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Research Article

Biodegradation of Nicotine and TSNAs by Bacterium sp. Strain J54

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Background: Microorganisms play an important role in reducing harmful substances in flue-cured tobacco. Numerous studies have been conducted to degrade nicotine by microorganisms.

Objectives: The present research deals with the isolation of a potent bacterial strain able to efficiently degrade nicotine and tobacco-specific nitrosamines (TSNAs) in flue-cured tobacco.

Material and Methods: Bacterial strain J54, capable of efficiently degrading nicotine and tobacco-specific nitrosamines (TSNAs), was isolated from tobacco leaves and identified. The strain J54 can use nicotine as the sole carbon and nitrogen source and could effectively degrade nicotine while growing in a nicotine isolation medium (NIM) medium.

Results: Compared with the control (CK), the total TSNAs content in the tobacco flue-cured eaves after being sprayed with a solution of the J54 strain was found to decrease by 26.22%. Therein, the degradation rates of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), and N′-nitrosoanabasine (NAB) were 24.01%, 26.27%, 28.6%, and 1.83%, respectively.

Conclusions: Bacterial strain J54, was isolated from tobacco leaves and identified as a bacterium, which is similar to *Bacillus altitudinis* based on its morphological and biochemical characteristics and by phylogenetic analysis based on 16S rRNA gene sequences. To our knowledge, this is the first report of the isolation and characterization of a *Bacillus* sp. strain that can efficiently degrade nicotine and TSNAs. The findings pave the way for the application of new biotechnologies for the degradation of nicotine and TSNAs by microorganisms.

Keywords: Bacillus sp. Strain, Nicotine biodegradation, Tobacco-specific nitrosamines.

1. Background

Nicotine, characterized by high toxicity, biomembrane penetrability, stable chemical structures (and thereby, non-degradability) (1), is an important precursor of carcinogenic tobacco-specific nitrosamines (TSNAs) (2). The toxic aspects of nicotine have been well known. Each year, about 600,000 people die as a result of nicotine. In addition, many tobacco wastes containing a high concentration of nicotine may pollute environments across the world (3). It is a pressing issue for China to tackle the various forms of harm caused by nicotine in tobacco is a, a country that is the largest producer of tobacco in the world. Previous research has isolated strains able to degrade nicotine from tobacco soil, leaves, and waste, such as *Pseudomonas* sp. Nic22 (4), *Aspergillus oryzae* 112822, and *Arthrobacter* sp. M2012083: however, these strains degrade different amounts of nicotine in different environments (5-7), therefore, the conditions for degrading nicotine with microorganisms remain to be explored.

Meanwhile, through a series of chemical reactions,

nicotine will lose a methyl group and thereby produce nornicotine which is further transformed into TSNAs (8). TSNAs with high carcinogenicity are among the main harmful substances that threaten human health and are nitrosylation products of tobacco alkaloids formed during the curing, aging, and burning of tobacco (9, 10). It is reported that the mortality of smokers is three times higher than that of those who have never smoked (11). Among the eight TSNAs that have been identified, N'-nitrosonornicotine (NNN) and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are highly carcinogenic (12). It has been demonstrated that the TSNAs content is extremely low, even undetectable, in fresh tobacco leaves. Considering this, it is presumed that TSNAs are produced due to microbial reduction of nitrate to nitrite and nitrogen oxides (NOX), which then react with tobacco alkaloids during the curing and storage of tobacco (13). In recent years, how to reduce the content of TSNAs in tobacco has been a key challenge to be solved by tobacco researchers and has become a key research topic of major tobacco companies. How to reduce and adjust the content of TSNAs in tobacco is vital for the production and development of tobacco, however, there is no effective natural degradation method available at present. The degradation of TSNAs by microorganisms has attracted increasing attention as it is highly efficient and simple in application. Numerous studies have been conducted to degrade nicotine with microorganisms. For example, *Pseudomonas putida* PC1, *Arthrobacter oxydans, Arthrobacter nicotinovorans,* and *Achromobacter insolitus* all exhibit nicotine-degrading ability (14). It is speculated that endophytic bacterial communities in tobacco have been used to taking nicotine as their growth substrates and applying biochemical reactions to decompose nicotine, (15): however, little is known about the genera of endophytes in tobacco leaves that may have biotransformative functions for nicotine. To the best of our knowledge, there is very little information on the degradation of TSNAs by endophytes of tobacco leaves. Other researchers have isolated an endophyte from tobacco leaves and identified it as a new *Bacillus* sp. strain J54 that can degrade nicotine and TSNAs. Moreover, the conditions for using the strain to degrade pure nicotine and degrade TSNAs in the curing process were characterized.

2. Objective

Isolation and identification of bacteria capable of degrading nicotine and tobacco-specific nitrosamines (TSNAs) from tobacco leaves

3. Materials and Methods

3.1. Isolation of Endogenous Nicotine-Degrading Bacterium

The collected tobacco leaves (mass 5 g) were washed with bacteria-free water and then soaked in 75% alcohol for 1 min. Then, the leaves were soaked in sodium hypochlorite solution for 3 min and 75% alcohol for 30 s, following by triplicate wash using bacteria-free water. The leaves were cut into pieces of 2–3 cm using sterilized scissors, placed in 45 mL phosphate buffer solution (0.1 M, pH 7.2), and exposed to ultrasonic for 30 min. The phosphate buffer solution was filtered through four layers of gauze to remove the tobacco leaves. After vacuum filtration of the filter liquor, the bacteria were collected on a filter membrane with a pore size of 0.2 μm (Whatman, Germany). Bacterial cells on the filter membrane were ultrasonic elution in 10 mL bacteria-free water and collected by centrifugation (13,000 rpm, 20 min). The pellet was re-suspended in 1 mL bacteria-free water and uniformly spread on solid NIM medium (6 $g.L^{-1}$ of Na_2HPO_4, 3 $g.L^{-1}$ of KH_2PO_4, 1 $g.L^{-1}$ of NH_4Cl, 0.5 $g.L^{-1}$ of NaCl, 0.12 $g.L^{-1}$ of $MgSO_4$, 0.1 $g.L^{-1}$ of $CaCl_2$, 0.5 $g.L^{-1}$ of nicotine, and 15 g.L-1 of agar), in which nicotine (Sigma-Aldrich, Germany) was added as the sole carbon source. The plates were incubated at 30 °C for 48–72 h. Single colonies were transferred, isolated, and purified, followed by incubation in the liquid NIM medium to verify whether, or not, the bacteria capable to grow with nicotine as a sole carbon source. The finally screened nicotine-degrading bacteria were preserved.

3.2. Screening TSNA-Related Bacteria

The M9 medium (6 $g.L^{-1}$ of Na_2HPO_4, 3 $g.L^{-1}$of KH_2PO_4, 1 $g.L^{-1}$ of NH_4Cl, 0.5 $g.L^{-1}$ of NaCl, 0.12 $g.L^{-1}$ of $MgSO_4$, 0.1 $g.L^{-1}$ of $CaCl_2$, and 15 $g.L^{-1}$ of agar) without glucose was sterilised and cooled to 45 °C. Afterward, NNK (at a final concentration of 0.1 $g.L^{-1}$) was added to the medium, which was then shaken and spread on a plate. Then, a sterilized toothpick was used to pick the screened nicotine-degrading bacteria for point incubation on a new plate. After being cultured for 48 h at 26 °C, strains that grew well were selected as target strains and preserved in 30% (v/v) glycerol at –80 °C for later use.

3.3. 16S rRNA Sequencing and Analysis

A TaKaRa miniBEST genomic DNA extraction kit was used to extract genomic DNA from the target strains, following the instructions provided by the

manufacturer. Universal primers of 27F (5'-AGA GTT TGA TCA TGG CTC AG -3') and 1492R (5'-ACG GTT ACC TTG TTA CGA CTT-3) were used for the amplify the 16S rRNA gene. The PCR products were checked in 1.2% agarose gel, purified using the Gel Extraction Kit, and then cloned to the vector using the pMDTM18-T kit (TaKaRa, Japan). White colonies were selected from the transferring plate and sent to Shanghai Invitrogen Biotech Co., Ltd for sequencing. The BLAST program was used to analyze the resulting sequences against nucleic acid data in the GenBank database. The initial alignment was conducted using the CLUSTAL-W sequence alignment software. Based on 16S rRNA gene sequences, a phylogenetic tree for strains with close genetic relations to the strain J54 was constructed in MEGA 3.0 software using NJ (neighbor-joining) method. One thousand times of bootstrap, Poisson correction, and pairwise deletion were performed during the construction of the phylogenetic tree.

3.4. Physiological and Biochemical Characteristics of the Strain

After being cultured for 36 h in the NA medium, the physiological and biochemical characteristics of J54 strain were analyzed according to Bergey's Manual of Systematic Bacteriology (16). Besides, the morphological characteristics of the bacteria were observed under an Axio Imager A2 microscope (Germany).

3.5. Detection of Degradation Rate of Nicotine

The purified strains were incubated in the LB culture solution and the bacterial concentration was measured to be 1010 CFU.mL^{-1} after two days. Some 50 µL strain solution was transferred to a 5 mL NIM liquid medium (containing 2.0 g.L^{-1} of nicotine), to be cultured on a shaker for 12 h at 30 °C at 200 rpm. Two replicates were established for all strains in a colorimetric assay using a Lambda 650 ultraviolet (UV)-visible spectrophotometer (Perkin Elmer, USA), to measure the OD600 value. Afterward, the bacterial solution was centrifuged at 12,000 rpm for 10 min. The supernatant was subjected to high-pressure liquid chromatography (HPLC) (Agilent 110 Series, USA) with Agilent chromatographic columns (5 µm, 4.6 × 150 mm). Mobile phases were set for gradient elution: 40%-50% methanol for 5 min, 50% methanol for 2 min, 100% methanol for 4 min, and 40% methanol for 5 min under conditions with a flow rate of 1 mL.min^{-1} and a sample size of 20 µL. The wavelength of the UV detector was set to 254 nm and the retention time was 4.9 min.

3.6. Detection of TSNAs

LB medium was used to culture the target strain J54 until the bacterial concentration reached 1 × 1010 CFU.mL^{-1}. After 10-fold dilution, the solution of bacteria was uniformly sprayed onto tobacco leaves. After being kept still for 1 h to ensure no visible trace of the solution was left on the surface, the tobacco leaves were put in a bulk curing barn for baking and the TSNAs content therein assayed after baking. A mass of 0.5 g of tobacco leaves was placed in a 50 mL beaker flask, to which 0.1 mL internal standard solution (2000 ng.mL^{-1}) and 30 mL ammonium acetate solution (100 mmoL.L^{-1}) were added. The mixed solution was oscillated using an oscillator for 30 min at 130 rpm, followed by filtering using a 0.22-µm syringe filter for aqueous phases into a 2 mL chromatographic bottle for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent 1200 Series, USA) (17). To separate TSNAs, the filtrate was loaded to a reversed-phase HPLC equipped with a Waters Symmetry Shield RP18 column (3.9 mm×150 mm i.d. ×5 µm), and separated at 65 °C using 0.1% acetate solution in 50% methanol and 50% water as the mobile phase (flow rate: 0.25 mL.min^{-1}). The electrospray ionization (ESI) source working in the positive ion mode was used to ionize the TSNAs. The ionized TSNAs were then detected in the tandem mass spectrometry operated with 5 kV spray voltage, 172 Pa sheath gas pressure, 34 Pa aux gas pressure, 350 °C capillary temperature, 0.2 Pa collision gas pressure, 10 V collision energy and 0.2 s scanning time. All mass spectral data were collected using the selected reaction monitoring (SRM) mode.Data analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), unless otherwise indicated.

4. Results

4.1. Cell and Colonial Morphologies of Strain J54

The nicotine isolation medium (NIM) medium (with nicotine as the sole carbon and nitrogen source for bacterial growth) was adopted to isolate bacteria that can degrade nicotine from tobacco leaves. According to the colonial morphologies, the isolated nicotine-degrading bacterial strain showed high community diversity (data not shown). The colonies were milk-white and had smooth surfaces (**Fig. 1A**). Microscopic observation showed that the strains were rod-shaped with the length of 2.0–5.0 µm and width of 0.6–1.0 µm, with spores formed on terminals or secondary

terminals, having mobile flagella all around (**Fig. 1B**). The strain was named J54 and further identified and characterized.

4.2. Identification of Bacterial Strain J54

16S rRNA gene sequencing was conducted for the strain J54 and the results were deposited in GenBank with accession number MN148770.1. Then, a phylogenetic tree was constructed according to the 16S rRNA sequences of relevant bacteria (**Fig. 2**). The 16S rDNA gene analysis of the strain J54 revealed that the strain showed an identity exceeding 99% with *Bacillus* spp., such as strains of *Bacillus altitudinis, Bacillus stratosphericus*, and *Bacillus* sp. (in *Bacteria*). As strain J54 was closely related to the above species, it was difficult to determine its specific characteristics at the species level. Despite this, it is determined, from the phylogenetic tree based on 16S rDNA sequences, that strain J54 belongs to a species of the genus of *Bacillus.*

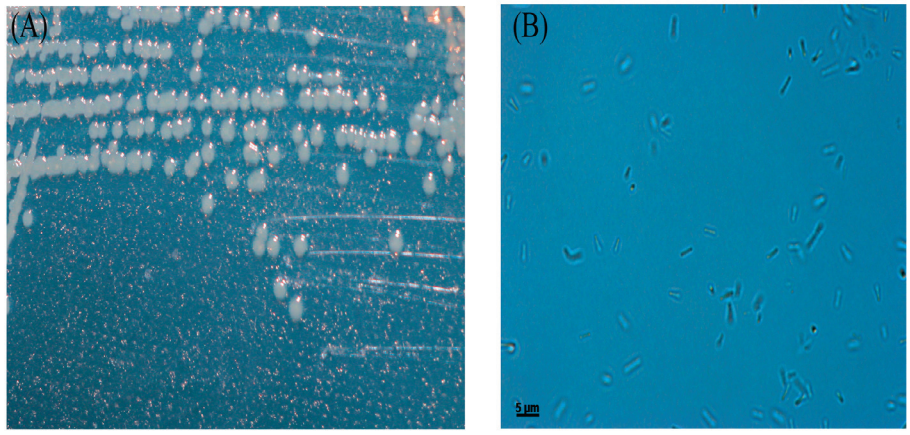

Figure 1. A) Colonial morphological of strain J54; B) Microscopic morphologies of strain J54 under the electron microscope

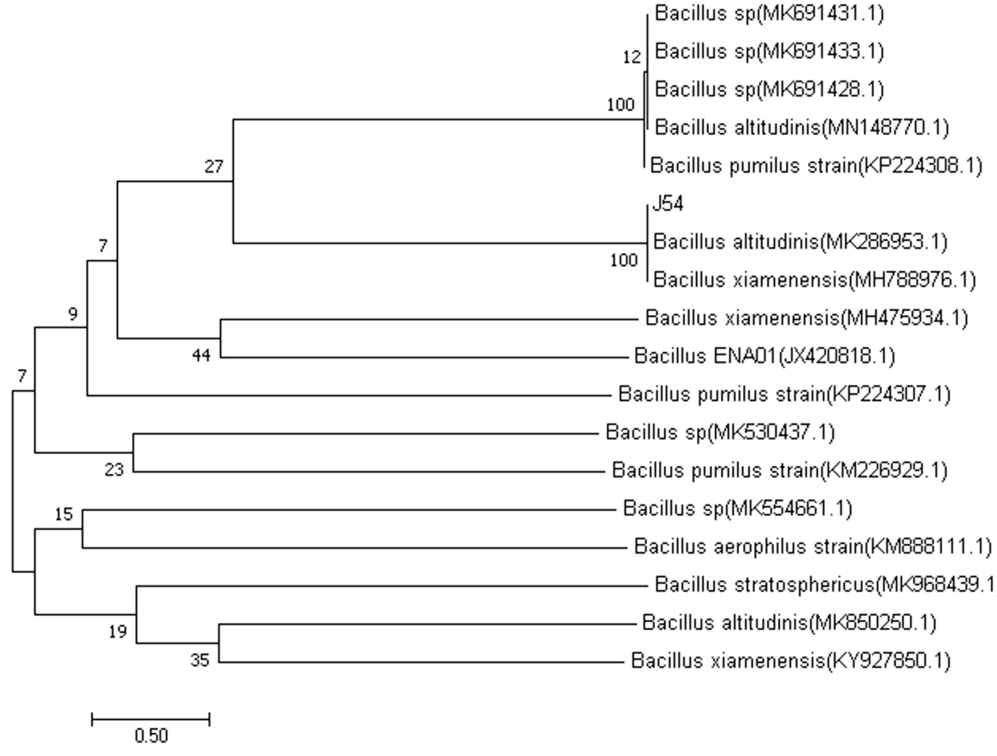

Figure 2. The phylogenetic tree of strain J54 based on 16S rDNA sequences (accession numbers of strains are given in parentheses)

4.3. Growth and TSNA Degradation Performance of Strain J54

After being incubated for 54 h in the NIM medium at 30 °C, Bacillus sp. strain J54 degraded more than 85% of the available nicotine. Meanwhile, as the concentration of nicotine in the NIM medium decreased, the OD_{600} value of the bacterial culture increased, and the two showed a close correlation. After being cultured, the strain was subjected to colorimetric assay using the Lambda 650 ultraviolet (UV)-visible spectrophotometer, and the detected OD value and the nicotine content (as measured by HPLC) are given in **Table 1** and **Figure 3**, which indicated that strain J54 could degrade nicotine in tobacco.

Compared with the CK, the tobacco flue-cured after being sprayed with a solution of strain J54 showed a significantly lower TSNA content. In tobacco leaves treated with strain J54, the TSNA content was decreased by 26.22%, with apparent degradation rates for NNK, NNN, NAB, and NAT at the same time (**Table 2**). Although it has been demonstrated that the screened strain was able to decrease the TNSA content in the tobacco, the mechanism of degradation, kinetic parameters of the degradation, activity, and modes of utilization of the strain remained unclear. Further research is needed to determine the mechanism of the action of the strain and the kinetic parameters of TSNA degradation, for the purpose to optimize the fermentation technology and to further enhance the degradation activity, broaden application prospects for the strain, and finally improve the safety of the raw materials of tobacco.

Table 1. Growth of the strain and nicotine concentration in the medium after incubated for 54 h

Treatment	OD	Nicotine (mg/L)
CK	0.513 ± 0.0003	1628.5 ± 56.9
J54	2.012 ± 0.0024	246.35 ± 12.3
Degradation ratio of nicotine	----	85%

Note: CK refers to the control treated with sterile distilled water of the same volume (100 mL). Degradation ratio of nicotine = ($Nicotine_{CK}$ – $Nicotine_{J54}$) / $Nicotine_{CK}$ * 100/100

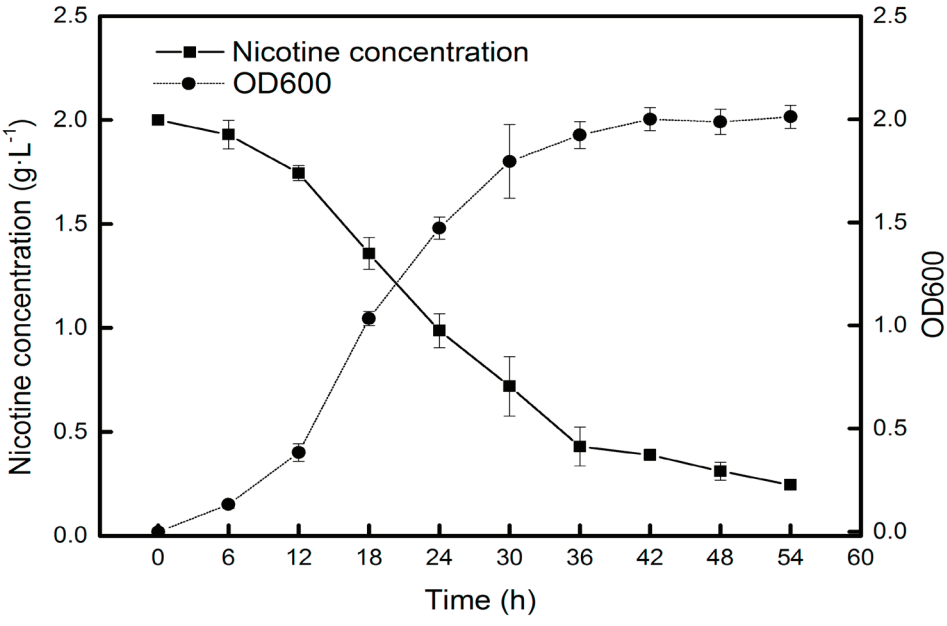

Figure 3. The growth and nicotine degradation of Bacillus sp. strain J54

5. Discussion

Tobacco-specific nitrosamines (TSNAs) have been a major concern to the public health community for decades. A decades-long industry effort through agricultural practices, plant breeding, and tobacco processing have been made to lower the content of nicotine in tobacco products and remove nicotine from tobacco wastes. Recently, microbiological technology provides a new perspective to study the reduction of nicotine in tobacco (17, 18), since the bacterial community residing in the tobacco leave and rhizosphere is capable of using nicotine as a growth substrate. In the present study, our data showed that the J54 strain isolated from the flue-cured tobacco consistently exhibited high degradation rates in NNN, NNK, and NAT levels. Furthermore, the morphological, physiological, and biochemical characteristics of the J54 strain are similar to Bacillus altitudinis (**Table 3**) (17).

Many nicotine-degrading bacteria of Bacillus spp. can utilize nicotine as the sole carbon and nitrogen source for growth (15, 18). The growth temperature condition for nicotine degradation in these species is typically at 25- 45 °C, with a pH range of 4–9 (**Table 3**). Most of these bacteria could grow at a concentration of up to 2.0 $g.L^{-1}$ nicotine. In our study, the J54 strain showed gram-positive and grew well at 30 °C in an environment with pH of 6.5. The tolerance of the strain was less than 9%. It was positive in gelatine liquefaction, nitrate reduction assay, starch hydrolysis test, methyl red (MR) test, and V.P test while negative in oxidase reaction and citrate utilization tests. It could produce acids by utilizing inositol, D-sorbitol, mannose, N-acetylglucosamine, and sucrose while could not use D-xylose, D-raffinose, maltose, and rhamnose. Some Bacillus species could degrade nicotine in tobacco leaves and showed potential application in tobacco processing. For example, Shivaji et al (2006) found that Bacillus altitudinis was capable of bioremediation and biodegradation in plants and played a favorable role in controlling of potato common scab in the agricultural sector (18). Moreover, it was found that TSNAs were formed due to the reaction of alkaloids and nitrates in the tobacco leaves, in which NNK was derived from nicotine, NNN was derived from nicotine and nor nicotine, and NAB and NAT were derived from neonicotinoids and anatabine, respectively, with the synthesis substrates used being similar to those used when synthesizing nicotine (8, 9). Therefore, nicotine and TSNAs were used as substrates in this research to screen a strain of microorganisms that can degrade the two at the same time, to provide more theoretical support for biodegradation of nicotine and TSNAs.

6. Conclusion

This is the first research to report the isolation and characterization of Bacillus sp. strain J54 capable of degrading nicotine and TSNAs. After being cultured for 12 h, strain J54, with an initial concentration of 2 $g.L^{-1}$, was able to degrade 85% of all available nicotine. For tobacco leaves that had been flue-cured after being sprayed with J54 strain solution, the total TSNA content therein was decreased by 26.22%, while the NNK, NNN, and NAT contents decreased by 24.01%, 26.27%, and 28.60%, respectively. Further research will be focused on the identification of molecular mechanisms involved in the biodegradation of Nicotine and TSNAs by Bacillus sp. strain J54.

Table 2. Influences of strain treatment on the ratio of degradation of TNSAs in flue-cured tobacco leaves

Treatment	NNK	NNN	NAB	NAT	Total TSNA content
CK	27.95 ± 4.89a	34.71 ± 6.47a	1.70 ± 0.19	44.65 ± 5.99a	108.95 ± 17.37a
J54	21.24 ± 1.70	25.59 ± 3.85	1.67 ± 0.23	31.88 ± 5.93	80.38 ± 10.64
Degradation ratio of TSNAs	24.01%	26.27%	1.83%	28.60%	26.22%

Each strain treatment involved three replicates. Mean ± SEM

[a] $P \leq 0.05$ versus in CK and J54 strain treatment

Degradation ratio of TSNAs = $(TSNA_{CK} - TSNA_{J54}) / TSNA_{CK} * 100/100$

Table 3. Physiological and biochemical characteristics of strain J54

Test characters	*Bacillus altitudinis*	Strain J54
Gram staining	+	+
Citrate utilisation	-	-
Oxidase reaction	-	-
Gelatine liquefaction	-	+
Starch hydrolysis	-	-
Nitrate reduction	+	+
Utilization of :		
N-acetylglucosamine	+	+
Sucrose	+	+
D-xylose	-	-
Maltose	-	+-
Rhamnose	-	-
Inositol	+	+
D-Sorbitol	+	+
Histidine	+	+
Methionine	+	+
pH 4	+	+
pH 9	+	+
Temperature 25 °C	+	+
Temperature 45 °C	+	+

Note: "+" and "–" separately refer to positive and negative reactions. The pH and temperature indicated the range of J54 strain growth.

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