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Successful Formulation and Application of Low-Temperature Bacterial Agents for Corn Stover Degradation

Sainan Zhang [1,†], Shengcai Han [1,2,3,†], Xiaofang Yu [1,2], Julin Gao [1,2,*], Shuping Hu [1,2], Naoganchaolu Borjigin [1,2], Qinggeer Borjigin [1,2], Jiangan Guo [1], Jianfei Bai [1], Bizhou Zhang [2], Zhiyuan Huang [2] and Yong Lei [2]

[1] College of Agriculture, Inner Mongolia Agricultural University, Hohhot 010019, China
[2] Key Laboratory of Crop Cultivation and Genetic Improvement of Inner Mongolia Autonomous Region, Hohhot 010019, China
[3] College of Horticulture and Plant Protection, Inner Mongolia Agricultural University, Hohhot 010011, China
* Correspondence: nmgaojulin@imau.edu.cn; Tel.: +86-13294815858; Fax: +86-(0471)-431-8142
† These authors contributed equally to this work.

Abstract: Solid bacterial agents are required to accelerate stover degradation in low-temperature areas. However, the laboratory-to-practice translation of bioprocessing techniques is hindered by high cost, poor practicality, and short shelf life. Using corn stover powder, starch, and bran as additives, we screened *Pseudomonas putida* and *Acinetobacter lwoffii*, which effectively degrades corn stover at low temperatures, to develop a sustainable and low-cost bacterial agent formula that ensures bacterial viability in low-temperature soil and storage. The optimal formulation included precipitates and additives at a 1:4 ratio, including corn stover powder, starch, and bran at a 4:3:9 ratio. The viable bacterial count with this formulation reached 7.5×10^{10} colony-forming units/g, with high lignocellulase activities. The degradation effect of the optimal formulation on stover and its components, in both lab soil culture simulation and the field environment, was significantly higher than that without bacterial agent application. This formulation had an outstanding effect on lignin. The optimal storage conditions included vacuum packing under 10% water content at 4 °C; the survival rate of viable bacteria reached 85.33% after 180 d. Given the global value of stover-return agriculture, our results offer a valuable strategy for application in low-temperature soils where stover degradation rates are otherwise low.

Keywords: corn stover; degradation; bacterial agents; formulation; storage

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1. Introduction

The advantages of decomposing corn residues ('stover return') in situ include improved soil fertility, increased soil organic carbon storage, and improved soil microbial environment [1,2]. We found that after the autumn harvest (1 October to 9 November) for 3 consecutive years (2017–2019), the average soil temperature of an experimental field representative of the soil in northern China (Chilechuan Modern Agricultural Expo Park, Baotou, Inner Mongolia, China) was 15 °C. Based on this finding, the soil temperature after the autumn harvest in North China can be considered to be 15 °C, with little annual change. Consequently, stover returned to the field cannot be fully degraded before sowing in the next spring, resulting in a series of problems, such as impaired agricultural operations and decreased crop emergence rates [3]. Solidified bacterial agents are powder or granule preparations comprising bacterial strains with biocompatible solid additives, which provide effective adsorption and protection of the strains during expansion, fermentation, and drying). Solidified bacterial agents that can efficiently degrade corn stover at low temperatures have become the preferred method of in situ stover return to ensure the efficient utilization of resources in low-temperature areas [4,5].

Solid compound bacterial agents, which are two or more compatible bacterial strains used to make solid bacterial agents, are associated with stable application effects, easy

storage, and easy transportation when compared to those in bacterial agents prepared in liquid media [6]. The process of preparing bacterial agents is a multi-step, combined process that includes various fermentation, formulation, and storage techniques. Therefore, strict quality control at each stage is critical for successful bacterial agent research, application, and production [7,8]. Formulation research is crucial for the successful preparation of bacterial agents and considerably influences their target effects and subsequent storage. The formulation of bacterial agents mainly includes the selection and compounding ratio of an additive (e.g., adsorbent and protective agent) [9]. The additive should be non-toxic, stable, and able to provide specific substrates to maintain strain vitality [10,11]. Additionally, additives must be environmentally friendly, easily accessible, and cost-effective for on-field applications [12]. A variety of additives can effectively improve the efficacy of bacterial agents; however, they can only achieve high biocompatibility at specific ratios [13]. The ideal bacterial agent formulation should provide a favorable microenvironment for the growth and survival of bacterial strains [14]. However, due to the current inappropriate type or amount of additives used, bacterial strain activity is not protected effectively, resulting in the inability of the bacterial agent to achieve the expected degradation effect in the complex soil environment. In addition, an inappropriate formulation can also lead to a sharp decline in the number of viable bacteria during storage, which limits the practical application of the bacterial agent [15]. Therefore, research on low-temperature active bacteria is needed to improve the corn stover degradation efficacy of bacterial agents at low temperatures, and formulations need to be developed to ensure their viability and activity.

In this study, we aimed to screen bacterial agent formulations that could degrade corn stover at low temperatures using immobilization methods. Lignocellulose is the main chemical component of corn stover husk; hence, it could act as an appropriate adsorbent for bacterial agent production [16]. We selected lignocellulose-rich corn stover powder as the adsorbent because the presence of lignin and its powdery physical structure supports its effective adsorption. Moreover, corn stover is a crop waste that is a renewable organic resource [17,18] and is environmentally friendly, easy to obtain, low-cost, and to a certain extent, can improve the carbon cycle, which makes it a good additive component of bacterial agents and source for green applications [19]. Starch was selected as the protective agent owing to its fine, dense structural characteristics that can protect microbial cells, minimize damage caused during lyophilization, and provide a favorable dormant environment for cells [20]. Moreover, starch can effectively retain moisture to improve microbial activity and enhance the colonization rate in corn stover, thereby improving the effectiveness of the bacterial agent. Bran was selected as a protective agent because it not only enhances the adsorption and encapsulation of strains, thus increasing the specific surface area of the bacterial agent [21], but it also provides a carbon source for the microorganisms that are conducive to the maintenance of microbial strain activity. Furthermore, compared to a single protective agent, multiple protective agents have synergistic effects that can improve freeze-drying and ensure microbial activity [22,23]. Based on the outstanding properties of the above additives, we tested the optimum bacterial agent formulation by experimenting with different additive ratios. Subsequently, the formulation's effects in low-temperature soil were identified. Moreover, storage conditions were studied with the purpose of extending shelf life and creating an efficient, stable, and suitable bacterial preparation for low-temperature areas.

2. Materials and Methods

2.1. Bacterial Species and Medium

The bacterial formulation GFF2 comprises 2 bacterial strains in the same proportions, namely, GF-40 (*Pseudomonas putida*; CGMCC No. 20521) and GF-45 (*Acinetobacter lwoffii*; CGMCC No. 20522). These strains were deposited in the China General Microbiological Culture Collection Center (CGMCC). The GenBank accession numbers for nucleotide sequences of GF-40 and GF-45 are SUB11224186 GF-40 ON063331 and SUB11224468 GF-45 ON064992, respectively.

The source of GF-40 and GF-45 was soil with 8 years of continuous stover return, located in Chilechuan Modern Agriculture Expo Park, Tumed Right Banner, Baotou City, Inner Mongolia, China. Screening of GF-40 and GF-45 by lignin or cellulose using the single carbon source approach was initiated using the combined dilution and restriction selection method.

We experimentally verified the stover degradation ability, enzyme activity and suitable survival conditions of the 2 strains in the early stage of the experiment. Briefly, a single bacterial inoculum was mixed with soil supernatant at a ratio of 1:400 (*v:v*), and 1 g of corn stover was added. Both the GF-40 and GF-45 strains degraded 18.35% and 21.31% of stover, respectively, after 14 d at 15 °C, which was significantly higher than that of the control treatments (no bacterial solution applied). Both GF-40 and GF-45 had high laccase activity of 47.4 U/L and 101.1 U/L, respectively, and GF-45 also had the ability to produce cellulose and hemicellulase. In addition, GF-40 and GF-45 had strong low-nitrogen tolerance, especially GF-45, which also had high reproductive efficiency under all 3 pH conditions (pH = 5, 7 and 9), indicating broad pH adaptability.

Luria-Bertani (LB) liquid medium was used for bacterial fermentation. Viable count measurement was conducted on LB solid agar medium. The medium was sterilized at 121 °C for 20 min prior to use.

2.2. Fermentation of Bacteria

The 2 bacterial strains were inoculated into 2 150-mL conical flasks containing 50 mL liquid medium and cultured in a shaker at 28 °C and 150 rpm. The optical density (OD) was measured using a spectrophotometer (PuxiGeneral Instrument Co., Ltd., Beijing, China). The fermentation broth was sampled every 2 h for 14 h, diluted, and dilution plating was used to enumerate colony-forming units to estimate the number of viable bacteria. Logarithmic growth periods with the highest number of viable bacteria were used as the appropriate fermentation time for the bacteria to ensure the high activity of the bacterial broth. The logarithmic growth periods for GF-40 and GF-45 were 2–12 and 6–12 h, respectively, whereas the number of viable bacteria reached a maximum when they were cultured for 10 h; the number of viable bacteria reached 3.1×10^8 and 2.9×10^8 CFU/mL, respectively, and the growth rates of both were 3.1×10^7 and 2.9×10^7 CFU/mL/h, respectively. Therefore, these periods were the optimal fermentation times for each bacterial strain (Figure S1).

2.3. Preparation of Dry Powder Bacterial Agents

Preparation of additives: The bacterial agent additive, which is a collective term for adsorbent and protective agents, was corn stover powder (adsorbent) produced by crushing corn stover and passing it through a 60-mesh sieve. The protective agents reduce the damage caused by the freeze-drying process to bacteria and improve the solubility and stability of freeze-dried products. The added material is collectively called a freeze-drying protective agent. The protective agent used was soluble potato starch and wheat bran. All additives were sterilized at 121 °C for 20 min in a sealed Petri dish before use.

Centrifugation of fermentation broth: Fermented broth was centrifuged at 11,292× *g* for 5 min at 4 °C (HC-3018R, Zhong Jia Co., Ltd., Zhengzhou, China) to obtain the precipitates, which had a mass of approximately 1 g (wet precipitates after centrifugation) per 50 mL broth.

Formulation settings: The mass (g) ratios of precipitates to additives were 1:8, 1:6, and 1:4 in 3 gradients: 8, 6, and 4 g additives added to 50 mL bacterial broth (25 mL of each of the 2 bacterial broths). The adjustment methods used for the 3 additives were single-use, combination, and mixing. The multiple (combination/mixing) additive blending methods had 5 gradient mass (g) ratios, 1:1, 1:2, 1:3, 3:1 and 2:1, set between the adsorbent and protective agent and between the 2 protective agents. The specific formulation of the bacterial agent is shown in Tables S1–S3. These bacterial agent formulations were freeze-dried in a vacuum freeze dryer LYO-0.5 (Dongfulong Technology Co., Ltd., Shanghai, China) for preparation. The 5-tube most-probable-number method (GB 20287-2006) was

used to measure the viable bacteria count of the bacterial agent after freeze-drying. All the above treatments were replicated 3 times, and the data are presented as the mean of the 3 replicates.

2.4. Lignocellulosic Enzyme Activity of Bacterial Agents

The lignocellulase activity of the formulations with the highest number of viable bacteria when using single (J1), 2 (J2), and 3 (J3) additives was determined. The formulation with the highest number of viable bacteria and enzyme activity was used as the optimal formulation for the bacterial agent.

Enzyme extract: The solid dry powdered bacterial agent culture broth was prepared by adding 10-fold (v/w) of sterile distilled water and shaking the mixture at 150 rpm and 28 °C for 60 min. Then, the solid additives and bacterial biomass were separated by centrifugation (11,292× g, 5 min, 4 °C), and the clarified supernatant was used for enzyme assays. The cell density of the crude enzyme solution extracted by the bacterial agent J1, J2 and J3 was approximately 1.39 × 10^9 CFU/mL, 5.45 × 10^9 CFU/mL and 7.50 × 10^9 CFU/mL, respectively.

Enzyme assay: Filter paper enzyme (FPA), β-glucosidase (CB), endonuclease (CX), and exonuclease (C1) activities were determined by the 3,5-dinitrosalicylic acid method (DNS method) in "Cellulose Enzyme Preparation" (QB/T 2583-2003) [24]; xylanase (Xyl) by the DNS method in "Determination of Xylanase Activity of Feed Additives-Spectrophotometric Method (GB/T 23874-2009)" [25]; laccase (Lac) by the ABTS method; lignin peroxidase (Lip) by the quinoa method; and manganese peroxidase (Mnp) by the DMP method [26].

2.5. Degradation Effect of Bacterial Agents

2.5.1. Laboratory Soil Culture Test

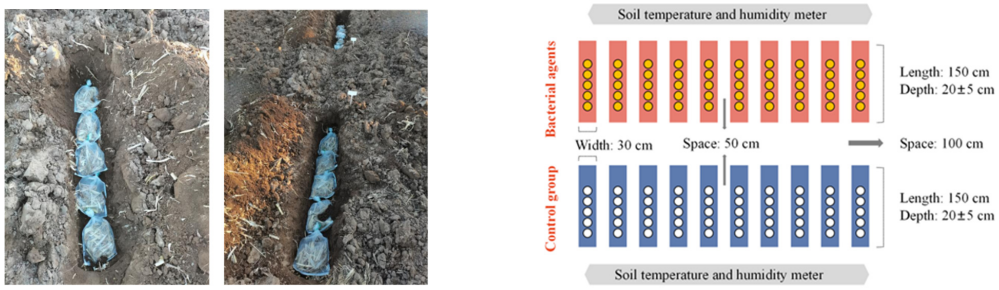
A 40-day soil culture test was conducted from 1 August to 10 September 2020 at the microbiology laboratory of the Mengxi Comprehensive Experiment Station for Maize (Chilechuan Modern Agriculture Expo Park, Tumed Right Banner, Baotou City, Inner Mongolia, China) of the Inner Mongolia Agricultural University. Specifically, 30 culture boxes (10 cm [l] × 10 cm [w] × 12 cm [h]) were filled with 800 g of soil to a height of 10 cm. Thereafter, 8 g of corn stover was placed in the middle (5 cm) of each box. In brief, the corn stover samples were cut into 2–3 cm segments, washed, and dried at 60 °C to a constant weight. Thereafter, each 8 g sample was packed into a gauze mesh bag (5.0 × 10.0 cm, sieve aperture size, 1 mm); this was done for a total of 30 bags. Thereafter, 2 bacterial agent application treatments were prepared by weighing 0.01 g of the bacterial agent with a viable bacterial count = 7.5 × 10^{10} CFU/g using a 10,000 ppm balance and placed in a tinfoil packet. Next, the foil packet was pinched slightly and sealed. The bacterial agent or the no-application treatment (control group) was set up, with 15 replicates per treatment. Further, the soil samples in each box were maintained at 20% water content and placed in an incubator at 15 °C to simulate a low-temperature environment. The degradation rates of the corn stover samples were then measured after 40 d.

2.5.2. Field Test

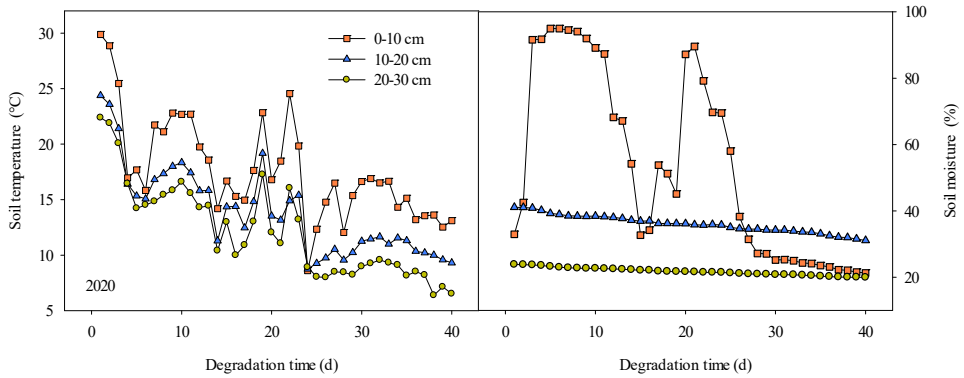
Two 40-d field tests were conducted from 1 October to 9 November 2020 and 2021 to verify the degradation effect of the bacterial agent. The experiment was conducted at the Mengxi Comprehensive Experimental Station of Maize Center. The time of the experiment was set as the degradable time period after the completion of stover return from the corn harvest and at temperatures close to the low-temperature conditions (15 °C) simulated in our laboratory-based pot experiment. Rainfall during the field test period in 2020 was 1.8 mm, 1.0 mm, and 1.3 mm on October 6, 17, and 20, respectively. There was no rainfall during the test period in 2021.

Corn stover was obtained during an annual collection and then cut into 4–5 cm segments, washed, and dried at 60 °C to a constant weight. Each 40 g of corn stover was packed into a gauze mesh bag (20 × 30 cm, sieve aperture size: 1 mm), and a total of

100 bags were collected. We set up 2 bacterial agent application treatments. In the first treatment, 0.05 g of the bacterial agent with a viable bacterial count = 7.5×10^{10} CFU/g was weighed with a 10,000 ppm balance, placed in a tinfoil packet, pinched tightly and sealed. The second treatment was a no-application treatment control group. The bacterial agent was spread evenly onto the surface of the stover, which was then covered with soil. The stover was buried at a depth of 20 ± 5 cm. Five bags of buried stover were considered a replicate at 1 location, with 10 replicates at different locations considered in total. The spacing between each treatment set-up was 100 cm, and the spacing between replicates was 50 cm. In addition, 2 soil Soil Temperature and Humidity Recorder (IZS-2X, Zhejiang Top Instrument Co., Ltd., Zhejiang, Chain) were used to determine the temperature and humidity of the soil layers at depths of 0–10 cm, 10–20 cm, and 20–30 cm during the experiment. The layout of the experiment is shown in Figure 1a. The average daily temperature and humidity of the soil during the experiments in 2020 and 2021 are shown in Figure 1b,c, respectively.



(**a**)



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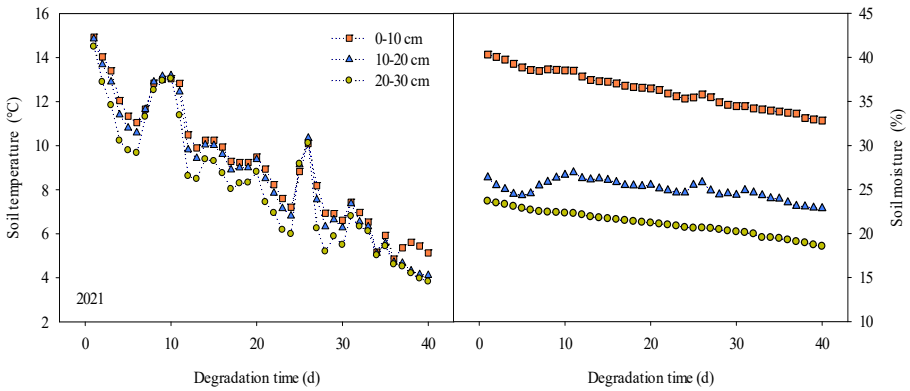


(**c**)

Figure 1. Field test layout and soil environment for bacterial agent assessment. (**a**) Layout of field tests. Temperature and moisture in different soil layers during (**b**) 2020 and (**c**) 2021.

In this study, in both the "Laboratory soil culture test" and "Field test," the bacterial agent treatment and treatment without bacterial agent were applied. The treatment without bacterial agents served as the blank control. The soil for both treatments was taken from the same site, and we controlled the consistency of other factors except for the bacterial agent in the experiment. Before the experiment, we used a 5-point sampling method to determine the base strength of the soil at the site where the soil was taken, and the information is summarized in Table 1.

Table 1. Soil fertility before sowing.

Years	Available N (mg/g)	Available P (mg/g)	Available K (mg/g)	Organic Matter (g/kg)
2020	55.36	6.67	96.54	16.96
2021	56.62	6.82	97.01	18.23

Each treated stover sample was put into a sieve (1-mm mesh), and the dirt was rinsed off slowly with running water to ensure that the stover was not lost. Subsequently, the corn stover was dried, powdered, and sieved through a 1-mm sieve. The cellulose, hemicellulose, and lignin fractions of the samples were determined using the method reported by Van Soest et al. [27,28].

The degradation rate was calculated as follows:

$$\text{corn stover degradation rate (\%)} = (W_0 - W_1)/W_0 \times 100\% \quad (1)$$

where W_0 and W_1 represent the mass of corn stover before degradation (g) and after degradation (g), respectively.

2.6. Storage Conditions of Bacterial Agents

Bacterial agents with a water content of 5, 10, 15 and 20% were stored at 2 temperature conditions: cold storage (4 °C) and room temperature (25 °C) in a vacuum and ordinary packaging. Samples were obtained at 0, 15, 30, 50, 70, 90, 120, and 180 d during the storage period to determine the number of viable bacteria in the bacterial agents. The water content, temperature, packaging mode, and maximum storage time for the bacterial agents were evaluated based on the viable bacterial counts [29]. Sterile fermentation broth under the same storage conditions was used as the control group.

$$\text{Survival rate of viable bacteria (\%)} = \text{number of viable bacteria during storage/initial number of viable bacteria} \times 100\% \quad (2)$$

2.7. Data Analysis

MS Excel 2010 (Microsoft Corp., Redmond, WA, USA): Experimental data are reported as the mean and standard deviation (SD). The results of 3 replicates per treatment were averaged to give a single value for each sample for use in subsequent statistical analyses.

IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA): The data were tested for normal distribution, and the relationships between the variables were tested using the Chi-squared test. Data that conformed were analyzed using analysis of variance (2-way) and the t-test. Duncan's multiple range test was used to compare treatment means; data that did not conform were tested by nonparametric tests (Kruskal–Wallis). Differences were considered significant at $p < 0.05$.

SigmaPlot 12.5 (Systat Software, San Jose, CA, USA), GraphPad Prism (GraphPad Software, San Diego, CA, USA), and ChiPlot (https://www.chiplot.online/, accessed on 3 September 2022) were used for graphical analyses.

3. Results and Discussion

3.1. Formulation Analysis of Bacterial Agents

Figure 2 shows the viable bacterial counts of the tested bacterial agents, which varied with the different types of additives and ratios of precipitates to additives. We did not observe a regular trend with an increase in the ratios of precipitates to additives or additive types, and individual specific formulations maintained high viable bacterial counts.

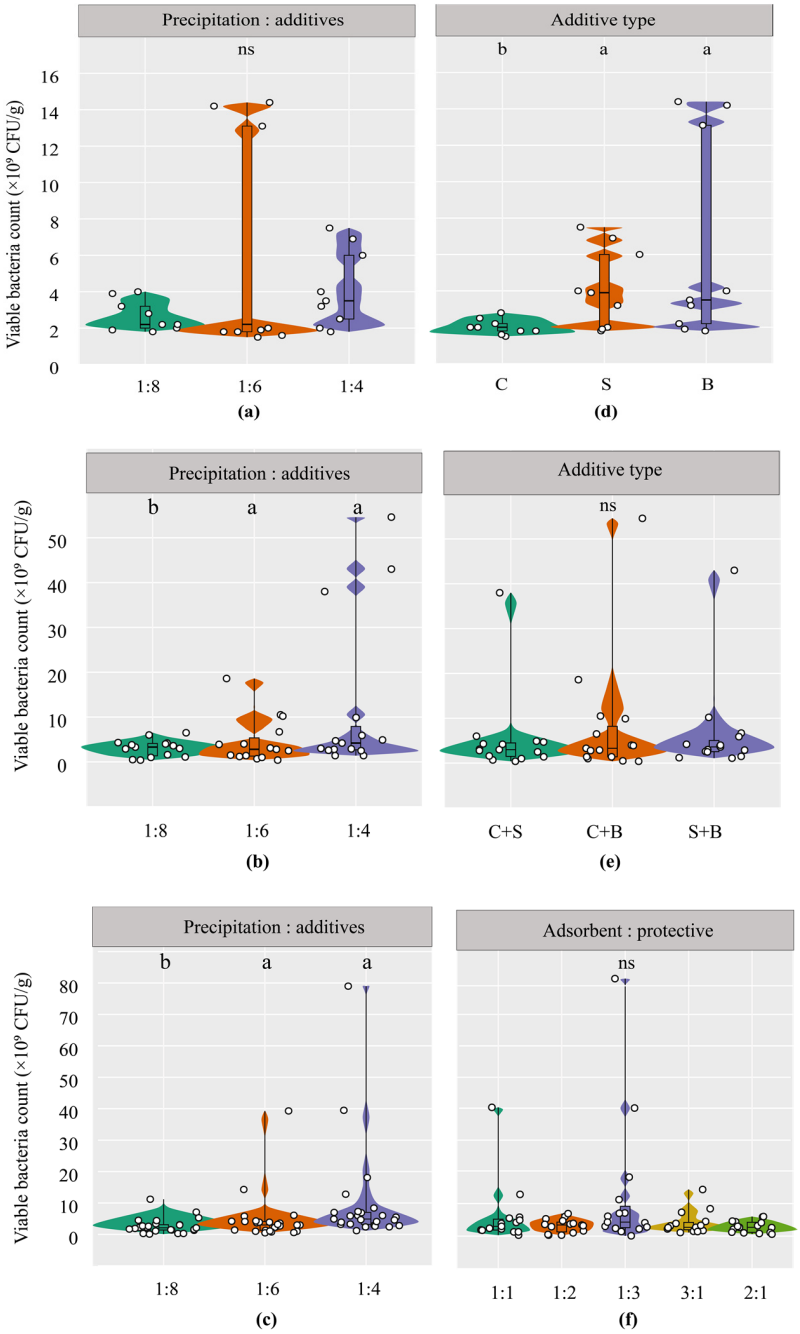


Figure 2. Viable bacterial count among the three ratios of precipitates to additives when (**a**) one, (**b**) two, and (**c**) three types of additives are used in the bacterial agent. (**d**) Viable bacterial count among the three additive groups, (**e**) three combination modes for the additives, and (**f**) five ratios of adsorbent to protective agents when combined with one, two, and three types of additives, respectively. C: corn stalk husk powder; S: starch; B: bran. ns: no significant difference. Significant differences at $p < 0.05$ are indicated by lowercase letters.

When a single additive was used, the bacterial agent had a generally low viable count (Figure 2a,d). Tables 2 and 3 show the nonparametric analyses of the viable counts of the bacterial agents prepared from different ratios of precipitate to additives and from different additive types. The results revealed that the viable bacterial counts of the bacterial agents with starch and bran as additives were significantly (n = 27, p = 0.024) higher than those of the agents with corn stover powder as an additive, and the differences between the different ratios of precipitate and additive were not significant. The formulation that maintained a relatively high viable count was the bacterial agent with bran as the additive and a precipitate:additive ratio of 1:6 (1.39×10^{10} CFU/g).

Table 2. Nonparametric analysis of viable bacteria counts at different proportions of precipitates and additives (CFU/g; Kruskal–Wallis).

Number of Additive Types	Precipitates:Additives			χ^2	p
	1:8	1:6	1:4		
One (n = 27)	2.2 (2.0, 3.6)	1.9 (1.7, 13.7)	3.5 (2.3, 6.5)	1.966	0.374
Two (n = 135)	3.3 (1.3, 4.3) ^{b}	2.9 (1.2, 6.5) ^{a}	4.3 (2.6, 9.6) ^{a}	8.706	0.013 *
Three (n = 75)	2.1 (1.4, 3.3) ^{b}	3.0 (1.2, 4.2) ^{a}	4.9 (3.1, 7.2) ^{a}	16.630	0.000 **

n: Sample number. χ^2: Chi-square value. Each value represents the median (25% percentile, 75% percentile). Significant differences at $p < 0.05$ are indicated by lowercase letters. * $p < 0.05$. ** $p < 0.01$.

Table 3. Nonparametric test of the viable count at different additives (CFU/g; Kruskal–Wallis).

Number of Additive Types	Types of Additives			χ^2	p
One (n = 27)	Corn stalk husk powder	Starch	Bran		
	2.0 (1.7, 2.4) ^{b}	3.9 (2.0, 6.5) ^{a}	3.5 (2.1, 13.7) ^{a}	7.430	0.024 *
Two (n = 135)	Corn stalk husk powder + starch	Corn stalk husk powder + bran	Starch + bran		
	2.9 (1.5, 4.6)	3.3 (1.4, 9.2)	3.5 (2.4, 5.7)	1.608	0.447

n: Sample number. χ^2: Chi-square value. Each value represents the median (25% percentile, 75% percentile). Significant differences at $p < 0.05$ are indicated by lowercase letters. * $p < 0.05$.

When two or three additives were used (Figure 2b,c,e,f), the overall number of viable bacteria was higher than that when a single additive was used. The number of viable bacteria was significantly higher (Table 2, two: n = 135, p = 0.013; three: n = 75, p = 0.000) when the ratio of precipitate to additive was 1:4 and 1:6 than when it was 1:8. Hence, the maximum viable bacterial count from the different additive combinations or mixing was obtained at a precipitate:additive ratio of 1:4. Based on the ratio analysis among the three combination modes for two additives, the formula with the relatively high viable bacterial count (5.45×10^{10} CFU/g) was corn stover husk powder and bran at a 3:1 ratio. Compared with the other formulas, the bacterial agent formula mixed with three additives, an adsorbent:protective agent ratio of 1:3, and a ratio between two protective agents of 1:3 (i.e., corn stover husk powder:starch:bran = 4:3:9) had a maximum number of 7.50×10^{10} CFU/g viable bacteria, which was higher than that observed for the other formulas.

Due to the selection and ratios of the additives' influence on the quality of bacterial agents during the preparation process, the immobilized additives became the focus of research in successful bioaugmentation [30]. Baoliang Chen et al. showed that the type of additive is the key factor affecting the target effect of the bacterial agent, and the additives are intended to offer a protective niche for inoculated microbes and hence reduce competition with indigenous microorganisms [31]. Tapia-Olivares et al. demonstrated that the physical interaction between cells and the surface of adhesives impacts the survival and viability of inoculants [32]. Bailey et al. used starch as an additive to prepare the

bacterial agents that enhanced the strain survival rate, water retention, and rhizosphere colonization rate compared to those in free microorganisms [33]. Cheng et al. found that the presence of bran in the additive of the bacterial agent greatly increased the percentage of bacterial biomass due to its availability as a supplementary carbon source for microorganisms [34]. In the present study, the number of viable bacteria in the optimal formula reached 7.5×10^{10} CFU/g, effectively verifying the feasibility of preparing bacterial agents using additives.

Interestingly, in this study, when there was only one additive, the type of additive significantly affected the viable bacteria count of the bacterial agent (Table 3), but the mass ratio of precipitate to additive had no significant effect on it, ranging from 1:4 to 1:8 (Table 2). When a variety of additives were used for the preparation of the bacterial agent, the effect of the ratio of bacterial precipitate to additives on the viable number of bacteria of the agent (Table 2) was greater than the effect of different ratios between additives on the viable number of bacteria of the agent (Tables 3 and 4). The number of viable bacteria was significantly higher when the ratio of precipitate to additive was small, and the highest value of viable bacteria appears in the ratio of 1:4. For this reason, we chose smaller ratios (1:2); however, too-small amounts of additives could not effectively adsorb and protect the bacteria. Moreover, the fluidity of the bacterial mixture was too great and increased the difficulty of freeze-drying. Therefore, we believe that in the preparation of multi-series additives, it is not the accumulation of additive types or the increase in their mass that makes a high-quality formulation, but rather a relatively small amount of additives on the basis of adsorption, protection of the bacterium cells, and to obtain a high rate of cell release during re-solubilization. However, it should be in a moderate range and should be combined with the needs of the technology.

Table 4. Nonparametric test of the viable bacterial count at different ratios between adsorbent and protective agents (CFU/g; Kruskal–Wallis).

Number of Additive Types	Adsorbent:Protective Agents					χ^2	*p*
	1:1	1:2	1:3	3:1	2:1		
Three (*n* = 75)	3.0 (1.8, 5.5)	3.4 (1.0, 4.7)	4.3 (2.4, 11.2)	2.7 (1.7, 4.5)	2.7 (1.1, 4.4)	4.617	0.329

n: Sample number. χ^2: Chi-square value. Each value represents the median (25% percentile, 75% percentile).

3.2. Lignocellulosic Enzyme Activity of Bacterial Agents

We determined the lignocellulase activity of the three treatment formulations with the highest number of viable bacteria using single, two, and three additives. We found that the enzyme activity of both the J2 and J3 treatments was significantly higher than that of the J1 treatment (Figure 3). The highest filter paper enzyme activity of cellulase was 1.30 U/mL, 1.97 U/mL, and 2.40 U/mL in the J1, J2, and J3 treatments, respectively; the xylanase enzyme activity in treatment J3 was 4.53 U/mL, which was 2.4 and 1 times higher than the enzyme activity in J1 and J2 treatments, respectively; the manganese peroxidase activity of ligninase was high in all three treatments, with the highest enzyme activity in J3 treatment (261.33 U/L). In summary, the J3 treatment had the highest number of viable bacteria and lignocellulase activity than did the other treatments, indicating it was the optimal bacterial formulation with the potential to degrade corn stover.

Lignocellulase activity is one of the important parameters to evaluate the degradation capacity of stover, which can directly reflect the intensity of microbial metabolism during the decomposition of lignocellulose [35]. Studies have shown that the structure of lignin is complicated, and lignin can prevent contact between degrading enzymes and cellulose or hemicellulose. Therefore, the degradation of lignin is generally regarded as a rate-limiting step [36]. Enzyme degradation of lignin is the main reaction in the humification process. The changes in lignin-degrading enzyme activities can reflect the rate and degree of humification. Lignin degradation requires a variety of oxidases and peroxidases, including LiP, MnP, and laccase. Laccase is a copper-dependent polyphenol oxidase that oxidizes

aromatic compounds and catalyzes the synthesis of humic acid from quinones and amino acids [37]. Zhu N et al. showed that high levels of FPase and xylanase degrade cellulose and hemicellulose to monosaccharides, providing a carbon source for microbial growth and that increased microbial biomass contributes to the production of lignin-degrading enzymes [38]. In summary, it is evident that the bacterial agent in this study has the potential to degrade corn stover due to its high level of lignocellulase.

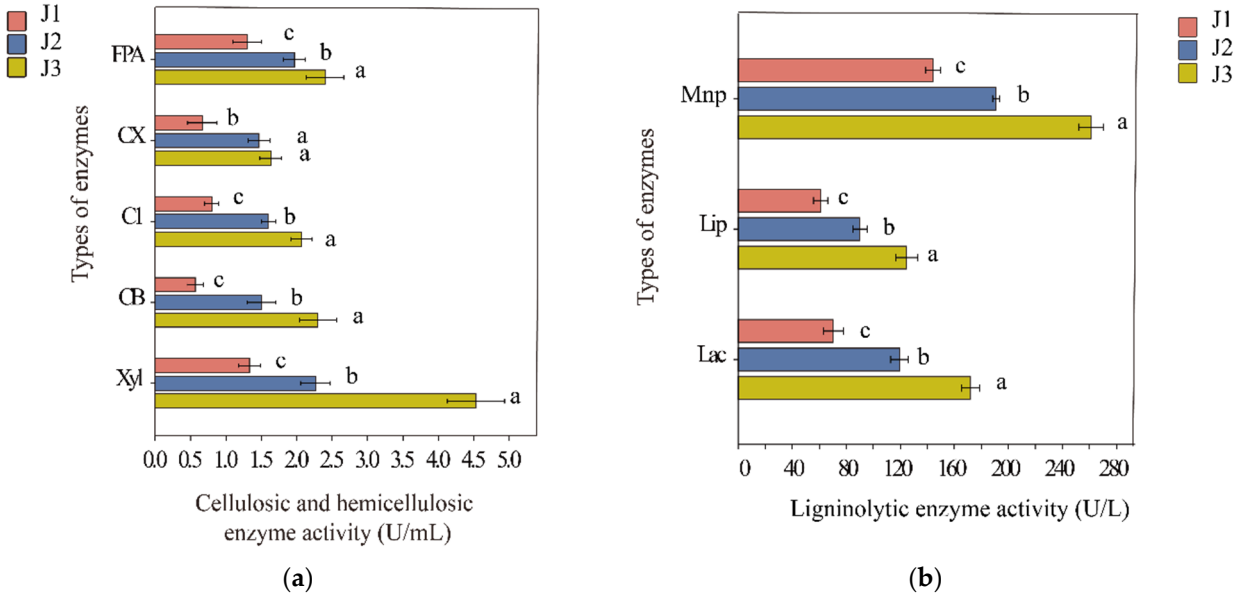


Figure 3. Lignocellulase activity of the tested bacterial agents. Cellulosic and hemicellulosic (**a**) and ligninolytic (**b**) enzyme activities of the tested bacterial agents. Different letters represent significant differences ($p < 0.05$).

3.3. Validation of the Degradation Effect of the Bacterial Agent on Corn Stover

To verify the degradation effect of corn stover by the optimal formulation of the bacterial agent, we conducted soil culture pot tests and field tests. Figure 4a shows the degradation rates of the bacterial agent on corn stover and its components in the laboratory test. The degradation rate of corn stover with the bacterial agent was 32.04%, which was 15.08% higher than that of the control group (16.96%). The degradation rates of cellulose, hemicellulose, and lignin in corn stover treated with the bacterial agent were 43.62%, 44.44%, and 55.91%, respectively, which were 22.51%, 24.74% and 41.92% higher than those in the control group, respectively. The overall structure of the corn stover without the bacterial agent remained integrated and compact, displaying no discernible change compared with that of the initial stover (Figure S2). However, the stover treated with the bacterial agent became loose, hollow, and fragmented.

The degradation effects of the bacterial agents on corn stover in the field tests in 2020 and 2021 are presented in Figure 4b,c. In 2021, the degradation rate of stover and its components by the bacterial agent was generally lower than that in 2020 due to the difference in soil temperature and moisture; however, the results of both experiments showed that the degradation rate of stover treated with the bacterial agent was significantly higher than those of the control group. In 2020 and 2021, the degradation rates of stover with the applied bacterial agent were 34.10% and 29.60%, which were 15.74% and 12.94% higher than those of the control group, respectively. In agreement with the laboratory results, the application of bacterial agents in the field environment was able to significantly degrade lignin compared with the control. In 2020 and 2021, the lignin degradation rates in the groups that received bacterial agent treatment were 46.80% and 34.80%, respectively, whereas the lignin degradation rates of the control groups were 13.08% and 9.48%, respectively.

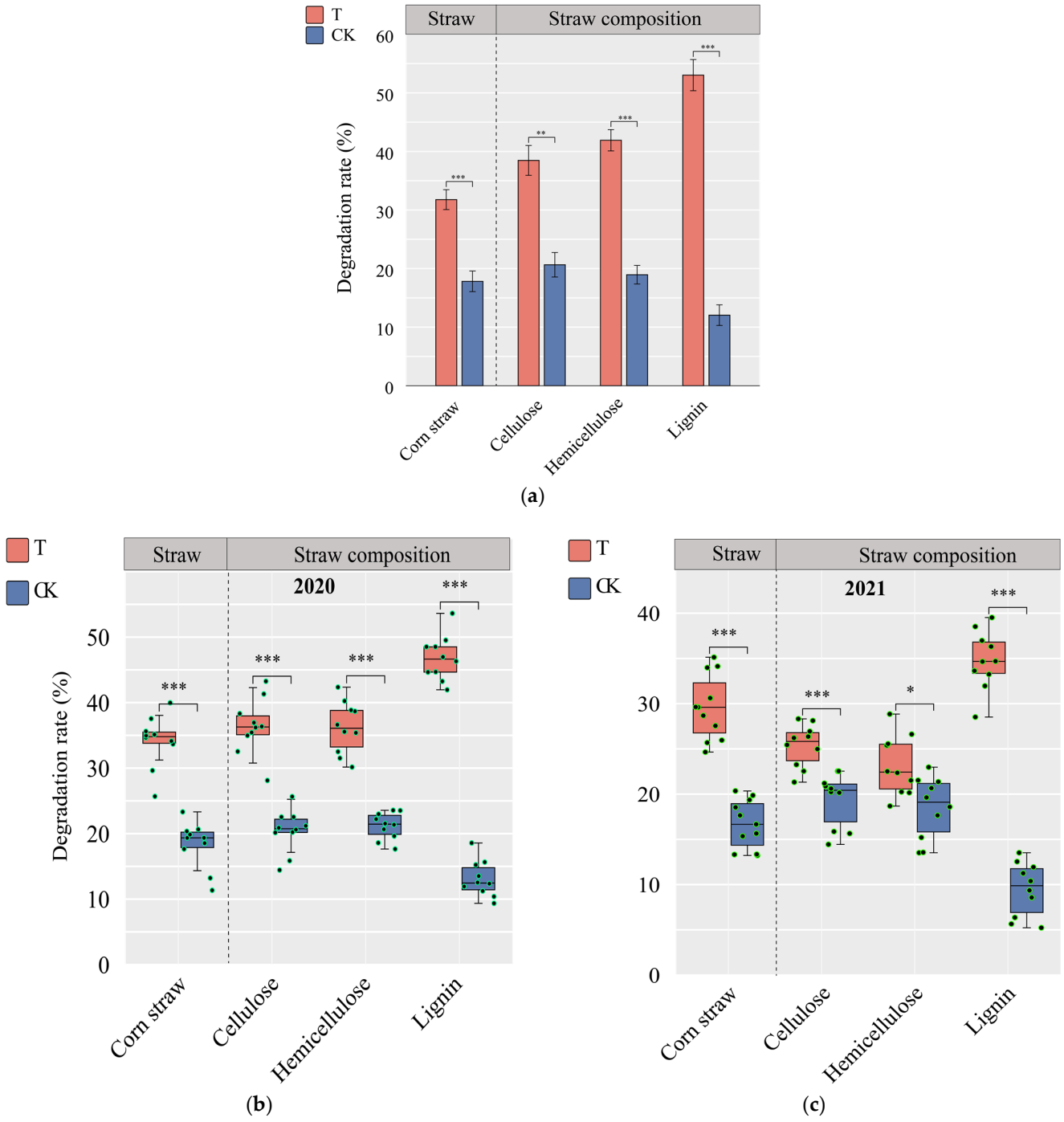


Figure 4. Effectiveness of the tested bacterial agents in the laboratory and in field conditions. Degradation rates of corn stover and its components in the (**a**) laboratory and in field tests conducted in (**b**) 2020 and (**c**) 2021. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T: bacterial agent application treatment. CK: control group.

Soil temperature and humidity varied during the two field tests, which were influenced by rainfall and climate. The relatively high temperature and humidity conditions in 2020 were favorable for the propagation of the bacterial strains; thus, the degradation effect achieved in this year was more significant than that in 2021. The climate in 2021 was unique in that overall temperature and humidity were low, especially in the initial stage of the experiment when the soil temperature was below 15 °C and gradually decreased as the experiment progressed. Although the degradation of stover and its components was

slightly poorer in 2021 than in the laboratory soil culture test, the effect of the bacterial agent applied in the same environment in both field tests was still significantly higher than that of the control group. In addition, the main components of stover dry matter are lignin, cellulose, and hemicellulose, which are the main factors that limit stover degradation. Lignin, in particular, has been a challenge for stover degradation research due to its complex and recalcitrant structure [36,39]. In the present study, the bacterial agent treatment significantly increased the degradation rate of stover components in both laboratory and field tests, maintaining the outstanding degradation ability of stover lignin in the field, which is important for bacterial agent applications, especially for stover degradation in low-temperature regions.

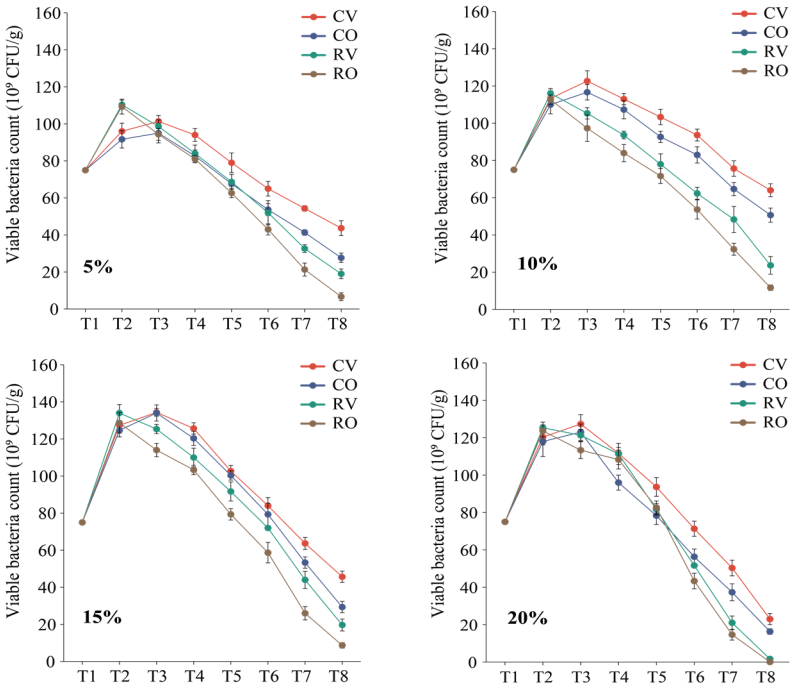
The strains in our study were *P. putida* and *A. lwoffii*, for which the degradation ability has been reported in previous studies. In a screening of numerous species with lignin-degrading activity in humus-rich forest black soil residues, Brossi et al. showed that Acinetobacter could efficiently degrade aromatic hydrocarbons and phenols, and it is one of the species that can use aromatic and aliphatic carboxylic esters as a sole carbon source [40]. Pseudomonas exhibits a great capacity for aerobic degradation and secretes extracellular enzymes (e.g., proteases and urease) at high levels, which can be involved in the metabolism of complex carbohydrates [41]. In addition, the substrate of the strain can induce or stimulate the production of corresponding enzymes; for example, as an additive to the bacterial agent, the lignocellulose-rich corn stover powder can stimulate, to an extent, the secretion of enzymes related to lignocellulose degradation [42]. This response explains the outstanding ability of the bacterial agent to degrade the components of corn stover observed in this study.

Our results demonstrated how the additives could effectively protect the activity of the bacterial strain in low-temperature soil. The efficient low-temperature adaptation prompted us to use temperature-restricted succession cultures in subsequent experiments to allow the strain to adapt to a lower-temperature environment and explore its potential to adapt to a larger range of low-temperature conditions for efficient stover return.

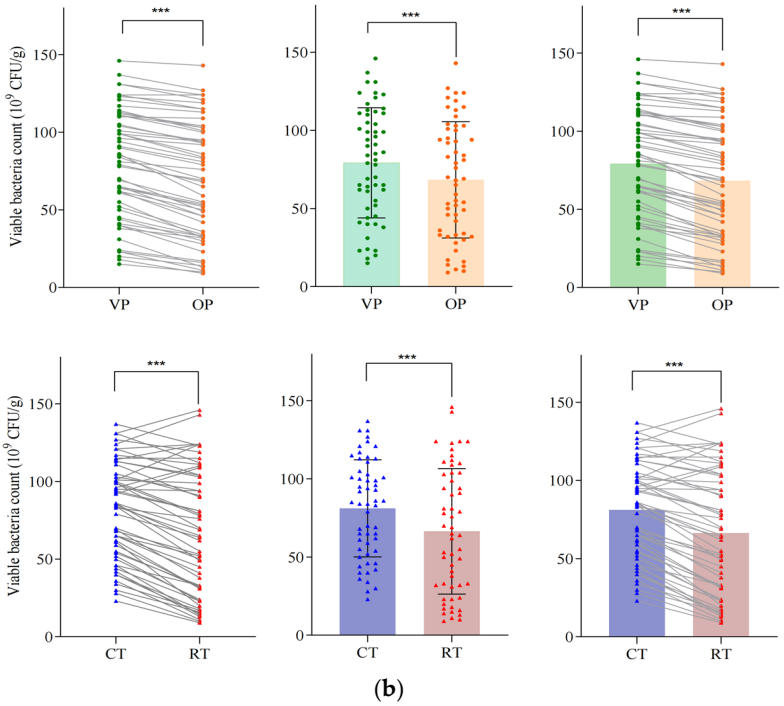
3.4. Analysis of Bacterial Agent Storage Conditions

The viable bacterial count of the bacterial agents with different moisture content and packaging methods all initially increased and then decreased under different storage temperature conditions (Figure 5a). The bacterial agent maintained a longer initial elevated count under cold storage conditions (30 d) relative to room temperature conditions (15 d). After 50 d of storage, the viable bacterial count for all treatments under cold and room temperature storage decreased but was still higher than the initial count. Following 180 d of cold storage with vacuum packaging and a water content of 10%, the bacterial agents had the highest number of viable bacteria at 6.40×10^{10} CFU/g, with a survival rate of 85.33%. For the bacterial solution stored under conditions similar to that of the control group (Figure S3), the number of viable bacteria began to decline from the initial stage, and the number of viable bacteria in the bacterial solution was 10^5 CFU/mL after 180 d. In addition, evaporation of the bacterial solution stored at room temperature occurred.

A t-test analysis (Figure 5b) was performed for each treatment to compare vacuum packaging versus ordinary packaging and cold storage versus room temperature storage. We observed that the number of viable bacteria under the cold storage condition was significantly higher than that under room temperature ($t = 3.78$, $p = 0.000$), and the viable count under vacuum packaging was significantly higher than that under ordinary packaging ($t = 2.76$, $p = 0.006$).



(**a**)



(**b**)

Figure 5. Changes in viable bacterial counts for bacterial agents under different storage conditions. (**a**) Changes in viable bacterial counts for bacterial agents with different water contents and packaging methods under different storage temperatures. CV: cold storage vacuum packaging; CO: cold storage ordinary packaging; RV: room temperature vacuum packaging; RO: room temperature ordinary packaging. T1, T2, T3, T4, T5, T6, T7, and T8: the storage times were 0, 15, 30, 50, 70, 90, 120, and 180 d, respectively. (**b**) Paired-sample *t*-test of the packaging methods and storage temperatures. VP: vacuum packaging. OP: ordinary packaging. CT: cold storage. RT: room temperature storage. *** $p < 0.001$.

During the preparation of the bacterial agent, the appropriate storage conditions were determined and combined with the long-term physical and chemical protection of the appropriate additive formula, which could effectively prevent the decay of viable bacteria and prolong the storage period of the bacterial agent. The trends of the number of viable bacteria under different water content and packaging preparations varied under cold storage and room temperature conditions, which indicated that in addition to the water content and additive formula, external conditions are key factors that affect the storage quality of bacterial agents.

Cold storage slows down the cell division and metabolism of bacteria as well as reduces water loss, nutrient consumption, and toxic metabolite accumulation [43,44]. Vacuum packaging can inhibit the breeding of miscellaneous bacteria, as well as reduce the number of microorganisms and the production of harmful volatile substances during storage, effectively maintaining the product's initial effect [45]. Additionally, considerably high or low water content can inhibit the growth of microorganisms and affect their activity [46]. In the present study, the number of viable bacteria in the vacuumed packaged bacterial agent stored at a low temperature was higher than that in the bacterial agent with ordinary packaging stored at room temperature. The bacterial agent with 10% water content had the highest number of viable bacteria under cold storage and room temperature compared to that of the other preparations, indicating that this was the optimal water content level for the survival of the bacteria. Under these conditions, the bacterial agent provided the requirements for microorganism survival.

In addition, except for the relatively rapid decrease in the number of viable bacteria in the bacterial agent with 35% water content stored at room temperature, the number of viable bacteria in the bacterial agent stored under other conditions remained at 1010 CFU/g after 180 d. Moreover, the number of viable bacteria in the bacterial solution decreased sharply with the extended storage time under cold storage and room temperature conditions due to factors such as nutrient depletion and hypoxia [8,47]. These results indicated that solid bacterial agents could effectively maintain bacterial activity and are more suitable for long-term storage than liquid bacterial solutions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041032/s1, Figure S1: OD_{600} values and viable bacterial counts during bacterial fermentation; Figure S2: Comparison of the degradation effect of the bacterial agent and control groups in the laboratory test conditions; Figure S3: Viable bacterial counts in bacterial solutions at different storage temperatures; Table S1: Formulation of bacterial agents with a single additive; Table S2: Formulation of bacterial agents with two types of additives; Table S3: Formulation of bacterial agents with three types of additives.

Author Contributions: Conceptualization: S.Z., S.H. (Shengcai Han), X.Y. and J.G. (Julin Gao); Data curation, S.Z.; Formal analysis, S.H. (Shuping Hu), N.B., Q.B., J.G. (Jiangan Guo), J.B., B.Z., Z.H. and Y.L.; Funding acquisition, S.H. (Shengcai Han), X.Y. and J.G. (Julin Gao); Methodology, S.Z.; Resources, S.H. (Shengcai Han); Software, S.Z.; Writing–original draft, S.Z.; Writing–review & editing, S.H. (Shengcai Han), X.Y. and J.G. (Julin Gao). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article or supplementary material.

Conflicts of Interest: The authors declare that they have no relevant financial or non-financial interests to disclose.

Abbreviations

CGMCC	China General Microbiological Culture Collection Center
LB	Luria-Bertani
OD	Optical density

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