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Removal of Heavy Metals and Production of Bioethanol by Green Alga *Scenedesmus obliquus* Grown in Different Concentrations of Wastewater

(Penyingkiran Logam Berat dan Pengeluaran Bioetanol oleh Alga Hijau *Scenedesmus obliquus* Hidup dalam Pelbagai Kepekatan Air Sisa)

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ABSTRACT

Algae have recently received a lot of attention as a new biomass source for the production of renewable energy and an important bioremediation agent. This study was carried out to evaluate the potential of green algae Scenedesmus obliquus *grow in different concentrations of wastewater and the improvement of cultivation conditions to produce biomass rich in sugar to produce bioethanol by fermentation processes. The highest sugar content of* S. obliquus *biomass was recorded for algae cultivated with 40 and 85% wastewater after 9 days under aeration condition with dark and light duration (44.5%). It was found that the highest removal efficiency of BOD and COD were 18% for* S. obliquus *grown under aeration condition. The highest ethanol efficiency of* S. obliquus *biomass hydrolysate was 20.33% at 4th day. The best condition of* S. obliquus *to grow efficiently was under aeration with light and dark durations, where it has high efficiency to remove heavy metals from wastewater in this condition.*

Keywords: Bioethanol; culture condition; fermentation; heavy metals; Scenedesmus obliquus*; wastewater treatments*

ABSTRAK

Alga pada masa ini telah mendapat perhatian sebagai sumber biojisim baru untuk penghasilan tenaga boleh baharu dan sebagai agen penting biopemulihan. Penyelidikan ini dijalankan untuk menilai potensi alga hijau Scenedesmus obliquus *membesar dalam pelbagai kepekatan air sisa dan dalam penambahbaikan keadaan penanaman untuk menghasilkan biojisim yang kaya dengan gula untuk menghasilkan bioetanol melalui proses penapaian. Kandungan gula tertinggi daripada biojisim* S. obliquus *direkodkan sebagai alga yang ditanam menggunakan 40 dan 85% air sisa selepas 9 hari di bawah keadaan pengudaraan dengan tempoh gelap dan terang (44.5%). Didapati bahawa kecekapan penyingkiran tertinggi BOD dan COD adalah 18% untuk* S. obliquus *membesar di bawah keadaan pengudaraan. Kecekapan tertinggi etanol hidrolisat biojisim* S. obliquus *adalah 20.33% pada hari ke-4. Keadaan terbaik untuk* S. obliquus *membesar dengan cekap adalah di bawah keadaan pengudaraan dengan tempoh terang dan gelap dan mempunyai kecekapan tinggi untuk membuang logam berat daripada buangan dalam keadaan.*

Kata kunci: Bioetanol; keadaan budaya; logam berat; penapaian; rawatan air kumbahan; Scenedesmus obliquus

INTRODUCTION

Algae can have a high biomass yield per unit area, high oil or starch content, do not require agricultural land (Sivasubramanian 2009). The major chemical pollutants in wastewater are nitrogen, phosphorus, heavy metals, detergents, pesticides and hydrocarbons. Of these chemicals, the most common nutrient limiting ones are nitrogen and phosphorus (Larsdotter 2006). Microalgae are highly efficient purification agents, they not only use inorganic nutrients (such as nitrogen and phosphorus) for their growth, they also contribute to purification by producing oxygen, removing metals and xenobiotic substances (Craggs et al. 1995). Microalgae have a great potential for the removal of nitrogen and phosphorus from wastewater (An et al. 2003). Wastewater treatment required high energy costs with mechanical aeration to provide oxygen to aerobic bacteria to consume the organic compounds in the wastewater, whereas algae based wastewater treatment, provides the oxygen for aerobic bacteria (Oswald 2003). *Chlorella vulgaris* and *S. obliquus, Spirulina* sp. and *Porphyridium purpureum* have the ability of producing high levels of carbohydrates instead of lipids as reserve polymers, These species are ideal candidates for the production of bioethanol, as carbohydrates from microalgae can be extracted to produce fermentable sugars (Nguyen & Vu 2012). Under stress conditions, such as nutrient starvation or high light intensity, some microalgal species accumulate carbohydrates in their biomass, which can increase to a significantly high level (a content of up to 65%) (Markou et al. 2013). *Scenedesmus obliquus* is a very versatile microalga as raw material for biofuels production (Miranda et al. 2012a). *S. obliquus* can grow in industrial wastewater of different origins showing good adaptation ability (Hodaifa et al. 2009, 2008). *Scenedesmus*, have been utilized as bioremediature agents in the removal of inorganic nutrients from polluted water to improve

quality (Martinez et al. 2000). Micro-algae are one of the most important sources of aquatic biomass and potentially represent a significant source of renewable energy (Wijffels & Barbosa 2010).

The objective of this study was to evaluate the growth of green alga *Scenedesmus obliquus* on different concentrations of wastewater and culture conditions for the utilization of carbohydrate source for bioethanol production. This current study also investigate the ability of microalgae in removing nitrogen, phosphorus, total alkalinity, ammonia, chloride, chemical oxygen demand (COD), 5-days Biochemical Oxygen Demand (BOD5) and metal ions from the waste waters.

Materials and Methods

The green microalga *Scenedesmus obliquus* was isolated from Damietta Nile branch in January 2011. Methods of isolation and purification of microalga in axenic cultures are based on serial dilution culture techniques and agar plate method as described by Venkataraman (1969). The alga was grown in 250 mL flasks containing 100 mL BG11 medium (Rippka et al. 1979) and all flasks were kept at room temperature (25 ± 1°C) under natural day light.

Characterization of wastewater sample

Wastewater was collected from Wastewater Company in Quesna-Egypt in February 2011 at 13:00 pm. The samples were stored, refrigerated and analyzed within few hours after arrival.

The microalga was cultivated under different concentrations of wastewater (0, 20, 40, 60, 80 and 85%). In order to find out the optimum culture condition, the cultures were subjected to three different conditions: Continues illumination of white fluorescent lamps (40 W) having 33.75 μ mol m^{-2} s^{-1} at 25-30°C; alternate (12:12 h) light and dark period at 25-30°C; and aerated condition by air pump under alternate light and dark period (12:12 h) at 25-30°C.

The measurement of physicochemical parameters

The physicochemical parameters including total alkalinity, phosphate, ammonia, chloride, chemical oxygen demand (COD) and 5-days biochemical oxygen demand (BOD5) were measured according to procedures described by Andrew et al. (2005). Nitrogen was determined using sodium salicylate method (Deutsche & Abwasser 1960). Analysis of the heavy metals (Pb^{+2}, Cd^{+2}, $Cu^{+2}$and Mn^{+2}) followed the direct aspiration into an air-acetylene flam using atomic absorption spectrophotometer type Perkin-Elmer spectrophotometer model 2380 (USA).

Algal growth including optical density (OD) at 660 nm (Wetherell 1961) was measured daily using a Unico UV-2000 spectrophotometer (USA), dry weight, the Neubauer Hemocytometer (Germany) counting chamber was used to determine the cell number (APHA 2005), and Reducing sugar concentration was estimated using the phenol-sulphuric acid method (Dubois et al. 1956; Krishnaveni et al. 1984).

Bioethanol production from microalga begins with the collection and drying of alga that has been cultivated under suitable environmental conditions (aeration under alternate light and dark period 12:12 h). The next step of the process, the alga mass is dried, ground and hydrolysed by 5% sulphuric acid autoclaving at 120°C for 20 min, and then pressed through the cheesecloth. The hydrolysed mass is fermented with a 63×10^4 cells/mL yeast in ocula size and a pH controlled to 4.5. *Saccharomyces cerevisiae* was used for ethanol conversion. Dry powdered *S. cerevisiae* (baker's yeast) was obtained from a local market. The inoculation media for yeast cultivation was prepared as 20 g/L glucose, 20 g/L yeast extract and 10 g/L peptone in a 1L Erlenmeyer flasks with distilled water (Atlas & Parks 1993). The solution was divided into 250 mL Erlenmeyer flasks where each flask contains 100 mL of solution with cotton plugs autoclaved at 121°C for 20 min. After sterilization, 2 g of common baker's yeast were transferred into each flask. The sealed flasks were then placed into a rotary shaker at 30°C for 24-26 h at 150 rpm. After incubation period, the cell suspension was aseptically collected by centrifugation (5 min at 2147 × g*)* stored at 4°C.

Yeast fermentation was carried out in 250 mL Erlenmeyer flasks (at pH4.6 and 30ºC) containing pretreated alga and supplemented with nutrients 0.9 g/L $(NH_4)_2SO_4$, 0.375 g/L yeast extract, 1g/L urea and the yeast inoculums (1.3×10^8/ mL) (Staniszewski et al. 2009). The flasks were closed with rubber stoppers through which hypodermic needles had been inserted for the removal of CO_2 produced during the experimental period (48 h). The samples were withdrawn after 24 h and for 5 days where, the ethanol content and residual sugars were analyzed. Ethanol was measured according to the method of Caputi et al. (1968). Conversion rate of the ethanol was calculated according to the ratio of produced ethanol and the initial sugar content in the fermentation medium (Caylak & Sukan 1998).

The identification and quantification of the sugars were done by high-performance liquid chromatography (HPLC) by Aminex NH2 25 cm, 4 mm column and with refraction UV detector 193 nm. The mobile phase was acetonitrile: Deionized water 75: 25 at a flow rate of 1.25 mL/min.

The data presented in the figures and tables as the average of at least three replicates per treatment and means ± standard error. Data were subjected to analysis, using one-way analysis of variance (ANOVA) using the SPSS (version, 16.0) least significant difference method (LSD) tests at the levels ≤ 0.05.

Results and Discussion

Scenedesmus obliquus was grown under continuous light illumination with different wastewater concentrations

showed that the highest values of growth was obtained at 40% wastewater in 9 days (14.08±1.48 10^4 cells/mL, 0.88±0.018 nm and 1.15 g/L), (Figures 1, 2, 3 and 4). The best reducing sugar contents of *S. obliquus* was obtained at 60% wastewater after 9 days (38.73%).

Estimation of growth through cell count (cells/mL), optical density (nm) and dry weight (g/L) of *S. obliquus* in alternate light and dark period (12:12 h) under different wastewater concentrations showed that the highest values of *S. obliquus* growth was obtained at 60% wastewater in 9th day (18±1.26 *10^4 cells/mL, 0.83±0.044 nm and 1.26 g/L) and the best result of reducing sugar contents of *S. obliquus* was shown at 40% wastewater after 9 days (44.217%) (Figures 5, 6, 7 & 8).

Similar results were observed by Richmond and Grobbelaar (1986) on *Spirulina platensis*. These observations were related to the growth of the microalgae under laboratory conditions and were significantly lower when compared with the growth observed under natural conditions, which probably was due to artificial illumination stress to the cultured microalgae. According to Rocha et al. (2003), artificial light can cause heating and difficulty in dissipation of energy to the atmosphere. Different light sources can vary the microalgal composition. In fluorescent lighting, the light is concentrated in a few preferred colors while sunlight has all the wavelengths in equal amount. The similar reports were done by Mercado et al. (2004). Artificial

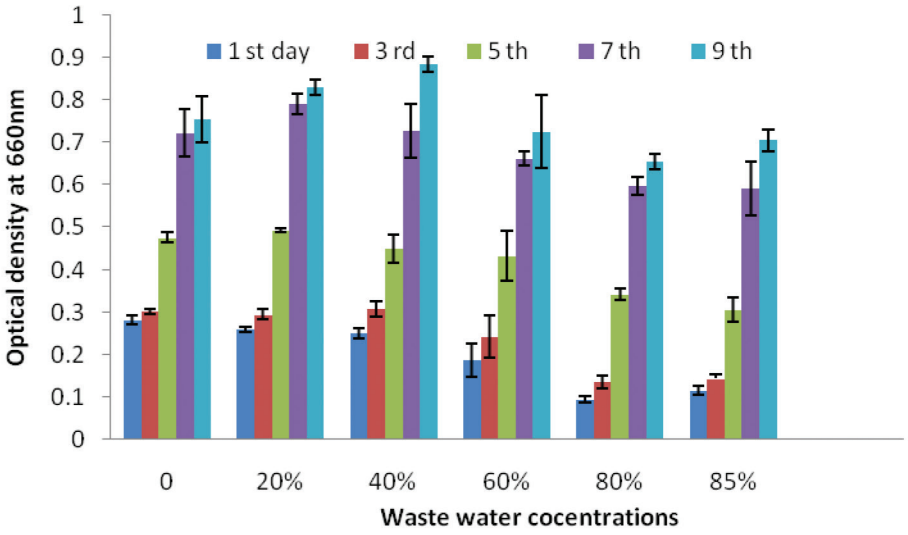

FIGURE 1. Effect of continuous light on *S. obliquus* growth measured as optical density (660 nm) under different concentrations of wastewater

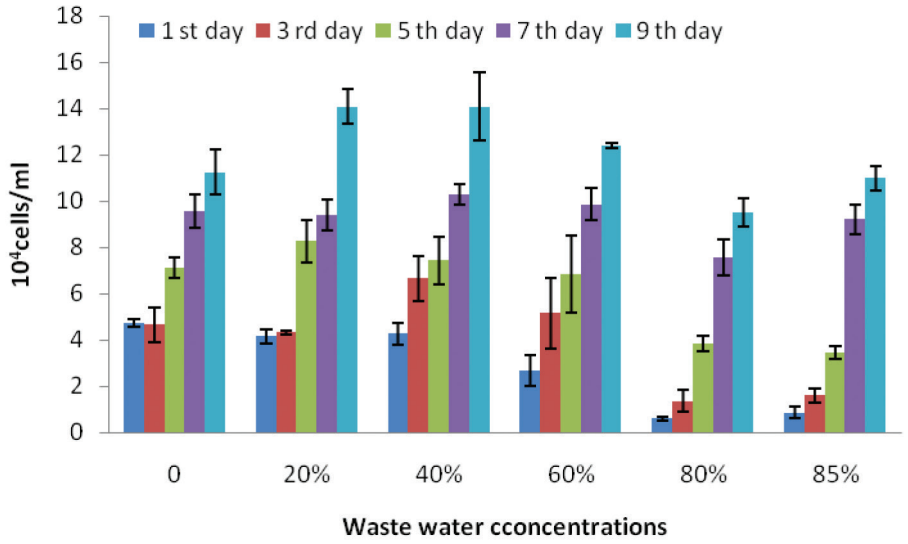

FIGURE 2. Effect of continuous light on growth of *S. obliquus* measured as cell count (10^4 cells/mL) with different concentrations of wastewater

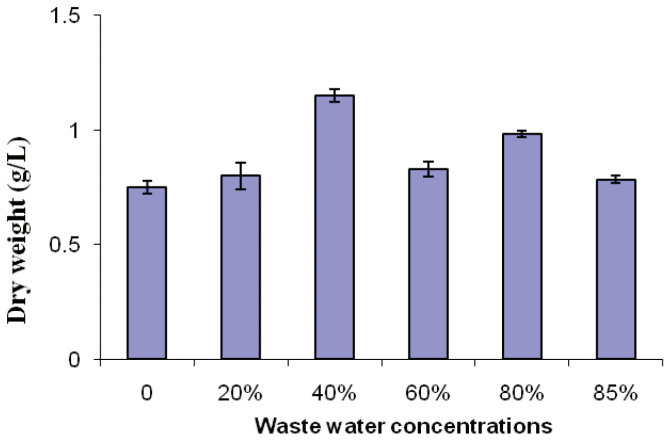

FIGURE 3. Effect of continuous light on dry weight (g/L) on *S. obliquus* growth in 9th day under different concentrations of wastewater

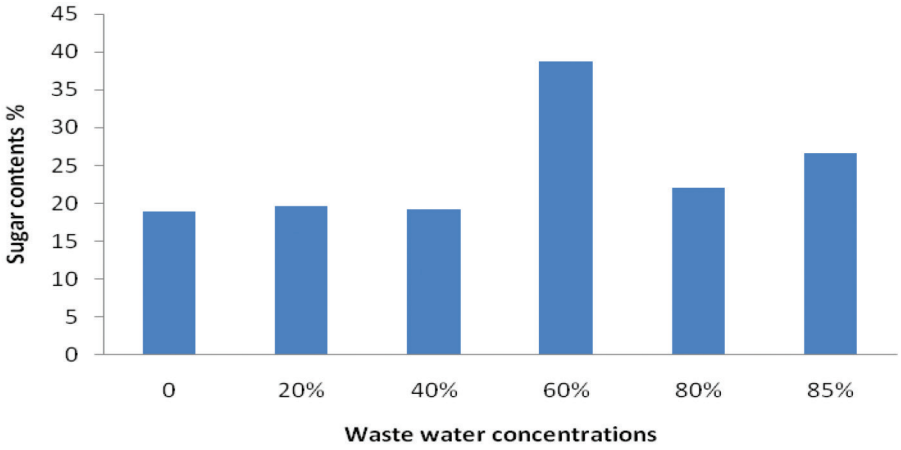

FIGURE 4. The percentage of reducing sugar contents of *S. obliquus* growth with different concentrations of wastewater after 9 days under continuous light

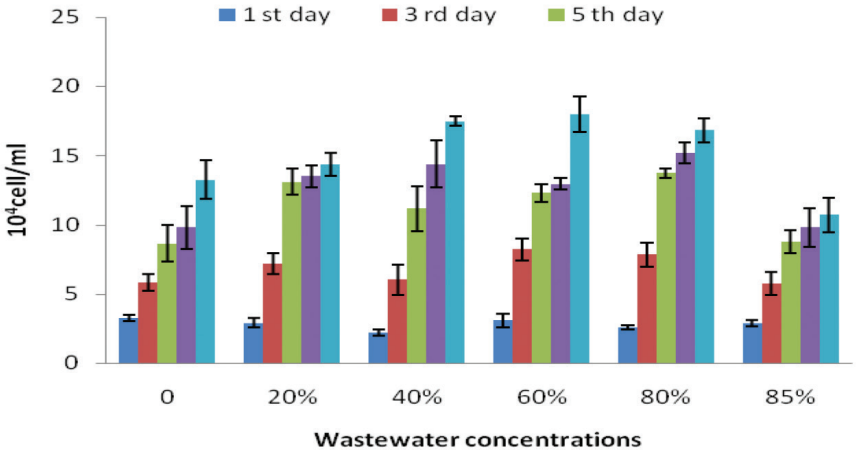

FIGURE 5. Effect of light and dark duration on growth of *S. obliquus* measured as cell count (10^4 cells/mL) with different concentrations of wastewater

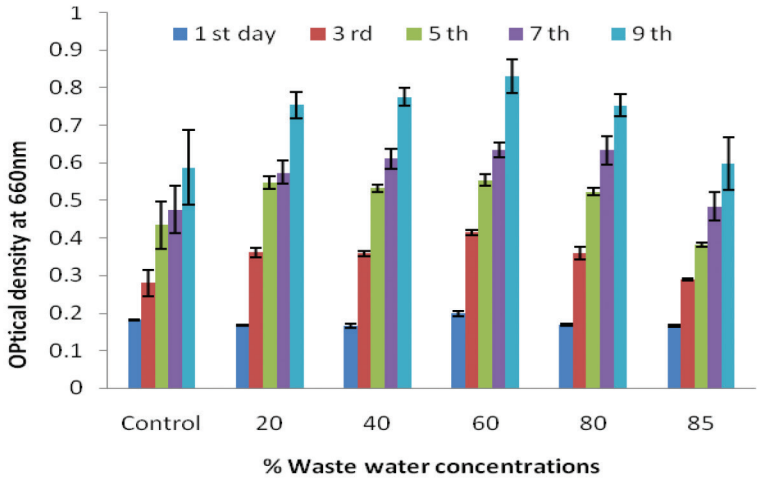

FIGURE 6. Effect of light and dark duration on *S. obliquus* growth measured as optical density (660 nm) with different concentrations of wastewater

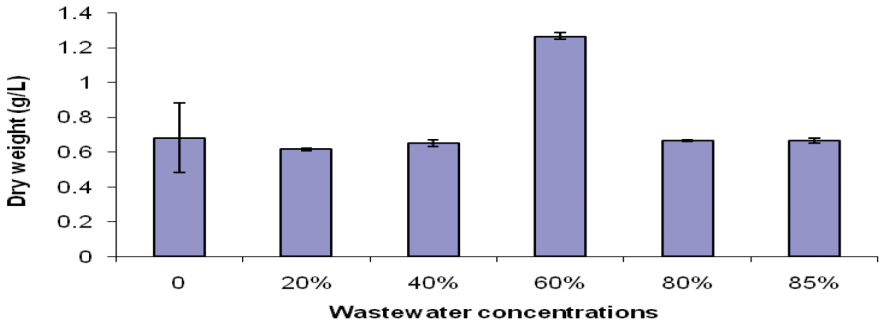

FIGURE 7. The percentage of reducing sugar contents of *S. obliquus* growth with different concentrations of wastewater after 9 days under light and dark duration

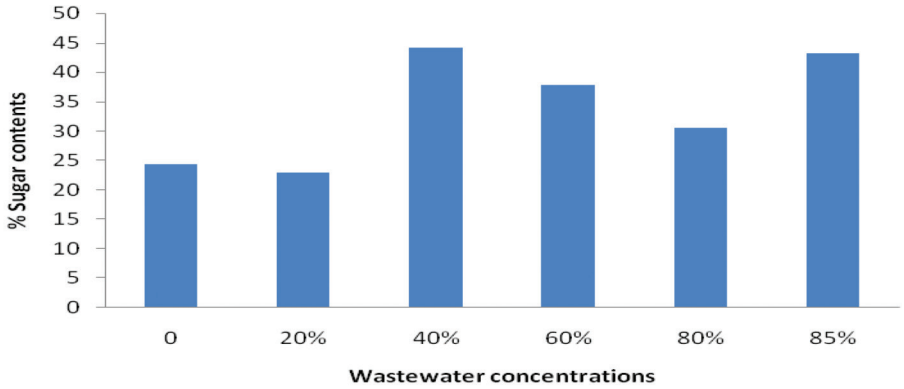

FIGURE 8. The percentage of reducing sugar contents of *S. obliquus* growth with different concentrations of wastewater after 9 days under light and dark duration

illumination employs fluorescent lamps exclusively for the cultivation of phototrophic algae at pilot scales stages and allows for continuous production but at significantly higher energy input. Thus the natural illumination would be preferable on the basis of overall cost compared with the artificial illumination which requires energy input for lighting (Brennan & Owende 2010). Weidang et al. (2008) has reported that light helps photosynthesis in producing chlorophyll and other metabolites in the algae. Furthermore, Laval and Mazliak (1995) found that some enzymes of the pentose cycle of photosynthesis and CO_2 fixation are inactive during illumination. The use of natural

conditions for microalgae production has the advantage of using sun light as a free natural resource. Under natural growth conditions phototrophic microalgae absorb sunlight and assimilate carbon dioxide from the air and nutrients from the environment. However, some authors (Bouterfas et al. 2006) suggested that the use of light/dark cycles allows for either an increase in final biomass concentration and/or a reduction in production costs. Seyfabadi et al. (2011) also suggested that the Light/dark cycle was more supportive for growth than other regimes, because cell number is sustained longer in exponential phase longer and photoperiodicity also save the consumption of light energy and increase light energy efficiency.

Figures 9, 10 and 11 show the cell count (cells/mL), optical density (nm) and dry weight (g/L) of *S. obliquus* cultivated in different wastewater concentrations using air pump under alternate light and dark period (12:12 h). Cell count, optical density and dry weight clearly indicated that the highest values of *S. obliquus* growth was obtained at 20% wastewater on the 9th day (35.62±2.52 *10^4 cells/mL, 2.34±0.26 nm and 1.36 g/L), respectively. The highest value of reducing sugar contents of *S. obliquus* was obtained at both 40 and 85% wastewater after 9 days (44.5%) (Figure 12).

Another parameter studied was aeration which can influence the microalgal growth. Hodaifa et al. (2010) studied the effects of aeration rates on the microalgae on growth and biomass composition. He concluded that the stress induced by stirring or by aeration of the cultures did not alter the final cell-protein and carbohydrate content, but the increase in aeration rates slowed the maximum specific growth rate of the cultures. The advantages of keeping the algal suspension in movement are numerous. The continuous mixing prevents sedimentation of the algal biomass (Stengel 1970) with all the negative effects to which this can lead to avoid thermal stratification and keeps the nutrients in active contact with algal cell surface leading to a stimulation of the nutrient uptake (Ukeles 1971), to a more effective utilization of incident light (Gates & Borchardt 1964), to remove photosynthetically generated oxygen and to ensure that cells experience alternating periods of light. It is well known that the

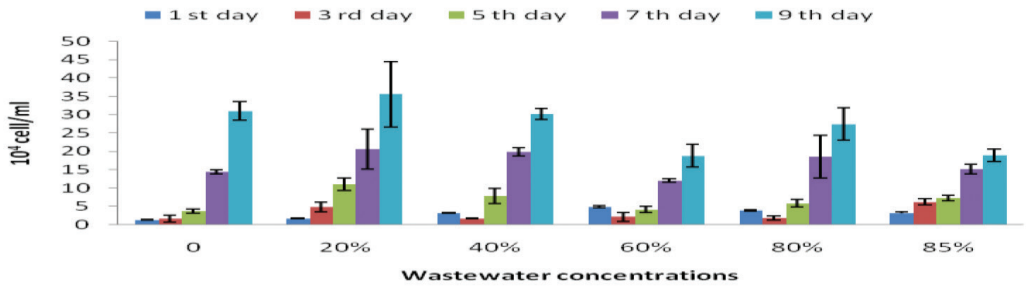

FIGURE 9. Effect of aeration on *S. obliquus* growth measured as Cell Count (10^4 cells/mL) with different concentrations of wastewater under light and dark duration

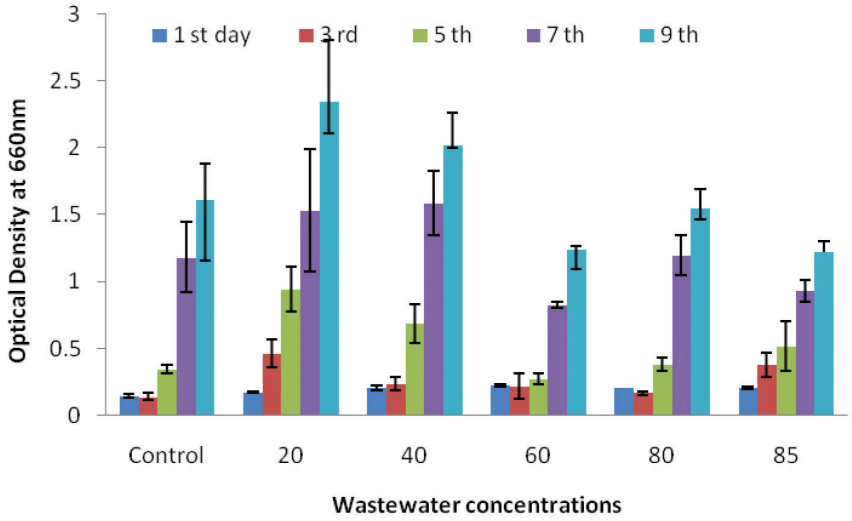

FIGURE 10. Effect of aeration on *S. obliquus* growth measured as optical density (660 nm) with different concentrations of wastewater under light and dark duration

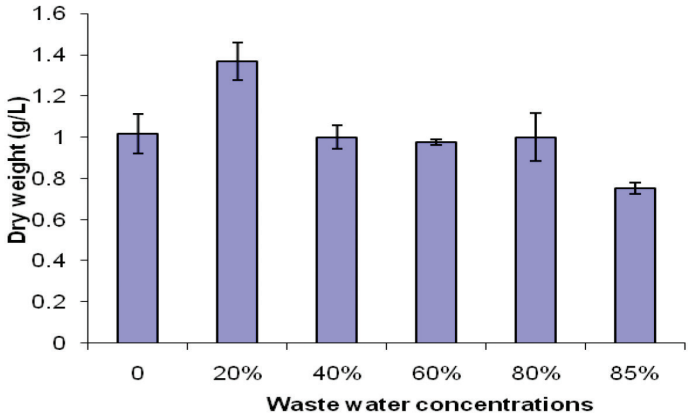

FIGURE 11. Effect of aeration on dry weight (g/L) of *S. obliquus* growth in 9th day with different concentrations of wastewater under light and dark duration

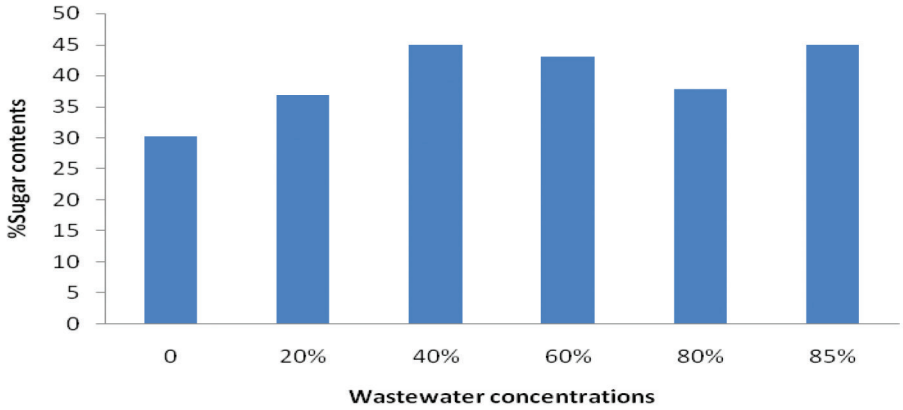

FIGURE 12. The percentage of reducing sugar contents of *S. obliquus* growth with different concentrations of wastewater after 9 days under aeration and light and dark duration

developing of algal growth and nutrient removal efficiency could be increased depending on the media composition and environmental conditions such as algal species, the light/dark cycle, cell concentration, aeration and retention time (Abdel Hameed 2007).

Lau et al. (1998, 1997), reported that *Chlorella* sp. and *Scenedesmus* sp. were common and effective species for the immobilization and nutrient removal purposes. It was found that the highest removal efficiency of BOD and COD were 18.05% for *S. obliquus* grown under aeration condition (Table l). This is because the introduction of oxygen contributes to the organic matter oxidation.

Also, with the culture aeration higher values of algal biomass growth were obtained due to increased photosynthetic activity of microalgae. Therefore, more oxygen was generated, helping to reduce the COD and making the treatment more effective (Travieso et al. 2008). Lee and Lee (2001) has reported that the microalgae can utilize nitrogen for their growth and phosphorus as a micro-nutrient essential for growth, which is taken up by algae as inorganic orthophosphate (PO_4^{-3}). The results obtained for nutrient removal were supported by the previous reports (Picot et al. 1991), where high rates of N and P removal were observed during the growth of *Scenedesmus* sp. under light/dark duration and aeration condition, respectively. *Scenedesmus* sp. were also able to remove and incorporate heavy metals, such as lead (Aksu & Kustal 1991), cadmium, copper or manganese (Chen et al. 1998), present in effluents and their use could be potentially more widespread. The presence of any chelating agent could reduce the adsorption ability of metals (Wu et al. 1999) for it is well known that metals in diluted chelated solutions are hard to remove (Yeh et al. 1995). This high capacity of adsorbing heavy metal ions was mainly due to the charged functional groups on the cell walls of microalgae, which can act as binding sites for metals (Gupta & Rastogi 2008). The best sugar content of *S. obliquus* biomass was when cultivated with 40% wastewater after 9 days under aeration condition with light and dark duration (44.5%) while the minimum sugar content was observed (18.485%) under continuous light without wastewater (control) after 9 days. Analysis of reducing sugar by HPLC using acids pretreatments of dry weight *Scenedesmus obliquus* grown under aeration condition with light and dark duration showed that the main contents of the hydrolysate were fructose, sucrose, lactose, glucose and inulins. The results

TABLE 1. Removal of physicochemical parameters and heavy metals from the wastewater by the microalga *S. obliquus* in different culture conditions

Conditions of growth / Reduction %	Light	Light and dark duration	Aeration with light and dark duration
BOD(	18.05	10.78	6.74
COD	18.05	10.7	6.74
Ammonia	78.84	49.25	24.67
Chloride	20	15	17.5
T.alkalinity	80.77	76.92	73.08
Pb^{+2}	41.2	41.2	58.8
Cd^{+2}	50	70	20
Cu^{+2}	62.5	87.5	87.5
Mn^{+2}	31.5	52.6	73.6
Phosphat	49.2	38.1	39.7
Nitrogen	14	17.3	14

BOD: Biological oxygen demand; COD: Chemical oxygen demand

TABLE 2. Experimental results for ethanol efficiency (%), ethanol yield (%), sugar residue (%) and optical density of yeast (nm) of *S. obliquus* biomass hydrolysate which used as raw material to ethanol yield in the fermentation process

Days	1st	2nd	3rd	4th	5th
Ethanol efficiency %	14.17	17.37	18.17	20.33	19.9
Ethanol yield %	7.1	8.7	9.1	10.2	10
Sugar residue %	0.4	0.56	0.56	0.41	0.49
Optical density of yeast	0.78	1.05	0.47	0.93	0.94

indicated that the dilute acid pretreatment improved the degradation of cellulose during the acids hydrolysis. The sulphuric acid was able to break the hydrogen bond among the cellulose, making them available for sulphuric acid and allowing hydrolysing glycosidic bonds to release sugars from cellulose and hemicelluloses (Binder & Raines 2010). H_2SO_4 (2 N) was the condition that yielded more sugars from *Scenedesmus obliquus* biomass without originating high concentrations of harmful compounds (Miranda et al. 2012a)

Table 2 shows that the highest bioethanol yield (10.2% g EtOH/gdw biomass) was observed on the 4th day. Therefore microalgal biomass was a potential feedstock for biofuel production (Markou et al. 2013) whereas S. *obliquus* was potentially a good source for bioethanol production (Miranda et al. 2012b).

CONCLUSION

The present results indicated that microalga *Scenedesmus obliquus* was an effective bioremoval for heavy metals (bioremediation). *S. obliquus* can also produce carbohydrates in large amounts over short periods of time. *S. obliquus* may be considered as promising feedstock candidate for bioethanol production. The highest sugar content was recorded for alga cultivated with 40 and 85% wastewater concentrations after 9 days under aeration condition and light duration of 44.5%. Bioethanol efficiency by fermentation of *S. obliquus* biomass hydrolysate was 20.33%. at day 4. The high efficiency of *S. obliquus* to remove heavy metals from wastewater was achieved under aeration condition with light and dark duration.

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