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RESEARCH ARTICLE

Effects of nitrate and phosphate on total lipid content and pigment production in *Botryococcus braunii* Kutzing KM-104

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Abstract

Botryococcus braunii KM-104 grown in modified CHU 13 medium was analyzed for its growth parameters at different concentrations of potassium nitrate and di-potassium hydrogen phosphate. The alga had a maximum lipid content (860 mg/L) recorded on 24th d at 1.98 mM KNO_3 with modified CHU 13 medium. The maximum concentration of chlorophyll a and b were 38.65 and 28.46 mg/L on 21st d at 9.89 mM KNO_3 and 13.78 mg/L of total carotenoid was recorded on 30th d at 11.87 mM KNO_3. From the study, it may be concluded that potassium nitrate at a concentration of 1.97 mM in modified CHU 13 medium can be used in mass cultivation of *B. braunii* for total lipid and biofuels production.

Keywords: *Botryococcus braunii*, modified CHU 13 medium, lipid, chlorophyll, total carotenoid, biofuels**.**

Introduction

Botryococcus braunii Kutzing is a green, autotrophic, pyramid shaped, colonial planktonic microalga found throughout the world in fresh and brackish water lentic ecosystem. It is widely used in the production of lipids in the form of hydrocarbons. The bulk of *B. braunii* lipids are located in the outer walls which surround the basal part of the cells and build up the matrix of the colonies. Around 95% of the hydrocarbons are stored in the outer walls. These external lipids are easily recovered by short extraction of the dry biomass with hexane as yellow to red, sometimes brownish and colored oil. In contrast, recovery of the internal lipids which are located in the membranes and in cytoplasmic inclusions requires prolonged extraction with chloroform–methanol mixture.

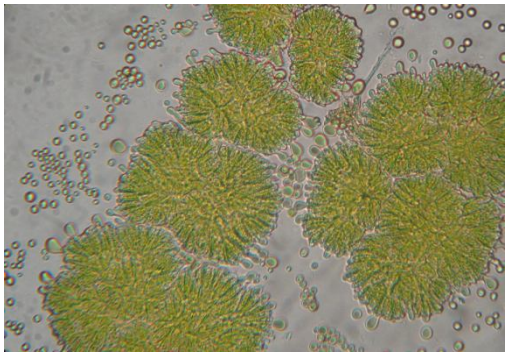
Based on hydrocarbons synthesis, *B. braunii* is classified into A, B and L races. Race A produces derivatives of fatty acids (C_{23} to C_{33} odd numbered *n*-alkadienes, mono, tri, tetra and pentaenes) (Metzger *et al*., 1990). Race B produces large amount of unsaturated hydrocarbons which are regarded as botryococcenes (C_{30} to C_{37} unsaturated hydrocarbons) and little amounts of methyl branched squalenes (Metzger and Largeau, 2005), while race L generates a single tetraterpenoid hydrocarbon identified as lycopadiene (Metzger *et al*., 1990). In addition, analytical investigations on external lipids of various strains of race A revealed several series of non-classical compounds such as aldehydes, alkenylphenols, epoxides and ether lipids. The knowledge on consequences of different cultural conditions and physico-chemical environments is important to tap the hydrocarbon production potential of *B. braunii*.

The cultural and physiological setup can influence hydrocarbon production of *B. braunii* (Dayananda *et al*., 2005). Yang *et al*. (2004) reported the influence of sulphite and bisulphite on the growth and hydrocarbon production of *B. braunii*. The macronutrients such as nitrogen, phosphorous, potassium and sulfate play a crucial role in the life cycle of plants. Thus, the present study is focused on the effects of KNO_3 and K_2HPO_4 on pigment and lipid production of *B. braunii*.

Materials and methods

Collection of samples: Botryococcus braunii Kutzing KM-104 (Fig. 1) was collected from Mandya district of Karnataka and maintained in modified CHU 13 medium and analyzed for different growth parameters at 25 ± 1°C at 30 µE $m^{-2}s^{-1}$ light intensity of 12/12 (light/dark) photo period for different concentrations (0.99, 1.98, 3.67 (control), 5.93, 7.91, 9.89 and 11.87 mM) of potassium nitrate and di-potasium hydrogen phosphate (0.29, 0.34, 0.46 (control), 0.40, 0.52, 0.57 and 0.63 mM) under laboratory conditions.

Fig. 1. *Botryococcus braunii* Kutzing KM-104 (X 40).



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Growth studies: Growth experiments was carried out in 500 mL Erlenmeyer flasks containing 225 mL of modified CHU 13 medium inoculated with 25 mL of optimally grown isolates of *Botryococcus braunii* KM-104 for a period of 30 d under laboratory conditions. At every 3 d of interval, 5 mL of sample was drawn and analyzed for the following parameters: Chlorophyll a and b, total carotenoid (Lichtenthaler, 1987), total protein (Bradford, 1976), total carbohydrate (Dubois *et al.,* 1956), total lipid (Folch *et al.,* 1956) and dry weight (g/L).

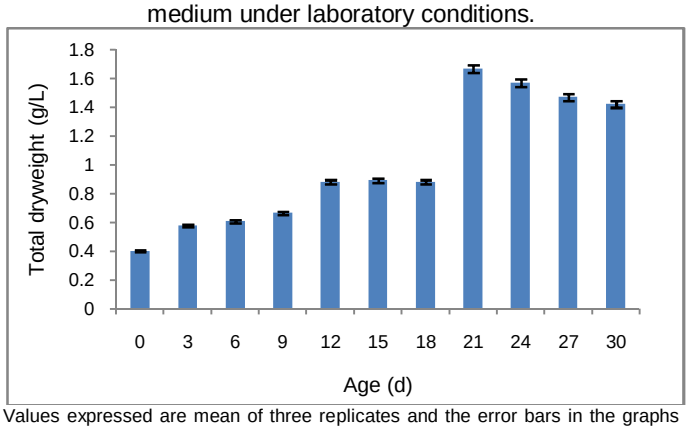
Effect of nitrate (KNO_3) and phosphate (K_2HPO_4): The experiments were carried out in 500 mL conical flasks amended with different concentrations of potassium nitrate (KNO_3) viz., 0.99, 1.98, 3.67 (control), 5.93, 7.91, 9.89 and 11.87 mM added in minus KNO_3 modified CHU 13 medium and inoculated *B. braunii* KM-104 and recorded different growth characteristics. Similarly, different concentrations of di-potassium hydrogen phosphate (K_2HPO_4-0.29, 0.34, 0.46 (control), 0.40, 0.52, 0.57 and 0.63 mM) were added in minus K_2HPO_4 basal medium and inoculated *B. braunii* KM-104 and recorded for different parameters under laboratory conditions.

Statistical analysis: Values expressed are mean of three replicates and the error bars in the graphs indicate standard deviations (±SD).

Results and discussion

Nitrate and phosphate act as a macronutrient for growth and synthesis of different metabolites on algae, fungi, and higher plants. In fact, all commercial plant fertilizers are labeled as to their NPK contents. Phosphorus makes up to 0.2% of a plant's dry weight but it is critical for energy conversion and genetic transfer. Inorganic orthophosphate controls enzyme activity, metabolic pathways and transport systems within the cell. Much of the research on phosphorus uptake mechanisms has been done on higher plants, but most researchers agree that there is a 'broad' similarity with algae (Schachtman *et al.,* 1998). In the natural environment, bacteria facilitate the release of inorganic phosphate from organic phosphate compounds. Most algae can tolerate phosphate up to the range of 50 µg/L (Becker, 1994). The nitrate source increases with different medium to enhance biomass concentration (Chittra and Benjamas, 2001). The loss of biomass was recorded, when the green alga *Botryococcus* sp. was exposed to nitrogen deficient condition and accumulated carbon metabolite as lipid (Yessang and Cheirsilp, 2011). In *Chlorella vulgaris*, lipid content increased from 20.9 to 55.9% while the concentration of nitrogen source decreased from 1.25 to 0.313 g/L (Yeh and Chang, 2011). The domestic sewage that has been pretreated by activated sludge treatment can be used as a medium for hydrocarbon production by *B. braunii*. Usage of sewage can reduce the cost of producing hydrocarbons.

Fig. 2. Growth of *B. braunii* KM-104 in modified CHU 13 medium under laboratory conditions.



Values expressed are mean of three replicates and the error bars in the graphs indicate SD.

Fig. 3. Effect of KNO_3 on chlorophyll a level of *B. braunii* KM-104.

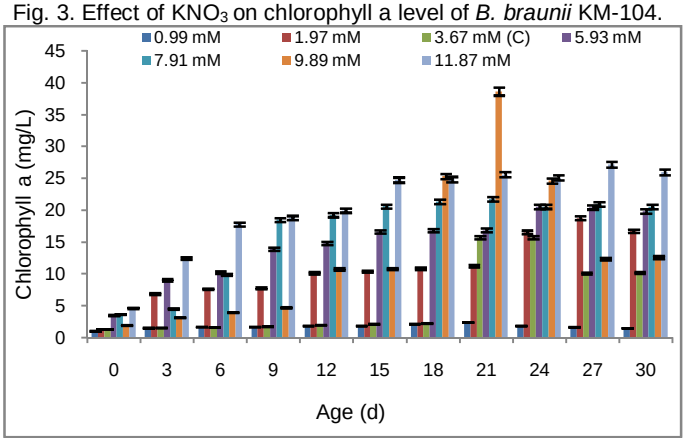


Fig. 4. Effect of KNO_3 on chlorophyll b level of *B. braunii* KM-104.

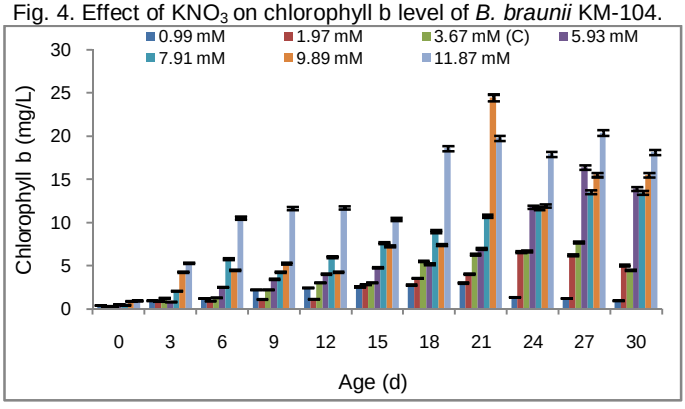


Fig. 5. Effect of KNO_3 on total carotenoid of *B. braunii* KM-104.

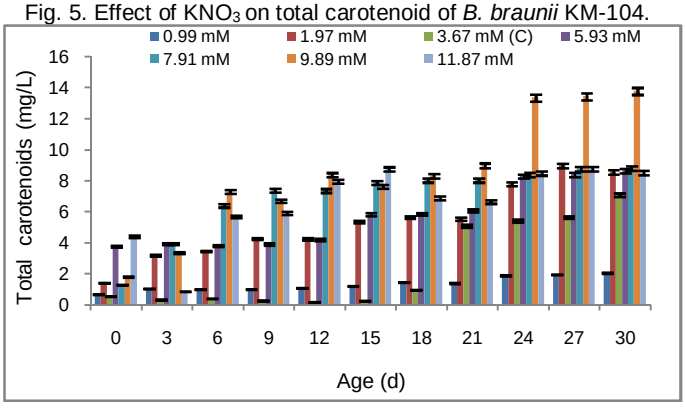


Fig. 6. Effect of KNO_3 on total protein of *B. braunii* KM-104.

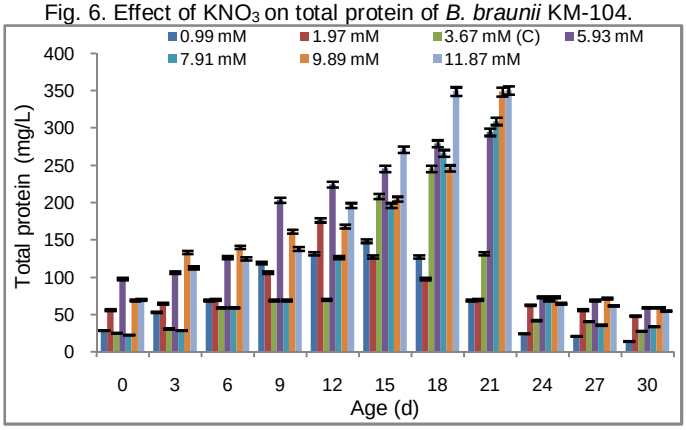


Fig. 7. Effect of KNO_3 on total carbohydrate of *B. braunii* KM-104.

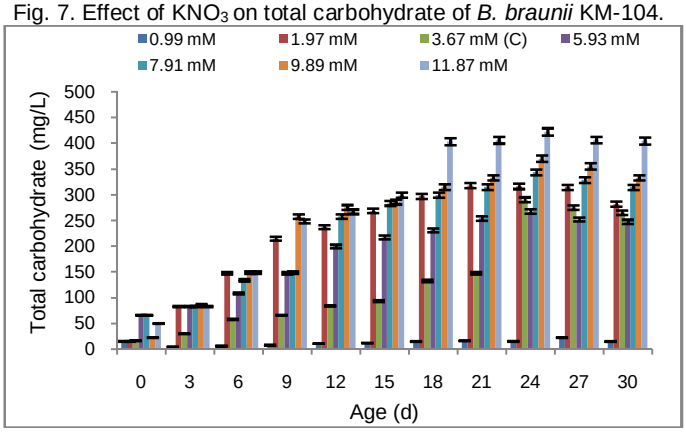


Fig. 8. Effect of KNO_3 on total lipid of *B. braunii* KM-104.

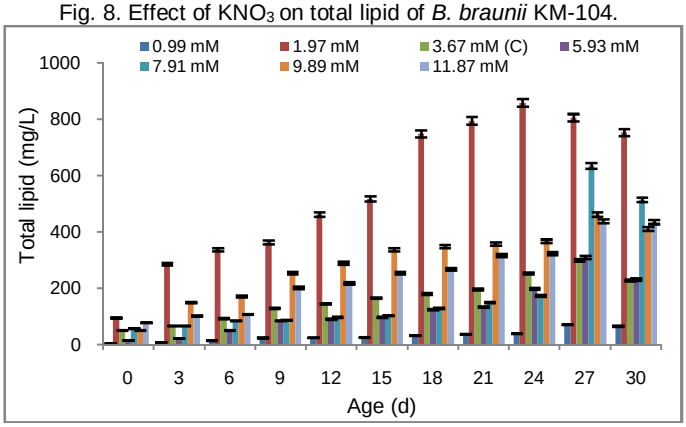


Fig. 9. Effect of K_2HPO_4 on chlorophyll a level of *B. braunii* KM-104.

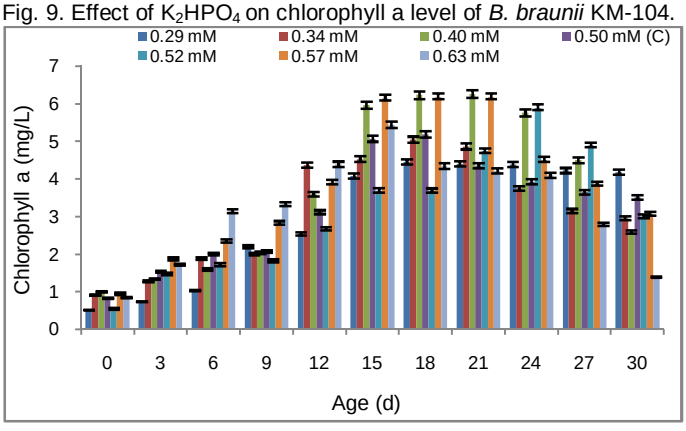


Fig. 10. Effect of K_2HPO_4 on chlorophyll b level of *B. braunii* KM-104.

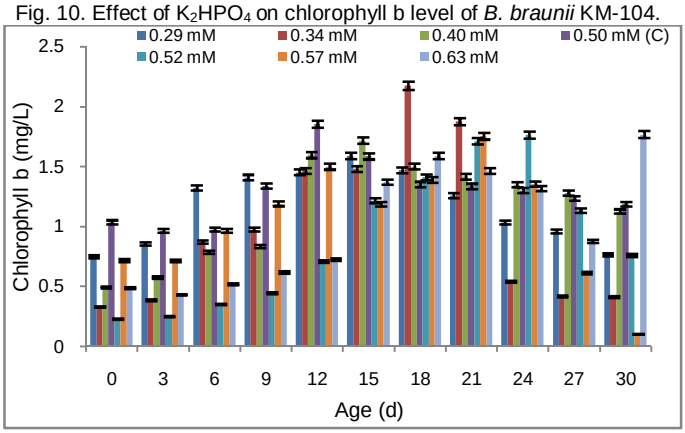


Fig. 11. Effect of K_2HPO_4 on total carotenoids of *B. braunii* KM-104.

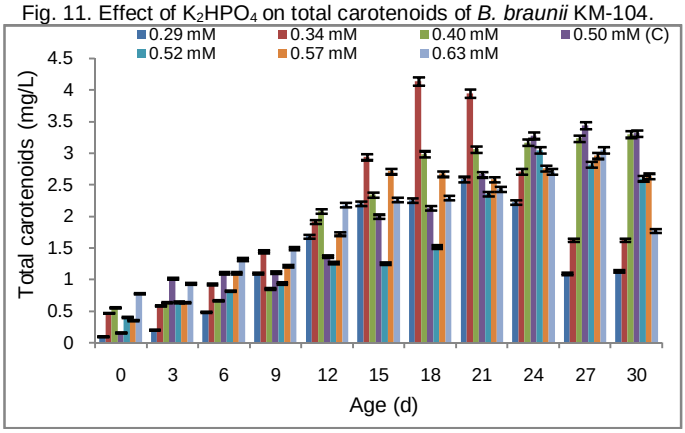


Fig. 12. Effect of K_2HPO_4 on total protein of *B. braunii* KM-104.

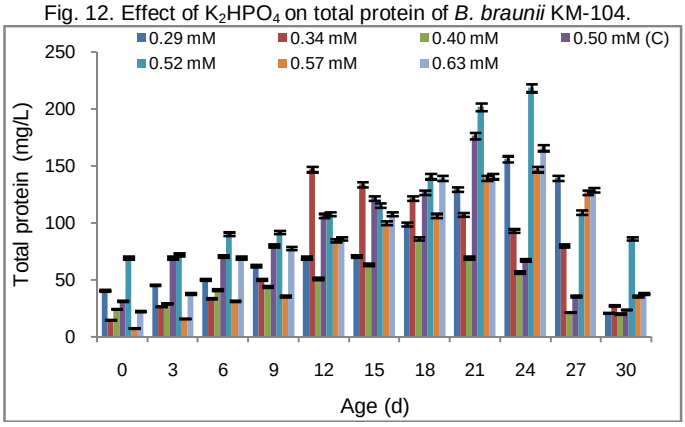


Fig. 13. Effect of K_2HPO_4 on total carbohydrate of *B. braunii* KM-104.

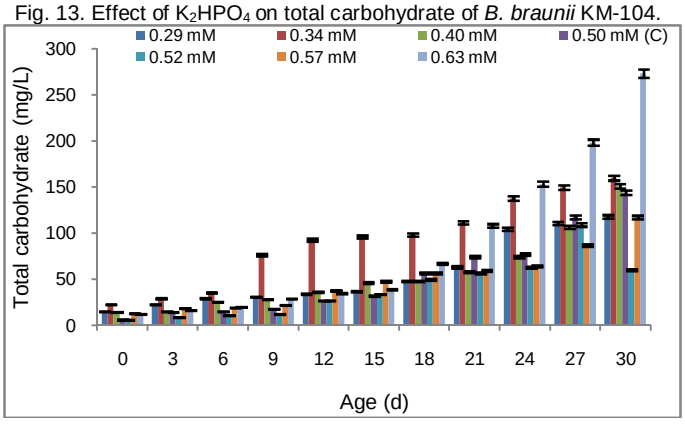
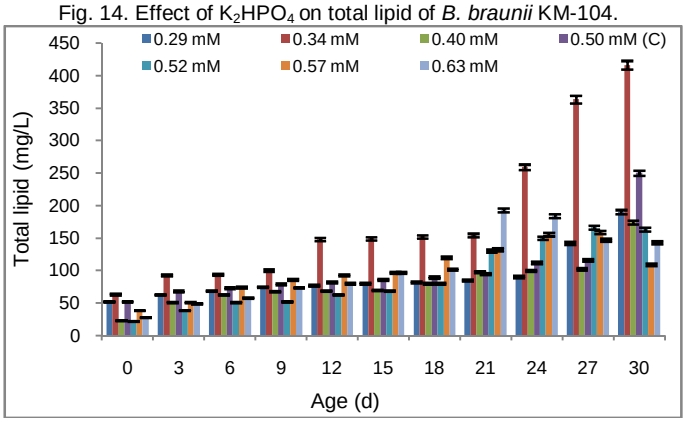


Fig. 14. Effect of K_2HPO_4 on total lipid of *B. braunii* KM-104.



Secondary stage-treated sewage (STS) has been characterized and found to contain a large amount of nitrogen (as nitrate) and phosphorus (as PO_4^{3-}). When *B. braunii* was cultured on STS aerated with 1% CO_2 at 25°C without controlling the pH, the nitrate content reduced from 7.67 gm^{-3} to a level below detection (<0.01 gm^{-3}). The alga was able to consume the phosphate present at quite low levels (0.02 gm^{-3}). Nitrate appeared to be consumed after nitrite but ammonium was not utilized. The growth rate was 0.35 kgm^{-3} per week and the hydrocarbon content was 53% compared with 58% in modified CHU 13 medium under the same conditions (Sawayama *et al*., 1992). The green alga, *Chlorella pyrenoidosa* was grown autotrophically in Foggs medium with different concentrations of KNO_3 (0, 0.05, 0.1, 0.2 and 0.4 g/L). Control was used to investigate the effect of nitrogen on growth and lipid content of the organism. The maximum concentration of biomass obtained was 0.316 g/L in 0.4 g of KNO_3 where as the maximum lipid content recorded was 28% in 0.05 g KNO_3 containing Foggs medium (Nigam *et al*., 2011). *Botryococcus braunii* was grown in Modified CHU 13 medium with different nitrogen sources such as sodium nitrate, ammonium nitrate, calcium nitrate and urea were studied for their growth and hydrocarbon production. Among the nitrates tested, potassium nitrate appeared to be more effective compared to calcium and sodium nitrates. The yield of biomass was 1.2 and 0.6 g/L with 32-38% of hydrocarbon production (Dayananda *et al*., 2006).

Botryococcus braunii grown in modified CHU 13 medium at different concentrations of potassium nitrate, di-potassium hydrogen phosphate, magnesium sulphate, ferric citrate and ferric citrate-citric acid maintained in the ratio of 1:10 by adding and keeping other constituents of the medium constant. Total biomass of 0.0195, 0.05, 0.02 and 0.0185 g/L were recorded for di-potassium hydrogen phosphate, potassium nitrate, magnesium sulphate and ferric citrate respectively (Dayananda *et al*., 2005). In the present study, the maximum total dry weight of 1.666 g/L was recorded on 21st d in modified CHU 13 medium for *B. braunii* KM-104 (Fig. 2).

The test alga grown in modified CHU 13 medium amended with different concentrations of potassium nitrate and di-potassium hydrogen phosphate under laboratory conditions. The alga had a maximum amount of total lipid as 860 mg/L on 24th d at 1.97 mM KNO_3. Similarly, maximum level of total protein and carbohydrate (351 and 423 mg/L) was recorded on 21st and 24th d. The maximum amount of chlorophyll a and b as 38.65 mg/L and 24.46 mg/L were registered on 21st d at 9.89 mM KNO_3 while, the maximum accumulation of total carotenoid of 13.78 mg/L was recorded on 30th d at 11.87 mM of KNO_3. In the case of di-potassium hydrogen phosphate, the maximum amount of total lipid (416 mg/L) was recorded on 30th d at 0.34 mM and the maximum total protein and carbohydrate level (218 and 273 mg/L) were recorded on 24th and 30th d at 0.52 mM and 0.63 mM respectively.

The maximum concentration of chlorophyll a and b were 6.43 mg/L and 2.35 mg/L recorded at 0.46 and 0.46 mM K_2HPO_4 on 21st and 24th d respectively while, maximum carotenoid (4.13 mg/L) was recorded at 0.34 mM K_2HPO_4 on 18th d (Fig. 3-14). The findings revealed that addition of nitrogen increased the pigment concentrations whereas, decreased nitrogen concentration enhanced lipid accumulation. Similarly, under phosphate deficient conditions, the lipid accumulation was high while a slight increase in phosphate concentration increased the pigment concentration.

Conclusion

Botryococcus braunii KM-104 grown at different concentrations of nitrogen and phosphate was analyzed at different concentrations of potassium nitrate and di-potassium hydrogen phosphate. The level of total lipid content increased up to one fold, chlorophyll a up to five folds, chlorophyll b up to eight folds and total carotenoid to three folds in potassium nitrate (1.97 mM) when compared to di-potassium hydrogen phosphate (0.34 mM) amended medium with control. Therefore, from the present study, it can be concluded that potassium nitrate at a concentration of 1.97 mM in modified CHU 13 medium can be used in mass cultivation of *Botryococcus braunii* KM-104 for total lipid and biofuels production.

Acknowledgements

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