Chapter 3

Material and methods

3.1 Composter design

Aerobic composting was conducted using cylindrical containers measuring 35 cm in height, 34 cm in diameter, and a total surface area of 5554.33 cm^2. These vessels had a capacity of 20 liters and were equipped with 104 evenly spaced 7 mm diameter holes across the entire surface, including the top and bottom regions, as shown in *Figure 3.1*. Within each container, a 35 cm long, 2.5 cm diameter air pipe with 4 mm diameter holes was positioned at the center and connected to an air pump. This air pump delivered a daily airflow of 0.6 L/min for ten minutes, fostering aerobic microbial activity.

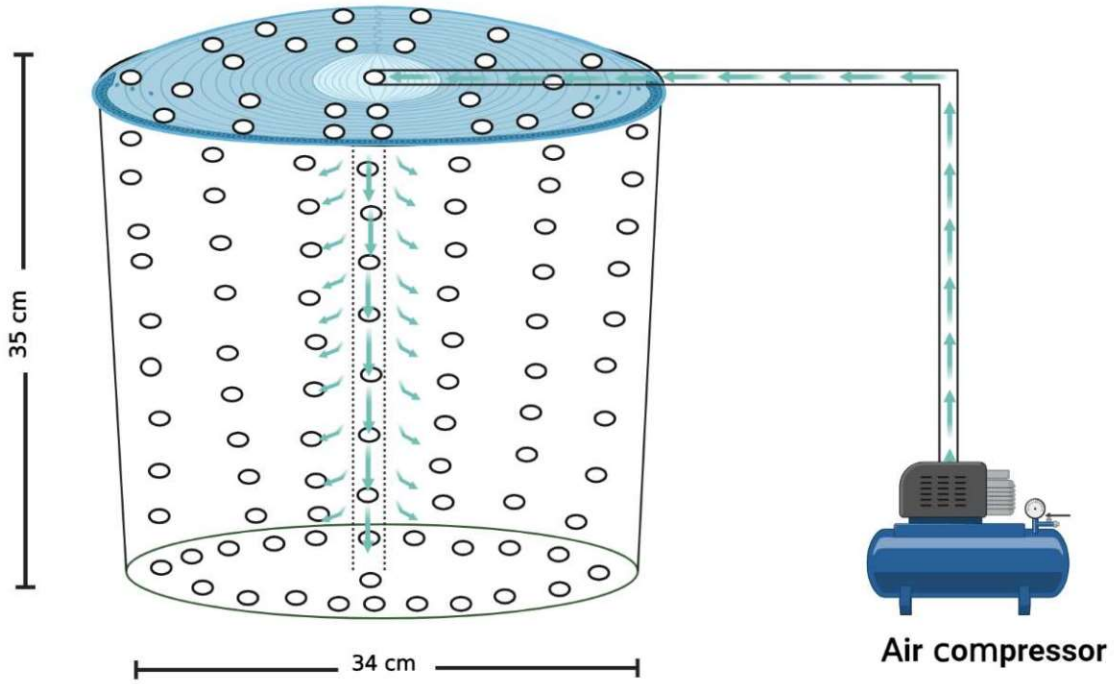

Figure 3.1. Schematic representation of a lab-scale composting unit (scale may not be proportional).

3.3 Sample collection and physicochemical parameter analysis

Compost samples were systematically collected from three distinct vertical sections: the upper portion (24 cm from the base), the middle section (12 cm from the base), and the lower segment (6 cm from the base). These collected samples were thoroughly homogenized to ensure uniformity for subsequent analysis of their physicochemical properties. Temperature measurements were recorded at each vertical tier using a calibrated digital thermometer to monitor thermal gradients within the compost reactor. Moisture content was determined by subjecting the samples to drying in a hot air oven (Equitron, Stream Series) at a controlled temperature to achieve constant weight, ensuring accurate moisture analysis. Nitrate levels were quantified through the method, while phosphate concentrations were deduced utilizing the stannous chloride treatment technique [51,52]. Moisture content, total solids (TS), and volatile solids (VS) were evaluated in line with the procedures stipulated in the Standard Methods for the Examination of Water and Wastewater [57]. Total volatile fatty acids (TVFAs) were deduced using the Montgomery spectrophotometry method [58]. pH values were measured using a portable digital pH meter (HI96107) from Hanna Instruments, India. For the quantification of lignin, cellulose, and hemicellulose content, an extractive-free RS sample was meticulously prepared using a modified Soxhlet extraction process. Benzene was employed as the solvent, in place of the usual toluene [55]. Intricate details further encompassed the determination of acid-insoluble lignin (AIL) through the Klason method and the assessment of acid-soluble lignin (ASL) via the UV spectrophotometric methodology [55][59]. Subsequently, RS's hemicellulose and cellulose content were examined after a thorough delignification of the solvent-extracted sample. This process was conducted by adhering to the sodium chlorite method [56]. The surface morphology of the samples was assessed with a scanning electron microscope (SEM) (EVO - Scanning Electron Microscope MA15/18, Carl Zeiss Microscopy Ltd.) for visualizing morphological traits. A desiccated powder sample (5 gm) was readied

through three-day drying at 105°C in a hot air oven. This sample was affixed onto a holder and imaged at 5X, 20X, and 100X magnifications. The software was used for image analysis. A comprehensive examination, including carbon, nitrogen, hydrogen, and sulfur content determination, was performed using a CHNS analyzer (EuroEA Elemental Analyser, EuroVector, Italy). The finely ground sample was placed in small tin capsules and precisely analyzed in the CHNS analyzer to record elemental composition.

3.4 Flow cytometry analysis

Flow cytometry analysis examined the changes and variations in the enhancement of microbial communities within the compost samples. The microbial community was sampled from vessels every 10 days. A 0.5 g sample was dissolved in 5 mL of phosphate-buffered saline (PBS-1X) and then centrifuged at 2000 rpm to remove debris [56]. The resulting suspension was filtered using a BD Falcon 12 × 75 mm tube with a 35 µm nylon mesh cell strainer cap (Catalog No. 352235). Flow cytometry analysis was conducted immediately using a Beckman CytoFlex Flow Cytometer equipped with blue (488 nm) and red (638 nm) lasers. Calibration of the flow cytometer was performed using CytoFlex Daily QC Fluorospheres (Beckman Coulter, Inc., USA), which emit fluorescence in the 410 nm to 800 nm range when excited by wavelengths of 405 nm, 488 nm, or 635 nm. The analysis captured 100,000 events with a threshold set at 20,000 and appropriate gain settings, enabling the identification and separation of individual cells with distinct characteristics from a heterogeneous population.

3.5 Microbial analysis

Samples for metagenomic analysis were collected at both the beginning and end stages of the composting process to capture the microbial community dynamics over time. To preserve the integrity of the DNA in these samples, they were mixed with autoclaved 50% glycerol, a cryoprotectant that prevents ice crystal formation, and stored at -80°C, ensuring long-term stability. Genomic DNA was extracted using a specialized kit designed for soil DNA extraction,

optimized to handle the complex and heterogeneous nature of soil matrices. The quality of the extracted DNA was then assessed using agarose gel electrophoresis, a technique that separates DNA fragments by size, providing a visual check for the integrity and purity of the DNA (Xploregen Discoveries Pvt. Ltd., Bangalore, Karnataka, India). For the metagenomic analysis, the V3-V4 variable regions of the 16S rRNA gene, which are informative for differentiating bacterial and archaeal taxa, were targeted using PCR amplification. Universal primers were used: the forward primer AGAGTTTGATCMTGGCTCAG and the reverse primer TTACCGCGGCKGCTGGCAC. Before the amplification, the concentration of the extracted DNA was quantified using spectrophotometry, which measures DNA absorbance at specific wavelengths to determine concentration and purity. Gel electrophoresis was again used to confirm DNA quality. To ensure successful downstream processing, further purification steps and additional PCR cycles were performed, incorporating Illumina barcoded adapters into the DNA fragments. These adapters are necessary for the preparation of sequencing libraries, which were then sequenced on an Illumina MiSeq platform using paired-end sequencing. This sequencing method reads each DNA fragment from both ends, improving the accuracy and completeness of the data. Quality control of the raw sequencing data was conducted using FASTQC, a tool for assessing the quality of high-throughput sequence data, and MULTIQC, which aggregates results from multiple samples. Adapters and low-quality reads were trimmed using TRIMGALORE to ensure clean data for analysis. The next stage involved processing the sequence data, starting with merging the paired-end reads to reconstruct the original DNA sequences. Chimeric sequences, which are artificial products of PCR, were eliminated to avoid misleading results. Operational Taxonomic Unit (OTU) abundance was computed, representing the diversity and quantity of different microbial taxa in the samples. QIIME (Quantitative Insights Into Microbial Ecology) and KRAKEN, robust bioinformatics tools, were employed for precise taxonomic assignment and estimation of abundance at the genus level. The analysis

of community composition covered a range of taxonomic classifications, including phylum, class, order, family, and genus, for both bacteria and archaea. This comprehensive approach provided a detailed understanding of the microbial communities present at different stages of composting. The entire metagenomic analysis was carried out by Biokart India Pvt. Ltd. in Bengaluru, India, ensuring thorough and accurate processing of the data.

3.6 Statistical analysis

Data visualizations were created using Origin Pro 2023b, comprehensive data analysis and graphing software, which illustrated mean values and their standard deviations, providing clear and accurate representations of the data distribution and variability. The software was also utilized for conducting detailed analyses to explore the relationships among various physicochemical properties and flow cytometric data, facilitating a deeper understanding of how these factors interact within the studied samples. The assessment of alpha diversity, a measure of species diversity within a particular habitat or ecosystem, was performed using a range of established metrics. These metrics included the Chao1 index (estimating species richness), the Shannon index (accounting for species abundance and evenness), the Simpson index (measuring the probability that two individuals from a sample belong to the same species), and Fisher's alpha (a parameter for modeling species abundance using the Fisher log-series distribution). Statistical analyses were employed to determine the significance of differences in diversity measures across different samples or conditions. These analyses included t-tests, used to compare the means of two groups, and Analysis of Variance (ANOVA), used to compare means across multiple groups. These statistical methods allowed for the identification of significant differences in alpha diversity metrics, providing insights into the richness and evenness of species within the studied environment. Overall, alpha diversity metrics offered a comprehensive view of the biodiversity within the habitat, revealing the richness (number of different species) and evenness (how evenly the individuals are distributed

among the species) of the species present. By employing these metrics and statistical analyses, the study was able to provide a detailed understanding of the biodiversity and its underlying patterns within the ecosystem. [60]. The Bray-Curtis index distance method was employed for a comprehensive beta diversity analysis focusing on the Genus taxonomic level. This method constructed a dissimilarity matrix, facilitating the quantification of sample differences based on their taxonomic composition[60]. Further, the Permutational MANOVA (PERMANOVA) statistical approach was harnessed to gauge the significance of variations in beta diversity across different groups or treatments.