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Fermentace Chlorella sp., perspektivního substrátu pro biotechnologické aplikace

Diplomová práce

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Vedoucí práce: doc. Ing. Tomáš Vítěz, Ph.D.

Ústav experimentální biologie
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Anotace

Fotosyntetické eukaryotické organismy, jako jsou mikrořasy, představují zdroje se širokou škálou biotechnologických aplikací. Rod *Chlorella* je skupina zelených řas s rostoucím vědeckým a komerčním zájmem. Vzhledem k tomu, že mohou růst fototrofně, heterotrofně, ale i mixotrofně, mohou být využity pro různé biotechnologie při odlišných podmínkách. *Chlorella* je již dlouho považována za kvalitní zdroj bílkovin a vyrábí se komerčně jako potrava pro lidi a zvířata, ale je také bohatá na obsah tuků, takže může být slibnou surovinou pro výrobu obnovitelných paliv, o které je v dnešní době velký zájem.

V této diplomové práci jsou popsány biotechnologické aplikace Chlorelly, přičemž zvláštní pozornost je věnována využití Chlorelly při výrobě bioplynu, kde byl proveden i fermentační experiment s využitím druhu *Chlorella pyrenoidosa* (s obohacením o lipidy a bez nich). Tato mikrořasa byla fermentovaná společně s různými inokuly ze zemědělské bioplynové stanice v Čejči a z fermentorů čistírny odpadních vod v Modřicích. Výsledky ukázaly zajímavé výtažky metanu, které se u každého inokula lišily, přestože obsah lipidů nehrál takovou roli jako byla očekávaná.

Anaerobní fermentace je složitý proces, do kterého jsou zapojená různé mikrobiální společenstva. Popis mikrobiálních změn po přidání řas do fermentoru není však dostatečně prozkoumán. Abychom pomohli popsat složení mikrobiální komunity s možnými změnami ve společenstvích po přidání řas, byla provedena sekvenční analýza, která nám umožnila zjistit, zda existují nějaké důležité vztahy mezi změnami mikrobiální komunity a vyšší produkcí biometanu.

Abstract

Photosynthetic eukaryotic organisms, such as microalgae, are resources with a wide range of biotechnological applications. A genus *Chlorella* is a group of green microalgae with growing scientific and commercial interest. Because they can grow phototrophically, heterotrophically but also mixotrophically, they can be used for different biotechnologies under different conditions. Chlorella has long been considered a quality source of protein and is produced commercially as food for humans and animals, but it is also rich in oil thus can be a promising feedstock for the production of renewable biofuels, which is of great interest today.

This diploma thesis describes the biotechnological applications of *Chlorella*, with special attention being paid to the use of *Chlorella* in biogas production, where a fermentation experiment was performed using the species *Chlorella pyrenoidosa* (with and without lipid enrichment). These microalgae were fermented together with various inoculums from the agricultural biogas plant in Čejč and from the fermenters of the wastewater treatment plant in Modřice. The results showed interesting yields of methane, which differed for each inoculum, although the lipid content did not play the role as expected.

Anaerobic fermentation is a complex process involving various microbial communities. However, the description of microbial changes after the addition of algae to the fermenter has not been sufficiently investigated. To describe the composition of the microbial community with possible changes in the communities after the addition of algae, a sequence analysis was performed to see if there were any important relationships between changes in the microbial community and higher biomethane production.

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Ředitel *ústavu* PřF MU Vám ve smyslu Studijního a zkušebního řádu MU určuje diplomovou práci s názvem:

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Oficiální zadání:
Cílem diplomové práce bude provést fermentační testy řas (Chlorella sp.) obohacených lipidy v laboratorních vsázkových fermentorech o objemu 3 litry. K fermentačním testům budou použita dvě různá inokula, ze zemědělské bioplynové stanice a z anaerobní stabilizace čistírenského kalu. V průběhu fermentačních testů bude měřen objem a kvalita vznikajícího bioplynu. Pro určení ovlivnění mikrobiomu po přídavku řas bude provedena izolace DNA z inokula a z fermentorů po ukončení fermentačních testů. Pro vyhodnocení změn mikrobiomu bude použita metoda NGS s využitím 16SrRNA primerů. Literatura bude k dipozici u vedoucího diplomové práce.

Vedoucí práce:	doc. Ing. Tomáš Vítěz, Ph.D.
Konzultant:	RNDr. Kateřina Sukačová
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Declaration

I declare that I have prepared the diploma thesis independently under the guidance of the supervisor using information sources that are cited in the thesis.

Brno, June 15, 2021 .....

Lívia Knapová

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Glossary

ABP	–	agricultural biogas plant
AcoD	–	anaerobic co-digestion
AD	–	anaerobic digestion
AVs	–	amplicon sequence variant
CGF	–	Chlorella growth factor
DAGs	–	diglycerides
DM	–	dry matter
FFAs	–	free fatty acids
MAGs	–	monoglycerides
oDM	–	organic dry matter
OTU	–	operational taxonomic unit
PE	–	population equivalent
PUFAs	–	polyunsaturated fatty acids
RUBISCO	–	ribulose-1,5-bisphosphate
SAFA	–	saturated fatty acids
SDB	–	SILVA database
TAGs	–	triglycerides
WWTP	–	wastewater treatment plant

1 Introduction

Microalgae are a diverse group of photosynthetic organisms with a simple cellular structure from which about 40 000 have already been described or analysed. Basically, we can distinguish between them according to their pigmentation and divide them into several major groups namely green, brown and red algae (Ru, et. al., 2020).

These simple organisms can grow in various environmental conditions. They have large surface to volume body ratio, which gives them the ability to take up a large number of nutrients and are able to produce a wide range of chemical products. Thanks to that and especially with their high ability to CO_2 fixation and high biomass production these organisms represent an interesting group of organisms for biotechnological exploitation. They are valuable sources for production of beneficial products and have been used in different processes and services with an important impact in food, pharmaceutical industry as well as in public health (Borowitzka, 2018).

Nowadays, microalgal biotechnology has shown a wide range of applications not only in human and animal nutrition but also as products used in the cosmetic industry or in agriculture as soil conditioning. In addition, they have been exploited for wastewater treatment and used as a biological tool for assessment of environmental toxicants, and most recently they have been studied for possible production of a „third“ generation of biofuels (Andrade & Andrade, 2017).

One group of microalgae which can be used as a model organism represents a genus of green single-cell algae *Chlorella* that was first discovered by Dutch microbiologist M. W. Beijerinck in 1890. He noticed the green colour of water in a small pond near the Delft River and isolated several species of algae, one named *Chlorella vulgaris* (Beijerinck, 1890). Since his discovery *Chlorella* sp. have been studied by several scientists. German cell physiologist Otto H. Warburg studied photosynthesis by using *Chlorella* sp. and later on by two American biochemists and biologists M. Calvin and A. A. Benson who were focusing their work on carbon dioxide assimilation in plants (Warburg, 1919; Calvin, 1962). In the 1960s, *Chlorella* was used as the first large-scale culture of the microalga for commercial purposes in Japan because it was initially seen as a protein-rich source of food (Iwamoto, 2003).

Since then, a lot of research has been done by using *Chlorella* strains, making them promising model organisms for various biotechnological applications (Borowitzka, 1999).

2 Description of genus *Chlorella*

2.1 Morphology and chemical composition

The name *Chlorella* comes from the Greek word *chloros*, which means green colour, and the Latin suffix *ella* refers to its microscopic size (Safi, et. al., 2014). This genus represents unicellular, simple, non flagellated green algae. They live in both aquatic and terrestrial habitats such as freshwater, marine water, soil and some of them are even symbiotic with lichens and protozoa (Graham & Graham, 1980; Watanabe, et. al., 2005).

Chlorella cells are more or less spherical or they have an ellipsoidal shape, and their cell size may range from 2 to 15 μm (in diameter). They contain nucleus, mitochondria, vacuoles, lipid bodies, one single chloroplast with a pyrenoid, the structure that helps to fixate CO_2 with the enzyme RUBISCO, ribulose-1,5-bisphosphate carboxylase /oxygenase (Liu & Chen, 2014).

Their cell walls are composed of a matrix and a rigid fibrillar structure, which is sometimes called a "rigid cell wall", but the structure differs across the species (Takeda, 1988). Some of the species are characteristic for the presence of glucose and mannose or glucosamine but it varies from species to species and it also serves as a characteristic feature to distinguish species from each other. For example, the sugar composition of *Chlorella zofingiensis* is 70 % glucose and 30 % mannose in its "rigid cell wall" and 65 % mannose, 30 % glucose, plus minor amounts of rhamnose and galactose in its matrix cell wall. Concerning the cell wall sugar composition of *Chlorella minutissima*, it presents 85 % glucose, 15 % mannose in its "rigid cell wall" and 70 % mannose, 20 % glucose, and 10 % galactose in its matrix cell wall (Rodrigues & Bon, 2011).

The chemical composition of *Chlorella* typically consists of the proteins, carbohydrates, lipids, pigments and vitamins in their cells. Numerous species of microalgae, including genus *Chlorella*, have shown to be rich in proteins, carbohydrates, lipids, and are able to synthesize other bio-active compounds. According to some research the composition of *Chlorella* is up to 50-70 % essential amino acids, 30 % polyunsaturated fatty acids, up to 8-14 % carotene and a fairly high concentration of vitamins B_1, B_2, B_3, B_6, B_{12}, E, K, D, among other microalgae but it also differs within a genus (Andrade & Andrade, 2017; Koyande, et. al., 2019).

Their composition also differs according to cultivation conditions or their age. Cells have more proteins under normal conditions. Proteins are involved in capital roles such as growth, repair, and maintenance of the cell as well as serving as chemical messengers, regulators of cellular activities etc. *Chlorella* sp. can utilize both essential and nonessential amino acids (Ru, et. al., 2020).

On the other hand, there is a greater accumulation of carbohydrates and lipids under stress conditions. Carbohydrates, in addition to their structural function in the cell wall, serve as energy and carbon reserves in microalgae. Lipids themselves play an essential role in the metabolism and growth of microalgae, serve as an energy and carbon reservoir too (Ru, et. al., 2020).

Generally, lipids in microalgae can be classified into neutral and polar lipids. Polar lipids are structural lipids that mainly contain polyunsaturated fatty acids (PUFAs). These lipids such as phospholipids and sterols build cell membranes and some of them (e.g., sphingolipids, inositol lipids) can behave as important intermediates in cell signalling pathways. Non-polar lipids, stored in cells in various ways, are triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs), free fatty acids (FFAs), hydrocarbons and pigments. Predominantly, polyunsaturated fatty acids (PUFA) have a structural function, while saturated fatty acids (SAFA) have a storage function. It has been reported that microalgae containing 80 % oil, grow 30 times slower than those containing 30 % oil (Deng, et. al., 2009). Lipids, especially TAGs, in microalgae were better studied because of their usage in biofuel production. During optimal growth conditions *C. vulgaris* can reach 5-40 % lipids per dry weight of biomass but on the contrary during unfavourable growth conditions (like nitrogen starvation) lipids content can reach even more than 58 % lipids per dry weight of biomass but it also differs between *Chlorella* strains (Safi, et. al., 2014).

Chlorella is mainly a photoautotrophic genus of algae, which is characterized by using chlorophyll *a* which is a major photosynthetic pigment responsible for oxygenic photosynthesis. In addition, it also has chlorophyll *b* that absorbs energy to help the photosynthesis process and other beneficial pigments like carotenoids (carotenes and xanthophylls). Commonly found carotenoids in *Chlorella* sp. are α- and β-carotenes, lutein, zeaxanthin, violaxanthin, neoxanthin. Keto-carotenoids such as canthaxanthin and astaxanthin are also found in some strains (Liu & Chen, 2014). Algae pigments can protect chlorophyll molecules from degradation and bleaching during heavy exposure to radiation and oxygen, and have various applications in the food industry on a commercial scale (Safi, et. al., 2014).

2.2 Taxonomy

In general, members of the *Chlorella* genus belong to the group of green algae *Chlorophyta* into class *Trebouxiophyceae* (order *Chlorellales*). Despite the fact that there are described more than one hundred species of this genus and new species are constantly being added, in some articles we can find that according to results from analyses of the small subunit of the ribosomal RNA gene (18S rRNA) this genus is reduced to only four *Chlorella* species such as: *C. vulgaris*, *C. lobophora*, *C. sorokiniana* and *C. kessleri* (Huss et al., 1999; Takeda, 1988).

Over time, numerous studies have been carried out to revise the systematics according to their nutritional requirements, morphological or structural features, serological reactions, the chemical composition of the cell wall, biochemical and molecular phylogenetic characteristics. At present, based on biochemical and molecular data the genus consists of five "true" *Chlorella* species: *Chlorella vulgaris*, *C. lobophora*, *C. sorokiniana*, *C. heliozoae* and *C. variabilis*. (Bock, et. al., 2011).

It is known that some algae evolved independently in different evolutionary lineages within *Chlorophyta*, *Trebouxiophyceae* and because of that other *"Chlorella"* species like: *"C." ellipsoidea*, *"C." mirabilis*, *"C." saccharophila* have been placed into genus *Chloroidium* (Tatyana et al., 2010).

But the whole species delineation in *Chlorella* genus shows to be problematic because the morphological characters separating individual species are scarce, not easy to observe, and as with more attention being paid to them new and new species are being described (Bock et al., 2011; Ahn, et. al., 2012).

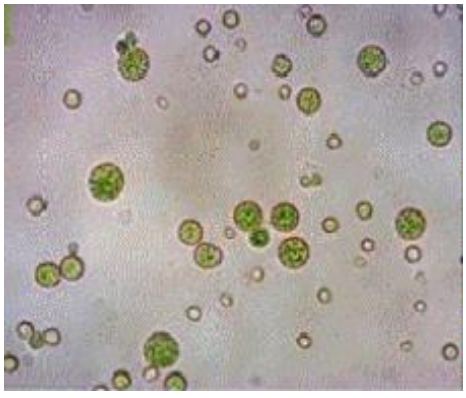


Figure 1. *Chlorella vulgaris* (Andrade & Andrade, 2017)

2.3 Reproduction

Commonly, *Chlorella* genus has no sexual life cycle and reproduces asexually through an autospore production but there is evidence that some of the species can reproduce sexually like *Chlorella variabilis* which showed the presence of meiosis-related genes (Blanc, et. al., 2010). Asexual reproduction goes on by maturing the autospores in the mother cell and rupturing and releasing matured autospores via mother cell's wall. The number of autospores derived from a single mother cell may vary greatly from 2 to 16 (Liu & Chen, 2014).

2.4 Growth and cultivation

Chlorella growth requires nutrients including carbon, nitrogen, phosphorus, sulphur as well as metals. Inorganic nutrients such as K, Ca, Mg, Fe, Cu, Zn, Mn, are needed in trace amounts (Golub & Voyevoda, 2013).

Carbon, the most important element of every living organism, is usually obtained from carbon dioxide, HCO_3^- or sugars (glucose, fructose, mannose etc.) that are widely used for *Chlorella* fermentation (Lam & Lee, 2013). *Chlorella* utilizes carbon dioxide from the atmosphere, but air contains only 0.04 $\%_{vol}$ CO_2 which is not sufficient to maintain rapid growth of *Chlorella*. Therefore, in some cases, the cultivation is enriched with CO_2 gas at the concentration of 1-5 $\%_{vol}$ to *Chlorella* cultures. On the other hand, higher levels of CO_2 may cause a decrease in pH of the medium thus inhibit or even block the algal growth. Optimal growth is obtained under lower concentrations of CO_2 but there are species that can grow well in the presence of its higher concentrations (Moroney & Somanchi, 1999).

The second important nutrient for *Chlorella* growth is nitrogen. *Chlorella* sp. are able to utilize nitrate, ammonia, and organic sources of nitrogen such as urea, glycine and amino acids. Different stains of *Chlorella* may favour different nitrogen sources for growth. But it was also indicated that *Chlorella* sp. prefer to use organic nitrogen and nitrate nitrogen in the choice of nitrogen sources rather than utilize ammonium nitrogen. In the study where were used $NaNO_3$, $CO(NH_2)_2$ and NH_4Cl as sources of nitrogen for their cultivation, it was shown that consumption of NH_4, will gradually decrease the pH of the culture, which may affect the absorption of CO_2 and even inhibit the growth of microalgae (Feng, et. al., 2020). Nitrogen concentration in the culture medium plays an important role in regulating algal growth

and metabolism. A high concentration of nitrogen may inhibit the growth of *Chlorella* sp. and on the other hand, nitrogen deficiency causes the decrease of protein levels, and promotes the accumulation of lipids within cells (Kendirlioğlu Şimşek & Cetin, 2019).

Phosphorus is the third essential nutrient required for the normal growth of *Chlorella*. It is involved in the formation of nucleic acid and cell membrane, as well as the formation of ATP that provides energy for cellular metabolism. The most used phosphorus source for algal cultivation is phosphate, either as $H_2PO_4^-$ or HPO_4^{2-}. As with nitrogen starvation, phosphorus deficiency causes lipid accumulation in cells too (Liang, et. al., 2013).

Sulphur is a structural element of proteins, enzymes, peptides, some amino acids, vitamins and coenzymes in microalgae cells and is also a component of many other organic cell compounds. The role of sulphur in the cells relates to a cell division process, protein metabolism, and fatty acid synthesis. Usually, it is provided in the form of SO_4^{2-} for algal growth. Sulphur deficiency is a stress factor for cells and leads to lipid accumulation and to a disorder of cell division in the culture medium. At the same time, its high concentration decreases medium pH thus has an inhibitory effect on the culture growth (Golub & Voyevoda, 2013).

Generally, *Chlorella* can be cultured photoautotrophically, heterotrophically or mixotrophically in open or closed culture systems.

2.4.1. Autotrophic growth

The common characteristic of microalgae is that they can convert solar light energy to chemical energy through photosynthesis by using chlorophyll *a*. Photosynthesis consists of light-dependent reactions and carbon dioxide fixation. The light-dependent reactions produce high-energy molecules ATP and NADPH, which are utilized in the Calvin–Benson cycle to fix carbon dioxide. *Chlorella* performs photosynthesis efficiently at a relatively low light intensity and becomes saturated when the light intensity reaches a certain value. Higher light intensity above the saturation value may inhibit the photosynthesis of the algae and even cause the destruction of chlorophylls (photobleaching) and cell deaths (Mohammad, et. al., 2016).

Chlorella is commonly cultivated outdoors in open ponds or closed photobioreactors. Open ponds (circular ponds and raceway ponds) are the most common way of microalgae biomass production. Circular ponds are typically made from reinforced concrete and raceway ponds from poured concrete, they are simply dug into the earth and lined

with a plastic liner to prevent the ground from soaking up the liquid. Open pond systems are the cheapest method with easy manipulation for large-scale biomass production. However, they do have some limitations because they require a strict environmental control to avoid the risk of pollution, water evaporation, contaminants, invading bacteria and the risk of growth of other algae species. In addition, temperature differences due to seasonal change cannot be controlled and CO_2 concentration and excess exposure to sunlight are difficult to manage. Moreover, some cells are not sufficiently exposed to sunlight because other cells floating near the surface cover them, leading to lower mass yields thus stirring of the medium is currently practiced but it is accompanied by high energy consumption (Feng, et. al., 2020).

To overcome some limiting factors in the open pond systems closed photobioreactors were implemented. This technology manages environmental conditions (pH, light intensity, temperature, carbon dioxide concentration) to obtain higher cell concentration as well as products that are more suitable for the production of pure pharmaceuticals, nutraceuticals and cosmetics. Multiple designs have been used and tested for its production such as flat-plate photobioreactor, tubular photobioreactor and column photo-bioreactor. Tubular PBR is one of the most popular designs. It is a system made up of tubes which can be placed in various ways and they are used for feeding the biomass with CO_2 by bubbling. The tubes are generally 20 cm or less in diameter and the thickness of their transparent walls is few millimetres, allowing appropriate light absorption. Fluorescent lights are used in case the tubes are not or not sufficiently exposed to sunlight (Acién, et. al., 2017).

2.4.2. Heterotrophic growth

Chlorella sp. can grow as well under heterotrophic conditions as photoautotrophically. The cultivation is performed by using bioreactors or fermenters, usually placed indoors without provision of light, with the addition of organic carbon sources. These organic carbon sources, including sugars, hydrolysed carbohydrates, acetate, and glycerol, serve as the solo carbon and energy sources to support the growth of *Chlorella*. Heterotrophic algal cultivation has the potential to solve the problems associated with open-pond algae production too. It prevents algae from unwanted contamination. In some studies, was shown that heterotrophically can be produced higher cell biomass than photoautotrophically. Because of the large requirement of organic compounds and bioreactors, the cost of heterotrophic cultivation

is much higher than photoautotrophic growth (O'Grady & Morgan, 2011; Shen, et. al., 2010).

2.4.3. Mixotrophic growth

Many *Chlorella* strains are able to grow robustly under mixotrophic conditions utilizing both CO_2 and organic carbon in the presence of light. Basically, it is a complementation of photoautotrophic growth with organic substrates, which can improve the growth rate, shorten the growth cycle, reduce biomass loss in dark a stage, and increase biomass and lipid productivity. Since organic compounds can be utilized under mixotrophic cultivation, light is not a limiting factor in the growth of microalgae and does not strictly depend on photosynthesis. Photoinhibition can be reduced when illumination levels are too low or too high. Organic carbons added into open ponds or PBRs can boost mixotrophic production and it has been also shown that *Chlorella* grows better under mixotrophic conditions. In a closed system, it offers better performance than an open system to maintain a monoculture under mixotrophic conditions because it can minimalize contamination. Specifically, comparing with heterotrophic growth, mixotrophic cultivation of microalgae yields higher productivities with identical organic carbon supply. Under mixotrophic cultivation, the cost reduction can be achieved thus it can be applied commercially (Mohammad, et. al., 2016; Wang, et. al., 2014).

3 Biotechnological application

3.1 *Chlorella* sp. as animal feed and dietary supplements for humans

In today's modern hectic world, people all around the world suffer from various diseases (obesity, high blood pressure, diabetes, and other heart-related problems). Their everyday choices (eating high-calorie food, visiting fast-foods frequently, lack of exercise etc.) may greatly contribute to that. For a healthy lifestyle, a balanced diet consisting of eating antioxidants, vitamins etc. is required. Microalgae due to their phototrophic growth (they are exposed to free-radical and high oxidative stresses) evolved natural protective systems such as antioxidants and pigments. These components and many others obtained from microalgae, represent a valuable source of nutrients used in human supplementation or serve as an essential animal nutrition (Bito, et. al., 2020).

Since the first commercial production of *Chlorella* in Japan, factories for its production have been constructed in countries such as Taiwan, Malaysia and Indonesia. Currently the alga is available in the form of capsules, tablets, dried powders and liquids for human consumption in many other countries. It can be mixed with candies, gums, noodles, breakfast cereals, wine, and other beverages (Spolaore, et. al., 2006). Moreover, *Chlorella* can be added into cookies to have a new and attractive appearance with better textural characteristics and its pigments can be also used as a natural colour additive for human food (Gouveia, et. al., 2007).

This alga contains immunostimulator-β-1,3-glucan which acts as a free radical scavenger and has another beneficial effect in reducing blood lipids. An additional product named '*Chlorella* growth factor' is also produced by this genus. In some literature, CGF was described as a hot-water extract of *Chlorella* that contains a mixture of proteins, nucleic acids, polysaccharides, and a variety of minerals. The extract has been shown to play a positive role in health care and disease prevention, such as boosting immune functions, preventing tumours and cancers, lowering blood pressure etc. (Liu & Chen, 2014). Moreover, in another source, it was described as a well-distributed agent for improving the growth of lactic bacteria (Chu, 2012).

Many studies have been performed with *Chlorella* sp. as a human supplement or food and have shown a positive effect on human health.

More specifically, the studies where *Chlorella* sp. have been shown to be excellent hepatoprotective and hypocholesterolemic agents that lower blood sugar and increase haemoglobin or that have preventive effects on cognitive decline in age-dependent dementia (Barrow & Shahidi, 2007; Nakashima, et. al., 2009).

Chlorella can be used as animal feed too. However, the alga is too expensive to use widely as a protein supplement in animal feed. Nevertheless, in many experiments, it was found that even a very low addition of *Chlorella* biomass that is economically accepted in food can positively influence the growth, fertility, weight, and overall health conditions of animals (Sousa, et. al., 2008). Promising results were also achieved in the use of *Chlorella* biomass as a carrier of organically bound selenium and iodine that play a substantial role in the thyroid hormone regulation in an organism (Kotrbáček, et. al., 2015).

Nutritional *Chlorella* sp. can be directly used in aquaculture because they improve growth, nutritional level and the amount of fish's pigmentation which can be important quality criteria determining the market value of fish. They eat organisms that contain carotenoids as microalgae because they are unable to synthesize them *de novo* and need them for their natural pigmentation (Liu & Chen, 2014).

Chlorella at very low percentages in feed benefits growth performance parameters of poultry. In this case, it also shows a promising application in poultry farming, especially when feeding hens to colour their egg yolks (Gouveia, et. al., 1996).

In general, using *Chlorella* sp. in animal feed or as human dietary supplement is showing a promising component for prevention or treatment of some serious health problems due to their richness in essential substances, provitamins and vitamins, antioxidants, pigments etc. (Sousa, et. al., 2008).

3.2 *Chlorella* sp. used in cosmetics

Awareness of the photoaging process, skin cancer (due to sun exposure) has caused an increased demand for various cosmetic products in the last decades. Thanks to the microalgal benefits their extracts have become part of them.

Nowadays they play a big role in skincare market because of their chlorophyll-a presence with a light absorption function which can be used as a sun protectant. They produce metabolites such as amino acids sporopollenin, scytonemin and mycosporine that act as natural ultraviolet (UV) blockers and attract commercial attention. We can find

their extracts in anti-aging creams, rejuvenating, refreshing, regenerative care products or hair care products etc. (Sharma & Sharma, 2017; Stolz & Obermayer, 2005). For instance, an extract from *Chlorella vulgaris* is used in skin care products because it boosts collagen synthesis thus supports skin tissue and reduces wrinkles (Wang, et. al., 2015).

3.3 Applications of *Chlorella* sp. in wastewater treatment

As urbanization and industrial development grow, so does the amount of waste. Large amounts of waste have been discharged into rivers, lakes or oceans that destroy the aquatic ecosystem. The dramatic increase in water consumption also produces a large amount of wastewater, which today leads to many environmental and ecological problems. In addition, the water scarcity dilemma is an undeniable issue and could be a major problem in the future that could seriously endanger human livelihoods. Therefore, attention is being paid to the proper use of the low level of available freshwater resources, and wastewater reuse technology is also being introduced, which is extremely important and of global importance. In this case, the use of microalgae proves to be advantageous (Fan, et. al., 2019; Zhao, et. al., 2018).

Wastewaters generally contain various organic waste materials, pathogenic or disease-causing agents, nutrients that stimulate plant growth, inorganic chemicals, minerals and sediments. It may also contain toxic compounds or toxic pollutants, but it varies according to the function of the specific place and the activities in the area (agriculture, industry, farms, etc.). Wastewater treatment has a stepwise approach which contains preliminary, primary, secondary, and tertiary and/or advanced treatment.

Preliminary treatment is the removal of coarse solids and other large materials often found in wastewater. This step helps to remove large, floating solids such as pieces of wood, cloth, paper, plastics, faecal matter etc. High density inorganic solids such as sand, gravel as well as metal, or glass are also removed.

Primary treatment is designed to remove organic and inorganic solids by the physical processes of sedimentation and flotation. The treatment utilizes clarifiers or settling tanks, which remove the settleable organics and settleable inorganic solids from the wastewater. Therefore, colloidal and dissolved constituents are not affected and the effluent from primary treatment contains mainly colloidal and dissolved organic and inorganic solids.

Additional removal of organics can be accomplished by secondary treatment. The secondary treatment process consists of the biological treatment of wastewater by utilizing a mixture of microorganisms that utilize the organic constituent for energy and growth in a controlled environment. Several aerobic biological processes are used for secondary treatment differing primarily in the way in which oxygen is supplied to the microorganisms and in the rate at which organisms metabolize the organic matter. It was also pointed out that biological oxidation systems can remove also pathogenic bacteria from sewage.

Tertiary treatment is aimed at removing ammonium, nitrate and phosphate from wastewater but advanced techniques can be added such as ion exchange, reverse osmosis, chemical precipitation for additional removal of organic and suspended solids, removal of nitrogen, phosphorus, other nutrient removal or removal of toxic materials (Abdel-Raouf, et. al., 2012; Sonune & Ghate, 2004).

The utilization of microalgae for wastewater treatment was first reported by American scientist W. J. Oswald in 1957 and since then it has been intensively studied (Oswald & Gotaas, 1957). It is because the composition of the wastewater is quite similar to the culture media that is usually maintained to produce microalgae. It contains carbon, nitrogen, phosphorus and other minor components necessary for their growth, although some other undesirable compounds (heavy metals, drugs, surfactants etc.) can be found there as well (Acién, et. al., 2016).

Using microalgae like *Chlorella* sp. has not been applied at a large scale so far but biotreatment with microalgae is very attractive because of their photosynthetic capabilities. During photosynthesis, microalgae release O_2 into the medium, which uses aerobic bacteria to degrade organic matter into CO_2 and it is later absorbed by microalgae again. Therefore, compared to conventional wastewater treatment plants, the use of algae and bacterial consortia for wastewater treatment offers the advantage of nutrient recycling (Tan, et. al., 2021). They are also very efficient at the fixation of inorganic nitrogen and phosphorus which is desirable in removing them for wastewater treatment. Recycling of N and P is associated with the production of feedstock for biofuels or high added-value products. Therefore, their energy consumption and production costs will be reduced (Molinuevo-Salces, et. al., 2019).

Advantageous is also their ability to tolerate other pollutants found in wastewater and together with bacteria can help to remove them. In the study, where *Chlorella vulgaris* and *Entherococcus* were used, significantly decreased levels of Cr, Cd, Cu, and Pb (Mubashar, et. al., 2020). In another study by using *Chlorella vulgaris* was Cr(IV) reduced

to Cr(III) and the total concentration of Cr decreased but accompanying features were increased temperature and pH (Xie, et. al., 2014).

The increase in the temperature, pH and dissolved oxygen (photo-oxidative damage of cells) can contribute to pathogen removal as well. It can be efficient on *Escherichia coli* that is typically occurring in wastewaters, but it was also reported that *Chlorella* sp. can be even efficient in helping to remove *Vibrio cholerae* (Mezrioui, et. al., 1994).

The microalgae were usually used in the tertiary stage of wastewater treatment, but their removal activity can be successful in secondary or even preliminary treated effluents. Despite *C. vulgaris*, was used *Chlorella sorokiniana* with the same efficiency of treatment (Asadi, et. al., 2019). Other species of *Chlorella* were used in wastewater treatment as well. For example, *C. zofingiensis* was better in nitrogen and phosphorus removal than was than *C. vulgaris* (Zhao, et. al., 2018).

In general, *Chlorella* sp. can remove a wide range of substances from wastewater and represent a suitable component for wastewater treatment without secondary pollution. Although *Chlorella* cultures were showing us a great potential in these studies, one major and practical limitation appeared and it is a separation of the algal biomass from the treated wastewater, which requires capital-intensive steps such as flocculation, flotation, filtration, or centrifugation. Due to that using immobilized *Chlorella* cells may be more advantageous because no harvest step is required. The removal efficiency of wastewater components by immobilized *Chlorella* was influenced by the type of culture, culture density and pH. In some cases, *Chlorella* was cocultured with other microalgae (Liu & Chen, 2014).

3.4 The use of *Chlorella* sp. as feedstock for biofuel production

The world's population is increasing year by year, which is why we are facing a higher consumption today (for both processed foods and industrial products), which is also putting pressure on the energy supply system. Energy for industrial, commercial and residential purposes, electricity generation and transportation is primarily supplied by fossil fuels (coal, oil and gas) and nuclear power (Morone & Cottoni, 2016).

Fossil fuels cover the energy demands of society very effectively. However, there are two major problems. Firstly, when fossil fuels are burned for energy, carbon dioxide is released into the atmosphere adding to global greenhouse gases. Therefore, the combustion of fossil

fuels is strongly linked to climate change (which leads to an increase in the average temperature of the Earth) thus cause environmental problems. And secondly, fossil fuel reserves have a finite nature. After the peak of their production, they will start to decline. Taken this in association with the current dramatic increase of worldwide population and with industrialized consumption plus overwhelming environmental concerns for greenhouse gas emissions have driven scientists and technologists to search for alternative or renewable sources of energy (Basu, 2013).

Bioenergy is basically the production of usable energy from biological feedstocks. In past decades, several biomass sources (sugar, starch, oil crops etc.) have been identified with increasing potential to be used as bioenergy called “Biofuels” or in some literature we can find them marked as "Agrofuels". They can be liquid fuels (e.g., bioethanol, biodiesel), gaseous fuels (e.g., methane, biohydrogen) or direct solid heat energy (e.g., firewood) (Dutta, et. al., 2014; Morone & Cottoni, 2016).

Biofuels are classified into different generations according to the wide range of raw materials (biomass feedstock) and production technologies being used (Sajjadi, et. al., 2018).

The “first” generation or conventional biofuels are fuels produced from food crops grown on arable land (corn, wheat, barley etc.). Sugar, starch, or vegetable oil obtained from crops is converted to biodiesel or bioethanol by transesterification or fermentation. As the production of the “first” generation biofuel is mainly derived from an agricultural food crop, it has induced social and economic conflict between utilisation of quality agricultural land for food or for cultivation of biofuel feedstock (Sajjadi, et. al., 2018).

The “second” generation of biofuels are fuels manufactured from various types of biomasses (e.g., derived from plant materials) but they can also include animal materials as well. Whereas first generation of biofuels compete with food crops about arable land, second generation biofuels are made from lignocellulosic biomass or woody crops, agricultural residues, or waste plant material (from food crops that have already fulfilled their food purpose). The feedstocks used to generate second-generation of biofuels include grasses, jatropha and other seed crops, waste vegetable oil, municipal solid waste etc. thus are non-edible and largely available. However, lignocellulosic biomass has different physical properties and chemical composition, therefore, it needs to be pre-treated before further processing and thermochemical and biological conversion processes are employed when producing biofuel (Fatma, et. al., 2018).

The “third” generation consists of algae. This generation of biofuel production involves the oil extraction and transesterification process. Algae convert solar into chemical energy and store it in the form of oils, carbohydrates and proteins. Therefore, they are identified as great potential feedstocks for biofuel production but have not been carried out on a commercial scale yet. The third generation is showing more advantages over the above mentioned generations. Firstly, high yield is expected because of their fast growth. Secondly, microalgae, unlike crop-based biofuels, does not cause a decline in food production, as it requires neither agricultural land nor fresh water. They can grow in saline water or wastewater too. Thirdly, algae tolerate various extreme weather conditions whereas the growth of higher plants depends on seasonal conditions, solar radiation and all other weather-based changes. And fourthly, the efficiency of oil production in algae was much higher than in traditional vegetable oil crops in the conducted studies (Liew, et. al., 2014; Wijffels & Barbosa, 2010).

Some articles describe these generations differently. For instance, “fourth” generation biofuel is the result of genetically engineered, highly yielding biomass with low lignin and cellulose contents (thus eliminating the issues present in the second generation biofuels production line) or metabolically engineered algae with high oil contents, increased carbon entrapment ability or improved cultivation, harvesting, and fermentation processes (Ziolkowska, 2020). In some publications, the fourth group of biomass includes various litoautotophic, methanotrophic, methylotrophic bacteria, fungi and yeast for biofuel production. Their high growth rate and relatively easy cultivation growth plus their diversity allow the use of different substrates for biofuel production. It is greatly advantageous, which makes them promising next generations of biofuels (Liao, et. al., 2016).

Overall, there may have been developed many more biofuel generations so far, but their system is not uniform. On the other hand, there is no doubt that microalgae form one of the most promising biomasses for biofuels and there is a growing interest in them. Among them, *Chlorella* sp. are of the greatest interest (Guccione, et. al., 2014).

3.4.1. Bioethanol production

Since ethanol (CH_3CH_2OH) can be used as a partial or complete substitute for motor fuel and can also be converted readily into ethene and related compounds, its production from indigenous and renewable

resources is now an attractive alternative strategy as alcoholic biofuel available in the current world market (Smith, 2009).

Nowadays, industrial alcohol production is largely synthetic, using petrochemical processes. Petrochemical ethanol is made by the hydration of ethene. On the other hand, bioethanol, nonpetroleum-based renewable and sustainable liquid fuel, is made by microbial or yeast fermentation of carbohydrates and fermentable sugars obtained from various plants from the first- or second-generation biofuels category or from microalgal biomass (third-generation biofuels, Ganesan, et. al., 2020).

The produced bioethanol can be used as a gasoline substitute in car, tractors, planes, and even boat engines. When comparing bioethanol with gasoline, bioethanol has a lower heating value thus more ethanol is required to obtain the same output as gasoline. The produced bioethanol can be directly used as a gasoline substitute in engines. When comparing bioethanol with gasoline, bioethanol has lower heating value thus more ethanol is required to obtain the same output as gasoline. But the higher octane number of ethanol leads to improved thermal efficiency and increased power. High oxygen content in bioethanol leads to cleaner combustion which represents lower emissions to the atmosphere. More differences are shown in the Table 1. (Ruan, et. al., 2019).

To improve the fuel quality, bioethanol can be blended with gasoline in a different concentration. Particularly, E5 is a mixture of 5 % ethanol and 95 % gasoline or E25 is mixture of 25 % ethanol and 75 % gasoline etc. It was estimated that bioethanol can reduce up to 90 % of CO_2 and 60 %-80 % of SO_2 emissions when blended with 95 % gasoline fuel, therefore it can be used as an octane number enhancer (Halder, et. al., 2019; Ruan, et. al., 2019).

Many studies on microalgae-based fuels have focused on the production of biodiesel rather than bioethanol due to the high lipid content relatively low carbohydrate content of some microalgae. But microalgal species from *Chlorella* genus belong to the high carbohydrate content group thus are preferable for bioethanol production. In the studies using *Chlorella vulgaris* microalgal hydrolysate was converted into ethanol at a yield of 89 % (Kim, et. al., 2014).

On the other hand, the study where *C. minutissima* was used proves that bioethanol production from microalgae is not still fully developed and the yields of bioethanol are low (Lakatos, et. al., 2019; Şerbetçioğlu, et. al., 2018).

Table 1. The comparison of gasoline and ethanol chemical and physical properties (Ruan, et. al., 2019)

Property	Gasoline	Ethanol
Formula	C_4 to C_{12}	C_2H_5OH
Molecular weight	100–105	46.07
Density at 15°C (kg/L)	0.69–0.79	0.79
Specific gravity (relative density) at 15°C	91	106–110
Freezing point (°C)	−40	−114
Boiling point (°C)	27–225	78
Vapor pressure at 38°C (kPa)	48–103	15.9
Specific heat (kJ/kgK)	2.0	2.4
Viscosity at 20°C (mPa s)	0.37–0.44	1.19
Lower heating value (MJ/L)	30–33	21.1
Flash point (°C)	−43	13
Auto-ignition temperature (°C)	257	423
Lower flammability limit (vol%)	1.4	4.3
Higher flammability limit (vol%)	7.6	19.0
Stoichiometric air-fuel ratio	14.7	9.0
Research octane number	88–100	108.6
Motor octane number	80–90	89.7

3.4.2. Biodiesel production

Conventional diesel, an important transportation fuel, is a by-product of the crude oil distillation process, which also results in the formation of petrol, and has a high aromatic and sulphur content. In contrast, biodiesel consists of methyl or ethyl esters of fatty acids produced by the transesterification of natural lipids. It is mainly derived from vegetable oil, animal fats, algal lipids, or waste grease through transesterification (alcoholysis) in the presence of alcohol (usually methanol) and alkali, acid agents, or enzymes (Gharabaghi, et. al., 2015).

Unlike bioethanol, in addition to physical properties, biodiesel also differs in its chemical composition from conventional diesel. Their differences are shown in the Table 2. When comparing both of them, biodiesel has significant advantages over diesel but also some disadvantages. Biodiesel has a higher flash point, which makes it safer for storage, handling, use, and transportation or a higher cetane number

which means that it has a better ignition quality. The use of biodiesel leads to a reduction of emissions of engines because it does not contain sulphur and aromatic compounds. However, biodiesel production is still more expensive than conventional diesel fuel it has more advantages over fossil fuel-derived diesel. Although it is renewable, it is also biodegradable (that can reduce environmental damage due to spillage) and biodiesel has also lower flashpoint and does not ignite. But on the other hand, biodiesel has similarly like bioethanol lower calorific value which means more biodiesel will be consumed to supply the same output, also higher pour point (it tends to gel up/solidify) and higher viscosity, which has a negative effect on fuel spray atomization (Tamalampudi & Fukuda, 2011).

Anyway, biodiesel can be used in home heating as well as in car engines, traction motors, railway locomotives, construction machinery, mining equipment, military carriers, etc. and it is usually blended with petroleum diesel, for instance, B2 which is a mixture of 2 % biodiesel, B5 (5 % biodiesel and 95 % regular diesel), B20 (20 % biodiesel and 80 % regular diesel) etc. (Morone & Cottoni, 2016).

There have been many research studies focusing on using *Chlorella* for biodiesel production in the past years. Since then, they have been researched in optimizing their growth condition in other to achieve a high yield of biodiesel. To give an example, optimizing autotrophic growth for this purpose with the use of *C. pyrenoidosa* (Singh, et. al., 2019). The heterotrophic growth of *C. protothecoides*, has shown very high cell densities, lipid and biomass productivities thus the high yield of biodiesel (Xu, et. al., 2006). It is well known that under certain conditions like nitrogen or phosphorus starvation, can be maintained higher lipid production resulting in higher biodiesel yield like in the case of *C. vulgaris.* Therefore, they are promising biodiesel production factories (Chávez-Fuentes, et. al., 2018).

Table 2. The comparison of diesel and biodiesel chemical and physical properties (Ruan, et. al., 2019)

Property	Diesel	Biodiesel
Carbon (%)	86.4	79.6
Hydrogen (%)	13.6	10.5
Oxygen (%)	–	8.6
Nitrogen (%)	–	1.3
C/H	6.5	7.6
n-Aliphatics (%)	67.4	15.2
Olephenics (%)	3.4	84.7
Aromatics (%)	20.1	–
Naphtens (%)	9.1	–
Density at288 K (kg/m^3)	816–840	820–897
Calorific value (MJ/kg)	42–46	32–43
Flash point (K)	323–371	408–469
Pour point (K)	238–258	260–289
Kinematic viscosity at 300 K (mm^2/s)	2.5–5.7	4–26
Cetane number	45–55	46–66

3.4.3. Biogas production

Natural gas plays a significant role in satisfying energy demands by providing mechanical, electrical and thermal energy. It is currently used by pipelines as a fuel source for domestic and industrial purposes. In addition, methane can be converted to methanol and used as a fuel in internal combustion engines.

Combustion of natural gas produces the lowest share of carbon dioxide compared to other fossil fuels and therefore plays an important role as a transitional fuel. Transition fuel in the context of a low-carbon fuel substitute for higher content fossil fuels (coal and oil) can reduce CO_2 emissions, it is affordable and offers benefits in its wide geographical distribution. Some literature doubts its importance, as it is still a fossil fuel and its role may be temporary, as natural gas still emits CO_2 emissions. On the other hand, biogas, also known as biomethane, can be used as a substitute for natural gas. Biogas, also known as biomethane, can be used as a substitute for natural gas (Smith, 2009; Aguilera & Aguilera, 2020; Gürsan & de Gooyert, 2021).

It is mainly produced from the anaerobic digestion of various organic substances (biomass feedstocks). The anaerobic digestion process can be generally divided into four steps:

1. Hydrolysis: the large organic polymers (proteins, fats, carbohydrates, etc.) are broken down into smaller molecules (amino acids, fatty acids, simple sugars, etc.).

2. Acidogenesis: acidogenic microorganisms (acidogenic bacteria) further break down the organic matter. They produce an acidic environment when creating ammonia, H_2, CO_2, H_2S as well as acids like acetic, propionic, butyric or others that are produced.

3. Acetogenesis: acetogens catabolize many of the products created in acidogenesis into acetic acid, CO_2, and H_2.

4. Methanogenesis: methanogens produce methane from the final products of acetogenesis, as well as from some of the intermediate products from hydrolysis and acidogenesis mainly through two pathways (Sawyerr, et. al., 2019):

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O \text{ (hydrogenotrophic pathway)}$$

or

$$CH_3COOH \rightarrow CH_4 + CO_2 \text{ (acetoclastic pathway)}$$

Consequently, biogas can be used in motor vehicles, house heating, and electricity generation. There are significant differences between the characteristics of biogas and natural gas which are shown in the Table 3. As bioethanol or biodiesel, using biogas can have its advantages and limitations too. Eco-friendly biogas reduces greenhouse emissions, and its feedstocks are renewable. This low-cost technology helps in reducing the pollution produced by organic wastes, it helps in alleviating the waste management problems plus it may improve water quality. On the other hand, its cost while considering large scale application is not that cheap. After refinement and compression, biogas still contains impurities. If the generated biofuel was utilized to power automobiles, it can corrode the metal parts of the engine. It is observed that the higher content of carbon dioxide (30 %-40 %) contributes to the much lower energy of the biogas compared to natural gas (Guo, et. al., 2015; Khayal, 2019).

Table 3. The comparison of natural gas and biogas composition (+ indicate that there could be some content but not detailed, Ruan, et. al., 2019)

Composition	Natural Gas	Biogas
Methane (%)	95	45–65
Ethane (%)	5	–
Propane (%)	+	–
Butane (%)	+	–
Carbon dioxide (%)	+	30–40
Hydrogen sulfide (%)	–	0.3–3
Ammonia (%)	–	0–1
Moisture (%)	–	0–10
Nitrogen (%)	+	0–5
Oxygen (%)	–	0–2
Hydrogen (%)	–	0–1

Because biogas is mainly composed of methane and CO_2, carbon dioxide from biogas is an excellent carbon source to induce high microalgal biomass production. Generation of biogas from microalgae *Chlorella* was successful because of its ability to endure the stressful biogas composition without affecting their physiological capacity to capture CO_2 and biosynthesize high-value metabolites (Ramos-Ibarra, et. al., 2019).

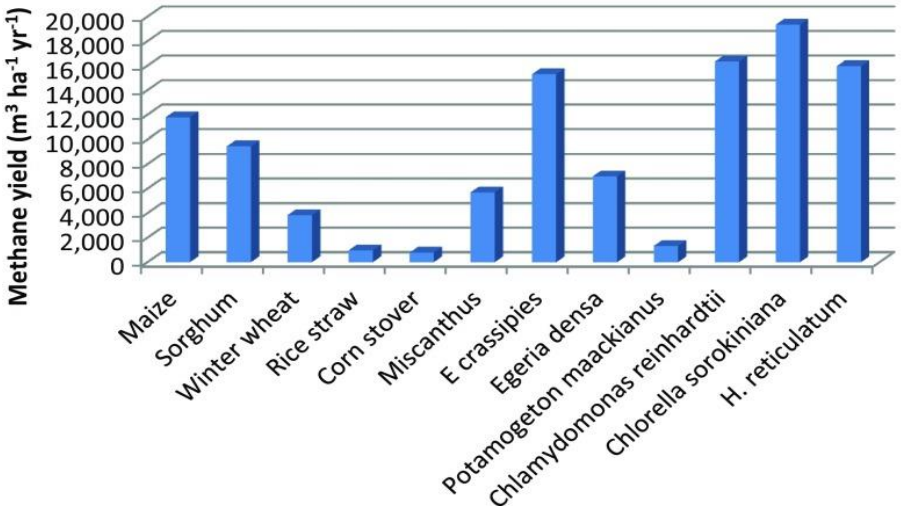
Although the use of microalgae may show great potential over other biomass feedstocks in biogas production (option for using wastewater and seawater, feasibility to recycle nutrients, maximum biomass utilization, minimum sludge production or lesser energy consumption etc.) there are some obstacles that should be considered.

Firstly, many microalgal species are characterized by high resistance toward degradation by anaerobic microorganisms because of their cell walls and *Chlorella* sp. contain cellulose, hemicellulose, pectin and variety of other sugars. To overcome this obstacle, enzymatic, physical or chemical pre-treatment is needed.

Secondly, as in the case of biodiesel or bioethanol production, growth conditions need to be adjusted for higher carbon/nitrogen ratios because it is low naturally and they are mostly high in protein. Low carbon/nitrogen ratios are harmful for fermentation processes because releasing of ammonium can lead to the formation of free ammonia, which is toxic for methanogenesis. And thirdly, the overall costs are high

thus this technology is still not implemented in a large scale commercial use (Klassen, et. al., 2015; Klassen, et. al., 2016).

Biogas production from microalgae has been intensively studied for decades, and the data showed theoretical considerations that algal biomass may have greater potential for biogas generation than other sources, Graph 1.



Graph 1. The comparison of methane yield of different types of biomass (Martínez-Gutiérrez, 2018).

4 Aim

The aim of this thesis was to perform fermentation tests of algae (*Chlorella* sp.*)* which were with and without lipid enrichment in laboratory batch fermenters and measured the volume and quality of the resulting biogas. Two different inoculums were used for the fermentation tests, from the agricultural biogas plant in Čejč, Czech Republic and from anaerobic stabilization of sewage sludge from the wastewater treatment plant in Modřice, Czech Republic. We assume that changes in the microbiome in the fermenters may occur after the addition of algae. To determine this effect DNA was isolated from the inoculums and from the fermenters after the fermentation tests were in the end. The NGS method using 16S rRNA primers was used to evaluate microbial diversity changes.

This diploma thesis focuses on the verification of the following hypotheses:

- Does the addition of algae affect the production of biogas?
- Does the inoculum affect the production and the composition of biogas?
- Does the lipid content in algae affect the composition of biogas?
- Does the lipid content in algae affect the amount of biogas produced?
- Does the addition of algae affect the composition of the microbial community in the fermenter?
- And if there are any changes, how will it affect the biogas production?

5 Materials and methods

5.1 Substrates and inoculums

5.1.1. Algae substrates

The dried *Chlorella* sp. samples with and without lipid enrichment were obtained from CzechGlobe, Czech Republic. Specifically, it was *Chlorella pyrenoidosa* Chick (Ippas C2) species (Sukačová, et. al., 2019).

5.1.2. Inoculum Modřice

The inoculum samples were collected at wastewater treatment plant 513,000 PE, Modřice, Czech Republic. The sample (50 L) was obtained from the anaerobic sewage sludge stabilization tank which is operated at mesophilic temperature (34 °C). After sewage sludge collection, solids were removed by filtration trough the sieve (mesh size 3 mm). Sludge samples characteristics were pH = 7.2; dry matter (DM) content 3.36 % and organic dry matter content (oDM) 60.48 %. The residual sample was stored in the freezer for further analysis.

5.1.3. Inoculum Čejč

This biogas plant is used for agricultural product processing. The input substrate is a mixture of maize silage and pig slurry (80/20, w/w %). The biogas plant is operated at mesophilic temperature condition (38 °C). The samples of maize silage inoculum were taken in 50 L plastic containers directly from the fermenter of the agriculture biogas plant in Čejč, Czech Republic. After digestate collection, solids (maize residues, etc.) were removed by filtration through the sieve (mesh size 3 mm). The residual leachate was analysed and used as inoculum for biomethanation tests. Leachate samples characteristics were pH = 7.28; organic dry matter content 3.54 % and organic matter content 69.73 %. The residual sample was stored in the freezer for further analysis.

5.2 Analytical methods

Dry matter content was determined by drying at 105 °C ± 5 °C to constant weight followed by cooling in a desiccator. Organic dry matter content was determined by incineration of the samples in a muffle furnace at 550 °C ± 5 °C using a furnace LMH 11/12 (LAC Ltd., Židlochovice, Czech Republic).

For pH, redox, conductivity determination multi-parameter instrument WTW 3410 (Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany) was used (Vítěz, et. al.,2021).

5.3 Biomethanation batch assay

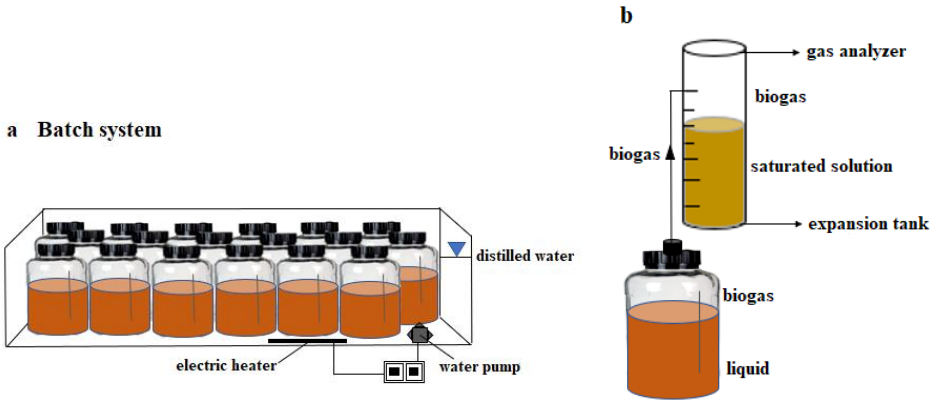


Figure 2. Design of biomethanation batch assay (Vítěz, et. al., 2021, edited by Knapová)

Biogas production and especially biomethane yield were determined experimentally using a batch system of anaerobic fermenters. For this assay, 18 batch fermenters made of glass were used. Each fermenter has 300 ml in volume and was placed in the water bath. Each fermenter plastic lid was equipped with fittings and connected through gas tight tubing to the glass gas holder. Each fermenter had its own gas holder for reading the biogas yield. Each gas holder was also equipped with a port for biogas composition measurement (Figure 2.).

On the first day (day 0) of the experiment, all 18 batch fermenters were filled up with 200 ml of the particular inoculum. The inoculum of maize silage originated from the agricultural biogas was placed in the first 9 fermenters and the next 9 fermenters were filled up with the sludge inoculum from the wastewater plant.

Subsequently, approximately 2 g of algae substrates with and without lipid contents were dosed into almost every fermenter except for the first two, 10 and 11 fermenters. Those were used as blank for indigenous inoculum biogas yield determination (Table 4.). The amount of the substrate doses was chosen as optimum to avoid overdosing of the whole system. Prior to the start of the biomethanation test, the fermenters were tempered to 25 °C ± 2 °C and kept within this range until the end of the experiment, which happened after 33 days. The biomethanation batch assay conditions are shown in the Table 5.

Table 4. Characteristics of each sample from batch assay

Designation of fermenter	**Type of the inoculum**	**Type of the sub-strate**	**Amount of sub-strate [g]**	**Dry matter (DM) [%]**	**Organic dry matter (oDM) [%]**
R1	ABP Čejč	-	-	3.54	69.73
R2	ABP Čejč	-	-	3.54	69.73
R3	ABP Čejč	*Chlorella* sp. with lipid content (2017)	1.90	99.20	97.55
R4	ABP Čejč	*Chlorella* sp. with lipid content (2017)	2.03	99.20	97.55
R5	ABP Čejč	*Chlorella* sp.	2.31	95.65	96.38
R6	ABP Čejč	*Chlorella* sp. without lipid content	1.99	96.13	95.58
R7	ABP Čejč	*Chlorella* sp.	1.97	96.13	95.58

		without lipid content			
R8	ABP Čejč	*Chlorella* sp. with lipid content	1.98	96.58	95.64
R9	ABP Čejč	*Chlorella* sp. with lipid content	2.02	96.58	95.64
R10	WWTP Modřice	-	-	3.36	60.48
R11	WWTP Modřice	-	-	3.36	60.48
R12	WWTP Modřice	*Chlorella* sp. with lipid content (2017)	2.01	99.20	97.55
R13	WWTP Modřice	*Chlorella* sp. with lipid content (2017)	2.06	99.20	97.55
R14	WWTP Modřice	*Chlorella* sp.	2.10	95.65	96.38
R15	WWTP Modřice	*Chlorella* sp. without lipid content	2.05	96.13	95.58

R16	WWTP Modřice	*Chlorella* sp. without lipid content	2.00	96.13	95.58
R17	WWTP Modřice	*Chlorella* sp. with lipid content	1.96	96.58	95.64
R18	WWTP Modřice	*Chlorella* sp. with lipid content	1.99	96.58	95.64

Table 5. The biomethanation batch assay characteristics

Parameter	**Value**
Number of fermenters	18
Fermenter volume	Total volume 300 ml; working volume 200 ml
Temperature	25°C ± 2°C
Stirring	Manual; daily
Dosage of substrates	2 g
Gas amount measurement	Liquid displacement method
Gas quality measurement	Gas analyser Dräger X-am 5600; infrared sensors CH_4 and CO_2 standard gas mixture for calibration (60 % CH_4/40 % CO_2)
Retention time	33 days

5.3.1. Biogas and methane content measurement

Produced biogas was measured daily by applying the liquid displacement method in accordance with standard VDI 4630, using the acidified saturated NaCl solution as a barrier solution. The methane content in biogas was determined using DRÄGER X-am 5600 gas analyser (Drägerwerk AG & Co. KGaA, Germany). Methane yield of substrates was deducted from the overall methane yield of individual fermenters processing the substrate + inoculum. That gave the actual amount of methane produced from the samples tested. Methane yield was converted to standard conditions (T_0 = 273.15 K, p_0 = 101.325 kPa).

Produced biogas and methane volumes were expressed in NL (normal litres). Methane yield was expressed as NmL (normal millilitre) per g of organic dry matter in the added inoculum.

Equation used for the calculation of the biogas volume was used and edited from (Karafiát, et. al., 2013; Trávníček, et. al., 2017):

$$GP = h * V * \left(\frac{273}{(t+273)} + 273\right) * \left(\frac{(p+kv-pW)}{1013.5}\right)[Nm3]$$

where
GP is the volume of biogas [Nm^3]
h is the height of gas in the glass cylinder [m]
V is cylinder volume [L]
t is the temperature [°C]
p is the pressure of the biogas [bar]
kv height of liquid [m]
pW is the pressure of water vapour [bar]

5.4 qPCR amplification of 16S rRNA gene and sequencing

The 16S rRNA gene is the most commonly used marker gene for description of both the whole bacterial and archaeal communities.

5.4.1. Sample collection and isolation of DNA

During the inoculum sample collection (day 0) at the WWTP Modřice and ABP Čejč, 2 x 50 ml from each collection were taken for DNA isolation and stored in the freezer until the DNA isolation was performed. Those samples gave us the information about the inoculum microbiome composition unaffected by addition of *Chlorella* sp. in batch fermenters during the biomethanation assay. After 33 days (in the end) of experiment, the samples for DNA isolation were collected from the residual 14 fermenters. As a control, 4 fermenters were used and the samples had already been stored in the freezer (2 x 50 ml). 50 ml of each sample from wastewater sludge and maize silage leachate with *Chlorella* sp. (with and without lipid enhancement) were taken from the mixtures (melled appropriately to the type of substrate sample) of the remaining 14 fermenters according to the Table 6. Those samples gave us the information about the possible change of inoculum microbiome composition affected by addition of *Chlorella* sp.

50 millilitres of liquid were collected to conical centrifuge tubes and stored in the freezer until the DNA isolation was performed.

DNA was extracted from 5 silage (R2A, R2B, R2C, R2D, R2E) and 5 sludge (R2F, R2G, R2H, R2I, R2J) samples by QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). This Kit was selected for the isolation of total DNA based on high yield, availability and frequent use in recent publications. The isolation was followed exactly according to the instructions of the standard protocol supplied by the manufacturer.

Table 6. Designation of DNA samples

Designation of DNA sample	**Designation of fermenter**	**Type of used inoculum**	**Type of added substrate**
R2A	R1	ABP Čejč	-
	R2	ABP Čejč	-
R2B	R3	ABP Čejč	*Chlorella* sp. with lipid content (2017)
	R4	ABP Čejč	*Chlorella* sp. with lipid content (2017)
R2C	R5	ABP Čejč	*Chlorella* sp.
R2D	R6	ABP Čejč	*Chlorella* sp. without lipid content
	R7	ABP Čejč	*Chlorella* sp. without lipid content
R2E	R8	ABP Čejč	*Chlorella* sp. with lipid content
	R9	ABP Čejč	*Chlorella* sp. with lipid content
R2F	R10	WWTP Modřice	-
	R11	WWTP Modřice	-
R2G	R12	WWTP Modřice	*Chlorella* sp. with lipid content (2017)
	R13	WWTP Modřice	*Chlorella* sp. with lipid content (2017)
R2H	R14	WWTP Modřice	*Chlorella* sp.

R2I	R15	WWTP Modřice	*Chlorella* sp. without lipid content
	R16	WWTP Modřice	*Chlorella* sp. without lipid content
R2J	R17	WWTP Modřice	*Chlorella* sp. with lipid content
	R18	WWTP Modřice	*Chlorella* sp. with lipid content

5.4.2. Quantitative PCR reaction and sequencing

After the isolation of DNA (what about algae removal), amplification of 16S rRNA was performed by using quantitative PCR reaction. For this reaction following primers were used as a template:

- 515F-Y Forward primer 5′-GTG YCA GCM GCC GCG GTA A-3′
- 806R Reverse primer 5′-GGA CTA CHV GGG TWT CTA AT-3′

This primer set targeted the V4 hyper-variable region of 16S rRNA thus provided immensely powerful ability to spot microbial taxa presented in a given sample and tended to correspond well to trends in overall gene content (Caporaso, et. al., 2011).

PCR amplification was performed in a 25 µl reaction volume containing KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), 0.3 µm primers and 0.4 µl of isolated DNA. The PCR program consisted of a hot start at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s with a 50 % thermal ramp, and extension at 72 °C for 30 s. PCR was terminated by a final extension at 72 °C for 5 min, Table 7.

After PCR, the amplification products were purified using and UltraClean PCR Clean-Up Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Next 5 µl of each of the purified PCR samples was tagged with NEXTERA indexes using the Nextera XT Indexes Kit (Illumina, San Diego, CA, USA) and the KAPA HiFi HotStart ReadyMix (Kapa Biosystems) in a 25 µl reaction volume according to the Illumina protocol. PCR products were purified using a GeneRead DNA size selection Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Samples were eluted with 17 µl of 1xTE buffer, quantified using the Kapa Library Quantification Kit (Kappa Biosystems), and diluted to 4 nM concentrations.

The final library was subjected to NGS using a MiSeq® Reagent Kit v3 (2 x 200 paired-end sequencing) and an Illumina MiSeq sequencer

according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Table 7. The PCR reaction settings

Reaction steps	Temperature	Cycle time	Repeat
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	30 s	↳ 30 cycles ↰
Primer application	60 °C	30 s	
Elongation	72 °C	30 s	
Final elongation	72 °C	5 min	

5.4.3. Sequences analysis

The raw sequences were processed by using the DADA2 package (version 1.16.) according to DADA2 pipeline tutorial. A set of Illumina sequenced paired-end fastq files was split (demultiplexed) into individual per-sample fastq files. Firstly, reads were filtered and trimmed (maximum of 5 ambiguous bases, expected error threshold of 5 and the last 20 bases truncated). Filtered reads were de-replicated and unique sequences were extracted. Identified sequencing errors were then removed using learned error rates and quality profiles of reads. Subsequently, reads were merged and chimeras removed. The DADA2 package was used to provides a native implementation of the naive Bayesian classifier method for assign taxonomy to the sequence variants. Final reads were clustered at 97 % identity against SILVA v138 16S reference database (Quast, et. al., 2013).

Overall, the end product is an amplicon sequence variant (ASV) table, a higher-resolution analogue of the traditional OTU table. ASVs were used because record the number of times each exact amplicon sequence variant was observed in each sample instead clustering similar sequences (into OTUs).

5.4.4. Statistical analysis

Using experimental data, basic mathematical operations were performed. Graphs were created in Microsoft® Excel®.

6 Results

6.1 The results from biomethanation assay

18 fermenters were monitored with two types of inoculums and five types of *Chlorella* sp. substrates, it is shown in the Table 4. in the materials and methods chapter. The volumes of biogas and methane were measured and calculated as described in the methodology, but the total volume of biogas and methane was corrected by deducting the inoculum biogas and methane production. Total volume of biogas and methane of different types of substrates together with the measured percentage composition of methane are shown in Table 8.

Table 8. Overall characteristics of each fermenter

Fermenter designation	**Type of the substrate**	**Total corr. volume of produced biogas [NL]**	**Total corr. volume of produced CH_4 [NL]**	**CH_4 [%vol]**
R1	-	0.32	0.25	44.70
R2	-	0.30	0.23	45.10
R3	*Chlorella* sp. with lipid content (2017)	1.13	0.83	57.60
R4	*Chlorella* sp. with lipid content (2017)	1.05	0.77	57.50
R5	*Chlorella* sp.	1.32	0.96	58.90
R6	*Chlorella* sp. without lipid content	1.17	0.86	58.10
R7	*Chlorella* sp. without lipid content	1.16	0.85	58.30

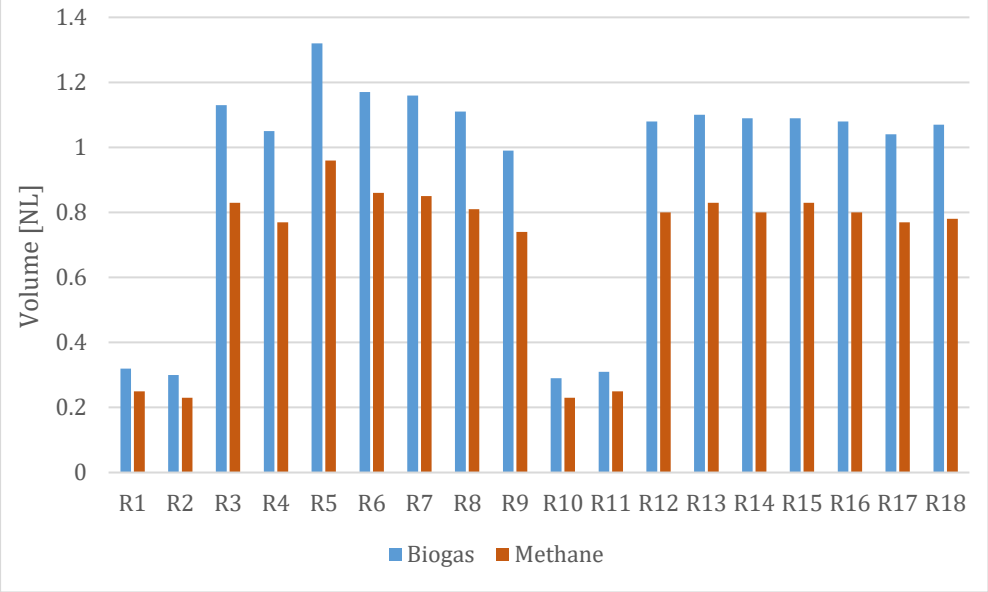
R8	*Chlorella* sp. with lipid content	1.11	0.81	57.80
R9	*Chlorella* sp. with lipid content	0.99	0.74	57.10
R10	-	0.29	0.23	47.60
R11	-	0.31	0.25	47.70
R12	*Chlorella* sp. with lipid content (2017)	1.08	0.80	58.60
R13	*Chlorella* sp. with lipid content (2017)	1.10	0.83	58.60
R14	*Chlorella* sp.	1.09	0.80	58.20
R15	*Chlorella* sp. without lipid content	1.09	0.83	58.50
R16	*Chlorella* sp. without lipid content	1.08	0.80	58.80
R17	*Chlorella* sp. with lipid content	1.04	0.77	58.50
R18	*Chlorella* sp. with lipid content	1.07	0.78	58.40

The results of biomethanation assay showed a significantly higher amount of produced biogas as well as an increase in the methane volume in all maize silage samples (R3, R4, R5, R6, R7, R8 and R9) and all sludge samples (R12, R13, R14, R15, R16, R17 and R18) after addition of algae compared to their control samples R1 and R2 (ABP Čejč inoculum) and R10 and R11 (WWTP Modřice inoculum).

Interestingly, in the silage samples R3, R4, R8 and R9 (where *Chlorella pyrenoidosa* with lipid enrichment was added) lower biogas and methane production was observed when comparing samples with ABP Čejč inoculum with algae between each other. On the other hand, the silage samples R5, R6 and R7 (where *Chlorella pyrenoidosa*

without lipid enrichment was added) showed higher increase of volume of produced biogas as well as methane volume (Graph 2.).

The samples with inoculum from wastewater treatment plant in Modřice with different *Chlorella pyrenoidosa* substrates showed comparably almost the same amount of produced methane and biogas (Graph 2.).



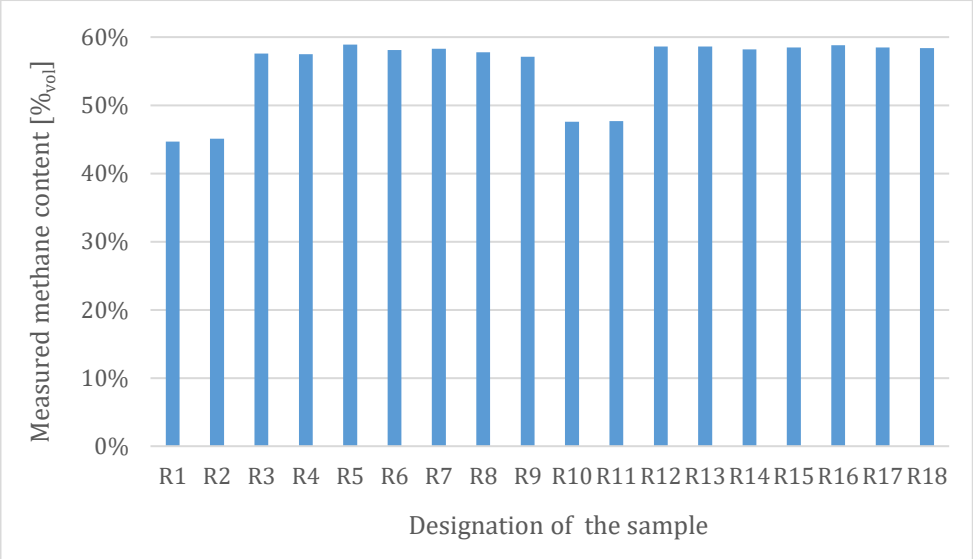
Graph 2. The total volumes of produced biogas and methane in the fermenters during fermentation process

The measurement of biogas composition in the R3, R4, R5, R6, R7, R8, R9 sample showed higher methane content when comparing to their control samples (R1 and R2). The same phenomenon was observed in the R12, R13, R14, R15, R16, R17 and R18 sample compared to their (R10 and R11) control samples (Graph 3.).

The differences between the individual samples with different substrates showed no or little changes in the biogas quality. The methane content in the last day of biomethanation assay fluctuated around 58 % ± 0.90 %. Control samples showed methane content 46.20 % ± 1.50 % in all samples (Graph 3.). As well as the increase of methane content was observed also increase of measured CO_2.

Chlorella pyrenoidosa biogas and methane yield were also recalculated in in all samples per unit of organic dry matter, the results are in the Table 9. Then the specific production of methane is shown in the Graph 4. The yields of samples with the same inoculum and

Chlorella biomass were always averaged together. The overall biogas yield per ton of substrate weight is shown in the Graph 5.

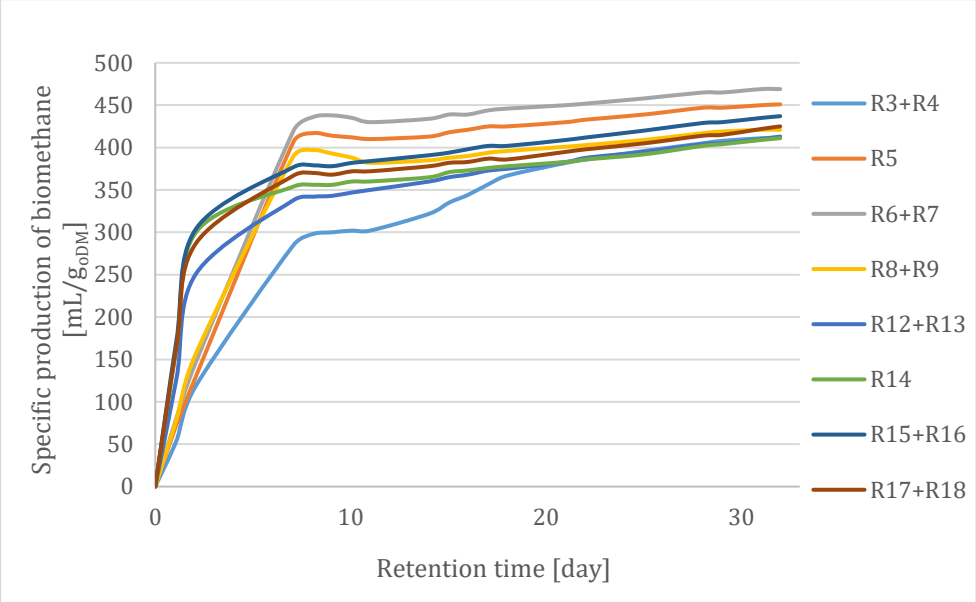


Graph 3. Determined biomethane content in all samples

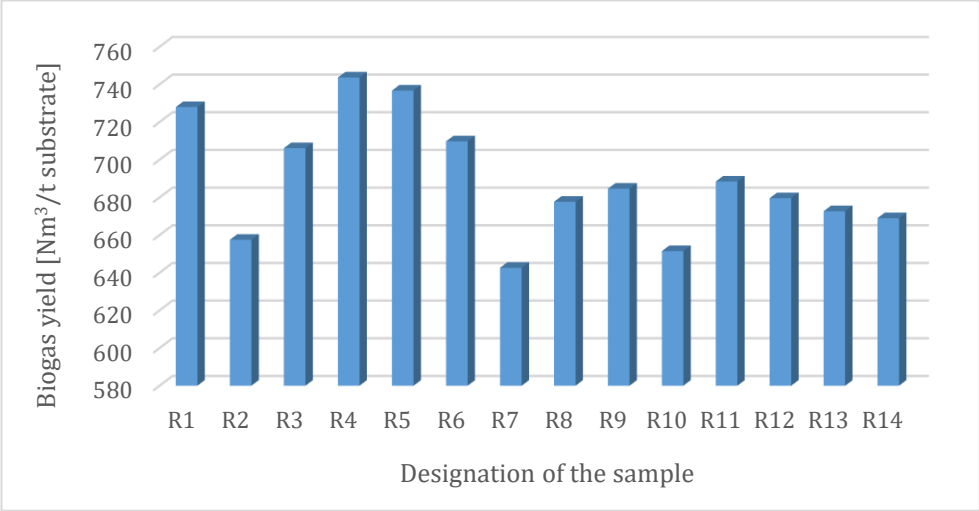
Table 9. *Chlorella pyrenoidosa* biogas and biomethane yields per unit of organic matter

Designation of fer-menter	**Type of biomass used**	**Biogas yield [mL/g_{oDM}]**	**Bio-methane yield [mL/g_{oDM}]**
R3	*Chlorella* sp. with lipid content (2017)	590	433
R4	*Chlorella* sp. with lipid content (2017)	534	391
R5	*Chlorella* sp.	620	451
R6	*Chlorella* sp. without lipid content	640	470
R7	*Chlorella* sp. without lipid content	638	467
R8	*Chlorella* sp. with lipid content	607	444
R9	*Chlorella* sp. with lipid content	531	397
R12	*Chlorella* sp. with lipid content (2017)	554	410

R13	*Chlorella* sp. with lipid content (2017)	553	415
R14	*Chlorella* sp.	562	411
R15	*Chlorella* sp. without lipid content	578	438
R16	*Chlorella* sp. without lipid content	587	435
R17	*Chlorella* sp. with lipid content	573	426
R18	*Chlorella* sp. with lipid content	582	423



Graph 4. Specific biomethane production of *Chlorella pyrenoidosa* samples



Graph 5. The overall biogas yield per ton of substrate weight

6.2 The results from sequencing

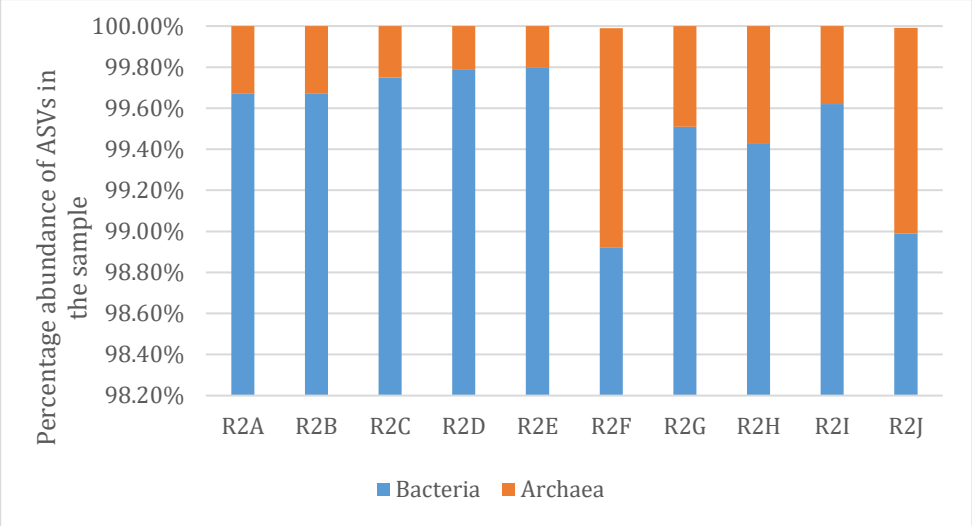
Quantitative PCR and sequence analysis of the gene for the 16S rRNA subunit provided a large amount of information about the composition of the microbial community of both inoculums from ABS Čejč and community of WWTP Modřice sample. Moreover, the results showed considerable variability in terms of composition and number of individual groups of microorganisms by using different *Chlorella* sp. substrates as well. The sequencing of the samples was performed exactly as described in the methodological part of the work.

6.2.1. Occurrence and composition of different kingdoms in the samples

The sequence analysis showed the presence of microorganisms from 3 different kingdoms namely *Bacteria*, *Archaea* and *Eukaryota*. The total data in their number is in the Table 10. It is shown that the incidence of bacterial and archaeal species is predominance. In the case of *Eukaryota*, it is speculative whether the sequences in the order of units of ASVs can be considered as positive samples. Therefore, the kingdom of *Eukaryota* is not statistically significant and will therefore not be further evaluated. The percentage composition of *Bacteria* and *Archaea* is shown in the Graph 6.

Table 10. The number of microorganisms from different kingdoms in samples

Designa-tion of the sample	The number of *Bacteria*	The number of *Archaea*	The number of *Eukaryota*	Total sum
R2A	105259	351		105610
R2B	95322	316		95638
R2C	89185	219		89404
R2D	90228	189		90417
R2E	96704	197		96901
R2F	88670	958	7	89635
R2G	94783	469		95252
R2H	83313	743		83792
R2I	86340	329		86669
R2J	96793	986	2	97781



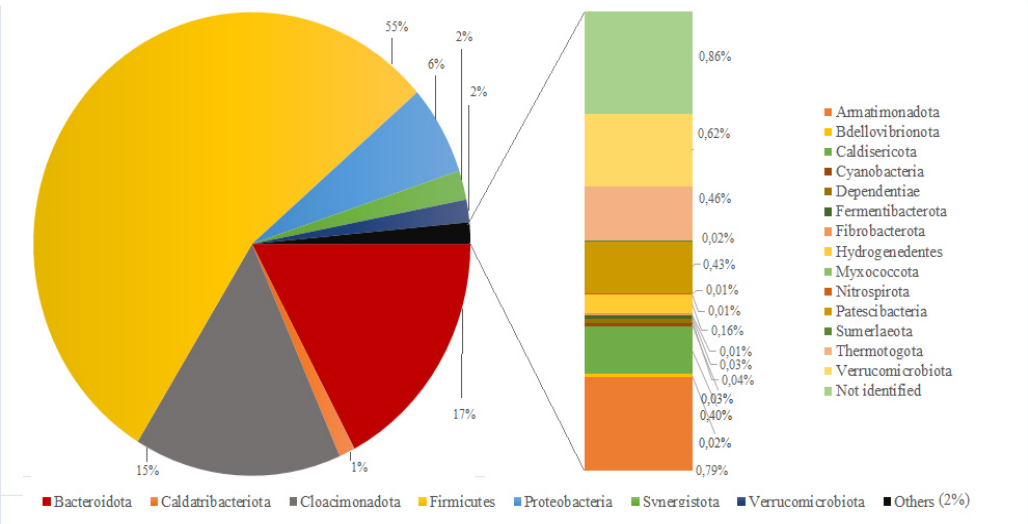
Graph 6. The percentage composition of *Bacteria* and *Archaea* kingdom in the samples

6.2.2. Occurrence and composition of different phyla in the control samples

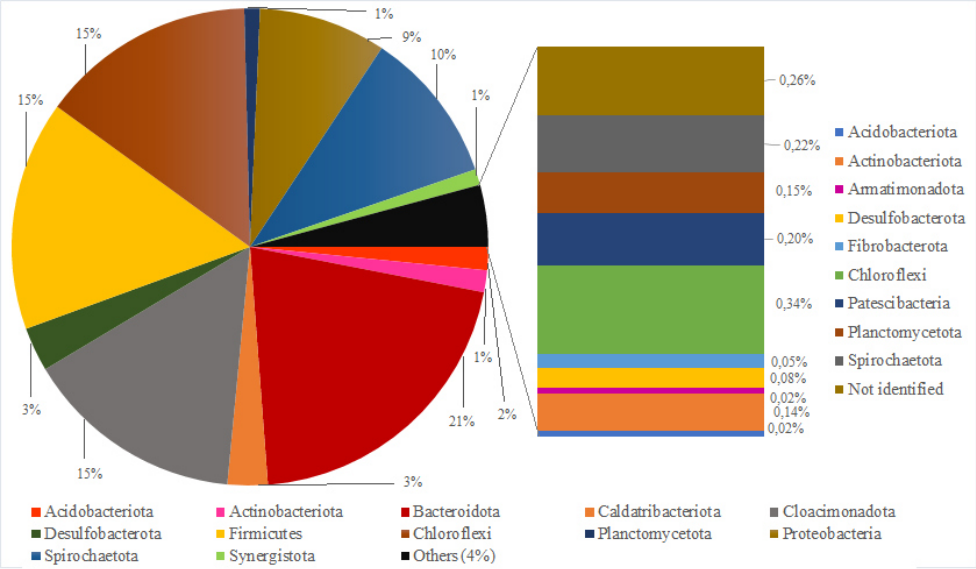
The sequence analysis of the inoculum sample taken the first day of experiment from the biogas silage leachate from ABP in Čejč showed high variation of different bacterial phyla (Graph 7.). A typical biogas community members, with a great ability to degrade a wide range of complex organic molecules, belonging to the bacterial phyla *Firmicutes*

(55 %) and *Bacteriodetes* (SDB *Bacteriodota,* 17 %) were found as most abundant. Representatives from the phyla *Proteobacteria* (6 %) or *Cloacimonadota* (15 %) were also found although in lower abundance. At lower levels were presented phyla such as *Synergistota* (2 %), *Verrucomicrobiota* (2 %), *Caldatribacteriota* (1 %) and phyla with prevalence under 1 %: *Chloroflexi* (0.34 %), *Spirochaeota* (0.22 %), *Patescibacteria* (0.20 %), *Planctomycetota* (0.15 %), *Actinobacteriota* (0.14 %), *Desulfobacterota* (0.08 %), *Fibrobacterota* (0.05 %), *Acidobacteriota* (0.02 %), *Armatimonadota* (0.02 %). Not identified was 0.26 %.

On the other hand, the sequence analysis of the inoculum taken directly from sewage sludge anaerobic stabilization tank at WWTP Modřice the first day of experiment too shows little higher variation of bacterial phyla (Graph 8.). *Bacteriodetes* (SDB *Bacteroidota*) was found with 21 % prevalence and 15 % has several phyla: *Firmicutes*, *Cloacimonadota* and the phylum *Chloroflexi* which has been shown to dominate in digesters operating with municipal wastewater. *Proteobacteria* were present with 9 % and approximately 10 % has also *Spirochaetota*. *Desulfobacterota*, *Caldatribacterota* were present with 3 %, *Acidobacteriota* (2 %) and with 1 % were *Actinobacteriota*, *Synergistota* and *Planctomycetota*. Other phyla such as: *Armatimonadota* (0.79 %), *Verrucomicrobiota* (0.62 %), *Thermotogota* (0.46 %), *Patescibacteria* (0.43 %) etc. were less represented (under 1 %) but more variable (it is shown in the Graph 8.).



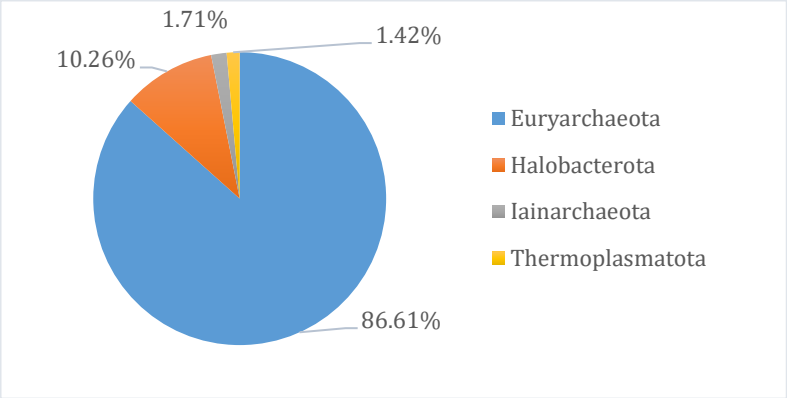
Graph 7. The percentage abundance of bacterial phyla in ABP Čejč sample



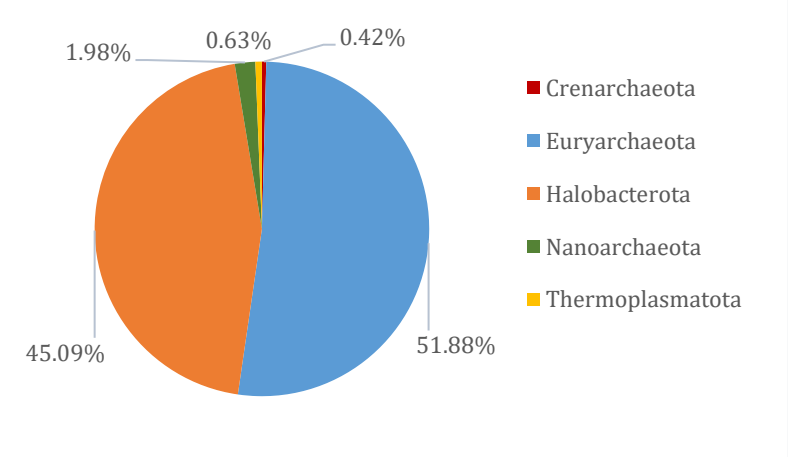
Graph 8. The percentage abundance of bacterial phyla in WWTP Modřice sample

The bacterial community clearly predominates over *Archaea* (Graph 6.), but nevertheless in some samples they have increased above 1 % and therefore archaeal community changes will be further evaluated in control samples and subsequently in all other samples. Methanogenic community usually represents a few percent of the whole microbial community in biogas digester but still performing critical role.

Sequences analysis of the control sample from ABP in Čejč showed the largest proportion of the archaeal phylum *Euryarchaeota* (86.61 %) instead of the control sample from WWTP in Modřice where it makes up only 51.88 %. *Halobacterota* showed higher abundance in sludge sample (45.09 %) than in maize silage leachate (10.26 %). Other phyla were less present from all archaeal community in the control samples (Graph 9. and Graph 10.).



Graph 9. The percentage composition of archaeal community in ABP Čejč sample



Graph 10. The percentage composition of archaeal community in WWTP Modřice sample

6.2.3. Occurrence and composition of different phyla in all samples

The results showed that phyla predominantly present were *Firmicutes*, *Bacteroidetes* (SDB *Bacteroidota)* and *Cloacimonadota* in all samples (Graph 11.).

When comparing samples where maize silage leachate was dosed with a control sample of pure inoculum (R2A), the samples (R2B, R2D, R2C and R2E) did not show large shifts in the composition of bacterial phyla but there are some significant changes.

In the R2B sample with addition *Chlorella* sp. with lipid enrichment is a higher increase of *Cloacimonadota* (15.29 % vs. 21.50 %), slight increase of *Firmicutes* (54.71 % vs. 55.69 %), *Chloroflexi* (0.34 % vs. 0.43 %) or other phyla with their abundance under 1 % of all

microbial community in R2B sample. On the other hand, the significant decrease was observed within phylum *Proteobacteria* where their number dropped from 6.21 % to 0.60 %. A smaller reduction in the number of microbial communities is also observed within *Bacteroidota* (17.18 % vs. 16.47 %), *Synergistota* (2.04 % vs. 1.68 %), *Verrucomicrobiota* (1.58 % vs. 1.09 %), *Caldatribacteriota* (1.18 % vs. 1.09%), *Desulfobacterota* (0.08 % vs. 0.04 %) or other phyla with their abundance lower than 1 %.

The sample R2C where *Chlorella* sp. without lipid enrichment was dosed showed increase in the number of *Firmicutes* (54.71 % vs. 58.70 %) and *Cloacimonadota* (15.29 % vs. 17.66 %) as well. The light increase was observed also with the phyla: *Synergistota* (2.04 % vs. 2.14 %), *Acidobacteriota* (0.02 % vs. 0.05 %), *Actinobacteriota* (0.14 % vs. 0.21 %) or *Spirochaetota* (0.22 % vs. 0.27 %). The abundance of *Bacteroidota* stayed almost unchanged (17.18 % vs. 17.40 %). As with the previous sample big decrease was observed in the phylum of *Proteobacteria* where the number dropped from 6.21 % to 0.33 %. There has been a light reduction in the number of *Verrucomicrobiota* sp. (1.58 % vs. 1.02 %), *Caldatribacteriota* (1.18 % vs. 1.11 %), *Desulfobacterota* (0.08 % vs. 0.06 %) and *Chloroflexi* (0.34 % vs. 0.28 %). The variation of other phyla also decreased (0.86 % vs. 0.68 %).

The R2D sample where *Chlorella* sp. without lipid enrichment was added did not also show huge difference from the control sample R2A. There was noted increased content of *Firmicutes* (54.71 % vs. 56.60 %), *Cloacimonadota* (15.29 % vs. 18.15 %), *Synergistota* (2.04 % vs. 2.77 %), *Verrucomicrobiota* (1.58 % vs. 1.79 %). The number of *Bacteroidota* stayed approximately unchanged (17.18 % vs. 17.40 %) as well as for example *Spirochaetota* (0.22 % vs. 0.22 %) or *Desulfobacterota* (0.08 % vs. 0.11 %). The difference was shown with phylum *Proteobacteria* where initial percentage 6.21 % (control sample) dropped to 0.68 % but slight decrease was observed with other phyla too.

The last sample R2E from fermenter filled with silage maize leachate with addition *Chlorella* sp. substrate but enhanced with lipid content show similarities with R2B sample (the sample with lipid enrichment too), for instance increase of phyla *Cloacimonadota* (15.29 % vs. 18.15 %) but *Frimicutes* (54.71 % vs. 54.57 %) stayed approximately the same. The results from sequencing show increased amount of: *Bacteroidota* (17.18 % vs. 21.04 %) which is comparable with R2C sample but decrease of *Proteobacteria* (6.21 % vs. 0.63 %) that is similar to R2B or R2D samples. Other phyla under 1 % stayed unchanged

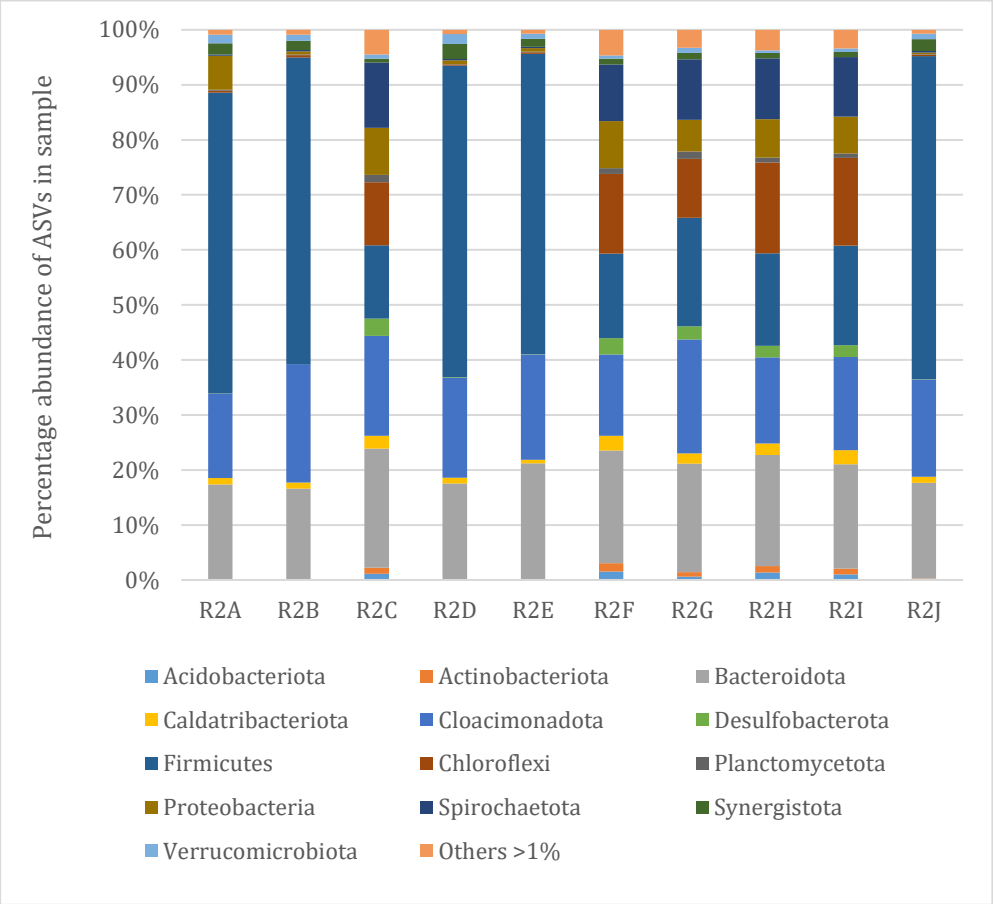
as *Desulfobacterota* (0.08 % vs. 0.08 %) or slightly decreased (0.86 % vs. 0.68 %).

The last samples R2F, R2G, R2H, R2I, R2J with different *Chlorella* sp. substrates from last 5 fermenters filled with sludge from anaerobic stabilization also did not show huge compositional changes between phyla.

When comparing R2G (sample with enhanced *Chlorella* sp.*)* to the control sample R2F, there is higher amount of *Firmicutes* (15.40 % vs. 19.69 %), *Cloaciminadota* (14.75 % vs. 20.76 %) and light increase of *Spirochaetota* (10.29 % vs. 11.01 %), *Synergistota* (1.06 % vs. 1.20 %) and *Verucomicrobiota* (0.61 % vs. 0.90 %). The sequences analysis showed slight decrease of phyla: *Bacteriodota* (20.52 % vs. 19.74 %), *Chloroflexi* (14.46 % vs. 10.73 %), *Proteobacteria* (8.56 % vs. 5.75 %), *Acidobacteriota* (1.55 % vs. 0.63 %), *Actinobacteriota* (1.47 % vs. 0.79 %) and others stayed approximately unchanged e.g., *Desulfobacterota* (2.97 % vs. 2.39 %).

Samples R2H and R2I showed almost the same prevalence of microbial communities and differ little from control sample. The R2H shows light increase of *Chloroflexi* phylum (14.46 % vs. 16.55 %) and little changes are also within R2I sample with the decrease of phylum *Bacteriodota* (20.52 % vs. 18.97 %), *Protheobacteria* (8.56 % vs. 6.70 %) and increase of *Firmicutes* (15.40 % vs. 18.09 %). Other phyla show similar values.

The sequences analysis of the last sample R2J (*Chlorella* sp. with lipid content) showed higher number of *Cloacimonadota* (14.75 % vs. 18.16 %) and light increase of *Spirochaeota* (10.29 % vs. 11.88 %), *Desulfobacterota* (2.97 % vs. 3.15 %), *Bacteroidota* (20.52 % vs. 21.67 %), *Synergistota* (1.06 % vs. 0.70 %) or *Verrucomicrobiota* (0.61 % vs. 0.77 %). The phylum of *Proteobacteria* stayed unchanged (8.56 % vs. 8.55 %). The phylum *Firmicutes* showed decline (15.40 % vs. 13.32 %) as well as phylum *Chloroflexi* (14.46 % vs. 11.49 %). Other phyla with abundance under 1 % stayed almost the same but with little decrease of their number (4.63 % vs. 4.45 %).



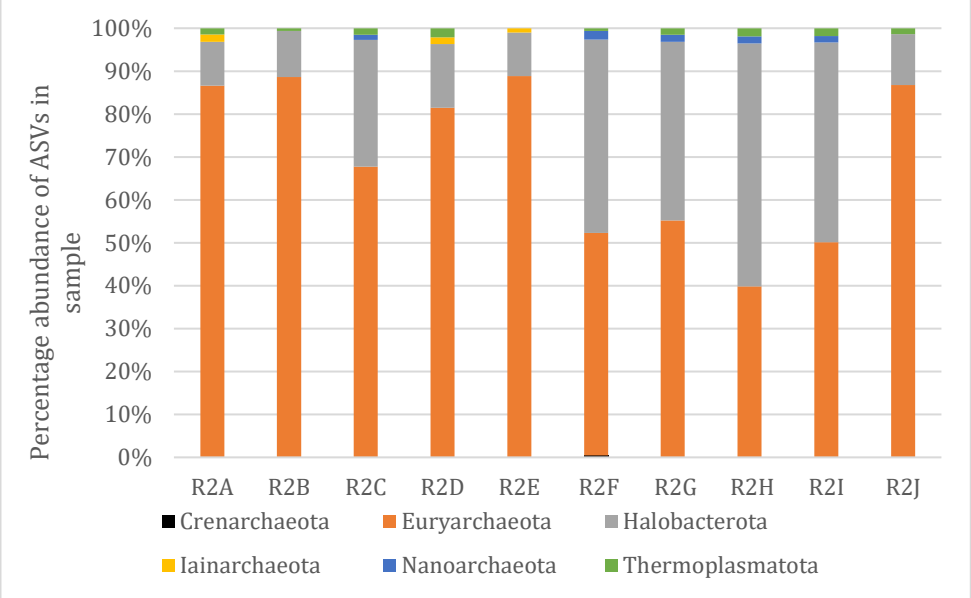
Graph 11. The percentage abundance of bacterial phyla in the samples

The sequences analysis showed that *Euryarchaeota* with *Halobacteria* are two main phyla in all present samples and the difference is only in their number. The phylum *Crenarchaeota* was not present in all samples R2A, R2B, R2C, R2D, R2E from ABS in Čejč, except of R2F control sample, *Crenarchaeota* was not also in sludge samples R2G, R2H, R2I, R2J. On the other hand, phylum *Nanoarchaeota* was present only in the sludge samples and phylum *Iainarchaeota* was present only in the silage leachate samples. The sequences analysis showed also presence of *Thermoplasmatota* in all samples but with difference in their number (Graph 12.).

Except for R2D where was decrease of *Euryarchaeota* observed (86.41 % vs. 81.48 %), in every sample with inoculum from ABP Čejč was showed increase, particularly in samples R2B (86.41 % vs. 88.61 %), R2E (86.41 % vs. 88.83 %) and in the sample R2C, the amount stayed approximately the same (86.41 % vs. 86.76 %). The number of *Halobacterota* increased in samples R2C (10.26 % vs. 11.87 %)

and R2D (10.26 % vs. 14.81 %). In the samples R2B (10.26 % vs. 10.76 %), R2E (10.26 % vs. 10.15 %) stayed almost unchanged. The number of *Iainarchaeota* slightly decrease in the samples R2D *Chlorella* sp. without lipid enrichment (1.71 % vs. 1.59 %), R2E (*Chlorella* sp. with lipid enrichment) sample (1.71 % vs. 1.02 %) and completely dropped to 0 % in the R2B and R2C sample. The content of *Thermoplasmatota* dropped in R2B sample (1.42 % vs. 0.63 %) and R2C sample (1.42 % vs. 1.37 %), increased in R2D (1.42 % vs. 2.12 %) and dropped to 0 % in R2E sample.

The samples from wastewater plant had higher content of *Halobacteria* and the highest was in R2H sample (45.09 % vs. 56.58 %). The lowest content was observed in R2J sample (42.09 % vs. 29.51 %). In the R2G was slightly lower (45.09 % vs. 41.58 %) and in R2I higher (45.09 % vs. 46.50 %). The content of *Euryarchaeota* was opposite the highest in R2J sample (51.88 % vs. 67.75 %), lowest in R2H (51.88 % vs. 39.87 %), slightly higher in R2G (51.88 % vs. 55.22 %) and lower in R2I (51.88 % vs. 50.15 %). The number of *Thermoplasmatota* increased in every sample compared to control R2F, R2G (0.63 % vs. 1.49 %), R2H (0.63 % vs. 1.88 %), R2I (0.63 % vs. 1.82 %), R2J (0.63 % vs. 1.52 %) showed in the Graph 12.



Graph.12 The percentage abundance of archaeal phyla in the samples

6.2.4. Occurrence and composition of different classes in all samples

Sequence analysis revealed the presence of a wide range of different classes. Those with a frequency above 1 % are shown in the Graph 13. The following classes were abundant in all samples: *Clostridia*, *Bacteroidetes* (SDB *Bacteroidota*) and *Cloacimonadia*.

In the class *Clostridia* from silage samples, there has been reduction in their number observed in a R2B sample (with the percentage 18.74 %), R2C (18.77 %) and R2E (17.43 %) comparing to R2A control (20.24 %). On the other hand, the sample R2D showed its increase to 21.11 %. The number of *Bacteroidia* stayed almost the same with approximately 17.42 % ± 0.01 % in the R2C and R2D sample but dropped in R2B to 16.50 % and increased in R2E sample to 21.07 % from original 17.21 % (R2A). The class *Cloacimonadia* showed increase from initial 15.34 % (R2A) in every silage sample to 21.57 % (R2B), 17.70 % (R2C), 18.19 % (R2D) and 19.18 % (R2E).

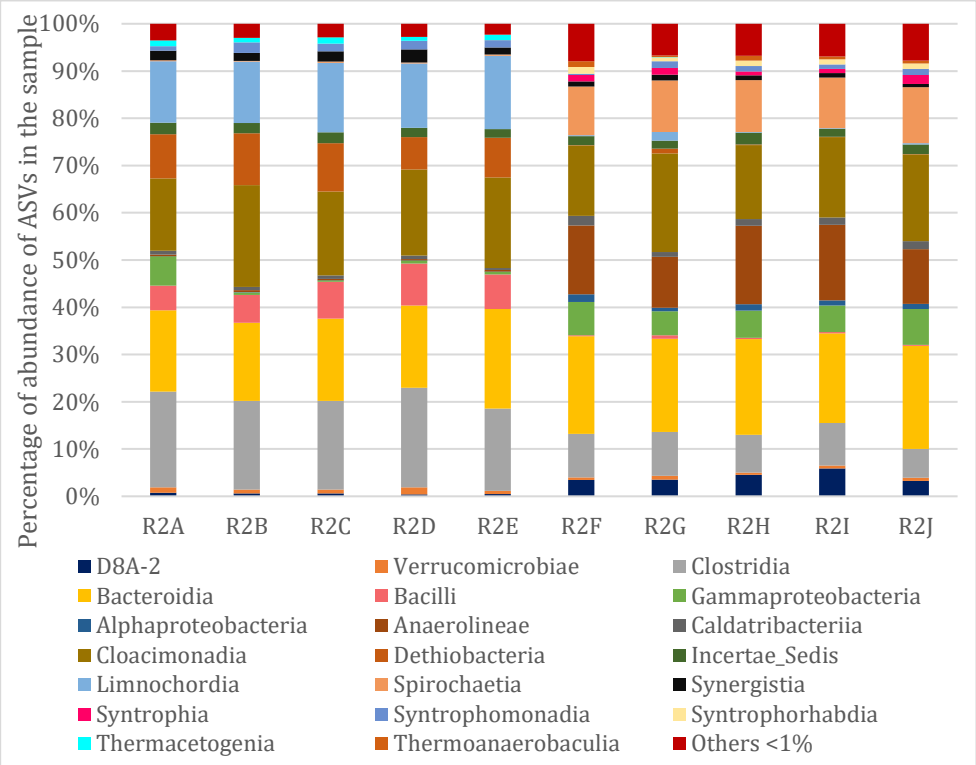
The sequences analysis showed presence of other classes in the silage samples too. The significant changes in classes above 1 % were in the classes: *Bacilli* where was increase observed from initial 5.22 % ASVs (R2A sample) to 5.90 % (R2B), 7.83 % (R2C), 8.89 % (R2D) and 7.29 % (R2E), *Verrucomicrobiae* from initial 1.16 % the decrease was observed R2B (0.87 %), R2C (0.78 %) and R2E (0.68 %) but increase in R2D sample to 1.46 %. In the class of *Gammaproteobacteria* the overall decrease was observed among the samples R2B (0.58 %), R2C (0.31 %), R2D (0.66 %), R2E (0.62 %) from original 6.22 %. The *Dethiobacteria* class, R2A (9.36 %) did not show large shifts in their numbers but changes were observed: R2B (10.97 %), R2C (10.23 %), R2D (6.84 %) and R2E (8.38 %). The changes within *Limnochordia* class (R2A sample with percentage 13.01 %) were also present: R2B (12.97 %), R2C (14.73 %), R2D (13.56 %) and R2E (15.52 %). The system analysed sample with name Incertae_Sedis (belongs to the phylum *Firmicutes*) with the number of ASVs in R2A (2.41 %), R2B (2.17 %), R2C (2.33 %), R2D (2.02 %) and R2E (1.83 %). The changes were also observed with classes: *Synergistia* R2A (2.04 %), R2B (1.68 %), R2C (2.14 %), R2D (2.78 %), R2E (1.50 %), *Syntrophomonadia* R2A (0.93 %), R2B (2.22 %), R2C (1.64 %), R2D (1.86 %), R2E (1.55 %) and *Thermoacetogenia* R2A (1.26 %), R2B (0.97 %), R2C (1.35 %), R2D (0.80 %), R2E (1.16 %). Other classes under 1 % dropped from the original percentage in control sample (R2A) 3.49 % to R2B (2.96 %), R2C (2.82 %), R2D (2.77 %) and in the R2E (2.30 %).

The highest abundant classes such as: *Clostridia*, *Bacteroidia* and *Cloacimonadia* showed little changes in sludge samples.

When comparing number of *Clostridia* in the control R2F (9.24 %) with other samples of sludge with addition of different *Chlorella* sp. substrates, it showed no change in the R2G (9.23 %) and R2I (9.01 %) samples, but the number decreased in R2H (7.98 %) and R2J (6.13 %). The abundance of *Bacteroidota* fluctuated among the samples with initial R2F (20.65 %) to R2G (19.75 %), R2H (20.27 %), R2I (18.96 %) and R2J (21.77 %). The increase within class of *Cloacimonadia* was observed R2F (14.91 %), R2G (20.87 %), R2H (15.70 %), R2I (16.97 %) and R2J (18.34 %).

The *Anaerolineae* and *Spirochaetia* classes were also highly abundant in the wastewater sludge samples but nevertheless *Spirochaetia* did not show significant changes in their number among different sludge samples (11,08 % ± 0,77 %). The percentage of ASVs of *Anaerolineae* among the samples was: R2F (14.55 %), R2G (10.76 %), R2H (16.63 %), R2I (15.99 %) and R2J (11.57 %).

The decline occurred in the class *Caldatribacteria* from initial number 2.02 % (R2F) to R2G (1.06 %), R2H (1.39 %), R2I (1.57 %) and R2J (1.72 %) or the decline was observed within *Gammaproteobacteria* (R2F sample with 6.99 %) in samples R2G (5.08 %), R2H (5.74 %), R2I (5.65 %) but the population increased in sample R2J (7.55 %). The system analysed sample with name DBA-2 (belongs to the phylum *Firmicutes*) with the number of ASVs in R2F (3.45 %), R2G (3.54 %), R2H (4.54 %), R2I (5.90 %) and R2J (3.24 %). There were also slight changes in the other classes, but they were not so significant.



Graph 13. The composition of different classes of the kingdom *Bacteria* in the samples

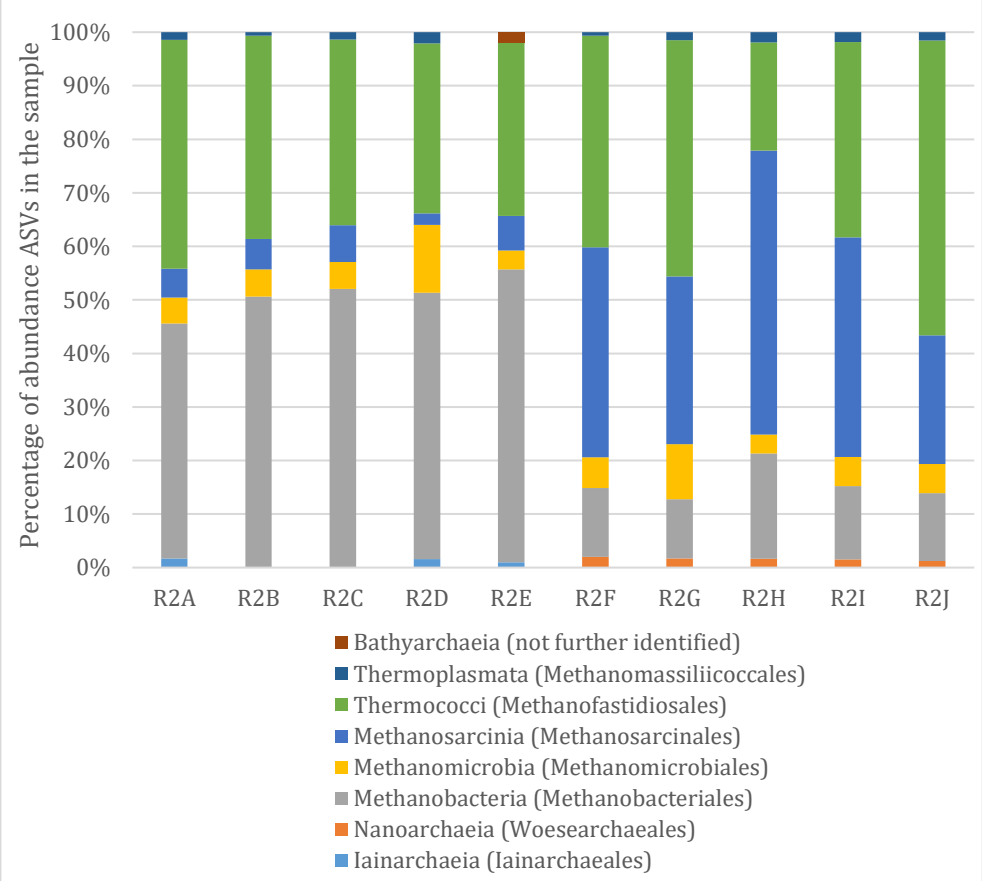
The sequences analysis revealed the presence of 8 classes of the kingdom of *Archaea* which were even further taxonomically divided into orders, as they were the only ones identified in the samples (Graph 14.).

The class of *Bathyarchaeia* was present only in R2E with 2 % but was not further identified. Mostly abundant classes were *Methanobacteria* (order *Methanobacteriales*), *Methanomicrobia* (order *Methanomicrobiales*), *Methanosarcinia* (order *Methanosarcinales*), *Thermococci* (order *Methanofastidiosales*) and *Thermoplasmata* (order *Methanomassiliicoccales*). The classes *Iainarchaeia* and *Nanoarchaeia* were present with low incidence. Class *Iainarchaeia* (order *Iainarchaeales*) was not present in sludge samples. On the other hand, *Nanoarchaeia* (order *Woesearchaeales*) was only present in them.

The silage samples contained higher amount of *Methanobacteria* sp. particularly R2A (44 %), R2B (51 %), R2C (52 %), R2D (50 %) and R2E (56 %). *Thermococci* sp. were also highly abundant with their percentage among samples: R2A (43 %), R2B (38 %), R2C (35 %), R2D (32 %) and R2E (33 %). The significant shift was observed within the class

of *Methanomicrobia* and especially in the R2D sample with 13 % compared to R2A (5 %). *Methanosarcinia* class changed amonged samples: R2A (5 %), R2B (6 %), R2C (7 %), R2D (2 %) and R2E (7 %) and *Thermoplasmata* stayed almost unchanged.

Methanobacteria increase in the sludge samples was observed mainly in R2H sample (13 % vs. 20 %). Little decrease was observed with R2G sample (13 % vs. 11 %), higher in R2I (13 % vs. 14 %) and R2J (13 % vs. 13%) stayed unchanged. The number of *Methanomicrobia*, *Methanosarcinia* and *Thermococci* differently fluctuated among the sludge samples, in particular *Methanomicrobia*: R2F (6 %), R2G (10 %), R2H (4 %), R2I (5 %) and R2J (5 %); *Methanosarcinia*: R2F (39 %), R2G (31 %), R2H (53 %), R2I (41 %) and R2J (24 %) and *Thermococci*: R2F (39 %), R2G (44 %), R2H (20 %), R2I (36 %) and R2J (55 %). The class of *Thermoplasmata* was not so significant.



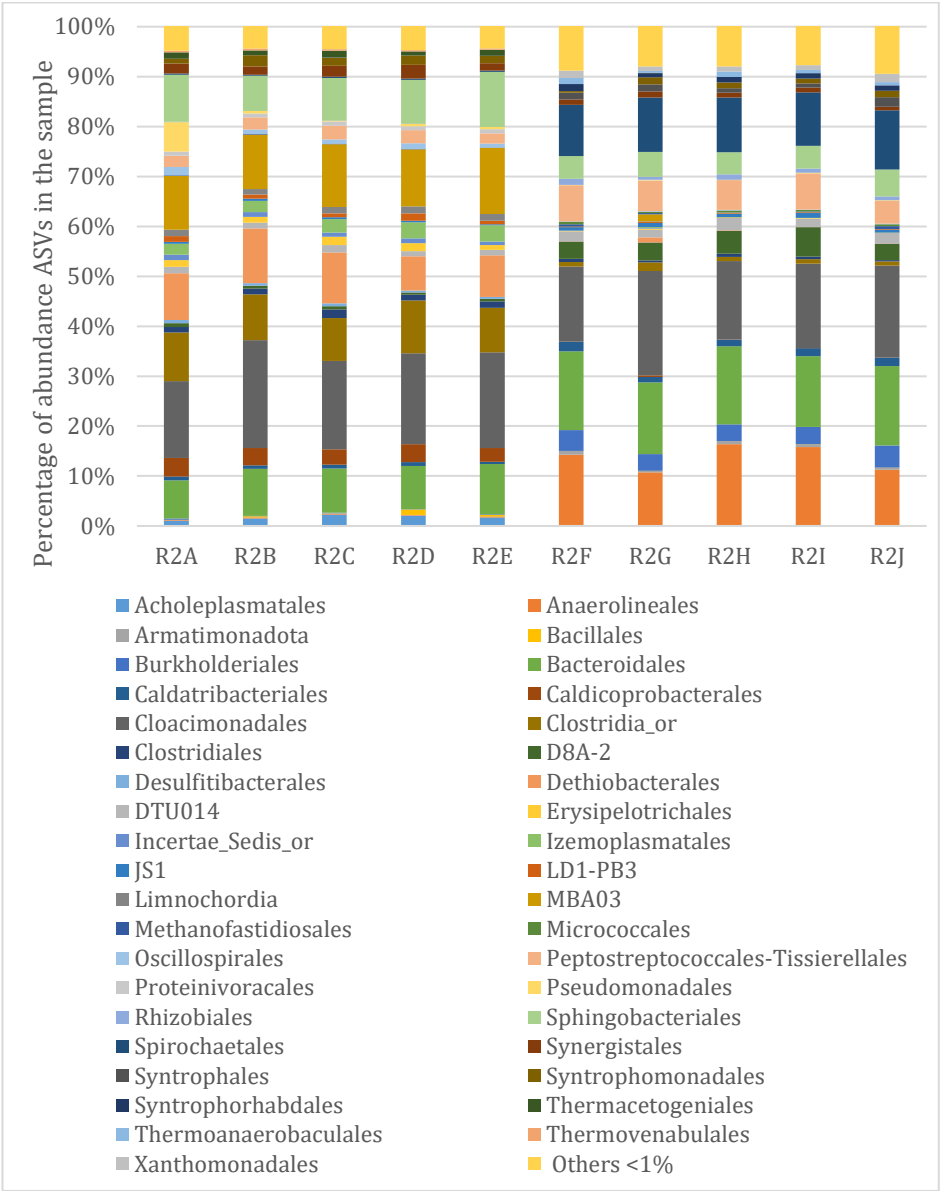
Graph 14. The composition of different classes and orders of the kingdom *Archaea* in the samples

6.2.5. Occurrence and composition of different orders in all samples

Sequence analysis at the level of orders pointed out some changes in the representation of individual orders, but there were not too many changes in their representation, Graph 15.

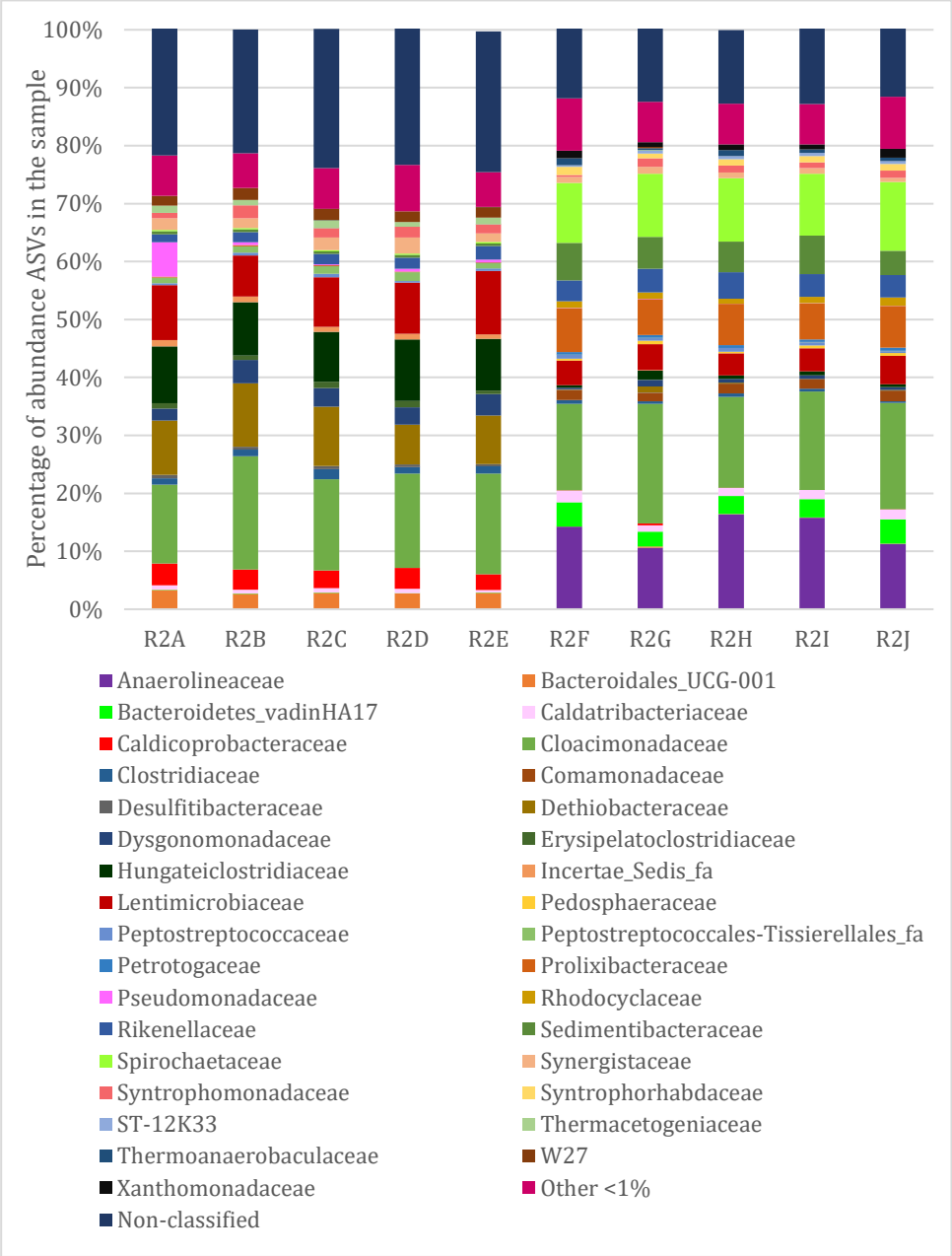
In the silage maize samples were observed changes in the numbers of orders: *Bacteroidales*, *Cloacimonadales* and *Pseudomonadales*. Compared to the reference sample R2A, there was an increase in the number of *Bacteroidales* particularly: R2A (8 %), R2B (9 %), R2C (9 %), R2D (9 %) and R2E (10 %). The percentage of ASVs of *Cloacimonadales* was: R2A (15 %), R2B (22 %), R2C (18 %), R2D (18 %) and R2E (19 %) and ASVs of *Pseudomonadales*: R2A (6 %), R2B (1 %), R2C (0 %), R2D (0 %) and R2E (0 %).

In the sludge samples significant changes observed within orders: *Anaerolineales*, *Bacteroidales* and *Cloacimonadales*. In the *Anaerolineales* order, there was an increase if its abundance observed in the samples R2H (14 % vs. 16 %) and R2I (14 % vs. 16 %) but as well decrease in the samples R2G (14 % vs. 11 %) and R2J (14 % vs. 11 %). *Bacteroidales* and *Cloacimonadales* showed more fluctuations among the samples, in particular *Bacteroidales*: R2F (16 %), R2G (14 %), R2H (16 %), R2I (14 %), R2J (16 %) and *Cloacimonadales*: R2F (15 %), R2G (21 %), R2H (16 %), R2I (17 %), R2J (18 %).

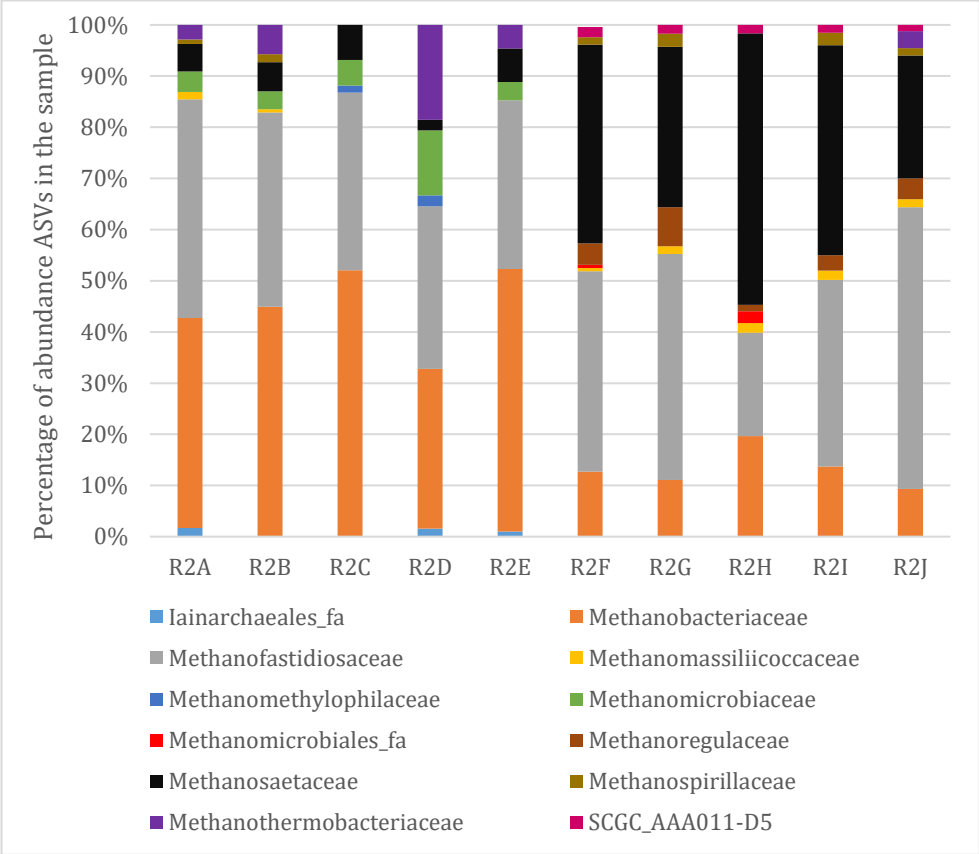


Graph 15. The composition of different orders of the kingdom *Bacteria* in the samples

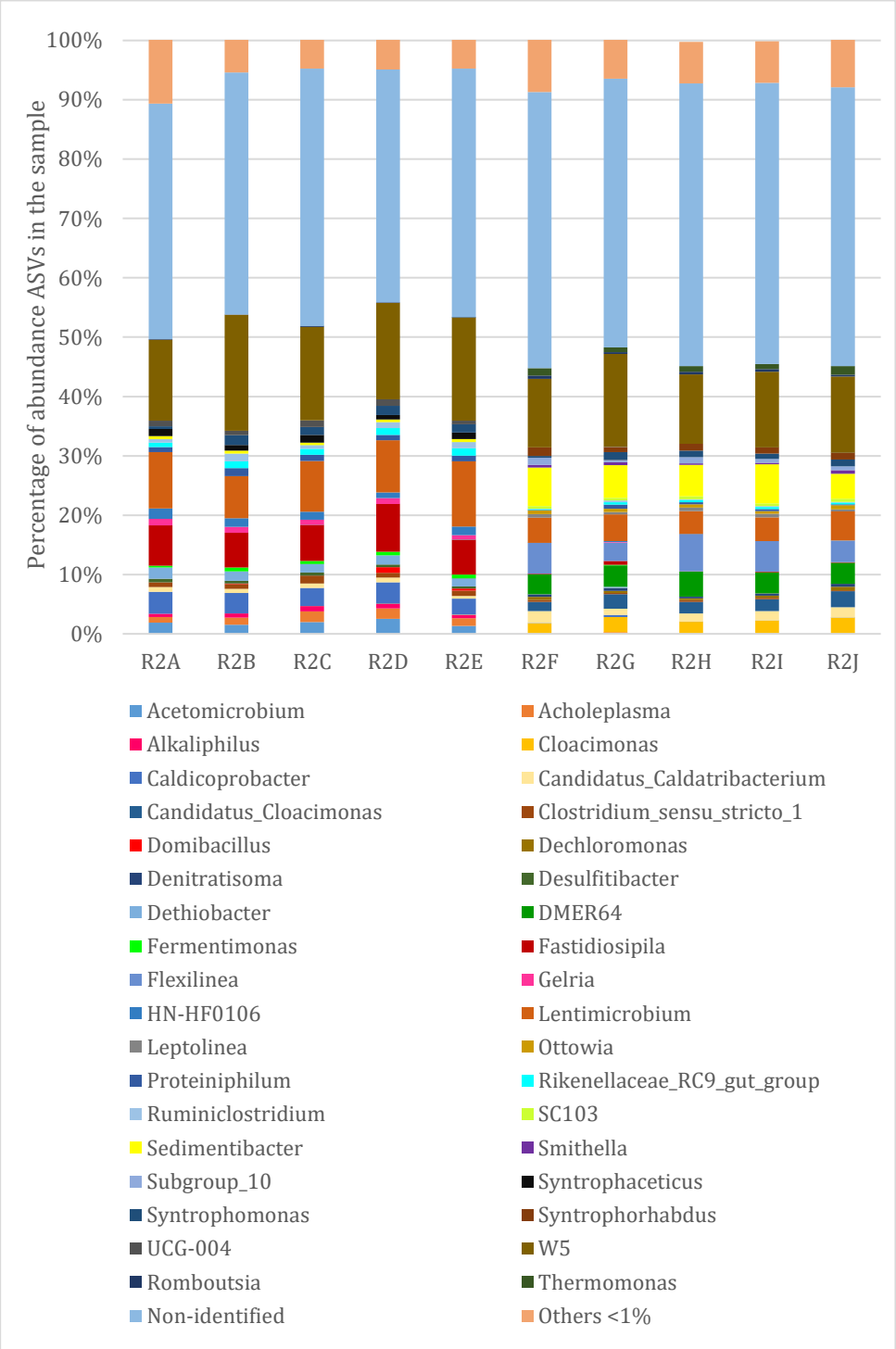
6.2.6. Occurrence and composition of different families and genera in all samples



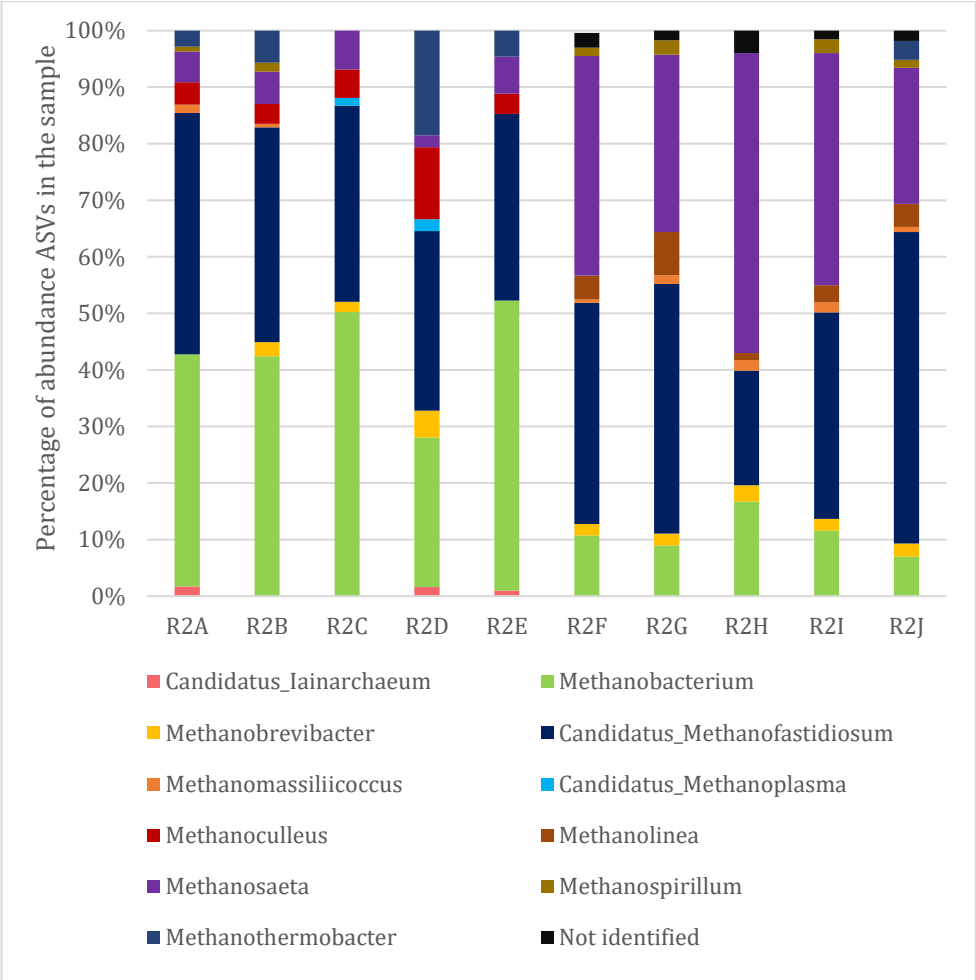
Graph 16. The composition of different families from *Bacteria* domain in all samples



Graph 17. The composition of different families from *Archaea* domain in all samples



Graph 18. The composition of present genera from *Bacteria* domain in all samples



Graph 19. The composition of present genera from *Archaea* domain in all samples

7 Discussion

7.1 The results from biomethanation assay and their meaning

In general, the use of microalgae monocultures has shown many pitfalls (lack of diversified micro-organisms, micro- and macro- nutrients etc.), among them the low C/N ratio is the most limiting thus the method of anaerobic co-digestion (AcoD) of microalgae and carbon-rich substrates proved optimalization of biomethanation process described in the wide range of literature (Cavinato, et. al., 2017; Hagos, et. al., 2017; Klassen, et. al., 2016). The co-digestion of *Chlorella* sp. with inoculums from ABS Čejč and WWTP Modřice, resulted in higher methane production compared to inoculum mono-substrates, which only support these studies.

The fermentation of *Chlorella pyrenoidosa* also showed higher biogas and methane production when was co-digested with maize silage leachate from biogas plant then with sewage sludge from anaerobic stabilization of wastewater treatment plant. Many articles using *Chlorella* sp. are not described in a connection with this observation particularly with inoculums from biogas plants. On the other hand, wastewater biogas production studies by using different *Chlorella* sp. are well described (Wang, et. al., 2013; Zhou, et. al., 2018; Zielinski, et al., 2020). The results therefore indicated that co-digestion of maize silage leachate may be more efficient in biogas production than sewage sludge under mesophilic conditions when co-cultivated with *Chlorella pyrenoidosa.*

Moreover, there has been studies conducted where it was shown that cultivating microalgae under stress condition (e.g., nitrogen, phosphorus starvation) can enhanced their lipid production thus improve the biogas production (Klassen, et. al., 2015). For this reason, the *Chlorella* sp. substrates differed with their lipid content. However, the results showed that the fermentation of *Chlorella pyrenoidosa* did not exceed the amount of biomethane produced in the samples where *Chlorella* sp. was used as a substrate without the addition of lipids. This was especially observed for inoculum samples from the biogas plant. And for the wastewater samples, there was no significant difference in the amount of methane produced between the individual *Chlorella* sp. substrates. The same effect was observed for the total biogas volume. The amount of biogas increased after the addition of algae,

and its composition showed significant higher methane content among all samples.

These results contradicted mentioned scientific research on this topic. The question is why it so but upon closer examination of the whole biomethanation process, various obstacles that affected the final results may have been limiting during anaerobic digestion such as: temperature, pH, retention time, pre-treatment of microalgae etc.

Firstly, the whole process took place under mesophilic conditions and the temperature was kept approximately the same till the end of the experiment. Mesophilic conditions were described as the most suitable for microalgae digestion whether other substrates like wastewater sludge needs more thermophilic conditions (Klassen, et. al., 2016; Thorin, et. al., 2017). It is debatable whether the increase of temperature would be more efficient when AcoD is performed. For instance, in a study where *Chlorella sorokiniana* was used in co-cultivation with wastewater activated sludge, it was shown that methane production increased during 35 °C but in the publication was changed the ratio of sludge to algae mixture (Beltrán, et. al., 2016). Therefore, AD process can be stabilized in mesophilic conditions.

Secondly, the process could be affected by pH because hydrolysis and acidogenesis are described as the most effective in a pH range 4.5-7 and acetogenesis and methanogenesis at 6.8-8.2 (Klassen, et. al., 2016). Measurement during the process is also possible and was performed only at the beginning of experiment. Although, in some articles, two-stage digestion is recommended, it does require more complex process setup than simple one-stage digestion (Ding, et. al., 2016).

Thirdly, it is hard to say if the 33 day of retention time is enough it depends on the degradability of microalgae by microbial community which is closely associated with their ability to degrade microalgae cell walls. The opinions vary among the studies but in majority articles was described retention time of 20-30 days (Beltrán, et. al., 2016; Solé-Bundó, et. al., 2019; Solé-Bundó, et. al., 2018).

Another reason may be the lack of pre-treatment of algae. Many algal cells are resistant to degradation by microbial communities, thus pre-treatment is necessary, but it was not performed in our experiment and it may be crucial (Córdova, et. al., 2018; Giang, et. al., 2019; Klassen, et. al., 2016; Sukačová, et. al., 2019).

Finally, the use of *Chlorella pyrenoidosa* (with or without lipid enrichment in general) showed a higher yield of both biogas and methane in our experiment compared to some other microalgae and *Chlorella* sp., Table 11.

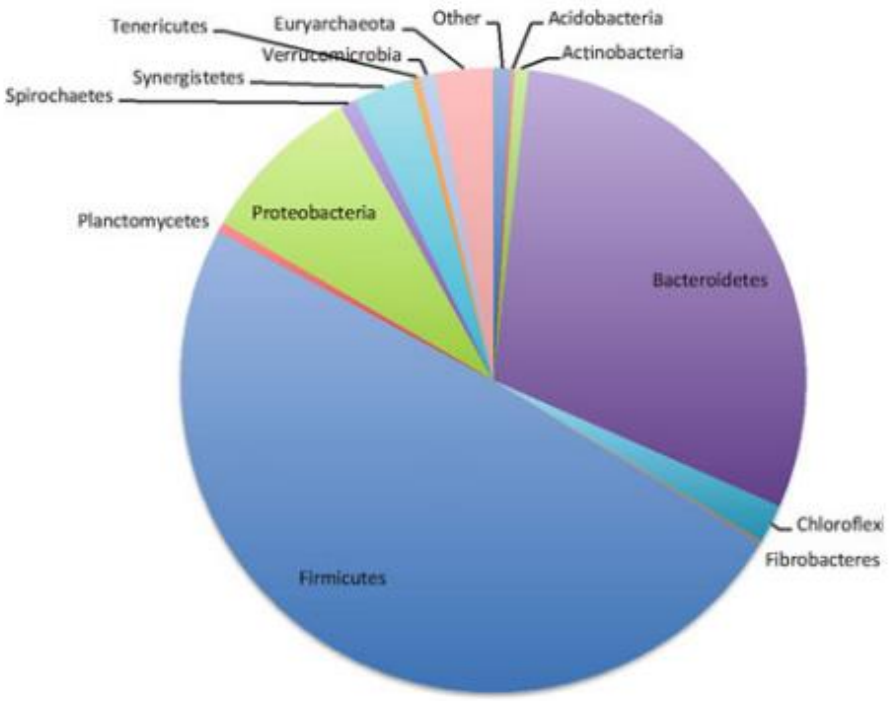
Table 11. The comparison of biogas and methane yields between different *Chlorella* sp. and other common microalgal species (Zabed, et. al., 2020)

Microalgal strains/ Biomass	T (°C)	Time (d)	Biogas yield (mL g^{-1} VS)	Methane yield (mL g^{-1} VS)
Chlamydomonas reinhardtii	38	32	587	387.42
Scenedesmus obliquus	38	32	287	177.94
Scenedesmus dimorphus	35	34–50	–	397
Dunaliella salina	38	32	505	323.2
Euglena gracilis	38	32	485	324.95
Chlorella kessleri	38	32	335	217.75
Arthrospira platensis	38	32	481	293.41
Chlorella pyrenoidosa	36	–	464	264.71
Chlorella vulgaris	36	–	369	195.64
Chlorella minutissima	36	–	340	166.12
Botrycoccus brauni (pretreated biomass)	30	45	–	521
Botryococcus braunii	35	34–50	–	343–370
Chroococcus sp.	36	30	487	267.36
Nannochloropsis oculata	35	30	–	204
Nannochloropsis salina (lipid extracted biomass)	37	40		130
Chlorella sorokiniana	30	42	–	298
Chlorella sorokiniana (pretreated biomass)	30	42	–	388
Chlorella sorokiniana	40–41	71	248	212
Isochrysis sp.	35	34–50	–	408
Chlamydomonas sp.	35	34–50	–	333
Macrocystis pyrifera	37	31	–	545

7.2 The results from sequencing and their meaning

The sequences analysis helped reveal both bacterial and archaeal community within maize silage leachate and wastewater samples. The occurrence of the bacterial and archaeal phyla was similar to those generally reported in the literature as a typical biogas community, Graph 20. with the prevalence of *Bacteroidetes* (SDB *Bacteroidota*) and *Firmicutes* in bacterial community and *Euryarchaeota* in the archaeal one (Schnürer, 2016).

However, representatives of other phyla with lower prevalence also corresponded to the literature, it turned out that the *Cloacimonadota* phylum also forms a significant part of biogas production community. The occurrence was 15 % in both control samples from ABS Čejč and WWTP Modřice.



Graph 20. The typical biogas community (Schnürer, 2016)

The results showed the presence of a wide range of bacterial orders, families and genera, but almost 50 % of the species in the genera were still unidentified. From the most important species according to literature were captured: within *Firmicutes* orders like *Bacillales* (family *Bacillaceae*), genus *Bacillus*, *Clostridiales* (family *Clostridiaceae*) genus *Clostridium_senso_stricto1*, *Peptostreptococcales-Tissierellales* (family *Peptostreptococcales-Tissierellales_fa*) genus *Alkaliphilus*, *Syntrophomonadales* (family *Syntrophomonadaceae*) genus *Syntrophomonas*, *Caldicoprobacterales* (family *Caldicoprobacteraceae*) genus *Caldicoprobacter*, *Desulfitibacterales* (family *Desulfitibacteraceae*) genus *Desulfitibacter*, *Dethiobacterales* (family *Dethiobacteraceae*) genus *Dethiobacter*, *Thermacetogeniales* (family *Thermacetogeniaceae*) genus *Syntrophaceticus* or other (*Oscillospirales*, *Erysipelotrichales, Limnochodiales* etc.).

In the phylum *Bacteriodota* mostly presence of orders *Bacteroidales* and *Sphingobacteriales* was revealed. Members of *Bacteroidales* belonged to the families: *Reikenellaceae* (mostly genus *Reikenellaceae_RC9_gut_group*), *Dysgonomonadaceae* (genus

Proteiniphilum), *Prolixibacteraceae* etc. *Sphingobacteriales* were mostly represented by *Lentimicrobiaceae* family (genus *Lentimicrobium*).

Members of *Cloacimonadota* phylum belonged to the *Cloacimonadales* order (family *Cloacimonadaceae*) and two genera were identified as *W5* and *Candidatus_Cloacimonas*.

Phylum *Proteobacteria* was represented by orders such as *Burkholderiales*, *Rhizobiales*, *Pseudomonadales* or *Xanthomonadales* but within these orders some species were less than 1 % abundant. On the other hand, sequences analysis showed high abundance from *Burkholderiales* (family *Rhodocyclaceae*) genus *Dechloromonas*, from *Rhizobiales* (family *Hyphomicrobiaceae*) genus *Hyphomicrobium* and from *Xanthomonadales* (family *Xanthomonadaceae*) genera like *Stenotrophomonas* or *Thermomonas*.

Sequence analysis captured the presence of prominent archaeal members of the phylum *Euryarchaeota* such as: orders *Methanobacteriales* (family *Methanobacteriaceae*) genus *Methanobacterium*, (family *Methanothermobacteraceae*) *Methanothermobacter*, or from the order *Methanofastidiosales* (family *Methanofastidiosaceae*) the presence of the genus *Candididastus_Methanofastidiosum*.

From *Halobacterota* were present orders: *Methanomicrobiales* (family *Methanomicrobiaceae*) genera *Methanoculles* or *Methanolinea*, (family *Methanospirilaceae*) genus *Methanospirillum* and order *Methanosarcinales* (family *Methanosaetaceae*) genus *Methanosaeta*.

Moreover, sequences analysis recorded the presence of another more abundant phylum *Themococci* namely order *Methanofastidiosales* (family *Methanofastidiosaceae*), designation of the genus *Candidatus_Methanofastidiosum* and these results are in accordance with findings in recent publications (Lee, et. al., 2020; Paddock, et. al., 2020; Wirth, et. al., 2015).

Regarding the representation of members of individual taxonomic units, sequence analysis showed that their percentage changed after the addition of algae. There has been an increase but also decrease of some taxa observed.

The increase was observed in the orders like *Clostridiales*, *Bacteroidales*, *Syntrophomonadales* or within archaeal community in *Methanobacteriales*, *Methanomicrobiales* or *Methanosarcinales* etc., described in the literature to have important function in the anaerobic digestion of algae, Figure 3.

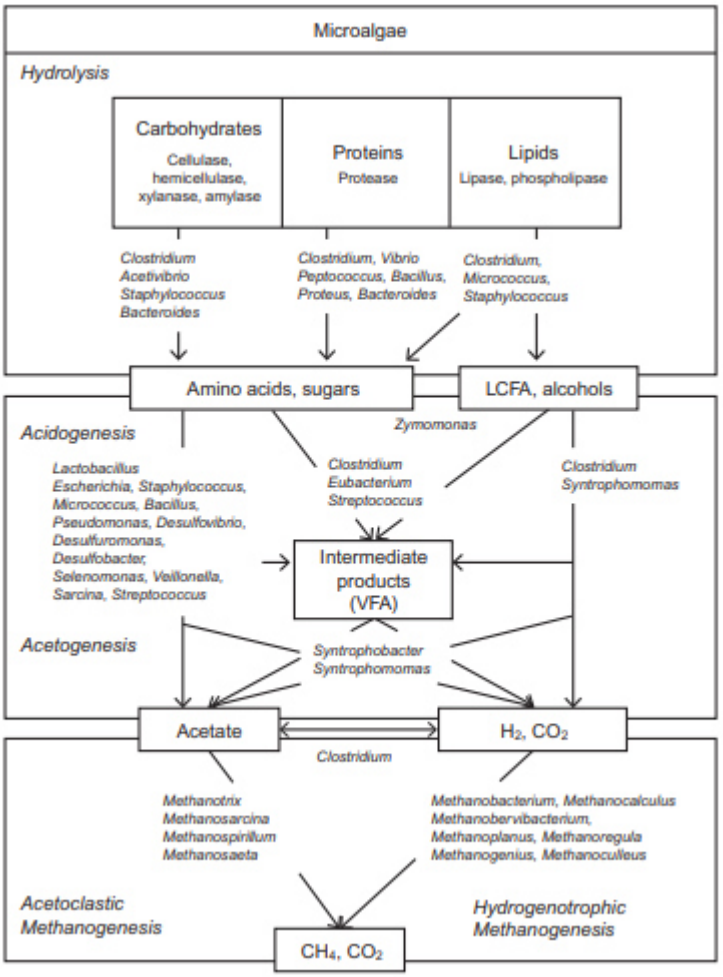


Figure 3. Anaerobic degradation of microalgae and genera of microbial community involved in the process (Cavinato, et. al., 2017)

7.3 The relationship between biogas production and changes in microbial community

The relationship between *Chlorella* degradation and methane production in wastewater samples is difficult to characterize because the volume of biogas was approximately the same and did not lead to a significant result.

On the other hand, in the samples with *Chlorella pyrenoidosa* and silage maize leachate, significant biogas production occurred in the R5 fermenter. Despite the sequence analysis provided a general overview of the composition of microbial taxonomic units

and the individual percentage composition of the microbial population, preferably at the level of phyla, classes, orders and families (in terms of genera, up to almost 50 % were not identified), in connection with other studies did not revealed any significant links in the influence of the microbial community on the degradation of microalgae thus a possible increase in methane production.

For example, in the literature were described *Methanosaeta*, *Methanobacterium* or *Methanospirrillum*) as the community associated with higher methane production. (Klassen, et. al., 2016; Lee, et. al., 2020; Li, et. al., 2017; Schnürer, 2016). When analysing archaeal composition in the highest biogas and methane volume sample (R5) where the maize silage and *Chlorella pyrenoidosa* without lipid enrichment was placed there was really higher number of present *Methanobacterium* (50 %) and *Methanosaeta* (7 %) but in the sample R2E with *Chlorella pyrenoidosa* with additional lipid content was even higher number of *Methanobacterium* present (51 %) with the same number of genus *Methanosaeta* (7 %) but the whole sample showed lower biogas and respectively methane yield. This observation is completely against the recent literature where lipid content is associated with higher methane yields (Lee, et. al., 2020).

When analysing bacterial composition changes among the co-digestion of *Chlorella* and silage maize samples, the sequences analysis did not show significant link between the higher production of biomethane and higher composition of some taxa. To give an example, *Bacteroidales* order which supports the growth of methanogens (Lee, et. al., 2020). When comparing sample with the highest volume of biomethane (R5) with the samples with lower biogas production (R8 and R9), there has been higher percentage abundance of *Bacteroidales* in (R8 and R9) sample (10 % vs. 9 %). And the similar discrepancies have been observed with other major biogas producers.

This result could be due to the inadequacy of algae pre-treatment which is mentioned in many articles as a great method for their better digestion by microorganisms which go hand in hand with the consequent higher methane yield (Córdova, et. al., 2018; Giang, et. al., 2019; Milledge, et. al., 2019).

8 Conclusion

The research of microalgae has become important in recent years because of their desirable properties, which can be used in a broad range of biotechnological applications.

Among them, the single-cell green algae genus *Chlorella* shows wide use in the cosmetic or food industry as a source of essential nutrients not only for humans but also for animals. The use of members of this genus proves to be advantageous in the application in wastewater treatment as they can use substances in wastewater for their growth and can help remove pollutants without secondary pollution. However, they are mostly studied for their possible use as a renewable source of biomass for biofuel (bioethanol, biodiesel, biogas etc.) production nowadays.

This thesis was focused on their exploitation, particularly in biogas production. The results from this work proved that the use of *Chlorella* sp., can serve as a suitable biomass substrate for biogas production. Co-digestion of *Chlorella pyrenoidosa* with different inoculums showed higher biogas and biomethane yield. Moreover, silage maize leachate can be more efficient for biogas production compared to using the sewage sludge.

Regarding to the biogas composition after the addition of algae, the methane content of the biogas has increased. What's even more interesting is that the difference between the use of *Chlorella* with or without lipid enrichment to boost methane yield was not proved however according to recent literature proper pre-treatment of algae could be used.

Anaerobic fermentation is a complex process consisting of different steps of cleavage of macromolecules by different microorganisms. Changes in the amount of present taxonomic units were demonstrated after the addition of algae but with no link to the assumption that higher biogas and biomethane yields are related to the presence of certain microbial communities.

In the end, the fermentation of a representant from the genus *Chlorella*, namely *Chlorella pyrenoidosa*, has shown a great potential in biomethane production compared to some other microalgae, but their use is much broader not only in biogas but generally in biofuels production and in other areas of industry as well. Therefore, certainly deserves further scientific research.

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