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Research Article

ISOLATION AND IDENTIFICATION OF NICOTINE UTILIZING BACTERIAL SPECIES FROM TOBACCO LEAVES

Ithisree Gaekwad * and Vinchurkar A.S.

Department of Zoology, D.B.F. Dayanand College of Arts and Science, Solapur, Maharashtra, India

*Corresponding Author Email: ithisreeg@gmail.com

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ABSTRACT

Nicotine degrading bacteria was isolated from crude unprocessed tobacco from local vendor of Solapur city. Using medium containing whole tobacco extract as the sole carbon and nitrogen source. One purified isolate with a high capacity for tobacco utilization was characterized for 16s rRNA and identified as *Stenotrophomonas maltophilia* by phylogenetic analysis and on morphological and biochemical features. The optimal culture condition of strain *Stenotrophomonas maltophilia* for tobacco utilization is pH 6.5 and 30–34 °C and 0.5 g/ L−4.5g/L of nicotine concentration in the inorganic salt medium, which is basically consistent with utilization of nicotine by the isolate. The growth and nicotine degradation of the isolate suggested that strain *Stenotrophomonas maltophilia* could utilize nicotine from the extract as sole source of carbon, nitrogen and energy. The thermal denaturation test indicated that the G + C content of the DNA was 55.11 mol%.

Keywords*:* Nicotine; Degradation; Bioremediation; *Stenotrophomonas maltophilia.*

INTRODUCTION

Nicotine is the principal alkaloid in leaves of most *Nicotiana* species[1]. Nicotine is a xenobiotic compound and not easily removed from the environment. A large amount of nicotine comes from the cigarette processing industries which are involved in processing of tobacco leaves. Compared with physical and chemical methods, biological methods using microbes are preferable because they are more efficient and less expensive [2, 3]. Recently, *Pseudomonas spp.*, a nicotine-degrading bacteria have received increasing attention due to their potential roles in tobacco and waste treatment [4-6]. Nicotine degradation by microorganisms have been studied almost 50 years ago by work performed with *Arthrobacter oxidans* (reclassified as *Arthrobacter nicotinovorans*)[7]. Another nicotine degrading bacterium,strain HF-1, was isolated from tobacco waste contaminated soil[8]. In this paper, potential tobacco utilizing bacteria was investigated from crude unprocessed tobacco. The isolated bacterium *Stenotrophomonas maltophilia* was then used to test its efficiency for its potential roles in degrading nicotine from tobacco extracts by using different concentration. *Stenotrophomonas maltophilia* is an aerobic, non fermentative, Gram-negative bacterium. It is an uncommon bacterium and human infection is difficult to treat [9]. Initially classified as *Pseudomonas maltophilia*, *S. maltophilia* was also grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in 1993[10]. The name signifies "a unit feeding on few substrates," based on the Greek roots stenos (narrow), trophos (one who feeds), and monas (a unit). Maltophilia means "affinity for malt," based on the Greek roots maltum (malt) and philia (affinity). *S. maltophilia* is ubiquitous in aqueous environments, soil and plants; it has also been used in biotechnology applications [11]. In immuno compromised patients, *S. maltophilia* can lead to nosocomial infections. *S. maltophilia* is the only species of *Stenotrophomonas* known to infect humans[12] whereas its closest genetic relatives are plant pathogens[13,14].

MATERIALS & METHODS

Isolation of nicotine degrading bacteria

The isolate of bacteria are grown in two media inorganic salt medium(ISM) & Luria Bertani medium. 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 (Crude tobacco extract, 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 per liter). Filter-sterilized tobacco extract 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 the extract used as the control.

Phenotypic, physiological and biochemical characterization of tobacco utilizing bacteria

The morphology of isolate was studied after the bacterium has grown for 24 hrs on Luria-Bertani (LB) medium [15]. The cell morphology was observed with a compound microscope. Conventional physiological and biochemical characteristic assays were determined by the procedures of Bergey's Manual of Determinative Bacteriology [16].

Genetic Identification

Genetic identification of isolated bacteria was performed according to nucleotide sequence of 16S ribosomal RNA (rRNA).

Bacterial 16S rRNA was amplified with universal primer 27F/1492R primers. PCR was performed in a reaction mixture with 1 µl of template DNA in 20 µl of PCR reaction solution and then performed for 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. included a positive control (*E. coli* genomic DNA) and a negative control in the PCR. The purified PCR product was sequenced by using 2 primers as described (Primer Name File). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Activity of tobacco utilizing bacteria

One pure isolate was obtained from the tobacco leaves. This isolate was analyzed for its tobacco utilizing property. Activities using a medium (ISM) containing crude tobacco extract as the sole carbon and nitrogen source was analyzed. The purified isolate was tested for its optimum activity and was analyzed for showing the highest tobacco utilizing activity. Comparison of nicotine-degrading activities of isolates to different tobacco extracts.

RESULTS

A total of 4 bacterial strains from crude unprocessed tobacco were grown on LB agar media and inorganic salt medium plates with nicotine as the sole carbon source. The isolate showed gram negative short rods, endospore forming bacterium. Colonies of isolate on Luria agars were white and circular entire cells were rod shaped and were motile with flagellum. Other physiological biochemical tests performed, the isolate was catalase positive, methyl red negative, VP negative, nitrate reductase negative, citrate negative and indole negative respectively.

The G+C content of strain was 55.1% as determined by the thermal denaturation procedure. The sequence similarity was observed of 99% homology as compared with 10 sequences as mentioned in the table of BLAST N report based on 16s rRNA sequence analysis and on morphological, physiological and biochemical characteristics. The strain was identified as *Stenotrophomonas Spp*. The bacterium was grown on different concentrations of Tobacco extract from 0.5mg/ml -5mg/ml. Maximum growth of the nicotine degrading bacteria was observed at4.0mg/ml- 4.5mg/ml conc. of the tobacco extract at 18°C and 37°C there was no growth at 5°C. The isolated organism could also degrade a dye Reactive red at 5mg/L conc. complete discoloration of the dye was achieved within 2 hours.

Table 1 Details of PCR Primer

	Primer Name	Type	Type2	Sequence (5 to 3)
1	518F	Universal	Forward	CCAgCAgCCgCggTAATACg
2	800R	Universal	Reverse	TACCAgggTATCTAATCC
3	27F	Universal	Forward	AgAgTTTgATCMTGGCTCAg
4	1492R	Universal	Reverse	TACggYTACCTTgTTACgACTT

Note:
Primer 1 & 2 for Sequencing Reference.
Primer 3 & 4 for PCR Amplification.

Table 2: Analysis report of GC content
sample1_contig_1 Report ::::::::::::::::::::::::::::::::

Analysis Report

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
sample1_contig_1	**1475**	**1393**	**1393**	**55.11864406779661**
sample1_R	748	735	733	54.81283422459893
sample1_F	916	912	912	55.24017467248908

Table 2: BLAST N Report
- Query name : sample1_contig_1
- Query length : 1475

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct. (%)	Strand
2	1474	Stenotrophomonas sp. U18 16S ribosomal RNA gene, complete sequence	JN000347.1	1513	1	1471	2691	1457	0.0	1469	1474	99	Plus/Plus
2	1474	Stenotrophomonas maltophilia strain pp5c 16S ribosomal RNA gene, partial sequence	GQ360071.1	1538	1	1471	2691	1457	0.0	1469	1474	99	Plus/Plus
15	1474	Stenotrophomonas maltophilia strain Dh 16S ribosomal RNA gene, partial sequence	HQ200414.1	1536	12	1470	2689	1456	0.0	1459	1460	99	Plus/Plus
7	1474	Stenotrophomonas maltophilia 16S rRNA gene, isolate 41	FN645727.1	1526	14	1480	2687	1455	0.0	1465	1469	99	Plus/Plus
2	1474	Uncultured marine bacterium clone Tc-42 16S ribosomal RNA gene, partial sequence	JF925027.1	1506	1	1471	2686	1454	0.0	1468	1474	99	Plus/Plus
2	1474	Stenotrophomonas maltophilia strain YHYJ-1 16S ribosomal	FJ765513.1	1509	1	1471	2686	1454	0.0	1468	1474	99	Plus/Plus

		RNA gene, partial sequence											
2	1474	Stenotrophomonas maltophilia strain 1.22 16S ribosomal RNA gene, partial sequence	EF426435.1	1503	1	1471	2686	1454	0.0	1468	1474	99	Plus/Plus
2	1474	Stenotrophomonas maltophilia 16S rRNA gene, clone M27	HE646776.1	1611	14	1484	2680	1451	0.0	1467	1474	99	Plus/Plus
15	1474	Stenotrophomonas maltophilia strain PSSB7 16S ribosomal RNA gene, partial sequence	FJ707375.1	1536	15	1473	2680	1451	0.0	1457	1460	99	Plus/Plus
2	1474	Uncultured bacterium partial 16S rRNA gene, clone SMQ138	AM930343.1	1503	1	1471	2680	1451	0.0	1467	1474	99	Plus/Plus

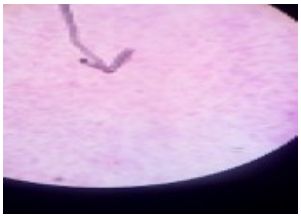

Figure 1: Microscopic observation *Stenotrophomonas maltophilia*

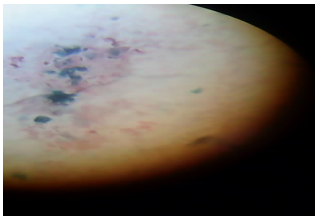

Figure 2: Endospore Staining *Stenotrophomonas maltophilia*

Figure 3: Tobacco utilization *Stenotrophomonas maltophilia*

Figure 4: Growth of *Stenotrophomonas maltophilia* at 5^0C

Figure 5: Growth of *Stenotrophomonas maltophilia* at 18^0C

Figure 6: Growth of *Stenotrophomonas maltophilia* 37^0C

DISCUSSION

In the present study, the strain capable of nicotine degradation was isolated and identified as *Stenotrophomonas maltophilia* according to the morphological and physiochemical characteristics and sequence analysis of 16S rRNA. Although other Pseudomonas strains have been previously reported to decompose nicotine, the strain *Stenotrophomonas maltophilia* still exhibited outstanding nature for nicotine utilization. It could endure up to 4500 mg/ L) nicotine in liquid culture, which is lower than that of P. sp. S16 (6000 mg/L) [5], but significantly higher than that observed from P. sp.HF-1 (1600 mg/ L) [8]. And degradation was achieved within 24 h at 1000 mg/ L) nicotine. By contrast, similar degradation efficiency achieved by *Achromobacter nicotinophagum* [17] or O. intermedium DN2 [18] required at least 36 h, few bacteria in genera *Alcaligenes*, *Pseudoxanthomonas*, *Pseudoxanthomonas* and *Delftia* were found in the soils. Moreover, *Arthrobacter* [19], *Ochrobactrum* [20] and *Alcaligenes* [21] have been reported to degrade nicotine, while *Pseudoxanthomonas*, *Sphingobacterium* and *Delftia* are reported to utilize nicotine for the first time. The characterization of genes and gene products involved in nicotine degradation in Pseudomonas can lead to a full understanding of this catabolic activity. The identification of genes involved in the nicotine degradation pathway increases the possibility to clone related genes from other organisms, including non cultivable environmental samples. 6-Hydroxy-3-succinoylpyridine (HSP) and 2, 5-dihydroxy-pyridine (DHP) in nicotine degradation are useful precursors for chemical synthesis [3, 9]. Knowledge of nicotine degradation genes such as hpo and hspB15 can be adapted in detoxification programs for tobacco wastes and synthesis of useful products of pharmaceutical importance. These genes may also be useful for the modification or degradation of alkaloid substances and other heterocyclic aromatic compounds[22].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 [4]. In the present study, we cloned two novel genes involved in the initial steps of nicotine degradation by strain HZN6, which differ from any other reported mechanisms [23]. First, strain CS3 showed its excellent nicotine resistance and degradation ability. It could endure up to 4000 mg /L) nicotine in liquid culture[24]. Nicotine is very harmful; therefore, finding methods to detoxify it is imperative. Microorganisms growing on tobacco leaves have adapted to nicotine as a growth substrate and developed biochemical strategies to degrade nicotine. Thus far, most studies on nicotine degradation have focused on bacteria and only a few have examined fungi. In this paper, an efficient nicotine-degrading fungus A. oryzae 112822 was isolated from tobacco leaves, and resting cells were used to investigate the pathway for nicotine degradation[25]. It is reported that some bacteria in genus Agrobacterium could degrade xenobiotics such as atrazine [26], quinoline- 4-carboxylic acid, 4-aminobenzenesulfonate, phenanthrene [27] and phenol [28]. But there is no published information in the literature about the genus capable of degrading nicotine until now. Therefore, the present work is the first report that Agrobacterium sp. has the ability to degrade nicotine [29].This

study is the first investigation of the optimization of a medium for enhancing the rate of nicotine degradation by applying a CCD experiment[17].

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