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ORIGINAL PAPER

Metabolic degradation of phenazine-1-carboxylic acid by the strain *Sphingomonas* sp. DP58: the identification of two metabolites

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Abstract The biotransformation of phenazine-1-carboxylic acid (PCA) by PCA-degrading strain *Sphingomonas* sp. DP58 yielded small quantities of metabolites and was demonstrated for the first time. The metabolites were isolated by using preparative high-performance liquid chromatography (HPLC). In addition, these were subsequently characterized by gas chromatography (GC)–mass spectrum (MS) after *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization and ^{1}H-nuclear magnetic resonance (NMR). They were identified as 4-hydroxy-1-(2-carboxyphenyl) azacyclobut-2-ene-2-carbonitrile (HPAEC) and 4-hydroxy-1-(2-carboxyphenyl)-2-azetidinecarbonitrile (HPAC). The two metabolites had transformational relationship between each other.

Keywords Biotransformation · *Sphingomonas* · PCA · HPAEC · HPAC

Introduction

Fluorescent pseudomonads are ubiquitous inhabitants of plant surfaces, and certain strains are considered promising agents for the biological control of plant diseases caused by phytopathogenic fungi and bacteria (Haas et al. 2000; Omar et al. 1988; Shaukat and Siddiqui 2003). Moreover, certain secondary metabolites such as phenazine compounds, which are produced by root-colonizing fluorescent pseudomonads, have been shown to play a significant role in the suppression of soil-borne diseases of important crop plants (Gurusiddaiah et al. 1986; Thomashow and Weller 1988). There are previous reports on the mechanism of the disease suppression existing in the literatures (Britigan et al. 1997; Hassan and Fridovich 1980).

Phenazine-1-carboxylic acid (PCA) (Fig. 1), which is one phenazine compound and isolated from the fermentation broth of *Pseudomonas* M18 (Hu et al. 2005), is an antibiotic that efficiently protects crops against a broad spectrum of soil-borne fungal phytopathogens. Its biosynthesis and regulation by some strains of fluorescent *Pseudomonas* spp. were reported previously (Dmitri et al. 2006). The strain *Sphingomonas* sp. DP58, which has been isolated in our laboratory, can degrade PCA and reduce its activity against soil-borne plant pathogens in the field (Yang et al. 2007). The study of PCA biodegradation mechanism is important for improving the effectiveness of PCA in preventing plant disease by inhibiting the degradation at some point.

In this study, the organism *Sphingomonas* sp. DP58 utilized PCA as a sole source of carbon, nitrogen and energy. Several metabolites were

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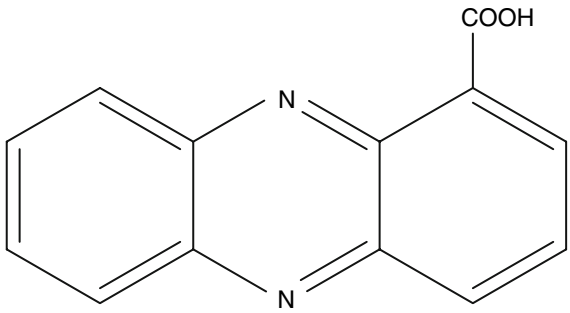

Fig. 1 Molecular structure of PCA

detected at different incubating times through analytical high-performance liquid chromatography (HPLC) and were then isolated by preparative HPLC. Afterwards, the metabolites were characterized by multiple analytical methods.

To our knowledge, this is the first investigation conducted on the intermediates or metabolites of PCA biodegradation. The identification of the metabolites is important for deducing the metabolic pathway of PCA and doing further research on the mechanism underlying PCA biodegradation.

Materials and methods

Cultivation medium and growth conditions

The experiments that examined the biodegradation of PCA were carried out in 250-ml sterilized flasks containing 50 ml of mineral salt medium. This medium had the following composition per liter of distilled water: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 10 g H_3BO_3, 10 g $MnSO_4$, 70 g $ZnSO_4$, 50 g $CuSO_4$, 10 g MoO_3. Afterwards, PCA was added as the sole source of carbon, nitrogen and energy at a concentration of 400 mg/l (1.79 mM).

PCA degradation and metabolite isolation

For turnover experiments, the cells of the strain *Sphingomonas* sp. DP58 were grown in 20-ml test tubes containing 5 ml of LB medium in an orbital shaker (220 rpm) at 28°C up to the late exponential growth phase. The cells were harvested by centrifugation (12,000 rpm for 5 min) and washed twice with the mineral salt medium. Meanwhile, 0.8 ml cell suspensions (with optical density at 600 nm of 5.0) were added in the flasks described above and incubated at 220 rpm and 28°C. Similar experiments were performed on incubations employing poisoned cells (10 mM NaN_3) (Keim et al. 1999) and incubations with active cells inoculated in the mineral salt mediums without PCA. These served as different controls.

This biotransformation was monitored and quantified by using the flasks, wherein at intervals, a set of triplicate flasks was removed and the cells were separated by centrifugation (12,000 rpm for 5 min) and filtration (0.46 μm microfiltration membrane). A small part of the supernatants (5 ml per flask) were analyzed directly by using analytical HPLC. The metabolites were then isolated from the remnant supernatants (45 ml per flask) through preparative HPLC (Shimadzu LC-8A) using the preparative column (Shimadzu C_{18}, 10 μm, 100 Å, 20 × 250 mm) eluted in 5 ml/min mobile phase of 35% methanol and 65% water with an ultraviolet (UV) light detector monitoring at 210 nm. The purified fractions were collected, evaporated under reduced pressure, flushed with N_2 and then used in the GC–MS and ^{1}H-nuclear magnetic resonance (NMR). The controls described above were also used in GC–MS after the same pretreatment.

Analytical methods

PCA and metabolites were detected and analyzed through analytical HPLC (Shimadzu LC-8A) using the analytical column (Kromasil C_{18}, 5 μm, 100 Å, 4.6 × 250 mm) eluted in 0.7 ml/min mobile phase of 40% methanol and 60% water with an UV light detector monitoring at 210 nm. The concentrations of PCA and metabolites were related with the HPLC peak areas by using purchased PCA and isolated metabolites as standard samples. The metabolites were transformed into trimethylsiloxy derivatives via a reaction with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99%) plus trimethylchlorosilane (TMCS) (1%) at 60°C for 1 h before GC–MS analysis (Farshtchi and Moss 1969; Farshy and Moss 1970; Gai et al. 2007; Hong et al. 2002; Yu et al. 1998). Meanwhile, GC–MS (Hewlett-Packard GCD 1800C) in EI mode at 70 eV used the DB-5 MS

column, and the following temperature programme was 60°C for 2 min → (10°C/min) → 300°C for 20 min. The ^{1}H-NMR spectrum was obtained using a Bruker Dpx 400 spectrometer (28°C, DMSO, 400.13 MHz) and D_2O was added for identifying active hydrogens.

Chemicals

Here, PCA (analytical purity ≥ 98%) isolated from the fermentation broth of *Pseudomonas* M18 was purchased from Shanghai Nongle Bio-Product Co., Ltd. BSTFA and TMCS were purchased from JK Chemical. All other commercially available chemicals were of analytical grade.

Results and discussion

PCA degradation and metabolite isolation

The strain *Sphingomonas* sp. DP58 degraded PCA, as detected by the analytical HPLC when grown in the mineral salt medium supplemented with 400 mg/l of PCA as the sole source of carbon, nitrogen and energy. The time course of the PCA degradation was followed. The PCA peak at 3.7 min decreased gradually. After 5 h, a new peak appeared at 7.2 min, and gradually increased for ~10 h. After 18 h, another peak at 6.8 min was observed in the analytical HPLC and it increased while the peak at 7.2 min decreased. After 40 h, the peak at 7.2 min disappeared and the peak at 6.8 min reached its maximum height without any additional changes afterwards (Fig. 2). As these phenomena were not observed in the controls, the results above indicate that there were two metabolites of PCA which were detectable in HPLC. The metabolite A (at 7.2 min) was derived from the PCA transformation and the metabolite B (at 6.8 min) was derived from the transformation of A.

The supernatants described above at different incubating times were used to isolate A and B by using the preparative HPLC. The supernatant at 15 h was used to isolate A, and the supernatant at 40 h was used to isolate B according to analytical HPLC results. A and B were obtained after completing the separations with the preparative HPLC using the C_{18} reversed phase column eluted with methanol–water (V:V = 35:65) and with a UV detector monitoring at 210 nm. The isolated A and B components were then verified by the analytical HPLC. For the isolated component B, there was just one peak, while in the analytical HPLC, it was observed that the isolated component A was mixed with a small amount of B (not shown).

Identification of PCA degradation metabolites

The mass spectra of trimethylsiloxy derivatives of component A were used to analyze its molecular structures (Fig. 3). The mass spectrometry data of Fig. 3 were summarized in Table 1. The partial losses of the molecular weight could reflect specific groups in the molecular structure. According to the diagnostic peaks in Fig. 3 and the molecular structure of PCA, the compound was identified as 4-hydroxy-1-(carboxyphenyl)azacyclobut-2-ene-2-carbonitrile (HPAEC) tentatively. Since the isolated component A was not pure enough according to the analytical HPLC as described above, ^{1}H-NMR could not be applied to confirm the molecular structure of component A.

Two gas chromatography (GS)–mass spectra of trimethylsiloxy derivatives of component B are shown in Fig. 4. The mass spectrometry data of Fig. 4 were summarized in Table 1.The losses of specific groups were helpful to identify the molecular structures. According to the diagnostic peaks in Fig. 4 and the molecular structures of PCA and HPAEC, the compound was identified as 4-hydroxy-1-(2-carboxyphenyl)-2-azetidinecarbonitrile (HPAC) tentatively. In addition, the corresponding ^{1}H-NMR spectrum [DMSO, 400.13 MHz, δ = 8.051(d, 1H, H1); 7.132 (t, 1H, H2); 7.050(t, 2H, H3 and H4); 2.755(t,1H,H7); 2.458(m,2H,H6); 2.160(t,1H,H5); 10.79(s,1H,Ha); and 6.99(s, 1H, Hb) ppm; $J_{H1–H2} = J_{H2–H3} = J_{H3–H4}$ = 7.7 Hz; $J_{H6–H7}$ = 9.6 Hz; $J_{H5–H6}$ = 8.8 Hz] (the ^{1}H signals at 10.79 and 6.99 ppm disappeared when D_2O was added) confirmed the molecular structure of HPAC (Fig. 5). Ha and Hb could be identified as active hydrogens because their signals disappeared when D_2O was added. The ^{1}H-NMR data of H6 were related with the molecular stereo-structure of HPAC (not shown in Fig. 5). It was supposed that there was one intramolecular hydrogen bond formed between

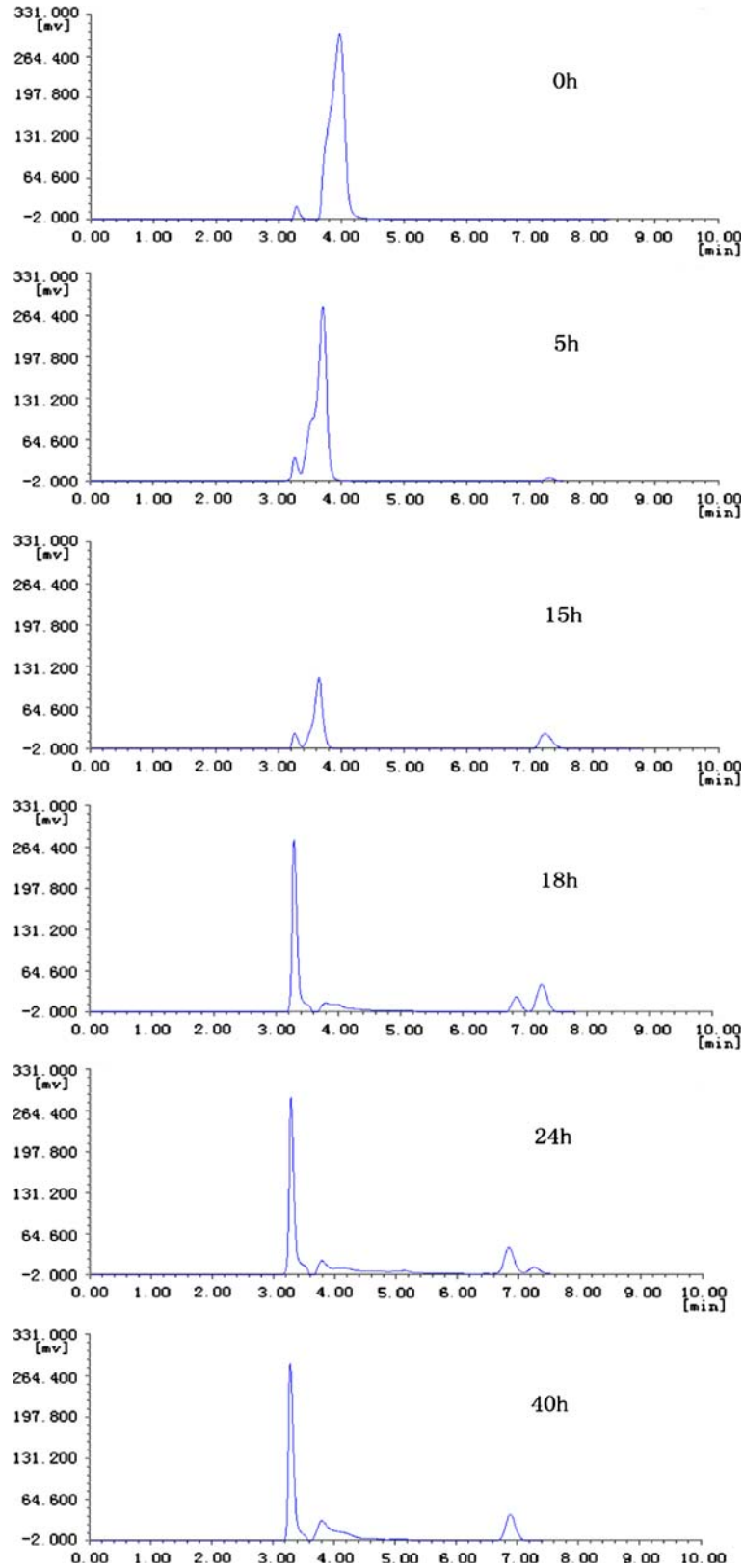

Fig. 2 Analytical HPLC of the supernatants of the cultures of *Sphingomonas* sp. DP58 in mineral salt mediums added with PCA (400 mg/l) incubated in an orbital shaker (220 rpm) at 28°C for 0, 5, 15, 18, 24, and 40 h

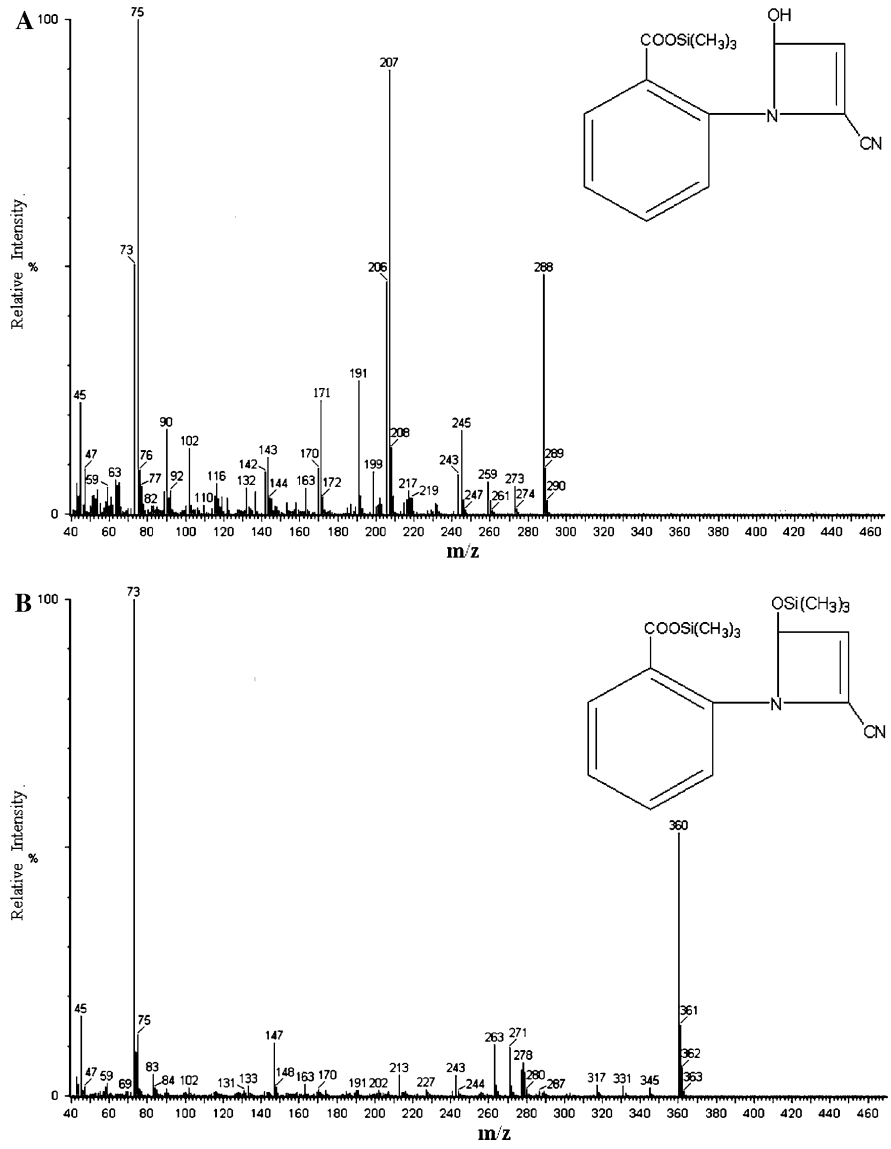

Fig. 3 Identification of HPAEC (two trimethylsiloxy derivatives) as a metabolite produced from PCA by *Sphingomonas* sp. DP58 through GC–MS

Table 1 GC–MS diagnostic fragments of HPAEC and HPAC (trimethylsiloxy derivatives)

Compound	Fragments [*m*/*z* (assignment)]
HPAEC (Fig. 3a)	288(M^+), 273(M^+–CH_3), 207[M^+–(HO–CH–CH=C–CN)], 171 [M^+–$COOSi(CH_3)_3$], 102[M^+–(CH=C–CN)–OH–H–$COOSi(CH_3)_3$], 90 [M^+–(HO–CH–CH=C–CN)–$COOSi(CH_3)_3$]
HPAEC (Fig. 3b)	360 (M^+), 345 (M^+–CH_3), 271 [M^+–$OSi(CH_3)_3$], 243[M^+–$COOSi(CH_3)_3$], 147[M^+–(CH=C–CN)–$OSi(CH_3)_3$–$Si(CH_3)_3$
HPAC (Fig. 4a)	290(M^+), 275(M^+–CH_3), 262(M^+–CN–2H), 261(M^+–CH_3–CH_2), 247 (M^+–CH_3–CN–2H), 206[M^+–(HO–CH–CH_2–CH–CN)–H], 173 [M^+–$COOSi(CH_3)_3$]
HPAC (Fig. 4b)	362(M^+), 347(M^+–CH_3), 273[M^+–$OSi(CH_3)_3$], 245[M^+–$COOSi(CH_3)_3$], 147 [M^+–(CH_2–CH–CN)–$OSi(CH_3)_3$–$Si(CH_3)_3$]

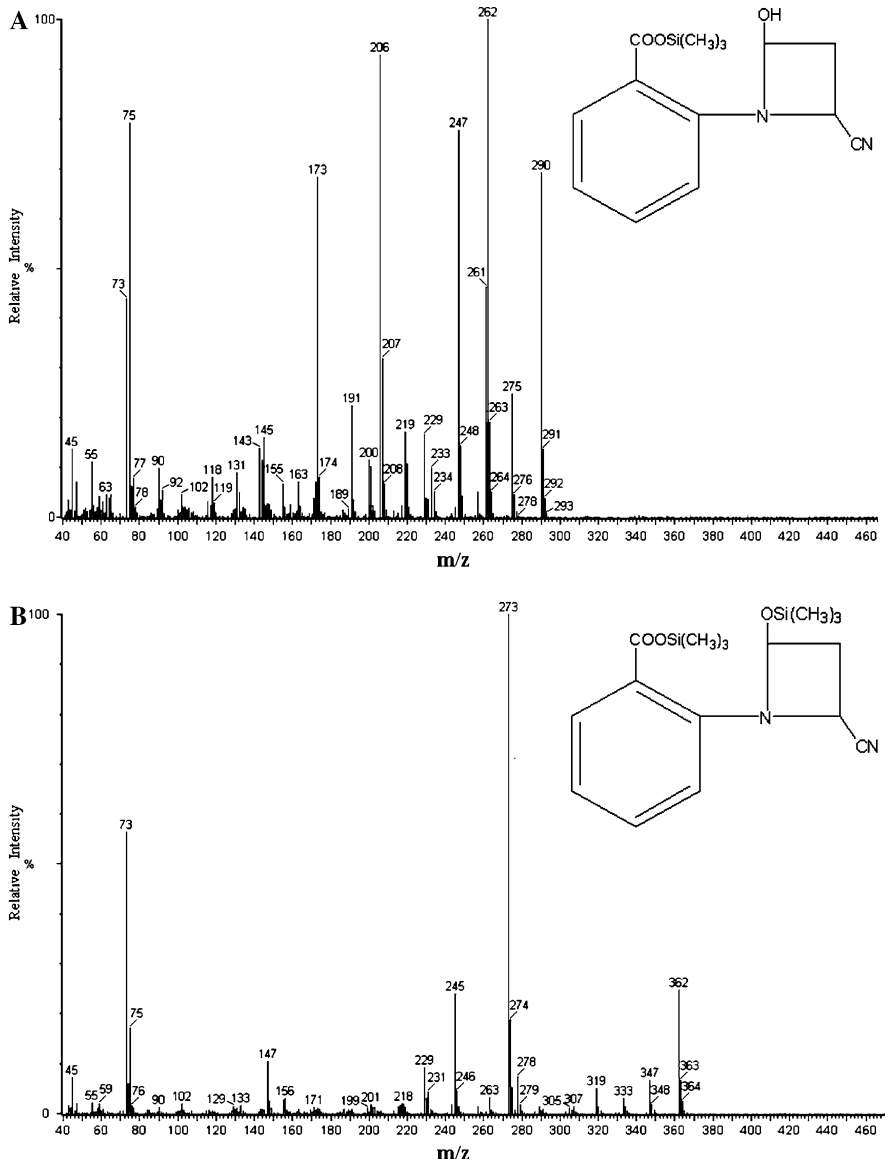

Fig. 4 Identification of HPAC (two trimethylsiloxy derivatives) as a metabolite produced from PCA by *Sphingomonas* sp. DP58 through GC–MS

the hydroxyl and the carboxyl according to the chemical shift of Hb. HPAC is structurally similar to some previously reported compounds (Almena et al. 1994; Fischer et al. 1960; Sulmon et al. 1985; Walker and Weaver 1960).

Biotransformation characteristics of PCA and proposed degradation pathway

By employing *Sphingomonas* sp. DP58, we were able to identify HPAEC and HPAC as two metabolites of PCA. The proposed pathway from PCA to HPAEC and HPAC is shown in Fig. 6. As can be seen, one C–N bond binding the carboxyl-substituted benzene ring and one nitrogen atom could be hydrolyzed and PCA was transformed into 6-(2-carboxyphenylimino)-2,4-cyclohexadien-1-one oxime. After that, the loss of the hydroxyl and the cleavage of the single bond in the cyclohexadiene ring led to the formation of the cyan (Johansen et al. 1997; Necdet 2004). Meanwhile, enol form could be formed after the hydrolyzation of another single bond in the cyclohexadiene ring. Thus, 4-hydroxy-2-(2-carboxyphenylimino)-3-butenenitrile was formed and it could be transformed into 4-oxo-2-(2-carboxyphenylimino)butanenitrile. There existed

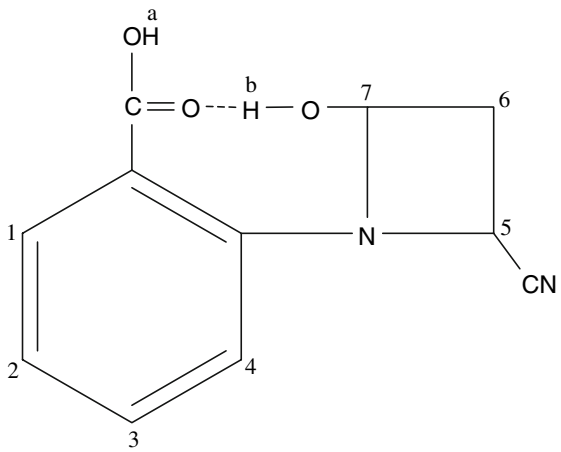

Fig. 5 Molecular structure of HPAC

keto–enol tautomerism between 4-hydroxy-2-(2-carboxyphenylimino)-3-butenenitrile and 4-oxo-2-(2-carboxyphenylimino)butanenitrile (Burdett and Rogers 1964; Tam et al. 1981). HPAEC was derived from the transformation of 4-hydroxy-2-(2-carboxyphenylimino)-3-butenenitrile along with the formations of the C–N single bond and the C–C double bond. Then HPAEC was transformed into HPAC as a result of hydrogen addition. We supposed that the carboxyl of PCA had affected the biotransformation, and specific oxime-metabolizing enzymes had led to the formation of the cyan according to some previous reports (Busk and Moller 2002; Hansen et al. 2001).

When the biotransformation was monitored over time (Fig. 7), it became evident that after 40 h of incubation at least 45% of the PCA had been transformed into HPAEC and about 18% of the PCA had been transformed into HPAC. HPAEC was transformed into HPAC partially. However, HPAC could not be utilized by *Sphingomonas* sp. DP58 because it didn't decrease even when HPAEC had been degraded completely. Thus, *Sphingomonas* sp. DP58 probably utilized other undetected metabolites as growth substrates, which were not derived from HPAC transformation but HPAEC transformation or other PCA-degrading pathways. Moreover, metabolite formation was observed neither in the poisoned controls nor in the controls in which the active cells were inoculated in the mineral salt mediums without PCA.

Because only 45% of the PCA had been transformed into HPAEC which was not transformed into HPAC completely but no other metabolites were observed, it's supposed that HPAEC and HPAC were main extracellular metabolites and the concentrations of other extracellular metabolites were too small to be

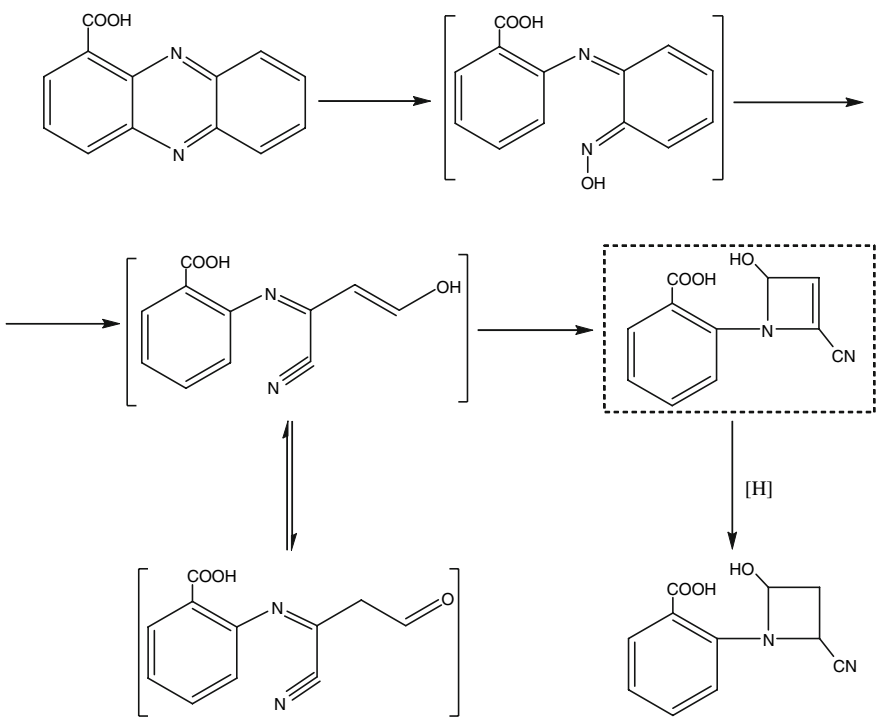

Fig. 6 Pathway proposed for the metabolism of PCA by *Sphingomonas* sp. DP58. The compounds in brackets were not detected and the compounds in *square dotted boxes* were tentatively identified

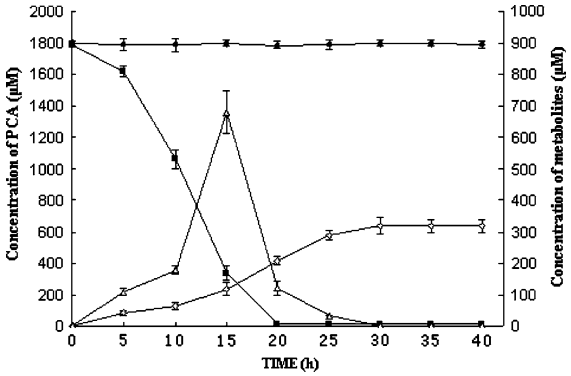

Fig. 7 Biotransformation of PCA by *Sphingomonas* sp. DP58. Symbols: ■, PCA; △, HPAEC; ◇, HPAC; ◆, PCA in poisoned control

detected. In the future, we should research intracellular metabolites of PCA including the metabolites derived from PCA absorbed into cells and the metabolites absorbed into cells after having been formed outside cells.

Conclusions

Overall, the results of this study indicate that PCA can be transformed into HPAEC and HPAC in sequence. The proposed pathway for PCA degradation is helpful to research in order to reveal the degradation mechanism so that we can improve the effectiveness of PCA in preventing plant disease by inhibiting the degradation in the future. According to the proposed pathway, it is supposed that the carboxyl of PCA would affect the biotransformation, and specific oxime-metabolizing enzymes would lead to the formation of the cyan. The results and the suppositions are beneficial to further researches.

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