Screening of Lipid Production in Marine Cyanobacteria and Microalgae Grown in Ossein Effluent for Biodiesel Production

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Abstract  Microalgae and Cyanobacteria are potentially diverse, dominant photosynthetic organisms in nature. Initially, eight strains belonging to Marine cyanobacteria and Microalgae, based on growth, were selected. *C. vulgaris* BDU G91771 was used to study the possibility of utilizing ossein effluents. The organism was inoculated in two different effluents (HTDS and LTDS) with seawater and nutrient dilutions, namely (effluent, effluent with seawater with N:P, The maximum lipid productivity was observed in 100%LTDS + Nutrients (Urea and superphosphate) with 16.8%. Lipid composition in fatty acids appeared to be suitable for biodiesel production. The results showed that the marine microalgae *C. vulgaris* BDU G91771 could be used to treat ossein effluents, and at the same time, to produce biodiesel sustainably.

Keywords: microalgae, cyanobacteria, *C. vulgaris*, HTDS, LTDS, Ossein Effluent, Lipid, Biodiesel

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1. Introduction

Biofuel production coupled with carbon dioxide sequestration through photosynthetic microorganisms has been a good process since the last century. [1] Microalgae and cyanobacteria, photosynthetic organisms, uptake nutrients and convert carbon dioxide into carbon-rich lipids [2]. Algae govern the consumption of CO₂ and they can remove contaminants and uptake the nutrients in industrial wastewater effluent; It also has been proven that nutrients in most types of wastewater, such as municipal wastewater, agricultural wastewater, food industry wastewater, etc., could be effectively utilized by microalgae [3]. The use of microalgae for effluent treatment has been employed for decades, as a low-cost process, especially in areas with increased ambient temperature and adequate sunshine throughout the year. [4] Ossein (decalcified bone) is the chief organic substance of the animal bone tissue obtained as a residue in the clarification process of the gelatin production system. High total dissolved solids (HTDS)-released on heat treatment of the decalcified bones and low total dissolved solids (LTDS) effluent-discharged after final washing and settlement of Ossein. [5] Microalgae-based advanced nutrient removal treatment has outstanding advantages, including cost-effective and environmentally friendly as no additional chemicals are required, while oxygen generation, carbon dioxide mitigation, and metal ion reduction can be realized at the same time; and potential utilization of the harvested microalgal biomass for production of food, feed, fuel, fertilizers, and fine chemicals. [6,7] Microalgae biomass contains products with high commercial importance like proteins, lipids, carbohydrates. [8] Nutrient availability significantly impacts the growth and propagation of microalgae and overall effects on their lipid and fatty acid composition, especially Lipids are one of the main components of microalgae. The interest in microalgae for oil production is due to some species’ high lipid content and the fact that lipid synthesis, especially of the non-polar TAGs, which are the best substrate to produce biodiesel, can be modulated by varying growth conditions. [9] Microalgae contain around 2-60 % lipids of the total cell dry weight depending on the species and growth conditions. Lipids derived from microalgae have been the focus of considerable interest because these oils contain fatty acid and triglyceride compounds. [10] To study the growth characteristics of eight strains based on the lipid selected, the best strain for the ossein effluent growth study, *C. vulgaris* used to remove nutrients from the effluent.
The use of both LTDS and HTDS effluent wastewater for microalgae biomass production is a plausible approach as a renewable and sustainable process removing nutrients and producing by-products economically. The study aims to demonstrate the performance of *C. vulgaris* in treating ossein effluent and estimating biomass and lipid simultaneously under different combinations under Laboratory conditions. Moreover, according to the experimental results to describe, growth, nutrients consumption and promote the strains further investigated in the Large scale cultivation.

### 2. Materials and Methods

#### 2.1. Culture Collection

A total of eight strains were obtained from the repository of the National Facility for Marine Cyanobacteria (Sponsored by DBT, Govt of India) for the study. Based on the screening of the chosen eight strains collected from various coastal areas.

#### 2.2. Light Microscopy

An inverted light microscope observed eight strains' cell size and morphological characteristics (unialgal, axenic cyanobacterial) (Leica DMI 3000B). A measuring ocular calibrated to the different magnification (10X, 40X, and 100X) was used to calculate cell sizes. At least 100 microalgal cells if each species were measured as its stationary period. [11]

#### 2.3. Selection of Suitable Marine Cyanobacteria and Microalgae for Maximum Lipid

To select the suitable organisms for sustainable bioenergy, eight organisms were screened based on growth and lipid analyses. 30 ml of the culture with high cell density were inoculated in triplicate into 250ml Erlenmeyer flasks containing 150 ml of ASN Medium prepared in sterilized conditions. Cultures were maintained at 27±2°C, with a photoperiod of 16:8 h light / dark cycles under a light intensity of 250 μmol photons m⁻² s⁻¹ provided by cool white fluorescent tubes and culture for Seven days. The cell count was measured every alternate day, and the growth rate of all eight strains was determined in triplicates for seven days following. [12]

#### 2.4. Effluent Sources

Calcium-rich ossein effluent was collected at three different clarification stages from the gelatin manufacturing industry, Pioneer Jellice Industries, Cuddalore, Tamil Nadu, India. Two effluents, namely, high total dissolved solids (HTDS) low total dissolved solids (LTDS), have been used for the present study. The effluent differed in amounts of the total dissolved solids (TDS), hence named as high TDS (HTDS) and low TDS (LTDS). The collected effluents are stored in black plastic cans at 4°C to avoid microbial growth till use. Autoclaved effluent was used for the experiments.

#### 2.5. Utilizing Industrial Ossein Effluent by *C. vulgaris* BDG 9177

Sterilized Erlenmeyer flasks (250 ml) were used for the experiments. The effluent's initial physicochemical analysis was estimated following the Standard Methods (APHA, 1975). The following treatments were employed to study the interaction of *C. vulgaris* with the Industrial Ossein effluent. Effluent uninoculated was taken as a control for physicochemical analysis (C). ASN medium inoculated with *C. vulgaris* BDUG91771 for the estimation of growth. Inoculation was made by adding a 5.0 ml uniform suspension of *C. vulgaris* BDG 91771 (0.5 OD). The experiment was conducted under controlled conditions (Temperature 25 ± 2°C with light intensities of 1200lux provided from overhead cool with fluorescent tubes). Effluents were diluted at different combinations Table 1. The growth of *C.vulgaris* BDG 91771 was studied in the rich cultures harvested on the 7th day. The filtered effluents (inoculated and control) were used for physicochemical analysis.

#### 2.6. Analysis of Physicochemical Properties

The un-inoculated effluent served as control. The clear supernatant was used for the analysis after centrifugation of the effluent-grown microalgal culture. The physicochemical parameters of the two types of effluents were analyzed on Initial and Final days for the experimental period. The total solids (TS), total suspended solids (TSS), and total dissolved solids (TDS) were evaluated according to the standard methods [13] by drying the sample at 100°C using pre-weighed crucibles. The nutrient removal by strains from the effluents was evaluated by determining the amount of dissolved oxygen (DO), total Kjeldahl nitrogen (TKN), nitrate, nitrite, ammonia, total phosphorus, inorganic phosphorus, calcium, and magnesium by following the standard methods for the examination of water and wastewater.

#### 2.7. Lipid Extraction Using Chloroform: Methanol Method (2:1) Method 1

Chloroform: methanol (2:1) extraction method was followed to ensure complete lipid extraction. Lipid extraction from algal cells requires cell membrane disruption physically or chemically. As it is a temperature tolerance study, the direct solvent extraction method has been used. The biomass and solvent mixture (chloroform: methanol (2:1)) containing RB flask equipped with a
condenser was refluxed for about two hours. In the extracted lipid, distilled water was added to remove water-soluble impurities. The lower chloroform layer containing lipid was carefully collected, and the upper layer was discarded. (for hexane and petroleum ether facilitated extraction, the top layer was collected) A moisture content of collected lipid was eliminated by passing it to the sodium sulfate bed. The lipid was transferred to the preweighed glass container and dried in a Rotary evaporator (Evator II), and the lipid weight was measured gravimetrically.

\[
\text{Lipid} \left( \% \right) = \frac{W_t - W_p}{D} \times 100
\]

Where,  
\(W_t\) – Total weight of dried lipid container  
\(W_p\) – Pre weight of the container  
\(D\) – Dry cell weight of algal biomass

In the transesterification process, the purified lipid was taken into Round Bottom (RB) flask and was preheated in a water bath to get rid of any water. The acid catalyst (3% sulfuric acid) was thoroughly dissolved in methanol, and the resulted mixture was poured into the preheated lipid at room temperature. The reaction setup was equipped with a reflux condenser, magnetic stirrer, thermometer, and reactions for 2 h at 65±10°C. After completion of the reaction, the mixture was carefully transferred to a separating funnel and allowed to stand for 30 min, a lower layer containing glycerol, unreacted methanol, and catalysts was drawn off, and the upper layer comprising methyl ester was collected. Methyl ester thus obtained was purified by repeated water wash until the lower water layer reached pH 7.0. The resulting purified methyl ester was dried at 70°C in a Rotary evaporator (Evator II) and used for gas chromatographic analysis.

### 2.8. Gas Chromatography (GC)

For the separation of FAMEs, one µl of transesterified samples dissolved in hexane used as a solvent was injected onto a gas chromatograph (Perkin Elmer Clarus 500) equipped with SP 2560 capillary column (100 m length) and Flame Ionization Detector. The chromatography conditions were; carrier gas flow rate -1 mL min⁻¹ (1 ml –nitrogen, 10ml – Hydrogen, 100ml –Zeroair), oven temperature 140-240°C; injection port temperature 260°C; detector temperature 260°C and the total run time was 45 min. Supelco, 37 component FAME mix, was used as a standard to identify the analytes. The response of each fatty acid separated on the chromatograph was determined as the peak area.

### 3. Results and Discussion

#### 3.1. Selection of Strains

Eight strains were obtained from the repository of the National Facility for Marine Cyanobacteria (Sponsored by DBT, Govt of India) for the study. The chosen strains are listed in Table 2.

#### 3.2. Validation of Strains Based on Morphological Characteristics

All the procured eight strains from the repository were further authenticated for their morphological characters under an inverted light microscope (Leica DMI 3000B) for morphological and unialgal conformity, and their microscopical images were illustrated below in Plate 1.

### Table 2. List of selected marine cyanobacteria and microalgal strains procured from the repository for growth and lipid production

| S.No | Strains                          | GPS data            |
|------|----------------------------------|---------------------|
| 1.   | Chlorella vulgaris BDUG91771     | 8° 45' 32.64” N 78° 08’ 09.13” E |
| 2.   | Chlorella sp BDUG 22101          | 8°42'20.1”N 78°07'35.5”E |
| 3.   | Chlorella sp BDUG 10101          | 9°32’02.8”N 78°54'41.5”E |
| 4.   | Chlorella sp BDUG 20601          | 8°46'29.4”N 78°08'29.6”E |
| 5.   | Oscillatoria laetevirens BDUG100891 | 12°13'13.9”N 79°58'21.9”E |
| 6.   | Phormidium sp. BDU 142231        | 13°16'55.3”N 93°01'38.0”E |
| 7.   | Plectonema sp. BDU 51111         | 8°45'12.7”N 78°08'17.2”E |
| 8.   | Lyngbya confervodies BDU142001   | 12°54'51.3”N 92°53'54.2”E |

Plate 1. (a-h) Bright field microscopical image images of marine microalgal strains depicting cell morphology (40X magnification)
3.3. Screening of Strains for Maximum Lipid Production

To identify a high lipid yielding marine candidate, the chosen eight strains on the 7th day in ASN III medium were estimated for their maximum lipid using 2:1 chloroform: methanol solvent mixture and expressed gravimetrically. Among the eight strains, *Chlorella sp.* _BDUG 91771_ possessed high lipid content of approximately 22%, which is of substantial value from a biodiesel perspective. Both marine microalgae and cyanobacteria equally competed for lipid content, of which cyanobacteria showed approximately 16%, which was comparatively lower than the lipid produced by microalgae.

| S.No | Chosen strains          | Lipid content (%) |
|------|-------------------------|-------------------|
| 1    | *Chlorella* sp. _BDUG 91771_ | 22.1              |
| 2    | *Chlorella* sp _BDUG 22101_  | 7                  |
| 3    | *Chlorella* sp _BDUG 10101_  | 3                  |
| 4    | *Chlorella* sp _BDUG 20601_  | 5.5                |
| 5    | *Oscillatoria* laetevirens _BDU100891_ | 16             |
| 6    | *Phormidium* sp. _BDU 142231_ | 14                 |
| 7    | *Plectonema* sp. _BDU 51111_ | 9                  |
| 8    | Lyngbya confervodies _BDU142001_ | 6.9              |

Table 4. Physiochemical parameters for the Effluent

| No. | Parameters         | HTDS (mg/l) | LTDS (mg/l)   |
|-----|-------------------|-------------|---------------|
| 1   | Color             | Slightly Off white | Slightly Yellowish (Transparent) |
| 2   | pH                | 8.9         | 8.6           |
| 3   | Phenolphthalein   | 46          | 70            |
|     | Total             | 8           | 20            |
| 4   | Dissolved oxygen  | 2.02        | 3.15          |
| 5   | Nitrate           | Nil         | Nil           |
| 6   | Nitrite           | Nil         | Nil           |
| 7   | Chloride          | 3249.9      | 2359.93       |
| 8   | Sulfate           | Nil         | Nil           |
| 9   | Total Phosphate   | Nil         | Nil           |
| 10  | Calcium           | 4000        | 400           |
| 11  | Magnesium         | 1760        | 200           |
| 12  | Ammonia           | 9           | 4             |

Microalgae and cyanobacteria could serve as feedstock for biodiesel production if they meet all the above requirements. They have high growth and provide a high lipid fraction to produce biodiesel. [14] Marine cyanobacteria exhibited polyvalence in lipid content across genera among the eight selected strains. The previous study reported that *Phormidium* might accumulate lipids up to 0.001 to 14, while *Phormidium angustissimum* is the lowest lipid producer of the genus. Likewise, The previous research showed *Phormidium sp.* _FW01, Phormidium sp. FW02, Oscillatoria sp. FW01_, and *Oscillatoria sp.* _FW02_ showed 6.7, 8.2, 10.2, and 9.4% lipids, respectively. In our present study, *P.valderianum* _BDU142231_ showed 14%, and *O.laetevirens* _BDU100891_ showed 16.2 % lipid. This was comparatively the same in the *Phormidium* and above in the *oscillatoria*. [15] The previous study reported that *S. platensis* resulted in the highest lipid production of 4.7%, grown in the medium of Zarrouk’s synthetic medium; compare these results [16]. *Plectonema* sp. _BDU 51111_ showed 9% of lipid produced in the ASN medium in our results. In this study, 4 are *Chlorella* sp., namely _BDUG 91771, BDUG 22101, BDUG 10101, BDUG 20601 Plectonema sp_ _BDU 51111_ and _L. confervodies_ _BDU 142001_ were obtained. When comparing the previous study in Phormidium, maximum lipids accounted for approximately 15% of total biomass mass. [17] Moreover, the previous study reported using five input cultivation variables (i.e., illumination flux, CO₂, NaNO₃ ) the maximum lipids was found 1.99 g L⁻¹ in stichococcus [18]. In the present study, *L. confervodies* _BDU142001_ showed 7% of lipid for the economical production of biofuels from microalgae, biomass, and lipid content significant role. In a previous study, *Chlorella* strains, among which *C. emersonii, C. minutissima,* and *C. vulgaris* increased lipid content of 63%, 56%, and 40% biomass by dry weight, respectively [19]. Our previous research reported that *C. vulgaris* _BDUG 91771_ was a potent strain to yield 22.2% lipid [20]. This research also showed 22% lipid in _C vulgaris_ _BDUG 91771_. The screening results manifestly confirmed that, of the nominated eight strains, Due to the high lipid content coupled with moderate growth, further optimization studies due to the biodiesel perspective were restricted to _C vulgaris_ _BDU91771_.

3.5. Growth of *C.vulgaris* _BDU91771_ in Ossein Effluent Streams and Combination with Seawater Supplemented with N:P Nutrient

Lipid content for *C.vulgaris* _BDU91771_ grown in different effluent and seawater combinations were tested and expressed gravimetrically as percentage lipid. The maximum lipid productivity was observed in both 100%LTDS + Nutrients (Urea and superphosphate) and LTDS with seawater and nutrients containing 16.8% on equal. The minimum lipid was observed in the LTDS + seawater with nutrient (1:1:1) combination. In the tested HTDS effluent condition, the maximum lipid production was about 14%. This indicates that lipid productivity was high in LTDS than HTDS effluent condition. (Figure 1).

3.6. Fatty Acid Profile of *C.vulgaris*

*BDU91771 in Ossein Effluent with the Combined Nutrients (Seawater+N:P) Effect*

The chosen high lipid yielding strain _C.vulgaris_ _BDU91771_ was further characterized for their fatty acid profile is defined ASN medium (control), low-cost seawater based medium and both the effluent (LTDS and HTDS) and the maximum lipid yielding condition, LTDS with seawater and nutrients (1:1:1).
In a previous study, Calcium-rich ossein effluent from a gelatin-producing industry was treated by *O. willei* BDU130791 and *P. valderianum* BDU20041. Results showed calcification which appears to be a plausible alternative to removing calcium in effluents and sequestration of CO₂ from the point source [5]. Our current study also uses the same ossein effluent for *C. vulgaris* BDU G91771. Previous research studied the growth rate and bulk, macro, and micronutrient removal capability of *C. vulgaris* in three different wastewaters (primary wastewater, PWW, secondary wastewater, SWW, and petroleum effluent, PE). Calcium, sulfur, magnesium, potassium was high in this waste effluent. It was removed 66% to 80% [21]. Various studies reported that using bioremediation options by microalgae is viable for treating wastewater. Some previous studies reported that phosphorus and nitrogen removal for wastewater injected CO₂ by *C. vulgaris*, respectively [22]. The lipid content increased to 34.1% [23]. In our current study, the maximum lipid content was 16% compared to the control of ASN medium, with 22% of lipid produced. The previous results in the study of *C. vulgaris* microalgae using wastewater had palmitoleic acid (C16: 1) of 30.54%, higher when compared with 10.2% photobioreactor and 10.4% open pond. Stearic acid (C18: 0) was 13.65% lower when compared with photobioreactor of 21.6% and open pond by 21.4%. There is no linolenic acid (C18: 3) when linolenic acid [18:3] at photobioreactor 14.9% and open pond 14.3%. In the current study, The fatty acid profile varied inevitably in the different conditions tested. The selected strain showed the predominance of middle and long-chain fatty acid, namely palmitic (C16),

![Figure 1. Percentage of Lipid content in different effluent conditions](image_url)

**Table 5. Fatty acid profile of lipid extracted from the selected conditions for the chosen strain *C. vulgaris* BDU91771 grown in ASNIII defined medium; seawater based low-cost medium (SW); 100% effluent (LTDS and HTDS); LTDS amended with seawater with N:P ratios**

| SL.No | Fatty acids (%)          | ASN  | SW   | LTDS | LTDS (1:1) | HTDS |
|-------|--------------------------|------|------|------|------------|------|
| 1     | CAPRIC ACID (C10:0)      | 0.95 | 1.87 | 0.56 | 0.74       | 1.01 |
| 2     | UNDECENOIC ACID (C11:0)  | 0.48 | 0.60 | 0.31 | 0.77       | 1.20 |
| 3     | MYRISTIC ACID (C14:0)    | 1.29 | 1.57 | 0.84 | 1.87       | 2.62 |
| 4     | Cis-10, PENTADECANOIC ACID (C15: 1) | 9.39 | 28.10 | 11.76 | 16.04 | 9.33 |
| 5     | PALMITIC ACID(C16:0)      | 10.59| 25.87| 13.85| 10.21      | 7.99 |
| 6     | PALMITOLEIC ACID (C16:1)  | 7.92 | 8.42 | 11.05| 7.90       | 7.04 |
| 7     | Cis -10, HEPTADECANOIC ACID (C17:1) | 12.37| 18.46| 5.83 | 7.55 | 12.58 |
| 8     | STEARIC ACID (C18:0)      | 11.57| 3.85 | 12.51| 9.07       | 6.53 |
| 9     | OLEIC ACID (C18: 1n9c)    | 13.67| 1.065| 18.27| 13.40      | 11.80 |
| 10    | LINOLEIC ACID (C18:3n3)   | 20.62| 0.96 | 17.25| 13.30      | 17.94 |
| 11    | UNIDENTIFIED              | 11.1 | 9.18 | 7.72 | 19.10      | 21.90 |
|       | Saturated fatty acids     | 24.88| 33.76| 28.07| 22.66      | 19.35 |
|       | Unsaturated fatty acids   | 63.97| 57.005| 64.16| 58.19      | 58.69 |
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