Chemostat Based Enrichment System: Nitrogen Loading Rates Impacts on Microalgal Communities and Intracellular Storage Compounds

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Abstract

In this study, the impacts of nitrogen loading rates on microalgal communities and intracellular storage compounds in a chemostat enrichment system were investigated. The chemostat was operated at a constant dilution rate of 0.5 d-1 with different nitrogen loading rates of 10, 20, 32.5, 42 and 63 mgN.l-1.d-1. The cultures with the lowest nitrogen loading rates showed nitrogen fixation capacity. Diverse microalgal communities were observed for nitrogen loading rates of 10 and 20 mgN.l-1.d-1. Chlorella sorokiniana and Chlorella vulgaris were dominant species at nitrogen loading rates of 42 and 32.5 mgN.l-1.d-1, respectively. Different species of Arthrospira platensis, Chlorella sp., Rhopalodia coexisted under the light-limited condition at NLR of 63 mgN.l-1.d-1. Lipids accumulation overtook starch accumulation at all nitrogen loading rates. mgN.l-1.d-1. Lipids accumulation overtook starch accumulation at all nitrogen loading rates. Specifically, the concentration of lipids in the enriched species cells, Chlorella sorokiniana was 4.2 times higher than the amount of accumulated starch. The results demonstrate that the approximately balanced addition of nutrients and light in chemostat enrichment culture could be an important trait to enrich a potential lipid production organism.

Introduction

In recent decades, biofuels have drawn considerable attention due to fossil fuel depletion and adverse climate effects of fossil fuel burning (Martins, et al., 2019; Kung, 2019; Kumar and Singh, 2020). Microalgae are introduced as a unique biomass feedstock for biofuels since they have several advantages including fast growth rates, resistance to extreme environmental conditions, feasible and eco-friendly large-scale production, and simple life cycle. Microalgae-derived biofuels production can be coupled with flue gas CO_2 mitigation, wastewater treatment, and high-value chemical compounds extraction. However, their production is not yet financially competitive with fossil fuels and conventional biofuels . Microalgal storage compounds which are the prime biofuel precursors, starch, and lipids, are used as the substrates for bioethanol and biodiesel production, respectively. Since these storage compounds' accumulation is not linearly delineated to the growth rate, one of the technical challenges in making biofuels cost-effective is to increase starch and lipids productivity. The most frequently reported approaches are nutrient starvation/limitation including nitrogen , phosphorus , and sulfur , salt stress (Takagi and Yoshida, 2006; Pancha et al., 2015) and light intensity stress . Nitrogen starvation is reported as the most convenient technique to enhance energy storage components . Most studies to date have been focused on two-stage production. In the first stage, microalgae are grown

in the non-limiting growth medium. In the second stage, microalgae are triggered by one of the above-mentioned nutrient limitations and/or stress approaches to induce storage polymer production . However, under stress, nutrient or light limitation conditions, along with the accumulation of storage compounds, the system is exposed to extended unfavorable environmental conditions, which lead to high expenses of metabolic energy and decreasing productivity . To overcome this problem, a single-stage continuous system (chemostat) with a nutrient-limited medium can be explored. The main advantage of using chemostat is that the liquid dilution rate controls the growth rate of the biomass with a defined limiting substrate. When steady-state conditions are reached at specific dilution rates, biomass productivity, medium concentrations, and intracellular biochemical composition remain constant . This permits the optimization of the dilution rate to a determined value for maximal storage compound productivity in microalgae .

Another major obstacle in the profitable production of biofuels and scale-up is to maintain the microalgae cultivation systems monoculture, due to high-priced sterilization of inlet streams and reactor system. On the other hand, the use of environmental water bodies as inoculum has this opportunity to introduce new suitable microalgae species for biodiesel production which are offered by nature's microbial diversity. The natural selection and competition is introduced by Environmental Biotechnology which is targeted at enrichment and maintaining a characteristic or function as an alternative to a specific species in a system in order to engineer the ecosystem rather than the organisms . Furthermore, several studies claimed that biomass yield improved in mixed-species growth systems relative to algal monocultures grown under the same resource supply conditions. Previous related studies have shown that the strategy of chemostat selection could be successfully applied to obtain a stable enrichment of a polyhydroxyalkanoates (PHA) storing microbial community .

The main objectives of this study were to investigate the impacts of different nitrogen concentrations in feeding medium on mixed microalgae communities in a chemostat reactor. Especially the starch and lipids productivities were monitored as they have the potential for the production of biofuels. Furthermore, the nitrogen loading rates (NLR) were designed to direct the culture to be under light- or nitrogen-limited conditions that elucidate the microalgae tendency to accumulate lipids or starch as a dominant intracellular energy reservoir compound. For all NLRs, the CO_2 supply was unlimited.

Materials and methods

Feed composition

Different ecological water-bodies of Netherlands ponds were inoculated in chemostat feeding medium with composition as follows (concentration in $mg.l^{-1}$): $MgSO_4.7H_2O$ (394.4); KCl (18.2); K_2HPO_4 (156.8); $CaCl_2.2H_2O$ (22.0); $FeCl_3.6H_2O$ (38.0); $NaEDTA.2H_2O$ (100.5); H_3BO_3 (46.4); $NaSiO_3.9H_2O$ (85.3) and 1 ml/L trace element solution. Except for the nitrogen source, all other nutrients concentrations were adjusted in a way to ensure the nitrogen limitation was the only possible nutrient limitation occurred in all experiments. For different NLRs, the concentration of $NaNO_3$ is shown in table 1.

The final trace elements composition in medium for all different nitrogen feeding regimes was as follows (concentration on µmol/L): $ZnSO_4.7H_2$ O (1.7); $MnCl_2.4H_2O$ (19.3); $CuSO_4.5H_2O$ (0.1); $CoCl_2.6H_2$ O (1.1); $NaMoO_4.2H_2O$ (2.0); Na_2SeO_4 (0.3); Na_3VO_4 (0.02).

Table 1

A non-sterile 2 L bioreactor (Applikon, Schiedam, the Netherlands) was used in a continuous mode for three months with average light intensity at photobioreactor's inner surface 375 $\mu mol.m^{-2}.s^{-1}$ provided by an LED lamp. The working volume of 1.5 L was used for all nitrogen loading rates. The pH of the culture was maintained at 7.5 using 1 M HCl and 1 M NaOH. The medium was aerated with enriched CO_2 (5%, v/v in N_2) at a flow rate of 46 $ml.min^{-1}$controlled by a mass flow controller (Brooks Instruments, Ede, the Netherlands). The outflow gas stream was cooled to 4 °C and sent to the Rosemount NGA off-gas analyzer

(Emerson, USA). The speed of stirrer and temperature were maintained at 350 rpm and 26 °C, respectively. Masterflex pump (Cole-Parmer, Vernon Hills, IL, USA) with two head was used at inflow and outflow rate of 0.52 $ml.min^{-1}$ to provide the constant dilution rate of 0.5 d^{-1} and HRT of 2 d. A Biocontroller ADI 1030 (Applikon, Schiedam, The Netherlands) continuously measured pH and DOT (Dissolved Oxygen Tension). All data were stored by PC with MFCS_win software (Sartorius Stedim Systems, Goettingen, Germany). A schematic overview of the experimental setup is shown in Figure 1.

Figure 1

Photobioreactor operation

First, the fresh medium of F_1 was inoculated as described in the Materials and methods section. The initial OD was 0.05 and the photobioreactor was run in batch mode for two days to reach the OD of 1.8. Then the fresh medium with the composition of F_1 was supplied to the reactor continuously to reach the steady-state point. Off-gas compositions and the biomass concentration were used for monitoring the steady-state condition, in which the variation of these parameters should be less than 10% for three hydraulic retention times. The different nitrogen loading rates were applied by supplying different nitrate concentrations in the fresh medium, F_1, F_2, F_3, F_4, and F_5.

Analytical methods

Samples were collected from the photobioreactor every day. All experiments were conducted in triplicate. Data were analyzed by an analysis of variance ($P<0.05$) and results were processed by a computer program: Excel software.

Nitrate measurement

The content of nitrate in the reactor was measured spectrophotometrically using Dr. Lange LCK 339 Nitrate cuvette tests (Hach Lange, Dusseldorf, Germany) .

Biomass measurements

The effluent from the reactor was collected in an ice bath for measuring biomass dry weight (DW). 300 ml of microalgae suspension was centrifuged, the supernatant was removed and the pellet was dried overnight in pre-weighed aluminum foil container at 104 °C. The optical density was measured at the wavelength of 680 nm every day. Biomass productivity was calculated by the following equation:

$$\text{Biomass Productivity } (mg.l^{-1}.d^{-1}) = \text{DW } (mg.l^{-1}) \times D\ (d^{-1}) \quad (1)$$

where DW and D are dry weight and dilution rate, respectively.

Lipids and starch analysis

The content of Lipids was measured following the PHB extraction protocol described by Johnson et al. with minor modification. Formaldehyde was not added to the samples and Myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid were applied as standards. Into digestion tubes, 50 µl of internal standard benzoic acid in 1-propanol (1 g in 50 mL), 1.5mL of 1-propanol: hydrochloric acid (4:1 v/v) and 1.5 ml of 1,2-dichloroethane were added to weighed freeze-dried biomass. The tubes were placed in a digester block at 100 °C for 2 h. Gas chromatography (model 6890N, Agilent, USA) equipped with a flame ionization detector on an HP Innowax column was used for extracted lipids analysis. For starch measurement, approximately 4-mg freeze-dried biomass heated with 0.6 M HCl for 3 h at 100 °C. After centrifugation and filtration with a 0.45-µm pore size filter (PVDF Membrane, Millipore, Ireland), the poly glucose concentration was measured by high-performance liquid chromatography with the specification of an Aminex HPX-87H column from

Bio-Rad (USA) (t=60 °C) coupled to an ultraviolet and a refractive index detector . The productivity of starch and lipids are calculated according to the following equations:

Starch Productivity ($mg.l^{-1}.d^{-1}$) = Starch Content($mg.l^{-1}$)×D(d^{-1}) (2)

Lipid Productivity ($mg.l^{-1}.d^{-1}$) = Lipid Content ($mg.l^{-1}$)×D(d^{-1}) (3)

Staining of microalgal cells

4 µl BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4- bora-3a,4a-diazasindacene) in anhydrous dimethyl sulfoxide (DMSO; 1 $mg.ml^{-1}$) was added to 0.2 mL of algal culture for visualizing the lipids droplets in the cells by Leica DM500B light microscope (Leica Microsystems, Wetzlar, Germany) equipped with fluorescence filtercube A. The excitation and emission wavelengths for monitoring BODIPY 505/515 fluorescence were 488 and 515 nm, respectively as described by Klock et al. (2016). Starch staining was conducted by adding 4µl of Lugol's solution to 0.2 ml of microalgal suspension . Different species present in the five NLR cultures were categorized based on the morphological microscopic cellular appearance by taking pictures with an above-mentioned microscope using 100 times magnification. Identification was based on the morphology of the individual cells following microscopic examination. The objects which were not morphologically consistent with algae, such as debris, bacteria, and particles were discarded.

PCR-DGGE Analysis

The microbial composition of chemostat enriched culture at different nitrogen loading rates was analyzed using the PCR-DGGE technique as described by Danesh et al. .

Results and discussion

The phototrophic microalgal chemostat enrichment cultures reached a steady-state for each nitrogen loading rate formerly detailed analysis was performed. The nitrogen concentration in the effluent dropped below the method detection limit for the NLRs of 10, 20, 32.5 and 42 $mgN.l^{-1}.d^{-1}$ which indicates that nitrogen supply limited growth. Therefore, these NLRs are considered nitrogen-limited conditions. On the other hand, the increase of the NLR to 63 $mgN.l^{-1}.d^{-1}$ caused an incomplete nitrogen uptake. At the NLR of 63 $mgN.l^{-1}.d^{-1}$ and in the presence of other nutrients (the phosphate concentration was measured every day, data are not shown), mixed microalgae could not consume all nitrogen supplied to the reactor, which indicates a different growth limitation in the system rather than nutrient deficiency. This was likely a light limitation which is attributed to the high biomass concentration that limits the light penetration into the microalgal culture. Therefore, NLR of 63 $mgN.l^{-1}.d^{-1}$ is considered as light-limited condition (for more explanation, see supplementary data 1). Similarly, Klok et al. (2013) reported that when residual nitrogen was observed in the effluent of *Neochloris oleoabundans*continuous culture system (0.066±0.002 $g.l^{-1}$ at the dilution rate of 1.15 ± 0.10 d^{-1}), the growth was limited by the light supplied to the system . At lower nitrogen supply rates, residual nitrate was non-detectable, indicating nitrogen-limited growth conditions.

Effects of Nitrogen Loading Rates on Present Species

Microscopic observations of species grown under the five nitrogen regimes are represented in Figure 2. According to the observed microscopic morphology, a coculture of species including *Chlorella sp.* , *Arthrospira platensis,* and *Scendesmus* was identified at both NLR of 10 and 20 $mgN.l^{-1}.d^{-1}$(Figure 2. A & B). *Chlorella sorokiniana* and *Chlorella vulgaris* were identified as dominant species at NLR of 32.5 and 42 $mgN.l^{-1}.d^{-1}$, respectively (Figure 2. C & D). Different species of*Arthrospira platensis* , *Chlorella sp.* , *Rhopalodia*coexisted under the light-limited condition at NLR of 63 $mgN.l^{-1}.d^{-1}$ (Figure 2. E). Genomic DNA was extracted to confirm the identity of microscopically observed species. The amplicons of extracted DNA were amplified by 18S rRNA and 16S rRNA. According to the 16S rRNA and 18S rRNA sequences, the morphologically

recognized microalgal species were closely related to PCR-DGGE identified species (Table 2). Besides some minor species were identified by the amplicon sequencing. The coexistence of different species at NLRs of 10, 20, and 63 $mg.l^{-1}.d^{-1}$ can be illustrated by biodiversity neutral theory which contends that diversity is due to the equivalent competitive ability of all species within the same functional group.

Table 2.

Regan et al. (1984) investigated the dominant microalgal species under different nitrogen and light limitations in continuous culture and reported dominance and coexistence of various species including diatoms, blue-green algae, and green flagellates. For dilution rates of 0.1-0.4 d^{-1}, at low inlet nitrate concentrations (7.5 $mgN.l^{-1}$), pennate diatoms (Nitzschia) were dominated (Regan and Ivancic, 1984). However, at higher inlet nitrate concentrations (37 $mgN.l^{-1}$), pennate diatoms (Nitzschia) and green flagellates (Tetraselmis) coexisted (Regan and Ivancic, 1984). Similarly, an N-limited chemostat culture was dominated by picocyanobacteria and a diverse group of diatoms using multispecies inoculum. In our study, cyanobacteria existed in F_5culture in which their presence may be attributed to their capacity to produce accessory pigments to harvest light more efficiently under light limiting conditions . This result also supports the suggestion that cyanobacteria can be introduced as shade organisms. This characteristic of cyanobacteria helps them compete with other species present in culture under light limitation. Aligned with this, coculture of different species of cyanobacteria, green algae and diatoms were enriched at the nitrogen loading rates of 20 and 10 $mgN.l^{-1}.d^{-1}$ (Table 2), which may conclude that the decrease of nitrogen loading rate and higher light availability also leads to the growth of nitrogen-fixing algae such as cyanobacteria in the algal community. In fact, the specific nature of cyanobacteria's water-soluble light-harvesting antenna complexes, phycobilisomes (PBs), dictates their photoprotection mechanisms. PBs gather light in a wide spectral range and transfer excitation energy to the photosystems. Cyanobacteria use the photoactive orange carotenoid protein (OCP) to control this energy flow . Diatoms species of*Navicula sp* ., *Nitzschia thermalis,* and *Rhopalodia gibba* could stay in the competition of F_1, F_2, and F_5 cultures, respectively. It can be pointed that diatoms contain the accessory pigments Chl c_1 + c_2, and the xanthophyll, fucoxanthin. Light energy absorbed by fucoxanthin utilized in photosynthesis with the same quantum efficiency as Chl a which makes diatoms be adaptable to low light growth conditions (NLR of 63 $mg.l^{-1}.d^{-1}$) (Tanada, 1951; Friedman and Alberte, 1984). Another study explained in detailed the photoprotection general features of diatoms exposed by harmless dissipation of excess energy which can be the purpose of diatoms presence at NLRs of 10 and 20 $mg.l^{-1}.d^{-1}$.

Based on the microscopic images (Figure 2, right pictures which are under fluorescence light), the accumulation of lipids is obvious at NLRs of 32.5 and 42 $mgN.l^{-1}.d^{-1}$, which are related to *Chlorella sorokiniana* and *Chlorella vulgaris* , the dominant species of F_3 and F_4 culture, respectively.

Figure 2

Effects of Nitrogen Loading Rates on Cell Growth, Lipids and Starch Accumulation

Figure 3 illustrates the consumed nitrogen to dry weight and active biomass ratios for the NLR of F_1 to F_5cultures. Active biomass is calculated by subtracting the values of ash, lipid, and starch from dry weight. The ratios of consumed nitrogen and biomass dry weight are approximately identical throughout the tested experimental conditions (F_1 to F_4). This may be attributed to the fact that the amount of nitrogen that is assimilated per produced biomass is approximately constant for these experiments. However, the consumed nitrogen to dry weight ratio for the nitrogen loading rate of 10 $mgN.l^{-1}.d^{-1}$ dropped considerably which indicates that the diatoms and/or cyanobacteria fix nitrogen (See Figure 3). Moreover, the approximately identical pattern was noticed for the consumed nitrogen to active biomass ratio (Figure 3).

Figure 3.

The dry weight concentration was measured to be 342 ± 11 $mg.l^{-1}$ at a NLR of 10 $mgN.l^{-1}.d^{-1}$ and increased approximately linearly to 868 ± 12 $mg.l^{-1}$ at NLR of 42 $mgN.l^{-1}.d^{-1}$ (Table 3). However, the microalgae

growth was limited by light supply for the nitrogen loading rate of 63 $mgN.l^{-1}.d^{-1}$ in which dry weight slightly increased to 895 ± 14 $mg.l^{-1}$.

Table 3.

According to Table 3, the content of lipids increased from 5.5 ± 0.3% to 14.0 ± 0.8% on the basis of dry weight, simultaneous with increasing NLR from 10 to 42 $mgN.l^{-1}.d^{-1}$ up to where light became the growth limiting factor. The highest amount of lipids were measured at NLR of 42 $mgN.l^{-1}.d^{-1}$, which is confirming the observation of lipid bodies by fluorescence microscopy (Figure 2). Contrary to our findings, Klok et al. reported the increase of lipids bodies inside of *N. oleoabundans* with a decrease in the nitrogen supply rate. Apparently, the lipid enhancement can be attributed to the decrease of growth rate which was implemented by dilution rate reduction in the experiments of Klok et al. (2013). Another pure culture study indicated a higher accumulation of lipids in *C. subellipsoidea* at lower nitrate addition rates, which was similarly induced by a decreased dilution rate . There was no significant difference in the starch, lipids and biomass concentration of F_4 and F_5 at 5% significant level (P-value>0.05). The highest lipids and starch concentration 121.2 ± 1.7 $mg.l^{-1}$ (14.0 ± 0.8 % on the basis of dry weight), 42 ± 4.5 $mg.l^{-1}$ (4.8 ± 0.5 % of dry weight), respectively were achieved under nitrogen limiting conditions of experiment F_4 (Table 3). The results confirm the hypothesis that chemostat cultures under nitrogen limiting conditions can lead to the accumulation of lipids and therefore it can be a good strategy to enrich the lipids accumulating algal communities. Interestingly we obtained significant lipids accumulation in our enrichment culture. This was not observed for algal enrichments under dynamic cultivation in a day-night cycle . Xiao et al. (2013) cultured*Nannochloropsis oceanica* using a medium with different nitrogen concentrations at constant dilution rates fed to a photobioreactor. They revealed that nitrogen limitation induced by a continuous medium with a nitrogen concentration of 0.23 $mgN.l^{-1}.d^{-1}$ at a dilution rate of 0.27 d^{-1} showed the highest lipids accumulation in*Nannochloropsis oceanica* cells up to 56.17 ± 0.21 % of dry weight with a value of 179.26 $mg.l^{-1}$.

Other studies were based on decreasing the nitrogen feeding rate by reduction of dilution rate of pure culture studies such as*Neochloris oleoabundans* , *Chlorella pyrenoidosa* , and*Coccomyxa subellipsoidea* . Klok et al. (2013) reported the accumulation of 14.8% total fatty acid on the basis of dry weight in*Neochloris oleoabundans* at a dilution rate of 0.33 d^{-1}. Another report revealed that *Coccomyxa subellipsoidea* under the lowest nitrate loading rate of 64.7 $\mu g.l^{-1}.h^{-1}$ with a dilution rate of 0.06 d^{-1} resulted in the highest lipids accumulation up to 30.7±1.3 $mg.l^{-1}$. Moreover, Wen et al. (2014) observed that the lipids content of *Chlorella pyrenoidosa* under low specific nitrogen loading rate of 7.9 $mg.l^{-1}.d^{-1}$ at a dilution rate of 0.48 d^{-1} was 34.7 % of DW. The main and notable advantage of this study is that the lipids and starch are measured in entire experiments which provide the chance to conclude that continuous mode cultivation resulted in higher lipid accumulation than the starch synthesis for all NLRs. Therefore, lipids are considered as dominant storage compounds in our chemostat enrichment system. The highest ratio of lipids to starch concentration was calculated for experiment F_3 with a value of 4.2. It corroborates the results of the continuous growth of *Nannochloropsis sp.* in which the content of lipids was doubled under nitrogen limitation . However, the greater increase of carbohydrates than lipids occurred for *Scenedesmus sp. AMDD* under nitrogen-limited continuous culture .

For *Chlorellasorokiniana* and*Chlorella vulgaris* , the dominant species of experiment F_3 and F_4, the trade-off between starch and lipids accumulation was reported . The lipids and starch contents of*Chlorella sorokiniana* were reported 7.2% and 16.2% on a dry weight basis in a complete medium batch mode cultivation. The latter species could increase simultaneously starch and lipids content to 40% and 30% of DW, respectively under batch sulfur-deficient condition . Another study revealed that under nonexistence of nitrogen and moderate aeration and light intensity in batch culture, the lipid content increased in *Chlorella vulgaris* cells up to 43% of dry cell weight which equates to lipids productivity of 77.8 $mg.l^{-1}.d^{-1}$.

Table 4.

The starch content of experiment F_4 in which*Chlorella vulgaris* was dominant decreased to 4.8±0.5% of DW and the lipids content increased up to 14.0±0.8% based on DW.*Chlorella sorokiniana* which was enriched at

a nitrogen loading rate of 32.5 $mgN.l^{-1}.d^{-1}$ had a starch and lipids content of 3.2±0.2% and 13.5±0.9% on a dry weight basis, respectively. Therefore, additionally, it can be concluded that continuous mode can promote the enrichment of species which tend to enhance the lipids content under unfavorable culture conditions . As illustrated in Table 4, there is a significant positive relationship between the NLRs, biomass, and lipids productivity ($mg.l^{-1}d^{-1}$) of F_1 to F_4 culture. Furthermore, a comparison between the biomass and lipids productivity of continuous pure microalgal production and that of our study revealed that mixed microalgae could provide admissible biomass and lipids productivity without the high expense of sterilization (Table 4). Mazzuca Sobczuk and Chisti (2010) reported the biomass and lipids productivity of 351 and 83 $mg.l^{-1}.d^{-1}$ for *Choricystis minor* at a dilution rate of 0.336 d^{-1}. Another study showed the maximum biomass productivity of 242.2 $mg.l^{-1}.d^{-1}$ and 82.5 $mg.l^{-1}.d^{-1}$ lipids productivity for the continuous production of*Nannochloropsis oceanica* . In the latter mentioned study, the nitrogen supply rate was 8.3 $mgN.l^{-1}.d^{-1}$ with a dilution rate of 0.27 d^{-1}. The maximum biomass and lipids productivity obtained in this study were 433.9 and 60.6 $mg.l^{-1}.d^{-1}$, respectively which are quite high. However, the process conditions optimizing are required which may result in higher lipid content, comparable to those reported for pure culture.

Conclusions

This research aimed at enrichment, growth, and accumulation of biopolymer storage compounds using a chemostat bioreactor operated at different NLRs. *Chlorella vulgaris,* and *Chlorella sorokiniana,* the enriched species of this study stored lipids over starch under the continuous growth conditions. Hence, this method is a high throughput screening to select the most suitable microalgae species for lipids production. These findings can be used in large scale microalgae cultivation. Future studies are required to optimize lipids productivity by adjusting cultivation parameters like dilution rate, pH, temperature and light intensity for simultaneous microalgae enrichment and growth, lipid production and biological nutrient removal.

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Table 1. Nitrate concentration in chemostat enrichment culture under different nitrogen loading rates.

Cultures	F_1	F_2	F_3	F_4	F_5
NLRs ($mgN.l^{-1}.d^{-1}$)	10	20	32.5	42	63
NaNO3 ($mg.l^{-1}$)	120	240	395	510	765

Table 2. Microscopic observations and PCR-DGGE identification of species present at different nitrogen loading rates.

NLR ($mgN.l^{-1}.d^{-1}$)	Microscopic Identification	PCR-DGGE Identification	GenBank Accession numbers	Identity
10	*Chlorella sp.* *Arthrospira platensis* *Scendesmus* *Navicula sp.*	*Chlorella sp. ArM0029B* chloroplast	KC994656.1	99
		Arthrospira platensis str. Algerian	KF290490.1	100
		Acutodesmus obliquus	AB917118.1	100
		Ochromonas sp. CCMP2740	EF165144.1	98
		Navicula sp. C20	FJ002226.1	87

NLR ($mgN.l^{-1}.d^{-1}$)	Microscopic Identification	PCR-DGGE Identification	GenBank Accession numbers	Identity
20	*Arthrospira platensis Scendesmus Chlorella sp. Chlorella vulgaris Nitzschia*	*Arthrospira platensis str. Algerian*	KF290490.1	97
		Acutodesmus obliquus	AB917118.1	99
		Chlorella sp. ArM0029B chloroplast	KC994656.1	96
		Chlorella vulgaris strain S708	KF981995.1	99
		Nitzschia thermalis isolate C17	FJ002224.1	85
32.5	*Chlorella sorokiniana*	*Chlorella sorokiniana* chloroplast, complete genome	KJ397925.1	99
42	*Chlorella vulgaris*	*Chlorella sp. ArM0029B* chloroplast, complete genome	KC994656.1	99
63	*Arthrospira platensis Chlorella sp. Rhopalodia*	Arthrospira platensis str. Algerian	16S	100
		Chlorella sp. ArM0029B chloroplast, complete genome	16S	99
		Rhopalodia gibba	AJ582391.1	86

Table 3. Summary of algal enrichment chemostats results. The dilution rate for all experiments was 0.5 d^{-1}.

Data are presented as mean ± SD of triplicates ($P < 0.05$)

Experiment	F_1	F_2	F_3	F_4	F_5
Nitrogen Loading Rate (NLR) ($mgN.l^{-1}.d^{-1}$)	10	20	32.5	42	63
Nitrogen Uptake Rate (NUR) ($mgN.l^{-1}.d^{-1}$)	10	20	32.5	42	43.35
Total DW ($mg.l^{-1}$)	342±11	434±18	682±20	868±12	895±14
NUR ($mgN.l^{-1}.d^{-1}$) / Total DW production rate ($mg.l^{-1}.d^{-1}$)	0.058	0.092	0.095	0.096	0.096
Lipids ($mg.l^{-1}$)	18.7±0.5	30.3±1.3	91.8±0.6	121.2±1.7	115.5±2.1
Lipids (w/w %)	5.5±0.3	7.0±0.8	13.5±0.4	14.0±0.8	12.9±0.8
Starch ($mg.l^{-1}$)	10.9±0.9	13.6±3.0	22.1±2.8	42.0±4.5	40.3±4.1
Starch (w/w %)	3.2±0.1	3.1±0.4	3.2±0.2	4.8±0.5	4.5±0.3

Table 4. Biomass and lipids productivity reported in literature dealing with chemostatic pure and mixed microalgal production with different dilution rates and N-source. Data are presented as mean ± SD of triplicates ($P < 0.05$) for this study.

Species	D (d^{-1})	N. Source	Nitrogen ($mgN.l^{-1}.d^{-1}$)	Biomass Prod. ($mg.l^{-1}.d^{-1}$)	Lipids Prod. ($mg.l^{-1}.d^{-1}$)	Reference
Mixed Microalgae	0.5	Nitrate	10	171±6	9.4±0.3	This Study
Mixed Microalgae	0.5	Nitrate	20	217±9	15.1±0.7	This Study
Mixed Microalgae	0.5	Nitrate	32.5	341±10	45.9±0.3	This Study
Mixed Microalgae	0.5	Nitrate	42	434±10	60.6±0.9	This Study
Mixed Microalgae	0.5	Nitrate	63	448±7	57.7±1.1	This Study
Chlorella minutissima	0.328	Nitrate	40.5	137	6	
Choricystis minor B. Fott	0.336	Nitrate	82.8	351	82	(Mazzuca Sobczuk and Chisti, 2010)
Coccomyxa subellip-soidea	0.18	Nitrate	0.2	63	1.7	
Nannochloropsis oceanica	0.27	Nitrate	0.23	86.2	48.4	
Nannochloropsis oceanica	0.27	Nitrate	0.45	196.3	69.2	
Nannochloropsis oceanica	0.27	Nitrate	8.3	242.2	82.5	
Scenedesmus sp. AMDD	0.7	Ammonium	13.8	510	41.8	
Chlorella pyrenoidosa	0.48	Nitrate	7.9	417.8 ± 26.9	144.9 ± 5.2	
Neochloris oleoabun-dans	0.53	Nitrate	34.9	660	79	
Neochloris oleoabun-dans	1.74	Nitrate	207	1290	118	

Figure Captions

Figure 1. Schematic overview of the chemostatic experimental setup: (1) Acid bottle; (2) Base Bottle; (3) the fresh medium container; (4) Effluent flask; (5) Gas bottle; (6) Cryostat; (7) Thermostat; (8) pH probe; (9) DO probe; (10) Applikon photobioreactor; (11) Stirrer motor with four-blade impellers; (12) Condenser; (13) Acid pump; (14) Base pump; (15) Two-head Masterflex pump using for feed and effluent; (16) Stirrer speed controller; (17) Air pump; (18) Off-gas analyzer; (19) A Bio Controller ADI; (20) Mass flow controller; (21) Digital monitor Flowmeter; (22) PC; (23) Light source

Figure 2. Microscopic pictures of species present in culture after reaching steady-state under different

nitrogen loading rate. Nitrogen loading rate for A, B, C, D and E series are 10, 20, 32.5, 42 and 63 $mgN.l^{-1}.d^{-1}$ corresponding to feed composition of F_1, F_2, F_3 and F_4 for nitrogen limitation and F_5 for light limitation, respectively. The left and right pictures are under normal and fluorescence light, respectively. The scale bar represents 30 μm.

Figure 3. The nitrogen to dry weight and active biomass (X) ratio for different nitrogen loading rates.

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`Figure 1. Schematic overview of the chemostatic experimental setup.docx` available at https://authorea.com/users/294364/articles/422682-chemostat-based-enrichment-system-nitrogen-loading-rates-impacts-on-microalgal-communities-and-intracellular-storage-compounds

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