Environ Geol (2008) 53:1339–1344
DOI 10.1007/s00254-007-0742-6

ORIGINAL ARTICLE

Comparative proteome analysis of butachlor-degrading bacteria

Jinhua Wang · Yitong Lu · Yingying Chen

Received: 16 January 2007 / Accepted: 12 March 2007 / Published online: 12 April 2007

Abstract A *Pseudomonas putida* strain, named ER1, was isolated from an agricultural soil and found to actively degrade the herbicide butachlor. The enzyme extracted from ER1 could degrade butachlor. Furthermore, incubation of ER1 in a medium containing 50 mg/kg of butachlor after 3 days resulted in the high butachlor-degrading enzyme activity of ER1. Response of ER1 to butachlor might be related to changes in protein composition at both quantitative and qualitative levels. Total proteins were extracted from control strain (incubated in the medium without butachlor) and the treated strain (incubated in the medium with butachlor). The proteins were separated by two-dimensional gel electrophoresis. Of the total number of ER1 protein, 11 spots were significantly changed under butachlor stress. Analysis by matrix-assisted laser desorption/ionization mass spectrometry and tandem mass spectrometry coupled with database searching allowed the function of some proteins which were similar to the hydrolases activity or oxidoreductase activity.

Keywords Butachlor · ER1 strain · Degradation · Protein · Two-dimensional gel electrophoresis

J. Wang · Y. Lu (✉) · Y. Chen
Department of Resource and Environment,
Shanghai Jiao Tong University,
2678 Qixin Road, Shanghai 201101, China
e-mail: ytlu@sjtu.edu.cn

J. Wang
e-mail: wangjinhua@sjtu.edu.cn

Y. Chen
e-mail: chenfirst_927@yahoo.com.cn

Introduction

The herbicide butachlor, *N*-(butoxymethyl)-2-chloro-2′,6′-diethyl acetanilide, is applied in agricultural fields to control weeds. It is now one of top three herbicides applied widely in China (Yu et al. 2003). Butachlor is a persistent pollutant in agricultural soil, posting potential threat to the agro-ecosystem and human health through food chains (Sapna et al. 1995; Debnath et al. 2002). As this pollutant may be a hazard to human health, much attention has been paid to its fate in soil and to the chemical and biological processes involved in its degradation (Min et al. 2002; Yu et al. 2003).

Microbial degradation is considered to be the primary mechanism for the dissipation of pesticides from soil (Sethunathan et al. 1973). A variety of bacteria isolated from soil have been shown to degrade/metabilize pesticides (Adhya et al. 1981; Mallick et al. 1999; Rousseax et al. 2001). In addition, the degrading bacteria can produce a battery of enzymes with different specificities, working together and the enzymes extract from prepared bacteria have been characterized with respect to the rate and specificity of pesticides (Hoskin et al. 1984; Brown et al. 1997). Since the degrading processes are directly executed by proteins, the state of degrading bacteria is essentially reflected in their proteome rather than genome, which is much more stable (Mullbry et al. 1986).

Proteomic analysis is a powerful tool to investigate the cellular responses to toxicants, and thus to uncover new acclimation mechanisms (Yan et al. 2005). Currently, 2-DE (two-dimensional gel electrophoresis) is a key separation technique in proteome analysis due to its advantage of simultaneous separation and display of thousands of proteins at a time. Therefore, 2-DE proteome analysis is becoming a popular method of choice to determine

differentially expressed proteins between proteome profiles in functional genomics. Protein expression mapping gives a global view of protein expression changes within cells under a defined condition versus a control condition. 2-DE proteome analysis is not the end of proteomics. Mass spectrometry (MS) is a powerful tool for mining more meaningful information from 2-DE proteome profiles due to its high sensitivity and high speed. The combination of 2-DE with mass spectrometry enables the identification of proteins on 2-DE patterns in a large scale (Shevchenko et al. 1996). Such 2-DE-based proteome analyses have already been performed for many microorganisms whose complete genome sequences are available (Jungblut et al. 2000; Eymann et al. 2004).

In this study, we isolated and characterized a strain that was capable of degrading butachlor. The degrading activity of the enzymes extracted from the bacteria incubated in medium with butachlor and without butachlor was determined. Furthermore, we analyzed the protein expression changes within bacteria under different conditions (with butachlor and without butachlor) by using 2-DE and MS. Using proteome tools we were able to characterize proteins whose expression enhanced significantly as a result of butachlor stress, which may be helpful to find an effective method for improving the biodegradation of butachlor.

Materials and methods

Enrichment cultures and identification of the bacterial isolate

Soil samples were collected from an agricultural soil in Shandong, China (burozem, 36°38′N, 117°E) that had a previous history treatment with butachlor. Two grams of soil samples were inoculated in mineral salt medium (MSM) containing butachlor (from 10 to 200 mg/l). MSM containing $MgSO_4 \cdot 7\ H_2O$, 0.2 g; K_2HPO_4, 0.1 g; $CaSO_4$, 0.04 g; $FeSO_4 \cdot 7\ H_2O$, 0.001 g; yeast extract, 0.05 g; distilled water, 1 l, were adjusted to pH 7.0 as described by Mallick et al. (1999) and revised. After 8 weeks, cultures were plated on MSM agar plates containing butachlor (200 mg/l). One isolate named strain ER1 showed the best growth on MSM and was selected for further characterization. Strain ER1 was identified by conventional methods based on substrate specificity and biochemical reactions (Buchanan et al. 1974).

Enzyme assays

ER1 strain was incubated in the medium without butachlor (the control) and with butachlor (50 mg/kg, the treated) after 3 days. Enzyme was extracted by the method described by Sims et al. (1986) with some modifications. Cells from a stationary-phase culture in defined medium were collected by centrifugation (10,000*g* for 15 min) and washed with 30 mM phosphate buffer (pH 7.0). The cells were disrupted by sonication (in an ice bath), and the debris was removed by centrifugation (12,000*g* for 30 min). Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Cell extracts stored at –20°C retained high levels of enzyme activity for 4 weeks or longer.

Enzyme activity analysis was according to the method described by Sins et al. (1986) with some modifications. Reaction mixtures contained crude cell extracts, phosphate buffer (30 mM, pH 7.0), and butachlor in a total volume of 3 ml. Enzyme activity was expressed in nanomoles of butachlor transformed per minute per milligram of protein.

Analysis of butachlor

Butachlor was extracted with petroleum ether and detected by gas chromatography. An HP5890 gas chromatograph system was coupled with a flame-ionization detector and fitted with a DB-17 column (30 m × 0.53 mm × 0.30 μm). The gas chromatograph was equipped with a split/splitless injector with electronic pressure control. The temperature program was as follows: oven temperature, 180°C; injector temperature, 185°C; and detector temperature, 250°C. Operating conditions: H_2 gas flow, 45 ml/min; air flow, 45 ml/min; N_2 carrier gas flow, 35 ml/min.

Preparation of samples for 2-DE

Preparation of bacterial protein was according to the methods described by Görg et al. (1988, 1997) with some modifications. The cells were collected as described above (see ''Enzyme assays'') and dried in a freeze dryer. Then, the cell sample was ground in a liquid nitrogen-cooled mortar, and the obtained powder was immediately suspended in 10% trichloroacetic acid (TCA) in acetone (–20°C) containing 0.2% DTT and kept at –20°C overnight. Following centrifugation (12,000*g*, 30 min, 4°C), the supernatant was discarded and the pellet resuspended in acetoned containing 0.2% DTT and kept at –20°C about 1 h. The sample was centrifugated again, the supernatant was discarded and the pellet was dried in a freeze dryer. And then, the pellet was solubilized in lysis buffer (9.5 M urea, 65 mM DTT, 4% w/v CHAPS, 2% v/v pH 3-10 IPG buffer) and centrifugated (12,000*g*, 60 min, 15°C) and protein concentrations were determined as described above (see ''Enzyme assays'').

2-DE analysis and image analysis

For 2-DE, 80 μg of proteins were loaded onto analytical and preparative gels, respectively. For IEF, the Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden) and pH 4–7 IPG strips (13 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 12 h in 250 μl rehydration buffer (8 M urea, 0.2% w/v DTT, 0.5% w/v CHAPS, 0.5% v/v pH 3–10 IPG buffer) containing protein samples. Focusing was performed in three steps: 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 10 h. The gel strips were equilibrated for 15 min in 10 ml equilibration buffer. SDS-PAGE was performed with 12.5% gels. The gels were run at 15 mA per gel for the first 30 min and followed by 40 mA per gel. Gels were stained with colloidal Coomassie Brilliant blue G-250 (Neuhoff et al. 1988). At least three replicates were performed for each sample.

The gels were scanned using ImageScaner 5.0 (Amersham Biosciences). Data were analyzed using ImageMaster 2D Platinum 5.0 software (Swiss Institute of Bioinformatics, etc.). Only those with significant and reproducible changes were considered to be differentially accumulated proteins.

In-gel digestion and MS analysis

Protein spots were excised from the preparative gels, washed three times with ultrapure water, destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) 20 min at room temperature. Then they were washed twice with deionized water, shrunk by dehydration in ACN. The samples were then swollen in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/μl trypsin at 4°C. After 30 min incubation, the gels were digested more than 12 h at 37°C. Peptides were then extracted twice using 0.1% TFA in 50% ACN.

MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Mass spectra were obtained in a mass range of 700–3,200 Da, using a laser operated at a 200 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV, and mass resolution was maximized at 1,500 Da. The TOF-TOF mass spectra were acquired by the data dependant acquisition method with 15 strongest precursor ions selected from one MS scan. MS accuracy was external calibrated with trypsin-digested peptides of horse myoglobin. Data from MALDI-TOF MS/MS were analyzed using MASCOT (Matrix Science, London) search engine against the SWISS-PROT, TrEMBL and NCBI database. The following parameters were used in the search: bacteria, trypsin digest with one missing cleavage, peptide tolerance of 100 mg/l, MS/MS tolerance of 0.6 Da and possible oxidation of methionine.

Data analysis

Data of the gel were analyzed using ImageMaster 2D Platinum 5.0 software (Swiss Institute of Bioinformatics, etc.). The other data were analyzed using the EXCEL and SAS software.

Results and discussion

Isolation and characterization of ER1

Enrichment cultures of the soil samples collected from an agricultural soil in China. The isolate, designated ER1, was identified as a *Pseudomonas putida*. This strain could grow in a MSM adding butachlor as a sole carbon and nitrogen source. When ER1 was incubated in a basal liquid medium at pH 7.0 and initial concentration of 50 mg/l butachlor after 3 days, over 80% of the initial butachlor was degraded. This result showed that strain ER1 was effective for degrading butachlor. *P. putida* are ubiquitous bacteria that engage in important metabolic activities in the environment, including element cycling and the degradation of biogenic and xenobiotic pollutants (Timmis 2002). Chablain et al. (1997) reported a soil psychrotrophic toluene-degrading *Pseudomonas* strain. Furthermore, Walia et al. (2002) isolated a *P. putida* strain OU83 from soil contaminated with substituted aromatics which could degrade dinitrotoluenes (DNTs).

Characterization of butachlor-degrading enzyme

P. putida had considerable potential for biotechnological applications, particularly in the areas of bioremediation (Dejonghe et al. 2001), biocatalysis (Schmid et al. 2001). Many studies had demonstrated that enzyme in bacteria cells including the species in *P. putida* was responsible for the degradation of many pollutants (Hoskin et al. 1984; Rousseax et al. 2001; Pazarlioglu et al. 2005). In this study, we tested the degradation of butachlor by the crude enzyme of ER1. In addition, we also tested butachlor as an inducer of butachlor degrading enzyme synthesis. There were significantly different in enzyme activities between the cells incubated with butachlor (the treated) and without butachlor (the controls). The activities of the enzyme protein were 9.4 nmol/min mg protein (the control) and 20.4 nmol/min mg protein (the treated). These data indicated that butachlor-degrading enzyme protein was not only constitutively expressed in ER1 and was also induced by butachlor. So it seemed that some enzyme proteins

benefit for the degrading of butachlor were induced by butachor.

Comparison of protein expression profiles in butachlor-treated and untreated ER1 cells

In order to compare changes between the constitutive proteins and the butachlor-induced protein, total proteins were extracted from ER1, separated by 2-DE and visualized by CCB staining (Fig. 1). The 2-DE gels were highly reproducible since the experiments performed on three to four replicates had a correlation coefficient varying from 0.67 to 0.89 for spot volumes (data not shown). Approximately 200 spots were detected on each CBB stained gel.

The analysis of the effect of butachlor on the protein composition of ER1 was made by comparing the protein patterns of control and treated samples. These comparisons were based on the volume of each spot (vol%) provided by ImageMaster 2D Platinum 5.0 software (Swiss Institute of Bioinformatics, etc.). We only considered the significant changes revealed by the statistical analysis performed on three replicates of 2-DE gels. Of the total bacteria proteins, 11 spots were found changed after treated by butachlor. The spots are numbered in Fig. 1. Of these variants 10 spots were found to be up-regulated (U1-U5, U7-U11), while one protein spot (spot N6) was only detected in butachlor-treated cells but not in butachlor-untreated cells. Representative examples of the same portions of the 2-DE gels from butachlor-treated and butachlor-untreated cells indicating the differentially expressed protein spots (spot U5 and N6) are shown in Fig. 2. Abundance ratio of the differentially accumulated up-regulated proteins after 3 days of butachlor stress treatment was showed in Fig. 3. The vol% of each spot was considered as the abundance of each spot. The abundance ratio of each spot was calculated by vol% in treated samples/vol% in control samples (Tr/Con). The abundance ratio of each spot was over three and the ratio of spot U5 was even over seven, which meant that these protein spots were significantly changed.

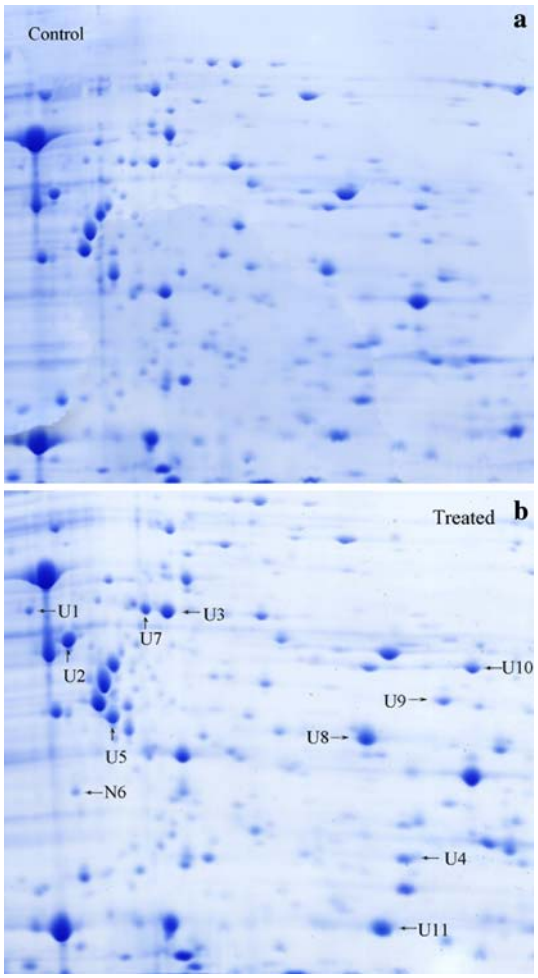

Fig. 1 Comparison of the protein patterns of control (incubated without butachlor) and treated (incubated with butachlor) ER1 strain. Proteins from ER1 were extracted and separated on a pH 4–7 cm IPG strip followed by 12.5% SDS-PAGE. Butachlor-increased spots are designated by *arrows* and *numbered* in the treated pattern

Identification by MALDI-TOF/TOF MS with PMF and/or MS/MS

To make the identification more confident and specific, the spots were excised from preparative 2-DE gels and

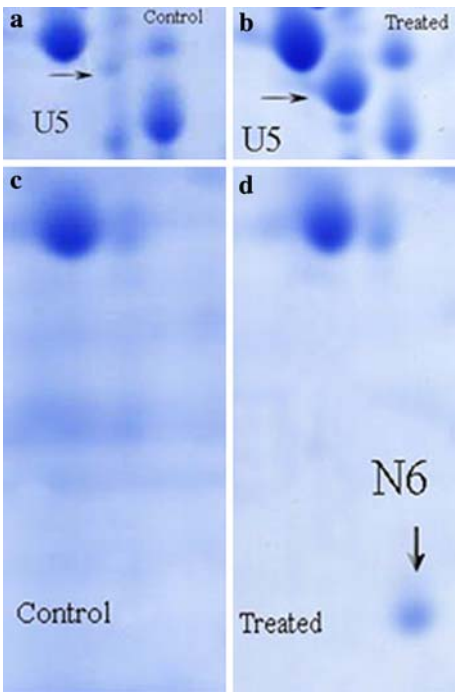

Fig. 2 The magnified comparison maps of up-regulated spot U5 and the new spot N6 of train ER1 in control and treated pattern

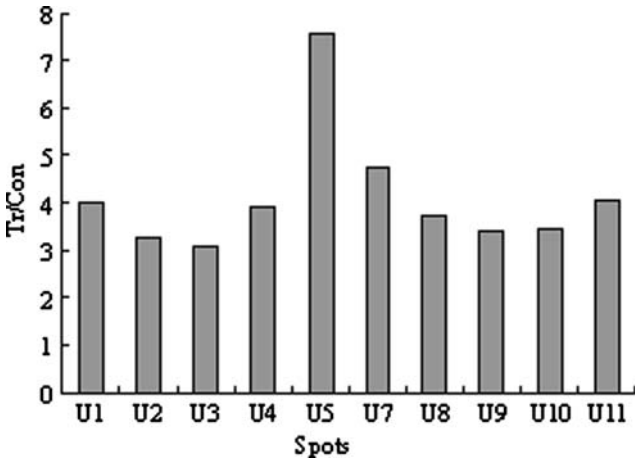

Fig. 3 Abundance ratio of the differentially accumulated up-regulated proteins after 3 days of butachlor stress treatment. The vol% of each spot was considered as the abundance of each spot. The abundance ratio of each spot was calculated by vol% in treated samples/vol% in control samples (Tr/Con)

analysed by MALDI-TOF MS (MS analysis of spot 5 was shown in Fig. 4). In order to identify their functions, they were subjected to database searching against SWISS-PROT, TrEMBL and NCBI using profound software.

Only four spots were similar to known proteins related to the metabolism of pollutants (Table 1), while others produced no mass spectra or gave no good matches. Spot U1 was identified as branched-chain amino acid ABC transporter which might have hydrolase activity. Hoshion and Kose (1990) reported that some bra genes could encode the high-affinity branched-chain amino acid transport system in *P. aeruginosa* PAO. The similar protein was also described in *P. putida* KT2440, and the protein might be related to metabolism of amino acids (Nelson et al. 2002). Amino acids are key intermediates in both carbon and nitrogen metabolism of bacteria. So in this study, the increase of amino acid ABC transporter protein might mean that the carbon and nitrogen metabolism of the butachor-degrading ER1 was improved with the stress of butachlor. Spot U2 and U3 were identified as oxidoreductase. Nelson et al. (2002) also reported some oxidoreductase that might be related to protect *P. putida* KT2440 from pollutants. Oxidoreductase played an essential role in cell defense against environmental stresses. Thapper et al. (2006) also reported that aldehyde oxidoreductase (AOR) activity had been found in a number of sulfate-reducing bacteria and the enzyme that was responsible for the conversion of aldehydes to carboxylic acids. Spot U5 was similar to the putative hydrolases in *P. putida* KT2440 (Nelson et al. 2002). Hydrolases were responsible for degradation of pollutants. Several bacterial strains that could use organophosphate (op)-pesticides as a source of carbon has been isolated from soil samples collected from diverse geographical regions and all these soil isolates synthesize an enzyme called parathion hydrolase (PH) (Mulbry et al. 1986; Somara and Siddavatam 1995; Siddavattam et al. 2006).

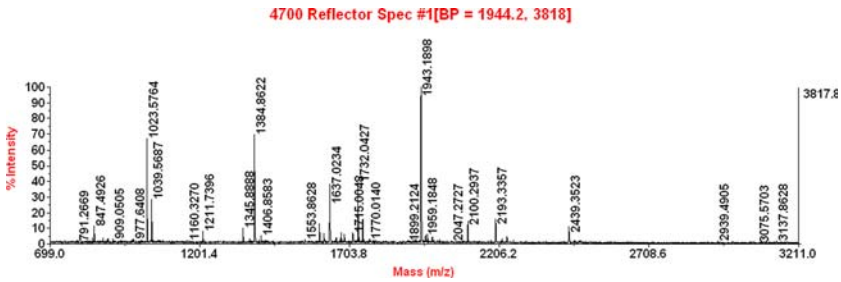

Fig. 4 Ms analysis of spot U5. The protein excised from gels was digested with trypsin and the resulting peptides were analyzed using a 4700 Proteomics Analyzer. A MALDI-TOF MS anlysis

Table 1 Butachlor-increased proteins of ER1 strain

Comparison of the MALDI-TOF obtained PMF with those available in SWISS-PROT, TrEMBL and NCBI. Only spot U1, U2, U3 and U5 were identified as the reported proteins related to the metabolism of pollutants

Spot no.	Similar protein name	Swiss-prot accession no.	Functions
U1	Branched-chain amino acid ABC transporter	Q88NR4	Hydrolase activity, acting on acid anhydrides
U2	2-hydroxy-3-oxopropionate reductase	Q2X806	Oxidoreductase activity
U3	Oxidoreductase, short-chain dehydrogenase/reductase family	Q2T8S2	Oxidoreductase activity, coenzyme binding
U5	Periplasmic binding protein, putative	Q88FX3	Hydrolase activity

Conclusion

A new butachlor-degrading bacterium identified as a *P. putida* strain has been isolated from an agricultural soil in China. We tested butachlor as a inducer of butachlor-degrading enzyme. The results indicated that the enzyme was not only constitutively expressed and was also induced by butachlor. Our proteomic analysis detailed the proteins related to the degradation of butachlor. Of these variants 10 spots were found to be up-regulated, while one protein spot was only detected in butachlor-treated cells but not in butachlor-untreated cells. The identified proteins were similar to the hydrolases activity or oxidoreductase activity. Further research is needed to study the function of the unknown up-regulated proteins and the new protein in this study. Furthermore, more experiments should do to understand the molecular bases involved in the regulation of the quantitative variations of butachlor-degrading proteins.

Acknowledgments This work is financially supported by National Key Basic Research Program of China (No. 2004CB418503), National Natural Science Foundation of China (No. 20337010) and Key Program of Basic Research of Shanghai City (No. 04JC14051).

References

Adhya TK, Barik S, Sethunathan N (1981) Hydrolysis of select organophosphorus insecticides by two bacteria isolated from flooded soil. J Appl Bacteriol 50:167–172

Brown HM, Van JAT, Carski TH et al (1997) Degradation of thifensulfuron methyl in soil: role of microbial carboxyesterase activity. J Agric Food Chem 45:955–961

Buchanan RE, Gibbons NE (1974) Bergey's manual of determinative bacteriology. The Williams & Wilkins Company, Baltimore

Chablain PA, Philippe G, Groboillot A et al (1997) Isolation of a soil psychrotrophic toluene-degrading *Pseudomonas* strain: influence of temperature on the growth characteristics on different substrates. Res Microbial 148:153–161

Debnath A, Das AC, Mukherjee D (2002) Persistence and effect of butachlor and basalin on the activities of phosphate solubilizing microorganisms in Wetland rice soil. Bull Environ Contam Toxicol 68:766–770

Dejonghe W, Boon N, Seghers D et al (2001) Bioaugmentation of soils by increasing microbial richness: missing links. Environ Microbiol 3:649–657

Eymann C, Dreisbach A, Albrecht D et al (2004) A comprehensive proteome map of growing *Bacillus subtilis* cells. Proteomics 4:2849–2876

Görg A, Obermaier C, Boquth G et al (1997) Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. Electrophoresis 18:328–337

Görg A, Postel W, Guenther S Friedrich C (1988) Horizontal two-dimensional electrophoresis with immobilized pH gradients using PhastSystem. Electrophoresis 9:57–59

Hoshino T, Kose K (1990) Cloning, nucleotide sequences, and identification of products of the *Pseudomonas aeruginosa* PAO bra genes, which encode the high-affinity branched-chain amino acid transport system. J Bacteriol 172:5531–5539

Hoskin FCG, Kirkish MA, Steinmann KE (1984) Two enzymes for the detoxification of organophosphorus compounds: sources, similarities, and significance. Fundam Appl Toxicol 4:165–172

Jungblut PR, Bumann D, Haas G et al (2000) Comparative proteome analysis of *Helicobacter pylori*. Mol Microbiol 36:710–725

Lowry OH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mallick K, Bharati K, Banerji A et al (1999) Bacterial degradation of chlorpyrifos in pure cultures and in soil. Bull Environ Contam Toxicol 62:48–54

Min H, Ye YF, Cheng ZY et al (2002) Effects of butachlor on microbial enzyme activities in paddy soil. J Environ Sci 14:413–417

Mulbry WW, Karns JS, Kearney PC et al (1986) Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium sp*. by southern hybridization with opd from *Pseudomonas diminuta*. Appl Environ Microbiol 51:926–930

Nelson KE, Weinel C, Paulsen IT et al (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4(12):799–808

Neuhoff V, Arold N, Taube D et al (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9:255–262

Pazarlioglu NK, Telefoncu A (2005) Biodegradation of phenol by *Pseudomonas putida* immobilized on activated pumice particles. Process Biochem 40:1807–1814

Rousseax S, Hartmann A, Soulas G (2001) Isolation and characterization of new gram-negative and gram-positive Atrazine degrading bacteria from different French soils. FEMS Microbiol Ecol 36:2–3

Sapna S, Natarajan P, Govindaswamy S (1995) Genotoxicity of the herbicide butachlor in cultured human lymphocytes. Mutat Res 344:63–67

Schmid A, Dordick JS, Hauer B et al (2001) Industrial biocatalysis today and tomorrow. Nature 409:258–268

Sethunathan N, Yoshida T (1973) A Flavobacterium sp. That degrades diazinon and parathion. Can J Microbiol 19:873–875

Shevchenko A, Wilm M, Vorm O et al (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Siddavattam D, Raju ER, Emmanuel Paul PV et al (2006) Overexpression of parathion hydrolase in *Escherichia coli* stimulates the synthesis of outer membrane porin OmpF. Pestic Biochem Phys 86:146–150

Sins GK, Sommers LE, Konopka A (1986) Degradation of Pyridine by *Micrococcus luteus* isolated from soil. Appl Environ Microbiol 51(5):963–968

Somara S, Siddavatam D (1995) Plasmid mediated organophosphorus pesticide degradation by *Flavobacterium balustinum*. Biochem Mol Biol Int 36:627–631

Thapper A, Rivas MG, Brondino CD et al (2006) Biochemical and spectroscopic characterization of an aldehyde oxidoreductase isolated from *Desulfovibrio aminophilus*. J Inorg Biochem 100:44–50

Timmis KN (2002) *Pseudomonas putida*: a cosmopolitan opportunist par excellence. Environ Microbiol 4:779–781

Walia SK, Ali-Sadat S, Brar R et al (2002) Identification and mutagenicity of dinitrotoluene metabolites produced by strain *Pseudomonas putida* OU83. Pestic Biochem Phys 73:131–139

Yan SP, Tang ZC, Su WA et al (2005) Proteomic analysis of salt stress–responsive proteins in rice root. Proteomics 5:235–244

Yu YL, Chen YX, Luo YM et al (2003) Rapid degradation of butachlor in wheat rhizosphere soil. Chemosphere 50:771–774