Optimization of Cultivation Condition of Newly Isolated Strain *Chlorella Sorokiniana* pa.91 for CO₂ Bio-Fixation and Nutrients Removal From Real Municipal Wastewater: Impact of Temperature and Light Intensity

Poone Yaqoubnejad

Babol Noshirvani University of Technology

Hassan Aminirad (✉ h.a.rad@nit.ac.ir)

Babol Noshirvani University of Technology <https://orcid.org/0000-0002-9403-9429>

Mohsen Taghavijeloudar

Babol Noshirvani University of Technology

Research Article

Keywords: Microalgae, *Chlorella sorokiniana* pa.91, Optimization, CO₂ bio-fixation, Wastewater nutrients removal,

Posted Date: February 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-195210/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Optimization of cultivation condition of newly isolated strain *Chlorella sorokiniana* pa.91 for CO₂ bio-fixation and nutrients removal from wastewater: Impact of temperature and light intensity

Poone Yaqoubnejad^a, Hassan Amini-Rad^{a*}, Mohsen Taghavijeloudar^a

^a Department of Environmental Engineering, Faculty of Civil Engineering Faculty, Babol University of Technology, 47148-7313, Babol, Iran

^b Department of Civil and Environmental Engineering, Seoul National University, 151-744, Seoul, South Korea

ABSTRACT

The cultivation conditions of a newly isolated strain *Chlorella sorokiniana* pa.91 were optimized for the first time by performing sixty batch cultivation experiments at various temperatures (20, 25, 30 and 35 °C) and light intensities (1000, 3000, 4000, 5000 and 7000 Lux) in three different culture mediums of BG-11, real settled municipal wastewater (RMWW) and synthetic wastewater (SWW). Additionally, to evaluate the capability of *C. sorokiniana* pa.91 in CO₂ bio-fixation and wastewater treatment, the microalgae was cultivated in a flat-plate photobioreactor (CO₂ = 16% and 0.6 vvm aeration) under the optimal condition. The optimization results suggested that at the culture conditions of 30 °C, 4000 Lux and RMWW (COD 211 mgL⁻¹) microalgae had the best performance in growth and biomass productivity. Maximum biomass concentration and productivity of 3.21 gL⁻¹ and 0.31 gL⁻¹d⁻¹ were achieved, respectively, by cultivation of *C. sorokiniana* pa.91 in the photobioreactor under the optimized condition. Experimental results showed that *C. sorokiniana* pa.91 has a high capacity of CO₂ bio-fixation (0.59 mgL⁻¹d⁻¹) and CO₂ removal rate (35.6 %). Moreover, using *C. sorokiniana* pa.91 could efficiently remove 74% of NH₃, 93% of NO₃⁻, 83% of PO₄⁻³ and 76% of COD from real municipal wastewater after eight days of cultivation in the photobioreactor.

Keywords: Microalgae; *Chlorella sorokiniana* pa.91; Optimization; CO₂ bio-fixation, Wastewater nutrients removal,

* Corresponding author. h.a.rad@nit.ac.ir, Department of Environmental Engineering, Faculty of Civil Engineering Faculty, Babol University of Technology, 47148-7313, Babol, Iran

1. Introduction

The increasing use of water in urban area due to increased urbanization, industrialization and population growth has led to generate large amount of municipal wastewater. The discharge of untreated wastewater into the ecosystem has caused several environmental problems due to its high pollutants concentrations such as nitrate, phosphate and chemical oxygen demand (COD) (AlMomani and Örmeci, 2016; De Francisci et al., 2018; Wang and Lan, 2011). Currently, several methods have been comprehensively investigated for municipal wastewater treatment including chemical/physical treatments such as electrochemical or chemical oxidation (Shahid et al., 2020) which can remove high concentration of COD, and biological treatments such as anaerobic sequencing batch reactor, constructed wetlands and lagoon trickling filter (Yang et al., 2019). However, most of the chemical/physical methods are not environmentally friendly and in the case of biological methods, removal efficient in phosphorus may not be satisfactory (Li et al., 2019). Moreover, the current treatment methods produce high amount of sludge which required significant energy demand resulting in increased carbon dioxide (Javed et al., 2019). The increased emissions of CO₂ in the atmosphere is another global crises leads to serious environmental problems (Yin et al., 2020). The total emission of CO₂ has increased from 98 to 395 PPMV (parts per million volume) since 1990 and expected to reach 500 PPMV by 2050 if no reaction is taken (Shahid et al., 2020). Several attempts have been made to reduce CO₂ concentration such as carbon capture, storage and utilization technologies (Nocito and Dibenedetto, 2020).

Meanwhile, microalgae has received great attention due to their enormous multiple applications for wastewater treatment, sustainable CO₂ bio-fixation and producing high-valuable bio-produces (Anto et al., 2020; Pugazhendhi et al., 2020). Microalgae can reduce the nutrients such as nitrogen and phosphorus from wastewater by consuming them that promote microalgae biomass growth rate (Watsuntorn et al., 2019). In addition to the wastewater

treatment, microalgae have a great potential for carbon mitigation and CO₂ bio-fixation due to their higher photosynthetic efficiency (40–50% more than earthy plants) (Chang et al., 2016). It has been reported previously that microalgae contributed to almost 40% of global CO₂ sequestration. For example, 1.83 kg of CO₂ can be utilized by approximately 1 kg of microalgae (Ng et al., 2017).

Several research have investigated the performance of different microalgae strains for treatment of municipal and industrial wastewaters. Among them some microalgae species which grow rapidly have shown an excellent performance in nutrient removal from wastewater includes *Chlorella* sp. (Wang et al., 2010), *Nannochloropsis* sp. (Jiang et al., 2011) and *Scenedesmus* sp. (Xu et al., 2015). According to Kumar et al., (2019), *Chlorella* sp. microalgae could remove significant amounts of nitrate, phosphate and COD up to 67%, 88% and 71% from sewage wastewaters (Kumar et al., 2019). In another attempt, Wang et al. (2017) has reported that *Neochloris aquatica* CL-M1 microalgae could achieve a removal rate of 81% of COD and 96% of NH₄⁺ from swine wastewater (Wang et al., 2017). Novoveská et al. (2016) used *scenedesmus dimorphus* microalgae for raw municipal wastewater treatment. They could achieve 75% of total nitrogen, 93% of phosphorus and 84% of COD removal efficiency (Novoveská et al., 2016). However, still development of a new species of microalgae aiming to achieve high CO₂ bio-fixation and nutrient removal with lower energy consummation and capital investment is required. In this regard, a new microalgae strain *chlorella sorokiniana* pa.91 that was isolated from dairy wastewater has been introduced in our previous work (Asadi et al., 2019). According to Asadi et al., (2020), *C. sorokiniana* pa.91 showed a high capability of lipid production in comparison with *chlorella vulgaris* microalgae (Asadi et al., 2020). Considering the significant capability of *C. sorokiniana* pa.91 in converting pollutants into lipid production, it is hypothesized that this new microalgae may has a high wastewater pollutant ability and CO₂ bio-fixation potential.

Regardless the microalgae species, the complicated interactions between environmental factors such as temperature and light intensity have substantially influence on microalgae growth rate (Di Capua et al., 2016). Therefore, culture conditions for various microalgae strains needs to be optimized to achieving maximum biomass concentration at reasonable cost (Devi and Parthiban, 2020). The key factors that have significantly influenced on photosynthesis intensity and biomass productivity includes temperature, the light intensity, carbon dioxide and pH (Bazdar et al., 2018). Among them, temperature is an important factor that affects morphology and physiology of microalgae cell through changes in its metabolic rate (Qu et al., 2019). Therefore, finding optimal temperature for each microalgae species is vital to have a best performance of growth in culture medium (Brindhadevi et al., 2021; Nogueira et al., 2015). However, the most expensive environmental factor in microalgae cultivation is light illumination due to the usage of light emitting diodes (LEDs) (Devi and Parthiban, 2020). It has been reported that with an increase in light intensity the biomass growth rate increases up to a photo-inhibitory level (Nzayisenga et al., 2020). Optimization of light intensity in addition to aiming to enhancement of biomass productivity, can reduce of operational energy expenditure (Fan et al., 2020). To the best of our knowledge, the optimum culture condition of *C. sorokiniana pa.91* and its capability for CO₂ bio-fixation and nutrient removal from municipal wastewater has not been investigated yet.

Therefore, in the present research, first the optimum condition for *C. sorokiniana pa.91* growth were investigated by cultivation of the microalgae at variable temperatures and light intensities in different culture medium. Then, the performance of *C. sorokiniana pa.91* for CO₂ bio-fixation and municipal wastewater treatment was comprehensively studied. To do this, the microalgae was cultivated in a flat plate photobioreactor using real municipal wastewater under optimum culture condition. Overall, this study provides an effective strategy for CO₂ bio-fixation and wastewater treatment by a new strain which had not been investigated before.

2. Materials and Methods

2.1. Microalgae strain and culture mediums

A new strain of *Chlorella sorokiniana* pa.91 which was previously isolated from Gela dairy wastewater treatment plant (Amol, Iran) was selected and used in this study (Asadi et al., 2019). This strain was recognized and analyzed phylogenetically based on 18S rRNA gene sequence (Asadi et al., 2019). Because of the possibility of impurities and contamination, three purification phases were performed in plates containing agar and BG-11 culture medium. The microalgae cells were inoculated into several 500 ml Erlenmeyer flasks of BG-11 medium to prepare larger volumes of microalgae. Real raw municipal wastewater influent (RMWW) after preliminary settling tank was collected from wastewater treatment plant (WWTP), Sari, Iran. After pretreatment of the wastewater was used as culture medium to evaluate the microalgae capacity for nutrient removal from real municipal wastewater. Additionally, two synthetic mediums including BG-11 and synthetic wastewater (SWW) were considered in the present work. The physicochemical characteristics of the culture mediums are listed in Table 1.

2.2. Microalgae cultivation experiments

2.2.1. Batch experiments for optimization of cultivation condition

To investigate the optimum cultivation condition for new specie *C. sorokiniana* pa.91, series of batch cultivation experiments were carried out in 250 mL Erlenmeyer flasks containing 150 mL of the culture mediums which were aerated by HAIEACO-500 air pump. The effect of temperature and light intensities on microalgae growth in the three culture mediums (BG-11, RMWW and SWW) was evaluated by performing cultivation tests at different temperatures (20, 25, 30 and 35 °C) and light intensities (1000, 3000, 4000, 5000 and 7000 Lux). In each cultivation test, one parameter was varied while maintaining the other parameters constant. Finally, the results of the sixty batch cultivation experiments were used to plot 3D optimization graphs by Surfer 11.2 software. All the experiments were carried out in triplicate.

2.2.2. Photobioreactor experiments for CO₂ bio-fixation and nutrient removal

After optimizing the growth conditions of *Chlorella sorokiniana* pa.91, the microalgae was cultivated in a pilot-scale flat-plate photobioreactor (FP-PBR) at the optimum condition to investigate its capacity for CO₂ bio-fixation and nutrient removal from real municipal wastewater. The PBR used in this study was 8 L glass flat-plate photobioreactor (340 mm length×100 mm width×400 mm height), equipped with a gas sparger at the bottom of the PBR. On the top of the photobioreactor, three holes were placed to measure CO₂ output. Liquid samples were also collected from a sample point at the side of the photobioreactor daily to determine temperature, biomass concentration, pH and nutrient concentrations. The light intensity was provided by white fluorescent lamp that exposed to back side of photobioreactor with cycle of Light/dark 12/12h. In addition, CO₂ gas (16%) was aerated by the sparger at the rate of 0.6 vvm into the photobioreactor to providing mixing.

2.3. Analytical method

2.3.1. Determination of biomass concentration and productivity

The cell dry weight was measured by using the optical density at 680 nm in a UV spectrophotometer (2800 UV/VIS UNICO, China). The biomass concentration was determined by washing and drying the samples of microalgae cells in an oven at 70 °C for 24 h (Arbib et al., 2014). The relation between OD₆₈₀ and biomass concentration was obtained according to Eq. 1, as follows:

$$C \text{ (g/L)} = 1.824 \text{ OD}_{680} \text{ (g/L)} + 0.043, (R^2 = 0.9889) \quad (1)$$

Where, C and OD₆₈₀ are biomass concentration and optical density at 680 nm, respectively. Furthermore, the biomass productivity (P_B) were calculated from changes in biomass concentration over time according to Eq. (2) as follows (Nayak et al., 2016):

$$P_B(gL^{-1}d^{-1}) = (C_f/C_0)/(t_f - t_0) \quad (2)$$

Where, P_B is biomass productivity and C_f and C_0 are the final and initial biomass concentration at time t_f and t_0 (day), respectively.

2.3.2. Nutrient removal

During the cultivation period, the concentration of main nutrients from the municipal wastewater influent such as phosphate (PO_4^{3-}), ammonia (NH_3), nitrate (NO_3^-) and sCOD were determined based on standard methods ([Association et al., 1912](#)). 50 mL sample of microalgae medium (RMWW) from the photobioreactor was collected and centrifuged at 3500 rpm for 15 min, then, the supernatant was used for measuring the nutrients concentration. The nutrient removal percentage and removal rates were calculated as Eqs (3) and (4):

$$\text{Removal percentage (\%)} = (C_0 - C_f)/C_0 \times 100 \quad (3)$$

$$\text{Removal rate (}gL^{-1}d^{-1}\text{)} = (C_0 - C_f)/(t_f - t_0) \quad (4)$$

Where, C_0 and C_f are the initial and final nutrient concentrations at day t_0 and t_f , respectively.

2.3.3 CO₂ gas analysis

The CO₂ gas concentration at the outlet of the photobioreactor were measured by using carbon dioxide analyzer gas detector RIKEN Model RX-515. During cultivation, the exhaust CO₂ concentration was measured by the sensor of gas analyzer. The CO₂ bio-fixation rate and CO₂ removal percentage in the photobioreactor were calculated by the Eqs (5) and (6) ([Basu et al., 2013](#)):

$$CO_2 \text{ fixation rate (}gL^{-1}d^{-1}\text{)} = 1.88 \times P_B \quad (5)$$

$$CO_2 \text{ removal (\%)} = \frac{CO_{2inlet} - CO_{2outlet}}{CO_{2inlet}} \times 100 \quad (6)$$

Where, $P_{overall}$ is the biomass productivity ($gL^{-1}d^{-1}$), CO_{2inlet} (%) and $CO_{2outlet}$ (%) are the

percentage of CO₂ concentration in the inlet and outlet gas, respectively. The pH and dissolved oxygen (DO) was measured daily by pH meter and dissolved oxygen meter (WA-2017SD LUTRON, Japan), respectively. Light intensity was measured in all experiments by a digital light meter (Mastech MS6610, USA).

Results and discussion

3.1. Optimization of cultivation conditions of *chlorella sorokiniana* pa.91

To promote the growth rate of *chlorella sorokiniana* pa.91 for the purposes of CO₂ bio-fixation and nutrients removal from wastewater, optimization of the culture conditions are required. Among different cultures parameters, temperature and light intensity are the two most important environmental factors that influence the microalgae growth (Singh and Singh, 2015). Therefore, the present research focused on the optimization of temperature and light intensity to enhance microalgae growth rate at different culture mediums.

3.1.1. Effect of temperature on the growth of *chlorella sorokiniana* pa.91

Temperature has a significant effect on microalgae growth through controlling the rate of chemical reactions, enzyme activities, diffusion rate in water and photosynthesis efficiency (Luo et al., 2017). According to previous studies, a range of 20-35 °C is an appropriate temperature for microalgae cultivation of many species (Khoo et al., 2019; Singh and Singh, 2015). Therefore, the effect of various temperatures (20 °C, 25 °C, 30 °C and 35 °C) on the growth rate of *C. sorokiniana* pa.91 in the BG-11 culture medium under light intensity 3000 Lux was investigated (Fig. 1). As shown in Fig. 1a, *C. sorokiniana* pa.91 could grow well in a wide range of 20 – 35 °C temperature. The microalgae growth rate increased by increasing temperature from 20 to 30 °C, then it started to detriment when the temperature further increased to 35 °C. This might be related to the lower photosynthesis efficiency and metabolic rate at low temperatures and sever oxidative damages at high temperatures (Ali et al., 2005).

The maximum biomass concentrations were obtained as 1.05, 1.2, 1.4 and 0.9 gL⁻¹ after 15 days of cultivation at temperatures of 20 °C, 25 °C, 30 °C and 35 °C, respectively (Fig. 1b). Therefore, the optimum temperature for cultivation of *C. sorokiniana pa.91* in BG-11 at 3000 Lux was 30 °C and the temperatures order for cell growth were observed as follow 30°C > 25°C > 20°C > 35 °C. The results suggested that *C. sorokiniana pa.91* has good temperature tolerance and is suitable for outdoor cultivation. Similarly, Qu et al. (2019), Ho et al. (2014) and Kitaya et al. (2005) obtained the optimum temperature of 30 °C, 35 °C and 29 °C for cultivation of *Parachlorella kessleri QWY28*, *Desmodesmus sp. F2* and *Euglena gracilis* produced maximum biomass productivity of 646, 762 and 59 mgL⁻¹d⁻¹, respectively (Ho et al., 2014; Kitaya et al., 2005; Qu et al., 2019).

3.1.2. Effect of light intensity on the growth of *Chlorella sorokiniana pa.91*

It is well documented that light intensity has a direct influence on microalgae metabolic rate and microalgae growth (Brindhadevi et al., 2021; Qu et al., 2019). Generally, the range of applied light intensity for different microalgae cultivation was reported between 1000 – 8000 Lux (equivalent to 15 – 150 μmolm⁻²s⁻¹) (Devi and Parthiban, 2020). Accordingly, the effect of variable light intensities (1000, 3000, 4000, 5000 and 7000 lux) on the growth of *C. sorokiniana pa.91* in BG-11 medium at 30 °C was investigated. As can be seen in Fig. 2a, during the two initial days *C. sorokiniana pa.91* growth had a similar trend at all light intensities (approximately 0.16 g L⁻¹) except 4000 Lux which was bit higher (0.24 g L⁻¹). This might be attributed to the delay phase which was not observed at 4000 Lux. However, after the delay phase when the growth rate entered the exponential phase, the growth rate increased rapidly with an increase in the light intensity up to 4000 Lux with maximum biomass concentration of 1.61 gL⁻¹. This might be due to the insufficient photons at low light intensities which cannot provide the light required for an appropriate photosynthesis process. However, further increase in light intensity led to a lower biomass concentration and decline in growth rate which

might be related to the photo-inhibition and cells damage due to heat accumulation (Devi and Parthiban, 2020; Ho et al., 2014). Fig. 2b shows the maximum biomass concentrations of *C. sorokiniana pa.91* obtained after 15 days of cultivation at different light intensities in BG-11 medium under 30 °C. The maximum concentrations of 0.99, 1.41, 1.61, 1.32, 1.18 gL⁻¹ were obtained at the light intensities of 1000, 3000, 4000, 5000 and 7000 Lux, respectively. The optimal condition for *C. sorokiniana pa.91* was achieved at 4000 Lux followed by 3000 > 5000 > 7000 > 1000 Lux at temperature of 30 °C. According to Devi & Parthiban. (2020), Maynardo et al. (2015) and Khoeyri et al. (2012), maximum biomass concentration of 1.76, 1.9 and 2.05 mgL⁻¹ were obtained for *Nostoc ellipsosporum* NCIM 2786, *Nannochloropsis* and *Chlorella vulgaris* microalgae at optimal light intensity of 4500, 5000 and 4000 Lux, respectively (Devi and Parthiban, 2020; Khoeyri et al., 2012; Maynardo et al., 2015).

3.1.3. Optimization of cultivation condition at different cultivation mediums

To investigate the practical application of the optimal condition for *chlorella sorokiniana pa.91* cultivation, it is essential to evaluate the maximum biomass production in different cultivation mediums, particularly in the real municipal wastewater (RMWW). Therefore, a series of *C. sorokiniana pa.91* cultivation experiments (60 tests) were carried out under various temperatures (20 °C, 25 °C, 30 °C and 35 °C) and light intensities (1000, 3000, 4000, 5000 and 7000 lux) in the three culture environments of BG-11, real municipal wastewater (RMWW) and synthetic wastewater (SWW) (Table 1) for 15 days. The maximum biomass concentration as a function of temperature and light intensity were plotted for the three culture mediums and presented in Fig. 3. According to Fig. 3, *C. sorokiniana pa.91* achieved the highest biomass concentration of 2.45 gL⁻¹ in RMWW, which was significantly higher than those obtained in BG-11 (1.61 gL⁻¹) and SWW (1.9 gL⁻¹) under the optimal condition of 30 °C and 4000 Lux. This might be related to the existence of the symbiotic bacteria such as Verrucomicrobium, Proteobacteria and Frimicutes in real municipal wastewater (RMWW) that can be favourable

to microalgae growth (Tandon and Jin, 2017; Toyama et al., 2018). The lower biomass concentration yield in the case of SWW (COD = 403 mgL⁻¹) might be attributed the low N/P rate (~ 1.8), high COD concentration (~ 403 mgL⁻¹) and turbidity which negatively affect the microalgae growth (Wen et al., 2017).

Moreover, a close monitoring of the interaction effects between temperature and light intensity on the biomass growth revealed a synergy between temperature and light intensity at higher values resulting in sub-optimal conditions. For instance at higher light intensity of 7000 Lux the optimal condition were obtained at 25 °C instead of 30 °C (Fig. 3). The results suggest that, in cold area with lower temperature microalgae cultivation at higher light intensity would be recommended. This synergistic effect might be related to the heat accumulation at higher light intensities which results in higher metabolic reaction of microalgae and carbon bio-fixation at lower temperature (Ho et al., 2014). However, at lower temperatures, the light intensity showed less effect on the growth rate, suggesting that *C. sorokiniana pa.91* growth rate was light independent at low temperature. A similar phenomena was previously observed (Nogueira et al., 2015).

3.2. Cultivation of *C. sorokiniana pa.91* in flat-plate photobioreactor

To further evaluate the capability of newly isolated strain *C. sorokiniana pa.91* in CO₂ bio-fixation and nutrient removal from real municipal wastewater, the microalgae was cultivated in a flat-plate photobioreactor under the optimized condition of 30 °C temperature and 4000 Lux light intensity, using RMWW as culture medium.

3.2.1. *C. sorokiniana pa.91* growth rate and productivity in the photobioreactor

Fig. 4 shows the biomass concentration and productivity of *C. sorokiniana pa.91* in the photobioreactor during 11 days of cultivation under optimal condition of 30 °C and 4000 Lux. According to Fig.4, *C. sorokiniana pa.91* growth rate was very low (approximately 0.8 gL⁻¹)

during the initial two days which might be due to being in adaptation phase. Similar behavior was observed by [Hinterholz et al. \(2019\)](#) and [Cheng et al. \(2020\)](#) when they cultivated *Chlorella PY-ZUI* and *P. Malhamensis* in the JTSP and FP photobioreactors, respectively ([Cheng et al., 2019](#); [Hinterholz et al., 2019](#)). However, after the third day microalgae growth entered to an exponential phase, which biomass concentration gradually increased up to 2.83 gL⁻¹ until the eighth day when maximum biomass productivity of 0.30 gL⁻¹d⁻¹ was obtained. Then, microalgae concentration and biomass productivity slightly decreased due to the decay period of microalgae in the photobioreactor with might be related to the higher microalgae concentration that prevented equal light receiving by all microalgae cells in the photobioreactor. The results of *C. Sorokiniana pa.91* cultivation in the photobioreactor showed that this microalgae can growth rapidly and reach to 3.21 gL⁻¹ and 0.31 gL⁻¹d⁻¹ biomass concentration and productivity, respectively, within eight days of cultivation under optimal condition (30 °C and 4000 Lux). These results suggesting that *C. Sorokiniana pa.91* might has high potential for CO₂ bio-fixation and wastewater treatment.

3.2.2. CO₂ bio-fixation efficiency of *C. Sorokiniana pa.91* in the photobioreactor

The capability of *C. Sorokiniana pa.91* specie for CO₂ absorption and O₂ generation was investigated in this study for the first time. [Fig. 5a](#) shows the CO₂ removal rate and dissolved oxygen (DO) concentration during *C. Sorokiniana pa.91* cultivation in the photobioreactor under the optimal condition. Generally, sufficient supply of carbon is required for the growth of microalgae ([Weissman et al., 1988](#)). The maximum CO₂ removal percentage and the dissolved oxygen concentration after eight days of cultivation were 35.6% and 1.66 mgL⁻¹, respectively. It is well-known that in the presence of sufficient light, microalgae cells can convert CO₂ to oxygen by photosynthesis process ([Lam et al., 2012](#)). According to the results of the present work, *C. Sorokiniana pa.91* showed a great capability for CO₂ absorption when cultivated under optimal cultivation condition.

Fig. 5b shows the average CO₂ bio-fixation rate through *C. Sorokiniana pa.91* and pH variation in the photobioreactor. The maximum CO₂ fixation rate was obtained at 0.59 gL⁻¹d⁻¹ when the maximum biomass productivity of 0.31 gL⁻¹d⁻¹ was achieved at the eighth day. The results of CO₂ bio-fixation showed a good agreement with that of biomass productivity (Fig. 4) indicating the demand of CO₂ increase with increasing biomass concentration. Several research have been proposed previously for CO₂ bio-fixation through cultivation of different microalgae species in PBRs. Cheng et al. (2019) cultivated *Chlorella PY-ZUI* in a novel JTSP photobioreactor, resulting in a maximum CO₂ fixation rate of 0.52 gL⁻¹d⁻¹ (Cheng et al., 2019). Xia et al. (2018) achieved a maximum CO₂ fixation rate of 0.1 gL⁻¹d⁻¹ by cultivation *Chlorella vulgaris FACHB-31* in their new designed PBR. Additionally, Fig. 5b shows the variation pH of culture medium during *C. Sorokiniana pa.91*. A dramatically decrease in pH was observed from 7.8 to 5.8 at the initial three days (adoption period) which might be attributed to the higher H⁺ concentration as results of rapid dissolution of CO₂ in the culture medium (Cheng et al., 2019; Xia et al., 2018). However, the medium pH gradually began to increase again and stabilize at 7 due to the dissolved CO₂ saturated in the medium by microalgae cells.

3.2.3. Nutrient removal efficiency of *C. Sorokiniana pa.91* in the photobioreactor

The consumption of nutrient from wastewater by microalgae cells promotes biomass growth rate and results in wastewater treatment. To assess the feasibility of microalgae based wastewater treatment using *C. sorokiniana pa.91*, the removal of nutrients such as ammonia (NH₃), nitrate (NO₃⁻), phosphate (PO₄⁻³) and chemical oxygen demand (COD) from real municipal wastewater influent (RMWW) was investigated in the photobioreactor under the optimized condition: 30 °C temperature and 4000 Lux light intensity (Fig 6).

Among different nutrients, nitrogen is the main source for microalgae growth (Daneshvar et al., 2019). Different forms of nitrogen in wastewater includes ammonium (NH₄⁺), ammonia (NH₃), nitrate (NO₃⁻), nitrite (NO₂⁻). It has been reported that ammonia (NH₃ = 34 mgL⁻¹) is

the main form of nitrogen in municipal wastewater influent. However, low concentration of NO_3^- (2.15 mgL^{-1}) and NO_2^- (0.06 mgL^{-1}) were also identified in the wastewater influent presumably due to the slightly nitrification as a result of accidental aeration. Fig. 6 (a) and (b) show the evaluation of ammonia (NH_3), nitrate (NO_3^-) from the real municipal wastewater (RMWW) by *C. sorokiniana* pa.91, respectively. As shown in Fig 6, *C. sorokiniana* pa.91 microalgae could remove ammonia and nitrate up to 74% and 93%, respectively after 10 days of cultivation. During the first two days, *C. sorokiniana* pa.91 was almost ineffective in removing ammonia and nitrate from wastewater due to the adoption period. However, after the adoption period (the 3rd day) the removal efficiency was remarkably increased until the 8th day in the photobioreactor. These results are in agreement with the maximum biomass concentration of microalgae was obtained after eight days cultivation (Fig. 4b). According to Fig 6a, NH_3 and NO_3^- concentration decreased to less than 10 and 0.2 mg L^{-1} within six days. The removal of nitrate (NO_3^-) was almost completed after the 5th days in photobioreactor under optimized condition. Although, in the presence of ammonia microalgae first consume NH_3 and then utilize other forms of nitrogen, *C. sorokiniana* pa.91 showed a good capacity for the removal of nitrate even at low dosage which was not seen in the other species of microalgae.

Phosphate is another nutrient that can be used for microalgae growth while the current treatment technologies cannot efficiently removed phosphate from wastewater (Watsuntorn et al., 2019). According to Fig. 6c the consumption of phosphate (PO_4^{3-}) by *C. sorokiniana* pa.91 showed a similar trend with that observed for ammonia. However, the final removal rate of the phosphate (83%) was higher than ammonia (73%) and less than nitrate (93%) which might be attributed to the initial concentration of the nutrients in the culture medium. As showed in Fig. 6c, the PO_4^{3-} concentration decreased markedly from 6.1 to less than 1 mg L^{-1} within 8 days of *C. sorokiniana* pa.91 cultivation in the photobioreactor under the optimized condition. The results suggested that *C. sorokiniana* pa.91 has high capacity of orthophosphate storing from

the medium. It should be noted that the achieved final concentration of phosphate is below the acceptable level of the surface water quality standard (1 P-mgL^{-1}). (China, 2002)

Chemical oxygen demand (COD) has been reported as the most challenging problem in the treatment process due to its re-increasing in the wastewater (Lee et al., 2019). As illustrated in Fig 6d, the COD was eliminated gradually from 211 to 50.7 mgL^{-1} resulted in 76.3% removal rate of COD during 10 days cultivation of *C. sorokiniana pa.91* in the photobioreactor. In comparison with the other nutrients, the COD showed relatively lower removal efficiency. Generally, microalgae release organic matter after utilization of carbon sources from the COD components which leads to increased COD in the culture medium (Mujtaba and Lee, 2017). However, it seems that the wastewater in this study has provided sufficient sCOD to the microalgae cells for metabolism process. The results suggested that using *C. sorokiniana pa.91* for municipal wastewater could reduce COD concentration below the standard for agriculture reuse water (50 COD-mgL^{-1}) (Shoushtarian and Negahban-Azar, 2020).

For comparison purposes, Table 2 presences the results of municipal wastewater treatment by *C. sorokiniana pa.91* used in this study and other microalgae species obtained from literature. Although there are many factors that affect microalgae based wastewater treatment, the removal efficiency of nutrients mainly depends on the specific adsorption capacity and cells yield of microalgae species (Yang et al., 2016). According to Table. 2, when *C. sorokiniana pa.91* was used for the treatment of municipal wastewater, the removal efficiency of 73%, 93%, 83% and 76% was achieved for ammonia (NH_3), nitrate (NO_3^-), phosphate (PO_4^{3-}) and COD, respectively, after 10 days of cultivation under optimal condition. In comparison with the other microalgae species, *C. sorokiniana pa.91* which has been investigated for municipal wastewater treatment for the first time in this study, showed higher utilization efficiency under optimized condition. The results suggested that the new specie of *C. sorokiniana pa.91* can be used as promising approach for the treatment of municipal wastewater.

Conclusion

The newly isolated microalgae *Chlorella sorokiniana* pa.91 showed a great potential for CO₂ bio-fixation and nutrients removal from real municipal wastewater. After a comprehensive investigation on the effect of culture condition on the growth rate of *C. sorokiniana* pa.91 in three different culture mediums, the optimal condition was achieved at 30 °C temperature and 4000 Lux light intensity in RMWW. Further, *C. sorokiniana* pa.91 was cultivated in a flat-plate photobioreactor (16% CO₂ and 0.6 vvm) at the optimized condition which produced a maximum biomass concentration of 3.21 gL⁻¹ after 10 days of cultivation. Moreover, a high CO₂ bio-fixation and removal rate of 1.66 mgL⁻¹ and 35.6% were achieved. Under optimal condition, *C. sorokiniana* pa.91 showed an excellent nutrient removal ability from wastewater by achieving removal rates of 73%, 93%, 83% and 76% for ammonia (NH₃), nitrate (NO₃⁻), phosphate (PO₄⁻³) and COD, respectively. The results of this study suggested a promising method enhancing CO₂ bio-fixation and municipal wastewater treatment.

Reference

- Ali, M.B., Hahn, E.-J., and Paek, K.-Y. (2005). Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in *Phalaenopsis*. *Plant physiology and Biochemistry* 43, 213-223.
- AlMomani, F.A., and Örmeci, B. (2016). Performance Of *Chlorella Vulgaris*, *Neochloris Oleoabundans*, and mixed indigenous microalgae for treatment of primary effluent, secondary effluent and centrate. *Ecological Engineering* 95, 280-289.
- Anto, S., Mukherjee, S.S., Muthappa, R., Mathimani, T., Deviram, G., Kumar, S.S., Verma, T.N., and Pugazhendhi, A. (2020). Algae as green energy reserve: Technological outlook on biofuel production. *Chemosphere* 242, 125079.
- Arbib, Z., Ruiz, J., Álvarez-Díaz, P., Garrido-Pérez, C., and Perales, J.A. (2014). Capability of different microalgae species for phytoremediation processes: Wastewater tertiary treatment, CO₂ bio-fixation and low cost biofuels production. *Water research* 49, 465-474.
- Asadi, P., Rad, H.A., and Qaderi, F. (2019). Comparison of *Chlorella vulgaris* and *Chlorella sorokiniana* pa. 91 in post treatment of dairy wastewater treatment plant effluents. *Environmental Science and Pollution Research* 26, 29473-29489.
- Asadi, P., Rad, H.A., and Qaderi, F. (2020). Lipid and biodiesel production by cultivation isolated strain *Chlorella sorokiniana* pa. 91 and *Chlorella vulgaris* in dairy wastewater treatment plant effluents. *Journal of Environmental Health Science and Engineering*, 1-13.
- Association, A.P.H., Association, A.W.W., Federation, W.P.C., and Federation, W.E. (1912). Standard methods for the examination of water and wastewater. (American Public Health Association.).

377 Basu, S., Roy, A.S., Mohanty, K., and Ghoshal, A.K. (2013). Enhanced CO₂ sequestration by a novel microalga:
378 *Scenedesmus obliquus* SA1 isolated from bio-diversity hotspot region of Assam, India. *Bioresource*
379 *technology* 143, 369-377.

380 Bazdar, E., Roshandel, R., Yaghmaei, S., and Mardanpour, M.M. (2018). The effect of different light intensities
381 and light/dark regimes on the performance of photosynthetic microalgae microbial fuel cell. *Bioresource*
382 *technology* 261, 350-360.

383 Brindhadevi, K., Mathimani, T., Rene, E.R., Shanmugam, S., Chi, N.T.L., and Pugazhendhi, A. (2021). Impact of
384 cultivation conditions on the biomass and lipid in microalgae with an emphasis on biodiesel. *Fuel* 284, 119058.

385 Chang, H.-X., Huang, Y., Fu, Q., Liao, Q., and Zhu, X. (2016). Kinetic characteristics and modeling of microalgae
386 *Chlorella vulgaris* growth and CO₂ biofixation considering the coupled effects of light intensity and dissolved
387 inorganic carbon. *Bioresource technology* 206, 231-238.

388 Cheng, J., Lai, X., Ye, Q., Guo, W., Xu, J., Ren, W., and Zhou, J. (2019). A novel jet-aerated tangential swirling-
389 flow plate photobioreactor generates microbubbles that enhance mass transfer and improve microalgal growth.
390 *Bioresource technology* 288, 121531.

391 China, E. (2002). Environmental quality standards for surface water. MEP, China, GB3838-2002.

392 Daneshvar, E., Zarrinmehr, M.J., Koutra, E., Kornaros, M., Farhadian, O., and Bhatnagar, A. (2019). Sequential
393 cultivation of microalgae in raw and recycled dairy wastewater: microalgal growth, wastewater treatment and
394 biochemical composition. *Bioresource technology* 273, 556-564.

395 De Francisci, D., Su, Y., Iital, A., and Angelidaki, I. (2018). Evaluation of microalgae production coupled with
396 wastewater treatment. *Environmental technology* 39, 581-592.

397 Devi, T.E., and Parthiban, R. (2020). Hydrothermal liquefaction of *Nostoc ellipsosporum* biomass grown in
398 municipal wastewater under optimized conditions for bio-oil production. *Bioresource Technology* 316, 123943.

399 Di Capua, F., Ahoranta, S.H., Papirio, S., Lens, P.N., and Esposito, G. (2016). Impacts of sulfur source and
400 temperature on sulfur-driven denitrification by pure and mixed cultures of *Thiobacillus*. *Process Biochemistry*
401 51, 1576-1584.

402 Fan, H., Wang, K., Wang, C., Yu, F., He, X., Ma, J., and Li, X. (2020). A comparative study on growth characters
403 and nutrients removal from wastewater by two microalgae under optimized light regimes. *Environmental*
404 *Technology & Innovation* 19, 100849.

405 Hinterholz, C.L., Trigueros, D.E.G., Módenes, A.N., Borba, C.E., Scheufele, F.B., Schuelter, A.R., and Kroumov,
406 A.D. (2019). Computational fluid dynamics applied for the improvement of a flat-plate photobioreactor
407 towards high-density microalgae cultures. *Biochemical Engineering Journal* 151, 107257.

408 Ho, S.-H., Chen, C.-N.N., Lai, Y.-Y., Lu, W.-B., and Chang, J.-S. (2014). Exploring the high lipid production
409 potential of a thermotolerant microalga using statistical optimization and semi-continuous cultivation.
410 *Bioresource technology* 163, 128-135.

411 Javed, F., Aslam, M., Rashid, N., Shamair, Z., Khan, A.L., Yasin, M., Fazal, T., Hafeez, A., Rehman, F., and
412 Rehman, M.S.U. (2019). Microalgae-based biofuels, resource recovery and wastewater treatment: a pathway
413 towards sustainable biorefinery. *Fuel* 255, 115826.

414 Jiang, L., Luo, S., Fan, X., Yang, Z., and Guo, R. (2011). Biomass and lipid production of marine microalgae
415 using municipal wastewater and high concentration of CO₂. *Applied energy* 88, 3336-3341.

416 Khoeyi, Z.A., Seyfabadi, J., and Ramezanpour, Z. (2012). Effect of light intensity and photoperiod on biomass
417 and fatty acid composition of the microalgae, *Chlorella vulgaris*. *Aquaculture International* 20, 41-49.

418 Khoo, K.S., Lee, S.Y., Ooi, C.W., Fu, X., Miao, X., Ling, T.C., and Show, P.L. (2019). Recent advances in
419 biorefinery of astaxanthin from *Haematococcus pluvialis*. *Bioresource technology* 288, 121606.

420 Kitaya, Y., Azuma, H., and Kiyota, M. (2005). Effects of temperature, CO₂/O₂ concentrations and light intensity
421 on cellular multiplication of microalgae, *Euglena gracilis*. *Advances in Space Research* 35, 1584-1588.

422 Kumar, P.K., Krishna, S.V., Naidu, S.S., Verma, K., Bhagawan, D., and Himabindu, V. (2019). Biomass production
423 from microalgae *Chlorella* grown in sewage, kitchen wastewater using industrial CO₂ emissions: comparative
424 study. *Carbon Resources Conversion* 2, 126-133.

425 Lam, M.K., Lee, K.T., and Mohamed, A.R. (2012). Current status and challenges on microalgae-based carbon
426 capture. *International Journal of Greenhouse Gas Control* 10, 456-469.

427 Lee, S.-A., Lee, N., Oh, H.-M., and Ahn, C.-Y. (2019). Enhanced and Balanced Microalgal Wastewater Treatment
428 (COD, N, and P) by Interval Inoculation of Activated Sludge. *Journal of microbiology and biotechnology* 29,
429 1434-1443.

430 Li, K., Liu, Q., Fang, F., Luo, R., Lu, Q., Zhou, W., Huo, S., Cheng, P., Liu, J., and Addy, M. (2019). Microalgae-
431 based wastewater treatment for nutrients recovery: a review. *Bioresource technology* 291, 121934.

432 Luo, Y., Le-Clech, P., and Henderson, R.K. (2017). Simultaneous microalgae cultivation and wastewater treatment
433 in submerged membrane photobioreactors: a review. *Algal research* 24, 425-437.

434 Maynardo, J.J., Doshi, V., Rajanren, J.R., and Rajasekaran, R. (2015). The optimization of light intensity and
435 drying temperature on lipid content of microalgae *Nannochloropsis oculata*. *Journal of Engineering Science*
436 *and Technology* 1, 112-121.

437 Mujtaba, G., and Lee, K. (2017). Treatment of real wastewater using co-culture of immobilized *Chlorella vulgaris*
438 and suspended activated sludge. *Water research* 120, 174-184.

439 Nayak, M., Karemore, A., and Sen, R. (2016). Performance evaluation of microalgae for concomitant wastewater
440 bioremediation, CO₂ biofixation and lipid biosynthesis for biodiesel application. *Algal Research* 16, 216-223.

441 Ng, I.S., Tan, S.I., Kao, P.H., Chang, Y.K., and Chang, J.S. (2017). Recent developments on genetic engineering
442 of microalgae for biofuels and bio-based chemicals. *Biotechnology journal* 12, 1600644.

443 Nocito, F., and Dibenedetto, A. (2020). Atmospheric CO₂ mitigation technologies: carbon capture utilization and
444 storage. *Current Opinion in Green and Sustainable Chemistry* 21, 34-43.

445 Nogueira, D.P.K., Silva, A.F., Araújo, O.Q., and Chaloub, R.M. (2015). Impact of temperature and light intensity
446 on triacylglycerol accumulation in marine microalgae. *biomass and bioenergy* 72, 280-287.

447 Novoveská, L., Zapata, A.K., Zabolotney, J.B., Atwood, M.C., and Sundstrom, E.R. (2016). Optimizing
448 microalgae cultivation and wastewater treatment in large-scale offshore photobioreactors. *Algal research* 18,
449 86-94.

450 Nzayisenga, J.C., Farge, X., Groll, S.L., and Sellstedt, A. (2020). Effects of light intensity on growth and lipid
451 production in microalgae grown in wastewater. *Biotechnology for biofuels* 13, 1-8.

452 Pugazhendhi, A., Nagappan, S., Bhosale, R., Tsai, P.-C., Natarajan, S., Devendran, S., Al Haj, L., Ponnusamy,
453 V.K., and Kumar, G. (2020). Various potential techniques to reduce the water footprint of microalgal biomass
454 production for biofuel—A review. *Science of the Total Environment*, 142218.

455 Qu, W., Zhang, C., Zhang, Y., and Ho, S.-H. (2019). Optimizing real swine wastewater treatment with maximum
456 carbohydrate production by a newly isolated indigenous microalga *Parachlorella kessleri* QWY28.
457 *Bioresource technology* 289, 121702.

458 Shahid, A., Malik, S., Zhu, H., Xu, J., Nawaz, M.Z., Nawaz, S., Alam, M.A., and Mehmood, M.A. (2020).
459 Cultivating microalgae in wastewater for biomass production, pollutant removal, and atmospheric carbon
460 mitigation; a review. *Science of the Total Environment* 704, 135303.

461 Shoushtarian, F., and Negahban-Azar, M. (2020). Worldwide regulations and guidelines for agricultural water

- reuse: a critical review. *Water* *12*, 971.
- Singh, S., and Singh, P. (2015). Effect of temperature and light on the growth of algae species: a review. *Renewable and sustainable energy reviews* *50*, 431-444.
- Tandon, P., and Jin, Q. (2017). Microalgae culture enhancement through key microbial approaches. *Renewable and Sustainable Energy Reviews* *80*, 1089-1099.
- Toyama, T., Kasuya, M., Hanaoka, T., Kobayashi, N., Tanaka, Y., Inoue, D., Sei, K., Morikawa, M., and Mori, K. (2018). Growth promotion of three microalgae, *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Euglena gracilis*, by in situ indigenous bacteria in wastewater effluent. *Biotechnology for biofuels* *11*, 1-12.
- Wang, B., and Lan, C.Q. (2011). Biomass production and nitrogen and phosphorus removal by the green alga *Neochloris oleoabundans* in simulated wastewater and secondary municipal wastewater effluent. *Bioresource technology* *102*, 5639-5644.
- Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., and Ruan, R. (2010). Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. *Applied biochemistry and biotechnology* *162*, 1174-1186.
- Wang, Y., Ho, S.-H., Cheng, C.-L., Nagarajan, D., Guo, W.-Q., Lin, C., Li, S., Ren, N., and Chang, J.-S. (2017). Nutrients and COD removal of swine wastewater with an isolated microalgal strain *Neochloris aquatica* CL-M1 accumulating high carbohydrate content used for biobutanol production. *Bioresource technology* *242*, 7-14.
- Watsuntorn, W., Ruangchainikom, C., Rene, E.R., Lens, P.N., and Chulalaksananukul, W. (2019). Comparison of sulphide and nitrate removal from synthetic wastewater by pure and mixed cultures of nitrate-reducing, sulphide-oxidizing bacteria. *Bioresource technology* *272*, 40-47.
- Weissman, J.C., Goebel, R.P., and Benemann, J.R. (1988). Photobioreactor design: mixing, carbon utilization, and oxygen accumulation. *Biotechnology and bioengineering* *31*, 336-344.
- Wen, Y., He, Y., Ji, X., Li, S., Chen, L., Zhou, Y., Wang, M., and Chen, B. (2017). Isolation of an indigenous *Chlorella vulgaris* from swine wastewater and characterization of its nutrient removal ability in undiluted sewage. *Bioresource technology* *243*, 247-253.
- Xia, A., Hu, Z., Liao, Q., Huang, Y., Zhu, X., Ye, W., and Sun, Y. (2018). Enhancement of CO₂ transfer and microalgae growth by perforated inverted arc trough internals in a flat-plate photobioreactor. *Bioresource technology* *269*, 292-299.
- Xu, J., Zhao, Y., Zhao, G., and Zhang, H. (2015). Nutrient removal and biogas upgrading by integrating freshwater algae cultivation with piggery anaerobic digestate liquid treatment. *Applied microbiology and biotechnology* *99*, 6493-6501.
- Yang, H., Deng, L., Wang, L., Zheng, D., Liu, Y., Wang, S., and Huang, F. (2019). Comparison of three biomass-retaining reactors of the ASBR, the UBF and the USR treating swine wastewater for biogas production. *Renewable Energy* *138*, 521-530.
- Yang, I.-S., Salama, E.-S., Kim, J.-O., Govindwar, S.P., Kurade, M.B., Lee, M., Roh, H.-S., and Jeon, B.-H. (2016). Cultivation and harvesting of microalgae in photobioreactor for biodiesel production and simultaneous nutrient removal. *Energy Conversion and Management* *117*, 54-62.
- Yin, Z., Zhu, L., Li, S., Hu, T., Chu, R., Mo, F., Hu, D., Liu, C., and Li, B. (2020). A comprehensive review on cultivation and harvesting of microalgae for biodiesel production: Environmental pollution control and future directions. *Bioresource technology* *301*, 122804.

Figures

Figure 1

The effect of temperature on (a) *C. sorokiniana* p. 91 biomass concentration and (b) maximum biomass concentration after 15 days of cultivation in BG-11 under 3000 Lux light intensity

Figure 2

The effect of light intensity on (a) *C. sorokiniana* pa. 91 biomass concentration and (b) maximum biomass concentration after 15 days of cultivation in BG-11 under 30 °C temperature

Figure 3

Optimization of *C. sorokiniana* pa.91 cultivation condition in (a) BG-11, (b) RMWW and (c) SWW culture mediums

Figure 4

Biomass concentration and productivity of *C. sorokiniana* p.91 cultivated in flat-plate photobioreactor using real municipal wastewater under optimal condition of 30 °C temperature and 4000 Lux light intensity

Figure 5

Variation of (a) CO₂ removal rate and dissolved oxygen concentration, (b) CO₂ bio-fixation and pH variation during cultivation *C. sorokiniana* p.91 in flat-plate photobioreactor using real municipal wastewater under optimal condition of 30 °C temperature and 4000 Lux light intensity

Figure 6

Removal rate and concentration of (a) NH₃, (b) NO₃⁻ (c) PO₄⁻³ and (d) COD during cultivation of *C. sorokiniana* p.91 in flat-plate photobioreactor using real municipal wastewater under optimal condition of 30 °C temperature and 4000 Lux light intensity

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [CULGraphicalAbstractCTEP.docx](#)