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New green biorefineries from cyanobacterial-microalgal consortia: Production of chlorophyll-rich extracts for the cosmetic industry and sustainable biogas

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ABSTRACT

Microalgae are promising and sustainable candidates for developing biorefineries to obtain valuable bioproducts and bioenergy. However, key challenges, such as low biomass production, high-cost harvesting, and non-efficient extractions, are restricting its large-scale production. Symbiotic relationships between microalgae and cyanobacteria can simultaneously mitigate these technical and economic restrictions. This research aims to develop sustainable and cost-effective biorefineries from two microalgae-cyanobacteria consortia to produce valuable ingredients for the cosmetic field (chlorophylls) and bioenergy (biogas). Solvent screening and cell disruption experiments were carried out to optimise the chlorophyll extraction protocols. Green solvents were chosen for both consortia. The mildness method (vortexing) was enough to achieve the maximum extraction level of chlorophylls (4.8 ± 0.2 mg/g) using 96% ethanol from a consortium composed of *Chlorella vulgaris*, *Tetraselmis* sp., and *Kamptonema* sp. The use of bead-beating was even more effective using water in the case of the consortium of *C. vulgaris* and *Arthrospira platensis* (13.5 ± 1.1 mg/g). High-potential antioxidant chlorophyll extracts were obtained for the cosmetic sector. As ethanol traces were found in the residual biomass of the *C. vulgaris*, *Tetraselmis* sp., and *Kamptonema* sp. consortium, the highest cumulative biomethane production (472 ± 32 $mLCH_4/gVS$) was achieved with this residual biomass, the corresponding value for the initial consortium being significantly lower (239 ± 32 $mLCH_4/gVS$). The study concludes through the overall mass balances of the best biorefineries that it is possible to recover up to 100% of both consortia weight as cosmetic ingredients, biogas, and fertilisers or cultivation media.

1. Introduction

Microalgae are promising and sustainable candidates for developing biorefineries to obtain valuable bioproducts and bioenergy in the context of a circular economy. However, some limitations and challenges must be overcome to scale up the technology from pilot to industrial scale. The most critical issues are low biomass production, high-cost harvesting stage, and non-efficient extraction of bioproducts and bioenergy. In this context, symbiotic relationships between microalgae and cyanobacteria can simultaneously mitigate these technical and economic restrictions. In addition, consortia of microalgae and cyanobacteria can result in higher production of valuable bioproducts with extraordinary biotechnological applications such as proteins, carbohydrates, lipids, vitamins, or pigments. Some of those bioproducts are ingredients for the cosmetic industry because of their potential as antioxidant, moisturising, photoprotective, and whitening agents (Nowruzi et al., 2020). In fact, there is a current concern for finding novel and natural antioxidants, as the ones obtained from microalga-cyanobacteria consortia, in the cosmetic sector to remove the skin effect of external irritants (pollution or UV radiation) and intrinsic ageing, as both induce the formation of reactive oxygen species that cause oxidative stress resulting in damaged skin (Jaffri, 2023).

Among the bioactive products obtained from microalgae and cyanobacteria with a potential application as antioxidants in the cosmetic

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industry, chlorophyll is a green and liposoluble pigment consisting of a tetrapyrrole chelated with magnesium at the centre. Thus, chlorophyll acts as an antioxidant, scavenging reactive oxygen species and, consequently, leading to healthier skin (Agustina et al., 2021). The global chlorophyll market size was US$ 252.19 million in 2021 and is forecast to reach US$ 504.10 million by 2030 (Polaris Market Research, 2022). Increasing demand from the cosmetic industry and consumer awareness of healthy cosmetics are some of the factors driving the growth of the global chlorophyll extract market.

Chlorophylls can be recovered from microalgae and cyanobacteria and their corresponding consortia either by using organic solvents such as methanol, ethanol, dimethylformamide (DMF), acetone, and diethyl ether or by supercritical fluid extraction (Halim et al., 2010). More recently, an aqueous solution of ionic liquid and surfactants was also used for chlorophyll extraction from microalga (Martins et al., 2021). However, green solvents that are sustainable, clean, safe, non-toxic, and yield higher chlorophyl content should be selected for the extraction of chlorophyll, particularly for its use in the cosmetic sector.

Chlorophylls are located in the chloroplasts of eukaryotic microalga cells and in the lamellae of cyanobacteria. Thus, effective chlorophyll extraction requires a pretreatment (e.g., microwave, sonication, homogenisation, grinding) to break the cell walls. In the case of consortia, which have strains with different cell wall structure and composition, chlorophyll extraction sets out the first challenge of developing a specific cell disruption method that allows releasing the chlorophylls within the cells of the strains of the consortia in the most economical, effective, and clean way. The breaking down of the cell wall depends, in turn, on the cell wall characteristics (rigidity, size, and surface properties) of the microalgae and cyanobacteria strains that composed the consortia, affecting the economic cost of chlorophyll extraction. For this reason, protocols for extracting chlorophylls should be optimised, focusing on the structure and nature of the cell walls of the consortia species.

Another challenge of using microalgae and cyanobacteria to obtain bioproducts such as chlorophyll is the high cost of biomass production. In this context, the sustainable biorefinery concept, in which different products can be obtained with minimum waste generation, appears to be a solution to improve the use of cyanobacterial-microalgal consortia as a resource to produce chlorophylls, exploiting the residual biomass to produce bioenergy.

Anaerobic digestion is nowadays one of the most sustainable processes to produce bioenergy from microalgae and cyanobacteria due to its high energy recovery rates and the possibility of avoiding preprocessing of the microalga biomass, such as drying, which is an economic and energy-demanding step. Biogas production from microalgae and cyanobacteria is still not considered profitable. However, a whole-integrated process constitutes a promising alternative to increase the overall efficiency and cost-effectiveness of the process. In this context, the research of microalga and cyanobacteria biorefineries has lately focused on the extraction of valuable pigments such as phycobiliproteins (Arashiro et al., 2020; Van Den Hende et al., 2016) and carotenoids (Espada et al., 2020; Goswami et al., 2022), together with the production of biogas from spent biomass. In addition, residual biomass obtained from *C. vulgaris* microalga after extracting a valuable fraction of chlorophylls, proteins, and lipids was used as feedstock for biogas production (Markou et al., 2022). However, past studies have not evaluated a biorefinery from microalgal-cyanobacteria consortia to obtain a chlorophyl-rich extract with suitable properties for the formulation of cosmetic products and bioenergy in form of biogas.

The present work raises a novel green biorefinery of two microalgae-cyanobacteria consortia. The study is focused on the extraction of chlorophyll and the assessment of the residual biomass to produce biogas. Sustainable chlorophyll extraction protocols are selected and optimised regarding cell disruption methods and suitable solvents, whereas chlorophyll-rich extracts are analysed to assess their potential as antioxidants for the cosmetic industry. This approach, starting from microalgae and cyanobacteria consortia, that includes chlorophyll extraction optimisation, the potential application of the chlorophyl-rich extract in the cosmetic industry, and biogas valorisation from the residual biomass, have not been previously studied so far.

2. Materials and methods

2.1. Cyanobacterial-microalgal consortia culture and characterisation

Marine Algaemass® and Chlospira® consortia were cultured and supplied by Microalgae Solutions (Madrid, Spain). The consortia were cultivated in flat photobioreactors with a temperature of 25 ± 2 °C, a photoperiod of 16:8, and a photon flux density of 100μmols/m^2s. Modified F/2 (Guillard's) medium was used for the consortia's growth. Marine Algaemass® is composed of *C. vulgaris* (10%), *Tetraselmis* sp. (30%), and *Kamptonema* sp. (60%). Chlospira® contains *C. vulgaris* (40%) and *A. platensis* (60%). Table 1 summarises the algae group, cell wall properties (structure and composition), and type of chlorophyll of the main species that constitute each consortium (D'Hondt et al., 2017; Graham et al., 2009; Read et al., 2007). Table 2 shows the biochemical composition, ashes, and elemental composition of both consortia.

Carbohydrates and proteins were measured according to methods described by Dubois et al. (1956) and Lowry et al. (1951), respectively. Lipids were determined according to a Bligh and Dyer (1959) modified protocol using a mixture of water:chloroform:methanol (2:2:1 (v/v)). Chloroform and methanol were supplied by Merck (Darmstadt, Germany). Ashes were determined by the method of Liu (2019), where the sample was calcined at 600 °C overnight in a muffle furnace (Nabertherm Lilienthal, Germany). Elemental composition was determined using a CHNS Flash 2000 analyser (Thermo Fisher Scientific. Waltham, MA, USA).

2.2. Chlorophyll extraction

Extraction of chlorophyll was optimised based on its different solubility in solvents and the use of cell disruption methods to enable chlorophyll release outside the cells.

2.2.1. Solvent screening

Four pure solvents were used: acetone, N,N-dimethylformamide (DMF), ethanol, and methanol. 80% acetone and 96% ethanol were used to increase the polarity of the corresponding pure solvents. All of them were analytical grade and supplied by Merck (Darmstadt, Germany). The screening of solvents was performed using the following methodology: 30 mg of biomass was stirred for 20 min in a Vortex device (IKA-Werke GmbH. Staufen, Germany) with 10 mL of the solvent, followed by ultrasound-assisted extraction for an additional 25 min in an Elmasonic P bath (Elma Schmidbauer GmbG. Singen, Germany), providing a maximum power of 738 W and a frequency of 37 kHz. The mixture was centrifuged (10000 rpm, 10 min) in a 5910 Eppendorf centrifuge (Hamburg, Germany).

2.2.2. Cell disruption

Different cell disruption methods were applied to improve the chlorophyll extraction using pure ethanol and ethanol 96%: vortexing (I), manual press (II), vortexing plus ultrasounds (III), and manual press plus vortexing coupled with ultrasounds (IV). Method I consisted of mixing the biomass (30 mg) and solvent (10 mL) and stirring the mixture during 20 min in a Vortex 3 device. In method II, the biomass was pressed with a manual hydraulic press (Specac Ltd. Orpington, UK) with a charge of 10 tons (24000 psi). The chlorophyll was extracted in method III by applying method I plus ultrasounds in an Elmasonic P bath at the following conditions: 738 W, 37 kHz, 50 °C and 25 min. Method IV includes the application of method II and III. As a cell disruption alternative (method V), a bead-beater (Biospect Products, Inc. Bartlesville, OK, USA) with glass beads and water was used (3min). After applying the above methods, the supernatant and the residual biomass were

Table 1
Group, cell wall properties, chlorophyll type of the species in the consortia.

Strain	Group (Common name)	Cell wall layers and composition	Chlorophyll type	Consortium
C. vulgaris	Chlorophyta (green algae)	Two Layers: - Layer II: Algaenan layer (Sporopollenin) - Layer I: cellulose fibrilar layer (mannoglucan, chitin-like glycans, glucosamine, rhamnose and galactose)	a, b	Marine Algaemass® Chlospira®·
***Tetraselmis* sp.**	Chlorophyta (green algae)	Two or three layers: - Cell wall theca composed of crystalline scales	a, b	Marine Algaemass®
A. platensis	Cyanobacteria (blue-green algae)	Four layers: - Layer IV: outer membrane - Layer III: proteinaceous fibrillar - Layer II: peptidoglycan - Layer I: fibrils	a	Chlospira®·
***Kamptonema* sp.**	Cyanobacteria (blue-green algae)	Tree Layers: - Layer III: mucilage (outer membrane) - Layer II: proteinaceous fibrillar - Layer I: peptidoglycan	a	Marine Algaemass®

Table 2
Characterisation of cyanobacterial-microalgal consortia (dry weight basis).

	Marine Algaemass®	Chlospira®
Biochemical Composition (wt%)		
Proteins	28.4 ± 1.4	39.9 ± 3.0
Carbohydrates	11.9 ± 0.6	23.7 ± 2.9
Lipids	28.5 ± 2.3	22.0 ± 0.2
Ashes	29.6 ± 1.3	14.9 ± 0.8
Elemental Analysis (wt%)		
C	31.4 ± 0.1	40.8 ± 0.1
H	4.74 ± 0.01	5.63 ± 0.01
N	6.02 ± 0.01	6.85 ± 0.05
S	1.24 ± 0.01	0.83 ± 0.06
C/N	5.22 ± 0.02	5.96 ± 0.05

separated by centrifugation (10000 rpm, 10 min) using an Eppendorf 5910 centrifuge (Hamburg, Germany).

2.3. Analysis of chlorophyll-rich extracts

2.3.1. Chlorophyll content

The amounts of chlorophyll *a* (Chla) and b (Chlb) extracted were measured by spectrophotometric absorption in a Cary 500 UV–Vis–NIR spectrophotometer (Varian, Inc. Palo Alto, CA, USA). According to Table 1, chlorophyll *c* and *d* are not present in the consortia. Since the estimation of chlorophyll concentration is affected by the solvent, different equations have been used. Equations (1)–(8) were used for acetone, 80% acetone, 96% ethanol, and methanol (Lichtenthaler and Wellburn, 1983), Equations (9) and (10) for ethanol (Rowan, 1989), and Equations (11) and (12) for DMF (Porra et al., 1989).

Acetone: Chla (μg/mL) = $11.75A_{662}$-$2.35A_{645}$ [Eq. 1]

Chlb (μg/mL) = $18.61A_{645}$-$3.96A_{662}$ [Eq. 2]

80% Acetone: Chla (μg/mL) = $12.21A_{663}$-$2.81A_{646}$ [Eq. 3]

Chlb (μg/mL) = $20.13A_{646}$-$5.03A_{663}$ [Eq. 4]

96% Ethanol: Chla (μg/mL) = $13.95A_{665}$-$6.88A_{649}$ [Eq. 5]

Chlb (μg/mL) = $24.96A_{649}$-$7.32A_{665}$ [Eq. 6]

Methanol: Chla (μg/mL) = $15.65A_{666}$-$7.34A_{653}$ [Eq. 7]

Chlb (μg/mL) = $27.05A_{653}$-$11.21A_{666}$ [Eq. 8]

Ethanol: Chla (μg/mL) = $13.7A_{665}$-$5.76A_{649}$ [Eq. 9]

Chlb (μg/mL) = $25.8A_{649}$-$7.6A_{665}$ [Eq.10]

DMF: Chla (μg/mL) = $12.00A_{663.8}$–$3.11A_{646.8}$ [Eq. 11]

Chlb (μg/mL) = $20.78A_{646.8}$–$4.88A_{663.8}$ [Eq. 12]

Where A_i is the absorbance at the specific wavelength (nm).

Chlorophyll extraction yields (Y) are expressed as the amount of chlorophyll extracted (mg) per g of biomass (dry weight basis), using the following expression:

$$Y\left(\frac{mg}{g}\right) = Chl\left(\frac{mg}{mL}\right)\cdot\frac{V(mL)}{W(g)} \quad \text{[Eq. 13]}$$

Where Chl is the concentration of chlorophyll (a or b) measured through Eqs. 1–12, V is the volume of solvent, and W is the weight of dry biomass.

2.3.2. Antioxidant capacity

Antioxidant capacity, in terms of hydroxyl radical scavenging activity of the extracts, was analysed using Smirnoff and Cumbes's method (Smirnoff and Cumbes, 1989). A mixture of 1 mL of 1.5 mM $FeSO_4$, 0.7 mL of 6 mM H_2O_2, and 0.3 mL of 20 mM sodium salicylate was prepared and incubated (1 h at 37 °C) with different concentrations of the extracts. All chemicals were analytical grade and supplied by Merck (Darmstadt, Germany). The scavenging activity of the hydroxyl radical was determined spectrophotometrically at 562 nm, and the antioxidant capacity (three replicates) was calculated using Equation (14):

$$Antioxidant\ capacity\ (\%) = \left(1 - \left(\frac{A_1 - A_2}{A_0}\right)\right)100 \quad \text{[Eq. 14]}$$

Where A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without sodium salicylate, and A_0 is the absorbance of the control. IC_{50} is the value that corresponds to the amount of extract necessary to cause an antioxidant capacity of 50%.

2.4. Analysis of residual biomass

The size distribution of cell debris of the residual biomass was characterised by laser diffraction using a Mastersizer 2000 apparatus (Malvern Panalytical, Ltd. Malvern, UK). The biochemical composition, ash content, and C/N ratio were measured according to procedures described in Section 2.1.

2.5. Biogas production from residual biomass

The biochemical methane potential (BMP) experiments of the consortia and residual biomasses after extraction were carried out in triplicate in 100 mL non-stirred glass bottles at mesophilic conditions (37 °C) using an oven (JP Selecta S.A. Barcelona, Spain) and monitored for 25 days. The inoculum was an anaerobic sludge kindly supplied from

Arroyo del Soto wastewater treatment plant, managed by Canal de Isabel II S.A. (Móstoles, Spain). In all the experiments, 44 mL of inoculum (68.8 ± 1.2% volatile solids (VS), 23 mL of MiliQ water, and the substrate (in a ratio of 2:1 in terms of VS of inoculum to substrate) were added. Bottles were flushed with N_2 each day after measurement to promote an anaerobic environment (Angelidaki et al., 2006).

Control experiments consisting of bottles containing only the inoculum were performed in triplicate, and the cumulative biomethane values offered by the inoculum were subtracted from the BMP test results of the consortia and residual biomass. Additionally, ethanol control experiments were carried out in triplicate as the residual biomass of Marine Algaemass® was impregned with ethanol. Thus, 521 μL of 96% ethanol were used as substrate. The volume of 96% ethanol corresponded to the remaining solvent after the chlorophyll extraction in the case of Marine Algaemass®, which was calculated by drying the residual biomass at 80 °C for 24 h.

Moisture, total solids (TS), and VS were analysed for the initial and residual biomasses and inoculum. Initial wet inoculum and residual biomass ($M_{INITIAL}$) were dried at 100 °C for 8 h to obtain TS. The moisture content (wt%) was determined with Equation (15). TS was calcinated to obtain total ashes (wt%). VS were calculated with Equation (16).

$$Moisture\ content(\%) = \frac{M_{INTIAL} - TS}{M_{INTIAL}} 100 \quad \text{[Eq. 15]}$$

$$VS(\%) = \frac{TS - total\ ashes}{TS} 100 \quad \text{[Eq. 16]}$$

Biomethane production was monitored daily during the first week and measured depending on the pressure values. The pressure inside the bottles was measured with a Pressure Sensor 400, and data were collected through Vernier Graphical Analysis software (Vernier Software & Technology. Beaverton, OR, USA). Biogas composition was analysed using a GC-4000A gas chromatography equipment (East & West Analytical Instruments, Inc. Beijing, China) fitted with a flame ionisation detector (FID), a thermal conductivity detector (TCD), and a Carboxen-1010 PLOT capillary column (30 m length, 0.53 mm i.d., 30 μm film thickness) (Supelco. Bellefonte, PA, USA). The carrier gas was helium at a flowrate of 4 mL/min. Biogas quality was determined according to Equation (17).

$$Biogas\ quality(\%CH_4) = \frac{ACH_4}{ACH_4 + ACO_2} 100 \quad \text{[Eq.17]}$$

Where ACH_4 and ACO_2 are the chromatogram peak areas corresponding to methane and carbon dioxide in the biogas sample, respectively.

The digestate was centrifuged (10000 rpm, 10 min) and filtered with a 0.45 μm Nylon filter, obtaining the soluble fraction of the digestate after BMP tests. Then, it was analysed in terms of Chemical Oxygen Demand (COD) and NH_4^+ using the Spectroquant® kits (Merck, Darmstadt, Germany). The cumulative methane production data were fitted to a first-order kinetic model (Jensen et al., 2011) yielding the hydrolysis kinetic constant.

2.6. Statistical analysis

All the experiments were performed in triplicate. Standard deviations were calculated, and statistical analyses were performed. Normality tests were performed to check data distribution with the Shapiro-Wilk test (p-value >0.05). The variance homogeneity was assessed using Levenne's test (p-value >0.05). One-way ANOVA tests were carried out since only one independent variable or factor was implied. In the results of solvent screening and cell disruption experiments, solvent was the factor, and the dependent variable was the chlorophyll yield. This test was also performed for the results of biogas production, as the factor was the type of biomass, and the dependent variable was the cumulative methane production. Tukey's HSD (honestly significant difference) test was carried out to find the data pairs in which we can observe significant differences. All statistics and graphs were performed using the R software (R Core Team, 2022).

3. Results and discussion

3.1. Chlorophyll extraction

The study of the chlorophyll extraction process from both consortia included the assessment of different solvents as well as the selection of the better pretreatment for cell disruption.

3.1.1. Solvent screening

A screening was performed using six organic solvents in order to evaluate their capability of extracting Chla and Chlb from the cells of the consortia Marine Algaemass® and Chlospira® (Fig. 1). Results are represented on a dry basis. The solvents were selected based on a previous study that pointed out that higher Chla yields were obtained with amphiphilic solvents of moderate polarity (Martins et al., 2021). Higher yields of total chlorophyll (Chla+Chlb) were obtained with Chlospira® in comparison to Marine Algaemass® for all solvents, which agrees with consortia composition. Both consortia include *C. vulgaris*, a microalga known for its high chlorophyl content that usually varies from 10 to 38 mg/g (Safi et al., 2014), but *C. vulgaris* is most abundant in Chlospira®. In addition, Chlospira® contains the cyanobacteria *A. platensis*, which likewise has significant values of chlorophyll contents (2.00–10.6 mg/g) (Ismaiel et al., 2016)]. However, *Tetraselmis* sp., the main species in Marine Algaemass®, has a lower chlorophyll content (1.00–8.14 mg/g) (Schüler et al., 2020).

The highest levels of total chlorophyll extracted were achieved using methanol in both consortia (8.8 ± 0.1 and 12.7 ± 0.4 mg/g for Marine Algaemass® and Chlospira®, respectively). Consequently, the use of methanol, together with ultrasonication and mixing, promotes damage to cell walls by solubilising cell membranes, causing cell disruption and allowing the dissolution of chlorophyll in the extraction solvent. The total chlorophyll yields were slightly lower using the most polar solvent, DMF, in the extraction of Marine Algaemass® (5.71 ± 0.33 mg/g) and Chlospira® (11.74 ± 0.72 mg/g), but the difference was not significant for Chlospira®.

The European Regulation on Cosmetic Products (Council Directive, 1223/2009/EC, 2009) includes DMF in Annex II of prohibited substances and methanol in Annex III of restrictive compounds (maximum concentration of methanol in ready-for-use preparation: 5%). Thus, although methanol and DMF are efficient solvents to extract total chlorophyll in both consortia, their toxic nature reduced their appeal to industrial chlorophyll extraction for the cosmetic sector and should be replaced by others. For instance, pure acetone has been extensively used as an extracting solvent since it strongly inhibits the formation of degradation products. However, the extraction yields achieved for total chlorophyll using this solvent were much lower (0.75 ± 0.05 and 3.9 ± 0.2 mg/g) than those obtained with methanol and DMF for Marine Algaemass® and Chlospira®, respectively. This is in line with previous research demonstrating that organic solvents with relatively high polarity, such as DMF and methanol, are more suitable for the extraction of chlorophyll than acetone (Lee et al., 2021). In this sense, acetone can be appropriate for some groups of microalgae. However, the strong walls of the Chlorophyta (*Tetraselmis* sp., *Chlorella* sp.) and cyanobacteria (*Kamptonema* sp., *A. platensis*), the main components of both consortia, are recalcitrant towards the extraction using this solvent.

Conversely, the use of 80% acetone increased the chlorophyll extraction yield (5.9 ± 0.3 and 9.1 ± 0.5 mg/g for Marine Algaemass® and Chlospira®, respectively) because the presence of 20% water increased the solvent polarity. Chlorophyll yields using 96% ethanol was similar (6.37 ± 0.03 and 9.7 ± 0.2 mg/g, respectively) to that obtained with 80% acetone. Pure ethanol yielded slightly lower values (5.0 ± 0.4 and 7.0 ± 0.3 mg/g) due its lower polarity. These results are consistent

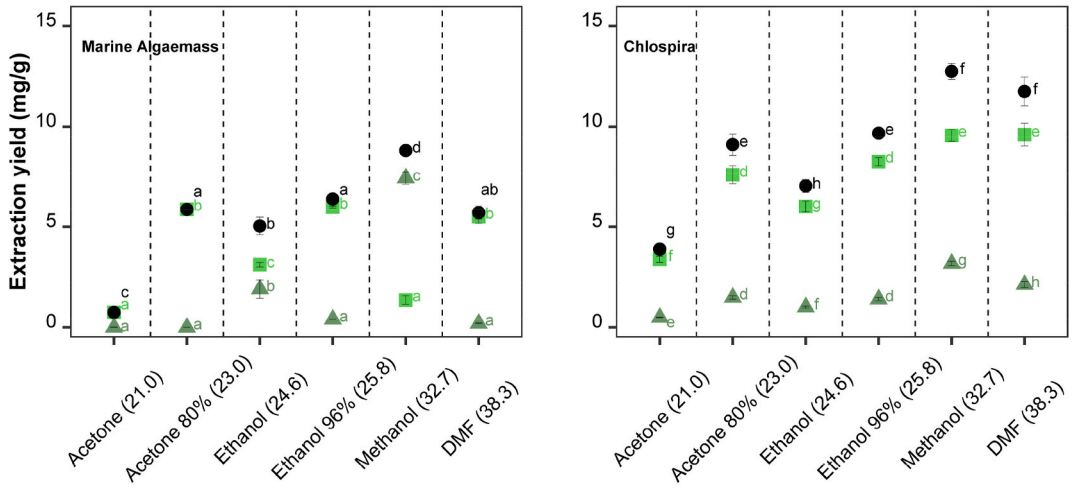

Fig. 1. Extraction yield (mg Chl/g dry biomass) of chlorophyll *a* (■), chlorophyll *b* (▲) and total chlorophyll (●) from Marine Algaemass® and Chlospira® using organic solvents and ultrasound-assisted extraction. Dielectric constant at 25 °C, in brackets. Letters show statistically significant differences between data in Marine Algaemass® (a to c) and in Chlospira® (d to h).

with previous findings (Halim et al., 2010). In the cosmetic industry, there is a need for environmentally friendly extraction methods. Ethanol is a preferred choice due to its non-toxic properties, cost-effectiveness, and ease of separation and reuse compared to other solvents used in this study.

Chla has been extracted to a greater extent than Chlb (Fig. 1) since the former is more abundant in the species that make up both consortia. In fact, Chla is the principal pigment in *C. vulgaris* (Marine Algaemass®

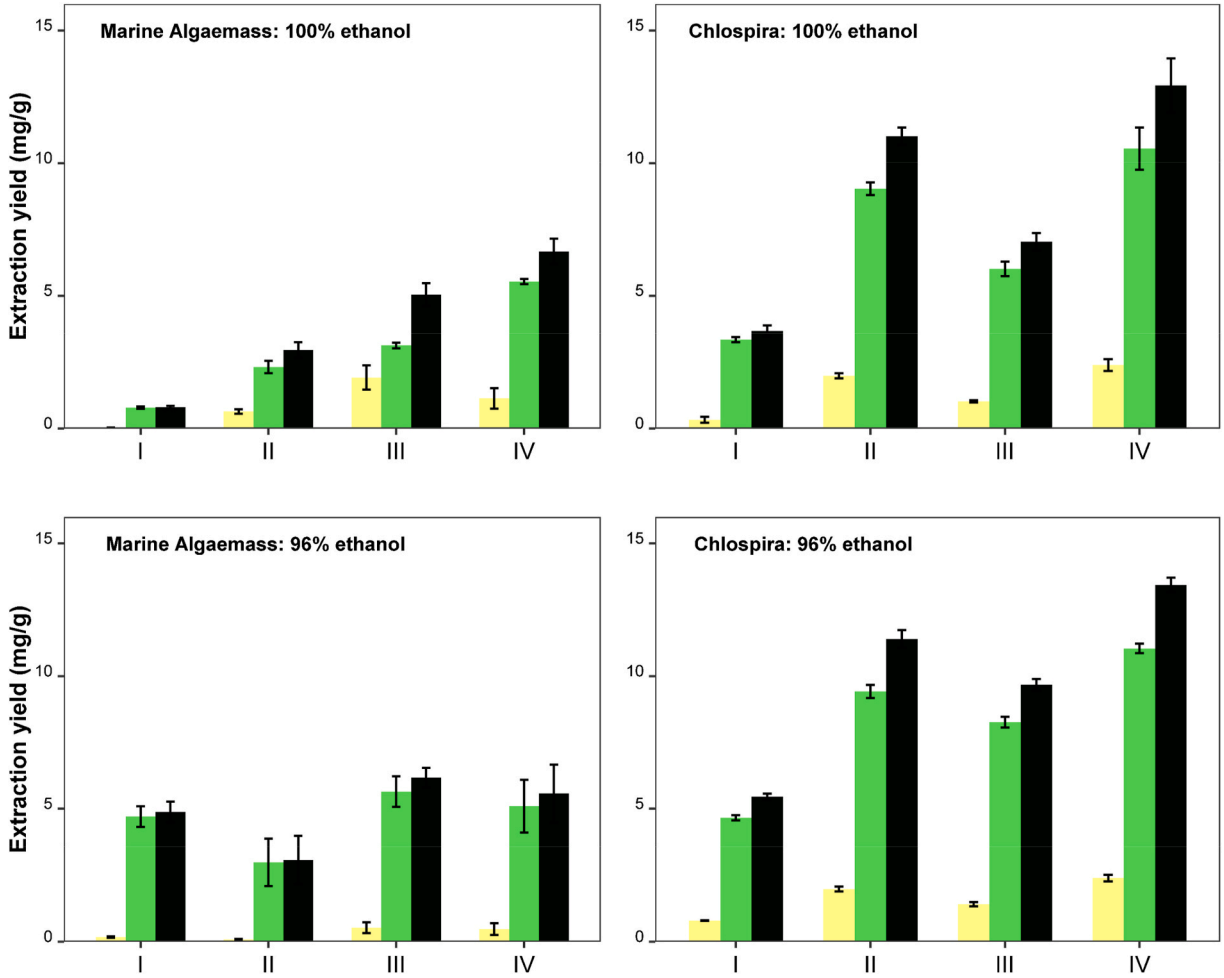

Fig. 2. Extraction yield (mg Chl/g dry biomass) of chlorophyll *a* (green), chlorophyll *b* (yellow) and total chlorophyll (black) from Marine Algaemass® and Chlospira® using 100% ethanol and 96% ethanol and different disruption methods. I: vortexing, II: manual press, III: vortexing+ultrasounds, and IV: press+vortexing+ultrasounds.

and Chlospira®) and *Tetraselmis* sp. (Marine Algaemass®), while Chlb is an accessory pigment that collects the energy to pass on to Chla. In addition, cyanobacteria, such as *Kamptonema* sp. (Marine Algaemass®) and *A. platensis* (Chlospira®), only contain Chla since they are unable to synthesise Chlb (Table 1).

The resulting residual biomass still retained green colouration, which meant that the solvents were not efficient enough to extract all the chlorophyll in the consortia. Thus, experiments on cell disruption were required to obtain higher chlorophyll yields.

3.1.2. Cell disruption

Different cell disruption methods were carried out using the green solvent ethanol (100 and 96%) to break cell walls and allow the solubilisation of chlorophylls. Fig. 2 shows the results of the extraction yields of Chla, Chlb, and total Chla+Chlb from both consortia. Statistical analyses were performed to find significant differences ($p < 0.05$) (see Supplementary files).

The extractions using 100% ethanol released different contents of chlorophyll in both consortia when different cell disruption methods were applied, meaning that the use of this solvent is not enough and a potent pretreatment to break cell walls is mandatory. In fact, the cell walls of the microalgae and cyanobacteria in the consortia used in this work are rigid and multilayered, as shown in Table 1. Thus, the release of chlorophyll (a+b) increased with the severity of the cell disruption procedure in both consortia. Method I (vortexing) was the least efficient in recovering chlorophyll (a+b), whereas method IV (press+vortexing+ultrasounds) was the best one for this purpose. Nonetheless, a higher amount of chlorophyll (a+b) was obtained from Chlospira® using pure ethanol in comparison to that obtained from Marine Algaemass®. For instance, the total chlorophyll yield was 6.7 ± 0.5 mgChl/g for Marine Algaemass® and 12.9 ± 1.0 mgChl/g for Chlospira®, using manual press+vortexing+ultrasounds (method IV). These results are related to the consortium composition of microalgae and cyanobacteria, as explained in the previous section.

When chlorophyll was extracted from Marine Algaemass® using 96% ethanol, all cell disruption methods studied offered similar amounts of total chlorophyll. Thus, the mildness method I (vortexing) was enough to achieve the maximum extraction level of chlorophyll (a+b) (4.8 ± 0.2 mgChl/g). This result elucidated that the determining factor for extracting chlorophyll from Marine Algaemass® was solvent selection. In fact, using 96% ethanol combined with the simplest cell disruption method I (vortexing) offered similar results to those obtained using pure ethanol (6.7 ± 0.5 mgChl/g) with the most disruptive method IV (press+vortexing+ultrasounds).

However, the cell disruption method was the driving factor for chlorophyll extraction from the consortium of Chlospira® using 96% ethanol since significant differences in the chlorophyll content of the extract were observed with different cell disruption methods. In this regard, the most severe method IV (press+vortexing+ultrasounds) was the most effective in extracting chlorophyll *a* and *b* (13.4 ± 0.3 mgChl/g). These results are consistent with the multilayered cell walls of the main strains of these consortia: *C. vulgaris* (two layers) and *A. platensis* (four layers). In particular, the outer cell wall of *C. vulgaris* includes a trilaminar algaenan, a highly resistant aliphatic polymer. In addition, the peptidoglycan layer of *A. platensis* also provides high rigidity.

An additional method (V), which involves the use of a bead-beating, was tested to obtain a rich-chlorophyll extract using an alternative procedure of cell breaking. Water was used instead of ethanol because this approach did not allow the use of volatile solvents. The extraction yields obtained using bead-beating and water were 5.03 ± 0.36 mgChl/g and 13.5 ± 1.1 mgChl/g for Marine Algaemass® and Chlospira®, respectively. The result for Marine Algaemass® was not statistically different from those obtained using methods I to IV with 96% ethanol as solvent, confirming the selection of method I (vortexing) since it is the most efficient and simplest. In the case of Chlospira®, similar results were achieved using method IV (manual press+vortexing+ultrasounds) with 96% ethanol and method V (bead-beating) with water. The latter was the most attractive for chlorophyl extraction from this consortium due to the use of the cleanest solvent (water) for the extraction of bioactive compounds.

Breaking cell walls leads to smaller particle cell sizes, so measurements of cell particle size were performed in order to confirm the disruption of cell walls using vortexing and 96% ethanol for Marine Algaemass® and bead-beating and water for Chlospira®. Fig. 3 shows the distribution of cell particle size for the consortia and the corresponding residual biomass. Differences in cell particle size distribution were observed when comparing the residual biomass measurements of both consortia. The particle sizes within the range of 0–2.5 μm were more abundant in the original biomass than in the corresponding residual biomass for Marine Algaemass®, which means that no hard disruptive methods are required to obtain high levels of chlorophyll using this consortium. Therefore, method I (vortexing) was effective enough to extract all the chlorophyll when using 96% ethanol. By contrast, significant differences in particle size distribution from 0 to 2.5 μm were observed between Chlospira® and its corresponding residual biomass after chlorophyll extraction. Thus, the number of particles within that range increased substantially after chlorophyll extraction. In addition, the variation of volume distribution of particle size (%) is also noticeable between the initial and residual biomass of Chlospira® in the 2.5–5 and 5–7.5 μm. These findings support the fact that severe cell breaking, such as that caused by bead-beating, is necessary with this consortium for chlorophyll extraction.

Chlorophyll extracts were also analysed in terms of antioxidant capacity to assess their potential application in the cosmetic industry. Three chlorophyll-rich extract doses (0.57, 1.00 and 1.92 mg) obtained with the best extraction protocols for each consortium were tested to find out the required quantity of extract that induced an antioxidant capacity of at least 50% (IC_{50}), and the results were compared with the antioxidant capacities reported in other works (Fig. 4). Only 0.57 mg were necessary to reach antioxidant capacity higher than 50% for Chlospira®'s extract (56.8 ± 3.6%). The antioxidant capacity increased significantly, reaching 100% for chlorophyll-rich extract doses of 1.00 and 1.92 mg. *C. vulgaris* is one of the species found in this consortium, and its antioxidant capacity has been studied in other work (Chatzikonstantinou et al., 2017), yielding lower values in terms of antioxidant capacity. Other authors (Wu et al., 2017) reported IC_{50} values for *A. platensis* extracts (a constituent of Chlospira® consortium) that were also lower than those obtained in this work for Chlospira®. Marine Algaemass® chlorophyll-rich extracts offered lower antioxidant capacity than Chlospira® ones at any dose. Hence, 1.92 mg were required for Marine Algaemass® extract to reach an antioxidant capacity higher than 50% (51.9 ± 2.1%). The higher antioxidant capacity of Chlospira® extract could be correlated with its chlorophyll content since it is richer in chlorophyll than Marine Algaemass®. It has been demonstrated that chlorophylls are a potent source of antioxidant activity (Pérez-gálvez et al., 2020). Thus, the chlorophyll-rich extracts of *Phormidium autumnale* were much more antioxidant than α-tocopherol. The use of water in Chlospira® extraction produces an extract with high levels of water-soluble carbohydrates, which in turn increases the antioxidant power (see Supplementary Files).

3.2. Anaerobic digestion of residual biomass after chlorophyll extraction

Residual biomass after chlorophyll extraction were used in anaerobic digestions in order to assess their potential to produce biogas under a biorefinery context. For comparison purposes, the potential of producing biogas was also evaluated using consortia before chlorophyll extraction. The kinetics of methane production and biogas quality for both consortia are represented in Fig. 5. The biomethane production yield was essentially the same for both initial consortia, achieving similar results for Marine Algaemass® and Chlospira® (239 ± 32 $mLCH_4/gVS$ and 299 ± 18 LCH_4/gVS, respectively), and higher than

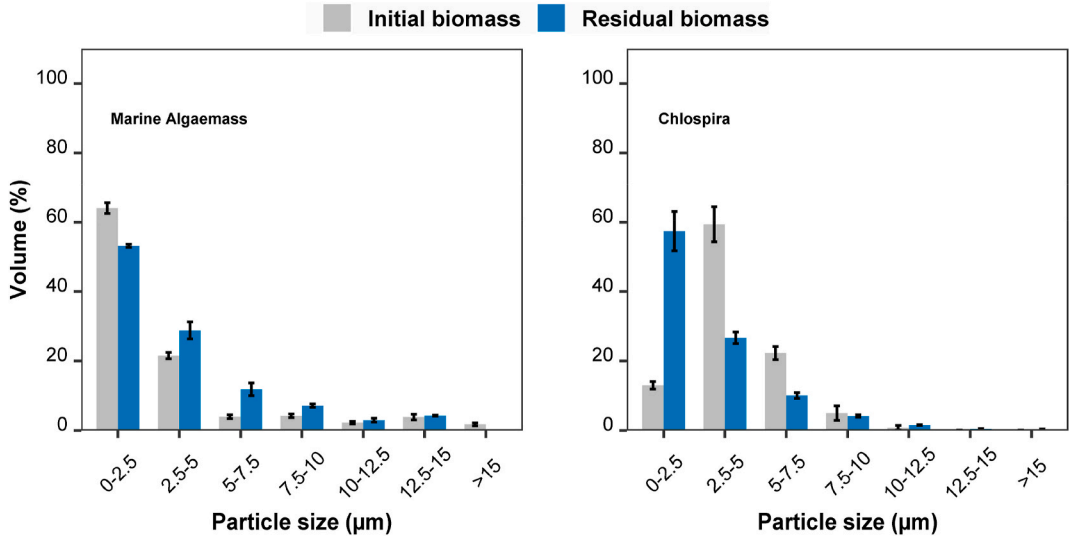

Fig. 3. Cell particle size distribution of consortia (grey) and residual biomass (blue) using vortexing and 96% ethanol for Marine Algaemass® and bead-beating and water for Chlospira®.

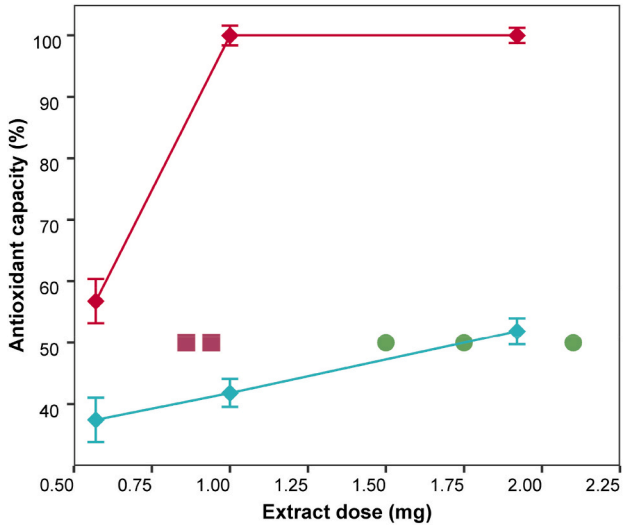

Fig. 4. Antioxidant capacities of the rich-chlorophyll extracts from Marine Algaemass® using vortexing and 96% ethanol (blue rhombus) and Chlospira® with bead-beating and water (red rhombus) at various doses and antioxidant activities of chlorophyll extracts described in other works for *A. platensis* (pink squares) (Wu et al., 2017) and *C. vulgaris* (green circles) (Chatzikonstantinou et al., 2017).

those reported in the literature for *Tetraselmis suecica* (Santos-Ballardo et al., 2015), a microalgal genera present in Marine Algaemass®, and for *C. vulgaris* (Jankowska et al., 2017), which is part of both Marine Algaemass® and Chlospira®.

The biomethane production using the residual biomass of Marine Algaemass® as substrate was significantly higher (472 ± 32 mLCH_4/gVS) in comparison to that obtained with the initial biomass of this consortium (239 ± 32 mLCH_4/gVS). This difference may be due to the presence of ethanol traces in the residual biomass of Marine Algaemass® after the chlorophyll extraction with this solvent. These results are in accordance with a previous study that pointed out a 30–150% increase in the methane production of ethanol-pretreated sludge with respect to untreated sludge (Refai et al., 2014). To assess the effect of this short-chain alcohol on biomethane production, a control experiment was performed without biomass, but using the same amount of ethanol measured in the residual Marine Algaemass®. The control experiment yielded 243 ± 10 mLCH_4/gVS, which explained the above observed results. In fact, when subtracting the effect of the ethanol present in the residual biomass of Marine Algaemass®, no significant differences were found between the biomethane production using the initial and residual biomass for this consortium. As shown Fig. 6A, this finding is in line with the high COD reduction observed between day 1 and day 25 of the anaerobic digestion for the residual biomass (83 ± 3%) in comparison to the COD reduction in the initial consortium (66 ± 5%). Increasing COD reduction during anaerobic digestion gives a direct indication that efficient methanogenesis is taking place (Kainthola et al., 2019). The C/N ratio of the initial Marine Algaemass® was 5.22 ± 0.02. However, the presence of ethanol in its residual biomass increased the C/N value to 12.3 ± 0.03, also explaining the observed increase in biomethane production. Besides, this higher C/N ratio in the residual biomass reduced the $NH4^+$ accumulation in comparison to the corresponding value in the initial biomass (Fig. 6B).

The methane production of the Chlospira® substrate was somewhat higher (299 ± 18 mLCH_4/gVS) than the one obtained with the corresponding residual biomass after extraction with water (240 ± 25 mLCH_4/gVS). Recent studies (Markou et al., 2022) using residual biomass from *C. vulgaris* after chlorophyll extraction showed a similar methane production (219 ± 30 mLCH_4/gVS). The methane production of Chlospira®, both fresh and residual, was lower than that obtained with the residual biomass of Marine Algaemass® (472 ± 32 mLCH4/gVS). The lower values of COD reduction observed during the anaerobic digestion of the initial and residual Chlospira® (Fig. 6A) also supported the lower methane production and biodegradability of these substrates compared to the spent biomass of Marine Algaemass®. Conversely, the C/N ratio for the Chlospira® residual biomass (8.9 ± 0.1) was higher than that of the initial consortium (5.96 ± 0.05), because aqueous extraction of chlorophylls led to a more efficient solubilisation of proteins in the chlorophyll extract (see Supplementary files). Although higher C/N ratios usually lead to methane production enhancement (Cerón-Vivas et al., 2019), excessively high C/N ratios, as in the case of the residual biomass of Chlospira®, involve a deficit of nitrogen content, which is necessary for bacterial growth and the proper development of methanogenesis reactions (Choi et al., 2020). The C/N values of the initial and residual Chlospira® are in accordance with their values of NH_4^+ increase (Fig. 6B) since lower C/N led to NH_4^+ accumulation.

Another important parameter to assess in the anaerobic digestion process is biogas quality (Fig. 5). It was observed that the concentration

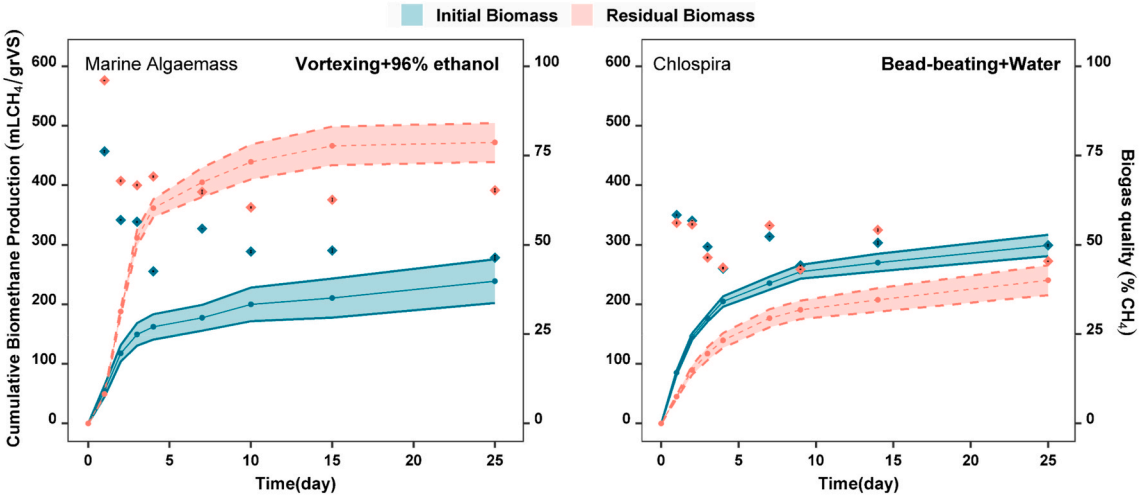

Fig. 5. Cumulative biomethane production (lines) and biogas quality (rhombus) of initial biomass (blue) and residual biomass (pink).

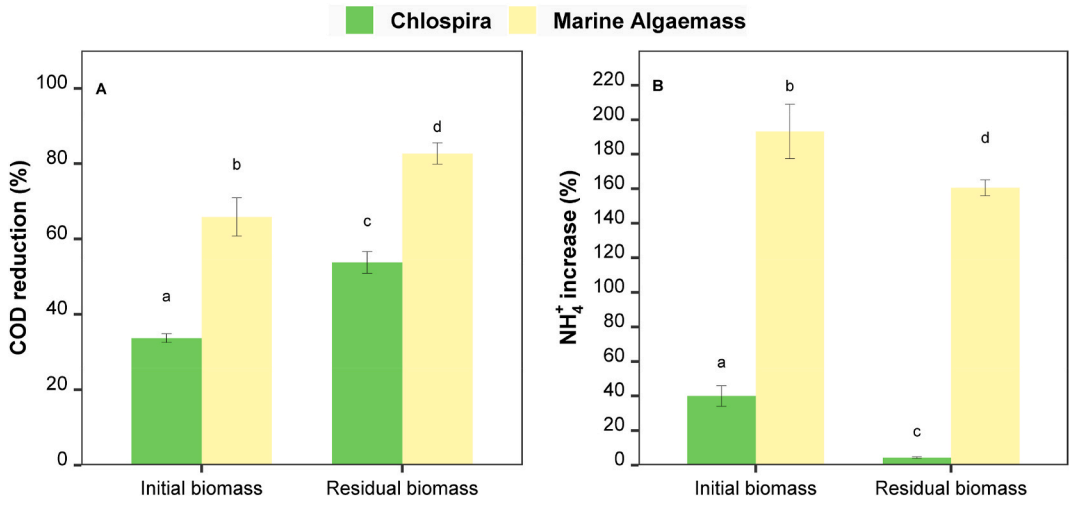

Fig. 6. Chemical oxygen demand (COD) reduction (A) and ammonium increase (B) from the initial time (day 0) to the end time (day 25) during the anaerobic digestion of Marine Algaemass® and Chlospira®. Letters show statistically significant differences.

of methane in the biogas decreased with time for both Marine Algaemass®-derived substrates, i.e., initial and residual biomass. Although the initial Marine Algaemass® offered a high-quality biogas (76% of CH_4) on the first day, it decreased to 46% on the last day of the experiment (day 25). However, the anaerobic digestion of the Marine Algaemass® residual biomass yielded excellent-quality biogas (96%, day 1) that dropped to 65% at day 25. Substrates from Chlospira® released biogas of a more stable quality along the time-course of the process, ranging from 43% to 58% of CH_4.

The above results accounted for the maximum potential production of methane once all the biodegradable biomass was digested. However, it's important to consider the rate at which this potential is reached in evaluating process effectiveness. Table 3 summarises the first-order kinetic constant of the hydrolysis stage of all substrates. Marine Algaemass®, both initial and residual, showed the highest hydrolysis constants (0.22 ± 0.01 and 0.26 ± 0.02 d^{-1}), while the corresponding values for Chlospira® were 0.06 ± 0.02 and 0.11 ± 0.01 d^{-1}, respectively. The values of the correlation coefficients (R^2) indicate that the model fits the experimental results thoroughly. The rate of hydrolysis was enhanced during the anaerobic digestion of the residual biomass for both consortia due to the disruption undergone by cell walls (Fig. 3). The effect of increasing hydrolysis rate by applying different pretreatments has been reported previously for different microalgal species (Oraby et al., 2023; Solé-Bundó et al., 2019). Comparing the kinetic behavior of both consortia, the results show that, although biogas production was similar for both consortia, the first-order kinetic constant was 3.7 times higher for Marine Algaemass® (0.22 ± 0.01 d^{-1}) than for Chlospira ® (0.06 ± 0.02 d^{-1}). In this case, Chlospira® is mostly composed (60%) of

Table 3
Kinetic constants for first-order hydrolysis model.

	Marine Algaemass®		Chlospira®	
	Initial Biomass	Residual Biomass	Initial Biomass	Residual Biomass
k [d^{-1}]	0.22 ± 0.01	0.26 ± 0.02	0.06 ± 0.02	0.11 ± 0.01
R^2	0.9980	0.9931	0.9501	0.9933

A. platensis, which is the species that contains the thickest cell wall (4 layers) of all the species present in both consortia (Table 1). This fact can hinder the degradation of the materials that make up the cell wall itself as well as the interior content of the cells partially damaged by the pretreatment of the biomass, yielding significantly lower hydrolysis rates during the anaerobic digestion of these cell types.

3.3. Overall mass balances

A better understanding of the potential improvements in terms of cleaner and sustainable production in both consortium biorefineries with the best results can be developed by representing their material flows as Sankey diagrams (Fig. 7), where all the flows were scaled per kilogram of each consortium. Herein, the width of the flow is proportional to the flow amount. By this approach, it is possible to recover up to 100% of both original consortia weight as cosmetic ingredients, biogas, and fertilisers or cultivation media after chlorophyl extraction using sustainable solvents (i.e. ethanol for Marine Algaemass® and water for Chlospira®) and anaerobic digestion of the spent biomass.

In the case of the Marine Algaemass® (Fig. 7A), 24.1% of this consortium was successfully transformed into a chlorophyll-rich extract for the cosmetic industry, whereas 56.6% is converted into biogas. The biogas, in turn, is composed of CH_4 (66.4%) and renewable CO_2 (33.6%). In a last step, the CO_2 could be separated from the product gas and recycled to the cultivation stage. Part of the methane could be applied to cover the heating needs of the processes. The solids obtained through chlorophyl extract filtration constitute 4.1% of the initial consortia and are composed of polysaccharides (see Supplementary files), which also have antioxidant properties (Yu et al., 2019) and thus are suitable for the cosmetic industry. The digestate obtained represents 15.2% of the consortium and is usually used as fertiliser or can be recirculated to the cultivation stage.

It can be observed in Fig. 7B that 52.8% of Chlospira® was effectively converted into a chlorophyll extract useful as a cosmetic ingredient, the biogas representing 25.6% of the original consortium (54.8% CO_2 and 45.2% CH_4). In this case, the solid fraction of polysaccharides is more abundant (16.8%), whereas the digestate constitutes only 4.8%

Both integrated designs from consortia constitute sustainable closed-loop systems to fully convert the consortia into valuable bioproducts and bioenergy in the context of a circular economy. However, techno-economic and life cycle assessments are required for both biorefineries to know their accurate economic and environmental sustainability.

4. Conclusions

Two biorefinery schemes were assessed to obtain chlorophyll-rich extracts and biogas from commercial microalgae-cyanobacteria consortia for the first time.

The first biorefinery comprised the ethanolic extraction of chlorophylls (4.8 ± 0.2 mg/g) from Marine Algaemass® consortia (composed of *C. vulgaris*, *Tetraselmis* sp., and *Kamptonema* sp.), which offered an IC_{50} value of 1.92 mg, followed by the anaerobic digestion of the residual biomass at mesophilic conditions to obtain a high-quality biogas (66.43% CH_4) and a high methane yield (472 ± 32 $mLCH_4/gVS$).

The second scheme employed aqueous media and a bead-beating disruption process to extract chlorophylls (13.5 ± 1.1 mg/g) from Chlospira® consortium (*C. vulgaris* and *A. plantensis*), resulting in a higher antioxidant capacity (IC_{50} = 0.54 mg) than that obtained in the first scheme due to higher amounts of chlorophylls and functional molecules such as carbohydrates, constituting a promising cosmetic ingredient for industry. The anaerobic digestion of the residual biomass produced 240 ± 25 $mLCH_4/gVS$ and a lower-quality biogas (45.24% methane) than Marine Algaemass® biorefinery. The higher cumulative biomethane production of the latter biorefinery was attributed to the presence of ethanol traces from the previous extraction step. When subtracting the ethanol effect, the residual biomass offered similar biomethane yields to those of the Chlospira® biorefinery.

From a sustainable point of view, the biorefinery schemes not only produce chlorophyll extract and biogas, but also a fraction of insoluble polysaccharides that can be employed as an additional cosmetic ingredient and a digestate from the anaerobic digestion with applications as biofertilizer or cultivation media. The carbon dioxide fraction from biogas could be recirculated into a microalgal culture. Therefore, the whole consortia biomass can be fully used, minimizing the generation of waste.

This work provides a reference for other biorefinery designs as it includes optimised protocols for chlorophyll extraction and residual biomass valorisation. However, additional work must be carried out, with special attention to the scaling-up of the processes described in this work, to overcome the limitations posed by the extrapolation from the results obtained at laboratory scale to industrial scale.

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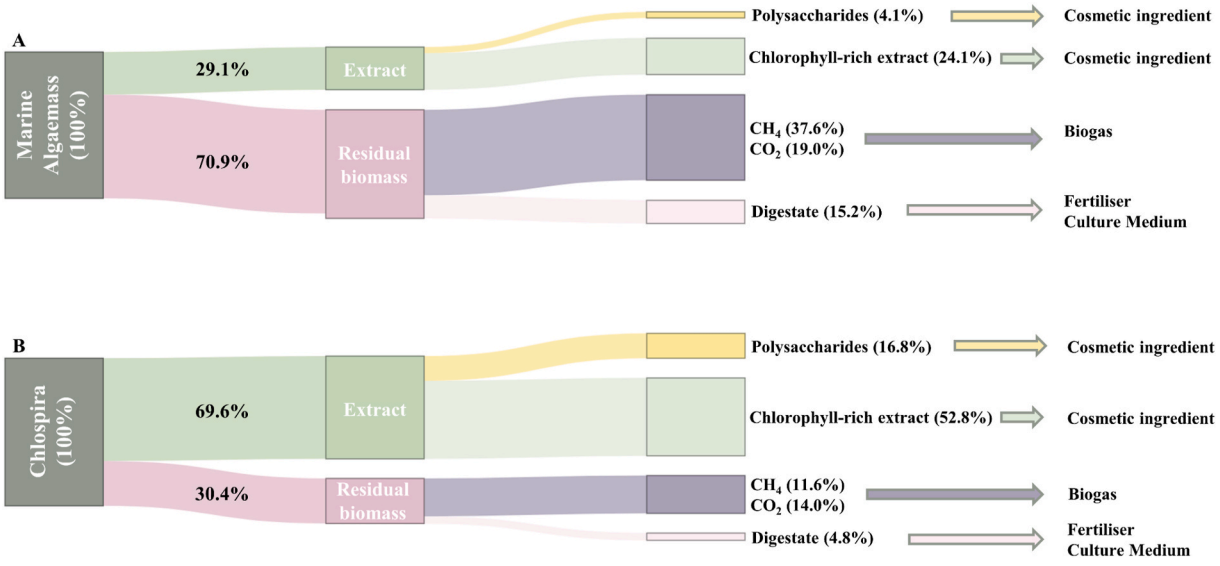

Fig. 7. Sankey diagrams for biorefineries of Marine Algaemass® (A) and Chlospira® (B).

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CRediT authorship contribution statement

Pilar Águila-Carricondo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Juan Pablo de la Roche Cadavid:** Conceptualization, Resources, Validation. **Pedro Luis Galán:** Conceptualization, Resources, Validation. **Luis Fernando Bautista:** Conceptualization, Resources, Supervision, Validation, Writing – review & editing. **Gemma Vicente:** Conceptualization, Resources, Supervision, Validation, Writing – review & editing, Project administration, Funding acquisition.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2023.139652.

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