Chapter 14

Anaerobic Digestion: I. A Common Process Ensuring Energy Flow and the Circulation of Matter in Ecosystems. II. A Tool for the Production of Gaseous Biofuels

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Abstract

Anaerobic digestion, a process that ultimately generates methane and carbon dioxide, is common in natural anoxic ecosystems where concentrations of electron acceptors such as nitrate, the oxidized forms of metals and sulphate are low. It also occurs in landfill sites and wastewater treatment plants. The general scheme of anaerobic digestion is well known and comprises four major steps: (i) hydrolysis of complex organic polymers to monomers; (ii) acidogenesis that results in the formation of hydrogen and carbon dioxide as well as non-gaseous fermentation products that are further oxidized to hydrogen, carbon dioxide and acetate in (iii) acetogenesis based on syntrophic metabolism and (iv) methanogenesis. Approaches to the analysis of methane-yielding microbial communities and data acquisition are described. There is currently great interest in the development of new technologies for the production of biogas (primarily methane) from anaerobic digestion as a source of renewable energy. This includes the modernization of landfill sites and wastewater treatment plants and the construction of biogas plants. Moreover, research effort is being devoted to the idea of separating hydrolysis and acidogenesis from acetogenesis and methanogenesis under controlled conditions to favour biohydrogen and biomethane production, respectively. These two stages occur under different conditions and are carried out in separate bioreactors.

Keywords: anaerobic digestion, microorganisms, hydrogen, methane, syntrophy, renewable energy

1. Introduction

Anaerobic digestion of biomass under mesophilic conditions (anaerobic microbial decomposition/degradation of organic matter), whose final products are methane and carbon dioxide, contributes to the energy flow and circulation of matter in ecosystems. It is a key process in the global carbon cycle that is promoted by the activity of many different groups of microorganisms. Anaerobic digestion commonly occurs in natural anoxic ecosystems such as freshwater sediments, wetlands, marshlands, paddy fields and deeper zones of marine sediments. The digestive tracts of animals, especially ruminants and termites, are also sites of methane production by this process. It is estimated that biological methanogenesis is responsible for more than 70% of total global methane emissions [1, 2].

Anaerobic decomposition of biomass to carbon dioxide and methane only occurs in anoxic environments with a low redox potential, i.e., where concentrations of other electron acceptors including nitrate, oxidized forms of metals such as Mn(IV) and Fe(III) or sulphate are low. The inhibition of anaerobic digestion by nitrate, oxidized metal ions and sulphate is determined by the redox potential. As shown in **Figure 1**, a decrease in redox potential leads to changes in the dominant type of anaerobic respiration towards low energy-yielding processes. The nature of the final electron acceptors present in an environment is a key factor in determining the ecological niches for particular microorganisms.

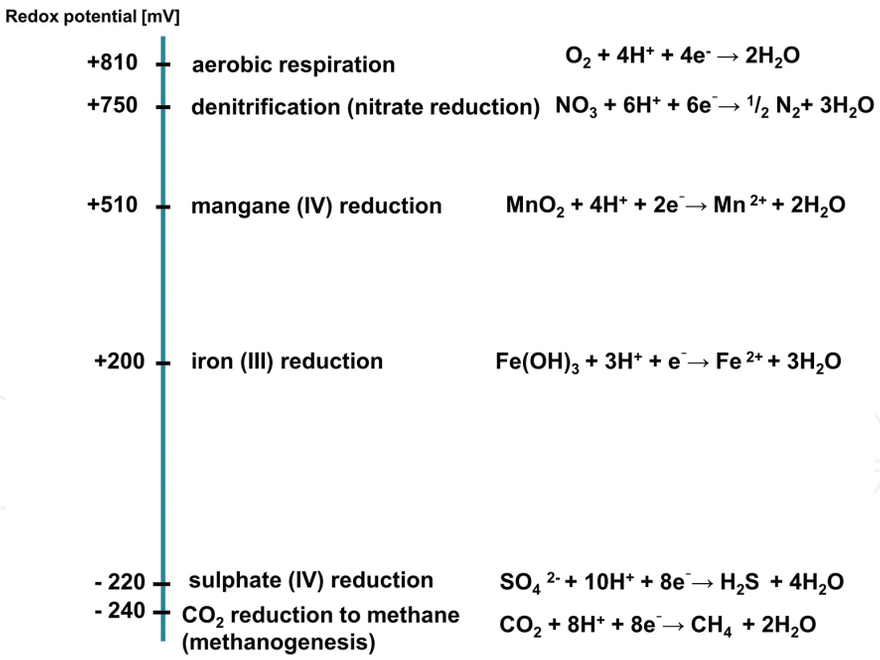

Figure 1. Redox potential for different types of final electron acceptors in anaerobic respiration and energy gain for microbial cells.

The general scheme of anaerobic digestion is well known (**Figure 2**). It is a complex process promoted by the interaction of many groups of microorganisms and has four major steps. The first is hydrolysis of complex organic polymers to monomers. The second step is acidogenesis

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that results in the formation of hydrogen and carbon dioxide as well as non-gaseous fermentation products, i.e., low-molecular weight organic acids (short-chain fatty acids) and alcohols. In the third step, known as acetogenesis, these non-gaseous products are further oxidized to hydrogen, carbon dioxide and acetate, mainly by syntrophic degradation processes. The fourth step is methanogenesis. The final two steps, acetogenesis and methanogenesis, are closely linked and involve syntrophic associations between hydrogen-producing acetogenic bacteria and hydrogenotrophic methanogens. These associations keep the hydrogen partial pressure sufficiently low to allow acetogenesis to become thermodynamically favourable. This process, referred to as interspecies electron transfer, is in fact a hydrogen/formate transfer. Acetate is a direct substrate for methanogenesis and can also be syntrophically oxidized to hydrogen and carbon dioxide [3–8].

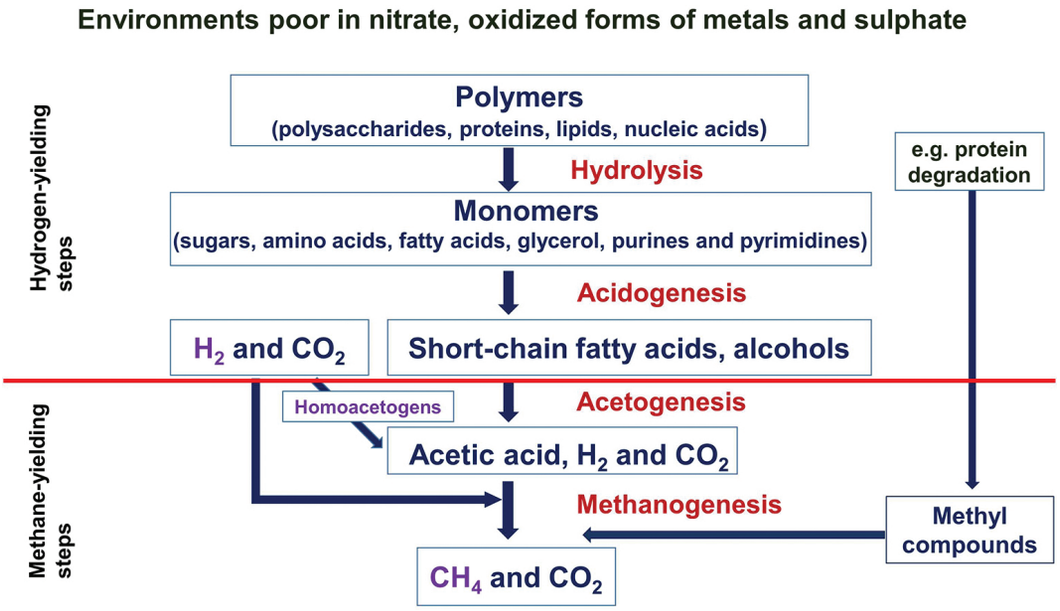

Figure 2. Scheme of anaerobic digestion of polymeric organic matter to methane and carbon dioxide.

Anaerobic digestion is common in landfill sites and anaerobic wastewater treatment plants. The process of anaerobic decomposition of biomass, such as energy crops or organic agro-waste, is commonly used to produce biogas as an alternative energy source. There is currently great interest in the development of new technologies for the modernization of landfills and wastewater treatment plants to control the release of biogas and collect methane to use as fuel. Moreover, for the purpose of innovative technologies based on microbial processes, it is desirable to build modern biogas plants where the hydrogen-yielding (hydrolysis and acidogenesis) and methane-yielding (acetogenesis and methanogenesis) stages of anaerobic digestion are separated to, respectively, favour the production of hydrogen and methane under controlled conditions. Optimization of methane or hydrogen and methane production from organic matter requires a good understanding of anaerobic digestion at the molecular level, namely the structure and diversity of microbial communities and metabolic pathways, leading to transformation of the organic substrate to the desired gaseous products.

2. Meta-omics approaches for exploring microbial communities

Current knowledge of microbial ecology and physiology, derived from culture-dependent techniques, is limited and incomplete because the majority of microorganisms have not been cultivated. It has been predicted that only 1% or less of all microorganisms present in natural ecosystems may be cultivated as a pure culture using standard methods [9]. Moreover, syntrophy is believed to be common in microbial communities, and syntrophic bacteria cannot be grown as a monoculture. However, culture-dependent techniques have permitted the isolation and characterization of some species involved in specific metabolic processes during anaerobic digestion, and numerous genomes have been sequenced. Data from genome sequence analyses supported by the results of physiological and biochemical studies on cultivated bacteria have provided hints as to which physiological groups of microorganisms are responsible for the key steps of anaerobic digestion. Information on methane-yielding microbial communities is now being obtained using culture-independent analytical techniques (**Figure 3**).

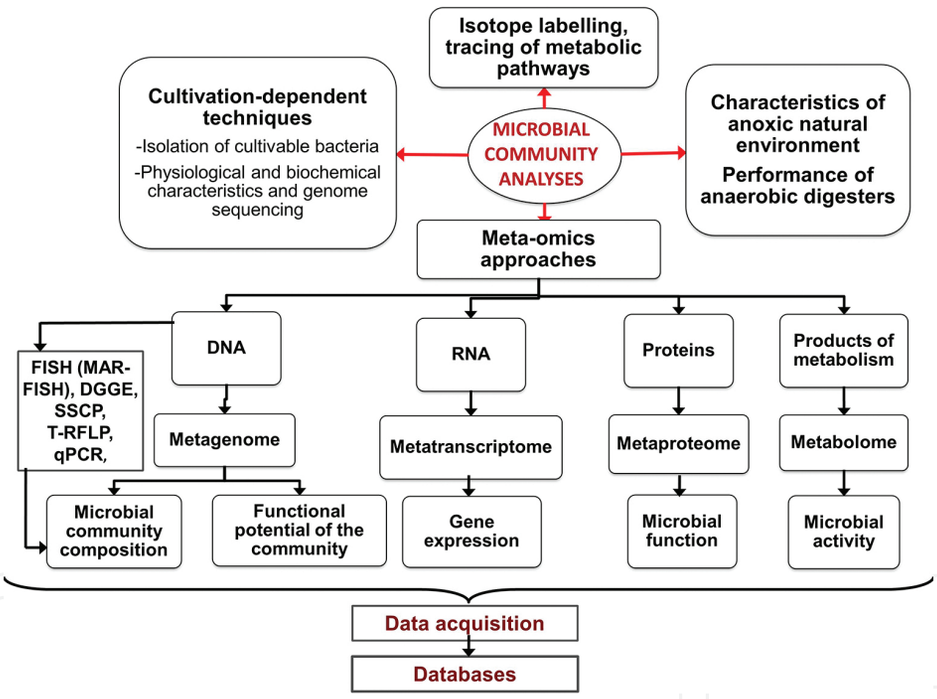

Figure 3. Culture-independent approaches to analyse methane-yielding microbial communities. FISH, fluorescence in-situ hybridization; MAR-FISH, microautoradiography combined FISH; DGGE, denaturing gradient gel electrophoresis; SSCP, single-strand conformation polymorphism; T-RFLP, terminal restriction fragment length polymorphism; qPCR, real-time quantitative PCR.

The recent increase in the number of culture-independent molecular biology techniques and bioinformatic tools for exploring microbial communities has helped to develop the field of meta-omics. Meta-omics encompasses metagenomics, metatranscriptomics, metaproteomics and metabolomics, based on analyses of, respectively, total DNA, mRNA, total proteins and metabolites isolated from the microbial communities [10–14]. Metagenomics shows microbial

potential by describing the genes present in a microbial community or ecosystem. Metatranscriptomics analyses gene expression and thus represents potential microbial function. Messenger RNA (mRNA) can be sequenced directly or used to generate cDNA (by reverse transcription) that is subsequently sequenced using metagenomics platforms. Metaproteomics is focused on microbial function—it investigates proteins expressed within a microbiome. Metabolomics analyses the intermediates and end-products of metabolism and then shows microbial activity.

The data generated by these novel methodologies have provided significant insights into the structure and function of microbial communities in both natural environments and man-made systems. However, meta-omics-based approaches do suffer from certain limitations: the variable extraction efficiency of DNA/RNA/protein/metabolites may affect the results, and reference databases used for comparative analyses often contain false or missing assignments of DNA and protein sequences or chromatography/mass spectrometry data. For example, metagenomic analyses always generate large numbers of sequences that are of low complexity, unclassified, not assigned or show no hits. Such unidentified reads usually constitute a significant proportion of the total reads, as discussed by Chojnacka et al. [15]. It is noteworthy that the limited number of microorganisms that can be propagated as pure cultures determines the number of sequenced reference genomes available for genomic studies. So far, only five genomes of syntrophic bacteria involved in acetogenesis have been sequenced: *Syntrophus aciditrophicus*, *Syntrophus wolfei*, *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum* and *Syntrophothermus lipocalidus*, as discussed by Li et al. [16].

Using metagenomic sequence data and genomic assembly procedures, it is possible to reconstruct genomes of bacteria that have not been cultivated. One example is a reconstruction of the genome of *Candidatus* Cloacimonas acidaminovorans [17]. This is a representative of the *Cloacimonetes*, a sub-dominant group of bacteria found in anaerobic mesophilic digesters and gut microflora. They are regarded as syntrophs capable of amino acid fermentation, propionate and butyrate oxidation as well as cellulose degradation and have never been grown in pure culture.

In the case of anaerobic digestion, the combined use of meta-omic approaches and isotope labelling techniques in both natural anoxic environments and bioreactors plus the analysis of reactor performance data will allow us to develop a fundamental understanding of the processes leading to methane production. Meta-omic data can also be used to validate commonly accepted theses.

Other cultivation-independent techniques include isolation of total DNA from microbial communities, amplification, cloning and sequencing of marker genes, most frequently 16S rRNA or others such as *gyrB* or *mcrA* for methanogenic *Archaea*; fluorescence in-situ hybridization (FISH) and its derivatives such as microautoradiography combined FISH (MAR-FISH); community fingerprinting by denaturing gradient gel electrophoresis (DGGE); single-strand conformation polymorphism (SSCP); terminal restriction fragment length polymorphism (T-RFLP) and real-time quantitative PCR (qPCR), as discussed by Dziewit et al. [18].

3. Hydrolysis and acidogenesis: the anaerobic digestion steps yielding short-chain fatty acids and hydrogen

3.1. Hydrolysis

Hydrolysis is the first step in the anaerobic decomposition of organic matter. It involves the conversion of polymeric organic matter (e.g., polysaccharides, lipids, proteins) to monomers (e.g., sugars, fatty acids, amino acids) by hydrolases secreted to the environment by microorganisms. Three key groups of hydrolases are involved in the process of anaerobic digestion: esterases, glycosidases and peptidases, which catalyse the cleavage of ester bonds, glycoside bonds and peptide bonds, respectively [19]. The bacteria most commonly associated with hydrolysis include representatives of the *Firmicutes* (*Clostridia, Bacilli*), *Bacteroidetes* and *Gammaproteobacteria* [20–22]. Usually, the same bacteria are also able to conduct acidogenesis, the second step in the decomposition of organic matter.

Metaproteomic analysis of microbial communities mediating the decomposition of dead plant material in forest leaf litter revealed fungi to be the main producers of extracellular hydrolytic enzymes, the most prominent of which are cellulolytic enzymes: exo- and endo-glucanases as well as β-glucosidases. Other hydrolases involved include phosphatases, pectinases, xylanases, lipases, amylases, chitinases and oxidoreductases. Moreover, the species of fungi – the main cellulase producers – changed depending on the season. In a sample collected in February, *Leotiomycetes* dominated, whereas in samples collected in May, *Eurotiomycetes*, *Dothideomycetes*, *Leotiomycetes* and *Sordariomycetes* were the most abundant fungal phyla. Interestingly, no bacterial hydrolases were detected [23].

3.2. Acidogenesis

3.2.1. Fermentation of sugars

During acidogenesis, the products of hydrolysis are converted to non-gaseous short-chain fatty acids, alcohols, aldehydes and the gases, such as carbon dioxide and hydrogen [3]. The dominant end-products of the fermentation process determine the type of fermentation (**Figure 4A**).

The main hydrogen-yielding fermentations under mesophilic conditions are butyric acid fermentation (*Clostridium*-type fermentation) and mixed-acid fermentation (*Enterobacteriaceae*-type fermentation). The common first step is glycolysis (the Embden-Meyerhof-Parnas pathway) when glucose is converted to pyruvate and NADH is formed (Figure 4B). In both types of fermentation, hydrogenases are responsible for hydrogen release. Hydrogenases are metalloenzymes that are divided into two major groups according to the metal in the prosthetic group of the active site: [FeFe] and [FeNi] hydrogenases [24, 25]. In the *Clostridium*-type fermentation, pyruvate is oxidized to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR) in the presence of ferredoxin (Fd). Reduced ferredoxin is also formed in the reaction with NADH catalysed by NADH:ferredoxin oxidoreductase (NFOR). Hydrogen is released, mainly by [FeFe] hydrogenases that catalyse proton reduction using electrons from ferredoxin.

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Acetyl-CoA is converted to non-gaseous end-products including short-chain fatty acids (acetate, butyrate, lactate, propionate), alcohols (mainly not only ethanol, but also butanol and propanol) and ketones (such as acetone) by a wide range of enzymes. An increased number of non-gaseous products of fermentation decreases the production of hydrogen during acidogenesis. The hydrogen concentration regulates the relative activities of PFOR and NFOR. A hydrogen partial pressure of >60 Pa inhibits NFOR activity and favours the formation of non-gaseous end-products. In contrast, PFOR is still active at hydrogen concentrations of up to 30,000 Pa [3, 26–29].

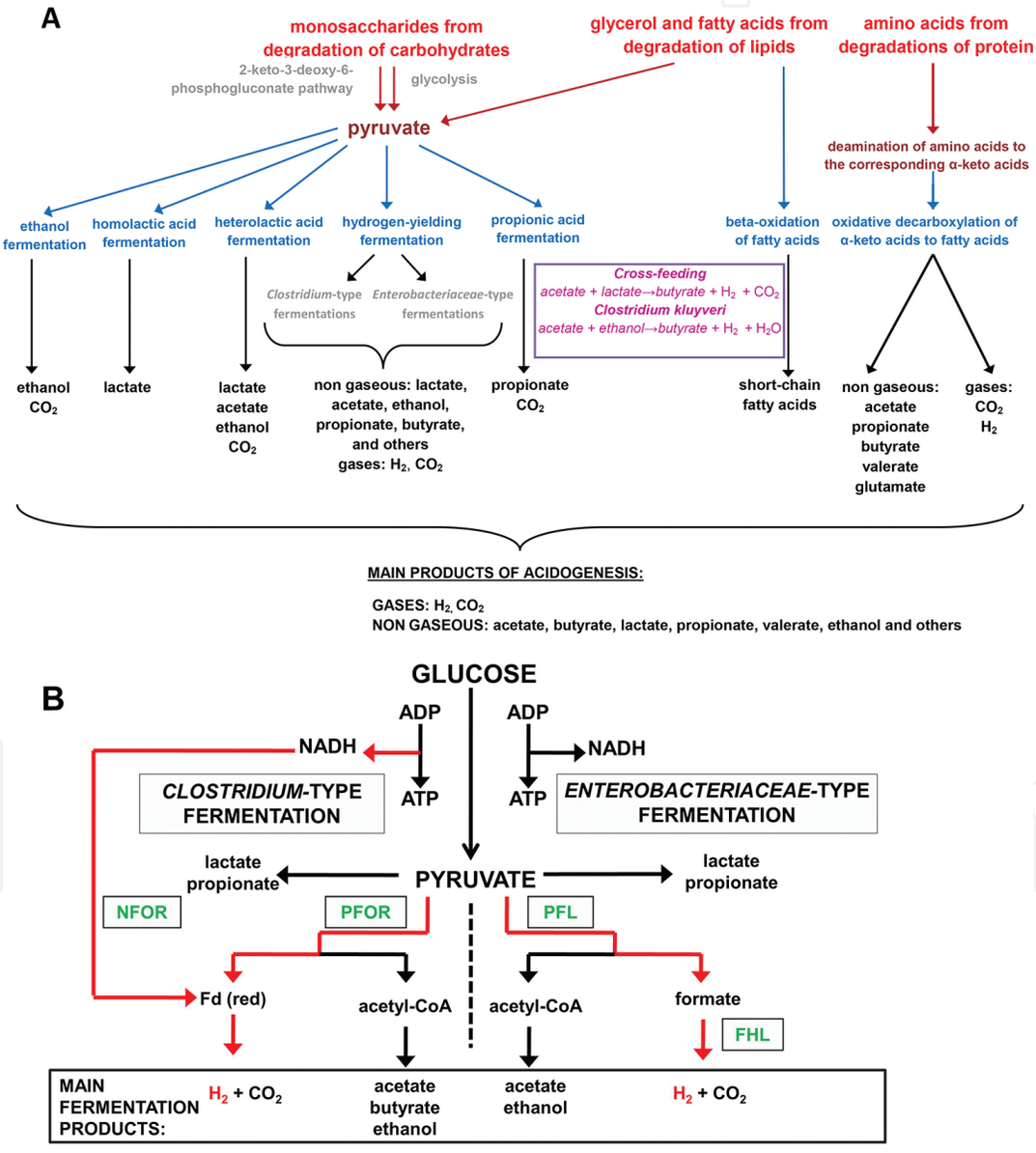

Figure 4. Metabolic pathways of acidogenesis: (A) general overview and (B) glycolytic hydrogen-yielding fermentations. PFOR, pyruvate:ferredoxin oxidoreductase; NFOR, NADH:ferredoxin oxidoreductase; PFL, pyruvate formate-lyase; FHL, formate hydrogen-lyase complex.

In the mixed-acid fermentation (also known as formic acid fermentation), pyruvate is converted to acetyl-CoA and formic acid by pyruvate formate-lyase (PFL). The formic acid can then be degraded into hydrogen and carbon dioxide by formate hydrogen-lyase (FHL) complex. One of the FHL subunits is the [FeNi] hydrogenase Hyd-3. There are two types of mixed-acid fermentation: the *Escherichia coli* type and the *Enterobacter* type. In the *Enterobacter*-type fermentation, hydrogen can also be generated through oxidation of NADH by NFOR in reactions similar to those described for the *Clostridium*-type fermentation. Non-gaseous products of the *Enterobacteriaceae*-type fermentation can include ethanol, short-chain fatty acids (formate, acetate, lactate, succinate) as well as acetoin and 2,3-butanediol [30–32].

Besides glycolysis, other pathways of pyruvate formation exist, e.g., the 2-keto-3-deoxy-6-phosphogluconate (Entner-Doudoroff) pathway. Two intermediates of glycolysis, glyceraldehyde-3-phosphate and fructose-6-phosphate, are also formed in the pentose phosphate pathway. Monosaccharides other than glucose can enter glycolysis or other pathways leading to pyruvate formation. Pyruvate can also be formed from glycerol [33].

In addition to the hydrogen-yielding fermentations, other fermentations occur during acidogenesis, including lactic, propionic and ethanol fermentations. Two types of lactic acid fermentation are distinguished: homolactic and heterolactic, whose products are, respectively, lactate only or lactate, ethanol, acetate and carbon dioxide.

3.2.2. Fermentation of amino acids

Members of the *Clostridiales* (families *Clostridiaceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae*), *Fusobacterales, Synergistetes* (*Aminobacterium colombiense*) and *Cloacimonetes* (*Candidatus* Cloacimonas acidaminovorans) are capable of amino acid fermentation [4, 7]. Amino acids are generally degraded to acetate, propionate, hydrogen and carbon dioxide, with the formation of butyrate and ammonia. This process involves NAD(P)- or FAD-dependent deamination of amino acids to the corresponding α-keto acids with subsequent oxidative decarboxylation of α-keto acids to fatty acids with CoA and phosphate derivatives (Figure 4A). The pathways of fermentation differ depending on the amino acid type [4]. Amino acid mixtures are often degraded by coupled fermentation of pairs of amino acids through the Stickland reaction (e.g., alanine and glycine in *Clostridium sporogenes*). Oxidation of one amino acid is coupled to the reduction in another in a single cell:

$$\text{alanine} + 2H_2O \rightarrow \text{acetate}^- + CO_2 + NH_4^+ + 2H_2$$

$$\text{glycine} + 2H_2O + H^+ \rightarrow 2CO_2 + NH_4^+ + 3H_2$$

$$\text{serine} + H_2O \rightarrow \text{acetate}^- + CO_2 + NH_4^+ + H_2$$

$$\text{threonine} + H_2O \rightarrow \text{propionate}^- + CO_2 + NH_4^+ + H_2$$

http://dx.doi.org/10.5772/64645

$$\text{histidine} + 4H_2O + H^+ \rightarrow \text{glutamate}^- + CO_2 + 2NH_4^+ + H_2$$

$$\text{proline} + 2H_2O \rightarrow \text{glutamate}^- + H^+ + 2H_2$$

$$\text{glutamate}^- + 2H_2O + H^+ \rightarrow \text{propionate}^- + 2CO_2 + NH_4^+ + 2H_2$$

$$\text{glutamate}^- + 2H_2O \rightarrow 2\ \text{acetate}^- + CO_2 + NH_4^+ + H_2$$

$$\text{aspartate}^- + 2H_2O + H^+ \rightarrow \text{acetate}^- + 2CO_2 + NH_4^+ + 2H_2$$

Notably, glutamate may be fermented through five different pathways by various bacterial species: Pathway 1—through 3-methylaspartate; Pathway 2—through 3-methylaspartate to acetate, propionate, carbon dioxide and ammonium; Pathway 3—through 2-hydroxyglutarate to acetate, butyrate, hydrogen, carbon dioxide and ammonium; Pathway 4—through 4-aminobutyrate to acetate, butyrate and ammonium and Pathway 5—through 5-aminovalerate to acetate, propionate, valeriate and ammonium [34].

3.2.3. Transformation of lipids during acidogenesis

The products of lipid hydrolysis are glycerol and long-chain fatty acids (Figure 4A). Glycerol can enter (i) a reductive pathway and be converted to 1,3-propanediol or (ii) an oxidative pathway and be transformed to phosphoenolopyruvate in a four-step process. Phosphoenolopyruvate can then be converted to succinate and propionate and/or to pyruvate. In the latter case, further transformations of pyruvate occur through glycolytic fermentations as described for sugars [33, 35]. Significant hydrogen production was observed when *Enterobacter aerogenes* [36] and *Klebsiella pneumoniae* [37] were grown on glycerol-rich media.

Long-chain fatty acids are transformed to acetate and hydrogen through the beta-oxidation pathway, requiring syntrophic cooperation between acetogens and methanogens (described in Section 2.3). However, long-chain fatty acids have an inhibitory effect on anaerobic digestion due to their adherence to microbial cell walls, which can block the passage of nutrients through the cell membrane and/or cause flotation of the cells.

3.2.4. Cross-feeding

Symbiotic interactions between lactic acid bacteria and butyrate-producing bacteria involving clostridia, called "cross-feeding", have been detected in the gastrointestinal tract (Figure 4A). Numerous observations in different animal models have described lactate and acetate conversion to butyrate by butyrate-producing intestinal bacteria, stimulated by lactic acid bacteria (for review, see Ref. [38]). The incubation of human microflora in media containing ^{13}C-labelled lactate revealed that butyrate was the major net product of lactate conversion [39]. Other studies performed using ^{2}H-labelled acetate and ^{13}C-labelled lactate showed that acetic and lactic acids are important precursors of butyrate production in human faecal samples [40].

The metabolic pathway of lactate and acetate utilization to produce butyrate proposed for *Eubacterium hallii* and *Anaerostipes caccae* involves the conversion of lactate to pyruvate by lactate dehydrogenase [41, 42]. The next steps are typical of hydrogen-yielding *Clostridium*-type fermentation. Pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA), which is subsequently routed to acetate and butyrate. Additional acetate is converted to acetyl-CoA. Hydrogen can be produced by both PFOR and NFOR complexes and hydrogenases. The conversion of lactate to butyrate is an important factor in maintaining homeostasis in gastrointestinal tracts.

It is commonly accepted that anaerobic digestion requires symbiotic interactions between specific groups of microorganisms. Some studies have indicated that lactic acid bacteria (LAB), often detected within mesophilic hydrogen-producing microbial communities, may support hydrogen production during acidogenesis. Based on our own research and the findings of other groups, we have considered the true role of LAB in bioreactors and their influence on hydrogen producers [38]. Our metagenomic survey of microbial communities in anaerobic bioreactors, performed using 454-pyrosequencing, revealed that *Clostridiaceae, Enterobacteriaceae* and heterolactic fermentation bacteria, mainly *Leuconostocaeae,* were the most dominant bacteria in hydrogen-producing consortia under optimal conditions for gas production. Furthermore, the complete consumption of lactic acid and predominance of butyric acid in the acidic effluent were observed [43].

An analysis of the hydrogen-yielding granular sludge using the FISH technique [44] revealed that *Streptococcus* spp. cells are located inside the granules, surrounded by *Clostridium* cells. This finding indicates the importance of *Streptococcus* spp. in sludge granule formation and the positive role they play within these microbial communities by stimulating hydrogen production.

Others researchers have examined the effects of lactic acid on hydrogen production by communities of fermentative bacteria. In one study, the complete consumption of lactic acid increasing hydrogen production and butyric acid formation was observed [45]. Subsequently, another group demonstrated that lactic acid increased the efficiency of hydrogen production [46]. FISH analysis revealed that *Clostridium* spp. were the dominant hydrogen producers in the examined system.

Many studies have examined the conversion of lactate and acetate to butyrate and hydrogen by clostridial species, and all point to pH as a critical factor for this process. It is noteworthy that the results of studies on gastrointestinal microflora indicate that acidity is a key regulatory factor in lactate metabolism. The pH may influence both bacterial growth and the development of specific groups of bacteria, as well as fermentation processes affecting the relative proportions of short-chain fatty acids (for review, see Ref. [38]).

A phenomenon analogous to cross-feeding observed in the gastrointestinal tract may occur in hydrogen-producing bioreactors and natural environments [38, 43] (Figure 4A).

Clostridium kluyveri ferments ethanol and acetate to butyrate and hydrogen (Figure 4A; for review, see Ref. [47]).

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4. Acetogenesis

4.1. The essence of acetogenesis

The two final steps of anaerobic digestion, acetogenesis (Stage III) and methane formation (Stage IV), are tightly connected. Acetogenesis supplies substrates for methanogens. Three groups of substrates for methane production and three types of methanogenic pathways have been recognized: (i) splitting of acetate (aceticlastic/acetotrophic methanogenesis); (ii) reduction in CO_2 with H_2 or formate and rarely ethanol or secondary alcohols as electron donors (hydrogenotrophic methanogenesis) and (iii) reduction in methyl groups of methylated compounds such as methanol, methylated amines or methylated sulphides (hydrogen-dependent and hydrogen-independent methylotrophic methanogenesis) [2, 48–51].

Due to the limited number of substrates for methanogenesis, methanogens are strictly dependent on partner microbes with which they form syntrophic systems. Syntrophy is a special type of symbiotic cooperation between two metabolically different types of microorganisms, which depend on one another for the degradation of a certain substrate, typically through the transfer of one or more metabolic intermediate. In this case, the partner microbes oxidize non-gaseous products of acidogenesis to acetate, carbon dioxide, hydrogen and formate that are directly utilized by the methanogens, making the entire syntrophic metabolism efficient and thermodynamically favourable. This is the essence of acetogenesis. The process of hydrogen or formate transfer (interspecies hydrogen/formate transfer) between acetogenic bacteria and methanogenic *Archaea* is an excellent example of syntrophy [4, 6, 7].

Under standard conditions, the oxidation of butyrate, propionate, acetate, ethanol and other non-gaseous products of acidogenesis, coupled to hydrogen or formate production, is endergonic, as demonstrated by the positive change in Gibbs free energy. However, when the oxidation processes are coupled to methane production, the conversion is energetically feasible (exergonic) due to the very low hydrogen partial pressure ensured by hydrogen-consuming methanogens (**Figure 5**). Oxidation of non-gaseous products of acidogenesis during acetogenesis is based on reverse electron transfer: the energetically unfavourable movement of electrons that requires the input of energy to drive the oxidation/reduction reaction (**Figure 5**). This involves multiple systems, most of which are membrane-located, comprising formate dehydrogenases (FDHs), ferredoxin:NAD^+ oxidoreductase, hydrogenases, *c*-type cytochromes, quinone reactive complexes, flavoprotein:quinone oxidoreductases and confurcating hydrogenases. Electron confurcation is a key process in reverse electron transfer. It involves a combined biochemical reaction using two dissimilar electron donors to generate a single product. Confurcating hydrogenases couple hydrogen production from reduced ferredoxin with hydrogen production from NADH [7]. The process responsible for energy conservation in syntrophically growing acetogens is called flavin-based electron bifurcation. Electron bifurcation is the reverse process whereby two products are formed, e.g., NADH and reduced ferredoxin from butyryl-CoA (see Section 4.2) [47, 52].

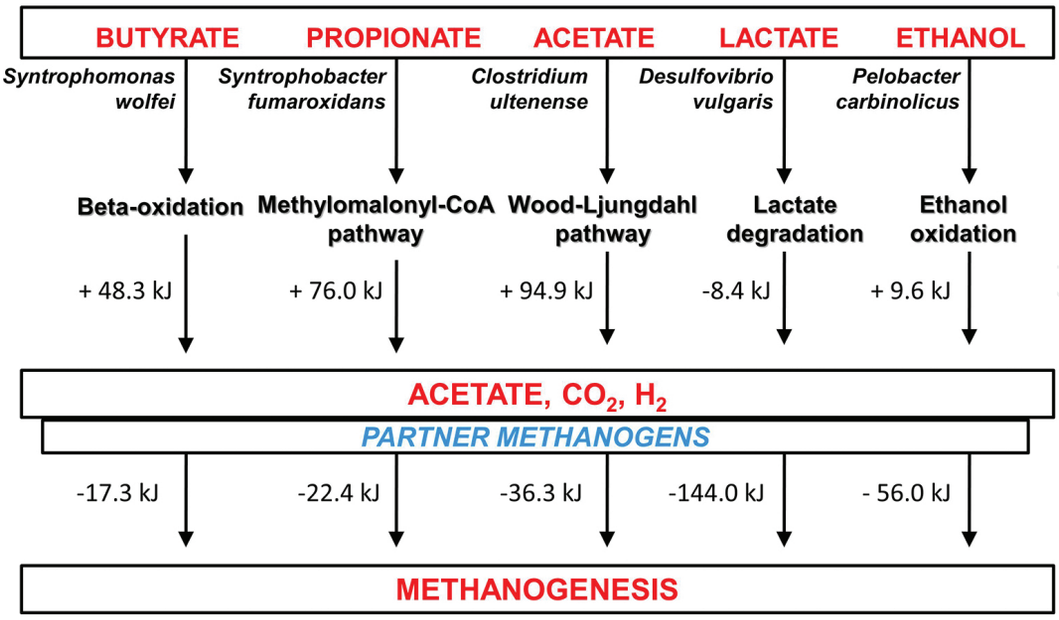

Figure 5. Syntrophic metabolism during acetogenesis—oxidation of non-gaseous products of acidogenesis based on reverse electron transfer in syntrophy with hydrogen-consuming methanogens. The $\Delta G^{0\prime}$ values for acetate, butyrate, propionate and ethanol come from the study of Kamagata [53] and those for lactate oxidation, coupled or uncoupled with the methanogen partner, from the study of McInerney and Bryant [54].

The second known mechanism of interspecies electron transfer in methanogen-yielding communities is direct transfer. This was described between *Geobacter* and *Shewanella* species as the electron donor and methanogen (the electron acceptor), respectively, in environments lacking Mn(IV) and Fe(III) compounds. In this case, pili and outer membrane *c*-type cytochromes are involved in the cell-to-cell electron transfer. Interspecies electron transfer in syntrophic methanogenic microbial communities has been recently reviewed [55].

Our current understanding of the microbial ecology and physiology associated with anaerobic digestion is restricted to culture-dependent techniques and thus is incomplete. The majority of microorganisms involved in the process of anaerobic digestion have yet to be cultivated. It is noteworthy that acetogenic bacteria are unable to grow without their syntrophic partners and cannot be cultivated as a monoculture. Thus, the mechanisms of acetogenesis are poorly characterized at the molecular level. Data derived using recently developed meta-omics approaches are likely to give a deeper insight into syntrophic metabolic pathways of anaerobic digestion.

4.2. Biochemistry of syntrophic oxidation of non-gaseous products of acidogenesis

The metabolic pathways utilized for syntrophic oxidation of common non-gaseous products of acidogenesis include beta-oxidation for butyrate, the methylmalonyl-CoA pathway for propionate, the Wood-Ljungdahl pathway for acetate, the pathway of lactate oxidation recognized in *Desulfovibrio* in the absence of sulphate and the pathway of ethanol oxidation recognized in the genera *Pelobacter* and *Desulfovibrio* in the absence of other electron acceptors.

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In the first reaction of butyrate oxidation, butyrate is activated with acetyl-CoA to butyryl-CoA by butyrate-CoA transferase. This is followed by the conversion of butyryl-CoA to crotonyl-CoA catalysed by butyryl-CoA dehydrogenase, to release electrons as hydrogen or formate, which requires ATP. This process is only possible by a reverse electron transport through electron transfer flavoprotein EtfAB and a membrane-anchored DUF224 protein to the menaquinone pool in the membrane, cytochromes and other electron transfer complexes, terminating at the formate dehydrogenase and hydrogenase/formate dehydrogenase complexes. Crotonyl-CoA is transformed to 3-hydroxy-butyryl-CoA by crotonase and then to aceto-acetyl-CoA by 3-hydroxybutyryl-CoA dehydrogenase. The latter reaction also yields electrons as hydrogen or formate due to reverse electron transfer and the activity of the NADH:hydrogenase/formate dehydrogenase complex. Aceto-acetyl-CoA is split into two moieties of acetyl-CoA by acetyl-CoA acetyltransferase: one is used for butyrate activation and the second is transformed to acetate by phosphotransacetylase and acetate kinase activity, accompanied by the release of ATP [52, 56, 57].

In the first reaction of propionate oxidation, propionate is activated with acetyl-CoA to propionyl-CoA by propionate-CoA transferase. This is then transformed to (S) methylmalonyl-CoA, (M) methylmalonyl-CoA, succinyl-CoA and succinate by, respectively, methylmalonyl-CoA decarboxylase, methylmalonyl-CoA epimerase, methylmalonyl-CoA mutase and succinyl-CoA synthetase. The final step generates ATP. The next reaction is the conversion of succinate to fumarate by fumarate reductase, which releases electrons. This is the first key reaction that requires reverse electron transport. Fumarate is transformed to malate by fumarate hydratase. Malate is then converted to oxaloacetate by malate dehydrogenase in the second key reaction coupled to reverse electron transport. Pyruvate formed from oxaloacetate by pyruvate carboxylase is then transformed to acetyl-CoA by pyruvate:ferredoxin oxidoreductase. Finally, acetyl-CoA is converted to acetate in the third step generating electrons during propionate oxidation. The oxidation of oxaloacetate to fumarate involves coupling menaquinone reduction, proteins encoded by cytochrome c gene homologues, cytochrome b:quinone oxidoreductases, formate dehydrogenases, and hydrogenases including confurcating [FeFe]-hydrogenases [7, 52, 55, 57].

Acetogens that synthesize acetate from hydrogen and carbon dioxide use the reductive carbon monoxide dehydrogenase/acetyl-CoA synthase pathway (reductive CODH/ACS) known as the Wood-Ljungdahl pathway. Acetate-oxidizing syntrophs use the same pathway in reverse (oxidative CODH/ACS). Electrons as hydrogen or formate are released in the reactions catalysed by the carbon monoxide dehydrogenase/acetyl-CoA synthase, methylene-tetrahydrofolate (methylene-THF) reductase and methylene-THF dehydrogenase formate dehydrogenase. Reverse electron transfer during acetate oxidation has yet to be confirmed. It is likely that the same electron transfer mechanism is used in both pathways (reductive and oxidative) [52, 58].

It is believed that ethanol is oxidized to acetaldehyde coupled to NADH formation. Subsequently, acetaldehyde is oxidized to acetate and reduced ferredoxin is formed. Ethanol-oxidizing *Pelobacter carbinolicus* possesses genes encoding membrane-bound ion-translocating

ferredoxin:NAD^+ oxidoreductase and a confurcating hydrogenase that could directly catalyse the oxidation of NADH and reduced ferredoxin to form hydrogen [7, 59].

The key reaction of syntrophic lactate oxidation in *Desulfovibrio* spp. is the conversion of lactate to acetyl-CoA (via pyruvate) by lactate dehydrogenase followed by pyruvate:ferredoxin oxidoreductase, in a reaction that requires reverse electron transfer. The membrane-bound Qmo (quinone-interacting membrane-bound oxidoreductase) complex, cytochromes (involving Hmc, high-molecular-weight cytochrome c complex), menaquinone, hydrogenases (Hyn, Hyd, Hys) and formate dehydrogenases are responsible for reverse electron transport and final hydrogen and formate release. Acetyl-CoA is further processed to acetate by phosphate acetyltransferase and acetate kinase or to ethanol by alcohol dehydrogenase [60].

Worm and co-workers analysed the genomes of the butyrate- or propionate-oxidizing syntrophs *Syntrophus aciditrophicus*, *Syntrophus wolfei*, *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum* and *Syntrophothermus lipocalidus*, and identified six syntrophy-specific functional domains [52]. These include the extra-cytoplasmic formate dehydrogenase (FDH) alpha subunit, as well as an FdhE-like protein and FDH accessory protein. The functions of the latter two proteins are tightly connected with FDH. This finding points to the important role of formate in interspecies electron transfer. The fourth domain was detected in CapA, a protein involved in capsule or biofilm formation that may facilitate syntrophic growth. The fifth domain is characteristic of FtsW, RodA and SpoVE proteins involved in membrane integration, cell division, sporulation and shape determination. The final domain was detected in a conserved site of ribonuclease P involved in tRNA maturation.

In the same study, functional domains involved in electron transfer were also identified [52]. These were found in the following proteins: cytoplasmic FDH, extra-cytoplasmic FDH, formate transporter, Fe-Fe hydrogenase, NiFe hydrogenase, Rnf complex, Ech complex, Etf alpha, Etf beta, Bcd, cytochromes c, cIII, b561 and b5 and the DUF224 protein complex.

Notably, the genomes of sulphate-reducing non-syntrophs were found to lack the syntrophy-specific domains. However, these domains are present in other sulphate reducers that have never been tested for syntrophy: *Desulfobacterium autotrophicum*, *Desulfomonile tiedjei* and *Desulfosporosinus meridiei* [52].

4.3. A model of methane-yielding granules

According to the model of methane-yielding granules proposed more than 25 years ago acetotrophic methanogens constitute a central core of the granule surrounded by acetogenic bacteria and hydrogenotrophic methanogens, and the external layer is composed of microorganisms responsible for acidogenesis. The physical distances (proximities) necessary for energetically favourable hydrogen transfer between acetogenic bacteria and hydrogenotrophic methanogens have been estimated from studies on the propionate-, propanol-, ethanol-oxidizing syntroph *Pelotomaculum thermopropionicum* and hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus*. The proximity needed for efficient interspecies hydrogen transfer depended on the substrate and was estimated at 2, 16 and 32 μm for propionate, ethanol and propanol oxidation, respectively. It is noteworthy that for the less

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energetically favourable syntrophic process ($\Delta G^{0'}$ = +76.0 kJ, +9.6 kJ and +3.0 kJ, respectively, for propionate, ethanol and propanol oxidation), a closer relationship, i.e., a shorter distance between syntrophic partners, is required (for review, see Refs. [53, 55]).

4.4. Syntrophic relationships between acetogenic bacteria and methanogens during anaerobic digestion

The most well-studied examples of syntrophic metabolism in methanogenic communities are described below. The *Syntrophomonadaceae*, a family from the order *Clostridiales*, are highly specialized syntrophic microbes found in methanogenic consortia that can oxidize butyric, propionic and long-chain fatty acids to acetic and formic acids with the production of hydrogen and carbon dioxide—the basic substrates for their partner methanogens [61–63].

The most frequently recognized butyrate oxidizers are representatives of the *Syntrophomonadaceae—Syntrophomonas wolfei, S. bryantii, S. curvata, S. sapovorans, S. palmitatica, S. cellicola, S. saponavida, S. erecta, S. zehnderi; Syntrophothermus lipocalidus, Thermosyntropha lipolytica* and representatives of the *Syntrophobacterales (Deltaproteobacteria)* and *Syntrophus acidotrophicus.* Proteins expressed specifically during syntrophic growth of *S. wolfei* with butyrate have been investigated by proteomic analysis [4, 6, 7, 56].

The propionate-oxidizing bacteria are members of the *Syntrophomonadaceae*—genus *Syntrophobacter* (*S. fumaroxidans, S. wolinii*) and *Smithella propionica* and of the *Peptococcaceae—Desulfotomaculum* (*D. thermocisternum, D. thermobenzoicum* subs. *thermosyntrophicum*) and *Pelotomaculum* (*P. thermopropionicum*) genera [4, 6, 7].

Li and co-workers [16] developed specific PCR assays for propionate-CoA transferase genes (*pct*) to identify and analyse propionate oxidizers in the methane-yielding microbial communities in anaerobic digesters treating various food industry wastes. In addition to *Syntrophobacter fumaroxidans*, six other distinct clusters of putative *pct* genes were detected. The diversity and abundance of the *pct* genes were determined by the nature of the feedstocks of the anaerobic digesters. There was little difference between the *pct* gene profiles of the granular sludge and the liquid phase in the same digester. These authors postulated that the feedstock is a critical factor influencing propionate metabolism in anaerobic digesters. It is noteworthy that such PCR assays may also be used to examine anaerobic decomposition of organic matter in natural environments.

Acetate is the major intermediate product during anaerobic digestion of organic matter to methane and carbon dioxide. It can be directly transformed to methane and carbon dioxide by acetoclastic methanogens (Section 2.4) or syntrophically oxidized to hydrogen and carbon dioxide. The latter reaction requires the participation of two microbial partners: an acetate-oxidizing bacterium and a hydrogenotrophic methanogen. Recognized acetate-oxidizing bacteria include members of the *Clostridia—Thermoacetogenium phaeum, Clostridium ultunense, Clostridium sporomusa, Syntrophaceticus schinkii, Tepidanaerobacter syntrophicus, Tepidanaerobacter acetatoxydans, Candidatus* Syntrophonatronum acetioxidans and *Moorella* sp., as well as *Deltaproteobacteria—Geobacter* spp. and *Thermotogae—Thermotogae lettingae* [4, 52]. Analyses using culture-independent techniques have revealed many other uncultured acetate oxidizers.

Ito and co-workers [64] used MAR-FISH combined with phylogenetic analysis of ^{13}C-labelled bacterial 16S rRNA and tracing of [2-^{14}C]-labelled acetate degradation to study metabolic pathways of acetate transformation in methanogenic sludge from an anaerobic digester fed with mineral medium containing powdered whole milk. These analyses identified *Synergistes* Group 4, belonging to the phylum *Synergistetes*, as the major acetate-utilizing group of bacteria. Moreover, acetate oxidizers were shown to win the competition with acetoclastic methanogens from the genus *Methanosaeta* for the utilization of acetate. At high acetate concentrations, the *Synergistetes* showed a lower affinity for acetate and higher utilization rate in comparison with *Methanosaeta*.

Lee and co-workers [8] presented evidence that in anaerobic digesters fed with a medium containing acetate as the sole carbon source, *Spirochaetes* syntrophically oxidize this substrate with hydrogenotrophic *Methanomicrobiales*. Quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR) targeting the 16S rRNA genes of cluster II *Spirochaetes* and methanogens (*Methanosaetaceae*, *Methanosarcinaceae*, *Methanomicrobiales* and *Methanobacteriales*) revealed that an increase in the former was correlated with higher numbers of *Methanomicrobiales.* High concentrations of hydrogen inhibited the activity of the *Spirochaetes*.

Synergistetes and *Spirochaetes* are frequently found in anaerobic digesters and natural environments, but little is known about their role in anaerobic digestion besides the fact that the latter are thought to be capable of glucose fermentation.

Interestingly, current knowledge concerning the oxidation of lactate in methanogenic consortia is limited to members of the *Desulfovibrio* genus. These species are capable of syntrophic growth on lactate and ethanol with hydrogenotrophic methane-producing partners in the absence of sulphate. As methanogenesis is thermodynamically unfavourable, such syntrophic metabolism is possible only when other electron acceptors such as sulphate are absent. Otherwise, sulphate reduction occurs. Lactate can also act as a substrate for the non-methanogen *Archaeoglobus*, a known sulphate reducer capable of oxidizing lactate to carbon dioxide [7, 60].

Recent studies on anaerobic digestion of molasses wastewater in an upflow anaerobic sludge blanket (UASB) reactor revealed the significant contribution of *Lactococcus* and *Methanosaeta* and their close interaction in methane production [65]. These authors analysed cDNA obtained by reverse transcription of RNA isolated from methane-yielding sludge samples. They proposed lactate as the major fermentation product, which is subsequently oxidized to acetate, a substrate for *Methanosaeta*.

Chojnacka and co-workers [15] hypothesized that a symbiotic interaction between lactic acid bacteria and clostridia, known as lactate cross-feeding (described in Section 2.2.3.), may also occur in methanogenic communities. Butyrate and hydrogen are the products of lactate transformation. The hydrogen and the products of further syntrophic butyrate oxidation constitute substrates for methanogenesis.

Ethanol is also effectively utilized by the methane-yielding microbial communities [15, 66]. Apart from *Desulfovibrio* species, other well-recognized syntrophic ethanol oxidizers are representatives of the *Deltaproteobacteria*, *Geobacter* and *Pelobacter*—well-known Fe(III) reduc-

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ers. To be an energetically effective reaction, the oxidation of ethanol to carbon dioxide and hydrogen also requires strict cooperation with hydrogenotrophic methanogens [4].

Members of the orders *Clostridiales* (*Clostridiaceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae* families), *Fusobacteriales* and phylum *Synergistetes* (*Aminobacterium colombiense*) are capable of amino acid fermentation [4, 7]. However, the transformation of amino acids to produce methane is only energetically possible in syntrophic association with hydrogenotrophic methanogens that scavenge hydrogen.

The *Cloacimonetes*, including Waste Water of Evry 1 (WWE1), are a sub-dominant group of bacteria found in mesophilic anaerobic digesters and gut microflora. So far, all attempts to cultivate representatives of the *Cloacimonetes* have failed, probably due to their need for obligatory symbiotic relationships with other microorganisms. However, the genome of a representative bacterium *Candidatus* Cloacimonas acidaminovorans has been reconstructed using metagenomic sequence data and genomic assembly procedures [93]. The candidate division WWE1 bacteria are regarded as syntrophs capable of amino acid fermentation, propionate and butyrate oxidation as well as cellulose degradation [7, 66].

Actinobacteria, *Chloroflexi* and *Plantomycetes* are often among the bacterial phyla detected in methane-producing anaerobic digesters and wastewater treatment plants. Their functional activities in methanogenic communities have not been well characterized. *Actinobacteria* and *Chloroflexi* are thought to hydrolyse and ferment carbohydrates. The contribution of *Chloroflexi* and *Plantomycetes* to butyrate oxidation was identified in experiments performed with [^{13}C]-labelled butyrate [62].

We have examined the microbial community processing an acidic effluent from molasses fermentation to methane in a UASB bioreactor [15]. Total DNA isolated from the methanogenic community formed in the reactor was sequenced by 454-pyrosequencing. The results revealed that the biodiversity of methanogenic sludge is significantly higher than that of the hydrogen-producing community. The ratio of *Bacteria* to *Archaea* in the methanogenic community was 4:1. The domain *Bacteria* was dominated by *Firmicutes* (~24%), *Bacteroidetes* (~21%), *Proteobacteria* (~9%), *Cloacimonetes* (~7.5%) and *Spirochaetes* (~7%). The *Firmicutes* were dominated by *Clostridia*, which constituted approximately 14% of all bacterial reads. The *Proteobacteria* were mostly represented by the delta and gamma subdivisions (~9 and ~1.5%, respectively), whereas the alpha and beta subdivisions were poorly represented (~0.5%). Other minor groups were *Actinobacteria* (~2%), *Chlamydiae* (~1%), *Synergistetes* (~1%) and *Chloroflexi* (~0.5%). A small number of reads were sequences from *Armatimonadetes, Negativicutes* and *Plantomycetes*. The low level of unfermented sugars and the abundance of *Clostridia* and *Bacteroidetes* suggested that these bacteria play a previously unrecognized role in acetogenesis, involving syntrophic oxidation of non-gaseous products of hydrogen-yielding fermentation. Moreover, an analysis of short-chain fatty acids revealed that butyric and lactic acids were the main substrates utilized in the methanogenic step.

Some of the aforementioned bacterial phyla are capable of oxidizing other compounds, including 1-propanol, benzoate, hydroxybenzoate, phenol and phthalates.

5. Methane formation

Methane formation, Stage IV of anaerobic digestion, is a complex process requiring specific enzymes and cofactors not found in other microorganisms. The course of the reaction depends on the substrates utilized by the methanogens. Three groups of substrates are recognized: (i) acetate, (ii) CO_2 and H_2 or formate, and rarely ethanol or secondary alcohols and (iii) methylated compounds including methanol, methylated amines and methylated sulphides. These substrates are, respectively, processed through three recognized pathways of methanogenesis: aceticlastic/acetotrophic, hydrogenotrophic and methylotrophic (hydrogen-dependent and hydrogen-independent) (**Figure 6**) [67]. Irrespective of the substrate, the final step in each methanogenic pathway is the reaction of methyl-coenzyme M (CH_3-S-CoM) and coenzyme B to produce heterodisulphide CoM-S-S-CoB and methane:

$$CH_3 - S - CoM + Co\,B \rightarrow CoM - S - S - CoB + CH_4$$

This reaction is catalysed by methylcoenzyme M reductase (Mcr), the key enzymatic complex of the methanogenic process. It possesses a unique prosthetic group, coenzyme F_{430}, containing nickel. CoM-S-S-CoB acts as the final electron acceptor during anaerobic respiration and is the key compound for energy gain by methanogens. Methane is a by-product of methanogen metabolism. The pathways of methanogenesis are in fact pathways of CoM-S-S-CoB synthesis.

Splitting of acetate (acetotrophic methanogenic pathway) involves the formation of acetyl-CoA, the transfer of methyl groups to tetrahydrosarcinopterin (H_4SPT) and the formation of methyl tetrahydrosarcinopterin CH_3-H_4STP. CH_3-S-CoM is formed in the reaction of CoM

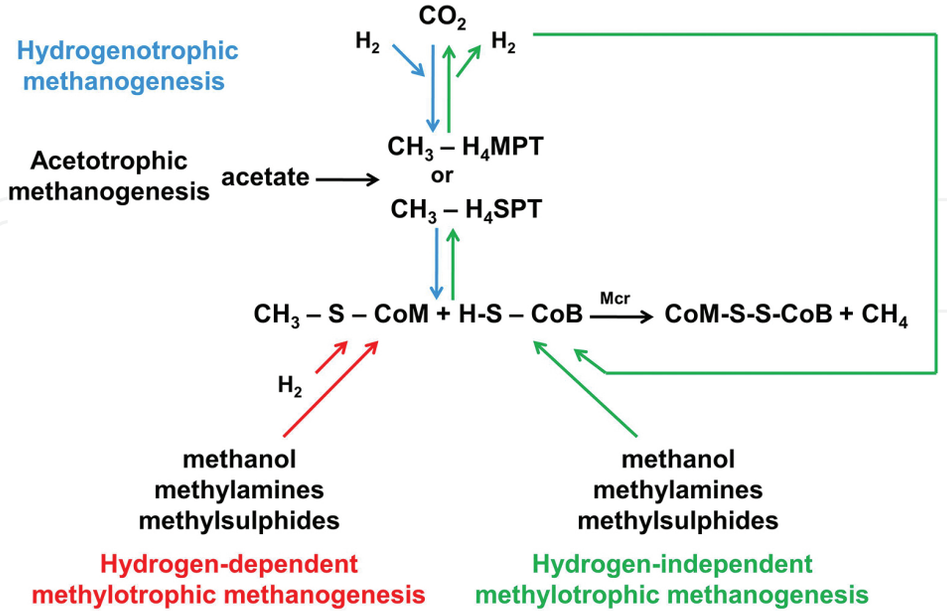

Figure 6. Pathways of methanogenesis. H-S-CoM, coenzyme M; H-S-CoB, coenzyme B; H_4MPT, tetrahydromethanopterin; H_4SPT, tetrahydrosarcinapterin.

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with CH_3-H_4STP. The electrons required to reduce CH_3-S-CoM to methane come from oxidation of the carboxyl group of acetate.

The formation of CH_3-S-CoM by the reduction in CO_2 with H_2, formate or alcohols constitutes the hydrogenotrophic methanogenic pathway. This pathway is comprised of the following steps: (i) the formation of formylmethanofuran (formyl-MFR) from methanofuran (MFR) and CO_2, (ii) the reaction of formyl-MFR and tetrahydromethanopterin (H_4MPT) to produce formyl tetrahydromethanopterin (formyl H_4MPT), (iii) the formation of methylene H_4MPT that in reaction with F_{420}, a derivative of 5′ dezaflavin, produces methyl H_4MPT and (iv) the reaction of methyl H_4MPT with CoM to generate CH_3-S-CoM. The electrons required to reduce CH_3-S-CoM to methane come from hydrogen, formate or alcohols.

In the methylotrophic pathway of methanogenesis, CH_3-S-CoM is formed by the direct transfer of methyl groups from methylated compounds to CoM. One methyl group bound to CoM is oxidized to CO_2 and hydrogen (in the form of $F_{420}H_2$ and reduced ferredoxin) to reverse the hydrogenotrophic pathway. The reducing equivalents are used to reduce CH_3-S-CoM to methane.

In the recently discovered process of hydrogen-dependent methylotrophic methanogenesis, CH_3-S-CoM is also formed through the direct transfer of methyl groups from methylated compounds to CoM. However, the electrons required to reduce CH_3-S-CoM to methane come from externally supplied hydrogen. Genomic analysis revealed that organisms generating methane by this process lack genes encoding the enzymes of hydrogenotrophic methanogenesis [50, 51].

The known cultured methanogens are strict anaerobes and comprise seven orders in the class *Euryarchaeota* of the domain *Archaea*: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales* [2, 48], *Methanocellales* [49] and *Methanomassiliicoccales* [50, 51]. Surprisingly, only two known genera, *Methanosarcina* and *Methanosaeta*, members of the order *Methanosarcinales*, are capable of methane production from acetate. Moreover, only *Methanosaeta* is strictly acetoclastic, whereas *Methanosarcina* is able to produce methane from acetate, CO_2 and H_2 and from methylated compounds. The recognized methylotrophic methanogens belong to the order *Methanosarcinales*. All other known methanogens produce methane by the reduction in CO_2 [1, 2, 48, 49, 67]. The known members of the *Methanomassiliicoccales* order are H_2-dependent methylotrophs. They use methylated compounds (mono-, di-, tri-methylamine and dimethylsulphide) as substrates for methanogenesis, and the methyl group is reduced by hydrogen [50, 51].

It has been estimated that 70% of methane is produced from acetate. When biomass is transformed into methane under mesophilic conditions in anaerobic digesters or natural environments, it is first fermented to acetate, carbon dioxide and hydrogen and formate, as well as short-chain fatty acids during acidogenesis. The theoretical maximum hydrogen yield during dark fermentation occurs with the conversion of one-third of the substrate to hydrogen and carbon dioxide and two-thirds of the substrate to acetate. Therefore, it follows that two-thirds of methane originates from acetate and one-third is from hydrogen, formate and carbon dioxide [2].

Culture-independent analyses of methanogenic communities (mainly from anaerobic digesters) based on cloning and sequencing of 16S rRNA and *mcrA* gene fragments or high-throughput DNA sequencing technologies have revealed that the contribution of methanogens performing the aceticlastic or hydrogenotrophic pathways depends on the substrate and the process conditions. *Methanomicrobiales*, represented by *M. marisnigri*, often predominate in methanogenic communities in biogas plants, indicating that methane is produced through the hydrogenotrophic pathway. This finding does not support the thesis that methane is produced primarily from acetate through the acetoclastic pathway [68]. We found that the hydrogenotrophic pathway of methane production was predominant in the bioreactor processing an acidic effluent from molasses fermentation to methane, and the order *Methanomicrobiales* dominated the archaeal community, constituting about 59%. The most abundant genus within this order was *Methanoculleus* represented by *M. marsigni* and *M. bourgensis*, while the second and the third most abundant genera were *Methanocorpusculum* and *Methanofollis*. Other representatives of this order were members of the genus *Methanoplanus* including the species *Methanoregula formicica, Methanosphaerula palustris* and *Methanospirillum hungatei*. Among the identified hydrogenotrophic methanogens were representatives of the *Methanobacteriales* including the genera *Methanobacterium, Methanococcales* and *Methanocellales*. *Archaea* conducting the aceticlastic pathway of methane production included the *Methanosarcinales* (~3.5%), represented by the genera *Methanosaeta* and *Methanosarcina*. Metagenomic analysis revealed a relatively large contribution of sequences assigned to the genus *Methanomassiliicoccus*, including *Methanomassiliicoccus luminyensis, Candidatus* Methanomassiliicoccus intestinalis and *Candidatus* Methanomethylophilus alvus.

It should be noted that the acetoclastic pathway provides only a small amount of energy available for growth:

$$CH_3COO^- + H^+ \rightarrow CO_2 + CH_4 \left(\Delta G^{0\prime} = -36\ \text{kJ / mol}\right)$$

In comparison, the hydrogenotrophic pathway produces fourfold more energy:

$$4H_2 + CO_2 \rightarrow CH_4 + H_2O \left(\Delta G^{0\prime} = -131\ \text{kJ / mol}\right)$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + H_2O\ (\Delta G^{0\prime} = -144.5\ \text{kJ / mol})\ [67]$$

Thus, the hydrogenotrophic pathway is much more energetically effective, and this may be one of the reasons for the dominance of the *Methanomicrobiales* order in the analysed communities. Moreover, as it was mentioned previously, acetate oxidizers such as *Synergistetes* successfully compete with acetoclastic methanogens belonging to the *Methanosaeta* for acetate [64].

An analysis of the substrate preferences of the recognized methanogenic *Archaea* revealed that hydrogen and carbon dioxide, methyl compounds and acetate are utilized by 74.5, 33 and 8.5% of the methanogens, respectively [69].

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In all methanogenic microbial communities examined by high-throughput DNA sequencing, the contribution of unidentified sequences is usually high. As phylogenetic analyses are dependent on comparison with DNA sequences present in databases and the majority of the recognized genera of methanogens produce methane through the hydrogenotrophic pathway, it is possible that acetoclastic methanogens are hidden among the unidentified sequences. Therefore, the apparent dominance of hydrogenotrophic methanogens such as *Methanomicrobiales* may only be due to our limited knowledge of methanogenic *Archaea*.

Recently, Dziewit and co-workers [18] described four novel molecular markers—other than 16S rRNA and *mcrA*—for the metagenomic analysis of methanogenic communities, with a particular focus on methylotrophic methanogens. These are the *mcrB, mcrG, mtaB* and *mtbA* genes encoding beta and gamma subunits of the methyl-CoM reductase, methanol-5-hydroxybenzimidazolylcobamide Co-methyltransferase and methylated [methylamine-specific corrinoid protein]:coenzyme M methyltransferase, respectively.

It is commonly recognized that methanogenic granular sludge is rich in minerals, mainly ferric sulphide and Ca-, Mg-, Na-, K- or Al-containing compounds. They constitute between 10 and 90% of the dry mass, depending on the composition of the wastes and nature of the methanogenic process [70]. The inorganic components of the extracellular matrix of methanogenic granules may inhibit some metabolic pathways and thus determine the processes leading to methane production by the microbial community. Both Al and K are undesirable elements in the methanogenic sludge due to their competition with other essential metals, inhibiting microbial growth and consequently their adverse effect on the methanogenic process. In contrast, Ca and Mg have a positive effect due to their promotion of the granulation process. Sodium plays a role in the formation of ATP and oxidation of NADH and then is essential for the growth of methanogens. However, high concentrations of Ca^{2+}, Mg^{2+} and Na^{+} ions cause inhibitory effects on methanogen activity. The optimum concentration of Ca^{2+} and Na^{+} ions for methane synthesis from acetate was found to be 200 and 230 mg/L, respectively, whereas a concentration of 8000 mg/L of either ion inhibited the process [71]. Interestingly, the combination of various elements can mitigate the toxicity of others, e.g., magnesium, sodium and ammonium counteract potassium toxicity. It is noteworthy that the acetoclastic pathway of methanogenesis and the oxidation of propionate are particularly sensitive to raised levels of certain minerals [71]. Moreover, it has been observed that inhibition of the acetotrophic pathway of methane formation is usually accompanied by inhibition of propionate oxidation [61].

6. Hydrogen and methane production in a two-stage anaerobic digestion

There is currently great interest in the development of new technologies for the production of energy from renewable sources, of which fermentation processes generating methane and hydrogen show great promise. Hydrogen-yielding fermentation is considered to be one of the most attractive alternative biological methods of hydrogen production. However, there are two major drawbacks: low productivity of the process and the formation of large amounts of

environmentally unfriendly non-gaseous fermentation products [29, 72]. The theoretical maximum hydrogen yield during *Clostridium*-type fermentation is four moles of hydrogen per mole of glucose, when all of the substrates are converted to acetic acid according to the following equation:

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 4\,H_2 + 2\,CO_2 + 2\,CH_3COOH$$

This gives the highest possible yield of hydrogen during dark fermentation. The complete oxidation of glucose provides 12 moles of hydrogen per mole of glucose:

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 12\,H_2 + 6\,CO_2$$

Theoretically, only one-third of the biomass can be converted to hydrogen by the process of hydrogen-yielding fermentation. In practice, this value is lower due to the formation of non-gaseous products such as organic acids and alcohols. For example, when the glucose is converted to butyrate, the hydrogen yield drops to two moles. It is estimated that the efficiency of hydrogen production must reach 60–80% to be economically attractive [73, 74]. This level of efficiency may be attained by using two-stage systems to achieve the transformation of substrates into hydrogen and methane. In such systems, the hydrogenic (hydrolysis and acidogenesis) and methanogenic (acetogenesis and methanogenesis) steps are performed separately under controlled conditions to favour biohydrogen and biomethane production, respectively. In the first stage, hydrogen-rich fermentation gas is produced, while in the second, the non-gaseous products of hydrogen fermentation act as substrates for methanogenic consortia. These two processes are carried out in separate bioreactors that differ in design and have different pH conditions and hydraulic retention times.

A growing number of reports describe the use of two-stage systems for hydrogen and methane production. Such systems have shown promise at the laboratory and pilot scales using various substrates including organic wastes, plant biomass, by-products of the food industry and pure hydrocarbons [66, 75–90]. Increases in energy recovery of up to 20–30% have been achieved using these systems compared to one-stage biogas-producing bioreactors [76, 78, 85, 90]. Effective biomethane production from non-gaseous fermentation products could make biological production of hydrogen through fermentation economically attractive. It has been estimated that by 2040, biohydrogen may be produced on an industrial scale [91].

The idea of two-phase anaerobic digestion as a method for the effective degradation of biomass to methane and carbon dioxide is not new [92]. The novel aspect is the co-production of hydrogen and methane. Many studies on the production of both hydrogen and methane by the anaerobic digestion of biomass have focused on the performance and efficiency of the entire process, but they have lacked any in-depth analysis of the microbial communities in the bioreactors where the two steps are performed. Recognition of the structure and diversity of the microbial communities capable of syntrophic cooperation in the transformation of

substrate to the desired gaseous products should facilitate the optimization of hydrogen and methane co-production from organic matter in two-stage systems.

Research on two-stage anaerobic digestion has been conducted in our laboratory for several years. We have developed and described a laboratory-scale two-stage anaerobic digestion system that produces hydrogen (in Stage 1) and methane (in Stage 2) from sucrose-rich by products of the sugar beet refining industry as the primary energy substrate under mesophilic conditions [15, 43]. Initially, hydrogen is generated through processes of acidogenesis in a three-litre packed bed reactor (PBR) by a hydrogen-yielding microbial community fermenting molasses. Subsequently, non-gaseous organic products from this first stage feed a 3.5-litre UASB reactor in which methane (biogas) is produced by a methane-yielding microbial community. A detailed molecular characterization of this two-stage anaerobic digestion system producing hydrogen and methane from sugar beet molasses was achieved using optimized DNA extraction protocols and high-throughput pyrosequencing (454 Roche) [15, 43].

Recently, the two-stage system for hydrogen and methane production described above has been successfully scaled up 10 times and is currently being trialled in a Polish sugar factory.

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