CHAPTER -4

MATERIALS & METHODS

4.1 Substrate

Willow dust was obtained from the National Textile Mill, Kharar, (Punjab) India.

4.2 Chemical and Physical Analysis

For physical characterization, sieve analysis of willow dust was carried out using sieves of Indian Standard of different sizes. Chemical characterization of willow dust was done according to standard methods as described below:

4.2.1 Moisture and Total Solids

Sample was dried in a hot air oven at 103-105±2°C to a constant weight. Calculations were done according to standard methods (APHA, 1985).

4.2.2 Volatile Solids and Ash

Sample was incinerated at 600±5°C for 5 h in a muffle furnace after drying to constant weight in an oven at 103-105±2°C. Loss in the weight was noted and calculations were made as per standard method (APHA, 1985).

4.2.3 Cellulose

Cellulose was estimated colorimetrically by a standard Anthrone method described by Updegraff (1969).
Reagents

1. Acetic-Nitric reagent: To 80 ml glacial acetic acid, 20 ml of distilled water and 10 ml of concentrated nitric acid was added.

2. Anthrone reagent: 0.2 g Anthrone was dissolved in 100 ml concentrated sulfuric acid. The reagent was chilled 2 h before use. The reagent was prepared fresh every time.

3. Sulfuric acid 67% (v/v): To 67 ml concentrated sulfuric acid, 33 ml of distilled water was added slowly.

4. Cellulose solution: 50 mg of pure dry cellulose powder was dissolved in 10 ml 67% sulfuric acid. The solution was diluted to 500 ml with distilled water in a volumetric flask to make 100μg cellulose/ml.

Procedure

About 0.1 g of oven dried sample of willow dust was weighed accurately and taken in a 150 x 18 mm test tube. The sample was moistened with distilled water and mixed with 5 ml acetic-nitric reagent on a cyclomixer by adding 1 ml at a time. The tube was placed in a boiling water bath for 30 min. to digest all the organic matter except cellulose. However, a precaution needs to take to see that the reflux action takes place without evaporation, for which a marble is put on the mouth of the tube. During this period, the water level was maintained in the bath equal to the liquid level in the tube. The tube was cooled to room temperature and centrifuged at 5000 rpm for 5 min. Decant off the supernatant and the residue was washed with distilled water by repeated centrifugation till it became colorless. To this, 10 ml of 67 % H₂SO₄ (v/v) was added in 1ml aliquots with intermittent mixing on a cyclomixer. Allowed to stand for 1 h. Diluted to 100 ml with distilled water and centrifuged at 2000 rpm for 10 min. to remove
precipitate or turbidity, if any. 0.1 ml of the diluted sample was taken in 150 x 18 mm test tube and to it, 4.9 ml distilled water was added. The tube was transferred to an ice bath and 10 ml ice cold Anthrone reagent was added to it gradually to avoid spurting. The tube was transferred to boiling water bath for 16 min. During boiling, the marble was placed on the mouth of the tube. The tube was cooled rapidly in an ice bath and allowed to stand for 10 min. at room temperature. The absorbance was read of the blue green color developed on a spectrophotometer (Spectronic-21, Milton Roy) at 620 nm against a reagent blank.

For the preparation of standard graph, pure cellulose powder was dried in a hot air oven at 103 -105±2°C for 6 h and placed in a dessiccator. 50 mg of it was dissolved in 10 ml 67 % H$_2$SO$_4$ (v/v) with gentle heat. The mixture was diluted to 500 ml in a volumetric flask. This solution contained 100 µg cellulose/ ml. 0.5, 1.0 1.5 and 2.0 ml of the standard stock solution was taken corresponding to the 50, 100, 150 and 200 µg cellulose in a set of four 150 x18 mm test tubes. To them 4.5, 4.0 3.5 and 3.0 ml of distilled water was added respectively, to make the total volume of 5 ml. A reagent blank was run along with the experimental. The color was developed with the cold Anthrone reagent as described above and read against the reagent blank. A standard graph of absorbance against the cellulose concentrations was plotted.

Calculations

<table>
<thead>
<tr>
<th>Calculation</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of the empty test tube</td>
<td>A</td>
</tr>
<tr>
<td>Weight of the test tube + dry sample</td>
<td>B</td>
</tr>
<tr>
<td>Cellulose in 100 ml sample</td>
<td>C</td>
</tr>
</tbody>
</table>

Cellulose in the dry sample (%) = $\frac{C}{(B-A)} \times 100$
4.2.4 Hemicellulose

Hemicellulose was estimated colorimetrically according to method proposed by Deschatelets and Yu (1986).

Reagent

1. Sulfuric acid 3% (w/v): To 1.7 ml concentrated sulfuric acid, 50 ml distilled water was added slowly and diluted to 100 ml in a volumetric flask.

2. Potassium hydroxide 10N: 56 g solid KOH was dissolved in 80 ml distilled water and diluted to 100 ml in a volumetric flask.

3. p-Bromo-aniline reagent: 4.0 g thiourea was mixed in 100 ml glacial acetic acid, the supernatant was decanted and 2.0 g p-Bromo-aniline was dissolved in the supernatant.

Procedure

2.0 g oven dried sample of willow dust was accurately weighed in a previously weighed beaker. To this, 20 ml of 3 % (w/v) sulfuric acid was added, mixed well and autoclaved at 121°C for 1 h. Then cooled to room temperature and neutralized by 10N KOH. The hydrolysate was centrifuged at 5000 rpm for 10 min. and the supernatant was diluted to 100 ml with distilled water.

For pentose assay, 0.2 ml of the diluted hydrolysate was added to 1 ml of p-Bromo-aniline reagent. A blank was prepared in the same manner. The test tubes were placed in a water bath at 70°C for 10 min., whereas the blanks were kept in dark at room temperature. After incubation at 70°C, the test tubes were cooled rapidly to room temperature and kept in the dark for 70 mins. The purpose of the blank was to make correct in for the furfural already present in the sample as well as for any possible coloration associated with the same. The
absorbance was read at 520 nm for all tests and corresponding blanks using a reagent blank for zero adjustment, on a spectrophotometer.

For the preparation of standard graph, 1.0 g of pure anhydrous xylose was dissolved in 50 ml distilled water and diluted to 100 ml in volumetric flask. The standard xylose solutions were prepared containing 20, 40, 60, 80, and 100 µg.ml⁻¹ by suitably diluting the stock solution. Rest of the procedure was done according to the method described above. The graph was used to find out the xylose in the hydrolysate of unknown sample.

Calculations

Absorbance due to pentoses \( A \)
Absorbance of the test \( B \)
Absorbance of the blank \( C \)
Weight of the original oven dry sample \( D \)

\[
A = B - C
\]

The amount of the pentose per ml of the hydrolysate was calculated from the standard graph.

\[
\text{Hemicellulose in the dry sample} \% = \frac{(A*100)}{D} * 100
\]

4.2.5 Lignin

Lignin was estimated by standard method described by Godbole (1986).

Reagent

1. Sulfuric acid 72% (v/v): To 72 ml concentrated sulfuric acid, 28 ml of distilled water was added slowly.
Procedure

0.1g of the sample was taken in a 50ml beaker, to which 1ml of 72% H₂SO₄ was added. The mixture was stirred well for 3 min. After 2h (from the time of sulfuric acid addition), 2.6 ml of distilled water was added. After an additional 4h, the sample was transferred to a 500ml refluxing flask containing 30ml distilled water. The solution was refluxed for 6h. The condenser was rinsed with 2.5ml distilled water. Total volume of the sample was made to 50ml with distilled water. The solution was filtered through preweighed whatman filter paper No. 1 and precipitates were washed with 5 portions of 20ml boiling distilled water. The precipitates were dried at 105±2°C in an oven and weighed.

4.2.6 Carbon, Hydrogen, Nitrogen & Oxygen

Carbon, hydrogen, nitrogen content was estimated on ash free basis by elemental analyzer model No.2400 (PERKIN-ELMER) and oxygen content was calculated by subtracting the carbon, hydrogen, nitrogen content from 100.

4.3 Anaerobic batch–fed digestion of willow dust

Laboratory scale experiments were carried out in single stage batch reactors to study the solid state anaerobic digestion of willow dust.

4.3.1 Apparatus

Each experiment was conducted in 1L capacity aspirator bottles with wide neck and bottom outlets to facilitate sampling without disturbing the system. The mouth of the bottles was tightly closed with a rubber stopper carrying glass tube for gas outlet. The gas outlet was connected to a brine displacement system for measuring gas volume: the system comprised two
graduated flasks connected in series with the digester. The brine was a 2.5\% aqueous solution of NaCl, adjusted to pH 1.0 with HCl, as in this solution; methane and carbon dioxide have negligible solubility (Van velsen, 1979).

4.3.2 Experimental design

The digestion was carried out using high influent volatile solid concentrations of willow dust i.e 220, 146, 110 and 88 kgVS.m\(^{-3}\) on dry wt. basis. Working volume of the each digester was kept 800 ml. To each digester, 20\% (w/w) inoculum was added, prepared as described below. pH of each digester was adjusted to 7.0 with 4M NaOH. Temperature of each digester was maintained at 37 ± 2\(^{\circ}\)C. The experiments were conducted for a duration of 30 days. Digesters were shaken manually twice every day.

4.3.3 Inoculum

To prepare the inoculum for the present study, batch anaerobic digestion of willow dust, using cattle dung slurry (collected from an active cattle manure digester) as an inoculum, was carried out. After the completion of the digestion process, the effluent from this batch digester was filtered and the filtrate was used as an inoculum for all the experiments. Volatile solid content of the inoculum was analysed along with willow dust before preparing the feeding mixture and adjusted the proportions for the desired final concentrations of volatile solids of willow dust.

4.3.4 Analytical Methods

For the determination of gas yield from each digester, the volume of biogas produced was monitored daily by the method of liquid displacement. The volume of water displaced from the flask was assumed to be equivalent to the volume of biogas production. Gas analysis of CO\(_2\) and CH\(_4\) was done with an
Orsat-Klein apparatus (No. 1004; Germany) according to the procedure of Laura & Idnani (1977). Samples of effluent slurry were withdrawn after 30 days from the outlet provided at the bottom of the digester in all the sets. The digesters were shaken manually before draining the samples. The effluent was analyzed for pH, which was monitored by a standardized pH meter kit (Century, CK 710) and for volatile solid content, estimated as described in section 4.2.2.

4.4 Chemical pretreatment of willow dust

4.4.1 Effect of alkali Pretreatment on the Chemical Constituents of Willow Dust

Alkali pretreatment of willow dust was carried out with different concentrations of NaOH (0.5%, 1%, 2%, 4%, 6%, 8% (w/v) NaOH) for 1 h at three different temperatures (10°, 20° & 35°C). The effect of pretreatment was studied on the cellulose, hemicellulose and lignin content of willow dust. The pretreated samples of willow dust were neutralized with 0.5N HCl. The samples were analyzed for cellulose, hemicellulose and lignin content according to standard methods described above in this chapter.

4.4.2 Residual NaOH left after the Alkali Pretreatment of willow dust

The pretreatment of willow dust with above mentioned concentrations of NaOH was carried out at 35°C for the rest of the experiments. The amount of residual NaOH in alkali treated samples of willow dust was determined as a function of concentration of NaOH used and of duration of pretreatment. For this, the pretreated samples of willow dust were washed with distilled water, until the washings were free of alkali and the collected washings of each sample were titrated with 0.5 N HCl, using phenol-red as an indicator.
4.4.3 Effect of Washing on the Hemicellulose Content of Pretreated Willow Dust

To study the effect of washing of the pretreated samples of willow dust, with water, on the hemicellulose content, the pretreated samples of willow dust were washed thoroughly with distilled water, until the washings were free of alkali. The washed pretreated samples of willow dust and the collected washings were analysed for the hemicellulose content as elaborated in section 4.2.4.

4.4.4 Structural analysis of untreated and pretreated willow dust

Untreated and pretreated samples of willow dust were scanned microscopically using a scanning electron microscope (JSM 6100, Jeol). Fibers of the oven dried samples of willow dust were spread over double adhesive tape, fixed on scanning electron microscope stubs. The loose particles were removed by tapping the stubs. The stubs were then coated with gold in a splutter unit (JFC 1000, Jeol) and examined under the scanning electron microscope using magnification in the range of X2500-3500 and photographed.

4.4.5 Crystallinity Index

Crystallinity index of the pretreated and untreated samples of willow dust was measured by X-ray diffraction using a Philips X-ray diffractometer PW 1729. The specimen was mounted horizontally, while the Geiger Counter moved in a vertical arc. A Cu Kα target with nickel filters was used. The sample was dried overnight at 80°C and stored in a desiccator. Care was taken in handling the samples to minimize exposure to the atmosphere, because adsorption of moisture from the air interferes with the X-ray diffraction patterns. The X-ray beam (35kV peak and 15mA) from the copper target was filtered through a nickel filter. The ground sample of willow dust was pressed onto the sample
holder and the diffraction patterns were obtained with in a 5 to 50° range. An X-ray crystallinity index was obtained by the height ratio of the peaks at 20 =22° and 20 = 18°. The following crystallinity index (CrI) proposed by Segal et al., (1959) was employed.

$$\text{CrI} = \left[ \frac{I_{002} - I_{am}}{I_{002}} \right] \times 100$$  \hspace{1cm} (4.1)

Where $I_{002}$ is the intensity of the 1.002 peak (at about 20 = 22°), and $I_{am}$ is the intensity at 20 = 18°. The 1.002 peak corresponds to the crystalline fraction and the $I_{am}$ intensity corresponds to the amorphous fraction.

4.4.6 Effect of alkali pretreatment on the Anaerobic Batch-fed Solid State Anaerobic Digestion of Willow Dust.

Laboratory scale batch reactors were used to evaluate the effect of pretreatment of willow dust, with different concentrations of NaOH, on the biogas yield, methane yield and the effluent slurry characteristics.

4.4.6.1 Experimental plan

Alkali pretreatment of willow dust was carried out with 0.5, 1, 1.5, 2, 3, 4, 6 & 8% (w/v) NaOH for 1 h at 35°C. After the pre-treatment, unreacted NaOH was neutralized with 3N HCl to pH 7.0. Working volume of each digester was kept 800 ml. To each digester, 20% (w/w) inoculum was added from an active anaerobic digester using willow dust as feed. Volatile solid concentration of each digester was kept at 146 kgVS.m⁻³. Temperature was maintained at 37 ± 2°C and batch fed anaerobic digestion of untreated and treated willow dust was carried out for 30 days.

4.4.6.2 Analytical Methods

Volume of gas production was measured daily and gas analysis was
carried out as described in section 4.3.4. Effluent slurry from each digester at the end of experiment was withdrawn and analysed for pH and volatile solid content.

4.5 Two Phase Anaerobic Digestion of Willow Dust

4.5.1 Acidogenesis of Willow Dust

Willow dust pretreated with 1% NaOH was used to study the acidogenic process of anaerobic digestion of willow dust in batch reactors. The experiments were conducted to study the effect of operational parameters such as (i) initial substrate concentration, (ii) temperature and (iii) pH on the concentration of volatile fatty acids produced and on the rate of acid production during the acidogenesis of the pretreated willow dust.

4.5.1.1 Experimental design

The experimental design was set so as to show the effect of one operational variable on the concentration and rate of volatile fatty acids produced, while keeping the other two variables constant. Three series of batch experiments were conducted in 1 liter aspirator bottles. Effect of four different pH, 5.0, 6.5, 7.0 and 8.0 was investigated, keeping the temperature and influent VS concentration of willow dust constant at 37±2°C and 146 kgVS.m⁻³ respectively. Four temperatures, 25, 35, 40 and 50°C were used, keeping the pH and substrate concentration constant at 6.5 and 146 kgVS.m⁻³. Four different influent substrate concentrations of willow dust, 88, 110, 146 and 220 kgVS.m⁻³ were used, while keeping the pH and temperature constant at 6.5 and 40°C. 20% (w/w) inoculum was used in all the digesters and each experiment was carried out for a duration of 10 days. The pH was monitored daily in each digester and a saturated solution of sodium bicarbonate was used, when the pH fell below the desired value.
To determine the rate of production of volatile fatty acids, the observed concentrations of volatile fatty acids (S) up to its maxima were plotted against time for all the experimental runs of this series. The data were fitted to the following first degree polynomial using linear regression technique:

$$S = A_o + A_1 t$$  \hspace{1cm} (4.2)

Where, $A_o$ and $A_1$ are the constants, $t$ is the time (days) and $S$ is the concentration of volatile fatty acids produced (g/l).

This functional relationship was observed in all the experiments carried out at different pH. The data when fitted to Eq.(4.2), yield the rate of the reaction ($R_{vfa}$), which is:

$$R_{vfa} = dS/dt = A_1$$  \hspace{1cm} (4.3)

The value of $A_1$ for each set of data corresponds to the rate of formation of volatile fatty acids (g/l.d$^{-1}$) with regression coefficient ($R^2$) in the range of 0.94-0.98.

In order to quantify the effect of temperature, a standard Arrhenius type equation was used:

$$\ln k = \ln k_o - \frac{E}{R'T}$$  \hspace{1cm} (4.4)

where $k$ is reaction velocity; $k_o$ is the frequency factor; $E$ is the activation energy (cal.mol$^{-1}$); $R'$ is the gas constant (1.987 cal.mol$^{-1}$K$^{-1}$) and $T$ is the absolute temperature (K).

In the following analysis, the reaction velocity is equal to the rate of product formation and is measured as g of acid produced.l$^{-1}$.d$^{-1}$, as described
Using linear regression technique, the parameters of the Eq.(4.4) were calculated as:

\[
\ln (r_p) = 9.58 - \frac{5597.93}{R'T} \quad R^2 = 1.0
\]  \hspace{1cm} (4.5)

The temperature correction factor \(Q_{10}\) was also calculated for this system. This indicates, how many times the overall reaction rate will increase, when the temperature increases by 10°C (Dinopolulu et al., 1988):

\[
Q_{10} = \frac{rate \text{ at } (T^o + 10^oC)}{rate \text{ at } T^o C}
\]  \hspace{1cm} (4.6)

### 4.5.1.2 Analytical Methods

Concentration of volatile fatty acids was measured every day by the method proposed by Barnett and Reid (1957). VFA were determined by steam distilling 5 ml of the supernatant on acidification and collecting 125 ml of distillate, which was titrated against standard NaOH solution to the phenolphthalein end point. Total volatile fatty acids were expressed as mg. of acetic acid per liter and calculated as:

\[
TVFA \text{ (mg acetic acid. l}^{-1}) = \frac{A * 1000 * N * (a - b)}{B}
\]  \hspace{1cm} (4.7)

where, \(A\) is equivalent weight of acetic acid; \(N\) is normality of NaOH; \(B\) is ml of sample taken; \(a\) is titer of sample, and \(b\) is titer of blank

### 4.5.2 Kinetics of Acid Phase

The experiments were carried out to find out the kinetic parameters of the acid phase of batch-fed anaerobic digestion of willow dust.
4.6.2.1. Determination of specific growth rate of acid forming microorganisms

Volatile suspended solids provide an indirect measure of viable organisms or biomass concentration (Chan and Pearson, 1970). Biomass concentration is the amount of biomass per unit volume. Specific growth rate ($\mu$) is the growth rate divided by the biomass concentration.

4.6.2.1.1. Experimental design

Willow dust was first treated with 1% (w/v) NaOH for 1h at 35°C. After the pretreatment, willow dust was neutralized to pH 7.0 with 3N HCl. The treated willow dust was then used for several batch experiments to find out the concentration of biomass (= VSS) at different initial substrate concentrations i.e. 48.88, 62.84, 88, 110, 146 & 220 kg VS.m$^{-3}$. As the specific growth rate depends upon the temperature and pH, thus each set of batch anaerobic acidogenesis of willow dust was conducted at different temperatures: 25, 35, 40, 45 and 55°C and pH: 5.5, 6.5, 7.0 and 8.0. The working volume of each digester was kept 800 ml and 20% (w/w) inoculum was added to each digester. The set of experiments conducted at different temperatures were set at pH 6.5 and the set of experiments conducted at different pH were set at temperature 40°C. All the experiments were conducted for a duration of 10 days.

4.6.2.1.2 Analytical Methods

Concentration of volatile suspended solids was estimated daily according to standard method (Anon, 1985) by filtering the samples on GF/C whatman filters, drying at 105±2°C and by subtracting the ash after keeping the dried samples for three hours in a muffle furnace at 600°C.

Concentration of VSS, which corresponds to the concentration of...
biomass (x) (Doran, 1995) was plotted as a function of time for each set of data. The data was fitted into the exponential form of equation given below:

\[ x = x_0 e^{\mu t} \]  

(4.8)

where, x is the concentration of biomass (g.l⁻¹) x₀ is the biomass concentration at time zero and \( \mu \) is the specific growth rate. Taking the natural logarithm of Eq.(4.8) gives:

\[ \ln x = \ln x_0 + \mu t \]  

(4.9)

A plot of \( \ln x \) vs. time (t) gives a straight line with slope \( \mu \). For each set of data of VSS, specific growth rate (\( \mu \)) was evaluated corresponding to each initial substrate concentration.

4.5.2.2 Determination of Kinetic parameters

The values of specific growth rate and the corresponding initial substrate concentration at all the temperatures and pH, described above, were fitted in various models described below:

(i) Monod model

The values of specific growth rates and the corresponding initial substrate concentrations at each temperature and pH were fitted to the following linearised form of the Monod equation:

\[ \frac{1}{\mu} = \left( \frac{1}{S_0} \right) \left( \frac{1}{K_s/\mu_{max}} \right) + \left( \frac{1}{\mu_{max}} \right) \]  

(4.11)

where, \( \mu \) is the specific growth rate (day⁻¹); \( \mu_{max} \) is the maximum specific growth rate (day⁻¹); \( S_0 \) is the influent substrate concentration (g.l⁻¹) and \( K_s \) is substrate saturation constant (g.l⁻¹). The plot of \( 1/\mu \) vs. \( 1/S_0 \) was plotted.
(ii) **Inhibition models**

Two types of inhibitions to the growth of micro-organisms in the fermentation media were studied which are product inhibition and substrate inhibition. The following model of product inhibition (volatile fatty acid inhibition model) given by Luengo and Alvarez (1988) was tested with the experimental data:

\[
\frac{dS}{dt} = -\frac{K_i \cdot S \cdot X}{A}
\]  \hspace{1cm} (4.12)

where, \(S\) is the substrate volatile solid concentration (g.l\(^{-1}\)); \(t\) is the time (days); \(X\) is the concentration of microorganisms (g VSS.l\(^{-1}\) of the liquid); \(A\) is the volatile fatty acid concentration (g.l\(^{-1}\)) and \(K_i\) is the kinetic constant of the model (day\(^{-1}\)).

The experimental data were also fitted to the substrate inhibition models. Substrate inhibition models proposed by the following workers were used for the present study. The model proposed by Andrews (1968) that depicts the effect of substrate concentration on specific growth rate, represented by the following equation (Eq.2.10), was tested with the experimental data:

\[
\mu = \frac{\mu_{\text{max}} \cdot S}{K_s + S(1 + S/K_i)}
\]  \hspace{1cm} (4.13)

where, \(K_i\) is substrate inhibition constant.

The following equation, which is another form of Eq. (4.13) given by Noack (1968) was analysed for the present data (Eq.2.11):

\[
\mu = \mu_{\text{max}} \cdot \frac{1}{1 + K_i \cdot S + S/K_{i,s}}
\]  \hspace{1cm} (4.14)

where \(K_{i,s}\) is substrate inhibition constant.
Aiba et al., (1968) proposed the following equation (Eq.2.12) for substrate inhibition, which was also applied to the experimental data:

\[ \mu = \mu_{\text{max}} \cdot \frac{S}{(K_S + S)} \cdot e^{-\frac{S}{K_i}S} \]  

(4.15)

From these models, kinetic parameter \( \mu_{\text{max}} \), \( K_S \) & \( K_i \) were determined by non-linear regression technique.

4.6 Methane Phase of the Anaerobic Digestion of Willow Dust

4.6.1 Substrate

The effluent from acid phase of anaerobic digestion of willow dust containing volatile fatty acids was used as substrate for the methane phase of the anaerobic digestion of willow dust.

4.6.2 Apparatus

Fixed film packed bed reactor was used to study the kinetics of methane phase of anaerobic digestion of willow dust. The reactor used was made of glass with an internal diameter of 5 cm and height of 30 cm and provided with a perforated tube for gas collection at its upper part. Four sampling ports were allocated along the reactor at length to diameter ratios of 1.5, 3.0, 4.5 and 6.0. Volume of packing was 0.184 liter and volume of the reactor was 0.589 liter. The reactor was packed with glass beads of 6-7 mm diameter. Glass beads were initially treated with hydrofluoric acid to increase surface roughness so as to facilitate the immobilization of the methanogenic microorganisms.

4.6.3 Inoculum

Inoculum used in these experiments was digested activated sludge
obtained from Distillery at Banur (Punjab). The inoculum was grown in batch reactors using volatile fatty acids as the substrate.

4.6.3.1 Methane Producing Capacity

To determine the methane producing capacity of the inoculum, 200 ml of inoculum was added to 1.7 liter of sodium acetate medium (1% sodium acetate) maintained at 37°C in a batch reactor of 2 liter capacity. Volume of gas evolved was measured by liquid displacement method and methane content was analyzed by Orsat-Klein apparatus. Liquid samples were centrifuged and analyzed for VSS as per standard method described in section 4.6.2.1.2.

4.6.4 Experimental design

For the immobilization of microbial biomass on the glass beads, initial circulation of the inoculum through the packed bed reactor was followed by continuous increase in the ratio of inoculum to substrate (3g.L⁻¹ concentration of VFA) i.e 100:0, 75:25, 50:50 and 25:75 for 10 days each. This was followed by 0:100, which continued upto 90 days. The temperature was kept constant at 37 ± 2°C. After the film development, experimentation commenced by feeding the liquid, containing volatile fatty acids, from the bottom and removing the effluent from the top and intermediate sampling parts of the reactor. The reactor was operated in a semi-continuous mode basis. The loading rates used were 3.0, 5.0, 8.0 and 10 kg.m⁻³ volatile fatty acids and each loading rate was run at different hydraulic retention times of 3, 5, 6 and 7 days corresponding to the 3.0, 1.8, 1.5 and 1.1 ml. h⁻¹ flow rate. The influent was pumped into the reactor with the help of peristaltic pump (Miclins, PP-10). The performance of the reactor in regards to the biogas yield, methane yield and the VFA reduction was evaluated.

4.6.5 Analytical methods
Every week, the development of biofilm on glass beads was monitored on oculometer, model GSZ-77-252, Getner. The rate of biofilm formation was calculated from the data of biofilm thickness as a function of time, which was fitted to the polynomial equation of the form:

\[ L_f = A_0 + A_1 \theta + A_2 \theta^2 + A_3 \theta^3 \]  

(4.16)

Where, \( L_f \) is thickness of biofilm (\( \mu \)m); \( \theta \) is time (days) and \( A_0, A_1, A_3 \) ... are polynomial constants.

By taking differential of Eq.(4.16), the values of these constants were measured by regression analysis.

Concentration of VSS was determined as described in section 4.6.2.1.2. Immobilised microbial biomass concentration on support particles were determined by washing microbial biomass from certain number of glass beads (about 20) with distilled water and the concentration was determined after filtering the washings and drying of filter paper (Rozzi et al., 1989).

The concentration of volatile fatty acids was measured by the method described in section 4.6.2.1.2. Gas was collected in a 500 ml graduated flask by liquid displacement method. Gas analysis was done using Orsat-Klein apparatus. \( pH \) was measured by the standardized \( pH \)-meter kit.

4.6.6 Evaluation of Kinetic Parameters

For most biochemical processes, the specific substrate utilization rate (\( r \)) has been expressed as a function of substrate concentration (\( S \)) as follows: (Hanemoes, 1983; Rittman et al., 1986 and Droste and Kennedy, 1987)(Eq.2.18)

\[ r = r_{\text{max}} \cdot \frac{S}{(K_s+S)} \]  

(4.17)
where, kinetic parameters, $r_{\text{max}}$ is the maximum specific substrate utilization rate and $K_S$ is the half-velocity coefficient.

However, these rates incorporate the mass transfer resistance effect, which is difficult to measure and thus, the measured reaction rates would not reflect the true or intrinsic rates (Doran, 1995). But, it is possible to measure the overall reaction rate, as the rate of disappearance of substrate from the bulk liquid must equal to the overall rate of conversion by the reaction. These rates are called observed or apparent rates. Thus, the equation (4.17) could be modified as follows:

$$r_{\text{obs}} = r_{\text{max \, app}} * S_b / (K_S^{\text{app}} + S_b) \quad (4.18)$$

where, $r_{\text{max \, app}}$ and $K_S^{\text{app}}$ are the apparent kinetic parameters and $S_b$ is the substrate concentration in the bulk liquid.

To estimate the observed rates of volatile fatty acid consumption, the following equation (Eq.2.20) given by Zaiat and Foresti (1997) was used for the present study:

$$R_{\text{VFA}} = - (\epsilon \cdot v_s)/D \cdot dS_{\text{VFA}}/d(L/D) \quad (4.19)$$

where, $R_{\text{VFA}}$ is observed rate of VFA consumption (g.l$^{-1}$.h$^{-1}$); $\epsilon$ is bed porosity; $v_s$ is liquid superficial velocity (cm.h$^{-1}$); $D$ is diameter of the reactor (cm); $S_{\text{VFA}}$ are VFA concentrations along the length of the reactor at different length to diameter ratio (g.l$^{-1}$).

To find out the values of $S_{\text{VFA}}$, the following exponential decay expression (Eq.2.21) was adjusted to the observed $S_{\text{VFA}}$ profile along the reactor as a function of $L/D$:

$$S_{\text{VFA}} = a_0 + a_1 \cdot e^{-(L/D) a_2}/(a_3) \quad (4.20)$$
where, $a_0, a_1, a_2$ and $a_3$ are the constants.

From equation (4.19) and (4.20), the values of $R_{VFA}$ and $S_{VFA}$ respectively, were calculated. These values of $R_{VFA}$ were correlated with the values of $S_{VFA}$ in order to obtain the best fit kinetic model.

**Monod model** was tested with the experimental data of $R_{VFA}$ and $S_{VFA}$. Hanes-Woolf plot of $S_{VFA}/R_{VFA}$ vs. $S_{VFA}$ was plotted.

The experimental data was further fitted to the First order kinetic model. $R_{VFA}$ vs. $S_{VFA}$ was plotted and the apparent kinetic constant at each influent substrate concentration and at each hydraulic retention time was evaluated from this model. However, according to Atkinson (1974), the apparent kinetic coefficient ($k_{1\text{app}}$), which can be defined in such a manner that both the mass transfer and kinetic contributions are combined, is not useful for simulation and reactor design, since, it is obtained under specific conditions of reactor operation. Thus, its extrapolation for other conditions, where reaction is only kinetic controlled, may lead to errors in the evaluation of the substrate utilization rates. Therefore, the determination of intrinsic kinetic coefficient is essential under conditions of no liquid or solid mass transfer resistance.

For the determination of first order intrinsic kinetic parameter ($k_1$), the following correlation given by Atkinson (1974) was used (Eq.2.26):

$$k_1 = \frac{(k_a a \cdot k_{1\text{app}})}{(k_a a - k_{1\text{app}})} \quad (4.21)$$

where, $k_a$ is the volumetric liquid-phase mass transfer coefficient (cm.h$^{-1}$) and $a$ is the interfacial area for mass transfer (cm$^{-1}$).
Interfacial area of mass transfer was estimated by the following Eq. (2.27):

$$a = \eta \cdot \frac{A_p}{V_l} \quad (4.22)$$

where, $V_l$ is the bulk liquid volume (cm$^3$); $A_p$ is the bioparticle area considering an equivalent sphere (cm$^2$) and $\eta$ is the number of bioparticles.

To estimate the value of liquid-phase mass transfer coefficient ($k_s$), the following correlation given by Bannet and Meyers (1974) for the immobilized reactors was used (Eq.2.28):

$$(k_s \cdot \frac{d_p}{D_e}) \cdot (\frac{\mu_l \cdot D_e}{\rho_l})^{-\frac{1}{3}} \cdot (\frac{V_s \cdot \rho_l \cdot d_p}{\mu_l})^{-\frac{1}{2}} = 1.9 \left( \frac{V_s \cdot \rho_l \cdot d_p}{((1-\varepsilon) \cdot \mu_l)} \right)^{0.5} \quad (4.23)$$

where, $v_s$ is the liquid superficial velocity (cm.h$^{-1}$); $\rho_l$ is the liquid density (g.cm$^3$); $d_p$ is the diameter of the bioparticle (cm); $\varepsilon$ is the bed porosity; $\mu_l$ is the liquid viscosity (g.cm$^{-1}$.h$^{-1}$) and $D_e$ is the substrate diffusion in the bulk liquid (cm$^2$.h$^{-1}$).

Another correlation given by Maccune and Wilhelm (1949) for fixed bed reactors was used to determine the value of $k_s$ (Eq.2.33):

$$J_d = 1.625 \cdot (Re)^{-0.507} \quad Re<120 \quad (4.24)$$

Finally, the volumetric coefficient of mass transfer ($k_s.a$) was estimated by the following equation (Zaiat and Foresti, 1997) (Eq.2.36):

$$k_s.a = 3 \cdot (1-\varepsilon) \cdot \frac{k_s}{\varepsilon \cdot R_p} \quad (4.25)$$

The values of intrinsic kinetic parameter ($k_l$) for each influent substrate concentration and retention time were determined using equation (4.21).
The intraparticle mass transfer effect was quantified by using the following equation (Eq.2.37) for spherical particles proposed by Bailey and Ollis (1985) for the first order kinetics:

$$\phi_{obs} = k_{1,app}.R_p / 9.D_c$$  \hspace{1cm} (4.26)

where, $D_c$ is the effective diffusivity in the bioparticle (cm$^2$.h$^{-1}$) and $\phi_{obs}$ is the observed Thiele modulus used to quantify the effect of mass transfer on the rate of reaction.

The influence of the mass transfer resistance on the overall specific substrate utilization rate for a first order kinetics was determined by the following equation (Eq.2.39):

$$\eta_{obs} = k_{1,app}/k_1$$ \hspace{1cm} (4.27)

where, $\eta_{obs}$ is the observed effectiveness factor and $k_1$ is the first order intrinsic kinetic parameter.