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Census of cultivable bacterial community in common effluent treatment plant (CETP) of tannery discharge and computational scrutiny on their leading residents

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Abstract:

Common effluent treatment plant (CETP) for tannery effluent, is the combination of physical, chemical and biological treatment to facilitate the degradation of industrial waste water. Obviously, the biomass which survives in this extreme environment may have the ability to utilize the effluent as the sole carbon source for its survival. The ultimate aim of the present investigation is to expose the microbial diversity in each stage of the CETP through the culture dependent way. Bacterial diversity in the effluent were analysed through 16S rRNA gene. The community study revealed the dominance of firmicutes and the dominant genus was *Bacillus* sp, with variable species diversity. Notably, Putative *Bacillus* sp, *B. firmus* and *B. licheniformis* were observed in all stages of treatment. The dominant residents were analysed by BProm and TF site scan to prove their uniqueness. This species richness indicates the capability of liveliness in treatment plant and whose can be exploited for treating the effluent by using modern molecular approach.

Keywords: CETP, Bacterial community, 16S rRNA, Tannery, Effluent, *Firmicutes*, *Bacillus* sp.

Abbreviation: CETP - Common Effluent Treatment Plant; PTIET - Pallavaram Tanners Industrial Effluent Treatment Company Ltd

Background:

In the world's foot wear production India occupies 10th position and is one of the highest producers of raw hides. As a result of increasing demand for leather and leather products to indigenous use and export, more than 2500 tanneries have been mushroomed in India. The quality leather was obtained from the raw hides through a complex and tedious process called tanning. Tanning process consumes a large quantity of lime, sodium sulphide, ammonium sulphate, sodium chloride, bactericides, vegetable tannins and chrome salts. This leads to the heavy discharge of high organic load, phenolic, tannins and heavy metals into the environment. When the industrial tannin discharged into water and soil it causes serious threat to biotic and abiotic factors, notably in developing countries **[1]**. Moreover tannin is resistant to microbial attack; thereby, it retards the growth of soil microbial communities and stops the biodegradation process **[2]**. Perhaps condensed tannin binds with proteins and it develops into complex, which is not

feasible for decomposition. Even though tannin has the antimicrobial property, many bacterial species developed various mechanisms and pathways for their degradation in natural habitats **[3]**. The degradation of gallotannin by an anaerobic bacterium *Achromobacter* sp was reported by Lewis and Starkey **[4]**. Deschamps *et al.,* **[5]** portrayed the ability of bacteria using tannic acid as the sole source of carbon and his group isolated 15 bacterial strains belong to *Bacillus* sp, *Staphylococcus* sp and *Klebsiella* sp.

Activated sludge is the cost effective method for the treatment of industrial waste waters. Sudden changes in the rate of organic load and chemicals to meet out the market demand makes the activated sludge process more complicated. To overcome this problem, the promising approach CETP was emerged. It was designed with the combination of the physical, chemical and biological process. The present study has been undergone in CETP functioning at Pallavaram, Chennai, Pallavaram Tanners Industrial Effluent Treatment Company Ltd (PTIET). In this, seven collection wells are located in various places to receive effluent from 152 tanneries, which is pumped to a collection tank, from there the treatment process was started **[6]**. This study unmask the biomass survive in each stage of tannery effluent treatment plant, also our prime focus is to evidence the predominant group of microbes in the treatment plant and to exploit it for tannin degradation by adopting molecular strategies.

Methodology:

Sample Collection, Isolation and Identification of bacterial isolates

The effluent samples were collected in sterilized containers from the raw effluent tank, primary treated, secondary treated, tertiary treated and soil from CETP, Pallavaram, Chennai, India. The supernatant was removed without disturb the biomass and 1 g of the biomass was suspended in 1ml of distilled water. From this crude, serial dilutions were made up to 10^{-7} for each stage of treatment including soil. Each dilution were spread on LB agar plates and incubated at 32˚C for overnight. The observed colonies were picked on the basis of the morphological features like size, shape, elevation, opacity, surface, texture and margin. Pure cultures were obtained through the quadrant streak and stored in the refrigerator for molecular analysis. It was subcultured 2 to 3 times to check the purity before DNA extraction.

Molecular investigation

The genomic DNA was extracted from bacterial isolates using bacterial genomic DNA isolation kit (Real Biotech Corporation). The amplification of 16S rRNA gene was performed with universal primers 27F (agagtttgatcmtggctcag) and 1492R (tacggytaccttgttacgactt) (Lane, 1991)**.** The reaction mixture contained 50ng of template DNA, 10X PCR buffer, 1.5mM $MgCl_2$, 2.5mM each dNTPs, 10 pmol of forward and reverse primer and 1.5 U of Taq DNA polymerase. The total mixture volume was 50µl and the program starts with initial denaturation at 94˚C for 3 mins, followed by denaturation at 94˚C for 30s, annealing at 55˚C for 1.5 minutes, extension at 72˚C for 2.5 minutes then the final extension at 72˚C for 5 minutes. From denaturation to extension step 35 cycles were repeated. The 16S product was resolved in 1.5% (w/v) agarose gel electrophoresis in 1X TAE buffer using 1 kb ladder as molecular weight marker and visualized by staining with ethidium bromide.

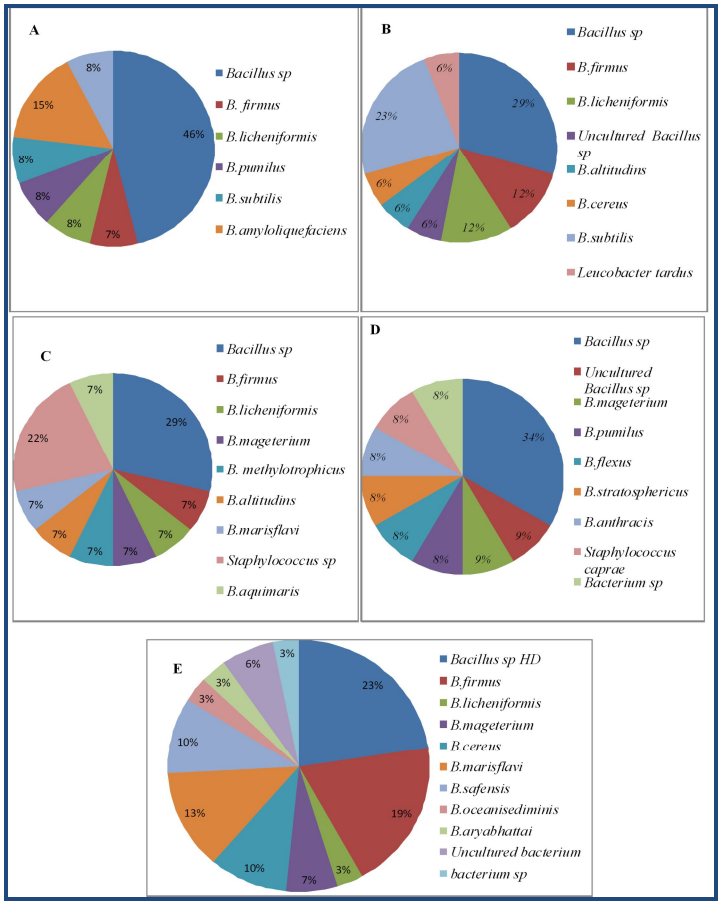

Figure 1: Statistical pie diagram represents the percentile of bacterial community in the **A)** Raw Effluent; **B)** Primary treated Effluent; **C)** Secondary treated Effluent; **D)** Tertiary treated Effluent; **E)** Soil.

Sequencing and In silico analysis

The amplicons were sequenced and the partial sequences obtained were compared with available data in GenBank using the BLAST. The sequences were deposited in GenBank public database under the accession numbers JN411558 - JN411575 and JN559869 - JN559926. In the total population, the dominant isolates were subjected to the promoter identification and the prediction of its differential promoter location in the isolates. The functional promoters were identified using the online software Bprom. The transcription binding factor present in the 16S rRNA gene was also identified using another online tool named TF scan site.

Result and Discussion:

The present study was performed to estimate the microbial population in the different stages of treatment in CETP. Samples were collected from the discharges of Raw Effluent tank, Primary Treated effluent, Secondary Treated effluent, Tertiary Treated effluent and Soil in Pallavaram CETP, Chennai. Those 5 different samples were worn to harvest the microbes in culture dependent way; LB media was used as a substrate to harvest the microbes. The microbes developed in the plate were picked for screening with respect to its morphological characteristics. The colonies were subcultured atleast thrice to

check the mixed profile and also to obtain the pure culture of the isolate. The pure cultures were subjected to genomic DNA isolation. The isolated DNA was checked for its quality and quantity to perform the PCR. 16S rRNA genes of the isolated bacterial colonies were amplified with the above said conditions; they were sequenced to identify the phylotype of the bacterial colonies in different stages. Microbial communities have been explored comprehensively in all states of soil **[7]**. However, in the recent decades the scientist has turned towards the analysis of microbial consortia of contaminated source. The classical reason behind this is to exploit the untapped microbial resource in contaminated sites, and the consortia tends to frame microbiome which codes for the superior activity to clean up the contaminating pollutants from the same site. Moreover, the microbiomes exist in the contaminated sites develops the resistivity which have the ability to convert complex pollutants into simpler harmless derivatives. On this basis, the microbiome based removal of pollutants from the industrial premises was practised in current situation **[8, 9]**. The number of colonies formed in the plate showed arbitrary growth in different stages of treatment.The number of colonies increased gradually from the initial stage (Raw effluent) to the final stage (Treated effluent and soil). The survey of the bacterial community in CETP revealed that, the dominant group belonging to firmicutes in all stages of the treated effluent. Around 90% of the *Bacillus* genera were occupied, which constitute different species in the different treatment plant. The Raw effluent consists of putative *Bacillus* sp, whose population was around 50% in total bacterial population **(Figure 1A).** Similarly, the treated effluent and soil also have the same percentage of population, but the percentage of putative *Bacillus* sp was variable around 30-40% in all stages of the sample. *Bacillus subtilis* as the second highly populated bacteria present in the primary treated effluent **(Figure1B).** In the secondary treated effluent *Staphylococcus sp* was the second dominantly existing bacteria **(Figure.1C).**The tertiary treated effluent has notable population variability. Other than Putative *Bacillus sp,* totally diversified bacterial colonies were also found **(Figure 1D).** In soil the *Bacillus firmus* ranks the second highly populated and *Bacillus safensis* leads the third of the same.

However, in this study, the CETP samples from treatment site was collected, which receives wastewater from various tanneries; this results in the complex diversity of bacteria in the CETP. One of the major tasks is to analyse the microbial flora and this is the first step in understanding the operational efficiency of these systems **[10]**. Even though, it was well recognized that cultivable microbes comprise a very low level of total diversity than the culture-independent. One of the ever best options was the culture-based method, whereby specific substrate enrichment of the community can be carried out **[11]**. The conventional fact is, the culture dependent method enhances the growth of dominant microbes in selected sample. So the majority of population diversity studies were carried out with both the aspects **[12]**. Here diversity calculation is based on 16S rDNA gene sequence analysis. Many 16S rRNA based molecular techniques have been developed for community-level analysis of differences in microbial composition associated with environmental, spatial or temporal changes **[13]**. Moreover, in the current scenario microarrays have been implied for community analysis in treatment plants. These are designed using either the 16S-23S rDNA intergenic spacer region **[14]** or the 16S rDNA gene **[15]**. However, results from this study demonstrate the importance of using both culture-based and culture-independent tools for the characterization of microbial diversity of a niche **[11]**. A coherent dominance of putative *Bacillus sp, B. firmus and B. licheniformis* were observed in all the stages of effluent, while uncultured *Bacillus sp* was observed in all the treated samples except raw effluent and secondary treated. The Firmicutes, *B. megaterium* and *B. methylotrophicus* showed their presence from secondary treatment because they were not seen in raw and primary treated samples. *Bacillus altitudins* was observed in the primary and secondary stage of the treatment plant, while *B. subtilis* present in the raw effluent and primary treated. Fascinatingly, *B.pumilus* and *B. marisflavi* were the members existing in the population without any continuity, *B. pumilus* were present in the raw effluent and in the tertiary treatment, *B. marisflavi* was found in the secondary treated and soil sample. Likewise, *B.cereus* was existing in the primary treated effluent and soil sample.

This is the first attempt to isolate and elucidate the fact of dominant microbes present in all stages of the CETP through culture dependent method, so samples were collected from the different stages of same CETP site. Correspondingly, in earlier studies it was insisted that *Bacillus pumilis* and *Bacillus polymyxa* are reported as the good producers of microbial tannase **[16]**. Similarly, the data of microbial diversity in aeration tanks revealed that there was an interesting notification regarding the presence of firmicutes in one aeration tank. Notably, in the same analysis on another aeration tank they observed firmicutes were not found, this clearly states that the microbial population varies with respect to several factors **[17]**. The current study is also highlighting the prevalence of the tannin tolerant microbes vary with respect to the CETP and its organic load, but the firmicutes was found in most of the CETP which has been confined in earlier studies on tannery effluents. The dominant occurrence of *Bacillus* sp was predicted in the study site, consistently in all stages starting from the raw effluent to the each step of treated effluent and finally in soil also, a similar dominance was reported earlier in chromium contaminated site **[18, 19]**. A unique set of bacteria were also found in each sampling stage of the CETP, for instance *B. amyloliquefaciens and Lysinibacillus fusiformis* in raw effluent, *Leucobacter tardus* in primary treated, *Staphylococcus sp* and *Bacillus aquimaris* in Secondary treated. A variety of diversified strains were present in the secondary treated sample such as *B.flexus, B.stratosphericus, B. anthracis, Staphylococcus caprae*, and *Bacterium sp*. Similarly soil also comprised of distinctly variable colonies such as *B.safensis, B.oceanisedminis, B.aryabhattai* and uncultured bacterium **(Figure 1E).** The lineage of the branches extended well enough with inter variable species in firmicutes and also a wide distribution of diversified firmicutes were visualized in the soil when compared to the others **(Figure 1E)** there is a percentage of cumulative bacterial diversity in the CETP, which elucidates the total diversity constitutes above 90 % of the population was firmicutes. In that a *Bacillus sp* occupies the dominance of 33%, *Bacillus firmus* was second dominant species resides about 11% in total population. Rather than these, the census of all other bacteria survives in the CETP was below the margin of 10%. This evidence propably sounds the validity of dominance of *Bacillus sp* and *Bacillus firmus,* those can be studied in detail to know how they are resistant towards the

tannin and oraganic load. The dominant bacteria can be implied in treatment process through enrichment of media, which enables them to have a prolonged stationary phase. A Novel tannase gene can be traced from those microbes, thus obtained gene can be over expressed and can be exploit the same to treat the organic pollutant from CETP.

As a final point, we look forward the *in silico* analysis of the isolates obtained, most probably all the isolates varied evidently in their promoters counts, site and in their location. But we scrutinize and accomplish the *in silico* data of the abundant microbes interspersed in the treatment setup. Accordingly the *Bacillus* sp*, Bacillus firmus and Bacillus licheniformis* were the isolates taken for the interpretation of *insilico* characterization, the results exposed the variable number of promoters in its site and location. The *Bacillus* sp procures 2 promoters, one was unpredictable and the other was ihf (integration host factor), in *Bacillus firmus* 3 different promoters namely rpoD19 and rpoD17 (Transcription factors) and pur R is a Purine regulation promoter. Then the *Bacilllus licheniformis* persist the following promoters Crp (C-reactive protein) and rpoD16 (Transcription factors**)** Usually the integration host factor – ihf is a nucleoid-associated which persuades the confirmation of the neighboring promoter sequences, and defined the specific DNA base sequence that rendered the promoter susceptible to this activation. Here, ihf binding sets the confirmation of neighboring sequences, thereby triggering promoter activation **[20]**. The promoter rpo family was closely associated with the RNA polymerase transcription initiation factor which closely related to sigma complex factor **[21]**. Crp superfamily of transcription factors that bind as homodimers to palindromic sequences of DNA, each subunit binding to one half-site.

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Supplementary material:

Table 1: Promoter and transcription biding site analysis of highly populated 16S rDNA sequence by using the online tool Bprom and TF scansite.

***Bacillus* sp**	***Bacillus firmus***	***Bacillus licheniformis***
Number of predicted promoters - 2 Threshold for promoters - 0.20 1. Promoter Pos: 154 LDF- 1.56 -10 box at pos. 139 GGATAGGAT Score 46 -35 box at pos. 119 GGGAAA Score -10 2. Promoter Pos: 642 LDF- 0.47 -10 box at pos. 627 GTGCAGAAA Score 3 -35 box at pos. 607 TTGAAA Score 60	Number of predicted promoters - 3 Threshold for promoters - 0.20 1. Promoter Pos: 511 LDF- 3.42 -10 box at pos. 496 TGCTACAAT Score 72 -35 box at pos. 477 ATGACC Score 14	Number of predicted promoters - 2 Threshold for promoters - 0.20 1. Promoter Pos: 195 LDF- 3.33 -10 box at pos. 179 AATTATAAA Score 36 -35 box at pos. 159 TTGATT Score 53 2. Promoter Pos: 691 LDF- 0.84 -10 box at pos. 676 CGGTGAAAT Score 45 -35 box at pos. 658 GTGAAA Score 27
Oligonucleotides from known TF binding sites: For promoter at 154: ihf: CTTCGGGA at position 115 Score - 12 No such sites for promoter at 642	Oligonucleotides from known TF binding sites: For promoter at 511: rpoD19: ACGTGCTA at position 493 Score - 12 rpoD17: GCTACAAT at position 497 Score - 8 purR: CGCGAGGT at position 529 Score - 6	Oligonucleotides from known TF binding sites: For promoter at 195: crp: GTTCAATT at position 175 Score - 6 rpoD16: TTATAAAA at position 181 Score - 9 No such sites for promoter at 691
Ihf – Integration host Factor	rpoD19 and rpoD17 Transcription factors purR – Purine regulation promoter	Crp - C-reactive protein rpoD16 - Transcription factors

Pos: Position