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Cultivation of Oleaginous Microalgae *Scenedesmus obliquus* on Secondary Treated Municipal Wastewater as Growth Medium for Biodiesel Production

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ABSTRACT

Local single cell microalgae isolated from a wastewater swamp and identified as *Scenedesmus obliquus* was used to determine its applicability for utilization of domestic wastewater for biomass and lipid production. Secondary treated domestic wastewater with or without mixing of growth medium was used to cultivate *S. obliquus* for the biomass and lipid production as a renewable feedstock for biodiesel. *S. obliquus* showed the highest OD when grown in 100% Bold's basal medium (BBM). *S. obliquus* utilized 95.2% and 78.5% of P and N contents, respectively, when grown in 25% WW+75% BBM mixture and the utilization efficiency of both elements decreased with the increasing wastewater portion in the mixture. Although the BBM displayed the highest dry biomass and lipid production (25.15% of the cell dry biomass). The lowest values were recorded for the uninoculated wastewater, followed by 100% wastewater enriched with *S. obliquus.* The obtained data revealed that the lipid classes of *S. obliquus* differs according to the cultivation medium and conditions. The highest percentage of C16-C18 fatty acids (54.76% from total lipids) were recorded in case of algae cultivated in 100% wastewater, followed by 46.96% in case of 100% BBM medium. These results suggest the utilization of mixtures containing a higher portion of secondary treated wastewater, such as 75% WW+25% BBM or 50% WW+50% BBM, could increase the economical production of the lipid-rich microalgae *S. obliquus* for biodiesel through saving water and nutrients.

Keywords: biodiesel; microalgae; *Scenedesmus*; municipal wastewater; biomass; oil contents; fatty acid compositions

INTRODUCTION

An acceptable alternative fuel should be readily available, competitive with fossil fuels, and environmentally accepted (Meher et al., 2006). Biodiesel has been the leading unconventional liquid biofuel for the past two decades (Meng et al., 2009). Additionally, most of the current commercially available biofuels are crop-based and classified as "first generation biofuels" which compete for arable land and freshwater (Maity et al., 2014). Microbial lipids are highly accumulated, with a ratio of more than 20% of their cell mass, by fast grown oleaginous microorganisms, such as microalgae and fungi, which are known as single cell oils (SCOs). SCOs are considered to be one of the most promising sources for biodiesel production (Meng et al., 2009). Microalgae represent an auspicious renewable alternative third-generation sustainable bioenergy source. It can efficiently produce huge amounts of biomass (7–20 times greater than soy or corn per land unit) with high lipid content (Maity et al., 2014; Bharathiraja et al., 2015; Lyon et al., 2015).

Implementing various microalgae as a sustainable feedstock for biodiesel production was introduced by Ahmed et al. (2011). They stated that several algal groups, including members of *Diatoms*, *Green algae*, and *Red algae* from both marine and freshwater sources, were reported to be suitable for utilization in the biodiesel production, according to their high biomass productivity and their lipid content. Among the microalgae groups, *Scenedesmus* sp. were found to be appro-

priate candidates for biodiesel production based on their high lipid productivity (24.66 mg/L/day), high biomass yield (0.9 g/L), as well as appropriate fatty acid profile (Jena et al., 2012). The accumulated lipids in *S. obliquus* were evaluated by Mandal and Mallick (2009) under several growth conditions.

On the other hand, microalgae can grow under various aquatic environments, such as fresh, brackish, marine, and wastewaters (municipal, industrial, agricultural, and domestic wastewaters), when adequate amounts of required nutrients are present (Mobin & Alam, 2014). Growing microalgae consumes a huge amount of water and inorganic nutritional elements that increase the production cost (Xin et al., 2010). Furthermore, the addition of organic carbon for many microalgal strains prompts their growth (Xu et al., 2006), but increases the biomass cost as well (Li et al., 2007). Many obstacles are faced by the mass production of microalgae, including the harvesting and nutrients cost. The harvesting challenges could be overcome using efficient low-cost methods, such as flocculation (Matter et al., 2016), while the nutrient cost could be reduced by recycling nutrients from wastewaters (Lv et al., 2017). Thus, sustainable and economically acceptable culturing medium for algae-based biodiesel production is still needed (Mandal & Mallick, 2011). Accordingly, several previous studies regarding the use of swine wastes, dairy manure, and other animal residues for the cultivation of microalgae have been carried out (Wilkie & Mulbry, 2002). Cultivation of microalgae in a nutrient-rich effluent as an inexpensive, readily available and cheap medium is expected to overcome the economic dilemma of biodiesel production as well as decrease the environmental problems arising from discharging nutrients into bodies of water. Utilization of wastewater for the cultivation of microalgae has several advantages, including providing an alternative source for the huge amounts water required for growing algae, which presents a supplemental source of nutrients, as well as decreases the load of contaminants, according to Ansari et al. (2017). On the other hand, numerous challenges are faced by the cultivation of microalgae in wastewater, such as the unbalanced N/P ratio, the extraordinary biological pollutants and competitors, low biomass and lipid content production, and the low level of effective nutrient elimination (Xin et al., 2010; Min et al., 2011; Zhang et al., 2012). Additionally, the wastewater treatment methods differ from one country to another, which make the quality of effluents differ as well. Thus, the aim of the current study was to evaluate the suitability of secondary treated municipal wastewater as a cheap growth medium for algal cultivation, lipid content and the composition of the lipid-rich microalgae *Scenedesmus* sp. for biodiesel production under the conditions found in Egypt.

MATERIALS AND METHODS

Wastewater collection

The wastewater used in this study was obtained from the abundant effluent of the Zenen municipal wastewater treatment plant in Giza, Egypt. The municipal wastewater in this plant was subjected to sedimentation and aeration as a secondary treatment step before being discharged into the irrigation canal. The secondary wastewater treatment sample was collected in a clean plastic container and transferred immediately to the lab. The Zenen municipal wastewater plant effluent has the following characteristics: pH, 7.9; COD, 95.8 mgL^{-1}; BOD, 71.5 mgL^{-1}; TSS, 81 mgL^{-1}; TOC, 1.8 mgL^{-1}; TP,1.28 mgL^{-1} and TN, 21.96 mgL^{-1}.

Microalgae isolation, purification and identification

The single-cell green microalgae were isolated from a swamp contaminated with wastewater located in the Gharbia governorate in Egypt. BBM was used for the isolation and maintenance of the microalgal isolate (Pizarro et al., 2006). It contained (per liter) 175 mg KH_2PO_4, 25 mg $CaCl_2.2H_2O$, 75 mg $MgSO_4.7H_2O$, 250 mg $NaNO_3$, 75 mg K_2HPO_4, 25 mg NaCl, 11.42 mg H_3BO_3, 1 mL from Microelement stock solution (which consist of: 8.82 g $ZnSO_4.7H_2O$, 1.44 g $MnCl_2.4H_2O$, 0.71 g MoO_3, 1.57 g, $CuSO_4.5H_2O$ and 0.49 g $Co(NO_3)_2.6H_2O$, per liter), 1 mL from Solution 1 (which consist of: 50 g Na_2EDTA and 3.1 g KOH, per liter) and 1 mL from Solution 2 (which consist of: 4.98 g $FeSO_4$ and 1 mL concentrated H_2SO_4, per liter), and final pH of 6.8.

The algae isolation and purification were performed by serial dilution and culturing on liquid BBM followed by plating on solid BBM. The incubation during the isolation process was

conducted at ambient temperature under continuous illumination with white fluorescent light (at intensity of 2000 Lux). The purity of the culture was ensured by regular observation under light microscope (Zeiss, Oberkochen, Germany). After purification of the microalgal isolate, microscopic examination was performed to identify the genus according to Bellinger and Sigee (2015). The identification of the species level was confirmed using molecular-based techniques.

DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

The total genomic DNA was extracted using a modified enzymatic lyses method (Bellinger & Sigee, 2015). The microalgae cells were harvested and dried using liquid nitrogen before incubation in CTAB buffer at 60°C for 1 h; afterwards, the lysozyme (20 mg/mL) and Proteinase K (1 mg/mL) were applied. The total genomic DNA was purified using isopropanol precipitation (Darwesh et al., 2014).

The extracted DNA was used as a template for PCR amplification using a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR was performed with a pair of universal primers targeting the 18S rRNA gene; EUK1 (5′-AGCGGAGGAAAAGAAACTA-′3) and EUK2 (5′-ACTAGAAGGTTCGATTAGTC-′3) as the forward and reverse primers, respectively, according to Rainer et al. (2007).

The final 50 µL reaction mixture contained 1× PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit of Taq DNA polymerase (NEB, England) and 5 µL template DNA.

The PCR amplification included an initial denaturation of the DNA at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec. Then, the mixture was kept for 10 min at 72°C for complete extension. The PCR product was purified by means of the QIA quick purification Kit (Qiagen, USA) and ran on an agarose gel to evaluate the purified 18S rRNA fragments for sequencing. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3730xl DNA analyzer (Applied Biosystems). The obtained 18S rRNA sequence was deposited in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared to those of other known species using the BLASTN program. The sequences were aligned using Jukes Cantor Model. The phylogenetic reconstruction was built using the neighbor-joining (NJ) algorithm with bootstrap values. The obtained sequences were deposited in GenBank under the accession no. KY621475.

Experimental design

The *Scenedesmus* strain was grown in a 250-mL conical flask containing 150 mL of media and incubated on an orbital shaker under continuous illumination to be utilized as the inoculum. Five mL of the inoculum was used for inoculation of the experimental flasks containing 150 mL of different treatments. In order to evaluate the capacity of the isolated microalgae and utilize wastewater as the growth medium for biomass and lipid production, the microalgal isolate was cultivated in different mixtures of wastewater and growth medium (BBM). A hundred percent of the BBM growth medium served as a control, as well as 100% wastewater inoculated with microalgae, which were implemented in different combinations. The percentages of wastewater/BBM mixtures were 25:75, 50:50 and 75:25, respectively. A wastewater treatment sample without *Scenedesmus* inoculation was consecutively carried out with other treatments to evaluate the native algal load of the utilized wastewater. All treatments were carried out in triplicate on an orbital shaker incubator (Gallenkamp, Germany) with a shaking rate of 120 rpm at ambient temperature under continuous illumination using white fluorescent light (1000 Lux). The samples were initially withdrawn for analysis at 2-day intervals.

Analytical methods

The growth rate of the microalgae was routinely assessed through measuring the optical density of the samples at 680 nm using a spectrophotometer (SHIMADZU UV-2401PC, Japan) as reported by Lee (2008). The chlorophyll *a* content was determined according to Richmond (2008) and Hosikian et al. (2010) with some modifications: 1 mL of algal culture was centrifuged at 10000 rpm for 10 min, the supernatant was discarded and the cells were re-suspended in 1 mL of 90% methanol and sonicated in an ultrasonic water bath at maximum power for 15 min. The mixture was vortexed for 5 min, centrifuged and the supernatant (contains chlorophyll) was transferred into a new tube. Additional extraction steps were performed with 1 mL of 90% methanol to extract almost all

of the chlorophyll from the cells. After the second extraction, the mixture was centrifuged again and the supernatant was transferred to the first portion of the extract. Finally, the volume was increased to 2 mL with 90% methanol, and the absorbance at 650 and 665 nm was recorded spectrophotometrically to calculate the chlorophyll *a* content according to the following equation:

$$\text{Chlorophyll } a \text{ (mgl}^{-1}) = (16.5 \times A_{665}) - (8.3 \times A_{650}) \quad (1)$$

The nitrate-nitrogen was estimated using the pyrogallol method with a modification of the method described by De-Nardo (1929). In brief, 1 mL of cells-free culture after centrifugation was placed in a test tube with 1 mL of pyrogallol solution (prepared by dissolving 3 g of Pyrogallol in 100 mL DW). Then, 2 mL of conc. sulfuric acid was added to the mixture and left for 30 min to allow for color formation. After adding 5 mL of DW, the developed color was measured on the spectrophotometer at 606 nm against the stander concentrations of sodium nitrate. Furthermore, ammonium nitrogen was measured colorimetricaly using Nessler's method (Basova et al., 2011) and the generated color was measured at 410 nm using a spectrophotometer (SHIMADZU UV-2401PC, Japan). Standard solutions of ammonium sulfate were measured and used to design the standard curve. Additionally, P content in all the samples was determined following the ascorbic acid method, in accordance with Akpor et al. (2007). The absorbance values of the preformed blue color were read at a wavelength of 880 nm using the spectrophotometer. The Potassium dihydrogen phosphate standards were read together with the samples.

Total lipid was determined according to the method described by Bligh and Dyer (1959) with the following modifications: 100 mL of microalgae culture were harvested by centrifugation at 6000 rpm for 20 min and re-suspended in 1 mL distilled water. The sample was then mixed with 2.5 mL chloroform. Afterwards, 5 mL methanol (1: 2 v/v) was subjected to sonication for 30 min at the maximum power. After sonication, the tubes were shacked with extraction solvents overnight. The next day, an additional portion of chloroform (2.5 mL) was added to each tube and the mixture was sonicated again for 30 min and vortexed. Then, 2.5 mL of distilled water was added to separate the chloroform and aqueous methanol layers by centrifugation at 4000 rpm for 10 min. The chloroform layer was gently removed from the bottom and a second extraction step was performed by adding 5 mL of chloroform and then the suspension was vortexed. The chloroform portions were collected and washed with 5 mL of 5% NaCl solution, after which the chloroform was evaporated in the oven at 50°C. The total lipids were measured gravimetrically.

GC characterization of lipid profile for microalgae samples

The fatty acid methyl esters were prepared for the GC analysis according to the method used by Ichihara and Fukubayashi (2010). In brief, the lipid samples were dissolved in toluene (0.20 mL), placed in screw-capped glass test tubes and mixed with 1.8 mL of 8.0% methanolic HCl (w/v) followed by heating at 100°C for 1 h. After cooling to room temperature, 1 mL of hexane was vortexed with the mixture for the extraction of FAMEs followed by the addition of 1 mL of water to enable the layer separation. The hexane layer was pipetted out and purified through a membrane filter for the GC analysis.

The methyl esters of the fatty acids were analyzed by a Gas Chromatography system (Hewlett Packard, HP 6890 series, United States) equipped with an Alltech BPX70 Capillary Column (60 m X 320 μm X 0.25 μm) coated with 70% poly silphenyl-siloxane supplied with flame ionizing detector. Fatty acid content was expressed as a percentage of the total fatty acids identified in the oil.

Statistical analysis

All experimental trials were performed in triplicate and the results are presented as the means ± standard deviation. Statistical analyses were performed using SPSS software 20.0. The comparisons of the mean values were conducted by means of one-way analysis of variance (ANOVA) followed by a Duncan's new multiple-range test for statistical significance. The differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and identification of microalgae

The isolation source was a sample of wastewater-contaminated swamp which showed algal growth that could be observed with the naked eye. A preliminary microscopic examination

of the water sample showed the domination of the genus *Scenedesmus*. Using the microscopic characterization, a microalgae isolate was collected and primarily identified as belonging to the genus *Scenedesmus***,** which was confirmed using molecular biology tools. The data derived from the BLAST analyses and phylogenetic tree (Fig. 1) were constructed based on 18S rRNA gene sequences of isolated microalgae, which indicated that the obtained isolate was close to *S. obliquus* with 99% similarity. The sequences of this strain were uploaded to GenBank and recorded under the accession number of KY621475. Many reports recorded the potential of using *Scenedesmus* microalgae as a feedstock for biodiesel production and the ability of this genus to grow on wastewater as a growth medium (Álvarez-Díaz et al., 2015; Mata et al., 2013). Hodaifa et al. (2008), Ji et al. (2015) and Álvarez-Díaz et al. (2015) reported the capacity of *S. obliquus* to grow under mixotrophic conditions of different wastewaters under illumination.

Growth rates of S. *obliquus* in different wastewater-medium mixtures

The growth of *S. obliquus* on Bold Basal Medium and wastewater, as well as their mixtures, were assessed using cultural optical densities (OD) and the chlorophyll *a* content. This experiment was performed to examine the suitability of such wastewater mixtures to give the maximum growth for the studied microalgae. The obtained results showed different growth parameters according to the mixtures of medium and wastewater (Fig. 2 and 3). The data obtained during the current study revealed that the optimum growth rate, dry biomass production, lipid content, as well as the percentage of C16-C18 fatty acids of the microalgae all belong to different types of medium. These results could be due to the different behavior of each parameter in response to the degree of stress, which is the percentage of WW in the growth medium. Previous studies showed that divers and the individual responses of the studied parameters affected the factors. Madkour et al. (2012) found the highest dry material, chlorophyll

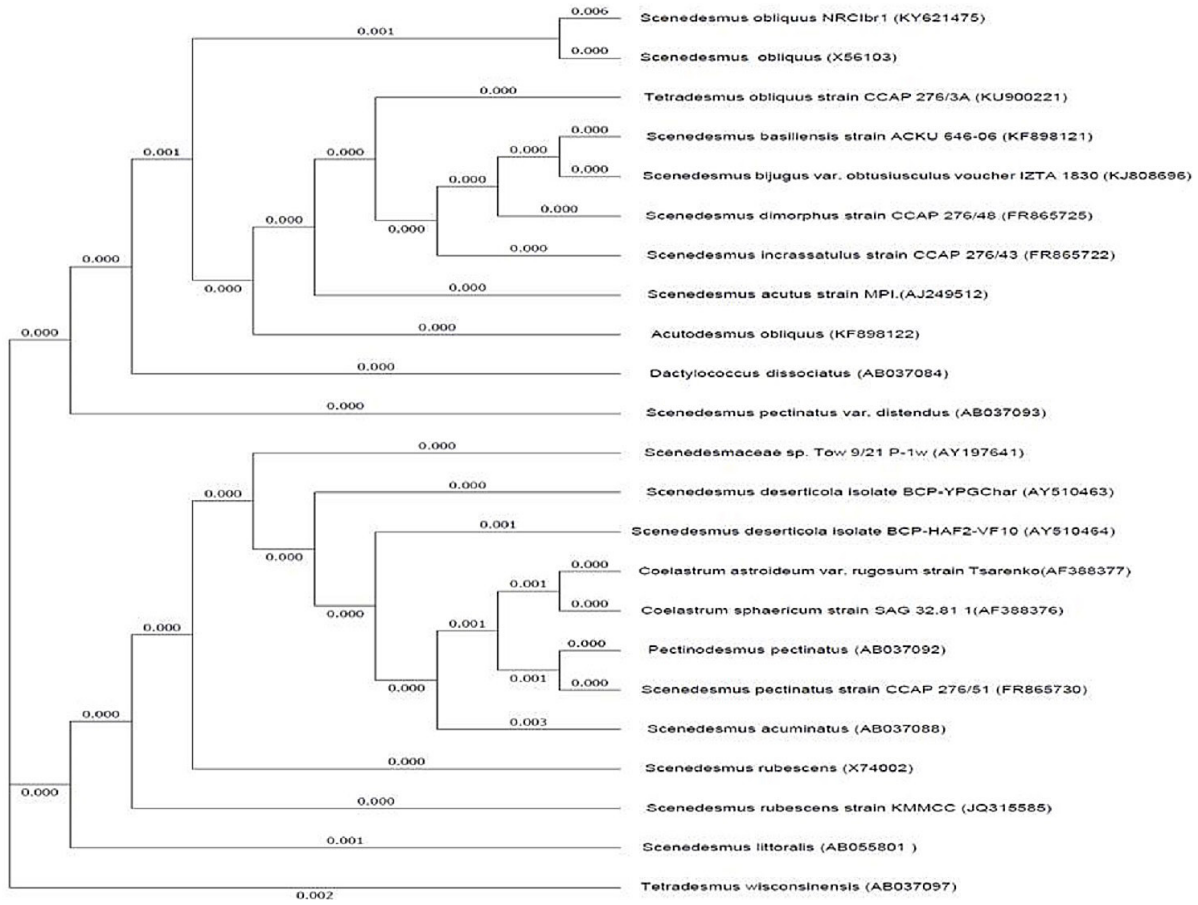

Fig. 1. Phylogenetic relationship between the obtained 18S rRNA sequence of *S. obliquus* and their related strains in GenBank. The scale bar represents the number of changes per nucleotide position (substitution/site)

content and protein yields were achieved via SM medium while utilizing urea and NH_4NO_3 as the N sources, providing the maximum lipid and carbohydrate content of *Spirulina* sp, respectively.

Optical density

During the 14 days of the experiment, the growth rate of *S. obliquus*, which was represented by the OD at 680 nm, showed a steady increase in BBM as the mono cultivation medium (Fig. 2a). By using wastewater as a component in the cultivation medium at 25% and 50%, the cultures showed a slightly higher density during the first 6 days than BBM (control). Afterwards, the growth curve became lower than BBM, 8 days after the experiment started (Fig. 2b and c). In the case of cultivation in 75% wastewater amended with 25% BBM, the growth trend was similar to the previously mentioned mixtures, but with lower values at the final 4 days of incubation (Fig. 2d). On the other hand, cultivation of *S. obliquus* on 100% wastewater showed a normal trend compared to BBM during the first 6 days; then, the growth decreased and its OD became approximately 28.7% of the BBM (Fig. 2e). However, the wastewater itself contained some native microalgae species that showed negligible growth when subjected to long term aeration and illumination (Fig. 2f). At the end of the cultivation period (day 12 and 14), the OD values of the medium treatment reached 100%, which was significantly higher than other treatments. A similar result was found by Toyub et al. (2008) when growing *S. obliquus* in BBM compared with sweetmeat factory waste media.

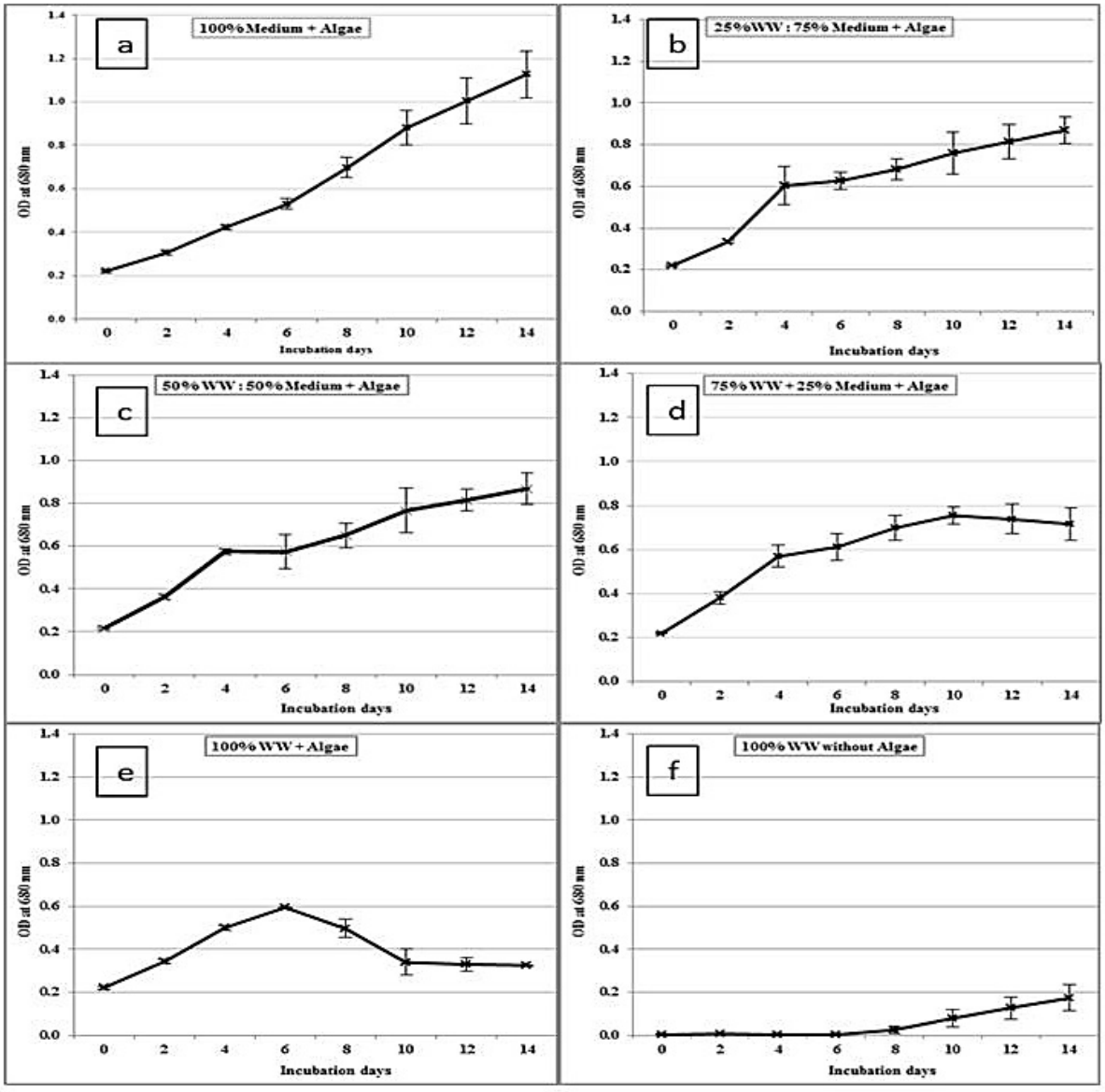

Fig. 2. Optical densities (OD_{680nm}) of *S. obliquus* cultivated on BBM, wastewater or their mixtures

The aptness of BBM for vegetative growth of *S. obliquus* was reported by Martinez-Jeronimo and Espinosa-Chavez (1994).

Chlorophyll a content

Chlorophyll *a (Chl a)*, a photosynthetic pigment, is the principal photochemically active compound, which functions as a receiver of light for photosynthesis. Therefore, the content of this pigment in microalgae reflects the photosynthetic activity and could indicate the growth rate (MacIntyre et al., 2002). In the current study, the chlorophyll *a* content has been implemented as a parameter to evaluate the growth of *S. obliquus* on different mixtures of pretreated municipal wastewater (WW) and BBM (Fig. 3a-f). Following the inoculation of *S. obliquus* into WW (100%), its chlorophyll content increased for 8 days and then declined until the end of the incubation period (Fig. 3a). The maximum chlorophyll *a* content (1.95 mgl^{-1}) was recorded when the WW was mixed with BBM growth medium in the ratio of 1:1 (V/V) (Fig. 3c). The chlorophyll *a* content reached its peak (1.44 mgl^{-1}) when changing the mixing ratio to 1:3 (WW: BBM), then started dropping up to the 10th day before starting to increase. Although the chlorophyll *a* content of *S. obliquus* grown on 100% BBM exceeded its value in 100% WW treatment media, it unexpectedly was not the leading treatment (Fig. 3a and e). On the other hand, the incubation of WW without inoculation showed almost zero chlorophyll *a,* which reflects the very low native algal content of the wastewater used in this experiment (Fig. 3f). Although the *Chl a* content in the 25% WW+75% BBM treatment were significantly higher compared to the experimental setup, after 10 days there was no significant difference between the BBM treatment and their wastewater mixtures.

The chlorophyll *a* content could be applied as a measurement for the algal weight and volume, which reflects the empirical link between the nutrient concentration and other biological phenomena in aquatic ecosystems (Berkman & Canov, 2007). The *Chl a* content is widely accepted as a parameter for algal biomass world-wide (Moore

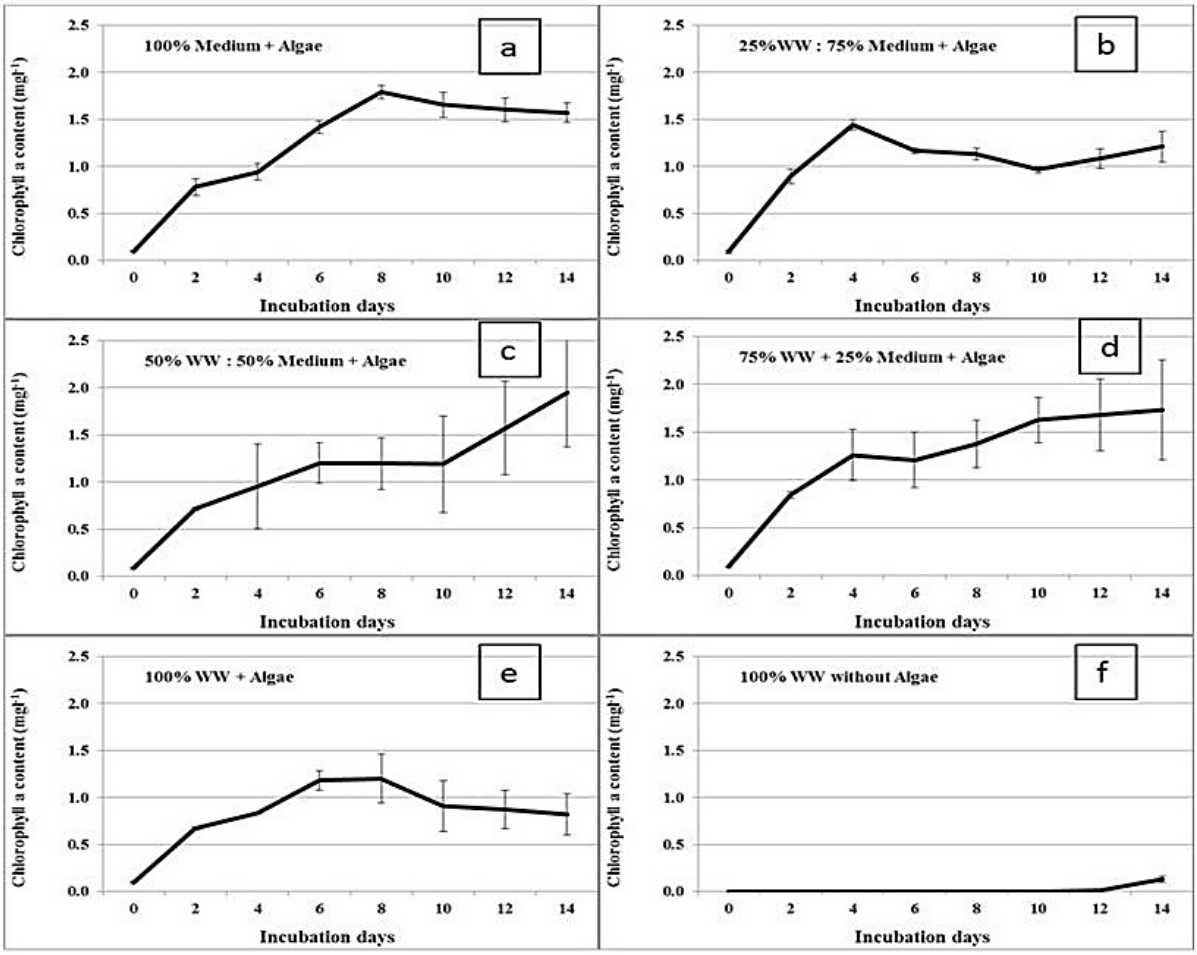

Fig. 3. Chlorophyll *a* content of *S. obliquus* cultivated on BBM, wastewater or their mixtures

& Schindler, 2008; Boyce et al., 2013). In the current study, the differences in the *Chl a* content among the treatments (Fig. 3) may refer to the utilization of nutrients during the growth period. The noted decline in the chlorophyll *a* content was associated with the reduction in the nutrient content (P and N) in the growth medium (Fig. 4 and 5). These results are in accordance with Juneja et al., (2013) who reported the influence of nitrogen and phosphorus deficiency on reducing the chlorophyll *a* and protein content of several microalgae genera, including *Scenedesmus.*

The presented data are the mean ± sd and the letters show significance at $P \leq 0.05$.

Nutrients utilization of wastewater-media cultivation mixtures

The nutrient content of the growth medium is one of the main factors affecting the microalgal growth, biomass and lipid production. As phosphorus and nitrogen are the major nutrients that can influence the growth and productivity of microalgae, their effect and utilization were studied in the present study.

Phosphorus content

The phosphorus utilization by *obliquus* for different wastewater-medium mixtures was presented in Figure 4. The data obviously declared that the concentration of P in the wastewater used in this experiment was very low, which may be owing to the primary treatment of municipal wastewater. The initial P concentration in some treatments varied due to the mixing rate of WW with the growth medium, which contained P in its composition. The P concentration in the WW was 1.27 ppm, which decreased to 0.3–0.4 ppm in 100% WW after 14 days of incubation with or without the algal inoculation.

The highest P content was in the 100% growth medium and decreased according to the mixing ratio. Generally, the P content in the mixed growth media sharply declined in all the treatments during the period of 2–4 days, except the mixing ratio of 25% WW+75% BBM, which declined quickly starting after the 4th day of incubation (Fig. 4). These results can explain the drop in the OD after 4–6 days of incubation in the WW-BBM mixtures (Fig. 3). Almost the entire P content was utilized when mixing the WW and BBM by the ratio of 75%: 25% (Fig. 4d), providing the most economic initial P concentration during the growth period. This result suggested that the P concentration was slightly higher than 15 ppm P (between 15 – 25 ppm), which could be enough for growing *S. obliquus* for 14 days. In his work on *Chlorella* microalgae that grow on municipal wastewater, Wang et al. (2013) stated that microalgae can grow well even in low P concentrations, suggesting that P may not be the limiting factor for its growth. Accordingly, the total nitrogen and phosphorus content are considered to be essential factors and directly influence the growth of the algal community composition and richness (Xu et al., 2010). Nitrogen and phosphorus have been reported to be major nutritional elements that govern the potentiality of a water source for algal growth (Abou-Shanab et al., 2013).

Nitrogen content

The Nitrate-N and ammonium-N are the major forms of nitrogen in wastewater that could influence the microalgal growth. These two forms of nitrogen were examined in this study to investigate the nitrogen content of the culture during growth. The ammonium nitrogen was not detected either in the initial wastewater or in the mixtures during the experiments. This result may be owing to the pretreatment of the wastewater utilized in this study. The removal of ammonium throughout the pretreatments of the municipal wastewater has been reported by Klieve and Semmens (1980).

The initial nitrate content in different wastewater-media mixtures varies because of the percentage of wastewater in the mixtures. *S. obliquus* utilized N after cultivation led to a gradual decrease in its content during the incubation period (Fig. 5).

The lowest N content was observed at the end of the incubation time while inoculating the microalgae strain in 100% wastewater. In this case, *S. obliquus* consumed almost all the N (92.17%) in the wastewater. On the other hand, the native community in the WW without inoculation utilized only 59.8% of the N content during the growing period. This result may be due to the low P content in the wastewater (Fig. 4-e and f), which causes the lower growth rate as shown in the OD and chlorophyll *a* content data (Fig. 2-e and Fig. 3-f). On the other hand, growing microalgae in the BBM for 14 days resulted in the reduction of its N content by 70%. The reduction in the N content when mixing WW with BBM was higher than its value when cultivating

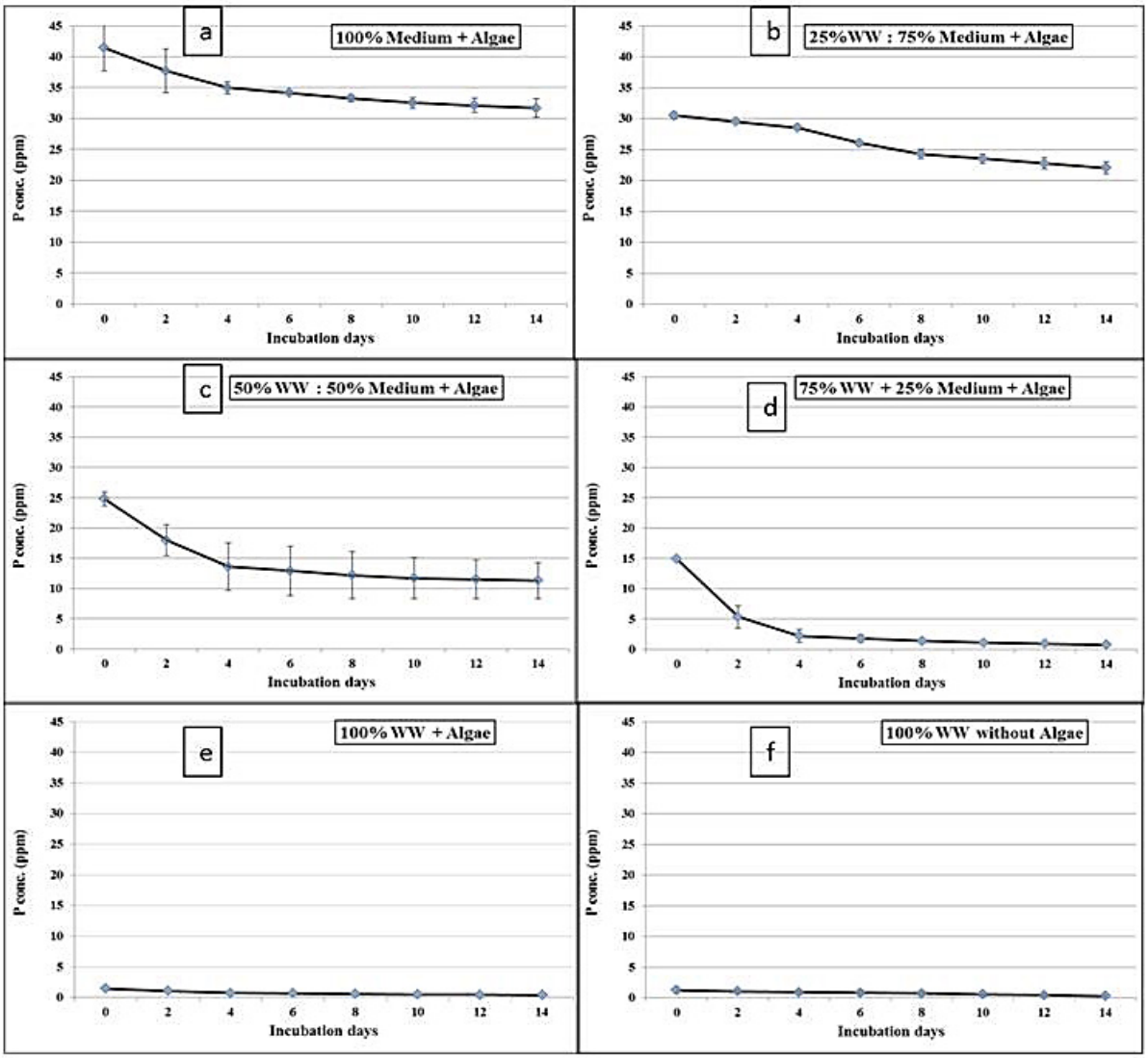

Fig. 4. Phosphorus content of different wastewater-media combinations during the cultivation of *S. obliquus*

microalgae in BBM. This finding is in accordance with the results of growth parameters (OD and chlorophyll *a*) in the first stage of the growth curve (6–8 days), suggesting that the abundance of N and P in the WW-BBM mixture improved the growth of *S. obliquus*. In this way, Vasileva et al. (2016) reported the importance of the nitrogen source and concentration in the growth media for the production rate and the biochemical composition of *Scenedesmus* sp.

Dry biomass production

The growth rate and biomass productivity of microalgae differed according to the growth condition (Fig. 6). The highest dry biomass was produced while growing *S. obliquus* on a medium composed of 75% WW and 25% BBM and 100% BBM, which reached 0.529 and 0.524 gl^{-1}, respectively. As expected, the lowest biomass production was recorded with 100% WW without algae inoculation treatment. The obtained data revealed that there were no significant differences ($P \leq 0.05$) in dry biomass among BBM and all the BBM-WW mixtures. These results indicate the suitability of wastewater for partial replacement of growth media for the production of adequate algal biomass.

The adequate existence of nutrients in both the 75% waste water and 25% algal growth medium, as well as 100% algal growth medium treatment, could explain the superior dry biomass production from the microalgae. While the algal growth medium is normally designed to provide the growing algae with sufficient and balanced nutrients, the wastewater could introduce several nutrients into the cultivated microalgae in satisfactory amounts (Vasileva et al., 2015; 2016).

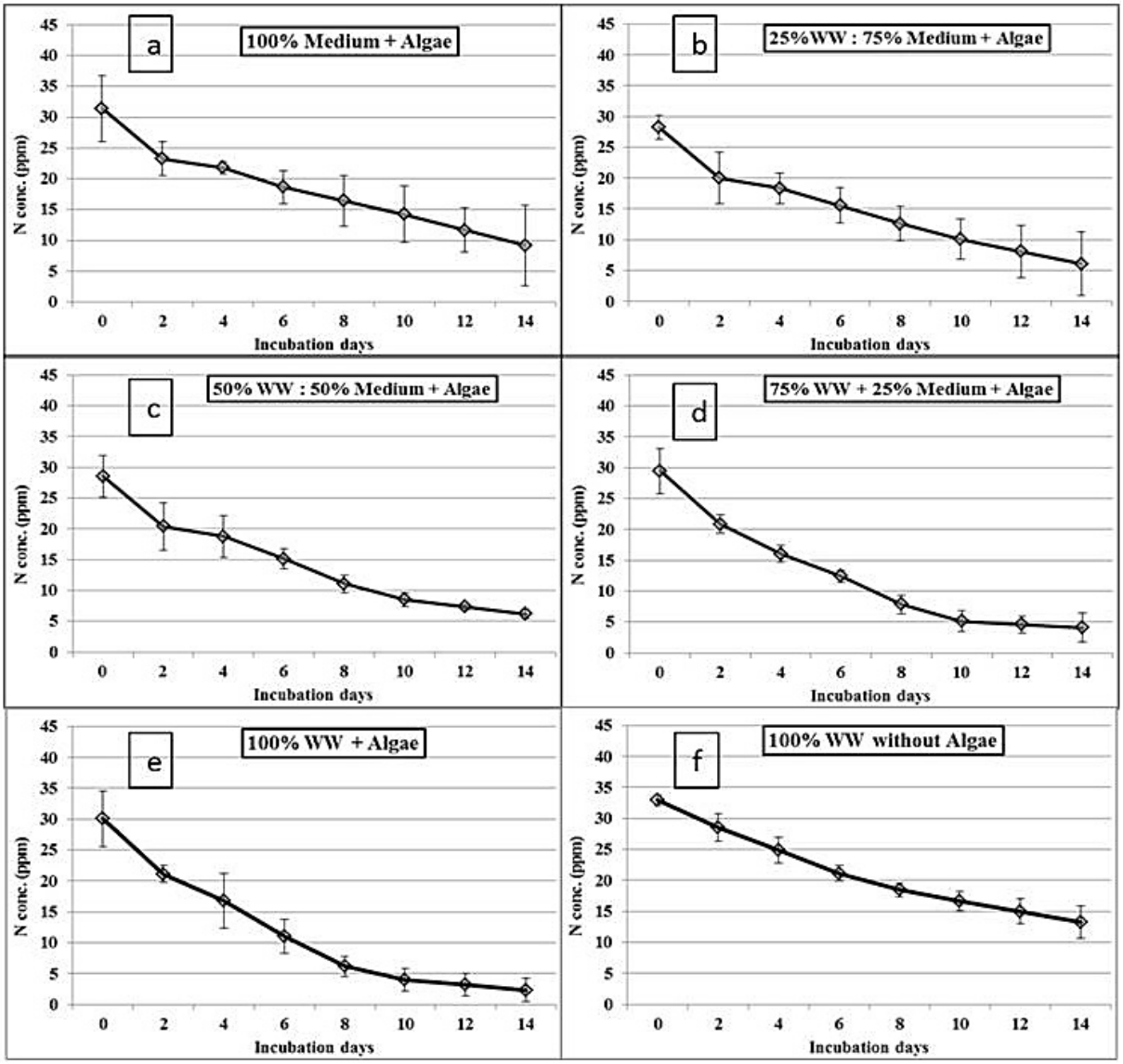

Fig. 5. Nitrogen (Nitrate) content in different wastewater-media combinations used for cultivation of microalgae *S. obliquus*

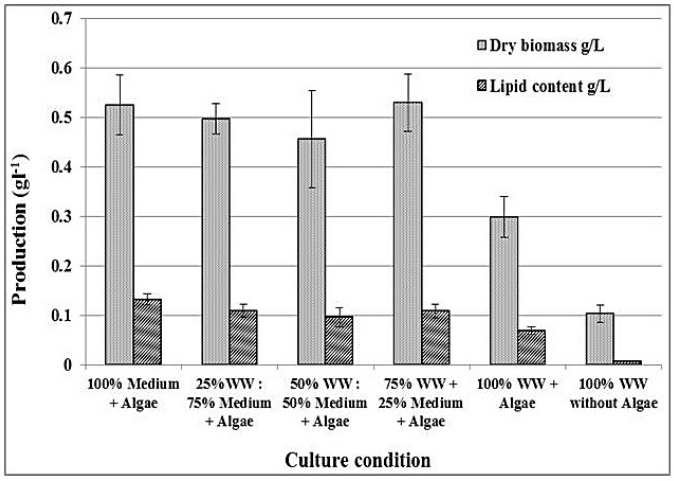

Fig. 6. Dry biomass and lipid content of *S. obliquus* cultivation on different wastewater-media combinations after 14 days of incubation

Lipid content

The algal biomass produced from the different treatments was utilized for lipid extraction. The amount of lipids produced by *S. obliquus* cultivated on WW, BBM and their mixtures are presented in Figure 6 and the percentage of the lipids contents are presented in Table 1. Table 2 compares the lipid content of some *Scenedesmus* spp. from the current and previous studies (Griffiths & Harrison, 2009; Rodolfi et al., 2009; Mata et al., 2010; Tang et al., 2011; Hakalin et al., 2014). The highest lipid accumulation was recorded when growing *S. obliquus* in 100% BBM growth medium, which may due to the highest accumulated biomass from this treatment at the end of growth period. The lipid content in this treatment reached 25.2% of the cell dry biomass. The second lipid accumulation percentage (22.7%) was recorded when the microalgae *S. obliquus* were grown on 100% wastewater. Out of the wastewater and growth medium mixtures, mixing 25% WW+75% BBM had the highest lipid content. Among the recorded variances in the lipid content of *S. obliquus,* there were no significant differences in its values among all the treatments, except for the 100% WW without inoculation, which exhibited the lowest lipid content. A higher lipid accumulation rate in the WW treatment corresponds to the nutrient-deprived conditions and resulted in nitrogen stress on the algal cells leading to increases in the lipid content. Such effects have been stated by Guldhe et al. (2014) and Ramanna et al. (2014), who reported high levels of lipids under nutrient stress.

Fatty acid composition of S. *obliquus* cultivated in different wastewater mixtures

The lipids extracted from the microalgae *S. obliquus* that were cultivated in different wastewater-media mixtures, as well as in BBM, were subjected to esterification and analyzed using GC. Table 3 explores the fatty acid profiles of algal lipids from different treatments.

The obtained results referred to the lipid profile of *S. obliquus*, which differs depending on the cultivation medium and conditions. The highest percentage of C16-C18 fatty acids (54.76% from total lipids) was recorded when the algae were cultivated in 100% wastewater, followed by 46.96% in the case of 100% BBM. Culturing *S. obliquus* on 25:75 WW-BBM produced the lowest C16–18 fatty acids among all the treatments. The increase in the appropriate lipid fraction (C16-C18) in the wastewater may refer to the starvation condition, while the increase in its content in BBM could be attributed to the balance in the composition of this medium.

Amin et al. (2013) stated that C16-C18 fatty acids are the ideal fraction for biodiesel production. El-Baz et al. (2016) studied the performance of the biodiesel obtained from *S. obliquus* in the diesel engine and recommended it as an environmentally-friendly biofuel.

Table 1. Lipid content (%) of *Scenedesmus obliquus* cultivation on different wastewater-media combinations

Culture condition	Lipid content (%)
100% Medium + Algae	$25.2^{a} \pm 1.8$
25% WW: 75% Medium + Algae	$21.9^{a} \pm 1.4$
50% WW: 50% Medium + Algae	$21.1^{a} \pm 3.6$
75% WW + 25% Medium + Algae	$20.4^{a} \pm 3.9$
100% WW + Algae	$22.7^{a} \pm 1.9$
100% WW without Algae	$6.8^{b} \pm 1.3$

Table 2. Lipid content of some *Scenedesmus* spp.

Scenedesmus species	Lipid content [% of dry weight]	Reference
Scenedesmus obliquus	20.4–25.2	Current study
	21 – 42	Griffiths & Harrison (2009)
	11 – 55	Mata et al. (2010)
	15.2- 24.4	Tang et al. (2011)
Scenedesmus dimorphus	26	Griffiths & Harrison (2009)
Scenedesmus quadricauda	18.4	Rodolfi et al. (2009)
Scenedesmus rubescens	18.5 – 23.2	Hakalin et al. (2014)

Table 3. Fatty acid composition of *S. obliquus* cultivation on different wastewater-media combinations

Fatty acid	Algal cultivation medium				
	100% BBM	25% WW: 75% BBM	50% WW: 50% BBM	75% WW: 25% BBM	100% WW
Palmitic acid C16 (0)	16.45	10.90	12.33	18.40	16.03
Palmitoleic acid C16 (1)	2.54	nd*	nd	nd	nd
Stearic acid C18 (0)	6.96	3.30	7.70	7.56	11.79
Oleic acid C18 (1)	10.18	6.60	11.85	12.53	19.46
Linoleic acid C18 (2)	7.65	3.20	3.78	4.80	5.60
Linolenic acid C18 (3)	3.18	1.60	0.90	3.00	1.88
Eicosanoic acid C20 (1)	2.90	1.50	2.20	2.90	3.03
Erucic acid C22 (1)	6.75	3.90	9.58	10.54	16.04
Lignoceric acid C24 (0)	4.76	26.97	16.02	nd	nd
Nervonic acid C24 (1)	8.61	nd	nd	11.40	nd
Hexacosanoic acid C26	13.57	23.90	16.60	11.55	10.73
Heptacosanoic acid C27	11.27	11.36	15.18	13.98	13.58
Undefined	5.18	6.77	3.86	3.34	1.86
Total	**100**	**100**	**100**	**100**	**100**

* nd – Not detected.

CONCLUSION

Secondary treated municipal wastewater represents a huge portion of wastewater that is discharged to water bodies. Thus, finding a practical way to utilize it to produce an eco-friendly product(s) is an important step for optimizing the benefits from one of the most abundant lost resources of water. Due to its relatively low organic content, light permeability and the existing mineral nutrients, secondary treated municipal wastewater could be utilized by mixotrophic microalgae as a low-cost growth medium to produce biomass-based biofuels. The results of the current study indicated no significant difference in the biomass production and lipid content of *S. obliquus* grown in either BBM or BBM-WW mixtures. These results suggest the utilization of mixtures containing a higher proportion of secondary treated wastewater, such as 75% WW+25% BBM or 50% WW+50% BBM, which could increase the economical production of microalgae for biodiesel. It saves water and nutrients as well. The growth of *S. obliquus* in wastewater could be improved by enhancing its nutrient content either by mixing wastewater and algal medium in certain ratios or by adding nutrients to wastewater. Further studies are needed to enhance the growth and lipid productivity of microalgae grown on wastewater as abundant cheap cultivation medium either in the lab scale and large scale.

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