ORIGINAL PAPER

UV-VIS ANALYSIS OF GRANULAR ACTIVATED ALGAE CHLOROPHYLL CONTENT

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Abstract*. Chlorophyll-a is the pigment whose concentration is an important indicator for the development of microalgae biomass. This study aims at evaluating the concentration of chlorophylls in the biomass of granular activated algae, using acetone and 90% ethanol for the extraction procedures applied. Concentrations of chlorophyll a, b and c were determined by applying three calculation types: methods proposed by Jeffrey and Humphrey for the extracts in acetone, Ritchie method for ethanolic extracts, and monochromatic method with acidification for ethanolic extracts. Experimental findings show that the solvent 90% ethanol is more efficient than acetone for extracting chlorophyll from biomass of granular activated algae. Also, by comparing results obtained by the two methods proposed for ethanolic extracts, it was found that the values obtained through the acidification method are influenced by the volume of hydrochloric acid added to the organic chlorophylls extract.*

Keywords: *Granular activated algae; chlorophyll; phaeophytin; phytopigment.*

1. INTRODUCTION

Chlorophylls are the most important class of bio-organic compounds that can absorb light radiation and convert it into chemical energy [1]. Light reactions generate energy-rich substances and biochemical reductants, used in the biosynthesis of carbohydrates and other compounds essential for photoautotrophic organisms such as photosynthetic bacteria, microalgae, and plants and some animals. Currently, are known more than 100 chlorophylls and most of them were found in green bacteria [2].

Based on the type of photosynthetic organism, chlorophylls can be divided into two categories: chlorophylls from oxygenated photosynthetic organisms (abbreviation - *Chls*) and chlorophylls (bacteriochlorophyll) from anoxygenic photosynthetic bacteria (abbreviation - *BChls*) [3,4]. The *Chls a, b, c (c1, c2, c3), d,* and *f* are found in oxygenated photosynthetic organisms such as superior plants, algae, and cyanobacteria, while photosynthetic bacteria

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such as green sulfur bacteria, violet bacteria, filamentous anoxygenic phototrophs, acid bacteria, and heliobacteria possess *BChls a, b, c, d, e* and *g* type of chlorophylls [5].

First, the *Chls a, b, c,* and *d* (Fig.1.) were the only known chlorophylls in oxygenated photosynthetic organisms, until the fifth type of chlorophyll. *Chl f,* was discovered in 2010, in a cyanobacterium in stromatolites from Shark Bay, Western Australia [6]. Another compound still disputed was reported by Harold H. Strain in 1948, a new chlorophyll named *Chl e*, whose properties are not yet fully defined, its chemical structure and functionality are still uncertain, and thus this chlorophyll is not (yet) recognized by the scientific community [7].

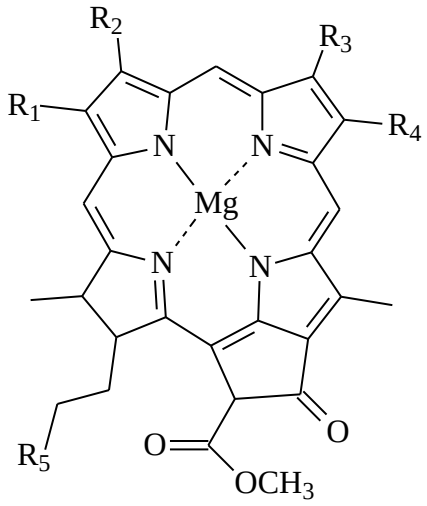

Figure 1. Structure of *Chlorophyll a, b, c, d, f* (adapted from Voloshin et al. [8])

Name	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**
chlorophyll a	CH_3	$CH=CH_2$	CH_3	C_2H_5	$CO\text{-}O\text{-}C_{20}H_{39}$
chlorophyll b	CH_3	$CH=CH_2$	CHO	C_2H_5	$CO\text{-}O\text{-}C_{20}H_{39}$
chlorophyll c_1	CH_3	$CH=CH_2$	CH_3	C_2H_5	CH=CH-COOH
chlorophyll c_2	CH_3	$CH=CH_2$	CH_3	$CH=CH_2$	CH=CH-COOH
chlorophyll c_3	CH_3	$CH=CH_2$	$CO\text{-}O\text{-}CH_3$	$CH=CH_2$	CH=CH-COOH
chlorophyll d	CH_3	CHO	CH_3	C_2H_5	$CO\text{-}O\text{-}C_{20}H_{39}$
chlorophyll f	CHO	$CH=CH_2$	CH_3	C_2H_5	$CO\text{-}O\text{-}C_{20}H_{39}$

All the photosynthetic algae contain mainly chlorophyll-a (Chl-*a*) [9], while accessory pigment chlorophyll-*b* (*Chl-b*) is less widely distributed being found in higher plants and green microalgae [10]. Chlorophyll *c* is found in traces in green algae, but it is abundant in diatoms and brown algae [11]. The chlorophyll *d* is mainly found in red seaweed [12]. The microalgae contain Chl-*a* as the abundant type of chlorophyll, while Chl-*b* is the minor species in these plants [13].

Granular activated microalgae can be a viable alternative for wastewater treatment [14-17]. The pigments involved in the photosynthesis process can be measured either by traditional spectroscopic technique or by chromatographic method, using HPLC, followed by UV-Vis spectrometry detection.

The accuracy of chlorophyll determinations is based on the careful application of each step of the recommended protocols that have been verified and issued by specialists as well as on a correct assessment and description of the limits of validity (LOV) of the method. Various procedures have been reviewed over time and several guidelines and recommendations for the determination of photosynthetic pigments in biomass separated from different types of aqueous solutions have been proposed (SCOR-UNESCO, 1966; BMB, 1979) [18]. The updated UNESCO publication "*Phytoplankton pigments in oceanography: guidelines to modern methods*" presents a very detailed review of phytoplankton pigments performed by the SCOR 78 Working Group on "*Determination of photosynthetic pigments in seawater*"

[19]. As this book contains the latest methodological developments and recommendations in the field, one may consider that it provides the benchmark for the study of pigments.

This study aims to determine the phytopigments content in granular activated microalgae biomass using two methods, each of them involving extraction of pigments in 90% ethanol and acetone, respectively. Also, for the investigation of chlorophylls (Fig. 2) concentrations, the study presents three types of calculations: (a) trichroic equations proposed by Jeffrey and Humphrey for the acetone extract; (b) trichroic equations proposed by Ritchie for ethanolic extract; (c) the monochromatic method with acidification of the ethanolic extracts.

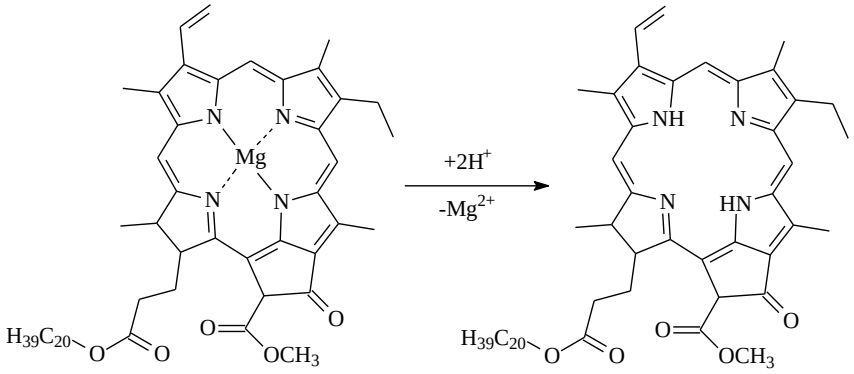

Figure 2. Chlorophyll to pheophytin transformation in acidic media.

2. MATERIALS AND METHODS

2.1. MATERIALS

All reagents were of analytical grade: ethanol (96%, evaporation residue ≤ 25 mg/L), acetone (purity ≥ 99.8%, evaporation residue ≤ 3 mg/L), hydrochloric acid (36%, high purity) are provided by Merck (Merck Millipore Burlington, MA, USA). Redistilled water (conductivity < 0.10 $\mu S \cdot cm^{-1}$ at 25 °C) was used for the experiments.

2.2. METHODS

In this research, the biomass from the same batch was studied. Generation of biomass was performed according to the procedure that is described herein. Mature activated algae granules were used for treatment of a synthetic municipal wastewater in a 1.5 L sequencing batch photobioreactor (BIOSTAT® A*plus*, Sartorius Lab Instruments GmbH, Goettingen, Germany). Inside the granular structures, microalgae biodiversity was mainly represented by *Chlorella* sp. and *Phormidium* sp. (Fig. 3). Each treatment batch was conducted for 24 hours including the following consecutive cycles: (1) biomass settling (1 min), (2) 1 L effluent withdrawn (5 min), (3) biomass feeding (5 min), and (4) wastewater treatment with a hydraulic retention time of 23 h 49 min by setting up a photoperiodicity to 12 h light – 12 h dark, with continuous stirring (speed 200 rpm). The light cycle has begun immediately after biomass feeding providing by a cool-white lamp with 3,980 lm placed in front of the photobioreactor to ensure at the outer vessel wall an irradiance of 200 $\mu mol/m^2/s$. The influent

had the following chemical composition: sodium acetate 500 mg/L; NH_4Cl 89 mg/L; K_2HPO_4 28 mg/L; $CaCl_2$ 40 mg/L; $MgSO_4 \cdot 7H_2O$ 75 mg/L; $FeSO_4 \cdot 7H_2O$ 5 mg/L; H_3BO_3 50 µg/L; $ZnCl_2$ 50 µg/L; $CuSO_4$ 5H2O 30 µg/L ; $MnSO_4 \cdot H_2O$ 50 µg/L, $(NH_4)_6Mo_7O_{24}$ 50 µg/L; $AlCl_3$ 50 µg/L; $CoCl_2 \cdot 6H_2O$ 50 µg/L; $NiCl_2$ 50 µg/L.

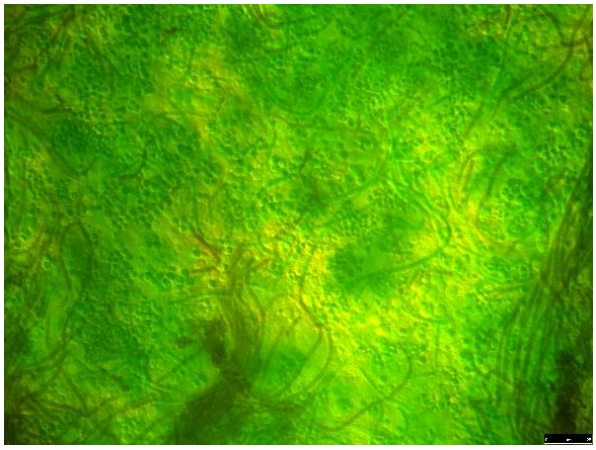

Figure 3. Light microscopy image of the microalgae populations distribution inside activated algae granules (200*x* magnification).

The BK-FD10 Biobase lyophilization system (Biobase Biodustry Co.Ltd., Shandong, China) was used to preserve the microalgae biomass. Filtered microalgae biomass samples were dried under vacuum at -110°C for 24 hours and stored at -81°C.

The measurements were performed by UV–Vis spectrophotometry using Evolution™ 260 Bio UV-Vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). UV-Vis spectroscopy was used to obtain the absorption spectra of microalgae biomass hydroalcoholic extract in 350 – 800 nm wavelength range. The readings were performed against blank (the solvent used in the chlorophylls extraction step) at 1 nm intervals, and using quartz cuvettes with an optical path of 10 mm.

Chlorophylls extraction procedure with ethanol 90% and light absorption spectra evaluation

An aliquot of lyophilized microalgae biomass of approx. 20 mg, weighed on analytical balance (PIONEER™, Ohaus GmbH, Nänikon Switzerland) was transferred in 15 mL tube with screw cap. For each sample, two aliquots were weighted to perform parallel tests with and without the addition of HCl.

In the next step, 10 mL of hydroalcoholic solvent (90% EtOH) was added to each tube, and then two ultrasound (45 kHz) and temperature (50 °C) cycles were applied; 1 minute vortex sessions after every 10 minutes were applied. Between the two ultrasound-temperature cycles, the samples were allowed to rest for approx. 30 minutes and after that were centrifuged for 5 minutes at 15000 rpm. The supernatants were used to obtain the visible absorption spectra in the 350 – 800 nm wavelength domain. After centrifugation, some of the liquid samples were acidified with 3M HCl solution to observe the visible absorption spectrum changes that could be associated with pH variation of the solution. The volume of 3M HCl solution added to acidify the supernatants was adjusted to obtain concentration of 1.5, 3, 4.5, and 6 mM HCl. The procedure was set to establish the optimal hydrochloric acid volume needed to estimate the concentration of chlorophylls in ethanolic extract. The UV-Vis spectra of acidified supernatants were recorded in the above-mentioned wavelength range, immediately after the addition of the acidic solution, and every other minute that followed, for a time frame of 20 minutes. Also, a final scan step was done after 24 hours in order to

evaluate potential time changes. It can be mentioned that up to the final scan, samples were stored in a refrigerator, in closed dark-brown containers.

Chlorophyll's extraction procedure with acetone and light absorption spectra evaluation

The procedure used to extract phytopigments from microalgae with acetone as solvent followed the same steps as the one detailed above for the ethanolic extraction solvent. Glass containers used for extraction were tightly closed to avoid loss of solvent during the preparation of the extract. After centrifugation, the absorption spectra of the supernatants were measured in the 350 – 800 nm wavelength range. To evaluate potential changes that may be produced by acidification of the extract, a calculated volume of 3M HCl solution was dropped in the samples. Thus, the final HCl concentration in the organic extract reached the value of 3 mM.

3. RESULTS AND DISCUSSION

Before starting the analysis of data obtained by the UV-Vis method, it is important to mention that the scanning performed immediately after acid addition, each minute after for a timeframe of 20 minutes, and then after 24 h showed no differences in the recorded UV-Vis spectra. This experimental finding led to conclusion that transformation of chlorophylls to pheophytins is a fast reaction.

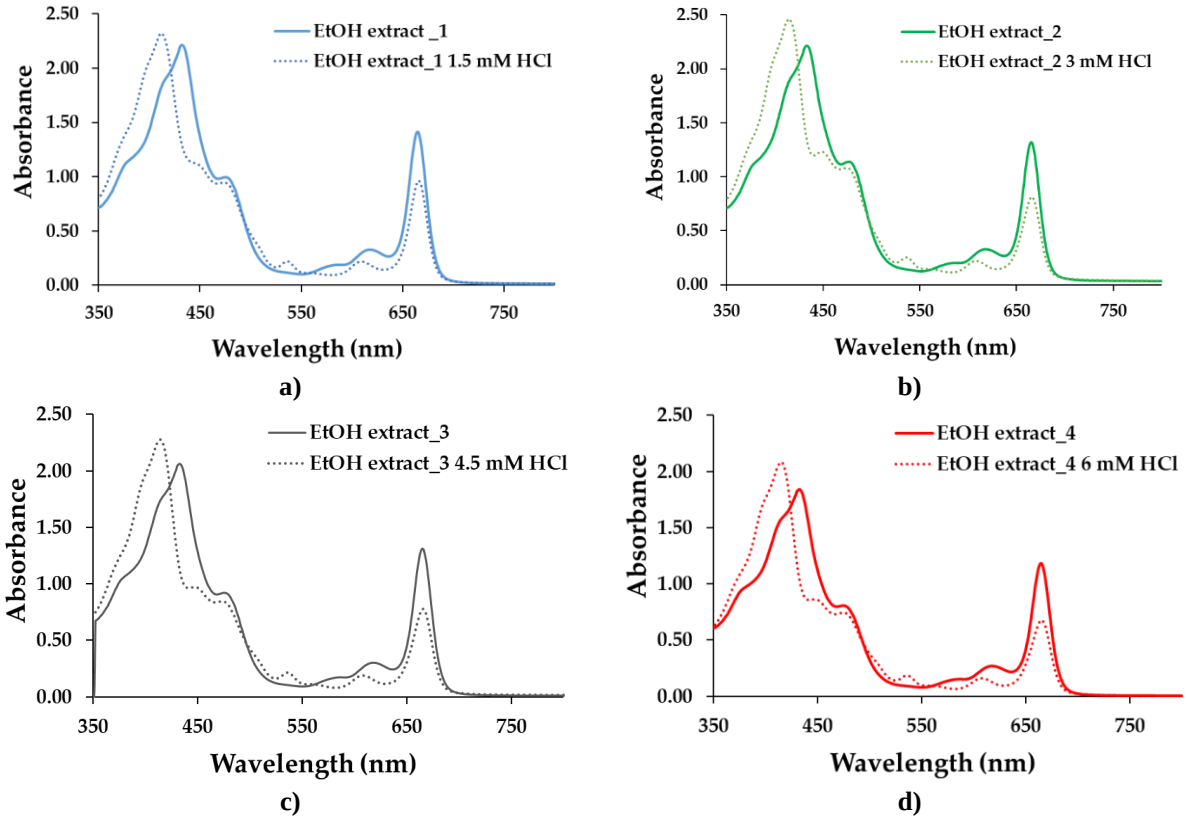

Figure 4. UV-Vis Spectra of ethanolic extracts obtained from microalgae biomass before (continuous lines) and after (dotted lines) acidification up to HCl concentrations of: a) 1.5 mM, b) 3.0 mM, c) 4.5 mM and d) 6.0 mM

Fig. 4 shows experimental findings from the UV-Vis study of the ethanolic extracts obtained from the microalgae. Each of the four plots shows a comparison of the UV-Vis spectra of ethanolic extracts obtained from the studied microalgae biomass without (continuous lines) and with (dotted lines) addition of hydrochloric acid at different concentrations (values indicated on each graph, in millimoles per litre/mM). It may be observed that the recorded spectra show characteristic absorption bands associated with phytopigments. Thus, the bands at 415 nm and 474 nm correspond to Soret bands of phaeophytin, the band at 540 nm is characteristic to chlorophylls and phaeophytin. Also, addition of hydrochloric acid leads to a decrease of the absorption band from 619 nm, and an increase of the absorption band from 540 nm, as reported by Lopes et al [20]. Both of these values are characteristic of wavelengths for chlorophylls and phaeophytins [20].

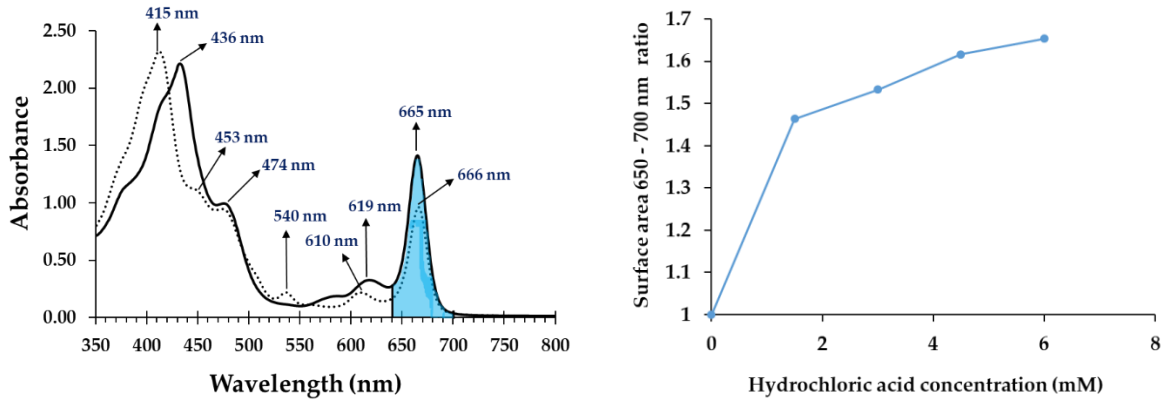

Figure 5. a) Comparative study of UV-Vis spectra for granular activated microalgae samples ethanolic extract vs. acidified ethanolic extract; b) Surface area (640 nm – 700 nm) ratio vs hydrochloric acid concentration.

To compare the effect of the progressive addition of hydrochloric acid to the chlorophyll extract samples, the areas under the absorption band around 665-666 nm in the wavelength range of 640 - 700 nm were calculated. Then, the ratio between the calculated area of the non-acidified solution and the one for the acidified solution (Fig. 5a, b) was determined. The plot indicated in Fig. 5b shows that the ratio between the two areas increases progressively with the increase of the hydrochloric acid concentration. In addition, from Fig. 5b, one may observe that this ratio has a tendency to reach a constant level when the addition of HCl lead to concentrations higher than 5 mM in the organic extract.

Fig. 6a shows results obtained from the UV-Vis study of the acetone extracts of phytopigments from the microalgae biomass. One may observe that addition of HCl to the acetone extract does not produce significant changes in the UV-Vis spectrum. In addition, by comparing the UV-Vis spectra in acetone with those recorded for the ethanolic extracts, it was observed that the appearance of the curves is similar. On the other hand, when biomass samples were subjected to ethanol extraction, the color of the phytopigments extract were more intense than for extracts obtained under the same experimental conditions, but with acetone as solvent (Fig. 6b). One of the reasons may be a residual acidity of the employed commercial reagents that lead to a slight phaeophytinization of chlorophylls, more pronounced for the acetone that was used as received, without dilution.

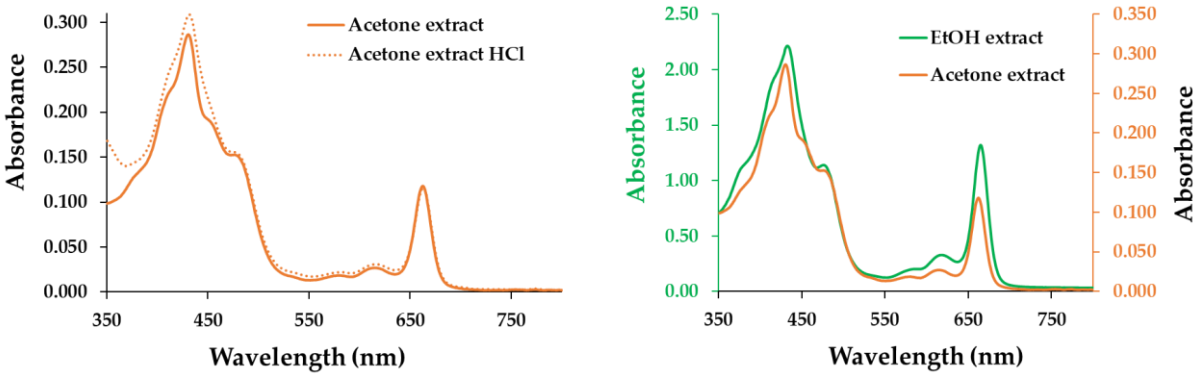

Figure 6. Recorded UV-Vis spectra for granular activated biomass extracts in a) acetone *vs.* acetone with 10 µL HCl 3M added to 10 mL extract and **b) acetone extract *vs.* ethanolic extract**

Calculation of chlorophyll content in the acetone extracts of granular activated microalgae biomass using the Jeffrey and Humphrey trichromatic equation

One method to assess the chlorophyll content of granular activated microalgae biomass samples is to calculate the concentrations of chlorophyll *a*, *b* and *c* according to the trichromatic equations proposed by Jeffrey and Humphrey [21]. By using the equations (1) - (3), adapted according to the Jeffrey - Humphrey, the concentrations of chlorophyll *a*, *b* and *c* in the acetone extracts obtained after centrifugation were determined. Then, the chlorophyll content in biomass is calculated according to equation (4).

$$[Chl\ a] = 11.85 \cdot (A_{664} - A_{750}) - 1.54 \cdot (A_{647} - A_{750}) - 0.08 \cdot (A_{630} - A_{750}) \quad (1)$$

$$[Chl\ b] = -5.43 \cdot (A_{664} - A_{750}) + 21.03 \cdot (A_{647} - A_{750}) - 2.66 \cdot (A_{630} - A_{750}) \quad (2)$$

$$[Chl\ c] = -1.67 \cdot (A_{664} - A_{750}) - 7.60 \cdot (A_{647} - A_{750}) + 24.52 \cdot (A_{630} - A_{750}) \quad (3)$$

$$c_{Chl} = \frac{[Chl] \cdot V_e}{m_{microalgae}} \quad (4)$$

where: A_{630}, A_{647}, A_{664}, A_{750} are the measured absorbances for acetone extract at 630 nm, 647 nm, 664 nm and 750 nm; V_e represents the volume of the acetone extract (mL) and $m_{microalgae}$ is the weighted mass of the lyophilized sample subjected to extraction with acetone (g). Concentrations calculated by this procedure are expressed in µg chlorophyll/mL extract.

Table 1. Chlorophyll concentration in acetone extract and dry biomass, calculated by Jeffrey and Humphrey procedure.

Chl type	Chlorophyl concentration in acetone extract [µg/mL]	Chlorophyl concentration [µg/g] in dry biomass
Chl a	1.283 ± 0.159	602.614 ± 13.453
Chl b	0.020 ± 0.005	9.509 ± 0.213
Chl c	0.013 ± 0.004	6.340 ± 1.541
total Chl	1.317± 0.108	618.464 ± 15.485

By comparing the calculated values as indicated in Table 1, one may observe that a significant mass of chlorophyll-a was extracted from the microalgae biomass, while at the same time, small amounts of *chlorophyll-b* and *chlorophyll-c* were transferred into acetone in the previously described extraction conditions.

Calculation of chlorophyll content in the ethanolic extracts of granular activated microalgae biomass using the Ritchie trichromatic equations

Most of the studies related to monitoring the presence of chlorophyll in ethanolic extracts obtained from microalgae report the *Chl a*, *Chl b* and *Chl c*, as existing in the biomass subjected to solvent extraction. Use of the trichroic equations (5) - (8) presented by Ritchie [22] represents one of the methods appropriate for estimation of chlorophyll content (μg/mL). In the end, concentrations of chlorophyll extracted in ethanol expressed as related to dry matter are calculated using equation (4).

$$[Chl\ a] = -0.9394 \cdot A_{632\,nm} - 4.2774 \cdot A_{649\,nm} + 13.3914 \cdot A_{665\,nm} \quad (5)$$

$$[Chl\ b] = -4.0937 \cdot A_{632\,nm} + 25.6865 \cdot A_{649\,nm} - 7.3430 \cdot A_{665\,nm} \quad (6)$$

$$[Chl\ c] = 28.5073 \cdot A_{632\,nm} - 9.9940 \cdot A_{649\,nm} - 1.9749 \cdot A_{665\,nm} \quad (7)$$

$$[Total\ Chl] = 23.4742 \cdot A_{632\,nm} + 11.4096 \cdot A_{649\,nm} + 4.0735 \cdot A_{665\,nm} \quad (8)$$

Table 2. Calculated concentrations of chlorophylls according to Ritchie's equations (5)-(8)

Chl type	Chlorophyl concentration in ethanolic extract (μg/mL)	Chlorophyl concentration (μg/g) in dry biomass
Chl a	15.474 ± 0.959	8310.702 ± 707.857
Chl b	0.111 ± 0.073	56.990 ± 37.506
Chl c	0.576 ± 0.299	298.483 ± 148.015
total Chl	16.159 ± 1.108	8664.938 ± 584.414

Table 2 shows the chlorophyll content calculated according to Ritchie trichroic equations, expressed in μg / mL for the ethanolic extracts and reported to dry matter mass (in μg/g). It was found that the largest variations for estimating the chlorophyll content were found for the case of chlorophyll *b* (equation 2) and chlorophyll *c* (equation 3).

Comparing the estimated values for photosynthetic pigments extracted with ethanol and respectively with acetone, one may observe that acetone seems to be a less efficient solvent in the process of separating pigments from granular activated microalgae biomass resulted from synthetic municipal wastewater treatment. Thus, for the studied biomass, the total estimated chlorophylls (in *μg* per *g* dry biomass) is approx. 14 times higher for the use of ethanolic solvent than for the use of acetone, in the same extraction conditions.

Calculation of chlorophyll content in the ethanolic extracts of granular activated microalgae biomass using the monochromatic method with acidification

The chlorophyll content can also be estimated using the equation (9) according to the working procedure involving the acidification of the phytopigments extract in ethanol [23].

$$[Chl\ a] = \frac{11.4 \cdot K \cdot [(A^n_{665} - A^n_{750}) - (A^a_{665} - A^a_{750})]}{d} \quad (9)$$

where: A^n_{665}, A^n_{750} are the absorbances of the ethanolic extract before acidification, A^a_{665}, A^a_{750} are the absorbances of the ethanolic extract after acidification, and *d* is the length of light path.

In this type of calculation, the concentration of phaeopigments in the ethanolic extract is estimated using equation (10), where signification of calculation parameters is the same as indicated for equation (9) of above.

$$[Phaeopigments\ a] = \frac{11.4 \cdot K \cdot [R \cdot (A^n_{665} - A^n_{750}) - (A^a_{665} - A^a_{750})]}{d} \quad (10)$$

R is maximum absorbance ratio of A^n_{665}/A^a_{665} in the absence of phaeopigments (Pheo-a) and it is equal to 1.7, while *K= R/(R-1)* = 2.43.

Table 3. Calculated concentrations of phytopigments for samples acidified at different HCl concentration

Phytopigment type	***Phytopigments concentrations in ethanolic extract (µg/mL)***			
[Chl a]	12.73	14.13	15.04	14.05
[Pheo-a]	5.49	0.82	-0.36	-1.04
[Chl a] + [Pheo-a]	18.22	14.95	14.68	13.01
HCl concentration in the ethanolic extract	*1.5 mM*	*3mM*	*4.5 mM*	*6 mM*

The values presented in Table 3 provide guidance on the optimal experimental procedure applicable for the acidification working procedure for 90% ethanol pigments extraction. Once the values obtained through this acidification procedure with the values obtained by using the Ritchie calculation method (Table 2), one may observe that similar numbers were obtained in the case of acidification of the ethanolic extracts up to 3mM and 4.5 mM.

On the other hand, the total pigments concentrations determined by the acidification procedure is close to the value calculated in Table 2 for the solution where 10 µL 3M HCl was added to 10 mL ethanolic extract (3 mM HCl solution). Therefore, one may conclude that the working procedure for the determination of chlorophyll by application of the extract's acidification method seems to be optimal at a ratio between the volume of added 3M HCl to the volume of the extract is close to 1:1000 (v/v).

4. CONCLUSIONS

This study aimed at establishing an optimal working procedure to determine the content of phytopigments extracted from the biomass of granular activated microalgae resulted from synthetic municipal wastewater treatment. The pigments were extracted with 90% ethanol and analytical grade pure acetone. The ethanolic extracts were more intensely colored, as confirmed by the recorded UV-Vis spectra. The shape of the UV-Vis spectra of the biomass acetone-extract was similar with the spectra of the ethanolic-extracts obtained by following the same extraction procedure for a biomass sampled from the same batch. The only difference was the higher absorption maxima for the ethanolic extracts. Acidification of the biomass extracts samples with 3M HCl led to significant changes in the UV-Vis spectra of the extracts in 90% EtOH, while, in the same conditions, the acetone-extracts did not show peaks shifts or variation of the absorption intensity.

Three types of calculations to estimate chlorophyll concentrations were used to determine the pigments concentrations. Thus, for the estimation of chlorophyll concentrations in the granular activated microalgae biomass acetone-extract the trichroic equations proposed by Jeffrey and Humphrey were used. The extraction of pigments in ethanol was evaluated

through two methods: calculation with trichroic equations proposed by Ritchie, and the monochromatic method with acidification of ethanolic extracts.

The concentrations values for the extracted pigments calculated for the acetone extract were lower compared to ethanolic extract. This indicates a better efficiency for the pigment's extraction from the microalgae biomass when the 90% ethanol is used. Calculated data obtained for samples subjected to ethanol extraction by Ritchie method, and extract acidification procedure, indicated that both methods can be applied to the analysis of pigments extracted with alcoholic solvents. Another conclusion is that acidifying of ethanolic extracts should be performed up to an optimal value of 3 mM HCl in the studied extracts.

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