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Microorganisms as Direct and Indirect Sources of Alternative Fuels

Anna Matuszewska

Abstract

The industrialization and economic growth during the XXth century had been supported by fossil fuels, but it is clear that they are limited and they cannot sustain the growing energy needs. There is urgency in finding renewable and efficient fuels. The solar energy is obviously the solution in long-term but without suitable methods of storage, it is impossible to use it as a primary source of energy. One of the most important form of solar energy capturing is biomass itself, including the cell mass of microorganisms. The potential of microbes in alternative fuels and energy production is still unexploited. There are several possible routes for using a single-celled organisms to harvest energy.

The scope of this chapter include the review of possible technologies based on application of microorganisms in fuels and energy generation. The reader will find the reliable descriptions of currently available biotechnologies along with the ones that could become important in the future, like the new born technologies that are developed only on the laboratory stage.

The biotechnologies described in this chapter have been divided into two main groups, regarding to the role that the microbes play in the production process:

• the technologies in which the microorganisms serve as a direct source of biomass for fuel production from accumulated intracellular oil (e.g. microalgae and other oleaginous microorganisms biomass - bacillus, fungi and yeast),

• the technologies in which the abilities of microorganisms to excreting some valuable chemicals make them the indirect source of alternative fuels (e.g. methanogenic fermentation, ethanolic fermentation, fermentation of syngas etc.).

Additionally the methods of producing electrical power in microbial fuel cells (MFC) have been included, as a third group. In MFC, bacteria convert the energy from chemical compounds to electricity, that could be used as the final product or as driving force for other processes e.g. hydrogen production by microbiologically assisted water electrolysis.

The pros and cons of different presented scenarios, in which the microorganisms are playing the leading role as energy and fuel producers, have been mentioned. By giving
the comprehensive basics for understanding the principles of wide range of processes, the author wants to introduce technologies that already exist and those which may be our future.

**Keywords:** Alternative fuels, microorganisms, microalgae, yeast, fungi, microbial fuel cells

### 1. Introduction

Since ancient times, humans used their surrounding nature as a source of food, medicine, energy and other valuable products. The discovery of coal and crude oil and their enormous energy potential has led people to focus their attention towards them. Oil and coal became the main raw materials for the production of energy and fuels for many years. They have been relatively cheap and easy to extract; moreover, the methods of their processing are well known. Rapid development in the industrial and automotive sectors caused crude oil resources depletion at a rapid rate. This resulted in the necessity of extracting crude oil from deeper layers, which caused increase in cost and influenced on the prices of petroleum products. Thus, the production of fuels from sources other than petroleum has become one of the main goals of humanity. The replacement of crude oil with other materials is very important for long-term energetic security and economic growth. Thus, once again the people turned their attention to nature and natural sources of energy such as water, sun or biomass. To meet the growing energy demand, people also began to use also microorganisms such as bacteria, microalgae, fungi and yeast. Today, these organisms are both raw material and producers of valuable substances used in many branches of industry, including production of fuels. Some microorganisms are able to convert waste biomass and biodegradable rubbish into desired products.

Due to the high diversity of microorganism species, their environmental requirements and eating habits, their ability to reproduce, yield of the desired compounds as well as their utilization and processing cannot be standardized as in the case of crude oil. There are several ways to obtain fuel products using microorganisms. Some of them are inexpensive, and today the production of biofuels is carried out on an industrial scale. Other technologies are in their infancy and require more time, more research, work of scientists and financial outlays to become ready for fuel production in the future.

In the literature, we can find some research involving microorganisms in production of biofuels, for example, production of biodiesel from microalgae or production of H₂ by bacteria. We can also find references that cover the revision of knowledge connected with particular methods of biofuel production or with processes taking place in the microbial bioelectrochemical systems (BESs). But there is a lack of literature, which includes the overall review of methods using microorganisms as a feedstock for the production of biofuels and as their producers.
2. Oleaginous microorganisms

Oleaginous microorganisms are species that are able to store oil in their cells, with the oil content excess of 20% biomass weight. This oil is commonly called microbial oil. It can be produced by some species of microalgae, bacteria, fungi and yeast. The main focus of research is on microalgae, fungi and yeast due to the yield of produced oil. Because the quantity of oil generated by bacteria is much lower, the interest in these organisms is not as high as for other microorganisms. The most advanced is work on technologies for producing biofuels from microalgae.

2.1. Microalgae

Microalgae are recognized as the oldest microorganisms present on Earth. They are present in all ecosystems and live under different environmental conditions. They are primitive unicellular form of plants. They do not have formed leaves, stems and roots. The cell structure of these species is very simple, which allows them to adapt to new environmental conditions relatively easy. The in-built chlorophyll in the cell of microalgae allows them to perform photosynthesis. But some species are heterotrophic and they require other sources of organic carbon and energy for growth. There are also mixotrophic microalgae, which, depending on the ambient conditions, can change their nutrition system from autotrophic to heterotrophic and vice versa.

Microalgae have a huge biodiversity; it is estimated that there are over 100 000 species of these organisms [1]. They differ among other species in cellular structure, life cycle and type of pigment. Two groups of microorganisms are classified as microalgae: prokaryotes and eukaryotes. The eukaryotes are divided into four main classes: the diatoms (Bacillariophyceae), the green algae (Chlorophyceae), the golden algae (Chlorophyceae) and the red algae (Rhodophyceae), whereas from prokaryotes to the microalgae group belong the cyanobacteria (Cyanophyceae) (Figure 1). Despite the large number of species of these algae, in practice, only about 15 species are used for large-scale production processes [2].

Under appropriate conditions such as temperature, the insolation level and nutrients, algae can grow abundantly. Typically, these organisms double their biomass for 24 hours, but their greatest growth phase (called exponential phase) is for 3.5 hours [3]. It is much higher than it is observed for agricultural crops or forestry. Because of such an intensive increase in biomass, microalgae need a lot of carbon, hydrogen and oxygen, which are taken from water and air. They also need minerals that are sources of nitrogen, potassium, calcium, magnesium, iron, sulphur, phosphorus, silicon and trace elements. All these elements are built in algae cells, so microalgae have become a source of these elements and of different valuable compounds such as pigments, lipids, sterols, fatty acids, starch, oils and others [4, 5].

Microalgae could be used to obtain different kinds of biofuels. The conversion of microalgae to energy can be realized in biochemical as well as thermochemical routes [6, 7] – Figure 2. We can obtain biodiesel from extracted microalgae oil, biomethane by anaerobic digestion of microalgae biomass, biohydrogen in dark fermentation stage or bioethanol by fermentation of
ethanol. Algae biomass can be converted into biofuels not only biochemically but also thermochemically in processes such as pyrolysis, gasification and liquefaction. Another way to get energy is direct combustion of biomass.

Regardless of the way of algae biomass conversion, the most important step is to provide the feedstock for a process. Microalgae are most often cultivated by special systems: open ponds and different kinds of photobioreactors [1]. The microalgae conversion process consists of several main steps: species selection, cultivation, harvesting, biomass concentration and algae pretreatment before conversion – Figure 3.

The very important stage in microalgae biomass production is the selection of the most appropriate species. These microorganisms can thrive in diverse environment such as fresh
water, brackish water or saline water. Currently, researches are being conducted on available species, but under artificial conditions these organisms lost their genuine properties due to their long-term cultivation. Such species are, for example, more sensitive to the environmental stress. In lab scale, the cells have more metabolites that improve the growth of algae [8]. On the other hand, in natural systems, microalgae can use other components that are not available under lab conditions. There is a lack of information about properties of species growing in isolate systems.

To select microalgae for any conversion process, the composition of cells and cell walls, productivity and the resistance to changes in temperature, pH, nutrition level, light or carbon supply should be taken into account. The kind of selected species and cultivation system (close or open ponds) decide the equipment, biomass yield, financial investment and so on. It should be remembered that microalgae production is much more expensive than oilseed crop production. These costs can be reduced, for example, using natural sunlight, CO$_2$ from industrial plants or nutrients (phosphates and nitrates) from sewage treatment plants or by changing the cultivation system.

2.1.1. Biodiesel

Generally, biodiesel is produced from oils extracted from oilseed crops or animals. These oils are esters of fatty acids and glycerine. Biodiesel is produced by transesterification, where the glycerine is replaced with other alcohol (most often methanol or ethanol) in the presence of a catalyst. The result of the transesterification process is a mixture of fatty acid methyl (or ethyl) esters. Since recently, microalgae are being considered as a potential source of oils for biodiesel production. They have the ability to accumulate lipids. Because lipids are basic raw material for biodiesel production, the oil content in cell should be taken into account for the microalgae selection process.
The oil content in cell depends on microalgae species and growth conditions [5, 8-10]. It can be as high as 80% dry weight. Examples of oil content of some algae cells are presented in Table 1. Microalgae need less land area for growing and have a higher yield of oil in comparison with oil crops. For example, oil yield from soybean is 446 L/ha, from palm oil 5950 L/ha, but from microalgae produced in photobioreactors, the oil yield is 58,700 L/ha for species with 30% oil by weight in biomass and 136,900 L/ha for species with 70% oil by weight in biomass [9].

<table>
<thead>
<tr>
<th>Species</th>
<th>Oil content (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botryococcus braunii</td>
<td>2575</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>2</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>1440/56</td>
</tr>
<tr>
<td>Dunaliella salina</td>
<td>625</td>
</tr>
<tr>
<td>Dunaliella primolecta</td>
<td>23</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>1771</td>
</tr>
<tr>
<td>Euglena gracilis</td>
<td>1420</td>
</tr>
<tr>
<td>Haematococcus pluvialis</td>
<td>25</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>740</td>
</tr>
<tr>
<td>Nannochloris sp.</td>
<td>2056</td>
</tr>
<tr>
<td>Nannochloropsis sp.</td>
<td>3168</td>
</tr>
<tr>
<td>Neochloris oleobundans</td>
<td>2965</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>2030</td>
</tr>
<tr>
<td>Prymnesium parvum</td>
<td>2238</td>
</tr>
<tr>
<td>Tetraselmis maculata</td>
<td>3</td>
</tr>
<tr>
<td>Spirulina maxima</td>
<td>69</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>1222/3555</td>
</tr>
<tr>
<td>Schizochytrium sp.</td>
<td>5077</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>923</td>
</tr>
</tbody>
</table>

Table 1. Oil content of some microalgae cells on the base of [1, 3, 9-11].

The difference between algae and other raw materials for biodiesel production is that the microalgae are microorganisms that generally live in water environments, and thus cultivation, harvesting and processing techniques are different. For mass diesel production, microalgae production is concentrated on one production unit where algae cells grow and then are separated from the growing medium and where the lipids are extracted from microalgae biomass. The biodiesel is produced in a way similar to existing processes and technologies used for other biodiesel feedstocks. Naturally, obtained oil can be used for production of other biofuels and then the transesterification reaction is replaced by other processes. For example, hydrotreating of vegetable oils is a quite modern way to produce very high-quality diesel fuels.
[12, 13]. The other way could be a thermal decomposition or cleavage of the triglycerides and other organic compounds presented in the feedstock to get alkanes, alkenes and other chemicals [14, 15].

As mentioned earlier, feedstock providing is the important step before microalgae biomass conversion. Taking these stages into account, the process of biodiesel production from microalgae will be extended with additional operations such as oil extraction and biodiesel production. Figure 4 shows the main stages of biodiesel production process.

Each biodiesel used as a car fuel should meet the requirements of the International Biodiesel Standard for Vehicles (EN14214), also in terms of oxidative stability. The disadvantage of microalgae oils is that they contain much more polyunsaturated fatty acids with four or more double bonds than vegetable oils [16]. Such oils are susceptible to oxidation during storage, and therefore, their use for biodiesel production is limited. Many microalgae oils cannot be directly used as automotive fuels because of their composition, but the quantity of unsaturated bonds can be reduced easily by partial catalytic hydrogenation of double bonds [9]. The composition of microalgae oils depends on algae species; therefore, the proper selection of cultivated microorganisms is more important. The advantages of algae biodiesel over vegetable oil is that it is derived from plants that do not compete with foods.

![Figure 4. Scheme of microalgae biodiesel production.](image)

2.1.2. Biogas

Apart from oils, microalgae in their cells also accumulate other components, for example, proteins, carbohydrates or starch, which are good nutrient for microorganisms producing biogas. Thus, these algae as well as maize silage or wastes from processing of fruit and
Vegetables can be a good feedstock for anaerobic digestion process. But comparing with other substrates in the literature, the studies on methane fermentation of microalgae are significantly less.

In recent years, two approaches to the production of biogas from microalgae have been investigated: (1) direct use of microalgae culture after concentration of the cells by filtration or centrifugation and (2) the use of cell residues, which remained after the extraction of oil or other components from microalgae.

Microalgae, due to the enormous growth, need very large amount of nitrogen and phosphorus to build their cells. In the case of nitrogen, annual demand is estimated between 8 and 16 tonnes/ha, which is 55 to 111 times greater than in the case of rapeseed [17]. It results in the fact that microalgae have a very big potential for the purification of water from the compounds containing N and P. The phosphorus and nitrogen accumulated in the cells remain in biomass after extraction of oil. Methane fermentation makes it possible to release these elements from the microalgae cells and then applying them as nutrients in the algae cultivation. It reduces costs of algae production, and the recovered biogas additionally improves the economy of the company.

In addition to the high content of N and P, microalgae contain many other minerals (Fe, Co, and Zn), which not only meet the nutritional requirements of anaerobic microorganisms, but also stimulate their growth. The content of above-mentioned compounds and minerals is differentiated and depends on the species of algae and conditions of their growth (in particular, the availability of nutrients). For example, deficiency of nitrogen in cultivation algae medium results in lower concentrations of proteins and higher concentrations of lipids. Thus, the yield of methane is also related to the type of microalgae used as a feedstock to the digestion process – Table 2 and Figure 5.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth conditions</th>
<th>Proteins [%]</th>
<th>Lipids [%]</th>
<th>Carbohydrates [%]</th>
<th>CH$_4$ [L CH$_4$/g VS$^{-1}$]</th>
<th>Before lipid extraction</th>
<th>After lipid extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella vulgaris</td>
<td></td>
<td>29</td>
<td>18</td>
<td>51</td>
<td>0.64</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>Low content of N</td>
<td>7</td>
<td>40</td>
<td>55</td>
<td>0.69</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Chlorella emersonii</td>
<td></td>
<td>32</td>
<td>29</td>
<td>41</td>
<td>0.74</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Chlorella emersonii</td>
<td>Low content of N</td>
<td>28</td>
<td>63</td>
<td>11</td>
<td>0.92</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Chlorella protothecoides</td>
<td></td>
<td>38</td>
<td>11</td>
<td>52</td>
<td>0.65</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Chlorella protothecoides</td>
<td>Low content of N</td>
<td>36</td>
<td>23</td>
<td>41</td>
<td>0.71</td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. The effect of low nitrogen content in the environment on the composition of three species of Chlorella and the theoretical methane potential before and after lipid extraction (adapted from [10]).
The yield of methane is determined not only by species of microalgae. It is important if to the fermentation process are used whole cells of or cells damaged, for example, by extraction of oils or other algae components. Methanogenic microorganisms have problem accessing the intracellular components because of the specific constitution of cell wall of algae (which can be different for various algae species). The destruction of the cell wall caused these components to become more available, resulting in an increase of biogas and methane yield. The extraction of lipids from microalgae cells before fermentation influences the composition of biogas. This treatment reduces lipids, which lowers methane yield (see Table 2) and increases the concentration of proteins. High content of proteins (as a result of extraction or due to kind of algae species) causes releasing of high amount of ammonium ions to fermentation medium. Higher concentration of those ions can inhibit fermentation process and, in extreme cases, may be toxic to the methanogenic organisms, especially at higher pH values. For example, fermentation of cyanobacteria, *Spirulina maxima*, which is rich in protein (contains up to 60% of proteins), resulted in release of large amounts of ammonia during hydrolysis (up to 7000 mg L$^{-1}$), resulting in withering anaerobic bacteria away [19, 20]. The advantage of biogas produced from microalgae is low hydrogen sulphide content in gaseous product.

When microalgae biomass is not subjected to any processes of destroying cell walls, the walls protect cells from the action of enzymes produced by hydrolysing bacteria. Thus, the biodegradation of the cells in the anaerobic process is weaker and biogas yield is smaller. Some species of microalgae can be much resistant to hydrolysis of their cell walls, for example, *Scenedesmus* and *Chlorella*. The cell walls of such algae have a multilayer structure and are mainly composed of organic compounds such as cellulose and hemicellulose, which are less biodegradable. Algal species devoid of the cell wall (e.g., *Dunalieila, Pavlova_cf*) or with a cell wall composed of glycoproteins (e.g., *Chlamydomonas, Euglena*) are more susceptible to microbiological degradation, resulting in higher yields of biogas and methane [21, 22]. The composition of the cell walls of microalgae is still poorly recognized and can considerably vary even within a given genus.
Theoretical yield of biogas is usually higher than real one because the bioavailability of feedstock determines biomass conversion during anaerobic digestion. Operations taken to increase the availability of the cellular content of microalgae for methanogenic bacteria allow for increasing the amount of gaseous product. As in the case, other types of biomass, degradation of the cell walls of microorganisms, which increases the availability of methanogenic bacteria to the contents of the cells and thereby affects the biogas productivity. The process of disintegration of microalgae can be carried out using different methods: thermal, mechanical, chemical or biological. The efficiency of used method depends on the parameters of the process (e.g., temperature, exposure time and dose of chemicals), as well as the characteristics of microalgae (the strength and structure of the cell wall and macromolecular composition of algae cells).

An important parameter during methane fermentation is the carbon (C) to nitrogen (N) ratio in biomass feedstock, which should range from 20/1 to 30/1. When this parameter is not properly balanced and the C/N is less than 20, the significant amounts of nitrogen (volatile fatty acids) are released, leading to their accumulation in the fermenter. Such a situation causes the inhibition of the methane production because of acidification of process environment. C/N ratio can be controlled by choosing the kind of substrates and their proportions in the mixture. Microalgae have low C/N value (below 10) [23], and it would be well to introduce an additional substrate rich in carbon (co-digestion) such as maize silage or sludge from sewage treatment plants. In such cases, not only the increase in methane yield was observed [24, 25], but there has been reported cases where the real yield was higher than the theoretical (synergy effect) [26, 27]. Co-digestion may also lead to the dilution of some compounds present in the microalgae biomass (e.g., sodium compounds) that have an inhibitory or toxic effect on the anaerobic bacteria.

2.1.3. Butanol and ethanol

Fermentation is a common process used commercially on a large scale to produce ethanol from crops containing sugar and starch. Microalgae accumulate polysaccharides in their cells. They are rich in various carbohydrates such as cellulose, starch, mannitol, agar and laminarin [28]. Some species contain a large amount (even over 50% of the dry weight) of starch and glycogen, which are essential for ethanol production. Such species include Chlorella, Chlamydomonas, Dunaliella, Scenedesmus and Spirulina [29, 30]. The examples of microalgae and the starch content in the cells are presented in Table 3. This causes these organisms can be used as a good raw material for alcohol production.

Ethanolic fermentation is performed mainly by yeast. Fermentation process for microalgae is similar to that for other plants. The biomass is ground down, and the polysaccharides are converted to monosaccharides. Then, the yeast breaks down the sugars and converts them to ethanol. Acetone–butanol fermentation is an anaerobic process of enzymatic degradation of saccharides to butanol, acetone, carbon dioxide and hydrogen. It is carried out by certain species of bacteria of the genus Clostridium (especially C. Butylicum and C. acetobutylicum).
Generally, the whole process of alcohol production consists of pretreatment of the biomass, saccharification, fermentation and product recovery.

<table>
<thead>
<tr>
<th>Species</th>
<th>% Starch (g/dry weight)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella vulgaris</td>
<td>37</td>
<td>[29]</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>17</td>
<td>[31]</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>17</td>
<td>[31]</td>
</tr>
<tr>
<td>Chlorococcum humicola</td>
<td>11</td>
<td>[32]</td>
</tr>
<tr>
<td>Nostoc muscorum</td>
<td>34</td>
<td>[30]</td>
</tr>
<tr>
<td>Oscillatoria obscura</td>
<td>13</td>
<td>[30]</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>23</td>
<td>[30]</td>
</tr>
<tr>
<td>Spirulina fusiformis</td>
<td>37-56</td>
<td>[30]</td>
</tr>
</tbody>
</table>

Table 3. Starch content in the microalgae cells.

Quantity of produced alcohols depends on the production process parameters and characteristic of used algae biomass. For instance, the microalgae species such as Chlorella and Chlorococcum are better raw materials for ethanol production than Chlamydomonas [28]. As in the case of biogas production, the yield of alcohol is also dependent on the microalgae biomass pretreatment. During these operations, the fermentable sugars are released from algae cells and become available for the fermentation process. The biomass, as in the case of methane fermentation, can be pretreated in physical, biological and chemical ways. Harun and Danquah [32] studied an acid exposure as a pretreatment method to release the carbohydrates from cells of Chlorococcum species. The highest ethanol yield was obtained when the microalgae (in concentration 15 g L\(^{-1}\)) were treated at 140°C with 1% (v/v) of sulphuric acid for 25 minutes. They found that during this pretreatment method the most important parameter that influences bioethanol production from microalgae is temperature. The other significant parameters are the acid concentration and the amount of microalgae loading.

Ethanol from microalgae can be produced in three ways: (1) from algae cell components (starch and saccharides) after their extraction or from cell wall components (cellulose) after enzymatic hydrolysis of walls, (2) some species of microalgae product ethanol during dark fermentation and (3) via genetic modification of some microalgae to direct production of ethanol [30].

In the first way, the starch can be extracted from the cells using mechanical (ultrasonic and steam explosion) or biological methods (dissolution of cell walls by enzymes). The starch after separation is used for fermentation process using the technology similar to other feedstock rich in starch.

Alcohol can be also produced from the cell residues after oil extraction [33]. Harun et al. investigated the possibility of using the cells of Chlorococcum species after oil extraction to ethanol production. In their experiment, the ethanol yield was about 3.8 g L\(^{-1}\) from 10 g L\(^{-1}\) of the substrate.
Some microalgae species are rich in starch and their cell walls contain cellulose. In case of such algae, the cellulase-producing microorganisms are used to convert this carbohydrate into simple sugar and then the all biomass can be fermented to ethanol. Depolymerization of cell walls (chemical and biological) increases the amount of monosugars and thus the yield of ethanol. Some microalgae contain other sugars (e.g., mannitol and laminarin). It must be remembered that not all anaerobic bacteria are able to ferment all kind of sugars (e.g., mannitol). Therefore, it is very important to know the algae cell composition and find appropriate microorganisms for fermentation process [30].

The stage that influences ethanol yield is a kind of a fermentation process. Harun et al. [34] investigated three of them: (1) separate hydrolysis and fermentation (SHF), (2) separate hydrolysis and co-fermentation (SHCF) and (3) simultaneous saccharification and fermentation (SSF) with different pretreatment of *Chlorococcum* sp. biomass (acid and enzymatic hydrolysis). They stated that acid SHF was the most effective method of ethanol production, and the fermentation process conducted in continuous way is more efficient than batch fermentation.

In lack of light and in presence of oxygen, microalgae convert starch or glycogen by oxidizing them to carbon dioxide. But in dark, under anaerobic conditions, the oxidation is incomplete and different products (such as hydrogen, ethanol, formic acid and acetic acid) are produced. The proportion of particular compounds depends inter alia on the species of microalgae. Microalgae whose cells contain polysaccharides composed of glucose (e.g., *Chlamydomonas*, *Chlorella*, *Microcystis*, *Oscillatoria*, and *Spirulina*) are able to produce ethanol in the dark under oxygen-free conditions easily. The yield of alcohol production can be enhanced by appropriate pH and temperature range [35, 36]. Ueno et al. [36] obtained the maximum productivity of ethanol (450 µmol g⁻¹ dry weight) from *Chlorococcum littorale* at 30°C. Beside the ethanol, the fermentation products were acetate, hydrogen and carbon dioxide. It was stated that ethanol productivity can be improved by adding methyl viologen, additionally resulting in decreased production of hydrogen (more electrons were involved in ethanol formation in the presence of methyl viologen, which is used as an electron acceptor and transfer catalyst in redox reactions). Hirano et al. [35] stated that intracellular ethanol production is simpler and less energy intensive in comparison with the conventional ethanol-fermentation process.

The processes of ethanol production by biomass pretreatment, extraction, fermentation and so on involve costs. It would be interesting if the microalgae can produce alcohol directly. Currently, intensive researches are conducted with the aim of increasing the accumulation of compounds (lipids, starch, alcohol, etc.) in photosynthetic organisms using genetic engineering. The glucose and other metabolites of algae are produced in Calvin cycle of photosynthesis. Using genetic engineering, the ethanol-producing genes from the ethanologenic bacterium *Zymomonas mobilis* were introduced to cyanobacterium *Synechococcus* sp. New species were able to produce ethanol in presence of light. The ethanol produced by the transformed cyanobacterium diffused from the cells into the culture medium [37]. This way of ethanol production is still in the research; there are more questions than answers.
2.1.4. Hydrogen

Microalgae have capacity for producing hydrogen by photobiological reaction. The hydrogen is produced by direct or indirect photolysis of water [38]:

Direct photolysis: \(2 \text{H}_2\text{O} \rightarrow \text{light} \rightarrow 2 \text{H}_2 + \text{O}_2\)

Indirect photolysis:

1. \(12 \text{H}_2\text{O} + 6 \text{CO}_2 \rightarrow \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{O}_2\)
2. \(\text{C}_6\text{H}_{12}\text{O}_6 + 12 \text{H}_2\text{O} \rightarrow 12 \text{H}_2 + 6 \text{CO}_2\).

During photosynthesis, water molecules are converted by microalgae into hydrogen ions (H\(^+\)) and oxygen. Then the hydrogen ions are converted into hydrogen with the use of hydrogenase enzymes. The presence of oxygen results in rapid inhibition of the hydrogenase enzymes and the hydrogen production process is impeded. Therefore, cultivation of microalgae for H\(_2\) production must be realized under anaerobic condition [29].

Photosynthetic production of hydrogen can be carried out with the use of two-stage method. In this process the photosynthetic generation of O\(_2\) and production of H\(_2\) are separated. In the first stage, the algae grow photosynthetically under normal conditions. During the second stage, the access to the sulphur is limited and microalgae are exposed to anaerobic conditions. Under S deprivation conditions, microalgae are fundamentally altering photosynthesis and cellular metabolism to survive. They consume internal starch and protein and produce hydrogen. This production process is limited with time, the yield of hydrogen decreases after 60 hours of production. In this method, the theoretical maximum yield of H\(_2\) production by green algae is 20g H\(_2\) m\(^{-2}\) d\(^{-1}\) [39]. The use of this method for hydrogen production does not generate any undesirable, toxic or environmentally harmful by-products.

Another method for hydrogen production is a continuous mode. In this mode, electrons and protons that are released during photosynthetic H\(_2\)O oxidation are directly recombined by the hydrogenase to produce hydrogen. Theoretically, such a process is for 33% more efficient than two-phase process because in the two-phase process, electrons and protons released from water are storage (e.g., as starch) before being use to H\(_2\) generation [40].

Technical and physiological parameters of microalgae cultivation influence hydrogen production efficiency. As described by Kruse and Hankamer [40], the important parameters are light intensity, chlorophyll concentration, culture mixing, pH and the interplay between these parameters.

2.1.5. Thermochemical conversion

Gasification, pyrolysis and liquefaction are basic processes of thermochemical conversion of microalgae biomass. Before these processes the valuable substances contained in the cells of algae are very often extracted. The principles of thermochemical conversion methods of microalgae are similar to these for other types of biomass.
Gasification is a process of partial oxidation of biomass with air, oxygen and/or steam at high temperatures, usually in the range 800-1000°C. The biomass is converted into gaseous product (syngas), which is the mixture of hydrogen, carbon oxide, carbon dioxide, methane and nitrogen. Nitrogen content can be reduced after mineralization to ammonia. Syngas has low calorific value (usually 4-6 MJ m⁻³) and can be used as a fuel for gas turbines or engines [41]. The syngas is a crucial intermediate resource for production of other compounds such as methanol, synthetic hydrocarbons, ethanol and others via the Fischer–Tropsch (FT) process. It can also be microbiologically fermented where the main products are ethanol, butanol, butyric acid, acetic acid and methane. Due to substantial water content in microalgae biomass, before gasification, the feedstock can be partially dried. There are only a few works reported in literature concerning the gasification of microalgae. Hirano et al. [42] partially oxidized *Spirulina* at temperatures of 850°C, 950°C and 1000°C and determined the composition of obtained gas in order to evaluate the theoretical yield of methanol. They stated that the gas composition depends on the process temperature. The highest theoretical yield of 0.64 g methanol from 1 g of the biomass was obtained by them for the gasification conducted at 1000°C. Additionally, they estimated energy balance (ratio of the energy of methanol produced to the total required energy), which was slightly disadvantageous. The greater part of energy is used for algae cultivation, thus the balance can be significantly improved by more efficient production of microalgal biomass. López-González et al. [43] investigated gasification process of chars obtained from the pyrolysis process of three microalgae *Scenedesmus almeriensis*, *Nannochloropsis gaditana* and *Chlorella vulgaris* with the use of thermogravimetric–mass spectrometric analysis. They stated that the indigenous mineral matter present in microalgae samples catalytically influences the gasification process. The metals in microalgae samples influenced the samples’ reactivity as well as the production of gases. The highest gas yields were obtained for *Scenedesmus* sample, which was characterized by high potassium content.

The other form of gasification of microalgae is catalytic supercritical water gasification SCWG – a kind of steam reforming. In this process, the thermal conversion of algae could be conducted for biomass with high moisture content (50-90%). SCWG is realized in lower temperatures (250-360°C), pressure about 20 MPa and in the presence of catalyst. The system is operated as a liquid-phase and the main product is a mixture of methane (50-60%) and carbon dioxide [44]. The problem in this method is presence of biomass trace components, which can react with the catalyst and significantly reduce its activity.

Catalytic supercritical water gasification conversion is characterized by a high chemical energy conversion efficiencies (up to 70-77%) and short time of reaction (order of minutes) so high rates of biomass conversion are possible on a much smaller area in comparison with anaerobic digestion. The SCWG process enables recovery of some nutrients from the microalgae biomass because the fluid shows low solubility for salts [45]. Cherad et al [46] gasified macroalgae *Saccharina latissima* in a batch reactor at 500°C and 36 MPa and studied the influence of biochemical content and ash on syngas composition. Such a process can also be used for microalgae. They stated that the presence of Ru/Al₂O₃ catalyst caused an increase in the yields of hydrogen (30%) and C1-C4 gases, and the gasification efficiency in comparison with a
process without this catalyst. Their results also indicated that the process water recovered from gasification of microalgae can be used as nutrients during microalgae cultivation.

Another thermochemical process of microalgae biomass is its liquefaction (HTL process), which is similar to SCWG. It is direct hydrothermal liquefaction in sub-critical water conditions and can be employed to convert wet algal biomass into liquid fuel. The process is realized at low temperature (300–350°C), high pressure (5–20 MPa) and in the presence of catalyst and hydrogen. Sub-critical conditions enable decomposition of biomass materials to shorter and smaller molecular materials (bio-oil) with a higher energy density [29]. Hydrothermal liquefaction is considered for being the most promising technique for conversion of wet algal biomass. It has been shown that the yield of bio-oil from HTL of whole biomass exceeds the lipid content in the raw material [47]. Disadvantages of hydrothermal liquefaction are the complexity and very high cost of the apparatus. Another way to produce bio-oil is pyrolysis - heating the biomass in the absence of air at 500°C, without or in the presence of a catalyst for very short time. The main products are bio-oil, charcoal and gas. The ratio of particular products depends on hot vapour residence time and temperature. The shorter the time, the higher the yield of the liquid product. The high biomass-to-liquid conversion (yield of bio-oil about 75%) can be achieved during flash pyrolysis (hot vapour residence time about 1 second, temperature 500°C) [29]. Since algae contain a lot of moisture content, a biomass must be initially dried [7], which significantly increases the cost of bio-oil production. The HTL process enables to avoid this cost.

The composition of bio-oil depends on a kind of algae biomass, method of its conversion and parameters of process. Algae-derived bio-oil contains large amounts of heteroatoms, such as N, S and O. It contains long chain fatty acids, resulting in high viscosity, so such products cannot be used directly as a fuel [48]. But it can be converted together with crude oil or independently to other valuable products. The microalgae bio-oil could contain some metals such as Fe, Mg, Ni and Zn, which are present in original algae cells. These metals can cause some difficulties for the upgrading process. Galafassi et al. [49] stated that it is possible to remove oxygen and metals from crude algae oils produced by HTL only by thermal means without the use of catalysts or hydrogen. Thermal treatment can reduce amount of acids in the bio-oil, decrease its viscosity and make it more volatile. The higher temperature of thermal treatment reduces the amount of trace metals present in bio-oil more effectively.

2.2. Others oleaginous microorganisms

Microbial oil (called also single-cell oil SCO) can be obtained not only from microalgae. Some microorganisms such as yeast and fungi (especially moulds) or bacteria can also be a source of it. However, the use of these organisms as a source of lipids for biofuels production still remains in the sphere of research. Oleaginous yeasts and moulds produce polyunsaturated fatty acid triacylglycerol, which is similar to vegetable oils. Produced triglycerides are rich in polyunsaturated fatty acid such as oleic (C18:1), linoleic (C18:2), palmitic (C16:0) and palmitoleic (C16:1). Because the fatty acid profile of microbial oils is similar to that of plant oils, oleaginous yeast and fungi can be a favourable feedstock for the biodiesel industry, but currently, the production of microbial oil from these organisms is expensive. Similar to
microalgae, yeasts accumulate reserve lipids as storage metabolites, especially they suffer a deficiency of nutrients, usually involving nitrogen, but with access to the carbon-containing components [49]. Thus, the lipid production depends on the carbon-to-nitrogen ratio (C/N). Nitrogen is used for production of nucleic acids and proteins, whereas carbon is necessary for energetic processes and synthesis of proteins, carbohydrates, nucleic acids and lipids. During nitrogen shortage, the growth of microorganisms slows down and production of nucleic acids, proteins and carbon is consumed for storage lipid synthesis [50].

In contrast to algae, yeast and fungi are not able to produce carbon compounds from CO$_2$, because they cannot carry out photosynthesis. They must obtain a carbon source (e.g., fat) from a medium in which they live. Oleaginous yeast or moulds growing in media, which contain fats as a carbon source, accumulate lipids in cells during primary anabolic growth. This accumulation process is not limited by presence of some nutrients in the cultivation environment. Although storage lipids are produced from glucose or other similar components, during second anabolic activity, the lack of some nutrients in the growth medium is a condition of accumulation process [51, 52]. In case of oleaginous microorganisms growing on fats, when the deficiency of fatty acids in growing medium have place, the organisms start to consume their own storage lipids for theirs metabolic requirements and growth.

The lipid content in oleaginous yeast and fungi depends inter alia on their species (Table 4) and growth conditions such as a type of carbon source, pH and temperature. Due to various accumulation of lipids, there are not many species of fungi and yeast, which can be applied as a feedstock for biodiesel production. For example, among over 600 species of yeasts, less than 30 are able to accumulate more than 25% of their biomass weight as oil [53]. Therefore, before using any yeast or fungi for biofuel production, the appropriate selection and characterization of oleaginous strains should be performed.

Fungi and yeast have several advantages over conventional plant and microalgae. Their cultivation is easy; they can be grown in bioreactors. Yeast and fungi have short life cycles, characterized by the rapid growth rates, which are unaffected by space, light or climatic variations. Their cultivation can be easily scaled up. They have been found to be robust microorganism that can grow on various substrates such as lignocellulosic biomass and agro-industrial residues, for example, glycerol, fats, whey and molasses [50, 51, 54]. Some species can also grow in sewage sludge, waste water or in salt water.

Extraction of lipids from fungi and yeast can be carried out with the methods that are used during oil extraction from microalgae, for example, cold extraction with solvents. Obtained oil can be used as a feedstock for biodiesel production by its transesterification. The amount of obtained oil can be increased by biomass pretreatment, but there is a lack of information about it. Tsigie et al. [57] treated Yarrowia lipolytica Po 1g yeast with subcritical water (SCW) method. It is an environment-friendly technique for increasing the amount of extractable lipids in microorganisms. Treatment is performed in water (in the liquid state) at temperatures ranging between 100 and 374°C under high pressure. This method enabled to increase the amount of extractable lipid from 51.53% to 84.75%. Results suggest that solvent extraction alone is not an effective method for the complete recovery of lipids from Yarrowia lipolytica Po 1g.
Table 4. Oil content in the oleaginous microorganisms cells [53, 55, 56].

<table>
<thead>
<tr>
<th>Species</th>
<th>Oil content (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yeast</strong></td>
<td></td>
</tr>
<tr>
<td>Candida curvata</td>
<td>58</td>
</tr>
<tr>
<td>Cryptococcus albidus</td>
<td>65</td>
</tr>
<tr>
<td>Lipomyces starkeyi</td>
<td>64</td>
</tr>
<tr>
<td>Rhodotorula glutinis</td>
<td>72</td>
</tr>
<tr>
<td>Rhizopus arrhizus</td>
<td>57</td>
</tr>
<tr>
<td>Trichosporon pullulans</td>
<td>65</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>36</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>57</td>
</tr>
<tr>
<td>Humicola lanuginosa</td>
<td>75</td>
</tr>
<tr>
<td>Mortierella isabellina</td>
<td>86</td>
</tr>
<tr>
<td>Mortierella alpina</td>
<td>40</td>
</tr>
<tr>
<td>Mortierella vinacea</td>
<td>66</td>
</tr>
<tr>
<td>Rhizopus stolonifer LGAM (9)1</td>
<td>28</td>
</tr>
</tbody>
</table>

Dai et al. [58] showed that biodiesel obtained by transesterification of oil from *Rhodotorula glutinis* yeast possessed similar composition to that from vegetable oil. Other promising species for the microbial oil production is *Yarrowia lipolytica*, which is used in the industry for the production of citric acid and protein [52]. However, in lipids production from oleaginous microorganisms is a lot of unknown, for example, connected with metabolism of these organisms (e.g., functional and structural properties of enzymes). Not enough is yet known about the effects of nutrients in culture medium on the quality of oil produced in cells. Some substrates, for example, molasses, raw glycerol lead to accumulation higher quantity of saturated fatty acids in comparison with rapeseed oil [59]. On the one hand, this may result in the improvement of the cetane number and oxidation stability; on the other hand, it adversely affects the low-temperature properties and viscosity. High cost of cultivation, especially substrate cost, causes that commercialization of fuel production from fungi and yeast will not quickly occur. Genetic engineering, by improvement in lipids accumulation in oleaginous microorganisms or by generation strains which are able to produce specific fatty acid compositions, can improve economics of microbial oil production.

Similar to fungi, some species of bacteria can also accumulate lipids in the form of triacylglycerol under some special environment, but this oil is different from other microbial oil. Generally, the oil produced by most bacteria is complex; only a few species produce oil that can be a raw material for fuel production, and some examples are presented in Table 5.
As stated by Gouda et al. [60], *Gordonia* sp. and *R. opacus* PD630 are able to accumulate oils under special conditions with maximum oil content over 70%, but the biomass is only 1.88 g L\(^{-1}\). The advantage of bacteria in comparison with microalgae is their higher growth rate and easier culture method [53].

The wealth of knowledge on the genetics and metabolic pathway of bacteria makes them ideal candidates to research with the use of metabolic and genetic engineering, because scientists know the genes that are responsible for the synthesis of fatty acid in bacteria [61]. So it is easier to “produce” new bacteria using DNA recombination. For instance, the well-known *Escherichia coli* was converted into oleaginous organisms by engineering their metabolism [62]. Such genetically modified *E. coli* could produce biodiesel (fatty acid esters) directly. Kalscheuer et al. [63] obtained fatty acid ethyl esters (FAEE) with concentrations of 1.28 g L\(^{-1}\) and FAEE content in cells was 26% of the cellular dry mass during fed-batch fermentation using renewable carbon sources. Although the yield was low, it opened a new perspective for biofuel production.

We can say that microbial oil might become one of the potential feedstock for biofuels production in our future. The advantages of it are renewability, fast growth rate of microorganisms and the fact that cultivation of these organisms does not take arable land. Use of genetic engineering and metabolic engineering can improve oil production by oleaginous microorganisms.

### 3. Fermentation as a process of biofuels production

Fermentation is a natural metabolic process, in which microorganisms (bacteria or yeast) obtain energy through conversion of organic compounds such as sugar and starch lipids, into simpler liquid or gaseous substances. The kind of final product depends on the metabolic pathway occurring within a cell. Some of these processes have been applied in a large industrial scale for the production of food or other valuable products. Some of these products can be used as biofuels, for example, ethanol, butanol, biomethane or hydrogen.
3.1. Alcohols

The conversion of carbohydrates (sugars and starch) with a general formula of \( C_x(H_2O)_y \) from different crops, for example, potatoes, corn and cereals, into bioethanol is a common process used commercially on a large scale. Bioethanol is an oxygen compound, which is the most studied as a fuel biocomponent. This alcohol is commonly added to gasoline as a component; it can also be used as E95 ethanol fuel to supply diesel engines [64] or as a hydrogen carrier for fuel cells. Researches were also conducted to use bioethanol as a biocomponent to diesel oil [65, 66], but disadvantages of that alcohol are limited miscibility with diesel oil and high affinity to water, which influence poor physical stability of fuel.

Ethanolic fermentation is generally performed with the use of yeast such as *Saccharomyces cerevisiae*. This process consists of several stages. During first stage sucrose is hydrolysed to glucose and fructose by yeast invertase enzyme.

\[
C_{12}H_{22}O_{11} + \text{invertase} \rightarrow 2 C_6H_{12}O_6
\]

Next, glucose molecule is broken down into two pyruvates \((CH_3COCOO^-)\), which are then broken down into two acetaldehydes and \(CO_2\). In the last stage, acetaldehyde molecules are converted into two ethanol molecules. The yeast enzyme that converts simple sugars to ethanol is called zymase, and these steps can be summarized using the following formula:

\[
C_6H_{12}O_6 + \text{zymase} \rightarrow 2 C_2H_5OH + 2 CO_2.
\]

The fermentation process is then followed by distillation and dehydration to anhydrous bioethanol. The technologies of bioethanol production from corn, potatoes or cereals are known very well.

Besides plants containing sugars or starch, lignocellulosic biomass can also be used for bioethanol production. Generally, the yield of ethanol in fermentation process depends on the ease with which the substrates can be decomposed to sugars. Starch is a biopolymer that is built from repeating glucose units and similarly to disaccharides and other oligosaccharides, is readily hydrolysed. The structure of lignocelluloses is more complex, and it is not easy to break it down into fermentable sugars. Before enzymatic hydrolysis, lignocellulosic biomass needs to be pretreated to decompose a complex matrix of cellulose, lignin and hemicelluloses [67]. This stage is complicated and needs a lot of energy. Its efficiency highly depends on the type of used pretreatment method and then influences enzymatic hydrolysis and subsequent stages [68]. The different pretreatment processes, the key factors that influence the efficiency and costs as well as advantages and disadvantages of methods of lignocellulosic biomass degradation were described in detail in literature, for example [69-71].

Ethanolic fermentation process, similarly as others biotechnological processes, can be conducted in batch, semi-continuous and continuous bioreactors. Continuous processes are more technologically advanced and have many advantages compared with the batch processes, for
example, lower operation requirements, lower costs of bioreactors, better control of process and higher productivities caused by higher concentration of yeast cells. This high cell concentra-
tion can be reached by better controlling of process parameters and through some technolo-
gical solutions such as immobilization techniques, recycling and recovery of microorganisms. One of the main disadvantages of the continuous processes is decrease in yeast ethanol productivity caused by long-term cultivation of cells under anaerobic conditions, and a part of substrate cannot be converted [67]. Alfenore et al. [72] indicated that aeration is an important parameter during fermentation. The presence of air increases the cell mass and ethanol productivity and causes reduction in glycerol production (the main by-product). It was stated that microaeration limited inhibiting effect of ethanol on cell growth. However, the presence of air leads to decrease in ethanol concentration.

Butanol is a four-carbon alcohol with a formula of $C_4H_9OH$. It is another compound produced by microorganisms that can be used as a fuel or fuel component. Due to the length of butanol’s chain, it is easier to blend with higher hydrocarbons, including gasoline. It has also other advantages in comparison with ethanol such as being much less corrosive, less evaporative and having lower water solubility [73]. The results of the research conducted by Yang et al. [74] showed that when the butanol concentration in gasoline is below 20% v/v, the engine power level maintains without any engine modifications. Higher concentrations required optimization of some operational parameters of engine, for example, advancing the spark timing.

Butanol has four isomers: $n$-butanol (butan-1-ol), sec-butanol (butan-2-ol), isobutanol (2-methylpropan-1-ol) and tert-butanol (2-methylpropan-2-ol). Not all the isomers are produced by microorganisms. The tert-butanol is received in refinery, whereas others can be obtained in biological processes. Contrary to ethanol in the conversion of biomass to butanol not yeast are involved, but suitable strains of bacteria.

In biological processes, butanol can be produced from the same biomass as ethanol. As a substrate, it can be used plants containing carbohydrates, for example, sugar cane, sugar beets, corn and wheat. More interest is focused on feedstocks that do not compete for food such as Miscanthus, Switchgrass, wood and crop waste, algae biomass, and food processing waste.

The first process, which was enabled to obtain $n$-butanol, was acetone-butanol-ethanol (ABE) fermentation. The products of ABE fermentation are solvents such as acetone, butanol and ethanol present in the ratio of 3:6:1. In this process, strains of anaerobic bacteria from the class Clostridium are involved. The best-studied and widely used species is Clostridium acetobutylicum; other species such as C. beijerinckii, C. aurantibutyricum or C. tetanomorphum also give good butanol productivity. The particular strains are different in their ratio of end products or in a kind of end products (e.g., the isopropanol is produced in place of acetone) [75]. The kind of strains used for fermentation is dependent inter alia on a used feedstock, the required end products and their ratio, and resistance to bacteriophages. It should be remembered that $n$-butanol is very toxic for microorganisms. Concentration of this alcohol cannot exceed 12 grams per litre of a fermenting broth; higher quantity $n$-butanol will inhibit the production of this alcohol by bacterial cells, but some improvements have been made to enable the production of $n$-butanol with final content of 20 grams per litre of broth [76]. Processes of $n$-butanol
separation from the broth and its purification are complex, difficult and expensive. Additionally, this process is characterized by a low conversion of glucose to butanol.

The ABE fermentation process is more complex than the production of ethanol. There are two main phases of fermentation. During the first one (acidogenesis phase), there are produced metabolites such as acetone, butyrate, hydrogen and carbon dioxide, which decreases the pH of the culture medium. During next phase (solventogenesis), the change in bacteria metabolism takes place; butanol, acetone, ethanol, H₂ and CO₂ are produced and pH of environment increases. When the glucose lacks in the culture medium (less than 10 grams per litre), the *Clostridium* produces only acids.

Besides glycolytic reactions in metabolic pathway for the production of acids and solvents, the reactions between pyruvate and butyryl-CoA take place. During the acidogenesis phase, the acetone is produced from acetyl-CoA and the butyrate from the butyryl-CoA. During the solventogenesis phase, both acetyl-CoA and butyryl-CoA are intermediates for the production of ethanol and butanol. Acetyl-CoA is the key intermediate for the synthesis of acetone. Some strains of *Clostridia*, for example, *Cl. beijerinckii* and *Cl. Aurantibutyricum*, reduce acetone to isopropanol in later stages [77]. Simplified pathway of ABE fermentation by *Clostridia* is presented on Figure 6.

![Figure 6. Simplified ABE fermentation diagram pathway (adopted from [78]).](image-url)
sec-Butanol is not obtained directly by fermentation. In the first step, some strains of bacteria convert sugars (from starch, cellulose or hemicelluloses) to an intermediate product by fermentation. In the next step, this product (directly in the fermented broth) is chemically converted to sec-butanol. The efficiency of conversion by fermentation as well as chemical conversion of intermediate product to sec-butanol is very high - over 90%. One tonne of this alcohol can be obtained from three tonnes of carbohydrates; it is much more than in case of other butanol isomers [76].

Obtaining higher yields, higher productivity of butanol or others isomers of this alcohol, better butanol tolerance of bacteria involves genetic manipulation of the metabolic pathway within bacteria or DNA code. Such actions make the butanol production more attractive to the industry. Use of genetically modified microorganisms of *Ralstonia eutropha* H16 enabled the production of isobutanol from CO$_2$. Wild-type *Ralstonia eutropha*, in the presence of carbon and under nutrient deficiency, produces polyhydroxybutylate (PHB) in cells; this compound is an intracellular material for carbon storage. In modified strains of these bacteria, the excess carbon was redirected from PHB storage for the production of isobutanol and 3-methyl-1-butanol [79]. Another example of modified bacteria is *Clostridium pasteurianum* MBEL_GLY2. This microorganism was engineered to use glycerol as a major carbon source for butanol production. This hyper–butanol-producing strain was able to produce 10.8 g L$^{-1}$ of butanol from 80 g L$^{-1}$ of glycerol, whereas the native bacteria was able to produce only 7.6 g L$^{-1}$ of butanol [80].

Progress in the efficiency of the butanol production (substrates, yield and productivity) and in the butanol separation from the broth decreases production costs and causes the production of butanol as a fuel to be more profitable.

### 3.2. Biogas

Methane fermentation is a well-recognized process, which is widely used for biogas production. It is one of the methods of organic waste management (e.g., livestock manure, sludge from sewage treatment plants or organic fraction of municipal waste). As a feedstock for this process, we can also use biomass from landfill or energy crops.

Biogas is produced by anaerobic microorganisms from organic substances during digestion. Methane and carbon dioxide are the reaction products, as well as small amounts of nitrogen, hydrogen and hydrogen sulphide. The process of methane fermentation is divided into several stages, conducted by several groups of interdependent microorganisms. Products of particular stages become a food for the next group of bacteria. Methane is a bacterial metabolic waste of the last fermentation step.

There are four main phases in the production of biogas: hydrolysis, acidogenesis, acetogenesis and methanogenesis - Figure 7. In the various phases, different groups of microorganisms that remain in syntrophic relationships are involved and have different environmental requirements [81].

At hydrolysis stage, the complex organic matter (e.g., carbohydrates, fats and proteins) is decomposed to simpler compounds (e.g., amino acids, sugars and fatty acids). In this process, extracellular enzymes (hydrolases) of bacteria break down the organic material by biochemical
reactions. Facultative anaerobic bacteria involved in this stage consume oxygen dissolved in water, which is usually supplied with substrate. In this way the anaerobic environment is ensured for further changes during methane fermentation. The duration of hydrolysis depends on the type of a raw material. Hydrolysis of carbohydrates takes several hours, whereas of proteins and fats takes several days. Lignocellulose and lignin are decomposed very slowly and their hydrolysis is incomplete.

During acidogenesis, the acidogenic bacteria convert the soluble products from the first stage to the lower volatile fatty acid (e.g., acetic, propionic, butyric and valeric acids), ketones, alcohols (methanol and ethanol) and gases (hydrogen and carbon dioxide). In contrast to the hydrolysis, the reactions of this phase take place within the bacterial cells. The bacteria involved in hydrolysis and acidogenesis are almost the same. They could be facultative anaerobic (e.g., Enterobacterium and Streptococcus) or obligatorily anaerobic bacteria (e.g., Bacillus, Clostridium, and Bifidobactrium).

The products of second stage are transformed by acetogenic bacteria into acetic acid, hydrogen and carbon dioxide (methanogenic substrates) at acetogenesis. Acetogenic bacteria are very sensitive to environmental changes and require long periods to adapt to new conditions. They are mandatory producers of H₂, but the growth of these bacteria is strongly inhibited by even small increase of hydrogen pressure. Acetogenic bacteria can only survive in symbiosis with genera that consume hydrogen, for example, hydrogenotrophic methanogens [81].

During the last stage (methanogenesis), methanogens, under strictly anaerobic conditions, convert the carbon dioxide, hydrogen and acetic acid to methane. Almost two-thirds of methane are produced from acetate or alcohols (acetoclastic methanogenesis) and one-third from reduction of carbon dioxide (hydrogenotrophic methanogenesis) [82].
The biogas composition depends on a type and a chemical composition of substrates, used technology and operational parameters. Methane fermentation process is extremely sensitive to changes in ambient conditions because of delicate balance between cooperating groups of involved microorganisms. There are a lot of factors that affect the biogas productivity, for example, temperature, pH, mixing, redox potential, nutrients (C/N/P ratio), inhibitors, trace elements, organic loading and hydraulic retention time [81, 83]. For example, too low C/N ratio leads to increased ammonia production and inhibition of methane production. On the other hand, too high C/N ratios cause lack of nitrogen and have a negative influence on bacterial metabolism [84]. Temperature of the process is also very important. The rate of the decomposition of organic substances increases when temperature increases till the optimal value is reached. The better the decomposition, the better the efficiency of biogas production. Increase in temperature over optimal value causes the protein denaturation and microorganisms’ death; so the rapid decrease in process rate takes place. There are three temperature ranges for methanogenic fermentation: psychrophilic, mesophilic and thermophilic [85].

Generally, the biogas production systems are technologies involving one or two technological steps. In one-step process, all phases of anaerobic digestion take place in one digester. In two- or multi-step process, the biochemical phases are physically separated and are conducted in different bioreactors. Very often, fermentation process is realized using a wet digestion technology. In this technology, the dry matter content is from 12% to 15% and the feedstock can be easily pumped and stirred. If the dry matter content exceeds 16%, the culture medium loses the ability to pump; so this process is called to be dry [86].

Similar to other biotechnological processes, different types of biodegradable substrates can be used for anaerobic digestion, including lignocellulosic. When the biomass contains a large amount of lignocellulose, the microorganisms are not able to decompose it. In this case, it is important to break these structures by biomass pretreatment. The destruction of the lignocellulose structure causes the biomass to become more available for microbes, which resulted in increase in biogas and methane yield. The methods of biomass pretreatment are similar to those described above for conversion of microalgae. The biomass for methane fermentation can be prepared mechanically, physically, chemically, biologically or using mixed methods [71, 87, 88].

The next step is a biogas purification and/or upgrading process. The purification/upgrading of biogas can be realized with different methods. The choice of method of a biogas treatment is dependent on the destination of the final product. To produce a hot water or steam in boilers, biogas needs to be filtrated to remove particles and purified from steam and sulphur compounds that affect corrosion (hydrogen sulphide). Similar purification should be conducted when we want to use biogas in cogeneration (CHP process), for the simultaneous generation of usable heat and electricity. In cogeneration, a power plant from 1 m³ of the biogas, 2.1 kWh of electricity and 2.9 kWh of heat could be generated [89]. Biogas can also be upgraded to the quality of natural gas and injected to gas grid or used as a vehicle fuel [90, 91]. In this case, in addition to removal of particulates, H₂S and water vapour, it is necessary to significantly reduce CO₂ content to meet the requirements of natural gas as Wobbe Index or calorific value. Biogas should be additionally cleaned from the trace components that are harmful to the
natural gas grid or vehicle engine. For instance, siloxanes could form SiO$_2$ under combustion conditions, which deposits at spark plugs, valves and cylinders causing the wear of surface. Ions such as Cl$^-$ and F$^-$ cause corrosion of engine elements. After cleaning operations, such gaseous product contains 95–97% CH$_4$ and 1–3% CO$_2$ [91].

Purification of biogas can be realized by different methods that were described in many publications [81, 90, 92]. Separation of H$_2$S can be realized with the use of:

• biological methods – commonly with the use of microorganisms such as *Thiobacillus* and *Sulfolobus*, which reduce the hydrogen sulphide to the elemental sulphur and sulphates;
• physical methods as (a) precipitation of sulphides using iron(II) or (III) chloride iron (II) sulphate, (b) absorption in iron chelate solution, (c) desulphurization with bog iron ore, and (d) adsorption on an activated charcoal.

CO$_2$ from biogas can be removed by physical methods such as high-pressure water wash, pressure swing adsorption, chemical adsorption (e.g., in amines), precipitation of CO$_2$, for example, by CaO, membrane separation and cryogenic separation.

Upgraded biogas can be used as a fuel in vehicles in the similar forms as a natural gas: compressed (CNG), liquid (LNG) and adsorbed (ANG).

As mentioned earlier, one of the products produced during first stages of anaerobic digestion is hydrogen. If this part of fermentation (the so-called dark fermentation) is conducted in separated bioreactor, we can get hydrogen, in addition to methane. The amount of obtained hydrogen highly depends on hydraulic retention time, the pH value and gas partial pressure [93]. The higher yield of H$_2$ is obtained when the feedstock is rich in carbohydrates. The main products of the dark fermentation are H$_2$ and CO$_2$, combined with other gases, such as CH$_4$ or H$_2$S, depending on the reaction process and the used substrate. From the model substrate, which is glucose, maximum 4 mole of H$_2$ are produced from 1 mole of glucose when additional final product is acetic acid:

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

When the end product is butyrate, the yield of hydrogen is two times smaller:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

Generally, the end product is a mixture of both acetate and butyrate, therefore the yield of 4 moles of H$_2$ per 1 mole of glucose cannot be achieved [94]. This fermentative process of hydrogen production has relatively low energy conversion efficiency, so the improvement of efficiency of H$_2$ generation is the main challenge for researchers.

The separation of methane fermentation process into two stages has also an additional advantage. Biogas from the second step of fermentation, collected in a separate container, is richer in CH$_4$ because it is not diluted with CO$_2$, which is produced mainly in the early stages.
of fermentation. This indicates that with suitable plan of multi-step process and by appropriate selection of the parameters, we can obtain two valuable gaseous fuels.

3.3. Gas fermentation

Direct fermentation of biomass to produce biofuels or other chemicals is not always possible and profitable. When the biomass contains a large amount of lignocelluloses, microbial enzymes or biomass pretreatment methods are not able to break effectively this structure into simple compounds. Biomass can also be converted into biofuels with the use of microorganisms, but in an indirect way. In first step biomass is gasified to produce gaseous mixture containing CO, CO₂, H₂, CH₄ and N₂ (the so-called producer gas). After cooling and purification from tars (e.g., by stripping with solvents or by catalytic gasification of tars), obtained gas can be upgraded to syngas. Next, the syngas can be subjected to the thermochemical FT process, where it is converted to liquid fuels in the catalyst presence. Another possibility is the fermentation of purified producer gas or syngas by microorganisms leading to valuable products such as ethanol [95]. An advantage of the fermentation process in relation to the FT process is its lower cost. The costs connected with FT thermochemical route are generally generated by metal catalysts, which are expensive because of their limitations in the robustness, flexibility and selectivity [96]. Biocatalysts, which are cells of microorganisms, are cheaper than metal catalysts. They are highly specific what improves product yield and simplifies product recovery. Furthermore, biocatalysts are more resistant to sulphur contamination (presence of H₂S, carbonyl sulphide, mercaptans in producer gas) than inorganic catalyst, and the H₂:CO ratio has no influence on it [97]. This hybrid thermo/biochemical process of biomass conversion has an additional advantage – organic feedstock, which is toxic for conventional fermentative microbes, can be fermented after gasification (difference in chemical composition of feedstocks is unimportant for biomass gasification). However, some disadvantages of gas fermentation, causing that this process is not commercialized, are its low productivity and limited mass transfer between gas and liquid phases [98]. The mass transfer can be improved by reducing the mass transfer resistance at the gas–liquid interface or by reducing the surface tension, increasing the gas solubility in the liquid. This can be achieved, for example, by increasing the speed of agitation, by increasing the gas flow rate, using impeller in stirred tank reactor or by special reactor configurations (e.g., bubble column reactor, air-lift reactor, trickle-bed reactor or immobilized cell reactor) [99]. To significantly improve the mass transfer rate, the fermentation process can be carried out using reducing surface tension chemicals such as bio-polymers (xanthan gum and dextran), bio-surfactants (biological detergents) and organic compounds (high carbon alcohols and perfluorocarbon compounds) [100]. Addition of 0.1 volume percent TYLOXAPOL™ detergent causes the CO mass transfer rate to increase to over 300%.

Some microorganisms need CO and H₂ (which are present in producer gas) for metabolism; these compounds are sources of carbon and energy for bacteria. Final products are different and depend inter alia on the strain of bacteria, which can be acetate, butyrate, formate, butanol, ethanol and hydrogen [101]. Similar to methanogenic bacteria, bacteria for gas fermentation have different necessities for an optimal growth. Some species live in moderate temperatures
30-40°C (mesophilic organisms) or in higher temperatures 55-60°C (thermophilic organisms). However, it was recognized that the optimal growth of some bacteria occurs at temperatures of 70-80°C. The high temperature of fermentation process is not a problem because syngas is obtained by gasification at a very high temperature (700-800°C), and it must be cooled before introducing into the bioreactor. However, the processes of gas fermentation with the use of thermophilic bacteria are still at the research stage. Gas-fermentative organisms can differ in optimal pH value (from 5.8 to 7.5) and time when they are doubling their biomass (from 1 hour to 140 hours). Similar to other fermentations, the efficiency of gas fermentation process depends on nutrient concentration, pressure and agitation speed. The content of trace metals in the reaction medium is also very important. Some of them can enhance the cell growth and ethanol production (e.g., Zn\(^{2+}\) and Fe\(^{2+}\)), whereas some elements may have a negative impact on the process (e.g., Cu and Mo) [99]. Examples of microorganisms and final products of fermentation process are presented in Table 6.

<table>
<thead>
<tr>
<th>Microorganisms species</th>
<th>Optimal growth conditions</th>
<th>Final products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium autoethanogenenum</td>
<td>T=37°C, pH=5.8-6.0</td>
<td>Acetate, ethanol</td>
</tr>
<tr>
<td>Clostridium carboxidivorans</td>
<td>T=38°C, pH=6.2</td>
<td>Acetate, ethanol, butyrate, butanol</td>
</tr>
<tr>
<td>Acetobacterium woodii</td>
<td>T=30°C, pH=6.8</td>
<td>Acetate</td>
</tr>
<tr>
<td>Butyribacterium methanobacterium</td>
<td>T=37°C, pH=6</td>
<td>Acetate, ethanol, butyrate, butanol</td>
</tr>
<tr>
<td>Rubrivivax gelatinosus</td>
<td>T=34°C, pH=6.7-6.9</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Moorella thermoacetica</td>
<td>T=55°C, pH=6.5-6.8</td>
<td>Acetate</td>
</tr>
<tr>
<td>Carboxydiethrombium pacificus</td>
<td>T=70°C, pH=6.8-7.1</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Thermincola carboxydiphila</td>
<td>T=55°C, pH=8</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Archaea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>T=37°C, pH=7.4</td>
<td>Methane</td>
</tr>
<tr>
<td>Methanothermobacter thermooautotrophicus</td>
<td>T=65°C, pH=7.4</td>
<td>Methane</td>
</tr>
<tr>
<td>Thermococcus strain AM4</td>
<td>T=82°C, pH=6.8</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Archaeoglobus fulgidas</td>
<td>T=63°C, pH=6.4</td>
<td>Acetate, formate, hydrogen sulphide</td>
</tr>
</tbody>
</table>

Table 6. Anaerobic microorganisms and final products of the fermentation process [101].

Synthesis of acetate, butyrate, ethanol and butanol by bacteria from syngas is realized via the acetyl-CoA pathway. Acetyl-CoA is produced in two major steps. During the first step, H\(_2\) is oxidized to 2H\(^+\) or CO to CO\(_2\) (with H\(_2\)O). Next, the CO\(_2\) and 2H\(^+\) are reduced to formate (HCOOH), which is then converted into methyl group through a series of reactions. In the second step, the methyl, carbonyl and the CoA groups take part in the synthesis of acetyl-CoA with the use of enzymes. Further reduction of acetyl-CoA produces acetate and ethanol. The butyrate and butanol are produced by reduction of acetoacetyl-CoA that is formed from two
acetyl-CoA molecules [101]. The reactions taking place during CO and CO₂ fermentation via acetyl-CoA pathway can be summarized [98] as follows:

\[
6\text{CO} + 3\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_5\text{OH} + 4\text{CO}_2 \quad (1)
\]

\[
2\text{CO}_2 + 6\text{H}_2 \rightarrow \text{C}_2\text{H}_5\text{OH} + 3\text{H}_2\text{O} \quad (2)
\]

\[
4\text{CO} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 2\text{CO}_2 \quad (3)
\]

\[
2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \quad (4)
\]

The composition of syngas is a very important factor for the yield of fermentation process. For instance, during fermentation to ethanol from CO alone, from Eq. (1), one-third of the carbon from CO can be theoretically converted to alcohol. Practically, not all carbon can be converted to ethanol, because a part of CO is oxidized during metabolic pathway of bacteria to produce CO₂ and reduce equivalents. For the mixture of H₂ and CO₂ in the ratio 3:1 (Eq. 2), theoretically all carbon can be converted to C₂H₅OH, but it is not possible to obtain this ratio of mentioned gases during gasification of biomass. H₂ molecule supplies H⁺ ions and electrons required in reactions of hydrogenase enzyme, leading to ethanol synthesis. Thus, in this case, a part of H₂ is used for the production of reducing equivalents [102].

Gas obtained from biomass contains different components, for example, gaseous compounds containing sulphur and nitrogen, tar, ethylene, acetylene and ash. They can affect the efficiency of gas fermentation, inhibiting the microbial catalyst, what influences the product yield. For example, the contamination of syngas by NO in concentrations above 40 ppm causes inhibition of hydrogenase enzyme and the cells of bacteria stopped consuming H₂. It changes the final product composition because CO is used in place of hydrogen for electron production rather than in product formation [103]. Similarly, acetylene is a strong inhibitor of hydrogenase and also affects the hydrogen consumption by cell [102]. Additionally, research results obtained by Datar et al. [102] indicated that production of ethanol is associated with growth of bacteria during producer gas fermentation. Ethanol was primarily produced once the cells stop growing.

Content of sulphur and nitrogen oxides in the producer gas can be reduced by the removal of N- and S- compounds from biomass. Reduction of the concentration of some elements in biomass can be achieved by its pretreatment. Turn et al. [104] studied fuel characteristics of sugarcane, which were subjected to a single milling and an initial milling, followed by leaching and a secondary milling. They stated that milling caused reduction of ash. They explained it by removal of K, Cl, S and N in the treatment operations. The second pretreatment method (milling combined with leaching) was more effective in the removal of the mentioned elements than the first one.
Some products can have an inhibiting effect. Najafpour et al. [105] indicated that during the batch fermentation of synthetic mixed gas (55% CO, 10% CO$_2$, 20% H$_2$ and 15% Ar) by *Rhodospirillum rubrum* ATCC 25903, the increase in the acetate content to 3g/L caused significant decrease in the conversion of acetate and CO and led to the reduction of H$_2$ production.

The yield of gas fermentation products depends on both the kind of biomass and the strain of microorganisms. Liu et al. [106] stated that replacing yeast extract with corn steep liquor in batch fermentation with the use of *Alkalibaculum bacchi* strain CP15 leads to increase in ethanol production by 78%. They also reported that fermentation of yeast extract under continuous conditions enabled to achieve C$_2$H$_5$OH concentration of 6 g/L, but when the corn steep liquor was a substrate, the maximum produced concentrations of ethanol, n-propanol and n-butanol were 8 g/L, 6 g/L and 1 g/L, respectively. High cell mass concentration above 5 g/L decreases ethanol production. Some microorganisms are known to convert syngas to ethanol, but very few can produce higher alcohols alone. The solution could be fermentation by mixed culture of bacteria [107]. Using *Alkalibaculum bacchi* and *Clostridium propionicum* to syngas fermentation results in over 60% more alcohol yield than with A. bacchi CP15 alone. Moreover, the mixed culture converted 50% more carboxylic acids (propionic acid, butyric acid and hexanoic acid) into their corresponding alcohols than the CP15 monoculture. The alcohol concentration can be increased also by appropriate composition of liquid phase. Najafpour and Younesi [108] examined the syngas fermentation using batch culture of *Clostridium ljungdahlii*. It was stated that initial presence of hydrogen and carbon dioxide in the liquid phase enhanced the ethanol concentration twice.

Main disadvantages of gas fermentation such as sterile conditions, slow reaction rate and mass transfer limitations cause that there is a lack of commercial plants. Most installations exist in the laboratory or pilot scale. Genetic engineering can help in faster progress regarding the implementation of this process by improving the sensitivity of involved microorganisms to the high concentrations of end products as well as the efficiency of the syngas conversion to valuable chemicals.

### 4. Energy and fuel production with microbial BESs

Many microorganisms are naturally capable of transporting electrons in and out of the cell – electrogens. In nature, this phenomenon is used for, for example, mineral reduction, but it can be exploited for harvesting electricity from or to provide electricity to microbial communities [109]. All bioelectrochemical systems (BESs) consist of anode, where the oxidation reaction occurs, and cathode, where the reductive reaction takes place. At least one of these processes is catalysed by microorganisms (microbial electrocatalysis), and it brings on the terms microbial bioanode and biocathode [110]. In bioanodes, exoelectrogenic bacteria anaerobically oxidize organic or inorganic matter, discharge electrons and transport them through the electron transport chain to the electrode surface directly or via mediators. The direct electron transport occurs by a contact with the electrode surface through conductive proteins that are the integral elements of the microorganism cell membrane (e.g., cytochrome). The indirect
electron transfer is realized through substances with redox properties that act as electron carriers and transport them from cell membrane to the anode surface [111]. In biocathodes, electrotrophs collect electrons from the cathode (directly or using mediators) and reduce different compounds such as organics, carbon dioxide, sulphate or nitrate [109, 110]. There is a wide range of BESs that have been developed for different processes such as power generation (microbial fuel cells, MFC), biofuels and biochemical production (BES), waste remediation (bioelectrochemical treatment systems BET), production of H₂ at low applied potential (microbial electrolysis cells, MEC) and others. The schemes of exemplary BES configurations are presented in Figure 8. The most common process based on microbial electrocatalysis is electricity production in MFCs [112, 113]. BESs can be inoculated with a wide spectrum of bacteria, for example, *Shewanella oneidensis*, *Geobacter sulfurreducens*, *Moorella thermoacetica*, *Clostridium ljungdahlii*, *Escherichia coli* or *Acetobacterium woodii* [114]. Microorganisms can be used as a monoculture or as a culture mixture.

MFC devices transform the chemical energy in organic matter into electrical power. Because the chemical energy from the oxidation processes is directly turned into the electricity instead of heat, the Carnot cycle with limited thermal efficiency can be omitted, as it is in conventional chemical fuel cells (more than 70%) [115]. MFCs, as this is a new technology, have tremendous potential because of their operational and functional advantages [115]:

- high conversion efficiency of direct conversion of energy contained in organic or inorganic substrate to electricity,
- efficient operation in ambient and even low temperatures (unlike the all other current bioenergy generation processes),
- the gaseous off-products of the process do not require the treatment,
- no energy input for aeration needed (passive aeration),
- applications in locations without the electrical infrastructure.

The selection of MFC apparatus components and design, along with the microbial consortia, is crucial for the performance of whole device and is limited by the specific requirements posed by the nature of the electrochemical processes and the bacteria. All materials that are used for MFC construction should be chemically stable, biocompatible and insensitive for impurities and additionally resistant to corrosion. What is more is that the materials for electrodes should have large active surface area, high porosity and conductivity. The structure of their surface should not impair the electron transport process. Of course, it is desired that they should be simultaneously cheap and easy to manufacture. The search for the perfect electrode material is still on-going – there is a great hope in graphene. So far, the most commonly used materials are carbon and graphite paper, cloth, fibres and meshes, reticulated vitreous carbon and stainless steel [113, 116].

Figure 9A presents the power density values (referred to anode active surface area) that were obtained in different MFC configurations. As it can be seen, the differences reach more than two orders of magnitude. Figure 9B presents the maximal values generated in MFC voltage and maximal power densities (referred to active cathode surface area) for different cathodes.
Figure 8. Schematic diagrams of exemplary two-chamber BESs configurations: A – microbial fuel cell (MFC), B – microbial electrolysis cell (MEC), C - electrosynthesis microbial cell (MES).
This clearly shows that change in even one MFC component has potentially enormous influence on the whole system efficiency.

MFCs enabled electricity production from biodegradable raw materials, for example, waste-water. They seem to be an alternative to expensive conventional aerated wastewater treatment. Huggins et al. [118] compared traditional aeration process in wastewater treatment plant with MFC. They indicated that MFC showed lower removal efficiency when the carbon oxygen demand (COD) concentration is high, but it is much more effective than aeration when the COD is low. It also significantly reduces sludge production (by 52–82% as compared with

![Figure 9.](image-url)
aeration), which can reduce the size of secondary clarifier and save the cost of sludge disposal. Furthermore, MFCs save 100% of aeration energy with extra electricity output.

Another application is that BESs uses them for underground contaminant remediation where the electrodes are an inexhaustible source of terminal electron acceptors for a groundwater environment. Process can be conducted with the use of a single or array electrodes without using enclosed containers. In such a solution, bacteria are stimulated to decomposition of underground pollutants and produce additional electricity. It eliminates chemicals, which are indispensable in conventional technologies and reduces cost of energy [119]. Remediating MFC technology can be used, for example, for removal of petroleum hydrocarbons by their oxidation. Morris et al. [120] indicate that anaerobic biodegradation of petroleum derivatives was importantly enhanced in an MFC (82% removal) in comparison with an anaerobically incubated control cell (31% removal) over 21 days at 30°C [120]. When the electrode is used as an electron donor, the oxidized contaminants, such as chromium, perchlorate, chlorinated solvents and uranium, can be reduced using the electrode as the electron donor [119].

MFCs can be easily adjusted to the generation of hydrogen, instead of producing electricity. Produced H₂ can be accumulated for different applications. When the anodic potential in the MFC device increases using an external voltage of about 0.23 V or more, the gaseous hydrogen is produced at the cathode by the reduction of protons (in the absence of oxygen in the cathode chamber) [117]. It is the so-called MEC. The main advantages of such a hydrogen production are [119, 121]:

- used external power is much lower (0.2 V) than used in traditional water electrolysis (1.23 V),
- no expensive catalysts needed on the anode,
- waste and renewable materials can be used as a substrates,
- the H₂ production rate can be three times higher than during dark fermentation.

Despite the big potential of microbial electrolytic production of hydrogen, the development of this technique is still at the laboratory scale. Successful application of the MEC is possible when the problems with low efficiency and with scalability of device are resolved.

In case of two-chamber devices, the chambers are separated by a membrane that is expensive and can increase the internal resistance at pH 7.0. The membrane also caused large potential losses associated with the exchange of anions and cations. It resulted in considerable increase in the value of applied voltage and decrease in the energy recovery [121]. On the other hand, one-chamber MECs have simpler construction (membrane-free), lower internal resistance and much higher yield of H₂, but produced hydrogen is consumed by methanogenesis to generate methane [119, 121].

MEC systems can be used for the production of other inorganic compounds in the cathode chamber. For example, Rozendal et al. [122] stated that phosphate can be recovered as struvite in a modified MEC. Single-chambered reactors are characterized by the low efficiency of phosphorus removal from wastewater (20–50%), and there is a problem with failure of cathode
because of scale accumulation [122]. Rozendal et al. [122] designed a two-chamber MEC with a fluidized bed to decrease scale formation on the cathode surface. This reactor promotes bulk phase struvite precipitation and protect electrode. Additionally, it has a high level of soluble phosphorus removal (70–85%), compared to 10–20% for the control (open circuit conditions). The method of struvite recovery with the use of MEC device is consuming less energy than conventional pH adjustment systems (e.g., aeration and chemical base addition). MEC also enables a production of hydrogen peroxide (H$_2$O$_2$) by reduction of oxygen at a cathode [123]. It was possible to obtain $\sim 1.9 \pm 0.2$ kg H$_2$O$_2$/m$^3$/day from acetate at an overall efficiency of 83.1 ± 4.8%, where the applied voltage was 0.5 V.

Microbial electrosynthesis systems (MES) enabled the production of organic compounds and fuels by reduction of CO$_2$ or other chemicals on the cathode. The concept of MES is quite new (2009–2010); the devices were elaborated when it was discovered that electrical current can change microbial metabolism. One of the generated products is acetate, which can be converted to liquid fuels, but this process is characterized by low rates and yields. Generally, acetogenic bacteria can reduce CO$_2$ to acetate using hydrogen as a donor of electrons. It was found that some acetogens, for example, *Geobacter* and *Anaeromyxobacter* species, accept electrons from graphite electrodes for chemical productions in place of hydrogen electron donor [124]. Therefore, Nevin et al. [124] investigated the possibility of carbon dioxide reduction to acetate using acetogenic microorganism *Sporomusa ovata*, where the electrons were delivered directly to the cells with a graphite electrode. They stated that the conversion of CO$_2$ and H$_2$O to an organic compound and oxygen:

$$2\text{CO}_2 + 2 \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{O}_2$$

is much similar to the reaction taking place during oxygenic photosynthesis. Marshall et al. [125] improved acetate yield by operating established biocathode in semi-batch mode at a potential of $\sim 590$ mV (versus SHE) for over 150 days. Maximum acetate production from CO$_2$ (as the only carbon source) was 17.25 mM day$^{-1}$ (1.04 g L$^{-1}$ d$^{-1}$). Beside acetate, the second main product was hydrogen. Steinbusch et al. [126] showed that obtained acetate can then be converted into ethanol by biological reduction with the use of electrode, in hydrogen stead. To stimulate acetate reduction at the cathode with the mixture of bacteria cultures, the addition of a mediator (methyl viologen) was required. At applied cathode potential $\sim 550$ mV, used mediator enhanced ethanol production 6-fold and increased ethanol concentration 2-fold compared with the control probe. Generally, the microbial electrosynthetic cells have great potential, especially in biofuel production, but there are still many technological as well as economical challenges to be solved before their implementation in industrial scale.

From among microbial BESs, the most advanced research is in the field of MFC, but there are also a lot of challenges facing researchers. Similarly as in other systems, there is a problem with upscale of MFC. With increasing size of MFC, the power density generally decreases. In bigger MFC devices, the distance between anode and cathode electrodes is also bigger, which influences increase in resistance and pH slope of solution [112]. The MFC can be connected parallel or in series to increase produced current and voltage, but there are also some problems,
especially when any cell is weak. The other problem connected with upscaling is high cost of membrane electrodes, which additionally should be resistant to degradation by microorganisms. It is also important to for example [112]:

- find cheaper materials for construction of reactors, electrodes, membranes etc.,
- improve metabolism of microorganisms for better extracellular transfer of electrons,
- improve catalytic efficiency of microorganisms,
- improve electrolyte conductivity,

and others. However, in the near future, the microbial BESs could revolutionize the market for the production of sustainable energy, fuels and chemicals.

5. Conclusions

Technological progress is inextricably linked to the increase in demand for energy. Rapid development of industrial and automotive sectors caused depletion of the deposits of fossil fuels, which resulted in the necessity of searching for alternative sources of energy. The microorganisms such as bacteria, microalgae, fungi and yeast can become allies in meeting the growing needs of people for fuels. These organisms can produce valuable substances (used as biofuels or as a substrates for their production) as a product of organism’s metabolism or can be a raw material for technologies converting their biomass into biofuels.

There are many ways in which fuels can be obtained with the use of microorganisms. At present, the greatest hopes are set on technologies that are well known as ethanolic fermentation or anaerobic digestion of biodegradable organic wastes to biogas. Researches in the area of these technologies are focused mainly on improving the efficiency of the process, the search for new substrates or on the methods of pretreatment of raw materials. Promising microorganisms seem to be unicellular algae. These organisms are source of different valuable compounds such as pigments, lipids, sterols, fatty acids, starch, oils and others. Microalgae are both producers and raw materials for biofuel production. Their conversion to energy can be realized in different biochemical as well as thermochemical routes. Microbial oil obtained from algae can be a substrate for the production of biodiesel or hydrocarbons. Also algae biomass (raw or after oil extraction) can be converted into fuels by different fermentations, gasification and liquefaction. At present, we know a lot about their metabolism, cultivation, harvesting and some technologies of their processing, but there are still a lot of problems demanding solution for improving the efficiency of microalgae cultivation, cost reduction, increasing oil content in the cells and others. Microalgae have additional advantages; they have a very big potential for the purification of water from nitrogen and phosphorus. These elements are necessary to build algae’s cells and due to the enormous growth, microalgae need very large amounts of N and P. Algae can also accumulate other elements dissolved in water, which may increase the scope of their application. Furthermore, algae are photosynthetic organisms, so they need significant amounts of CO\textsubscript{2} during growth. It caused that these organisms can be
used to capture carbon dioxide from different gases (biogas, producer gas and exhaust gas). The above-mentioned advantages of algae indicate that these organisms can be used multifaceted. They can be a valuable source of biochemicals, may be used in the purification processes of water or gases or to recover some elements from water. Microalgae seem to be an ideal raw material for biorefineries.

Application of other microorganisms (bacteria and fungi) in fuels and energy production for example direct hydrocarbon production by bacteria or microbial BESs, seems to be very interesting issue, however, in the nearest future these technologies probably will not be implemented in a larger scale. The researches in the field of metabolism of these organisms, efficiency of valuable compounds production, systems for cultivation or for energy production are in their infancy and require a lot of time before they become commercially viable.

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