American Journal of Biochemistry and Biotechnology

Original Research Paper

Production of *Bacillus Subtilis* as Biological Fertilizer by Biotransformation of Corn Steep Liquor

[1,2]Xiaojie Ren, [1]Baoyue Liu, [1]Zhuangzhuang He, [3]Xiaolong Wang, [4]Yubin Zhao, [5]Mario Jolicoeur and [1,4]Xinhe Zhao

[1]School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China
[2]Shandong Fuyang Biotechnology Co. Ltd., Dezhou, Shandong, China
[3]China Food Fermentation Industry Research Institute Co., Ltd., Beijing, China
[4]Luzhou Bio-Technology Co. Ltd, Linyi, Shandong, China
[5]Research Laboratory in Applied Metabolic Engineering, Department of Chemical Engineering, École Polytechnique de Montreal, Montreal, Quebec, Canada

Article history
Received: 28-03-2022
Revised: 22-08-2022
Accepted: 05-10-2022

Corresponding Author:
Xinhe Zhao
School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China
Email: zhaoxinhe@sdut.edu.cn

Abstract: Corn steep liquor is a kind of intractable organic waste liquid produced during the processing of corn starch. But it is rich in nutrients, soluble protein, and amino acids and it could be used as a nitrogen source in the fermentation industry. Many starch processing enterprises cannot directly utilize corn steep liquor and choose to discharge it directly, thus causing environmental pollution. Studies have shown that high ammonia nitrogen in corn steep liquor can promote the increase of microbial biomass. *B. subtilis* is the dominant strain of microbial flora in corn steep liquor, it is a classic strain in the research and industrial production of biological bacterial fertilizer. In this study, the dominant *B. subtilis* 3301, which is suitable for high-density cultivation in corn steep liquor, was screened first. Then the optimal fermentation parameters, like corn steep liquor concentration, pH, and inoculation amount of the bacteria were analyzed by a three-factor and three-level Response Surface (RSM) analysis experiment. The highest cell density was obtained when the concentration of corn steep liquor is 145 $g \cdot L^{-1}$, pH is 7 and the inoculation amount is 8. Then the dynamic changes of *B. subtilis* cultured in corn pulp and beef extract peptone medium (NB) were compared under response surface optimization fermentation conditions. The results showed that *B. subtilis* cultured in corn pulp medium was significantly higher than in NB medium. Finally, the fermentation effect of fed-batch glucose on the culture of *B. subtilis* in corn steep liquor was investigated and the results showed that when feeding with glucose, the number of viable bacteria can reach 1.75×10^{10} $CFUmL^{-1}$, which is significantly higher than the number of viable bacteria cultured without feeding glucose. All of the above results show that the optimized medium using cheap corn-steep liquor shows a good production level. The research results provide a theoretical reference for further improving the biomass of biological bacterial fertilizer and at the same time provide a practical reference for the industrial production of biological bacterial fertilizer to reduce the cost of raw materials, alleviate the pollution of corn steep liquor to the environment and promote the further development of the crop processing industry.

Keywords: Corn Steep Liquor, Biological Bacterial Fertilizer, *B. Subtilis*, Biotransformation, High-Density Fermentation, Fed-Batch Fermentation

Introduction

Corn is one of the three major food crops in the world and has played an important role in agricultural production. With the continuous increase in China's economic level, the economic value of corn continues to increase (Chunxiang, 2021). The deep processing of corn obtains a large amount of corn starch through soaking,

crushing, screening, drying, concentration, washing, extraction, and other processes. In addition, crude fiber feed, corn gluten meal, corn steep liquor, and other bulk by-products are also produced (Jiqiang *et al*., 2011). Among them, CSL (CSL) is the main by-product of corn starch production by wet process, which is a viscous and acidic slurry (Sarwar and Ajmal Khan, 2004). As corn starch leftovers, some high-quality CSL is used in the feed and biological fermentation industry and reprocessed into CSL powder (Hongyu and Li, 2017). It will lead to the existence of a large number of unqualified CSLs, which seriously limits its application in various industries. Especially in recent years, the implementation of environmental protection and CSL quality standards has increased, resulting in the accumulation of a large number of CSL wastes in enterprises. This waste seriously pollutes the environment and has become a shackle for the development of many corn processing enterprises (Choi *et al*., 2013). However, CSL is rich in a large number of amino acids, vitamins, growth factors (Hongmei and Sen, 2021), a variety of proteins, soluble sugars, minerals, and other nutrients (Martinez-Burgos *et al*., 2021), the cost is low. Therefore, it has great application potential in agriculture.

With the development of biotechnology, the application of CSL involves food, pharmacy, biochemistry, environmental protection, and other fields, especially in biological agriculture. For example, many scholars are adding it to the feed to enhance nutrition (Ying *et al*., 2013). The rich microorganisms can reduce the growth of the lettuce root system (Chinta *et al*., 2014) and they can be used to repair polluted soil (Salam and Ishaq, 2019). Therefore, we can study the utilization value of corn pulp as microbial raw material to produce biological bacterial fertilizer, so that it can be reused, truly "turn waste into treasure", benefit more starch processing enterprises and promote the green development of agriculture. In addition, in recent years, China has made biological fertilizers with various functions by using microorganisms, which can not only improve fruit quality, and increase the yield of agricultural products, but also reduce environmental pollution and provide more possibilities for the development of organic agriculture (Wu *et al*., 2021).

Biological bacterial fertilizer is a microbial living product that promotes the absorption of nutrients by plants through the life activities of microorganisms and enables plants to obtain specific fertilizer effects (Jun *et al*., 2006). The rational application of biological bacterial fertilizer can not only improve the soil conditions of crops but also help to enhance the disease resistance and stress resistance of crops. According to previous studies, *B. subtilis*, as a dominant probiotic in the original CSL flora (Zhuangzhuang, 2021), has the advantages of strong temperature adaptability, high tolerance to osmotic pressure, it can quickly revive and growth, and strong secretory enzymes (Ziheng *et al*., 2021). Although there are many reports on the use of CSL to produce biological bacterial fertilizer, there are few reports on the optimization of the fermentation method of *B. subtilis* using CSL to produce biological bacterial fertilizer.

Therefore, this research screened *B. subtilis* suitable for high-density cultivation in CSL and continuously optimized the fermentation parameters. Simultaneously glucose was fed to simulate another by-product of starch processing enterprises-crystallized glucose, to explore the effect of glucose on the growth of *B. subtilis* in the CSL medium. At the same time, it can also improve the market reuse of the crystalline glucose mother liquor and make data reference for further expanding the market of CSL to produce biological bacterial fertilizer.

Materials and Methods

Experiment Materials

The fermentation strain was *B. subtilis*, which was preserved by the fermentation engineering laboratory of the Shandong University of Technology. Strain storage adopts the 30% glycerin preservation method, mix 30% autoclaved glycerin with *B. subtilis liquid culture* according to a volume ratio of 1:1. Store it in the -70°C refrigerator, and transfer once every 2~3 years. Corn Steep Liquor (CSL), a by-product of corn starch production, was provided by Luzhou Biotechnology Co., Ltd. The pH of CSL was 4.2±0.1, the Amino Acid Nitrogen (AAN) content was 9.71±0.18 g/L, the moisture content was 67.14±3.3%, the total sugar content was 4.52±0.1% and the reducing sugar content was 40.33±0.92 g/L, the ash content was 7.46±0.3% and the soluble phosphorus content was 4.39±1.18 g/L (Xinhe *et al*., 2020).

Preparation of Culture Medium and Seed Solution

Beef peptone (NB) medium (Ligang, 2002): 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 to 200 g/L and then adjusted to pH 7 with potassium hydroxide and sterilized at 121 °C for 20 min.

Two rings of *B. subtilis* were inoculated into NB medium and the seed liquid was obtained by shaking culture at 37°C and 160 r/min for 18-20 h (measured by the pre-stage *B. subtilis* growth curve measurement test).

Shake Flask Culture Experimental Design

To screen for *B. subtilis* suitable for high-density culture in CSL, different *B. subtilis* subspecies' seed cultures were inoculated into 200 g/L CSL with 1% (v/v) inoculum, respectively. After incubating for 48 h at 37°C with shaking at 160 r/min (MQD-B1CG, Minquan Technology Instruments Co., Ltd), samples were taken to determine the number of viable bacteria, amino acid nitrogen content, soluble phosphorus, reducing sugar, and pH.

According to the screening, the dominant *B. subtilis* strains were obtained and the related factors such as CSL

concentration, pH, and inoculation amount were studied by single-factor analysis. Fermentation for 48 h was performed by filling 50 mL of sterilized CSL medium in a 250 mL flask. To determine the optimal CSL concentration for the high-density culture of *B. subtilis*, the CSL concentration ranged from 50, 100, 150, 200, and 250 g/L, respectively. To determine the optimal initial pH, the pH of the medium was controlled at 6–8 by adding 1M KOH before sterilization. To optimize the inoculum size, the medium was fermented at the inoculum size of 2.5, 5, 7.5, 10, and 12.5%. To determine whether the addition of glucose can promote the high-density fermentation of *B. subtilis*, the effect of adding 20g/L glucose and pure CSL medium was compared. All the culture was carried out for 48 h at 37°C with shaking at 160 r/min and the number of viable bacteria was determined after culture. All experiments were repeated three times to determine their validity.

After the initial range of the culture variables was determined by the single-factor experiment, a three-factor and three-level response surface analysis experiment were performed using the Box-Behnken experimental design (Table 1). A total of 17 experimental combinations were included, including 5 central experiments and 12 factorial experiments. Finally, the number of viable bacteria was used as the response value to carry out regression analysis on the response value of the 17 experimental points and the regression equation of the main relevant culture parameters was established.

The Box-Behnken experimental design data was analyzed using the following second-order polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}$$
$$X_2X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

In the formula, Y is the predicted response value; b_0 is an intercept; b_1 b_2 b_3 is a linear coefficient; b_{12}, b_{13}, b_{23} are the cross-product coefficients; b_{11}, b_{22}, b_{33} are quadratic coefficients; X_1 X_2 X_3 are input variables.

To verify the prediction results of response surface optimization, comparing the growth of *B. subtilis* in NB medium and CSL medium, according to the optimal level predicted by response surface optimization, *B. subtilis* was inoculated into NB medium and CSL medium with optimized concentration respectively, shake flasks were cultured at 37°C at 160 r/min for 60 h and samples were taken every 6-12 h to determine the number of viable bacteria and pH after cultivation.

Fed-Batch Fermentation

Batch cultivation in a 5 L fermenter: According to the optimization results of the response surface design experiment, the *B. subtilis* seed liquid was inoculated to a 5 L automatic fermenter (T&J Bio-engineering Co., LTD. Shanghai) for batch cultivation. The 5 L fermenter was filled with 3 L of fermentation medium, the initial ventilation was 4 NL/min (NL is the volume of gas at 0 degrees Celsius and 1 standard atmosphere), the stirring speed was 200 r/min, the initial pH was controlled at 6.5 (InPro 3100/120 Pt100, Mettler Toledo International Trading Co., Ltd) and the culture temperature was 37°C.

The suitable pH range for the growth of Bacillus is neutral and acidic. During the fermentation process, the consumption of amino acid substrates leads to an increase in pH. Therefore, neutralizers need to be added for adjustment after the pH exceeds 7. In addition, a certain amount of foam will be produced during the fermentation process. Properly increasing the rotating speed can remove the foam, but it will not significantly affect the growth of microorganisms. Therefore, after culturing for 12 h, the stirring speed was adjusted to 300 r/min, after the pH was raised to 7 by the fermentation itself, 2 m potassium hydroxide and sulfuric acid were added to control the pH to 7.

Compare the effect of glucose fed-batch on the fermentation process of *B. subtilis*, after 12 h of fermentation, glucose with a concentration of 40% was fed by feeding at a flow rate of 10 mL/h and the feeding period was 24 h. Samples were taken every 3 h to determine the number of viable bacteria, amino acid nitrogen content, soluble phosphorus, reducing sugar, and pH during the fermentation process. The fermentation culture was defoamed with polypropylene glycol.

Indicator Measurement and Statistical Analysis

The number of effective viable bacteria was determined by the dilution plate method and the number of viable bacteria per mL of fermentation broth was calculated; the amino acid nitrogen was determined according to the formaldehyde colorimetric method (Zhang *et al*., 2018), $\triangle AAN = CN\text{-}CN_0$ and the corresponding value is obtained by the amino acid nitrogen content produced by unit mass of CSL; The dissolved phosphorus was measured by molybdenum blue spectrophotometry (Zhu *et al*., 2012), $\triangle P = (CP_0\text{-}CP)/CP_0$ and the corresponding value was obtained by the utilization rate of soluble phosphorus; The reducing sugar was measured by the DNS method (Deng and Tabatabai, 1994) and the corresponding value is obtained by the utilization rate of reducing sugar, $\triangle RS = (CS_0\text{-}CS)/CS_0$; pH is measured using pH acidity meter; The amount of dissolved oxygen and acid-base flow was measured online by the fermenter system; the concentration of glucose and glutamic acid was measured by a biosensor (SBA-40C, Institute of Biology, Shandong Academy of Sciences).

Table 1: Factors and level coding table of box-behnken experimental design

	Coded level of variables		
Factors	-1	0	1
X_1: CSL/gL $^{-1}$	100.0	150.0	200.0
X_2: Inoculation volume/%	5.0	7.5	10.0
X_3: pH	6.5	7.0	7.5

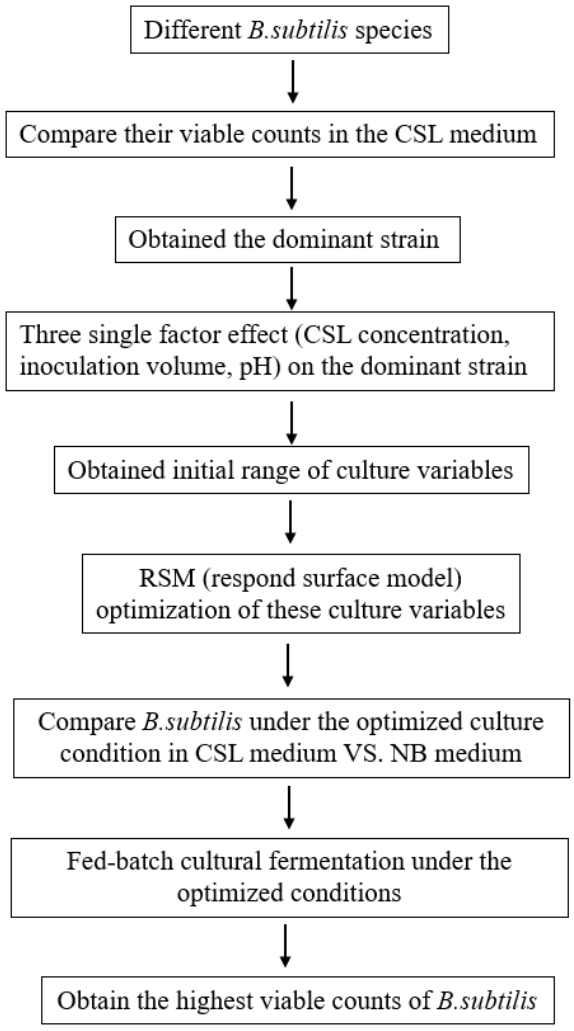

Fig. 1: Mind map of the design of the experiments in this study

Design Expert. 8.26 software was used for experimental design and regression analysis of the data. The univariate data were evaluated by ANOVA followed by Duncan's multiple range test using SPSS 12.0. The difference between the different treatment results is statistically significant ($P<0.05$).

A mind map in Fig. 1 shows the content of the experiments in this study for a clear review of this article.

Results

Screening of B. Subtilis Strains

To screen the dominant strains of *B. subtilis* fermented at high density in CSL, 8 strains of *B. subtilis* sp. that has been used for production were collected from industrial production. The fermentation characteristics of 8 strains in CSL were compared, as shown in Table 2. It can be seen from Table 2 that *B. subtilis* 3301 obtained the maximum viable count of 39.0×10^8 CFU mL^{-1} in pure CSL culture, while strains A330 and N52 also obtained relatively high viable counts, reaching 37.5×10^8 CFU mL^{-1} and 35×10^8 CFU mL^{-1} respectively. Strain A308 had the lowest number of viable bacteria in CSL, only 15.5×10^8 CFU mL^{-1}, and the pH of its fermentation broth was also the lowest, only 6.7. The dynamic changes of protein hydrolysis and amino acid nitrogen utilization of CSL by different strains were reflected by △the AAN value. The content of amino acid nitrogen produced by strain 3301 after fermenting CSL was also the highest, which was 2296.7 mg/L. At the same time, the fermentation broth of strain Y07 also contained a higher level of amino acid nitrogen content, which was 2172.9 mg/L, showing strong protein decomposition ability. △P represents the dynamic changes of insoluble phosphate dissolution and phosphorus utilization in CSL by different strains. Strain N52 has a high utilization rate of phosphorus in CSL and the utilization rate of dissolved phosphorus is 22.2%, which can be used for the decomposition and utilization of CSL phytate. Strain A441 had the highest utilization rate of reducing sugar in CSL, reaching 62.3%. Because the number of viable bacteria is a key indicator for evaluating the quality of microbial inoculants (Xinhe *et al*., 2019), the strain *B. subtilis* 3301 was selected as the starting strain in this study to utilize the CSL for high-density fermentation to produce bio-bacterial fertilizers.

Single Factor Optimization of Biological Bacterial Fertilizer Production

To further determine the fermentation process parameters of *B. subtilis* 3301 using CSL as raw material in high-density fermentation, this study only considered CSL as the only fermentation medium and investigated the effect of concentration of CSL, inoculum amount, initial pH value on the growth of *B. subtilis*. The single-factor optimization test was carried out with the number of viable bacteria of *B. subtilis* as an indicator. The measurement results are shown in Fig. 2.

It can be seen from the experimental results that when the concentration of CSL is 150 g/L, the viable count of *B. subtilis* is the highest, reaching 41×10^8 CFU/mL, indicating that the concentration of CSL is most suitable for the growth of *B. subtilis* at this time; It should because when the concentration of CSL is too low, it cannot meet the basic nutritional requirements for the growth, while when the concentration is too high, the osmotic pressure is too high and the content of inorganic salts increases, which is not conducive to the high-density growth.

When the inoculation amount was 7.5%, the viable count of *B. subtilis* was the highest, reaching 39.5×10^8 CFU/mL. Too high or too low an inoculation amount was not conducive to the growth of *B. subtilis*. If the inoculation amount is too low, the delayed growth period is too long, which is not conducive to the rapid reproduction of *B. subtilis*; If the inoculation amount is too high, the *B. B. subtilis* grows rapidly and the strains compete for nutrients and the pH rises too fast, which inhibited the high-density fermentation.

Table 2: Fermentation characteristics of different *B. subtilis* strains in pure culture in CSL

Strain	Viable count/10^8 CFU/mL	△ AAN/mg/L	△ P/%	△ RS/%	pH
B. subtilis A308	15.5±4.2	1543.9±105.2	1.2±0.06%	46.7±2.84%	6.7±0.41
B. subtilis 3301	39.0±1.4	2296.7±107.9	6.7±0.02%	44.7±7.32%	7.9±0.38
B. subtilis N52	37.5±4.2	1633.7±52.1	22.2±1.01%	60.4±2.65%	8.5±0.45
B. subtilis Y07	21.5±2.8	2172.9±81.2	9.7±0.75%	52.4±1.87%	8.7±0.12
B. subtilis A441	18.5±4.9	1806.2±69.5	10.7±0.92%	62.3±4.39%	7.7±0.24
B. subtilis A330	35.0±7.0	1929.5±30.2	**-0.9±0.02%**	59.4±1.93%	7.1±0.32
B. subtilis N53	30.5±0.7	1880.4±52.4	7.4±0.36%	50.3±2.16%	7.5±0.18
B. subtilis Y15	31.5±2.1	1518.7±53.2	9.2±0.57%	52.1±1.28%	7.4±0.31

Note: Viable count represent the number of colonies that appeared on the plate by coating the fermentation culture; △ AAN: Represents the variation of the amino acid level in the fermentation culture; △ P: Represents the variation of phosphate level in the fermentation culture; △ RS represents the variation of reducing sugar level in the fermentation culture; pH: Represent the pH level in the culture; ± standard deviation; The statistical difference analysis shows $p<0.05$ for all the detected indicators

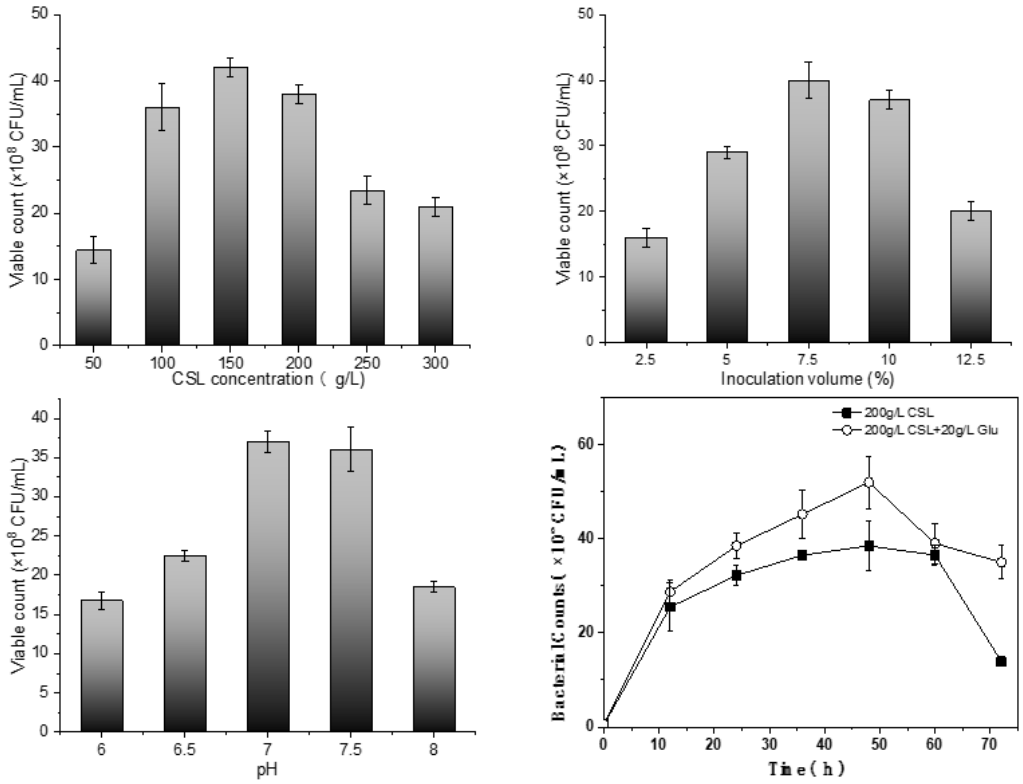

Fig. 2: The effect of different culture parameters on the viable count of *B. subtilis* Note: Significant differences (p values) between CSL concentration groups, inoculation volume groups, pH groups and time groups were $p = 0.01$, $p = 0.03$; $p<0.01$ and $p = 0.02$ respectively

When the initial pH was adjusted to 7, the number of viable bacteria of *B. B. subtilis* was the highest, reaching 34.5×10^8 CFU/mL, which was not significantly different from that at pH 7.5. If the pH is too low, it is not conducive to the growth of *B. subtilis*, which affects the activity of enzymes in the bacteria and hinders the normal growth and metabolism of the bacteria. If the pH is too high, the pH of the fermentation will be higher in the later stage and the bacteria will be autolyzed in advance. Comparing the effect of adding glucose on the growth of *B. subtilis*, it can be seen that the addition of an appropriate amount of glucose can increase the viable count of *B. subtilis*. The CSL medium supplemented with glucose reached the highest number of viable bacteria when *B. subtilis* was cultured for 48 h, reaching 50×10^8 CFU/mL. In the later fermentation process, feeding glucose can significantly increase the number of viable bacteria in high-density fermentation.

Therefore, when the CSL concentration was 150 g/L, the inoculum amount was 7.5% and the initial pH was adjusted to 7, the viable count of *B. subtilis* reached the highest value respectively, which could be used as the optimal level of fermentation parameters.

Response Surface Optimization for the Production of Biological Bacterial Fertilizers

The appropriate levels of CSL concentration, inoculum size, and pH were determined by a single-factor experiment. According to the Box- Behnken principle, a 3-level response surface test design was carried out for the three response variables using the number of viable bacteria as the response value through the Design -Expert software. The test results and the predicted values obtained by the fitting equation are shown in Table 3 and the regression coefficient and variance analysis were shown in Table 4.

Using Design-Expert software for fitting, the ternary quadratic regression equation is obtained as: $Y = 44.00 + 0.63X_1 + 3.38X_2 + 0.75X_3 + 0.50X_1X_2 + 2.25X_1X_3 + 0.25X_2X_3 - 4.50X_1^2 - 7.50X_2^2 - 6.25X_3^2$. Among them, the quadratic multinomial regression equation is analyzed by variance and the results are shown in Table 4. It can be seen from Table 4 that the model $P<0.01$, the model is extremely significant. Lack of fit is not significant ($P = 0.4074>0.05$), the model's coefficient of determination $R^2 = 0.9717$; the model's adjusted determination coefficient R^2 Adj = 0.9353, indicating that the regression equation is well fitted, the model is established and can be used in analyzation and prediction. In the model, the inoculum size of X_2 was $P<0.01$, indicating that the inoculum size had a significant effect on the number of viable bacteria. According to the F value, it can be judged that the order of the influence of three factors on the number of effective viable bacteria is inoculum size > pH > CSL concentration. In addition, the interaction effect X_1 X_3 $P<0.05$ indicated that the interaction between CSL concentration and pH had a significant effect on the results ($P<0.05$), while the quadratic terms had significant effects on the results ($P<0.01$).

Interaction between Response Surface Factors

According to the above regression fitting equation, fix any factor at a constant value (take the 0 levels), draw the response surface curve (Fig. 3), and obtain the effect of the interaction of the other two factors. Further study the interaction between the relevant variables and identifies the optimal point. In Fig. 3, when the fixed pH was 7, the CSL concentration was increased from 100 $g \cdot L^{-1}$ to 200 $g \cdot L^{-1}$, and the inoculum amount was increased from 5 to 10%, the number of viable *B. subtilis* was all first increase and then decrease. The vertex of the surface is the point of the maximum viable count of *B. subtilis*. Similarly, other surface plots reflect the interaction of pH and inoculum and pH and CSL concentration, respectively. According to the quadratic regression equation, when the CSL concentration was 145 $g \cdot L^{-1}$, the pH was 7 and the inoculation amount was 8, the maximum viable count of *B. subtilis* was 4.5×10^9 CFU/mL. To verify the accuracy of the predicted value of the model, the verification experiment was carried out under the conditions of the optimal fermentation parameters and repeated three times.

Table 3: Box-behnken experimental design and response results for three variables

	Factor and real levels			Y Biomass × 10^9 CFU/mL	
Runs	X_1 CSL ($g \cdot L^{-1}$)	X_2 Inoculation volume (%)	X_3 pH	Actual	Predicted
1	100	5.0	7.0	2.7±0.09	2.85±0.22
2	200	5.0	7.0	2.9±0.12	2.88±0.13
3	100	10.0	7.0	3.4±0.05	3.43±0.21
4	200	10.0	7.0	3.8±0.07	3.65±0.09
5	100	7.5	6.5	3.5±0.13	3.41±0.02
6	200	7.5	6.5	3.0±0.21	3.08±0.04
7	100	7.5	7.5	3.2±0.74	3.11±0.32
8	200	7.5	7.5	3.6±0.32	3.69±0.14
9	150	5.0	6.5	2.7±0.84	2.64±0.22
10	150	10.0	6.5	3.2±0.36	3.26±0.30
11	150	5.0	7.5	2.8±0.56	2.74±0.37
12	150	10.0	7.5	3.4±0.28	3.46±0.27
13	150	7.5	7.0	4.2±0.47	4.40±0.54
14	150	7.5	7.0	4.4±0.32	4.40±0.22
15	150	7.5	7.0	4.6±0.76	4.40±0.39
16	150	7.5	7.0	4.5±0.42	4.40±0.27
17	150	7.5	7.0	4.3±0.21	4.40±0.20

Note: Runs represent different experimental groups' numbers; Factor and real levels represented three parameters and their input levels which were designed by the Box- Behnken experimental design, including X_1 (CSL concentration), X_2 (Bacillus inoculation volume in the CSL medium) and X_3 (the culture pH); Y (biomass) represented the biomass yield under the designed experiment parameter conditions, the actual value is the experimental output and predicted value represent the data outputted by the model. The significant difference in the biomass yield between each experimental group, $P<0.05$

Table 4: Regression coefficient model and variance analysis table

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	Significance
Model	660.87	9	73.43	26.700	0.0001	**
X_1 - CSL	3.12	1	3.12	1.140	0.3218	NS
X_2 -Inoculation volume	91.12	1	91.12	33.140	0.0007	**
X_3 -pH	4.50	1	4.50	1.640	0.2416	NS
$X_1 X_2$	1.00	1	1.00	0.360	0.5655	NS
$X_1 X_3$	20.25	1	20.25	7.360	0.0300	*
$X_2 X_3$	0.25	1	0.25	0.091	0.7718	NS
X_1^2	85.26	1	85.26	31.000	0.0008	**
X_2^2	236.84	1	236.84	86.120	<0.0001	**
X_3^2	164.47	1	164.47	59.810	0.0001	**
Residu l	19.25	7	2.75			
Lack of Fit	9.25	3	3.08	1.230	0.4074	NS
Pure error	10.00	4	2.50			
Cor total	680.12	16				

Note: Df. degrees of freedom; *. Significant difference (P<0.05); **. An extremely significant difference (P<0.01); NS. Not significant; model's determination coefficient $R^2 = 0.9717$; the adjusted coefficient of determination of the model $R^2_{Adj} = 0.9353$

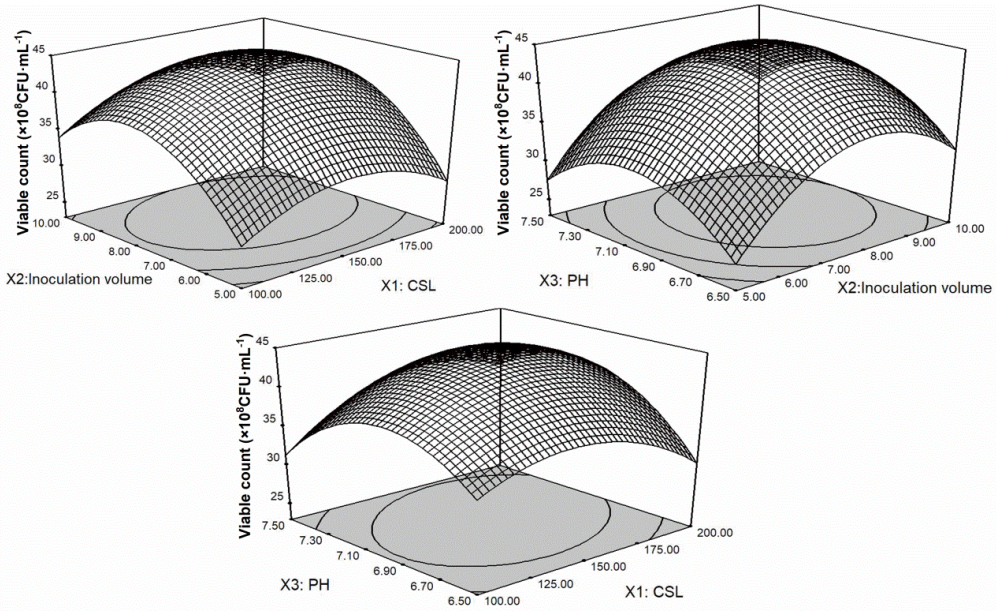

Fig. 3: Interaction effect between variables on the viable count of *B. subtilis*

Growth Comparison of B. Subtilis in NB Medium and CSL Medium

CSL, as a high-quality and inexpensive nitrogen source, can be used as a medium for *B. subtilis*. NB medium is often used as a seed medium for bacteria (Guoqing *et al*., 2016). Under the fermentation conditions optimized by response surface, we compared the dynamic changes of CSL and NB medium when culturing *B. subtilis*, as shown in Fig. 4. It can be seen that the viable count of *B. subtilis* cultured in CSL medium is significantly higher than that in NB medium. When cultured in CSL, *B. subtilis* reached the highest biomass at 48h, 4.5×10^9 CFU/mL, while *B. subtilis* reached the highest viable count at about 6h when cultured in NB medium, it can reach 1.2×10^9 CFU/mL. This may be because the NB medium is a fast-acting medium and the nutrient content is not as rich as that of the CSL medium, which causes *B. subtilis* quickly reached the highest viable count, but then decreased due to insufficient nutrients. In addition, the pH value of the fermentation broth of the NB medium was significantly higher than that of the CSL medium. which indicates that too high a pH was not conducive to the growth of *B. subtilis*. Therefore, CSL of suitable concentration can be used as an inexpensive medium, it also can be used as an alternative seed medium for *B. subtilis*, which not only increases the number of viable bacteria of *B. subtilis* but also reduces the cost of producing seed media.

Fed-Batch Fermentation for Biological Bacterial Fertilizer Production

Based on the factor level of response surface optimization, a pilot test was carried out in a 5 L fermenter. As the previous result, the growth of *B. subtilis* can be significantly promoted with the addition of glucose in the early shake flask culture. Therefore, to explore the effect of feeding glucose on the culture of *B. subtilis* in the fermentation tank, 40% glucose was used to simulate the high-density fermentation of *B. subtilis* by combining glucose crystallization mother liquor with CSL medium and the fermentation characteristics of *B. subtilis* by fed-batch fermentation of CSL were explored. The dynamic changes of viable bacterial counts during the *B. subtilis* culture process are shown in Fig. 5. In the test group, from 0 to 12 h, no glucose was fed and *B. subtilis* grows rapidly. After 12 h, glucose was fed in and the viable count of *B. subtilis* was significantly higher (up to 1.75×10^{10} CFU/mL) than the control group (the viable count could reach 1.2×10^{10} CFU·mL^{-1}in CSL culture). What is interesting, the glucose addition could only promote the biomass of *B. subtilis*, but the number of viable bacteria reached the highest value both at about 33 h with and without glucose feeding.

Figure 6 is the dynamic change of fermentation characteristics of *B. subtilis* in CSL fed or not fed with glucose. Figure 6a shows the changes in the content of amino acid nitrogen and glutamic acid during the fermentation process. It can be seen that the content of amino acid nitrogen showed a trend of first increasing and began to decrease after reaching the maximum value at 36 h. The content of amino acid nitrogen after feeding glucose is lower than that of the CSL medium without feeding glucose, this is because the feeding of glucose greatly increases the number of viable bacteria of *B. subtilis*, resulting in the utilization of amino acid nitrogen by bacteria for their growth. At 0~10 h, the glutamate content decreased continuously, which was directly utilized by *B. subtilis* for cell growth and at 10~36 h, the glutamate content increased continuously, reached the highest value at 36 h, and then began to decrease. *B. subtilis* can produce protease to decompose macromolecular proteins in CSL into amino acids, increasing free amino acid content (Hashem *et al*., 2019; Yijie and Zuozhong, 2019; Rocky-Salimi *et al*., 2016). With the increase in cell density, free amino acids in CSL are used for microbial growth, resulting in a decrease in free amino acid nitrogen content (Zhuangzhuang, 2021).

Figure 6b shows the dynamic changes in soluble phosphorus content during the fermentation process. The content of soluble phosphorus decreases with the increase of fermentation time. Phosphorus can provide a phosphorus source for cell growth, which fully shows that the organic phosphorus in CSL can be decomposed and utilized by *B. subtilis* (Rocky-Salimi *et al*., 2016), at the same time, it can effectively promote the growth of *B. subtilis*.

Figure 6c shows the changes in dissolved oxygen and acid-base flow rates detected in real-time during the fermentation process. The DO in the fermentation broth decreased continuously from 0 to 10 h and finally, the dissolved oxygen was almost zero. Increased ventilation still cannot meet the oxygen demand of *B. subtilis*, so DO is one of the factors limiting the high-density growth of *B. subtilis* in the bioreactor.

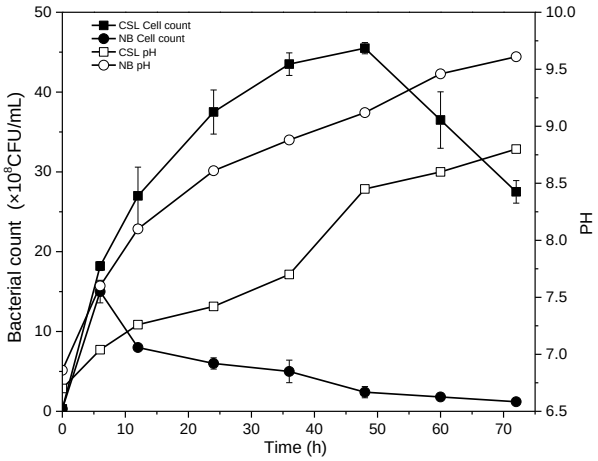

Fig. 4: Growth comparison of *B. subtilis* in NB medium and CSL medium

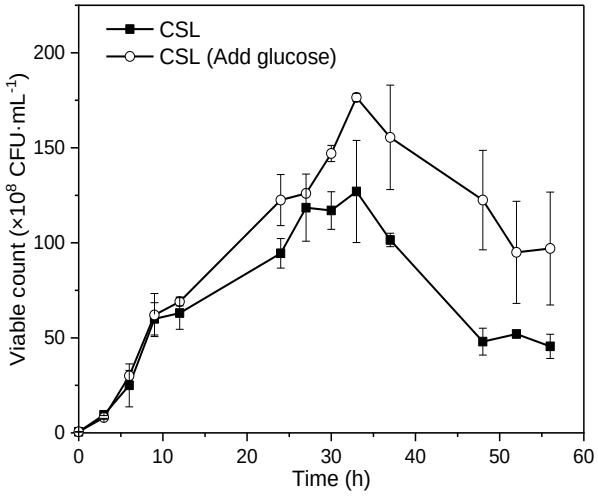

Fig. 5: Comparison of viable counts of *B. subtilis* in fed-batch culture with feeding glucose and without feeding

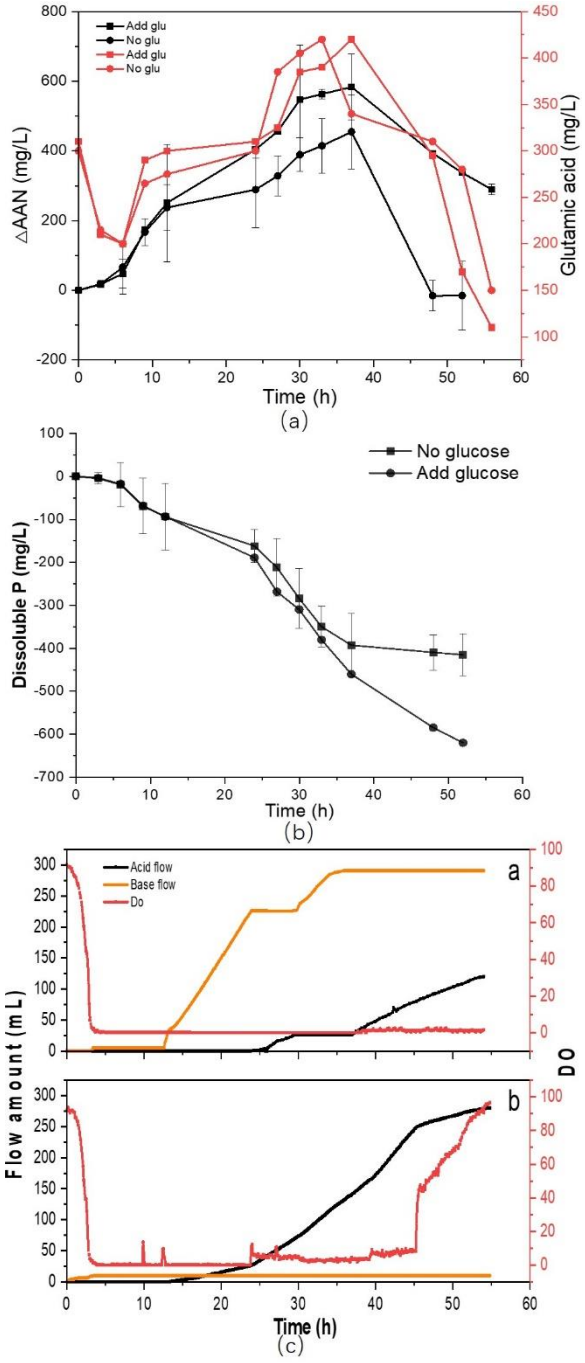

Fig. 6: Characteristic analysis of batch fermentation of *B. subtilis* in CSL medium with or without feeding glucose (a: Changes of amino acid nitrogen and glutamate content during fermentation; b: dynamic changes of soluble phosphorus content during fermentation; c: Changes of acid flow, base flow into the bioreactor, and the dissolved oxygen level in the bioreactor)

From 0 to 15 h, the metabolism of *B. subtilis* is used to increase the pH value in the fermentation broth. When the pH is 7, the pH is controlled by adding an acid-base regulator. Therefore, the acid-base flow rate is 0 at this stage. After 15 h, the pH of the fermentation broth without adding glucose continued to rise, resulting in the continuous increase of acid flow addition until the acid flow addition increased slowly after 45 h. At this time, the DO value began to rise and the number of viable bacteria of *B. subtilis* decreased, which led to the increase of dissolved oxygen in the fermentation broth. After feeding glucose, the C/N ratio increased, since glucose is a carbon source, so when we flow added 40 g/L glucose into the fed-batch culture, the C/N ratio increased. Although carbon sources could provide the skeleton of biosynthesis, they could produce a lot of organic acid intermediates. Therefore, the pH value of the culture will decrease and was detected by the pH indicator. That's why we adjusted the pH in the later fermentation stage and increased the amount of alkali flow. After 36 h, the feeding of glucose was stopped. It was still high and the pH began to rise, resulting in an increasing amount of acid flow the DO value was always at the zero level, indicating that the growth of *B. subtilis* was still very vigorous at this time.

Discussion

CSL is a kind of refractory corn starch processing organic waste liquid. It is rich in nutrients and is often used as a nitrogen source in the fermentation industry. There are many studies on its resource utilization. For example, (Yang *et al*., 2013) used CSL as an alternative source to produce 2,3-butanediol and ethanol from *B. subtilis*; Zhang and Jia (2018) used CSL and other corn processing waste to increase butanol production with *Clostridium* SE-2, Germec *et al*. (2020) used CSL as an alternative nitrogen source to study the production of inulinase and the optimization of fermentation medium. However, its nitrogen content is too high, and excessive addition is not conducive to the formation of fermentation secondary metabolites. But high ammonia nitrogen can be beneficial to the increase of biomass, so CSL is used for high-density fermentation of functional microorganisms. Deng and Tabatabai (1994) used high-throughput sequencing technology to find the dominant flora of the microbial flora in CSL, which was *B. subtilis*. At the same time, *B. subtilis* is a class of classical and common bacteria widely used in biological fertilizers and it has been proved that it can effectively promote the growth of plants (Hofer *et al*., 2018). Therefore, CSL is used as an inexpensive medium for high-density cultivation of *B. subtilis*. But different species of *B. subtilis* subsp. grow in CSL differently. Therefore, this study screened a dominant strain *B. subtilis* 3301 from industrial production, which can reach 39.0×10^8 CFU/mL in pure culture in CSL, which has obvious advantages compared with other studies. Zhu *et al*. (2012) used waste mushroom substrate to produce biological bacterial fertilizer and the

density of the prepared biological bacterial fertilizer reached 5.6 × 10^8 CFU/g. Fenfen (2016) used cassava alcohol wastewater to ferment to produce microecological bacterial fertilizer, the number of effective viable bacteria reached 1.34 × 10^9 CFU/mL. Meanwhile, the content of amino acid nitrogen produced by strain 3301 after fermenting CSL was also the highest, which was 2296.7 mg/L. Therefore, it is a very good candidate to produce high-density bio-fertilizer using CSL.

In the process of industrial production of refined glucose, after the glucose is obtained by evaporation and concentration, the crystals are separated by stirring and cooling and centrifugation and the remaining part is the crystalline sugar mother liquor (Hong *et al*., 2018). Crystallized sugar mother liquor and CSL are both starch sugar leftovers. Combining the two can effectively increase the added value of corn starch enterprises. In this study, glucose was used to simulate the glucose crystallization of mother liquor. It was found that the addition of glucose could significantly promote the growth of *B. subtilis* and at the same time, it could reduce the high pH caused by high nitrogen fermentation of CSL. Yubin *et al*. (2019) and others used crystalline sugar mother liquor and CSL to ferment *LactoB.* with high density, which produced a higher number of viable bacteria. Therefore, the high-density fermentation of *B. subtilis* using CSL also has a good application prospect.

CSL is an inexpensive nitrogen source and its carbon source can easily become a limiting nutrient source for cell growth during high-density fermentation. Too high or too low carbon source concentration is not conducive to the progress of fermentation production. The feeding method not only conforms to the fluid properties of the mother liquor itself but also can better promote the high-density growth of submerged fermentation. With the rapid development of biological bacterial fertilizers, the use of CSL as a single medium for fermentation production of biological bacterial fertilizers will also face many challenges and need to be further studied.

Conclusion

In this study, a strain of *B. subtilis* 3301, which grows well in CSL, was screened from industrial strains. Through response surface optimization, when the CSL concentration was 145 g·L^{-1}, pH was 7 and the inoculum amount was 8%, the viable count of *B. subtilis* could reach the highest of 4.5 × 10^9 CFU/mL. At the same time, the addition of glucose can significantly increase the viable count of *B. subtilis*. The viable count of *B. subtilis* after single CSL fermentation in a 5L fermenter can reach 1.2 × 10^{10} CFU/mL and the number of viable bacteria was up to 1.75 × 10^{10} CFU/mL after feeding 40% glucose at the same time. Therefore, CSL is an inexpensive medium for the high-density fermentation of *B. subtilis*, which not only realizes the resource utilization of waste but also reduces the production cost of *B. subtilis* biological fertilizer. This study confirmed the feasibility of using CSL as raw material to ferment *B. subtilis* and opened new ideas for the industrial production of *B. subtilis*. Finally, there are also some limitations in this study. We noticed that the range of pH and rotational speed in the fermentation experiment was set based on our experience in the experimental process and had certain limitations. In addition, corn steep liquor is rich in nutrients, we only studied the utilization characteristics of one type of bacteria, hoping to provide ideas for other research on the use of corn steep liquor to produce biological bacterial fertilizers, to study the biological characteristics of other bacteria and to apply their specific function.

Acknowledgment

Thanks to Luzhou Bio-Technology Co. Ltd. for providing corn steep liquor as raw material.

Funding Information

Promote Scientific Research Cooperation and High-level Talent Training Projects with Canada, Australia, New Zealand, and Latin America (study abroad fund in the Americas 2022-1007). Shandong Provincial Natural Science Foundation, China (ZR2020MC201). Chongqing technological innovation and application development key projects (cstc2019jscx-gksbX0113). China Post-Doctoral Science Foundation (2019M662362).

Author's Contributions

Xiaojie Ren, Baoyue Liu and Zhuangzhuang He: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Xiaolong Wang and Yubin Zhao: Participated in manuscript preparation.

Mario Jolicoeur and Xinhe Zhao: Ameliorated the manuscript and contributed to the funding support.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

References

Chinta, Y. D., Kano, K., Widiastuti, A., Fukahori, M., Kawasaki, S., Eguchi, Y., ... & Sato, T. (2014). Effect of corn steep liquor on lettuce root rot (Fusarium oxysporum f. sp. lactucae) in hydroponic cultures. *Journal of the Science of Food and Agriculture*, *94*(11), 2317-2323. https://onlinelibrary.wiley.com/doi/abs/10.1002/jsfa.6561

Choi, J. D. R., Jang, Y. S., Cho, J. H. (2013). Characterization and evaluation of corn steep liquid in acetone-butanol-ethanol production by *Clostridium acetobutylicum* [J]. *Biotechnology & Bioprocess Engineering*, 2013, 18(2), 266-271.

Chunxiang, L. (2021). Current status of corn planting and efficiency of new technology application [J]. *Agricultural Technology Extension*, 2021, (10), 30-31.

Deng, S. P., & Tabatabai, M. A. (1994). Colorimetric determination of reducing sugars in soils. *Soil Biology and Biochemistry*, *26*(4), 473-477. https://doi.org/10.1016/0038-0717(94)90179-1

Fenfen, W. (2016). Research on the production of microbial fertilizer using cassava alcohol wastewater [D. Shijiazhuang: Hebei University of Science and Technology, 2016.

Germec, M., Gürler, H. N., Ozcan, A., Erkan, S. B., Karahalil, E., & Turhan, I. (2020). Medium optimization and kinetic modeling for the production of Aspergillus niger inulinase. *Bioprocess and Biosystems Engineering*, *43*(2), 217-232. https://link.springer.com/article/10.1007/s00449-019-02219-1

Guoqing, H., Yingmin, J., & Lixiao, D. (2016). Food Microbiology [M]. Beijing: China Agricultural University Press, 2016.8.

Hashem, A., Tabassum, B., & Abd_Allah, E. F. (2019). Bacillus subtilis: A plant-growth promoting rhizobacterium that also impacts biotic stress. *Saudi Journal of Biological Sciences*, *26*(6), 1291-1297. https://doi.org/10.1016/j.sjbs.2019.05.004

Hofer, A., Hauer, S., Kroll, P., Fricke, J., & Herwig, C. (2018). In-depth characterization of the raw material corn steep liquor and its bioavailability in bioprocesses of Penicillium chrysogenum. *Process Biochemistry*, *70*, 20-28 https://doi.org/10.1016/j.procbio.2018.04.008

Hong, W., Shuwen, Y., & Fuan, Z. (2018). Effects of crystalline glucose mother liquor on lysine fermentation process [J]. *Fermentation Science and Technology Communications*, 2018, 47(03), 184-188.

Hongmei, D., & Sen, D. (2021). Corn steep liquor and other organic materials combined with soda alkaline soil improvement effect and humus characteristics. *Chinese Journal of Agricultural Environmental Science*, 2021,40 (10), 2159-2166.

Hongyu, L., & Li, X. (2017). Nutritional characteristics and application of corn steep liquor [J]. *Feed Research*, 2017, 19(5), 9-18.

Jiqiang, W., Qiang, L., & Aiqin, L. (2011). Nutritional characteristics of by-products of corn processing and its application effect in poultry industry [J]. *Guangdong Feed*, 2011, 20(05), 30-33.

Jun, L., Xin, J., & Li, L. (2006). The development of microbial fertilizers and the maintenance of soil biological fertility [J]. *China Soil and Fertilizer*, 2006(4), 1-5.

Ligang, Z. S. (2002). Physiological characteristics test of FM strain and optimization of shaker fermentation conditions. *Journal of Microbiology*, 2002, 22(6) 12-14.

Martinez-Burgos, W. J., Sydney, E. B., de Paula, D. R., Medeiros, A. B. P., de Carvalho, J. C., Molina, D., & Soccol, C. R. (2021). Hydrogen production by dark fermentation using a new low-cost culture medium composed of corn steep liquor and cassava processing water: Process optimization and scale-up. *Bioresource Technology*, *320*, 124370. https://doi.org/10.1016/j.biortech.2020.124370

Rocky-Salimi, K., Hashemi, M., Safari, M., & Mousivand, M. (2016). A novel phytase characterized by thermostability and high pH tolerance from rice phyllosphere isolated Bacillus subtilis BS 46. *Journal of Advanced Research*, *7*(3), 381-390. https://doi.org/10.1016/j.jare.2016.02.003

Salam, L. B., & Ishaq, A. (2019). Biostimulation potentials of corn steep liquor in enhanced hydrocarbon degradation in chronically polluted soil. *3 Biotech*, *9*(2), 1-20. https://link.springer.com/article/10.1007/s13205-019-1580-4

Sarwar, M., & Ajmal Khan, M. (2004). Influence of ruminally protected fat and urea treated corncobs ensiled with or without corn steep liquor on nutrient intake, digestibility, milk yield and its composition in Nili-Ravi buffaloes. *Asian-Australasian Journal of Animal Sciences*, *17*(1), 86-93. https://www.animbiosci.org/upload/pdf/17_14.pdf

Wu, Q. M., Zhang, J. M., Li, Y. Y., & Zhang, Y. (2021). Recent advances on the mechanism of beneficial microbial fertilizers in crops. *Biotechnology Bulletin*, *37*(5), 221. http://biotech.aiijournal.com/EN/10.13560/j.cnki.biotech.bull.1985.2020-0846

Xinhe, Z., Zhuangzhuang, H., & Hongjun, L. (2019). Research progress on the development of biological bacterial fertilizer from agricultural product processing waste [J]. *China Soil and Fertilizer*, 2019, (06), 14-21.

Xinhe, Z., Zhuangzhuang, H., Yubin, Z., Chengye, M., & Xiaojie, R. (2020). Kinetic simulation of amino acid metabolism of butanol produced from the fermentation of corn steep liquor. *Transactions of the Chinese Society of Agricultural Engineering*. 2020, 36(18) 263-274.

Yang, T. W., Rao, Z. M., Zhang, X., Xu, M. J., Xu, Z. H., & Yang, S. T. (2013). Effects of corn steep liquor on production of 2, 3-butanediol and acetoin by Bacillus subtilis. *Process Biochemistry*, *48*(11), 1610-1617. https://doi.org/10.1016/j.procbio.2013.07.027

Yijie, L., & Zuozhong, Y. (2019). Research progress on the main mechanism and application of *B. subtilis*[J]. *Sichuan Forestry Science and Technology*, 2019, 40(04), 126-130

Ying, Z., Ping, Z., & Hui, Z. (2013). The role of corn steep liquor in amino acid fermentation industry [J]. *China Brewing*, 2013, 32(11), 18-22.

Yubin, Z., Jing, W., & Jianting, G. (2019). Preparation of LactoB. acidophilus from corn starch sugar by-products [J]. *Food and Feed Industry*, 2019, (07), 51-55.

Zhang, J., & Jia, B. (2018). Enhanced butanol production using Clostridium beijerinckii SE-2 from the waste of corn processing. *Biomass and Bioenergy*, *115*, 260-266. https://doi.org/10.1016/j.biombioe.2018.05.012

Zhang, Y., Yang, G., & Li, D. (2018). Determination of Amino Acid Nitrogen in Soy Sauce by Colorimetry[J]. *China Condiment*, 2018, 43(06), 146-149.

Zhu, H. J., Sun, L. F., Zhang, Y. F., Zhang, X. L., & Qiao, J. J. (2012). Conversion of spent mushroom substrate to biofertilizer using a stress-tolerant phosphate-solubilizing Pichia farinose FL7. *Bioresource Technology*, *111*, 410-416. https://doi.org/10.1016/j.biortech.2012.02.042

Zhuangzhuang, H. (2021). Research on the use of corn steep liquor to develop microbial fertilizer based on symbiotic fermentation technology [D]. Master's thesis of Shandong University of Technology, 2021.06.12.

Ziheng, X., Zhikang, W., & Ziyun, C. (2021). The effect of biological bacterial fertilizer on the root configuration and root morphology of *A. chinensis* [J]. *China Soil and Fertilizer*, 2021 (4), 258-266.