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BIO-OIL PRODUCTION POTENTIAL OF GREEN MICROALGAE ISOLATED FROM WASTEWATER BODY

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Abstract

Bio-oil production potential of green microalgae isolated from nearby wastewater body has been investigated. Physiochemical conditions that favours microalgal growth in terms of biomass productivity, lipid and carbohydrate contents has been explored through experimental work for further mass scale cultivation and yield optimization. Microalgal cultures MASP-2 and MASP-4 were found to have commanding influence for lipid and carbohydrate content under selective physiochemical conditions [(Higher irradiance 70-100 $\mu Em^{-2}s^{-1}$, long light exposure (16 hrs to 24 hrs), elevated CO_2 levels (2-3%), salinity (7.5-8.5 pH), Nitrogen and Phosphorus limited media (Half strength as in BBM media) and high concentrations of chelates (Na_2-EDTA, 5-fold strength) and micronutrient (Fe and Mg, Double strength)]. Culture conditions that optimize the microalgal growth for improved lipid and carbohydrate contents has been standardized, considering economic and energy inputs for commercial scale cultivation and product recovery.

Introduction

Cosmopolitan growth nature, source of biofuel, higher biomass yield and CO_2 mitigation (carbon sequestration) are the few attributes that makes microalgae as one of the major candidates of biological research in energy, environment and biotechnology domains. Eloka-Eboka& Inambao (2017) highlights the potential of microalgae as green energy feedstock. Algal growth is found to be the best option for wastewater treatment coupled with sustainable biofuel production in comparison to higher plants (Singh & Singh, 2014). Studies indicate possibility of culturing algae in wastewater for biofuel production due to the presence of high nutrient levels (NPK) and adds economic viability as well (Teichner, 2015). Apandi *et al.,* (2017) performed cultivation of *Scenedesmus sp.* for protein and lipid content using wet market wastewater. Ramachandra *et al.,* (2013) studied lipid prospects of algae (*Euglena* sp.*, Spirogyra* sp. and *Phormidium* sp.) grown in Indian wastewater systems. In the present investigation, the major objective was to perform the biochemical modulation studies on microalgae isolated from wastewater bodies w.r.t. Bio-oil and Bio-ethanol productivity. Therefore, Green microalgae were isolated from the nearby wastewater body and cultured under different physiochemical conditions to investigate the lipid (for bio-oil) and carbohydrate (bio-ethanol) yields. The screening of microalgae (MASP-1, MASP-2, MASP-3 and MASP-4) were based on the preliminary survey report and attributes like native abundance, growth rate, biomass productivity, lipid & carbohydrate content, mass scale cultivations, etc. Environmental conditions affect intricate metabolic pathways of a cell (Zhang *et al.,* 2013). Algal growth is affected by various physiochemical parameters like Carbon dioxide, pH, light/irradiance, temperature, mixing /aeration, Salinity, etc., directly or indirectly (Singh & Singh, 2014). Manipulation of microalgal biomass composition can be achieved through different stress dependent cultivation conditions (Markou *et al.,* 2013). Metabolic plasticity of the screened microalgae pertaining to higher bio-oil and lipid content has been explored under variable physiochemical conditions like growth regimes (Autotrophic, Heterotrophic, Mixotrphic), light intensity & photoperiod, carbon dioxide conc. & pH, nutrient stress (N, P), chelating agents, micronutrients (Mn, Cu, Fe and Zn), etc.

Materials & Methods

Microalgal species were isolated from the nearby wastewater body (at different sampling points) using serial dilution and plating technique (Job Gopinath *et al.,* 2014). The identification of microalgae cultures were performed based on the standard monographs and literature survey (Prescott 1954, 1962; Randhawa, 1959; Philipose, 1967; Bellinger & Sigee, 2010; Singh & Chaudhary, 2011; Gouda *et al.,* 2015; Vijayan & Ray, 2015; Barupal & Narayan, 2016; http://algalweb.net/Islay03.htm; https://www.landcareresearch.co.nz/home). Pure cultures were maintained under fed batch system in Erlenmeyer flasks under aseptic and controlled conditions on a modified synthetic BBM medium (Bold, 1949; Bischoff & Bold, 1963) in a growth chamber under 16/8-hr light/dark cycle (to obtain synchronous culture, Dominic *et al.,* 2009) illuminated at 2500 lux irradiance (Aguoru & Okibe, 2015) provided by cool white fluorescent tubes at 25±2°C temperature (Waghmare *et al.,* 2016). The microalgae cultures were harvested at the onset of stationary phase to study the growth (cell density), lipid and carbohydrate content. Cell mass of microalgal monocultures has been determined spectrophotometrically (El-Ardy *et al.,* 2012) as well as gravimetrically (Munir *et al.,* 2015; Visca *et al.,* 2017). To quantify the microalgal biomass on dry weight basis, the harvested microalgal cells were centrifuged at 5000 rpm for 10 min at 4°C followed by drying of cellular pellets in incubator at 80°C (Luo *et al.,* 2015). Dried algal biomass further processed and stored as described by Waghmare *et al.,* (2016) till further analysis. Lipid content in air-dried biomass of microalgal species was analyzed by Bligh & Dyer (1959) method (as described by Nigam *et al.,* 2011) and Sulfo-Phospho-Vanillin colorimetric method (Park *et al.,* 2015). Total Carbohydrate content was determined colorimetrically by the phenol-sulfuric acid method using a standard curve of glucose (Dubois *et al.,* 1956). Estimation of Carbohydrates was also carried out by Anthrone method (Xue, 1985) as adapted by Luo *et al.,* (2015).

Results and Discussion

Microalgae can be cultivated under autotrophic, mixotrophic or heterotrophic modes (Oliveria *et al.,* 2017). Autotrophic microalgal growth achieved in modified BBM media under 16L/8D cycle at 50 µE $m^{-2}s^{-1}$ irradiance while Heterotrophic conditions has been maintained in phototrophically raised microalgal cultures (obtained at exponential growth phase) using modified BBM media enriched with 1% (w/v) glucose as organic carbon source, without illumination. Heterotrophically raised microalgae under illumination were served as Mixotrophic cultures. MASP-1 and MASP-3 favoured heterotrophic growth regimes. Heterotrophic microalgal cultures showed higher lipid content (% dry wt) (Fig.1) and Mixotrophically raised cultures showed higher Carbohydrate content (% dry wt) (Fig.2). Microalgae can fix CO_2 either through atmosphere, soluble carbonates or industrial discharges (Wang *et al.,* 2008; cited by Singh & Singh, 2014). Microalgae cultures were grown in the modified BBM media with (1, 2, 3% levels of CO_2 served as treatments) and without (served as control) external source of CO_2. The variation in pH in all the treatment cultures were recorded between 7.8 to 8.5, while in control cultures it was found below 8. The exponential phase was observed clearly between 8-15 days while doubling time ranges from 2-5 days in treatment and control cultures. At 1% CO_2 concentration, microalgae cultures MASP-1, MASP-2, MASP-3 and MASP-4 showed 12.2%, 58%, 27%, 27.8% higher biomass content w.r.t. control cultures. A/c to Eloka-Eboka& Inambao (2017), 1% (v) of CO_2 addition promoted algal growths in comparison with ambient CO_2 concentrations. Similar trend was followed by microalgal cultures MASP-2 and MASP-4 at 2% CO_2 concentration. With respect to control cultures, 67% higher biomass content, 67.33% higher lipid (% dry wt

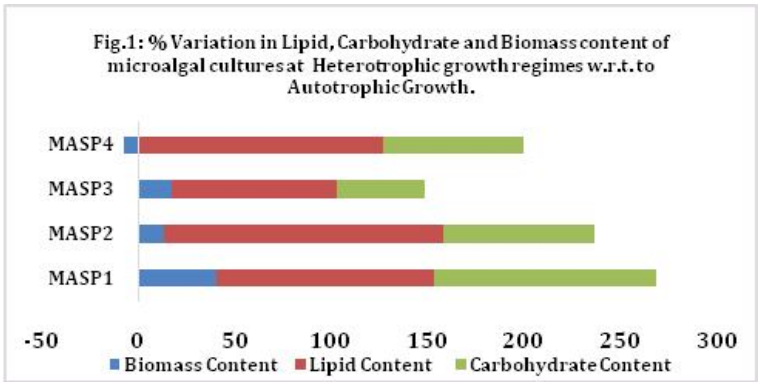


Fig.1: % Variation in Lipid, Carbohydrate and Biomass content of microalgal cultures at Heterotrophic growth regimes w.r.t. to Autotrophic Growth.

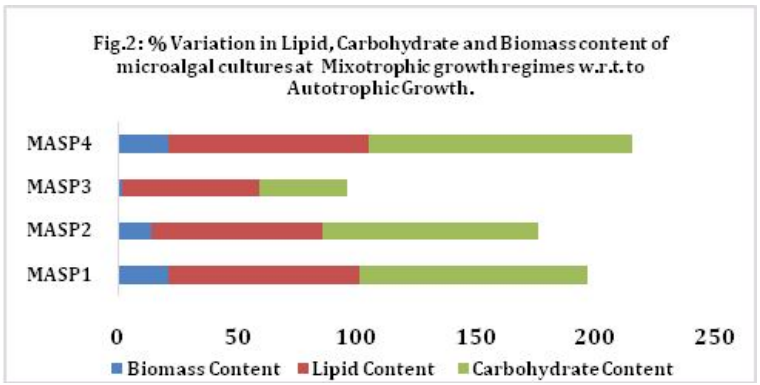
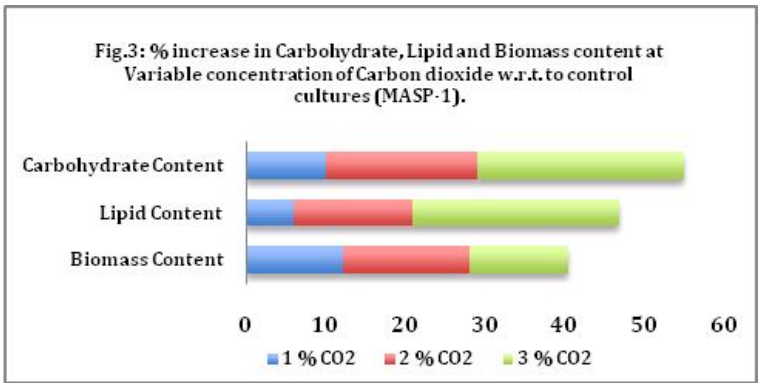
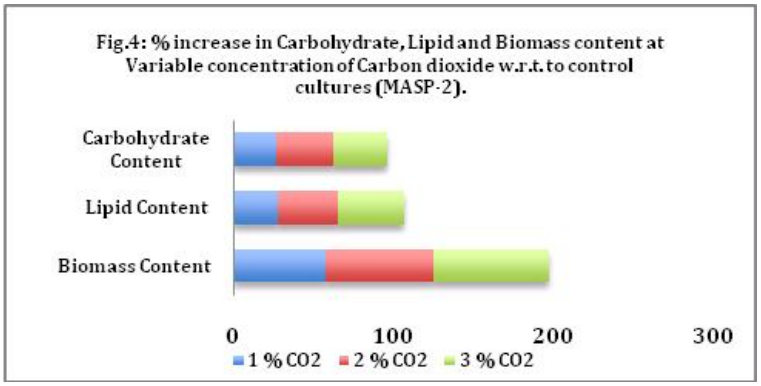
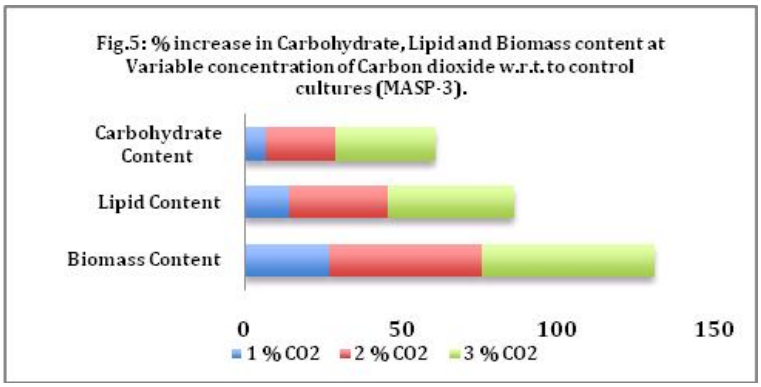
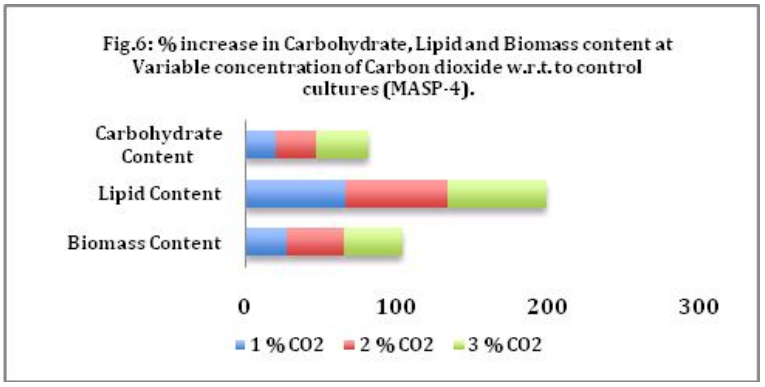


Fig.2: % Variation in Lipid, Carbohydrate and Biomass content of microalgal cultures at Mixotrophic growth regimes w.r.t. to Autotrophic Growth.





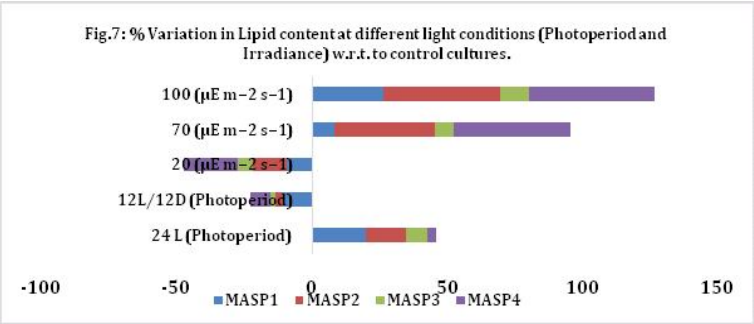


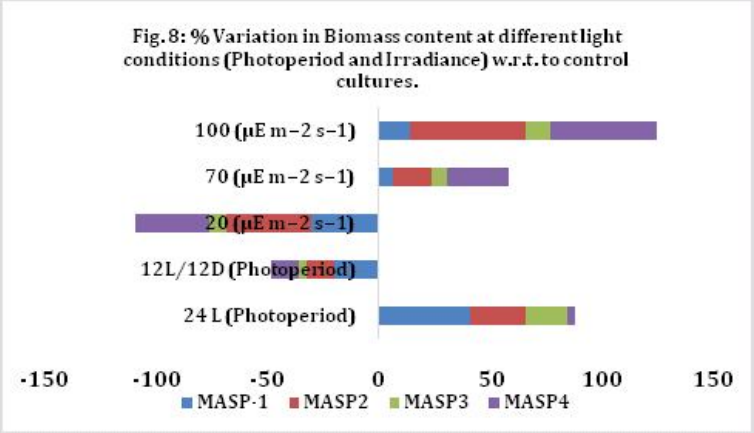


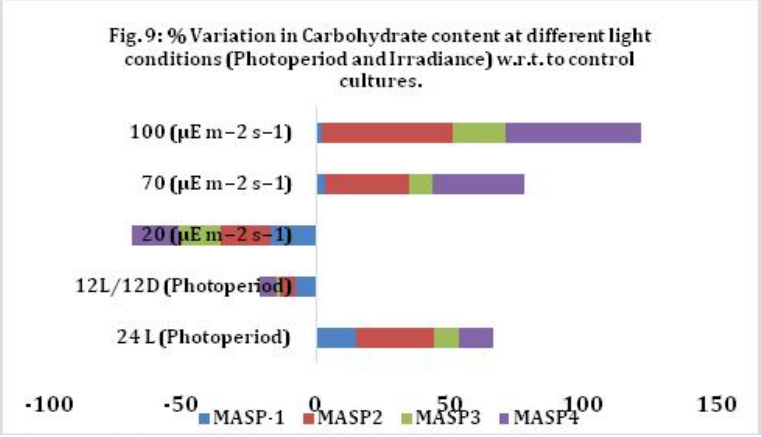
basis) and 35% higher carbohydrate (% dry wt basis) content was recorded in microalgae cultures MASP-2, MASP-4 and MASP-2 respectively. Maximum Biomass[a], Lipid[b] and Carbohydrate[c] content were recorded at 3%[a] (MASP-2), 2%[b](MASP-4) and 2%[c](MASP-2) & 3%[c](MASP-4) CO_2 concentration respectively (Fig 3-6).

Light is an essential factor for microalgal growth under autotrophic as well as mixotrophic modes of cultures (Oliveria *et al.,* 2017). Light and temperature influence metabolism, enzymatic activities and cell composition of algae (Munir *et al.,* 2015). Higher algal growth rates have been reported under artificial fluorescent lights in comparison to sunlight (Munir *et al.,* 2015). Microalgal growths at 12h L:12h Dphotoperiod and 24h continuous illumination were served as treatments while at 16h L/8h D cycle was served as working control. Similarly, microalgal cultures at light irradiances of 20, 70 and 100 µE m^{-2} s^{-1} were served as treatments while light irradiance of 50 µE m^{-2} s^{-1} was served as working control under 16h L/8h D cycle. Biomass production, Lipid and Carbohydrate contents were analyzed with respect to the working controls. As far as the effect of photoperiodic conditions concerned, Biomass (mg/l), Lipid (% dry wt) and Carbohydrate (% dry wt) content showed increasing trend in the microalgal cultures raised at 24 h of continuous illumination while decreasing trend in treatment cultures raised at 12h L/12h D cycle w.r.t. the controlled cultures (at 16h day/8h night cycle) (Fig. 7-9). Effect of irradiance at variable light intensities has also studied. With respect to the lipid content recorded at 50 µE m^{-2} s^{-1} (working control), the highest proportional increase in lipid was observed in microalgal cultures MASP-4 (46.5%) followed by MASP-2 (42.85%) at irradiance of 100 µE m^{-2} s^{-1}. Microalgal cultures *i.e.* MASP-1 showed over 3 times higher lipid content and MASP-3 showed over twice carbohydrate content on shifting light irradiance from 70 µE $m^{-2}s^{-1}$ to 100 µE $m^{-2}s^{-1}$. However, Carbohydrate content (% dry wt) in microalgal culture MASP-1 showed higher values at irradiance of 70 µE m^{-2} s^{-1} instead of 100 µE m^{-2} s^{-1}.

Variation in carbon and nitrogen sources directly affects biomass and lipid productivities in microalgae (Aguoru & Okibe, 2015). A/c to El-Kassas (2013), Nitrogen and Phosphate limited cultures could be applied to increase lipid and carbohydrates production in microalgae (*Picochlorum* sp.). Higher carbohydrate-enriched biomass has been reported in some microalgal species under nutrient starvation or high light intensity conditions (Markou *et al.,*

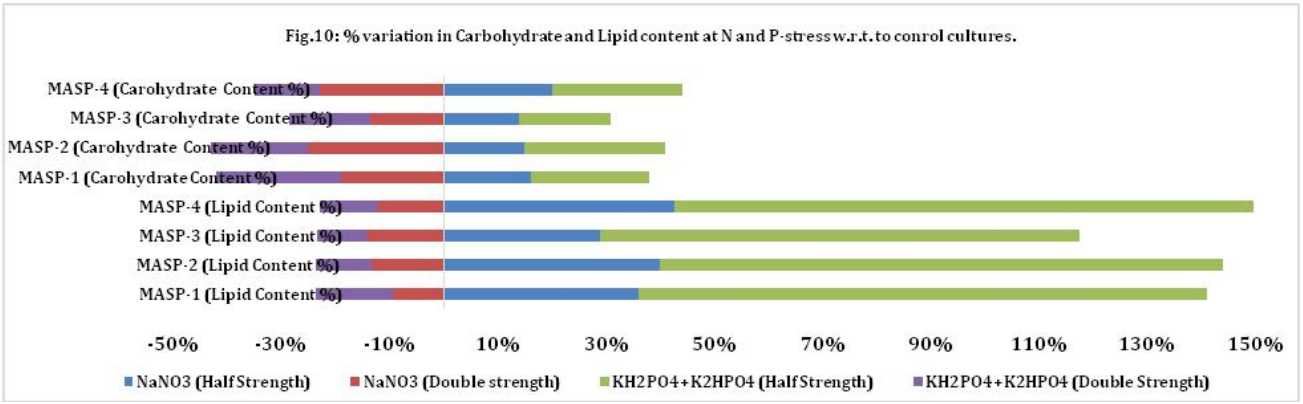






2013; cited by Markou *et al.,* 2012, Gonzalez-Fernandez *et al.,* 2012), suitable for bioethanol production (Markou *et al.,* 2013). Nutrient stress elevates percentage lipid content in microalgae due to the shifting of lipid metabolism from membrane lipid synthesis to neutral lipid storage (El-Kassas, 2013). To study the effect of Nitrogen (N) and Phosphorous (P) stress on Lipid and Carbohydrate content in microalgae, nutrient status *i.e.* $NaNO_3$ and KH_2PO_4+K_2HPO_4 content in BBM media was modified as half strength and double strength. Increase in Lipid and Carbohydrate production has been recorded at half strength N & P concentrations w.r.t. to control concentrations (Fig. 10). Double strength N & P concentrations were found to have inhibitory effect on Lipid and Carbohydrate contents in all the microalgal cultures in comparison to control cultures. Maximum accumulation of Lipid (42.7%) was recorded in MASP-4 followed by MASP-2 (42%) in Half-strength $NaNO_3$ (w.r.t. to control culture at 0.25g/l $NaNO_3$). Overall, N-and P-stress (Half-strength) favours lipid accumulation in microalgae cultures MASP-4, MASP-2 followed by MASP-1 and MASP-3.

Miwa & Morizane (1988) studied the effect of chelating agents on the growth of BGA (*Anabaena macrospora*) and reported 10 times higher cell numbers in EDTA enriched complete medium in comparison to EDTA-free CT medium, thus recommended the use of chelators like EDTA for the healthy growth of alga. Effect of disodium EDTA complex (Na_2-EDTA) has been studied on selected (based on overall biomass, lipid and carbohydrate productivity) microalgal cultures *i.e.* MASP-2 and MASP-4. Selected microalgal cultures were grown autotrophically at BBM media with Na_2-EDTA (0.01g/l standard conc. served as control), without Na_2-EDTA (0.00g/l) and excess Na_2-EDTA (0.5g/l) under controlled conditions of 70 $mEm^{-2}s^{-1}$ irradiance, 24h continuous illumination and 2% CO_2. At excess Na_2-EDTA, MASP-2 and MASP-4 showed increase in Lipid content by 9.67% and 11.69% w.r.t to control cultures respectively. Overall, both the microalgal cultures were found to have tolerance to higher concentrations of Na_2-EDTA.Dou *et al.,* (2013) studied the effect of trace elements on the Lipid productivity and Fatty acid composition of *Nannochloropis oculata* and showed that the addition of Fe^{3+}, Zn^{2+}, Mn^{2+}, Mo^{6+}, EDTA and the deletion of Cu^{2+} & Co^{2+} can increase the lipid productivity. Effect of Mn^{2+}, Co^{2+} and H_2O_2 on biodiesel production potential of *Chlorella vulgaris* has been studied by Battah *et al.,* (2014) and reported that Mn^{2+} deficiency resulted in growth inhibition. To study the effect of micronutrients (Fe, Cu, Zn and Mn), selected microalgae (MSAP-2 and MSAP-4) were cultured in BBM media modified with different concentrations of above micronutrients as follows ([a,b,d]stock solution volume 1ml/l;[c] acidified iron stock solution (1 ml/l) (concentrations expressed as fold change w.r.t control concentrations): [a]Cu as $CuSO_4.5H_2O$: 0mg (0x), 0.079mg (Control), 0.15mg (2x), 0.75mg (5x); [b]Mn as $MnCl_2.4H_2O$: 0mg (0x), 1.81mg (Control), 4mg (2x); [c]Fe and [d]Mg as $FeSO4.7H_2O$ + $MgSO_4.7H_2O$: (0mg+0mg)



(0x), 0.075+0.0049mg (Control), 0.15mg+0.001mg (2x). Copper at lower and higher concentrations were found to play inhibitory role in both the microalgal cultures for Lipid and Carbohydrate content. MASP-4 microalgal cultures without copper in BBM showed 1.2% decrease in lipid content, while 2.6% increase in lipid content was recorded in MASP-2 cultures w.r.t to control concentration. There was not much significant variation in lipid content of MASP-2 and MASP-4 were observed at control and 2-fold concentrations of Manganese. MASP-2 and MASP-4 at 2-fold (2x) concentration of Mn showed increase in lipid content by 4.13% and 3.27 % respectively w.r.t to control cultures. The increased values of lipids and carbohydrates in microalgal species can be due to the active participation of Mn^{2+} associated enzymes in metabolism (Coates *et al.* 1972; Clarkson & Hanson, 1980; Burnell, 1988; cited by Battah *et al.,* 2014). However, BBM media without Mn has showed inhibitory effect on microalgal cultures, due to the affected nitrate utilization (Zhang *et al.,* 2012; cited by Dou *et al.,* 2013). Battah *et al.,* (2014) also reported growth inhibition under Mn^{2+} deficient conditions. Iron is an essential micronutrient for plant growth including algae and one of the growth limiting factor for algal growth due to its physiochemical properties (like pH) (Kean *et al.,* 2015). Fe^{3+} is an important component of nitrate and nitrite reductase and controls the rate of photosynthesis as well (Dou *et al.,* 2013). Bioavailability of Iron is also important for efficient oil production process in algal cells (Kean *et al.,* 2015). Iron with Magnesium have some determinantal effect on the lipid content of microalgal species, as observed in the present study. Fe and Mg at 2-fold (2x) concentrations found to increase lipid content by 12.17% (MASP-2) and 14.64% (MASP-4) respectively w.r.t to control cultures. BBM media without Fe and Mg decreased lipid content in MASP-2 and MASP-4 by 3.5% and 6.15% w.r.t. control cultures.

Conclusion

Exhaustion of fossil fuels, increasing energy demands and inflating oil prices, necessitates towards exploring alternative energy sources (Munir *et al.,* 2015). Higher growth rate, lipids and carbohydrates levels in microalgae attracts researchers to derive biofuels and other value added products from microalgal feedstocks (Eloka-Eboka & Inambao, 2017). Scientists emphasized on the selection of native algal species, algal species with high growth rate coupled with greater lipid content for their efficient use as a biodiesel source (Munir *et al.,* 2015). Therefore, biochemical modulation studies to achieve higher lipid content has been carried out on native microalgal species isolated from nearby wastewater body. A/c to Teichner (2015), improvements in growth parameters are required for feasibility of algal biofuels. Lipid overproduction is an essential strategy to scale up the microalgal biodiesel production and can be achieved through nutrient starvation, light intensity, temperature, carbon dioxide, salinity stress, and metal stress (Zhu *et al.,* 2016). A/c to Singh & Gu (2010), minimizing the operational and maintenance cost along with maximization of oil-rich microalgae production is the key for successful and economically viable commercialization of microalgae-based fuels. Culture conditions that optimize the microalgal growth for improved lipid and carbohydrate contents has been standardized, considering economic and energy inputs for commercial scale cultivation and product recovery. Higher irradiance 70-100 $\mu Em^{-2}s^{-1}$, long light exposure (16 hrs to 24 hrs), elevated CO_2 levels (2-3%), salinity (7.5-8.5 pH), Nitrogen and Phosphorus limited media (Half strength as in BBM media) and high concentrations of chelates (Na_2-EDTA, 5-fold strength) and micronutrient (Fe and Mg, Double strength) favours microalgal productivity in terms of lipid and carbohydrate contents. Microalgal cultures (MASP-2 and MASP-4) accumulate good level of carbohydrate and lipid under standardized physiochemical conditions that can further modulated for bio-ethanol and bio-oil production respectively. Further studies pertaining to mass scale

production of microalgal biomass under standardized conditions (based on observations from experimental work), analysis of dried microalgal biomass for Lipid and Carbohydrate content followed by extraction and characterization of Bio-oil are underway.

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