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Bacterial Isolation and Purification of Biodegradable Paclobutrazol and Its Ability Evaluation

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Abstract

Paclobutrazol can inhibit the elongation of stems. In practical application, Paclobutrazol enters the soil, causing environmental damage and pollution. By studying the ability of bacteria purified and separated from soil containing paclobutrazol to degrade paclobutrazol, the residue of paclobutrazol in soil was reduced and the environmental pollution was reduced. Methods: The strains were purified and separated by dilution coating plate method and plate scribing method, and the strains were identified by PCR, BLAST comparison and phylogenetic tree analysis. Then, the carbon source control experiment and gas chromatography (GC) were used to analyze the degradation ability of paclobutrazol. Results: The strain isolated and purified by plate streaking and limited dilution method had the highest similarity with Micrococcus yunnanensis strain Yim 65004, and the homology was 99.76% after removing the redundant sequence. From day 1 to day 11, the concentrations of paclobutrazol in the bacterial liquid were determined by gas chromatography (GC) to be 6.49g/L, 6.04g/L, 4.92g/L, 3.68g/L, 3.00g/L and 1.89g/L respectively, which showed that the degradation rate of paclobutrazol by the bacteria was 6.99% in turn with time.

Keywords

Paclobutrazol, Purification and Separation, Gas Chromatography, Degradation Rate.

1. INTRODUCTION

1.1. Research overview of paclobutrazol

1.1.1 Physicochemical properties of paclobutrazol

Paclobutrazol, also known as chlorbutazole, referred to as PP333, its chemical name is (2 RS,3 RS)-1- (4-chlorophenyl) -4, 4-dimethyl-2 - (1h-1,2, 4-triazole-1-yl) pentylene 3-ol, its molecular weight is 293.8. Polybulozole is white crystal in appearance, melting point 164℃, boiling point 384℃, vapor pressure 0.0019mPa (20℃), solubility in water is 22.9mg/L (20℃), soluble in methanol, acetone, xylene and other organic solvents.

1.1.2 Mechanism of action and application of paclpbutrazol

It is mainly manifested in the effects on the roots and leaves of plants [1]. First, the efficacy of paclpbutrazol is mainly manifested as: inhibiting the growth of taproots, making obvious difference in total fresh weight between lateral roots and absorbent roots, and increasing the thickness of absorbent roots [2]. The second reason is that the effect of polyphobuzole on the stem is mainly because it can prevent the synthesis of the growth promoter gibberellin in the plant, thus limiting the length of the cells.

Paclpbutrazol can be used as plant growth regulator, which is the most widely used triazole regulator in agricultural production [3], and can also be widely used as a fungicide.

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1.2. On the degradation of paclpbutrazol

1.2.1 Degradation of paclpbutrazol in soil

Polybutrazole is mainly used as a plant growth regulator in agricultural production. In Fucheng Ophiopogon planting in Santai County, Mianyang City, it is usually sprayed directly on the soil surface. The digestion half-life of paclpbutrazol in soil varies from 6.1-618 days [4], which is related to the initial dose, soil properties and planted crops. According to its physical and chemical properties, polybulozole has a high lipid solubility (LogP=3.11) and is easily adsorbed by soil particles, and the content of organic matter in soil will affect its digestion rate.

1.2.2 Traditional removal of paclpbutrazol

1.2.2.1 Water washing method

Washing method is a basic method that can remove other pollutants and pesticide residues on vegetables and fruits, mainly used on leaf vegetables. It can also be used in various Chinese medicinal materials [5].

1.2.2.2 Chemical method

Thermal chemical methods include direct incineration method, direct pyrolysis method, indirect pyrolysis method, non-incineration heat treatment method, etc. [6]. This kind of method is usually used for the degradation of organic matter in pesticide waste salt, and it is not used for general Chinese medicinal materials, fruits and vegetables.

1.2.2.3 Supercritical fluid extraction technique

Supercritical fluid extraction technology refers to a method of extracting active substances from liquid and solid phases by using supercritical fluid as extractant. In the field of pesticide residue analysis, Caprield et al. [7] began to use methanol as a supercritical fluid extraction agent in 1986 to study and extract bound pesticides and their metabolites from soil and plant samples.

1.2.3 Degradation of paclpbutrazol by microbial agent

Microorganisms have species, physiological and genetic diversity, and can degrade pesticides by secreting various degrading enzymes [8]. Among them, bacteria is one of the main microbial groups that degrade pesticides.

1.3. Introduction of micrococcus yunnanensis

Micrococcus yunnanensis [9] is a species of endophytic actinomyces. Actinobacteria is a Gram-positive bacterium that has a high C+ G level in its DNA, hence the name "actinobacteria". Plant endophytic actinomycetes not only have antibacterial, anti-inflammatory and anticancer pharmacological effects, but also have many functions such as promoting plant growth and improving plant stress resistance. These strains will be a new strain resource base that has not yet been explored, and will have great development and utilization value in medicine, agriculture and forestry.

2. EXPERIMENTAL MATERIALS AND METHODS

2.1. Experimental reagents and instruments

2.1.1 Experimental raw materials and reagents

The soil samples used in this experiment were taken from the fields containing polybutoxazole in Fucheng Ophiopogon producing area, Santai County, Mianyang City, Sichuan Province.

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Table 1. Main experimental reagents

Reagent Name	Reagent Specification	Manufacturer
Sodium hydroxide	AR	Chengdu Jinshan Chemical Reagent Co., LTD
Peptone	RG	Chengdu Colon chemical Co., LTD
Glucose	LR	Chengdu Colon chemical Co., LTD
AGAR	LR	Chengdu Colon chemical Co., LTD
Anhydrous ethanol	AR	Chengdu Colon chemical Co., LTD
Beef extract	RG	Chengdu Colon chemical Co., LTD
Ethyl acetate	AR	Chengdu Colon chemical Co., LTD
Paclpbutrazol standard (purity ⩾ 99%)	C16-P10793-250mg	Shanghai Chuangsai Technology Co., LTD

2.1.2 Experimental instruments and equipment

Table 2. Main experimental instruments and equipment

Instrument and equipment Name	Model	Manufacturer
Clean bench	SW-CJ-1FD	Suzhou Antai Air Technology Co., LTD
Autoclave	TOMY SX-700	TOMY KOGYO CO., LTD
Electronic analytical balance	FA2004	Ningbo Yinzhou Huafeng instrument factory
Electronic scales	ALC-210.4	Sartorius technology Instrument Co., LTD
Centrifuge	Thermo	Shanghai Yiheng Scientific Instrument Co., LTD
B220 thermostatic water bath	B220	Shanghai Yarong biochemical instrument factory
Gas chromatograph	SHB-Ⅲ	Zhengzhou Great Wall Science and Trade Co., LTD

2.1.3 Preparation of medium

The preparation operation is as follows:

(1) Prepare the solution

Add a portion of the required water to the container, weigh the various raw materials according to the formula of the medium, add them in order to dissolve them, and finally replenish the required water.

(2) Adjust pH value

pH test paper (or pH potentiometer, hydrogen ion concentration colorimeter) is used to detect the pH value of the medium.

(3) Filtration

Using filter paper, gauze or cotton cloth, the mixed medium is filtered out at high temperature.

(4) Packaging

The filtered media should be repackaged. To prepare the beveled culture solution, the culture solution needs to be separately loaded into the test tube.

(5) Add cotton plugs

After repacking, use a cotton stopper to close the hole in the tube or bottle.

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(6) Prepare plate medium

Put the just sterilized conical bottle and petri dish with the culture medium on the experimental platform, and light the alcohol lamp, about 10 ml per dish, so that the bottom of the dish is full.

2.2. Experimental method

Experimental procedures: purification, isolation and strain identification → determination of the degradation ability of paclpbutrazol by controlled carbon source experiment → determination of the degradation ability by gas chromatography (GC).

2.2.1 Purification and isolation of strains

Strains resistant to paclpbutrazol were isolated and purified from soil, and the specific steps of separation and purification were shown in 2.1.3

2.2.2 Strain identification (PCR identification, BLAST comparison and evolutionary tree mapping)

According to Scientific Compass company, the isolated bacterial strain was named HY1. After genome extraction and PCR amplification of the DNA sequence, through BLAST comparison and evolutionary tree drawing, to complete the identification of strains.

2.2.3 Determination of degradation ability of paclpbutrazol

2.2.3.1 Control carbon source test to detect degradation ability

The strain was diluted and coated on paclpbutrazol inorganic salt solid medium. After 48 hours of culture, the strain could use paclpbutrazol as carbon source for growth. The degradation capacity can be judged according to the concentration of polyphobulozole in the final medium.

2.2.3.2 The degradation capacity was detected by gas chromatography (GC)

(1) In order to evaluate the degradation effect of paclpbutrazol by bacteria, the degradation rate was compared by blank control in this experiment:

Table 3. Grouping of paclpbutrazol degradation experiments

Group	difference
1 set	15% paclpbutrazol pesticide (100mg), HY1 bacterial solution (50ml)
2 sets	15% paclpbutrazol pesticide (100mg)

(2) Both were cultured at the same time, and the degradation time was used as the GC determination node: Original, 3d, 5d, 7d, 9d, 11d.

(3) Making standard working curve: The five concentrations of 1.5, 3.0, 6.0, 15.0, 30.0mg/L were used as nodes to make standard working curve by external standard method, so as to obtain the relationship between the content of polybulozole and the column height of the gas chromatogram.

(4) Liquid culture bacterial solution: After the inoculated bacterial solution was cultured in a shaking table for 3d, 10ml of the culture solution was taken and the wavelength was 600nm to determine the absorbance. 10ml was taken from the original bacterial solution for determination at each time node. After 2ml bacterial solution was centrifuged for 1min, the supernatant was obtained and the gas phase was determined

(5) The temperature program is set as follows: the initial temperature is 120℃, and it rises to 260℃, at a rate of 5℃,per minute for 10 minutes. The samples were filtered with a 0.45 μm

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microporous filter membrane. The ion source temperature is 230℃, the transmission line temperature is 280℃, the injection mode is no shunt, the injection temperature is 20.0℃, the injection time is 1 minute and the injection volume is 1 μL.

The formula for the residual concentration of paclpbutrazol is as follows:

Residual concentration X= (A*c*V)/(As*m) (Equation 2-2)

X- Residual amount of paclpbutrazol in the sample, expressed in g/L

Peak area of paclpbutrazol in A- sample solution

Peak area of paclpbutrazol in AS-standard working solution

c-Concentration of polybulobutazole in standard working solution, expressed in μg/ml

V- final constant volume of sample liquid, unit is ml

m- The mass of the sample represented by the final sample, expressed in g

The degradation rate formula is as follows:

Degradation rate =(M2-M1)/m1*100% (Formula 2-2)

m2- Next day's polybulobutenol content, expressed in g/L

m1- The amount of polybulobutenol on day one, expressed in g/L

2.3. Data processing and analysis

All experiments were repeated in parallel for three times. Excel 2010 and origin software were used for data processing and statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Purification and isolation of strains

3.1.1 Preliminary screening of strains

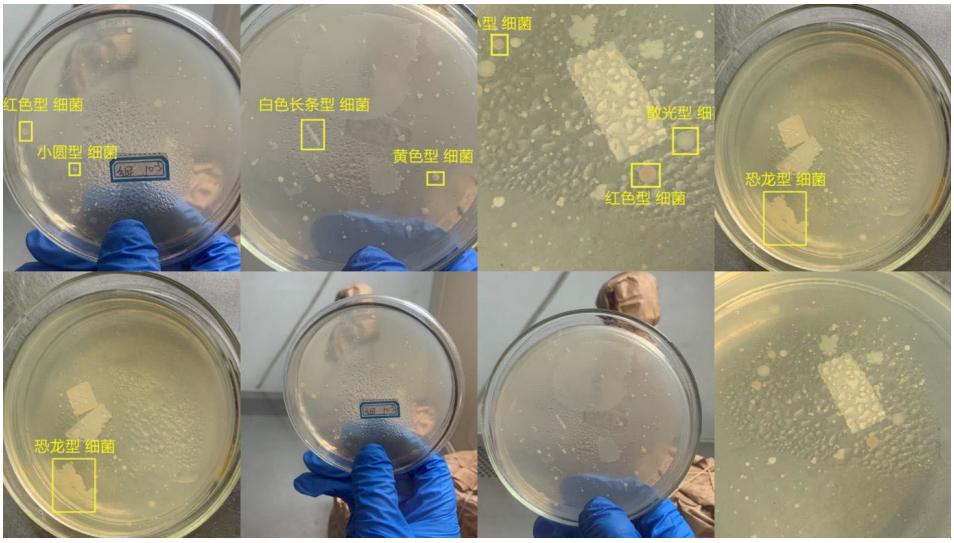

Figure 1. Color and morphology of 8 species of bacteria

After 48 hours of cultivation in the constant temperature incubator, it can be seen from Table 3 and Figure 1 that the growth of other bacteria except dinosaur type was relatively good. Most of the above growth conditions are better, small bacteria and small circular bacteria should be the same strain.

3.1.2 The results of scribing culture of strains

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The 8 kinds of bacteria preliminarily screened were observed after the first culture for 48h by using plate scribing method. As shown in FIG. 2, yellow and red spherical bacteria were taken as the results after the initial screening and as the object of subsequent experimental research. In this experiment, yellow bacteria were selected for subsequent identification and degradation effect test.

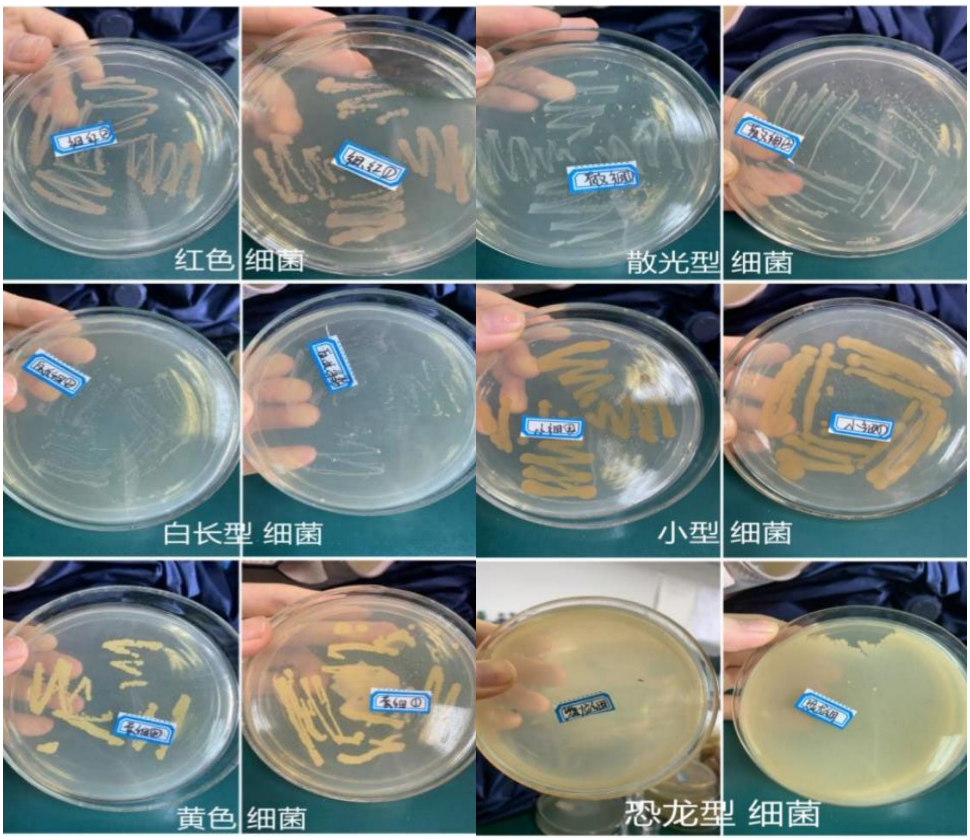

Figure 2. Streak culture diagram of strains

3.2. Strain identification result

3.2.1 Extraction and PCR amplification of bacterial genome

According to agarose gel electrophoresis, the swimlane from the left of the image is HY1, the blank control, and the rightmost swimlane is DL5000 DNA Marker. The sample has obvious bands between about 1500 bp, which proves that the 16SrDNA region of the sample is successfully amplified.

3.2.2 Blast comparison and morphological observation results

(1) The sequencing results were spliced with ContigExpress, and the inaccurate parts at both ends were removed.

(2) The concatenated sequences were compared in the ncbi database, using the rRNA_typestrains/16S_ribosomal_RNA database.

(3) Select the species with the highest homology. The strain HY1 had the highest similarity with Micrococcus yunnanensis strain YIM 65004, and the homology was 99.76% after removing the cephalic and cephalic excess sequences.

3.2.3 Evolutionary tree test results

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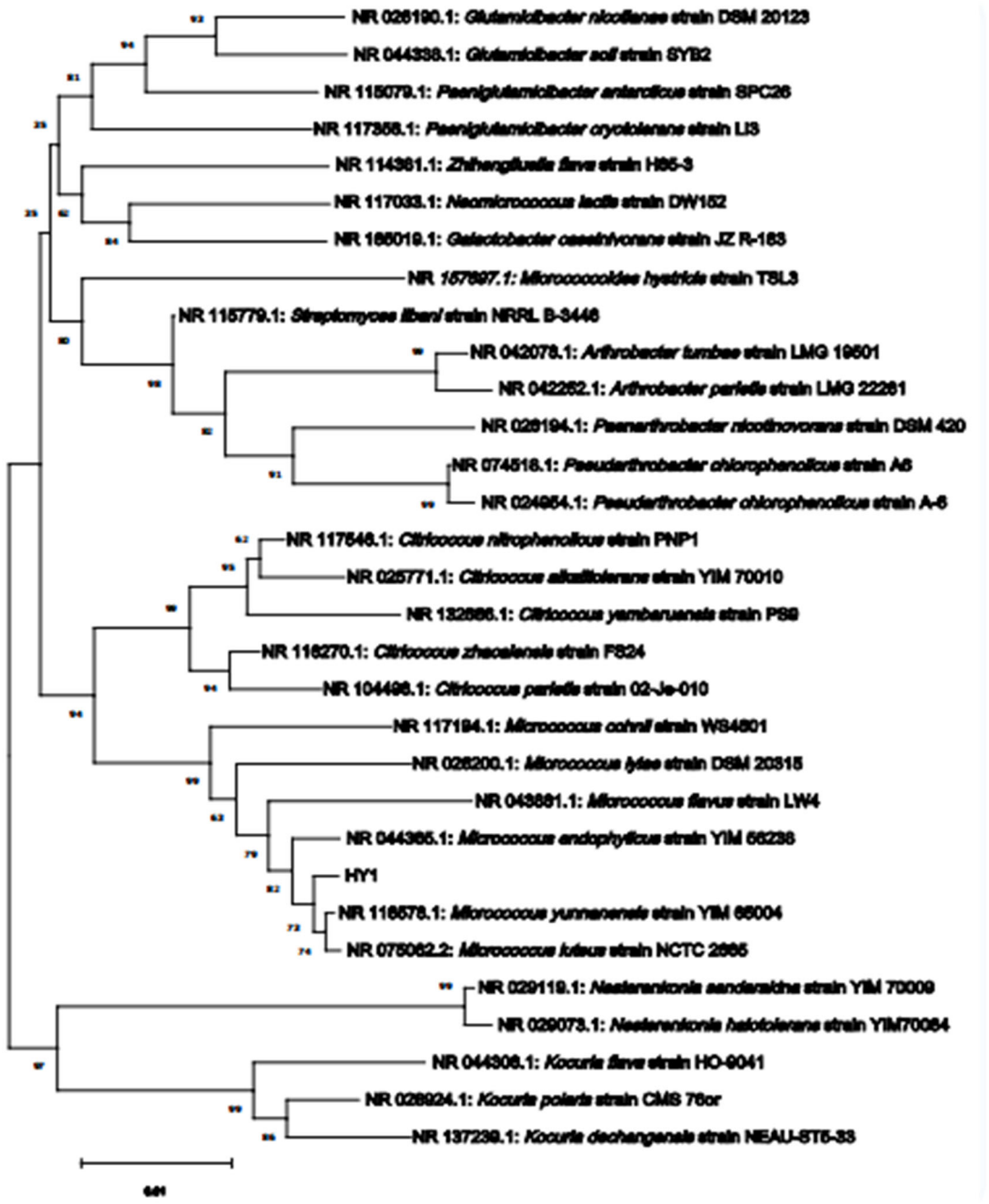

Figure 3. Evolutionary tree test results of strains

As shown in Figure 3, strain HY1 and Micrococcus yunnanensis strain YIM 65004 have the highest similarity, and the homology is 99.76% after removing the head and tail redundant sequences. Homology with Micrococcus aloeverae strain AE-6 is 99.68%, homology with Micrococcus luteus strain NCTC2665 is 99.60%. According to the results of the evolutionary tree, HY1 strain could be classified into Micrococcus.

3.3. Determination of degradation ability of polyphobulozole

3.3.1 The degradation ability was detected by controlled carbon source experiment

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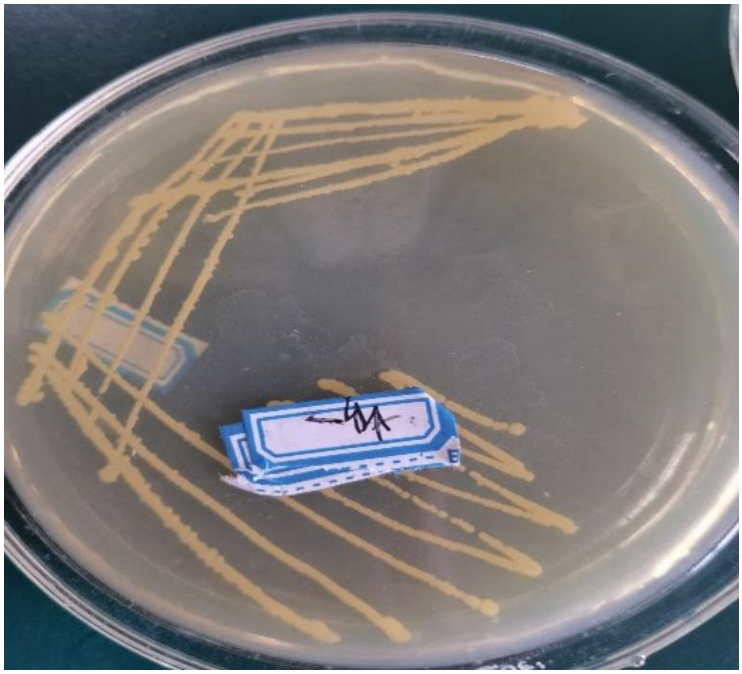

Figure 4. Bacteria can grow in medium containing paclpbutrazol as a carbon source

3.3.2 The degradation ability was detected by gas chromatography (GC)

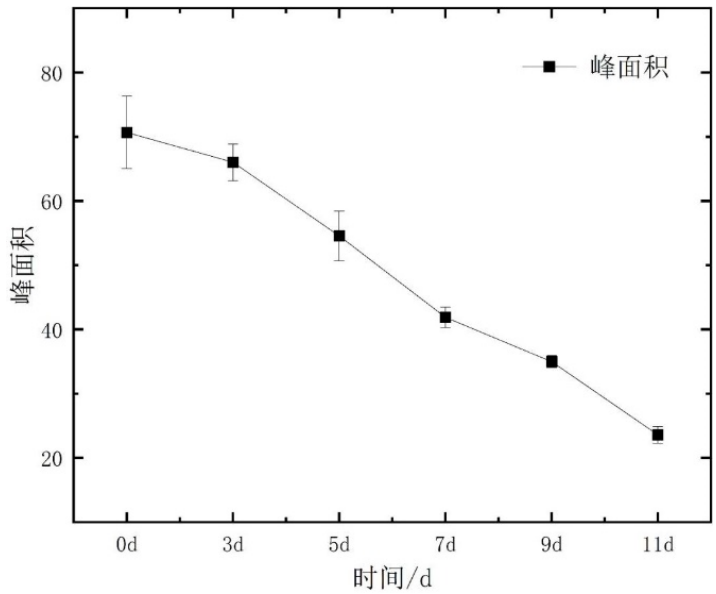

Figure 5. Change of average peak area of liquid containing pleurobutenazole over time

As shown in Figure 5, the average peak area of the liquid containing polylobulozole decreased with the passage of time. The peak area decreased the fastest from the 3rd day to the 7th day, indicating that the bacterial degradation of polybulobutrazole was the highest and the degradation ability was the strongest during this period. It was also consistent with the OD600 value of the bacterial solution, and the bacteria in the culture solution were in a stable growth stage, reaching the maximum bacterial concentration in culture.

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Table 4. Results of the change and degradation rate of polybulobulozole in bacterial solution with time

Time/day	Paclpbutrazol concentration g/L	Relative degradation /% (compared to the previous ratio)	Degradation rate /%
0	6.49		
3	6.04	6.99	6.99
5	4.92	18.53	24.23
7	3.68	25.21	43.33
9	3.00	18.36	53.73
11	1.89	37.03	68.68

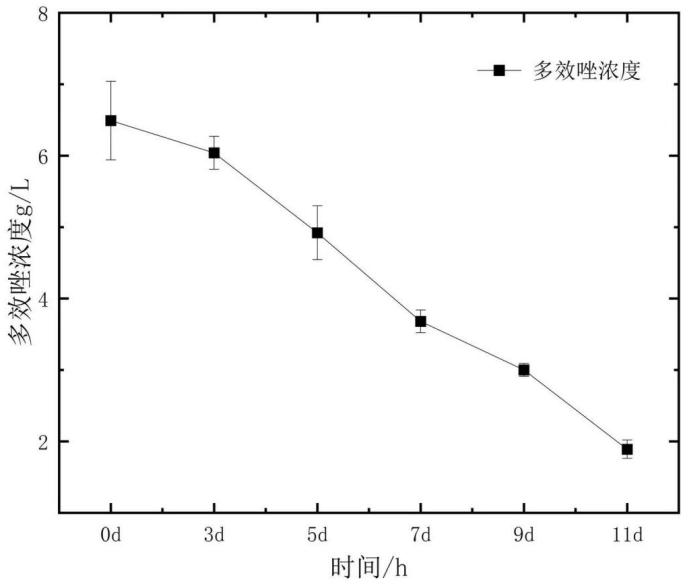

Figure 6. Change of paclpbutrazol concentration in bacterial solution with time

As shown in Table 4 and Figure 6, the concentration of paclpbutrazol in bacterial solution showed a decreasing trend as time went on. The results were consistent with the average peak area of the bacteria solution containing polybulobulozole as shown in Figure 5. From day 3 to day 7, the concentration of polybulobulozole decreased at the fastest rate, indicating that the bacteria in the bacteria solution had the most degradation amount and the strongest degradation ability of polybulobulozole during this period. On the 11th day, the degradation rates of paclpbutrazol were 6.99%, 24.23%, 43.33%, 53.73% and 68.68%, indicating that the bacteria had a good ability to degrade paclpbutrazol, and its degradation ability increased with the increase of the number of bacteria.

4. CONCLUSION

In this paper, 8 strains with good growth conditions were screened through strain isolation and purification experiments, and then measured by gas chromatography. Finally, the degradation rate of the strain was calculated. It was found that the concentration of paclpbutrazol continued to decline over time, and the degradation rate reached 68.68% on the 11th day, so the pesticide paclpbutrazol residue had good degradation effect. To provide some theoretical support for the efficient degradation of paclpbutrazol microbial agents.

This paper actively responds to China's policy of strengthening agriculture and benefiting agriculture, focuses on promoting the research of microorganisms in the field of degrading pesticides, and based on experimental projects, can vigorously develop the practical application

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of their strains, promote the reform of China's agricultural pesticide system, build an environmentally friendly society, and increase economic benefits. Microbial degradation is the most important and thorough way to purify pesticides in soil. The biological remediation method that uses organisms themselves or biological products to degrade pollutants has the advantages of non-toxicity, no residue, etc. It is a relatively cheap and effective way. When microbial microbial agents are used as fertilizers, they have different inhibitory effects on crop diseases and pests. Due to the particularity of the isolated strains, this paper provides a new idea for solving the residues of paclpbutrazol and promotes the development of pesticide agriculture in China.

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REFERENCES

[1] S. Zhang, W.J. Yuan, X.M. Song : The impact of paclobutrazol on plant physiology and ecology and its application prospects in the field of desertification, Research on temperate forestry, Vol.5(2022) No.1, p.6-11.

[2] X.M. Song, W.J. Yuan, Y. Yu : The effect of paclobutrazol on the structural characteristics of caragana seedlings, Resources and Environment in Arid Areas, Vol.31(2017) No.12, p.184-188.

[3] T. Yang, L.R. Wei: High performance liquid chromatography analysis of 25% paclobutrazol suspension, Sichuan Chemical Industry, Vol.14(2007) No.4, p.28-30.

[4] B. Wang, L. Lang, Y.B. Ji: Research progress on the removal technology of pesticide residues in traditional Chinese medicine, Heilongjiang Science and Technology Information, Vol.68(2012) No.36, p.26.

[5] Y.Q. Zheng, Z.Y. Liu, L. Li: Degradation of organic matter in pesticide waste salt by Thermochemistry method, Environmental Pollution and Prevention, Vol.43(2021) No.7, p.822-828.

[6] Y.Y. He: The value and application of microbial fertilizer in agricultural production, Industrial Microbiology, Vol.52(2023) No.2, p.8-10.

[7] M. Jafari, Z.F. Rokhbakhsh: Cytotoxic and antibacterial activities of biologically synthesized gold nanoparticles assisted by Micrococcus yunnanensis strain, Biocatalysis and Agricultural Biotechnology, Vol.15(2018) No.7, p.245-253.