Enhancement of Algal Biomass Accumulation Using Undiluted Anaerobic Digestate

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***Abstract*—Microalgae are unicellular photosynthetic organisms with industrial and economic perspectives. Their cultivation has a great deal of potential for many applications. Microalgae possess high growth and nutrient uptake rates, carbon capture and biomass generation. The cultivation of microalgae needs to be improved in order to reduce the cost of the produced biomass. Anaerobic digestion is a waste-to-energy technology employing a consortium of anaerobic microorganisms and resulting in continuous production of methane-rich biogas, with intermittent release of effluent digestate rich in undigested solids, organic and inorganic compounds and metal salts. The results in this work demonstrate the possibility of improving algal biomass accumulation using different anaerobic digestates and suitable algal strains. We investigated the growth and development of seven microalgal species – green, blue-green, red and yellow-green in the resultant digestate from methane production. The digestate was decolorized with active charcoal and used as medium in non-diluted and diluted with water (1:1) form. Most abundant growth was registered after two weeks of extensive cultivation for the green algae *Scenedesmus acutus,* followed by *Klebsormidium flaccidum,* and yellow-green *Trachydiscus minutus.* Further intensive cultivation of *Scenedesmus acutus* was performed and good growth was confirmed by estimating the average specific growth rate (μ=0.30 d^{-1}) and pigment accumulation (total pigment content 7.45% of DW).Our results showed that the digestate, obtained straight after a biomethane generating anaerobic process, with no additional water, is a suitable cultivation medium. Compared to the control with a standard medium, it enhanced the growth of *Scenedesmus acutus* 6.5 times in one week. This is a good precondition for a reliable application of this digestate in practice for production of algal biomass with its metabolites, instead of being released as a waste.**

Index Terms*—microalgae, digestate, growth rate, pigments, *Scenedesmus acutus

I. Introduction

Microalgae are fast-growing phototrophic microorganisms which utilize light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus etc.) [1] And in turn synthesize valuable biomass compounds, such as lipids, proteins, carbohydrate and pigments [2], [3]. These compounds find application in various industries: pharmaceutical, food industry, agriculture, green chemistry and the bioenergy sectors [4]. This makes the reduction of the price of the biomass production essential for the further development of the algal biotechnology. Among the major costs for algal cultivation are the ones for nutrients, such as nitrogen and phosphorous. These components could be found in large quantities in wastewaters from different industries [5], [6]. Microalgae offer an elegant solution to tertiary and quandary treatments due to their ability to use inorganic nitrogen and phosphorus for their growth [7], which is supprorted by their high capacity of inorganic nutrient uptake [8].

In the last decades, biogas production has developed rapidly. The major fraction in this anaerobic fermentation is the liquid digestate. Typically, anaerobic digestate is characterized by high levels of macro and microelements [9]. As the amount of nutrients in the digestate cannot be significantly reduced during the anaerobic digestion [10], it needs to be effectively recycled afterwards. This leads to the idea of utilizing the product by microalgae instead of discharging it into the environment [11], [12]. There are reports of successful cultivation of microalgae such as *Chlorella*, *Scenedesmus*, *Phaeodactylum* and *Pavlova* in anaerobic digestate [13]-[15].

Selection of appropriate strains is critical for producing valuable biomass [16]. Examples of targeted biosynthesis are the species *Klebsormidium* and *Stigeoclonium*, which accumulate valuable unsaturated fatty acids such as C18: 2 and C18: 3 in large quantities [17]. A main requirement for sustainable digestate treatment is a high biomass accumulation, therefore, the next step is the selection of suitable conditions for algae cultivation [6]. It is well known that each microalgal strain has its favorable growth conditions, such as pH, light, temperature, salinity, and the preferred nitrogen form and N / P ratio [2], [18]-[20]. The aim of this work was to find a perspective algal strain that grows well on

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undiluted digestate from anaerobic digestion of agro-waste during biogas production. This study presents a possible solution for reducing the costs of algal cultivation, and production of biomass and valuable metabolites.

II. Materials and Methods

A. Biological Material and Cultivation Conditions

For our experiments, seven microalgal strains: green algae *Scenedesmus acutus*, *Scenedesmus sp. BGP*, *Klebsormidium flaccidum* and *Coelastrella sp. BGV*, yellow-green alga *Trachydiscus minutus*, red alga *Porphyridium aerugineum*, blue-green alga *Synechocystis salina* were used. All of the abovementioned algae are deposited in the collection of Laboratory of Experimental Algology, Institute of Plant Physiology and Genetics, BAS, Sofia, Bulgaria.

B. Extensive Cultivation

Extensive algae cultivation was carried out at room temperature and natural daylight for a period of 14 days. A standard culture medium of Setlik, modified by [21], was used as a control. Three variants of anaerobic digestates from a methanogenic laboratory bioreactors were tested as nutrient media for algal cultivation: Variant 1: digestate from a mesophilic anaerobic process (37 ℃) with substrate wheat straw 10 g L^{-1}, chemically pretreated; Variant 2: digestate with substrate wheat straw (37 ℃) and Variant 3: digestate from a thermophilic process (55 ℃) with substrate wheat straw. The liquid digestate was subjected to clarification by adsorption with 8g 100mL^{-1} active charcoal (Flika) overnight at room temperature for all the three variants.

1) Chemical pretreatment

The digestate was treated with 28% NH_4OH, polyethylene glycol-4000 (3%) and water in a ratio of 1:0.5:20: NH_4OH: H_2O. The mixture underwent heating at 90 ℃ for 5h in a water bath. This was followed by rinsing till neutral pH.

a) Cell count

To evaluate the growth and development of the investigated strain, cell count was carried out using a Burker counting chamber.

b) Elemental cnalysis

Elemental composition of the obtained digestate was performed with EuroEA 3000 automatic analyzer.

C. Intensive Cultivation

An initial algal culture density of 0.8 mg. mL^{-1} dry weight (DW) was used for all experiments. Cultivation was carried out at 25 ℃ and continuous illumination (132 µmol photons m^{-2} s^{-1}). A carbon source was provided by bubbling sterile 2% CO_2 (v/v). The standard culture medium of Setlik [21], was used for the control cultivation. Variant 1 of the digestate was used in non-diluted and 50% diluted form for all the treatments.

1) Dry weight and specific growth rate

The growth was evaluated by the increase in algal biomass. The DW (mg mL^{-1}) was determined gravimetrically. Algal suspensions (3×5 mL each) were filtered through Whatman GF/C glass filters (Whatman International Ltd, Maidstone, UK), rinsed with tap water to eliminate salts and oven dried at 105 ℃ till a constant weight. The specific growth rate [µ] was calculated using the following formula:

$$\mu = ln(m_t2/m_t1)/t2 - t1 \text{ [22],}$$

where m_t are the dry weights at the different days ($t_1 = 0$ and $t_2 = 6$).

2) Pigment content

Pigments - chlorophyll a, chlorophyll b, carotenoids, were measured spectrophotometrically at 665 nm, 645 nm and 460 nm, respectively, using a T70 UV/Vis (PG Instruments Ltd, Leicester, UK) spectrophotometer after extraction with boiling methanol. Using the absorbtions, the pigment content was calculated, employing the Mackiney formulas [23].

D. Data Analysis A

The experiments were conducted in triplicate. The data were presented as the means ±SD. The difference between the treatments was statistically analyzed by oneway ANOVA followed by the Bonferroni's post hoc test at significance level of $p<0.05$, using GraphPAD InStat Software (San Diego, CA, USA).

III. Results and Discussion

A. Extensive Cultivation of Microalgae on Digestate

In order to investigate the growth abilities of microalgal strains in anaerobic digestate as a medium, batch experiments were conducted at extensive cultivation conditions for a period of 14 days. On Fig.1 is presented the growth of the 7 strains in Variant 1 of digestate, determined by the increased number of algal cells for all the strains. The graph shows that most of the algae were growing well on this waste product. The representatives of green microalgae showed the highest increase. The maximal growth was registered for *Scenedesmus acutus*, followed by *Klepsormidium flaccidum* ($p>0.05$). Good growth was also observed for the eustigmatophycean alga *Trachydiscus minutus* ($p<0.05$). Blue-green and red microalgae showed the slowest growth.

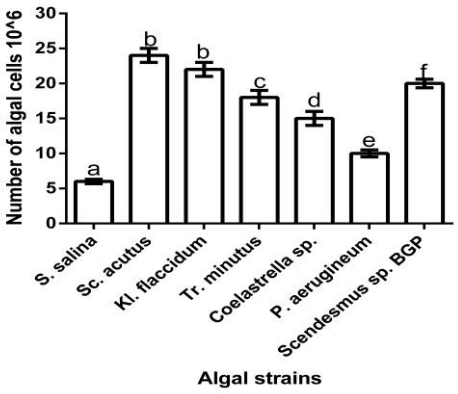

Figure 1. Growth of 7 different microalgal species on digestate from a mesophilic metanogenic process. Means with different lowercase letters are significantly different ($P < 0.05$) between the different algal strains.

Therefore, the subsequent experiments were performed with the alga that showed the greatest increase in cell mass (about 25-fold) - *Scenedesmus acutus*. It was grown in all the three variants of the anaerobic digestate, described in the Materials and methods section.

The results showed that Variant 1 of the digestate is the most suitable cultivation medium (Fig. 2). According to the cell count, there was no significant difference in the growth of *Scenedesmus acutus* in this medium compared to the control variant (p>0.05) at the end of the cultivation. Slightly lower algal growth was observed in Variant 2 followed by Variant 3. The elemental analysis indicated that when Variant 1 was used, the assimilation of N, H and C was also the highest (Table I).

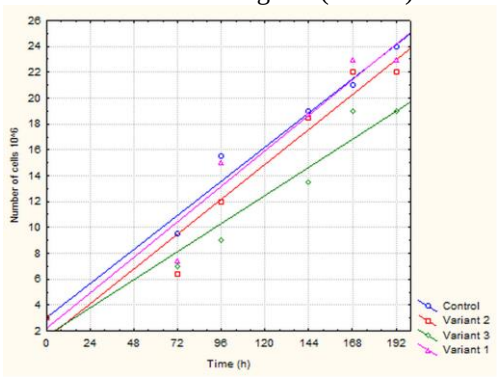

Figure 2. Growh of *Scenedesmus acutus* in different digestate variant.

TABLE I. ELEMENTAL ANALYSIS OF DIGESTATES

	Carbon, %	Nitrogen, %	Hydrogen, %
Control	12.70±0.97	2.79±0.16	2.33±0.21
Variant 1	7.68±0.54	1.65±0.11	1.49±0.11
Variant 2	12.33±1.04	2.70±0.23	2.41±0.19
Variant 3	8.57±0.63	1.86±0.17	1.86±0.15

B. Intensive Cultivation

Based on this, we used Variant 1 of the digestate (in non-diluted and 50% diluted form) as a cultivation medium for the intensive cultivation of the chosen alga – *Scenedesmus acutus* (Fig. 3, Fig. 4).

The results acquired were promising for both diluted and non-diluted digestate. Over the 144-hour cultivation period, the DW of the cultures grown in the undiluted digestate reached values of 5.2±0.2 mg mL^{-1}, even exceeding the amount of accumulated biomass in the standard medium (control sample).

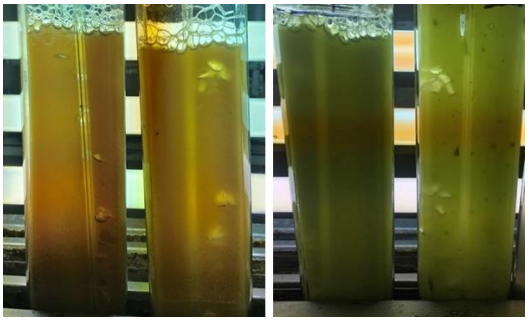

Figure 3. Intensive cultivation: at the beginning (left photo) and after 6 days of cultivation (right photo) in 100% digestate - first tube and. 50% digestate - second tube.

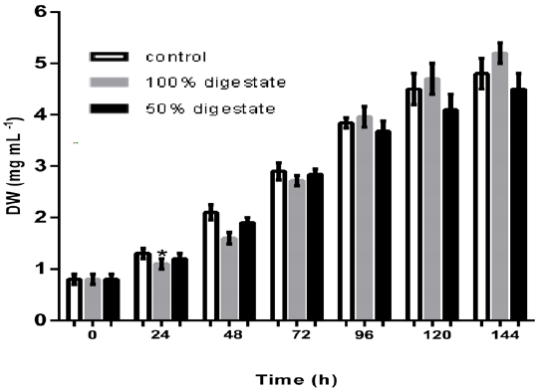

Figure 4. Intensive growth of *Scenedesmus acutus* in Variant 1 of digestate (100% and 50%). *p < 0.05 indicates significant difference from control values.

Following the dynamics of the process, initially (till 72h) a higher increase in the variant with 50% diluted digestate was registered (Fig. 4). In our opinion, this is due to the greater clarity of the diluted digestate at the beginning of the cultivation period. At the end of the exponential phase the elevated optical density due to the increased number of cells and the depleted nutrients, lead to decreased growth rate of *Scenedesmus* in this sample (Fig. 4).

Fig. 5 illustrates the average growth rate over the whole period. The highest average μ (d^{-1}) was recorded in the sample with undiluted digestate (0.30 d^{-1}) followed by the control (0.29 d^{-1}) and the variant with 50% dilution (0.28 d^{-1}).

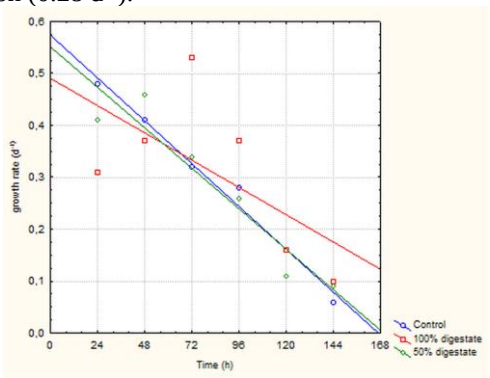

Figure 5. Specific growth rate of *Scenedesmus acutus* in digestate.

C. Pigment Analyses

The good growth and development of the microalgal culture of *Scenedesmus acutus* was proved by estimation of its pigment content.

The absorbance spectra peaks were obtained from methanolic extracts of intensively cultivated algal samples (Fig. 6). The results for the pigment composition of *Scenedesmus acutus* (Fig. 6, Table I) correlated with the growth of the algae and the increasing biomass production, which were highest in the samples, cultivated in the undiluted digestate.

Table II illustrates the results for the quantitative composition of the pigments from the samples, harvested at the 144th h of the intensive cultivation of *Scenedesmus acutus*. The values for chlorophylls and carotenoids are similar for control and undiluted variants (Table II).

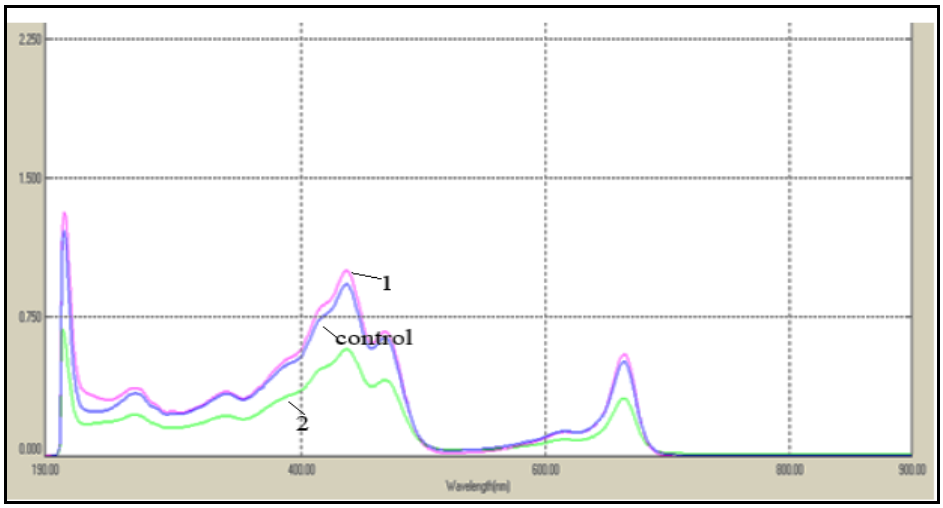

Figure 6. Specrophotometric estimation of absorption of pigments at the end of intensive cultivation. Green line-sample 2, grown in 50% diluted digestate; violet –control sample, grown in standard medium; pink – sample 1, grown in undiluted digestate.

TABLE II PIGMENT CONTENT (% OF DW)

	chl. "a"	chl."b"	carotenoids
control	2.8 ±0.1	1.2 ±0.2	3.15 ±0.37
100% digestate	2.9 ±0.1	1.3 ±0.2	3.25 ±0.28
50% digestate	2.1 ±0.1	0.8 ±0.1	2.5 ±0.16

The results obtained for the best growth in digestate for the green algae are confirmed by other authors [24], [25].

So far, most studies for growth of algae in digestate have been performed with large dilutions of 5 to 30 times [12], [24]. This reduces the efficiency many times, including addition of pure water in the cultivation process. In our work, we used activated charcoal to reduce the color of the digestate, thus were able to achieve substantial growth both with 50% dilution and without dilution (Fig. 4 and Fig. 5). At the end of the exponential phase in the non-diluted digestate was registered better growth of *Scenedesmus acutus*, about 1.16 times higher than diluted variant.

In the study of [26] was reported that the average specific growth rates of *Chlorella* and *Micractiniumin* are about 0.13 d^{-1} and 0.14 d^{-1}. The scientific team of [12] obtained a specific growth rate of 0.24 d^{-1} for the alga *Phaeodactylum tricornutum*. Our results showed μ of 0.30 (d^{-1}) on average over the entire cultivation period for the undiluted variant, which is 1.25 times higher than the reported by [26]. It should be noted that neither during the extensive, nor the intensive cultivation, any additional components were added to the culture medium.

Reference [27] reported an achieved yield of algal biomass in the range of 1.7 to 2.1 mg mL^{-1} after 8 days of intensive cultivation of *Chlorella* sp. in 50% diluted anaerobically digested sludge. Our results are two times higher, as over a 6-day cultivation period the DW of *Scenedesmus acutus* was 5.2±0.2 mg ml^{-1} without dilution, and 4.5±0.3 mg mL^{-1} with 50% dilution. This result is in agreement with a previous study of [28], claiming that the strains associated with this species were most effective when grown with substrates derived from waste water.

IV. CONCLUSION

The approach combining algal biomass production with utilization of digestate from a methane generating process creates a cost-effective management of an integrated biotechnological process. Moreover, the use of microalgae for waste remediation has an additional advantage. We proved the possibility of enhancing algal biomass production choosing the appropriate conditions for the anaerobic digestion process, as well as for the extensive and intensive cultivation of the algal strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J. I. and L. K. planned the project; L. K. provided the digestate for the experiments; J. I. and I. V. conducted the experiments, analyzed the data and wrote the article. All authors had approved the final version.

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