REVIEW OF LITERATURE
2.1 Anaerobic Digestion: an Overview

Anaerobic digestion is a promising method for treatment of municipal solid waste. When an organic material enters into a digester, it contains a large fraction of suspended solids and complex soluble matter. Under anaerobic conditions, these large organic molecules are converted, mainly, into methane (\(\text{CH}_4\)) and carbon dioxide (\(\text{CO}_2\)). A variety of microorganisms coexist in anaerobic digesters and their concerted activity is necessary for the complete bioconversion of organic materials to methane (Fernandez et al., 2000).

Therefore, an understanding of the microbial community structure and dynamics in anaerobic processes is a basic requirement for fundamental improvement of anaerobic digestion technology. Nonetheless, due to the limited knowledge of the microbial ecosystem involved, field-scale anaerobic digesters have been empirically set up and operated to avoid failure, resulting in over-dimensioned digester volumes (Hori et al., 2006).

The process of anaerobic digestion proceeds in three main stages; (i) hydrolysis, (ii) acid formation and (iii) methanogenesis.

![Figure 1: Degradation steps of anaerobic digestion process](image)

The overall biochemical reaction can generally be simplified to:

\[
\text{Organic material} \rightarrow \text{CH}_4 + \text{CO}_2 + \text{H}_2 + \text{NH}_3 + \text{H}_2\text{S}
\]
2.1.1 Hydrolysis

Hydrolysis is the first step in the anaerobic biodegradation process. It involves the conversion of the complex waste (particulate and soluble polymers) into soluble products by the extracellular enzymes secreted by the hydrolytic bacteria. The once complex insoluble organic polymers become more easily available for use by the acidogenic bacteria in the next stage. Proteins present in the waste are converted into amino acids, fats into long chain fatty acids and carbohydrates into simple sugars.

2.1.2 Acid Formation

2.1.2.1 Acidogenesis

In acidogenesis, the organic monomers of sugars and amino acids released earlier are degraded by the fermentative bacteria to produce volatile fatty acids (VFA) namely propionic, butyric and valeric acids, together with acetate, hydrogen (H₂), carbon dioxide (CO₂). The degradation of amino acids also produces ammonia (NH₃). Examples of different products from glucose fermentation are shown in Table 1.

<table>
<thead>
<tr>
<th>PRODUCTS</th>
<th>REACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>( C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 )</td>
</tr>
<tr>
<td>Propionate + Acetate</td>
<td>( 3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2CH_2COOH + 2CO_2 + 2H_2O )</td>
</tr>
<tr>
<td>Butyrate</td>
<td>( C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 )</td>
</tr>
<tr>
<td>Lactate</td>
<td>( C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH )</td>
</tr>
<tr>
<td>Ethanol</td>
<td>( C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 )</td>
</tr>
</tbody>
</table>

Courtesy: (Batstone et al., 2002)
2.1.2.2 Acetogenesis

During the acetogenesis step, the obligate hydrogen producing acetogens (OHPA) break down both long chain fatty acids (LCFA) and volatile fatty acids (VFAs), producing acetate, carbon dioxide and hydrogen (Table 2). The Fatty acid oxidizing bacteria thrive in anaerobic digesters rich in LCFA and they exhibit a high tolerance against the toxic and inhibitory effects of fatty acids.

Bacteria affiliated with the phyla *Bacteriods* and *Spirochaetes* plays an important role in LCFA degradation.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>REACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid</td>
<td>$\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightleftharpoons \text{CH}_3\text{COOH} + 3\text{H}_2 + \text{CO}_2$</td>
</tr>
<tr>
<td>i-butyric acid</td>
<td>$\text{CH}_3(\text{CHCH}_3)\text{COOH} + 2\text{H}_2\text{O} \rightleftharpoons 2\text{CH}_3\text{COOH} + 2\text{H}_2$</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>$\text{CH}<em>3\text{CH}</em>{2}\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightleftharpoons 2\text{CH}_3\text{COOH} + 2\text{H}_2$</td>
</tr>
<tr>
<td>i-valeric acid</td>
<td>$\text{CH}_3(\text{CHCH}_3)\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons 3\text{CH}_3\text{COOH} + \text{H}_2$</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>$\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{CH}_3\text{COOH} + \text{H}_2$</td>
</tr>
</tbody>
</table>

Table 2: Conversion of Volatile Fatty Acids to Acetic acid

On contrary to OHPA, Homoacetogenesis refers to metabolic activity of Obligate Hydrogen consuming Acetogenic bacteria which has attracted much attention in recent years because of its final product acetate, an important precursor to methane generation. The responsible bacteria are either autotroph or heterotroph. The autotroph homoacetogens utilize a mixture of hydrogen and carbon dioxide, with carbon dioxide serving as a carbon source for cell synthesis. Some homoacetogens can use carbon monoxide as a carbon source. The heterotrophic homoacetogens on the other hand, use organic substrate such as formate and methanol as a carbon source while producing acetate as the end product (Table 3).
Table 3: Metabolic Activity of Homoacetogens

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>PRODUCTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ + H₂</td>
<td>CH₃COOH + H₂O</td>
</tr>
<tr>
<td>4CO + 2H₂O</td>
<td>CH₃COOH + H₂O</td>
</tr>
<tr>
<td>4HCOOH</td>
<td>CH₃COOH + 2CO₂ + 2 H₂O</td>
</tr>
<tr>
<td>4 CH₃OH + 2CO₂</td>
<td>3CH₃COOH + 2CO₂</td>
</tr>
</tbody>
</table>

Courtesy: (Batstone et al., 2003)

2.1.3 Methanogenesis

Generally, the methanogenic substrates include acetate, methanol, hydrogen or carbon dioxide, formate, methanol, carbon monoxide, methylamines, methyl mercaptans, and reduced metals. According to Veeken et al. (2000), methane is produced from acetic acid, hydrogen and carbon dioxide as well as directly from other substrates of which formic acid and methanol are the most important.

The reactions defined as methyl type and carbon dioxide, including formic acid, methanol, and methylamine, oxidation of hydrogen, carbon monoxide and acetate are shown respectively in Table 4: Reaction (1) to (6) (Siang, 2006):

Table 4: Substrate Utilization by Methanogens

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>REACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid</td>
<td>4HCOO+ 4H+ ⇌ CH₄+3CO₂ +2H₂O</td>
</tr>
<tr>
<td>Methanol</td>
<td>4CH₃OH ⇌ 3CH₄+ CO₂ +2H₂O</td>
</tr>
<tr>
<td>Methylamine</td>
<td>4(CH₃)3N +H₂O ⇌ 9CH₄+3CO₂+6H₂O +4NH₃</td>
</tr>
<tr>
<td>Oxidation of hydrogen</td>
<td>4H₂+ CO₂ ⇌ CH₄ + 2H₂O</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>4CO + 2H₂O ⇌ CH₄ + 3CO₂</td>
</tr>
<tr>
<td>Acetate</td>
<td>CH₃COOH ⇌ CH₄ + CO₂</td>
</tr>
</tbody>
</table>

Courtesy: (Siang, 2006)

In an anaerobic digester, methanogenesis takes place via two ways; one through hydrogenotrophic methanogenesis producing methane by utilizing H₂ and CO₂ by the hydrogen-
consuming bacteria in a syntrophic co-culture with the OHPA bacteria and others by methanogenic aceticlastic bacteria which grow on acetate as substrate, releasing methane and carbon dioxide. Of these, acetic acid (CH$_3$COOH) and the closely related acetate are the main precursors to methane production accounting to about 75% of the methane production in natural environment (e.g. lake sediments). In anaerobic digester ecosystem the population level of hydrogen-utilizing methanogens is the same as or greater than that of methanogens utilizing acetate (Zuikus, 1980).

2.2 Process Balance

In a well balanced anaerobic digestion process, all products of a previous metabolic stage are converted into the next one without significant build up of intermediary products. The overall result is a nearly complete conversion of the anaerobically biodegradable organic material into end products like methane, carbon dioxide, hydrogen sulfide and ammonia (Adrie Veeken et al., 2000).

Methanogenic organisms in anaerobic digester make up the main final stage of anaerobic reaction and hence they are most important to anaerobic stabilization of different substrates. They grow very slowly during anaerobic processes, so the metabolism of these methanogens is usually the rate limiting in the anaerobic treatment.

The non-methanogens are also significant bacterium. The state of dynamic equilibrium of methanogenic and non-methanogenic bacteria maintains and stabilizes organic waste removal efficiency in anaerobic treatment system (Sponza and Cigal, 2008). Acidogens are generally fast growers and show very little sensitivity to the variation in environment conditions, during such an instance as in an anaerobic digester, the rate of hydrolytic and fermentative activity far exceeds the rate of acetogenic conversion of fermentation intermediates. The above condition leads to the process inhibition since methanogens are intrinsically slow growers, they do not consume di-hydrogen at the rate at which it is produced, leading to the accumulation of electron or hydrogen. This results in the formation of various other fermentation products like propionate, butyrate, lactate, succinate, and alcohols, wanting to rid of the electrons or hydrogen. The reactor pH then decreases as the levels of this fermentation products continue rising. The methane forming bacteria are pH sensitive and can only usually tolerate more or less neutral pH values in the narrow range of 6.5 to 7.5 (Mateescu and Constantinescu, 2011).
If the fermentation products accumulate to inhibitory levels, the decomposition of substrate will cease. It is therefore very important to understand that the overall fermentation is dependent largely on the particular bacteria species present and to a lesser extent its environmental conditions. There has to be a balance between activities of bacteria that form organic acids, carbon dioxide, and di-hydrogen (or formate) and the methanogenic bacteria which utilize these substrates.

2.3 Interaction between Microbial Groups

For application of anaerobic processes to the treatment of solid waste, understanding the structures and metabolic functions of the microbial communities responsible for degradation is important (Andrea Macmuller et al., 2003).

As mentioned previously the anaerobic digestion is a process that evolves at least four different groups of microorganisms. Each group contains diverse organisms responsible for different metabolic tasks, distinguishing characteristic of this anaerobic consortium is that different species of anaerobic microorganisms degrade one organic compound interactively, sharing energy and carbon sources from the compound (Sekiguchi et al., 2001).

These organisms have developed specific kind of interdependent relationship called syntrophy, special kind of symbiotic cooperation of mutual dependence of the partner bacteria with respect to energy limitation where neither partner can exist without the other and together they exhibit a metabolic activity that neither one could accomplish on its own. In this unique cooperation between two metabolically different types of microorganisms they depend on each other for degradation of a certain substrate for energetic reasons (Schink, 1997).

This unique cooperation between the microorganisms involved in the methanogenesis has evolved due to the need to utilize the energy obtained from the electron donor substrate more efficiently. For a concerned microorganism, the overall anaerobic degradation is a process with very low energy yield compared to the aerobic degradation (Figure 2). The main reason is that the electron acceptor in this case is the carbon dioxide and not oxygen like in the aerobic degradation. Carbon in the carbon dioxide is in the most highly oxidized state with a COD: C ratio of zero. Since the energy available depends on the oxidation state of the substrate and indicates the electrons available for removal as it is oxidized, with carbon dioxide as electron acceptor the amount of free energy available is very low.
Figure 2: Thermodynamic Consideration in Aerobic and Anaerobic Degradation of Glucose

![Thermodynamic Consideration in Aerobic and Anaerobic Degradation of Glucose](image)

Courtesy: (Kranet M, 1989)

The fermentative bacteria are composed of a very complex mixture of many bacterial species. Enumeration of these bacteria in sewage and organic waste digesters and in lake sediments showed that most are obligate anaerobes but some facultative anaerobes, such as *Streptococci* and *Enterobacteria* may also be in small numbers in some ecosystems. Microbial diversity in biogas digesters is as great as that of rumen, wherein, seventeen fermentative bacterial species have been reported to play an important role in the production of biogas. It is also evident that the nature of the substrate determined the type of the fermentative bacteria present in the digester.

Higher proteolytic population was noted in poultry waste-fed digesters while amylolytic microorganisms were found to dominate cow dung fed anaerobic digesters (Preeti Rao *et al.*, 1993). Among fermentative organisms, *Bacteroides succinogens*, *Bacteroides cellulosolvens* and *Acetivibrio cellulolyticus*, *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Eubacterium cellulosolvens* and among Clostridium, *Clostridium cellulosolvens*, *Clostridium cellulovorans*, *Clostridium cellubiovarum*, *Clostridium thermocellum*, were predominant.
Ramasamy et al., (1991) observed that a clear differentiation existed in the type of cellulolytic bacterial distribution in rumen and biogas digester. In rumen, *Ruminococcus* sp. alone accounted for 60% of the total population, in the biogas digester the predominant species belonged to the genera *Bacteroides* and *Clostridium* rather than the genus *Ruminococcus*.

Most of these bacteria adhere to the substrate prior to extensive hydrolysis, while the digester slurry contained higher cellulolytic population; the outlet of the digester recorded the least cellulolytic population. Furthermore, Ramasamy et al., (1990) reported that the particulate-bound cellulolytic bacteria were the predominant group in the slurry of the digester. It was observed that out of the total cellulolytic population of $42 \times 10^4$ ml$^{-1}$ of slurry, the particulate-bound bacteria accounted for $34 \times 10^4$ ml$^{-1}$ of slurry. Furthermore, the particulate-bound bacteria predominated up to 20th day of initiation of biogas digester. It is also known that the particulate-bound bacteria showed direct relation to the biogas yield from the digester (Figure 3).

**Figure 3:**

**Particulate Bound Bacteria and its Relation with Biogas Yield.**

![Graph showing particulate bound bacteria and its relation with biogas yield.](Image)

Courtesy: (Ramasamy et al., 1990)

Reductive halogenation of various halogenated aliphatic and aromatic compounds is the next important process by fermentative group of organisms. Thauer et al., (1997) observed that these reactions are thermodynamically feasible and can support growth. Anaerobic degradation of aromatic compounds also depend on hydrogen-consuming bacteria, Mountford and Bryant,
(1982) isolated a bacterium which oxidized benzoate to acetate in obligate co-culture with methanogens or sulphate-reducing bacteria. Phenol at 1000 ppm concentration inhibits the gas production up to six weeks, which increases with prolonged incubation; however, a mixed culture consortia enriched with phenol utilizes the phenol immediately, showing an increase in gas production without any lag phase.

Obligately hydrogen-producing acetogenic bacteria are one of the important groups in biogas digesters, these organisms oxidize the fatty acids that are longer than acetate to acetate and thereby help methanogens to release energy from the substrate in the form of methane. Boone and Bryant, (1980) isolated *Syntrophobacter wolinii* which beta-oxidized propionate to acetate, and later McInerney *et al.*, (1981) isolated *Syntrophomonas wolfei* which beta-oxidized C₄ to C₇ fatty acids (butyric acid).

But these reactions are favorable only if the hydrogen partial pressure is below 10⁻³ atmosphere. Under high hydrogen partial pressure, acetate formation is reduced and the substrate is converted to propionic acid, butyric acid and ethanol rather than methane and thus the accumulation of propionic acid resulted in acidification of digester which inhibited further methane formation because of its toxicity to methanogens. Subsequently, Nagamani *et al.*, (1994) developed a consortia comprised of syntrophic co-cultures in association with hydrogen-utilizing methanogens, which stabilized methane production from castor-oil-cake-fed digester. Earlier, Meher and Ranade (1993) isolated a propionate-degrading bacterium in association with *M. formicicum* from cattle dung-fed digesters. Meher *et al.*, (1996) also reported the presence of butyrate-degrading syntrophic co-culture in biogas digesters fed with cattle dung. Though these organisms occurred at a pH < 6.0 and below 45ºC, methanogenesis was observed at pH > 6.5 and above 40ºC.

Examples of propionate oxidizing mesophilic bacteria are – *Syntrophobacter wolinii*, *S.pfennigi* and *S.fumaroxidans* and *Smithella propionica*. Example of thermophilic bacteria capable of oxidizing propionate in a co-culture is only *Desulfotomaculum thermocisternum*.

Temperature may also affect the thermodynamics of syntrophic propionate-oxidation. Using the van ‘t Hoff’ equation it can be calculated that hydrogen formation becomes energetically more favorable at higher temperatures, whereas hydrogen consumption by the methanogens becomes less favorable (De Bok *et al.*, 2003)(Table 5). However, since diffusion
coefficients become higher and diffusion gradients steeper, it can be expected that propionate conversion rates are higher at elevated temperatures.

Table 5: **Syntrophic Oxidation of Propionate in Methanogenic Ecosystems**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>REACTION EQUATION</th>
<th>$\Delta \rightarrow G^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>35°C</strong></td>
</tr>
<tr>
<td>1</td>
<td>Propionate$^-$ + 3H$_2$O $\rightleftharpoons$ Acetate$^-$ + HCO$_3^-$ + H$^+$ + 3H$_2$</td>
<td>+76.1</td>
</tr>
<tr>
<td>2</td>
<td>Propionate$^-$ + 2HCO$_3$ $\rightleftharpoons$ Acetate$^-$ + H$^+$ + 3HCOO$^-$</td>
<td>+72.2</td>
</tr>
<tr>
<td>3</td>
<td>H$_2$ + ¼ HCO$_3^-$ + ¼ H$^+$ $\rightleftharpoons$ ¼ CH$_4$ + ¾ H$_2$O</td>
<td>-33.9</td>
</tr>
<tr>
<td>4</td>
<td>Formate$^-$ + ¼ H$_2$O + ¼ H$^+$ $\rightleftharpoons$ ¼ CH$_4$ + ¾ HCO$_3^-$</td>
<td>-32.6</td>
</tr>
<tr>
<td>5</td>
<td>Acetate$^-$ + H$_2$O $\rightleftharpoons$ CH$_4$+ HCO$_3^-$</td>
<td>-31.0</td>
</tr>
<tr>
<td>6</td>
<td>Propionate$^-$ + H$_2$O $\rightleftharpoons$ 1,¼ CH$_4$+ 1,¼ HCO$_3^-$ + ¼ H$^+$</td>
<td>-56.4</td>
</tr>
</tbody>
</table>

Courtesy: (De Bok et al., 2003)

Hydrogen-consuming acetogenic bacteria are the minor groups involved in fermentative reactions in biogas digesters. Their activity in the formation of acetate by reduction of CO$_2$ accounted for less than 5 per cent of the total acetate formed. However, a substantial activity by these groups of organisms should definitely increase methane formation, as acetate is the preferred substrate for *Methanosarcina barkeri*, *Methanobacterium formicicum* followed by *Methanobacterium ruminantium* the predominant methanogens in biogas digester in cattle waste-fed digester.

Methanogens possess very limited metabolic repertoire, using only acetate or C$_1$ compounds (H$_2$ and CO$_2$, formate, methanol, methylamines or CO), with methane being the end product of the reaction. Of the methanogenic genera, *Methanosarcina* sp. and *Methanosaeta* sp. form methane by aceticlastic reaction, whereas the apparent $K_m$ for methane formation from acetate for *Methanosaeta* sp. was ≤ 1mM and for *Methanosarcina* species it was 3–5 mM. Therefore, while faster-growing *Methanosarcina* sp. is predominant in high-rate shorter-retention digesters wherein the acetate concentration is higher, while the *Methanosaeta* sp. are predominant in low-rate, slow-turnover digesters.
Both carbon-dioxide-reducing and aceticlastic-methanogens play an important role in maintaining stability of the digester. The failure in a biogas digester can occur if carbon dioxide-reducing methanogens fail to keep pace with hydrogen production. Whereas apparent $K_m$ for hydrogen consumption in methanogenic environments is near $10^{-2}$ atmosphere, hydrogen consumption must be below $10^{-3}$ atmosphere for oxidation of fatty acid to acetate, and thus the carbon dioxide-reducing methanogens in a biogas digester are greatly under saturated for hydrogen. Despite this, hydrogen in biogas digester can build up rapidly to levels inhibitory to methanogenesis due to failure of the activity of hydrogen-scavenging organisms, shifting the fermentation products away from acetate. Moreover, failure of aceticlastic methanogens to keep up with acetic acid production results in the accumulation of fatty acids, resulting thereby in failure of the digester.

Interesting morphological differences was observed between pure cultured and co-cultured methanogens cells that is the length of co-cultured cells were one third to one quarter as long as those of pure cultured cells. More over the co-cultured cells had a cell wall that was at least three times thicker than that of cells from pure culture. More over the growth of co-culture was much lower than that of pure culture. The most likely cause of these morphological differences is the hydrogen concentration between the two culture conditions that is under co-culture conditions, the partial hydrogen pressure is kept very low as a result of interspecies hydrogen transfer between the acetate oxidizing hydrogen producing syntrophs and Methanobacterium thermoautotrophicus. The above Methanobacterium may increase their cell wall thickness under natural low hydrogen concentration conditions existing in anaerobic digesters. Without any appropriate pre-treatments (to thin out their cell wall or remove), those methanogens in their natural habitats would hardly be detected by Fluorescence In-Situ Hybridization (FISH), resulting in an imprecise estimation of their abundance and spatial distribution. Several techniques have recently been developed for in-situ visualization of microbes in natural habitats, such as FISH and in-situ PCR. However, these techniques involve the permeation of catalytic molecules into the specimen cytoplasm for their reactions, such as horse radish peroxidase and DNA polymerase, which have higher molecular weights than oligonucleotide probes (Nakamura et al., 2006).

Many of the species such as Methanobacterium bryantii, Methanobacterium thermoautotrophicum and Methanobacterium formicicum are capable of autotrophic growth that
used carbon dioxide as a sole carbon source. Both carbon dioxide reducing and acetoclastic methanogen play an important role in maintaining stability of the digesters. The failure in the biogas digester can occur if carbon dioxide reducing methanogens fail to keep pace with hydrogen production.

Acetate is essential for some species such as *Methanobrevibacter ruminantium* strain M1, *Methanobrevibacter smithii* strain Ps, and *Methanogenium cariaci* strain JRI serving as a major source of carbon and providing about 60% of the total cell carbon in *Methanogenium ruminantium* strain M1 even when large amount of organic carbon are present in the medium. Acetate is an important substrate for methanogens and about 60% to 90% of the methane produced from organic matter in sewage digesters and lake sediments is produced via the methyl group of the acetate. Only a few methanogenic species are believed to degrade acetate and only Methanosarcina species have been isolated in pure culture. Preferred energy sources such as hydrogen or methanol may regulate the degradation of acetate by *M. berkeri* in a manner that resembles catabolic repression.

Agitation of enrichments during microbial exponential growth resulted in unexpected stagnation (growth and methane production stops) and relatively stable growth will be observed when the culture is left without agitation, moreover addition of ferric chloride, stabilizes the propionate degrading enrichments (Hirayuki Imachi *et al.*, 2000).

### 2.4 Potential Feedstock for Anaerobic Digestion

Despite the importance of urban waste degradation in landfills, relatively little information is available on the environmental factors that simultaneously influence the urban waste degradation.

#### 2.4.1 Urban Waste (Municipal Solid Waste)

Anaerobic digestion is an attractive treatment strategy for the municipal solid waste as the waste stream contains a large proportion of organic materials, of about 60%. Of this percentage a high fraction includes waste paper (22%) followed by kitchen organics (16%) and garden trimmings (22%) whereas the remaining constituents are non organics such as plastics, textiles and rubber, rubble, soil, metal, glass, sanitary and hazardous materials (Street and Zydenbos, 2004).
Mata-Alvarez (2003) explained that the organic fraction of urban waste is usually obtained from the commingled or unsorted waste in three main pathways includes mechanical selection, source sorting and separate collection wherein the principal component of urban waste comprises high fraction of biodegradability. In addition, the organic fraction of municipal solid waste has been considered as a potential source for biogas production (Gunaseelan, 1997).

The biodegradation kinetics of urban waste has been reported to be 5-10 times larger for source-sorted municipal solid waste compared to mechanically-sorted ones (Mata-Alvarez et al., 1990). When waste was sorted at the source, a maximum kinetic constant, k of 3.53 d⁻¹ is reported whereby 406 L CH₄/kg VS is produced and achieving 98% biodegradation. The higher biodegradation and methane yield with source-sorted waste were due to absence of non-biodegradable portions namely large amounts of suspended solids, small pieces of plastic, wood, paper, etc. which were otherwise found with the mechanically-sorted MSW.

Chanakya et al., (2009) tested the decomposition patterns of vegetables, fruits, fresh leaf litter and paper as feedstock. The vegetable and fruit wastes fermented rapidly in an anaerobic digester, marking their suitability as feedstock. On the other hand, leaf litter and newspaper fermented slowly with poor process stability and moderate biodegradation, achieving only 25-50% decomposition in 30 days. Anaerobic digestion of the organic fraction of municipal solid waste (OF-MSW) has been demonstrated to proceed best using digested sludge as inoculum than if inoculated with cattle manure, which causes a restricted removal of organic matter and methane yield (Forster-Carneiro et al., 2006).

The maximal methane can be generated by decomposition of OFMSW under optimal pH, temperature and initial total solids concentration (Liu et al., 2008). The municipal organic waste steam has been successfully digested at pilot scale under thermophilic conditions, achieving 80% volatile solids degradation, methane yield of 300-400 m³ CH₄/ton VS at a retention time of 15 days (Davidsson et al., 2007).

It is worthwhile to note that according to Tchobanoglous et al., (1993) the biodegradability of the organic fractions of urban waste can be best described in relation to both volatile solids (VS) and lignin content. The use of VS alone in describing the biodegradability of the organic fraction of waste is often misleading, since the waste is maybe high in VS but low in biodegradability like newspaper and plant trimmings which are high in lignin (hardly biodegradable) content.
For practical purposes, the organic waste components in urban waste are often classified as high, medium or low degradability. Generally, a substance or a compound is biodegradable if it can be decomposed by the action of microorganisms which use this compound as their energy source and as carbon source (Angelidaki, 2002).

The variation in waste generation especially its composition and quantity as well as the collection regime changes the setting for microbial growth within the rubbish pile. These factors and others created the non-homogenous nature of the municipal solid waste. The heterogeneity of the solid waste poses challenges for the researcher intending to conduct analysis on the waste.

The other substrates utilized for anaerobic digestion includes agricultural waste, Energy crops, Industrial waste water and municipal organic waste.

Among the agricultural wastes, pig and cow slurry, chicken manure and farmyard manure are of primary concern because of the detrimental effect on the environment wherein the natural waste degradation leads to release of greenhouse gases in addition to eutrophication of surface water resources (Vedrenne et al., 2008).


Among energy crops, maize has been shown to be an excellent energy crop, capable of producing methane between 0.211 L CH\(_4\)/g VS (Raposo et al., 2006) to 0.300 L CH\(_4\)/g VS (Pereira et al., 2009). Huge amounts of agricultural raw materials like plants and livestock are processed in the food industries. During processing, wastes and wastewater are produced which are typically high in organic matter, making these waste products feasible feedstock for anaerobic digestion. Some examples include wastewater from meat processing industry (Luste et al., 2009), soybean (Yu et al., 1998) and orange juice (Siles et al., 2007) processing and spent apples from producing apple juice (Frederic et al., 2007).

### 2.4.2 Co-digestion

Co-digestion can be defined as one of the advantages of the anaerobic technology where several wastes with complementary characteristics are combined in a single treatment (Fernandez et al., 2005). One of the objectives of co-digestion is to improve the methane production of feedstock which otherwise if digested on their own would produce a low methane yield either due to its low biodegradability (e.g. due to lignin) or presence of inhibitory
compounds like potassium and lipids. Anaerobic digesters were originally designed for operation using sewage sludge and manures. Sewage and manure are not, however, the material with the most potential for anaerobic digestion, as the biodegradable material has already had much of the energy content taken out by the animals that produced it. Therefore, many digesters operate with co-digestion of two or more types of feedstock. For example, in a farm-based digester that uses dairy manure as the primary feedstock, the gas production may be significantly increased by adding a second feedstock, e.g., grass and corn (typical on-farm feedstock), or various organic byproducts, such as slaughterhouse waste, fats, oils and grease from restaurants, organic household waste, etc. The digestion of food waste and cow manure has been shown to improve by the addition of oily wastes (Neves et al., 2009). The co-digestion with fat increases the amount of biogas produced according to the organic loading imposed (Fernandez et al., 2005). Fat from animal origin (94% degraded) performs no different to fat of vegetable origin (97% degraded), which is of special interest in the industrial application of co-digestion process, where the co-substrates may be variable.

Another basis for co-digestion is when seasonal variations could be affecting the supply of anaerobic digestion feedstock, especially the case for agricultural waste and industrial by-products. Carballa et al., (2007) found that vegetables like cabbage, celery, tomato and potato can be co-digested indicating the possibility of treating different fruit and vegetable wastes together in one plant. The highest methane potential of 465 L CH₄/kg VS was achieved for a combination of tomato, potato and cabbage while the least was observed with tomato, potato and celery mixture, producing just 235 L CH₄/kg VS.

2.5 Pretreatment

Although anaerobic digestion is a mature technology for waste treatment some limitations are present which has to be addressed for improving reactor performance. One of the limitations is related to poor biodegradability of suspended solids which corresponds to around 50% of the chemical oxygen demand as in the case of municipal waste water (Foresti et al., 2006), making hydrolysis the limiting step of the anaerobic process. Hydrolytic organisms secrete enzymes to the bulk liquid where they are adsorbed onto a particle or react with a soluble substrate, while the organism may also attach to a particle, produce enzymes in its vicinity and benefit from soluble products released by the enzymatic reaction. Thermal treatment of the
substrate can enhance anaerobic digestion by accelerating the solubilization of the particulate organic matter as summarized in the table.

Most of the studies reported an optimal temperature in the range from 160 to 180ºC and treatment time from 30 to 60 min, while thermal treatment at moderate temperature (70ºC) lasted from hours to several days (Lu et al., 2008). Treatments at temperatures of 70ºC or 121ºC led to a 20 to 48% biogas production increase and treatments at 160-180ºC led to a 40 to 100% biogas production increase. The 160-180ºC pre-treatments are thus most efficient to enhance sludge anaerobic digestion, but they also lead to more dispersed results in terms of biogas production (Bougrier et al., 2008).

2.6 Batch Reactors

Various AD processes are used in practice and these can be divided into two types: suspended- or attached- growth anaerobic systems. The attached growth anaerobic processes include upflow packed-bed reactor process, upflow expanded-bed reactor process and fluidised-bed reactor process, etc. In addition, covered anaerobic lagoons are used in some cases (Tchobanoglous et al., 2003). Anaerobic digesters can be operated in batch, semi-continuous or continuous modes. In semi-continuous or continuous operations, the maximum growth rate can be constantly achieved at steady-state by controlling the feed rate (Boe, 2006). In the batch operation mode, the steady-state cannot be achieved as the concentrations of components in the digester are not constant with time (Klass, 1984). Batch reactors are loaded with feedstock, subjected to reaction, and then are discharged and loaded with a new batch. The batch systems may appear as in-vessel landfills but in fact achieve much higher reaction rates and 50- to 100% higher biogas yields than landfills for two reasons. First, the continuous re-circulation of the leachate and second, they are operated at higher temperatures than landfills (Vandevivere, 1999).

Batch processes offer the advantages of being technically simple, inexpensive and robust, however, the disadvantage includes settling of material to the bottom thus inhibiting digestion.

2.7 Parameters Influencing Anaerobic Digestion

2.7.1 Temperature

Temperature is one of the most important factors affecting the microbial activity within anaerobic digesters. There are mainly two temperature ranges suitable for anaerobic digestion of
organic fraction of urban waste for the production of methane. The mesophilic and thermophilic
temperature ranges are 20-40°C and 50-65°C with the optimum temperature of 35°C and 55°C respectively. Methanogens are more sensitive to temperature than any other organism in the anaerobic digester.

Acidogenesis has been reported to proceed at reasonable rates at temperatures as low as 21°C or lower, whereas, methane production rates at temperatures below 30°C have been found to be relatively slow (Gerardi, 2003). Higher temperatures have been reported to result in higher hydrolysis, fermentative and methanogenesis rates (Gerardi, 2003). Operation at a thermophilic temperature has some advantages over conventional mesophilic anaerobic sludge digesters, including, increased pathogen destruction and higher hydrolysis, acidogenesis and methanogenesis rates, and therefore less capital cost as a result of smaller fermenter size, increased efficiency with respect to the amount of organic matter destroyed, improved solid-liquid separation. However, a higher sensitivity to organic loading and temperature changes, increased concentrations of VFA in the reactor effluent and poor process stability due to reduced conversion of propionate to acetate have been reported in thermophilic sludge digesters (Han et al., 1997; Ghosh, 1987; Han and Dague, 1997). Information in the literature shows the greater influence of temperature on the microbial ecology of anaerobic digesters. Pender et al. (2004) observed that temperature elevation caused a change in the methanogenic population from a *Methanosaeta sp.* dominated community during mesophilic operation (35°C) to *Methanosarcina sp.* dominance. In general, methanogenic diversity has been reported to be greater in reactors operating under mesophilic conditions (Karakashv et al., 2005; Sekiguchi et al., 1999; Pender et al., 2004). Pender et al. (2004) reported a substantial increase of *Methanomicrobiales*, upon temperature increase from mesophilic to thermophilic conditions.

Anaerobic digestion of sludge and methane production at municipal wastewater treatment plants is mostly performed in the mesophilic range. At industrial wastewater treatment plants, thermophilic operation is sometimes preferred (Gerardi, 2003) due to limited space and the need for higher reaction rates in the anaerobic digesters. As regards to psychrophilic anaerobic digesters, although preliminary lab-scale studies showed the feasibility of using psychrophilic anaerobic reactors to treat low-strength waste-waters, their application are very limited (Lettinga et al., 2001). Currently, there is little information on the microbial populations of psychrophilic anaerobic reactors. It has been reported that under psychrophilic conditions, as with mesophilic
conditions, methanogenesis was mainly achieved through acetate cleavage by acetoclastic methanogens, especially *Methanosaeta sp.*, which was present in higher numbers in comparison to hydrogenotrophic methanogens (McHugh et al., 2003).

There is controversy regarding the survival and acclimatization of mesophilic species at thermophilic conditions or shift in microbial population in phased anaerobic digesters. Vandenburgh and Ellis (2002) performed a specific methanogenic activity test on both mesophilic (35°C) and thermophilic (55°C) biomasses of a temperature-phased digester. The methanogenic activity of the thermophilic biomass was as high as 0.2 and 1 g CH\(_4\)/g VSS/d when the serum bottles were incubated at 35 and 55°C, respectively. For the mesophilic biomass, the specific methane production rate at the mesophilic temperature was 0.4 and at the thermophilic temperature was 1 g CH\(_4\)/g VSS/d. As the specific methane production rates were almost similar at 35°C and 55°C for the biomass taken from thermophilic and mesophilic reactors they concluded that there were not necessarily a variation in microbial populations from mesophilic to thermophilic reactor. A similar conclusion was drawn by Song et al., (2004), indicating survival and activity of mesophilic organisms in a thermophilic digester. However, no microbiological examinations were performed in both cases. On the other hand, a number of investigations performed using molecular techniques show variations in the diversity of microbial consortia after temperature changes (Delbes et al., 2001; McMahon et al., 2001; Pender et al., 2004; McHugh et al., 2003).

There is little advantage in fermenting waste at 60 rather than at 55°C. Increasing the fermentation temperature from 55 to 60°C, in fact, may result in a lower net energy production due to the higher heating requirements to maintain the fermenter temperature and for municipal refuse the biogas production was found to peak dramatically at 35 and 55°C (Varel et al., 1980).

Growth conditions for hydrolytic, fermentative and hydrogenotrophic methanogenic populations are optimal at temperatures between 55 and 65°C. Therefore, the first steps in anaerobic digestion of solid wastes containing lignocelluloses materials are accelerated by operation at thermophilic temperatures. Thermophilic acetoclastic methanogens however exhibit maximal growth rates at 55°C and rapidly decreasing activities at temperatures higher than 57°C, so that a compromise temperature of 55°C is usually employed for single stage thermophilic digestion. The efficiency of methane production at mesophilic (30 to 45°C) and thermophilic (50 to 65°C) temperatures were compared in a four liter laboratory fermenters at a long and short
retention time (3 days and 18 days respectively), the results indicated that the effect of temperature on the rates of methane production was most noticeable at short retention time, yet temperature less than 60°C may be optimal for maximizing these rates.

2.7.2 Hydraulic retention time (HRT)

HRT is one of the main and controllable operational parameters that can influence the dominance of different microbial species, the kinds of intermediates produced by different microbial groups present and the rate of hydrolysis, fermentation and gasification reactions in digesters. Hydraulic retention time (HRT) of 14 days is optimum for biogas production from cattle dung and a reversion time of 10-20 days for mesophilic temperature and shorter for thermophilic temperature. There is a minimum retention time below which efficient fermentation ceases, due to the washout of the microbial population. Studies on the effect of retention time on the fermentation of sludge at 35°C showed that protein and carbohydrate fermenting bacteria grew rapidly and that these substrates were rapidly degraded to fatty acids even at retention time of less than 1 day. However, the fermentation of fatty acids did not occur until the retention time was greater than 5 days or more due to the slow growth of the fatty acid degrading bacteria.

The composition of the waste plays an important role when fermenters are operated at high loading rates and short retention time, this is because, at a shorter retention time the organic acid accumulation is higher and if less ammonia is available (nitrogen for buffering capacity) will lead to less alkality and less pH values. The concentration of acid generally increases with increase in loading rate.

2.7.3 Carbon to Nitrogen Ratio (C/N)

The relationship between the amount of carbon and nitrogen present in organic materials is represented by the C/N ratio. Optimum C/N ratios in anaerobic digesters are between 20 -30. A high C/N ratio is an indication of rapid consumption of nitrogen by methanogens and results in lower gas production. On the other hand, a lower C/N ratio causes ammonia accumulation and pH values exceeding 8.5, which is toxic to methanogenic bacteria. Optimum C/N ratios of the digester materials can be achieved by mixing materials of high and low C/N ratios, such as organic solid waste mixed with sewage or animal manure.
2.7.4 Total Solids Content/Organic Loading Rate

The total solids contained in a reactor play an influential part in the anaerobic process performance. Organic loading rate (OLR) is a measure of the biological conversion capacity of the Anaerobic Digester system. An anaerobic digester fed above its sustainable OLR results in low biogas yield due to accumulation of inhibiting substances such as fatty acids in the digester slurry. In such a case, the feeding rate to the system must be reduced. OLR is a particularly important control parameter in continuous systems. Many plants have reported system failures due to overloading.

An increase in total solids loading in a batch reactor from 15% and 20% to 25% saw the gas production decreasing correspondingly. Total inhibition occurred at 30% total solids loading. This was probably an effect of overloading, with a methane production lower than the control. The contact of substrate and bacteria is likely to decrease at increasing total solids concentration. Insufficient loading rate could reduce the digester performance due to the lack of nutrients for microbial growth.

2.7.5 Volatile Fatty Acids (VFAs)

Concentration of organic acids in a fermenter is a sensitive parameter used to determine fermenter stability. Branched chain volatile acids and ammonia are produced from branched chain amino acids present in protein or peptides by fermentative anaerobic bacteria, but none are known to oxidize fatty acids. The major volatile acids produced are acetic, propionate and butyric, while others may be present but not in detectable amounts.

Volatile Fatty Acids are the most important intermediate products and most of the terminal product methane is derived from VFAs. Before being converted to methane, VFAs are transformed to acetic acid, and the conversion rates vary in the decreasing order of acetic acid > butyric acid > propionic acid. It has been suggested by Marchaim and Krause (1993) that the propionic acid to acetic acid ratio can be used as an indicator to digester imbalance and a ratio greater than 1.4 could indicate impending digester failure.

For a normal anaerobic fermentation process, concentration of volatile fatty acids in terms of acetate should not exceed 2000 mg/L, propionic acid is believed to be the most toxic volatile fatty acid appearing in anaerobic digestion and its oxidation to acetic acid is the slowest among all volatile organic acids (VFAs). Among the VFA, propionic is one of the strongest
inhibitor and the concentration of around 1000 mg/L is considered as toxic and can cause digester failure. The reason for propionate accumulation could possibly be due to its slow rate of oxidation but the reaction can be made thermodynamically possible if hydrogen concentration in the digester is maintained at a favorable range between $10^{-4}$ and $10^{-6}$ atmospheres. In such cases the microbial consortia play an important role, consequential the close proximity of the microbial consortia of hydrogen producers with hydrogen consumers allows propionate conversion to proceed at much higher rates because of the fact that hydrogen partial pressure within the biofilm or aggregate is markedly lower than in bulk liquid. Moreover, it was reported that the microbial consortia proximity could efficiently reduced propionate and a single-phase non-mixed reactor enhances the close microbial consortia proximity.

2.7.6 pH

Even though, the pH and VFA are linked to each other but their relation depends on the waste composition which may differ from the type of waste and the environmental conditions of anaerobic digestion process. Microbial activities in anaerobic digester are greatly influenced by pH, an optimum pH value for anaerobic digestion lies between 6.4 and 7.2 (Chugh et al., 1999).

During digestion, the two processes such as acidification and methanogenesis require different pH levels for optimal process control. The retention time of digestate affects the pH value and in batch reactor acetogenesis occurs in rapid pace. Acetogenesis can lead to accumulation of large amounts of organic acids resulting in pH below 5.

Excessive generation of acid can inhibit methanogens. Increasing the pH level is necessary and this can be done with the use of basic solutions like NaOH or KOH. In addition, the degradation of protein through the release of ammonia has a buffering capacity. In which as digestion reaches the methanogenesis stage, the concentration of ammonia increases and the pH value can increase to above 8. Once the methane production is stabilized, the pH stays between 7.2 and 8.2.

2.7.7 Alkalinity

Ammonia is a by-product of decomposition of nitrogenous matter, like protein, phospholipids, lipids and nucleic acids and is usually found in two forms: the ammonium ion ($\text{NH}_4^+$) and free ammonia (FA) ($\text{NH}_3$) (Haug, 1993). Ammonia is beneficial to anaerobic
processes since nitrogen is an essential nutrient for anaerobic microorganisms but becomes inhibitory at concentrations between 1.7 to 14 g/L (Chen et al., 2008).

Ammonia’s inhibitory activity occurs at a concentration of 1500 mg/L which leads to increase in pH up to 8.5 which is toxic to methanogens. Ammonia accumulates because there is no mechanism for its biodegradation under methanogenic conditions. The problem can be overcome either by diluting the digester content with water; or by adjustment of feedstock C/N ratio (Mata-Alvarez, 2003).

Organic acids, pH and alkalinity are related parameters that influence digester performance. Under conditions of overloading and the presence of inhibitors, the methanogenic activity may possibly be inhibited; especially if the organic acids are produced in fast rate. This will result in the accumulation of acids, depletion of buffer and reduction in pH, if uncorrected via pH control and reduction in feeding, pH will drop to levels which will stop the fermentation process.

A normal healthy volatile acid to alkalinity ratio is 0.1. An increase to ratio of 0.5 indicates the onset of failure and a ratio of 1.0 or higher is associated with total failure. The alkalinity needed to neutralize VFA is calculated by multiplying 0.833 times the VFA concentration (mg/L as acetic acid).

2.7.8 Substrate

It has been implied that the composition of the substrate could affect the evolution of quantitative methanogenic community structure in an anaerobic process suggesting that more attention is required for quantitative as well as qualitative approaches on microbial communities for fundamental understanding of anaerobic processes, particularly under dynamic or transitional conditions (Changsoo Lee et al., 2009).

2.7.9 Inoculum

A viable seed can be derived from the environments in which the anaerobic methanogenic decomposition of organic compounds occurs naturally, for example, anaerobic sewage digesters, anaerobic lake sediments or from animal faeces. Inoculum material added into organic substrate plays an essential role for start-up of anaerobic digesters (Loper et al., 2004). In
anaerobic conditions the use of acclimatised organisms eliminated the need for extended incubation periods.

Different types of inoculum act more or less towards enhancing the decomposition of biomass to biogas generation, the amount of methane produced seemed proportional to the initial inoculum, the higher percentage of inoculum given, the higher production of biogas (Foster Carneiro et al., 2008), and substrate to inoculum ratio significantly affected the biogas production rate (Budiyono et al., 2009; Liu et al., 2009). In order to reach high biogas yield in biogas systems, type and quality of inoculums must be known and considered prior to start the operation of any anaerobic digester. The inoculums concentration affects the rate of biodegradation, the occurrence and duration of a lag period and the susceptibility of the degradation organisms to inhibitory effects (e.g. due to oxygen, toxic test materials, etc.).

Some of the frequently used inoculums materials are sewage sludge and cattle dung due to their methanogens content and high biogas potential. It has been noticed that methanogens loading of sewage sludge is higher than of cattle dung sample. Therefore, municipal sewage sludge is suitable for being used as inoculums in anaerobic digesters, due to its high level of methanogens content which can dominate over the other antagonistic microbial species contained in the organic substrate (Mateescu and Constantinescu, 2011).

However, past studies have shown that seed from a presumably more active cellulolytic environment such as the rumen (Chynoweth et al., 1993; O’Sullivan and Burrel, 2007), and anaerobic sediments containing decomposing kelp (Chynoweth, 1981) have not exhibited better decomposition rates compared to a sludge seed. Therefore, it seems reasonable that, for the case of anaerobic degradability testing where a standardized procedure is preferable, using seed sourced from a local wastewater treatment sludge digester would be suitable.

The proportion of active biomass and the diversity of this biomass (hydrolytic biomass versus methanogenic) contained in each inoculum may have caused the observed differences.

A low seed volume is desired to minimise biogas production which may blur the contribution from the substrate. A low seed volume also increases the allowable size of the substrate sample. However, a restricted seed amount can cause process overloading due to the overproduction of volatile acids (Angelidaki and Sanders, 2004).
2.7.10 Mixing

Suitable mixing is essential for optimum performance of the AD system. Mixing provides intimate contact between the digested material and active biomass, yielding uniformity of chemical (such as substrate, intermediate and final products) and physical (such as temperature) conditions throughout the digester, and preventing the formation of surface scum layers and the deposition of solids on the bottom of the tank (Appels et al., 2008). Natural mixing occurs to some extent in the digestion tank, due to the rise of gas bubbles. However, this is not sufficient for optimum performance; therefore, auxiliary mixing is needed. Prasad et al., (2008) evaluated the effect of mixing on anaerobic digestion of manure in laboratory-scale and pilot-scale experiments at 55°C. They found that in comparison with continuous mixing, intermittent and minimal mixing strategies improved methane production by 1.3% and 12.5%, respectively.

2.7.11 Inhibitors

Bio-methanogenesis is sensitive to several group of inhibitors (Table 7), the toxic effect of inhibitory compounds depends upon on its concentration and the ability of the bacteria to acclimatize to its effects. The inhibitory concentration depends upon different variables including pH, hydraulic retention time, temperature and the ratio of the toxic substance concentration to the bacterial mass concentration. Methanogenic populations are usually influenced by dramatic changes in their environment but an acclimatized to otherwise toxic concentration of many compounds.

Heavy metals such as copper, nickel, chromium, zinc, lead, etc. in small quantities are essential for the growth of bacteria but their higher concentration has toxic effects. Likewise, detergents including soap, antibiotics, organic solvents, etc. inhibit the activities of methane producing bacteria and the addition of these substances into the digester should be avoided.

High concentration of volatile fatty acids accompanied by low pH may inhibit methanogenesis. A problem with research on inhibition of microbial processes by VFA is that a VFA concentration increase results in a pH decrease. Therefore, it cannot be distinguished if VFA and/or pH are inhibiting the microbial process.
### Table 6:

**Concentration of Inhibitor in Anaerobic Digester.**

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>CONCENTRATION (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic compound</td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>2400</td>
</tr>
<tr>
<td>Heavy metals</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>160</td>
</tr>
<tr>
<td>Iron</td>
<td>1750</td>
</tr>
<tr>
<td>Cadmium</td>
<td>180</td>
</tr>
<tr>
<td>Copper</td>
<td>170</td>
</tr>
<tr>
<td>Chromium$^{2+}$</td>
<td>450</td>
</tr>
<tr>
<td>Chromium$^{6+}$</td>
<td>530</td>
</tr>
<tr>
<td>Nickel</td>
<td>250</td>
</tr>
<tr>
<td>Metals</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>2500 - 8000</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1000 - 3000</td>
</tr>
<tr>
<td>Potassium</td>
<td>2500 - 12,000</td>
</tr>
<tr>
<td>Sodium</td>
<td>3500 - 8000</td>
</tr>
<tr>
<td>Sulphide</td>
<td>600</td>
</tr>
</tbody>
</table>

Considering the anaerobic nature of methanogens, the toxic effect of nitrate can be attributed to its strong oxidizing property, among the cations Na$^+$, Ca$^{++}$ and Mg$^{++}$ exhibit toxicity in the descending order.

### 2.8 Measuring Anaerobic Decomposition

Anaerobic biodegradability can be ascertained by the amount of substrate transformed or the intermediates and end products developed by the different microorganisms groups. The volume of biogas produced also gives an indication of anaerobic decomposition. A number of methods are available to measure anaerobic biodegradability. Although these techniques vary in terms of the test procedure, measuring system and results, they can generally be simplified into three categories. This classification is based on the equipment used to measure anaerobic
biodegradability and activity including those that use (i) a variety of chemical analysis techniques to quantify substrate depletion or the formation of intermediates and end products, and (ii) microbiological based methods and (iii) gasometric measurement techniques to measure the amount of biogas produced.

2.8.1 Chemical Oxygen Demand (COD)

Substrate concentrations can be quantified by using either a particular substrate as such or a substrate-specific parameter. The dissolved organic carbon (DOC), chemical oxygen demand (COD) and total organic carbon (TOC) are generally applicable to assess the consumption of the whole substrate. However, the evolution from one component to another by anaerobic biodegradation may affect only a small change in COD, TOC or DOC, making the change harder to detect. Moreover, the complex nature of the test sludge and the tendency of some test chemicals to absorb to sludge particulate material may upset the suitability of the method to monitor substrate conversion (Birch et. al., 1989). That said, this does not mean that these gross measurements (as well as volatile solids, VS) are incapable of telling when there is substrate conversion to gas (CO2/CH4). But overall, a continuous reduction in COD over the period of 35 days HRT is a possible sign of healthy and stable anaerobic digestion. The more the percentage of reduction in COD the better the performance of the digester.

2.8.2 Analysis based on Microbiological Methods

2.8.2.1 Coenzyme F420

Methanogens induce UV blue-green auto-fluorescence, quantifiable under an auto-fluorescence microscopy. Methanogens with a high content of F420 such as the hydrogen-utilizing methanogens are visible. Exceptions apply to some methanogens which are not visually clear such as the acetate-utilizing methanogens that cannot be counted at all or the genus Methanosarcina which are found in clumps made of many individual cells (Solera et al., 2001a).

A high correlation was evident between the methane production and methanogenic population in thermophilic anaerobic reactors using auto-fluorescence microscopy as the method of analysis (Solera et al., 2001b). This method benefits over traditional microbiological methods as it can provide predictive value of the sample’s potential methanogenic activity without the need for culturing. A later study by Demirel and Yenigun (2006) found that numbers of auto-
fluorescent methanogens decreased during start-up. The type of substrate used and/or the variations of the organic loading rate (OLR) may have affected the morphology of the auto-fluorescent methanogens during start-up. The technique is not capable for distinguishing between live and dead microorganisms. In addition, the accuracy of results is easily influenced by fading of the auto-fluorescent cells and the presence of weakly or non-fluorescent cells (Solera et al., 2001a).

2.8.3 Gas quantification system

The total gas and methane productions when related to organic matter are direct indicators of the rate and extent of conversion. Gas yields are related to organic matter added which is expressed as VS and this is also known as specific gas production. These data are typically reported as gas volume per weight of volatile solids. Gas yield is directly proportional to the process efficiency. However, it is also important to note that a low gas/methane yield does not necessarily indicate a deficient performance but it is simply due to a low biodegradability of the substrate used. The methane yield is preferred over the gas yield because pH changes in the reactor can cause change in release or uptake of carbon dioxide that are unrelated to degradation. The use of volatile solids permits the calculation of a material balance between the feed, effluent solids and gas.

Methane production rate is a measure of process kinetics and is determined as volume of methane per volume of reactor per day. This parameter is a product of loading rate (kg/m$^3$/day) and methane yield (m$^3$/kg VS added). Methane content of the gas is a good indicator of stability. Since methanogenic activity is the key factor leading to imbalance, a reduction of methane gas content is a key performance parameter and has employed as an on-line control parameter (Chynoweth et al., 1994).

The volume of biogas generated can be determined either through volumetric or manometric means. In Volumetric method we measure the biogas volume by keeping the pressure constant and in the latter we can measure the pressure increase by keeping the volume constant. In volumetric methods, the biogas produced is allowed to move into an external collection system that measures the volume produced. This may be as simple as a lubricated glass syringe that is inverted straight onto the lid of the reactor, first introduced by Owen et al., in 1979. The overpressure generated inside the reactor forces the piston to expand, balancing the
pressure buildup to atmospheric pressure. The volume of biogas is read off straight and then either injected back into the serum bottle, or discarded to the atmosphere.

Alternatively, liquid displacement device can be employed. The set-up usually consists of a suitable vessel filled with a barrier solution and inverted in a reservoir. The biogas from the reactor passes to this liquid filled vessel thus displacing an equivalent liquid volume. The amount of biogas is determined either by graduation or by weighing the weight of liquid displaced.

The water displacement devices which make up the majority of volumetric gas metering methods are cheap and involve simple laboratory apparatus like water cylinders and plastic tubings. The manual approach is often cumbersome and time consuming making extended incubation period harder. This is due to the need for constant evacuation and resetting of the water level as large gas volumes cannot be catered by the small reservoir liquid available for the volume displacement. The automatic approach is less labour intensive but is more prone to problems associated with reservoir levels, corrosion, and complexity in managing CO₂ solubility and algae growth (Guwy, 2004). Corrosion is especially an issue with electronic solenoid valves and may be lessened using a siphon valve instead. The bubble counter is easily influenced by the liquid volume and viscosity where any variation may affect the bubble size thus the volume measured.

Regardless of which gasometric method, whether volumetric or manometric, the dissolution of CO₂ into water and its effects to the measurement is a recurring phenomenon. It is widely accepted that a considerable fraction of carbon dioxide can dissolve in water while methane hardly does. Factors such as pressure, pH, ratio of headspace-to-liquid volume, temperature and the complex thermodynamic equilibrium established between carbon dioxide and carbonate/bicarbonates of calcium and magnesium determines the solubility of CO₂. To reduce errors associated with CO₂ solubility, large increase of pressure should be avoided and can be done by venting the accumulated gas regularly. Another option is through use of a suitable barrier solution such as highly acidic or saline to avoid CO₂ diffusion in the liquid. An alkaline solution is an alternative if only methane needs to be measured since all the carbon dioxide in the off gas will be absorbed.
2.9 Identification of Microorganisms in Anaerobic Digesters

The study of microbial ecology requires identification of microorganisms based upon a comprehensive classification system that reflects the evolutionary relatedness of microorganisms (Hofman Bang et al., 2003). In any detailed study of microbial ecology in, the following three major objectives have to be achieved:

1) Identification and classification of microorganisms,
2) Quantification of microbial abundance, and
3) Quantification and identification of activity.

Morphology and other phenotypic traits have traditionally been used for identification and quantification of microbial populations (Hofman-Bang et al., 2003). Grotenhuis et al., (1991) microscopically counted cell numbers of methanogens and identified acetoclastic methanogens based on morphology, and hydrogenotrophic methanogens by visualizing auto-fluorescence at 420 nm. Culturing of methanogens is difficult due to their low growth rates and fastidious nutritional and environmental requirements.

Phylogenetic analysis allows the identification of microorganisms based on a molecular sequence, eliminating the need for cultivation. This implies that nucleic acids sequences can be retrieved from an environmental sample, sequenced, and compared to known sequences for identification of related organisms (Hofman-Bang et al., 2003).

The molecular biology approach is based on the RNA of the small ribosomal subunit (srRNA for prokaryotes) or their corresponding genes. The RNA molecule was chosen due to its obvious advantages, namely (i) being universal and abundant in all living beings (10^3 to 10^5 ribosomes/cell) (ii) molecule highly preserved throughout evolution (iii) sufficiently long gene sequence to produce statistically relevant data and (iv) gene can be easily sequenced with current technology (Sanz and Kochling, 2007). The current techniques most widely used include cloning of 16S rDNA, denaturant gradient gel electrophoresis (DGGE) and Fluorescent in situ hybridization (FISH).

2.9.1 Denaturant Gradient Gel Electrophoresis (DGGE)

Denaturant gradient gel electrophoresis (DGGE) helps determine the dominant members of a microbial community with the medium phylogenetic resolution without having to go through a laborious method as the cloning of 16S rDNA. This is generally so if knowledge of the
exact phylogenetic information is less desirable. With the DGGE technique, band patterns that directly reflect the genetic biodiversity of the sample are generated, following the various mobility on a gel of denatured DNA-fragments of similar size but with different nucleic acid sequences.

When DGGE analysis was used for studying the effect of various dilution rates on carbohydrate fermentation in a continuous flow stirred tank reactor, a population shift in the microbial community occurring with changing dilution rate (Ueno et al., 2001) was observed. Majority of the microorganism’s population detected were that of closely related thermophilic anaerobic bacteria. Another study demonstrated the applicability of DGGE for showing impact of temperature on bacterial community structure and diversity for bioreactors treating pharmaceutical wastewaters (Lapara et al., 2000). Three distinct microbial communities were observed with a reduction in number of bacterial populations seen under elevated, thermophilic reactor temperature.

The DGGE technique is very valuable for following dynamic modification in microbial communities, especially involving a large number of samples. It allows a rapid and simple examination of band patterns. However, the small number of detected bands restricts the number of identifiable species. The phylogenetic relations are thus less reliable, since the sequences of the bands involve only a small region (200-600 base pairs) of the 16S rRNA gene.

2.9.2 Fluorescence In-Situ Hybridization (FISH)

*Fluorescence in situ hybridization* (FISH) is a technique that uses fluorescently labeled phylogenetic oligonucleotide hybridization probes to detect specific microbial groups. The microbial cells having 16S rRNA sequences are hybridized with the fluorescent-dye labeled probes and detected under a microscope. The microbial abundance and/or activity are determined from the extent of hybridization. Stahl et al., (1995) have earlier distinguished probes suitable for identifying methanogenic microorganisms at different taxonomic levels (order, family and genus). Their work sets the ground for further application of FISH in the area of anaerobic digestion. Such applications include the determination of methanogenic activity in thermophilic-dry anaerobic reactors by comparing the amount of methane generated with the size of methanogenic population (Montero et al., 2008). The capability of FISH to illustrate the effects of high free ammonia concentrations on the performances of anaerobic bioreactors has also been
verified (Calli et al., 2005). The propionate degrading acetogenic bacteria were more sensitive to free ammonia than methanogenic archaea.

The test itself is suitable for routine analysis as it is fast and easy to perform without use of highly trained and specialized personnel. However, problems may arise if the probe for the desired bacterial taxon or group is not available. In addition, the user needs to be familiar with the target microorganisms, its ecosystem and the rRNA sequence is necessary prior to using FISH technique.

2.10 Methanogens as Key Organism in Anaerobic Digestion

Methanogens plays a pivotal role as a source for alternate form of energy, for xenobiotic pollution control, enhancing meat yield in cattle industry, global distribution of methane in the earth’s atmosphere and recently health implications in humans.

Methanogens are of great interest due to their unique physiological characteristic (e.g., low growth rate, high susceptibility to external conditions, limited substrate utilization range) has they are the only organisms which produce a hydrocarbon (viz., methane) as a major catabolic product. Methanogens are extremely sensitive to temperature and pH fluctuations and are inhibited by high levels of volatile fatty acids (Gavala et al., 2003).

Though they are grouped into several genera, only a few species of methanogens are known to play an important role in digestion of organic matter in anaerobic fermenters. In this regard the knowledge of the habitat, ecology and taxonomy of methanogens have to be better understood for isolation, cultivation, identification and utilization of methanogenic strains for improving the performance of the anaerobic digesters.

2.11 Habitat and Ecology of Methanogen

Methanogens are diverse group of strict anaerobes which are widely distributed in nature, but confined to strictly anaerobic environments. They can be found as an important member of microbiological consortia in variety of natural environment like permanently flooded soils, sediments, subterranean formations, waste streams, hot springs, peat bogs, decomposing algal mats, landfills, sewage-sludge digesters, bioreactors, fermenters constructed to process and dispose the wastes and also in the gastrointestinal tract of marine and land animals, insects and human gut, etc. Detectable methane production occurs in about one-third of the adult human
population. Genus Methanobrevibacter which uses hydrogen to reduce carbon dioxide to methane, is responsible for almost all of the methane produced in the human intestine (Khosro Issazadeh et al., 2013). Flooded Rice paddy soils are largely anoxic and typically methanogenic, they are estimated to contribute about 25% of the total budget of global methane emissions and therefore have a major impact on world climate due to their contribution to the greenhouse effect (Regine Grobkopf et al., 1998).

The abundance of methanogens in the above habitats depends on the limiting or absence of the electron acceptors such as oxygen, nitrate, iron and sulphate (Khosro Issazadeh et al., 2013). In the above habitats, the methanogens occupy the terminal niche in the transfer of electrons generated by the anaerobic degradation of organic matter (Shivadutta Singh and Mazumder, 2010).

2.12 Taxonomy of methanogens

All living cells have been classified into three main lines of evolutionary descent based on comparative analyses of sequences of the small subunit of ribosomal RNA. The phylogenetic domains are Archaea (formerly known as archaebacteria), Bacteria and Eukarya (Conway de Macario and Macario, 2003). The Archaea are further divided into phyla, with the most prominent being the Crenarchaeota and Euryarchaeota. Methanogens constitute a major taxonomic and phenotypic group within the Euryarchaeota (Trevan et al., 1987; Conway de Macario, and Macario, 2003; Watanabe et al., 2004).

Substrates and products of catabolism are a major part of the taxonomic descriptions of non-methanogenic bacteria, but other methods are required for methanogenic bacteria because of their restricted catabolism and their extreme phylogenetic diversity. Phenotypic characters are useful for taxonomy of methanogens include immunological, polyamine, lipid, cell wall, and protein analysis characters (Boone and Whitman, 1988).

Methanogens are morphologically very diverse, ranging from 0.4 μm to 1.7 μm in size. They occur as rods, cocci, spirilla, filaments, and plate shapes (Karakashev et al., 2005).

2.13 Phylogeny of Methanogens

The methanogens are phylogenetically diverse, they are classified into six orders: Methanobacterales, Methanocellales, Methanococcales, Methanomicrobiales,
Methanosarcinales, and Methanopyrales. Prokaryote phylogenetic is based on the results of comparative analysis of the evolutionary conservative 16s rRNA genes. The methanogen orders are based on 16s rRNA gene sequence similarities of less than 82% (Liu and Whitman, 2008). The orders are divided into 13 families (< 88-93% 16s rRNA gene sequence identity) and 31 genera (< 93-95% 16S rRNA gene sequence identity). A sequence identity of 98% or less is evidence for a separate methanogen species (Whitman et al., 2006).

Methyl coenzyme M reductase is an alternative phylogenetic marker for methanogens. Methyl coenzyme M reductase consists of two alpha (mcrA), two beta (mcrB), and two gamma (mcrG) subunits. The subunits are phylogenetically conserved (Hallam et al., 2003a) and on the basis of the comparison of available 16S rRNA and mcrA gene sequences of methanogens, the mcrA gene was demonstrated to be an alternative phylogenetic marker showing similar relationships as those seen based on the 16S rRNA gene (Luton et al., 2002).

2.13.1 Methanobacteriales

The order Methanobacteriales contains two families and five genera. Members of the order Methanobacteriales generally utilize H₂/CO₂ for methanogenesis although some can also use formate or secondary alcohols (Whitman et al., 2001). The Methanobacteriales have a cell wall composed of pseudomurein with the family Methanothermaceae also having a protein surface layer (Whitman et al., 2001). Morphologically members of this order can be rod or coccoid in shape. Members of the genus Methanosphaera can only produce methane by the reduction of methanol with hydrogen. This is the only genus outside of the order Methanosarcinales that can utilize methanol for methanogenesis (Whitman et al., 1992).

2.13.2 Methanococcales

This order contains two families and four genera. Both genera of the family Methanocaldococcaceae contain hyperthermophiles, as does the genus Methanothermococcus of the family Methanococcaceae. Members of this order use H₂/CO₂ for methanogenesis and have cell walls with a protein S-layer (Whitman et al., 2001).

2.13.3 Methanomicrobiales

The order Methanomicrobiales contains three families and eleven genera. Members of the order Methanomicrobiales utilise H₂/CO₂ for methanogenesis. Some species can also use
formate or secondary alcohols as electron donors. Acetate is not a substrate for methanogenesis; however, many species require acetate as a carbon source (Whitman et al., 2001). This is the most morphologically varied of the methanogenic orders; they can be coccoid, rod, plate or spiral in shape (Whitman et al., 2001). Cell walls have a glycoprotein S-layer and may also have an exterior sheath (Whitman et al., 2001).

### 2.13.4 Methanocellales

The order *Methanocellales* contains one family and one genus (Euzéby, 2011). This order was previously known as Rice Cluster I (RC-1) and, was known only from 16S rRNA gene sequences from rich paddy soil until the first representative was isolated in 2008, *Methanocella paludicola* (Sakai et al., 2008). Both species can utilize H₂/CO₂ and formate for methanogenesis (Sakai et al., 2008; Sakai et al., 2010).

### 2.13.5 Methanosarcinales

The order *Methanosarcinales* consists of three families. Members of the order *Methanosarcinales* are the most metabolically versatile of all the methanogens. They can produce methane by the disproportionation of methyl-group containing compounds, the fermentation of acetate and the reduction of CO₂ with H₂ (Whitman et al., 2001). However, they do not utilize formate. All methanogen that can utilize methylated compounds without hydrogen are found in this order. They also have diverse morphologies including cocci, pseudosarcinae, and sheathed rods. Members of the genus *Methanosaeta* (*Methanothrix*) are the only methanogens that utilize only acetate for methanogenesis. Cell walls of the *Methanosarcinales* have an S-layer (Whitman et al., 2001).

### 2.13.6 Methanopyrales

The order *Methanopyrales* is represented by only one species, the hyperthermophilic *Methanopyrus kandleri* (Euzéby, 2011). H₂/CO₂ is used for methanogenesis and cell walls contain pseudomurein (Whitman et al., 2001). *M. kandleri* has a temperature range of 84–110°C, it is found in marine hydrothermal systems (Kurr et al., 1991).
2.14 Cultivation of Methanogens

Biomethanation process in a properly run digesters is much more efficient, giving maximum yield at minimal cost. During digestion the enumeration and identification of methanogens at short intervals could help implement measures to increase gas production at maximal rates and avoid deviations in the balance of the microbial flora that could lead to inefficient digestion.

Methanogens is among the fastidious microorganism and with no standard method of detecting or culturing them, cultivating methanogen in a laboratory is a challenging, laborious and time-consuming process.

Methanogens are difficult to isolate as many are not amenable to laboratory cultivation and some require long incubation times for growth to occur, as obligate anaerobes, methanogens are sensitive even to low levels of oxygen (< 10 ppm) (Garcia et al., 2000). Methanogens need to be cultured in pre-reduced media and require a redox potential of about –330 mV for growth (Lange & Ahring, 2001). The insensitivity of methanogens to certain antibiotics has been used to advantage in the culture of methanogens by eliminating contaminating bacteria (Garcia et al., 2000).

2.14.1 Enrichment Technique

It is known that enrichment cultures select for fast-growing organisms with high growth yields and those best able to grow in the growth medium used for the cultivation. This means that the organisms isolated are not necessarily of numerical significance in the habitat under study. In addition, since these growth conditions favor organisms best adapted to the medium, there is also no guarantee that the organisms obtained have any significance in the biogeochemical processes within the environment under study. These problems, together with the general labor and time intensiveness of many cultivation studies, have meant that molecular approaches to studying microbial populations recently have gained popularity (Grobkopf et al., 1998). However, this approach is still in the phase of methodological evolution especially for heterogenous environments such as anaerobic digester, so the correct interpretation of the data obtained is still difficult.

Enrichment cultures are selective in different ways due to the media used. Organisms of numerical significance but with low growth rates, even if the latter are of smaller numerical
importance in the habitat under investigation. An alternative to enrichment cultures is cultivation by dilution series. The advantage of this technique is that, at high dilution levels, numerically dominant organisms can be isolated, given that they are able to grow in the medium selected (Liesack et al., 1997).

2.14.2 Roll Tube Method (Hungates Technique)

The breakthrough in an attempt for culturing anaerobic microorganism came with the Hungate anaerobic methods (Hungate et al., 1967). Then and there variant modifications were made in accordance with convenience and requirements and of studies and to the particular needs or talents of different groups of workers (Bryant, 2009). Modifications of Hungate technique described by Bryant have been most widely used for study of methanogens (Zeikus, 1977). Improved agar bottle techniques (Hermann et al., 1986) modified from Hungates roll tube method by Uffen and Wolfe (Ulfen et al., 1970) and latter by Braun (Braun et al., 1979) are also being practiced by many laboratories which also are inexpensive.

Anaerobic hood and an alternative for evacuation and gassing (Doddena, 1978) another modification of traditional roll tube methods is less laborious, less expensive and easy to perform method for culturing of anaerobic specially methanogen communities, as developed by Balch and Wolfe, 1976. Even the present researchers with anaerobic laboratory works still carries out variant modifications of traditional techniques in order to facilitate accordingly with working environmental factors (Shivadutta singh and Mazumder, 2010).

All the culture technique is based on the principle of creating an anaerobic environmental condition for culture of samples. Unlike other culture techniques, roll tube method is inexpensive and can be followed at any laboratory without much difficulty, but the technique needs well trained expertise with repeated practice.

The nutrient media for methanogens may typically contain ammonium chloride, potassium phosphate, magnesium sulphate, sodium phosphate, calcium chloride di-hydrate, yeast extract, sodium carbonate, potassium di-hydrogen phosphate, sodium bicarbonate, magnesium chloride, resazurin, ferrous chloride, and sodium chloride at varying concentrations (Raposo et al., 2006; Zhang et al., 2003; and Wu et al., 1995).

The corresponding trace elements may include ferrous sulphate, manganese sulphate, cobalt chloride, calcium chloride hexahydrate, zinc sulphate, copper sulphate, boric acid, sodium
molybdate, sodium chloride, sodium selenate and nickel chloride (Raposo et al., 2006; and Zhang et al., 2003).

Speece (1988) reported that the nutrients, in decreasing order of importance, are: nitrogen, sulfur, phosphorous, iron, cobalt, nickel, molybdenum, selenium, riboflavin and vitamin B$_{12}$. Ammonium appears to serve as the nitrogen source for all methanogens. Most methanogens utilize sulfide as a sulfur source, but some can utilize cysteine. Nickel is an essential component of bacterial ureases and in bacterial enzymes which convert H$_2$-CO$_2$ to acetate. It has been reported that 75% of methane is produced from acetate conversion (Evans, 2001). In addition to nickel, iron and cobalt must be supplemented to achieve high microorganism (volatile suspended solids, VSS) concentration.

### 2.14.3 Uncultivable Methanogens

Molecular approaches allow the use of informational molecules like rRNA for the direct characterization of environmental communities, for inference of the amounts of total and specific microbial biomass, and even location of specific species within a given environment (Nealson, 1997). The retrieval of nucleic acid sequences from environmental samples is used to investigate microbial communities without cultivation. The nucleic acid fraction that is obtained ideally represents the whole microbial diversity present in the digester sample.

Among the recognized archaeal taxa there are some novel phylogenetic groups detected only by culture-independent studies. For example, Zoige cluster I (ZC-I, Zoige wetlands, Tibetan Plateau) is an uncultured methanogen cluster known from 16S rRNA and mcrA gene sequencing (Grosskopf et al., 1998). Other Archaeal lineages suspected of containing methanogens, for example, the Mediterranean Sea Brine Lake candidate division 1 (MSBL1) which has been identified in Mediterranean hyper-saline basins by 16S rRNA gene based analysis (van der Wielen et al., 2005) could be a uncultivable methanogen.

### 2.15 Need of Methanogenic Consortium

Methanogenic community transition is very important, particularly during a start-up period because a successful start-up is critical for long-term stable and efficient digester operation (Show et al., 2004).
### 2.16 Methane Mitigation

A human intervention to reduce the sources or enhance the sinks of greenhouse gases (GHG) is referred to as mitigation. Methane mitigation is a human intervention to reduce the methane concentration in the atmosphere.

The methane is removed naturally from the atmosphere in three ways: these methods, commonly referred to as sinks, are oxidation by chemical reaction with tropospheric hydroxyl ion, oxidation within the stratosphere, and microbial uptake by soils.

In spite of their important role in removing excess methane from the atmosphere, the sinks cannot keep up with global methane production. Since, enteric methane (CH$_4$) is the most important contributor of GHG emissions in ruminant production, it is essential to look for alternatives to reduce CH$_4$ from live-stock emissions and by doing so contribute to less GHG. Methane mitigation is effective in one of two ways: either a direct effect on the methanogens, or an indirect effect caused by the impact of the strategy on substrate availability for methanogenesis, usually through an effect on the other microbes of the rumen.

Many methane mitigation strategies proposed have indeed multiple modes of action including chemical suppression and biotechnological interventions have been investigated to attenuate methane production. However, there is a growing concern over the use of chemical inhibitors in animals used for the human consumption, and a possibility of developing chemically resistant methanogens and in this regard the researches are now focused on developing biological strategies to solve the problem.

Wright et al., (2004) conducted an in-vivo assessment of two formulations of methanogen vaccine in sheep to reduce methane emissions. They reported that of the two vaccines tested, the formulation with fewer antigenic targets resulted in a significant reduction (7.7%) of methane emissions compared with a control group, the significant decrease in methane emissions observed were due to specific activity of anti-methanogen secretory antibodies delivered to the rumen via saliva.

On the basis of the influence of methanogen vaccines in the reduction of rumen methane emission a number of researchers also have shown that IgY antibodies can be used for passive immunization by oral administration of hen egg yolk (IgY) to control methane emission.
Figure 4:

Global Anthropogenic Methane Emissions are Projected to Increase by 23% to 7,904 Million Metric Tons by 2020

(Courtesy: Bartos, 2009)

It may achieve the similar or better results as like the specific antibodies induced by methanogen vaccines. Furthermore, the yolk of eggs from laying hens immunized with the target antigen is shown to be an inexpensive and convenient source for polyclonal antibodies.