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

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Research

Abstract

Background: *Bacillus subtilis* naturally produces large amounts of 2,3-butanediol (2,3-BD) as the main byproduct during poly-γ-glutamic acid (γ-PGA) fermentation using carbon sources. 2,3-BD is a promising platform chemicals in various industries, and co-production has great economic benefits. Thus, co-production of poly-γ-glutamic acid (γ-PGA) and 2,3-butanediol (2,3-BD) by *Bacillus subtilis* were investigated for the first time.

Results: In this study, a novel *Bacillus subtilis* CS13 was isolated that can efficiently co-production of γ-PGA and 2,3-BD. The fermentation medium and culture parameters by *B. subtilis* CS13 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 pH 6.5 and 37 °C. A medium composed of 119.83 g/L sucrose, 48.85 g/L L-glutamic acid, 21.08 g/L ammonium citrate, and 3.21 g/L $MgSO_4 \cdot 7H_2O$ was optimized by response surface methodology (RSM). The results show that the yields of γ-PGA and 2,3-BD reached 27.79 ± 0.87 g/L at 24 h and 57.05 ± 1.28 g/L at 84 h with the optimized medium, respectively.

Conclusions: To our knowledge, the co-production of 2,3-BD and γ-PGA will reduce the costs of production and separation in theory and provide a new perspective for industrial production of γ-PGA and 2,3-BD. *B. subtilis* CS13 as a generally recognized as safe (GRAS) strain, has great promise for the co-production of 2,3-BD and γ-PGA.

Background

Sucrose is a disaccharide composed of one molecule of a glucose unit linked to one molecule of a fructose unit by a glycosidic bond. It is the most abundant disaccharide in nature because it is present in the tissues of many plants as a way to store energy [1]. Sucrose is mainly derived from sugar cane and sugar beet, and is a cheap carbon source for biochemical production through microbial fermentation [2, 3].

B. subtilis consists of a sucrose metabolic system that hydrolyzes sucrose to glucose-6-phosphate and fructose and then synthesizes pyruvate by glycolysis [4]. Pyruvate is converted by acetolactate synthase (ALS) and acetolactate decarboxylase (ALD) to acetoin, which is then reduced to 2,3-butanediol (2,3-BD) by 2,3-BD dehydrogenase (BDH). 2,3-BD as an important chemical has applications in food, pharmaceutical, cosmetics, fine chemical and other industries. Additionally, 2,3-BD can be used as a fuel due to its high heat energy [5, 6]. In recent years, microbial fermentation for 2,3-BD production has been reported in a series of bacteria species, such as Klebsiella [7], Enterobacter [8], Paenibacillus [9], and Bacillus [10–12]. B. subtilis is a GRAS (generally regarded as safe) strain and also is considered to be a promising microbe for the production of 2,3-BD [13, 14].

In general, B. subtilis is also a poly-γ-glutamic acid (γ-PGA) producer [15, 16]. B. subtilis converts pyruvate to α-ketoglutarate via tricarboxylic acid cycle (TCA), followed by the production of glutamic acid as the

precursor to the synthesis γ-PGA. Most of the B. subtilis belong to the glutamate-dependent strains, and as such, only supplementation of exogenous L-glutamic acid can produce γ-PGA. In addition, adding citric acid to enhance the TCA cycle is an effective strategy to increase the γ-PGA production [17]. 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 nontoxicity to humans and the environment [18–20].

Due to the metabolic pathways, an attempt to produce 2,3-BD and γ-PGA simultaneously was proposed and investigated for the first time. In theory, low dissolved oxygen could enhance 2,3-BD production [14]; meanwhile, the produced γ-PGA increases the viscosity of the broth and thus decreases the dissolved oxygen. 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 [21]. To separate γ-PGA, large amounts of ethanol usually need to be added to precipitate the γ-PGA after removing the biomass by centrifugal [22]. 2,3-BD dissolved in ethanol could be separated from water by aqueous two-phase extraction [21]. Co-production of 2,3-BD and γ-PGA would have various advantages in industrial production, such as reducing the utilization of fossil fuels and the production and separation costs.

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. This study provides a new perspective on theoretical research and industrial applications.

Methods

Isolation and identification of the 2,3-BD and γ-PGA co-producing strain

B. subtilis strains were isolated from the Chung-kook-jang sauce, which is a traditional Korean food, purchased at a local market (Jeongeup-si, South Korea). Samples of 10 grams 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 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$_4$Cl, 1 g/L KH$_2$PO$_4$, 0.5 g/L MgSO$_4$·7H$_2$O, 0.04 g/L FeCl$_3$·6H$_2$O, 0.15 g/L CaCl$_2$·2H$_2$O, 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 2,3-BD and γ-PGA in the broth were measured, and the strains with the highest γ-PGA and 2,3-BD yield were chosen for further study.

The 16S rDNA gene of the isolated strains was amplified, sequenced, and analyzed as previously described [23]. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0.

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

The isolated strain was first cultured in the 50 ml tubes containing 15 ml basal medium at 37 °C, 200 rpm for 12 h. Then, the optical density of the seed cultures were 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 influence of dissolved oxygen on the 2,3-BD and γ-PGA co-production was investigated by controlling the agitation at 150, 200, and 250 rpm. Flasks were incubated at 37 °C with an initial pH of 6.5, and the agitation that promoted the highest γ-PGA production was used for all subsequent steps of the investigation. The effects of temperature were studied at 30, 34, 37, 42, and 45 °C at the determined optimum agitation 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 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. Incubation was conducted at the determined optimum agitation and temperature. The optimum agitation, 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, sodium citrate, ammonium chloride, potassium nitrate, sodium nitrate, yeast extract (YE), peptone, beef extract, and corn steep liquor (CSL) 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 the Plackett-Burman design

The Plackett-Burman design (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 agitation, temperature, and initial pH were used. Eight variables including sucrose, L-glutamic acid, ammonium citrate, KH2PO4, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, 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 shown in Table 1. The true values of each variable and its analysis of variance (ANOVA) are shown in Table 2.

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 2a

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

Factors	Concentration (g/L) Lower	Concentration (g/L) Higher	Mean square	Coefficient estimate	Standard error	F-value	P-value
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
$R^2 = 0.9941$ $R^2_{(adj)} = 0.9784$ $R^2_{(Pred)} = 0.9056$							

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	16440.63	< 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
$R^2 = 0.9998$ $R^2_{(adj)} = 0.9993$ $R^2_{(Pred)} = 0.9971$							

Optimization of the medium composition by response surface methodology

A face-centered central composite design (FCCD) was used for the response surface methodology (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 the other components in the medium were fixed at the same concentration described in Sect. 2.1. 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.

Table 3

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$_4$·7H$_2$O (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

Analytical methods

The biomass was determined according to the standard calibration curve between the OD$_{600}$ and dry cell weight (DCW) by measuring the OD$_{600}$ of the broth [23].

γ-PGA was purified by the ethanol precipitation method as previously described [23]. The concentration of γ-PGA was determined by an Agilent 1100 high-performance liquid chromatography (HPLC) system using a PL aquagel-OH gel permeation chromatogram (GPC) column (300 × 7.5 mm; Polymer Laboratories Ltd., UK) and refractive index detector (RID). 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, citric acid, glucose, and fructose in the broth was 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$_2$SO$_4$ and HPLC grade water, respectively, and the flow rate was 0.6 ml/min.

Results And Discussion

Isolation and identification of the 2,3-BD and γ-PGA co-producing strain

Based on the characteristics of the colonies which were highly mucoid, fifteen 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. Moreover, strain CS13 produced the highest concentration of γ-PGA (9.58 ± 0.28 g/L) (Additional file 1:Table S1). In addition, high concentrations of by-products were found, and one was identified as 2,3-BD during an analysis of residual sugars in the fermentation broth (Additional file 2: Fig. S1). Strain CS11 produced the highest yield of 2,3-BD (15.97 ± 0.51 g/L), and strain CS13 could yield 14.16 ± 0.45 g/L of 2,3-BD. 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. 1. CS13 formed 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.

B. subtilis CS13 is a glutamate-dependent γ-PGA producer, and no γ-PGA was produced in the medium in the absence of L-glutamic acid. The yield of γ-PGA could be controlled by the concentration of exogenous L-glutamic acid thereby controlling the 2,3-BD concentration, which is an interesting phenomenon. Until now, several B. subtilis strains have been isolated from various materials, which can produce either γ-PGA or 2,3-BD, but their co-production has rarely been studied.

Effect of culture parameters for the co-production of 2,3-BD and γ-PGA
Effect of agitation
To investigate the effect of dissolved oxygen on the effect of the γ-PGA and 2,3-BD co-production in flasks, various agitations at 150, 200, and 250 rpm were used for the shakers. The results are shown in Fig. 2a. The biomass showed an increase as the agitation increased up to 250 rpm; however, the highest concentrations of γ-PGA (9.79 ± 0.13 g/L) and 2,3-BD (14.20 ± 0.34 g/L) were obtained at 200 rpm, and there was no significant difference between agitation at 200 and 250 rpm. The low agitation (150 rpm) obviously inhibited the yield of γ-PGA and 2,3-BD.
The high viscosity of the fermentation broth led to a low efficiency of oxygen transfer, and a higher agitation speed improved the oxygen and nutrient supply to the cells. On the other hand, the high viscosity fermentation broth caused the phenomenon of "out-of-phase" operating conditions, significantly reducing the oxygen transfer and mixing intensity [24]; thus, similar results were obtained at 200 and

250 rpm. Previous studies have found that higher dissolved oxygen is beneficial to the production of γ-PGA while lower dissolved oxygen is favorable for 2,3-BD synthesis [14, 25]. To explore the effect of dissolved oxygen and maximize the yield of γ-PGA and 2,3-BD, further research will be carried out in a fermentor. In this study, 200 rpm was used for all subsequent experiments.

Effect of temperature

Figure 2b shows the fermentation results at different temperatures. The biomass decreased from 5.23 ± 0.17 g/L to 3.24 ± 0.09 g/L with increasing temperature from 30 to 45 °C. The high biomass at lower temperatures might be caused by active energy metabolism [15]. The maximum concentration of γ-PGA reached 10.34 ± 0.32 g/L at 45 °C, and the concentration of 2,3-BD was 12.64 ± 0.42 g/L; meanwhile, the highest concentration of 2,3-BD (14.45 ± 0.48 g/L) was obtained at 37 °C with 9.76 ± 0.29 g/L of γ-PGA. The high temperature (45 °C) is favorable for γ-PGA production, but not conducive for 2,3-BD synthesis. 2,3-BD and γ-PGA production are temperature-dependent because of the dependence of the enzyme activity. Perego et al. found that butanediol production increased to the highest value when the temperature was increased to 37 °C and decreased over 37 °C [26]. This phenomenon was consistent with our results. Interestingly, the highest γ-PGA production was obtained at 45 °C. According to the reported literature, each strain has its optimum temperature. B. subtilis (chungkookjang) could produce γ-PGA at 30 °C [27]. Most strains produce γ-PGA at an optimum temperature of 37 °C, such as B. subtilis NX-2, B. subtilis HB-1, and B. subtilis CGMCC 2108 [25, 28, 29]. The optimum temperature for B. subtilis GXA-28 to produce γ-PGA was 45 °C [15], consistent with our result. The high temperature (45 °C) increased the activity of isocitrate dehydrogenase (ICDH) and glutamate dehydrogenase (GDH), which led to an enhanced γ-PGA production. Moreover, the high temperature can reduce the molecular weight of γ-PGA, decrease the viscosity, and improve mass transfer. Considering the production of 2,3-BD, the temperature of 37 °C was chosen as the optimum temperature.

Effect of pH

pH is important in fermentation; thus, the synthesis of γ-PGA and 2,3-BD were investigated at all the tested pH values (5.0−8.5). The results show that although the biomass was increased with the increase of pH from 5.0−8.5, the production of γ-PGA and 2,3-BD was significantly changed (Fig. 2c). B. subtilis CS13 showed the highest γ-PGA (9.66 ± 0.31 g/L) and 2,3-BD (14.37 ± 0.45 g/L) production at pH 6.5, followed by 6.0 and 7.0. Nevertheless, γ-PGA and 2,3-BD co-production can adapt to a wide range of pH from 6.0 to 7.5, and more acidity and alkalinity all affect the biosynthesis of γ-PGA and 2,3-BD.

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 [30]. 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 was maybe caused by the high viscosity of broth promoting metabolic flux from acetoin to 2,3-BD. Finally, pH 6.5 was set as the pH for the next experiments.

Effect of carbon sources

The effect of different carbon sources on the γ-PGA and 2,3-BD co-production was investigated by adding sucrose, glucose, fructose, glycerol, maltose, and lactose to the medium at 30 g/L individually, and the results are shown in Fig. 2d. All the tested carbon sources could promote cell growth, but the yield of γ-

PGA and 2,3-BD were different. In the case of sucrose and glucose, a high yield of 2,3-BD (14.49 ± 0.47 and 14.33 ± 0.46 g/L) was attained, and the yield of γ-PGA was 9.78 ± 0.32 and 9.65 ± 0.28 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 12.24 ± 0.39 g/L of γ-PGA accumulated; but interestingly, glycerol is unfavorable for the synthesis of 2,3-BD, and only 6.93 ± 0.23 g/L of 2,3-BD was produced.

Most of the γ-PGA producers prefer glucose and glycerol as carbon sources. Glucose is utilized mainly to supply the required energy and as a substrate for cell growth during γ-PGA production [31]. Glycerol is used not only as a substrate but also has some functions to enhance the γ-PGA synthesis, such as stimulate polyglutamyl synthetase, improve the permeability of cell membranes, and decrease the broth viscosity [32]. In our results (Fig. 2d), glycerol obviously increased the yield of γ-PGA but reduced the yield of 2,3-BD compared to sucrose or glucose. Actually, glycerol has been used in the production of 2,3-BD by B. amyloliquefaciens, and improved 2,3-BD production through metabolic manipulation [33]. In the utilization of sucrose, B. subtilis NX-2 showed a good advantage to improve the γ-PGA production [16]. Additionally, sucrose was a popular carbon source for production of 2,3-BD [13]. 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.

Effect of nitrogen sources

Figure 2e 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 synthesis 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 (11.68 ± 0.38 g/L) and 2,3-BD (16.32 ± 0.53 g/L) production. Citrate improved the production of γ-PGA suggesting that citrate enhanced the production of α-ketoglutarate as the precursor for glutamate and γ-PGA in the TCA metabolism [34]. Sodium citrate as a nitrogen source showed a poor result for γ-PGA production (4.99 ± 0.16 g/L), which was caused by the lack of ammonium in the medium. B. subtilis are capable of forming glutamate only in the presence of ammonium and 2-oxoglutarate in vitro [35, 36]. The result also suggests the glutamate used to synthesize γ-PGA comes from an external sucrose and metabolic synthesis. In the present study, B. subtilis CS13 was able to use ammonium sulfate and ammonium chloride for γ-PGA production (Fig. 2e), which is consistent with B. subtilis TAM-4 [37], B. subtilis NX-2 [31] and B. subtilis HSF1410 [36] but different from B. methylotrophicus SK19.001 [38]. Nitrogen sources promote cell growth and γ-PGA synthesis but are strain dependent, which may be caused by differences in metabolism. The results also proved that ammonium citrate was the best for 2,3-BD production, perhaps the high viscosity led to the metabolism necessary for the 2,3-BD synthesis. Previous research also found that ammonium citrate showed significant effects on 2,3-BD production in B. amyloliquefaciens B10-127 [39], but the mechanism is not clear.

Screening of significant nutrients by Plackett-Burman design for co-production

In the present study, B. subtilis CS13 produced the highest yield of γ-PGA (22.37 ± 0.56 g/L) and 2,3-BD (26.23 ± 0.62 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 2a), 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 coefficient 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 2b). The coefficient of determination (R^2), which equaled 0.9941 and 0.9998 for γ-PGA and 2,3-BD, indicating 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. The model equations for the γ-PGA and 2,3-BD yield were obtained from the PBD experiments:

$$Y_{(\gamma\text{-PGA})} = 17.53 + 2.59X_1 + 1.62X_2 + 1.16X_3$$

$$Y_{(2,3-BD)} = 16.83 + 7.26X_1 - 0.20X_2 + 0.49X_3 + 0.78X_5 - 0.19X_6 - 0.24X_7$$

where X_1, X_2, X_3, X_5, X_6, and X_7 represent sucrose, L-glutamic acid, ammonium citrate, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $MnCl_2 \cdot 4H_2O$ in the coded level, respectively.

Previous reports have found that L-glutamic acid and citric acid as precursors successfully improve the γ-PGA production [17]; moreover, B. subtilis CS13 belongs to this group. Metallic ions have been shown to have an important role in γ-PGA synthesis. K^+ and Ca^{2+} could increase the activity of enzymes around 2-oxoglutarate in the γ-PGA biosynthesis branch [29, 40]; Fe^{3+} could enhance the expression of γ-PGA synthetase genes (pgs ABC) [41]. However, for B. subtilis CS13, these metallic ions do not affect the γ-PGA production (Table 2b), probably because of the low concentrations of metal ions. L-glutamic acid showed a negative effect only on the margins for 2,3-BD production ($P = 0.0378$); perhaps, L-glutamic acid enhanced the TCA metabolism pathway, and the real reason needs to be further studied. 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+} [42]. Therefore, $MgSO_4 \cdot 7H_2O$ showed a positive and significant effect on the 2,3-BD synthesis.

Optimization of the medium composition by response surface methodology

Sucrose, L-glutamic acid, ammonium citrate, and $MgSO_4 \cdot 7H_2O$ were used for further optimization with a face-centered central composite design (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 3.

Response surface of the γ-PGA yield

The regression models in the form of ANOVA are given in Table 4a. 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$_4$·7H$_2$O (X_5^2) had a significant influence on the γ-PGA production (Table 4a). The effect of the MgSO$_4$·7H$_2$O 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 of as a function of the sucrose concentration, and beyond this point, sucrose had an inhibitory effect (Table 4a). According to ANOVA, the following polynomial quadratic equations were obtained in coded level:

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

Table 4a

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 citric	7.25	0.63	0.088	52.51	< 0.0001
X_5: MgSO$_4$·7H$_2$O	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	1.0×10^{-4}	-2.5×10^{-3}	0.093	7.248×10^{-4}	0.9789
X_2X_3	5.59	-0.59	0.093	40.54	< 0.0001
X_2X_5	0.046	0.054	0.093	0.34	0.5713
X_3X_5	0.12	0.088	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		

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 citric	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	7.562×10^{-4}	6.875×10^{-3}	0.21	1.100×10^{-3}	0.9740
X_1X_5	0.8	0.18	0.21	0.73	0.4069
X_2X_3	0.074	-0.068	0.21	0.11	0.7469
X_2X_5	3.903×10^{-3}	-0.016	0.21	5.683×10^{-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	0.069	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
$R^2 = 0.9984$	$R^2_{(adj)} = 0.9969$		$R^2_{(Pred)} = 0.9890$		

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. 3). Figure 3a-3c 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 40−100 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. 3b, 3d, 3f). Figure 3a, 3d, 3e shows that a high γ-PGA yield could be achieved if the concentration of L-glutamic acid is from 30−50 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 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. 3c, 3e, 3f).

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 27.83 g/L, which was obtained with a sucrose of 100.46 g/L, L-glutamic acid of 50.45 g/L, and ammonium citrate of 21.14 g/L.

Response surface of the 2,3-BD yield

The 2,3-BD concentration varied from 13.94 to 56.78 g/L for the 30 experiments shown in Table 3. 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 4b). 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 citrate and $MgSO_4 \cdot 7H_2O$ ($X_3 X_5$), squared term of sucrose (X_1^2), ammonium citrate (X_3^2), and $MgSO_4 \cdot 7H_2O$ (X_5^2) (Table 4b). The polynomial quadratic equation in the coded level was given by:

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

Figure 4a, 4b, 4c deposited the interaction effect of the 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 40−120 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 the 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. 4d). L-glutamic acid inhibited the production of 2,3-BD due to the negative linear coefficient (Table 4b) thus decreased the 2,3-BD concentration with an increase in the L-glutamic acid from 30− 70 g/L (Fig. 4e, 4f). The predicted highest value of 2,3-BD was 57.63 g/L, which was obtained at a sucrose of 120 g/L, L-glutamic acid of 30 g/L, ammonium citrate of 24.51 g/L and $MgSO_4 \cdot 7H_2O$ of 3.64 g/L.

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

The maximum γ-PGA and 2,3-BD yields were predicted at different concentrations for each variable. L-glutamic acid at a middle level was best for the γ-PGA production whereas it decreased the 2,3-BD concentration. Fortunately, the sucrose concentration at 100.46−120 g/L and ammonium citrate concentration at 21.14−24.51 g/L were within the optimum response regions of the γ-PGA and 2,3-BD yield. 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 119.83 g/L sucrose, 48.85 g/L L-glutamic acid, 21.08 g/L ammonium citrate, and 3.21 g/L $MgSO_4 \cdot 7H_2O$. In this condition, the γ-PGA and 2,3-BD yield predicted by design expert 8.0.6 software were 27.52 and 56.78 g/L, respectively. At the optimum level, the highest yields of γ-PGA and 2,3-BD were 27.79 ± 0.87 and 57.05 ± 1.28 g/L (Fig. 5), which were close to the predicted values. 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 [1]. Furthermore, the fructose metabolism rate was higher than that of glucose. All of the sugars were exhausted at 84 h, and 57.05 ± 1.28 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-butanediol was 0.475 g $_{2,3-BD}$/g $_{sucrose}$ (Fig. 5a). As shown in Fig. 5b, the maximum γ-PGA concentration reached 27.79 ± 0.87 g/L at 24 h of fermentation, and then

showed a slow decline, suggesting the γ-PGA depolymerase was activated [43] and "out-of-phase" operating conditions occurred [24]. Additionally, the productivity of γ-PGA was 1.16 g/L/h, which was higher than in some other studies in similar conditions. Furthermore, fed batch operation could be used to achieve a high γ-PGA and 2,3-BD production.

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. At present, the γ-PGA and 2,3-BD are processed mainly according to the properties between the two compounds. Firstly, trichloroacetic acid (TCA) solution is added to the fermentation broth to separate the cells and proteins by centrifugal; 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.

Conclusion

The present study constructed a fermentation system for the co-production of γ-PGA and 2,3-BD by B. subtilis CS13. Sequential optimization of the γ-PGA and 2,3-BD co-production was carried out. Under the optimum conditions of 119.83 g/L sucrose, 48.85 g/L L-glutamic acid, 21.08 g/L ammonium citrate, and 3.21 g/L $MgSO_4 \cdot 7H_2O$, the maximum γ-PGA and 2,3-BD concentration was 27.79 ± 0.87 g/L and 57.05 ± 1.28 g/L, respectively. This is the first report on γ-PGA and 2,3-BD co-production, which favored maximizing the use of substrates and facilitated efficient downstream recovery in the industrial production of chemicals.

Abbreviations

γ-PGA:poly-γ-glutamic acid; 2,3-BD:2,3-butanediol; B. subtilis:Bacillus subtilis; GRAS:generally regarded as safe; PBD:Plackett-Burman design; RSM:response surface methodology; FCCD:face-centered central composite design; YE:yeast extract; CSL:corn steep liquor; ANOVA:analysis of variance; DCW:dry cell weight; HPLC:high-performance liquid chromatography; GPC:gel permeation chromatogram; RID:refractive index detector; ICDH:socitrate dehydrogenase; GDH:glutamate dehydrogenase; TCA:trichloroacetic acid.

Additional Files

Additional file 1:Table S1. Screening of γ-PGA and 2,3-BD co-producters.

Additional file 2:Fig. S1. Fermentation broth analysis and 2,3-BD identification by HPLC.

Declarations

Authors' contributions

DXW performed the experiments. DXW and MHJ designed the experiments, analyzed the data and drafted the manuscript. HMK, SBL, DHK and MHJ designed and guided the study, editing of manuscript. All authors read and approved the final manuscript.

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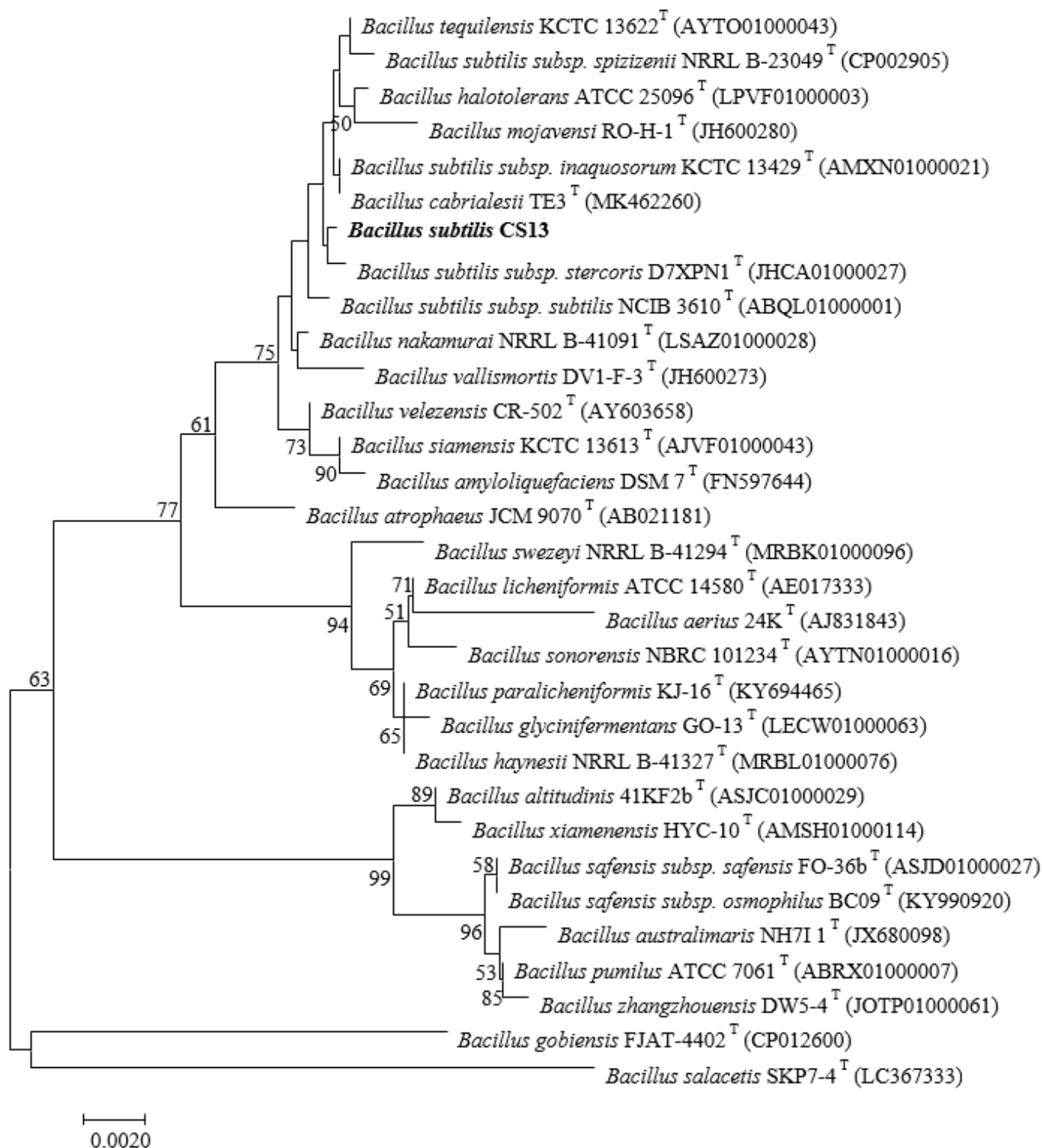
References

1. Reid SJ, Abratt VR. Sucrose utilisation in bacteria: genetic organisation and regulation. Appl.Microbiol Biotechnol. 2005;67:312-21.

2. Jiang M, Dai WY, Xi YL, Wu MK, Kong XP, Ma JF, Zhang M, Chen KQ, Wei P. Succinic acid production from sucrose by *Actinobacillus succinogenes* NJ113. Bioresour Technol. 2014;153:327- 32.

3. Zhang JZ, Yu L, Lin M, Yan QJ, Yang ST. n-Butanol production from sucrose and sugarcane juice by engineered *Clostridium tyrobutyricum* overexpressing sucrose catabolism genes and *adhE2*. Bioresour Technol. 2017;233:51-7.

4. Fouet A, Arnaud M, Klier A, Rapoport G. *Bacillus subtilis* sucrose-specific enzyme II of the phosphotransferase system: Expression in *Escherichia coli* and homology to enzymes II from enteric bacteria. Proc Natl Acad Sci USA. 1987;84:8773-7.

5. Syu MJ. Biological production of 2,3-butanediol. Appl Microbiol Biotechnol. 2001;55:10-8.

6. Zeng AP, Sabra W. Microbial production of diols as platform chemicals: Recent progresses. Curr Opin Biotechnol. 2011;22:749-57.

7. Guragain YN, Vadlani PV. 2,3-Butanediol production using *Klebsiella oxytoca* ATCC 8724: Evaluati-on of biomass derived sugars and fed-batch fermentation process. Process Biochem. 2017;58:25-34.

8. Zhang CY, Peng XP, Li W, Guo XW, Xiao DG. Optimization of 2,3-butanediol production by *Enterobacter cloacae* in simultaneous saccharification and fermentation of corncob residue. Biotechnol Appl Biochem. 2014;61:501-9.

9. Gao G, Xu H, Li QJ, Feng XH, Li S. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce *R,R*-2,3- butanediol. Bioresour Technol. 2010;101:7076-82.

10. Jurchescu IM, Hamann J, Zhou XY, Ortmann T, Kuenz A, Prüße U, Lang S. Enhanced 2,3-butanediol production in fed-batch cultures of free and immobilized *Bacillus licheniformis* DSM 8785. Appl Microbiol Biotechnol. 2013;97:6715-23.

11. Andrade CJ, Andrade LM, Bution ML, Dolder MAH, Fábio F, Barros C, Pastore GM. Optimizing alternative substrate for simultaneous production of surfactin and 2,3-butanediol by *Bacillus subtilis* LB5a. Biocatal Agric Biotechnol. 2016;6:209-18.

12. Yang TW, Rao ZM, Zhang X, Lin Q, Xia HF, Xu ZH, Yang ST. Production of 2,3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J Basic Microbiol. 2011; 51:650-8.

13. Feng J, Gu YY, Yan PF, Song CJ, Wang Y. Recruiting energy-conserving sucrose utilization pathways for enhanced 2,3-butanediol production in *Bacillus subtilis*. ACS Sustainable Chem. Eng. 2017;5:11221-5.

14. Fu J, Wang ZW, Chen T, Liu WX, Shi T, Wang GL, Tang YJ, Zhao XM. NADH plays the vital role for chiral pure D-(-)-2,3-butanediol production in *Bacillus subtilis* under limited oxygen conditions. Biotechnol Bioeng. 2014;111:2126-31.

15. Zeng W, Chen GG, Wang QL, Zheng SF, Shu L, Liang ZQ. Metabolic studies of temperature control strategy on poly(γ-glutamic acid) production in a thermophilic strain *Bacillus subtilis* GXA-28. Bioresour Technol. 2014;155:104-10.

16. Zhang D, Feng XH, Zhou Z, Zhang Y, Xu H. Economical production of poly(γ-glutamic acid) using untreated cane molasses and monosodium glutamate waste liquor by *Bacillus subtilis* NX-2. Bioresour Technol. 2012;114:583-8.

17. Kunioka M, Goto A. Biosynthesis of poly(γ-glutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in *Bacillus subtilis* IFO3335. Appl Microbiol Biotechnol. 1994;40:867-72.

18. Bajaj IB, Singhal RS. Poly (glutamic acid)-an emerging biopolymer of commercial interest. Bioresour Technol. 2011;102:5551-61.

19. Ogunleye A, Bhat A, Irorere VU, Hill D, Williams C, Radecka I. Poly-gamma-glutamic acid: production, properties and applications. Microbiol. 2015;161:1-17.

20. Inbaraj BS, Wang JS, Lu JF, Sao FY, Chen BH. Adsorption of toxic mercury (II) by an extracellular biopolymer poly-(gamma-glutamic-acid). Bioresour Technol. 2009;100:200-7.

21. Jiang B, Li ZG, Dai JY, Zhang DJ, Xiu ZL. Aqueous two-phase extraction of 2,3-butanediol from fermentation broths using an ethanol/phosphate system. Process Biochem. 2009;44:112-7.

22. Do JH, Chang HN, Lee SY. Efficient recovery of γ-poly(glutamic acid) from highly viscous culture broth. Biotechnol Bioeng. 2001;76:219-23.

23. Wang DX, Hwang JS, Kim DH, Lee SB, Kim DH, Joe MH. A newly isolated *Bacillus siamensis* SB1001 for mass production of poly-γ-glutamic acid. Process Biochem. 2019; https://doi.org/10.101 6/j.procbio.2019.11.034.

24. Büchs J, Lotter S, Milbradt C. Out-of-phase operating conditions, a hitherto unknown phenomenon in shaking bioreactors. Biochem Eng J. 2001;7:135-41.

25. Xu ZQ, Feng XH, Zhang D, Tang B, Lei P, Liang JF, Xu H. Enhanced poly(γ-glutamic acid) fermentation by *Bacillus subtilis* NX-2 immobilized in an aerobic plant fibrous-bed bioreactor. Bioresour Technol. 2014;155:8-14.

26. Perego P, Converti A, Borghi MD. Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by *Bacillus licheniformis*. Bioresour Technol. 2003;89: 125-31.

27. Ashiuchi M, Kamei T, Baek DH, Shin SY, Sung MH, Soda K, Yagi T, Misono H. Isolation of *Bacillus subtilis (chungkookjang)*, a poly-γ-glutamate producer with high genetic competence. Appl Microbiol Biotechnol. 2001;57:764-9.

28. Zhu F, Cai J, Zheng Q, Zhu XC, Cen PL, Xu ZN. A novel approach for poly-γ-glutamic acid production using xylose and corncob fibres hydrolysate in *Bacillus subtilis* HB-1. J Chem Technol Biotechnol. 2014;89:616-22.

29. Huang BQ, Qin PY, Xu ZW, Zhu RY, Meng YH. Effects of $CaCl_2$ on viscosity of culture broth, and on activities of enzymes around the 2-oxoglutarate branch, in *Bacillus subtilis* CGMCC 2108 producing.
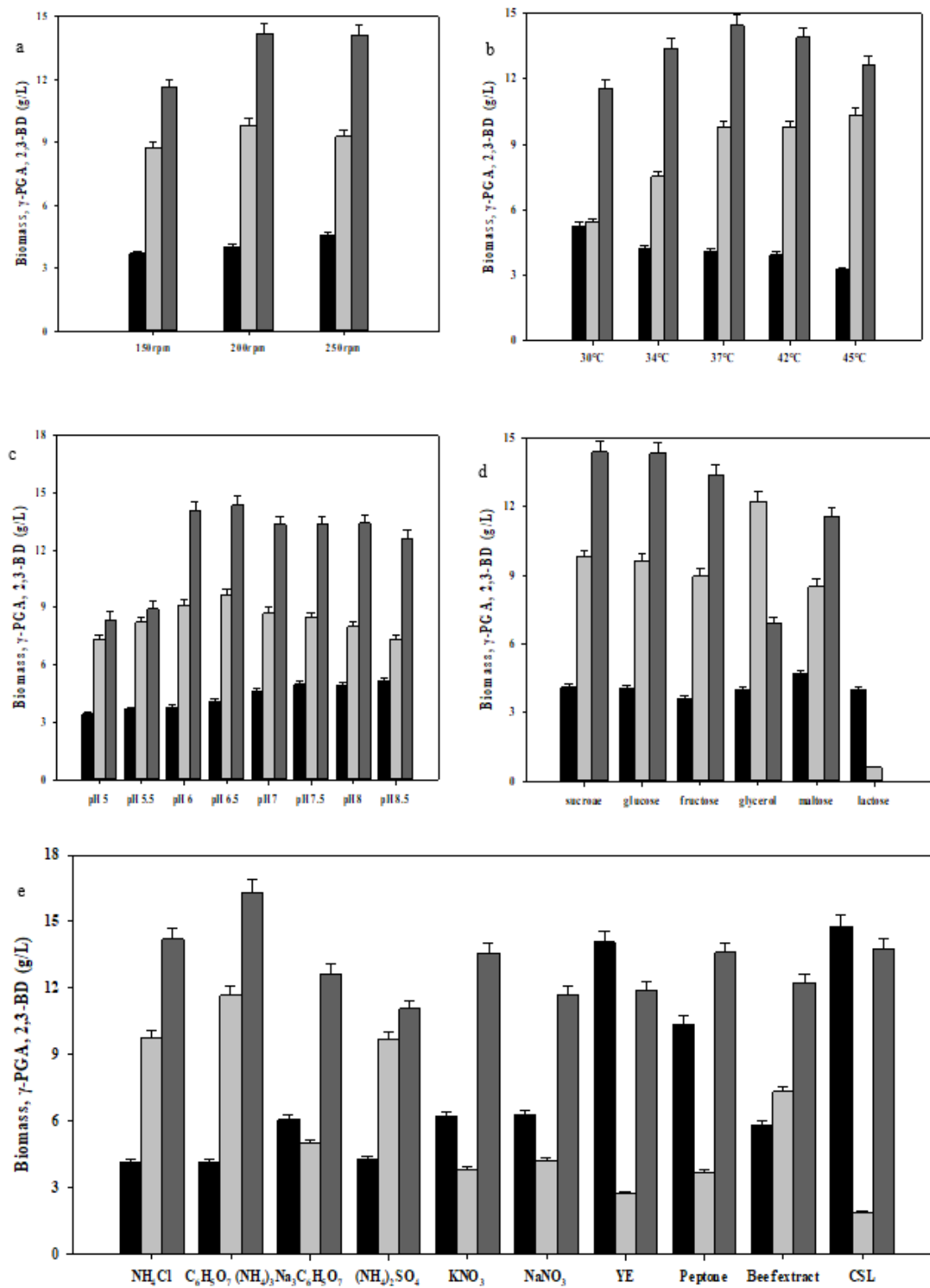
Bioresour Technol. 2011;102:3595-8.

30. Zhu F, Cai J, Wu XT, Huang J, Huang L, Zhu JZ, Zheng Q, Cen PL, Xu ZN. The main byproducts and metabolic flux profiling of γ-PGA-producing strain *B. subtilis* ZJU-7 under different pH values. J Biotechnol. 2013;164:67-74.

31. Yao J, Xu H, Shi NN, Cao X, Feng XH, Li S, Ouyang PK. Analysis of carbon metabolism and improvement of γ-polyglutamic acid production from *Bacillus subtilis* NX-2. Appl Microbiol Biotechnol. 2010;160:2332-41.

32. Du GC, Yang G, Qu YB, Chen J, Lun SY. Effects of glycerol on the production of poly(γ-glutamic acid) by *Bacillus licheniformis*. Process Biochem. 2005;40:2143-7.

33. Yang TW, Rao ZM, Zhang X, Xu MJ, Xu ZH, Yang ST. Enhanced 2,3-butanediol production from biodiesel-derived glycerol by engineering of cofactor regeneration and manipulating carbon flux in *Bacillus amyloliquefaciens*. Microb Cell Factories. 2015;14:1-11.

34. Ashiuchi M. Occurrence and biosynthetic mechanism of poly-gamma-glutamic acid. In: Hamano Y, editors. Amino-Acid Homopolymers Occurring in Nature. Microbiology Monographs; 2010.p.77-93.

35. Gunka K, Commichau FM. Control of glutamate homeostasis in *Bacillus subtilis*: a complex interpiay between ammonium assimilation, glutamate biosynthesis and degradation. Mol Biol. 2012;85:213-24.

36. Ren YY, Huang BQ, Meng YH, Wei LN, Zhang C. Metabolic and phylogenetic analyses based on nitrogen in a new poly-γ-glutamic acid-producing strain of *Bacillus subtilis*. Biotechnol Lett. 2015; 37:1221-6.

37. Ito Y, Tanaka T, Ohmachi T, Asada Y. Glutamic acid independent production of poly (γ-glutamic acid) by *Bacillus subtilis* TAM-4. Biosci Biotech Biochem. 1996;60:1239-42.

38. Peng YY, Jiang B, Zhang T, Mu WM, Miao M, Hua YF. High-level production of poly(γ-glutamic acid) by a newly isolated glutamate-independent strain, *Bacillus methylotrophicus*. Process Biochem. 2015;50:329-35.

39. Yang TW, Zhang X, Rao ZM, Gu SH, Xia H., Xu ZH. Optimization and scale-up of 2,3-butanediol production by *Bacillus amyloliquefaciens* B10-127. World J Microb Biot. 2012;28:1563-74.

40. Zeng W, Liang ZQ, Li Z, Bian YX, Li ZH, Tang Z, Chen GG. Regulation of poly-γ- glutamic acid production in *Bacillus subtilis* GXA-28 by potassium. J TaiWan Inst Chem Eng. 2016;61:83-9.

41. Feng J, Shi QS, Zhou G, Wang LL, Chen AM, Xie XB, Huang XM, Hu WF. Improved production of poly-γ-glutamic acid with low molecular weight under high ferric ion concentration stress in *Bacillus licheniformis* ATCC9945a. Process Biochem. 2017;56:30-6.

42. Poulsen C, Stougaard P. Purification and properties of Saccharomyces cerevisiae acetolactate synthase from recombinant *Escherichia coli*. Eur J Biochem. 1989;185:433-9.

43. Yao J, Jing J, Xu H, Liang JF, Wu Q, Feng XH, Ouyang PK. Investigation on enzymatic degradation of γ-polyglutamic acid from *Bacillus subtilis* NX-2. J Mol Catal B-Enzym. 2009;56:158-64.

Figures

Figure 1

Phylogenetic relationship of Bacillus subtilis CS13 and other Bacillus strains based on neighbor-joining tree analysis of the 16S rDNA gene.
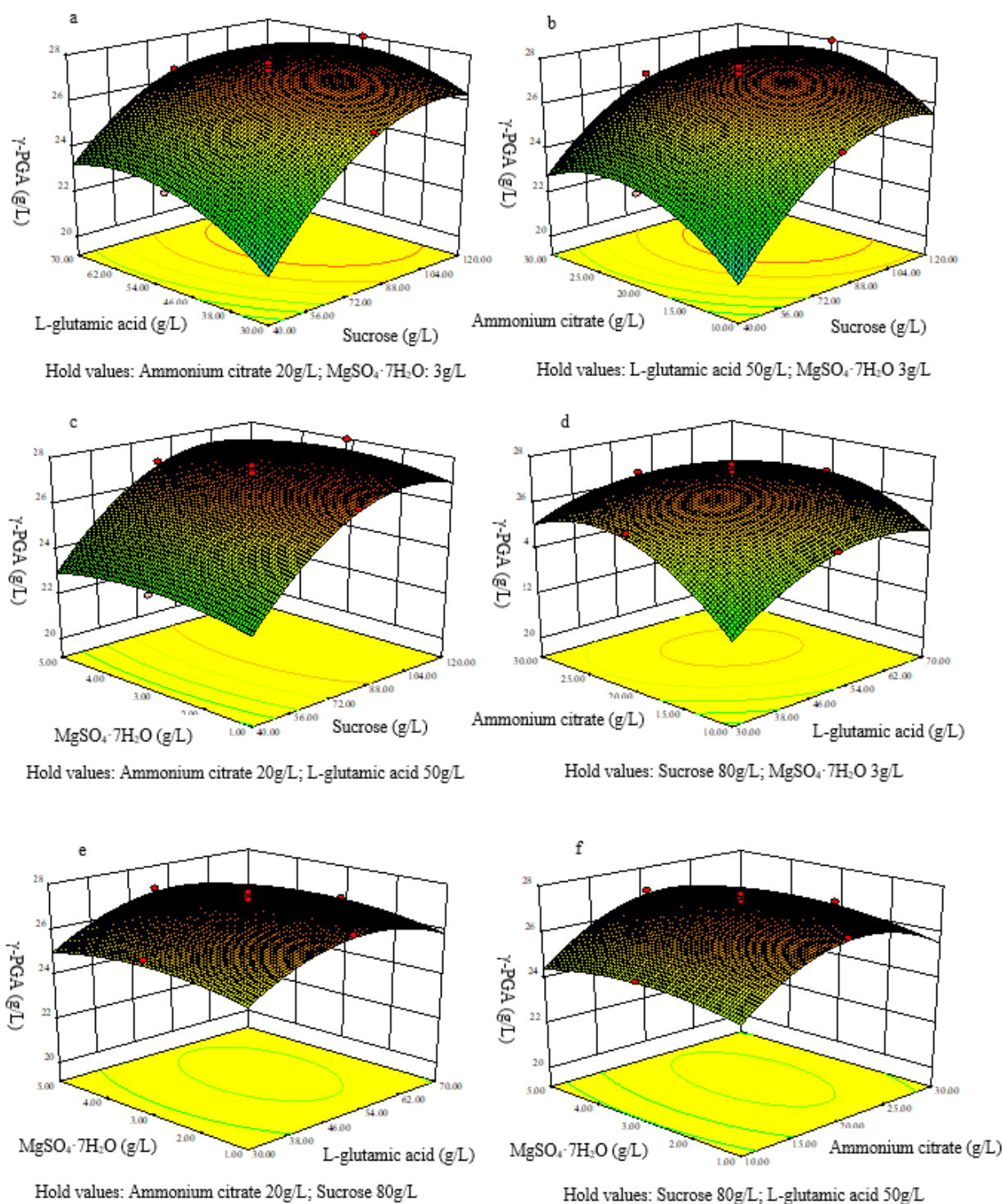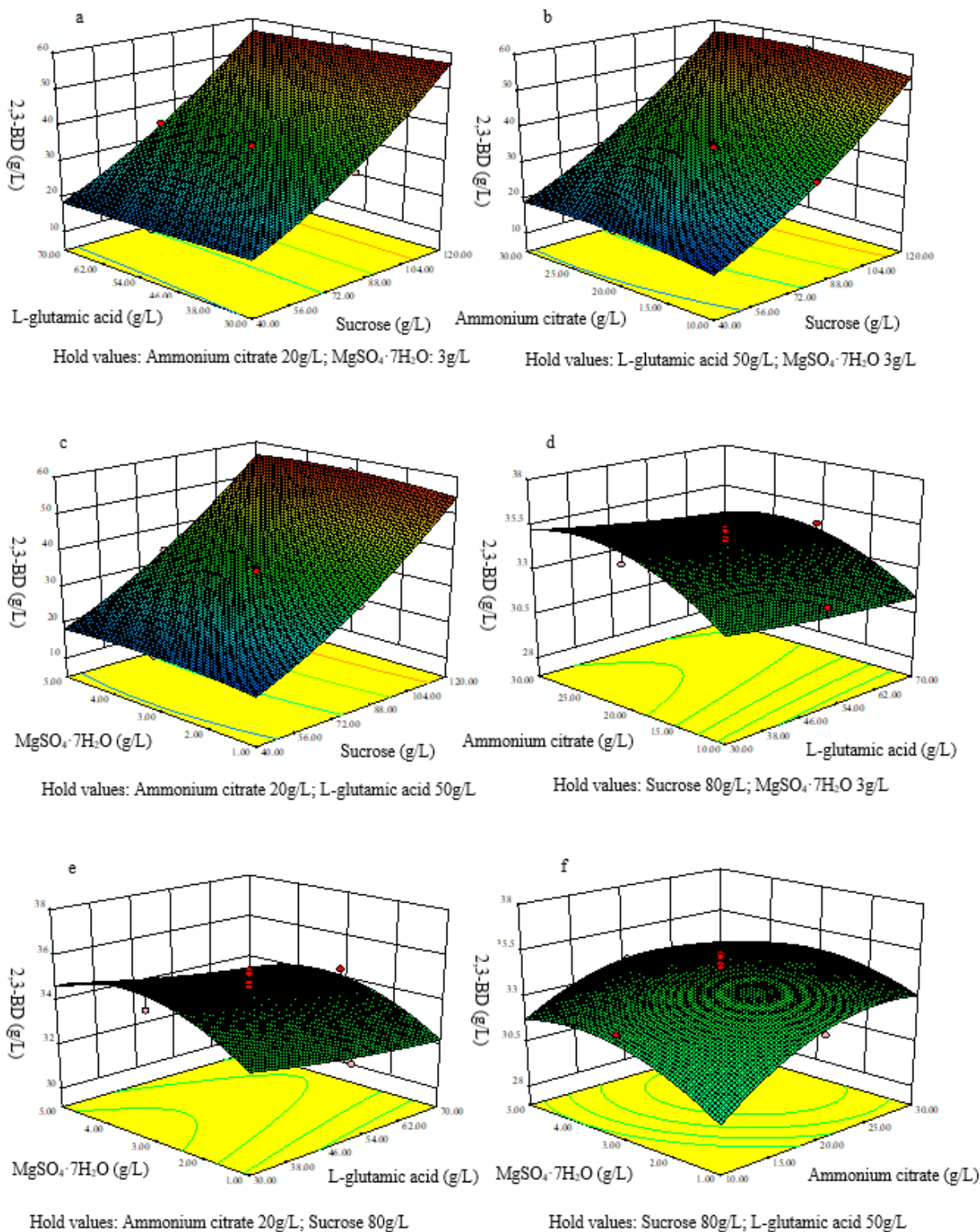
Figure 2

Phylogenetic relationship of Bacillus subtilis CS13 and other Bacillus strains based on neighbor-joining tree analysis of the 16S rDNA gene.
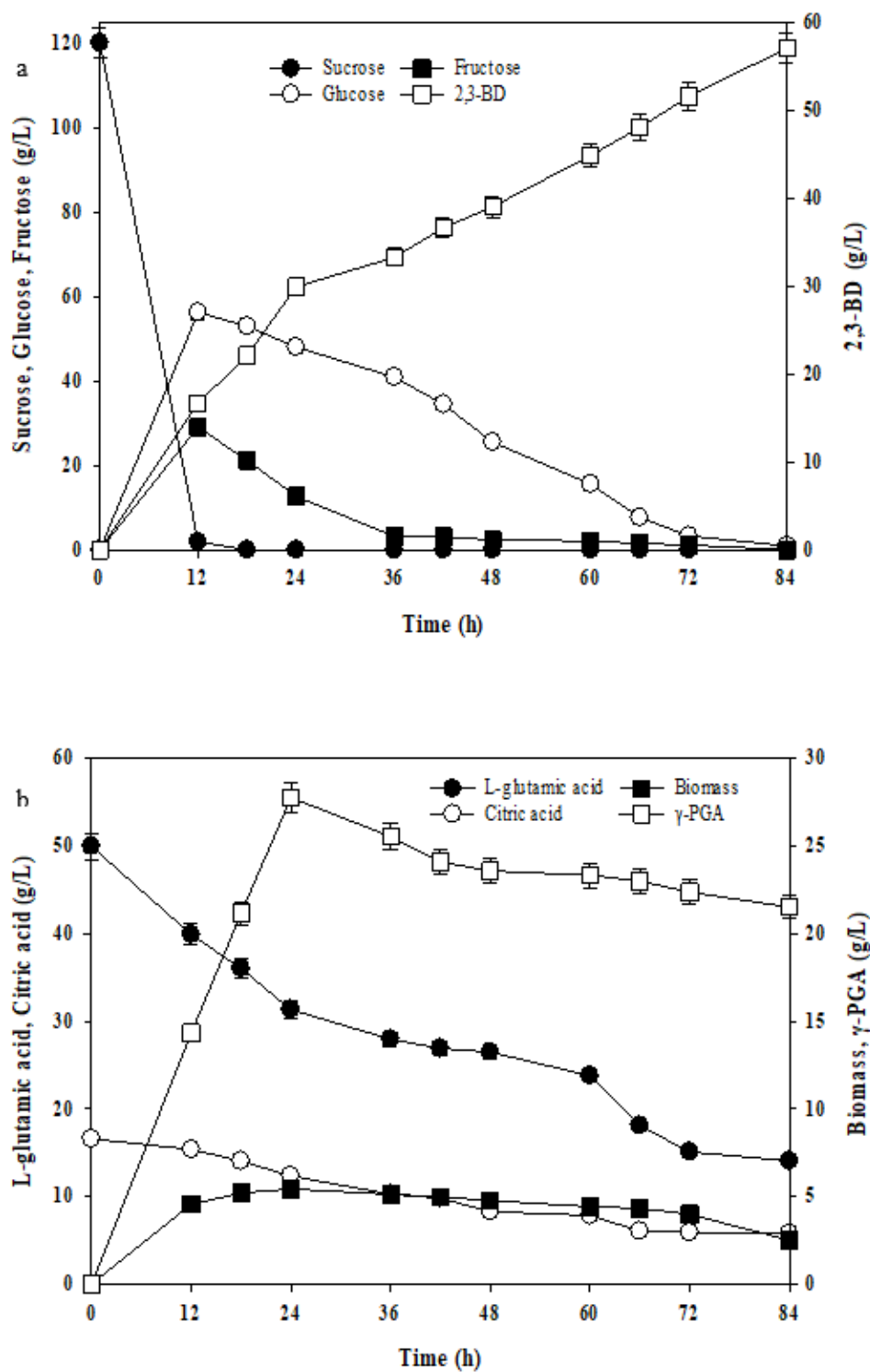
Figure 3

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 MgSO4·7H2O and sucrose, (d) effect of ammonium citrate and L-glutamic acid, (e) effect of MgSO4·7H2O and L-glutamic acid, (f) effect of MgSO4·7H2O and ammonium citrate.

Figure 4

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 MgSO4·7H2O and sucrose, (d) effect of ammonium citrate and L-glutamic acid, (e) effect of MgSO4·7H2O and L-glutamic acid, (f) effect of MgSO4·7H2O and ammonium citrate.

Figure 5

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.

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