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# Valorization of hydrolyzable tannins by microbes

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## Abstract

Due to the great interest in producing bioactive compounds for functional foods and biopharmaceuticals, it is important to explore the microbial degradation of potential sources of target biomolecules. Gallotannins are polyphenols present in nature, an example of them is tannic acid which is susceptible to enzymatic hydrolysis. This hydrolysis is performed by tannase or tannin acyl hydrolase, releasing in this way, biomolecules with high- added value. Samples were collected from various locations throughout Mumbai. They were enriched in minimal media containing tannic acid as a substrate. Tannic acid-degrading organisms were isolated. Plate assays, such as the bore well method, will reveal the zone of degradation, and cultures with a higher zone of degradation will be subjected to further shake flask or fermentation studies. TLC and HPLC will be used to determine whether tannic acid degrades into gallic acid or pyrogallol. The degradation potential of the isolate's organisms will be used in the production of byproducts with enormous industrial application potential.

Keywords: Valorization, Tannins, Gallic acid, HPLC, Biodegradation.

## Introduction

Valorisation is a process that converts toxic waste into usable, high-value, and environmentally friendly raw materials. It is regarded as a key mechanism for organic chemical elimination in natural systems due to its environmental tolerance and adaptability. As a result, microbial metabolic potentials are widely used in a wide range of industrial applications, including environmental pollution decommissioning and the production of industrial grade Bio products.

The process primarily employs biocatalysts, either as complete cells or as enzymes, to produce a diverse range of bio products (s). In comparison to chemical technologies, the biotransformation process provides superior control over numerous process parameters, the ability to genetically alter microorganisms, higher yields, credibility, safety, and reusability.

Pollution of water and soil from tannin-rich systems is a serious environmental risk, particularly in developing countries (Issa Mohammed, 2016)Tannins are polyphenolic compounds with high molecular weight (500-3000 Da) that occur in plants and are one of the major constituents of tanning industry effluents that are toxic to plants, animals, and microorganisms, posing a potential threat to both human

health and the environment (Van De Lagemaat & Pyle, 2001)Tannins are considered nutritionally undesirable because they inhibit digestive enzymes and interfere with vitamin and mineral absorption. The ingestion of large amounts of tannins may have negative health consequences. However, a small amount of the right kind of tannins may be beneficial to human health (Gu et al., 2003)

They distinguish themselves from other phenolics by their ability to precipitate proteins from solutions, form strong complexes, and interact with other macromolecules such as starch, cellulose, and minerals(Belmares et al., 2004), (Lekha & Lonsane, 1997)

Tannins are classified as secondary compounds in plants due to their absence from metabolic pathways. They are the second most common type of plant phenolic after lignin. Because they have a lot of phenolic hydroxyl groups, they can form massive complexes with proteins and, to a lesser extent, with other macromolecules like celluloseand pectin (Mcleod, 2023), (Mueller-Harvey et al., 2010)

## Degradation Products of Tannic Acid (Gallic Acid):

The trihydroxy benzoic acid (also known as gallic acid) has the formula $C_6H_2(OH)_3CO_2H$ (Fig 1.1) It belongs to the phenolic acid family. Gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants contain it. It's a white solid, but due to incomplete oxidation, most samples are brown. Gallates are the salts and esters of gallic acid.

(Polewski et al., 2002)) describes it as a yellowish white crystal with a molecular mass of 170.12 g/mol, a melting point of 250°C, and a water solubility of 1.1 percent at 20°C. Gallic acid has high antioxidant, anti-inflammatory, antimutagenic, and anticancer effects (Kim et al., 2002)). (Mirvish et al., n.d.) In the presence of metal ions, gallic acid is also known to have a pro-oxidant characteristic that is concentration dependant. Gallic acid has been shown to cause apoptosis in cancer cell lines thanks to its pro-oxidant properties (Yen et al., n.d.), (Sergeant, 1990). Gallic acid's preventive function in chemically induced carcinogenesis has also been confirmed by research (Giftson Senapathy et al., 2011)

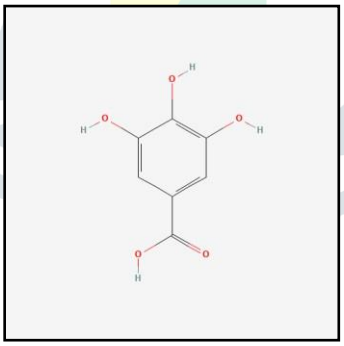


**Figure 1.1. Chemical Structure of Gallic Acid (PubChem)**

### Material and Methods

1. Sample collection
   Polyphenols, such as tannins derived from logs, barks, and leaves, are constantly deposited in the soil surrounding lumberyards. Microflora near the logs can degrade phenolic compounds like tannins. Keeping these considerations in mind, soil from a lumber yard containing tannic acid degrading microflora was cultured on Bushnell and Hans' tannic acid-containing medium for enrichment over week.
2. Screening of the isolates
   After enrichment, the sample was isolated on Bushnell and Han's media with 0.5% tannic acid and incubated at 37 C for 24hrs. the isolated organisms were further purified on LB agar and preserved by cryo preservation.

3. Plate assay by agar cup method
   To check the tannase production by the organisms the isolates were screened on St, B& H media with 0.5% tannic acid and incubated same as above (Kumar et al., 2010)

4. Molecular characterization
   The isolates which shows more zone of inhibition around the colony wee further selected for gDNA and plasmid extraction by general protocol and gel electrophoresis was done. The 16Sr RNA gene of the selected culture were amplified using Polymerase Chain Reaction in PCR Thermocycler.

**Table 1.1 Primers used for amplification**

| Loci | Primer Name | Primer Sequence 5' to 3' |
|---|---|---|
| 16S rRNA | 8F | AGAGTTTGATCCTGGCTCAG |
| | 907R | CCGTCAATTCMTTTRAGTTT |

5. Shake flask studies
   The two isolates selected for 16sRNA sequencing was used for shake flask studies. The isolates were inoculated in st. B & H medium with 0.5% tannic acid. It was kept in shaker in 24hrs and after every 3 hr of interval the sample were aliquoted and further it was taken for gallic acid estimation, TLC ,and HPLC.

6. Gallic acid estimation
   The assay works on the principle of formation of chromogen between gallic acid and rhodamine in the presence of KOH which provides the alkaline condition for chromogenic substrate formation and stopping the overall reaction between gallic acid and rhodanine. The absorbance is measured at 520nm.

7. Thin Layer Chromatography Quantitative Determination of Gallic Acid

   Supernatant from shake flask studies run on Aluminium TLC sheet in which Ethyl acetate : Chloroform :Formic acid (3 : 7 : 0.2) mobile phase was used.

8. High-Pressure Liquid Chromatography (HPLC) for Quantitative Determination of Gallic Acid

- Supernatant from shake flask studies was used for HPLC analysis by gradient method in which twosolvents and two standards was used i.e. Solvent A (D/W + 0.1% Formic Acid), Solvent B (Aceto Nitrile
  :D/W) (50:50) + 0.1% Formic Acid, Gallic Acid (1mg/mL), Tannic Acid (1mg/mL), Tannic Acid + GallicAcid (1mg/mL).

**Table 1.2 Method parameters for HPLC analysis**

| | |
|---|---|
| Solvent A | D/W + 0.1 % Formic acid (v/v) |
| Solvent B | ACN: D/W 50:50 (v/v) + 0.1% Formic Acid |
| Mode | Gradient |
| Injection Volume | 20 µL |
| Injection Mode | Automated |
| Detection Volume | 280nm |

**Table 1.3 Gradient Condition for HPLC**

| Time (in Minutes) | Solvent A | Solvent B |
|---|---|---|
| 0.00 | 95 | 5 |
| 2.00 | 95 | 5 |
| 5.00 | 55 | 45 |
| 5.02 | 0 | 100 |
| 10.00 | 0 | 100 |
| 10.02 | 95 | 5 |
| 15 | 95 | 5 |
| 20µL | Injection mode | Automated |

## Results and Discussion

### PLATING:

All the concentration were serially diluted up to $10^{-8}$ and pour plated on Luria Bertani agar with 0.5% Tannicacid.


**Fig1.2 Growth of Colonies observed on plates with Luria Bertani agar with 0.5% Tannic cid from plating enriched samplewith 0.5% Tannic Acid for dilution $10^{-6}$(A) ,$10^{-8}$(B) ,$10^{-10}$ (C) and 0.8% Tannic Acid for dilution $10^{-6}$(D) ,$10^{-8}$(E) ,$10^{-10}$ (F)**

### ISOLATION OF PURE CULTURE:

Isolation of pure cultures were carried out by streaking the colonies on Luria Bertani Agar . Total 16 pure isolateswere obtained and labelled as C1,C2, C3, C4,C5,C6, C7, C8.)

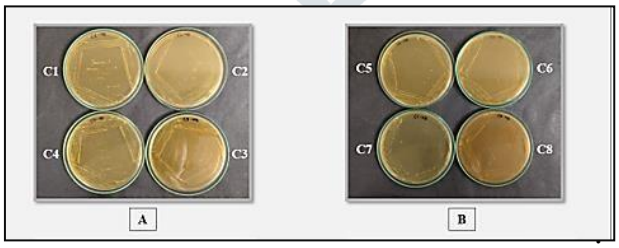



**Fig.1.3 Pure isolates of the 16 colonies labelled as C1 – C8. C1-C4 (A), C5-C8 (B)**

### SCREENING ASSAY:

Screening assay was carried for cultures (C1 to C16) on Bushnell and Haas (B&H) Agar with 0.5 % Tannic acid(pH 5.5) to observe zone of degradation.


**Fig.1.4. Zone of Degradation observed around the colonies (C1-C8) on Bushnell and Haas (B&H) Agar with 0.5 % Tannicacid (pH 5.5)**

## DNA Sequencing and Species Identification

- Species identification was done using BLAST.
- Following were the sequences obtained by DNA sequencing
- The length of C2 and C9 was found to be 950 bp and 934 bp

>STRAIN_C2

CTAACACATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAA

TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGA

CCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAC

GGCTCACCTAGGCGATGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA

GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT

ATGAAGAAGGGCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCATT

TGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT

TAATCGGACTTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGTTCAAC

CTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGTGGTGA

AATGCCTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG

AAAGCTTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGT

GCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCCCGGTTGAGT

CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT

TACCTGGTCTTGACATCCACAGAACTT

**Fig.1.5 FASTA Sequence of strain C2**

**Table 1.4 Confirmed genus and species data obtained from sequencing and using BLAST**

| Isolated code | Organism identified | Percent identify (%) |
|---|---|---|
| C2 | *Enterobacter cloacae* | 98.64% |

## GALLIC ACID ESTIMATION:

Gallic acid was estimated using Spectrophotometric assay reported by (Sharma et al., 2000)2000.Gallicacidestimation of supernatant from shake flask studies was carried out.

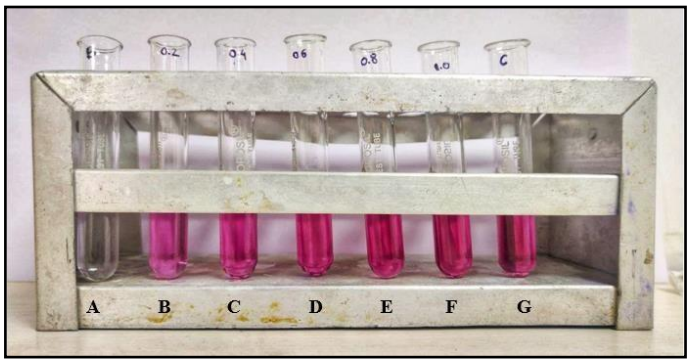
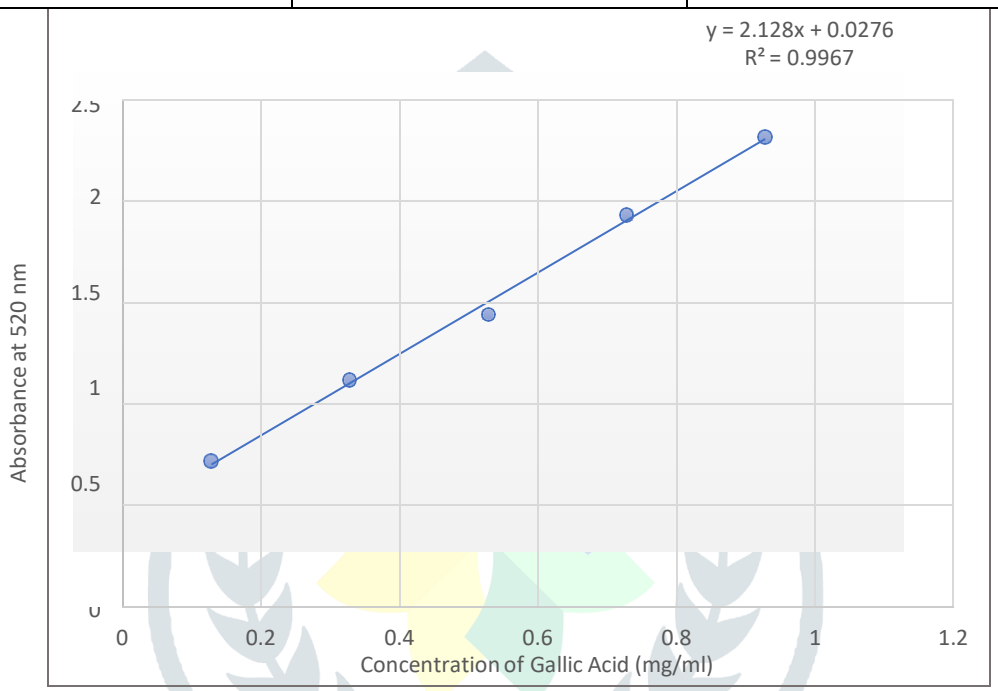



**Fig 1.6 Representation of Spectrophotometric assay for Standard Gallic acid (1mg/mL). Blank (A), 0.2 mg/mL (B), 0.4 mg/mL(C), 0.6 mg/mL (D), 0.8 mg/mL (E), 1.0 mg/mL (F),Control (G)**


**Fig 1.7 Representation of Spectrophotometric assay for Supernatant extracted from shake flask studies. 3hrs (A), 6hrs (B),12hrs (C), 24hrs (D), 36hrs (E), 48 hrs (F).**

**Table 1.5 Linearity for Standard Gallic acid (1mg/mL) at 520 nm**

| Concentration of stock Gallic acid (1mg/mL) | O.D at 520nm | Standard - Blank |
|---|---|---|
| 0.2 mg/mL | 0.564 | 0.469 |
| 0.4 mg/mL | 0.989 | 0.894 |
| 0.6 mg/mL | 1.331 | 1.236 |
| 0.8 mg/mL | 1.853 | 1.758 |
| 1.0 mg/mL | 2.26 | 2.165 |
| Control | 1.048 | - |
| Blank | 0.095 | - |




**Graph 1.1. Linearity curve for standard Gallic acid (1mg/mL)**

- From the Graph (Graph 5.10) linearity equation was obtained as follows
- Y= 2.128(X)+0.0276
- Using this equation Gallic acid was estimated for both the cultures *Enterobacter cloacae* and
  *Enterococcus fecalis*.

**Table 1.6 Gallic Acid Estimation for *Enterobacter cloacae***

| Sample | O.D at 520 nm | Control | Y | M | C | Y-C | Y-C/M | x Dilution Factor | mg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 3 hrs | 1.244 | 1.12 | 0.124 | 2.128 | 0.0276 | 0.0964 | 0.0453 | 0.453007519 | 5.5300752 |
| 6 hrs | 1.267 | 1.12 | 0.147 | 2.128 | 0.0276 | 0.1194 | 0.0561 | 0.561090226 | 5.6109023 |
| 12 hrs | 1.613 | 1.12 | 0.493 | 2.128 | 0.0276 | 0.4654 | 0.2187 | 2.187030075 | 21.870301 |
| 24 hrs | 1.491 | 1.12 | 0.371 | 2.128 | 0.0276 | 0.3434 | 0.1614 | 1.613721805 | 16.137218 |
| 36 hrs | 1.534 | 1.12 | 0.414 | 2.128 | 0.0276 | 0.3864 | 0.1816 | 1.815789474 | 18.157895 |
| 48 hrs | 1.363 | 1.12 | 0.243 | 2.128 | 0.0276 | 0.2154 | 0.1012 | 1.012218045 | 10.12218 |

## Thin Layer Chromatography:

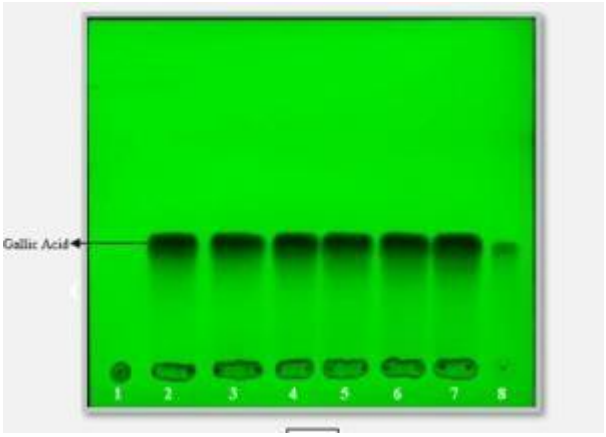



**Fig 1.8 TLC plates loaded with Culture supernatant of *Enterobacter cloacae* Lane 1 Shows standard Tannic acid, Lane 2 toLane 7 consists supernatant from 3 hours to 48 hours sampling, Lane 8 shows the standard Gallic acid .**

**Table 1.7 Rf Values for *Enterobacter cloacae* from TLC plates**

| ***Enterobacter cloacae*** | | | |
|---|---|---|---|
| **Hours** | **Distance travelled by sample (cm)** | **Distance Travelled by Solvent in (cm)** | **Rf** |
| 3 hours | 3.1 | 7 | 0.443 |
| 6 hours | 3.1 | 7 | 0.443 |
| 12 hours | 3.1 | 7 | 0.443 |
| 24 hours | 3 | 7 | 0.429 |
| 36 hours | 3 | 7 | 0.429 |
| 48 hours | 2.9 | 7 | 0.414 |
| Gallic acid standard | 2.8 | 7 | 0.4 |

## High Pressure Liquid Chromatography:

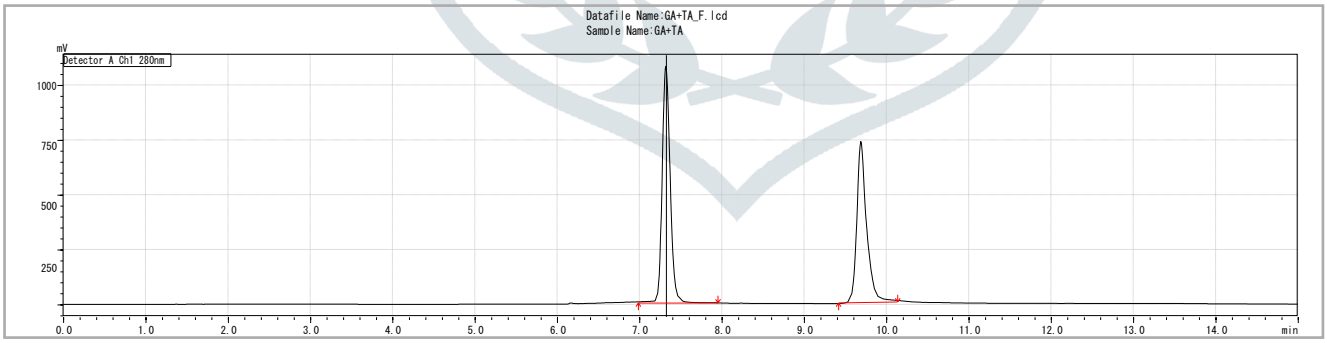



**Fig. 1.9.1 HPLC Chromatogram for Standard Gallic acid + Tannic Acid (1mg/mL)**

**Table 1.8.1 Table showing peak area and retention time for Std. Gallic acid + Tannic acid**

| **Peak#** | **Ret. Time** | **Area** | **Height** | **Area%** |
|---|---|---|---|---|
| 1 | 7.323 | 7178555 | 1076687 | 54.669 |
| 2 | 9.693 | 5952469 | 731622 | 45.331 |
| Total | | 13131024 | 1808309 | 100 |

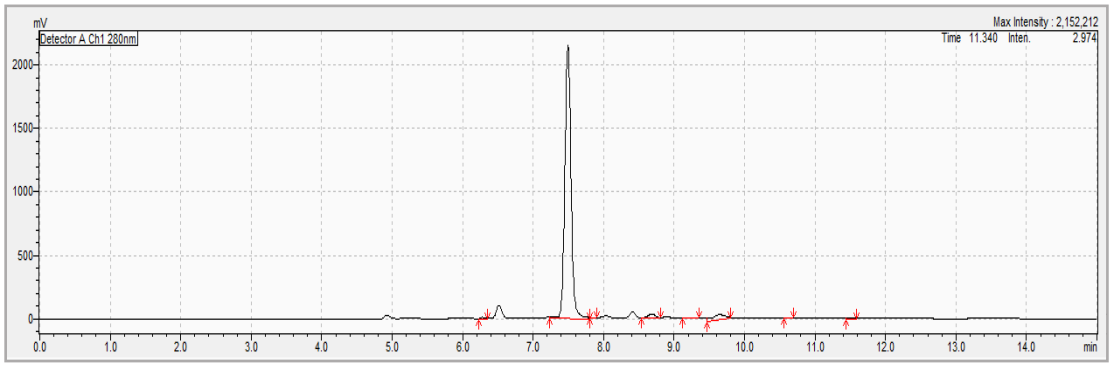



**Fig 1.9.2 HPLC Chromatogram of the sample from *Enterobacter cloacae* (6hrs)**

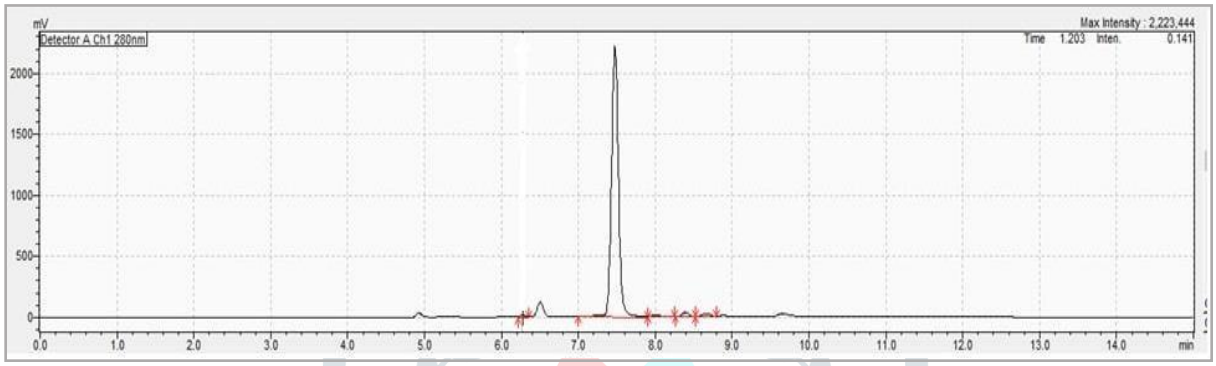
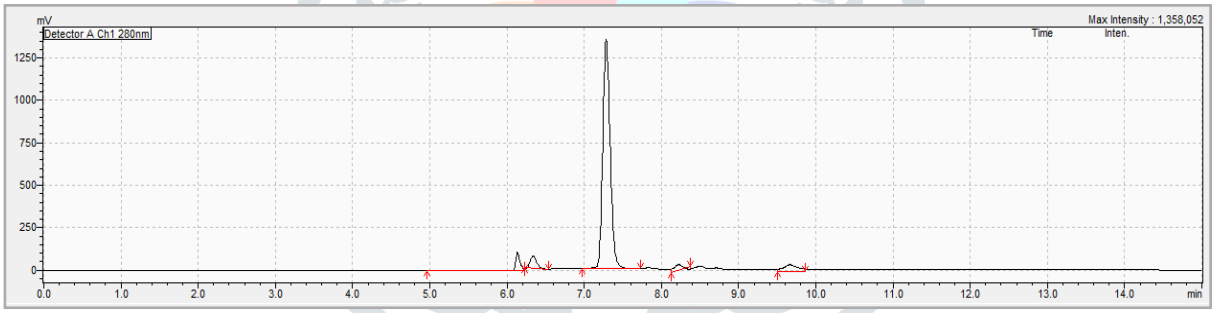



**Fig 1.9.3 HPLC Chromatogram of the sample from *E***




***nterobacter cloacae* (12hrs)**

**Fig 1.9.4 HPLC Chromatogram of the sample from *Enterobacter cloacae* (24hrs)**

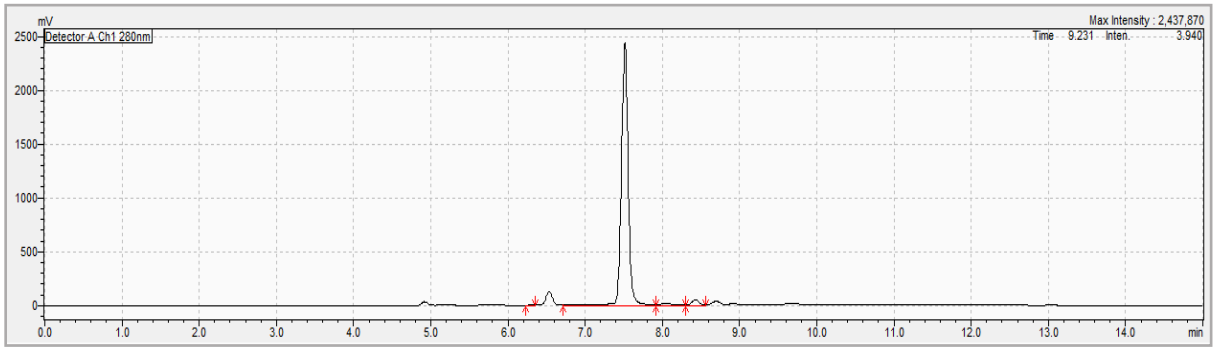



**Fig 1.9.5 HPLC Chromatogram of the sample from *Enterobacter cloacae* (48hrs)**

**Table 1.8.2 Table showing peak area for the sample *Enterobacter cloacae* (3hrs-48hrs)**

| Sample (*Enterococcus cloacae*) | Area |
|---|---|
| 3hrs | 12354712 |
| 6hrs | 12773629 |
| 12hrs | 16707451 |
| 24hrs | 14622812 |
| 36hrs | 13685296 |
| 48hrs | 12973522 |

## Discussion

The soil sample was obtained from lumber yards. The sample was first enriched in 25 mL Bushnell and Haas medium containing 0.2% Tannic acid. t he primary enrichment sample was further enriched in varying concentrations (0.2%, 0.4%, 0.6%, 0.8%, and 1.0%) of the substrate Tannic Acid. All concentrations were serially diluted up to 10-2 before being pour plated on Luria Bertani agar with 0.5% Tannic acid 9 (fig 1.1) . Bacterial colonies grew in all concentrations (0.2%, 0.4%, 0.6%, 0.8%, and 1.0%) of the sample. The number of colonies on the plate decreased as the concentration of tannic acid increased. For pure culture isolation, all concentrations

(0.2% - 1.0%) were serially diluted up to 10-6,10-8,10-10 and pour plated on Luria Bertani with 0.5% agar withantibiotics.

Plate assays were performed on Bushnell and Haas (B&H) media, Minimal Media, Minimal Broth (Davis) with 0.5% Tannic Acid and pH 5.5 with 5 random cultures (C2, C9, C10, C12, C13) and zone of degradation was observed. The highest zone of degradation was observed in Bushnell and Haas (B&H) media with C2, which had a zone of degradation of 16mm (fig 1.3). The genomic DNA of C2 was extracted from all cultures using the CTAB method, and the DNA samples were aged. Under the Gel documentation system, DNA was observed as fluorescent bands. The isolates were identified based on differences in the conserved 16S rRNA region. By comparing the homology in the 16S rRNA gene to the standard NCBI Nucleotide database, their species was identified. The 16S rRNA gene was isolated from gDNA of selected cultures for identification using universal primers 8F and 907R. The length of the sequence for C2 was 950, which is the entire sequence of this species' 16S rDNA ( fig 1.4) depicts the sequencing data. The sequence was then compared to other sequences in the GenBank/NCBI database using the sequence comparing software BLAST. The nucleotide sequence of strain C2 was found to be highly similar (98.64%) to that of *Enterobacter cloacae*.

Gallic Acid estimation was carried out with the help of spectrophotometric assay for both the strains. It was observed that the sampling for 12 hours showed the highest gallic acid production i.e., 21.87 mg/mL (fig 1.6) for*Enterobacter cloacae.*

TLC of supernatant sampling for 3 hour, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours , was carried for Quantative analysis of gallic acid. The bands of gallic acid were observed under UV Transilluminator at 254nm for both the cultures (fig 1.7). The Rf values for *Enterobacter cloacae* were between 0.41-0.44 compared to standard gallic acid that was 0.4

HPLC analysis for the samples (3hrs – 48hrs) of *Enterobacter cloacae* and *Enterococcus fecalis* were carried out. Comparing the retention time of standard gallic acid (1mg/mL) (fig 1.8.1) with the Samples of *Enterobacter cloacae* presence of gallic acid was confirmed. Highest gallic acid production was observed for 12 hours sampling with peak area of 16707451 for *Enterobacter cloacae* respectively (fig 1.8.3). Based on comparative analysis of CFU/mL, Spectrophotometric assay and HPLC analysis it was found that *Enterobacter cloacae* was able to produce 21.87 mg/mL of gallic acid at 12 hours with CFU/mL of 35.6 x $10^{-4}$ and a peak area 16707451.

## Conclusion

Tannins are polyphenolic secondary metabolites of higher plants that are commonly used in leather tanning, textile dyes, ink production, food antioxidants, and rubber coagulants. Tannins are thus a significant component of the waste generated by these industries. Tannic acid is a commercially available gallotannin form. Tannic acid is completely degraded enzymatically to gallic acid and/or ellagic acid by tannin acyl hydrolase (EC 3.1.1.20), also known as tannase. Given the industrial importance of gallic acid, the current study was conducted to produce and quantitatively analyse gallic acid from a potent bacterial strain.

Despite the fact that there have been several studies on bacterial and fungal degradation. Fungal species have clearly been extensively studied and exploited for gallic acid production in comparison to bacterial species. Indeed, most researchers in the last 150 years of Gallic acid production research (1867-2017) have painted a simple picture of Gallic acid production from fungi rather than bacteria. However, the bacterial species responsible for Gallic acid production demonstrates tremendous industrial potential.

We concentrated on Enterobacter Cloacae in this study. The first part of the study involved collecting, enriching, and plating soil samples from lumber yards on medium containing the substrate tannic acid. This work resulted in the isolation of 16 potential candidates, who were then screened using a quick, simple modified plate assay to select the best tannic acid degraders. Visual evaluation and zone of degradation were used to assess the selected candidates' ability to degrade tannic acid. Out of the 16 isolates, two gave the maximum zone of degradation, namely Enterobacter Cloacae, which gave a zone of degradation of 16mm and was identified as Enterobacter Cloacae using 16S rRNA sequencing.

To determine which of them was the most potent gallic acid producer, qualitative and quantitative gallic acid estimation assays were performed using Thin layer chromatography and High-Pressure Liquid Chromatography, which confirmed the presence of gallic acid in the culture filtrate. The method developed by Sharma et al 2000 was modified for quantitative estimation of gallic acid. Gallic acid production by Enterobacter Cloacae was 21.87 mg/mL. Based on the results of the cell count analysis, spectrophotometric assay, and HPLC analysis, Enterobacter Cloacae is a potent producer of gallic acid.

The current research has helped to identify Enterobacter Cloacae as a promising candidate for gallic acid production. Gallic acid, which has enormous potential in the global market, has seen an increase in demand in recent years. The findings presented in this chapter will undoubtedly add to our current understanding of microbial gallic acid production. To summarise, the current study is a modest attempt to demonstrate the use of Enterobacter Cloacae to produce gallic acid in a cost-effective, safe, and effective manner for the general public.

## References

1. Belmares, R., Contreras-Esquivel, J. C., Rodríguez-Herrera, R., Coronel, A. R., & Aguilar, C. N. (2004). Microbial production of tannase: An enzyme with potential use in food industry. *LWT - Food Science and Technology*, *37*(8), 857–864. https://doi.org/10.1016/j.lwt.2004.04.002
2. Giftson Senapathy, J., Jayanthi, S., Viswanathan, P., Umadevi, P., & Nalini, N. (2011). Effect of gallic acid on xenobiotic metabolizing enzymes in 1,2-dimethyl hydrazine induced colon carcinogenesis in Wistar rats
   - A chemopreventive approach. *Food and Chemical Toxicology*, *49*(4), 887–892. https://doi.org/10.1016/j.fct.2010.12.012
3. Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., & Prior, R. L. (2003). Screening of Foods Containing Proanthocyanidins and Their Structural Characterization Using LC-MS/MS and Thiolytic Degradation. *Journal of Agricultural and Food Chemistry*, *51*(25), 7513–7521. https://doi.org/10.1021/jf034815d
4. Issa Mohammed, Y. H. (2016). Isolation and Characterization of Tannic Acid Hydrolysing Bacteria from Soil. *Biochemistry & Analytical Biochemistry*, *s3*. https://doi.org/10.4172/2161-1009.1000254
5. Kim, D. O., Lee, K. W., Lee, H. J., & Lee, C. Y. (2002). Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *Journal of Agricultural and Food Chemistry*, *50*(13), 3713–

3717. https://doi.org/10.1021/jf020071c

6. Kumar, R., Kumar, A., Nagpal, R., Sharma, J., & Kumari, A. (2010). A novel and sensitive plate assay for screening of tannase-producing bacteria. *Annals of Microbiology*, *60*(1), 177–179. https://doi.org/10.1007/s13213-010-0022-7
7. Lekha, P. K., & Lonsane, B. K. (1997). Production and Application of Tannin Acyl Hydrolase: State of the Art. In *Advances in Applied Microbiology* (Vol. 44, Issue C, pp. 215–260). https://doi.org/10.1016/S0065-2164(08)70463-5
8. Mcleod, M. N. (2023). *Plant Tannins-their role in forage quality [1974]*.
9. Mirvish, S. S., Ca, A., Wallcave, L., & Shubik, P. (n.d.). *Induction of Mouse Lung Adenomas by Amines or Ureas Plus Nitrite and by N-Nitroso Compounds: Effect of Ascorbate, Gallic Acid, Thiocyanate, and Caffeine*. http://jnci.oxfordjournals.org/
10. Mueller-Harvey, Reed, I.;, & Hartley, J. D.; (2010). Characterisation of phenolic compounds, including flavonoids and tannins, of ten Ethiopian browse species by high performance liquid chromatography [1987].*Journal Article*.
11. Polewski, K., Kniat, S., & Sławińska, D. (2002). GALLIC ACID, A NATURAL ANTIOXIDANT, IN AQUEOUS AND MICELLAR ENVIRONMENT: SPECTROSCOPIC STUDIES. In *Current Topics in Biophysics* (Vol. 26, Issue 2).
12. Sergeant, J. (1990). *The cognitive-energetic model: an empirical approach to Attention-Deficit Hyperactivity Disorder* (Vol. 13). Plenum press. www.elsevier.com/locate/neubiorev
13. Sharma, S., Bhat, T. K., & Dawra, R. K. (2000). A spectrophotometric method for assay of tannase using rhodanine. *Analytical Biochemistry*, *279*(1), 85–89. https://doi.org/10.1006/abio.1999.4405
14. Van De Lagemaat, J., & Pyle, D. L. (2001). Solid-state fermentation and bioremediation: development of a continuous process for the production of fungal tannase. In *Chemical Engineering Journal* (Vol. 84).
15. Yen, G.-C., Duh, P.-D., & Tsai, H.-L. (n.d.). *Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid*. www.elsevier.com/locate/foodchem