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Isolation and Characterization of *Lacticaseibacillus paracasei* with Nicotine-Degrading and Catechu Stain-Reducing Potential

Bhavika Chauhan and Nileshkumar Pandya*

Department of Microbiology, Arts, Science & Commerce College, Kamrej Cross Road, Surat, Gujarat-394185, India (Affiliated to Veer Narmad South Gujarat University, Surat)

**Corresponding author*

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ABSTRACT

The increasing consumption of gutkha, a smokeless tobacco product, poses severe public health risks due to its high nicotine, areca nut alkaloids, slaked lime and catechu—substances known to cause serious oral and systemic health problems. Nicotine is the major addictive and toxic compound, while catechu and lime contribute to severe staining of oral tissues and public spaces. This study investigates the probiotic bacterium *Lacticaseibacillus paracasei* for its potential to degrade nicotine and reduce catechu-induced stains. *L. paracasei* was isolated from fermented milk products and identified based on morphological and molecular characteristics. Nicotine degradation and stain-reducing activities were quantified spectrophotometrically. The *Lacticaseibacillus paracasei* isolate exhibited significant nicotine degradation efficiency and noticeable reduction of catechu stains. These findings suggest that *L. paracasei* can serve as a potential bio-agent for developing probiotic formulations or modified gutkha products aimed at reducing tobacco-related toxicity and improving public hygiene.

Introduction

Gutkha, commonly known as *pan masala with tobacco*, is extensively used across South Asia and the Indian subcontinent (Changrani & Gany, 2005).

It consists of tobacco, areca nut (seed of the areca palm), slaked lime (calcium hydroxide), catechu (an extract from *Acacia catechu*), and various spices and sweeteners (Strickland, 2002). The mixture is typically placed between the cheek and gum and chewed or sucked for its stimulant effect (CDC, 2007; Willis *et al.,* 2014).

The key addictive and toxic compound in gutkha are nicotine, chemically identified as 3-(1-methyl-2-pyrrolidinyl) pyridine—a hygroscopic oily alkaloid that is colorless to pale yellow (Hossain & Salehuddin, 2013). Nicotine is one of the most potent neurotoxins among tobacco alkaloids (The Merck Index, 1989), with related compounds such as nornicotine, anabasine, and anatabine present in smaller amounts (Hoffmann & Hecht, 1985). Exposure to nicotine causes a wide range of harmful effects, including carcinogenicity, mutagenicity, and teratogenicity (Yildiz, 2004). It can alter cellular functions by generating oxidative radicals, inducing apoptosis and hyperplasia, modifying hormonal secretion, and disrupting enzymatic regulation (Yildiz, 2004; Yildiz *et al.,* 1998; Qiao *et al.,* 2005). Nicotine rapidly crosses biological membranes and the blood–brain barrier, leading to increased heart rate, arterial pressure, and endothelial dysfunction (Campain *et al.,* 2004; Schievelbein *et al.,* 1982; Sabha *et al.,* 2000).

Other components of gutkha also contribute to its toxicity. Slaked lime, obtained from shells or limestone (WHO, 2012), is corrosive and can cause throat pain, vomiting, and systemic collapse when ingested (Do & Vu, 2020). Long-term use of areca nut combined with lime has been linked to organ damage and oral submucous fibrosis (Do & Vu, 2020). Catechu, a tannin-rich extract used as an astringent, produces reactive oxygen species (ROS) under alkaline conditions, leading to oxidative stress and DNA damage (Nair *et al.,* 2004; Chadha & Yadav, 2011). Catechu exhibits both mutagenic and clastogenic properties (Sankhla *et al.,* 2018). Similarly, alkaloids present in areca nut—arecoline, arecaidine, guvacine, and guvacoline—have been identified as potential biological carcinogens (Sankhla *et al.,* 2018), though a few conflicting reports claim medicinal value (Keshava Bhat, 2018).

Microbes naturally found in tobacco waste have been shown to degrade nicotine during fermentation, but such species are not suitable for human application due to safety concerns. Hence, isolating safe probiotic microorganisms capable of degrading nicotine provides a novel, biocompatible alternative.

In addition to toxicity, gutkha contributes to unsightly and unhygienic staining of public areas due to catechu and lime residues. Developing probiotic strains capable of reducing both nicotine and stain-forming pigments could serve as an effective harm-reduction and sanitation-improvement strategy**.**

Therefore, the present study focuses on the isolation, characterization, and application of probiotic bacteria to (i) degrade nicotine within gutkha and (ii) reduce its stain-forming ability, offering a dual-benefit biotechnological approach for improving public health and environmental hygiene.

Materials and Methods

Sample Collection

For isolation of probiotics, various fermented milk sources such as Yakult, Amul Probiotic buttermilk, Mother dairy Probiotic buttermilk and Curd were selected. All medicated products were collected from local Dairy store from Surat, Gujarat, India.

Isolation of Probiotics / Microorganisms

For isolation of probiotics, buttermilk, curd, and Yakult sample were streaked on sterile MRS agar plate and incubated at 37 °C for 48 hrs. After the incubation period, developed colonies were transferred and streaked on the new agar plates repeatedly until a pure isolate was obtained. Isolates were transferred individually on sterile MRS agar slant and preserved at 4°C for further use.

Enrichment culture and isolation of nicotine reducing microorganisms

Microorganisms isolated as above were enriched for determining their nicotine reducing activity. The isolates were added into sterile Bushnell Haas (BH) broth containing nicotine 4 mg/ml, in which nicotine was the sole source of carbon and nitrogen, nicotine was sterilized separately by filtration and added into media before inoculation and inoculated broth were incubated at 37 °C.

After 2 days, the culture was used as inoculum and streaked onto the solid BH agar plates containing 4 mg/ml nicotine. After incubation at 37°C for 72 hrs, colonies began to appear on the plates and these isolates were selected for this study.

Screening of microorganisms for Nicotine reducing activity

Different isolates obtained on nicotine containing agar plates were screened for their nicotine reducing activity on sterile BH broth containing 8 mg/ml nicotine. Isolates were inoculated in sterile BH broth with nicotine and incubated at 37 °C and determined cell density and nicotine reducing activity at 2 hrs intervals. Un-inoculated BH broth containing 8 mg/ml nicotine was used as negative control. During the incubation time, aliquots of inoculated broth were taken at 2 hrs intervals and measured the cell density at 600 nm by using spectrophotometer. The samples were also taken for determining nicotine reducing activity.

Analytical method for determining Nicotine reducing activity of isolates

Nicotine reducing activity in inoculated BH broth was determined by spectrophotometric, and FTIR method. At predetermined time intervals, the samples of inoculated broth were taken and cells were removed by centrifugation at 10,000 rpm for 10 min. Nicotine reducing activity were determined by using supernatant.

Spectrophotometric Analysis

The procedure for measuring reduction in Nicotine concentration involved a spectrophotometric assay that was modified from a method published by Omara & Attaf, in 2014. The supernatant was taken 1 ml & mixed with 1.5 ml of 10^{-3} M acidic Cerric Sulphate [Ce (VI)] solution and mixture was heated in a water bath at 80°C for 5 min then allow it to cool down and 1.6 ml of 10^{-3} M Amranth dye was added and made final volume to the 10 ml with double distilled water and the absorbance was measured at 519 nm.

FTIR Analysis

Nicotine reduction was identified through FTIR analysis of the supernatant. Spectra were collected in the 4000–400 cm^{-1} region at 4 cm^{-1} resolution, averaging 32 scans per sample, with a background run prior to each batch.

Screening of microorganisms for Stain reducing activity

For determining Stain reducing activity, Microorganisms isolated on MRS agar plate were inoculated into sterile Bushnell has (BH) broth containing catechu 10 mg/ml and incubated at 37 °C. After incubation, the stain reduction was detected by taking absorbance at 500nm in spectrophotometer.

Microbial Identification of potent isolate using 16S rRNA sequencing

DNA was isolated from the culture and the quality of DNA was evaluated on 1.0% Agarose gel electrophoresis; a single band of high-molecular weight DNA was observed. Fragment of 16S rRNA gene was amplified by PCR using universal primers. A single discrete PCR amplicon band was observed when resolved on Agarose gel electrophoresis. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 27F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer (Sanger Sequencer).

The 16S rRNA sequence was used to carry out BLAST analysis with the NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple sequence alignment software programs.

Results and Discussion

Nicotine reducing microorganisms are most important because they can be down toxic effect of nicotine, among them probiotic bacteria are more important for removing nicotine in human being. The main drawback of nicotine reducing microbes isolated from tobacco waste is that it cannot be directly apply on human.

On the other hand, probiotic bacteria offer an advantage for reduction of nicotine concentration from smokeless tobacco products. Therefore, in the present study attempts were made to isolate nicotine reducing bacteria from various probiotic sources by screening for their nicotine reducing activity and by using most potential one, further study carried out.

Sample collection

Source-Various fermented milk sources such as Yakult, Amul Probiotic buttermilk, Mother dairy Probiotic buttermilk and Curd were selected for this study.

Microbial strains in various probiotic sources

The microbial strain and active ingredient in probiotic sources were mentioned here according to information given on leaflet of probiotic product and many microbial strains in curd, as described by Hawaz E. (2014).

Isolation of probiotics / Microorganisms

Total ten isolates were obtained on the MRS agar plates from various medicated probiotic sources.

Enrichment culture and isolation of nicotine reducing probiotics

After 2 days of Isolate addition into liquid nicotine medium, turbidity observed in broth indicating their growth. The microorganisms acquired adaptation to nicotine metabolism by enrichment of isolates on 4 mg/ml nicotine containing medium. The colony formation on solid BH agar plates containing 4 mg/ml nicotine as sole source of carbon and nitrogen after 72 hrs indicating they possessed nicotine reducing activity. Total 4 nicotine reducing activity possessing probiotics were obtained, whereas remaining isolates were unable to form visible colony on the nicotine agar plate showing that isolates were incompetent to reduce nicotine concentration.

Screening of microorganisms for nicotine reducing activity

Total four isolates showing colony formation on the BH agar plates containing 4 mg/ml nicotine were selected for screening of nicotine reducing activity. All isolates inoculated into sterile BH broth containing 8 mg/ml nicotine. Among them those showing maximum nicotine reducing activity were selected for this investigation, named as isolate 3 (One isolate was selected).

Analytical method for determining nicotine reducing activity of isolates

Nicotine reducing activity of microorganisms was determined by spectrophotometric and FTIR method.

Spectrophotometric Analysis

The cell growth and nicotine reducing activity of isolates were expressed by absorbance at 600 nm & at 519 nm respectively. The nicotine reducing activity of isolates were determined by spectrophotometric method according to that isolate 3 possessed maximum nicotine reducing activity. The cell growth of isolates into nicotine medium was determined by taking optical density of broth at 600 nm. The absorbance at 600 nm of nicotine broth inoculated with isolate 3 was found 0.635 at 0 hr and 0.805 after 6 hrs of incubation.

The absorbance at 519 nm of nicotine broth was found 0.823 at 0 hr and 0.684 after 6 hrs of inoculation with isolate 3. After 6 hrs, maximum optical density of isolate 3 inoculated broth at 600 nm was shown its better ability to stand with nicotine in compare to other isolates. The depletion of absorbance of nicotine medium at 519 nm was suggested that nicotine concentration decreased in the broth.

A minimal optical density at 519 nm of nicotine medium inoculated with strain isolate 3 after 6 hrs was manifested that its maximum nicotine reducing activity in compare to other. Figures 1 & 2 showed that a significant correlation was observed between the increase in cell growth and decrease in nicotine concentration in the medium.

FTIR Analysis

Nicotine reducing activity of isolates was also determined by FTIR method. Detection of nicotine reducing activity of isolates by FTIR analysis were shown in figure 3 and 4 in which figure 3 indicated FTIR of nicotine broth without microbe inoculation as control and FTIR spectrum of nicotine broth inoculated with isolate 3 was shown in figure 4.

The reduction in intensity or disappearance of peaks signifies the consumption or breakdown of the original functional groups.

The FTIR analysis clearly suggested that microorganisms possessed nicotine reducing activity.

Based on the preliminary assessments, spectrophotometric analysis and FTIR spectroscopy collectively demonstrated that isolate 3 exhibited the maximum nicotine reducing activity. Consequently, this superior performance in the reduction assay led to its selection for subsequent studies.

Screening of microorganisms for Stain reducing activity

The spectrophotometric technique was used to determine the microorganisms' stain-reducing activity. Absorbance at 500 nm was used to express the isolates' stain-reducing potential.

The spectrophotometric method was used to determine the stain reducing activity of the isolates, and the results showed that isolates 3 possessed the highest stain reducing activity.

Table.1 Microbial strains in various probiotic sources

Probiotic source	Microbial strain
Probiotic Buttermilk	*Bifidobacterium bifidum* *Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Lactobacillus reuteri* *Bifidobacterium animalis* *Lactobacillus plantarum*
Curd	*Lactobacillus casei* *Lactobacillus brevis* *Lactobacillus acidophilus* *Lactobacillus delbrueckii* *Lactobacillus leichmannii* *Lactobacillus fermentum* *Lactobacillus coagulans* *Lactobacillus lactis* *Saccharomyces spp.*
Yakult	*Lactobacillus casei*

Figure.1 Cell growth of isolates in nicotine containing medium

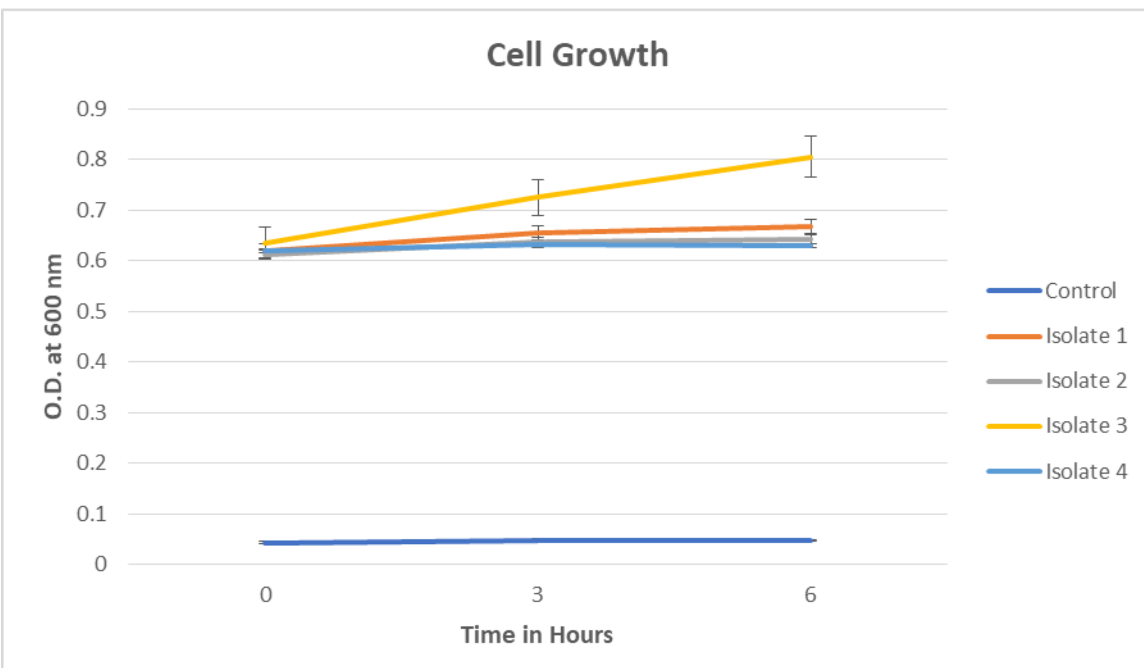


Figure.2 Nicotine reducing activity of isolates in nicotine containing medium.

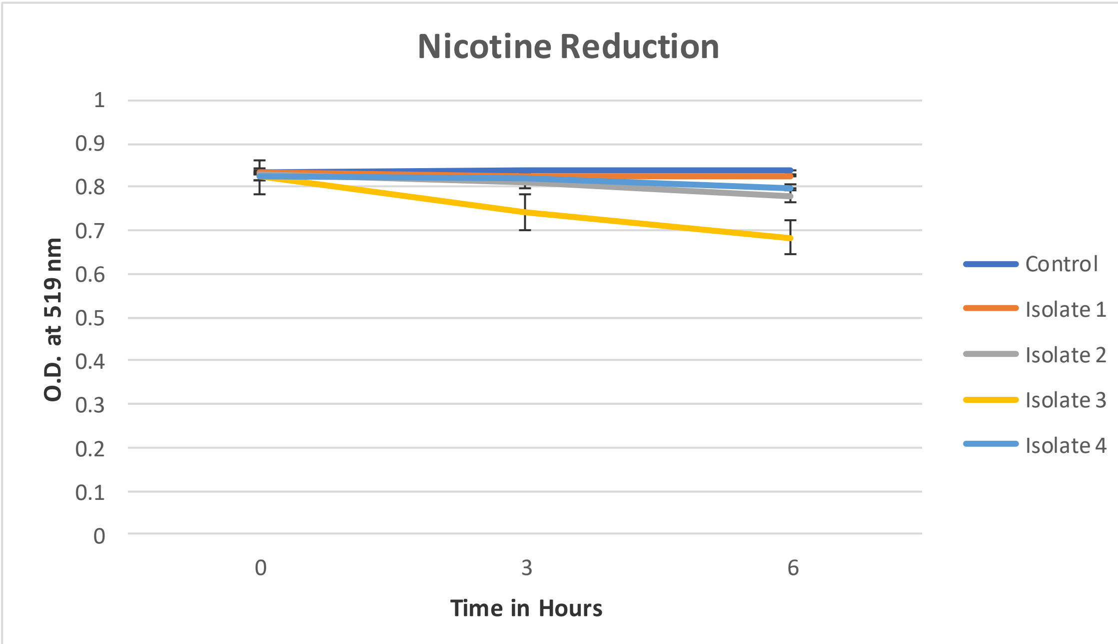


Figure.3 FTIR analysis of nicotine medium.

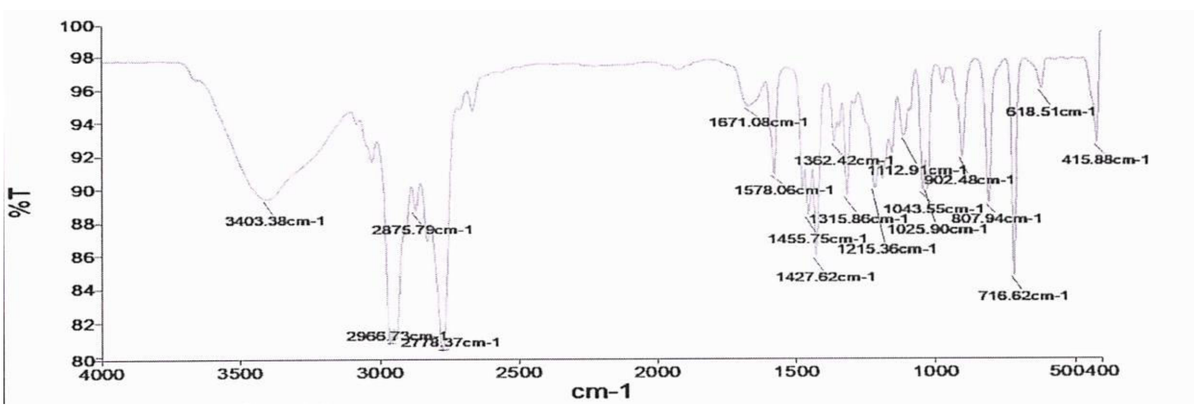


Figure.4 FTIR analysis of nicotine reducing activity of isolates 3.

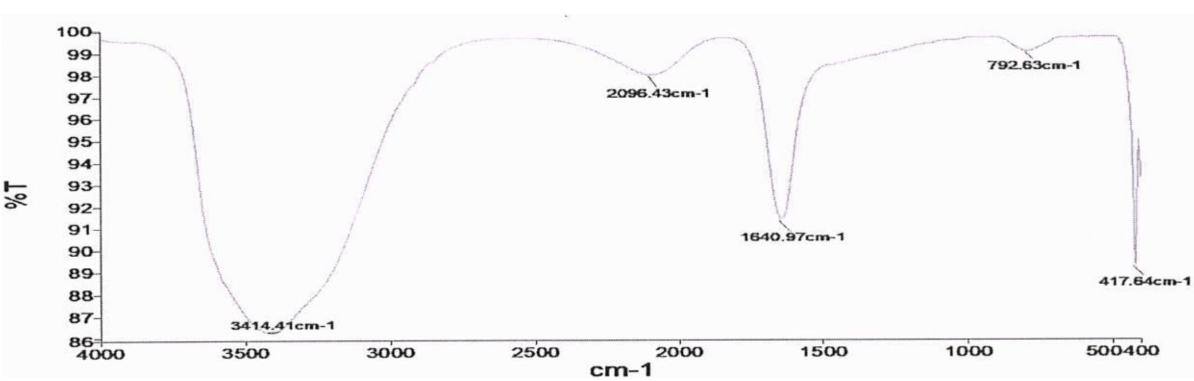


Figure.5 Stain reducing activity of isolates in stain solution.

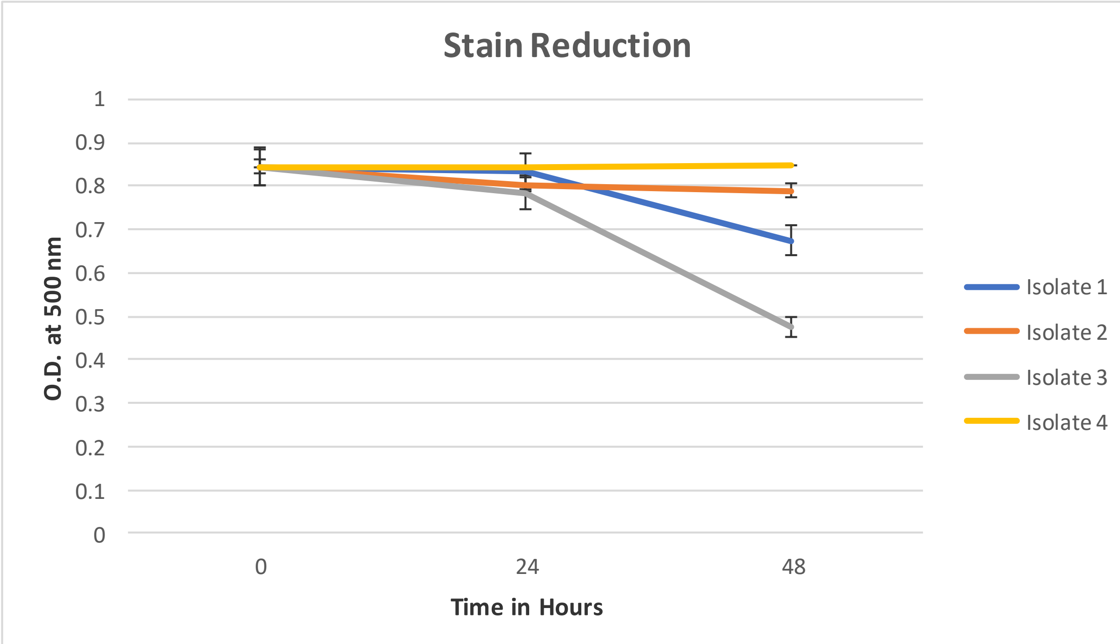
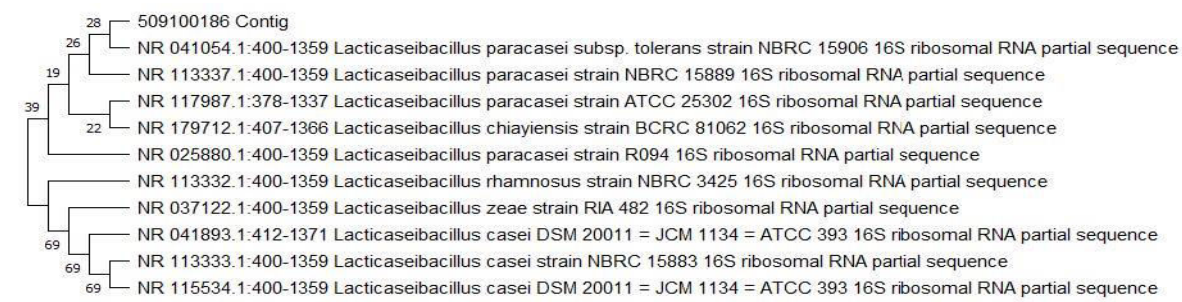


Figure.6 Molecular Phylogenetic analysis



The stain solution's absorbance at 500 nm was 0.845 at 0 hours and 0.475 after 48 hours of inoculation with isolate 3 respectively. The stain solution's lower absorbance at 500 nm was interpreted as a sign of a drop in stain concentration. After 48 hours, the stain solution inoculated with isolate 3 showed a minimal optical density at 500 nm, indicating its maximum stain reduction activity in comparison to the others.

Microbial Identification of potent isolate using 16S rRNA sequencing

Sample labelled as isolate 3 is found to be *Lacticaseibacillus paracasei* based on nucleotide homology analysis & phylogenetic analysis.

Generated Sequences from the Microbial Sample

>509100186_Contig

GCGTGAGTGAAGAAGGCTTTCGGGGTCGTAAAA
CTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTA
ACTGTTGTCGGCGTGACGGTATCCAACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTATCCGGATTTATT
GGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGT
CTGATGTGAAAAGCCCTCGGCTTAACCGAGGAA
GCGCATCGGAAACTGGGAAACTTGAGTGCAGA
AGAGGACAGTGGAACTCCATGTGTAGCGGTGAA
ATGCGTAGATATATGGAAGAACACCAGTGGCGA
AGGCGGCTGTCTGGTCTGTAACTGACGCTGAGG

CTCGAAAGCATGGGTAGCGAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGATGAATGCT
AGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGC
AGCTAACGCATTAAGCATTCCGCCTGGGGAGTA
CGACCGCAAGGTTGAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCTTTTGATCACCTGAGAGATCAGGTTTC
CCCTTCGGGGGCAAAATGACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTATGACTAGT
TGCCAGCATTTAGTTGGGCACTCTAGTAAGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGATGGTACAACGAGTT
GCGAGACCGCGAGGTCAAGCTAATCTCTTAAAG
CCATTCTCAGTTCGGACTGTAGGCTGCAACTCG
CCTACACGAAGTCGGAATCGCTAGTAATCGCGG
ATCAG

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 814 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. In conclusion, the present study demonstrates that *Lacticaseibacillus paracasei* possesses the unique ability to degrade nicotine and reduce catechu-induced staining. The isolate from promising medicated probiotic source ensuring its survival in oral and gut environments.

Its nicotine-degrading activity indicates potential for detoxifying tobacco alkaloids, while its capacity to reduce catechu stains suggests a practical application in improving oral hygiene and public sanitation. These findings highlight *L. paracasei*as a potential bioagent for developing probiotic-based formulations or modified gutkha products aimed at reducing nicotine toxicity and promoting cleaner, safer consumption practices. Further in vivo and formulation-based studies are recommended to validate its efficacy and ensure safety for consumer use.

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Author Contributions

Bhavika Chauhan: Investigation, formal analysis, writing—original draft. Nileshkumar Pandya: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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