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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Pezzolesi L., Samori' C., Zoffoli G., Xamin G., Simonazzi M., Pistocchi R. (2023). Semi-continuous production of polyhydroxybutyrate (PHB) in the Chlorophyta Desmodesmus communis. ALGAL RESEARCH, 74, 1-11 [10.1016/j.algal.2023.103196].

Availability:
This version is available at: https://hdl.handle.net/11585/954156 since: 2024-03-21

Published:
DOI: http://doi.org/10.1016/j.algal.2023.103196

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(Article begins on next page)

15 September 2024

This is the final peer-reviewed accepted manuscript of:

Laura Pezzolesi, Chiara Samorì, Giorgia Zoffoli, Giulia Xamin, Mara Simonazzi, Rossella Pistocchi, “Semi-continuous production of polyhydroxybutyrate (PHB) in the Chlorophyta Desmodesmus communis”, Algal Research, 74, 2023, 103196.

The final published version is available online at: https://doi.org/10.1016/j.algal.2023.103196.

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1 **Semi-continuous production of polyhydroxybutyrate (PHB) in the Chlorophyta *Desmodesmus***
2 ***communis***
3
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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.

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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 μm [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±1°C and continuous aeration with filtered (0.22 µm) air from the bottom of the flasks at a
143 flow rate of about 150 mL min^{-1}, with a 16 h light and 8 h dark photoperiod and light at about 200
144 µmol m^{-2} 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±1°C with a 16 h light and 8 h dark photoperiod and provided with low light at 20-30
148 µmol m^{-2} s^{-1} and continuous aeration with filtered (0.22 µm) air at a flow rate of about 150 mL min^{-}
149 ^{1}. Culture growth was followed as dry weight (DW) and maximum quantum yield for the evaluation
150 of the photosynthetic efficiency (PAM fluorometer). After 2, 4 or 7 days, aliquots of the culture (200
151 mL) were centrifuged, and the resulting pellet was stored at -20°C for PHB analysis. The nutritionally
152 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±1°C with a 16 h light and 8 h dark photoperiod, low light at 20-30 µmol m^{-2} s^{-1} and continuous
166 aeration with filtered (0.22 µm) air at a flow rate of about 1 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°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 $$DW\ (g\ L^{-1}) = \frac{W_t\ (g) - W_0\ (g)}{V\ (L)}$$

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 × 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 × 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_2 detector. The COD was calculated as grams of oxygen per liter by comparing the signal areas
216 related to O_2 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-butenoic 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^{-1}. Mass spectra were recorded under electron ionization (70 eV) at a
234 frequency of 1 scan s^{-1} 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^{-1}).
236 PHB content was expressed on biomass weight basis (g PHB/g biomass %). Specific PHB
237 productivity (g PHB g $biomass^{-1}$ d^{-1}) was calculated based on the cultivation period (d) as previously
238 reported [33] following the equation:

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

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 ± 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 synthetize 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 (ØP) 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 ØP
265 medium (Fig. 1). *D. communis* cultured in all the other conditions produced PHB in a lower amount
266 than ØP 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 ØP 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 ± 0.00 vs 0.05 ± 0.01 g PHB/g
311 biomass/d, corresponding to 0.015 ± 0.005 and 0.016 ± 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 ± 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 ± 2.7% w/w; PHB
329 productivity of 0.023 ± 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 ± 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 ± 1.1% proteins, 24.1 ± 2.1% polysaccharides, 9.0 ± 1.5% lipids, and 23.1 ± 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_2/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 β-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 ± 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 ± 0.4% *vs* 55.6 ± 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
457 **Acknowledgements**
458 This study was supported by the ALMAIDEA project founded by the University of Bologna.
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**

470 [1] S.F. Siqueira, M.I. Queiroz, L.Q. Zepka, E. Jacob-Lopes, Introductory Chapter: Microalgae
471 Biotechnology - A Brief Introduction, in: Microalgal Biotechnol., 2018: pp. 1–11.
472 https://doi.org/10.5772/intechopen.73250.

473 [2] J. Trivedi, M. Aila, D.P. Bangwal, S. Kaul, M.O. Garg, Algae based biorefinery—How to
474 make sense?, Renew. Sustain. Energy Rev. 47 (2015) 295–307.
475 https://doi.org/10.1016/j.rser.2015.03.052.

476 [3] S.Y.A. Siddiki, M. Mofijur, P.S. Kumar, S.F. Ahmed, A. Inayat, F. Kusumo, I.A. Badruddin,
477 T.M.Y. Khan, L.D. Nghiem, H.C. Ong, T.M.I. Mahlia, Microalgae biomass as a sustainable
478 source for biofuel, biochemical and biobased value-added products: An integrated
479 biorefinery concept, Fuel. 307 (2022) 121782. https://doi.org/10.1016/j.fuel.2021.121782.

480 [4] S. Onen Cinar, Z.K. Chong, M.A. Kucuker, N. Wieczorek, U. Cengiz, K. Kuchta, Bioplastic
481 Production from Microalgae: A Review, Int. J. Environ. Res. Public Health. 17 (2020) 3842.
482 https://doi.org/10.3390/ijerph17113842.

483 [5] B.T. Dang, X.T. Bui, D.P.H. Tran, H. Hao Ngo, L.D. Nghiem, T.K.D. Hoang, P.T. Nguyen,
484 H.H. Nguyen, T.K.Q. Vo, C. Lin, K. Yi Andrew Lin, S. Varjani, Current application of algae
485 derivatives for bioplastic production: A review, Bioresour. Technol. 347 (2022) 126698.
486 https://doi.org/10.1016/j.biortech.2022.126698.

487 [6] R. Madadi, H. Maljaee, L.S. Serafim, S.P.M. Ventura, Microalgae as contributors to produce

488 biopolymers, Mar. Drugs. 19 (2021) 466. https://doi.org/10.3390/MD19080466.
489 [7] S.S. Costa, A.L. Miranda, M.G. de Morais, J.A.V. Costa, J.I. Druzian, Microalgae as source
490 of polyhydroxyalkanoates (PHAs) — A review, Int. J. Biol. Macromol. 131 (2019) 536–547.
491 https://doi.org/10.1016/j.ijbiomac.2019.03.099.
492 [8] N. Mal, G.G. Satpati, S. Raghunathan, M.A. Davoodbasha, Current strategies on algae-based
493 biopolymer production and scale-up, Chemosphere. 289 (2022) 133178.
494 https://doi.org/10.1016/j.chemosphere.2021.133178.
495 [9] Z.A. Raza, S. Abid, I.M. Banat, Polyhydroxyalkanoates: Characteristics, production, recent
496 developments and applications, Int. Biodeterior. Biodegrad. 126 (2018) 45–56.
497 https://doi.org/10.1016/j.ibiod.2017.10.001.
498 [10] B. Singh Saharan, D. Sharma, Bioplastics-For Sustainable Development: A Review, Int. J.
499 Microb. Resour. Technol. Accept. 1 (2011) 11–23.
500 [11] C. Gioia, G. Giacobazzi, M. Vannini, G. Totaro, L. Sisti, M. Colonna, P. Marchese, A. Celli,
501 End of Life of Biodegradable Plastics: Composting versus Re/Upcycling, ChemSusChem. 14
502 (2021) 4167–4175. https://doi.org/10.1002/cssc.202101226.
503 [12] B. McAdam, M.B. Fournet, P. McDonald, M. Mojicevic, Production of polyhydroxybutyrate
504 (PHB) and factors impacting its chemical and mechanical characteristics, Polymers (Basel).
505 12 (2020) 2908. https://doi.org/10.3390/polym12122908.
506 [13] Y.J. Sohn, J. Son, S.Y. Jo, S.Y. Park, J.I. Yoo, K.A. Baritugo, J.G. Na, J. il Choi, H.T. Kim,
507 J.C. Joo, S.J. Park, Chemoautotroph *Cupriavidus necator* as a potential game-changer for
508 global warming and plastic waste problem: A review, Bioresour. Technol. 340 (2021)
509 125693. https://doi.org/10.1016/j.biortech.2021.125693.
510 [14] S. Venkata Mohan, M. Venkateswar Reddy, G. Venkata Subhash, P.N. Sarma, Fermentative
511 effluents from hydrogen producing bioreactor as substrate for poly(β-OH) butyrate
512 production with simultaneous treatment: An integrated approach, Bioresour. Technol. 101
513 (2010) 9382–9386. https://doi.org/10.1016/j.biortech.2010.06.109.

514 [15] J. Mozejko-Ciesielska, K. Szacherska, P. Marciniak, *Pseudomonas* Species as Producers of
515 Eco-friendly Polyhydroxyalkanoates, J. Polym. Environ. 27 (2019) 1151–1166.
516 https://doi.org/10.1007/s10924-019-01422-1.
517 [16] K. Amulya, M. Venkateswar Reddy, S. Venkata Mohan, Acidogenic spent wash valorization
518 through polyhydroxyalkanoate (PHA) synthesis coupled with fermentative biohydrogen
519 production, Bioresour. Technol. 158 (2014) 336–342.
520 https://doi.org/10.1016/j.biortech.2014.02.026.
521 [17] G. García, J.E. Sosa-Hernández, L.I. Rodas-Zuluaga, C. Castillo-Zacarías, H. Iqbal, R. Parra-
522 Saldívar, Accumulation of PHA in the Microalgae *Scenedesmus* sp. under Nutrient-Deficient
523 Conditions, Polymers (Basel). 13 (2021) 131. https://doi.org/10.3390/polym13010131.
524 [18] J. Il Choi, S.Y. Lee, Process analysis and economic evaluation for poly(3-hydroxybutyrate)
525 production by fermentation, Bioprocess Eng. 17 (1997) 335–342.
526 https://doi.org/10.1007/s004490050394.
527 [19] C. Samorì, M. Basaglia, S. Casella, L. Favaro, P. Galletti, L. Giorgini, D. Marchi, L.
528 Mazzocchetti, C. Torri, E. Tagliavini, Dimethyl carbonate and switchable anionic
529 surfactants: Two effective tools for the extraction of polyhydroxyalkanoates from microbial
530 biomass, Green Chem. 17 (2015) 1047–1056. https://doi.org/10.1039/c4gc01821d.
531 [20] G. Mannina, D. Presti, G. Montiel-Jarillo, J. Carrera, M.E. Suárez-Ojeda, Recovery of
532 polyhydroxyalkanoates (PHAs) from wastewater: A review, Bioresour. Technol. 297 (2020)
533 122478. https://doi.org/10.1016/j.biortech.2019.122478.
534 [21] P. Sharma, V.K. Gaur, S.H. Kim, A. Pandey, Microbial strategies for bio-transforming food
535 waste into resources, Bioresour. Technol. 299 (2020) 122580.
536 https://doi.org/10.1016/j.biortech.2019.122580.
537 [22] R. Sirohi, J.S. Lee, B.S. Yu, H. Roh, S.J. Sim, Sustainable production of
538 polyhydroxybutyrate from autotrophs using CO_2 as feedstock: Challenges and opportunities,
539 Bioresour. Technol. 341 (2021) 125751. https://doi.org/10.1016/j.biortech.2021.125751.

540 [23] R. Carpine, G. Olivieri, K.J. Hellingwerf, A. Pollio, A. Marzocchella, Industrial production
541 of poly-β-hydroxybutyrate from CO_2: Can cyanobacteria meet this challenge?, Processes. 8
542 (2020) 1–23. https://doi.org/10.3390/pr8030323.
543 [24] J. Możejko-Ciesielska, R. Kiewisz, Bacterial polyhydroxyalkanoates: Still fabulous?,
544 Microbiol. Res. 192 (2016) 271–282. https://doi.org/10.1016/j.micres.2016.07.010.
545 [25] D.G. Gradíssimo, L.P. Xavier, A.V. Santos, Cyanobacterial polyhydroxyalkanoates: A
546 sustainable alternative in circular economy, Molecules. 25 (2020) 1–23.
547 https://doi.org/10.3390/molecules25184331.
548 [26] A.K. Singh, L. Sharma, N. Mallick, J. Mala, Progress and challenges in producing
549 polyhydroxyalkanoate biopolymers from cyanobacteria, J. Appl. Phycol. 29 (2017) 1213–
550 1232. https://doi.org/10.1007/s10811-016-1006-1.
551 [27] S.M. Abdo, G.H. Ali, Analysis of polyhydroxybutrate and bioplastic production from
552 microalgae, Bull. Natl. Res. Cent. 43 (2019) 1–4. https://doi.org/10.1186/s42269-019-0135-
553 5.
554 [28] R.J. Wicker, H. Autio, E. Daneshvar, B. Sarkar, N. Bolan, V. Kumar, A. Bhatnagar, The
555 effects of light regime on carbon cycling, nutrient removal, biomass yield, and
556 polyhydroxybutyrate (PHB) production by a constructed photosynthetic consortium,
557 Bioresour. Technol. 363 (2022) 127912.
558 [29] N. Krasaesueb, A. Promariya, W. Raksajit, W. Khetkorn, Inactivation of phosphate regulator
559 (SphU) in cyanobacterium *Synechocystis* sp. 6803 directly induced acetyl phosphate pathway
560 leading to enhanced PHB level under nitrogen-sufficient condition, J. Appl. Phycol. 33
561 (2021) 2135–2144. https://doi.org/10.1007/s10811-021-02460-w.
562 [30] S.S. Costa, A.L. Miranda, B.B. Andrade, D. de J. Assis, C.O. Souza, M.G. de Morais, J.A.V.
563 Costa, J.I. Druzian, Influence of nitrogen on growth, biomass composition, production, and
564 properties of polyhydroxyalkanoates (PHAs) by microalgae, Int. J. Biol. Macromol. 116
565 (2018) 552–562. https://doi.org/10.1016/j.ijbiomac.2018.05.064.

566 [31] S. Samantaray, N. Mallick, Impact of Various Stress Conditions on Poly-β-Hydroxybutyrate
567 (PHB) Accumulation in *Aulosira fertilissima* CCC 444, Curr. Biotechnol. 4 (2015) 366–372.
568 https://doi.org/10.2174/2211550104666150806000642.
569 [32] A. Kaewbai-ngam, A. Incharoensakdi, T. Monshupanee, Increased accumulation of
570 polyhydroxybutyrate in divergent cyanobacteria under nutrient-deprived photoautotrophy:
571 An efficient conversion of solar energy and carbon dioxide to polyhydroxybutyrate by
572 Calothrix scytonemicola TISTR 8095, Bioresour. Technol. 212 (2016) 342–347.
573 https://doi.org/10.1016/j.biortech.2016.04.035.
574 [33] M. Simonazzi, L. Pezzolesi, P. Galletti, C. Gualandi, R. Pistocchi, N. De Marco, Z.
575 Paganelli, C. Samorì, Production of polyhydroxybutyrate by the cyanobacterium cf.
576 *Anabaena* sp., Int. J. Biol. Macromol. 191 (2021) 92–99.
577 https://doi.org/10.1016/j.ijbiomac.2021.09.054.
578 [34] F. Hempel, A.S. Bozarth, N. Lindenkamp, A. Klingl, S. Zauner, U. Linne, A. Steinbüchel,
579 U.G. Maier, Microalgae as bioreactors for bioplastic production, Microb. Cell Fact. 10
580 (2011) 1–6. https://doi.org/10.1186/1475-2859-10-81.
581 [35] A.P.A. Cassuriaga, B.C.B. Freitas, M.G. Morais, J.A.V. Costa, Innovative
582 polyhydroxybutyrate production by *Chlorella fusca* grown with pentoses, Bioresour.
583 Technol. 265 (2018) 456–463. https://doi.org/10.1016/j.biortech.2018.06.026.
584 [36] S.K. Das, A. Sathish, J. Stanley, Production of Biofuel and Bioplastic from *Chlorella*
585 *pyrenoidosa*, Mater. Today Proc. 5 (2018) 16774–16781.
586 https://doi.org/10.1016/j.matpr.2018.06.020.
587 [37] R. Robert, P.R. Iyer, Isolation and Optimization of PHB (Poly-β-hydroxybutyrate) Based
588 Biodegradable Plastics from <i>Chlorella vulgaris<i>, J. Bioremediation Biodegrad. (2018)
589 2–5. https://doi.org/10.4172/2155-6199.1000433.
590 [38] C. Safi, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Morphology, composition,
591 production, processing and applications of *Chlorella vulgaris*: A review, Renew. Sustain.

592 Energy Rev. 35 (2014) 265–278. https://doi.org/10.1016/j.rser.2014.04.007.
593 [39] M.C. Chan, S.H. Ho, D.J. Lee, C.Y. Chen, C.C. Huang, J.S. Chang, Characterization,
594 extraction and purification of lutein produced by an indigenous microalga *Scenedesmus*
595 *obliquus* CNW-N, Biochem. Eng. J. 78 (2013) 24–31.
596 https://doi.org/10.1016/j.bej.2012.11.017.
597 [40] F. Di Caprio, P. Altimari, F. Pagnanelli, Integrated biomass production and biodegradation of
598 olive mill wastewater by cultivation of *Scenedesmus* sp., Algal Res. 9 (2015) 306–311.
599 https://doi.org/10.1016/j.algal.2015.04.007.
600 [41] L. Wang, M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, R. Ruan, Cultivation of green
601 algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant,
602 Appl. Biochem. Biotechnol. 162 (2010) 1174–1186. https://doi.org/10.1007/s12010-009-
603 8866-7.
604 [42] M. Radkova, M. Stoyneva-Gärtner, I. Dincheva, P. Stoykova, B. Uzunov, P. Dimitrova, C.
605 Borisova, G. Gärtner, *Chlorella vulgaris* H1993 and *Desmodesmus communis* H522 for low-
606 cost production of high-value microalgal products, Biotechnol. Biotechnol. Equip. 33 (2019)
607 243–249. https://doi.org/10.1080/13102818.2018.1562381.
608 [43] M.M. Zaharieva, D. Zheleva-Dimitrova, S. Rusinova-Videva, Y. Ilieva, A. Brachkova, V.
609 Balabanova, R. Gevrenova, T.C. Kim, M. Kaleva, A. Georgieva, M. Mileva, K. Yoncheva,
610 N. Benbassat, H. Najdenski, A.D. Kroumov, Antimicrobial and Antioxidant Potential of
611 *Scenedesmus obliquus* Microalgae in the Context of Integral Biorefinery Concept, Molecules.
612 27 (2022) 519. https://doi.org/10.3390/molecules27020519.
613 [44] G. Samorì, C. Samorì, F. Guerrini, R. Pistocchi, Growth and nitrogen removal capacity of
614 *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in
615 view of urban wastewater treatment: Part I, Water Res. 47 (2013) 791–801.
616 https://doi.org/10.1016/j.watres.2012.11.006.
617 [45] G. Samorì, C. Samorì, R. Pistocchi, Nutrient removal efficiency and physiological responses

618 of *Desmodesmus communis* at different HRTs and nutrient stress condition using different
619 sources of urban wastewater effluents, Appl. Biochem. Biotechnol. 173 (2014) 74–89.
620 https://doi.org/10.1007/s12010-014-0792-7.
621 [46] S.P. Chu, The Influence of the Mineral Composition of the Medium on the Growth of
622 Planktonic Algae: Part I. Methods and Culture Media, J. Ecol. 30, No. 2 (1942) 284–325.
623 [47] APHA, Standard Methods for the Examination of Water and Wastewater, Stand. Methods.
624 (1995). https://doi.org/ISBN 9780875532356.
625 [48] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J.
626 Biochem. Physiol. 37 (1959) 911–917. https://doi.org/dx.doi.org/10,1139/cjm2014-0700.
627 [49] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin
628 phenol reagent., J. Biol. Chem. 193 (1951) 265–275. https://doi.org/10.1016/0304-
629 3894(92)87011-4.
630 [50] S. Myklestad, A. Haug, Production of carbohydrates by the marine diatom *Chaetoceros*
631 *affinis* var. willei (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture
632 medium, J. Exp. Mar. Bio. Ecol. (1972). https://doi.org/10.1016/0022-0981(72)90041-X.
633 [51] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for
634 determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
635 https://doi.org/10.1021/ac60111a017.
636 [52] F. Abbondanzi, G. Biscaro, G. Carvalho, L. Favaro, P. Lemos, M. Paglione, C. Samorì, C.
637 Torri, Fast method for the determination of short-chain-length polyhydroxyalkanoates (scl-
638 PHAs) in bacterial samples by In Vial-Thermolysis (IVT), N. Biotechnol. 39 (2017) 29–35.
639 https://doi.org/10.1016/j.nbt.2017.05.012.
640 [53] Ø. Hammer, D.A.T. a. T. Harper, P.D. Ryan, PAST: Paleontological Statistics Software
641 Package for Education and Data Analysis, Palaeontol. Electron. 4 (2001) 1–9.
642 https://doi.org/10.1016/j.bcp.2008.05.025.
643 [54] C. Kourmentza, J. Plácido, N. Venetsaneas, A. Burniol-Figols, C. Varrone, H.N. Gavala,

644 M.A.M. Reis, Recent advances and challenges towards sustainable polyhydroxyalkanoate
645 (PHA) production, Bioengineering. 4 (2017) 55.
646 https://doi.org/10.3390/bioengineering4020055.
647 [55] R. De Philippis, A. Ena, M. Guastiini, C. Sili, M. Vincenzini, Factors affecting poly-β-
648 hydroxybutyrate accumulation in cyanobacteria and in purple non-sulfur bacteria, FEMS
649 Microbiol. Lett. (1992). https://doi.org/10.1016/0378-1097(92)90309-C.
650 [56] E.S. Salama, M.B. Kurade, R.A.I. Abou-Shanab, M.M. El-Dalatony, I.S. Yang, B. Min, B.H.
651 Jeon, Recent progress in microalgal biomass production coupled with wastewater treatment
652 for biofuel generation, Renew. Sustain. Energy Rev. 79 (2017) 1189–1211.
653 https://doi.org/10.1016/j.rser.2017.05.091.
654 [57] M. Ciebiada, K. Kubiak, M. Daroch, Modifying the cyanobacterial metabolism as a key to
655 efficient biopolymer production in photosynthetic microorganisms, Int. J. Mol. Sci. 21
656 (2020) 1–24. https://doi.org/10.3390/ijms21197204.
657 [58] V. Dutt, S. Srivastava, Novel quantitative insights into carbon sources for synthesis of poly
658 hydroxybutyrate in Synechocystis PCC 6803, Photosynth. Res. 136 (2018) 303–314.
659 https://doi.org/10.1007/s11120-017-0464-x.
660 [59] L. Pezzolesi, M. Mazzotti, S. Vanucci, R. Pistocchi, Assimilation of inorganic nitrogen for
661 scaling up *Desmodesmus communis* (Scenedesmaceae) biomass production, J. Appl. Phycol.
662 (2019). https://doi.org/10.1007/s10811-019-01814-9.
663 [60] D.M. Arias, J. García, E. Uggetti, Production of polymers by cyanobacteria grown in
664 wastewater: Current status, challenges and future perspectives, N. Biotechnol. 55 (2020) 46–
665 57. https://doi.org/10.1016/j.nbt.2019.09.001.
666 [61] G. Kavitha, C. Kurinjimalar, K. Sivakumar, M. Kaarthik, R. Aravind, P. Palani, R.
667 Rengasamy, Optimization of polyhydroxybutyrate production utilizing waste water as
668 nutrient source by *Botryococcus braunii* Kütz using response surface methodology, Int. J.
669 Biol. Macromol. 93 (2016) 534–542.

670 [62] S.M. Haase, B. Huchzermeyer, T. Rath, PHB accumulation in *Nostoc muscorum* under
671 different carbon stress situations, J. Appl. Phycol. (2012). https://doi.org/10.1007/s10811-
672 011-9663-6.
673 [63] R. Bhati, N. Mallick, Carbon dioxide and poultry waste utilization for production of
674 polyhydroxyalkanoate biopolymers by *Nostoc muscorum* Agardh: a sustainable approach, J.
675 Appl. Phycol. 28 (2016) 161–168. https://doi.org/10.1007/s10811-015-0573-x.
676 [64] M.A. Yaakob, R.M.S.R. Mohamed, A. Al-Gheethi, G.A. Ravishankar, R.R. Ambati,
677 Influence of nitrogen and phosphorus on microalgal growth, biomass, lipid, and fatty acid
678 production: An overview, Cells. 10 (2021) 393. https://doi.org/10.3390/cells10020393.
679 [65] R. Srinivasan, A. Mageswari, P. Subramanian, C. Suganthi, A. Chaitanyakumar, V. Aswini,
680 K.M. Gothandam, Bicarbonate supplementation enhances growth and biochemical
681 composition of Dunaliella salina V-101 by reducing oxidative stress induced during
682 macronutrient deficit conditions, Sci. Rep. (2018) 6972. https://doi.org/10.1038/s41598-018-
683 25417-5.
684 [66] K. Samadhiya, A. Ghosh, R. Nogueira, K. Bala, Newly isolated native microalgal strains
685 producing polyhydroxybutyrate and energy storage precursors simultaneously: Targeting
686 microalgal biorefinery, Algal Res. 62 (2022) 102625.
687 https://doi.org/10.1016/j.algal.2021.102625.
688 [67] S.G. Mastropetros, K. Pispas, D. Zagklis, S.S. Ali, M. Kornaros, Biopolymers production
689 from microalgae and cyanobacteria cultivated in wastewater: Recent advances, Biotechnol.
690 Adv. 60 (2022) 107999.
691 [68] A.E.M.M.R. Afify, G.S. El Baroty, F.K. El Baz, H.H. Abd El Baky, S.A. Murad,
692 *Scenedesmus obliquus*: Antioxidant and antiviral activity of proteins hydrolyzed by three
693 enzymes, J. Genet. Eng. Biotechnol. 16 (2018) 399–408.
694 https://doi.org/10.1016/j.jgeb.2018.01.002.
695 [69] J.M.L. Dias, P.C. Lemos, L.S. Serafim, C. Oliveira, M. Eiroa, M.G.E. Albuquerque, A.M.

696 Ramos, R. Oliveira, M.A.M. Reis, Recent advances in polyhydroxyalkanoate production by
697 mixed aerobic cultures: From the substrate to the final product, Macromol. Biosci. 6 (2006)
698 885–906. https://doi.org/10.1002/mabi.200600112.
699 [70] L.S. Serafim, P.C. Lemos, M.G.E. Albuquerque, M.A.M. Reis, Strategies for PHA
700 production by mixed cultures and renewable waste materials, Appl. Microbiol. Biotechnol.
701 81 (2008) 615–628. https://doi.org/10.1007/s00253-008-1757-y.
702 [71] M.H. Jau, S.P. Yew, P.S.Y. Toh, A.S.C. Chong, W.L. Chu, S.M. Phang, N. Najimudin, K.
703 Sudesh, Biosynthesis and mobilization of poly(3-hydroxybutyrate) [P(3HB)] by Spirulina
704 platensis, Int. J. Biol. Macromol. 36 (2005) 144–151.
705 https://doi.org/10.1016/j.ijbiomac.2005.05.002.
706 [72] K. Sudesh, K. Taguchi, Y. Doi, Effect of increased PHA synthase activity on
707 polyhydroxyalkanoates biosynthesis in *Synechocystis* sp. PCC6803, Int. J. Biol. Macromol.
708 30 (2002) 97–104. https://doi.org/10.1016/S0141-8130(02)00010-7.
709 [73] G. Kavitha, C. Kurinjimalar, K. Sivakumar, P. Palani, R. Rengasamy, Biosynthesis,
710 purification and characterization of polyhydroxybutyrate from *Botryococcus braunii* kütz,
711 Int. J. Biol. Macromol. (2016). https://doi.org/10.1016/j.ijbiomac.2016.04.086.
712 [74] M.M.A. Nur, A. Yuliestyan, F. Irfandy, T.M. Setyoningrum, Nutritional factors influence
713 polyhydroxybutyrate in microalgae growing on palm oil mill effluent, J. Appl. Phycol. 34
714 (2022) 127–133. https://doi.org/10.1007/s10811-021-02654-2.
715 [75] P. Kumari, B.R. Kiran, S.V. Mohan, Polyhydroxybutyrate production by *Chlorella*
716 *sorokiniana* SVMIICT8 under Nutrient-deprived mixotrophy, Bioresour. Technol. 354
717 (2022) 127135.
718 [76] M.M. Mourão, D.G. Gradíssimo, A.V. Santos, M.P.C. Schneider, S.M.M. Faustino, V.
719 Vasconcelos, L.P. Xavier, Optimization of polyhydroxybutyrate production by amazonian
720 microalga *Stigeoclonium* sp. B23, Biomolecules. 10 (2020) 1628.
721 https://doi.org/10.3390/biom10121628.

722 [77] M.M. Mourão, L.P. Xavier, R. Urbatzka, L.B. Figueiroa, C.E.F. da Costa, C.G.B.T. Dias,
723 M.P.C. Schneider, V. Vasconcelos, A.V. Santos, Characterization and biotechnological
724 potential of intracellular polyhydroxybutyrate by *Stigeoclonium* sp. B23 using cassava peel
725 as carbon source, Polymers (Basel). 13 (2021) 687. https://doi.org/10.3390/polym13050687.
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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

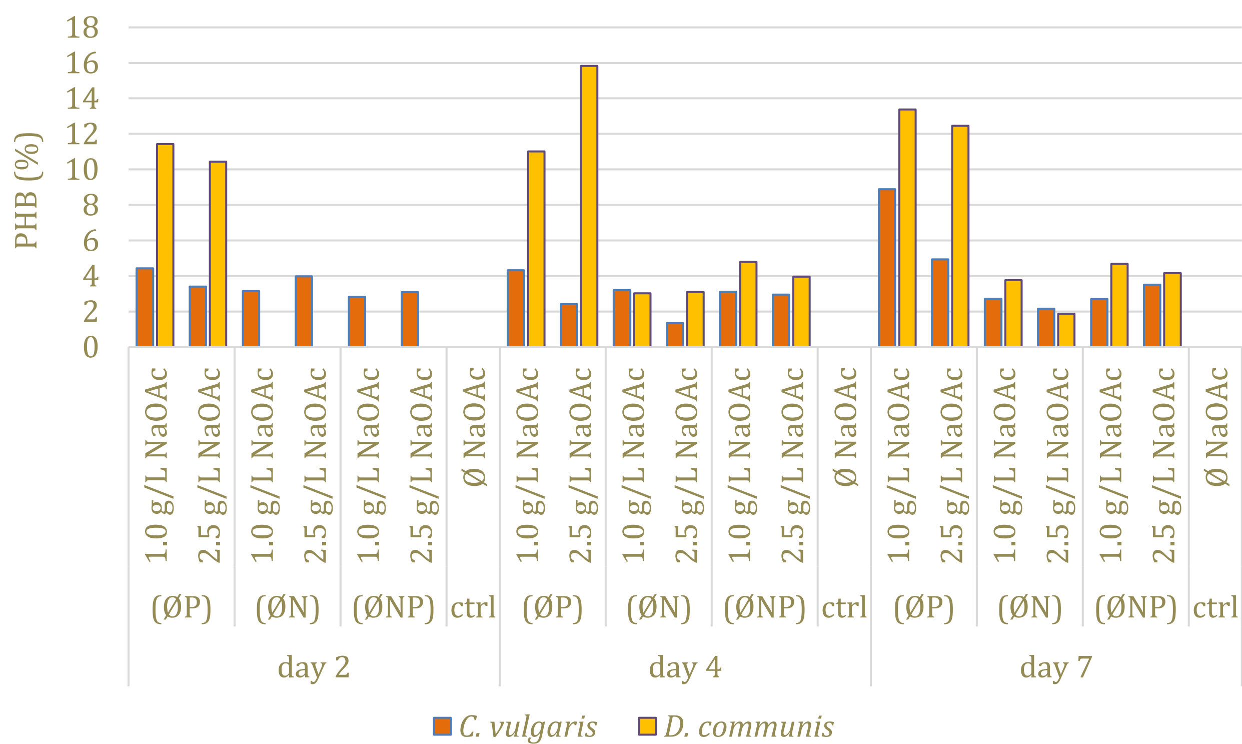


Fig. 1 – PHB amount (% w/w) accumulated by *D. communis* and *C. vulgaris* in the screening test with nutrient depletion (ØN, ØP, or both ØNP) and different sodium acetate concentrations (NaOAc, 1 or 2.5 g/L). crtl: control condition with nutrients.

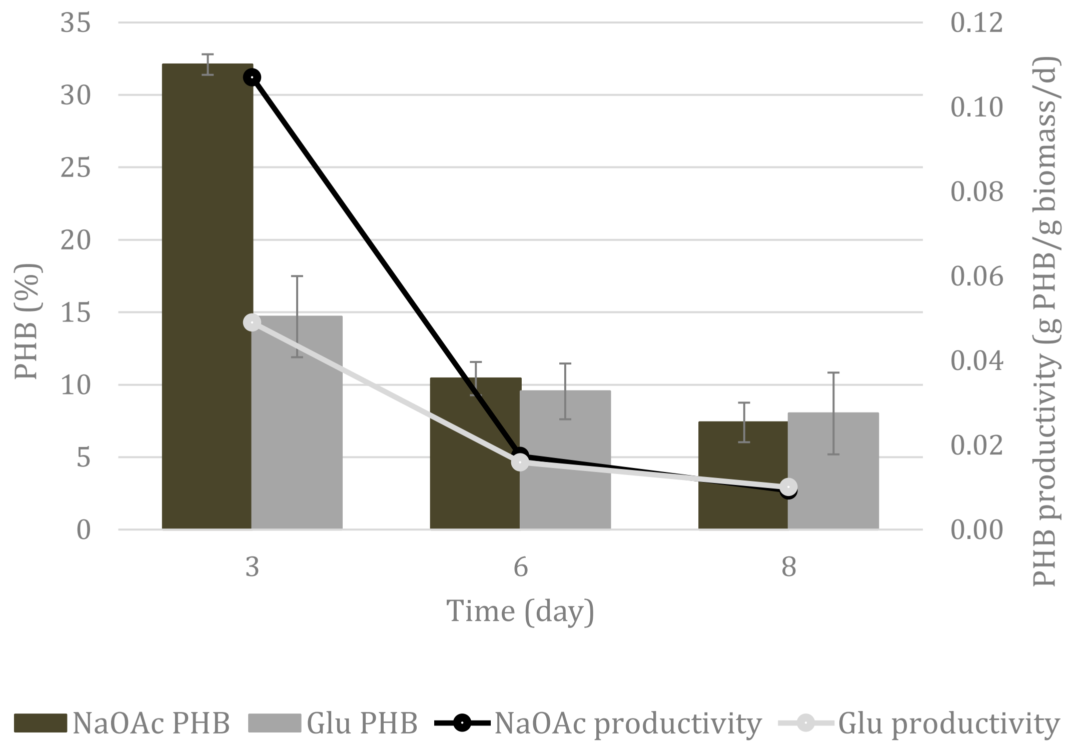
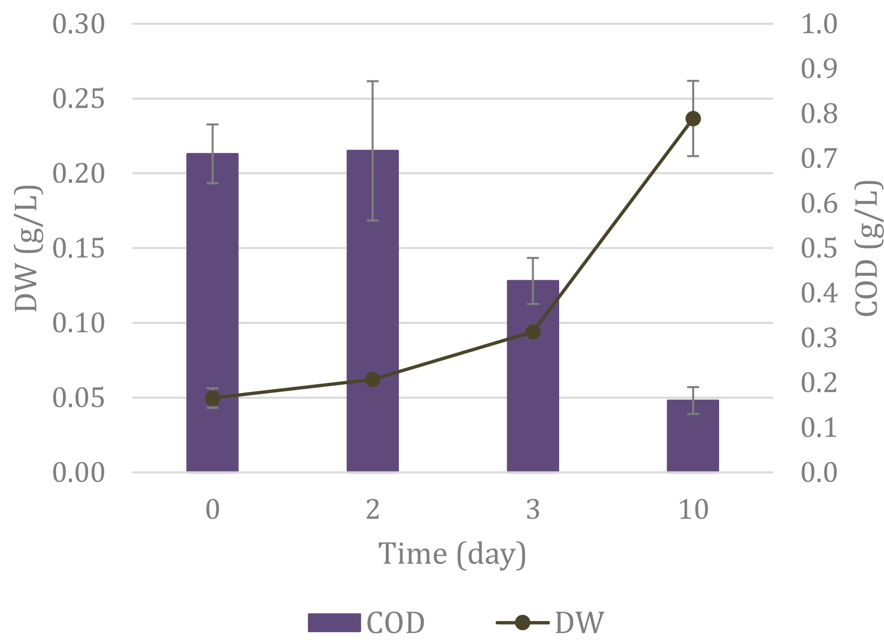


Fig. 2 – PHB amount (% w/w) and productivity ($g_{PHB}/g_{biomass}/d$) in *D. communis* grown for 8 days with sodium acetate (NaOAc) and glucose (Glu) as carbon sources.

a)



b)

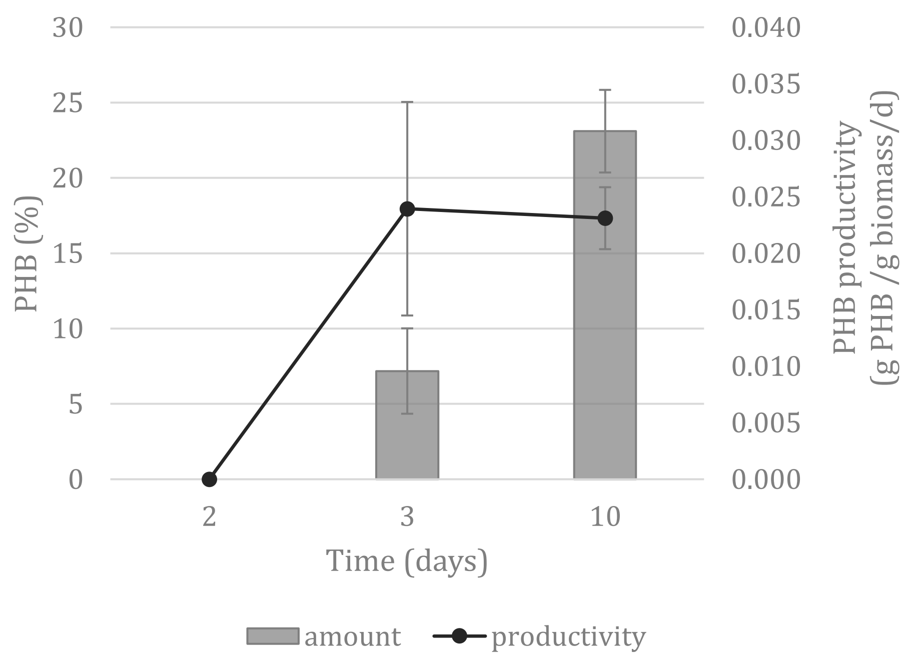


Fig. 3 – a) *D. communis* growth (expressed as dry weight, DW, g/L) and COD values of the medium after biomass filtration (gO_2/L) and b) PHB amount (% w/w) and productivity ($g_{PHB}/g_{biomass}$/d) in the batch cultivation test (10L PBR).

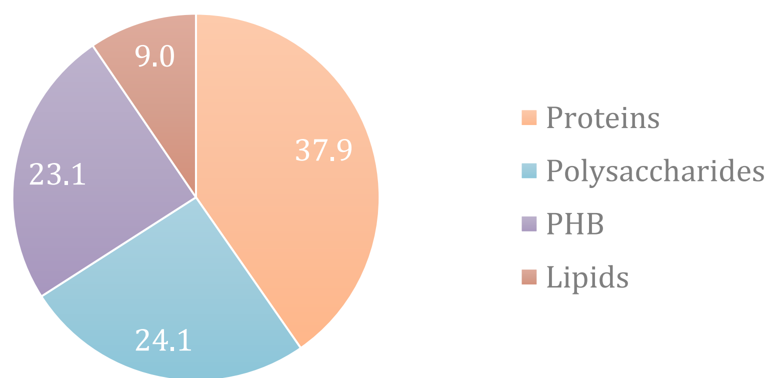
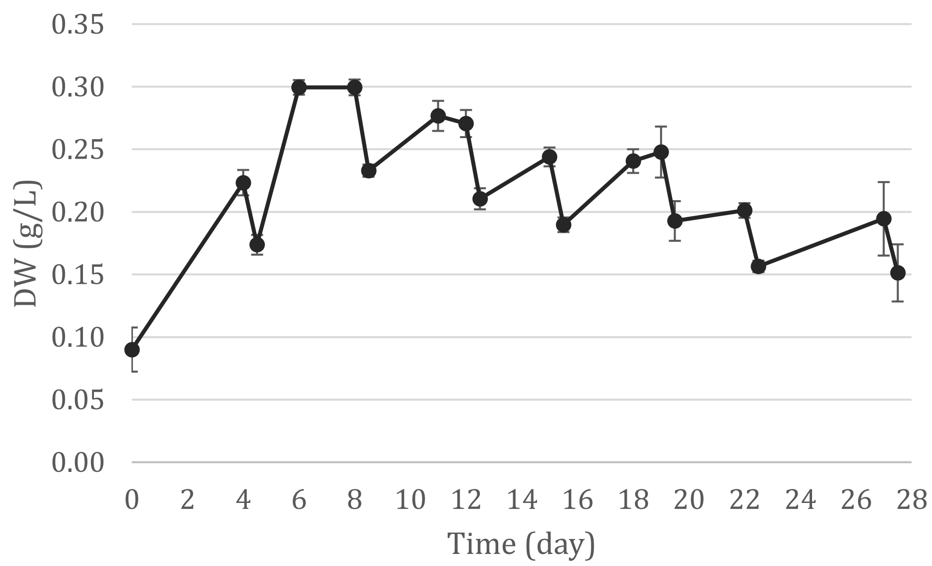


Fig. 4 – Biomass composition (%) of *D. communis* at day 10 in the batch cultivation test (10L PBR).

a)



b)

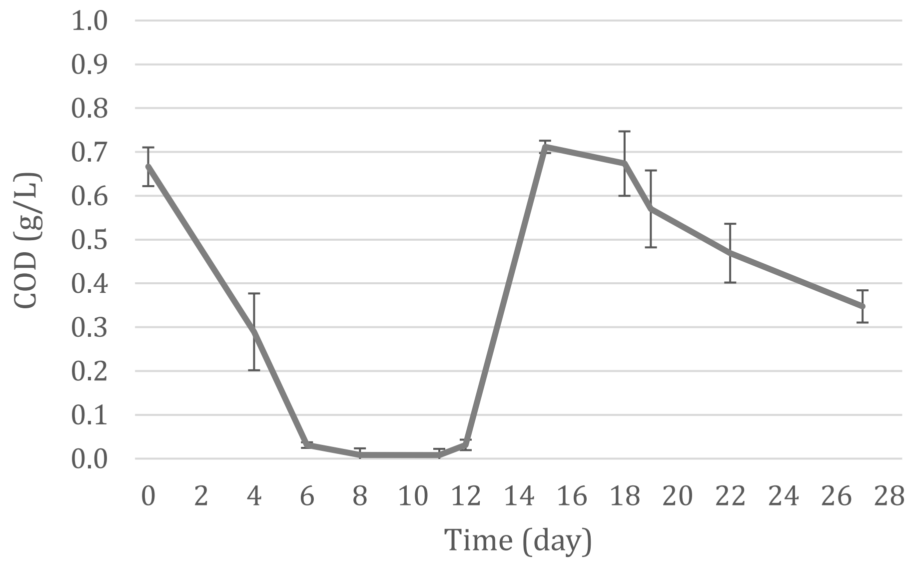
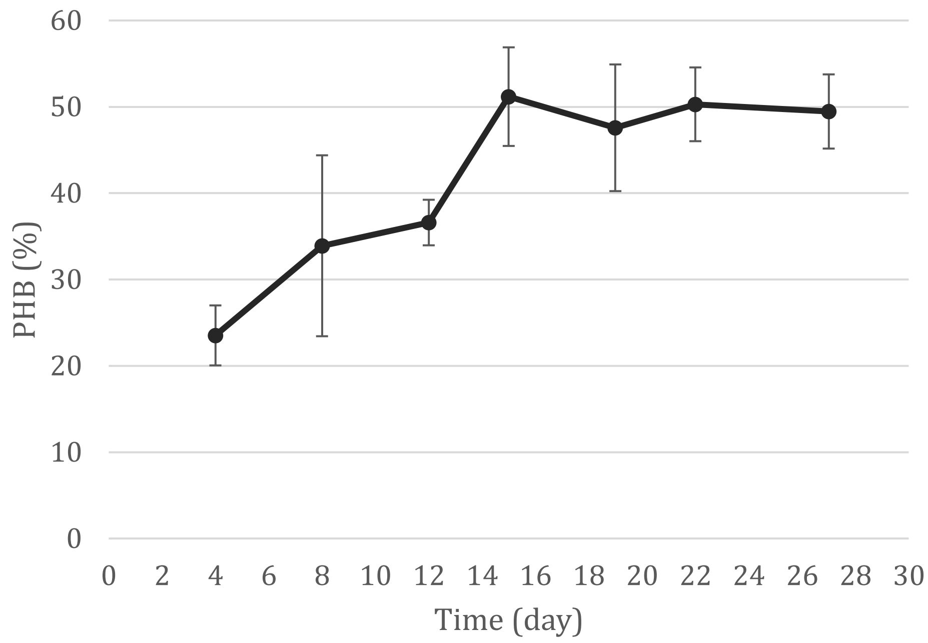


Fig. 5 – a) Semi-continuous growth of *D. communis* in the batch cultivation test (10L PBR) expressed as dry weight (DW, g/L); b) COD values of the medium after biomass filtration (gO_2/L).

a)



b)

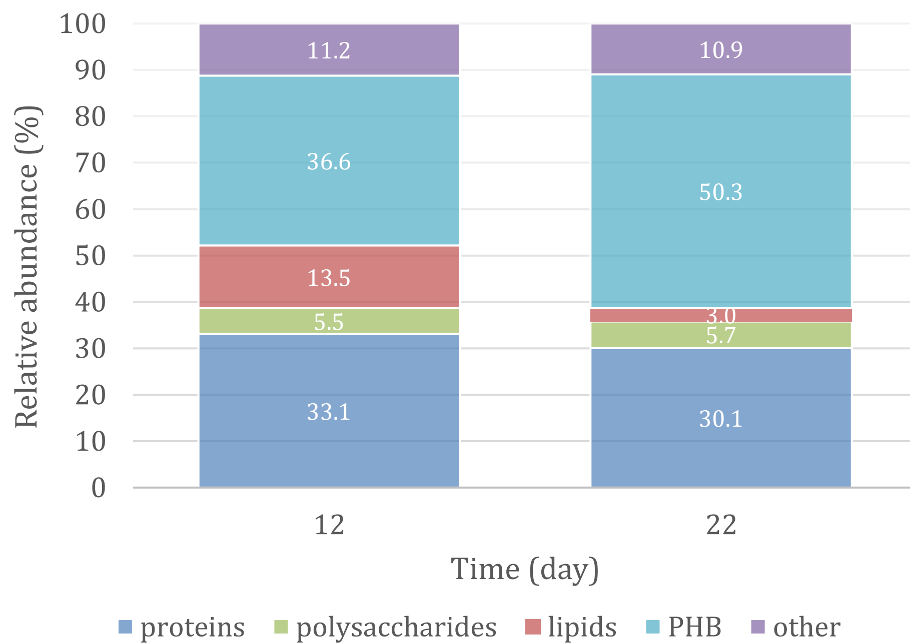


Fig. 6 – a) PHB amount (% w/w) and b) biomass composition (%) of *D. communis* after 12 and 22 days during the semi-continuous cultivation in a 10L PBR.

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