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# Study on the Characterization and identify pollutants of distillery wastewater degrading bacteria

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***Abstract - In this study outcome from morphological and biochemical tests (such as those for shape, arrangement, colonies, temperature, growth, form, margin, elevation, indole production, methyl red, Voges-Proskauer, citrate utilisation, starch hydrolysis, urease production, hydrogen sulphide production, catalase activity, lactose, glucose, and sucrose fermentation) were used to identify and characterise pure bacterial isolates chosen for this study.***

***Keywords - Distillery wastewater, Bacteria, Pollutants, Characterization, Identify***

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## INTRODUCTION

Stricter environmental rules are requiring distilleries to upgrade their present practises in addition to reducing pollutants. When it comes to eliminating organic contaminants from aqueous waste generated by the chemical processing sector, biological treatment is typically the less expensive and more economical option. While many physical & chemical processes only collect pollutants or transport them from one medium (e.g., from liquid to air) to another, making their final destiny throughout the atmosphere unpredictable, other toxins may be entirely biodegraded (mineralized) via these systems. Consequently, biological treatment techniques have earned a reputation for being a successful approach for the treatment of wastewaters with a high organic load, as those generated by wine distilleries. Among the different biological treatment techniques accessible, the anaerobic treatment procedure is often used for the distillery effluents treatment for methane recovery. Methane-rich biogas may one day provide the plant with some of its energy needs. The system has several advantages, such as high strength wastewater disposal, low energy consumption, low sludge generation, and biogas generation; however, it also has several disadvantages, such as regular digester instabilities caused by the development of undesirable compounds, long digester start-up time, long retention time, and low conversion of organic constituents to biogas

The waste material produced during the distillation of ethyl alcohol from molasses is known as distillery wastewater (Barrow and Feltham 1993). Distillery wastewater harms the environment due to its alkaline composition, high BOD, COD, and TDS, among other factors. There is a lot of dark brown molasses wasted wash in the effluents of distilleries that use molasses (MSW) (Bergey's, 2005). There are about 319 distilleries in India, each of which can produce 3.25 × 109 l of alcohol and release effluent at a rate of 40.40 x 1010 l annually. A conventional molasses-based distillery produces wasted wash (15L) for every litre of alcohol produced. It is hazardous to marine life and vegetation because the coloured component of distillery effluent decreases photosynthetic activity and depletes dissolveO2 in water sources.

## METHODOLOGY

### Biochemical Characterization of isolated bacterial strains

According to the procedures outlined in Cowan and Steel's Manual for Identification of Medical Bacteria, the bacterial strains DS3, DS4, and DS5 were morphologically and biochemically characterised (Barrow and Feltham 1993).

### Molecular characterization of bacterial isolates

- **Genomic DNA Isolation**

Each bacterial culture was cultured in GPYM broth that had been altered with distillery effluent for a 48-hour incubation period in order to separate the genomic DNA of isolated bacterial strains. Following the alkaline lysis procedure, the complete genomic DNA from overnight-grown cells was extracted (Kapley et al., 2001). To pellet the bacterial cells, 5

mL of each log phase bacterial culture was centrifuged at 5,000 rpm for 5 min at 4 °C. The resultant bacterial pellet was then gently vortexed in 200 L of 0.5 N NaOH to lyse the bacteria before being resuspended in 200 L of 10 mM phosphate buffer (pH 7.0). To separate the pellet from the bacterial cell debris, the lysate was neutralised by adding 100 L of 1M Tris (pH 7.5) and centrifuged at 10,000 rpm for 15 min at 4 °C. A volume adjustment of up to 500 L was made to the supernatant. The resulting supernatant was colourless and transparent; it was kept at -20 °C and utilised in subsequent experiments.

**PCR amplification and cloning of 16 S rDNA gene**

The 16S rDNA gene was amplified from about 5 L of genomic DNA using the universal eubacterial primers (27F) 5'-AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5'- CGGTTACCTTGTTACGACTT-3', yielding a 1,500 bp product (Narde et al. 2004). In a final reaction volume of 50 L, the reaction mixture included 100 ng template, 1x PCR buffer, 200 M of each dNTP, 3.0 mM MgCl2, 25 pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer). The thermocycling procedures (Applied Biosystems, USA's Veriti® 96-Well Thermal Cycler) were as follows: denaturation at 94 °C for 1 min, then annealing at 45 °C for 1 min, and extension at 72 °C for 2 min.

Using a 1 Kb DNA ladder (Merk, Biosciences, India) as a molecular weight marker, the PCR-amplified 16S rDNA gene product was electrophoresed across a 1.2% (w/v) agarose gel in 1X TAE buffer and visualised by staining with ethidium bromide (EtBr). Using primer 27F, the PCR products were sequenced and gel purified using a gel extraction kit from Merk, Biosciences, India. When exposed to agarose gel electrophoresis, the restriction digestion of pure plasmid DNA produced with 10 units of EcoR1 for 2 hours at 50 °C in 1X reaction buffer containing 60 mM Tris-Cl, pH 7.9, 1.5 M NaCl, and 60 mM MgCl2 revealed the presence of an anticipated 1.5 kb insert.

**16S rDNA gene sequencing and phylogenetic tree analysis**

The PCR products were sequenced using primers 27F and 1492R as reported in the preceding section, and then gel purified using a gel extraction kit (Merk, Biosciences, India). ABI PRISM® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit was used to carry out the sequencing reactions, which employed primer 27 F to amplify the 16S rDNA gene. This product was then gel purified and used as a template (Applied Biosystems, USA). The resulting partial sequences were analysed using the online BLAST tool at www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997), which provided information on the identity of the bacterial isolates. The 16S rDNA sequences obtained were also deposited in GenBank databases under the accession numbers MF182113, MF967441, and MF182114 for bacteria DS3, DS4, and DS5, respectively. The phylogenetic tree was created using the neighbour-joining method using Kimura-2-parameter distances in MEGA-4 software.

**Detection and characterization of ligninolytic enzymes (laccase and MnP)**

For the produced bacterial cell-free extract, filtered bacteria were cultivated in 250 mL Erlenmeyer flasks with 100 mL of pH 7.0 medium and incubated at 35 °C for 24 hours. After the incubation time, 2.0 mL of the sample was removed from the flask and centrifuged for 15-20 minutes at 10,000 rpm at 4 °C to harvest the material. In order to assess the enzymatic state, the resulting supernatant was also employed as extracellular enzymes. These collected cells (75 mg/mL) were suspended in a potassium phosphate buffer (50 mmol/L) with a pH of 7.4 and then sonicated (Sonics-Vibracell ultrasonic processor, USA), maintaining the sonifier output at 40 (amps), providing 7 strokes after each of 30 sec, with a 1 min interval at 4 °C. The supernatant from the centrifuged homogenate, which had been at 10,000 rpm for 20 min, was employed as a source of crude enzyme.

**SDS-PAGE and molecular weight determination of ligninolytic enzyme**

A spacer plate should be placed on top of a short plate. On a level surface, insert both plates into the casting frame. Make sure the casting frame's "legs" are pointing downward. Check that the plates are level on the bottom by clamping the casting frame.

**RESULT AND DISCUSSION**

**Biochemical Characterization of isolated bacterial strains**

The first morphological identification of the isolated bacterial strains from distillery wastewater and sludge samples led to the designation of DS3 and DS4 as gramme positive (+ve) coccus and rod shape, respectively, and DS5 as gramme negative (-ve) and rod form. The bacterial strains DS3, DS4, and DS5 passed the motility tests. While DS4 produced favourable findings, bacterial strains DS3 and DS5 showed acceptable results for ornithine consumption but poor results for catalase and sorbitol. Additionally, testing for lactose fermentation, H2S, and citrate utilisation for DS3, DS4, and DS5 came out negative. While the lysine consumption test findings for these strains, DS3, DS4, and DS5, were contradictory, the urease test was positive for all. Additionally, DS3 and DS5 both reported negative findings, despite the fact that DS5's test results for arabinose were inconsistent. The nitrate reduction test result for bacterial strains DS3 has been positive, whereas those for DS4 and DS5 have been negative (Table 1 and Fig. 1).

**Table 1: Secondary biochemical findings of probable bacterial strains that have been isolated for initial identification**

| Biochemical test | Bacterial strains | | |
|---|---|---|---|
| | DS3 | DS4 | DS5 |
| Catalase | +ve | +ve | +ve |
| Indole test | +ve | -ve | -ve |
| Amylase test | +ve | +ve | -ve |
| Citrate utilization | -ve | -ve | -ve |
| Lysine utilization | V | V | V |
| Ornithine utilization | +ve | V | +ve |
| Urease | +ve | +ve | +ve |
| Phenylalanine deamination | V | -ve | -ve |
| Nitrate reduction | +ve | -ve | -ve |
| $H_2S$ production | -ve | -ve | -ve |
| Glucose | +ve | +ve | -ve |
| Adonitol | -ve | -ve | -ve |
| Lactose | -ve | -ve | -ve |
| Arabinose | -ve | V | -ve |
| Sorbitol | -ve | +ve | -ve |

**(+ve): positive; -ve: (negative); V: variables results**

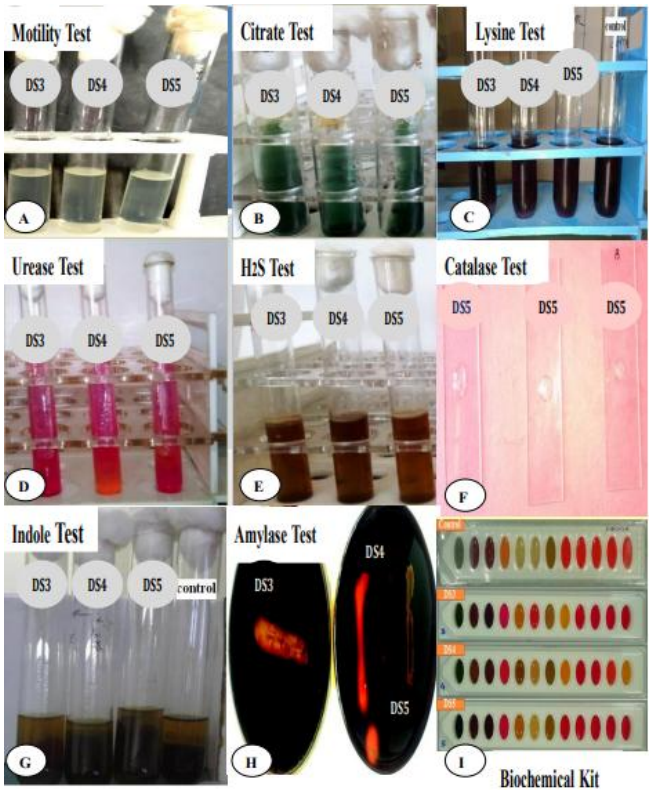



**Figure 1: Different biochemical reactions shown by isolated bacterial strains DS3, DS4, and DS5; A: Motility Test; B: Citrate Test; C: Lysine Test; D: Urease Test; E: H2S Test; F: Catalase Test; G: Indole Test; H: Amylase Test; I: Biochemical Kit (For other Biochemical Test).**

**16S rDNA gene sequence analysis**

Furthermore, the 1500 bp long 16S rDNA gene sequences (Fig. 2A) from the DS3, DS4, and DS5 bacteria have shown the most similarity to the uncultured Alcaligenaceae bacterial clone De 110a, Bacillus megaterium strain, and Staphylococcus saprophyticus strain ATCC15305, respectively. Five Alcaligenaceae species, one Bacillus species, and ten Staphylococcus species were collected from GenBank and used to build the phylogenetic tree using the neighbour-joining method (Tamura et al., 2007) (Fig. 3). A few unrelated sequences were included to the tree as a control to highlight the link distance between the isolates. Because of their similarities in the 16S rDNA sequencing, the bacterial strains DS3, DS4, and DS5 were identified as Staphylococcus saprophyticus, Bacillus megaterium sp., and Alcaligenaceae sp., respectively. Additionally, the partial 16S rDNA gene sequences from DS3, DS4, and DS5 were submitted to GeneBank's public data bank with the accession codes MF182113, MF967441, and MF182114, respectively.

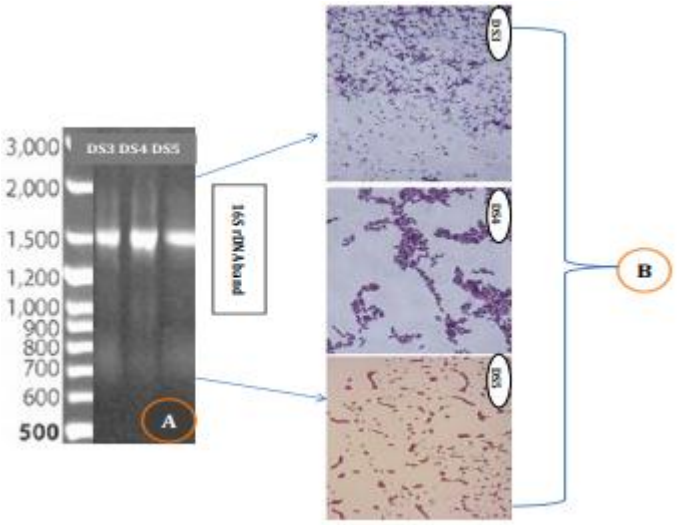



**Figure 2 (A): 16S rDNA gene band from isolated bacterial strains amplified by PCR; (B): and morphological characteristics of isolated bacteria strains DS3, DS4, and DS5.**

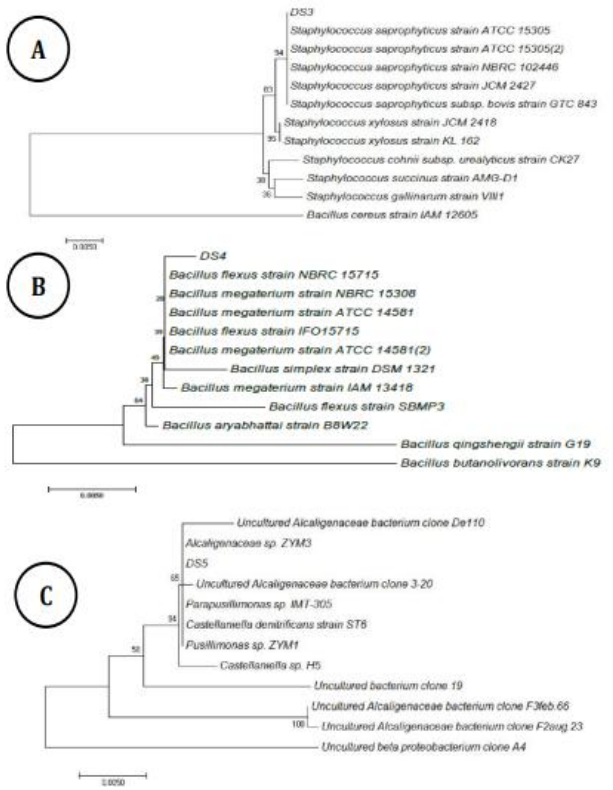
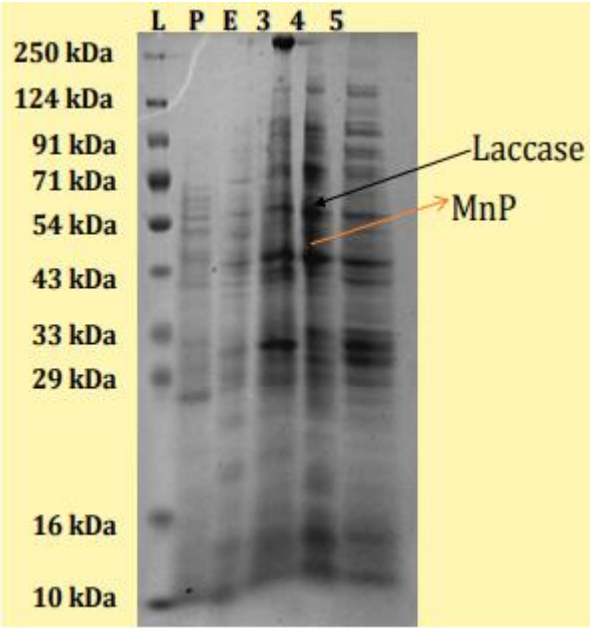



**Figure 3: Based on the 16S rDNA gene sequence, a phylogenetic tree comprising isolated bacterial strains and related species was created (A): DS3; (B): DS4); (C): DS5**

This analysis thus determined that the bacterial strains DS3, DS4, and DS5 were Staphylococcus saprophyticus, Bacillus megaterium sp., and Alcaligenaceae sp., respectively, with accession numbers MF182113, MF967441, and MF182114 (Bharagava et al., 2009; Bharagava and Chandra, 2010).

**Molecular weight determination of ligninolytic enzyme**

Three of the nine identified bacterial strains—DS3, DS4, and—then demonstrated MnP activity by changing the medium's colour from deep orange to light yellow (discussed in previous chapter). Additionally, in the partially purified enzyme's denaturing SDS-PAGE electrophoresis, all three bacterial strains formed a band of laccase and MnP with molecular weights of 65 and 43 kDa, respectively (Fig. 4)




**Figuure 4: Analysis of crude enzyme Laccase and MnP synthesis using SDS-PAGE by *Staphylococcus saprophyticus* (DS3), *Bacillus megaterium* sp. (DS4), and *Alcaligenaceae* sp. (DS5)**

The most promise is held by laccases, which have a broad spectrum of specificity and well-studied ligninolytic enzymatic activity. Laccases may be employed in a variety of industrial applications (Chandra and Chowdhary, 2015). Due to their structural features, which include carbohydrate concentrations of 15–30%, molecular weights of 60–90 kDa, and acidic isoelectric points around pH 4.0, laccases have remarkable enzymatic stability. (2006) Baldrian There are about 100 different types of compounds that may serve as substrates for laccases, and these substances differ from one laccase to another. Various types of compounds may be oxidised by laccases. Similar to this, only a few microbes have been discovered for the distillery effluent's MnP activity-dependent melanoidin metabolization (Bharagava et al., 2009).

**CONCLUSION**

The study's findings indicated that the three putative bacterial strains (DS3, DS4, and DS5) employed in the creation of the consortia were Staphylococcus saprophyticus, Bacillus megaterium sp., and Alcaligenaceae sp. (accessions numbers MF182113, MF967441, and MF182114). In addition, some bacteria species may secrete ligninolytic enzyme, which may be responsible for degrading contaminants in distillery effluent. SDSPAGE electrophoresis of partly purified enzyme exhibits yield band of laccase and MnP with the molecular weight 65 and 43 kDa, respectively, confirming their identities as the ligninolytic enzymes laccase and MnP. Therefore, these enzymes may play a role in

the breakdown of melanoidins and other phenolic and colouring chemicals found in distillery effluent.

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