Saudi Journal of Biological Sciences 28 (2021) 3981–3988

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Evaluation of high salinity adaptation for lipid bio-accumulation in the green microalga *Chlorella vulgaris*

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ARTICLE INFO

Article history:
Received 15 February 2021
Revised 31 March 2021
Accepted 4 April 2021
Available online 20 April 2021

Keywords:
Chlorella vulgaris
Salinity stress
Vitamin B
Oil content
FAMEs
Fuel proprieties

ABSTRACT

Aiming at the reutilizing wastewater for algal growth and biomass production, a saline water rejected from reverse osmosis (RO) facility (salinity 67.59 g L^{-1}) was used to cultivate the pre-adapted green microalga *Chlorella vulgaris*. The inoculum was prepared by growing cells in modified BG-11 medium, and adaptation was performed by applying a gradual increase in salinity (56.0 g L^{-1} NaCl and 125 ppm $FeSO_4{\cdot}7H_2O$) to the culture in 200 L photobioreactor. Experiments using the adapted alga were performed using original-rejected water (ORW) and treated rejected water (TRW) comparing with the recommended growth medium (BG-11). The initial salinity of ORW was chemically reduced to 39.1 g L^{-1} to obtain TRW. Vertical photobioreactors (15 L) was used for indoor growth experiments. Growth in BG-11 resulted in 1.23 g L^{-1}, while the next adaptation growth reached 2.14 g L^{-1} of dry biomass. The dry weights of re-cultivated *Chlorella* after adaptation were 1.49 and 2.19 g L^{-1} from ORW and TRW; respectively. The cellular oil content was only 12% when cells grown under control conditions verses to 14.3 and 15.42% with original and treated water, respectively. Induction of stress affected the fatty acid methyl esters (FAMEs) profile and the properties of the resulting biodiesel. The present results indicated that induction of stress by high salinity improves the quality of FAMEs that can be used as a promising biodiesel fuel.

1. Introduction

Freshwater ecosystems worldwide have experienced increasing salinity mainly as a result of long-term droughts, overly intensive agriculture, rising seawater levels and inappropriate water management strategies (Canedo-Arguelles et al., 2018). Water with high salinity can adversely affect habitats and livestock, although some-aquatic plants, halophytes and microorganisms can survive under such conditions (Hou et al., 2017). Although, second-generation biofuel feedstock doesn't compete with human food or land, the costly pretreatment is the main disadvantage (Elsayed et al., 2018, 2019). In recent years, microalgae have received increasing attention as a sustainable feedstock for biofuel, that can grow in wastewater or saline water without competing with human resources (Abomohra et al., 2019, 2021; Wang et al., 2020; Touliabah and Almutairi, 2021). Extensive research has been carried out to enhance lipid accumulation in microalgae in order to enhance the economic feasibility of biodiesel production. Such strategies include selection and improvement of microalgal strains (Sheehan et al., 1998), metabolic engineering (Sun et al., 2019), salinity optimization (Abomohra et al., 2020a), plant growth regulators (Esakkimuthu et al., 2020), CO_2 supplementation (Tu et al., 2019), and magnetic field stress (Shao et al., 2018). However, extreme high salinity of water adversely affects microorganisms, mainly microalgae, because it inhibits their growth and production of pigments and oils increases protein decomposition, and alters the accumulation of specific bio-active substrates. Algae can be grouped into halophilic and halotolerant based on their tolerance to salt, and they all produce metabolites both for protection from salt injuries and to equilibrate with the surrounding osmotic pressure (Abomohra et al., 2020b). However, many species of microalgae are tolerant to great variations in salinity, and their chemical composition can be affected (Ding et al., 2013). Organisms that

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.04.007
1319-562X/

can grow under unfavorable conditions, including saline water, are promising sources of chemical compounds valued by humans (Arora et al., 2019). Indeed, although salinity greatly influences macroalgal growth, many efforts have been made to reduce its impact. The physiological response of microalgae varies among species, and some freshwater microalgae can grow well under marginally saline conditions. For example, *Botryococcus* sp. can survive in 3.0 M NaCl and has maximal growth at 0.25 M NaCl (Vazquez-Duhalt and Arredondo-Vega, 1991). In addition, a halophilic *Dunaliella salina* KSA-HS022 was isolated from a hypersaline lagoon with salinity average of 45.3‰ and was able to grow at elevated salinity up to 250‰ (Abomohra et al., 2020b).

High salinity is known to cause both hyper-ionic and hyper-osmotic effects in plants, leading to membrane disorganization, metabolic toxicity and increase of reactive oxygen species (ROS). Thus, DNA mutation, protein denaturation, lipid peroxidation and chlorophyll bleaching as well as the loss of membrane integrity could potentially damage the function of photosystems (Leshem et al., 2007; Almarashi et al., 2020). Salinity stress affects algae and plants through osmotic and ionic stresses. Water deficit can lead to increased osmotic stress due to the increased concentration of Na^+ and Cl^- that subsequently negatively affects the uptake of other mineral nutrients causing ionic imbalances or stress (Ashraf and Harris, 2004). Several plant metabolites were recognized to be used as growth stimulators in which help plant as well algae to overcome adverse effect of biotic and abiotic stress. For instance, nitrogen containing compounds such as amino acids, amides, protein, and quaternary ammonium compounds have been found helpful in osmoregulation (Grumet et al., 1985) and in tolerance of ion toxicity under salt stress (Turhan et al., 2008). Recently, Esakkimuthu et al. (2020) evaluated the application of p-coumaric acid as a suggested novel growth regulator founded predominantly in phenolics-rich streams to enhance the biodiesel yield from microalgae, which showed positive impact on biomass and lipid accumulation to overcome the stress conditions. The way these compounds are accumulated is species-specific and spanning from only one to several different compounds (Teixeira and Pereira, 2007).

The production of osmo-protectants or compatible solutes, such as mannitol and proline, can aid the cell to uptake water from the surrounding environment by lowering the internal cell water potential. The later is synthesized intracellularly from glutamate using ATP and NADPH. The trade-off of the energy-demanding process of maintaining proper osmotic conditions is manifested in the reduced growth rates and decreased photosynthetic electron transport activities (Annan, 2014). Photosynthetic organisms possess a number of various antioxidative enzymes that are involved in the detoxification of ROS and the avoidance of damage under salt stress (Hediye Sekmen et al., 2007). Organisms with high levels of constitutive and induced antioxidants have been reported to have greater resistance to oxidative damage (Shi and Bao, 2007). ROS cause lipid peroxidation and production of highly toxic lipid derivatives, which in turn can modify cell functions and even may lead to cell death (Marnett, 2000; Almarashi et al., 2020).

The use of microalgal cultures as a source of chemical energy products for biofuel feedstocks, including biodiesel (Gomaa, 2018; El-Sheekh et al., 2019; Gomaa et al., 2019; Almutairi et al., 2020), alcohols (El-sayed et al., 2017), methane (Geng et al., 2020), hydrogen (Sharma and Arya, 2017), and crude bio-oil (Wang et al., 2018) was previously evaluated and confirmed as a promising approach. Among different studied microalgal species, *C. vulgaris* cells can accumulate lipid up to 56 %dw when grown under stress conditions (Illman et al., 2000), which are appropriate feedstock for high-quality biodiesel production (Han et al., 2016). Microalgae are renewable eco-friendly feedstocks as they fix CO_2 that might be useful in reducing the level of the greenhouse gases (Darda et al., 2019; Abomohra et al., 2020b). In recent years, the production of fuel from microalgae has received a great attention due to the increasing energy prices, greenhouse gases emissions, and depletion of fossil fuels (Xiong et al., 2008; Damiani et al., 2010). However, evaluation of the biodiesel potential from microalgae grown on wastes is necessary to obtain a cost-effective microalgal biomass for commercial scale production (Chisti, 2008; Abomohra et al., 2016). Hence, the high cost of biodiesel production and the low lipid content of microalgae are the main bottlenecks for commercial applications (Chi et al., 2019). One of most efficient and economical approaches towards reducing the total cost of biodiesel production is by enhancing lipid accumulation in the microalgal cells by growth on waste- or saline-water (Yahya et al., 2018).

Optimization of growth conditions were reported as effective methods to increase microalgal lipids such as nitrogen limitation, high salinity, high light intensity, static magnetic field, ionized radiation, and high temperature (Converti et al., 2009; Small et al., 2012; Almutairi, 2020a, 2020b; Rugnini et al., 2020). However, the simultaneous occurrence of both high lipid productivity and content is difficult to achieve because the biomass productivity of microalgae is low when the concentration of nutrient is low (Xin et al., 2010). Nevertheless, very few microalgal oils are satisfactory for biodiesel production (Guschina and Harwood, 2006). Gopinath *et al.* reported that biodiesel produced from unsaturated fatty acids (FA) emits more nitrogen oxides with thermal efficiency lower than the biodiesel produced from saturated FA (Gopinath et al., 2010). The nature of the FA and the alcohol used for fatty acid esters preparation influences the properties of the biodiesel (Abomohra et al., 2020b). Therefore, evaluation of the properties of microalgal oils and FA is essential when trying to develop a suitable biodiesel fuel.

Growth of *C. salina* was enhanced progressively by increasing salt levels up to 1 M NaCl, where it reached 2-folds of the control value and was approximately comparable to the control at 2 M NaCl. Lipid peroxidation as an oxidative stress parameter showed a marked and progressive increase due to elevation of salinity of *C. vulgaris* medium reaching about 61% over the control at 0.8 M NaCl. In *C. salina*, the amount of malondialdehyde (MDA) content decreased up to 1.0 M NaCl and increased marginally by about 17% at 2 M NaCl. In reverse osmosis technique, rejected water contains all of the initial salt of original water and the environment hazarded concerning dilution and safe discarding not fully functioned. Therefore, the objective of this study was to determine the potential of reutilizing rejected water of reverse osmosis process by the green microalga *Chlorella vulgaris* as a feedstock for biodiesel production.

2. Materials and methods

2.1. Microalga and inoculum preparation

The freshwater microalga C. vulgaris (Chlorophyceae) was provided by the Algal Biotechnology Unit, National Research Centre, Cairo, Egypt. Then cultured to obtain the proper inoculum using modified BG-11 growth medium (Supplementary data) containing 17.6 mM nitrogen (Stanier et al., 1971). All experiments were performed at a nitrogen concentration of 17.6 mM, and urea concentration of 0.53 g L^{-1} was used instead of 1.5 g L^{-1} sodium nitrate (El-Shafey et al., 1999). Aeration was supplied by oil-free compressed air and light was provided from one side light bank (12 lamps × 40 W per each one) and the used light intensity at the center of the column was 120 μ.E. Heterotrophic growth was achieved by addition of 15 mM sodium acetate and 0.25 ml L^{-1} acetic acid. Sub-culturing was performed within a Plexiglass photobioreactor

containing 200 L BG-11 growth medium as described in the previous study (El-Sayed and El-Sheekh, 2018).

2.2. Salinity adaptation

The inoculum grown in 200 L photobioreactor was subjected to hypersaline adaptation. Sodium chloride (2.0 g) and 4.5 ppm $FeSO_4{\cdot}7H_2O$ were daily added to the culture. In addition, nitric acid (0.117 ml L^{-1}) was also daily added, reaching N content to 24.7 ppm which equal 1/10 of the initial nitrogen content of BG-11. Overall, after starting the culture using BG11, culture finally received 56 g L^{-1} of NaCl, 125 ppm $FeSO_4{\cdot}7H_2O$ and 691.6 ppm N during the whole adaptation period (28 days). Furthermore, vitamins including B1 (200 mg L^{-1}); B6 (1.0 mg L^{-1}) and B12 (1.0 g L^{-1}) were added at the first cultivation time of adaptation process.

2.3. Experimental growth media

In addition to the BG-11 medium described in the previous section, two additional growth media were also examined (Table 1). The first medium was the original-rejected water (ORW) from a reverse osmosis (RO) facility and the second medium was treated rejected water (TRW). Both of the examined waters were analyzed as described by Chapman and Pratt (1978). Chemical desalination was performed as described by (El-Sayed, 2004a, 2004b).

Growth experiments were performed in 14 L transparent Plexiglass tubes (2 m long, 11 cm diameter, and 6 mm thickness containing the examined growth medium and pre-adopted microalga. The pre-adopted cells were harvested and washed two times by the proper examined water. A light bank on one side provided the light irradiation at 120 μmol m^{-2} s^{-1}. Growth was allowed to proceed for 15 days from the starting time. Biomass was harvested by allowing cells to settle for 24 h and then centrifugation (4000 rpm for 3 min) and drying at 45 °C in a circulated oven until constant weight.

2.4. Growth measurement

Dry weight (g L^{-1}) was determined by filtration of a defined volume of algal culture through a pre-weighted membrane filter (0.45 μm), followed by drying the filters in an oven at 105 °C for 30 min. Growth rate (g L^{-1} d^{-1}), doubling time (n), and percent of increase (%) were calculated as described by (Pirt, 1975).

2.5. Lipid extraction fatty acid profile

Oil extraction was performed by overnight soaking of dewatered algal samples within cellulose extraction thimbles (33 × 94 mm). The Soxhlet technique (1.0 L flask) was then performed using 750 ml of a non-polar solvent mixture (n-hexane: isopropanol, 3:2, *v:v*). The thimbles were then water washed, dried at 105 °C for 60 min, and re-weighed. Fatty acid methyl esters were obtained based on acid hydrolysis procedures and gas chromatography (GC) system (GC Perkin Elmer Auto System XL) equipped with a flame ionization detector (FID) was used for fatty acid identification and determination of *trans*-methylated FA.

2.6. Fuel properties

The cetane number (CN); saponification value (SV); iodine value (IV); degree of unsaturation (DU); long-chain saturated fatty acid (LCSF) and cold filter plugging point (CFPP) were determined using equations from Mittelbach and Remschmidt (2004).

2.7. Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean ± standard deviation. The experimental results were analyzed by one-way ANOVA using the statistical software STATISTICA 8 at P value < 0.05.

3. Results

3.1. Growth adaptation of Chlorella vulgaris

Growth was measured as a dry weight using different growth media of original BG-11 and those adapting media, which showed different growth patterns (Fig. 1). The highest dry weight (2.22 g L^{-1}) was observed in the adapted culture at the 26th day. On the other hand, the dry weight in BG-11 reached a maximum value of 1.06 g L^{-1} at the 28th day of growth. Growth rate of BG-11 culture possessed a constant increase pattern as compared with the adapted culture, which possessed a negative increase during the initial growth periods then returned to high values. Such observation might be ascribed to the high salinity effect and the algal cells entered a long adaptation time after it received about 32 g L^{-1} of NaCl.

In addition, *Chlorella* biomass production was determined as dry weight and biomass productivity at the end of growth period (Fig. 2), where it showed the superior values in the treated rejected water (TRW) comparing to those grown in BG-11 or original rejected water (ORW). Growth of *Chlorella* in ORW medium led to lower dry weight accumulation (1.49 g L^{-1}). However, adaptation of the cells to high NaCl concentration increased the rate of dry weight accumulation, biomass productivity, and the chlorophyll content. Overall, the dry weight of cultures after vegetative growth was 1.23 g L^{-1} in control (BG-11) medium, 2.22 g L^{-1} in the adaptive medium, 1.49 g L^{-1} in ORW medium, and 2.19 g L^{-1} in TRW medium. On the other hand, the TRW medium was slightly alkaline (Table 1) and had high nitrogen content due to the desalination processes. These conditions enhanced dry weight accumulation (2.19 g L^{-1}). However, both biomass and lipid content are very important parameters for economical production of biodiesel from microalgae. Therefore, lipid content and production were further evaluated as shown in the next section.

3.2. Oil content and FAMEs profile

The oil content also varied according to the culture medium. Oil content of the optimally grown *C. vulgaris* was reduced to be about 12.0%, comparing to 14.3% and 15.4% for those grown in original rejected and treated rejected-waters; respectively (Fig. 3). In particular, the total SFA content was about 20% in control medium,

Table 1
The main chemical components (g L^{-1}) of the different media and wastewater used in the present study.

Medium	pH	TDS	N	P	Na	Cl
BG-11	7.3	1.408	0.2465	0.0297	0.0340	0.0272
ORW	7.5	67.59	0.039	0.042	12.8	37.79
TRW	8.2	39.1	0.205	0.09	9.13	11.05

Original-rejected water (ORW), Treated rejected water (TRW)

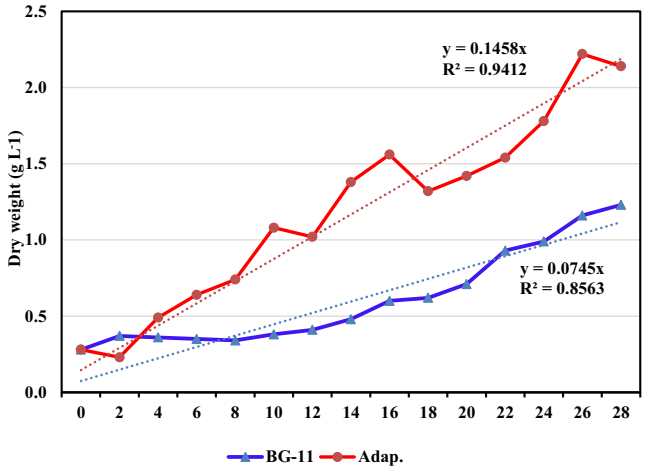

Fig. 1. Dry weight (g L^{-1}) of *Chlorella vulgaris* during adaptation growth.

but exceeded 28% for the other media. Thus, the reduction of nitrogen stress during growth appeared to increase the oil content. Regarding fatty acid profile, the present results showed relatively lower concentrations of saturated fatty acids (SFAs) in C. vulgaris oil, with a maximum of 29.53% lipids in the TRW, when the P level was 50% lower (Table 2). While the lowest SFAs of 19.7% was recorded in the control medium. In addition, the present results showed C16:1, C18:1, and C14:0 ratio of 3:4:1 in the control medium. The present study showed that *Chlorella* cells produced a considerable amount of oleic acid, suggesting its bio-oil suitability for good biodiesel quality (Section 4).

3.3. Biodiesel properties

Biodiesel properties produced by *C. vulgaris* when grown under different conditions were evaluated (Table 3). The DU represents the weight percentage of monounsaturated and polyunsaturated fatty acids. Growth of *C. vulgaris* in ORW and TRW reduced the DU from 146.78 to 78.04 and 80.37, respectively. Results indicated that biodiesel of *Chlorella* grown in control medium has an IV of 100 g I_2/100 g oil, with lower IVs when grown in ORW and TRW (94.7 and 90.2 g I_2/100 g oil, respectively). On the other hand, growth in ORW media increased the SV (194.4) above that of the control medium (182.77021). Interestingly, growth of *C. vulgaris* in TRW showed increase of CN from 53.6 in the control BG-11 medium to 55.8. In addition, CFPP increased from 0.7497 to 1.6513 in BG-11 and TRW, respectively (Table 3).

4. Discussion

The growth of microalgae is widely affected by the composition of the growth medium. The maximum dry weight was observed in the adapted culture by the 11th day. The continuous enhancing effect on algal growth could be attributed to the nutrition strategy

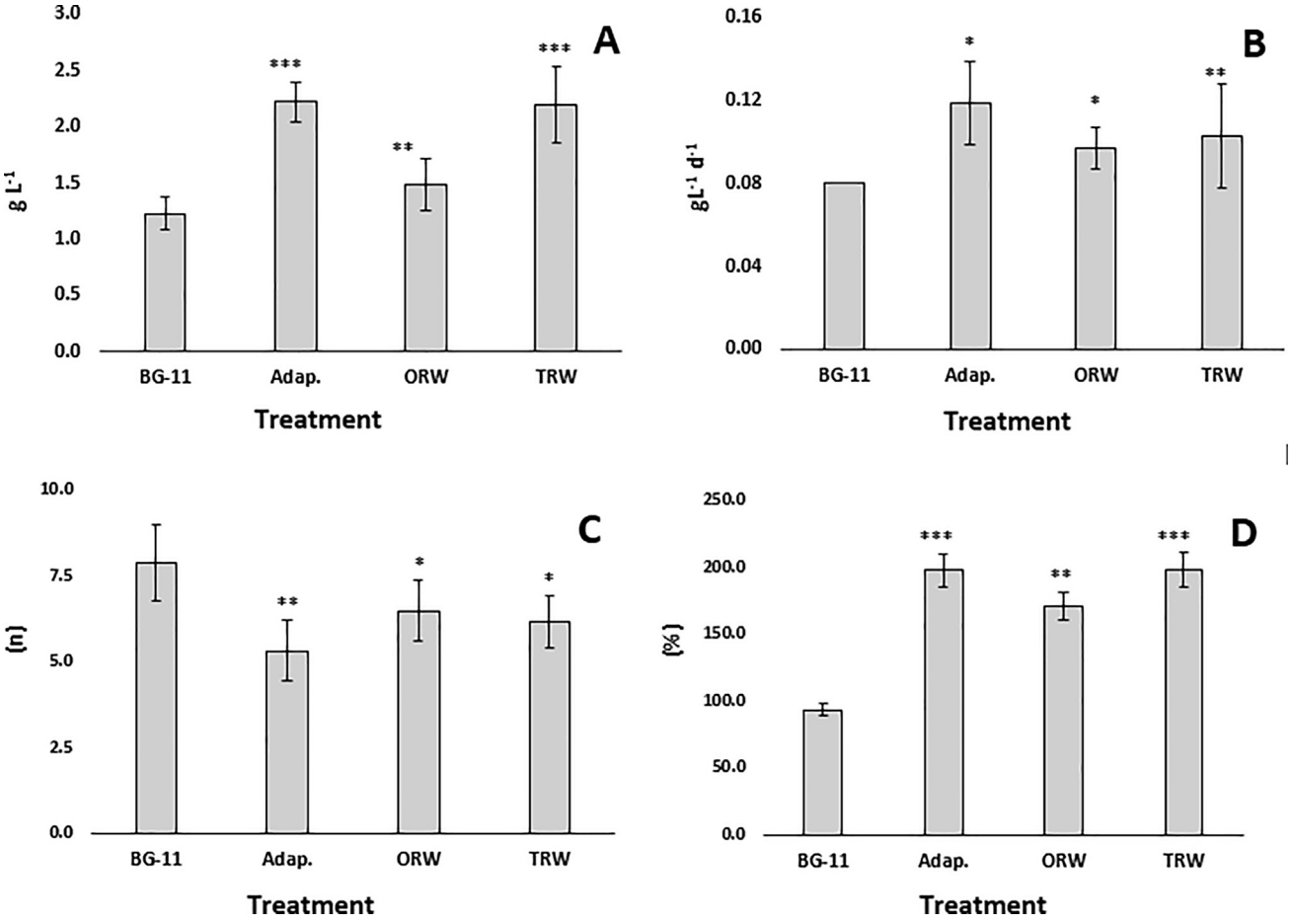

Fig. 2. A) Dry weight, B) growth rate and C) doubling time and D) percentage increase of *C. vulgaris* grown in different water types. The error bars show the standard deviation of three replicates. A single (*), double (**) and triple (***) asterisk indicate respectively significant ($P < 0.05$), highly significant ($P < 0.01$) and very highly significant ($P < 0.001$) differences with the control.

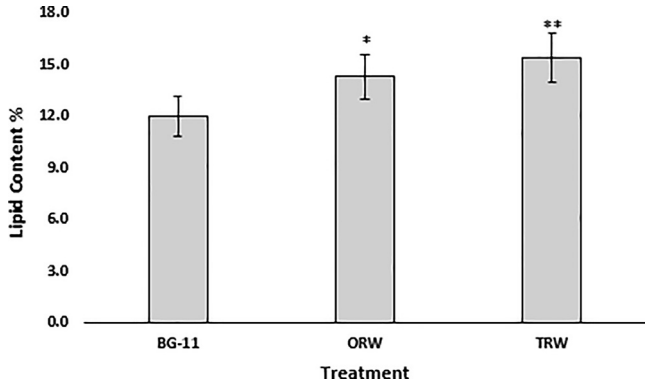

Fig. 3. Lipid content of *C. vulgaris* grown in different water types. The error bars show the standard deviation of three replicates. A single (*) and double (**) asterisk indicate respectively significant ($P < 0.05$) and highly significant ($P < 0.01$) differences with the control.

in concern nitric acid as continuous nitrogen feeding with buffering action and also due to the presence of vitamin mixture. The fast nitrogen utilization might lead to retardation of growth as present in cultures grown under the recommended growth medium (BG-11). In addition, high salinity results in algal cells entered a long adaptation time, which resulted in longer lag phase in the adapted culture. However, implementation of vitamin in algal growth medium might mitigate the adverse effect of hyper-salinity of the medium. It was previously reported that some algal species have lost a gene of the biosynthetic pathway of this cofactor and as a result some *Chlorophyta* and *Cryptophyta* members need thiamine supplemented in the culture whereas other algal species need combinations of three B vitamins (cobalamin, thiamine and biotin) (Croft et al., 2006; Tandon, 2017). Some vitamins are essential for autotrophic algal cells. For biotin and thiamine, the loss of one or more key biosynthetic enzymes requires an exogenous supply, while cobalamin biosynthesis is absent in algae because of the loss of a cobalamin -independent methionine synthase. In open cultivation, cobalamin comes from a symbiotic relationship with bacteria (Croft et al., 2005).

Chlorella growth was superior in treated rejected water (TRW) comparing with those grown in BG-11 or original rejected water (ORW). The sustainable enhancing effect on growth dry weight accumulation during adaptation process could be ascribed to the continuous supplementation of nitrogen through daily nitric acid addition besides buffering action and hardness solubility. Moreover, vitamin B mixture might play an important role against the injury effect of hyper-salinity and algae stimulate vitamins parallel to growth conditions, where optimum growth, biomass and total B vitamins of the marine diatom *Skeletonema costatum* produced was achieved by culturing at salinity of 24 g L^{-1} and light intensity 34 µmol m^{-2} sec^{-1} (Suantika et al., 2016). Further, many marine algae are vitamin B12-rich, however the source is unknown. Previous reports point that certain algae can *de novo* synthesize cobalamin (Carlucci and Bowes, 1970); others have been shown to require exogenous cobalamin for growth in culture (Kraepelin, 1977); implying that many algae cannot synthesize it. As early mentioned by El-Sayed et al. (2010); chemical desalination leads to precipitation of some salts mainly ammonia, sodium, magnesium and chlorine and the remainder of desalination contain an adequate amount of them to save the next algal growth. It was reported that dissolved vitamin B12, thiamine, and biotin can be found in many seawaters samples within the ranges which influence the growth rate, cell size, and chlorophyll *a* content of *Cyclotella nana*, *Monochrysis lutheri*, and *Amphidinium carterae*, respectively. The response of algae *in situ* to vitamins may be similar (Carlucci and Silbernagel, 1969). Type of the salt stress based on nutrients balance could increase the obtained biomass of algae (El-Sayed et al., 2008); where the green alga *Scenedesmus* sp. successfully grown under 4 folds salinity of the recommended growth medium (El-Sayed et al., 2001).

NaCl led to delays in growth, especially at higher concentrations (1 to 2%), and to chlorophyll and protein decomposition and massive accumulation of carotenoids and oils. In addition, nitrogen limitation, high light irradiation, and free radical production can stimulate carotenogensis (El-Sayed, 2004a, 2004b). The present results indicated that use of adequate nitrogen content, a regular light regime, and slow adaptation to high NaCl ameliorated the adverse effects of high NaCl concentration on growth. Lower dry weight was recorded in ORW medium, while adaptation to high NaCl concentration increased the dry weight accumulation and the chlorophyll content. This might be attributed to the utilization of sodium ions by microalgal cells within organic metabolites to overcome the adverse effect of sodium.

For economical production of biofuel from microalgae, biomass as well as lipid content are very important parameters. Growth medium should provide the inorganic elements that constitute

Table 2
Fatty acid methyl esters produced by *C. vulgaris* grown in different water types.

Fatty acid	C No.	BG-11			ORW			TRW		
Caprylic	C8:0	0.54	±	0.08	0	±	0.00	0.09	±	0.01
Capric	C10:0	0.26	±	0.01	0.18	±	0.01	0.19	±	0.02
Lauric	C12:0	0.46	±	0.01	0.19	±	0.03	0.22	±	0.02
Myristic	C14:0	1.29	±	0.13	0.54	±	0.10	0.65	±	0.10
Myristoleic	C14:1	6.42	±	0.43	6.04	±	0.90	6.22	±	0.80
Palmitic	C16:0	12.16	±	1.10	26.03	±	2.10	24.29	±	3.60
Palmitoleic	C16:1	2.88	±	0.43	2.12	±	0.09	1.59	±	0.09
Stearic	C18:0	3.64	±	0.42	0.58	±	0.05	3.69	±	0.23
Oleic	C18:1	32.81	±	2.50	34.05	±	3.70	22.6	±	1.60
Linoleic	C18:2	12.14	±	1.40	9.42	±	1.10	13.6	±	1.20
Linolenic	C18:3	1.32	±	0.18	1.03	±	0.10	2.12	±	0.09
Arachidic	C20:0	0.26	±	0.02	0.24	±	0.03	0.31	±	0.00
Gadoleic	C20:1	2.74	±	0.43	2.27	±	0.21	3.06	±	0.20
Arachidonic	C20:4	4.12	±	0.53	4.37	±	0.76	2.19	±	0.18
Behenic	C22:0	1.14	±	0.16	1.16	±	0.05	0.09	±	0.00
Erucic	C22:1	2.03	±	0.21	1.54	±	0.06	2.36	±	0.20
Docosahexaenoic	C22:6	4.09	±	0.64	4.21	±	0.67	4.36	±	0.50
SFA		19.75	±	1.60	28.92	±	2.10	29.53	±	2.50
USFA		68.55	±	3.80	65.05	±	4.50	58.10	±	3.90

Original-rejected water (ORW), Treated rejected water (TRW)

Table 3
Prediction of the fuel properties of bio-oil produced by *C. vulgaris* grown in water types.

Medium	SV	IV	CN	DU	LCSF	CFPP
BG-11	182.77	100.095	53.6414	146.78	5.006	0.7497
ORW	194.411	94.6807	53.0714	78.04	4.873	1.1675
TRW	182.755	90.1689	55.8771	80.37	4.719	1.6513
Europe (EN 14214)		$\leq$ 120	$\geq$ 51			− 20 to − 5
US (ASTM D6751-08)			$\geq$ 47			− 5 to − 13

ORW = original rejected water and TRW = treated rejected water.
CN = cetane number; SV = saponification value; IV = iodine value; DU = degree of unsaturation; LCSF = long-chain saturated fatty acid and CFPP = cold filter plugging point.

the algal cell. Many of freshwater algae showed a good defense strategy towards the increased levels of ROS under salinity stress by increasing the activity of protective enzymes responsible for the detoxification and elimination of ROS. For example, Farghl et al., (2015) indicated the importance of antioxidants induction for the tolerance mechanism of *Chlorella* species to salinity as demonstrated by growth behavior. The satisfactory growth during the vegetative phase seems to be the main reason for the greater growth during carotenogenesis due to the presence of large quantities of organic matter. Similarly, Xu et al. (2012) reported the induction of osmotic pressure by NaCl and the effects of salt stress on cell growth and lipid production in *C. vulgaris* which depended on time of exposure and concentration of NaCl. In particular, NaCl greatly affected the cellular growth since it was added at the beginning of cultivation, which consequently had a significant negative effect on lipid accumulation. Furthermore, a gradual increase of NaCl concentration, with a low salt concentration used during the growth phase and high concentration during the stationary phase, can maximize lipid yield and minimize the negative effects of NaCl on cell growth. On the other hand, the TRW medium was slightly alkaline and showed high nitrogen content due to the desalination processes. These conditions enhanced the dry weight accumulation in this medium. In that context, the elevated levels of nitrogen, phosphorus, and potassium are known to increase microalgal growth (El-Sayed et al., 2008).

Lipid content also varied according to the culture medium, where original rejected and treated rejected-waters enhanced the lipid content over the control. The enhancing effect of treated rejected water on lipid accumulation might be attributed to the initial nutrients mainly nitrogen with high media reaction value (pH) as a result of desalination process (El-Sayed et al., 2010). Results also confirm that reduction of nitrogen stress during growth appeared to increase the oil production. It is documented that under stressful conditions, the entirety of the carbon produced are converted to oil (Lardon et al., 2009) and other substances that function in microalgal tolerance and defense. Sheehan et al. (1998) suggested that the reason for the increased oil content under stress was that nutrient deprivation led to starvation and a decrease in the production rate of all cellular compounds, but production of oil remained high. Nonetheless, high oil content is easy to achieve in a variety of microalgae (Chisti, 2007). For example, *Botryococcus braunii* was reported to have an oil content of 86% (Brown et al., 1969). However, the main obstacle in using *B. braunii* for industrial-level production of biodiesel is its low biomass productivity and poor growth rate (Xin et al., 2010).

Biodiesel quality is directly related to CN, an indicator of a fuel's ignition quality in an engine (Stournas et al., 1995). The present results showed lower content of SFAs in *Chlorella* oil. Indeed, polyunsaturated fatty acids (PUFAs) that contain at least four double bonds are subject to oxidation, emit more nitrogen oxides, and have a thermal efficiency lower than biodiesel rich in saturated fatty acids. Thus, PUFAs are less acceptable for biodiesel production (Chisti, 2007). Previous research proposed the ideal C16:1, C18:1, and C14:0 mixture to be used for biodiesel production should have a 5:4:1 ratio. Such a biodiesel would have very low oxidative potential (Stournas et al., 1995). The present results indicated a ratio of nearly 3:4:1 in control cultures, which confirms that further optimization is required to achieve a more ideal ratio of these fatty acids. In addition, the proper ratio of SFAs and UFAs is very important when using microalgal oils as a biodiesel feedstock (Deng et al., 2009). In that context, Gouveia and Oliveira (2009) and Abomohra et al. (2020b) suggested that other oils should be added to microalgal oil to improve its quality. Other studies found that oils with high content of oleic acid exhibit a satisfactory balance of fuel properties (quality of ignition, combustion heat, CFPP, oxidative stability, viscosity, and lubricity). All these factors are affected by the FAMEs profile, and manual addition of oleic methyl ester in a previous study improved the oxidative stability and lowered the melting temperature (Prabakaran and Ravindran, 2012). Interestingly, *Chlorella* cells produced a considerable amount of oleic acid, suggesting its bio-oil as suitable feedstock for good quality biodiesel production.

Biodiesel properties produced by *C. vulgaris* grown under different conditions were estimated. The IV is the total unsaturation within a mixture of FA is limited up to 120 g I_2/100 g in the European biodiesel standard (EN 14214, 2008). This limit eliminates some promising oil sources to be used as feedstock for biodiesel (Mittelbach and Remschmidt, 2004). A drawback of high content of unsaturated fatty acids is that their combustion leads to polymerization of glycerides and the development of deposits or worsening of lubrication (Schenk et al., 2008). The present results indicated lower IVs when *C. vulgaris* is grown in ORW or TRW, which is advantageous for higher biodiesel quality. Microalgae commonly produce polyunsaturated fatty acids with at least four double bonds, which are susceptible to oxidation, emit more nitrogen oxides, and have a thermal efficiency lower than biodiesel produced from saturated fatty acids, which restricts the acceptability of microalgal bio-oil for biodiesel production (Chisti, 2007). The oxidative potential of such biodiesel would be very low (Schenk et al., 2008). Moreover, a low CN in the control culture is related to more highly unsaturated components (C18:2 and C18:3). Furthermore, oxidation stability decreases as the content of polyunsaturated methyl esters increases. However, the worst CFPP values were reported for biodiesels rich in long carbon chains with saturated methyl esters. Therefore, it is important to generate the proper percentages of saturated and unsaturated FA when using microalgae as a biodiesel feedstock. Overall, the present study concluded that *C. vulgaris* is a suitable feedstock for biodiesel because of its high oil content and high levels of palmitic and oleic acids. In addition, cultivation of *C. vulgaris* on TRW could enhance the biodiesel characteristics.

5. Conclusion

The energy crisis and the increasing salinization of fresh water are closely related problems. It may be possible to resolve both problems by using hyper-saline water after chemical desalination to stimulate bio-oil production in microalgae. Biofuel matrix can

be obtained from algal biomass through the production of bioalcohol from carbohydrates, biogas from proteins and biodiesel from oils. In addition, biogas can be obtained from all of the mentioned fractions. The main goals of future research are to optimize the desalination method using proper microalgal strain and strategy used to stimulate carotenogenesis. In addition, large-scale open cultivation of microalgae using seawater might be efficient approach for dual purpose of seawater desalination and biofuel production in arid and semi-arid regions such as Kingdom Saudi Arabia.

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.

Acknowledgments

This work was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University (Jeddah, Saudi Arabia) by grant No. D-122-662-1441. The authors acknowledge the DSR for technical and financial support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.04.007.

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