hoffman, T., Zhu, Y., Jahn, D., 1998. Nitrogen and oxygen regulation of *Bacillus subtilis* nasDEF encoding NADH-dependent nitrite reductase by TnrA and ResDE. Journal of Bacteriology 180, 5344–5350.

Papagianni, M., 2012. Recent advances in engineering the central carbon metabolism of industrially important bacteria. Microbial Cell Factories 11, 50.

Ramos, H.C., Hoffmann, T., Marino, M., Nedjari, H., Presecan-Siedel, E., Dreesen, O., Glaser, P., Jahn, D., 2000. Fermentative metabolism of *Bacillus subtilis*: physiology and regulation of gene expression. Journal of Bacteriology 182, 3072–3080.

Shi, F., Xu, Z.N., Cen, P.L., 2006a. Optimization of γ-polyglutamic acid production by *Bacillus subtilis* ZJU-7 using a surface-response methodology. Biotechnology and Bioprocess Engineering 11, 51–257.

Shi, F., Xu, Z.N., Cen, P.L., 2006b. Efficient production of poly-γ-glutamic acid by *Bacillus subtilis* ZJU-7. Applied Biochemistry and Biotechnology 133, 271–282.

Shih, I.L., Van, Y.T., 2001. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresource Technology 79, 207–225.

Wu, Q., Xu, H., Shi, N.N., Yao, J., Li, S., Ouyang, P.K., 2008. Improvement of poly(γ-glutamic acid) biosynthesis and redistribution of metabolic flux with the presence of different additives in *Bacillus subtilis CGMCC 0833*. Applied Microbiology and Biotechnology 79, 527–535.

Zhang, H.L., Zhu, J.Z., Zhu, X.C., Cai, J., Zhang, A.Y., Hong, Y.Z., Huang, J., Huang, L., Xu, Z.N., 2012. High-level exogenous glutamic acid-independent production of poly-(γ-glutamic acid) with organic acid addition in a new isolated *Bacillus subtilis* C10. Bioresource Technology 116, 241–246.