Bioconversion of poly-γ-glutamic acid (γ-PGA) from fulvic acid powder produced from the wastewater of yeast molasses fermentation

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Research

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Abstract

Background: Molasses is a wildly used feedstock for fermentation, but it poses a severe wastewater-disposal problem worldwide. Recently, the wastewater produced by yeast during molasses fermentation is being processed into fulvic acid (FA) powder as a fertilizer for crops, but it consequently induces a problem of soil acidification after being directly applied in soil. In this study, the low-cost FA powder was bioconverted into a value-added product, γ-PGA, by a glutamate independent producer, Bacillus velezensis GJ11.

Results: With FA powder, the substrates of sodium glutamate and citrate sodium used in medium were decreased around one third. Moreover, FA powder could completely substitute Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Fe$^{3+}$ in the fermentation medium. In the optimized FA powder fermentation medium, the γ-PGA was produced with its maximum concentration at 42.55 g/L and a productivity of 1.15 g/(L·h), while only 2.87 g/L was produced in the medium without FA powder. Hydrolyzed γ-PGA could trigger induced systemic resistance (ISR), e.g. H$_2$O$_2$ accumulation and callose deposition, against the pathogen infection in plants. Further investigations found that the ISR triggered by γ-PGA hydrolyzates was dependent on the ethylene signalling and NPR1.

Conclusions: To our knowledge, this is the first report of using the industry waste, FA powder, as a sustainable substrate for the microbial synthesis of γ-PGA. This bioprocess can not only develop a new way of FA powder as a cheap feedstock for producing γ-PGA, but also help to reduce pollution from the wastewater of yeast molasses fermentation.

Background

Due to the petroleum crisis, renewable sources, such as bioethanol, have been explored and used as alternative energy in recent years [1]. Molasses is one of the main raw materials for producing bioethanol through yeast fermentation [2–4], because molasses contains many easily-fermentable sugars (e.g. primarily sucrose, glucose, and fructose), and is abundant, low-cost and highly available for the fermentation industry [5–7]. Thereby, molasses is widely used for microbial conversion into different value-added products, such as bioethanol, baker's yeast, amino acids, butanol, organic acids, single cell proteins, etc [8–11]. However, the fermentation poses a severe waste disposal problem worldwide [12], because most wastewater is discharged into the environment without appropriate pre-treatment, aggravating the dire water pollution situations, and contributing to severe environmental pollution [1, 13, 14].

In China, the wastewater produced during molasses fermentation can be processed into a biological product, fulvic acid (FA) powder, by dry-spraying. This technology can produce approximately 200 thousands of tonnes of FA powder every year. The FA powder is rich in fulvic acid (> 45%) as well as other nutrients, such as N (> 3%), P (> 0.5%), K (> 12%), and amino acids (> 6%). Thus, it can be used as a fertilizer for crops. Additionally, processing wastewater into FA powder is favorable for reducing the
pollution generated during molasses fermentation. FA is an important component of organic matters in soil, which can influence plant growth and microorganism activity [15, 16]. Otherwise, FA has efficacy in controlling several plant diseases and protecting plants against abiotic stresses [15, 17–19]. However, application of FA powder can lead to the soil acidification because it contains organic acids. On the other hand, FA powder is soluble, and contains many nutrients, such as N, P, K, carbon sources, and amino acids, which makes it a kind of cheap raw material (~ 1500 ¥ per ton) for microbial conversion into value-added products. By fermentation, the organic acids in FA power can be consumed by microorganisms as carbon sources except for FA, which contains a high amount of oxygen-rich and carbon-poor functional groups, and is hard to be used by microbes [18]. Thereby, the fermented FA powder becomes an ideal fertilizer with a minimized side effect on soil acidification. Moreover, the FA powder can be bioconverted into some value-added products by fermentation, which will probably encourage people to convert the wastewater produced during the molasses fermentation into FA powder instead of polluting the environment.

*Bacillus* is ubiquitously distributed in the natural environment. Among *Bacillus*, many species, such as *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. velezensis* are popularly used as hosts for producing fermentation-products (*e.g.* poly-γ-glutamic acid (γ-PGA)). γ-PGA is a natural anionic polymer of D/L-glutamic acids linked together via amide bonds between the α-amino group and the γ-carboxylic acid group, resulting in numerous properties, such as holding water, biodegradability and non-toxicity [20, 21]. It has been widely used in food, medicine, cosmetic, and agriculture nowadays [22]. In agriculture, γ-PGA is a new environmental-friendly fertilizer synergist, which is able to improve plant in uptake of N, P, and K [23]. However, its use in agriculture is hindered due the low yield and high cost of the production when compared with conventional materials. This is because producing γ-PGA via fermentation generally needs glutamate, as well as other high-cost components. Thereby, low-cost feedstock (*e.g.* FA powder) is urgently needed to overcome economical and sustainable obstacles of biotechnological production of γ-PGA.

There are two types of *Bacillus* strains that can produce γ-PGA: one requires an external supply of glutamate and the other does not [21]. We previously isolated a strain of *B. velezensis* GJ11 from soil with an excellent activity to trigger the induced systemic resistance (ISR) in plants [24]. Interestingly, this strain could produce γ-PGA in a glutamate independent manner. The aim of our study was to use GJ11 for microbial conversion of FA powder into γ-PGA with low cost, and investigate whether γ-PGA could be used as an activator to trigger ISR in plants [25]. We found that FA powder could reduce the cost of γ-PGA production around one third, and the γ-PGA produced by GJ11 could trigger ISR against pathogens infection via the ethylene signaling and NPR1 in plants.

**Results**

**GJ11 producing γ-PGA by a glutamate-independent manner**
Without sodium glutamate added in the medium, GJ11 produced γ-PGA at about 25.0 g/L. With the increase of sodium glutamate added in the medium, the γ-PGA production was also increased in the broth. However, the γ-PGA production was not increased continuously when the sodium glutamate concentration was more than 40.0 g/L (Fig. 1a). This was consistent with the result of SDS-PAGE (Fig. 1b). Thereby, GJ11 is a glutamate-independent γ-PGA producer, and an addition of glutamate can further improve its γ-PGA production. We further used gel permeation chromatography to determine the molecular weight of γ-PGA produced by GJ11. The retention time of standard γ-PGA was about 8.39 min, while the retention time of γ-PGA produced by GJ11 was about 6.92 min (Fig. 1c). Thereby, the molecular weight of γ-PGA produced by GJ11 is higher than that of the γ-PGA standard with a molecular weight of 580 kD.

**Compatibility of GJ11 to FA powder**

FA powder was dissolved in water as the medium for culturing GJ11. The results showed that the biomass of GJ11 was gradually increased with the increase of FA powder (< 40 g/L), indicating that FA powder could provide nutrients to support the bacterial growth. After FA powder was increased to more than 40 g/L, the biomass of GJ11 was decreased (Fig. S1a). We further found that, with an increase of FA powder used for culturing GJ11, the pH value of the medium was decreased due to the presence of organic acids (Fig. S1b). The low pH value suppressed the bacterial growth. Thereby, we adjusted the pH value of the medium to 7.0, and found it was later decreased to ~ 6.5 after sterilization (Fig. S1c).

The original fermentation medium with FA powder was adjust to pH 7.0 for culturing GJ11. As shown in Fig. S1d, the biomass of the culture reached its maximum when FA powder was used at 40 g/L. When the concentration of FA powder was more than 40 g/L, the biomass decreased dramatically when FA powder added in the fermentation medium increased. The γ-PGA production reached the highest value, ~ 42 g/L, when no FA powder was added into the fermentation medium. Then, the production decreased as FA powder added in the fermentation medium increased. Thereby, the excessive FA powder is unfavorable for the bacterial growth and γ-PGA production.

**Effects of carbon and nitrogen sources on γ-PGA production**

We found that FA powder was unfavorable for GJ11 to produce γ-PGA in the original fermentation medium. Thereby, we further detected whether FA powder (40 g/L) could substitute some nutrients in order to reduce the cost of γ-PGA production. Glucose is known as the most efficient carbon source for producing γ-PGA currently. Thus, we studied whether FA powder could substitute glucose in the fermentation medium. As shown in Fig. 2a, the biomass and γ-PGA production in the broth were both decreased without an addition of glucose. Under the glucose concentration of 70 g/L, the biomass and γ-PGA production were both increased when the concentration of glucose added in the medium increased. When the glucose concentration was more than 70 g/L, the biomass and γ-PGA production was no longer increased with the increase of glucose. Besides glucose, citrate acid is regarded as a common carbon source for γ-PGA production. As shown in Fig. 2b, the increase of citrate sodium could improve γ-PGA
production rather than biomass. This was probably due to the fact that citrate acid was mainly contributed to biosynthesizing glutamate, a monomer for biosynthesizing γ-PGA. When the concentration of citrate sodium was more than 10 g/L, the production of γ-PGA was no longer increased with the increase of citrate sodium added in the medium.

Although GJ11 is a glutamate-independent γ-PGA producer, addition with glutamate could improve its γ-PGA production. As shown in Fig. 2c, the biomass was not significantly influenced by addition with sodium glutamate, but the γ-PGA production was increased with the increase of sodium glutamate added in the medium. The highest γ-PGA production was obtained when sodium glutamate was added at a final concentration of 10 g/L, and higher concentrations of sodium glutamate could not improve but reduce γ-PGA production in the broth.

The glutamate-independent producer can use inorganic nitrogen sources for biosynthesizing γ-PGA [21]. As shown in Fig. 2d, an addition of NaNO₃ could improve both biomass and γ-PGA production in the broth. When the concentration of NaNO₃ was more than 2 g/L, the biomass was no longer increased with the increase of NaNO₃, but the γ-PGA production was still increased with the increase of NaNO₃ (< 12 g/L). However, another inorganic nitrogen source, NH₄Cl, could neither improve γ-PGA production nor increase biomass in the broth (Fig. 2e). Moreover, the bacterial growth was inhibited by NH₄Cl when it was added into the medium at a final concentration more than 2 g/L. Additionally, the biosynthesis of γ-PGA was reduced when NH₄Cl was added into the medium at a final concentration more than 4 g/L.

**Effects of inorganic salts on γ-PGA production**

Inorganic salts have been reported to be important for γ-PGA production [26, 27]. As shown in Fig. 3a, KH₂PO₄ could improve both biomass and γ-PGA production. However, when the concentration of KH₂PO₄ was more than 0.3 g/L, the biomass was no longer increased with the increase of KH₂PO₄ added in the medium. Moreover, the biomass was reduced when the concentration of KH₂PO₄ was more than 0.7 g/L. For γ-PGA production, the excessive KH₂PO₄ (> 0.9 g/L) was also unfavorable for GJ11 to produce γ-PGA.

It has been reported that Mn²⁺ can improve cell growth, prolong cell viability, and assist the utilization of different carbon sources and increase γ-PGA production [26]. As shown in Fig. 3b, an addition of MnSO₄ could not significantly improve γ-PGA production, indicating that Mn²⁺ in the FA powder was enough for GJ11 to produce γ-PGA. Moreover, with the increase of MnSO₄ added in the medium, γ-PGA production in the broth was gradually decreased.

Mg²⁺ has been reported to be necessary for the activity of PgsBCA in biosynthesizing γ-PGA [27]. Our results showed that an addition of MgSO₄ could not improve biomass and γ-PGA production. When the concentration of MgSO₄ increased, γ-PGA production in the broth was gradually reduced (Fig. 3c). Thereby, an addition of Mg²⁺ is not necessary for γ-PGA production in the medium with FA powder.
We further investigated whether an addition of Ca\(^{2+}\) could improve γ-PGA production. We found that an addition of CaCl\(_2\) could not significantly improve biomass and γ-PGA production in the medium with FA powder. Moreover, the excessive Ca\(^{2+}\) inhibited γ-PGA production (Fig. 3d). The results indicated that Ca\(^{2+}\) was already enough for producing γ-PGA in the medium with FA powder.

As shown in Fig. 3E, an addition of FeCl\(_3\) in the medium with FA powder was favorable for improving biomass rather than γ-PGA production. However, excessive Fe\(^{3+}\) (> 0.02 g/L) could not further improve biomass in the broth.

**Orthogonal test for optimizing fermentation medium**

On the basis of our results, we further optimized the fermentation medium with orthogonal test. Glucose, citrate sodium, sodium glutamate, NaNO\(_3\), KH\(_2\)PO\(_4\), and FA powder were selected for further optimization according to the orthogonal experiments (L\(_{18}(3^7)\)). As shown in Table 2, glucose, NaNO\(_3\), sodium glutamate, and citrate sodium improved γ-PGA production, while FA powder was negative for γ-PGA production. According to the R-value obtained from the orthogonal tests, we found that γ-PGA production was successively affected by sodium glutamate, NaNO\(_3\), citrate sodium, FA powder, glucose, and KH\(_2\)PO\(_4\). The optimal combination of the medium was A\(_3\)B\(_3\)C\(_2\)D\(_1\)E\(_3\)F\(_3\), corresponding to 80 g/L glucose, 20 g/L NaNO\(_3\), 0.7 g/L KH\(_2\)PO\(_4\), 20 g/L FA powder, 20 g/L sodium glutamate, and 20 g/L citrate sodium, which resulted in the highest γ-PGA production of 35.54 g/L. On the other hand, glucose and FA powder improved the bacterial biomass, while NaNO\(_3\), sodium glutamate, citrate sodium, and KH\(_2\)PO\(_4\) were unfavorable for the bacterial growth. On the basis of R-value, we found that the biomass was successively affected by glucose, NaNO\(_3\), citrate sodium, FA powder, sodium glutamate, and KH\(_2\)PO\(_4\). The bacterial growth was negatively related with γ-PGA production. In this study, we mainly focused on γ-PGA production. Thus, our optimized medium contained 80 g/L glucose, 20 g/L NaNO\(_3\), 0.5 g/L KH\(_2\)PO\(_4\), 20 g/L FA powder, 20 g/L sodium glutamate, and 20 g/L citrate sodium. Compared to original fermentation medium, the cost of sodium glutamate and citrate sodium were both decreased around one third due to an addition of FA powder in the medium. Moreover, FA powder could be a substitute for NH\(_4\)Cl, MgSO\(_4\), MnSO\(_4\), CaCl\(_2\), and FeCl\(_3\) in the original fermentation medium.
Table 2
Results of Orthogonal Experiment

| Treatment | A | B | C | D | E | F | γ-PGA (g/L) | OD<sub>600</sub> |
|-----------|---|---|---|---|---|---|-------------|-----------------|
| 1         | 1 | 1 | 1 | 1 | 1 | 1 | 15.39       | 4.91            |
| 2         | 1 | 2 | 2 | 2 | 2 | 2 | 22.08       | 3.04            |
| 3         | 1 | 3 | 3 | 3 | 3 | 3 | 27.14       | 2.15            |
| 4         | 2 | 1 | 1 | 2 | 2 | 2 | 22.51       | 5.27            |
| 5         | 2 | 2 | 2 | 3 | 3 | 2 | 26.83       | 5.52            |
| 6         | 2 | 3 | 3 | 1 | 1 | 2 | 25.53       | 3.61            |
| 7         | 3 | 1 | 2 | 1 | 3 | 2 | 29.86       | 6.88            |
| 8         | 3 | 2 | 3 | 2 | 1 | 3 | 26.69       | 7.03            |
| 9         | 3 | 3 | 1 | 3 | 2 | 1 | 22.78       | 7.38            |
| 10        | 1 | 1 | 3 | 3 | 2 | 2 | 17.69       | 4.98            |
| 11        | 1 | 2 | 1 | 1 | 3 | 3 | 33.06       | 3.00            |
| 12        | 1 | 3 | 2 | 2 | 1 | 1 | 21.68       | 4.42            |
| 13        | 2 | 1 | 2 | 3 | 1 | 3 | 19.09       | 5.20            |
| 14        | 2 | 2 | 3 | 1 | 2 | 1 | 20.87       | 4.12            |
| 15        | 2 | 3 | 1 | 2 | 3 | 2 | 29.21       | 4.45            |
| 16        | 3 | 1 | 3 | 2 | 3 | 1 | 24.31       | 7.01            |
| 17        | 3 | 2 | 1 | 3 | 1 | 2 | 22.23       | 7.20            |
| 18        | 3 | 3 | 2 | 1 | 2 | 3 | 35.54       | 4.43            |
| K1        | 138.44 | 128.86 | 145.19 | 160.25 | 130.62 | 131.87 |
| K2        | 144.04 | 151.76 | 155.08 | 146.48 | 141.48 | 146.60 |
| K3        | 161.42 | 161.88 | 142.24 | 135.77 | 170.41 | 164.04 |
| k1        | 23.07  | 21.48  | 24.20  | 26.71  | 21.77  | 21.98  |
| k2        | 24.01  | 25.29  | 25.85  | 24.41  | 23.58  | 24.43  |
| k3        | 26.90  | 26.98  | 23.71  | 22.63  | 28.40  | 27.34  |
| R         | 3.83   | 5.50   | 2.14   | 4.08   | 6.63   | 5.36   |

Optimization of fermentation conditions for γ-PGA production
On the basis of optimized fermentation medium, we detected the effect of medium pH on γ-PGA production. As shown in Fig. 4a, with the increase of original pH value of medium, the γ-PGA production was gradually decreased. At pH 7.0, the biomass achieved its highest value. This result was consistent with a previous literature [21]. We also investigated the effect of liquid volume on γ-PGA production, and found the production was decreased when the liquid volume increased (Fig. 4b). The biomass reached its highest amount when the liquid volume was 50 mL which was loaded in a 250 mL flask. However, excessive liquid volume was unfavorable for the bacterial growth. As shown in Fig. 4c, the γ-PGA production and biomass were both influenced by the inoculation amount. When the inoculation amount was more than 3%, the γ-PGA production was gradually decreased with the increase of inoculation amount. Similarly, the biomass was decreased with the increase of inoculation amount (> 5%).

**Verification of optimized fermentation medium and conditions for γ-PGA production**

We cultured GJ11 in the optimized fermentation medium and conditions, and found glucose in the medium was not significantly consumed by GJ11 during the first 12 h, corresponding to our result that no γ-PGA was accumulated in the broth. During 12–36 h, the biomass was dramatically increased, corresponding to a rapid decrease of residual glucose in the broth. Consistently, γ-PGA was rapidly biosynthesized in this period, with a maximum production of 41.47 g/L and a high productivity of 1.15 g/(L·h). During 36–48 h, the biomass, residual glucose, and γ-PGA production had no significant change. During 48–96 h, the biomass was increased again, accompanied by a decrease of residual glucose in the broth. In this period, γ-PGA production was gradually decreased (Fig. 5a), suggesting that some γ-PGA was consumed as carbon and nitrogen source for the bacterial growth. FA in the medium could not be used by GJ11, which was consistent with the previous report [18]. However, other organic acids could be gradually consumed by GJ11, accompanied by a gradually increased pH value of broth (Fig. 5b).

We further verified the influence of FA powder on biomass and γ-PGA production in the optimized formula. We found that the optimized fermentation medium with 20 g/L FA powder could produce γ-PGA at 42.55 g/L, while the medium without FA powder only produced 2.87 g/L γ-PGA (Fig. 5c). These results indicated that FA powder was important for γ-PGA production in our optimized formula because it probably contained some nutrients for the bacterial cells to produce γ-PGA.

**HR induced by γ-PGA and its hydrolyzates**

Lei et al. have reported that γ-PGA could protect plants against abiotic stress, such as high and low temperature [28]. In our study, we used γ-PGA purified from the fermentation broth of GJ11 to treat plants, then invested whether HR could be triggered in the leaves. The results showed that γ-PGA could not induce neither HR nor ISR to protect plants form the pathogen (Pst DC3000) infection (Fig. 6a and b). We deduced that this might be due to the high molecular weight of γ-PGA. Thus, we digested γ-PGA into γ-PGA hydrolysates with smaller molecular weights. We found that, as hydrolysis time increased, more and more γ-PGA was digested into the hydrolysates with smaller molecular weights (Fig. 6c).
After hydrolysis, the solution was adjusted to pH 7.0, then used for injecting the tobacco leaves. As shown in Fig. 6d, the 5 h hydrolysates could trigger HR in the leaves significantly. Consistently, the plants with ISR triggered by irrigating roots with 5 h hydrolysates showed a significant resistance against the pathogen \((Pst \ DC3000)\) infection (Fig. 6e). We further found that 5 g/L \(\gamma\)-PGA hydrolysates was more effective for inducing the resistance of plants against \(Pst \ DC3000\) infection (Fig. 6f).

**H2O2 accumulation and callose deposition induced by \(\gamma\)-PGA hydrolysates**

Many activators produced by beneficial microorganisms can trigger ISR to protect plants from pathogens infection by eliciting defence-related responses, such as ROS (e.g. \(H_2O_2\)) accumulation and callose deposition [24]. In order to know whether \(\gamma\)-PGA hydrolysates could induce the defence-related responses, the roots of \(A. \ thaliana\) were treated with \(\gamma\)-PGA hydrolysates, then infected by \(Pst \ DC3000\). The results showed that treatment with \(\gamma\)-PGA hydrolysates alone could not induce significant \(H_2O_2\) accumulation (Fig. 7a) and callose deposition (Fig. 7b) in the leaves. However, inoculation with pathogen alone could elicit \(H_2O_2\) accumulation and callose deposition. Our further investigation showed that pre-treatment with \(\gamma\)-PGA hydrolysates could elicit mild but effective plant immunity to rapidly response to \(Pst \ DC3000\) infection, accompanied by significant \(H_2O_2\) accumulation (Fig. 7a) and callose deposition (Fig. 7b) in the leaves.

We used different defense-compromised lines of \(Arabidopsis\), including NahG, jar1-1, ein2, and npr1, to detect the possible signals induced by \(\gamma\)-PGA hydrolysates. Compared to the control (water), after inoculation with \(Pst \ DC3000\) for 12 h, the lines, including Col-0, NahG, and jar1-1, were all observed to have \(H_2O_2\) accumulation (Fig. 7c) and callose deposition (Fig. 7d), while the lines, such as ein2 and npr1, were not. After 24 h, pre-treatment with \(\gamma\)-PGA hydrolysates significantly enhance \(H_2O_2\) accumulation (Fig. 7c) and callose deposition (Fig. 7d) in the lines Col-0, NahG, and jar1-1, rather than in the lines ein2 and npr1. These results suggested that the ISR induced by \(\gamma\)-PGA hydrolysates is dependent on NPR1, and the ET signal, rather than the SA and JA signals in plants.

We further recovered the pathogen in different lines, and found that pre-treatment with \(\gamma\)-PGA hydrolysates could significantly reduce the amount of \(Pst \ DC3000\) in Col-0, NahG, and jar1-1 when compared to that in CK (Fig. 7e). However, the pathogen amounts recovered in ein2 and npr1 was similar between the group pre-treated with \(\gamma\)-PGA hydrolysates and the control (CK). The results further verified that the ISR induced by \(\gamma\)-PGA hydrolysates is dependent on the ET signaling and NPR1 in plants.

**Discussion**

In the industrial bioprocess, molasses is a cheap and widely used carbon source for microbial fermentation, which can be used to produce various kinds of highly valuable metabolites [29]. However, the wastewater produced during molasses fermentation can lead to an environmental pollution if it is directly discharged into the environment without appropriate pre-treatment. Since molasses contains a
large amount of fulvic acid (FA), the wastewater from molasses fermentation can be made into powder by dry-spraying, which can be used as a fertilizer for crops [15, 16]. However, fertilizing with FA powder can result in soil acidification. In this study, we managed to use FA powder in microbial fermentation. After fermentation, some organic acids in the FA powder were consumed as carbon sources by microorganism. Thus, microbial fermentation can reduce the negative influence of FA powder on soil acidification. On the other hand, FA powder has been potentially used as a low-cost feedstock for fermentation to produce highly valuable metabolites, such as γ-PGA [22]. In agriculture, γ-PGA is regarded as a new environmental-friendly fertilizer synergist, as well as an activator to induce resistance against plant diseases [23]. However, due to low yield and high cost of production, γ-PGA is difficult to be wildly used in agriculture. Our study found that FA powder could be used as an alternative feedstock to produce γ-PGA at a low cost. After fermentation, most of organic acids were consumed to produce γ-PGA, but FA was difficult to be used by GJ11 because of its high amount of oxygen-rich and carbon-poor functional groups [18]. Thereby, FA, together with γ-PGA, remained in the broth to promote plant growth and protect crops from stresses. These results indicated that the fermentation broth containing both FA and γ-PGA could be a better and more effective fertilizer for crops.

Previously, we isolated a strain, B. velezensis GJ11, which could produce acetoin to trigger ISR in plants [24]. Here, we found it could also produce γ-PGA efficiently by using glucose and ammonium chloride as substrates in a glutamate-independent manner. However, most of these producers have low yield of γ-PGA. Thus, people began to use glutamate to enhance the yield of this polymer, resulting in a significant increase of the cost. In order to produce γ-PGA at a low cost, we tested whether FA powder could substitute some nutrients in the original formula. We firstly determined whether GJ11 was compatible to FA powder. Due to the low pH of FA powder medium, increasing the concentration of FA powder resulted in a gradual decrease in pH value of the medium, which was unfavorable for the bacterial growth. However, the decrease could be reversed by increasing the original pH value of the medium with NaOH. pH is an important environmental factor for γ-PGA fermentation. It has been reported that pH 6.5 can support a high γ-PGA production, whereas pH 7.0 is favorable for cell growth [26]. Thereby, in order to enhance γ-PGA production, the medium pH should be maintained at 7.0 for the first 24 h of culturing to obtain a maximum biomass, then shifted to 6.5 to maximize the γ-PGA production.

Although FA powder could promote the growth of GJ11 at a certain concentration, it reduced γ-PGA production in the broth. Thereby, we further optimized the medium formula with FA powder to improve γ-PGA production and reduce the production cost. Generally, glucose is used as a preferred carbon source for γ-PGA production [21]. In our study, an addition of glucose could improve both biomass and γ-PGA production in the medium with FA powder, indicating that FA powder alone is not enough to provide carbon sources for producing γ-PGA. Therefore, FA powder could not substitute glucose in the original fermentation medium. Production of γ-PGA often requires the supplementation of glutamate, resulting in an increase in the overall cost of production [15]. Although GJ11 is a glutamate-independent producer, our study showed that an addition of glutamate could significantly increase γ-PGA production. Interestingly, FA powder could partially substitute glutamate in the original fermentation medium. Glutamate-independent producer can de novo synthesize glutamate monomer for biosynthesis of γ-PGA.
via the TCA cycle [30]. Thereby, citrate acid is able to enhance γ-PGA production by joining the TCA cycle directly to elevate the level of α-ketoglutarate. In this way, more glutamate are generated to produce γ-PGA [21, 31]. In this study, we tested whether FA powder could substitute citric acid. According to our results, FA powder with organic acids could partially substitute citric acid in the medium for producing γ-PGA.

GJ11 can use inorganic nitrogen sources to synthesize glutamate for γ-PGA production. In this study, we found that FA powder could substitute NH₄Cl rather than NaNO₃, indicating that FA powder mainly contains NH₄⁺ rather than nitrate. Mg²⁺ is necessary for γ-PGA production because the activity of PgsBCA that is responsible for biosynthesis of γ-PGA is dependent on it [27]. Mn²⁺ is important for the stereochemical and enantiomeric composition of γ-PGA [26]. Ca²⁺ and Fe³⁺ are also needed for high production of γ-PGA. In this study, we found that FA powder could substitute all of these ions listed above. As a result, an addition of 20 g/L FA powder could decrease the cost of glutamate and citrate acid around one third, and substitute NH₄⁺, Mg²⁺, Mn²⁺, and Fe³⁺ when compared to the original fermentation medium. Thereby, the formula of medium with FA powder is low-cost for producing γ-PGA via fermentation.

Although *B. subtilis* and *B. licheniformis* have been reported to be promising native bacteria for commercial production of γ-PGA most frequently [21], our study proved that *B. velezensis* GJ11 could be potential for producing γ-PGA. With the optimized fermentation medium and conditions, the γ-PGA production reached a maximum concentration of 42.55 g/L, and a high productivity of 1.15 g/(L·h). In this formula, FA powder is essential because the medium without FA powder had a much lower production of γ-PGA which was only 2.87 g/L.

After fermentation, the pH value of the broth was significantly increased and FA was slightly consumed by GJ11. Thereby, the broth containing both γ-PGA and FA with a high pH value could be a better and more effective fertilizer than that containing FA alone. Although γ-PGA is generally used as a fertilizer synergist in agriculture, it has been reported to have the ability to protect crops from plant diseases (e.g. *Fusarium* root rot) [32]. We hypothesized that γ-PGA might act as an activator to induce resistance against the pathogen infection in plants. Plant activators that can induce defense response have attracted increasing attentions due to their potentials in controlling plant diseases whilst reducing the environmental burdens. Their action mechanisms can activate a complex signaling network, including the pathways regulated by SA, ET, JA, etc [19]. In our study, we further investigated whether γ-PGA could trigger resistance against the pathogens infection in plants. We found that γ-PGA with high molecular weight could not effectively trigger the resistance against *Pst* DC3000 infection, but the γ-PGA hydrolyzates were effective to induce the defense response (e.g. HR, H₂O₂ accumulation and callose deposition) against pathogen infection. Our results also revealed that γ-PGA hydrolyzates mainly triggered the induced systemic resistance (ISR) via ET signaling and NPR1. Thereby, in addition to acting as a fertilizer synergist, γ-PGA is potential to be used as a new activator to trigger the defense response against plant diseases.
Conclusions

We used the industry waste, FA powder, as a sustainable substrate for microbial synthesis of some biotechnological products, e.g. γ-PGA. This technology can not only alleviate the soil acidification induced by directly returning FA powder into soil, but also develop a new application of FA powder as a cheap raw material for producing γ-PGA at a low cost. Moreover, the novel use of FA powder as a raw material is favorable for reducing the possible pollution induced by wastewater from yeast fermentation with molasses. Thereby, the bioprocess of converting FA powder to highly valuable products, such as γ-PGA, is circular economic.

Materials And Methods

Strains, mediums, and chemicals

The strain used for fermentation was B. velezensis GJ11 [24]. The lines of Arabidopsis thaliana, including a wild-type line Col-0, and four defense-compromised mutants which were npr1 (nonexpressor of PR proteins), jar1-1 (jasmonic acid (JA) - insensitive line), ein2 (ethylene (ET) - insensitive line), and NahG (salicylic acid (SA) - degrading transgenic line) [33], were gifts from Prof. Yan S, Huazhong Agricultural University, Wuhan, Hubei, China. FA powder was bought from Lesaffre, Guangxi, China. All other chemicals were of analytical grade supplied by Sinopharm Chemical Reagent (China).

The basic fermentation medium (pH 7.4) contained (g/L): glucose 70.0, citrate sodium 30.0, NaNO₃ 12.0, NH₄Cl 8.0, K₂HPO₄ 0.7, CaCl₂ 0.3, MgSO₄·7H₂O 1.0, MnSO₄·H₂O 0.07, and FeCl₃·6H₂O 0.08. The original fermentation medium was prepared with the basic fermentation medium and sodium glutamate (30.0 g/L).

Production of γ-PGA by GJ11

We determined whether GJ11 could produce γ-PGA by a glutamate-independent manner. GJ11 was cultured in LB medium overnight, then inoculated in 50 mL of basic medium or the basic medium with a concentration gradient of glutamate (10, 20, 30, 40, 50, 60 and 70 g/L, respectively) in a 250 mL flask and incubated at 37 °C and 180 rpm for 36 h. After the incubation, the samples in different broths were collected for determining γ-PGA production.

Detecting compatibility of GJ11 to FA powder

GJ11 was cultured in LB medium overnight, then inoculated in 50 mL of concentration - gradient FA powder medium (1, 5, 10, 20, 40, 60 and 100 g/L, respectively) in a 250 mL flask at a ratio of 3% (v/v), for further culture at 37 °C and 180 rpm for 24 h. After the incubation, the samples in different broths were collected for counting the colony-forming unit (cfu) of GJ11.

Assaying influences of FA powder on γ-PGA production and biomass
FA powder was added into the original fermentation medium at different concentrations (0, 10, 20, 40, 60, 100 g/L, respectively). Then, the pH value of the medium was adjusted to 7.0 using 6 M NaOH solution. GJ11 was cultured in LB medium overnight, and transferred into 50 mL of the fermentation mediums with FA powder in a 250 mL flask at a ratio of 3%, for further culture at 37 °C and 180 rpm for 36 h. After the incubation, the samples were collected for determining γ-PGA production and biomass of the broth.

**Optimization of fermentation medium**

In the fermentation medium, the concentration of FA powder was set at 40 g/L, then the impacts of glucose, citrate sodium, sodium glutamate, NaNO₃, KH₂PO₄, NH₄Cl, MgSO₄, MnSO₄, CaCl₂ and FeCl₃ on γ-PGA production and biomass were investigated, respectively. In detail, glucose was added into the medium at a final concentration of 0, 10, 30, 50, 70, 90, 110 and 130 g/L, citrate sodium was added at a final concentration of 0, 5, 10, 20, and 30 g/L, and sodium glutamate was added at a final concentration of 0, 5, 10, 20, and 30 g/L respectively. NaNO₃ was added into the medium at a final concentration of 0, 2, 4, 8, 12, 16, 20 and 24 g/L, and NH₄Cl was added at a final concentration of 0, 2, 4, 6 and 8 g/L, respectively. The concentration of KH₂PO₄ was set at 0, 0.3, 0.5, 0.7, 0.9, 1.1 and 1.3 g/L, respectively. MnSO₄ was added into medium at a final concentration of 0, 0.01, 0.03, 0.05 and 0.07 g/L, MgSO₄ was added at a final concentration of 0, 0.2, 0.4, 0.8 and 1 g/L, CaCl₂ was added at a final concentration of 0, 0.1, 0.2, 0.3 and 0.4 g/L, and FeCl₃ was added with a final concentration at 0, 0.02, 0.04, 0.08 and 0.1 g/L, respectively. All other factors were held constantly.

On the basis of above optimization, the orthogonal test was set up for further optimizing the fermentation medium. Glucose, citrate sodium, sodium glutamate, NaNO₃, KH₂PO₄ and FA powder were selected for the orthogonal experiment design (L18(3⁷)) (Table 1).

| Factorial level | A-glucose g/L | B-NaNO₃ g/L | C-KH₂PO₄ g/L | D-FA power g/L | E-L-glutamate g/L | F-citrate sodium g/L |
|-----------------|---------------|-------------|--------------|---------------|------------------|---------------------|
| 1               | 60            | 12          | 0.5          | 20            | 5                | 5                   |
| 2               | 70            | 16          | 0.7          | 30            | 10               | 10                  |
| 3               | 80            | 20          | 0.9          | 40            | 20               | 20                  |

**Optimization of culture conditions**

On the basis of the orthogonal experiments, we further studied the effect of culture conditions on γ-PGA production. To study the impact of pH on γ-PGA production, the initial pH value of medium was set at 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. To study the impact of ventilation on γ-PGA production, several 250 mL flasks containing 25, 50, 75, 100 and 125 mL medium, respectively, were prepared for culturing GJ11. The
amounts of inoculation were set at 1%, 3%, 5%, 7% and 10% (v/v), respectively, for producing γ-PGA. All other factors were held constantly.

**Detecting γ-PGA production and molecular weight, biomass, residual glucose, pH and FA of broth**

The biomass of GJ11 was assayed by detecting the OD$_{600}$ value of broth. γ-PGA production of the broth was determined by the cetyltrimethylammonium bromide (CTAB) turbidimetry method, and the SDS-PAGE with methylene blue staining [30, 32, 34]. Residual glucose in the broth was determined using SBA-40D Bio-analyzer (Shandong Academy of Sciences, China) [35]. The pH value of broth was detected using a pH meter. The content of fulvic acids was determined by the KMnO$_4$ oxidation method. After dissolution, fulvic acids were valued by titration with ferrous ammonium sulphate and N-phenyl anthranilic acid, which could indicate the end point [17, 36]. The γ-PGA molecular weight was measured by using gel permeation chromatography with a RI-10 refractive-index detector and a SuperposeTM 6 column (Shimadzu Corp) [37].

**Analysis of hypersensitive reaction (HR) induced by γ-PGA and its hydrolyzates**

γ-PGA was recovered from the GJ11 broth [20]. 20 µL of γ-PGA in gradient concentration (1, 5, 10 and 15 g/L, respectively) was used for injecting the tobacco leaves with an 1 mL syringe without needles. After 24 h, the hypersensitive response (HR) was detected via trypan blue staining [24]. After irrigating tobacco roots with γ-PGA (1, 5, 10 and 15 g/L, respectively) at 5 ml per seedling in a pot (one plant per pot, 10 pots per group) for 3 days, the pathogen of *Pseudomonas syringae* pv. *tomato* DC3000 (termed as *Pst* DC3000, $1 \times 10^8$ cfu mL$^{-1}$) was used for infecting tobacco plants by spraying the leaves evenly. The leaves were collected three days after the infection, then sterilized and homogenized for spreading plates. After incubation, the bacterial colony was counted for calculating the *Pst* DC3000 content per gram of fresh leaf (cfu g$^{-1}$) [24].

The γ-PGA solution (5 g/L) was adjusted to pH 2.0, then incubated at 80 °C for 0, 1, 2, 3, 4, 5, 6, 7 and 8 h, respectively. After that, the pH value of solution was adjusted to 7.0 with 6 M NaOH, then the samples were collected for analysis of hydrolyzates using SDS-PAGE [32]. After hydrolysis, the γ-PGA hydrolyzates were detected for the activity to trigger the hypersensitive response, and the ISR against *Pst* DC3000 infection as above.

**Detecting cellular defensive responses induced by γ-PGA hydrolyzates**

Different lines of *A. thaliana* seedlings (6-week old, 10 seedlings per group), including Col-0, NahG, npr1, jar1-1, and ein2, were irrigated with 5 mL of γ-PGA hydrolyzates (5 g/L of γ-PGA was hydrolyzed for 5 h), then infected with *Pst* DC3000 by spraying the leaves as above. After that, the leaves were collected and
stained with DAB to detect the accumulation of $\text{H}_2\text{O}_2$, a kind of reactive oxygen species (ROS), in plants [24]. Additionally, callose deposition of the leaves was also detected by our previous methods [24].

**Statistical analysis**

All experiments were repeated in triplicates. The data between two groups were compared using Student's $t$ test.

**Abbreviations**

FA
fulvic acid; γ-PGA: poly-γ-glutamic acid; SDS-PAGE: dodecyl sulfate sodium salt (SDS)-Polyacrylamide gel electrophoresis; HR: hypersensitive response; ISR: induced systemic resistance; ET: ethylene; SA: salicylic acid; JA: jasmonic acid; CK: control.

**Declarations**

**Authors’ contributions**

YL and JW completed performed the major experiments. GQ and LK proofed data and text and gave guidance for formatting and submission of the paper. XZ helped with analyzing the results. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

All data generated during this study are included in this published article and its additional file.

**Consent for publication**

All the authors consented on the publication of this work.

**Ethics approval and consent to participate**

Not applicable.

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Figures
Figure 1

γ-PGA produced by GJ11. a: Effect of sodium glutamate on γ-PGA production. b: SDS-PAGE analysis of γ-PGA produced by GJ11 in the medium with sodium glutamate. Lane S: Commercial γ-PGA standard; Lane 1 - 8: γ-PGA produced by GJ11 in the medium with 70, 60, 50, 40, 30, 20, 10, 0 g/L of sodium glutamate, respectively. c: Gel permeation chromatography analysis of the molecular weight of γ-PGA produced by GJ11.
Figure 2

Effect of carbon and nitrogen sources on biomass and γ-PGA production. a: glucose; b: citrate sodium; c: glutamate sodium; d: NaNO3; e: NH4Cl.
Figure 3

Effect of inorganic salts on biomass and γ-PGA production in the medium with FA power. a: KH2PO4; b: MnSO4; c: MgSO4; d: CaCl2; e: FeCl3.
Figure 4

Optimization of fermentation conditions for γ-PGA production. a: Effect of pH on cell biomass and γ-PGA production. b: Effect of loading liquid volume on cell growth and γ-PGA production. c: Effect of inoculation amount on cell growth and γ-PGA production.
Figure 5

Optimized fermentation medium and conditions for γ-PGA production. a: Time-course of γ-PGA fermentation. b: Variation of pH value and FA content in the broth. c: Influence of FA power on biomass and γ-PGA production in the optimized formula.
Figure 6

Effect of γ-PGA and its hydrolysates on triggering HR against Pst DC3000 infection in tobacco plants. a: Effect of γ-PGA on triggering HR in tobacco leaves; b: Effect of γ-PGA on triggering ISR against Pst DC3000 infection in tobacco plants. c: SDS-PAGE analysis of hydrolysates of γ-PGA. The pH of γ-PGA solution (5 g/L) was adjusted to 2.0, then hydrolyzed at 80°C for 0, 1, 2, 3, 4, 5, 6, 7 and 8 h, respectively. d: Effect of γ-PGA hydrolysates on triggering HR in tobacco plants. 5-1 - 5-10: 5 g/L γ-PGA were hydrolyzed for 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h at pH 2.0 and 80°C. e: HR triggered by γ-PGA with hydrolysis for different time to protect plants from Pst DC3000 infection. f: Effect of γ-PGA hydrolysates at different concentrations on triggering HR against Pst DC3000 infection. * indicates significant difference from control (CK, treated with water).
Figure 7

H2O2 accumulation and callose deposition in the leaves treated with γ-PGA hydrolyzates. a: H2O2 accumulation in the leaves of wild-type line of Arabidopsis pre-treated with γ-PGA hydrolyzates following with infection by Pst DC3000. b: Callose deposition in the leaves of wild-type line of Arabidopsis pre-treated with γ-PGA hydrolyzates following with infection by Pst DC3000. c: H2O2 accumulation in the leaves of mutant lines of Arabidopsis pre-treated with γ-PGA hydrolyzates following with infection by Pst DC3000. d: Callose deposition in the leaves of mutant lines of Arabidopsis pre-treated with γ-PGA hydrolyzates following with infection by Pst DC3000. e: Pathogen amounts recovered from the leaves of mutant lines of Arabidopsis pre-treated with γ-PGA hydrolyzates following with infection by Pst DC3000. * indicates significant difference from control (CK, pre-treated with water).

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