Bacillus amyloliquefaciens uses mixed sugar to synthesize γ-PGA

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Abstract. Poly-γ-glutamic acid (γ-PGA) is an anionic polymer synthesized by microorganisms. It has very strong water solubility, edible property and biocompatibility. It is non-toxic to humans and the environment. It has wide application prospect in agriculture, food industry, pharmacy and environment. However, its fermentation cost is high, and its fermentation medium needs a large amount of carbon and nitrogen sources such as glucose, citric acid and glutamic acid, while lignocellulose As one of the most abundant biomass resources on the planet, it can hydrolyze glucose and xylose. Therefore, it is possible to select suitable fermentation strains and utilize lignocellulose to optimize culture conditions to improve the efficiency of γ-PGA synthesis. In this paper, a self-screening bacillus amyloliquefaciensC1 was studied and found that the bacteria can use xylose as a carbon source for growth and metabolism. Using bacillus amyloliquefaciensFZB42 as a control to study in depth, two strains of bacteria were cultured in M9 containing 10g/L xylose, and when the culture time reached 44h, the strain C1 had almost completely consumed 10g/L of xylose, while the strain FZB42 Only consumed 2g/L of xylose. In response to this phenomenon, we will be Cl bacteria can effectively use the nature of xylose used in polyglutamic acid fermentation experiments, 60g/L of xylose as a carbon source can produce polyglutamic acid 14.52g/L, while the same concentration Of glucose as carbon source, can produce polyglutamic acid 9.64g/L.

1. Introduction
γ-Polyglutamic acid is an anionic polyamino acid formed by microorganisms such as Bacillus bacteria in nature and polymerized by γ-amide bond formed between α-amino group and γ-carboxyl group by L-glutamic acid. γ-glutamic acid has excellent water-soluble, super-absorbent and biodegradable, degradation products of pollution-free glutamic acid, is an excellent environment-friendly polymer materials, can be used as a water-retaining agent, heavy metal ions Adsorbent, flocculant, sustained release agent and drug carrier, etc., have great commercial value and social value in cosmetics, environmental protection, food, medicine, agriculture, desert control and other industries. The fermentation medium needs to add a large amount of carbon and nitrogen sources such as glucose, citric
acid and glutamic acid. In order to reduce the production cost, it is necessary to seek cheap and easy-to-obtain biomass resources as substrates for producing \( \gamma \)-PGA; lignocellulose is one of the most abundant biomass resources on Earth and is also an ideal resource and energy for the future. Hydrolysis can generate glucose and xylose, synthesize \( \gamma \)-PGA with glucose and xylose, and routinely optimize its fermentation medium, improve their synthesis efficiency and provide a new way for comprehensive utilization of lignocellulose resources.

As a biological macromolecule material with wide application prospects, \( \gamma \)-PGA mainly includes the following ways: chemical synthesis, enzymatic conversion and microbial fermentation. The molecular weight of chemically synthesized polymers is significantly smaller than that of native \( \gamma \)-PGA, and the technology required for chemical synthesis and extraction methods is more advanced and costly than for biosynthesis. And microbial fermentation method is simple and convenient, easy to control and operation, and high yield of \( \gamma \)-PGA, suitable for large-scale production.

Dilute acid hydrolysis is a more mature woody biomass hydrolysis method. Dilute acid hydrolysis process using two-step method: the first step of dilute acid hydrolysis at a lower temperature, in the process, the hemicellulose is very easy to be hydrolysed to five-sugar products such as xylose; the second step acid hydrolysis Is carried out at a higher temperature, acid re-acid hydrolysis residual solids (mainly microcrystalline cellulose), to obtain fermentable hydrolyte glucose. During the reaction, hemicellulose is relatively easy to hydrolyze, its rate constant increases with the increase of acid concentration and temperature, and the conversion of xylose is also very high.

2. Materials and methods

2.1. Experimental Materials
Strain C1 (laboratory screening), FZB42 (laboratory deposit) used in the paper; medium used: LB solid medium, M9 liquid medium, fermentation medium (g/L).

2.2. Experimental method

2.2.1. Growth curve determination. The same microorganism in different culture conditions, the growth curve is not the same. Bacillus amyloliquefaciens belongs to the bacterium and has the same four typical growth stages of lag phase, exponential phase, stable phase and decay phase as all single-cell microbial cultures. Bacillus amyloliquefaciens in the exponential phase has the characteristics of high density of bacteria, strong growth ability and strong reproductive capacity, which facilitates rapid expansion of bacillus amyloliquefaciens. Therefore, in order to facilitate the rapid expansion culture of bacillus amyloliquefaciens, it is necessary to understand the growth curve of bacillus amyloliquefaciens under certain conditions. Meanwhile, in order to compare the growth ability of bacillus amyloliquefaciens C1 in xylose medium, we selected the model strain bacillus amyloliquefaciens FZB42 (model strain) as a reference strain.

The activated two strains of bacteria C1 and FZB42 were inoculated into 50ml M9 medium containing 250ml flask, 37 °C, 220r/min shaker culture 16h, as a seed solution.

The medium of 10 g/L xylose, 10g/L glucose and 5g/L xylose and 5g/L glucose as carbon source were prepared respectively. \( \gamma \) Inoculation amount inoculated on the above three different carbon sources of the medium, Shake in the ultra-clean Taichung, respectively, sub-packed in sterile flasks flask, with a marker pen culture time: 0, 2, 4, 6, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48h. The control test tube was not added to the bacterial liquid, stored in a refrigerator at 4 °C, and the rest of the test tube was incubated in a constant temperature shaker (37 °C, 220 r/min) until the test tube was removed. The starting culture medium was taken as the control, and the OD600 value of the cells was measured at 600nm wavelength. The experimental data were recorded and the growth curve was drawn by taking the shaker culture time as the abscissa and the OD600 value as the ordinate.
2.2.2. **Monosaccharide content in culture medium.** In order to understand the depletion ability of bacillus amyloliquefaciens C1 on different carbon sources, the content of residual sugars in different culture media was detected by HPLC to achieve the goal.

Chromatographic conditions: High-performance liquid chromatography (HPLC) analysis of the system using an SCL-10A system consisting of an LC-10ATvp pump and a RID-10A differential detector using a HPX-87H column (300 x 7.8 mm, Aminex HPX-87H Ion Exclusion column, injection volume 20ul, 0.005 mol/L sulfuric acid as mobile phase, flow rate 0.6mL/min, differential detector temperature 35 °C, column temperature 60 °C.

The column should be cleaned and regenerated when there is a high column pressure or when trailing of chromatographic peaks in the HPLC assay. Column cleaning method: 5% acetonitrile 0.005 mol/L sulfuric acid solutions at a flow rate of 0.2mL/min reverse washing column 4h, with cleaning solution 2 at the same flow rate continued reverse column 12h, the positive column, with Mobile phase (0.005mol/L sulfuric acid) to 0.2mL/min flow rate to the baseline stable, the column was cleaned.

Long-term use of the column will reduce the efficiency, the need to regenerate the column. Column regeneration can be carried out after washing the column, can also be carried out separately. The regeneration method is as follows: the column is connected directly, with 0.025 mol/L sulfuric acid solution at a flow rate of 0.2mL/min run 7 ~ 8h, and then mobile phase (0.005mol/L sulfuric acid) to 0.2mL/min flow rate to baseline stable, Column regeneration is completed.

Detection of the remaining sugar content in the medium: strain culture reference 2.2.1, sampling time: 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 44 h, the sample after centrifugation, according to 2.2.2.1 HPLC chromatographic conditions of injection, the detection of two samples of each peak area of sugar, the use of standard curve, calculate the sugar concentration.

2.2.3. **Bacillus amyloliquefaciens C1 uses different sugar sources to produce polyglutamic acid.** Inoculation loop Pick a flat plate single colonies were inoculated into LB liquid seed medium liquid medium, incubated at 37 ° C under 220rpm for 16 hours, inoculated 5% inoculum into liquid shake flask fermentation medium at 30 ° C under 170 rpm Shaker training for 72 hours. In order to study the fermentation ability of bacillus amyloliquefaciens using different carbon sources, 60g/L glucose, 60g/L xylose and 30g/L glucose and 30g/L xylose mixed sugar were respectively added into fermentation medium to produce polyglutamic acid Amino acids.

2.2.4. **Polyglutamic acid extraction and purification.** After the fermentation broth was centrifuged at 15, 000g for 20min, the supernatant was taken, 4 times the volume of precooled absolute ethanol was added, and the mixture was allowed to stand overnight and centrifuged at 12,000g. The precipitate was dissolved in deionized water and then dialyzed in deionized water overnight Molecular weight 9,000 Da) and lyophilized to give a solid starting material. The initial extract was dissolved in deionized water, centrifuged at 12,000 g, and the supernatant was added with 20 mg/mL proteinase K and dialyzed in deionized water overnight. Centrifugation was performed as described above and the supernatant was lyophilized to obtain a solid purified sample of γ-PGA. °C save.

3. **Results and analysis**

3.1. **Strain growth curve determination results**

In this experiment, bacillus amyloliquefaciens shaker culture OD600 values based on to examine the growth curve. Figure 1 shows the growth curve of bacillus amyloliquefaciens C1 and FZB42 in M9 medium with xylose as carbon source. Figure 2 shows the growth curve of bacteria C1 in different carbon sources.
It can be seen from Figure 1, C1 bacteria in the 0 ~ 10h, OD600 value increased slowly, indicating that cells in the lag phase, slow growth of bacteria, is the adaptation of bacteria to the environment; in the next 10 ~ 20h, Bacillus amyloliquefaciens grew rapidly at the logarithmic phase, and the number of the cells rapidly increased and reached a maximum quickly. During this period, the cell activity was the strongest and the growth was vigorous. At about 20h, the number of cells is larger and the number of viable cells is the most, which is suitable for culturing or fermenting as a kind of bacteria, which can shorten the growth or fermentation period; the OD600 value does not change much within 20-36h, Bacillus subtilis began to produce spores after a constant number of viable cells. After 36h, the OD600 of the cells started to decrease, the number of the cells started to decrease, the bacteria started to age, and bacteriolytic phenomena occurred and entered the decay period. Therefore, should be selected within 10 ~ 20h Bacillus subtilis culture solution as a seed solution, to expand the culture. Compared with FZB42, it is obvious that after entering the logarithmic phase, the bacterial cell concentration of C1 strain is obviously higher than that of the control strain FZB42, that is, the growth ability of C1 strain using xylose as a carbon source is stronger than that of the control strain FZB42. Figure 2 shows that
bacillus amyloliquefaciens C1 has different growth ability in different carbon sources, and its growth is more vigorous with glucose as carbon source. The use of xylose as a carbon source of growth is relatively poor. When mixed glucose and xylose were used as carbon sources, the growth curve fluctuated, possibly due to the presence of significant carbon metabolism inhibition (CCR) in the metabolic pathway. CCR refers to the preferential use of readily available carbon sources (usually glucose) by microorganisms in the mixed carbon source fermentation, and the carbon source and its metabolites inhibit the microbial expression of genes related to the metabolism of other non-available carbon sources (eg xylose) and Enzyme activity, thus affecting the efficiency of non-efficient use of carbon sources.

3.2. Standard curve drawing
According to the HPLC detection results of xylose and glucose standard samples, standard curve was drawn by Excel software. Table 1 and Table 2 are the HPLC detection peak areas of xylose and glucose at different concentrations respectively.

### Table 1. Xylose standard sample concentration and HPLC detection peak area

| Xylose concentration x (g/L) | 1.0  | 2.5  | 5.0  | 7.5  | 10   |
|-----------------------------|------|------|------|------|------|
| Peak area y                 | 352539 | 894283 | 1806708 | 2609632 | 3530658 |

According to Table 2, we can fit the regression equation $y = 350708x + 15082$, $R^2 = 0.9995$.

### Table 2. Glucose standard concentration and HPLC detection peak area

| Glucose concentration x (g/L) | 1.0  | 2.5  | 5.0  | 7.5  | 10   |
|------------------------------|------|------|------|------|------|
| Peak area y                  | 352539 | 894283 | 1806708 | 2609632 | 3530658 |

According to Table 2, we can fit the regression equation $y = 364057x + 9862.7$, $R^2 = 0.9994$.

3.3. Bacillus amyloliquefaciens C1, FZB42 on xylose, glucose utilization
In order to understand the ability of bacillus amyloliquefaciens C1 to utilize xylose, the experiment also took the standard strain FZB42 as the control strain. Figure 3 and Figure 4, respectively, two strains of xylose and glucose utilization, Figure 5 is C1 bacteria on the use of mixed sugar.

![Figure 3. Clostridium C1, FZB42 utilization of xylose](image-url)
As can be seen from Fig. 3 and Fig. 4, the ability of utilization of glucose by bacillus amyloliquefaciens C1 and FZB42 are basically the same, but there is more difference in the utilization of xylose, and the utilization ability of C1 bacteria is obviously stronger than that of the model bacterium FZB42. As can be seen from Fig. 5, the concentration of glucose rapidly decreased from 0 to 15h, indicating that the bacteria C1 is now growing with glucose as a carbon source; and the content of xylose varies from 0 to 20h is very small. After 20h, Sugar content began to decline, the rate of decline more slowly, this phenomenon is the carbon metabolite inhibitory effect (CCR). How to relieve CCR inhibition is also a hot research direction in the field of scientific research.
3.4. *Bacillus amyloliquefaciens C1* uses different carbon sources to produce polyglutamic acid

In the experiment, polyglutamic acid was fermented by adding 60g/L xylose, 60g/L glucose and 30g/L xylose and glucose respectively in the fermentation medium. Polyglutamic acid the case of fermentation, the data is shown in Table 3.

**Table 3. Bacillus amyloliquefaciens C1 polyglutamic acid production with different carbon sources**

| Carbon source         | Glutamic acid | γ-PGA production |
|-----------------------|---------------|------------------|
| 60g/L Xylose          | 0g/L          | 4.56g/L          |
| 60g/L glucose         | 0g/L          | 4.20g/L          |
| 60g/L Xylose          | 10g/L         | 14.52g/L         |
| 60g/L glucose         | 10g/L         | 9.64g/L          |
| 30g/L Xylose +30g/L glucose | 10g/L | 11.98g/L         |

As can be seen from the data in the table, *Bacillus amyloliquefaciens C1* can still be fermented to produce polyglutamic acid without the precursor glutamic acid, the yield is relatively low, and the yield in the 60g/L xylose system is 4.56g/L, the yield in a system to which 60 g/L of glucose was added was 4.20 g/L. After adding 10g/L precursor glutamic acid, polyglutamic acid production increased significantly. L-PGA alone could be fermented by using xylose alone as carbon source system; 9.64 g/L γ-PGA could be fermented by using glucose alone as carbon source; 11.98 g/L γ-PGA could be fermented by mixed sugar system. In terms of yield, xylose is more suitable as a carbon source for glutamic acid fermentation by bacillus amyloliquefaciens C1.

4. Summary

(A). By measuring the growth curves of bacillus amyloliquefaciens C1 and FZB42 in M9 medium with xylose as carbon source, it can be seen that the Bacillus subtilis culture solution within 10-20 h should be selected as the seed liquid for enlarging culture. After entering the logarithmic phase, the bacterial concentration of C1 strain was significantly higher than that of the control strain FZB42. The growth ability of C1 strain using xylose as a carbon source was stronger than that of the control strain FZB42.

(B). Bacillus amyloliquefaciensC1 showed different growth capacities in different carbon source media by measuring the growth curve of bacteria C1 in different carbon source media. When using glucose as carbon source, the growth of bacillus amyloliquefaciens C1 was more vigorous, while using xylose as growth potential of carbon source relatively poor. When glucose and xylose mixed sugar were used as a carbon source, the growth curve fluctuated due to the inhibitory effect of the sugar metabolites.

(C). the standard strain FZB42 was used as a control strain to study the utilization of xylose and glucose by the two strains. The ability of utilization of glucose by bacillus amyloliquefaciens C1 and FZB42 was basically the same, but the utilization of xylose was quite different. However, the carbon metabolite inhibitory effect (CCR) existed when fermenting with glucose xylose mixed sugar as carbon source.

(D). In the experiment, polyglutamic acid was fermented by adding 60g/L xylose, 60g/L glucose and 30g/L xylose and glucose respectively in the fermentation medium. Polyglutamic acid Case of fermentation, from experimental data can be drawn, adding 10g/L precursor glutamate, polyglutamic acid production increased significantly. Xylose is more suitable as a carbon source for glutamic acid fermentation by bacillus amyloliquefaciens C1.

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