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Research Article

Removal of Nitrogen and Phosphorous from Synthetic Wastewater by *Oscillatoria* sp. Cultivated in Vertical Tubular Photobioreactor under Natural Environmental Conditions

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Abstract: A newly isolated cyanobacterium *Oscillatoria* sp. from the Sub-Himalayan region of India was assessed for nutrient removal efficiencies. It was cultivated in synthetic wastewater under uncontrolled natural environmental conditions. The filaments of cyanobacterium formed entangled mats which easily floated on the surface of the medium. This characteristic feature was exploited for easy harvesting of the biomass that is hitherto a bottleneck for the industrial scale oil production from microalgae. The cultures of *Oscillatoria* sp. grown in synthetic wastewater removed 93.02% of 76 mg/L nitrate and 94.11% of 51 mg/L phosphate. The fatty acid profiling of 10-day-old cultures showed the dominance of saturated and monounsaturated fatty acids. The percentages of predominant fatty acids found in *Oscillatoria* sp. were 25.25% myristoleic acid (14:1w5c), 23.3% myristic acid (14:0), 16.81% palmitoleic acid (16:1w7c), 14.88% palmitic acid (16:0) and 4.765% hexadecanoic acid (16:1 w5c). The biomass productivities of 0.065 g/L/day and 0.05 g/L/day were attained by the cultures grown in synthetic wastewater and control BG-11 growth media respectively. This mesocosm study was conducted under the influence of natural sunlight, uncontrolled temperatures and the presence of biological contaminants namely, bacteria and small ciliates. The main objective of the study was to mimic the outdoor conditions, which is the way forward for achieving economic and environmental sustainability in microalgae biomass production.

***Keywords*:** autoflotation, cyanobacteria, nitrate, oscillatoria, phosphate, wastewater

1. Introduction

Microalgae including cyanobacteria are the natural scavengers of the inorganic compounds in nutrient-loaded wastewater sources [1-2]. The nutrients loading in sewage and industrial effluents, and run-offs from intensive agriculture and livestock farming to the natural water bodies such as lakes, ponds rivers and even sea cause eutrophication; which subsequently leads to the proliferation of undesirable algae blooms [3]. The proliferation of harmful blooms in any water body depends on the concentrations and ratios of different nutrients namely N, P, and Si that discriminately favor the growth of different groups of algae. For example, the low N:P (<16:1) could promote the

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growth of toxin-producing flagellated microalgae over diatoms which are detrimental to Salmon fish [4]. Apart from the nutrient status, the increasing temperature [5] and salinity in the water bodies resulting from global warming and the rise in sea levels respectively, favor the proliferation of toxin-producing cyanobacteria. The blooms forming cyanobacteria outcompete other phytoplanktons at temperatures exceeding 25 °C and due to halotolerant characteristics, cyanobacteria also survive high salinities of the inland water bodies [6].

As cyanobacteria are prokaryotic in nature, they possess morphological and biochemical characteristics that make them suitable for nitrogen and phosphorous removal under different types of environmental and nutrient conditions. The dominance of various species of cyanobacteria in eutrophic lakes is due to their abilities to assimilate various forms of nitrogen such as NO_3^-, NO_2^-, NH_3, free atmospheric nitrogen, to form gas vesicles and aggregates or mats and to resist herbivory by grazers [7]. With respect to biochemical composition, the lipids sourced from cyanobacteria have useful applications for pharmaceutical and biodiesel production [8]. However, the production of favorable quantities and suitable fatty acid profiles in algae are generated through modulations in light, pH, photobioreactor design, mode of operation, temperature [8] and nutrients [9-10].

High inputs of inorganic nutrients, light and temperature are the major costs associated with microalgae biomass production. Therefore microalgae cultivation done in indoor environments, under controlled illumination, and constant temperature, is an economically and environmentally unsustainable process. Another high-energy input process associated with biomass production is dewatering or harvesting [11], which is a major drawback related to the commercialization of algal biofuels [12]. Conventionally, microalgae biomass is separated from the medium through one or two rounds of sedimentation, followed by centrifugation [13]. Sedimentation settlement depends upon multiple factors such as size, shape, cell density, biochemical composition and age of microalgae. Species of microalgae possessing needlelike or cylindrical cell shapes are found to be reluctant to the settlement, thus making the process inefficient [13]. In this respect, mat-forming cyanobacteria such as *Oscillatoria* can easily be concentrated prior to biomass harvesting via filtration or centrifugation. In the present study, the mat formation in *Oscillatoria* allowed the auto-flotation of the cyanobacteria cultures which were then dewatered through manual filtration.

In this paper, we have reported a mesocosm study on the newly isolated species of *Oscillatoria*. The cyanobacterium was grown in a vertical tubular photobioreactor to assess its performance for nutrient removal from synthetic wastewater under natural light and temperature conditions. The objective was to establish a sustainable mode of microalgae cultivation by utilizing the naturally available light and outdoor temperature conditions. This significantly cut down the costs associated with the cultivation process. An efficient removal of nutrients from wastewater with simultaneous production of biomass rich in desirable fatty acids can further reduce the cost of algal oil production [14]. The present study is a preliminary report on the utilization of mat-forming cyanobacterium *Oscillatoria*, under uncontrolled ambient environmental conditions for producing fatty acid-rich biomass.

2. Materials and methods

2.1 *Cyanobacterium Culture*

The experimental organism was isolated from the water sample collected from the Pakibhatan Bird and Wildlife Sanctuary, Teesta River, Gajaldoba, West Bengal, India 26.752088N, 88.583181E (Figure 1). The culture was maintained in the BG-11 (N+) medium containing 0.04 g/L K_2HPO_4, 0.075 g/L$MgSO_4.7H_2O$, 0.036 g/L $CaCl_2.2H_2O$, 0.006 g/L citric acid, 0.006 g/L ferric ammonium citrate, 0.001 g/L EDTA-Na_2, 0.02 g/L Na_2CO_3, 0.0028 g/L H_3BO_3, 0.0018 g/L $MnCl_2.4H_2O$, 0.0002 g/L $ZnSO_4.7H_2O$, 0.0003 g/L $Na_2MoO_4.2H_2O$, 0.00008 g/L $CuSO_4.5H_2O$, 0.00005 g/L and $Co(NO_3)_2.6H_2O$ and the culture flasks were placed on the window seal that exposed it to the natural light, photoperiod and temperature conditions.

Figure 1. The sampling location, a) Satellite image taken from Google maps; b) photograph of the wetland water body from where the water was taken for algae isolation

2.2 *Effect of synthetic wastewater on cyanobacterium*

Synthetic wastewater (SWW) [15] was prepared with addition of sucrose to obtain the chemical oxygen demand (COD) unit of 100 mg/L. This COD concentration represents the expected levels of organic compounds remained in the wastewater after primary anaerobic and aerobic treatments. The standard BG-11 (N+) medium was used as control treatment. To study the uptake rate of nitrate, 0.1 g/L $NaNO_3$ was added to both the media. The experiment was set up in the in-house built air lift vertical tubular photobioreactors (Figure 2) and was kept near the North facing window which provided approximately 12:12 of photoperiod and average 25 °C of temperature. The reactor tubes included one without inoculum BG-11 control, two biological treatments for BG-11, one without inoculum SWW control and two biological treatments for SWW, as shown in Figure 2. Each tube of the reactor contained 800 ml culture medium. The tubes with BG-11 and SWW biological treatments were inoculated with 50 ml cyanobacteria culture which was equivalent of 10 mg dry weight biomass. The cell counting method could not be adopted for measuring the growth as the long filaments of cyanobacteria spanned multiple squares of the haemocytometer counting grid. The biomass productivities were calculated using the gravimetric method as explained in next section.

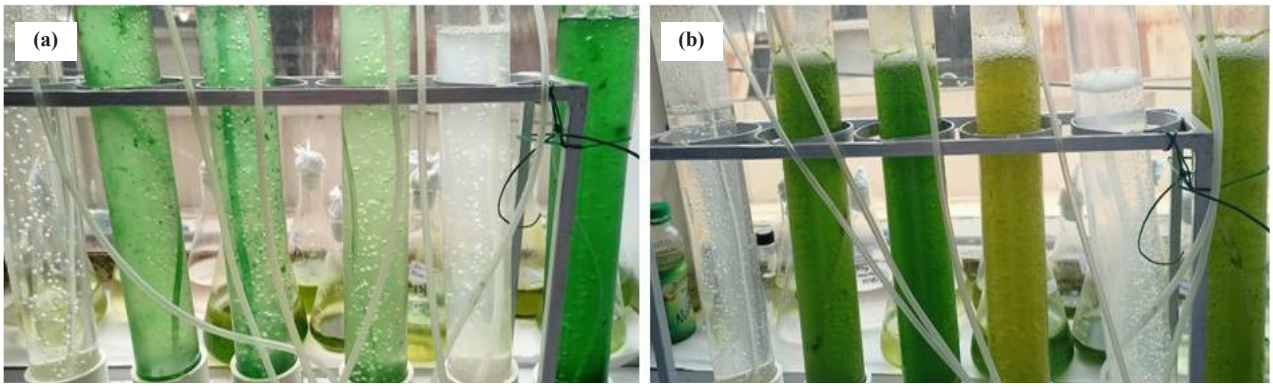

Figure 2. The air-lift tubular photobioreactors were placed near the window to provide natural sunlight and uncontrolled temperature conditions. The tubes from left to right in both images are as follow: i) BG11 control (without inoculums), ii-ii) BG-11 a-b, iv) SGW a, v) SGW control (without inoculums), vi) SGW b. a) After 3 days of cultivation; b) After 10 days of cultivation

2.3 *Biomass sampling for dry weight, nitrate and phosphate measurements*

From each of the experimental tubes, 50 ml aliquots were drawn for biomass and nutrient measurements. Both nitrate and phosphate cultures were filtered using Whatman filter paper and 10 ml filtrate was taken for the nutrient analysis. The analytical tests were performed using the Hanna reagent kits for the nitrate-cadmium reduction (HI93728-01) and phosphate-ascorbic acid (HI93713-01) methods. The measurements were taken at the onset and at the end of the experiment using a multiparameter Environmental Photometer (HI83306-02, Hanna Instruments). For the dry weight gravimetric measurements, 30 ml culture was filtered through Whatman filter paper and the biomasses were dried overnight at 70 °C. The biomass productivities were calculated from initial and final values.

2.4 *Gas chromatographic analysis of fatty acids methyl esters*

The dried biomass samples were saponified using 1 ml of methanolic sodium hydroxide (45 g NaOH in 150 ml methanol and 150 ml distilled water) by heating for 30 minutes in a boiling water bath followed by methylation (325 ml 6N hydrochloric acid in 275 ml methyl alcohol) by heating at 80 °C for 10 minutes. The methyl esters were extracted into the organic phase using a mixture of hexane and methyl tert-butyl ether followed by base wash using aqueous sodium hydroxide solution. The fatty acid methyl esters were analyzed through a flame ionization detector by running the samples in Agilent Gas Chromatograph and the peaks were identified using the Sherlock MIS Software [16]. The Gas chromatography conditions were set as follows: temperature program ramped from 170 °C to 270 °C at 5 °C per minute using hydrogen as the carrier gas, nitrogen as make-up gas and air to support the flame. The final ballistic increase to 300 °C allowed cleaning of the column (25 mm × 0.2 mm phenyl methyl silicone fused silica capillary column) by holding for 2 minutes.

3. Results and discussions

The newly isolated strain of cyanobacterium is an unbranched filamentous belonging to the family *Oscillatoria*ceace and has been morphologically identified as *Oscillatoria* sp. due to gliding movement and the lack of sheath around the filaments (Figure 3). Our isolation studies found that this is the most common cyanobacterium strain, living in the water bodies of district Darjeeling, located in the sub-Himalayan northeastern region of India. The species-level identification is beyond the scope of this study. *Oscillatoria* is among the dominant genus of the *Oscillatoria*ceae family of cyanobacteria. As it is found colonizing the plastic waste in the water bodies in various parts of India; it can play critical roles in domestic wastewater treatment and waste management [17].

The main factors that make microalgae suitable for wastewater treatment include rapid growth rate, low production cost, potential to remove pollutants and a high tolerance to extreme environmental conditions. Some commonly used genera are *Chlorella, Scenedesmus, Desmodesmus, Botryococcus, Nannochloropsis* [18]*, and Chlorococcum* [19]. In the present research, we used the cyanobacterium culture without any acclimatization in the synthetic wastewater. Interestingly, the cyanobacterium attained biomass productivity in the synthetic wastewater at par with the control BG-11 (N+) growth medium (Table 1). The intent was to; utilize the algae to treat the blackwater effluent originating after the successive anaerobic and aerobic treatment processes. The experiment was run under conditions that mimicked the outdoor environmental conditions, by placing the reactor near the window which on average provided 12 hours 30 minutes of natural day length. The experiment was conducted in the last week of April 2023 with average temperatures of 25-26 °C.

The experimental tubes were not fully closed and were bubbled with unfiltered air which encouraged an initial growth of bacteria. The bacteria could have contributed to the oxidization of sucrose in the synthetic wastewater medium. As a result, we observed a longer lag phase for *Oscillatoria* cells growing in the synthetic wastewater. The oxidation of complex organic compounds by bacteria contributes towards the production of inorganic compounds such as CO_2, NH_4^+, and PO_4^{3-} which are photosynthetically utilized by microalgae for biomass production [18].

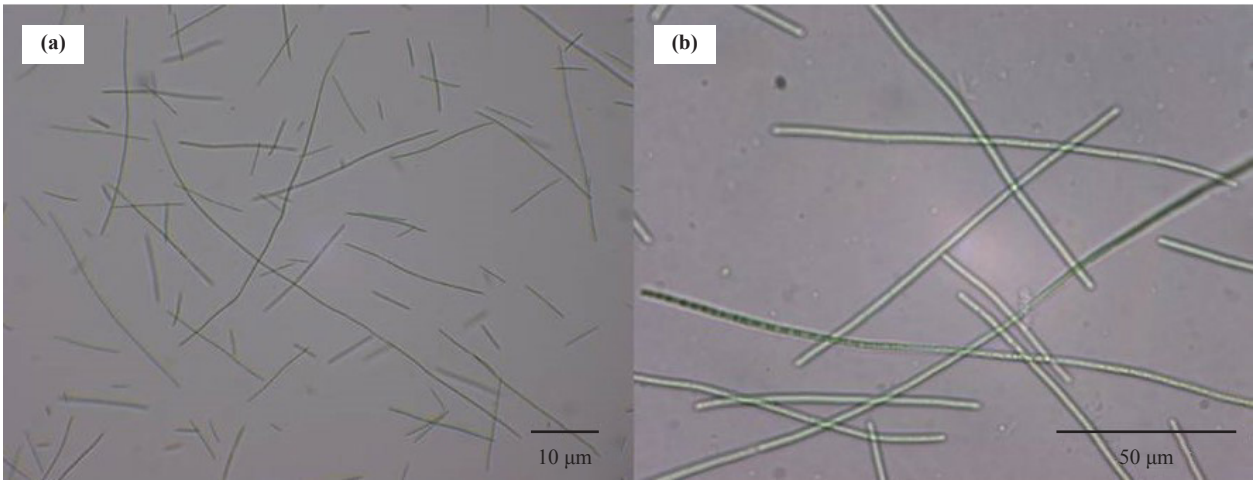

Figure 3. Microscopic images of the isolated strain of Oscillatoria sp. a) Filaments under 100 X magnification; b) Filaments under 400 X magnification

Microalgae can utilize both organic and inorganic compounds present in the wastewater [18]. Within the scope of this study, we measured the uptake of inorganic nutrients namely nitrate and phosphate by microalgae. Since we were cultivating the microalgae under an uncontrolled environment, we had anticipated bacterial growth. With this rationale, we maintained two control reactor tubes that were not inoculated with the cyanobacteria. The no-organisms BG-11 and SGW controls allowed us to examine the microalgae-specific reduction in (Figure 2a) nitrate and phosphate concentrations. The nutrient levels in the two controls at the onset of the experiment and at the time of final measurements (after 9 days of cultivations) did not show much difference, and therefore these values were used to derive the % removal of nutrients by the cyanobacteria. With respect to the nitrate, we did not observe a marked difference in BG-11 and synthetic wastewater nutrient media (Table 1). Even though the synthetic wastewater was highly enriched, the cyanobacterium showed a remarkable removal efficiency of 94% of the phosphates (Table 1).

Table 1. Comparison of biomass productivities and nutrient uptake efficiencies of ***Oscillatoria*** sp. in BG11 growth medium and synthetic wastewater. * Measurements were taken after 9 days of cultivation. In controls, there were no inoculums and therefore the biomass productivities and removal of nutrients are shown as nil (negligible) and NA (not applicable)

Reactor tube	BG control	SWW control	BG	SWW
Biomass productivity (g/L/day)*	nil	nil	0.05 ± 0.007	0.065 ± 0.021
NO_3 (mg/L)*	83.5	76	6.3 ± 2.12	5.3 ± 2.26
NO_3-N (mg/L)*	19	17	1.4 ± 0.56	1.2 ± 0.56
NO_3 (% removal)	NA	NA	92.45	93.02
PO_4 (mg/L)*	23	51	8.2 ± 1.7	3 ± 0.57
P (mg/L)*	7	17	2.6 ± 0.56	1.0 ± 0.28
PO_4 (% removal)	NA	NA	64.34	94.11

The symbiotic microalgae-bacteria associations have been exploited for the treatment of municipal wastewater [20]. The growth of bacteria in such studies depends on the amount of organic matter or the COD levels of the wastewater. In the present study, the COD of 100 mg/L led to an initial proliferated growth of bacteria which subsided in a day or two, followed by the profuse growth of cyanobacterium. The tubular photobioreactor used in this study achieved similar areal biomass productivity of 6.5 g/m^2/day as obtained in a raceway-biofilm reactor hybrid growth system (6.1-6.79 g/m^2/day) treating real municipal wastewater with microalgae-bacteria assemblages containing COD, NH_3-N, NO_3-N and P of 116 mg/L, 37.3, 1.6 and 5.2 respectively [21]. With respect to the nutrient removal rates, our study with synthetic wastewater achieved considerably higher percentage removals for both N and P. For comparisons, the *Oscillatoria* tenuis grown in secondary effluents of municipal wastewater containing lower amounts of P (0.8 mg/L), showed 82.9% removal efficiency [22].

In addition to the high nutrient removal potential, *Oscillatoria* species display favorable traits for easy harvesting using filtration and auto floatation (Figure 4). The species of *Oscillatoria* are excellent candidates for treating wastewater owing to their higher growth rates as compared to other cyanobacteria and large size which allow easy solid-liquid separation at the harvesting stage [23]. When utilizing microalgae for algae-based wastewater treatment systems, the operation costs are increased due to harvesting challenges with small-size strains [24]. In this regard, the filamentous mat-forming strains of *Oscillatoria* should be considered as economical and favorable species for the secondary wastewater treatment processes.

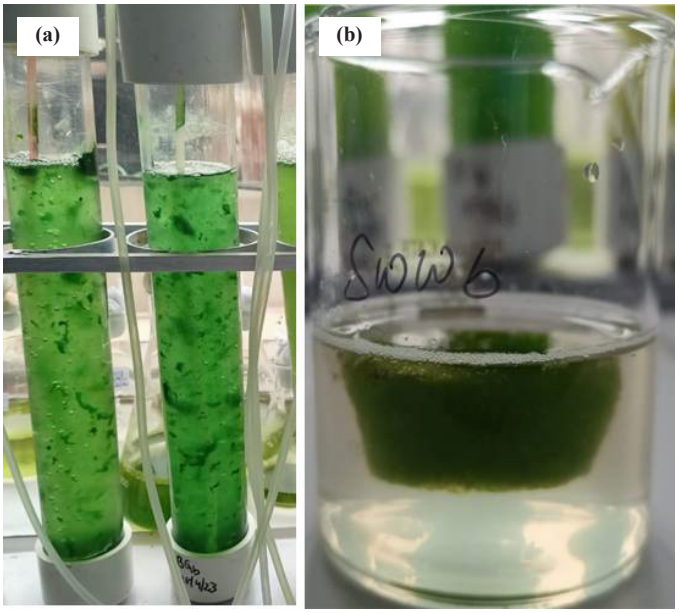

Figure 4. The mat forming cultures of Oscillatoria show excellent autoflotation. a) Culture tubes showing mat formation of the cyanobacterium filaments; b) beaker showing floating cyanobacterial mat

Microalgae including cyanobacteria are a rich source of lipids and fatty acids which are potential feedstock for valuable commercial products, particularly transportation fuel and nutraceuticals [25]. The *Oscillatoria* culture maintained in our laboratory in BG-11 medium showed 19.75% dry weight lipids (unpublished data) that conform to the reported values found in the literature. The two species *Oscillatoria* chlorina and *Oscillatoria* limosa showed similar percentages of total lipid content of 16% and 16.6% [26]. There occurs a wide variation in the reported values of total lipid content in different species of *Oscillatoria* ranging from 4-31.9%, which largely depend on the abiotic factors and most of these have been reported across different regions of India [8].

The fatty acid profiles in the total lipids of *Oscillatoria* were studied after 10 days of cultivation in both BG-11 (N) control and synthetic wastewater media. The cyanobacteria grown in SWW showed higher percentages of commonly occurring fatty acids namely lauric, myristoleic, mystic, palmitoleic, and arachidonic acid (Table 2). The percentage of

saturated palmitic acid was considerably reduced in SWW. The number of fatty acid methyl esters detected in SWW was slightly higher than in the control medium. The GC method and the fused silica column allowed us to identify more fatty acid methyl esters than usually reported in the literature. Our study confirms (Table 2) the earlier reports, where predominant fatty acids found in *Oscillatoria* were 16:0, 16:1, 18:1, and 18:3, based on which, the species have also been considered as promising biodiesel feedstock [27]. The hexadecanoic acid (16:0) methyl ester or palmitic acid is one of the dominant fatty acids found in microalgae [28]. Palmitic is a small saturated carbon chain and owing to its low oxidation and melting point; it is considered suitable for biodiesel production [8].

Table 2. Percentage composition of fatty acid methyl esters of ***Oscillatoria*** grown under different nutrient regimes. In parenthesis are the commonly found fatty acids known in algae

Fatty acid peak	IUPAC name	BG-11 (N)	SWW
10:0	Decanoic acid	-	0.11± 0.000
11:0 iso	9-Methyldecanoic acid	0.14 ± 0.000	0.24 ± 0.028
11:0	Undecanoic acid	-	0.14 ± 0.000
10:0 2OH	2-Hydroxydecanoic acid	-	0.04 ± 0.000
12:0 iso	10-Methylundecanoic acid	-	0.32 ± 0.000
12:0	Dodecanoic acid **(Lauric acid)**	0.945 ± 0.148	1.235 ± 0.134
13:0 iso	11-Methyldodecanoic acid	-	0.34 ± 0.042
13:0 anteiso	10-Methyldodecanoic acid	0.07± 0.000	0.29 ± 0.057
13:1 w3c	(10Z)-10-Tridecanoic acid	0.325± 0.021	0.35 ± 0.000
14:0 iso	12-Methyltridecanoic acid	-	0.525 ± 0.120
14:0 anteiso	11-Methytridecanoic acid	0.57 ± 0.127	0.715 ± 0.191
14:1 w5c	(9Z)-9-Tetradecanoic acid **(Myristoleic acid)**	**20.735 ± 5.183**	**25.25 ± 0.976**
14:0	Tetradecanoic acid **(Myristic acid)**	**20.485 ± 2.114**	**23.345 ± 2.708**
15:1 iso w6c	(8Z)-13-Methyl-8-Tetradecanoic acid	0.11 ± 0.000	0.115 ± 0.064
15:0 iso	13-Methyltetradecanoic acid	0.655 ± 0.276	1.01 ± 0.311
15:0 anteiso	12-Methyltetradecanoic acid	0.38 ± 0.170	0.41 ± 0.071
15:1 w6c	(9Z)-9-Pentadecanoic acid	0.285 ± 0.092	0.24 ± 0.042
16:0 aldehyde	Palmitaldehyde	0.33 ± 0.240	-
15:0	Pentadecanoic acid	0.42 ± 0.113	0.57 ± 0.071
16:1 w7c alcohol	(9Z)-9-Hexadecen-1-ol	0.56 ± 0.396	0.24 ± 0.014

Table 2. (cont.)

Fatty acid peak	IUPAC name	BG-11 (N)	SWW
16:3 w6c	(4Z-7Z-10Z)-Hexadecatrienoic acid	0.8 ± 0.608	0.39 ± 0.000
16:0 anteiso	13-Methylpentadecanoic acid	-	0.235 ± 0.007
16:0 iso	14-Methylpentadecanoic acid	0.235 ± 0.148	0.13 ±0.000
16:1 w7c	(9Z)-9-Hexadecanoic acid **(Palmitoleic acid)**	**15.19 ± 0.283**	**16.815 ± 1.803**
16:1 w5c	(11Z)-11-Hexadecanoic acid	3.635 ± 0.078	4.765 ± 0.163
16:0	Hexadecanoic acid **(Palmitic acid)**	**25.655 ± 4.815**	**14.88 ± 1.471**
16:0 10-methyl	10-Methylhexadecanoic acid	0.79 ± 0.849	0.56 ± 0.000
17:1 iso w9c	(7Z)-15-Methyl-7-Hexadecanoic acid	0.23 ± 0.042	-
17:0 iso	15-Methylhexadecanoic acid	0.3 ± 0.000	0.16 ± 0.000
17:0 cyclo w7c	cis-9,10-Methylene-Hexadecanoic acid	0.26 ± 0.170	0.29 ± 0.085
17:0	Heptadecanoic acid **(Margaric acid)**	0.225 ± 0.134	0.15 ± 0.000
18:3 w6c	(6Z,9Z,12Z)-Octadecatrienoic acid **(GLA)**	-	0.715 ± 0.035
18:2 w6c	(6Z,9Z)-Octadecadienoic acid **(LA)**	-	0.35 ± 0.311
18:1 w9c	(9Z)-Octadeceneoic acid **(Oleic acid)**	0.27 ± 0.226	0.39 ± 0.014
18:1 w7c	(11Z)-Octadecenoic acid	1.3 ± 0.000	2.295 ± 0.219
18:1 w5c	(13Z)-Octadecenoic acid **(cis-13-Oleic acid)**	3.07 ± 0.000	0.24 ± 0.000
18:0	Octadecanoic acid **(Stearic acid)**	0.31 ± 0.212	0.345 ± 0.205
18:1 w7c 10-methyl	(11Z)-10-Methyl-11-Octadecenoic acid	0.09 ± 0.000	0.35 ± 0.014
19:1 w7c	(12Z)-Nonadecenoic acid	-	0.13 ± 0.042
20:4 w6c	(5Z,8Z,11Z,14Z)-Icosatetraenoic acid **(Arachidonic acid)**	0.09 ± 0.000	0.255 ± 0.035

The reported biomass productivities and lipid content reported in the literature are generally measured on a smaller scale under controlled and optimized abiotic conditions. However, when the microalgae are cultivated in outdoor conditions, both biomass and lipid or fatty acid productivities become variable due to constantly changing environmental conditions. The mandatory requirement for microalgae or cyanobacterial biodiesel to become an economical substitute is outdoor cultivation using wastewater. Therefore, we need more studies that are conducted under uncontrolled natural conditions, so that we can assess the real performance of microalgae under the variable influences of both abiotic and biotic factors. The use of wastewater can reduce the biomass and affect lipid composition in microalgae [29]. Nutrient limitation and temperature can alter the lipid profiles in microalgae [30]. As the present study was only conducted in the

month of April, the future scope of this work is to assess the fatty acid profiles under the influence of seasonal variations in light and temperature. In our future studies, we would optimize the synthetic wastewater medium for enhancing the biomass and lipid productivities using varied sources of organic carbon such as acetate.

4. Conclusions

The present mesocosm study was undertaken to determine the potential of filamentous mat-forming cyanobacterium *Oscillatoria* sp. to achieve competitive growth in synthetic wastewater with COD of 100 mg/L under natural environment conditions. The newly isolated species of cyanobacterium equally performed well in both the BG-11 (N+) control medium and the synthetic wastewater which also contained 19.7 mg/L of NH_3. The cultures grown in synthetic wastewater showed considerably high percentage removal for phosphorous and produced comparatively higher amounts of predominant fatty acids than in the control BG-11 (N+) medium. The present study found that *Oscillatoria* sp. attained higher growth in synthetic wastewater under natural conditions and is a suitable candidate to grow in outdoor cultivation systems such as raceway ponds or high-rate algal ponds that are mandatory for economical nutrient removal from wastewater.

Conflict of interest

All the authors listed in this manuscript hereby declare that they have NO affiliations with any other organization or entity with any financial or non-financial interests. We also certify that there is NO conflict of Interest. The research undertaken for this manuscript has been solely funded by Climate Survival Solutions, Inc.

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