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Isolation of nicotine-degrading bacterium *Pseudomonas* sp. Nic22, and its potential application in tobacco processing

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ABSTRACT

Nicotine-degrading bacteria were isolated from tobacco leaves and the tobacco field soil using medium containing nicotine as the sole carbon and nitrogen source. One purified isolate with a higher capacity for nicotine degradation was identified and named *Pseudomonas* sp. Nic22 based on 16S rDNA sequence and on morphological and biochemical features. The optimal culture condition of strain *Pseudomonas* sp. Nic22 for nicotine degradation is pH 6.5 and 30–34 °C. This strain can decompose nicotine from different tobacco samples including Burley, Oriental and Zimbabwe LB_4. The crude enzyme extract of *Pseudomonas* sp. Nic22 also decomposed nicotine and significantly improved the quality of tobacco leaf.

1. Introduction

Nicotine is the principal alkaloid in leaves of most *Nicotiana* species (Doolittle et al., 1995) and contributes significantly to smoking properties. Although cigarette smoke contains more than 4000 chemicals, the alkaloid nicotine has shown to be linked to vascular diseases in smokers (Hawkins et al., 2004). Moreover, large quantities of wastes with high concentrations of nicotine are generated during the tobacco-manufacturing process and other activities involving tobacco (Briški et al., 2003). These wastes can pollute the environment, imperil the health of human beings and disrupt the ecological balance when they directly enter the ecosystem without any prior treatment.

The toxic effects of nicotine have been known for a long time. As a result, intensive efforts have been paid to reduce the nicotine content in tobacco products and their associated wastes. Compared with physical and chemical methods, biological methods using microbes are preferable because they are more efficient and less expensive (Clarke and Staley, 1967; Larson et al., 1980; Lenkey, 1989). Recently, Meher et al. (1995) developed a process for the biodegradation of tobacco wastes under anaerobic conditions, leading to a reduction in environmental pollution. Moreover, their treatment generated biogas with the potential of becoming a valuable energy source. Because these treatment methods depend directly on microbial abilities to degrade nicotine, they are effective at reducing the nicotine content during tobacco processing and waste treatment.

Nicotine degradation by microorganisms have been studied almost 50 years ago by work performed with *Arthrobacter oxidans* (reclassified as *Arthrobacter nicotinovorans*) by Decker and Bleeg (1965), Hochstein and Rittenberg (1959, 1960), Richardson and Rittenberg (1961) and Gherna et al. (1965). Moreover, a nicotine-degrading plasmid (pAO1) has been isolated from *A. nicotinovorans* (Brandsch and Decker, 1984), and the gene structure of pAO1 and related mechanism in molecular biology of nicotine degradation were partially elucidated (Brandsch et al., 1986; Schenk et al., 1998; Igoli and Brandsch, 2003). Recently, *Pseudomonas* spp., another nicotine-degrading bacteria have received increasing attention due to their potential roles in tobacco and waste treatment (Wang et al., 2004, 2007; Ruan et al., 2005). However, little is known about the distribution of these microorganisms in tobacco fields and tobacco leaves, and their potential roles in tobacco processing. In this paper, potential nicotine-degrading bacteria were investigated from tobacco fields and from flue-cured tobacco leaves. The isolated organisms were then used to test for their potential roles in degrading nicotine of different tobacco extracts and in improving cigarette quality by reducing the nicotine content.

2. Materials and methods

2.1. Material and samples

L-nicotine (>99%) was purchased from Sigma–Aldrich (USA). Yeast extract and peptone were purchased from Oxoid Ltd. (England). Other reagents including

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NaH_2PO_4, NaOH, KH_2PO_4, NaCl, KCl, etc. were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai).

The tobacco leaves and soil samples used to isolate the nicotine-degrading bacteria were collected from tobacco-producing regions of Zimbabwe and Yunnan province in China.

2.2. Nicotine and related metabolites analysis

The nicotine concentration in samples was analyzed by high-pressure liquid chromatography (HPLC) (Waters 2690, Japan) equipped with a Nova-pak C-18 column (5 μm, 3.9 × 150 mm) and an ultraviolet (UV) detector operating at a wavelength of 260 nm. The column was eluted with a mixture of acetonitrile:distilled water:triethylamine (20:80:0.2, v/v/v) at a flow rate of 0.7 ml min^{-1} and at room temperature (18–22 °C). Quantitative data were obtained by comparing the peak areas of the query compounds with that of standards of known concentrations.

Nicotine and related metabolites in the culture was determined by gas chromatography/mass spectrum (GC/MS) analysis. GC-MS analysis was performed using HP 6890 GC/5975 MS (Agilent Technologies, Santa Clara, California). The MS measurements were made with electron energy of 70 eV. Source temperature was 200 °C and mass scan ranges were 30–450 amu. Peak identifications of the components in the gas chromatograms were carried out by comparing the mass spectral data and the GC retention times with those of authentic known compounds.

2.3. Isolation of nicotine-degrading bacteria

To isolate nicotine-degrading bacteria, tobacco leaves were washed using 50 mM NaH_2PO_4/NaOH buffer (pH 7.0) and the collected wash fluids were plated on Luria-Bertani medium and incubated at 30 °C for 2 days. For soil samples, serial dilutions were plated on the same medium. The inorganic salt medium (ISM) was used for screening nicotine-degrading bacteria (per litre: 4 g nicotine, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 ml 0.1% $MnSO_4$, 3 ml 0.1% $FeSO_4$, 3 ml 1% KCl, pH adjusted to 6.5 using 0.5 M NaOH). Filter-sterilized nicotine was added to the medium and candidate bacterium was cultivated in ISM for 2 days at 30 °C with shaking at 220 rpm. The ISM without nicotine was used as the control. The concentration of nicotine in the medium was analyzed using HPLC.

2.4. Comparison of nicotine-degrading activities of isolates Nic15, Nic22 and Nic51 to different tobacco extracts

Tobacco extract medium (TEM) was used to further determine the nicotine-degrading activity of the three isolates (Nic15, Nic22 and Nic51). TEM contains (per litre) 1 ml tobacco extract, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 ml 0.1% $MnSO_4$, 3 ml 0.1% $FeSO_4$, and 3 ml 1% KCl, adjusted to pH 6.5 using 0.5 M NaOH. The tobacco extracts used here were prepared as follows. A tobacco leaf:distilled water ratio of 1:10 (w v^{-1}) were mixed and incubated at 60 °C for 2 h. After incubation, the filtrates were collected. Each isolate was cultivated in TEM for 2 days at 30 °C with shaking at 220 rpm. The TEM without inoculation is used as the control. The concentration of nicotine was analyzed using HPLC.

2.5. Identification of strain Nic22

Morphological and biochemical characteristics of strain Nic22 were determined following the methods described by Dong and Cai (2001). After the genomic DNA of strain Nic22 was isolated, its 16S rDNA gene was amplified using the forward primer P1 (5′-ACGGTTACCTTGTTACGACTT-3′) and the reverse primer P2 (5′-AGAGTTTGATCCTGGCTCAG-3′) as described by Weisburg et al. (1991). The partial 16S rDNA sequence has been submitted to the GenBank database with the accession number EF541115. This sequence was compared to known sequences found in the GenBank database using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). The neighbor-joining (NJ) (Saitou and Nei, 1987) method (implemented in MEGA2.0, Kumar et al., 2001) was used for phylogenetic analysis with the model of Kimura-2-Parameter. The robustness of the tree topology was assessed by bootstrap analysis, with 1000 resembling replicates.

2.6. Effect of temperature and pH on nicotine-degrading activity of Pseudomonas sp. Nic22

The optimal temperature for nicotine-degradation for strain Nic22 was determined by culturing in ISM for 2 days with shaking at 28, 30, 32, 34 and 36 °C, respectively. The concentrations of nicotine at different incubation temperatures were then analyzed using HPLC. Similarly, to determine optimal pH, *Pseudomonas* sp. Nic22 was cultivated in ISM at pH values 5.0–7.5 and nicotine concentrations were analyzed using HPLC.

2.7. Bacterial growth and nicotine degradation analysis

Pseudomonas sp. Nic22 was cultivated in ISM for 2 days at 30 °C with shaking at 220 rpm. The culture was sampled every 3 h after an initial growth for 8 h. The TEM without inoculation used as the control. Bacterial growth was determined at 600 nm using a 752 spectrophotometer (Shanghai) and the concentration of nicotine was analyzed using HPLC.

2.8. Analysis of nicotine and related metabolites

Nicotine and related metabolites in the culture were determined as follows: the culture was sampled and the cells were removed by centrifugation at 8000 rpm for 10 min at 4 °C. The supernatant was extracted with dichloromethane, and the dichloromethane layer was concentrated by vacuum. A portion of the sample was used for GC/MS analysis.

2.9. Effect of crude enzyme extract on nicotine degradation of the upper tobacco leaf

To determine the effect of crude enzyme extract on nicotine degradation, crude enzyme extracts were prepared as follows. Five hundred milliliters of bacterial culture grown as above in ISM was collected and the supernatant fluid was removed by centrifugation at 5000 rpm for 10 min at 4 °C. The cells were then washed twice with 50 ml NaH_2PO_4/NaOH buffer (50 mM, pH 7.0) and re-suspended in the 50 ml of same buffer. The cells were broken apart using ultrasonic forces, the unbroken cells were removed and the crude enzyme extract (supernatant fluid) was collected by centrifugation at 8000 rpm for 10 min at 4 °C.

Crude enzyme extract was mixed with tobacco sample (Yuxi B_3F) in a ratio of 2%, 5% and 10% (v m^{-1}), respectively. The mixtures were incubated at 30 °C and kept relative humidity at 25% for 2 days. For negative controls, the same volume of NaH_2PO_4/NaOH buffer (50 mM, pH 7.0) was mixed with tobacco sample. The concentration of nicotine was analyzed using HPLC. After treatment, tobacco leaves treated with different concentrations of crude enzyme extracts were prepared into cigarettes for quality evaluation by professional smokers.

3. Results

3.1. Isolation of nicotine-degrading bacteria

A total of 389 pure isolates were obtained from tobacco leaves and soil samples. These isolates were all analyzed for their nicotine-degrading activities using a medium (ISM) containing nicotine as the sole carbon and nitrogen source. And 18 nicotine-degrading isolates were isolated (Table 1). Among them, three purified isolates showing the highest nicotine-degrading activity were obtained and named Nic15, Nic22 and Nic51, respectively. More than 70% nicotine was degraded by isolates Nic22, Nic51 and Nic15 after cultivating for 2 days at 30 °C with shaking at 220 rpm.

3.2. Comparison of nicotine-degrading activities of isolates Nic15, Nic22 and Nic51 to different tobacco extracts

Nine different tobacco samples collected from different tobacco producing-regions and breeds of tobacco were chosen as substrates

Table 1
Isolation of nicotine-degrading bacteria from tobacco leaves and soil samples

Isolates	Tobacco samples	Color of culture	The final nicotine concentration (g l^{-1})	Degree of nicotine degradation (%)
Control	–	–	3.95	–
Nic5	Yiliang[b] X_3F	Opalescent	2.03	48.6
Nic15	Tobacco soil	Opalescent	0.30	92.4
Nic22	Tobacco soil	Green	1.06	73.1
Nic45	Henan[b] B_2F	Opalescent	1.72	56.5
Nic51	Tobacco soil	Green	1.09	72.4
Nic67	Zimbabwe[a] L_2MFT	Opalescent	1.91	51.6
Nic74	Zimbabwe[a] L_{10}	Gray	2.43	38.5
Nic112	Sanming[b] B_3F	Gray	2.24	43.3
Nic116	Sanming[b] B_3F	Gray	2.72	26.1
Nic210	Songming[b] B_3F	Gray	2.24	44.3
Nic213	Songming[b] C_3F	Gray	2.17	45.1
Nic234	Baoshan[b] CX_1K	Gray	2.17	45.1
Nic269	Yuxi[b] C_3F	Opalescent	2.49	37.0
Nic274	Yuxi[b] X1F	Gray	2.20	45.3
Nic323	Qujing[b] C_2F	Opalescent	2.08	47.3
Nic325	Qujing[b] C_2F	Gray	2.22	43.8
Nic338	Qujing[b] C_3F	Gray	2.22	43.8
Nic355	Honghe[b] B_1F	Opalescent	1.86	52.9

[a] Tobacco samples collected from Zimbabwe.
[b] The main tobacco-producing regions of Yunnan in China.

Table 2
Comparison of the nicotine-degrading activity of isolates Nic15, Nic22 and Nic51

Tobacco samples	Control ($g\,l^{-1}$)		Nic15 ($g\,l^{-1}$)		Nic22 ($g\,l^{-1}$)		Nic51 ($g\,l^{-1}$)	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Burley	1.46	1.48	0.23	0	0.23	0.23	0.21	0
Oriental	0.37	0.37	0	0	0	0	0	0
Tobacco powder K	0.85	0.86	0.69	0.36	0.38	0	0.55	0.23
Tobacco powder H	1.31	1.31	0.86	0.60	0.37	0	0.89	0.20
Zimbabwe LB_3	1.69	1.71	1.67	1.50	1.38	0.55	1.48	0.96
Zimbabwe LB_4	0.96	1.06	0.98	0.92	0.71	0	0.82	0
Tobacco K	0.90	0.92	0.88	0.84	0.56	0	0.82	0
Tobacco H	1.11	0.97	1.16	0.88	0.91	0	1.02	0
Tobacco Y	0.83	0.83	0.82	0.80	0.61	0	0	0

to compare the nicotine-degrading capacity of isolates Nic15, Nic22 and Nic51. HPLC analyses suggested that after digestion, the nicotine concentrations in these samples ranged between 0.37 and 1.71 $g\,l^{-1}$. The Oriental tobacco contained the lowest concentration of nicotine (0.37 g^{-1}), whereas the tobacco from Zimbabwe (LB_3) and burley contained high concentrations of nicotine (more than 1.46 $g\,l^{-1}$). Isolates Nic15, Nic22 and Nic51 can decompose the nicotine after cultivating in TEM for 2 days at 30 °C with shaking at 220 rpm. However, they showed different nicotine-degrading capacity to different samples. The nicotine in Oriental tobacco was found the easiest to degrade while that from Zimbabwe (LB_3) and Burley more difficult to degrade. Among the three strains, strain Nic22 showed a higher nicotine-degrading capacity than Nic15 and Nic51 (Table 2), nicotine in seven different media was degraded completely by strain Nic22 after cultivating for 2 days.

3.3. Identification of strain Nic22

Strain Nic22 was identified as a gram-negative rod without spore or capsule and was a facultative anaerobe. This strain could use nitrate, urea and ammonium, produced catalase and oxidase, could not form indole and sulfurated hydrogen by decomposing tryptophan or sulfur-containing amino acids (e.g. cystine and methionine), and both the methyl red test and Voges-Proskauer test were negative. Strain Nic22 can utilize various carbon sources including sucrose, glucose, starch, maltose and mannose, and grow rapidly on medium containing nicotine as the sole carbon and nitrogen source. Colonies of strain Nic22 on ISM plates was opalescent, convex, and mucous. Interestingly, Nic22 produced pigments on ISM plate, and the color changed from virescent to carmine after 2 days.

Strain Nic22 was identified as *Pseudomonas* sp. Nic22 according to the morphological and biochemical characteristics (Holt et al., 1994). Multiple alignments revealed that the 16S rDNA sequence of *Pseudomonas* sp. Nic22 was closely related to that of *Pseudomonas putida* (AY574282, 98.7% of similarity) (Wang et al., 2007), *Pseudomonas fulva* (D84015, 98.3% of similarity), and *Pseudomonas* sp. HF-1 (AY823996, 98.1% of similarity) (Ruan et al., 2005). Phylogenetic analysis using the 16S rDNA gene (GenBank accession no. EF541115) confirmed its affinity to genus *Pseudomonas* (Fig. 1). *Pseudomonas* sp. Nic22 has been deposited in the China General Microbiological Culture Collection Center (CGMCC1998).

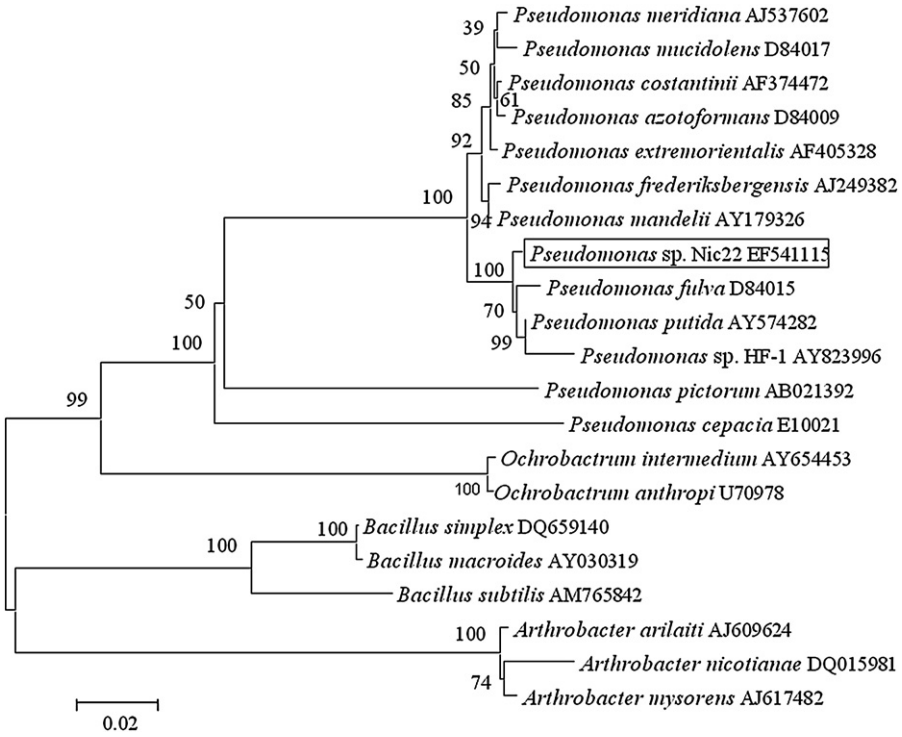

Fig. 1. Phylogenetic analysis of *Pseudomonas* sp. Nic22 and related species by the neighbor-joining approach. Bootstrap values (%) are indicated at the nodes. The scale bars represent 0.02 substitutions per site.

3.4. Effect of temperature and pH on nicotine degradation

The optimum culture temperature and optimal pH for nicotine degradation by *Pseudomonas* sp. Nic22 were 30–34 °C and 6.5, respectively. The nicotine-degrading activity was stable when *Pseudomonas* sp. Nic22 was cultivated at 30–34 °C, 160 rpm, and the activity decreases when the culture temperature was below 28 °C and above 34 °C (Fig. 2). The nicotine-degrading activity increased gradually when pH changed from 5.0 to 6.5, whereas the nicotine-degrading activity decreased with an increasing pH from 6.5 to 7.5 (Fig. 3).

3.5. Degradation of nicotine by Pseudomonas sp. Nic22

Pseudomonas sp. Nic22 grew well in ISM when nicotine was used as the sole carbon and nitrogen source. The cell population increased rapidly after cultivating at 30 °C, 160 rpm for 8 h. At the same time, the concentration of nicotine decreased sharply, suggesting *Pseudomonas* sp. Nic22 could effectively decompose and utilize nicotine for growth (Fig. 4).

Nicotine and related metabolites in the culture was determined (Fig. 5). From the results of GC-MS analysis, pure nicotine could be degraded by *Pseudomonas* sp. Nic22 effectively (Fig. 5). After culturing for 24 h, majority nicotine (96.5%) was degraded, and some harmful compounds including myosmine and cotinine were found (Fig. 5B). However, nicotine was decomposed almost completely (99.9%), and other intermediates also were decomposed completely (Fig. 5C) after treating for 60 h. Moreover, the results of GC-MS of tobacco extracts (Fig. 6) showed nicotine in tobacco extract was also decomposed quickly, and only trace intermediates of nicotine, such as 2,3′-dipyridyl and cotinine were found in TSM (Fig. 6B).

3.6. Effect of crude enzyme extract on the upper tobacco leaf

The upper tobacco leaf (Yuxi, B_3F) contained a high concentration of nicotine (3.54%) and was effectively degraded after treatment with a crude enzyme extract (Table 3). Specifically, 13.3%, 24.1% and 33.1% of the nicotine was degraded after treatment with 2%, 5% and 10% of the crude enzyme extract, respectively. Moreover, crude enzyme extract-treated tobacco leaves had a better smoking quality than the untreated original. For example, the aroma components of tobacco were not lost, the stimulator became softer, and the taste was better. Therefore, the crude enzyme extract of *Pseudomonas* sp. Nic22 can decompose nicotine and significantly improve the quality of tobacco leaf.

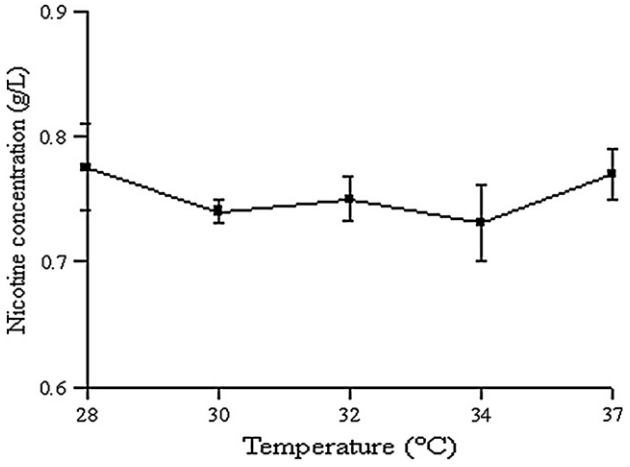

Fig. 2. The effect of temperature on nicotine degradation. Error bars: standard error (SE).

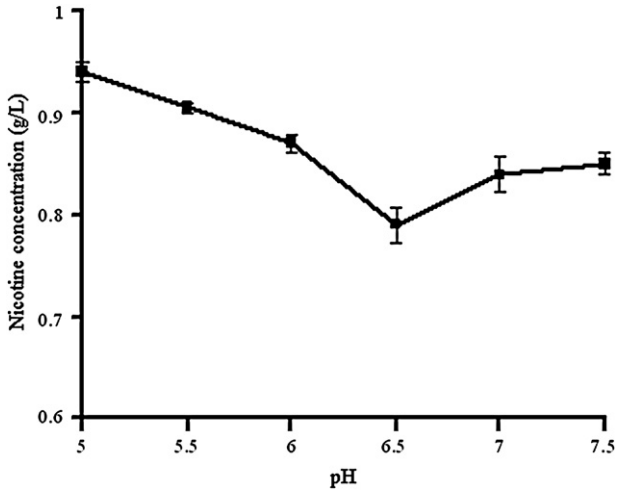

Fig. 3. The effect of PH on nicotine degradation. Error bars: standard error (SE).

4. Discussion

Nicotine, a major alkaloid synthesized as the L-isomer in tobacco plants, plays a critical role in smoking addiction. One side, it is responsible for smoking properties; and on the other, toxic aspects of the nicotine are also well known. Recently, nicotine degradation by microorganisms has received increasing attention because microorganisms have shown great potentials in reducing the harmful effects of nicotine levels in tobacco and in detoxifying tobacco wastes (Civilini et al., 1997; Wang et al., 2004). More important, biodegradation methods with microorganisms can selectively reduce the nicotine content of tobacco without deleteriously modifying its desirable smoking properties (Gravely et al., 1985).

In this study, 18 isolates capable of degrading nicotine were isolated from tobacco leaves and soil samples from tobacco-planting fields. All these isolates could utilize nicotine as the sole carbon and nitrogen source for growth. Among these strains, their nicotine-degrading capacity varied widely. The culture broth containing nicotine-degrading bacteria and nicotine changes color to opalescent, green and gray after bacterial growth (Table 1). The color changes in media containing nicotine have been described previously and may reflect the different types of pathways involved in nicotine degradation. Giovannozzi-Sermanni (1959) reported that *A. nicotinovorans* isolated from the storage of tobacco leaves was capable of metabolizing nicotine and converting the medium first

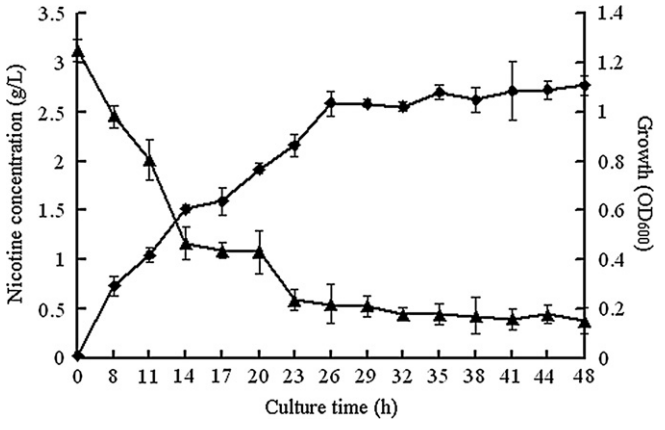

Fig. 4. Utilization of nicotine as the sole carbon source for growth (30 °C, pH 6.5, 160 rpm) by *Pseudomonas* sp. Nic22. Error bars: standard error (SE) ▲ indicates degradation of nicotine, and ◆ indicates cell growth.

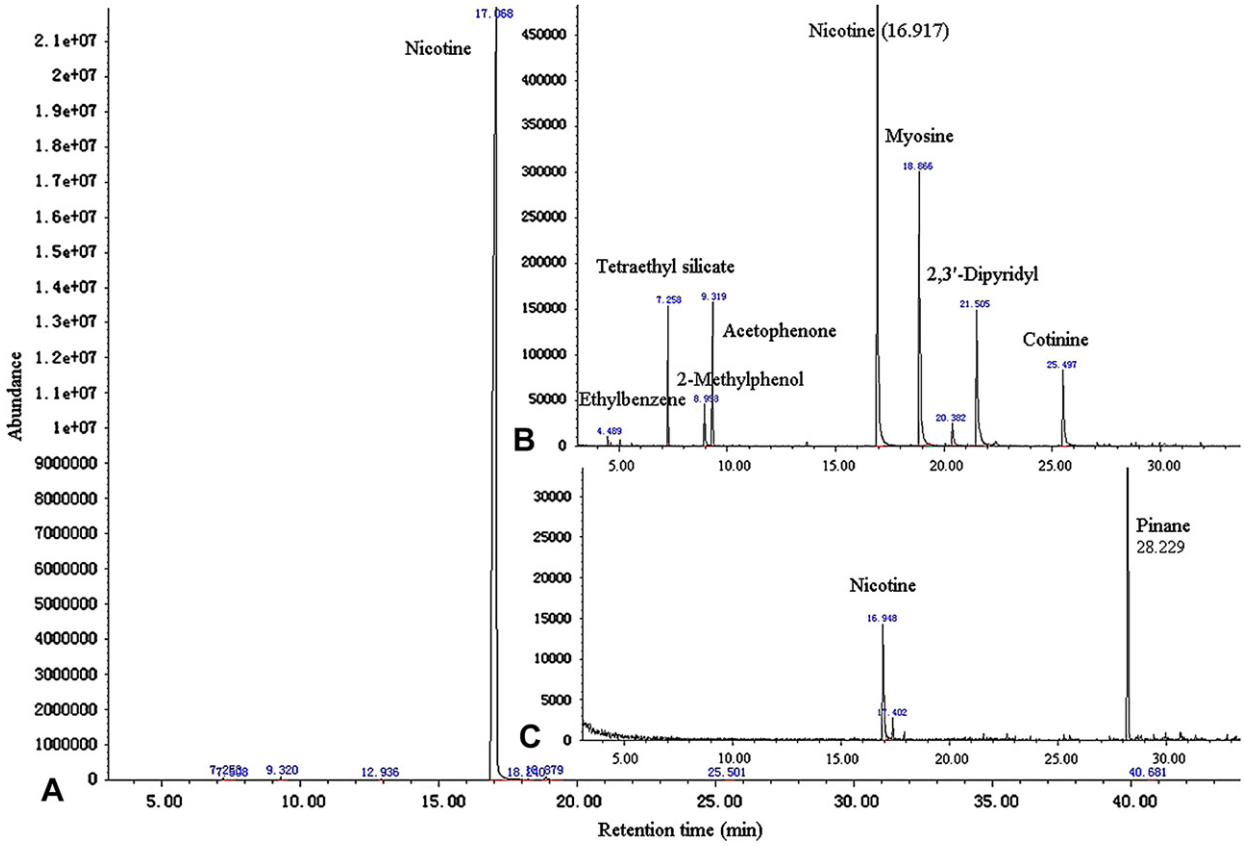

Fig. 5. GC-MS analysis of pure nicotine and related metabolites. (A) Analysis of pure nicotine (Sigma–Aldrich, USA). (B) Nicotine was degraded by *Pseudomonas* sp. Nic22 for 24 h. (C) Nicotine was degraded by *Pseudomonas* sp. Nic22 for 60 h.

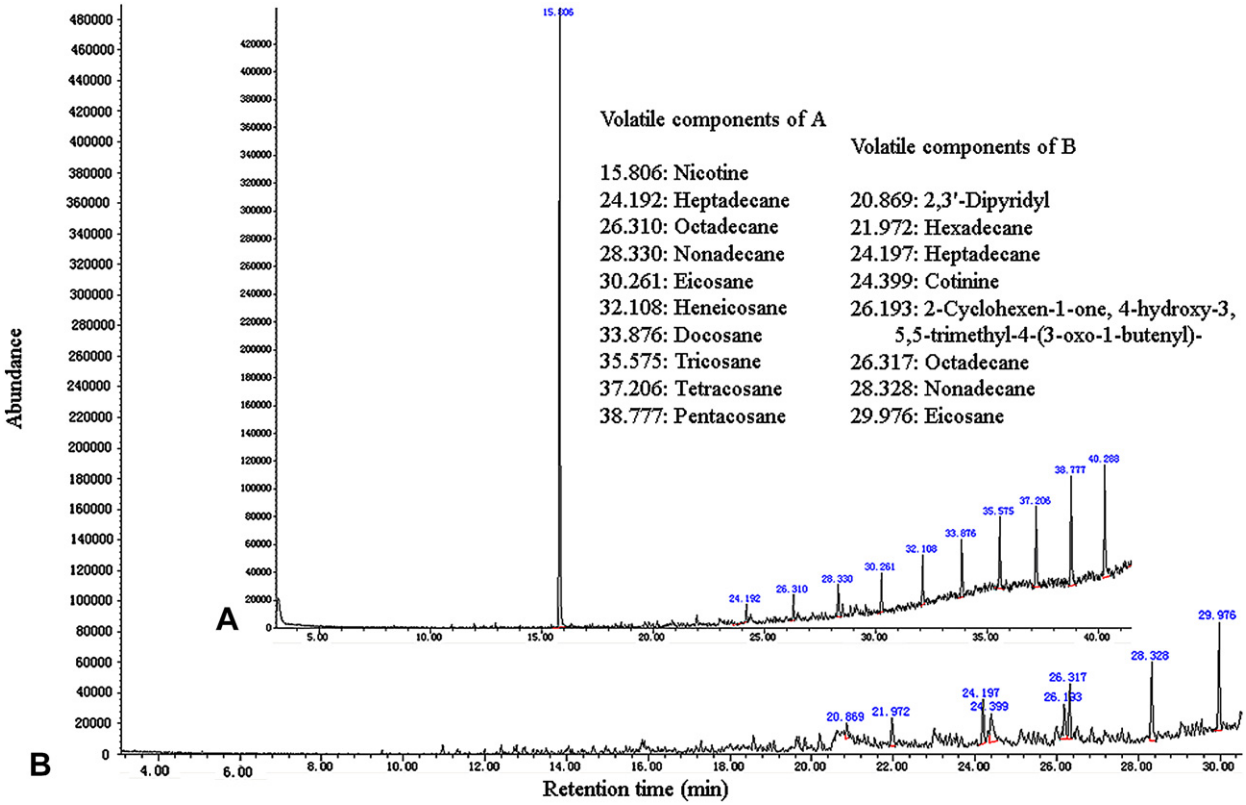

Fig. 6. GC-MS analysis of nicotine and volatile components in tobacco extracts. (A) Nicotine and other volatile components in tobacco extracts. Tobacco extract was prepared according to the method described in Section 2. (B) *Pseudomonas* sp. Nic22 was cultured in TSM for 48 h.

Table 3
Effects of crude enzyme extracts on upper tobacco leaves

Items	Control	Tobacco sample 1	Tobacco sample 2	Tobacco sample 3
Crude enzyme extract (%)	0	2	5	10
Nicotine concentration (%)	3.54	3.07	2.69	2.37
Degree of nicotine degradation (%)	0	13.3	24.1	33.1

to yellow and then to carmine. Ruan et al. (2005) also described a *Pseudomonas* sp. strain HF-1 that transformed nicotine through a different pathway to produce virescent pigments. These nicotine-degrading strains provided a basis for studying further the degradation pathway of nicotine in different bacteria.

Table 2 showed that strains Nic15, Nic22 and Nic51 have different nicotine-degrading capacity to tobacco samples collected from different producing-regions and breeds of tobacco. Among them, strain Nic22 showed a higher nicotine-degrading capacity than isolates Nic15 and Nic51, and nicotine in seven of nine samples were decomposed completely by strain Nic22 in 2 days. This result suggested strain Nic22 could degrade nicotine from a broad range of tobacco samples.

The optimal pH and temperature (Figs. 2 and 3) of nicotine degradation by *Pseudomonas* sp. Nic22 was consistent with those reported by Ruan et al. (2005). From Figs. 4 and 5, the nicotine in culture could be decomposed effectively by *Pseudomonas* sp. Nic22, and the degradation of nicotine was significantly dependent on the growth of *Pseudomonas* sp. Nic22. Moreover, the nicotine of the upper tobacco (Yuxi, B_3F) could be decomposed effectively by crude enzyme extract of *Pseudomonas* sp. Nic22, and the quality of tobacco leaf was significantly improved (Table 3).

At present, the detailed metabolic pathway of nicotine in *Pseudomonas* spp. has not yet been fully elucidated. Recently, a putative pathway of nicotine degradation in *Pseudomonas putida* was proposed by Wang et al. (2007), and some intermediates including 2′-hydroxynicotine, 3-pyridinebutanal, 3-succinoylpyridine, 6-hydroxy-3-succinoylpyridine and 2,5-dihydroxypyridine were detected. However, different intermediates including myosmine, 2,3′-dipyridyl and cotinine were found (Fig. 5B), fortunately, these harmful compounds could be degraded by *Pseudomonas* sp. Nic22. Which suggested that nicotine could be decomposed by different metabolic pathways in *Pseudomonas* spp. (Brandsch, 2006; Ruan et al., 2005; Wang et al., 2007), although these *Pseudomonas* spp. shared close phylogenetic relationship (Fig. 1). Comparing to the pure nicotine, *Pseudomonas* sp. Nic22 also could decompose effectively nicotine in tobacco extract, and less intermediates of nicotine were found (Fig. 6).

One care for biodegradation of the tobacco nicotine using microorganism is their diverse metabolic pathway (Brandsch, 2006; Chiribau et al., 2006). Some metabolic intermediates including cotinine may be also harmful for health or environment. So it is necessary to further study the metabolic pathway for *Pseudomonas* sp. Nic22 and their products before deploying the bacterium into cigarettes industry. Moreover, understanding the degradation route of nicotine for *Pseudomonas* sp. Nic22 may provide a novel eyeshot for using nicotine-degrading microorganisms to improve quality of tobacco leaves, or to decontaminate environments contaminated by tobacco wastes by a green environment-friendly way.

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