JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 2419–2425 2419
DOI:10.1021/jf903787s

Metabolism of the Neonicotinoid Insecticides Acetamiprid and Thiacloprid by the Yeast *Rhodotorula mucilaginosa* Strain IM-2

Yi-Jun Dai,† Wei-Wei Ji,† Ting Chen,† Wen-Jian Zhang,† Zhong-Hua Liu,† Feng Ge,‡ and Sheng Yuan*,†

†Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, College of Life Science, Nanjing Normal University, Nanjing 210046, People's Republic of China, and ‡Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection, Nanjing 210042, People's Republic of China

A yeast identified as *Rhodotorula mucilaginosa* strain IM-2 was able to degrade acetamiprid (AAP) and thiacloprid (THI) in sucrose mineral salt medium with half-lives of 3.7 and 14.8 days, respectively, while it did not degrade imidacloprid and imidaclothiz. Identification of metabolites indicated that *R. mucilaginosa* IM-2 selectively converted AAP and THI by hydrolysis of AAP to form an intermediate metabolite IM 1-3 and hydrolysis of THI to form an amide derivative, respectively. Metabolite IM 1-3 had no insecticidal activity, while the THI amide showed considerable insecticidal activity but was 15.6 and 38.6 times lower than the parent THI following oral ingestion and a contact test against the horsebean aphid *Aphis craccivora*, respectively. The inoculated *R. mucilaginosa* IM-2 displayed biodegradability of AAP and THI in clay soils.

KEYWORDS: Acetamiprid; biodegradation; half-life; *Rhodotorula mucilaginosa*; thiacloprid

INTRODUCTION

Neonicotinoid insecticides are one of the most important commercial insecticides used worldwide. They are systemic broad-spectrum insecticides and act as a contact and stomach poison against sucking insect pests, such as aphids, whiteflies, leaf- and plant-hoppers, thrips, some micro-lepidoptera, and a number of coleopteran pests. The molecules exhibit a novel mode of action because they are agonists of the nicotinic acetylcholine receptor (nAChR), leading to paralysis and death of pest organisms (*1*−*7*). This selectivity of neonicotinoids to insect nAChR is attributed to the pharmacophore group nitroimine (=N−NO_2) in imidacloprid (IMI), thiamethoxam (THIA), clothianidin (CLO), and dinotefuran, to cyanoimine (=N−CN) in acetamiprid (AAP) and thiacloprid (THI), and to nitromethylene (=C−NO_2) in nitenpyram because they have a much higher affinity for insects as compared to vertebrate nAChRs, and the loss of the nitro or nitrile group can completely reverse the selective toxicity of neonicotinoids for insects over vertebrates (*8*). Therefore, biotransformation of the neonicotinoid pharmacophore moiety has been a particular focus in plant and mammalian systems (*9*, *10*). Enzyme studies on the nitroimine moiety revealed that several human P450 monooxygenases reduced IMI to the nitroso (=N−NO) derivative in an oxygen-sensitive manner. An aldehyde oxidase from rabbit liver cytosol converted IMI to nitroso and amino (=N−NH_2) metabolites (*1*, *11*), and human liver aldehyde oxidase reduced CLO and THIA to their corresponding nitroso metabolites (*12*).

In microbial metabolism of neonicotinoid insecticides, Pandey et al. (*13*) recently reported that the bacterium *Pseudomonas* sp. 1G transformed the nitroimine group in IMI and THIA to nitroso, imine (=NH), and urea (=O) metabolites. The bacterium *Leifsonia* sp. PC-21 metabolized IMI to imine and urea metabolites (*14*). The two bacteria attacked the so-called "magic nitro" nitroimine group. Our previous study indicated that the bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788 always attacked the carbon atom neighbor of the tertiary amine moiety with hydroxylation of IMI, THI, and imidaclothiz (IMT) as well as N-demethylation of AAP (asterisks designated in **Figure 1**) rather than the pharmacophore moieties nitroimine and cyanoimine (*15*−*17*). Although the metabolism of AAP and THI has been studied in honeybee, mice, spinach, and soils (*9*, *10*, *17*−*20*), the metabolism of the cyanoimine group in AAP and THI is still poorly understood in comparison to the nitroimine group. In addition, AAP and THI were quickly eliminated in soils, and microbial metabolism is considered the major degrading pathway (*19*, *20*). In the present study, we studied a soil-isolated yeast strain IM-2 identified as *Rhodotorula mucilaginosa*, which selectively degraded AAP and THI by hydrolysis to form IM 1-3 and THI amide, respectively. IM 1-3 had no insecticidal activity, and the activity of THI amide was 15.6 and 38.6 times lower than the parent THI against the horsebean aphid *Aphis craccivora* following oral ingestion and contact test, respectively. Soil bioremediation indicated that *R. mucilaginosa* IM-2 could degrade AAP and THI *in situ*.

MATERIALS AND METHODS

Chemicals. IMI and AAP samples (> 97% purity) were provided by the Nanjing Pesticides Factory, China. IMT was provided by the Nantong

*To whom correspondence should be addressed. E-mail: yuansheng@njnu.edu.cn.

Published on Web 01/29/2010

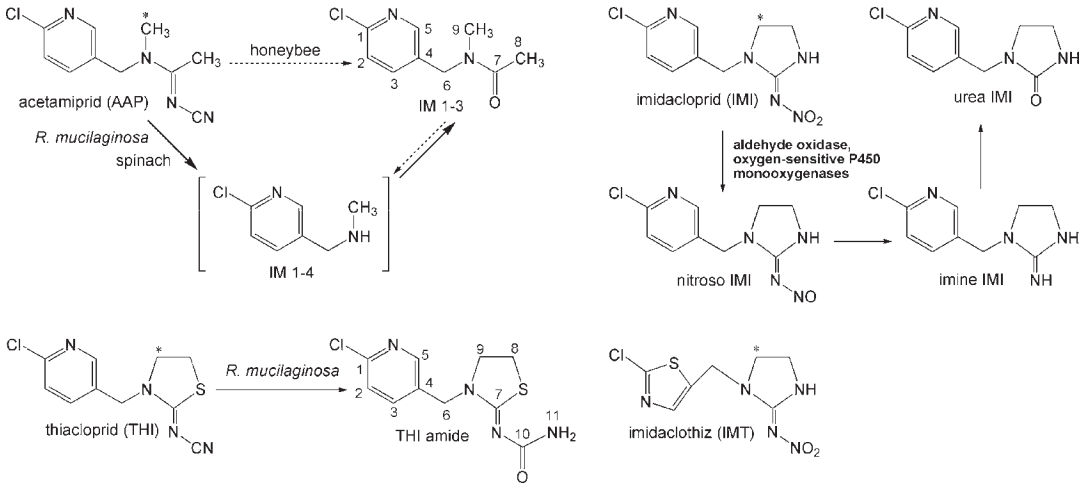

Figure 1. Molecular structure of the neonicotinoid insecticides acetamiprid, thiacloprid, imidacloprid, imidaclothiz, and their metabolites. Asterisks designate the site of hydroxylation of IMI, THI, and IMT as well as N-demethylation of AAP by *S. maltophilia* CGMCC 1.1788. The dashed lines represent the formation of IM 1-3 in honeybee because of direct oxidative cleavage of AAP and subsequent N-deacetylation to form IM 1-4 (*18*). The thick lines represent the formation of IM 1-3 in spinach via cleavage of AAP to IM 1-4, which was further acetylated to IM 1-3 (*10*). The bracket designates the hypothetical but not the observed intermediate.

Jiangsu Agrochemical and Chemical Limited Corporation, China (>95% purity). THI was provided by the Tianjin Renong Pesticide Industry Co., Ltd., China (>97%). Acetonitrile used for high-performance liquid chromatography (HPLC) analysis was HPLC-grade and purchased from E. Merck, Germany. Piperonyl butoxide and methimazole were purchased from the Sigma-Aldrich Corporation, St. Louis, MO. The other solvents and reagents were analytical-grade from commercial sources.

Media. The medium for biotransformation was sucrose mineral salt medium (MSM) (*21*) containing 20.0 g of sucrose, 10.0 g of NH_4Cl, 1.36 g of KH_2PO_4, 2.13 g of Na_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 10 mL of metal solution in 1.0 L of distilled H_2O (pH 7.5). The metal solution contained 0.40 g of $CaCl_2 \cdot 2H_2O$, 0.30 g of H_3BO_3, 0.04 g of $CuSO_4 \cdot 5H_2O$, 0.10 g of KI, 0.20 g of $FeSO_4 \cdot 7H_2O$, 0.40 g of $MnSO_4 \cdot 7H_2O$, 0.20 g of $NaMoO_4 \cdot 2H_2O$, and 10.0 mL of concentrated HCl in 1.0 L of distilled H_2O. The medium for cultivating yeast was YEPD medium that consisted of 20.0 g of peptone, 10.0 g of yeast extract, and 20.0 g of glucose in 1.0 L of distilled H_2O (pH 6.0).

Cultivations. Cultivations were carried out at 30 °C in a 100 mL flask containing 10 mL of YEPD broth and incubated in a rotary shaker at 220 rpm. After cultivation for 24 h, the culture broth (0.1 mL) was then inoculated into a 100 mL flask containing 10 mL of sucrose MSM broth supplied with biotransformation substrate and incubated under the same conditions.

Identification of the Yeast. The yeast was identified by morphological analysis and confirmed by a 28S rRNA gene sequence (*22*). Nucleotide sequence data were deposited with the GenBank database. The sequence obtained was analyzed using the BlastN alignment algorithm to compare it to sequences in the GenBank database.

Biotransformation of AAP, THI, IMI, and IMT. Bioconversion was conducted by adding 500 mg/L AAP and IMI and 200 mg/L THI and IMT (near maximum solubility of THI and IMT) into sucrose MSM broth, in which the transformation was carried out under standard cultivation conditions for the indicated time. Before sampling, sterilized double-distilled water was added to the biodegradation broth up to the initial weight and the samples were centrifuged at 10000*g* for 10 min to remove cell residues. The supernatant was collected, filtered, and diluted to an appropriate volume for the analysis of substrates and biodegradation products. The effects of the cytochrome P450 inhibitor piperonyl butoxide (*23*, *24*) and flavin monooxygenase inhibitor methimazole (*25*) on AAP and THI biotransformation were examined. The cultures were precultivated for 24 h, and then piperonyl butoxide and methimazole solution in acetone at a final concentration of 1 mmol/L was added to the culture broth. After continual cultivation for 120 h, the samples were collected for HPLC analysis. Optical density (OD) was measured at 600 nm by a 2802S UV/vis spectrophotometer (Unico Instruments Co., Ltd., Shanghai, China).

Preparation of the Metabolites of AAP and THI. A single colony of the isolate was inoculated into a 100 mL flask containing 30 mL of YEPD broth, which was then incubated at 30 °C on an orbital shaker at 220 rpm for 24 h. The culture broth was transferred to a 5 L fermenter (Eastbiotech Co., Zhenjiang, China) with 3.5 L of sucrose MSM broth containing 500 mg/L AAP or 200 mg/L THI. During cultivation at 30 °C, the fermenter was stirred at 300 rpm and constantly aerated with an air flow rate of 3 L/min. After biotransformation of about 2 weeks, the cells were removed from the broth by centrifugation at 10000*g* for 20 min and the supernatant was collected. For preparation of the metabolite of AAP, the supernatant was extracted twice with an equal volume of ethyl acetate. For preparation of the THI metabolite, the supernatant was first extracted twice with $^1/_{10}$ volume of dichloromethane to remove the remaining substrate THI and then the water phase was extracted with an equal volume of ethyl acetate. All of the ethyl acetate extracts were dehydrated with anhydrous sodium sulfate, filtered using a 0.22 μm (pore size) ultrafiltration membrane, and then concentrated using a vacuum rotary evaporator. The concentrated extracts were dissolved in a solution of acetonitrile/water (35:65 for THI and 30:70 for AAP, v/v) and further purified using an Agilent preparative HPLC system equipped with an Eclipse XDB-C18 column (21.2 × 150 mm, 7 μm, Santa Clara, CA). The column was first eluted with water/acetonitrile (65:35 for THI and 70:30 for AAP, v/v) at a flow rate of 20 mL/min, and the peaks at 235 nm for AAP and 242 nm for THI were determined using a G1365B MWD detector. The elution, of which the purity was confirmed by HPLC analysis, was subsequently vacuum-dried using a vacuum rotary evaporator.

Bioassays of the Metabolites of AAP and THI. Oral ingestion and contact bioassay against the horsebean aphid *A. craccivora* for metabolites of AAP and THI were tested using the methods described by Nauen et al. (*26*) and were conducted at the National Pesticide Research and Development South Centre, Nanjing, China.

Biodegradation of AAP and THI by *R. mucilaginosa* IM-2 in Soil. Soil samples were provided by the Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection of China. The physicochemical properties of the soil were pH 5.29; organic matter, 9.94 g/kg; clay, 45.8%; sand, 31.9%; silt, 22.3%; and texture clay and field capacity moisture content, 40%.

For soil biodegradation, 10 g of sterilized soil samples was transferred and spread uniformly in Petri plates (10 cm inner diameter) and then the stock solutions of AAP and THI (200 mg/L) were added to obtain a final concentration of 20 mg/g soil, respectively. The yeast precultured for 4 days in sucrose MSM broth was collected and resuspended in fresh sucrose MSM broth, and then a 1 mL suspension was inoculated into the above soil samples (the final amount of yeast was 10^8/g of soil). An additional 2 mL of sterilized distilled water was supplied. The controls

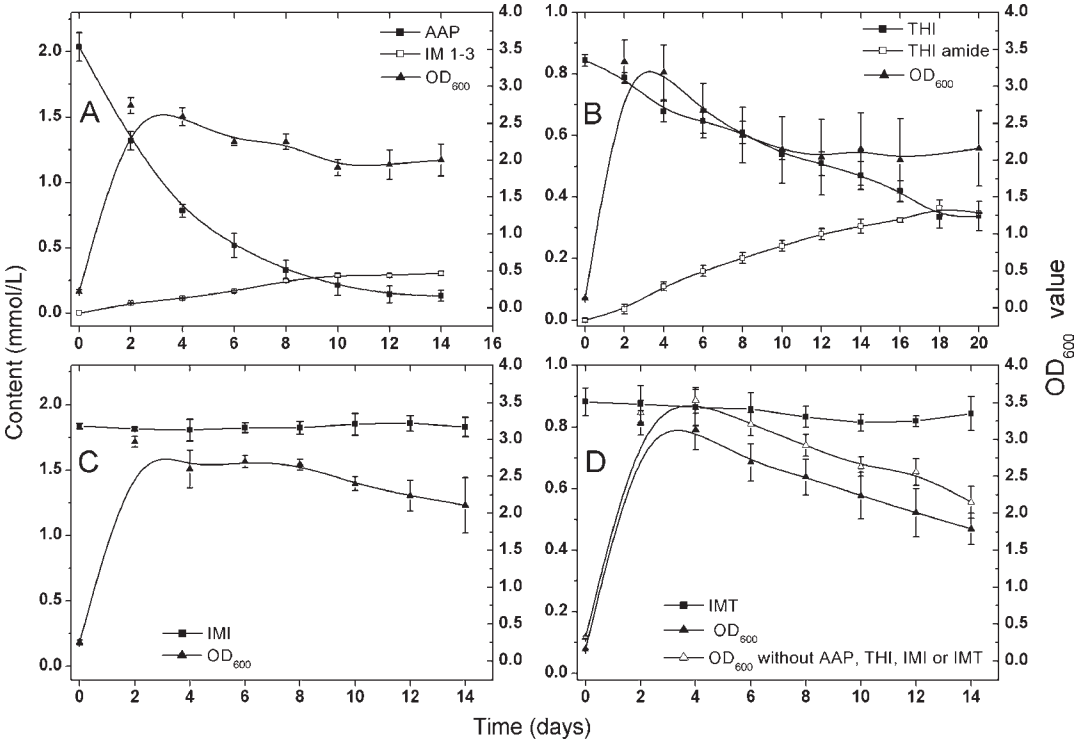

Figure 2. Time courses of cell growth and degradation of AAP, THI, IMI, and IMT by *R. mucilaginosa* IM-2. Under the biotransformation conditions described in the Materials and Methods, the substrate control without inoculated yeast did not degrade AAP and THI. Therefore, spontaneous decomposition of AAP and THI was negligible. A, B, C, and D represent degradation of AAP, THI, IMI and IMT by *R. mucilaginosa* IM-2, respectively. Cell growth was measured at 600 nm by a spectrophotometer. Bars indicate standard deviation (SD) ($n = 9$). The open triangle plots represent the parallel control of cell growth without substrate AAP, THI, IMI, or IMT.

were set with an additional 3 mL of sterilized distilled water. All samples were incubated at 30 °C in darkness and with 80% moisture. Soil sample extractions were performed according to the method by Gupta and Gajbhiye (*27*). The residues of AAP and THI were estimated by HPLC analysis.

HPLC, Liquid Chromatography−Mass Spectrometry (LC−MS), and Nuclear Magnetic Resonance (NMR) Spectra Analysis. An Agilent 1200 HPLC system equipped with an HC-C18 column (4.6 × 250 mm, 5 μm, Agilent Technologies, Santa Clara, CA) was employed for the analysis of the substrates and their metabolites. Elution was carried out at a flow rate of 1 mL/min, with the mobile phase containing water and acetonitrile as well as 0.01% acetic acid. The proportion of water and acetonitrile was 70:30 for AAP, 65:35 for THI and IMT, and 75:25 for IMI. The signal was monitored at wavelengths of 235, 242, 266, and 269 nm for AAP, THI, IMT, and IMI, respectively, with the Agilent G1314A UV detector.

LC−MS analysis was conducted using a Waters Quattro Micro LC−MS system equipped with an electrospray ion source that was operated in the positive-ion mode. ^{13}C and ^{1}H NMR spectra of the biotransformation products were obtained in DMSO-d_6 using a Bruker AV-400 spectrometer (Switzerland) operating at 100 and 400 MHz, respectively. Chemical shifts were referenced against internal tetramethylsilane (TMS). Techniques including ^{1}H and ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum coherence (HSQC), and $^{1}H-^{1}H$ correlation spectroscopy (COSY) spectra were used to assign chemical shifts to protons and carbon atoms.

Half-Life of AAP and THI. Half-life was determined by plotting $\ln(I/I_o)$ against time according to the equation

$$\ln(I/I_o) = -kt$$

where I_o and I represent initial and residual concentrations. The half-life was calculated with the equation

$$t_{1/2} = (\ln 2)/k$$

where $t_{1/2}$ is the half-life and k is the apparent elimination constant. In all cases, the first-order equation provided a satisfactory fit for the data ($r > 0.9$), providing the basis for the half-life calculation (*28*).

RESULTS

Identification of the AAP-Degrading Microorganism. The yeast strain IM-2 was initially isolated from AAP-degrading soils. Its clones exhibited a red color on the YEPD plate, and the typical yeast budding divisions were observed under microscopy. Therefore, the yeast strain IM-2 belonged to the genus, *Rhodotorula*. A 28S rRNA gene sequence analysis further confirmed that strain IM-2 had 99% similarity to *R. mucilaginosa*. Thus, the strain was identified as a species of *R. mucilaginosa*. The accession number of the nucleotide sequence data of *R. mucilaginosa* IM-2 is EF490690 in the GenBank database.

Biodegradation of AAP, THI, IMI, and IMT by *R. mucilaginosa* IM-2. Biodegradation of AAP was primarily optimized in AAP-containing YEPD broth, MSM broth with or without additional sucrose, as well as sucrose MSM broth, which did not contain ammonium chloride. *R. mucilaginosa* IM-2 exhibited the highest AAP biodegradability in sucrose MSM broth; therefore, sucrose MSM broth was selected as the medium for further study. Measurement of the OD_{600} value indicated that the growth of *R. mucilaginosa* IM-2 was significantly inhibited by the addition of 200 mg/L THI or IMT in sucrose MSM broth, and enhanced inhibition was observed by the addition of 500 mg/L AAP or IMI (**Figure 2**). The time course of biodegradation of AAP (**Figure 2A**) showed that *R. mucilaginosa* IM-2 degraded 93.5% AAP when incubated for 14 days. The half-life of AAP degradation fitted first-order dissipation kinetics ($R = 0.99$) and was calculated to be 3.4 days. *R. mucilaginosa* IM-2 degraded 59.9% THI when incubated for 20 days (**Figure 2B**). The half-life of THI

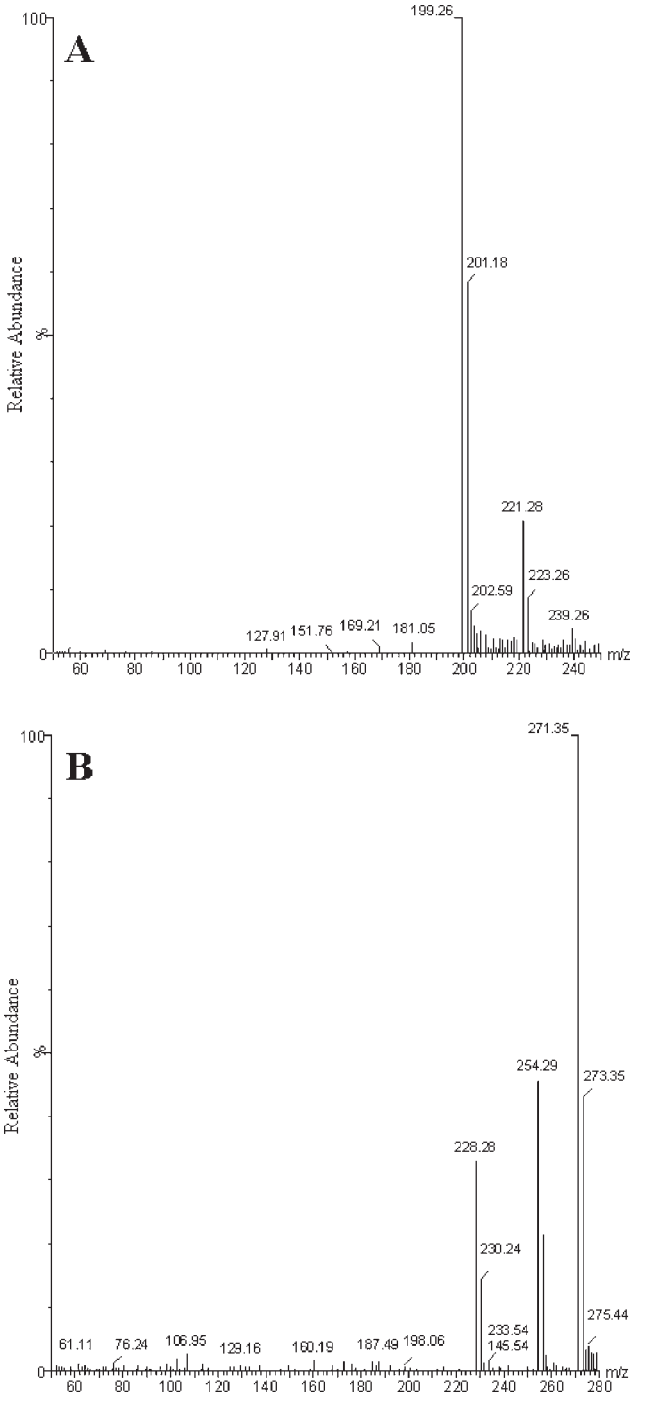

Figure 3. Mass spectrum of (A) AAP and (B) THI metabolite transformed by *R. mucilaginosa* IM-2. The $^{35}Cl/^{37}Cl$ isotope was observed in mass spectrum analysis.

degradation was 14.8 days ($R = 0.99$). However, under the same conditions, *R. mucilaginosa* IM-2 did not degrade IMI (**Figure 2C**) or IMT (**Figure 2D**) ($p > 0.05$). The bacterium *S. maltophilia* CGMCC 1.1788 was previously proven to transform all four of these neonicotinoid insecticides via hydroxylation of IMI, THI, and IMT and by N-demethylation of AAP (*16*). *R. mucilaginosa* IM-2 only degraded AAP and THI, suggesting chemical selectivity of the yeast *R. mucilaginosa* IM-2 in the biotransformation of neonicotinoid insecticides.

Identification of the Metabolites of AAP and THI. During the biotransformation, a polar metabolite was observed by HPLC analysis at a retention time of 4.8 min for THI and 5.6 min for AAP, respectively, while a comparable peak was not found in the controls containing inoculated yeast alone and the substrate alone. The metabolite was not the same as the metabolite IM 2-1 (retention time of 6.4 min) and 4-hydroxy THI (retention time of 6.1 min) following transformation of AAP and THI by *S. maltophilia* CGMCC 1.1788, respectively (*16*, *17*). LC−MS analysis indicated that the AAP metabolite showed a protonated parent ion (M + H) at *m*/*z* 199 and a sodiated adduct (M + Na) at *m*/*z* 221 (**Figure 3A**). The THI metabolite displayed a protonated molecule (M + H) at *m*/*z* 271 and two fragment ions (M − NH_2) at *m*/*z* 254 and (THI imne + H) at *m*/*z* 228 (**Figure 3B**).

Table 1. ^{13}C and ^{1}H NMR Chemical-Shift Assignments for the Biotransformation Product of AAP in DMSO-d_6

position	^{13}C NMR spectrum	^{1}H NMR spectrum
C1	149.4	
C2	124.5	7.47 (d), 1H, J = 8.2 Hz
C3	139.5	7.69 (d), 1H, J = 8.2 Hz
C4	133.2	
C5	149.5	8.29 (s), 1H
C6	47.3	4.90 (s), 2H
C7	170.8	
C8	21.9	2.05 (s), 3H
C9	36.1	2.94 (s), 3H

Table 2. ^{13}C and ^{1}H NMR Chemical-Shift Assignments for the Biotransformation Product of THI in DMSO-d_6

position	^{13}C NMR spectrum	^{1}H NMR spectrum
C1	149.8	
C2	124.7	7.48 (d), 1H, J = 8.2 Hz
C3	140.1	7.80 (dd), 1H, J = 2.4, 8.2 Hz
C4	132.3	
C5	150.1	8.40 (d), 1H, J = 2.4 Hz
C6	49.1	4.70 (s), 2H
C7	168.4	
C8	22.0	3.02 (t), 2H, J = 7.5 Hz
C9	46.9	3.52 (t), 2H, J = 7.5 Hz
C10	163.7	
N11		6.28 (s), 1H, 6.55 (s), 1H

The structure of the purified metabolites was further characterized by NMR. **Table 1** lists the data of chemical-shift assignments for the metabolite of AAP. The ^{13}C spectrum showed that there were only nine carbon atoms, one carbon atom fewer than the parent AAP, and the carbon atom of the cyano group (δ 118.0) was not observed in the ^{13}C spectrum. In combination with the mass spectrum result, it can be concluded that the AAP metabolite transformed by *R. mucilaginosa* was the metabolite IM 1-3, which was reported in the metabolism of AAP in honeybee, mice, and spinach (*9*, *10*, *18*). As shown in **Table 2**, two new protons (δ 6.28 and 6.55) were observed in the ^{1}H NMR spectrum of the THI metabolite. HMBC and HSQC analysis indicated that these two protons had no correlation with the carbon atom. Therefore, they were assigned to the protons of the amide moiety. The number of carbon atoms was identical to the parent THI; however, the chemical shift of the C10 carbon atom of THI in the ^{13}C NMR spectrum was not observed, and a new chemical shift (δ 163.7) appeared in the metabolite, which could be assigned to the carbon atom of the amide moiety. Therefore, THI was converted by *R. mucilaginosa* to form THI amide, a major metabolite in soil metabolism (*20*).

The formation of metabolites IM 1-3 and THI amide (panels **A** and **B** of **Figure 2**) indicated that only 15.8% of reduced AAP (1.90 mmol/L) was converted to IM 1-3 (0.30 mmol/L) after degradation for 14 days. It seems that degradation via IM 1-3 is a

Table 3. Efficacy of IM 1-3 and THI Amide against the Aphid *A. craccivora* Following Oral Ingestion and the Dip Test Bioassay (72 h)[a]

mode of activity	compounds	LC_{50}[b] (mg/L)	FL 95%[c]	slope
contact (dip test)	IM 1-3	>40		
	AAP	0.19	0.14−0.27	2.28
	THI amide	5.79	2.01−16.27	1.05
	THI	0.15	0.10−0.21	2.02
oral ingestion	IM 1-3	>40		
	AAP	0.11	0.05−0.24	1.48
	THI amide	2.49	1.93−3.20	2.81
	THI	0.16	0.13−0.20	1.62

[a] The bioassay was replicated 3 times. The number of aphids exceeded 30 in each independent experiment. The total number of pests was more than 100. [b] LC_{50} = 50% lethal concentration. [c] FL 95% = 95% fiducial limits.

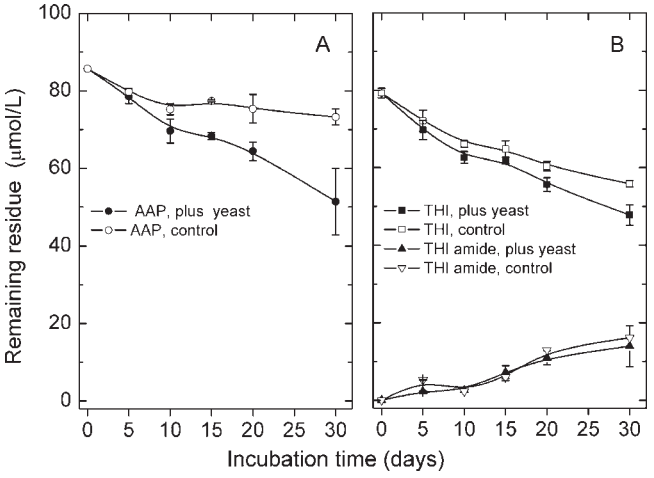

Figure 4. Biodegradation of (A) AAP and (B) THI by *R. mucilaginosa* IM-2 in clay soils. The texture clay soils were sterilized before use. The initial concentration of AAP and THI was 20 mg/g of soil. The recoveries of AAP and THI were 95.4 and 100.2%, respectively. Because recoveries were more than 80%, the residue data had not been corrected for the recoveries. The tested soils were inoculated with 10^8/g of soil of *R. mucilaginosa* IM-2, which was preincubated in sucrose MSM broth for 4 days. Bars indicate SD (n = 9).

partial metabolic pathway for AAP by *R. mucilaginosa* IM-2. Although *R. mucilaginosa* IM-2 had a lower biodegradation ability for THI than AAP, it had a higher molar transformation efficacy because 68.6% reduced THI (0.51 mmol/L) was converted to the corresponding amide metabolite (0.35 mmol/L). Apparently, hydrolysis to form the amide metabolite was the main metabolic pathway for THI.

We reported that N-demethylation of AAP and hydroxylation of THI were mediated by the P450 enzyme system (*16*, *17*). The biodegradation of AAP and THI by *R. mucilaginosa* was also examined following inhibition by the P450 monooxygenase inhibitor piperonyl butoxide and the flavin monooxygenase inhibitor methimazole. The results showed that neither substrate reduction nor metabolite production was affected by piperonyl butoxide or methimazole, indicating that the biotransformation of AAP and THI by *R. mucilaginosa* was not mediated by monooxygenase systems.

Laboratory Bioassays of IM 1-3 and THI Amide against the Aphid *A. craccivora*. In bioassays, replacement of the crucial pharmacophore cyanoimine in AAP with the urea group resulted in IM 1-3, which was inactive at the tested concentrations (LC_{50} > 40 mg/L) (**Table 3**). The THI amide exhibited considerable activity, with LC_{50} values of 2.49 and 5.79 mg/L, but its activity was 15.6 and 38.6 times lower than the parent compound THI following oral ingestion and the contact test, respectively (**Table 3**). The THI imine analogue with the loss of a cyano group dramatically decreased the binding affinity to fruit fly head nAChRs (*2*), and the 4-ketone THI imine was inactive against the aphid *A. craccivora* (*17*). Substitution of the nitrile group with an amide group does not lead to thorough deactivation but reduces the insecticidal activity by at least 1 order of magnitude.

Biodegradation of AAP and THI by *R. mucilaginosa* in Soils. The recoveries of AAP and THI from soil samples were more than 95%; therefore, the residue data were not corrected for the recoveries. As shown in **Figure 4A**, the inoculated *R. mucilaginosa* IM-2 exhibited apparent AAP-degrading ability in clay soils ($p < 0.01$). The soils inoculated with *R. mucilaginosa* IM-2 reduced 40.0% AAP (7.6 mg/g soil), while the controls only reduced 14.5% AAP (2.8 mg/g soil) after incubation for 30 days. No AAP metabolites were detected in the tested soils and controls. The soils with additional *R. mucilaginosa* IM-2 reduced 39.7% THI (8.0 mg/g soil), and the controls reduced 29.6% THI (5.9 mg/g soil) (**Figure 4B**); therefore, *R. mucilaginosa* IM-2 also biodegraded THI in clay soils ($p < 0.05$). Surprisingly, in the soils without microbial activity, the formation of THI amide was detected. Hence, the formation of THI amide in soils should be combined with soil microbial hydrolysis and chemical production.

DISCUSSION

The commercial neonicotinoid insecticides differ considerably with respect to soil use: AAP is limited (+), and THI is not relevant (−), while IMI is broad (+++). These differences are mainly due to soil stability (*4*). For example, IMI persisted with a half-life often greater than 100 days in southern European soil (*29*) and between 36 and 44 days in Indian field soils (*30*). AAP was degraded rapidly by aerobic soil metabolism, with a half-life ranging from 1 to 8.2 days in studies of various U.S. and European soils, and varied from 16 to 17 days under field conditions in Indian soils (*31*). The half-life of THI in soils under field conditions in northern Europe ranged from 9 to 27 days and ranged from 10 to 16 days in southern European soil (*20*). Our recent data indicated that, in the same soils, the half-lives of AAP and THI were less than 5 days, while that of IMI and IMT exceeded 40 days, in laboratory conditions (unpublished data). In comparison to the above data, AAP and THI are more easily degraded in soils. Microbial metabolism of AAP and THI in soil is considered the main elimination route (*19*, *20*). From the present results, the easily attacked cyanoimine pharmacophore in AAP and THI is more prone to hydrolysis by microbes to form the urea and amide metabolite, respectively. Surprisingly, the formation of THI amide was detected in sterilized soils at a level of 1.6 mg/g of soil on the 15th day (**Figure 4B**), which implies that THI amide is partially formed from soil chemical production. In comparison to sterilized soils, the level of THI amide in unsterilized soils was 14 mg/g of soil following the initial application of 20 mg/g of soil of THI (unpublished data); thus, microbial hydrolysis is still predominant.

The metabolic pathway of AAP has been studied in honeybee, mice, spinach, and soils (*9*, *10*, *18*, *19*). Two different metabolic pathways for IM 1-3 formation were presented: Brunet et al. (*18*) suggested that AAP was metabolized in honeybee by primary oxidative cleavage of the cyanoimine group to form IM 1-3 and subsequent N-deacetylation to form IM 1-4, while Ford and Casida (*10*) proposed that AAP in spinach was first cleaved at the $N(CH_3)-C(CH_3)CN$ linkage to form IM 1-4, which was subsequently acetylated to IM 1-3 (see **Figure 1**) (*10*). The metabolite

IM 1-4 was not detected in HPLC and positive-ion LC−MS analysis, and the formation of IM 1-3 was not mediated by the monooxygenase system. Therefore, these findings suggest that the observed metabolite IM 1-3 is a product of the hydrolysis of AAP via an IM 1-4 intermediate. However, limited by the fact that no metabolite was detected following soil biodegradation by the inoculated yeast, the primary metabolic pathway of AAP by *R. mucilaginosa* in soil is still unclear and needs further study.

Besides soil stability, bioefficacy of metabolites is another important factor affecting the method of neonicotinoid insecticide application. Nauen et al. (*26*, *32*) reported that one of the plant metabolites, olefin IMI, had 10- and 16-fold greater activity against whiteflies and aphids, respectively. They speculated that, after soil application, a few of the biologically active metabolites originating from the parent compound acted in concert with the remaining parent compound IMI to provide good control and long-lasting residual activity against plant-sucking pests in certain crops. In the soil biodegradation of THI, the formation of the amide metabolite is the main metabolic pathway (*20*) but the insecticidal activity of THI amide is more than 10 times lower than the parent THI; therefore, biotransformation of THI via hydrolysis is the mechanism of decreasing bioefficacy. It is possible that the vulnerability of the cyanoimine pharmacophore involving chemical and microbial action results in lower soil stability and detoxifying metabolism and reduces the applicability of neonicotinoids containing cyanoimine moiety in soil.

Yeast *R. mucilaginosa* is commonly found in air, soil, and lakes (*33*), and *R. mucilaginosa* IM-2 was proven to biodegrade AAP and THI in soils. Therefore, *R. mucilaginosa* may participate in the soil decomposition of AAP and THI *in situ*. However, in comparison to the biodegradation of AAP and THI in natural soils with half-lives of less than 5 days, the biodegradation of AAP and THI by inoculated pure *R. mucilaginosa* in soils had longer calculated half-lives of 43 days. We speculate that, in addition to yeast *R. mucilaginosa*, there may be diverse AAP- and THI-degrading microbes in natural soils, which might act in combination with *R. mucilaginosa* to quickly degrade AAP and THI. This speculation is partially supported by a recent study in our laboratory where the bacterium *Variovorax* sp., isolated from THI-degrading soils, exhibited a high ability to hydrolyze THI to amide in phosphate buffers (data are not shown).

In the study on the enzyme mechanism of neonicotinoid insecticides, IMI has been thoroughly elucidated but there is a lack of understanding of the mechanisms for AAP, THI, and IMT (*1*). Schulz-Jander et al. (*34*) reported that rabbit liver cytosol with effective nitroreduction of IMI was inactive in the metabolism of AAP and THI and thereby suggested that the "imidacloprid nitro reductase" pathways were not available for AAP and THI. The hydrolysis of the nitrile group in the herbicide dichlobenil by *Variovorax* sp. DSM 11402 was proven to be mediated by the enzyme nitrile hydratase (*35*, *36*). THI can be hydrolyzed to THI amide by the same genus *Variovorax* sp. and yeast *R. mucilaginosa*. Therefore, the hydrolysis of THI to the amide metabolite by *R. mucilaginosa* may also be mediated by nitrile hydratase. Because nitrile hydratase and the AAP-degrading enzyme have not been reported in *R. mucilaginosa* (*37*), gene cloning and expression are underway to help understand the biochemical mechanism involved in the biotransformation of AAP and THI.

In conclusion, our work first reported that the yeast *R. mucilaginosa* could degrade the neonicotinoid insecticides AAP and THI in culture and soil. The present study will help in the design of new neonicotinoids and elucidate the applicability of neonicotinoid insecticides.

ACKNOWLEDGMENT

We are thankful for the NMR analysis by Dr. Jia-Hong Zhou from the Analytical Center at the Nanjing Normal University.

LITERATURE CITED

(1) Tomizawa, M.; Casida, J. E. Neonicotinoid insecticide toxicology: Mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 247–268.

(2) Tomizawa, M.; Lee, D. L.; Casida, J. E. Neonicotinoid insecticides: Molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *J. Agric. Food Chem.* **2000**, *48* (12), 6016–6024.

(3) Tomizawa, M.; Talley, T. T.; Maltby, D.; Durkin, K. A.; Medzihradszky, K. F.; Burlingame, A. L.; Taylor, P.; Casida, J. E. Mapping the elusive neonicotinoid binding site. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (21), 9075–9080.

(4) Elbert, A.; Haas, M.; Springer, B.; Thielert, W.; Nauen, R. Applied aspects of neonicotinoid uses in crop protection. *Pest Manage. Sci.* **2008**, *64*, 1099–1105.

(5) Talley, T. T.; Harel, M.; Hibbs, R. E.; Radic, Z.; Tomizawa, M.; Casida, J. E.; Taylor, P. Atomic interactions of neonicotinoid agonists with AChBP: Molecular recognition of the distinctive electronegative pharmacophore. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (21), 7606–7611.

(6) Ihara, M.; Okajima, T.; Yamashita, A.; Oda, T.; Hirata, K.; Nishiwaki, H.; Morimoto, T.; Akamatsu, M.; Ashikawa, Y.; Kuroda, S. Crystal structures of *Lymnaea stagnalis* AChBP in complex with neonicotinoid insecticides imidacloprid and clothianidin. *Invertebr. Neurosci.* **2008**, *8* (2), 71–81.

(7) Matsuda, K.; Kanaoka, S.; Akamatsu, M.; Sattelle, D. B. Diverse actions and target-site selectivity of neonicotinoids: Structural insights. *Mol. Pharmacol.* **2009**, *76* (1), 1–10.

(8) Tomizawa, M.; Casida, J. E. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* **2003**, *48*, 339–364.

(9) Ford, K. A.; Casida, J. E. Chloropyridinyl neonicotinoid insecticides: Diverse molecular substituents contribute to facile metabolism in mice. *Chem. Res. Toxicol.* **2006**, *19* (7), 944–951.

(10) Ford, K. A.; Casida, J. E. Comparative metabolism and pharmacokinetics of seven neonicotinoid insecticides in spinach. *J. Agric. Food Chem.* **2008**, *56* (21), 10168–10175.

(11) Dick, R. A.; Kanne, D. B.; Casida, J. E. Identification of aldehyde oxidase as the neonicotinoid nitroreductase. *Chem. Res. Toxicol.* **2005**, *18* (2), 317–323.

(12) Shi, X.; Dick, R. A.; Ford, K. A.; Casida, J. E. Enzymes and inhibitors in neonicotinoid insecticide metabolism. *J. Agric. Food Chem.* **2009**, *57* (11), 4861–4866.

(13) Pandey, G.; Dorrian, S. J.; Russell, R. J.; Oakeshott, J. G. Biotransformation of the neonicotinoid insecticides imidacloprid and thiamethoxam by *Pseudomonas* sp. 1G. *Biochem. Biophys. Res. Commun.* **2009**, *380* (3), 710–714.

(14) Anhalt, J. C.; Moorman, T. B.; Koskinen, W. C. Biodegradation of imidacloprid by an isolated soil microorganism. *J. Environ. Sci. Health, Part B* **2007**, *42* (5), 509–514.

(15) Dai, Y. J.; Yuan, S.; Ge, F.; Chen, T.; Xu, S. C.; Ni, J. P. Microbial hydroxylation of imidacloprid for the synthesis of highly insecticidal olefin imidacloprid. *Appl. Microbiol. Biotechnol.* **2006**, *71* (6), 927–934.

(16) Chen, T.; Dai, Y. J.; Ding, J. F.; Yuan, S.; Ni, J. P. N-Demethylation of neonicotinoid insecticide acetamiprid by bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788. *Biodegradation* **2008**, *19*, 651–658.

(17) Zhao, Y. J.; Dai, Y. J.; Yu, C. G.; Luo, J.; Xu, W. P.; Ni, J. P.; Yuan, S. Hydroxylation of thiacloprid by bacterium *Stenotrophomonas maltophilia* CGMCC1.1788. *Biodegradation* **2009**, *20* (6), 761–768.

(18) Brunet, J. L.; Badiou, A.; Belzunces, L. P. *In vivo* metabolic fate of [^{14}C]-acetamiprid in six biological compartments of the honeybee, *Apis mellifera* L. *Pest Manage. Sci.* **2005**, *61* (8), 742–748.

(19) Tokieda, M.; Ozawa, M.; Gomyo, T. Methods of determination of acetamiprid and its degradation products in soil by gas chromatography. *J. Pestic. Sci.* **1999**, *24*, 181–185.

(20) Krohn, J. Behaviour of thiacloprid in the environment. *Pflanzenschutz-Nachrichten Bayer* **2001**, *54* (2), 281–290.
(21) Schackmann, A.; Müller, R. Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. *Appl. Microbiol. Biotechnol.* **1991**, *34* (6), 809–813.
(22) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M. G. D., Sninsky, J., White, T., Eds.; Academic Press: San Diego, CA, 1990; Vol. 18, pp 315−322.
(23) Joussen, N.; Heckel, D. G.; Haas, M.; Schuphan, I.; Schmidt, B. Metabolism of imidacloprid and DDT by P450 CYP6G1 expressed in cell cultures of *Nicotiana tabacum* suggests detoxification of these insecticides in Cyp6g1-overexpressing strains of *Drosophila melanogaster*, leading to resistance. *Pest Manage. Sci.* **2007**, *64* (1), 65–73.
(24) Iwasa, T.; Motoyama, N.; Ambrose, J. T.; Roe, R. M. R. M. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot.* **2004**, *23* (5), 371–378.
(25) Tomasi, I.; Artaud, I.; Bertheau, Y.; Mansuy, D. Metabolism of polychlorinated phenols by *Pseudomonas cepacia* AC1100: Determination of the first two steps and specific inhibitory effect of methimazole. *J. Bacteriol.* **1995**, *177* (2), 307–311.
(26) Nauen, R.; Ebbinghaus-Kintscher, U.; Schmuck, R. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). *Pest Manage. Sci.* **2001**, *57* (7), 577–586.
(27) Gupta, S.; Gajbhiye, V. T. Persistence of acetamiprid in soil. *Bull. Environ. Contam. Toxicol.* **2007**, *78* (5), 349–352.
(28) Suchail, S.; De Sousa, G.; Rahmani, R.; Belzunces, L. P. *In vivo* distribution and metabolisation of ^{14}C-imidacloprid in different compartments of *Apis mellifera* L. *Pest Manage. Sci.* **2004**, *60* (11), 1056–1062.
(29) Krohn, J.; Hellpointner, E. Environmental fate of imidacloprid. *Pflanzenschutz-Nachrichten Bayer* **2002**, *55*, 3–26.
(30) Sarkar, M. A.; Roy, S.; Kole, R. K.; Chowdhury, A. Persistence and metabolism of imidacloprid in different soils of West Bengal. *Pest Manage. Sci.* **2001**, *57* (7), 598–602.
(31) Gupta, S.; Gajbhiye, V. T. Persistence of acetamiprid in soil. *Bull. Environ. Contam. Toxicol.* **2007**, *78* (5), 349–352.
(32) Nauen, R.; Reckmann, U.; Armborst, S.; Stupp, H. P.; Elbert, A. Whitefly-active metabolites of imidacloprid: Biological efficacy and translocation in cotton plants. *Pest Manage. Sci.* **1999**, *55* (3), 265–271.
(33) Libkind, D.; Brizzio, S.; Ruffini, A.; Gadanho, M.; van Broock, M.; Paulo Sampaio, J. Molecular characterization of carotenogenic yeasts from aquatic environments in Patagonia, Argentina. *Antonie van Leeuwenhoek* **2003**, *84* (4), 313–322.
(34) Schulz-Jander, D. A.; Leimkuehler, W. M.; Casida, J. E. Neonicotinoid insecticides: Reduction and cleavage of imidacloprid nitroimine substituent by liver microsomal and cytosolic enzymes. *Chem. Res. Toxicol.* **2002**, *15* (9), 1158–1165.
(35) Holtze, M. S.; Sorensen, S. R.; Sorensen, J.; Aamand, J. Microbial degradation of the benzonitrile herbicides dichlobenil, bromoxynil and ioxynil in soil and subsurface environments—Insights into degradation pathways, persistent metabolites and involved degrader organisms. *Environ. Pollut.* **2008**, *154* (2), 155–168.
(36) Nielsen, M. K. K.; Holtze, M. S.; Svensmark, B.; Juhler, R. K. Demonstrating formation of potentially persistent transformation products from the herbicides bromoxynil and ioxynil using liquid chromatography−tandem mass spectrometry (LC−MS/MS). *Pest Manage. Sci.* **2007**, *63* (2), 141–149.
(37) Prasad, S.; Sharma, D.; Bhalla, T. Nitrile- and amide-hydrolysing activity in *Kluyveromyces thermotolerans* MGBY 37. *World J. Microbiol. Biotechnol.* **2005**, *21* (8−9), 1447–1450.

Received for review May 31, 2009. Revised manuscript received January 10, 2010. Accepted January 19, 2010. We are thankful for the support from the National Science Foundation of China (30970040), the Key Basic Research Program of Jiangsu Higher Education Institution of China (06KJA21016), the Program of Jiangsu Higher Education Institution of China (09KJB180003), and the Professional and Public Project of National Environmental Protection (200809092).