

**Karolina DZIOSA, Monika MAKOWSKA**

Institute for Sustainable Technologies – National Research Institute, Radom  
karolina.dziosa@itee.radom.pl, monika.makowska@itee.radom.pl

## **MONITORING OF *CHLORELLA* *sp.* GROWTH BASED ON THE OPTICAL DENSITY MEASUREMENT**

### **Key words**

*Chlorella* *sp.*, microalgae, optical density, biomass, nutrients, phosphorus deficiency.

### **Abstract**

A study on the possibility of using optical density measurement by UV-Vis spectrophotometry to control the intensity of multiplication of freshwater microalgae *Chlorella* *sp.* cells under phosphorus deficiency conditions. A properly equipped laboratory included a reactor filled with an aqueous solution of synthetic culture medium containing nitrogen and phosphorus compounds as well as other macro- and microelements necessary for the growth of algae. The growth of algae biomass under the experimental conditions also required to provide appropriate parameters, such as elevated temperature, availability of light, water, and carbon dioxide. The efficiency of algae biomass growth was measured due to optical density, defined as the absorption of visible radiation at 686 nm. At the same time, changes were measured in the content of nutrients in the culture medium, which were the result of metabolic processes. Based on the results of the experiments, it was found that the factor limiting the growth of microalgae is phosphorus. Once it is depleted, nitrogen is no longer assimilated by algae cells. Additionally, optical density is also decreased.

## Introduction

A global growth in energy demand led to the exploitation of nonconventional renewable energy sources. One of these examples of a renewable energy source is a biomass of algae [1, 2]. Its cells live in aquatic environments or in humid spaces, either in salt or fresh water. Since they are an autotrophic organism, they function as producers of organic matter. They are able to efficiently use less fertile areas more than energetic plants such as rapeseed or soya [3]. They are also capable of growing in wastewater. Algae can assimilate carbon dioxide as well as phosphorous and nitrogen derived from air pollutants, and municipal and industrial sewage [4–6].

For the proper growth of algae, not only is nonorganic carbon required but also nitrogen and phosphorous [7, 8]. Nitrogen is one of the elements of amino acids as well as nucleic acids (building blocks of proteins), chlorophylls, and plant hormones. The presence of phosphorous plays a significant role in metabolic processes of living organisms through the energetic balance regulation and other intracellular processes. Phosphorous deficiency contributes to the decline in the efficiency of marine ecosystems, thus even a small amount of phosphorous in aquatic ecosystem is the cause of an increased growth of algae [9]. Algae biomass is produced in the process of eutrophication in water tanks. In order to avoid an oxygen deficiency, the biomass needs to be constantly removed. Algae can be used in the bio-sorption and bioaccumulation, in the process of toxic metal ions concentration and in wastewater treatment [10–12].

The growth of an algal population can be divided into several major phases that follow one after the other (Fig. 1):

- Adaptation to environmental conditions,
- Intensive growth of algal biomass,
- Declining growth,
- Stationary phase, and
- Algal cell death.

These phases are characterized by a specific metabolism and the course of the photosynthesis process. From the viewpoint of the efficiency of culture, a particularly important role is played by the intensive growth phase. Total growth of algae biomass depends on its duration.

The estimated investment and operating costs associated with the implementation of technology and the production of algae biomass in Poland has maintained at a very high level. This is mainly due to unfavourable climatic conditions. Therefore, the most important factor in determining the scale of algae biomass use is the economic viability of their production. Much of the cost is associated with heating, exposure, and dehydration of biomass, as well as the control of production (including the cost of laboratory analysis). The sources of savings in this area include the use of fast and simple methods for monitoring metabolic processes [15–17].

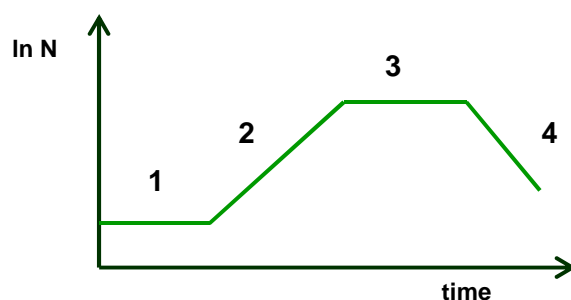


Fig. 1. Growth curve of an algal population under batch culture conditions ( $N$  – dry weight, 1 – adaptation, 2 – intensive growth, 3 – stationary phase, 4 – cell death), based on [13–15]

The aim of the study was to investigate the possibility of the use of optical density measurements to control the efficiency of microalgae biomass growth under a phosphorus deficiency. Due to the high capacity to adapt to changing environmental conditions and culture conditions, freshwater algae *Chlorella* sp. (single-cell green algae) were selected to carry out these experiments. Achieving this objective required the following: the preparation of a suitable laboratory stand, the determination of the chemical composition of the culture medium and other conditions of the experiment (temperature, mixing and light intensity, photo-period), as well as investigation of the intensity of multiplication of microalgae cells based on spectrophotometric measurements.

### Research objectives and methods

A laboratory culture was inoculated with microalgae *Chlorella* sp from the Culture Collection of Baltic Algae (Institute of Oceanography of the University of Gdańsk). During initiation of the culture, 20 cm<sup>3</sup> inoculum (BA0025) was introduced into the reactor containing culture medium. Figure 2 presents a microscopic image of algal cells before the inoculation.

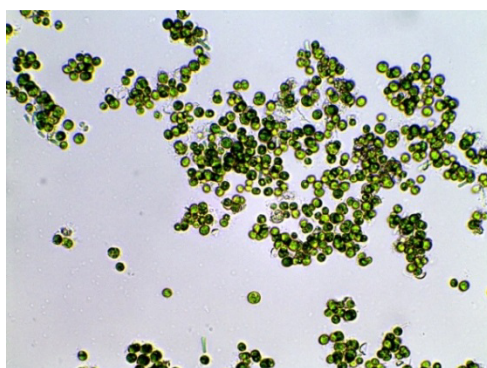


Fig. 2. Microscope image of algal cells (magnification 40×)

The culture medium was an aqueous solution of BG-11 synthetic medium (Table. 1) containing nitrogen (sodium nitrate) and phosphorus compounds (potassium hydrogen phosphate) easily assimilated by the algae as well as macro- and microelements essential for their growth. The medium was prepared with p.a. chemicals.

Table 1. Composition of the culture medium [18]

N <sup>o</sup>	Component	Content
1	NaNO <sub>3</sub>	1.5 g/dm <sup>3</sup>
2	K <sub>2</sub> HPO <sub>4</sub>	0.04 g/dm <sup>3</sup>
3	MgSO <sub>4</sub> ·7H <sub>2</sub> O	75 mg/dm <sup>3</sup>
4	CaCl <sub>2</sub> ·2H <sub>2</sub> O	36 mg/dm <sup>3</sup>
5	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> ·1H <sub>2</sub> O	6 mg/dm <sup>3</sup>
6	C <sub>12</sub> H <sub>22</sub> FeN <sub>3</sub> O <sub>14</sub>	6 mg/dm <sup>3</sup>
7	Na <sub>2</sub> CO <sub>3</sub>	20 mg/dm <sup>3</sup>
8	Na-EDTA	1 mg/dm <sup>3</sup>
9	H <sub>3</sub> BO <sub>3</sub>	2.86 mg/dm <sup>3</sup>
10	MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.81 mg/dm <sup>3</sup>
11	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.22 mg/dm <sup>3</sup>
12	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.39 mg/dm <sup>3</sup>
13	CuSO <sub>4</sub> ·5H <sub>2</sub> O	79 µg/dm <sup>3</sup>
14	Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	49.4 µg/dm <sup>3</sup>

Experiments were carried out in a cylindrical glass reactor equipped with a water jacket, a circulating thermostat, and mechanical stirrer with a PTFE anchor-shaped propeller blade (Fig. 3). Adequate water circulation facilitates the assimilation of CO<sub>2</sub> and nutrients by algae. Table 2 summarizes parameters of the experiment.



Fig. 3. Laboratory stand for the algae cultivation

Table 2. Parameters of the algae cultivation

N <sup>o</sup>	Parameter	Value
1	working volume	2 dm <sup>3</sup>
2	temperature	28±0.5°C
3	mixing intensity	160 rpm
4	light intensity	780–800 lux
5	photoperiod	16h/8h (light/dark)
6	time	42 days

Free gas exchange between the content of the reactors and surroundings was provided due to two outlets in the top of the cover in which were placed cellulose plugs. Two fluorescent lamps with chrome reflectors (power 2×18 W) in the vertical position, approximately 20 cm from the reactor, were the source of light. Throughout the culture process, observations of the cells were made using optical microscope Opta-Tech MK 358 (magnification 40×).

The efficiency of biomass growth was controlled by measuring the optical density (OD), which is defined as the absorption of visible radiation (adsorption peak of chlorophyll is at about 680 nm). The optimal wavelength (686 nm) was determined based on the UV spectrum. The research was carried out using a UV-VIS 6000 HACH DR 6000.

Assimilation of biogenic substances was analysed based on the content of phosphorous and nitrogen in the culture medium. The sample was taken from the reactor and subjected to filtration using filter paper MN 619 (φ 150 mm, thickness 0.17 mm, weight 75 g/mm<sup>2</sup>) in order to obtain a clear, colourless solution. Subsequently, total nitrogen bound TNb (according to EN-ISO 11905-1 Determination of nitrogen. Method using oxidative digestion with peroxodisulfate) and phosphorous (according to EN ISO 6878 Determination of phosphorus. Ammonium molybdate spectrometric method) in the culture medium was determined using cuvette tests HACH LANGE at the wavelength of 345 and 880 nm.

Samples for analysis were taken at regular time intervals (every few days). All results presented in the work are the arithmetic mean of results from three parallel measurements. The standard deviation was calculated.

## Results and discussion

Figure 4 shows the effect of algae biomass growth assessed based on the optical density OD<sub>686</sub>. At the same time, the changes in the content of nitrogen and phosphorous in the culture medium, which was the result of metabolic processes, were monitored (Fig. 5 and Fig. 6). The initial value of D<sub>686</sub> and the contents of nitrogen and phosphorous were determined after 1 hour from the initiation of microalgae culture (data in Figs. 4–6 refer to the solution of the culture medium and inoculum).

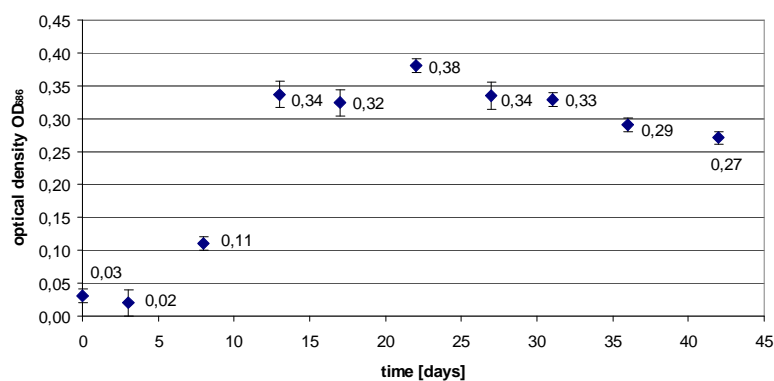


Fig. 4. Growth of an algae biomass during the experiment (results of OD686 measurement)

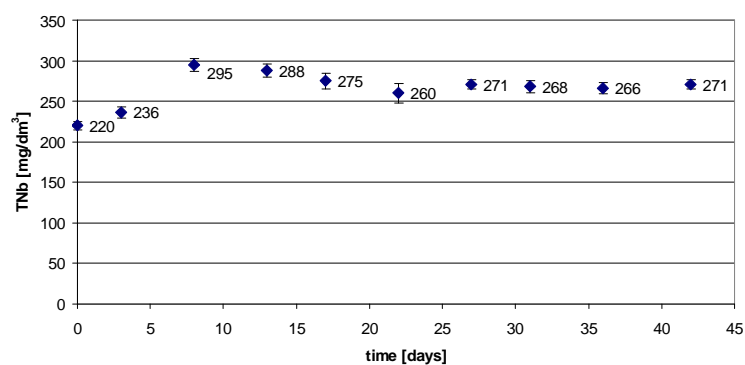


Fig. 5. Content of nitrogen in the filtrate of culture medium

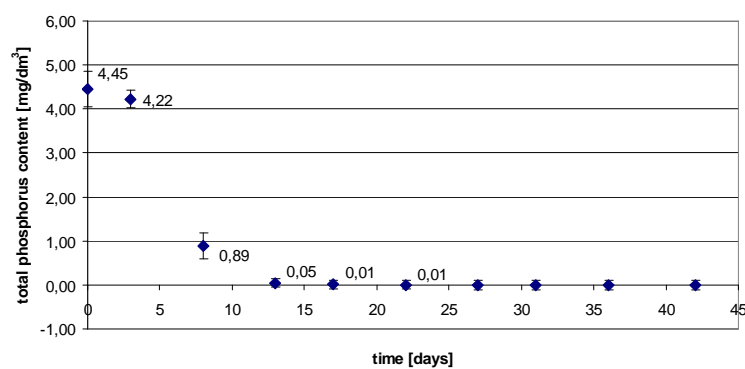


Fig. 6. Content of nitrogen in the filtrate of culture medium

Based on the data presented in Figs. 4–6 and microscopic observations, it was found that at the initial stage of the culture algae cells were being adapted to the new environmental conditions, although they earlier stayed at anabiosis state. This led to inhibition of the cell division, which was associated with photo-inhibition, particularly during the first three days. At the time, the optical density as well as nitrogen and phosphorous contents varied very slightly. The next step involved the acceleration of metabolic processes. The increase in optical density until the 8th day of the culture was relatively low (Fig. 4), but the microalgae assimilated the highest amount of phosphorous (about 74.8% of the initial phosphorus content in the culture medium). This resulted in a significant change in optical density on the following days (8–13 days). Its value increased three-fold (from 0.11 to 0.34) and the nitrogen content of 295 mg/dm<sup>3</sup> decreased to 288 mg/dm<sup>3</sup>. Changes in optical density and nutrient contents were statistically significant.

The subsequent stage involved the limitation and stabilization of the biomass growth. Most likely, together with the cell population densities while the content of phosphorus in the substrate decreased, it was followed by a deterioration of the oxygen supply and a reduction in the access to light [14]. The process of new cell formation was increasingly associated with old cell death and autolysis. After 22 days of algae culture, the depletion in the content of phosphorous was observed (Fig. 6). It caused an inhibition of nitrogen assimilation within the cells (Fig. 5) and decrease in optical density (Fig. 4).

Previous works regarding the growth of microalgae biomass and wastewater treatment using algae has resulted in various, sometimes contradicting, conclusions. Some authors found that algal growth was depended on the initial nutrient content in the culture medium and others that high concentration of the nutrients in wastewater inhibited algal growth [11, 19]. Wang C. et al. [19] suggest that phosphorus might be not limiting factor to algal growth, because *Chlorella* sp. grew well when this element's concentration was very low. Beuckels A. t al. [20] showed the positive effect of nitrogen availability on the accumulation of phosphorus in microalgae cells. Our work was conducted under laboratory conditions using standard synthetic medium. These experiment results show that the phosphorus deficiency inhibits the growth of *Chlorella* sp. These results are consistent with the work [21]. The influence of phosphorus on the growth of algae biomass certainly depends on the species, culture conditions, and other environmental parameters, which should be considered when comparing the test results.

## Summary

Spectrophotometric analyses enabled the assessment of the biomass growth efficiency of microalgae *Chlorella* sp. in laboratory conditions based on the optical density measurements as measured by absorption wavelength at 686 nm. Control of the OD686, conducted simultaneously with the determination of

nitrogen and phosphorus content in the culture medium, enables the assessment of metabolic processes. Based on the obtained results, conclusions can be drawn on various stages of the growth of the algae population. Based on the results of the investigations, it was found that a the growth-limiting factor for microalgae was phosphorus, which caused the reduction of the assimilation of nitrogen by cells and a decrease in the optical density when exhausted, the consequence of which was to achieve phase dying.

Further research is required for the optimization of microalgae culture conditions, including the development of the composition of the synthetic medium, with the proper ratio of nitrogen and phosphorus. This is necessary to adapt these conditions to the expected results, which in perspective will allow for the intensification of metabolic processes and increasing the productivity of algae biomass.

## References

1. Bharathiraja B. et al.: Aquatic biomass (algae) as a future feed stock for bio-refineries: A review on cultivation, processing and products. *Renewable and Sustainable Energy Reviews*, 2015, 47, 634–653.
2. Ajanovic A.: Renewable fuels – A comparative assessment from economic, energetic and ecological point-of-view up to 2050 in EU-countries. *Renewable Energy*, 2013, 60, 733–738.
3. Schlarb-Ridley B., Parker B.: A UK road map of algal technologies. NERC-TSB Algal Bioenergy-SIG, 2013.
4. Kumar A. et al.: Enhanced CO<sub>2</sub> fixation and biofuel production via microalgae: recent developments and future directions. *Trends in Biotechnology*, 2010, 28, 371–380.
5. Lu Q. et al.: Mitigating ammonia nitrogen deficiency in dairy wastewater for algae cultivation. *Bioresource Technology*, 2016, 201, 33–40.
6. Lu Q. et al.: Growing *Chlorella* sp. on meat wastewater for nutrient removal and biomass production. *Bioresource Technology*, 2015, 198, 189–197.
7. Lu W., Wang Z., Wang X., Yuan Z.: Cultivation of *Chlorella* sp. using raw dairy wastewater for nutrient removal and biodiesel production: Characteristics comparison of indoor bench-scale and outdoor pilot-scale cultures. *Bioresource Technology*, 2015, 192, 382–388.
8. Rowley W.M.: Nitrogen and phosphorous biomass-kinetic model for *Chlorella vulgaris* in a biofuel production scheme. Airforce Institute of Technology, 2010.
9. Bednarz T., Starzecka A., Mazurkiewicz-Boroń G.: Microbiological processes accompanied by algal and blue-greens water blooms. *Wiadomości Botaniczne*, 2002, 16, 45–55 (in Polish).



10. Urbańska M., Kłosowski G.: Algae as biosorption material – removing and recycling of heavy metals from industrial wastewater. *Ochrona Środowiska i Zasobów Naturalnych*, 2012, 51, 62–77 (in Polish).
11. Hill C.L.: Examination of nitrogen to phosphorous ratio in nutrient removal from wastewater through *Chlorella vulgaris*. University of Arkansas, 2014.
12. Ahn Ch.H., Park J.K.: Critical factors affecting biological phosphorous removal in dairy wastewater treatment plants. *Journal of Civil Engineering*, 2008, 12 (2), 99–107.
13. Barsanti L., Gualtieri P.: *Algae. Anatomy, Biochemistry, and Biotechnology*. Taylor&Francis, 2006.
14. Kozieł W., Włodarczyk T.: Algae – biomass production. *Acta Agrophysica*, 2011, 17(1), 105–116 (in Polish).
15. Sivakumar R., Rejendran S.: Growth measurement technique of microalgae. *International Journal of Current Science*, 2013, 7, 52–54.
16. Rodrigues L. et al.: Algal density assessed by spectrophotometry: A calibration curve for the unicellular algae *Pseudokirchneriella subcapitata*. *Journal of Environmental Chemistry and Ecotoxicology*, 2011, 3(8), 225–228.
17. Nowak B., Bojarska M., Skowroński J., Piątkiewicz W.: Antibacterial and antifouling properties of polypropylene membranes modified with zinc oxide nanowires. *Membranes and Membrane Processing in Environmental Protection*. Polish Academy of Science 2014, 118, 209–220.
18. Belotti G. et al.: Effect of *Chlorella vulgaris* growing conditions on bio-oil production via fast pyrolysis. *Biomass and Bioenergy*, 2014, 61, 187–195.
19. Wang C.: Nitrogen and phosphorus removal from municipal wastewater by the green algae *Chlorella* sp. *Journal of Environmental Biology*, 2013, 34(2), 421–425.
20. Beuckels A., Smolders E., Muylaert K.: Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. *Water research*, 2015, 77, 98–106.
21. Sławik-Dembiczak L., Kwarciak-Kozłowska A.: The application of algal growth potential test to the outflow canal of wastewater treatment plant. *Mat. konf. Eko-Dok*, 2014. (in Polish)

### **Monitorowanie przyrostu biomasy mikroalg *Chlorella* sp. na podstawie pomiaru gęstości optycznej**

#### **Słowa kluczowe**

*Chlorella* sp., mikroalgi, gęstość optyczna, biomasa, substancje biogenne, niedobór fosforu.

**Streszczenie**

Zbadano możliwość zastosowania spektrofotometrycznego pomiaru gęstości optycznej do kontroli intensywności namnażania komórek mikroalg słodkowodnych *Chlorella sp.* w warunkach niedoboru fosforu. Przygotowano odpowiednie stanowisko laboratoryjne zasilone w roztwór wodny pożywki syntetycznej zawierającej związki azotu i fosforu oraz inne makro- i mikroelementy niezbędne do wzrostu alg. Przyrost biomasy alg w warunkach prowadzenia eksperymentu wymagał także zapewnienia odpowiednich parametrów, w tym podwyższonej temperatury, oświetlenia oraz dostępu wody i dwutlenku węgla. Efektywność przyrostu biomasy w trakcie hodowli oceniano na podstawie pomiaru gęstości optycznej, definiowanej jako absorpcja promieniowania w zakresie widzialnym (686 nm). Równocześnie kontrolowano zmiany zawartości substancji biogenych w podłożu hodowlanym, będące wynikiem zachodzących procesów metabolicznych. Na podstawie wyników przeprowadzonych badań stwierdzono, że składnikiem limitującym wzrost mikroalg był fosfor, po wyczerpaniu którego nastąpiło zahamowanie przyswajania azotu przez komórki oraz spadek gęstości optycznej.