Biodegradation of Nicotine Obtained from Tobacco Powder Using *Pseudomonas aeruginosa Sp*

Tamilmani, Muthukumar, Saravanan, Karventhan, Dr.Saravanan, Dr.Ramesh Babu, Dr.Ragunathan

Abstract—**Cigarette smoking may lead to lung cancer due to the continuous deposition of nicotine alkaloids with carbon in the lungs. Nicotine degrading bacteria was isolated from soil using Nicotine as the sole carbon source. Two microbes were obtained from the soil which were already mixed with tobacco powder in the two different cigarettes grown in the Nicotine medium and named as A and B. These strains were analysed using Thin layer chromatography, The bacterial strain A showed effective degradation and so it was selected as the desired isolate. The isolates were identified as Pseudomonas aeruginosa sp. by biochemical tests. The degradation was analysed using UV-Visible Spectrophotometer and Fourier transform infrared spectroscopy (FTIR). These results showed that the degradation was effectively done by Pseudomonas aeruginosa sp.**

Index Terms— ***Cigarette, Inorganic Salt Media, Nicotine degradation, Pseudomonas aeruginosa sp***

I. INTRODUCTION

Nicotine is the most widely used additive drug in the world. Cigarette used for smoking contains more than 4000 chemicals, the alkaloid nicotine has shown to be linked to vascular diseases in smokers [6]. Nicotine is not readily degradable as well as highly toxic and hence, classified as ''toxic and hazardous waste'' [4].

When the nicotine content exceeds 500 mg/kg dry weight, it is considered as toxic. During the processing of tobacco large quantities of wastes that contain high concentration of nicotine have been accumulated [2]. Tobacco can pollute the environment, imperil the health of human beings and disrupt the ecological balance when they directly enter the ecosystemwithout any prior treatment. At least seven lakhs people die due to tobacco related diseases in India every year. Besides, tobacco alkaloids affect human health; they also pollute the environment and disrupt the ecological balance when they enter the soil without any treatment [9].

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Tamilmani, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Muthukumar, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Saravanan, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Karventhan, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Dr.Saravanan, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Dr.Ramesh Babu, Department of Biotechnology, Adhiyamaan College of Engineering (Autonomus), Hosur, India
Dr.Ragunathan, Centre for Bioscience and Nanoscience Research, Coimbatore, India

Owing to the heterocyclic structure of nicotine and its alkaloids, it is easily soluble in water, acts as a serious water pollutant. Various methods are available to detoxify the toxic and hazardous substances in a polluted environment.

Both physical and chemical methods are very expensive and ineffective in waste disposal management. Biological methods are efficient as well as cost-effective and therefore, play an important role in the clean-up of toxic and hazardous wastes in the contaminated environment.

Specific group of soil bacteria have the ability to degrade tobacco alkaloid wastes [1]. These nicotinophilic bacteria could use nicotine as a sole carbon, nitrogen and energy source for their growth. Pseudomonad bacteria such as *Pseudomonas sp., P. convexa* and *P. Putida* [11] have been identified as nicotinophilic bacteria.

Recently, Pseudomonas sp., another nicotine-degrading bacterium has received increasing attention due to its potential role in tobacco and waste treatment [8] [10] [11]. 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 context, the efficiency of potential nicotine-degrading bacteria *Pseudomonas aeruginosa sp.* were investigated.

II. METHODS

A. Extraction of Nicotine

Nicotine can be extracted from tobacco powder obtained from cigarette using methylene chloride. 50g of tobacco powder was mixed with a solution of concentrated ammonium hydroxide (0.9g/ml). 4.5g/ml had been diluted with 13 ml of sterile distilled water and the mixture was incubated for 30 minutes at room temperature on shaking condition. After incubation 125 ml of methylene chloride solution was added and the suspension was allowed to stand for an additional 30 minutes without agitation. Then it was filtered through Wattmaan filter paper and tobacco powder was then pressed [7].

B. Isolation of bacteria

Nicotine-degrading bacteria was isolated from the soil samples collected from Coimbatore which was already mixed with tobacco powder obtained from cigarettes were serial diluted to obtain 10^{-2}, and 10^{-3} planted on Luria-Bertani medium and incubated at 30° C for 2 days. An inorganic salt medium (ISM) was used for screening nicotine-degrading bacteria. Filter-sterilized nicotine was added to the medium and candidate bacterium was cultivated in ISM for 2 days at 30° C on a shaker maintained at 220 rpm (Fig.1). The ISM without nicotine was used as the control [3].

Fig.1. Culture of Pseudomonas and Bacillus

C. Identification of bacteria

The isolates were identified by gram staining and biochemical tests viz. Methyl red test, Indole production test, Urease test, Catalase test, Citrate utilization test, Nitrate reduction test, Oxidase test, Voges Proskauer (VP) test.

D. DNA isolation

Prepare LB broth of about 10ml. The organism isolated from the soil was cultured on the LB broth. The culture was maintained at 30° C for about 2 days. The pellet was collected by centrifuging at 6000rpm for about 5 minutes. 700 µl of saline EDTA (TE buffer) and 20 µl of lysozyme were added. The mixture was incubated at 37° C for about 30 minutes. Then it was mixed by inverting the eppendorf tube. To this 150 µl of 10 % SDS was added. Again it was incubated in water bath at 65°C for about 15 minutes. To this Phenol, Chloroform and Isooxyl alcohol in the ratio 25:24:1 were added respectively. Then it was centrifuged at 12000 rpm for about 10 minutes. To the aqueous layer about 0.2 volume of sodium acetate and 5 volume of iso propanol were added. Then it was again centrifuged at 12000 rpm for 10 minutes. To the pellet 700 µlof 100% ethanol was added and centrifuged at 6000rpm for 6minutes. Again to the pellet 500 µl of 70 % ethanol was added and centrifuged at 6000 rpm for 5 minutes. To the pellet, 50 µl of 1X TE buffer was added. Thus obtained pellet was run in a 0.8 % Agarose gel.

E. **16s** RNA Amplification

Genomic DNA was extracted by using standard method. Amplification of 16S rRNA gene was carried out using DNA of bacteria as template with forward primer (5'-CGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GACGGGCGGTGTGTACAA-3'). PCR reaction were carried out by adding 2µl of Template DNA, 8µl of PCR mater mix, 2µl of primer, 5µl of PCR buffer, 5µl of nuclease free water.

F. Biodegradation study

Thin Layer Chromatography

The Thin layer Chromatographic plates were taken. They were used for control, sample1 and sample2. The samples were withdrawn and centrifuged at 6000 rpm for 5 minutes. The clear supernatant was used to measure degradation. The plates are allowed to run in a solution of methylene: chloroform: acetic acid in the ratio of 5:2:1 for about 5 minutes. Then the plates were sprayed with 1 % Ninhydrine solution. The plates were viewed under the UV-Transilluminator. It is showed in Fig.2.

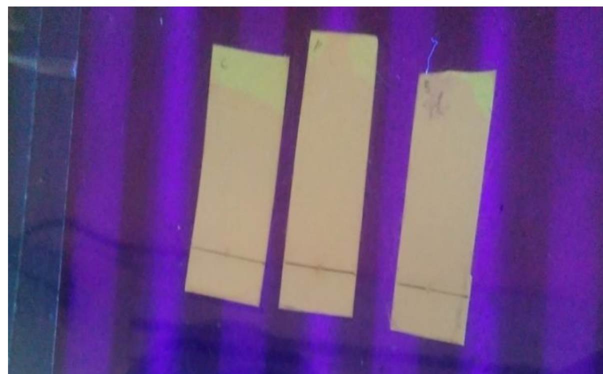

Fig.2. Thin layer chromatographic plates showing the degradation of the control and sample1 and 2

Colorimetric assay

Isolated test organisms were inoculated onto 100 ml of sterile nicotine broth and incubated for 37° C for 24 hours. After incubation 3 ml was dispensed into clean cuvettes for the observation of i.e.,change in the intensity of colour of the inoculated broth at 500nm using UV-Visible spectrometer (ELICO SC 159) and this procedure was repeated for 2 days. The absorbances were obtained at 500 nm.

Bruker

The FTIR used here is BRUKER .The sample was obtained by filtering the culture using Wattmaan filter paper and then air dried. Then the samples were sent for analysing the degradation character.

G. Phylogenetic Analysis

The partial 16s RNA sequences were submitted to the GenBank database with the accession number. This sequence was compared to know the sequence found in the GenBank database using Blast (http://www.ncbi.nlm.nih.gov/BLAST). The Distance tree method was used for phylogenetic analysis. [3]

RESULTS AND DISCUSSION

a) Screening of bacteria

The nicotinophilic bacteria that degrades nicotine in soil were isolated, out of which one strain showed highest zone of clearance around its colonies. That strain was selected for analysing the degradation.

b) Thin layer chromatography

In thin layer chromatography it has been found that the sample 1 has moved higher as compared to the sample 2. This shows that degradation was rapid and effective in sample1.

c) Biochemical tests

The biochemical tests confirmed that the isolates are *Pseudomonas aeruginosa sp.* (Table 1)

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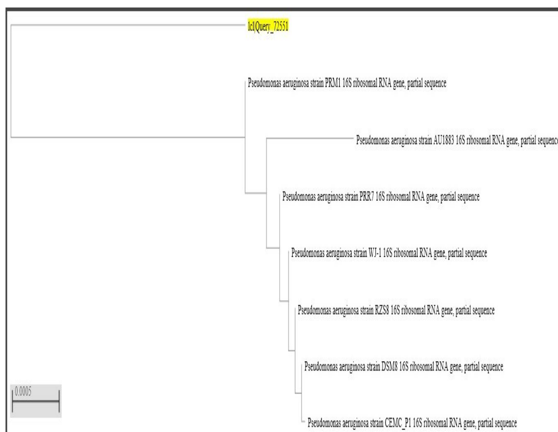

fig.3. Phylogenetic relationship of 16s RNA of *Pseudomonas aeruginosa sp.*, with sequence from gene bank

Table 1: Biochemical test results

Test	Result
Gram staining	Negative
Indole production test	Negative
Methyl Red test	Negative
Voges Proskauer test	Negative
Citrate utilization test	Positive
Urease test	Negative
Nitrate reduction test	Positive
Catalase test	Positive
Oxidase test	Positive

Table 2: Degradation of nicotine by strain of *Pseudomonas aeruginosa*

	Absorbance at 500nm	
	Day 1	**Day 2**
Control	0.482	0.077
Sample 1	1.107	0.292
Sample 2	1.021	1.777

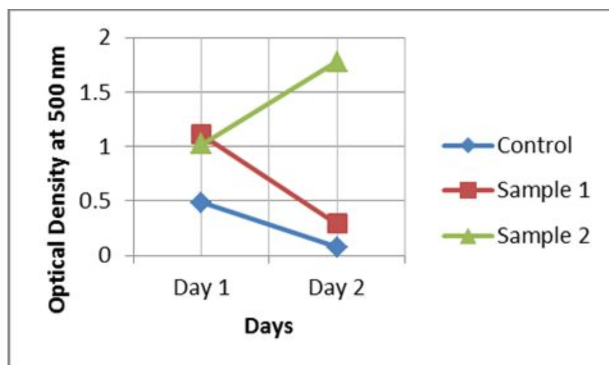

Fig.4. Optical Density obtained at 500 nm between an interval of 24 hrs

d) Colorimetric Assay

The sterile nicotine broth was inoculated with *Pseudomonas aeruginosa* sp. and *Bacillus* sp. The absorbances were note using UV-Visible spectrophotometer at a time interval of 24 hours. The absorbance obtained at 500 nm using UV-Visible spectrophotometer was given in Table 2. Based on the absorbance value, sample 1 showed decrease in value after a time interval of 24 hours. This showed that *Pseudomonas aeruginosa* sp. is effective for nicotine degradation.

e) FTIR

The FTIR spectrum of the control (Fig.5) nicotine showed vibration peak at 3105.61cm^{-1}attributed due to C-H stretch of alkenyl and stretching peak at 2546.38cm^{-1}, 3245.96cm^{-1}was due to O-H group. The FTIR spectrum of Treated A (Fig.6) showed stretching peak for the O-H group at 3345.67cm^{-1}. The FTIR spectrum of Treated B showed stretching peak for the O-H group at 3203.44cm^{-1}. The Treated A showed increase in stretching wave number by 99.71cm^{-1} as compared to control and the Treated B (Fig.7) showed decrease in stretching wave number. The increase in –OH stretching wave number may due to degradation of nicotine molecules. Therefore Treated A showed effective degradation.

f) Phylogenetic Analysis

The obtained strain was identified as *Pseudomonas aeruginosa* according to morphology and biochemical test. Multiple alignments revealed that the 16s rRNA sequence of *Pseudomonas aeruginosa sp.* was closely related to that of *Pseudomonas aeruginosa* (PRM1, 99% of similarity), *Pseudomonas aeruginosa* (PRR7, 99% of similarity), *Pseudomonas aeruginosa* (WJ-1, 99% of similarity), *Pseudomonas aeruginosa* (RZS9, 99% of similarity), *Pseudomonas aeruginosa* (DSM8, 99% of similarity) and *Pseudomonas aeruginosa* (CEMC_P1, 99% of similarity). Phylogenetic analysis using the 16s rRNA gene conform its affinity to genus *Pseudomonas.*

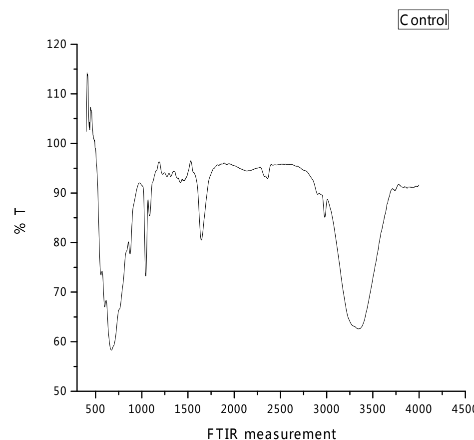

Fig.5. FTIR spectrum of Control

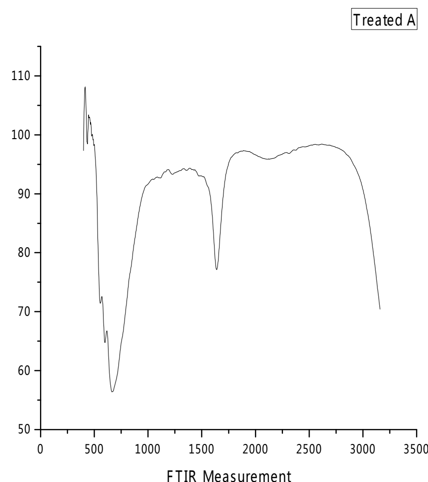

Fig.6. FTIR spectrum of Treated A

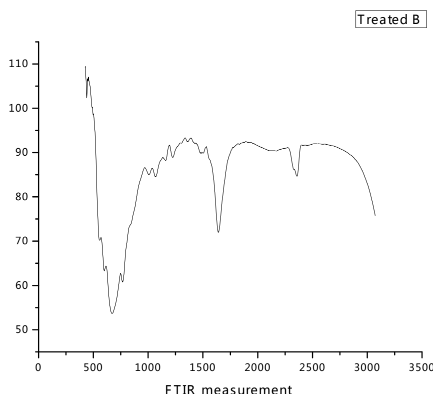

Fig.7. FTIR spectrum of Treated B

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