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Comparative Study of Defined Media for the Growth of *Chlorella vulgaris* BDUG91771 as a Biodiesel Feedstock

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A B S T R A C T

To overcome the variation of lipid productivity of *Chlorella vulgaris*, the objective of the present research was to investigate the effect of various growth medium compositions on *C. vulgaris* BDUG91771 in an attempt to enhance its growth and lipid production using batch culture conditions. Two defined media (ASN III and F/2) were evaluated for the growth of *C. vulgaris* BDUG91771 and F/2 medium ranks the best in terms of cell density. Various physical parameters (Light intensity, Temperature and pH) were optimized in varying ranges in the selected F/2 medium. The overall lipid productivity for the chosen strain *C. vulgaris* BDUG91771 was about 25% at 30µmol $m^{-2}s^{-1}$ light intensity, 25±2°C temperature and pH 7. The study holistically identifies the suitable growth medium and optimal culturing physical parameters for the lipid production in *C. vulgaris* BDUG91771.

***Key words*:** *Chlorella vulgaris* BDUG91771, ASN III and F/2 medium, Optimization of growth and lipid, Light intensity, Temperature, pH

Increased energy demand globally, has necessitated universal scientists to find an alternative sustainable renewable energy. One of the best choices is biodiesel, which is non-toxic, biodegradable, and eliminates less level of pollutants. Microalgae are matchless biodiesel feedstock substitute which is blooming economically [1]. Microalgae has various advantages over other sources, such as having high lipid productivity, devoid of any seasonal changes, less water and land requirement and greater photosynthetic efficiency, which make it more sustainable. Besides autotrophic growth, microalgae also grow well on various cheap carbon sources such as wastewater. This significance, has made microalgal cultivation a promising strategy, thereby reducing water, nutrient and land footprint. Algae represent a diverse group with heterogeneous physiological complex, demanding different growth requirements in view of exhibiting its value-added compounds [2].

On the other hand, sustainable microalgal production has several technological and economic obstacles that must be overcome. Still, microalgal large scale cultivation is in research and it is being optimized in various segments such as physiology, production, photobioreactors' assessment, biomass recovery, product development and applications. Temperature, light intensity, nutrients, CO_2 concentrations, and pH are also key factors in influencing algal growth [3].

Microalgae require both macronutrients and micronutrients for their growth and metabolic function. Types of algal medium are determined by the chemical composition of these nutrients. Several growth media abound for the growth of algae, and still are important to identify the suitable medium for the maximal growth [4]. The study was to evaluate the most suitable medium between two defined medium for cultivation of *C. vulgaris* BDUG91771 as biodiesel feedstock. The present investigation also identifies the optimal physical parameter suitable for lipid production for the chosen biodiesel feedstock.

MATERIALS AND METHODS

Source of the organism

Chlorella vulgaris BDUG9771, a unicellular, microalgae was obtained from the National Repository for Microalgae and Cyanobacteria (NRMC), Bharathidasan University, Tiruchirappalli, India. The strain was cultured in artificial seawater nutrient (ASN III) medium [Rippika et al., 1967] and maintained under continuous aeration and white fluorescent lighting at an intensity of 20 µmol photon m^2 s^{-1} at 25 ± 2 °C in a controlled culture room. The strain was observed under an inverted light microscope (Leica DMI 3000B) for morphological and unialgal conformity following standard taxonomic keys [5].

Growth media

In order to test for preferential growth medium of the chosen strain, the samples were subjected to two different media of varying nutrient composition as stated.

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ASN III medium: NaCl – 25g; $MgSO_4 \cdot 7H_2O$-3.5g; $MgCl_2 \cdot 6H_2O$ -2.0 g; $NaNO_3$ -0.75g; $K_2HPO_4 \cdot 3H_2O$ -0.75g; $CaCl_2 \cdot 2H_2O$- 0.5g; Ferric ammonium citrate -3.0 mg; Mg EDTA- 0.5g; Vitamin B_{12} - 10.0 µg; A-5 trace minerals: H_3BO_3- 2.86 g; $MnCl_2 \cdot 4H_2O$ -1.81g; $ZnSO_4 \cdot 7H_2O$ -0.22g; $NaMoO_4 \cdot 2H_2O$- 0.39g; $CuSO_4 \cdot 5H_2O$ – 0.079g; $Co(NO_3)_2 \cdot 6H_2O$- 49.4 mg; Distilled water: 1.0 L.

F/2 medium: $NaNO_3$ -- 0.75g; $NaH_2PO_4 \cdot H_2O$- 0.05g; trace minerals (1ml) $ZnSO_4 \cdot 7H_2O$ - 23 mg; $MnSO_4 \cdot H_2O$ – 152 mg; $Na_2MoO_4 \cdot 2H_2O$ – 7.3 mg; $CoSO_4 \cdot 7H_2O$ – 14mg; $CuCl_2 \cdot 2H_2O$ – 6.8 mg; $Fe(NH_4)_2(SO_4)2 \cdot 6H_2O$ – 4.6g; $Na_2EDTA \cdot 2H_2O$ – 4.4g; Vitamin B_{12} – 0.0016g; Biotin-0.002g; Thiamine – 0.003g; Distilled water: 1.0 L.

The growth media were prepared based on their compositions, 200ml ASN III and F/2 medium were transferred into 500ml Erlenmeyer flasks and sterilized at 121°C for 15min and cooled to room temperature (25°C) prior to use. The chosen strain was inoculated in these two media and maintained under continuous aeration and white fluorescent lighting at an intensity of 20 µmol photon m^2 s^{-1} at 25 ± 2°C in a controlled culture room. All tests were carried out in triplets.

Growth measurement

Growth of *C. vulgaris* BDUG91771, in the selected two different defined media was determined in terms of optical density (OD). OD was observed at 595nm using UV-visible spectrophotometer (Cary 100 Bio, USA). The OD was observed for a period of seven days at the regular growth conditions [6].

Optimization of physical factors for maximum lipid production

The strain grown in best optimized growth medium was further evaluated for impact of physical factors on lipid content was studied by incubating uniform mid-log phase culture of *C. vulgaris* BDUG91771 at (i) 20, 50, 100 µmol photon m^2 s^{-1} light intensities, (ii) 4, 15, 27, 37(±2°C) temperatures (iii) different pH ranges (unbuffered) 5, 7, 9 (±0.2). In all the experiments, the rest of the culture conditions remain unaltered. Organism incubated at 20 µmol photon m^2 s^{-1}, 27 ± 2°C, pH 7 ± 0.2 served as control. Lipid content was estimated in triplicates for the chosen strain *C. vulgaris* BDUG91771.

Lipid estimation

At endpoint growth, the chosen strain grown in the varying physical factors organisms were extracted for total lipid using a binary solvent system described by [7]. The solvent mixture, chloroform: methanol (2:1) was added to dried algal biomass, and repeated the extraction process until lipid was extracted completely. Added water to the crude lipid, and the lower layer-comprising lipid was carefully collected. The moisture content of the collected lipid was eliminated by passing it through the sodium sulfate bed. Solvent containing lipid was evaporated in the rotary evaporator (Evator II), and total lipid was measured gravimetrically using the formula:

$$\text{Lipid (\%)} = [R_1\text{-}R_0 / \text{dry weight (g)}] *100$$

Where R_0 and R_1 are the container weight before and after evaporation, respectively. The resultant value is expressed as lipid in percentage.

RESULTS AND DISCUSSION

Selection of suitable cultivation media for maximum growth of C. vulgaris BDUG91771

The chosen strain *C. vulgaris* BDUG91771 examined for their morphology under an inverted light microscope (Leica DMI 3000B), and the micrographs were shown in (Fig 1). The taxonomic character includes a small spherical cell that has a size of 5-10uM viewed under 40X magnification.

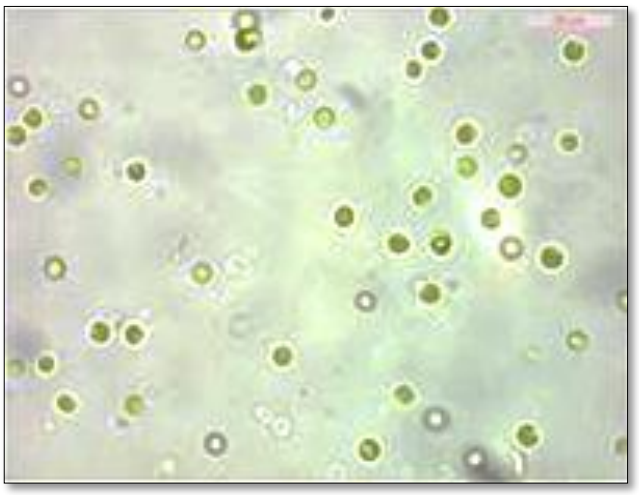

Fig 1 Bright field microscopical image of *C. vulgaris* BDUG91771 depicting cell morphology (40X magnification)

In order to study the efficiency of cultivation media on the growth of, *C. vulgaris* BDUG91771, grown in two different defined culture medium such as ASN III and F/2 were compared and evaluated in terms of growth for the period of seven days. When, growth in terms of cell density (595 nm) was evaluated, *C. vulgaris* BDUG91771 showed slightly profound growth in F/2 medium compared to ASN III medium (Fig 2).

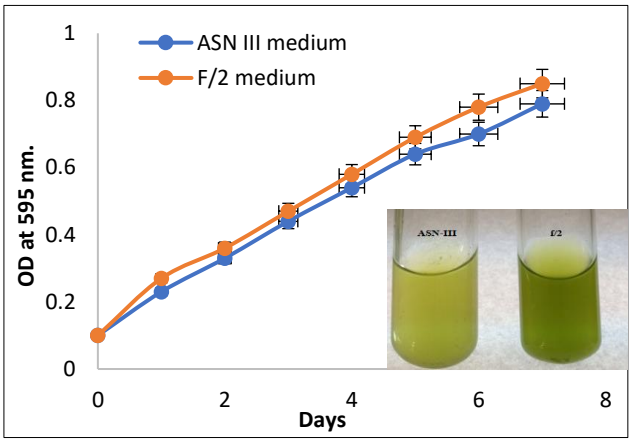

Fig 2 Comparative growth of *C. vulgaris* BDUG91771 in terms of cell density cultivated in defined media (ASN III and F/2) for a period of seven days; Light 1500 Lux; Temp: 37 ± 2°C; pH: 7.0

It is stated that the elements like N, P K, Mg, Ca, Fe, Mn and Zn are essential for the growth of microalgae. In the present study, both ASN III medium and F/2 medium possessed equal amount of $NaNO_3$ as Nitrogen source. Nitrogen and phosphorus are the major nutrients for microalgal metabolism, and therefore, optimum concentration of these nutrients promotes the synthesis of biochemical components in algae [8]. Nitrogen is responsible for biosynthesis protein and nucleic acid, whereas Phosphorus (P) is an essential numerous metabolic alleyway as well as a structural frame of phospholipids, nucleotides and energy outlay (ATP synthesis) in microalgae [9].

Certain other nutrients in appropriate quantities are needed in culture media for selected algae to multiply. Ferric iron concentration was higher in F/2 medium (tenfold higher than ASN III medium) which resulted in higher cell density

production (Fig 1), when compared to ASN III medium. It is noted that iron was one of the vital elements in algal growth and acts as the redox catalyst in photosynthesis and nitrogen assimilation, and participate in the electron transport reactions in photosynthetic organisms [10] which is demonstrated in the present study.

Trace elements such as Mn, Cu, Zn, Co and B are known to support the algal cell accumulation in small amount but retard cell growth when in excess [11]. The ASN III medium contained comparatively high concentration of these micronutrients which is responsible for less growth of the selected organism *C. vulgaris* BDU91771. Fairly, higher concentration of EDTA, ease the availability of trace elements for growth of the organism in low concentrations [12] which is evidenced in the present study in F/2 medium, to that of ASN III medium.

Microalgae require complex vitamins as growth promoting substances namely, vitamin B12, thiamine and biotin. F/2 medium is rich in vitamin supplement than ASN III medium which enhances the growth of *C. vulgaris* BDUG91771 comparatively. It is also reported by [13] that in environment under mixotrophic condition, certain bacteria can promote micro-algal growth by excreting growth-promoting compounds or vitamins (e.g., thiamine, biotin, etc.). The overall nutrient details revealed slight nutrient modifications in F/2 medium namely dosage of ferric ion, EDTA and vitamin supplementation have fairly increased the growth of the chosen strain *C. vulgaris* BDUG91771 than ASN III medium. Hence further optimization for maximum lipid production was restricted only to F/2 medium.

Optimization of physical factors influencing the lipid content of marine microalga C. vulgaris BDUG 91771

To enhance lipid production in the selected strain *C. vulgaris* BDUG91771 in F/2 defined medium, further optimization was done with the following physical parameters light intensity, temperature and pH.

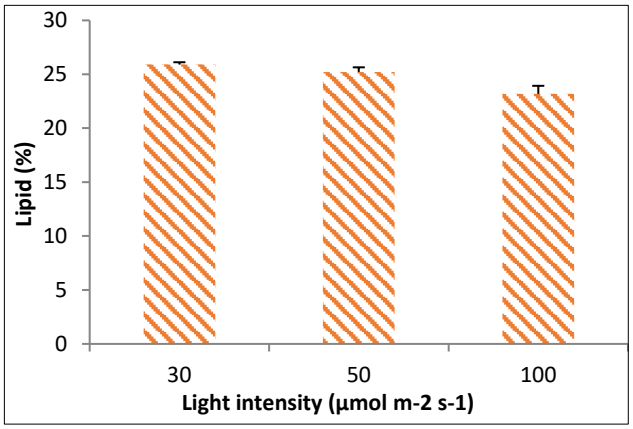

Fig 3 End point lipid content with varying light intensities in *C. vulgaris* BDUG91771 grown in F/2 medium under optimal growth conditions

Effect of light intensities on lipid content

The lipid content of marine microalga *C. vulgaris* BDUG91771 was positively influenced by the intensity of continuous illumination from 30 to 100µmol $m^{-2}s^{-1}$. When estimated at a light intensity of 30µmol $m^{-2}s^{-1}$the selected strain showed 23%, while higher light intensities 50µmol $m^{-2}s^{-1}$ and 100µmol $m^{-2}s^{-1}$ revealed 21.8 and 21% lipid respectively (Fig 3) and was inversely proportional to the increase in light intensities. According to [14] the reduced lipid content under high light irradiance is owing to the reduction or inactivation of chloroplastidial activity of microalgae when exposed to higher light illumination. According to [15] light quality determines the amount of energy available to photosynthetic organisms to conduct their metabolic activities. Numerous studies with microalgae of various groups suggest that pigments, unsaturated fatty acids, carbohydrates, and protein content all change in response to increased or decreased light intensity. Variations in the light/dark regime impose changes in the cellular content of lipids which is observed in the present finding.

Effect of temperature on lipid content

At the selected light intensity of 20µmol $m^{-2}s^{-1}$ for the chosen *C. vulgaris* BDUG91771 in F/2 medium, four different temperature regimes (4, 15, 27 and 37°C) were used to determine the ideal temperature for maximal lipid content. Under the selected light condition 20µmol $m^{-2}s^{-1}$, 27±2 °C proved to be the optimal temperature for lipid production (26%) followed by 37±2 °C (20%)> 15±2 °C (16%) and at least 4±2 °C (13%). Least lipid synthesis was noticed at 4±2°C (Fig 4).

As reported by Renaud *et al*. [16], the higher temperature above 35°C was found to decrease the growth of *Cryptomonas sp*., and *Rhodomoonas sp*. NT19, and *Isochrysis sp*. respectively. The inferior lipid production in microalgae under higher temperatures is owing to the disruption of cell metabolism, and further, cessation of cell proliferation by irreversible enzyme damage was also seen under extreme temperatures [16]. In the present study, as *C. vulgaris* BDUG 91771 showed high lipid at slightly low temperature regime of 27 ± 2°C than the other temperature regime tested. According to [17], membrane fluidity could have been increased lipid, thereby microalgae to acclimatize.

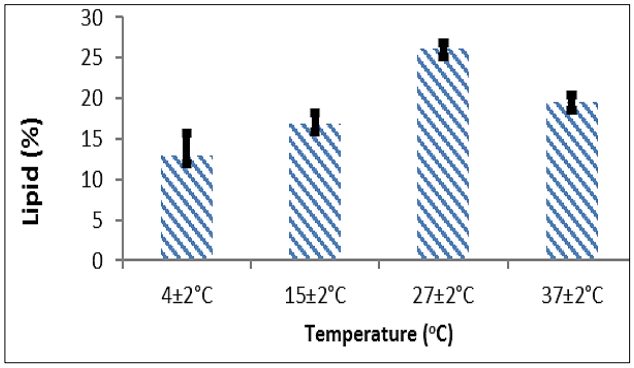

Fig 4 End point lipid content with varying Temperature in *C. vulgaris* BDUG91771 grown in F/2 medium under optimal growth conditions

Effect of pH on lipid content

With selected light (20µmol $m^{-2}s^{-1}$) and temperature (conditions, the pH influence on were studied for the maximum lipid yield of the organism *C. vulgaris* BDUG 91771. Interestingly the tested pH does not seem to have effect on lipid production (Fig 5). Irrespective of the pH range, the lipid productivity remained the almost the same @ 25%.

Marine microalga *C. vulgaris* BDUG 91771 irrespective of the acidic, neutral, alkaline ranges showed almost the same amount of lipid content of 25%. Similarly, in the report of [18] variation in the pH of culture suspension did not make any significant changes in the lipid content of *Nannochloropsis salina*. Further, stated that different pH values namely 4, 5, 6, and 7 did not unveil drastic changes in the algal lipid content. The high pH cultivation system offers several benefits such as low microbial contamination, permit to utilize the non-portable water, and high CO_2 mass transfer [19].

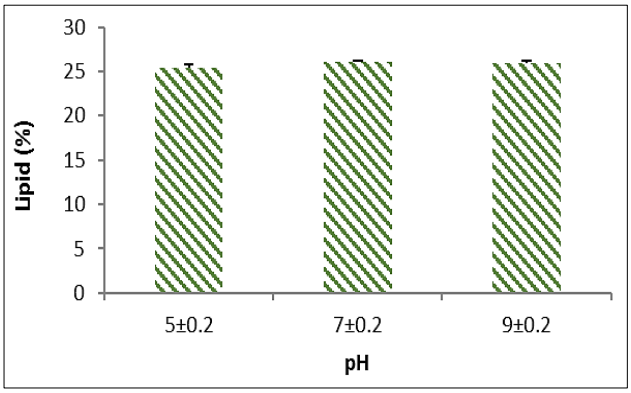

Fig 5 End point lipid content with varying pH in *C. vulgaris* BDUG91771 grown in F/2 medium under optimal growth conditions

Henceforth, a two-stage culture strategy was recommended (1) the algae is cultured in nutrient-sufficient conditions to obtain a maximized or increased dry biomass as quickly as possible, (2) In cultural stage, the growing conditions (both physical and chemical) are modulated to trigger the accumulation of lipids. However, some physical conditions such as light intensity, temperature and pH may also bring significant effects of biomass and lipid production. Further study is required with a focus on one or two particular nutrients (with alteration in concentration) in one type of medium, and other factors remaining unchanged each time to find out the induce effect of nutrients to the biomass and lipid accumulation.

CONCLUSION

Selecting an appropriate growth medium is mandatory for the maximum biomass and lipid accumulation. The present study compared the two defined medium namely ASN III and F/2 medium for the lucrative growth of *C. vulgaris* BDUG91771 under optimized conditions. The comparative analysis revealed the superiority of F/2 medium for the enhanced growth of the organism in view of excess ferric iron, optimal concentrations trace elements and supplementation of vitamins as growth promoting substances. Further optimization with physical parameters in the selected medium showed the holistic lipid production strategy. Thus, the study focused a two-stage culture strategy to obtain a maximize dry biomass first, and later modify the growth conditions to trigger the accumulation of algal lipids.

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