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Original article

Potential of diatom consortium developed by nutrient enrichment for biodiesel production and simultaneous nutrient removal from waste water

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ABSTRACT

Because of the decreasing fossil fuel supply and increasing greenhouse gas (GHG) emissions, microalgae have been identified as a viable and sustainable feedstock for biofuel production. The major effect of the release of wastewater rich in organic compounds has led to the eutrophication of freshwater ecosystems. A combined approach of freshwater diatom cultivation with urban sewage water treatment is a promising solution for nutrient removal and biofuel production. In this study, urban wastewater from eutrophic Hussain Sagar Lake was used to cultivate a diatom algae consortium, and the effects of silica and trace metal enrichment on growth, nutrient removal, and lipid production were evaluated. The nano-silica-based micronutrient mixture Nualgi containing Si, Fe, and metal ions was used to optimize diatom growth. Respectively, N and P reductions of 95.1% and 88.9%, COD and BOD reductions of 91% and 51% with a biomass yield of 122.5 mg L^{-1} day^{-1} and lipid productivity of 37 mg L^{-1} day^{-1} were observed for cultures grown in waste water using Nualgi. Fatty acid profiles revealed 13 different fatty acids with slight differences in their percentage of dry cell weight (DCW) depending on enrichment level. These results demonstrate the potential of diatom algae grown in wastewater to produce feedstock for renewable biodiesel production. Enhanced carbon and excess nutrient utilization makes diatoms ideal candidates for co-processes such as CO_2 sequestration, biodiesel production, and wastewater phycoremediation.

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1. Introduction

The level of atmospheric CO_2 is rising because of increased anthropogenic emissions of CO_2. Given the elevated energy demand and limited accessibility to fossil fuels, there is an urgent need to explore renewable, ecofriendly, and cost-effective alternative fuel sources. Algae have high oil content and show rapid biomass production. They can grow on non-cultivable land using wastewater; thus, in contrast to land-based plant sources, they do not compete for land and water for biofuel production. Microalgal biomass can also be used to produce high-value biomolecules (Milledge, 2011). Based on these properties, microalgae are a potential alternative for biofuel production.

Microalgae can utilize low-quality water such as agricultural runoff and municipal or domestic wastewater as a growth medium and source of nitrogen, phosphorus, and minor nutrients. Thus, growing algae in wastewater is economical and environmentally friendly alternative; it can also reduce the cost for nutrients and fresh water for mass culturing while providing a method for wastewater treatment (Oswald, 1988). The quantity of wastewater generated by a city is directly proportional to the amount of water consumed. The use of microalgae for CO_2 mitigation, wastewater treatment, and biofuel production has the potential to maximize the impact of microalgal biofuels on climate change; however, many crucial aspects such as the isolation of algal strains with high growth rates and nutrient uptake, integration of algal growth systems with wastewater systems, improved algal harvesting, and life cycle analysis must be further explored to maximize the potential of algal biofuels.

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Table 1
Showing different nutrient enrichment tested in the experimental study.

Experimental variation	Nutrient source	Concentration
Control	Domestic and industrial waste water(DIW)	18 mg L^{-1} N, 3.8 mg L^{-1} P
Silicate	DIW + Si2	35 mg L^{-1} Si
Silicate + trace metal	DIW + Si2 + trace metal	35 mg L^{-1} Si + Fe + Trace metal solution as in F/2 Si medium (Guillard and Ryther, 1962)
Nualgi	DIW + Si + trace metals	1 ml l^{-1} Nualgi

Benthic diatoms contribute greatly to reduce nutrient level and increases O_2 levels in wastewater bodies and enhances the benthic food web. Diatoms are estimated to contribute ~40% of total primary production in Oceans, which is equal to the biomass of all tropical rain forests. Diatoms due to their efficient carbon concentrating mechanisms (CCM) play a significant role in carbon sequestration to the deep ocean and are major contributors to the "biological carbon pump" (Bowler et al., 2009). Although diatoms exhibit numerous characteristics required for biofuel production such as an elevated growth rate, rich lipid content, ability to grow under diverse environmental conditions, and species diversity, they are the least-represented species in mass-scale experiments for biofuel and biomolecule production (Hildebrand et al., 2012). The productivity of diatoms in natural environments is largely influenced by the availability of silica but a limited iron supply is also known to negatively influence diatom growth in oceans (Takeda, 1998). Iron and silica can readily form complexes when dissolved in water, which may not be readily available for diatoms thus, enrichment experiments should evaluate metal ion bioavailability. At a high Si:P ratio, diatoms are known to dominate other microalgal species such as blue-green algae (Holm and Armstrong, 1981) This means that Si and Fe enrichment can help shift the nutrient balance towards diatom dominance. Mesocosm experiments conducted by many researchers have stressed on the importance of Si in producing an algal community dominated by diatoms (Litchman, 2007). Therefore, in the present study, we assessed the effects of silica, Fe, and trace metals, which are the three main medium components in triggering diatom growth in wastewater. We analyzed the potential of a diatom consortium developed using nutrient enrichment with Si, Fe, and trace metals grown in urban wastewater for nutrient removal and biomass and lipid production. We also explored the potential of the nano-nutrient mixture Nualgi to trigger diatom growth and lipid production.

2. Methods

2.1. Sample collection and study area

Hussain Sagar lake is situated in the heart of the cities Hyderabad and Secunderabad and is fed by four major inlets. The lake covers an area of 5.7 km^2 with an average depth of 5.00 m. It was a major drinking water source for the city till 1920. Due to urbanization of the city, sewage and industrial wastewater were discharged into lake, greatly contributing to cultural eutrophication of water. Water samples were collected from the lake to test physiochemical parameters. Samples for preparing the diatom consortium were collected using standardized protocols (Kelly et al., 1998).

2.2. Growth studies

Two sets of experiments were conducted in same time and conditions to evaluate growth and lipid productivity of algae in wastewater and the effect of algal growth on nutrient removal. Our goal was to grow an algae consortium dominated by diatom algae. We want to develop a diatom consortium rather than using a single strain because using different strains of diatoms isolated from the same water can eliminate issues related to adaptation and the time required to establish pure cultures. We used the patented commercial micronutrient mixture Nualgi®, which has an alumina-modified nano-silica base coated with inorganic salts of the major nutrient Fe and trace metals including Mn, Co, S, Ca, Mg, Zn, and B (US patent application No.: 70275856). The diatom consortium was prepared by dislodging diatoms growing on rock samples collected from lake water followed by culture for 30 days in filtered lake water with silica enrichment using Nualgi® 1 mL L^{-1} with 20% exchange of wastewater every 5 days. The resulting culture contained an algal consortium dominated by diatoms; this was used as an inoculum for further experiments. Different experimental variations were studied by conducting enrichment experiments (Table 1). The cultures were grown in a culture room at 26 ± 2 °C with a 12-h:12-h light:dark cycle at a light intensity of 100 μmol photons m^{-2} s^{-1}. The cultures were hand-shaken twice per day. All experiments were conducted in triplicate. Growth kinetics was studied by cell counting and determining the specific growth rate (Furnas, 2002) and measuring biomass at stationary phase. Diatom samples were counted at 0.1 mm depth using a hemocytometer and compound microscope.

2.3. Diatom identification

Identification of diatoms in the consortium before and after Si enrichment was carried out following standard protocols (Taylor et al., 2007) and using taxonomic guides (Prescott, 1962).

2.4. Analysis of physiochemical parameters

Physico-chemical parameters of water such as dissolved oxygen, pH, total hardness, biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids, total nitrogen, total phosphate, and electrical conductivity were analyzed before and after the growth period using standard methods (APHA, 1985).

2.5. Fatty acid profiling

Total lipids were extracted according to the modified Bligh and Dyer protocol of Folch method (Bligh and Dyer, 1959) for algal lipids. The lipids extracted from each sample were analyzed by gas chromatography-mass spectrometry (Suman et al., 2012).

2.6. Statistical analysis

Growth data, represented the mean ± standard deviation, was statistically analyzed. SPSS version 21.0 software (SPSS, Inc., Chicago, IL, USA) was used for all the statistical analysis. One-way ANOVA was used to compare the means between groups to identify the significance level of variations.

3. Results and discussion

3.1. Growth studies and nutrient enrichment

Nutrient enrichment with Si favors diatom growth, as diatoms require silica for cell wall biogenesis. Enrichment with Nualgi, a silica-based nutrient mixture containing Fe and metal ions, resulted in a higher specific growth rate (μ) and cell number of 0.18 and 2.89×10^6 cell mL^{-1}, respectively; in the absence of Nualgi, these values were 0.11 and 0.93×10^6 cell mL^{-1} (Fig. 1). In cultures with silica enrichment of 35 mg L^{-1}, the cell concentration increased to values similar to that in Nualgi-enriched cultures; however, after the 4th day, a slight decrease was observed in the growth rate and cell concentration, which remained at 0.14 and 1.5×10^6 cell mL^{-1}, respectively in the stationary phase. Cultures enriched with silica and a trace metal mixture showed the same pattern, but had a lower specific growth rate and cell number, with values of 0.15 and 1.5×10^6 cell mL^{-1}, respectively, compared to Nualgi-enriched cultures. The differences in growth may be attributed to better availability of silica and trace metals present in Nualgi because of its nano-form, as nano-nutrients are less prone to precipitation when they are present in water compared to in their typical form. These specific growth rate and cell numbers

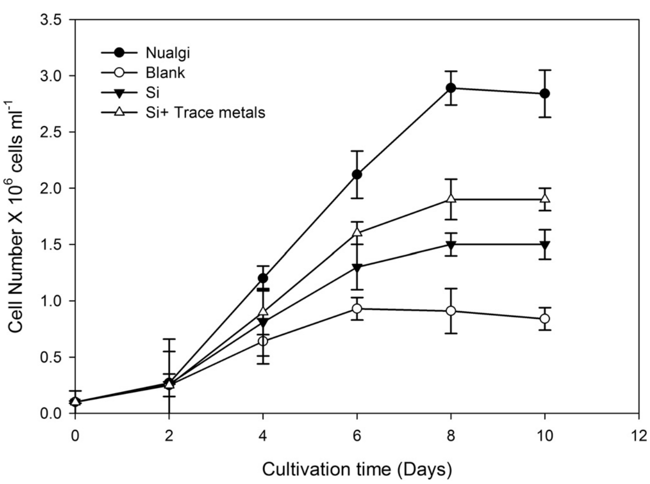

Fig. 1. Growth curve of diatom consortium after inoculation in waste water enriched with Nualgi 1 ml L^{-1}, Silica, Silica + trace metal solution and blank with no enrichment. Bars indicate SD.

are equivalent to the values reported by Suman et al. (2012) however, the previously reported values were observed in pure cultures of marine diatom strains. Additionally, the Nualgi used previously was in powdered form, but this substance is now available only in liquid form. Thus, we have optimized the Nualgi concentration to achieve maximum growth and found that 1 mL L^{-1} was the optimum concentration. From the biomass generated upon reaching the stationary phase, we calculated biomass productivity and found values of 122 and 28 mg L^{-1} day^{-1} in Nualgi and blank cultures, respectively; in Si-enriched cultures, this value was 51.2 mg L^{-1} day^{-1} and in Si and trace metals was 66.2 mg L^{-1} day^{-1} (Table 2). Although the biomass productivity found in this study was low compared to that of previous studies (Table 3), these values were obtained by using a mixture of diatom strains rather than single-species cultures grown in a laboratory; thus, additional optimization is required to reach maximum productivity. In a similar study by Mooij et al. (2015) on silica replete and deplete batch reactors with a mixed algal consortium, Si excess resulted in cultures with diatom *Nitzschia* dominance; in Si-depleted medium, green algae *Chlamydomonas* dominated the culture (Mooij et al., 2015) supporting that silica enrichment resulted in an algal consortium dominated by diatoms. In freshwater, silica is less soluble than amorphous silica. Algal species dominance is effected by numerous elements like temperature, light, pH, nutrient composition adaptation of algae, grazers, and parasites (Benemann, 2003) Attempts to introduce single species have failed because of the contamination of natural species in many open pond algae cultures. Thus, single-species algal culturing techniques require further analysis. The use of a consortium of one dominant algal group has not been widely examined, but exhibits distinctive advantages such as the reduction in time required to develop unialgal cultures and the prevention of cross-contamination by other algal groups. In natural water bodies, diatom algae have been extensively studied for their ability to act as water quality indicators. Depending on nutrient concentration, diatom diversity and density differ, which may be advantageous for growing algae in wastewater, as the growth conditions of different species with different nutrient loads do not require optimization. A single diatom

Table 2
Comparison of growth and lipid production parameters during different nutrient enrichments.

Parameter	Blank	Nualgi	Si	Si + Fe + Trace
Cell no $\times 10^6$ ml^{-1}	0.9	2.89	1.5	1.9
Dry weight g l^{-1}	0.23	0.98	0.41	0.53
Biomass productivity mg L^{-1} day^{-1}	28.7	122.5	51.2	66.2
Total lipid yield g l^{-1}	0.02	0.29	0.06	0.11
Lipid % DCW	11.9	30.13	25.1	21.5
Lipid productivity mg L^{-1} day^{-1}	2.5	37	7.5	13.75

Table 3
Type of water and nutrient enrichment with biomass and lipid percentage achieved in previous studies in comparison with present study.

Type of water	Algal strain	Biomass g L^{-1}	Lipid content % DCW	Reference
MWW[b] + 15%CO_2	*C. vulgaris*	0.29	30	Ji et al. (2013)
MWW + 15%CO_2	*Ourococcus multisporus*	0.31	31	Ji et al. (2013)
1° Treated	Mixed	0.025	28	Ip et al. (1982)
1° Treated + CO_2	Mixed	0.27	09	Woertz et al. (2009)
Facultative pond (STP)	*Euglena* sp.	0.5	24.6	Mahapatra et al. (2013)
Lake	*Phormidium* sp.	0.3	8.8	Mahapatra et al. (2013)
DWW[c] + Mixotrophy + CO_2	Mixed	3.4[a]	28.2	Devi and Mohan (2012)
MWW + IWW[d]	Mixed	0.14	11.9	This study
MWW + IWW	Diatom consortium	0.9	30.13	This study

[a] Through OD at 650 nm.
[b] MWW - municipal waste water.
[c] Domestic waste water.
[d] IWW - industrial waste water.

consortium containing different species can grow under diverse nutrient loads. Thus, mixed diatom species were evaluated for biomass and lipid productivity. This is the first study to evaluate the use of a diatom consortium for nutrient removal.

3.2. Diatom species diversity

Collected water samples were analyzed for diatom diversity and the effect of Si and trace metal enrichment using Nualgi to trigger diatom diversity was studied (Table 4). Nualgi enhanced diatom growth by increasing the number of diatom cells and also the diatom diversity. Flasks without Nualgi were dominated by green algae species like *Scenedesmus dimorphus* and *Chlorella vulgaris*, blue-green algae mainly of *Microcystis* sp., and six different species of diatoms in which *Cyclotella meneghiniana*, *Gomphonema lanceolatum*, and *Nitzschia palea*. In samples containing Nualgi *Achnanthidium exiguum*, *Navicula crytocephala*, *Cymbella tugidula*, *Navicula gracilis*, and *Pleurosigma elongatum* were the dominant species, with a total of 30 different diatom species identified. Dominant species such as *C. meneghiniana* and *N. palea* in samples with no Si enrichment were identified as pollution-tolerant species and in Nualgi-added samples dominant species such as *Achnanthidium exiguum* and *Navicula crytocephala* were less pollution-tolerant (Kobayasi and Mayama, 1989). This variation in diatom diversity clearly specifies that addition of Nualgi reduced excess nutrient levels by promoting diatom growth. Algae dominace in natural environments was controlled by ratio of major nutrients like N, P and Si. Diatoms dominate at a high Si:P ratio, green algae at low Si:P and high N:P ratios, and cyanobacteria at low Si:P and low N:P ratios (Tilman et al., 1986) Thus, changes in nutrient concentration by nutrient enrichment resulted in increased diatom species diversity.

Table 4
Showing relative abundance and diversity of microlagal species before and after Si + trace metal enrichment with Nualgi.

Diatom species	Abundance	
	With Nualgi	Without Nualgi
Achnanthidium exiguum	***	–
Amphora ovalis	*	–
Cocconeis placentula	**	–
Cymbella aspera	*	–
Cymbella tugidula	**	–
Cymbella tumida	*	–
Cyclotella meneghiniana	**	***
Eunotia minor	*	–
Eunotia pectinalis	*	–
Fragilaria ulna	*	–
Fragilaria sp.	*	–
Gomponema lanceolatum	*	***
Gomphonema parvulum	*	**
Gomphonema affine	*	–
Gomphonema pseudoaugur	**	–
Gomphonema gracile	*	–
Gomphone maminutum	*	–
Gomphonema undulatum	*	–
Gyrosigma nodiferum	**	–
Navicula crytocephala	***	–
Navicula sigmatifera	**	–
Nitzschia intermedia	*	–
Nitzschia palea	**	***
Nitzschia thermalis,	*	*
Nitzschia linearis	*	–
Nitzschia frustulum	*	–
Navicula gracilis	**	–
Navicula sp.	–	*
Pleurosigma elongatum	**	–
Pleurosigma salinarum,	–	*
Pinnularia boreanis	*	–
Synedra ulna	*	**
Staphanodiscus sp.	**	–
Staphanodiscus hantzschii	*	–
Scenedesmus dimorphus	–	***
Chlorella vulgaris	–	**
Microcystis sp.	–	**

–: Not present.
* Present.
** Abundant.
*** Dominant.

3.3. Physiochemical parameters

Water quality parameters of the lake have been well-documented by numerous researchers over many years. Lake water quality has deteriorated gradually because of pollution from both industrial and domestic wastewater entering the lake without proper treatment (Chandra et al., 2012). In this study, nitrate concentration of the lake increased from 14 to 18.9 mg L^{-1} and phosphate concentration raised from 2 to 3.87 mg L^{-1} from 2008 to 2014, respectively. This increase in nutrient levels led to an imbalance in water nutrients, further triggering enormous growth of blue-green algae which is harmful to the environment (Chislock et al., 2013). BOD levels also increased alarmingly from 90 to 212 mg L^{-1} and COD increased from 30 to 350 mg L^{-1}.

3.4. Nutrient removal

The removal efficiency of the two main nutrients N and P as well as other key parameters such as COD and BOD were calculated. The maximum N removal percentage was observed in Nualgi-enriched cultures (95.1%), followed by Si and trace metals (69.3%), Si (52.1%), and blank (32.3%). A P removal efficiency of 88.9% was observed in the Nualgi culture, while the Si, Si and trace

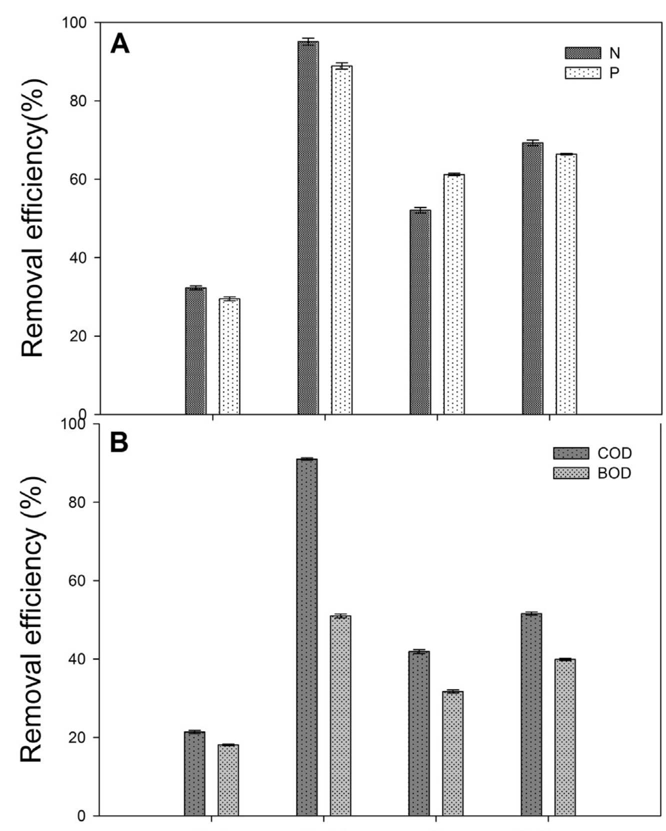

Fig. 2. Total nitrate (TN) (A), Total phosphate (TP) (A), Chemical oxygen demand (COD) (B) and Biological Oxygen Demand (BOD) (B) reduction from waste water by Diatom consortium under different Nutrient enrichments. Bars indicate SD.

Table 5
Type of algal cultures and waste water along with N and P removal efficiencies achieved in previous studies in comparison with present study.

Algal strain	Water type	Culture type	Removal time (d)	TN initial con	TN % removal	TP initial con	TP % removal	Reference
C. vulgaris	IWW[b]	Batch	5–9	3–36	30–95[a]	112	20–55	González et al. (1997)
S. dimorphos	IWW	Batch	9	–	–	112	20–55	González et al. (1997)
S. obliquos	MWW	Batch	0.2–8	27	79–100[a]	12	47–98	Ruiz-Marin et al. (2010)
P. tricornatum	MWW	Continuous	14	498–835	8–100	76	100	Craggs et al. (1995)
Diatom consortium	MWW + IWW	Batch	4–6	18.9	95	3.87	88.9	This study

[c]MWW - municipal waste water.
[a] Ammonia N - NH_2^+-N.
[b] IWW - industrial waste water.

metals, and blank samples showed removal efficiencies of 61.2%, 66.4%, and 29.5%, respectively. (Fig. 2A). COD and BOD reductions of 91% and 51% (Fig. 2B), respectively, were achieved over a period of 10 days in Nualgi-enriched samples, while in the absence of Nualgi, this percentage was significantly lower. The removal efficiencies achieved in this study correlated with the percentages achieved with other species of algae both as single and mixed species (Table 5). Enrichment with C (carbon) and N resulted in the highest COD removal and 66% N removal and 65% P removal were observed following N and P enrichment in mixed cultures (Devi and Mohan, 2012) These values are lower than those achieved with Si enrichment in this study. Many researchers has studied nutrient removal by green algal species like *C. vulgaris* and *Scenedesmus obliquus*, but few studies have examined diatoms, which are one of the most dominant algal species in a variety of wastewaters under varied climatic conditions.

3.5. Lipid studies

Lipid content was estimated in stationary phase cultures after 8 days of cultivation. In cultures containing Nualgi, the highest lipid % per dry cell weight reached 30.13 ± 2.37%, whereas in the control culture, this value was 7.58 ± 1.98%. In silica- and Si and trace metal-enriched culture, these values were 25.1 ± 1.17% and 21.5 ± 1.87%, respectively (Table 5). According to the manufacturer, Si content in Nualgi was lower compared with f/2 Si medium concentration of 35 mg L^{-1}. However, silica in the form of sodium metal silicate easily forms a precipitate, and thus enhanced bioavailability of nano-silica in Nualgi may have resulted in increased biomass. Silica limitation is a known factor that enhances the accumulation of lipids in diatoms species. Devi and Mohan (2012) conducted a similar study to examine the effects of N, P, and C enrichment on algal growth and lipid production in wastewater using mixed algal cultures. P enrichment increased centric and pinnate diatom species, resulting in an increased lipid percentage. This is consistent in our study, in which diatom rich biomass showed a higher lipid percentage compared to that in control cultures. A maximum lipid productivity of 37 mg L^{-1} day^{-1} was observed in Nualgi-enriched cultures (Table 2). In this study, lipid content was estimated at the end of the growth phase, while in previous studies on diatom *Phaeodactylum tricornutum*, the maximum lipid and EPA production was obtained in the early stationary phase (Yongmanitchai and Ward, 1991). Algal species or biomass with at least 30% lipid per dry cell weight is ideal for biofuel production. Based on the lipid percentage obtained by using Nualgi enrichment, which is 30.13% lipid per dry weight, this method can be used as a possible enrichment procedure for mixed cultures for biodiesel production.

3.6. Fatty acid profiling

Gas chromatography-mass spectrometry analysis revealed the presence of 13 different fatty acid methyl esters (Table 6). Major

Table 6
Relative percentage of individual fatty acids and total lipid content in cultures grown with Nualgi, Si 35 mg/l and Si + Trace metal (same as F/2 medium concentration) compared to control (domestic waste water).

Fatty acids	Control	Si	Si + Trace metals	Nualgi
14:0	8.21	7.09	8.01	8.46
15:0	1.94	2.11	2.08	1.93
16:0	29.08	22.37	20.1	18.11
18:0	2.73	2.55	3.01	2.87
16:1($n-7$)	27.71	23.6	25.7	27.89
18:1($n-9$)	4.91	1.2	2.1	0.78
18:1($n-7$)	0.82	0.8	0.9	0.80
16:3($n-4$)	12.09	10.3	10.9	11.80
18:2($n-6$)	1.29	1.89	2.1	1.40
18:3($n-6$)	1.91	1.6	1.9	1.77
20:3($n-6$	1.24	2.27	3.1	1.37
20:4($n-6$)	3.98	9.12	7.9	8.19
20:5($n-3$)	4.09	15.1	15.3	14.63
Saturated	41.96	34.12	33.2	31.37
Mono-saturated	33.44	25.6	25.7	29.47
Poly-unsaturated	24.6	40.28	40.28	39.16
Total lipid content (% DCW)	11.92	25.1	21.5	30.13

fatty acids found in control medium were saturated with palmitic acid (C16:0), which was dominant at 29.08%, followed by monosaturated palmitoleic (C16:1) at 27.71%. In Nualgi-enriched cultures, poly-unsaturated eicosapentaenoic acid (C20:5($n-3$)) was the major fatty acid at 24.63%, followed by palmitic acid (C16:0) at 18.11% and palmitoleic (C16:1) at 17.89%. In Si-enriched cultures, palmitic acid was the major fatty acid, while in the culture containing Si and trace metals, palmitoleic was the major FA. These results are in accordance with those of a similar study of mixed cultures (Mohan et al., 2011) In Si-enriched cultures, there was a slight increase in polyunsaturated fatty acid content (PUFA), while in the blank culture, saturated fatty acid content was high. This increase in PUFA content in Si-enriched cultures may be attributed to the dominance of diatoms rich in PUFA content (Hu et al., 2008). Although higher un-saturation is not ideal for biodiesel production, the higher percentage of lipid content along with higher biomass in Si-enriched cultures may be ideal for greater lipid production, and lipids rich in PUFA, particularly EPA, are highly demanded in the nutraceutical and food supplement industries.

4. Conclusions

Overcoming current challenges in algae production will benefit both the biofuel and wastewater treatment fields. Further studies are needed to fully explore this collaborative potential. Using wastewater as a source for nutrients in combination with the production of algae-based byproducts can overcome several major limitations in this field. Optimum utilization of existing infrastructure of wastewater treatment facilities as well as urban lakes, ponds and water channels for algae production can reduce capital costs and scalability issues. This study highlights the capability of using a diatom consortium isolated from wastewater to achieve high growth and lipid productivity. The highest growth rate and high lipid percentage and EPA content were observed in Nualgi-enriched medium. The results of this study demonstrate that the diatom consortium developed by using Si enrichment is cost-effective and less time-consuming for sustainable biodiesel production using wastewater.

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