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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Simultaneous production of poly-γ-glutamic acid and 2,3-butanediol by a newly isolated *Bacillus subtilis* CS13

Dexin Wang[1,2] · Hyangmi Kim[3] · Sungbeom Lee[4,5] · Dae-Hyuk Kim[2] · Min-Ho Joe[1]

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Abstract

Bacillus subtilis naturally produces large amounts of 2,3-butanediol (2,3-BD) as a main by-product during poly-γ-glutamic acid (γ-PGA) production. 2,3-BD is a promising platform chemical in various industries, and co-production of the two chemicals has great economic benefits. Co-production of γ-PGA and 2,3-BD by a newly isolated *B. subtilis* CS13 was investigated here. The fermentation medium and culture parameters of the process were optimized using statistical methods. It was observed that sucrose, L-glutamic acid, ammonium citrate, and $MgSO_4 \cdot 7H_2O$ were favorable for γ-PGA and 2,3-BD co-production at culture pH of 6.5 and 37 °C. An optimal medium composed of 119.8 g/L sucrose, 48.8 g/L L-glutamic acid, 21.1 g/L ammonium citrate, and 3.2 g/L $MgSO_4 \cdot 7H_2O$ was obtained by response surface methodology (RSM). The results show that the titers of γ-PGA and 2,3-BD reached 27.8 ± 0.9 g/L at 24 h and 57.1 ± 1.3 g/L at 84 h with the optimized medium, respectively. γ-PGA and 2,3-BD production by *B. subtilis* CS13 was significantly enhanced in fed-batch fermentations. γ-PGA (36.5 ± 1.1 g/L, productivity of 1.22 ± 0.04 g/L/h) and 2,3-BD concentrations (119.6 ± 2.8 g/L, productivity of 2.49 ± 0.66 g/L/h) were obtained in the optimized medium with feeding sucrose. The co-production of 2,3-BD and γ-PGA provides a new perspective for industrial production of γ-PGA and 2,3-BD.

Key points
- *A strategy for co-production of γ-PGA and 2,3-BD was developed.*
- *The culture parameters for the co-production of γ-PGA and 2,3-BD were studied.*
- *RSM was used to optimize the medium for γ-PGA and 2,3-BD co-production.*
- *36.5 g/L γ-PGA and 119.6 g/L 2,3-BD were obtained from the optimum medium in fed-batch fermentation.*

Keywords Poly-γ-glutamic acid · 2,3-Butanediol · *Bacillus subtilis* CS13 · Response surface methodology

Introduction

Production of bulk chemicals and biopolymers by microbial fermentation has aroused widespread interest in industry due to its safety and environmental friendliness. Natural microorganism metabolism is a complex process and generally accumulates various by-products along with the target products. Therefore, screening microorganisms with high yield target

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✉ Min-Ho Joe
jomino@kaeri.re.kr

[1] Radiation Utilization and Facilities Management Division, Korea Atomic Energy Research Institute, 29 Geumgu-gil, Jeongeup 56212, Republic of Korea

[2] Department of Bioactive Material Sciences, Center for Fungal Pathogenesis, Institute for Molecular Biology and Genetics, Jeonbuk National University, Jeonju 54896, Republic of Korea

[3] Bacteria Research Team, Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea

[4] Radiation Research Division, Korea Atomic Energy Research Institute, 29 Geumgu-gil, Jeongeup 56212, Republic of Korea

[5] Department of Radiation Science and Technology, University of Science and Technology, Daejeon 34113, Republic of Korea

products has been an inevitable research direction for researchers. Moreover, bioengineering technologies have been developed to minimize the concentration of by-products or maximize the concentration of the target products (Guo et al. 2014). However, most researches for microbial production of bulk chemicals or polymers are just limited in laboratory scale due to the high cost of fermentation process and subsequent purification steps (Lee et al. 2011).

Under certain circumstances, two or more metabolites can accumulate simultaneously in organisms, and co-production strategies have been developed in a variety of microorganisms to make the processes more efficient, such as co-production of 3-hydroxypropionic acid and 1,3-propanediol (Huang et al. 2013), simultaneous production of butanol and acetoin (Liu et al. 2015), and simultaneous production of surfactin and 2,3-butanediol (2,3-BD) (Andrade et al. 2016). The co-production strategies realized the redox balance in cultivation and improved the mass yields and product values.

Bacillus subtilis is a poly-γ-glutamic acid (γ-PGA) producer (Zeng et al. 2014; Zhang et al. 2012). γ-PGA is a biopolymer consisting of L- and D-glutamic acid units and has applications in medicine, food, agriculture, cosmetics, and wastewater treatment industries due to its excellent properties such as water solubility, super absorption, biodegradability, and non-toxicity to humans and the environment (Bajaj and Singhal 2011; Luo et al. 2016). γ-PGA production by *B. subtilis* is an L-glutamic acid–dependent process. Meanwhile, *B. subtilis* consists of a 2,3-BD synthesis pathway. Pyruvate is converted to acetoin with the catalysis of acetolactate synthase (ALS) and acetolactate decarboxylase (ALD), and the latter is reduced to 2,3-butanediol (2,3-BD) by 2,3-BD dehydrogenase (BDH) (Fig. 1). 2,3-BD is an important chemical and has applications in food, pharmaceutical, cosmetics, fine chemical, and other industries. In addition, 2,3-BD can be used as a fuel due to its high heat energy (Zeng and Sabra 2011). In recent years, microbial route of 2,3-BD production has been reported in a series of bacteria

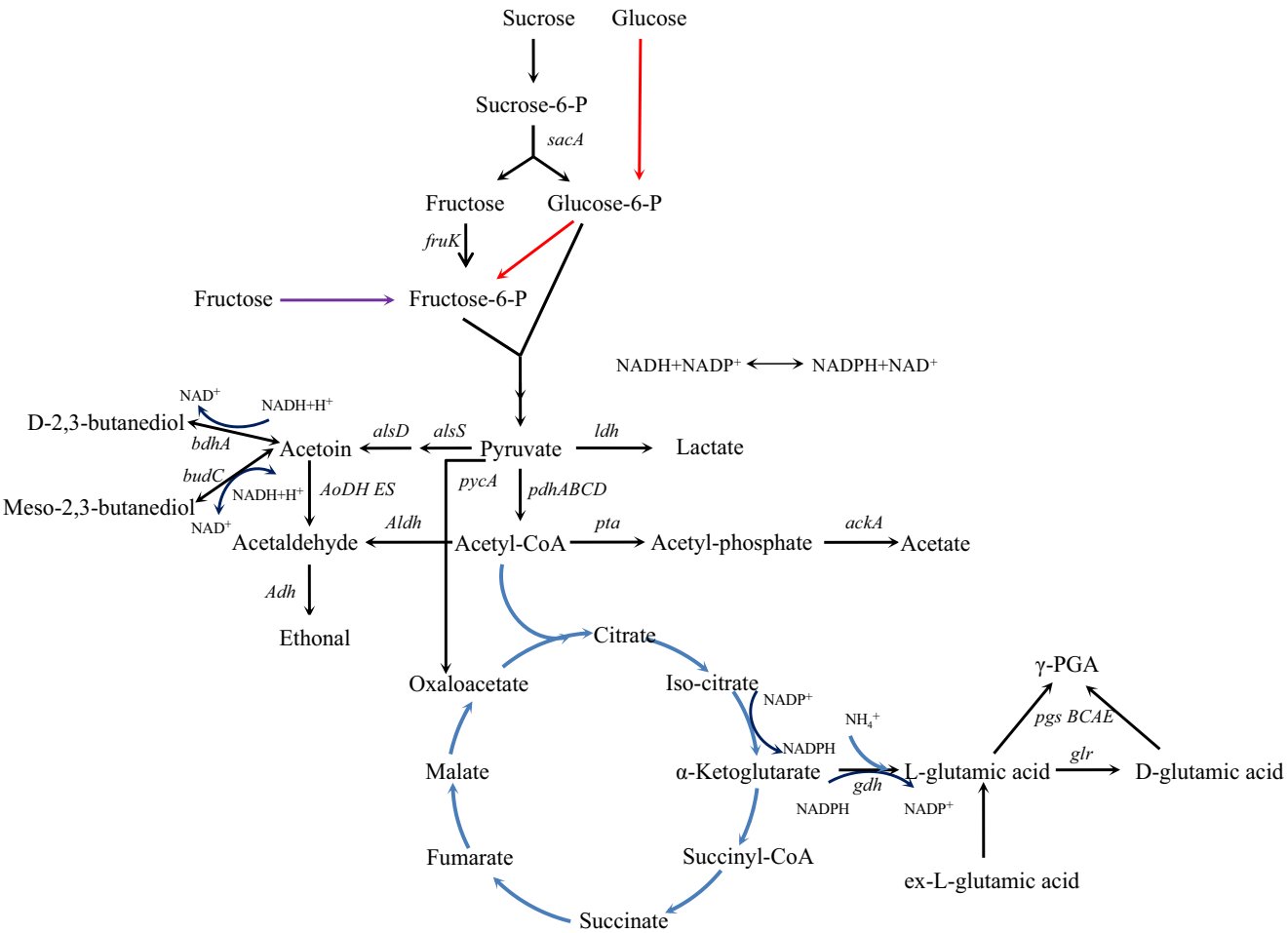


Fig. 1 The metabolic pathways of 2,3-BD and γ-PGA co-production in *B. subtilis* CS13. The coding genes of enzymes around pyruvate branch for 2,3-BD and γ-PGA production: lactate dehydrogenase (*ldh*); acetolactate synthase (*alsS*); acetolactate decarboxylase (*alsD*); D-butanediol dehydrogenase (*bdhA*); butanediol dehydrogenase (*budC*); pyruvate dehydrogenase (*pdhABCD*); glutamate dehydrogenase (*gdh*); glutamate racemase (*glr*); γ-PGA synthetase (*pgsBCAE*)

species, such as *Klebsiella* spp. (Guragain and Vadlani 2017), *Enterobacter* spp. (Zhang et al. 2014), *Paenibacillus* spp. (Gao et al. 2010), and *Bacillus* spp. (Białkowska et al. 2016; Andrade et al. 2016; Yang et al. 2013). *B. subtilis* is a GRAS (generally regarded as safe) bacteria and is considered to be a promising microbe for the production of 2,3-BD (Feng et al. 2017; Fu et al. 2014).

The production of 2,3-BD from acetoin requires oxidation of NADH to NAD^+. Glutamate dehydrogenase (GDH) is the key enzyme of glutamic acid metabolism and mainly uses NADPH as a coenzyme (Meng et al. 2016). The decrease of NADPH will increase the NADH accumulation, which was beneficial for 2,3-BD production (Fig. 1). Fermentative production of γ-PGA is an aerobic process, while 2,3-BD synthesis is inhibited at aerobic conditions (Luo et al. 2016; Fu et al. 2016). However, the γ-PGA broth has high viscosity, which decreases the dissolved oxygen and leads to the metabolic flux from carbon sources mainly into 2,3-BD synthesis pathway (Fu et al. 2014). Therefore, simultaneous production of 2,3-BD and γ-PGA was proposed and investigated for the first time. Temperature and pH affect the activities of related enzymes, which in turn affect γ-PGA and 2,3-BD production. Besides, carbon sources, nitrogen sources, and inorganic salts have a significant effect on the γ-PGA or 2,3-BD production individually by various *Bacillus* species (Bajaj and Singhal 2011; Zeng et al. 2014; Yang et al. 2012; Yang et al. 2013). Thus, medium components can be manipulated by statistical methods to improve the yield of γ-PGA and 2,3-BD. Statistical methods are more rapid, reliable, and reduce the total number of experiments.

In this paper, a new *B. subtilis* strain was isolated and identified, which can co-produce 2,3-BD and γ-PGA. Detailed research on the effects of the medium components and cultivation conditions on the 2,3-BD and γ-PGA co-production was carried out. The Plackett–Burman design (PBD) was used to identify significant factors affecting the 2,3-BD and γ-PGA co-production. An optimal initial media, which could maximize the co-production of 2,3-BD and γ-PGA, was obtained by the response surface methodology (RSM). As far as we know, this is the first report of 2,3-BD and γ-PGA co-production. The present study provides a new perspective on theoretical research and industrial applications.

Materials and methods

Isolation and identification of the γ-PGA-producing strain

B. subtilis strains were isolated from Chung-kook-jang sauce, which is a traditional Korean food, purchased at a local market (Jeongeup-si, South Korea). Samples of 10 g were diluted in 90 mL of distilled water and boiled for 5 min. The suspension was diluted 10^{-1} to 10^{-6}, and an aliquot (200 μL) of each suspension was spread on basal agar medium plates, containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L glucose, 20 g/L L-glutamic acid, and 15 g/L agar. After incubation at 37 °C for 48 h, high-viscosity and high-mucosity colonies were isolated as potential strains and purified on new agar plates. Pure colonies were inoculated into 15-mL tubes containing 3 mL of liquid basal medium and cultured for 12 h at 37 °C with agitation at 200 rpm, and 1% (*v*/*v*) of the samples was transferred into 50 mL of fermentation medium (pH 6.5) composed of 30 g/L glucose, 30 g/L L-glutamic acid, 5 g/L NH_4Cl, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.04 g/L $FeCl_3 \cdot 6H_2O$, 0.15 g/L $CaCl_2 \cdot 2H_2O$, 0.12 g/L $MnCl_2 \cdot 4H_2O$, and 0.5 g/L NaCl in 250-mL baffled flasks. The cells were incubated at 37 °C and shaken at 200 rpm for 24 h. The concentrations of γ-PGA in the broth were measured, and the strains with the highest γ-PGA yield were chosen for further study.

The 16S rDNA gene of the isolated strain was amplified, sequenced, and manually edited with the BioEdit software as previously described (Wang et al. 2020). The sequence was used for comparison with reported type strains from the EzBioCloud database (https://www.ezbiocloud.net/). The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0.

Culture conditions for co-production of γ-PGA and 2,3-BD

The isolated strain was first cultured in 50-mL tubes containing 15 mL basal medium at 37 °C and 200 rpm for 12 h. Then, the optical density of the seed cultures was adjusted to an OD_{600} of 5.0 ± 0.1, and 1% (*v*/*v*) was inoculated into 250-mL baffled flasks containing 50 mL of fresh fermentation medium and cultured for 24–36 h according to the experiment conditions. All experiments were performed independently in triplicates.

The effects of temperature were studied at 30, 34, 37, 42, and 45 °C with the agitation of 200 rpm and pH 6.5. The effects of pH on the 2,3-BD and γ-PGA co-production were carried out at an initial pH of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Incubation was conducted at the determined optimum temperature. The optimum temperature and initial pH were fixed for all subsequent experiments.

The effect of different carbon sources on the 2,3-BD and γ-PGA co-production was investigated by individually replacing glucose with sucrose, fructose, glycerol, maltose, and lactose at 30 g/L and keeping the others at the same levels. The optimal carbon source was selected for further study.

Various nitrogen sources including ammonium chloride, ammonium citrate, ammonium sulfate, sodium nitrate, potassium nitrate, yeast extract, peptone, and corn steep liquor were added to the fermentation medium at 5 g/L individually to

investigate their effects on the 2,3-BD and γ-PGA co-production. The nitrogen source supporting the maximum production of 2,3-BD and γ-PGA was used for all subsequent experiments.

Screening of significant nutrients by PBD

The PBD as an efficient technique was used for screening of the main factors that significantly influenced the 2,3-BD and γ-PGA co-production. In the present study, the optimum temperature and initial pH were used. Eight variables including sucrose, L-glutamic acid, ammonium citrate, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $FeCl_3{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, and $CaCl_2{\cdot}2H_2O$ were used to generate the experiment design by the Design-Expert 8.0.6 software (Stat-Ease Inc., Minneapolis, MN, USA). Each variable at two values (+ 1 for high and − 1 for low) were chosen and shown in Table 1. The true values of each variable and its analysis of variance (ANOVA) are shown in Table 2.

Optimization of the medium composition by response surface methodology

A face-centered central composite design (FCCD) was used for the RSM to optimize the levels of significant variables in the optimization of the 2,3-BD and γ-PGA production. The chosen variables were sucrose, L-glutamic acid, ammonium citrate, and $MgSO_4{\cdot}7H_2O$ identified by PBD (Table 2), while 1 g/L KH_2PO_4, 0.04 g/L $FeCl_3{\cdot}6H_2O$, 0.15 g/L $CaCl_2{\cdot}2H_2O$, 0.12 g/L $MnCl_2{\cdot}4H_2O$, and 0.5 g/L NaCl in the medium were fixed. The effect of each variable on the 2,3-BD and γ-PGA production was studied at three coded levels (− 1, 0, + 1), and a total of 30 experiments including six replicates at the central point were conducted. The coded and real values are shown in Table 3. The 2,3-BD and γ-PGA concentrations were measured in triplicates and as the average of the maximum as the response after 84 h. The data obtained from the RSM for the 2,3-BD and γ-PGA co-production were subjected to ANOVA using the Design-Expert 8.0.6 software. The experimental results of the RSM were fitted to the second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} {X_i}^2$$

where Y is the predicted response; β_0 is the intercept term; β_i is the linear coefficient; β_{ij} is the quadratic coefficient; β_{ii} is the squared term, and X_i, X_j are the coded independent variables.

Fed-batch fermentation

Fed-batch fermentation was carried out in a 3-L fermentor (FMT-ST-D03; Bio System Engineering & Machine

Table 1 Plackett–Burman design for variables with coded values and the response of the γ-PGA and 2,3-BD

Run	X_1 Sucrose	X_2 L-Glutamic acid	X_3 Ammonium citrate	X_4 KH_2PO_4	X_5 $MgSO_4{\cdot}7H_2O$	X_6 $FeCl_3{\cdot}6H_2O$	X_7 $MnCl_2{\cdot}4H_2O$	X_8 $CaCl_2{\cdot}2H_2O$	Y_1 γ-PGA (g/L)	Y_2 2, 3-BD (g/L)
1	− 1	− 1	− 1	1	− 1	1	1	− 1	11.33	8.41
2	− 1	1	1	− 1	1	1	1	− 1	17.6	10.24
3	− 1	− 1	− 1	− 1	− 1	− 1	− 1	− 1	12.34	8.8
4	1	− 1	1	1	− 1	1	1	1	19.16	23.46
5	− 1	1	1	1	− 1	− 1	− 1	1	17.51	9.66
6	1	1	1	− 1	− 1	− 1	1	− 1	22.37	23.48
7	− 1	1	− 1	1	1	− 1	1	1	15.89	9.47
8	1	− 1	− 1	− 1	1	− 1	1	1	17.18	24.46
9	1	− 1	1	1	1	− 1	− 1	− 1	20.49	26.23
10	− 1	− 1	1	− 1	1	1	− 1	1	14.98	10.83
11	1	1	− 1	1	1	1	− 1	− 1	20.54	24.44
12	1	1	− 1	− 1	− 1	1	− 1	1	20.95	22.48

Table 2 Actual values of the variables in PBD and ANOVA for the production of γ-PGA by *B. subtilis* CS13

Factors	Concentration (g/L)		Mean square	Coefficient estimate	Standard error	*F* value	*P* value
	Lower	Higher					
Model			16.19	17.53	0.15	63.21	0.0029
X_1: sucrose	20	60	80.29	2.59	0.15	313.46	0.0004
X_2: L-glutamic acid	20	40	31.3	1.62	0.15	122.19	0.0016
X_3: Ammonium citrate	5	15	16.05	1.16	0.15	62.68	0.0042
X_4: KH_2PO_4	1	3	0.021	− 0.042	0.15	0.081	0.7941
X_5: $MgSO_4{\cdot}7H_2O$	0.5	1.5	0.76	0.25	0.15	2.97	0.1834
X_6: $FeCl_3{\cdot}6H_2O$	0.04	0.12	0.12	− 0.1	0.15	0.48	0.5366
X_7: $MnCl_2{\cdot}4H_2O$	0.12	0.36	0.90	− 0.27	0.15	3.50	0.1581
X_8: $CaCl_2{\cdot}2H_2O$	0.15	0.45	0.083	0.083	0.15	0.33	0.6083

$R^2 = 0.9941$, $R^2_{(adj)} = 0.9784$, $R^2_{(Pred)} = 0.9056$

Company; Korea) with 0.9 L optimized fermentation medium at 37 °C, and 100 mL seed culture as previously described was inoculated. The aeration and the agitation speed were maintained at 3 L/min and 600 rpm, respectively; the pH was automatically controlled at 6.5 ± 0.1 by adding NH_4OH or 2 M HCl. Bottles of sucrose solution (80%, *w*/*v*) were added into the fermentor when the residue sugar concentration was about 20–30 g/L.

Analytical methods

The biomass was determined according to the standard calibration curve between the OD_{600} and dry cell weight by measuring the OD_{600} of the broth (Wang et al. 2020).

γ-PGA was purified by the ethanol precipitation method as previously described (Wang et al. 2020). The concentration of γ-PGA was determined by gel permeation chromatography system using an Agilent 1100 high-performance liquid chromatography (HPLC) system equipped with a PL aquagel-OH MIXED-H column (300 × 7.5 mm, 8 μm; Agilent Technologies, Inc., UK) and refractive index detector. The mobile phase was HPLC-grade water at a flow rate of 1.0 mL/min. The γ-PGA yield was determined by the standard curve between the area peak and purified γ-PGA (Bioleaders Corporation, Daejeon, South Korea).

The concentrations of 2,3-BD, acetoin, glucose, and fructose in the broth were measured by HPLC with an Aminex HPX-87H column and sucrose with the Aminex HPX-87P column (300 × 7.8 mm; Bio-Rad, USA) at 65 °C. The mobile phase consisted of 5 mM H_2SO_4 and HPLC-grade water, respectively, and the flow rate was 0.6 mL/min. L-Glutamic acid was measured using a Chirex® 3126 (D)-phenicillamine column (250 × 4.6 mm; Phenomenex Inc., Torrance, CA, USA) and a UV detector (254 nm) as described before (Wang et al. 2020).

Table 3 Actual values of the variables in PBD and ANOVA for the production of 2,3-BD by *B. subtilis* CS13

Factors	Concentration (g/L)		Mean square	Coefficient estimate	Standard error	*F* value	*P* value
	Lower	Higher					
Model			80.61	16.83	0.057	2094.30	< 0.0001
X_1: sucrose	20	60	632.78	7.26	0.057	16,440.63	< 0.0001
X_2: L-glutamic acid	20	40	0.49	− 0.20	0.057	12.68	0.0378
X_3: Ammonium citrate	5	15	2.84	0.49	0.057	73.84	0.0033
X_4: KH_2PO_4	1	3	0.16	0.12	0.057	4.12	0.1353
X_5: $MgSO_4{\cdot}7H_2O$	0.5	1.5	7.33	0.78	0.057	190.50	0.0008
X_6: $FeCl_3{\cdot}6H_2O$	0.04	0.12	0.42	− 0.19	0.057	10.86	0.0459
X_7: $MnCl_2{\cdot}4H_2O$	0.12	0.36	0.71	− 0.24	0.057	18.46	0.0232
X_8: $CaCl_2{\cdot}2H_2O$	0.15	0.45	0.13	− 0.10	0.057	3.33	0.1656

$R^2 = 0.9998$, $R^2_{(adj)} = 0.9993$, $R^2_{(Pred)} = 0.9971$

Results

Isolation and identification of the γ-PGA-producing strain

Based on the characteristics of the colonies which were highly mucoid, 15 strains (CS1–CS15) were picked up from the agar plates. These strains were transferred into the fermentation medium, and eight strains showed a γ-PGA production ability. Of these strains, strain CS13 produced the highest concentration of γ-PGA (9.6 ± 0.3 g/L) (Table S1). In addition, high concentration of 2,3-BD (14.2 ± 0.5 g/L) was found during an analysis of residual sugars in the fermentation broth (Fig. S1). Hence, the idea of the co-production γ-PGA and 2,3-BD arose due to strain CS13.

The 16S rDNA gene sequence of CS13 showed the similarity to *B. subtilis* subsp. *subtilis* NCIB 3610 (99.93%), *B. subtilis* subsp. *stercoris* D7XPN1 (99.92%), *B. tequilensis* KCTC 13622 (99.85%), and *B. subtilis* subsp. *inaquosorum* KCTC 13429 (99.85%) when blasting the sequence on EzBioCloud. A phylogenetic tree was constructed based on the 16S rDNA sequence and is shown in Fig. 2. CS13 formed

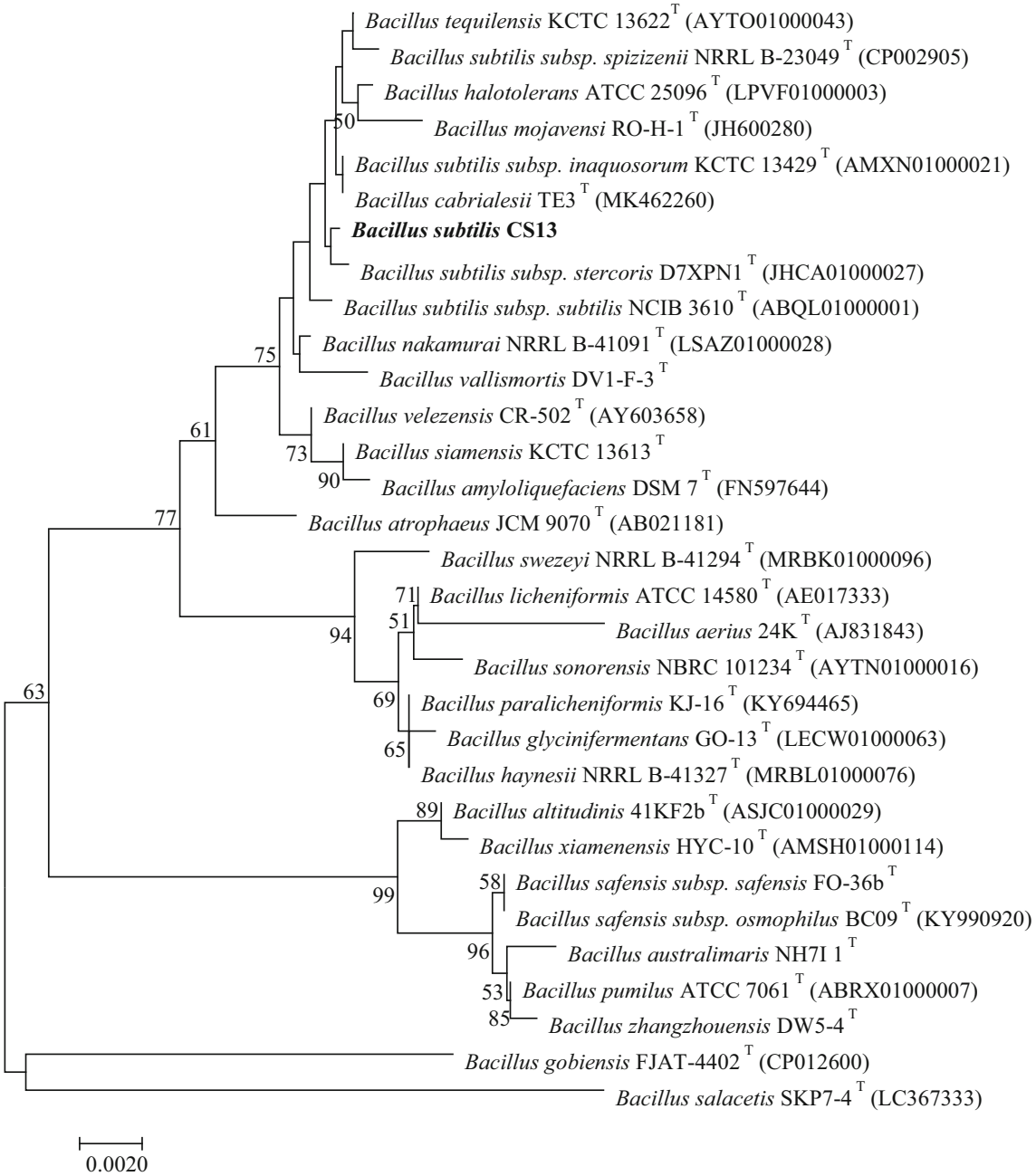


Fig. 2 Phylogenetic relationship of *Bacillus subtilis* CS13 and other *Bacillus* strains based on neighbor-joining tree analysis of the 16S rDNA gene. Bootstrap values greater than 50 are indicated at branch nodes. Bar, 0.0020 substitutions per nucleotide position

a cluster with *B. subtilis* subsp. *stercoris* D7XPN1 and was classified as the species *B. subtilis*. The 16S rDNA gene sequence of *B. subtilis* CS13 was deposited in GenBank with a gene ID of MG722817. *B. subtilis* CS13 was deposited at the Korean Collection for Type Cultures with the accession number KCTC 14094 BP.

Effect of temperature and pH for co-production of 2,3-BD and γ-PGA

As shown in Fig. 3a, the biomass decreased from 5.2 ± 0.2 g/L to 3.2 ± 0.1 g/L with increasing temperature from 30 to 45 °C. The maximum concentration of γ-PGA reached 10.3 ± 0.3 g/L at 45 °C, and the concentration of 2,3-BD was 12.6 ± 0.4 g/L; meanwhile, the highest concentration of 2,3-BD (14.5 ± 0.5 g/L) was obtained at 37 °C with 9.8 ± 0.3 g/L of γ-PGA. The high temperature (45 °C) is favorable for γ-PGA production, but not conducive for 2,3-BD synthesis. Although the biomass was increased with the increase of pH from 5.5 to 8.0, the production of γ-PGA and 2,3-BD was significantly changed (Fig. 3b). *B. subtilis* CS13 showed the highest γ-PGA (9.7 ± 0.3 g/L) and 2,3-BD (14.4 ± 0.5 g/L) production at pH 6.5, followed by 6.0 and 7.0. Considering the production of 2,3-BD, the temperature of 37 °C and pH 6.5 were chosen as the optimum temperature and pH.

Effect of carbon sources and nitrogen sources for co-production of 2,3-BD and γ-PGA

All the tested carbon sources could promote cell growth, but the yield of γ-PGA and 2,3-BD were different (Fig. 3c). In the case of sucrose and glucose, a high yield of 2,3-BD (14.5 ± 0.5 and 14.3 ± 0.5 g/L) was attained, and the yield of γ-PGA was 9.8 ± 0.3 and 9.7 ± 0.3 g/L, respectively. Lactose was good for cell growth but did not affect the γ-PGA and 2,3-BD co-production. Glycerol was the best carbon source for the γ-PGA production, and 12.2 ± 0.4 g/L of γ-PGA accumulated; but interestingly, glycerol is unfavorable for the synthesis of 2,3-BD, and only 6.9 ± 0.2 g/L of 2,3-BD was produced. Thus, considering the yield of the 2,3-BD, sucrose as a cheap carbon source was chosen for the γ-PGA and 2,3-BD co-production.

Figure 3d shows the effects of different nitrogen sources on the γ-PGA and 2,3-BD co-production of *B. subtilis* CS13. All the nitrogen sources could be used by *B. subtilis* CS13 to synthesize 2,3-BD. In general, the strain preferred to utilize organic nitrogen sources showing improved cell growth and the inorganic nitrogen sources showed an enhanced γ-PGA production. Among the nitrogen sources tested, ammonium citrate yielded the highest γ-PGA (11.7 ± 0.4 g/L) and 2,3-BD (16.3 ± 0.5 g/L) production.

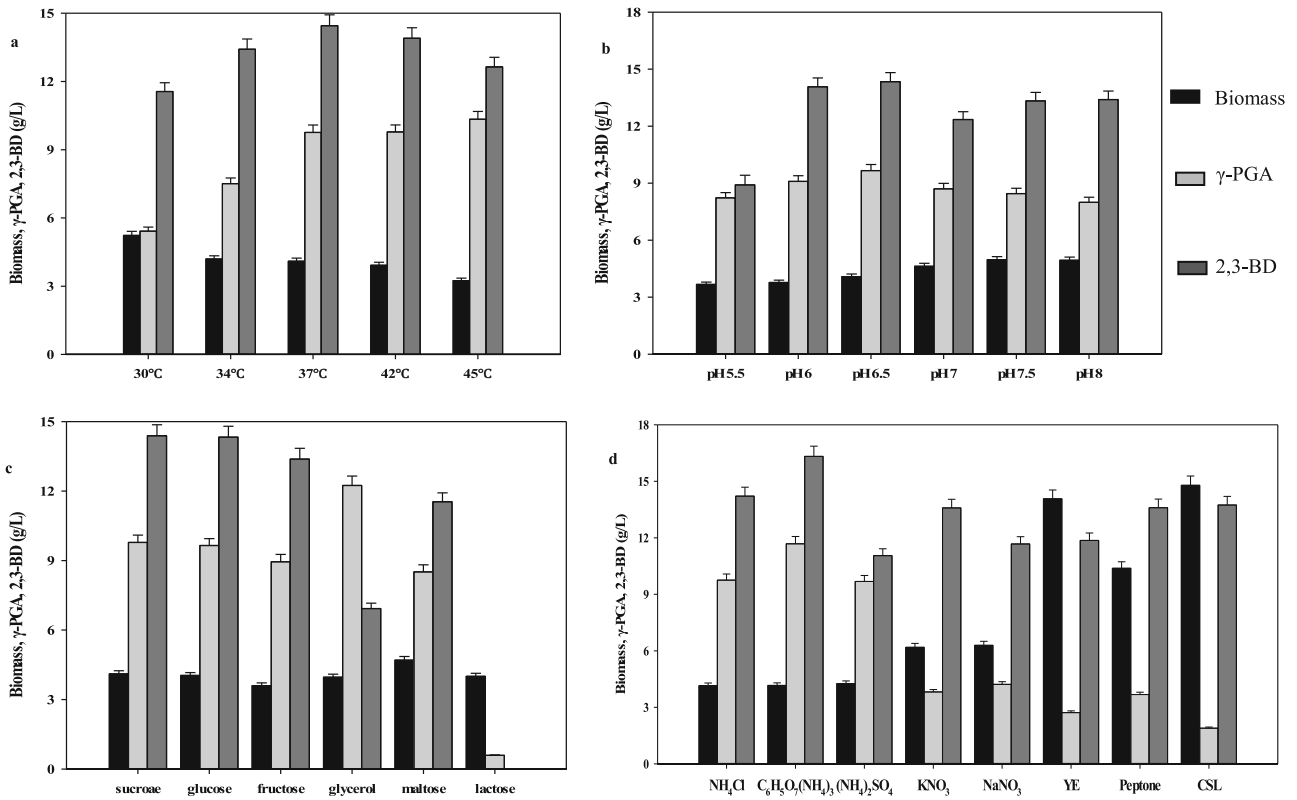


Fig. 3 Effect of (**a**) temperature, (**b**) pH, (**c**) carbon sources, and (**d**) nitrogen sources for the co-production of 2,3-BD and γ-PGA by *B. subtilis* CS13

Screening of significant nutrients by PBD for co-production

In the present study, *B. subtilis* CS13 produced the highest yield of γ-PGA (22.4 ± 0.6 g/L) and 2,3-BD (26.2 ± 0.6 g/L) in combinations 6 and 9 (Table 1), and the ANOVA is shown in Table 2. Three variables, namely, sucrose, L-glutamic acid, and ammonium citrate, influenced the γ-PGA fermentation process significantly ($P < 0.05$) and showed a positive coefficient (Table 2), suggesting that the levels for these variables can be increased to improve the γ-PGA production. In the process of 2,3-BD fermentation, sucrose, L-glutamic acid, ammonium citrate, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $MnCl_2 \cdot 4H_2O$ showed a significant effect. The coefficients of L-glutamic acid, $FeCl_3 \cdot 6H_2O$, and $MnCl_2 \cdot 4H_2O$ were negative, and lower concentrations of these chemicals are suggested for future experiments (Table 3). The coefficient of determination (R^2), which equaled 0.9941 and 0.9998 for γ-PGA and 2,3-BD, indicates that 99.41% and 99.98% of the variability in the response could be explained by the model. The high values of the adjusted determination coefficient ($R^2_{adj} = 0.9787$; 0.9993) imply a high significance of the model.

Optimization of the medium composition by RSM

Sucrose, L-glutamic acid, ammonium citrate, and $MgSO_4 \cdot 7H_2O$ were used for further optimization with a FCCD to maximize the γ-PGA and 2,3-BD production. The averages of the maximum values of γ-PGA and 2,3-BD were used as the responses after an 84-h fermentation with 30 experiments in triplicate, and the experimental design is shown in Table 4.

Table 4 The FCCD of the variables in coded and actual values with their response for co-production of γ-PGA and 2,3-BD

Run	Sucrose (g/L)	L-Glutamic acid (g/L)	Ammonium citrate (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	PGA (g/L)	2,3-BD (g/L)
1	− 1 (40)	− 1 (30)	1 (30)	− 1 (1)	20.29	20.15
2	− 1 (40)	− 1 (30)	1 (30)	1 (5)	20.74	17.67
3	1 (120)	1 (70)	− 1 (10)	− 1 (1)	24.47	51.5
4	1 (120)	− 1 (30)	1 (30)	1 (5)	25.09	56.78
5	0 (80)	0 (50)	0 (20)	0 (3)	27.34	34.64
6	1 (120)	1 (70)	1 (30)	− 1 (1)	23.35	54.29
7	0 (80)	0 (50)	0 (20)	0 (3)	27.66	34.42
8	1 (120)	1 (70)	1 (30)	1 (5)	23.78	55.63
9	1 (120)	− 1 (30)	− 1 (10)	1 (5)	23.16	54.14
10	0 (80)	0 (50)	− 1 (10)	0 (3)	25.01	32.45
11	− 1 (40)	− 1 (30)	− 1 (10)	− 1 (1)	17.75	13.94
12	− 1 (40)	− 1 (30)	− 1 (10)	1 (5)	17.36	18.2
13	0 (80)	0 (50)	0 (20)	0 (3)	26.48	35.22
14	− 1 (40)	1 (70)	− 1 (10)	− 1 (1)	20.51	13.94
15	1 (120)	− 1 (30)	− 1 (10)	− 1 (1)	23.16	52.28
16	0 (80)	0 (50)	0 (20)	− 1 (1)	26.79	32.45
17	1 (120)	− 1 (30)	1 (30)	− 1 (1)	25.02	55.43
18	− 1 (40)	1 (70)	1 (30)	− 1 (1)	21.51	16.96
19	0 (80)	0 (50)	1 (30)	0 (3)	26.47	33.63
20	− 1 (40)	1 (70)	− 1 (10)	1 (5)	20.72	15.2
21	0 (80)	0 (50)	0 (20)	0 (3)	27.51	35.39
22	− 1 (40)	1 (70)	1 (30)	1 (5)	21.82	17.37
23	0 (80)	0 (50)	0 (20)	0 (3)	26.68	34.83
24	0 (80)	1 (70)	0 (20)	0 (3)	26.54	34.44
25	1 (120)	0 (50)	0 (20)	0 (3)	27.99	56.38
26	0 (80)	0 (50)	0 (20)	0 (3)	26.87	34.06
27	− 1 (40)	0 (50)	0 (20)	0 (3)	23.24	19.22
28	0 (80)	0 (50)	0 (20)	1 (5)	26.98	33.89
29	0 (80)	− 1 (30)	0 (20)	0 (3)	25.76	34.73
30	1 (120)	1 (70)	− 1 (10)	1 (5)	24.51	53.23

Response surface of the γ-PGA yield

The regression models in the form of ANOVA are given in Table 5. The "Model *P* value" (< 0.0001) implies the models are significant. The values of the determination coefficient (R^2 = 0.9918) indicate a 99.18% variability in the γ-PGA yield. The high values of the adjusted determination coefficient (R^2_{adj} = 0.9842) advocate a good term fit for the models. The "Lack of Fit *P* value" (0.8992) indicates that the "Lack of Fit" was insignificant relative to the pure error. The "*P* value" (< 0.005) showed the significant influence of the coefficients. Among the model terms, sucrose (X_1), L-glutamic acid (X_2), ammonium citrate (X_3), the interaction term of sucrose and L-glutamic acid (X_1X_2), sucrose and ammonium citrate (X_1X_3), L-glutamic acid and ammonium citrate (X_2X_3), squared term of sucrose (X_1^2), L-glutamic acid (X_2^2), ammonium citrate (X_3^2), and $MgSO_4{\cdot}7H_2O$ (X_5^2) had a significant influence on the γ-PGA production (Table 5). The effect of the $MgSO_4{\cdot}7H_2O$ and its interaction between the other variables on the γ-PGA yield were not significant. The variables such as sucrose with a positive linear coefficient (2.03) indicate that the production of γ-PGA increased with the increasing concentration of sucrose. Whereas the negative squared coefficient of sucrose (− 1.77) suggests the existence of a maximum as a function of the sucrose concentration, and beyond this point, sucrose had an inhibitory effect (Table 5). According to ANOVA, the following polynomial quadratic equations were obtained in coded level:

$$Y_{(\gamma\text{-PGA})} = 27.24 + 2.03X_1 + 0.49X_2 + 0.63X_3 - 0.55X_1X_2 - 0.38X_1X_3 - 0.59X_2X_3 - 1.77X_1^2 - 1.23X_2^2 - 1.64X_3^2 - 0.50X_5^2$$

The surface response plots for the optimization of the fermentation conditions of γ-PGA were generated by holding two constants at the central point while keeping another two variables within the experiment range (Fig. 4). Figure 4a–c shows there were significant interactions of sucrose concentration with other parameters on the γ-PGA yield. The γ-PGA concentration (21–27 g/L) increased significantly when the sucrose concentration increased from 40 to 100 g/L. However, if the sucrose concentration is above 100 g/L, it will decrease the γ-PGA yield, which may be caused by the substrate limitation. A similar effect for ammonium citrate was observed; the ammonium citrate increased to the optimum point increased the γ-PGA production to the maximum level, and a further increase in the ammonium citrate decreased the γ-PGA production (Fig. 4b, d, f). Figure 4a, d, and e shows that a high γ-PGA yield could be achieved if the concentration of L-glutamic acid is from 30 to 50 g/L. The increase of the L-glutamic acid concentration could decrease the γ-PGA yield because L-glutamic acid at too high of a concentration could not be used by the strain and substrate limitation occurs. The γ-PGA yield did not change as the $MgSO_4{\cdot}7H_2O$ concentration increased due to its insignificant effect (Fig. 4c, e, f).

Ignoring the effect of $MgSO_4{\cdot}7H_2O$, the maximum value of γ-PGA was predicted by the "Numerical Optimization" tool of the Design-Expert 8.0.6 software. The predicted maximum γ-PGA yield was 27.8 g/L, which was obtained with a sucrose of 100.5 g/L, L-glutamic acid of 50.5 g/L, and ammonium citrate of 21.1 g/L.

Response surface of the 2,3-BD yield

The 2,3-BD concentration varied from 13.9 to 56.8 g/L for the 30 experiments shown in Table 4. The model was highly significant as the "Model *P* value" (< 0.0001). The determination coefficient (R^2 = 0.9984) and adjusted determination coefficient (R^2_{adj} = 0.9969) suggest good fits were achieved by the model (Table 6). The variables with obvious effect were sucrose (X_1), L-glutamic acid (X_2), ammonium citrate (X_3), $MgSO_4{\cdot}7H_2O$ (X_5), interaction term of ammonium

Table 5 The ANOVA for the γ-PGA production of FCCD

Factors	Mean square	Coefficient estimate	Standard error	*F* value	*P* value
Model	17.97	27.24	0.12	130.22	< 0.0001
X_1: sucrose	74.38	2.03	0.088	539.10	< 0.0001
X_2: L-glutamic acid	4.38	0.49	0.088	31.75	< 0.0001
X_3: ammonium citrate	7.25	0.63	0.088	52.51	< 0.0001
X_5: $MgSO_4{\cdot}7H_2O$	0.095	0.073	0.088	0.69	0.4189
X_1X_2	4.77	− 0.55	0.093	34.60	< 0.0001
X_1X_3	2.31	− 0.38	0.093	16.75	0.0010
X_1X_5	1.00×10^{-4}	-2.50×10^{-3}	0.093	7.25×10^{-4}	0.9789
X_2X_3	5.59	− 0.59	0.093	40.54	< 0.0001
X_2X_5	0.046	0.05	0.093	0.34	0.5713
X_3X_5	0.12	0.09	0.093	0.89	0.3610
X_1^2	8.10	− 1.77	0.23	58.67	< 0.0001
X_2^2	3.94	− 1.23	0.23	28.53	< 0.0001
X_3^2	6.99	− 1.64	0.23	50.67	< 0.0001
X_5^2	0.64	− 0.50	0.23	4.65	0.0477
Lack of fit	0.092			0.40	0.8992
R^2 = 0.9918	$R^2_{(adj)}$ = 0.9842		$R^2_{(Pred)}$ = 0.9756		

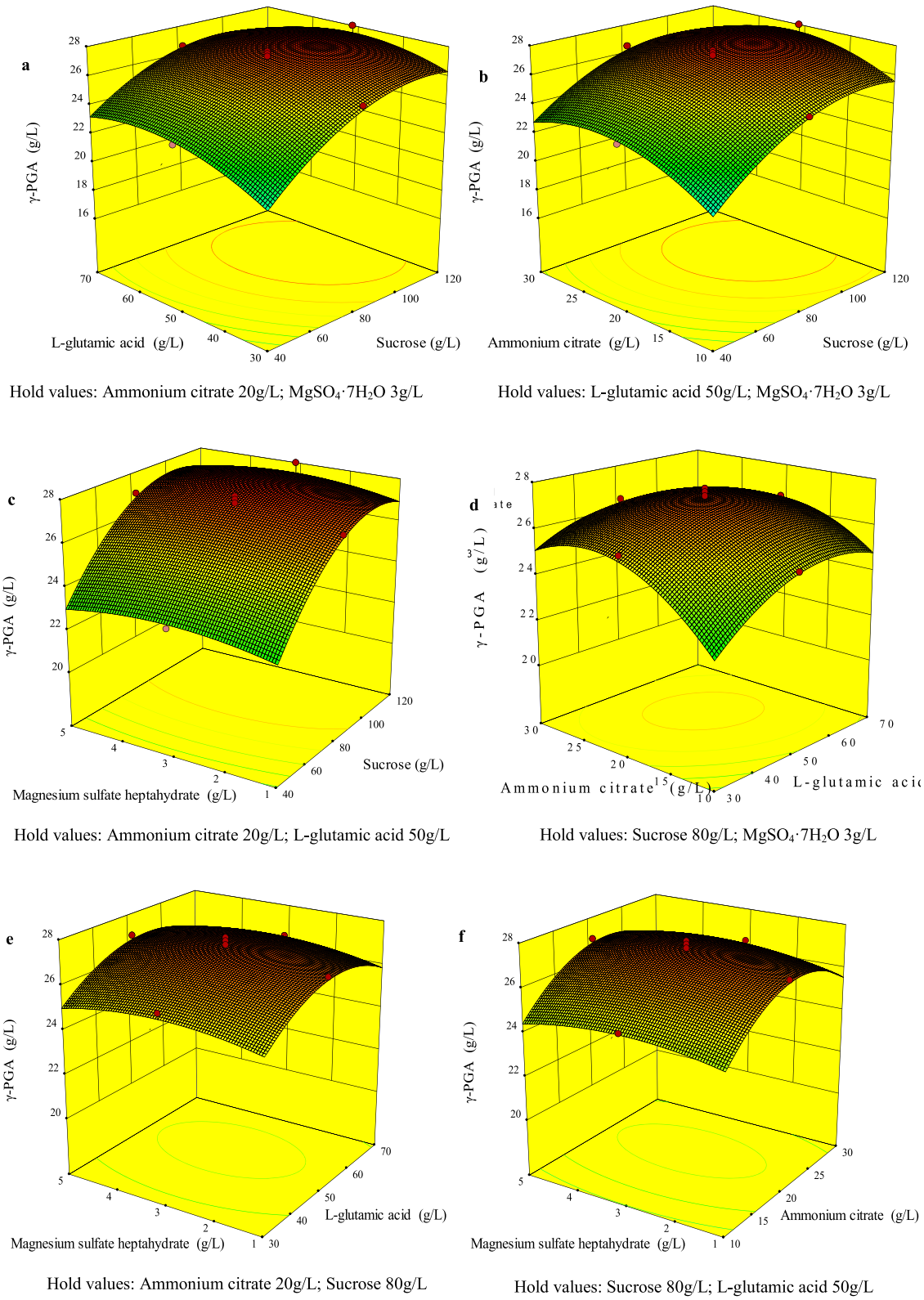


Fig. 4 Response surface and contour plots for γ-PGA production by *B. subtilis* CS13. (**a**) Effect of L-glutamic acid and sucrose, (**b**) effect of ammonium citrate and sucrose, (**c**) effect of $MgSO_4·7H_2O$ and sucrose, (**d**) effect of ammonium citrate and L-glutamic acid, (**e**) effect of $MgSO_4·7H_2O$ and L-glutamic acid, (**f**) effect of $MgSO_4·7H_2O$ and ammonium citrate

Table 6 The ANOVA for the 2,3-BD production of FCCD

Factors	Mean square	Coefficient estimate	Standard error	*F* value	*P* value
Model	456.51	34.61	0.26	664.10	< 0.0001
X_1: sucrose	6309.76	18.72	0.20	9179.15	< 0.0001
X_2: L-glutamic acid	6.43	− 0.60	0.20	9.36	0.0080
X_3: ammonium citrate	29.47	1.28	0.20	42.87	< 0.0001
X_5: $MgSO_4{\cdot}7H_2O$	6.93	0.62	0.20	10.08	0.0063
X_1X_2	0.39	0.16	0.21	0.57	0.4609
X_1X_3	7.56×10^{-4}	6.87×10^{-3}	0.21	1.10×10^{-3}	0.9740
X_1X_5	0.8	0.18	0.21	0.73	0.4069
X_2X_3	0.074	− 0.07	0.21	0.11	0.7469
X_2X_5	3.90×10^{-3}	− 0.02	0.21	5.68×10^{-3}	0.9409
X_3X_5	4.51	− 0.53	0.21	6.55	0.0218
X_1^2	29.08	3.35	0.52	42.30	< 0.0001
X_2^2	0.047	0.13	0.52	0.07	0.7968
X_3^2	5.15	− 1.41	0.52	7.49	0.0153
X_5^2	4.24	− 1.28	0.52	6.18	0.0252
Lack of fit	0.91			3.68	0.0816
$R^2 = 0.9984$	$R^2_{(adj)} = 0.9969$		$R^2_{(Pred)} = 0.9890$		

citrate and $MgSO_4{\cdot}7H_2O$ (X_3X_5), squared term of sucrose (X_1^2), ammonium citrate (X_3^2), and $MgSO_4{\cdot}7H_2O$ (X_5^2) (Table 6). The polynomial quadratic equation in the coded level was given by:

$$Y_{(2,3\text{–BD})} = 34.61 + 18.72X_1 - 0.60X_2 + 1.28X_3 + 0.62X_5 - 0.53X_3X_5 + 3.35X_1^2 - 1.41X_3^2 - 1.41X_3^2 - 1.28X_5^2$$

Figure 5a, b, and c deposited the interaction effect of L-glutamic acid, ammonium citrate, $MgSO_4{\cdot}7H_2O$, and sucrose on 2,3-BD production, respectively. The 2,3-BD concentration increased significantly when the sucrose concentration increased from 40 to 120 g/L, different from the other variables, and a high concentration of sucrose did not inhibit the 2,3-BD production within the experiment range. An increase in ammonium citrate and $MgSO_4{\cdot}7H_2O$ increased the 2,3-BD production gradually, and at a higher ammonium citrate and $MgSO_4{\cdot}7H_2O$ concentration, the trend was reversed but with a less significant tendency (Fig. 5d). L-Glutamic acid inhibited the production of 2,3-BD due to the negative linear coefficient (Table 6) and thus decreased the 2,3-BD concentration with an increase in L-glutamic acid from 30 to 70 g/L (Fig. 5e, f). The predicted highest value of 2,3-BD was 57.6 g/L, which was obtained at a sucrose of 120 g/L, L-glutamic acid of 30 g/L, ammonium citrate of 24.5 g/L, and $MgSO_4{\cdot}7H_2O$ of 3.6 g/L.

Optimization and validation of the medium for the γ-PGA and 2,3-BD co-production

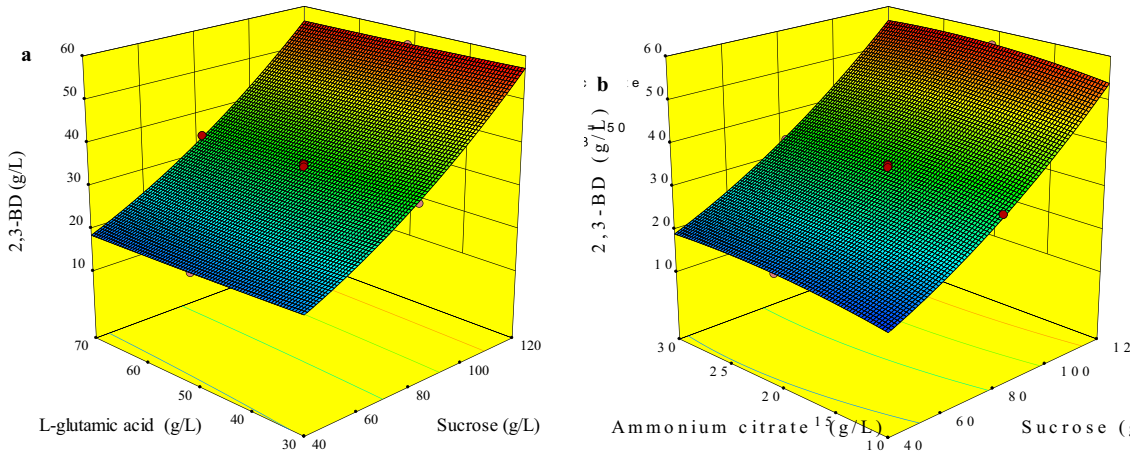
The optimum medium composition was calculated by a numerical iteration procedure using the Design-Expert 8.0.6 software. The optimum conditions for the maximum γ-PGA and 2,3-BD co-production was found to be 119.8 g/L sucrose, 48.9 g/L L-glutamic acid, 21.1 g/L ammonium citrate, and 3.2 g/L $MgSO_4{\cdot}7H_2O$. In this condition, the γ-PGA and 2,3-BD yields predicted by Design-Expert 8.0.6 software were 27.5 and 56.8 g/L, respectively. At the optimum level, the highest yields of γ-PGA and 2,3-BD were 27.8 ± 0.9 and 57.1 ± 1.3 g/L (Fig. 6), which were close to the predicted values.

Fed-batch fermentation

To further verify the potential application of γ-PGA and 2,3-BD co-production strategy, fed-batch culture was performed primarily. Figure 7a illustrates the changes of residual sugars, γ-PGA, 2,3-BD, biomass, and L-glutamic acid in the fed-batch process. The maximum biomass reached 12.2 ± 0.4 g/L at 60 h. The maximum γ-PGA concentration of 36.5 ± 1.1 g/L was obtained at 30 h with a productivity of 1.22 ± 0.04 g/L/h and the conversion rate of L-glutamic acid to γ-PGA up to 1.04 $g_{\gamma\text{-PGA}}/g_{\text{L-glutamic acid}}$. The highest concentration of 2,3-BD reached 119.6 ± 2.8 g/L at 48 h with a 2,3-BD yield (0.48 $g_{2,3\text{-BD}}/g_{\text{sucrose}}$) and productivity of 2.49 ± 0.06 g/L/h. Moreover, *B. subtilis* CS13 produced a mixture of meso-2,3-BD and D-2,3-BD in the proportion of 8.7:1.3 at 48 h (Fig. 7b). The production of acetoin begins after 24 h, and the final titer of acetoin was 13.8 ± 0.6 g/L (Fig. 7b).

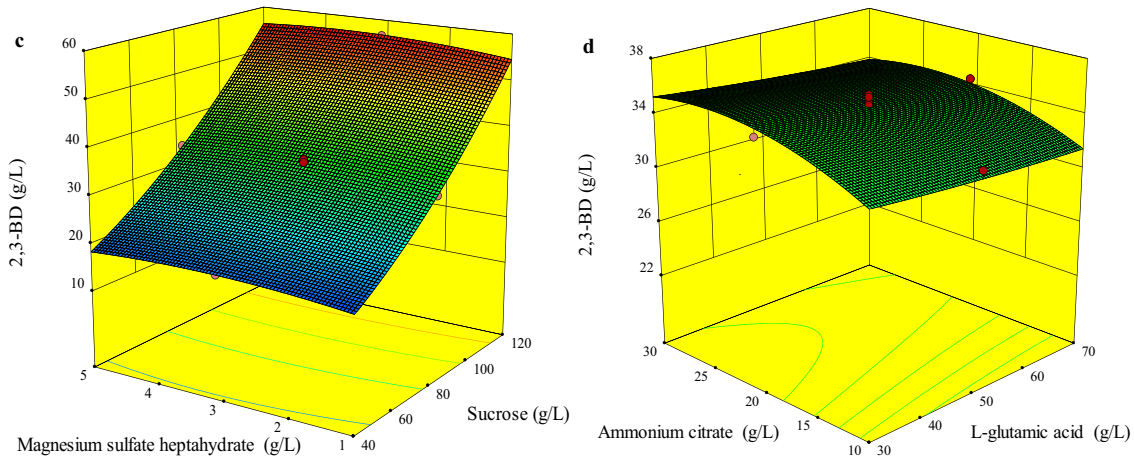
Discussion

2,3-BD and γ-PGA production is temperature dependent because of the dependence of the enzyme activity. The high biomass at lower temperatures might be caused by active energy metabolism (Zeng et al. 2014). Perego et al. found that butanediol production increased to the highest value when the



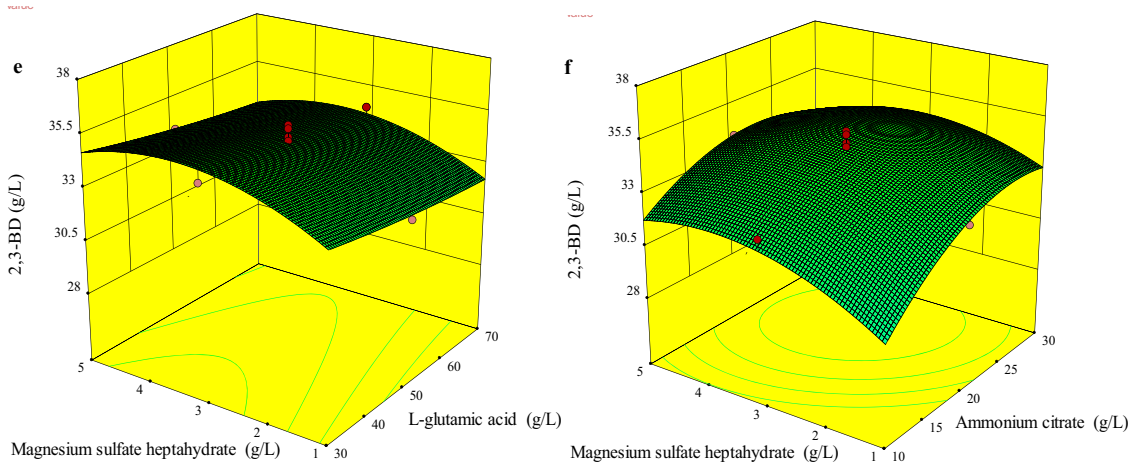
Hold values: Ammonium citrate 20g/L; $MgSO_4 \cdot 7H_2O$ 3g/L

Hold values: L-glutamic acid 50g/L; $MgSO_4 \cdot 7H_2O$ 3g/L



Hold values: Ammonium citrate 20g/L; L-glutamic acid 50g/L

Hold values: Sucrose 80g/L; $MgSO_4 \cdot 7H_2O$ 3g/L



Hold values: Ammonium citrate 20g/L; Sucrose 80g/L

Hold values: Sucrose 80g/L; L-glutamic acid 50g/L

Fig. 5 Response surface and contour plots for 2,3-BD production by *B. subtilis* CS13. (**a**) Effect of L-glutamic acid and sucrose, (**b**) effect of ammonium citrate and sucrose, (**c**) effect of $MgSO_4 \cdot 7H_2O$ and sucrose, (**d**) effect of ammonium citrate and L-glutamic acid, (**e**) effect of $MgSO_4 \cdot 7H_2O$ and L-glutamic acid, (**f**) effect of $MgSO_4 \cdot 7H_2O$ and ammonium citrate

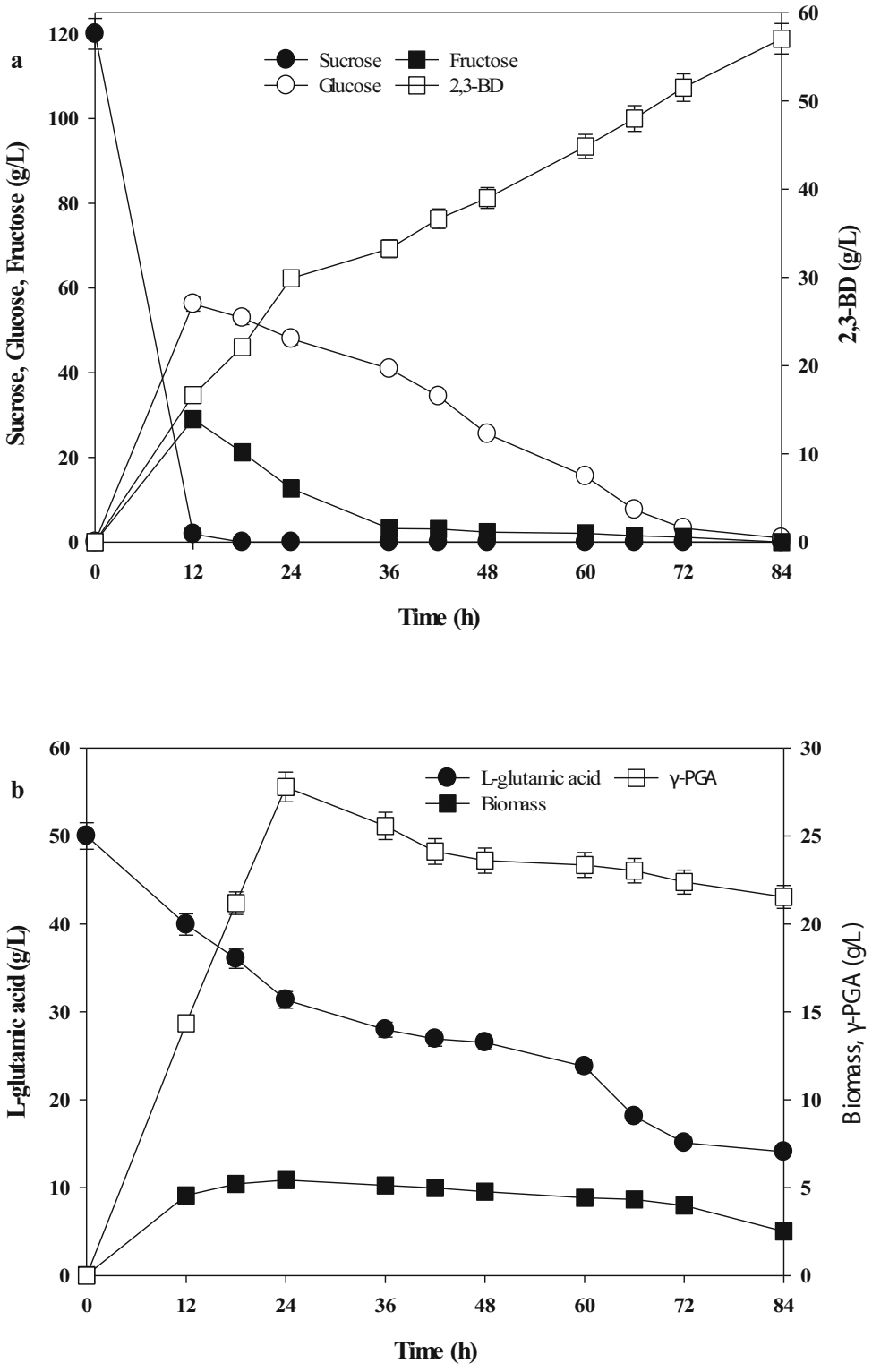


Fig. 6 Time courses of (**a**) sucrose, glucose, fructose, 2,3-BD; (**b**) L-glutamic acid, citric acid, biomass, and γ-PGA production under the optimized medium

temperature was increased to 37 °C and decreased over 37 °C (Perego et al. 2003). This phenomenon was consistent with our results. Interestingly, the highest γ-PGA production was obtained at 45 °C. According to the reported literature, the high temperature (45 °C) increased the activity of isocitrate dehydrogenase (ICDH) and glutamate dehydrogenase (GDH), which led to an enhanced γ-PGA production (Zeng et al. 2014). Moreover, the high temperature can reduce the molecular weight of γ-PGA, decrease the viscosity, and improve mass transfer.

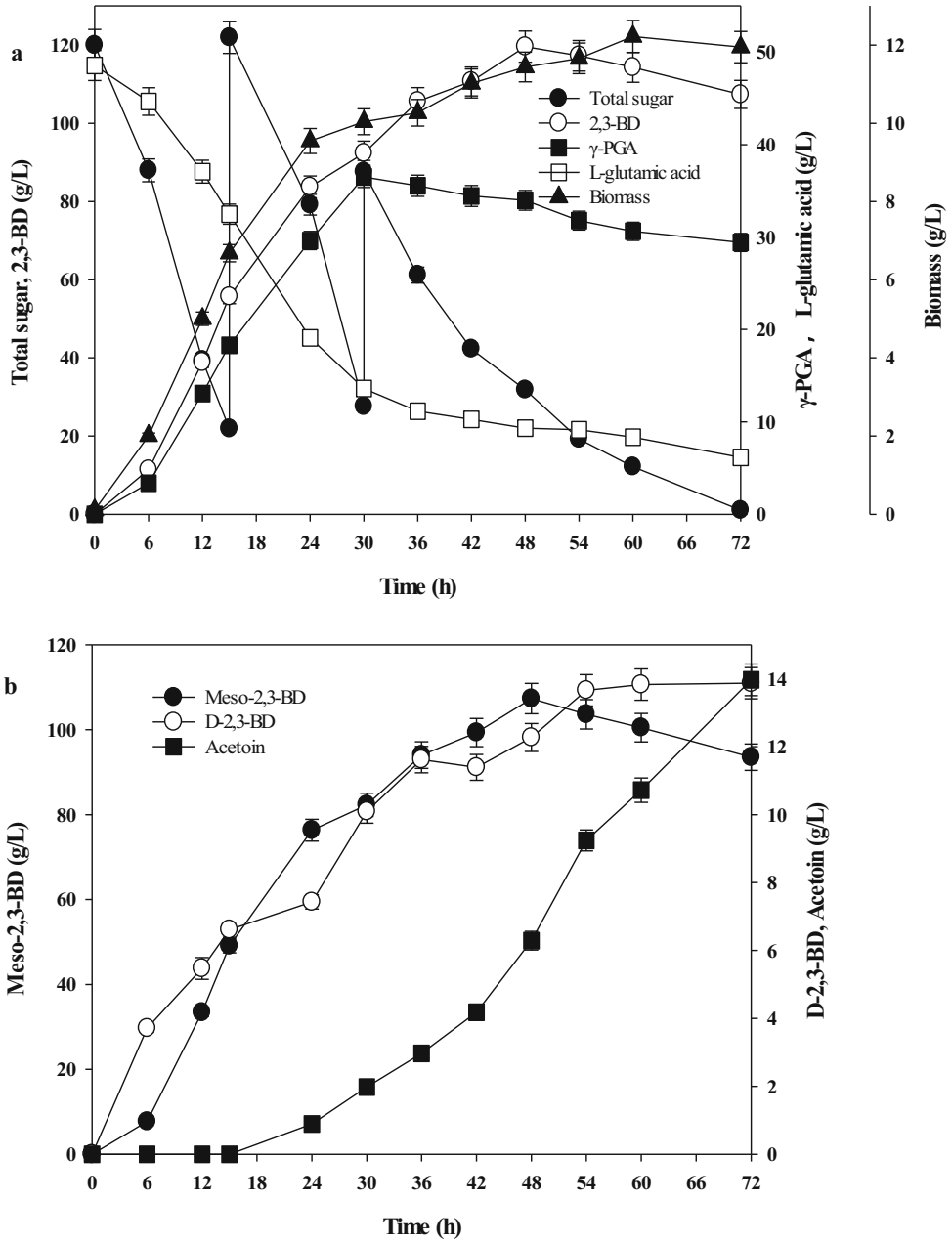


Fig. 7 Time courses of (**a**) total sugar, 2,3-BD, γ-PGA, L-glutamic acid, biomass; (**b**) meso-2,3-BD, D-2,3-BD, and acetoin production under the optimized medium in fed-batch fermentation

pH influences bacterial metabolism and the formation of products. Zhu et al. found that 2,3-BD was a major by-product at pH 6.5 and 7.3 during γ-PGA fermentation. In contrast, the synthesis of 2,3-BD was limited, and acetoin had a high concentration at pH 5.7 (Zhu et al. 2013). However, for the *B. subtilis* CS13 in this study, a low concentration of acetoin (< 0.5 g/L) was detected, and the low pH only decreased the yield of 2,3-BD, which may be caused by the high viscosity of broth promoting metabolic flux from acetoin to 2,3-BD.

Most of the γ-PGA producers prefer glucose and glycerol as carbon sources. In our results (Fig. 3c), glycerol obviously increased the yield of γ-PGA but reduced the yield of 2,3-BD compared with sucrose or glucose. Sucrose is mainly derived from sugar cane and sugar beet, and is a cheap carbon source for biochemical production through microbial fermentation (Jiang et al. 2014; Zhang et al. 2017). Additionally, sucrose was a popular carbon source for production of 2,3-BD (Feng et al. 2017). Nitrogen sources promote cell growth and γ-PGA synthesis but are strain dependent, which may be caused by differences in metabolism. Previous reports have found that L-glutamic acid and citric acid as precursors successfully improve γ-PGA production (Ashiuchi 2010); moreover, *B. subtilis* CS13 belongs to this group. Ammonium citrate improved the production of γ-PGA and 2,3-BD (Fig. 3d), suggesting that citrate enhanced the production of α-ketoglutarate as the precursor for glutamate and γ-PGA in

Table 7 2,3-BD production by *B. subtilis* strain

Strain	Culture	Production (g/L)	Productivity (g/L/h)	Conversion rate ($g_{2,3\text{-BD}}/g_{\text{carbon source}}$)	Reference
B. subtilis 168 (BSF9)	Fed batch	103.7	0.46	0.48	(Fu et al. 2016)
B. subtilis ACR	Batch	40.9	0.68	0.39	(Yang et al. 2013)
B. subtilis LOCK 1086	Fed batch	75.73	0.66	0.31	(Białkowska et al. 2016)
B. Subtilis FJ-1-CEG	Batch	44.6	0.37	0.45	(Feng et al. 2017)
B. subtilis JCM10629	Batch	21.2	0.29	0.35	(Tanimura et al. 2016)
B. subtilis CS13	Fed batch	119.6	2.49	0.48	This work

the TCA metabolism (Ashiuchi 2010). NH_4^+ enhanced glutamate metabolism conversion more of NADPH to $NADP^+$, therefore, it increased the NADH generation and promoted the synthesis of 2,3-BD (Fig. 1). L-α-Acetolactate synthase is a key enzyme from pyruvate to acetoin and 2,3-BD, and the activity of the enzyme is dependent on Mg^{2+} (Poulsen and Stougaard 1989). Therefore, $MgSO_4{\cdot}7H_2O$ showed a positive and significant effect on 2,3-BD synthesis (Tables 3 and 6).

This design successfully utilizes the metabolic pathways and fermentation conditions of γ-PGA and 2,3-BD. Sucrose was rapidly hydrolyzed to glucose and fructose before 12 h due to the sucrose utilization systems (Reid and Abratt 2005). All of the sugars were exhausted at 84 h, and 57.1 ± 1.3 g/L 2,3-BD was obtained with a productivity of 0.68 g/L/h, and the conversion rate of sucrose to 2,3-BD was 0.48 $g_{2,3\text{-BD}}/g_{\text{sucrose}}$ (Fig. 6a). As shown in Fig. 6b, the maximum γ-PGA concentration reached 27.8 ± 0.9 g/L at 24 h of fermentation, and then showed a slow decline, suggesting the γ-PGA depolymerase was activated (Yao et al. 2009). Furthermore, compared with the batch culture in flasks, fed-batch operation achieved a high γ-PGA (36.5 ± 1.1 g/L) and 2,3-BD (119.6 ± 2.8 g/L) production. Meanwhile, the productivity of γ-PGA and 2,3-BD improved from 1.16 ± 0.04 g/L/h to 1.22 ± 0.04 g/L/h and 0.68 ± 0.02 g/L/h to 2.49 ± 0.06 g/L/h, respectively. The fermentor overcomes the substrate shortages and provides comfortable fermentation conditions (dissolved oxygen and pH) which are crucial to γ-PGA and 2,3-BD production. Our results proved that co-production of γ-PGA and 2,3-BD has potential application in industry, and further research will focus on the fermentation conditions on a large-scale fermentor.

Many *B. subtilis* species have the ability of produce γ-PGA and 2,3-BD individually. Until now, the highest concentration of 2,3-BD (103.7 g/L) was produced by *B. subtilis* 168 with a productivity of 0.46 g/L/h (Table 7). *B. subtilis* NX-2, *B. subtilis* MJ80, and *B. subtilis* ZJU7 have good advantages in the final titers of γ-PGA, but the productivities were lower than *B. subtilis* CS13 (Table 8). Compared with these previously reported systems, the co-production by *B. subtilis* CS13 has the highest final 2,3-BD level and highest productivities of γ-PGA and 2,3-BD. This might be caused by the co-production of γ-PGA and 2,3-BD in the optimized medium which balanced the production and consumption of cofactors perfectly (Fig. 1). Actually, 2,3-BD is the major by-product during γ-PGA production by *Bacillus* species. Previous researches have focused on reducing the production of 2,3-BD by regulating metabolic flux during γ-PGA production, enhancing NADPH generation (Cai et al. 2017), supplementing

Table 8 γ-PGA production by *B. subtilis* strain

Strain	Culture	Production (g/L)	Productivity (g/L/h)	Conversion rate ($g_{\gamma\text{-PGA}}/g_{\text{glutamic acid}}$)	Reference
B. subtilis NX-2	Fed batch	42	0.35	1.05	(Yao et al. 2010)
B. subtilis BL53	Batch	23.5	0.98	–	(de Cesaro et al. 2014)
B. subtilis CGMCC 0833	Batch	27.7	0.38	0.94	(Wu et al. 2010)
B. subtilis MJ80	Batch	75.5	1.05	1.08	(Ju et al. 2014)
B. subtilis D7	Batch	24.9	0.69	–	(Lee et al. 2014)
B. subtilis ZJU7	Fed batch	54	0.84	–	(Chen et al. 2010)
B. subtilis CS13	Fed batch	36.5	1.22	1.04	This work

nitrate (Li et al. 2014), and controlling the pH in the γ-PGA fermentation process (Zhu et al. 2013). Compared with previous studies, we believe that the co-production of 2,3-BD with a value-added γ-PGA is a more novel direction.

This research not only provides a new strategy to obtain γ-PGA and 2,3-BD simultaneously but also demonstrates the potential for industrial scale. The design utilizes the properties of the products, reduces the production cost, and simplifies the steps of industrial separation and purification. In the fermentation downstream processing, 2,3-BD is difficult to separate from the fermentation broth due to its high boiling point (180–184 °C) and high affinity for water (Jiang et al. 2009). The steam stripping and vacuum distillation methods consume a large amount of energy, preventing its application in the industrial separation of 2,3-BD (Shao and Kumar 2011). A novel aqueous two-phase extraction showed a good advantage because of the low energy, but it requires a large amount of ethanol (Jiang et al. 2009; Li et al. 2010). To separate γ-PGA, large amounts of ethanol usually need to be added to precipitate the γ-PGA after removing the biomass by centrifugation (Luo et al. 2016). 2,3-BD dissolved in ethanol could be separated from water by aqueous two-phase extraction (Jiang et al. 2009; Li et al. 2010). First, trichloroacetic acid (TCA) solution is added to the fermentation broth to separate the cells and proteins by centrifugation; second, γ-PGA is obtained through alcohol precipitation and drying and then, the alcohol and 2,3-BD mixture are obtained by aqueous two-phase extraction. The mixture could be used as a fuel or by distillation separation. The co-production of 2,3-BD with a value-added γ-PGA improves the economics of the fermentation process and facilitates its application.

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Author's contributions D.X.W. performed the experiments. D.X.W. and M.-H.J. designed the experiments, analyzed the data, and drafted the manuscript. H.M.K., S.B.L., D.-H.K., and M.-H.J. designed and guided the study, and edited the manuscript. All authors read and approved the final manuscript.

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 conflicts of interest.

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