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Semi-continuous production of polyhydroxybutyrate (PHB) in the Chlorophyta *Desmodesmus communis*

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1 **Semi-continuous production of polyhydroxybutyrate (PHB) in the Chlorophyta *Desmodesmus***  
2 ***communis***

3  
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## 27 **Abstract**

28 Polyhydroxyalkanoates (PHAs) are promising alternatives that accumulate as energy and carbon  
29 storage material in various microorganisms, including bacteria and microalgae, being biodegradable  
30 and suitable for a wide variety of applications. Among these compounds, the most prevalent and well-  
31 characterized biopolymer is polyhydroxybutyrate (PHB), which belongs to the short-chain PHAs.

32 The present study was designed to evaluate algae-based PHB production in two Chlorophyta  
33 (*Desmodesmus communis* and *Chlorella vulgaris*) under a two-phase nutritional mode of cultivation,  
34 namely a phototrophic growth phase (PGP) and a mixotrophic stress phase (MSP) with N,P-depleted  
35 media and organic carbon supply (i.e., glucose or sodium acetate, NaOAc). The highest PHB  
36 productivity (0.11 g PHB/g biomass/d; 0.015 g PHB/L/d), corresponding to 32.1% w/w of  
37 intracellular PHB, was observed for *D. communis* after 3 days of cultivation under mixotrophic  
38 conditions in batch cultures (e.g., low light, phosphorus-free medium, 1 g/L of NaOAc). A scaled-up  
39 cultivation (10 L) was set up to evaluate for the first time PHB yields and biomass composition in a  
40 semi-continuous system. A PHB content of 34% w/w was achieved on day 8, corresponding to a  
41 maximum PHB productivity of 0.10 g PHB/g biomass/d (or 0.011 g PHB/L/d), which increased up  
42 to 54% w/w on day 15. The biomass was composed of about 30% w/w proteins, 6% w/w  
43 polysaccharides, and 11 % w/w lipids, which can be valorised from a biorefinery perspective. The  
44 scaled-up *D. communis* cultivation in 10 L PBRs confirmed the potential utilization of this algal  
45 species for PHB production with productivity up to 2-times higher than those reported for several  
46 cyanobacterial species and similar to the maximum value obtained with batch cultures in previous  
47 works performed with Scenedesmaceae.

## 48 **Keywords**

49 *Desmodesmus communis*; polyhydroxyalkanoates; algal biomass; microalgal cultivation;  
50 mixotrophy.

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## 55 **1. Introduction**

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

78 Bacteria are efficient PHA-producing organisms; thus, their commercial exploitation is widespread  
79 using continuous fermentation processes which provide controlled conditions, high productivity, and  
80 uniform product quality, along with low investment costs [12]. Different bacterial species can store  
81 PHAs within the cytoplasm in granules ranging in size from 0.2 to 0.5  $\mu\text{m}$  [9], some of which, such  
82 as wild type or genetically modified *Cupriavidus necator*, *Bacillus* sp., *Alcaligenes* sp., *Azotobacter*  
83 sp., and *Pseudomonas* sp., are commercially exploited [13–16]. The PHAs microbial production  
84 needs the addition of an organic substrate to the medium. Indeed, the chemical composition of the  
85 resulting polymer depends on the latter and can be manipulated by varying the carbon source [17].  
86 Commercialization and industrialisation of PHAs are difficult because they are three-times more  
87 expensive than conventional plastics [7]: the cost of the carbon source used for feeding PHA-  
88 accumulating bacteria contributes significantly to the final PHA price (up to 30%), accounting 70-  
89 80% of total raw material cost [18], as well as the downstream phase for recovering PHA from  
90 microbial biomass that can contribute up to 50% [19]. The use of waste can abate the costs related to  
91 the use of the raw material, as well as the use of recyclable solvents/additives for PHA extraction  
92 (e.g., [20–23]). Photosynthetic organisms, such as cyanobacteria and microalgae, can also produce  
93 PHAs as a response to nutrient deficiency (i.e., nitrogen, phosphorus, [6,8,24]) and in the presence of  
94 a carbon source, which can be obtained also from wastewater [20,25]. PHAs are naturally present in  
95 microalgae, even if in a lower amount than that of bacteria [7,17,26]. However, this concentration  
96 can be increased under suitable culture conditions that are strain specific. Furthermore, microalgae  
97 boast rapid growth, low space and water requirements and the capability to use sunlight as an energy  
98 source [7]. Most of the studies performed with various microalgae strains concentrates on altering  
99 growth conditions to maximize and optimize polyhydroxybutyrate (PHB) production, which is the  
100 most studied, commercially exploited, and well-characterized short-chain homopolymer of PHAs  
101 (e.g., [17]; and other studies in Table 1). Significant factors that contribute to the accumulation of  
102 PHB in microalgae biomass are: presence of organic carbon (such as acetic acid, pentoses, etc., [7,27],  
103 the photoperiod and light exposure [28], limitation of nitrogen and phosphorus [17,29–31], limitation

104 of certain heavy metals (Ni and Cu) and dissolved gas transfer resistance in the culture [31]. So far,  
105 about 100 strains of eukaryotic and prokaryotic (cyanobacterial) microalgae have been identified as  
106 capable of photoautotrophically accumulating PHB (see [32] and studies in Table 1). Considerable  
107 quantities have been found in several cyanobacterial or algal species, e.g., *Synechococcus subsalsus*  
108 (16%) and *Spirulina* sp. (12%) [30]; cf. *Anabaena* sp. (up to 46%) [33]; recombinant *Phaeodactylum*  
109 *tricornutum* (11%) [34]. Most of the studies concern cyanobacteria, while more recent papers  
110 investigate the production and identification of PHAs in Chlorophyta (Table 1). Among tested  
111 species, *Chlorella* sp. [35–37] and *Scenedesmus* sp. [17] achieved the best concentrations (27-29%  
112 w/w). Both genera are of great commercial interest due to their resistance to harsh environmental  
113 conditions; moreover, these species are commonly exploited for a wide number of industrial  
114 applications (i.e., food, nutraceutical, bioremediation), and have been extensively studied [38,39].  
115 Additionally, they could grow using wastewater as a source of nutrients (e.g., anaerobic effluents  
116 from digested wastewater), lowering environmental footprint and production costs [40,41]. Among  
117 Scenedesmaceae, *Desmodesmus communis* has great potential either for high protein [42] and  
118 bioactive compounds accumulation [43] or in wastewater treatment processes [44,45].

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

123

## 124 **2. Materials and methods**

### 125 *2.1. Microalgae isolation and identification*

126 Two different chlorophytes were chosen and tested for the first time for PHAs production due to their  
127 growth performance and resistance to different culture conditions as attested in previous studies.

128 *Desmodesmus communis* strain used in this study was isolated from a freshwater pond in the province

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

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

133

## 134 2.2. Experimental design

### 135 2.2.1. PHB production by chlorophytes

136 A first set of experiments aimed to select the growth factors for boosting PHB production by *D.*  
137 *communis* and *C. vulgaris*, focusing on the lack of nutrients (nitrates and/or phosphates), on the  
138 addition of a carbon source at different concentrations (1 or 2.5 g/L NaOAc), and on the growth period  
139 (2, 4, 7 days). Each strain was grown in a two-phase nutritional mode of cultivation, namely a  
140 phototrophic growth phase (PGP) for optimizing the inoculum, and a mixotrophic stress phase (MSP).  
141 In the PGP the batch cultures (800 mL in 1 L flasks) were grown with a modified CHU13 medium  
142 [46] at  $20\pm 1^\circ\text{C}$  and continuous aeration with filtered ( $0.22\ \mu\text{m}$ ) air from the bottom of the flasks at a  
143 flow rate of about  $150\ \text{mL min}^{-1}$ , with a 16 h light and 8 h dark photoperiod and light at about  $200$   
144  $\mu\text{mol m}^{-2}\ \text{s}^{-1}$ , and without pH control. In the MSP phase, the batch cultures (100 mL inoculum and  
145 700 mL medium to have an initial biomass concentration of about 0.05 g/L) were grown without  
146 nitrates, phosphates or both nutrients, and with the addition of 1 or 2.5 g/L of NaOAc; the cultures  
147 were kept at  $20\pm 1^\circ\text{C}$  with a 16 h light and 8 h dark photoperiod and provided with low light at  $20\text{-}30$   
148  $\mu\text{mol m}^{-2}\ \text{s}^{-1}$  and continuous aeration with filtered ( $0.22\ \mu\text{m}$ ) air at a flow rate of about  $150\ \text{mL min}^{-1}$ . Culture growth was followed as dry weight (DW) and maximum quantum yield for the evaluation  
149 of the photosynthetic efficiency (PAM fluorometer). After 2, 4 or 7 days, aliquots of the culture (200  
150 mL) were centrifuged, and the resulting pellet was stored at  $-20^\circ\text{C}$  for PHB analysis. The nutritionally  
151 balanced CHU13 medium was used as a control.

153

### 154 2.2.2 PHB production by *D. communis* using different carbon sources



155 To find the best conditions to optimize PHB production in the MSP phase, *D. communis* was selected  
156 and grown testing the effect of different carbon sources (1 g/L glucose or NaOAc) and growth times  
157 (3, 6 or 8 days) in a modified CHU13 medium without phosphates. Cultures (800 mL in 1 L flasks)  
158 were prepared in triplicates with an initial biomass concentration of about 0.07 g/L and cultivated as  
159 in the first experiment. DW, maximum quantum yield and PHB production were evaluated.

#### 160 2.2.3. Scaled-up fed-batch cultivation of *D. communis*

161 The scale-up of *D. communis* cultivation was performed with a fed-batch growth experiment using  
162 10 L bubble column photobioreactors (PBRs). After the PGP phase, cultures were prepared in  
163 triplicates with an initial biomass concentration of about 0.05 g/L using 1 g/L NaOAc and a modified  
164 CHU13 medium without phosphates and cultivated as in the previous experiments. PBRs were kept  
165 at  $20\pm 1^\circ\text{C}$  with a 16 h light and 8 h dark photoperiod, low light at  $20\text{-}30\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  and continuous  
166 aeration with filtered ( $0.22\ \mu\text{m}$ ) air at a flow rate of about  $1\ \text{L min}^{-1}$ . Culture dry weight (DW),  
167 maximum quantum yield, and the Chemical Oxygen Demand (COD) of the medium without algae  
168 were analysed. After 2, 3 or 10 days, aliquots of the culture (400 mL) were centrifuged, and the pellet  
169 was stored at  $-20^\circ\text{C}$  for the analysis of intracellular PHB and biomass composition (proteins,  
170 polysaccharides, and lipids) at day 10.

#### 171 2.2.4. Scaled-up semi-continuous cultivation of *D. communis*

172 The semi-continuous cultivation of *D. communis* was performed using the same conditions and  
173 reactors described in section 2.2.3. The growth was followed for 27 days. During cultivation, the algal  
174 biomass dry weight, maximum quantum yield, and COD of the medium without algae were  
175 monitored; after 12 days, NaOAc was restored (1 g/L), as it resulted as depleted. Part of the culture  
176 (2 L) was harvested every 3-4 days (days 4, 8, 12, 15, 19, 22 and 27), and replaced with NaOAc (2  
177 g) and 2 L of fresh medium without phosphate. The harvested algal biomass was collected by  
178 centrifuge to determine PHB content and to characterize the biomass composition in terms of proteins,  
179 polysaccharides, and lipids (days 12 and 22), as well as for PHB characterization.

180

### 181 2.3. Algal biomass characterization

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

185 Algal biomass dry weight was calculated following the equation:

$$186 \quad DW (g L^{-1}) = \frac{W_t (g) - W_0 (g)}{V (L)}$$

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

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

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

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

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

202

### 203 2.4. Photosynthetic efficiency measurement

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

208

### 209 *2.5. Chemical Oxygen Demand (COD) analysis*

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

213 The culture filtrate obtained from the dry weight analysis was injected directly into the instrument,  
214 where it was completely oxidized at 1200°C under air/nitrogen flow and constantly analysed with an  
215 O<sub>2</sub> detector. The COD was calculated as grams of oxygen per liter by comparing the signal areas  
216 related to O<sub>2</sub> consumption with those of a known standard solution of glucose (1000-10000 ppm  
217 range).

218

### 219 *2.6. PHB analysis*

#### 220 *2.6.1. PHB content*

221 PHB content in algal biomass was determined according to the procedure reported in the literature  
222 for bacterial PHB quantification named “in-vial thermolysis” (see [52]). Briefly, freeze-dried algal  
223 samples (10 mg) or standard PHB (1–2 mg; Biomer, Germany) were charged in screw-cap vials (4  
224 mL volume, 50 mm high) and then placed on a heating plate at 350°C. At this temperature, PHB  
225 depolymerized into (E)-2-butenic acid (i.e., crotonic acid) that was used as the molecular fingerprint  
226 of PHB for the quantitative analysis. After 20 min, the vials were removed from the heating plate and  
227 let cooling down to RT before adding the internal standard (2-ethylbutanoic acid, 0.1 mL of a solution  
228 5000 ppm in acetonitrile). The sample was then diluted with acetonitrile (4 mL) and analyzed by GC–  
229 MS to quantify the amount of crotonic acid in each sample, and then the amount of PHB [52].

230 GC–MS analysis was performed using an Agilent 7820A gas chromatograph connected to an Agilent  
231 5977E quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were  
232 separated on a DBFFAP polar column (30 m length, 0.25 mm i.d., 0.25 µm film thickness), with  
233 helium flow of 1 mL min<sup>-1</sup>. Mass spectra were recorded under electron ionization (70 eV) at a  
234 frequency of 1 scan s<sup>-1</sup> within the 29–450 *m/z* range. The temperature of the column was set to 50°C  
235 (5 min) and increased to 250°C (10°C min<sup>-1</sup>).

236 PHB content was expressed on biomass weight basis (g PHB/g biomass %). Specific PHB  
237 productivity (g PHB g biomass<sup>-1</sup> d<sup>-1</sup>) was calculated based on the cultivation period (d) as previously  
238 reported [33] following the equation:

$$239 \quad \text{PHB productivity} \left( \frac{\text{g PHB}}{\text{g biomass d}} \right) = \frac{\text{PHB content} \left( \frac{\text{g PHB}}{\text{g biomass}} \% \right)}{\text{cultivation period (d)}}$$

240

#### 241 2.6.2. PHB extraction and characterization

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

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

250

#### 251 2.7. Statistical analysis

252 Differences among samples were tested by multivariate analysis of variance (ANOVA) using PAST  
253 2.17 software [53]. Levene's tests were performed to verify the homogeneity of the variance and

254 Tukey's tests were used for pairwise comparisons. Data are reported as mean values  $\pm$  standard  
255 deviations of triplicates.

256

### 257 **3. Results and discussion**

#### 258 3.1. PHB production in two chlorophytes

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

263 On the other hand, *D. communis* accumulated a higher amount of PHB than *C. vulgaris* already from  
264 day 2 (11.4% with 1 g/L NaOAc, 10.4% with 2.5 g/L NaOAc), but only when grown in the  $\emptyset P$   
265 medium (Fig. 1). *D. communis* cultured in all the other conditions produced PHB in a lower amount  
266 than  $\emptyset P$  medium. PHB productivity values resulted up to 3-times higher with *D. communis* than with  
267 *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  
268 biomass/d with 2.5 g/L of NaOAc) and decreased in time (Fig. S1), probably due to still non-  
269 optimized conditions. In most studies investigating the production of PHB by Chlorophyta, the  
270 highest percentages (3-30% w/w) were obtained after at least 14 days (see references summarized in  
271 Table 1), corresponding to a low PHB productivity.

272 As concerns the composition of the medium, promising results were observed when *C. vulgaris* and  
273 *D. communis* were grown in a  $\emptyset P$  medium (Fig. 1), confirming that PHB biosynthesis by microalgae  
274 is promoted by a nutritional deficiency of key nutrients, as occurs for bacteria and cyanobacteria  
275 [32,54]. While nitrogen deprivation has been extensively investigated to boost PHB synthesis in both  
276 heterotrophic and phototrophic microorganisms, phosphorus limitation may also play a key role.  
277 Specifically, a lack of phosphorus could affect the balanced intracellular formation of ATP and  
278 NADPH, on which the PHB biosynthesis pathway depends [33,55]. Nevertheless, the increase in  
279 PHB due to the deficiency of a specific nutrient, or a combination of two or more, would appear to

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

291

### 292 3.2. *D. communis* batch cultures

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

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

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

306 time and the importance of identifying the peak of PHB production, which could vary among algal  
307 species and depend on growth conditions. Indeed, most studies on the production of PHB by  
308 Chlorophyta (summarized in Table 1) reported the peak of PHB production after at least 14 days of  
309 cultivation. Consequently, the PHB productivity of *D. communis* resulted higher with NaOAc than  
310 with glucose (Fig. 2), especially after 3 days of cultivation ( $0.11 \pm 0.00$  vs  $0.05 \pm 0.01$  g PHB/g  
311 biomass/d, corresponding to  $0.015 \pm 0.005$  and  $0.016 \pm 0.006$  g PHB/L/d, respectively), and resulted  
312 comparable to the maximum value of 0.24 g PHB/L (corresponding to 0.017 g PHB/L/d) previously  
313 reported for *Scenedesmus* sp. after 14 days of cultivation under mixotrophic conditions [17].  
314 Furthermore, PHB productivity obtained in the present work for an eukaryote alga resulted almost  
315 two-times higher than the one previously reported (i.e., 0.06 g PHB/g biomass/d) for the  
316 cyanobacterium cf. *Anabaena* sp. grown at low light, in a ØP medium and with the addition of NaOAc  
317 [33]. It is also important to note that even though the dry weight was higher in the samples grown  
318 with the addition of glucose (Fig. S4A), samples grown with the addition of NaOAc maintained a  
319 better photosynthetic efficiency throughout the study period (Fig. S4B).

320 After the optimization of PHB production in *D. communis*, scaled-up cultivation (10 L PBR) was  
321 established. *D. communis* biomass (DW, g/L) grown with the previously optimized mixotrophic  
322 condition slightly increased over time (Fig. 3A), reaching the maximum value of  $0.24 \pm 0.03$  g/L after  
323 10 days of cultivation, contrarily to the small-scale cultures where the same biomass yield was  
324 achieved after 3-4 days of cultivation (Fig. S4A), suggesting that the biomass productivity was not  
325 optimized. Furthermore, photosynthetic efficiency (Table 2) decreased over time, evidencing that  
326 algal cells switched to the mixotrophic metabolism which boosts the biosynthesis of PHB. PHB  
327 content in the algal biomass was quantified after 2, 3 and 10 days of cultivation (Fig. 3B) and resulted  
328 comparable to the content obtained at a small scale (PHB content of  $23.1 \pm 2.7\%$  w/w; PHB  
329 productivity of  $0.023 \pm 0.03$  g PHB/g biomass/d at day 10). Algal cells started to accumulate PHB on  
330 the third day of cultivation ( $7.2 \pm 2.8\%$  w/w), then the content increased but PHB productivity  
331 maintained constant, although still not optimized, probably due to the decrease of NaOAc after day

332 3, as attested by the COD values measured in the medium (Fig. 3A). Indeed, various studies on the  
333 production of PHB by photosynthetic organisms suggested important interrelations between the PHB  
334 biosynthetic pathway and those of the central carbon metabolism [57]. Moreover, it was proved that  
335 in the PHB accumulating cyanobacterium *Synechocystis* PCC 6803 grown photosynthetically under  
336 N-depletion, up to 87% of the carbon contained in PHB derived from intracellular carbon reserves  
337 [58].

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

349

### 350 3.3. *D. communis* semi-continuous cultivation

351 When *D. communis* has been cultivated in a semi-continuous mode in 10 L PBRs under the optimized  
352 mixotrophic conditions, the biomass yield reached 0.2-0.3 g/L, with a partial collection of the biomass  
353 every 3-4 days (Fig. 5A). Conversely, COD values in the medium decreased during the cultivation as  
354 a result of NaOAc consumption (Fig. 5B). The addition of NaOAc at day 12, when it was depleted,  
355 restored the COD value at about 0.7 gO<sub>2</sub>/L, which subsequently decreased while cells continued to  
356 grow. As for the biomass productivity, significant differences were observed among different stages  
357 of the algal cultivation (ANOVA,  $p < 0.05$ ); in particular, pairwise comparisons highlighted a clear



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

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

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

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

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

410 compounds, including antioxidants such as lutein and  $\beta$ -carotene [43] or protein hydrolysates having  
411 antioxidant activity as well as antiviral due to amino acid residues such as methionine and arginine  
412 [68]. Moreover, wastewaters could be used to culture *D. communis* (or other Chlorophyta such as  
413 *Chlorella* spp.) as attested in previous studies (e.g., [44,45]) leading to the formation of mixed algal  
414 microbial consortia. Production of PHAs by mixed cultures has been widely studied as PHA-  
415 accumulating organisms are selected by the dynamic operating conditions imposed to the reactor  
416 [69,70].

417

#### 418 3.4. PHB extraction and characterization

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

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

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

438

#### 439 **4. Conclusions**

440 Chlorophyta are considered an ideal feedstock for a wide number of biotechnological applications,  
441 and among all, the production of PHAs is one of the most innovative. In the present work, the potential  
442 of *Desmodesmus communis* as PHB-producing organism has been explored, optimized and scaled-up  
443 in 10 L PBR under a mixotrophic stress phase with phosphate depletion and NaOAc as carbon supply.  
444 A PHB productivity up to 0.11 g PHB/g biomass/d (0.015 g PHB/L/d) and a PHB content of about  
445 50% (w/w) was achieved, together with about 30% (w/w) of proteins.

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

456

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459

#### 460 **Conflict of interest**

461 The authors declare no competing financial interest.

462

463 **Contributions**

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

468

469 **Reference**

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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
<i>Botryococcus braunii</i>	Autotrophic	40% CHU13 medium 60% sewage wastewater	40	7.5	n.a.	∅	≈ 20 (% w/w)	15	[61]
<i>Botryococcus braunii</i>	Autotrophic	CHU13 medium	25	n.a.	Photoperiod 12 h of light, 30 μmol/m <sup>2</sup> /s of light intensity	∅	16.4 (% w/w)	30	[73]
<i>Botryococcus braunii</i> 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/m <sup>2</sup> /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]
<i>Chlorella fusca</i> LEB 111	Mixotrophic	BG-11 medium N deficiency (- 50%)	30	n.a.	Photoperiod 18 h of light, 28 μmol/m <sup>2</sup> /s of light intensity	Arabinose	14 (% w/w) Productivity 0.03 g/L/d	10	[35]
					Photoperiod 18 h of light, 9 μmol/m <sup>2</sup> /s of light intensity	Xylose	16.2 (% w/w) Productivity 0.02 g/L/d		
					Photoperiod 6 h light, 28 μmol/m <sup>2</sup> /s of light intensity	Xylose	17.4 (% w/w) Productivity 0.03 g/L/d		
<i>Chlorella pyrenoidosa</i>	Autotrophic	Fogg's medium	n.a.	n.a.	80 Lux of light intensity	∅	27 (% w/w)	14	[36]
<i>Chlorella sorokiniana</i> SVMIICT8	Mixotrophic, constant aeration	Modified Bold's medium	n.a.	7.0	Photoperiod 12 h of light, 200 μmol/m <sup>2</sup> /s of light intensity	Sodium acetate	29.5 (% w/w) Productivity 0.28 g/L (0.0175 g/L/d)	16	[75]
<i>Chlorella vulgaris</i> 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
<i>Coelastrrella</i> sp., <i>Ettlia texensis</i>	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)	<i>Coelastrrella</i> sp. 15.18 (% w/w) <i>E. texensis</i> 13.55 (% w/w)	3	[66]
						Sucrose (10 g/L)	<i>Coelastrrella</i> sp. 15.08 (% w/w) <i>E. texensis</i> 13.46 (% w/w)		
<i>Scenedesmus</i> sp. UTEX 1589	Mixotrophic	BG-11 medium N and P deficiency NaCl (0.5 g/L)	20	8.2	100 µmol/m <sup>2</sup> /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)		
<i>Stigeoclonium</i> sp. B23	Autotrophic or Mixotrophic	BG-11 medium standard or N deficiency	25	7	Photoperiod 12 h of light	∅ or sodium acetate (0.82 g/L) or sodium bicarbonate (0.42 g/L)	Up to 2.5 (% w/w)	30	[76]
<i>Stigeoclonium</i> sp. B23	Autotrophic, continuous and intermittent aeration	Z8 medium N deficiency (-75%)	25	7	10–30 µmol/m <sup>2</sup> /s of light intensity	∅	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	$0.654 \pm 0.030$
2	$0.540 \pm 0.014$
3	$0.494 \pm 0.019$
10	$0.456 \pm 0.038$

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

<b><math>\Delta</math>Time (day)</b>	<b>Productivity (g PHB/L/d)</b>	<b>Productivity (g PHB/g biomass/d)</b>
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

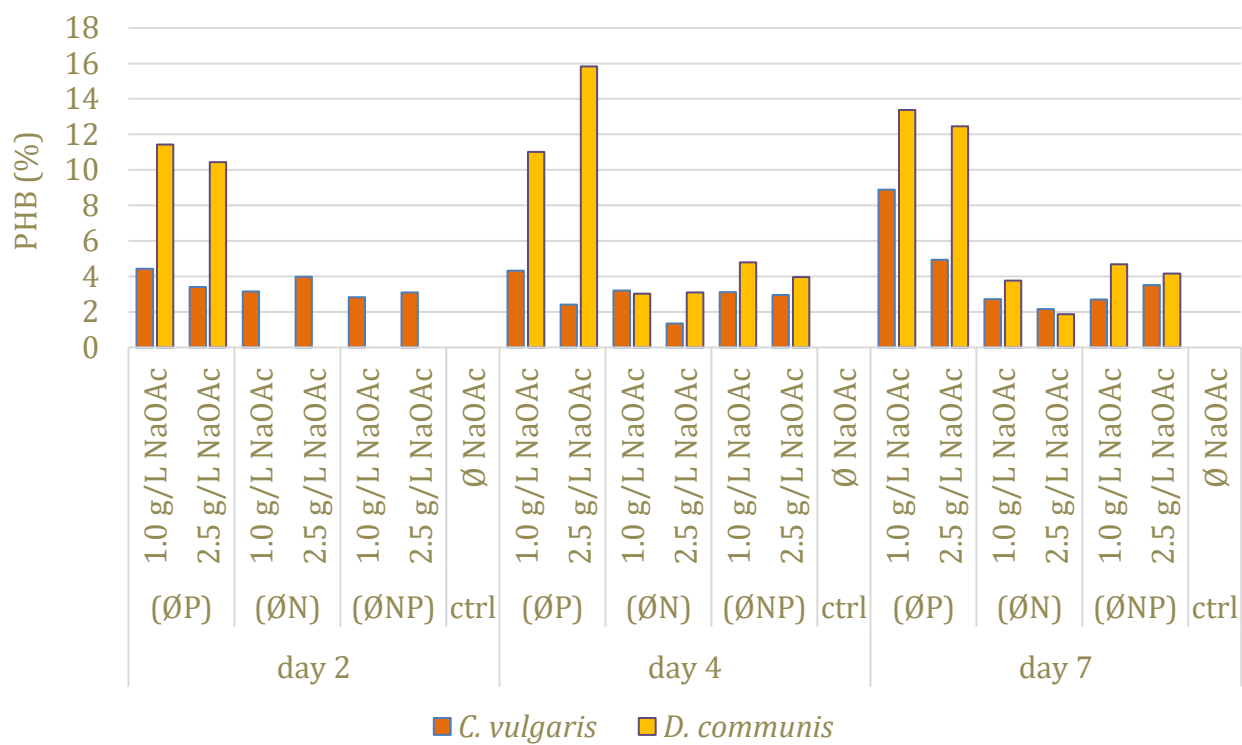


Fig. 1 – PHB amount (% w/w) accumulated by *D. communis* and *C. vulgaris* in the screening test with nutrient depletion (ØN, ØP, or both ØNP) and different sodium acetate concentrations (NaOAc, 1 or 2.5 g/L). ctrl: control condition with nutrients.

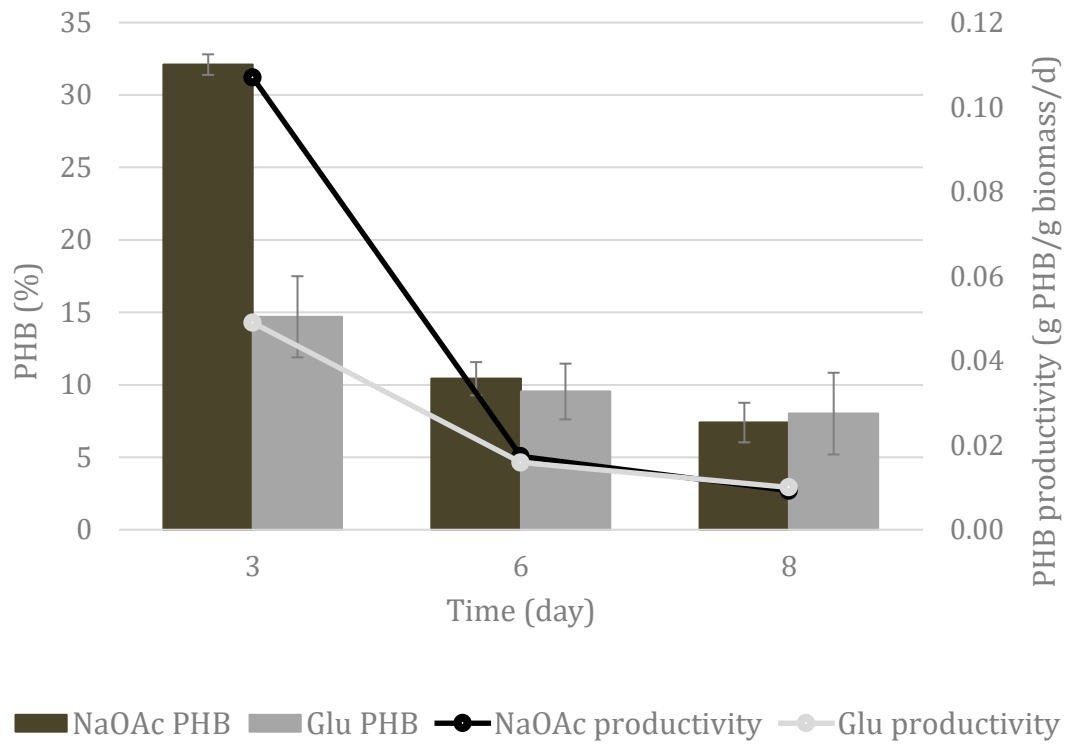
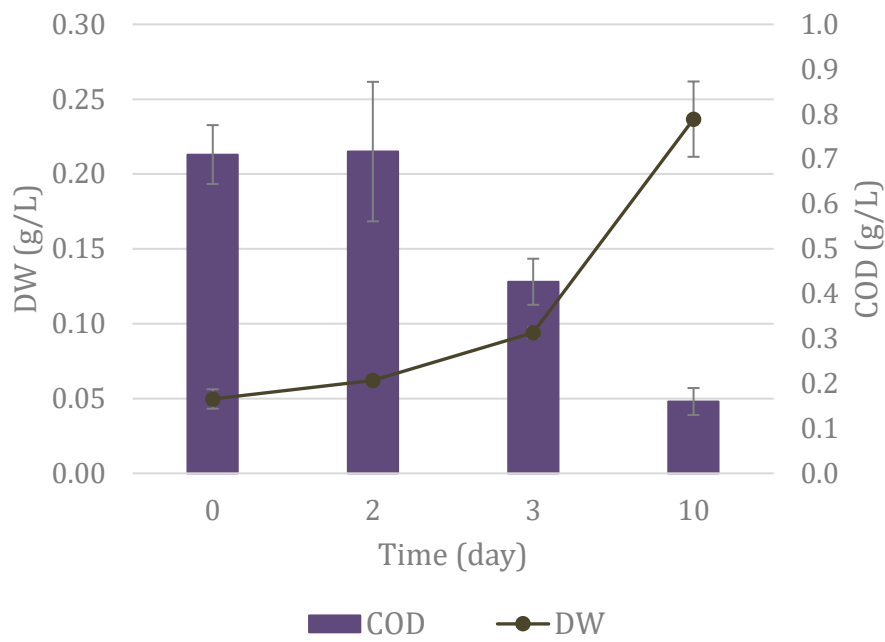


Fig. 2 – PHB amount (% w/w) and productivity ( $\text{g}_{\text{PHB}}/\text{g}_{\text{biomass}}/\text{d}$ ) in *D. communis* grown for 8 days with sodium acetate (NaOAc) and glucose (Glu) as carbon sources.

a)



b)

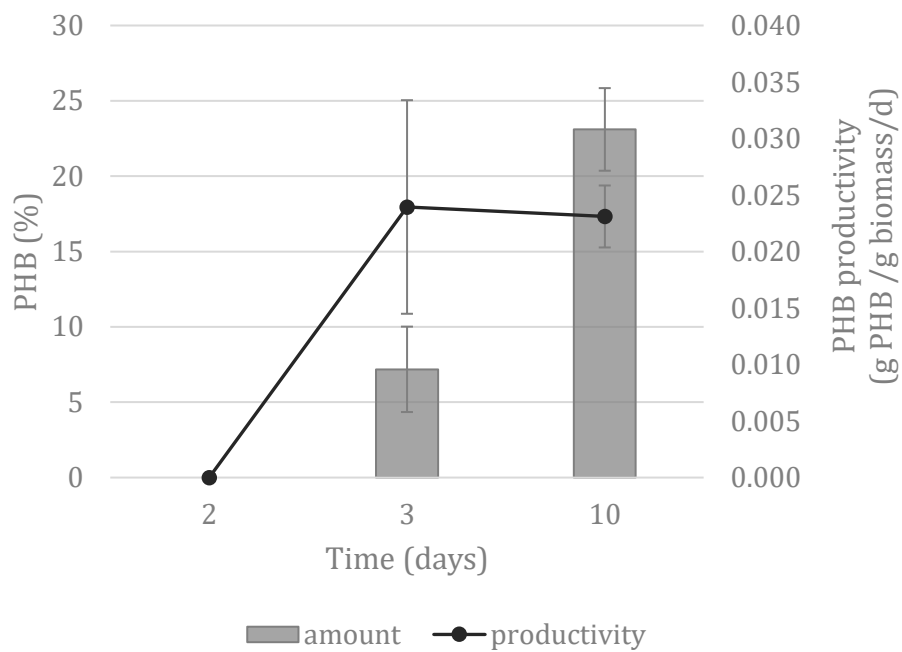


Fig. 3 – a) *D. communis* growth (expressed as dry weight, DW, g/L) and COD values of the medium after biomass filtration ( $\text{gO}_2/\text{L}$ ) and b) PHB amount (% w/w) and productivity ( $\text{g}_{\text{PHB}}/\text{g}_{\text{biomass}}/\text{d}$ ) in the batch cultivation test (10L PBR).

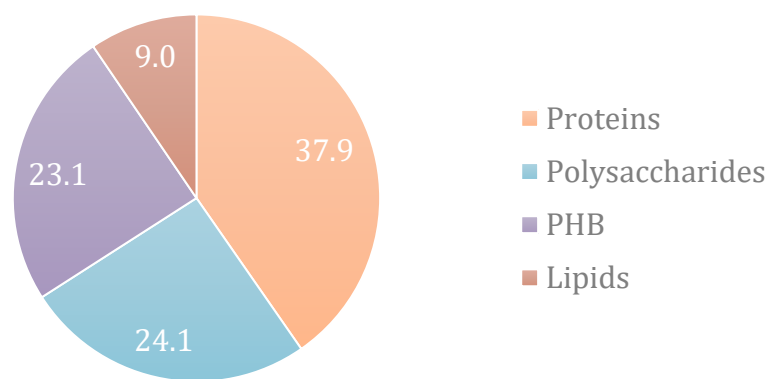
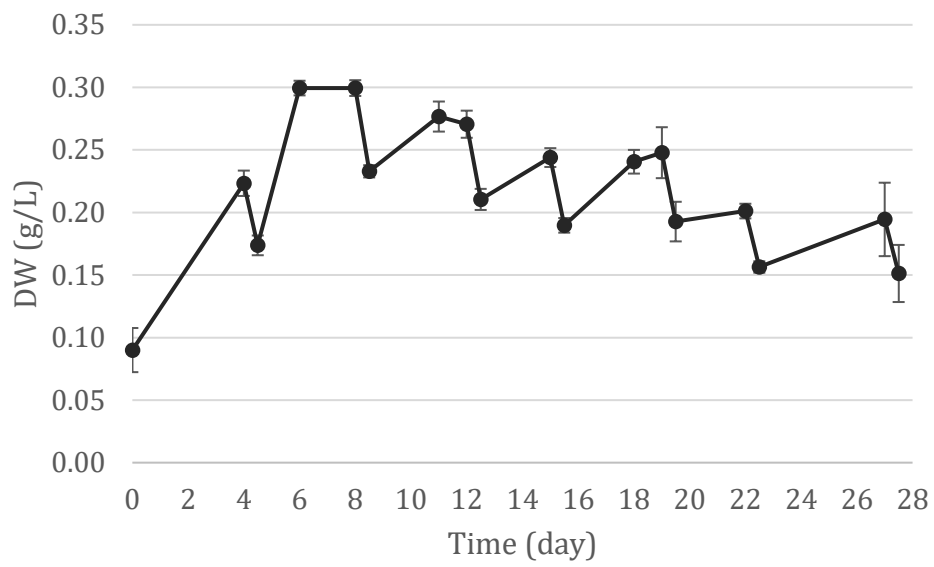


Fig. 4 – Biomass composition (%) of *D. communis* at day 10 in the batch cultivation test (10L PBR).

a)



b)



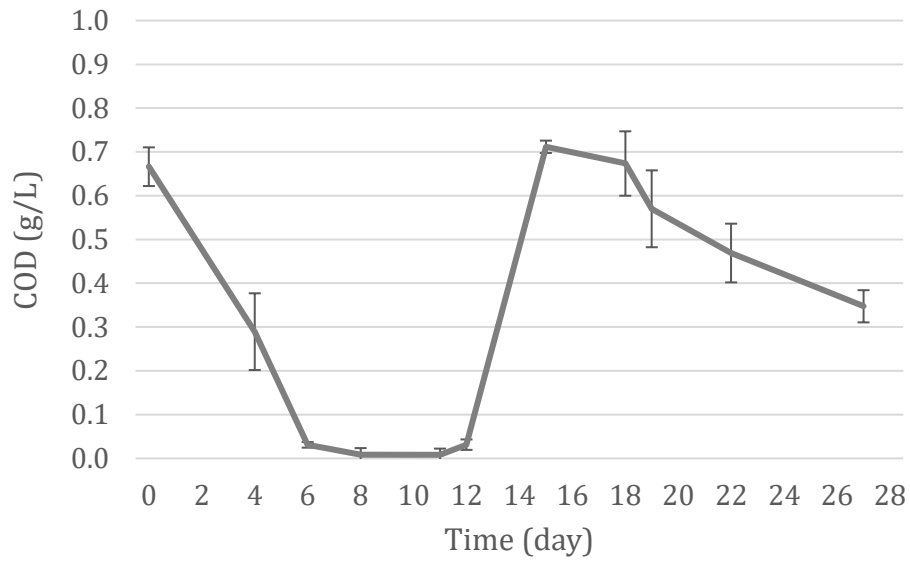
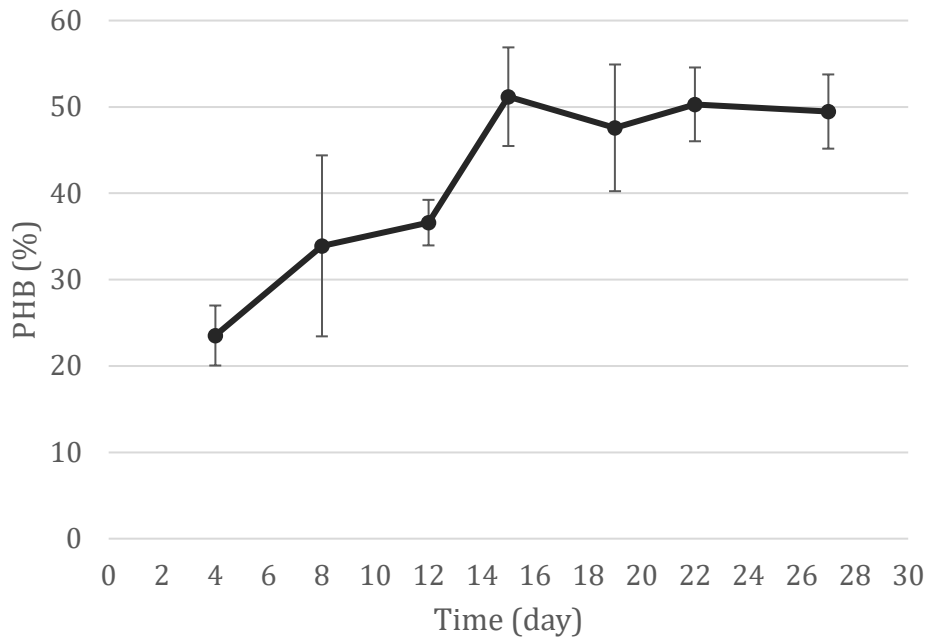


Fig. 5 – a) Semi-continuous growth of *D. communis* in the batch cultivation test (10L PBR) expressed as dry weight (DW, g/L); b) COD values of the medium after biomass filtration (gO<sub>2</sub>/L).

a)



b)

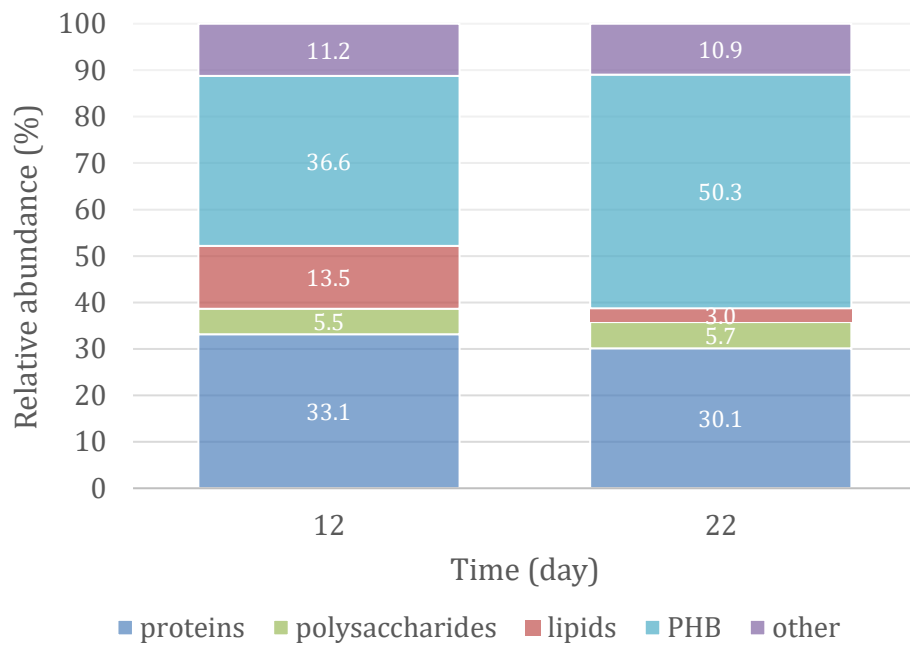


Fig. 6 – a) PHB amount (% w/w) and b) biomass composition (%) of *D. communis* after 12 and 22 days during the semi-continuous cultivation in a 10L PBR.



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