Chapter 3

Materials and methods

3.1. Collection of substrate and inoculum

Rice straw was collected from an agricultural farm at Banaras Hindu University, Varanasi, Uttar Pradesh, India, in the month of November 2022 at the end of the harvesting period and immediately cut using an electric fodder cutting machine and brought to the lab. RS was first air-dried, then dried at 60 °C for 24 hours in a hot air oven (Equitron, Stream Series) to reduce moisture content until a constant weight was obtained and processed into smaller sizes using an electrical grinder (Wonderchef, Nutri-blend thunder). The rice straw was then stored in airtight plastic bags at room temperature to avoid degradation until further use for experiments. The fresh cow dung (CD) was collected from a dairy farm at Banaras Hindu University, Varanasi, India (25° 15' 19.82" N, 82° 59' 42.46" E). Mesophilic anaerobic digester slurry (ADS) was collected from a local biogas plant in Varanasi (25° 11' 33.95" N, 82° 51' 19.06" E), reportedly using cow dung mixed with water in a 1:1 ratio. Both CD and ADS were used in the study for comparison of inoculum sources. For the fungal treatment, two fungal strains, including *Pycnoporus sanguineus* (MTCC 137) and *Trichoderma logibrachiatum* (MTCC 2478), were used, which were purchased from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, Haryana. All the reagents used in this study were of analytical grade. All the samples and reagents were prepared in deionised water (MilliQ, Millipore). The physicochemical characteristics of inoculum and substrate are shown in *Table 3.1*.

Table 3.1. Physical methods of pretreatment of rice straw for improved methane generation

Characteristics*	**Rice straw (RS)**	**Cow Dung (CD)**	**Anaerobic Digester Slurry (ADS)**
Total Solids (TS, %wt)	93.51 ± 0.19	18.3 ± 0.66	7.47 ± 0.72
Volatile Solids (VS, %wt)	84.10 ± 0.39	14.83 ± 0.51	4.45 ± 0.45
VS/TS	0.90 ± 2	0.81 ± 0.87	0.60 ± 0.62
Moisture content (%)	6.50 ± 0.20	81.70 ± 0.66	92.53 ± 0.72
Cellulose (%)	43.30	-	-
Hemicellulose (%)	36.37	-	-
Lignin (%)	8.53	-	-
Silica (% of ash)	45.60		
Carbon (%)	41.38	42.06	29.27
Hydrogen (%)	6.43	-	5.31
Nitrogen (%)	<1%	<1%	<1%

* Numbers are mean ± standard deviation from three replicates

3.2. Thermal, alkali, and fungal pre-treatment of rice straw

The rice straw was treated thermally by double autoclaving it at 121 °C and 15 psi. Alkali treatment was performed by soaking the rice straw in 1% and 2% NaOH prepared in deionized water separately for 24 hours. Low concentrations were chosen to minimize the toxic effects of alkali. After 24 hours, the rice straw was washed thoroughly with deionized water until the pH tested neutral.

The rice straw was fungal treated with two different fungi, namely, *Pycnoporus sanguineus* and *Trichoderma longibrachiatum.* The pure cultures of *P. sanguineus* and *T. logibrachiatum* were grown on potato dextrose agar (PDA) plates in an incubator at 30 °C for 7 days. Subsequently, four agar medium pieces with fungal hyphae, measuring 1 cm in diameter, were introduced into 100 mL Erlenmeyer flasks containing 50 mL of potato dextrose broth. The flasks were then sealed with cotton plugs and placed in an orbital shaker, set at 30 °C, and agitated at 150 rpm for 7 days [141]. After a 7-day incubation period, the hyphae were separated by centrifuging the liquid broth at 6000 rpm for 15 minutes (Heraeus Fresco 21 centrifuge, Thermo Scientific, USA). The separated hyphae were then suspended in autoclaved deionized water to achieve a moisture content of 75% (based on total solids in rice straw). These fungal suspensions were subsequently inoculated onto autoclaved rice straw in 250 mL Erlenmeyer flasks, ensuring thorough mixing and sealing of the flasks with cotton plugs. The rice straw was fungal treated for 10, 20, 30 and 40 days to check the effect of time on the efficiency of AD.

All the pretreated samples of rice straw were first dried at 60 °C for 24 hours prior to use in anaerobic digestion to ensure the right F/M based on VS.

3.3. Experimental setup for anaerobic digestion

3.3.1. Comparison of raw versus digested manure for AD of rice straw

100 mL each of obtained inoculum sources, CD (mixed with water in a 1:1 ratio), and ADS were incubated in 500 mL glass serum bottles, sealed, and purged with 99.9% pure nitrogen at 25 per square inch (psi) pressure for 5 minutes and incubated at 37 ± 1 °C for three days in a BOD incubator (OLSC-103-17, Ocean Life Science Corporation) for acclimatization. The bottles containing only CD and ADS were labeled as CD_PC and ADS_PC, respectively, and served as controls. After 3 days, 20 g rice straw as feedstock

was added to bottles containing CD and ADS in triplicates and were named CD_RS and ADS_RS. The working volume of all the bottles was made up to 350 mL with distilled water. All the serum bottles were sealed with rubber stoppers, crimped with aluminium seals, and purged using 99.9% pure nitrogen gas at 25 per square inch (psi) pressure for 5 minutes and incubated at 37 ± 1 °C in the BOD incubator and digested for 45 days.

3.3.2. Comparison of thermal, alkali, and fungal pre-treatments for AD enhancement of rice straw

The thermal, alkali, and fungal-treated rice straw was inoculated in 500 mL serum bottles containing acclimatized ADS as inoculum in an F/M ratio of 1 on a VS basis. The volume of all the serum bottles was made up to 350 mL to obtain 5% TS. The AD bottles containing thermally treated rice straw were named "Autoclaved." The AD bottles containing alkali-treated rice straw were named "NaOH 1% and NaOH 2%," respectively, based on the concentration of NaOH taken for pretreatment. The AD bottles containing *Pycnoporus sanguineus* treated rice straw were labeled as PS-10D, PS-20D, PS-30D, and PS-40D depending on the duration of treatment, i.e., 10, 20, 30, and 40 days, respectively. Similarly, the AD bottles containing *Trichoderma longibrachiatum* treated rice straw were labelled as TL-10D, TL-20D, TL-30D, and TL-40D, respectively. All the bottles were sealed, purged with nitrogen, and incubated in a BOD incubator as per the conditions described in the last section.

3.3.3. Efficiency of fungal treatment at higher feedstock concentration

While comparing the different pretreatments, it was observed that fungal treatment gave the lowest biogas and methane production values. This may be attributed to the fact that fungi have their own nutritional requirements and depend on the substrate for their growth and maintenance. While feeding on the organic matter, fungi penetrate the straw with their

mycelia. The breakdown of the organic matter makes the structure of rice straw easily accessible to the AD microbes. The initial higher biogas and methane production may be attributed to the same. The drop in production after that may be a reason for the depletion of available carbon. To test the efficiency of fungal treatment, anaerobic digestion was carried out by increasing the content of rice straw. After acclimatization, bottles with only inoculum were the negative control (NC), while bottles with 20 g of untreated rice straw added to acclimatized inoculum were the positive control (PC). Separate bottles labeled as PS and TL contained 20 g rice straw treated with *P. sanguineus* and *T. logibrachiatum*, respectively. The working volume in all bottles was increased to 350 mL with distilled water. Experiments were conducted in triplicates. Each serum bottle was tightly sealed with rubber stoppers, secured with aluminium seals, purged with nitrogen gas for 5 minutes, and placed in a BOD incubator at a temperature of 37 ± 1 °C for 45 days. Biogas production was measured every three days throughout the experiment using a water displacement column, and cumulative values were reported in mL/g VS.

For all the experiments, biogas and methane production were measured every 3 days using a water displacement column and gas chromatography, respectively. Cumulative biogas and methane are reported in mL/g VS, and biogas and methane yield recorded every three days are reported in mL.

3.4. Analytical methods

Standard Methods for the Examination of Water and Wastewater [142] were used to determine moisture content, total solids (TS), and volatile solids (VS) present in the feedstock and inoculum sources. Samples for sCOD and cellulase analysis were withdrawn every 3 days using a syringe with a 16-gauge needle and then centrifuged at 12000 revolutions per minute (rpm) (Heraeus, Fresco 21, Thermo Scientific, USA) for 10

minutes. To determine sCOD, the supernatant was mixed with potassium dichromate and sulphuric acid and boiled in a COD digester for 90 minutes. The sample was cooled, and a few drops of ferroin indicator were added, and the sample was titrated against 0.1N ferrous ammonium sulphate (FAS) until the color changed to red. The volume of FAS was noted to achieve the end point [143]. The sCOD was expressed in mg/L and was calculated using the following formula:

$$sCOD = \frac{8000(b-s)N}{Sample\ volume}$$

where,

b = volume of FAS used in the blank sample

S = Volume of FAS used in the original sample

N = Normality of FAS

Cellulase activity was measured by using carboxymethyl cellulose (CMC) as a substrate and supernatant from the centrifuged samples as a source of enzyme. After incubation for 30 minutes, the glucose formed as a product was estimated using the 3,5-dinitro salicylic acid (DNSA) method by measuring absorbance at 540 nm [144]. Carboxymethyl cellulase activity (CMCase), which is the combination of exoglucanase and endoglucanase, can be measured as the quantity of enzyme needed to generate one micromole of glucose in one minute.

Xylanase activity was estimated using the method described by Fatma et. al, by measuring xylose formed from Beechwood xylan using the 3,5-dinitro salicylic acid (DNSA) colorimetric method by measuring absorbance at 540 nm [145]. The cellulase and xylanase activities were measured in U/mL, where U represents the enzyme necessary to release 1 μmol of product per minute.

The activity of lignin peroxidase (LiP) enzyme was measured by the oxidation of the azure B dye method (Arora and Gill, 2001). The LiP enzyme activity was quantified by expressing one unit as a decrease of 0.1 optical density (O.D.) units per minute per millilitre of the culture filtrate at 651 nm [146]. Total VFAs were determined using the Montgomery method, a spectrophotometric method that quantifies the carboxylic esters present in a sample [147].

The methane content of biogas was determined using a gas chromatograph (Nucon-5765, Nucon Engineers, India) equipped with a flame ionization detector (FID) and a PORAPAK-Q column operated at a detector, injector, and oven temperature of 180°C, 120°C, and 90°C, respectively.

VFAs were determined by the spectrophotometric method based on quantifying the carboxylic esters present in a sample, also known as the Montgomery method [147]. AD samples were first filtered from a 0.22 μm nylon filter, then 100 μL of this sample was transferred to dry test tubes to which 300 μL of ethylene glycol followed by 40 μL of 19.2 N sulphuric acid was added, and the mixture was heated for 3 minutes in a boiling water bath. After cooling down the mixture, 100 μL of 10% hydroxylamine hydrochloride solution and 400 μL NaOH (4.5 N) followed by 2 mL ferric chloride (5% prepared in 1N sulphuric acid) were added. Absorbance was taken at 495 nm by NanoDrop One^{C} (Thermo Scientific).

To determine lignin, cellulose, and hemicellulose, the rice straw sample was dried in a hot air oven at 60 °C for 3 days to obtain a constant weight and then ground to 1-5 mm particle size. An extractive-free sample of rice straw was prepared by the Soxhlet extraction method described by Jung et al. [148], except that toluene was replaced by benzene as the solvent. Lignin content in the extractive free sample in terms of acid-

insoluble lignin (AIL) was determined by the Klason method, and acid-soluble lignin (ASL) was determined by the UV spectrophotometric method [148]. Holocellulose content in rice straw was determined after the delignification of the extractive-free sample with the sodium chlorite method as described by Rabemanolontsoa and Saka [149]. The content of cellulose present in holo-cellulose was determined according to the testing method for alpha, beta, and gamma cellulose in pulp (KS M 7044 method) [150]. The weight of hemicellulose was determined by deducting the weight of cellulose from the weight of holocellulose.

3.5. Instrumental analysis

The visualization of morphological changes and understanding of the elemental composition of the rice straw samples was performed using a Scanning Electron Microscope (SEM) (EVO - Scanning Electron Microscope MA15/18, Carl Zeiss Microscopy Ltd.) equipped with Energy dispersive spectroscopy (EDS) (51N1000, Oxford Instruments Nanoanalysis). An analysis of changes in functional groups was conducted using a Fourier Transform Infrared (FTIR) spectrophotometer (Nicolet iS5, Thermo Electron Scientific Instruments LLC). The KBr was mixed with the sample and pressed to form a disc. The infrared spectra were evaluated using the percentage transmittance mode in the 4000 to 500 cm^{-1} frequency range with a resolution of 4 cm^{-1}.

3.6. Metagenomic analysis

Samples for metagenomic analysis were collected at the beginning and the end of the anaerobic digestion experiment in sterile microcentrifuge tubes and were mixed with autoclaved 50% glycerol and stored at -80 °C until processed for deoxyribonucleic acid (DNA) extraction. DNA extraction was carried out using a commercially available genomic DNA kit for soil (Xploregen Discoveries Pvt. Ltd., Bangalore, Karnataka,

India). The integrity of DNA was tested using agarose gel electrophoresis. Polymerase chain reaction (PCR) was carried out for the amplification of the V3-V4 variable regions of extracted DNA was carried out using universal primers 16s forward (AGAGTTTGATGMTGGCTCAG) and reverse (TTACCGCGGCMGCSGGCAC) primers for the identification of bacteria and archaea. Extracted DNA from the samples were subjected to NanoDrop and gel check before being taken for PCR amplification. 40 ng of extracted DNA was amplified along with 10 pM of each primer. PCR amplification was performed with the following conditions: an initial denaturation step at 95 °C for 3 minutes, followed by annealing at 60 °C for 15 seconds, elongation at 72 °C for 2 minutes, and final extension at 72 °C for 10 minutes and then hold at 4°C. After that, the amplicons from each sample were purified with Ampure beads to remove unused primers, and an additional 8 cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantified using a Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2x300PE v3 sequencing kit.

Raw data QC was done using FASTQC and MULTIQC. Trimming of adapters and low-quality reads was carried out by TRIMGALORE. The trimmed reads were further taken for processing, including merging of paired-end reads, chimera removal, and OTU abundance calculation, and estimation correction was achieved by QIIME and KRAKEN workflows. This workflow enables highly accurate investigations at the genus level. The community composition analysis was conducted at taxonomic levels, including phylum, class, order, family, and genus for bacteria and archaea. Metagenomic analysis was carried out at Biokart India Pvt. Ltd., Bengaluru, India. The pictorial description for the metagenomic analysis work-flow is described in *Figure 3.1*.

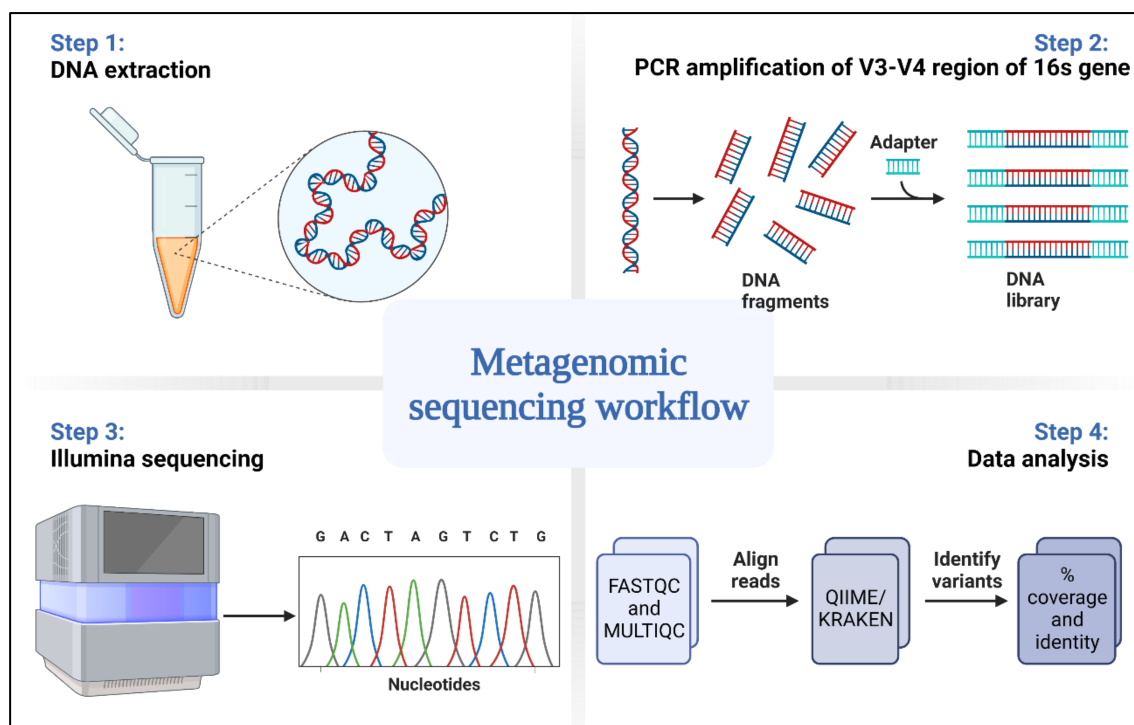

Figure 3.1. Steps for the metagenomic analysis (created in www.biorender.com)

3.7. Flow cytometric fingerprinting

The anaerobic microbial community was sampled every 3 days for flow cytometry analysis from each serum bottle after measuring biogas using an 18-gauge needle and syringe and processed using the method outlined by Dhoble et al., 2016, with some modifications. Before analysis, samples from each serum bottle were mixed with an equal volume of phosphate buffer saline (PBS-1X). The resulting mixture underwent two rounds of centrifugation at 2000 rpm, pooling the supernatants from each step. The pellet was washed three times with PBS, then resuspended in PBS. The suspension was filtered using a BD Falcon 12 × 75 mm tube with a cell strainer cap featuring a 35 µm nylon mesh (Catalog No. 352235) to remove debris. Analysis was conducted immediately using a Beckman CytoFlex Flow Cytometer equipped with blue (488 nm) and red (638 nm) lasers. For calibration, CytoFlex Daily QC Fluorospheres (Beckman Coulter, Inc., USA) with fluorospheres of approximately 3 µm size and emitting fluorescence in the range of

410 nm to 800 nm when stimulated by 405 nm, 488 nm, or 635 nm wavelengths were used as an internal standard. A total of 100,000 events were collected during the analysis.

3.8. Statistical analysis

Redundancy analysis (RDA), correlation analysis, and other statistical analysis were performed in OriginPro 2023b (Origin Lab Corporation, MA, US). Evaluation of significance was conducted in Excel 2019 (Microsoft, WA, USA) employing a t-test, and a p-value ≤ 0.05 was considered significant. OTU abundance was calculated using QIIME and KRAKEN workflows. Alpha diversity refers to the diversity of species within a single habitat or ecosystem. The beta diversity was constructed at the taxonomic level of Genus with the Bray-Curtis index distance method based on Permutational MANOVA (PERMANOVA) statistical method.