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Short Communication

Effect of nitrogen concentration on lipid productivity and fatty acid composition of *Monoraphidium* sp.

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HIGHLIGHTS

- Nutrient concentrations in optimum amount increases growth rate along with accumulation of lipids.
- Increased productivity and lipid profile determine an appropriate time for harvesting.
- Algal growth with lower nitrogen concentration is beneficial for enhancing lipid productivity in large scale production.

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ABSTRACT

Green algae, *Monoraphidium* sp. T4X, was isolated locally, in New Delhi, India and identified as a potential source of biofuel. The study focuses on the effect of nutritional amendments and their uptake rates with respect to growth and change in fatty acid composition of the species. The lipid productivity and fatty acid profile were investigated and compared under six different nitrogen concentrations. Of the tested concentrations, cultures with nitrate concentration 0.36 g/l exhibited higher lipid productivity (0.18 g/l/day) with optimum content of all fatty acid compositions (SFA = 37.22, MUFA = 39.19, PUFA = 23.60) with appropriate biodiesel properties. The right phase for harvesting microalgae was also investigated on the basis of the growth curve.

1. Introduction

Globally, there has been rising interest in using microalgae as a potential source of biofuel. This is due to higher growth rates and the potential to accumulate higher amounts of lipids than conventional oil crops in per unit area. Microalgae are also known for their metabolic flexibility which means that by varying the cultivation conditions of algae, regulation of variation in biochemical composition of the biomass can be achieved (Pascal et al., 2012).

The major parameters determining the possibility of using microalgae as a potential source of biofuels are economic viability achieved through biomass productivity, lipid productivity and type of fatty acids. While it is documented in the literature that lipid production is more under stress conditions and stress can also be created by nutrient deficiency, studies on nutrient removal efficiency and uptake rates are limited. Therefore, more knowledge is required to investigate over it, such that it can be applied to understand the mechanism of growth and lipid accumulation.

Previous studies considering growth and lipid productivity from freshwater microalgae have been largely performed on *Chlorella* and *Scenedesmus* sp. Studies on lipid production in *Monoraphidium* sp. are limited and major studies on this species are restricted to carotene production. To the best of our knowledge there has been no study which defines the right phase to extract lipids from algal biomass.

In the present study, *Monoraphidium* sp. T4X, was selected for studying the lipid productivity on selected medium with appropriate nutritional amendments. This strain of algae was selected because of its higher growth rates, compared to other strains that were isolated. Its effect on removal of nutrients was also evaluated. Furthermore, the right time to harvest and extract lipids from algal biomass was determined.

2. Methods

2.1. Microalgal strain and culture conditions

Microalgal strain, *Monoraphidium* sp. T4X, was collected and isolated from a fresh water pond in India Habitat Centre, New Delhi, India (28.5897°N, 77.2249°E). The strain was maintained in

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liquid BG11 medium consisting of (g/l): K_2HPO_4 (0.04), $MgSO_4{\cdot}7H_2O$ (0.075), $CaCl_2{\cdot}2H_2O$ (0.036), Citric acid (0.006), Ferric Ammonium Citrate (0.006), $NaHCO_3$ (0.02), Na_2EDTA (0.001), $NaNO_3$ (1.5). The pH of the medium was adjusted to 7.5. The medium was sterilized at 121 °C for 15 min. *Monoraphidium* sp. cultured in BG11 medium (Kaushik, 1987) was maintained in a 250 ml glass jar as stock, incubated at 25 °C ± 2.

2.2. Experimental procedure

Experiments were performed in three essentially similar batches differentiated only for consumption in further analysis. The factors differentiating the three batches were based on time and phase at which they were harvested for further analysis; first batch was harvested after a period of 15 days (a period after the beginning of exponential phase). Second batch was harvested after 25 days (exponential phase) and harvesting was done after 35 days for the third batch (start of stationary phase). These harvests were made depending on the growth curve of *Monoraphidium* sp. T4X. Batch experiments were performed and removal of nitrogen and phosphorous were evaluated.

Medium BG11 with six different concentrations of sodium nitrate (g/l) – 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0, giving N:P ratio of 0.153, 0.307, 1.53, 3.07, 4.61 and 6.15 respectively. The nitrate concentration corresponding to 0.036, 0.072, 0.36, 0.72, 1.09 and 1.45 respectively, were added into 1 l Erlenmeyer flask and working volume was kept as 800 ml. Strain was inoculated into the solution at a proportion of 10% (v/v). Culture flasks were operated under 16:8, light and dark conditions at an rpm of 160.

2.3. Analytical method

2.3.1. Nutrient analysis

Culture samples (4 ml) for growth and nutrient measurements were taken at an interval of 2 days until it was harvested. Collected samples were filtered through glass fibre filters for analysis. Nutrients were analysed photometrically using standard methods from American Public Health Association (APHA). Nutrient uptake rates (NUR, mg-Nutrient g^{-1} d^{-1}) which refers to the nutrient taken up by unit mass of algae and Nutrient removal efficiency (NRE, %), were calculated as follows:

$$\mathrm{NUR} = (C_o - C_t)V/\mathrm{DW}/t;$$

$$\mathrm{NRE} = 100 - (100 \times C_t/C_o)$$

where, C_o and C_t are the nutrient concentrations (mg/l) at the beginning of the experiment and end of the experiment respectively, V is the volume of water (l), DW is algal dry weight (g) and t is the time interval (days).

2.3.2. Esterification and fatty acid analysis

The three batches of samples were harvested by continuous centrifugation (5000 rpm for 5 min) after completion of their respective time. Concentrated algal samples was frozen overnight at −20 °C and freeze dried under vacuum. Algal mass was accurately weighed and lipid extraction was carried out according to Folch et al. (1957) method.

Lipid content (%) and lipid productivity (g/l/day) were calculated as follows;

$$\text{Lipid content } (C_{\mathrm{lipid}}) = (\text{wt. of lipid}/\text{wt. of sample}) \times 100$$

$$\text{Lipid productivity} = (C_{\mathrm{lipid}} \times \mathrm{DCW})/t$$

where, C_{lipid} is the lipid content (%), DCW is dry cell weight (g/l) and t is the time interval (days).

Lipid extracts were converted to methyl esters with methanolic HCl and hexane. Fatty acid methyl esters (FAME) were prepared by adding 1 ml of concentrated HCl with 5 ml methanol. The mixture was heated at 80–90 °C in a water bath for 30 min. Hexane (1 ml) was added into the vial after methylation. Top hexane layer containing methyl esters were placed into GC vials for GC analysis (Agilent 6890N (USA)) equipped with a DB-5 column (0.2 mm ID, 30 m, 0.25 mm film by Agilent). The temperature program was started at 2 °C and was increased by 50 °C min^{-1} to 250 °C. Peaks were integrated with Chemstation and identified by comparison of retention times with pure standard (Sigma). System performance was checked with blanks and standard samples prior to analysis. Concentration was expressed in mg/ml which was converted to percentage. All the tests were performed in triplicates. Degree of unsaturation is calculated to determine their potential as a biofuel on the basis of a significant biofuel property, cetane number (Ramos et al., 2009).

$$\text{Degree of unsaturation } (\mathrm{DU}) = \mathrm{MU} + 2(\mathrm{PU})$$

where, MU is the percentage of monounsaturated fatty acids and PU is the percentage of polyunsaturated fatty acids.

3. Results and discussion

3.1. Effect of nitrogen concentrations on growth kinetics

Many microalgal species when induced with stress are known to accumulate considerable quantities of lipid leading to high yields of oil. But previous studies have stated that high lipid content is usually accompanied by lower growth rates which often lead to decreased biomass and lipid productivities (Huerlimann et al., 2010). There are two important traits which are responsible for increasing lipid productivity. Firstly the high lipid efficiency with moderate growth as in *Botryococcus braunii* where the lipid content reached to 50% with 28 mg/l/day of biomass productivity (Dayananda et al., 2007). Secondly, low lipid content but high cell growth as in *Chlorella vulgaris* which accumulated 20% of lipid content with 19 h doubling time (Griffiths and Harrison, 2009). Therefore, it is important to consider lipid productivity along with biomass productivity to evaluate the overall performance of the strain being induced.

In this study, the twin effects of lipid content and biomass were used as an assessment method to evaluate overall lipid production per unit of volume under different nutrient concentrations. Growth rates for *Monoraphidium* sp. T4X grown in six different nitrate concentrations for a period of 15 days are given in Table 1. It is clearly evident that the growth rate of this species is not significantly affected by nitrate concentrations. It can also be observed from Table 1 that with nitrate concentrations in the range,

Table 1
Biomass, lipid content and lipid productivity of *Monoraphidium* sp. T4X under six different nitrate concentration.

NO_3^- concentration (mg/l)	0.036	0.072	0.36	0.72	1.09	1.45
Biomass (g)	0.15 ± 0.02	0.16 ± 0.01	0.2 ± 0.00	0.19 ± 0.02	0.23 ± 0.02	0.21 ± 0.01
Biomass productivity (mg/l/day)	10	11	13	13	15.3	14
Specific growth rate (day^{-1})	0.064	0.089	0.075	0.075	0.083	0.052
Lipid content (%)	18.42 ± 0.4	15.57 ± 0.8	14.30 ± 0.5	11.85 ± 0.1	11.57 ± 0.6	10 ± 0.7
Lipid productivity (g/l/day)	0.18	0.16	0.19	0.15	0.17	0.14

0.036–1.09 g/l, the growth rates were not affected but at concentration – 1.45 mg/l, there was decline both in the biomass and lipid production, although, biomass productivity was still higher than the ones at lower nitrate concentration. But growth rate with concentration 1.45 mg/l, is much lower when compared to others. Thus, it can be concluded that after a certain level of tolerance, higher nutrient concentrations become toxic for algal survival. As previously stated, this is because there is increase in the activity of nitrate reductase at higher concentrations of nitrate leading to enhanced production of nitrite and ammonia that are accumulated in vivo. Therefore, the accumulated nitrite and ammonia act as toxins, resulting in decrease in biomass production (Jeanfils et al., 1993).

3.2. Effect of N:P ratio on nutrient removal and nutrient uptake

Nitrogen and phosphorous uptake rates were significantly dependent on the nitrate concentration ($P > 0.05$). The N and P uptake rates decreased from 4.76 to 3.70 mg N g^{-1} d^{-1} and from 0.35 to 0.17 mg P g^{-1} d^{-1}, respectively with the increasing nitrate concentrations (Table 2). It was evident from one way ANOVA that the N and P uptake rates for nitrate concentration having N:P ratio lower than 3.07 were significantly higher than the ones above this. Dong et al. (2011), reported that the N:P ratio had a substantial effect on nutrient usage and found that N:P ratio of 7.4, the lowest concentration studied in the range 7.4–28.5 was optimum for macroalgae *Laminaria japonica*. Similarly, Aslan and Kapdan (2006) found N:P ratio of 8.1 was favourable for *C. vulgaris*. In our study, we have observed that the nutrient uptake rate with N:P ratio 0.153 is highest. But if we evaluate in accordance with the growth rate and lipid productivity, N:P ratio of 1.53 giving optimum biomass and highest lipid productivity should be considered, since, this ratio is also statistically significant.

It was observed that, as nitrogen concentration is lowered, there is an increase in consumption of phosphates from the medium. Thus, it can be said that nitrate concentration can have a significant effect on phosphorous usage. This is an intriguing observation which needs to be further investigated.

3.3. Effect of N:P ratio on lipid productivity

Fatty acid composition is an important and apposite parameter for determining the capability of a species for being a potential candidate for biodiesel production. Therefore, fatty acid profiles of *Monoraphidium* sp. under six different nitrate concentrations have been investigated (Table 3). Nitrate and phosphate concentrations being the major basic elements often limit growth as well as lipid accumulation and seem to have their own independent affect on the algal strain. Quantitative differences were observed in lipid productivity and fatty acid profiles on the six concentrations of nitrate. Previous studies have indicated, that with an increase in the nutritional stress, a general trend towards the accumulation of lipids is observed as the algae alter their biosynthetic pathways to produce storage lipids (Hu et al., 2008). Herein, we can see that there is no evident trend in the profile of individual fatty acids. But, if we consider them together as saturated, monounsaturated and polyunsaturated fatty acids, it can be said that nitrate concentration 0.36 mg/l has an optimum content of all the fatty acid compositions (SFA = 37.22, MUFA = 39.19, PUFA = 23.60) needed to accomplish the appropriate biodiesel properties in accordance with lipid productivities (Table 1). Also, a significant biodiesel quality, cetane number, which is the measurement of fuel's ignition delay, is associated with high content of saturated fatty acids such as palmitic acid and stearic acid. The concentrations associated with higher degrees of unsaturation as observed in Table 3, lead to lower cetane numbers.

In the study, we can see that nitrate concentration of 0.36 g/l can be used since it has an optimum mixture of saturated fatty

Table 3
Comparision of fatty acid profile of *Monoraphidium* sp. T4X under six different N:P ratios.

Nitrate concentration	0.036	0.072	0.36	0.72	1.09	1.45
Saturated	*3.91*	*17.24*	*37.22*	*21.73*	*28.54*	*22.36*
Monounsaturated	*66.72*	*41.95*	*39.19*	*45.74*	*49.08*	*37.79*
Polyunsaturated	*29.37*	*40.81*	*23.60*	*32.53*	*22.38*	*39.85*
DU	125.47	123.58	86.38	110.79	93.85	117.49

Table 4
Comparision of fatty acid profile of *Monoraphidium* sp. T4X, under three different phases of growth.

Days	15	25	35
NO_3^-	0.36 mg/l		
C14	3.84	8.67	4.11
C15	2.48	1.84	–
C16	2.44	3.87	4.95
C17	9.08	11.01	1.63
C18	6.86	10.82	6.65
C20	7.01	3.42	3.52
C23:0	5.51	–	2.57
C14:1	3.13	4.81	5.35
C15:1	11.39	3.54	3.48
C16:1	1.35	8.21	1.72
C17:1	7.99	1.85	3.42
C18:1	2.74	4.41	7.04
C20:1	–	–	4.38
C21:1	3.47	2.14	4.35
C22:1	4.62	4.36	2.88
C24:1	4.49	6.11	6.41
C18:2 TRANS	3.49	2.75	2.60
C18:3 (6)	4.61	15.89	15.15
C18:3 (3)	2.84	1.57	5.42
C22:2	6.01	–	1.71
C20:2	6.65	1.62	5.28
C20:3	–	–	2.44
C20:4	–	3.10	4.93
Saturated	*37.22*	*39.64*	*23.43*
Monounsaturated	*39.19*	*35.42*	*39.03*
Polyunsaturated	*23.60*	*24.93*	*37.54*
DU	*86.38*	*85.29*	*114.11*

Table 2
Removal efficiency and uptake rates of *Monoraphidium* sp. T4X under six different nitrate concentrations, based on initial and final concentrations of nitrogen and phosphorous.

NO_3^- concentration (mg/l)	N:P ratio	N concentration (mg/l)		NRE (%)	NUR (mg N g^{-1} d^{-1})	P concentration (mg/l)		NRE (%)	NUR (mg P g^{-1} day^{-1})
		Initial	Final			Initial	Final		
0.036	0.153	15.28 ± 0.09	1.02 ± 0.01	93.36	4.76	1.4 ± 0.11	0.34 ± 0.00	75.71	0.35
0.072	0.307	16.61 ± 0.09	3.07 ± 0.01	81.52	4.23	1.47 ± 0.01	0.42 ± 0.02	71.43	0.33
0.36	1.53	17.16 ± 0.01	4.22 ± 0.01	75.41	3.40	1.37 ± 0.02	0.43 ± 0.02	68.61	0.25
0.72	3.07	31.19 ± 0.02	17.22 ± 0.00	44.79	3.68	1.49 ± 0.06	0.7 ± 0.10	53.02	0.21
1.09	4.61	35.42 ± 0.01	19.25 ± 0.02	45.65	3.51	1.5 ± 0.01	0.56 ± 0.04	62.67	0.20
1.45	6.15	49.47 ± 0.06	33.92 ± 0.00	31.44	3.70	1.24 ± 0.00	0.53 ± 0.01	57.26	0.17

Table 5
Biomass, lipid content, lipid productivity and removal efficiency and uptake rates of *Monoraphidium* sp. T4X, under three different phases of growth based on initial and final concentrations of nitrogen and phosphorous.

Days	Biomass (g)	Lipid content (%)	Lipid productivity (g/l/day)	N concentration (mg/l)		NRE (%)	NUR (mg N g^{-1} d^{-1})	P concentration (mg/l)		NRE (%)	NUR (mg N g^{-1} d^{-1})
				Initial	Final			Initial	Final		
15	0.2 ± 0.00	14.3 ± 0.5	0.19	17.1 ± 0.07	4.2 ± 0.02	75.39	3.40	1.3 ± 0.05	0.4 ± 0.01	68.61	0.25
25	0.2 ± 0.01	15.0 ± 0.2	0.17	19.5 ± 0.09	2.7 ± 0.01	86.20	1.75	1.3 ± 0.06	0.2 ± 0.04	79.14	0.19
35	0.4 ± 0.01	16.2 ± 0.38	0.19	20.2 ± 0.01	2.2 ± 0.04	89.11	0.94	0.5 ± 0.02	0.1 ± 0.06	81.36	0.06

acids and monounsaturated fatty acids, which can lead to an optimum cetane number and can be in order to accomplish the set standards for biodiesel properties. But it can also be observed that nitrate concentration 0.036 g/l has higher monounsaturated fatty acids which could lead to higher cetane number, but the amount of saturated fatty acids, accounting only from palmitic acid, is too low. Hence, media with nitrate concentration, 0.36 g/l can be considered for culturing *Monoraphidium* sp. T4X.

3.4. Effect of different harvest time on fatty acid composition

The medium with the optimum nitrate concentration (0.36 g/l) as concluded above is taken and compared on the basis of different harvest time. The three batches harvested at different phases of the growth cycle (15, 25 and 35 days) are evaluated on the basis of fatty acid profile. As per the observations of lipid productivity, nutrient uptake rate and lipid profile, it can be inferred that both phases (15 days and 25 days) can be taken into consideration for harvesting the biomass on different grounds. If there is a need of an optimum removal of nutrients with slightly higher lipid productivity, as in case of treating wastewater, which mainly focuses on removal of nutrients from wastewater, the harvest can be done after a period of 15 days, keeping in mind the minimum limit to which it has to be brought down, such that, the remaining nutrients make the wastewater fit for agricultural and other similar uses. But, if the main focus revolves only around the lipid productivity, then, a period of 25 days for growth can be observed. Also, it was observed that with increase in cultivation time, there was a slight increase in palmitic acid (C16:0) and oleic acid (C18:1) content (Table 4). Harvesting after 25 days leads to an increased production of fatty acids that are responsible for its application as biofuel. The increase in fatty acid content after 25 days is observed due to an increase in the nutrient uptake from the medium (Table 5), leading to building up a stress due to nutrient depletion. If we consider them as saturated, monounsaturated and polyunsaturated fatty acids, higher saturated and monounsaturated fatty acids are present in both phases (15 days and 25 days). The degree of unsaturation and the culture time taken to attain comparable content of fatty acids are similar. Also, lipid productivity of both phases is comparable (Table 5). But, it is evident that with a decline in the nutrient availability, there was reduction in the nutrient uptake. Thus, it can be inferred that the harvest to obtain biomass with optimum growth, lipid productivity and fatty acid composition can be done as early as 15 days if the removal of nutrients is of utmost importance and can be done after 25 days, if lipid productivity is the sole focus.

4. Conclusion

Growth and lipid productivity of green algae, *Monoraphidium* sp. T4X, was tested for an optimum nitrate concentration in BG11 medium for its potential as a biofuel feedstock. The results of the study suggest an optimum nutrient concentration and appropriate harvest time, hence reducing the cost for production of *Monoraphidium* sp. T4X. The findings open a window into researching and manipulating dosage requirement of nutrients for large scale production with novelty of stating the right time to harvest algal culture and allow us to investigate further on the prospect of practically culturing and harvesting algal culture on every day basis yielding optimum growth and lipid productivity.

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