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Characterization of a strain of *Sphingobacterium* sp. and its degradation to herbicide mefenacet

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Abstract: A bacterium (designated strain Y1) degrading acetanilide herbicide mefenacet was isolated from aerobic sludge. Based on the analyses of partial 16S rRNA gene, cellular fatty acid and BIOLOG-GN, and general physiological and biochemical characteristics, strain Y1 was identified as *Sphingobacterium multivolum*. Strain Y1 was able to degrade mefenacet used as sources of carbon and energy. Degradation of mefenacet was accompanied by producing the metabolites N-methylaniline and an unidentified compound with molecular weight 205, indicating a metabolic pathway of mefenacet initiated by hydrolysis of amido bond.

Keywords: herbicide; mefenacet; *Sphingobacterium*; biodegradation

Introduction

The acetanilide herbicide mefenacet(2-(1,3-benzothiazol-2-yloxy)-N-methylacetanilide), which is used for pre- and post-emergence control of annual weeds in paddy soil, is a relatively new and promising herbicide in agricultural field and developed rapidly in China(Lu, 2000). Mefenacet has been used widely and repeated in other Asian countries(Hirahara, 1997; Yang, 2000) and is frequently detected in river or lake water(Okamura, 1999; 2002). Synthetic agrochemicals, such as butachlor(Min, 2001), trifluralin(Min, 2001) and mefenacet can affect the microbial activity and cause an overall toxic effect on the environment. Therefore, their residue in soil may act as potential environmental hazards and disturb the natural ecological equilibrium(Alexandre, 2000). Ecotoxicologial studies have suggested that mefenacet could cause shortening of height and root length of the rice plant and disturb the balance of active oxygen in the body of rice plant(Zhang, 2001a; 2001b). Mefenacet is also harmful to shrimp and algae, while it has a little effect on *Daphnia magna* (Okamura, 1999; 2002; Shigehisa, 1998). Mefenacet can be absorbed and desorbed by soil(Kaori, 2002; Fajardo, 2000), but its degradation in agricultural soils occurs predominantly by microbiological processes (Zhang, 2001a; 2001b).

Currently, to our knowledge, there are no reports on microbial degradation of mefenacet although many studies have described about degradation or mineralization of other herbicides such as isoproturon, atrazine, N'-(2, 4-dimethylphenyl)-N-methylformamidine, and 2, 4-dichlorophenoxyacetic acid and so on (Sebastian, 2001; Edward, 2000; Zhu, 2000; Tohru, 2001; Mary, 1998; Elisa, 1998; Tixier, 2002; Diego, 1997). The present paper describes the isolation and characterization of a strain of *Sphingobacterium* sp. (designated strain Y1) from a wastewater treatment plant in China, which can degrade mefenacet effectively.

1 Materials and methods

1.1 Chemicals

Mefenacet (90% purity, 4 mg/L water solubility at 20℃) was provided by the Department of Plant Protection, Zhejiang University. The molecular structure of mefenacet is presented as follows(Fig.1). CH_2Cl_2 was used as a solvent extracting mefenacet for GC/MS measurement grade. The other chemical agents used were all of analytical grade.

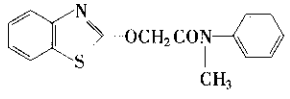

Fig.1 Molecular structure of the acetanilide herbicide mefenacet

1.2 Growth media

Medium Ⅰ used for enrichment and pure culture consisted of the mineral salt medium (MS) and mefenacet. The MS was as follows(g/L): KH_2PO_4 0.4, K_2HPO_4 0.4, NH_4Cl 1, $MgCl_2$ 0.1, Na_2SO_4 1.425, $FeSO_4 \cdot 7H_2O$ 0.025, trace element solution 10 ml. Composition of trace element solution was described by Min *et al*. (Min, 2001). Supersaturated water solution of mefenacet after autoclaved was added to dissolve the above substances to one liter. Then pH was adjusted to 7.2 with 4.0 mmol/L NaOH solution. Medium Ⅱ for degradation of mefenacet was supplemented yeast(0.5 g/L) in medium Ⅰ.

1.3 Enrichment culture

Aerobic sludge was taken from Hangzhou Sibao Wastewater Treatment Plant, Zhejiang Province, China. Sterilized 100 ml flasks containing 20 ml medium Ⅰ were inoculated with 2 ml aerobic sludge. The flasks were incubated in the dark at 28℃. 0.1 ml of enrichment culture

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in medium Ⅰ was inoculated into 20 ml of fresh mefenacet-containing medium Ⅰ. A stable enrichment bacterial culture with higher purity was obtained after more than 15 subcultures had been performed.

1.4 Isolation, characterization and identification

A mixed bacterial culture was plated on Petri dishes with medium Ⅰ supplemented with 2% agar and incubated for 2—3 d at 28℃. Colonies were picked up and screened for their ability to degrade mefenacet in pure culture. Several strains of bacteria degrading mefenacet were isolated, among which a strain(designated Y1) that could degrade mefenacet effectively was characterized and identified by sequencing of the partial 16S rRNA gene, analysis of the cellular fatty acids (Lu, 1997), and analysis of the BIOLOG GN and physiological tests. Alignment of the partial 16S rRNA gene sequence was performed with sequences deposited in the GenBank database (National Center for Biotechnology Information) using CLUSTAL W. version 1.8. A neighbor-joining method (Neighbor-Joining/ UPGMA, version 3.6) from the PHYLIP software package was used to estimate relatedness(Higgins, 1991; Maidak, 2001) and constructed phylogenetic tree.

1.5 Preparation of inoculum

Prior to the pure culture degradation studies with the isolated strain Y1, strain Y1 was grown in 250 ml flasks containing 100 ml medium Ⅰ at 28℃. The liquid culture of strain Y1 with a density of 10^8 cells ml^{-1} was prepared for inoculation, as determined by plate counts on Luria-Bertani media.

1.6 Inoculation of BIOLOG-GN microplates

Strain Y1 was grown on LB agar medium and incubated for 48 h at 28℃. Colonies from slant medium were suspended in the sterilized water and the inoculum density was adjusted to the recommended turbidity of 0.03. One hundred and 50 μl of this suspension was pipetted into each prefilled, dried well of a BIOLOG-GN microplates(Shona, 1999). Microplates were incubated at 20℃ without shaking and read everyday for a week by measuring absorbance (A) at 590 nm on an automated microplate reader(BIOLOG Co., USA). The A_{590} of the well with no carbon source was used as a background control. After comparing data obtained with those from the BIOLOG database, a 7 d incubation time was chosen as giving the most reliable results for strain Y1. Data were collected and analyzed by the BIOLOG bacteria automatic identification apparatus.

1.7 Degradation of mefenacet

The liquid culture of strain Y1 was inoculated to the 150 ml flasks containing 50 ml of medium Ⅱ (inoculation rate 3%). The flasks were incubated in the dark at 28℃ on a shaker at 200 r/min. Degradation of mefenacet was monitored by measuring the concentration of mefenacet as described by Lu *et al*.(Lu, 2000). Mefenacet and its metabolites were measured with GC/MS TRACE-2000 produced by THERMOQUEST Co. using a capillary column HP-5 (30 m × 0.25 mm × 0.25 μm). The instrument operation condition was as follows: The energy of the ionizing beam was 70 eV, the scanning mass range was 350—400 m/z in 0.9 s^{-1} with interval of 0.1 second, the temperature of the injection chamber was held at 240℃, the initial temperature for the column was 50℃ for 3 min, then programmed at 15℃/min to a final temperature of 240℃, the temperatures of transfer line and of the MS ion source were held at 250℃ and 200℃, respectively. At least triplicate samples were prepared and sterile controls were included in all experiments.

2 Results and discussion

2.1 Degradation of mefenacet and its metabolites

Mefenacet was efficiently degraded by strain Y1 with 89% of degradation rate within 6 d, however, the degradation rate of mefenacet was slow afterwards(Fig.2).

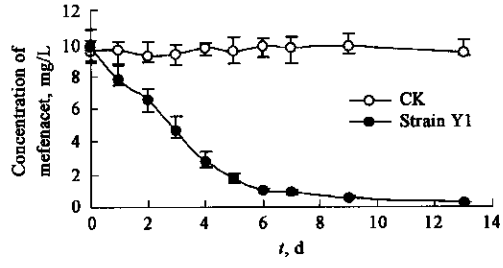

Fig.2 Degradation of mefenacet by strain Y1 with 3% of inoculation rate at pH 7.0 and 28℃

MS spectrograms of samples from standard mefenacet and its degradation derivatives are shown in Fig.3. It was found that N-methylaniline was one of the metabolites of mefenacet, which appeared at 8.13 min retention time in the chromatogram and was the first intermediate detected.

2.2 Characterization and identification of mefenacet-degrading bacteria

Several mefenacet-degrading bacteria which grew well on the medium Ⅱ supplemented with 2% agar were isolated, among which strain Y1 was characterized further. Strain Y1 was a gram-negative, non-spore-forming rod with a width of 0.6 to 0.8 μm and a length of 0.6 to 4 μm. Filaments were easily observed on the surface of small cells of strain Y1, however, hardly on the surface of large cells under the same magnitude(Fig.4). It is catalase positive, arginine bihydrolase negative. This bacterium could oxidize glucose, cellobiose, D-fructose, galactose, lactose, maltose, sucrose, fucose, xylose, raffinose, sorbose and rhamnose but not inositol, sorbitol, mannose, ethanol (3%) and did not hydrolyze gelatin and amylum. It is positive in tests for indole production but negative for acetic acid oxidization and denitrification. Optimal growth was observed at 28℃ and at pH 7.0 for 24 h on LB medium. It forms yellow colonies within 2—3 d at 20℃ when growing on medium Ⅰ, Ⅱ or

LB. The degrading ability of strain Y1 to mefenacet was very stable and still retained well even after several generations of nonselective LB medium. Analysis of whole-cell fatty acid revealed that the I-2-OH-C15:0 existed in the strain Y1(Fig. 5), which is typical fatty acid for the genus *Sphingobacterium*(Dong, 2001).

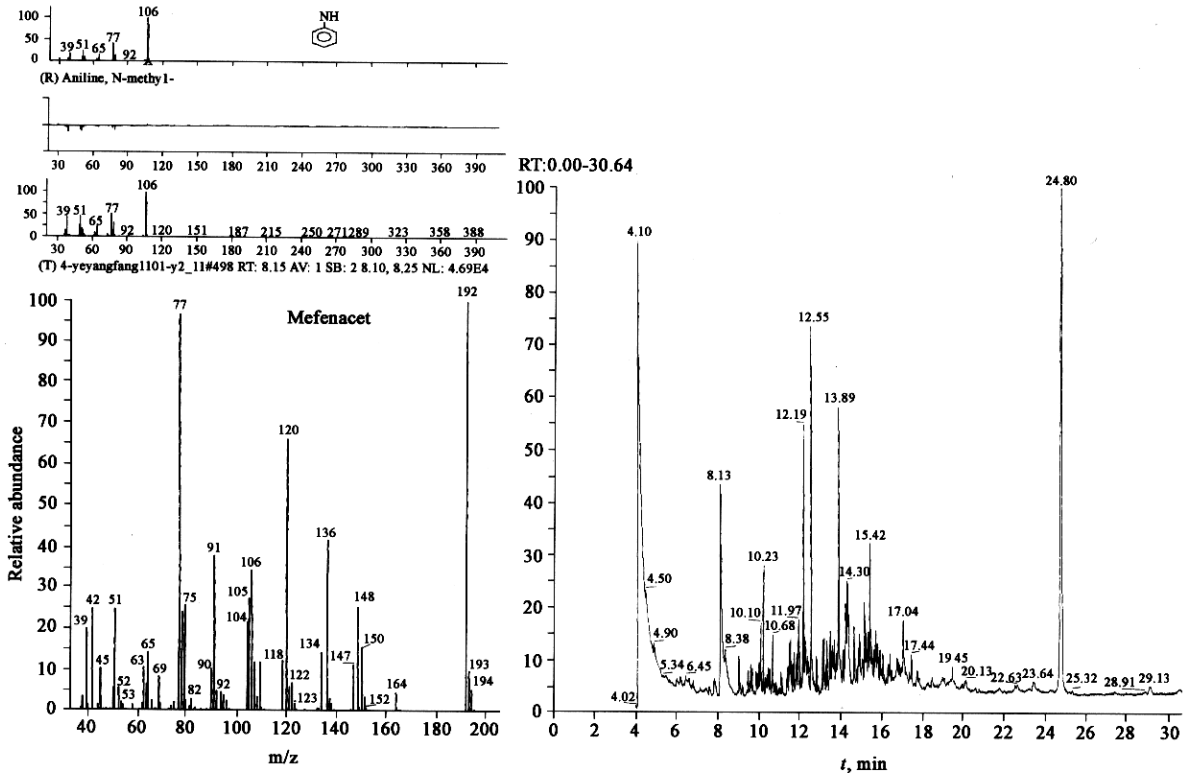

Fig.3 Mass spectra of a N-methylaniline analytical standard and of mefenacet, the compound at 8.13 min retention time was identified as N-methylaniline, and that at 24.77 min retention time was mefenacet

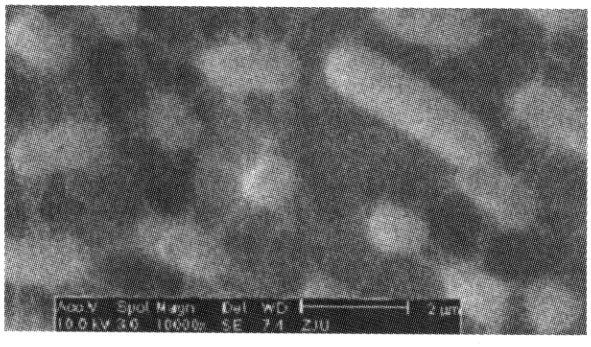

Fig.4 Electron micrographs of strain Y1 cell(10000×)

Upon comparison of a partial 16S rRNA gene sequence from the GenBank Database, the highest degree of similarity (99%) was obtained with that of *Sphingobacterium multivorum* OM-A8. Alignment of the partial 16S rRNA gene sequences revealed a close phylogenetic relationship to several *Sphingobacterium* sp.(Fig. 6).

The growth of strain Y1 was observed in BIOLOG-GN microplates and the developed colors are shown by O.D in Table 1. Strain Y1 was identified as *Sphingobacterium multivorum* by the BIOLOG bacteria automated identification apparatus. This result was completely consistent with that of analysis of 16S rRNA.

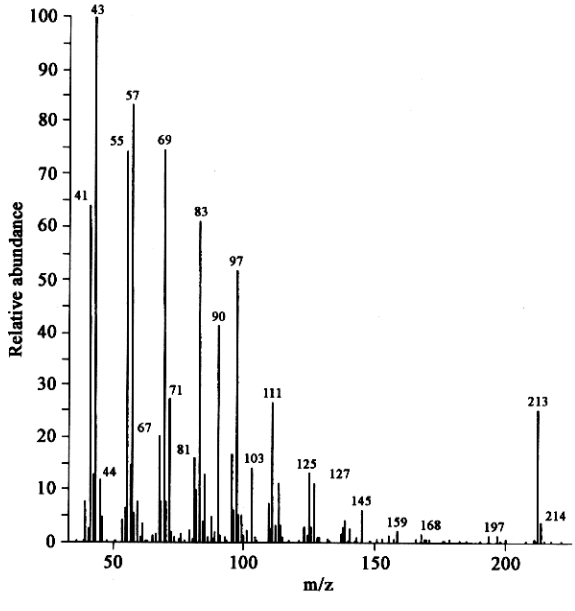

Fig.5 Mass spectra of fatty acid I-2-OH-C15:0 with retention time 24.87 min

Strain Y1 was able to efficiently degrade the mefenacet. The sequencing of partial 16S rRNA gene, the characteristic cellular fatty acid composition and the analysis of BIOLOG-GN strongly suggested that strain Y1 was belonged to the genus *Sphingobacterium*. Strain Y1 was phylogenetically

related to a previously characterized *Sphingobacterium* sp. F1 which was able to degrade the fluorobenzene (Carvalho, 2002). However, the 16S rRNA gene sequence of strain Y1 was the most similar to that of *Sphingobacterium multivorum* (99%). The findings of *Sphingobacterium* sp. able to degrade xenobiotic organic compounds are rarely reported and it is evident that the degrading ability of the genus *Sphingobacterium* needs to be studied further. To our knowledge this is the first report on biodegradation of herbicide mefenacet as a source of carbon and energy by *Sphingobacterium multivorum*.

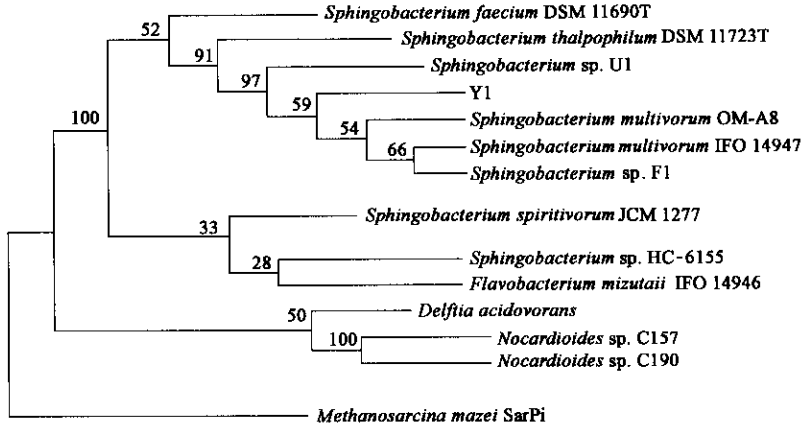

Fig.6 Phylogenetic tree based on the 16S ribosomal DNA sequences data showing the relationships of strain Y1 with the most closed related bacteria identified in the GenBank Database. Included in the analysis are *S. faecium* DSM 11690T, *S. thalpophilium* DSM 11723T, *S.* sp. U1, *S. multivorum*, *S. multivorum* IFO 14947, *S.* sp. F1, *S. spiritivorum* JCM 1277, *S.* sp. HC-6155, *F. Mizutaii* IFO 14946, *Delftia acidovorans*, *Nocardioides* sp. C157, *Nocardioides* sp. C190, and *M. mazei* Sarpi as an outlier

Table 1 Utilization of 95 carbon substrates by strain Y1 using BIOLOG microplate

Carbon substrate	OD_{590}	Carbon substrate	OD_{590}	Carbon substrate	OD_{590}
Water	0.045	Turanose	1.457	D-alanine	0.065
α-cyctodextrin	0.846	Xylitol	0.050	L-alanine	0.062
Destrin	1.450	Methyl pyruvate	0.057	L-alanylglycine	0.057
Glycogen	1.229	Mono-methyl-succinate	0.106	L-asparagine	0.110
Tween 40	0.080	Acetic acid	0.076	L-aspartic acid	0.088
Tween 80	0.066	Cis-aconitic acid	0.078	L-glutamic acid	0.072
N-acetyl-D-galactosamina	0.648	Citric acid	0.063	Glycyl-L-aspartic acid	0.085
N-acetyl-D-glucosamine	1.107	Formic acid	0.066	Glycyl-L-glutamic acid	0.118
Adonitol	0.054	D-galactonic acid lactone	0.063	L-histidine	0.143
L-arabinose	0.093	D-galacturonic acid	0.058	Hydroxy-L-proline	0.145
D-arabitol	0.061	D-gluconic acid	0.061	L-leucine	0.127
D-cellobiose	1.420	D-glucosaminic acid	0.055	L-qrnithine	0.074
L-erythritol	0.080	D-glucuronic acid	0.937	L-phenylalanine	0.096
D-fructose	2.024	α-hydroxy butyric acid	0.058	L-proline	0.251
L-fucose	0.070	β- hydroxy butyric acid	0.065	L-pyroglutamic acid	0.116
D-galactose	0.847	γ- hydroxy butyric acid	0.560	D-serine	0.071
Gentiobiose	1.224	*p*-hydroxy phenylactic acid	0.108	L-serine	0.101
α-D-glucose	1.222	Itaconic acid	0.084	L-threonine	0.083
m-inositol	0.050	α-keto butyric acid	0.066	D, L-carnitine	0.081
α-D-lactose	1.291	α-keto glutaric acid	0.062	γ-amino butyric acid	0.113
Lactulose	1.143	α-keto valeric acid	0.073	Urocanic acid	0.140
Maltose	1.190	D, L-lactic acid	0.050	Inosine	0.141
D-mannitol	0.052	Malonic acid	0.061	Uridine	0.116
D-mannose	1.151	Propionic acid	0.058	Thymidine	0.107
D-melibiose	1.430	Quinic acid	0.067	Phenyethylamine	0.134
β-methyl-D-glucoside	1.239	D-saccharic acid	0.067	Putrescine	0.115
D-psicose	0.064	Sebacic acid	0.057	2-aminoethanol	0.108
D-raffinose	1.326	Succinic acid	0.097	2,3-butanediol	0.110
L-rhamnose	0.067	Bromo succinic acid	0.161	Glycerol	0.194
D-sorbitol	0.054	Succinamic acid	0.091	D, L-α-glycerol phosphate	0.127
Sucrose	1.266	Glucuronamide	0.072	Glucose-1-phosphate	0.223
D-trehalose	1.188	L-alaninamide	0.057	Glucose-6-phosphate	0.109

Although BIOLOG was initially developed for identification of clinically important bacteria, its use has since been extended to classification, characterization, and diversity studies of other microorganisms and also microbial communities(Shona, 1999). There was considerable agreement between BIOLOG and partial 16S rRNA sequencing in the identification of strain Y1 in this experiment and these two methods even assigned it to the same species, although significant discrepancies between these two methods have been addressed (Shona, 1999), which is why BIOLOG does not be used more widely. While precise identification of strain Y1 should be based on results from as wide a range of techniques as possible, our study has shown the value of BIOLOG coupled with 16S rRNA analysis for characterization and identification of strain Y1.

Mefenacet was degraded rapidly by *Sphingobacterium multivorum* Y1. On the 2nd day after inoculation of strain Y1, the accumulation of metabolites was detected by GC/MS. According to the structure of mefenacet, it seemed that the amido bond was cleaved by strain Y1. The existence of N-methylanilide, a metabolite detected firstly, proved this hypothesis. Therefore, degradation of mefenacet was initiated by the cleavage of amido bond, indicating that hydrolysis appeared to be the first step in mefenacet degradation by *Sphingobacterium multivorum* Y1.

The concentration of mefenacet did not decrease or decrease very slowly after 7 d incubation, so mefenacet will be incompletely degraded by *Sphingobacterium multivorum* Y1 only within a week. The nature of the metabolic pathway in the degradation of mefenacet by *Sphingobacterium multivorum* Y1 remains to be elucidated in future.

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