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Biodegradation of Imazapyr by Free Cells of *Pseudomonas fluorescene Biotype II* and *Bacillus cereus* Isolated from Soil

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Pesticides are used in control of pests of man, animals and plants with the result that many of these chemicals reach soil and persist for long period causing harm to soil microorganisms and plant growth. Organic pesticides applied to soil may be used as substrates by microorganisms and undergo degradation resulting in the formation of new compounds (Screenivasulu and Apama 2001). Losses of efficacy due to enhanced or accelerated rates of biodegradation have been documented for a variety of soil-applied pesticides. It is generally accepted that this is due to proliferation of soil microorganisms that use pesticides as C or N sources, or both, for growth, which forms the foundation of bioremediation technique (Shelton and Doherty 1997).

Imazapyr developed by American Cynamid Company is a broad-spectrum postemergence herbicide with longer residual activity. It can control annual and perennial grasses, sedges, broadleaf weeds, shrubs and deciduous trees (Christensen 1992; Winfield and Bannister 1988; James et al. 1997). Now it has been registered temporarily in June 2002 in China mainland. Many previous researches mainly focused on its environmental behavior in soil (Pusino et al. 1997; Azzouzi et al. 1998; Jenkins et al. 2000) and water (Mansour et al. 1999; Elazzouzi et al. 1999). However, little information is available concerning its microbial degradation (Cox 1996). Such questions as the imazapyr-degrading bacterial species in soil, their degradation kinetics and application of the dominant imazapyr-degrading bacteria to reduce its pollution quickly in field have not been reported. In this paper, two imazapyr-degrading bacterial strains were isolated and identified. Their degrading characteristics were also investigated.

MATERIALS AND METHODS

Imazapyr (99.6% pure) was kindly provided by Shanghai Branch, BASF (China) Co., Ltd, and was monitored by HPLC to assure that no interference impurity had occurred. Dichloromethane and acetonitrile used in this study were analytical, HPLC grade purchased from Tianjin Chemical Reagents Factory.

Loamy sand soil [pH 6.71, OMC (organic matter content) 19.8 g/kg, CEC (cation exchangeable capacity) 13.28 cmol(+)/kg] was collected at 5-10 cm in depth from the field sprayed by imazapyr many times to control weeds in 2001 in Zhejiang province, Southeastern China. The fresh field soil was immediately brought to the laboratory after collection, removing large pieces of plant materials, stones and

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visible soil fauna. A subsample of the soil was air-dried, ground, passed through a 2-mm sieve and preserved at 4℃. 10 g portion of soil was taken into pre-sterilized test tubes and maintained at 60% maximum water holding capacity (WHC) (Venkateswalu et al. 1977). Acetonitrile solution of imazapyr was added to the soil sample separately to obtain a final concentration of 50 ppm. Soil enrichments were developed by eight subsequent additions of 50 ppm at 7-day intervals.

Soil suspensions were prepared with the imazapyr-enriched soil sample. Dilutions of soil suspension were prepared up to 10^{-7} dilution. To isolate bacteria capable of degrading imazapyr, suspensions of 10^{-7} dilution were placed on nutrient agar medium (NAM) and kept at 30℃ for 24 h. NAM was composed of beef extract 3 g, peptone 5 g, sodium chloride 5 g, agar 15 g (per liter of deionized water) and the final pH was adjusted to 7.0-7.2. After 24 h of incubation, single colony was selected according to the different morphological and cultural characteristics, then purified and tested for their ability to use imazapyr as the sole source of carbon in mineral salts medium (MSM) amended with imazapyr (50 ppm). MSM which we used contained 1 g of sodium chloride, 1 g of dipotassium hydrogen phosphate, 3 g of potassium dihydrogen phosphate, 0.5 g of magnesium sulfate in per liter of deionized water. The final salt solution was sterilized and pH was adjusted to 7.0-7.2. Two isolated strains which occurred predominantly and exhibited a greater potential in degrading imazapyr were isolated and identified by automatic bacterial identification system (Yingdi 2001).

Bacterial cell suspensions were prepared by growing the culture to high cellular densities at 30℃. The cells were harvested by centrifugation at 10000 g for 10 min. Supernatants were discarded and the cells were suspended in sterile distilled water. Bacterial cell densities were measured at 486 nm (OD_{486}). The cell density, which is at OD_{486}=0.2, at the beginning of experiment was maintained unless otherwise mentioned as inoculum in degradation studies. The rate and extent of imazapyr degradation by the isolated strains were studied by inoculating the culture suspension to 50 ml sterilized MSM supplemented with 50 ppm imazapyr (Siddaramappa et al. 1973). Controls were maintained without bacterial inoculation. At regular time intervals, triplicate samples were removed for each treatment, pH of the sample was adjusted to 5 with 0.1 mol/L HCl and then extracted three times with 20 ml of dichloromethane each time. The dichloromethane phase was dehydrated by passing over anhydrous sodium sulfate, evaporated using rotary vacuum evaporator and the volume was made up to 5 ml with acetonitrile for estimation by HPLC.

An Agilent model HP1100 liquid chromatograph, equipped with DAD (diode array detector) and chromatography workstation, was used to determine the residue of imazapyr. The chromatographic conditions were as follows: column, 10 μm YWG-C_{18} (250 mm × 4.6 mm i.d); mobile phase, acetonitrile - water - 0.2%H_3PO_4 (70 : 25 : 5 v/v); flow rate 1 ml/min; detection wavelength, 234 nm; injection volume, 20 μl. An external standard method was used for calibration. Under the above-mentioned conditions, retention time of imazapyr was 3.3 min.

RESULTS AND DISCUSSION

The indigenous microbial communities in soil were enriched in laboratory at 30 ℃ to use imazapyr as carbon source. Imazapyr-degrading bacteria were isolated by the method of serial dilutions and plating from soil enriched with imazapyr.

Based on the different morphological and cultural characteristics, 14 bacterial strains designated as ZJX-1 to ZJX-14 were isolated. Their degrading abilities to use imazapyr as the sole source of carbon were tested by its degradation rate after 48 h of incubation. The results in Table 1 showed that among 14 isolated

Table 1. Degradation of imazapyr by the isolated strains.

No	Degradation rate in 48 h (%) (±SD)	No	Degradation rate in 48 h (%) (±SD)	No	Degradation rate in 48 h (%) (±SD)
CK	4.9 ± 0.1	ZJX-5	81.4 ± 5.9**	ZJX-10	10.5 ± 0.7
ZJX-1	4.3 ± 0.3	ZJX-6	2.2 ± 0.1	ZJX-11	9.9 ± 0.6
ZJX-2	5.8 ± 0.4	ZJX-7	6.6 ± 0.4	ZJX-12	6.9 ± 0.5
ZJX-3	10.4 ± 0.8	ZJX-8	7.4 ± 0.5	ZJX-13	7.1 ± 0.6
ZJX-4	7.8 ± 0.8	ZJX-9	86.6 ± 6.4**	ZJX-14	12.7± 1.0

SD denotes standard deviation, ** marked differences are significant at $P<0.01$

strains only ZJX-5 and ZJX-9 were capable of degrading imazapyr quickly. When fortified at the initial concentration level of 50 ppm, ZJX-5 and ZJX-9 could degrade 81% and 87% imazapyr, respectively, after 48 h of incubation which were significantly higher than that of other isolated strains and control. The strains were identified as *Pseudomonas fluorescenes biotype II* and *Bacillus cereus*, respectively, using automatic bacterial identification system (Yingdi 2001). The confidence probabilities of the judgement were 90% and 79%, and the judgment results showed to be very good and acceptable.

The effect of initially fortified concentration on degradation was summarized in Table 2. ZJX-5 and ZJX-9 showed highly degrading ability to imazapyr, which could have a degradation rate of more than 70% at the fortified level of 50 or 100 ppm after 48 h of incubation, but degradation rate decreased rapidly with the increasing of imazapyr concentration. ZJX-5 and ZJX-9 could degrade 33% and 21% of imazapyr when fortified at the level of 200 ppm, and 5% and 9% at the level of 300 ppm. Therefore, increase in imazapyr concentration appeared to lead to its slower degradation rate. Results also showed that the highest tolerant concentration for two separate bacterial strains to imazapyr was about 200 ppm. If the initial concentration was over it, the activities of both bacterial strains would be inhibited severely.

Table 2. Degradation of imazapyr by ZJX-5, ZJX-9 and their mixed culture.

Initial residues (ppm)	ZJX-5 (48 h)		ZJX-9 (48 h)		Mixed culture bacteria	
	Residue (ppm) (±SD)	Degradation rate (%)	Residue (ppm) (±SD)	Degradation rate (%)	Residue (ppm) (±SD)	Degradation rate (%)
48.7	9.1 ± 0.6	81.4	6.5 ± 0.5	86.6	1.6 ± 0.9	96.6
97.6	27.8 ± 1.4	71.5	23.6 ± 1.7	75.8	17.1 ± 1.3	83.4
202.5	136.3 ± 18.8	32.7	160.2 ± 10.0	20.9	61.7 ± 5.8	68.8
287.4	274.5 ± 16.9	4.5	261.2 ± 17.1	9.1	203.6 ± 6.1	31.3

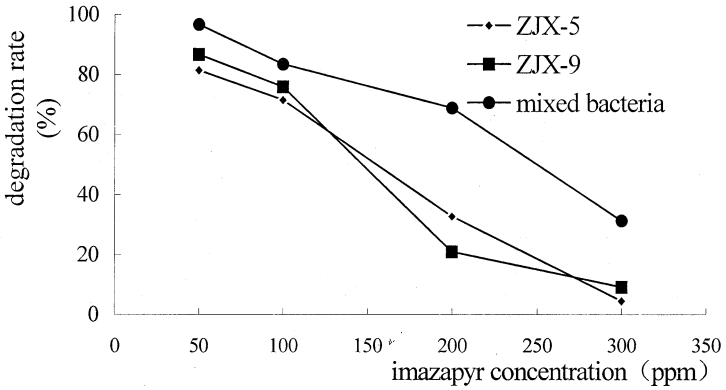

Figure 1. Degradation of imazapyr by the single and mixed bacteria (ZJX-5 and ZJX-9)

The research on degradation of the mixed bacteria is especially important in that they play an important role in degrading organic pollution in soil (Qimei et al. 2000). ZJX-5 and ZJX-9 were mixed in equal proportion of their original suspensions, diluted it to OD_{486}=0.2 and the subsequent operations were the same as their single ones. The degradation ability of the mixed culture bacteria was superior to each separate one, especially at the higher fortified concentration of 200-300 ppm (Figure 1) and the degradation rate is about 2-7 times as that of their separate one. The possible reason could be the presence of co-metabolism between the two strains.

An attempt was made to determine the toxic concentrations of imazapyr employing the step-up levels of 50, 100, 200, 300, 400 ppm by inoculating the mixed culture bacteria in MSM. The results in Figure 2 demonstrated that 50 ppm was completely degraded within 60 h of incubation whereas 100 ppm degraded after 72 h of incubation. There was an increase of 24 h in complete degradation when imazapyr was supplemented at 200 ppm. However, the initiation of imazapyr degradation was delayed until 24 h in MSM treated with 300 ppm, complete degradation was observed at 144 h after incubation. There was no onset of degradation at 400 ppm concentration of imazapyr, and appeared to be toxic to the mixed culture bacteria. The above results showed that the lag period occurred during degradation of imazapyr was directly proportional to increasing imazapyr concentration, and the level of imazapyr that is toxic enough to totally inhibit degradation is 400 ppm. Based on the first-order kinetics equation, the half-lives were calculated to be 8.9, 13.4, 12.8 and 17.0 h, respectively, at 50, 100, 200 and 300 ppm concentration of imazapyr which demonstrated that the degradation clearly decreased with the increase of initial concentration. However, as can be seen from figure 2, the degradation curve did not conform to first-order kinetics equation at 400 ppm of imazapyr because no occurrence of degradation was observed (Fig 2).

Two highly effective imazapyr-degrading bacteria were obtained from soil, both of which had highly degrading ability for imazapyr in MSM. From the stand point of environmental contamination of pesticides, the present observation of

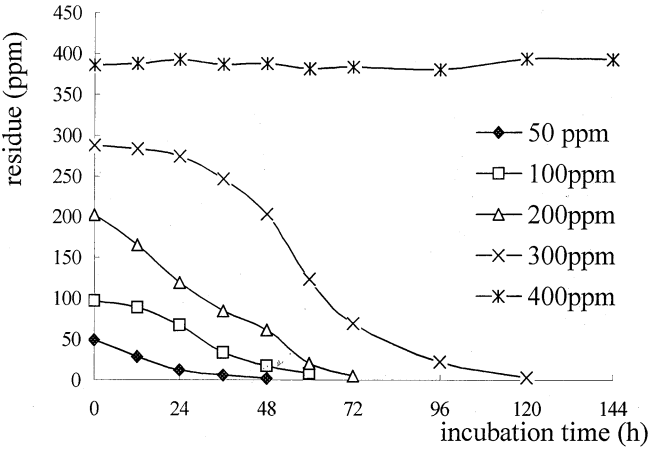

Figure 2. Degradation of imazapyr by the mixed culture

using free cells for remediation of imazapyr may be exploited further in environmental biotechnology for the effective detoxicification of the imazapyr contaminated areas. In addition, more considerations should be given in application of two imazapyr-degrading bacteria in the fields.

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