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Research Paper

Studies on the Effect of Nitrogen Nutrition on Growth and Lipid Content of Microalga *Botryococcus braunii KSV 1*

Biofuels

KEYWORDS : Microalgae, Lipids, Biofuel, Nitrogen depletion, biomass and lipid productivity.

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ABSTRACT

Sodium nitrate at a concentration of 17.6mM (1X) is the preferred nitrogen source for enhancing the biomass and lipid productivity of Botryococcus braunii KSV1 in BG -11 medium over different concentrations of other nitrogen sources viz., potassium nitrate, ammonium sulphate, ammonium nitrate and urea. Optimal biomass productivity (0.0521g/L/day) and lipid productivity (around 10 mg/L/day) for this microalga were obtained with 1.0X and 0.75X strength BG-11 medium respectively. Botryococcus braunii KSV1 grew better in BG-11 medium at strength 1.0X, whereas 0.5X - 0.75X strengths increased lipid production.

1. INTRODUCTION:

Microalgae are recognized biofuel feed stocks since 1940s (Harder & von Witsch, 1942) though the concerted efforts on the commercial exploration came up during last 2 decades only. With relatively short generation time, low space requirement and potential for high lipids accumulation, microalgae are superior resources for biofuel production (Li et al., 2008a; Li et al., 2008b; Li et al., 2014; Singh &Mallick, 2014) and alternatives of petroleum, the hydrocarbon feedstock (Bajhaiya et al., 2010). Amongst the several oil producing microalgae (Rodolfi et al., 2009), *Botryococcus* is one high lipid accumulating fresh water microalga (Chisti, 2008; Raja et al., 2008; Hannon et al., 2010; Picazo-Espinosa et al., 2011, Kommu et al., 2014), the productivity of which can be optimized through trials on various parameters. The major obstacle of high production costs can be addressed by maximizing algal lipid productivity.

Nutrient stresses/ limitations have been reported to enhance lipid production in several microalgae including *Botryococcus, Isochrysis, Dunaliella, Spirulina* species (Thomas et al., 1984; Maced & Alegre, 2001; Sharma et al., 2012; Rukminasari, 2013; Singh & Malik, 2014). Zhila et al., 2005 assessed the influence of nitrogen deficiency on the lipid classes, and fatty acid composition of the lipids in *B. braunii*. N & P starvation, while reducing the algal growth proved to be an efficient strategy for stimulating lipid production (Singh & Malik, 2014). Sharma et al. (2012) detailed the impact of nitrogen starvation on lipid accumulation in different microalgal species exhibiting lipid contents between 15 -40%.

The present study is an attempt to identify an appropriate nitrogen source and level for optimal biomass and lipid production by *Botryococcus braunii* KSV1, the free floating lipid producing microalga described by us from North Gujarat (Kommu et al., 2014).

2. MATERIALS & METHODS:

2.1 Organism and Culture Conditions:

Botryococcus braunii KSV1 maintained on BG-11 agar (Kommu et al., 2014) was cultivated in 50 mL of BG-11 medium (http://www.ccap.ac.uk/media/pdfrecipes.htm collected on 29.05.2012) in 250 mL flasks by incubatingat 20±1°C under 5.0±0.2 k lux light intensity with 16:8 hrs of light: dark cycles at 120 RPM. 10 mL of growth obtained after 7 days was used as inoculum for 100 mL BG-11 medium in 250 mL flasks incubated at identical conditions.

2.2 Nitrogen sources:

Taking the Nitrogen source and content of BG-11(17.6 mM/L sodium nitrate (SN), as the standard (X), the various nitrogen sources: ammonium sulphate (AS), ammonium nitrate (AN), potassium nitrate (PN), and urea (U) were substituted in BG-11 medium on nitrogen content basis for 0.5X, 1.0X and 2.0X concentrations of BG-11 nitrogen (Varsharani et al., 2011; Amin et al., 2013). 0.5X, 0.75X, 1.0X, 1.5X, 2.0X, 2.5X and 3.0X amounts of sodium nitrate were also tried (Table1) by taking BG-11 contents in volumes proportionate to achieve 0.5X, 0.75X, 1.0X, 1.5X, 2.0X, 2.5X and 3.0X levels. The flasks containing 100 mL of the varying N source/ content containing BG-11 media were inoculated with 10 mL of 7 days growth of *Botryococcus braunii* KSV1 in standard BG-11 medium and incubated for 4 weeks at parameters as in 2.1.

2.2 Determination of Growth & Lipid Content:

The incubated cultures were centrifuged 2-3 times with washings by distilled water and the biomass was oven dried. Biomass weights were recorded on alternate days and lipids in the biomass were extracted on 10^{th}, 20^{th} and 28^{th} day using Chloroform: Methanol: Water (10:5:1) mixture initially, followed by mixture of Chloroform: Methanol (1:1) as per the method of Ryckebosch et al. (2011). Cell debris, if any, in lipid fraction was filtered off through Whatman No.1 filter paper with solvent washings. Solvent evaporation resulted in gravimetric determination of Lipids. All the experiments were carried out in triplicate. Biomass productivity and lipid productivity were calculated using the formulae *(a) Biomass productivity* = $(X_t-X_0)/$ t where X_0 is initial biomass and X_t is biomass after incubation at t days of incubation *(b) Lipid Productivity* = $(L_t-L_0)/$ t where L_0 is initial lipid content and L_t is total lipid content after t incubation period.

3. RESULTS & DISCUSSION:

3.1Effect of Nitrogen Nutrition on Biomass and Lipid Content of *Botryococcus braunii* KSV1:

The data on the effect of various nitrogen sources on the Biomass and Lipid yields of microalga *Botryococcus braunii* KSV1 is presented in Table 1 and Fig. 1. The growth of *Botryococcus-braunii* KSV1 was best in BG-11 medium with sodium nitrate (**1.0X**), closely followed by that in potassium nitrate (**0.5X).** The treatments containing ammonium sulphate (**2.0X**), ammonium nitrate (**1.0X**) and sodium nitrate (**1.5X**) followed in descending order. The rest of the treatments showed appreciably low growth and low lipid yields. KSV1 preferred sodium nitrate over other nitrogen sources as was also observed in *Neochloris isoleoabundancs* by Li et al. (2008a), in another strain of *Botryococcus braunii* by Tyagi et al. (2014) and in *Chlorella* by Leesing et al. (2014). The biomass optima of 0.146 g/100mL and optimum biomass productivity of 0.0521g/ L / day for *Botryococcus braunii* KSV1 was observed in BG-11 with standard (1X) i.e. 17.6mM/ L sodium nitrate concentration. The biomass increased and lipid yields declined at N levels above or below the N level of BG-11 (0.5X/ 0.75X).

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Nitrogen level of the KSV1 cultivation BG-11 medium had an inverse relationship with the lipid yields by KSV1, (**0.5X / 0.75X** BG-11 with SN or **1.0X** BG-11 with ammonium sulphate yielding the highest lipid content). These were followed by the yields in BG-11 with urea (**0.5X)**/BG-11 with potassium nitrate (**0.5X)** and BG-11 with ammonium nitrate (**1.0X**) respectively (Table1 and Fig.1). An optimal lipid productivity of 10.0 mg/L/day was obtained here. Ammonium nitrate resulted in very low lipid yields at all strengths suggesting that it is not a suitable nitrogen source for lipid synthesis by *Botryococcus braunii* KSV1. Identical to the results obtained by Thomas et al., 1984; Macedo & Alegre, 2001; Yeesang and Cheirsilp, 2011; Sharma et al., 2012; Rukminasari, 2013, the results with our strain of *Botryococcus braunii* indicate that low nitrogen in the medium enhances the lipid yields. Potassium nitrate resulted in highest biomass yield at 0.5X level but the lipid yields were moderate.

Botryococcus, *Chlorella, Neochloris, Nitzschia spp.* and *Scenedesmus* have been shown to prefer nitrate nitrogen over ammonical N both for enhanced cell growth and lipid production (Dayananda et al., 2006 Li et al., 2008b; Lin and Lin, 2011; Supriya et al., 2012; Yadavalli et al., 2012). However, Amin et al., 2013 reported urea, as optimal nitrogen source for *Chlorella* and *Scenedesmus* spp. indicating the suitability of urea as nitrogen source for growth and lipid metabolism in microalgae differing with species and even strains.

Ammonium sulphate exhibited little or no impact on lipid synthesis by *B. braunii* even at 2.0X concentrations, while ammonium nitrateresulted in low yields, though the growth was not so much affected in both the cases. Organic nitrogen in form of urea was also utilized well by KSV1. Lipid biosynthesis in *Botryococcus braunii* KSV1 does not seem to be a growth dependent metabolism.

Table1: Effect of Nitrogen Nutrition on the Growth and Lipid Yields by *Botryococcus braunii* KSV1

NS	Strength	Biomass (Dry Weight)		Lipid		
		g/100mL	*Productivity g/L/day	g/100mL	*Lipid%(g/100g biomass)	*Productivity mg/L/day
AS	**0.5X**	0.127±0.004	0.0454	0.027±0.003	21.23±1.493	9.64
	1.0X	0.134±0.003	0.0478	0.028±0.002	20.69±0.899	10.00
	2.0X	0.138±0.003	0.0494	0.025±0.002	18.31±1.335	8.93
AN	**0.5X**	0.128±0.003	0.0456	0.014±0.002	11.21±0.920	5.00
	1.0X	0.138±0.004	0.0492	0.016±0.002	11.60±1.160	5.71
	2.0X	0.131±0.004	0.0469	0.015±0.001	11.17±0.260	5.36
PN	**0.5X**	0.144±0.003	0.0514	0.025±0.003	17.57±1.382	8.93
	1.0X	0.134±0.002	0.0478	0.024±0.002	17.69±1.274	8.57
	2.0X	0.134±0.002	0.0480	0.020±0.001	15.13±0.644	7.14
U	**0.5X**	0.134±0.003	0.0478	0.026±0.003	19.68±1.429	9.29
	1.0 X	0.130±0.005	0.0463	0.021±0.003	16.42±1.296	7.50
	2.0X	0.130±0.003	0.0463	0.023±0.002	17.98±1.173	8.21
SN	**0.5X**	0.123±0.003	0.0440	0.028±0.002	22.70±1.082	10.00
	0.75X	0.130±0.003	0.0463	0.028±0.001	21.59±0.289	10.00
	1.0X	0.146±0.003	0.0521	0.026±0.003	17.81±1.778	9.29
	1.5X	0.137±0.002	0.0489	0.023±0.002	17.03±0.870	8.21
	2.0X	0.133±0.002	0.0475	0.020±0.002	15.28±0.924	7.14
	2.5X	0.130±0.003	0.0465	0.020±0.001	15.34±0.419	7.14
	3.0X	0.123±0.002	0.0438	0.019±0.001	15.49±0.630	6.79

(AS- Ammonium Sulphate, AN- Ammonium Nitrate, NS-Nitrogen Source, PN- Potassium Nitrate, SN- Sodium Nitrate, U- Urea, X-Nitrogen Concentration equivalent to 17.6mM Sodium Nitrate in BG-11 medium; *-on dry weight basis)

3.2 Effect of different Strengths of BG-11 medium on Biomass and Lipid content of *Botryococcus braunii* KSV1:

The effect of the strength of BG-11 medium on the biomass and lipid yields of *Botryococcus braunii KSV1* can be assessed from the data presented in Tables 2 & 3 and Figures 2 &. The optimal strength of BG-11 medium for growth of *Botryococcus braunii* is 1.0X while that for enhanced lipid yield is 0.5X/ 0.75X. The impact of nutrient starvation and other stresses on lipid accumulation by algae was detailed by Sharma et al. 2012.The conditionsfavouring the increase of biomass have been reported to be not conducive in the enhancement of lipid content and particularly nitrogen starvation/ limitation is an often documented as effective approach for enhanced lipid production in Algae (Illman et al., 2000; Rodolfi et al., 2009; Chen et al., 2011; Nigam et al., 2011; Sharma et al., 2012). Behaviour of *B. braunii* has followed the same pattern of relationship.

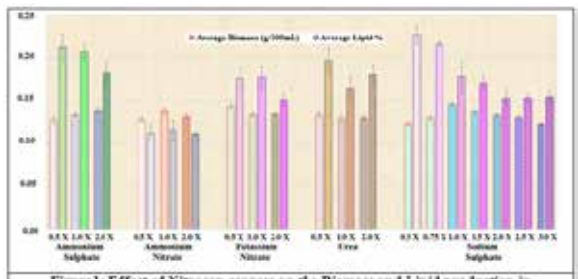

Figure1: Effect of Nitrogen sources on the Biomass and Lipid production in *Botryococcus braunii* KSV1. (AS- Ammonium Sulphate, AN- Ammonium Nitrate, PN- Potassium Nitrate, U- Urea, SN- Sodium Nitrate, X- Nitrogen Concentration equivalent to 17.6mM Sodium Nitrate in BG-11 medium)

The data indicates growth of *Botryococcus braunii* KSV1 in pulses of flashes rather than as steady and constant growth. Lipid productivity in *B. braunii* KSV1 is not a growth related metabolism. Lipid biosynthesis is known to be enhanced with higher NADPH consumption under growth limiting conditions provided by nutrient starvation (Sharma et al., 2012).

The total lipid of *Botryococcus braunii* KSV1 but also the lipid (%) in the biomass is higher in 0.5X BG-11 medium compared to 0.75X/ 1.0X/ 2.0X concentration media in this strain of *Botryococcus braunii* KSV1. The rates of lipid biosynthesis in the first 10 days, next 10 days & the last incubation period remains almost constant in case of 0.5X BG-11 medium.

Table 2: Effect of Strength of Normal BG-11 Medium Strength on Growth of *Botryococcus braunii* KSV1 (Biomass as dry weight in g/100mL)

Incubation Period (days)	0.5X	0.75X	1.0X	2.0X
0	0.010±0.0000	0.010±0.0000	0.010±0.0000	0.010±0.0000
2	0.017±0.0058	0.017±0.0058	0.017±0.0058	0.017±0.0058
4	0.023±0.0058	0.027±0.0058	0.027±0.0058	0.023±0.0058
6	0.027±0.0058	0.030±0.0100	0.033±0.0058	0.033±0.0058
8	0.033±0.0058	0.037±0.0058	0.040±0.0100	0.043±0.0058
10	0.043±0.0058	0.047±0.0058	0.050±0.0100	0.053±0.0058
12	0.047±0.0058	0.050±0.0100	0.060±0.0100	0.060±0.0100
14	0.057±0.0058	0.070±0.0100	0.070±0.0100	0.073±0.0058
16	0.063±0.0058	0.083±0.0058	0.077±0.0058	0.087±0.0058
18	0.073±0.0058	0.087±0.0058	0.093±0.0058	0.097±0.0058
20	0.083±0.0058	0.093±0.0058	0.103±0.0058	0.103±0.0058

22	0.093±0.0058	0.107±0.0058	0.113±0.0058	0.110±0.0100
24	0.100±0.0100	0.113±0.0058	0.123±0.0058	0.117±0.0058
26	0.107±0.0115	0.123±0.0058	0.133±0.0058	0.123±0.0058
28	0.120±0.0100	0.133±0.0051	0.147±0.0058	0.133±0.0058

(X- Strength of standard BG-11 medium components)

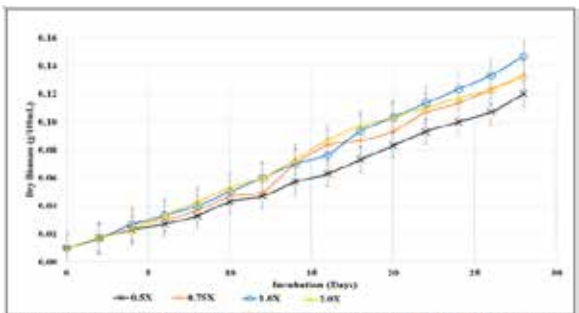

Figure 2: Effect of BG-11 medium strength on growth (biomass as dry weight) in g/100mL of *Botryococcus braunii* KSV1. 0.5X, 0.75X, 1.0X & 2.0X- Strength of BG-11 Medium)

Even towards the end of the fermentation cycle 0.5X BG-11 medium exhibits higher lipid productivity. It needs to be seen if enhancement of further fermentation cycle would result in cost effective lipid production or not.

Table 3: Effect of strength of normal BG-11 medium and incubation period on lipid productionby*Botryococcus braunii* KSV1

Incubation Days	Strength of BG-11 Media	Total Lipid Yield (g/ 100mL)	Lipid% (g/ 100g biomass)	Lipid Productivity mg/L/day
10	**0.5X**	0.0083±0.0006	19.33±1.15	8.30
	0.75X	0.0087±0.0006	18.67±1.15	8.70
	1.0X	0.0093±0.0006	19.06±3.06	9.30
	2.0X	0.0093±0.0006	17.56±0.77	9.30
20	**0.5X**	0.0170±0.0010	20.42±0.72	8.50
	0.75X	0.0163±0.0006	17.56±1.46	8.15
	1.0X	0.0173±0.0006	16.79±0.37	8.65
	2.0X	0.0163±0.0006	15.82±0.31	8.15
28	**0.5X**	0.0280±0.0017	23.37±1.05	10.00
	0.75X	0.0283±0.0015	21.25±0.42	10.11
	1.0X	0.0260±0.0010	17.73±0.35	9.29
	2.0X	0.0207±0.0006	15.51±0.59	7.39

(0.5X, 0.75X, 1.0X & 2.0X- Strengths of BG-11 medium)

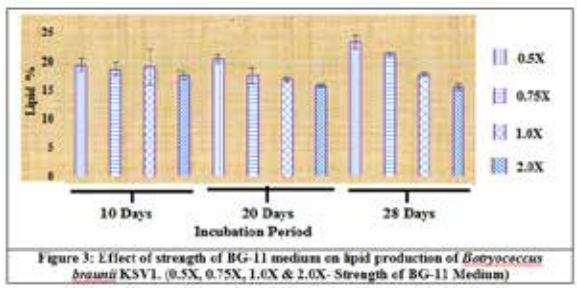

Figure 3: Effect of strength of BG-11 medium on lipid production of *Botryococcus braunii* KSV1. (0.5X, 0.75X, 1.0X & 2.0X- Strength of BG-11 Medium)

4. CONCLUSIONS:

Botryococcus braunii KSV1 exhibited preference towards sodium nitrate as nitrogen nutrition source at 17.6mM concentration for the optimal growth and enhanced lipid yields in BG-11 medium. Normal strength (1.0X) of BG-11 medium proved to be the best combination for enhanced biomass of *Botryococcus braunii* whereas BG-11 medium at 0.5X - 0.75X strengths led to increased lipid production (~10.0mg/L/day). Low nitrogen levels/ nitrogen limitation/ starvation were found to enhance the lipid yields, productivity and biosynthesis rate, but led to slightly lower algal growth.

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