



# Efficient utilization of monosaccharides from agri-food byproducts supports *Chlorella vulgaris* biomass production under mixotrophic conditions

Francesca Angelini<sup>a,1</sup>, Erika Bellini<sup>a,1</sup>, Angela Marchetti<sup>b</sup>, Gaia Salvatori<sup>b</sup>,  
Marianna Villano<sup>b,c</sup>, Daniela Pontiggia<sup>a,c</sup>, Simone Ferrari<sup>a,c,\*</sup>

<sup>a</sup> Department of Biology and biotechnologies "Charles Darwin", Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy

<sup>b</sup> Department of Chemistry, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

<sup>c</sup> Research Center for Applied Sciences to the Safeguard of Environment and Cultural Heritage (CIABC), Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

## ARTICLE INFO

### Keywords:

*Chlorella vulgaris*  
Agri-food waste  
Microalgae  
Mixotrophy  
Biomass production

## ABSTRACT

Microalgae are promising resources for the sustainable production of biofuels, feed, and high-value chemicals. Several strains can grow heterotrophically or mixotrophically on multiple organic substrates even if the high cost associated to their use can hinder scalability and economical sustainability of the overall process. The use of agri-food waste biomass hydrolysates might make the cultivation procedure more sustainable, while at the same time valorising underutilized by-products. In this study, *Chlorella vulgaris* biomass production and sugar utilization was investigated during mixotrophic cultivation on hydrolysates of two inexpensive and widely available recalcitrant agri-food waste biomasses: barley straw (BS) and citrus processing waste (CPW). CPW hydrolysate supported enhanced biomass production, compared to BS digestate, likely because of the presence, besides glucose, of significant amounts of galactose, which is rapidly metabolized by the algae. Notably, when pure monosaccharides were provided as sole organic carbon, growth stopped before complete sugar consumption. Arrested growth in presence of pure monosaccharides correlated with a drastic drop in extracellular pH, which appears to depend on both carbon and nitrogen sources. Our results show that mixotrophic cultivation of *C. vulgaris* on BS or CPW hydrolysates results in more efficient conversion of organic carbon into biomass, compared to growth on pure sugars, indicating that these agri-food by-products can be utilized as valid feedstocks for sustainable algal biomass production.

## 1. Introduction

Microalgae are photosynthetic microorganisms, both prokaryotic and eukaryotic, that can be employed for the production of feed, fuel and high-value biomolecules [1]. Due to their simple structure and high cell surface/volume ratio, microalgae can grow up to 100 times faster than higher plants, doubling their biomass in less than one day [2]. In addition, compared to plants, they assimilate CO<sub>2</sub> more efficiently [3], require smaller areas of cultivation and do not need herbicides and pesticides [4,5]. Moreover, they tolerate a wide range of temperatures, salinities, pH values and different light conditions [6]. Owing to these features, current industrial cultivation of microalgae finds applications in many sectors, like production of nutraceuticals, pharmaceuticals, dietary supplements, and bioenergy [7–9].

The major hurdle to commercialization of microalgal products is the high cost of biomass production and extraction of the valuable products [10,11]. These costs can be minimized by increasing algae productivity, e.g. optimizing light use efficiency, integrating waste streams, decreasing infrastructural costs and improving industrial scale algae production logistics [12–15]. In addition, the availability and form of nutrients, particularly carbon, followed by nitrogen and phosphorous, greatly affects biomass accumulation and product yield [16]. For many species, biomass yield can be increased by providing organic carbon sources under heterotrophic (in dark) or mixotrophic (in light) cultivation modes [17]. Mixotrophic metabolism allows simultaneous assimilation of CO<sub>2</sub> and organic carbon [18–21], reducing the dependence on a single energy source [22] and increasing carbon flow to biomass [23]. However, an abundant and economically viable carbon source is

\* Corresponding author at: Department of Biology and biotechnologies "Charles Darwin", Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy.  
E-mail address: [simone.ferrari@uniroma1.it](mailto:simone.ferrari@uniroma1.it) (S. Ferrari).

<sup>1</sup> These authors contributed equally.

required to pursue sustainable biorefinery processes-based microalgae. In this context, agricultural residues and by-products of the food industry offer substantial potential for this purpose, owing to their cost-effectiveness, extensive availability, and the imperative need for disposal. For example, world cereal production in 2022 corresponded to around 2.8 million tons [24], with straw representing up to 50 % of the dry weight of crops. Another example of abundant agri-food by-product is citrus processing waste (CPW), representing around 44–60 % of the mass of fruits employed in orange juice production [25,26]. Approximately 10 million tons of wet CPW is annually generated worldwide by the orange processing industry alone [27]. Its easy fermentability makes it extremely pollutant [28], and its proper disposal requires significant investment [29]. Since agri-food by-products mostly consist of plant cell wall polysaccharides (cellulose, hemicelluloses and pectin), they represent a potential source of simple sugars for microalgae cultivation, that can be obtained by hydrolysis of the raw biomass [30–33]. Depending on polysaccharide composition, not all biomass originating from agriculture residues exhibit facile conversion into simple sugars; on the contrary, some require specific treatments, either chemical or physical, to mitigate recalcitrance to the saccharification process. Although this clearly affects the cost of the overall process of large-scale algal cultivation, within the context of a circular economy, the expense associated with enzymatic treatment represents a necessary investment to utilize certain exceptionally recalcitrant biomasses that would otherwise persist as waste materials to dispose of. In addition to sugars, agri-food waste hydrolysates contain other nutrients, such as organic and inorganic nitrogen, phosphates, potassium, and vitamins, that can improve algal biomass production [37,38]. Among microalgal species able to grow under mixotrophic conditions, those belonging to the genus *Chlorella* can accumulate large amounts of lipids, proteins, and other value-added products and have good productivity under different regimes [34,35]. Many studies demonstrated the efficient cultivation of *Chlorella* spp. using both hydrolysates of different waste biomasses [36–38] and pure sugars [39–41], even if with different biomass yields. For instance, *C. pyrenoidosa* shows enhanced mixotrophic growth in the presence of rice straw or sugarcane bagasse hydrolysates, compared to pure Glc at the same starting concentrations of reducing sugars [42,43]. Researchers have also investigated the potential of CPW as a substrate for mixotrophic growth of *Chlorella* strains [44,45]. Among these, *C. vulgaris*, a highly resistant, fast-growing green algae can use different organic carbon sources, such as glycerol, glucose, or acetate [46] to synthesize compounds of significant industrial value, including phenolics, chlorophyll, lutein and other carotenoids, linolenic acid, and vitamins [47–49]. *C. vulgaris* has one of the highest market values, among commercialized microalgae, expected to reach USD 412.3 million by 2028, and an actual yield of 5000 tons of dry matter per year [50]. Moreover, *C. vulgaris* is widely used in the food and feed industries, but also in the cosmetic and pharmaceutical fields [49].

In this study, we investigated the use of very different agri-food biomass as source of simple sugars for algae mixotrophic growth: barley straw (BS), representative of homogeneous but recalcitrant lignocellulosic materials, and CPW, a mixture of citrus peels, leaves, pulp and branches, representative of a very heterogeneous but more digestible biomass. This work demonstrates that both tested materials represent suitable sources of organic carbon for *C. vulgaris*, here chosen as model organism for its robustness and industrial value. Moreover, we found that algae use the monosaccharides present in the hydrolysates more efficiently than when cultured on single pure sugars, which likely contributes to support enhanced growth.

## 2. Material and methods

### 2.1. Production of biomass hydrolysates

Dried barley (*Hordeum vulgare*) straw (BS) was kindly provided by Luigi Cattivelli (Consiglio per la ricerca in agricoltura e l'analisi

dell'economia - Centro di ricerca per la genomica vegetale, Fiorenzuola d'Arda, Italy). BS was micronized and sieved to 1 mm particles and stored at room temperature. Fresh tangerine (*Citrus tangerina*) processing waste (CPW) was obtained from a juice producer from Santa Flavia (Palermo, Italy). CPW was oven-dried, and stored at  $-20^{\circ}\text{C}$ . Before use, dried CPW was manually ground. Orange peels (OP), dried and ground using a trash compactor, were obtained from Daniel Valentin Savatin (University of Tuscia, Viterbo, Italy).

For saccharification, BS and CPW were autoclaved and resuspended at 5 % (w/v) in a solution containing 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.15 supplemented with 1 % (v/v) Celluclast 1.5L (cellulases from *Trichoderma reesei* ATTC 26921) (Sigma-Aldrich, St. Louis, USA) and 1 % (v/v) Pectinex 3XL (pectinases from *Aspergillus niger* P-2736) (Sigma-Aldrich). Saccharification was performed in 250 mL-Erlenmeyer flasks in constant agitation at  $37^{\circ}\text{C}$  for seven days. The digestates were filtered with sterile Miracloth, incubated for 15 min at  $75^{\circ}\text{C}$  in a heated bath to inactivate the enzymes, and centrifuged for 5 min at  $10,000 \times g$ . The supernatant was collected for subsequent experiments.

Extraction of sugars from OP was performed incubating the autoclaved biomass (10 % w/v) in distilled water for 3 h at  $30^{\circ}\text{C}$ . Then, the solution was centrifuged for 5 min at  $10,000 \times g$  and, the extract was filtrated with  $0.22 \mu\text{m}$  filters to avoid any contamination.

### 2.2. *Chlorella vulgaris* cultivation

*C. vulgaris* strain 211-11 (SAG Culture Collection of Algae, Göttingen University, Germany) was kindly provided by Prof. Roberto Bassi (Department of Biotechnologies, University of Verona). *C. vulgaris* was maintained on Tris-acetate-phosphate (TAP) agar plates (20 mM Tris base, 0.51 mM  $\text{K}_2\text{HPO}_4$ , 0.41 mM  $\text{KH}_2\text{PO}_4$ , 7.5 mM  $\text{NH}_4\text{Cl}$ , 0.4 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.34 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1 mL  $\text{L}^{-1}$  glacial acetic acid, and 1 mL  $\text{L}^{-1}$  of Hutner's trace elements solution) at pH 6.8 [93], and every four weeks a small amount of an algal colony was streaked to a new plate. Unless otherwise stated, all experiments were performed at a  $22^{\circ}\text{C}$  with a light intensity of  $100 \mu\text{E m}^{-2} \text{s}^{-1}$ , 16 h light/8 h dark photoperiod.

For liquid cultures, a single colony of *C. vulgaris* was inoculated into 250 mL-Erlenmeyer flasks containing liquid TAP medium and precultured for one week on an orbital shaker at 120 rpm. The cells were collected by centrifugation for 5 min at  $2000 \times g$  at the end of the period and were resuspended to a final density of  $\sim 1 \times 10^6$  cells  $\text{mL}^{-1}$  in TP medium (i.e. TAP without acetate). Exogenous carbon sources (either pure monosaccharides or biomass digestates) were sterilized with  $0.22 \mu\text{m}$  filters before addition to the medium, and pH was adjusted to  $\sim 6.8$ . The monosaccharides used in this work were purchased from Sigma-Aldrich. Biomass digestates were added to the cultures to a final concentration of 20 % (v/v). The antibiotics ampicillin and cefotaxime ( $50 \mu\text{g mL}^{-1}$  and  $150 \mu\text{g mL}^{-1}$ , respectively) were added to the medium at the beginning of each experiment to avoid any contamination.

Growth in mixotrophy was carried out in 250 mL-Erlenmeyer flasks on an orbital shaker at 120 rpm or, where stated, in a MultiCultivator OD-1000 system (PSI Photon System Instruments, Brno, Czech Republic), in tubes containing 80 mL of culture, with air insufflation during the whole cultivation time.

### 2.3. Monosaccharide analysis

Monosaccharide analysis was performed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a ICS3000 system equipped with a  $3 \times 150$  mm anionic exchange column CarboPac PA20 with guard column (Thermo Fisher, Chelmsford, MA, USA). Samples (diluted 1:20 in ultra-pure water) were filtered using  $0.22 \mu\text{m}$  nylon Costar Spin-X centrifuge tube filters (Costar #8169) and subjected to isocratic elution with 6 mM NaOH at a flow rate of 0.4 mL/min for 23 min followed by a gradient up

to 800 mM NaOH for 20 min. Monosaccharides were detected using the PAD set on waveform A, according to the manufacturer's instructions. Peaks were identified and quantified by comparison to a standard mixture containing known concentrations of arabinose (Ara), galactose (Gal), xylose (Xyl), mannose (Man), galacturonic acid (GalA) and glucose (Glc), using the Chromeleon software package (Thermo Fisher, Chelmsford, MA, USA).

#### 2.4. Nitrogen and phosphorus analysis on biomass digestates

Quantification of dissolved nitrogen species was carried out upon filtration of the liquid samples through 0.45  $\mu\text{m}$  porosity syringe filters. Total N (i.e., both organic and inorganic nitrogen) was determined using the Test 0-92 Nanocolor TN<sub>b</sub> 60 kit (Macherey-Nagel - GmbH & Co., Düren, Deutschland). Ammoniacal nitrogen (i.e., the sum of ammonia and ammonium nitrogen) was quantified by the Nessler spectrophotometric method, measuring the absorbance of reacted samples at 420 nm wavelength with a Shimadzu Spectrophotometer UV-1800 [94].

Nitrates and nitrites were quantified with a Dionex ion chromatograph (Sunnyvale, CA, USA) ICS-1000 IC with conductivity cell detector, equipped with Dionex AS-40 autosampler, a pre-column Dionex IonPac™ AG14 (4 × 50 mm), a Dionex IonPac™ AS14 IC column, and a suppressor AESR 500 4 mm (Thermo Fisher Scientific Chelmsford, MA, USA). The eluent phase consisted of a 3.5 mM Na<sub>2</sub>CO<sub>3</sub> and 1.0 mM NaHCO<sub>3</sub> solution in deionized water (flow rate of 1.2 mL/min).

Total phosphorus amount was determined following the validated method by the Italian Institute for Environmental Protection and Research [95]. In detail, 700  $\mu\text{L}$  of oxidizing solution (50 g/L K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>; 30 g/L H<sub>2</sub>BO<sub>3</sub> and 14 g/L NaOH in deionized water) and 30 mg of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> were added to 5 mL samples, then autoclaved (120 °C, 30 min) and left to cool at room temperature. After oxidation, to quantify total phosphorus, 150  $\mu\text{L}$  of reducing solution (70 g/L C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>; 0.3 g/L Na<sub>2</sub> EDTA and 6 mL HCOOH) were added to 5.7 mL of samples. After 2 min of incubation at room temperature, 150  $\mu\text{L}$  of reagent mixture [0.34 g KOOC(CHOH)<sub>2</sub>COOSb ½H<sub>2</sub>O, 8.1 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>4H<sub>2</sub>O, 100 ml H<sub>2</sub>SO<sub>4</sub> concentrated, density 1.84, in a final volume of 500 ml dH<sub>2</sub>O] were added to each sample, then incubated for 5 min. Total phosphorus content was spectrophotometrically measured at 882 nm. The data were calculated against a calibration curve built with standard solutions of KH<sub>2</sub>PO<sub>4</sub> (from 0 to 1 mg/L P) in distilled water, subjected to the procedure described above.

#### 2.5. Total protein, lipid, and carbohydrate content of *C. vulgaris* biomass

Determination of total carbohydrate content in algal biomass was conducted using the phenol-sulfuric acid method [51], with slight modifications. In brief, 10 mg of lyophilized microalgae were suspended in 200  $\mu\text{L}$  of a 5 % (v/v) phenol solution in deionized water, to which 1 mL of concentrated sulfuric acid was added. The samples were moved to a heating block at 100 °C for 15 min covered with aluminum foils. The absorbance of the solutions at 500 nm was spectrophotometrically measured in polystyrene microtiter plates. Sugar concentrations were calculated against a calibration curve built with standard solutions of Glc (from 0 to 40  $\mu\text{g } \mu\text{L}^{-1}$ ) in distilled water, subjected to the procedure described above.

Protein content in algal biomass was determined as described by Chia and colleagues [52], with slight modifications. Briefly, dry biomass was resuspended at 1 % w/v in 0.5 M NaOH. The extraction was carried out for 120 min at 100 °C in a block heater. The extracted proteins were obtained by collecting the supernatant after sample centrifugation at 1000 ×g for 10 min. Total protein concentration was determined using the method of Bradford [53] with bovine serum albumin (BSA) as standard.

Total lipids extraction was performed following the method of [54] with slight modifications as described elsewhere [55]. Algal biomass was ground using a mixer mill for 1 min at 30 Hz, then suspended in

chloroform:methanol (2:1 v/v), and centrifuged at 2200 ×g for 3 min. The supernatant was recovered, and the pellet was repeatedly washed by suspension in chloroform:methanol (2:1 v/v) and centrifuged at 2200 ×g for 3 min, until it turned whitish. The pooled supernatants were treated with 0.1 M HCl, 0.5 % MgCl<sub>2</sub> and centrifuged at 2200 ×g for 3 min to separate the proteins from the total lipids. The lower phase, containing lipids, was then recovered, dried by solvent evaporation, and weighed.

#### 2.6. Statistical analysis

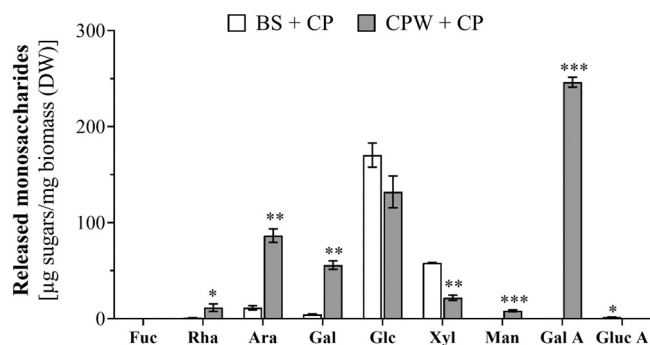
Data analyses were performed using Graph-Pad Prism 8.2.1 program (GraphPad software Inc., San Diego, CA, USA) and data were reported as means ± standard deviation (SD). Three independent experiments were performed for each test, and significance was assessed by Student's *t*-test or ANOVA One-way test followed by Tukey's post-hoc test, unless otherwise stated.

### 3. Results and discussions

#### 3.1. Characterization of agri-food waste hydrolysates

The potential of BS and CPW as sources of sugars for mixotrophic growth of *C. vulgaris* was initially assessed. The monosaccharides released after enzymatic saccharification using a cocktail of commercial cellulases and pectinases, namely Celluclast 1.5 L and Pectinex (henceforth, CP), were separated and quantified by high-performance anion-exchange chromatography/pulsed amperometric detection (HPAEC-PAD).

Under the tested condition, we observed efficient conversion of BS into Glc (about 170 mg g<sup>-1</sup> DW, Fig. 1). This yield was about 3.0- and 1.6-fold less than that obtained with dilute acid and ethanol organosolv pretreatments, respectively, as reported in previous studies [56,57], but still sufficient to allow further dilution in the algal culture media. In addition to Glc, saccharification of BS released other monosaccharides that could have different effects on algal growth. The high amount of Xyl released in the hydrolysate (about 60 mg g<sup>-1</sup> DW, Fig. 1) confirmed that Xyl-containing hemicelluloses (most likely arabinoxylans) are the most abundant polysaccharides in BS, beyond cellulose [58,59]. The presence of Ara and Gal, though in lesser amounts than Glc and Xyl (Fig. 1), was also consistent with previous reports [58,60], whereas the absence of detectable GalA indicated that BS contains negligible amounts of pectins.



**Fig. 1.** Monosaccharides released after saccharification of waste biomasses. Barley straw (BS, white bars) and citrus processing waste (CPW, grey bars) were incubated (biomass content: 5 % w/v) for seven days in a solution containing 1 % (v/v) Celluclast 1.5 L + 1 % (v/v) Pectinex (CP). The amounts of monosaccharides released in the solution were quantified by HPAEC-PAD, using pure sugars as standards. Bars represent mean amounts of monosaccharide released per mg of biomass dry weight ± SD (n = 3). Asterisks indicate statistically significant differences between BS and CPW, according to Student's *t*-test (\*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001).

**Table 1**

Monosaccharide amount in the commercial enzyme preparations. The amounts of monosaccharides present in a solution of 1 % v/v Celluclast 1.5 L + 1 % v/v Pectinex were quantified by HPAEC-PAD, using pure sugars as standards. Values are reported as mg/ml (mean  $\pm$  SD; n = 3) (n.d. not detected).

Fuc	Rha	Ara	Gal	Glc	Xyl	Man	Gal A	Gluc A
n.d.	n.d.	n.d.	0.009 $\pm$ 0.001	0.030 $\pm$ 0.018	0.020 $\pm$ 0.004	n.d.	n.d.	n.d.

**Table 2**

Content of total phosphorus, total nitrogen, and nitrogen sources in biomass hydrolysates. Barley straw (BS) and citrus processing waste (CPW) were incubated (biomass content: 5 % w/v) for seven days in a solution containing 1 % (v/v) Celluclast and 1 % (v/v) Pectinex (BS + CP, CPW + CP) or in the absence of enzymes (BS, CPW). Total phosphorus (P), total nitrogen (N), ammoniacal nitrogen (sum of ammonia and ammonium nitrogen), nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>) were determined in three independent samples and their mean concentration  $\pm$  SD (mg/L) in the hydrolysates is indicated. N.d., not detected. Different letters indicate statistically significant differences at p < 0.05 (one-way ANOVA, followed by Tukey's post-hoc test).

	Total P (mg/L)	Total N (mg/L)	N-NH <sub>4</sub> <sup>+</sup> (mg/L)	NO <sub>3</sub> <sup>-</sup> (mg/L)	NO <sub>2</sub> <sup>-</sup> (mg/L)
BS	2.34 $\pm$ 0.07 <sup>a</sup>	281.7 $\pm$ 12.6 <sup>a</sup>	57.7 $\pm$ 1.2 <sup>a</sup>	n.d.	n.d.
BS + CP	3.45 $\pm$ 0.28 <sup>b</sup>	502.7 $\pm$ 10.0 <sup>b</sup>	223.0 $\pm$ 12.2 <sup>b</sup>	n.d.	n.d.
CPW	3.55 $\pm$ 0.33 <sup>b</sup>	642.7 $\pm$ 29.6 <sup>b</sup>	172.7 $\pm$ 22.9 <sup>b</sup>	n.d.	n.d.
CPW + CP	3.99 $\pm$ 0.40 <sup>b</sup>	1150 $\pm$ 83.9 <sup>c</sup>	656.0 $\pm$ 54.7 <sup>c</sup>	n.d.	n.d.

Saccharification of CPW also yielded significant amounts of Glc, found in concentrations comparable to those observed in BS digestates (Fig. 1). In addition, high levels of Gal, Ara and GalA were detected, whereas Xyl and Man were present only in much lower concentration (Fig. 1), confirming that pectin and hemicelluloses are the major components of this biomass [61]. For both BS and CPW, saccharification did not release significant amounts of Fuc or GlcA (Fig. 1).

The obtained results indicate that both BS and CPW digestates are good sources of Glc, and that CPW has the potential to yield significant amounts of other sugars that might be used by algae as carbon sources. Notably, the release of monosaccharides from the enzymes in the absence of substrates appeared negligible, compared to those released upon biomass digestion (Table 1), indicating that most sugars found in the digestates derive from biomass saccharification.

In addition to the available carbon sources, nitrogen and phosphorus also deeply affects algal biomass production and composition, influencing protein, lipid, and carbohydrate synthesis [62–64]. While microalgae usually prefer NH<sub>4</sub><sup>+</sup>, since less energy is required for its uptake [65–70], they can still utilize other forms of nitrogen, including nitrate, nitrite and organic sources such as urea, yeast extract and free amino acids [71–74]. BS and CPW digestates contained significantly greater amounts of total N compared to those released merely by incubating them in control solutions (Table 2). Interestingly, the bioavailable forms of nitrogen exhibited variations; nitrates and nitrites were undetected, whereas a substantial release of ammoniacal nitrogen was observed from saccharified biomass (Table 2). This data can be explained not only by an increased release of endogenous N from the biomass because of enzymatic hydrolysis, but also by the enzyme's direct contribution to the total N present in the digestates.

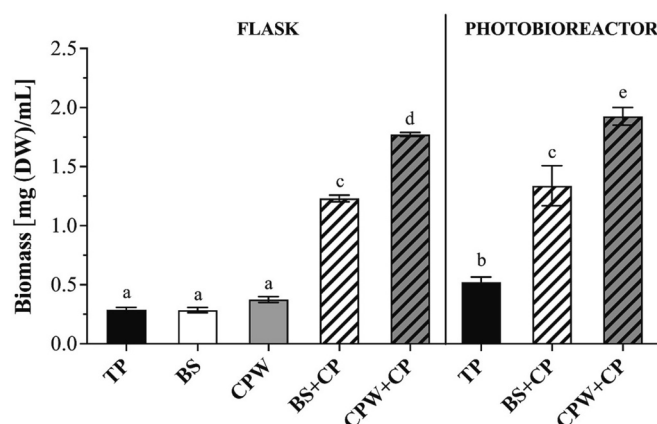
In addition to N, phosphorus (P) is also an essential element for algal physiology, which can be assimilated in the form of both polyphosphates and orthophosphates [63]. No significant differences in P released in solution could be observed between saccharified and untreated CPW, whereas saccharification seemed to improve the release of this element from BS (Table 2). This finding underscores the potential of these waste biomasses not only as a good source of organic carbon, but also of inorganic nutrient such as N and P.

### 3.2. Mixotrophic growth of *Chlorella vulgaris* in media supplemented with biomass digestates

The effect of BS and CPW digestates on *C. vulgaris* growth was thus investigated. The algae were cultivated in the light for seven days in TP medium alone or supplemented with 20 % (v/v) digestates, either in photobioreactors with air insufflation or in shaking flasks. As expected,

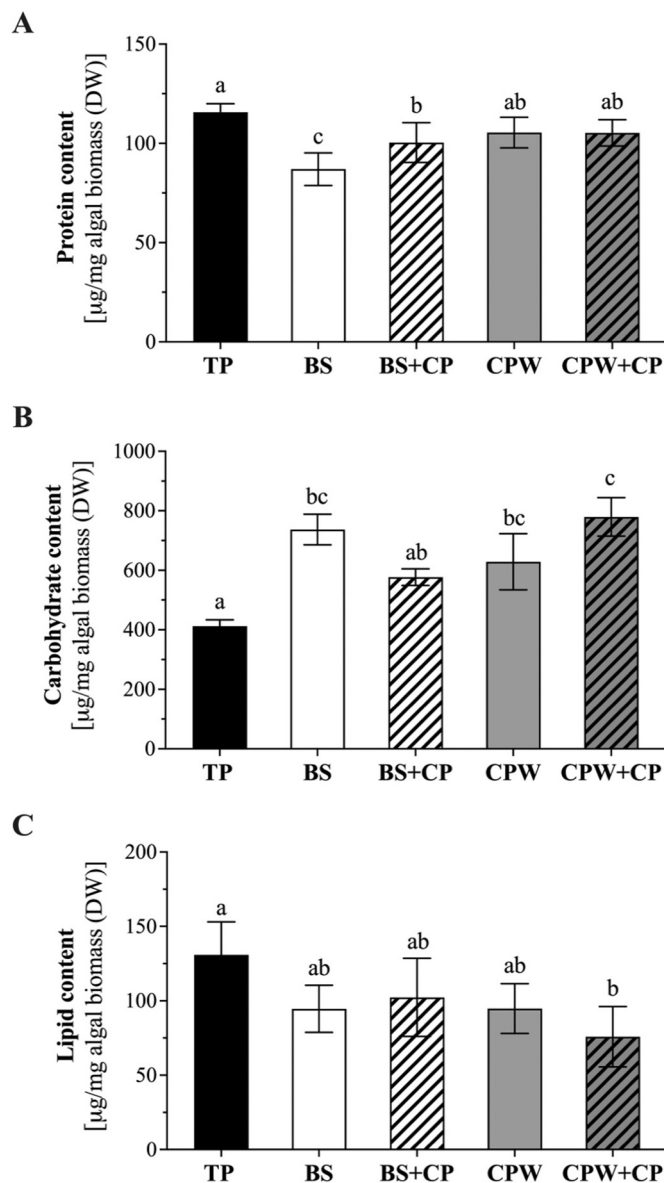
photoautotrophic growth resulted in greater biomass production in the photobioreactor than in flasks (Fig. 2), as air insufflation promotes photosynthetic activity by improving dissolved inorganic carbon availability in the medium [75,76]. Biomass production on digestates was four- to six-fold greater than under photoautotrophic conditions, regardless of air bubbling (Fig. 2). Notably, the total biomass obtained in the presence of CPW hydrolysates was significantly greater than with BS digestates, regardless of the method of cultivation (Figs. 2, S1). No significant increase in final dry biomass compared to photoautotrophy was observed when the algae were grown in TP supplemented with the solutions obtained from undigested biomasses (Fig. 2). These results indicate that the monosaccharides released in both biomass digestates can efficiently support the growth of *C. vulgaris* under mixotrophic conditions, with a more pronounced effect in the case of CPW.

The influence of BS and CPW hydrolysates on the composition of the algal biomass was then evaluated. Total protein content (Fig. 3A) was not markedly affected by the addition of CPW hydrolysate, and only slightly reduced in the case of BS hydrolysate. In contrast, total carbohydrate content was markedly increased in algae grown on both biomass



**Fig. 2.** Biomass production of *Chlorella vulgaris* mixotrophically grown on waste biomass hydrolysates. *C. vulgaris* was cultivated in the light for seven days either in shaking flasks with no air insufflation or in a photobioreactor under continuous air bubbling. The algae were grown using TP medium alone (black bar) or supplemented with 20 % (v/v) of solutions obtained incubating barley straw (BS, white bars) or citrus processing waste (CPW, grey bars) in the presence (CPW + CP; BS + CP) or absence (CPW, BS) of cellulases and pectinase. Bars represent mean dry cell weight per mL of culture  $\pm$  SD (n = 3). Different letters indicate statistically significant differences at p < 0.05, according to one-way ANOVA followed by Tukey's post-hoc test.





**Fig. 3.** Biochemical composition of *Chlorella vulgaris* biomass grown on biomass digestates. *C. vulgaris* was cultivated for seven days on TP medium alone (black bar) or TP supplemented with 20 % (v/v) of solutions obtained incubating barley straw (BS, white bars) or citrus processing waste (CPW, grey bars) in the presence (CPW + CP; BS + CP; striped bars) or absence (CPW, BS) of cellulases and pectinase. Algal biomass composition was evaluated in terms of total proteins (A), carbohydrates (B) and lipids (C). Bars represent means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$ , according to one-way ANOVA, followed by Tukey's post-hoc test.

digestates compared to photoautotrophic growth, with a greater effect in the case of the CPW digestate (Fig. 3B). This result is not unexpected, as most of the excess sugars provided to algae under mixotrophic conditions are stored as reserve polysaccharides in plastids or used for the biosynthesis of cell wall polysaccharides [77,78]. Lipid algal production was slightly reduced in the presence of the biomass hydrolysates (Fig. 3C), especially in the case of CPW + CP, compared to autotrophic growth, consistently with the observed increased carbohydrates accumulation (Fig. 3B). As N starvation is required to increase lipid accumulation in several microalgae [34,79–81], our results are not surprising, also considering the greater N content in the CPW hydrolysate (Table 2). Taken together, these data indicate that BS and CPW digestates are suitable sources of organic carbon for *C. vulgaris* biomass

production, but cultivation conditions should be optimized according to the microalgal biomass end use. For instance, if the main goal is lipid production, an approach would be to shift from nutrient-rich to N starvation conditions after reaching an adequate cell density, as widely described in literature [82]. On the other hand, CPW digestate appears to be preferable to BS digestate, both in terms of total biomass obtained, and in terms of protein and carbohydrate content of the obtained algal biomass.

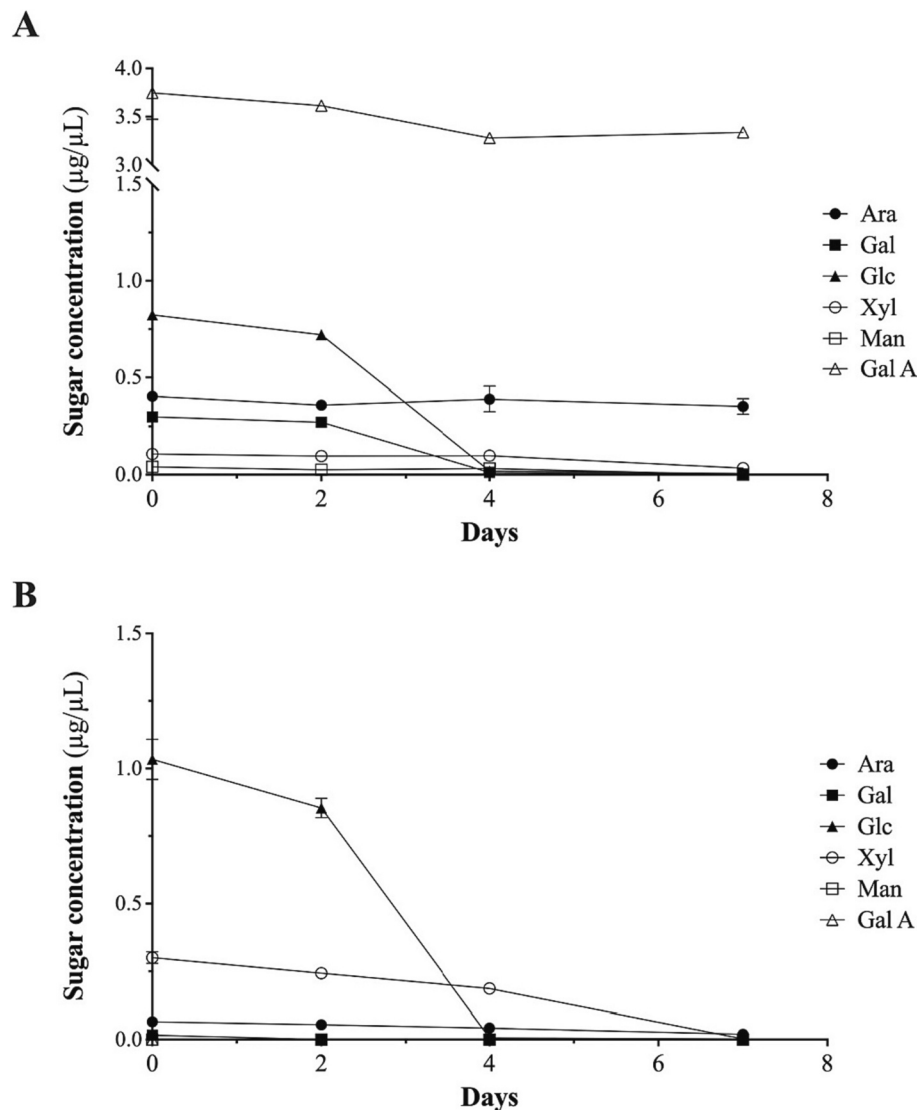
The difference in algal biomass yield observed on BS and CPW digestates is possibly due to differences in their monosaccharide composition, as they contain comparable amounts of Glc, but CPW hydrolysate also contains significant amounts of Gal (about 50 % of Glc content, Fig. 1). When algae were grown on CPW hydrolysates, Glc and Gal were indeed completely removed from the medium after four days (Fig. 4), indicating that *C. vulgaris* can efficiently uptake and metabolize both hexoses. In contrast, levels of Ara and, to a lesser extent, GalA in the culture medium remained mostly unaffected until the end of the experiment (Fig. 4A), indicating that these monosaccharides are not actively uptaken by *C. vulgaris*. Algae grown on BS hydrolysates also rapidly consumed all the available Glc, whose concentration was below detection limits at the fourth day of cultivation (Fig. 4B). Xyl appeared to be uptaken by *Chlorella* very slowly up to day 4 but was completely removed from the medium at day 7 (Fig. 4B), suggesting an uptake of this monosaccharide only once Glc is no more available. It was previously shown that, in *C. kessleri*, Glc and Xyl share the same sugars transport system, even if with greater affinity to Glc. If this is the case also in *C. vulgaris*, it is expected that Xyl can be efficiently uptaken from hydrolysates only after Glc is completely removed from the medium (Fig. 4). This is consistent with recent work showing that *C. vulgaris* mixotrophically grown on sweet sorghum bagasse hydrolysate assimilates Xyl only after Glc is exhausted [36]. In contrast to Xyl, Ara, despite its low initial levels, was still present after seven days of growth, confirming that this sugar is not efficiently utilized by the algae (Fig. 4B).

These results indicate that the cocktail of monosaccharides released by the enzymatic hydrolysis of biomass can efficiently support the growth of *C. vulgaris* under mixotrophic conditions, and that those present in CPW hydrolysates can be more efficiently utilized by the alga to produce biomass. The achievement of stationary phase after 4 days of cultivation (Fig. S1) can be indeed explained by the complete consumption of the monosaccharides suitable for growth. Continuous or semi-continuous growth system might allow sustained production of biomass for longer periods of time.

### 3.3. Impact of pure monosaccharides on *Chlorella vulgaris* mixotrophy

To assess the specific influence of the main monosaccharides present in the hydrolysates on *C. vulgaris* growth, algae were cultured in mixotrophy on TP medium supplemented with pure sugars (Ara, Gal, Glc, Xyl or GalA), and culture optical density (OD) and algal biomass dry weight were determined (Fig. 5A–B). These experiments confirmed the ability of both Glc and Gal to efficiently support *C. vulgaris* growth, as previously reported for other algal strains [39,83]. Interestingly, Gal ensured slightly enhanced OD and biomass production, compared to Glc (Fig. 5), with a final dry biomass production of about 1.1 and 0.9 g/L, respectively. However, after five days of cultivation, no further increase in OD could be observed (Fig. 5A).

Regarding the other tested sugars, GalA and Ara did not result in any significant effect, while Xyl completely inhibited growth and biomass accumulation (Fig. 5A–B). This pentose is well known to inhibit photosynthesis in green algae, arresting cell division and inducing bleaching of the culture within a few days [39,84]. In *C. sorokiniana*, half of the Xyl assimilated is converted into D-xylulose through multiple reactions requiring NADPH [85]. In particular, NADPH generated during the first stage of photosynthesis is used for D-xylulose biosynthesis, with consequent decrease of the pool of reducing power available for carbon fixation, which might explain the negative effects of Xyl on



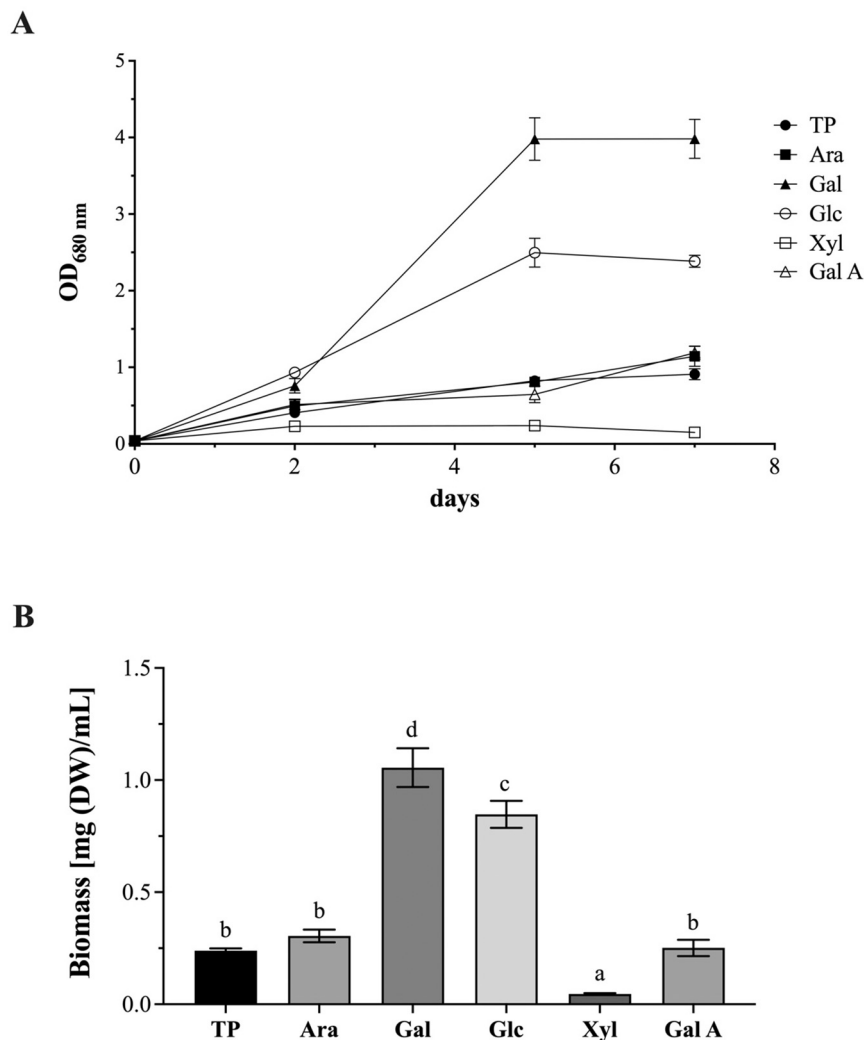
**Fig. 4.** Consumption of monosaccharides from waste biomass hydrolysates during *Chlorella vulgaris* mixotrophic growth. *C. vulgaris* was cultivated for seven days on TP medium supplemented with 20 % (v/v) hydrolysate of citrus processing waste (A) or barley straw (B). Sugar concentration in the medium was measured at the indicated times by HPEAC-PAD. The experiment was repeated in triplicate. The lines are provided as guidelines.

photoautotrophy [85]. It is very likely that similar mechanisms also hinder *C. vulgaris* growth in the light in the presence of Xyl.

The differences in terms of biomass accumulation and growth observed when *C. vulgaris* is grown on different monosaccharides could be explained not only by different energetic requirements for their metabolism, but also by a specific uptake mechanism (Fig. 6). In *C. kessleri* three monosaccharide/H<sup>+</sup> symporters have been reported for the transport across the plasmalemma of Glc and other pentoses and hexoses, though with different affinities. In particular, the inducible HUP1, HUP2 and HUP3 symporters are all able to transport Glc (and, with a reduced affinity, Xyl [85]), but only HUP2 can transport Gal, indeed with greater rates than Glc [86]. Our observation that Gal is consumed more rapidly than Glc can thus be explained by a more efficient internalization system (Fig. 6B, C) that favors the intake of the former sugar under mixotrophic conditions. However, neither Glc or Gal could be completely removed from the medium when provided as pure sugars, in contrast to what observed when the algae were grown in the presence of BS or CPW hydrolysates (Fig. 4A–B). This indicates that sugar availability is not the only limiting factor restricting prolonged growth in the light when pure monosaccharides are provided as sole organic carbon sources.

In contrast to Glc and Gal, GalA levels in the culture medium did not significantly change during the entire period of culture (Fig. 6E), confirming that *C. vulgaris* cannot uptake it from the medium, as also observed for algae grown on CPW hydrolysates (Fig. 4A). On the other hand, both Xyl and Ara were actively uptaken by the algae (Fig. 6A, D), despite their negative (Xyl) or negligible (Ara) effect on growth, indicating that, when provided as sole organic carbon sources, they can be internalized by the algal cells but do not contribute to biomass production.

Sugars uptake seems to influence culture medium pH in a manner highly specific to the kind of monosaccharide, as previously reported [87,88]. A rapid acidification of the culture medium (from pH 7.0 to 3.0) was observed when algae were grown on Glc or Gal, with the former monosaccharide inducing the strongest and quickest effect (Fig. 6B, C), whereas cultivation on not metabolically active sugars (Xyl, Ara, GalA) did not affect extracellular pH (Fig. 6A, D, E). Notably, active consumption of Glc and Gal in the light was accompanied not only by a sharp decrease in medium pH, but also by a rapid bleaching of the culture (Fig. S2). This phenomenon could contribute to the growth arrest observed in the light before complete removal of these sugars from the medium (Fig. 6B, C), as previously suggested by other authors



**Fig. 5.** *Chlorella vulgaris* growth and biomass production using pure monosaccharides as organic carbon sources. *C. vulgaris* was cultivated in the light for seven days in TP medium supplemented with the indicated monosaccharides at a final concentration of 0.25 % (w/v). (A) Optical density was monitored at 680 nm at the indicated times. Lines are provided as guidelines. (B) Cell dry biomass was assessed after seven days of growth and reported as mg DW/mL. Bars represent means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$ , according to one-way ANOVA, followed by Tukey's post-hoc test. All experiments were repeated in triplicate.

[87,89,90].

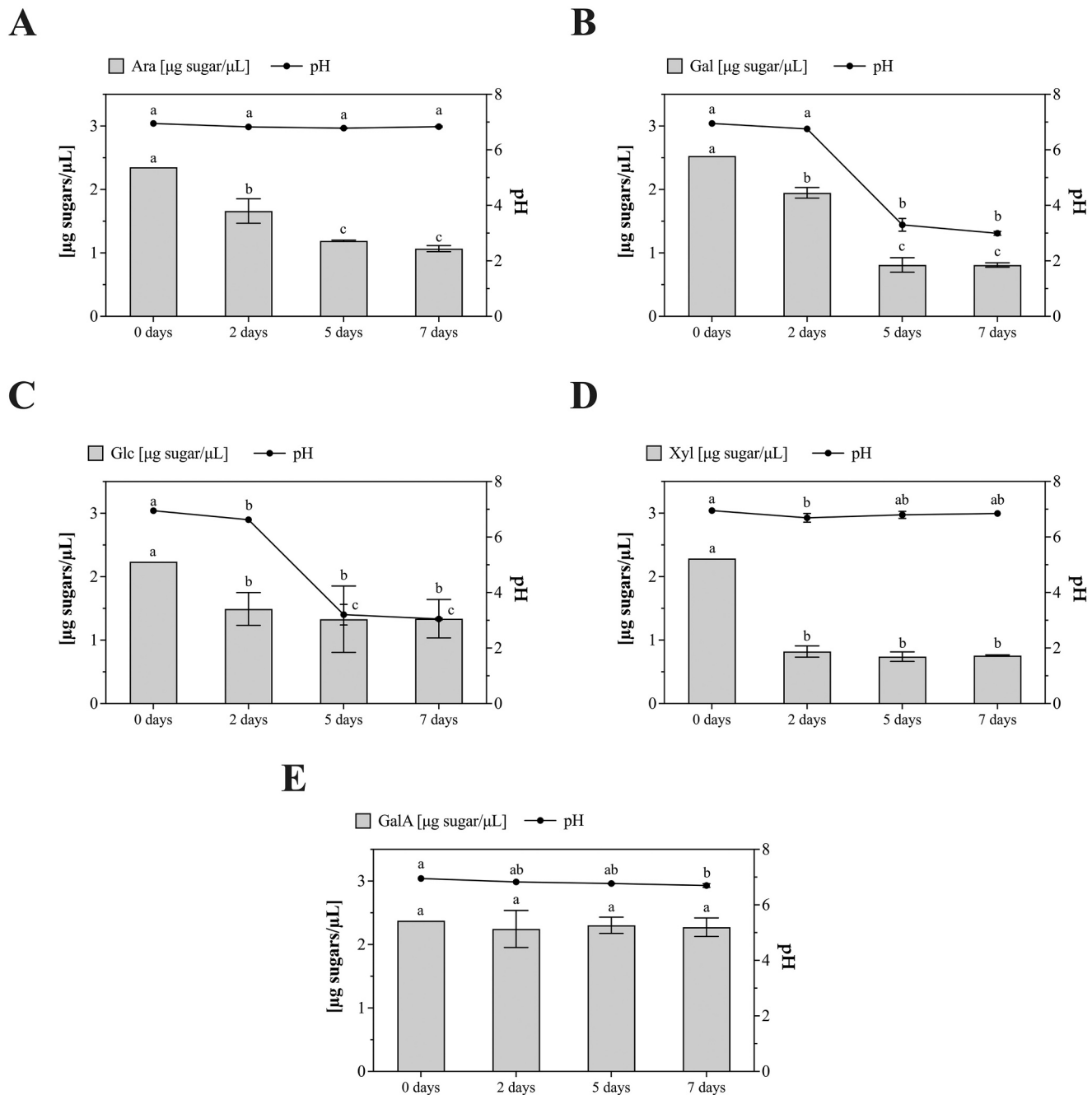
Intriguingly, extracellular pH of *C. vulgaris* cultivated on waste biomass hydrolysates decreased to a much lesser extent, reaching pH 5.0 after seven days of cultivation (Fig. 7). No significant pH change was observed when algae were grown in TP medium alone or supplemented with solutions derived from undigested biomass (Fig. 7), confirming that the decrease of extracellular pH correlates with sugars consumption (Fig. 6). However, since both Glc and Gal present in the hydrolysates were completely utilized (Fig. 4), other factors might account for the limited pH reduction observed under these conditions, compared to that observed when the algae are grown on pure monosaccharides.

A major player in pH shift has been shown to be the nitrogen source in the medium, which can lead to significant pH decrease, as when algae are cultivated in media containing ammonia (as it is the case of TP used in this work), or to alkalization, as when media containing  $\text{NO}_3^-$  (as in the case of the commercial cultivation medium BG11 [67]) are used. Therefore, it is expected that the presence of Glc or other metabolically active sugars in the culture medium, promoting the consumption of either  $\text{NH}_4^+$  or  $\text{NO}_3^-$ , would also increase the ionic fluxes associated with the uptake of the available nitrogen source, resulting in a reduction or increase of medium pH, respectively. Indeed, when *C. vulgaris* was

mixotrophically grown in BG11 medium supplemented with Glc, an increase in extracellular pH could be observed (Fig. S3A) accompanied by a significantly increased accumulation of biomass (Fig. S3B), that was even greater than that observed when algae were grown in TAP, which is considered the preferred medium for biomass production of this species.

Thus, the efficient use of pure Glc for mixotrophic growth of *C. vulgaris* is, at least in part, limited by the presence of ammonia as the main nitrogen source, which causes a sharp acidification of the medium and consequent arrest of the growth. The availability of alternative nitrogen sources can however alleviate the negative effects of ammonia on medium pH, allowing sustained growth and increased biomass yield. We can therefore speculate that the presence of other forms of nitrogen in CPW and, to a lesser extent, BS hydrolysates might contribute to increase biomass production, compared to mixotrophic growth on pure Glc or Gal. We cannot however rule out that other nutrients released in the hydrolysates might promote growth. For instance, citrus peels fatty acids were recently reported to increase total *Chlorella* biomass and lipid content [91]. It is likely that the high biomass yield observed during cultivation on CPW hydrolysate is dependent on the effects of multiple components, including sugars, free amino acids, and fatty acids.

The results reported here indicate that digestates obtained upon

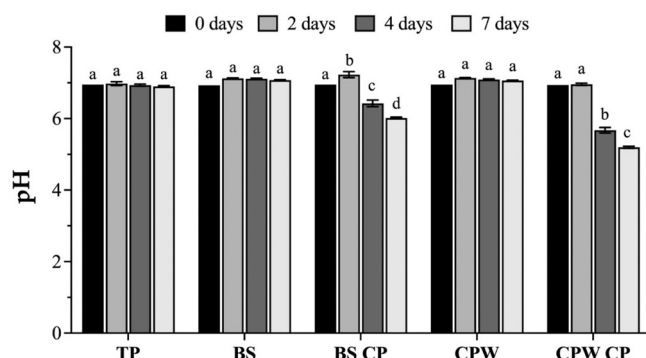


**Fig. 6.** *Chlorella vulgaris* sugars consumption when cultured on pure monosaccharides as organic carbon and medium pH changes. *C. vulgaris* was cultivated in the light for seven days in TP medium supplemented with the indicated monosaccharides at a final concentration of 0.25 % (w/v). Bars represent sugar content as means  $\pm$  SD ( $n = 3$ ) of  $\mu\text{g sugar}/\mu\text{L}$  (left y axis); lines stand for pH value (right y axis). Different letters indicate statistically significant differences at  $p < 0.05$ , according to one-way ANOVA, followed by Tukey's post-hoc test.

enzymatic saccharification of different agri-food byproducts can efficiently sustain *C. vulgaris* mixotrophic growth. Enzymatic hydrolysis of lignocellulosic material has long been proposed as an efficient and environmentally sound method for generating sugars for fermentation processes and production of added value products in a biorefinery context [92]. However, since lignocellulosic biomass is naturally recalcitrant to enzymatic hydrolysis, the cost of enzymes can hinder economic viability of this process. A biorefinery approach using low-cost feedstock, such as waste biomass, coupled to the production of high value biochemicals, might help make the cultivation of microalgae from biomass digestates more competitive. On the other hand, some less recalcitrant by-products of agriculture and food industry might yield

significant amounts of Glc without the need for enzymatic saccharification. We found that untreated BS and CPW, not subjected to saccharification, exhibited only minimal yield of Glc, compared to saccharified biomasses (Fig. S4), indicating the need for enzymatic digestion for efficient release of monosaccharides from these feedstocks. However, orange peels (OP) derived from juice production appear to be much less recalcitrant: OP macerated in distilled water at 30 °C for 3 h yielded an extract containing approximately  $10.25 \pm 1.6 \text{ mg/ml}$  of Glc. This extract was capable to sustain the mixotrophic growth of *C. vulgaris* to levels comparable to pure Glc (Fig. S5). This suggests that, depending on their composition, different agri-food byproducts could provide significant amounts of sugars for algal cultivation upon saccharification





**Fig. 7.** Changes in extracellular pH during *Chlorella vulgaris* cultivation on biomass digestates. *C. vulgaris* was cultivated in the light for seven days on TP medium supplemented with 20 % (v/v) of digestate (C). Medium pH was measured at the indicated times. Bars represent means  $\pm$  SD ( $n = 3$ ). For each culture condition, different letters indicate statistically significant difference at  $p < 0.05$ , according to one-way ANOVA, followed by Tukey's post-hoc test.

with low enzymatic loading, or even without the need of any saccharification.

#### 4. Conclusion

In conclusion, we have shown here that different agri-food waste biomasses can be used as valuable sources of organic carbon, efficiently supporting *C. vulgaris* growth under mixotrophic condition with minimal impact on protein and carbohydrate content. Our results also indicate that CPW hydrolysate is a more attractive substrate than BS hydrolysate, since it allows greater biomass production (Fig. 2), probably thanks to a wider range of metabolically active monosaccharides and, possibly, to the presence of additional nutrients, including suitable nitrogen sources that do not interfere with sugar uptake. Extracting sugars from such biomasses in many instances requires saccharification, though in some cases, as with OP, a simple extraction with hot water appears suitable to obtain sufficient sugars to sustain algal growth. In any case, the costs associated with enzymatic pretreatment can be offset by optimizing the entire process, e.g., adopting a biorefinery approach that allows for the extraction of multiple value-added products from the microalgal biomass, or optimizing growth conditions for the targeted production of a specific product, such as applying N starvation and/or adopting a semi-continuous system, thus making the whole process economically and environmentally sustainable.

#### CRediT authorship contribution statement

Francesca Angelini and Erika Bellini performed most of the work, performing experiments and analysis of data. Daniela Pontiggia contributed to HPEAC-PAD experiments and data analysis. Angela Marchetti and Gaia Salvatori performed nitrogen determination experiments and data analysis. Marianna Villano collaborated in the analysis of data and writing the paper. Simone Ferrari supervised the work, participated to the revision of data, discussion, and preparation of the final documents.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

#### Acknowledgment

*C. vulgaris* strain 211-11 was a kind gift of Roberto Bassi (University of Verona, Italy). BS was provided by Luigi Cattivelli (Council for Agriculture Research and Economics - Genomics Research Center, Fiorenzuola d'Arda, Italy). We are grateful to Federica Taddei (Council for Agriculture Research and Economics - Research Centre for Engineering and Agro-Food Processing, Rome, Italy) for assistance in BS grinding and sieving, and to Daniel Valentin Savatin (University of Tuscia, Viterbo, Italy), for providing OP.

This work was supported by the Italian Ministry of University (project n. ARS01\_00881 "ORIGAMI - Integrated biorefinery for the production of biodiesel from microalgae", call PON 2017, awarded to S. F., and project "A global approach to third generation biorefineries", call for funding competitive projects of Inter-university research consortia ex D.M. 1049 of 29/12/18, awarded to SF); by Regione Lazio (project n. A0375-2020-36720 "Alternative use of agri-food waste in a circular economy context", call LazioInnova for Research Group Projects 2020, awarded to SF); by the National Civil Aviation Authority (project "Alternative fuels for civil aviation", awarded to SF), and by Sapienza University of Rome ("Progetti di Ricerca 2022 - Progetti Medi" grant n. RM12218161B8A750 awarded to S.F., and "Progetti di Avvio alla Ricerca di tipo 2 2022" grant n. AR2221816A1EE1C1 awarded to E.B.). This study was carried out within the AgriTech National Research Center and received funding from the European Union Next-Generation EU [PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) - MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 - D.D. 1032 17/06/2022, CN00000022]. This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2023.103358>.

#### References

- [1] G. Kumar, A. Shekh, S. Jakhu, Y. Sharma, R. Kapoor, T.R. Sharma, Bioengineering of microalgae: recent advances, perspectives, and regulatory challenges for industrial application, *Front. Bioeng. Biotechnol.* 8 (2020), <https://doi.org/10.3389/fbioe.2020.00914> (accessed August 30, 2023).
- [2] M.K. Lam, K.T. Lee, A.R. Mohamed, Current status and challenges on microalgae-based carbon capture, *Int. J. Greenh. Gas Control* 10 (2012) 456–469, <https://doi.org/10.1016/j.ijggc.2012.07.010>.
- [3] J.C.M. Pires, M.C.M. Alvim-Ferraz, F.G. Martins, M. Simões, Carbon dioxide capture from flue gases using microalgae: engineering aspects and biorefinery concept, *Renew. Sust. Energ. Rev.* 16 (2012) 3043–3053, <https://doi.org/10.1016/j.rser.2012.02.055>.
- [4] S.A. Khan, M.Z. Rashmi, S. Hussain, U.C. Prasad, Banerjee, prospects of biodiesel production from microalgae in India, *Renew. Sust. Energ. Rev.* 13 (2009) 2361–2372, <https://doi.org/10.1016/j.rser.2009.04.005>.
- [5] M.M. Pacheco, M. Hoeltz, M.S.A. Moraes, R.C.S. Schneider, Microalgae: cultivation techniques and wastewater phycoremediation, *J. Environ. Sci. Health A* 50 (2015) 585–601, <https://doi.org/10.1080/10934529.2015.994951>.
- [6] L. Barsanti, P. Coltell, V. Evangelista, A.M. Frassanito, V. Passarelli, N. Vesentini, P. Gualtieri, Oddities and curiosities in the algal world, in: V. Evangelista, L. Barsanti, A.M. Frassanito, V. Passarelli, P. Gualtieri (Eds.), *Algal Toxins: Nature, Occurrence, Effect and Detection*, Springer Netherlands, Dordrecht, 2008, pp. 353–391, [https://doi.org/10.1007/978-1-4020-8480-5\\_17](https://doi.org/10.1007/978-1-4020-8480-5_17).
- [7] I. Barkia, N. Saari, S.R. Manning, Microalgae for high-value products towards human health and nutrition, *Mar. Drugs* 17 (2019) 304, <https://doi.org/10.3390/md17050304>.
- [8] M.I. Khan, J.H. Shin, J.D. Kim, The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products, *Microb. Cell Factories* 17 (2018) 36, <https://doi.org/10.1186/s12934-018-0879-x>.
- [9] M.U. Saeed, N. Hussain, A. Shahbaz, T. Hameed, H.M.N. Iqbal, M. Bilal, Bioprospecting microalgae and cyanobacteria for biopharmaceutical applications, *J. Basic Microbiol.* 62 (2022) 1110–1124, <https://doi.org/10.1002/jobm.202100445>.
- [10] L. Amer, B. Adhikari, J. Pellegrino, Technoeconomic analysis of five microalgae-to-biofuels processes of varying complexity, *Bioresour. Technol.* 102 (2011) 9350–9359, <https://doi.org/10.1016/j.biortech.2011.08.010>.

- [11] Y. Chisti, Biodiesel from microalgae, *Biotechnol. Adv.* 25 (2007) 294–306, <https://doi.org/10.1016/j.biotechadv.2007.02.001>.
- [12] Y. Li, M. Horsman, N. Wu, C.Q. Lan, N. Dubois-Calero, Biofuels from microalgae, *Biotechnol. Prog.* 24 (2008) 815–820, <https://doi.org/10.1021/bp070371k>.
- [13] A. Sun, R. Davis, M. Starbuck, A. Ben-Amotz, R. Pate, P.T. Pienkos, Comparative cost analysis of algal oil production for biofuels, *Energy* 36 (2011) 5169–5179, <https://doi.org/10.1016/j.energy.2011.06.020>.
- [14] Z. Yin, L. Zhu, S. Li, T. Hu, R. Chu, F. Mo, D. Hu, C. Liu, B. Li, A comprehensive review on cultivation and harvesting of microalgae for biodiesel production: environmental pollution control and future directions, *Bioresour. Technol.* 301 (2020), 122804, <https://doi.org/10.1016/j.biortech.2020.122804>.
- [15] Y. Zhu, L. Li, Multi-layered regulation of plant cell wall thickening, *Plant Cell Physiol.* 62 (2021) 1867–1873, <https://doi.org/10.1093/pcp/pcab152>.
- [16] T.J. Johnson, S. Katuwal, G.A. Anderson, L. Gu, R. Zhou, W.R. Gibbons, Photobioreactor cultivation strategies for microalgae and cyanobacteria, *Biotechnol. Prog.* 34 (2018) 811–827, <https://doi.org/10.1002/btpr.2628>.
- [17] R.E. Lee, *Phycology*, Cambridge University Press, Cambridge, 1980.
- [18] Y.-K. Lee, S.-Y. Ding, C.-H. Hoe, C.-S. Low, Mixotrophic growth of *Chlorella sorokiniana* in outdoor enclosed photobioreactor, *J. Appl. Phycol.* 8 (1996) 163–169, <https://doi.org/10.1007/BF02186320>.
- [19] F.J. Marquez, N. Nishio, S. Nagai, K. Sasaki, Enhancement of biomass and pigment production during growth of *Spirulina platensis* in mixotrophic culture, *J. Chem. Technol. Biotechnol.* 62 (1995) 159–164, <https://doi.org/10.1002/jctb.280620208>.
- [20] T. Ogawa, S. Aiba, Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*, *Biotechnol. Bioeng.* 23 (1981) 1121–1132, <https://doi.org/10.1002/bit.260230519>.
- [21] J. Wang, H. Yang, F. Wang, Mixotrophic cultivation of microalgae for biodiesel production: status and prospects, *Appl. Biochem. Biotechnol.* 172 (2014) 3307–3329, <https://doi.org/10.1007/s12010-014-0729-1>.
- [22] H.-Y. Ren, R.-N. Xiao, F. Kong, L. Zhao, D. Xing, J. Ma, N.-Q. Ren, B.-F. Liu, Enhanced biomass and lipid accumulation of mixotrophic microalgae by using low-strength ultrasonic stimulation, *Bioresour. Technol.* 272 (2019) 606–610, <https://doi.org/10.1016/j.biortech.2018.10.058>.
- [23] N. Pang, X. Gu, S. Chen, H. Kirchhoff, H. Lei, S. Roje, Exploiting mixotrophy for improving productivities of biomass and co-products of microalgae, *Renew. Sust. Energ. Rev.* 112 (2019) 450–460, <https://doi.org/10.1016/j.rser.2019.06.001>.
- [24] F. FAO, *Food Outlook: Biannual Report on Global Food Markets*, FAO, Rome, 2018.
- [25] J. Ángel Siles López, Q. Li, I.P. Thompson, Biorefinery of waste orange peel, *Crit. Rev. Biotechnol.* 30 (2010) 63–69, <https://doi.org/10.3109/07388550903425201>.
- [26] W. Widmer, W. Zhou, K. Grohmann, Pretreatment effects on orange processing waste for making ethanol by simultaneous saccharification and fermentation, *Bioresour. Technol.* 101 (2010) 5242–5249, <https://doi.org/10.1016/j.biortech.2009.12.038>.
- [27] D.A. Zema, P.S. Calabró, A. Folino, V. Tamburino, G. Zappia, S.M. Zimbone, Valorisation of citrus processing waste: a review, *Waste Manag.* 80 (2018) 252–273, <https://doi.org/10.1016/j.wasman.2018.09.024>.
- [28] B. Ruiz, X. Flotats, Citrus essential oils and their influence on the anaerobic digestion process: an overview, *Waste Manag.* 34 (2014) 2063–2079, <https://doi.org/10.1016/j.wasman.2014.06.026>.
- [29] S. Suri, A. Singh, P.K. Nema, Current applications of citrus fruit processing waste: a scientific outlook, *Appl. Food Res.* 2 (2022), 100050, <https://doi.org/10.1016/j.afres.2022.100050>.
- [30] K.W. Chew, S.R. Chia, P.L. Show, T.C. Ling, S.S. Arya, J.-S. Chang, Food waste compost as an organic nutrient source for the cultivation of *Chlorella vulgaris*, *Bioresour. Technol.* 267 (2018) 356–362, <https://doi.org/10.1016/j.biortech.2018.07.069>.
- [31] A.P.C. da Rosa, L. Moraes, E.G. de Moraes, J.A.V. Costa, Fatty acid biosynthesis from *Chlorella* in autotrophic and mixotrophic cultivation, *Braz. Arch. Biol. Technol.* 63 (2020), e20180534, <https://doi.org/10.1590/1678-4324-2020180534>.
- [32] A.P. Peter, K.W. Chew, A.K. Koyande, S. Yuk-Heng, H.Y. Ting, S. Rajendran, H.S. H. Munawaroh, C.K. Yoo, P.L. Show, Cultivation of *Chlorella vulgaris* on dairy waste using vision imaging for biomass growth monitoring, *Bioresour. Technol.* 341 (2021), 125892, <https://doi.org/10.1016/j.biortech.2021.125892>.
- [33] L.H. Sipauba-Tavares, B. Scardoli-Truzzi, D.C. Fenerick, M.G. Tedesque, Comparison of photoautotrophic and mixotrophic cultivation of microalgae *Mesostigma gracile* (Chlorophyceae) in alternative culture media, *Braz. J. Biol.* 80 (2019) 914–920, <https://doi.org/10.1590/1519-6984.226548>.
- [34] T. Chen, J. Liu, B. Guo, X. Ma, P. Sun, B. Liu, F. Chen, Light attenuates lipid accumulation while enhancing cell proliferation and starch synthesis in the glucose-fed oleaginous microalga *Chlorella zofingensis*, *Sci. Rep.* 5 (2015), 14936, <https://doi.org/10.1038/srep14936>.
- [35] Q. Yu, H. Wang, X. Li, Y. Yin, S. Qin, B. Ge, Enhanced biomass and CO<sub>2</sub> sequestration of *Chlorella vulgaris* using a new mixotrophic cultivation method, *Process Biochem.* 90 (2020) 168–176, <https://doi.org/10.1016/j.procbio.2019.11.022>.
- [36] N. Arora, G.P. Philippidis, Insights into the physiology of *Chlorella vulgaris* cultivated in sweet sorghum bagasse hydrolysate for sustainable algal biomass and lipid production, *Sci. Rep.* 11 (2021) 6779, <https://doi.org/10.1038/s41598-021-86372-2>.
- [37] G.F. Ferreira, L.F. Ríos Pinto, R. Maciel Filho, L.V. Fregolente, Effects of cultivation conditions on *Chlorella vulgaris* and *Desmodesmus* sp. grown in sugarcane agro-industry residues, *Bioresour. Technol.* 342 (2021), 125949, <https://doi.org/10.1016/j.biortech.2021.125949>.
- [38] E. Trevisan, R.F.B. Godoy, F.A.D. Radomski, E.L. Crisigiovanni, K.B.Z.F. Branco, P. A. Arroyo, *Chlorella vulgaris* growth in different biodigested vinasse concentrations: biomass, pigments and final composition, *Water Sci. Technol.* 82 (2020) 1111–1119, <https://doi.org/10.2166/wst.2020.192>.
- [39] S. Chai, J. Shi, T. Huang, Y. Guo, J. Wei, M. Guo, L. Li, S. Dou, L. Liu, G. Liu, Characterization of *Chlorella sorokiniana* growth properties in monosaccharide-supplemented batch culture, *PLoS One* 13 (2018), e0199873, <https://doi.org/10.1371/journal.pone.0199873>.
- [40] A.K. Patel, J.M. Joun, M.E. Hong, S.J. Sim, Effect of light conditions on mixotrophic cultivation of green microalgae, *Bioresour. Technol.* 282 (2019) 245–253, <https://doi.org/10.1016/j.biortech.2019.03.024>.
- [41] S.J. Sim, J. Joun, M.E. Hong, A.K. Patel, Split mixotrophy: a novel cultivation strategy to enhance the mixotrophic biomass and lipid yields of *Chlorella protothecoides*, *Bioresour. Technol.* 291 (2019), 121820, <https://doi.org/10.1016/j.biortech.2019.121820>.
- [42] P. Li, X. Miao, R. Li, J. Zhong, *In situ* biodiesel production from fast-growing and high oil content *Chlorella pyrenoidosa* in rice straw hydrolysate, *Biomed. Res. Int.* 2011 (2011), e141207, <https://doi.org/10.1155/2011/141207>.
- [43] J. Mu, S. Li, D. Chen, H. Xu, F. Han, B. Feng, Y. Li, Enhanced biomass and oil production from sugarcane bagasse hydrolysate (SBH) by heterotrophic oleaginous microalga *Chlorella protothecoides*, *Bioresour. Technol.* 185 (2015) 99–105, <https://doi.org/10.1016/j.biortech.2015.02.082>.
- [44] R. Katiyar, B.R. Gurjar, A. Kumar, R.K. Bharti, S. Biswas, V. Pruthi, A novel approach using low-cost *Citrus limetta* waste for mixotrophic cultivation of oleaginous microalgae to augment automotive quality biodiesel production, *Environ. Sci. Pollut. Res.* 26 (2019) 16115–16124, <https://doi.org/10.1007/s11356-019-04946-0>.
- [45] W.-K. Park, M. Moon, M.-S. Kwak, S. Jeon, G.-G. Choi, J.-W. Yang, B. Lee, Use of orange peel extract for mixotrophic cultivation of *Chlorella vulgaris*: increased production of biomass and FAMES, *Bioresour. Technol.* 171 (2014) 343–349, <https://doi.org/10.1016/j.biortech.2014.08.109>.
- [46] R. Li, J. Pan, M. Yan, J. Yang, W. Qin, Effects of mixotrophic cultivation on antioxidant and lipid accumulation of *Chlorella vulgaris* in wastewater treatment, *Int. J. Phytoremediation* 22 (2020) 638–643, <https://doi.org/10.1080/15226514.2019.1701982>.
- [47] T. Fábryová, J. Čheľ, D. Kubáč, P. Hrouzek, D.L. Vu, L. Tůmová, J. Kopecký, Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC), *Algal Res.* 41 (2019), 101574, <https://doi.org/10.1016/j.algal.2019.101574>.
- [48] A. Gille, U. Neumann, S. Louis, S.C. Bischoff, K. Briviba, Microalgae as a potential source of carotenoids: comparative results of an in vitro digestion method and a feeding experiment with C57BL/6J mice, *J. Funct. Foods* 49 (2018) 285–294, <https://doi.org/10.1016/j.jff.2018.08.039>.
- [49] I.T.K. Ru, Y.Y. Sung, M. Jusoh, M.E.A. Wahid, T. Nagappan, *Chlorella vulgaris*: a perspective on its potential for combining high biomass with high value bioproducts, *Appl. Phycol.* 1 (2020) 2–11, <https://doi.org/10.1080/26388081.2020.1715256>.
- [50] W. Levasseur, P. Perré, V. Pozzobon, A review of high value-added molecules production by microalgae in light of the classification, *Biotechnol. Adv.* 41 (2020), 107545, <https://doi.org/10.1016/j.biotechadv.2020.107545>.
- [51] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350–356, <https://doi.org/10.1021/ac60111a017>.
- [52] M.A. Chia, A.T. Lombardi, M. da Graça Gama, C.C. Parrish Melão, Combined nitrogen limitation and cadmium stress stimulate total carbohydrates, lipids, protein and amino acid accumulation in *Chlorella vulgaris* (Trebouxiphyceae), *Aquat. Toxicol.* 160 (2015) 87–95, <https://doi.org/10.1016/j.aquatox.2015.01.002>.
- [53] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254, [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- [54] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917.
- [55] S. Savio, S. Farrotti, D. Paris, E. Arnaiz, I. Díaz, S. Bolado, R. Muñoz, C. Rodolfo, R. Congestri, Value-added co-products from biomass of the diatoms *Staurosirella pinnata* and *Phaeodactylum tricornutum*, *Algal Res.* 47 (2020), 101830, <https://doi.org/10.1016/j.algal.2020.101830>.
- [56] Y. Kim, A. Yu, M. Han, G. Choi, B. Chung, Enhanced enzymatic saccharification of barley straw pretreated by ethanolsolv technology, *Appl. Biochem. Biotechnol.* 163 (2011) 143–152, <https://doi.org/10.1007/s12010-010-9023-z>.
- [57] B.C. Saha, M.A. Cotta, Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol, *New Biotechnol.* 27 (2010) 10–16, <https://doi.org/10.1016/j.nbt.2009.10.005>.
- [58] T. Persson, J.L. Ren, E. Joëlsson, A.-S. Jönsson, Fractionation of wheat and barley straw to access high-molecular-mass hemicelluloses prior to ethanol production, *Bioresour. Technol.* 100 (2009) 3906–3913, <https://doi.org/10.1016/j.biortech.2009.02.063>.
- [59] L. Rosgaard, S. Pedersen, A.S. Meyer, Comparison of different pretreatment strategies for enzymatic hydrolysis of wheat and barley straw, *Appl. Biochem. Biotechnol.* 143 (2007) 284–296, <https://doi.org/10.1007/s12010-007-8001-6>.
- [60] I.A. Panagiotopoulos, R.R. Bakker, T. de Vrije, E.G. Koukios, Effect of pretreatment severity on the conversion of barley straw to fermentable substrates and the release of inhibitory compounds, *Bioresour. Technol.* 102 (2011) 11204–11211, <https://doi.org/10.1016/j.biortech.2011.09.090>.

- [61] P. Li, J. Xia, Z. Nie, Y. Shan, Saccharification of orange peel wastes with crude enzymes from new isolated *Aspergillus japonicus* PJ01, *Bioprocess Biosyst. Eng.* 39 (2016) 485–492, <https://doi.org/10.1007/s00449-015-1531-3>.
- [62] X.-Y. Liu, Y. Hong, Q.-Y. Zhai, G.-P. Zhao, H.-K. Zhang, Q. Wang, Performance and mechanism of *Chlorella* in swine wastewater treatment: roles of nitrogen-phosphorus ratio adjustment and indigenous bacteria, *Bioresour. Technol.* 358 (2022), 127402, <https://doi.org/10.1016/j.biortech.2022.127402>.
- [63] M.A. Yaakob, R.M.S.R. Mohamed, A. Al-Gheethi, R. Aswathnarayana Gokare, R. R. Ambati, Influence of nitrogen and phosphorus on microalgal growth, biomass, lipid, and fatty acid production: an overview, *Cells* 10 (2021) 393, <https://doi.org/10.3390/cells10020393>.
- [64] N. Yodsuwan, S. Sawayama, S. Sirisansaneeyakul, Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom *Phaeodactylum tricornutum*, *Agric. Nat. Resour.* 51 (2017) 190–197, <https://doi.org/10.1016/j.anres.2017.02.004>.
- [65] J.C. Goldman, Biomass production in mass cultures of marine phytoplankton at varying temperatures, *J. Exp. Mar. Biol. Ecol.* 27 (1977) 161–169, [https://doi.org/10.1016/0022-0981\(77\)90136-8](https://doi.org/10.1016/0022-0981(77)90136-8).
- [66] J.U. Grobbelaar, Algal nutrition: mineral nutrition, in: *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*, 2004, pp. 97–115.
- [67] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: metabolism and potential products, *Water Res.* 45 (2011) 11–36, <https://doi.org/10.1016/j.watres.2010.08.037>.
- [68] X.-M. Shi, X.-W. Zhang, F. Chen, Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources, *Enzym. Microb. Technol.* 27 (2000) 312–318, [https://doi.org/10.1016/S0141-0229\(00\)00208-8](https://doi.org/10.1016/S0141-0229(00)00208-8).
- [69] P.J. Syrett, I. Morris, The inhibition of nitrate assimilation by ammonium in *Chlorella*, *Biochim. Biophys. Acta BBA Spec. Sect. Enzymol. Subj.* 67 (1963) 566–575, [https://doi.org/10.1016/0926-6569\(63\)90277-3](https://doi.org/10.1016/0926-6569(63)90277-3).
- [70] Y. Zheng, J. Shi, M. Tu, Y.-S. Cheng, Chapter one - principles and development of lignocellulosic biomass pretreatment for biofuels, in: Y. Li, X. Ge (Eds.), *Advances in Bioenergy*, Elsevier, 2017, pp. 1–68, <https://doi.org/10.1016/b.s.aibe.2017.03.001>.
- [71] E.V. Armbrust, J.A. Berges, C. Bowler, B.R. Green, D. Martinez, N.H. Putnam, S. Zhou, A.E. Allen, K.E. Apt, M. Bechner, M.A. Brzezinski, B.K. Chaal, A. Chiovitti, A.K. Davis, M.S. Demarest, J.C. Detter, T. Glavina, D. Goodstein, M.Z. Hadi, U. Hellsten, M. Hildebrand, B.D. Jenkins, J. Jurka, V.V. Kapitonov, N. Kröger, W. W.Y. Lau, T.W. Lane, F.W. Larimer, J.C. Lippmeier, S. Lucas, M. Medina, A. Montsant, M. Obornik, M.S. Parker, B. Palenik, G.J. Pazour, P.M. Richardson, T. A. Ryneerson, M.A. Saito, D.C. Schwartz, K. Thamatrakoln, K. Valentin, A. Vardi, F. P. Wilkerson, D.S. Rokhsar, The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism, *Science* 306 (2004) 79–86, <https://doi.org/10.1126/science.1101156>.
- [72] E.W. Becker, *Microalgae: Biotechnology and Microbiology*, Cambridge University Press, 1994.
- [73] G.-Q. Chen, F. Chen, Growing phototrophic cells without light, *Biotechnol. Lett.* 28 (2006) 607–616, <https://doi.org/10.1007/s10529-006-0025-4>.
- [74] E. Ganuza, T. Benítez-Santana, E. Atalah, O. Vega-Orellana, R. Ganga, M. S. Izquierdo, *Cryptocodinium cohnii* and *Schizochytrium* sp. as potential substitutes to fishery-derived oils from seabream (*Sparus aurata*) microdiets, *Aquaculture* 277 (2008) 109–116, <https://doi.org/10.1016/j.aquaculture.2008.02.005>.
- [75] F.G. Acien Fernández, C.V. González-López, J.M. Fernández Sevilla, E. Molina Grima, Conversion of CO<sub>2</sub> into biomass by microalgae: how realistic a contribution may it be to significant CO<sub>2</sub> removal? *Appl. Microbiol. Biotechnol.* 96 (2012) 577–586, <https://doi.org/10.1007/s00253-012-4362-z>.
- [76] G. Salbitani, F. Bolinesi, M. Affuso, F. Carraturo, O. Mangoni, S. Carfagna, Rapid and positive effect of bicarbonate addition on growth and photosynthetic efficiency of the green microalgae *Chlorella sorokiniana* (Chlorophyta, Trebouxiophyceae), *Appl. Sci.* 10 (2020) 4515, <https://doi.org/10.3390/app10134515>.
- [77] M.A. de Carvalho Silvello, I. Severo Gonçalves, S. Patrícia Held Azambuja, S. Silva Costa, P. Garcia Pereira Silva, L. Oliveira Santos, R. Goldbeck, Microalgae-based carbohydrates: a green innovative source of bioenergy, *Bioresour. Technol.* 344 (2022), 126304, <https://doi.org/10.1016/j.biortech.2021.126304>.
- [78] S. Vyas, A. Patel, E. Nabil Risse, E. Krikigianni, U. Rova, P. Christakopoulos, L. Matsakas, Biosynthesis of microalgal lipids, proteins, lutein, and carbohydrates using fish farming wastewater and forest biomass under photoautotrophic and heterotrophic cultivation, *Bioresour. Technol.* 359 (2022), 127494, <https://doi.org/10.1016/j.biortech.2022.127494>.
- [79] A.M. Illman, A.H. Scragg, S.W. Shales, Increase in *Chlorella* strains calorific values when grown in low nitrogen medium, *Enzym. Microb. Technol.* 27 (2000) 631–635, [https://doi.org/10.1016/S0141-0229\(00\)00266-0](https://doi.org/10.1016/S0141-0229(00)00266-0).
- [80] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M. R. Tredici, Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, *Biotechnol. Bioeng.* 102 (2009) 100–112, <https://doi.org/10.1002/bit.22033>.
- [81] M. Takagi, K. Watanabe, K. Yamaberi, T. Yoshida, Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris* sp. UTEX LB1999, *Appl. Microbiol. Biotechnol.* 54 (2000) 112–117, <https://doi.org/10.1007/s002530000333>.
- [82] G. Mujtaba, W. Choi, C.-G. Lee, K. Lee, Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions, *Bioresour. Technol.* 123 (2012) 279–283, <https://doi.org/10.1016/j.biortech.2012.07.057>.
- [83] X.-B. Tan, X.-C. Zhao, L.-B. Yang, J.-Y. Liao, Y.-Y. Zhou, Enhanced biomass and lipid production for cultivating *Chlorella pyrenoidosa* in anaerobically digested starch wastewater using various carbon sources and up-scaling culture outdoors, *Biochem. Eng. J.* 135 (2018) 105–114, <https://doi.org/10.1016/j.bej.2018.04.005>.
- [84] K.A. Hassall, Xylose as a specific inhibitor of photosynthesis, *Nature* 181 (1958) 1273–1274, <https://doi.org/10.1038/1811273a0>.
- [85] Y. Zheng, X. Yu, T. Li, X. Xiong, S. Chen, Induction of D-xylose uptake and expression of NAD(P)H-linked xylose reductase and NADP<sup>+</sup>-linked xylitol dehydrogenase in the oleaginous microalga *Chlorella sorokiniana*, *Biotechnol. Biofuels* 7 (2014) 125, <https://doi.org/10.1186/s13068-014-0125-7>.
- [86] R. Stadler, K. Wolf, C. Hilgarth, W. Tanner, N. Sauer, Subcellular localization of the inducible *Chlorella* HUP1 monosaccharide-H<sup>+</sup> symporter and cloning of a co-induced galactose-H<sup>+</sup> symporter, *Plant Physiol.* 107 (1995) 33–41, <https://doi.org/10.1104/pp.107.1.33>.
- [87] A. Huang, L. Sun, S. Wu, C. Liu, P. Zhao, X. Xie, G. Wang, Utilization of glucose and acetate by *Chlorella* and the effect of multiple factors on cell composition, *J. Appl. Phycol.* 29 (2017) 23–33, <https://doi.org/10.1007/s10811-016-0920-6>.
- [88] L. Sijtsma, A.J. Anderson, C. Ratledge, 7 - alternative carbon sources for heterotrophic production of docosahexaenoic acid by the marine alga *Cryptocodinium cohnii*, in: Z. Cohen, C. Ratledge (Eds.), *Single Cell Oils*, second edition, ACS Press, 2010, pp. 131–149, <https://doi.org/10.1016/B978-1-893997-73-8.50011-6>.
- [89] M. Matsuka, E. Hase, Metabolism of glucose in the process of “glucose-bleaching” of *Chlorella protothecoides*, *Plant Cell Physiol.* 6 (1965) 721–741, <https://doi.org/10.1093/oxfordjournals.pcp.a079144>.
- [90] J. Wang, T. Rosov, P. Wensel, J. McGowen, W.R. Curtis, A preliminary implementation of metabolic-based pH control to reduce CO<sub>2</sub> usage in outdoor flat-panel photobioreactor cultivation of *Nannochloropsis oceanica* microalgae, *Algal Res.* 18 (2016) 288–295, <https://doi.org/10.1016/j.algal.2016.07.001>.
- [91] K.G. Jahromi, Z.H. Koochi, G. Kavooosi, A. Shahsavari, Manipulation of fatty acid profile and nutritional quality of *Chlorella vulgaris* by supplementing with citrus peel fatty acid, *Sci. Rep.* 12 (2022) 8151, <https://doi.org/10.1038/s41598-022-12309-y>.
- [92] N. Mosier, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresour. Technol.* 96 (2005) 673–686, <https://doi.org/10.1016/j.biortech.2004.06.025>.
- [93] J. Kropat, A. Hong-Hermesdorf, D. Casero, P. Ent, M. Castruita, M. Pellegrini, S. S. Merchant, D. Malasarn, A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*, *Plant J.* 66 (2011) 770–780, <https://doi.org/10.1111/j.1365-3113.2011.04537.x>.
- [94] American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*, 19th Edition, American Public Health Association, Inc, New York, 1995.
- [95] APAT - IRSA/CNR Manuali e Linee Guida 29, 2003, Metodi analitici per le acque, ISBN: 88-448-0083-7.