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Cultivation of microalgae - *Chlorella sorokiniana* and *Auxenochlorella protothecoides* - in shrimp boiling water residues

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ABSTRACT

Based on the ability of microalgae to purify industrial processing waters, the overall aim of this study was to evaluate whether currently wasted shrimp processing waters could be used as microalgal growth media to produce new protein-enriched food and feed ingredients. Low molecular weight (LMW) fractions of shrimp boiling water (SBW) which had been pre-flocculated using alginate (AL), carrageenan (CA), chitosan (CH) or Superfloc C-592 to recover shrimp protein via flotation, were used for cultivation of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* to produce a protein-enriched microalgal biomass. CH-derived media induced the highest growth rates for both species with A. *protothecoides* out-performing *C. sorokiniana*. A. *protothecoides* best assimilated phosphate-phoshorous (P-PO_4) and total phosphorous (TP) in all media; ≤63 mg/L and ≤ 45 mg/L after 4 days, respectively. In upscaled aerated cultures of *A. protothecoides* in CH- and AL-derived media, P and TP uptake increased up to 85 and 127 mg/L, respectively. Further, 63% of the free amino acids (AA) were assimilated in both waters. Biomasses derived from SBW contained 37–43% protein and 15.0–17.4% fatty acids (FA) per DW; with 38–40% essential AA (EAA) and 21.3–22.5% polyunsaturated FA (PUFA), respectively Corresponding numbers for biomass cultivated in control media were 11 and 53%, protein and FA, respectively, and with 38% and 15.6% EAA and PUFA, respectively. Ability of A. *protothecoides* to assimilate TP and AA, and to generate a protein-rich biomass from LMW-fractions derived from SBW was thus revealed for the first time, and paves the way for a SBW-based biorefinery comprising chemical, physical and microbial processes to produce multiple products.

1. Introduction

Microalgae are known as important production organisms for macro- and micronutrients with wide applications in feed, food, pharmaceuticals and biofuel [1]. Microalgae typically grow on inorganic nitrogen and phosphorous and can potentially produce more biomass per acre than terrestrial plants. It has been reported that the biomass produced by a number of microalgae species range from 6.6 to 43 g dry weight (DW)/m^2/d while soy bean yield 135 g/m^2/season [2,3]. However, microalgae cultivation is an highly costly and resource-intensive process partly due to the fertilizer use, resulting in a significant carbon footprint when inputs are not carefully sourced [2]. Two inputs that can significantly influence the cost and environmental impact of cultivations are the nutrient demand and the demand for fresh water resources [4].

Effluents emerging from food processing, livestock, and slaughterhouses, contain various forms of nitrogen, carbon and phosphorous, which can potentially be utilized as valuable nitrogen and phosphorous sources, thereby reducing the cost of cultivation media and environmental impacts associated with use of chemical fertilizers. Additionally, by assimilation of phosphorous which is anticipated to be a limiting resource by the end of the century [5], this element will be biologically mediated or recycled. Furthermore, the use of effluents as cultivation media reduces the water footprint which is significant if a cultivation requires freshwater and if it is located in low latitudes [6]. Thus, altogether, integrating effluents in cultivations can contribute to more sustainable production of microalgal biomass.

Effluents or wastewaters from various food industry sectors have, in a limited number of studies, been evaluated as cultivation media of different microalgae species such as *Chlorella* sp., *Scenedesmus* sp. and *Spirulina* sp. [7–13], and similarly, effluents connected to livestock and fish handling have been used [14]. To completely replace nutrients for microalgal cultivation, the effluent needs to have balanced

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concentrations of phosphorous and nitrogen and in an appropriate ratio [15]. There is also a requirement for micronutrients such as minerals, trace metals and vitamins that may not be provided in adequate concentrations. Thus to provide more balanced nutrient levels, different side streams generated in the same plant can be mixed [7,16]. In addition to inorganic nutrients, industrial effluents can also contain organic carbon and nitrogen sources that can support microalgal growth if appropriate mixotrophic strains are selected. This could result in high growth rates compared to solely autotrophic metabolism even when irradiances are low [17]. Depending on the intended use of the microalgal biomass, the type of effluent used for cultivation must be selected with great care. Utilizing nutrients from manure or municipal waste for instance could make biomass unsuitable for human or animal consumption due to the risk of pathogens or harmful compounds present in these streams. Effluents derived from the food industry, if maintained in a food grade state, represent safer sources of nutrients for producing high-value microalgal biomass suitable as food ingredients. There is however a limited number of reports available in this area [9,18].

Shrimp boiling water (SBW) is generated during industrial steaming of shrimps intended for subsequent peeling; roughly in a volume of 1 m^3 per 1 tonnet of fresh shrimp. Due to its abundance in protein, we successfully recovered a protein-rich biomass using a flocculation-based technique followed by dissolved-air-flotation, DAF [19,20]. A residual fraction, referred to as low-molecular-weight (LMW) fraction, remained from this protein recovery, was less abundant in protein but high in inorganic nitrogen and phosphorous. We here hypothesized that the obtained shrimp-derived LWM fraction could be a suitable media for microalgae growth. Given the flocculant-dependent nature of the shrimp protein recovery process, it was however anticipated that the precise nutrient profile of the LMW-fraction would differ based on flocculant used, thereby affecting the microalgae growth profile and final biomass composition.

In the first part of the present study, LMW-fractions generated during pilot scale flocculations and DAF of industrial SBW using several different flocculants were screened for their ability to support growth and nutrient uptake of *Chlorella sorokiniana* SAG 211-8k and *Auxenochlorella protothecoides* SAG 211-7a at small scale. Both these species have earlier been shown to successfully utilize organic carbon and nitrogen in wastewaters during their growth while accumulating high protein levels (>40%, DW) or carotenoid pigments under certain cultivation conditions [21–23]. The best performing strain and LMW-fraction were then evaluated in greater detail in upscaled batch cultures in terms of nutrient uptake and biomass composition. To the best of our knowledge, the present study is the first to report that chemical and physical methods for protein recovery can been combined with microalgal production to maximize resource recovery from shrimp processing water.

2. Material and methods

2.1. Material

SBW was provided by Räkor & Laxgrossisten AB, Gothenburg, Sweden. The flocculants tested for protein recovery were chitosan (CH), alginate (AL), λ carrageenan (CA), and Superfloc C-592 (C-952) [19,20]. CH (86% deacethylated) was purchased from GTC BIO Corporation (Qingdao, China). AL (Manucol® DM) and CA (Viscarine® GP-109NF) were purchased from FMC Food and Nutrition (PA, USA). C-592 was provided by Kemira (Helsingborg, Sweden).

Liquid residual LMW-fractions were generated during the protein recovery from SBW using different flocculants, i.e. CH, AL, CA and C-592 combined with DAF. The pre-flocculation of SBW (500–600 L scale) with AL and CA (0.5 and 0.45 g/L, respectively) was carried out at pH 4 using a method first established on lab scale [19]. CH flocculation of SBW was performed at pH 6 using 0.5 g CH/L. The reason for using different pH adjustments for the flocculation treatments was connected to the characteristics of their functional groups; for CH the functional group is positively charged whereas those of AL and CA are negatively charged. Thus, in order to have proteins with opposite charges interacting with the flocculants, pH of SBW was adjusted to 6 and 4, respectively. Flocculation with C-592 was performed at the native pH of the SBW (8.6–8.9) at a final concentration of 5 g/L. Flotation was performed immediately after flocculation using a 376 L flotation unit in stainless steel (Bio-Aqua A/S, Denmark) equipped with a pneumatic scraper with a rubber blade as recently described [20]. A shrimp protein-enriched foam was collected with the scraper, while the LMW-fraction was tapped off and stored at −80 °C until further use in microalgal cultivations.

2.2. Microalgae cultivation

The growth of two freshwater microalgae species, *Chlorella sorokiniana* SAG 211-8 k (referred to as *C. sorokiniana*) and *Auxenochlorella protothecoides* SAG 211-7a (referred to as *A. protothecoides*) from SAG Culture Collection of Algae (Göttingen, Germany) were assessed in LMW-fractions, here after referred to as LMW-media. The axenic strains were received as slants on solid Polytoma medium with glucose. Stock cultures of microalgae strains were maintained in Bold's Basal Media +3 N + V (BBM) and sub-cultured every 2 weeks at room temperature [24]. The mineral content in this BBM medium per liter were: 0.75 g $NaNO_3$, 0.057 g K_2HPO_4, 0.175 g KH_2PO_4, 12.5 mg $CaCl_2{\cdot}2H_2O$, 37.5 mg $MgSO_4{\cdot}7H_2O$, 12.5 mg NaCl, 4.98 mg $Na_2EDTA{\cdot}2H_2O$, 0.582 mg $FeCl_3{\cdot}6H_2O$, 0.246 mg $MnCl_2{\cdot}4H_2O$, 0.017 mg $ZnCl_2$, 0.012 mg $CoCl_2{\cdot}6H_2O$, and 0.024 mg $Na_2MoO_4{\cdot}2H_2O$. Additionally, the vitamin content per 1 L was as follows: 1.2 mg thiamine HCl, 0.01 mg biotin, and 0.01 mg cyanocobalamin. The BBM medium was generally used in control cultivations throughout the entire study to allow comparison of microalgae performance with that obtained in the SBW-derived medium. Upon upscaling, cultures were transferred to 25 mL after which, to 75 mL tissue culture flasks with hydrophobic coating and a sterile 0.2 um air filter vented cap (Sarstedt Hamburg, Germany). At the 75 mL scale, culture media was amended with addition of organic additive medium (NPM) [25,26], which included the following per L: 1 g sodium acetate, 6 g glucose, 3 g sodium succinate hexahydrate, 4 g neopeptone, 1 g bacto-tryptone and 0.1 g yeast extract. This was done at 10 mL/L of media. The purpose of supplementation was to allow cultures to acclimate their metabolism for the assimilation of organic compounds.

Prior to inoculation into test media, the pH of LMW-media was adjusted to 7 and, to avoid any limitation of vitamins and trace minerals, it was fortified with vitamin solution (1 L of medium contained 1.2 mg thiamine HCl, 0.01 mg biotin, and 0.01 mg cyanocobalamin) and trace metal l solution (1 L of medium contained 4.98 mg $Na_2EDTA{\cdot}2H_2O$, 0.582 mg $FeCl_3{\cdot}6H_2O$, 0.246 mg $MnCl_2{\cdot}4H_2O$, 0.017 mg $ZnCl_2$, 0.012 mg $CoCl_2{\cdot}6H_2O$, and 0.024 mg $Na_2MoO_4{\cdot}2H_2O$) to yield similar levels as in the BBM medium. The media were subsequently sterilized using a 0.2 μm polyethersulfone (PES) membrane filter (Thermo Scientific, Waltham, MA, USA) mounted on a sterile filtration glassware. Cultivation experiments were conducted in two stages as follows:

2.2.1. Primary batch cultivation in flask

A 25 mL portion of each LMW-media was transferred into a 75 mL tissue culture flask (Sarstedt, Hamburg, Germany). Each LMW-media was inoculated to a starting OD_{750} of 0.1 with each strain separately, in duplicate. One flask from each LMW-media was kept as blank during cultivation. The flasks were placed on a modified shaker (160 rpm) equipped with an up-ward facing light array providing light from underneath the flasks. The light array consisted of cold white LEDs strips with an output at 5600 K and the LED strips were organized in a 1 × 2 cm grid. The light output was approximately 100 μM photons m^{-2} s^{-1}. The light:dark cycle was set to 18:6 h. The cultivation was performed without aeration such that the limited availability in carbon dioxide and slightly less intense illumination would promote mixotrophic growth on organic carbon in the LMW-media. Cultivation was monitored for 8

days, optical density at 750 nm (OD_{750}) was read on days 0, 2, 4, 6 and 8. Filtered and uninoculated LMW-media was used as the blank for OD measurements. Growth rates were determined as the slope of the Ln (OD_{750}) data versus time. For the maximal specific growth rate, the time points of the initial maximal linear phase in the Ln (OD_{750}) data were identified and the slope was calculated by linear regression. Samples were taken at days 0, 4 and 8 for nutrient analyses.

2.2.2. Upscaled batch cultivation in 1 L bottles under aeration

Based on the results from growth and nutrient removal, *A. protocoides* was chosen for upscaled cultivation with improved conditions for growth (higher light intensity and improved air transfer). 800 mL of LMW-media was transferred into a 1-L universal bottle and inoculated with seed culture of *A. protocoides* to yield a starting OD_{750} of 0.1. A control media of BBM supplemented with 100 mL/L NPM was also tested. The cultivation continued until the growth was leveled off, i.e., for 8 days, with constant stirring (150 rpm) and aeration of waters. The system consisted of three parallel magnetic stirrers set between perpendicular light racks so that all bottles were illuminated evenly from two sides. Each light rack consisted of 2 (4 in total) 8 W T8 cool white LED tubes (OSRAM) and cultures were grown under a 18:6 h light: dark cycle at 150 μM photons s^{-1} m^{-2}. Each bottle was equipped with a gas inlet supplying filtered (0.2 μm) and ambient air to be bubbled through the cultures at a rate of 0.1 L/L culture/min. Cultivation was performed in duplicate for alginate-derived LMW-media and triplicate for chitosan-derived LMW-media. Samples of media were taken at days 0, 2, 4, 6 and 8 for nutrients analyses. Microalgae biomass samples were harvested at days 4 and 8 by centrifugation at 4000 ×*g* for 5 min. The biomass pellet was washed with MilliQ water twice prior to freezing and subsequent freeze-drying.

2.3. LMW -media characterization

2.3.1. Protein and total nitrogen (TN) measurements

The protein content of LMW-media from flocculation were measured following the method of Lowry, Rosebrough, Farr and Randall [27] modified by Markwell, Haas, Bieber and Tolbert [28] using serum bovine albumin as standard in the concentration range of 10–100 mg/L. Absorbance was read at 660 nm using a Cary60 BIO UV–vis spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia). For waters used in the upscaled trial, total nitrogen (TN) measurement was performed using a nitrogen analyzer (LECO TruMac N, MI, USA). All analyses were performed in duplicate

2.3.2. Phosphate-phosphorous and total phosphorous content

Phosphate-phosphorous (P-PO_4) was measured of LMW-media as previously described by Qvirist, Carlsson and Andlid [29]. A 500 μL aliquot of the sample was mixed with 0.9 mL of 5% sodium dodecyl sulphate, after which 1 mL of 1.25% of ammonium molybdate solution in 2 M HCl was mixed in, followed by adding 0.1 mL of 1 g/L of ascorbic acid. After a 30 min incubation at room temperature, absorbance at 700 nm was read.

Total phosphorous (TP) was measured after acidic digestion using acidic persulfate as previously described [30] with minor modifications. In brief, to 0.5 mL sample in a 11 mL glass tube with a Teflon lined cap, a 4 mL persulfate digestion reagent (0.015 M potassium persulfate ($K_2S_2O_8$) in 0.018 M sulfuric acid (H_2SO_4)) was added, after which the tubes were autoclaved at 121 °C for 75 min. Prior to phosphate-phosphorous determination as described above, samples were neutralized. Acidic digestion was performed in duplicates whereas phosphate-phosphorous determination was carried out in triplicates.

2.3.3. Ammonia content

The ammonia content of LMW-media was measured using an ammonia assay kit (AA0100, Sigma, USA) in a 96-well microplate. Briefly, 20 μL of sample and 200 μL of ammonia assay reagent were mixed, after which 2 μL L-glutamate dehydrogenase solution was added and incubated for 5 min. The absorbance at 340 nm was recorded before and after the 5 min incubation. All analyses were performed in duplicate.

2.3.4. Free amino acid (AA) content

A 500 μL filtered (syringe filter, PES, 0.2 μm) sample of the LMW-media was mixed with an equal volume of trichloroacetic acid (TCA) solution (7.5% *w/v*) and maintained on ice. The mixture was vortexed and centrifuged at 12,000×*g* for 10 min. Free AA were measured as previously described by Özcan and Şenyuva [31] using LC/APCI-MS. A 2 μL sample was injected to an LC-MS system (Agilent 1100 HPLC, Waldbron, Germany) using a C18 Phenomenex column (250 μm × 4.6 μm × 3 μm), coupled to an Agilent 6120 quadrupole in the SIM positive mode (Agilent Technologies, Germany). The peak area was compared against an AA standard mix (Ref# NCI0180. 20,088, Thermo Scientific-Pierce, Rockford, IL, USA)). The analyses were done in duplicate.

2.3.5. Content of organic compounds - glucose, lactate, acetate and glycerol

To measure the presence of organic compounds in the media during cultivation, high-performance liquid chromatography (HPLC) was performed to separate compound of interest and quantified by refractive index. An Ultimate 3000 (Dionex) HPLC system was utilized, with an Aminex-87H column (300 mm × 7.8 mm, 9 um) and a guard column (50 mm × 7.8 mm, 9 um). Eluent was 5 mM H_2SO_4 at a flow rate of 0.8 mL min^{-1} with the columns maintained at 80 °C. Twenty μL of each sample was injected. Samples were filtered to 0.22 um and diluted in MilliQ prior to analysis. Five-point standard curves of analytical grade glucose, sodium lactate, sodium acetate and glycerol were used for quantification. All analyses were performed in duplicate.

2.4. Microalgal biomass biochemical profile

2.4.1. Fatty acid (FA) content

FA were extracted and methylated as previously described by Laurens, Quinn, Van Wychen, Templeton and Wolfrum [32]. A known amount of freeze-dried and powdered biomass was weighed into a furnaced glass tube and suspended in 400 μL of chloroform, after which 200 μL internal standard (heptadecanoic acid 100 μL/mL) was added to the tube. Sample was sonicated on ice for 1 h and transesterification was performed by 0.75 mL of HCl/MeOH (5% *v/v*) at 90 °C for 90 min. After cooling, FA methyl esters (FAMEs) were extracted by addition of 2 mL hexane and vigorous mixing for 30 s followed by shaking at 300 rpm for 20 min. After centrifugation at 2000×*g* for 5 min, the upper phase was transferred into a clean tube and the extraction was repeated one more time. After evaporation of hexane, measurement of FAs were carried out by GC-–MS using an Agilent Technologies 7890 A GC system connected to Agilent Technologies 5975 inert MSD (Kista, Sweden) as previously described [33]. Quantification was performed using a 37 component FAME standard mix (Supelco, Bellefonte, USA). Masshunter Quantitative Analysis software (version B.09.00, Agilent Technologies) was used for acquisition and quantification of FAME peaks against those of the standard. FA analyses were done in triplicate.

2.4.2. Total protein and AA contents

The total protein content of microalgal biomass was determined colorimetrically (750 nm) using the DC protein kit (Bio-Rad) following a sequential hot trichloroacetic acid and alkaline extraction of the biomass [34]. The only modification was that samples were bead-beaten for 2 min at 30 Hz (QIAGEN Tissuelyser II) prior to TCA extraction. A standard curve of bovine serum albumin in the range of 0.225–1.35 mg/L was used for calibration. To measure the AA content of microalgal biomass, a known amount of freeze-dried and powdered biomass was mixed with 4 mL of 6 N HCl followed by flushing with nitrogen gas for 30 s; hydrolysis was performed by maintaining tubes at 110 °C for 24 h, after which hydrolysed samples were filtered (syringe filter, PES, 0.2

μm) and diluted prior to AA determination using LC/APCI-MS as described previously (Section 2.3.4). All analyses were performed in duplicate.

2.5. Statistical analysis

Statistical differences among sample means were determined by Analysis of Variance (ANOVA) at $p \leq 0.05$ using the statistical package of MINITAB Ver. 18. The values used in these calculations, and the values reported in Figures and Tables are mean values from the two or three replicate cultivations. The standard deviation from the cultivation replicates is also depicted in Figures and Tables. The number of analytical replicates was two or three as stated in each analytical section, and mean values from these replicate analyses were used to calculate the variations between different cultivations.

3. Results and discussions

3.1. Characteristics of LMW-fractions used for microalgae cultivations

Protein, P-PO_4, TP, and ammonia contents were evaluated in the LMW-fractions generated after flocculation with CH, AL, CA and C-592 (Table 1). The native pH of the fractions varied from 4 to 8.7 as flocculation was performed at pH ranges compatible to the optimum pH of the flocculants in the respective flocculation process [19,20]. The protein content was 3.3, 2.7, 1.9 and 5.8 g/L for CH, AL, CA and C-592-derived fractions, respectively, with the protein content of CA- and C-592-derived fractions being significantly lower and higher ($P \leq 0.05$), respectively, compared to the other two. TN were 0.7 and 1.3 for CA- and AL-derived fractions, respectively, compared to 0.9 and 2.1 g/L in CH- and C-592-derived fractions, respectively; nitrogen originated from CH- and C-592-derived fractions contribute partly to the TN. The P-PO_4 content ranged 101–147 mg/L, whereas the TP concentration ranged between 126 and 251 mg/L with the AL-derived fraction containing the highest amount (251 mg/L) ($P \leq 0.05$). TP in AL-, CA- and CH-derived fractions was 1.8-, 1.6- and 1.2-fold higher compared to the respective P-PO_4 content. The ammonia content in all waters was in the range of 17–68 mg/L. In comparison, TP, TN and ammonia content measured in various dairy liquid side streams which were used for cultivation of *C. Vulgaris* were found to be in the range of 22–1012, 935–3570, and 24–429 mg/L, respectively [35]. For different effluents from meat processing industries which were used for similar purposes, the ranges for TP, TN and ammonia contents varied greatly; between 5 and 46, 64–327 and 2–193 mg/L, respectively [7]. Evidently, each type of side stream possesses specific characteristics that results from the type of raw material and the nature of pre-processes. However, none of the effluents mentioned here was subjected to pre-removal of proteins.

Table 1
Composition of LMW-fractions resulting from flocculation and flotation treatment of SBW using flocculants as indicated from naming of the waters.

LMW-fraction	pH*	Protein content (g/L)	TN (g/L)	P-PO_4 (mg/L)	TP (mg/L)	Ammonia (mg/L)
CH	6.0	3.3 ± 0.2^{b}	0.9 ± 0.0^{bc}	106.0 ± 1.6^{b}	126.4 ± 1.6^{d}	33.0 ± 4.5^{bc}
AL	4.0	2.7 ± 0.0^{b}	1.3 ± 0.2^{b}	139.2 ± 2.1^{a}	251.9 ± 3.0^{a}	40.8 ± 2.5^{b}
CA	4.0	1.9 ± 0.1^{c}	0.7 ± 0.0^{c}	101.2 ± 1.4^{b}	162.4 ± 1.5^{c}	17.1 ± 0.9^{c}
C-592	8.7	5.8 ± 0.2^{a}	2.1 ± 0.2^{a}	147.7 ± 3.2^{a}	207.1 ± 5.7^{b}	68.0 ± 5.2^{a}

Data shown are mean from three replicate analyses ± standard deviation. Prior to cultivations, the LMW-fractions were fortified with vitamins ** and trace metals **to the same levels as the control media. AL (alginate); CA (carrageenan); CH (chitosan); C-592 (Superfloc C-592).
Means with different superscript letters within the same column indicate significant differences among media ($P \leq 0.05$).
* This was the native pH of the SBW.
** The content of vitamins per liter of medium were: 1.2 mg thiamine HCl, 0.01 mg biotin, and 0.01 mg cyanocobalamin; and the content of trace metals per liter of medium were as follows: 4.98 mg $Na_2EDTA{\cdot}2H_2O$, 0.582 mg $FeCl_3{\cdot}6H_2O$, 0.246 mg $MnCl_2{\cdot}4H_2O$, 0.017 mg $ZnCl_2$, 0.012 mg $CoCl_2{\cdot}6H_2O$, and 0.024 mg $Na_2MoO_4{\cdot}2H_2O$.

3.2. Screening of LMW-media during cultivation of C. sorokiniana and A. protothecoides

Four LMW-media were screened as cultivation media of two strains, *C. sorokiniana* and *A. protothecoides*, grown with ambient air for 8 days (Fig. 1). Of the four LMW-media, both species grew on LMW-media derived from AL, CA and CH flocculations but not on the fraction from C-592 flocculation. This could be due to the adverse effect from residual C-592 in the cultivation media [36]. The highest growth rates and final OD of both microalgae species were found in CH- and AL– media (Fig. 1A-B). The exponential growth curves of both species are presented in supplementary Fig. S1. The *A. protothecoides* strain out-performed *C. sorokiniana* in terms of final OD. Both strains showed comparable growth rates which averaged 0.7 d^{-1} and 1.0 d^{-1} in the AL– and CH-media, respectively (Table 2). The maximum growth rate of *A. protothecoides* when cultivated in the CH-media, 2.4 d^{-1}, was higher in comparison to earlier reported growth rates for this species, 0.49 d^{-1}, when cultivated in municipal wastewater (containing TP: 211 mg/L and NH_3-N: 91 mg/L) [37]. Cultivation of *Chlorella* sp. in the effluent collected from a sewage pool of a seafood company (containing 121 TN and 57 mg/L TP) for 19 days also resulted in maximum growth of 0.04 d^{-1} [10]. These media contained high levels of N and P; thus, the reduced growth could be due to the presence of contaminants/inhibitors carried over e.g., from cleaning processes in these complex waste streams as opposed to the studied LMW-media which originated solely from processing of shrimp.

Removal of P-PO_4, TP, protein, and ammonia were measured to investigate the capacity of the microalgae species in bioremediating those nutrients. Reduction of P-PO_4 by both strains was significant ($p < 0.05$) until day 4 for all media except for the C-592-derived one; the P-PO_4 reduction ranged from 16 to 47%, in which *A. protothecoides* showed the best results (Fig. 1E-F). Only the P-PO_4 uptake from the CA-media was significantly ($p < 0.05$) higher at day 8 than at day 4, reaching 56% with *A. protothecoides*. The P-PO_4 content was reduced by 30–62 mg/L in the three media at day 8 as compared to the initial content at day 0. Largest P-PO_4 removal was achieved by the *A. protothecoides* strain in the CH media, with a reduction of up to 62 mg/L at day 8.

With regards to TP, the highest removal (86 mg/L in 8 days) was observed by *A. protothecoides* cultivated in the AL-media. Removal of TP in AL- and CA-media was significant both at day 4 and day 8 ($p < 0.05$); however, for the CH-media, uptake of TP was only significant at day 8. Reduction of TP in the AL-media was 1.8- and 1.5-fold higher as compared to that of P-PO_4 for *A. protothecoides* and *C. sorokiniana* strains, respectively. For the CA-media, TP reduction was 1.2-fold for both strains assuming that organic P was more accessible to the strains in the AL-media as compared to in CA and CH-media. The TP removal in the present study over an 8-day cultivation was more efficient compared to earlier reported removal of 18 and 33 mg TP/L achieved during growth of *Chlorella* sp. for 19 days in seafood wastewater collected from a sewage pool under diluted or aerated conditions, respectively [10].

The protein content of the final media made up from AL, CA- and CH-fractions ranged between 1.4 and 2.3 g/L, and no significant change was observed during growth of *A. protothecoides* in these media. However, the protein content of the CA-media at day 8 showed a significant increase ($p < 0.05$), which could be related to protein release caused by cell death [38].

Ammonia was not assimilated by any of the strains, instead, the content increased 3.5–17-fold up to day 4 for AL-, CA- and CH-media;

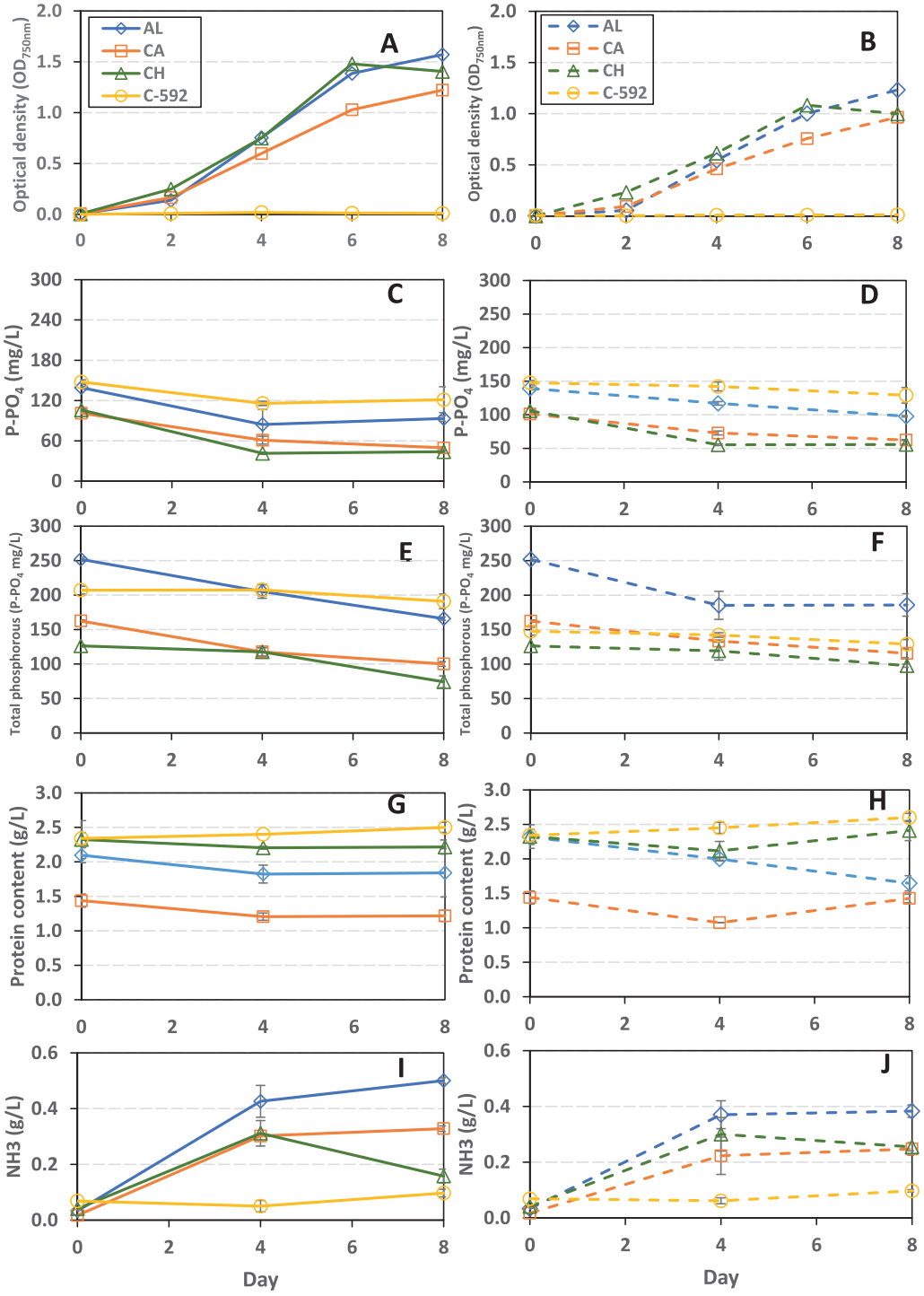

Fig. 1. Growth curves (A and B), P-PO4 (C and D), TP (E and F), protein (G and H) and NH_3 (I and J) of A. protothecoides (solid line) and *C. sorokiniana* (dash line) grown in AL-, CA-, CH- and C-592-derived LMW media during primary batch cultivation. Data points represent the means of duplicate cultivations with error bars showing the standard deviation. AL (alginate); CA (carrageenan); CH (chitosan); C-592 (Superfloc C-592).

largest changes were found for both strains in AL-media. The increase was probably due to ammonia generation during AA metabolism, e.g., deamination. It is also possible that some AAs are not transported into the cells but extracellularly deaminated releasing ammonia as previously reported for *Dunaliella* spp. [39]. However, the ammonia content stabilized after day 4 without further significant changes ($p < 0.05$) in both species except for the CH-media, in which, the ammonia content declined in the presence of *A. protothecoides*, reaching 158 mg/L. The obtained results could be due to the inherent characteristics of these cultivated media brought about by the initial protein recovery process.

3.3. Upscaled batch cultivation of A. protothecoides in CH– and AL-media

Based on the combined results on growth and removal of $P\text{-}PO_4$, TP as well as TN in the screening, *A. protothecoides* was cultivated in 1 L of AL- and CH-media as well as control BBM media under aeration and agitation while studying protein, $P\text{-}PO_4$, TP, TN and ammonia changes

Table 2
Growth rates *of C. sorokiniana* and *A. protothecoides* during primary batch cultivation in CH-,AL-, CA- and C-592-derived LMW media. pH measured on day 8 of cultivation is also presented.

Strain	Type of LMW media	Max. growth rate (d^{-1})	Average growth rate (d^{-1})	Final pH
A. protothecoides	CH	2.38 ± 0.05^{a}	1.05 ± 0.00^{a}	9.0
C. sorokiniana	CH	1.91 ± 0.08^{ab}	0.93 ± 0.03^{ab}	8.5
A. protothecoides	AL	1.31 ± 0.03^{c}	0.72 ± 0.00^{c}	9.0
C. sorokiniana	AL	1.67 ± 0.06^{bc}	0.72 ± 0.03^{c}	8.4
A. protothecoides	CA	1.48 ± 0.11^{bc}	0.66 ± 0.04^{c}	9.2
C. sorokiniana	CA	1.63 ± 0.18^{bc}	0.66 ± 0.04^{bc}	8.6
A. protothecoides	C-592	0.49 ± 0.10^{d}	0.10 ± 0.02^{d}	7.6
C. sorokiniana	C-592	0.33 ± 0.07^{d}	0.19 ± 0.05^{d}	7.6

Values are means of duplicate cultivations ± standard deviation.
Means with different superscript letters within the same column indicate significant differences among samples ($p \leq 0.05$). AL (alginate); CA (carrageenan); CH (chitosan); C-592 (Superfloc C-592).

Table 3
Growth dynamics of *A. protothecoides* in control BBM medium and in AL- and CH-media during upscaled batch cultivation.

Medium	Maximal specific growth rate[a] (d^{-1})	Average growth rate[b] (d^{-1})	Final pH
Control BBM	1.541 ± 0.018 (2)	0.482 ± 0.004	7.7
CH-media	1.459 ± 0.067 (2)	0.654 ± 0.016	8.5
AL-media	1.444 ± 0.150 (2)	0.595 ± 0.026	8.6

Growth experiments were performed in triplicate for CH- (chitosan) media and duplicate for AL- (alginate) media. Initial pH for all media was pH 7. Values shown are means ± standard deviation. For maximal specific growth, the number in brackets is the duration (in days) exponential growth was sustained.
[a] Exponential growth rate is determined over the period where the r^2 of the slope of logarithmically treated data is greater than 0.99.
[b] The average growth rate is calculated over 8 days.

in the media over time (Fig. 2A-F). The strain went immediately into exponential growth in all three media until day 2 (Table 3). The growth in the control BBM medium, was almost ceased after 2 days while

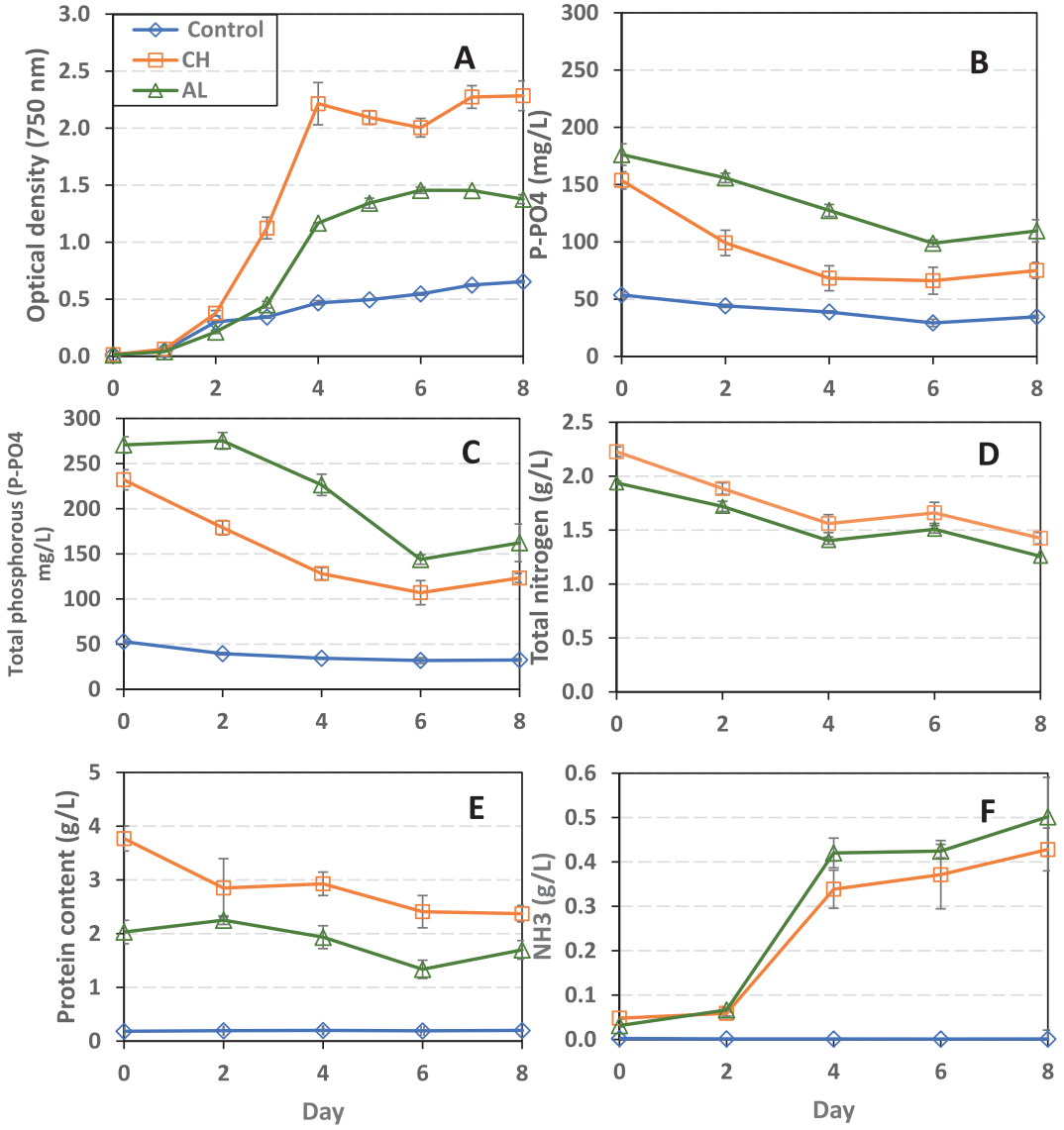

Fig. 2. Growth curves (A) and nutrient assimilation; P-PO4 (B), total P (C), total nitrogen (D), protein (E) and NH_3 (F) of *A. protothecoides* during 8 days of upscaled (800 mL) batch cultivation in control and in CH- and AL-media. Growth experiments were performed in triplicate (except for AL-media which was done in duplicate) and samples were analyzed in triplicate. Bars represent mean values for each treatment with error bars showing standard deviation. CH (chitosan); AL (alginate).

slightly slower growth continued up to day 4 in both AL- and CH- media. In both LMW-based media higher final pH as compared to the BBM medium was as a result of better growth in these cultures. In the CH–media, growth ceased after day 4 and OD_{750} values fluctuated until day 8. In contrast, in the AL-media, a trend of growth was visible until day 6, after which OD_{750} values fluctuated until day 8 (Fig. 2A) (exponential growth curve is presented in supplementary Fig. S2). Stirring and aeration of cultures in the upscaled batch cultivations improved growth of *A. protothecoides* compared to the initial screening experiments which subsequently resulted in the formation of more biomass in the CH-media, at upscaled conditions (OD 2.2, day 4) compared to in the screening cultivation (OD 1.5, day 6) (Fig. 2A vs Fig. 1A). In the AL-media, the *A. protothecoides* strain showed similar final biomass levels in both experiments, yet with higher rate in the upscaled cultivation compared to the initial cultivation.

The $P\text{-}PO_4$ content was reduced gradually in the CH- and AL-media until day 4 and 6, respectively, followed by slightly increased concentrations until day 8. The $P\text{-}PO_4$ removal was greater during cultivation in the CH-media compared to the AL-media from day 0 to day 4. The $P\text{-}PO_4$ removal in the CH-media did not change significantly ($p < 0.05$) from day 4 to 6 but there was a slight increase until day 8, in agreement with larger biomass formation in this fraction. In total, the reduction of $P\text{-}PO_4$ in CH- and AL- media was 43 and 56%, respectively. The cultivation in both AL- and CH– media showed 55 and 48% removal of TP, respectively, up to day 6 (Fig. 2C). TN content decreased by 37 and 36% in both media with the main decrease occurring until day 4, which was in agreement with microalgal growth. Protein content decreased by 27 and 32% in the AL- and CH-media, respectively, until day 6 of cultivation.

The ammonia content increased 9- and 16-fold in CH- and AL-media, respectively, over the entire cultivation period, with a 5- and 9-fold increment between days 2 and 4. The latter could indicate major cell activities pertaining to generation of ammonia during assimilation of organic nitrogen [40]. Ammonium ions (NH_4^+) and unionized ammonia

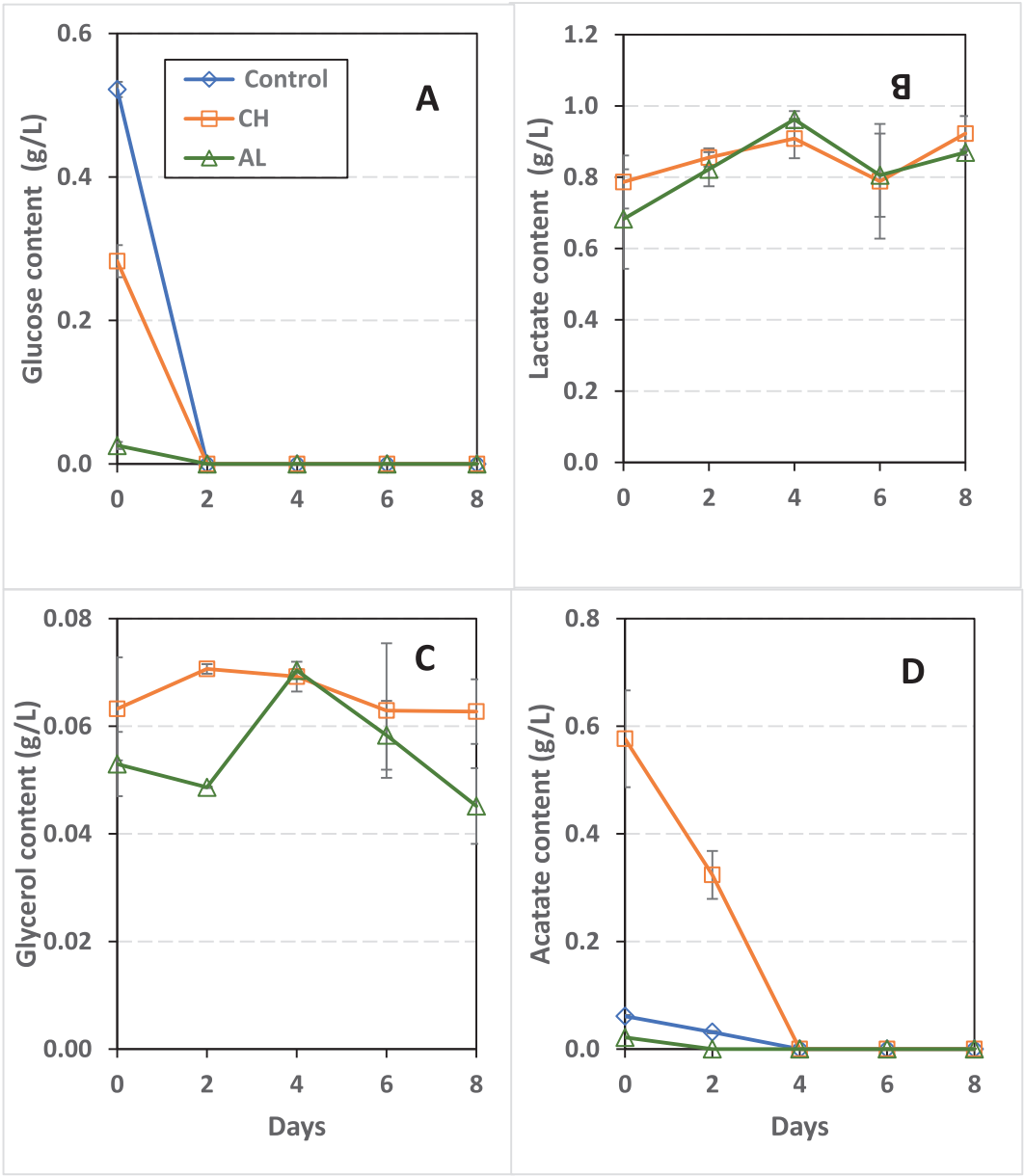

Fig. 3. Profile of media components; glucose (A), lactate (B), glycerol (C) and acetate (D) during upscaled batch cultivation of *A. protothecoides* in control-, CH- and AL-media. Data points represent mean values for the replicate cultivations ($n = 3$ for control- and CH-media, $n = 2$ for AL-media) with error bars showing standard deviation. Control BBM cultures contained no lactate or glycerol. CH (chitosan); AL (alginate).

(NH_3) are in equilibrium in an aqueous solution, therefore, at any given time both NH_4^+ and NH_3 are basically present in the process- or wastewaters. The NH_3 amount is substantially pH-dependent which is calculated as a function of TN, pH and temperature [40]. The dissociation constant (pKa) of the ammonia/ammonium reaction is about 9.3 depending on e.g., salinity and temperature, whereas at pH >9, the ammonia toxicity is mostly attributed to NH_3 On the other hand, at pHs <8, any toxicity effects are more likely related to ammonium ions whereas in the pH-range 8–9 the toxicity is due to an accumulative effect of both ammonia and ammonium [41]. In general, the ratio of unionized ammonia to ammonium ion increases by 10-fold per each unit increase in pH [41]. Earlier studies on ammonia toxicity indicated that unionized ammonia (NH_3) is the predominant form of nitrogen which is toxic to microalgae [42]. The EC_{50} of ammonium for growth can be as low as 0.5 g/L (30 μM) for some classes of microalgae [41]. Ammonia, in our study, was observed to reach such concentrations, thereby, may have retarded growth of *A. protothecoides*. Further investigations into the relation between ammonia concentration and *A. protothecoides* growth can help indicate if ammonia limits biomass production.

Findings on the assimilation of organic carbon sources (glucose, lactate, glycerol and acetate) indicated that all glucose was consumed very quickly during the first two days (Fig. 3). Interestingly, in the CH-media, acetate, which was relatively abundant, was also fully assimilated to below detection limits (<10 mg/L) by *A. protothecoides*. Glycerol and lactate in both the CH- and AL-media were maintained at constant levels and thus were not assimilated by *A. protothecoides*. It was earlier reported that glycerol can increase the growth and biomass production of 12 microalgal strains, and such effect was microalgal strain dependent [43]. Glycerol, in another study, increased in biomass productivity for *Chlamydomonas debaryana*, *C. sorokiniana* and *Micractinium* sp. [44]. It has been found that *Auxenochlorella* and other closely related strains can utilize glycerol, however, there is not any report on lactate as a suitable substrate for microalgae [45]. It is possible that the concentration of glycerol in these media was below the detectable threshold for this microalga and its assimilatory metabolism has therefore not been upregulated. A potential strategy for further improvements of biomass productivities would be to supplement the tested media with additional organic carbon; in this case, glucose or acetate appear favorable as demonstrated in several studies [45–47]. Such carbon sources could potentially be derived from other process water side streams.

Quantification of free AA in CH- and AL-media at day 0 and day 8 of cultivation showed that all AA were significantly ($p < 0.05$) assimilated by *A. protothecoides,* except for aspartic acid + asparagine (ASX) in both fractions and lysine in the alginate-derived fraction (Table 4). Proline was the dominant AA with concentrations of 847 and 421 mg/L in CH- and AL-media, respectively, followed by glycine, alanine, glutamic acid and leucine. Total free AA in the CH-media was 1.6-fold higher than that in the AL-media. In both cases, 37% of the initial free AA content was taken up by *A. protothecoides*, equal to 970 and 603 mg/L in the CH- and AL-media, respectively, which could be related to the larger biomass generation in the former. Uptake of essential AA (EAA) was superior to the uptake of non-essential AA (NEAA), 86 and 80% of EAA in comparison to 53 and 56% of NEAA, in the CH- and AL-media, respectively. The greatest reduction occurred for valine and leucine. It is possible that uptake of AA was induced by the other compounds present in the two media. Cho et al. [48] reported on up to 150-fold induction of alanine uptake by *Chlorella* in the presence of glucose, whereas aromatic and acidic AAs were not affected by preincubation of the cells with glucose. Microalgal cells under ammonia-induced stress alleviate it by converting ammonia to AAs, e.g., to glutamine/glutamate but there is not much evidence showing it is necessarily exported [41,49]. In the present study, GLX content increased in the media during cultivation. If cells are unhealthy due to high ammonia, this could also induce cell leakage explaining why some AA were at higher levels at day 8. Also, the high ammonia could cause cell lysis that in turn could result in an increase in some AAs [41]. It is generally assumed that microalgae will preferentially take up N sources in the following order: ammonium > nitrate > urea > organic-N. It is likely that the microalgae, in our study are inhibited by the high ammonium levels or have yet to change their metabolism to start assimilating it effectively. The efficient uptake of AA and increase in ammonia content could be due to various pathways resulting from specific nature/characteristics of the evaluated fractions, which would need in depth studies beyond this work.

Table 4
AA concentration (mg/L) in AL- and CH-media measured at day 0 and 8 of cultivation using *A. protothecoides*.

	AA (mg/L)			
	CH-media		AL-media	
	Day 0	Day 8	Day 0	Day 8
LYS	103.0 ± 4.3	59.5 ± 16.9	62.0 ± 25.7*	58.1 ± 5.9*
ARG	49.8 ± 5.3	7.3 ± 1.1	26.7 ± 4.2	8.7 ± 0.5
HIS	16.0 ± 6.7	3.9 ± 0.1	19.1 ± 2.2	3.9 ± 0.3
GLY	358.3 ± 8.4	76.7 ± 8.6	360.1 ± 10.0	213.3 ± 9.8
SER	39.4 ± 3.5	4.4 ± 0.4	37.8 ± 3.6	3.5 ± 0.5
ALA	279.1 ± 28.5	102.7 ± 11.2	159.4 ± 21.1	44.7 ± 6.8
THR	57.7 ± 4.2	11.8 ± 2.4	31.3 ± 3.0	3.6 ± 0.8
GLX	189.8 ± 18.6	240.2 ± 27.2	116.9 ± 13.9	173.2 ± 17.2
ASX	46.4 ± 3.8*	52.3 ± 8.5*	29.3 ± 3.4*	32.7 ± 2.3*
PRO	847.3 ± 39.6	381.4 ± 76.1	421.4 ± 55.9	37.5 ± 14.5
VAL	129.1 ± 17.2	6.2 ± 1.8	68.2 ± 11.4	3.0 ± 0.1
MET	62.1 ± 7.0	2.0 ± 0.2	34.5 ± 6.0	2.8 ± 0.3
TYR	60.9 ± 9.2	4.9 ± 0.2	52.9 ± 10.7	4.6 ± 0.2
ILE	95.4 ± 12.3	3.6 ± 0.5	49.7 ± 8.7	2.5 ± 0.6
LEU	155.5 ± 35.6	3.5 ± 0.3	86.6 ± 14.4	2.8 ± 0.5
PHE	86.9 ± 10.9	2.2 ± 0.2	53.1 ± 7.9	1.7 ± 0.2
TEAA	705.9	92.8	404.7	78.3
TNEAA	1874.7	877.4	1207.9	525.3
TAA	2580.6	970.3	1612.5	603.6

Data are means ± standard deviation of 3 replicate cultivations in CH media and 2 replicate cultivations in AL media.
Means within the same row for each media were significantly different between day 0 and 8 ($P \leq 0.05$) except for those labeled with *. GLX, glutamic acid and glutamine; ASX, aspartic acid and asparagine; TAA, total amino acid; TEAA, total essential amino acid; TNEAA, total non-essential amino acid, CH (chitosan) and AL (alginate).

3.4. Biochemical composition of *A. protothecoides* biomass

The biochemical composition of *A. protothecoides* biomass harvested at days 4 and 8 of cultivation is summarized in Fig. 4. The protein content of *A. protothecoides* biomass harvested from CH- and AL-media and both days varied from 35 to 43% per DW whereas total FA was in the range of 15–17%. In biomass from the control BBM medium, total FA and protein levels were 35% and 53%, as well as 19 and 11%, at day 4 and 8, respectively. The high protein content of microalgae species from the *Chlorellaceae* family is well recorded, and up to 55% protein per DW has been reported [21]. For all media, the protein content decreased by day 8 whereas the total FA content increased, and this effect was particularly y pronounced for biomass harvested from the control BBM cultures (Fig. 4). The increase in FA content and decrease in protein content are characteristic of cells that were exposed to nitrogen starvation. Nitrogen in the control BBM medium was provided as nitrate at 62 mg/L. The nitrogen in the two used process water fractions were mainly in the form of AA which were not fully consumed during the cultivation (Table 4). Furthermore, there was an increase in extracellular ammonium (Fig. 2), thus, cells were exposed to no or very mild starvation conditions. The slight increase in FAs from day 4 to 8 in both biomasses is possibly related to oxidative stress rather than nitrogen limitation, which can also influence lipid metabolism. Increased oxidative stress results in the diversion of excess reductant to FA metabolism as a means of dissipating excess energy [50,51]. High extracellular ammonia concentrations is one such cause of increased oxidative stress, brought about by several complex changes in cellular metabolism [52],

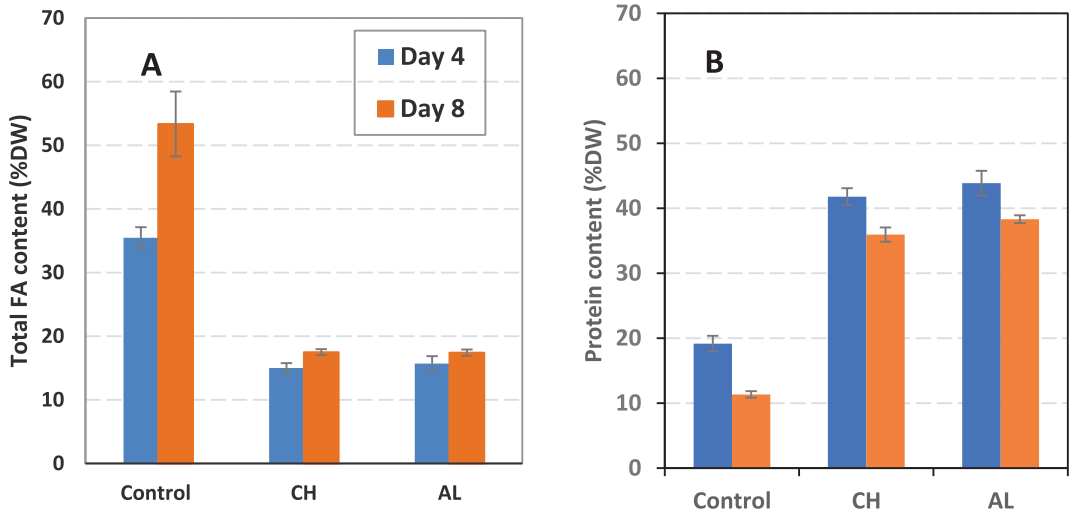

Fig. 4. Total FA (A) and protein content (B) of dry *A. protothecoides* biomass. Data shown are mean values from triplicate (CH (chitosan)) and duplicate (AL (alginate)) cultivations and error bars show standard deviation among these.

however there are no studies, so far, demonstrating a clear link between ammonia induced-stress and lipid accumulation in microalgae.

Table 5
AA content (mg/g DW) in *A. protothecoides* biomass harvested at day 8.

	AA (mg/g)			FAO/WHO adult (mg/g protein)
	Control	CH-media	AL-media	
LYS	5.08 ± 1.10^{C}	20.64 ± 0.54^{B}	22.32 ± 0.16^{A}	45
ARG	8.78 ± 1.39^{B}	20.80 ± 0.73^{A}	21.76 ± 0.31^{A}	
HIS	4.53 ± .67^{B}	8.19 ± 0.26^{A}	8.59 ± 0.15^{A}	15
GLY	5.60 ± 1.16^{C}	19.90 ± 0.69^{B}	21.60 ± 0.78^{A}	
SER	6.83 ± 1.51^{B}	18.25 ± 0.08^{A}	19.30 ± 0.25^{A}	
ALA	11.70 ± 2.80^{B}	35.13 ± 0.47^{A}	37.59 ± 0.68^{A}	
THR	5.29 ± 1.23^{B}	18.62 ± 0.86^{A}	19.34 ± 0.56^{A}	23
GLX	10.01 ± 2.21^{B}	42.39 ± 0.60^{A}	43.71 ± 0.66^{A}	
ASX	9.33 ± 1.71^{B}	34.25 ± 1.28^{A}	35.41 ± 0.44^{A}	
PRO	4.99 ± 1.13^{C}	39.35 ± 1.15^{A}	29.03 ± 2.17^{B}	
VAL	5.74 ± 1.46^{B}	21.71 ± 2.49^{A}	23.07 ± 0.43^{A}	39
MET	0.00 ± 0.00^{B}	0.37 ± 0.61^{AB}	1.01 ± 0.60^{A}	17
TYR	3.37 ± 0.57^{B}	9.89 ± 0.68^{A}	10.01 ± 0.41^{A}	
ILE	4.22 ± 1.13^{B}	14.63 ± 0.26^{A}	15.03 ± 0.56^{A}	30
LEU	8.88 ± 2.25^{B}	34.52 ± 0.31^{A}	35.96 ± 0.62^{A}	59
PHE	4.74 ± 1.21^{B}	19.45 ± 0.63^{A}	20.30 ± 0.53^{A}	19
TAA	102.53	361.63	367.48	
TEAA	38.48	138.11	145.63	
TEAA/ TAA	0.38	0.38	0.40	

Data are means ± standard deviation for biomass from 3 replicate cultivations in control media and the chitosan-derived LMW fraction (CH-media) and from 2 replicate cultivations in the alginate-derived LMW fraction (AL-media). Means with different superscript letters within the same row indicate significant differences among media ($P \leq 0.05$). TAA, total amino acid; TEAA, total essential amino acid; CH (chitosan); AL (alginate).

The total amino acid (TAA) content of the biomass at day 8 mirrored the protein data in that biomasses from control media, CH- and AL-media contained 10.2, 36.2 and 36.7% TAA per DW, respectively (Table 5). GLX was the predominant AA (42.4 and 43.7 mg/g) in biomass harvested from CH- and AL-media followed by proline, alanine, leucine, aspartic acid, arginine and lysine. Among EAA, leucine was found to be the predominant, followed by valine and lysine after cultivation in CH- and AL-media. The proportion of EAA to TAA in all biomasses was in the same range, 0.38–0.40. This EAA proportion is higher compared to that reported for *Nannochloropsis* (0.3) [53] as well as *Chlorella vulgaris* KAV 1000 (0.28) and *C. sorokiniana* UTEX 1230 (0.29) [54]. It was also slightly lower than that reported for the macroalgae *Saccharina latissima* (0.40–0.47O [55] and *C. vulgaris* 87/1 (0.47) [56] as well as beef and fish (0.47–0.48) [57]. Content of individual EAAs, except for methionine, which is susceptible to acid hydrolysis during the sample preparation, were well above the requirement for human nutrition [58]. Thus, the *A. protothecoides* strain could be of relevance as a food protein source, given the protein bioavailability is satisfactory, which needs to be evaluated. We recently showed that without cell disintegration, protein accessibility of *Nannochloropsis oculata* in an in vitro digestion model was very low [59]. *A. protothecoides* biomass could also be relevant from a feed perspective due to the good balance of EAA. Fish meal has always been an essential part of aquaculture feed formulation due to a well-balanced EAA content, which is lacking in plant protein counterpart, i.e., soya protein. Provided cultivation as well as harvest conditions with low CO_2 and water footprints are used, *A. protothecoides* could thus be a more sustainable, yet EAA-dense, replacement of fish meal in aquaculture feed formulation. Toxicological analysis is however required to confirm the suitability of the microalgal biomass as a feed ingredient.

The FA profile of harvested biomasses from day 4 and 8 is presented in Table 6 (supplementary). Monounsaturated FAs (MUFA) were the major portion, of which C18:1 n-9 (oleic acid) was the most abundant followed by C18:1 trans Δ^9 (n-9) (elaidic acid) and, C16:1. After 4 and 8 days, polyunsaturated FA (PUFA) contributed to up to 20.6% and 22.5% of total FA, respectively, with almost all PUFA being from the n-6 family. Biomasses harvested from AL- and CH-media contained significantly higher n-6 PUFA compared to the control. Both MUFA and n-6 PUFA increased from day 4 to day 8; with the changes for the n-6 PUFA being highest. The n-3 PUFAs, e.g., α-linolenic acid, EPA and DHA, made up 0.3% of total FA in the control, and 0.6–0.8% of total FA in biomasses harvested from AL- and CH-media. The control biomass had less n-3 PUFA than the other two biomasses which is possibly due to lower

nitrogen and phosphorous content in the control media. The large increment in C18:0, C18:1 and C18:2 in control biomass at day 8 could be a result of nutrient starvation, and likely the FAs were in the form of triglycerides as previously reported in other oleaginous microalgae [60,61].

4. Concluding remarks

A. protothecoides was successfully cultivated in LMW fractions generated during AL- and CH-induced protein flocculation and flotation of SBW, illustrating a promising use for this currently wasted process water side stream. During growth, *A. protothecoides* significantly reduced available P-PO_4, TP, TN and free AAs, except glutamate, in both CH- and AL-media; however, the amount of ammonia increased during cultivation. The produced microalgae biomass contained up to 43% protein and 17.4% total FA per DW, and the EAA was well above human requirements. FA were dominated by MUFAs and revealed a high n-6/n-3-ratio. The produced biomasses may be of use in both for food and feed; although for the microalgae biomass, nutrient bioavailability should first be documented.

Compared to a typical microalgae cultivation system based on tap water, the superiority of using process water residues, as studied in this paper, lies in two aspects; firstly, the consumption of fresh water is reduced by up to 0.11 m^3 per 1 kg of dry biomass, secondly, remediation of the process water as a source of N and P replaces the need to add fertilizers, which enhances the sustainability and energy balance of the biomass production [2]. It is estimated [2] that in a case where 88 g N and 12 g P are required to produce 1 kg of microalgal biomass, the global warming potential associated with the production of the required N/P-fertilizers amount to 0.82 and 0.04 kg CO2- equivalents [2]. Also, Canter et al. calculated that to meet 23% of the total US advanced fuel production (79 billion L/year) using microalga, the cultivation of this biomass would require up to 22% and 18% of the total N and the total P, respectively, available in the US [62]. The increased fertilizer prices resulting from a raised demand will also significantly impact other sectors such as agriculture. It is therefore imperative to maximize the recycling of residual nutrients lost from food processing operations in microalgae cultivations. Overall, our findings reveal that such a strategy can be integrated in a 2-step protein production platform combining chemical, physical and microbial principles to render values from side streams which currently incurs a cost to shrimp processors to discharge.

CRediT authorship contribution statement

Bita Forghani Methodology, Conceptualization, Formal analysis, Roles/Writing - original draft.

Joshua J. Mayers Conceptualization, Methodology, Writing - review & editing, Formal analysis.

Eva Albers Conceptualization, Methodology, Writing - review & editing.

Ingrid Undeland Funding acquisition, Conceptualization, Project administration, Resources Writing - review & editing, Methodology.

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 data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102753.

References

[1] D. Das, Algal Biorefinery: An Integrated Approach, 2015.
[2] J.J. Mayers, A. Ekman Nilsson, E. Svensson, E. Albers, Integrating microalgal production with industrial Outputs—Reducing process inputs and quantifying the benefits, Ind. Biotechnol. 12 (2016) 219–234.
[3] L.A. Johnson, P.J. White, R. Galloway, Soybeans: Chemistry, Production, Processing, and Utilization, Elsevier, 2015.
[4] E.-S. Salama, M.B. Kurade, R.A.I. Abou-Shanab, M.M. El-Dalatony, I.-S. Yang, B. Min, B.-H. Jeon, Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation, Renew. Sust. Energ. Rev. 79 (2017) 1189–1211.
[5] D. Cordell, A. Rosemarin, J.J. Schröder, A.L. Smit, Towards global phosphorus security: a systems framework for phosphorus recovery and reuse options, Chemosphere 84 (2011) 747–758.
[6] B. Guieysse, Q. Béchet, A. Shilton, Variability and uncertainty in water demand and water footprint assessments of fresh algae cultivation based on case studies from five climatic regions, Bioresour. Technol. 128 (2013) 317–323.
[7] Q. Lu, W. Zhou, M. Min, X. Ma, C. Chandra, Y.T.T. Doan, Y. Ma, H. Zheng, S. Cheng, R. Griffith, P. Chen, C. Chen, P.E. Urriola, G.C. Shurson, H.R. Gislerød, R. Ruan, Growing chlorella sp. On meat processing wastewater for nutrient removal and biomass production, Bioresour. Technol. 198 (2015) 189–197.
[8] A.P. Abreu, B. Fernandes, A.A. Vicente, J. Teixeira, G. Dragone, Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source, Bioresour. Technol. 118 (2012) 61–66.
[9] G. Hodaifa, M.E. Martínez, S. Sánchez, Influence of pH on the culture of Scenedesmus obliquus in olive-mill wastewater, Biotechnol. Bioprocess Eng. 14 (2009) 854–860.
[10] F. Gao, Y.-Y. Peng, C. Li, G.-J. Yang, Y.-B. Deng, B. Xue, Y.-M. Guo, Simultaneous nutrient removal and biomass/lipid production by chlorella sp. In seafood processing wastewater, Sci. Total Environ. 640 (2018) 943–953.
[11] L. Jiang, H. Pei, W. Hu, Y. Ji, L. Han, G. Ma, The feasibility of using complex wastewater from a monosodium glutamate factory to cultivate Spirulina subsalsa and accumulate biochemical composition, Bioresour. Technol. 180 (2015) 304–310.
[12] C. Song, X. Hu, Z. Liu, S. Li, Y. Kitamura, Combination of brewery wastewater purification and CO2 fixation with potential value-added ingredients production via different microalgae strains cultivation, J. Clean. Prod. 268 (2020), 122332.
[13] S. Gupta, R.A. Pandey, S.B. Pawar, Microalgal bioremediation of food-processing industrial wastewater under mixotrophic conditions: kinetics and scale-up approach, Front. Chem. Sci. Eng. 10 (2016) 499–508.
[14] K. Duangjan, B. Kumsiri, C. Pumas, Lipid production by microalga Scenedesmus sp. AARL G022 in the cultivation with effluent from chicken manure biogas plant, Desalin. Water Treat. 57 (2016) 27191–27198.
[15] J.J. Mayers, K.J. Flynn, R.J. Shields, Influence of the N: P supply ratio on biomass productivity and time-resolved changes in elemental and bulk biochemical composition of nannochloropsis sp, Bioresour. Technol. 169 (2014) 588–595.
[16] Q. Lu, W. Zhou, M. Min, X. Ma, Y. Ma, P. Chen, H. Zheng, Y.T.T. Doan, H. Liu, C. Chen, P.E. Urriola, G.C. Shurson, R. Ruan, Mitigating ammonia nitrogen deficiency in dairy wastewaters for algae cultivation, Bioresour. Technol. 201 (2016) 33–40.
[17] D. Morales-Sánchez, O.A. Martinez-Rodriguez, J. Kyndt, A. Martinez, Heterotrophic growth of microalgae: metabolic aspects, World J. Microbiol. Biotechnol. 31 (2015) 1–9.
[18] M. Amini, H. Younesi, A.A. Zinatizadeh Lorestani, G. Najafpour, Determination of optimum conditions for dairy wastewater treatment in UAASB reactor for removal of nutrients, Bioresour. Technol. 145 (2013) 71–79.
[19] B. Forghani, R. Bordes, A. Ström, I. Undeland, Recovery of a protein-rich biomass from shrimp (Pandalus borealis) boiling water: a colloidal study, Food Chem. 302 (2020), 125299.
[20] B. Forghani, A.-D.M. Sørensen, J. Johannesson, T.C. Svendsen, I. Undeland, Flocculation and flotation to recover protein-enriched biomasses from shrimp boiling and peeling process waters-a case study, ACS Sustain. Chem. Eng. 8 (2020) 9660–9668.
[21] H. Safafar, P. Uldall Nørregaard, A. Ljubic, P. Møller, S. Løvstad Holdt, C. Jacobsen, Enhancement of protein and pigment content in two chlorella species cultivated on industrial process water, J. Mar. Sci. Eng. 4 (2016) 84.
[22] S. Rani, C.S.P. Ojha, Chlorella sorokiniana for integrated wastewater treatment, biomass accumulation and value-added product estimation under varying photoperiod regimes: a comparative study, J. Water Process Eng. 39 (2021), 101889.
[23] S. Shinde, S.S. Lele, Statistical Media Optimization for Lutein Production From Microalgae Auxenochlorella protothecoides SAG 211-7A, 2010.
[24] R.A. Andersen, Algal Culturing Techniques, Elsevier, 2005.
[25] R.R. Guillard, Culture of phytoplankton for feeding marine invertebrates, in: Culture of Marine Invertebrate Animals, Springer, 1975, pp. 29–60.
[26] R.R.L. Guillard, J.H. Ryther, Studies of marine planktonic diatoms. I. Cyclotella nana hustedt and Detonula confervacea cleve, Can. J. Microbiol. 8 (1962) 229–239.

[27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[28] M.A.K. Markwell, S.M. Haas, L. Bieber, N. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.
[29] L. Qvirist, N.-G. Carlsson, T. Andlid, Assessing phytase activity–methods, definitions and pitfalls, J. Biol. Methods 2 (2015).
[30] E.A. Dayton, S. Whitacre, C. Holloman, Comparison of three persulfate digestion methods for total phosphorus analysis and estimation of suspended sediments, Appl. Geochem. 78 (2017) 357–362.
[31] S. Özcan, H.Z. Şenyuva, Improved and simplified liquid chromatography/atmospheric pressure chemical ionization mass spectrometry method for the analysis of underivatized free amino acids in various foods, J. Chromatogr. A 1135 (2006) 179–185.
[32] L.M. Laurens, M. Quinn, S. Van Wychen, D.W. Templeton, E.J. Wolfrum, Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification, Anal. Bioanal. Chem. 403 (2012) 167–178.
[33] L.R. Cavonius, N.-G. Carlsson, I. Undeland, Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods, Anal. Bioanal. Chem. 406 (2014) 7313–7322.
[34] S.P. Slocombe, M. Ross, N. Thomas, S. McNeill, M.S. Stanley, A rapid and general method for measurement of protein in micro-algal biomass, Bioresour. Technol. 129 (2013) 51–57.
[35] Q. Lu, W. Zhou, M. Min, X. Ma, Y. Ma, P. Chen, H. Zheng, Y.T. Doan, H. Liu, C. Chen, Mitigating ammonia nitrogen deficiency in dairy wastewaters for algae cultivation, Bioresour. Technol. 201 (2016) 33–40.
[36] Kemira, Chemical Flocculants, 2021.
[37] W. Zhou, Y. Li, M. Min, B. Hu, H. Zhang, X. Ma, L. Li, Y. Cheng, P. Chen, R. Ruan, Growing wastewater-born microalga auxenochlorella protothecoides UMN280 on concentrated municipal wastewater for simultaneous nutrient removal and energy feedstock production, Appl. Energy 98 (2012) 433–440.
[38] K.D. Bidle, P.G. Falkowski, Cell death in planktonic, photosynthetic microorganisms, Nat. Rev. Microbiol. 2 (2004) 643–655.
[39] C.A. Murphree, J.T. Dums, S.K. Jain, C. Zhao, D.Y. Young, N. Khoshnoodi, A. Tikunov, J. Macdonald, G. Pilot, H. Sederoff, Amino acids are an ineffective fertilizer for Dunaliella spp. growth, Front. Plant Sci. 8 (2017) 847.
[40] A.C. Anthonisen, R.C. Loehr, T. Prakasam, E. Srinath, Inhibition of nitrification by ammonia and nitrous acid, J. Water Pollut. Control Fed. (1976) 835–852.
[41] Y. Collos, P.J. Harrison, Acclimation and toxicity of high ammonium concentrations to unicellular algae, Mar. Pollut. Bull. 80 (2014) 8–23.
[42] T. Källqvist, A. Svenson, Assessment of ammonia toxicity in tests with the microalga, Nephroselmis pyriformis, chlorophyta, Water Res. 37 (2003) 477–484.
[43] K. Paranjape, G.B. Leite, P.C. Hallenbeck, Strain variation in microalgal lipid production during mixotrophic growth with glycerol, Bioresour. Technol. 204 (2016) 80–88.
[44] K.C. Park, C. Whitney, J.C. McNichol, K.E. Dickinson, S. MacQuarrie, B.P. Skrupski, J. Zou, K.E. Wilson, S.J.B. O'Leary, P.J. McGinn, Mixotrophic and photoautotrophic cultivation of 14 microalgae isolates from Saskatchewan, Canada: potential applications for wastewater remediation for biofuel production, J. Appl. Phycol. 24 (2012) 339–348.
[45] V. Turon, C. Baroukh, E. Trably, E. Latrille, E. Fouilland, J.P. Steyer, Use of fermentative metabolites for heterotrophic microalgae growth: yields and kinetics, Bioresour. Technol. 175 (2015) 342–349.
[46] H. Rismani-Yazdi, K.H. Hampel, C.D. Lane, B.A. Kessler, N.M. White, K.M. Moats, F.C. Thomas Allnutt, High-productivity lipid production using mixed trophic state cultivation of auxenochlorella (Chlorella) protothecoides, Bioprocess Biosyst. Eng. 38 (2015) 639–650.
[47] A. Patel, L. Matsakas, U. Rova, P. Christakopoulos, Heterotrophic cultivation of auxenochlorella protothecoides using forest biomass as a feedstock for sustainable biodiesel production, Biotechnol. Biofuels 11 (2018) 169.
[48] B.-H. Cho, N. Sauer, E. Komor, W. Tanner, Glucose induces two amino acid transport systems in chlorella, Proc. Natl. Acad. Sci. 78 (1981) 3591–3594.
[49] J. Muñoz-blanco, J. Cardenas, Changes in glutamate dehydrogenase activity of Chlamydomonas reinhardtii under different trophic and stress conditions, Plant Cell Environ. 12 (1989) 173–182.
[50] K. Yilancioglu, M. Cokol, I. Pastirmaci, B. Erman, S. Cetiner, Oxidative stress is a mediator for increased lipid accumulation in a newly isolated Dunaliella Salina strain, Plos One 9 (2014) e91957-e91957.
[51] O. Osundeko, A.P. Dean, H. Davies, J.K. Pittman, Acclimation of microalgae to wastewater environments involves increased oxidative stress tolerance activity, Plant Cell Physiol. 55 (2014) 1848–1857.
[52] A. Bittsánszky, K. Pilinszky, G. Gyulai, T. Komives, Overcoming ammonium toxicity, Plant Sci. 231 (2015) 184–190.
[53] J.A. Gerde, T. Wang, L. Yao, S. Jung, L.A. Johnson, B. Lamsal, Optimizing protein isolation from defatted and non-defatted nannochloropsis microalgae biomass, Algal Res. 2 (2013) 145–153.
[54] C.D. Lane, D.A. Coury, F.T. Allnutt, Composition and potential products from auxenochlorella protothecoides, Chlorella sorokiniana and Chlorella vulgaris, Ind. Biotechnol. 13 (2017) 270–276.
[55] M. Abdollahi, J. Axelsson, N.-G. Carlsson, G.M. Nylund, E. Albers, I. Undeland, Effect of stabilization method and freeze/thaw-aided precipitation on structural and functional properties of proteins recovered from brown seaweed (Saccharina latissima), Food Hydrocoll. 96 (2019) 140–150.
[56] H.J. Morris, A. Almarales, O. Carrillo, R.C. Bermúdez, Utilisation of chlorellavulgaris cell biomass for the production of enzymatic protein hydrolysates, Bioresour. Technol. 99 (2008) 7723–7729.
[57] F.W. Sosulski, G.I. Imafidon, Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods, J. Agric. Food Chem. 38 (1990) 1351–1356.
[58] WHO, FAO, UNU, Protein and Amino Acid Requirements in Human Nutrition, 2007.
[59] L.R. Cavonius, E. Albers, I. Undeland, In vitro bioaccessibility of proteins and lipids of pH-shift processed nannochloropsis oculata microalga, Food Funct. 7 (2016) 2016–2024.
[60] D. Simionato, M.A. Block, N.La Rocca, J. Jouhet, E. Maréchal, G. Finazzi, T. Morosinotto, The Response of Nannochloropsis gaditana to nitrogen starvation includes De Novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus, Eukaryot. Cell 12 (2013) 665–676.
[61] Z. Sun, Z.-G. Zhou, H. Gerken, F. Chen, J. Liu, Screening and characterization of oleaginous chlorella strains and exploration of photoautotrophic Chlorella protothecoides for oil production, Bioresour. Technol. 184 (2015) 53–62.
[62] C.E. Canter, P. Blowers, R.M. Handler, D.R. Shonnard, Implications of widespread algal biofuels production on macronutrient fertilizer supplies: nutrient demand and evaluation of potential alternate nutrient sources, Appl. Energy 143 (2015) 71–80.