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Cultivation of Microalgae *Chlorella* sp. and *Scenedesmus* sp. as a Potentional Biofuel Feedstock

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The growth of two robust algae strains *Chlorella* sp. and *Scenedesmus* sp. growing in Lithuanian lakes was investigated with the aim to obtain optimum conditions for biomass cultivation for biofuel production in the Lithuanian environment. Samples were taken from different nitrogen sources and of different concentrations, with addition of various concentrations of CO_2 and in the presence of salt. The best biomass productivity was achieved using urea as a nitrogen source or modified growing medium BG11 with decreased concentration of $NaNO_3$. The positive impact on the growth of biomass was achieved by aeration with CO_2 (especially with concentration of 24%). Additional research into the removal of pollutants, such inorganic salts of nitrogen and phosphorus and organic materials from wastewater using microalgae has revealed good possibilities of using both algae strains in wastewater treatment plants. A content of oil in *Chlorella sp.* and *Scenedesmus sp.* has suggested their potential use as biodiesel feedstock.

Key words: *microalgae, biomass, growing conditions, CO_2 fixation.*

1. Introduction

The study of different aspects related to the behavior of microalgae has received renewed interest due to the wide field of application of these microorganisms. Algae cultures have been principally developed as an important source of many products, such as aquaculture feeds, human food supplements, and pharmaceuticals (Pulz and Gross, 2004; Apt et al. 1999) and they have been suggested as a very good candidate for fuel production (Shenk 2008).

Algae are a large and diverse group of simple typically autotrophic organisms, ranging from unicellular to multicellular forms. The advantages of algae over the other available feedstock of biodiesel fuel are the following: rapid growth rate and productivity (microalgae can produce 50 times more biomass compared to higher plants) (Li et al. 2008); no competition for land with crops (different types of microalgae are able to grow in a variety of environmental conditions, even on the limited areas of land (Mata et al. 2010); no competition with food market (Pokoo-Aikins et al. 2010); high oil content (oil yield in microalgae can exceed 75% by weight of dry biomass (Christi, 2007; Hu 2008); when used for biodiesel fuel production algae can simultaneously reduce CO_2 content in exhaust gases, minimize contamination – wastewater treatment from inorganic salts, such NH_4^+, NO^{3-}, PO_4^{3-}, using them as nutrient materials (Mata et al. 2010; Rodolfi et al. 2009). Such ability of algae can be considered a new possibility of using cheap methods for feeding of algae including filtrate of landfills or liquid fraction of digestate.

Various species of algae, among them seaweeds and freshwater organisms, are cultivated for biofuel production. Selecting algae strains for biomass conversion into the energy, attention is focused on those which are robust, highly productive, etc. (Spolaore et al. 2006). Strains with relatively high lipid content are very attractive for biodiesel fuel production (Rodolfi et al. 2009). Currently biodiesel fuel is produced mostly from rapeseed oil which is also used for food purposes (Montrimaitė et al. 2010). In order to reduce a negative influence of biodiesel fuel production on food consumption protection, it is

necessary to look for new raw materials for biofuel production.

Previous studies have demonstrated that lipid content in some microalgae increases during different cultivation conditions such as nitrogen deprivation (Illman et al. 2000; Hsieh and Wu 2009), high light intensity (Khotimchenko and Yakovleva 2005), and salt concentration (Araujo et al. 2011). The present study was focused on the cultivation of two robust green algae strains *Chlorella* sp. and *Scenedesmus* sp. extracted from Lithuanian lakes. The main objectives of the work were to investigate algae biomass growth under different nitrogen (N) sources and concentrations, various concentrations of CO_2 and salinity with the aim to obtain optimum conditions for biomass cultivation for biofuel production.

An investigation was also carried out into removal efficiency (reduction of pollutants) from liquid waste (liquid fraction of digestate after biogas production), which is rich in inorganic nitrogen (major part of ammonium) and phosphorus. This study was undertaken to explore the possibilities of *Chlorella* sp. and *Scenedesmus* sp. to reduce environmental pollution and lighten the operations of wastewater treatment plants.

2. Material and Methods

Algal monocultures. Monocultures of two common in Lithuanian lakes algae species, *Chlorella* sp. and *Scenedesmus* sp., were cultivated in BG11 medium (Stainier R. Y. et al. 1971) in the laboratory from 29 to 50 days with different inorganic nitrogen, carbon dioxide and salinity sources and concentrations. Algal strains were cultivated in the temperature controlled condition at 28 ^{o}C under illumination of white fluorescent lamps for approximately 8 hours daily. The cultures were hand shaken once or twice a day to avoid sticking.

Effect of N deficiency on algal growth. The nitrogen deficient medium was provided by the addition of $NaNO_3$, 0.75 g l^{-1} to result in a medium with N content of 0.1235 g l^{-1}or by the addition of urea, 0.1 g l^{-1} to result in a medium with N content of 0.0466 g l^{-1}.

Effect of the bio fixation of carbon dioxide. The effect of four CO_2 concentrations (6, 12, 18 and 24% of $CO_{2)}$ was investigated on the growth of algae biomass. The culture was aerated by a supply of CO^2 and nitrogen gas mixture at the same gas flow rate (2.08 l min^{-1}) for about 30 min every 48 h.

Effect of salt on algal biomass. The experiment was carried out with three different salinities (30, 35, 40 g l^{-1}) of BG11 medium with a deficient amount of $NaNO_3$ (0.75 g l^{-1}) with *Clorella* sp. These conditions were chosen to simulate sea water salinity. The salinity was adjusted by the addition of commercial NaCl (Poch, Poland).

Biomass concentrations. The growth of algae and biomass concentration was monitored by measuring optical density at a wavelength of 530 nm. Quantitative analysis of algae was performed in Goriajev chamber and biomass was estimated using stereometric method-appropriate geometrical volume (Jankavičiūtė G., 1996).

Analytical method. The wet extraction method modified by Bligh and Dyer (1959) was used to extract the lipids from microalgae biomass. The fatty acid composition of algae oil was determined according to the requirements of the standards LST EN ISO 5508 and LST EN ISO 5509. The analysis was carried out using Shimadzu GC -17A Gas Chromatography equipped with BPX – 70, 120 mm column and flame ionization detector. Fatty acids were qualified by comparing their retention time with a number of standard ones.

Main parameters (pollutants) of liquid digestate, such as total nitrogen (TN), total phosphorus (TP) and biological oxygen demand (BOD), were estimated according to the standard LST EN 25663:2000, spectrometric and electrochemical methods, respectively.

3. Results and Discussion

Algae, as a source of biofuel, offer many advantages over traditional biofuel crops including the potential to be grown on the marginal land, the use of water sources not suitable for agriculture (e.g. high salt content could be tolerated), as well as high growth rates, and relatively high lipid content (Wawrik B., Harriman B. 2010).

A wide variety of nitrogen sources, such as ammonia, nitrate, nitrite and urea, can be used for growing microalgae (Becker 1994). In the experimental data, urea and $NaNO_3$ were used to investigate the effect of the source of N and concentration of $NaNO_3$ (1.5 g l^{-1}, 0.75 g l^{-1}) on the growth of biomass.

Fig. 1 illustrates the effect of the *N* source and concentration on the *Chlorella* sp. biomass growth. As shown in Fig. 1 the initial growth of *Chlorella* sp. biomass at an early lag phase was very similar to that when the source of *N* was $NaNO_3$, despite that the concentrations were different (Fig. 1. curves 1 and 2). A sharp increase in concentration was characteristic of the growth medium with the initial $NaNO_3$ concentrations of 1.5 g l^{-1} during the lag phase of the cultivation. The biomass growth for the medium with initial $NaNO_3$ of 0.75 g l^{-1} was not so sharp but noticeable. The critical day of the biomass growth for the both media was identified as day 30, when the biomass concentration started to decrease rapidly until it reached a constant concentration In the case of urea, the sharp growth of biomass concentrations was noticed in the lag phase period, when the lag phase period of growth was very slight and stable till day 40.

Decrease in $NaNO_3$ concentrations in the medium and increase in algal metabolism products led to a slight decrease in the biomass amount. But an increase in N source concentration leads to decreasing

of lipid content in the cells (Hsieh and Wu 2009; Yeesang 2011). According Hsieh and Wu (2009) the critical urea concentration was observed at 0.1 g l^{-1} when the biomass grew intensively and had a higher lipid content compared to that cultivated with a sufficient nitrogen amount. Moreover, urea was reported to be the best nitrogen source of culturing *Chlorella* (Becker 1994). Consequently, an optimized supply of urea or deficiency of $NaNO_3$ is considered to be a cultivation strategy for microalgae lipid production.

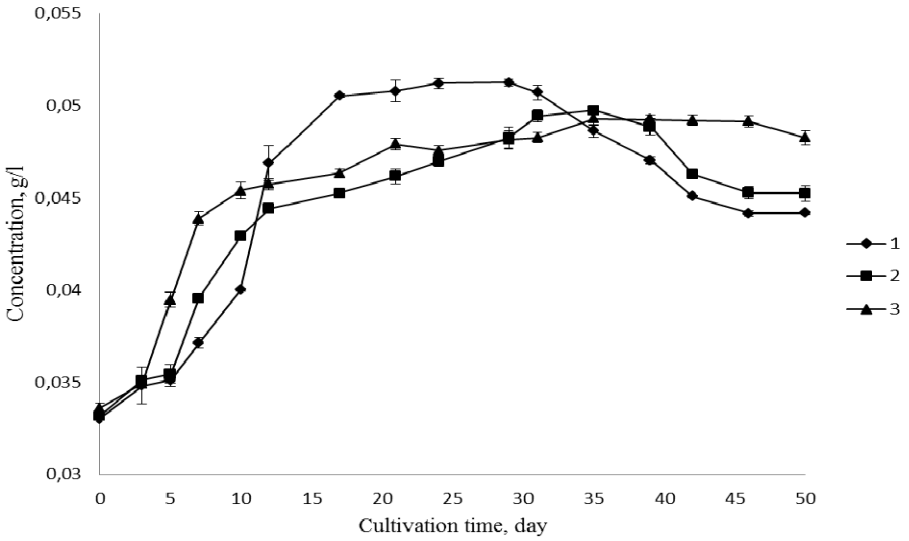

Fig. 1. Effect of N concentration and source on the Chlorella sp. biomass growth, cultivated in medium BG11, $NaNO_3$, 1.5 g l^{-1} (N concentration 0.247 g l^{-1}) (1); in modified medium $NaNO_3$, 0.75 g l^{-1} (N concentration 0.1235 g l^{-1}) (2) and in medium with urea, 0.1 g l^{-1} (N concentration 0.0466 g l^{-1}) (3)

In the application of algae to remove carbon dioxide (CO_2 biofixation) and energy production, tolerance to the CO_2, is of great importance (Tang et al. 2011). High levels of CO_2 are favorable to accumulation of total lipids and polyunsaturated fatty acids.

In the present study *Chlorela* sp. and *Scenedesmus* sp. were cultivated in BG11 medium under different CO_2 concentrations. The effects of different CO_2 concentrations on the growth of both microalgae are shown in Figs 2 - 3. In a subsequent study period (5-19 days) the algae biomass growth increased proportionally to the increase in CO_2 concentration. The slight growth and its proportional increase depending on the CO_2 concentration were characteristic of the latest algae biomass growth period (Fig.2, curves 2-5). A similar effect of CO_2 concentrations on *Scenedesmus* sp. growth was also determined (Fig. 3). The highest concentrations of biomass were detected in the medium under CO_2 concentration of 24% and reached the maximum biomass of 0.2 g l^{-1} and 0.12 g l^{-1} in *Chlorella* sp. and *Scenedesmus* sp., respectively.

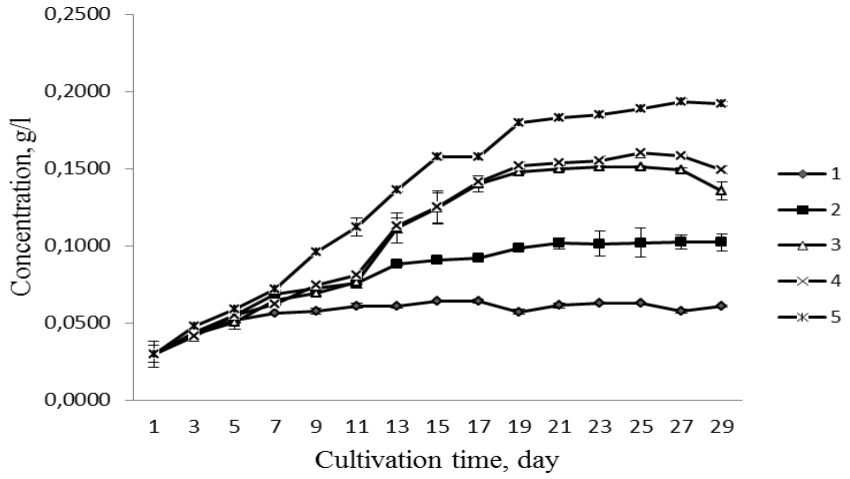

Fig. 2. Time course of biomass concentration of Chlorella sp. cultivated in medium BG11 under 0% (1), 6% (2), 12% (3), 18% (4), 24% (5) CO_2 concentrations

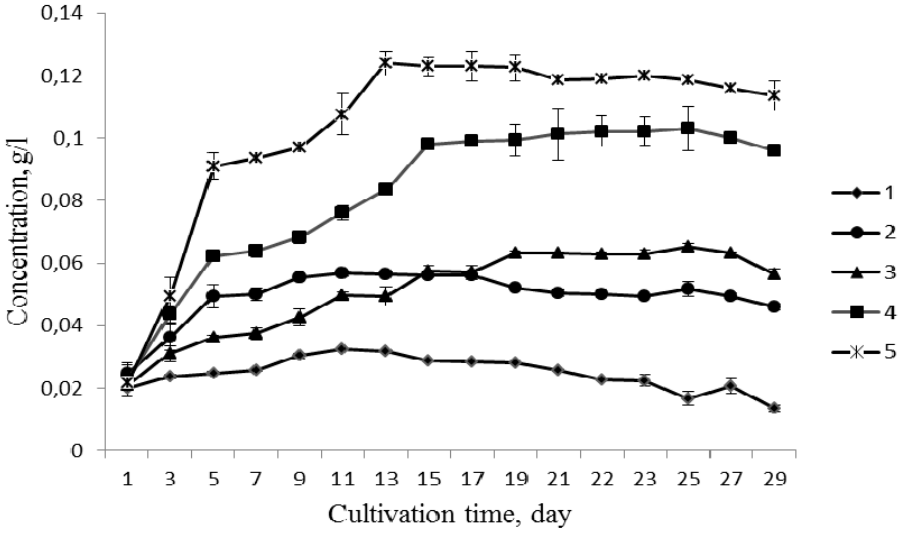

Fig. 3. Time course of biomass concentration of Scenedesmus sp. cultivated in medium BG11 under 0% (1), 6% (2), 12% (3), 18% (4), 24% (5) CO_2 concentrations

Literature repots on the CO_2 effect are greatly contradictory. Some of them showed that the concentration of CO_2 aeration above 5% could be harmful to microalgae cells and inhibit the microalgae growth (Chiu et al. 2008). Tang et al. (2011) reported that *Scenedesmus obliquus* and *Chlorella pyrenoidosa* could grow well under CO_2 concentrations ranging from 5% to 30%. They even could grow at the CO_2 level of 50%. Ota et al. (2009) reported the maximum biomass concentration of highly CO_2 tolerant *C. littorale* was about 0.5 g/l at the CO_2 concentration of 50% after 14 days cultivation.

It may be concluded that aeration of CO_2 is significantly superior due to the differences of cultivation conditions. During cultivation under the CO_2 flow, the bubbles and turbulent flow formed by aeration are helpful to enhance CO_2-culture medium contact and diffusion, and employ a carbon source for photosynthesis (Zhao B. et. al. 2011). But excessive turbulent flow conditions may affect the normal growth of microalgae biomass due to abnormal metabolism of cells.

The relationship between the average pH value of the culture medium and the cultivation time is illustrated in Fig. 4. The pH value ranged from 6.42 to 7.08 under the CO_2 concentration of 24% (Fig. 4, curve 1) while microalgae *Chlorella vulgaris* maintained the maximum growth rate in a wide range of pH between 6.0 and 9.0, but started to be inhibited from pH 5 (Fig. 4, curve 2) (Yun et al. 1996).

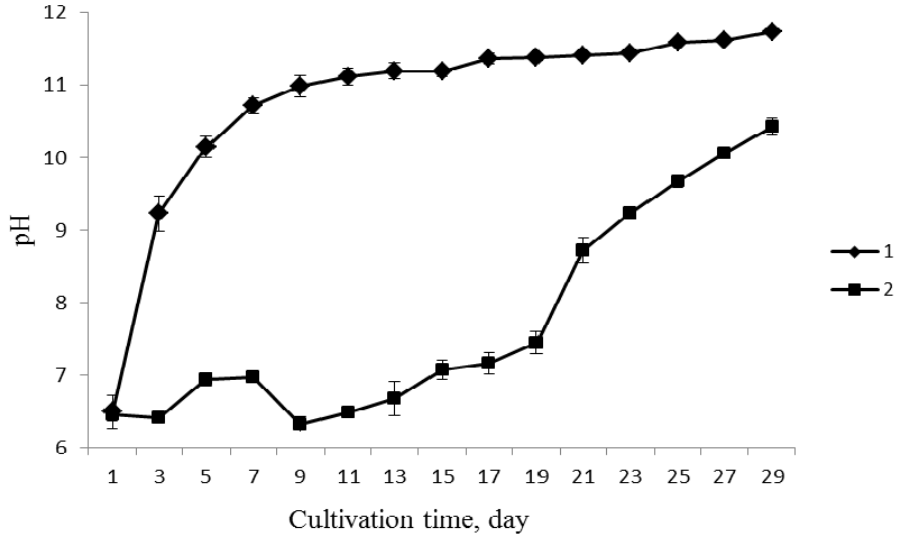

Fig. 4. Comparison of pH values of Chlorella sp. in the culture medium under 0% CO_2 (1) and with 24% CO_2 (2)

Many microalgae species can grow in brackish water or seawater, thereby avoiding demand for fresh water (Araujo 2011). Salinity is one of the environmental factors determining accumulation of lipids in algal cells. In this study the growth of *Chlorella* sp. biomass affected by salinity was investigated (Fig. 5). Algae survived under investigated conditions, but the biomass growth decreased with a salinity increase. Differences between biomass concentrations were not significant

while the salinities used were 35 g l^{-1} and 40 g l^{-1} (Fig. 5, curves 3, 4) compared to the concentrations obtained when salinity was 30 g l^{-1} (Fig 5, curves 2).

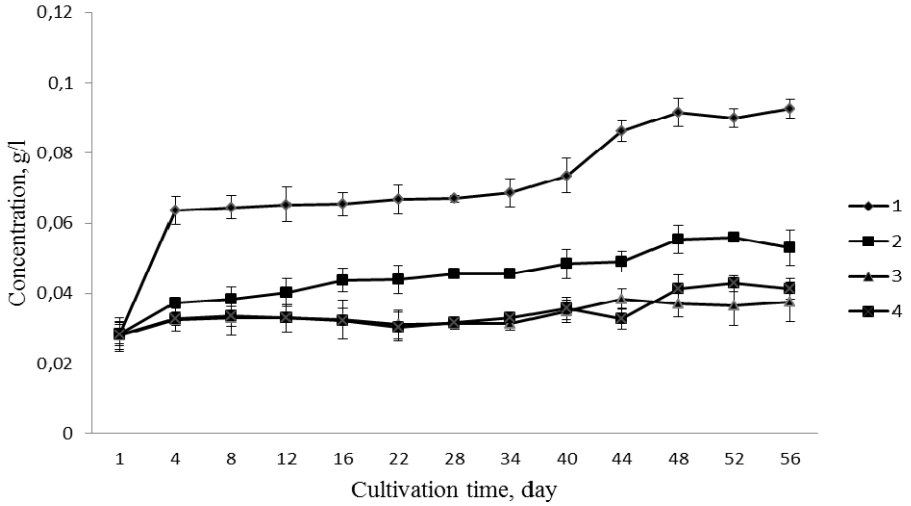

Fig. 5. Time course of biomass concentration of Chlorella sp. cultivated in medium BG11 at salinities 0 g l^{-1} (1), 30 g l^{-1} (2), 35 g l^{-1} (3), 40 g l^{-1} (4)

The removal efficiency of nitrogen and phosphorus from liquid digestate of both algae strains was tested during the cultivation time (20 days), (Table 1). The removal efficiency of N and P of *Chlorella* sp. and *Scenedesmus* sp. reached relatively high values: TN– 91% (for both algae), TP – 94.7% and 95.6%, respectively. During the algae cultivation period major part of organic pollutants was consumed. When growing *Chlorella* sp. BOD was reduced up to 87.1% and *Scenedesmus* sp. removed the mentioned pollutants to 92.1%. Comparing to the research data results of other scientists, in our study the removal of total nitrogen from sewage was achieved better. Wang et al. (2009) reported that removal of TN was approximately 80% and TP about 90% but such results were obtained in the sewage with less concentration of TN (approximately 6.0 times) and TP (approximately 1.5 times), cultivation time of algae was twice shorter.

Lipid composition during normal nutrition was analyzed by means of gas chromatography and the results are shown in Table 2. For comparisonsake the composition of fatty acids of rapeseed oil is also given. The results of the studies have shown that the contents of saturated fatty acids in algae oil are higher than those of rapeseed oil as well as the contents of monounsaturated fatty acids are quite lower. The contents of polyunsaturated fatty acids are very similar to algae oil and rapeseed oil. According to the requirements of the Lithuanian Standard LST EN 14214, the contents of linolenic acid methyl ester in biodiesel fuel should not exceed 12%.

Therefore, it can be forecast that biodiesel fuel produced from algae oil will meet the requirements of the standard concerning linolenic acid methyl ester contents. Also it can be expected that produced biodiesel fuel due to the fatty acid composition will meet the standard requirements for oxidation stability and iodine value.

Table 1. Algae removal efficiency of nitrogen and phosphorus from liquid digestate

Algae strains	Composition of digestate						Efficiency of removal of pollutants, %		
	Before algae cultivation			After algae cultivation					
	BOD (mgO_2/l)	TN (mgN/l)	TP (mgP/l)	BOD (mgO_2/l)	TN (mgN/l)	TP (mgP/l)	BOD_7 (mgO_2/l)	N_t (mgN/l)	P_t (mgP/l)
Chlorella sp	237	429	124	30.47	39.2	6.59	87.14	90.86	94.68
Scenedesmus sp	237	429	124	18.66	40.00	5.36	92.12	90.07	95.68

Table 2. Comparison of lipid composition

Fatty acids	Composition (%), of total fatty acids		
	Clorella sp.	*Scenedesmus*	*Rapeseed*
Saturated	48.9	51.9	5.4
Monounsuturated	20.9	17.5	58.3
Polyunsuturated	23.7	27.4	36.3
Trans isomers	4.9	2.1	
Omega-3	5.0	17.7	
Omega-6	12.5	9.3	
Linolenic	2.3	10.6	5.0-13.0

4. Conclusions

The investigation into the cultivation conditions of two robust algae *Chlorella* sp. and *Scenedesmus* sp. strains extracted from Lithuanian lakes has shown that the best results of the algae biomass growth could be achieved when cultivation medium BG11 is modified with urea or a decreased concentration of $NaNO_3$ and the growing biomass is aerated under CO_2 (concentration of 24 %). The fatty acid composition of oil in both cultures has conformed to the requirements and conclusion can be made that it is a promising resource for biofuel production. Besides, both algae strains *Chlorella* sp. and *Scenedesmus* sp. could be used in wastewater treatment as an additional removal agent of pollutants such as nitrogen and phosphorus.

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Potencialių biodegalų žaliavų – žaliadumblių *Chlorella* sp. ir *Scenedesmus* sp. – auginimas

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(gauta 2011 m. liepos mėn.; atiduota spaudai 2011 m. rugsėjo mėn.)

Žaliadumblių augimo proceso metu sukauptų riebalų viena iš galimų panaudojimo sričių – biodegalų gamyba. Siekiant išsiaiškinti optimalias dumblių auginimo sąlygas, tirta azoto šaltinio ir koncentracijos, CO_2 koncentracijos ir druskingumo įtaka Lietuvos ežeruose augančių žaliadumblių *Chlorella* sp. ir *Scenedesmus* sp. biomasės augimui, siekiant dumblius panaudoti biodegalų gamybai. Ištyrus azoto šaltinio ir koncentracijos auginimo terpėje įtaką, nustatyta, kad didžiausia biomasės koncentracija pasiekiama kaip azoto šaltinį naudojant karbamidą arba sumažintą $NaNO_3$ kiekį. Papildomas CO_2 dujų tiekimas taip pat skatino biomasės augimą. Ištirtas dumblių gebėjimas surišti nuotekų neorganinius (fosforo ir azoto) ir organinius teršalus. Gautų dumblių aliejų cheminė sudėtis leidžia įvertinti dumblius kaip potencialią biodegalų žaliavą.