Studies on the Growth of *Chlorella vulgaris* in Culture Media with Different Carbon Sources

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Dedicated to the memory of Professor Dr. Valentin Koloini

Diminishing oil reserves, rising oil prices and a significant increase in atmospheric carbon dioxide levels have led to an increasing demand for alternative fuels. Microalgae have been suggested as a suitable means for fuel production because of their advantages related to higher growth rates, higher photosynthetic efficiency and higher biomass production, compared to other terrestrial energy crops. During photosynthesis, microalgae can fix carbon dioxide from different sources, including the atmosphere, industrial exhaust gases and soluble carbonate salts. To determine the most optimal conditions for the growth of *Chlorella vulgaris* in order to produce lipids that can be transformed into biodiesel fuel, different nutritional conditions were investigated. For this purpose, three media, namely Jaworski's medium, an enriched solution from modified Dual Solvay process and natural mineral water, were prepared and analyzed for biomass production, chlorophyll content and lipid content. The best growth resulted in an enriched solution from the modified Solvay process. This medium was diluted in different dilution ratios (1:100, 1:50, 1:10) and the best results were obtained in a medium diluted in a 1:10 ratio on the fifth day of culturing ($3.72 \cdot 10^6$ cells mL^{-1}; 4.98 μg mL^{-1} chlorophyll *a*).

Key words:
Carbon dioxide, microalgae, *Chlorella vulgaris*, batch culture, lipid content

Introduction

There is a growing consensus among governments, scientists, and industrial organizations of developed countries, about the threat of climate changes due to the greenhouse effect caused by enormous emissions of anthropogenic carbon dioxide (CO_2) into the atmosphere.[1]

A variety of strategies can be adopted to limit and reduce CO_2 emissions. These include improving the efficiency of energy production, substituting carbon-rich fossil fuels such as coal and oil, with natural gas and other energy sources that contain less carbon or are carbon-free, and developing technologies to capture CO_2 in view of reutilization and/or sequestration. Concerning the latter approach, there is a range of potentially attractive technologies for CO_2 capture based on physical and chemical absorption methods, cryogenic and membrane separation processes, and biological fixation.[2]

Biological CO_2 approaches have drawn much attention because they lead to production of biomass energy in the process of CO_2 fixation through photosynthesis.[3] Plants and photosynthetic microorganisms are capable of performing this process. They use solar energy to convert CO_2 and water into biomass.

Microalgae, a group of fast-growing unicellular or simple multicellular microorganisms, offer several advantages, including higher photosynthetic efficiency, higher growth rates and higher biomass production compared to other energy crops.[2,4,5,6] Microalgae can fix CO_2 from different sources, which can be categorized as CO_2 from the atmosphere, CO_2 from industrial exhaust gases, and fixed CO_2 in the form of soluble carbonates ($NaHCO_3$ and Na_2CO_3).[2] Many microalgal strains have been reported to have the ability to accumulate large quantities of lipids. Allard and Templier (2000) have extracted lipids from a variety of freshwater and marine microalgae and reported that the lipid content varied from 1 to 26 % (*C. vulgaris* 14–22 %).[7] Nitrogen limitations were observed to induce the increase of lipid content in some chlorella strains, including *C. vulgaris* (57.9 %).[8,9] Traditional analysis of lipid content in biological

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samples was conducted by solvent extraction and gravimetric determination. This method has some advantages because of the simplicity of procedures, inexpensiveness as regards equipment and chemicals, but on the other hand, organic solvents are required. Lately, supercritical fluid extraction as an alternative extraction method is being introduced.[10] Its main disadvantages are high investment costs for the required equipment.

Microalgae *Chlorella vulgaris* was chosen as a subject for this research due to its easy growth in a relatively low-priced media without the necessity of utilizing very specific compounds and its significant lipid content, as lipids are the most desirable component from the energy point of view. Many different studies have been conducted with this strain.[11–14]

The Solvay process, employing a dual alkali approach, uses ammonia as catalyst to aid the reaction of CO_2 with sodium chloride for the production of sodium carbonate. The reaction of the Solvay process can be represented with the following equation:

$$NaCl + H_2O + NH_3 + CO_2 \rightarrow NaHCO_3 + NH_4Cl$$

After the reaction, sodium bicarbonate, which is fairly insoluble, is separated by filtration. Sodium carbonate is subsequently obtained by heating sodium bicarbonate. The ammonia is recovered by reacting ammonium chloride with lime, $Ca(OH)_2$, where limestone serves as the source of lime.

$$2NH_4Cl + Ca(OH)_2 \rightarrow 2NH_3 + CaCl_2 + 2H_2O$$

Among others, the use of limestone for the regeneration of ammonia renders the process ineffective, mainly because of the consumption of limestone, the production of CO_2, and extensive energy requirement during calcination. In the Solvay process, for every two moles of CO_2 that is captured from power plants, one mole of CO_2 from the calcination of limestone is released.[15]

In this work, we focused merely on the first step of the Solvay process in which bicarbonate and ammonium ions are formed. Both these ions are used as a source of carbon and nitrogen for the nutrition of microalgae.

The purpose of this work was to establish the growth of *C. vulgaris* in a medium where bicarbonate ions serve as a source of CO_2 for photosynthesis. Besides carbon, other nutrients essential for algal growth were added.[16] An investigation was based on batch experiments carried out in a lab in order to optimize the medium composition for the best microalgal biomass yield, and consequently for the economic efficiency of the process if used in an up-scale for biofuel production.

Materials and methods

Microalgal culture and preparation of inoculum

The culture of *Chlorella vulgaris* (SAG 211-12) was purchased from the Collection of Algal Cultures at the University of Göttingen (Germany). The culture of *C. vulgaris* from agar slants was aseptically inoculated into several 200 mL Erlenmeyer flasks containing 150 mL of liquid Jaworski medium. The medium consisted of the following components (per litre of distilled water): 36 mg $Na_2HPO_4 \cdot 12H_2O$, 80 mg $NaNO_3$, 12.4 mg KH_2PO_4, 20 mg $Ca(NO_3)_2 \cdot 4H_2O$, 50 mg $MgSO_4 \cdot 7H_2O$, 15.9 mg $NaHCO_3$, 2.25 mg EDTAFeNa, 2.25 mg $EDTANa_2$, 2480 μg H_3BO_3, 1390 μg $MnCl_2 \cdot 4H_2O$, 1000 μg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 40 μg cyanocobalamin ($B_{12}$), 40 μg thiamin HCl ($B_1$) and 40 μg biotin. The flasks were exposed to direct sunlight and room temperature until they reached sufficient density of microalgae for inoculation in further experiments.

Culture media

The first set of experiments was carried out in three different media: the aforementioned Jaworski medium, the solution from the modified Solvay process, and natural mineral water Donat^{Mg} (Rogaška Slatina, Slovenia).

Preparation of solution from the modified Solvay process

During the preparation of this culture medium, we used only the first step of the Solvay process; therefore we named it the pure solution from the modified Solvay process. In a weak solution of sodium chloride (2 g L^{-1}) in distilled water, the ammonia was blown in until pH value reached 10.7. Then, carbon dioxide was blown in the alkaline solution until the desired pH value of 6.8 was reached.

An enriched solution from the modified Solvay process differed from the pure solution in the supplements added. These supplements were all constituents of Jaworski's medium, with the exception of $NaHCO_3$. The effect of bicarbonate concentration on growth, lipid content and chlorophyll content was investigated by dilution of the enriched solution from the modified Solvay process. Dilutions at ratios 1:10, 1:50 and 1:100 were applied in the second set of experiments.

Analysis of the solution from the modified Solvay process

According to the ISO 5664 (1984)[17] method, the initial concentration of NH_4^+ was 1282 mg L^{-1}.

The ratio of bicarbonate and carbonate in the reaction solutions and the concentrations of bicarbonate and carbonate were determined from the ^{13}C NMR spectra of the reaction solutions. ^{13}C NMR spectra were recorded on a Brucker DPX 300 spectrometer operating at 75.475 MHz with 17480 scans. First, the standard solution was prepared from 13.10 mL H_2O, 0.79 mL D_2O, 71.4 mg $NaHCO_3$ and 44.9 mg acetone. The f = 1.78 resulted from the ^{13}C NMR spectrum of the standard solution. The known amount of the acetone was added to the reaction solution before measuring of the ^{13}C NMR spectra. About 10 % of D_2O was added for the NMR lock. Since the resonance of pure bicarbonate is at 160.82 ppm and the carbonate at 168.24 ppm,[1] the percentage of the bicarbonate in the bicarbonate/carbonate mixture was calculated as:

$$\% \, HCO_3^- = 100(168.24 - \delta(sample))/(168.24 - 160.82),$$

where $\delta(sample)$ is the chemical shift of the bicarbonate-carbonate ^{13}C NMR resonance of the reaction solution.

The concentrations of carbonate and bicarbonate $C(HCO_3^-/CO_3^{2-})$ in the reaction solutions were calculated using the following equation:[18]

$$C(HCO_3^-/CO_3^{2-}) =$$

$$= 1.78 \; C(\text{acetone}) \cdot I(HCO_3^-/CO_3^{2-})/0.5 \; I(\text{acetone}),$$

where C(acetone) is the concentration of the acetone added to the reaction solution, and $I(HCO_3^-/CO_3^{2-})/I$(acetone) is the ratio of intensities of the resonance of bicarbonate-carbonate and the methyl resonance of the acetone.

Preparation of culture medium with natural mineral water

The natural mineral water Donat^{Mg} contains the following cations, anions and non- disintegratable compounds (per litre of Donat^{Mg}): 1.05 mg NH_4^+, 3.3 mg Li^+, 1500 mg Na^+, 13 mg K^+, 1030 mg Mg^+, 380 mg Ca^{2+}, 6.8 mg Sr^{2+}, < 0.1 mg Fe^{2+}, 0.17 mg Mn^{2+}, 0.23 mg F^-, 59 mg Cl^-, 0.29 mg Br^-, 0.08 mg I^-, < 2 mg NO_3^-, < 0.007 mg NO_2^-, 2400 mg SO_4^{2-}, < 0.02 mg HPO_4, 7700 mg HCO_3^-, 16.6 mg metaboric acid, 156 mg metasilicic acid, approximately 3800 mg dissolved carbon dioxide. This medium was prepared according to Golob (1998).[19] By mixing in a vessel with a large enough surface, the dissolved carbon dioxide was eliminated. The natural mineral water was diluted with distilled water at a 1:2 ratio. Then, 6 g $CaCl_2 \cdot 6H_2O$ and 0.5 g NH_4Cl per litre of natural mineral water were added. This medium was diluted with distilled water at a 1:50 ratio, and 80 mg $NaNO_3$ and 36 mg $Na_2HPO_4 \cdot 12H_2O$ per litre were added.

Culture conditions

Experiments were carried out in 3000 mL Erlenmeyer flasks containing 1750 mL of each individual medium with initial inoculum of approximately 10^5 cells mL^{-1}. The alga was grown in batch culture in a growth chamber at 25 °C under 12/12 hour light/dark cycles. Illumination was provided using PAR fluorescent lights. The light intensity was between 100–120 μE m^{-2} s^{-1}. The flask contents were continuously stirred with a magnetic stirrer at 150 rpm. Samples were collected every 24, 48 or 72 hours, depending on the sets of experiments. The cell number, chlorophyll content, and lipid content were monitored.

Microalgal cell counting and dry weight

A direct microscopic count (cells mL^{-1}) was performed on a sample of microalgal suspension using a Bürker-Türk counting chamber (Brand, Germany) and a Nikon Eclipse 80i microscope (Nikon Corporation, Japan). Optical density of the microalgal suspension was measured by absorbance at 550 nm (A_{550}) in an HP 8452 UV/Visible Spectrophotometer. The spectrophotometer was blanked with each medium, respectively. At the end of the experiments, 100 mL of the culture broth was removed from every flask, respectively. The samples were filtered through glass microfibre discs (Sartorius stedim biotech, Göttingen, Germany) and the dry weights of pellets were measured after drying at 105 °C for 2 hours.

pH and light measurement

The sample pH was directly determined using a pH meter (model 211, Hanna Instruments). The pH meter was calibrated daily. Light intensity was measured with the Li-Cor measuring device.

Chlorophyll content determination

To determine chlorophyll content of microalgal cells, two different methods were applied: the spectrophotometric technique and the fluorometric tehnique.

The sample of microalgal suspension was centrifuged for 10 minutes at 13000 rpm. The supernatant was decanted and the pellet resuspended in 90 % methanol. Chlorophyll was then extracted from the sample during one hour of incubation in a water bath at 50 °C. The sample was again centrifuged for 10 minutes at the same speed.

For the spectrophotometric determination of chlorophyll, the absorbance of light green supernatant was measured at two wavelengths, 665 (A_{665}) and 750 nm (A_{750}), using the HP 8452 UV/Visible spectrophotometer. The spectrophotometer was

blanked with methanol. The chlorophyll content of the sample was calculated using the following formula:[20]

$$chlorophyll\ a = \frac{13.9 \cdot (A_{665} - A_{750}) \cdot V_{MeOH}}{V_{sample} \cdot l}\ (\mu g\ mL^{-1})$$

where V_{MeOH} is the methanol volume (mL), V_{sample} is the sample volume (mL) and l is the width of cuvette (cm).

For fluorometric determination of chlorophyll *a*, the fluorescence at an excitation wavelength of 430 nm and an emission wavelength of 663 nm was measured. Fluorescence measurements were carried out using a Jasco FP 750 spectrofluorometer. The apparatus was blanked with methanol. The chlorophyll *a* content of the sample was calculated using the calibration curve that was made with chlorophyll *a* from alga *Anacystis nidulans* (Sigma) as standard.

Lipid extraction and measurement of lipid content by fluorescent spectrometry

For the determination of lipid content, a fluorescent spectrometric method was applied. The samples of 12 mL were taken from each culture, respectively, centrifuged for 10 minutes at 4000 rpm, then 8 mL of supernatants was removed and the rest of the samples were stored at –20 °C. Low temperatures and subsequent thawing caused a disruption of microalgal cells. For extraction, 4 mL of hexane were added, sealed up, and mixed well. Lipids were extracted during 45 minutes of incubation in a water bath at 45 °C. During and at the end of incubation, the samples were vigorously mixed on a vortex mixer. After separation in two phases, 1.5 mL of light fraction (lipids in hexane) was taken for staining with 5 μL of $7.8 \cdot 10^{-4}$ mol L^{-1} Nile Red (Sigma) dissolved in acetone according to Elsey (2007), who reported on using Nile Red, a lipid-soluble fluorescent probe revealing several characteristics advantageous to *in situ* screening used for lipid measurement.[21]

The samples were then excited at 486 nm and the emission was measured at 570 nm using a Jasco FP 750 spectrofluorometer. Emission intensities were recorded over a period of 300 seconds. For easier comparison of lipid content in different media, sample emission intensities were compared at determined time points (150 seconds).

Supercritical CO_2 extraction of microalgal lipids

In order to acquire sufficient microalgal biomass for supercritical fluid extraction of lipids, the algal growth was carried out in a 10 L vessel with a ten times diluted and enriched solution from the modified Solvay process containing nutrients that are present in Jaworski's medium, with the exception of $NaHCO_3$. The system was aerated at an air-flow rate of 270 L h^{-1}. Other culturing conditions were the same as in the previous sets of experiments.

After six days of growth, microalgal cells were harvested by sedimentation in the dark for 3 days, when clear supernatants were decanted, and by centrifugation (Rotanta 460 R, Hettich Zentrifugen) of the residues of supernatants and sediments together at 4000 rpm for 10 minutes. The sediments were collected and dried using a freeze-drier (CHRIST, Alpha 2–4 LSC). We used lyophilisation as the drying method because of losses and alteration of lipid composition caused by conventional drying at different temperatures.[9]

Supercritical CO_2 extraction of lipids from microalgae *Chlorella vulgaris* was carried out using a flow apparatus. The freeze-dried microalgal biomass (360 mg) was submitted to supercritical CO_2 at a pressure of 300 bar and temperature of 50 °C. The extraction was carried out in consecutive batches. The fluid amount (CO_2) was measured with a gas meter. The lipids from supercritical extracts were determined gravimetrically, weighing the glass test tube before and after extraction.

Results and discussion

Effect of different culture media on growth of *Chlorella vulgaris*

The growth of microalgae *Chlorella vulgaris* in different culture media was primarily followed by counting algal cells under the microscope (Fig. 1).

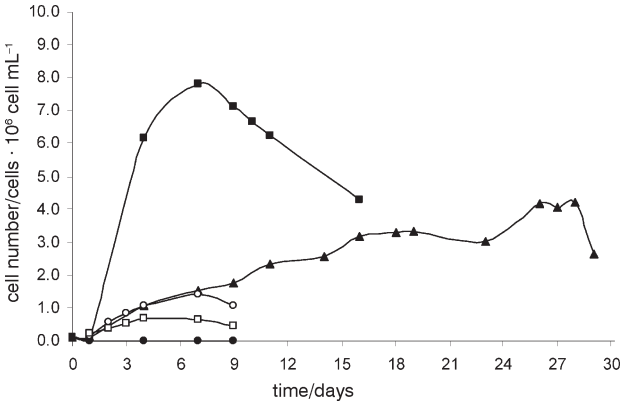


Fig. 1 – *Comparison of growth curves of microalgae Chlorella vulgaris cultured in different culture media: Jaworski's medium (▲), an enriched solution from modified Solvay process with dilution in a 1:10 ratio (■), a pure solution from modified Solvay process with dilution in a 1:10 ratio (□), a natural mineral water medium (●), a diluted natural mineral water medium with added supplements (○)*

The Jaworski medium was chosen due to its well-defined composition favourable for the culture green algae, and on the basis of preliminary experiments that showed relatively good growth of different algal species in this medium. The growth was tracked for 29 days due to slow increasing of microalgal biomass, and because the maximum cell number had not yet been determined until day 26 of culturing. The fastest growth was obtained in an enriched solution from the modified Solvay process. The maximum cell number ($7.8 \cdot 10^6$ cell mL^{-1}) was reached on the seventh day. After that day, the cell number decreased rapidly. In comparison with the pure solution from the modified Solvay process, which contained only $NaHCO_3$ and NH_4Cl as nutrients necessary for algal growth, the maximum cell number in the enriched solution was more than ten times higher. No evident growth was present in the culture medium with natural mineral water. Due to high turbidity resulting from precipitation, microalgal growth did not even start. When diluted and adjusted to proper ion strength with an addition of sodium nitrate and phosphate, the growth in the first few days was comparable with that in Jaworski's medium, but after day 7 the cell number decreased. The cell number was six-times lower compared to the cell number in the enriched solution from the modified Solvay process; therefore we did not followed it any further, as media with poor biomass yield was not of interest to us.

Effect of bicarbonate concentration on growth, chlorophyll content, and lipid content

Bicarbonate concentration of 1.05 g L^{-1} in the pure solution from the modified Solvay process was determined by the ^{13}C NMR analysis. Compared to the result (1.15 g L^{-1}) obtained by titration of a solution with 0.1 mol L^{-1} HCl in the presence of methyl orange as indicator, both methods showed relatively good mutual agreement. Also, by means of ^{13}C NMR analysis, a 99.8 % portion of bicarbonate was determined.

Fig. 2 shows the growth of algae in batch cultures under different concentrations of an enriched solution from the modified Solvay process, as the maximum cell number in this medium was outstanding and had been reached within a short period of five days. The best growth resulted in the least diluted medium (1:10), and the poorest growth resulted in the most diluted medium (1:100). The maximum cell number in all three media was reached around the fifth day of culturing.

Every time a sample was taken, the pH value of the medium was determined. Numeric results regarding pH values in different diluted media on the first and ninth day of cultivation of microalgae

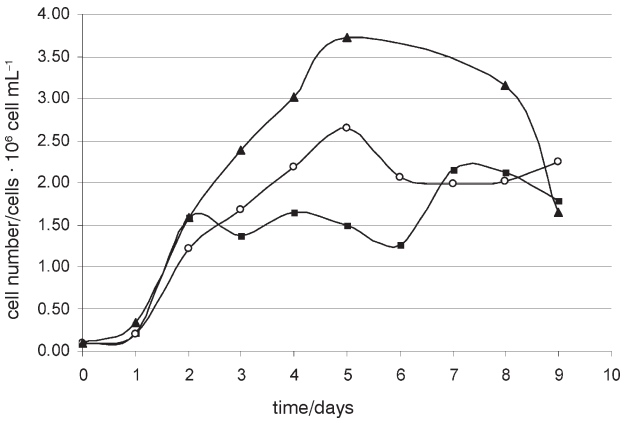


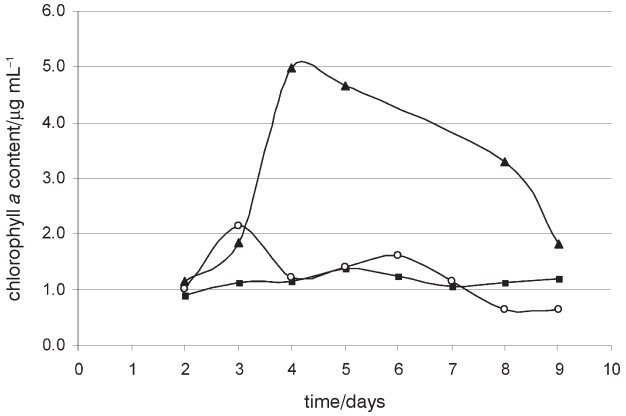
Fig. 2 – *Comparison of growth curves of microalgae Chlorella vulgaris cultured in an enriched solution from modified Solvay process at different dilution ratios: 1:10 (▲), 1:50 (○), 1:100 (■)*

Chlorella vulgaris are presented in Table 1. After diluting the medium, the pH value slightly decreased due to more added water. Gradually, the pH value increased until day nine of cultivation, probably due to consumption of bicarbonate ion in the medium as a sole source of carbon for algal growth and photosynthesis. This is in compliance with Sorensen *et al.* (1996), who maintained that, since algae use CO_2(aq) from bicarbonate to compensate the lack of CO_2 from gas supply, this results in an increase of pH.[22]

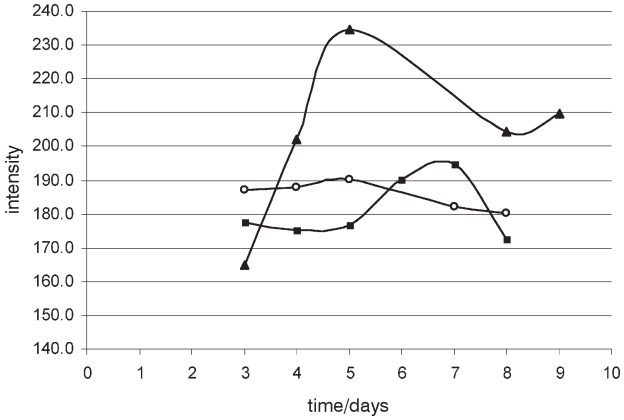
Table 1 – *pH values of different diluted media during the experimental period*

Dilution ratio	pH value	
	1st day	9th day
1:10	7.10	9.56
1:50	7.07	9.38
1:100	7.03	9.07

Chlorophyll *a* content (Fig. 3) was measured from the second day on, since a slight increase in cell number was observed due to the lag phase of microalgal cells. As in the case of cell number, the highest chlorophyll *a* content was obtained in the medium with a 1:10 dilution ratio, and a peak in chlorophyll *a* content was observed on day five of the experiment. Chlorophyll measurements did not directly coincide with direct cell counts. The discrepancy in chlorophyll concentration is likely due to the variability of levels within individual cells, and not as a result of changes in the overall biomass. With regard to the growth curve of a sample

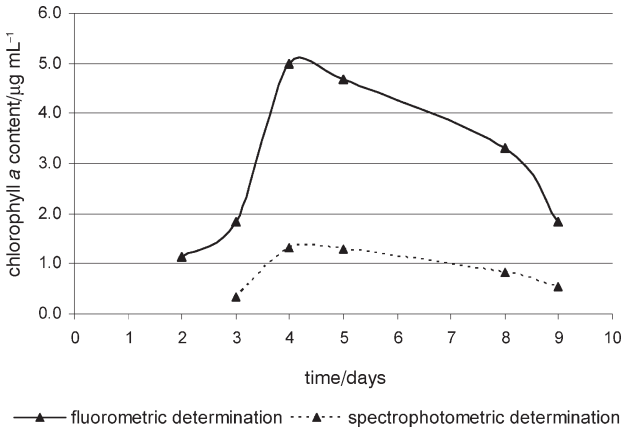


F i g . 3 – *Comparison of chlorophyll a content of microalgae Chlorella vulgaris cultured in an enriched solution from modified Solvay process at different dilution ratios: 1:10 (▲), 1:50 (○), 1:100 (■). Results shown here were obtained by fluorometric technique.*



F i g . 5 – *Comparison of emission intensities of Nile Red excited at 486 nm and recorded at 570 nm at determined time point 150 seconds after addition to a light fraction of hexane extracts of lipids from microalgal cells (Chlorella vulgaris) cultured in an enriched solution from modified Solvay process at different dilution ratios: 1:10 (▲), 1:50 (○), 1:100 (■)*

with a 1:50 dilution ratio, the cell number peaked on day five, but an increase in chlorophyll *a* content occurred on days three and six. Both methods, spectrophotometry and fluorometry, which were applied for chlorophyll determination, showed a similar pattern (Fig. 4). The chlorophyll concentrations measured fluorometrically were higher as this method is more sensitive. These optical methods can significantly under- or overestimate chlorophyll *a* concentration, partly because of the overlap of absorption and fluorescence bands of co-occurring accessory pigments, and partly due to chlorophyll degradation products.[23]



F i g . 4 – *Comparison of chlorophyll a content determined by spectrophotometric and fluorometric techniques in microalgal cells of Chlorella vulgaris cultured in an enriched solution from the Solvay process diluted in a 1:10 ratio*

Fig. 5 shows the relative lipid content in cells of microalgae *Chlorella vulgaris* cultured in an enriched solution from the modified Solvay process at different dilution ratios. The highest intensities were detected in a sample of medium diluted at a 1:10 ratio. Peak emission intensity was obtained on the fifth day of cultivation as reported for cell number and chlorophyll *a* content. In the medium with the dilution ratio of 1:50, all measurements were in a narrow range with a slight increase on day five. In the medium with the dilution ratio of 1:100, the curve of emission intensity peaked during days six and seven as previously reported for cell number. For absolute lipid measurements per unit cell, one needs to develop the requisite calibration curve that correlates fluorescence to lipid content, whether determined gravimetrically or by means of lipid standards.

Supercritical extraction of microalgal lipids

Supercritical extraction with CO_2 was performed in 360 mg of freeze-dried microalgal biomass. Extraction was carried out at 300 bar (30 MPa) and 50 °C (323.1 K) in nine batches in a cumulative time of 240 minutes. In this time, the cumulative amount of CO_2 used was 295.68 L. The yield of supercritical extraction of lipids was only 1.69 %. Mendes *et al.* (2003) obtained from about 20 % of lipids in *Chlorella vulgaris* at severe conditions (35 MPa, 328.1 K), and establishing that the concentration of lipids was higher when crushed cells were used in the supercritical extraction. The crushing is important, because the cell wall is structurally a polymer (sporopollenin) derived from carotenoid polymerisation,[24] which makes the extraction of the internal compounds difficult.

Conclusion

Despite several reports discussing different media for cultivation of microalgae *Chlorella vulgaris*, as far as the authors know, none of them discusses

the application of the principles of the Solvay process for CO_2 fixation through photosynthesis carried out by microalgae. If compared with the growth of microalgae in Jaworski's medium, the results showed a faster growth with a two times higher biomass yield in a solution from the modified Solvay process enriched with essential elements required for microalgal nutrition and are the same as in Jaworski's medium. The maximum cell number was reached already on the fifth day. The best results were obtained in the medium diluted in a 1:10 ratio. It was found that the chlorophyll *a* and the lipid contents also peaked around day five.

The yield of lipids from freeze-dried microalgal biomass extracted with supercritical CO_2 at 30 MPa and 50 °C was low. With this method, we only qualitatively proved the presence of lipids in microalgal cells of *Chlorella vulgaris* cultured under normal nutrition. However, an improvement in the efficiency of extraction and consequently quantification of lipids will be the aim of further research, along with the production of microalgal biomass with higher lipid content as the result of exposure to nitrogen starvation. These parameters are of vital importance for the application of the system in a larger scale.

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