Optimizing the Fermentation Conditions and Determining the Disease Control and Growth Promotion Function of *Bacillus Amylolyquefaciens* 3-5

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Abstract

Bacillus spp. are helping to develop towards sustainable agriculture and have become a research hotspot in the field of plant pathology because they have great development potential such as control fungal plant diseases. *Bacillus Amyloliquefaciens* 3-5 was used as antagonistic bacteria in this experiment. The optimal medium for solid-state fermentation of *B. amyloliquefaciens* 3-5 contained bran (35%), rice hull powder (40%), corn gluten (20%), bean flour (15%), corn starch (1.5%), beef extract (2.5%) and MgSO_4 (1.5%), and the optimal fermentation conditions included an inoculum of 6%, a solids content of 36 g/L, a feed-to-water ratio of 1:1, a fermentation temperature of 32 °C, an initial pH of 7.0 and fermentation time of 44 h. When the dosage of the *B. amyloliquefaciens* 3-5 agent was 10%, the thick stems, root length and plant height of cucumber were significantly higher than those of the control ($P \leq 0.05$), and the growth rates were 77.45 %, 35.77 %, and 53.33 %, respectively in pot experiments. Compared with the control, and the preventive effect and therapeutic effect on cucumber *Fusarium* wilt were 72.09 % and 48.83 % by the application of *B. amyloliquefaciens* 3-5 agent—which showed that cucumber *Fusarium* wilt was successfully controlled by a newly isolated strain of *B. Amyloliquefaciens* 3-5. These results suggested that the prevention and control effect of *B. amyloliquefaciens* 3-5 agent on cucumber *Fusarium* wilt could not be underestimated.

1. Introduction

Cucumber, a popular vegetable with young people, is an economically important crop. Vascular wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) is one of the most destructive soil-borne diseases causing major economic losses worldwide (Qiu et al. 2012). Pathogens of the genus *Fusarium* have a particularly wide host range, including potato, tomato, pepper, bean, pea, chickpea, banana, strawberry, cotton, and melon, and are among the most damaging soilborne pathogens in crop production systems. They usually infect plants through the roots, and cause damping-off, root rot, and vascular wilt (Cha et al. 2016).

Chemical control with fungicides has been the most common control strategy for diseases caused by *Fusarium* spp. However, their extensive use has not only posed threat to pathogen resistance and the resurgence of the disease but endangered human health (Feng et al. 2021). Moreover, as a matter of common knowledge that the application of chemical fungicides leads to the selection for resistant strains, which limits their long-term efficacy (Janga et al. 2017). Furthermore, owing to the persistence or survival of *F. oxysporum* in the soil over long periods of time and the wide hosts range of *F. oxysporum* it is so difficult to control this disease using chemical control method (Park et al. 2020).

Therefore, safe and efficient methods for prevention and control of diseases caused by *Fusarium* wilt are badly needed. Biocontrol, as an eco-security, safe and sustainable method of controlling plant infections, represents a promising approach to protect crops from the pathogen (Compant et al. 2005; Haas and Keel. 2003; Hass and Défago. 2005; Lugtenberg and Kamilova. 2009). In particular biocontrol of *Fusarium* wilt by bacterial antagonists has been extensively studied. For example, *B. subtilis* SQR9 was found to control *Fusarium* wilt of cucumber by colonizing plant roots and producing lipopeptides (fengycin and bacillomycin) that are effective against *F. oxysporum* (Cao et al. 2011). *B. velezensis* RC 218 reduced

disease severity of *Fusarium* head blight (FHB) caused by *Fusarium graminearum* and reduced accumulation of associated mycotoxins (Palazzini et al. 2016). “Amfissis” trees treated with *Paenibacillus alvei* K165 had significantly lower disease severity and lower relative AUDPC (Area Under the Disease Progress Curve) values than those of untreated, diseased plants (Markakis et al. 2016). *Streptomyces griseorubens* E44G was found to possess chitinolytic activity and was able to reduce the severity of *Fusarium* wilt of tomato and increase tomato growth and yield (Rashad et al. 2017). Finally, isolates of the fungus *Trichoderma asperellum* which had high levels of chitinase and β -1,3-glucanase activities, strongly inhibited the mycelial growth of *F. oxysporum* f. sp. *lycopersici* (El Komy et al. 2015). As everyone knows, microorganisms are an almost unlimited source of natural products, many of which have potential therapeutic uses. In the context of the current study, the bacterium *B. amyloliquefaciens* has been widely used as a probiotic in the field of biological control that its antibacterial compounds play a vital role in the prevention and control of plant, livestock and poultry diseases (Wang et al, 2020), and it has the advantages of safe, efficiency and green environmental protection.

An increasing number of literature indicated a series of advantages of endophytes. Kang et al. (2007) detailed the growth-promoting characteristics of endophytes, while Kloepper et al. (2004) and Senthilkumar et al. (2007) demonstrated the disease-inhibiting traits of endophytes. The nature of endophytes in strengthening the defence mechanism of crops to various plant diseases was researched upon by Bargabus et al. (2002), Mishra et al. (2006) and Bakker et al. (2007). In the early stage of the laboratory, endophytic strain 3–5 with magnificent antagonistic effect against *Streptomyces galilaeus*, the pathogen of potato scab, was screened by plate confrontation method and identified as *Bacillus amyloliquefaciens*. *B. amyloliquefaciens* 3–5 has good antagonistic effect on many pathogens such as *F. oxysporum* f. sp. *cucumerinum*, *F. solani*, *F. avenaceum*, *F. oxysporum* f. sp. *potato*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *chili*, *F. solani* f. sp. *chili*, *Alternaria solani*, *A. tenuissima* and *Colletotrichum coccodes*, which showed *B. amyloliquefaciens* 3–5 has great application potential. It would be necessary to know “solid-state fermentation” from previous literature before the experiment started, which the level of production through solid-state fermentation depends not only on the performance of the strain itself, but on the provision of suitable fermentation conditions to fully utilize its production capacity (Kubiak et al, 2019). Research on fermentation process optimization is particularly important for its can make full use of the potential of strains, improve production efficiency and reduce production cost (Wang et al, 2018). Therefore, the solid-state fermentation conditions of *B. amyloliquefaciens* 3–5 were optimized, its antagonistic effect on FOC and other plant pathogenic fungi was determined, and its function of disease prevention and growth promotion was demonstrated in this experiment. The microbial agent was evaluated, and its growth promoting effect and disease control effect on cucumber *Fusarium* wilt were discussed. It not only provided the basis for the development of multifunctional *B. amyloliquefaciens* 3–5, but also laid the foundation for the development of preventive microbial agents.

2. Materials And Methods

2.1. Test materials

2.1.1 The cucumber varieties

'Xinjinyan No. 4', produced by Xinxiang seed industry in Henan Province, purchased from the Sandboat seed market in Anning District, Lanzhou City, Gansu Province.

2.1.2 The antagonistic bacteria

B. amyloliquefaciens 3–5 was isolated from commercial healthy potato tubers, and it is now stored in the laboratory of Plant Pathogenic Bacteria and Bacterial Diversity, College of Plant Protection, Gansu Agricultural University.

2.1.3 The test medium

Seed medium : NB medium (beef peptone 3 g / L, yeast extract 3 g / L, lactose 20 g / L, sodium chloride 3 g / L).

NB medium : beef peptone 3 g / L, yeast extract 3 g / L, lactose 20 g / L, sodium chloride 3 g / L, agar 18 g / L.

NA medium : beef extract 5 g / L, peptone 10 g / L, sodium chloride 5 g / L, agar 18 g / L.

PDA plate : potato 200 g / L, glucose 20 g / L, agar 20 g / L.

2.1.4 The test materials and reagents

Carrier (wheat bran, rice bran and raw corn straw), Filler (powdered rice hulls), Matrix carbon source (corn meal, corn starch and sugar beet pulp), Matrix nitrogen source (soybean flour and oil residue), Carbon source (sucrose, maize starch, soluble starch, lactose, maltose and glucose), Nitrogen sources (urea, $(\text{NH}_4)_2\text{SO}_4$, KNO_3 , NH_4Cl , yeast extract and beef extract)and Inorganic salts (NaCl , MgSO_4 , MnSO_4 , FeSO_4 and KH_2PO_4). The above raw materials are all commercially available.

2.2. Test methods

2.2.1 Microorganisms growth conditions

In this study, a functional strain *B. amyloliquefaciens* 3–5 was transferred from – 20°C frozen stocks to NB agar plates at 28°C for pre-culture before being used as inoculum for solid substrate fermentations.

2.2.2 Preparation of seed cultures

To prepare seed cultures, *B. amyloliquefaciens* 3–5 colonies were inoculated from fresh NB plates in 250-mL flasks containing 50 mL of liquid NB medium. The flasks were incubated at 28°C with shaking at 180 rpm for 24 h.

2.2.3 Single matrix screening

In this experiment, bran, corn stalk, rice bran, corn flour, corn starch, soybean powder, oil residue and beet pulp residue were selected as the base medium, and the seed solution was inoculated into 20 g substrate

with the ratio of material to water of 1 : 1 at 10 % (v / m). The seed solution was placed in an incubator at a constant temperature of 28°C, and after fermentation for 36 h, it was removed and dried in an oven at 65°C. 1 g of fermented substrates were mixed with 99 mL of sterilized water containing some beads in a 250-mL flask and stirred for 20 min at room temperature. The number of viable germs (cfu / g) was then counted using the standard dilution plate counting method (Turner and Backman 1991). All experiments were repeated three times, and the optimum substrate combination and hair were selected based on the number of viable bacteria fermentation conditions.

2.2.4 Base matrix optimization

In the selected single substrate, wheat bran and rice husk flour were used as the main material, and corn flour and soybean powder were used as auxiliary materials. According to the mass ratio of main and auxiliary materials, four levels were determined 90 % : 10 %, 80 % : 20 %, 70 % : 30 % and 60 % : 40 % (Table 1). Count the number of viable bacteria after fermentation, and finally determine the best mass ratio of basic substrate.

Table 1
Factors and levels of orthogonal experiment (L16(4⁴))

No,	Combination	Matrix quality(g)			
		Bran	Rice husk	Corn meal	Bean flour
1	B ₁ R ₁ C ₁ B ₁	4.5	4.5	0.5	0.5
2	B ₁ R ₂ C ₂ B ₂	4.5	4	1	1
3	B ₁ R ₃ C ₃ B ₃	4.5	3.5	1.5	1.5
4	B ₁ R ₄ C ₄ B ₄	4.5	3	2	2
5	B ₂ R ₁ C ₂ B ₃	4	4.5	1	1.5
6	B ₂ R ₂ C ₁ B ₄	4	4	0.5	2
7	B ₂ R ₃ C ₄ B ₁	4	3.5	2	0.5
8	B ₂ R ₄ C ₃ B ₂	4	3	1.5	1
9	B ₃ R ₁ C ₃ B ₄	3.5	4.5	1.5	2
10	B ₃ R ₂ C ₄ B ₃	3.5	4	2	1.5
11	B ₃ R ₃ C ₁ B ₂	3.5	3.5	0.5	1
12	B ₃ R ₄ C ₂ B ₁	3.5	3	1	0.5
13	B ₄ R ₁ C ₄ B ₂	3	4.5	2	1
14	B ₄ R ₂ C ₃ B ₁	3	4	1.5	0.5
15	B ₄ R ₃ C ₂ B ₄	3	3.5	1	2
16	B ₄ R ₄ C ₁ B ₃	3	3	0.5	1.5

2.2.5 Carbon source optimization

Six carbon sources (sucrose, corn starch, soluble starch, lactose, maltose, and glucose with a mass of 1 %) were added to the optimal substrate, and fermented in a constant temperature incubator at 28°C. After 36 h, the number of viable cells was determined, and the other assay methods were the same as those in Sect. 2.2.3.

2.2.6 Nitrogen optimization

Six nitrogen sources (urea, (NH₄)₂SO₄, KNO₃, NH₄Cl, yeast extract and beef extract with 0.5 % mass) were added to the optimum substrate and fermented in a constant temperature incubator at 28°C. After 36 h, the

number of viable cells was determined. The other test methods were the same as those described in Sect. 2.2.3.

2.2.7 Optimization of the inorganic salt

Five inorganic salts (NaCl, MgSO₄, MnSO₄, FeSO₄ and KH₂PO₄ with 0.05 % mass) were added to the optimum base medium, and fermented in a constant temperature incubator at 28°C. After 36 h, the number of viable cells was determined, and the other test methods were the same as in Sect. 2.2.3.

2.2.8 Optimum proportion of fermentation substrate components

Carbon sources were adjusted four levels of 1 %, 1.5 %, 2 % and 2.5 % ; nitrogen sources were adjusted to 4 levels of 1 %, 1.5 %, 2 % and 2.5 % ; inorganic salts were adjusted to four levels of 0.5 %, 1 %, 1.5 % and 2 %, and then orthogonal test was performed(Table 2). According to the number of viable cells to determine the optimum ratio of fermentation substrate composition, and other test methods were the same as those in Sect. 2.2.3.

Table 2
Factors and levels of orthogonal experiment (L16(4³))

No.	Combination	Matrix percentage(%)		
		Carbon source	Nitrogen source	Inorganic salt
1	C ₁ N ₁ M ₁	1	1	0.5
2	C ₁ N ₂ M ₂	1	1.5	1
3	C ₁ N ₃ M ₃	1	2	1.5
4	C ₁ N ₄ M ₄	1	2.5	2
5	C ₂ N ₁ M ₂	1.5	1	1
6	C ₂ N ₂ M ₁	1.5	1.5	0.5
7	C ₂ N ₃ M ₄	1.5	2	2
8	C ₂ N ₄ M ₃	1.5	2.5	1.5
9	C ₃ N ₁ M ₃	2	1	1.5
10	C ₃ N ₂ M ₄	2	1.5	2
11	C ₃ N ₃ M ₁	2	2	0.5
12	C ₃ N ₄ M ₂	2	2.5	1
13	C ₄ N ₁ M ₄	2.5	1	2
14	C ₄ N ₂ M ₃	2.5	1.5	1.5
15	C ₄ N ₃ M ₂	2.5	2	1
16	C ₄ N ₄ M ₁	2.5	2.5	0.5

2.2.9 Optimization of the inoculation quantity

In the optimum medium, the seed solutions were added at 6 %, 8 %, 10 %, 12 % and 14 % of the inoculation amount respectively, and fermented in an incubator at a constant temperature of 28°C. After 36 h, the number of viable cells was determined, and other test methods were the same as in Sect. 2.2.3.

2.2.10 Optimization of solid addition

In the optimal matrix, the seed solutions of 5 g, 7 g, 9 g, 11 g and 13 g were added as solids to a 250 mL triangular flask, and fermented in a constant temperature incubator at 28°C. After 36 h, the number of viable cells was determined. The other test methods were the same as in Sect. 2.2.3.

2.2.11 Material-water ratio optimization

In the optimum medium, the seed liquid was added with the ratio of material to water of 1 : 0.6, 1 : 0.8, 1 : 1.0, 1 : 1.2 and 1 : 1.4, respectively, and fermentation was carried out in an incubator with constant temperature at 28°C. After 36 h, the number of viable cells was determined, the other test methods were the same as in Sect. 2.2.3.

2.2.12 Optimization of fermentation temperature

Five temperature levels were set for the optimum substrate at 24°C, 28°C, 32°C, 36°C and 40°C, respectively. After 36 h, the number of viable bacteria was determined, the other test methods were the same as in Sect. 2.2.3.

The above four factors were orthogonally designed as shown in Table 3.

Table 3
Factors and levels of orthogonal experiment (L25(4⁴))

No.	Combination	Levels of conditions			
		Inoculum size(%)	Solid addition(g)	Material water ratio	Fermentation temperature(°C)
1	I ₁ S ₁ M ₁ F ₁	6	5	1: 0.6	24
2	I ₁ S ₂ M ₂ F ₂	6	7	1: 0.8	28
3	I ₁ S ₃ M ₃ F ₃	6	9	1: 1.0	32
4	I ₁ S ₄ M ₄ F ₄	6	11	1: 1.2	36
5	I ₁ S ₅ M ₅ F ₅	6	13	1: 1.4	40
6	I ₂ S ₁ M ₂ F ₃	8	5	1: 0.8	32
7	I ₂ S ₂ M ₃ F ₄	8	7	1: 1.0	36
8	I ₂ S ₃ M ₄ F ₅	8	9	1: 1.2	40
9	I ₂ S ₄ M ₅ F ₁	8	11	1: 1.4	24
10	I ₂ S ₅ M ₁ F ₂	8	13	1: 0.6	28
11	I ₃ S ₁ M ₃ F ₅	10	5	1: 1.0	40
12	I ₃ S ₂ M ₄ F ₁	10	7	1: 1.2	24
13	I ₃ S ₃ M ₅ F ₂	10	9	1: 1.4	28
14	I ₃ S ₄ M ₁ F ₃	10	11	1: 0.6	32
15	I ₃ S ₅ M ₂ F ₄	10	13	1: 0.8	36
16	I ₄ S ₁ M ₄ F ₂	12	5	1: 1.2	28
17	I ₄ S ₂ M ₅ F ₃	12	7	1: 1.4	32
18	I ₄ S ₃ M ₁ F ₄	12	9	1: 0.6	36
19	I ₄ S ₄ M ₂ F ₅	12	11	1: 0.8	40
20	I ₄ S ₅ M ₃ F ₁	12	13	1: 1.0	24
21	I ₅ S ₁ M ₅ F ₄	14	5	1: 1.4	36
22	I ₅ S ₂ M ₁ F ₅	14	7	1: 0.6	40

No.	Combination	Levels of conditions			
		Inoculum size(%)	Solid addition(g)	Material water ratio	Fermentation temperature(°C)
23	I ₅ S ₃ M ₂ F ₁	14	9	1: 0.8	24
24	I ₅ S ₄ M ₃ F ₂	14	11	1: 1.0	28
25	I ₅ S ₅ M ₄ F ₃	14	13	1: 1.2	32

2.2.13 Optimization of initial pH of fermentation

In the optimal medium, 13 initial fermentation pH values were set at 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10. After 36 h of fermentation, the number of viable cells was determined, the other test methods were the same as in Sect. 2.2.3.

2.2.14 Optimization of fermentation time

Under the conditions of optimum inoculation amount, solid loading amount, solid-liquid ratio, temperature and pH, the fermentation time was adjusted to 8 levels of 20 h, 24 h, 28 h, 32 h, 36 h, 40 h, 44 h and 48 h, and the number of viable cells was measured every 4 h to determine the optimum fermentation time.

2.2.15 Determination of the growth promoting effect of *B. amyloliquefaciens* 3–5 on cucumber

The substrate soil (provided by Gansu Lvneng Agricultural Science and Technology Co., Ltd.) and vermiculite were thoroughly mixed at a ratio of 1:1 (w/w) after autoclaved at 121°C for 2 h, and supplemented with fertilizers to which 3–5 bacteriocin was added according to the mass ratio of bacteriocin to culture medium (the mixture of substrate soil and vermiculite) of 0.25%, 0.5%, 1.0%, 5.0%, 10.0% and 20.0%. The soil (200 g) was distributed in plastic pots ($\Phi = 12$ cm), and moisture was maintained at 90% of the maximum water-holding capacity of the soil by daily addition of sterilized water. After mixing, it was placed in a 12 cm × 12 cm plastic flower pot (200 g per pot), without *B. amyloliquefaciens* 3–5 agent as a control. Cucumber seeds with full grains were selected for germination (soaked at 45°C for 2 hours) and planted in each pot (four plants per pot) after germination. When the fourth true leaf of the cucumber seedlings had grown out, the fresh weight (g), dry weight (g), stem thickness (cm), root length (cm) and plant height (cm) of each plant were measured (after being killed in 105°C oven and dried at 70°C to constant weight). This experiment was repeated three times.

2.2.16 Determination of the control effect of *B. amyloliquefaciens* 3–5 on cucumber fusarium wilt

Disease suppression experiments on tomato seedlings were conducted as previously reported (Asaka and Shoda 1996; Mizumoto et al. 2006). The healthy cucumber seeds with equal growth vigor were planted in a plastic flower pot ($\Phi = 12$ cm) containing a nutrient soil with the dosage of the microbial agent was 10.0 % (nutrient soil was mixed with vermiculite at a ratio of 1 : 1). When the cucumber seedlings had grown out of the fourth true leaf, the experiment was conducted.

(1) Prevention experiment : The soil on the surface of the flower pot was plucked off with a sterile dissector, and the bacterial agent which constituted 10.0 % of the mass of the nutrient soil in the flower pot was applied to the surrounding plants. The bacterial agent was covered with the previously plucked soil. After 3 days, the soil was inoculated and pricked 10 cm from the stem base of the plant, and the spore suspension of FOC with the concentration of 10^7 cfu / mL was dipped with defatted cotton and spread evenly on the microinjury site.

(2) Treatment test : 10 cm from the stem base of the plant, a pinprick injury was inoculated and the spore suspension of FOC with a concentration of 10^7 cfu / mL was inoculated with defatted cotton. After 3 days, 10.0 % of nutrient soil was applied around the plant in the flower pot and the soil was covered with the previously released bacterial agent.

(3) Control : The spore suspension of FOC containing 10^7 cfu / mL was used as control.

Each treatment set 10 seedlings, repeated three times. After inoculation, humidification with preservative film for 48 hours, and the incidence of cucumber wilt was studied after 15 days, and the disease index and control effect of 3–5 on cucumber wilt were calculated.

The incidence and severity of wilt were scored on a 0–5 scale; 0 = healthy, 1 = 1–3 leaves curled and yellowed, 2 = > 3 leaves curled and deformed, 3 = chlorosis and early wilting of the plant, 4 = necrosis and wilting of the entire plant and 5 = dead or almost dead (Sanogo et al., 2016). Disease incidence (DI) was calculated as the percentage of infected plants to the total number of plants in each block and was assessed when disease occurred (> 20% of leaves wilted).

$$DI = \sum (\text{number of diseased plants} \times \text{representative grade}) / (\text{total number of investigated plants} \times \text{highest representative grade}) \times 100 ;$$

$$\text{Control effect (\%)} = (\text{control disease index} - \text{treatment disease index}) / \text{control disease index} \times 100.$$

2.2.17 Statistical analysis

Three independent experiments were performed to check the reproducibility of results. Data obtained were expressed as the mean $\pm$ standard deviation (SD) and analyzed statistically using the computer software

package SPSS 24.0 program. Multiple comparisons were performed on the data using the one-way ANOVA program, with 0.05 set as the threshold significance level for Duncan's test (Li et al., 2010).

3. Results

3.1 Matrix optimization

3.1.1 Single Matrix Screening

To explore the effect of the feeding ingredients on the fermentation, we have chosen three kinds of carriers, carbon sources and nitrogen sources respectively. As shown in the Fig. 1, in the single substrate base medium with bran as carrier, the number of viable cells of *B. amyloliquefaciens* 3–5 was the highest, which was 31.25×10^{10} cfu / g, which was significantly higher than that of the carrier rice bran and corn straw. In the single substrate base medium with corn flour as carbon source, the number of viable cells was the greatest (8.65×10^{10} cfu / g), which was significantly higher than that of corn starch and sugar beet residue. In the single substrate medium with soybean powder as nitrogen source, the number of viable bacteria was the greatest at 4×10^{10} cfu / g, which was significantly higher than that of the nitrogen source oil residue. Therefore, wheat bran as carrier, corn flour as carbon source and soybean flour as nitrogen source were the best substrate combination for basic medium of *B. amyloliquefaciens* 3–5.

3.1.2 Base matrix optimization

Following optimization the base matrix, our results showed that at aratio of bran : rice husk powder : corn flour : soybean powder of 3.5 : 4 : 2 : 1.5, the number of viable cells of strain 3–5, was the highest, was 9.97×10^{12} cfu / g, which was significantly higher than that of the other composite substrate combinations, indicated that the optimum combination for solid-state fermentation of *B. amyloliquefaciens* 3–5 were 35 % bran, 40 % rice husk powder, 20 % corn flour and 15 % soybean powder, respectively (Table 4).

Table 4
Screening of strain 3–5 composite matrix

No.	Combination	Matrix quality(g)				Viable bacteria counts ($\times 10^{12}$ cfu/g)
		Bran	Rice husk	Corn meal	Bean flour	
1	B ₁ R ₁ C ₁ B ₁	4.5	4.5	0.5	0.5	2.192
2	B ₁ R ₂ C ₂ B ₂	4.5	4	1	1	2.089
3	B ₁ R ₃ C ₃ B ₃	4.5	3.5	1.5	1.5	1.905
4	B ₁ R ₄ C ₄ B ₄	4.5	3	2	2	1.080
5	B ₂ R ₁ C ₂ B ₃	4	4.5	1	1.5	0.762
6	B ₂ R ₂ C ₁ B ₄	4	4	0.5	2	1.064
7	B ₂ R ₃ C ₄ B ₁	4	3.5	2	0.5	0.522
8	B ₂ R ₄ C ₃ B ₂	4	3	1.5	1	1.000
9	B ₃ R ₁ C ₃ B ₄	3.5	4.5	1.5	2	1.445
10	B ₃ R ₂ C ₄ B ₃	3.5	4	2	1.5	9.970
11	B ₃ R ₃ C ₁ B ₂	3.5	3.5	0.5	1	1.502
12	B ₃ R ₄ C ₂ B ₁	3.5	3	1	0.5	1.772
13	B ₄ R ₁ C ₄ B ₂	3	4.5	2	1	3.115
14	B ₄ R ₂ C ₃ B ₁	3	4	1.5	0.5	0.814
15	B ₄ R ₃ C ₂ B ₄	3	3.5	1	2	1.609
16	B ₄ R ₄ C ₁ B ₃	3	3	0.5	1.5	0.994
K1		5.449	5.635	4.313	3.974	
K2		2.510	10.451	4.673	5.779	
K3		11.016	5.420	3.873	10.110	
K4		4.898	3.634	11.015	3.899	

Note: K1 represents the average value of the four tests at the first level of each factor; K2 represents the second level of each factor and the mean value of the four tests; K3 represents the average value of the third level four tests of each factor; K4 represents the fourth level of each factor and the average of the four trials.

No.	Combination	Matrix quality(g)				Viable bacteria counts ($\times 10^{12}$ cfu/g)
		Bran	Rice husk	Corn meal	Bean flour	
Best organization		K3	K2	K4	K3	
Note: K1 represents the average value of the four tests at the first level of each factor; K2 represents the second level of each factor and the mean value of the four tests; K3 represents the average value of the third level four tests of each factor; K4 represents the fourth level of each factor and the average of the four trials.						

3.2 Optimization of solid fermentation substrate

3.2.1 Carbon source optimization

For the different carbon sources that we tested, our results showed that *B. amyloliquefaciens* 3–5 had the highest number of viable cells (2.48×10^{13} cfu / g) in the solid matrix with corn starch as carbon source which was significantly higher than that of other carbon sources (Fig. 2).

3.2.2 Nitrogen source optimization

The influence of different nitrogen sources on the visible counts was shown in the Fig. 3. The visible number of the strain in the solid matrix with yeast extract as nitrogen source was 0.87×10^{13} cfu / g ; In the solid matrix with beef extract as nitrogen source, the number of viable cells of strain 3–5 was the largest, which was 1.21×10^{13} cfu / g. In conclusion, the viable count of beef extract with nitrogen source was significantly higher than the visible counts of urea, $(\text{NH}_4)_2\text{SO}_4$, KNO_3 , NH_4Cl and yeast extract with nitrogen source.

3.2.3 Inorganic salt optimization

The suitable inorganic salt was critical for the viable cells growth because of it is a useful ingredient in media for the cultivation microorganisms. As shown in the Fig. 4, the number of viable cells of strain 3–5 was the largest when in the solid medium with MgSO_4 as inorganic salt, was 2.13×10^{13} cfu / g, which was significantly higher than that of NaCl , MnSO_4 , FeSO_4 and KH_2PO_4 .

Table 5
Screening of carbon source, nitrogen source and inorganic salt

No.	Combination	Matrix percentage(%)			Viable bacteria counts ($\times 10^{13}$ cfu/g)
		Carbon source	Nitrogen source	Inorganic salt	
1	C ₁ N ₁ M ₁	1	1	0.5	0.023
2	C ₁ N ₂ M ₂	1	1.5	1	0.146
3	C ₁ N ₃ M ₃	1	2	1.5	0.423
4	C ₁ N ₄ M ₄	1	2.5	2	0.247
5	C ₂ N ₁ M ₂	1.5	1	1	2.345
6	C ₂ N ₂ M ₁	1.5	1.5	0.5	0.955
7	C ₂ N ₃ M ₄	1.5	2	2	1.243
8	C ₂ N ₄ M ₃	1.5	2.5	1.5	2.415
9	C ₃ N ₁ M ₃	2	1	1.5	0.413
10	C ₃ N ₂ M ₄	2	1.5	2	1.538
11	C ₃ N ₃ M ₁	2	2	0.5	1.507
12	C ₃ N ₄ M ₂	2	2.5	1	2.407
13	C ₄ N ₁ M ₄	2.5	1	2	0.375
14	C ₄ N ₂ M ₃	2.5	1.5	1.5	0.317
15	C ₄ N ₃ M ₂	2.5	2	1	0.867
16	C ₄ N ₄ M ₁	2.5	2.5	0.5	0.483
K1		0.210	0.790	0.740	
K2		1.740	0.740	0.890	
K3		1.470	1.010	1.440	
K4		0.510	1.390	0.850	
Note: k1 represents the average value of the four tests at the first level of each factor; K2 represents the second level of each factor and the mean value of the four tests; K3 represents the average value of the third level four tests of each factor; K4 represents the fourth level of each factor and the average of the four trials.					

No.	Combination	Matrix percentage(%)			Viable bacteria counts ($\times 10^{13}$ cfu/g)
		Carbon source	Nitrogen source	Inorganic salt	
Best organization		K2	K4	K3	
Note: k1 represents the average value of the four tests at the first level of each factor; K2 represents the second level of each factor and the mean value of the four tests; K3 represents the average value of the third level four tests of each factor; K4 represents the fourth level of each factor and the average of the four trials.					

Results of optimization of parameters mentioned in the Table 5 depicted that the number of visible bacteria of strain 3–5 was highest when corn starch : beef extract : magnesium sulfate = 1.5 : 2.5 : 1.5, with 2.415×10^{13} cfu / g. In summary, corn starch with 1.5 % carbon source, beef extract with 2.5 % nitrogen source and magnesium sulfate with 1.5 % inorganic salt were chosen as the optimum carbon, nitrogen and inorganic salt combination in solid matrix of *B. amyloliquefaciens* 3–5.

3.2.4 Optimization of fermentation conditions

As we expected, when the inoculation amount was 6 %, the solid-solid addition amount was 36 g / L, the solid-water ratio was 1 : 1 and the temperature was 32°C, the viable count of *B. amyloliquefaciens* 3–5 was the largest, which was 4.545×10^{13} cfu / g, which was significantly higher than that of other combinations. The results showed that the optimum fermentation conditions for strain 3–5 were as follows : a 6 % inoculation rate, a solid addition rate of 36 g / L, solid-liquid ratio of 1 : 1 and temperature of 32°C (Table 6).

Table 6
Optimization of fermentation conditions

No.	Combination	Levels of conditions				Viable bacteria counts ($\times 10^{13}$ cfu/g)
		Inoculum size(%)	Solid addition(g)	Material water ratio	Fermentation temperature($^{\circ}$ C)	
1	I ₁ S ₁ M ₁ F ₁	6	5	1:0.6	24	1.330
2	I ₁ S ₂ M ₂ F ₂	6	7	1:0.8	28	1.885
3	I ₁ S ₃ M ₃ F ₃	6	9	1:1.0	32	4.545
4	I ₁ S ₄ M ₄ F ₄	6	11	1:1.2	36	0.560
5	I ₁ S ₅ M ₅ F ₅	6	13	1:1.4	40	1.055
6	I ₂ S ₁ M ₂ F ₃	8	5	1:0.8	32	4.433
7	I ₂ S ₂ M ₃ F ₄	8	7	1:1.0	36	0.760
8	I ₂ S ₃ M ₄ F ₅	8	9	1:1.2	40	0.668
9	I ₂ S ₄ M ₅ F ₁	8	11	1:1.4	24	1.237
10	I ₂ S ₅ M ₁ F ₂	8	13	1:0.6	28	0.175
11	I ₃ S ₁ M ₃ F ₅	10	5	1:1.0	40	1.100
12	I ₃ S ₂ M ₄ F	10	7	1:1.2	24	0.933
13	I ₃ S ₃ M ₅ F ₂	10	9	1:1.4	28	0.453
14	I ₃ S ₄ M ₁ F ₃	10	11	1:0.6	32	0.078
15	I ₃ S ₅ M ₂ F ₄	10	13	1:0.8	36	0.172
16	I ₄ S ₁ M ₄ F ₂	12	5	1:1.2	28	0.702
17	I ₄ S ₂ M ₅ F ₃	12	7	1:1.4	32	0.698
18	I ₄ S ₃ M ₁ F ₄	12	9	1:0.6	36	0.270

Note: k1 represents the first level of each factor and the mean value of the five tests; K2 represents the second level of each factor and the mean value of the five trials; K3 represents the third level of each factor and the average value of the five trials; K4 represents the fourth level of each factor and the average value of the five trials; K5 represents the fifth level of each factor and the average of the five trials.

No.	Combination	Levels of conditions				Viable bacteria counts ($\times 10^{13}$ cfu/g)
		Inoculum size(%)	Solid addition(g)	Material water ratio	Fermentation temperature($^{\circ}$ C)	
19	I ₄ S ₄ M ₂ F ₅	12	11	1:0.8	40	2.913
20	I ₄ S ₅ M ₃ F ₁	12	13	1:1.0	24	2.737
21	I ₅ S ₁ M ₅ F ₄	14	5	1:1.4	36	0.248
22	I ₅ S ₂ M ₁ F ₅	14	7	1:0.6	40	0.328
23	I ₅ S ₃ M ₂ F ₁	14	9	1:0.8	24	2.567
24	I ₅ S ₄ M ₃ F ₂	14	11	1:1.0	28	3.140
25	I ₅ S ₅ M ₄ F ₃	14	13	1:1.2	32	0.485
K1		1.878	1.543	0.437	1.760	
K2		1.435	0.924	2.377	1.274	
K3		0.912	1.701	2.456	2.028	
K4		1.463	1.586	0.670	0.403	
K5		1.354	0.826	0.739	1.214	
Best organization		K1	K3	K3	K3	
Note: k1 represents the first level of each factor and the mean value of the five tests; K2 represents the second level of each factor and the mean value of the five trials; K3 represents the third level of each factor and the average value of the five trials; K4 represents the fourth level of each factor and the average value of the five trials; K5 represents the fifth level of each factor and the average of the five trials.						

3.2.5 Optimization of initial pH of fermentation

The pH value was an essential parameter to ensure the normal reproduction and metabolism of microorganisms, having great influence on fermentation. As shown in the Fig. 5, the number of viable cells of strain 3–5 increased with the initial pH of fermentation. When the pH value was 4.5, the viable count grew to reach a stable phase and the viable count grew at a faster speed when the pH value was 6; the number of viable cells, reached a maximum of 4.568×10^{13} cfu / g, was significantly higher than that at other pH values when the initial pH of fermentation was 7. When the pH value was between 7.5 and 10, the number of viable count was obviously decreased. Therefore, the initial pH value of fermentation was 7, which was the optimal initial pH of the strain.

3.2.6 Optimization of fermentation time

The fermentation time also was one of the key of parameters. As shown in the Fig. 6, the number of viable cells of strain 3–5 changed as fermentation time increased. The number of viable bacteria was approach 0 when the fermentation time was less than 36 h. When the fermentation time was 40 h, the viable count was dramatic increase. Until the fermentation time was 44 h, the number of viable bacteria was 2.835×10^{12} cfu / g, which was significantly higher than that of other fermentation times, and then significantly decreased. Obviously, 44 h was the strain's best fermentation time.

3.3 Determination of the growth promoting effect of *B. amyloliquefaciens* 3–5 on cucumber

The results of the pot control showed that the growth of cucumber plants after inoculation with different concentrations of biocontrol bacteria 3–5 differed significantly from the control. Cucumber plant height and underground dry weight were significantly higher than those of the control ($P < 0.05$) when the dosage of the microbial agent was 5 % ~ 10 %, and the maximum plant height and underground dry weight were 26.05 cm and 0.22 g when the dosage of the microbial agent was 10 %. When the dosage of the microbial agent was 10 %, the root length was 23.58 cm, which was significantly higher than other treatments and control groups. When the dosage of the microbial agent was 0.25 % – 20 %, the aboveground fresh weight and stem diameter of the plant were better than those of the control group, and the data showed that the aboveground fresh weight and stem diameter of the plant increased with dosage. When the dosage of the microbial agent was 10 %, the growth promotion effect was significant, and the aboveground fresh weight of the plant was 3.55 g, with 0.23 cm thick stems (Table 7).

Table 7
Effect of 3–5 on the growth of cucumber plants

Growth parameter	Inoculant dosage						CK
	0.25%	0.5%	1%	5%	10%	20%	
Plant fresh weight (g)	1.41 ± 0.053c	1.32 ± 0.078c	1.63 ± 0.105c	2.41 ± 0.086b	3.55 ± 0.591a	2.70 ± 0.226b	1.13 ± 0.082c
Plant dry weight (g)	0.12 ± 0.006b	0.10 ± 0.010b	0.13 ± 0.009b	0.16 ± 0.010b	0.22 ± 0.047a	0.15 ± 0.008b	0.13 ± 0.010b
Thick stems (cm)	0.16 ± 0.020bc	0.19 ± 0.015abc	0.20 ± 0.002ab	0.21 ± 0.024ab	0.23 ± 0.017a	0.18 ± 0.019abc	0.14 ± 0.021c
Root length(cm)	18.10 ± 1.320ab	13.12 ± 0.875b	16.78 ± 0.650ab	18.77 ± 1.285ab	23.58 ± 5.128a	18.45 ± 0.801ab	16.79 ± 0.650ab
Plant height (cm)	13.78 ± 0.320cd	13.02 ± 0.509d	15.38 ± 0.614c	21.37 ± 0.634b	26.05 ± 1.045a	21.15 ± 1.014b	13.20 ± 0.442d
Note: Different lowercase letters in the same column mean significantly different at p<0.05 level with Duncan's new multiple range least.							

3.4 Determination of the control effect of *B. amyloliquefaciens* 3–5 on cucumber fusarium wilt

The results of pot control showed that the disease index of prevention and treatment was significantly lower in the pot control group than in the control group. The treatment effect was 48.83 %, and the prevention effect was 72.09 %.The prevention effect overperformed the treatment effect (Table 8).

Table 8
Control effect of 3–5 on cucumber Fusarium wilt in pot experiment

Treatment	Disease index /%	Control effect /%
CK	71.67	–
Preventive effect	20.00	72.09
Therapeutic effect	36.67	48.83

4. Discussion

Modern chemical fungicides were less harmful than those of older generations, but agrochemicals in general still bring environmental harm such as health, environmental and ecological problems, severe toxicity, accumulation in the food chain and long degradation times (Vandenberghe et al. 2021). Thus, the development of environmentally friendly alternatives has been encouraged over the years. Bioproducts with low environmental impact and multiple mechanisms of action have emerged, and they can be of plant origin such as essential oils which have shown antifungal activities, among others (Vandenberghe et al. 2021), or they can be antagonistic living organisms (Leiter et al. 2017) as well as their natural metabolites. These bioproducts with antifungal activities are called biofungicides, and they can be produced in several ways.

Solid-state fermentation (SSF) has enormous potential because of its lower production investment, shorter fermentation time and higher yield of secondary metabolites (Vandenberghe et al. 2021). It was eported that the differences in physiological changes mediated by submerged fermentation (SmF) and SSF can cause differences in microbial molecular behavior that affect product formation and yield (Barrios-González et al. 2008). Compared to SmF, SSF offers the advantages of lower energy consumption, lower cost, and simpler techniques (Zhang et al. 2014). Whatever the fermentation technique, high spore yields were often achieved based on careful experimental designs for industrial exploitation. A considerable increase in the production of alpha-amylase by *B. amyloliquefaciens* in flask fermentation using statistical methods was reported by Zhao et al. (2011). The substrate used in the present study, i.e. wheat bran, was reported to be potent substrate for the production of alpha-amylase under SSF (Ramachandran et al. 2004). Therefore, the development of low-cost and highly efficient technologies for the production of spore-forming probiotics became necessary. It was generally accepted that SSF has several biotechnological advantages; it was a simple and low-investment process as it required low energy consumption, cheap plant raw materials can be used as growth substrates, and after SSF, the product can be lyophilized directly without centrifugation.

It also provides higher productivity of fermentation and higher concentration of products (Holker et al. 2004).

The development of efficient technologies for the production of spore-forming bacteria requires understanding of the physiological mechanisms that regulate *Bacilli* growth and sporulation, as well as elucidation of the conditions that favor both processes. Sporulation of *Bacillus* spp. depends on medium composition, pH, aeration, temperature, and other factors (Ren et al. 2018; Posada-Urbe et al. 2015). Limited information is available in the literature on the effect of lignocellulosic substrates on sporulation of bacilli. Among the four growth substrates tested for submerged fermentation of *Bacillus* spp. KKU02 and KKU03, cassava root supported sporulation better than basal nutrient broth (Wangka-Orm et al. 2014). These strains formed 8.32×10^8 spores ml^{-1} and 1.35×10^8 spores ml^{-1} , respectively, of an optimal cassava concentration of 100 g L^{-1} . A combination of tapioca with lactose in a nutrient medium for submerged cultivation of *B. amyloliquefaciens* subsp. B128 resulted in a spore yield of 5.92×10^8 spores ml^{-1} (Posada-Urbe et al. 2015). The reasonable way to increase spore production is to create optimal cultivation conditions that promote maximum vegetative cell accumulation followed by effective sporulation. One of the valid approaches is to supplement the culture medium with a suitable nitrogen source at the optimal concentration. To achieve this goal, several abundantly available agro-industrial by-products were tested. Among these growth substrates, bran followed by rice bran and corn straw, provided particularly high yields of spores. These results suggest that a distinctive feature of *B. amyloliquefaciens* 3-5 is its ability to use various low-cost lignocellulosic materials as growth substrates for high-yield spore production. Similar results were reported for submerged cultivation of *B. subtilis* where ammonium sulfate (4.54%) in combination with corn flour (1.2%) resulted in maximum spore production (Khardziani et al. 2017). Furthermore, using a response surface method, Rao et al. (2007) optimized the composition of the medium for maximum spore production of *B. amyloliquefaciens* subsp. B128 and showed that a mixture of $(\text{NH}_4)_2\text{SO}_4$ and peptone gave the highest yield of spores at concentrations of 1.8 and 8.0 g L^{-1} , respectively. In this study, we attempted to reduce the cost of probiotics production by using low cost raw materials as medium components by optimizing of the medium for significant increase in spore yield.

Recently, several studies have investigated the antagonistic activity of different bacterial strains on phytopathogenic fungi in postharvest fruits, so their use can be considered as an alternative in controlling fungal diseases by chemical methods (Ma et al. 2019). *B. amyloliquefaciens* 3-5 as an endophytic bacterium with good biocontrol potential. In this study, its fixed fermentation conditions were optimized. The results showed that wheat bran was the carrier, corn flour was the carbon source and soybean meal was the nitrogen source. 35% bran, 40% rice husk meal, 20% corn meal and 15% soybean meal were selected as the composite matrix for solid-state fermentation of strain 3-5. The best carbon source was 1.5% corn starch, 2.5% beef paste and 1.5% magnesium sulfate. The optimal fermentation conditions were 6% inoculation, 36 g/L solid addition, 1:1 ratio of material to water, 32°C temperature, pH7 and 44 h fermentation time. The results of this study provided the basis for the production of the bactericide of *B. amyloliquefaciens* 3-5, and also provided the Micrabiace resources for the biological control of cucumber *Fusarium* wilt.

It is widely known that *Fusarium* wilt, as a destructive and economic disease, is very difficult to control by classical methods because there is no effective chemical treatments. In addition, it is not practical to control the soil-borne diseases with chemicals, biological control is a realistic approach for the management of *Fusarium* wilt. For all these reasons, there is an urgent need for plant disease control in agriculture.

In this research, *B. amyloliquefaciens* 3-5 showed that preventive effect on cucumber *Fusarium* wilt and therapeutic effect was 72.09 % and 48.83 % by applying *B. amyloliquefaciens* 3-5 agent by pot culture experiments under greenhouse conditions. *B. amyloliquefaciens* 3-5 agent developed in this laboratory significantly reduced the incidence and severity index of cucumber *Fusarium* wilt and can partially reduce the economic losses. Thus, *B. amyloliquefaciens* 3-5 agent can effectively control cucumber *Fusarium* wilt. Biocontrol agents developed by antagonistic strains do not pollute the environment, which is beneficial for maintaining the balance of the ecosystem and promoting agriculture. At present, this experiment only studied the development of *B. amyloliquefaciens* 3-5 indoor research for disease prevention, growth promotion. However, the application of *B. amyloliquefaciens* 3-5 agent in the field needs further investigation.

Abbreviations

FOC: *Fusarium oxysporum* f. sp. *cucumerinum*

DI: Disease incidence

SD: Standard deviation

SSF: Solid-state fermentation

SmF: Liquid fermentation

Declarations

Ethics Approval and Consent to Participate

Not applied.

Consent for Publication

Not applied.

Availability of Data and Materials

Data and materials available on request from the authors. The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

All authors declare that there is no conflict of competing interests in this original article.

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Authors' Contributions

All authors contributed to the study conception and design. Ting Ma conceived and designed research. Chengde Yang wrote the reviews. Ting Ma and Fengfeng Cai conducted experiments. Lingxiao Cui contributed new reagents. Ting Ma and Yidan Wang analyzed data. Ting Ma wrote the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

Single substrate screening for solid fermentation of strain 3-5 Note: Different lowercase letters in the fig mean significantly different at $p \leq 0.05$ level with Duncan's new multiple range least, the same below.

Figure 2

The effect of carbon source on the number of viable bacteria

Figure 3

Effect of nitrogen source on the number of viable bacteria

Figure 4

Effects of inorganic salts on the number of viable bacteria

Figure 5

Effect of initial pH on the number of viable bacteria

Figure 6

Effect of fermentation time on the number of viable bacteria