International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 14 (4), pp. 001-007, April, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolation and characterization of *Rhodococcus* sp.BX2 capable of degrading bensulfuron-methyl

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Accepted 30 July, 2019

Bensulfuron-methyl (BSM) is a widely applicable sulfonylurea herbicides. However, BSM remains in environment for extended period of time (over 100 days) and causes a series of environmental problems. In this study, BX2, a strain presents high BSM degradation abilities, was isolated from an activated sludge sample using a continuous enrichment method and determined to be a member of the genus Rhodococcus based on its physiobiochemical characteristics and 16S rRNA gene sequence. Strain BX2 degradated 92% BSM from an initial concentration of 20 mg/L within a 7- day incubation at 35℃. The specific degradation rate increased with an increase in the initial BSM concentration and the maximum specific degradation rate obtained was 0.146 mg/(mg•d) at 140 mg/L. BSM degradation by strain BX2 can be presented by Haldane's inhibition equation and the kinetic parameters were: vm ax = 16.75 mg/(mg•d), ks = 7809.36 mg/L and ki = 2.41 mg/L, respectively. These results indicated that the strain BX2 could be an excellent candidate for bioremediation of BSM-contaminated environments due to its high BSM-degradative ability.

Key words: Bensulfuron-methyl (BMS); *Rhodococcus* sp.BX2; Isolation; Biodegradation.

INTRODUCTION

Bensulfuron methyl (BSM) (Londax| methyl 2-[[[[[(4, 6-dimethoxypyrimidin-2-yl) amino] carbonyl] amino] sulfonyl] methyl] benzoate) is a sulfonylurea herbicide that has experienced an exponential increase in usage due to its ability to control weeds at very low dosage (2 to 70 100 g/ha), high herbicidal activity, good crop selectivity and low toxicity to humans and animals. BSM has been used to control broad-leaved weeds and several grass types in a variety of cereal crops in California, Japan, and many regions of the world (Mabury et al 1996; Okamoto et al., 1998).

Although BSM is typically applied at a low dose, low-level residues were still detected in major agricultural drains and rivers. Okamoto et al. (1998) reported that the BSM concentration in certain rivers and lakes of Japan was as high as 0.1 to 2.3 mg/L. The BSM concentration in the groundwater near rice paddy fields in Italy reached 0.02 mg/L (Wei et al., 1998). BSM remains in the environment for an extended period of time (over 100 days) when applied under particular climatic and or pedologic conditions. BSM residues in soil or agricultural drains can cause a series of severe environmental problems (Nicosia et al 1991; Xie et al 2004). Previous studies suggested that BSM residues can alter aspects of the biomass, activity and structure of soil microbial community (El-Ghamry et al., 2002; Gigliotti et al., 1998; Hou et al., 2007; Saeki and Toyota, 2004, Xie et al., 2004) and have significant adverse effects on the growth of aquatic ferns, algae and rice (Aida et al., 2006; Tao et al., 2008; Wu et al., 2007; Yue et al., 2006).

Hence, there is a great need for exploring potential degradation processes to reduce the BSM residue levels in the environment. Microbial degradation and chemical

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hydrolysis are the main pathways of BSM degradation in soil, but bacteria with BSM-degradative capabilities are not frequently reported. Zhu et al. (2005) isolated a strain BH belonging to the genus *Brevibacterium* that has the ability to degrade BSM. Recently, strain LS was found to be capable of utilizing quinelorac and BSM, individually and in combination, as carbon and energy sources (Lu et al., 2008). Due to the modest collections of such bacteria, knowledge about BSM degradation is limited. Consequently, it is of significance to find more bacterial species that have a wider availability, high environmental endurance and strong degradation ability.

In this study, we isolated a bacterium strain capable of using BSM as the sole carbon source from an activated sludge sample and studied its growth and ability to degrade BSM and analyzed the experimental data by Haldane's inhibition model.

MATERIALS AND METHODS

The activated sludge sample that contained BSM-degrading bacteria was collected from the activated sludge tank of a wastewater treatment plant of Jiangsu hormone institute located in the city of Jintan, China. BSM (purity ≥ 96.74%) was purchased from Jiangsu hormone institute, acetonitrile (HPLC grade) was obtained from YUWANG GROUP. (Shandong, China). All other chemical reagents used for the preparation of media and other biochemical studies were of the analytical grade purity commercially available.

Media

The carbon-free minimal salt medium (MSM) for the enrichment consisted of the following components (per liter): $Na_2HPO_4 \cdot 12H_2O$ 6.8 g, KH_2PO_4 3.0 g, $(NH_4)_2SO_4$ 1.0 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.24 g, $CaCl_2 \cdot 2H_2O$ 0.02 g. The medium was adjusted to pH 7.0 with NaOH and then autoclaved at 121°C for 25 min.

Isolation and culture conditions

A continuous enrichment method was used to isolate potential BSM-degrading bacteria, One gram of the activated sludge sample was added to an Erlenmeyer flask (250 ml) containing 100 ml MSM and BSM (100 mg/L), incubated at 30°C on a rotary shaker at 150 rev/min for about 7 days. One milliliter of this enrichment culture was then subcultured five times into fresh MSM containing 100 mg/L BSM every 7 days. BSM removal was measured by HPLC in the culture from the fifth transfer. The enrichment culture capable of degrading BSM was serially diluted in MSM, and transferred to fresh MSM containing 100 mg/L BSM. The loss of BSM was again measured over time. The highest dilution that still presented degradation ability of BSM was spread onto fresh MSM containing 100 mg/L BSM. After incubation at 30°C for 3 days, the colonies were selected to verify their degrading capabilities. One strain, designated BX2, was selected for further investigation.

Identification of the strain BX2

Morphology and physiological characterizations

Cell morphology of the strain BX2 was observed with a light microscope (Leica DM4000 B, German) and transmission electron characterization tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Holt, 1994).

16S ribosomal Deoxyribonucleic acid (rDNA) sequence determination and phylogenetic analysis

The genomic DNA for polymerase chain reaction (PCR) was extracted according to the method described previously (Cheng and Jiang, 2006). The PCR amplification of 16S rRNA gene was carried out with a set of universal primers: 5'-AGAGTTTGATCCTGGCTCAG-3'(16S rRNA gene position 8 to 27 of *E. coli*) and 5'-TACGGTTACCTTGTTACGACTT-3') (16S rRNA gene position1510 to 1492 of *E. coli*) in a thermal cycler (Bio-Rad, USA) under the following conditions: 5 min at 94°C, 30 cycles of 1min at 94°C, 2 min at 48°C, 3 min at 72°C, and one final step of 10 min at 72°C.The amplified PCR products containing the 16S rRNA gene were sequenced by TaKaRa Biotechnology (Dalian, China). Database searches were conducted with the Blast algorithm provided by the National Center for Biotechnology Information. The 16S rRNA gene sequence determined and reference sequences obtained from GenBank databases were aligned using multiple-sequence alignment software CLUSTAL W 1.81. The neighbor - joining (N - J) method, Tamura et al. (2007) was used for phylogenetic analysis. The robustness of the tree topology was assessed by bootstrap analysis with 1000 resembling replicates. The nucleotide sequence coding for the 16S rRNA gene of the strain BX2 (14 19 bp) was deposited in the GenBank database with the accession No. FJ517612.

The environmental factors analysis

Effects of environmental factors on the growth of the strain BX2 were investigated in 250 ml Erlenmeyer flasks. Each flask contained 100 ml MSM, 100 mg/L BSM and 1 ml of enriched culture (OD ≈ 1.0). Tests to determine the effect of temperature were first examined at six levels: 20, 25, 30, 35, 40 and 45°C, at pH 7.0. These were followed by tests of 7 different pH levels from pH 4 to 10 at the identified optimal temperature. The growth was defined as an increase in OD_{600} during the logarithmic growth phase.

Degradation of BSM by the strain BX2 in mineral salt medium

BSM degradation experiments were carried out at the optimal pH and temperature in 250 ml Erlenmeyer flasks at 150 rev/min. Each flask contains 1ml of enrichment culture and 100 ml MSM with BSM concentration varying from 20 to 300 mg/L. Fermentation broths were sampled at intervals of 24 h for BSM and biomass analyses. The experiments were carried out in triplicate, with both uninoculated flasks and flasks without BSM serving as controls. Uninoculated flasks with BSM were used for monitoring any abiotic loss of substrates during incubation. The specific degradation rate was calculated from the obtained degradation curves by dividing the slope of the degradation curve by the associated concentration of the biomass.

Quantification of BSM by HPLC

BSM concentrations of all samples were analyzed by high perfor-mance liquid chromatography system (HPLC, AgelienT 1100 with an U.V. detector). Samples were prepared as described by Luo et al. (2008), 10 µl was used in the injection. Separation was carried out in a ZORBAX SB-C18 reverse-phase column (4.6 mm × 250 um × 5 um). The mobile phase was a mixture of acetonitrile and Ultra pure water (pH3.0 adjusted with aceticum) (70:30, V/V), and the flow rate set at 1 ml/min. BSM was detected at 254 nm and identified by

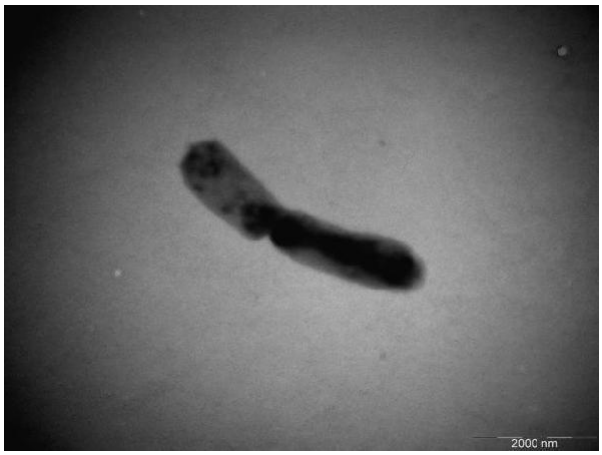

Figure 1. The transmission electron micrograph of strain BX2.

microscope (Philips CM10, Netherlands). Physiological comparison of HPLC retention times.

RESULTS AND DISCUSSION

Isolation and identification of the strain BX2

Isolation of BSM-degrading strains

Various microbial strains were isolated from the activated sludge sample through enrichment culture with BSM as the sole carbon source. High performance liquid chromatography (HPLC) was used to select strains capable of degrading BSM. Among those isolates, a colony with the maximum BSM-degrading potential, designated BX2, was selected as the best strain for further studies.

Morphological and physiobiochemical characteristics of the strain BX2

Strain BX2 (size range: 1.4 to 1.6 µm × 0.8 µm) showing the typical rod–coccus growth cycle usually found among strains of the genus *Rhodococcus* and related taxa, such as *Gordonia*. Upon cultivation on standard nutrient agar, the strain BX2 exhibited salmon pink, smooth and edge-trim colonies. Cell was Gram-positive, non-acid-fast, non-flagellated, and non-motile. No spores were detected (Figure 1). The detailed physiobiochemical characteristics of the strain BX2 were investigated and compared with some type strains of the species of the genus *Rhodococcus* as shown in Table 1.

16S rRNA gene sequencing and phylogenetic analysis

To determine the phylogenetic position of the strain BX2, the 16S rRNA gene sequence was compared with those of type strains of the genus *Rhodococcus* and the related genus, such as *Gordonia,* retrieved from GenBank, Phylo-genetic relationships could be inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known bacteria, and the approximate phylogenetic position of the strain is shown in Figure 2. It is evident that strain BX2 formed a separate branch along with *Gordonia rubripertinctus* (AY771328) (homology, 100.0%), and was found to be similar to *Rhodococcus pyridinicvorans (*AF459741*)* (homology, 100.0%) and *Rhodococcus rhodochrous* (X80624) (homology, 99.0%) Shen et al. (2005) classified *G. rubripertinctus* to the genus *R*hodococcus based on 16S rRNA gene sequencing and phylogenetic analysis, and combining physiological, biochemical characteristics, that the strain BX2 was identified as a strain of the genus *R*hodococcus, and named *Rhodococcus* sp.BX2. Actually, *Rhodococcus* species are widespread in nature and can be isolated from various habitats like soils and sea water. They are equipped with a large number of enzymatic activities, unique cell wall structure and suitable biotechnological properties. This makes *Rhodococcus* strains suitable for use as industrial organisms, primarily for the biotransformation and biodegradation of many organic compounds (Bell et al., 1998).

The genus *R*hodococcus was regarded as one of the most promising groups of organisms suitable for the biodegradation of compounds not easily transformed by other organisms (Warhurst and Fewson., 1994). So far only *Pseudomonads* and related bacteria have been reported to possess comparable biodegradation abilities (Martínková et al., 2009). The xenobiotic compounds metabolized by *R*hodococci cover a wide range of structural groups, including aliphatic and aromatic hydro-carbons, oxygenates, halogenated compounds including polychlorinated biphenyls, nitro aromatics, heterocyclic compounds, nitriles and various herbicides. Many of these substrates are complex synthetic molecules with remark-able stability and toxicity. The significance of *Rhodococci* in environmental biotechnology was discussed in the review characterizing the genus *Rhodococcus* (Bell et al., 1998) and in the reviews concerned primarily with the degradations of recalcitrant compounds by *Rhodococci* (Larkin et al., 2005). *Rhodococci* can thus be applied in environmental remediation and in the pharmaceutical and chemical industries (Geize and Dijkhuizen, 2004; Larkin et al., 2005).

Effect of environmental

factors *Temperature*

In order to design the effective and efficient conditions to accelerate BSM degradation, it is necessary to determine the optimal temperature for the cell growth and the BSM degradation. Figure 3 reveals that the strain BX2 is a

Table 1. Comparison of phenotypic characteristics for the strain BX2 and some type strains of species of the genus *Rhodococcus* by conventional chemical tests.

Characteristic	1	2	3	4	5	6
Shape	R-C	EB-R-C	EB-R-C	H-R-C	H-R-C	R-C
Colony color	Pink to red	ND	ND	ND	ND	salmon pink
H2S production	+	-	+	+	+	+
Hydrolysis of:						
Gelatin	+	ND	ND	ND	ND	-
Tween 80	+	+	+	+	+	+
Starch	+	-	-	-	-	-
Urea	+	-	+	-	-	-
Acid production from:						
D-Glucose	+	+	-	+	w	+
Maltose	-	+	-	-	-	+
Mannitol	+	+	+	+	-	+
Sorbitol	+	+	+	+	-	+
Glycerol	+	+	+	+	-	+
Utilization of:						
Sodium acetate	+	+	+	+	w	+
Sodium benzoate	+	+	-	+	+	+
Sodium succinate	+	+	+	+	-	+
Sodium Citrate	+	+	+	+	-	+
Growth at 4°C	-	-	+	-	-	-
Antibiotic susceptibility:						
Lincomycin	Resistant	ND	ND	ND	ND	Resistant
Rifampicin	Susceptible	ND	ND	ND	ND	Susceptible

1, *G. rubropertincta*; 2, *R. rhodochrous*; 3, *R.pyridinicvorans*; 4, *R. ruber*; 5, *R.coprophilus*; 6, BX2. +, positive; -,negative ND, not determined; w, weak positiveAll strains are positive for catalase, nitrate reduction, tween 80 hydrolysis, D-fructose products acid; and negative for V-P, MR test, oxidase, casein hydrolysis, rhamnose, galactose, lactose, xylose, arabinose and starch products acid.

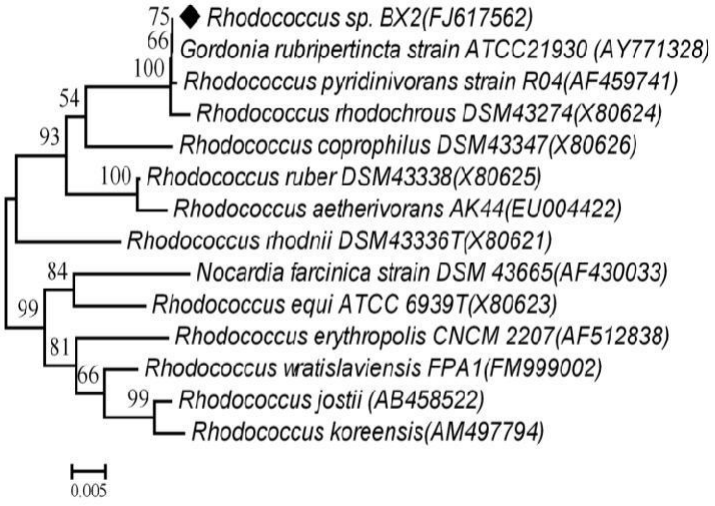

Figure 2.16S rDNA phylogenetic tree for strain BX2 calculated by the neighbor joining method. The numbers at the branch nodes are bootstrap values based on 1000 resamplings. Only bootstrap values greater than 50% are shown. Scale bar: nucleotide divergence of 0.5%. Nucleotide sequence accession number for each reference strain is indicated in brackets.

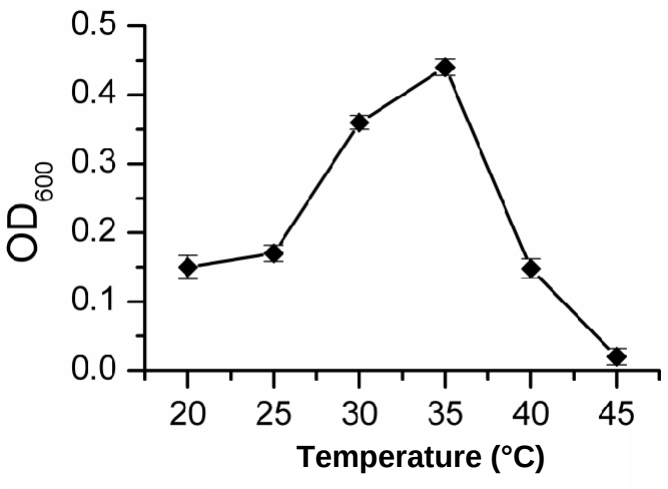

Figure 3. Effect of temperature on cell growth. Biomass was derived from the cell density of *OD*600 and the exponential cells growth period.

mesophilic bacterium. The optimum growth temperature was between 30 and 35°C. However, growth decreased sharply above 35°C and complete growth inhibition occurred at 45°C.

pH

The relationship between growth and pH is shown in Figure 4. The growth of the strain BX2 increased quickly when pH increased from 6.0 to 10.0. The highest growth was observed at pH 8.0.

Characterization of BSM degradation rates

Different initial BSM concentrations were assessed as shown in Figure 5. It can be seen that the strain BX2 has strong BSM degradation capacity, especially at low substrate concentration (20 mg/L). When the initial BSM concentration was 20 mg/L, the strain BX2 could degrade BSM more than 92% within 7 days. No appreciable loss of BSM was observed in the sterile control. BSM concentrations detected in most contaminated soil and discharged wastewater are less than 20 mg/L, so the strain BX2 has a great potential in alleviating practical BSM pollution.

With an initial substrate concentration of 200 mg/L, obvious inhibition appeared. The specific degradation rate increased with an increase in BSM initial concentration and the maximum specific degradation rate was obtained at 0.146 mg / mg·d with a BSM initial concentration of 140 mg/L.

The specific degradation rate decreased at higher BSM concentrations owing to substrate inhibition. The experimental results could be well described by Haldane's inhibition model [Equation (A.1)]:

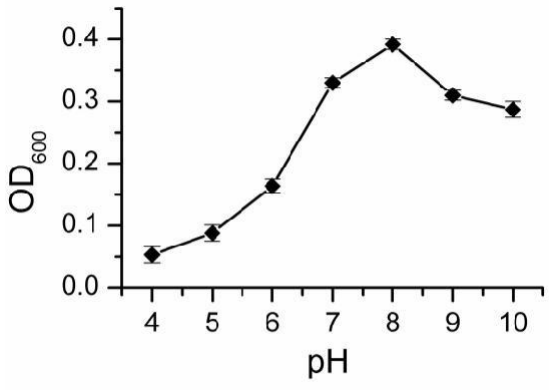

Figure 4. Effect of pH on cell growth. Biomass was derived from the cell density of *OD*600 and the exponential cells growth period.

$$v = \frac{v_{max} S}{k_s + S + S^2 / k_i} \quad (A.1)$$

Where v denotes the specific degradation rate of BSM (mg/(mg·d)); v_{max} , the maximum specific degradation rate of BSM (mg/(mg·d)); k_S , the half-saturation constant for BSM degradation kinetics (mg/L); k_i , the inhibition constant for BSM degradation kinetics (mg/L).

Kinetic parameters for BSM degradation by cells were determined from experimental data in terms of Eq. (A.1) were v_{max} = 16.75 mg/ (mg·d), k_S = 7809.36 mg/L, and k_i = 2.41 mg/L. Figure 6 shows that the substrate inhibition model reasonably describes the relationship between the specific degradation rate and the BSM concentration. Similar substrate inhibition model was also found in BSM degradation by *Brevibacterium* sp.BH (Luo et al., 2008).

Conclusions

The BSM-degradative strain BX2 was successfully isolated from an activated sludge sample. It was identified as a member of the genus *Rhodococcus* based on morphological and physiological characteristics and 16S rRNA gene sequencing. In studies of aerobic BSM biodegradation under favorable conditions, it has been shown that the strain BX2 presented high BSM degradation abilities. The corresponding kinetic parameters of degradation were calculated based on the experimental data and simulated by Haldane's equation. The highest v_{max} was16.75 mg/ (mg·d). This kinetics could be good prediction of the degradative abilities of the BX2 strain.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Fei Ye and Dongmei Li,

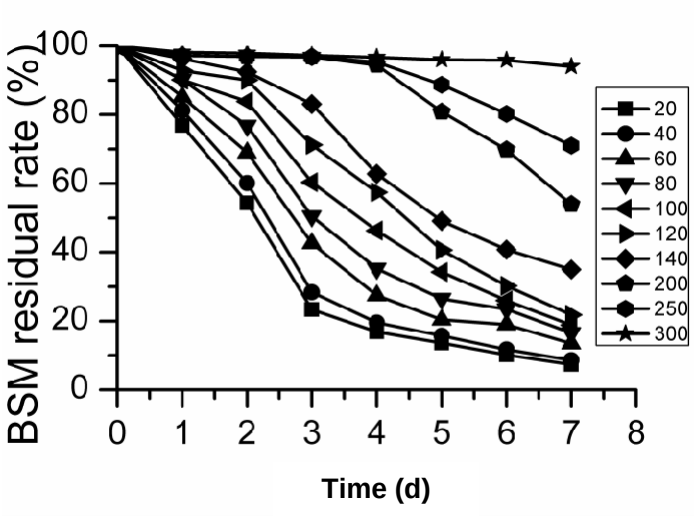

Figure 5. Effect of BSM concentration (mg/L) on the degradation by strain BX2 (37°C, pH 8.0, 150 rev/min).

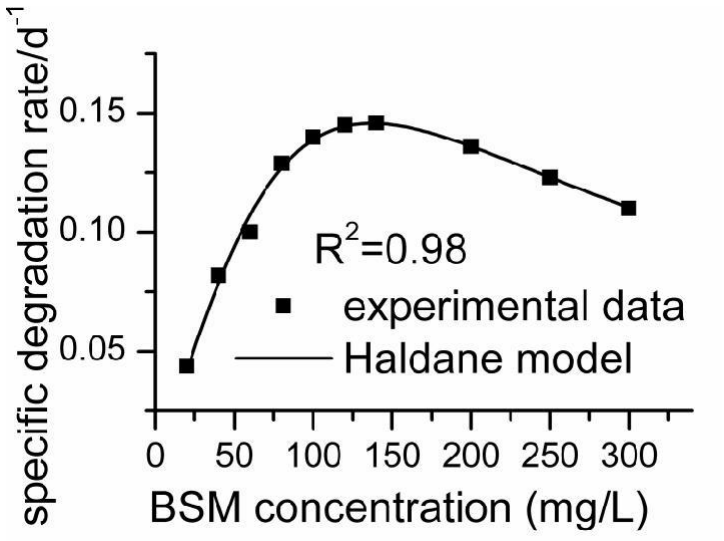

Figure 6. The dependence of specific degradation rate on initial BSM concentration by strain BX2. The software Origin8.0 is employed to process the experimental data.

Basic chemistry Lab, Department of applied chemistry, College of Science, Northeast Agricultural University, for providing high performance liquid chromatogram (HPLC) and excellent technical assistance. Authors are also grateful to Jianfeng Wu, Department of Environmental Health Sciences, University of Michigan School of Public Healt, and Mindy Hatsuko Mizobefor critical reading of the manuscript and their good advice on English version of this paper. This work was supported by Science and Technology Research Project of Heilongjiang Education Bureau (No.11541015); Postdoctoral Science and Research Foundation of Heilongjiang (No. LBH-Q09158) The National Key Technology R and D Program of China (No. 2007BAD87B03); Innovative Team Foundation of Northeast Agricultural University (CXT003-1-3) in China. This work was also supported by Natural Science Foundation of Heilongjiang (No.C201021).

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