Establishment of Fed-batch Fermentation Conditions for Biocontrol Bacteria B579

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Abstract: *In order to increase the cell concentration in fermentation, feeding strategy and feeding amount were optimized. The result showed that glucose feedback feeding strategy is the best one, and glucose concentration should be controlled at 6.0 g/L during the fermentation of B. subtilis B579. The final cell concentration was 9.5×10^9 CFU/mL, which represented a 1.9-fold increase when compared with those of before optimized. Feed-batch fermentation in 7L fermenter was conducted under the following condition: temperature 37°C, pH 7.0, DO 10%, then supplemented glucose to the fermenter during the fermentation, and kept the glucose concentration at 6.0 g/L, after 35 h fermentation the final cell concentration was 3.9×10^{10} CFU/mL, which represented a 3.8-fold increase compared with those of before optimized.*

Keywords: *Bacillus subtilis; Fed-batch fermentation; Glucose feedback feeding strategy.*

Introduction

The gram-positive bacteria, like *B. subtilis*, have been studied intensively in recent years, because many *B. subtilis* strains are considered to be safe biocontrol agents [1]. As *B. subtilis* has the characteristics of omnipresence, thermal tolerance, rapid growth, and ready formation of resistant spores, it is considered to be good biological control agent. *B. subtilis* is not only an organism known for protease production [2], but also a good bacteria used for fungicide production. *B. subtilis* has a direct antagonistic activity not only by producing antibacterial protein [3], but also by producing various hydrolytic enzymes (for example, chitinase, β-1, 3-glucanase) and antibiotics such as iturin A and surfactin [4, 5, 6]. From the large amount of antimicrobials produced, lipopeptides stand among the most representative. *Bacillus* lipopeptides may be divided into three families – iturin, fengycin and surfactin. These antifungal lipopeptides are either linear or cyclic. They frequently contain amino acid residues, which are unique and not commonly found in proteins, with high stability to pH, heat and protease [7]. It could inhibit the growth of some relative bacteria [8]. These antifungal peptides have been proved safe to people and no pollution to environment [9, 10].

So, they have high potential for being used in biological control, food antisepsis, medicine, and so on.

B. subtilis B579 (deposition number CGMCC No. 2270) was isolated from rhizosphere of cucumber in Tianjin, China. It could effectively inhibit the growth of several species pathogenic fungi, and exhibited a broad spectrum antifungal ability [11]. In our previous studies, genes involved in the biosynthesis of six antifungal compounds were detected in genomic DNA of B579. Two kinds of lipopeptide antifungal compounds were identified by HPLC and LC-MS. The crude antifungal compounds were stable to pH, thermal, protease *K* and detergents [12]. The related genes for biosynthesis of bacilysin, sublancin, subtilosin were detected by PCR, but they were not been detected by HPLC and LC-MS which need us for further study. They probably have been produced too little to separation and identification. The separation of antifungal compounds and the effect of these antifungal compounds on the morphology of the pathogens are under extensive investigation in our laboratory. So, high cell concentration fermentation might helpful for antifungal compounds separation.

Formulation of powder preparation of B579 endospores had been developed (Chinese patent registration number: 200810153180). However, live cell concentration in formulation need to be improved. Increased cell concentration in fermentation was one of the most effective methods. Some isolates of *Bacillus* have been successfully commercialized and marketed, such as the Gustafson product Kodiak is widely used for suppression of cotton disease in the US [13]. As the interest in biological control of soil-borne plant pathogens has increased considerably in the last few decades, we believe that *B. subtilis* will be used more in production agriculture and horticulture based on the recent progress shown in implementing microbial inoculants.

So, the primary objective of this study was to construct the optimal conditions for high density fermentation of *B. subtilis* B579. It will establish well rationale for antagonistic substance isolation and high concentration powder preparation.

Materials and methods

Microorganisms and growth conditions

B. subtilis B579 was grown in LB broth at 37°C in a rotary shaker with 180 r/min for 24 h. They were maintained on LB agar plate at 4°C. Fermentation medium was used with (g/L): yeast extract, 6.0; soluble amylum, 11.0; glucose, 6.0; soybean cake powder, 13.9; beef extract, 8.9; and maizena, 12.6. The pH was adjusted to 7.5 prior to autoclaving.

Conditions of fed-batch fermentation in shake flask

Seed of B579 was inoculated into fermentation medium with a inoculation amount of 7%. Each shake flask (250 mL) was filled with 30 mL medium. The bacteria were grown in fermentation medium at 37°C in a rotary shaker with 180 r/min for 24 h. Four different feeding amounts were contained 0, 3.5, 6.0, and 8.0 g/L. Feeding began from the fifth to twelfth hours after the fermentation started, which is from the early period of exponential phase to the later period of log phase. The glucose was supplemented when its concentration was below 3.0 g/L.

Three feeding strategy were selected, which contained constant speed feeding strategy, variable speed feeding strategy, and glucose feedback feeding strategy. For constant speed feeding strategy, glucose concentration was detected once an hour, and then adjusted to 6.0 g/L. Variable speed feeding strategy was conducted according to the fermentation kinetics

curve (Fig. 1). Feeding was started when the growth rate begin to decrease at log phase. Glucose feedback feeding strategy was conducted according to the glucose concentration. Glucose concentration was detected, and the glucose was supplemented to 6.0 g/L when its concentration was below 3.0 g/L.

The best feeding strategy and feeding amount were selected according to the results of plate count of each treatment. Three replicates were made for each treatment.

Conditions of fed-batch fermentation in fermentor

Seed of B579, which was grown in LB broth at 37°C in a rotary shaker with 180 r/min for 14 h, was inoculated into the fermentor (7 L) with the amount of 7%. Loading coefficient of the fermentor was 0.7. The best feeding strategy and feeding amount were used according to the results of above. The growth conditions were the same as in the shake flask.

Determination of glucose concentration in medium

Broth after proper dilution was centrifuged for 2 min with 12000 r/min. Supernatant was used for determination of glucose concentration in medium using biosensor analyzer (SBA-40C).

Results

Fermentation kinetics curve before optimization

For better understanding the relationship between cell growth and glucose consumption during the course of fermentation, fermentation kinetics curve of *B. subtilis* B579 was made using 7 L fermentor (Fig. 1). There were rapid cell growth and glucose consumption from the early period of exponential phase to the period of log phase (5-12 h). Glucose concentration was decreased to a minimum at stationary phase. The cell growth was effected by the low concentration of glucose in this phase. So, feeding was conducted at fifth to twelfth hours after fermentation started.

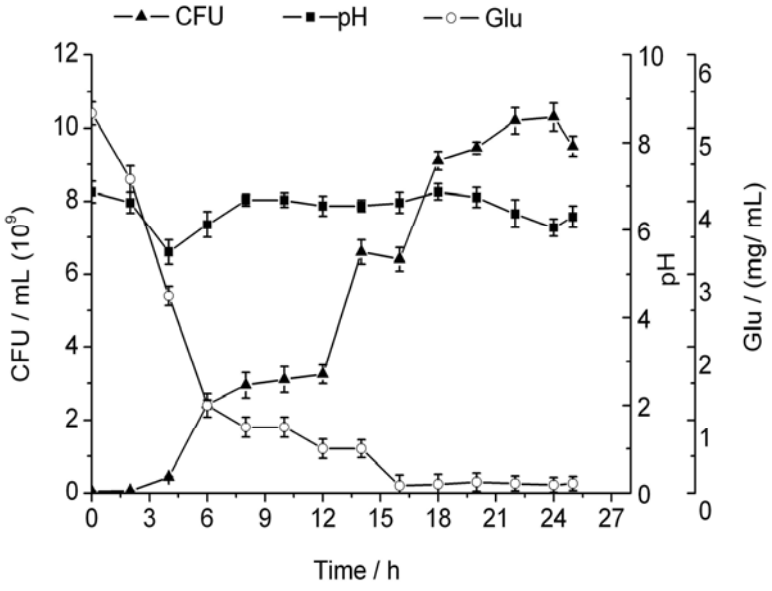

Fig. 1 Fermentation kinetics curve of *B. subtilis* B579 before optimization

Feeding amount optimization

The proper feeding amount was selected according to the fermentation results conducted in shake flask. As shown in Fig. 2, there was the highest cell concentration when the feeding amount achieved 6.0 g/L. The cell concentration was as high as 7.3×10^9 CFU/mL. The cell could not obtain sufficient available carbon sources at log phase when the feeding amount less than 6.0 g/L. However, when the feeding amount more than 6.0 g/L, carbon-nitrogen ratio in the medium was overbalanced and cell osmotic pressure was increased. So, the proper feeding amount for bacteria B579 was 6.0 g/L.

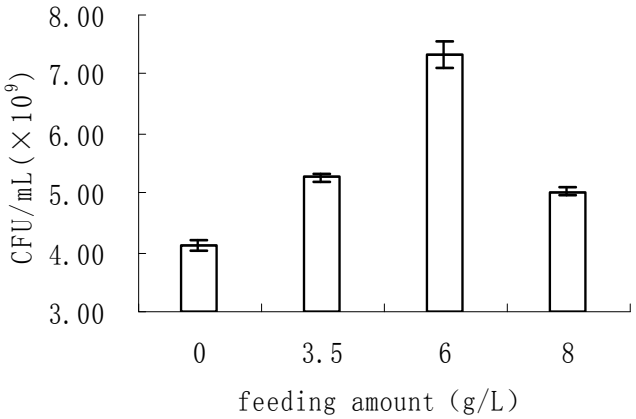

Fig. 2 Effects of supplement feeding amount on cell growth

Feeding strategy optimization

Three feeding strategy, which contained constant speed feeding strategy, variable speed feeding strategy, and glucose feedback feeding strategy, was compared. As shown in Fig. 3, glucose feedback feeding strategy had the highest cell concentration, which was as high as 9.5×10^9 CFU/mL. Contrary, constant speed feeding strategy exhibited the least cell concentration, which was only 7.3×10^9 CFU/mL. Variable speed feeding strategy showed the cell concentration of 8.6×10^9 CFU/mL. This strategy was conduted according to the bacteria growth curve and the glucose consumption curve (Fig. 1).

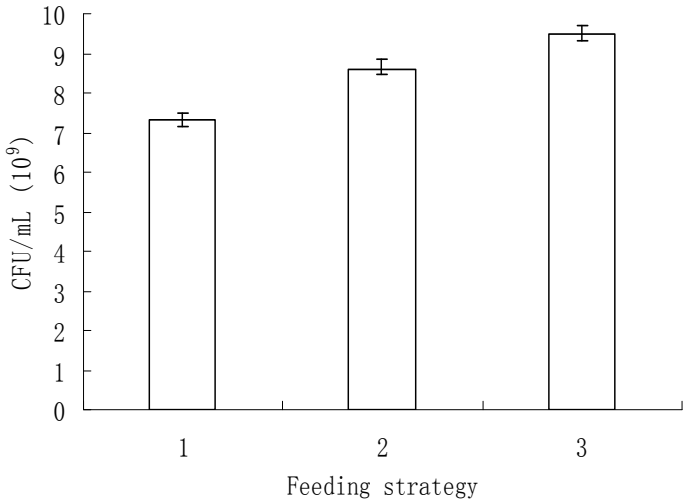

Fig. 3 Effects of different feeding strategy on cell growth

Note: Column 1: Constant speed feeding strategy;
Column 2: Variable speed feeding strategy; Column 3: Glucose feedback feeding strategy.

Fed-batch fermentation using 7 L fermentor

Under the optimal conditions obtained above, fed-batch fermentation of B579 was conducted (Fig. 4). The pH was adjusted to 7.0-7.5 in fermentation process. Glucose feedback feeding strategy was used, and the glucose concentration was detected and maintained to 6.0 g/L from 5-12 h after the fermentation started. Dissolved oxygen (DO) was adjusted to 10%. Under this condition, cell concentration was as high as 3.9×10^{10} CFU/mL, which represented a 3.8-fold increase compared with those before optimized.

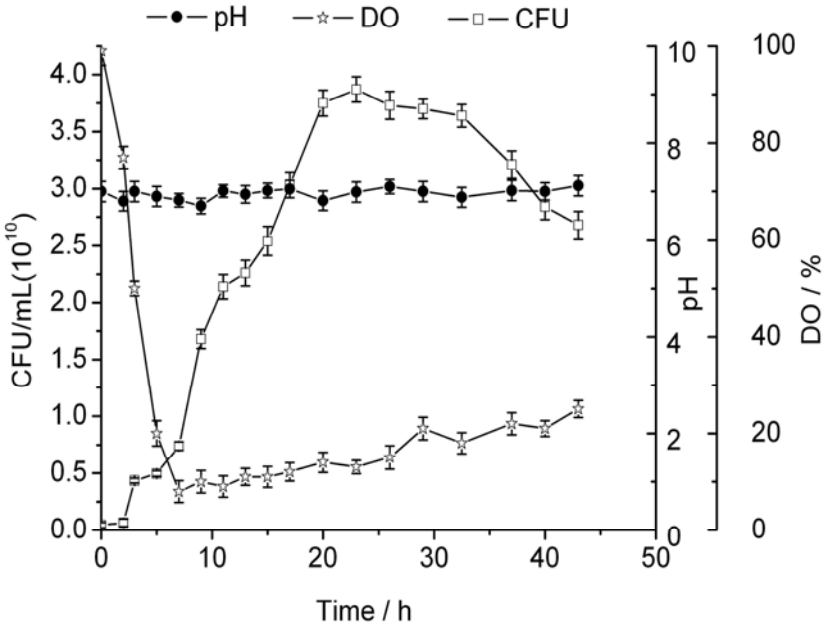

Fig. 4 Fed-batch fermentation process of B579 in 7 L fermentor

Conclusion

On the basis of fermentation process analysis, feeding was conducted from the early period of exponential phase to the period of log phase (5-12 h after fermentation started), because there was a rapid cell growth and glucose consumption. The proper feeding amount for bacteria B579 was 6.0 g/L. Glucose feedback feeding strategy was the best feeding strategy. Under the optimal conditions, cell concentration was as high as 3.9×10^{10} CFU/mL, which represented a 3.8-fold increase compared with those before optimized.

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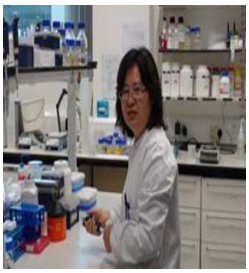

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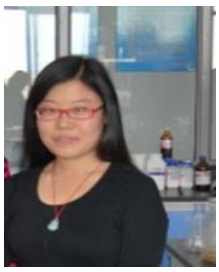

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