ejpmr, 2016,3(4), 315-321

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

MICROBIAL REDUCTION OF NICOTINE AND EFFECT ON ORAL FLORA

G. Krishna Kumari* and M. Thangavel

P.G and Research Department of Microbiology, Sree Narayana Guru College, Coimbatore, TN, India.

***Correspondence for Author: G. Krishna Kumari**

P.G and Research Department of Microbiology, Sree Narayana Guru College, Coimbatore, TN, India.

Article Received on 04/02/2016 **Article Revised on 25/02/2016** **Article Accepted on 16/03/2016**

ABSTRACT

Tobacco is a product prepared from the leaves of tobacco plants. Alkaloid Nicotine is a major addictive drug which is responsible for disease, disability and death. Nicotine utilizing organisms were isolated from various soil samples and the organisms were identified as *Pseudomonas sp* and *Bacillus sp.* To study the effect of nicotine on oral flora, organisms were isolated from oral region and those organisms identified as *Staphylococcus sp, Streptococcus sp, Pseudomonas sp, Klebsiellasp and Proteus sp.Pseudomonas sp,* was found to effectively degrading nicotine(0.31 OD at 48 hrs) than *Bacillus sp*(0.25 OD at 48hrs). Isolates both *Pseudomonas* and *Bacillus sp* showed increase in the pH from 7 to 9 at 24hrs. Dissolved Oxygen of first day of incubation was obtained as 5.65 and at 3rd day of incubation was2.54 and Biological Oxygen Demand was calculated as 3.1.Effect of nicotine on normal flora was studied and *Streptococcus* showed maximum effect(25mm zone at 100microliters) and *Staphylococcus*(14mm zone) against crude nicotine extract and *Pseudomonas* exhibits resistance against nicotine.

KEYWORDS: Tobacco, Nicotine, Oral Flora.

INTRODUCTION

Nicotine is the alkaloid component of tobacco and constitutes about 3% of the dry mass of tobacco leaves-Nicotinatabacum. Nicotine, a major alkaloid synthesized L-isomer in tobacco plant, plays a critical role in smoking addiction. Current smoking patterns persists, tobacco will kill around 100 million in next 50 years. Nicotine accounts for at least 30%of cancer death and 87% of lung cancer death (ShuNing Wang *et al*., 2007). By mediating cholinergic receptors in the central nervous system nicotine results in addiction (Amstrong*et al*., 1998). Nicotine is a pyridine alkaloid (ArunBahl*et al*., 1992) one or more nitrogen heterocyclic rings are present in their structure. The nicotine is oily liquid and Colorless at boiling point 245.5^{o}C. It has a tobacco like smell and burning alkali taste, its soluble in water as well as organic solvents. On exposure to air it rapidly turns brown due to oxidation(K.S. Tewari*et al*., 1994). Nicotine is a deadly poison, even two or three drops internal ingestion may leads to death with in few minutes. Nicotine stimulates central nervous system and large doses causes depression, respiratory paralysis and death. Nicotine is ten times more potent than heroin in its addictive properties. They are carcinogenic compounds with poly nuclear aromatic hydrocarbons, tar, vinyl chlorides and co carcinogenic such a catechol phenol cresol. It also contains cilio toxin and pulmonary irritation along with radioactive compound (Stephen Mulcahi., 2006). Nicotine is also a significant toxic waste product in tobacco production(Novotny *et al*., 1999).

Methods of Nicotine Extraction

A non recyclable powdery, nicotine containing waste is formed during tobacco production which has an average nicotine content of 18g per kg dry weight(Civilini*et al*., 1997). Chemical physical treatment have been extensively used in denicotinisaction that is the removal of nicotine from tobacco industry wastes(Ireland *et al*., 1980, Lenkey 1989). Biological treatment with microbes also have potential for nicotine degradation(Civilini*et al*., 1997). Bacterial community residing in the tobacco rhizosphere has presumably adapted to use nicotine as a growth substrate and developed biochemical strategies decompose this organic heterocyclic compound. *Arthrobacter* nicotine (Hochstein *et al*., 1958). *Arthrobacterglobiformis* (Maeda and kiaski, 1981), *Cellulomonassp*(Gravely *et al*., 1978) and *Ochrobactrumintermedium*(Yuan *et al*., 2005) can degrade nicotine Arthrobactersp were reported to extract carbon, nitrogen and energy the breakdown of nicotine(Schenk *et al*., 1998). *Ochrobactrumintermedium* DN2 was used to degrade nicotine in tobacco waste extracts. It was found that nicotine degradation by the strain DN2 efficiently at 30^{0}C – 37^{0}C and pH 7(Y.J. Yuan *et al*., 2006).

Various methods of nicotine extraction by B.S. Bahl*et al*., 1992 explained that dry leaves are finely powdered and extracted with dilute acid solution. To the acid extract is added sodium hydroxide solution and steam distilled. The oil layer so obtained is crude nicotine which is purified by fractional distillation in vacuum.

Tobacco leaves of high nicotine content are crushed and extracted with cold water. The hydrocarbons present in extract are removed by acidifying the solution and extracting with ether. The residual solution then made alkaline and the free nicotine is extracted with ether(K.S. Tewari*et al*., 1994). The use of ultra-sonication for the release of nicotine into the water has been shown to expedite the extraction process by rupturing nicotine cells with in the tobacco matrix, green uncured tobacco can be used. Tobacco was cut, water and lime were added and juice expressed using sugarcane technology. This almost contains 0.1% to 1% nicotine. These extracts are then processed to concentrate the nicotine (N.Rustica).

Organic solvent extraction can be used it with treating with ammonia then extracted with other solvents such as trichloroethylene, benzene, gasoline, kerosene, diethyl ether or Freon. In case of organic solvents immiscible with water, the extracts can be re extracted with aqueous sulphuric acid solution until the desired concentration is reached (Tabuchi.T, 1955). Dry distillation technique can also be successfully practiced on the waste tobacco; preliminary investigations into the choice of solvent included a isooctane or heptane system. This is efficient in yielding 64% reduction in leaf nicotine. The methyl ethyl ketone-brine extraction system, extracted with cold acidic brine which helps to obtain the 85% reduction in leaf nicotine. The brine extract was made alkaline with sodium hydroxide and warmed; distinct upper phase was obtained (ShuNing Wang *et al*., 2007). It was shown that 20%nicotine giving a recovery of 82%from the leaf. In nicotine extraction from leaf by Freon 11, tobacco chopped and mixed with water after 30 mins. Freon11 was added again and allowed to stand for 30 minutes at 20^0C, then filtered through a cheese cloth. The Freon extracted with citric acid. It was felt that the nicotine could be extracted if the tobacco were treated with alkali strong enough to release bound nicotine (Kenneth Todar, 2007). The ammonium hydroxide was added to the chopped tobacco leaves and after 30 minutes methylene chloride was added. This was filtered through cheese cloth. Methylene chloride helps in excellent removal of nicotine (J.R. Reid, 1997). Microbial destruction of nicotine leads to increase in nitrate concentration in soil flora due to conversion of an alkaloid to nitrate (Batham, 1927). Oxidation of nicotine by crude extracts which has been prepared from several independently grown batches of usually oxidized nicotine at slow but definite rate (L.I. Hochestein*et al*., 1958).

Nicotine Degradation

The isolation of nicotine degrading bacteria, isolated using nicotine agar medium were inoculated in different concentration of nicotine and observed for the degradation of nicotine with HPLC method (Hailei Wei *et al*., 2008). Cells harvested was centrifuged and washed with sodium phosphate buffer. The path way of nicotine degradation was proposed to be from nicotine to 2,5 dihydroxypyridine through the intermediates N-methylmyosmine, 2-hydroxy nicotine, pseudo oxy nicotine, 3 phyridinebutanol, c-oxo, succinopydirine and 6-hydroxy-3-succinoyl pyridine, Methylmyosmine, 2,5 dihydroxypyridine and succinic acid were detected during the nicotine degradation and 1-Butanone, 4-Hydroxy-1-(3-pyridinyl) was found to be a novel product of nicotine degradation (ShuNing Wang *et al*., 2007, N.Chaudhary*et al*., 2007, Sponza, 2002., Munari, 1986., Saunders and Blume, 1981). Nicotine degrading bacterium HF1, isolated from tobacco contaminated soil, which can use nicotine as a sole source of carbon, nitrogen and energy. Pigments were also observed during degradation and helps in bioremediation of the environments (Ruan A *et al*., 2006). *Pseudomonas sp* and *Arthrobacter*carries the ability to breakdown nicotine into carbon, nitrogen and energy. This breakdown starts at pyridine ring and goes for demethylation results in formation of 6-Hydroxy-3-succinoylpyridine (RoderichBrandsch, 2005).

Microbial Oral Flora

The presences of nutrients, epithelial debris and secretions make the mouth a favorable habitat for a great variety of bacteria. Oral bacteria includes*Streptococci, Lactobacillus, Staphylococcus* and *Corynebacteria*with a great number of anaerobes especially bacteroids. The mouth presents a succession of different ecological situations with age. The host provides nutrients and habitat to microbes in turn oral flora synthesis vitamins and immunity by inducing low levels off circulating and secretory antibodies that may cross react with pathogens. Finally, the oral bacteria exert microbial antagonism against non indigenous species by production of inhibitory substances such as fatty acids, peroxides and bacteriocins(Knneth Todar, 2007).

Vitamins deficiencies, especially vitamin K and B12, increased susceptibility to infectious diseases, poorly developed immune system and lack of natural antibody or natural immunity to infectious diseases may leads to lack of normal flora in some persons. The oral cavity may be considered an ideal microbial incubator. It possess temperature of 35^0C-36^0C and has an abundance of moisture, an excellent supply of various types of food and differences in oxygen tension (Frankenburg W.G *et al*., 1955).

Gram positive cocci*Streptococcus* most commonly found in the oral cavities. *S mutants* involved in plaque formation and initiation of dental carries. From throat these organisms may spread to tissues leading to otitis media, mastoditis and streptococcal pneumonia (www.action bioscience.org).*Staphylococcus aureus* is found to be one of the pyogenic agent. The toxin produced will cause ritters syndrome, toxic epidermal necrolysis, localized bullous impetigo and may result in toxic shock syndrome(R. Ananthanarayanan*et al*., 1978). *Lactobacillus* in oral cavity may contribute to acid formation that leads to dental carries(Kenneth Todar, 2007). *Klebsiella* occurs as commensal and as well as

pathogens and cause pyogenic infections, speticaemia, necrosis, abscess formation. Some strains of *Pseudomonas* are pathogenic and cause various diseases in man. The growth of these bacteria occurs at a wide range of temperature from 15 to 42^{0}C. Blue pus, suppurative otitis etc was supposed to occur. *Proteus* also found in the oral microflora(R. Ananthanarayanan*et al*., 1978). Two types of *Bacteriods* like pigmented and non pigmented found in the mouth(Kaiser *et al*., 1996).

Effect of Nicotine on oral flora

Nicotine known to have effects on the oral cavity of smokers, especially on periodontal tissue(Karina Cogo*et al*., 2008). Average dose of nicotine delivered by smoking one cigarette is about 0.5mg fatal dose of nicotine is 60mg and causes respiratory muscle paralysis. In respiratory system local irritation, impairedciliary motion which impacts the auto cleaning mechanism of the lungs(Robert Shubinski., 2006). Nicotine content of tobacco may cause the imbalance of oral flora and may leads to various diseases. Based on the above background information the present study was planned for the extraction and biological degradation of nicotine to control its harmful effects on human health.

MATERIALS AND METHODS

Isolation of Nicotine degrading organisms

Samples were collected from desired regions such as oral and various soils. Rhizosphere region of the tobacco plants sandy soils from different areas other than cultivation regions. Oral samples were collected using sterile swabs. 1g of soil sample were suspended in 100ml of sterile distilled water and 0.1 ml of soil suspension was spread over the surface of nicotine medium(NIM) plates. Then the plates were incubated at 27^{0}C for 72 hours to obtain well isolated colonies which are capable of utilizing nicotine.

Extraction of Nicotine: Nicotine can be extracted from tobacco plants using methylene chloride. 50g of coarsely chopped tobacco leaves were mixed with a solution of the concentrated ammonium hydroxide(0.9g/ml). 4.5 g/ml had been diluted with13ml of sterile distilled water and the mixture incubated for 30minutes at room temperature at shaking condition. After incubation 125ml of methylene chloride solution added and the suspension was allowed to stand for an additional 30minutes without agitation. Then it was filtered through cheese cloth and tobacco was then pressed. The filtrate consists of nicotine and was used in NIM medium preparation and also to detect the effect of nicotine in the oral flora.

NIM media(pH-7.0)
Na2HPO4-6g
KH4PO4-3g
NH2Cl-1g
Nacl-0.5g
Mgso4-0.12gCaCl2-0.5g
Agar-15g
Distilled water-1000ml.

Isolation and Identification of Oral flora: Sample collected using sterile swabs were inoculated on various selective media such as MacConkey Agar, Blood Agar, EMB Agar, Pseudomonas Agar and Nutrient Agar. And then incubated at37^{0}C for 24h and after incubation observed for bacterial growth. The isolated organisms were subjected to cultural characteristics and biochemical observation.

Charecterization of Nicotine Degradation

a. Colorimetric assay: Isolated test organisms were inoculated onto 100ml of sterile nicotine broth and incubated for 37^{0}C for 24hrous. After incubation 3ml was dispensed into clean cuvetts for the observation of change in the intensity of colour of the inoculated broth at 620nm and this procedure repeated on a constant interval of 3hours.

Nicotine broth(pH-7.0)
Extracted nicotine- 1ml
Distilled water-100ml.

b. Effect on pH

Isolated test organisms were inoculated onto 100ml of sterile nicotine broth, prepared with the pH of 7.0 and incubated for 37^{0}C for 24hrous. pH of the media was checked at a regular interval of 3hours.

c. Biological Oxygen Demand: Sterile nicotine broth inoculated with sample was kept for 24hours incubation at 37^{0}C. The broth was collected without bubbles in 250ml BOD bottle. 2ml of manganese sulphate and alkaline iodide-azide solution added and mixed well. Brown coloured precipitate formed was then allowed to settle down. To that 2ml of concentrated sulfuric acid added to dissolve the brown precipitate. 25ml of the treated sample was then drawn and added with 2 drops of starch solution and titrated against thiosulphate solution till the color changes from pale blue to pale yellow or straw color. The procedure performed again after 3 days of incubation. Biological oxygen demand can be calculating dissolved level of oxygen(DO) by using the formula,

BOD in mg/ml = D1-D2
D1 – Initial DO in the sample
D2 – DO after 3 days of incubation.
Estimation of DO:

$$\text{DO mg/ml} = \frac{\text{CD} \times M \times E \times 1000 \times 0.698 \times Vt}{Vs}$$

CD (correlation displacement of sample when reagents are added).

$$\text{CD} - \frac{\text{Volume of bottle}}{\text{Volume of bottle} - \text{Volume of reagent.}}$$

M – molarity of thiosulphate

E – equivalent weight of oxygen
1000 – to express in litres

0.698 – to covert ppm to mg of oxygen
Vt– titre value

Vs– volume of sample used for titrant

Reagents used
Manganoussulphate solution
Dissolved 364g of MnSO4 in distilled water.

Alkaline iodide – azide solution
700 g of KOH and 150g of KI in distilled water and mix then make up to 1L. then dissolve 10g of sodium –azide in 40ml of distilled water.

Sodium thiosulphate(0.025N)
Dissolve 6.205g sodium thiosulphate in distilled water. Sodium hydroxide 1 pellet added as preservative.

Starch indicator
1g of starch added in 200ml of distilled water.

Effect of Nicotine on normal flora
The effect of nicotine on normal flora was evaluated further by using Antibiotic sensitivity test- Kirby Bauer method. Well diffusion method performed in Muller-Hinton medium(pH-7.2-7.4) which is designed for rapid determination of the efficacy of a drug by measuring the zone of inhibition that result from diffusion of the agents into the medium surrounding the well. A sterile cotton swab was dipped into the overnight incubated broth culture and swab on the sterile MH agarsurface uniformly. The wells were then created using well cutter. Different dilution of nicotine was added to the wells created and then incubated at 37C for 24hours. After incubation the plates were observed for the presence of zone of inhibition.

RESULTS
Isolation and identification of Nicotine degrading organisms: Various soil samples were collected for isolating nicotine utilizing organisms using NIM media inoculated with test samples and incubated at 37C for 24hours and plates were observed for well isolated colonies. Sample collected from rhizosphere area showed 6morphologically different colonies and sample from sea shore area found that no growth on the media. The isolates obtained from various samples were identified using various biochemical tests and the isolates were identified *as Pseudomonas sp and Bacillus sp.*

Isolation and Identification of Oral flora: Oral samples were obtained using sterile swabs and swabbed on nutrient agar plates and incubated at 37C for 24hours. Well isolated colonies with morphologically different types were obtained and identified using various biochemical tests. The isolates were identified as *Staphylococcus, Streptococcus, Pseudomonas, Klebsiella and Proteus*.

Charecterization of Nicotine Degradation
a. Colorimetric assay
Sterile Nicotine broth was inoculated with test organisms which are identified as Psuedomonas and Bacillus sp. After incubation observed that the increase in the turbidity when compared to the control. Pseudomonas sp was found that to be more effectively degrading nicotine was found 0.19 OD at 24hours and increased to 0.28 at 30hours .maximumdegradtation was observed at 48th hour of 0.31 OD and subsequent incubation leads to decreases to 0.28 OD. In case of Bacillus sp 0.20at 24th hour and it becomes maximum to 0.25 and after decreased to 0.20 at 54th hour incubation.

b. Effect on pH
Isolated and identified test organisms were inoculated onto sterile Nicotine broth (pH-7.0)and incubated at 37C for 24hours to observe the change in the pH of the media. Pseudomonas sp showed an increase in pH to 9.0 with increase in the growth and after there was a decline to 8.0 in the pH at 24 hours. Bacillus p also leads to change in the pH of media to 9.0 from 7.0 at 24 hours of incubation. Then the pH changes to 8.0 and starts decline.

c. Biological Oxygen Demand
Test organisms inoculated in sterile NIM media and incubated at 37C for 24hours. After incubation the dissolved oxygen was calculated by using the formula

$$DO\ mg/ml = \frac{CD \times M \times E \times 1000 \times 0.698 \times Vt}{Vs}$$

Dissolved oxygen content was found to be 5.65. the incubation continued further and at 3rd of incubation the dissolved oxygen was again calculated in similar way as 2.54. The DO of the sample was then calculated as 3.1 by using the above mentioned formula. The biological oxygen demand was calculated by the formula and it is observed that the BOD was increased during incubation time BOD in mg/ml = D1-D2(D1 – Initial DO in the sample; D2 – DO after 3 days of incubation).

Biological oxygen demand indicates the organism are able to survive and utilize the nicotine there by the nicotine degradation occurs.

Effect of Nicotine on normal flora
Isolated and identified test organisms were swabbed on sterile MH agar surface and after wells are created by using well cutter. The crude nicotine extract added in different concentration of 50 and 100microliters with positive control of streptomycin and negative control of distilled water was added and incubated at 37C for 24hours. The results were observed for the presence of clear zone. *Streptococcus sp* produced 25mm at 100 microliters and 15 mm in 50 microliters. *Staphylococcussp* showed a zone of 14mm in 100 microliters and no zone in 50 microliters. *Proteus sp*showed 23mm in 100 microliters and16mm in 50 microliters. *Klebsiellasp* found no zone at 50 microliters and 3mm zone in 100 microliters. *Pseudomonas sp* was showed resistance against nicotine. They produced no zone off clearance in both 50 and 100 microliters concentration.

DISCUSSION

Nicotine utilizing organisms were isolated from various soil samples such as normal soil, sea shore and rhizosphere soil using sterile NIM agar medium. Non pigmented colonies were isolated from rhizosphere soil sample and the isolates were identified as Pseudomonas sp. Some nicotine degrading organisms were isolated from tobacco waste contaminated soil(Ruan A *et al*) using nicotine medium and both pigmented and non pigmented colonies were isolated from rhizosphere tobacco plant was identified as Pseudomonas sp and Arthrobactersp(Haileie*t al*., 2008).

Few organisms were isolated from tobacco or sewage waste water using MI agar medium. Sample from tobacco are efficient when subjected to nicotine degradation using MI agar medium and few number of isolates were obtained and identified as Bacillus sp(N. Chaudhaary*et al*., 2007). The isolated and identified organisms which are effectively degrading nicotine are Pseudomonas sp and Bacillus sp were subjected to calorimetricassay at 620nmand Pseudomonas was found to be more effective in nicotine degradation at 48th hour (0.31) than Bacillus on nicotine degradation at 30th hour(0.25) Bacillus salvei and Bacillus circulans were found to grow best at a temperature of 37C and at 50C respectively(N.Chaudhaary*et al*., 2007).

Sterile Nicotine media inoculated and incubated with test organisms and observed that pH of the culture broth was periodically increased from pH7.0 to 9.0 at 48th hour and maintained same pH. In case of Bacillus the pH was maximum at 24th hour incubation and maintained upto 30th hour and there was decrease in the pH. Bacillus cereus and Lactobacillus sp were utilizing nicotine at maximum of pH7.0 and sudden increase in pHwhich indicates nicotine degradation(N. Chaudhaary*et al*., 2007). Nicotine utilizing organisms were studied for nicotine degradation using HPLC for the intermediates obtained (Haileie*t al*., 2008). Nicotine effluent was sterilized and inoculated with Pseudomonas and after 24th hour and 72th hour of incubation BOD was calculated. Dissolved oxygen of first day incubation found to be 5.65 and at the third day of incubation 2.54 was measured and BOD was calculated as 3.11.

Normal oral floral organisms were isolated using sterile swabs and identified as *Staphylococcus sp, Streptococcus sp, Proteus sp, Pseudomonas sp*and *Klebsiella sp*. Antibiotic sensitivity test well diffusion method was performed using MH agar plates with the isolated and identified organisms by using different concentration of nicotine extraction. *Staphylococcus sp* showed 14mm zone of clearance at 100 microliters and in 50 microliters. *Streptococcus* showed the greater zone of clearance of 25mm and 15mm obtained in 100 and 50 microliters respectively. The *Proteus sp* obtained 23mm at 100 microliters. And no zone found at 50 microliters of nicotine. *Pseudomonas sp* showed no zone at both 100 and50 microliters which indicates no degradation of nicotine. The present study helps to practice a biological method in nicotine reduction. The soil flora was maintained by nicotine reduction. The organisms obtained and studied in this study are most common organisms and its effect on oral flora is important to study since the imbalance in the normal flora occurred due to the use of nicotine containing chewing gums, tobacco, cigarettes etc. It is advisable not to use any nicotine substrate sue to its harm.

REFERENCES

1. Amstrong D.W., Wang X., Ercal N. Enantiomeric composition of nicotine in smokeless tobacco, medicinal products and commercial reagents. Chiriality., 1998; 10: 587-591.
2. Ananthanarayanan, R., C.K.J. Paniker., Ed 5. 1978. The text book of microbiology, 178-180.
3. Arun Bahl, Nithin Bahl. Modern Chemistry., 1992; 3: 156-159.
4. Batham, H. N. Nitrification in soil.II. Soil science, 1927; 24: 187-203.
5. Brandsch, R., Hinkkanen, A. E and Decker, K. Plasmid mediated nicotine degradation in Arthrobacteroxidans. Arch Microbiol, 1982; 132: 26-30.
6. Charles. S. Pavis., Pierre.A and Nowakowski, J. 2000. Antimicrobial activity of nicotine against a spectrum of bacterial and fungal pathogens. J. Med. Miicrobiol., 49: 675-676. PMID: 10882095.
7. Chaudhary, N., Qazi. J.I., Gill. A. Isolation and optimization of tobacco decomposing Baci; us and Lactobacillus sp. Caspean journal of environmental science., 2007; 5: 45-49.
8. Civilini, M., Domenis, C., Sebastianutto, N and de Berfoldi, M. Nicotine decontamination of tobacco agro industrial waste and its degradation by microorganisms. Waste Man Res., 1997; 15: 349-358.
9. Ertel. A., Eng. R and Smith, S.M. 1991. Differential effect of cigarette smoke on the growth of bacteria found in humans.
10. Faitelowitz, A. Bacterial decomposition of tobacco as leading to formation of bases in the presence of water. Biochem. J. London., 1927; 21: 262-264.
11. Frankenburg, W.G and Vaitekunas, A.A and Zacharias. R.M., Chemical studies on nicotine degradation by microorganisms derived from the surface of tobacco seeds. Arch. Biochem. Biophys., 1955; 58: 509-512.
12. Gherna, R.L., Richardson, S.H and Rittenberg, S.C. The bacterial oxidation of nicotine. VI. The metabolism of 2, 6-dihydrxypseudooxynicotine. J Biol Chem., 1965; 240: 3699-3674.
13. Gravely, L.E., Geiss V.L., Newton R. P. Process for maximizing growth and nicotine degradation acitivity of microorganisms. United states patent, 1978; 4011: 141.
14. Haileiwei., Liping Lei., Zhenyuan XIA., Shuo LIU., Xingzhong LIU., Peigui LIU., Characterization of a novel aerobic nicotine biodegrading strain of

Pseudomonas putida. Ann. Microbiol., 2008; 58: 41-45.
15. Hochstein L.I., Rittenberg S.C. The bacterial oxidation of nicotine: I. Nicotine oxidation by cell-free preparations. J. Biol. Chem., 1958; 234: 151-156.
16. Hochstein L.I., Rittenberg S.C. The bacterial oxidation of nicotine: II. The isolation of the first product and its identification as 1, 6-hydroxynicotine.J.Biol. Chem., 1959; 234: 156-162.
17. Hylin J.W. 1959. The microbial degradation of nicotine II. The mode of action of Achromobacternicotinophagum. Arch Biochem Biophys, 83: 528-537.
18. Ireland M.S., Larson. T.M., Moring T.M. Nicotine transfer process. US Patet No, 1980; 4215706.
19. Kaiser. J.P., Feng. Y and Bollag, J.M. Microbial metabolism of pyridine, quinolone, acridine and their derivatives under aerobic and anaerobic conditions. Microbiol Rev 1996; 60: 483-498.
20. Karina Cogo, Michelle Franz Montan, Christians de Cassia Bergamaschi, Eduardo D. Andrade, Pedro LuizRosalen and Franscisco Carlos Groppo. Invitro evaluation of the effect of nicotine, cotinine and caffeine on oral microorganisms. Can. J. Microbiol., 2008; 54: 501-508.
21. Kenneth Todar 2007. Bacterial flora of humans.
22. Lie, L.P., Wang Y., Wei H.L., LIU X. Z. isolation and characterization of nicotine degrading bacterial strain LI.J. Agri. Biotech., 2007; 15: 721-722.
23. Lenkey A.A. Nicotine removal process and product produced thereby; mixing with alkaline agent in aerobic environment. US Patent No, 1989; 4848373.
24. Maeda S., Kisaki T.1981. Identification of nicotine-1'N-Oxide degrading bacteria. Agr. Biol. Chem., 45: 565-569.
25. Munari M. Quantitative determination of the nicotine content in protein extracted from tobacco. Tobacco journal international., 1986; 2: 128-132.
26. Novotny, T.E and Zhao. F. Consumption and production waste: another externality of tobacco use. Tob Control, 1999; 8: 75-80.
27. Roderich Brandsch Microbiology and biochemistry of nicotine degradation. Appl. Microbiol. Biotechnol., 2006; 69: 493-498. Doi:10. 1007/s 00253-005-0226-0. PMID:16333621.
28. Robert D and Cole P. 1979. Effect of tobacco and nicotine on the growth of Haemophilus influenza invitro. J. Clin. Pathol., 32: 728-731.
29. Robert Shubiski. 2006. Components of tobacco smoke.
30. Reid, J.R. 1977. Extraction of nicotine from tobacco. Lorillard research center green shoro.
31. Richard, P. Newton. Vernon L. Geiss., John. N. Jewell. Lawrence E. Gravely.1977.Process for reduction of nicotine content of tobacco by microbial treatment.
32. Ruan, A., Min H., Peng X., Huang Z2005. Isolation and characterization of Pseudomonas sp strain HF-I, capable of degrading nicotine. Res. Microbiol., 156: 700-706.
33. Ruan A., Min H and Zhu. W. 2006. Studies on biodegradation of nicotine by Arthrobactersp strain HF-2. J. Environ. Sci. Health B., 41: 1159-1170. Doi:10. 1080/03601230600856934. PMID:16923598.
34. Rustica, N., Extraction of nicotine from the tobacco by water extraction method.
35. Saunders, J.A., Blume. D.E. 1981. Quantitation of major tobacco alkaloids by HPLC. Chromatog., 205: 147-154.
36. Schenk, S., Hoelz. A., Kraub. B and K. Decker. Gene structures and properties of enzymes of the plasmids-encoded nicotine establism of Arthrobacteroxidans. J. Mol Biol., 1998; 284: 1323-0329.
37. ShuNing Wang., Zhen Liu., Hong Zhi Tang., Jing Meng and Ping Xu. Characterization of environmentally friendly nicotine degradation by Pseudomonas putita biotype A strain S16. State Key Laboratory of Microbial Technology., 2007; 1556-1565.
38. Sponza D.T., Toxicity studies in a tobacco industry biological treatment plant. Water, air and pollution., 2002; 134: 137-164.
39. Stephan Mulcahy. Toxicology of Cigarette smoke and environmental tobacco smoke.
40. Tabuchi T. Part II. Degradation of nicotine. J. Agr. Chem. Soc. Japan, 1955; 29: 219-225.
41. Tabuchi T. Microbial degradation of nicotine and nicotinic acid. I. Isolation of nicotine degrading bacteria and their morphological and physiological properties. J. Agr. Chem. Soc. Japan, 1954; 28: 807-810.
42. Tabuchi T. Microbial degradation of nicotine and nicotinic acid III. Degradation of nicotine. 2. J. Agric Chem Soc Jpn, 1955; 29: 222-225.
43. Tewari. K.S., Mehrotra. F.N. Vishnoi. N.K., Organic Chemistry., 1994; 4: 320-332.
44. Wada, E and Yamsaki. K. Degradation of nicotine by soil bacteria. J. Am. Chem Soc., 1954; 76: 155-157.
45. Wada, E and Yamasaki K. Degradation of nicotine by soil bacteria. J. Am. Chem Soc., 1953; 76: 155-157.
46. Wang, S.N., Xu P., Tang. H.Z., Meng. J., Liu, X.L Huang. J., Chen. H., Du. Y and Blankespoor. H.D. Biodegradation and detoxification of nicotine in tobacco solid waste by a Pseudomonas sp. Biotechnol Lets., 2004; 26: 1493-1496.
47. Wang, S.N., Xu P., Tang. H.Z., Meng. J., Liu, X.L and Ma. C.O. 2005. Green route to 6-hydroxy-3-Succinoyl-Pyridine from nicotine of tobacco waste by whole cells of a Pseudomonas sp. Environ Sci Technol, 39: 6877-6880.
48. Weber, K The decomposition of tobacco during the fermentation of tobacco. Mitt. Gebiete Lebenam. U. Hyg., 1935; 26: 214-249.

49. Xie X.T., Zheng P., Zhu W.M., Zhao G.L. Separation and determination of nicotine in total particulate matter of cigarette gas by HPLC. Chinese J Anal. Chem., 2000; 28-1085-1087.
50. Yuan Y.J., Lu. Z.X., Huang. L.J., Bie. X.M., Lu. F.X and Li.Y. Optimization of a medium for enhancing nicotine biodegradation by Ochrobactrumintermedium DN2. J. Appl. Microbiol., 2006; 101: 691-697.
51. Yuan Y.J., Lu. Z. X., Huang. L. J., Bie. X. M., Lu. F. X and Li.Y. Isolation and preliminary characterization of a novel nicotine degrading bacterium Ochrobactrumintermedium DN2. Int. Biodeter. Biodegr., 2005; 56: 45-50.
52. Med-Microbial-vol-49.2000.
53. www.actionbioscience.org.