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Enhanced cultivation and lipid production of isolated microalgae strains using municipal wastewater

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ABSTRACT

Cultivation of microalgae in wastewater is strongly related to the selection of suitable species for a specific type of wastewater. Thus, this study presented the cultivation of isolated microalgae strains, *Acutodesmus obliquus* CN01 and *Desmodesmus maximus* CN06 using municipal wastewater, along with *Chlorella vulgaris* NIES-1269 as the control species. The highest growth rate of 0.23/day was achieved by *D. maximus* CN06, while all species exhibited excellent nutrient removal efficiencies. High removal of NH_3-N was demonstrated by all strains and complete total phosphorus removal was observed to be fastest in *A. obliquus* CN01 at 3.8 mg P/gDCW.day, followed by *D. maximus* CN06 and *C. vulgaris* NIES-1269, respectively. Lipid productivity was found to be highest in *D. maximus* CN06 at 3.43 mg/L.day which majorly consisted of hexadecanoic (C16:0) and oleic (C18:1) acids. The extracted fatty acids indicated good potential to be applied for biodiesel production. This study has shown that cultivation of isolated microalgae strain in municipal wastewater is beneficial for high nutrient removals and lipid accumulation.

1. Introduction

The worldwide industrialization and urbanization have led to increasing generation of wastewater in many sectors, including manufacturing industries, agricultural businesses and domestic activities (Nagarajan et al., 2020). Release of excessive amount of nutrients from wastewater into water bodies has resulted in eutrophication and deteriorated water quality in the environment (Li et al., 2019). Currently, municipal and domestic wastewater are usually treated using conventional activated sludge system, which possesses several limitations such as high energy requirement and large production of sludge wastes (Nancharaiah and Kiran Kumar Reddy, 2017). Wastewater that are typically rich in nutrients (i.e. nitrogen and phosphorus) are recently considered as potential bioresources that may be utilized to shift perception

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towards the concept of circular bioeconomy (Heidrich et al., 2011). For instance, although nitrogen is abundantly available in atmosphere, the production of fertilizer is an energy-intensive process, whereby chemical synthesis of ammonia require total energy consumption of 78 kJ/kg (Gellings and Parmenter, 2016). On the other hand, phosphorus is a finite resource with increasing markets, whereby the synthesis production of phosphate fertilizer may require up to 230 kJ/kg energy. Thus, recycle of phosphorus from wastewater is a promising alternative to achieve sustainable practice and meet the increasing phosphorus demands (Cooper et al., 2011; Soltangheisi et al., 2019). Therefore, there are growing interests in utilizing abundantly produced wastewater as the substrate in microalgae cultivation, whereby the system is able to simultaneously remove organic and inorganic pollutants while achieving high microalgae growth (Mohd Udaiyappan et al., 2017).

Microalgae cultivation in wastewater exhibited many advantages, such as lower capital and operational costs as well as significantly reduced energy requirements compared to the conventional wastewater treatment system (Acién et al., 2016; Whitton et al., 2015). Moreover, microalgae-based wastewater treatment system demonstrated high nutrient removal efficiency in treating different types of wastewaters, such as poultry, soybean processing and domestic wastewaters (Chen et al., 2020; Ling et al., 2019; Okpozu et al., 2019; Shen et al., 2020). The diversity in microalgae species allows for various cultivation conditions, such as in acidic or alkaline pH, higher or lower temperature and high salinity, which may be useful for cultivation in industrial wastewater with complex characteristics (Nagarajan et al., 2020). Furthermore, microalgae biomass cultivated in wastewater may be further utilized for numerous applications, including biofuel or biodiesel production and as an alternative for animal and aquaculture feed (Raheem et al., 2015). During cultivation of microalgal biomass in commercialized media, the costs associated with the media preparation as well as harvesting and downstream processes may hinder its application for the biofuel and biodiesel production (Huang et al., 2010). For instance, cultivation cost of microalgae in commercialized media may reach up to $200 per 1 kg biomass and it was reported that 3 tons of water, 0.33 kg of nitrogen and 0.71 kg of phosphate are required to successfully produced 1 kg of biodiesel (Ahmad et al., 2022; Huang et al., 2022). This issue could be addressed by cultivation of microalgae species using wastewater, whereby the costs of media preparation may be eliminated. Thus, cultivation of microalgae in wastewater is a promising field, prompting more investigations, especially in finding the most suitable microalgae species for a certain type of wastewater.

During microalgae cultivation using wastewater, selection and optimization of suitable microalgae strains are important to address the bottlenecks of large-scale cultivation. Previous studies have extensively researched on screening and isolation of new microalgae species for efficient cultivation and excellent nutrient removal efficiencies (Shen et al., 2020; Ye et al., 2020). A study by Ye et al. (2020) reported the isolation of five *Scenedesmus* spp. and three *Desmodesmus* spp. from Hexi Corridor region, China. The isolated strains demonstrated high growth rate in synthetic wastewater containing high total organic carbon (875 mg/L) and total nitrogen (52 mg/L). Chen et al. (2020) utilized a locally isolated *Desmodesmus* sp. from piggery wastewater in Fujian, China, whereby the respective strain was able to treat piggery wastewater and exhibited self-flocculating properties. In addition, Ling et al. (2019) argued that by utilizing locally isolated microalgae strain, the microalgae biomass productivity and wastewater removal efficiency were significantly improved.

Therefore, this study aims to (i) demonstrate the cultivation of isolated microalgae strains of *Acutodesmus obliquus* CN01 and *Desmodesmus maximus* CN06 in actual municipal wastewater, which has not been reported previously. It was expected that the isolated species could outperform *Chlorella vulgaris* NIES-1269, which is a widely known species for microalgae-based wastewater treatment process (Min et al., 2011; Tejido-Nuñez et al., 2020). Furthermore, this study also aims to (ii) reveal the ability of these strains to accumulate lipid followed by (iii) characterization of extracted fatty acids from microalgae cells for prospective applications, including for biodiesel and biofuel productions.

2. Materials and methods

2.1. Microalgae species and wastewater sample

In this study, a total of three microalgae strains, namely *A. obliquus* CN01, *D. maximus* CN06 and *C. vulgaris* NIES-1269 were utilized. The first two species were isolated from Hulu Langat River, Selangor, Malaysia by Yahya et al. (2018). Meanwhile, *C. vulgaris* NIES-1269 was obtained from the National Institute of Environmental Science (NIES), Japan, and was used as a control species due to its renowned ability to treat wastewater. All microalgae species were continuously grown using AF6 medium prior to be used in the experimental procedures. The municipal wastewater sample was collected from the grit chamber in Pantai 1 Sewage Treatment Plant (STP), Kuala Lumpur, Malaysia. These samples were filtered and autoclaved (HV-85 II, Hirayama, Japan) in order to remove any suspended solids and possible bacterial contamination. The samples were kept at 4 °C until further experiments. The characteristics of autoclaved wastewater samples are summarized in Table 1.

2.2. Experimental set-up and conditions

The collected municipal wastewater samples were used as a substrate during the cultivation of selected microalgae species. Each microalgae species was subjected for cultivation in AF6 medium and municipal wastewater for comparison

Table 1
Characteristics of autoclaved municipal wastewater samples and AF6 medium used as the substrate for microalgae cultivation process.

Parameters	Autoclaved wastewater (mg/L)	AF6 medium (mg/L)
COD	130 ± 44	33
Total nitrogen (TN)	29.2 ± 5	47
Total phosphorus (TP)	4.8 ± 0.3	6.2
NH_3–N	14.9 ± 1.5	0
NO_2^-–N	3 ± 1.7	34
NO_3^-–N	13.3 ± 5.5	13
pH	7.2 ± 0.5	6.6

between controlled and experimental conditions, respectively. *A. obliquus* CN01, *D. maximus* CN06. and *C. vulgaris* NIES-1269 cultivated in AF6 medium were labeled as R_{1-c}, R_{2-c} and R_{3-c}, accordingly. Meanwhile, *A. obliquus* CN01, *D. maximus* CN06 and *C. vulgaris* NIES-1269 grown in wastewater were named as R_1, R_2 and R_3, respectively. All microalgae culture were inoculated at the same initial inoculation density of 50 mg/L in 300 mL medium. Continuous aeration with 0.1 L/min flowrate was provided along with 24 h illumination using white fluorescent lamp with light intensity of 80 μmol/m^{2}s. The cultivation was conducted in room temperature (i.e., 24 ± 2 °C) for a total of 12 days experimental period. Samples were collected every two days for the analyses of microalgae growth and nutrient removal efficiencies.

2.3. Analytical methods

2.3.1. Determination of microalgae growth

Microalgal growth was analyzed based on the optical density (OD) at 750 nm and dry cell weight concentration. Every two days, 1 mL microalgae culture was analyzed using a UV–Vis spectrophotometer (UV-1900, Shimadzu, Japan) for measurement of OD_{750nm}. The specific growth rate of microalgae species was determined according to Eq. (1), whereby X_1 and X_2 indicate absorbance value at t_1 and t_2, respectively. Concurrently, 10 mL of microalgae culture was filtered through a pre-weighed GF/A grade filter paper (M_1) using a vacuum filtration set. Filter paper containing the retentate was dried at 105 °C for 1 h, and let cool in a desiccator. The weight of dried sample and filter paper was recorded as M_2, and the dry cell weight was calculated using Eq. (2).

$$\text{Specific growth rate }\left(\text{day}^{-1}\right) = \frac{\ln\left(X_2/X_1\right)}{t_2 - t_1} \tag{1}$$

$$\text{Dry cell weight (mg/L)} = \frac{M_2 - M_1}{V} \tag{2}$$

2.3.2. Nutrient removal efficiency

A 10 mL microalgae culture was filtered using a glass microfiber (GF/A grade) filter paper, and the filtrate was analyzed for the characteristics. Analyses of wastewater included the chemical oxygen demand (COD), total nitrogen (TN), ammoniacal nitrogen (NH_3–N) and total phosphorus (TP) concentrations. All analyses were conducted according to the Standard Method for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012). COD concentration was measured using the potassium dichromate method (standard method 5220-D), while analyses of TN and NH_3–N were conducted using the persulfate digestion (standard method 4500-N) and the salicylate methods (standard method 4500-NH_3). In addition, the molybdovanadate and persulfate digestions method (standard method 4500-P) were applied for analysis of TP concentration.

2.3.3. Total lipid and fatty acid extraction

Total lipid extraction was conducted according to modified Folch's method (Folch et al., 1957), whereby 50 mL microalgae culture was harvested by centrifugation at 10,000 × g for 5 min. The supernatant was discarded and pelleted microalgae cells were stored at −80 °C. The stored microalgae pellets were then lyophilized using a freeze dryer (DC41B, Yamato, Japan) overnight and the lyophilized biomass was weighed and re-suspended in 6 mL ratio of methanol:chloroform mixture (i.e., 1:2 v/v). A 100 μl C17 fatty acid was added as an internal standard with the concentration of 500 μg/mL, and the solution was thoroughly mixed using a vortex mixer. The solution was centrifuged at 10,000 × g for 10 min, and the supernatant was transferred to a new glass tube. The supernatant was then mixed with 1.25 mL of 0.1 M KCl and centrifuged at 10,000 × g for 10 min. The bottom layer was extracted and inserted into a rotary evaporator (VC-15 Taitec, Japan) for half to one hour. The weight of dried lipid was recorded accordingly.

Extraction of fatty acid methyl ester (FAME) was performed according to Kotajima et al. (2014). The extracted lipid was mixed with 4 mL ratio of 0.6 N methanol:HCl, and the mixture was kept in a 100 °C water bath (Isotemp GPD20, Fisher, United States) for 1 h. Once the sample is cooled to room temperature, 4 mL of hexane was added, and the upper layer was extracted. The remaining layer was re-extracted with 2 mL hexane and 2 mL distilled water, and the formed upper layer was then extracted. All solutions were inserted into a rotary evaporator for 2 h. Lastly, 300 μL of hexane was added, and the sample was filtered using a 0.22 µm hydrophilic filter. The filtrate was stored at −80 °C until further analyses. The extracted fatty acid was analyzed using gas chromatograph with flame-ionization detector (GC-FID) [GC-2030, Shimadzu, Japan] using an RT-2560 column (Shimadzu, Japan). The carrier gas used was helium with split injection mode at 50 split ratio. The initial temperature was 140 °C and held for 2 min, which was then increased to 225 °C with a ramping rate of 4 °C/min and held for 7 min. The temperature was then further increased to 240 °C with a ramping rate of 2 °C/min and held again for 10 min (Othman et al., 2019). The FAME content was calculated according to the peak area of the total FAME chromatogram. In addition, biodiesel properties of the extracted fatty acid were estimated using Biodiesel Analyzer© Ver 2.2 (retrieved from http://www.brteam.ir/biodieselanalyzer on June 17, 2021).

2.3.4. Statistical analysis

The data showed are presented as mean ± standard deviation and analyzed using One-way analysis of variance (ANOVA) with significance level set at $P < 0.05$ to identify the significant difference between sets of data. The analyses were conducted using Microsoft Excel 365 and IBM SPSS software.

3. Results and discussion

3.1. Microalgae growth

The biomass growth after 12 days of experimental period was depicted in Fig. 1. The highest to lowest biomass accumulation were R_{2-c}, R_2, R_1, R_{1-c}, R_{3-c} and R_3. It was observed that *A. obliquus* CN01 cultivated in wastewater (R_1) could outgrown the cultivation in AF6 medium (R_{1-c}) as indicated by the dry cell weight concentration and the specific growth rate. However, *D. maximus* CN06 grown in municipal wastewater (R_2) achieved slightly lower growth rate at 0.23/day as compared to cultivation in AF6 medium (R_{2-c}) with growth rate of 0.28/day. In addition, cultivation of *C. vulgaris* NIES-1269 in both AF6 (R_{3-c}) and wastewater (R_3) exhibited a lower growth rate and biomass accumulation at less than 500 mg/L after 12 days of cultivation period. The results highlighted that the isolated species achieved significantly better biomass accumulation during cultivation in wastewater, especially when compared to the commercial *C. vulgaris* NIES-1269. Moreover, it can be concluded that the collected municipal wastewater contained sufficient nutrients, i.e., Carbon, Nitrogen and Phosphorus, which are essential for microalgae growth (Ferreira et al., 2019).

The *D. maximus* CN06 in AF6 medium (R_{2-c}) was observed to have highest biomass accumulation at 785 mg/L, followed by *D. maximus* CN06 in wastewater (R_2) at 705 mg/L. Nevertheless, the statistic analysis showed no significant difference in terms of the biomass accumulation of *D. maximus* CN06 in both AF6 medium and wastewater ($P > 0.05$). Meanwhile, biomass accumulation of *A. obliquus* CN01 in municipal wastewater (R_1) was reported at 617.5 mg/L which was higher than during cultivation in AF6 medium (R_{1-c}) with biomass accumulation of 540 mg/L. A similar results were obtained from the statistic analysis, whereby no significant difference were observed between R_1 and R_{1-c} ($P > 0.05$). Thus, the results support the initial hypothesis that the isolated strains have the ability to be cultivated in municipal wastewater. The comparison of microalgal growth between *A. obliquus* CN01 (R_1) and *D. maximus* CN06 (R_2) in wastewater highlighted that *D. maximus* achieved significantly higher growth rate and biomass accumulation.

The isolation of *A. obliquus* and *D. maximus* have been reported from various environmental sample, including industrial wastewater, freshwater and soil samples (Chen et al., 2020; Lee et al., 2021; Ling et al., 2019). The green algae, or the phylum Chlorophyta have been reported to have the ability to tolerate high concentrations of organic and inorganic pollutants, such as heavy metals and pharmaceutical compounds (García et al., 2018; Mohd Udaiyappan et al., 2017). Nevertheless, the utilization of the isolated strains in this study for cultivation in municipal wastewater has not been demonstrated. Utilization of municipal wastewater for microalgae cultivation is a promising field since municipal wastewater readily available throughout the year and municipal wastewater in this study allows for direct utilization without dilution process. However, various factors may influence the growth of microalgae in wastewater, for instance nitrogen to phosphorus (N/P) ratio which directly correlates with the nutrient uptake in microalgal metabolism (Beuckels et al., 2015). The N/P ratio of AF6 medium used as the control medium was 7.5 with TN and TP concentrations of 47 mg/L and 6.2 mg/L, respectively. Meanwhile, N/P ratio in municipal wastewater sample was 5.1 with lower TN and TP concentrations as shown in Table 1. Therefore, the overall lower biomass growth during cultivation in municipal wastewater may be influenced by lower TN and TP concentrations in municipal wastewater. This was in agreement with previous study by Ling et al. (2019), whereby a lower growth rate of *Scenedesmus obliquus* was reported at lower NH_3–N concentration in modified BG-11 medium.

In terms of growth rate, the highest growth rate during microalgae cultivation using municipal wastewater was observed in *D. maximus* CN06 which is consistent with the report of Ye et al. (2020), whereby *Desmodesmus* sp. exhibited higher biomass concentration as compared to *Scenedesmus* sp. during cultivation in synthetic wastewater. A study by Hernández-García et al. (2019) also reported higher growth of *Desmodesmus* sp., reaching 1.95 g/L as compared to *S. obliquus* when cultivated using leachate as substrate. *Desmodesmus* sp. has also been reported to have high self-flocculating ability and exhibited high biomass concentration at 1.76 g/L when cultivated in undiluted piggery wastewater (Chen et al., 2020). The ability of *D. maximus* to survive in different types of wastewater may be influenced by the structure of its cell wall with a thick inner polysaccharide layer (Gorelova et al., 2015). The thick cell wall in *D. maximus* CN06 play important roles for microalgae growth during stress condition, specifically when harmful substances present in the substrate. However, another study has demonstrated that a large portion of genetic and metabolic aspects of *Desmodesmus* genus remains unidentified and require extensive research and studies to reveal any hidden potential application of this genus (Neofotis et al., 2016).

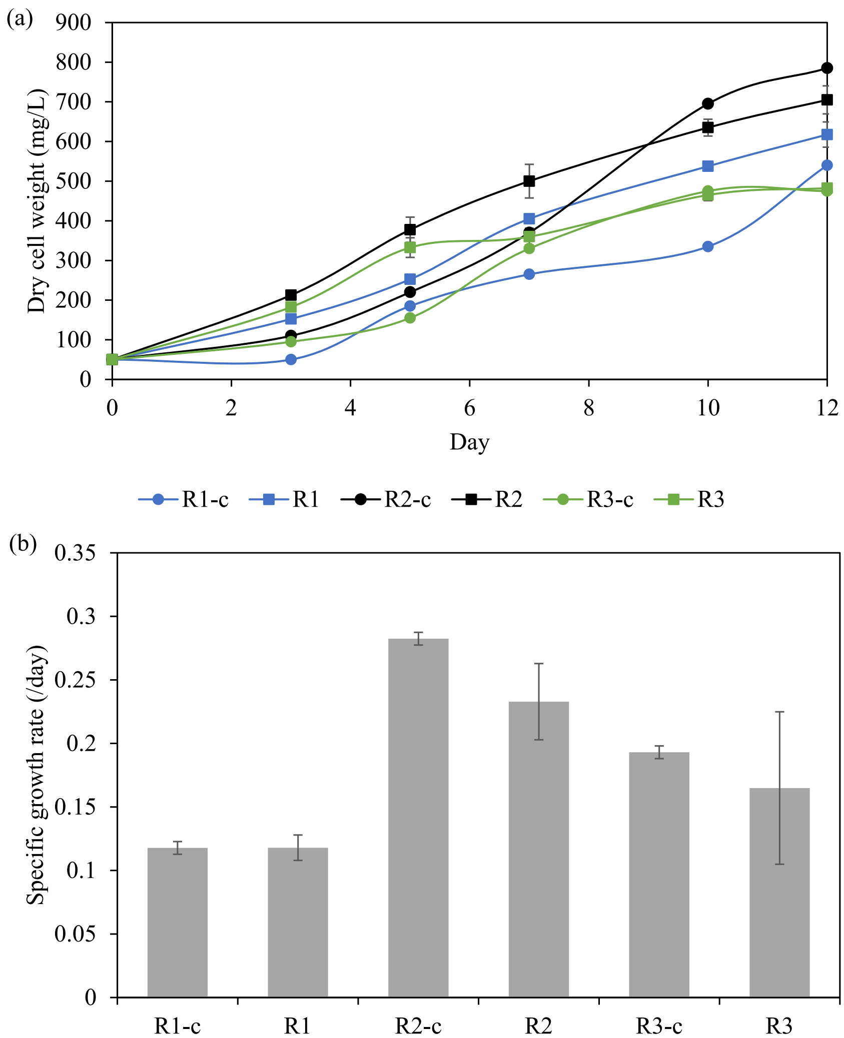

Fig. 1. Growth of microalgae. (a) dry cell weight and (b) specific growth rate of all microalgae species. R_1: *A. obliquus* CN01, R_2: *D. maximus* CN06. R_3: *C. vulgaris* NIES-1269. -c: culture in AF-6, without -c: culture in wastewater. Each data represents mean ± standard deviation.

3.2. Nutrient removal efficiencies

Observation of nutrient removals was conducted based on the nitrogen and phosphorus removal efficiencies as shown in Fig. 2. The highest total nitrogen removal efficiency of 96% was observed in *A. obliquus* CN01, followed by *D. maximus* CN06 at 91% and lastly *C. vulgaris* NIES-1269 at 90% removal efficiencies. Moreover, complete NH_3–N removal was observed in *A. obliquus* CN01 after 10 days of cultivation period. Meanwhile, *D. maximus* CN06 and *C. vulgaris* NIES-1269 exhibited a lower NH_3–N removal efficiency at approximately 78%. Microalgae has the ability to directly assimilate ammonia in wastewater, whereby ammonium is incorporated into amino acid glutamine by using glutamate and ATP (Liu et al., 2017). Oxidized nitrogen forms, including nitrate and nitrite are converted into ammonium, for instance nitrate is reduced into nitrite by the enzyme nitrate reductase, followed by nitrite conversion into ammonium by the enzyme nitrite reductase, respectively (Strohm et al., 2007). Therefore, all inorganic nitrogen forms are converted into ammonium in microalgal cells. The complete removal of NH_3-N by *A. obliquus* CN01 indicated the high ability of these isolated species to assimilate ammonium into the cells (Hernández-García et al., 2019). Although highest nitrogen removal was observed in *A. obliquus* CN01, the biomass accumulation was observed to be higher in *D. maximus* CN06 during cultivation in municipal wastewater. These results indicated that *D. maximus* CN06 may take up the other forms of nitrogen, such as NO_3-N and NO_2-N that were highly abundance in wastewater for their cell metabolism (Ji et al., 2014). Nevertheless, this study demonstrated an excellent nitrogen removal from municipal wastewater while simultaneously produced high biomass concentration which comparable with previous reports. Although highest nitrogen removal was exhibited by *A. obliquus* CN01, no significant difference ($P > 0.05$) was detected when compared to the other two species, indicating the suitability of all microalgae species in removing nitrogen from municipal wastewater.

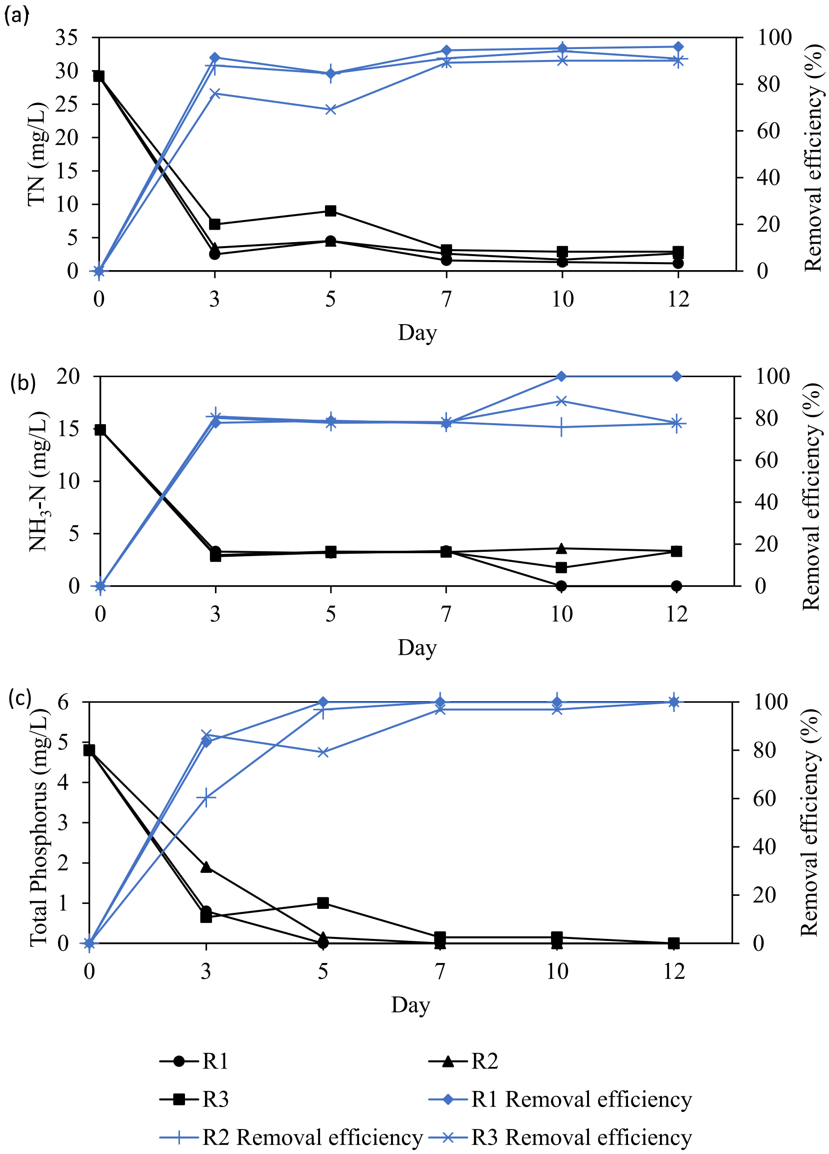

Fig. 2. Removal efficiencies of (a) TN, (b) NH_3–N and (c) TP by *A. obliquus* CN01 (R_1), *D. maximus* CN06. (R_2) and *C. vulgaris* NIES-1269 (R_3) which demonstrated excellent nitrogen removals and complete TP removal by all species.

A. obliquus was able to treat various types of wastewater, including piggery wastewater, soybean processing effluent, leachate and municipal wastewater (Chen et al., 2020; García et al., 2018; Kim et al., 2016). A significant reduction of NH_3–N concentration in saline piggery wastewater was demonstrated using *A. obliquus*, whereby the NH_3–N concentration was decreased from 644 mg/L to 299 mg/L (Kim et al., 2016). However, during treatment of piggery wastewater using two microalgae species, *C. minutissima* and *A. obliquus*, it was reported that by the end of the experiment, *Chlorella* sp. was highest in abundance and take over the initial inoculated species, thus, confirming a high tolerance of *Chlorella* sp. towards high organic and inorganic pollutants in piggery wastewater. Meanwhile, treatment of soybean processing wastewater by *S. obliquus* was able to achieve 95% total nitrogen removal efficiency while simultaneously promote high lipid accumulation by applying nitrogen deficiency stage towards the end of the cultivation period (Shen et al., 2020). An optimized treatment of leachate mixed with municipal wastewater was achieved using *S. obliquus* and *Desmodesmus* spp., whereby 81% NH_3–N removal efficiency was reported in 7% leachate ratio. Higher leachate ratio resulted in decreasing biomass growth and nutrient removal efficiencies.

The analyses of phosphorus removal efficiency highlighted that complete phosphorus removal was achieved in all three microalgae species. However, the fastest phosphorus uptake was demonstrated by *A. obliquus* CN01 at 3.8 mg P/gDCW·day followed by *D. maximus* CN06 and *C. vulgaris* NIES-1269 at 1.37 mg P/gDCW·day and 0.82 mg P/gDCW·day, respectively.

Table 2
Summary of lipid content, production and productivity of selected species after cultivation in AF6 and wastewater. Each data represent mean ± standard deviation.

Sample	Lipid content (%)	Lipid production (mg/L)	Lipid productivity (mg/L·d)
R_{1-c} (*A. obliquus* CN01 in AF6)	31.45 ± 1.66	16.98 ± 1.75	1.42 ± 0.31
R_1 (*A. obliquus* CN01 in wastewater)	26.3 ± 1.70	16.632 ± 2.53	1.39 ± 0.34
R_{2-c} (*D. maximus* CN06 in AF6)	28.62 ± 2.37	22.47 ± 2.62	1.87 ± 0.19
R_2 (*D. maximus* CN06 in wastewater)	57.82 ± 1.29	41.115 ± 3.36	3.43 ± 0.28
R_{3-c} (*C. vulgaris* NIES-1269 in AF6)	15 ± 2.37	7.125 ± 1.30	0.59 ± 0.23
R_3 (*C. vulgaris* NIES-1269 in wastewater)	46.38 ± 2.20	22.415 ± 2.19	1.87 ± 0.22

It was highlighted that high phosphorus removal in microalgae-based wastewater treatment system is achieved through phosphorylation process, in which inorganic phosphate is incorporated into cellular organic compounds such as DNA, RNA and lipids (Cai et al., 2013). Moreover, several microalgae species are also capable to accumulate polyphosphate (polyP) and store it as intracellular polyphosphate (Schmidt et al., 2016). The ability of microalgae species to accumulate polyphosphate supports the biomass growth during P starvation as demonstrated by previous study whereby 100% phosphorus removal was reported during cultivation of *Desmodesmus* sp. EJ9-6 in anaerobic digestion wastewater (Ji et al., 2014). Previous reports also highlighted municipal wastewater treatment using different microalgae species such as *S. obliquus* and *Desmodesmus* sp. and demonstrated excellent phosphorus removal averaging at more than 90% removal efficiencies. Moreover, indoor cultivation of *S. obliquus* using municipal wastewater reached 90%–93% phosphorus removal efficiency (Ling et al., 2019). However, it was reported that during outdoor semi-continuous cultivation using municipal wastewater, the phosphorus removal efficiency slightly decreased to 70%.

3.3. Lipid production

Lipid production from microalgae-based wastewater treatment system is gaining popularity over the last decade as it can minimize the production cost compared to the cultivation in synthetic medium (Ferreira et al., 2019). It was reported that cultivation of microalgae using synthetic medium required \$6.95/L biomass as compared to the \$3.9/kg biomass of *Chlorella* sp. cultivated in poultry wastewater (Richardson et al., 2012). The summary of lipid content, production and productivity in this study were listed in Table 2. After 12 days of cultivation period, the highest lipid content was accumulated in R_2 at 57% followed by R_3 at 46%. Subsequently, the highest lipid productivity was observed in R_2 at 3.43 mg/L.d followed by R_3 and R_{2-c}at 1.87 mg/L.d. Fig. 3 showed the comparison on the lipid content, production and productivity of each species during cultivation in AF6 medium and municipal wastewater, whereby different lowercase letter indicated a significant difference while a same lowercase letter assigned to the data indicated that there is no significant difference. The results highlighted that cultivation using municipal wastewater significantly impacted the lipid production in *D. maximus* CN06 and *C. vulgaris* NIES-1269 in a positive manner ($P < 0.05$). Meanwhile, for *A. obliquus* CN01, no significant difference ($P > 0.05$) was observed for lipid accumulation after cultivation in AF6 medium and municipal wastewater. The observations revealed a significantly higher lipid accumulation by *D. maximus* CN06 cultivated in municipal wastewater (R_2).

It has been reported that nutrient depletion may induce lipid accumulation in microalgae species (Ling et al., 2019). The nitrogen and phosphorus contents in municipal wastewater used as the substrate was found to be lower than the nitrogen and phosphorus concentrations in AF6 medium. Moreover, the high total nitrogen and phosphorus removal efficiencies have subsequently induced nutrient depletion stage from day 5–7 that lead to lipid accumulation in the microalgae species. It was argued that microalgae cultivation is divided into two stages, namely the nutrient sufficient stage and the nutrient depletion stage, in which during the nutrient sufficient stage, microalgae biomass increased rapidly, while during the nutrient deficiency stage, lipid accumulation increased significantly (Shen et al., 2020). The highest biomass growth and lipid accumulation for cultivation in municipal wastewater was achieved in *D. maximus* CN06, whereby there was 50% increase in lipid content and 45% increase in lipid productivity as compared to the cultivation using AF6 medium. The increase of lipid content in *D. maximus* cultivated in wastewater was also observed by Hernández-García et al. (2019) by using a mixture of wastewater and leachate.

The lipid accumulation in this study was in agreement to a study conducted by Arbib et al. (2014) and Cabanelas et al. (2013) achieving up to 27% lipid accumulation during cultivation of *C. vulgaris* in municipal wastewater. The *D. maximus* CN06 cultivated in wastewater (R_2) was also able to accumulate higher lipid content compared to a report by Diniz et al. (2017) and Ye et al. (2020) with lipid accumulation ranging from 10% to 16%. Moreover, the results of this study have demonstrated higher lipid accumulation by *D. maximus* CN06 as compared to the cultivation of the same isolated strain using AF6 medium added with azide (Yahya et al., 2018). Nevertheless, lipid accumulation in *A. obliquus* CN01 was lower compared to previous studies using optimized synthetic medium for lipid accumulation (Othman et al., 2019; Yahya et al., 2018). Therefore, it may be concluded that *D. maximus* CN06 has highest ability of lipid production during cultivation using municipal wastewater as previously shown in Table 2.

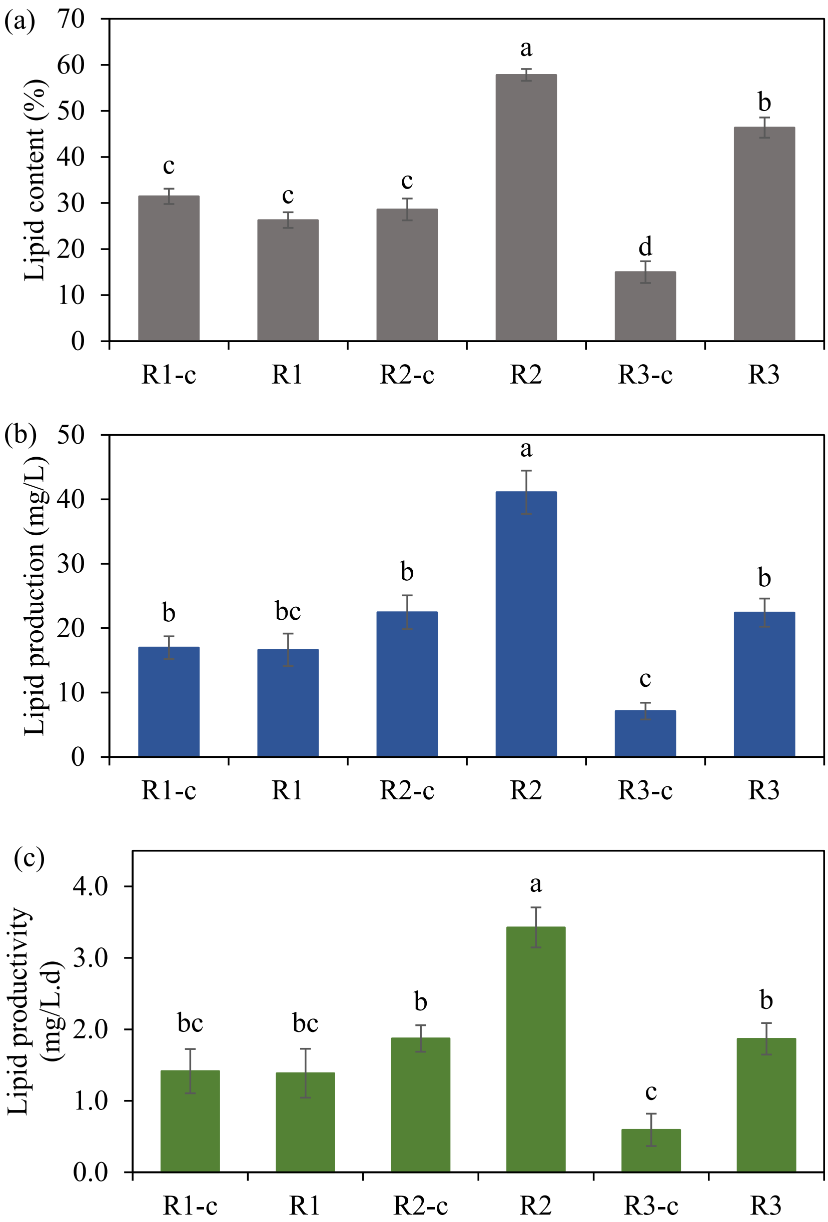

Fig. 3. Comparison of (a) lipid content, (b) lipid production and (c) lipid productivity of selected species. R_1: *A. obliquus* CN01, R_2: *D. maximus* CN06. R_3: *C. vulgaris* NIES-1269. -c: culture in AF-6, without -c: culture in wastewater. Each data represent mean ± standard deviation and different lowercase letter indicate significant difference (P < 0.05).

3.4. FAME composition

Fig. 4 depicted the composition of different types of FAME, including saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) obtained in this study. The cultivation of *A. obliquus* CN01 in municipal wastewater resulted in significant increase of SFA and MUFA while decreasing the PUFA in overall fatty acid composition. It was found that the longer chain SFA such as heneicosanoic acid (C21:0) was increased in R_1 whereby *A. obliquus* CN01 were cultivated in municipal wastewater. In addition, production of oleic acid (C18:1) was increased significantly. Similar results were found in *C. vulgaris* NIES-1269, whereby the production of oleic acid (C18:1) was significantly higher in R_3 as compared to R_{3-c}, resulting in significant increase of MUFA percentage during cultivation in municipal wastewater. Meanwhile, cultivation of *D. maximus* CN06 in municipal wastewater has significantly decreased the production of MUFA and PUFA while simultaneously increase the production of SFA, such as hexadecenoic acid (C16:0) and octadecanoic acid

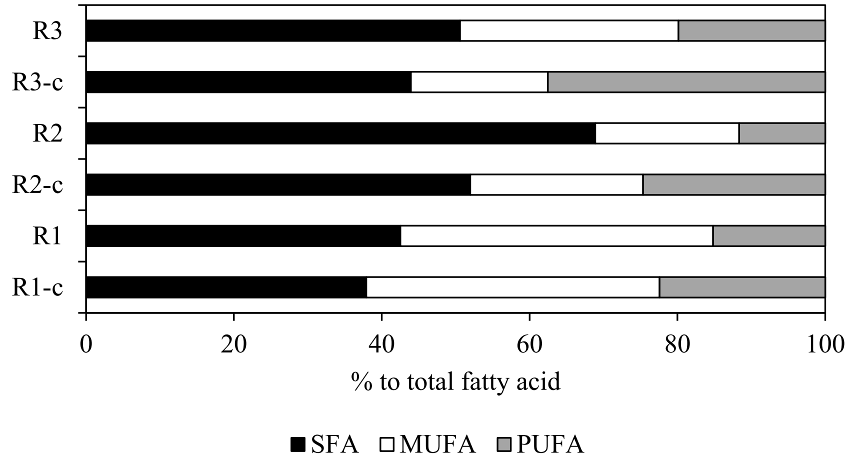

Fig. 4. Percentage of different types of FAMEs indicating compositional change of fatty acids during cultivation of selected species in municipal wastewater. R_1: *A. obliquus* CN01, R_2: *D. maximus* CN06. R_3: *C. vulgaris* NIES-1269. -c: culture in AF-6, without -c: culture in wastewater.

Table 3
Properties of biodiesel of *D. maximus* CN06 cultivated in municipal wastewater (R_2) as compared with previous studies according to vehicular biodiesel standard of ASTM D6751 and EN 14214.

Properties	ASTM D6751	EN 14214	R_2 (*D. maximus* CN06 in wastewater)	Okpozu et al. (2019)	Chen et al. (2020)
Cetane value	47 (min)	51 (min)	63	75	53
Kinematic viscosity (mm^2/s)	1.9–6.0	3.5–5.0	3.7	3.8	4.35
Cloud point (°C)	[a]	≤5	4	−12.5	1.96
Iodine values ($gI_2/100$ g)	[a]	120 (max)	42	57	113.1

[a]Not specified for designated properties.

(C18:0). Hexadecanoic acid (C16:0) was found to be the dominant fatty acid in R_2 accounted for 56% of the total FAMEs, followed by oleic acid (C18:1) at 18%.

Oleic acid (C18:1) play important roles in biodiesel properties from microalgae biomass (Kaur et al., 2012). A high proportion of this MUFA may lead to excellent cold start performance of an engine (Shen et al., 2020). Moreover, in terms of production and storage, oleic acid exhibited better low-temperature performance (Chen et al., 2020). The biodiesel properties of FAMEs from *D. maximus* CN06 cultivated in wastewater (R_2) exhibited high cetane number of 63, which was comparable with cetane number of biodiesels from other types of biomass (Okpozu et al., 2019; Piloto-Rodríguez et al., 2013). The kinematic viscosity, cloud point and iodine values of FAMEs from R_2 are summarized in Table 3. These values have fulfilled the authoritative standard by American Society of Testing Materials (ASTM D6751) and European Standard EN 14214, indicating that *D. maximus* CN06 cultivated in municipal wastewater have high potential to be used in biodiesel production. In terms of health benefits, oleic acid may be used to prevent cardiovascular, autoimmune and metabolic diseases, as well as controlling growth of cancer cells (Pozzobon et al., 2020). Meanwhile, linoleic acid (C18:2) exhibited valuable health properties in preventing cardiovascular diseases, metabolic syndrome and type 2 diabetes (Marangoni et al., 2020). Therefore, cultivation of *D. maximus* CN06 in municipal wastewater serves as promising alternatives for production of many valuable compounds, taking into account the strict control of potential contamination and other ethical issues, especially when the products were used for human consumption, e.g., health supplement.

4. Practical applications and future research prospects

This study has presented the application of locally isolated microalgae species, namely *A. obliquus* CN01 and *D. maximus* CN06 for the cultivation in actual municipal wastewater, which successfully outgrown the commercial *C. vulgaris* NIES-1269 in terms of the growth rate and nutrient removal efficiencies. Utilization of locally isolated strains for cultivation in wastewater is beneficial for cost-effective microalgae cultivation process. In addition, the ability of microalgae cells to accumulate lipid was achieved during cultivation in municipal wastewater, thus, allowing sustainable resource recovery processes. Future research may focuses on the upscaling process for cultivation of isolated microalgae species in wastewater, by implementing suitable design of photobioreactor in laboratory and pilot scales. Moreover, sustainability assessment, e.g. life cycle assessment (LCA), may be conducted to ensure the long-term application of the cultivation process.

5. Conclusions

The highest specific growth rate of 0.23/day was demonstrated by isolated strain *D. maximus* CN06. In addition, up to 96% of total nitrogen was removed using all isolated microalgae strains with complete total phosphorus removal. High lipid productivity of 3.43 mg/L·day was also exhibited by *D. maximus* CN06, whereby the extracted fatty acids have high potential to be used as biodiesel in accordance to the requirements by the US and European standards. This study has demonstrated successful cultivation of isolated microalgae strains using locally sourced wastewater that simultaneously enhanced nutrient removal efficiencies and lipid productions by the selected strains.

CRediT authorship contribution statement

Laila Dina Amalia Purba: Conceptualization, Investigation, Resources, Writing – original draft. **Fatin Syahirah Othman:** Validation. **Ali Yuzir:** Writing – review and editing, Supervision, Funding acquisition. **Shaza Eva Mohamad:** Writing – review & editing. **Koji Iwamoto:** Writing – review & editing. **Norhayati Abdullah:** Supervision, Writing – review & editing. **Kazuya Shimizu:** Writing – review & editing. **Joni Hermana:** Writing – review & editing.

Declaration of competing interest

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

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