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# Heavy metal removal and effects of nitrogen sources on *Botryococcus* sp. NJD-1 for wastewater treatment under heterotrophic conditions

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**ABSTRACT**

Increasing urbanization and industrialization have increased toxic levels of pollutants in the environment. Heterotrophic cultivation of microalgae eliminates the light requirement and provides the possibility to achieve fast cells growth and efficient removal of pollutants from wastewaters. In this study, effects of heavy metals (Cu and Cr) and N sources (i.e., $NaNO_3$, $NH_4NO_3$, $NH_4Cl$, and urea) on *Botryococcus* sp. NJD-1 were investigated. Results showed that the biomass yield decreased as the initial concentrations of heavy metal increased, ranging from 8.37 g $L^{-1}$ to 6.32 g $L^{-1}$ (on Cu) and 7.85 - 6.27 g $L^{-1}$ (on Cr). *Botryococcus* sp. NJD-1 removed Cu and Cr in the range of 78- 86% and 95 -100%, respectively. Concerning N sources experiments, high biomass yields of 2.44, 2.41, 1.47, and 0.72 g $L^{-1}$ for $NaNO_3$, urea, $NH_4NO_3$, and $NH_4Cl$ were achieved, respectively. $NaNO_3$ exhibited the highest N/P/C removal reaching 69.9, 60.8, and 100%. Regarding growth ability and nutrients removal, the assimilation order of $NaNO_3$ > urea > $NH_4NO_3$ > $NH_4Cl$ for NJD-1 was obtained. This strain exposed a potential capacity to remove heavy metals, produce high biomass, and assimilate different N sources which proves a remarkable role of wastewater-born NJD-1 in wastewater-based bioremediation.

**Keywords:** Microalgae; Heavy metals; Nitrogen sources; Heterotrophic cultivation; Bioremediation.

## 1. INTRODUCTION

Heavy metals (HM), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Cooper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn), Mercury (Hg), Nickel (Ni), Lead (Pb), Selenium (Se), Zinc (Zn), …) are in general toxic to all living organisms though some like Co, Ni and Zn are at very low concentrations essential for life [1]. Human activities are the most contributors to their release to environment. Due to their higher toxicities, higher bioaccumulation in human body and food chain, nature of non-biodegradability, and most likely carcinogenicities to humans, heavy metals pollution has become a global issue of great concern [2]. Their non-biodegradability property renders the clean-up of contaminated water and soil more challenging. The traditionally existing techniques to remove or recover heavy metals such like sedimentation, flocculation, absorption and cation/anion exchange, complexation, precipitation, oxidation/reduction is hindered by the high cost, low selectivity, incomplete removal, toxic slurries generation to name few [3]. The use of microbial (including microalgae) activity and uptake approach is the preferred alternative which is both economically and environmentally sound, emerging recently as the most desirable technology in remediation process [2].

In past years, microalgae have been ideal candidates for the selective removal and concentration of heavy metals due to their properties like high tolerance to heavy metals, ability to grow both autotrophically and heterotrophically, large surface area/volume ratios, phototaxy, phytochelatin expression and potential for genetic manipulation [4]. Since years, the capability of microalgae (living or non-living biomass) to sorb toxic metals has been recognized and well documented by several researchers [4]. For example, Onyancha, Mavura, Ngila, Ongoma and Chacha [5] evaluated the ability of two live algae *Spirogyra condensata* and *Rhizoclonium hieroglyphicum* in removing Cr from tannery and synthetic wastewaters while Han, Wong, Wong and Tam [6] studied Cr removal from synthetic solutions using dead cells of *Chlorella miniate* previously grown in domestic wastewater. Heavy metals removal from tannery effluents was also investigated by [7] using *Scenedesmus* sp. and found capable to reach 96% removal efficiencies. *Chlorococcum* sp. was reported to effectively remove Cr (67%) and Cu (75%) from aqueous solutions [8]. Algal isolates (*Anabaena variabilis*, *Aulosira* sp., *Nostocmuscorum*, *Oscillatoria* sp. and *Westiellopsis* sp.) were grown in sewage and reported able to completely remove Cr, Cd, Ni and Pb [9]. The growh and heavy metals (Cu, Co, Pb and Zn) accumulation potential by *Scenedesmus bijuga* and *Oscillatoria quadripunctulata* in both sewage and petrochemical effluents was investigated by Ajayan, Selvaraju and Thirugnanamoorthy [10] and both species showed high level of heavy metal removal efficiency. In the investigation on the remediation potential of two algal isolates *Chlorella vulgaris* and *Chlorella salina* for treating sewage and mixtures of sewage, sea and well waters, the removal efficiency of Zn, Cu, Mn, Ni, Co, Fe and Cr in the range of 13.6-100% was obtained [11].

In wastewater treatment industry, microalgae cultures offer a classy alternative to the post-secondary treatment steps due to their ability to effectively use wastewater nutrients (nitrogen and phosphorus) for growth, and remove efficiently heavy metals and toxic organic compounds from wastewater [12]. Microalgae are well known to efficiently use nutrients for valuable biomass production, and even under highly unfavorable growth conditions, they are capable to thrive and produce valuable biomass components such as lipids (oils), carbohydrates and proteins, the feedstocks that can be converted into bio-fuels and other useful materials [13]. Moreover, the use of microalgae (i) is in accordance with natural ecosystem principles, environmentally sound and feasible at low cost, (ii) has no secondary pollution effect as the produced biomass is reused and (iii) allows for an efficient nutrient recycling [12]. Still, the obstacles for the scale-up application of microalgae in wastewater treatment lie on the large working area required for an efficient light harvesting and the low biomass growth restricted to the shading effect among microalgae, as these treatments are commonly conducted photoautotrophically. In this sense, heterotrophic cultivation of microalgae in which the organic substrates are used as the sole carbon and energy sources so as to eliminate the requirement for light, provides the possibility to achieve fast growth and efficient removal of nutrients from wastewaters [14]. Compared to photoautotrophic approach, heterotrophic cultivation of microalgae is with a simple operation and daily maintenance and with higher volumetric biomass productivities [14].

In particular, for algae-based nutrient assimilation process, N, P, and C macro-elements are very important. All organisms require basic nutrients for growth and multiplication, and most microalgae can meet all their cellular needs for their growth with a few key compounds; macronutrients, micronutrients (trace elements) and vitamins [15]. The growth abilities and biochemical compositions of microalgae can be greatly affected by the concentration of nitrogen in the culture medium. Hong et al. [16] reported that limiting the nitrogen concentration in the culture medium, the protein synthesis will be decreased, but lipid and carbohydrates storages will be increased. Most microalgae utilize various inorganic and organic nitrogen sources (i.e., nitrate, nitrite, ammonium, urea, yeast extract and so on). Each N source is assimilated into amino acids by different pathways after being reduced to the ammonium form which is mostly used by several algae, due to less energy requirement for assimilating into amino acids. In contrary, some microalgae including *Dunaliella tertiolecta* and *Botryococcus braunii* prefer using nitrate instead of ammonium for their growth [17]. *Chlorella* species apart from nitrate substrate, they can also effectively consume organic nitrogen sources such as yeast extract, glycine, urea, peptone etc. [18]. It is known that algal cells growth, pollutants removal, and biochemical compositions can change due to the type of nitrogen source supplemented in the culture medium.

However, there still a limited number of published studies conducted under heterotrophic conditions for heavy metals removal from wastewater culture medium and biomass accumulation. Besides, since N sources can change the growth parameters and nutrients removal efficiencies from species to species, it is reasonable to compare various N sources and select the most promising for algal strains in line to enhance the biomass productivity and pollutants removal from wastewaters. Therefore, the current study, utilized a newly isolated wastewater-born microalga strain *Botryococcus* sp. NJD-1 for heavy metals and nutrients removal from wastewater under heterotrophic cultivation. Also, the effects of different nitrogen sources (i.e., nitrate, urea, and ammonium) on algal growth abilities and nutrients removal were investigated.

## 2. MATERIALS AND METHODS

### 2.1. Microalgal strain and cultivation conditions

Microalgal strain *Botryococcus* sp. NJD-1 (with accession KY656445) previously isolated from domestic wastewater by Liang et al. [14], was used in this study. This strain was selected among others due to its performance in biomass production, pollutants removal from wastewaters, lipids accumulation, and its adaptation to different organic compounds [14]. The modified Bold-3N medium (mBold-3N) was used as the growth medium in all experiments. Initial inoculums size was adjusted to about 0.22 g $L^{-1}$. The effects of heavy metals on algal growth properties were evaluated. Five different scenarios i.e., Control (without heavy metal concentration), 0.1, 1, 5, 10, and 20 mg $L^{-1}$ of Cu and Cr, separately were added in mBold-3N medium. In order to explore the effects of different N sources on growth ability and nutrients removal for *Botryococcus* sp. NJD-1, sodium nitrate ($NaNO_3$), ammonium nitrate ($NH_4NO_3$), ammonium chloride ($NH_4Cl$), and urea ($CO(NH_2)_2$) were utilized. The concentration (in g $L^{-1}$) of 0.750 ($NaNO_3$), 0.353 ($NH_4NO_3$), 0.472 ($NH_4Cl$), and 0.265 $CO(NH_2)_2$ were supplemented in mBold-3N medium. The initial nitrogen concentration for each N source was estimated at 123.5 mg N $L^{-1}$ (8.82 mM). *Botryococcus* sp. NJD-1 cells were inoculated in 250 mL with modified Bold-3N medium, containing different N sources. The concentration of 10 g $L^{-1}$ glucose was supplemented in the synthetic wastewater media as the source of energy. The cultivation conditions were at initial pH 7, 150 rpm rotation, and 30°C under heterotrophic conditions for 8 days. All experiments were carried out in triplicates

## 2.2. Analytical methods and calculations

The algal biomass concentration was determined by measuring the optical density (OD) and the dry weight (DW) was estimated after calibration between OD and DW value. The optical density values were estimate via Microplate Reader (model SpectraMax 190, Molecular Devices) as previously reported [14]. The concentration of heavy metals in wastewater culture medium was determined by a multi-channel inductively coupled plasma optical emission spectrophotometer, ICP-OES (model PS-4; Baird Co., Bedford, MA, USA). Briefly, 4 mL samples were collected daily from algae cultures. After centrifugation, the supernatant was diluted with 10 volumes of 5% $HNO_3$ prior to ICP-OES analysis. The concentrations of P and N were estimated by ascorbic method and colorimetric technique, respectively while the concentration of glucose was determined spectrometrically using dinitrosalicylic acid routine [19]. According to our previous study, the relationship between $OD_{685}$ and algal concentration of *Botryococcus* sp. NJD-1 (P, g DW $L^{-1}$) is shown in Eq. (1):

$$\mathrm{OD}_{685} = 10.18\,P - 1.7475 \qquad \left(R^2 = 0.9952\right) \qquad (1)$$

in which OD and *P* are optical density and dry biomass, respectively.

The biomass productivity (*BP*) was calculated through the Eq. (2). The specific growth rate ($\mu$) was estimated by the Eq. (3).

$$BP\left(g\ L^{-1} d^{-1}\right) = \left.\frac{(x_2 - x_1)}{(t_2 - t_1)}\right|\ \max \qquad (2)$$

$$\mu\ (d^{-1}) = \left.\frac{\left(\ln x_2 - \ln x_1\right)}{\left(t_2 - t_1\right)}\right|\ \max \qquad (3)$$

in which $x_1$ and $x_2$ are the dry cell weights (g $L^{-1}$) on days $t_1$ (start of the exponential phase) and $t_2$ (maximum of exponential phase), respectively. All experiments were conducted in triplicates. Nutrient removal efficiencies (*RE*, %) were calculated using Eq. (4). The rate of nutrient removal (*RR*) was calculated according to Eq. (5). Total organic carbon (TOC) was measured by a total organic carbon analyzer (model TOC-L CPH, Shimadzu, Japan).

$$RE = 100\ x\ \frac{(S_0 - S_t)}{S_0}\Bigg|\max \qquad (4)$$

$$RR = \frac{(S_0 - S_t)}{(t_t - t_0)}\Bigg|\max \qquad (5)$$

where *RE* is the removal efficiency of N, P, C, Cu, and Cr; $S_0$ is the initial concentration and $S_t$ is the maximum concentration at initial ($t_0$) and final ($t_t$) time (days), respectively.

### 2.3. Statistical analysis

In order to decide statistically significant differences between growth circumstance and strain, all experiments were performed in triplicates. The OrginPro 8 software was used to run analysis. Average values of the results of three parallel experiments (with two analytical replications in each). The data are mean ± standard deviation of three determinations and indicated as error bars.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of heavy metals on microalgal strain performance

The growth parameters of *Botryococcus* sp. NJD-1 in different initial concentrations of synthetic wastewater solutions were presented in Fig. 1. The maximum biomass concentrations of 8.37 ± 0.023, 8.23 ± 0.075, 8.14 ± 0.091, 7.84 ± 0.181, and 6.32 ± 0.102 g $L^{-1}$ were obtained when 0.1, 1, 5, 10, and 20 g $L^{-1}$ of the initial concentrations were used, respectively. For Cr metal, the biomass yield were 7.85 ± 0.101, 7.64 ± 0.271, 6.80 ± 0.014, 6.99 ± 0.132, and 6.27 ± 0.087 at the same initial concentrations as Cu above. As shown in Fig. 1, it is clear that the biomass concentrations (DW) of *Botryococcus* sp. NJD-1 decreased as the initial concentrations of HM increased. At 0.1 mg $L^{-1}$ initial Cu and Cr concentration, biomass production is higher than that obtained at higher initial concentrations (20 g $L^{-1}$). The ability of *Botryococcus* sp. NJD-1 to remove toxic HM from wastewater samples after 8 days cultivation was shown in Table 1. The data indicated that the %removal efficiency differed according to the types of HM utilized. Table 1 shows the percentage HM removal efficiencies of NJD-1 for different concentrations of Cu and Cr, respectively. Microalgal strain NJD-1 exhibited the highest removal efficiency ranging from 80 to 86% and 95-100% of Cu and Cr, respectively. For Cu metal, the highest %removal (88%) was observed at initial concentration of 20 mg $L^{-1}$ while the lowest %removal (78%) was detected at 20 mg $L^{-1}$ initial concentration. In other hands, from 0.1 to 10 mg $L^{-1}$ of Cr initial concentrations, NJD-1 showed the complete removal efficiency at concentrations range of 0.1–10 mg $L^{-1}$ reaching 100%. But a little decrease in %removal was observed at 20 mg $L^{-1}$ initial concentration where 95% of Cu reduction was reached. As shown in Table 2, the results of this work are comparable or even higher to those reported in literature (Table 1). It was obvious that the heterotrophic microalgae used in this study exhibited high ability to remove HM from aqueous samples.

**Table 1:** Different microalgae-based removal of heavy metals from different wastewaters

| **Medium** | **Heavy metal** | **Microalgal strain** | **Removal efficiency** | **Reference** |
|---|---|---|---|---|
| Synthetic solutions | Cu<br>Cr | *Botryococcus* sp. NJD-1 | 78-86%<br>95-100% | This study |
| Synthetic and tannery wastes | Cr | [a]*Spirogyra condensate* and *Rchizoclonium hieroglyphicum* | ≥ 75% | [5] |
| Synthetic solutions | Cr | [b]*Chlorella miniate* grown in domestic wastewater | Cr(III) 75% and Cr(VI) 100% | [6] |
| Tannery wastewater | Cr, Cu, Pb, Zn | *Scenedesmus* sp. | Cr-81.2-96%, Cu-73.2-98%, Pb-75-98% and Zn-65-98%) | [7] |
| Synthetic solutions | Cr and Cu | [b] *Chlorococcum* sp. | Cu (43–75%), 67% Cr | [8] |
| Sewage | Cr, Cd, Ni, Pb | *Anabaenavariabilis*, *Aulosira* sp., *Nostocmuscorum*, *Oscillatoria* sp. and *Westiellopsis* sp. | 100% | [9] |
| Sewage wastewater and Petrochemical effluents | Cu, Co, Pb and Zn | *Oscillatoria quadripunctulata* | 37-50, 20.3-33.3, 34.6-100 and 32.1-100% | [10] |
| | | *Scenedesmus bijuga* | 60-50, 29.6-66, 15.4-25 and 42.9-50% | |
| Sewage, sea water, well water, and their mixtures | Zn, Cu, Mn, Ni, Co, Fe and Cr | *Chlorella salina* and *Chlorella vulgaris* | 13.61–100 % | [11] |

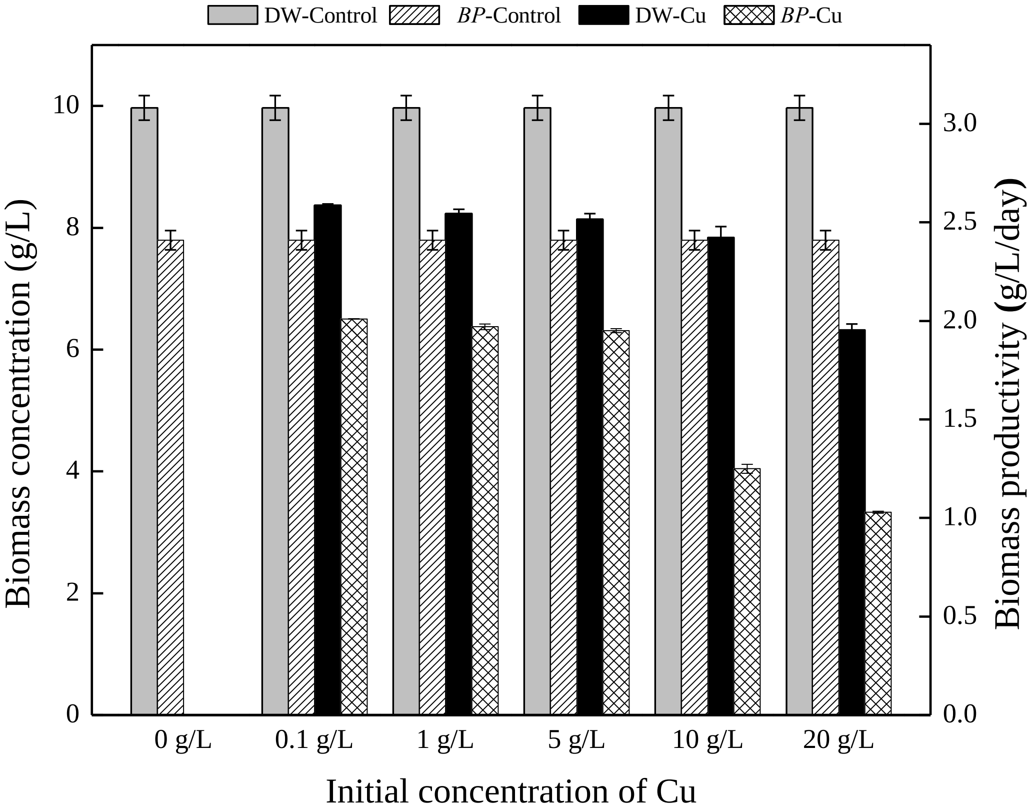


**Fig. 1** Growth parameters of *Botryococcus* sp. NJD-1: Biomass concentration (g DW $L^{-1}$) and biomass productivity (g $L^{-1}$ $day^{-1}$) of the control (without initial concentration of Cu) and Cu, respectively, obtained when *Botryococcus* sp. NJD-1 strain was cultured under heterotrophic cultivation in mBold-3N media for 7 days.

Additionally, HM with high initial concentrations also reached high amount of removal efficiencies. The same phenomenon was witnessed by El Sheekh et al. [11] where *Chlorella salina* and *Chlorella vulgaris* were cultured in different water samples wherein the media with very high metal initial concentrations also removed higher amount of metals. In contrary, the lowest removal efficiency for Cr (95%) was observed at 20 mg $L^{-1}$ initial concentration. The same observation has been reported in El-Sheekh et al. [11] wherein some metal uptake were independents on the peripheral concentration of metals. The microalga strains used here showed high tolerance to high HM concentrations. This is probably due the type and concentrations of HM utilized, chemical composition of the synthetic medium, and the nature of microalgae strain used *Botryococcus* sp. NJD-1 (a pyramid-shaped microalga with the cell body of 6 to 10 µm long, and 3 to 6 µm wide). From previous studies, it was reported that diverse metal uptake depends upon the physiological state, the nature and charge of the cell wall polysaccharides, and the type of biosorbent of the algal cells used [11]. Also, the biosorption of metals depends on many factors such as cellular structure, storage polysaccharides, cell wall, and extracellular polysaccharides [6].

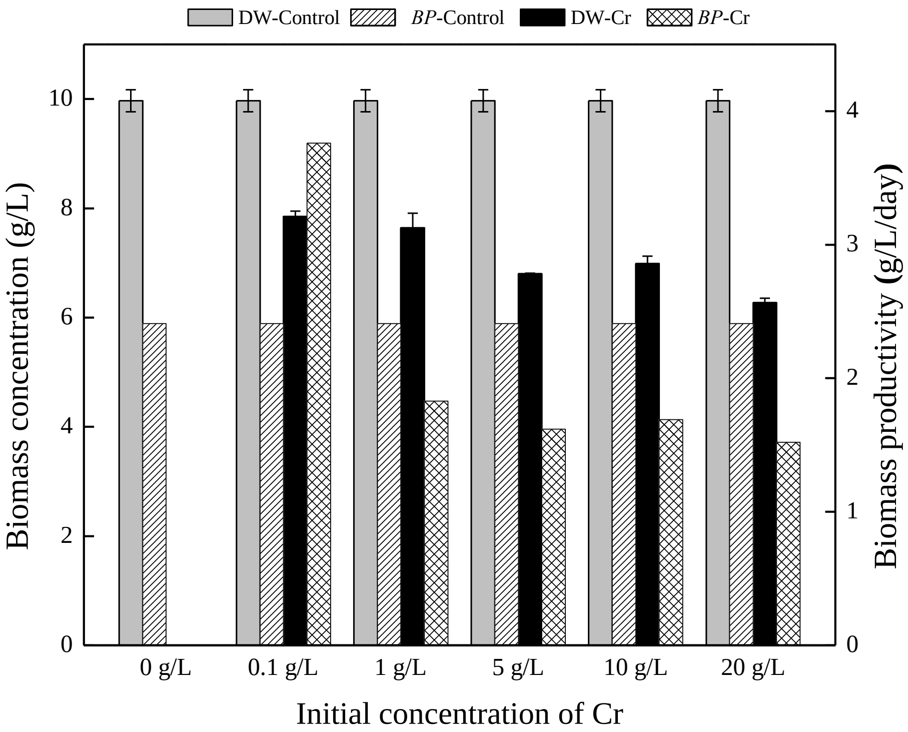


**Fig. 2** Growth parameters of *Botryococcus* sp. NJD-1: Biomass concentration (g DW $L^{-1}$) and biomass productivity (g $L^{-1}$ $day^{-1}$) of control (without initial concentration of Cr) and Cr, respectively, obtained when *Botryococcus* sp. NJD-1 strain was cultured under heterotrophic cultivation in mBold-3N media for 7 days.

### 3.2. Effects of nitrogen sources on microalgal strain performance

Four different nitrogen sources Sodium nitrate ($NaNO_3$), Ammonium chloride ($NH_4Cl$), Ammonium nitrate ($NH_4NO_3$), and Urea ($(NH_2)_2CO$) were utilized to investigate the influence of nitrogen source on heterotrophic growth and wastewater pollutants (N, P, and C) removal in heterotrophic conditions as presented in Fig.3 and Table 2. Results indicated that the *Botryococcus* sp. NJD-1 can utilize all the nitrogen sources but their biomass concentrations and N/P/C removal efficiencies showed major differences. Among above nitrogen sources, $NaNO_3$ was the best substrate obtaining the maximum biomass concentration of 2.44 g $L^{-1}$, at $6^{th}$ day of cultivation. The biomass productivity was 1.26 g $L^{-1}$ $day^{-1}$ while its specific growth rate was 0.72 $d^{-1}$. The urea also exhibited the highest biomass yield reaching 2.41 g $L^{-1}$ with the biomass productivity of 0.95 g $L^{-1}$ $day^{-1}$, while the growth rate attained was 0.44 $day^{-1}$. Nevertheless, in all cultures supplemented with $NH_4Cl$ and $NH_4NO_3$, the inhibition of the cell growth was observed. $NH_4Cl$ and $NH_4NO_3$ were also acceptable as nitrogen sources which obtained low biomass concentrations (0.72 and 1.47 g $L^{-1}$), low biomass productivities of 0.87 and 0.94 g $L^{-1}day^{-1}$, and specific growth rates of 0.29 and 0.41$d^{-1}$, respectively.

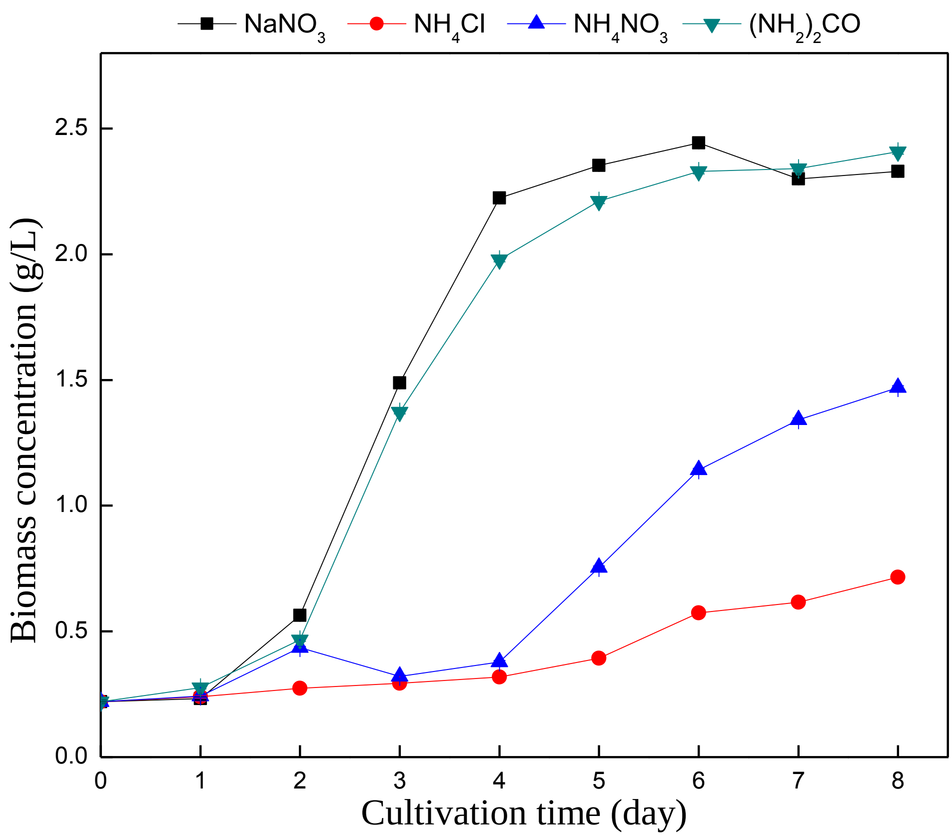


**Fig. 3** Growth ability of *Botryococcus* sp. NJD-1 expressed in biomass yield (g DW $L^{-1}$) incubated in different nitrogen sources for 7 days under heterotrophic conditions.

**Table 2:** Performance of *Botryococcus* sp. NJD-1 strain after 8-d cultivation in synthetic wastewater, 30 ˚C, pH 7 and 150 rpm in shaker incubator

| N sources | DCW (g $L^{-1}$) | *BP* (g $L^{-1}$ $d^{-1}$) | *μ* ($d^{-1}$) | $NO_3$-N | | $PO_4$-P | | C | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *RE* (%) | *RR* (g $L^{-1}$ $d^{-1}$) | *RE* (%) | *RR* (g $L^{-1}$ $d^{-1}$) | *RE* (%) | *RR* (g $L^{-1}$ $d^{-1}$) |
| $NaNO_3$ | 2.44 | 1.26 | 0.72 | 69.9 | 34.3 | 60.8 | 7.1 | 100 | 0.170 |
| $NH_4Cl$ | 0.72 | 0.87 | 0.29 | a | a | 61.5 | 7.1 | 32.7 | 0.045 |
| $NH_4NO_3$ | 1.47 | 0.94 | 0.41 | 43.4 | 24.3 | 61.5 | 7.1 | 59.2 | 0.099 |
| $(NH_2)_2CO$ | 2.41 | 0.95 | 0.44 | 18.1 | 10.7 | 61.0 | 7.2 | 97.1 | 0.165 |

“n.d” means not determined.

Many microalgae prefer $NH_4^+$ rather than nitrate/nitrite forms. Nitrate and nitrite have to be reduced to $NH_4^+$ prior to its utilization but $NH_4^+$ as the reduced form of N, can be directly assimilated into amino acids within algal cells [20]. In contrary, NO3- was observed as more favorable for the growth of Botryococcus sp. NJD-1 which enhanced 3-fold higher biomass yield in NaNO3 compared to NH4Cl. Similar results were also reported [17]. Under ammonium-based N sources supplementation, *Botryococcus sp.* NJD-1 cells were inhibited, which indicate that $NH_4^+$ was toxic to the cells at the given concentration (123 mg $L^{-1}$). In addition to this, the fact that ammonium ($NH_4^+$) N sources reached low growth rates is probable due to the existence of ammonia ($NH_3$) in the culture medium in the form of ammonium ions. It is known that under acidic conditions (< pH 7), $NH_3$ exist in $NH_4^+$ form and algal cells cannot assimilate ammonium directly. As the pH of the medium increases during cultivation, there is a high probability that $NH_3$ can volatilize, thus inhibiting the alga cells from growing [20]. Urea which is cheap compared to other N sources can be reduced to $NH_4^+$ and bicarbonate by microalgae in order to be easily assimilated [20]. The wastewater-born green alga *Botryococcus sp.* NJD-1 showed high cells growth in urea supplementation.

Concerning pollutants removal, Table 2 summarized the variation of growth parameters and nutrients removal efficiencies of NJD-1 from MBG-11 medium, in which $NaNO_3$ substrate reached high $NO_3$-N, $PO_4$-P, and C removal efficiencies of 69.9, 60.8, and 100%, respectively. Very low N removal rates were detected on $NH_4NO_3$ and $(NH_2)_2CO$ achieving 33.4 and 18.1%, in that order. There are a lot of components that can contribute to the total nitrogen such as ammonium, nitrite, organic nitrogen and ammonia. It was reported that algae can assimilate frankly different forms of inorganic nitrogen (i.e., nitrate, nitrite, and ammonia) [21]. The fact that only (18.1 – 33.4%) for $NH_4NO_3$ and $(NH_2)_2CO$ of total N was removed in this study, indicating there were still some organic compounds that could not be converted to $NO_3$-N or assimilated by algae in the culture solution. As microalgae require N for nucleic acid, phospholipid, and protein synthesis, they can assimilate $NH_4$-N, $NO_3^-$ and simple organic nitrogen including acetic acid, urea, and amino acids presented in wastewaters [22]. The algal strain removed > 60% of P for all four nitrogen sources used in this study. The lowest C removal was observed with $NH_4Cl$ (32.7%). Regarding the growth abilities and biomass production of *Botryococcus* NJD-1 strain, the nitrogen sources used in this work can be listed follows: $NaNO_3$ > $(NH_2)_2CO$ > $NH_4NO_3$ > $NH_4Cl$. Considering the N removal, $NaNO_3$ > $NH_4NO_3$ > $(NH_2)_2CO$ series is observed. For P removal efficiency, $NaNO_3$ > $NH_4NO_3$ > $NH_4Cl$ > $(NH_2)_2CO$ was obtained and $NaNO_3$ > $(NH_2)_2CO$ > $NH_4Cl$ > $NH_4NO_3$ series was listed for C removal as well.

Microalgae need P to grow and produce which is used for nucleic acids, phospholipids storage and ATP. Likewise, P in the form of inorganic orthophosphate is assimilated by microalgae, possibly as $H_2PO_4$ and $HPO_4$. Phosphorous (demonstrating about 1-3% of microalgae in DW), as a major macronutrient, plays a significant role in cellular metabolic routes including production of nucleic acids, DNA, energy transfer and probably forms various functional constituents and structures essential for development and growth [23]. Then, orthophosphate is merged into organic compounds through several kinds of phosphorylation procedures. Growing algae in P enriched wastewater, may display a competent uptake of P deposited into polyphosphate individuals subjective to multiplicity conditions like temperature, light intensity and pH, or nutrients concentration revel in wastewater. The ways these environmental and process conditions have affected the uptake have not been decided by researcher Richmond [23]. Again, the N:P ratio can possibly play a significant role in nitrogen and phosphorus elimination during wastewater remediation as it evaluates the prospective productivity and also maintaining the dominance in culture medium [23]. Table 2 showed that *Botryococcus* NJD-1 removed >97% of C pollutant when $NaNO_3$ and $(NH_2)_2CO$ substrates were used. Those results are much higher than the reported studies in which 85.3% of TOC removal efficiency in wastewater treatment workshop treated by *Scenedesmus obliquus* was obtained [24] and 91% TOC removal was recorded in the algal/bacterial bioreactor at 10 days of hydraulic-retention time when treating domestic wastewater [25]. Also, the degradation of organic compounds in which 80.8% COD removal efficiency in the heterotrophic growth of *Chlorella* sp. was achieved [26].

## 4. CONCLUSIONS

In this study, effects of heavy metals and nitrogen sources on *Botryococcus* sp. NJD-1 were evaluated. As the initial concentrations of HM increased in wastewater, Botryococcus sp. NJD-1 growth rates decreased. The highest removal efficiency up to 86% Cu was obtained while >95% removal of Cr was achieved by *Botryococcus* sp. NJD-1. Sodium nitrate was preferred for algal cell growth rather than ammonium-based nitrogen sources. Among four N sources utilized, $NaNO_3$ resulted in the highest biomass productivity (1.26 g $L^{-1}day^{-1}$) followed by urea (0.95 g $L^{-1}day^{-1}$) while $NH_4Cl$ obtained the lowest biomass productivity (0.26 g $L^{-1}day^{-1}$). This wastewater-born microalga presented elevated capacity to produce high biomass concentrations, remove HM and nutrients from wastewater. Also, NJD-1 is able to assimilate either inorganic or organic nitrogen sources. It seems to be a promising strain for effective advanced wastewater bioremediation and algal biomass production for biofuel.

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