Isolation and characterization of microalgae strains able to grow on complex biomass hydrolysate for industrial application

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ABSTRACT

Producing algae biomass economically remains one of the chief bottlenecks for commercializing algae products. The aim of this work is to identify new strains of algae that can grow on cost effective media derived from cellulosic waste streams, characterize the potential of these strains to produce compounds of high industrial value, and identify those strains capable of facile genetic transformation. Here we report, out of 45 strains initially isolated, three were selected based on their ability to efficiently grow on organic waste material (corn stover hydrolysate) as a carbon source; *Chlorococcum sp.*, *Desmodesmus sp.*, and *Chlamydomonas debaryana*. Untargeted metabolomics was performed on each strain, identifying several metabolites of high relative abundances that are of commercial interest, such as lactic acid, butane-2,3-diol, amino acids, tartaric acid, triacylglycerols, and lipid species containing different, mono- and polyunsaturated fatty acids, depending on the strain and growth condition. The strains also produced carbohydrates of industrial relevance. *Chlorococcum sp.* was found to be genetically transformable using standard simple transformation protocols. These results suggest that with further development, these strains could open the door to economic production of high value commercial compounds utilizing waste streams from cellulosic biomass.

1. Introduction

Microscopic algae are a diverse group of photosynthetic eukaryotes (microalgae) and prokaryotes (cyanobacteria), with a history of commercial production dating back to the 1960s [1]. Considering the number of species already recognized, and the estimated number of species still undescribed [2,3], there are relatively few species of algae that have been commercially developed [1,4]. The potential to identify, characterize, and establish new strains with commercial value is a rich field for investigation.

There has been significant research progress in the field of algal genetic engineering over the last decades, including the development of many molecular tools and methods [5–7]. Research has advanced faster in cyanobacteria compared with microalgae, facilitated by the lower complexity of these prokaryotes and the relative ease of genetic transformation of certain cyanobacterial species [8]. Nevertheless, the gap has been narrowing between the two groups, with important advancements for microalgae genetic engineering [9]. Microalgae offer certain advantages over cyanobacteria, such as different metabolite accumulation patterns, and the ability to perform post-translational modifications on proteins, ensuring adequate activity and functionality of complex recombinant eukaryotic proteins, allowing for the organisms to serve as a platform for the production of protein-based therapeutics [10–13]. For any newly recognized strain, one key factor to evaluate is whether it can be genetically transformed through an established protocol, with successful recombinant protein expression. This technology opens the door for future strain improvement through genetic engineering. In the case of eukaryotic algae, more advanced DNA delivery methods are required, and the entire process requires careful design and optimization of the expression cassettes, with additional considerations for cell morphology, cell wall structure, transgene silencing, *etc.* [6,7,9,14].

Microalgae have the potential to be the source of a wide range of compounds of interest, depending on the species, which may be of use for different industrial sectors [15], including the food, feed, nutraceutical, and cosmetic industries [16,17], and they may serve as base ingredients for the manufacturing of renewable plastics [16,18–20], and

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the production of biofuels [16]. Metabolic profiles are often different in algae grown under heterotrophic or autotrophic modes, due to a variety of factors, including metabolic flux rerouting [21]. Heterotrophic conditions may allow, for example, for the increased accumulation of storage lipids and carbohydrates, without compromising overall growth rates [21].

Heterotrophic growth has several other advantages over autotrophic cultivation, such as higher cell densities and biomass productivities, leading to reduced production facilities requirements, as well as reduced harvesting costs. Heterotrophic (fermentation) systems are also more easily controlled compared to the open pond systems widely used for autotrophic cultivation. Mixotrophic cultivation can have similar advantages as heterotrophic growth conditions, compared to autotrophic growth, but the additional significant cost of the photobioreactor systems used for mixotrophic cultivation has to be taken into account during production process design.

For cultivation of algae on organic carbon substrates there are a number of considerations including: that many algae are strictly autotrophic with a limited number of species identified that are able to grow on organic carbon substrates; that algae are selective for the organic carbon compounds they are able to metabolize; and that the cost of pure organic substrates can be quite significant [1,14,21]. A potential solution to overcome these limitations is to select algae strains that are able to efficiently assimilate organic carbon substrates present in cost-effective biomass waste materials coming from agriculture and available in large quantities, such as lignocellulosic biomass waste. Indeed, several groups have focused their research on using these low-cost waste materials for algae cultivation [22–24]. Generally, cellulosic biomass has to be pretreated to break down complex inaccessible polysaccharide structures and release the simple sugars which are utilized by algae. The two main monomeric sugars released after pretreatment of cellulosic waste are glucose and xylose [23,24]. Glucose is often a preferred substrate for heterotrophic growth, as assimilation of xylose can be a major challenge, frequently leading to growth inhibition or toxic effects [25,26]. Therefore, identifying strains able to metabolize both glucose and xylose to a significant degree without negative impact on the cultures, would be of great relevance in further reducing media cost.

Our objective in this study was to characterize new strains of algae, isolated from Southern California's Imperial Valley and San Diego area, as well as strains procured from culture collections, to identify those that could meet the following selection criteria: (1) have the ability to efficiently grow on economical organic waste material as a carbon source (2) have the potential to produce metabolites of commercial interest, and (3) have the possibility to be easily genetically transformed. To identify strains that meet these criteria, we characterized isolated strains for those able to rapidly grow on corn stover hydrolysate, and then investigated the relevant metabolites produced in the identified strains under different cultivation modes, thus providing a catalogue of potential commercial strains and associated products. Finally, the algae selected were tested for their ability to be genetically transformed, and for recombinant protein expression.

2. Materials and methods

2.1. Screening on corn stover hydrolysate and its main sugar components

2.1.1. Algae strains

The algae strains screened were Southern California isolates from the Imperial Valley and San Diego area. The newly characterized novel strains (*Chlorococcum sp.* and *Desmodesmus sp.*) were isolated specifically from the following location: 33.195724, −115.567250, Duck Club Pond. This area is characterized by a monthly average air temperature through the year ranging from 11 to 35 °C, and a monthly average solar radiation from 134 to 361 W/m^2. A more detailed information can be accessed at the CIMIS website (https://cimis.water.ca.gov/).

In addition, some of the strains screened were procured from the following culture collections: Culture Collection of Algae at the University of Texas at Austin (UTEX), Austin, Texas, USA; Chlamydomonas Resource Center at the University of Minnesota, St. Paul, Minnesota, USA; Culture Collection of Algae and Protozoa (CCAP), Oban, UK; Culture Collection of Algae at the University of Göttingen, (SAG), Göttingen, Germany; Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Tsukuba, Japan.

2.1.2. Culture conditions

The Southern California isolates were purified using standard microbiological techniques [27]. The initial screening of 45 strains was performed at room temperature on agar plates containing modified Bold's Basal Medium with 5 g/l yeast extract as a nitrogen source, in heterotrophic conditions in the dark, and in mixotrophic conditions at continuous illumination under fluorescent lights at 100 μmol photons/ m^{2}s. The selection was first performed on the pure chemical substrates, glucose and xylose, separately, at 10 g/l concentration. Subsequently, the 24 strains able to grow on any of the substrates were tested on corn stover hydrolysate. The enzymatically pretreated corn stover waste was provided by the National Renewable Energy Laboratory (NREL). Supplementary Table 1 shows the main potential organic carbon sources in the hydrolysate. The hydrolysate was diluted 36 times: 28 ml of the substrate was added to a total volume of 1 l of modified Bold's Basal Medium, targeting an initial glucose and xylose concentration in the growth medium of approximately 10 and 4 g/l, respectively. Six selected strains that showed a good growth on the biomass hydrolysate on agar plates were then tested on this substrate in shake flask batch cultures in the same conditions as described for the agar plates (Table 1). A working culture volume of 100 ml was used in 250 ml flasks, with the agitation rate set to 130 rpm. Prior to inoculation precultures were grown autotrophically. The experiment was then repeated in triplicates with the 3 strains of the final selection. Each screening experiment lasted 20 days and was performed in sterile conditions. The sterility of the cultures was confirmed at the end of each experiment by plating of the algae on agar plates with added glucose, or *via* microscopic observations.

2.1.3. Cell dry weight measurement

Biomass concentration was determined gravimetrically according to Zhu and Lee, 1997 using 2 to 5 ml of culture sample volume depending on the estimated biomass concentration, and 100 ml of Milli-Q water for washing [28].

2.1.4. Determination of glucose and xylose concentration

Glucose and xylose concentrations were measured enzymatically, glucose *via* the glucose oxidase/peroxidase (GOPOD), and xylose by the xylose mutarotase/β-xylose dehydrogenase (XMR/β-XDH) method using kits K-GLUC and K-XYLOSE from Megazyme.

2.2. Strain identification

Strain identification was done *via* ITS sequencing. ITS sequences of the nuclear rDNA obtained from single isolates were amplified by PCR using the forward primer (ITS1), TCCGTAGGTGAACCTGCGG, and the reverse primer (ITS4) TCCTCCGCTTATTGATATGC designed based on conserved nucleotide sequences of the nuclear 18S and 28S rDNA regions respectively [29]. The cells were lysed by boiling at 95 °C for 5 min prior to amplification. PCR products were purified, and the sequences were obtained by the Sanger method using Eton Bioscience, Inc.'s services (https://www.etonbio.com/). The sequences obtained were checked for homology using GenBank's (National Center for Biotechnology Information) Basic Local Alignment Search Tool (BLAST). Two reference sequences were selected per strain based on query cover and percent identity. A phylogenetic tree was constructed with the obtained and reference sequences *via* the Neighbor-Joining method using the software Geneious Prime (version 2022.1.1).

Table 1

Growth of algae strains on glucose, xylose, and corn stover hydrolysate in the light or dark.

Strain ID	Strain name	Origin	Glucose	Xylose	CSH
CCAP 11/41	*Chlamydomonas asymmetrica*	CCAP	+	−	(+)
NIES 2207	*Chlamydomonas asymmetrica*	NIES	+	−	(+)
UTEX LB 2609	*Chlamydomonas asymmetrica*	UTEX	−	−	
UTEX231*	*Chlamydomonas debaryana*	UTEX	+	−	(+)
CC 1419	*Chlamydomonas eugametos*	CC	−	−	
CC 1873	*Chlamydomonas eugametos*	CC	−	−	
CC 3349	*Chlamydomonas globosa*	CC	−	−	
SAG 24.72	*Chlamydomonas inflexa*	SAG	−	−	
UTEX B730	*Chlamydomonas mexicana*	UTEX	−	−	
CC 55	*Chlamydomonas moewusii*	CC	−	−	
CC 958	*Chlamydomonas moewusii*	CC	−	−	
CC 1875	*Chlamydomonas moewusii*	CC	−	−	
CC 1904	*Chlamydomonas moewusii*	CC	−	−	
CC 1373c	*Chlamydomonas smithii*	CC	−	−	
NIES 2234	*Chlamydomonas rapa*	NIES	−	−	
CPA	*Chlamydomonas pseudagloe*	SD	+	−	(+)
CPC	*Chlamydomonas pseudococcum*	SD	+	−	(+)
WG	*Chlorella sp.*	SD	+	−	−
WG7	*Chlorella sp.*	SD	+	−	(+)
BFS32	*Coelastrella sp.*	SD	+	−	(+)
DA25	*Desmodesmus armatus*	SD	+	−	(+)
PK25	*Parachlorella kessleri*	SD	+	−	(+)
IV006*	*Chlamydomonas sp.*	IV	+	−	(+)
IV031		IV	+	−	(+)
IV033		IV	+	−	(+)
IV041		IV	−	−	
IV055*	*Chlorella sp.*	IV	+	−	(+)
IV112		IV	+	−	(+)
IV113		IV	+	−	(+)
IV118		IV	+	−	(+)
IV131		IV	+	−	(+)
IV132*	*Chlorococcum sp.*	IV	+	(+)	(+)
IV139*	*Chlorococcum sp.*	IV	+	−	(+)
IV157		IV	+	−	(+)
IV161		IV	−	−	
IV162		IV	−	−	
IV164		IV	−	−	
IV168		IV	−	−	
IV172		IV	−	−	
IV173		IV	−	−	
IV220		IV	−	−	
IV233		IV	+	−	(+)
IV238		IV	+	−	(+)
IV241*	*Desmodesmus sp.*	IV	+	(+)	(+)
IV244		IV	−	−	

SD: isolate form San Diego area; IV: isolate form Imperial Valley area, CSH: corn stover hydrolysate; +: pure substrate utilization, (+): substrate utilization from lignocellulosic biomass hydrolysate.

2.3. Metabolomics

2.3.1. Untargeted polar metabolite analysis and lipidomics

The strains selected in Section 2.1 were grown autotrophically and heterotrophically in shake-flask cultures. In the dark the same medium was used as described in Section 2.1.2, with 10 g/l glucose as carbon source. In the light the cells were grown on modified Bold's Basal Medium containing 10 g/l sodium bicarbonate, and with 0.6 % CO_2 concentration adjusted in the growth chamber. The light, agitation rate, and temperature conditions were as described in Section 2.1.2. The biomass was harvested at late exponential phase. The separated pellet and supernatant were stored at −80 °C until analysis.

The preparation and analysis of the samples were performed at the University of California, Riverside Metabolomics Core Facility. Biphasic metabolite extractions from the freeze-dried supernatant and pellet were performed as described by Hollin el al., 2022, with some modifications [30]. Briefly: To break up the pellets, the samples were sonicated for 5 min, then vortexed for 1 h. To reach phase separation 250–270 μl LC-MS grade water was added per ml extraction buffer, followed by sonication for 2 × 5 min, with vortexing in between and at the end, then centrifugation at 1399 *g* for 30 min. 200 μl of the top organic layer resulting from the biphasic extraction were collected, dried under nitrogen stream, and prepared for lipidomics analysis by LC-MS. 200 μl of the aqueous bottom layer were dried under nitrogen stream for chemical derivatization and polar metabolite analysis by GC–MS. Ribitol was used as an internal standard.

The equipment and reagents used for LC-MS analysis were as described by Hollin el al., 2022 [30]. Data analysis was performed with Progenesis Qi software (Nonlinear Dynamics, Durham, NC). A Thermo 1300 GC coupled to a Thermo Fisher ISQ7000 mass spectrometer, equipped with a 30 m × 0.25 mm × 0.25 μm Thermo TG5SilMS column was used for the GC–MS analysis. Helium was the carrier gas. Data analysis was performed with MS-DIAL software (Yokohama City, Kanagawa, Japan).

2.3.2. Carbohydrate analysis

The strains were cultivated as described in Section 2.3.1 in autotrophic conditions, harvested at late exponential phase and kept at −80 °C until analysis. Sample pellet and supernatant preparation and analysis were performed at the University of California, San Diego GlycoAnalytics Core. The supernatant was dialyzed against deionized water, and freeze-dried. Inositol was used as internal standard. Samples were methanolyzed (1 M MeOH-HCl for 18 h at 80 °C), re-Nacetylated, and TMS derivatized. GC–MS analysis was performed by an Agilent Technologies 7820 GC system attached to 5975 MS detector, equipped with Restek-5MS capillary column. Helium was the carrier gas.

2.4. Testing for genetic transformation and protein expression

2.4.1. Genetic transformation

The selected strains were cultivated in mixotrophic conditions using modified Bold's Basal Medium with 5 g/l yeast extract as nitrogen source and glucose added at 20 g/l concentration, in shake flask cultures until reaching mid-exponential phase. The light, agitation rate, and temperature conditions were as described in Section 2.1.2. The cultures were diluted back with fresh medium the day before transformation. Each strain to be transformed, including the positive control *C. reinhardtii* CC124, was harvested, washed and resuspended in MAX Efficiency™ Transformation Reagent for Algae (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's guide, adding an extra washing step. All centrifugation steps were performed at 15 °C. Cells were processed in sterile conditions. 1 μg of linearized DNA was mixed with 250 μl of cell suspension for each strain, and incubated on ice for 10 min. Genetic transformation was performed through electroporation *via* a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA) in a 4 mm cuvette, with a time constant of 3 ms and two different field strengths: 1.25 and 2 kV/cm. Cells were allowed to recover for 24 h for the former and 48 h for the latter field strength setting, in 10 ml fresh medium containing 40 mM of sucrose, using the same light, temperature and agitation rate settings as for culturing. Cells were then harvested and resuspended in 2 ml of fresh media without sucrose. Half of the suspension was spread on plates containing 30 μg/ml hygromycin B and 10 μg/ml zeocin, the other half was spread on plates containing only 30 μg/ml hygromycin B. Antibiotic concentrations were chosen based on previous antibiotic sensitivity testing. After 7 to 11 days of incubation at continuous illumination at 100 μmol photons/m²s and room temperature individual colonies were transferred to 96-well cell culture plates containing modified Bold's Basal Medium with added glucose and incubated at the same light and temperature settings for 3 days. 3 colonies of the wild type strains were included in the plates as negative control.

2.4.2. Testing for protein expression

2.4.2.1. Fluorescence measurements. An Infinite® 200 PRO microplate reader (Tecan Group, Männedorf, Switzerland) was used to measure mClover fluorescence of the individual colonies after 3 days of incubation in the 96-well cell culture plates (Section 2.4.1), as described by Sproles et al., 2022 [31]. The clones selected based on the fold of induction were then grown in shake-flask cultures using the same media, light, temperature and agitation settings as described in Section 2.4.1. For each clone 1 ml of culture was harvested by centrifugation at late-exponential phase, and the pellet was frozen at −80 °C and stored for western blotting.

2.4.2.2. Western blotting. Pellets were allowed to thaw on wet ice for 2 min. Sonication was applied (4 cycles of 10 s on, 10 s off, at 20 %). Western blotting was performed as described by Berndt et al., 2021, with some minor modifications [11]. Namely: No BaseMuncher was added to the BugBuster® Protein Extraction Reagent. After separation the proteins were transferred *via* wet transfer to a nitrocellulose membrane (at 200 mA for 60 min). TBST was used for washing. Recombinant protein detection was by using goat anti-GFP conjugated to alkaline phosphatase (ab6661, Abcam, Boston, MA, USA) diluted in Haycock's solution at 1:10000.

## 3. Results and discussion

3.1. Strain screening on corn stover hydrolysate and its main sugar components

In the first step of strain selection, a total of 45 algae strains were screened for growth on glucose and xylose separately (Table 1; Fig. 1, Panel A and B). Our primary goal was to select strains in the dark, however testing was also performed in the light with the intention to provide additional information in case there is an interest in the future for an industrial process designed for mixotrophic conditions utilizing one of the selected strains. Out of the 45 strains screened, 15 were *Chlamydomonas* strains from public algae culture collections, while the remaining algae were local isolates from Southern California, either from the San Diego or the Imperial Valley area. Local isolates, especially from the desert areas, may have features which are beneficial in biological contamination control, such as high temperature or high salt resistance, that could be relevant when organic carbon sources are used for cultivation. The reason for putting emphasis on *Chlamydomonas* strains was from the perspective of future strain improvement, with the expectation that the genetic tools developed for the model organism, *Chlamydomonas reinhardtii* [32], would be more easily transferred into a species of the same genus, than to a genetically more distant species.

Of the initial 45 strains screened, 24 in total were able to grow on

A

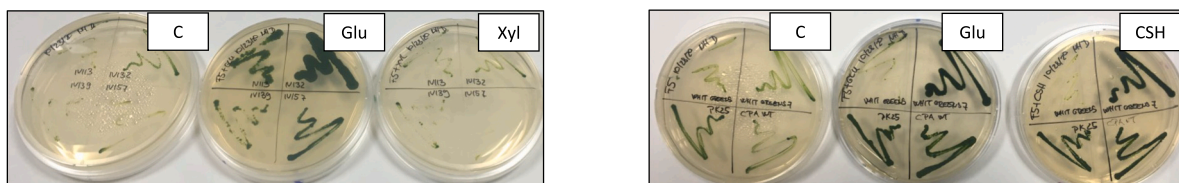| Strain ID	Origin	Glu	Xyl	CSH
CCAP 11/41	Culture collection	+	-	+
NIES 2207		+	-	+
UTEX231		+	-	+
CPA	San Diego isolates	+	-	+
CPC		+	-	+
WG		+	-	-
WG7		+	-	+
BFS32		+	-	+
DA25		+	-	+
PK25		+	-	+
IV006	Imperial Valley isolates	+	-	+
IV031		+	-	+
IV033		+	-	+
IV055		+	-	+
IV112		+	-	+
IV113		+	-	+
IV118		+	-	+
IV131		+	-	+
IV132		+	(+)	+
IV139		+	-	+
IV157		+	-	+
IV233		+	-	+
IV238		+	-	+
IV241		+	(+)	+
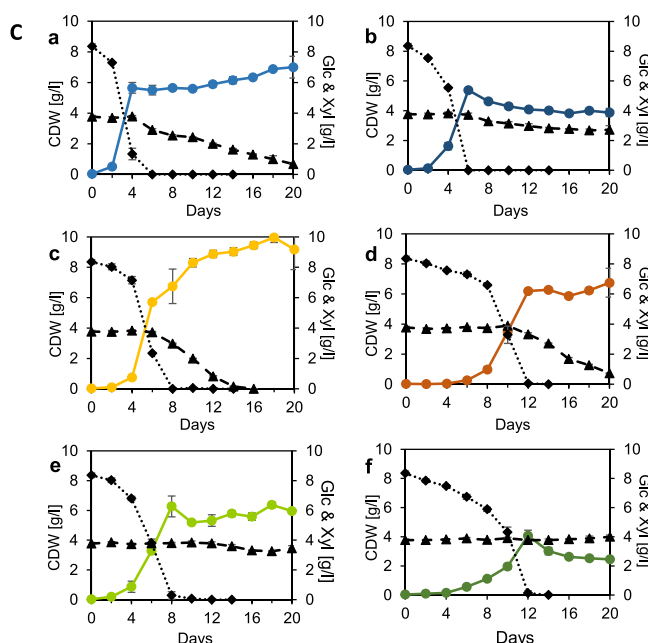
Fig. 1. Panel A and B Strain selection on glucose (Glu), xylose (Xyl), and corn stover hydrolysate (CSH) on agar plates, (C – control); Panel C Evolution of the cell dry weight, and the glucose and xylose concentration in the culture medium of *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana* grown on corn stover hydrolysate in the light (a), (c), (e), respectively, and in the dark (b), (d), (f), respectively; CDW: cell dry weight (colored lines); Glc: glucose (dotted lines); Xyl: xylose (dashed lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

glucose in the light or in the dark (Fig. 1, Panel A). Of the *Chlamydomonas* strains two *C. asymmetrica* strains, *C. debaryana*, *C. pseudogloe*, *C. pseudococcum*, and a *Chlamydomonas sp.* isolated from Imperial Valley, were able to assimilate glucose. None of the strains screened was able to grow on xylose as a sole carbon source under our experimental conditions.

In the next step, the 24 strains able to assimilate glucose were subjected to screening on crude corn stover hydrolysate. The growth on the lignocellulosic substrate was comparable to the growth on glucose, with the exception of *C. pseudococcum* (CPC), which showed signs of significant growth inhibition in the light (image not shown), and *Chlorella sp.* (WG), which was not able to grow either in the light or dark on corn stover hydrolysate. Six selected strains that showed a good growth on corn stover hydrolysate (Fig. 1, Panel A and B) on agar plates were tested in shake flask cultures (marked with an asterisk in Table 1). A local *Chlorella vulgaris* isolate, CV25, was selected as a positive control, as *C. vulgaris*, a commercially grown species, was previously reported to grow on lignocellulosic biomass hydrolysate [23]. *Chlamydomonas reinhardtii*, strain CC124, was used as negative control due to its documented inability to grow on glucose. All six strains showed higher biomass productivity and biomass density than CV25 (data not shown). Even though none of the strains appeared to be able to utilize xylose as a sole carbon source during the first step of the screening, based on visual observations (Fig. 1, Panel A and B), when grown on corn stover hydrolysate in shake flask cultures, 2 strains: IV132 and IV241 were observed to take up xylose to a significant degree. The initial xylose medium concentration was reduced by at least 50 % in a period of 20 days in the light and, for IV241, also in the dark, determined by measuring the residual sugar concentration in the medium at the end of the experiment (Fig. 1, Panel C).

Based on a combination of factors such as biomass productivity, maximum biomass density, xylose uptake from the lignocellulosic biomass hydrolysate, predicted ease of genetic transformation, and the potential to apply genetic tools already established, 3 strains were selected as potential industrial strains and were subjected to further characterization. 2 were Southern California isolates: IV132 (*Chlorococcum sp.*) and IV241 (*Desmodesmus sp.*), and one was a strain from a culture collection: UTEX231 (*Chlamydomonas debaryana*). For strain identification refer to Section 3.2.

Fig. 1, Panel C shows the kinetics of the changes in cell dry weight and in glucose and xylose concentration in the medium over time, both in the dark and in the light, for the three selected species grown on corn stover hydrolysate. The mean biomass productivities were 2.6, 2.5, and 1.4 g/ld in the light; and 1.3, 1.3, and 0.6 g/ld in the dark for *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana*, respectively. As for the maximum biomass densities, the values were 6.9, 10, and 6 g/l in the light; and 5.4, 6.2, and 4.1 g/l in the dark were measured for *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana*, respectively. Higher biomass productivities and maximum biomass concentrations observed under mixotrophic than under heterotrophic conditions for the selected strains are consistent with what was observed by other authors [33]. A possible reason for this may be that the presence of both inorganic and organic carbon in mixotrophic regime has a synergistic metabolic effect, leading to increased cellular biosynthetic capacity [34]. The biomass yield on a substrate depends on the organism [35]. For xylose consumption, the *Chlorococcum sp.* strain utilized 82 % of the monosaccharide present in the medium in the light, and 29 % in the dark, over a period of 20 days. *Desmodesmus sp.* metabolized 100 % of the xylose in mixotrophy and 81 % in heterotrophic growth. In the case of *C. debaryana* there was 14 % xylose consumed in the light, and no significant consumption of xylose in the dark. Biomass productivities, maximum biomass densities, and xylose consumption of the selected strains, were significantly higher than the values measured for the positive control, *C. vulgaris*, indicating greater organic carbon substrate utilization. *C. vulgaris* appeared to be under stress in the experimental conditions, especially in the light, marked by low biomass productivity,

low maximum biomass concentration, reduced pigment content (visual observation in the light), and low carbon substrate consumption. The biomass productivities for *C. vulgaris* were 0.1 and 0.2 g/ld; the maximum biomass densities were 1.1 and 1.8 g/l in the light and in the dark, respectively. Xylose consumption was not significant for this strain either in mixotrophic or in heterotrophic conditions. No significant growth was observed for the negative control strain, *C. reinhardtii*, in the dark, and in the light a maximum biomass density of 1.5 g/l was reached throughout the course of the experiment, most likely only as a result of autotrophic metabolism.

As shown on Fig. 1, Panel C both *Chlorococcum sp.* and *Desmodesmus sp.* followed a diauxic growth pattern when grown in the light: glucose appeared to be the preferential substrate, being metabolized first for rapid growth. After most of the glucose was consumed, xylose concentrations slowly declined, with a simultaneous gradual increase in biomass. In the case of *Desmodesmus sp.* the minor biomass increase after both glucose and xylose have been exhausted from the medium might be due to the assimilation of another organic carbon source present in corn stover hydrolysate, or it might as well be due to photosynthetic growth. In the dark there was also a reduction in xylose concentration only after glucose was depleted from the medium, though to a lesser extent in *Chlorococcum sp.*, and with no simultaneous increase in the biomass concentration. In the case of *C. debaryana*, as only minimal xylose consumption was observed either in the light or in the dark, after glucose was assimilated from the media the algae culture entered into stationary phase.

The observed sequential utilization of glucose and xylose by both *Chlorococcum sp.* and *Desmodesmus sp.* might be due to carbon catabolite repression, limiting the use of xylose while glucose is present [36]. This sugar assimilation pattern was observed for *C. vulgaris* as well [37]. It is likely that the xylose transport mechanism in these strains is similar to what was observed by Zheng et al., 2014 for *Chlorella sorokiniana*, *i.e.* via a glucose-inducible hexose/H+ symporter, as no substrate uptake was observed when the cultures were grown on pure xylose [38]. A higher affinity of the transporter to glucose would also support the observed diauxic growth pattern, as high glucose concentration would outcompete xylose uptake. It may also be possible that the presence of another sugar in the hydrolysate, such as arabinose, induces xylose utilization, as it was reported in the case of some filamentous fungi, in which xylose and arabinose metabolism are tightly linked [39]. The more significant xylose uptake in the light compared to the dark may be due to the extra reducing power generated in the form of NADPH, necessary for xylose assimilation, during the light-dependent reaction of photosynthesis [26,38]. In the dark, xylose taken up by the cells does not appear to support growth and may be converted into secondary metabolites.

3.2. Strain identification

Fig. 2 shows a phylogenetic tree constructed based on the internal transcribed spacer (ITS) sequences of the 5 local isolate strains screened in shake-flask cultures (Section 3.1), and on the reference strain sequences selected using GenBank's BLAST. The 5 strains belong into 2 classes of the phylum Chlorophyta: *Chlorophyceae* (IV006, IV132, IV139, IV241) and *Trebouxiophyceae* (IV055). 2 sequenced strains with already existing genetic tools were included in the tree as a reference: *Chlamydomonas reinhardtii* (AF033286.1) and *Chlorella vulgaris* (FM205832.1), representing the *Chlorophyceae* and the *Trebouxiophyceae*, respectively. *Tetraselmis striata* (HE610129.1), with a sequenced genome and existing genetic tools, belonging to the class *Chlorodendrophyceae* of the phylum Chlorophyta, closely related to the other 2 classes, was selected as an outgroup species. Bootstrap confidence levels are indicated next to the nodes. Branch lengths indicate genetic change, expressed in units of substitutions per site of the sequence alignment. Supplementary Table 2 shows the statistics of the BLAST hits.

As stated in Section 3.1, based on a set of criteria, one of which is their relatedness to a strain with existing genetic tools, the local isolates
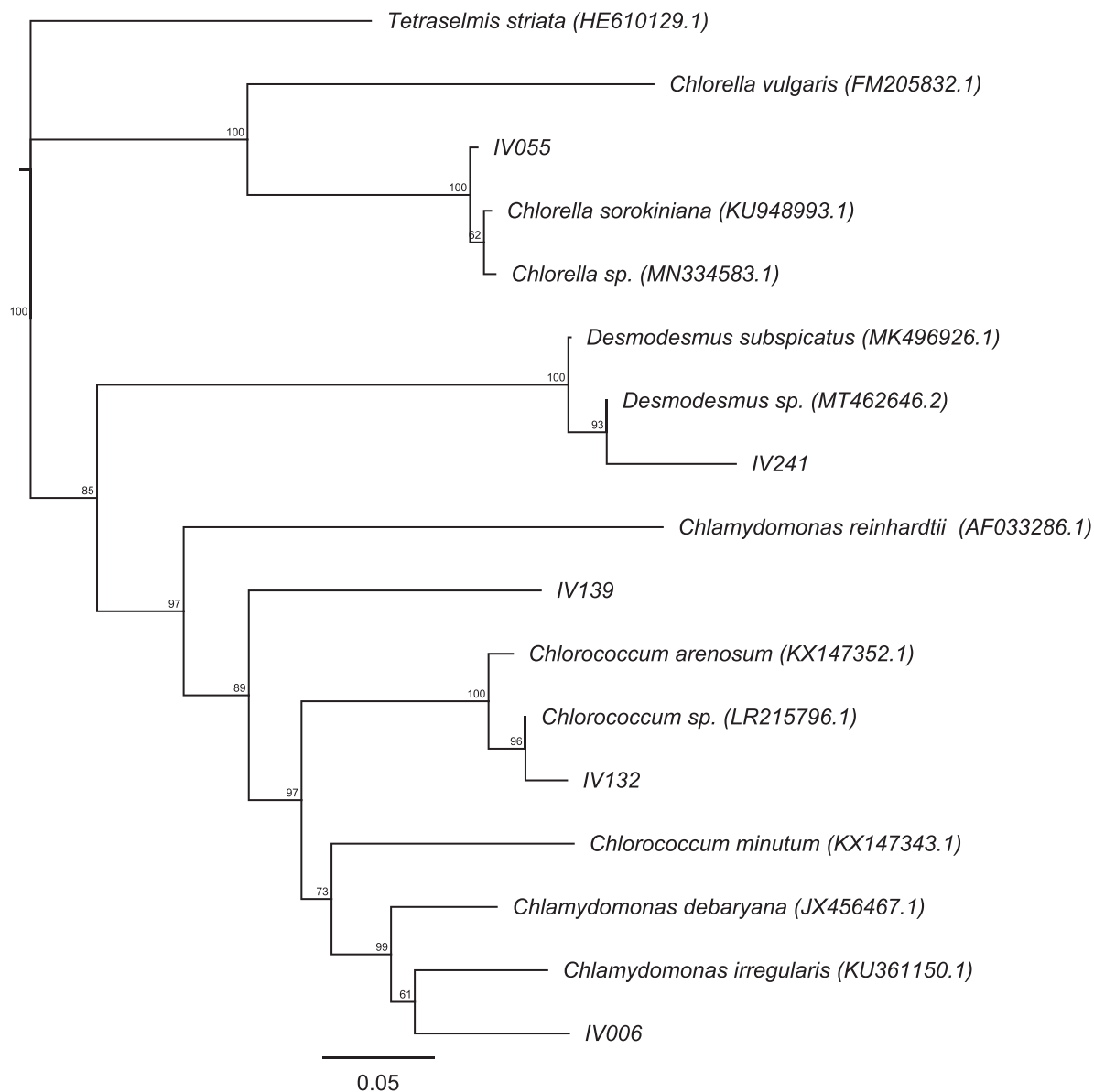
Fig. 2. Phylogenetic tree showing the downselected Southern California isolates.

IV132 and IV241 were selected for testing for transformation and protein expression, and for metabolic profiling. Based on the ITS sequencing results IV132 belongs to the genus *Chlorococcum*, and IV241 to the genus *Desmodesmus*, being in the same class as *Chlamydomonas reinhardtii*. The strains were registered at the UTEX algae collection under accession numbers UTEX 3181 *Chlorococcum sp.* and UTEX 3224 *Desmodesmus sp.*

3.3. Metabolomics

Even though the growth parameters of *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana* on corn stover hydrolysate appeared to be higher in mixotrophy than in heterotrophy (Section 3.1), we chose to perform metabolomics analysis on the selected strains in heterotrophic conditions, considering the lower reactor costs in this mode at industrial scale. The substrate used for biomass production for this analysis was glucose, due to the variability of corn stover hydrolysate composition. Results were compared to metabolomics performed on algae biomass produced under autotrophic conditions (Figs. 3 and 4).

As shown in Table 2, the main metabolites identified in this analysis that were highly abundant, and of potential industrial interest, were

certain amino acids [40], dicarboxylic acids [41], hydroxy monocarboxylic acids [19], short chain diols [20], triacylglycerols, and some lipid species rich in mono- and polyunsaturated fatty acids [42], depending on the algal strain and growth condition. The relevant metabolites that will be described in the following may be valuable for the food, feed, nutraceutical, pharmaceutical, chemical industries, or for the production of biofuels or bioplastics [18–20].

We observed in general more significant metabolite accumulation in heterotrophic conditions than in autotrophic mode, likely due to increased carbon flux partitioning under the carbon rich heterotrophic cultivation mode [21]. The heterotrophic cultures, especially of *Chlorococcum sp.* and *C. debaryana*, had elevated levels of the mixed acid fermentation products derived from pyruvate: butane-2,3-diol and lactic acid (Fig. 3), which have a role in achieving reducing power balance under limited oxygen availability [20], suggesting that these cultures may have been under low oxygen concentrations. The levels of these fermentation products were especially high for *Chlorococcum sp.* Tartaric acid levels in heterotrophic conditions were quite elevated for *Desmodesmus sp.* and *C. debaryana*.

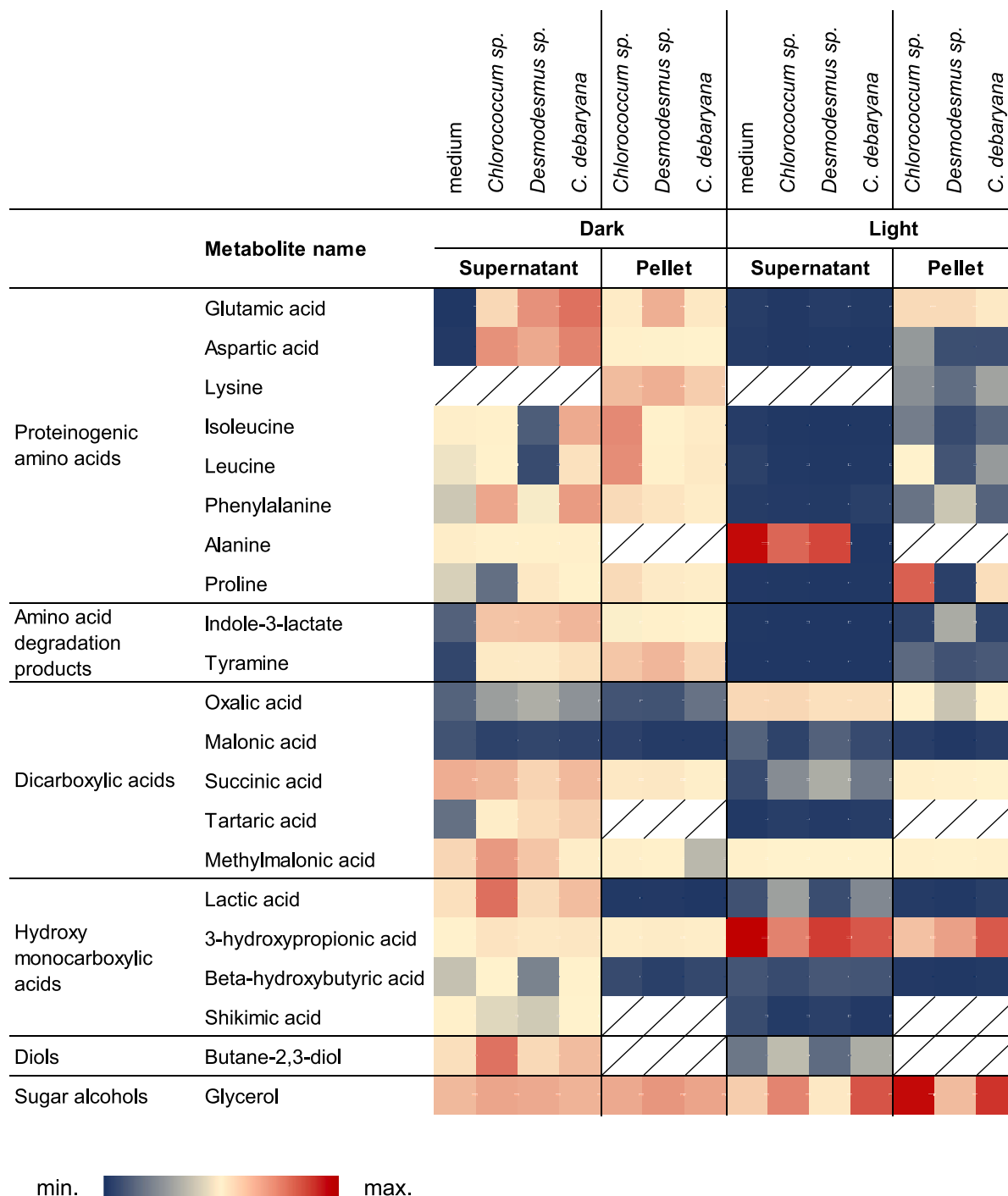The relative abundances of certain amino acids were also high in the

Fig. 3. Heatmap showing the relative abundances normalized by internal standard of the relevant polar metabolites selected for *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana* grown in autotrophic and heterotrophic conditions.

heterotrophic cultures, with some of these being secreted. Glutamic acid, aspartic acid, and lysine levels were elevated for all the strains. Isoleucine, leucine, and phenylalanine abundances were high in the case of *Chlorococcum sp.* and *C. debaryana*, and proline levels were elevated for *Chlorococcum sp.* and *Desmodesmus sp.* The relative pool sizes of these compounds depend on the interplay between protein and amino acid synthesis, and their degradation [43]. In our case, amino acid accumulation may be a result of *de novo* synthesis from glycolytic intermediates, such as phosphoenolpyruvate and pyruvate, or from intermediates of the TCA cycle, namely oxaloacetate and α-ketoglutarate. High amino acid concentrations can also be a result of protein degradation. Highly active

pathways of protein/amino acid degradation are indicated by the accumulation of indole-3-lactate and tyramine (Fig. 3), resulting from the catabolism of tryptophan and tyrosine, respectively, and of methylmalonic acid, the latter having especially high levels in the case of *Chlorococcum sp.*

In the late exponential phase, when the samples were taken, the heterotrophic cultures were possibly in a metabolic shift from exogenous carbon utilization for rapid biomass growth towards the synthesis of storage compounds, such as triacylglycerols, as indicated by the accumulation of these compounds for all the strains (Fig. 4) [44]. This is also suggested for *Chlorococcum sp.* and *C. debaryana* by the elevated
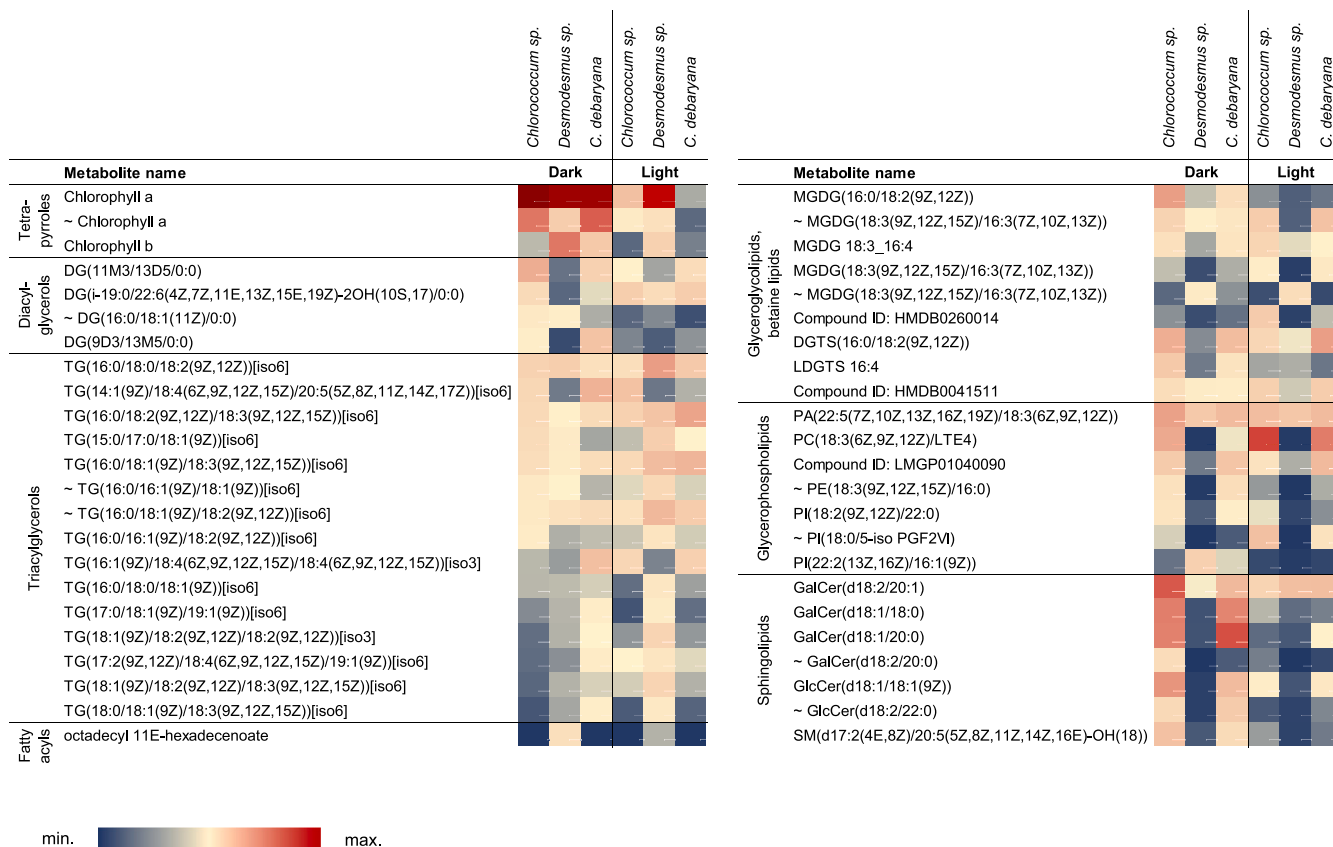
Fig. 4 heatmap — metabolite names (columns: Chlorococcum sp., Desmodesmus sp., C. debaryana under Dark; then Chlorococcum sp., Desmodesmus sp., C. debaryana under Light)

Left panel

Tetra-pyrroles
- Chlorophyll a
- ~ Chlorophyll a
- Chlorophyll b

Diacyl-glycerols
- DG(11M3/13D5/0:0)
- DG(i-19:0/22:6(4Z,7Z,11E,13Z,15E,19Z)-2OH(10S,17)/0:0)
- ~ DG(16:0/18:1(11Z)/0:0)
- DG(9D3/13M5/0:0)

Triacylglycerols
- TG(16:0/18:0/18:2(9Z,12Z))[iso6]
- TG(14:1(9Z)/18:4(6Z,9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]
- TG(16:0/18:2(9Z,12Z)/18:3(9Z,12Z,15Z))[iso6]
- TG(15:0/17:0/18:1(9Z))[iso6]
- TG(16:0/18:1(9Z)/18:3(9Z,12Z,15Z))[iso6]
- ~ TG(16:0/16:1(9Z)/18:1(9Z))[iso6]
- TG(16:0/18:1(9Z)/18:2(9Z,12Z))[iso6]
- TG(16:0/16:1(9Z)/18:2(9Z,12Z))[iso6]
- TG(16:1(9Z)/18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))[iso3]
- TG(16:0/18:0/18:1(9Z))[iso6]
- TG(17:0/18:1(9Z)/19:1(9Z))[iso6]
- TG(18:1(9Z)/18:2(9Z,12Z)/18:2(9Z,12Z))[iso3]
- TG(17:2(9Z,12Z)/18:4(6Z,9Z,12Z,15Z)/19:1(9Z))[iso6]
- TG(18:1(9Z)/18:2(9Z,12Z)/18:3(9Z,12Z,15Z))[iso6]
- TG(18:0/18:1(9Z)/18:3(9Z,12Z,15Z))[iso6]

Fatty acyls
- octadecyl 11E-hexadecenoate

Right panel

Glyceroglycolipids, betaine lipids
- MGDG(16:0/18:2(9Z,12Z))
- ~ MGDG(18:3(9Z,12Z,15Z)/16:3(7Z,10Z,13Z))
- MGDG 18:3_16:4
- MGDG(18:3(9Z,12Z,15Z)/16:3(7Z,10Z,13Z))
- ~ MGDG(18:3(9Z,12Z,15Z)/16:3(7Z,10Z,13Z))
- Compound ID: HMDB0260014
- DGTS(16:0/18:2(9Z,12Z))
- LDGTS 16:4
- Compound ID: HMDB0041511

Glycerophospholipids
- PA(22:5(7Z,10Z,13Z,16Z,19Z)/18:3(6Z,9Z,12Z))
- PC(18:3(6Z,9Z,12Z)/LTE4)
- Compound ID: LMGP01040090
- ~ PE(18:3(9Z,12Z,15Z)/16:0)
- PI(18:2(9Z,12Z)/22:0)
- ~ PI(18:0/5-iso PGF2VI)
- PI(22:2(13Z,16Z)/16:1(9Z))

Sphingolipids
- GalCer(d18:2/20:1)
- GalCer(d18:1/18:0)
- GalCer(d18:1/20:0)
- ~ GalCer(d18:2/20:0)
- GlcCer(d18:1/18:1(9Z))
- ~ GlcCer(d18:2/22:0)
- SM(d17:2(4E,8Z)/20:5(5Z,8Z,11Z,14Z,16E)-OH(18))

min. — max.

Fig. 4. Heatmap showing the normalized relative abundances (total ion current) of the relevant lipid species selected for *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana* grown in autotrophic and heterotrophic conditions; ~: similar to.

Table 2
Single metabolites and metabolite groups of high relative abundance (+ to +++) of potential industrial interest produced by the selected strains in the light and in the dark.

Metabolite name	Industrial use	Chlorococcum sp.		Desmodesmus sp.		C. debaryana	
		Dark	Light	Dark	Light	Dark	Light
Amino acids							
Glutamic acid	Food [40,49]	+	+	+++	+	+++	+
Aspartic acid	Food [49], pharmaceutical, chemical, green (polymers) [54]	++		++		+++	
Isoleucine	Food, feed, pharmaceutical [40]	++				++	
Leucine	Food, feed, pharmaceutical, cosmetics [49]	++				+	
Phenylalanine	Food, feed, [40,49] pharmaceutical [55]	++		+		++	
Lysine	Food, feed, pharmaceutical [40,49]	++		++		+	
Proline	Food, pharmaceutical, [56]	+	+++	+		+	+
Dicarboxylic acids							
Oxalic acid	Food, pharmaceutical, chemical, green [57]		+				
Succinic acid	Food, pharmaceutical, chemical, agriculture, [58] green (polymers) [18]	+	+		+		+
Tartaric acid	Food, [41] pharmaceutical, chemical, construction [59]	+		+		+	
Hydroxy monocarboxylic acids							
Lactic acid	Food, pharmaceutical, cosmetic, chemical, green (polymers) [19]	++		+		+	
3-Hydroxypropionic acid	Chemical, green (polymers) [51]	+		+		+	
Butane-2,3-diol	Chemical, green (polymers) [20]	++		+		+	
Lipids							
Chlorophylls	Food, pharmaceutical, cosmetics [60]	+++	+	+++	++	+++	+
Triacylglycerols	Green [19]	++	++	++	+++	+++	+++
Lipids rich in mono- & polyunsaturated fatty acids	Food, feed, [61] green (polymers) [18]	+++	++	++	++	+++	+++

abundance of free amino acid pools, as mentioned before, and by a somewhat more significant accumulation of diacylglycerols, which are compounds at the crossroad of storage lipid and membrane lipid synthesis [42].

The most abundant fatty-acyl moieties for the three strains in different lipid classes were: C16:0, C18:1, C18:2, and C18:3. Indeed, these were identified as the major fatty acids for other algal species as well in the class of the *Chlorophyceae* [16]. Significant polyunsaturated fatty acids also present were: C16:3, C17:2, C18:4, C22:5, C22:6 for all the strains; C16:4, C20:5 for *Chlorococcum sp.* and *C. debaryana*; and C22:2 for *Desmodesmus sp.*

In autotrophic conditions in general, low accumulation of metabolites was observed, as mentioned before, with the exception of glutamic acid, especially for *Chlorococcum sp.* and *Desmodesmus sp.*, a key compound in nitrogen assimilation [45]; proline for *Chlorococcum sp.*, and *C. debaryana*; and triacylglycerols, especially for *Desmodesmus sp.* Proline often has a role in osmoregulation, high osmolarity leading to the accumulation of this amino acid [45]. It is possible that increased relative abundance of proline may be due to the presence of high concentration of sodium bicarbonate in the autotrophic medium. The stress-response of the different species studied may differ, and it is possible that in the case of *Desmodesmus sp.* high concentration of bicarbonate in the medium caused more significant triacylglycerol accumulation [16]. Lower levels of chlorophyll in the light than in the dark (Fig. 4), may also indicate that the cultures were under a certain degree of stress, chlorophyll degradation possibly being caused by the reactive oxygen species generated [46].

Metabolic engineering approaches may be applied to reroute the carbon fluxes in the selected strains in order the increase the accumulation of compounds of economic importance, such as hydroxy monocarboxylic acids, *i.e.* shikimic acid, beta-hydroxybutyric acid, and 3-hydroxypropionic acid; and dicarboxylic acids, *i.e.* malonic acid, oxalic acid, and succinic acid.

Fig. 5 shows the monosaccharide composition of the biomass and of the exopolysaccharides of the three strains cultivated in autotrophic conditions, giving an indication on possible polysaccharide composition. The monosaccharide profiles of *Chlorococcum sp.* and *C. debaryana* are comparable to each other, both in the case of the biomass and the exopolysaccharides, while *Desmodesmus sp.* differs from the other two strains in this regard. A possible reason for this is that *Chlorococcum sp.* and *C. debaryana* are phylogenetically closer to each other (Section 3.2), and are more distant from *Desmodesmus sp.* This might also explain why the metabolite accumulation patterns of *Chlorococcum sp.* and *C. debaryana*, as previously described, were more similar to each other.

The main monosaccharide in the pellet for all the strains was glucose, likely mainly from starch. The second most abundant monosaccharide was arabinose for *Chlorococcum sp.* and *C. debaryana*, and mannose for *Desmodesmus sp.*, most likely largely from the cell wall. The cell wall composition of microalgae is very diverse. Members of the *Chlorophyceae* class were shown to produce a variety of structures including cellulose-pectin complexes and arabinogalactan proteins [47].

As for the exopolysaccharides: the main monosaccharide for *Chlorococcum sp.* and *C. debaryana* was arabinose, just as in the pellet. For *Desmodesmus sp.* it was glucose, and then mannose. The exopolysaccharides of the Chlorophytes show a degree of heterogeneity across species, however the main monosaccharide was often found to be galactose, and in some cases glucose or rhamnose. Arabinose was also observed to be present in higher concentrations, although typically not as the main monosaccharide [17].

Further research on the polysaccharide composition and biological activities may be of interest to evaluate the applicability of these compounds for the food, feed, nutraceutical or other industries.

In general, the primary microalgal products are considered to be biofuel related compounds [19] and animal feed. Also, microalgae have shown success in production of correctly folded and functional complex recombinant mammalian proteins. The utilization of algae as a
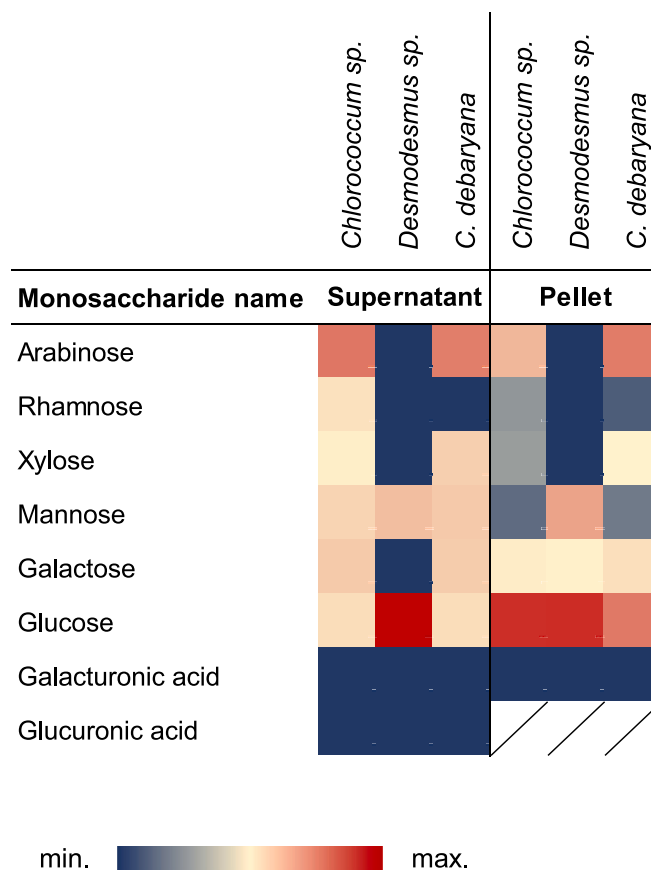

Fig. 5. Heatmap showing the monosaccharide composition of the biomass and the exopolysaccharides secreted by *Chlorococcum sp.*, *Desmodesmus sp.*, and *C. debaryana* grown in autotrophic conditions.

production platform for high value therapeutics is deemed as financially viable business model [10–13,48]. On the other hand, in order to have an economically feasible algal industry besides the efforts in reducing the costs of the upstream and downstream processes, primary algal products should be presented to the market combined with their by-products. As a summary, the compounds highlighted above are of high industrial relevance. More specifically glutamic acid is widely used as a flavor enhancer; lysine as a feed additive for swine, poultry and fish, among others. The primary use of the branched-chain amino acids leucine and isoleucine is as a supplement to enhance strength for athletes. Aspartic acid and phenylalanine are ingredients of the sweetener Aspartame [40,49]. Many of these metabolites can be used as precursors for the production of biodegradable bio-based polymers, such as succinic acid, lactic acid, and 3-hydroxypropionic acid, precursors for polyurethane [18], polylactic acid [19], and poly(3-hydroxypropionate) [50], respectively. Butane-2,3-diol can be used as liquid fuel, moreover it can be chemically converted into 1,3-butadiene, which enters into the manufacturing of rubber [20].

3.4. Testing for genetic transformation and protein expression

As no genome sequence information was available for *Chlorococcum sp.*, *Desmodesmus sp.*, or *C. debaryana*, a green fluorescent protein (*mClover*) expression vector (pAES5) designed for nuclear transformation of the phylogenetically related model organism *Chlamydomonas reinhardtii* was used for the transformation [31]. This vector allows for double selection on hygromycin B and zeocin. Panel A of Fig. 6 shows the colonies formed 10 days after transforming pAES5 into cells, using electroporation with a field strength of 1.25 kV/cm, of *Chlorococcum sp.*, with selection on media containing 30 μg/ml
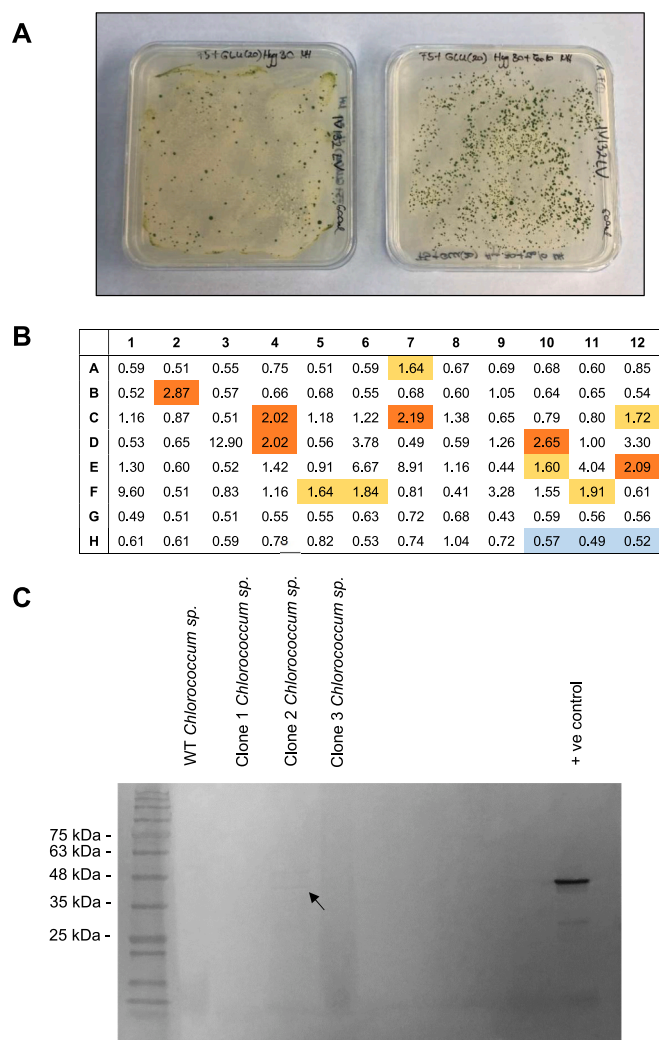
A

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.59	0.51	0.55	0.75	0.51	0.59	1.64	0.67	0.69	0.68	0.60	0.85
B	0.52	2.87	0.57	0.66	0.68	0.55	0.68	0.60	1.05	0.64	0.65	0.54
C	1.16	0.87	0.51	2.02	1.18	1.22	2.19	1.38	0.65	0.79	0.80	1.72
D	0.53	0.65	12.90	2.02	0.56	3.78	0.49	0.59	1.26	2.65	1.00	3.30
E	1.30	0.60	0.52	1.42	0.91	6.67	8.91	1.16	0.44	1.60	4.04	2.09
F	9.60	0.51	0.83	1.16	1.64	1.84	0.81	0.41	3.28	1.55	1.91	0.61
G	0.49	0.51	0.51	0.55	0.55	0.63	0.72	0.68	0.43	0.59	0.56	0.56
H	0.61	0.61	0.59	0.78	0.82	0.53	0.74	1.04	0.72	0.57	0.49	0.52

C

Fig. 6. Panel A Representative plate showing colonies formed after transformation on media containing 30 μg/ml hygromycin B (left) and 30 μg/ml hygromycin B + 10 μg/ml zeocin (right) for *Chlorococcum sp.*; Panel B mClover fluorescence intensities normalized to chlorophyll fluorescence, expressed in relative fluorescence units (RFU), for selected putative transformant clones of *Chlorococcum sp.* Negative control values are highlighted in blue, values showing a fold of induction in the range of 2.8–3.8 are highlighted in yellow, and values in the range of 3.8–5.8 are highlighted in orange.; Panel C Western blot characterizing the expression of mClover in selected clones of *Chlorococcum sp.* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hygromycin B (left) and 30 μg/ml hygromycin B + 10 μg/ml zeocin (right). Unexpectedly, in the case of single selection 1750 colonies/μg DNA were obtained, while 8500 colonies/μg DNA were obtained with the double selection [as a reference, Wang et al., 2019 obtained a transformation efficiency of 2000–6000 transformants/μg exogenous DNA for *C. reinhardtii* with a square-wave electroporation method, using a paromomycin-resistance cassette] [51]. We expected just the opposite of what we observed, namely lower colony count for double antibiotic selection than for single selection [31]. We have repeated the genetic transformation procedure, and we got a confirmed reproducibility of our results. The reason for the observed phenomenon might lie in the mode of action of zeocin, an antibiotic causing DNA double-strand breaks, and its effect on *Chlorococcum sp.* in the applied concentration. The product of the *Sh ble* gene forms a complex with zeocin, preventing the action of the antibiotic on the DNA. Resistance to zeocin is proportional to the expected expression level of the *Sh ble* gene, due to the stoichiometric

relationship between the antibiotic and the gene product. Partial DNA cleavage may occur when the *Sh ble* expression is low [52]. It has been reported that exposure to zeocin can have a mutagenic effect in non-transgenic algae [53]. Survival may happen by adaptation involving double-strand break repair, genotypic modification, altering the cellular metabolism *etc.* [53]. It is therefore possible that, in the applied dose, zeocin had a similar effect either on the low expressing transformants, or on the cells which did not have DNA integration.

Panel B of Fig. 6 shows the normalized mClover fluorescence measurements for a set of colonies picked from double selection. Highlighted in yellow and orange are the potential high mClover expressing transformants, with a fluorescence fold induction of 2.8–3.8 and 3.8–5.8, respectively. Panel C of Fig. 6 shows the western blot analysis for 3 *Chlorococcum sp.* clones selected based on the mClover fluorescence measurements. A faint signal can be observed at a size of ~41 kDa for clone 2, the size of the mClover fused with Ble, indicating production of the recombinant protein. A very faint band shows on the western blot for *Chlorococcum sp.* transformant in comparison to *C. reinhardtii* transformant, likely a result of the gfp gene used having been codon optimized to *C. reinhardtii* and not for *Chlorococcum sp.* Also, the AR1 promoter used is well characterized for high expression in *C. reinhardtii*, which may not be true for *Chlorococcum sp.*

The presented data indicate that *Chlorococcum sp.* is transformable following standard protocol, even using the genetic toolbox established for *C. reinhardtii*. Nevertheless, sequencing of the strain will be necessary to optimize the vector design, and improve transformation efficiency. In contrast to *Chlorococcum sp.*, we did not get a significant number of colonies on the selection media after transformation in the case of *Desmodesmus sp.* and *C. debaryana*, hence the protocol, and vectors used, will need to be further optimized for these strains.

4. Conclusions

In the present work we characterized wild isolates and culture collection green algae to identify those capable of robust growth on inexpensive media derived from corn stover hydrolysate. Of the 45 strains initially characterized, three were selected based on their ability to efficiently grow on the corn stover hydrolysate as a carbon source: *Chlorococcum sp.*, *Desmodesmus sp.*, and *Chlamydomonas debaryana*. Metabolomic analysis of these strains identified several metabolites of high relative abundances that are of commercial interest. These results suggest that with further development, these strains could open the door to economic production of high value commercial compounds utilizing waste streams from cellulosic biomass. These potential products could be economically viable for the green polymer, biofuel, pharmaceutical, feed, construction, food, and cosmetics industries. We have succeeded in the genetic manipulation of one of these strains, *Chlorococcum sp.*, utilizing standard genetic engineering protocols and vectors. As a next step, sequencing of the selected strains, quantifying the relevant metabolites, and providing a genetic toolbox for each of these, could allow for the development of new commercially viable strains of green algae for the production of a variety of highly valuable products.

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CRediT authorship contribution statement

Amr Badary: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. **Nora Hidasi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. **Simone Ferrari:** Funding acquisition, Supervision, Writing – review & editing. **Stephen P. Mayfield:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

Data availability

Data will be made available on request.

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