Materials and Methods
3.1. EXPERIMENTAL REQUIREMENTS

The materials used and the methods adopted for determining the toxicity reduction of *Calotropis gigantea* (L.) R. Br. during biomethanation are given below.

3.2. PLANT MATERIAL USED

**CALOTROPIS GIGANTEA (L.) R. Br.** [Plate 3.1]

The plant description, ecological distribution, phytochemistry, economic values and medicinal properties have been already discussed in section 2.7.

3.3. BIOGAS POTENTIAL OF CALOTROPIS

3.3.1. Collection and preparation of biomass

*Calotropis* twigs were collected from the campus playgrounds of Urumu Dhanalakshmi College, Tiruchirappalli, and authenticated through the Rapinat Herbarium St.Joseph’s college Tiruchirappalli. The leaves were treated with 2 per cent (w/v) solution of sodium hydroxide at a temperature of 80°C for overnight to augment digestion (Jimenez *et al.*, 1989). The treated leaf material was sun dried, finally oven dried at 60°C and ground into a fine powder to be used as a substrate for laboratory scale digester. This powdery substrate is hereafter referred to as the biomass.

3.3.2. Source of inoculum

*Calotropis* biomass does not contain cellulolytic and/or methanogenic bacteria responsible for biogas generation. Hence fresh cow dung (corresponding to 17% dry matter) was used as seeding material (Chakraborthy, 2002) with *Calotropis*.

From a local dairy farm, cow dung was collected as a single cattle source. The fresh dung was diluted with two volumes of water (w/v). After homogenization, the slurry was passed through a 1mm mesh sieve to remove the debris.

3.3.3. Substrates for fermentation

Cow dung and water in the ratio 1:2 (w/v) served as a control substrate (Ranade *et al.*, 1987; Kasali, 1990 and Chakraborthy *et al.*, 1996).

The experimental substrate consisted of solid matter and water in the ratio 1:2 (w/v). The solid matter contained cowdung (corresponding to 17 per cent dry matter) and *Calotropis* powder in the ratio 1:4 (w/w) (Chakraborthy, 2002). The quantities of organic carbon in the
Plate 3.1. *Calotropis gigantea* Habit
control substrate and experimental substrate were maintained to be approximately equal (Humphries, 1956). The pH of the above two substrates were adjusted to 6.8.

3.3.4. Biogas generation and quantification

Anaerobic batch fermentor

The control and the experimental substrates were subjected to fermentation in batch fermentors assembled in the laboratory (Plate 3.2a & b). Two different reactors were maintained for the control and the experimental substrates.

Air tight glass reagent bottles of 2.5 litre capacity (12cm diameter and 25cm height) were taken as reactor vessels. A separate gas outlet with a metal ball valve was fixed at the top of the reactor vessels. It was fitted to a rubber tube of 5mm diameter. The other end of the rubber tube was connected to the gas bottle containing 5 per cent (w/v) NaOH solution with thymol blue as an indicator. NaOH solution was chosen, because it absorbs CO₂ and allows CH₄ to pass through it. The blue color of the indicator disappeared when the CO₂ absorption capacity of the solution was exhausted. Replacement with a fresh batch of NaOH solution was done.

The digester was kept inside the Tempo water bath cum shaker, and it was shaken at 50 rpm until the completion of biogenesis. The temperature of the water bath was maintained at 37°C. The raise in the level of substrate and appearance of effervescence confirm the initiation of methanogenesis. The emitted biogas was collected by the downward displacement of water. The gas accumulated in a graduated measuring jar containing water. The volume occupied by the biogas was recorded for every 6 hours. The average values of the duplicate fermentors were recorded. Flux is the difference between the quantity of methane generated by the control (cow dung) and the experimental substrate (Calotropis biomass).

3.4. SOME PHYTOCHEMICAL COMPONENTS OF COW DUNG AND CALOTROPIS BIOMASS BEFORE AND AFTER BIOMETHANATION

3.4.1. Estimation of cellulose (Updegraff, 1969)

Reagents

i) Acetic acid + nitric acid mixture (150ml of 80% acetic acid + 15ml of concentrated nitric acid).
Plate 3.2 a. Schematic Representation of the Laboratory Bioreactor for Biomethanation

Plate 3.2 b. Laboratory Bioreactor Designed for Biomethanation
ii) Anthrone reagent (0.2g of anthrone dissolved in 100ml of concentrated sulphuric acid) prepared freshly and chilled for 2 hours before use.

**Procedure**

Five ml of reagent (i) was added to 100 ml of reagent (ii) in a test tube and thoroughly mixed using a vortex mixture [REMI- CM 101]. The tube was kept in a water bath at 100°C for 20 minutes. The samples were centrifuged at 4000-5000 rpm for 15-20 minutes in a centrifuge [REMI- C29] and the supernatant liquid was discarded. The residue was washed two or three times with distilled water and recentrifuged. The washed residue was treated with 67% sulphuric acid and allowed to stand for one hour. One ml of the above solution was diluted to 100ml. To 1ml of the diluted solution in a test tube, 4ml of distilled water was added and cooled in a ice bath. Then 10ml of cold anthrone reagent was added by layering with a pipette and mixed well. The tubes were placed back in ice bath after mixing. A marble piece was placed in each of the test tubes and kept in boiling water and cooled in ice bath for 2-3 minutes and placed at room temperature for 5-10 minutes. The optical density was measured at 620 nm against a reagent blank in a spectrophotometer (SYSTRONICS 104). The cellulose content was calculated in percentage from the standard curve. The samples of cow dung and biogas residue before and after biogas production were analyzed. All the estimations were repeated six times.

**3.4.2. Estimation of hemicellulose and lignin** (Goering and Van Soest, 1975)

**Reagents**

i) **Cold neutral-detergent solution**

An amount of 18.61g of disodium ethylenediaminetetraacetate dehydrate and 6.8g of sodium borate tetrahydrate were taken in a beaker and dissolved in distilled water by heating, and to this 30g of sodium lauryl sulphate and 10ml of 2-ethoxy ethanol were added. To this, 4.5g of disodium hydrogen phosphate was added, the volume made upto 1 litre and the pH was adjusted to 7.0.

ii) **Decahydro naphthalene**

iii) **Sodium sulphite**

iv) **Acetone**

v) **Cold acid detergent solution**

Twenty gram of acetyl trimethyl ammonium bromide in 1 litre of 1N sulphuric acid.
vi) 72% Sulphuric acid

Procedure

One g of Neutral Detergent Fibre (NDF) was placed into a refluxing flask and 100ml of cold neutral detergent solution was added. To this 2ml of decahydronapthalene and 0.5g of sodium sulphite were added. The mixture was refluxed for 60 minutes. The contents were filtered through a sintered glass crucible (G2) by suction and washed twice with hot water followed by acetone. The residue was then transferred to a crucible, dried at 100°C for 8 hours. The weight of dried residue gives NDF, expressed in wt/wt basis. Similar experiments were carried out separately, with cow dung and biogas residues. All the experiments were conducted in 6 replicates.

One gram of Acid Detergent Lignin (ADL) sample with 100ml cold acid–detergent solution was used for estimation. Refluxing was done as detailed for NDF. Further analysis was carried out following the procedure detailed for NDF estimation. The hemicellulose content in percentage was determined by subtracting ADL from the NDF. Thus,

\[ \text{NDF} - \text{ADL} = \text{Hemicellulose} \]

Estimation of lignin

The dried acid detergent fibre was treated with 72% sulphuric acid for three hours, then filtered, washed thoroughly with distilled water to make it free from acid, dried at 100°C and weighed. The residue was then ignited in a muffle furnace at 550°C for 3 hours cooled and weighed. The ADL content was calculated by the loss of weight upon ignition.

3.4.3. Estimation of organic carbon (Walkley and Black, 1934)

Procedure

One gram of fresh leaf material was weighed and transferred to a 500ml conical flask. 20ml of 1N potassium dichromate and 20ml of concentrated sulphuric acid were added and the contents were mixed gently. The mixture was allowed to stand for 20-30 minutes at room temperature.

The blank was also run in the same manner. The solution was diluted by adding 200ml of distilled water and 10ml of 85% orthophosphoric acid and 3 drops of diphenylamine
indicator. The contents were titrated against 0.5M, ferrous ammonium sulphate solution. The change of colour from blue to brilliant green was taken as the end point. The experiment was conducted in 6 replicates for each sample.

**Calculation**

1ml of 1N K₂Cr₂O₇ = 0.003g of carbon

Volume of potassium dichromate =20ml

Blank titer value = BTV

Titer Value=TV

Moisture content = M

Percentage of organic carbon = \[20 - \frac{20xTVx.003x100x100}{BTVxWt \text{ of sample} x (100-M)}\]

**3.4.4. Estimation of total nitrogen** (Humphris, 1956)

One g of dry powder, 5g of catalyst mixture (0.5g CuSO₄ and 4.5g K₂SO₄) and 10ml of H₂SO₄ were taken in a sand bath at 400°C and digested for 1 hour. A clear apple green solution indicated the complete digestion. The solution was cooled for 15 minutes, 15ml of distilled water and 20 ml of 40% NaOH were added and distilled. Ammonia, thus released, was collected in 20ml of 4% boric acid, which was titrated against 0.1N H₂SO₄ using a drop of indicator (2.5ml of methyl red and 2.5ml of methylene blue dissolved in 500ml of 4% boric acid). The end point was the appearance of pink colour. From the titre value the percentage of nitrogen was calculated as follows:

A blank titration was done without the sample. 0.01N H₂SO₄ is equivalent to 0.14mg nitrogen. Blank value was subtracted from the titer value.

Percentage of organic nitrogen= \[\frac{[V₂-V₁]xNx0.014x100]}{W}, \text{ where}\]

\[V₁ = \text{Volume of 0.01N H₂SO₄ used to neutralize the blank}\]

\[V₂ = \text{Volume of 0.01N H₂SO₄ used to neutralize the distillate of the sample}\]

\[N = \text{Normality of standard acid (H₂SO₄)}\]

\[W = \text{Weight of the sample in grams}\]

The experiment was carried out in 6 replicates for the samples.
3.4.5. Estimation of volatile fatty acids (Rand et al., (1976))

Reagents

i) Silicic acid

A volume of 250ml of acid was suspended in distilled water and allowed to stand for 15 minutes. The supernatant liquid portion was decanted. The treatment was repeated several times. The washed acid was thoroughly dried in an air-oven at 103°C for 30 minutes and then stored in a desiccator.

ii) Chloroform-butanol reagent

Three hundred ml of chloroform, 100ml 1-butanol and 80ml 0.5N H₂SO₄ were well mixed in a separating funnel and allowed to stand for some time till inorganic and organic layers were formed distinctly. The lower organic layer was drained off through a fluted filter paper and stored in a dry bottle.

iii) Thymol blue indicator solution

Eighty mg of thymol blue was dissolved in 100ml of absolute methanol.

iv) Phenolphthalein indicator solution.

Eighty mg of phenolphthalein was dissolved in 100ml absolute methanol.

v) Concentrated sulphuric acid

vi) Standard sodium hydroxide:

Sodium hydroxide of 0.02N was prepared by drawing out 20ml of IN NaOH stock solution into 100ml water and equally diluted with absolute methanol. The solution was then standardized.

Procedure

i) Pretreatment of sample

The crushed samples were vacuum filtered enough to obtain 10-15ml of sludge in a test tube. A few drops of thymol blue were added as indicator to it followed by concentrated H₂SO₄ in drops till the color changed from red to blue (pH 1.0 to 1.2).
ii) Column chromatography

An amount of 12 g of silicic acid was placed in a Gooch glass crucible and suction was applied to pack the column. Five ml of acidified sample was pipetted out and distributed uniformly over the surface of the column. Finally, 65ml of chloroform-butanol reagent was used to elute the column. Fresh column were used for each sample.

The eluent was supplied with nitrogen immediately and then titrated with standard NaOH solution (0.02N). The end point was the disappearance of pink colour.

Five ml of acidified distilled water was prepared and was placed on the column and extracted with 65ml chloroform - butanol reagent and titrated.

Calculation

Total organic acid (mg/l as acetic acid)

\[
\text{Total organic acid (mg/l as acetic acid)} = \frac{(a-b) \times N \times 60,000}{\text{Vol. of sample in ml}}
\]

\[a = \text{Volume of NaOH in ml consumed by sample},\]
\[b = \text{Volume of NaOH in ml consumed by blank},\]
\[N = \text{Normality of NaOH} \]

The estimations were carried out in 6 replicates for each sample.

3.4.6. Estimation of total phosphorus (Jackson, 1973)

Reagents

i) **Triple acid mixture:** Concentrated nitric acid, sulphuric acid and perchloric acid in 9:2:1 (V/V) ratio.

ii) **Ammonium molybdate:** Five grams of ammonium molybdate dissolved in 10ml of distilled water

iii) **Ammonium metavanadate (0.25%):** 1.25g of ammonium metavanadate was dissolved in 300ml of hot water with 250ml of con. HNO₃.

Procedure

Dried sample (500mg) was taken in a flat bottomed flask and digested with 15 ml of triple acid mixture over a hot plate till ash white digest was obtained. The digested material was filtered through a filter paper, and the volume was made up to 50ml.
To this 1.25ml of ammonium molybdate and 1.25ml of ammonium metavanadate (0.25%) were added. The absorbance of the colour thus developed, was read in a Spectronic-20 colorimeter at 540nm against a reagent blank. The amount of phosphorus was calculated by referring to the calibration curve with potassium dihydrogen phosphate expressed in percentage. 6 replicates sampling were performed.

**Calculation**

\[
\text{Percentage of phosphorus} = \frac{X_{\text{ppm}} \times 50 \times 100 \times 100}{1000000 \times 5 \times 0.5}
\]

where \(X\) is the sample reading

3.4.7. Estimation of total potassium (Jackson, 1973)

**Preparation of standard potassium solution**

The standard potassium solution was prepared by dissolving 1.9079g of potassium chloride in distilled water and making up to 100ml. Then 10ml of the solution was diluted to 1000 ml and used as standard solution.

**Procedure**

Dried sample (500mg) was digested with 15ml of triple acid mixture of HNO\(_3\), H\(_2\)SO\(_4\) and HClO\(_4\) in the ratio of 9:2:1 (v/v). The digestion was carried out till a white mixture was obtained. The flask was cooled and the contents were made up to 50ml with distilled water.

Different concentrations of the standard potassium chloride solution were fed into flame photometer [ELICO-CL361] and the standard curve was prepared by the transmittance recorded using potassium filter. The sample was also fed in the same manner and from the calibration curve; the percentage of potassium in the unknown was calculated.

**Calculation**

\[
\text{Percentage of phosphorus} = \frac{X_{\text{ppm}} \times 50 \times 100 \times 100}{1000000 \times 5 \times 0.5}
\]

where \(X\) is the sample reading
3.5. BACTERIAL DIVERSITY IN THE RHIZOPLANE/RHIZOSPHERE

3.5.1. Isolation and enumeration of bacteria from rhizoplane

The root sample of *Calotropis* was collected along with the soil in a sterile polythene bag and brought to the laboratory. The rhizoplane soil was separated from the root, with the help of a sterile brush. 10g of soil sample was weighed aseptically, added to 100ml of sterile distilled water, and was shaken for 15 minutes on a mechanical shaker [REMI - RS 24 BL]. After shaking, serial dilutions were prepared (10^{-2} to 10^{-6}), and 0.1 ml of each dilution was transferred to sterile Petri plates having nutrient agar medium in 6 replicates [Streak plate method] (Sabitharani *et al.*, 2003). After inoculation the plates were incubated at 37°C for 24 hours. All the inoculated plates were observed for the number and distribution of colonies from each dilution (10^{-2} to 10^{-6}). The bacterial colony morphology, consistency, pigmentation, margin and elevations were carefully examined. Later some of the cells from the colonies were subcultured into suitable media (Blood agar media plates for *S. aures* and *B. subtilis*, EMB agar for *E. coli*, Mac Conkey agar for *P. aeruginosa*) and incubated for 48-72 hours at 37°C. The plates were observed for the growth of pure colonies. Based on the morphological character and their biochemical reactions the bacterial genera were identified.

3.5.2. Isolation of bacteria from rhizosphere

Soil sample was collected a little away (about 5 cm) from the rhizoplane soil for rhizosphere soil analysis. For isolation and enumeration of microbes identical protocols were followed as that of rhizoplane soil.

Results

Microbial count in rhizoplane and rhizosphere soils were calculated by following the formula

\[
\text{Number of microorganisms/g of soil} = \frac{\text{Number of colonies}}{\text{Dilution factor} \times \text{Volume of the sample taken}}
\]

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3.6. EVALUATION OF ANTIMICROBIAL ACTIVITY OF *CALOTROPIS* ON THE RHIZOSPHERE BACTERIA

3.6.1. Plant material

The healthy mature *Calotropis* leaves were washed with tap water followed by distilled water several times, dried under shade and hot air. The air dried material was powdered and stored in an airtight glass container.

3.6.2. Preparation of extracts

The plant extract was prepared by the method of Alade and Irobi (1993) with minor modification. The air dried powdered leaf material was extracted with 80% methanol twice constantly. The extract was filtered through Whatman No.1 filter paper. The filtrate was further concentrated in a vacuum rotary evaporator. The residue was weighed and stored at 4°C until further use.

3.6.3. Test organisms

Four test organisms *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis* were obtained from the rhizosphere area of *Calotropis* plant by serial dilution technique. The organisms were subcultured in their appropriate media. Based on the gram reaction and the biochemical tests the organisms were identified and confirmed.

3.6.4. Culture media

The tested strains were subcultured twice for activation in Erlenmeyer flasks (125ml) containing 25ml of Muller Hinton broth (NCCLS, 1982) at 37°C for 24 hours. Optical density was determined at 600nm. The culturing was stopped at 0.8 to 1.0 OD₆₀₀ range (equivalent to 10(CFU) and used for antimicrobial testing).

3.6.5. Screening of antibacterial activity

The agar well diffusion method (Perez *et al.*, 1990) was adopted for this assay. An amount of 23g of ready made (Hi-media) Muller – Hinton agar was suspended in 1000ml of distilled water and dissolved completely. The medium was autoclaved at 15 psi for 20 minutes. The medium was poured into petri plates under aseptic conditions in a laminar air flow chamber and left to solidity. These Petri plates were inoculated with 0.1ml of 24 hour
old cultures of concentrated test organisms. After inoculation cups were scooped out with 3mm wide sterile cork borer and the lids of the dishes were replaced. To each cup, 20μl sample (plant extract suspension) of different concentrations (200 – 2000 μg) was added (Onkar et al., 1995). Antimicrobial activity was evaluated by measuring the zone of inhibition against each test organism, (Minimum Inhibitory Concentration test (Rios et al., 1988)). The plates were incubated at 37°C for 24 - 48 hours. After the incubation period, the diameter of the inhibition zone of each well was measured. Triplicates were maintained in each extract and the average values were calculated for the eventual antimicrobial activity. Simultaneously the standard antibiotics (Ampicillin 30μg) were tested against the bacteria.

3.7. QUANTITATIVE DETERMINATIONS OF SECONDARY METABOLITES

Calotropis leaf sample was collected from Urumu Dhanalakshmi College campus playgrounds. The leaf was allowed to air dry in a sheltered area at 25-30°C. The air dried leaf samples were ground in a mixi, and the milled samples were stored at ambient temperature in sealed plastic bags for subsequent analysis.

Similarly the residue obtained after methanogenesis, was allowed to air dry in the sheltered area at 25-30°C until moisture was exhausted. The sample was ground, and it was stored in a plastic bag for subsequent analysis. The Calotropis leaf powder and the biogas residue were subjected for qualitative identification of the following secondary metabolites with appropriate standards (Table 3.1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Secondary metabolites</th>
<th>Reference Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flavonoid</td>
<td>[Catechin] (Wang et al., 2003)</td>
</tr>
<tr>
<td>2.</td>
<td>Terpenoid</td>
<td>[γ-Terpinene] (Dubber and Kanfer, 2006)</td>
</tr>
<tr>
<td>3.</td>
<td>Alkaloid</td>
<td>[Histamine] (Shen and Shao, 2006)</td>
</tr>
<tr>
<td>4.</td>
<td>Steroid</td>
<td>[Stigmasterol] (Nair et al., 2006)</td>
</tr>
</tbody>
</table>
Chromatographic condition/analytical determination

The contents of secondary metabolites were qualitatively measured by high pressure liquid chromatography (HPLC).

The instrument is a JASCO series liquid chromatograph system comprising vacuum degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector. The column used was a C.18 reversed phase [HYPOSIL] 5 μm C18 (250mm x 4.2mm) guard column.

3.7.1. Terpenoids (Dubber and Kanfer, 2006)

3.7.1.1. Preparation of sample

Five g of *Calotropis* leaf powder and residue were weighed separately. Each sample was extracted with a mixture of 30ml of ethyl acetate and 10ml of water. The mixture was allowed to settle. The organic layer was separated and washed with three 10ml portions of water. The combined aqueous layers were extracted with 30ml ethyl acetate. The combined ethyl acetate extracts were mixed with equal volume of chloroform and filtered. The filtrate was evaporated to dryness under vacuum to afford a yellow residue, which was dissolved in 3ml methanol. Finally the sample solution was filtered through 0.45μm membrane filter, and the filtrate was employed for HPLC analysis.

3.7.1.2. Preparation of standard γ - terpinene

The standard solution was prepared by dissolving 20μg of γ - terpinene in 10μl mobile phase solvent.

3.7.1.3. Mobile phase solvent system

The mobile phase was prepared by mixing water, methanol and 2-propanol in a ratio of 72.5: 17.5:10 (w/v). The flow rate was 1ml min⁻¹. The detector was set 220nm. The sample injection volume was 10μl. The column temperature was set at 30°C. The peaks were identified by comparing their retention times with the authentic standards.
3.7.2. Flavonoids (Wang et al., 2003)

3.7.2.1. Preparation of sample

Five g of milled *Calotropis* leaf powder was accurately weighed and extracted with 40 ml of a solution of ethanol/water [10:90 v/v] by sonication for 20 minutes. The extract was filtered into a 50 ml volumetric flask and made up to 50 ml with the same solvent. Approximately 4 ml of sample solution was filtered through a 0.45 μm membrane filter and the filtrate was used for the HPLC analysis.

3.7.2.2. Preparation of standard Catechin

The standard catechin solution was prepared by dissolving 20 μg of catechin in 10 μl of mobile phase solvent.

3.7.2.3. Mobile phase solvent system

The mobile phase consisted of 0.1 per cent orthophosphoric acid in water (v/v) [eluent A] and 0.1 per cent orthophosphoric acid in methanol [eluent B]. Elution was performed at a solvent flow rate of 1 ml/min. The column was maintained at 30°C. The sample injection volume was 10 μl. Peaks were identified by comparing their retention times and UV spectra measured in the 200-400 nm range with authentic standards.

3.7.3. Steroids (Nair et al., 2006)

3.7.3.1. Preparation of sample

Five g of dried leaf powder and the residue were taken in 100 ml volumetric flask in triplicates. Later 8 ml of methanol was added and mixed well. The mixture was ultrasonicated for 20 minutes and was allowed to cool for 5 minutes. Thereafter the volume was made up to 100 ml with methanol. 100 ml of methanol solution was concentrated to 10 ml and kept in a 10 ml volumetric flask, followed by the addition of 1 ml of 500 μg/ml cholesterol solution (internal standard solution). The volume was made up to 10 ml with methanol and the solution was vortexed for 1 minute. Finally the sample solution was filtered through 0.45 μm membrane filters (Millipore). The filtrate was employed for the HPLC analysis.
3.7.3.2. Preparation of standard Stigmasterol

The standard solution was prepared by dissolving 20µg stigmasterol in 10µl mobile phase solvent.

3.7.3.3. Mobile phase solvent system

The mobile phase consists of a solvent mixture of acetonitrile and tert-butyl methyl ether (7/3 v/v). It was pumped at a flow rate of 1ml/min and the detection of stigmasterol was done by UV adsorption at 235nm. The sample injection volume was 10µl. The peaks were identified by comparing their retention times with the authentic standards.

3.7.4. Alkaloids (Shen and Shao, 2006)

3.7.4.1. Preparation of sample

Three sets of Calotropis leaf powder and residue were each prepared in a 50ml caped conical flask. The alkaloid was extracted with 40ml boiling water for 30 minutes in a thermostated boiling water bath at 90°C and shaken once in every 20 minutes. The extract was filtered through Whatman No.1 filter paper. 1ml of the filtrate was then diluted to 4ml with ultrapure water. The sample solution was finally filtered through a 0.45µm filter paper and the filtrate was directly injected for HPLC analysis.

3.7.4.2. Preparation of standard histamine

The standard histamine solution was prepared by dissolving 20µg histamine in 10µl of mobile phase solvent.

3.7.4.3. Mobile phase solvent system

The HPLC analysis was performed on an analytical cartridge system, using the mobile phase ether 5% (v/v) acetonitrile solvent containing 0.05% (v/v), phosphoric acid (85%) (Goto et al., 1996). The flow rate was 1.0 ml/min. 10µl was injected. The column temperature was set at 30°C and the monitored wavelength was 220nm. The peaks were identified by comparing their retention times with authentic standards.
3.8. Statistical analysis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Statistical tests</th>
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<tr>
<td>1. Biomethanation</td>
<td>- Student’s ‘t’ test</td>
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<tr>
<td>2. Biochemical compounds</td>
<td>- Students paired ‘t’ test</td>
</tr>
<tr>
<td>(Cellulose, hemicellulose, lignin and volatile fatty acids)</td>
<td></td>
</tr>
<tr>
<td>3. Macronutrients</td>
<td>- Students paired ‘t’ test</td>
</tr>
<tr>
<td>(C, N, P, K and C: N ratio)</td>
<td></td>
</tr>
<tr>
<td>4. Antibacterial activity</td>
<td>- Correlation analysis</td>
</tr>
<tr>
<td>(Concentration Vs Zone of inhibition)</td>
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</tr>
</tbody>
</table>

For all the above analyses SPSS Packages (Version 11) was used.