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Phycoremediation of Slaughterhouse Wastewater Using Microalgae for Nutrient Recovery and Biodiesel Production

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Abstract
The microalga Chlorella sorokiniana was grown in Bold's basal medium (BBM) as a control and wastewater enriched with BBM elements (WW+). Cultivation in WW+ showed the highest dry weight and optical density over which grown in BBM. So, rapid growth in WW++ was accompanied by significant increasing in lipid content. Due to lipid accumulation in WW++, it showed the maximum significant lipid productivity of 48.36 mg/L at the last day of incubation period. The present results confirmed that C. sorokiniana grown in secondary effluent slaughterhouse wastewater offers real potential for future application in wastewater treatment and biodiesel production.
Keywords: Microalgae, Biodiesel, Waste water

1. Introduction

Growth of world's population demand increase of the food requirement and consequently more energy is required. Using of traditional energy is correlated with more pollution and with running time the energy availability will be declined. Thus, the search for sustainable and renewable fuels is increasingly important nowadays due to the depletion of fossil fuels and also to protect the environment form the pollutant emissions associated to traditional energy.

Biofuel is defined as a fuel that are generated from processing conducted on biological materials (Lee & Lavoie, 2013). There are four biofuels generations are identified as: . They are characterized by their sources of biomass, their limitations as a renewable source of energy, and their technological progress. In summary, various generations of biofuels are defined as follows, (Bowyer et al., 2018)

- First-generation biofuels are directly related to a biomass that is generally edible by humans.
- Second-generation biofuels are defined as fuels produced from a wide array of different feedstock, ranging from lignocellulosic feedstocks to municipal solid wastes.
- Third-generation biofuels are, at this point, related to algal biomass but could, to a certain extent, be linked to utilization of CO_2 as feedstock.
- Fourth- generation biofuels are envisioned as sustainable fuels, derived from engineered biological materials, to achieve high levels of energy efficiency and environmental performance.

Biodiesel is a diesel fuel consisting of mono-alkyl esters of long-chain fatty acids that are generally made by the transesterification of lipids in animal fat or vegetable oils such as soybean, sunflower, rapeseed, and oil palm, (Williams & Laurens, 2010).

Also, microalgae are currently considered a promising feedstock for biodiesel production due to their GHG fixation ability, rapid growth rate and high production rate of lipid. Besides, the biodiesel production from microalgae is expected to be 15 to 300 times higher compared to conventional crop plants which are usually harvested once or twice a year while microalgae possess a very short harvesting cycle (around 12 days, depending on the species and cultivation method) which allows continuous harvesting throughout the year, (Nadiah et al., 2018).

Microalgae are single cellular, fast growing, rich in lipids that use carbon dioxide and other nutrients for its growth and serve as an efficient feedstock for biomass production , (Nayak & Ghosh, 2019).

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Microalgae can utilize the pollutants from wastewater as substrate and act as a renewable feedstock for biodiesel production. In addition, many microalgae can be grown in seawater/wastewater which reduces dependence on freshwater supplies for large-scale production of microalgae and thus minimizes competition for resources with traditional agriculture (Slocombe et al., 2015).

Therefore, a number of researches have been carried out to reduce the production cost of microalgae by implementing dual application of microalgae for phytoremediation and biomass production. This can be done by converting freely available nutrient from wastewater (particularly nitrogen, N and phosphate, P) into microalgae biomass with concomitant carbon dioxide (CO2) sequestration via photosynthesis process (Noue et al 1992; Green et al., 1995). Besides, most of the microalgae species are able to grow under nutrient-rich environments by absorbing the nutrients and metal from the wastewater, which make them an extremely attractive means for sustainable and low-cost wastewater treatment (De-Bashan and Bashan 2010; Hoffmann 1998; Mallick 2002).

However, the N:P ratio in wastewater should be in an optimum range as it plays an important role for successful microalgae cultivation (Salama et al., 2017).

Green microalgae especially Chlorella Spices are ideally suited to play a dual role of treating wastewater and biomass production by converting wastewater nutrients to biomass and lipid, (Rawat et al., 2011).

The release of untreated wastewater poses serious environmental challenges to the receiving water bodies as the wastewater contain high levels of organic materials, nutrients as well as toxic metals, which require costly chemical- based treatments to remove them in order to prevent from the risks of eutrophication, (De-Bashan, Bashan Y., 2010), (Mulbry et al., 2008) and (Olguí , 2003). It has been appreciated for some years now that biological treatment using microalgae in wastewater is considered as the most environmentally compatible and the least expensive of wastewater treatment methods , (Rawat et al., 2011) and (Mantzavinos and Kalogerakis , 2005).

Nearly 71% of the Earth's surface is covered by water and currently they are being polluted by human activities as more waste are being disposed into rivers and coasts without any prior treatment , (Sonune and Ghate , 2004). Once water is contaminated, it is unsafe for human and animal consumption due to toxic or harmful compounds that are contained in the water. Eventually, this causes water-related diseases to occur, which responsible for 80% of all illnesses and deaths in developing countries. Hence, proper wastewater treatment need to be intensively encouraged to reduce the contaminants to acceptable levels or meeting the recommended microbiological and chemical quality before it is being discharged to water bodies, (Sonune and Ghate , 2004).

Wastewater treatment is one of the most important environmental conservation processes to improve the quality of the discharged wastewater by making it more appropriate for reuse and to prevent water pollution. Currently, coupling microalgae-based treatment processes focuses on the production of microalgae biomass and the removal of inorganic nutrients from various type of wastewaters have been studied widely as it is considered to be a feasible method for saving large amount of nutrients and water required for microalgae cultivation,(Nadiah et al., 2018)

The key objectives of this work are to evaluate Chlorella Sorokiniana microalgae through the growth using economic media from natural resources . In addition, lipids production of microalgae are studied to evaluate its potential as a biodiesel feedstock.

1. Materials and Methods

1.1. Sample collection and preparation

Raw untreated feedstock wastewater sample was collected from slaughterhouse located at El-Moneeb region, Giza, Egypt. The samples were collected in sterilized bottles and kept directly at 4 °C after dilution with distilled water in the ratio 1:10. After that, the sample was analyzed initially and after 14 days of incubation based on the following parameters: Chemical Oxygen Demand (COD), Ammonia nitrate (NH3 – N), Nitrate nitrogen (NO3-N), total phosphate (TP), K , Na, Ca, Mg and heavy elements like (Ni, Pb, Cd and Cu).

1.1.1. Microalgae cultivation

Chlorella Sorokiniana BENHA721_ABO4 was selected to be used due to its high content of lipids. The fresh water microalga was procured from Faculty of Science, Tanta University. The used culture media for microalgae cultivation was Bold's Basal Medium (BBM) which is used as a control with the main component as illustrated in table 1.

1.1.2. Cultivation and growth conditions

Culture of *Chlorella Sorokiniana* microalgae was 10 ml so it was considered a seed culture. It was centrifuged at 4100 rpm then the pellet was incubated with 100ml of Bold's Basal Medium (BBM) in Flask 250 ml. Optical density (OD_{680}) and cell counting were measured daily for 14 days as an exploratory attempt for monitoring algal growth using a Spectrophotometer and Microscopic devices, respectively. The results of growth algal for seed

cultures were illustrated as shown in the following figure (1).

Table (1) : The components of Bold's Basal Medium

Stock	Stock solution	ml/ Litre
KH_2PO_4	8.75 g/500 ml	10 ml
$CaCl_2.2H_2O$	1.25 g/500 ml	10 ml
$MgSO_4.7H_2O$	3.75 g/500 ml	10 ml
$NaNO_3$	12.5 g/500 ml	10 ml
K_2HPO_4	3.75 g/500 ml	10 ml
NaCl	1.25 g/500 ml	10 ml
$Na_2EDTA.2H_2O$	10 g/L	1 ml
KOH	6.2 g/L	
$FeSO_4.7H_2O$	4.98 g/L	1 ml
H_2SO_4 (concentrated)	1 ml/L	
H_3BO_3	5.75 g/500 ml	0.7 ml
Trace Metal Solution		
Substance		g/Litre
H_3BO_3		2.86 g
$MnCl_2.4H_2O$		1.81 g
$ZnSO_4.7H_2O$		0.222 g
$Na_2MoO_4.2H_2O$		0.390 g
$CuSO_4.5H_2O$		0.079 g
$Co(NO_3)_2.6H_2O$		0.0494 g

Reference: Stein, J. (ED.) Handbook of Phycological methods. Culture methods and growth measurements. Cambridge University Press. 448 pp.

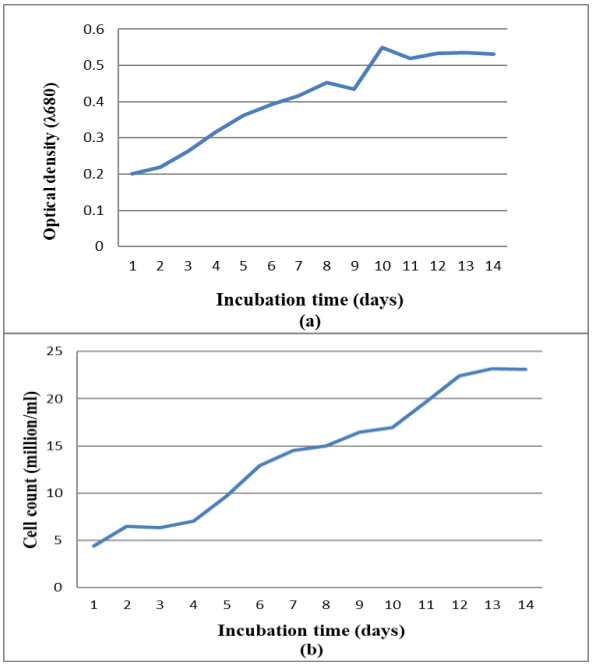


Fig (1): Growth curves by monitoring of optical density (a) and cell number (b) of *Chlorella sorokiniana* grown for 14 days on synthetic medium (BBM) as a seed culture before carrying out the main experiment.

1.1.3. Experimental setup

The experimental setup consists of two growth media treatments, namely BBM as a control and wastewater enriched with BBM elements (WW+). Three replicates were used for every growth treatment. Cultures were incubated in 25 ± 2 °C and light intensity was 100 µmol photons m^{-2} s^{-1}.

1.2. Biomass and lipid determination

Algal growth were monitored daily in two growth media treatment by measuring dry weight (DW), optical density ($OD_{\lambda 680}$), cell counting (CC), pH , electrical conductivity (EC), total dissolved solids (TDS), chlorophyll and carotenoids .

1.2.1. Biomass productivity

Biomass productivity was calculated according to (Abomohra et al., 2013) as follows:

$$\textbf{Biomass productivity } (\text{g } L^{-1}\text{day}^{-1}) = \frac{DW_f - DW_i}{t}$$

where the DW_f represents dry weight (g L^{-1}) after time (t) of incubation and DW_i represent dry weight (g L^{-1}) at the day of incubation.

1.2.2. Total lipids

The total lipid content was calculated in the final of incubation using chloroform: methanol (2:1) according to (Bligh and Dyer, 1959). Lipid extracts were dried in oven at 80 °C in a pre-weighed glass vials.

1.3. Measurement Parameters

1.3.1. Optical density

It was calculated daily for monitoring growth algal using spectrophotometer device with wavelength 680 nm according to (Mahmoud-aly et al., 2018).

1.3.2. Chlorophyll and total carotenoids determination

Chlorophyll $_{(a)}$, chlorophyll $_{(b)}$ and total carotenoids were determined using spectrophotometer- UV device and calculated according to the following equations:

Chll(a) = (12.47 X $A_{\lambda 665}$) – (3.62 X $A_{\lambda 649}$)

Chll (b) = (25.06 X $A_{\lambda 649}$) – (6.5 X $A_{\lambda 665}$)

Total Carote = ((1000 X $A_{\lambda 480}$) – (1.29 X $chll_{(a)}$) – (53.78 X $chll_{(b)}$)) / 220

where $A_{\lambda 665}$ and $A_{\lambda 649}$ represents absorption maxima (λ, nm) of both chlorophyll (a) and (b) with wavelength (λ) 665 and 649 and $A_{\lambda 480}$ represents absorption maxima (λ, nm) of total carotenoids with wavelength (λ) 480, (Mahmoud-aly et al., 2018)

1.3.3. Cell number

Cells number was calculated according to (Mahmoud-aly et al., 2018) at the following equation:

Cell number (million / ml) = Average of readings NO. **X** dilution factor **X** 10^4

1.3.4. Total dissolved solids, electrical conductivity and pH measurements

Total dissolved solids and electrical conductivity were measured daily using (T.D.S , EC meter). Also, pH was measured daily for monitoring the acidity and alkalinity of culture using (Benchtop pH Meter kit (Orion STAR A212, Thermo Scientific)).

1.4. Statistical analysis

Results are presented as mean ± standard deviation (SD) from three replicates for both treatments of experiment.

2. Results and discussion

In the present work, BBM was selected as a control medium because it has been extensively used as an effective growth medium for different freshwater microalgae. *Chlorella sorokiniana* was grown in BBM as a control and enriched wastewater (WW^{++})

2.1. Growth parameters

All cultures started the exponential phase after 1 day of incubation. *C. sorokiniana* grown in BBM started the stationary phase on the 6th day, where there was a slow growth increase then ended on the 10^{th} day with the beginning of the declined phase during the whole incubation period.

2.1.1. Dry weight, optical density and cell number

Growth indicators are considered very important for biomass production. They include dry weight, optical density and cell number. In addition, dry weight indicates the weight of both living and dead cells while cell density indicates only the number of viable cells,(Mahmoud-aly et al., 2018) . As well as, the more dry weight the more optical and cell density.

Figures (2) showed that enrichment of wastewater with additional nutritional elements of BBM enhanced the algal growth and retarded the stationary phase due to excess availability of nutrients. As well as, cell number and optical density started to reduce at the 10th day of the whole incubation period. This result signed that the maximum growth for C. sorokiniana algae was 2.10 gL^{-1} and 1.22 gL^{-1} for WW++ and BBM , respectively.

As well as , the growth curve for WW+ was noticed with the highest average values over BBM where optical density (OD_{680}) gave the greatest results in WW+ over BBM with average values 1.90 and 0.95 respectively on the 10th day as shown in figure (2).

Also, the highest average values for cell number in BBM was 35.48 million cell/mL on the 10th day of incubation. But cell number for WW+ has never measured because there was an obstacle during the vision of microscopic for cells as a result of water characteristics of WW+.

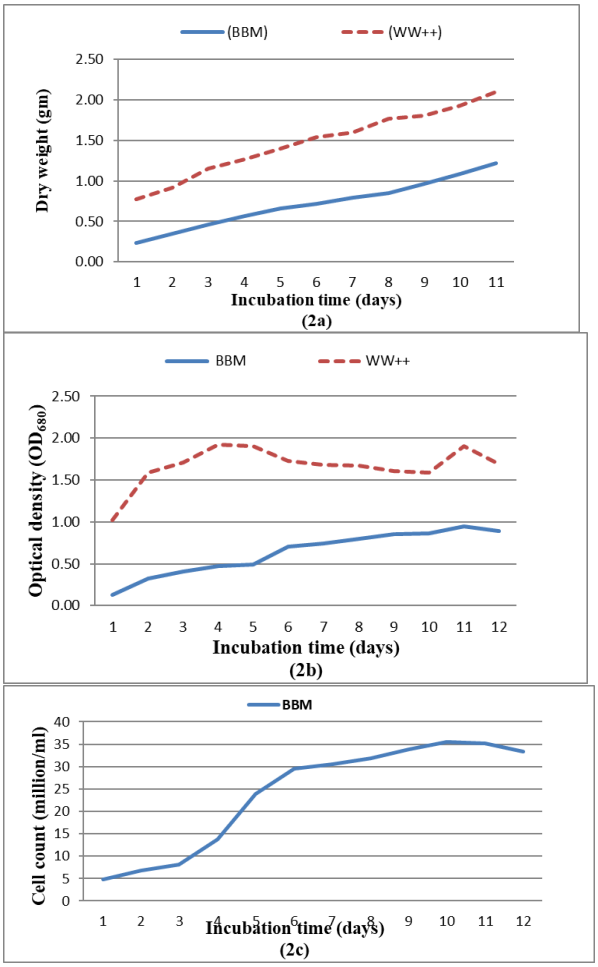


Fig. (2) Growth curves by monitoring of dry weight (2a) optical density (2b) and cell number (2c) of *Chlorella sorokiniana* grown for 11 days on synthetic medium (BBM) and enriched wastewater (WW^{++}).

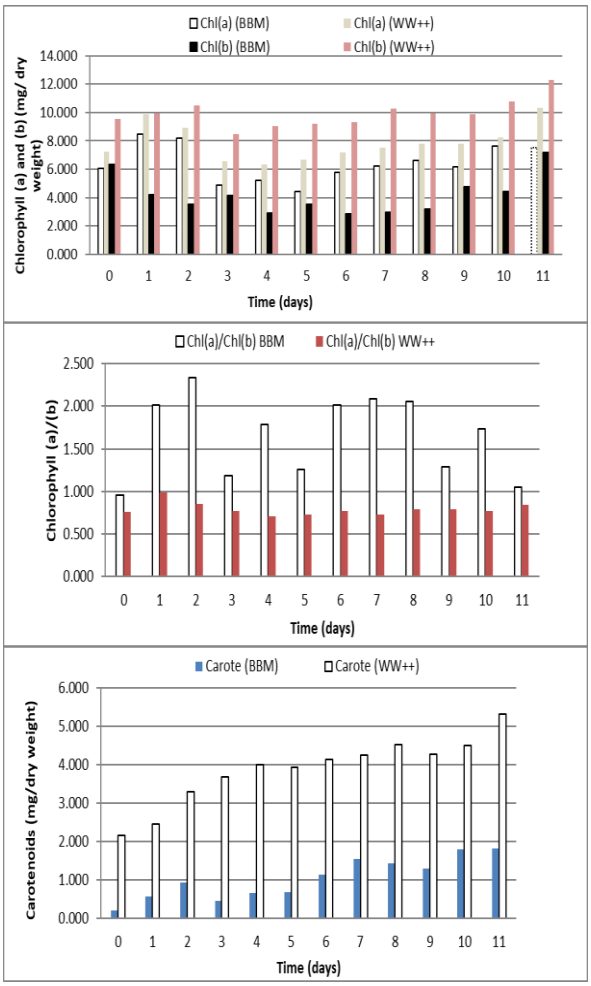
2.1.2. Photosynthetic pigments (Chlorophyll and Carotenoids)

The quantities of Chl a, Chl b and total Carotenoids were measured daily for strain grown under both media conditions from zero time to 11 days of the whole of incubation period.

The main observations can be concluded as dramatic increasing of chl (a), chl(b) and

Carotenoids contents for WW^{++} over BBM treatment. In addition, carotenoids played an important role in increasing the average values for both treatments after the first day to reach the highest average values 1.83 and 5.32 mg/ dry weight at the last day for BBM and WW^{++}, respectively. But chl(a) and chl(b) started to increase after the first day then decrease to reach the highest average values for chlorophyll (a) 7.52 and 10.35 mg/ dry and for chlorophyll (b) 7.19 and 12.29 mg/ dry weight at the last day for BBM and WW^{++}, respectively as showed in fig (3).

This can be interpreted by photodegradation of photosynthetic pigments under the experimental light intensity in use which may be due to high optical density and dry weight for algae.



Figures (3) Growth columns by monitoring of Chlorophyll (a), (b) and carotenoids of *Chlorella sorokiniana* grown for 11 days on synthetic medium (BBM) and enriched with wastewater (WW^{++}).

2.1.3. Power of hydrogen (pH)

Microalgae have the capability to grow over a wide pH range, but the suitable pH for growth is largely species-dependent. The growth parameters of C. sorokiniana at different culture pH were figured in fig (4). The range of pH in BBM was 7.7 to 8.8 but in the WW^{++} was 7.14 to 8.44. So, the range of pH is considered ideal for growth of algae through two media, BBM and WW^{++} as shown in fig (4).

This result is accepted with (Zheng et al., 2013) who were signed that pH did not play a significant role in the biomass yield (the amount of produced biomass per gram of sugar consumed), which indicated that the capability to convert glucose to algal biomass for C. sorokiniana might not be sensitive to pH (in the range of 6.0–9.0).

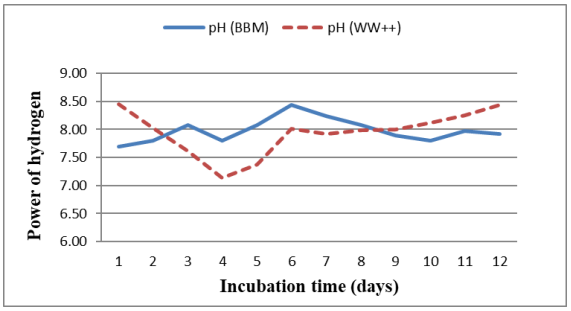


Fig (4) pH curve by monitoring pH daily for both treatment BBM and WW^{++}

2.1.4. Electrical conductivity and total dissolved solids

Conductivity (EC) and total dissolved solids (TDS) are water quality parameters, which are used to describe salinity level. Figs (5) and (6) described the correlation between EC and TDS that the more TDS the more EC. In addition, EC and TDS curves reached to the maximum average values for BBM and WW++ on the 10th day of the whole incubation period then started to reduce through declined phase.

These results showed that TDS is related with cell number where when TDS reached to the greatest average values, cell number started to reduce through declined phase. However, WW++ recorded the greatest average values over BBM on the 10th day although average values for WW++ were less than BBM in the first days of the period of incubation.

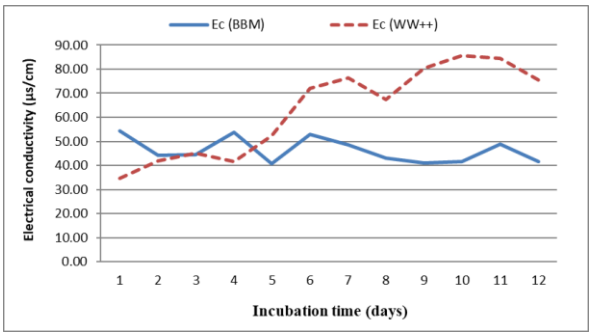


Fig (5) EC curve by monitoring EC daily for both treatment BBM and WW^{++}

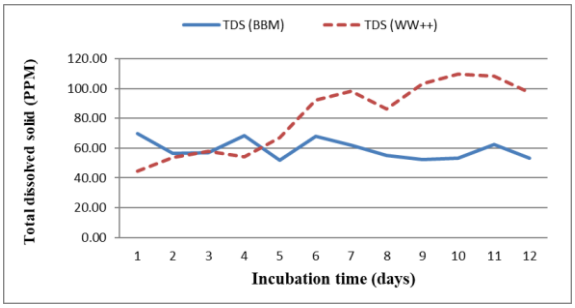


Fig (6) TSD curve by monitoring TDS daily for both treatment BBM and WW^{++}

2.2. Lipid extraction

The total lipid content was calculated in the final of incubation using chloroform : methanol (2:1) according to Bligh and Dyer. Lipid extracts were dried in oven at 80 °C in a pre-weighed glass vials. The values result of total lipids at the last day of the incubation period are shown in the following table (2).

Table (2) Total lipids for both treatment BBM and WW^{++}.

Parameter	Media	
	BBM	WW^{++}
Total lipids (mg/L)	33.2	48.37
Standard deviation (±)	4.56	5.44

3. Conclusion

Cultivation of microalgae using wastewater has received considerable attention around the globe as a platform to remove inorganic nutrients from the wastewater and producing microalgae biomass for biodiesel production. This can be done by converting freely available nutrients from the wastewater (particularly nitrogen, N and phosphorus, P) into microalgae biomass with concomitant carbon dioxide (CO2) sequestration via photosynthesis process. This research includes a new direction for technological improvements to improve the commercialization potential of microalgae biodiesel. Furthermore, microalgae biodiesel could help to reduce greenhouse gas (GHG) emissions to the atmosphere through the replacement of fossil diesel as it is made from renewable resources. Hence, this paper aims to reveal an in-depth analysis and discussions on the process of microalgae-based wastewater treatment as well as microalgae cells disruption technology to improve lipid extraction efficiency.

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