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Study on Optimizing Nutrition and Fermentation Conditions for Compound *Bacillus spp.*

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Abstract

In order to improve the spore yield of compound *Bacillus spp.* (*B. amyloliquefaciens*, *B. laterosporus* and *B. megaterium*), the effects of nutrient conditions including carbon source, nitrogen source, mineral salt and fermentation conditions including the inoculum age, inoculation amount, loading volume of liquid and initial pH on the spore yield were studied. The results indicated that the optimized medium was glucoses 20 g/L, soybean meal 30.0 g/L, K_2HPO_4 1.0 g/L; fermentation temperature is 37˚C, the inoculum age 12 h, initial pH 7.0, 2% inoculation amount, loading volume of liquid 20 mL/250 mL. Under the optimized conditions of culture medium and fermentation for compound *Bacillus spp.*, spore yield was 10.24 times more than the initial medium, and the spore formation rate reached more than 90%.

Keywords

Compound *Bacillus spp.*, Spore Yield, Medium Optimization, Fermentation Optimization

1. Introduction

Bacillus spp., which has broad spectrum antibacterial activity and strong resistance to stress, is applied abundantly to scientific research and practical production. The types of Bacillus currently used in biological control research are mainly *B. subtilis*, *B. thuringiensis*, *B. amyloliquefaciens*, *B. cereus*, *B. megate-*

rium, *B. licheniformis* and *B. polymyxa* [1]. It colonizes in plant rhizosphere, body surface, roots and stems, competes with pathogens for nutrients around the plant, and secretes antibacterial substances to inhibit the growth of pathogenic bacteria, thus achieving the purpose of prevention and control [2].

With the application of Bacillus in agriculture, it has become one of the research hotspots to exploit Bacillus agents which have higher yield and are easy to produce in recent years [3] [4] [5]. The optimization of the medium is the precursor of the fermentation process. The suitable medium formula and culture conditions can not only significantly increase the biomass of the strain or the yield of the spore, but also reduce the production cost. It could provide scientific basis for large-scale production of microbial fertilizer through the optimization of the medium. Based on the initial culture medium, the optimal fermentation medium and conditions of compound *Bacillus spp.* were determined by single factor experiments and orthogonal experiments to lay the foundation for further large-scale application.

2. Materials and Methods

2.1. Strains

Compound *Bacillus spp.* (6 biocontrol strains: ZM9 YH-22 ZH XS-1 3-10 BG2) were deposited in College of Life Sciences, Hubei University. It has great prevention and control effect on tobacco bacterial wilt and black shank. Among them, ZM9, YH-22 and XS-1 are *Bacillus amyloliquefaciens*; 3-10, ZH are *Bacillus licheniformis*; BG2 belongs to *Bacillus megaterium*.

2.2. Cultures

YSP medium: sucrose 20 g/L, Yeast extract 5 g/L, Tryptone 10 g/L, pH 7.0~7.2; LB medium: tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0~7.2; NA medium: Glucose 20 g/L, Tryptone 5 g/L, Beef extract 3 g/L, pH 7.0~7.2; NB medium: tryptone 10 g/L, Beef extract 3 g/L, NaCl 5 g/L, pH 7.0~7.2. Above were 4 initial culture formula of "optimized medium".

2.3. Selection of Different Substitutes and Their Concentration of the Medium

The selection of carbon sources and concentration optimization: YSP medium was used as the initial medium, and other components of the medium were kept unchanged except for the carbon source. The sucrose in the medium was replaced by equivalent amount of maltose, glucose, glycerol, lactose, soluble starch and maize starch. Selecting the carbon source concentrations of 10, 15, 20, 25, 30 g/L for flask fermentation and then taking the bacteria solution at 80˚C water bath for 15 min at the end of fermentation.

The selection of nitrogen sources and concentration optimization: maintaining the optimized carbon source in the initial fermentation medium, and keeping other components of the medium unchanged except for the nitrogen source.

The tryptone in the medium was replaced by equal amount of bean pulp, fish meal, $(NH_4)_2SO_4$, peanut meal and NO_3NH_4. Selecting the nitrogen source concentrations of 10, 15, 20, 25, 30, 35, 40 g/L for flask fermentation and then taking the bacteria solution at 80˚C water bath for 15 min at the end of fermentation.

The selection of inorganic salt sources and concentration optimization: adding 0.5 g/L $CaCO_3$, $FeSO_4$, $MgSO_4$, $ZnSO_4$ and K_2HPO_4 to the culture medium respectively. Selecting the inorganic salt source concentrations of 0.1, 0.3, 0.5, 0.7, 0.9 g/L for flask fermentation and then taking the bacteria solution at 80˚C water bath for 15 min at the end of fermentation.

2.4. Optimization of Fermentation Conditions

Set different strain ages in "optimized medium": 8 h 10 h 12 h 14 h 16 h; inoculum amount: 1% 2% 3% 4% 5%; initial pH: 6.0 6.5 7.0 7.5 8.0; loading volume: 20 ml/250 ml 30 ml/250 ml 40 ml/250 ml 50 ml/250 ml 60 ml/250 ml. With the above exploration, we could obtain the effects of fermentation conditions on the biomass of compound *Bacillus spp.*

2.5. Orthogonal Experiment Design

On the basis of single factor experiment, we could design orthogonal experiment of three-factor three-level of glucose, bean pulp and K_2HPO_4 which has great influence on the fermentation of compound *Bacillus spp.* as well as three-factor three-level of inoculums size, initial pH and loading volume (**Table 1**). Thus, further optimize the formula and fermentation conditions of the medium.

3. Results and Discussion

3.1. Effect of initial Medium on Biomass of Bacillus

The spore biomass of compound *Bacillus spp.* after fermentation of 4 initial mediums was shown in **Figure 1**. The highest yield of spores was produced in YSP medium (13.8×10^8 CFU/mL). It was significantly higher than the other three medium. So YSP medium was selected as the initial fermentation medium for Bacillus complex.

3.2. The Optimization of Culture Conditions of Compound *Bacillus spp.*

3.2.1. Effects of Carbon Sources and their Concentrations on the Biomass of Compound *Bacillus spp.*

The effect of different carbon sources on the biomass of compound *Bacillus spp.* was shown in **Figure 2**. Among 7 organic carbon sources, the enhancement of sucrose and glucose on the biomass of bacillus complex was evidently higher than other carbon sources followed by soluble starch, lactose, glycerol and maltose while the biomass of bacillus complex was the lowest as maize starch was carbon source. Since the cost of glucose is much lower than that of sucrose, glucose is selected as the carbon source for culture optimization. With the rise of glucose concentration, the biomass of compound *Bacillus spp.* increased in

Table 1. Factors and their levels employed in orthogonal test.

	Levels	Factors		
		A (glucose, g/L)	B (bean pulp, g/L)	C (K_2HPO_4, g/L)
Medium optimization orthogonal test	1	30	40	1.0
	2	20	35	2.0
	3	15	30	2.5
	Levels	Factors		
		A (inoculum size, %)	B (initial pH)	C (loading volume, mL/250 mL)
Fermentation condition optimization orthogonal test	1	2	7	20
	2	3	7.5	30
	3	4	8	40

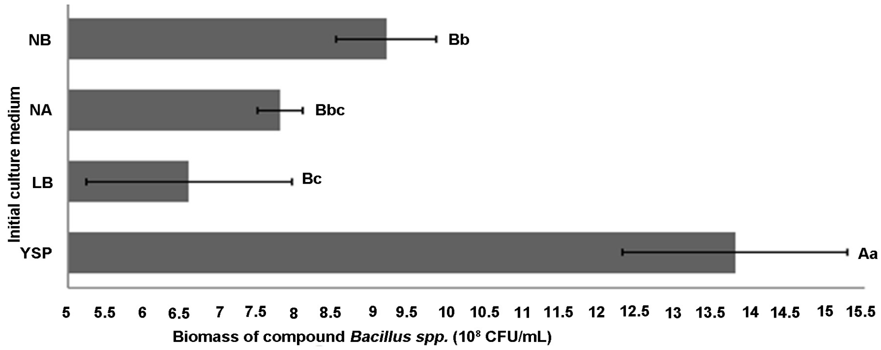

Figure 1. Effects of different initial culture media on the spore yield of compound *Bacillus spp.*

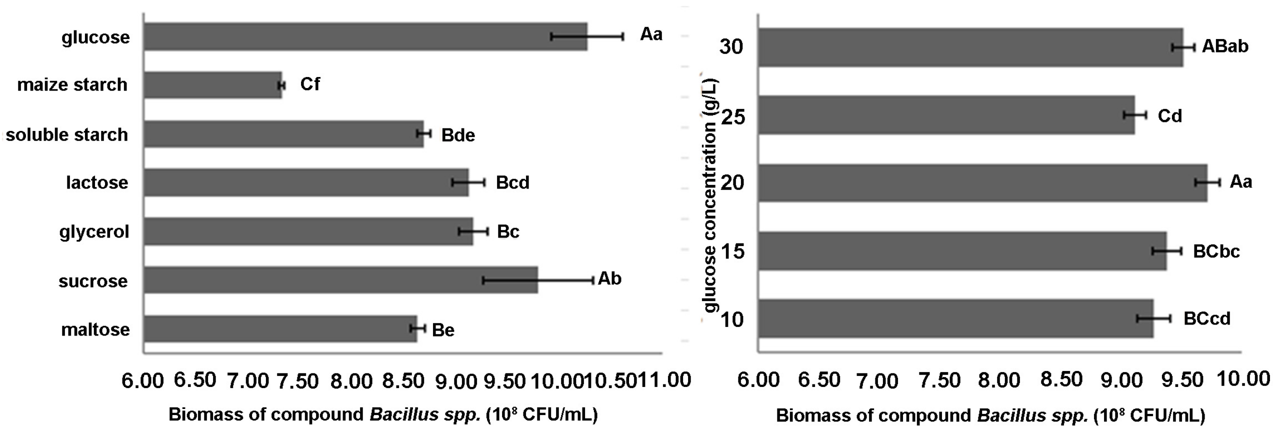

Figure 2. Effects of different carbon source and different concentration of glucose on the spore yield of compound *Bacillus spp.* Note: Different capital letters indicated that each treatment reached a very significant difference ($p < 0.01$). Different lowercase letters represented significant difference within treatments ($p < 0.05$). It was the same below.

initial stage and then declined. When the glucose concentration was 20 g/L, the biomass of compound *Bacillus spp.* was much higher than other concentration conditions.

3.2.2. Effects of Nitrogen Sources and their Concentrations on the Biomass of Compound *Bacillus spp.*

As was shown in Figure 3, compound *Bacillus spp.* was more suitable to utilize organic nitrogen sources compared with inorganic nitrogen sources. Among the organic nitrogen sources, the utilization of bean pulp and fish meal was significantly higher than other nitrogen sources while inorganic nitrogen sources such as $(NH_4)2SO_4$ and NO_3NH_4 were unbeneficial to the formation of bacteria spores. When the bean pulp concentration was 30 g/L, the biomass of compound *Bacillus spp.* reached the highest than that of when the bean pulp concentration was less than 30 g/L. There was no significant difference when the bean pulp concentration was above 30 g/L, indicating that the high concentration of bean pulp might be too viscous to facilitate the transfer of dissolved oxygen.

3.2.3. Effects of Mineral Salt Sources and their Concentrations on the Biomass of Compound *Bacillus spp.*

It could be seen from Figure 4, among 5 different mineral salt, the promoting effect of K_2HPO_4 on the biomass of compound *Bacillus spp.* was dramatically higher than that of another 4 inorganic salt. So, K_2HPO_4 was selected to serve as inorganic salt of compound *Bacillus spp.* The biomass was much higher when the concentration of K_2HPO_4 was 1 g/L than that of 0.5 g/L and 1.5 g/L. There was no significant difference when K_2HPO_4 was above 2 g/L.

3.2.4. Orthogonal Experiment of Culture Condition Optimization

According to the effects of different concentrations of glucose, bean pulp and K_2HPO_4 on the biomass of compound *Bacillus spp.*, the optimal concentration of the three substances was selected for orthogonal test. It could be seen from the range value of Table 2 that the most important factor affecting the biomass of compound *Bacillus spp.* was glucose (A), followed by bean pulp (B) and K_2HPO_4 (C) had the least influence on the biomass of compound *Bacillus spp.*. From the results of mean value, the best combination was A2B3C1. So, the optimal culture condition was: glucose 20 g/L, bean pulp 30 g/L and $K_2HPO_4 \cdot 3H_2O$ 0.5 g/L. The results of orthogonal test were analyzed by variance analysis (Table 3). And the importance of each factor was judged in the light of F value. The importance of the factors was A: glucose > B: bean pulp > C: K_2HPO_4, which was the same as the range analysis. The production of optimized Bacillus L5 was 128.00×10^8 CFU/mL.

3.3. Optimization of Fermentation Conditions of Compound *Bacillus spp.*

The effects of strain age, inoculum size, initial pH and loading volume on the biomass of compound *Bacillus spp.* at 37˚C were measured (Figure 5). Results indicated that under the conditions of different strain age, the biomass of compound *Bacillus spp.* showed a tendency of rising firstly and falling subsequently. The biomass was evidently higher at 12 h than other periods, demonstrating that compound *Bacillus spp.* was the most vigorous in the logarithmic growth phase

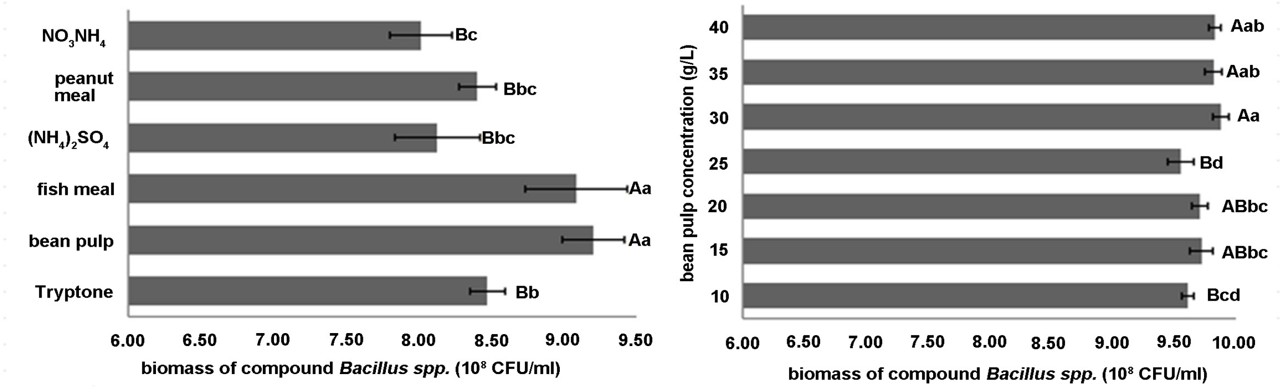

Figure 3. Effects of different nitrogen source and different concentration of soybean meal on the spore yield of compound *Bacillus spp.*

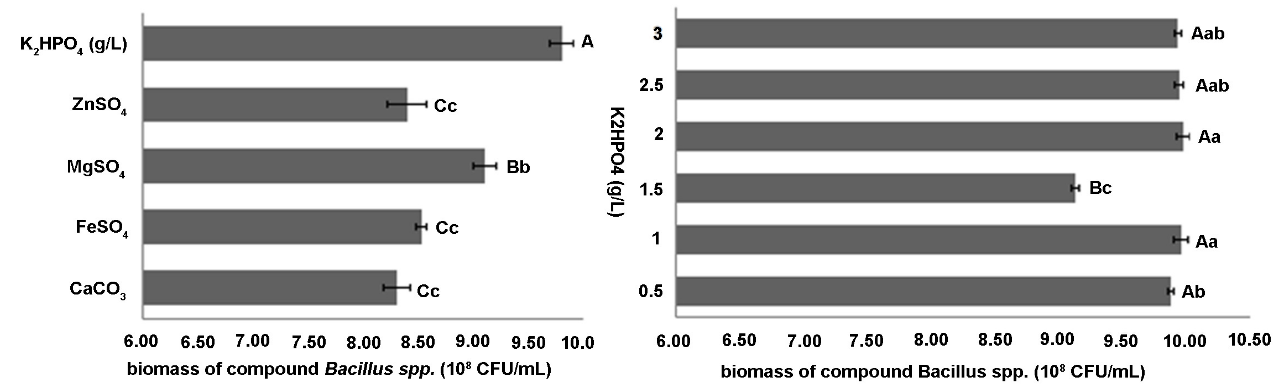

Figure 4. Effects of different mineral salts and different concentration of K_2HPO_4 on the spore yield of compound *Bacillus spp.*

Table 2. Results of orthogonal test of culture conditions.

Numbers	Experimental factors and levels			Biomass of compound *Bacillus spp.* (10^8 CFU/mL)
	A (glucose, g/L)	B (bean pulp, g/L)	C (K_2HPO_4, g/L)	
1	30	40	1.0	57.00
2	30	35	2.0	82.00
3	30	30	2.5	53.00
4	20	40	2.0	96.67
5	20	35	2.5	102.00
6	20	30	1.0	128.00
7	15	40	2.5	71.00
8	15	35	1.0	79.00
9	15	30	2.0	85.00
Mean value 1	64.00	74.89	88.00	Range Analysis
Mean value 2	108.89	87.67	87.89	
Mean value 3	78.33	88.67	75.33	
Range R	44.89	13.78	12.67	

at 12 h. Therefore, in the optimization of fermentation conditions, the orthogonal test of three factors three levels of inoculum size, loading volume and initial pH which have great effect on fermentation were tested apart from temperature and strain age.

Table 3. The variance analysis of orthogonal experiment.

Experiment	Factors	Sum of squares of deviations	Degrees of freedom	Mean square	F	Sig.
Medium optimization orthogonal test	A (glucose)	9462.30	2	4731.15	55.78	0.000
	B (bean pulp)	1062.30	2	531.15	6.26	0.008
	C (K_2HPO_4)	954.30	2	477.15	5.63	0.012
	Deviation	1696.30	20	84.81		
	Total	13175.19	26			
Fermentation condition optimization orthogonal test	A (inoculum size)	13460.40	2	6730.20	18.35	.00
	B (initial pH)	11104.33	2	5552.17	15.14	.00
	C (loading volume)	4293.16	2	2146.58	5.85	.01
	Deviation	7334.82	20	366.74		
	Total	36192.70	27			

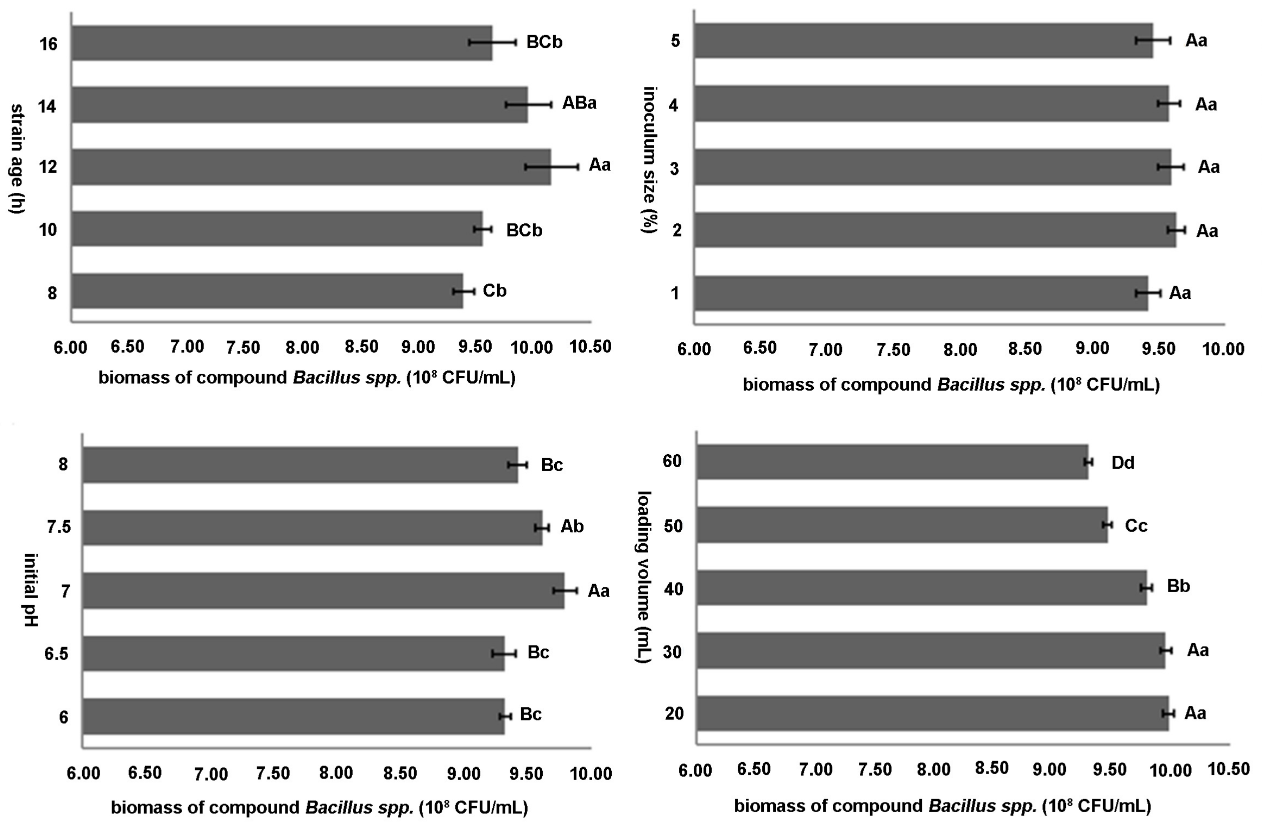

Figure 5. Effects of different fermentation conditions on the spore yield of compound *Bacillus spp.*

The results of orthogonal test were analyzed by range analysis (Table 4) and F value, the significance of all factors on the biomass of compound *Bacillus spp.* was inoculum size ≈ initial pH > loading volume. The combination of optimal fermentation condition was: strain age 12 h, inoculum size 2%, initial pH 7.0 and loading volume 20 mL/250 mL, cultivating for 48 h with 240 r/min at 37˚C. The yield of spores reached 141.33×10^8 CFU/mL, and its formation rate was over 90%, which was 10.24 times higher than the initial medium.

Table 4. Results of orthogonal test of fermentation conditions.

Numbers	Experimental factors and levels			Biomass of antagonistic bacteria (10^8 CFU/mL)
	A (inoculum size, %)	B (initial pH)	C (loading volume, ml/L)	
1	2	7	20	141.33
2	2	7.5	30	80.67
3	2	8	40	30
4	3	7	30	37.33
5	3	7.5	40	36.67
6	3	8	20	30
7	4	7	40	54.33
8	4	7.5	20	38.33
9	4	8	30	24
Mean value 1	84.00	77.66	69.89	Range Analysis
Mean value 2	34.67	51.89	47.33	
Mean value 3	38.89	28.00	40.33	
Range R	49.33	49.66	29.55	

4. Conclusion and Discussion

Effective components in *Bacillus* preparations were spores with marvelous stress resistance. Thus, the ability of spores formation and the yield of spores were of great importance to the applied potentials. Study had shown that the formation of spores was affected by nutrients and environmental factors. It was one of the crucial point to produce high-yield of Bacillus preparations that optimized the liquid fermentation conditions to increase the biomass and spore yield of the cells [6] [7].

In this study, the effects of culture conditions (carbon sources, nitrogen sources, inorganic salts) and fermentation conditions (strain age, inoculums size, loading volume and initial pH) on the yield of compound *Bacillus spp.* were analyzed by single-factor experiment and orthogonal test. Carbon source is one of the main components in microbiological medium, which provides nutrients and energy for microbial anabolism, growth and reproduction. With analysis of different carbon sources, glucose is optimal for the growth of compound *Bacillus spp.* The result was same with Song Kawei who has confirmed that the best carbon source of *Bacillus subtilis* B68 was glucose [8]. Nitrogen source provides substances of basic structure of the microorganism and nitrogen required for metabolism. Besides, it can also serve as energy source when carbon source is insufficiently supplied. Within organic nitrogen sources, there are not only abundant soluble protein, peptide and free amino acids, but also sugars, fats, inorganic salts, vitamins, growth factors and precursors of metabolite synthesis. This study shows that organic nitrogen is more eligible for the growth of compound *Bacillus spp.* Inorganic salts are essential for the growth of microorganisms and the synthesis of metabolites. It promotes the growth and metabolic synthesis of microbes at lower concentrations, while exhibits distinct inhibition at higher concentrations. It proves that $K_2HPO_4 \cdot 3H_2O$ is the better one for the utilization of compound *Bacillus spp.* In addition to the essential nutrients of the microorganisms, the medium should also determine the appropriate percentage between the ingredients to ensure maximum yield. At present, the common medium optimization methods are mainly Orthogonal Design, Plackett-Burman Design, Full Factorials Design, Uniform Design and Response Surface Methods based on single factor level test. Hong Peng (2013) designed orthogonal experiment to acquire the optimal medium based on glucose and yeast extract as carbon and nitrogen sources respectively. As a result, the antibacterial ability of *Bacillus amyloliquefaciens* improved by 37.3% [9] [10]. Xin Jian adopted the method of BBD response surface to optimize the fermentation conditions of *P. aeruginosa* and *P. putida* at the shake flask level. The optimized biomass reached 104.73 × 108 CFU/mL, which was 16.70 times higher than the initial medium [11].

We have optimized culture and fermentation conditions of compound *Bacillus spp.* The medium formula is as follows: glucose 20.0 g/L, bean pulp 30.0 g/L, and $K_2HPO_4 \cdot 3H_2O$ 1.0 g/L; the fermentation conditions: strain age 12 h, inoculums size 2%, temperature 37˚C, initial pH 7.0 and loading volume 20 mL/250 mL. Under optimized fermentation medium and conditions, the spore yield of compound *Bacillus spp.* reached 141.33×10^8 CFU/mL, which was 10.24 times higher than the initial medium, and the formation rate of spores was over 90%. It provides a technical basis for its application in crop planting. The economic cost of fermentation is greatly reduced.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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