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High-density Fermentation of *Bacillus subtilis* with Corn Steep Liquor as an alternative Substrate

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Abstract: *The main purpose of this research was to reduce the production cost of high-density fermentation of Bacillus subtilis. The method was to find cheap and easily available raw materials with high efficiency. In this study, Bacillus subtilis was used as the starting strain. A single factor experiment was conducted to explore the effects of different concentrations of corn steep liquor(CSL) on the fermentation characteristics of Bacillus subtilis without adding other nutrients. It showed that the effect of high-density fermentation of Bacillus subtilis was the best, the number of viable bacteria could exceed 3.9×10^9 CFU/mL, and the content of amino acid nitrogen was the highest per unit mass of CSL when the concentration of CSL was 100~200 g/L. This conclusion provides reference for the high-density fermentation of Bacillus subtilis with CSL as raw material and the realization of resource utilization of CSL.*

Key words: *Corn steep liquor; Microecological inoculants; Bacillus subtilis; Fermentation characteristics; High-density fermentation*

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I. Introduction

The microecological inoculum is a live inoculum product made from beneficial microorganisms through fermentation and other special processes based on the principles of microecology, and is widely used in animal husbandry, aquaculture, agricultural production and other fields[1]. *Bacillus subtilis* is one of the bacterial species allowed to be used in probiotics[1]. *Bacillus subtilis* is a mesophilic aerobic gram-positive bacteria, which can produce auxin, antibacterial peptides and other active substances to inhibit pathogenic microorganisms and promote plant growth [2, 3]. At the same time, it has the characteristics of safety, non-toxicity, strong stress resistance, and easy colonization in the rhizosphere of plants. It is widely used in plant microecological preparations[4]. In addition, *Bacillus subtilis* can secrete a variety of enzymes such as protease, lipase, phytase, etc., which has been used for the decomposition and utilization of waste and the preparation of organic and biological inoculants[2, 4, 5]

At present, there are many studies on improving the density viable count of *Bacillus subtilis*. For example, Hu et al. optimized the composition of carbon source, nitrogen source and inorganic salt in the fermentation medium of this bacterium, and the number of spore reached 2.42×10^9 CFU/mL[6]; Song et al. determined the best components of the fermentation medium, glucose 1.0%, beef extract 1.5%, K_2HPO_4 0.5%, and the concentration of viable bacteria in the fermentation broth reached 3.98×10^9 CFU/mL[7]. However, high-cost raw materials such as corn flour and peptone are mostly used for fermentation production. Therefore, it is necessary to find raw materials that are cheap, readily available, and have high production efficiency to reduce the production cost of high-density fermentation. CSL is a type of high-concentration organic wastewater produced by the concentration of soaking water after corn starch processing [8]. Nearly 10 million tons of CSL are produced in China each year, and its processing and utilization have become the shackles of starch production enterprises[9]. As we all know, CSL is often used as a cheap nitrogen source for the fermentation industry[10]. For example, Yang et al. used CSL as an alternative nitrogen source to produce 2,3-butanediol and ethanol by *Bacillus subtilis*[11]; Zhang et al. used CSL and other corn processing waste to increase butanol production with Clostridium SE-2 [12]. However, its high-concentration ammonia nitrogen is limited in its utilization for the synthesis of metabolites, and CSL can only be added in small amounts as an alternative nitrogen source, which is difficult to solve the current situation that the high yield of CSL needs to be used urgently.

In order to solve the problems of difficult to use, low added value of CSL, and the disadvantages of the traditional high production cost of *Bacillus subtilis*, this study evaluated the possibility of pure cultivation of *Bacillus subtilis* in high-concentration CSL without adding other nutrients, and explored the fermentation

characteristics of different concentrations of CSL to cultivate *Bacillus subtilis*. It is expected to provide a reference for further using CSL to increase the density of *Bacillus subtilis*, reduce the production cost of microbial inoculants and the resource utilization of waste.

II. Materials And Methods

2.1 Test materials

The tested strain is *Bacillus subtilis* 3301, preserved by the Fermentation Engineering Laboratory of Shandong University of Technology. Corn starch wastewater——Corn steep liquor (CSL) is provided by Luzhou Biotechnology Co., Ltd.

2.2 Preparation of medium and seed culture

Nutrient broth (NB) medium: beef extract 3.0 g/L, peptone 10.0 g/L, NaCl 5.0 g/L, pH 7. CSL medium: CSL was diluted with distilled water at different concentrations (50 g/L, 100 g/L, 150 g/L, 200 g/L, 300 g/L, 400 g/L, 500 g/L) and adjusted the pH to 7 with KOH. Sterilized at 121°C for 20 minutes.

Taken 2 loops of *Bacillus subtilis* from the oblique interview tube and inoculate it into NB medium, and cultured it with shaking at 37℃and 160 r/min for 18-20 h to obtain seed culture solution.

2.3 Cultivation of *Bacillus subtilis* with different concentrations of CSL

10% (v/v) seed solution was inoculated into CSL of different concentrations (50 g/L, 100 g/L, 150 g/L, 200 g/L, 300 g/L, 400 g/L, 500 g/L). The culture medium was shaken and cultured at 37°C and 160 r/min for 72 h. Samples were taken every 12 h to observe the viable cell count, amino acid nitrogen content, soluble phosphorus, reducing sugar content, and pH of the CSL fermentation broth at various levels.

2.4 Analytical methods

The effective viable cell count was determined by the dilution plate method, and the viable number was calculated per mL of fermentation broth [13]. Amino acid nitrogen was determined according to formaldehyde colorimetry [14], the corresponding value was obtained by the amino acid nitrogen content produced by unit mass of CSL(∆AAN), $\Delta AAN = (C_N-C_{N0})/m_{CSL}$. The dissolved phosphorus was determined by molybdenum blue spectrophotometry [15], the soluble phosphorus utilization rate(∆P Ratio) was the corresponding value, $\Delta P\ Ratio = (C_{P0}-C_P)\ /C_{P0}$. Reducing sugar was measured by DNS method[16], the reducing sugar utilization rate(∆R.S Ratio) was the corresponding value, $\Delta R.S\ Ratio = (C_{S0}-C_S)\ /C_{S0}$. pH was measured with a pH meter. All experiments were repeated three times.

III. Results and analysis

3.1The viable count of *Bacillus subtilis* cultured with CSL in different concentrations

The viable count is a key indicator to evaluate the quality of microbial agents[9]. The *Bacillus subtilis* seed solution was inoculated into CSL of different concentrations for pure culture, and process changes of the viable count were studied on the growth of *Bacillus subtilis* at different concentrations of CSL without adding other nutrients, as shown in Figure 1. Among the CSL used, the cell mass was the best when 200 g/L CSL was used to culture *Bacillus subtilis*, and the cell mass reached about 3.9×10^9 CFU/mL at about 48 h. At the same time, as the concentration of CSL increases, the maximum viable bacteria count decreased. Especially, the viable count continues to decrease with the fermentation time when the concentration exceeds 500 g/L and finally was lower than 10^7 CFU/mL, which was lower than the initial inoculation amount. It can be seen that high-concentration CSL inhibited significantly the growth of *Bacillus subtilis*. In addition, with the increase of the concentration, the time of the maximum cell biomass also gradually gone back. When the CSL concentration was 400 g/L, the biomass reached the maximum in 60 h. Especially, the viable count reached the highest value at 12 h after inoculation when the concentration of CSL was 50 g/L. It showed that low-level CSL could be added to the bacterial culture medium as an alternative available nitrogen source for rapid cell growth. A suitable CSL concentration can be used as a substrate to cultivate *Bacillus subtilis* to obtain a higher cell number.

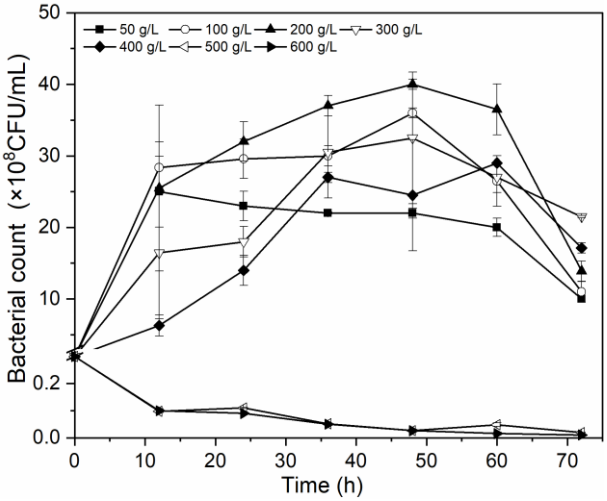

Fig. 1 The dynamic change of the viable count of *Bacillus subtilis* cultured in CSL

3.2Dynamic changes of ∆AAN **in CSL of different concentrations by *Bacillus subtilis***

CSL is a typical protein waste, which is rich in amino acids, protein and other nutritional factors[17]. During the fermentation of *Bacillus subtilis*, more protease can be produced to decompose the protein as free amino acids in CSL, which increases the amino acid content in the microecological agent of *Bacillus subtilis*[18]. The process changes of ∆AAN in the fermentation broth when *Bacillus subtilis* fermented CSL with different concentrations were studied, as shown in Figure 2. Among the CSL of different concentrations used, the ∆AAN reached a maximum of 7 $mg \cdot L^{-1} \cdot g^{-1}$ in the late fermentation stage, and tends to be stable in the later stage when the concentration of CSL was 200 g/L, and the ∆AAN of 300 g/L CSL still increased in the late fermentation stage. However, the cell mass of *Bacillus subtilis* reduced at the later stage of fermentation when the concentration of CSL was 300 g/L (Figure 1), indicated that amino acid nitrogen was not a limiting factor for cell mass reduction. In addition, 50 g/L CSL produced the highest amino acid nitrogen at 0~48 h, and finally decreased with the increase of fermentation time. At the same time, the ∆AAN continued to decrease as the concentration of CSL increased. It showed that the protein in low-concentration CSL was completely hydrolyzed in the fermentation process, and the amino acid produced was used for cell growth, so the ∆AAN was reduced in the later stage of fermentation. When the concentration of CSL was too high (more than 400 g/L), the ∆AAN increased in the early stage and tends to be stable. Too high concentration of CSL had too high osmotic pressure in the matrix, which was not conducive to cell growth and made it difficult for its nutrients to be degraded and utilized. Therefore, the ∆AAN of low-concentration CSL decreased in the late fermentation stage, which was not enough to support the cell growth of *Bacillus subtilis*; when the CSL concentration was 100-200 g/L, the ∆AAN presented a higher increase.

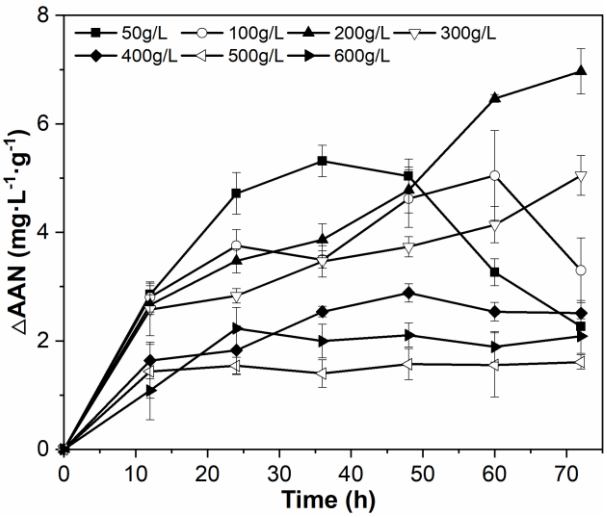

Fig. 2 The dynamic change of AAN content of *Bacillus subtilis* when cultured in CSL

3.3 Effect of CSL concentration on pH dynamics of *Bacillus subtilis* fermentation process

pH is one of the main parameters that need to be tested in the industrial production process. During the fermentation process, pH is an important factor that directly affects metabolism. The process changes of pH in the fermentation broth were studied when culturing *Bacillus subtilis* with different concentrations of CSL. It can be seen from Figure 3 that as the concentration of CSL increases, the overall pH value after fermentation shows a gradual downward trend. Among the different concentrations of CSL used, the pH value gradually increased as the fermentation time progresses when the concentration of CSL was lower than 400 g/L. The lower the CSL concentration, the higher the pH increased; When the concentration was higher than 400 g/L, the pH value gradually decreased as the fermentation time progresses. CSL is a high-protein organic waste liquid. When the C/N in the medium was low, it was beneficial to the growth of microbial cell mass, but correspondingly, the pH of the fermentation liquid increased. The amino acids in CSL were utilized by *Bacillus subtilis* to produce more extracellular alkaline substances, leading to an increase in pH. High-density cultivation of *Bacillus subtilis* caused the pH to be too high or even above 8.5 in CSL if the pH is not controlled, which may adversely affect the later fermentation.

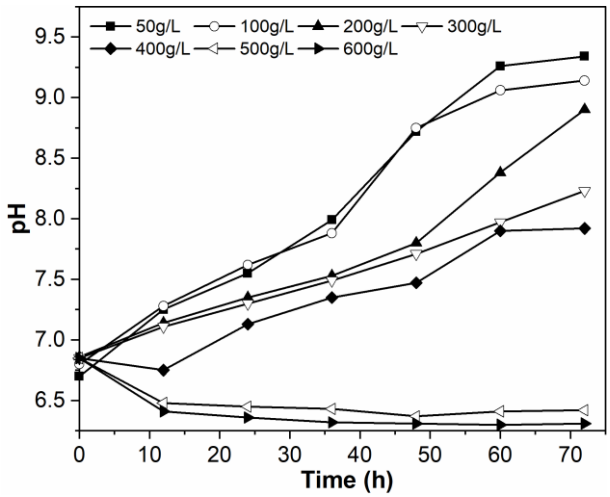

Fig. 3 Changes in pH during the cultivation of *Bacillus subtilis* with different concentrations of CSL

3.4 The dynamic change of ΔP Ratio of *Bacillus subtilis* cultured in CSL

CSL is not only rich in protein resources, but also rich in phosphorus. Phosphorus in CSL exists in the form of phytic acid. Phytic acid can chelate with Ca, Mg and protein in CSL to form insoluble salts, which affect the utilization of nutrients by microorganisms[19]. The process changes of the ΔP Ratio were studied during the fermentation of CSL with different concentrations by *Bacillus subtilis*. It can be seen from Figure 4 that the ΔP Ratio gradually increases in CSL during the fermentation process, and tends to be stable in the later stage of fermentation. It showed that *Bacillus subtilis* could make good use of the solubilized phosphorus for its own cell growth in CSL. At the same time, the ΔP Ratio was negative in the fermentation broth of low-concentration CSL in the early stage of fermentation, indicated that *Bacillus subtilis* may convert part of the insoluble phosphate into soluble phosphate in CSL [5], especially, the increase of soluble phosphorus is the most obvious when the concentration of CSL is lower than 100 g/L. In the late fermentation stage, *Bacillus subtilis* used a large amount of solubilized phosphorus, leading to an increase in ΔP Ratio. When the concentration of CSL was 100 g/L, the ΔP Ratio was the highest after 36 hours. Therefore, *Bacillus subtilis* converted a part of the insoluble phosphate in CSL in the early stage of fermentation, and it could convert the phosphorus in the form of phytic acid into phosphate for cell utilization. At the same time, it indicated that the solubilized phosphorus of CSL served as a sufficient phosphorus source for *Bacillus subtilis* and effectively promoted cell growth.

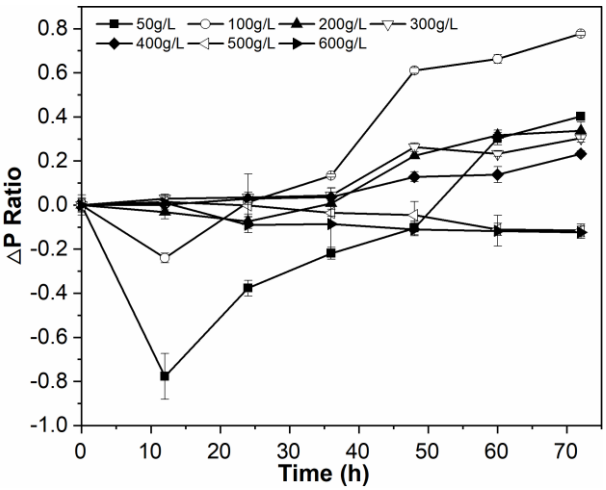

Fig. 4 The dynamic change of ∆P Ratio of *Bacillus subtilis* cultured in CSL of different concentrations

3.5Dynamic changes of ∆R.S Ratio of *Bacillus subtilis* cultured with CSL in different concentrations

Although CSL is often used as a cheap nitrogen source for fermentation, it still contains about 4% reducing sugars, which can provide part of the carbon source for the growth of *Bacillus subtilis*. Figure 5 shows the dynamic changes of reducing sugar utilization when using different concentrations of CSL to cultivate *Bacillus subtilis*. It can be seen that as the fermentation time increased, the ∆R.S Ratio gradually increased, and finally stabilized. 0~12 h was the time period during which the ∆R.S Ratio increased rapidly. When the concentration of CSL was 100 g/L, the ∆R.S Ratio was significantly higher than other concentrations. As the concentration of CSL increased, the ∆R.S Ratio gradually decreased. When the concentration of CSL exceeds 400 g/L, the ∆R.S Ratio was lower than 0.4. At the same time, as the concentration increased, the time was gradually delayed when the reducing sugar utilization rate entered the stable phase.

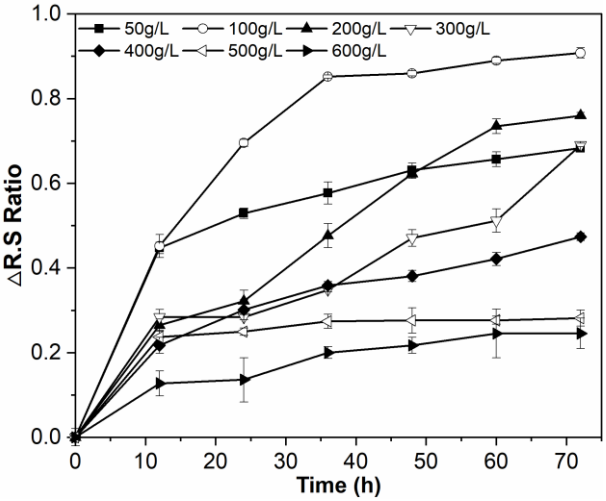

Fig. 5 Dynamic changes of ∆R.S Ratio of *Bacillus subtilis* cultured with different concentrations of CSL

IV. Conclusion

Corn starch leftovers-CSL was used as a raw material to explore the fermentation characteristics of *Bacillus subtilis* in different concentrations of CSL. When the concentration of CSL was 100~200 g/L, *Bacillus subtilis* had the best high-density fermentation effect. Its viable cell count could exceed 3.9×10^9 CFU/mL, and the ∆AAN was higher. This study confirmed the feasibility of fermentative production of *Bacillus subtilis* using CSL as a by-product of starch processing, which opened up ideas for the industrial production of *Bacillus subtilis* and the comprehensive utilization of starch processing by-products.

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