

**Anna Sikora\*, Anna Detman, Aleksandra Chojnacka, Damian Mielecki**

Institute of Biochemistry and Biophysics Polish Academy of Sciences,  
Pawińskiego 5a Street, 02-106 Warsaw, Poland

\* annaw@ibb.waw.pl

## **Selected issues of anaerobic digestion based on the studies on hydrogen- and methane yielding bioreactors**

### **Abstract**

Anaerobic digestion of organic matter results from the metabolic activity of many groups of microorganisms. Interactions between microorganisms during acidogenesis, acetogenesis and methanogenesis, source of inoculum, type of feedstock and operational conditions determine metabolic pathways in bioreactors and consequently the efficiency of fermentation processes. In innovative installations it is desirable to separate acidogenesis from acetogenesis and methanogenesis to favour respectively the production of biohydrogen or biomethane under controlled conditions.

**Keywords:** microorganisms, biohydrogen, methane, anaerobic conditions, metabolism.

### **1. Introduction**

Anaerobic digestion, whose final products are methane and carbon dioxide, is a common process in natural anoxic ecosystems such as water sediments, wetlands or marshlands where the concentration of other electron acceptors such as sulphate, nitrate, Mn(IV) or Fe(III) is low. It is running in landfills and waste-water treatment plants. Digestive tracts of animals are also a source of methane. The general scheme of anaerobic digestion is commonly known, however it is still poorly recognized on the molecular level. It is a complex process that results from the interaction of many groups of microorganisms, requiring four major steps. The first one is hydrolysis of complex organic polymers (e.g. polysaccharides, lipids, proteins) to monomers (sugars, fatty acids, amino acids) by fermentative bacteria. The second step is acidogenesis that results in formation of hydrogen and carbon dioxide as well as non-gaseous fermentation products, i.e. low-molecular-weight organic acids and alcohols. These products are further oxidized to hydrogen, carbon dioxide and acetate. This step is called acetogenesis and involves mainly syntrophic degradation of non-gaseous fermentation products. The forth step is methanogenesis. The two last steps, acetogenesis and methanogenesis are closely related and involve syntrophic associations between hydrogen-producing acetogenic bacteria and hydrogenotrophic methanogens. These associations keep the hydrogen partial pressure low enough to allow acetogenesis to become thermodynamically favourable. This process is referred to as interspecies hydrogen transfer. Acetate is a direct substrate

for methanogenesis. It can be also syntrophically oxidized do hydrogen and carbon dioxide (Thauer et al., 2008; Sieber et al., 2012; Mao et al., 2015; Sikora et al., 2017).

Process of anaerobic digestion of biomass is used to produce biogas as an alternative energy source. Currently there is great interest in the development of new technologies aimed at producing energy from renewable sources, of which biomethane and biohydrogen production show great promise. For the biotechnological purpose the idea of separation of hydrogenic (hydrolysis and acidogenesis) and methanogenic (acetogenesis and methanogenesis) steps under controlled conditions was arisen to favour biohydrogen and biomethane production, respectively. In the first stage, hydrogen-rich fermentation gas is produced, while in the second stage, the non-gaseous products of hydrogen fermentation act as substrates for methanogenic consortia. These two processes are carried out in separate bioreactors of different type with different pH conditions and hydraulic retention times (HRTs). The key problems of dark fermentation are low hydrogen yields (theoretical maximum — 4 moles of  $H_2$ /mole of glucose and generation of large quantities of non-gaseous organic products remain. It is noteworthy that effective biomethane production from non-gaseous fermentation products could make biological production of biohydrogen *via* fermentation economically attractive. Furthermore the mechanisms of particular steps of anaerobic digestion are similar in natural environments as well as in anaerobic bioreactors aimed at gaseous fuels production (Schievano et al., 2014; Sen et al., 2016; Jarunglumert et al., 2018; Guwy et al., 2011).

## 2. Molecular studies on anaerobic digestion

Recently, there has been a rapid development in culture-independent techniques (meta-omics approaches such as metagenomics, metatranscriptomics, metaproteomics, metabolomics) for exploring microbial communities which have led to a new insight into their structure and function in both natural environments and anaerobic digesters. The current trends involve the combined use of meta-omic approaches and detailed reactor performance data as well as isotope labelling techniques that allow us to develop a fundamental understanding of the processes occurring in AD. Those activities are aimed to improve biogases production and increase the share of renewable energy in total energy consumption. Analysis of many studies on metagenomes of microbial communities from anaerobic digesters shows that: (i) contribution of methanogens in the methane-yielding microbial communities is relatively small, below 20%; (ii) the most abundant phyla of bacteria are usually: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*; (iii) methanogenic archaea are dominated by acetotrophs or hydrogenotrophs with a certain contribution of methylotrophs; (iv) substrate, operational conditions such as temperature, pH, ammonia concentration, etc. shape the structure, percentage distribution of specific taxons and functioning of the community of microorganisms; (v) it is important to describe interactions within microbial communities and assign functions in AD steps to specific groups of microbes; (vi) the majority of sequences are not classified at the genus level confirming that most of the microorganisms are still unrecognized (Guo et al., 2015; Cai et al., 2016; Campanaro et al., 2016; Kleinstеuber, 2018).

### 3. Selected issues based on the studies on hydrogen- and methane-yielding bioreactors

#### 3.1. Methane production from lactate-rich substrate

Lactate, a product of acidic fermentation, is an important intermediate in anaerobic digestion of organic matter. Various types of silage are common feedstocks for commercial biogas plants. Silage technology has been applied to biomass storage in biogas production and ensilaging is regarded as a means of increasing methane yield from anaerobic digestion. Lactic acid is a common product of the acidogenic step in two-stage anaerobic digesters. Many studies clearly show that lactate is effectively utilized by methane-producing microbial communities. Studies done on pure cultures of *Acetobacter woodii* and *Desulfovibrio vulgaris* have constituted milestones in the research on the anaerobic lactate oxidation. Recently, we have presented a study where methane-yielding microbial communities instead of pure cultures of microorganisms were used to process lactate-rich artificial media to collect data allowing the description of the metabolic transformation of lactate during the acetogenic and methanogenic steps of AD in methane-yielding bioreactors. The artificial media were intended to imitate a mixture of acidic products in the anaerobic environments/anaerobic digesters where lactate fermentation dominates. Among Archaea present in the bioreactors the order *Methanosarcinales* predominated. The acetoclastic pathway of methane formation was further confirmed by analysis of the stable carbon isotope composition of methane and carbon dioxide. The domain *Bacteria* was represented by *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Synergistetes*, *Actinobacteria*, *Spirochaetes*, *Tenericutes*, *Caldithrix*, *Verrucomicrobia*, *Thermotogae*, *Chloroflexi*, *Nitrospirae* and *Cyanobacteria*. Available genome sequences of species and/or genera identified in the microbial communities were searched for genes encoding the lactate-oxidizing metabolic machinery homologous to those of *Acetobacterium woodii* and *Desulfovibrio vulgaris*. Furthermore, genes for enzymes of the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) were present in the microbial communities. In conclusion we postulate that when a lactate-rich substrate is processed by a methane-yielding microbial community, the main end product of the acetogenic step is acetate, which is then utilized by acetotrophic methanogens (Detman, Mielecki et al., 2018).

#### 3.2. Inhibition of hydrogen production during acidogenic step of anaerobic digestion

Dark fermentation, which forms part of the acidogenic step of anaerobic digestion, is an alternative biological method of bio-hydrogen production. Several well-recognized factors can reduce bio-hydrogen production: (i) an increase in hydrogen partial pressure that inhibits the activity of NADH: ferredoxin oxidoreductase (NFOR), an enzyme that catalyzes the formation of reduced ferredoxin in the reaction with NADH according to the equation  $\text{NADH} + \text{Fd} \rightarrow \text{NAD}^+ + \text{FdH}$ ; (ii) metabolic shift — the accumulation of acids causing a drop in pH in the bioreactor, which is unfavourable for bio-hydrogen production (the optimal pH for this process is around 5); (iii) coexistence of hydrogen-consuming microorganisms such as methanogens and acetogens; (iv) substrate competition between microorganisms including the replacement of hydrogen fermentation by lactic acid or ethanol fermentation; (v) excretion of antibacterial factors such

as bacteriocins by some bacteria, inhibiting the growth of other bacteria; (vi) various toxic or inhibitory compounds. Recently, the presence of ascomycetous yeasts in the hydrogen-producing microbial community has been shown as another factor that can seriously inhibit bio-hydrogen production during acidogenesis, while having no influence on the methane-yielding steps of anaerobic digestion. This inhibitory effect appears to depend on substrate competition and the production of antibacterial compounds (aforementioned factors iv and v) (Detman, Chojnacka et al., 2018).

#### 4. Conclusions

Biomass conversion to gaseous biofuels is the effect of complex interactions between microorganisms. These processes occur due to the microbial enzymatic machinery involved in specific metabolic pathways. Linking structure and function of microbial communities, understanding interactions within microbial communities, searching for syntrophic cooperation between microorganisms are crucial for attempts to optimize production of biomethane or co-production of biohydrogen and biomethane from organic matter in two-stage systems.

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### **Acknowledgement**

*This project is funded by the National Centre for Research and Centre Development (grant number BIOSTRATEG2/297310/13/NCBiR/2016).*

