Media engineering in marine diatom Phaeodactylum tricornutum employing cost-effective substrates for sustainable production of high value renewables

Mohammed Rehmanji[1], Asha Nesamma[1], Nida Khan[2], Tasneem Fatma[2], and Pannaga Jutur[1]

[1]International Centre for Genetic Engineering and Biotechnology New Delhi
[2]Jamia Millia Islamia Central University

January 3, 2022

Abstract

Phaeodactylum tricornutum is a marine diatom, and well-studied model of unicellular microalga. This diatom contains a wide range of high-value renewables (HVRs) with high commercial relevance owing to their importance in human nutrition and health. In this study, we screened P. tricornutum for biomass, eicosapentaenoic acid (EPA) and fucoxanthin production under photoautotrophic and mixotrophic condition with various substrate combinations. Results highlights that culture supplemented with glycerol and urea lead to enhanced biomass, biochemical and HVR production. Further continuous feeding of urea in glycerol supplemented medium results in an increase in biomass yield (0.77 g L-1) by ~ 2-fold. Additionally, continuous feeding of urea channelizes the carbon flux towards biosynthesis of fatty acids increasing FAME content by ~2-fold as compared to the control conditions. Overall EPA and fucoxanthin production was 27 mg L-1 and 11 mg L-1 (~2 & 4 fold) in urea fed cultures respectively. Present study demonstrates efficient valorization of cost-effective substrates such as glycerol and urea for the production of high-value renewables in P. tricornutum.

1. Introduction

The transition from biofuels to bio-product based economy, demands a suitable feedstock with capability of producing multiple high-value renewables (HVRs). Microalgae are by far the most abundant primary producers responsible for photosynthetic conversion of light energy and carbon dioxide (CO_2) into sustainable renewables[1]. Recent advances in bioprocess technology supports the development of microalgal cell factories for establishing environmentally sustainable manufacturing of HVRs. In this regard *Phaeodactylum tricornutum* , a unicellular, marine pennate diatom, is considered as a potential feedstock for the production of biofuel and HVRs such as[2, 3], eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)[4], chrysolaminarin[5], fucoxanthin[6, 7], etc., and can be considered as a suitable microalgal cell factory for sustainable biorefinery processes[8].

One of the major fatty acid in *P. tricornutum* is the ω-3 fatty acid EPA (C20:5), having a significant commercial importance in pharmaceutical and nutraceutical industries[9, 10]. These long chain polyunsaturated fatty acids (LC-PUFAs) have a bioactive role against a variety of disorders, including coronary heart disease, thrombosis, and recently in prospective adjuvant therapy in COVID-19-related cardiovascular problems[11-14]. EPA content in *P. tricornutum* is reported upto 3-5 % of DCW (dry cell weight) with an average productivity of 56 mg $L^{-1}D^{-1}$, highlighting a potential alternate vegan source of ω-3 fatty acid production[15]. Commercially EPA from *P. tricornutum* is available in global market *via*brand name SIMRIS® ALGAE OMEGA-3, containing 50 mg of EPA per capsule (www.simris.com/pages/ingredients).

Additionally, fucoxanthin (1% - 6% DCW) an important HVR, is the primary carotenoid produced in *P.tricornutum*[16][6]. During photosynthesis, xanthophylls acts as a light harvesting pigment connected to fucoxanthin-chlorophyll a/c-proteins (FCP), which are an integral part of the thylakoids[17]. Furthermore, due to its unique structure, fucoxanthin has various major bioactivities such as anti-oxidant, anti-obesity, anti-cancer properties, and it has been found to be an effective treatment for chronic disorders such as Alzheimer's[18-21]. Commercial source of fucoxanthin is primarily brown seaweeds, which are difficult to meet market demands due to low productivity, low quality, and high cost. The amount of fucoxanthin generated by *P. tricornutum* is substantially higher than brown seaweeds, making it a potential choice for commercial production[22].

Media engineering strategies for microalgae cultivation have recently acquired appeal as feasible techniques for achieving high HVR output[7, 23]. Though the high costs of substrates, is a major impediment for generating commercially viable product [24]. Mixotrophy, on the other hand, represents an innovative methodology for HVRs production in *P. tricornutum*[25, 26]. It appears challenging to attain high biomass, EPA, and fucoxanthin content simultaneously; hence, designing an appropriate strategy for the mixotrophic cultivation of*P. tricornutum* is critical for the commercial co-production of HVRs.

P. tricornutum grows mixotrophically and has been reported to grow efficiently on glucose, fructose, mannose, lactose and glycerol[27]. Furthermore, various nitrogen sources such as nitrate, nitrite, ammonia, and urea has been employed for its cultivation[28]. It has been reported that cultivation of *P. tricornutum* on glycerol supplemented medium yields 0.4 g $L^{-1}D^{-1}$ of biomass and 8.5 mg $L^{-1}D^{-1}$ of EPA[29].Furthermore, utilizing urea as sole nitrogen source resulted in a considerable enhancement in EPA content (26 mg g^{-1})[28-30]. Compared to the conventional nitrogen source i.e. sodium nitrate ($NaNO_3$), urea is the cost effective and environmental friendly substrate for the cultivation of microalgae and thus the production of HVRs[28, 31]. The nutrient costs for the large scale cultivation of *P. tricornutum* on F/2 medium (with $NaNO_3$) approximately to be USD 0.15 kg^{-1} biomass, whereas its cost decreased to half i.e. USD 0.07, kg^{-1} biomass when grown on a modified medium (with urea) (Cui et al., 2021). Whereas crude glycerol, a by-product of biodiesel processing, reported for the production of β-carotene and DHA from *Schizochytrium limanicum*and *Blakeslea trispora*[32]. As a result, using these substrates might be a viable method for sustainable production of biomass, and HVRs from *P. tricornutum* .

The aim of this study is to examine the effect of glycerol, urea, $NaNO_3$ and their various combinations for the production of HVRs in *P. tricornutum* , with a primary focus on EPA along with fucoxanthin. Our preliminary screening revealed that glycerol (0.1M) and urea (441 M) could be used as low-cost substrate for the generation of biomass, lipids, carbohydrates and EPA. Moreover, feeding additional urea to the culture supplemented with glycerol led to significant enhancement of biomass, EPA, and fucoxanthin production. In this context, we highlight an alternate strategy, beneficial for the sustainable co-production of various HVRs from *P. tricornutum* .

2. Experimental section

2.1 Microalgal strain and pre-culture conditions

The marine diatom, *Phaeodactylum tricornutum* UTEX 646, was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES) in Tsukuba, Japan. The microalgae were grown in Erlenmeyer flasks (1000 mL) containing 400 mL medium (minimal medium F/2)[33] for 4 days, under a light regime of 16:8 h and an illumination of 30 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at 24 °C. The F/2 medium was prepared in artificial seawater as described previously[34, 35].

2.2 Experimental design

Different substrates and various combinations along with concentrations used for initial experiments are mentioned in **Table S1 (Supporting information)** . Glycerol and urea concentrations were selected based on previous literature[29, 30]. Inoculation was carried out using the inoculum in linear growth phase as described earlier with a biomass of 0.1 g L^{-1}. The organic nutrients (urea and glycerol) were sterilized

by filtration through 0.2 µm membrane filters and added to the autoclaved medium in sterile conditions. Subsequently the effect of urea feed was investigated by cultivating *P. tricornutum* on glycerol + urea or M3. Urea was added as feed according to the consumption profile, before its complete exhaustion from the medium (**Table S2, Supporting information)** . Sampling was carried out at regular time intervals of 2 days for further analysis and growth was monitored by cell count using hemocytometer[36] and dry weight analysis (DCW). Parameters such as specific growth rate and doubling time were calculated as described previously[37, 38].

2.3 Estimation of organic and inorganic substrates

Residual sugars quantified using high-performance liquid chromatography (HPLC) which includes the Aminex HPX-87H Ion Exclusion column (300 × 7.8 mm) attached with a refractive index (R.I.) detector (Agilent Techologies, USA)[39]. The supernatant was collected every 48 h and was diluted to 100m× with 4 mM sulphuric acid. Sample of 10 uL injected into the column maintained at 40 ºC using 4 mM H_2SO_4 as the mobile phase with a flow rate of 0.3 mL min^{-1} and a run time of 50 min [40]. The peak area of standard glycerol (Sigma Aldrich Pvt. Ltd., USA) used as reference. Nitrate uptake by the cells was measured spectrophotometrically[41]. Briefly, 1 mL of culture was pelleted down at room temperature (RT), and supernatant was diluted 50-fold with deionized water. The residual nitrate content was determined by measuring the absorbance at 220 nm. Urea estimation was done as described in Jung et al[42, 43]. Briefly supernatant was used directly to perform the assay, 50 µL of supernatant was transferred into a clear flat-bottom 96-well plate. Then 200 µL of freshly prepared working reagent was added and mixed quickly by gently rocking the plate. The reaction was incubated for 1 h at room temperature. Optical densities (OD) at 430 nm were measured on the plate reader for measuring the urea content in the medium.

2.4 Biochemical characterization

Biochemical analysis of all the samples were done for analyzing the changes in composition, i.e., total proteins, carbohydrates, and lipids content subjected to various growth conditions. The sulfo-phospho-vanillin test was used to estimate the total lipid content[44]. Briefly, 0.5 mg cells were pelleted and resuspended into 100 µL of distilled water. The cells were treated with 2 mL of 98 % H_2SO_4 and incubated at 100 °C for 10 minutes. After cooling for 5 minutes on ice, 5 mL of phosphovanillin reagent (0.6 g vanillin in 10 mL ethanol and 90 mL distilled water and 400 mL of 85 % phosphoric acid) was added, and the samples incubated for 10 minutes at 37 °C, absorbance was measured at 530 nm. Total carbohydrate was estimated using a modified phenol-sulfuric acid technique [45]. Around 0.25 mg cells were hydrolyzed; with 0.2 mL of 98 % H_2SO_4 at RT for 1 hour; following that, 5 % of phenol was added along with 1 mL of H_2SO_4 and incubated at RT for 20 minutes and the absorbance was measured at 490 nm. Protein content was quantified using a modified biuret technique. After pelleting 0.5 mg cells, 1 mL of extraction buffer (25 % NaOH in 1N methanol) was added. The reaction mixture was incubated at 80 °C for 15 minutes. To eliminate debris, the sample was cooled to RT and centrifuged. The supernatant was then treated with $CuSO_4$ solution (0.21 % $CuSO_4$ in 30 % NaOH) and kept at RT for 10 minutes before being measured at 310 nm[46].

2.5 Quantification of total fatty acid methyl esters (FAMEs)

A modified Bligh-dyer technique was adapted from shaikh et al[35] for extracting total fatty acid methyl esters (FAMEs). The hexane layer was injected into an Agilent 6890 gas chromatograph (GC) instrument, which was connected to a triple quadrupole outfitted with an OMEGAWAX 250 column (30 m x 0.25 m x 0.25 m). The GC-MS running conditions were as stated by Kareya et al [47]. Concisely, 2 L of the sample was run in the split mode (1:10) for 25 minutes, with the beginning oven temperature set at 150 °C and a simultaneous escalation of 10 °C min^{-1} to 240 °C. For quenching, helium (He) and nitrogen (N_2) gas were employed at flow rates of 2.25 mL min^{-1} and 1.5 mL min^{-1}, respectively. The data was collected using the Agilent 7000D triple quadrupole selective mass detector, which has a scan range of 30 to 550 amu. The produced peaks were compared to NIST libraries for identification and alignment based on retention indices as well as mass spectral similarity (cut-off hits with R-values greater than 600 were selected).

2.6 Quantification of fucoxanthin in *P. tricornutum*

Fucoxanthin was quantified using high-performance liquid chromatography (HPLC), and the extraction was performed as reported in Paliwal et al[48]. Briefly, 0.5 mg cells were centrifuged and resuspended in 1 ml of 100% methanol. For pigment extraction, the cell suspension briefly vortexed with glass beads for 20 minutes. The supernatant was collected and analyzed by HPLC-UV (Agilent Infinity series 1,260 HPLC, Agilent Technologies, Santa Clara, CA, United States). Running condition were as follows, C30 column (4.6 x 250 mm, 5 mm) was used to run the samples, at 35 °C with the binary solvent system as the mobile phase, which included methanol as primary solvent A and methyl tert-butyl ether (MTBE) as solvent B. The run conditions were as follows: 2–20 % B for the first 10 minutes, then 20 % B (10–12 minutes), 20–80 % B (12–30 minutes), 80 % B (30–32 minutes), and 80–2 % B (32–35 minutes)[49]. Pigments were measured at 437 nm and identified by comparing the retention duration of DHI standards from Hrsholm, Denmark.

2.7 Statistical Analysis

Experimental runs were conducted in triplicate **(** the mean values were presented as ±SE), comparing each condition in the time course for a period of 10 days. Statistical analyses such as ANOVA and t-test were performed using Microsoft excel for determination of significance.

3. Results

3.1. Biomass production of *P. tricornutum*

Initial screening for measuring the growth and nutrient consumption in photoautotrophic and mixotrophic conditions was performed. *P. tricornutum* was grown with an initial biomass concentration of 0.1 g $L^{-1}$and attained a maximum biomass i.e. 0.77 g L^{-1} (˜2-fold higher) in M4 condition in 10 days. Additionally higher growth rates were observed in the mixotrophic cultures (**Table 1)** . Overall, the combined effect of mixotrophy and urea feeding clearly show a higher growth rate and biomass in *P. tricornutum* . Previous reports have shown the effect of mixotrophy on growth of *P. tricornutum* which correlates with our results [25, 27, 50, 51].

Substrate consumption reflects biomass productivity, thus nutrient uptake rates were measured (**Figure S1, S2, S3, S4, Supporting information)** . *P. tricornutum* consumed around 3 g L^{-1} glycerol (10^{th} day), 75 mg L^{-1} $NaNO_3$ (6^{th} day), and 26 mg L^{-1} urea (4^{th} day) (**Figure S1, Supporting information**). Further, in the presence of glycerol, consumption rate of nutrients increased resulting in the exhaustion of $NaNO_3$ by 4^{th} day (M1) and urea by 2^{nd} day. Mixotrophic mode of *P. tricornutum* enhanced the nutrient uptake rate was previously reported by Villanova et al.,[51]. Interestingly, urea is completely consumed in the medium by 4^{th} day (P2, P3, M2, M3), whereas $NaNO_3$ is exhausted by 6^{th} (P1) day of cultivation (**Figure S2** , **S3, Supporting information**). This also justifies decrease in biomass and growth rate in P3 culture conditions as compared to P1and P2. Hence, a strategy based on feeding urea was designed to maintain a high biomass rate on a cost-effective substrate (M4). Urea was fed into the medium at the early log phase at 36^{th} hour (before urea depletion) according to the 12-hour urea consumption profile (**Table S2** , **Figure S4, Supporting information)** . Growth profile in M4 medium improved as compared to the P1 and M3, highlighting it as effective nitrogen source for the growth of *P. tricornutum* . Effect of different nitrogen sources on growth of *P. tricornutum* , highlighted urea as the best source due to the increase in biomass yield and lower substrate cost[28]. It can be observed that *P. tricornutum* prefers urea as nitrogen source and consumes it more efficiently than $NaNO_3$ (**Figure S2, S4, Supporting information**).

3.2. Biochemical analysis

The volumetric concentration of biochemical constituent were estimated to understand the influence of various growth conditions on cellular constituents of *P. tricornutum* (**Table 1**). Biochemical constituents were significantly enhanced in M4 condition. Protein titer in M4 condition was maximum i.e., ˜362 mg L^{-1} (˜2.5-fold increase) and with higher biomass productivity (**Figure 1)** . In addition, increased protein concentrations were observed in photoautotrophic cultures with 2 nitrogen sources and combined mixotrophic medium supplemented with mixed nitrogen source (P2 and M2) (**Table 1)** . Microalgae under the influence of nitrogen rich environment (feed cultures) channelize the photosynthetically fixed carbon to protein synthesis

to support growth and division, however, exactly an opposite trend is seen in nitrogen deprived conditions, where cell division ceases and fraction of carbon allocation to storage molecules increases at the expense of protein synthesis [28].

The maximum lipid titer was 227 mg L^{-1} and 208 mg L^{-1} in M4 and M3 cultures respectively (**Table 1)** . Our study highlights that glycerol and urea supplementation enhanced lipid productivity which is quite interesting, as many microalgae are known to accumulate lipids in response to N limitation[52]. This can be justified by the addition of glycerol; *P. tricornutum* in lipid accumulation phase uses glycerol as a carbon source and hence channelize the carbon directly towards lipid production, moreover feeding nitrogen leads to more biomass, which in turn reflects in increasing the productivity of biochemical constituents. The result clearly indicates that the strategy of urea recharge *via* feeding in glycerol supplemented medium can effectively produce biomass and lipids.

Cultures supplemented in M3 condition showed higher carbohydrate production i.e., 164 mg L^{-1}, and was enhanced to 231 mg L^{-1} (˜1.5-fold increase) in M4 (**Table 1)** . Carbohydrate are primary storage reserves of diatoms, one the first photosynthetic product of the Calvin–Benson cycle and serves as a precursor for various cellular components[53]. Therefore, carbohydrate accumulation and conversion into other metabolites is necessary for cell survival. It was observed that addition of glycerol switches the cellular metabolism towards carbohydrate production in*P. tricornutum*[52]. Compared to lipid production, the carbohydrate content was less in glycerol supplemented cultures, which clearly demonstrates the channeling of carbon flux towards TAG production[54]. In this context, feeding *P. tricornutum* with a cost-effective nitrogen source like urea, resulted in higher protein, lipid, carbohydrate and biomass production. Highlighting the potential of our substrates to enhance the overall productivity of *P. tricornutum* and to further use in a biorefinery-based system.

3.3 FAME analysis

FAME profile showed ˜ 2-fold increase in glycerol supplemented cultures (M1, M2, M3, M4), where ˜97 mg L^{-1} of lipid was obtained in the M4 condition, which is ˜ 40 % higher as compared to P1 and 8 % as compared to M3 cultures (**Table 2)** . Glycerol in the form of reduced carbon source affects lipid metabolism by mimicking most of the effects of nitrogen limitation[52, 55], hence the storage molecules i.e. triacylglycerol (TAGs) are accumulated in M3 culture. Moreover, significant variations were observed in FAME profile (% total fatty acid) in different culture conditions (**Table 2)** . Saturated and monounsaturated fatty acid percentage were higher in P1, P3, M1, M2 and M3 supplemented cultures, whereas P2 and M4 cultures showed a higher polyunsaturated fatty acid percentage. EPA being the major long chain fatty acid in *P. tricornutum* was highest among all other fatty acid species in all the conditions but ˜2-fold increase of EPA % of total fatty acid (TFA) was observed in M4, accounting for 28% of TFA (**Table 2).** These results imply that feeding with cost effective nitrogen source can be used as an effective strategy to enhance the omega-3 production in *P. tricornutum.*

3.4. Enhancement of EPA and fucoxanthin production in *P. tricornutum.*

Total EPA and fucoxanthin content is represented in **Figure 2A & B.** The EPA content increased from 9 mg L^{-1} to 27 mg L^{-1} in M4 condition within 10 days **(Table 3)** , which accounts for a ˜3-fold increase compared to P1 control and ˜ 2-fold increase in compare to M3 conditions. Content wise on 6^{th} day, EPA was higher in initial log phase accounting to 37 mg g^{-1} in the M2 condition, whereas M1 and M3 condition showed 34 mg g^{-1} of EPA (**Figure 2A)** . Furthermore, EPA content was higher in culture supplemented with only urea (P3) when compared to $NaNO_3$ (P1) on 4^{th} and 6^{th} day (**Figure 2A)** . Increase in EPA content in the initial growth phase was reported earlier in the presence of urea[28] and glycerol along with urea in *P. tricornutum*[29, 30, 56].

Along with EPA, fucoxanthin content was measured in various culture conditions as depicted in **Figure 2B.** Higher fucoxanthin production was observed in M4 conditions i.e., 11 mg L^{-1} on 10^{th} day of experiment (**Table 3)** . This showed a ˜ 4-fold increase in fucoxanthin productivity compared to P1 and M3 conditions. Fucoxanthin production was 6.62 mg L^{-1} in medium supplemented with P2 on 10^{th} day, whereas highest

content was 21.9 mg g^{-1} on 6^{th} day of cultivation in the P1 condition (**Figure 2B)** . Addition of glycerol limited the nitrogen content in the medium, and thus a decrease in the fucoxanthin content is observed in glycerol supplementation (M1 and M3) (**Figure 2B)** , although higher production is observed in M2 condition compared to M1 and M3 conditions, which can be justified by the presence of two nitrogen source and residual N in the medium until 10^{th} day (**Figure S1, S2, S3, Supporting information)** . Fucoxanthin being a primary carotenoid is highly influenced by nitrogen content in the medium[57]. Previous reports highlights higher fucoxanthin content in *P.tricornutum*cultivated in nitrate-enriched medium[7, 16], thus feeding cultures with urea, increased biomass and the biomass associated metabolites i.e. EPA and fucoxanthin.

4. Discussion

Microalgae derived HVRs are progressively playing a significant role in the production of cosmeceuticals, medicinal alternatives and high-value foods [58].*P. tricornutum* as a cell factory for marketable products which majorly includes omega 3 PUFAs like EPA and light harvesting pigment fucoxanthin, [8]. Due to their high pharmaceutical and nutritional relevance, these HVRs currently have a high market value for example the market size of omega 3 ingredients in 2019 exceeded USD 2.3 billion and is estimated to grow at over 7.2% compound annual growth rate (CAGR) between 2020 and 2026 (www.gminsights.com). Moreover, revenue generated from fucoxanthin was USD 100 million in 2019, and is expected to reach USD 123 million by 2025, with a CAGR of 3.5% (www.wboc.com).

Sustainable co-production of these HVRs is a challenging task in terms of economic feasibility. Therefore, to enhance production, modification in the cultivation parameters can be employed as an effective strategy. Our preliminary analysis highlighted the effect of different substrates (glycerol, urea, and $NaNO_3$) and their different combination on growth and biochemical constituents. Optimization of medium with glycerol and urea (M4) addition is a suitable, sustainable and economic approach, due to its beneficial effect on cellular metabolism, biomass and HVRs production enhanced. Furthermore, since the nitrate is depleted from the medium in the early phases of growth, employing a strategy to feed urea in culture medium before exhaustion proved to be more feasible in terms of enhancing HVR production.

Screening highlighted M4 as a suitable condition to achieve higher biomass and better growth rate in *P. tricornutum* . A combined effect of mixotrophy and urea feeding resulted in higher biomass compared to cultures without urea feeding. Mixotrophy metabolism reportedly engages both respiration and photosynthesis at the same time[26]. Transport activities between the chloroplast and mitochondria, as well as the physical connection between the two organelles, have an intense energetic exchange during mixotrophy in *P. tricornutum*[52]. Moreover, mixotrophy enhances the nutrient uptake rate as observed in glycerol-supplemented (M1 and M3) medium, which enhances nitrogen uptake (**Figure S1, S2, S3, Supporting information)** .

The increase in biomass reflects the assimilation of C and N in cellular biochemical components. Higher protein content was obtained in the medium supplemented with M2 and M4 combinations depicting conversion of excess nitrogen into cellular proteins. Currently microalgal proteins are in demand as functional foods due to its high nutritional value, and health benefits. Additionally utilization of whole algal biomass as super foods or healthy foods is promoted worldwide in order to maintain a balance diet[59]. Therefore higher protein production in *P. tricornutum* makes it a suitable candidate for nutraceutical applications. Moreover, carbohydrate productivities reached to 231 mg L^{-1} in M4 condition (**Table 1)** . Anticancer activity of polysaccharides derived from*P. tricornutum* along with antibacterial, antioxidant, and antiviral properties is well demonstrated[60, 61]. Polysaccharides from P. *tricornutum* have a high commercial potential and a wide range of applications in different industrial sectors.

Biodiesel is considered as a desirable energy source, an exceptional alternative to fossil fuels. According to the previous reports the FAME, profile of *P. tricornutum* meets the requirements of international biodiesel standards, showing that it could be a good alternative for biodiesel production[2]. Our results are in correlation with these studies showing a high percentage of C16, C16:1, C18:1 (%TFA). Moreover, higher FAME yields obtained in *P. tricornutum* on cost effective substrates, indicates *de novo*fatty acid synthesis

in presence of glycerol. These fatty acids are main components of storage lipids, which justifies its higher accumulation in the form of TAGs in the mixotrophic conditions[26, 56]. Feeding additional nitrogen shifted the fatty acid metabolism towards membrane lipid synthesis i.e., EPA showing a higher percentage of PUFAs in feed conditions (**Table 2**).

The expense of nutrients is an inevitable liability in the production of algal biomass (4-8 % of total cost)[62]. As a result, the usage of recovered nutrients from secondary streams, such as glycerol from the biodiesel sector and urea, ammonia from wastewater, may be included into the biorefinery framework for the long-term synthesis of HVRs. Although previous reports highlights the use of glycerol as a C source for microalgae production[63] and the recovery of nutrients (N and P) by digestate pretreatment[64], lesser data is available on the combined effect of replacing both C and N with secondary streams. The validity of such a strategy has been introduced in our study by applying it on production of HVRs.

In M4 condition, higher EPA (27 mg L^{-1}) was accumulated along with TAG (92 mg L^{-1}) (**Figure 2A, Table 2).** Indicating that glycerol triggers metabolic changes resembling not only nitrogen depletion but also promote growth[56]. An increase in EPA content on initial growth days (**Figure 2A)** indicate the necessity of structural lipids in log phase but as nitrogen depletes in the medium EPA content did not change (**Figure 2A)** in the respective conditions, though urea-fed cultures maintain the EPA pool throughout the experiment. EPA being one of the main structural lipids required during the growth supporting conditions, whereas is incorporated into TAGs when the cells are under nitrogen stress. Hence, to maintain high EPA content in stress condition use of glycerol as carbon source can be ideal, additionally feeding with the nitrogen source can enhance the productivity *via* ., higher biomass yields.

Along with EPA, fucoxanthin content highlights a significant increase in the productivity especially during feed conditions. Fucoxanthin is associated with photosynthetic machinery and is enhanced as the growth increases. Nitrogen on the contrary in the form of urea positively regulates the photosynthetic machinery in feed condition (M4) reflected by the higher biomass production. Hence, in the condition supplemented with nitrogen substrates (P1, P2, and P3) fucoxanthin content was higher (**Figure 2B)** as compared to glycerol-supplemented cultures (M1, M2, and M3). Glycerol is found to be directly entering glycolysis*via* glycerol kinase producing dihydroxyacetone phosphate and thus channelizing flux towards lipid synthesis rather than carotenoid production [52].

Our findings highlight the effect of feeding nitrogen to the glycerol-supplemented cells (M4), higher nitrogen content results in increasing the biomass and thus enhancing growth related metabolites like EPA and fucoxanthin. Presence of glycerol in the medium upregulates the EPA biosynthesis *via* regulating enzyme like stearoyl desaturase [56] and the effect of feeding strategies of various nutrients on biomass, and lipid productivities[65-67] is mentioned in previous reports. In conclusion, this study successfully highlights an alternative application of feeding nutrients in mixotrophic mode to increase HVRs like EPA and fucoxanthin. Further, the ability of *P. tricornutum* to grow mixotrophically using glycerol as the main carbon source and urea as an additional nitrogen source can be applied in the biorefinery approach for recovering carbon and nitrogen from waste effluents to produce HVRs.

Statements and Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by grants from Department of Biotechnology (BT/PB/Center/03/2011-Phase II), Ministry of Science and Technology, Government of India (GoI), New Delhi, India. Fellowship(s) for MR, and AAN are supported from Department of Biotechnology (DBT), New Delhi, India, is duly acknowledged.

Data availability statement: All the data within the main article and supplementary material are provided.

Authors Contributions

Mohammed Rehmanji : Conceptualization, Methodology, Investigation, Analysis, Writing - Original Draft. **Asha Arumugam Nesamma:** Methodology, Investigation, Analysis, Writing - Original Draft, Writing - review & editing. **Tasneem Fatma** : Supervision, Writing - review & editing. **Nida Jamil Khan:** Supervision, Writing - review & editing**. Pannaga Pavan Jutur** : Conceptualization, Funding acquisition, Supervision, Methodology, Validation, Project administration, Writing - review & editing.

References

[1] R. Singh, P. Parihar, M. Singh, A. Bajguz, J. Kumar, S. Singh,*Front. microbiol* **2017** , *8* .

[2] M. Branco-Vieira, S. S. Martin, C. Agurto, M. A. Santos, M. A. V. Freitas, N. S. Caetano, *Energy Procedia* **2017** ,*136* , 369-373.

[3] X. Hao, L. Luo, J. Jouhet, F. Rébeillé, E. Maréchal, H. Hu,*Biotechnol. Biofuels* **2018** , *11* , 312.

[4] A. Pudney, C. Gandini, C. K. Economou, R. Smith, P. Goddard, J. A. Napier, *Sci. Rep* **2019** , *9* , 11444.

[5] M. A. Caballero, D. Jallet, L. Shi, C. Rithner, Y. Zhang, G. Peers, *Algal Res* **2016** , *20* , 180-188.

[6] F. Derwenskus, B. Schäfer, J. Müller, K. Frick, A. Gille, K. Briviba, *Chem. Ing. Tech* **2020** , *92* , 1780-1789.

[7] R. Yang, D. Wei, *Front. Bioeng. Biotechnol***2020** *, 8* .

[8] T. Butler, R. V. Kapoore, S. Vaidyanathan, *Trends Biotechnol* **2020** , *38* , 606-622.

[9] J. Gupta, R. Gupta, *Curr. Pharm. Biotechnol***2020** , *21* .

[10] M. Rehmanji, S. Suresh, A. A. Nesamma, P. P. Jutur, in Microbial and Natural Macromolecules (Eds. S. Das, H. R. Dash), Academic Press **2021** , Chp 26.

[11] Z. Szabó, T. Marosvölgyi, É. Szabó, P. Bai, M. Figler, Z. Verzár, *Front. Physiol* **2020** , *11* .

[12] P. Weill, C. Plissonneau, P. Legrand, V. Rioux, R. Thibault,*Biochimie* **2020** , *179* , 275-280.

[13] W.-H. Schunck, A. Konkel, R. Fischer, K.-H. Weylandt,*Pharmacol. Ther* **2018** , *183* , 177-204.

[14] O. Saidane, L. Semerano, J. Sellam, *Jt. Bone Spine***2019** , *86* , 9-12.

[15] R. Vaezi, J. A. Napier, O. Sayanova, *Mar. Drugs***2013** , *11* , 5116-5129.

[16] D. D. McClure, A. Luiz, B. Gerber, G. W. Barton, J. M. Kavanagh, *Algal Res* **2018** , *29* , 41-48.

[17] K. Gundermann, M. Schmidt, W. Weisheit, M. Mittag, C. Büchel,*Biochim Biophys Acta Bioenerg* **2013** , *1827* , 303-310.

[18] W. Fu, K. Wichuk, S. Brynjólfsson, *New Biotechnol***2015** , *32* , 547-551.

[19] J. Peng, J.-P. Yuan, C.-F. Wu, J.-H. Wang, *Mar. Drugs***2011** , *9* , 1806-1828.

[20] C. Vílchez, E. Forján, M. Cuaresma, F. Bédmar, I. Garbayo, J. M. Vega, *Mar. Drugs* **2011** , *9* .

[21] S. Xiang, F. Liu, J. Lin, H. Chen, C. Huang, L. Chen, *J. Agric. Food Chem* **2017** , *65* , 4092-4102.

[22] G. Rajauria, B. Foley, N. Abu-Ghannam, Int. *Food Res. J***2017** , **99** , 995-1001.

[23] L. F. Wu, P. C. Chen, A. P. Huang, C. M. Lee, *Bioresour Technol* **2012** , *113* , 14-18.

[24] L. Oliver, T. Dietrich, I. Marañón, M. C. Villarán, R. J. Barrio, *Resources* **2020** , *9* , 148.

[25] M. C. Cerón Garcí, J. M. Fernández Sevilla, F. G. Acién Fernández, E. Molina Grima, F. García Camacho, *J. Appl. Phycol***2000** , *12* , 239-248.

[26] V. Villanova, C. Spetea, *Physiol. Plant* **2021** ,*173* , 603-611.

[27] M. C. Cerón-García, F. Camacho, A. Mirón, J. M. Fernandez-Sevilla, Y. Chisti, E. Molina-Grima*, J. Microbiol. Biotechnol* **2006** , *16* , 689.

[28] J. P. Fidalgo, A. Cid, J. Abalde, C. Herrero, *Cah. Biol. Mar* **1995** , *36* , 165-173.

[29] M. C. Cerón Garcıa, A. Sánchez Mirón, J. M. Fernández Sevilla, E. Molina Grima, F. Garcıa Camacho, *Process Biochem***2005** , *40* , 297-305.

[30] Y. Cui, S. R. Thomas-Hall, E. T. Chua, P. M. Schenk, *J. Phycol* **2021** , *57* , 258-268.

[31] P. Pérez-López, S. González-García, C. Allewaert, A. Verween, P. Murray, G. Feijoo, *Sci. Total Environ* **2014** , 991-1002.

[32] M. Bindea, B. Rusu, A. Rusu, M. Trif, L. F. Leopold, F. Dulf,*Microb. Cell Factories* **2018** , *17* , 97.

[33] R. R. Guillard, J. H. Ryther, *Can. J. Microbiol***1962** , *8* , 229-239.

[34] M. S. Kareya, I. Mariam, K. M. Shaikh, A. A. Nesamma, P. P. Jutur, *Front. Plant Sci* **2020** , 11.

[35] K. M. Shaikh, A. A. Nesamma, M. Z. Abdin, P. P. Jutur,*Biotechnol. Biofuels* **2019** , *12* , 182.

[36] R. Guillard, M. Sieracki, *Algal Culturing Techniques***2005** , 239-252.

[37] M. Levasseur, P. A. Thompson, P. J. Harrison, *J. Phycol***1993** , *29* , 587-595.

[38] R. Singh, C. Paliwal, A. A. Nesamma, A. Narula, P. P. Jutur,*Front. Mar. Sci* **2020** , 7.

[39] I. Mariam, M. Kareya, A. Nesamma, P. Jutur, *Algal Res***2021** , *55* , 102285.

[40] A. Patel, U. Rova, P. Christakopoulos, L. Matsakas,*Biotechnol. Biofuels* **2019** , *12* , 255.

[41] Y. Collos, F. Mornet, A. Sciandra, N. Waser, A. Larson, P. J. Harrison, *J. Appl. Phycol* **1999** , *11* , 179-184.

[42] D. Jung, H. Biggs, J. Erikson, P. U. Ledyard, *Clin. Chem***1975** , *21* , 1136-1140.

[43] R. J. X. Zawada, P. Kwan, K. L. Olszewski, M. Llinas, S.-G. Huang, *Biochem Cell Biol* **2009** , *87* , 541-544.

[44] S. K. Mishra, W. I. Suh, W. Farooq, M. Moon, A. Shrivastav, M. S. Park, *Bioresour Technol* **2014** , *155* , 330-333.

[45] C. Paliwal, I. Pancha, T. Ghosh, R. Maurya, K. Chokshi, S. V. Vamsi Bharadwaj, *Bioresour Technol* **2015** , *197* , 363-368.

[46] Y. Chen, S. Vaidyanathan, *Anal. Chim. Acta***2013** , *776* , 31-40.

[47] M. S. Kareya, I. Mariam, G. H. Rajacharya, A. A. Nesamma, P. P. Jutur, *Biofuel Bioprod Biorefin* **2021** .

[48] C. Paliwal, P. P. Jutur, *Chem. Eng. Sci* **2021** ,*418* , 129413.

[49] B. Gleize, M. Steib, M. André, E. Reboul, *Food Chem***2012** , *134* , 2560-2564.

[50] X.-J. Liu, S.-S. Duan, A.-F. Li, K.-F. Sun, *J. Integr. Plant Biol* **2009** , *51* , 272-278.

[51] V. Villanova, D. Singh, J. Pagliardini, D. Fell, A. Le Monnier, G. Finazzi, *Front. Plant Sci* **2021** , *12* , 642199.

[52] V. Villanova, A. E. Fortunato, D. Singh, D. D. Bo, M. Conte, T. Obata, *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **2017** ,**372** , 20160404.

[53] B. Gao, A. Chen, W. Zhang, A. Li, C. Zhang, *J. Ocean Univ. China* **2017** , *16* , 916-924.

[54] F. Daboussi, S. Leduc, A. Maréchal, G. Dubois, V. Guyot, C. Perez-Michaut, *Nat. Commun* **2014** , *5* , 3831.

[55] M. Su, G. D'Imporzano, D. Veronesi, S. Afric, F. Adani,*J. Biotechnol* **2020** , *323* , 73-81.

[56] T. K. Marella, R. Bhattacharjya, A. Tiwari, *Microb. Cell Factories* **2021** , *20* , 135.

[57] S. Xia, B. Gao, J. Fu, J. Xiong, C. Zhang, *J. Biosci. Bioeng* **2018** , 126.

[58] K. Rahman, *Microalgae Biotechnology for Food, Health and High Value Products* , Springer, Germany, **2020** .

[59] A. K. Koyande, K. W. Chew, K. Rambabu, Y. Tao, D.-T. Chu, P.-L. Show, *Food Sci. Hum. Wellness* **2019** , *8* , 16-24.

[60] I. Michalak, K. Chojnacka, *Eng. Life Sci* **2015** ,*15* , 160-176.

[61] S. Yang, H. Wan, R. Wang, D. Hao, *PeerJ* **2019** ,*7* , e6409.

[62] F. G. Acién, J. M. Fernández, J. J. Magán, E. Molina,*Biotechnol. Adv* **2012** , *30* , 1344-1353.

[63] D. Veronesi, A. Ida, G. D'Imporzano, F. Adani, *Chem. Eng. Trans* **2015** , *43* , 1201-1206.

[64] C. Ledda, A. Schievano, S. Salati, F. Adani, *Water Res***2013** , *47* , 6157-6166.

[65] H. F. Jin, B. R. Lim, K. Lee, *J. Environ. Sci. Health A***2006** , *41* , 2813-2824.

[66] D. Soletto, L. Binaghi, A. Lodi, J. C. M. Carvalho, A. Converti, *Aquac. Res* **2005** , *243* , 217-224.

[67] M. Takagi, K. Watanabe, K. Yamaberi, T. Yoshida, *Appl. Microbiol. Biotechnol* **2000** , *54* , 112-117.

Figure Legends

Figure 1. Time course profile of *P. tricornutum* biomass concentration in **Photo1 (P1):** Photoautotrophic control culture with additions of 882 µM sodium nitrate ($NaNO_3$);**Photo 2 (P2):** Photoautotrophic control culture with additions of 441 µM urea and 882 µM sodium nitrate ($NaNO_3$);**Photo 3 (P3):** Photoautotrophic control culture with additions 441 µM of urea; **Mixo 1 (M1):** Mixotrophic culture with additions of 0.1M glycerol and 882 µM sodium nitrate ($NaNO_3$);**Mixo 2 (M2):** Mixotrophic culture with additions of 0.1M glycerol, 441 µM of urea and 882 µM sodium nitrate ($NaNO_3$); **Mixo 3 (M3):** Mixotrophic culture with additions of 0.1M glycerol and 441 µM of urea; **Mixo 4 (M4):**Mixotrophic culture with additions of 0.1M glycerol, feeded with 441 µM of urea post 36 hours of cultivation. Values indicate mean (n = 3) with standard error; * indicate statistical significance by one-way analysis of variance (ANOVA), p-value < 0.05.

Figure 2A. Quantification of EPA content (mg g^{-1}) of *P. tricornutum* cultivated in different conditions, **Photo1 (P1):** Photoautotrophic control culture with additions of 882 µM sodium nitrate ($NaNO_3$);**Photo 2 (P2):** Photoautotrophic control culture with additions of 441 µM urea and 882 µM sodium nitrate ($NaNO_3$);**Photo 3 (P3):** Photoautotrophic control culture with additions 441 µM of urea; **Mixo 1 (M1):** Mixotrophic culture with additions of 0.1M glycerol and 882 µM sodium nitrate ($NaNO_3$);**Mixo 2 (M2):** Mixotrophic culture with additions of 0.1M glycerol, 441 µM of urea and 882 µM sodium nitrate ($NaNO_3$); **Mixo 3 (M3):** Mixotrophic culture with additions of 0.1M glycerol and 441 µM of urea. Values indicate mean (n = 3) with standard error; * indicate statistical significance by one-way analysis of variance (ANOVA), pvalue < 0.05.

Figure 2B. Quantification of fucoxanthin content (mg g^{-1}) of *P. tricornutum* cultivated in**Photo1 (P1):** Photoautotrophic control culture with additions of 882 µM sodium nitrate ($NaNO_3$); **Photo 2 (P2):**Photoautotrophic control culture with additions of 441 µM urea and 882 µM sodium nitrate ($NaNO_3$);

Photo 3 (P3): Photoautotrophic control culture with additions 441 µM of urea;**Mixo 1 (M1):** Mixotrophic culture with additions of 0.1M glycerol and 882 µM sodium nitrate ($NaNO_3$); **Mixo 2 (M2):** Mixotrophic culture with additions of 0.1M glycerol, 441 µM of urea and 882 µM sodium nitrate ($NaNO_3$); **Mixo 3 (M3):** Mixotrophic culture with additions of 0.1M glycerol and 441 µM of urea conditions. Values indicate mean (n = 3) with standard error; * indicate statistical significance by one-way analysis of variance (ANOVA), pvalue < 0.05.

Table Legends

Table 1. Performance of microalgal dry biomass concentration on day 10, specific growth rate, carbohydrate titer, protein titer, and lipid titer of *P. tricornutum* under **Photo1 (P1):**Photoautotrophic control culture with additions of 882 µM sodium nitrate ($NaNO_3$); **Photo 2 (P2):** Photoautotrophic control culture with additions of 441 µM urea and 882 µM sodium nitrate ($NaNO_3$); **Photo 3 (P3):** Photoautotrophic control culture with additions 441 µM of urea; **Mixo 1 (M1):** Mixotrophic culture with additions of 0.1M glycerol and 882 µM sodium nitrate ($NaNO_3$); **Mixo 2 (M2):** Mixotrophic culture with additions of 0.1M glycerol, 441 µM of urea and 882 µM sodium nitrate ($NaNO_3$); **Mixo 3 (M3):** Mixotrophic culture with additions of 0.1M glycerol and 441 µM of urea; **Mixo 4 (M4):**Mixotrophic culture with additions of 0.1M glycerol, feeded with 441 µM of urea post 36 hours of cultivation. Values indicate mean (n = 3) with standard error; * indicate statistical significance by one-way analysis of variance (ANOVA), pvalue < 0.05.

Table 2. Total fatty acid methyl esters (FAMEs) (% FAME profile) using gas chromatography-mass spectrometry (GC-MS) of *P. tricornutum* sp. in the presence of glycerol, $NaNO_3$, and urea. Values indicate mean (n = 3) with standard error; * indicate statistical significance by

one-way analysis of variance (ANOVA), p-value < 0.05.

Table 3. Eicosapentaenoic acid and fucoxanthin titer (mg L^{-1}) in *P. tricornutum* sp. in **Photo1 (P1):** Photoautotrophic control culture with additions of 882 µM sodium nitrate ($NaNO_3$); **Photo 2 (P2):** Photoautotrophic control culture with additions of 441 µM urea and 882 µM sodium nitrate ($NaNO_3$); **Photo 3 (P3):** Photoautotrophic control culture with additions 441 µM of urea; **Mixo 1 (M1):** Mixotrophic culture with additions of 0.1M glycerol and 882 µM sodium nitrate ($NaNO_3$); **Mixo 2 (M2):** Mixotrophic culture with additions of 0.1M glycerol, 441 µM of urea and 882 µM sodium nitrate ($NaNO_3$); **Mixo 3 (M3):** Mixotrophic culture with additions of 0.1M glycerol and 441 µM of urea; **Mixo 4 (M4):**Mixotrophic culture with additions of 0.1M glycerol, feeded with 441 µM of urea post 36 hours of cultivation condition. Values indicate mean (n = 3) with standard error; * indicate statistical significance by one-way analysis of variance (ANOVA), p-value < 0.05.

Table 1.

Parameters/Conditions	**P1**	**P2**	**P3**	**M1**	**M2**	**M3**
Specific growth rate (day^{-1})	0.26 ± 0.01	0.25 ± 0.03	0.22 ± 0.02	0.37 ± 0.03*	0.32 ± 0.04*	0.38
Biomass (g L^{-1})	0.35 ± 0.03	0.36 ± 0.03	0.32 ± 0.01	0.54 ± 0.02*	0.53 ± 0.06*	0.46
Lipid (mg L^{-1})	55.85 ± 0.89	61.77 ± 1.73	60.97 ± 1.17	167.66 ± 4.41*	152.85 ± 4.09*	208
Carbohydrates (mg L^{-1})	88.83 ± 2.45	86.91 ± 5.37	97.5 ± 6.12	93.83 ± 3.01*	124.5 ± 2.71*	164
Proteins (mg L^{-1})	169.49 ± 10.50	194.06 ± 1.20	132.10 ± 0.75	148.78 ± 1.59*	217.52 ± 2.20*	138

Total Fatty acid (% FAME)	**Total Fatty acid (% FAME)**	**Total Fatty acid (% FAME)**	**Total Fatty acid (**
Fatty acid	**P1**	**P2**	**P3**
C14	7.6 ± 0.01	7.6 ± 0.04	7.7 ± 0.15
C16	15.8 ± 0.03	14 ± 0.01	17.5 ± 0.21*
C16:1	20.2 ± 0.46	17.4 ± 0.39	23.1 ± 0.5
C16:2	4.7 ± 0.03	7.5 ± 0.03*	4.2 ± 0.14
C16:3	6.2 ± 0.03	7.8 ± 0.03	4.9 ± 0.11

C16:4	NA	1.1 ± 0.02	NA
C18	4.8 ± 0.15*	3.7 ± 0.02	2.6 ± 0.08
C18:1	11.8 ± 0.69	9.1 ± 0.09	11.8 ± 0.01
C18:2	1.2 ± 0.01	3.7 ± 0.09	3.6 ± 0.32
C18:3	2.3 ± 0.11	2.5 ± 0.16	2.3 ± 0.12
C18:4	2.7 ± 0.11	1.7 ± 0.01	2.0 ± 0.14
C20:4	NA	NA	NA
C20:5	18.1 ± 0.04	18.4 ± 0.15	15.4 ± 0.22
C24	4.5 ± 0.06	5.7 ± 0.24	4.7 ± 0.11
SFA	32.8 ± 0.05	31.0 ± 0.24	32.5 ± 0.54
MUFA	32.0 ± 0.22	26.4 ± 0.48	35.0 ± 0.51
PUFA	35.2 ± 0.27	42.6 ± 0.24	32.5 ± 0.04
Total FAME (mg L^{-1})	54.2 ± 0.28	50.6 ± 0.62*	51.7 ± 0.24

Table 2.

Table 3.

Conditions	**EPA concentrations (mg L^{-1})**	**Fucoxanthin concentrations (mg L^{-1})**
P1	9.09 ± 0.23*	5.44 ± 0.22*
P2	11.28 ± 0.33	6.62 ± 0.81
P3	10.44 ± 0.12	3.66 ± 0.44
M1	11.00 ± 0.17	3.05 ± 0.17
M2	14.29 ± 0.34	4.01 ± 0.87
M3	12.6 ± 0.37*	2.88 ± 0.93*
M4	27.07 ± 0.38*	11.01 ± 0.75*

Figure 1.

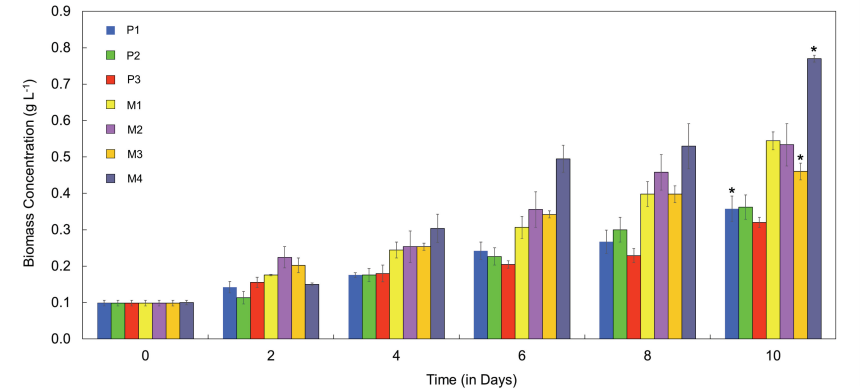

Figure 2A.

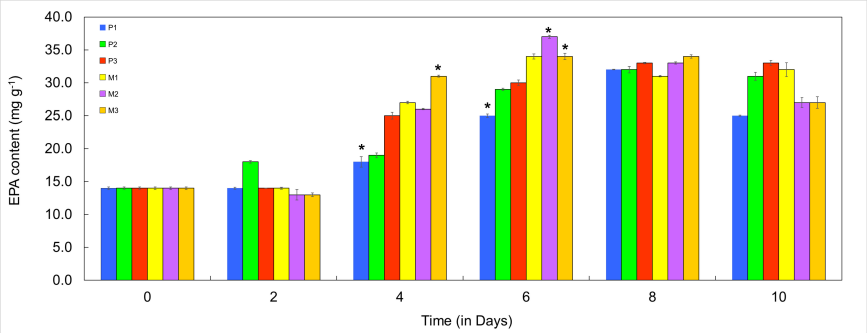

Figure 2B.