Growth phase-dependent biochemical composition of green microalgae: Theoretical considerations for biogas production

Marek Klin*, Filip Pniewski, Adam Latała

University of Gdańsk, Institute of Oceanography, Laboratory of Marine Plant Ecophysiology, al. Marszałka Piłsudskiego 46, 81-378 Gdynia, Poland

ABSTRACT

One of the most efficient and promising technique for biofuel production from microalgae biomass is an anaerobic fermentation. The goal of this work was to investigate changes in the biochemical composition during the long-term cultivation period of 15 green microalgal strains originating from the Baltic Sea. Subsequently, their theoretical methane potential (TMP), which is strictly determined by an algal growth phase and thus physiological state, was established. Based on the full spectrum of changes in the percentage share of lipids, carbohydrates, and proteins in biomass, it was shown that the TMP values differed among strains as well as fluctuated during cultivation. The common trend, i.e., lipids accumulation and proteins breakdown in the late growth phase, was observed for most of the strains; others, however, preferred carbohydrates as storage material. The TMP data obtained herein allows developing a strategy for the design and production of algal biomass biochemically suited for fermentation.

1. Introduction

The idea of algae biomass as a new substrate for various commercial uses is continually gaining more interest. The goal is clearly defined, i.e. to use locally adapted microalgal strains for sequestering atmospheric carbon dioxide CO_2, a reaction driven by sunlight and then controlled release of this energy with a carbon dioxide balance close to zero. On one hand this gives a possibility to deal with the current ecological problems such as global warming and water pollution, on the other provides high quality biomass which can be a source of lipids, carbohydrates, proteins and various biologically active compounds which can be used in pharmaceutical, cosmetics and food industry etc. (Khan et al., 2018). Algal biomass also harbors a potential to be used as a substrate for biofuel production. However, not all techniques of transforming algal compounds into energy sources maintain their previously announced potential due to the still un-optimized process of pretreatment e.g. biomass dewatering or complicated lipid extraction for the production of biodiesel (Dong et al., 2016). One of the most promising technique for biofuel production is anaerobic fermentation (Mussgnug et al., 2010), an enzymatic break down of organic compounds carried out by methanogenic microorganisms in the absence of oxygen (Klassen et al., 2016; Sakarika and Kornaros, 2019). The most important product of the decomposition of organic matter is methane (CH_4), a combustible gas which in fact is already widely used in power engineering. A significant advantage of the use of biomass for fermentation is the lack of necessity for advanced dewatering nor further chemical extraction of desired compounds (Ras et al., 2011). The efficiency of methanogenic bacteria that produce biogas depends on the biochemical composition of the substrate used. Green algae are commonly recognized as efficient organisms in mass algal cultures due to their biochemical composition and the fact that it can be easily altered under stress conditions (Gilmour, 2019). That corresponds well with the features of the optimal fermentation substrate – with high lipids content due to their high energy capacity and thus theoretical methane potential (TMP) (1.014 L $CH_4g^{-1}VS$) (Sialve et al., 2009), but not exceeding 40% share in dry biomass (DW), so as not to slow down the first stage of fermentation – hydrolysis. Proteins are desired at the lowest possible concentration due to the formation of toxic ammonia during their decomposition (Kwietniewska and Tys, 2014; Gonzalez-Fernandez et al., 2015) and having significantly lower biomethane yield than lipids (TMP 0.446 L $CH_4g^{-1}VS$). Carbohydrates are the least energetically valuable (0.415 L $CH_4g^{-1}VS$), however, are decomposed relatively without inhibitors (Donius et al., 1976; Sialve et al., 2009) so are considered as a fine fermentation substrate.

Some information on the experimental biochemical methane potential (BMP) and biomass preparation, the so-called pretreatment methods can be found regarding green algal strains (Ganesh Saratale et al., 2018). TMP and BMP values for microalgae are also increasingly

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* Corresponding author.
  E-mail address: klin.m@outlook.com (M. Klin).

being compared with the results for conventional fermentation substrates (e.g. agricultural waste) as a potential new plant substrate. In fact, BMP tests may encounter difficulties in complete substrate fermentation. Usually, the resulting methane yield is lower than the previously calculated theoretical gas volume due to the imperfection of the process and the presence of a protective cellulose cell wall in algae cells (Klassen et al., 2016). Frequently, those are individual data without prior insight in the possible wide plasticity of the microalgal biochemical composition. According to our best knowledge there is only one author (Córdova et al., 2018) showing the direct relationship between this parameter and growth phase of selected strain in a single experimental setup. This work aims in understanding TMP fluctuations resulting from metabolism of a strain and growth phase so that so that optimization of the future BMP tests will be developed on the most physiologically suitable version of this biomass. Conducting long-term stationary culture with simultaneous biochemical monitoring of biomass is the basis for calculating and indicating the highest possible biomass TMP value. The TMP tests are therefore the recommended step towards exploring the full potential of microalgal biomass potential in anaerobic digestion.

The aim of this work was to review locally occurring Baltic green algae strains in terms of their potential use as a source of substrate for the fermentation process. We have adopted the perspective of the use of biomass in a specific physiological state resulting from the changes in biochemical composition corresponding to subsequent growth phases. The multiplicity of data obtained in long-term, monitored cultures works in favor of selection of the appropriate strain and the culturing phase with the biochemical composition meeting the conditions set for an optimal fermentation substrate: high content of storage materials (lipids, carbohydrates) and low content of proteins.

Fifteen strains of green algae were grown for over 30 days and their growth rate was estimated. Lipids, carbohydrates and proteins as well as photosynthetic pigments content were also monitored so as to evaluate the changes in biochemical composition of the strains with respect to their growth phase.

2. Materials and methods

2.1. Microalgal strains

For the screening research 15 strains (full list Table 1) of Baltic green microalgae belonging to the following genera were used: *Chlorella*, *Monoraphidium*, *Kirchneriella*, *Oocystis* and *Scenedesmus*. Selected strains are part of the Culture Collection of Baltic Algae (CCBA) at the University of Gdansk and maintained as non-axenic monocultures. They were isolated from the brackish water samples collected in the Gulf of Gdansk located in the southern part of the Baltic Sea.

2.2. Algal cultivation and sampling

The batch cultures were performed in 300 ml wide-mouth glass flasks. They were filled with 200 ml of artificial seawater-based f/2 medium with salinity of 8 PSU and capped with a cotton wool plug for gas exchange. F/2 medium composition: $NaNO_3$ 75 mg L^{-1}, $NaH_2PO_4 \cdot H_2O$ 5 mg L^{-1}, $Na_2SiO_3 \cdot 9H_2O$ 30 mg L^{-1}, $FeCl_3 \cdot 6H_2O$ 3.15 mg L^{-1}, $Na_2EDTA \cdot 2H_2O$ 4.36 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 180 µg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 22 µg L^{-1}, $CoCl_2 \cdot 6H_2O$ 10 µg L^{-1}, $CuSO_4 \cdot 5H_2O$ 9.8 µg L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 6.3 µg L^{-1}, thiamine · HCl (vitamin B1) 200 µg L^{-1}, biotin (vitamin H) 0.1 µg L^{-1}, cyanocobalamin (vitamin B12) 0.1 µg L^{-1} (Guillard, 1975).

After autoclaving (20 min, 1 atm, 121 °C), 100 µl of dense algal culture (cells were in the logarithmic growth phase) of each strain was transferred into the fresh medium and flasks were kept on phytotron shelves illuminated with 150 µmol $m^{-2}s^{-1}$ with photoperiod of 16/8 (light/dark), at the temperature of about 23 °C $\pm$ 1 °C. During the 30-day culturing period, beginning with the day 8th, every 2 days 3 ml aliquots of the culture were taken for optical density measurements. Subsequently they were divided into three 1 ml subsamples for lipids, carbohydrates and proteins quantification. Every 6 days additional 5 ml sample was taken for photosynthetic pigments analysis. Cultures of each strain were performed in triplicates.

2.3. Algal growth and dry biomass

The growth of the algae was monitored by measuring optical density of the suspension at the wavelength of 750 nm in a 1 cm glass cuvette. Separately, for each strain, the correlation curves for dry biomass (DW) and optical density were prepared. A known volume of a culture was filtered through a pre-weighed GF/C Whatman glass filters which were then dried for 12 h at 90 °C and weighed again afterwards. The specific growth rates (μ; d^{-1}) were calculated using the optical density values according to the equation provided by Guillard (1973).

$$\mu = (\ln E_1 - \ln E_0)\Delta t^{-1}$$

where E_0 and E_1 are the absorbances at the beginning and at the end of the time interval, respectively, and $\triangle t$ is the length of the time interval [days; d^{-1}].

2.4. Total lipids, carbohydrates and protein assays

For each assay 1 ml of culture was centrifuged at 8000*g* for 3 min, supernatant was discarded and the remaining cell pellet was frozen at −60 °C until further processing.

2.4.1. Lipids

Quantitative measurements of lipids were performed using colorimetric sulpho-phospho-vanilin reaction (SPV method) introduced by Chabrol and Charonnat (1937) and modified for algal material by Klin et al. (2018). To extract lipids thawed cell pellets were resuspended in 400 µl methanol and mixed vigorously with glass beads for 15 min. Secondly, 800 µl chloroform was added and mixing procedure was repeated (Folch et al., 1957). Resulted cells extract was centrifuged and without disturbing the cell debris transferred to a new test tube followed by the addition of 300 µl of 0.8% NaCl solution. After the two liquid layers had stabilized, the upper water-methanol phase was discarded. The lower layer was dried under nitrogen stream at 50 °C. Subsequently, 300 µl of concentrated H_2SO_4 was added and samples were heated at 90 °C for 10 min. Next, 1000 µl of SPV reagent (1.2 g vanillin per 1 L of 68% phosphoric acid) was added and the test tubes

Table 1
Growth rates and dry biomass at final 30th day of culture. Data shown as mean $\pm$ SD, n = 3.

Strain	Growth rate (µ) d^{-1}	sd	Dry biomass (mg L^{-1})	sd
Oocystis submarina BA-1	0.34	± 0.02	465.4	± 15.2
Chlorella vulgaris BA-2	0.74	± 0.04	552.6	± 17.0
Monoraphidium contortum BA-5	0.71	± 0.03	495.7	± 11.4
Scenedesmus armatus BA-6	0.53	± 0.06	426.9	± 17.1
C. minutissima BA-12	0.72	± 0.02	442.4	± 27.6
M. convolutum BA-17	0.36	± 0.02	424.6	± 16.6
C. fusca BA-18	0.48	± 0.01	515	± 24.0
Chlorella sp. BA-20	0.58	± 0.09	506.8	± 15.5
C. vulgaris BA-46	0.85	± 0.06	419.1	± 14.9
Kirchnereilla obesa BA-51	0.34	± 0.02	395	± 11.5
C. vulgaris BA-80	0.46	± 0.02	455.5	± 13.1
Monoraphidium sp. BA-165	0.81	± 0.01	520	± 6.6
Chlorella sp. BA-167	0.56	± 0.04	515.9	± 13.0
O. cf. *submarina* BA-172	0.34	± 0.00	519.1	± 7.2
Haematococcus sp. BA-179	0.34	± 0.01	421.1	± 26.8

were incubated at 36 °C for 5 min. The absorbance of the final solution was measured at 530 nm. Lipids concentration was estimated from the calibration curve, which was developed using as a source of lipid high-purity soybean oil (Sigma).

2.4.2. Carbohydrates

Total carbohydrates content was analyzed by modified phenol-sulphuric acid method adapted to microplate format (Dubois et al., 1956) First, 1000 μl of 9.1% H_2SO_4 (Masuko et al., 2005) and glass beads were added to the thawed samples in order to disrupt cell walls, extract sugars and perform hydrolysis of the intracellular starch and structural polysaccharides (de Souza et al., 2017). Samples were mixed vigorously in thermomixer for 15 min, held at 100 °C for 60 min and then again mixed for 15 min at room temperature. Solution was clarified from cell debris by centrifugation (as described above) and 50 μl of supernatant was transferred into microplate wells. Next, using multichannel pipette 200 μl of concentrated H_2SO_4 was added to each well then 50 μl of 5% phenol solution was rapidly added. For completing and unifying the reactions in all wells, the plate was incubated in the oven for 5 min at 90 °C after which the absorbance was measured at 488 nm wavelength. Calibration curve was prepared using glucose solution.

2.4.3. Proteins

Total proteins content was analyzed by modified microbiuret reaction adapted to microplate format (Itzhaki and Gill, 1964). Briefly, 1000 μl of extracting reagent (0.5 N NaOH) and glass beads were added to thawed cell pellets. Samples were mixed vigorously in thermomixer for 15 min, held at 100 °C for 60 min and again mixed for 15 min in room temperature (Rausch, 1981). Solution was clarified from cell debris by centrifugation and 200 μl of supernatant was transferred into microplate wells. Next, using multichannel pipette 100 μl of staining reagent (0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH) was added to each well. Microplate was incubated for 5 min at 30 °C followed by absorbance measurements at 310 nm wavelength. Bovine serum albumin (BSA) was used as the standard for calibration.

2.5. Photosynthetic pigments

Photosynthetic pigments analysis was performed by spectrophotometric method (Jeffrey and Humphrey, 1975). 5 ml of algal culture was filtered through GF/C Whatman filter and placed in 5 ml 90% acetone followed by manual grinding until filter was completely defragmented. Resulted mixture was kept at −20 °C for 1 h for pigments extraction. After centrifugation, the extract was transferred to a 1 cm glass cuvette and the absorbance was measured at wavelengths as in the following equations (Jeffrey and Humphrey, 1975; Strickland and Parsons, 1972):

Chlorophyll a − chl a (μg ml^{-1})

$$= [11.93(E_{664} − E_{750}) − 1.93(E_{647} − E_{750})] \cdot vV^{-1}L^{-1}$$

Carotenoids − car(μg ml^{-1}) = $[4.0(E_{480} − E_{750})] \cdot vV^{-1}L^{-1}$

E_n – absorbance value, n – wavelength [nm], v – acetone volume used for extraction [ml], V – sample volume [ml], L – optical path length [cm].

2.6. Theoretical methane potential (TMP)

The formula adopted by Sialve et al. (2009) and revised by Heaven et al. (2011) was used to calculate theoretical methane potential (TMP):

$$TMP = \frac{1}{100} A \times C_L \times B \times C_P \times C \times C_C \tag{1}$$

where three quantified biomass components that make up its calorific value are characterized by a specific methane yield for lipids (A; 1.014 L $CH_4 g^{-1}VS$), proteins (B; 0.446 L $CH_4 g^{-1}VS$), carbohydrates (C;

0.415 L $CH_4 g^{-1}VS$); C_L, C_P, C_C are the percentage shares of lipids, proteins and carbohydrates, respectively, in algal dry biomass.

2.7. Statistical analysis and software

All experiments were conducted using three independent biological replicates and two technical replicates for the analytical methods. Data are expressed as mean ± standard deviation (SD). Student's t-tests were performed at a significance level of 0.05. Statistical analysis and graphs were created with the program OriginPro, Version 2019. OriginLab Corporation, Northampton, MA, USA.

3. Results and discussion

3.1. Growth rate, dry biomass, total proteins, lipids, carbohydrates

3.1.1. Growth rate, dry biomass

Growth rates of cultured microalgae are significant from the economic point of view as they allow reliable comparison and possibly rejection of slow-growing strains in the perspective of mass culturing. Specific growth rate (μ) is an effective predictor of the final biomass estimation but not as unfailing as it turned out in our study as the strain with the highest growth rate reached one of the lowest final biomass. Out of 15 strains of tested green algae the highest growth rates (d^{-1}) were noted for BA-46 (0.85 d^{-1}), BA-165 (0.81 d^{-1}), BA-2 (0.71 d^{-1}), BA-5 (0.71 d^{-1}) while the lowest for BA-1 (0.34 d^{-1}), BA-172 (0.34 d^{-1}) and BA −179 (0.34 d^{-1}) (Table 1). The final highest DW on the 30th day (Table 1, Fig. 1) was accumulated by i.e. BA-2 (552.6 mg L^{-1} DW) and BA-165 (520 mg L^{-1} DW), which coincided with their high growth rate values, i.e. 0.74 d^{-1} and 0.81 d^{-1}, respectively. The strain BA-46, an expected efficient biomass producer based on its high growth rate value (0.85 d^{-1}), reached only 419.1 mg L^{-1}DW, whereas BA-172 produced 519.1 mg L^{-1}DW biomass despite its low growth rate (0.34 d^{-1}). Optical density monitoring (750 nm) is a convenient indirect measure of biomass concentration, however it can lead to misinterpretation if not verified by other methods, like used in this work-biomass yield (Griffiths et al., 2011). Agustí (2005) noticed that larger cells have a lower growth rate but in the later phase they are not limited as much by self-shading as fast-growing small cells. The rate of growth correlated with cell size which has been observed in our previous study (Klin et al., 2018). Bigger size cells of strains such as *Oocystis* genus (92–129 μm^3) grew slower (0.24–0.35 d^{-1}) in comparison to small-size strains such as BA-12 (~18 μm^3) reaching 0.5 d^{-1}. This could explain the large final biomass of strain BA-172 despite its slow cell division.

3.1.2. Proteins

Proteins, as a structural material in plant cells, perform various functions: are the basis for cell growth, enzymatic reactions and build light-harvesting pigments (Kim et al., 2016). There are also free form proteins found inside the cells and the proteins quantity and composition is species-specific.

In this study, the percentage content of proteins in DW was strictly dependent on the strain, but in most cases, it had a downward trend with the aging of a culture, which could be explained by the depletion of nutrients (mainly nitrogen) in the medium. Regarding all strains, protein content ranged from 2% in BA-12 to 42% DW of maximum values observed in BA-6 (Fig. 1). There was a rapid decrease in the share of proteins in biomass in the last days of culturing for BA-1 (to 13%), BA-17 (to 13%), BA-18 (to 4%), and BA-20 (to 9%) with a simultaneous increase in carbohydrates or lipids. On the other hand, a continuous accumulation of proteins in biomass for the BA-80 strain was observed up to 24% on day 30th with only slight increase in lipids and mediocre in carbohydrates. Typical values observed here (around 15–20% of DW) in active growth phase were relatively lower than some listed in literature for green algae e.g. *Chlorella pyrenoidosa* 57%, *C.*
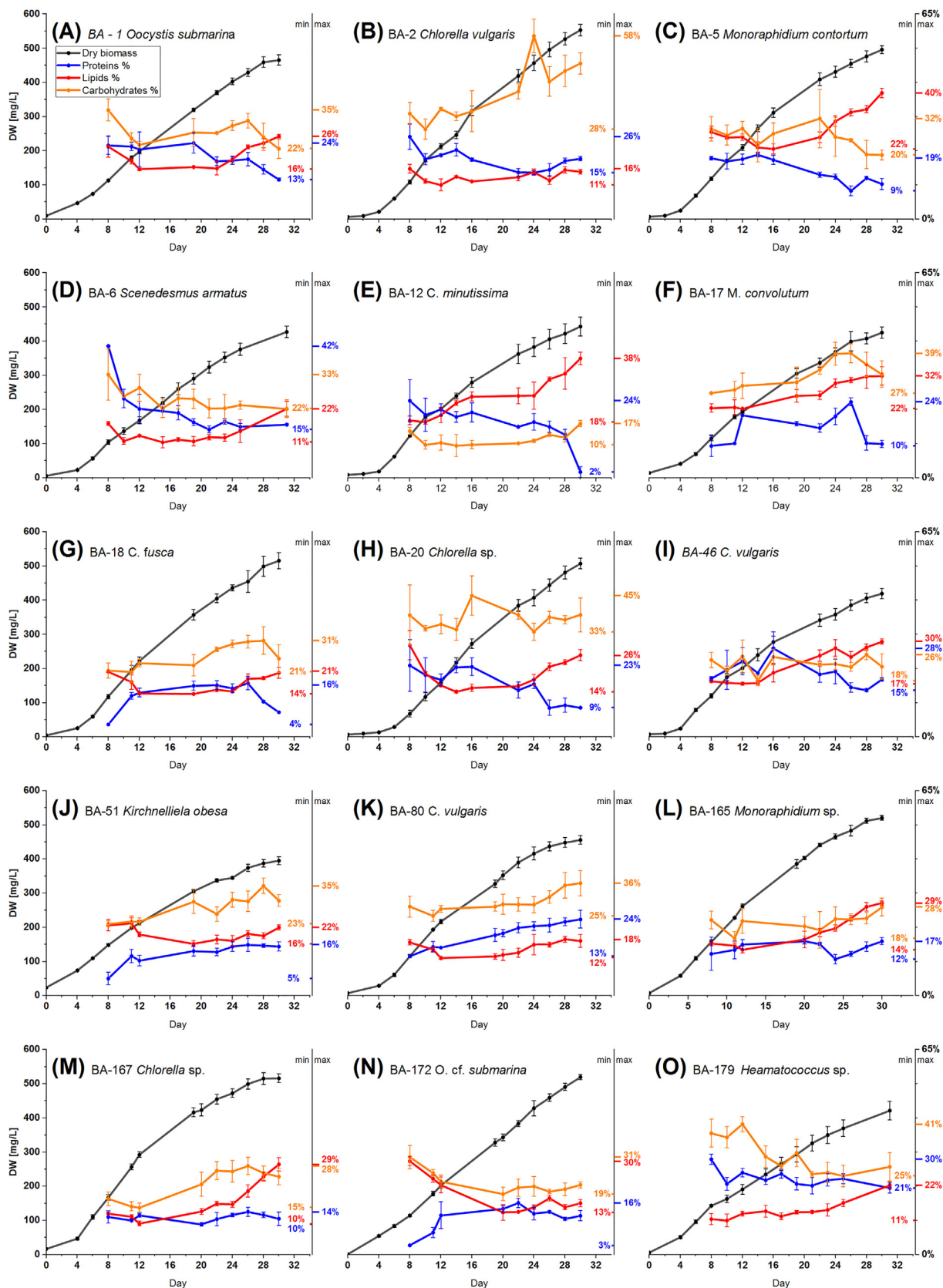
Fig. 1. Dry biomass (starting from initial day) and percentage share of lipids, carbohydrates and proteins during 30 days culture (starting at day 8th). Values present at the right scale represent minimum and maximum percentage share of biomass compoments during whole culture period. Data shown as mean ± SD, n = 3.
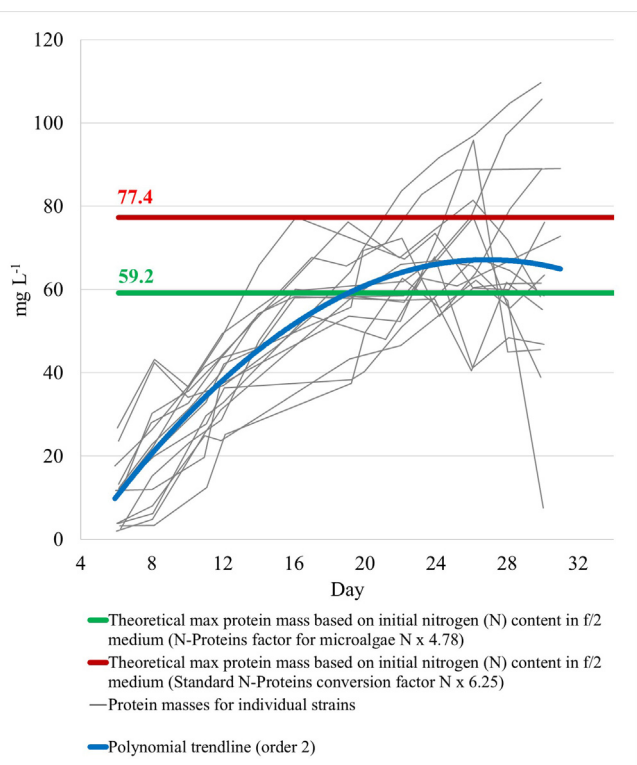
Fig. 2. Trend of changes in protein mass over time for all strains. The maximum theoretical masses of proteins resulting from the amount of N in the f/2 medium were marked. Data based on mean mass $\pm$ SD, n = 3.

vulgaris 51–58% (Becker, 2007), *Scenedesmus* sp. 47.75% (Pancha et al., 2014). Crisóstomo-Vázquez et al. (2016) drew attention to the fact that much information in literature on protein content is based on the estimation of total elemental nitrogen in biomass which may lead to its overestimation. Nucleic acids, amines, glucosamides, and cell wall materials also contain nitrogen but would not make dye-protein complex in colorimetric methods (Becker, 2007).

In batch type cultures (based on dH_2O) the single source of nutrients (including the required for protein synthesis - nitrogen) is their initial addition to water in the form of salts. In this study, a f/2 medium with a sodium nitrate content of 75 mg L^{-1} was used. Nitrogen constitutes 16.5% of weight of $NaNO_3$ salt, which gives 12.4 mg of elementary nitrogen in the litre of the medium. As the salts concentrations is known, it allows estimation of the theoretical maximal mass of proteins in case all nitrogen was used by microalgae cells for the synthesis of biomass. Fig. 2 shows the actual production of proteins by all strains during culture along with theoretical maximal mass of proteins,

calculated using two N to proteins conversion factors: the standard conversion factor (6.25) and factor proposed by Templeton and Laurens (2015) formulated for microalgae (4.78). As indicated by the trend for all strains, active protein synthesis proceeds until about the 20th day of culture and stabilizes at protein mass at about 70 mg L^{-1}. This value is intermediate to the theoretical values for the standard conversion factor (77.4 mg L^{-1}), described in the literature as likely to be overestimated, and for the conversion factor for microalgae (59.2 mg L^{-1}) (Templeton and Laurens, 2015). Achieving a mass of proteins containing all the nitrogen added in the nutrient solution and stabilization of protein synthesis on the 20th day indicates the depletion of free nitrogen in the medium and the initiation of physiological stress caused by its deficiency. This is also demonstrated by rapid metabolic changes after the 20th day and the rearrangement of the percentage content of the storage materials in DW, as described in the Section 3.1.4.

3.1.3. Photosynthetic pigments

The content of photosynthetic pigments was strongly dependent on the phase of cultivation. In a few cases, (*C. vulgaris* BA-2, *M. contortum* BA-5, *Chlorella* sp. BA-20), the high content of photosynthetic pigments in the early growth phase appeared to be preceding later high total productivity of the storage materials. Among all tested strains, three i.e. BA-2, BA-5 and BA-20, were characterized by the highest concentration of chlorophyll *a* and carotenoids (Fig. 3). The concentration of pigments, both chlorophylls and carotenoids as well, decreased as the culture went into the late growth phase, in agreement with Ruivo et al. (2011). Chlorophyll *a* is a nitrogenous macromolecule and cells grown in media with sufficient concentration of nitrogen contain high amount of this pigment. However, when the nitrogen availably is decreasing, chlorophyll *a* is readily used as intracellular nitrogen source to further support biomass production e.g. (Dean et al., 2010; Ruivo et al., 2011). As a result, nitrogenous macromolecules (including chlorophylls), proteins and peptides are transformed into storage materials, i.e. lipids and carbohydrates (Chen et al., 2017). The results obtained in this study further support this notion as the strains with the highest content of pigments were also characterised by the highest amount of storage materials (BA-2 64%, BA-5 60%, BA-20 64%) (Fig. 3).

3.1.4. Lipids, carbohydrates

Lipids (mostly triacylglycerols) serve as the major storage component for fatty acids in eukaryotic cells, and thus for energy reservoir molecules. Their content in DW depends mainly on the strain's metabolic pathways but is further altered by growth phases of the culture and exposure to stress conditions like nitrogen depletion which is currently the most widely studied phenomenon (Adams et al., 2013; Schwenk et al., 2013). The dynamics of lipids content were similar for the vast majority of strains; however, their attained minima and maxima were characterized by significant differences. Strains BA-5, BA-12 and BA-17 showed the highest lipid content 22–40%, 18–38% and
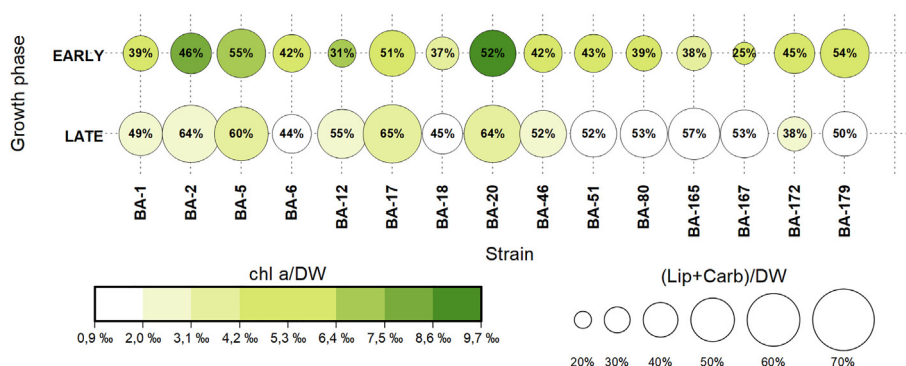

Fig. 3. Concentration of chlorophyll *a* and percentage share of total lipids and carbohydrates in dry biomass (DW) in early and late phase of growth. Data based on mean DW, mass of chlorophyll *a*, lipids and carbohydrates at day 6th and 30th, n = 3.

17–30% DW, respectively, while in the BA-2 strain lipids content ranged from 11 to 16% DW. For the majority of strains (13/15) a certain pattern consisting of three phases of biochemical characteristics was observed (Fig. 1).

- The early phase occurred between 8 and 12th day during which the lipids content (expressed as a percentage of DW) was constant or slightly decreased before reaching the next phase. Hodgson et al. (1991) also noted elevated levels of lipids in the early phase of cultivation and explained this by the ongoing photosynthesis despite the inhibition of cell division after inoculation into a new medium.
- The middle phase (day 12–20th) was characterized by the constant or slightly increasing lipids content. This phase coincided with the phase of active growth and it seemed to represent an optimal physiological state of cells without stress factors and a lipids content characteristic for the species under non-limiting conditions.
- Finally, the late phase (22–30th day) when lipid content gradually increased and reached its maximum concentration indicating that cells responded to nutrients depletion.

The remaining two strains were characterized by a relatively stable percentage of lipids in DW throughout whole (BA-2) or after a marked lipid decline in the initial phase cultivation period (BA-172). In the first case, this was related to the carbohydrate metabolism, which is discussed further while in the second case, probably the late phase was not reached, as shown by the continuous steep growth line in the last days of culture (Fig. 1).

Schwenk et al. (2013) examined the changes in lipid contents in some Baltic microalgae strains covering various taxonomic groups. The percentage lipid content for green algae was within the range of ca. 6% (logarithmic phase)–35% (stationary phase) lipids in dry biomass, which was also observed here. The dependence of the lipid content on the availability of nutrients has also been confirmed.

Carbohydrates are another group of energy-rich molecules closely linked to lipids metabolism. Green algal carbohydrates consist mostly of starch stored in chloroplasts and cell wall material – cellulose (lignin free). As lipids, starch accumulation is usually triggered by nutrients depletion (Hanifzadeh et al., 2018). However, studies have shown that one type of energy-rich molecules is usually preferred at the expense of the other as the most important precursor for triacylglycerol synthesis is glycerol-3-phosphate, a catabolite from glucose (Ho et al., 2012; Siaut et al., 2011).

In the studied cultures, with one exception (BA-12, carbohydrates 10–17%, lipids 18–38%), carbohydrates content was constantly higher than lipids content and its peak values was reached in early and/or middle phase (day 8–20th) (Fig. 1). The percentage of carbohydrates for most strains ranged from 10% to 58% DW. In contrast to lipids, in the late phase carbohydrates in most cases remained stable or began to decline (BA-1, BA-5, BA-17, BA-18, BA-51, BA-167) with few exceptions that showed rapid (BA-2) and slight increase (BA-12, BA-80, BA-165). This data showed the general preferences of examined strains towards lipids, but also clearly indicated those investing in starch: BA-2 (28–58% DW), BA-20 (33–45% DW), and BA-179 (25–41% DW).

The carbohydrates content determined for the studied strains were like those observed by other authors, e.g. *C. vulgaris* 51% (Illman et al., 2000), *S. obliquus* 51.8% (Ho et al., 2012), *C. vulgaris* 52%, *Scenedesmus* sp. 45.74% (Pancha et al., 2014).

3.1.5. Lipids and carbohydrates volumetric productivity (P_V)

The average daily production of lipids for 15 strains was 4.9 ± 1.4 mg and 6.6 ± 2.8 mg per liter for carbohydrates. However, the average value is the result of high deviations during culture and it is not a reliable base for inference. Fig. 4 presents fluctuations of lipids and carbohydrates volumetric productivity (P_V) values for 5 selected strains (highest TMP) based on lipids/carbohydrates mass changes between two consecutive time points of culture (ca. 2-days periods) and
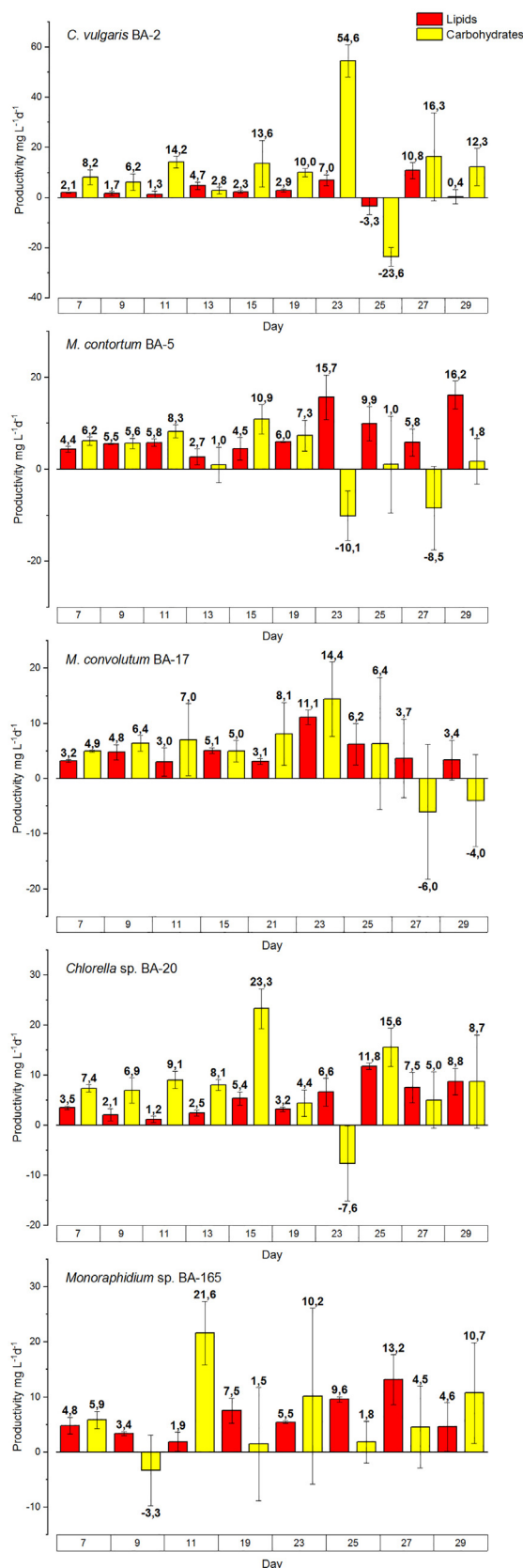

Fig. 4. Lipids and carbohydrates volumetric productivity summary for 5 selected strains based on 11 two-day periods. Data based on mean mass of lipids and carbohydrates between two consecutive culture points (ca. 2-days periods) ± SD, n = 3.

averaged to mg $L^{-1}d^{-1}$. Until 21/23th, the daily P_V of storage materials was relatively low due to culture still being in the phase of active growth and at relatively low density of biomass per volume unit. During this period, the P_V of carbohydrates over lipids was higher as starch serves as an efficient source of energy during growth. With cultures entering the late phase, productivity increased, due to the higher number of cells contained in a volumetric unit but was also accompanied by a shift in metabolism towards the accumulation of storage substances caused by the depletion of nutrients. As mentioned above, in the later phase the differences between strains in the final content of storage materials sharpen and thus: BA-2 produces carbohydrates throughout the period and intensifies the production at a later phase; BA-5 produces both forms of energy and intensifies lipids production at the expense of carbohydrates (negative productivity value). The case of strain BA-17 shows yet another mechanism, i.e. the rapid accumulation of storage substances around 23rd day, after which lipids production decreased and carbohydrates were broken down. The BA-5 strain, before it was determined with the highest lipid productivity, 16.2 mg $L^{-1}d^{-1}$, also showed a production rate almost three times lower only 4 days earlier whereas BA-2 increased carbohydrates 5.5 times during the same period. It allows concluding that in order to know the true potential in the production of a given storage compound, a culture should fully response to physiological stress (as the depletion of nutrients) after the initial optimum growth and increase in the density of biomass. Additionally, the daily volumetric productivity, especially for carbohydrates, (Fig. 4) shows high fluctuations in the period of adaptation to the conditions of nutrient deficiency. Therefore, too early termination of culture may lead to not capturing an important change in the metabolism of storage compounds.

The problem of calculating volumetric lipids/carbohydrates productivity has been comprehensively addressed by Xu and Boeing (2014). One of the author's conclusions appropriable in this discussion is that it is impossible that an isolated population synchronously reach maximum biomass productivity and maximum lipid content. Therefore, the use of the traditional formula ($P_V = \mu \times Q$, where P_V is the volumetric productivity [mg $L^{-1} d^{-1}$], μ–growth rate [d^{-1}], Q–lipids/carbohydrates yield [mg L^{-1}]) is burdened with a high error. In this work, numerous data from consecutive days of the culture allow to learn the productivity for different periods of culture that account for the overall, average productivity. As has been shown, it is likely to observe a radical increase in the value of lipids productivity at the stage of transition of the culture to the late stage of growth. The density of biomass at the period of passing to the late phase is dictated by the initial amount of nutrients available in the medium (shown in Section 3.1.2). The use of richer medium (e.g. BG-11 containing 1500 mg L^{-1} $NaNO_3$ versus f/2 75 mg L^{-1} $NaNO_3$ used in this study) could positively affect the maximum cell density (so-called "carrying capacity"), thereby increasing their P_V. However, for some strains, high macroelements concentrations can act inhibitive and introduce an undesirable stress factor (Andersen, 2005). Also, in highly dense cultures, the previously mentioned phenomenon of cell self-shading may be an additional factor influencing the rate of growth phase transformations (Hewes, 2015). Since the aim of this work was to compare the course of undisturbed growth of strains in optimal conditions in the sense of nutrient availability, the f/2 medium has been chosen due to optimal range of nutrients concentration for green algae. The listed problems resulting from the lack of data on lipid mass at various culturing points forcing to use standard P_V formula and the number of combinations of culturing conditions (e.g. medium), direct comparison of values among other studies was considered invalid, so it has been limited to the strains grown under equal conditions in this study. This approach based on raw lipids and carbohydrates mass data also drastically reduces the probability of error resulting from the use of the model or formulas listed in reviews (Griffiths and Harrison, 2009; Xu and Boeing, 2014).

3.2. Theoretical methane potential (TMP)

3.2.1. TMP% based on biochemical compounds percentage shares

The theoretical methane potential (TMP) is widely recognized as an indication of the maximum methane production expected from a specific substrate (Klassen et al., 2016). If the exact substrate composition is known, methane production can be predicted by a stoichiometric equation, balancing the total conversion of the organic material to CH_4 and CO_2 under anaerobic conditions. However, often the exact atomic composition of the feedstock is unknown, and only the approximate content of the main organic compounds is known. Mohamed et al. (2018) examined three theoretical models for obtaining fast TMP test results; (1) the BMPthAtC model which uses empirical relationships based on the chemical composition of the substrate, (2) the BMPthCOD model based on the Chemical Oxygen Demand (COD) of the substrate and (3) the BMPthOFC model, used in this study, which uses the percentages of the various compounds in the substrate (carbohydrates, lipids and proteins). On the basis of tests carried out on different substrates, it was shown that the BMPthOFC model is more precise (error between 4 and 7%) than BMPthAtC and BMPthCOD models, respectively (error between 5 and 15%).

Table 2 contains all calculated TMP% values per gram of DW based on percentage share of biochemical biomass components. It should be noted that this is the value per unit of biomass without considering the growth rate of the given strain and therefore its productivity in time. This summary showed differences between strains and allowed for distinguishing the growth stage corresponding to the maximum methane yield. TMP values were most strongly correlated with the content of lipids that have the highest energy charge, and thus they were considered as the most suitable fermentation substrate (Gonzalez-Fernandez et al., 2015). A good example is the BA-167 strain which TMP% showed the lowest value among all tested strains, i.e. 0.26 L CH_4g^{-1}TS in the initial phase of cultivation. This was caused by a low lipid content constituting only 10% of the dry biomass. Together with the increasing lipid content in the next phases, the value of TMP% also increased reaching 0.44 L CH_4g^{-1}TS. A similar trend was noted for the strain BA-165 with an even higher TMP% value of 0.49 L CH_4g^{-1}TS at the end of the culturing period. The BA-5 strain biomass characterized by the highest lipid content (22–40%) maintained the expected high potential methane yield (on days 8th and 30th, 0.48 L CH_4g^{-1}TS and 0.54 L CH_4g^{-1}TS, respectively) with a slight decrease in the middle

Table 2
The theoretical methane potential (TMP% L CH_4g^{-1}TS) values based on lipids, carbohydrates and proteins percentage content in dry biomass (DW) for each day of culture. Red color indicates low, while green the highest TMP% values. Data based on mean percentage share of lipids, carbohydrates and proteins in DW at each day, n = 3.

Day ↓	Strain : BA -														
	1	2	5	6	12	17	18	20	46	51	80	165	167	172	179
8	0.48	0.42	0.48	0.51	0.35	0.37	0.32	0.51	0.37	0.34	0.31	0.33	0.26	0.44	0.41
10	0.39	0.32	0.45	0.36	0.31	0.36	0.32	0.43	0.37	0.39	0.32	0.28	0.23	0.38	0.36
11	nd	0.35	0.47	0.36	0.34	nd	nd	0.39	0.36	nd	0.30	nd	nd	nd	0.42
12	0.36	0.37	0.42	0.30	0.37	0.37	0.30	0.38	0.36	0.34	0.33	0.32	0.22	0.38	0.37
16/19	0.38	0.35	0.42	0.32	0.40	0.44	0.30	0.44	0.37	0.37	0.34	0.35	0.27	0.27	0.35
22	0.36	0.37	0.46	0.30	0.38	0.45	0.34	0.39	0.38	0.35	0.35	0.34	0.32	0.30	0.37
24	0.40	0.46	0.48	0.28	0.39	0.55	0.34	0.40	0.44	0.37	0.38	0.36	0.33	0.30	0.34
26	0.45	0.37	0.49	0.31	0.45	0.58	0.39	0.42	0.44	0.39	0.39	0.41	0.38	0.32	0.36
28	0.42	0.43	0.50	0.32	0.45	0.52	0.36	0.44	0.46	0.41	0.43	0.46	0.41	0.29	0.38
30	0.41	0.44	0.54	0.38	0.46	0.51	0.34	0.46	0.45	0.41	0.43	0.49	0.44	0.31	0.43

phase of culturing (0.42 L $CH_4g^{-1}TS$). Carbohydrates accumulating strains (BA-2, BA-20, BA-80 and BA-179) due to the lower energy value of sugars also had a proportionally lower maximum methane potential (0.31–0.51 L $CH_4g^{-1}TS$). Finally, the highest value of $TMP^\%$ (0.58 L $CH_4g^{-1}TS$) was calculated for BA-17 strain around day 26th (lipids 32%, carbohydrates 39% and proteins 24%) just before fast decline to 0.51 L $CH_4g^{-1}TS$ in the last 4 days of culturing along with the decrease (24% to 10%) in previously high protein content. Observing $TMP^\%$ values with the case of the *M. convolutum* BA-17 strain as an example shows that if lower percentage share of proteins in dry biomass isn't obligatory for optimal fermentation it is worth monitoring and terminating the cultures at the right moment of peak $TMP^\%$'s value. It reached the highest value (0.58 $CH_4g^{-1}TS$) 4 days before the end of culture, and gradually decreased as the proteins were decomposed. If the highest possible C/N ratio in the substrate is recommended, it is worth prolonging the culture and thereby increasing the share of carbon, and reducing nitrogen share through proteins breakdown. Taking this perspective, biomass rich in carbohydrates and lipids would have been preferred for effective fermentation rather than TMP potent protein-rich biomass.

Due to various extraction and quantification techniques as well as the culture period of sampling from the culture, direct comparison of microalgal species between authors happens to be questionable (Laurens et al., 2012). Also, Heaven et al. (2011) draws attention to the proper identification of a type of solids (total solids TS or volatile solids VS) as this information is occasionally missing in publications. The sum of lipids, carbohydrates and proteins reaching 95% (and above) of dry matter stays in conflict with reports providing information on the ash content in dry biomass of green algae which is about 5–18% of dry matter (Liu et al., 2015). The total share of the three biochemical components in biomass should be taken into account as it informs about the complementary part of the biomass which is not attributed to any of the tested classes of compounds, but might represent even about 20% of DW (BA-5, 29% total share). The strains tested in this study are distinguished by an expected total share of organic biomass components not exceeding 95% of dry biomass.

3.2.2. *TMP^g based on biochemical compounds actual masses produced*

The TMP^g values in Fig. 5 were calculated based on the actual mass of lipids, carbohydrates and proteins produced at the final 30th day of culturing for each strain. It represents the theoretical maximum volume of biomethane which would be yielded if the biomass was transferred directly to fermentation and fermented completely. Three strains BA-5, BA-165 and BA-2 revealed the highest TMP^g values 0.267 L $CH_4g^{-1}VS$, 0.255 L $CH_4g^{-1}VS$ and 0.244 L $CH_4g^{-1}VS$, respectively. Their growth rates were high (Table 1) and biochemical profile shifted towards storage materials (Fig. 1). BA-5 produced high quantity of lipids (L-198 mg L^{-1}; C-105.5 mg L^{-1}; P-55.2 mg L^{-1}), BA-165 medium–high quantity of three of components (L-152.5 mg L^{-1}; C-146 mg L^{-1}; P-

89.6 mg L^{-1}) while BA-2 was the most efficient producer of carbohydrates (L-82.8 mg L^{-1}; C-272.8 mg L^{-1}; P-105.7 mg L^{-1}). It showed that the *C. vulgaris* BA-2 producing low-calorie substrate for fermentation, i.e. carbohydrates (max 58% DW), produces it efficiently enough to approach TMP^g value of the parallel growing *M. contortum* BA-5 containing twice more calorific fuel – lipids constituting up to 40% of DW. These strains could provide an equally productive source of two opposite types of energy substrates (lipids and carbohydrates) and at the same time provide a basis for interesting studies on the influence of their proportions on the efficient fermentation process (BMP tests).

4. Conclusions

The review of growth rates and the spectra of biochemical changes in biomass of green algae during long-term cultures provided valuable data for the evaluation of microalgal biomass as a stand-alone substrate for anaerobic digestion. Characteristic metabolism features of energetic compounds (lipids and carbohydrates) in individual strains were revealed. Combining fluctuations of the TMP index with the high volumetric productivity of energy compounds initiated by the stress factor enables the planning cultivation of biomass designed for its efficient decomposition into biomethane. The results provide guidance for deliberate BMP tests further exploring the potential of microalgal biomass.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Author contributions

All authors contributed to the preparation of the manuscript: Marek Klin (klin.m@outlook.com) was responsible for experiment concept and design, conducting biochemical analysis as well as interpretation of the data. Filip Pniewski critically revised the article for important intellectual content and linguistic quality. Adam Latała provided biological materials, substantive guidance and approved final form of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
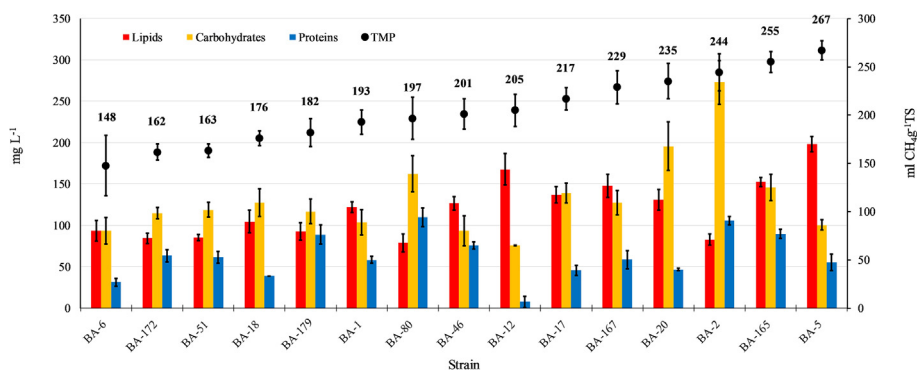
Fig. 5. Theoretical methane potential (TMP^g ml $CH_4g^{-1}VS$) values calculated on mass of lipids, carbohydrates and proteins at final 30th day of culture for each strain. Data based on mean mass $\pm$ SD, n = 3.

Department of Marine Ecosystem Functioning at the Gdansk University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.122875.

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