Bulgarian Chemical Communications, Volume 53, Issue 1 (pp. 105 -116) 2021 *DOI: 10.34049/bcc.53.1.5347*

A new Bulgarian strain of *Scenedesmus* sp.– identification, growth, biochemical composition, and oil recovery

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Received: November 2, 2020; Accepted: December 4, 2020

In the present study a new Bulgarian strain of *Scenedesmus* sp. was taxonomically and molecularly identified as *Scenedesmus obliquus*. The most favorable cultivation conditions in terms of temperature, light intensity and growth phase were determined. The biochemical composition analyses showed that the strain is among the best producers of proteins, carbohydrates and lipids from the genus. The algal oil was recovered from the biomass applying conventional extraction methods and solvents with varying polarity. The highest cumulative yield of 28 % of dry weight was achieved by a two-step Soxhlet with *n*-hexane and ethanol, on a biomass pretreated by sonication. GC-MS analyses of the oils showed that saturated and polyunsaturated fatty acids content changed within (19.5-27.3) % and (15.7-23.8) %, respectively, and GC-FID analyses of fatty acids demonstrated the dominance of oleic and palmitic esters. The results obtained reveal the feasibility of the newly isolated strain as a renewable resource for the biofuel industry.

Keywords: *Scenedemus* sp., growth, lipid extraction methods, fatty acid composition, biofuels

INTRODUCTION

Algae, as a biological resource, have been the focus of prolific research in the last years and are advertised as the most suitable and sustainable feedstock for producing green energy (as the whole process is carbon-neutral in nature) and value-added compounds.

More than one million algae species exist and the diversity of compounds produced by them is estimated to be over 10 times greater than those produced by land plants [1] and despite their potential to help changing the economy and society in general from oil-based to bio-based systems they are still a highly unused resource. Microalgae are regarded as a revolutionary raw material and an alternative, the so-called third generation feedstock. That is because they possess essential advantages over conventional land plants, e.g. about 10–50 times higher biomass productivities and higher CO_2 fixation rate, but also because arid or low-quality agricultural land is used for their cultivation.

In recent years, the biorefinery (BioRef) concept is believed to be one of the most important tools towards the development of a sustainable circular economy. The diversity in composition of biomass/biowaste offers huge perspectives to industry as their BioRef processing can deliver a spectrum of marketable products and energy.

It is well known that algae biomass composition is influenced by the cultivation parameters and varies greatly in different algae representatives. Hence, the composition of the specific algae biomass could be used as an indicator of the spectrum of potential products that can be recovered, e.g. either energy and/or non-energy related and bioactives, and, subsequently, the multitude of possible techniques that can be applied to realize the particular valorization desired. Therefore, the application of algae biomass in a one feedstock-multiproduct BioRef platform fits perfectly within the circular economy concept.

Among the over 150 000 algal species identified in the world [2], green unicellular eukaryotic algae (*Chlorophyceae*) and in particular *Scenedemus* sp., are subjected to various studies due to their effective nutrient uptake, high photosynthetic efficiency, rapid reproduction and relatively simple culture maintenance, as well as commercial and pharmaceutical value [3, 4]. In the past decade, a number of species – *S. obliquus, S. dimorphus, S. quadricauda, S. protuberans* – to name just a few, have been a subject of extensive research from the viewpoint of their application as excellent renewable, sustainable, and economical sources in

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the food, pharmaceutical and biofuels industries [5-7].

One of the strains among the *Scenedemus* species, *S. obliquus*, has been a target of considerable research owing to its rich biochemical composition that opens a number of different avenues for application of its biomass. *S. obliquus* was firstly reported to evolve hydrogen in dark or light under anaerobic conditions [8] that triggered scientific interest to the strain. Since then, it was demonstrated that this organism accumulates up to 45 % w/w triacylglycerol (TAG) under nitrogen (N) starvation and hence can be considered to be among the most promising microalgae species for sustainable biofuel applications [9]. Moreover, taking into consideration that for example at present, over 95 % of the biodiesel produced globally is from edible vegetable oils, recovered from plants grown on agricultural land, while *S. obliquus* oil is not used in food industry, and hence the species biomass can be used as a feedstock to produce biofuels without any threat to food production. It has also been shown that *S. obliquus* possesses antioxidant and antitumor activity since it produces the bioactives astaxanthin and lutein [10, 11].

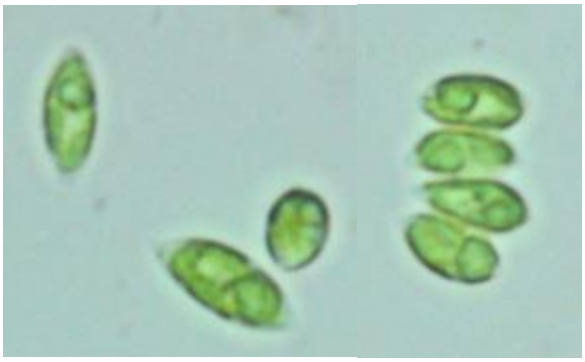
Recently, a new previously unidentified strain of the genus *Scenedesmus* was isolated from a rainwater puddle in Sofia, Bulgaria at an average temperature of 20 °C, and named *Scenedesmus* sp. BGP. Preliminary studies revealed that it possesses metabolic plasticity towards the application of different N sources in the nutrient medium while preserving the growth rate [12]. However, the potential of the *Scenedesmus* sp. BGP as a renewable bioresource is still far from being examined. Consequently, additional research and analyses are required to gather quality new information that could serve as a basis for assessing and outlining the capabilities of this totally unused at present biomass as a potential viable feedstock in a future BioRef targeted at products with multiple and diversified applications.

The main objectives of our work were to: 1. Conduct taxonomic identification of the new Bulgarian strain; 2. Examine the influence of cultivation conditions and factors like temperature and light intensity on its growth and biochemical composition; 3. Study the influence of solvents on the yield and composition of the algae oil extracts recovered from the strain biomass by conventional extraction methods; 4. Perform a preliminary assessment of the potential of the above extracts as a source of biofuel related compounds.

MATERIALS AND METHODS

Algal strain collection and taxonomic identification

A rain water sample from Sofia, Bulgaria was collected and provided in July 2013. Among the microalgae (*Haematococcus pluvialis*) present, an unidentified strain, of genus *Scenedesmus*, was found, and was taxonomically and molecularly analyzed. The taxonomic identification was performed following the classification system adopted by Komárek and Fott [13]. Microphotography and light microscopy were done using an Olympus BX50 microscope equipped with HI 100x/1.35 corr. objective (Picture 1).



Picture 1. Image of monoalgal culture of *Scenedesmus* sp. BGP containing single cells and four-cell cenobia

To perform DNA isolation and PCR analysis the following procedures were adopted. Monoalgal, non-axenic culture of *Scenedesmus* sp. BGP was cultivated at 25°C and 8000 Lux light illumination for 96 h. Pellets of microalgae samples were collected following centrifugation and stored at -80 °C. By applying a Tissue Lyser II (Qiagen) the samples were disintegrated, the DNA was isolated by GeneJT Plant Genomic Purification Kit (Thermo Scientific) and used as a template for PCR amplification of the internal transcribed spacer (ITS) region using universal primers [14] ITS1 (5'AGCGGAGGAAAAGAAACTA) and ITS4 (5'TACTAGAAGGTTCGATTAGTC). In the PCR reaction the Phusion High-Fidelity DNA Polymerase master mix (Thermo Scientific) was used and the experimental setting included 30 s denaturation at 98 °C. After that 35 cycles at 98 °C for 10 s, 57 °C for 30 s and 72 °C for 15 s and final extension at 72 °C for 3 min (Quanta Biotech QB-96) were performed. The PCR fragment was purified using a GeneJET Gel Extraction Kit (Thermo Scientific). After that it was sequenced using Macrogen Europe B.V. services. The sequences obtained were constructed and manually edited using Vector NTI v. 10 (Life Technologies). The

proximity of the Internal Transcribed Spacers (ITS) sequences with other sequences deposited at the GenBank was assessed by Basic Local Alignment Search Tool (BLAST). The ITS sequence plus the selected pools of the GenBank retrieved sequences were utilized for the construction of phylogenetic tree using MEGA 4.1 [15].

Cultivation conditions

Monoalgal, non-axenic cultures of *Scenedesmus* sp. BGP were grown autotrophically on a temperature block [16]. The block uses a constant water flow to maintain gradually increasing temperatures in its different parts. On the top of the block cool-white fluorescent lamps were attached and provided the continuous light for the cultivation. This equipment enables to set and maintain the desired temperatures (15, 20, 25, 30, 35 °C) simultaneously at two different light intensities – 132 μmol photons m^{-2} s^{-1} unilateral (conditionally marked as LLI – low light intensity) and 2 × 132 μmol photons m^{-2} s^{-1} bilateral (HLI – high light intensity). Individual flasks of 100 ml with the culture were grown for each temperature and light intensity. The algae were cultured in the modified nutritive medium of Setlik [17, 18], diluted to ¼ of its original concentration, routinely used in the lab for green algae cultivation, but only with one *N* source – urea with preserved equimolar quantity. All cultures were continuously bubbled with air enriched with 2 % CO_2. The experimental cultures were harvested after 144 hours. Cells were collected by centrifugation (5000 × g, 20 min), rinsed three times with distilled water, frozen, and stored at -70 °C until analyzed.

Growth and specific growth rate

The growth of *Scenedesmus* sp. BGP was assessed by the increment in the dry weight (DW) of the algal biomass in g L^{-1} which was determined gravimetrically. For this purpose, algae suspensions (3 × 5 ml each) were filtered through Whatman GF/C glass filters (Whatman International Ltd, Maidstone, UK), rinsed with tap water to eliminate salts and oven dried at 80 °C to a constant weight.

The specific growth rate (μ) was calculated as follows [19]:

$$\mu = \ln(mt_2/mt_1)/t_2–t_1 \quad (1)$$

where mt_1 and mt_2 represent the *Scenedesmus* cells DW at the starting day of the experiment (t_1) (t_1=0) and at the final day of the experiment (t_2) (t_2=4).

Biochemical composition

Protein content

Total protein content (g L^{-1}) was measured following the method of Lowry [20], with Bovine serum albumin (BSA, Sigma-Aldrich) as a standard. The algal suspension was flooded with hot methanol (1:1, Merck), then the mixture was centrifuged. To the residue 4 ml of 1N NaOH (Honeywell Fluka) were added. The reaction was conducted on a water bath for 20 min, followed by centrifugation. The supernatant, ($NaCO_3$ + (Na-K tartrate+$CuSO_4$) and Folin-Chiocalteu reagent (Sigma-Aldrich) formed the final mixture, followed by absorption measurement.

Carbohydrate content

Total carbohydrates (g L^{-1}) were estimated by the phenol-sulfuric acid method using glucose (99.5%, Sigma-Aldrich) as a standard [21]. To 0.5 ml of algal suspension 0.5 mL of 5% phenol solution and 2.5 mL of concentrated H_2SO_4 (95-97%, Merck) were added After 30 min incubation, the absorption was measured.

Lipid content

Two methods were employed to determine the lipid content. The method described by Petkov [22] is suitable for extraction of lipids for all classes of microalgae. However, in a parallel study co-authored by the same author [23], it was ascertained that on green microalgae another safer and quicker method can be used. In Petkov's method [22], the centrifuged algae biomass was extracted *via* a boiling solution of chloroform+methanol (2:1) on a reverse condenser. The extract was filtered in order to remove the cell fragments. Next, chloroform (> 99 %, Merck) and methanol were separated by a solution of 11.5 % NaCl (to 1/5 of the volume of the extract, > 99 %, Sigma-Aldrich). On a rotary vacuum evaporator, back-extraction with chloroform was performed at (40–45) °C and the extract was dried by applying Na_2SO_4 (> 99 %, Sigma-Aldrich). The lipid quantity (g L^{-1}) was determined gravimetrically.

Following the second method advocated by Petkov and Dilov [23], the algae suspension was centrifuged, then algae biomass was extracted twice with hot ethanol (1:20, 96%, Chimspectar) under reflux. The ethanol extract was evaporated and back-extracted with chloroform. The latter was removed by evaporation at (40–45) °C on a rotary vacuum evaporator. Lipids were gravimetrically quantified (g L^{-1}) following Petkov and Dilov [23].

Extraction techniques

Conventional extraction techniques, characterized by low equipment cost and easy operation, were applied to recover the algal oil from the biomass. Solvents with different polarity were used. Also, in certain cases, mild pretreatment methods – flooding with boiling ethanol and ultrasonication – were applied.

The procedure adopted for the sample preparation was the following: The harvested biomass was centrifuged at 5500 × g for 15 min and then frozen at -22 °C until lyophilization. In our case, lyophilization was conducted on a LGA 05 lyophilizer (Janetzki, Leipzig, Germany) at +20 °C for 24-30 h until the sample was completely dry. The lyophilized biomass was conserved at 4 °C in dark.

Petkov and Dilov and Petkov extraction methods

The extraction techniques are described in the sections *Lipid content*, *Two-step atmospheric extraction with stirring.*

The experiments were carried out on a temperature-controlled magnetic stirrer. Two solvents were tested: *n*-hexane (99 % Honeywell/Riedel-de Haen), and ethanol (96 %, Chimspectar). For step 1 the procedure was the following: 1 g of pre-ground lyophilized algae was mixed with 30 ml of *n*-hexane in a flask. The extract was recovered and dried. In step 2, the residual biomass was dried, weighed and ethanol quantity was calculated to satisfy 1:30 (biomass:ethanol) ratio. The contact time for both steps was 60 min.

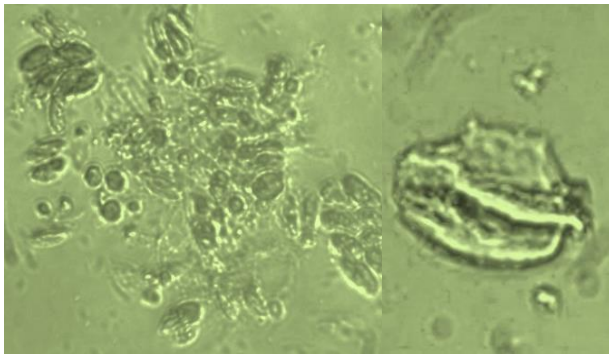
*Soxhlet extraction*s

Soxhlet extraction is an effective method that guarantees high yield and continuous contact with fresh solvent, which is easily recovered afterwards by evaporation, etc. Still, the use of an organic solvent (e.g. *n*-hexane), the relatively high temperatures and long extraction times which might increase the possibilities of thermal degradation of heat sensitive compounds, are some of its drawbacks. In our Soxhlet experiments the influence of biomass pretreatment and solvents on the oil yield and composition were studied.

With regard to the biomass pretreatment, it was noted that the selection of a disruption technique is exceedingly specific and depends on the strain and structural morphology of each microalgal cell wall, and on the nature of the products expected to be recovered and/or their application [24]. In this study, two pretreatment methods were used: Flooding twice with boiling ethanol and ultrasonication. The latter is favored by some authors, who have pointed out its effectiveness in enhancing the extraction yield of various intracellular compounds including lipids [24]. Still, on the other end are authors (see for example [25]) who state that the microwave oven method is more effective for lipid extraction than sonication.

One-step Soxhlet with ethanol

In this case, the biomass pretreatment was as follows: two grams of algae biomass were placed in the extractor thimble and flooded twice with 40 ml of boiling ethanol. The solution gradually turned green and after cooling for about 15 min was transferred to a round-bottom flask and another 70 ml of ethanol were added, hence the total volume was 150 ml (solvent/solid ratio 75 ml/1 g). The working volume of the Soxhlet extractor was 70 ml. The process time was 8 hours. The criterion for stopping the Soxhlet extraction was a change in the color of the solution – it slowly faded from green to light yellow. The biomass and the extract were separated by filtration with Millipore (0.22 μm). The extract obtained was separated from the solvent by a rotary vacuum evaporator (Heidolph/ Germany) at $T = 40\ ^0$C and $p = 175$ mbar. Picture 2 shows an image of a disrupted *Scenedesmus* sp. BGP single cell after flooding the biomass with boiling ethanol.



Picture 2. Image of *Scenedesmus* sp. BGP cells after sonification

Two-step Soxhlet extraction

In step 1 the solvent used was *n*-hexane. Firstly, the biomass mixed with *n*-hexane was sonicated in an ultrasonic disintegrator UD 20 (Techpan, Warsaw) with ultrasonic field of 8-24 kHz. The optimum operating parameters were determined in a series of preliminary sonications at different resonance, cycles and time. After each sonication, the sample treated was examined by a microscope to establish the level of cell disintegration. As a result, the procedure adopted was the following: in a volumetric flask two grams of algae biomass were mixed with 30 ml of *n*-hexane, and subjected to five consecutive ultrasonications at the maximum ultrasonic field level, followed by a cool-down

period in ice water for 1 min. Total time of pretreatment was 10 min.

After the sonication pretreatment, the whole quantity was transferred to the extractor thimble and additional 120 ml of *n*-hexane were added in the round-bottom flask to achieve a solvent volume of 150 ml (solvent/solid ratio 75 ml/1 g). After 4 hours, the process was stopped and the extract was filtered through Millipore (0.22 μm). After drying, the residual biomass from step 1 was extracted with 150 ml of 96 % ethanol. The second step was stopped after 8 hours and the liquid extract obtained was filtered and dried.

GC analyses of the extracts obtained

Fatty acids (FAs) composition of selected extracts was determined by gas chromatography (GC) of methyl esters (FAME). The methodology is discussed in details by Taneva *et al.* [26] and is briefly summarized here. Each sample was transmethylated with 1% sulfuric acid in methanol. The FAME were purified by preparative silica gel thin-layer chromatography (TLC) using hexane-acetone (100:6, v/v) as a mobile phase. The gas chromatograph was Shimadzu 17A (Shimadzu, Japan) equipped with a flame ionization detector and Supelcowax-10 column (100 m × 0.25 mm × 0.25 μm, SUPELCO). The temperature gradient of the column was 4 K min^{-1} in the range T = (433 – 543) K. Upon reaching the maximum temperature desired, it was sustained for 20 min. The temperature of the injector was 533 K and that of the detector– 553 K. Split injection mode (1:50) was used, each sample was 15 μg and helium was used as a carrier gas at a 1.1 ml min^{-1} flow rate. The peaks identification was according to retention times of the reference FAME. The qualitative analysis of selected extracts was carried out by GC-MS. The gas chromatograph was Agilent 7890B equipped with Agilent 5977A mass selective detector, the column - DB-5 MS ((5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm × 0.25 μm I.D.) and helium was used as a carrier gas. The temperature of the injector and detector was 533 K. The temperature of the column – 333 K, held for 4 min, the temperature gradient was 3 K, held for 1 min up to reaching 573 K. The FAs were identified by interpreting and comparing their mass spectra to existing databases. The relative content of FAs was expressed as percentage (%) of individual fatty acid to total fatty acids.

Statistical analysis

All experiments were conducted in three independent biological replicates and each measurement had three replicates. The data were presented as the means ± standard deviation. The significance of differences between the treatments was evaluated by ONE WAY analysis of variance (ANOVA) and Bonferroni's post hoc test using GraphPAD InStat software (San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Taxonomic identification

Monoalgal culture of *Scenedesmus* sp. BGP contained single cells and four-cell cenobias. In the lag phase, more single cells were observed, while in the exponential phase the culture was dominated by cenobias. The morphological analysis showed that the investigated cells were non-motile and zoospores were not observed. The cells had elongated shape with approximate length of 12 × width of 5 μm. After performing the morphological analysis, the newly isolated strain was identified as *Scenedesmus obliquus* (Turpin) Kutzing [2].

The sequence comparison results are presented on Fig. 1. From the BLAST search performed and from the phylogenetic tree it can be deduced that the strain has the closest proximity in terms of likelihood to the *Tetradesmus obliquus* strain *Scenedesmus obliquus*. From the algae data base [2] it can be confirmed that right now *S. obliquus* (Turpin) Kützing 1833 is considered a synonym of *Tetradesmus obliquus* (Turpin) [27], which substantiates our taxonomic and molecular analyses. Hence, to be consistent with the initially given name in what follows the Bulgarian strain is referred to as *S. obliquus* BGP.

Effect of temperature and light intensity on the growth and specific growth rate of Scenedesmus obliquus BGP

Temperature and light are the basic parameters that affect the photosynthesis and respiration, therefore, the biomass productivity. Although it is known that the influence of light on the growth and productivity depends on temperature and *vice versa* [28], still, the combined effect of these factors has been less studied than their individual impact [29, 30].

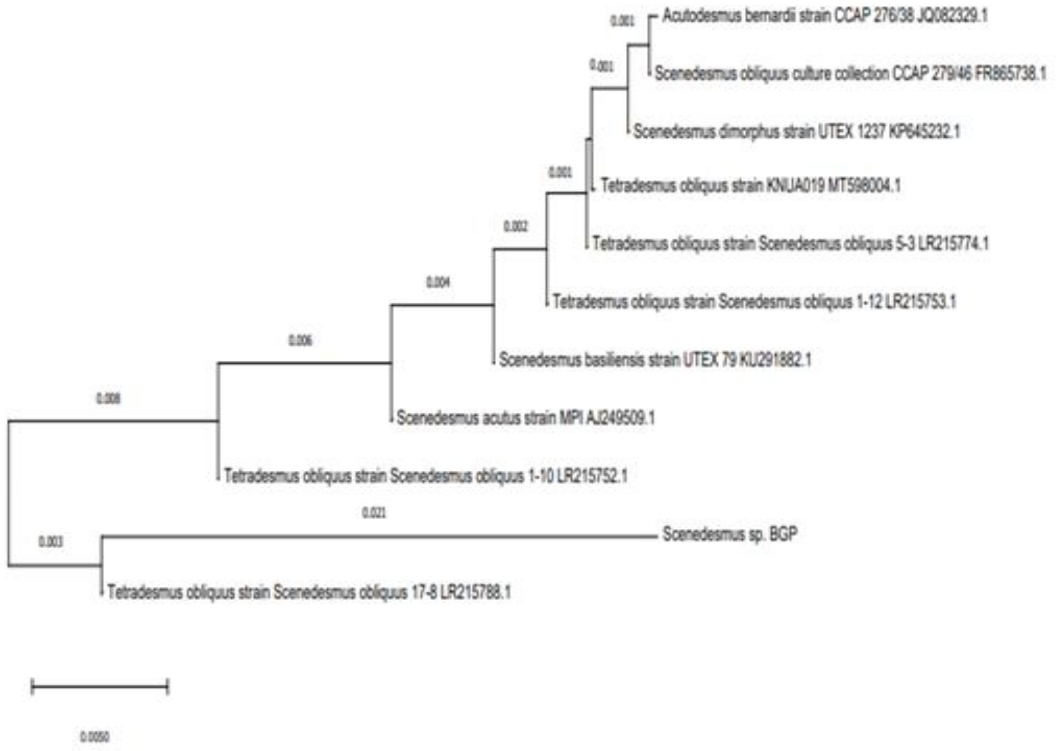


Fig. 1. Maximum likelihood tree. Numbers at the nodes indicate bootstrap probabilities (> 50 %) of maximum likelihood analyses (1000 replicates). The scale bar represents 0.02 % difference in nucleotide sequences.

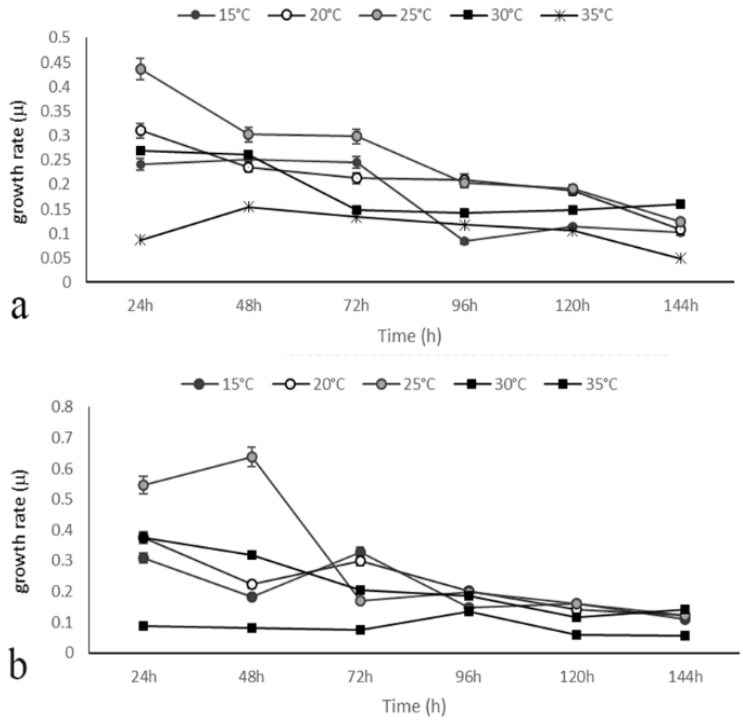


Fig. 2. Dependence of the accumulated biomass on the cultivation temperature and time of *Scenedesmus obliquus* BGP at LLI (a) and HLI (b).

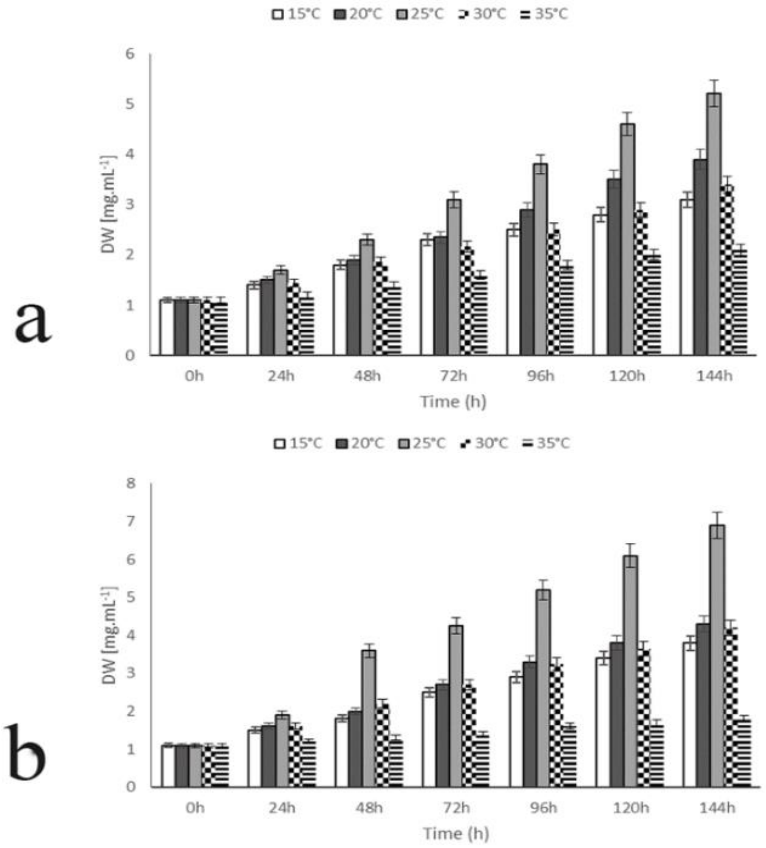


Fig. 3. Dependence of the specific growth rate on the cultivation temperature and time of *Scenedesmus obliquus* BGP at LLI (a) and HLI (b).

It was demonstrated for the first time in the present work that *S. obliquus* BGP grew well within the temperature range of (15–30) ° C at both light intensities on modified Setlik medium (Figure 2). The highest biomass yield and the maximal growth rate were observed at 25 °C at both LLI and HLI (optimal temperature) – 5.2 and 6.9; $\mu = 0.26$ and 0.31, respectively (Figure 2 and Figure 3). As briefly discussed previously, *S. obliquus* BGP has a natural habitat of lower temperatures than many other representatives from the same species. For example, *S. dimorphus* responded to changes in the cultivation temperature in a different way. Just like the Bulgarian strain, the lowest and the highest growth rate and dry biomass weight were observed at (15 and 25) °C, respectively (the results for 35 °C are not taken into consideration, Figure 2). However, in the case of *S. obliquus* BGP, the highest temperature (35 °C) along with the high light intensity were particularly unfavorable growth conditions as was observed with some other microalgae. A similar conclusion can be made when it comes to the specific growth rate. In the work of Duan *et al*. [31], during the cultivation of *S. obliquus*, $\mu = 0.26$ was reached just at the 48th hour of cultivation, and then the values of μ rapidly decreased.

Effect of temperature and light intensity on the biochemical composition of Scenedesmus obliquus BGP

The optimal temperature promotes best the protein synthesis, although protein production was higher at HLI than at LLI at all temperatures tested - 2.4 g L^{-1} and 1.9 g L^{-1}, respectively (Figure 4).

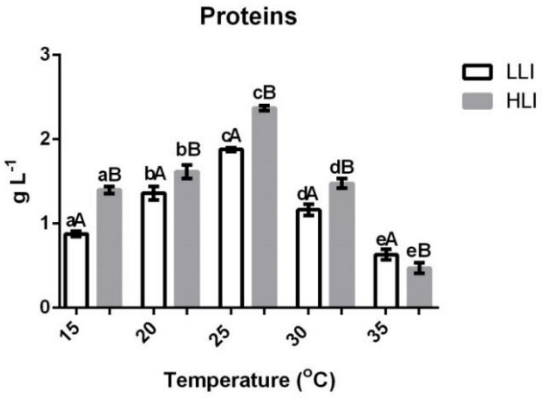


Fig. 4. The joint effect of temperature and light intensity on the protein content of *Scenedesmus obliquus* BGP. Means with different lowercase letters are significantly different ($P < 0.05$) between temperatures for a specific light intensity. Different capital letters indicate significant difference for a specific temperature between LLI and HLI.

The quantity of carbohydrates at the suboptimal temperatures was almost the same regardless of the light intensity. The highest yield was achieved when conditions of 25 °C (both light intensities - 2 g L^{-1} and 2.4 g L^{-1}, respectively) and 30 °C (HLI; 2.4 g L^{-1}) were applied. (Figure 5).

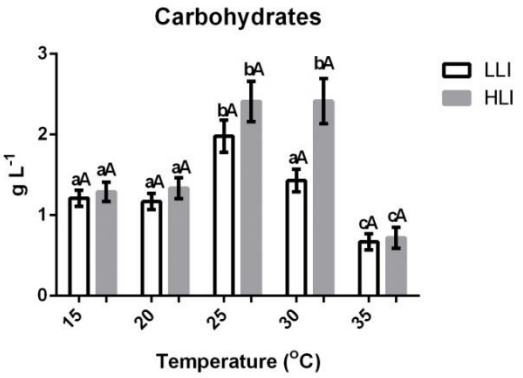


Fig. 5. The joint effect of temperature and light intensity on the carbohydrate content of *Scenedesmus obliquus* BGP. Means with different lowercase letters are significantly different (P < 0.05) between temperatures for a specific light intensity. Different capital letters indicate significant difference for a specific temperature between LLI and HLI.

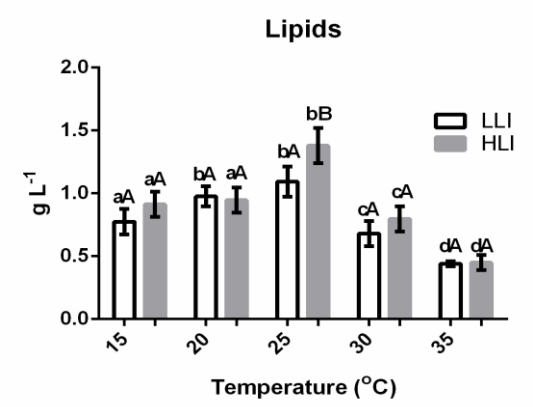


Fig. 6. The joint effect of temperature and light intensity on the lipid content of *Scenedesmus obliquus* BGP. Means with different lowercase letters are significantly different (P < 0.05) between temperatures for a specific light intensity. Different capital letters indicate significant difference for a specific temperature between LLI and HLI.

All suboptimal and the optimal temperature stimulated the lipid synthesis compared to 30 and 35°C. 25 °C was the only temperature investigated where the light intensity significantly stimulated the accumulation of lipids – from (1.1 to 1.4) g L^{-1} (Figure 6**)**. Turns out that the strain is among the best producers of lipids from *Scenedesmus* sp. reported in the literature, being inferior only to *S. protuberans* (up to 29 % according to [5]), *S. dimorphus* (up to 40 %, [32]) and few others. However, if most of the representatives are considered for their actual yield, the picture changes. For example, in the research of Gris *et al.* [33] it is reported that the lipid content is 40 % of DW, which is about 1.3 g L^{-1}, compared to 1.4 g L^{-1} and 20 % of DW in our study. The purpose of the study of Gris *et al.* [33] was to increase the lipid accumulation by applying stress (high light intensity). Another approach to this can be the one of Mandal and Mallick [34] who applied N-deficiency in order to increase the lipid content of *S. obliquus* (Trup.) Kütz, although at the expense of the growth. In contrast, *S. obliquus* BGP has a higher actual yield compared to the other representatives. Another advantage of our strain is the fact that unlike *S. obliquus* CNWN, for example, the increase of the light intensity positively affects the accumulation of lipids by 30 % [35], while the algal growth is preserved. The above considerations served as an impetus to study further the Bulgarian strain advantages and potential as a viable BioRef feedstock targeted at biofuel production.

Influence of extraction techniques and solvents on the lipid yield

Commonly, based on the polarity of a lipid head-group, it can be classified as either neutral/non-polar (e.g. TAGs) or as polar/complex (e.g. phospholipids). Microalgae lipids normally comprise neutral and polar lipids, and other complex lipophilic materials. The yield and composition of total lipids are influenced by a number of factors, e.g. biological nature of the algal biomass, its pretreatment, particular technique and nature of the solvents employed in their recovery, etc. Pertaining to the latter, as demonstrated by authors who studied solvents' efficiency concerning recovery of the different lipid classes (see for example [36]), low-polarity solvents are best for the extraction of neutral lipids, while polar solvents should be the choice when lipids from chloroplasts and membranes, which contain polar lipids like glycolipids and phospholipids, are to be recovered. Still, it is pointed out that in a direct extraction of the algal biomass by polar solvents a joint recovery of polar and certain neutral lipids could be realized. The latter are lipids that might form complexes with polar lipids, which also form hydrogen bonds with proteins in the cell membrane. Nonpolar solvents cannot extract such complexed neutral lipids since they cannot break those hydrogen bonds. Polar organic solvents, on the other hand, are capable of breaking the lipid-protein complexes and consequently polar and complexed neutral lipids are recovered from the cells [36].

The lipid yields shown in Table 1 were calculated according to:

$$Yield\ (\%) = \frac{mass\ of\ extract\ (g)}{mass\ of\ sample\ (g)} * 100 \qquad (2)$$

Petkov and Dilov and Petkov method

The yield according to Petkov and Dilov [23] method was (25 ± 4.2) %, while that of Petkov - (27±1.4) %. The higher yield achieved by the latter method can be explained within the light of the above discussion. On the one hand, methanol, the solvent with the highest polarity $E_T^N = 0.762$ [37] among all solvents used in our study, is supposed to be more effective in the recovery of polar and complexed neutral lipids than ethanol ($E_T^N = 0.654$). On the other - chloroform ($E_T^N = 0.259$) promotes the extraction of non-polar lipids which might not be extracted by the polar ethanol used in Petkov and Dilov method. Notwithstanding, the advantages of Petkov and Dilov method, which is suitable for green algae only, are not just its price and speed, but the most important is the fact that it is GRAS.

Two-step atmospheric extraction with stirring

In step 1 *n*-hexane ($E_T^N = 0.09$) was chosen because of its proven selectivity to non-polar lipids. The yield achieved, as shown in Table 1, was quite low - on average 2.4 %. That came as no surprise as firstly no pretreatment of the algal biomass was performed. Hence, the lipids remained entrapped within the cell walls which hindered the contact with the solvent. Secondly, *n*-hexane, because of its very low polarity, is totally incapable of recovering complexed neutral lipids. In contrast, the yield of step 2 was 15.2 %, which can be explained taking

Table 1. Experimental conditions and yields for the extraction techniques employed.

Extraction method	Solvent	Temperature (°C)	Extraction yield (wt %)
Petkov and Dilov, 1987	Ethanol	70	25±4.2
Petkov, 1990	Methanol:chloroform (2:1)	61	27±1.4
Two-step atmospheric with stirring	*n*-Hexane (step 1) Ethanol (step 2)	60 70	2.4 ± 0.34 15.2±0.31 Cumulative yield: 17.6
Soxhlet	Ethanol	78	23.6±2.8
Two-step Soxhlet	*n*-Hexane (step 1) Ethanol (step 2)	68 78	11.1±0.5 16.9±1.6 Cumulative yield: 28

Table 2. Fatty acid composition from FAME GC-FID analysis of selected extracts expressed as relative percent of total fatty acids identified.

Fatty acid identified		Extract recovered			
		1	2	3	4
12:0	Lauric	traces	traces	1.0	traces
14:0	Myristic	0.2	0.2	0.5	0.2
15:0	Pentadecylic	0.1	0.1	0.1	0.1
16:0	Palmitic	17.5	22.3	18.8	24.1
16:1	Palmitoleic	3.9	5.0	3.8	5.7
16:2	Hexadecadienoic	6.9	3.1	2.6	3.6
16:3	Palmitolinolenic	6.1	3.0	2.4	3.5
16:4	Palmitidonic	traces	2.4	1.7	3.0
17:0	Margaric	0.1	0.2	0.2	0.3
18:0	Stearic	1.6	2.7	3.2	2.2
18:1 (n-9)	Oleic	28.6	32.8	38.7	27.6
18:1 (n-7)	Vaccenic	1.0	0.7	0.6	0.8
18:2 (n-6)	Linoleic	16.3	14.3	13.5	14.8
18:3 (n-6)	Gamma-Linolenic	-	0.8	0.6	1.0
18:3 (n-3)	Alfa-Linolenic	17.7	9.5	9.1	9.9
18:4	Stearidonic	-	1.9	1.9	2.0
20:0	Arachidic	traces	0.1	0.1	0.1
20:1	Gondoic	-	0.3	0.4	0.4
22:0	Behenic	-	0.2	0.6	0.3
22:1	Erucic	-	0.4	0.2	0.4
SFA		19.5	25.8	24.5	27.3
MUFA		33.5	39.2	43.7	34.9
DUFA		23.2	17.4	16.1	18.4
PUFA		23.8	17.6	15.7	19.4

into consideration that polar ethanol is the solvent for the recovery of polar and complexed lipids from the biomass residue already depleted to a certain extent from non-polar lipids.

Soxhlet extractions

The yield achieved by the one-step Soxhlet with ethanol is commensurable with the yield of Petkov and Dilov (1987) method but lower than that of Petkov's method (Table 1). The reasons for that were briefly discussed previously.

The yield of step 1 of the two-step Soxhlet was 11.1 %, which is over 4 times higher than that of the atmospheric extraction with stirring applying the same solvent *n*-hexane. Apparently, the increase in the yield is a result of the biomass pretreatment, which caused thinning and disruption of the cell walls. Hence, the mass transfer and diffusion of lipids into the solvent was accelerated. The yield of the second step Soxhlet was 16.9 %, just slightly higher than that of the analogous atmospheric with stirring step, regardless of the fact that no pretreatment of the biomass was performed in the latter case. The cumulative yield of the two-step Soxhlet was about 28 %, which is 1.5 times higher than the cumulative yield of atmospheric with stirring, at the expense of the very low yield of its step 1, and commensurable with Petkov's method. However, it is greener than the latter, as the use of methanol is avoided [38]. Yet, it should be taken into consideration that the Soxhlet process time and volume of the solvents required are considerably higher than those of the other methods examined.

Composition of the extracts recovered depending on the techniques and solvents employed

Microalgae, in general, produce predominantly FAs with chain lengths of 16 and 18 carbon atoms (though there are species that can make FAs of up to 24 carbon atoms in length), both saturated and highly unsaturated. *S. obliquus* BGP is not an exception – about 90 % of its total lipid fraction is formed by C_{16} and C_{18} acids as can be deduced from the FA profile of the extracts recovered, applying different techniques and solvents, and shown in Table 2. Table 2 also displays the relative percentage of saturated (SFA), mono- (MUFA), di- (DUFA) and poly-unsaturated (PUFA) fatty acids in all four extracts, respectively.

Oleic acid, a MUFA, is the dominant FA. The highest (38.7 %) percentage was registered in the *n*-hexane extract (extract 3); the lowest - 27.6 % - in extract 4 recovered by ethanol from the already partially depleted biomass matrix, respectively. The latter is commensurable with the oleic acid percentage in extract 1. The FA with the second-highest relative percent is the saturated palmitic acid. Extract 4 stands out with 24.1 % of palmitic acid, while extract 1 registers just 17.5 %. In the latter, alpha-linolenic, a PUFA, is not only the second dominant FA, but has the highest percentage among all four extracts analyzed. Although an analogous trend is observed for linoleic acid, a DUFA, for which the highest percentage (16.3 %) is registered in extract 1, still it should be noted that the percentage is commensurable with those of the other three extracts.

Further examination of the FA profiles displayed in Table 2 can help assessing, on the basis of some general considerations, the potential of the *S. obliquus* BGP extracts recovered, as a viable biosource of biofuel-related compounds. Firstly, because oxidation has a detrimental effect on some fuel properties including cetane number (CN), therefore, the higher the content of esters of unsaturated, and particularly PUFAs, in a fuel, the higher is its proneness to oxidation. So, on the one hand, the presence of PUFAs lowers the CN and oxidation stability and is, therefore, undesirable for any fuel. On the other hand, however, it is useful since PUFAs are characterized with lower melting points which improve the cold-flow [39-41]. Also, if the relative rate of oxidation as a function of the degree of unsaturation for methyl esters of the respective FAs is examined it increases as follows: oleic < linoleic < linolenic < stearidonic (Table 2). Taking into consideration the relative percent of each of the above FAs in the extracts recovered, then the extract least prone to oxidation is extract 3, while the most susceptible – extract 1, in which the highest percentages of PUFAs and unsaturated FAs are registered even though it doesn't contain stearidonic acid. Secondly, the lower the PUFA:SFA ratio is in an extract, the more suitable it is as a source for biofuels production. If the corresponding PUFA:SFA ratios for the extracts are calculated, it appears that extract 1 is the only one with PUFA:SFA > 1, while extract 3 has the lowest PUFA:SFA = 0.64. Therefore, based on the above general considerations, extract 3 stands out among the rest with its capacity, either as a stand-alone or mixed with other carefully chosen extracts, as a source for biofuel production.

The experiments performed in our study revealed that *S. obliquus* BGP has a potential as a viable biosource of energy-related compounds. Consequently, that first initial study should be further expanded and intensified in order to outline the possible new horizons to the future applications and uses of this totally unutilized algal strain.

Also, the results of the above analysis can serve as a starting point towards the development of a future strategy targeted at designing a BioRef for algal biomass processing that can deliver a broad spectrum of products by performing a careful selection and sequencing of techniques, operating conditions and solvents. Products with compositions that could either be used directly for biofuels production or mixed with other to realize the delicate desired balance of FAs, will guarantee oxidative stability and CN without compromising the flow, lubricity and viscosity of a high-quality biofuel [39, 42].

CONCLUSIONS

The study presents the biotechnological advantages of the new strain *Scenedesmus obliquus* BGP. The ability to maintain substantial growth and balanced biochemical composition in a broad temperature range and under high light intensity indicates that it can be cultivated on a large scale with substantial reduction of cultivation costs. Moreover, its valuable components accumulate under conditions favorable to growth rather than in response to stress. Finally, because of the richness of BGP biochemical composition in lipids it can be exploited as an excellent, totally unused at present bioresource of compounds suitable for biofuels production.

Acknowledgements: *This work was supported by the National Science Fund, Ministry of Education and Science [grant number КП-06-ОПР04/1]. The research team is also grateful to Prof. Jaromir Lukavsky from the Institute of Botany, Trebon, Czech Republic, for conducting the morphological identification of the strain.*

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