Production of biodiesel from wastewater grown *Chlorella minutissima*

Shailendra Kumar Singh, Ajay Bansal*, M K Jha & Rahul Jain
Department of Chemical Engineering, Dr. B. R. Ambedkar National Institute of Technology, Jalandhar 144 011, India

Received 30 April 2012; accepted 22 July 2013

The present study has been undertaken to assess integrated algal harvesting in sewage wastewater for biodiesel feedstock. *Chlorella minutissima* microalgae are grown in wastewater from local hotel sewage drain and produced biomass is used to produce biodiesel. *C. minutissima* effectively removes the wastewater nutrients nitrogen and phosphorus with the 99.19% and 96% removal respectively. Content of oil in produced algal biomass is drastically reduced i.e. 4% in response to 57% of total dry biomass reported in literature. The extracted oil mainly consists of small chain fatty acids (C-16:0, C-18:1, C-18:2, C-18:3). Transesterification reaction shows that maximum fatty acid methyl ester (FAMEs) concentration is converted from free fatty acids of oil during 1-2 h at 65°C.

**Keywords:** Algae, Biodiesel, *Chlorella minutissima*, Sewage, Transesterification, Wastewater treatment

During last two decades, due to rapid industrialization and economic development, energy crisis, pollution and global warming are emerged as most important challenges in front of world. They are interlinked proportionally and have led to an increased awareness regarding necessity to adopt sustainable solution for energy alternatives with less pollution rate. Sustainability and environmental concerns are major issues in the world today. It is now being taken seriously because of escalating price of petroleum and, more significantly, emerging concern about global warming that is associated with burning fossil fuels. Biofuel is one of the sustainable energy options that can contribute future reductions in ecological footprint of human energy requirements. However, current biofuel feedstocks are not sufficient to replace fossil fuels entirely. New sustainable scientific solutions are needed to increase the production of biofuels; minimize negative impact on environment or food supply; and to use less energy to grow, extract and burn fuels than total energy they supply. Algae are among the most promising and renewable non-food-crop-based alternative. They possess several characteristics that could propel them to the forefront of the renewable fuels industry and provide sustainable solution for energy crisis, air pollution and climate. Compared with terrestrial plants, algae have a high oil content and growth rate; mass algal cultivation can be performed on unexploited lands using saline water in arid regions, thus avoiding competition for limited arable lands. Algae can also act as a sink for carbon dioxide because they absorb it for photosynthesis. At present algal biofuel production has not been commercialized due to high costs associated with production, harvesting, and preparation for biodiesel. Harvesting cost is one of the major limitations on viability of this technology. Algae harvesting in wastewater may be a significant advance towards reduction in constraints on economic viability. Utilizing harmful nutrients (primarily nitrogen and phosphorus) as a food source, algae hold promise of being able to accomplish nutrient removal with a net energy savings to water treatment system. Biodiesel production from wastewater grown algae could be commercially viable and ecologically sustainable solution for mitigating grave threats of global energy demand. According to biodiesel standard published by American Society for Testing Materials, biodiesel from micro algal oil is similar in properties to standard biodiesel, and is also more stable according to their flash point values. Among potential algal species, *Chlorella minutissima* microalgae have been proved to have high lipid content (57% of dry weight biomass) as well as its nutrient removal potential to treat wastewater in their studies. The idea of using algae as a source of fuel is not new but very few literature are found.

* Corresponding author.
E-mail: bansala@nitj.ac.in
regarding biodiesel from wastewater grown algae. The main objective of present work is to evaluate the potential of wastewater grown microalgae *C. minutissima* for biodiesel feedstock. It may provide new insights into the promising directions for treating wastewater using microalgae with biodiesel production.

**Experimental Procedure**

**Chemicals**

For transesterification and extraction experiments, sulfuric acid (98%), and HPLC grade chloroform, n-hexane, methanol were obtained from Sigma Aldrich (St. Louis, MO) and used as received. Individual fatty acid methyl ester standards (all greater than 95% purity) including methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C 16:0), and methyl docosahexaenoate (C 22:6) standards were obtained from Sigma Aldrich and used for gas chromatography calibrations. For culture media, chemicals were obtained from BD biosciences (San Jose, CA). Double distilled water was used for stock and working solution preparation.

**Algal strain and wastewater samples**

A pure strain of *Chlorella minutissima* was procured from Centre for Blue Green Algae, Indian Agricultural Research Institute (IARI), New Delhi, India. Algal strain was grown in a complex BG-11 medium on tissue culture rack (Vista Biocell Pvt. Ltd., India) adjusted at pH of 7.0 under an illumination 6500 ± 300 lux with regime 16:8 h (light: dark) at 27 °C. Sewage wastewater was taken from the drain of local hotel. After collection wastewater samples were filtered from Whatman filter paper No. 1 in order to remove large suspended solid particles and then exposed in UV light for sterilization.

**Algal growth in sewage wastewater**

*C. minutissima* cells were grown photo–autotrophically in different ratio of sterilized sewage wastewater to distilled water (20:80, 40:60, 60:40, 80:20 and 100:0) under above mentioned conditions. *C. minutissima* growth in experimental samples was measured by the estimation of chlorophyll ‘A’ concentration (mg.L⁻¹) in algae using hot methanol extraction method. The flasks were incubated statically at 27 ± 2 °C for 7 days. Experimental flasks (500 mL) were shaken twice in a day to facilitate proper mixing.

**Nutrients removal analysis**

The removal of nutrients (nitrogen and phosphorous) in wastewater was measured spectrophotometrically using LaMotte water analysis kits at the end of 15 days. Total nutrient removal was calculated by following formula:

\[
\text{Nutrient removal} = \left(1 - \frac{N_e}{N_o}\right) \times 100 \quad \text{(1)}
\]

where \(N_o\) and \(N_e\) are the concentrations of nutrient at first day and last day of experiment respectively.

**Algal oil extraction**

*C. minutissima* cells were collected from all experimental flasks. Algal cells residues were twice centrifuged (3000 g, 10 min) and washed with double distilled water. Wastewater grown algal biomass (2.5 g) was subjected to hydro-distillation for 4 h in closed type Soxhlet extractor apparatus (Fig. 1) using methanol as a solvent. The pale yellow colored oil was collected with solvent. Solvent was evaporated using vacuum evaporator apparatus. Amount of extracted oil was calculated by following formula:

\[
W_a = W_o - W_r \quad \text{(2)}
\]

where \(W_a\), \(W_o\) and \(W_r\) all weights of extracted algal oil (g), oil with solvent after extraction (g) and recovered solvent (g) from evaporation respectively.

**Fatty acid analysis of algal oil**

Extracted algal oil constituents were analyzed using a Gen Tech (HP 5890 GC coupled with 5972 MSD) GC-MS system equipped with flame ionization detector using CP-SIL-5 capillary column (50 m x 0.32 mm i.d., 0.25 µm film thickness). Nitrogen was
used as carrier gas at 162.1 kpa inlet pressure with 3.0 mL/min purge flow. Temperature range used was 80 - 230 °C. The total program time of instrument was 70 min. The injector and detector temperatures were 270 °C and 280 °C respectively. The injector volume of sample was 0.2 µL. The oil was injected neat with split injection mode having split ratio of 1:80. The final confirmation of constituents was made by computer matching of mass spectra of peaks with Wiley and NIST [National Institute of Standards (NIST), USA] libraries mass spectral databases. Relative amounts of individual components are based on GC peak areas.

**Transesterification of algal oil**

Acid value of extracted oil was measured using standard ASTM protocol. Measured acid value was more than 2, and hence acid catalysed transesterification method was carried out in round two-neck flask. Flask was heated to reaction temperature by using a constant temperature water bath. A standard reaction mixture consisted of 1:10 ratio of algal oil: methanol and 1% concentrated sulfuric acid was used. The reaction mixture was heated at 65 °C without stirring. Transesterification reaction in reaction mixture was monitored by measuring fatty acid methyl ester (FAMEs) composition which was converted from fatty acids of algal oil at different time intervals using HPLC (Waters, USA) apparatus. The FAME standards were prepared in a hexane solution at concentrations of 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 and 0.3 mg mL\(^{-1}\). This linear range of concentration standards was used to develop a standard curve equation by which all unknown fatty acid concentrations were determined. Octocosane, (C28:0) (0.1 mg mL\(^{-1}\)) was used as an internal standard. This was done to ensure sample measurements remained within calibrated range.

**Results and Discussion**

**Algal growth in wastewater**

*C. minutissima* cells growth in five wastewater dilutions (20, 40, 60, 80 and 100%) were measured and the results are plotted in Fig. 2. Algae in wastewater dilutions 40, 60 and 80% show similar growth curves. No lag phases were observed in all of five wastewater dilutions, indicating that *Chlorella minutissima* could adapt well in nutrient reach sewage wastewater. Similar growth patterns, with exponential phases in first 9 days followed by stationary phases in next 6 days are present for all dilutions, except pure wastewater, in which exponential phase lasted till 15 days. Results elucidate strong relationship between nutrient levels and algal growth. It is found that algal growth is significantly enhanced in wastewater without dilution because of its much higher levels of nitrogen and phosphorus than other four wastewater dilutions. Results of this study indicate that pure hotel sewage wastewater is best media for algal growth. Other studies have also shown that algal cells grow better in wastewater after primary settling than in the secondary effluent because of nutrients rich medium. Thus, algal ponds with high inoculums might be more suitable to be installed as a secondary rather than a tertiary treatment process. In addition, wastewater with very high nutrient load is found to promote rather than inhibit algal growth, which serves as basis for applying algal process in municipal water treatment plant in a new way to manage nutrient load.

**Nutrients removal analysis**

After 15 days of algal growth, treated pure wastewater sample was analyzed for nutrient removal. It is found that harmful nutrient ammonia-nitrogen is reduced (99.19%) significantly (Table 1). In fact, *C. minutissima* could use ammonia-nitrogen as
primary nitrogen source. The relative constancy of uptake, irrespective of nitrogen source, is tentatively considered to be due to use of ammonia-nitrogen for the production of amino groups which helps in nitrogenous metabolism. This homoeostasis of nitrogen assimilation enables it to maximize growth in changing environmental conditions.

More than 96% phosphorus is removed in wastewater at a comparable ratio before and after algal growth (Table 1). Results indicate that rapid uptake of phosphorus is an ability of algae species *Chlorella* to synthesize and accumulate polyphosphates in their bodies. Table 1 shows the inorganic N/P ratios of wastewater before and after algal cultivation. Compared to optimal inorganic N/P ratio (6.8–10) for algae growth, effluent shows N/P ratio of 10.41 which is in range, although it drops to 2.49 at the end of experiment.

However, unbalanced N/P ratio of wastewater affects neither nitrogen nor phosphorus removal, suggesting that both N/P ratios and absolute levels of N and P must be considered in evaluating the effects of nutrient compositions on algal growth. Other studies also reveal that nutrient removal efficiency is related to level of nutrients in wastewater and extent of nutrients utilized by algal growth or incorporated into algal tissues.

**Soxhlet extraction of wastewater grown algal biomass**

The yield of algal oil from wastewater grown algal biomass (2.5 g) by hydro-distillation method using closed type Soxhlet extractor apparatus is found to be 4% v/w (0.1 g). Algal oil content is very much less than that already reported (57%) in literature. Results suggest that cellular total fatty acid (TFA) content decreases due to high nitrogen concentration in wastewater. Therefore, nitrogen limitation could be an important factor to stimulate fatty acid biosynthesis in *Chlorella* cells for higher cellular TFA content. Nitrogen limitation decreases cellular glycolipid content in thylakoid membrane, which activates acyl hydrolase enzyme to stimulate hydrolysis of glycolipid and phospholipid, resulting in an increase in intracellular content of acetyl-CoA, which is used for enhanced fatty acid biosynthesis.

**Fatty acid analysis of algal oil**

GC-MS analysis of algal oil shows the presence of 4 major fatty acids and some minor compounds shown in Fig. 3. The major components in GC-MS are methyl palmitate (C16:0), at retention time 29.02 min, methyl oleate (C18:1) at retention time 28.21 min, methyl linoleate (C18:2) at 29.70 min and methyl linolenate (C18:3) at 27.62 min. Other studies show that *Chlorella* species contains majority of short-chain fatty acids (C14–C18) which are main components of biodiesel.

**Transesterification of algal oil**

Extracted oil has more fatty acid than triglycerides (acid value more than 2) and therefore acid catalyzed transesterification of extracted algal oil was carried out on water bath without stirring. Transesterification
reaction is analytically monitored by measuring converted FAMEs in reaction mixture using HPLC. HPLC spectra show peaks with retention time 0-5 min which are identified as FFA (free fatty acids), 5-10 min retentione time as FAME (fatty acid methyl ester), and some unidentified peaks at 10-15 min. Results provide information about relation between reaction time and FAMEs concentration. At longer reaction time FAMEs are converted back into fatty acids\(^{27}\). Maximum FAMEs (57% of total FFAs) are produced during 1-2 h reaction time (Fig. 4).

**Conclusion**

Present study shows that microalgae *C. minutissima* has potential to treat wastewater nutrients nitrogen and phosphorus effectively with percentage removal 99.19% and 96% respectively. N/P ratio drops from 10.41 to 2.49, showing a severe phosphorus limitation for algal growth. This suggests that both N/P ratios and the absolute levels of N and P must be considered in evaluating the effects of nutrient compositions on algal growth. The oil content of produced algae is reduced (4% of total dry biomass) from that already reported\(^5\) (57%), suggesting that nitrogen limitation is an important operational variable for high oil yield. Further optimization of nitrogen concentration in media composition is necessary for achieving a high TFA yield. It is low-cost and easy to manipulate compared to other factors influencing intracellular lipid formation. The extracted oil mainly consists of short chain fatty acids (C-16:0, C-18:1, C-18:2, C-18:3) which is an ideal combination for efficient biodiesel. So, it is vital to choose *C. minutissima* species as materials of biodiesel production. Analysis of monitoring transesterification reaction has shown that maximum fatty acid methyl ester (FAMEs) concentration converted from free fatty acids of oil is found in 1-2 h at 65 °C and 1% H\(_2\)SO\(_4\). Integrated algal harvesting in sewage wastewater for biodiesel feedstock appears to be a sustainable solution for environment and energy challenges.

**References**

4. Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels, ASTM D6751 - 11b, DOI: 10.1520/D6751-11B.