Chapter 3

Methodology and experiment planning

This chapter focuses on experimental designs and their associated methods that accomplish the objectives. Accordingly, the study has been divided into four phases, and the details of the designs pertaining to the protocols of the specific phases have been described in succeeding chapters and enclosed publications.

Phase I: Studying the variations in decomposition process of lignocellulosic waste materials with species reference to microbial community structure

In this phase of the research spent mushroom straw (SMS) was taken as a suitable representative of lignocellulosic wastes. The reason for taking SMS as the sample has been elaborated in Chapter 4. For the purpose of the study, the SMS was collected from the oyster mushroom (*Pleurotus ostreatus*) product unit of Defense Research and Development Organization (DRDO), Defence Research Laboratory (DRL), Tezpur, Assam, India. Well-grown adult (clitelleted) specimens of three epigeic earthworm species namely *Eisenia fetida, Eudrilus eugeniae,* and *Perionyx excavatus* were collected from the vermiculture unit of the Department of Environmental Science, Tezpur University. Both the composting and vermicomposting batches were prepared with different treatment combinations along with the Cow dung. The efficiency assessment of various earthworm species in terms of the end product was enumerated by different microbial and physic-chemical attributes. The overall details of phase I has be represented in a Schematic diagram **Fig 3.1.**The details of the experimental setup along with analysis are described in **chapter 4.**

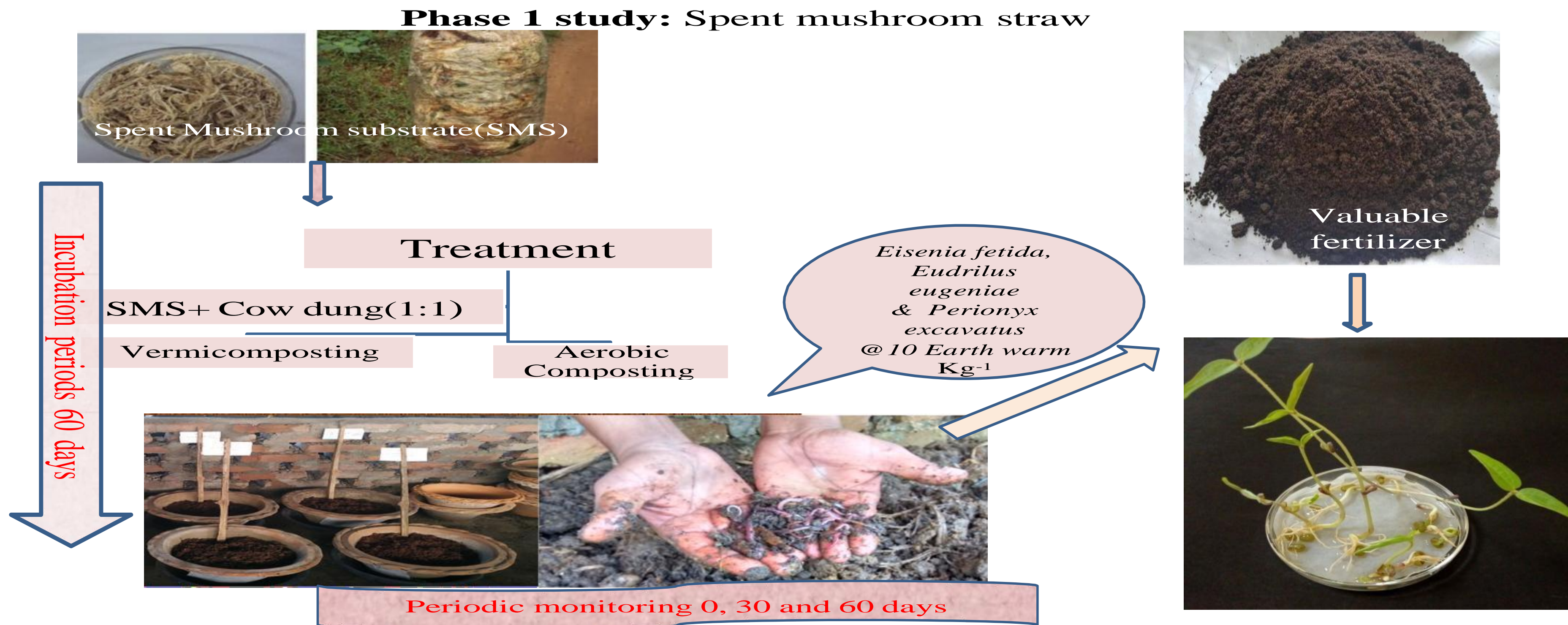

Fig. 3.1: Schematic representation of the work done under phase I

Phase II: Effect of earthworm stocking density on microbial profile and end product quality in vermibeds composed of kitchen vegetable waste, banana stem, and cow dung

In this phase, kitchen vegetable waste (KVW) and banana stem (BS) were selected as representatives of lignocellulosic wastes. The justification of such selection has been provided in Chapter V. The main aim of this phase of the study was to evaluate the effects of initial stocking density of earthworms (*Eisenia fetida* and *Eudrilus eugeniae*) on end product quality, humification process, and microbial community profiles in the vermibeds in comparison to aerobic composting.. The optimum efficiency assessment was enumerated on the basis of Physico-chemical [1] and biochemical attributes [2, 3].

The details of the experiment are described in **chapter V.**

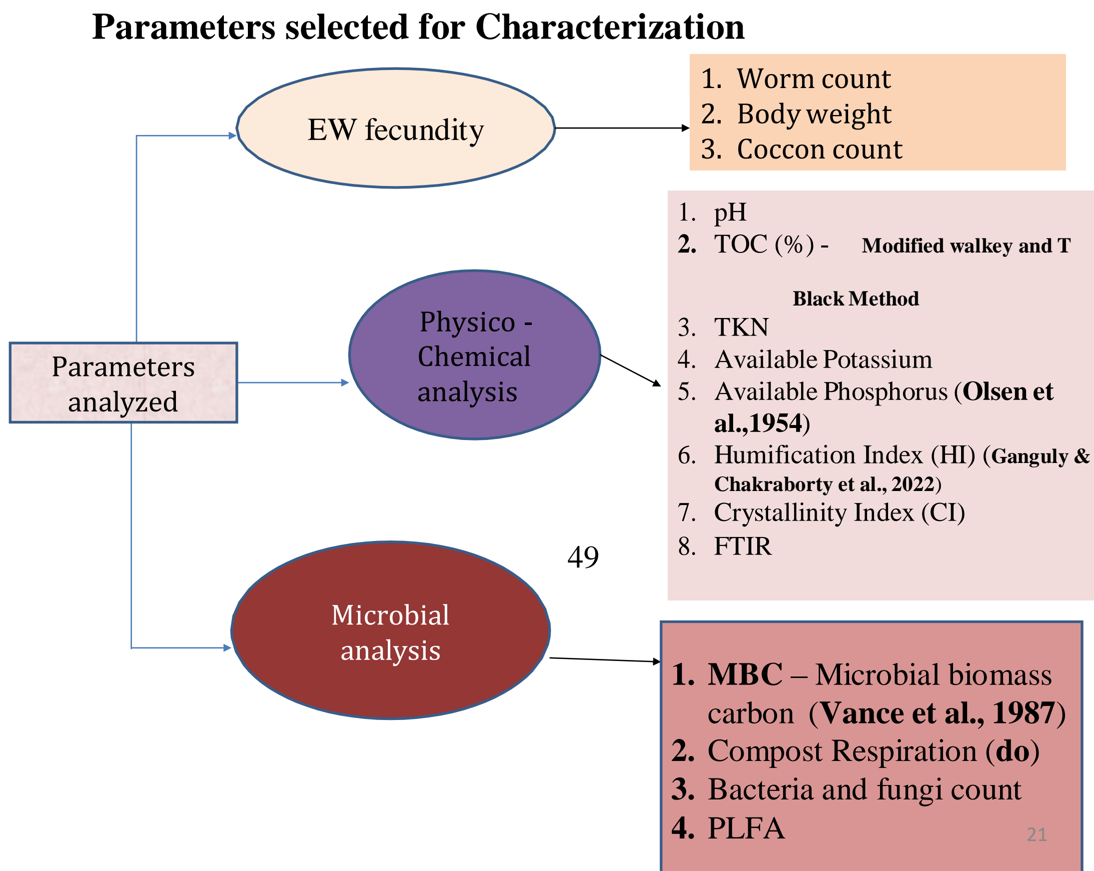

Fig. 3.2: Schematic representation of the work done under phase II

Phase III: Polyaromatic hydrocarbons (PAHs) detoxification routes by *Eisenia fetida* and *Eudrilus eugeniae*

This experiment was designed to evaluate the PAH degradation kinetics in vermicomposting system. The details of the experimental plan has been elaborated Chapter 6 of the thesis. In short, 13 designated PAHs were spiked to cow dung based vermicomposting substrates in different concentrations and incubated with two earthworm species (*Eisenia fetida* and *Eudrilus eugeniae*). Subsequently, the temporal variation of the concentrations of the spiked-PAHs was assessed in the vermibeds as well as in the earthworm body. The PAH degradation profile was further evaluated with the help of few evolved equations and apportionment budget of the compounds was enumerated. Appropriate measures were also taken for quality assurance and control (QA & QC) of the method of estimation of the PAHs through HPLC-based techniques. The details of the QA & QC approach (i.e., method detection limits (MDLs) and relative standard errors (RSEs)) for the all the 13 PAHs have been presented in Chapter 6 of the thesis.

Phase IV: Designing innovative continuous-flow mechanized vermireactor and performace assessment

This phase deals with the comparison of different vermireactors towards the smarter and more sanitized end products. The different types of vermireactors were designed and their maturity and end product quality were analyzed. In short, the design of the continuous-flow mechanized vermireactor was developed with the help of Mr. Rakesh Bhadra, Assistant Professor, Dept. of Mechanical Engineering, Tezpur University and a prototype was prepared. This performance of this prototype was evaluated in comparison with two types of conventional vermireactors. The detail of the experiment is described in **Chapter 7.**

Following parameters were used to derive a feasible understanding about the performance of the developed technology:

Chemical parameters:

1. pH (APHA, 1998)[1]
2. Total nitrogen (TN) [7]
3. Total organic C (TOC) [8]
4. Available phosphorous (Av. P) [9], and
5. Available potassium (Av. K)
6. Mn, Zn, Cd followed the DTPA extraction method

Among the biological properties, the following parameters will be analyzed.

Compost respiration, Microbial Biomass Carbon, enzymes, humic compounds, and 16s rRNA sequencing-based metagenomic analyses.

Quality assurance and quality control: All analyses were performed following the general quality control (QC) guidelines published by Tezpur University. A monitoring system without adequate QA/QC runs the risk of not being able to control the quality of data, and not being able to assure accuracy and precision.

Sample storage and preservation

After collecting Compost and vermicompost samples firstly air dried, grounded, and sieved according to NCR 13 and AOAC recommendations **[7]**. Subsequently, air-dried samples were stored in a plastic bottle with numbers and dates accordingly. Before storing the samples, plastic bottles were cleaned and rinsed with reagent water, and dried. Most of the samples were analyzed within 2-3 days post collection. In the case where storage was required, samples were stored at 4o C for a maximum of 28 days after collection.

Purity of chemicals, reagents, lab wares

All the used chemicals were of GR grade (guaranteed reagent) with certified 90-98% purity. For the preparation of regents, ultrapure water from the water purification system (Sartorius Stedim, Germany) was used. On the day of analysis, freshly individual reagents were prepared. The plasticware and glassware were washed with reagent water, rinsed with double distilled water, and oven-dried prior to every use.

Calibration procedures

Used analytical instruments were calibrated beforehand and adjusted accordingly. Before the beginning of the new analysis, the calibration was verified for new samples. This procedure includes one blank and several standards. Some instruments like pH meter do not require blanks so several standards were taken on a regular basis. Standard reference materials (SRMs) that offer certified values of analytes were used throughout the experiments to ensure the accuracy of the analysis. The highest purity in all standards (for pH meter, electrical conductivity meter, UV-Vis spectrophotometers, ICP-OES, AAS) was maintained.

Initial demonstration of performance

The initial demonstration of performance was used to characterize instrument performance (determination of linear calibration ranges) and laboratory performance i.e. determination of method detection limits prior to performing analyses.

Linear calibration range (LCR)

The LCR for major instruments like High-performance liquid chromatography (HPLC), UV-Vis spectrophotometer, Kjeltec N analyzer, ICP-OES, and AAS was initially determined and verified every 6 months or whenever a significant change in instrument response was observed. Generally, linearity verification was done by using 1 blank and 3 standards. For HPLC, the standard was injected after every 5 samples to maintain the accuracy of the results. In general, the linearity was re-established in the case when verification data exceeded the initial values by ± 10%.

3.5.6 Method detection limit (MDL)

To establish MDLs either reagent water (blank) or standard solutions for different analytes. To determine MDL values were determined for different analyses 7 replicates of aliquots of working standard solutions or fortified reagent water were analyzed following the entire analytical methods.The MDLs were calculated using the following formula:

$$MDL = t \times S$$

Where t= student's t value for a 99% confidence level a standard deviation estimate with n-1 degrees of freedom [t= 3.14 for seven replicates]. S = standard deviation of the replicate analyses. MDLs should be determined twice every six months. A list of MDLs for different analytes is given in the appendix.

Operations and maintenance

All the analytical instruments and equipment optimized conditions were maintained with all records of correct operation, calibration, and troubleshooting. Guidelines followed for daily operations of the following laboratory equipment were as follows:

Equipment: HPLC, UV-VIS Spectrophotometers, pH Meter & probes Conductivity Meter, Balances, and Automated Pipettes.

Calibrations

HPLC: Initially The instrument was warmed up for about 15-20 minutes before every use. After the warm-up, the column and other pipes were cleaned with mildly warm ultrapure water and purged for 30 minutes. The solvents were freshly prepared and utmost care was taken to ensure maximum purity. To minimize the error rate the machine was calibrated with various concentrations of standards. To verify the authenticity of the results, standards were injected after every 5 samples.

Maintenance: Yearly twice maintenance done by professionals.

UV-VIS Spectrophotometers: Verification of calibration was done after the initial warm-up of the instrument, every 30 samples analyzed.

Maintenance: Yearly maintenance yearly once by professionals.

ICP-OES: Verification of calibration was done after the initial warm-up of the instrument at every 30 samples analyzed. If periodic QC samples fail to be within control limits then a recalibration is used to be conducted.

Maintenance: Yearly maintenance once by professionals.

pH Meter & probes: Calibrating of the instrument at pH 4, 7, and 9 with standard solutions was done at every 100 samples.

Maintenance: Checking regular probes and ensuring that electrodes were filled.

Conductivity Meter: The conductivity probes were first cleaned with double distilled water before and after every use. The surfaces of the probes were usually wiped with high-absorbent paper towels to ensure their dryness. The instrument was frequently calibrated with known solutions supplied with the instrument.

Balances: Checking the balance was necessary daily before use. To nullify the error in measurement the balances were kept in air-tight cabinets. By weighing authentic weights the precision of the balances was routinely checked.

Maintenance: Necessary cleaning was performed with ethanol after every use. The dust and fallen chemicals during weighing was vacuum cleared. The instrument was serviced and calibrated by certified company engineers once in a year.

Automated Pipettes: calibration of used pipettes was done every 15 days.

Maintenance: Adequate cleaning was done after every use.

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