Detection of Bacillus Amyloliquefaciens C1 Xylose Metabolism Related Enzyme

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Abstract. Lignocellulose resources and their hydrolyzate contain a large amount of mixed carbon sources such as glucose and xylose, but Bacillus is less effective in utilizing xylose. In most bacillus, xylose is first converted to xylulose by xylose isomerase (XI) and subsequently catalyzed by xylulokinase (XK) to form 5-phospho-xylulose to enter the pentose phosphate pathway. In this paper, a self-screening bacillus amyloliquefaciens C1 was studied and found that the bacteria can make good use of 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. To understand the efficient use of xylose by C1 bacteria, we selected two of the most important enzymes in xylose metabolism, xylose isomerase and xylulokinase. The data showed that strain XI and XK enzyme of strain C1 were higher than that of strain FZB42. Among them, C1 strain XI and XK enzyme could reach 0.200U/mg and 0.516U/mg respectively, which were 1.6 times and 2.4 times that of FZB42 respectively. The results showed that XI, XK high enzyme is one of the reasons that bacillus amyloliquefaciens C1 can effectively utilize xylose.

1. Introduction

Lignocellulose is a huge source of renewable earth resources on Earth, and is currently being used industrially far below reserves, opening up other uses for the remaining lignocellulose. In addition, the current industrial fermentation raw materials are still mainly grain. With the development of biotechnology, the scale of industrial fermentation is expanding day by day. The demand for grain is also rapidly increasing. The fermenting industry and people have begun to compete for food. The use of rich lignocellulose degradation products instead of food as a raw material for fermentation is the best way to solve this problem, which has become the consensus of academia and society. However, there is a clear Carbon Catabolite Repression (CCR) in the metabolic pathway of Bacillus sp. CCR means that microorganisms preferentially utilize a quick-acting carbon source (usually glucose) in the fermentation of a mixed carbon source, and the carbon source and its metabolites inhibit the microorganism's
expression and enzyme resistance to other non-available carbon metabolism-related genes, thereby affecting Non-effective carbon source utilization efficiency. XynA (encoding xyloside permease), xynB (encoding β-xylosidase), xylA (encoding xylose isomerase) and xylB (coding is xylulokinase) constitutes a xylose metabolism operon. Is regulated at the transcriptional level and works by the following three mechanisms: (a). Regulates the expression of Xylose-sensitive Repressor (XylR) - encoding gene xylR. (B). CCR mediated by CcpA interacting with phosphorylated Hpr.

Hemicellulose is one of the main components of lignocellulose. In addition to the problem of pretreatment of lignocellulose, lignocellulose degradation products as a fermentation raw material still have another technical problem that an industrial fermentation strain is needed to fully utilize various kinds of lignocellulose degradation products Sugar, and has good industrial performance. It is a biological problem whether or not to use alternative strains of industrial sugars such as xylose instead of other pentose sugars. Unfortunately, there are very few industrial strains that can utilize xylose as the fermentation material. In order to solve the above problems, genetic engineering methods have been used to transform existing microorganisms to obtain genetically engineered bacteria capable of utilizing various components of lignocellulose hydrolyte and stably and efficiently producing industrial products.

2. Experiment method

2.1. Experimental medium used and preparation methods

(A). LB solid medium: tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar powder 15 g/L

(B). M9 liquid medium:

①. Preparation of 1M MgSO4: MgSO4 • 7H2O 2.46g plus double distilled water 10ML dissolved, autoclaved spare;

②. Preparation of 1M CaCl2: CaCl2 • 6H2O 2.1 91g plus double distilled water 10ML dissolved, autoclaved spare;

③. Then make 5×M9 salt solution: Na2PO4 • 7H2O 12.8g, KH2PO4 3.0g, NaCl 0.5g, NH4Cl 1.0g; Add double distilled water 200ml dissolved 121 degrees sterilization 15 minutes, Note: The above three were separately prepared, respectively Bottle, can be sent together to high pressure;

④. Preparation of 20% glucose solution: 4g glucose plus double distilled water 20ml dissolved, 0.22 micron filter sterilized;

(C). Fermentation medium (g/L): citric acid 2.5, glutamic acid 10, NH4Cl 6.8, glycerol 30, K2HPO4 1, MgSO4.7H2O 0.5, FeCl3.6H2O 0.02, CaCl2 0.2, MnSO4.H2O 0.05, pH 7.2.

2.2. Experimental method

2.2.1. Detection of remaining sugar in culture medium. Strains were sampled at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 44 h after sample centrifugation. Samples were taken according to HPLC chromatographic conditions to detect the presence of two sugars Peak area, the use of standard song, calculate the sugar concentration.

2.2.2. Enzyme Detection of Xylose Isomerase and Xylulose Kinase

(A). Crude enzyme solution extraction

The bacterial cells were collected at 8, 16, 24, 32 hours after culture at 32h, then centrifuged at 9000 rpm and 4 °C for 5 min to obtain the cells. First, the cells were washed twice with 2 mL of 0.1 M pH 7.0 phosphate buffer solution (precooling at 4 °C) and the cells were resuspended in 2 mL of the same buffer. Second, lysozyme was added to the suspension at a final concentration of 10 mg/mL and incubated in a constant temperature water bath at 37 °C for 20 min. Then, the cells were broken by ultrasonic wave, and the conditions of ultrasonic breaking were as follows: 130W, 20 kHz; Pulse on 2s; Pulse off 2s; the total breaking time was 20min. Finally, centrifuged at 9000 rpm for 30 min at 4 ° C to remove impurities such as bacteria, and the supernatant is the crude enzyme solution, placed on ice spare.
(B). Protein concentration test
Protein concentration is detected by: BCA protein quantitative analysis kit.

(C). Detection of xylose isomerase (XI) enzyme
In this study, the activity of the heterologous expressed xylose isomerase was analysed using the coupled xylose isomerase-sorbitol dehydrogenase method, a fast and accurate xylose isomer Enzyme force determination method. The principle of the determination of the enzyme force is based on the xylose produced by the xylose isomerization and the xylulose is reduced to xylitol by the commercial D-sorbitol dehydrogenase and NADH. By calculating the NADH in the reaction system 340nm at the rate of change of absorbance to determine xylose isomerase enzyme, the determination principle is as follows:

\[
\text{D-xylose} \xrightarrow{\text{Xylose isomerase}} \text{D-xylulose} \xrightarrow{\text{D-sorbitol dehydrogenase, NADH}} \text{xylitol} + \text{NAD}^+ 
\]

Xylose isomerase assay: 100 mM, pH 7.5 Tris-HCl, 500 mM xylose, 10 mM MgCl₂, crude enzyme solution 10 μl, 1 U sorbitol dehydrogenase (Sigma), 0.15 mM NADH, (Ie time scan at 340 nm). The specific activity of an enzyme is defined as the number of micromol L⁻¹ of NADH converted per minute per mg of enzyme protein, ie, U mg⁻¹ protein.

(D). Xylulokinase (XK) enzyme assay
Xylulose kinase enzyme using continuous monitoring method, select the linear reaction period to calculate the enzyme force, the determination principle is as follows:

\[
\text{D-xylulose + ATP} \xrightarrow{\text{Xylulokinase}} \text{D-xylulose-5-P + ADP} \\
\text{ADP + phosphoenolpyruvate} \xrightarrow{\text{Pyrivate kinase}} \text{pyruvate + ATP} \\
\text{Pyruvate + NADH} \xrightarrow{\text{Lactate dehydrogenase}} \text{D-lactate + NAD}^+
\]

According to the above reaction, when these coupling reactions reach equilibrium, the rate of xylulose conversion by the substrate of xylulose kinase in the reaction system is the same as the rate of change of NADH in the system.

Xylulose kinase enzyme detection method: 1mL reaction solution (50mM tris-HCl, pH7.5, 2mM MgCl₂, 0.2Mm NADH, 8.5mM D-xylulose, 0.2mM PEP, 10U pyruvate kinase, 10U lactate dehydrogenase, 2 mM ATP and appropriate amount of enzyme solution) at 34OunI detected NADH was oxidized (ie at 340nm do time scan). The specific activity of an enzyme is defined as the number of micromol L⁻¹ of NADH converted per minute per mg of enzyme protein, ie, U mg⁻¹ protein.

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. As shown in Figure 1.

![Figure 1. Growth curve](image)

Note: C1, FZB42 growth curve in xylose medium; X, G, X + G represent the growth curve in different media
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. Bacillus amyloliquefaciens C1 in different carbon source medium, the growth ability is different, the use of glucose as a carbon source its growth more exuberant! 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 fermentation of mixed carbon sources, and the carbon sources and their metabolites inhibit the microbial expression of genes related to the metabolism of other non-available carbon sources such as xylose and Enzymatic, 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. Figure 2 and figure 3, respectively, for their standard curve.

![Figure 2. Xylose liquid chromatography standard curve](image2)

![Figure 3. Glucose liquid chromatography standard curve](image3)
According to Figure 2, the fitting regression equation $y = 350708x + 15082$, $R^2 = 0.9995$; according to Figure 3, the fitted regression equation $y = 364057x + 9862.7$, $R^2 = 0.9994$.

3.3. Enzyme assay

In order to study the ability of bacillus amyloliquefaciens to utilize xylose by enzyme, we selected two enzymes that are important in xylose metabolism pathway, xylose isomerase (XI) and xylulokinase (XK). Bacillus amyloliquefaciens C1 and FZB42 were cultured in medium with different carbon sources. Enzyme was selected for three time samples. Table 1 and Table 2 respectively corresponded to the results of XI and XK.

**Table 1. XI enzyme assay results**

| Carbon source | Time (h) | X | G | X+G |
|---------------|----------|---|---|-----|
| C1            | 10       | 0.096 | ND | ND |
| FZB42         | 0.035    | ND | ND | ND |
| C1            | 20       | 0.198 | 0.107 | 0.028 |
| FZB42         | 0.107    | ND | ND | 0.010 |
| C1            | 30       | 0.200 | 0.126 | 0.106 |
| FZB42         | 0.126    | ND | ND | 0.038 |

**Table 2. XK enzyme assay results**

| Carbon source | Time (h) | X | G | X+G |
|---------------|----------|---|---|-----|
| C1            | 10       | 0.202 | ND | ND |
| FZB42         | 0.091    | ND | ND | ND |
| C1            | 20       | 0.494 | 0.196 | 0.112 |
| FZB42         | 0.196    | ND | ND | 0.039 |
| C1            | 30       | 0.516 | 0.213 | 0.204 |
| FZB42         | 0.213    | ND | ND | 0.098 |

Note: Specific activity units are U/mg; ND: no activity detected.

In Bacillus sp., Xylose is first converted to xylulose by xylose isomerase (encoded by the xylA gene) and subsequently catalyzed by xylulokinase (encoded by the xylB gene) to form 5-phospho-xylulose to pentose phosphate Sugar pathway, and then involved in intracellular anabolism. The two Bacillus sp. Strains FZB42 (a model strain of bacillus amyloliquefaciens) and the enzymatic properties of C1 showed that they all had the ability to metabolize xylose, but the enzyme of strain C1 was significantly higher than that of FZB42. The C1 strain has strong ability of xylose metabolism.

However, in the medium containing both xylose and glucose, FZB42 and C1 strains did not detect xylose metabolism-related enzyme properties (XI and XK) in the early stages of growth but showed activity after a period of time, indicating that wood The sequence of glucose metabolism exists in the sequence of expression. Reported in the literature lignocellulose resources and their hydrolyte contains a large amount of glucose, xylose and other mixed carbon sources, but Bacillus sp. Its use efficiency is still low, one of the main reasons is the carbon metabolism inhibition (Carbon Catabolite Repression, CCR) Hindered Bacillus sp. From fully utilizing xylose in lignocellulose resources. CCR refers to the microbial preferential use of carbon sources (usually glucose) in the fermentation of mixed carbon sources, and the carbon sources and their metabolites inhibit the expression of non-available carbon sources (such as xylose) metabolic genes, affecting non-available carbon Source utilization efficiency. The enzymatic properties of this study are in good agreement with the above conclusions. Therefore, in the later research, we can improve the metabolic efficiency of mixed sugar and even lignocellulose by regulating the transcriptional level of CCR related factors.

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 Cl strain was significantly higher than that of the control strain FZB42. The growth ability of Cl strain using xylose as a carbon source was stronger than that of the control strain FZB42.

(B). Bacillus amyloliquefaciens C1 in different carbon source medium, the growth ability is different, the use of glucose as a carbon source its growth more exuberant! 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 fermentation of mixed carbon sources, and the carbon sources and their metabolites inhibit the microbial expression of genes related to the metabolism of other non-available carbon sources such as xylose and Enzymatic, thus affecting the efficiency of non-efficient use of carbon sources.

(C). the ability of Bacillus amyloliquefaciens C1 and FZB42 to use glucose was basically the same, but the use of xylose was more different. The ability of C1 bacteria was obviously stronger than that of the model strain FZB42.

(D). Bacillus amyloliquefaciens C1 and FZB42 were grown in medium with different carbon sources. Three time-laden samples were selected for enzyme detection. Under the same conditions, strain XI and XKenzyme were higher than strain FZB42. The two strains of XI, XK activity with parallel, that XI activity higher strains, its corresponding XK activity is also high. Strains began to use xylose, corresponding XI, XK that showed activity.

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