



Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass

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ABSTRACT

Microalgae synthesize a variety of potentially high-value compounds. Due to their robust cell wall, cell disruption is necessary to improve extraction of these compounds. While cell disruption methods have been optimized for lipid and protein extraction, there are limited studies for other bioactive compounds. The present study investigated the effect of freeze-drying combined with sonication or ball-milling on the extraction of antioxidant and plant biostimulating compounds from *Chlorella* sp., *Chlorella vulgaris* and *Scenedesmus acutus*. Both cell disruption methods resulted in higher extract yields from the biomass compared to freeze-dried biomass using 50% methanol as a solvent. Antioxidant activity of *Chlorella* extracts was generally higher than freeze-dried extracts based on the diphenylpicrylhydrazyl (DPPH) and β -carotene linoleic acid assays. However, the effectiveness of each treatment varied between microalgae strains. Sonication resulted in the highest antioxidant activity in *Chlorella* sp. extracts. Ball-milling gave the best results for *C. vulgaris* extracts in the DPPH assay. Both cell disruption methods decreased antioxidant activity in *S. acutus* extracts. Plant biostimulating activity was tested using the mung bean rooting assay. Damaging the membrane by freeze-drying was sufficient to release the active compounds using water extracts. In contrast, both cell disruption methods negatively affected the biological activity of the extracts. These results indicate that bioactive compounds in microalgae are sensitive to post-harvest processes and their biological activity can be negatively affected by cell disruption methods. Care must be taken to not only optimize yield but to also preserve the biological activity of the target compounds.

1. Introduction

Microalgae contain a variety of molecules including proteins, lipids, carbohydrates, polyunsaturated fatty acids, amino acids, pigments, vitamins, secondary metabolites and hormones. These can be used for different applications including biofuel production, animal nutrition, food additives, nutraceuticals, cosmetics, pharmaceuticals and plant growth biostimulants (Chew et al., 2017; Petruk et al., 2018; Show et al., 2017). These compounds are located within the cell and may be bound to membranes and/or cell wall. Cell wall structure varies greatly between species as well as being influenced by environmental conditions and stage in the life-cycle (Bernaerts et al., 2018). An important

criterion when selecting strains for mass cultivation is a robust cell wall in order to survive the sheer stresses associated with mixing in open ponds and photobioreactors (Brennan and Owende, 2010). However, a tough cell wall acts as a barrier for compound extraction, restricting the entry of organic solvents into the cell and limiting extraction of intracellular compounds (Bernaerts et al., 2018; Kim et al., 2016). For example, protein extraction was more effective in microalgae species with a fragile cell wall (70–80% of total proteins were recovered from *Porphyridium cruentum* and *Arthrospira platensis*) compared to species with a tough cell wall (28–43% of total proteins were recovered from *Chlorella vulgaris*, *Nannochloropsis oculata* and *Haematococcus pluvialis*; Safi et al., 2013). Thus, due to small cell size and the tough but pliable

Abbreviations: AA, ascorbic acid; BHT, butylated hydroxytoluene; DPPH, diphenylpicrylhydrazyl; DW, dry weight; IBA, indole-3-butyric acid; MACC, Mosonmagyaróvár Algal Culture Collection; RSA, radical scavenging activity

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cell wall of mass cultivated microalgae, extraction of the target compound(s) is greatly improved if the cells are disrupted (Lee et al., 2012; Show et al., 2017).

Cell disruption methods are classified into two broad groups, namely mechanical and non-mechanical. There are numerous mechanical techniques based on different mechanical forces such as shear force (bead milling, high-speed homogenization), liquid-shear force (high-pressure homogenization, microfluidization), energy transfer via waves (ultrasound, microwave), current (pulse electric field) or heat (thermolysis, autoclaving). Non-mechanical techniques encompass cell lysis with chemical agents (solvents, detergents, chelating agents, acids and alkalis), enzymes and osmotic shock (reviewed in Günerken et al., 2015). As this adds to the overall production costs and affects the downstream processing steps, the selected cell disruption method(s) need to maximize yield from the microalgae biomass at a low operational cost while ensuring the quality of the extracted product is retained. Each disruption method has merits and drawbacks such as cost, upgrading from laboratory to industrial scale and external contamination of the end product (Chew et al., 2017; Günerken et al., 2015; Lee et al., 2012). Non-mechanical methods are less destructive than mechanical methods as they only perforate or increase the permeability the cell wall and membrane rather than breaking the cell wall. They are also more biologically specific but their drawbacks include longer process times, high chemical costs, degradation and denaturing of compounds by acids and alkalis, toxicity of solvents and the formation of unwanted side-products and impurities. The shear forces used in mechanical methods can generate heat and cause pressure changes. This can affect the quality of the end product through production of free radicals, degradation of the active constituents or the formation of impurities (Günerken et al., 2015; Lee et al., 2012, 2017).

There is a large body of research focused on optimizing methods for lipid extraction, including extraction from moist microalgal paste rather than dried biomass. Such research encompasses maximizing lipid yield as well as ways to improve net energy use, reduced costs and downstream purification and transesterification processes (Chatsungnoen and Chisti, 2016; Kim et al., 2016; Subhash et al., 2017). In comparison, there is limited research on suitable methods for the extraction of other bioactive compounds from microalgae (Günerken et al., 2015). For example, a four-step extraction protocol was designed for the marine flagellate *Isochrysis galbana* where extraction of carotenoids (mainly fucoxanthin) was optimized with simultaneous extraction of lipids as well as a carbohydrate- and protein-rich fraction with antioxidant activity (Gilbert-López et al., 2015).

To date, only a few microalgae-derived compounds are produced commercially e.g. astaxanthin, β -carotene and the fatty acid docosahexaenoic acid (Alhatab et al., 2019). Aspects of extraction, isolation and fractionation of high-value components need to be studied in order to improve the yield of target compounds. *Chlorella* and *Scenedesmus* strains are prime candidates for mass culture due to their relatively fast growth rates, high lipid content and the robust nature of the cell (Brennan and Owende, 2010). Previous studies highlighted the potential biological activities of five *Scenedesmus* strains and three *Chlorella* strains, including antioxidant, antimicrobial and acetylcholinesterase inhibitory activity (Aremu et al., 2014, 2016). Microalgae also have plant biostimulating activity. For example, *Acutodesmus dimorphus* extracts triggered faster seed germination and promoted growth and floral production in tomato (Garcia-Gonzalez and Sommerfeld, 2016) and *Chlorella sorokiniana* suspensions improved wheat growth (Kholssi et al., 2019). The aim of the present study was to investigate the effect of various cell disruption methods, namely freeze-drying combined with sonication or ball-milling on the extraction of antioxidant and plant biostimulating compounds from *Chlorella* and *Scenedesmus* strains.

2. Materials and methods

2.1. Microalgae cultures, growth conditions and experimental design

Three axenic microalgal strains from the Mosonmagyaróvár Algal Culture Collection (MACC), namely *Chlorella* sp. MACC-519, *Chlorella vulgaris* MACC-755 and *Scenedesmus acutus* MACC-677 were selected for the present study. Each strain was inoculated from agar cultures into two flasks containing 250 ml liquid Zehnder-8 nutrient medium (Staub, 1961). The cultures were grown at $25 \pm 2^\circ\text{C}$ in a 12:12 h light:dark photoperiod and illuminated from below with $130 \mu\text{mol}/(\text{m}^2 \text{ s})$ and aerated with 20 l/h 1.5% CO_2 -enriched sterile air during the light phase. After 7 days, the suspension cultures were used to inoculate four flasks per strain, each containing 250 ml Zehnder-8 medium. The cultures were maintained in the described conditions for a further 7 days. The experiment was initiated by inoculating 100 flasks containing 250 ml Zehnder-8 medium at a starting biomass of 10 mg/l for each strain. The experiment ran for 12 days in the described growth conditions and was repeated three times for each strain.

2.2. Growth and macromolecule measurements

During the experiment, 28 flasks (in total) per strain were harvested on day 0, 3, 5, 10 and 12 to monitor growth. Samples (5–20 ml) were immediately filtered through Whatman GF/C glass fibre filters (5 cm diameter) that had been dried at 105°C for 2 h, cooled in a desiccator and weighed. The algae-loaded filters were again dried at 105°C for 2 h, cooled and weighed. The biomass of the suspension was calculated as mg DW/l. Samples (2 ml) were preserved with Lugol's solution for quantification of cell number and cell area (size). At least 500 cells/sample were measured using an Olympus BX60 microscope and analysed with Stream Motion software (Olympus Optical Co. Ltd, Tokyo, Japan).

The algal biomass from different growth phases was harvested from 72 flasks (in total) on day 5 and 10 for the extraction of biochemical and cell disruption experiments. Between 1–10 l were harvested depending on the strain and harvest day. The harvested biomass was centrifuged ($2150 \times g$ for 15 min at room temperature) and the pellet immediately freeze-dried (Christ Gamma 1–15). Crude protein content was quantified using a Kjeldahl method and the lipid content by hydrolysis and sequential solvent elution as previously described (Ördög et al., 2013). Carbohydrate content was quantified using a method based on the MSZ 6830/26 Hungarian Standard as previously described (Ördög et al., 2016) which utilized the Luff-Schoorl method with titration.

2.3. Cell disruption methods

The freeze-dried biomass produced from each microalgae strain was divided into three portions for cell disruption treatment as follows:

- i) freeze-dried biomass (Christ Gamma 1–15) without further cell disruption;
- ii) freeze-dried biomass (2 g DW) was resuspended in 100 ml distilled water (20 g DW/l distilled water) and sonicated (Virtis VirSonic 600, 200 W) for 3 min and then immediately freeze-dried again. The temperature of the extract increased from 22°C to 31°C during sonication;
- iii) freeze-dried biomass (2 g DW) was resuspended in 100 ml distilled water (20 g DW/l distilled water) and ball-milled (Retsch MM400, 25 Hz) for 30 min where 15 g glass pearl (0.50–0.75 mm size) were added per 30 ml suspension. Following milling, the samples were freeze-dried. The temperature was maintained at a constant 22°C during ball-milling.

The % intact cells after the cell disruption treatments was estimated by microscopic examination (Olympus BX60 microscope). At least 500 cells/sample were examined in the freeze-dried sample. The number of intact cells were counted in the same volume of cell disrupted samples.

Membrane damage was determined by measuring electrical conductivity of living algal suspensions and freeze-dried and disrupted biomass samples harvested on day 5 and day 10. Suspension cultures (2 l) were centrifuged (2150 x g for 15 min at room temperature) and the supernatant discarded. The pellet containing the living cells was re-suspended in 600 ml distilled water at 3 g DW/l. Freeze-dried, sonicated and ball-milled biomass samples were re-suspended in 600 ml distilled water at 3 g DW/l. The samples were divided into 3 x 200 ml portions and gently stirred with a magnetic stirrer for 30 min at room temperature before the electric conductivity was measured (Hanna HI2300).

2.4. Biological activity

The effect of the cell disruption treatments on the biological activity of the biomass was tested using various bioassays.

2.4.1. Antioxidant activity

The dried biomass was extracted in 50% aqueous methanol (50 mg DW/ml). In order to not further disrupt the cells, the extract was gently shaken at 60 rpm for 2 h at 25 °C, left overnight at room temperature and then shaken for a further 30 min. The extracts were then vacuum concentrated at 1250 rpm (Univapo 100/150 H, Uniequip, Germany) for 15 min at room temperature. The supernatant was removed and dried under nitrogen. The resulting residue was weighed to determine extract yield. The residue was then suspended in 50% MeOH at 50 mg/ml. The extracts were tested in two *in vivo* antioxidant systems – the diphenylpicrylhydrazyl (DPPH) free radical scavenging assay and the β -carotene-linoleic acid assay.

The DPPH free radical scavenging assay was performed as described by Moyo et al. (2010). Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as standard antioxidants for comparison. The final concentration of the extracts and standards in the assay was 0.5 mg/ml. The % free radical scavenging activity (% RSA) was determined by the decolouration of the DPPH solution over 30 min in the dark. Three technical replicates were used per sample.

The β -carotene-linoleic acid assay as described by Moyo et al. (2010) was performed using BHT as a positive control. The final concentration of the extracts and standard in the assay was 0.4 mg/ml. Antioxidant activity (%) was calculated as the rate of β -carotene bleaching with absorbance readings taken every 30 min for 180 min when the samples were heated at 50 °C in the dark. Four technical replicates were used per sample.

2.4.2. Rooting activity

Plant biostimulating activity of the biomass was measured using the mung bean rooting bioassay (Crouch and van Staden, 1991). Mung beans (*Vigna radiata*) were germinated in moist vermiculite at 26 ± 1 °C in 16:8 h light:dark photoperiod and 120 μmol (m^2 s) light intensity. On day 7, water extracts of the treated biomass were made at 10 mg DW/ml and shaken at 60 rpm for 3 h at 25 °C. These were immediately diluted to 1, 2, 3 and 4 mg DW/ml solutions and left overnight at room temperature. On day 8, uniform cuttings (12 cm stem length) with two leaves were placed in the prepared solutions for 6 h, then rinsed and transferred to clean vials containing water. There were 5 cuttings per vial and two vials per solution (total of 10 cuttings). Distilled water was included as the control and indole-3-butyric acid (IBA) at 10^{-8} – 10^{-3} M as a positive control. The cuttings were placed in the growth conditions described above. The number of roots were recorded 9 days after the pulse treatment.

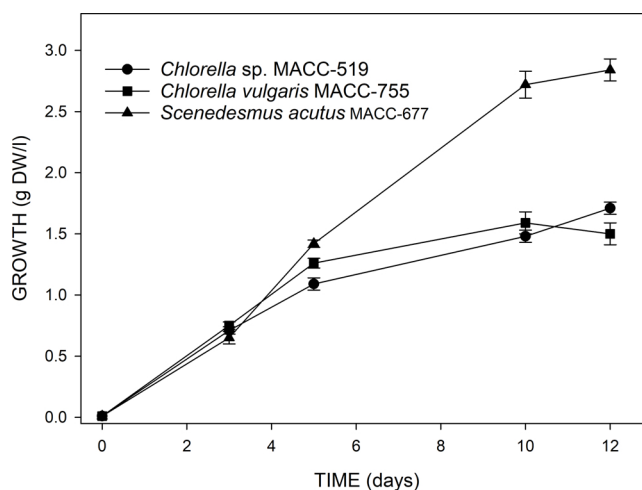


Fig. 1. Growth of three microalgal strains in culture. Results are shown as mean \pm SE (n = 3) from samples collected over three experiments.

2.5. Statistical analysis

One-way ANOVA followed by the *post hoc* Tukey test were used to detect significant differences ($P < 0.05$) in growth (cell number and area), conductivity, yield, antioxidant activity and rooting activity in the samples of each microalgal species. One-way ANOVA followed by the pairwise Holm-Sidak test were used to detect significant differences between the protein, lipid and carbohydrate content on day 5 and day 10 for each strain (SigmaPlot v. 13). General Analysis of Variance was performed to determine the effect of the cell disruption method, day of microalga harvest and extract concentration on rooting in mung beans cuttings for the three strains (Genstat Release 18.2).

3. Results

3.1. Growth and macromolecule content

The two *Chlorella* strains had similar growth rates, entering the stationary growth phase after 5 days in culture (Fig. 1). In both strains, cell area decreased significantly with culture age (Table 1). In comparison, *S. acutus* had a higher biomass (DW) with the cultures entering the stationary growth phase after day 10 (Fig. 1). Cell area increased significantly once in the stationary phase (Table 1).

The protein content decreased from day 5 to day 10 in the three species with this decrease being significant in both *Chlorella* sp. and *S. acutus*. Although not significant, lipid content increased in all three species from day 5 to day 10. The carbohydrate content decreased significantly with culture age in *Chlorella* sp. but did not change in *C. vulgaris* and *S. acutus*. *Chlorella* sp. had the highest lipid content and *S. acutus* the lowest lipid content of the three investigated microalgae. The sum of these macromolecules accounted for 68–89% of the total biomass weight (Supplementary Table).

3.2. Cell disruption

Microscopic examination of the cultures revealed that no cells were ruptured following freeze-drying. The cell wall of approximately 10–20% of the cells was ruptured after the sonication treatment and up to 70–80% of the cells had a ruptured cell wall due to the milling treatment (Supplementary Figure).

Cell membrane permeability as measured by electrical conductivity, was significantly increased following freeze-drying compared to the living cells in all three strains harvested on day 5 and day 10. Additional cell disruption treatments of sonication or milling of the dried biomass further increased membrane permeability. Sonication

Table 1

Cell number and size of three microalgae strains over the 12 day experimental period. Results are shown as mean \pm SE (n = 3) from samples collected over three experiments. Mean values with different letters indicate significant differences for each species (P < 0.05).

	Culture Age				
	Day 0	Day 3	Day 5	Day 7	Day 12
<i>Chlorella</i> sp. MACC-519					
Cell number (ml)	(1.37 \pm 0.10) $\times 10^6$ ^a	(9.83 \pm 0.03) $\times 10^7$ ^b	(1.62 \pm 0.02) $\times 10^8$ ^e	(1.56 \pm 0.01) $\times 10^8$ ^d	(1.31 \pm 0.02) $\times 10^8$ ^c
Cell area (μm^2)	11.03 \pm 0.37 ^b	8.98 \pm 0.07 ^{ab}	7.93 \pm 0.07 ^a	8.97 \pm 0.05 ^{ab}	9.87 \pm 0.11 ^{ab}
<i>Chlorella vulgaris</i> MACC-755					
Cell number (ml)	(2.31 \pm 0.14) $\times 10^6$ ^a	(9.79 \pm 0.04) $\times 10^7$ ^b	(1.50 \pm 0.01) $\times 10^8$ ^c	(1.91 \pm 0.02) $\times 10^8$ ^d	(1.51 \pm 0.02) $\times 10^8$ ^c
Cell area (μm^2)	11.13 \pm 0.21 ^a	10.68 \pm 0.15 ^a	8.95 \pm 0.08 ^b	7.20 \pm 0.11 ^d	7.93 \pm 0.05 ^c
<i>Scenedesmus acutus</i> MACC-677					
Cell number (ml)	(5.70 \pm 0.02) $\times 10^5$ ^a	(1.50 \pm 0.13) $\times 10^7$ ^b	(2.26 \pm 0.03) $\times 10^7$ ^b	(3.77 \pm 0.03) $\times 10^7$ ^b	(4.53 \pm 0.04) $\times 10^7$ ^b
Cell area (μm^2)	27.16 \pm 0.86 ^a	24.49 \pm 0.79 ^a	25.02 \pm 0.94 ^a	25.39 \pm 0.65 ^a	32.16 \pm 0.84 ^b

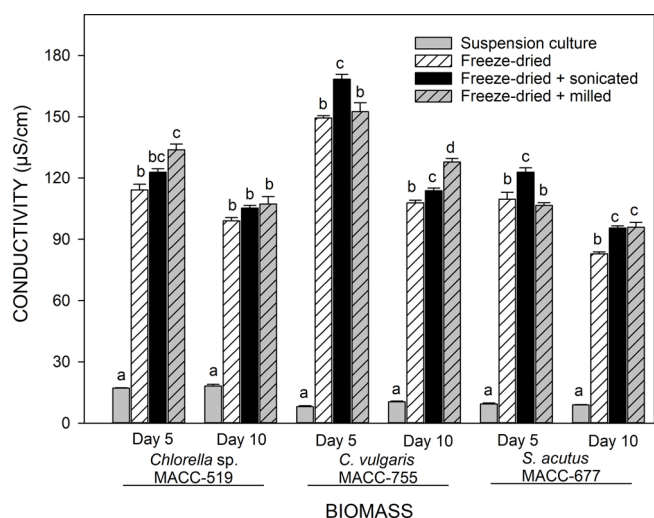


Fig. 2. Effect of cell disruption treatments on membrane permeability in three microalgae species harvested on day 5 and day 10. Following centrifugation and cell disruption, samples were resuspended in distilled water (4.08 $\mu\text{S}/\text{cm}$) at 3 g DW/l. Results are shown as mean \pm SE (n = 3) from samples harvested from three experiments. Different letters indicate significant differences (P < 0.05) within each species.

significantly increased membrane permeability in *C. vulgaris* and *S. acutus* harvested on day 5. Milling significantly increased membrane permeability in *Chlorella* sp. harvested on day 5 and *C. vulgaris* harvested on day 10. Both cell disruption treatments significantly increased membrane permeability in *S. acutus* harvested on day 10 (Fig. 2).

3.3. Extract yields

The extract yield following extraction with 50% methanol was significantly affected by the cell disruption treatment. For all three strains, the freeze-dried biomass had a significantly lower yield compared to the other treatments. Additional cell disruption treatment improved the efficiency of the solvent extraction. In *C. vulgaris*, extract yield was significantly higher from the milled samples compared to the sonicated samples. A similar trend was apparent in the *Chlorella* sp. and *S. acutus* biomass although the yields were not significantly different between the sonicated and milled samples (Fig. 3).

3.4. Biological activity

3.4.1. Antioxidant activity

Cell disruption treatments affected the antioxidant activity of the microalgae extracts with the effectiveness of the treatment varying

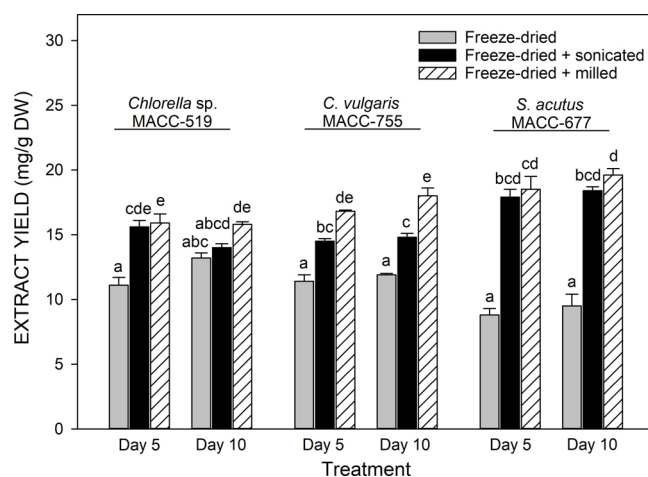


Fig. 3. Effect of cell disruption treatments on extract yield following extraction with 50% methanol in three microalgae species harvested on day 5 and 10. Results are shown as mean \pm SE (n = 3) from samples harvested from three experiments. Different letters indicate significant differences (P < 0.05) within each species.

between strains. In *Chlorella* sp., the extracts obtained from the biomass subjected to sonication and milling showed significantly higher biological activity compared to the freeze-dried samples. Sonication resulted in extracts with the highest activity in both the DPPH (Fig. 4A) and β -carotene assays (Fig. 4B). In *C. vulgaris*, milling produced extracts with the highest activity in the DPPH assay (Fig. 4A) while the cell disruption methods did not enhance the activity of the extracts in the β -carotene assays (Fig. 4B). In contrast, extracts obtained from the *S. acutus* biomass subjected to the cell disruption methods had similar activity in the DPPH assay compared to the freeze-dried samples (Fig. 4A) and significantly decreased activity in the β -carotene assay (Fig. 4B).

Antioxidant activity of the microalgae samples was also affected by culture age. In the two *Chlorella* strains, activity was lower in the DPPH assay in samples harvested on day 5 compared to day 10 but remained similar in the β -carotene assay. In contrast, antioxidant activity in the DPPH assay was significantly higher in the older *S. acutus* samples and were slightly lower in the β -carotene assay (Fig. 4).

3.4.2. Rooting activity

The microalgal extracts had biostimulatory activity, significantly increasing rooting in mung bean cuttings. *Chlorella* sp. was the most effective, generally producing the highest number of roots in the mung bean cuttings (Fig. 5A) with the activity being equivalent to 10–50 mg/l IBA (Fig. 5B). *C. vulgaris* extracts also had good rooting activity (Fig. 5C) being equivalent to 5–10 mg/l IBA (Fig. 5D). *S. acutus* extracts produced the lowest number of roots (Fig. 5E), being equivalent to 0.5–2 mg/l IBA (Fig. 5F). In most instances, increasing concentrations

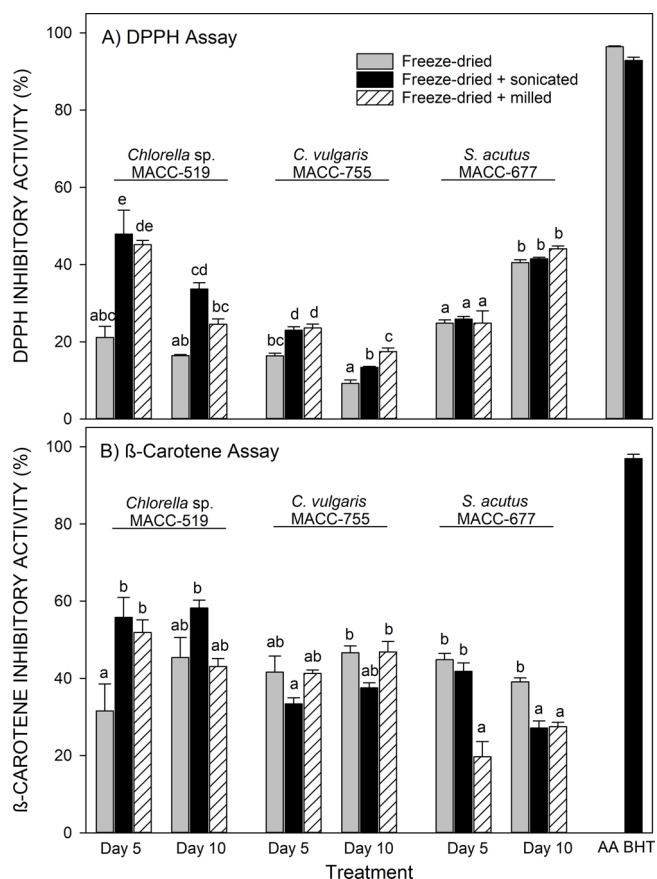


Fig. 4. Antioxidant activity of cell disrupted samples of three microalgae species tested in the A) DPPH free radical scavenging assay and B) β -carotene-linoleic acid assay. Results are shown as mean \pm SE ($n = 3$) from samples harvested from three experiments. Different letters indicate significant differences ($P < 0.05$) within each species. AA = ascorbic acid; BHT = butylated hydroxytoluene.

of microalgal extract significantly increased the biostimulatory activity of the extracts with the highest root number recorded in cuttings treated with either 3 or 4 mg DW/ml extracts. Cell disruption treatments significantly influenced the activity of the extracts in *Chlorella* sp. and *S. acutus* (Table 2). In both *Chlorella* strains, the lowest rooting activity was recorded in the cuttings treated with the milled microalgal biomass (Fig. 5A and C). In *S. acutus* samples, the milled samples harvested on day 10 had decreased rooting activity when applied to the mung bean cuttings (Fig. 5E).

4. Discussion

Biomass accumulation was initially similar for the three strains used in the present study but by day 5, the two *Chlorella* strains had entered the stationary growth phase while *S. acutus* continued growing exponentially until day 10 (Fig. 1). This growth trend was similar to previous studies investigating biological activity in five *Scenedesmus* strains and three *Chlorella* strains where *Scenedesmus* cultures entered the stationary phase by day 10 (Aremu et al., 2014) and *Chlorella* strains reached stationary growth a bit earlier (Aremu et al., 2016). As expected, the biochemical content of the microalgae changed with culture age as the growth conditions became limiting. Quantification of the macromolecule content showed the protein content decreased and the lipid content increased over time (Supplementary Table).

The harvested biomass was dried to a powder by freeze-drying. During lyophilization, intracellular ice-crystals form. Due to the low temperature, the ice crystals are sublimated, dehydrating the sample.

This process causes some damage to the cell membrane as the intracellular water expands upon freezing, making the cell membrane more porous although the cell wall is not ruptured (Guldhe et al., 2014; Lee et al., 2012, 2017). Electrical conductivity measures the extraction of ionic intracellular components and microelements as well as amino acids and water soluble proteins (Grimi et al., 2014) and is an indication of membrane damage. In the present study, electrical conductivity significantly increased in the freeze-dried biomass compared to the suspension cultures (Fig. 2), indicating that lyophilization caused some damage to the cell membrane, enabling certain compounds to be extracted. This suggests that the damage caused by freeze-drying was sufficient to extract the majority of the water soluble, low molecular weight compounds from the *Chlorella* and *Scenedesmus* samples. Cell disruption methods of sonication and ball-milling further disrupted the cells as seen by the increased cell debris when viewed microscopically (Supplementary Figure). Electrical conductivity increasing significantly in these samples (Fig. 2).

Cell wall structure and composition influences the effectiveness of the cell disruption method (Günerken et al., 2015) and hence extraction of compounds from the microalgal cells. The cell wall of green microalgae can be divided into two groups, namely a low resistant cell wall or a highly chemical resistant cell wall (Dunker and Wilhelm, 2018). *Chlorella* and *Scenedesmus* species have a highly resistant cell wall (Dunker and Wilhelm, 2018). Sonication is more suited to microalgae species with a less-resistant cell wall (Alhatab et al., 2019). In the present study, 30 min ball-milling was more effective than 3 min sonication, disrupting a higher percentage of cells (Supplementary Figure). Similarly, high pressure homogenization was the most effective method to disrupt *Chlorococcum* sp. (74% of cells disrupted) which has a thick cell wall, followed by sulphuric acid treatment (33%), bead beating (18%) and sonication the least effective (5%; Halim et al., 2012). For *Nannochloropsis oculata*, 60 s laser treatment was the most effective cell disruption method (97%), followed by microwave at 90 °C for 20 min (95%), blender for 20 min (93%), waterbath treatment at 90 °C for 20 min (88%) and sonication for 20 min the least effective (68%; McMillan et al., 2013).

In the present study, both sonication and ball-milling significantly increased the extract yield compared to freeze-dried samples, indicating that some method of cell disruption is necessary to maximize extraction. In *Chlorella* sp. and *S. acutus*, extract yield was similar regardless of which cell disruption method was used while ball-milling resulted in a significantly higher yield in *C. vulgaris* (Fig. 2). The difference in the extract yields between the two *Chlorella* strains used in the present study may be due to the great diversity in their cell wall structure (Alhatab et al., 2019). Most *Chlorella* species have a fibrillary cell wall with the main component being glucose, other species have a cellulosic cell wall structure and others have a trilaminar structure composed of algaenan (also called soropollenin). Algaenan is a highly acid and base resistant biopolymer containing high molecular weight long chain saturated fatty acids (Alhatab et al., 2019; Dunker and Wilhelm, 2018). *C. vulgaris* has a cell wall that is predominately natural sugars which form a rigid cell wall (reviewed in Alhatab et al., 2019). *Scenedesmus* strains usually have a bilayered cell wall. The rigid outer layer consists of a trilaminar structure of algaenan. The inner cell wall layer has a microfibrillar structure composed of mannose and polysaccharides (Alhatab et al., 2019; Allard and Templier, 2001; Lee et al., 2017).

There is interest in the untapped pharmacological and pharmaceutical potential of microalgae as a source of high value biologically active compounds such as antioxidants, anti-inflammatory, anti-angiogenic and anti-hypertension compounds. Their applications include ingredients in functional foods, antifoulants and novel UV-sunscreens (Show et al., 2017). The present study measured antioxidant activity of the *Chlorella* and *Scenedesmus* extracts in two assays. Based on previous studies on *Chlorella* and *Scenedesmus*, 50% aqueous methanol was used in the present study as it gave higher extract yields compared to more polar solvents although antioxidant activity was lower than in

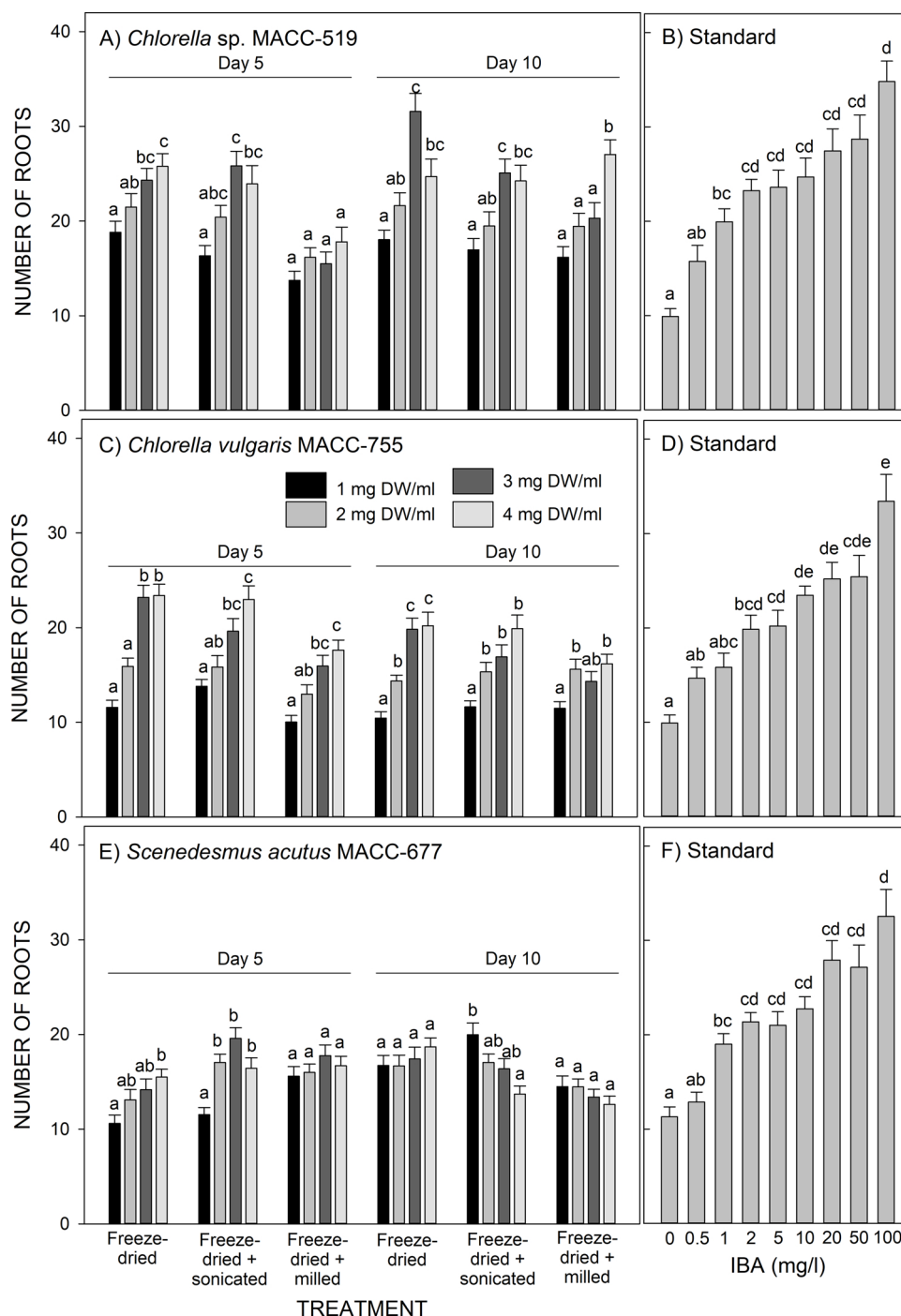


Fig. 5. Rooting activity of cell disrupted samples of A) *Chlorella* sp. MACC-519, C) *Chlorella vulgaris* MACC-755 and E) *Scenedesmus acutus* MACC-677 tested in the mung bean rooting assay. Results are shown as mean \pm SE (n = 30) from microalgae biomass harvested from three experiments. B, D and F) Indole-3-butyric acid (IBA) standards were included in each assay. Different letters indicate significant differences ($P < 0.05$) due to extract concentration for each cell disruption method.

dichloromethane extracts (Aremu et al., 2016). All extracts had antioxidant activity with the extracts generally being more active in the β -carotene assay (0.4 mg DW/ml final extract concentration) compared to the DPPH assay (0.5 mg DW/ml final extract concentration). This trend was similar to previous experiments with three *Chlorella* and five *Scenedesmus* strains tested using the same assays (Aremu et al., 2014, 2016). All photosynthetic organisms produce antioxidant compounds to mitigate oxidative stress. Antioxidant activity has been extensively reported in microalgae as they produce natural antioxidant compounds such as chlorophyll, carotenoids (lutein and zeaxanthin), tocopherol, peptides and polysaccharides (Choochote et al., 2014; Klein et al.,

2012). For example, the waste-product following starch extraction from *C. sorokiniana* had good antioxidant activity due to the presence of several carotenoids, chlorophylls and saturated and polyunsaturated fatty acids (Petruk et al., 2018). The presence of such compounds may account for the antioxidant activity measured in the present study.

The method of cell disruption significantly affected the antioxidant activity of the extract. In *Chlorella* sp., the sonicated samples had the highest activity in both assays while sonication resulted in decreased activity of *C. vulgaris* samples in the β -carotene assay. In contrast, both cell disruption treatments had no effect on the activity of the *S. acutus* extracts in the DPPH assay and significantly decreased the activity in

Table 2
General Analysis of Variance of the effect of microalga extracts on the rooting of mung bean cuttings.

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance	F-probability
<i>Chlorella</i> sp. MACC-519					
Day of harvest (D)	1	772.08	772.08	12.55	< 0.001
Disruption method (DM)	2	3100.17	1550.09	25.20	< 0.001
Concentration (C)	3	6219.43	2073.14	33.70	< 0.001
D X DM X C	17	2873.08	169.00	2.75	< 0.001
Residual	696	42754.83	61.52		
Total	719	55706.24			
<i>Chlorella vulgaris</i> MACC-755					
Day of harvest (D)	1	220	220	0.19	0.664
Disruption method (DM)	2	151	75	0.06	0.938
Concentration (C)	3	2690	897	0.77	0.513
D X DM X C	17	25,187	1482	1.27	0.207
Residual	696	813,314	1169		
Total	719	841562			
<i>Scenedesmus acutus</i> MACC-677					
Day of harvest (D)	1	57.23	57.23	1.84	0.176
Disruption method (DM)	2	234.02	117.01	3.76	< 0.024
Concentration (C)	3	276.32	92.11	2.96	< 0.032
D X DM X C	17	3069.20	180.54	5.80	< 0.001
Residual	696	21674.17	31.14		
Total	719	25310.93			

the β -carotene assay (Fig. 4). The difference in biological activity of the various samples due to the method of cell disruption used may be due to a number of factors. For example, while ball-milled samples produced the highest yields, the active antioxidant compounds could have been diluted by non-active compounds or inhibitory compounds could have been made available for extraction. Similarly, hot water extracts (80 °C for 20 min) gave higher yields than ethanol extracts for two *Chlorella* sp. and *Chlorococcum* sp. However, ethanol extracts had greater antioxidant activity. This was attributed to algae containing high levels of water-soluble components such as soluble polysaccharides, proteins and peptide which would have been extracted with the hot water treatment (Choochote et al., 2014).

The decreased activity of the sonicated *C. vulgaris* samples in the β -carotene assay may indicate that the active antioxidant compounds were heat sensitive and were partially degraded during the sonication process. In the present study, although samples were only sonicated for a relatively short time (3 min), the temperature increased rapidly from 22 °C to 31 °C. In contrast, as the temperature was controlled during ball-milling and remained at a constant 22 °C, a longer disruption method (30 min) was used, resulting in a higher % of cells being broken (Supplementary figure). This could have led to increased leakage of the active compounds. In addition, many pigments are light sensitive and the more effective ball-milling could have exposed some of the antioxidant pigments to light and air so that they were degraded faster during storage prior to testing in the assays.

Microalgae have potential as biofertilizers as an environmentally friendly alternative to synthetic agrochemicals. In addition to essential nutrients, they also contain secondary metabolites such as plant hormones and antimicrobial compounds that are active against phytopathogens (Costa et al., 2019; Ördög et al., 2004). Their application to crops is associated with greater nutrient uptake, increased crop biomass and improved yields. In the present study, aqueous extracts were prepared by gentle shaking and overnight extraction. These extracts were tested for plant biostimulating activity using the mung bean rooting assay. All *Chlorella* extracts stimulated rooting in the mung bean cuttings in a dose-dependent manner with rooting increasing with increasing extract concentrations. The best rooting response was obtained with the freeze-dried *Chlorella* extracts with the milled extracts being the least active (Fig. 5A and C). *S. acutus* extracts had the least biostimulating activity with sonicated and milled samples having a negative response at increasing extract concentrations (Fig. 5E). The mung bean rooting assay has been successfully used to monitor biological activity

in variety of seaweed-derived biostimulants (Stirk and van Staden, 1997). Rooting in mung beans is stimulated in a dose-dependent manner by various plant growth regulators. In the present assay, auxin i.e. IBA, was used as a positive control for comparative purposes as rooting in many plants is stimulated by auxin. However, rooting in this assay is also stimulated by other plant growth regulators such as karrikinolide, a smoke-derived butenolide (Jain et al., 2008) and eckol, a phlorotannin present in the seaweed *Ecklonia maxima* (Rengasamy et al., 2015). Microalgae, including *Chlorella* and *Scenedesmus* species, contain a variety of endogenous plant growth hormones such as auxins, cytokinins, gibberellins and brassinosteroids (Stirk et al., 2013a, b). Some of these hormones may account for the rooting activity measured in the present study.

Some cell disruption methods may cause product degradation (Alhattab et al., 2019). The results of the present study indicate that the membrane damage caused by freeze-drying was sufficient to allow the plant biostimulating compounds to be extracted from the samples. The decreased biostimulating activity of the disrupted samples compared to the freeze-dried samples may be due to the active compounds being oxidised or degraded faster during storage prior to testing in the mung bean assay. The dose-dependent inhibitory effect of the sonicated and milled *S. acutus* samples with increasing extract concentration could also be due to micronization of cell debris that remained in the samples. This debris could potentially block the xylem vessels of the mung bean cuttings, thereby reducing uptake of the active compounds in the extract.

Cell size can also affect the effectiveness of the cell disruption treatment (Alhattab et al., 2019). There were also differences in the biological activity of the biomasses harvested on day 5 and day 10 when tested in the two antioxidant assays and the mung bean rooting assay in the present study. This may be attributed to changes in the cellular constituents, including changes in the macromolecule content (Supplementary Table) as well as cell size. The cell area of the two *Chlorella* strains decreased with culture age while *S. acutus* increased significantly once in the stationary growth phase (Table 1).

Selection of the optimum method of cell disruption is dependent on the microalgae species biology (cell wall characteristics and cell size), the target compound and its application. Other considerations include operational conditions such as temperature and micronization of cell debris which can complicate downstream processes during fine separation and purification of the high-value product (Grimi et al., 2014; Kim et al., 2016; Lee et al., 2017). The present study highlighted the

effect of freeze-drying combined with two mechanical cell disruption methods on the biological activity of *Chlorella* and *Scenedesmus* strains. Both sonication and ball-milling resulted in higher extract yields from the biomass when 50% methanol was used as a solvent. Antioxidant activity of the extracts (tested in the DPPH and β -carotene linoleic acid assays) varied depending on microalgae species, cell disruption method and assay. Cell disruption methods generally improved the antioxidant activity in the *Chlorella* extracts and decreased activity in the *S. acutus* extracts. Both cell disruption methods negatively affected the plant biostimulating activity of the microalgae samples when tested in the mung bean rooting assay. Damaging the membrane by freeze-drying was sufficient to release the active compounds using water extracts. These results indicate that potentially valuable bioactive compounds in microalgae are sensitive to post-harvest processes and their biological activity can be negatively affected by the method of cell disruption. Care must be taken when optimizing extract yield to also preserve the biological activity of the target compounds and to reduce impurities in the extracts.

Author contributions

WAS was involved in the experimental design, performed the biological activity assays and wrote the manuscript; PB cultured the microalgae; MV performed the biological activity assays; CL quantified protein, lipid and carbohydrate content; ZM was involved in the experimental design; JvS was involved in the experimental design and edited the manuscript; VÖ conceived the original concept, was involved in the experimental design and edited the manuscript.

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jbiotec.2019.10.012>.

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