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 (Ilman 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 CO2 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 °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 NaNO3, 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 CO2 concentrations (6, 12, 18 and 24% of CO2) was investigated on the growth of algae biomass. The culture was aerated by a supply of CO2 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 NaNO3 (0.75 g l⁻¹) with Chlorella 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 Goriàjev 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.

The experiment was planned to study the influence of the concentration of NaNO3 (0.75 g l⁻¹) on the growth of algae biomass. 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 NaNO3, 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 NaNO3 concentrations of 1.5 g l⁻¹ during the lag phase of the cultivation. The biomass growth for the medium with initial NaNO3 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 biomass concentration was noticed in the lag phase period, when the lag phase period of growth was very slight and stable till day 40.

Decrease in NaNO3 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.

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 Chlorella 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.
Literature reports on the CO\textsubscript{2} effect are greatly contradictory. Some of them showed that the concentration of CO\textsubscript{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\textsubscript{2} concentrations ranging from 5\% to 30\%. They even could grow at the CO\textsubscript{2} level of 50\%. Ota et al. (2009) reported the maximum biomass concentration of highly CO\textsubscript{2} tolerant *C. littorale* was about 0.5 g/l at the CO\textsubscript{2} concentration of 50\% after 14 days cultivation.

It may be concluded that aeration of CO\textsubscript{2} is significantly superior due to the differences of cultivation conditions. During cultivation under the CO\textsubscript{2} flow, the bubbles and turbulent flow formed by aeration are helpful to enhance CO\textsubscript{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\textsubscript{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).

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.
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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).

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

<table>
<thead>
<tr>
<th>Algae strains</th>
<th>Composition of digestate</th>
<th>Efficiency of removal of pollutants, %</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before algae cultivation</td>
<td>After algae cultivation</td>
</tr>
<tr>
<td></td>
<td>BOD (mgO₂/l)</td>
<td>TN (mgN/l)</td>
</tr>
<tr>
<td>Chlorella sp</td>
<td>237 429 124</td>
<td>30.47 39.2 6.59</td>
</tr>
<tr>
<td>Scenedesmus sp</td>
<td>237 429 124</td>
<td>18.66 40.00 5.36</td>
</tr>
</tbody>
</table>

Table 2. Comparison of lipid composition

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Composition (%), of total fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorella sp</td>
</tr>
<tr>
<td>Saturated</td>
<td>48.9</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>20.9</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>23.7</td>
</tr>
<tr>
<td>Trans isomers</td>
<td>4.9</td>
</tr>
<tr>
<td>Omega-3</td>
<td>5.0</td>
</tr>
<tr>
<td>Omega-6</td>
<td>12.5</td>
</tr>
<tr>
<td>Linolenic</td>
<td>2.3</td>
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</table>
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 – žaliadiumblų Chlorella sp. ir Scenedesmus sp. – auginimas

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Žaliadiumblų augimo proceso metu sukauptų riebalų viena iš galimų panaudojimo srūvių – biodegalų gamyba. Siekiant išsiaiškinti optimalias dumblių auginimo sąlygas, tirta azoto šaltinio ir koncentracijos, CO2 koncentracijos ir druskingumo įtaka Lietuvos ežeruose augančių žaliadiumblų 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 pasiekiamo kaip azoto šaltinį naudojant karbamidą arba sumažinant NaNO3 kiekį. Papildomas CO2 dujų tiekimas taip pat skatino biomasės augimą. Ištirtas dumblių gebėjimas suringti nuotekų neorganinius (fosforo ir azoto) ir organinius teršalus. Gautų dumblių aliejų cheminė sudėtis leidžia įvertinti dumblius kaip potencialią biodegalų žaliavą.