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DEGRADATION OF NICOSULFURON BY *Bacillus subtilis* YB1 AND *Aspergillus niger* YF1

© 2012 X. H. Lu*, Z. H. Kang*, B. Tao*, Y. N. Wang*, J. G. Dong**, J. L. Zhang*

*College of Plant Protection, of Agricultural University of Hebei, Baoding 071001, China

**College of Life Sciences of Agricultural University of Hebei, Baoding 071001, China

e-mail: zhangjinlin@hebau.edu.cn

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The optimal degrading conditions for the nicosulfuron degradation by *Bacillus subtilis* YB1 and *Aspergillus niger* YF1, and site of their action on nicosulfuron were studied. The results showed that the degradation efficiency of free cells of *B. subtilis* YB1 and *A. niger* YF1 was respectively 87.9 and 98.8% in basic medium III containing 2 mg/l of nicosulfuron after inoculation with 1 ml of culture containing 2.3×10^7 CFU ml^{-1} and incubation for 5 days at 35°C. Moreover, the degradation rate of nicosulfuron by the mixture of microorganisms was much higher than for every of them taken separately in the same conditions. The mass spectrometric analysis of the products degraded by *B. subtilis* YB1 revealed that the sulfonylurea bridge in nicosulfuron molecule had been broken. Extracellular (EXF) and endocellular (ENF) fractions obtained from bacterium and fungus were tested for the ability to degrade nicosulfuron. The degradation efficiency of fractions extracted from *B. subtilis* YB1 was 66.8% by EXF and 15.8% by ENF, but neither EXF nor ENF extracted from *A. niger* YF1 had the activity of degrading nicosulfuron.

Nicosulfuron, one kind of the sulfonylurea herbicides, is mainly used for weed control in corn field. The effective application dose for it is much lower than that of the traditional herbicides [1–3]. The nicosulfuron moves easily in soil and causes groundwater contamination [4–6]. Even low remains of nicosulfuron in the soil may still cause phytotoxicity to some sensitive crops.

The bioremediation is one of the effective approaches to improve the environment conditions, especially to manage herbicides pollution. According to previous studies, Gu et al. [7] isolated the ethametsulfuron-methyl-degrading bacterium *Pseudomona*s sp. SW4 from contaminated soil. Luo et al. [8] reported on *Brevibacterium* sp. degrading bensulfuron-methyl; Xu et al. [1] isolated 3 strains (*Pseudomonas* sp. D61, D66 and *Bacillus* sp. D713) from pyrazosulfuron-ethyl contaminated soil and identified the main pyrazosulfuron-ethyl biodegradation products as pyrazosulfuron acid by liquid chromatography–mass spectroscopy and MS–MS techniques. Ma et al. [9] studied and reported the degradation characteristics, pathways and products of chlorimuron-ethyl by strain *Pseudomonas* sp. LW3; Valle et al. [10] described the biodegradation of azimsulfuron and its degradation products. Zhang et al. [11] found that *Klebsiella jilinsis* 2N3 was able to degrade sulfonylurea herbicides, including the nicosulfuron, but only with 8% degradation efficiency.

It is well known that the most biodegradation pathways on the pesticides are environmental enzymatic degradation [12]. Preparation of degrading enzymes can better to manage poor environment conditions than the use of the corresponding microbes, and the activity of degrading enzyme is much higher than the microorganism itself. Many studies on enzymatic degradation of pesticides were reported in literatures. Huang et al. [2] reported cell-free extract of S113 identified as *Methylopila* sp. was able to metabolize metsulfuron-methyl and other sulfonylurea herbicides and proved that enzymes played an important role in the decontamination of this compound. Pizzul et al. [13] revealed that the pure manganese peroxidase, laccase, lignin peroxidase and horseradish peroxidase could degrade glyphosate and other pesticides.

Recently, *Aspergillus niger* YF1 and *Bacillus* sp. YB1 were isolated in laboratory of Department of pesticide (Agricultural University of Hebei, Baoding, China) as efficient microorganisms to degrade nicosulfuron [14]. The aim of the study is to investigate the degradation characteristics of two strains and mechanism of the nicosulfuron degradation by *Bacillus* sp. YB1. The results obtained could be helpful in providing the theoretical evidence for controlling environment pollution of nicosulfuron. Furthermore, the potential for the use of strains *Bacillus subtilis* YB1 and *Aspergillus niger* YF1 in the treatment of soil polluted with the nicosulfuron need to be thoroughly investigated.

MATERIALS AND METHODS

Microorganisms, culture media and chemicals. The tested strains were *Bacillus* sp. YB1 and *Aspergillus niger* YF1 isolated and stored at the laboratory of Plant

Protection Institute of Pesticides of Hebei Agricultural University (China). Main apparatus were Agilent 1200 LC and HPLC–MS/MS (Thermo Electron Corp., San Jose, CA, USA); 94.87% nicosulfuron original drugs were provided by the Institute for Drug Control of Hebei Province (China).

A potato dextrose agar medium (PDA) containing (g/l): potato – 200, glucose – 20, agar – 20, was used for the activation of strains. The potato dextrose medium (PD) was the PDA medium without agar. Basic medium I contained (g/l): KH_2PO_4 – 0.5, K_2HPO_4 – 0.5, NaCl – 0.5, $MgSO_4$ – 0.5 and glucose – 5. In some experiments basic I medium was supplemented with 0.5 g/l of NH_4Cl (medium II) or 5 g/l of glucose (medium III). The described above 4 types of culture media were used for the degradation of nicosulfuron by *B. subtilis* YB1 and *A. niger* YF1. Luria-Bertani (LB) medium containing (g/l): yeast extract – 5.0, peptone – 10, NaCl – 5.0, was used for the bacterial culture to produce enzyme. Methanol and acetonitrile were chromatographic pure grade and other inorganic and organic chemicals were analytical grade.

Characteristics of the nicosulfuron degradation by *B. subtilis* YB1 and *A. niger* YF1. To detect the effect of different media on degradation of nicosulfuron, 1 ml suspension of microorganism was inoculated into 250 ml Erlenmeyer flask containing 100 ml of liquid medium with 2 mg/l of nicosulfuron and incubated for 5 days at 30°C. To detect the effect of nicosulfuron concentration on degradation, 1 ml bacterial or fungal suspension was inoculated into the optimal III medium with nicosulfuron at concentrations of 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l, respectively. To reveal the effect of temperature, 1 ml suspension of microorganism was inoculated to the optimal III medium with 2.0 mg/l of nicosulfuron. The samples were separately cultivated at 15, 20, 25, 30, 35 and 40°C for 5 days. All treatments described above were compared with two controls: one with nicosulfuron without microorganism and another with microorganism without nicosulfuron. Each treatment was carried out in triplicate. Samples were extracted and determined as the following method below.

Analysis of microbial samples treated with nicosulfuron by HPLC. In order to quantity the nicosulfuron in samples, the culture medium was centrifuged at 12000 × g for 10 min after 5 days of incubation at 30°C. 20 ml supernatant was transferred to a 250 ml glass separation funnel and extracted with 40 ml of dichlormethane thrice for the liquid-liquid distribution. The dichlormethane was evaporated by 40°C water bath under a stream of nitrogen, and redissolved in 5 ml of methanol. After filtration, 2 μl sample was subjected to HPLC analysis and the degradation rate of nicosulfuron was calculated.

Agilent Technologies 1200 series HPLC system (USA) consisted of a quaternary pump and a UV spectrophotometric detector. The separation was performed using an Agilent reversed phase C_{18} column (150 × 4.0 mm, 5 μm). The acetonitrile-water-glacial acetic acid mixture at the ratio of 30 : 70 : 0.05 (v : v : v) was used for elution detected at 240 nm and 1.0 ml min^{-1}, the temperature of the column was 30°C.

Dynamics of the nicosulfuron degradation by *B. subtilis* YB1 and *A. niger* YF1. Two treatments were conducted by inoculating 1 ml suspension of strains to 250 ml Erlenmeyer flask containing 50 ml of running water with 0.2% glucose and nicosulfuron (2 mg/l) placed in static culture at 30°C and inoculating 1 ml of bacterial or fungal suspension to the same liquid medium, with the 1 : 1 (v : v) mixed, respectively. Two controls were set up as described above. The samples were withdrawn at regular intervals of 12 h and extracted as the protocol described above. Degradation kinetics equation was calculated as following formula:

$$C_t = C_0 e^{-Kt} \rightarrow \ln C_t = \ln C_0 - Kt \rightarrow t_{1/2} = \ln 2/K,$$

where K is the degradation constant; t is reaction time; C_0 is the initial concentration of pesticide solution at the start of response; C_t is the concentration of pesticide solution when reaction time is t, and $t_{1/2}$ is half-life of pesticide.

Preparation of extracellular and endocellular microbial fractions degrading nicosulfuron. The *B. subtilis* YB1cells were inoculated to the plate with solid LB medium and incubated at 30°C for 24 h. Then a single colony was transferred to 500 ml Erlenmeyer flask containing 200 ml LB liquid medium and shaked at 30°C and 150 rpm for 72 h. The cells were separated by centrifugation at 5000 × g for 30 min at room temperature. The culture supernatant was further filtered using 0.22 μm membrane of vacuum filtration to remove the bacteria and concentrated by ammonium sulfate precipitation (80% saturation, w/v). It was left statically for overnight at 4°C and centrifuged at 8000 × g for 30 min to discard the supernatant. To resuspend the proteins, 10 ml of phosphate-buffered saline (PBS, 20 mM, pH 7.0) was added. Denatured proteins were removed by centrifugation and the crude enzyme was obtained. It was dialyzed against the same buffer which was changed several times until the ammonium sulfate was utterly removed. The solutions were concentrated 5-fold with polyethylene glycol 20000 (PEG 20000) and the concentrated solution was designated as the extracellular fraction (EXF). In addition, cells were washed twice with 40 ml of 20 mM PBS, pH 7.0, and then centrifuged at 8000 × g for 30 min to obtain sediment, which was resuspended with the same PBS and broken intermittently by ultrasonic cell crusher for 10 min on ice. After centrifugation at 10 000 × g for 30 min the supernatant was designated as the endocellular fraction (ENF). The protein content in crude extracts was determined colorimetrically at 595 nm by Bradford method [15] with the BSA as a standard protein.

The *A. niger* YF1 cells were inoculated to PDA plate and incubated at 35°C for 48 h. Then a fungal

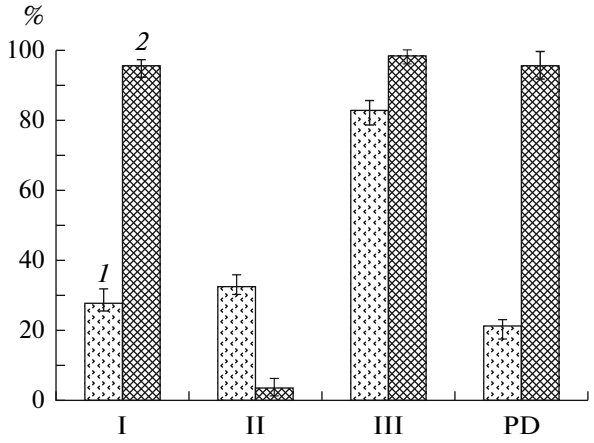

Fig. 1. Biodegradation of nicosulfuron by *B. subtilis* YB1 (*1*) and *A. niger* YF1 (*2*) in different culture media - I-III, PD.

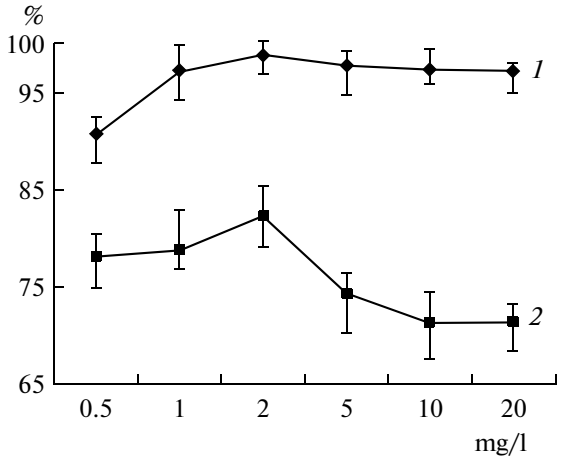

Fig. 2. The effect of different nicosulfuron concentrations of on its biodegradation by *B. subtilis* YB1 (*2*) and *A. niger* YF1 (*1*).

cake was transferred to 500 ml Erlenmeyer flask containing 200 ml of PD liquid medium and shaked at 35°C and 150 rpm for 5 days. The culture medium and mycelium were separated by filtration through filter paper. EXF was obtained by ammonium sulfate precipitation as the described above. The mycelium was squeezed between filter-paper sheets and grinded into powder with liquid nitrogen. Then it was resuspended with 20 mM PBS, pH 7.0 and centrifuged at 10000 × g for 30 min to obtain the supernatant as the ENF.

Nicosulfuron-degradating activity was measured in the reaction medium containing 2.7 ml of nicosulfuron (2 mg/l) and 0.3 ml of diluted enzyme after incubation at 30°C for 60 min. The reaction was stopped by adding 0.2 ml of 1.0 M HCl, the amount of nicosulfuron was determined by HPLC and compared with a control containing 0.3 ml inactive enzyme. The treatment was carried out in triplicate. One unit (U) of the crude enzyme was defined as its amount required to degrade 1 μg of nicosulfuron per min under the conditions described above.

Determination of the nicosulfuron degradation products by *B. subtilis* YB1. Nicosulfuron (200 mg/l) was added to 250 ml Erlenmeyer flask containing 50 ml of basic medium III, inoculated with 2 ml of suspension of *B. subtilis* YB1 (2.3×10^7 CFU ml^{-1}) and incubated for 10 days at 30°C. Then culture medium was filtered, the filtrate was extracted with an equal volume of dichloromethane 3 times, organic phase was dried over anhydrous Na_2SO_4, finally the extracts were concentrated into 2 ml in a rotary vacuum evaporation at 40°C. The residue was dissolved in 5 ml of methanol and determined by HPLC.

The HPLC–MS/MS was used. The HPLC system was equipped with a C_{18} column (150 × 2.1 mm, 5 μm) and the UV detector was operated at a wavelength of 240 nm. The mobile phase consisted of acetonitrile and 0.05% glacial acetic acid at a flow rate of 200.0 μl min^{-1}. The gradient conditions were 0–7–10–15 min, with acetonitrile 15–10–10–15% respectively. Mass spectrum (MS) analysis was carried out using electron impact ionization at 350°C and 15 eV with a mass scanning range from 100 to 400 m/z.

RESULTS AND DISSCUSSION

Effects of different media on the nicosulfuron degradation by *B. subtilis* YB1 and *A. niger* YF1. The effect of different culture media on the nicosulfuron degradation by both microorganisms was tested, and the results (Fig. 1) showed that in the basic medium III containing carbon-nitrogen the highest degradation rate for *B. subtilis* YB1 and *A. niger* YF1 was, 82.1 and 98.2%, respectively. On the other hand, the lowest degradation rate for *B. subtilis* YB1 was 20.9% in the PD medium and 3.5% for *A. niger* YF1 in basic medium (without glucose). Therefore, the basic medium III was more appropriate for the degradation of nicosulfuron by bacterium and fungus. The basic medium III was not only with carbon sources but also with nitrogen sources, as well as its ingredients were simple, available and low-cost.

Effects of different concentrations of nicosulfuron on its degradation by *B. subtilis* YB1 and *A. niger* YF1. The degradation nicosulfuron curves (Fig. 2) showed that degradation rate depended on its concentration. The degradation ability of microorganisms in the medium containing 0.5–2.0 mg/l of nicosulfuron was significantly higher than at concentration 5–10 mg/l. In the basic medium III containing 2 mg/l of nicosulfuron, the highest degradation rate for *B. subtilis* YB1 and *A. niger* YF1 was 82.5 and 98.5%, respectively. With the increase in the concentration, the degradation ability of strains decreased. When the concentration was 10 mg/l, the degradation rate reduced to 71.3% and 97.4% respectively. The results showed that the *B. subtilis* YB1 cells were more suitable for degradation of nicosulfuron at low concentration (0.5–2.0 mg/l), although some studies indicated that degradation abili-

ties of many microorganisms were stronger on the higher concentration of sulfonylurea herbicides in environment and poorer on the lower's [2, 7, 9, 11]. Previous studies showed that the most of microorganisms were better for degrading of sulfonylurea taken in high concentration in comparison with its low concentration in environment. This study discovered that the low concentration (2 mg/l) of nicosulfuron was the most optimal for strains *A. niger* YF1 and *B. subtilis* YB1 to degrade this compound. It is important that nicosulfuron presents at lower levels in nicosulfuron-used soil, nicosulfuron-contaminated water, pesticide manufacturers and treated industrial wastewater, but even if the concentration is very low, it still causes chemical injury to sensitive crops. Thus, *B. subtilis* YB1 and *A. niger* YF1 could be practically significant to degrade nicosulfuron.

Effects of temperature on the nicosulfuron degradation by *B. subtilis* YB1 and *A. niger* YF1. The temperature was an important factor influencing the microbial degradation. The results obtained (Fig. 3) showed that the different temperature could obviously affect the nicosulfuron degradation by both microorganisms. When the temperature was at 15°C, the degradation rates for *B. subtilis* YB1 and *A. niger* YF1 were only 6.4% and 40.9%, respectively. With the increase in the temperature, the degradation rate of nicosulfuron also increased. When the temperature reached 35°C, it was the highest, 87.9% and 98.5%. However, when the temperature reached 40°C, the degradation rate of *B. subtilis* YB1 decreased to 83% and that of *A. niger* YF1 stayed at level 98.5%. Therefore, too low or too high temperature suppresses the degradation of nicosulfuron by bacterium and the low temperature supresses it by fungus. The optimal temperature for both microorganisms was 35°C, however, at 25–30°C, the degradation activity remained high.

Determination of degradation dynamic of nicosulfuron by free cells of *B. subtilis* YB1 and *A. niger* YF1. The results illustrated degrading reaction of nicosulfuron obeyed first-order kinetic models [16–18]. Degradation rates of nicosulfuron by single microorganism and mixture of them and the kinetic parameters of the nicosulfuron degradation were displayed in Table. The results proved that the degradation rate by the mixture was higher than by single microorganism, and it was speculated that a mixture of two strains promoted the

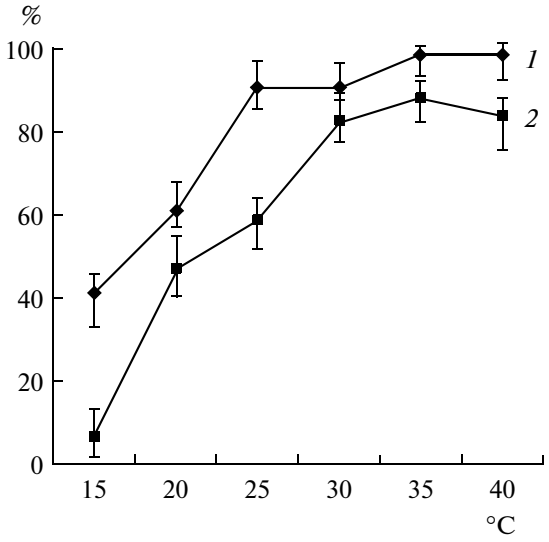

Fig. 3. The effect of different temperatures on biodegradation of nicosulfuron by *B. subtilis* YB1 (*2*) and *A. niger* YF1 (*1*).

degradation of nicosulfuron. Due to low levels of nicosulfuron in polluted environment, bacterium and fungus were inoculated to the culture medium containing a small quantity of glucose in running water to determine the degradation dynamic. Results revealed that the degradation rate of the mixed microorganisms was much higher than that of single one. The bioremediation of mixed strains has great developing prospects in future [19–20]. In this study, only glucose was added into the running water to provide nutrition for the growth and reproduction of the microorganisms, hence, the optimal conditions of degradation kinetics of nicosulfuron by mixed strains need further study. At the same time, the toxicity of degradation products to humans, animals and environments is necessary to be tracked and monitored.

Determination of degradation dynamic of nicosulfuron by partially purified microbial EXF and ENF. Through precipitation of the supernatant with ammonium sulfate, crushing cells by ultrasonic or grinding cells with liquid nitrogen, corresponding EXF and ENF were extracted, which were diluted to the same concentration of protein with 20 mM PBS (pH 7.0). The degradation activities were measured as described

The kinetic parameters for degradation of nicosulfuron by *B. subtilis* YB1 and *A. niger* YF1 in basic medium III

Strains	Kinetics equation of degradation	Rate constants of degradation		
		r*	K	$T_{1/2}$(d)
B. subtilis YB1	$C = 2.0e^{-0.00274t}$	0.9883	0.00274	6.5929
A. niger YF1	$C = 2.0e^{-0.0136t}$	0.9900	0.01360	4.9908
A. niger YF1+ *B. subtilis* YB1	$C = 2.0e^{-0.0380t}$	0.9958	0.03800	3.9633

* Correlation coefficient.

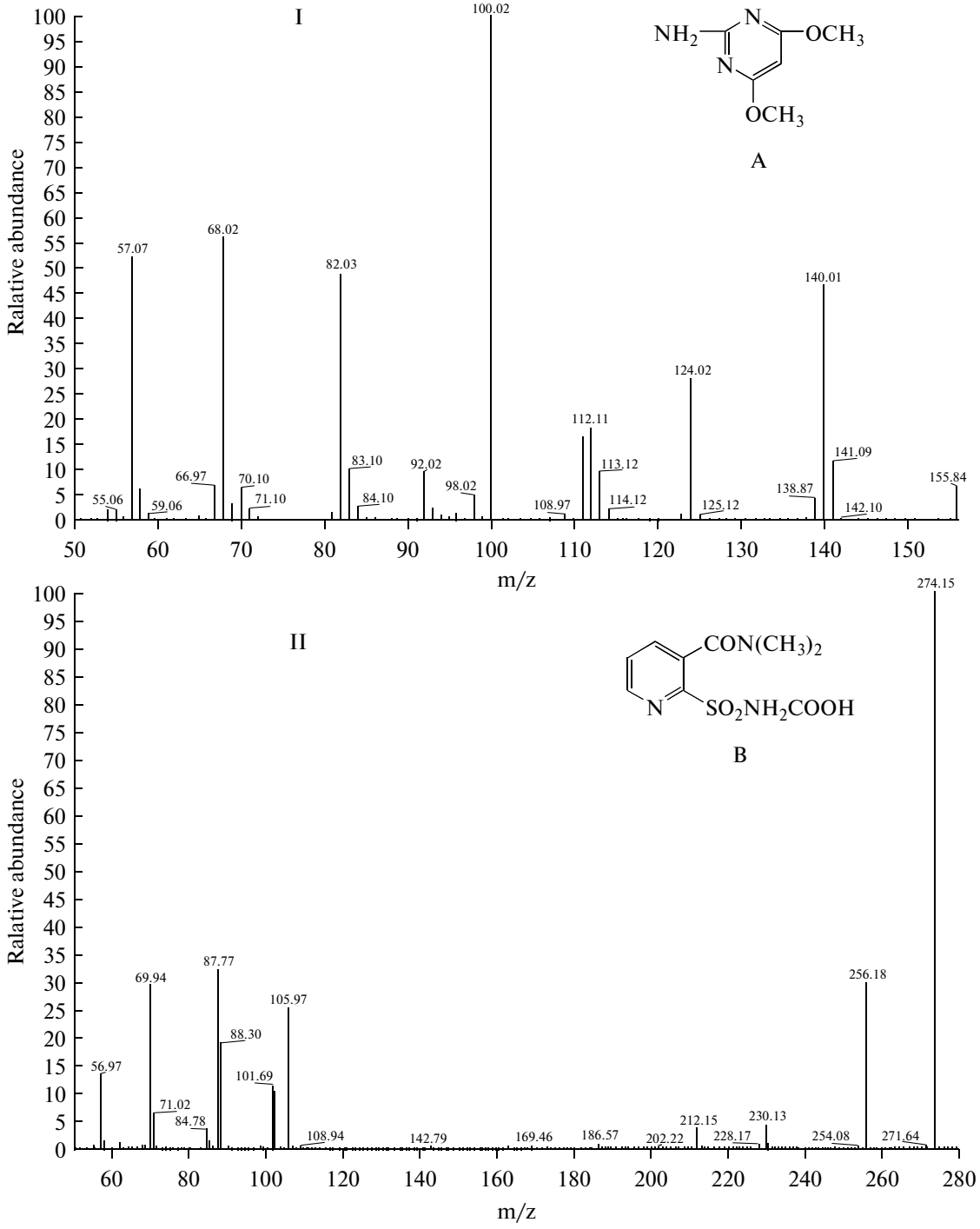

Fig. 4. Mass spectra of the metabolites A (I) and B (II) of nicosulfuron transformed by the *B. subtilis* YB1.

above. The results showed that *B. subtilis* YB1's ENF was capable of degrading nicosulfuron at 15.8% (2 mg/ml of nicosulfuron for 2.7 ml) in 60 min; the EXF had a higher degradation activity of nicosulfuron (about 66.8%). However, the EXF and ENF of *A. niger* YF1 had no the nicosulfuron degradation. It is known that the effective ways of biodegradation of pesticides include enzymatic and non-enzymatic degradation in environment. Most microorganisms degrade pesticides by exuding enzymes. This work built a foundation for constructing genetically engineered bacteria.

Degradation mechanism of nicosulfuron by *B. subtilis* YB1. Fig. 4 showed total ion chromatograms on degradation products of nicosulfuron. Product A

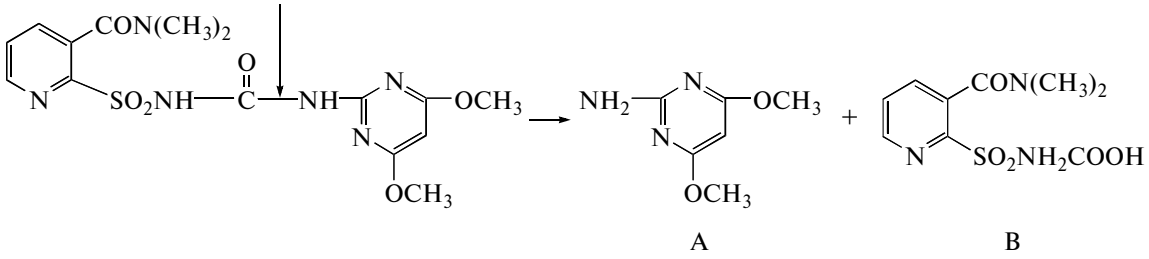

Fig. 5. Biodegradation pathway of nicosulfuron by *B. subtilis* YB.

(Fig. 4a) showed prominent protonated molecular ion at m/z 155.8. Product B (Fig. 4b) showed prominent protonated molecular ions at m/z 274.1. Based on this information, products A and B were identified as sulfonamide and heterocyclic amine, respectively. So the possible pathway of the nicosulfuron degradation is the cleavage of the sulfonylurea bridge (Fig. 5). The results proved that the degrading pathway was different between the microbial metabolic and hydrolysis mechanisms of nicosulfuron. Although the hydrolysis mechanism is also the cleavage of sulfonylurea bridge, the products are corresponding sulfonamide, amino heterocyclic compounds and CO_2 [1]. According to previous reports [1, 8, 9, 11, 21], the cleavage of sulfonylurea bridge seemed to be the common degrading pathway of sulfonylurea herbicides, yielding the corresponding hydrolysis products of sulfonamide and heterocyclic amine [22, 23]. In our work, the active site of nicosulfuron degradation by *B. subtilis* YB1 was preliminarily studied, which provided a theoretical basis for controlling pesticides-contaminated environment. Because only two major peaks of degradation products were determined by mass spectrometry, the remaining products need further separation and determination. The products and site of the nicosulfuron degradation by *A. niger* YF1 need further study.

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