Review Article

Microbial Ecology of Anaerobic Digesters: The Key Players of Anaerobiosis

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Anaerobic digestion is the method of wastes treatment aimed at a reduction of their hazardous effects on the biosphere. The mutualistic behavior of various anaerobic microorganisms results in the decomposition of complex organic substances into simple, chemically stabilized compounds, mainly methane and CO$_2$. The conversions of complex organic compounds to CH$_4$ and CO$_2$ are possible due to the cooperation of four different groups of microorganisms, that is, fermentative, syntrophic, acetogenic, and methanogenic bacteria. Microbes adopt various pathways to evade from the unfavorable conditions in the anaerobic digester like competition between sulfate reducing bacteria (SRB) and methane forming bacteria for the same substrate. *Methanosarcina* are able to use both acetoclastic and hydrogenotrophic pathways for methane production. This review highlights the cellulosic microorganisms, structure of cellulose, inoculum to substrate ratio, and source of inoculum and its effect on methanogenesis. The molecular techniques such as DGGE (denaturing gradient gel electrophoresis) utilized for dynamic changes in microbial communities and FISH (fluorescent in situ hybridization) that deal with taxonomy and interaction and distribution of trophic groups used are also discussed.

1. Introduction

Methanogenesis is complex, redox biochemical reactions occurring under anaerobic conditions. Under symbiotic effects of various anaerobic and relatively anaerobic bacteria, multimolecular organic substances are decomposed into simple, chemically stabilized compounds—methane and carbon dioxide [1]. Generally, this process consists of liquefaction and hydrolysis of insoluble compounds and gasification of intermediates. This is accompanied by a partial or complete mineralization and humification of organic substance [2]. An advantage of the process of anaerobic digestion is the production of biogas, a high energy fuel which may be used to produce environmental-friendly energy. It is basically for this reason that scientists and power industry companies have been interested in anaerobic digestion for almost 140 years. Biotechnology of biogas production usually refers to digestion of various types of organic wastes, food industry wastewater, sewage sludge, animal excrements or organic fraction of municipal wastes, and so forth. In some countries, subjected to anaerobic digestion are plants deliberately grown for this purpose, for example, maize [3, 4]. Currently in many European countries, the production of biomass as a substrate for the biogas plants is well established. In the most extreme European case, the German government has adopted measures in 2011 to reduce even monoculture maize production for energy purposes [5, 6].

Digestion connected with biogas production may play a triple part. First, it is a method of converting the energy contained in biomass into a useful fuel (biogas) which may be stored and transported. Second, it is a method of recycling of organic wastes into stable soil additives, that is, valuable liquid fertilizer and energy. Third, it is a method of wastes treatment aimed at a reduction of their hazardous effects on the environment [7].

Biogas is a digester gas arising from the activity of a consortium of anaerobic bacteria which decompose organic matter. Its composition depends on the type of raw material subjected to the digestion process and on the method of conducting this process and is as follows. Methane CH$_4$...
enzymes participating in their degradation [13,14]. The remaining part of the com-
pounds remains in their primary state because of the lack of biodegradation. The remaining part of the com-
setics, that is, cellulose and cellu-
cottons is considered of hydrolytic bacteria. Hydrolysis of hardly decomposable fatty acids. This stage of the methanogenesis passes through acetates, CO₂, and H₂, whereas other aci-
dogenesis products play an insignificant role. As a result of these transformations, methanogens may directly use the new products as substrates and energy source. Accumulation of electrons by compounds such as lactate, ethanol, propionate, butyrate, and higher volatile fatty acids is the bacterial response to an increase in hydrogen concentration in the solution. The new products may not be used directly by methanogenic bacteria and must be converted by obligatory bacteria producing hydrogen in the process called acetogene-
thesis. Among the products of acidogenesis, ammonia and hydrogen sulfide which give an intense unpleasant smell to this phase of the process should also be mentioned [8, 13, 17].

The acid phase bacteria belonging to facultative anaerobes use oxygen accidentally introduced into the process, creating favorable conditions for the development of obligatory anaerobes of the following genera: Pseudomonas, Bacillus, Clostridium, Micrococcus, or Flavobacterium.

2.2. Acidogenesis (Acidification Phase). During this stage, the acidifying bacteria convert water-soluble chemical substances, including hydrolysis products, to short-chain organic acids (formic, acetic, propionic, butyric, and pentanoic), alcohols (methanol, ethanol), aldehydes, carbon dioxide, and hydrogen. From decomposition of proteins, amino acids and peptides arise, which may be a source of energy for anaerobic microorganisms. Acidogenesis may be two-directional due to the effects of various populations of microorganisms. This process may be divided into two types: hydrogenation and dehydrogenation. The basic pathway of transformations passes through acetates, CO₂, and H₂, whereas other acido-
genesis products play an insignificant role. As a result of these transformations, methanogenes may directly use the new products as substrates and energy source. Accumulation of electrons by compounds such as lactate, ethanol, propionate, butyrate, and higher volatile fatty acids is the bacterial response to an increase in hydrogen concentration in the solution. The new products may not be used directly by methanogenic bacteria and must be converted by obligatory bacteria producing hydrogen in the process called acetogenesis. Among the products of acidogenesis, ammonia and hydrogen sulfide which give an intense unpleasant smell to this phase of the process should also be mentioned [8, 13, 17].

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2.3. Acetogenesis. In this process, the acetate bacteria including those of the genera of Syntrophomonas and Syntrophobacter convert the acid phase products into acetates and hydrogen which may be used by methanogenic bacteria [18]. Bacteria like Methanobacterium suboxydans account for decomposition of pentanoic acid to propionic acid, whereas Methanobacterium propionicum accounts for decomposition of propionic acid to acetic acid. As a result of acetogenesis, hydrogen is released, which exhibits toxic effects on the microorganisms which carry out this process. Therefore, a symbiosis is necessary for acetogenic bacteria with autotrophic methane bacteria using hydrogen, hereinafter referred to as syntrophy [9, 18]. Acetogenesis is a phase which depicts the efficiency of biogas production, because approximately 70% of methane arises in the process of acetates reduct-

Consequently, acetates are a key intermediate product of the process of methane digestion. In acetogenesis phase, approximately 25% of acetates are formed and approximately 11% of hydrogen is produced in the wastes degradation process [18].

2.4. Methanogenesis. This phase consists in the production of methane by methanogenic bacteria. Methane in this phase of the process is produced from substrates which are the products of previous phases, that is, acetic acid, H₂, CO₂, and formate and methanol, methylamine, or dimethyl sulfide. Despite the fact that only few bacteria are able to produce methane from acetic acid, a vast majority of CH₄ arising in
the methane digestion process results from acetic acid conversions by heterotrophic methane bacteria [19]. Only 30% of methane produced in this process comes from CO₂ reduction carried out by autotrophic methane bacteria. During this process, H₂ is used up, which creates good conditions for the development of acid bacteria which give rise to short-chain organic acids in acidification phase and consequently to too low production of H₂ in aceticogenic phase. A consequence of such conversions may be gas rich in CO₂, because only its insignificant part will be converted into methane [20, 21].

3. Cooperation of Microorganisms of Methane Fermentation Process

Conversions of complex organic compounds to CH₄ and CO₂ are possible owing to the cooperation of four different groups of microorganisms and are presented in Table 1. These microorganisms may be counted among primary fermentation bacteria, secondary fermentation bacteria (syntrophic and acetogenic bacteria), and two types of methanogens belonging to domain Archaea. These microorganisms occur in natural environment and fulfill various roles during the process of anaerobic degradation of wastes [13]. Syntrophy is a form of symbiosis of two metabolically different groups of bacteria, which enables degradation of various substrates [9, 11].

Cooperation of the population of microorganisms enables synthesis of certain products which are then used by another group of bacteria. The bacteria belonging to domain Archaea, which are involved in the production of methane, exhibit synergistic relationships with other populations of microorganisms. They may develop only when hydrogen is used by hydrogenotrophs. Such cooperation between microorganisms producing hydrogen and using hydrogen was defined as interspecific transfer of hydrogen [13]. Syntrophy between microorganisms producing and using hydrogen allows for the growth and activity of these microorganisms.

The phenomenon of interspecific transfer of hydrogen enables the growth of the so-called syntrophic bacteria (Syntrophomonas, Syntrophospora, and Syntrophobacter) which oxidise such compounds as propionate and butyrate and thereby obligatorily use hydrogen as electron acceptor. The reaction of butyrate oxidation, carried out by these microorganisms, may occur only when low pressure of H₂ is maintained [11, 22]. Syntrophic bacteria cannot grow in the form of pure cultures but only when accompanied by microorganisms using hydrogen produced by them, for example, methanogenic archaons. So syntrophy is a process in which the compound’s decomposition occurs, with participation of two or more microorganisms and none of them can use this compound separately. Syntrophic bacteria produce adenosine triphosphate (ATP) as a result of substrate phosphorylation or oxidation used in their metabolism of not only fatty acids but also alcohols produced by other microorganisms as a result of digestion. These compounds are converted to acetate and hydrogen which are used by methanogenic archaons [9]. Bacteria of Syntrophomonas genus oxidise butyric acid and caproic acid to CH₃COOH and H₂. Species belonging to this genus oxidise also pentanoic and enanthetic acids to CH₃COOH, CO₂, and H₂. Oxidation of propionate is also an important stage of methanogenesis process in which syntrophic consortia of acetogenic bacteria and methanogenic archaons obligatorily participated [11].

Most of the propionate-oxidising syntrophic bacteria, so far isolated from different environments, belonged to Syntrophobacter genus from δ-proteobacteria group. CH₃COOH and H₂ produced by the mentioned bacteria are used by methanogenic archaons to produce methane [12]. Species of Syntrophobacter genus are able to use sulfates as electron acceptor in the process of propionate oxidation. Syntrophic oxidation of propionate is not limited to gram-negative bacteria but also to gram-positive bacteria in which a similar phenomenon was observed. Two thermophilic, gram-positive species of syntrophic bacteria oxidising propionate have been described as follows: Desulfitomaculum thermobenzoicum subsp. thermosyntrophicum and Desulfovibrio [9]. Desulfitomaculum, similarly to Syntrophobacter may use sulfates as electron acceptors. Desulfovibrio may participate, along with Methanobacterium genus, in forming CH₃COOH and H₂ in the process of anaerobic respiration and digestion, using sulfate and lactate to make these products. The Desulphovibrio compete with methane forming bacteria by using the same substrate and produce H₂S and hinder the methane formation as shown in Figure 1.

3.1. Substrate to Inoculum Ratio. Inoculum to substrate ratio (ISR) has been shown to affect the methane yield, methane production rate, and the consumption of VFAs. ISR is generally presented on VS basis. ISR affects the occurrence and duration of lag phase (extracellular hydrolysis), methanogenesis, VS/COD reduction, and susceptibility of the microorganisms of the inhibitory effects [23, 24]. For a stable process, the ISR is a major parameter affecting the process of anaerobic digestion and should be higher than 1 in terms of VS. References [25, 26] have reported changes in the hydrolysis time of cellulose with changing the inoculums concentration. The optimum inoculum to substrate ratio depends on the source of inoculum. The different sources of inoculums will have

| Microorganisms       | Electron donor       | Electron acceptor | Product      | Reaction type |
|----------------------|----------------------|-------------------|--------------|---------------|
| Fermentative bacteria| Organic carbon       | Organic carbon    | CO₂          | Fermentation  |
| Syntrophic bacteria  | Organic carbon       | Organic carbon    | H₂           | Acidogenesis  |
| Acetogenic bacteria  | Organic carbon/H₂     | CO₂              | CH₃COOH      | Acetogenesis  |
| Methogenic bacteria  | Organic carbon/H₂     | CO₂              | CH₄          | Methanogenesis|
**Figure 1:** Many different groups of bacteria within the anaerobic digester often compete for the same substrate and electron acceptor. Methane is produced by methane-forming bacteria and a variety of acids and alcohols are produced by sulfate reducing bacteria. Hydrogen is used with sulfate (SO$_4^{2-}$) by sulfate-reducing bacteria and hydrogen sulfide (H$_2$S) is produced. [41].

different metabolic activities so the optimum ratio required for optimum anaerobic digestion of a particular feed may vary using inoculums from different sources. An optimum ISR ensures the presence of the groups of microorganisms required for the complete anaerobic digestion [27]. Lower ratio of inoculums to feed can lead to accumulation of VFAs and result in inhibition of methanogenesis [23, 28]. ISR is one of the crucial parameters but unfortunately is not included in the experimental design by many researchers [29]. The ISR can affect the methane production rate or methane yield. The kinetics of methane production were dependent on the concentration of inoculums used; however, the final yield was the same [30].

The amount of inoculum used per tonne of waste for starting the anaerobic process was shown to have a relevant effect on both biogas and biomethane production. For a waste-to-inoculum ratio ranging from 1:1 to 1:3 (w/w), the energy production increased from about 100 kWh/t to about 380 kWh/t of waste organic fraction. Consequently, the investment costs also rise, going from about 180 €/t to more than 370 €/t of treated municipal solid waste organic fraction. The economic optimization analysis showed that the waste-to-inoculum ratio that minimized the treatment cost ranged from 1:1.5 to 1:2.5, being strongly influenced by the plant size (i.e., electrical efficiency) and by the Green Certificate value. Considering the cost of producing electrical energy, the optimum ratio was 1:2, leading to a cost of 0.26 €/kWh, during the investment period, and of 0.14 €/kWh, after the investment period [31].

Inoculation will not be of much use for the fermentation of easily biodegradable waste. For initiating methanogenesis of such wastes, it is strongly recommended not to homogenize inoculums and waste, which will dissipate and degrade the active methanogens. Multiple loading of the waste to the inoculum is superior to single shot loading if the total organic loading rate is the same. It is suggested that an appropriate inoculation method could initiate methanogenesis rapidly even at a low inoculation rate [32].

Within days of incubation of MSW at 55°C, methane was produced at a high rate. In an attempt to narrow down which components of typical MSW contained the thermophilic methanogens, vacuum cleaner dust, banana peel, kitchen waste, and garden waste were tested as inoculum for thermophilic methanogenesis with acetate as the substrate. Results singled out grass turf as the key source of thermophilic acetate degrading methanogenic consortia. Within 4 days of anaerobic incubation (55°C), anaerobically incubated grass turf samples produced methane accompanied by acetate degradation enabling successful start-up of thermophilic anaerobic digestion. Stirring of the culture was not conducive for successful start-up as it resulted specifically in propionate accumulation [33].

**4. Methanogens—Key Microorganisms of the Methane Fermentation Process**

Methanogens as absolutely anaerobic microorganisms inhabit anaerobic environment ecosystems, such as tundras, marshlands, rice fields, bottom deposit, swamps, sandy lagoons, tanks where wastewater is decomposed, sewage sludge, solid wastes landfills, and ruminants’ stomach [15, 16, 34]. These microorganisms are particularly sensitive to changes in temperature and pH, their development being inhibited by a high level of volatile fatty acids and other compounds, that is, hydrogen, ammonia, and sulphur in the environment [34].

Among methanogenic microorganisms, we can distinguish psycho-, meso- and thermophilic microorganisms. Mesophilic and thermophilic bacteria described in literature exhibit high activity within temperatures, respectively, 28 to 42°C and 55 to 72°C. So far, no anaerobic psychrophilic bacterium has been found which would exhibit activity at a temperature lower than 25°C. Temperature is very important for methanogenic bacteria, due to a limited temperature resistance of their enzymatic structures.

Methanogenic bacteria usually develop in inert conditions, with environmental pH from 6.8 to 7.2. This, however, does not mean that methanogenesis does not occur in environments of acid or alkaline reaction. Methanogens which decompose acetates (Methanosarcina barkeri and Methano-sarcina sp.) were isolated from environments of approximately pH 5, while methylotrophic and hydrogen-oxidising methanogens were found in strongly alkaline ecosystems [16]. Methanogenic bacteria belong to chemolithotrophs, because they are capable to use CO$_2$ as a source of carbon [16, 34].

Methanogens are an important group of microorganisms for several reasons, the most important being their ability to process organic matter to methane. Methanogenic bacteria are used in anaerobic decomposition of wastewater, as a part of the wastes treatment system. Sedimentation processes are also used in stabilization of primary and secondary sludge arising in the process of aerobic wastewater treatment. These microorganisms arouse also some interest in pharmaceutical
Table 2: Kinetics characteristics of various methanogens.

| Parameter          | Methanoseta | Methanosarcina | Reference |
|--------------------|-------------|----------------|-----------|
| $U_{\text{max}}$ (d$^{-1}$) | 0.20        | 0.60           | [50, 52, 58] |
| $K_s$ (mg COD L$^{-1}$) | 10–50       | 200–280        | [50, 52, 58, 59] |
| $NH_4^+$ (mg L$^{-1}$) | <3000       | <7000          | [56, 57, 61] |
| Na$^+$ (mg L$^{-1}$) | <10,000     | <18,000        | [65]       |
| pH range          | 6.5–8.5     | 5–8            | [64, 66, 158, 159] |
| pH shock          | <0.5        | <0.8–1         | [64]       |
| Temperature range (°C) | 7–6.5      | 1–70           | [44, 160, 161] |
| Acetate concentration (mg L$^{-1}$) | <3000       | <15000         | [22, 52, 58, 59, 64, 66, 71, 145] |

Table 3: Typical reactions carried out by methanogens during anaerobic process [11].

| Reaction carried out by methanogens |
|------------------------------------|
| Hydrogen: 4H$_2$ + CO$_2$ → CH$_4$ + 2H$_2$O |
| Acetate: CH$_3$COOH → CH$_4$ + CO$_2$ |
| Formate: 4HCOOH → CH$_4$ + CO$_2$ + 2H$_2$O |
| Methanol 4CH$_3$OH → 3CH$_4$ + CO$_2$ + 2H$_2$O |
| Carbon monoxide: 4CO + 2H$_2$O → CH$_4$ + 3HCO$_3$ |
| Trimethylamine: 4(CH$_3$)$_3$N + 6H$_2$O → 9CH$_4$ + 3CO$_2$ + 4NH$_3$ |
| Dimethylamine: 2(CH$_3$)$_2$NH + 2H$_2$O → 3CH$_4$ + CO$_2$ + 2NH$_3$ |
| Methylamine: 4(CH$_3$)$_2$NH$_2$ + 2H$_2$O → 3CH$_4$ + CO$_2$ + 4NH$_3$ |
| Methyl mercaptans: 2(CH$_3$)$_2$S + 3H$_2$O → 3CH$_4$ + CO$_2$ + H$_2$S |
| Metals: 4Me$_0$ + 8H$^+$ + CO$_2$ → 4Me$^{++}$ + CH$_4$ + 2H$_2$O |

industry, because they may constitute a source of vitamin B12 [35].

4.1. Taxonomy and Morphology of Methanogens. All alive organisms, based on analysis and comparison of conservative phylogenetic features, through analysis of 16S and 18S rRNA, were classified to three main taxonomic units of the living world. Three phylogenetic domains may be distinguished: Archaea, Bacteria and Eukarya. According to analysis of sequence 16S rRNA, [7] 4133 methanogenic bacteria were classified to domain Archaea. Among the microorganisms within domain Archaea, four groups are distinguished, the most visible being Crenarchaeota and Euryarchaeota. In taxonomic and phenotypic respect, methanogens belong to Euryarchaeota group [36]. Methanogenic bacteria are divided into 4 classes, 5 orders, 9 families, and 26 genera [11]. As mentioned earlier, according to assumed classification of live organisms, methanogens are archeons. Unlike bacteria, methanogens do not have a typical peptidoglycan (murein) skeleton and are characterized by different metabolism. Besides, methanogens' cytoplasmatic membrane consists of lipids composed of isoprenoid hydrocarbons glycerol lipids. Methanogene ribosomes exhibit a size similar to that of eubacteria ribosome, but the sequence of principles in ribosome RNA, especially 16S rRNA, is completely different [37]. Methanogens are largely differentiated morphologically. Methanogens exhibit almost all shapes occurring in bacteria: cocci (Methanococcus), rods (Methanobacterium), short rods (Methanobrevibacter), Spirillaceae (Methanospirillum), sarcina (Methanosarcina), and filiforms (Methanothrix). The size of these microorganisms ranges from 0.3 to 7.4 μm [21]. The properties of selected methanogens are presented in Table 2. Typical reactions carried out by methanogens during anaerobic processes are shown in Table 3.

4.2. Substrates Used by Methanogenic Bacteria. Methanogens process a limited quantity of simple organic substrates, the most important of which are CH$_3$COOH, H$_2$, and CO$_2$ [13]. Most methanogenic bacteria are able to use H$_2$ and CO$_2$ for their growth (Table 3), although certain species process CH$_3$COOH, CH$_3$NH$_2$, and HCOOH [8]. Owing to used substrates, methanogens are divided into two groups [11].

4.2.1. Hydrogenotrophic. This group of microbe uses only H$_2$ and CO$_2$. Partial pressure of hydrogen is an important parameter which defines stability and disturbances in the anaerobic digestion process. Therefore, the activity of hydrogenotrophic methanogens is essential for stability and efficiency of the digestion process. Efficiency and activity of hydrogenotrophic methanogens are important in both anaerobic processing of simple, soluble types of substrates (such as acetate, ethanol, dextrose, and propionate) and various types of wastes (e.g., oil).

4.2.2. Acetotrophic. It reduces methyl groups, such as species of Methanosarcinales genus which use simple compounds for their growth, that is, acetate. Acetate is one of the most
important substrates for methanogenic bacteria, because over 70% of biomethane comes from processing of acetic acid. Acetotrophic methanogens are obligatory anaerobes which process acetate to methane and carbon dioxide. The activity and efficiency of this group of microorganisms are critical in processing of biomass of complex organic compounds [38]. Methanogenic bacteria binding hydrogen were found to belong to family Methanobacteriaceae [3].

Manure is a complex type of substrate composed of hydrocarbons, proteins, and acids. Characteristics of the population of bacteria and archaeans in anaerobic thermophilic processing of manure indicated a dominance of two species: Methanoculleus thermophilicus (hydrogenotrophic) and Methanosarcina thermophila (acetotrophic). The main hydrogenotrophic microorganisms participating in anaerobic processing of fruit and vegetable wastes comprise Methanospheara stadtmannae and Methanobrevibacter wolfinii [39].

Counted among acetotrophic methanogens should be the species belonging to genus Methanosarcina. It was found out that during anaerobic processing of sewage sludge and manure, the number of microorganisms of Methanoseta genus decreased with increasing acetate in the environment, with simultaneous intensive growth of the bacteria belonging to Methanosarcina genus which are acetotrophic methanogens [20]. The studies on the dynamics of the population of anaerobic microorganisms participating in degradation of municipal wastes and sewage sludge indicated that Methanoseta concilii was a dominant species among acetotrophic methanogens [22].

The rate of the development of digestive bacteria depends on the type of applied substrate. If the substrates are carbohydrates, the rate of bacteria generation in acid phase amounts to 5 h; whereas in the case when fats are the substrate, the time is prolonged to approximately 72 h. The generation time, in acetogenesis phase, for bacteria using propionic and fatty acids reaches, 84 and 131 h, respectively. In the methanogenesis phase, the generation time ranges between 15 and 85 h [40].

4.2.3. Methanosarcina: The All Rounder Microbe for Anaerobic Digestion. The methanogenic archaea are responsible for the final and critical step of anaerobic digestion, as they produce valuable methane. One of the major drawbacks of anaerobic digestion is the sensitivity of the methanogenic consortium to different environmental factors. An abrupt change in pH, an increase in salt or organic matter concentration, an alteration of the loading rate, or the introduction of a toxic compound often causes system failure [47–49]. Overloading is a frequent problem in anaerobic digestion since it leads to accumulation of fatty acids, as these are no longer efficiently removed by the methanogens. This is because of their low growth rates, compared to the acidogenic and acetogenic bacteria, which cause the uncoupling of the acetogenic bacteria and the methanogens [50]. Overloading thus causes an accumulation of fatty acids to concentrations which may have a toxic effect on the methanogens [48]. It also lowers the pH to suboptimal values, since the optimal pH range for methanogens lies between 6.8 and 7.5 [50, 51]. Taking these aspects into account, anaerobic digestion in continuously stirred tank reactors (CSTR) commonly operates at organic loading rates (OLR) below their optimum capacity to avoid overloading and sludge retention times (SRT) in the order of 20 days or more and to avoid washout of the methanogens [51]. It is however reported that Methanosarcina sp. have high growth rates (i.e., doubling times in the order of 1.0 to 1.2 days) and are tolerant to sudden changes in pH of around 0.8–1.0 units caused by overloading, compared to the other methanogens, which have doubling times of minimum 4–6 days and tend to be affected by a pH shock of 0.5 units or even less [52–54]. Methanosarcina sp. are able to use both the acetoclastic and the hydrogenotrophic methanogenesis pathway, making them more tolerant to specific inhibitors of the acetoclastic pathway, such as fluoracetate and methyl fluoride, compared to Methanosaeta sp. [53, 55]. They are tolerant to levels of ammonium up to 7000 mg TAN L⁻¹ (total ammonia nitrogen) as well [56, 57]. These Methanosarcina sp. are therefore able to achieve stable growth at low retention times (even as low as 4 days), high organic loading rates, and high levels of, for example, ammonium [52, 57].

4.2.4. Methanosarcina as Inoculum Species for Bioaugmentation. Severe impairment of the methanogenic community terminates the methane production [47–49]. Regeneration of the anaerobic digester can then be achieved by interruption of the feeding of the reactor and allowing the methanogens to restore or by replacing them entirely by anaerobic biomass in the digester. Neither option is economically very easy, thus enforcing the need for other solutions to solve the problem. The addition of an inoculum rich in Methanosarcina sp. can offer perspectives to tackle the problem. The specific features of the Methanosarcina sp. (i.e., high tolerance to several impairments, as described above) make them suitable for establishing the regeneration of the anaerobic digester if methanogenesis has ceased due to environmental factors. The disadvantage of the addition of Methanosarcina sp. rich anaerobic sludge lies within the limited removal of the residual acetate, which has accumulated most likely due to the uncoupling of the acetogenic bacteria and the methanogens, because of the high Ks value of Methanosarcina sp. for acetate [52, 58, 59]. The addition of a Methanosarcina sp. rich inoculum can initiate two different scenarios in the anaerobic digester. In the first case, the Methanosarcina sp. could start to grow fast, because of a high Umax and cause the methanogenic community to evolve towards a Methanosarcina sp.-dominated community with enhanced stability. In the other case, the Methanosarcina sp. could temporarily grow, ameliorating the conditions which initially caused the methanogenesis to cease and then decline again in abundance or disappear totally from the system, because of the regained dominance of Methanosaeta sp. at concentrations of acetate below 100 mg L⁻¹ [52, 59]. Both scenarios, however, will revive the methanogenesis in the anaerobic digester.

Anaerobic digesters normally host a diverse methanogenic community, containing both acetoclastic and hydrogenotrophic methanogens, thus enhancing reactor stability
value of VFA, especially acetate, in the output, because of the high Ks of this system will be the elevated concentrations of residual in the order of 20–30 days [51]. The disadvantage however compared to conventional anaerobic digesters, which have an SRT because of the low sludge retention time of 3–5 days, to be the dominant hydrogenotrophic methanogens at both pathways, that is, the direct cleavage of acetate to CH₄ and 

\[
\text{CH}_4 + \text{CO}_2 \rightarrow \text{H}_2 + \text{CO}_2
\]

\[
\text{Acetate} \rightarrow \text{Volatile fatty acids} \rightarrow \text{Syntrophic acetogenic bacteria (SAB)} \rightarrow \text{CO}_2 + \text{H}_2 \rightarrow \text{Hydrogenotrophic methanogens (HM)} \rightarrow \text{CH}_4 + \text{CO}_2
\]

**Figure 2:** Methanogenesis in anaerobic digestion as documented for normal waste treatment reactor systems [42]. The SAB consist mostly of Clostridium sp. at both mesophilic and thermophilic conditions [43]. The hydrogenotrophic methanogens in both mesophilic and thermophilic anaerobic digesters belong to the two orders of Methanobacterales and Methanomicrobiales [44].

[60, 61]. Methanosarcina sp. are however able to convert both acetate and CO₂ with H₂ to methane [55]. Other compounds can also be converted to methane by Methanosarcina sp., as mentioned above [55, 62, 63]. The ability of Methanosarcina sp. to convert these compounds to methane thus makes the presence of other methanogens in the anaerobic digester redundant.

It can therefore be stated that an anaerobic digester, entirely based on Methanosarcina sp., would still be able to achieve stable methanogenesis. The creation of an anaerobic digester entirely based on Methanosarcina, under normal nonstressed growth conditions, would require a decrease of the SRT to a value as low as 3–5 days, since Methanosarcina sp. have low doubling times, compared to Methanosaeta sp. [55, 59]. Yet taking into account their tolerance to several impairments (e.g., high ammonium and salt concentrations and high concentrations of VFA, correlated to a high OLR), an anaerobic digester with a retention time below 5 days would be based almost entirely on Methanosarcina and be able to handle severe impairments and organic loading rates [57, 64–66]. A Methanosarcina sp. based anaerobic digester would be able to handle higher amounts (Figure 2). Proposed robust methanogenesis system is based on syntrophic acetate oxidizing (SAO) bacteria and Methanosarcina for intensive energy production reactor systems. The SAB consist mostly of Clostridium sp. at both mesophilic and thermophilic conditions [43]. Syntrophic acetate oxidizing bacteria can be located in both mesophilic (Clostridium ultunense and Syntrophacectis schinkii) and thermophilic (Thermacetogenium phaeum and Thermotoga lettingae) conditions [18, 57, 61, 67–69]. Methanosarcina and Methanobacterales are postulated to be the dominant hydrogenotrophic methanogens at both mesophilic and thermophilic conditions of organic waste, because of the low sludge retention time of 3–5 days, compared to conventional anaerobic digesters, which have an SRT in the order of 20–30 days [51]. The disadvantage however of this system will be the elevated concentrations of residual VFA, especially acetate, in the output, because of the high Ks value of Methanosarcina sp. for acetate, imposing the need.

In anaerobic digestion, methanogenesis occurs through two pathways, that is, the direct cleavage of acetate to CH₄ and CO₂ (acetoclastic methanogenesis) and the reduction of CO₂ with hydrogen gas (hydrogenotrophic methanogenesis). The contribution of each pathway to the methanogenesis depends on the inoculum sludge, the organic substrate, and the process conditions [11]. It is to be expected that acetate, which is formed during acetogenesis from, for example, propionate, ethanol, and lactate, will be directly converted to methane by the acetoclastic methanogens. Hydrogen gas and CO₂, formed during hydrolysis, acidogenesis and acetogenesis, will be converted to methane by hydrogenotrophic methanogens (Figure 1). Yet part of the acetate can be oxidized to hydrogen gas and CO₂, depending on the reactor conditions, by syntrophic acetate oxidizing (SAO) bacteria, instead of being directly converted to methane by the acetoclastic methanogens [67, 70]. Syntrophic acetate oxidizing bacteria have been detected in both mesophilic (Clostridium ultunense and Syntrophacectis schinkii) and thermophilic (Thermacetogenium phaeum and Thermotoga lettingae) conditions [18, 43, 57, 61, 67, 68]. The pH range of these syntrophic acetate oxidizing bacteria varies between 6.0 and 8.0, with a pH of 7 being the optimal value. Several Clostridium sp., that is, potential acetate oxidizing partners in the syntrophic relationship, are however still able to grow at a pH of 5.0, thus making syntrophic acetate oxidizing bacteria able to remain active at slightly or even heavily deteriorated conditions in anaerobic digestion [43, 69]. The syntrophic oxidation of acetate to H₂ and CO₂ is however thermodynamically very unfavorable (DG₀₀ = 104.6 kJ mol⁻¹). As a result, syntrophic acetate oxidation can only take place at very low values of H₂ partial pressure, that is, between 2.6 and 74 Pa [67]. Only in this narrow interval of hydrogen gas concentrations, the syntrophic acetate oxidation and the subsequent hydrogenotrophic methanogenesis (SAO-HM) coupling are thermodynamically possible [67]. Hence these acetate oxidizing bacteria require partner organisms, which they find in hydrogenotrophic methanogens [61, 67]. Nevertheless, this indirect conversion of acetate to methane has the disadvantage that the available energy has to be divided over two organisms instead of one in the case of the direct cleavage of acetate to methane and CO₂, thus questioning the benefit of a two-organism system. The answer lies in
the sensitivity of the acetoclastic Methanoseta sp. towards different impairments causing a shift in acetate degradation from direct acetate cleavage towards syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis [70]. This shift of a Methanoseta sp. dominated system towards a hydrogenotrophic methanogens dominated methanogenic community has been reported at elevated ammonium concentrations in the range of 3000 mg TAN L$^{-1}$ [57, 61, 70]. An increase in the organic loading rate, causing the accumulation of fatty acids, especially acetic acid, also leads to a shift towards syntrophic acetate oxidation and hydrogenotrophic methanogenesis [70, 71]. An increase of the accumulated acetate concentration up to a value of 3000 mg COD L$^{-1}$ can be considered as the threshold for the shift in the methanogenesis pathway [58, 71]. The reactor temperature influences the dominant pathway as well, as acetoclastic methanogenesis is favored at mesophilic conditions, whereas SAO-HM becomes the dominant pathway at thermophilic temperatures [58, 70]. The free energy change of the syntrophic oxidation of acetate and subsequent hydrogenotrophic methanogenesis amounts to 36 kJ mol$^{-1}$ at 25°C and 42 kJ mol$^{-1}$ at 58°C, thus explaining the dominance of syntrophic acetate oxidation at elevated temperatures [18]. High ammonium concentrations and elevated acetate levels seem to suppress the growth of Methanoseta sp. and therefore enhance the SAO-HM. Yet, Methanosarcina is also able to tolerate these enhanced concentrations and is both acetoclastic and hydrogenotrophic [52, 55]. Given this information, the coupled growth of syntrophic acetate oxidizing bacteria and Methanosarcina sp. may enhance reactor stability. Elevated concentrations of fatty acids and ammonium lead to a rise in the abundance of both SAO and Methanosarcina sp., although at the cost of a severe decline in the abundance of Methanoseta sp., demonstrating the tolerance of this partnership to several environmental factors [57, 70]. The success of this partnership can possibly be due to the formation of nanowires between the SAO and Methanosarcina. It has already been reported that nanowires can be formed between Geobacter sp. and methanogens in anaerobic digesters and it seems therefore not unlikely that other species may succeed in achieving direct interspecies electron transfer in anaerobic digesters [72, 73]. The unique S-layer of Methanosarcina sp. together with the possibility of energy conservation by means of direct electron transfer makes the SAO-HM partnership very suitable for DIET [74]. A robust methanogenic process can therefore be established, based on the interaction between SAO and Methanosarcina sp. with acetoclastic methanogenesis by Methanosarcina sp. at low OLR and ammonium concentrations and syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis by Methanosarcina sp. at elevated OLR and ammonium concentrations (Figure 2). An anaerobic digester based on this system will not require a population change at abrupt impairments but only a change in methanogenesis physiology by Methanosarcina sp., which will greatly decrease the response time of anaerobic digesters to perturbations.

4.2.5. Cellulosic Microorganism. Cellulose is the most abundant biopolymer on earth and also a significant part of solid wastes. The cellulosic waste can be anaerobically digested to methane, which is a source of energy for heating, cooking, electricity, or car fuel [6]. Depolymerization followed by solubilization of the polymers is the first step in anaerobic digestion of solid wastes. After that, cellulose degradation products (i.e., cellobiose and higher order soluble cellobiose trins) can be converted to methane and carbon dioxide through acidogenesis, acetogenesis, and methanogenesis processes [75, 76]. The methanogenesis phase is normally considered as the rate-limiting step for anaerobic digestion of easy-to-digest substrates, because of the slow growth rate of the methanogenic bacteria. However, during anaerobic digestion of cellulose, several studies have rather pointed out the bacterial hydrolysis phase as the rate-limiting step [77]. Consequently, there is considerable interest in developing efficient pretreatments of cellulose to increase the rate of solubilization during anaerobic digestion. Two major questions need answers before this progress can be realized. Firstly, the structural features of cellulose need to be altered to enhance its solubilization rate. Secondly, what impact would augmented cellulose solubilization have on the rest of the digestion process? To meet the first question, (a), alteration of crystallinity, accessible internal surface area, and degree of polymerization, three major physicochemical and structural parameters, may increase the digestibility of cellulosic substrates [78, 79]. Although evaluation of these fine-structure parameters and their relative importance to cellulose hydrolysis is not easy; the crystallinity and accessible internal surface area have been reported to be the most important factors influencing the enzymatic hydrolysis rate of cellulose. Several studies have shown that the crystallinity of cellulose is the most important factor, with amorphous cellulose being more ready for digestion by fungal enzymes than high-crystalline cellulose [76, 78]. Nevertheless, other studies have reported that their available surface area is a more important factor in utilization of cellulose by whole-cell systems [80, 81]. The mechanism by which cellulose digestion occurs is by means of anaerobic microorganisms excreting cellulosome on the outside surface of their cell wall and attaching themselves to the surface of the cellulose particles, and hence causing hydrolysis. Therefore, cellulosome is more efficient compared to the free fungal cellulase system [82, 83]. The available surface area for hydrolysis bacteria to be attached is therefore an important determinant of the hydrolysis rate. Moreover, the degree of crystallinity does not have a significant impact on the rate of cellulose degradation by ruminal microorganisms [81]. It was later confirmed [84] that no significant difference between the solubilization rates of microcrystalline cellulose and amorphous cellulose exists. In consequence, the effect of cellulose structure on its solubilization rate in anaerobic digestion has not been adequately elucidated and thus question (a) above still needs to be tackled. Addressing the second question (b), it is known that in anaerobic digestion the degradation steps are linked and influence each other. However, this network is still not fully characterized [85] having recently investigated the digestion of microcrystalline cellulose at high organic loading.
rates (OLR) between 5.7 and 34.3 gVS/L. They increased the OLR gradually. Because of high adaptation level of microbial biocenosis, with every increase of OLR, the only sign of system stress was increasing total degradation time [85].

4.3. Enzymatic Hydrolysis of Plant Cell Wall. Because plant cell walls are relatively complex structures, a great variety of enzymes are needed for extensive enzymatic hydrolysis. Only three readily cultivable species of ruminal bacteria (Ruminococcus albus, Ruminococcus flavefaciens and Fibrobacter succinogenes) are found in high numbers in the rumen and are capable of rapid growth on cellulose [86]. The most extensively studied strains of these species appear to degrade cellulose at rates similar to that in the rumen itself (0.1 h

extensivelystudiedstrainsofthesespeciesappeartodegrade
and are capable of rapid growth on cellulose [86]. The most

bactersuccinogenes
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losomes of
complexes that display similarity to the well-studied cellu-

fibrolytic enzymes appear to be organized into cellulosomal

hydrolases, most of which are retained on the cell surface.

This species can produce more than 100 polysaccharide

enzymes are needed for extensive enzymatic hydrolysis.

sugars are linked together by different plant compounds and this association may affect

fermentation of the resulting glycerol; the long-chain free fatty acids are typically not fermented, although unsaturated acids may be reduced or isomerized [96] prior to their absorption.

4.3.1. Structure of Cellulose. Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellulobiose. These form long chains (or elemental fibrils) linked together by hydrogen bonds and van der Waals forces. Cellulose is usually present as a crystalline form and a small amount of nonorganized cellulose chains forms amorphous cellulose. In the latter conformation, cellulose is more susceptible to enzymatic degradation [97]. Cellulose appears in nature to be associated with other plant compounds and this association may affect its biodegradation. Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is formed of D-xyllose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic, and D-glucuronic acids. Sugars are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds. The main difference between cellulose and hemicellulose is that hemicellulose has branches with short lateral chains consisting of different sugars and cellulose consists of easily hydrolyzable oligomers. Lignin is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall. It is present in the cellular wall to give structural support, impermeability, and resistance against microbial attack and oxidative stress. It is an amorphous heteropolymer, non-water soluble and optically inactive that is formed from phenylpropane units joined together by non-hydrolyzable linkages. This polymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl propanol). This heterogeneous structure is linked by C-C and aryl-ether linkages, with aryl-glycerol and β-aryl ether being the predominant structures [98]. The metabolism of various organics for biogas generation has been given in Figure 3.

5. Different Inocula for Biogas Production

Different stages of anaerobic digestion contain considerably varied species of symbiotic microorganisms, which can be broadly classified as two groups: acidogens and methanogens. Since the methanogenic reaction is believed to be the rate-limiting step in the overall anaerobic digestion, proper
control of the methanogenic phase has been a key factor in the successful operation of most anaerobic processes [99]. Specifically, methane production is carried out by methanogenic groups belonging to Archaea, which are known to have very sensitive growth and activity behaviors concerning different substrates and operation conditions [56]. The stable formation of the methanogenic community determines the success or failure in anaerobic digestion systems treating wastewaters including highly concentrated organic and toxic materials (e.g., highly concentrated ammonia or acetate), such as swine wastewater.

5.1. Effect of Inoculum Source. The inoculum used for anaerobic digestion plays a vital role in the way it will degrade the organic matter and produce biogas and also affect the amount of biogas in some cases [100]. The inocula from different sources are normally utilized under the same operating conditions such as mesophilic temperature and around neutral pH, the respective microbial communities vary in many aspects like profile of extracellular enzymes, species profile, biofilm forming behavior, nutritional requirements, and physiological characteristics [26]. Microbial activities and chemical composition of inoculum’s consortia greatly affect the performance of biogas production particularly from lignocellulosic biomass [20]. The source of inoculum not only affects the amount of biogas production but also influences the kinetics of the process of anaerobic digestion. It has been reported in the variation in the amount and kinetics of biogas production during solid state anaerobic digestion of corn stover using inocula from different origins [28]. The selection of inocula to startup a full scale digester is crucial and plays an important role in overcoming the acidification. If an active inoculum is selected, process failure can be avoided during the startup phase [101]. The selection of source of the inocula is based on the activity; hence selection of the active inocula will reduce the amount of the inocula required for the operation of a full scale digester and consequently the volume of the digester [102]. Origins of inocula used by many researchers are from anaerobic digester treating municipal wastewater sludge, cattle manure, food waste, or combinations. However, the inocula from anaerobic digester treating municipal wastewater sludge are expected to have diverse groups of active microorganisms hence being suitable for many different sources of microorganisms. Inoculum source is also a very important operational parameter. Also, it is crucial for the selection of waste/inocula ratio as well as the assessment of anaerobic biodegradability of solid wastes [103]. In dry-thermophilic digestion, the inoculum source and the total solid percentage selected are responsible for accomplishing rapid onset of a balanced microbial population. The systematicness of inoculation, the percentage of inoculation, and wetting procedure differ between processes proposes by several authors. The percentage of inoculation for acidogenic fermentation of organic urban wastes is approximately 30% weight/weight (w/w) [104]. In case of the anaerobic biodegradability of solid waste, the use of a highly active anaerobic inocula or animal inoculum waste will reduce significantly the experimental time or reduce the amount of inocula required in full scale batch digesters and consequently the corresponding digester volume [105].

The effect of inoculum source on anaerobic thermophilic digestion of separately collected organic fraction of municipal solid wastes was investigated [106]. Laboratory scale reactors (V:1.1L) were evaluated using six different inocula sources: (1) corn silage (CS); (2) digested restaurant waste mixed with rice hulls (RH_OFMSW); (3) cattle excrement (CATTLE); (4) swine excrement (SWINE); (5) digested sludge (SLUDGE); and (6) SWINE mixed with SLUDGE (1:1) (SWINE/SLUDGE). Sludge is the best inoculum source for anaerobic thermophilic digestion of the treatment of organic fraction of municipal solid waste at dry conditions (30% TS). Over 60 days operating period, it was confirmed that SLUDGE reactor can achieve 44.0% COD removal efficiency and 43.0% VS removal. In stabilization phase,
SLUDGE reactor showed higher volumetric biogas generated of 78.9 mL/day (or 35.6 mL CH₄/day) reaching a methane yield of 0.53 L CH₄/g VS. Likewise, SWINE/SLUDGE and SWINE were also good inocula [106].

Inocula play an important role in anaerobic reactor startup by balancing the populations of syntrobacter and methanogens. Such balance makes syntrophic metabolism thermodynamically feasible in anaerobic digestion. The effect of inocula on performance of dairy manure digestion after the examinations of microcolonies in the granular, nongranular, and dairy manure suggests that syntrophic occurrence of methanogens and syntrophobacter in granular inocula was common while it was less visible in nongranular and completely absent in dairy manure. The scanning electron microscope and transmission electron microscope images show the variations in the juxtaposition and bacterial population level in different inocula and these were linked with biogas production in different treatments, granular sludge, nongranular sludge, and mixed culture from anaerobic lagoon and the control did not receive exogenous inocula, demonstrating the importance of syntrophic association and population level of different groups of bacteria in anaerobic digester performance [107].

Cellulose solubilization is the rate determining step in the anaerobic digestion of organic solid waste [3, 77, 108]. An increase in the rate of solubilization should lead to an increase in the overall efficiency of the anaerobic digestion process. Reactor studies on cellulose solubilization rates are typically conducted in anaerobic, mesophilic environments with approximately neutral pH and may be inoculated from a range of source environments including landfill leachate, manure, sewage sludge, anaerobic digesters, or the rumen [109–118]. Despite similarities in the operating conditions of reactors inoculated from different source environments, the respective microbial communities may differ in many respects, including their species profile, biofilm architecture, nutritional requirements, particle colonization, and hydrolysis rates. Microbial communities in cellulyotic environments are highly complex with interactions among numerous trophic groups required to carry out the digestion process [113, 116, 119–121]. *Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens* are dominant cellulose degrading bacteria in the rumen [122], while *Clostridium* species tend to be dominant in landfills and anaerobic digesters [111, 123].

Cellulose solubilization kinetics from controlled batch digestions were investigated [26]. Separate measurements and analyses were performed for sessile biomass (biofilms) and planktonic biomass (free suspensions). Experiments were conducted using either leachate enriched on cellulose or rumen fluid as inocula to assess if the effect of biomass concentration was consistent for microbial populations from different source environments in the batch. The correlation between solubilization rate and sessile biomass was statistically the same for the rumen and leachate inoculated reactors indicating that at low concentration ratios of inocula to cellulose, the rate of cellulose solubilization is dependant primarily on sessile biomass concentration rather than the species profile of the cellulytic community [26]. The effect of various inocula on biogas production has been shown in Table 4.

The results of thermophilic methanogens in turf used as inoculums show that *Methanoculleus* sp. regarded as hydrogenotrophic and *Methanosarcina* sp. regarded as aceticlastic methanogens were present in turf tested. However, active aceticlastic methanogens were present in turf soil only. The thermophilic methanogens were present in various turf grass species: *Stenotaphrum secundatum*, *Cynodon dactylon*, and *Zoysia japonica*. A dried and pulverized grass extract that could be generated can serve as a readily storable methanogenic inocula for thermophilic anaerobic digestion. The methanogens could also be physically extracted into an aqueous suspension, suitable for inocula [124].

Sea wrack (dislodged sea grasses and seaweeds) was used in biogas production. Fresh water scarcity in island communities where sea wrack could accumulate led to seawater utilization as liquid substrate. Three microbial seeds cow manure (CM), marine sediment (MS), and sea wrack-associated microflora (SWA) were explored for biogas production. The average biogas produced was 2172 ± 156 mL (MS), 1223 ± 308 mL (SWA), and 551 ± 126 mL (CM). Though methane potential (396.9 mL CH₄/g volatile solid) computed from sea wrack proximate values was comparable to other feedstocks, the highest methane yield was low (MS = 94.33 mL CH₄/g volatile solid). Among the microbial seeds, MS proved to be the best microbial source in utilizing sea wrack biomass and seawater. However, salinity (MS = 42%) observed exceeded average seawater salinity (3). Hence, methanogenic activity could have been inhibited. This is the first report on sea wrack biomass utilization for thalassic biogas production [125].

The biomethanization of food waste had three different total solid percentages (20%, 25%, and 30%) with two different inocula percentages (20–30%) of mesophilic municipal sludge. The best performance for food waste biodegradation and methane generation was the reactor with 20% of total solids and 30% of inocula gives rise to an acclimation stage with acetogenic/acetogenic activity between 20 and 60 days and methane yields of 0.49 L CH₄/g VS [126].

The conducted BMP test explored the influence of using two different anaerobic inocula sources on the digestion of food waste: digested sludge from a municipal wastewater treatment plant and from a digester treating the organic fraction of municipal solid wastes. The inoculum from an anaerobic digester treating municipal wastewater sludge was superior to the inocula from anaerobic digester treating food waste in digesting food waste [127].

Fish waste (FW) obtained from a fish processor was ensiled for biogas production. The FW silages were prepared by mixing FW with bread waste (BW) and brewery grain waste (BGW), and the quality of the prepared silages was evaluated. The biogas and methane yield for FW silages after 96 days was calculated to be 671–763 mL/g VS and 441–482 mL/g VS, respectively [128].

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Table 4. Effects of different anaerobic inocula on biogas production from food waste (FW) obtained from a fish processor and ensiled for biogas production. The FW silages were prepared by mixing FW with bread waste (BW) and brewery grain waste (BGW), and the quality of the prepared silages was evaluated. The biogas and methane yield for FW silages after 96 days was calculated to be 671–763 mL/g VS and 441–482 mL/g VS, respectively [128].
Table 4: Various inocula along various substrates and their potential for biogas production.

| Inocula                              | Pretreatment | Substrate                              | S/I ratio | Biogas production | Methane production | Reference |
|---------------------------------------|--------------|----------------------------------------|-----------|--------------------|--------------------|-----------|
| Cow manure                           | NG           | Sea wrack (dislodged sea grasses and sea weeds) | NG        | 551 ± 126 mL       | 396.9 mL CH₄ g⁻¹ VS (volatile solid) | [125]     |
| Marine sediments                     | NG           | Sea wrack                              | NG        | 2172 ± 156 mL      |                    | [125]     |
| Sea wrack associated microflora      | NG           | Sea wrack                              | NG        | 1223 ± 308 mL      |                    | [125]     |
| Municipal Sludge                     | NG           | Food waste                             | NG        |                    | 0.49 L CH₄ g VS.   | [126]     |
| Municipal wastewater sludge          | Y            | Food waste                             | Y         | 241 mL CH₄ g VS    | 221 mL CH₄ g VS    | [127]     |
| Inocula from food waste              | Y            | Preincubated Primary sludge            | Y         | 283 mL CH₄ g VS    | 231 mL CH₄ g VS    | [127]     |
| Inocula from WWTP                    | Y            | Nonincubated Primary sludge            | Y         |                    | 230 mL CH₄ g VS    | [127]     |
| Digester sludge                      | NG           | Bulrush                                | NG        | 464.3 g COD/g total solids |                  | [162]     |
| Digester sludge                      | Y            | Bulrush                                | Y         |                    | 14.5%              | [162]     |
| Rumen fluid                          | NG           | Bulrush                                | NG        |                    | 362.8 g COD/g TS   | [162]     |
| Rumen fluid                          | Y            | Bulrush                                | Y         |                    | 18.5%              | [162]     |
| Mesophilic anaerobic digester        | Y            | Ensiling                               | Y         | 671–763 mL/g VS    | 441–482 mL/g VS    | [128]     |
| Thermophilic anaerobic sludge        | Y            | Mixing with distillery wastewater      | Y         |                    | 259.46 mL/g VS     | [131]     |
| Mesophilic activated sludge          | Y            | Biological thermal ultrasound          | Y         | Negligible 46–62%  |                    | [163]     |
| Mesophilic activated sludge          | Y            | Microalgae                             | Y         | 188–395 mL         |                    | [163]     |
| Rumen fluids                         | NG           | Cattle manure                          | NG        | 17.64 23.51 35.27 70.54 | 191 mL/g VS       | [164]     |
| Sludge digester                      | NG           | Municipal solid waste                  | NG        | 2 g VM_inoculum/g VM_waste | 150 mL/g DM⁻¹       | [165]     |
A mesophilic lignocellulolytic microbial consortium BYND-5, established by successive subcultivation, was applied to enhance the biogas production. The degradation efficiency of BYND-5 for rice straw was more than 49.0 ± 1.8% after 7 days of cultivation at 30°C. Various organic compounds, including acetic acid, propionic acid, butyric acid, and glycerin, were detected during biodegradation. The diversity analysis of BYND-5 was conducted by ARDRA (amplified ribosomal DNA restriction analysis) of the 16S rRNA clone library. Results indicated that bacterial groups represented in the clone library were the Firmicutes (5.96%), the Bacteroidetes (40.0%), Deferribacteres (8.94%), Proteobacteria (16.17%), Lentisphaerae (2.13%), Fibrobacteraceae (1.7%), and uncultured bacterium (25.1%). Additionally, the enhancement of biogas yield and methane content was directly related to the pretreatment with BYND-5. The microbial community identified herein is a potential candidate consortium for the degradation of waste lignocelluloses and enhancement of biogas production under the mesophilic temperature conditions.

High activity levels and balanced anaerobic microbial communities are necessary to attain proper anaerobic digestion performance. Therefore, this work was focused on the kinetic performance and the microbial community structure of six full-scale anaerobic digesters and one lab-scale codigester. Hydrolytic (0.6–3.5 g COD g⁻¹ VSS d⁻¹) and methanogenic (0.01–0.84 g COD g⁻¹ VSS d⁻¹) activities depended on the type of biomass, whereas no significant differences were observed among the acidogenic activities (1.5–2.2 g COD g⁻¹ VSS d⁻¹). In most cases, the higher the Bacteroidetes and Archaea percentages the higher the hydrolytic and the methanogenic activity, respectively, in the biomasses. Hydrogenotrophic methanogenic activity was always higher than acetoclastic methanogenic activity, and the highest values were achieved in those biomasses with lower percentages of Methanoseta. In sum, the combination of molecular tools with activity tests seems to be essential for a better characterization of anaerobic biomasses.

In the study conducted by Regueiro et al. [130], a stable thermophilic microbial consortium with high cellulose-degradation ability was successfully constructed. Those several species of microbes coexisted in this consortium were proved by DGGE (denaturing gradient gel electrophoresis) and sequence analysis. The cooperation and symbiosis of these microbes in this consortium enhanced their cellulose-degradation ability. The pretreatment of cassava residues mixing with distillery wastewater prior to anaerobic digestion was investigated by using this microbial consortium as inocula in batch bioreactors at 55°C. The experimental results showed that the maximum methane yield (259.46 mL/g-VS) of cassava residues was obtained through 12 h of pretreatment by this microbial consortium, which was 96.63% higher than the control (131.95 mL/g-VS). In addition, it was also found that the maximum methane yield is obtained when the highest filter paper cellulase (FPase), carboxymethyl cellulase (CMCase), and xylanase activity and soluble COD (sCOD) are produced [131].

### 6. Molecular Techniques in Microbial Community Dynamics

The increased popularity of denaturant gradient gel electrophoresis (DGGE) is reflected by the increasing number of studies that use the technique. It is based on the differing mobility on a gel of denatured DNA-fragments of the same size but with different nucleic acid sequences, thus generating band patterns that directly reflect the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species [132].

The most important application of DGGE is monitoring dynamic changes in microbial communities, especially when many samples have to be processed. There are multiple applications of DGGE related to anaerobic digestion processes: studies on differences between mesophilic and thermophilic reactors, demonstrating the lower biodiversity in thermophilic reactors used for the treatment of residual waters generated by the pharmaceutical industry [133], and analysis of the changes observed in the bacterial diversity of an anaerobic digester for treating urban solid waste [134]. Studies on the changes in bacterial communities in a continuous stirred tank reactor (CSTR) in response to dilution rate [135, 136] monitored changes in an ethylbenzene-degrading bacterial consortium in enrichment cultures under anaerobic, sulfate reducing conditions. By monitoring the predominant bacterial species over a period of 127 days, they identified a dominant bacterium that was present throughout the whole incubation period and most likely to be the microorganism responsible for ethylbenzene degradation. Both spatial and temporal changes in microbial community profiles were monitored by [137] in a study of expanded granular sludge bed (EGSB) reactors for the treatment of oeleic acid. With this approach, the researchers were able to add another dimension to the analysis and compare the change in microbial communities in different layers of the sludge bed, as well as changes over the time. The start-up phase of garbage composters under fed batch operation was studied [138]. DGGE fingerprints revealed a significant shift in the bacterial population from ubiquinone containing microorganisms to Actinobacteria during the observation period.

Although DGGE (or TGGE: temperature gradient gel electrophoresis) is by far the most widely used genetic fingerprinting technique in molecular ecology, it is not the only method based on PCR amplification of nucleic acids and separation of the product mixture by electrophoresis. An alternative approach is the generation of SSCP (single strand conformation polymorphism) patterns; a technique that has been employed to study anaerobic digesters both on laboratory and industrial scales [39, 139] investigated the microbial community in a two-phase anaerobic bioreactor for the treatment of vegetable wastes with SSCP to assess changes in the composition of the bacterial and archaeanal biomasses. Methanogenic communities can be investigated with denaturing gradient gel electrophoresis (DGGE) analysis. The DGGE method is one of the most widely used fingerprinting technique, with the benefit of describing microbial communities by the recovery and sequencing of amplification products [140]. Therefore, it would be beneficial to figure out
the methanogenic process performance that methanogens were identified through 16S rRNA-based molecular methods like DGGE.

Reactor performance and microbial community dynamics were investigated during solid state anaerobic digestion (SS-AD) of corn stover at mesophilic and thermophilic conditions. Thermophilic SS-AD led to faster and greater reductions of cellulose and hemicelluloses during the first 12 days compared to mesophilic SS-AD. However, accumulation of volatile fatty acids (VFAs) was 5-fold higher at thermophilic than mesophilic temperatures, resulting in a large pH drop during days 6 to 12 in the thermophilic reactors. Culture-based enumeration revealed 10–50 times greater populations of cellulolytic and xylanolytic microbes during thermophilic SS-AD than mesophilic SS-AD. DGGE analysis of PCR amplified 16S rRNA genes showed dynamic shifts, especially during the thermophilic SS-AD, of bacterial and archaeal populations over the 38 days of SS-AD as a result of acclimation of the initial seed microbial consortia to the lignocellulosic feedstock. The findings of this study can guide future studies to improve efficiency and stability of SS-AD.

The microbial community dynamics were investigated in thermal acidogenesis using mesophilic sludge through DGGE (denaturing gradient gel electrophoresis) used for the monitoring of the microbial community. The bacterial community consists of *Pseudomonas mendocina*, *Bacillus halodurans*, *Clostridium hathiforme*, *Gracilibacter thermotolerans* and *Thermonas haemolytica*. Among these *B. halodurans*, *G. thermotolerans*, and *T. haemolytica* are reported to ferment carbohydrates therмотolerantly. In contrast, *P. mendocina* disappeared in the acidogenesis process because of its mesophilicity. The most thermophiles detected as DGGE bands could grow catalyzing carbohydrates metabolism in swine wastewater to produce volatile fatty acids thermotolerantly [130]. The combination of molecular tools, such as DGGE, gene sequencing, and FISH, with microbial activity test seems to be essential for a better characterization of anaerobic biomass [130].

An excellent way to overcome some of the problems of studying microbial populations of a microcosm without resorting to traditional methodology is to use fluorescent probes. These are short sequences of DNA (16–20 nucleotides) labeled with a fluorescent dye. These sequences recognize 16S rRNA sequences in fixed cells and hybridize with them in situ (DNA-RNA matching) (Figure 4). The specificity of the probe enables detection/identification on any desired taxonomic level, from domain down to a resolution suitable for differentiating between individual species. The main shortcoming of this technique lies in the lack of availability of probes targeting the desired bacterial taxon or group. Although it is possible, in theory, to design the most apt probe for each application thanks to the growing rRNA sequence databases (16/18S and 23/28S rRNA), it may be impossible to develop a probe that specifically detects certain groups of microorganisms that share metabolic properties (e.g., sulfatereduction or halo-respiration). Furthermore, some previous knowledge of the expected microorganisms in the sample is often required to apply this method successfully. To target a particular species, a specific probe must be ready or its 16S rRNA sequence must be available.

The study led by Stahl with pure cultures of methanogenic Archaea [141] formed the basis for all subsequent work in this area. In this pioneering study, many of the probes currently used to identify methanogenic microorganisms at different taxonomic levels (order, family, and genus) were described. The authors themselves used these probes to locate and quantify methanobacteria living in different anaerobic digesters [142]. Ever since the first studies on UASB reactors in the early 1980s, granular sludge has been popular with researchers from different fields of anaerobic digestion. Despite efforts and multiple articles, including guidelines for start-up of UASB reactors with granulation [143, 144] and mechanism and models [145, 146], the phenomenon of granulation is not well understood. Molecular techniques to analyze this form of biomass were introduced in the last decade and they have contributed greatly to the knowledge of its microbiology. After the pioneering work by [147] and the excellent contribution by [148] on the structure, composition, and distribution of different microbial groups, a number of aspects of this remarkable microcosm have been corroborated, disproved, and explained.

Publications that further illustrate the application of FISH in anaerobic digestion have dealt with the interaction and distribution of trophic groups, such as sulfate reducing bacteria and methanogenic Archaea in methanogenic/sulfidogenic reactors [149], or differentiation between hydrogenotrophic
and acetoclastic methanobacteria, and within this group between *Methanoseta* and *Methanosarcina* [150, 151]. The influence of feeding on granule structure and microbial composition has been also assessed by FISH [152]. Other works have emphasized the dependence of the settling behavior of UASB granules on their microbial composition and the decisive role of the positioning of methanogenic Archaea in granules during sedimentation or flotation [153]. A recent paper by 150 mentioned earlier when discussing the use of 16S rDNA-sequencing for the identification of microorganisms deserves highlighting again, as it dealt with filamentous bacteria that cause sludge bulking in UASB reactors and the involvement of members of the Chloroflexi phylum in this phenomenon. The authors reported an *in situ* hybridization based survey of the distribution of these microorganisms in thermophilic, as well as mesophilic UASB sludge granules. The authors [154] operated UASB reactors for the treatment of ammonia rich landfill leachates and were able to corroborate the impact of high ammonia loads on the microbial community of the sludge granules with FISH. The same authors [56] in a subsequent work also with UASB reactors operated under high ammonia concentrations found that the differences in the efficiencies of reactors were correlated with the proportion of resistant and sensitive microorganisms dominated in the reactors.

The most recent approaches combine complementary techniques. The authors [152] have studied the microbial composition and structure of different types of granules in a UASB reactor that treated wastewater from a brewery. The authors used FISH, DGGE, cloning, and electron microscopy to gain insight into the structure, function, and physical appearance of methanogenic granules. The use of multiple techniques was necessary to elucidate the structure-function relationship of the different granules. The authors [155] studied in depth the microbial community of granules from a reactor treating paper mill wastewater with a similar approach. *In situ* hybridization has been also used as a molecular tool to describe microbial communities in other anaerobic wastewater treatment systems beside UASB reactors. A few noteworthy publications include Plumb's analysis of the microbial composition of the biomass inside an anaerobic baffled reactor [155, 156].

We will bring this section to a close with two final comments. First, we should remember that FISH is exclusively a taxonomic method that is most commonly used to examine whether members of a specific phylogenetic affiliation are present in a sample. It cannot, however, reveal information about the function or metabolic features of the microorganisms, although these characteristics can sometimes be deduced from comparison of the microorganisms detected with phylogenetically related bacteria. With a view to overcoming this limitation, [157] have presented an approach that combined FISH with microautoradiography using substrates labeled with 3H or 14C. This combination allowed them to determine metabolic activities and simultaneously identify the microorganisms involved. This strategy can be useful for analyzing the structure and function of microbial communities, though it makes the technique much more complex.

The second closing remark refers to the quantitative aspects of FISH. The possibility of quantitative results represents a big advantage over the other molecular techniques, but this only applies to homogeneous and evenly distributed samples. The bacterial count per region of the microscopic grid should lie between 30 and 150. Between 10 and 20 regions should be counted to ensure statistically significant cell counts. Nonideal samples and fluorescent background (a common phenomenon with environmental and sludge samples) can make cell counting by fluorescence microscopy a tedious and time consuming process that can be influenced by the judgment of the operator and his or her experience. Truth in this case is in the eye of the beholder, and a standardized and automatic procedure would be preferred. This is possible with a laser confocal microscope or an epifluorescence microscope coupled to a digital camera and a computer workstation to analyze the pictures. The system usually requires expensive software, which makes it less accessible, although free simple software can be found on the Internet (Image J, Image Pro Plus). Confocal microscopy on its own is of course already an extraordinarily powerful tool for examining the three-dimensional structure and texture of the microbial aggregates (granules, biofilms) that develop in wastewater treatment systems [130].

Concurrent utilization of multiple molecular techniques is a common practice for studying microbial community structure, function, and dynamics. For example, a combination of FISH and microautoradiography (FISH-MAR), FISH-SIP, and microarrays can be used to evaluate microbial structure and function. Figure 5 illustrates how different molecular techniques can be used to analyze microbial community structure, function, and metabolic transformation separately or together. FISH, DGGE, ribosomal inter-genic spacer analysis (RISA), T-RFLP, clone libraries, DGGE, and T-RFLP are the monitoring techniques that have been used most frequently for microbial community structure analysis, with DGGE and T-RFLP being the procedures most commonly used concurrently with other techniques. Several recent molecular techniques that have been used for microbial identification and detection microarray are few of the most advanced and sophisticated techniques.

7. Conclusions

Anaerobic digestion is a complicated reduction process involving a number of biochemical reactions under anaerobic conditions. This digestion processes convert biomass to energy, used in recycling of organic wastes and reduction of hazardous effects on the environment. Hydrolysis, acidogenesis, acetogenesis, and methanogenesis are different stages of anaerobic digestion. Biogas is possible due to the different groups of microorganisms, namely, fermentative, syntrophic, acetogenic, and methanogenic bacteria. Methanogens are the vital microbes and have decisive role for biomethane production and consist of hydrogenotrophic and acetotrophic and the substrates such as CH₃COOH, H₂, and CO₂. Methanogenesis occurs through two pathways: the direct cleavage of
acetate to CH₄ and CO₂, known as acetoclastic methanogenesis and hydrogenotrophic methanogenesis reducing the CO₂ with hydrogen gas. The share of each pathway to the methanogenesis depends on the inoculum sludge, the organic substrate, and the process conditions.

Cellulose is the most abundant biopolymer on earth and is also a significant part of solid wastes. The cellulose waste can be digested anaerobically to methane which is a main source of energy. Plant cell walls are relatively complex structures; a great variety of enzymes are needed for extensive enzymatic hydrolysis. Three cultivable species of ruminal bacteria (Ruminococcus albus, Ruminococcus flavefaciens, and Fibrobacter succinogenes) are found at high numbers in the rumen and are capable of rapid growth on cellulose.

Inocula play an important role in anaerobic reactor startup by balancing the populations of syntrobacter and methanogens. This balance makes syntrophic metabolism thermodynamically feasible in anaerobic digestion. The source of inocula not only affects the amount of biogas production but also influences the kinetics of the process of anaerobic digestion.

Different molecular techniques such as DGGE and FISH are being utilized for studying the microbial dynamic changes in the microbial communities for anaerobic digester. Microbial communities can be investigated with DGGE analysis. This method is one of the most widely used fingerprinting technique with the benefit of describing microbial communities by the recovery and sequencing of amplifications products. FISH is exclusively a taxonomic technique, that is, most commonly used to examine whether members of a specific phylogenetic affiliation are present in the sample. However, it cannot reveal information about the function or metabolic features of the microorganisms. The quantitative aspect of the FISH is an advantage while comparing it with other molecular techniques and it is applicable only to homogenous and evenly distributed samples. The present molecular techniques are a common practice for studying microbial community structure, function, and dynamics. Various combinations of FISH and microautoradiography (FISH-SIP), FISH-SIP and microarrays can be used to evaluate microbial structure and function.

**Conflict of Interests**

It is declared that the authors have neither any conflict of interests nor financial gain from this paper.

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