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Materials and Methods

The present investigation deals with eco-friendly bioconversion methods of cocoa shell and jack fruit peel waste, various biocomposting treatments, pit composting, vermicomposting, total microbial load, physico-chemical, morphological characteristics of biocompost, different biometric characters, biochemical parameters, yield attributes, leghaemoglobin content in the nodules of the test crops, pre and post-harvest soil status, antibacterial, antioxidant activity, phytochemical screening and mineral composition of selected test crops and statistical analysis. The methods adopted are presented in this chapter.

PHASE I

3.1 Collection of Agroindustrial wastes

The agroindustrial waste cocoa shell and jack fruit peel waste was collected in large amount from Calicut and the Wayanad district of Kerala. The collected wastes were chopped into small pieces. It was sun-dried and stored in gunny bags.

3.1.1 Collection of Microorganisms

The microorganism *Pleurotus eous* and *Pleurotus florida* spawn were collected from Tamil Nadu Agricultural University (TNAU), Coimbatore and earthworms *Eudrilus eugeniae* Kinberg were obtained from KVK (Krishi Vigyan Kendra), Coimbatore.

3.1.2 Preparation of Biocomposting units

The biocomposting process of cocoa shell and jack fruit peel waste consist of pit composting (30 days) and vermicomposting (60 days)**.**

Preparation of Cocoa Shell Waste (CSW) Biocompost

The discarded cocoa shells were treated to different degradation processes to produce high-quality biocompost (Plate - 8).

Biocompost 1 (C_1)

The sundried 2 kg of cocoa shell waste was transferred to C_1 pit. This process was repeated till the heap reaches a height of above 1m. Water was sprinkled on the heap to preserve its moisture content. During 30 days of composting, rotating was done manually

every week to hasten the decomposition process. After 30 days, C_1 is permitted to undergo the vermicomposting process.

Biocompost 2 (C_2)

2 Kg of cocoa shell waste and 20 g of *Pleurotus eous* were added to the C_2 pit. It was left to decompose for 30 days. Vermicomposting was implemented after 30 days of pit composting. Water was sprinkled on the heap to keep the moisture content up. Rotating was done manually once a week for 30 days to expedite the decomposition process.

Biocompost 3 (C_3)

Sundried cocoa shell waste was used to fill C_3 pit and 20 g of *Pleurotus florida* spawn was distributed evenly. 2 kg of cocoa shell waste was put over this layer. This technique was continued until the heap reached a height of above 1m. Water was sprinkled on the heap to keep the moisture content up. To speed up the decomposition process, rotating was done manually once a week for the first 30 days of composting, and then the vermicomposting technique was implemented.

Biocompost 4 (C_4)

Sundried cocoa shell waste, 10 g *Pleurotus eous* and 10 g *Pleurotus florida* spawn were uniformly placed in C_4 pit. This layer was covered with 2 kg of cocoa shell waste. This process was carried out until the heap reached a height of 1m. To keep the moisture content high, water was sprayed over the heap. Rotating was done manually once a week for the first 30 days of composting to speed up the breakdown process, and then the vermicomposting approach was applied.

Preparation of Jack fruit peel waste (JFPW) Biocompost

Biocomposting pits of Jack fruit peel waste (C_5, C_6, C_7&C_8) was followed using the same technique as described for C_1, C_2, C_3, and C_4 of cocoa shell waste.

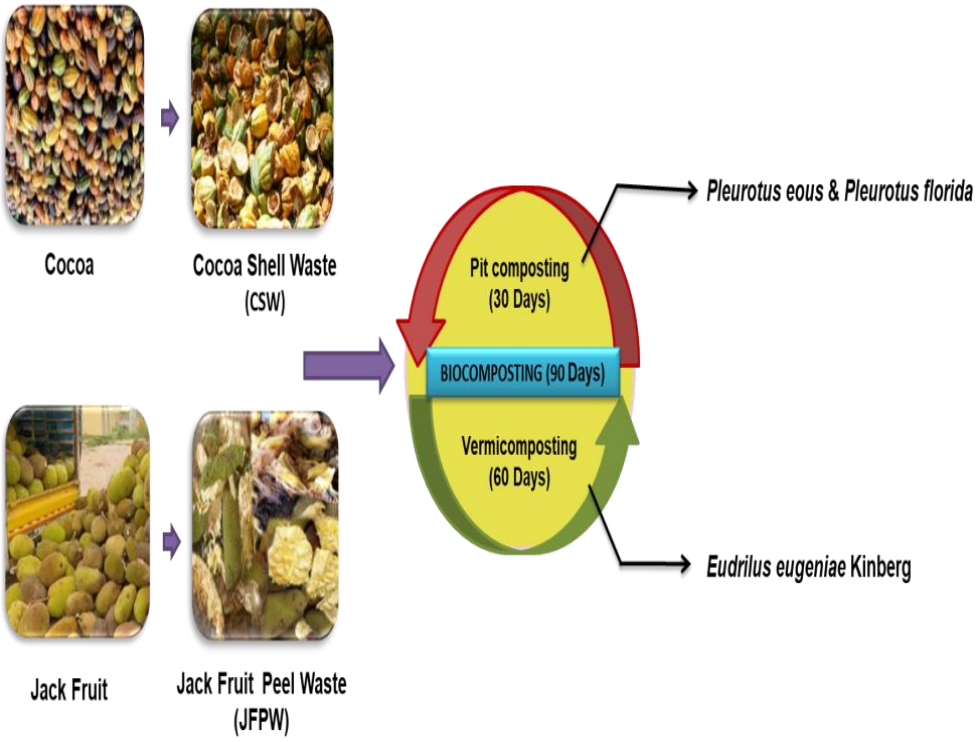
3.1.3 Pit composting

2 feet in length and 4 square feet in width of eight pits were used for pit composting. They were named C_1 (biocompost 1- Raw cocoa shell + *Eudrilus eugeniae* 5 t/ha^{-1}),C_2 (Biocompost 2 -Raw cocoa shell + 20 g *Pleurotus eous*+*Eudrilus eugeniae* 5 t/ha^{-1}),C_3 (Biocompost 3- Raw cocoa shell+ 20 g *Pleurotus florida*+*Eudrilus eugeniae* 5 t/ha^{-1}),C_4 (Biocompost 4 -Raw cocoa shell+10 g *Pleurotus eous* +10 g *Pleurotus florida*+*Eudrilus eugeniae* 5 t/ha^{-1}), C_5 (Biocompost 5 -Raw Jack fruit peel+ *Eudrilus eugeniae* 5 t/ha^{-1}),C_6

(Biocompost 6 -Raw Jack fruit peel + 20 g *Pleurotus eous*+*Eudrilus eugeniae* 5 t/ha^{-1}),C_7 (Biocompost 7 - Raw Jack fruit peel + 20 g *Pleurotus florida* +*Eudrilus eugeniae* 5 t/ha^{-1}) and C_8 (Biocompost 8 - Raw Jack fruit peel +10 g *Pleurotus eous*+10 g *Pleurotus florida*+*Eudrilus eugeniae* 5 t/ha^{-1}).

PLATE - 7

Schematic Representation of Biocomposting of CSW and JFPW



3.1.4. Preparation of Vermicomposting trays by *Eudrilus eugeniae* Kinberg

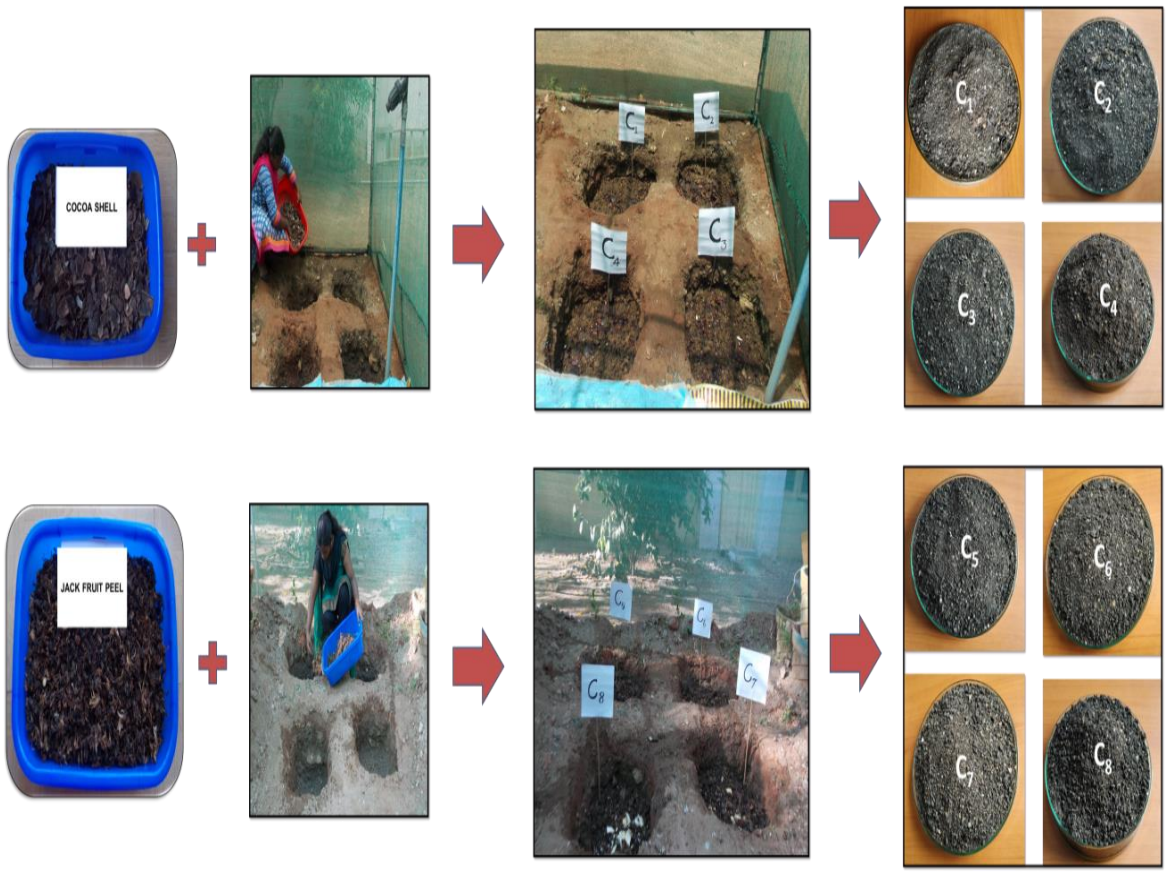
After 30 days of pit composting C_1-C_8 biocomposting units were transferred into eight plastic trays (40×20×20 cm). Small holes were made at the bottom to ensure air circulation in the vermicompost bin and drain the excess water in the experimental trays.

Pre-digested cocoa shell (C_1,C_2,C_3&C_4) and jack fruit peel (C_5,C_6,C_7&C_8) biocomposting units were taken for vermicomposting. Vermibeds are prepared by pre-decomposed CSW and JFPW and cow dung slurry. 30 healthy, *Eudrilus eugeniae* Kinberg (exotic earthworms) were inoculated into respective designated eight experimental trays. The worms entered the media immediately after the inoculation.

All the trays were covered on the top by jute cloth and water was sprayed regularly twice a day to maintain the moisture content. These experimental units were kept undisturbed in the shady place for 60 days. At the end of vermicomposting, the biocomposting units turned dark black and brown colour on 90 days and the earthworms were separated by sieving.

PLATE - 8

Preparation of CSW and JFPW Biocompost



3.2 Microbial Population during Biocomposting

One gram of each sample was taken in sterile conical flasks containing 9ml of distilled water, shaken for 30 min in vortex mixer and used as stock from which various dilutions were prepared to range from 10^1 to 10^7 with sterile distilled water as described by Kannan (1996). One ml each of the dilutions of 10^7 (bacteria), 10^4 (fungi) and 10^2 (actinomycetes) from each sample was transferred to sterile petri plates containing nutrient

agar medium (bacteria), potato dextrose agar medium (fungi) and Ken-Knights agar medium (actinomycetes) respectively. Bacteria, fungi and actinomycetes were cultured in triplicates and incubated for one day**,** three days and seven days respectively. After incubation, the total microbial colony was counted from the respective growth medium. The microbial load was recorded at regular intervals of 30 days from 0 to 30, 30 to 60 and 60 to 90 days. The viable colony count was done with the help of colony counter.

3.2.1 Physico-chemical Analysis of raw and Prepared Biocompost

The physicochemical characterization of raw and biocomposted samples were analysed on the following standard procedures.

Physical parameters

pH and electrical conductivity of the raw and biocomposted CSW and JFPW were estimated by ELTOP digital pH meter and Deluxe conductivity meter 602.

Chemical parameters

Chemical Parameters	**Reference**
Lignin	Goering and Vansoest, (1975) Appendix -1
Cellulose	Updegroff, (1969) Appendix - 2
Organic carbon (%)	Wakley and Black, (1934) Appendix - 3
Total Nitrogen (%)	Humphries, (1956) Appendix - 4
Total Phosphorus (%)	Jackson, (1973) Appendix – 5
Total Potassium (%)	Jackson, (1973) Appendix – 6
Calcium and magnesium (%)	Jackson, (1973) Appendix -7

3.2.2 FTIR analysis

The active surface functional groups present in the raw and biocomposted cocoa shell and jack fruit peel were identified using FTIR (MIRacle 10 Shimadzu). The samples were scanned in the range of 400-4000 cm^{-1}.

3.2.3 Scanning Electron Microscopy Analysis (SEM)

The surface morphological changes in raw and biocomposted cocoa shell and jack fruit peel samples were analysed using SEM (MIRA3, TESCAN) operated between 5.0 and 10.0 kV. The prepared samples (CSW, JFPW, C_1,C_2,C_3,C_4,C_5,C_6,C_7&C_8) were put inside the microscope's vacuum column, and once the vacuum was formed, a high-intensity beam of electrons was focused on the surface of the sample. These high-energy beams contact the atoms in each sample and produce secondary electrons on the surface. These electrons were examined by a detector, which produces images of the surface.

PHASE II

3.3 Pot Culture Experiment

A Pot culture experiment was conducted with cowpea (*Vigna unguiculata* (L.) Walp.Var. Co(CP)7, Yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc. Var.NS-621), Shankhpushpi (*Clitoria ternatea* L.) and Red amaranth (*Amaranthus tricolor* L. Var.Arun) as the test crops to evaluate the effect of biocomposted cocoa shell and jack fruit peel.

3.3.1 Collection of Seeds

Seeds of *Vigna unguiculata* (L.) Walp Co (CP) 7 were collected from the Department of Pulses, Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu. Seeds of *Vigna unguiculata* subsp.*sesquipedalis* (L.) Verdc (Var. NS-621) and *Amaranthus tricolor* L (Var. Arun) were collected from Krishi Bhavan, Kozhikode, Kerala and *Clitoria ternatea* L were collected from Arya Vaidya Sala Herb Garden, Malapuram, Kerala.

3.3.2 Authentication of Test crops

The plant samples, *Vigna unguiculata* (L.) Walp. (Identification No: BSI/SRC/5/23/2022/Tech/395) Annexure -1,*Vigna unguiculata* subsp.*sesquipedalis* (L.) Verdc (Identification No BSI/SRC/5/23/2022/Tech/188) Annexure - 2,*Clitoria ternatea* L. (Identification No : BSI/SRC/5/23/2022/Tech/186) Annexure - 3, *Amaranthus tricolor* L. (Identification No: BSI/SRC/5/23/2022/Tech/187) Annexure - 4, has been authenticated by Dr.M.U Sharief, Scientist F & Head of office, Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore.

3.3.3 Treatment Details

The experiments were carried out with control and eight treatments using biocomposted Cocoa shell waste (CSW) and jack fruit peel waste (JFPW). The biocomposed treatments composition and their cod were as detailed below.

Treatments code	Treatments composition	Biocompost
C	Control (Without treatment)	Control
T_1	Raw CSW + *Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 1
T_2	Raw CSW+ 20 g *Pleurotus eous*+*Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 2
T_3	Raw CSW+ 20 g *Pleurotus florida*+*Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 3
T_4	Raw CSW+10 g *Pleurotus eous*+10 g *Pleurotus florida*+ *Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 4
T_5	Raw JFPW+ *Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 5
T_6	Raw JFPW + 20 g *Pleurotus eous*+*Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 6
T_7	Raw JFPW +20 g *Pleurotus florida*+*Eudrilus eugeniae* 5t/ha^{-1}	Biocompost 7
T_8	Raw JFPW +10 g *Pleurotus eous*+ 10 g *Pleurotus florida* + *Eudrilus eugeniae* 5 t/ha^{-1}	Biocompost 8

3.3.4 Treatment Application and Pot Culture Experiment

The bulk soil samples were collected from the uncontaminated area of thondamuthur, Coimbatore. One hundred eight pots were filled with 6 kg of red sandy soil. The biocompost was applied to the respective pots and mixed thoroughly. The greenhouse was used for conducting pot culture experiments. Twenty seeds of cowpea, yardlong bean, shankhpushpi and red amaranth were sown in each pot. Three replications were used for each treatment. After germination four healthy plants were maintained per pot.

3.4 Evaluation of Vegetative Characters of Test Crops

Vegetative characteristics of test crops included root length (cm), shoot length (cm), number of leaves, number of branches, number of nodules, plant fresh weight (g) and plant dry weight (g) for cowpea at 25 DAS, 35 DAS, and 45 DAS, yardlong bean at 25 DAS, 50 DAS, and 75 DAS, shankhpushpi at 30 DAS, 60 DAS and 90 DAS, and Red amaranth at 25 DAS and 35 DAS. The number of flowers per plant was recorded on 45 DAS (Cowpea),65 DAS (Yardlong bean) and 90 DAS (Shankhpushpi) respectively.

3.4.1 Evaluation of Yield Parameters of Test Crops

The yield parameters of the test crops included the number of pods per plant, number of seeds per pod, length of the pod (cm), weight of the pod (g), weight of the seeds per pod (g), fresh weight of pod (g) and dry weight of pod (g) recorded in cowpea at 65 DAS, yardlong bean at 90 DAS and shankhpushpi at 120 DAS. Yield characteristics of red amaranth were examined for root length (cm), shoot length (cm), number of leaves per plant, plant fresh weight (g) and plant dry weight (g) on 50 DAS. The four test crops were uprooted from the respective pots in the above mentioned days after sowing (DAS).

PHASE III

3.5 Biochemical Analysis

Biochemical parameters analysed in leaves and seeds of selected test crops.

3.5.1 & 2 Estimation of Protein and Carbohydrate

Leaves: Estimated on cowpea (25, 35 and 45 DAS), yardlong bean (25,50 and 75 DAS), Shankhpushpi (30,60 and 90 DAS) and Red amaranth at (25 DAS and 35 DAS).

Seed: Estimated on cowpea (65 DAS), yardlong bean (90 DAS), Shankhpushpi (120 DAS) and Red amaranth at (50 DAS). Protein determined by the method of Lowry *et al*., (1951) and Carbohydrate based on the method of Hedge and Hofreiter, (1962) as given in Appendix - 8 & Appendix - 9.

3.5.3. Estimation of Chlorophyll

Chlorophyll a, chlorophyll b and total chlorophyll estimated on vegetative stage of cowpea (25 DAS, 35 DAS & 45 DAS) yardlong bean (25 DAS, 50 DAS &75 DAS) and shankhpushpi (30 DAS, 60 DAS & 90 DAS) leaves. Chlorophyll a, chlorophyll b and total chlorophyll in vegetative stage and yield stages of red amaranth was estimated according to the method by Arnon, (1949) as described in Appendix-10.

3.5.4 Estimation of Leghaemoglobin content

Leghaemoglobin content in nodules of Cowpea and Yardlong bean estimated on vegetative days assessed by the method of Appleby and Bergersen, (1980) as given in Appendix-11.

3.6. Estimation of Crude protein

Crude protein content in seeds of Cowpea at 65 DAS and Yardlong bean at 90 DAS was determined by the method of AOAC (2016) as elaborated in Appendix-12.

3.7 Pre and Post-harvest Soil Analysis

Soil analysis	Reference	Method
Available nitrogen	Subbiah and Asija 1956	Alkaline permanganate (Appendix -13)
Available phosphorus	Jackson 1973	Colorimetry (Appendix -14)
Available potassium	Standford and English 1949	Flame photometry (Appendix -15)

PHASE IV

3.8 Antibacterial activity

The antibacterial properties of selected plant aqueous and methanol extracts were determined using the standard method of Kirby-Bauer technique (Bauer *et al*., 1996), as detailed in Appendix-16. Mueller-Hinton agar was used to assess the antibacterial activity of cowpea and yardlong bean seed, shankhpushpi, and red amaranth leaf. Antibacterial activity of test crops was evaluated using pathological strains such as *Escherichia Coli* and *Staphylococcus aureus.*

3.9. Assessment of Antioxidant activity of Test crops

Antioxidant assays were performed on best treatment (T_8) and control-grown test crop seeds (Cowpea and Yardlong bean) and leaves (Shankhpushpi and Red amaranth). The ability of test crop aqueous and methanol seeds and leaf extracts to scavenge free radicals was assessed by DPPH radical scavenging activity, hydrogen peroxide scavenging activity, nitric oxide radical scavenging activity and reducing power assay.

The dried powdered seeds and leaves of the chosen test crops were progressively extracted in a soxhlet extractor with methanol and water. The sample was dried in a hot air oven before extraction with the subsequent solvent each time. The sample was then macerated in hot water for 24 hours while being occasionally stirred, and the aqueous extract was filtered. Using a rotary evaporator to concentrate the various solvent extracts, they were then air dried. Before further investigation, the extracts were freeze-dried and kept in desiccators.

3.9.1 DPPH Radical Scavenging Activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity) radical scavenging assay was carried out in aqueous and methanol extracts (seed and leaf) of test crops was estimated by the method of Mensor *et al.,* (2001) as given in Appendix - 17.

3.9.2 Hydrogen peroxide Scavenging Activity

Hydrogen peroxide scavenging activity of aqueous and methanol seeds and leaf extracts of test crops was carried out according to the method of Ruch *et al*., (1989) as described in Appendix - 18.

3.9.3 Nitric oxide Radical Scavenging Activity

Nitric oxide radical scavenging activity of aqueous and methanol seeds and leaf extracts of test crops was performed by Green *et al.,* (1982) as elaborated in Appendix-19.

3.9.4 Reducing Power Assay

Reducing power assay was carried out in aqueous and methanol seed and leaf extracts of test crops was assessed based on the method of Oyaizu, (1986) as described in Appendix -20

3.10 Phytochemical Assessment

The active secondary metabolites present in aqueous, ethyl acetate, and methanol extracts of *Clitoria ternatea* L. and *Amaranthus tricolor* L. leaves were determined using a standard technique (Harborne 1998). The preliminary phytochemical tests for alkaloids, anthraquinones, amino acids, flavonoids, glycosides, phenols, steroids, saponins, tannins, and terpenoids were performed in accordance with the techniques given in Appendix -21.

3.10.1 Preparation of Plant Extract

Medicinally active plant of *Clitoria ternatea* L. (Shankhpushpi) and *Amaranthus tricolor* L **(**Red amaranth**)** leaves were selected for phytochemical screening. The best treatment (T_8-Raw Jack fruit peel +10 g *Pleurotus eous*+10 g *Pleurotus florida+Eudrilus eugeniae* 5 t/ha^{-1}) under grown Shankhpushpi and Red amaranth leaves were used in this experiment. Leaves were collected, cleaned, and dried in the shade and dried for 24 hours in a hot air oven at 60^{o}C. The dried leaves were pulverised with an electric blender to obtain a fine leaf powder. The phytochemical elements of this fine powder were examined. The plant was extracted overnight with aqueous, ethyl acetate, and methanol extracts and filtered through Whatmann No.1 filter paper. On these extracts, qualitative tests were performed.

3.10.2 Mineral Composition Analysis of Test crops

Mineral Analysis was carried out in best treatment (T_8- Raw Jack fruit peel +10 g *Pleurotus eous*+10 g *Pleurotus florida+Eudrilus eugeniae* 5 t/ha^{-1}) and control leaves. The dry and fine leaf powder of cowpea, yardlong bean, shankhpushpi and red amaranth were analyzed for sodium**,** phosphorus, potassium, calcium, iron, magnesium and zinc respectively. Among this sodium and calcium were determined using Flame photometer. Potassium, iron, magnesium and zinc content in leaves were estimated using Atomic Absorption Spectrophotometer (AAS) 4129 as described by (Subba Rao, 2003) Appendix-22. Phosphorus is determined by using Nitro-vanado-molybdate method (Jackson, 1958) as given in Appendix -23.

Statistical Analysis

The experimental findings are provided in the form of tables and graphs (Figure) created with Microsoft Excel (Version 2013) and Origin (Version 6.0) software. The data obtained from the microbial population during biocomposting, as well as various biometrical, yield parameters, biochemical characters, antibacterial and antioxidant activity of test crops, were subjected to SPSS (Version sigma stat 3.1) statistical analysis (one-way and two-way ANOVA) and conclusions were drawn based on the results. The program output results reveal if the differences between the tests are substantial. These values are provided in the respective tables and Figures (graph) in chapter 4 (Results and discussion).