Productivity and Bioenergy Potential Assessment of Microalgae from Waste Stabilization Ponds

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Abstract: This study focused on the characterization of microalgae for bioenergy production and waste water treatment nutrient removal (from waste stabilization ponds). The dry biomass, lipid content and fatty acids in the microalgae were evaluated using centrifuge, Solvent extraction method and Gas Chromatography analysis method respectively. The Diatom numulloides sp with high lipid content and high productivity makes it suitable for future development by growing it in photo bioreactors to treat both municipal and industrial waste water and to produce biofuel making more benefits to human beings.

Keywords: Microalgae from waste stabilization ponds, Diatom numelloides, Bioenergy from Microalgae, Photo Bioreator

1. Introduction

Microalgae are group of unicellular or simple multicellular photosynthetic microorganisms. It’s divided in four groups (red, green, brown and diatoms microalgae). Microalgae are one of most important plant in kingdom plants, it has high photosynthetic process. So algae can be grown very fast and it has high reproduction.

Microalgae can be used for pollution control and also as an energy source as well as food, fertilizers, cosmetics, pharmaceuticals and aquacultures. So microalgae cultivation is one of a good technology for wastewater treatment. Microalgae cultivation in wastewater is for wastewater treatment, pollution control and production of energy from biomass. Microalgae cultivation is also useful in solving the environmental problems such as global warming as it consumes carbon dioxide in Photosynthesis process to produce oxygen and glucose.

Algae can be grown in open ponds system, closed ponds system, photo bioreactors, marine environment and wastewater. However, closed pond system can be control by all the conditions such as CO₂, gases, water, sun light supply and fertilizers (for example: photo bioreactors). Additionally, the microalgae can be grown (green and brown) in both seawater and wastewater in closed pond system when all the conditions are under control. Studies on microalgae have been carried out since the 1980s [1], but in recent years their importance has grown fast, the reason is that microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. Moreover, compared to other biofuels sources, such as traditional crops and wood, microalgae have several advantages: they grow extremely fast, reaching high areal and volumetric productivities, they do not require arable land, being able to use waste water, they can directly capture CO₂ released by industries and they overtake the food vs. fuel debate and their ability to grow in a wide range of conditions,
resisting to severe temperatures, pH, and salinity makes them even more attractive. Recently, algae have received a lot of attention as a new biomass source for the production of biofuels.

2 Microalgae sample collection

The microalgae species were collected from different waste stabilization ponds are listed in the Table

| Sl.No | Location of the pond | Capacity of the pond (m³) | Purpose of the pond |
|-------|----------------------|--------------------------|---------------------|
| 1     | Oxidation pond 1 IITM, Chennai | 120000 | Sewage Treatment |
| 2     | Oxidation pond 2 IITM, Chennai | 120000 | Sewage Treatment |
| 3     | VIT – Pond Vandalur, Chennai | 11113 | Waste Water Treatment |
| 4     | Mangalam Pond Pulikundram | 183143 | Waste Water Treatment |
| 5     | Kankyo Cleantech Aminjikarai, Chennai | 132000 | Waste Water Treatment |
| 6     | Sewage Treatment Plant, Chithathur | 150000 | Sewage Treatment |
| 7     | Parry Nutraceuticals Bose road, Chennai | 3581 | Waste Water Treatment |

2.1 Identification of Microalgae Species

Identification of microalgae was carried out by means of microscopic analysis, using an optical microscope. The classification of species were identified by placing a drop of water to fill the well and place coverslip on top of slide and it were identified by recording the observations utilizing the pond identification sheet for quick reference.

2.2 Isolation of Microalgae Using Suitable Algal Media

All the algae strains for the present study were isolated and kept in Phychospectrum Environmental Research Centre (PERC) Laboratory. These algal strains were grown in 200 mL glass tubes (2.5 cm in diameter) with different nutrient medium at room temperature, and light intensity was set at 150 μmol/(m²·s) from the 4th day. The CO₂ concentration was set as 0.5% (by volume) in the bubbling air, and initial cell concentration was set at 0.5 (OD₆₈⁰). Microalgae were isolated using algal media listed in Table 2 depending on the type of microalgae species identified. Stock solutions were made up by accurately weighing the prescribed amount of nutrient and dissolving in a specified volume of distilled water in a volumetric flask. Some nutrients readily dissolved and others need heat and stirring to fully dissolve.
Table 2.2 BBM, BG-11 & PAAP Nutrient Medium Ingredients

| Ingredients | BBM Media (mg/L) | BG - 11 Media (mg/L) | PAAP Media (mg/L) |
|-------------|-----------------|---------------------|-------------------|
| NaNO3       | 025.00          | 150.00              | 002.00            |
| CaCl2.2H2O  | 002.50          | 036.00              | 00.540            |
| MgSO4.7H2O  | 007.50          | 075.00              | 00.500            |
| K2HPO4      | 007.50          | 040.00              | -                 |
| KH2PO4      | 017.50          | -                   | 00.780            |
| NaCl        | 002.50          | -                   | -                 |
| Citric acid | -               | 006.00              | -                 |
| Ferric ammonium citrate | - | 006.00 | - |
| EDTA (disodium salt) | - | 001.00 | - |
| Na2CO3      | -               | 020.00              | -                 |
| Trace metals solution | BBM Media (g/L) | BG - 11 Media (mg/L) | PAAP Media (mg/L) |
| ZnSO4.7H2O  | 008.82          | 022.20              | 0.008             |
| MnCl2.4H2O  | 001.44          | 181.00              | 0.060             |
| MoO3        | 000.71          | -                   | -                 |
| CuSO4.5H2O  | 001.57          | 079.00              | 0.002             |
| Co(NO3)2.6H2O | 000.49        | 049.40              | -                 |
| H3BO3       | -               | 286.00              | -                 |
| NaMoO4:2H2O | -               | 039.00              | -                 |
| Na/SiO3     | -               | -                   | 0.700             |
| fl-glycerophosphoric acid | - | - | 0.100 |
| FeC13 - 6H2O | - | - | 0.020 |
| CoCl2. 6H2O | - | - | 0.004 |
| Na2MoO4.2H2O | - | - | 0.138 |
| Cyclo acid (buffer) | - | - | 50.00 |

The composition of f/2 Nutrient Media are represented in Table 3 with the concentration of Trace Metal Solutions
Table 2.3 f/2 Nutrient Medium Ingredients

| Ingredients                        | f/2 Media (mg/L) |
|------------------------------------|------------------|
| NaNO₃ (75.0 g/L dH₂O)              | 01.00            |
| Na₂HPO₄·H₂O (5.0 g/L dH₂O)         | 01.00            |
| Na₂SiO₃·9H₂O (30.0 g/L dH₂O)       | 01.00            |
| f/2 Trace Metal Solution           | 01.00            |
| f/2 Vitamin Solution               | 00.50            |
| Vitamin Solution                   |                  |
| 0.5ml/1L Distilled water           |                  |
| Vitamin B12 (1.0 g/L dH₂O)         | 001.00           |
| Biotin (0.1 g/L dH₂O)              | 010.00           |
| Thiamine HCl                       | 200.00           |
| Trace Metal mix                    |                  |
| 1ml/1L Distilled water             |                  |
| FeCl₃·6H₂O                         | 03.15            |
| Na₂EDTA·2H₂O                       | 04.36            |
| CuSO₄·5H₂O (9.8 g/L dH₂O)          | 01.00            |
| Na₂MoO₄·2H₂O (6.3 g/L dH₂O)        | 01.00            |
| ZnSO₄·7H₂O (22.0 g/L dH₂O)         | 01.00            |
| CoCl₂·6H₂O (10.0 g/L dH₂O)         | 01.00            |
| MnCl₂·4H₂O (180.0 g/L dH₂O)        | 01.00            |

2.3 Growth of Microalgae
In this study, the microalgae were grown in conical flasks where unicellular algae grow continuously by a process known as cell division. Each cell enlarges and divides into two daughter cells that subsequently grow and divide yielding a culture that increases exponentially. Growth slows as the algal population becomes more crowded. Most oil-producing microalgae have an optimal growth temperature of 25 to 30°C. Selection of algal strains grown at high temperatures, such as 40°C, may be needed, for example, in South China, Thailand, etc. The lipid content of microalgae is strongly influenced by temperature and depends on the strains. An increase in temperature from 20 to 25°C will double the lipid content of Nannochloropsis oculata (from 7.9 to 14.9%), while an increase from 25 to 35°C caused a decrease in the lipid content of Chlorella vulgaris, from 14.71 to 5.90% [8].

Nutrients are expected to be depleted, metabolites build, and light penetration decreases because of self-shading. The cultures that have reached their stationary phase for the current conditions will not increase in density. The time for algae to divide and the maximum density attained depend upon several factors such as pH, Water temperature, Salinity, Nutrients and
CO₂ Aeration. Growth of unicellular algae was measured in terms of cell numbers in a given volume of water.

2.4 Characterization of Microalgae Species
The algae was isolated and transferred to culture room and a day later the growth of algae was monitored by counting the cells through a hemocytometer. Microalgae were characterized by quantifying the Lipid content, Protein and carbohydrates quantity. It produces many different kinds of lipids, triglycerides and diglycerides, phospholipids and glycolipids, hydrocarbons and others [19]. The dry biomass weight was determined by obtaining pellet of the species grown in the nutrient media in cake formation after 20 days and 40 days from isolation procedure from which the dry biomass weight is obtained after evaporation of moisture content with the help petri plates and that dry biomass is weight in mg/L.

2.5 Solvent Extraction of Lipids
The biomass pellet was transferred to an 8mL vial glass using methanol acetyl chloride. After adding 1mL hexane, the vials were capped tightly and the lipids were extracted at 100°C for 60 minutes. The content was transferred to 15mL centrifuge tube and centrifuged at 1800g for 5 min at room temperature. The upper layer is separated and weighed to 0.1mg precision to determine the amount of lipids extracted.

2.6 FAME Analysis
To determine the fatty acids, analysis was carried out using Gas Chromatography equipped with a flame ionization detector with Mass spectrometer. 30mg biomass was extracted with 2mL of methanol acetyl chloride as internal standard (IS) to the extraction mix and the samples were heated at 100°C for 60min. the upper layer was collected and filtered using syringe filter prior to analysis by Gas Chromatography. Separation was achieved on a capillary column and the temperature was programmed to hold at 50°C for 1 min and then rise to 175°C.

3. Results and Discussions
The type of microalgae species obtained from different waste stabilization ponds are listed in Table 3.1. The microscopic image of each species is shown in Figure 3.1

| Name of the Pond                              | Name of the species obtained            |
|-----------------------------------------------|----------------------------------------|
| Oxidation pond, IIT Madras                    | Chlorococcum sp                        |
| VIT Sewage treatment pond, Vandalur           | Diatom numuloides sp                   |
|                                             | Chlorococcum sp                        |
|                                             | Diatom Muelleri sp                     |
|                                             | Diatom Scutellum sp                    |
| Sewage Treatment Plant, Perungudi             | Diatom Platycephala sp                 |
|                                              | Diatom Lyra sp                         |
|                                              | Anthrospira sp                         |
| Location                        | Algae Species                  |
|--------------------------------|--------------------------------|
| Sewage Treatment Plant, Chithathur | Oscillatoria sp                |
| Sewage Treatment Plant, Pulikundram | Chlorella Vulgarisis sp        |
| Parry Nutraceuticals, Chennai   | Chlorella Vulgarisis sp        |
|                               | Spirulina sp                   |

(a) Chlorococcum sp  
(b) Spirulina & Diatom sp  
(c) Chlorococcum sp  
(d) Diatom sp  
(e) Anthrospira sp  
(f) Oscillotoria sp
The microalgae species obtained from different waste stabilization ponds varies with respect to its growth factor such as Nitrogen and Phosphorus concentration. It is observed that Diatom species and Chlorococcum species are commonly found in waste stabilization ponds which are rich in nutrients as shown in Table 4. The Diatom species was obtained diversely in the Sewage Treatment Pond in Perungudi which has Total Nitrogen content and Phosphorus of $92.80\text{mg/L}$ and $26.30\text{mg/L}$ respectively which is the highest among all the other ponds in this study. Similarly the Total Nitrogen content and Phosphorus in IIT Madras Oxidation Pond is $49.30\text{mg/L}$ and $19.20\text{mg/L}$ which stands next to Perungudi sewage treatment pond in Nutrient Concentration with the same Chlorococcum species.

### 3.1 Cell Growth Count of Microalgae Species

The growth of algae was monitored by counting the cells through a hemocytometer where the count was observed and represented in Table 3.2 from average of 4 quadrants.

| Name of the Species                        | (Initial stage) cells/mL | (20 days Growth) cells/mL | (40 days Growth) cells/mL |
|-------------------------------------------|--------------------------|---------------------------|---------------------------|
| Chlorococcum sp                           | $64.00 \times 10^4$     | $92.40 \times 10^4$      | $153.00 \times 10^4$     |
| Diatom numulloides sp                     | $25.50 \times 10^4$     | $66.20 \times 10^4$      | $106.00 \times 10^4$     |
| Chlorococcum sp                           | $62.00 \times 10^4$     | $86.00 \times 10^4$      | $92.00 \times 10^4$     |
| Diatom mulleri sp                         | $36.00 \times 10^4$     | $41.00 \times 10^4$      | $149.00 \times 10^4$     |
| Diatom surtellum sp                       | $39.00 \times 10^4$     | $52.00 \times 10^4$      | $162.00 \times 10^4$     |
| Diatom Plateycephala                      | $43.00 \times 10^4$     | $62.00 \times 10^4$      | $96.00 \times 10^4$     |
| Diatom Lyra sp                            | $52.00 \times 10^4$     | $82.00 \times 10^4$      | $106.0 \times 10^4$     |
| Anthrospirasp                             | $19.00 \times 10^4$     | $26.00 \times 10^4$      | $86.00 \times 10^4$     |
| Oscillotoriasp                            | $10.00 \times 10^4$     | $32.00 \times 10^4$      | $97.00 \times 10^4$     |
| Chlorella sp                              | $72.00 \times 10^4$     | $106.00 \times 10^4$     | $162.00 \times 10^4$     |
| Spirulina sp                              | $62.00 \times 10^4$     | $86.00 \times 10^4$      | $102.00 \times 10^4$     |

### 3.2 Dry Biomass Productivity of Microalgae Species

The dry biomass weight was determined after 20 days and 40 days from isolation procedure and weighed in mg/L as tabulated in Table 3.3.
Table 3.3 Dry Biomass Weight of Microalgae species

| Name of the Species          | (20 days Growth) | (40 days Growth) |
|------------------------------|------------------|------------------|
|                              | mg/L             | mg/L             |
| *Chlorococcum sp*            | 0.01             | 132.00           |
| *Diatom numulloides sp*      | 0.30             | 109.50           |
| *Chlorococcum sp*            | 18.50            | 175.50           |
| *Diatom mulleri sp*          | 41.55            | 408.82           |
| *Diatom surtellum sp*        | 20.45            | 374.40           |
| *Diatom Plateycephala*       | 14.96            | 198.70           |
| *Diatom Lyra sp*             | 21.91            | 302.45           |
| *Anthrospirasp*              | 10.25            | 182.75           |
| *Oscillotoriasp*             | 0.55             | 217.34           |
| *Chlorella sp*               | 27.50            | 475.23           |
| *Spirulina sp*               | 15.99            | 132.41           |

The dry biomass weight of *Chlorococcum* species after 40 days growth is 132.00mg/L which was obtained from Oxidation pond in IIT, Madras. The dry biomass weight of *Chlorococcum* species is 280.00mg/L [1]. The dry biomass weight completely depends upon the cell growth count and the concentration of Nutrients in the media. The cell growth count of *chlorococcum* species in BBM Media is 153.00 cells/mL x 10⁴ as shown in Table 3.3

3.3 Lipid Content of Microalgae Species

The lipid content in each microalgae species were weighed to 0.1mg precision and determined in mg/L as shown in Table 3.4

Table 3.4 Lipid content of Microalgae Species

| Name of the Species          | Lipid Content (mg g⁻¹ dwt) (Trial 1) | Lipid Content (mg g⁻¹ dwt) (Trial 2) | Average Lipid Content (mg g⁻¹ dwt) |
|------------------------------|---------------------------------------|---------------------------------------|-----------------------------------|
| *Chlorococcum sp*            | 055.00                                | 056.00                                | 054.00                            |
| *Diatom numulloides sp*      | 130.00                                | 122.00                                | 126.00                            |
The lipid content of *Diatom numulloides* sp was found to be the microalgae species with maximum lipid content among other microalgae species in this study with 126.00 mg g\(^{-1}\) dwt in 40 days growth with dry biomass productivity 109.50 mg/L. The lipid content of same species is 92.30 mg g\(^{-1}\) dwt with dry biomass productivity 112.00 mg/L [1]. The study shows that the lipid content of the species is proportional to the dry biomass weight obtained.

### 3.4 Fatty Acid Content of Microalgae Species

The Fatty acid content in each microalgae species were weighed to 0.1 mg precision and determined in mg/L as shown in Table 3.5.

The Fatty acid content of *Diatom* sp was found to be the Microalgae species with maximum Fatty Acid content among other microalgae species in this study with 85.23 mg g\(^{-1}\) dwt in 40 days growth with dry biomass productivity 109.50 mg/L. The Fatty acid content of same species is 32.60 mg g\(^{-1}\) dwt with dry biomass productivity 170 mg/L [1]. The study shows that the Fatty Acid content of the species is proportional to the Dry biomass weight obtained.

The Lipid content and Fatty Acid analysis of obtained microalgae species varies with respect to the dry biomass weight gained after each day. The dry biomass weight depends on the growth of the same microalgae species and it is clear that higher the Nutrient concentration in media, higher the productivity.
Table 3.5 Fatty Acid content of Microalgae Species

| Name of the Species     | Fatty Acid Content (mg g⁻¹ dwt) | Fatty Acid Content (mg g⁻¹ dwt) | Average Fatty Acid Content (mg g⁻¹ dwt) |
|------------------------|----------------------------------|----------------------------------|-----------------------------------------|
|                        | (Trial 1)                        | (Trial 2)                        |                                          |
| Chlorococcum sp        | 055.00                           | 056.00                           | 054.00                                  |
| Diatom numuloides sp   | 130.00                           | 122.00                           | 126.00                                  |
| Chlorococcum sp        | 043.21                           | 063.49                           | 053.34                                  |
| Diatom mulleri sp      | 052.85                           | 056.18                           | 054.51                                  |
| Diatom surtellum sp    | 093.98                           | 083.31                           | 088.64                                  |
| Diatom Plateycephala   | 112.09                           | 115.87                           | 113.98                                  |
| Diatom Lyra sp         | 103.03                           | 091.34                           | 097.19                                  |
| Anthrospirasp          | 049.55                           | 026.17                           | 037.85                                  |
| Oscillotoriasp         | 114.73                           | 133.68                           | 124.20                                  |
| Chlorella sp           | 111.06                           | 068.74                           | 089.91                                  |
| Spirulina sp           | 036.97                           | 027.95                           | 030.92                                  |

3.5 Characteristics of The Microalgae Species From Waste Stabilization Ponds

The bioenergy production potential is measured by the parameters such as lipid content and fatty acids for all the microalgae species. The characteristics of microalgae from different waste stabilization ponds are tabulated in Table 3.6 and represented as a chart in Figure3.2 to compare its bioenergy production potential and thus the species with maximum Lipid content as well as Fatty acid content is considered as highly potential microalgae for Bioenergy production among the obtained species from the waste stabilization ponds.

Table 3.6 Characteristics of the Microalgae species from waste stabilization ponds

| Name of the Species     | Average Lipid Content (mg g⁻¹ dwt) | Average Fatty Acid Content (mg g⁻¹ dwt) |
|------------------------|-------------------------------------|-----------------------------------------|
| Chlorococcum sp        | 54.00                               | 39.39                                   |
| Diatom numuloides sp   | 126.00                              | 85.23                                   |
|微藻名称          | 培养基 | 光合单位 | 呼吸单位 |
|------------------|--------|----------|----------|
| Chlorococcum sp  | BG11   | 53.34    | 51.00    |
| Diatom mulleri sp| f/2    | 54.51    | 18.00    |
| Diatom surtellum sp | f/2   | 88.64    | 33.35    |
| Diatom Plateycephala | f/2 | 113.98   | 69.00    |
| Diatom Lyra sp   | f/2    | 97.19    | 62.00    |
| Anthrospirasp    | BBM    | 37.85    | 21.00    |
| Oscillotoriasp   | f/2    | 124.20   | 77.00    |
| Chlorella sp     | BBM    | 89.91    | 45.00    |
| Spirulina sp     | BBM    | 30.92    | 21.00    |

Figure 3.2 Characteristics of the Microalgae species from waste stabilization ponds

3.6 Nutrient Removal in Waste Water By Microalgae Treatment

The Nutrient concentration such as Total Kjeldahl Nitrogen and Phosphorous are tabulated in Table 3.7 for the respective Sewage Treatment Plant. The sewage treatment pond in Perungudi was found with highest nutrient of about 92.80 mg/L Nitrogen and 26.30 mg/L Phosphorus content before algal treatment among all the ponds in this study. The species obtained from this pond was also found with maximum cell growth when compared with other species in the study with 162.00 x 10^4 cells/mL.
Table 3.7 Nutrient removal in waste water by Microalgae treatment

| Name of the/pond | Before Treatment (Inlet) | During Treatment (Pond) | After Treatment (Outlet) |
|------------------|--------------------------|-------------------------|--------------------------|
|                  | TKN (mg/L) | P (mg/L) | TKN (mg/L) | P (mg/L) | TKN (mg/L) | P (mg/L) |
| Oxidation pond 1 | 49.30      | 19.20    | 33.60      | 17.60    | 20.19      | 10.62    |
| IITM, Chennai    |             |          |            |          |            |          |
| VIT – Pond       | 21.80      | 10.20    | 16.60      | 9.17     | 9.23       | 6.28     |
| Vandalur, Chennai|             |          |            |          |            |          |
| STP, Perungudi   | 92.80      | 26.30    | 66.48      | 16.20    | 19.19      | 6.12     |
| Sewage Treatment |             |          |            |          |            |          |
| Plant, Chithathur|             |          |            |          |            |          |
| Mangalam Pond    | 46.21      | 19.56    | 29.60      | 10.61    | 17.20      | 9.60     |
| Pulikkundram Parry|          |          |            |          |            |          |
| Nutraceuticals,  | 12.20      | 5.72     | 9.27       | 4.20     | 6.60       | 4.61     |
| Chennai          |             |          |            |          |            |          |

Next to Perungudi pond, the Oxidation Pond in IIT, Madras was found with highest nutrient of about 49.30 mg/L Nitrogen and 19.20 mg/L Phosphorus content before algal treatment. Similar to the species obtained in Perungudi pond, the species from IIT pond has maximum cell growth of about 106.00 x 10^4 cells/mL.

4. Conclusions

From the experiments conducted for the microalgae stains it is seen that the biomass growth was always directly related to the removal in nutrients and algal lipid content. From the results obtained it was identified that *Diatom numuloides sp* has the highest lipid content and Fatty acid content among the other species and thus it is considered to be the microalgae species with greater bioenergy productive potential microalgae species. It was also observed that the Pond in which the *Diatom numuloides sp* was obtained has the highest nutrient concentration and that concentration was removed effectively by the respective algal growth.

The *Diatom numuloides sp* exhibited the highest growth rate associated with nutrient removal. Finally the algal lipid content and Fatty acid varied with time and higher values were observed on day 40 of cultivation compared to day 20. The dry biomass weight of *Diatom numuloides sp* after 40 days growth is 109.50 mg/L, which was obtained from Sewage Treatment Pond, Perungudi. The Dry biomass weight completely depends upon the cell growth count and the concentration of Nutrients in the media. The cell growth count of *Diatom numuloides sp* in f/2 Media is 106.00 x 10^4 cells/mL at 25°C.

*Diatom numuloides sp* was found to be the microalgae species with maximum lipid content among other microalgae species in the study with 126mg g^-1 dwt in 40 days growth with dry biomass productivity 109.50mg/L. The Fatty acid content of *Diatom sp* was found to be the microalgae species with maximum Fatty Acid content among other
microalgae species in this study with 85.23 mg g⁻¹ dwt in 40 days growth. The study shows that the lipid content of the species is proportional to the dry biomass weight obtained.

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