



Poly- γ -glutamic acid production by simultaneous saccharification and fermentation using corn straw and its fertilizer synergistic effect evaluation

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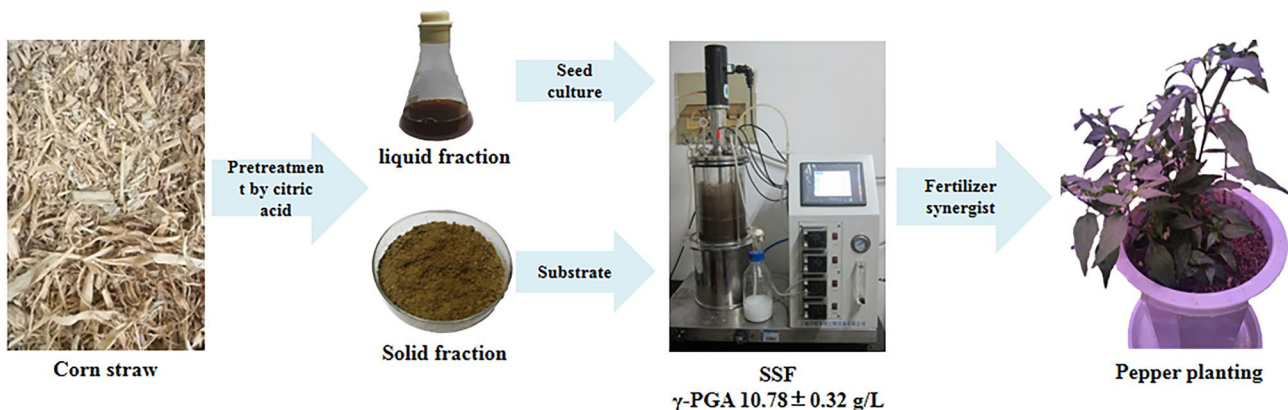
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Abstract

Agricultural wastes rich in lignocellulosic biomass have been used in the production of poly- γ -glutamic acid (γ -PGA) through separate hydrolysis and fermentation (SHF), but this process is complicated and generates a lot of wastes. In order to find a simpler and greener way to produce γ -PGA using agricultural wastes, this study attempted to establish simultaneous saccharification and fermentation (SSF) with citric acid-pretreated corn straw. The possibility of *Bacillus amyloliquefaciens* JX-6 using corn straw as substrate to synthesize γ -PGA was validated, and the results showed that increasing the proportion of glucose in the substrate could improve the γ -PGA yield. Based on these preliminary results, the corn straw was pretreated using citric acid. Then, the liquid fraction (xylan-rich) was used for cultivation of seed culture, and the solid fraction (glucan-rich) was used as the substrate for SSF. In a 10-L fermenter, the maximum cumulative γ -PGA concentration in batch and fed-batch SSF were 5.08 ± 0.78 g/L and 10.78 ± 0.32 g/L, respectively. Moreover, the product from SSF without γ -PGA extraction was used as a fertilizer synergist, increasing the yield of pepper by 13.46% ($P < 0.05$). Our study greatly simplified the production steps of γ -PGA, and each step achieved zero emission as far as possible. The SSF process for γ -PGA production provided a simple and green way for lignocellulose biorefinery and sustainable cultivation in agriculture

Graphic abstract



Keywords Simultaneous saccharification and fermentation (SSF) · Agricultural wastes · Biorefining process · Poly- γ -glutamic acid (γ -PGA) · Fertilizer synergist

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Introduction

Poly- γ -glutamic acid (γ -PGA) is a linear biomacromolecule polymerized with D- or L-glutamic acid monomers via γ amide bonds [1]. Owing to novel characteristics, including absorbability, water-solubility, biodegradability and innocuity, γ -PGA has been successfully applied in agriculture, cosmetics industry, sewage treatment and other fields [2]. Especially in agriculture, studies have shown that γ -PGA is an efficient fertilizer synergist and plant regulator. Applying γ -PGA in wheat planting increased the yield by 7.17%, while improving nitrogen utilization efficiency and soil microbe diversity [3]. γ -PGA could promote plant nitrogen metabolism by changing the $\text{Ca}^{2+}/\text{CaM}$ signaling pathway, thereby improving the growth of Chinese cabbage [4]. γ -PGA was also a good cryoprotectant, which could relieve the growth inhibition of rape seedlings under cold stress [5].

γ -PGA is mainly prepared via fermentation using various *Bacillus* strains, while glucose and glycerol are the mostly common used carbon sources [6, 7]. However, glucose and glycerol are expensive, which lead to a relatively high price of γ -PGA and hinder its large-scale application. To reduce the production cost of γ -PGA, it is necessary to find a more economical carbon source. Lignocellulosic biomass is rich in carbohydrate and reserves in the forms of wastes or by-products in agriculture and forestry such as corn straw [8]. In China, large amount of corn straws are generated every year, leading to a huge potential environmental burden [9]. As renewable and cheap material, recent studies have attempted to use lignocellulosic biomass for γ -PGA production by separate hydrolysis and fermentation (SHF). In SHF, the lignocellulosic biomass was first converted to monosaccharides followed by submerged fermentation using the hydrolysate as the carbon source. After a two-step hydrolysis using sulfuric acid and enzyme, the rice straw was transformed to a carbon source for γ -PGA fermentation by *Bacillus subtilis* NX-2, and the yield reached 73.0 ± 0.5 g/L [10]. Corncob fibers were treated with dilute sodium hydroxide and hydrochloric acid sequentially, the hydrolysate was applied in γ -PGA production by *Bacillus subtilis* HB-1, resulting in a 24.92 g/L cumulative production [11]. The corncob hydrolysate was also used by *Bacillus amyloliquefaciens* C1, and the final γ -PGA yield was 6.56 ± 0.27 g/L [12].

Although SHF has been successfully employed during production, sulfuric acid, hydrochloric acid, and sodium hydroxide were used in the hydrolysis of lignocellulosic biomass [10–12]. This would produce a lot of harmful inhibitors to microorganisms such as furfural and 5-hydroxymethyl furfural (5-HMF) [13], leading to

a great consumption of clean water to wash and calcium hydroxide or lime for detoxification [10–12]. These operations increased the complexity of the process and consumed massive labor, while generating a lot of wastewater and insoluble matters that might pollute the environment. So, the technologies for simple and green conversion of lignocellulose into γ -PGA are urgently needed. Simultaneous saccharification and fermentation (SSF), which integrates hydrolysis and fermentation in one reactor, is a relatively simple process to utilize lignocellulosic biomass as main feedstock [14]. During SSF, sugars are released from lignocellulosic biomass and simultaneously consumed by the microorganism for fermentation, reducing the number of reactors occupied, as well as saving a lot of labor and time inputs [15, 16]. Currently, SSF has been successfully applied in the fermentation of various chemical products, such as bio-ethanol [16], lactic acid [17], succinic acid [18], and butanediol [19]. Moreover, in the pretreatment of lignocellulosic biomass, the use of organic carboxylic acids produced less fermentation inhibitors [20, 21], and the pretreated substrates could also be directly used for SSF without detoxification [22]. This would further simplify the fermentation process, while reducing the generation of sewage and waste.

To overcome the disadvantages of complex steps and pollutant emission in SHF, we established an SSF process for γ -PGA production using corn straw pretreated by citric acid in this study. The SSF process integrated γ -PGA fermentation and corn straw hydrolysis, which extremely simplified the process of γ -PGA production from lignocellulose-rich agricultural waste. The corn straw pretreated by citric acid was fermented directly, which avoided the waste generation from detoxification. Moreover, SSF products could increase the yield of pepper, showing the synergistic effect of fertilizer. So, our results provided a new way for the clean bioconversion and green recycling of corn straw.

Materials and methods

Microorganism

Bacillus amyloliquefaciens JX-6 was used for γ -PGA fermentation. The strain was isolated from the field in Chengdu (Sichuan Province, China) and deposited at China General Microbiological Culture Collection Center with the accession number of CGMCC No. 13715. Our previous study showed that the strain JX-6 could use agricultural wastes (corn stalk and soybean meal) to produce γ -PGA through solid-state fermentation, and the maximum yield was 116.88 ± 5.05 g/kg [23].

Flask experiments

To obtain the seed culture, *B. amyloliquefaciens* JX-6 suspension was inoculated into a 500-mL Erlenmeyer flask. The flask was filled with 100 mL sterilized LB broth and incubated at 200 rpm for 15 h at 32 °C.

The subsequent γ -PGA fermentations in this study were all performed in a two-stage cultivation: (1) For cell growth, each 500-mL Erlenmeyer flask was filled with 100 ml sterilized fermentation medium (in g/L: $(\text{NH}_4)_2\text{SO}_4$, 10; K_2HPO_4 , 1; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.02; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; CaCl_2 , 0.2; xylose/glucose, or glucose, or xylose, 50; pH 6.5–7.5) and inoculated with 5% seed culture (v/v), followed by incubated at 240 rpm for 24 h at 32 °C; (2) For γ -PGA production, 20 mL precursor solution (L-glutamic acid and citric acid) was supplemented at 24 h, the final concentration of L-glutamic acid was 15 g/L and the citric acid was 5 g/L. Subsequently, the mixture was cultured for an additional 48 h.

Corn straw pretreatment

Corn straw collected from field (Shifang, Sichuan Province, China) was milled into 40 mesh particle and dried at 60 °C. The fiber determination instrument CXC-06 (Xinrui Instruments Co., Shanghai, China) was used to analyze the organic composition of straw powder. The straw powder was soaked in a 1.5% citric acid solution (w/v). The initial ratio of liquid-to-solid was 10:1 (w/w). This straw powder slurry was autoclaved at 121 °C for 3 h. Subsequently, the slurry was centrifugated at 4800 rpm for 15 min. The solid fraction was used as substrate for SSF, and the liquid fraction was used for seed culture cultivation. The composition of solid fraction was also analyzed by CXC-06, and the liquid fraction was tested by HPLC (Shimadzu, Kyoto, Japan).

SSF and fed-batch SSF

In all SSF experiments, 10 g/L $(\text{NH}_4)_2\text{SO}_4$ and 5 g/L peptone were dissolved in the liquid fraction from the pretreatment to make seed medium. Each 500-mL Erlenmeyer flask was filled with 100 mL seed medium and inoculated with 2% of *B. amyloliquefaciens* JX-6 suspension. Then, the flasks were incubated at 200 rpm for 24 h at 32 °C.

Solid fraction from the pretreated corn straw was suspended in a neutral cellulase (EC 3.2.1.4, 284.00 FPU/g, Heshibi Biotechnology Co. Ltd., Ningxia Province, China) solution. The total water insoluble solids (WIS) content of this slurry was 20% (w/v), while the titer of enzyme was 40 FPU/(g WIS).

A 10 L bioreactor (Yangge, Shanghai, China) was used for the batch SSF experiments. According to previous SSF studies [18, 24], and considering that γ -PGA is a highly

energy-consuming biological process, the SSF operating parameters were set as follows. The total WIS content of SSF was set as 8%, 10%, and 12%, while total enzyme loading was 40 FPU/(g WIS), and the working volume was 4 L. The medium (in g/L: $(\text{NH}_4)_2\text{SO}_4$, 10; K_2HPO_4 , 1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.02; CaCl_2 , 0.2) was autoclaved and supplemented with different amounts of enzyme and substrate slurry. The batch SSF was performed in three stages: (1) The pretreated corn straw powder was enzymatically pre-hydrolyzed at pH 5.5 for 10 h at 50 °C with agitation at 200 rpm. (2) 5% of seed culture was inoculated into the bioreactor after pre-hydrolysis, and this point was recorded as time point 0 h. The temperature of batch SSF was 32 °C, and the aeration rate was 2 vvm, as well as the agitation speed was 400 rpm. The pH was maintained at 6.5–7.0 by automatic addition of 2 mol/L HCl or 2 mol/L NaOH. This stage lasted for 24 h. (3) For γ -PGA production, 500 mL precursor solution (L-glutamic acid and citric acid) was supplemented at 24 h for subsequently 48 h of γ -PGA fermentation.

The fed-batch SSF was initially started with 10% (w/v) WIS content in 4 L of working volume. After addition of precursor solution, 580 mL of substrate and enzyme mixture was manually added at 24 h, 48 h and 72 h. The final working volume was up to 6.25 L and WIS content was 12% (w/v).

Testing of synergistic effects of fertilizer and fermentation broth

To investigate the possible synergistic effects of fed-batch SSF on fertilizer, the experiment of pepper cultivation was conducted in greenhouse. The experiment lasted from June 10 to September 25 under natural conditions. Each plastic pot was filled with 4 kg of perlites and humus (the ratio was 9:1 v/v). The pot experiment included two different treatments: (1) In the γ -PGA group, the soil matrix (perlites and humus) was added with 200 mL of fed-batch SSF products after ultrasonication; (2) In the control group, the soil matrix was mixed with the 200 mL pure medium of fed-batch SSF, which was not inoculated with *B. amyloliquefaciens* JX-6. After transplanting, the pepper seedlings in two groups were fertilized with 100 mL identical nutrient solution every two days (0.31 mM KNO_3 , 0.25 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.5 mM KH_2PO_4 , 0.5 mM NH_4NO_3 , 0.5 μM $\text{Fe} \cdot \text{NaEDTA}(\text{pH}5.5)$, 1.75 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.06 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.125 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 8.75 μM H_3BO_3 , 0.025 μM $(\text{NH}_4)_2\text{MoO}_4$, 0.00125 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$). The experiment was carried out in four replicates. The date of first flowering, first harvest and final production were recorded. In each

group, the yield, water content and length of peppers were determined.

Analysis methods

During the shake-flask experiments with mixed sugars and all SSF experiments, bacterial samples were collected every 8 h to monitor the cell density, γ -PGA concentration, residual sugars and L-glutamic acid in the broth.

The fermentation samples were serially diluted using sterilized water. Then, 100 μ L aliquots were plated on LB plates in triplicates. After 18 h incubation at 37 °C, the number of colonies on each plate was counted to calculate the cell density.

To precipitate γ -PGA, the samples were centrifuged at 10,000 rpm for 20 min, and the supernatant after centrifugation was mixed with 4 volumes of absolute alcohol. Then, the mixture was spun down at 12,000 rpm for 10 min to collect γ -PGA. Subsequently, the γ -PGA was dissolved in ultra-pure water and measured by HPLC as previous description [25].

Residual sugars (glucose and xylose) were measured by HPLC (LC-20A, Shimadzu Co, Japan) with a InertSustain@NH₂ column and an RI-10A refractive-index detector. The mobile phase was 75% (v/v) acetonitrile solution and the flow rate was 1.0 mL min⁻¹. The biosensor SBA-40D (Shandong Academy of Sciences, China) was used to determine the concentration of residual L-glutamic acid. All experiments were carried out in triplicates. The one-way ANOVA function of the software SPSS 19.0 was used to compare the values of different groups.

Results

Utilization of different sugars for γ -PGA production with *B. amyloliquefaciens* JX-6

Corn straw is rich in lignocellulose which mainly composed of cellulose, hemicellulose and lignin. The major components of cellulose and hemicellulose are corresponding glucan and xylan, whereas, the content of other sugars are very low. To explore the possibility of using corn straw for γ -PGA production by *B. amyloliquefaciens* JX-6, glucose and xylose, as well as their mixture were used to perform a flask fermentation test. The mixed sugar consisted of glucose and xylose in a ratio of 37:20, which was identical with the ratio of cellulose and hemicellulose in corn straw. As shown in Table 1, all three sugars could be used as carbon sources for cell growth and γ -PGA synthesis. The highest yield of γ -PGA was obtained in fermentation with glucose, which was 19.13 ± 1.18 g/L, followed by mixed sugar group (16.03 ± 0.85 g/L). Additionally, Glucose

Table 1 Effects of different sugars on γ -PGA production

Sugar	γ -PGA g/L	Cell density 10 ¹⁰ cfu/mL
Glucose	19.13 \pm 1.18	7.50 \pm 1.15
Xylose	15.30 \pm 0.63 ^a	3.40 \pm 1.01 ^a
Mixed sugar*	16.03 \pm 0.85 ^a	6.87 \pm 1.44

*The mixed sugar consists of glucose and xylose in a ratio of 37:20, and all initial concentrations of sugars were 50 g/L. Data were the averages of three replicates

^aThere was significant difference compared with glucose group ($P < 0.05$)

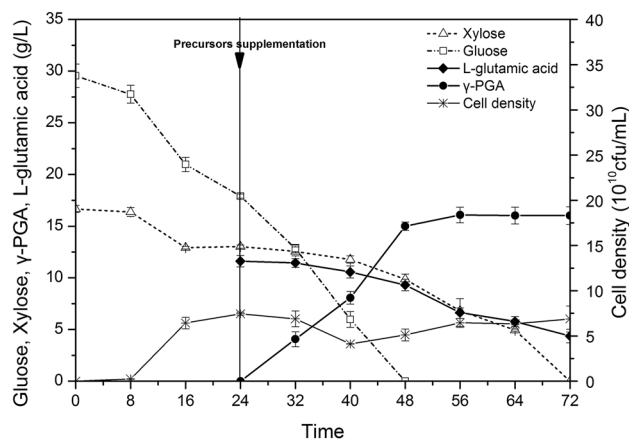


Fig. 1 Batch fermentation of γ -PGA by *B. amyloliquefaciens* JX-6 using xylose/glucose mixture. γ -PGA fermentation was performed in two-stage cultivation steps: bacteria reproduction was sustained for 24 h, followed by γ -PGA production after supplementation of precursor solutions (citric acid and L-glutamic acid). The results were derived from the average of three independent tests

was most conducive to cell growth and the final cell density in the fermentation broth was $(7.5 \pm 1.15) 10^{10}$ cfu/mL, which was higher than that in sugar mixture group $((6.87 \pm 0.44) 10^{10}$ cfu/mL). Given the lowest γ -PGA yield (15.30 ± 0.63 g/L) and cell density $((3.40 \pm 1.01) 10^{10}$ cfu/mL), we could infer that xylose was not suitable for *B. amyloliquefaciens* JX-6 to synthesize γ -PGA.

Fermentation profile of *B. amyloliquefaciens* JX-6 using mixed sugars to produce γ -PGA

In order to further understand the characteristics of γ -PGA fermentation with corn straw, the fermentation process of *B. amyloliquefaciens* JX-6 using mixed sugar was continuously monitored and sampled every 8 h (Fig. 1). The fermentation profile indicated that the consumption of glucose and xylose was not synchronous. Compared with xylose, glucose was preferentially utilized by *B. amyloliquefaciens* JX-6. The glucose in the fermentation broth was depleted at 48 h, and

the average consumption rate was 0.62 ± 0.02 g/(L·h), while xylose was finally exhausted at 72 h and the average consumption rate was only 0.23 ± 0.04 g/(L·h). After precursor supplementation at 24 h, the γ -PGA began to accumulate, and the final γ -PGA concentration reached 16.03 ± 0.85 g/L. Before glucose depletion, γ -PGA was rapidly synthesized, and the γ -PGA average synthesis rate was 0.63 ± 0.02 g/(L·h) during 24–48 h. However, xylose was the only carbon source after 48 h, which was not favored by JX-6. Thus, the production of γ -PGA was almost stagnated, and the average synthesis rate was only 0.04 ± 0.01 g/(L·h). In addition, the cell density in fermentation broth reached the peak value of $(7.45 \pm 0.16) 10^{10}$ cfu/mL at 24 h, then dropped to the valley with glucose consumption. As the γ -PGA synthesis stalled after 48 h, the titer of bacteria re-elevated and the final cell concentration was $(6.88 \pm 1.44) 10^{10}$ cfu/mL.

Effect of glucose/xylose ratio on γ -PGA fermentation

In SSF, the pretreatment of lignocellulosic biomass is essential to improve the production efficiency. However, the contents of glucan and xylan in substrate severely varied under different pretreatment. To find a suitable pretreatment method, the optimum ratio of glucose and xylose was investigated. Flask-fermentation experiments were performed using 50 g/L of glucose/xylose mixtures at five different ratios (9/1, 7/3, 5/5, 3/7, 1/9), and the result was shown in Fig. 2. The yield of γ -PGA under a 1: 9 ratio of glucose/xylose mixture was 12.89 ± 0.40 g/L, which was the lowest among the five groups. As the proportion of glucose in the fermentation medium increased, the final γ -PGA concentration gradually improved. When the ratio of glucose/xylose in the mixed sugar reduced to 9: 1, the yield of γ -PGA was

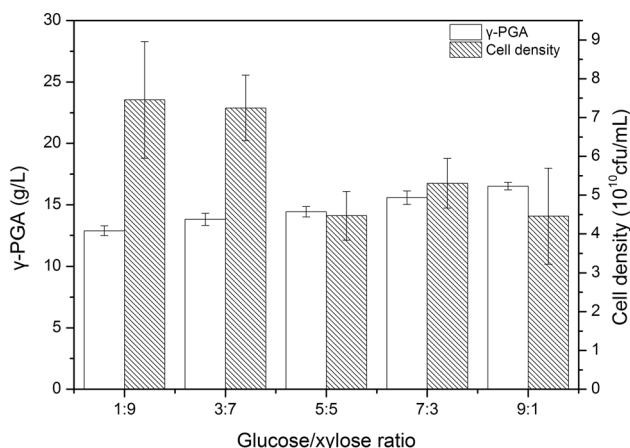


Fig. 2 Effects of glucose/xylose ratio on γ -PGA production. The initial concentration of each sugar mixture was 50 g L^{-1} , and the glucose/xylose proportion was at five different ratios (from 1:9 to 9:1). The results were derived from the average of three independent tests

16.52 ± 0.32 g/L, which was 28.16% higher than the 1: 9 group. The change of glucose/xylose ratio in the mixed sugar has no identical influence on final cell density. In the groups with a high proportion of xylose, the final cell densities were $(7.46 \pm 1.50) 10^{10}$ cfu/mL and $(7.25 \pm 0.85) 10^{10}$ cfu/mL at the glucose/xylose ratios of 1: 9 and 3: 7, respectively. As the further increase of glucose, the final cell densities slightly decreased, which values at the glucose/xylose ratios of 5:5, 7: 3 and 9: 1 were $(4.47 \pm 0.63) 10^{10}$ cfu/mL, $(5.31 \pm 0.64) 10^{10}$ cfu/mL, and $(4.45 \pm 1.23) 10^{10}$ cfu/mL, respectively.

Pretreatment of corn straw with citric acid

Based on the previous results, citric acid was selected for high-pressure steam pretreatment of corn straw. Corn straw was mainly composed of 36.77% cellulose, 19.52% hemicellulose and 15.06% lignin. After citric acid pretreatment, the cellulose and lignin content in pretreated straw (the solid fraction from the pretreatment) separately increased to 52.45% and 23.32%, while the hemicellulose decreased to 5.84% (Table 2). Compared with corn straw, the ratio of cellulose (rich in glucan) to hemicellulose (rich in xylan) was elevated to 52.45:5.84, which was close to 9:1. After filtration, the liquid fraction from the pretreatment contains 10.82 g/L xylose, 1.13 g/L glucose and residual 8.20 g/L citric acid, the hemicellulose of corn straw was degraded into monosaccharides and released.

γ -PGA production by batch SSF of different substrate contents

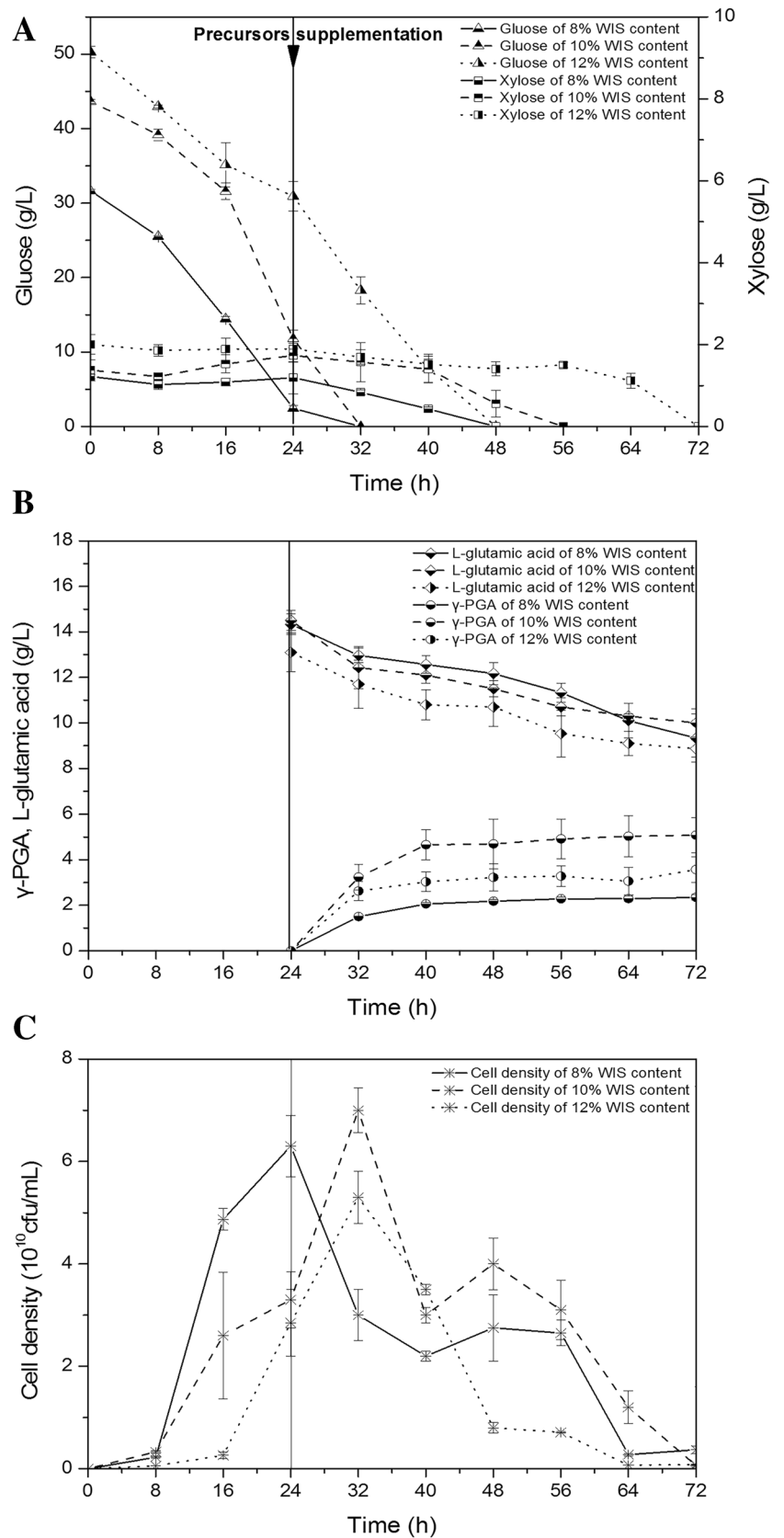
In a 10 L bioreactor, the batch SSF process for γ -PGA production was established and the effects of different contents of water insoluble solids (WIS) were studied. The seed culture was cultivated with the liquid fraction from corn straw pretreatment as the carbon source, and the solid part from pretreated straw was used as substrate for SSF. The batch SSF was performed in three stages: the pre-hydrolysis of substrate, the cell growth, and the γ -PGA production. The consumption of free sugars, the synthesis of γ -PGA and the growth of cells were all different for batch SSF under various WIS contents (8%, 10% and 12%) (Fig. 3).

Table 2 Components of corn straw after pretreatment with citric acid

Component	% Total dry weight (w/w)	
	Corn straw	Pretreated straw (Solid fraction)
Cellulose	36.77 ± 1.00	52.45 ± 2.23
Hemicellulose	19.52 ± 1.56	5.84 ± 0.78
Lignin	15.60 ± 1.42	23.32 ± 1.64

Data were the averages of three replicates

Fig. 3 γ -PGA fermentation profiles of batch SSF using citric acid-pretreated corn straw under different substrate contents: **a** the concentration of glucose and xylose, **b** the concentration of γ -PGA and L-glutamic acid, **c** the cell density. The results were derived from the average of three independent tests



After the pre-hydrolysis stage, the concentration of free glucose in the fermentation broth increased coordinately with the WIS content (31.63 ± 0.21 g/L, 43.66 ± 0.26 g/L and 50.28 ± 0.79 g/L, respectively), whereas, the xylose concentrations were very low (less than 2 g/L). During the subsequent cell growth process, the free glucose was quickly consumed with the increase of cell titer, while the xylose concentration remained constant. In the fermentation broth with 8% and 10% WIS, the free glucose could not be detected after 32 h, while the negative result in 12% WIS group was observed at 48 h. Moreover, the xylose depletion time point was delayed as the WIS content increased (at 48 h, 56 h and 72 h, respectively). The highest γ -PGA yield, 5.08 ± 0.78 g/L, was attained by batch SSF under 10% WIS content. In the 8% and 12% WIS content group, the corresponding eventual γ -PGA concentrations were 2.35 ± 0.11 g/L and 3.57 ± 0.57 g/L, respectively (Fig. 3b). In all batch SSF experiments, the duration of continuous γ -PGA synthesis was relatively short, and the yield of γ -PGA did not increase after 40 h. The maximum cell density (7.00×10^{10} cfu/mL) was also attained in 10% WIS content at 32 h. The peak cell density of 8% and 12% WIS content were 6.30×10^{10} cfu/mL and 5.30×10^{10} cfu/mL,

exhibited at 24 h and 32 h, respectively (Fig. 3c). A decrease in cell density during late fermentation stage was observed in SSF under all WIS contents, and the final cell density of three groups had little difference.

γ -PGA production by fed-batch SSF

To improve the γ -PGA yield of SSF, the fed-batch SSF process was established based on the result of batch SSF. The start of fed-batch SSF was identical with the batch trial under 10% WIS content, then the slurry of the substrate and enzyme was supplemented at 24, 48 and 72 h after inoculation, while the final WIS content in the reactor reached 12% (Fig. 4). Similarly, free glucose in the fed-batch SSF broth was also consumed quickly and could not be detected after 32 h. The free xylose in the fermentation broth was depleted at 88 h, which was 32 h delayed compared with batch SSF under 10% WIS content. The eventual concentration of γ -PGA was 10.78 ± 0.32 g/L, which was 2.14 folds than batch SSF. In fed-batch SSF, the yield of γ -PGA over total substrate was 0.10 ± 0.02 g/(g substrate), which was 3 folds higher than the value in batch SSF (0.03 ± 0.0012 g/(g substrate)). During the fed-batch SSF, γ -PGA was continuously synthesized between 24 and 96 h. The maximum recorded cell density was 9.45×10^{10} cfu/mL at 88 h, which was greater than the batch SSF. Particularly, no significant cell density decrease was observed in the later stage of fed-batch SSF.

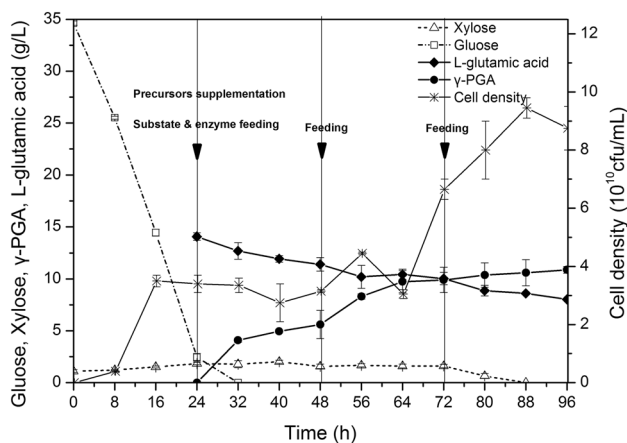


Fig. 4 γ -PGA production by fed-batch SSF using citric acid-pre-treated corn straw. The fed-batch SSF was started at 10% and completed at 12% substrate content. 580 mL of substrate/enzyme mixture was manually fed at the 24th, 48th and 72nd hour. The results were derived from the average of three independent tests

Fertilizer synergistic effect of fermentation product from fed-batch SSF

To study the fertilizer synergistic effect of fermentation product from fed-batch SSF, the mixture sterilized by ultrasonication was applied to the pot experiment of pepper cultivation directly. The results showed that the average yield of the γ -PGA applied group was 65.31 ± 0.31 g/pot, which was increased by 13.46% compared with the control ($P < 0.05$, Table 3). The fermentation broth could also promote the average length of peppers. The length of peppers in the γ -PGA group was 14.8 ± 0.61 cm, while in the control group was only 14.0 ± 0.67 cm. There was a little difference in water content of peppers between two groups, the values of

Table 3 Promotion effects of fermentation broth from SSF on peppers cultivation

Groups	Yield (g per pot)	Length of peppers (cm)	Water content (%)	First flowering date	First harvesting date	Final harvesting date
γ -PGA	65.31 ± 0.31^a	14.8 ± 0.61	83.75 ± 0.59	July 8	July 17	September 20
Control	57.56 ± 0.57	14.0 ± 0.67	82.60 ± 1.01	July 8	July 19	September 11

Data were the averages of four replicates

^a There was significant difference compared with control group ($P < 0.05$)

the γ -PGA group and the control group were $83.75 \pm 0.59\%$ and $82.60 \pm 1.01\%$, respectively. In two groups, the first flowering date of pepper was same (July 8th). However, the peppers after γ -PGA application possessed a prolonged picking period: the first harvesting date of the γ -PGA group was July 17th, two days earlier than the control group, while the final harvesting date was September 20th, which was nine days later than the control group.

Discussion

To reduce the production cost of γ -PGA, lignocellulosic biomass has been used for γ -PGA fermentation [10–12]. Previous researches were mainly carried out through SHF, which was complicated and not environmentally friendly [10, 11]. In this study, the fermentation characteristics of *B. amyloliquefaciens* JX-6 using glucose/xylose mixtures to produce γ -PGA were explored. Subsequently, a SSF process was successfully established for γ -PGA production using citric acid pretreated corn straw as the main carbon source. The fermentation product from SSF without γ -PGA extraction was directly applied as a fertilizer synergist, which successfully improved the yield of the peppers. Our study established the SSF process of γ -PGA production using corn straw for the first time, which provided a simple and clean bioconversion pathway for agricultural wastes rich in lignocellulose.

The proportions of the main components in lignocellulose are different among diverse plants [26]. Unlike rice straw, the hydrolysate of corn straw mainly harbored xylose and glucose, and other sugars were barely detected [10, 26, 27]. Thus, the mixture of xylose and glucose were used to mimic corn straw for γ -PGA fermentation. The results indicated that *B. amyloliquefaciens* JX-6 had the ability of using corn straw as the main carbon source for γ -PGA fermentation. Similar to *B. amyloliquefaciens* C1 and *B. subtilis* NX-2, *B. amyloliquefaciens* JX-6 preferred glucose than xylose during γ -PGA synthesis using mixed sugars [10, 12]. This phenomenon is common in most microorganisms, especially in *Bacillus* species, which is mainly caused by carbon catabolite repression (CCR) [28, 29]. In *Bacillus* species, CCR exhibits as the bias to use a preferred sugar (usually glucose) when multiple carbon sources exist, causing a lag in the consumption of other carbon sources (such as xylose) [30]. This was primarily the result of gene expression regulated by metabolite profiles of the preferred sugar or its intermediate metabolites, thereby affecting the metabolism of other carbon sources [12, 30, 31]. Also due to CCR, the γ -PGA fermentation using pure glucose was more efficient than using glucose/xylose mixtures [32].

Lignocellulosic biomass has a complex structure, which hinders the access of cellulase and reduces the efficiency

of hydrolysis [8]. Therefore, it is necessary to pretreat the lignocellulosic biomass to destroy its structure and improve the release of sugar in SSF [15]. Our results of fermentation using mixed sugars at different glucose/xylose ratios revealed that the hemicellulose (xylan) in the lignocellulosic biomass should be removed as much as possible to increase the proportion of glucan in the substrate of SSF. Finally, we chose citric acid for the high-temperature steaming pretreatment of corn straw. Citric acid has relatively strong acidity with the pK_{a1} of 3.13, which can promote the degradation of hemicellulose [8]. This could avoid CCR cleverly, resulting in a favorable environment for strain *B. amyloliquefaciens* JX-6. Besides, using organic acid in pretreatment would generate fewer inhibiting factors compared to inorganic acid, such as sulfuric acid [20, 21], and citric acid itself was not toxic to microorganisms. So, the pretreated corn straw could be directly used in fermentation without detoxification, which reduced a large number of pollutants and massive labor and time input due to the detoxification of the hydrolysates [22]. In addition, the liquid fraction from pretreatment, which containing xylose and residual citric acid, was used for seed cultivation of SSF to avoid waste of resources and pollution to the environment. Previous studies have shown that this operation was conducive to make full use of all the sugars in the lignocellulosic biomass as well as domesticate the strains to improve their resistance to inhibitors in the lignocellulosic biomass [33]. In short, compared to sulfuric acid and sodium hydroxide were used in SHF, the use of citric acid for corn straw pretreatment make our process more concise and environmentally friendly.

As described, batch SSF was successfully established with 8%, 10% and 12% WIS contents in a 10 L bioreactor. Due to the CCR, *B. amyloliquefaciens* JX-6 preferred glucose in the substrate. Therefore, the initial consumption of xylose meant that glucose was not sufficient, and the xylose exhaustion indicated that the substrate could no longer provide fermentable sugars. In the SSFs with 8% and 10% WIS contents, xylose was depleted at 56 h and 48 h, respectively, indicating that the substrates in these two groups were unsuitable to support long-term fermentation. However, when the WIS content elevated to 12% in our trial, both γ -PGA yield and cell density decreased. This was probably attributed to a higher fermentation broth viscosity [34] and inhibitor titer [35] as the increase of substrate content, thus inhibiting bacteria growth and metabolism. The fed-batch SSF was also established. Compared with batch SSF, fed-batch SSF is a better choice for γ -PGA production under high substrate content, γ -PGA synthesis maintained long period (about 72 h) and the cell density did not significantly decrease. These results indicated that the supplementation of enzymes and substrates correspondingly solved the shortage of substrates and the inhibition caused by high WIS contents in batch SSF. Moreover, we could infer that the viscosity of

the fermentation broth is maintained at a lower level due to the feeding of new WIS multiple times [36]. Also, the inhibitory effect from the hydrolysis of lignocellulosic biomass is reduced as a result of both gradual adaption of the bacteria and biological detoxification [24]. Compared with traditional SHF, the SSF process is a simpler way to produce γ -PGA. The process of using rice straw to synthesize γ -PGA using *Bacillus subtilis* NX-2 includes six steps [10], while the SHF with corncob as carbon source using *Bacillus subtilis* HB-1 consists of seven steps [11]. In our study, the SSF process integrated fermentation and hydrolysis. This extremely simplified strategy only required three steps: pretreatment, filtration, and fermentation. This results in the conservation of time, equipment, energy and labor, as well as reducing the cost of γ -PGA production [15, 16].

Similar to previous studies [37], the fermentation product from our fed-batch SSF can promote the yield and quality (length) of peppers, which indicate that corn straw can be used to prepare high-quality fertilizer synergists through the SSF process. In it, γ -PGA could increase fertilizer utilization, improve plant metabolic pathways, and promote soil microorganisms [3–5], while the corn straw could also enhance soil properties [38]. The traditional strategy to improve the efficiency of fertilizers is adding urease and nitrification inhibitors or coating the fertilizers [39, 40]. However, these two fertilizer synergists may pollute the environment: the inhibitors are toxic to animals and some microorganisms [41], and the polymers used for coating are generally not biodegradable [42]. In contrast, the γ -PGA in the SSF fermentation broth is nontoxic and biodegradable, which can be an environmental friendly fertilizer synergist. In China, corn straw is one of the largest agricultural wastes causing great pressure on the environment [43, 44], the effectively disposal of these straws is an urgent problem. Studies showed that composting technology was the most effective way to use straw as a resource [9], but the economic profit of organic fertilizer was limited and could not attract much attention [45]. The price of ordinary organic fertilizer prepared from straw and manure is 650 RMB/t, while the price of agricultural γ -PGA fermentation broth is as high as 9,000 RMB/t (<https://www.1688.com/>). It is calculated that 0.12 t of straw, 0.017 t of cellulase, 0.023 t of citric acid, 0.015 t of L-glutamic acid, and 0.01 t of $(\text{NH}_4)_2\text{SO}_4$ are required to produce 1 t of SSF fermentation broth, and the total material investment cost is about 1037 RMB/t. Now, the abuse of chemical fertilizers in Chinese agriculture has caused many soil and ecological problems, and there is a need for green and efficient fertilizer synergists [46]. Our study provided a new technology to produce high-value fertilizer synergists from agricultural waste, which was helpful to the recycling of straws and the development of sustainable agriculture.

However, the yield of γ -PGA obtained by our SSF was lower than that of reported SHF [10, 11]. The production efficiency of SSF can be improved by some optimization measures, such as screening effective pretreatment methods for lignocellulosic biomass [18, 47], selecting suitable substrate content [18], developing a reasonable feeding strategy [24, 36], and so on. In the next work, we will optimize the production conditions of SSF to increase production, and try to establish a larger production process.

Conclusion

In this study, a SSF using corn straw pretreated by citric acid to synthesize γ -PGA was established, and the products were proved to have fertilizer synergistic effect. Compared with the traditional SHF process, SSF greatly simplified the production steps, and each step achieved zero emission as far as possible. Based on our results, SSF with corn straw could be a promising strategy to produce γ -PGA, which also be a simple and green way for lignocellulose biorefinery and sustainable cultivation in agriculture. However, the γ -PGA yield of SSF process was slightly lower, we will optimize the production conditions to increase production and try to establish a large-scale production process in next work.

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Declarations

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

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