

Nicotine degradation enhancement by *Pseudomonas stutzeri* ZCJ during aging process of tobacco leaves

Lei Zhao · Chenjing Zhu · Yang Gao ·
Chang Wang · Xuanzhen Li · Ming Shu ·
Yuping Shi · Weihong Zhong

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Abstract Nicotine is a key harmful component of tobacco and cigarettes, and the development of low-nicotine cigarettes is of increasing importance in the market. The objectives of this study are to isolate native nicotine-degrading strains and evaluate their feasibility for nicotine reduction during the aging (or fermentation) of tobacco leaves. A novel nicotine-degrading strain was isolated and identified as *Pseudomonas stutzeri* ZCJ based on its 16S rDNA sequence and morphological-biochemical characteristics. In submerged cultures, *P. stutzeri* ZCJ could tolerate 4.5 g/L nicotine and completely degrade 1.5 g/L nicotine within 24 h at 37°C and pH 7.4. The addition of glucose (1 g/L) could improve nicotine degradation by *P. stutzeri* ZCJ in submerged cultures. After submerged culturing, the cell suspension of *P. stutzeri* ZCJ could be utilized to improve nicotine reduction in tobacco leaves during solid-state fermentation. The nicotine content of tobacco leaves decreased by as much as 32.24% after 7 days of solid-state fermentation by *P. stutzeri* ZCJ, suggesting the industrial application potential of the native

strain to enhance nicotine degradation during the aging of tobacco leaves.

Keywords Nicotine degradation · *Pseudomonas stutzeri* · Solid-state fermentation · Tobacco leaves

Abbreviations

HPLC High performance liquid chromatography
ESEM Environmental scanning electron microscope
rRNA Ribosomal ribonucleic acid
DNA Deoxyribonucleic acid
PCR Polymerase chain reaction

Introduction

Nicotine is a key harmful component of tobacco and cigarettes. Thus, low-nicotine cigarettes have seen increased market demands due to health considerations by many smokers, and various technologies for low-nicotine cigarette preparation have been explored. The widely applied strategy for reducing nicotine in cigarettes is mixing high-nicotine content tobacco leaves with low-nicotine ones. However, this strategy is limited by the need for more experimental efforts to integrate, control, and deal with varying tobacco materials. Thus, simpler and more effective technologies are still necessary to reduce the nicotine content of tobacco and cigarettes.

Biological treatment is a viable method of nicotine removal, and numerous studies have demonstrated nicotine degradation by microbes. To date, several microorganisms, including *Arthrobacter* sp. (Hochstein and Rittenberg 1959; Kodama et al. 1992; Ruan et al. 2006), *Pseudomonas*

L. Zhao · C. Zhu · C. Wang · X. Li · W. Zhong (✉)
College of Biological and Environmental Engineering,
Zhejiang University of Technology, Hangzhou 310032,
People's Republic of China
e-mail: whzhong@zjut.edu.cn

Y. Gao · M. Shu (✉)
Technology Center, China Tobacco Zhejiang Industrial Co. Ltd.,
Hangzhou 310009, People's Republic of China
e-mail: smdoc@126.com

Y. Shi (✉)
The Second Affiliated Hospital of Zhejiang University School of
Medicine, Hangzhou 310009, People's Republic of China
e-mail: shiyuping007@hotmail.com

sp. (Chen et al. 2008; Civilini et al. 1997; Ruan et al. 2005; Wang et al. 2004; Zhong et al. 2010), *Cellulomonas* sp. (Civilini et al. 1997), *Ochrobactrum intermedium* (Yuan et al. 2007; Yuan et al. 2005), *Rhodococcus* sp. (Gong et al. 2009), *Ensifer* sp. (Lei et al. 2009), *Agrobacterium* sp. (Wang et al. 2009b), *Apergillus oryzae* (Meng et al. 2010), *Acinetobacter* sp., and *Sphingomonas* sp. (Wang et al. 2011), have been reported to be capable of degrading nicotine. These microbes appear to play an important role in the manufacturing process of tobacco by altering the nicotine content of the final product and treating nicotine pollution (Brandsch 2006). The molecular basis of nicotine degradation has clearly been elucidated and well understood (Baitsch et al. 2001; Chiribau et al. 2005; Ganas et al. 2009, 2008; Igloi and Brandsch 2003; Lei et al. 2009; Tang et al. 2008, 2009, 2011; Wang et al. 2005, 2007, 2009a, 2011, Wei et al. 2009). The bacterial diversities of tobacco leaves have been estimated by 16S rRNA gene sequence analysis (Huang et al. 2010), with the results demonstrating that a large number of bacteria, including a large quantity of uncultivable species, exist on tobacco leaves. Most of these bacteria play important roles in tobacco aging (or fermentation), which is typically carried out for 2 years to improve the quality of flue-cured tobacco leaves.

During the past two decades, there have been increasing reports for the development of the solid state fermentation (SSF) in bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues (Pandey et al. 2000; Madeira et al. 2011; Joshi et al. 2011). However, applications of such microbes to enhance nicotine reduction in tobacco leaves are limited. Lei et al. (2008) reported that *Artheobacter* spp. (isolated from soil) treatment reduced the nicotine content of tobacco leaves during flue-curing after the harvest period. However, few reports have been published on the isolation of native strains to enhance nicotine reduction during the aging of tobacco leaves. In addition, more novel nicotine-degrading strains (especially native strains on tobacco leaf) with stronger abilities to degrade nicotine and suitable for application in tobacco material treatment must be found. Therefore, the present study aims to isolate native strains and evaluate the feasibility of their application in nicotine degradation during the solid-state fermentation of tobacco leaves. To the best of our knowledge, this is the first report on nicotine degradation by native strains during the solid-state fermentation of tobacco leaves.

Materials and methods

Chemicals and media

(-)-Nicotine (98%) isolated from tobacco was purchased from Weifang Three Power Group Co., Ltd. (Shandong,

China). Tobacco leaves, supplied by China Tobacco Zhejiang Industrial Co., Ltd. (Hangzhou, China), were obtained from Bijie, Guizhou, China.

Genomic DNA extraction and PCR kits were purchased from the Invitrogen Corp., Shanghai representative office. The 16S rDNA primers were designed and synthesized by the same.

The basic inorganic salt medium (BSM; pH 7.0) was composed of (g/L): Na_2HPO_4 (5.57), KH_2PO_4 (2.44), K_2SO_4 (1.0), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.0004), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.001), CaCl_2 0.001, and nicotine (added at different concentrations when necessary).

The enrichment medium (pH 7.0) contained (g/L): Na_2HPO_4 (5.57), KH_2PO_4 (2.44), $(\text{NH}_4)_2\text{SO}_4$ (1.0), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.0004), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.001), CaCl_2 (0.001), and nicotine (10.0).

Both media were sterilized in an autoclave for 20 min at 121°C.

Isolation and characterization of nicotine-degrading bacteria

Tobacco leaves (10 g) from Bijie, Guizhou were added to 100 mL physiological saline. The mixture was incubated for 30 min at 30°C and 180 rpm. Then, 1.0 mL of the culture was transferred into 100 mL enrichment medium and then kept under the same incubation conditions for 48 h. After enrichment, the culture was spread onto the solid BSM plates containing 2 g/L nicotine and incubated at 30°C for 48 h. The colonies were picked up and streaked onto a new plate repeatedly until a pure isolate was obtained. The cell morphology was examined under a transmission electron microscope. Physiological characteristics were determined using standard methods.

The genomic DNA of *Pseudomonas stutzeri* ZCJ was extracted via rapid extraction kit (Guangzhou Dongsheng Biotechnology Co. Ltd., China). The 16S rDNA sequence was amplified via PCR using Taq polymerase (Takara, Japan) and universal primer sequences (forward primer: 5'-agagtttgatcctggctcag-3'; reverse primer: 5'-cggctacctgt-tacgacttc-3'). The PCR conditions were as follows: 5 min at 94°C, 34 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final incubation at 72°C for 10 min, stored at -20°C. The amplified DNA sequence was determined by Shanghai Invitrogen Biological Technique Co. The resultant 16S rRNA gene sequences of strain ZCJ was submitted to Genbank under an accession number of FJ815158.1. Related sequences were obtained from the GenBank database using BLAST. The 16S rRNA gene sequences determined and reference sequences obtained from GenBank databases were aligned using CLUSTALX software. A phylogenetic tree was constructed using MEGA4 software.

Analysis of nicotine content

For the submerged fermentation, cells were removed by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was then used to determine nicotine content via HPLC using an SPD-10AVP/lc-10AVP instrument (SHIMADZU, Japan) equipped with an Agilent SB-C18 column (4.6 mm × 150 mm). A mixture of methanol (HPLC grade) and 0.1 mol/L KH₂PO₄ (10:90 by volume, pH = 3.0) was used as the mobile phase, and the flow rate was 1 mL/min. Ultraviolet detection was conducted at 254 nm, and sampling quantities were 5 µL.

For solid-state fermentation, nicotine was extracted from tobacco leaves using distilled water at 37°C and 180 rpm for 30 min. The solid waste was removed by centrifugation at 12,000 rpm for 5 min at 4°C, and the supernatant was used for nicotine content determination by HPLC as described above.

Effects of culture conditions on nicotine degradation through submerged fermentation of *P. stutzeri* ZCJ

The initial concentration of nicotine in BSM usually affects its degradation rate. A 2-mL cell suspension of *P. stutzeri* ZCJ was inoculated into 100 mL BSM containing different concentrations of nicotine and then incubated at 37°C and 180 rpm for 48 h. Changes in nicotine concentration in the broth were determined. Each sample was repeated in triplicate.

The culture temperature and initial pH of the culture media may also affect nicotine degradation by *P. stutzeri* ZCJ. The effect of culture temperature on nicotine degradation was determined by inoculating 1 mL of the cell suspension of *P. stutzeri* ZCJ into 100 mL BSM containing 1.5 g/L nicotine and then incubating at 25, 30, 34, and 37°C for 24 h. The nicotine concentration in the broth was subsequently determined. Each sample was repeated in triplicate. The effect of initial BSM pH on nicotine degradation was also examined by inoculating 1 mL of the cell suspension of *P. stutzeri* ZCJ into 100 mL BSM containing 1.5 g/L nicotine at different initial pH (6.0, 6.5, 7.0, 7.5, and 8.0). Nicotine concentration was determined after incubation at 37°C and 180 rpm for 24 h.

The effects of various trace elements, carbon sources, and nitrogen sources on nicotine degradation by *P. stutzeri* ZCJ were also evaluated. The effect of trace elements was determined by inoculating 1 mL of the cell suspension of *P. stutzeri* ZCJ into 100 mL BSM containing 1.5 g/L nicotine and various trace elements [NiCl₂·6H₂O (0.004 g/L), Na₂MoO₄ (0.006 g/L), ZnSO₄·7H₂O (0.1 g/L), and CuCl₂·4H₂O (0.05 g/L)]. The nicotine concentration in the culture was determined after incubation at 37°C and 180 rpm for 24 h.

The effect of additional carbon source substances was detected by inoculating 1 mL of the cell suspension into 100 mL BSM containing 1.5 g/L nicotine and 1.0 g/L carbon source substances (glucose, sucrose, and maltose, respectively). The nicotine concentration of the culture was determined after incubation at 37°C and 180 rpm for 24 h.

The effect of additional nitrogen source substances was determined by inoculating 1 mL of the cell suspension into 100 mL of BSM containing 1.5 g/L nicotine and 1.0 g/L nitrogen source substances (potassium nitrate, ammonium sulfate, peptone, and ammonium nitrite, respectively). The nicotine concentration was determined after incubation at 37°C and 180 rpm for 24 h.

Effect of culture conditions on nicotine degradation by solid-state fermentation of *P. stutzeri* ZCJ in flasks

The nicotine degradation activities of *Pseudomonas* sp. ZUTSKD and *P. stutzeri* ZCJ were compared. Five milliliters of the cell suspensions of ZUTSKD and *P. stutzeri* ZCJ containing the same quantity of biomass were inoculated into a flask with 10 g of treated tobacco leaves, respectively. The nicotine concentrations of the tobacco leaves were then determined after 7 day of incubation at 37°C.

The effect of culture temperature on nicotine degradation of tobacco leaves was determined. All the flasks were divided into two groups, which were incubated at 30 and 37°C. Flasks without cell suspensions were set as the control group. The nicotine concentration of tobacco leaves was determined after 7 days of incubation.

The effect of additional glucose on nicotine degradation via solid-state fermentation by *P. stutzeri* ZCJ was also evaluated. A mixture of 3 mL of the cell suspension (26.60 mg in dry weight) and 2 mL of a solution, containing 0, 2, 4, and 6 g/L glucose was inoculated into flasks with 10 g of treated tobacco leaves. The final concentration of glucose was 0, 0.8, 1.6, and 2.4 g/L. All cultures were incubated at 37°C for 7 days. Cultures incubated at the same temperature without the cell suspension were set as the control group.

The size of the cell suspension may influence nicotine degradation. Therefore, 5 and 10 mL of the cell suspensions containing the same dry weight of *P. stutzeri* ZCJ (12.67 mg) were inoculated into flasks containing 10 g of treated tobacco leaves. The nicotine concentration was determined after 7 days of incubation at 37°C.

Nicotine degradation via solid-state fermentation in shallow 1 L dishes

All of the tobacco leaves were cut into uniform pieces before treatment. Then, 10 g of tobacco leaves were put in a shallow 1-L dish (25 cm × 14.5 cm × 6.5 cm), on which 5 mL of the *P. stutzeri* ZCJ cell suspension (dry cell

Table 1 Three batches of solid fermentation under different conditions in a shallow 1L dish

	Original water ^a (mL)	Additional water each time (mL)	Interval time for water addition (h)	Inoculum density (total), mg/ml (g)	Total water added (mL)
Batch 1	7	3.50	48	15.70 (0.11)	17.50
	10	5.00		10.97 (0.11)	25.00
Batch 2	7	3.50	24	17.20 (0.12)	28.00
	10	5.00		12.04 (0.12)	40.00
Batch 3	7	4.25	48	15.00 (0.11)	19.75
	10	4.25		10.50 (0.11)	22.75

^a Original water was mixed with the total inoculum to produce a cell suspension for spraying onto the surface of tobacco leaves

weight: 340 mg) was sprayed before incubation at 37°C. After 7 days of fermentation, the tobacco leaves were sampled and mixed with 200 mL of distilled water in a flask with continuous shaking at 180 rpm for 30 min. The mixture was centrifuged at 12000 rpm for 10 min, and the supernatant was used to determine nicotine content by HPLC. Three batches of solid fermentation under different incubation conditions were conducted (Table 1). For each sampling point, the samples were randomly collected from three points in the shallow dish.

The investigation of bacterial distribution on the surface of tobacco leaves before and after treatment was performed using a Philips FEI XL-30 ESEM.

Results

Isolation and identification of nicotine-degrading bacteria

The isolated Gram-negative and spore-negative strain ZCJ has the strongest nicotine-degrading ability among all strains isolated from tobacco leaves. Electron microscope observations showed that strain ZCJ has short, rod-shaped cells with one flagellum attached to one terminal of the cell (Fig. 1); its colonies on the BSM agar plate were white, smooth surfaced, and rumple-edged. The physiological characteristics of strain ZCJ (data not shown) were similar to that of *P. stutzeri*. Similarity analysis of the partial 16S rRNA gene sequence of strain ZCJ (Genbank accession number: FJ815158.1) revealed that it had 99% similarity to *P. stutzeri*. Phylogenetic tree analysis (Fig. 1) demonstrated that strain ZCJ has a closer relationship with *P. stutzeri* than all other reported strains. Therefore, strain ZCJ was identified as *P. stutzeri*. ZCJ and then preserved at the China Center for Type Culture Collection (Access No. CCTCC NO. M209064).

Effect of culture conditions on nicotine degradation via submerged cultures of *P. stutzeri* ZCJ

The effect of culture conditions on nicotine degradation by *P. stutzeri* ZCJ was evaluated to improve the activity of nicotine degradation.

First, the effect of initial nicotine concentration was evaluated after each 12 h culture in flasks at 37°C and 180 rpm. Figure 2a shows that *P. stutzeri* ZCJ completely degraded 2.5 and 1.5 g/L nicotine within 36 and 24 h, respectively. The *P. stutzeri* ZCJ cultured on BSM agar plates grew only when the nicotine concentration was below 4.5 g/L, and no significant growth of *P. stutzeri* ZCJ was observed when the nicotine concentration increased above 4.5 g/L.

The effect of initial pH on nicotine (1.5 g/L) degradation by *P. stutzeri* ZCJ was also evaluated. Figure 2b shows that *P. stutzeri* ZCJ could degrade nicotine under the initial pH range of 6.0–8.0. The optimal pH was 7.5, at which point 97% nicotine was degraded within 16 h.

The effect of cultivation temperature on nicotine degradation by *P. stutzeri* ZCJ was evaluated after 24 h of cultivation, with 1.5 g/L nicotine added as the sole carbon source. Figure 3a shows that the nicotine degradation rate increased with increasing cultivation temperature; the optimal temperature was 37°C.

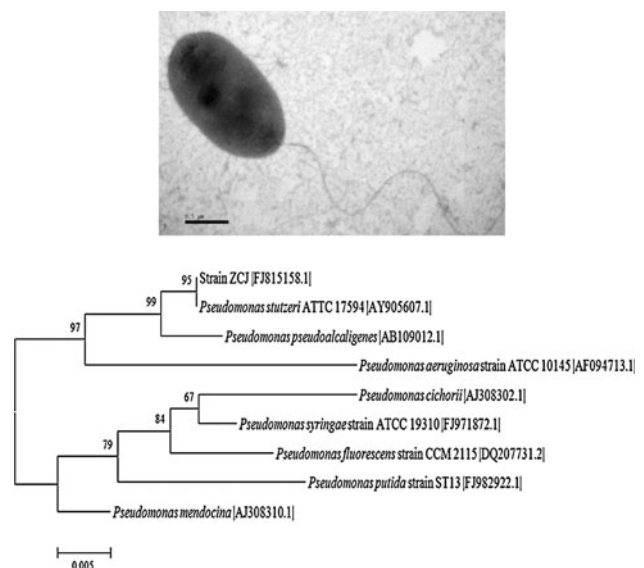


Fig. 1 Electron micrograph (30000×) and phylogenetic tree analysis based on 16S rRNA gene sequence of *Pseudomonas stutzeri* ZCJ

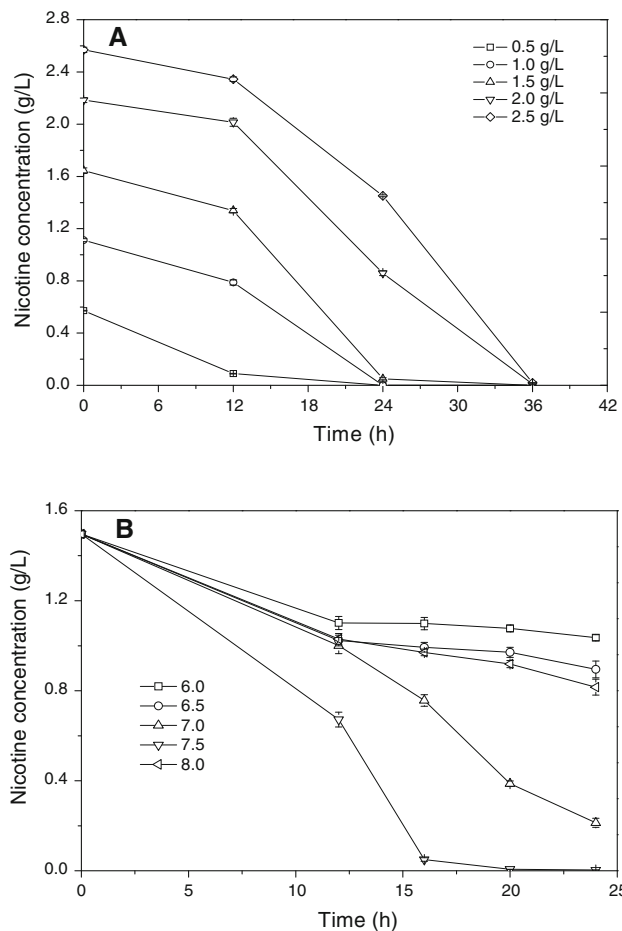


Fig. 2 Effect of **a** initial nicotine concentration and **b** initial pH on nicotine degradation in BSM by *Pseudomonas stutzeri* ZCJ at 37°C and 180 rpm

Trace elements usually play an important role in nicotine degradation by microbes (Ruan et al. 2005; Wang et al. 2011). Hence, the effect of trace elements on *P. stutzeri* ZCJ was evaluated. Figure 3b shows that the addition of $ZnSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$ had no significant influence on nicotine degradation, whereas the addition of Na_2MoO_4 and $CuCl_2 \cdot 4H_2O$ inhibited nicotine degradation.

The effects of additional carbon and nitrogen sources on nicotine degradation by *P. stutzeri* ZCJ were evaluated to enhance nicotine degradation. Figure 3c shows that glucose significantly improved nicotine degradation, whereas sucrose and maltose inhibited it. Glucose, the most available carbon source, promoted cell growth and increased the nicotine degradation rate. In contrast, sucrose and maltose, which are difficult to completely utilize within a short period of time, caused slow cell growth and inhibition of nicotine utilization. The effect of additional nitrogen sources on nicotine degradation by *P. stutzeri* ZCJ is shown in Fig. 3d. The addition of KNO_3 , $(NH_4)_2SO_4$, and peptone

showed no significant effect on nicotine degradation, whereas $NaNO_2$ inhibited it.

Effect of culture conditions on nicotine degradation via solid-state fermentation by *P. stutzeri* ZCJ in flasks

The effect of different strains, culture temperature, glucose, and cell suspension amount on nicotine degradation was investigated to evaluate further the potential of *P. stutzeri* ZCJ in nicotine degradation via solid-state fermentation in flasks.

The nicotine-degrading activities of *Pseudomonas* sp. ZUTSKD and *P. stutzeri* ZCJ in tobacco leaves were compared. Figure 4a shows that *P. stutzeri* ZCJ exhibits slightly higher nicotine-degrading activity in tobacco leaves than ZUTSKD. ZUTSKD, an isolate from tobacco waste, has been previously applied in the nicotine removal of tobacco waste extracts (Zhong et al. 2010). *P. stutzeri* ZCJ is an isolate directly isolated from the tobacco leaves in stock. As a native strain, *P. stutzeri* ZCJ may be safer and more suitable for use than ZUTSKD in terms of nicotine degradation in tobacco leaves. Therefore, *P. stutzeri* ZCJ was selected for the following experiments.

As shown in Fig. 3a, *P. stutzeri* ZCJ could grow at 25–37°C, and its degradation rate increased with increasing temperature. However, overly high or overly low temperatures could lead to higher costs when the strain is applied to the real industry. Therefore, 30 and 37°C culture temperatures were compared to determine their effect on nicotine degradation via solid-state fermentation of *P. stutzeri* ZCJ. Figure 4b shows that the degradation rate is 15.8% higher at 37°C than that at 30°C, suggesting that the cell suspension spray could be conducted during summer when the temperature is high enough to hasten nicotine degradation in tobacco leaves.

The addition of glucose shows no significant effect on nicotine degradation in solid-state fermentation (Fig. 4c), which is different from the results of submerged fermentation (Fig. 3C). This difference in results could be attributed to the complexity of tobacco leaf composition, suggesting that the addition of glucose to the tobacco leaves is not necessary for the solid-state fermentation of *P. stutzeri* ZCJ.

The size of the cell suspension showed significant effects on nicotine degradation by *P. stutzeri* ZCJ (Fig. 4d). When 10 and 5 mL of the cell suspension was used for inoculation, the degradation rates were 30.7 and 16.9%, respectively. However, tobacco leaves easily mildewed when 10-mL cell suspensions were used. This may be attributed to the increase in humidity caused by the higher water content of the 10-mL suspension compared with the 5-mL cell suspension. Thus, a good ratio of tobacco leaves

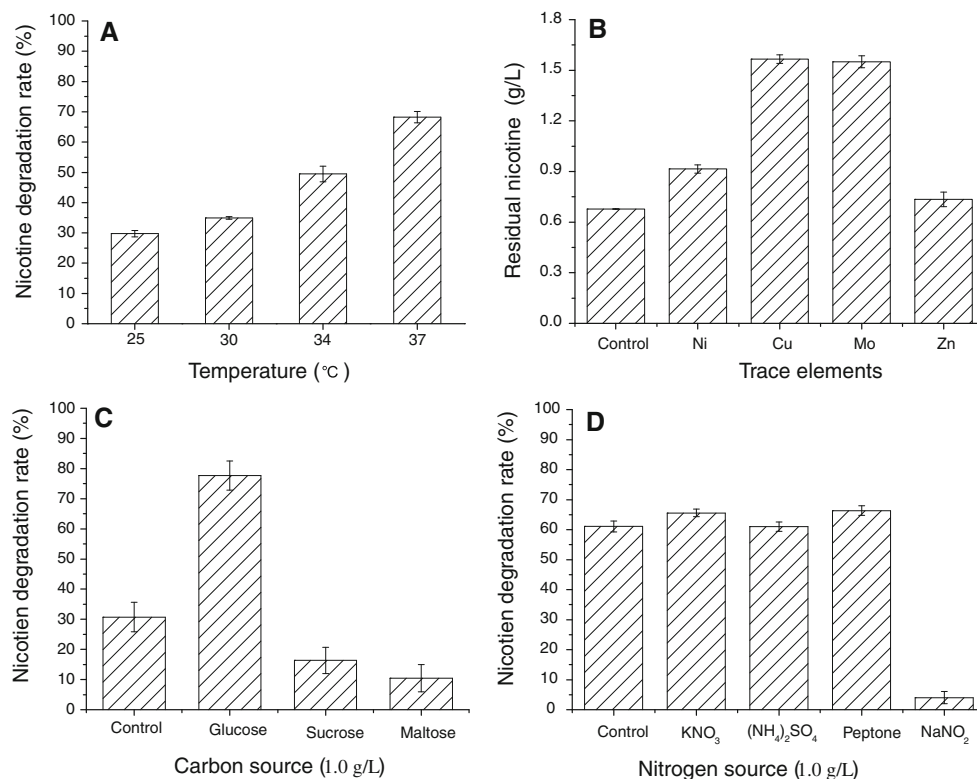


Fig. 3 Effect of **a** culture temperature, **b** different additional trace elements, **c** 1.0 g/L additional carbon source substances, and **d** 1.0 g/L nitrogen source substances on nicotine degradation in BSM by *Pseudomonas stutzeri* ZCJ

to cell suspension to use for scaling up for practical application appeared to be 2:1 (w/v).

Nicotine degradation via solid-state fermentation by *P. stutzeri* ZCJ in shallow 1-L dishes

The results of three batches of solid fermentation in shallow 1-L dishes are shown in Fig. 5, in which A, B, and C are the results of batches 1, 2, and 3, respectively (Table 1). The nicotine degradation rate was significantly improved by the addition of *P. stutzeri* ZCJ to the three batches. In addition, the low quantity of water was favorable for nicotine degradation. The nicotine degradation rates of A, B, and C with 7 mL water (25.93, 23.65, and 32.24%, respectively) were higher than those with 10 mL water (19.06, 17.46, and 22.13%, respectively). The degradation rate of batch 3 was the highest (Fig. 5c) among the three batches, suggesting that a larger inoculum size and a suitable water quantity improves nicotine degradation. Comparing the results of batches 1 and 3 (Fig. 5a and c), the addition of 4.25 mL of solution resulted in higher degradation effects. Comparing the results of batches 1 and 2 (Fig. 5a and b), a suitable interval time for water addition appeared to be 48 h. Thus, the optimal process conditions for shallow 1-L dishes were found to be: 7 mL original

solution, 4.25 mL additional solution, and 48 h interval time for water addition. However, the optimization of cultivation conditions was still necessary to further improve the degradation of nicotine in tobacco leaves by *P. stutzeri* ZCJ during solid fermentation.

Bacterial distribution on original tobacco leaves and tobacco leaves to which the *P. stutzeri* ZCJ cell suspension was added were examined via an environmental scanning electron microscope (ESEM). A small quantity of bacteria was found attached to or growing on the original tobacco leaves (Fig. 6a and b). This result is in accordance with the phenomenon where the nicotine concentration of original tobacco leaves was observed to decrease after a long period of aging (fermentation) or under incubation conditions (Fig. 5). However, the nicotine degradation rate was very low due to the small amount of nicotine-degrading microorganisms on the original tobacco leaves. After the addition of the *P. stutzeri* ZCJ cell suspension, the amount of bacterial cells on the tobacco leaves obviously increased (Fig. 6c and d), most of which presented as short-rod cells similar to that of *P. stutzeri* ZCJ. The significant increase in the amount of *P. stutzeri* ZCJ could decrease the nicotine content of tobacco leaves after 7 d of culture (Fig. 5), suggesting that *P. stutzeri* ZCJ could grow on tobacco leaves and enhance nicotine degradation.

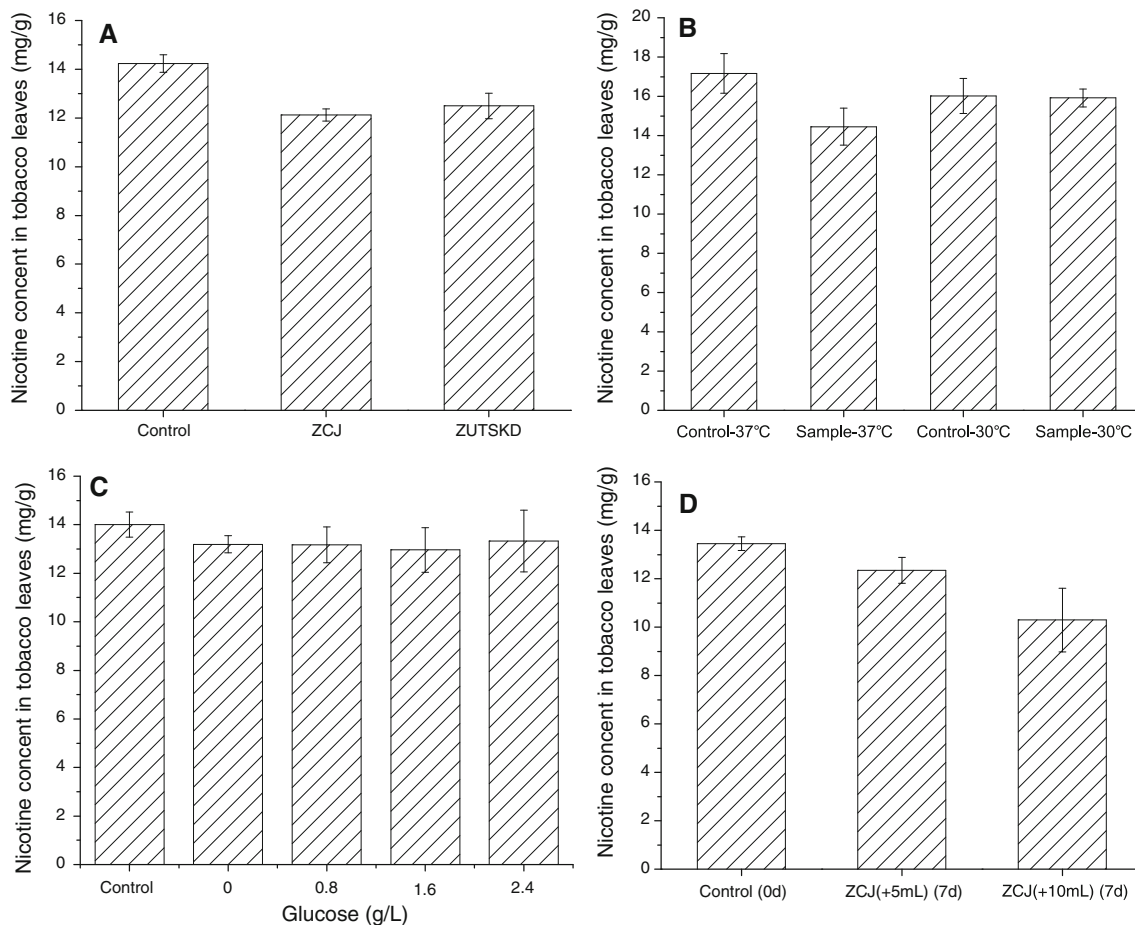


Fig. 4 Effect of **a** different strains, **b** culture temperature, **c** additional glucose as carbon source, and **d** cell suspension with different liquid volume on nicotine degradation by solid state fermentation at 37°C for 7 days

Discussion

A novel nicotine-degrading strain was isolated from flue-cured tobacco leaves from Bijie (Guizhou, China) and identified as *P. stutzeri* ZCJ based on its 16S rRNA gene sequence and morphological-biochemical characteristics. *P. stutzeri* ZCJ showed great nicotine-degrading activity in submerged cultures. It can tolerate 4.5 g/L nicotine and completely degrade 1.5 g/L nicotine within 24 h at 37°C and pH 7.4. Moreover, *P. stutzeri* ZCJ suspensions could improve nicotine degradation in tobacco leaves by solid-state fermentation. The nicotine content of tobacco leaves decreased by as much as 32.24% after 7 days of solid-state fermentation by *P. stutzeri* ZCJ. These results are in agreement with those of Huang et al. (2010), who found that *Bacillus* spp. and *Pseudomonas* spp. are two dominant genera in flue-cured tobacco leaves. *Pseudomonas* spp. is the most easily isolated nicotine-degrading microorganism from tobacco leaves or waste (Chen et al. 2008; Civilini et al. 1997; Ruan et al. 2005; Wang et al. 2004; Zhong et al. 2010); it could break down nicotine in tobacco leaves

without losing their desirable flavor, taste, and smoking properties (Chen et al. 2008; Li et al. 2010). Thus, native *Pseudomonas* spp. may play the main role in nicotine degradation during the aging of tobacco leaves. In present study, *P. stutzeri* ZCJ, a native strain, shows potential in increasing nicotine degradation during the aging of flue-cured tobacco leaves.

Compared with the reported strains, *P. stutzeri* ZCJ is not the best strain for nicotine degradation in submerged cultures (Zhong et al. 2010). However, information on the ability of the reported strains to degrade nicotine in tobacco leaves (solid -state fermentation) is limited. A study (Lei et al. 2008) on nicotine reduction in tobacco leaves reported that *Arthrobacter* spp. isolated from soil sample successfully reduces nicotine content during the flue-curing period. In this study, nicotine was soaked in the *Arthrobacter* spp. suspension for 3–5 s during a sequential flue-curing period. Results show that approximately 28–39% of the nicotine in the tobacco leaf was reduced, and the quality of the tobacco leaf was improved. However, in a cigarette factory, nicotine content reduction would be

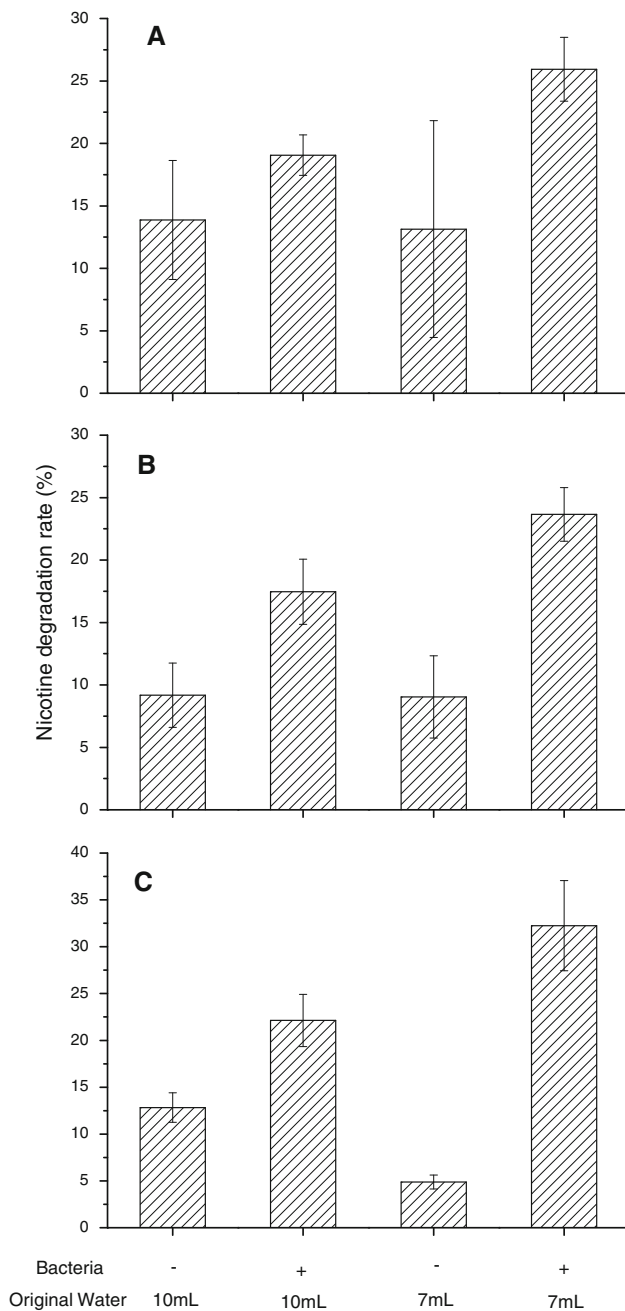


Fig. 5 Three batches **a**, **b**, **c** of nicotine degradation by *Pseudomonas stutzeri* ZCJ in a shallow 1 L tray at 37°C for 7 days

better during the aging (fermentation) period. Thus, nicotine degradation in tobacco leaves during an artificial fermentation process was evaluated in the present study. Under the selected conditions above, the nicotine content in tobacco leaves decreased by 32.24% after 7 days of solid-state fermentation with *P. stutzeri* ZCJ. Furthermore,

compared with nicotine-degrading *Pseudomonas* sp. ZU-TSKD isolated from tobacco waste (Zhong et al. 2010), *P. stutzeri* ZCJ showed better nicotine reduction capability (Fig. 4a). As a native strain, *P. stutzeri* ZCJ is more suitable for enhancing nicotine degradation during the aging of tobacco leaves. Further optimization of the nicotine degradation conditions including different native strains is necessary to scale up the aging process and improve the quality of the resulting tobacco leaves.

Huang et al. (2010) and Su et al. (2011) reported recently that bacterial communities on un-aged tobacco leaves were different from those on aging flue-cured tobacco leaves. Bacterial species, including nicotine-degrading strains, obviously varied with the kind and source of tobacco. Thus, construction of a library of native nicotine-degrading microorganisms isolated from different kinds and sources of tobacco is necessary in future research. With this library, selecting the corresponding native strain to enhance nicotine reduction in any given tobacco leaf would be easy. However, optimal conditions for nicotine reduction would vary with the native strain used. Thus, a series of experiments is also necessary to optimize the nicotine-degrading conditions for different strains in the library. In addition, the metabolic pathway and the safety of potential inter metabolites of varying strains need being investigated clearly before their industrial application (Chen et al. 2008; Li et al. 2010).

Moreover, nicotine was not the sole nutrient for the inoculated strains. The growth of *P. stutzeri* ZCJ on the surface of tobacco leaves might consume some other nutrients, such as glucose which showed improved effect on nicotine degradation as an additional carbon source (Fig. 3c). In the present study, the change of reducing carbohydrates content after aging process was also detected and was not significant to influence the taste of final treated tobacco (data not shown). However, the effect of the *P. stutzeri* ZCJ on tobacco tar or proteins was not detected. In one hand, the strain ZCJ did not show activity on tar degradation. In another hand, protein such as peptone showed no significant effect on the nicotine degradation by *P. stutzeri* ZCJ (Fig. 3d). Thus, *P. stutzeri* ZCJ might have no role in the change of tobacco tar and protein. In the future study, it was also necessary to evaluate more effects mentioned above of nicotine degrading microbes before application.

In summary, the present study proved for the first time that *P. stutzeri* ZCJ is a putative native *Pseudomonas* strain that can enhance nicotine reduction during the aging of tobacco leaves from Bijie (Guizhou, China). The experimental results indicate the need for further studies.

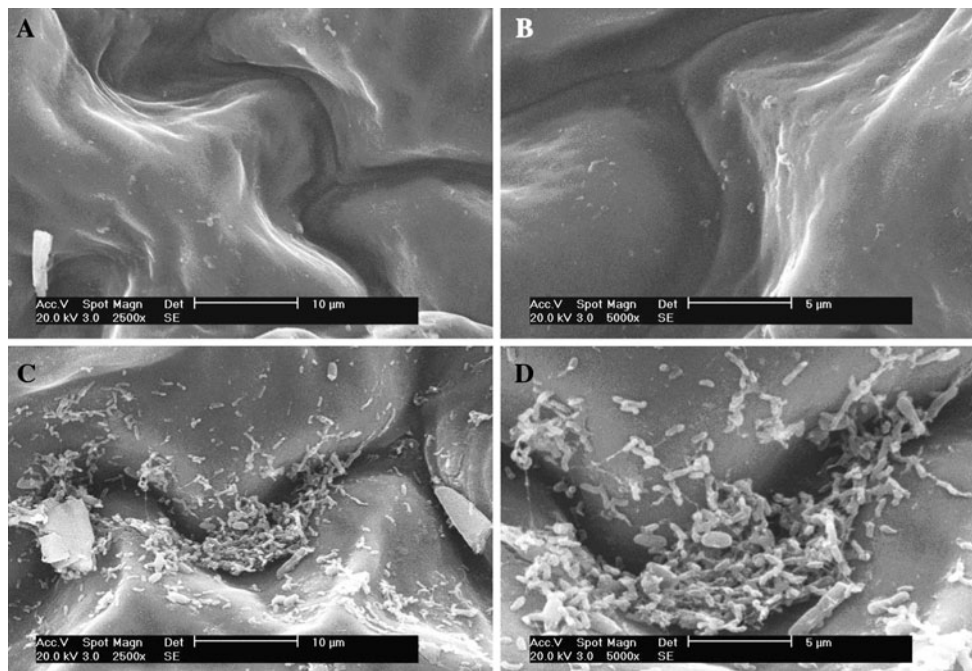


Fig. 6 Bacterial distribution on tobacco leaf surface **a** and **b** without and **c** and **d** with cell suspension of inoculated *Pseudomonas stutzeri* ZCJ. **a** and **c** 2500 \times , **b** and **d** 5000 \times

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