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A critical look into different salt removal treatments for the production of high value pigments and fatty acids from marine microalgae *Chlorella vulgaris* (NIOT-74)

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ABSTRACT

The prime challenge in seawater culture of microalgae for high value biomolecules production is presence of salt. Hence, twelve different salt removal treatments were evaluated for their impact on the lutein, total carotenoid, chlorophyll yields and fatty acid profile of marine microalgae *Chlorella vulgaris* (NIOT-74). The effectiveness of different treatments on salt removal was also visualized with the aid of Scanning Electron Microscope (SEM). Among the tested treatments, washing the algal biomass with 0.5 % HCl augmented the lutein (11.56 mg/g) and total carotenoid yield (60.88 mg/g) 1.82 and 1.86 fold respectively, in comparison to untreated control. Highest chlorophyll content (30.64 mg/g) was noticed in the distilled water wash treatment. Different salt removal treatments also impacted the fatty acid profile and degree of unsaturation of the fatty acids significantly. This study thus, signified the importance of salt removal treatments for the commercial production of biomolecules from marine microalgae cultured in natural seawater.

1. Introduction

With greater health awareness the global market demand for natural and organic products have surged several folds. Needless to say, the microalgal biotechnology has carved a niche in the animal feed and nutraceutical industry which is valued at $ 31.3 billion and 198.7 billion annually as of 2016 and predicted to grow to 34.2 billion and 285 billion respectively by 2021 [1,2]. Major share of this market is contributed by carotenoids like lutein, astaxanthin and beta-carotene and oleo fatty acids. Of the 700 reported carotenoids, the xanthophyll carotenoid lutein stands out due to its ever increasing global market demand as natural colorant [3], poultry and fish feed supplement [4], cosmetic and health supplement [5]. Being an integral part of eye retina and lens [6], lutein has exhibited diverse biological activities like prevention of AMD (Age related Macular Degeneration) [7], onset and progression of cataracts [8], retinal neural damage [9] and antiglycoxidative effect [10]. The remarkable bioactivities of lutein are due to its antioxidant activity, which is attributed to its conjugated double bond chemical structure [11]. The potent antioxidative properties of lutein has boosted its market as a health supplement to prevent or delay chronic diseases like AMD [12], atherosclerosis [13] and cardiovascular diseases [12]. Conventionally, marigold flowers are commercially used as prime source of lutein. Nevertheless, lutein present in marigold is esterified and 50 % of its weight corresponds to the attached fatty acids. Additionally, lutein production from marigold is subjected to the vagaries of season, planting area, land cost and high manpower cost [14]. Under this circumstance lutein production from microalgae has emerged as a viable alternative. Algal derived lutein distinguishes itself over the conventional source by its presence in free non-esterified form and production throughout the year [14]. Green algae especially, *Chlorella* sp. have established their efficient ability to grow and accumulate lutein under phototrophic, mixotrophic [15] and heterotrophic conditions [16,17]. Apart from being a good source of lutein, *Chlorella* species have also successfully demonstrated their capability of being a source of over 2000 metric tons/year of human health products [18], nutraceutical tablets [19], omega-3 fatty acids [20], recombinant protein [21], biopharmaceuticals and cosmetics [22].

Besides these biological applications *chlorella* biomass can also be used for the production of chlorophyll and as a feed stock for biofuel production. Chlorophyll is yet another algal photosynthetic pigment with a potential market in pharmaceutical products [23]. Chlorophyll, due to its well established bioactivity of wound healing, stimulating tissue growth, antioxidant and antimutagenic activities have found pharmaceutical applications in the treatment

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of ulcers, oral sepsis and proctology [24]. The unique role of chlorophyll derivatives in the prevention of cancer is attributed to its ability to trap mutagens in the gastrointestinal tract [25]. Added to these biological significance, *Chlorella* also has a inherent ability to accumulate high lipid content. This tendency has transformed this genus into a potential source of biofuel feed stock and polyunsaturated fatty acids (PUFA) [26]. The estimated market value for PUFA produced from microalgae is USD 140/Kg. Microalgal fatty acids are revered over fish oil due to the advantage of being devoid of heavy metal contaminants. Additionally, research evidences have also certified the exemplary beneficial effect of algal fatty acids against inflammation and wide range of cardio vascular diseases [20]. Microalgae being a source of multiple commercially valuable products in the present day market driven biotechnology industrythe concept of using microalgae as biorefinery model for the co-production of multiple high value products to improve the process economics is gaining much relevance [27]. The key challenge in mass culture of marine microalgae for the co-production of multiple products is to select strains which can grow well under highly fluctuating light, temperature and salinity variations under industrially relevant outdoor conditions. *Chlorella* is one of the few microalgal species which has proven its ability to grow under fluctuating outdoor conditions. Despite, microalgae being potential source of several commercially important bioactive molecules like protein, phenolics, long chain fatty acids and carotenoids, the commercial production of marine microalgae is still in dispute due to the prohibitive downstream cost which amounts to 70–80 % of the total cost. Hence, seawater culture of microalgae is gaining importance for commercial production of carotenoids and co-products in recent days due to lower energy and nutrient inputs when compared to freshwater culture [28]. Furthermore, growing microalgae in seawater curtails the freshwater requirement considerably.

The major contributer of algal downstream cost is algal dewatering or harvest. At present microalgal harvesting is done using mechanical, chemical, biological and electrical or a combination of two or more of these processes [29]. Regardless of the harvesting process employed, the major constraint in production of biomolecules from marine microalgae is the presence of salts in the biomass [30]. The increase in salt content of algal biomass impacts the actual algal biomas weight and intervenes in the subsequent downstream processes [28]. Earlier research work on ethanol and high value biochemical production from seawater cultivated microalgae have also evidenced the negative impact of salt content on fermentation and downstream processes [31]. Therefore, pre-treatment washing of the biomass is vital when it is used for the production of nutraceutical and other high value products [32]. Conventionally, seawater cultivated algal biomass is washed with deionized water to remove salts from cultivation. Though this method can be somewhat effective under laboratory conditions it can be prohibitively expensive when extended to commercial operations [33]. This study thus evaluated twelve different salt removal treatments and appraised its impact on the lutein, carotenoid and chlorophyll content. The study also focussed on the effect of salt removal treatment on fatty acid profile and degree of saturation.

2. Materials and methods

2.1. Microalgal species

The marine microalgae *Chlorella vulgaris* (Chlorophyceae) used in this study is from the marine microalgal culture collection of National Institute of Ocean Technology, Chennai, India (Strain Code –NIOT-74, NCBI accession number: KM403398). The microalgal strain was maintained initially under laboratory conditions in 3.0 l Haufkin flasks containing sterile f/2 medium [34]. Scale up culture was performed indoor in 300 l Bubble column photobioreactors with custom designed medium 1 (urea-0.1 g/L; NaH_2PO_4-0.01 g/l and FeEDTA-5 mg/l). The indoor cultures were maintained at 24–26 °C. On day 10, when the biomass concentration reached 0.5 to 0.75 g/l the cultures were transferred to paddle wheel operated outdoor raceways in a three step process: **Step1.** *C. vulgaris* culture grown in **custom designed medium 1**(urea-0.1 g/L; NaH_2PO_4-0.01 g/l and FeEDTA-5 mg/L) for 6 days in inoculation pond 1 of 2.77 ton capacity; **Step2.** *C. Vulgaris* from inoculums pond 1 transferred to inoculum pond 2 (8.32 ton capacity) and maintained in **culture medium 2** (urea-0.5 g/L; NaH_2PO_4-0.05 g/l and FeEDTA-10 mg/L for 6 days; **Step 3: t**he culture was transferred to grow out raceway pond of 25 ton capacity (Fig. 1) and grown for 20 days in **custom designed medium 3** (urea-1.0 g/l; NaH_2PO_4-0.1 g/l and FeEDTA-20 mg/l) until the culture reached a biomass concentration of 2.0 g/l. The rotation speed of the paddle wheel aerators were maintained at 15 rpm for continuous mixing circulation of the culture medium. The culture temperature in the raceway ranged from 29.1 to 30.3 °C during the culture operation. The salinity and pH of the raceway culture ranged from 33.9 to 35.0 and 7.60 to 8.09 respectively.

2.2. Experimental set up

2.2.1. Biomass concentration and treatment

The biomass was concentrated from 40 l of *Chlorella vulgaris* (NIOT-74) cultured in 25 ton open raceway. The biomass obtained was equally distributed among twelve different treatments (Table 1). Each treatment was done in triplicate. The biomass for each treatment was placed in a 50 ml Falcon tubes and washed with 20 ml of the respective buffer to eliminate the inorganic salts.

2.3. Extraction and quantification of pigments

2.3.1. HPLC analysis of lutein

Lutein content was determined using reverse phase High Performance Liquid chromatography (RP-HPLC-Shimadzu-LC2010 with UV detector, Japan) following the method of Leema et al., [35]. For sample preparation, a pre-weighed amount of lyophilized (-52 °C, Virtis, USA) *C. vulgaris* biomass (20 mg) was suitably disrupted and saponified at 40 °C for 30 min by the addition 2 ml of 10 M KOH with 2.5 % ascorbic acid. The reaction was stopped by cooling in ice and lutein was extracted by the addition of 18 ml of methanol:

Fig. 1. Outdoor mass culture of marine *Chlorella vulgaris* (NIOT-74) in 25 ton raceway ponds.

Table 1
Chlorella vulgaris biomass subjected to different salt removal treatments.

Salt neutralization experiment	
T1	0.5 M Ammonium bicarbonate
T2	6 N HCl
T3	3 N HCl
T4	2% HCl
T5	0.5 % HCl
T6	Distilled water
T7	0.5 M Ammonium bicarbonate + 6 N HCl + Distilled water
T8	0.5 M Ammonium bicarbonate + Distilled water
T9	6 N HCl + Distilled water
T10	2% HCl + Distilled water
T11	0.5 % HCl + 0.5 M Ammonium bicarbonate
T12	No wash

dichloromethane (3:1 v/v). The mixture was centrifuged at 2500 rpm for 15 min for removing the supernatant, rotary evaporated (Buchi, Switzerland) and reconstituted with 2 ml of HPLC mobile phase. The samples from different treatments and standard lutein (Sigma, USA) were filtered through a 0.22 μM syringe filter (acrodisc) prior to injection. The lutein content from different treatments were analyzed using reverse phase C-18 column (Phenomenex, Luna, USA, 4.6 × 25 × 250 mm, 5 μm particle size) with isocratic solvent system methanol: dichloromethane: acetonitrile:water (67.5:22.5:9.5:0.5, v/v) at a flow rate of 1 mL/min at 450 nm. The calibration curve for standard lutein (Sigma, USA) was prepared for identification and quantification of lutein present in the extract.

2.3.2. HPLC analysis of chlorophylls and carotenoids

The method for chlorophyll and carotenoid analysis were determined using RP-HPLC (Shimadzu LC2010, Japan) according to method of Wright et al., [36]. For chlorophyll sample preparation, a pre-weighed quantity of lyophilized (−52 °C, Virtis, USA) *C. vulgaris* biomass (10 mg) was suitably disrupted and extracted with 5 ml of cold acetone (90 %) overnight at 4 °C. For carotenoid sample preparation, pre-weighed quantity of lyophilized (−52 °C, Virtis, USA) *C. vulgaris* biomass (20 mg) was suitably disrupted and extracted with a solvent mixture consisting ethanol:n-hexane: water at a ratio of 1:6:2. The extracts for chlorophyll and carotenoid content determination were vortexed, centrifuged at 3000 rpm for 10 min and the supernatant was rotary evaporated (Buchi, Switzerland) and reconstituted in 2 mL HPLC grade methanol and stored at −20 °C until quantification using HPLC. The samples from different treatments and standards were filtered through a 0.22 μM syringe filter (acrodisc) prior to injection. The carotenoid content and chlorophyll content from different treatments were analyzed using a tenary gradient solvent systemat 450 nm for carotenoids and 436 nm for chlorophyll using a Phenomenex, USA Luna C18 reversed-phase column (4.6 mm × 250 mm, 5 μm particle size) fitted with a guard column (C18, 3.0 mm × 4.0 mm). The flow rate was maintained at 1 mL/min. The solvent systems were as follows:

Solvent A: 80:20 Methanol: 0.5 M ammonium acetate (aqueous pH 7.2 v/v)

Solvent B: 90:10 acetonitrile: water (v/v)

Solvent C: Ethyl acetate.

The gradient system adopted is as depicted in Table 2. The calibration curves for standard carotenoids (Sigma, USA), including lutein, zeaxanthin, neoxanthin, astaxanthin, canthaxanthin, cryptoxanthin, alpha caroteneand beta carotene were prepared for identification and quantification of each carotenoid present in the extracts. The calibration curves for standard chlorophylls (a,b,c, DHI water quality Institute, Horsholm, Denmark) were also prepared for identification and quantification of chlorophylls present in the extracts.

Table 2
Time series of HPLC program for carotenoid and chlorophyll determination.

Time (min)	Flow rate (ml min^{-1})	% A	% B	% C	Condition
0	1.0	100	0	0	Injection
4	1.0	0	100	0	Linear gradient
18	1.0	0	20	80	Linear gradient
21	1.0	0	100	0	Linear gradient
24	1.0	100	0	0	Linear gradient
29	1.0	100	0	0	Equilibration

2.4. Fatty acid profile

Fattyacid methyl esters (FAMEs) from all treatments were prepared by direct transesterification method of Lepage and Roy [37] and suitably modified by Chiu et al., [38]. Lyophilized cells (100 mg) from different treatments were incubated with a solvent mixture (methanol: acetyl chloride, solvent: sample ratio 20:1, v/w at 80 °C) for 45 min for the production of FAMEs which were then extracted with hexane. The FAMEs were characterized using Gas chromatography Mass spectrometry (Agilent, GC 7980, USA) equipped with split/splitless injector coupled with a mass detector. Both systems were controlled by MSD ChemStation, version E.02.02.1431 (Agilent Technologies, Inc.USA). GC capillary column (Agilent, HP-5 MS (30 m, 0.25 mm i.d., 0.25 μm film thickness) was used to separate the FAMEs. Samples were filtered with 25 mm dia (0.22 μm PTFE syringe filters (Pal Gelman, Germany) prior to injection into GC–MS. Helium (99.999 % purity) was used as carrier gas at a flow rate of 1 mL/min. Initial column temperature was set at 80 °C for 5 min, progressively raised to 260 °C at 2 °C/min and held for 20 min. The injector and detector were kept at 260 °C. An injection volume of 1 μl at a split ratio of 50:1 was injected. Data were collected in full scan mode from 50 to 1000*m/z* .Fatty acid profiles of *C. vulgaris* (NIOT-74) biomass subjected to different treatments were construed by comparison with a 37 FAME mix standard (37 FAME mix, C4-C24; Supelco, USA) and heptadecanoic acid (Sigma) as internal standard. Fatty acids were identified by comparing their retention time and area with fatty acid methyl standards and quantified using standardized area method. The fatty acid contents were expressed as weight percentages, % w/w (mg fatty acids/100 mg of sample).

2.5. Scanning Electron microscope

The physical and structural changes in the integrity of freeze dried *C. Vulgaris* cells subjected to different salt removal treatments were visualized by scanning electron microscope (SEM). To improve the electronic conductivity during imaging the cells from different treatments were placed on the conductive double layer carbon tapes mounted on aluminium stubs and gold sputter coated (Jeol Smart coat model P/N781186455, JEOL, Japan) by plasma under vacuum for a thickness of 200A. The samples were retained in silica gel filled desiccators until SEM imaging. Imaging of the specimens was performed by SEM (Model, JEOL-JSM IT 500, JEOL, Japan) equipped with secondary detector on an accelerating voltage of 0.3 −30 kV.

2.6. Statistical analysis

All experiments were performed in triplicates. Data were presented as mean ± SD. Analysis of variance (ANOVA) was performed for the comparison of the results under different treatments conditions. The significant means were subjected to

post hoc analysis using Tukey(HSD) test using the statistical program SPSS version 22.0 (IBM Co., Armonk, NY, USA). The confidence limit was set at $P < 0.05$.

3. Results and discussion

3.1. Effect of different salt removal treatments on lutein content

Lutein content from microalgal biomass is dependent on the pretreatment process employed [39]. Hence, the impact of different salt removal treatments on the lutein content was evaluated (Figs. 2 and 3). Interestingly, the *C. vulgaris* biomass subjected to 0.5 % HCl (T5) treatment exhibited the highest lutein content (11.56 ± 0.31 mg/g; Fig. 3). This lutein content was 1.82 fold higher than the control without any wash (T12; 6.35 ± 0.44 mg/g). Nevertheless, when the acid concentration was increased to 1 % (T10) the lutein content declined to 9.75 ± 0.75 mg/g and further increase to 2 % HCl (T4) to 6 N HCl (T2) resulted in a steep decline in lutein content from 5.92 ± 0.19 mg/g to 0.35 mg/g. When the biomass was washed with 0.5 M ammonium bicarbonate (T1), the lutein content was 8.33 ± 0.54 mg/g however, when the concentration of ammonium bicarbonate was reduced by the addition of distilled water (T8) the lutein content reduced to 6.69 ± 0.48 mg/g. Similarly, combination of 0.5 M ammonium bicarbonate with 0.5 %HCl (T11) showed a lutein content of 6.91 ± 0.40 mg/g. Notably, the combination treatment of 0.5 M ammonium bicarbonate+ 6 N HCl + distilled water (T7) resulted in very low lutein content (2.52 mg/g).

Very few studies have previously compared different salt removal treatments on lutein extraction yield for seawater cultured microalgae. The algal biomass subjected to 0.5 % HCl had higher lutein content when compared to other types of washing used. The better lutein yield obtained by mild acid treatment could be substantiated by the multifaceted action imparted by mild acid in the form of salt removal, cell wall disruption and better solvent biomass interaction. Conversely, when the biomass is subjected to lutein extraction without any wash, the lutein content reduced considerably due to poor solvent biomass interaction and presence of salt which further reiterates the significance of salt removal treatments for lutein extraction [40]. Nonetheless, when the acid concentration was increased from 2% to 6 N the lutein content declined drastically to 0.35 mg/g. This lower lutein content noticed at high acid concentrations could be attributed to the degradation effect brought forth by strong acids as was evidenced for another xanthophyll carotenoid astaxanthin by Singh et al., [41]. Furthermore, the biomass treated with 0.5 M ammonium bicarbonate gave a moderately high lutein content (8.33 ± 0.54 mg/g). The better lutein content obtained with 0.5 M ammonium bicarbonate might have been due to the neutralizing effect imparted by ammonium bicarbonate on carotenoid extractions as well documented by earlier research [42]. The present

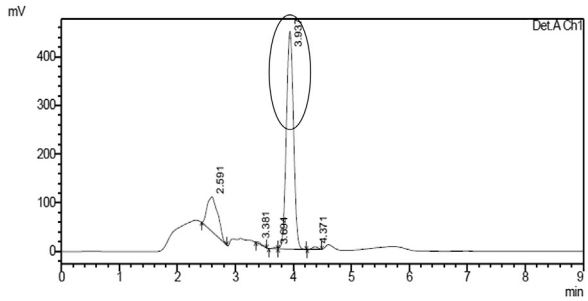

Fig. 2. HPLC chromatogram of lutein extracted from *Chlorella vulgaris* (NIOT-74) cultured in seawater.

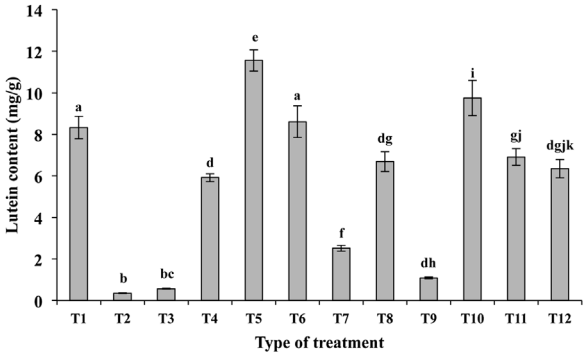

Fig. 3. The lutein content of *Chlorella vulgaris* (NIOT-74) subjected to different salt removal treatments: (T1). 0.5 M Ammonium bicarbonate; (T2). 6 N HCl; (T3). 3 N HCl; (T4). 2% HCl; (T5). 0.5 % HCl; (T6). Distilled water (DW); (T7). 0.5 M Ammonium bicarbonate + 6 N HCl + DW; (T8). 0.5 M Ammonium bicarbonate + DW; (T9). 6 N HCl + DW; (T10). 2% HCl + DW; (T11). 0.5 % HCl + DW; (T12). No wash. The values represent mean ± S.D. Different alphabets indicate significant differences between treatments ($p < 0.05$).

study demonstrated that 0.5 % HCl can be advocated as pretreatment step for extracting lutein from seawater cultured microalgae. In comparison to marigold flowers, marine microalgae cultivation is not limited to seasons and demands lesser land and labour requirement hence, several species of the green microalgae like *Chlorella* have emerged as alternate source of lutein production as they offer several advantages like having higher lutein content in free form and higher biomass productivity [19]. Despite these prospects, commercial lutein production from microalgae has not taken in a big way thus far due to high downstream processing cost. The higher lutein yield (1.82 fold higher) obtained after suitable wash with 0.5 % HCl might thus improve the prospects of lutein production from microalgae.

3.2. Effect of different salt removal treatments on carotenoid content

The HPLC analysis of carotenoids extracted from lyophilized *C. vulgaris* (NIOT-74) subjected to different treatments showed eight conspicuous peaks comprising lutein, zeaxanthin, neoxanthin, canthaxanthin, cryptoxanthin, violaxanthin, alpha carotene and beta carotene (Fig. 4). Xanthophyll carotenoid lutein is the prime carotenoid reported in chlorophycean microalgae accordingly, lutein contributed the highest percentage to the total carotenoid content. As indicated by Borges et al., [30], in seawater cultivation of microalgae when the biomass is processed without proper washing the salt content from cultivation adds to the algal biomass and inflates the actual biomass weight. Correspondingly, lack of estimation or removal of salt content in the algal biomass might lead to underestimation of the product yield denoted in mg/g.

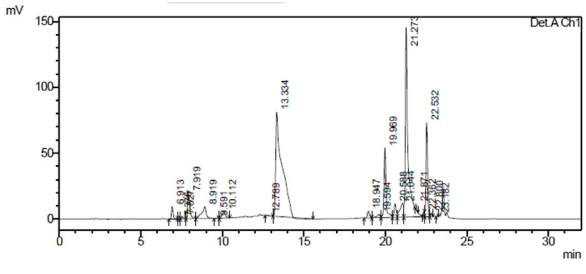

Fig. 4. HPLC chromatogram of carotenoids extracted from *Chlorella vulgaris* (NIOT-74) cultured in seawater (**peak 1**-Neoxanthin, RT-6.9 min; **peak 2**-Canthaxanthin, RT-7.91; **peak 3-**Violaxanthin, RT-8.91; **peak 4**-zeaxanthin, RT-12.78; **peak 5**-lutein, RT-13.33; **peak 6-**cryptoxanthin, RT-18.94; **peak 7**-alpha carotene, RT-19.96; **peak 8**-Chlorophyll a, RT-21.27**; peak 9-**Chlorophyll-b, RT-22.53; **peak 10-**Betacarotene, RT-22.53).

Eventually, the salts form a barrier preventing penetration of solvent into biomass leading to less efficient carotenoid extraction [42]. Hence, the study focused on the impact of different salt removal treatments on the carotenoid content. Fig. 5 shows the carotenoid content of *C. vulgaris* (NIOT-74) subjected to different treatments. As visualized in Fig. 5, the different treatments had a significant ($P < 0.05$) difference in the carotenoid content. Of the 12 different treatments T5, the algal biomass washed with 0.5 % HCl recorded the highest carotenoid content (60.88 ± 4.31mg/g). The carotenoid content observed in treatment T5 with 0.5 % HCl was 1.86 fold higher than that observed in untreated control (32.61 ± 3.01 mg/g). Nevertheless, further increase in HCl concentration to 2% (T4) resulted in 24.1 % reduction in carotenoid content. Still higher concentrations of acid 3N (T3) and 6N (T2) lead to carotenoid loss of 95.79 % and 97.48 %, respectively. Similarly, very low carotenoid content (1.35 ± 0.13 mg/g) was observed in combination treatment 6N HCl + distilled water (T9). Another treatment which gave a comparatively good carotenoid content (58.14 ± 4.26 mg/g) was with 0.5M ammonium bicarbonate (T1). Interestingly, dilution of 0.5M ammonium bicarbonate with distilled water (T8) reduced the carotenoid content to 56.17 ± 3.51 mg/g. Though, treatments like 0.5 % HCl and 0.5M ammonium bicarbonate wash gave a good carotenoid yield individually, the combination treatment (T11, 0.5 % HCl + 0.5M ammonium bicarbonate) gave a very low carotenoid yield 19.66 ± 0.90 mg/g. Notably, the combination treatment with 0.5M ammonium bicarbonate + 6N HCl + distilled water (T7) also showed a relatively low yield (6.21±0.32 mg/g). Washing the algal biomass with distilled water (T6) which is normally practiced for microalgae cultured in seawater gave a carotenoid content, 30.08 % lower than the carotenoid content observed for 0.5 % HCl wash (Fig. 5).

The presence of salt in seawater cultured *C. vulgaris* (NIOT-74) biomass can interfere with the carotenoid yield as was noticed in the lower carotenoid yield from biomass without any wash. Conversely, the biomass subjected to 0.5 % HCl treatment showed the maximum carotenoid yield substantiating the efficiency of dilute acids to remove the salts adhered to cell wall and facilitate optimal solvent penetration [39]. Similar to the present study, Singh et al. [41], have also demonstrated the efficiency of dilute (0.5 %) HCl treatment for enhanced carotenoid yield from *Thraustochytrium* sp. [41]. Nevertheless, higher acid concentration (6 N) resulted in lower carotenoid yield, this might have been due to degradation of carotenoids reported at higher acid concentration [43]. Use of 0.5 M ammonium bicarbonate exhibited a carotenoid yield 1.78 fold better than the control without any wash treatment. The better carotenoid yield brought forth by ammonium bicarbonate must have been due to the neutralizing effect imparted by ammonium bicarbonate [42]. Treatment with distilled water gave a carotenoid yield 1.76 fold better than control, but it was significantly ($P < 0.05$) lower than the treatments with ammonium bicarbonate and 0.5 % HCl indicating the inefficiency of distilled water to completely remove the salts from biomass [30]. These findings further corroborates the importance of appropriate salt removal treatment like 0.5 % HCl for carotenoid extraction from seawater cultured marine microalgae.

3.3. Effect of different salt removal treatments on chlorophyll yield

Chlorophyll is yet another valuable bioactive compound that can be extracted from microalgal biomass. Hence, the study also probed the salt removal treatment method suited for using the biomass for chlorophyll production. Highest chlorophyll content was noticed in distilled water (T6) wash treatment (30.64 ± 2.16 mg/g; Fig. 6). Treatment with 0.5 M ammonium bicarbonate gave chlorophyll content (19.67 ± 0.79 mg/g) lower than distilled water wash however, it did not differ significantly from the control without any wash (T12). Treatment with HCl (0.5 %–6 N, T2 to T5) reduced the chlorophyll content from 10.83 to 0.54 mg/g). Correspondingly, the combination treatments 2% + distilled water (T10, 16.71 ± 1.14 mg/g) and 6 N + distilled water (T9, 5.25 ± 0.28 mg/g) also resulted in lower yield than control. The combination treatments with ammonium bicarbonate + distilled water +6 N HCl gave the lowest chlorophyll content (T7, 0.32 ± 0.014 mg/g). Likewise, the combination of 0.5 M ammonium bicarbonate + distilled water (T8; 8.49 ± 0.76 mg/g) also gave a lower chlorophyll content than control. Interestingly, the combination treatment 0.5 M ammonium bicarbonate + 0.5 % HCl gave a chlorophyll content slightly higher than control (T11, 20.81 ± 1.18 mg/g).

Chlorophyll, like other algal pigments has a potential market value in the biotechnology industry [44]. For effective extraction of chlorophyll, the organic solvents should penetrate effectively through the cell membrane and dissolve the lipids and lipoproteins bound to it [45]. Hence, the study also focused on the ability of different salt removal treatments on the chlorophyll content. Highest chlorophyll content was obtained with distilled water wash. This might have been due to the highly reactive nature of chlorophyll. Furthermore, chlorophyll is reported to be susceptable to degradation when exposed to excess light, temperature, acid and bases [45]. Evidently, in the presence of weak acids chlorophyll-a gets converted to phaeophytin by the replacement of magnesium ion with two hydrogen atoms [46]. This elucidates the lower chlorophyll content witnessed in the biomass treated with 0.5 % HCl. Additionally, being a first order reaction this conversion to phaeophytin has been reported to increase with the

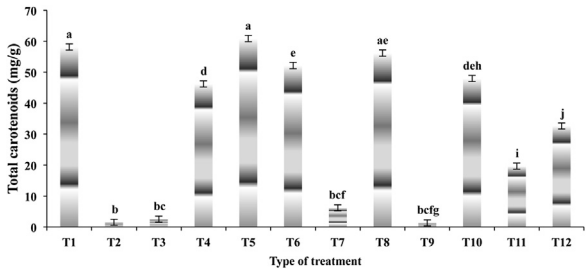

Fig. 5. Total carotenoid content of *Chlorella vulgaris* (NIOT-74) subjected to different salt removal treatments: (T1). 0.5 M Ammonium bicarbonate; (T2). 6 N HCl; (T3). 3 N HCl; (T4). 2% HCl ; (T5). 0.5 % HCl; (T6). Distilled water (DW); (T7). 0.5 M Ammonium bicarbonate + 6 N HCl + DW; (T8). 0.5 M Ammonium bicarbonate + DW; (T9). 6 N HCl + DW; (T10). 2% HCl + DW; (T11). 0.5 % HCl + DW; (T12). No wash. The values represent mean ± S.D. Different alphabets indicate significant differences between treatments ($p < 0.05$).

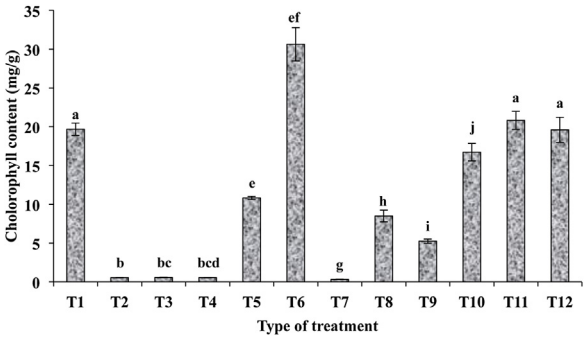

Fig. 6. Chlorophyll content of *Chlorella vulgaris* (NIOT-74) subjected to different salt removal treatments: (T1). 0.5 M Ammonium bicarbonate; (T2). 6 N HCl; (T3). 3 N HCl; (T4). 2% HCl; (T5). 0.5 % HCl; (T6). Distilled water (DW); (T7). 0.5 M Ammonium bicarbonate + 6 N HCl + DW; (T8). 0.5 M Ammonium bicarbonate + DW; (T9). 6 N HCl + DW; (T10). 2% HCl + DW; (T11). 0.5 % HCl + DW; (T12). No wash. The values represent mean ± standard deviation. Different alphabets indicate significant differences between treatments ($p < 0.05$).

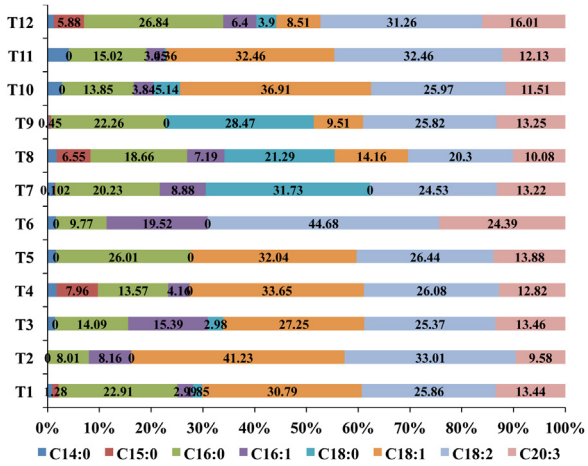

Fig. 7. Fatty acid profile of *Chlorella vulgaris* (NIOT-74) subjected to different salt removal treatments: (T1). 0.5 M Ammonium bicarbonate; (T2). 6 N HCl; (T3). 3 N HCl; (T4). 2% HCl ; (T5). 0.5 % HCl; (T6). Distilled water (DW); (T7). 0.5 M Ammonium bicarbonate + 6 N HCl + DW; (T8). 0.5 M Ammonium bicarbonate + DW; (T9). 6 N HCl + DW; (T10). 2% HCl + DW; (T11). 0.5 % HCl + DW; (T12). No wash. The values are average of triplicates.

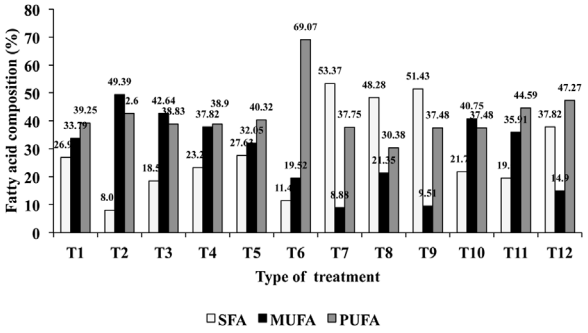

Fig. 8. Fatty acid composition of *Chlorella vulgaris* (NIOT-74) subjected to different salt removal treatments: (T1). 0.5 M Ammonium bicarbonate; (T2). 6 N HCl; (T3). 3 N HCl; (T4). 2% HCl ; (T5). 0.5 % HCl; (T6). Distilled water (DW); (T7). 0.5 M Ammonium bicarbonate + 6 N HCl + DW; (T8). 0.5 M Ammonium bicarbonate + DW; (T9). 6 N HCl + DW; (T10). 2% HCl + DW; (T11). 0.5 % HCl + DW; (T12). No wash. The values are average of triplicates.

concentration of the HCl [47]. Corroborating with the above reports, the chlorophyll content decreased with the increase in acid concentration from 0.5% to 6 N. Hager and Shansky [43] have also documented the conversion of chlorophyll-a to phaeophytin-a at 0.1 M HCl and its subsequent conversion and severe degradation to divalent cations of phaeophytin at 4 N HCl. This accounts for the severe lowering of chlorophyll content noticed at 3 M and 6 M acid treatments. Chlorophyll, like other algal pigments fetches a considerable market share in pharmaceutical products especially, in the treatment of ulcers, oral sepsis and proctology [24]. The study presented herein has established that algal biomass aimed at the production of chlorophyll should be washed with distilled water rather than with weak acids (0.5 % HCl) for achieving maximum chlorophyll yield.

3.4. Effect of salt removal treatments on FAME components

Microalgal fatty acids are now considered as crucial raw materials for several algal based industries [48]. Fig. 7 shows the fatty acid profile obtained from twelve different salt removal treatments. The fatty acids which were predominant in the untreated (T12) *C. vulgaris* biomass were palmitic acid (C16:0; 26.84 %), palmitoleic acid (C16:1; 6.40 %) stearic acid (C18:0; 3.90 %), oleic acid (C18:1;8.51 %), linoleic acid (C18:2; 31.25 %) and eicosatrienoic acid (C20:3; 16.02 %). The saturated fatty acids (SFA) contributed 37.82 %, monounsaturated fatty acids (MUFA) accounted 14.90 %, and polyunsaturated fatty acids (PUFA) comprised 47.27 % (Fig. 8). Significant differences in the relative abundance of SFA, MUFA and PUFA was noticed between the different treatments. Treatment with 6 N HCl wash had the least percentage of SFA (8.1 %) and higher percentage of MUFA: 49.39 % and PUFA: 42.60 %. With the decrease in HCl concentration from 6 N to 0.5 % (T2 to T5) the SFA increased from 8.1 to 27.63%; while MUFA percentage decreased from 49.39 to 32.05 %. Interestingly, the PUFA percentage did not differ significantly with the concentration of HCl used for washing the biomass. The biomass subjected to distilled water wash (T7) displayed a very low SFA: (11.41 %) and MUFA (19.52 %). The PUFA percentage (69.07 %) was extremely high for this treatment. Different combination treatments for salt removal were also scrutinized for their effect on the fatty acid profile. The combination of 6 NHCl + distilled water (T9) enhanced SFA percentage to 56.34 %, but MUFA was significantly lowered to 9.51 % and PUFA did not alter significantly and remained at 39.06 %. The combination treatment, 2% HCl + distilled water (T10) gave a lower SFA of 21.77 %, whereas MUFA increased to 40.75 % and the PUFA percentage (37.48 %) did not alter significantly. Notably, the combination treatment, 0.5 % HCl + distilled water (T11) exhibited SFA of 19.50 %, MUFA of 35.91 % and PUFA of 44.59 %. Apart from these treatments, the biomass were also tested with ammonium bicarbonate (T1) wash to evaluate the influence of its neutralizing effect on the fatty acid abundance and degree of saturation. Ammonium bicarbonate wash gave SFA about 26.92 %, MUFA of 33.79 % and PUFA of 39.29 %. Notably, the combination treatment of ammonium bicarbonate with distilled water (T8) improved the SFA significantly to 48.28 % and lowered the MUFA (21.35 %) and PUFA (30.38 %). The combination of 6 NHCl + ammonium bicarbonate + distilled water gave SFA of 53.36 % and a very low MUFA of 8.88 %, while the PUFA percentage (37.75 %) did not differ significantly.

As reported in earlier reports, *C. vulgaris* (NIOT-74) grown in open raceway ponds in natural sweater had predominantly short chain fatty acids (SCFAs) between 16–18 carbon lengths, which are recognized as suitable for biodiesel production [49,50]. Fatty acid profile, especially the characteristics of FAME like carbon chain, length and number of double bonds directly influence the biodiesel fuel properties of viscosity, ignition quality, oxidative stability and cold flow property [51]. Higher proportion of saturated fatty acids (SFAs) is reported to provide better oxidative stability and storage property. Conversely, the higher SFA percentage has been reported to affect the cold flow property. In the present study, the type of salt removal treatments had a very significant impact on the SFAs and MUFAs. The highest concentration of acid (6 N HCl) reduced the saturated fatty acids and with the decrease in acid concentration from 6 N to 0.5 % HCl resulted in increase of SFAs from 8.1 to 27.63%. In conformity with the present study Rosli et al. [50], have reported the SFA of *C. vulgaris* grown in nutrient rich waste water to be 26.1 %. In the same way, distilled water treatment also reduced the SFAs to 11.41 %. This might have been due to the rupture of cells and degradation of fatty acids [30]. On the contrary, higher percentage of saturated fatty acid is not favoured for fuel properties as it would result in poor cold flow properties leading to crystallization and clogging of fuel filters at high temperature [52]. Therefore, combination treatment (T9) which yielded SFA of 56.34 % might not be ideal for biodiesel production. A considerably (35 %) higher MUFA content is recommended for obtaining better oxidative stability, cold flow properties in biodiesel production from microalgae [53]. Corroborating to this, MUFA recorded in the

0.5 % HCl wash and 0.5 M ammonium bicarbonate wash ranged from 32.16 % to 33.79 %. In concurrence Rosli et al. [50], have also reported a MUFA content of 35.4 % in *C. vulgaris* cultivation in nutrient rich waste water. The proportion of C16:0, which is a reserve lipid (triglyceride), [30] was low in treatments with very high acid concentration (6 N HCl) and distilled water. From the results, it is evident that the presence of salts in the algal biomass could modify the fatty acid profile and percentage of saturated and unsaturated fatty acids too. Washing the algal biomass with 0.5 M ammonium bicarbonate or 0.5 % HCl resulted in fatty acid profile ideal for biodiesel production. The study also highlighted the fact that the choice of wash treatments is highy dependant on the destined use of microalgal biomass.

3.5. Characterization of treated algal biomass using scanning electron microscope

The morpologial and structural changes that accompanied different salt removal treatments were visualized with the aid of scanning electron microscope (SEM). The SEM images of *C vulgaris* biomass not subjected to any salt removal treatments revealed the presence of salts (Fig. 9). The wash with 0.5 % HCl signified the obvious absence of salt and least damage to the integrity of the algal cell wall (Fig. 9). Albeit, increase (2–3 %) in HCl concentration caused structural changes and the highest concentration (6 N) tested resulted in severe damage to cell walls and clumping of the damaged cells. Similarly, combination treatments with a higher

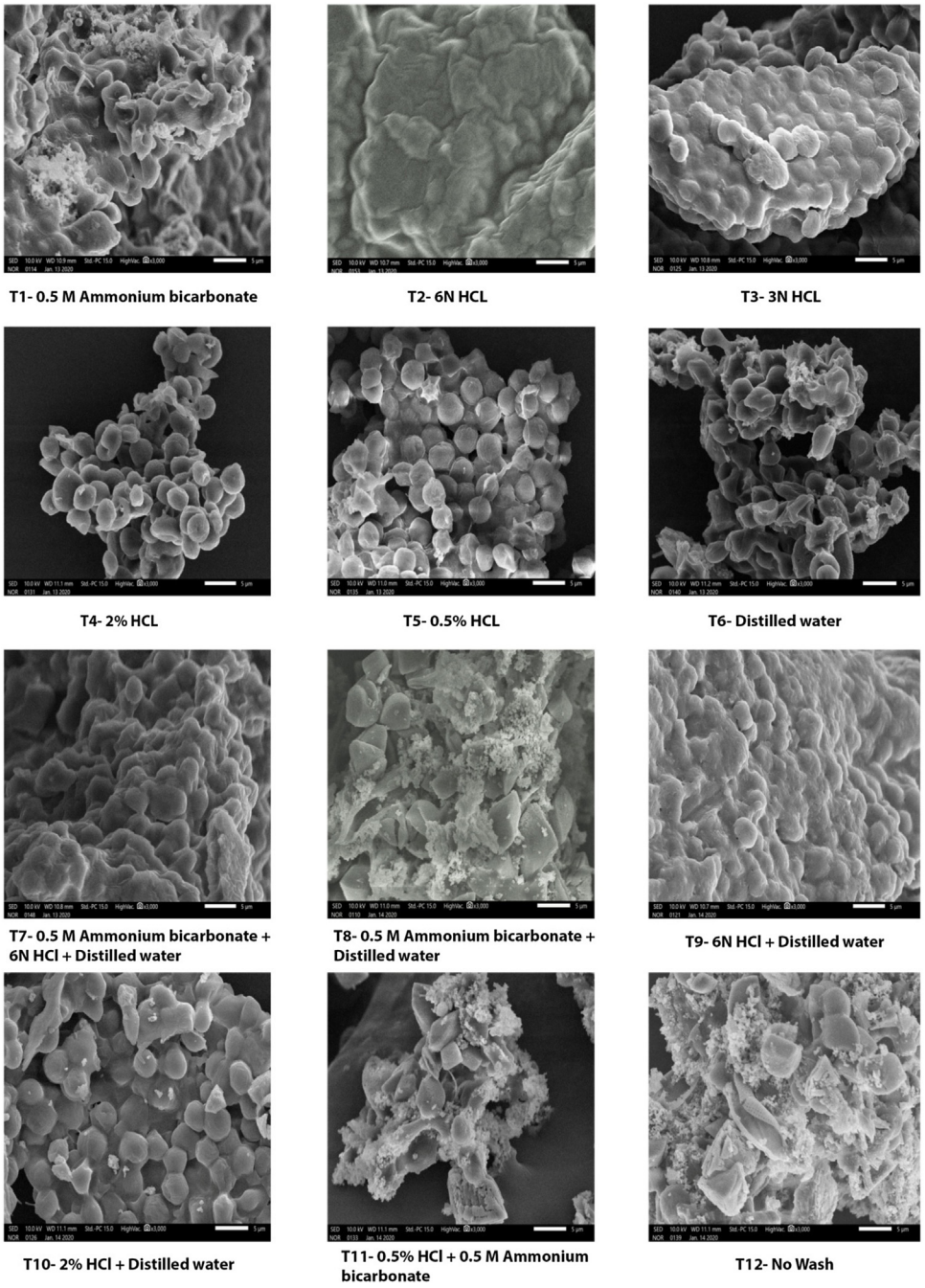

Fig. 9. Scanning electron microscope images showing freeze dried *C. vulgaris* biomass subjected to different salt removal treatments. The scale bar represents 5 μm.

acid concentration exhibited severe structural damage to cell wallwhile, dilution with distilled water showed incomplete removal of salt and severe shrinkage and pitting to the spherical shape indicating (Fig. 9) the inefficiency of distilled water to completely remove the salts from biomass [30]. Earlier research reports have established the *Chlorella c*ell wall to be composed of an outer cell wall formed of algaenan and a thinner microfibrillar wall composed of cellulose and chitin-like glycan amino sugars [54]. Substantial research reports have also evidenced the *Chlorella* cell wall disruption brought forth by acid wash treatments as a phenomenon caused by the interaction of H^+ions of acids with the glycosidic bonds of cell wall components [55]. This might have caused the severe damage to cell wall apparent herein under the increased acid concentrations. In accordance with our findings, Sarip et al. [56], have also reported severe cell wall damage of *Chlorella* cells treated with 6 N HCl. Though the *Chlorella* biomass treated with 0.5 M ammonium bicarbonate showed a relatively better carotenoid and lutein yield the SEM micrographs depicted the presence of salt and shrinkage of cells. Furthermore, the combination treatments of 0.5 M ammonium bicarbonate with 6 N HCl displayed more cellular damage, while dilutions with distilled water evidenced the presence of salt. These findings further substantiates the significance of appropriate salt removal treatment like 0.5 % HCl for the extraction of lutein and other carotenoids from seawater cultured marine microalgae.

4. Conclusion

The results of the study demonstrated the significance of salt removal in seawater cultured microalgal biomass aimed at the production of high value pigments and fatty acids. The presence of salt interfered with the lutein, carotenoid, chlorophyll yield of *C. vulgaris* cultured in open raceway. The presence of salts also modified the fatty acid profile. The study probed into twelve different treatments for salt removal and found that wash with 0.5 % HCl augmented the lutein and carotenoid yield 1.82 and 1.86 fold respectively. The better yield obtained for these high value pigments will thus reduce the production cost and improve the prospects for commercial production of these pigments from seawater cultured *C. vulgaris* as downstream process is one of the important contributor of production cost.

Contribution of authors

J.T, T.P., and G.D., conceived and designed the experiments. J.T., and T.P., carried out all the wash treatment experiments and HPLC quantification of lutein and carotenoids. D.M., contributed to FAME estimation. T.S.K., contributed to mass culture of marine algae in raceway. J.T., contributed to the interpretation of the results and took the lead in writing the manuscript. G.D. contributed significantly to the critical revision of the manuscript. All authors provided critical feedback and helped to shape the research, analysis and manuscript.

Author declaration

We (J. T. Mary Leema, T. Persia Jothy, D.Magesh Peter, T.S.Kumar and G. Dharani) confirm that the manuscript has been read and approved by all named authors and that there are no other persons who does not satisfy the criteria for authorship are listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us and further affirm our agreement for submission of the manuscript for peer review.

Declaration of Competing Interest

No conflicts, informed consent, human or animal rights applicable.

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