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Abstract

Polyhydroxyalkanoates (PHAs) are promising alternatives that accumulate as energy and carbon storage material in various microorganisms, including bacteria and microalgae, being biodegradable and suitable for a wide variety of applications. Among these compounds, the most prevalent and well-characterized biopolymer is polyhydroxybutyrate (PHB), which belongs to the short-chain PHAs. The present study was designed to evaluate algae-based PHB production in two Chlorophyta (*Desmodesmus communis* and *Chlorella vulgaris*) under a two-phase nutritional mode of cultivation, namely a phototrophic growth phase (PGP) and a mixotrophic stress phase (MSP) with N,P-depleted media and organic carbon supply (i.e., glucose or sodium acetate, NaOAc). The highest PHB productivity (0.11 g PHB/g biomass/d; 0.015 g PHB/L/d), corresponding to 32.1% w/w of intracellular PHB, was observed for *D. communis* after 3 days of cultivation under mixotrophic conditions in batch cultures (e.g., low light, phosphorus-free medium, 1 g/L of NaOAc). A scaled-up cultivation (10 L) was set up to evaluate for the first time PHB yields and biomass composition in a semi-continuous system. A PHB content of 34% w/w was achieved on day 8, corresponding to a maximum PHB productivity of 0.10 g PHB/g biomass/d (or 0.011 g PHB/L/d), which increased up to 54% w/w on day 15. The biomass was composed of about 30% w/w proteins, 6% w/w polysaccharides, and 11 % w/w lipids, which can be valorised from a biorefinery perspective. The scaled-up *D. communis* cultivation in 10 L PBRs confirmed the potential utilization of this algal species for PHB production with productivity up to 2-times higher than those reported for several cyanobacterial species and similar to the maximum value obtained with batch cultures in previous works performed with Scenedesmaceae.

Keywords

Desmodesmus communis; polyhydroxyalkanoates; algal biomass; microalgal cultivation; mixotrophy.

1. Introduction

Microalgae are considered promising organisms for various biotechnological applications in the function of metabolic characteristics that make them an important source of compounds to be explored through sustainable processes [1]. Nowadays, research is focused on the production of bio-components for biomaterials, due to the reduced economic attractiveness of other sectors (i.e., cosmetic and nutraceutical industries), where a biorefinery strategy has been proposed to improve the feasibility and sustainability of the processes [2,3]. Since the type and amount of the synthesized compounds are determined not only by the organism itself but also by chemical, physical and biological factors that can be fine-tuned and, consequently, influence the growth and the synthesis of the cell constituents [4], the selection of the cultivation mode and the producing organism is essential. Recently, microalgae have been proposed as a potential biomass source for sustainable bioplastic or biopolymers production, such as starch and polyhydroxyalkanoates [4–8]. Polyhydroxyalkanoates (PHAs) are a family of natural polyesters which possess various thermoplastic and elastomeric properties [9]. PHAs are produced intracellularly as carbon and energy storage by a wide variety of photosynthetic and heterotrophic organisms (i.e., bacteria, cyanobacteria), resulting in biobased and biodegradable polymers. The degradation of these biopolymers occurs in soil, compost, and marine sediment, and depends on various factors, such as temperature, pH, moisture, microbial activity, exposed surface area and molecular weight of the compound itself [10]. In addition, PHAs are non-toxic, not soluble in water, and have good resistance to UV light)[9]; even if PHAs currently cover niche applications in the plastic market, they could be good substitutes for conventional plastics with the potential to be used in a wide range of applications like packaging, medicine, or agriculture, in particular in the specific tailored cases in which leaving a biodegradable plastic in the environment (soil or water), where it carries out its function, is unavoidable (i.e. mulch films or fishing nets [11]).

Bacteria are efficient PHA-producing organisms; thus, their commercial exploitation is widespread using continuous fermentation processes which provide controlled conditions, high productivity, and uniform product quality, along with low investment costs [12]. Different bacterial species can store PHAs within the cytoplasm in granules ranging in size from 0.2 to 0.5 μm [9], some of which, such as wild type or genetically modified *Cupriavidus necator*, *Bacillus* sp., *Alcaligenes* sp., *Azotobacter* sp., and *Pseudomonas* sp., are commercially exploited [13–16]. The PHAs microbial production needs the addition of an organic substrate to the medium. Indeed, the chemical composition of the resulting polymer depends on the latter and can be manipulated by varying the carbon source [17].

Commercialization and industrialisation of PHAs are difficult because they are three-times more expensive than conventional plastics [7]: the cost of the carbon source used for feeding PHA-accumulating bacteria contributes significantly to the final PHA price (up to 30%), accounting 70-80% of total raw material cost [18], as well as the downstream phase for recovering PHA from microbial biomass that can contribute up to 50%[19]. The use of waste can abate the costs related to the use of the raw material, as well as the use of recyclable solvents/additives for PHA extraction (e.g., [20–23]). Photosynthetic organisms, such as cyanobacteria and microalgae, can also produce PHAs as a response to nutrient deficiency (i.e., nitrogen, phosphorus, [6,8,24]) and in the presence of a carbon source, which can be obtained also from wastewater [20,25]. PHAs are naturally present in microalgae, even if in a lower amount than that of bacteria [7,17,26]. However, this concentration can be increased under suitable culture conditions that are strain specific. Furthermore, microalgae boast rapid growth, low space and water requirements and the capability to use sunlight as an energy source [7]. Most of the studies performed with various microalgae strains concentrates on altering growth conditions to maximize and optimize polyhydroxybutyrate (PHB) production, which is the most studied, commercially exploited, and well-characterized short-chain homopolymer of PHAs (e.g., [17]; and other studies in Table 1). Significant factors that contribute to the accumulation of PHB in microalgae biomass are: presence of organic carbon (such as acetic acid, pentoses, etc., [7,27], the photoperiod and light exposure [28], limitation of nitrogen and phosphorus [17,29–31], limitation

of certain heavy metals (Ni and Cu) and dissolved gas transfer resistance in the culture [31]. So far, about 100 strains of eukaryotic and prokaryotic (cyanobacterial) microalgae have been identified as capable of photoautotrophically accumulating PHB (see [32] and studies in Table 1). Considerable quantities have been found in several cyanobacterial or algal species, e.g., *Synechococcus subsalsus* (16%) and *Spirulina* sp. (12%) [30]; cf. *Anabaena* sp. (up to 46%) [33]; recombinant *Phaeodactylum tricornutum* (11%) [34]. Most of the studies concern cyanobacteria, while more recent papers investigate the production and identification of PHAs in Chlorophyta (Table 1). Among tested species, *Chlorella* sp. [35–37] and *Scenedesmus* sp. [17] achieved the best concentrations (27-29% w/w). Both genera are of great commercial interest due to their resistance to harsh environmental conditions; moreover, these species are commonly exploited for a wide number of industrial applications (i.e., food, nutraceutical, bioremediation), and have been extensively studied [38,39]. Additionally, they could grow using wastewater as a source of nutrients (e.g., anaerobic effluents from digested wastewater), lowering environmental footprint and production costs [40,41]. Among Scenedesmaceae, *Desmodesmus communis* has great potential either for high protein [42] and bioactive compounds accumulation [43] or in wastewater treatment processes [44,45].

In the present work, the chlorophytes *Chlorella vulgaris* and *Desmodesmus communis* were tested as PHB-producing organisms. *D. communis* was selected to be scaled-up in batch and semicontinuous systems, to understand whether this species could be exploited in industrial biorefining processes aimed at a microalgae-based PHB production.

2. Materials and methods

2.1. Microalgae isolation and identification

Two different chlorophytes were chosen and tested for the first time for PHAs production due to their growth performance and resistance to different culture conditions as attested in previous studies. *Desmodesmus communis* strain used in this study was isolated from a freshwater pond in the province

of Forlì-Cesena (Emilia Romagna, Italy) in February 2009 and identified at the species level as previously described [44].

Chlorella vulgaris (strain CCAP211/11B) was isolated in Delft (Holland) and was purchased from the Culture Collection of Algae and Protozoa (CCAP).

2.2. Experimental design

2.2.1. PHB production by chlorophytes

A first set of experiments aimed to select the growth factors for boosting PHB production by *D. communis* and *C. vulgaris*, focusing on the lack of nutrients (nitrates and/or phosphates), on the addition of a carbon source at different concentrations (1 or 2.5 g/L NaOAc), and on the growth period (2, 4, 7 days). Each strain was grown in a two-phase nutritional mode of cultivation, namely a phototrophic growth phase (PGP) for optimizing the inoculum, and a mixotrophic stress phase (MSP). In the PGP the batch cultures (800 mL in 1 L flasks) were grown with a modified CHU13 medium [46] at 20±1°C and continuous aeration with filtered (0.22 µm) air from the bottom of the flasks at a flow rate of about 150 mL min^{-1}, with a 16 h light and 8 h dark photoperiod and light at about 200 µmol m^{-2} s^{-1}, and without pH control. In the MSP phase, the batch cultures (100 mL inoculum and 700 mL medium to have an initial biomass concentration of about 0.05 g/L) were grown without nitrates, phosphates or both nutrients, and with the addition of 1 or 2.5 g/L of NaOAc; the cultures were kept at 20±1°C with a 16 h light and 8 h dark photoperiod and provided with low light at 20-30 µmol m^{-2} s^{-1} and continuous aeration with filtered (0.22 µm) air at a flow rate of about 150 mL min^{-1}. Culture growth was followed as dry weight (DW) and maximum quantum yield for the evaluation of the photosynthetic efficiency (PAM fluorometer). After 2, 4 or 7 days, aliquots of the culture (200 mL) were centrifuged, and the resulting pellet was stored at -20°C for PHB analysis. The nutritionally balanced CHU13 medium was used as a control.

2.2.2 PHB production by *D. communis* using different carbon sources

To find the best conditions to optimize PHB production in the MSP phase, *D. communis* was selected and grown testing the effect of different carbon sources (1 g/L glucose or NaOAc) and growth times (3, 6 or 8 days) in a modified CHU13 medium without phosphates. Cultures (800 mL in 1 L flasks) were prepared in triplicates with an initial biomass concentration of about 0.07 g/L and cultivated as in the first experiment. DW, maximum quantum yield and PHB production were evaluated.

2.2.3. Scaled-up fed-batch cultivation of *D. communis*

The scale-up of *D. communis* cultivation was performed with a fed-batch growth experiment using 10 L bubble column photobioreactors (PBRs). After the PGP phase, cultures were prepared in triplicates with an initial biomass concentration of about 0.05 g/L using 1 g/L NaOAc and a modified CHU13 medium without phosphates and cultivated as in the previous experiments. PBRs were kept at 20±1°C with a 16 h light and 8 h dark photoperiod, low light at 20-30 µmol m^{-2} s^{-1} and continuous aeration with filtered (0.22 µm) air at a flow rate of about 1 L min^{-1}. Culture dry weight (DW), maximum quantum yield, and the Chemical Oxygen Demand (COD) of the medium without algae were analysed. After 2, 3 or 10 days, aliquots of the culture (400 mL) were centrifuged, and the pellet was stored at -20°C for the analysis of intracellular PHB and biomass composition (proteins, polysaccharides, and lipids) at day 10.

2.2.4. Scaled-up semi-continuous cultivation of *D. communis*

The semi-continuous cultivation of *D. communis* was performed using the same conditions and reactors described in section 2.2.3. The growth was followed for 27 days. During cultivation, the algal biomass dry weight, maximum quantum yield, and COD of the medium without algae were monitored; after 12 days, NaOAc was restored (1 g/L), as it resulted as depleted. Part of the culture (2 L) was harvested every 3-4 days (days 4, 8, 12, 15, 19, 22 and 27), and replaced with NaOAc (2 g) and 2 L of fresh medium without phosphate. The harvested algal biomass was collected by centrifuge to determine PHB content and to characterize the biomass composition in terms of proteins, polysaccharides, and lipids (days 12 and 22), as well as for PHB characterization.

2.3. Algal biomass characterization

Algal biomass was estimated as dry cell weight (DW, g/L). Culture sub-samples were filtered onto pre-weighed glass fiber filters (Whatman GF/C, 1.2 µm pore size); the filters were then dried at 105°C for at least 1 hour or until constant weight [47].

Algal biomass dry weight was calculated following the equation:

$$DW\ (g\ L^{-1}) = \frac{W_t\ (g) - W_0\ (g)}{V\ (L)}$$

where W_t and W_0 are the weight (g) of the filter after drying and before sample filtration, respectively, while V is the volume of filtered culture (L).

Periodical algal cell observations were performed using an inverted optical microscope (Axiovert S 100) at 320x magnification.

Lipids were extracted from freeze-dried samples (100 mg) with a mixture of methanol (1 mL) and dichloromethane (2 mL) for 2 h at 50-60°C under magnetic stirring. The extraction was repeated three times, then the solvent phases were collected, centrifuged at 3000 × g for 10 min, and dried under nitrogen. After complete solvent evaporation, total lipids were measured gravimetrically [48].

Protein determination was performed on freeze-dried algal biomass (10–15 mg). The samples were extracted with 3.0 mL NaOH (0.5 M) and incubated at 90°C for 8 min under magnetic stirring, then transferred in ice for 2 min, and subsequently centrifuged (2550 × g, 10 min). The resulting supernatant was collected, and the extraction procedure was repeated three-times. Protein content was determined on the collected supernatant with the Folin phenol reagent [49].

Intracellular polysaccharides were extracted from freeze-dried samples (5–10 mg) [50] and quantified spectrophotometrically through the phenol-sulfuric acid colorimetric reaction [51].

2.4. Photosynthetic efficiency measurement

A pulse-amplitude modulated fluorometer (101-PAM connected to a PDA-100 data acquisition system and equipped with a blue high-power LED Lamp Control unit HPL-C; H. Walz, Effeltrich, Germany) was used to measure the maximum quantum yield of the PSII, as an indirect measure of photosynthetic efficiency, as previously reported [33].

2.5. Chemical Oxygen Demand (COD) analysis

The COD of *D. communis* cultures was measured by thermal oxidation at 1200°C with detection of the oxygen consumption using a COD analyser QuickCODLab (LAR Process Analyzer AG) following the ASTM D6238-98 method.

The culture filtrate obtained from the dry weight analysis was injected directly into the instrument, where it was completely oxidized at 1200°C under air/nitrogen flow and constantly analysed with an O_2 detector. The COD was calculated as grams of oxygen per liter by comparing the signal areas related to O_2 consumption with those of a known standard solution of glucose (1000-10000 ppm range).

2.6. *PHB analysis*

2.6.1. *PHB content*

PHB content in algal biomass was determined according to the procedure reported in the literature for bacterial PHB quantification named “in-vial thermolysis” (see [52]). Briefly, freeze-dried algal samples (10 mg) or standard PHB (1–2 mg; Biomer, Germany) were charged in screw-cap vials (4 mL volume, 50 mm high) and then placed on a heating plate at 350°C. At this temperature, PHB depolymerized into (E)-2-butenoic acid (i.e., crotonic acid) that was used as the molecular fingerprint of PHB for the quantitative analysis. After 20 min, the vials were removed from the heating plate and let cooling down to RT before adding the internal standard (2-ethylbutanoic acid, 0.1 mL of a solution 5000 ppm in acetonitrile). The sample was then diluted with acetonitrile (4 mL) and analyzed by GC–MS to quantify the amount of crotonic acid in each sample, and then the amount of PHB [52].

GC–MS analysis was performed using an Agilent 7820A gas chromatograph connected to an Agilent 5977E quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated on a DBFFAP polar column (30 m length, 0.25 mm i.d., 0.25 µm film thickness), with helium flow of 1 mL min^{-1}. Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 29–450 *m/z* range. The temperature of the column was set to 50°C (5 min) and increased to 250°C (10°C min^{-1}).

PHB content was expressed on biomass weight basis (g PHB/g biomass %). Specific PHB productivity (g PHB g $biomass^{-1}$ d^{-1}) was calculated based on the cultivation period (d) as previously reported [33] following the equation:

$$PHB\ productivity\ \left(\frac{g\ PHB}{g\ biomass\ d}\right) = \frac{PHB\ content\ \left(\frac{g\ PHB}{g\ biomass}\%\right)}{cultivation\ period\ (d)}$$

2.6.2. PHB extraction and characterization

Algal freeze-dried samples (100 mg) were extracted twice with dichloromethane (50 mL) for 2 h under reflux and magnetic stirring. The solvent phase was separated from the extracted biomass by centrifuging at 4000 rpm for 2 min, and then evaporated under nitrogen atmosphere. The extracted PHB was washed several times with acetone and then dried overnight at 40°C. PHB yield was calculated gravimetrically on algal biomass weight basis (g PHB/g biomass %).

The elemental composition of the extracted PHB was determined using an elemental analyzer (Thermo Scientific, Flash2000, Organic Elemental Analyzer) by means of the flash combustion technique.

2.7. Statistical analysis

Differences among samples were tested by multivariate analysis of variance (ANOVA) using PAST 2.17 software [53]. Levene's tests were performed to verify the homogeneity of the variance and

Tukey's tests were used for pairwise comparisons. Data are reported as mean values ± standard deviations of triplicates.

3. Results and discussion

3.1. PHB production in two chlorophytes

C. vulgaris was able to synthetize PHB at different growth conditions and PHB content increased over time (Fig. 1), but it never reached high percentages (< 10%). The highest PHB amount was obtained after 7 days of cultivation under phosphate depletion (ØP) and in the presence of 1 g/L of NaOAc (8.9%), then a progressive decrease in PHB productivity values was observed (Fig. S1).

On the other hand, *D. communis* accumulated a higher amount of PHB than *C. vulgaris* already from day 2 (11.4% with 1 g/L NaOAc, 10.4% with 2.5 g/L NaOAc), but only when grown in the ØP medium (Fig. 1). *D. communis* cultured in all the other conditions produced PHB in a lower amount than ØP medium. PHB productivity values resulted up to 3-times higher with *D. communis* than with *C. vulgaris* (0.022 vs 0.057 g PHB/g biomass/d with 1 g/L of NaOAc; 0.017 vs 0.052 g PHB/g biomass/d with 2.5 g/L of NaOAc) and decreased in time (Fig. S1), probably due to still non-optimized conditions. In most studies investigating the production of PHB by Chlorophyta, the highest percentages (3-30% w/w) were obtained after at least 14 days (see references summarized in Table 1), corresponding to a low PHB productivity.

As concerns the composition of the medium, promising results were observed when *C. vulgaris* and *D. communis* were grown in a ØP medium (Fig. 1), confirming that PHB biosynthesis by microalgae is promoted by a nutritional deficiency of key nutrients, as occurs for bacteria and cyanobacteria [32,54]. While nitrogen deprivation has been extensively investigated to boost PHB synthesis in both heterotrophic and phototrophic microorganisms, phosphorus limitation may also play a key role. Specifically, a lack of phosphorus could affect the balanced intracellular formation of ATP and NADPH, on which the PHB biosynthesis pathway depends [33,55]. Nevertheless, the increase in PHB due to the deficiency of a specific nutrient, or a combination of two or more, would appear to

be strain specific, as demonstrated, for instance, in an extensive study of over 130 strains of cyanobacteria [32]. Additionally, higher values of dry weight (Fig. S2) and photosynthetic efficiency (Fig. S3) were observed in *D. communis* cultures grown with the ØP medium than in other conditions, highlighting the importance of nitrogen to sustain algal growth and the mixotrophic nature of this species. Although nitrogen deprivation could trigger the synthesis of specific compounds in microalgae (e.g. lipids, PHB), this can result in a decline of the growth rate and of the biomass productivity, thus affecting the productivity yields of the target compounds [30]. Finally, no marked differences were observed between the treatment with 1 and 2.5 g/L of NaOAc; thus, considering that the use of an exogenous carbon source constitutes one of the main costs of the entire PHB production process [56] and that the use of a lower amount of substrate would reduce the cultivation costs, 1 g/L of NaOAc was selected for the subsequent tests.

3.2. *D. communis* batch cultures

To investigate the impact of the carbon source on PHB production, *D. communis* was cultivated in the presence of NaOAc and glucose and tested under the same conditions in batch cultures. Results shown in figure 2 demonstrated that NaOAc was a better carbon source than glucose to promote PHB production (ANOVA, $p < 0.05$), contrarily to what has been described for *Scenedesmus* sp., capable of accumulating 30% PHB when grown in the presence of 1 g/L of glucose under stressed conditions (low light, ØP medium, [17]). A possible explanation could rely on the fact that NaOAc, contrarily to glucose, can be directly used in the synthesis of PHB, which is closely linked to the glycolysis process [57].

As in the previous experiment, the PHB amount reached the highest percentage during the first days of cultivation (32.1 ± 0.7% NaOAc; 14.7 ± 2.8% Glu) and then decreased over time.

Pairwise comparisons evidenced significant differences between the two carbon sources at day 3 and among the cultivation periods for the samples treated with NaOAc ($p < 0.001$). These results confirmed the ability of *D. communis* to produce a relevant amount of PHB (> 30%) in a very short

time and the importance of identifying the peak of PHB production, which could vary among algal species and depend on growth conditions. Indeed, most studies on the production of PHB by Chlorophyta (summarized in Table 1) reported the peak of PHB production after at least 14 days of cultivation. Consequently, the PHB productivity of *D. communis* resulted higher with NaOAc than with glucose (Fig. 2), especially after 3 days of cultivation (0.11 ± 0.00 vs 0.05 ± 0.01 g PHB/g biomass/d, corresponding to 0.015 ± 0.005 and 0.016 ± 0.006 g PHB/L/d, respectively), and resulted comparable to the maximum value of 0.24 g PHB/L (corresponding to 0.017 g PHB/L/d) previously reported for *Scenedesmus* sp. after 14 days of cultivation under mixotrophic conditions [17]. Furthermore, PHB productivity obtained in the present work for an eukaryote alga resulted almost two-times higher than the one previously reported (i.e., 0.06 g PHB/g biomass/d) for the cyanobacterium cf. *Anabaena* sp. grown at low light, in a ØP medium and with the addition of NaOAc [33]. It is also important to note that even though the dry weight was higher in the samples grown with the addition of glucose (Fig. S4A), samples grown with the addition of NaOAc maintained a better photosynthetic efficiency throughout the study period (Fig. S4B).

After the optimization of PHB production in *D. communis*, scaled-up cultivation (10 L PBR) was established. *D. communis* biomass (DW, g/L) grown with the previously optimized mixotrophic condition slightly increased over time (Fig. 3A), reaching the maximum value of 0.24 ± 0.03 g/L after 10 days of cultivation, contrarily to the small-scale cultures where the same biomass yield was achieved after 3-4 days of cultivation (Fig. S4A), suggesting that the biomass productivity was not optimized. Furthermore, photosynthetic efficiency (Table 2) decreased over time, evidencing that algal cells switched to the mixotrophic metabolism which boosts the biosynthesis of PHB. PHB content in the algal biomass was quantified after 2, 3 and 10 days of cultivation (Fig. 3B) and resulted comparable to the content obtained at a small scale (PHB content of 23.1 ± 2.7% w/w; PHB productivity of 0.023 ± 0.03 g PHB/g biomass/d at day 10). Algal cells started to accumulate PHB on the third day of cultivation (7.2 ± 2.8% w/w), then the content increased but PHB productivity maintained constant, although still not optimized, probably due to the decrease of NaOAc after day

3, as attested by the COD values measured in the medium (Fig. 3A). Indeed, various studies on the production of PHB by photosynthetic organisms suggested important interrelations between the PHB biosynthetic pathway and those of the central carbon metabolism [57]. Moreover, it was proved that in the PHB accumulating cyanobacterium *Synechocystis* PCC 6803 grown photosynthetically under N-depletion, up to 87% of the carbon contained in PHB derived from intracellular carbon reserves [58].

D. communis biomass harvested at day 10 was characterized as main compounds (w/w, Fig. 4), and resulted in 37.9 ± 1.1% proteins, 24.1 ± 2.1% polysaccharides, 9.0 ± 1.5% lipids, and 23.1 ± 2.7% PHB. These results highlighted *D. communis* potential as a PHB-producing organism and its ability to accumulate high percentages of other important compounds, such as proteins [42], exploitable in many industrial applications [59]. Furthermore, other studies revealed *D. communis* potential in environmental applications such as the bioremediation of wastewater [44,45] since, as well as other Scenedesmaceae, it is characterized by high biomass productivity and nutrient removal efficiency, even when subjected to stressful conditions. These results open the possibility to investigate the production of PHB by *D. communis* in a circular economy perspective, perhaps using wastewater as a nutrient source, as already reported for some cyanobacteria species [7,60] and the Chlorophyta *Botryococcus braunii* [61].

3.3. *D. communis* semi-continuous cultivation

When *D. communis* has been cultivated in a semi-continuous mode in 10 L PBRs under the optimized mixotrophic conditions, the biomass yield reached 0.2-0.3 g/L, with a partial collection of the biomass every 3-4 days (Fig. 5A). Conversely, COD values in the medium decreased during the cultivation as a result of NaOAc consumption (Fig. 5B). The addition of NaOAc at day 12, when it was depleted, restored the COD value at about 0.7 gO_2/L, which subsequently decreased while cells continued to grow. As for the biomass productivity, significant differences were observed among different stages of the algal cultivation (ANOVA, $p < 0.05$); in particular, pairwise comparisons highlighted a clear

difference between the first phase, in which the algal biomass was still increasing, and the subsequent phase in which cell density reached and maintained a plateau. PHB yield in the first 12 days of the culture was 24-35% (w/w), comparable to those previously found, then values increased to about 50% (w/w) and maintained constant till the end of the semi-continuous cultivation (day 27, Fig. 6A). PHB productivity was influenced by the biomass concentration and ranged between 0.054 and 0.096 g PHB/g biomass/d (corresponding to 0.006 and 0.011 g PHB/L/d, respectively) when the growth was optimized and NaOAc was not depleted (Table 3). A few studies reported PHB productivity in chlorophytes and the values obtained are of the same order of magnitude as the ones found in the present study (Table 1). However, it must be highlighted that *D. communis* was here cultivated in fed-batch mode in 10 L PBR, whereas in previous studies cultures were characterized by lower volumes and run in batch mode. Indeed, the results of the present work consolidate the high resistance of *D. communis* and its potential for large-scale cultivation, since it has been successfully cultivated under stressful mixotrophic conditions in fed-batch mode for almost one month.

Conversely, a considerable amount of research has been done on PHB production by cyanobacteria species, and the range of productivity found in cyanobacteria species is wide and highly variable, even within the same strain. For example, Haase et al. (2012) reported a maximum intracellular PHB content of 145.1 mg/L in *Nostoc muscorum*, which corresponded to productivity of 0.0062 g/L/d; while in Bhati and Mallick (2016) the same species was cultured for the accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), obtaining productivity of 0.1104 g/L/d.

The algal biomass was characterized at different growth periods (days 12 and 22) (Fig. 6B): the lipid content (13.5 and 3% at day 12 and 22, respectively) was lower than that achieved for the same strain under autotrophic conditions [59], in accordance also with the results obtained for cyanobacteria tested for PHB production at similar growth conditions [33]. The decrease in lipids found along the growth occurred as a direct consequence of PHB accumulation with time, since the carbon used for biosynthesizing storage lipids was feasibly diverted to PHB synthesis, as previously reported [30]. The polysaccharide and protein contents were stable over time (about 5-6% and 30-33%,

respectively); such values were in line with the polysaccharide and protein content found for the same strain under autotrophic conditions [44,59]. The concentration of organic carbon, nitrate and phosphate in the culture medium strongly affects the lipid, carbohydrate, and protein content of microalgal biomass that considerably vary depending on the species. Regarding the effect of P depletion, a positive effect for starch and lipids (i.e., FAMEs) was reported for some species [64]. Limited phosphorus concentrations could support continuous cell growth as well as enhance lipid accumulation in microalgae by switching photosynthetic carbon partitioning toward energy-rich storage macromolecules. Stress caused by the nutrients depletion is currently the most commonly used strategy to trigger the accumulation of energy storage metabolites in microalgae; however, their productivity is limited due to the decline of photosynthetic activity caused by the generation of ROS under nutrient depletion conditions which impairs the photosynthetic apparatus, and the consequent compromised biomass production [65]. In addition, three green algae species, *Coelastrella* sp., *Pectinodesmus* sp. and *Ettlia texensis*, were cultured in the same conditions to induce biopolymer accumulation [66]. It emerged that in *Coelastrella* sp. and *Pectinodesmus* sp. (both belonging to the family of Scenedesmaceae) PHB production was negatively correlated to lipid synthesis and closely related to protein synthesis, as here observed for *D. communis*. On the contrary, in *E. texensis* (a Chlorophyta belonging to the order of Chlamydomonadales) PHB synthesis was closely related to the biosynthetic pathways of lipids, therefore both lipid and PHB production can proceed together under optimized culturing conditions.

Although PHB productivities reported for microalgae are critically lower compared to those reported for bacterial species (e.g., [7,26]; studies in Table 1), the use of photosynthetic species for PHB production could be advantageous from a biorefinery perspective, as the cultivation of cyanobacteria or microalgae can result in the co-extraction of value-added compounds like pigments and antioxidants other than the biopolymer, making the whole process more profitable [67]. Hence, the integral use of all microalgal biomass will allow the realization of market competitive microalgae technology. *Scenedesmus* spp. are known to be promising species in synthesizing biologically active

compounds, including antioxidants such as lutein and β-carotene [43] or protein hydrolysates having antioxidant activity as well as antiviral due to amino acid residues such as methionine and arginine [68]. Moreover, wastewaters could be used to culture *D. communis* (or other Chlorophyta such as *Chlorella* spp.) as attested in previous studies (e.g., [44,45]) leading to the formation of mixed algal microbial consortia. Production of PHAs by mixed cultures has been widely studied as PHA-accumulating organisms are selected by the dynamic operating conditions imposed to the reactor [69,70].

3.4. PHB extraction and characterization

PHB was extracted from *D. communis* biomass after 4 and 12 days (Fig. 7), resulting in a high recovery (95%) of a light-green coloured polymeric film with a low purity (90-95%). As previously observed for other photosynthetic organisms (e.g., cyanobacteria, [33,71,72]), the association between PHB granules and thylakoid membranes could be responsible for the challenging purification of PHB from photosynthetic pigments (chlorophyll *a*). Similarly, both the colour of the recovered film in the present study and its elemental composition highlighted the presence of N-containing contaminations like proteins or pigments (i.e., chlorophyll *a*) that could not be removed after solvent extraction and further purification with acetone. Nitrogen content (1.1 ± 0.1%) may suggest a strong association between the biopolymer and the chloroplasts within *D. communis* cells which persist even by applying a pre-treatment of algal biomass with acetone to remove pigments before the extraction with dichloromethane (data not shown). As expected by the coloured contamination, the composition in terms of carbon in the extracted PHB was slightly but significantly different from the one of commercial PHB (54.2 ± 0.4% *vs* 55.6 ± 0.8%, respectively).

Thus, it can be deduced that the extraction process plays an important role in the purity level of the recovered product. Hence, differences in PHB elemental composition are not dependent on the producer algal species but more likely on the recovery method, giving evidence of the importance to optimize the PHB extraction process by these photosynthetic organisms. Indeed, the procedures

employed to extract PHAs have a strong influence on the monomeric sequences and therefore on the product's physical properties [67].

4. Conclusions

Chlorophyta are considered an ideal feedstock for a wide number of biotechnological applications, and among all, the production of PHAs is one of the most innovative. In the present work, the potential of *Desmodesmus communis* as PHB-producing organism has been explored, optimized and scaled-up in 10 L PBR under a mixotrophic stress phase with phosphate depletion and NaOAc as carbon supply. A PHB productivity up to 0.11 g PHB/g biomass/d (0.015 g PHB/L/d) and a PHB content of about 50% (w/w) was achieved, together with about 30% (w/w) of proteins.

In conclusion, this study i) confirmed the capacity of the Chlorophyta *D. communis* to produce PHB, with productivity up to 2-times higher than those reported for several cyanobacterial species and similar to the maximum value obtained in previous works performed with Scenedesmaceae, and ii) emphasized its potential, also in an industrial production perspective, being the first study where semi-continuous microalgal cultivation was performed for PHB production. Further investigations should be addressed to better understand the physiological mechanisms that allow *D. communis* to produce PHB, and whether the algal microbiota could play a relevant role in PHB production. Moreover, the development of extraction and purification methods suitable for obtaining high-quality PHB is mandatory. In addition, considering *D. communis* ability to bioremediate wastewater, its exploitation in a circular economy perspective could be promising.

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Conflict of interest

The authors declare no competing financial interest.

Contributions

LP: Conceptualization, Data Curation, Supervision, Writing - Original Draft, Writing—Review & Editing; **CS**: Conceptualization, Data Curation, Supervision, Writing—Review & Editing; **GZ**: Investigation, Visualization; **GX**: Investigation, Visualization; **MS**: Writing—Review & Editing; **RP**: Writing—Review & Editing. All authors approved the final manuscript.

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Table 1 – PHB production in Chlorophyta. List of studies found in the literature.

Species	Growing condition	Medium	Temperature (°C)	pH	Light condition	Carbon source	PHB	Growth time (days)	References
Botryococcus braunii	Autotrophic	40% CHU13 medium 60% sewage wastewater	40	7.5	n.a.	Ø	≈ 20 (% w/w)	15	[61]
Botryococcus braunii	Autotrophic	CHU13 medium	25	n.a.	Photoperiod 12 h of light, 30 µmol/m²/s of light intensity	Ø	16.4 (% w/w)	30	[73]
Botryococcus braunii SAG 807-1	Mixotrophic	60% BG-11 40% POME (palm oil mill effluent) salinity 1 PSU	30	7.5	Photoperiod 12 h of light, 70 µmol/m²/s of light intensity	Sodium acetate or D-glucose or glycerol	Up to 35 (% w/w) (with addition of 10 mg/L Fe-EDTA)	n.a.	[74]
Chlorella fusca LEB 111	Mixotrophic	BG-11 medium N deficiency (- 50%)	30	n.a.	Photoperiod 18 h of light, 28 µmol/m²/s of light intensity	Arabinose	14 (% w/w) Productivity 0.03 g/L/d	10	[35]
					Photoperiod 18 h of light, 9 µmol/m²/s of light intensity	Xylose	16.2 (% w/w) Productivity 0.02 g/L/d		
					Photoperiod 6 h light, 28 µmol/m²/s of light intensity	Xylose	17.4 (% w/w) Productivity 0.03 g/L/d		
Chlorella pyrenoidosa	Autotrophic	Fogg's medium	n.a.	n.a.	80 Lux of light intensity	Ø	27 (% w/w)	14	[36]
Chlorella sorokiniana SVMIICT8	Mixotrophic, constant aeration	Modified Bold's medium	n.a.	7.0	Photoperiod 12 h of light, 200 µmol/m²/s of light intensity	Sodium acetate	29.5 (% w/w) Productivity 0.28 g/L (0.0175 g/L/d)	16	[75]
Chlorella vulgaris PB 1-6 (axenic cultures)	Mixotrophic	CHU13 medium P deficiency	27	n.a.	Photoperiod 14 h, 3000 Lux of light intensity	Sodium acetate (1 g/L)	< 3 (% w/w)	14	[37]

Table 1 (continued)

Species	Growing condition	Medium	Temperature (°C)	pH	Light condition	Carbon source	PHB	Growth time (days)	References
Coelastrella sp., *Ettlia texensis*	Mixotrophic	Modified BG-11 medium (0.04 g/L P and 1.5 g/L N)	27	n.a.	Photoperiod 12 h of light, 3000 Lux of light intensity	Galactose (10 g/L)	*Coelastrella* sp. 15.18 (% w/w) *E. texensis* 13.55 (% w/w)	3	[66]
						Sucrose (10 g/L)	*Coelastrella* sp. 15.08 (% w/w) *E. texensis* 13.46 (% w/w)		
Scenedesmus sp. UTEX 1589	Mixotrophic	BG-11 medium N and P deficiency NaCl (0.5 g/L)	20	8.2	100 µmol/m²/s of light intensity	Glucose (4 g/L)	17.14 (% w/w) Productivity 0.120 g/L (0.0086 g/L/d)		[17]
		BG-11 medium N and P deficiency NaCl (2.0 g/L)				Glucose (1 g/L)	26.25 (% w/w) Productivity 0.171 g/L (0.0122 g/L/d)		
		BG-11 medium P deficiency NaCl (0.5 g/L)				Glucose (1 g/L)	29.92 (% w/w) Productivity 0.239 g/L (0.0171 g/L/d)		
Stigeoclonium sp. B23	Autotrophic or Mixotrophic	BG-11 medium standard or N deficiency	25	7	Photoperiod 12 h of light	Ø or sodium acetate (0.82 g/L) or sodium bicarbonate (0.42 g/L)	Up to 2.5 (% w/w)	30	[76]
Stigeoclonium sp. B23	Autotrophic, continuous and intermittent aeration	Z8 medium N deficiency (-75%)	25	7	10–30 µmol/m²/s of light intensity	Ø	12.16 (% w/w) Productivity 0.098 g/L (0.0022 g/L/d)	45	[77]

Table 2 – Maximum quantum yield of *D. communis* in the batch cultivation test (10L PBR).

Time (day)	Yield
0	0.654 ± 0.030
2	0.540 ± 0.014
3	0.494 ± 0.019
10	0.456 ± 0.038

Table 3 – PHB productivity achieved in the semi-continuous cultivation test (10L PBR).

ΔTime (day)	Productivity (g PHB/L/d)	Productivity (g PHB/g biomass/d)
0-4	0.008	0.072
4-8	0.011	0.096
8-12	0.003	0.031
12-15	0.006	0.054
15-19	0.007	0.063
19-22	0.001	0.013
22-27	0.004	0.034

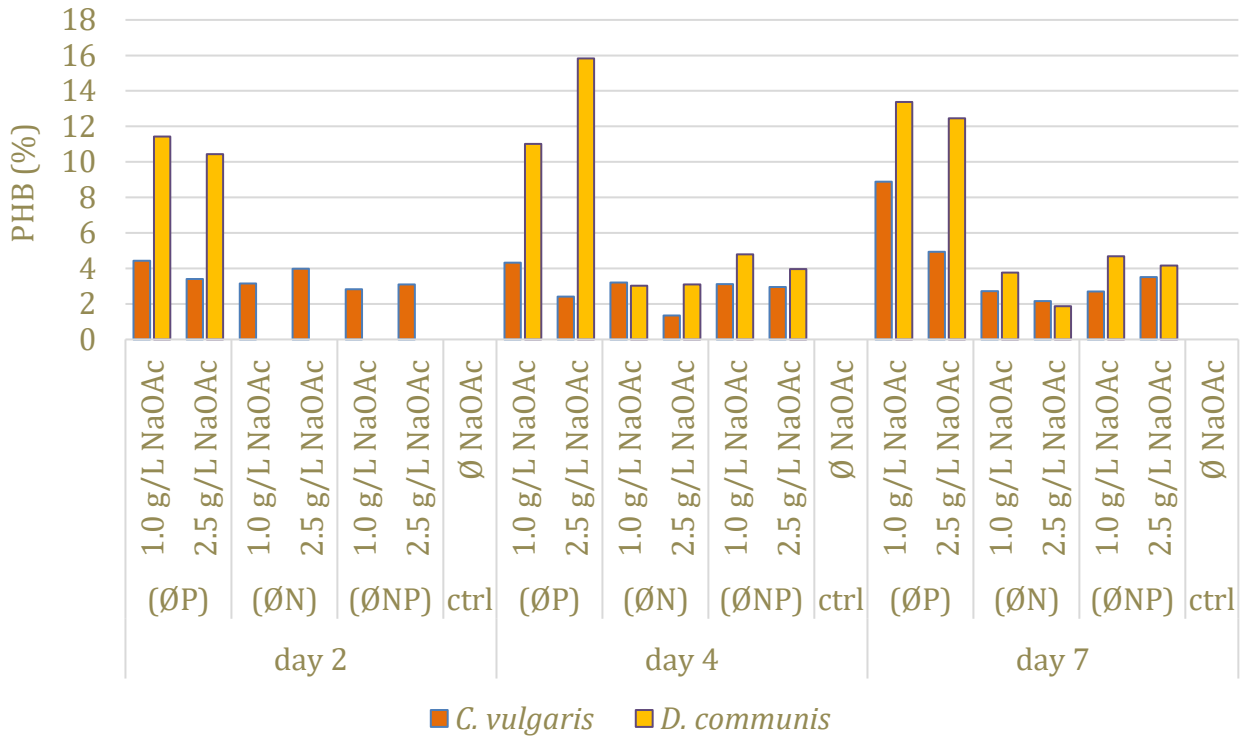


Fig. 1 – PHB amount (% w/w) accumulated by *D. communis* and *C. vulgaris* in the screening test with nutrient depletion (ØN, ØP, or both ØNP) and different sodium acetate concentrations (NaOAc, 1 or 2.5 g/L). crtl: control condition with nutrients.

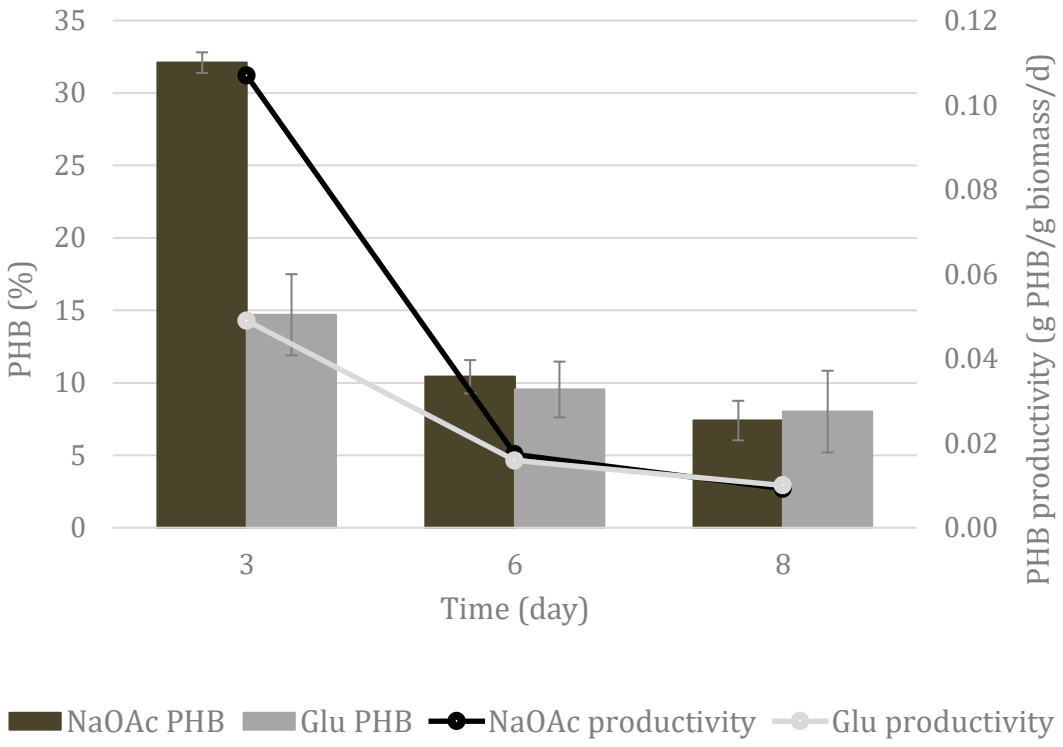
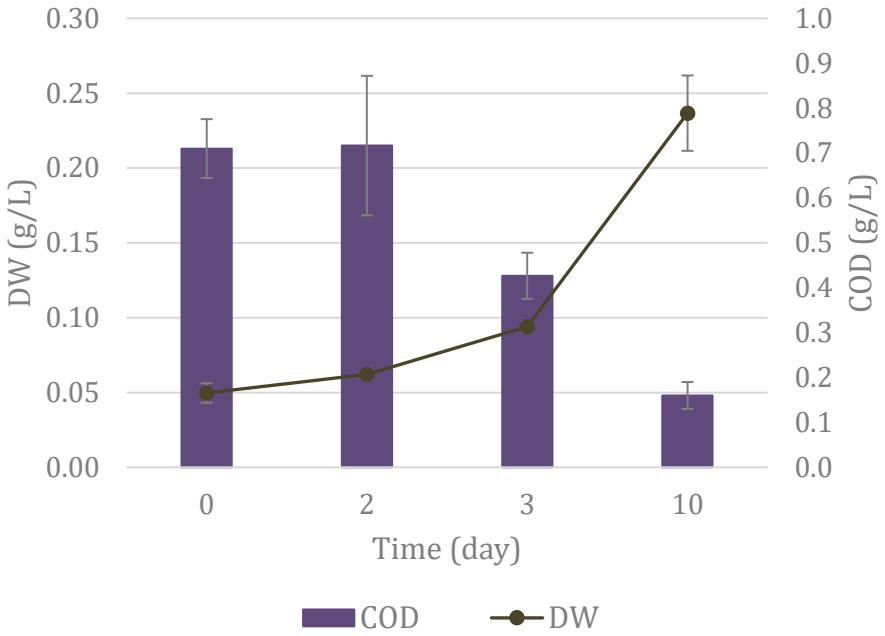


Fig. 2 – PHB amount (% w/w) and productivity ($g_{PHB}/g_{biomass}/d$) in *D. communis* grown for 8 days with sodium acetate (NaOAc) and glucose (Glu) as carbon sources.

a)



b)

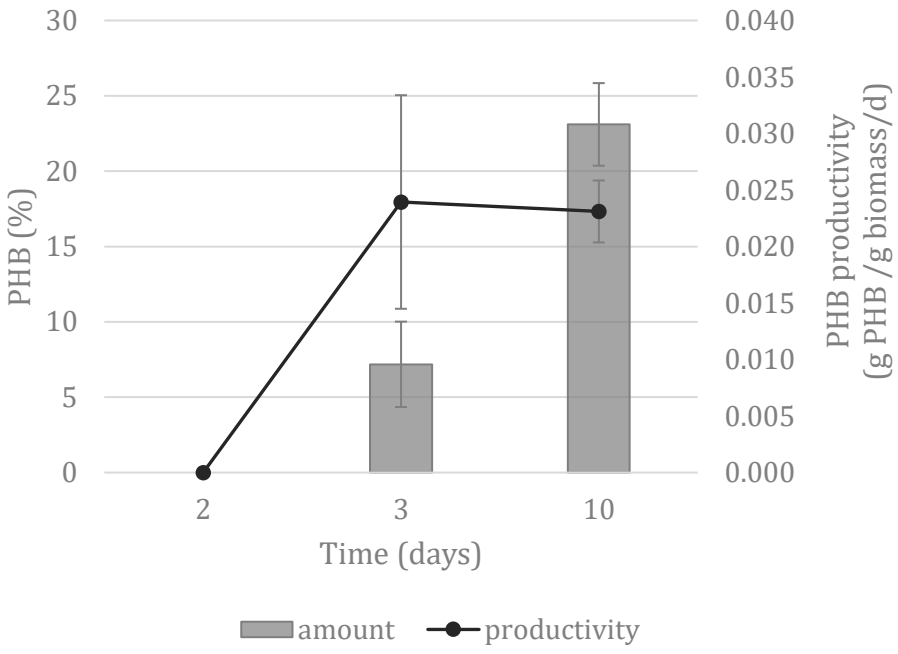


Fig. 3 – a) *D. communis* growth (expressed as dry weight, DW, g/L) and COD values of the medium after biomass filtration (gO_2/L) and b) PHB amount (% w/w) and productivity ($g_{PHB}/g_{biomass}/d$) in the batch cultivation test (10L PBR).

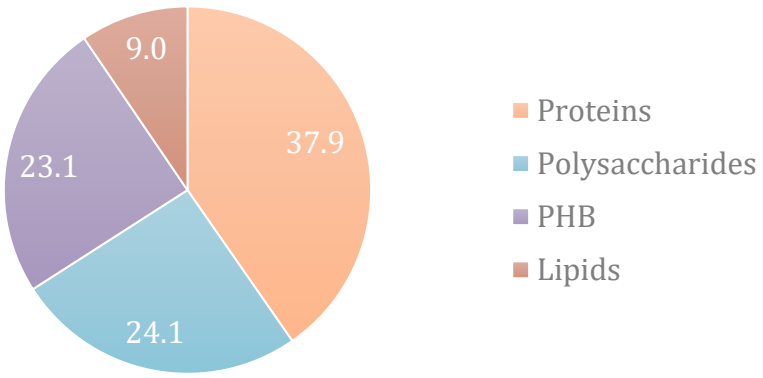
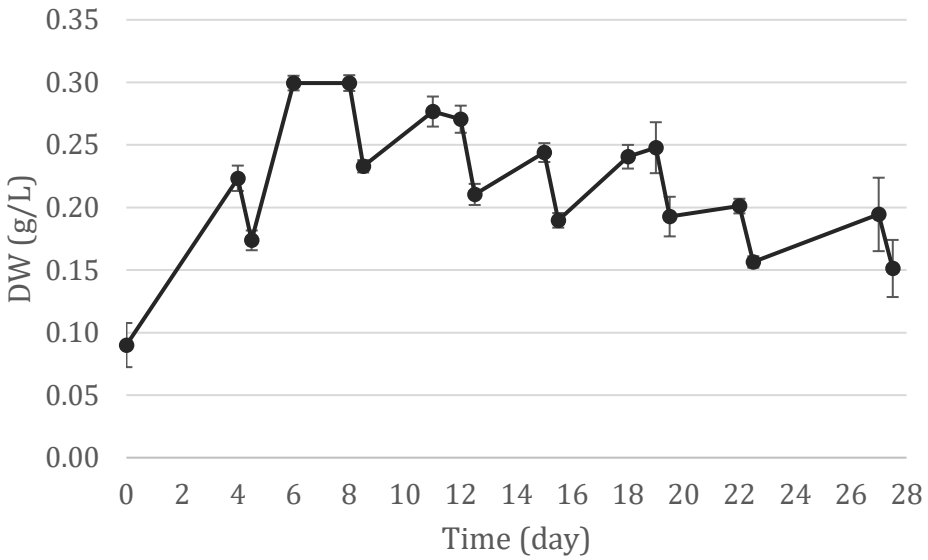


Fig. 4 – Biomass composition (%) of *D. communis* at day 10 in the batch cultivation test (10L PBR).

a)


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DW (g/L)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (day)

b)

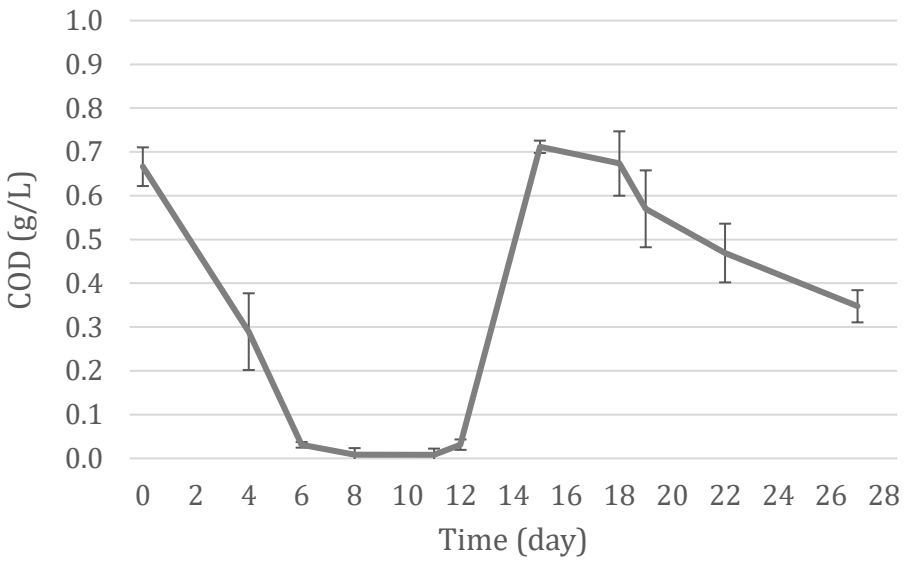
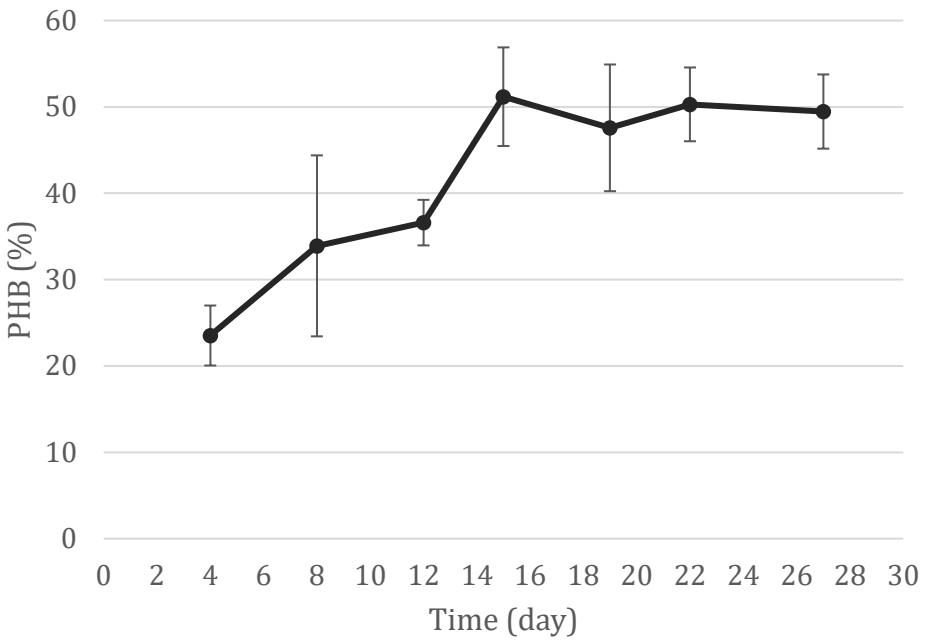


Fig. 5 – a) Semi-continuous growth of *D. communis* in the batch cultivation test (10L PBR) expressed as dry weight (DW, g/L); b) COD values of the medium after biomass filtration (gO_2/L).

a)



b)

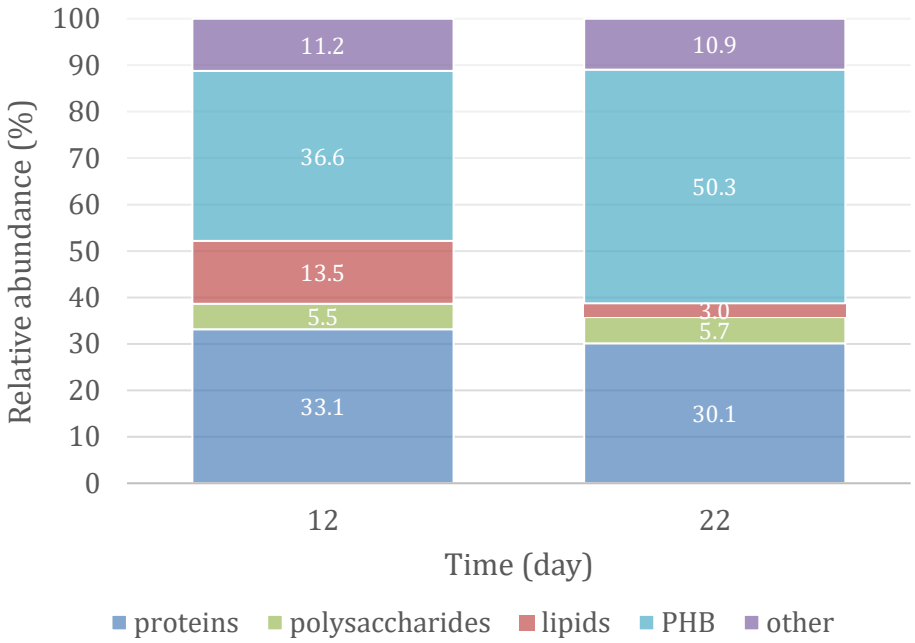


Fig. 6 – a) PHB amount (% w/w) and b) biomass composition (%) of *D. communis* after 12 and 22 days during the semi-continuous cultivation in a 10L PBR.

Fig. 7 – PHB extracted from *D. communis* biomass during the semi-continuous cultivation test.