Biodegradation of Nicotine in Aqueous Extract from Waste Tobacco by *Pseudomonas sp. ZUTSKD*

SHU Ming[1], YANG Jun[1], ZHU Chen-Jing[2], ZHONG Wei-Hong[2]

1. China Tobacco Zhejiang Industrial Co., Ltd., Hangzhou, China;
2. Zhejiang University of Technology, Hangzhou, China;

Outlines

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1. Introduction

The tobacco manufacturing process often produce solid or liquid wastes containing a high concentration of nicotine. Since nicotine is soluble in water, it can be easily transported into ground-water. Therefore, tobacco wastes have been classified as ‘toxic and hazardous wastes’ by European Union Regulations. It is required to control the pollution of tobacco wastes all over the world.

Nowadays, all tobacco wastes could be utilized as materials for the production of reconstituted tobacco leaf (RTL). Because of its advantageous economic impact on the manufacturing cost of cigarettes, reconstituted tobacco leaf technology has been gradually introduced into the tobacco industry.

In China, more than five special reconstituted tobacco leaf factories have been built up during the past decade. These factories collected large quantities of waste tobacco, including tobacco plant stems, leaf scraps and dry tobacco dust etc., to produce reconstituted tobacco leaf for cigarettes factories. For example, more than ten thousand tons of reconstituted tobacco leaf per year was processed only in Hangzhou Liqun Environment Protecting Co., Ltd.

- The process of reconstituted tobacco leaf would be one of the most promising technologies to control the pollution by tobacco wastes, and to easily control the nicotine content in cigarettes. However, high content of nicotine, together with high content of reducing sugars, is present in waste tobacco extract (WTE), which is used for smearing back onto reconstituted tobacco leaf, and of which all manufacturers are attempting to reduce the nicotine content.
- Biological treatment could be a viable alternative for nicotine removal and a large quantity of work has been carried out on nicotine degradation by microbes. So far, several microorganisms that are able to degrade nicotine have been isolated from the environment. However, novel strains with stronger ability in nicotine degradation, especially suitable for application in waste tobacco extract treatment, are still required.
- In addition, co-metabolism of nicotine and reducing-sugars might play an important role in the nicotine biodegradation in waste tobacco extract. However, few literatures about co-metabolism and nicotine degradation in waste tobacco extract could be found in the past decade.

The objectives of this study were 1) to isolate new strain that might be suitable for waste tobacco extract treatment, 2) to test the effect of co-metabolism of reducing sugars on the nicotine degradation, and 3) to evaluate the potential of newly isolated strain in waste tobacco extract treatment.

2. Materials and Methods

2.1 Chemicals and media

Nicotine (99 %) standard sample was purchased from WeiFang Three Power Group Co. Ltd. (Shandong, China).

Waste tobacco leaf and waste tobacco extract were supplied by China Tobacco Zhejiang Industrial Co. Ltd.. The contents of solid substrate, nicotine and reducing sugar in WTE were 54.04% (w/w, dry weight), 1.54 % (w/w) and 12.57 % (w/w), respectively.

The basic inorganic salt medium (BSM) contained: Na_2HPO_4 5.57 g, KH_2PO_4 2.44 g, $MgCl_2 \cdot 6H_2O$ 0.2 g, $MnCl_2 \cdot 4H_2O$ 0.0004 g, $FeCl_3 \cdot 6H_2O$ 0.001 g, K_2SO_4 1 g, $CaCl_2$ 0.001 g in 1 L of distilled water, the original pH of medium was adjusted to 7.0~7.5.

Domestication medium: gradually increased concentration of nicotine and glucose was added into BSM respectively to domesticate the wild strain to be osmosis resistant strain.

2.2 Analytical method

The concentrations of nicotine in all the liquid samples were analyzed by high-performance liquid chromatography (HPLC) (SPD-10AVP, SHIMADZU, Japan) equipped with Agilent SB-C18 (4.6 mm×150 mm). The mixture of methanol and 0.1 mol/L KH_2PO_4 (10:90 by volume, pH was adjusted to 3.0) was used as fluent phase with the flow rate of 1.0 ml/min, UV detector wavelength 254 nm, sampling quantity 5 μL.

Cells were harvested by centrifugation (12 000 rpm, 10 min, 4 ℃) from 5 ml of the liquid culture each time. The optical density (OD) of bacterial culture was determined at 600 nm using a 752 spectrophotometer (China) after the cells were washed for three times with potassium phosphate buffer (pH 7.0), and then re-suspended in the same volume of the same buffer.

The method of DNS (3,5-dinitrosalicylic acid) was employed to detect the changes in the concentration of reducing sugar. DNS could react with reducing sugar and result in the color change that could be detected by the absorption at 540 nm measured by spectrophotometer (SP-756, Shanghai Spectrum Instrument Co Ld.). Sucrose concentration was detected by UV-spectrophotometer according to the method reported by Zan. The absorbance of nicotine at 291nm was subtracted from the total data.

2.3 Strain identification and cultivation conditions

The nicotine degrading strain *ZUTSKD* studied in this report was isolated from waste tobacco. It has been deposited in the China Center for Type Culture Collection (CCTCC), which is at the Wuhan University (Hubei, China), with the accession number of CCTCCM207083.

Cell morphology of strain *ZUTSKD* was observed by a transmission electron microscope (JEM-1200EX, Japan). Physiological characteristics were determined by standard methods. The Biolog microstation (GN) (Biolog Hayward, CA, USA) was used to identify the carbon source utilization of the isolate.

100 mL of the medium was transferred to a 250 mL Erlenmeyer flask and autoclaved at 121℃ for 20 min (if sugars added, at 112℃ for 15 min). Then, a certain amount of filter-sterilized nicotine was supplemented to the cooled medium after sterilization, according to experimental requirements. All experimental cultures were incubated at 200 rpm and 30℃.

Chromosomal DNA of the isolate was extracted through boiling procedure. The 16S rDNA (ribosomal deoxyribonucleic acid) genes were amplified using PCR (polymerase chain reaction) with *Taq* polymerase, and the universal primer pair of 20F (5′-agagtttgatggctca-3′) and 1500R (5′-cggctaccttgttacgacttc-3′) modified by ourselves were based on that of Weisburg et al., and were determined by Shanghai Invitrogen Biological Technique Company.

The partial 16S rDNA sequence of strain *ZUTSKD* was deposited in the GenBank database under a accession number of EF538425. Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI) using the BLAST search program.

The 16S rDNA sequences determined and reference sequences obtained from GenBank databases were aligned using multiple sequence alignment software CLUSTAL W 1.81.

3. Results and Discussion

3.1 Identification of strain *ZUTSKD* capable of nicotine degradation

One Gram-negative bacterial named *ZUTSKD* (Fig 1), which showed strong ability in nicotine degradation, was isolated from waste tobacco.

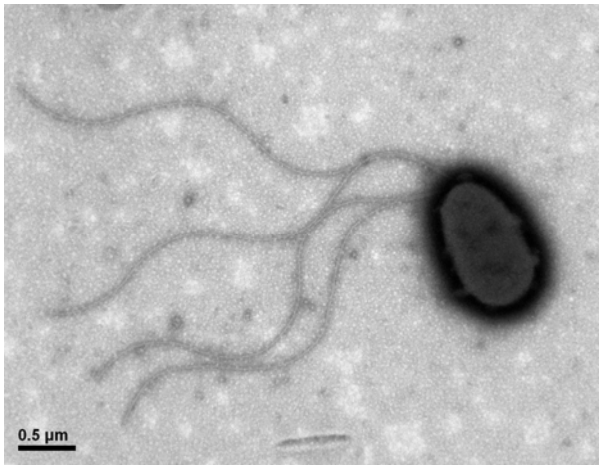

Fig.1 Electron micrograph of strain *ZUTSKD* (30000×)

• A phylogenetic tree

Based on standard morphological, physiological characters and nucleotide sequence analysis of enzymatically amplified 16S rDNA, strain *ZUTSKD* was identified as a member of the genus *Pseudomonas.*

A phylogenetic tree was constructed with MegAlign software of DNASTAR based on the 16S rDNA sequences of 11 strains close to strain *ZUTSKD* (Fig. 2). Therefore, the isolate was designated as *Pseudomonas* sp. *ZUTSKD*.

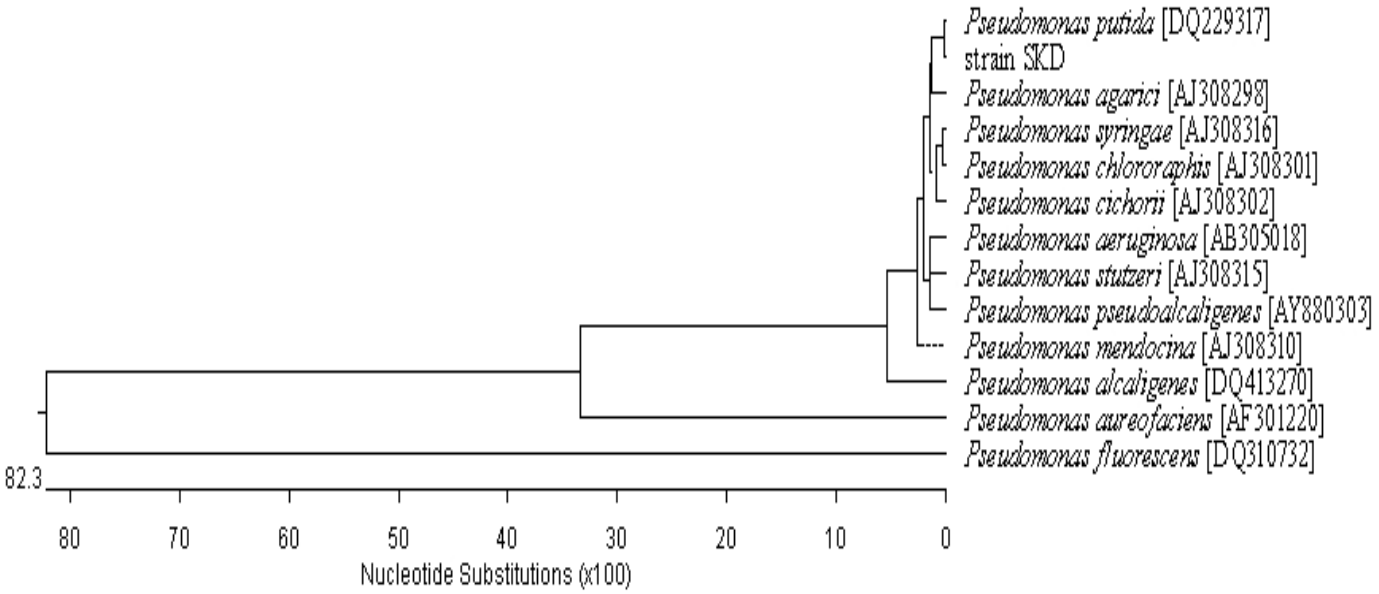

Fig.2. Phylogenetic tree based on 16S rDNA sequences using DNASTAR software.

• Characteristics of strain *ZUTSKD* capable of nicotine degradation

Pseudomonas sp. *ZUTSKD* could grow utilizing nicotine as sole resource of carbon, nitrogen and energy in BSM. Both cell growth and nicotine degradation were observed at a range of nicotine concentration from 2 to 5.8 g/L.

However, strain *ZUTSKD* exposed to 6.5 g/L nicotine in BSM exhibited neither cell growth nor nicotine degradation, suggesting that the concentration limit of strain *ZUTSKD* could be 5.8 g/L (Fig 3).

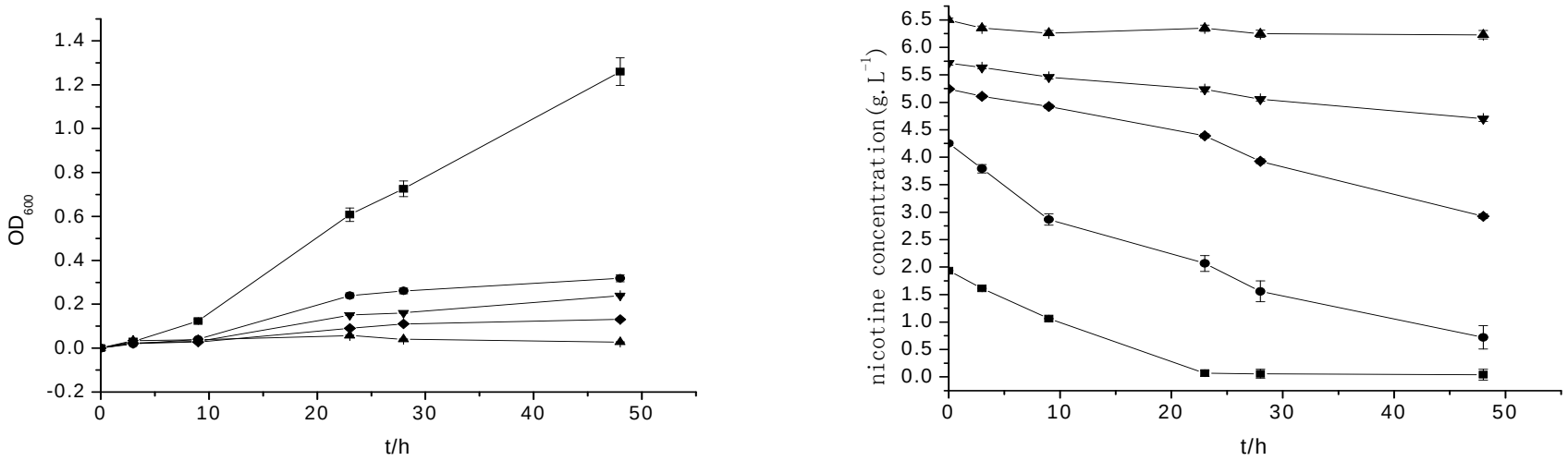

Fig.3 Effect of nicotine concentration on the growth and nicotine degradation of Strain *ZUTSKD*.
2 g•L^{-1}（■）， 4 .3g•L^{-1}（●）， 5.3 g•L^{-1}（◆）， 5.8 g•L^{-1}（▼）， 6.5 g•L^{-1}（▲）

3.2 Nicotine degradation by strain *ZUTSKD* in sugar containing medium

Due to high concentration of reducing sugars in waste tobacco extract, such as glucose, the effect of glucose on nicotine degradation by strain *ZUTSKD* in basic inorganic salt medium (BSM) was investigated before direct application in waste tobacco extract (WTE).

It could be found that addition of glucose at lower concentration in the range 1-10 g/L could significantly improve cell growth within 24 h and the optimal concentration range was 4-8 g/L (Fig 4 A).

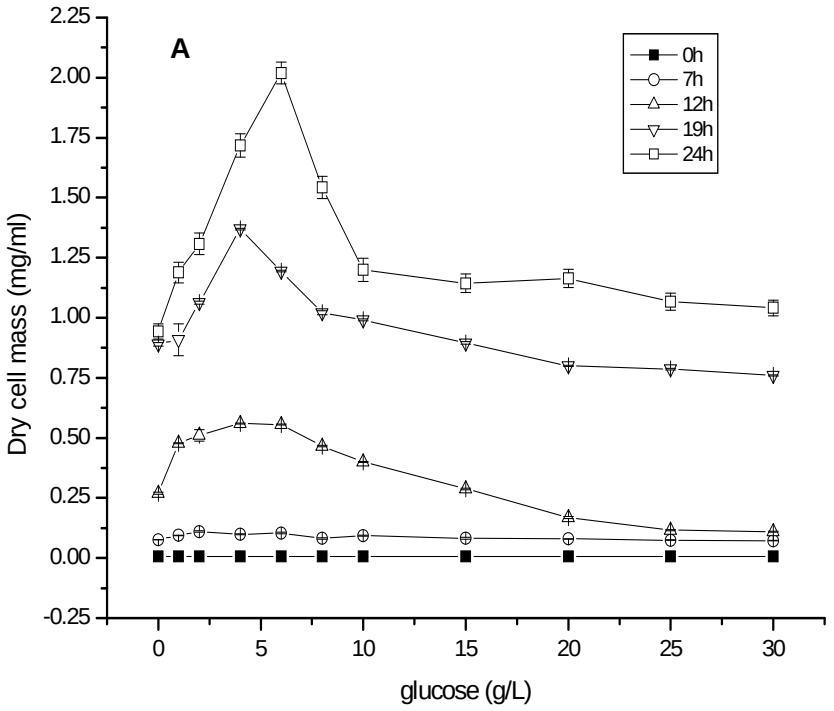

Fig.4(A) Effect of additional glucose concentration on the growth of *Pseudomonas* sp. *ZUTSKD*.

• The cell growth, nicotine degradation and glucose concentration

The enhancement of nicotine degradation was also detected at a range of glucose concentration from 1 to 10 g/L, in which above 99 % nicotine in BSM was degraded within 24 h (Fig 4 B).

However, the nicotine degradation would be inhibited by additional glucose at a concentration above 10 g/L, at which only 80 % or less nicotine could be degraded in BSM within 24 h.

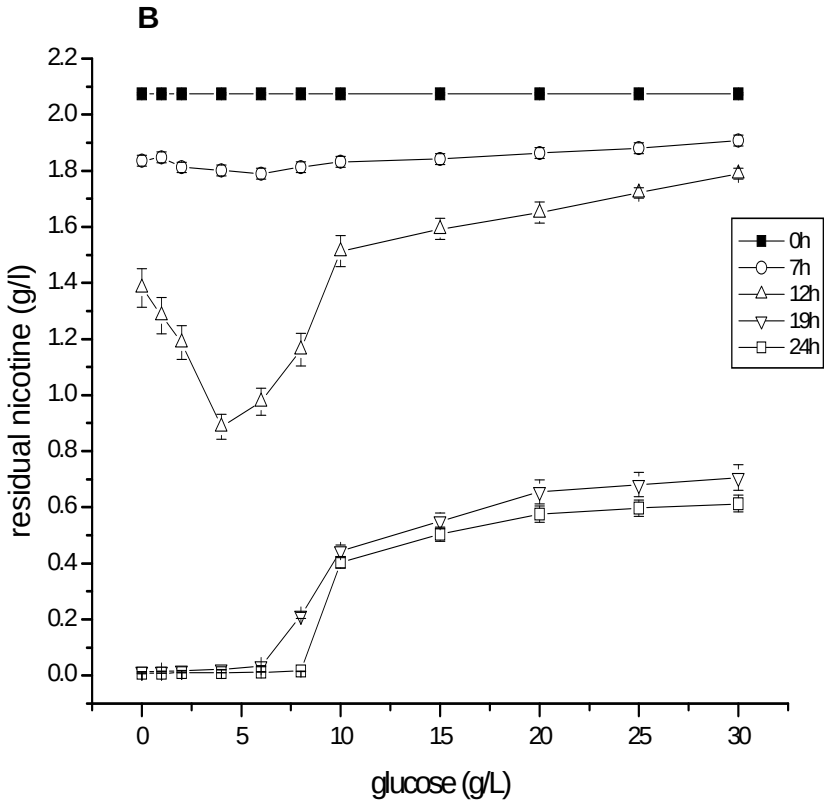

Fig.4(B) Effect of additional glucose concentration on nicotine degradation of *Pseudomonas* sp. *ZUTSKD*.

• The cell growth, nicotine degradation and glucose concentration

Fig 4 C showed the residual glucose change in BSM. It was found that the availability of glucose was low when additional glucose increased and that 4 g/L of glucose would be enough for the growth of strain *ZUTSKD*, with which above 75% glucose could be utilized after 24 h cultivation.

It could be concluded that glucose concentration in the range 1-10 g/L could promote the cell growth and nicotine degradation while glucose at a concentration above 10 g/L would slightly inhibit the nicotine degradation, and that glucose availability would decrease with the increase of glucose concentration.

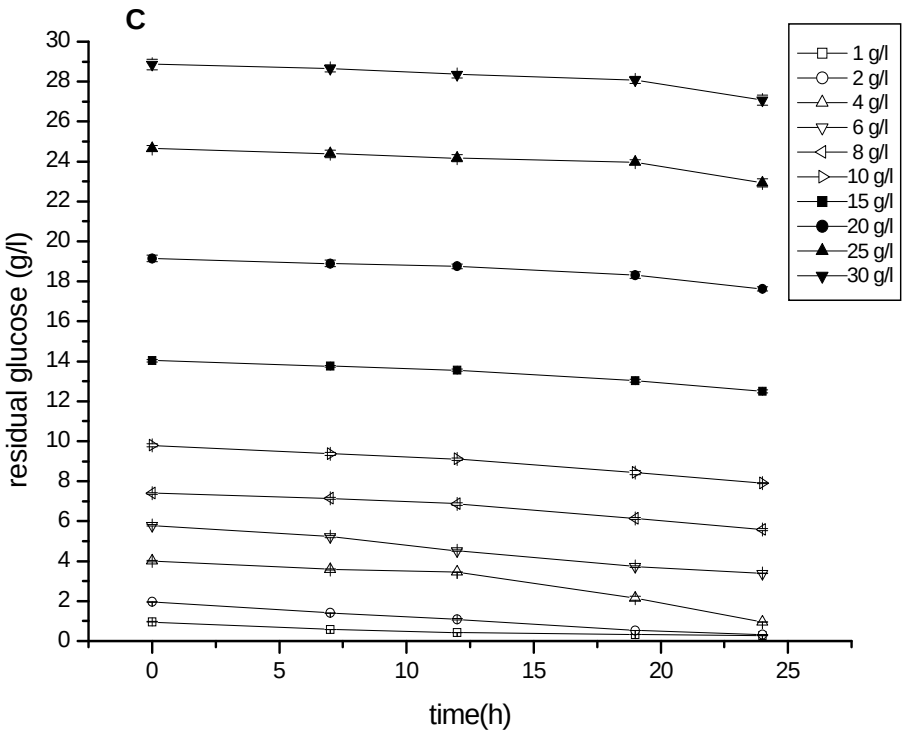

Fig.4(C) Effect of additional glucose concentration on glucose consumption of *Pseudomonas* sp. *ZUTSKD*.

3.3 Direct degradation of nicotine in WTE by *Pseudomonas* sp. *ZUTSKD*

- In Fig.5, it could be found that the significant nicotine degradation occurred only in waste tobacco extract (WTE) diluted 10% (v/v), in which 78% and 92% nicotine could be degraded after 24 h and 48 h cultivation respectively.
- However, in the WTE diluted 20% , 30% and 40% , only 14% or less nicotine could be degraded.
- It could be concluded that *Pseudomonas* sp. *ZUTSKD* could degrade the nicotine in WTE. However, the tolerance of higher concentration of reducing sugars need to be further increased.

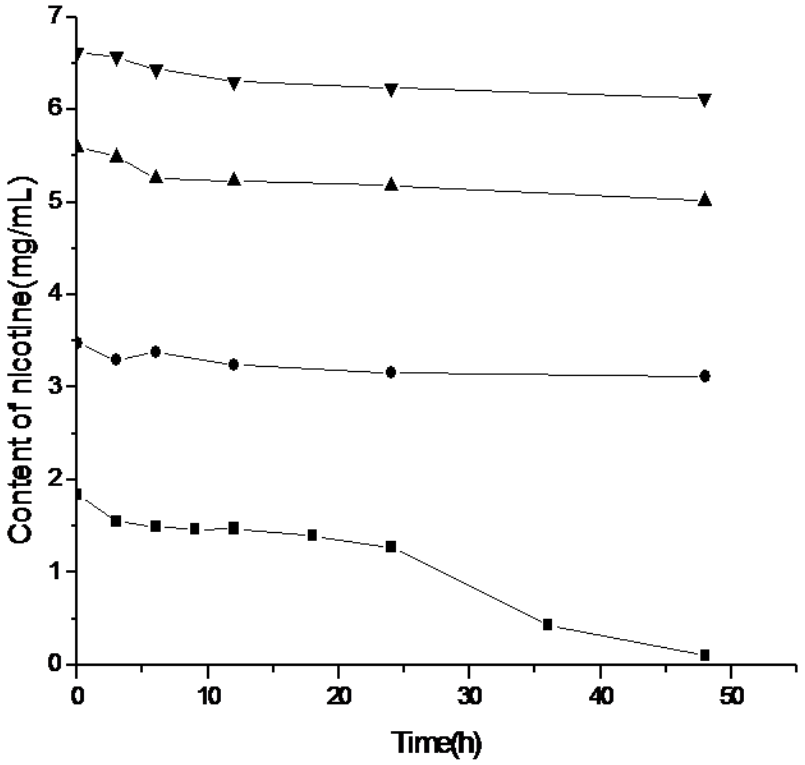

Fig 5 Degradation of nicotine in WTE by *Pseudomonas* sp. *ZUTSKD*.
10% WTE（■），20% WTE（●），30% WTE（▲），40% WTE（▼）

4. Conclusions

- The strain *ZUTSKD* isolated from waste tobacco was identified as belonging to *Pseudomonas*. *Pseudomonas sp. ZUTSKD* could grow utilizing nicotine as sole source of carbon, nitrogen and energy. Its resistant limit of nicotine was 5.8 g/L, which is at the highest level of limit among reported strains.

- *Pseudomonas sp. ZUTSKD* could degrade nicotine by co-metabolism with reducing sugars. However, glucose at a high concentration above 10 g/L would inhibit the cell growth and nicotine degradation.

- It would be hard for strain *ZUTSKD* to degrade nicotine completely and rapidly in waste tobacco extract. However, in waste tobacco extract diluted 10% with reducing sugars, more than 90% nicotine could be degraded by strain *ZUTSKD* within 48 h.

So *Pseudomonas sp. ZUTSKD* would be potential for application in waste tobacco extract treatment for the reduction of nicotine content in reconstituted tobacco leaf.

Thank you!