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Culture of Microalgae *Chlamydomonas reinhardtii* in Wastewater for Biomass Feedstock Production

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Abstract The objective of this research was to develop large-scale technologies to produce oil-rich algal biomass from wastewater. The experiments were conducted using Erlenmeyer flasks and biocoil photobioreactor. *Chlamydomonas reinhardtii* was grown in artificial media and wastewaters taken from three different stages of the treatment process, namely, influent, effluent, and centrate. Each of wastewaters contained different levels of nutrients. The specific growth rate of *C. reinhardtii* in different cultures was monitored over a period of 10 days. The biomass yield of microalgae and associated nitrogen and phosphorous removal were evaluated. Effects of CO_2 and pH on the growth were also studied. The level of nutrients greatly influenced algae growth. High levels of nutrients seem to inhibit algae growth in the beginning, but provided sustained growth to a high degree. The studies have shown that the optimal pH for *C. reinhardtii* is in the range of 7.5. An injection of air and a moderate amount of CO_2 promoted algae growth. However, too much CO_2 inhibited algae growth due to a significant decrease in pH. The experimental results showed that algal dry biomass yield reached a maximum of 2.0 g L^{-1} day^{-1} in the biocoil. The oil content of microalgae of *C. reinhardtii* was 25.25% (*w*/*w*) in dry biomass weight. In the biocoil, 55.8 mg nitrogen and 17.4 mg phosphorus per liter per day were effectively removed from the centrate wastewater. Ferric chloride was found to be an effective flocculent that helps the algae settle for easy harvest and separation from the culture media.

Keywords Microalgae culture · Biomass production · Feedstock · Oil yield · Biodiesel · Wastewater treatment · Nitrogen and phosphorus removal

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Introduction

Rapid growth in the biofuels industry recently has put tremendous pressure on food and animal feed supplies and agricultural land uses. In order for the biofuels industry to sustain and continue to grow, new non-food or non-feed biomass feedstocks must be explored and developed. The oil content and biomass production from algae is far superior to that of terrestrial plants such as soybean, corn, etc. [1–5]. Living in an aquatic environment, algae have better access to resources such as water, CO_2, mineral, and nutrients compared with terrestrial plants. Some algal species have 50–60% (dry biomass weight) of their total mass dedicated to lipids [6, 7]. The Aquatic Species Program sponsored by the US Department of Energy [6] estimated that algal oil yield of over 10,000 gallons per acre per year is possible compared with 50 to 100 gallons per acre per year for traditional oil crops such as soybean. Algal biomass produces both lipids that can be used to make biodiesel and starch for production of ethanol. High algal growth rates and oil content indicate that algal biomass has the potential to be used for biofuel production. Production of algae as biomass feedstock would not compete with food and feed crop land uses [8–11].

Human activities have increased the entry of chemical and biological contaminants, particularly nitrogen and phosphorus, into the water system through agricultural practices, urbanization, and industrialization. These activities have been shown to cause eutrophication of aquatic systems [12]. Numerous international studies have focused on nitrogen and phosphorus removal from wastewater using both physical and chemical methods [12, 13], which is costly and technically challenging. Recent studies demonstrated that microalgae have the potential for removing nitrogen and phosphorus from wastewater. These nutrients can be incorporated into algae cell biomass and subsequently removed from the wastewater. Algal treatment of wastewater, mediated through a combination of nutrient uptake, elevated pH, and dissolved oxygen concentration, can offer a more ecologically safer, cheaper, and more efficient means to remove nutrients and metals from wastewater than conventional tertiary treatment [13–16]. With increasingly stringent regulations and limits on wastewater discharge, modification of conventional processes must be made to meet these new requirements. These process modifications will require substantial capital investment and would also likely incur higher operating costs.

Built on previous research, the present study took a creative approach coupling the removal of nitrogen and phosphorus from wastewaters, with the culturing of algae to reduce the cost of algal biomass and oil production and wastewater treatment. This creates a win–win situation, which may enhance the economic viability of making algae as a biomass feedstock for biofuels production.

Materials and Methods

Algae Culture

Chlamydomonas reinhardtii was supplied by the Chlamydomonas Center at the University of Minnesota (Minneapolis, MN, USA) and cultured under continuous light (120 μmol photons per square meter per second) at 25±1 °C. A volume of 10% of algal seed was inoculated into 100 mL of culture media and expanded to 500 mL and then to 2 L to provide enough algae for the biocoil.

Algal Culture Media

Artificial and wastewater culture media were used in this study. Algae were cultured in the artificial culture media containing the following ingredients: $NaNO_3$, 2.94 mM; $CaCl_2{\cdot}2H_2O$, 0.17 mM; $MgSO_4{\cdot}7H_2O$, 0.30 mM; K_2HPO_4, 0.43 mM; KH_2PO_4, 1.29 mM; NaCl, 0.43 mM; and $CaCO_3$, 0.05 mM. The quantities of other minor ingredients including soil water, GR^+ medium, vitamin B_{12}, biotin vitamin solution, and thiamine vitamin solution used in the media followed the recipes provided in the Culture Collection of Algae of University of Texas [17] (http://www.utex.org). Wastewaters, namely, influent, effluent, and centrate, were taken in different stages of the treatment from the St. Paul Metro plant (St. Paul, MN, USA). They varied in the amount of N and P as shown in Table 1. Trace elements were added and included the following ingredients: $Na_2EDTA{\cdot}2H_2O$, 2 mM; $FeCl_3{\cdot}6H_2O$, 0.36 mM; $MnCl_2{\cdot}4H_2O$, 0.21 mM; $ZnCl_2$, 0.037 mM; $CoCl_2{\cdot}6H_2O$, 0.0084 mM; and $Na_2MoO_4{\cdot}2H_2O$, 0.017 mM.

Growth in Erlenmeyer Flasks

Artificial media containing trace element ingredients in 100- and 200-mL volumes were introduced to 250- and 500-mL Erlenmeyer flasks, respectively, for inoculation. Five thousand-milliliter flasks containing 2,000 mL of different kinds of wastewaters with added trace elements were used in the algal culture. The growth flask tests were carried out in triplicate at 25±1 °C under continuous illumination with six Gro-Lux agricultural fluorescent lights which was maintained using 0.2 M NaOH and flushed mixed gases of air and CO_2 with 0.05 L min^{-1}. This lighting provided an approximate irradiance of 120 μmol photons per square meter per second measured with a Li-1400 model LI-190 Quantum meter sensor. The lights were 45 cm above the flasks. All of the flasks were manually mixed four times daily.

Algal Growth in the Biocoil

The centrate in its full strength was used for scale-up experiments using the biocoil. The biocoil was constructed from two 16-m-long clear polyvinyl tubes (19-mm ID, 25-mm OD, and 9.0-L volume) coiled around a 1.4-m-high and 0.4-m diameter seven-iron bar frame. The total volume of the algae culture solution was 15 L, 9 L of which was contained in the coil and the other 6 L was held in the storage container which was maintained using 0.2 M NaOH at 25±1 °C. Light was supplied from inside the coil by six 1.2-m-high Gro-Lux agricultural fluorescent lights. The lights were placed 3 cm away from the reactor tubing. This lighting provided an approximate irradiance of 220 μmol photons per square meter per

Table 1 The chemical compositions in the wastewater from the Metro Plant.

Compositions	Influent	Effluent	Centrate
TKN[a] (mg/L)	64.00	14.30	128.60
NH_3 (mg/L)	49.92	8.78	67.00
TP[b] (mg/L)	6.92	1.25	120.60

[a] Total Kjeldahl nitrogen

[b] Total phosphorus

second measured with a Li-1400 model LI-190 Quantum meter sensor [18–20]. Dayton carbonator pump was employed to circulate the solution of algae with 1.8-L min^{-1} flow rates.

Algae Harvest and Separation

Algal cultures were harvested by mixing ferric chloride stock solution with 100 g Fe^{+++} per liter at different concentrations (Table 2) with the algae cultures. The solution was placed in square containers mounted to a Phipps & Bird model 7790-400 stirrer. The solution was homogenized at 300 rpm for approximately 30 s, after which it was continuously mixed at 20 rpm for 10 min. After mixing, the solution was then poured into 1 L graduated cylinders, respectively, to observe separation and settling time.

Analytical Methods

Optical densities were measured at 680 nm using GenesysTM-5 spectrophotometer. The specific growth rates of the cultures were determined by calculating the doubling time in exponential growth from semi-log cell density plots. The specific growth rate (μ) was determined using the equation:

$$\mu = \frac{Ln(N_2/N_1)}{t_2 - t_1}$$

The growth rate calculation is according to the equation, where N_1 and N_2 are defined as biomass at time 1 (t_1) and time 2 (t_2), respectively.

The PH value of the culture solution was measured with an Accumet AB15 pH meter. Cell densities were determined with a Neubauer hemocytometer. The total Kjeldahl nitrogen (TKN) was determined using the digestion and titration method and TP using the stannous chloride method [21]. The total dry biomass was determined as follows: Whatman glass (2.5 cm) filters were dried in 80 °C oven for 4 h and weighed to four decimal places on an analytical balance. Twenty milliliters of algal culture was filtered until the filters were dry using a Millipore filtration apparatus. The filters were then washed with 10 ml of 0.65 M ammonium solution to remove excess salts and dried at 80 °C for 4 h and weighed to one ten-thousandth of a gram.

Algal Oil Content Determination

Total algal oil content was determined using the method of Kates and Volcani [22]. A dry algal sample 5–6 g was extracted with 250 mL acetone using a Soxhlet extractor. The

Table 2 Dosages of Fe^{+++} added for algae separation.

Jars series	Algae sample volume (L)	Fe^{+++} (100 g/L) added (mg/L)	Sediment time (min)
1	1	0.00	–[a]
2	1	0.05	–[a]
3	1	0.10	35
4	1	0.25	15

[a] The algal cells did not settle down

sample was extracted for 2.5 h at a condensation rate of two to four drops per second. The thimble was dried at 80 °C for 4 h to a constant weight. Percent oil was determined by mass difference.

Results and Discussion

Algae Growth in Different Wastewater Media

The composition of wastewater discharged from industrial facilities and households is complex and varies continuously. Nitrogen and phosphorus are two main components in wastewater, which are capable of supporting algae growth. The algae were inoculated into the influent, the effluent, and the centrate. The centrate was diluted to three different concentrations so that the effect of nutrient levels on the growth could be studied. The results are shown in Fig. 1. The slowest growth rate was found on the effluent. Algae growth on the effluent began to decline after 7 days. This may be attributed to the initial low level of nutrients in the effluent where most of nutrients had been removed during the treatments in the wastewater treatment plant. Algae cannot grow without a plentiful supply of N and P. Growth rate in the influent was slightly faster than in the effluent. The likely cause of this is that the concentrations of N and P were higher in the influent than the effluent. For the centrate, the growth characteristics varied with dilution levels. At 100%, the growth on the centrate was slow in the beginning, but increased after 96 h where it reached an OD of 2.5. At 50% of the centrate, the growth was rapid in the beginning and remained constant after 7 days. The growth at 75% of the centrate was in between that in 50% and 100% of the centrate. It appeared that there were some initial inhibitory factors in the centrate. After a few days of growth, either the inhibitory factors diminished or the algae adjusted to the adverse conditions in the centrate. Figure 2 shows the results obtained when *C. reinhardtii* was cultured on 100% centrate in the biocoil for 1 month on a

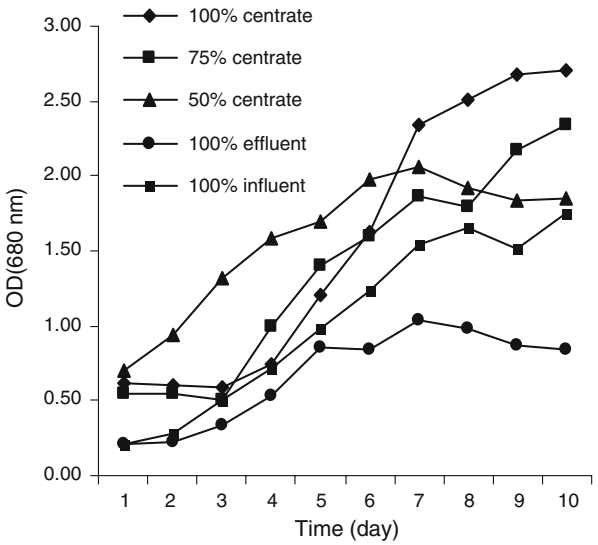

Fig. 1 The growth curve of *C. reinhardtii* algae in different kinds of wastewaters in batch cultivation

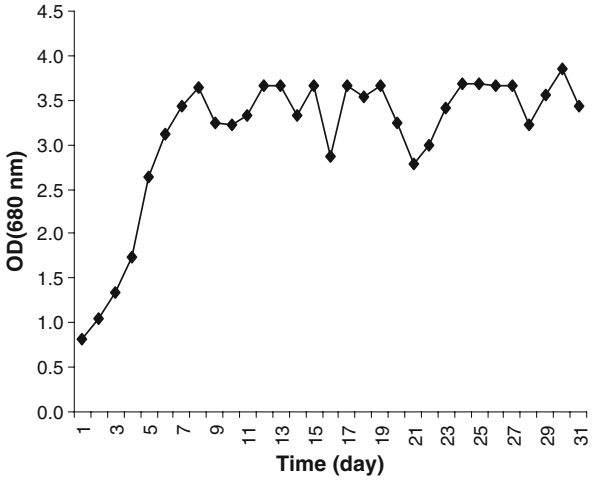

Fig. 2 Growth curve of *C. reinhardtii* grown in 100% centrate wastewater in the biocoil

continuous basis. Four liters of the cell suspension sample was taken out for analysis once per day, and the same amount of the centrate was added back to the reactor after each sample was taken. *C. reinhardtii* grew very fast in the first 9 days and maintained approximately the same growth rate for the remainder of the experiment.

Table 3 also shows that *C. reinhardtii* grown in the biocoil system had a higher dry biomass yield (2.0 g L^{-1} day^{-1}) and significantly higher cell densities and oil content than in the flasks systems. This may be attributed in part to a greater light exposure and intensity inside the polyvinyl tubing compared to the exposure of the cultures in the conical flasks. Meanwhile, the circulation of algae culture solution improved the situation of algae growth in the biocoil than in the flasks.

Effects of pH and CO_2 Level on Algae Growth

Under controlled light and temperature conditions, *C. reinhardtii* grew best at pH7.5, which was maintained using 0.2 M NaOH (Fig. 3) on 100% centrate in flasks, [3, 23, 24]. A mixture of air and CO_2 was injected into the bottoms of medium through aeration pipes to provide an additional carbon source at 0.05 L m^{-1} with the rubber stopper. The effect of CO_2 on algae (*C. reinhardtii*) growth is shown in Fig. 4 (on 100% centrate in flask). Air and 33.33% CO_2 (*v*/*v*) seemed to increase growth, while higher levels of CO_2 caused a pH decrease, resulting in stunting growth rates and potential lethal harm. Another factor that

Table 3 Productivity and N, P removal of *C. Reinhardtiiin* in flasks and coil-PBR.

The types of PBR	Specific growth rate (day^{-1})	Cell concentration (cells mL^{-1})	N removal (mg L^{-1})	P removal (mg L^{-1})	Productivity (g L^{-1} day^{-1})	
					Biomass	Oil
Flasks	0.346±0.06	5.4×10^7	40.36±0.04	15.08±0.02	0.82±0.04	0.136±0.01
			22.18±0.04	13.12±0.03		
Biocoil	0.564±0.05	7.6×10^7	55.80±0.03	17.40±0.04	2.00±0.03	0.505±0.02

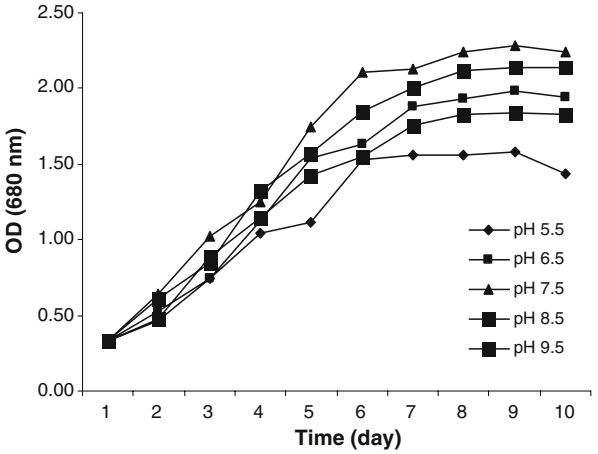

Fig. 3 The results of *C. reinhardtii* growth in 100% centrate with different pH conditions in batch cultivation

must be considered is the agitation effect of gas bubbling. Air flushed into the medium increased the growth rate of *C. reinhardtii*, which may be attributed to the mixing effect created by the turbulent air. This also minimized the amount of unstirred layers and increased gas exchange and nutrient availability [25] and increases the amount of light the algae cells receive.

Removal of Nitrogen and Phosphorus from Wastewater

In Erlenmeyer flasks, N and P concentrations in the centrate decreased with time (Figs. 5 and 6). We observed 42.2% to 55.0% removal of N and 12.5% to 15.4% removal of P, depending on two different levels of the initial concentrations of NH_4–N and PO_4–P in the centrate wastewater after 10 days of culturing. In the biocoil, approximately 83.0% of the N and 14.45% of the P were removed from the centrate wastewater with 100% concentration

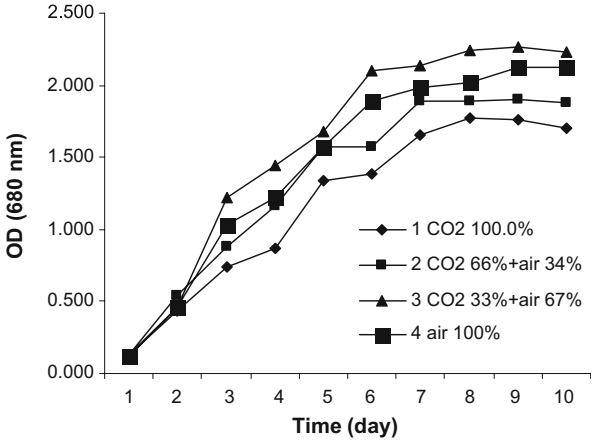

Fig. 4 The results of *C. reinhardtii* growth in 100% centrate with different CO_2 concentrations in batch cultivation

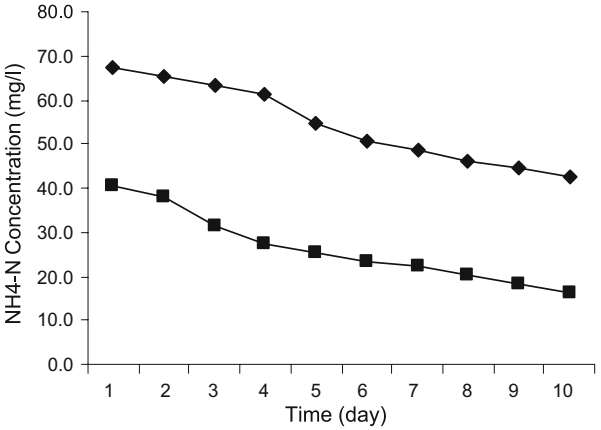

Fig. 5 Changes in NH_4–N *C. reinhardtii* growth in 50% centrate on batch cultivation

(Table 3). The main reason for less removal of initial N and P loadings could be the lower growth rate caused by the limited light transmission so that not enough algae cells absorbed N and P used to produce the biomass. Furthermore, other parameters such as N/P ratio may not be optimized for the best performance of *C. reinhardtii* in terms of N and P removal in the Erlenmeyer flasks compared with the biocoil.

Algae Oil Yield

The microalgae species used in the experiment, *C. reinhardtii*, contained 25.25% oil in the biocoil and 16.6% in the flasks (Table 3). The oil content was determined through extraction procedures previously described. While this percentage is less than desired for large-scale biofuel production, this was not the sole focus of the project. The experimental objective was to find a microalgae that could treat wastewater, produce large amounts of biomass, and generate enough oil to be used in biofuel production. Future studies will focus on identifying algae species with higher oil contents.

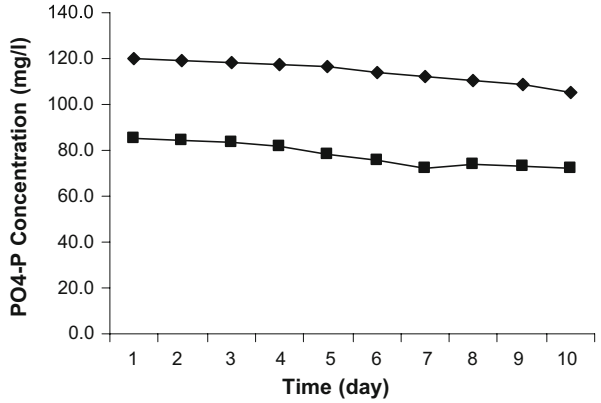

Fig. 6 Changes in PO_4–P *C. reinhardtii* growth in 100% centrate on batch cultivation

Algae Harvest and Separation

One of the major problems in the cultivation of unicellular algae as biomass feedstock for biofuel and oil production is the lack of economical methods for harvesting the relatively dilute suspensions. These unicelluar algae are 5–10 μm in diameter. In our study, ferric chloride was used as reagent for algal cell flocculation. Our experiments showed that ferric chloride addition can effectively precipitate algae and allow it to settle out (Table 2). A higher ferric chloride dose produced a much clearer supernatant. In terms of estimating improved solids capture, the optical densities were 2.58, 2.16, 0.20, and 0.08 at 680 nm as Fe^{+++} increased from 0.00, 0.05, 0.10 to 0.25 mg L^{-1}, respectively. This corresponded to 96.9% and 92.2% reductions in $OD_{680\ nm}$, presumably with proportional reductions in algal mass due to chemical addition and settling. However, the separation costs and process alternatives still need to be thoroughly investigated and analyzed.

Conclusions

The primary objective of this study was to develop creative methods to produce an economically and environmentally sustainable feedstock from algae grown on nutrients from wastewater. This is expected to create a win–win situation where water condition is improved through the elimination of phosphorus and nitrogen, while renewable energy is generated using the oil from the algae to produce biofuels. In this study, only one species of algae was screened and tested. Future experiments will focus on large-scale cultivation and biomass production for the purposes of nitrogen and phosphorus removal from the wastewater which can be accomplished using other species. The biomass yield of algae species *C. reinhardtii* reached a maximum of 2.0 g (dry weight) per liter per day and an oil content of 25.25% when cultured in the wastewater using the biocoil. Experimental results indicated that *C. reinhardtii* can remove up to 55.8 mg nitrogen and 17.4 mg phosphorus per liter per day in the biocoil over a period of 1 month.

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