THE POSSIBILITY OF BIOGASS PRODUCTION FROM Chlorella ALGAE

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Master´s thesis

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CONTENT

1. INTRODUCTION

In today's world, where we face growing challenges related to the environment and energy sustainability, the research of alternative energy sources becomes a key issue. One of the challenging tasks before us is to ensure energy independence while at the same time reducing the harmful impact on the environment. The increasing global demand for sustainable and renewable energy sources has led to a growing interest in biogas production as an environmentally friendly alternative. Biogas, primarily composed of methane, is produced by the anaerobic fermentation of organic materials and offers a promising route to reducing greenhouse gas emissions and mitigating climate change. In this context, the topic of this paper investigates the possibility of biogas production from *Chlorella* algae. *Chlorella* is a microscopic green algae that stands out for its fast growth and high nutrient content. So far, it has been recognized for its value as a source of high-quality proteins and oils, but more and more research points to its enormous potential for biogas production. Biogas is a renewable source of energy obtained through the anaerobic process of decomposition of organic matter, and it can be used for the production of electricity, thermal energy or as fuel for vehicles. In this paper, we will write about the *Chlorella* algae itself, its usability and the potential of its use for the purpose of biogas production. The biogas production experiment will be presented. This study investigates the possibilities of using *Chlorella* algae as a substrate for biogas production and compares its efficiency with traditional substrates such as cattle manure. The research investigates various aspects of biogas production, including methane production dynamics, methane production efficiency and the impact of substrate composition on biogas quality. By investigating the viability of *Chlorella* as a substrate for biogas production, this study contributes to broader efforts to develop sustainable and renewable energy sources. The findings promise a breakthrough in the use of *Chlorella* algae in the field of bioenergy, offering a greener and more sustainable alternative to meet our energy needs.

2. LITERATURE REVIEW

2.1. MICROALGAE

„Phycologists regard any organisms with chlorophyll a and a thallus not differentiated into roots, stem and leaves to be an alga (Lee, 1989.; Richmond, 2004)“. Light-emitting microorganisms known as microalgae are defined as single-celled organisms that use solar energy during the process of photosynthesis for their growth and development (Sarmidi and Prabandono, 2017.). Microalgae are capable of using sunlight to convert carbon dioxide and water into organic compounds through photosynthesis, similar to plants (Sen Tan et al., 2020). These tiny organisms play a key role in the food chain, serving as the primary food source for a variety of aquatic species, such as zooplankton and small fish (James, 2012.). In addition, microalgae are known for their fast growth rates, which makes them valuable for a variety of applications: Dietary supplements: Some microalgae, such as *Spirulina* and *Chlorella*, are cultivated for their high nutritional content (James, 2012.). They are rich in vitamins, minerals and proteins and are used as dietary supplements (Sangeetha et al., 2023.). Production of biofuels: microalgae can produce lipids (oils) that can be converted into biodiesel. They are considered a potential sustainable source of biofuel (Sarmidi and Prabandono, 2017.). Environmental remediation: Microalgae can help purify wastewater by absorbing nutrients such as nitrogen and phosphorus, reducing pollution in water bodies (Liu et al., 2021.). Carbon capture: They can sequester carbon dioxide from the atmosphere during photosynthesis, making them the subject of research for carbon capture and storage (CCS) technologies (Liu et al., 2021). Biotechnology: Microalgae are used in a variety of biotechnological applications, including the production of drugs, cosmetics and chemicals (De Luca et al, 2021.). Because of their versatility and potential environmental benefits, microalgae have received significant attention in recent years for their applications in areas such as sustainable agriculture, renewable energy, and environmental conservation. Light-emitting microorganisms known as microalgae are defined as single-celled organisms that use solar energy during the process of photosynthesis for their growth and development. These organisms are divided into two distinct categories: *cyanobacteria* and *photosynthetic* protists (James, 2012.). The common characteristic of these two groups of microalgae is their invisibility with the naked eye, and a microscope is needed to observe them. Unlike higher plants, microalgae do not have roots or stems (James, 2012.). *Cyanobacteria (Cyanophyceae)* (FIGURE 1) are the main representatives of prokaryotic microalgae and were the first bacteria to start producing oxygen (Britannica, 2023.). They do not contain a nucleus or cell organelles, but carry out the process of photosynthesis on thylakoid membranes and play an important role in relation to atmospheric nitrogen (Britannica, 2023.). On the other hand, eukaryotic microalgae belong to the category of photosynthetic protists. Unlike cyanobacteria, eukaryotic microalgae contain a nucleus and cell organelles, including chloroplasts, where the process of photosynthesis takes place (Thore et al., 2023.). The most important representatives of eukaryotic microalgae are green microalgae (*Chlorophyta and Bacyllariophyta*) (FIGURE 2 AND 3) and *flagellates* (*Cyanophyceae*) (FIGURE 4) (Thore et al., 2023.). Microalgae quickly adapt to adverse conditions due to their simple cellular structure, and you can find them in various environments such as rivers, seas, lakes and streams. In addition, they grow successfully in extremely high and low temperatures, as well as in salty and alkaline lakes. To date, about 30,000 species of microalgae have been identified and classified, although it is believed that there are about 50,000 of them on Earth. Most of these studied and classified microalgae belong to green microalgae, precisely 77%, while 8% belong to cyanobacteria. Microalgae play a key role in the creation of organic matter that supports life on Earth, and also produce about half of the oxygen present in the atmosphere (Sangeetha and Thangadurai, 2023).

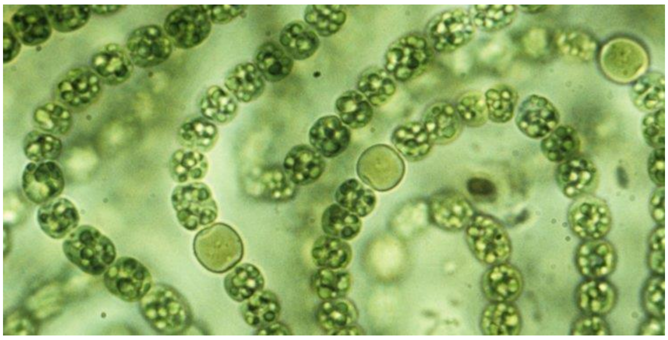


FIGURE 1: *Cyanobacteria*

SOURCE: https://vpchothuegoldenking.com/hr/why-did-an-oxygen-catastrophe-occur-on-earth-and-how-did-the-moon-affect-it/

FIGURE 2: Green microalgae *(Chlorophyta)*

SOURCE:

http://protist.i.hosei.ac.jp/PDB/Images/Chlorophyta/Unidentified_Chlorococcales/sp_04.html

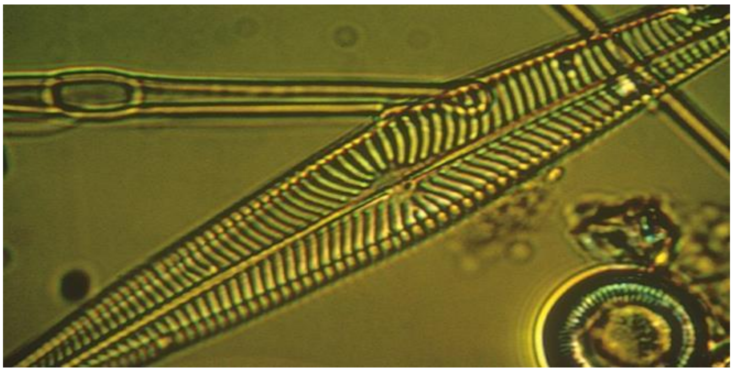


FIGURE 3: *Bacyllariophyta*

SOURCE: https://supercurioso.com/descubre-todo-lo-que-se-esconde-en-una-gota-agua-de-mar/

FIGURE 4: *Cyanophyceae*

SOURCE: https://www.thepondguy.com/weed-control-guide/?p=PPCBINGA&msclkid=6a622d8da440147eff3b497b183842b0&utm_source=bing&utm_medium=cpc&utm_campaign=ECI%20-%20%20Bing%20-%20DSA%20-%20Product%20Type%20%5BNBC%5D%20%5BCONS%5D&utm_term=weed%20control&utm_content=Weeds-%20New

2.2. CULTIVATION OF MICROALGAE

Cultivation systems for microalgae usually start with laboratory cultivation, which may later evolve into industrial cultivation. During the cultivation of microalgae, maintaining optimal conditions for growth is crucial. This includes the control of various parameters such as temperature, CO2 and O2 concentration, pH, nutrient availability and mixing speed in the aqueous medium (Liu et al., 2013.). The inoculation process marks the beginning of microalgae cultivation, where initial pure cultures are created. These pure cultures are usually isolated from the mother cultures. After the starter culture populations reach the stationary phase of growth, which usually occurs between 7 and 14 days, subcultures of larger volume are developed from them. During further development and reaching the stationary phase, these subcultures are transferred to even larger volumes, often exceeding the threshold of 50 liters (Liu et al., 2013.). This procedure of gradually increasing the volume of cultures enables the controlled scaling of microalgae production from smaller laboratory measures to industrial levels, which is crucial for the economical and efficient production of biofuels and other products from microalgae. The growth of microalgae (Figure 5.) can be divided into five phases:

1) Induction phase/lag phase

2) Exponential phase

3) Phase of growth deceleration

4) Stationary phase

5) Dying/death phase

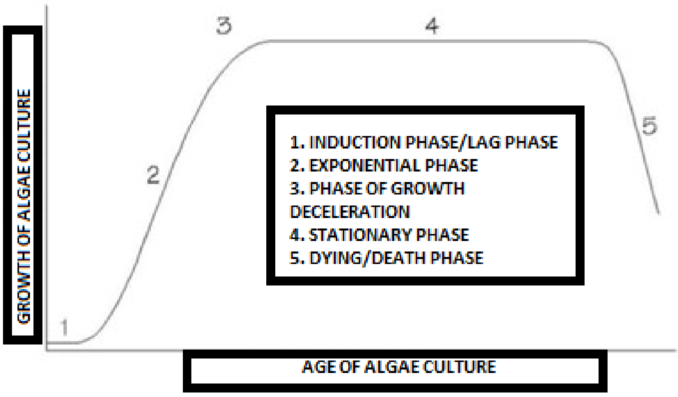


FIGURE 5: Growth stages of cultured microalgae

SOURCE: Author

In the cultivation of microalgae, they go through several different stages. First there is a lag phase in which the cells grow in size. This is followed by an exponential phase in which the microalgae population multiplies intensively (Richmond, 2013.). The third phase, slow growth, is characterized by the slowing down of cell division due to light limitation. In this phase, cell division slows down due to various factors that limit growth (Richmond, 2013.). The fourth phase, the stagnation phase, indicates that the cell growth rate is zero because nutrients have been used up (Richmond, 2013.). Then comes the fifth phase, the dying phase of the culture, which occurs due to depleted nutrients, contamination or a change in pH (Richmond, 2013.). In the process of growing microalgae, bioreactors play a key role. There are two basic types of bioreactors: open and closed bioreactors. Open bioreactors, as shown in Figure 6, are artificial pools that are integrated into the environment (Dowens and Hu, 2013.). They are usually made of plastic or concrete and often have an elliptical shape. In these bioreactors, the water medium containing the microalgae must be constantly stirred to prevent the microalgae from settling at the bottom of the pool and to provide the microalgae with all the necessary nutrients and light (Bosak, 2017.). To achieve constant mixing, a paddle wheel is often placed at the end of the pool to generate current (Dowens and Hu, 2013.). Open bioreactors have their advantages, including cheap and simple construction and the ability to build large pools (Bosak, 2017.). However, the daily biomass yield from these bioreactors is relatively low, which makes the total production of microalgae limited (Bosak, 2017.). In addition, microalgae in open bioreactors are exposed to external conditions, which can cause challenges such as rain, drought and changing brightness. Also, pathogen contamination can occur as microalgae come into contact with the atmosphere. Despite these challenges, open bioreactors are still widely used due to their simplicity and lower cost (Dowens and Hu, 2013.).

FIGURE 6: Open microalgae cultivation system

SOURCE: https://www.engr.colostate.edu/~marchese/algae-n2o.html

Another type of bioreactors are closed bioreactors, also known as photobioreactors. In this microalgae cultivation system, the microalgae pass through transparent plastic tubes that can be placed vertically (as shown in Figure 7) or horizontally (as shown in Figure 2.16) (Liu et al., 2013.). Also, microalgae can be grown in bags between flat plates (as shown in Figure 8). In this cultivation system, contamination by pathogenic microorganisms is minimal, but there are some challenges in production. For example, in tubular towers, microalgae can deposit on the inner walls of the tubes, creating clumps that can hinder microalgae growth and decay. If the tube walls cannot be easily cleaned, light can have difficulty penetrating the tubes, which slows down the growth of algae (Dowens and Hu, 2013.). Also, high costs are a challenge of this type of cultivation, although they bring double the yields compared to open systems. This is due to the precise control of the conditions that have a positive effect on the process of growing microalgae. Despite the higher production costs compared to open systems, closed systems are becoming more and more attractive due to high yields and the possibility of complete control of the cultivation conditions and cultivation of different types of microalgae (Bosak, 2017.). This is particularly important in the context of increased interest in the production of biofuels and other products from microalgae (Saad et al., 2019).

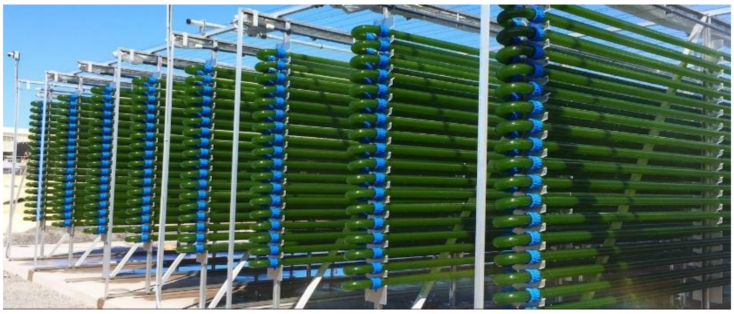


FIGURE 7: Closed photobioreactors in the form of vertically placed tubes and flat panels

SOURCE: http://www.variconaqua.com/products-services/phyco-flow/

FIGURE 8: Photobioreactors in the form of horizontally placed tubes, vertical flat panels and lyophilized biomass of microalgae

SOURCE: https://www.researchgate.net/figure/Commercial-microalgae-biomass-cultivation-in-flat-panel-photobioreactors-courtesy-of_fig1_264116497

Cultivation of microalgae can have an additional benefit in reducing gas emissions and purifying wastewater. Microalgae are effective in removing nitrogen, phosphorus and heavy metals such as arsenic (As), cadmium (Cd) and chromium (Cr) from aqueous solutions (Bosak, 2017.). Since gas emission control and wastewater treatment is often an expensive and technically challenging process, the use of wastewater as a source of nutrients for the cultivation of microalgae, while simultaneously purifying this wastewater, provides additional ecological and economic advantages (Qiao et al., 2020.). This approach enables the integration of microalgae cultivation with wastewater treatment, which reduces the costs of wastewater treatment and at the same time enables the production of useful products such as biofuels or high-value chemicals from microalgae. This can contribute to a more sustainable approach to the industry and help reduce the negative impact on the environment (Bosak, 2017.).

2.3. *CHLORELLA*

Chlorella represents a group of single-celled green microalgae that has garnered significant attention in both scientific research and commercial sectors (Wiley & Sons, 2013). „Chlorella is any alga of the genus *Chlorella* living in both aquatic and terrestrial habitats (Wiley & Sons, 2013)". Thanks to its straightforward life cycle, robust growth potential, and photosynthetic capabilities, *Chlorella* shares metabolic pathways akin to those found in higher plants. Consequently, it has long served as a valuable model organism for comprehending photosynthesis and carbon assimilation in higher plants (Iwamoto, 2004.). Furthermore, *Chlorella* boasts an high protein content, constituting up to 70% of its dry cell mass, alongside a rich reservoir of minerals, vitamins, and carotenoids, rendering it a compelling food source for both humans and animals (Iwamoto, 2004.). Indeed, *Chlorella* continues to be cultivated for human health products and livestock feed in various countries, including Germany, Japan, China, and several other Asian nations (Reyes et al., 2020.). Moreover, *Chlorella* exhibits the capacity to accumulate substantial amounts of storage neutral lipids, primarily in the form of triacylglycerol, when subjected to stress conditions, such as intense light or nitrogen deficiency (Li et al., 2016.). This inherent feature positions *Chlorella* as a promising candidate organism for the production of lipid-based biofuels (Saramidi and Prabandono, 2017.).

2.4. MORPHOLOGY AND TAXONOMY

Chlorella, first described by Beijerinck in 1890 with *Chlorella vulgaris* as the type species, encompasses a diverse group of microalgae (Kai Ru et al., 2020.). These microscopic organisms typically exhibit spherical or ellipsoidal shapes, with diameters ranging from 2 to 10 μm (Li et al., 2016.). They thrive in various environments, including freshwater, seawater, and soil, and can exist as free-living entities or engage in symbiotic relationships with lichens and protozoa (Kai Ru et al., 2020.). *Chlorella* reproduces asexually through autospore production, a process involving three phases: growth (cell size increase), ripening (preparation for mitosis), and division (mother cell splitting into daughter cells), with 2–16 autospores released upon mother cell wall rupture (Kai Ru et al., 2020.). Microscopic examinations have revealed essential structural feat ures of *Chlorella* (Kai Ru et al., 2020.). These include a cup-shaped chloroplast situated peripherally in the cytoplasm, occupying approximately half of the cell volume (Li et al., 2016.). The nucleus is positioned near the cytoplasmic membrane, facing the opening of the chloroplast, while mitochondria assume an ovoid shape and closely associate with the chloroplast (Kai Ru et al., 2020.). A pyrenoid, typically surrounded by a starch sheath, is commonly found in *Chlorella* species. In some instances, like *C. variegate* and *C. luteoviridis*, the pyrenoid may contain pyrenoglobuli, which are lipid-containing globules (Kai Ru et al., 2020.). *Chlorella* is characterized by a rigid cell wall, the structure of which can vary significantly among species (Kai Ru et al., 2020.). Some *Chlorella* species possess two layers in their cell walls—a mono or trilaminar outer layer and a microfibrillar inner layer—while others consist of just a microfibrillar layer (Kai Ru et al., 2020.). In scientific literature, over 100 *Chlorella* strains have been documented (Kai Ru et al., 2020.). Attempts have been made to classify *Chlorella* species based on various biochemical and physiological characteristics, such as hydrogenase activity, secondary carotenoid production, and tolerance to acids or salts (Kai Ru et al., 2020.). These efforts resulted in the assignment of strains into distinct taxa. Furthermore, phylogenetic analysis using 18S rRNA has led to a revision of the *Chlorella* genus, revealing it as a polyphyletic assemblage dispersed across two classes of *Chlorophyta*, namely *Chlorophyceae* and *Trebouxiophyceae* (Kai Ru et al., 2020.).

2.5. BIOCHEMICAL COMPOSITION

Under favorable growth conditions, *Chlorella* can produce up to 60% of protein, dry cell mass. It contains all the essential amino acids needed for human and animal nutrition (Canelli et al., 2020.). In addition to its high protein content, *Chlorella* biomass typically contains about 10-15% carbohydrates and 12-15% lipids when grown optimally. The major lipids found in *Chlorella* include C16 and C18 fatty acyl groups, such as C16:0, C16:2, C18:1, C18:2, and C18:3. Chlorella is also a rich source of vitamins, including provitamin A, vitamins B1, B2, B6, B12, C and E, as well as folic acid, niacin and pantothenic acid (Canelli et al., 2020.). It contains essential minerals such as sodium, potassium, calcium, magnesium, iron, manganese, zinc and copper. In terms of pigments, *Chlorella* contains chlorophyll a and b, along with various carotenoids such as β-carotene, lutein, zeaxanthin, violaxanthin, neoxanthin, and antheraxanthin (Canelli et al., 2020.). Lutein is the most abundant carotenoid in *Chlorella* cells, often reaching up to 0.45% of the cell's dry weight (Canelli et al., 2020.). However, it is worth noting that ketocarotenoids such as astaxanthin and canthaxanthin are primarily found in C. zofingiensis and are not commonly present in other Chlorella species or strains. The chemical and biochemical composition of *Chlorella* can vary significantly among different species or strains (Canelli et al., 2020.). For example, protein, carbohydrate, and lipid contents were observed to vary greatly among the five *Chlorella-C species. vulgaris, C. emersonii, C. protothecoides, C. sorokiniana and C. minutissima* (Canelli et al., 2020.). Furthermore, the biochemical composition of *Chlorella* is influenced by growing conditions, including nutrient availability and environmental factors (Canelli et al., 2020.). Nitrogen-rich conditions promote growth and protein production, while nitrogen deficiency can lead to reduced protein content and increased accumulation of starch and lipids (Canelli et al., 2020.). Light intensity and temperature also play a key role in influencing the biochemical composition of Chlorella.

2.6. BIOGASS

In an era marked by environmental concerns and the pursuit of sustainable energy sources, biogas has emerged as a promising and environmentally friendly alternative. Biogas is a versatile and renewable form of energy that is generated through the anaerobic digestion of organic materials, such as agricultural waste, sewage, and various organic byproducts (Kougias and Angelidaki, 2018.). This natural process involves the breakdown of organic matter by microorganisms in the absence of oxygen, resulting in the production of a gaseous mixture primarily composed of methane (CH4) (55-70%) and carbon dioxide (CO2) (30-45%), along with trace amounts of other gases. Biogas holds great significance due to its potential to address several critical challenges simultaneously (Chattopadhyay et al., 2009.). It offers an eco-friendly means of waste management by converting organic waste materials into valuable energy. Furthermore, biogas production can significantly reduce greenhouse gas emissions when compared to the decomposition of organic matter in landfills, which releases methane—a potent greenhouse gas—directly into the atmosphere. The applications of biogas are diverse and include electricity and heat generation, fuel for vehicles, and as a source of renewable energy in various industries. Moreover, biogas can be upgraded to biomethane, a high-purity form of methane, which can be injected into natural gas grids or used as a cleaner fuel for transportation (Al Seadi et al., 2008.).

2.7. POTENTIAL OF BIOGAS

The global potential for energy production from biomass is considered extremely high, but currently only a small part of this potential is being used. This is concluded on the basis of various assessments and studies that are made using different scenarios and assumptions. The European Biomass Association (AEBIOM) estimates that biomass energy production can increase from 72 million tonnes of oil equivalent (Mtoe) in 2004 to 220 Mtoe by 2020 (van Foreest, 2012). The greatest potential for such an increase lies in the use of agricultural biomass. According to their estimates, there are 20 to 40 million hectares of land in the European Union that can be used for energy production without negatively affecting the food supply (van Foreest, 2012.). Also, the German Institute for Energy and Environment claims that biogas production could satisfy the total consumption of natural gas, whereby purified biogas, also known as biomethane, would be fed into the gas network (Abdalla et al., 2022). Estimating the potential of biogas production in Europe is challenging due to the various factors and assumptions that must be taken into account. This includes the availability of agricultural land that can be used for energy production, the productivity of energy crops, the yield of methane from the raw material and the energy efficiency of the final utilization of biogas.

FIGURE 9: European gas pipeline network and potential corridors (marked in yellow) suitable for production and injecting biomethane into the pipeline system
SOURCE: Thrän , Seiffert, Müller-Langer, Plättner, Vogel, 2007.

2.8. ANAEROBIC DIGESTION (AD)

Anaerobic Digestion (AD) is a biochemical process in which complex organic compounds are broken down by the action of different types of bacteria in anaerobic conditions (without the presence of oxygen). Anaerobic decomposition is a natural process that occurs every day in nature, for example in sea sediment, during the digestion of ruminants or during the formation of peat. In biogas plants, the results of the AD process are biogas and digestate. In cases where the AD process is used for a homogeneous mixture of two or more different substrates, such as manure and organic waste from the food industry, the process is called co-digestion. Co-digestion is the most common method of biogas production (Al Seadi et al., 2008.).

2.8.1. SUBSTRATES FOR ANAEROBIC DIGESTATION

For the production of biogas, different types of biomass can serve as substrates. The following categories of substrates are commonly used:

A) Manure and slurry: These are the by-products of animal husbandry that are often used for biogas production.

B) Residues and by-products from agricultural production: This includes plant residues and other materials generated during agricultural activities.
C) Degradable organic waste from agriculture and the food industry: This includes residues of plant and animal origin that are suitable for anaerobic decomposition.
D) The organic part of municipal waste and waste from the catering industry: These are food residues of plant and animal origin that can be used for biogas production.
E) Sludges: These are by-products from various industrial processes, such as wastewater treatment.
F) Energy crops: This includes crops such as corn, sorghum and various types of grass and clover, which are grown specifically for the production of biogas (Bajpai, 2017.).

Examples of these substrate categories are shown in Figures 10, 11 and 12.

FIGURE 10: Municipal waste delivered to a biogas plant (Germany)

SOURCE: Rutz, 2008.

FIGURE 11: Waste from restaurants

SOURCE: Rutz, 2008.

FIGURE 12: Corn silage

SOURCE: Rutz, 2008.

The use of animal excrement for anaerobic digestion (AD) brings several advantages due to its characteristics:

A) Presence of anaerobic bacteria: Animal feces naturally contain anaerobic bacteria that are key to the AD process.

B) High water content: Fertilizers have a high percentage of water (usually between 4% and 8% dry matter), which makes them an excellent solvent for other substances and allows them to mix well with other substrates.
C) Economy and availability: Animal excrement is a cheap and readily available material because it is collected as farm waste.

During the past years, other substrates for AD have been tested, especially the so-called energy crops, which are grown exclusively for energy production. These crops include annual plants such as various types of grasses, corn and turnips, and may also include perennial plantations of woody species such as willow and poplar. However, before woody species can be used in AD, they need to be processed to remove lignin, and this technology is still under development. Substrates for AD are classified according to dry matter content (DM), methane yield and other criteria. Based on these classifications, the appropriate AD process is selected. For example, substrates with less than 20% ST are used for "wet digestion," which includes materials such as manure and slurry, which have a high percentage of water. On the other hand, when the substrate has a DM content of 35% or more, the process is called "dry digestion" and is typical for AD of energy crops and silage. The choice of substrate and their proportions depends on the content of dry matter and the presence of sugar, fat and proteins in the material (Bajpai, 2017.).

2.8.2. AD PARAMETERS

The efficiency of anaerobic digestion (AD) depends on a number of key parameters, therefore it is extremely important to ensure optimal conditions for the development of anaerobic microorganisms in the process. Some of these key parameters include:

A) Lack of oxygen: Anaerobic microorganisms grow and are active in the absence of oxygen. It is therefore crucial to ensure that there is no oxygen inflow into the digester, in order to maintain anaerobic conditions.
B) Temperature: Temperature has a significant effect on the speed of digestion. Different types of anaerobic microorganisms function optimally at different temperatures, so the temperature is often controlled to achieve the best efficiency.
C) pH value: Most anaerobic microorganisms prefer a slightly acidic or neutral environment. Maintaining the appropriate pH value in the digester is therefore important to the process.

D) Nutrient supply: Anaerobic microorganisms require certain nutrients for their growth and metabolism. Ensuring the availability of these nutrients is important for the optimal activity of microorganisms.
E) Intensity of mixing: The intensity of mixing inside the digester plays a role in ensuring a homogeneous environment and an even distribution of microorganisms and substrate.
F) Presence of inhibitors: The presence of certain substances, such as toxic chemicals or heavy metals, can inhibit the growth and activity of anaerobic microorganisms.

All these parameters should be carefully monitored and controlled to ensure maximum efficiency of anaerobic digestion and biogas production (Majkovčan, 2012).

2.8.3. HYDROLYSIS

Hydrolysis is the initial step of anaerobic digestion, in which complex organic substances, such as fats, cellulose, starch and proteins (polymers and biopolymers), are broken down into smaller units (monomers and oligomers) with the help of hydrolases, enzymes that work in this process. Methanogenic bacteria are not capable of directly degrading insoluble organic polymers, so they are first transformed into soluble derivatives, which enables other bacteria to further degrade them. Polymeric carbohydrates, lipids, amino acids and proteins are converted into glucose, glycerol, fatty acids, purines, pyridines and other individual monomers. All these reactions take place within the digestate mixture. Hydrolytic bacteria have an extremely high specific growth rate in the process of anaerobic decomposition, with a doubling time of only 30 minutes, which is significantly faster compared to acetogenic and methanogenic bacteria (Majkovčan, 2012).

2.8.4. ACIDOGENESIS

In the acidogenesis phase, hydrolysis products are transformed into methanogenic compounds such as water, carbon dioxide, acetates, formates and methanol, as well as compounds such as propionates, butyrates, aldehydes and alcohols. This transformation takes place with the participation of acidogenic bacteria. Fatty acid oxidation takes place more slowly compared to other reactions that are relatively faster. The high partial pressure of hydrogen promotes the formation of methanogenic products. Acidogenic bacteria transform sugars and amino acids into organic acids, ammonia, hydrogen sulfide and carbon dioxide (Majkovčan, 2012).

2.8.5. ACETOGENESIS

During acetogenesis, methanogenic bacteria cannot make organic compounds directly to transform into methane, but they turn them into methanogenic compounds. Volatile fatty acids which have carbon chains longer than two units and alcohol with more than one carbon molecule they oxidize into acetate, hydrogen and carbon dioxide. A negative phenomenon is the formation of hydrogen which increases the partial pressure in the bioreactor. This phenomenon causes inhibition of metabolism acetogenic bacteria. In order for acetogenic bacteria to produce hydrogen, a low partial pressure of the environment, and methanogens and Desulfovibrio have the ability to consume hydrogen from the environment. An increase in the partial pressure in the digester causes changes in the metabolic action of acetogenic bacteria. Under such conditions, they produce butyrate, propionate, valerate, caproate instead of acetate. Methanogens, despite the amount of hydrogen produced, do not can use the resulting substances. Interactions between different groups of bacteria are partial explainable. Acetates are the most important fermentation intermediates (Rohlik, 2016).

2.8.6. METANOGENESIS

The last and slowest stage of anaerobic decomposition is known as methanogenesis. In this phase, methane is produced through the methane fermentation process, and this happens in several ways. As much as 70% of methane is created from acetate, while the remaining part is created by the conversion of hydrogen and carbon dioxide. This process of reducing carbon dioxide to methane is exothermic and releases energy. Methanogenesis is also a very sensitive stage in the fermentation process and a complete stoppage of methane production can occur due to mismanagement of the digester, variations in temperature or oxygen ingress. Although methanogenesis can be disrupted, acidogenic bacteria still continue to produce acids, leading to acidification of the digestate. In such situations, one of the solutions is adding manure, which increases the digestate volume and reduces the acid content. The optimal pH value for the efficient operation of methanogenic bacteria is between pH 7.0 and 7.8, which means that neutral to slightly alkaline conditions are favorable (Vögeli et al., 2014).

2.9. MICROALGAE AS THIRD GENERATION FUEL

Over time, interest in the production of biofuels from renewable energy sources is growing. These include well-known sources such as solar energy, geothermal energy and wind energy. In addition, energy can be obtained from biomass. Biomass, as shown in Figure 13, is defined as organic matter that results from the decomposition of plant and animal organisms. To release and use energy from biomass, it is necessary to burn that organic matter (Bosak, 2017).

FIGURE 13: Biomass

SOURCE: https://noticias.usm.cl/2011/02/11/biomasa-en-chile-una-fuente-viva-y-natural-de-energia/

In addition, with the proper exploitation of biomass, it is possible to produce biofuels that are used as a substitute for fossil fuels in transport and household use. Biofuels can be classified into three different categories according to their source: first generation biofuels, second generation biofuels and third generation biofuels. The category of first-generation biofuels includes biodiesel, biogas and bioethanol, which are often obtained from the oils of various oilseeds, such as soybean (Figure 14) and rapeseed (Figure 15) (Vučko, 2017.).

FIGURE 14: Soybean

SOURCE: https://www.worldatlas.com/articles/largest-soybean-producing-countries.html

FIGURE 15: Rapeseed

SOURCE: https://www.flickr.com/photos/sarfrazh/8962899277

These biofuels have a positive impact on the environment because they emit fewer greenhouse gases compared to gasoline and conventional diesel fuel. Looking objectively, first-generation biofuels are not an ideal solution due to a number of negative aspects associated with them. For example, if crops such as maize are mostly grown for biofuel production, this may result in increased market prices for these crops, which may negatively impact food availability and prices in the food industry. Also, for the cultivation of such crops, pesticides are often used, which have a harmful effect on the environment and biological diversity. The second generation of biofuels uses wood raw materials (as shown in Figure 16) (Vučko, 2017.).

FIGURE 16: Wood biomass

SOURCE: https://energetika.ba/jos-je-puno-prostora-za-koristenje-biomase-u-hrvatskoj/

These biofuels are considered more economical than those of the first generation. Also, they are less harmful to the environment because they are produced using waste from the wood industry, which reduces the negative impact on the forest and the environment. However, it is important to note that the production process of second-generation biofuels is technologically demanding, which results in an increase in production costs and, finally, the price on the market. The last category is third-generation biofuels, which includes microalgae biomass (as shown in Figure 17). Several types of biofuels can be produced from microalgae, including bioethanol and biogas (Vučko, 2017.).

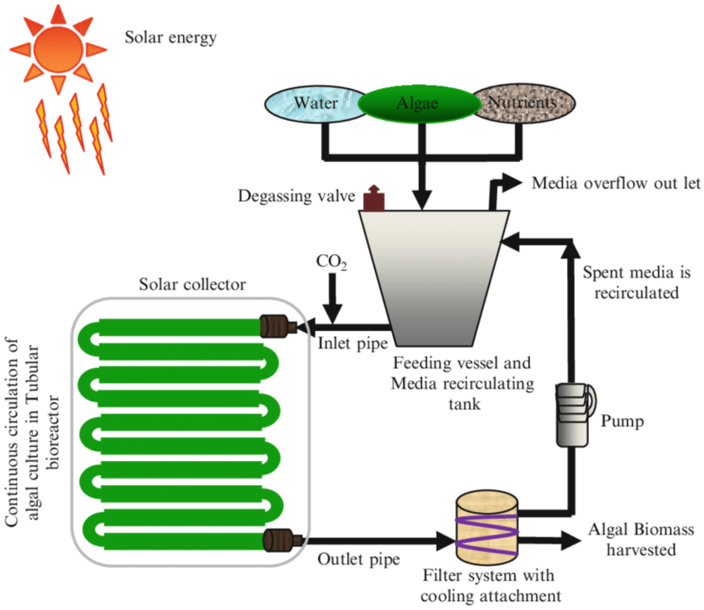


FIGURE 17: Schematic representation of algal bomass production n tubular photobioreactor

SOURCE: https://www.researchgate.net/figure/Schematic-representation-of-algal-biomass-production-in-tubular-photobioreactor_fig3_268209303

Microalgae produce bioethanol through fermentation, while biogas is obtained through anaerobic digestion (as shown in Figure 18) (Pereira, 2017.). Some microalgae even release biohydrogen. In addition, due to the high content of oil in microalgae, they can be used to produce biodiesel as fuel for cars. Primarily, the use of microalgae biomass for the production of biodiesel would not significantly affect the prices of food products on the market. In addition, the cultivation of microalgae requires significantly less water compared to the cultivation of agricultural crops (Pereira, 2017.). Microalgae also accumulate a high percentage of lipids during their growth, which means that they have a higher energy value compared to plant biomass. Biofuel produced from microalgae releases only a small amount of CO2 and other greenhouse gases during combustion compared to fossil fuels and first-generation biofuels (Pereira, 2017.).

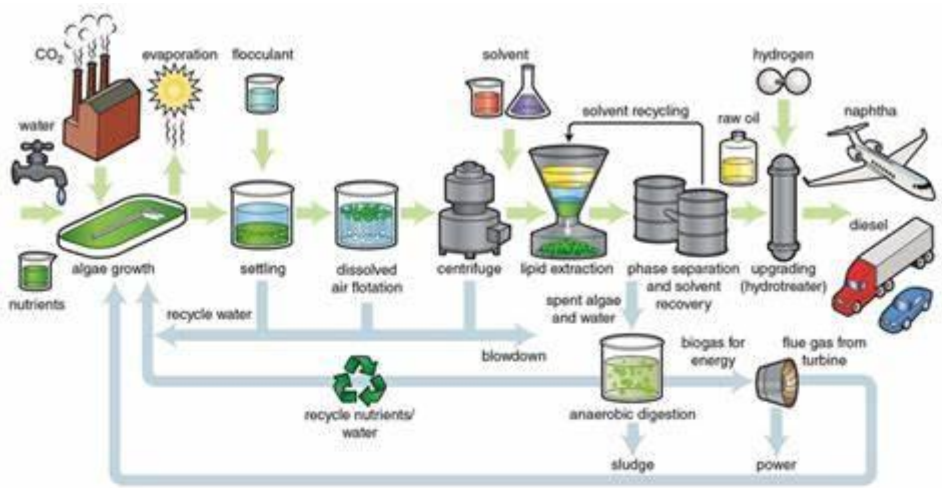


FIGURE 18: Biofuel extraction process from algal biomass

SOURCE: https://contest.techbriefs.com/2019/entries/sustainable-technologies/9555-0517-103210-solving-the-algae-problem

It is also important to note that microalgae use large amounts of CO2 for their growth, often taken from industrial processes, which can help reduce greenhouse gas emissions and simultaneously increase the production of microalgae biomass, although this may reduce the importance of the industry. Considering that microalgae are unicellular organisms that reproduce rapidly every few hours, their biomass can be collected daily. It can be argued that microalgae, as a source of biodiesel, are the only organisms that can completely replace conventional diesel derived from fossil fuels. Microalgae with a high oil content are particularly desirable for biodiesel production. However, it is important to note that the costs of producing biodiesel from microalgae are extremely high. Production of biodiesel from microalgae can be as much as 20 times more expensive compared to production from traditional crops. This means that companies involved in the production of biodiesel from microalgae may face challenges in the market, unless oil and natural gas prices increase significantly, which would increase the appeal of renewable energy sources such as microalgae (Pereira, 2017.).

3. MATERIALS AND RESEARCH METHODS

For the research, fresh samples of slurry from the "Orlovnjak" beef farm and Chlorella algae produced by the company PHYOX d.o.o. were used. The aim of the research was to determine the biogas potential of Chlorella microalgae and to compare the yield of the amount of biogas by manipulating the amount of microalgae.

3.1. ANAEROBIC FERMENTATION METHOD

Anaerobic fermentation was carried out in discontinuous bioreactors with a volume of 1 L under thermophilic conditions (55˚C) in a bath for a period of up to 21 days. The gas produced during fermentation was collected in graduated two-liter beakers and its produced amount was read daily. In the beakers where the biogas is collected, there is a supersaturated NaCl solution. The beakers are connected to the bioreactors via PVC pipes.

Substrates for further research, i.e., anaerobic fermentation, are mixed in the following mixtures:

I - control group K:

- fresh cattle manure 500 g (100%) was collected from the dairy cow farm Orlovnjak;

II – experimental group A1:

- 500 g of fresh cattle manure + 5 g of dry Chlorella algae

III - experimental group A2:

- 500 g of fresh cattle manure + 10 g of dry Chlorella algae

All groups were set up in three repetitions.

3.2. DETERMINATION OF DRY MATTER CONTENT

The content of dry matter in the samples was determined by drying 100 g of fresh sample matter in an oven at 75°C for 24 hours, then for an additional 3 hours at a temperature of 105°C (Thompson, 2001). The total dry matter was calculated from the data of the fresh sample and the dry sample after drying:

$$\text{Total dry matter (\%)} = [\text{net dry matter (g)} \div \text{net fresh sample (g)}] \times 100$$

FIGURE 19: Dryer

SOURCE: Author

3.3. DETERMINATION OF ASH AND ORGANIC MATTER CONTENT

The total content of ash and organic matter was determined by annealing at 550°C for 2 hours (Thompson, 2001) in an annealing furnace, and samples of dry matter after drying at 75°C and the following formulas were used:

ash (%) = [net mass of ash after 550°C (g) ÷ net dry sample (g)] × 100

organic matter = [1 – net ash after 550°C (g) ÷ net dry sample (g)] × 100

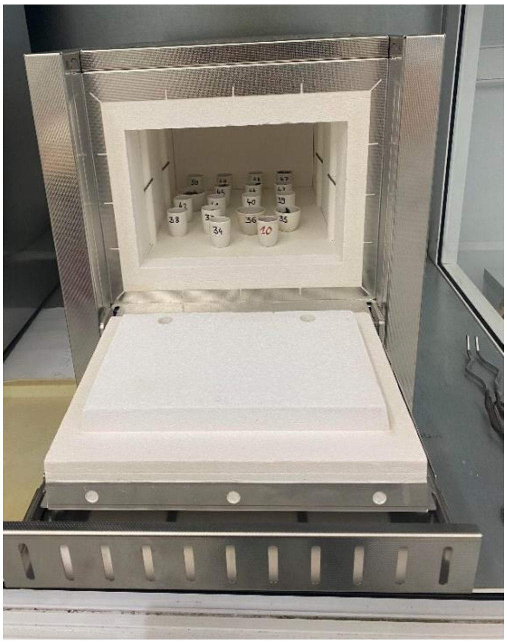


FIGURE 20: Annealing furnace

SOURCE: Author

3.4. DETERMINATION OF PH

The pH was determined directly in the samples by electrochemical measurement.

Gas composition analysis

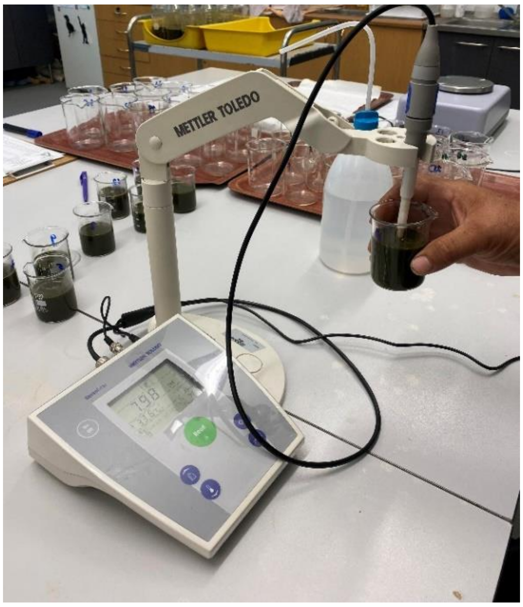


FIGURE 21: pH meter

SOURCE: Author

Using the Optima 7 biogas gas detector, the biogas composition and calorific value were determined.

FIGURE 22: Gas detector

SOURCE: Author

FIGURE 23: Measurement of gas composition

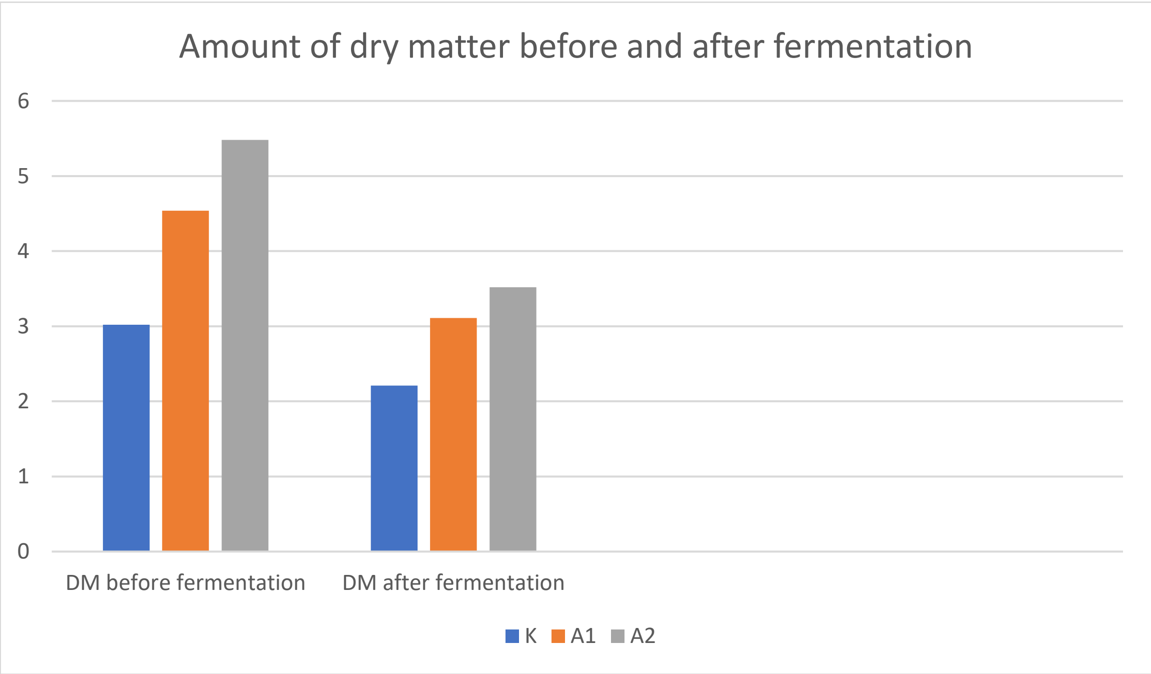
SOURCE: Author

The research was conducted in the Laboratory for Biomass and Renewable Energy Sources at the Faculty of Agrobiotechnical Sciences in Osijek.

4. RESEARCH RESULTS

4.1. AMOUNT OF DRY MATTER (DM)

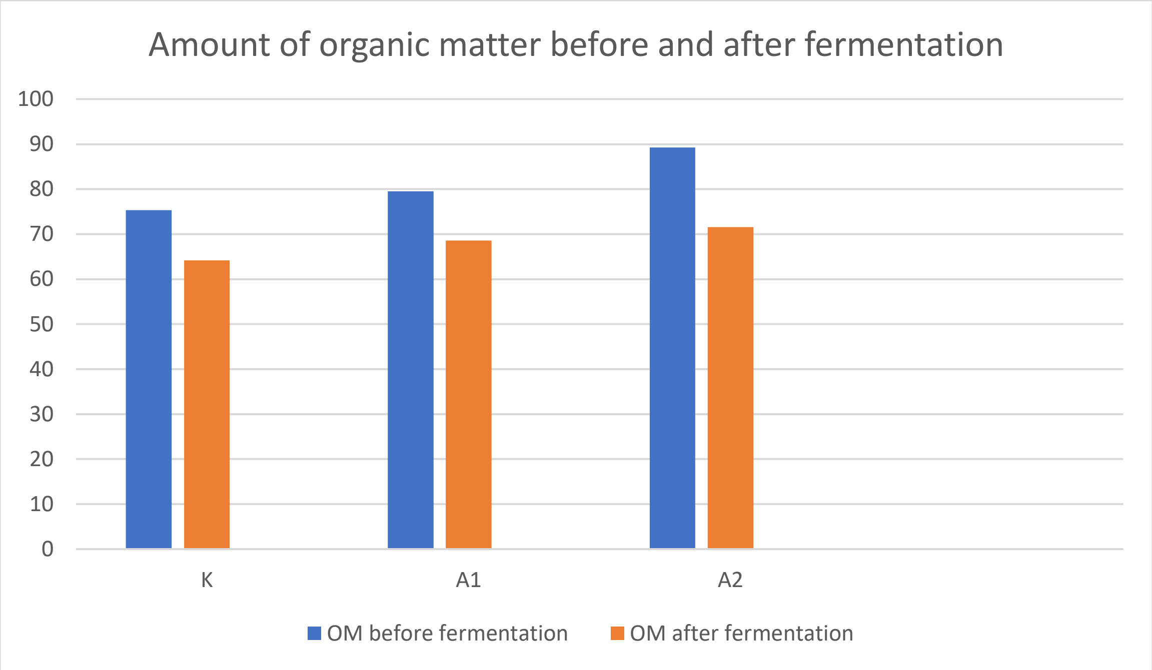
The percentage of dry matter in beef manure (control group K) before fermentation was 3.02%, in the mixture of beef manure and 5g of algae (experimental group A1) the dry matter was 4.54%, in the mixture of beef manure and 10g of algae (experimental group A2) dry matter was 5.48%, and the percentage of dry matter in algae was 94.22%. After fermentation, the percentage of dry matter in beef manure (control group (K)) was 2.21%, in the mixture of beef manure and 5g of algae (experimental group A1) the dry matter was 3.11%, in the mixture of beef manure and 10g of algae (experimental group A2) dry matter was 3.52%. Due to the mixing of 5g and 10g of dry algae with 500g of fresh beef manure, which has a lower concentration of dry matter, the concentration of DM changes and it has a higher value at the beginning compared to the end of the research. Graph 1 shows the concentration of OM at the beginning and at the end of anaerobic fermentation.



GRAPH 1: Amount of dry matter before and after fermentation

4.2. AMOUNT OF ORGANIC MATTER (OM)

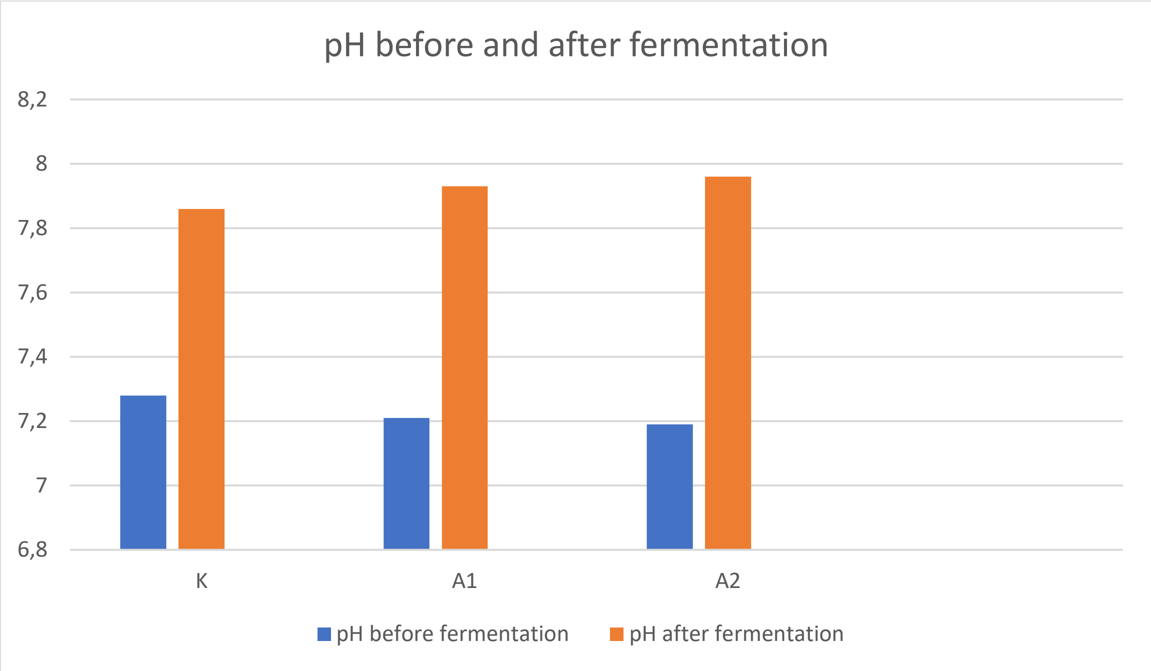
The percentage of organic matter in beef manure (control group K) before fermentation was 75.36%, in the mixture of beef manure and 5g of algae (experimental group A1) the organic matter was 79.52%, in the mixture of beef manure and 10g of algae dry matter was 81.27% (experimental group A2), and the percentage of organic matter in algae was 89.24%. After fermentation, the percentage of organic matter in beef manure (control group K) was 64.20%, in the mixture of beef manure and 5g of algae (experimental group A1) the dry matter was 68.55%, in the mixture of beef manure and 10g of algae dry matter was 71.54% (experimental group A2). Due to the decomposition of organic substances during anaerobic fermentation, its reduction occurs. Graph 2 shows the concentration of OM at the beginning and at the end of anaerobic fermentation.



GRAPH 2: Amount of organic matter before and after fermentation

4.3. pH VALUE

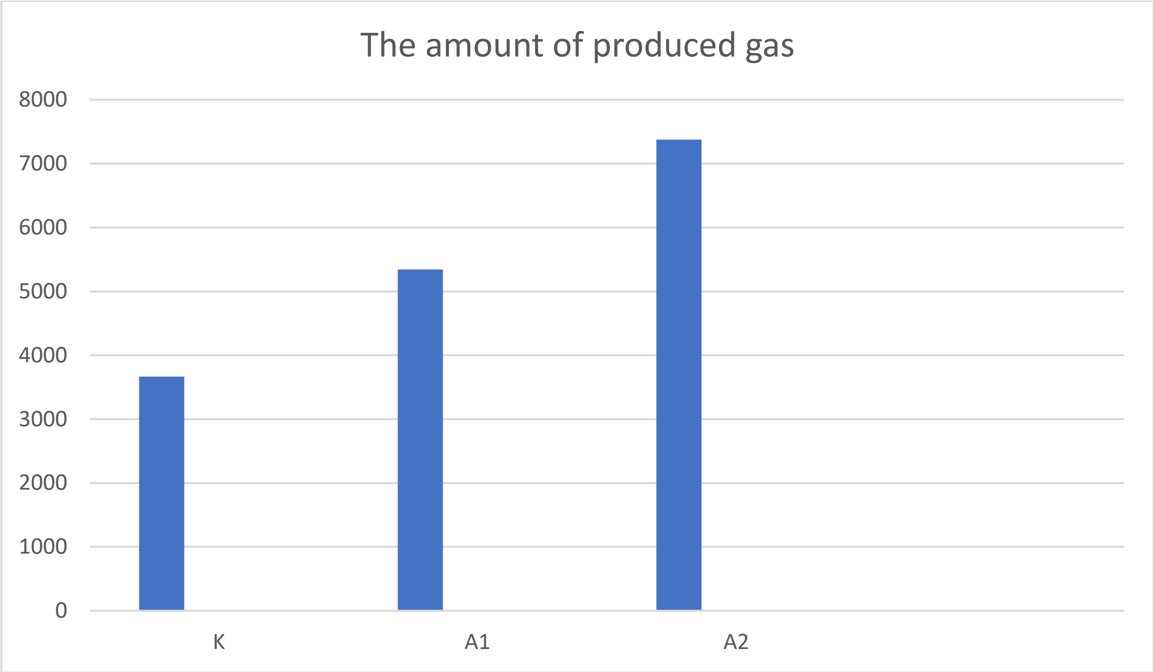
Acidity level is a key factor during anaerobic fermentation. The pH value of the substrate has a significant influence on the growth and development of methanogenic microorganisms and depends on the bicarbonate alkalinity, the partial pressure of CO_2 and the concentration of volatile fatty acids. The process of methanogenesis takes place best in the range of pH values from 6.6 to 7.6. During anaerobic fermentation, the pH value is not constant, but varies within the range of 5.5 to 8.2. The obtained pH value at the beginning of anaerobic fermentation for the control group is 7,28, and in the experimental groups it ranges from 7,21 to 7,19. After fermentation, the pH value increased in all groups and the pH value for control group was 7,86, in the mixture of beef manure and 5g of algae pH was 7,93, in the mixture of beef manure and 10g of algae pH was 7,95. Values are shown in the graph 3.



GRAPH 3: pH before and after fermentation

4.4. THE AMOUNT OF PRODUCED GAS

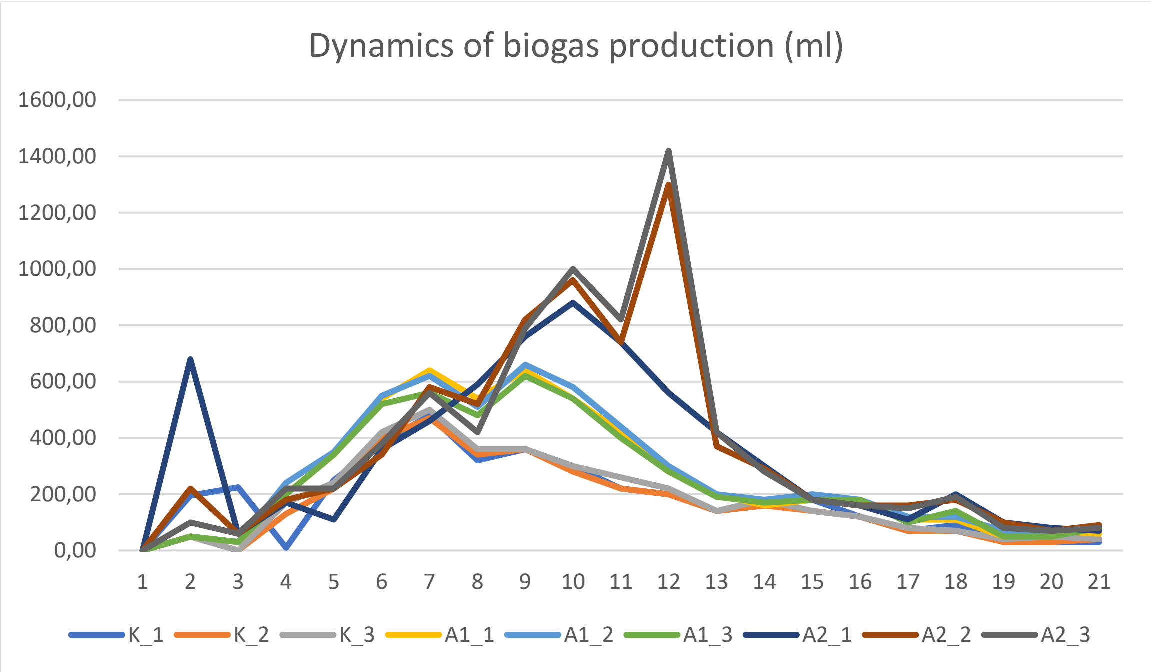
The total amount of biogas produced during the retention time of 21 days from 500ml of substrate is shown in Graph 4. The obtained results show that all experimental groups exceeded the biogas production of the control group, whose average value was 3666,67 ml/500ml. The total average amount of biogas in the experimental groups ranges from 5343,33 ml to 7376,67ml/500 ml, which is shown in Graph 4. That is, 7.33 ml/g was produced per gram of fresh sample in the control group, in the experimental groups the amount of gas per gram ranges from 335.33 to 371.00 ml/g.



GRAPH 4: Average values of the total amount of biogas produced in control samples and experimental samples of beef manure and algae

4.5. DYNAMICS OF BIOGAS PRODUCTION

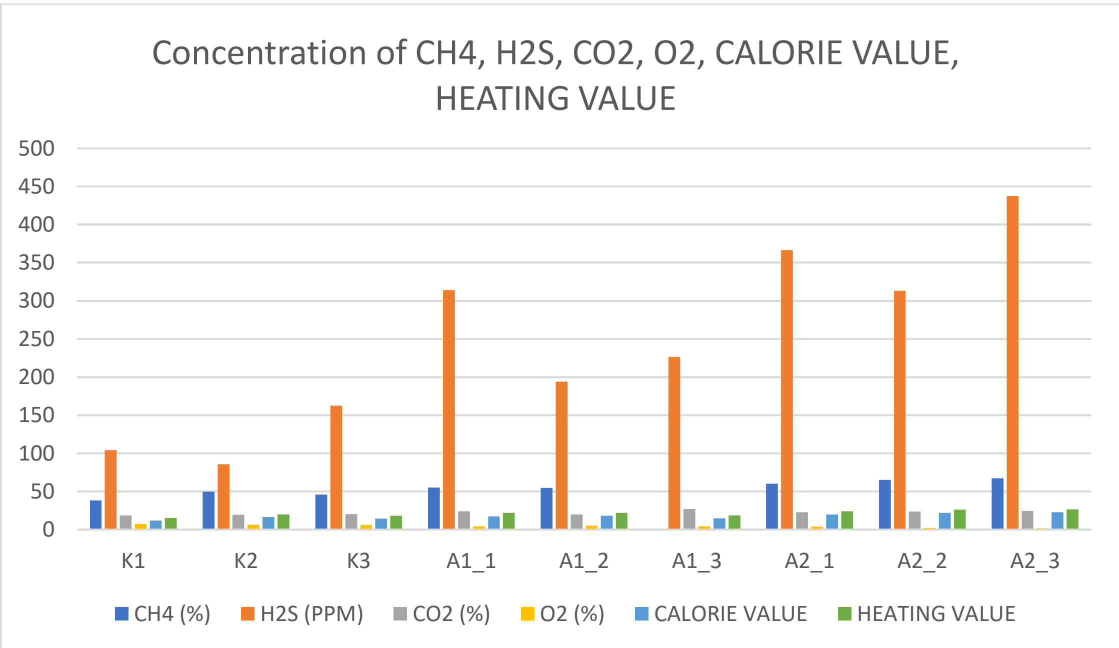
The dynamics of biogas production was monitored over a period of 28 days. Three samples were placed from each group. According to Graph 5, it can be seen that in all groups, the most intensive production takes place in the first 12 days, and it reaches its peak between the ninth and twelfth days of anaerobic fermentation. Beef manure with 10g of algae achieves the most intensive production between the eleventh and thirteenth day of anaerobic fermentation.



GRAPH 5: Dynamics of biogas production in control groups and experimental groups of beef manure and algae

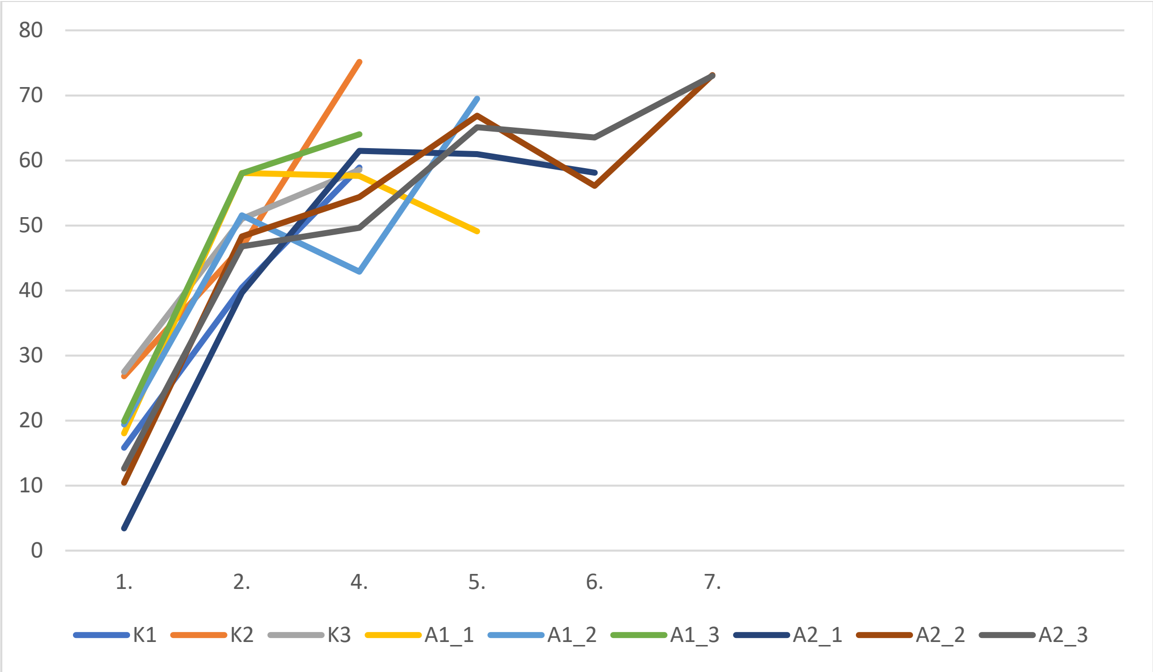
4.6. COMPOSITION OF GAS

During the research, three to six analyzes of the produced gas were carried out for each group. Graph 6 shows the average concentration values.



GRAPH 6: Average concentration values of gas

The growth of methane concentration is intense in the first 9 days, when it reaches values over 50%. The maximum concentration of methane was recorded on the 16th day of anaerobic fermentation in group A2, second sample, and was 73.12% (Graph 7).



GRAPH 7: Methane concentration values during anaerobic fermentation

5. DISCUSSION

In global production, the basic raw materials for biogas production via anaerobic digestion include animal manure (36%), agricultural waste (30%) and municipal solid waste (34%) according to a 2018 study by Valijanian et al. In biotechnology, the term "substrate" is used to describe the raw material component that is consumed during a microbial process. In the context of biogas technology, the term "substrate" replaces the term "raw material", as described in a 2009 study by Krička et al. The type of raw material has a significant influence on the configuration of the bioreactor, its design, mode of operation and bacterial physiology. For example, waste with a high content of dry matter and polymer compounds requires a completely different bioreactor design compared to wastewater containing easily degradable compounds. Hemicellulose, fats, and proteins can be degraded in just a few days, while sugars with low relative molecular weight, volatile fatty acids, and alcohols undergo degradation quickly, often within a few hours, as shown by a 1998. study by Steffen et al.

In this research, as a raw material for the production of biogas, we used agricultural and food waste, that is, the remains of wine production. Considering the growing need for thermal energy, especially in agriculture, the energy need is becoming more and more important. The use of agricultural residues for heating buildings, drying agricultural products and general energy utilization of agricultural residues is becoming an important way of energy production in certain parts of Croatia. Economic viability plays a crucial role in any biogas production project, as it is essential to demonstrate the project's profitability when compared to other potential uses of the same raw materials. For instance, utilizing agricultural raw materials for biogas production directly competes with their use in food and foodstuff production. When food resources are diverted towards biogas production, leading to reduced availability for food production, it can result in an increase in the market prices of these food products. One solution to address this issue is to identify alternative raw materials that do not compete with food production. In this research, *Chlorella* algae, known for its rapid growth and reproductive capabilities that do not pose a threat to nutrition, were studied alongside the control group (cattle manure) as a raw material for biogas production. Cattle manure has a low dry matter concentration, with a level of 3.02% dry matter in our study. On the other hand, Chlorella has a significantly higher percentage of dry matter compared to beef slurry, and it amounts to 94.22%. A higher content of dry matter represents a more favorable nutrient for bacteria, which results in their faster activity and production of larger amounts of methane, as shown in the research of Šalamon et al. from 1983.

The dry matter concentration changes during the study, with initially higher values due to mixing 5 and 10 grams of Chlorella with 500 grams of fresh beef slurry. Also, after anaerobic fermentation, the concentration of dry matter decreases in all experimental groups, including the control group. Before fermentation, the experimental group of cattle manure with 10 grams of algae had the highest concentration of dry matter, and the same group retained the highest proportion of dry matter even after fermentation. The amount of volatile and organic matter, with an optimal ratio with ash ranging from 80-60%, as described in the work of Benčević from 1993, shows that the conditions are favorable for the production of biogas in this research. Before fermentation, mix cattle manure with 10 grams of algae had the highest proportion of organic matter, amounting to 81.27%, while cattle manure had the lowest proportion of 75.36%. After fermentation, the results achieved remain relatively similar, a reduction in the proportion of organic matter ranging from 11 to 18% compared to the initial value before fermentation.

The pH value, the degree of acidity, plays a key role in the process of anaerobic fermentation and is an important parameter that affects the development of methanogenic bacteria. To recognize the process of methanogenesis, we can rely on pH values that range from 7.19 to 7.28. The degree of acidity largely depends on bicarbonate alkalinity, CO_2 partial pressure and volatile fatty acid concentration. During the process of anaerobic fermentation, the pH value is not constant and varies within the limits of 5.5 to 8.2. Before fermentation, the control group K had the highest pH value, which was 7.28. After fermentation, the highest pH value among the experimental groups was mixed cattle manure with 10g of algae, reaching a pH value of 7.96. Before fermentation, the same experimental group, mix cattle manure with 10g of algae, had the lowest pH value of 7.19, while after fermentation, the lowest pH value was the control group of cattle manure, with a value of 7.82. Methane can be produced in the pH range between 5.5 and 8.5, although the optimal range for the methanogenesis process is between pH 7 and pH 8. The total pH value inside the bioreactor depends on the partial pressure of carbon dioxide and the proportion of acidic and basic compounds.

From 500 ml of substrate during the retention time of 21 days, we obtained the total amount of produced biogas. The control group produced an average value of 3666.67 ml of biogas. However, the experimental groups outperformed the control group, with average values varying between 5160 and 7600 ml of biogas. The highest production value, 7600 ml, was shown by the group of mix cattle manure with 10 g of algae, while the lowest value, 5160 ml, was shown by the group of mix cattle manure with 5 g of algae.

In all groups, the dynamics of biogas production were monitored over a 21-day period, with the most intensive production observed after the sixth day. The peak of biogas production was reached between days 19 and 21 during anaerobic fermentation.

Methane produced through anaerobic fermentation of organic matter supports combustion and can be a suitable replacement for fossil fuels due to its energy value (Višković, 2008). Carbon dioxide is generated by burning biomass, released into the atmosphere, and cannot be converted into other harmless compounds. The atmospheric carbon dioxide load when using biomass is negligible because the amount of emitted carbon dioxide is equivalent to the amount absorbed during plant growth (Potočnik et al., 2002).

Throughout the research, 3 to 6 gas production analyzes were conducted for each group. In the first 7 days, methane concentration showed intensive growth, reaching values of over 50%. The maximum methane concentration was recorded on the 17th day of anaerobic fermentation in the cattle manure with 10g of algae group, amounting to 73.12%. The highest methane production per gram of dry organic matter (mlCH4/gDM) at 1000 ml was achieved by cattle manure with 10g of algae. A higher content of dry organic matter and a higher fat content in the substrate resulted in a higher percentage of methane. Substrates dominated by carbohydrates yielded a lower amount of produced biogas and lower methane content because the degradation of carbohydrates produces more carbon dioxide. Biogas with a high methane content has a higher energy value and is essential for energy production.

6. CONCLUSION

The study investigated the biogas production potential from *Chlorella* algae and compared it with cow manure as a substrate. The research monitored biogas production over a period of 21 days, observing that the most intensive production occurred after the sixth day, with a peak between days 19 and 21 during anaerobic fermentation. Methane, a valuable component of biogas, was found to be significantly produced, with concentrations exceeding 50% in the first seven days of fermentation. The highest concentration of methane (73.12%) was recorded on day 17 in the group that used manure with 10 g of algae. The study pointed out that the highest production of methane per gram of dry organic matter (mlCH4/gDM) was achieved in cattle manure with 10 g of algae group, reaching 1000 ml. This highlights the potential of *Chlorella* algae as a substrate for efficient biogas production. The research showed that substrates with a higher proportion of dry organic matter and a higher proportion of fat resulted in a higher percentage of methane production. Using biogas, which is primarily composed of methane, as an energy source has a lower environmental impact compared to fossil fuels, as it helps reduce carbon dioxide emissions. This is in line with the growing need for sustainable energy sources. In conclusion, this study demonstrates the potential of *Chlorella* algae as a substrate for biogas production, demonstrating its efficiency in methane generation, which is promising as a renewable and environmentally friendly energy source. The findings suggest that *Chlorella* algae could be a valuable resource in the field of bioenergy production, contributing to the shift towards more sustainable energy solutions. Further research and development in this area could unlock the full potential of *Chlorella* algae for biogas production.

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8. SUMMARY

Biogas is a product produced by anaerobic digestion, where different microorganisms break down organic matter through different metabolic pathways. Biogas has a large potential source of energy, and biogas production technology is favorable due to environmental protection and reduction of greenhouse gases. In the Republic of Croatia, there is only one factory that grows Chlorella (PHYOX D.O.O.). With a high dry matter content, Chlorella is a potentially good source of substrate for biogas production. Bovine slurry as a control substrate has a low concentration of dry matter, i.e. 3.02% dry matter. The average dry matter of Chlorella is 94.22%. The proportion of volatile and organic matter is within high limits, which is important for biogas production. In all groups, the dynamics of production was monitored over a period of 21 days. The control group produced an average value of 3666.67 ml of biogas. The control group was surpassed by the experimental groups where the average value ranges from 5160 to 7600 ml of biogas. The highest production of methane per gram of dry organic matter (mlCH 4 /gOT) in the amount of 1000 ml was achieved by the experimental group of cattle manure with 10g of dry algae.

9. INSET

List of abbreviations used

FULL NAME	ABBREVIATION
Dry matter	DM
Organic matter	OM
Control group	K
Experimental group A1	A1
Experimental group A2	A2
Gram	g
Mililiter	ml

10. LIST OF FIGURES

11. LIST OF GRAPHS

BASIC DOCUMENTATION CARD

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THE POSSIBILITY OF BIOGASS PRODUCTION FROM *Chlorella* ALGAE

Lucija Magdić

Abstract: Biogas is a product produced by anaerobic digestion, where different microorganisms break down organic matter through different metabolic pathways. Biogas has a large potential source of energy, and biogas production technology is favorable due to environmental protection and reduction of greenhouse gases. In the Republic of Croatia, there is only one factory that grows Chlorella (PHYOX D.O.O.). With a high dry matter content, Chlorella is a potentially good source of substrate for biogas production. Bovine slurry as a control substrate has a low concentration of dry matter, i.e. 3.02% dry matter. The average dry matter of Chlorella is 94.22%. The proportion of volatile and organic matter is within high limits, which is important for biogas production. In all groups, the dynamics of production was monitored over a period of 21 days. The control group produced an average value of 3666.67 ml of biogas. The control group was surpassed by the experimental groups where the average value ranges from 5160 to 7600 ml of biogas. The highest production of methane per gram of dry organic matter (mlCH 4 /gOT) in the amount of 1000 ml was achieved by the experimental group of cattle manure with 10g of dry algae.

Keywords: biogas, algae, Chlorella, cattle manure, anaerobic fermentation

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