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RESEARCH ARTICLE

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Isolation and screening of microorganisms capable of degrading nicosulfuron in water

Abstract In order to screen and isolate microorganisms capable of degrading nicosulfuron, five strains of microorganisms coded as YF1, YB1, YB2, YB3, and YB4 that can take nicosulfuron as the only source of carbon, nitrogen, and energy were obtained by enrichment culture. Of the five strains, YF1 was a fungus and the others were bacteria. All of the microorganisms were inoculated into the culture media with different concentrations of nicosulfuron and shaking culture was performed for 5 days at 30°C and 150 r·min^{-1}. High Performance Liquid Chromatography (HPLC) was used to detect the concentration of nicosulfuron and calculate the degradation efficiency. The results showed that the degradation rates of the five strains of microorganisms were higher in low concentrations than in high concentrations of nicosulfuron. YF1 had the highest degradation rate of 80.31%, followed by YB1 and YB2 with degradation rates of 78.18% and 73.72%, respectively. However, YB3 and YB4 had lower degradation rates of 36.82% and 25.75%, respectively. Upon primary identification of the three strains of microorganisms with higher degradation rates, it was discovered that YF1 was *Aspergillus niger*, while YB1 and YB2 were *Bacillus* sp.

Keywords nicosulfuron, microbial degradation, enrichment culturing

1 Introduction

Nicosulfuron is a kind of sulfonylurea herbicide applied at post-emergence in maize. Nicosulfuron has a broad and super-high function against annual gramineous plants, such as broad-leaved weeds and sedge weeds (Morton et al., 1991; Yang et al., 1998). Nicosulfuron has been widely used in China because of its low dosage, effectiveness, and safety to maize (Su, 2003). However, nicosulfuron has strong transferability, which can easily cause groundwater to be contaminated (Zhang et al., 2006). High concentrations of nicosulfuron exist in industrial wastewater from manufacturers producing this kind of herbicide. Therefore, it is important to study the degradation of nicosulfuron in water.

It has been shown that microbial degradation is one of the most important approaches to decomposing sulfonylurea herbicide residue in the environment (Joshi et al., 2003). Kulowski et al. (1997) had isolated microorganisms such as *Streptomyces griseolus*, *Aspergillus niger*, *Penicillium* sp. and so on, which were capable of degrading sulfonylurea herbicide. Joshi isolated three strains of microorganisms from soil, including *Streptomyces griseolus*, *Aspergillus niger*, and *Penicillium* sp., which were capable of decomposing chlorsulfuron, and *Aspergillus niger* and *Penicillium* sp., hydrolyzing the sulfonylurea bridge and producing homologous sulfonylureas and heterocycle (Joshi et al., 1985). Shen et al. (2002) screened thirty strains of microorganisms capable of decomposing metsulfuron from the moist soil tamed by bacteria, fungi and actinomycetes, with metsulfuron as the only source of carbon. Wang et al. (2003) reported that *Penicillium* sp. could remediate soil polluted by metsulfuron. So far, no reports on biodegradation of nicosulfuron have been published. In this study, we isolated five strains of microorganisms capable of degrading nicosulfuron from the wastewater drained by pesticide plants, of which three strains with a high ability were screened. The objectives of this study were to isolate and screen the strains of microorganisms with highly efficient degradation of nicosulfuron by enrichment culturing, and try to provide a theoretical basis for the possibility of bioremediation.

2 Materials and methods

2.1 Sample source

Experimental water samples were taken from wastewater treatment pools of nicosulfuron pesticide plants.

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Medicament was 94.87% nicosulfuron (Shandong Jingbo Agrochemicals Company).

2.2 Culture media

The enrichment medium was made of 10 g peptone, 1 g NaCl, 1 g KH_2PO_4, 1.0 g dextrose, and 1000 mL distilled water at pH 7.0. The basic medium was made of 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g NaCl, 0.5 g $MgSO_4$, and 1000 mL distilled water at pH 7.0. The potato dextrose agar medium was made of 200 g potato, 20 g peptone, 20 g agar and 1000 mL distilled water.

2.3 Isolation of the microorganisms

2.3.1 Taming and enrichment culture of the microorganisms

Nicosulfuron was added into prepared water samples at the concentration of 500 mg·kg^{-1}, then cultured at 30°C and 150 r·min^{-1}. After being cultured for fourteen days, 5 mL tamed water was put into the enrichment medium (50 g) at the nicosulfuron concentration of 200 mg·kg^{-1}, and cultured with shaking for 7 days at 30°C and 150 r·min^{-1}. Thereafter 5 mL of the liquid medium was transferred to a 50 g enrichment medium (the concentration of nicosulfuron was 200 mg·kg^{-1}), and cultured under the above conditions. The process was then repeated five times.

2.3.2 Isolation and identification of the microorganisms

The liquid medium with microorganisms was spread on PDA plates containing 400 mg·kg^{-1} nicosulfuron, cultured at 30°C for 48–96 hours to separate the single clones with transparent circle around the PDA plates, which continued to be cultured, and the strains were conserved. If they were fungi, they could be primarily identified based on their characteristics of colonial morphology and structure, spore size and so on (Wei et al., 1979); if bacteria, they could be identified according to their colonial morphology and color, biological characteristics, physiological and biochemical tests including Gram reaction referring to Bergey's Manual of Determinative Bacteriology (Buchanaan and Gibbons, 1974).

2.4 Calculation of the degradation efficiency of nicosulfuron

2.4.1 The chromatographic conditions of nicosulfuron by HPLC

Agilent 1200 LC High Performance Liquid Chromatography (HPLC) was Zorbax Eclipse XDB-C-18 (125 mm × 4.0 mm, I.D 5 μm). The temperature of the column was 30°C. The wavelength was 240 nm. The mobile phase was acetonitrile-water-glacial-acetic acid at the ratio of 30:70:0.05 (v/v). Flow rate was 1.0 mL·min^{-1}. The injection volume was 2 μL.

2.4.2 Drawing the calibration curve of nicosulfuron

0.1336 g nicosulfuron was diluted with methanol into 1000 mg·kg^{-1}, then rediluted with methanol into 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg·kg^{-1}, following the conditions described in 1.4.1 to determine the relative quantity of nicosulfuron. The concentration of nicosulfuron and the peak area were used as the abscissa and the vertical, respectively, to draw the standard work curve.

2.4.3 Determination of the added recovery of nicosulfuron in the basic medium

Nicosulfuron was added into 50 g basic mediums to create the concentrations of 0.2, 2 and 200 mg·kg^{-1}, and then cultured with shaking at 30°C and 150 r·min^{-1} for 2 hours. The mediums were extracted using the same volume of dichloromethane and divided into the organic phase, which was dehydrated using anhydrous sodium sulfate. Finally the extract was concentrated into 2 mL in a rotary vacuum evaporator at 40°C, removed from the solvent under N_2, and redissolved in methanol (5 g). Each treatment was replicated 3 times. The method of 1.4.1 was used to calculate the quantity of nicosulfuron, the added recoveries and the coefficients of variation.

2.4.4 Determination of the degradation efficiency of nicosulfuron

All of the isolated strains were inoculated on PDA plates, cultured for 3 days at 30°C, suspended using aseptic water, and thereafter centrifuged at 4000 r·min^{-1} for 5 min. The precipitate was washed using aseptic water, and recentrifuged. The precipitate was diluted using aseptic water to make into a suspension at 2.35×10^7 CFU·mL^{-1}. One milliliter suspension was inoculated into 50 g basic mediums at nicosulfuron concentrations of 2, 10 and 200 mg·kg^{-1}. The uninoculated mediums containing nicosulfuron were used as control. Each treatment was replicated 3 times. All of the treatments were cultured under the conditions of 30°C at 150 r·min^{-1} for 5 days. The content of nicosulfuron was determined with the method given in 1.4.1, and the degradation efficiency of nicosulfuron was calculated by the following formula:

$$\text{Degradation rate of nicosulfuron (\%)} = \frac{\text{content of control } (\text{mg}\cdot\text{kg}^{-1}) - \text{content of treatment } (\text{mg}\cdot\text{kg}^{-1})}{\text{content of control } (\text{mg}\cdot\text{kg}^{-1})} \times 100$$

3 Results and analysis

3.1 Isolation and purification of microorganisms

Microorganisms in the provided water were tamed and enrichment cultured at high concentration of nicosulfuron, and were inoculated on PDA plates containing 400 mg·kg^{-1} nicosulfuron. After they were cultured for 48 hours, transparent circles appeared around some microorganisms (Fig. 1). The degrading strains coded as YF1, YB1, YB2, YB3, and YB4 were conserved. According to the results of the degradation efficiency of the isolated strains, three strains of microorganisms with higher degradation rates were preliminarily identified. The results showed that, YB1 and YB2 were rod-shaped bacterium which could produce spores (Fig. 2); of the two, YB1 was brown, with irregularly compressed colonies, and YB2 had raised and folded light yellow colonies. The biological characteristics, as well as the results of biochemical and physiological tests of the two strains are shown in Table 1. By referring to Bergey's Manual of Determinative Bacteriology (eighth edition), YB1 and YB2 were preliminarily identified as *Bacillus* sp. After being cultured for 48 hours, YF1 was found to be a fungus with a light yellowish colony (Fig. 1), and so was identified as *Aspergillus niger*.

3.2 Calculation of the degradation efficiency of nicosulfuron

3.2.1 Calibration curve of nicosulfuron

Our test results (Fig. 3) show that the regressive equation of the calibration curve of nicosulfuron was $y = 3.4648x - 4.0895$, and the correlation coefficient was 0.9998 ($n = 9$). Thus, our method could quantitatively analyse the contents of nicosulfuron in wastewater.

3.2.2 Determination of the added recoveries of nicosulfuron in the basic medium

Figure 4 shows that nicosulfuron could be separated from impurities under the provided conditions, the retention time was about 5.3 minutes, the average recovery rate was higher than 90%, and the relative standard deviation was less than 8% (Table 2). Therefore, nicosulfuron could be extracted from basic mediums with dichloromethane.

3.2.3 Determination of degradation efficiency of nicosulfuron

Table 3 shows that the degradation efficiency of low concentration was higher than that of high concentration. In the basic medium containing 2 mg·kg^{-1} nicosulfuron, the degradation efficiency of YF1 was the highest, about 80.31%, followed by YB1 and YB2, whose degradation rates were 78.18% and 73.72%, respectively. The degradation rates of YB3, YB4 were only 36.82% and 25.75%, the lowest. The degradation rates of YF1, YB1, YB2, YB3 and YB4 were lower in the basic medium containing 10 mg·kg^{-1} nicosulfuron than in 2 mg·kg^{-1}. However, the lowest degradation rates were observed in the basic medium containing 200 mg·kg^{-1} nicosulfuron for all the five strains.

4 Discussion

Bioremediation is a valid environmental biological technology for purging organic contaminants. The goal was to use various viable methods to accelerate and enhance the capability of microorganisms to degrade organic contaminants. Using microorganisms to degrade residual herbicides in water and soil is feasible and of great significance in practical application. Moreover, there have been many

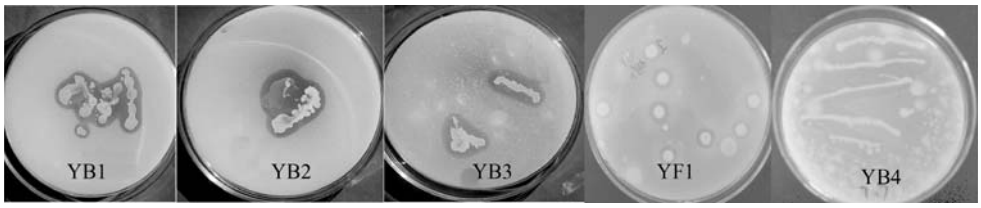

Fig. 1 Degrading rings formed by strains on agar plates

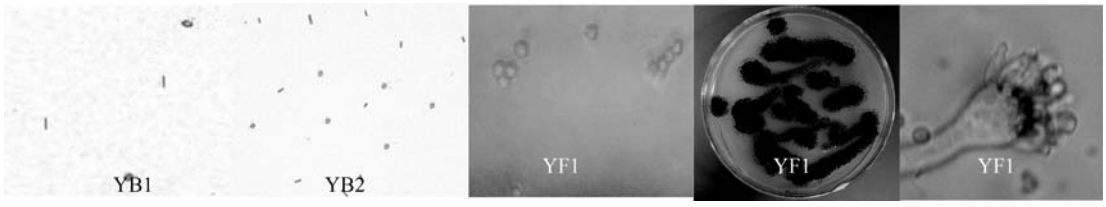

Fig. 2 Morphological characters of colonies, hyohae and spores of strains

Table 1 Physiological and biochemical characteristics of strains capable of degrading nicosulfuron

strains	YB1	YB2
Gram reaction	+	+
cellular shape	rod	rod
movable or not	movable	movable
catalase	+	+
Methyl red test	−	−
Voges-Proskauer test	+	+
fermentation of glucose	+	+
hydrolytic starch	+	+
citrate utilization test	+	+

Note: "+"means positive; "−"means negative.

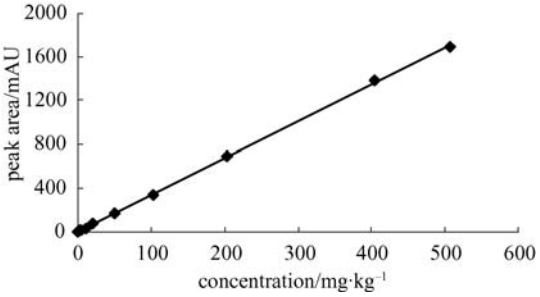

Fig. 3 Calibration curve of nicosulfuron

successful application examples (Wang et al., 2003; Zhang et al., 2002; Yan et al., 2005). Nowadays, there are two effective routine methods to degrade pesticides: one is to use isolation and screening to obtain strains from long-polluted environments; the other is by enrichment culturing to obtain active strains (Yi et al., 2000; Wu et al., 2004; Liu and Li, 2003; Mandelbaum et al., 1995). If strains are isolated from the polluted environment directly, there will be numerous varieties of microorganisms. This will increase the workload for screening the target microorganisms.

Table 2 Recovery and variation coefficients of nicosulfuron fortified in culture medium

fortified concentration/mg·kg^{-1}	average recovery/% ($X \pm S$)	$C.V.$/%
0.2	96.32 ± 2.6	3.2
2	98.58 ± 6.7	7.6
200	94.02 ± 2.8	3.3

Note: All the recoveries are averages of three replications; S means the standard deviation.

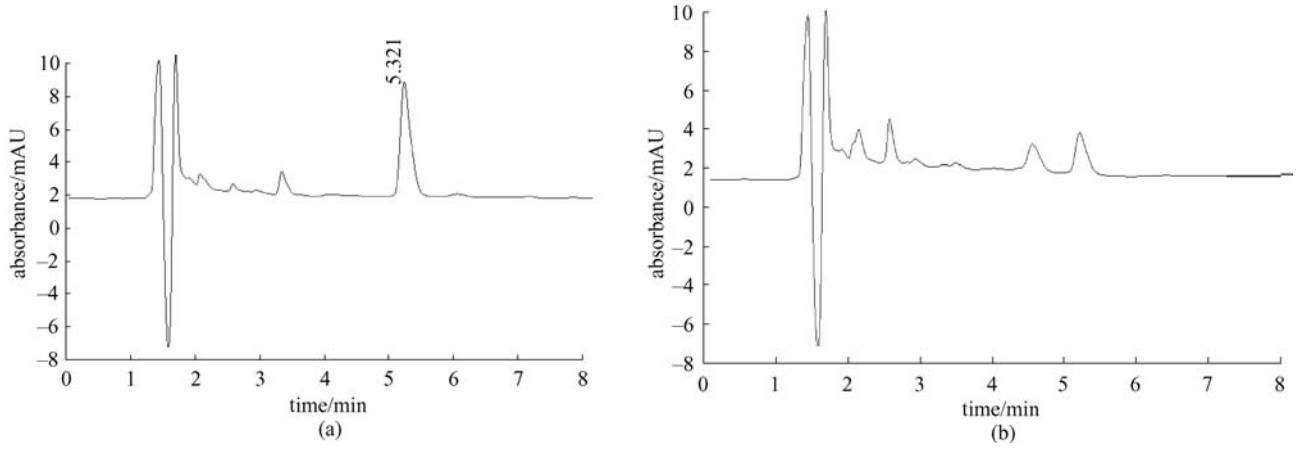

Fig. 4 High Performance Liquid Chromatograms of nicosulfuron under different conditions
Note: A represents CK; B represents the inoculated strain of YB1.

Table 3 Degradation rates of strains at different concentration of nicosulfuron

concentration of nicosulfuron/ mg·kg^{-1}	degradation rates of different strains/%				
	YF1	YB1	YB2	YB3	YB4
2	80.31****a	78.18****a	73.72***a	36.82**a	25.75*a
10	62.12****b	70.42*****b	37.39***b	30.50**b	19.86*b
200	51.50***c	22.84**c	12.63*c	10.22*c	9.23*c

Note: The different letters in the same column indicate significantly different at $P = 0.05$. The different * in the same row indicate statistically significant difference at $P = 0.05$.

Therefore, our study collected water samples polluted by nicosulfuron for a long time to screen target microorganisms by taming the microorganisms in water in the medium, in which nicosulfuron was used as the only source of carbon, nitrogen, and energy. After being tamed for 5 generations, microorganisms using nicosulfuron as nutrient substances would be the dominant populations. Through screening, we found that the varieties of microorganisms in the tamed medium on the PDA plates were far less than in water samples. After being cultured in the enrichment medium, microorganisms in the liquid medium were spread on the non-transparent PDA plates containing nicosulfuron. Several days later, some microorganisms formed transparent circles due to their decomposition of nicosulfuron. This method can easily screen out the active decomposing strains and reduce the workload.

The integrated evaluation of degradation efficiency of the strains shows that the degradation capability of the bacteria is lower than that of fungus, a conclusion which needs further tests to confirm. In our test, strains with a better degradation capability were screened and identified according to their characteristics of colonial morphology, namely: color, biological characteristics, and biochemical and physiological tests including Gram reaction. However, further identification of the microorganisms using 16S rDNA sequence analysis is needed since we are still in lack of their genic data. The optimum conditions for degradation and the degradation mechanism also need further study.

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