RESEARCH ARTICLE

Remediation effect of microbial agents on vegetable soil pollution

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To solve the problem of pesticide accumulation in vegetable soil while increasing the vegetable planting area in China, this study analyzed the remediation effect of microbial agents on different types of pesticide pollution. Multiple target microbial strains were investigated through bacterial sludge preparation and high-throughput screening. The target bacterial strains were purified and identified using bacterial 16S rRNA sequencing. Microbial preparations were then used in field experiments to determine the remediation effect of microbial preparations on different types of pesticide contamination. The best bacterial strains for remediation of different types of pesticide pollution were explored. The results showed that *Bacillus mycoides* had a degradation rate of 85.63% for acetamiprid and 57.81% for triazolone, making it the best bacterial strain in remediation of the same type of pollution compared to the other bacterial strains. The results suggested that *Australian bacillus*, uncultured prokaryotes, and *Bacillus mycoides* had better pollution remediation effects in pesticide pollution. This study provided an important reference for selecting microbial agents for the treatment of soil pesticide pollution.

Keywords: soil pollution; microbial preparations; pollution remediation; fosthiazate; chlorothalonil; triazolone; acetamiprid.

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Introduction

To solve the problem of vegetable supply cycle in China, vegetable cultivation facilities have gradually modernized in recent years, and the cultivation area has also increased year by year. However, with the application of modern technology, the use of pesticides has become more widespread and frequent. Although this approach has resulted in increased vegetable yield and disease resistance in the short term, it has also brought about a significant negative impact as the gradual accumulation of pesticide residues in the soil [1]. This accumulation has a dual impact on the environment and the agricultural sector with resulting in the pollution of soil used for the cultivation of vegetables and having a detrimental effect on the health of crops and the efficiency of the entire vegetable production chain, which ultimately leads to a considerable reduction in the quantity and quality of vegetable products [2]. In addition, vegetable soil pollution (VSP) may cause the accumulation of harmful substances in vegetables, posing potential hazards to human health. Long-term consumption of these contaminated vegetables may lead to various health problems such as chronic poisoning, endocrine disorders, *etc*. [3]. Therefore, VSP has had an undeniable impact on the business chain of vegetable farmers and the health of users. Given this, how to effectively control and reduce pesticide residues in vegetable soil has become an important issue that urgently needs to be

addressed [4]. Conventional repair techniques encompass both physical and chemical methods, which frequently prove costly and exhibit restricted efficacy. The scope of application of physical repair methods is constrained and challenged to implement on a broad scale. While chemical repair methods are effective, their high cost renders large-scale implementation impractical [5]. Therefore, the use of microbial remediation methods has gradually become a new and highly anticipated approach [6]. In comparison to conventional techniques, microbial remediation offers a cost-effective solution with notable benefits in environmental conservation and sustainability. Its potential for diverse applications and further advancement is evident [7]. This method is not only cost-effective but also relatively simple to operate and does not disrupt the chemical element balance in the soil, which makes it a more efficient remediation method [8]. Microbial remediation technology can effectively degrade pollutants in soil without causing secondary pollution, which protects the environment and promotes soil health and the development of sustainable agriculture [6].

Currently, the pollutant remediation can be divided into three types as physical remediation, chemical remediation, and microbial remediation. Osman *et al.* analyzed the application of biochar in soil environmental remediation and found that biochar could play a role in removing pollutants, effectively improve resource utilization, protect soil and surrounding water environment, and promote the growth of animals and plants [9]. Wang *et al*. focused on various types of environmental remediation materials such as modified dolomite and hydrogel and investigated the remediation effects for arsenic and antimony pollution in heavy metal soil pollution. The results showed that the combined application of modified dolomite and hydrogel was helpful to the leaching of arsenic and antimony from heavy metal contaminated soil. It also improved the exchange capacity of cations and aggregates in polluted soil, providing assistance for the enhancement of microorganisms in the soil [10]. New *et al*. applied nanotechnology to soil remediation and developed a nano soil remediation technology that contributed to the development of photocatalytic and chemical degradation methods for polluted soil, thereby benefiting the health status of soil organisms and flora and fauna. The method had higher real-time monitoring performance than that of other repair techniques [11]. Further, Chen *et al.* analyzed the relationship between vetiver and soil microorganisms during soil remediation and found that vetiver possessed the capacity to adapt to extreme climatic and soil conditions including high salinity and high acidity. Consequently, vetiver could play a role in the remediation of organic-contaminated soil. By analyzing plant tolerance and microbial assisted adaptation, the mechanism of action between vetiver and soil microorganisms was explored [12]. Research on microbial remediation has been continuously deepening in recent years. Elnahal *et al*. analyzed the role of microbial agents in biological fertilizers and biopesticides and encouraged farmers to use microbial control agents to reduce pollutants caused by excessive use of pesticides and fertilizers [13]. Mishra *et al*. analyzed the effectiveness and limitations of traditional bioremediation techniques and developed a novel remediation method using biotechnology. The results suggested that the combination of omics technology and culture medium with microbial electrochemical technology could achieve pollutant remediation and improve environmental remediation efficiency [14]. Li *et al*. also analyzed the role of microbial electrochemistry in soil pollution remediation and found that microbial electrochemistry had the characteristics of better control and less susceptibility to secondary pollution. This study discussed the mechanisms of electron release, transmission, and reception, and suggested that microbial electrochemical methods could effectively improve soil materials and achieve highly controlled remediation of sediment pollution [8]. Zhao *et al*. addressed the compound pollution of polycyclic aromatic hydrocarbons and fluoroquinolones in agricultural environments using molecular

Table 1. Pesticide information.

Pesticide	Molecular formula	Molecular weight (g/mol)
Acetamiprid	$C_{10}H_{11}Cl\ N_4$	233.64
Chlorofluazuron	$CHCl\ F_3NO_3$	457.63
Triazolone	$C_{14}H_{16}Cl\ N_3O_2$	237.86
Fosthiazate	$C_9H_{18}NO_3PS_2$	294.52
Chlorothalonil	$C_8N_2Cl_4$	277.17

dynamics methods and external field measures to promote the degradation of compound pollution. By screening out new enzymes with excellent degradation performance, the degradation of composite pollutants had been achieved [15].

To further explore the remediation effects of microorganisms, this study prepared microbial preparations for different types of pesticide contamination in vegetable soil to investigate the remediation effect of different bacterial strains. This study tested different formulations for different types of pesticide soil pollution and would provide reference value for land pollution control.

Materials and methods

Microbial and soil sample preparation

The soil samples were collected from an agricultural intensive area in Shouguang City, Shandong, China. The sampled region has been known for its intensive agricultural activities and frequent utilization of pesticides through historical pesticide use records and on-site soil testing results of significant pesticide residues in the soil. The samples were suspended and washed with clean water, inoculated into LB medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA), incubated in IKA KS 4000i Shaking Incubator (IKA Werke GmbH&Co. KG, Stauffen, Germany) at 30℃, 200 rpm, for 48-72 h. The bacterial cells were collected through centrifugation and the concentration was calculated as below.

$$C_m = \frac{m_p}{V_S} \quad (1)$$

where C_m was the concentration of bacterial precipitation. M_p was the mass of bacterial precipitation. V_s was the volume of suspension. This study screened five commonly used pesticides including fosthiazate, chlorofluazuron, chlorothalonil, triazolone, and acetamiprid (Table 1). 100 mg/L of each selected pesticide solution was added to basic salt culture medium, LB culture medium, and sterile water, respectively, and then different types of bacterial sludge were added. The mixture was continuously incubated at 30℃ for 7 days. The bacterial condition was determined by measuring the Optical Density (OD) value and calculated as below.

$$\Delta OD = \frac{OD_E - OD_S}{t_c} \quad (2)$$

where ΔOD was the rate of change in OD value. OD_E and OD_S were the OD values after and before the end of cultivation. t_c was the cultivation time. After high-throughput screening, the bacterial cells were further isolated and purified. Each degrading strain was then labeled. The labeled degrading bacteria were analyzed based on their molecular characteristic states. A single bacterial colony was selected and placed in 50 mL of culture medium, incubated at 34℃, 170 rpm for 15 hours. The first level seed liquid was obtained and mixed with 50 mL culture medium in a volume ratio of 1% before continuing incubation

under the same conditions for another 15 hours to obtain the second level seed liquid. 20 L of culture medium was put into a sterilized fermentation tank for 30 minutes of fermentation treatment. After the temperature of cultural medium was cooled down to below 40℃, the seed solution was injected into the tank at a volume ratio of 1% with adding defoamer and incubated at 30℃, 350 rpm, for 28 h. During the fermentation, once a large amount of foam was observed in the fermentation tank, more sterilization defoamer was added until the foam changed back to normal condition. The bacterial growth during the fermentation process was calculated as follows.

$$N(t) = N_0 e^{ut} \quad (3)$$

where $N(t)$ was the number of bacterial cells at time t. N_0 was the initial number of bacterial cells. μ was a specific growth rate. During the preparation process, the bacterial strain that reached its logarithmic phase was transferred to the fermentation environment, maintaining a volume ratio between 0.5% and 1%, and cultured to logarithmic phase again to obtain fermentation strain. The fermentation strains were reinoculated into the culture medium of the seed tank in a volume ratio of 5% - 10% and cultured until the logarithmic phase to obtain the seed liquid that was then inoculated into the culture medium in a volume ratio of 5% - 10% for final production. The calcium powder was added after completion of the fermentation before drying treatment to obtain the degraded microbial material.

Bacterial strain identification

The bacterial genomic DNA was extracted using QIAGEN DNA extraction kit (QIAGEN, Hilden, Germany) following manufacturer's instruction. The gene of 16s rRNA was amplified using the primers of 27F (5'- AGA GTT GAT CCT GCT CAG - 3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T - 3'). The polymerase chain reaction (PCR) consisted of 23.4 µL of Premix, 2.5 µL of upstream primer, 2.0 µL of downstream primer, 2.0 µL of DNA template, 2.5 µL of bovine serum albumin (BSA), and 17.6 µL of ddH_2O to a total of 50 µL using PCR kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Bio Rad T100 Thermal Cycler (Bio Rad, Hercules, CA, USA) was employed for PCR amplification with the routine amplification program. The PCR products were checked using electrophoresis and purified for subsequent DNA sequencing by BGI (Shenzhen, Guangdong, China). The sequencing results were processed using Chromas 1.62 software (https://technelysium.com.au/wp/chromas/) for sequence interval analysis and DNAMAN 5.2.2 (https://www.lynnon.com/dnaman.html) for the concatenation of forward and reverse complementary sequences to generate complete sequences [16]. MEGA 5 software (https://www.megasoftware.net/) was used to determine and identify the species of the screened strains. The identified bacterial strains were stored in glycerol at -80℃.

Field trial setup

The field experiment was conducted in a cucumber seedling field located in Shouguang Vegetable Industry Group Co., Ltd.'s plastic greenhouse (Shouguang, Shandong, China). The seedlings were planted on east-west ridges with the large ridge (operating row) of 80 cm and small ridge (planting row) of 40 cm paralleled. The double row parallel planting had a plant spacing of 25 cm, while a single row planting of about 9 plants, which were divided the experiment into a total of 18 plots with 16 plots as the sample area, 1 plot as a blank (water) control area, and 1 plot as a positive control area that did not contain microbial agents, but rather water containing mixed pesticides. The degradation rates of each experimental plot were calculated as follows.

$$DR = \frac{(Pca - Boa) - (Sample + Boa)}{Pca - Boa} \cdot 100\% \quad (4)$$

where DR was the degradation rate. *Boa* was the blank control area. *Pca* was the positive control

Table 2. Molecular identification of bacterial strains.

Pesticide	Bacterial strain	Strain number	Bacterial strain name
Fosthiazate	1	3-12	*Bacillus megalobus*
	2	11-6	*Bacillus subtilis*
Chlorofluazuron	1	2-37	*Bacillus thuringiensis*
	2	23-4	*Bacillus subtilis*
	3	2-33	*Proteolytic Bacillus*
	4	2-17	*Australian Bacillus*
Chlorothalonil	1	63-2	*Bacillus cereus*
	2	57-2	*Bacillus megalobus*
	3	21-7	*Uncultured prokaryotes*
	4	44-3	*Proteolytic Bacillus*
Triazolone	1	23-4	*Bacillus endophyticus*
	2	35-1	*Bacillus subtilis*
Acetamiprid	1	3-18	*Bacillus subtilis*
	2	66-3	*Bacillus endophyticus*
	3	25-2	*Bacillus pseudomycoides*

area. The loss of microbial agents was caused by drip irrigation. The experiment started after the plant reached 5-leaf stage. The pesticides were applied following with the subsequent addition of microbial agents with 5 days interval. The bacterial agent was applied twice during the experiment. The moisture around the roots and stems was well maintained. 6 plants in the middle of each row were selected as sampling plants. The pesticide residue amount was calculated as below.

$$R = \frac{\left(A_{EI} - A_B\right) \cdot V}{\varepsilon \cdot l \cdot m_s} \quad (5)$$

where A_{EI} was the absorbance of the extraction solution. A_B was the background absorbance. V was the total volume of the solution. ε was the molar extinction coefficient of pesticides. l was the optical path of the colorimetric dish. m_s was the quality of soil samples. About 100 g of soil sample was collected using a sampling shovel from 5 - 15 cm around the cucumber rhizosphere. The sample was stored at -20℃ for one week and cleaned up to remove the foreign objects such as crushed stones, residues, and plant roots and stems before being grounded and sieved through a 1 mm soil sieve. 20 g of the sample was dissolved in 10 mL of acidified acetonitrile solution and mixed with 0.5 mL of 3M HCl before mixing with excess NaCl and an equal volume of ethyl acetate. The sample was then centrifuged at 3,000 rpm for 20 minutes and the upper organic phase was recovered. After extraction twice, dehydration treatment was performed. The sample was dissolved and filtered again after dehydration. During the field experiment, VSP degradation efficiency was determined on different samples, and the differences in degradation efficiency between samples of different bacterial strains were compared.

Results and discussion

Identification of bacterial strains

A total of 14 spore like strains and 1 unnamed strain were identified in this study (Table 2), which were classified and arranged according to the names of the targeted pesticides.

Degradation effect of acetamiprid

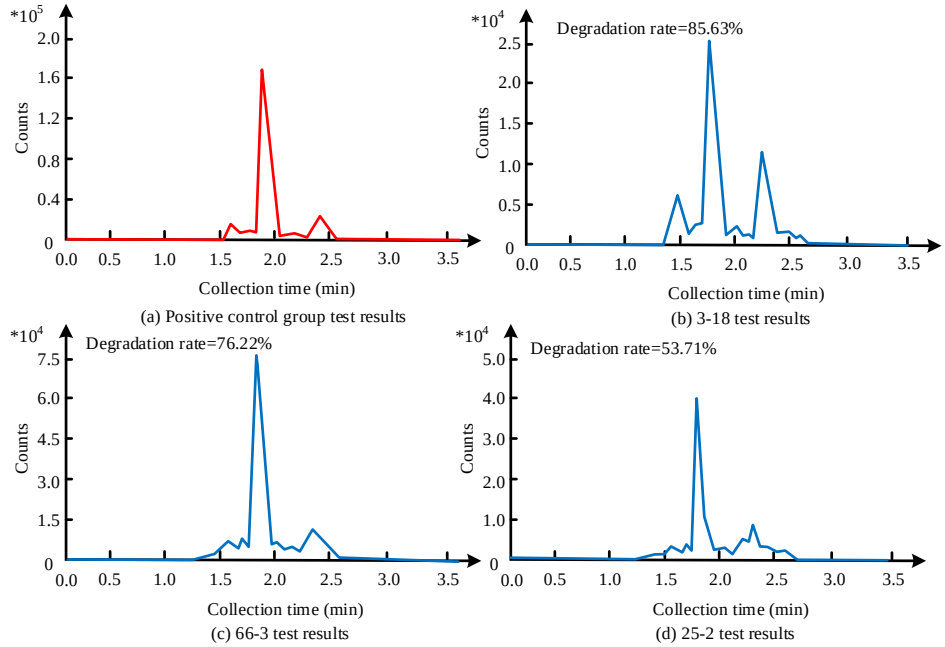

Figure 1. Degradation effect of acetamiprid.

In field experiments, targeted analysis was conducted based on the type of pesticide pollution. The acetamiprid degradation effects among the samples of positive control, 3-18 (*Bacillus mycoids*), 66-3 (*Endophytic bacillus*), and 25-2 (*Bacillus pseudomycoides*) showed that all four samples demonstrated a relatively consistent time change cycle on the detection timeline with the peak of detection reaching around 1.8 minutes and the detection point reaching 0 values around 2.5 minutes. The results showed that the degradation rate of *Bacillus mycoids* was 85.63%, while *Endophytic bacillus* was 76.22%, and *Bacillus pseudomycoides* was 53.71% (Figure 1). *Bacillus mycoides* showed the highest degradation rate compared to the others, which confirmed that *Bacillus mycoides* had the best degradation effect in treating imidacloprid pollution.

Degradation effect of Triazolone.

Among the samples of positive control, 23-4 (*Endophytic bacillus*), and 35-1 (*Bacillus mycoids*), the degradation detection line of the three samples showed a certain consistency in the detection time change cycle with the detection peak at around 2.5 minutes and reaching the 0 value detection point at around 2.6 minutes. *Endophytic bacillus* had a degradation rate of 40.22%, while *Bacillus mycoids* had a degradation rate of 57.81% (Figure 2). Therefore, *Bacillus mycoides* had a higher degradation rate and a greater degradation advantage in triazolone type pesticide pollution than that of *Endophytic bacillus*. From the pesticide residue detection standard curve (Figure 2d), the value of R^2 was 0.9936, indicating a relatively high recovery efficiency. *Bacillus mycoides* exhibited a high degradation efficiency in the degradation of triazolone pollution and had significant advantages.

Degradation effect of chlorofluazuron

The samples of positive control, 2-37 (*Bacillus thuringiensis*), 23-4 (*Bacillus subtilis*), 2-33 (*Proteolytic bacillus*), and 2-17 (*Australian bacillus*) showed the similar results in the detection time, reached the peak of detection between 12 and 15 minutes, and reached the zero detection point at approximately 13 minutes. The detection line showed the characteristic of a sudden increase, reaching a

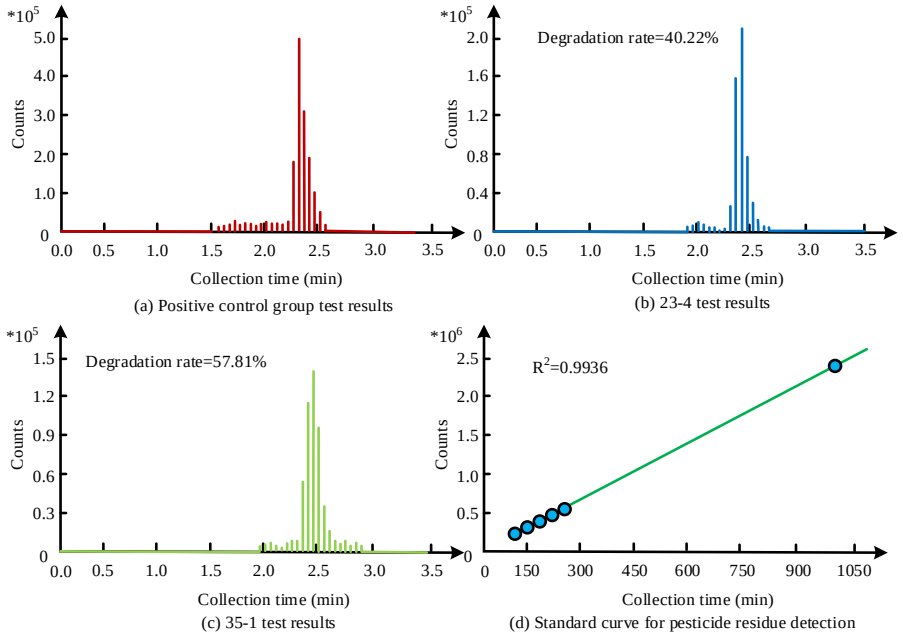

Figure 2. Degradation effect of triazolone.

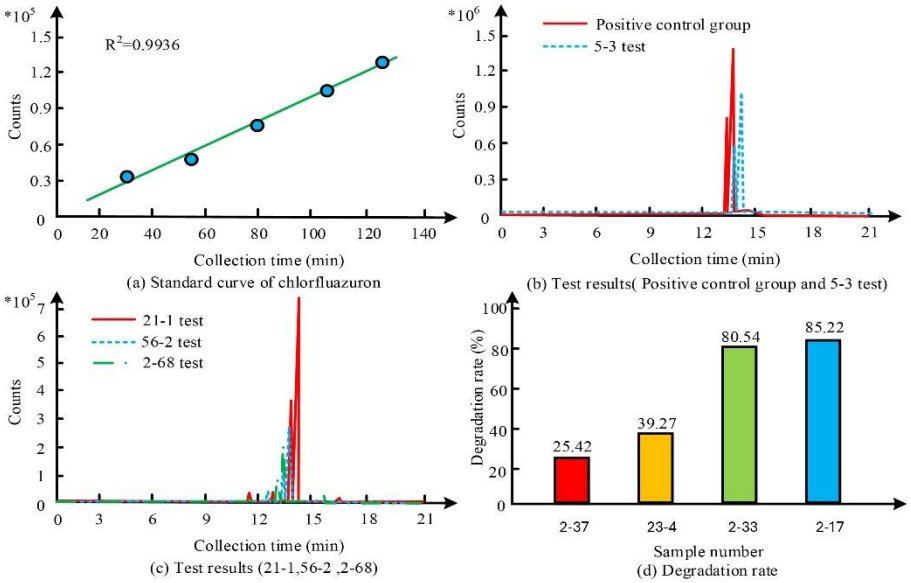

Figure 3. Degradation effect of chlorofluazuron.

peak in a short period, and then a sudden decrease, reaching a detection point with a value of 0. The degradation rates of all four samples showed a trend of increasing step by step with the degradation rate of *Bacillus thuringiensis* as 25.42%, *Bacillus subtilis* as 39.27%, *Proteolytic bacillus* as 80.54%, and *Australian bacillus* as 85.22% (Figure 3). *Australian bacillus and Proteobacter* demonstrated the higher degradation rates in the degradation of chlorpyrifos than that of *Bacillus subtilis* and *Bacillus thuringiensis* with *Australian bacillus* displaying the best degradation effect of chlorpyrifos.

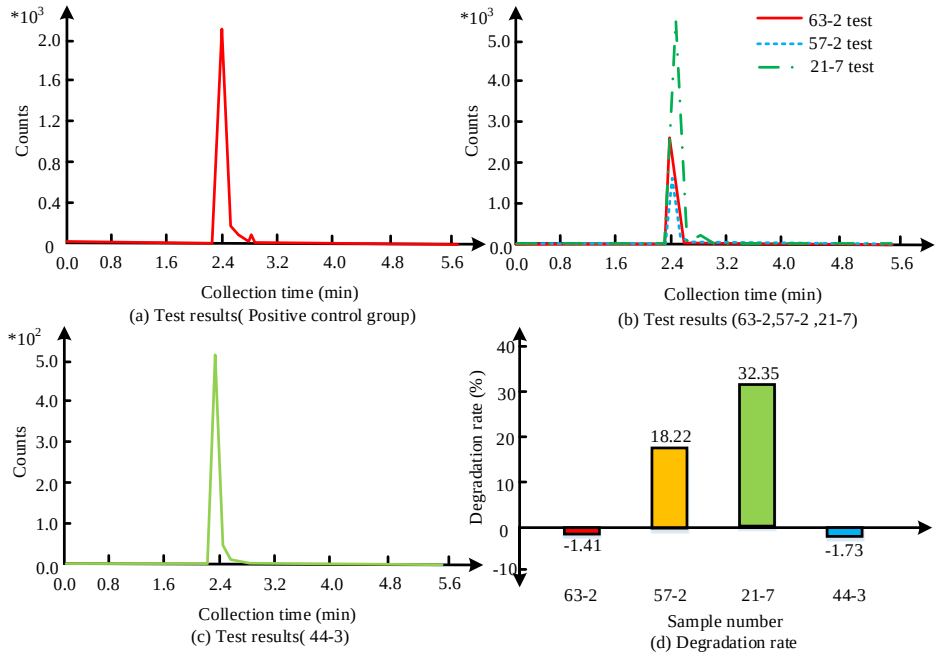

Figure 4. Degradation effect of chlorothalonil.

Degradation effect of chlorothalonil

The comparison results of samples 63-2 (*Bacillus cereus*), 57-2 (*Bacillus megalobus*), 21-7 (uncultured procaryotes), 44-3 (*Proteolytic bacillus*), and positive control showed that the detection lines of each sample reached their peak at 2.4 minutes and a zero value detection point around 2.7 minutes. The detection line of each sample demonstrated the characteristic of rapidly increasing to the highest point and then suddenly decreasing to 0 after reaching the highest point. The degradation rates of *Bacillus cereus* and *Proteolytic bacillus* were -1.41% and -1.73%, while *Bacillus megalobus* and uncultured procaryotes were 18.22% and 32.35%, respectively (Figure 4). Uncultured prokaryotes showed the highest degradation rate which was far superior to *Bacillus cereus*, *Bacillus megalobus*, and *Proteolytic bacillus*. The results confirmed that the uncultured procaryotes had the best effect in the degradation of chlorothalonil contamination.

Degradation effect of fosthiazate

The comparison between samples of positive control and 3-12 (*Bacillus megalobus*) and 11-6 (*Bacillus mycoides*) was shown in Figure 5. The detection peak appeared at around 2.8 minutes for each sample, and reached the zero value detection point at around 3.2 minutes. The results demonstrated the characteristics of suddenly increasing to the highest point and suddenly dropping to the detection point of 0 numerical value. The degradation rate of *Bacillus megalobus* was -1.36%, while the degradation rate of *Bacillus mycoids* was 30.71%. The degradation rate of *Bacillus mycoides* was higher than the others with the best degradation effect against Fosthiazate pesticide pollution. The results showed that *Bacillus mycoides* exhibited high degradation efficiency in treating thiazole phosphine pollution and was the most effective degradation strain.

This study confirmed the effectiveness of specific microbial preparations in remediation of specific pesticide contamination. Although the pollution remediation effect of microbial agents had been confirmed, the research still had some limitations, which included that this study mainly focused on universal planting environments, but the effectiveness of microbial preparations might

vary slightly in extreme planting environments. Special research on extreme environments would be the future research direction.

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