Biological Forum – An International Journal **16(8): 312-317(2024)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Harnessing the Potential of *Chlorella vulgaris* for Sustainable Bioplastic Production

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(Received: 30 May 2024; Revised: 25 June 2024; Accepted: 22 July 2024; Published: 14 August 2024)
(Published by Research Trend)

ABSTRACT: Global plastic pollution has surged over the past decades, contributing significantly to plastic pollution, which poses a severe environmental threat to ecosystems and human health. As a sustainable alternative, bioplastics derived from microalgae offer a promising solution, reducing reliance on fossil fuels and minimizing the environmental impact of plastic waste. This study explores the utilization of microalgae, specifically focusing on *Chlorella vulgaris*. Selected for its rapid cell division and superior biomass yield, this strain was cultivated in BG 11 medium under constant light (700-800 Lux), achieving optimal growth within 10 days. Biomass growth was measured at the optimal density, and microalgal concentrations were determined using a Neubauerhaemocytometer. Spectrophotometry quantified chlorophyll content, while DNA was assessed through UV spectrophotometry. The DNA was isolated and 18S regions amplified by PCR and subjected to BLAST to confirm the microalgae species. The microalgal biomass was processed to create bioplastic with inherent biodegradability, confirmed by the Sudan black dye test, establishing its environmental friendliness. Further, large scale production can be done in fermenters to develop a biodegradable plastic and evaluate its environmental benefits.

Keywords: Microalgae, biomass, Biodegradable, Poly Hydroxy Butyrate, Sudan Black Dye Test.

INTRODUCTION

The escalating global production of plastic waste has led to a pervasive issue of plastic pollution on a worldwide scale (Bhat *et al.,* 2021). Addressing this environmental challenge necessitates innovative solutions that go beyond conventional recycling practices. While recycling plastic waste remains a crucial aspect, it alone cannot provide a comprehensive resolution. It is imperative to simultaneously focus on diminishing the use of fossil-based plastics to ensure sustainability (van den Oever and Molenveld 2017).

Bio-based plastics have emerged as a promising alternative to traditional fossil-based plastics, gaining popularity in various markets. These bioplastics, derived from natural raw materials, offer a biodegradable option compared to conventional petrochemical-based plastics, thus contributing to environmental preservation and reducing dependence on fossil reserves (Asgher *et al.,* 2020). Biomass polymers, such as starch and cellulose, serve as starting materials for the production of bioplastics like polylactic acids (PLAs) and cellulose acetate (CA) (Subasri *et al.,* 2021). However, it is important to note that these molecules are sourced from food crops and may not present a universally viable alternative.

Microalgae are increasingly being used in bioplastic production due to their sustainable and eco-friendly properties. They have a high growth rate, can thrive in diverse environments, and do not require arable land, thus avoiding competition with food crops (Gouveia & Oliveira 2009). Additionally, microalgae are highly efficient in water use and can accumulate significant amounts of lipids, which are crucial for producing bioplastics (Singh *et al.,* 2016; Chong *et al.,* 2021). They can also utilize waste nutrients and help reduce carbon emissions by capturing carbon dioxide from the atmosphere (Singh & Ahluwalia 2013; Zhang and Liu 2021). This makes microalgae a promising alternative to traditional petrochemical- based plastics, offering a more sustainable solution for plastic production.

Chlorella, a green algae genus with notable protein content, has demonstrated potential in biomass–polymer blends due to its high crack resistance and thermal stability. Studies indicate that blending Chlorella with additives and polymers is essential for achieving optimal results in commercial applications (Onen Cinar *et al.,* 2020). Notably, *Chlorella vulgaris* has been found to yield higher quality bioplastics compared to Spirulina (Thomas *et al.,* 2019).

Dianursanti's research emphasizes the importance of compatibilizer ratios, particularly a 6% concentration of maleic anhydride, in enhancing the quality of produced PVA (polyvinyl alcohol)–*Chlorella vulgaris* composites (Ismail *et al.,* 2019). Additionally, starch granules derived from *C. sorokiniana microalgae* biomass, with a high gelatinization temperature, offer an attractive option for starch-based bioplastic production (Gifuni *et al.,* 2017). Furthermore, pre-treatment techniques, such as ultra-sonic

homogenization, have been shown to improve the homogeneity and surface features of Chlorella–PVA blends, presenting an alternative for food packaging applications (Ali *et al.,* 2021). PHB, a biodegradable thermoplastic with properties similar to propylene, was produced using *Chlorella vulgaris* and optimized under conditions including different media, aeration, phosphate levels and sodium acetate concentration (Setyorini and Dianursanti 2021).

Bioplastics like PLA, derived from renewable biofuels, play a significant role in internal carbon footprint reduction. PLA and thermoplastic starch exhibit considerable potential as eco-friendly packaging materials, with applications ranging from compost bags to industrial packaging. Their biodegradability contributes to reducing global warming, while the integration of nanotechnology enhances their thermodynamic and gas barrier properties, making them ideal for maximizing shelf life in food packaging (Shaikh *et al.,* 2021).

New bio-based green polymers, such as corn-based PLA and polyhydroxyalkanoates (PHAs), have achieved commercial status and show promise in eco-friendly automotive components, being both compostable and recyclable. In contrast, conventional plastics, predominantly petroleum-based, pose severe environmental issues, including non-biodegradability, greenhouse gas emissions, and pollution. Plastic pollution has led to significant harm to marine and terrestrial ecosystems, causing harm to millions of seabirds and aquatic animals (Wani *et al.,* 2021).

Bioplastics, sourced from biological materials like potato, corn, sugarcane, and banana peels, present an environmentally friendly and biodegradable alternative to petroleum-based plastics. These biodegradable plastics break down into carbon dioxide, water, and inorganic compounds, mitigating environmental impact (Shah *et al.,* 2021).

Bioplastics offer several advantages over conventional plastics, including biodegradability, a lower carbon footprint, versatility, unique mechanical and thermal characteristics, energy efficiency, and societal acceptance (Bhat *et al.,* 2021; Narancic *et al.,* 2020; Singh & Verma 2020). However, challenges persist in competing with certain engineering polymers in terms of processing capacity, mechanical robustness, thermal resistance, and stability (Fredi & Dorigato 2021). Efforts to hasten the decomposition of biodegradable plastics in outer environments involve the design of efficient plastic-digesting microorganisms. Micro-spray-based screening systems have proven effective, enabling the identification of new microorganisms capable of digesting conventional plastics (Shin *et al.,* 2021).

The objective of this study is to isolate polyhydroxybutyrate (PHB) from *Chlorella vulgaris.* The research involves the initial standardization of culture parameters for *Chlorella vulgaris*, followed by the isolation of bioplastics from the microalgae. Research is needed to optimize the extraction process of PHB from *Chlorella vulgaris* to enhance yield and quality of the bioplastic. Few studies compare the PHB production efficiency of *Chlorella vulgaris* with other microalgae, highlighting a gap in understanding its potential. There is limited data on using *Chlorella vulgaris*- derived PHB in industries like automotive or aerospace, where it's mechanical and thermal properties are crucial. Specific studies on the long-term environmental impact and degradation of PHB from *Chlorella vulgaris* under different conditions are lacking.

MATERIALS AND METHODS

Microalgal Cultivation: The microalgal species chosen for this study is a strain of *Chlorella vulgaris*, previously isolated and stored at the Scire Science R & D laboratory, KINFRA Kalamassery, Kerala, India.

Culturing Media, Composition, and Conditions: The culture broth for algae was prepared, and culture tubes were incubated with a working volume of 200mL of BG 11 medium. The culture medium, consisting of 200ml, was prepared and introduced into culture tubes. These tubes were then subjected to optimal conditions, maintaining a temperature of 24±1ºC, light intensity ranging from 700-800 lux, and a photoperiod of 16/18 hours, over a period of 14 days.

Microscopic Examination: Microscopic observation commenced after 5 days of incubation. Using a light microscope (ZEISS primo star) at 40X magnification, daily examinations were conducted to scrutinize the growth and multiplication of microalgal cells.

Maximum Absorbance: **O**ptical absorbance was determined at 700nm using a LAB India spectrophotometer. For cell counting, microalgal concentration was assessed using a Neubauerhaemocytometer, and cell density (cells/mL) was computed using the formula.

$$\text{Cell no. of Cell density} = \frac{\text{Counted cells}}{\text{Volume of square}} \times \text{Dilution factor}$$

Chlorophyll Content Determination: Chlorophyll content in microalgal cells was determined using spectrophotometric techniques. A 10-minute centrifugation at 13000 rpm (Centrifuge HERMLE-Z 3242), 90% methanol reconstitution, and UV spectrophotometry at 750 nm assessed chlorophyll, with methanol as the blank.

Nucleotide Blast: In the molecular sequencing of microalgae, DNA isolation followed Doyle and Doyle's method, with UV spectrophotometry and agarose gel analysis ensuring yield and purity. The 18S region was PCR-amplified, sequenced, and subjected to BLAST analysis for comparative evaluation against the NCBI database.

PHB Content Analysis Test.

Sudan Black Dye Test. The algal culture was heat-fixed on a glass slide, stained with 0.3% Sudan Black B stain in 60% ethanol for 10 minutes, rinsed with water, counter stained with 0.5% safranine for 5 minutes, and observed under a microscope at 1000X magnification.

Extraction of polyhydroxybutrate (PHB). PHB was extracted by the method described by Robert & Iyer (2018). A 100mL sample centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded, and the

pellets were treated with 10mL of sodium hypochlorite and incubated at 30°C for two hours. After incubation, the mixture was centrifuged at 5000rpm for 15 minutes and then washed with distilled water and methanol.

The pellet was dissolved in 5ml of boiling chloroform. The chloroform solution was concentrated to a small volume. Cold methanol was added, and the sample was refrigerated overnight. The precipitated PHB was collected via centrifugation.

For bioplastic production, 0.0334g of extracted PHB from *Chlorella vulgaris,* 0.0334g sorbitol, 0.0334g gelatin, and 1.1133ml 2% glycerol solution were thoroughly mixed. The mixture was heated to 95°C with continuous stirring and then poured into a dried petri plate. The time required for plastic separation depended on room temperature of the room, taking several days. After complete drying, the bioplastic was carefully separated from the petri plate using a scalpel.

RESULTS AND DISCUSSION

The microalgal species, a strain of *Chlorella vulgaris, was* cultured. Fig. 1 illustrates the microalgal culture on the initial and 10th days post-inoculation. In Fig. 2, a microscopic image of *Chlorella vulgaris* is presented under 45X magnification. Enumeration of both individual cells and colonies was conducted utilizing a haemocytometer.

The highest absorbance and chlorophyll content were observed on Day 10, as depicted in Fig. 3. Consequently, the cell count determined through a hemocytometer is illustrated in Fig. 4. Chlorella is rich in protein, dietary fibre, essential amino acids, minerals and trace elements, omega-3 fatty acids and polyunsaturated fatty acids (n3-PUFAs), calcium, iron, magnesium, zinc, and vitamins D and E (Sandgruber *et al.,* 2021). *Chlorella vulgaris*, rich in alkaloids, flavonoids, and phenols, exhibits strong antioxidant activity and high total phenolic content, with acetone extracts showing the most significant effects, indicating its potential as a dietary supplement for free radical scavenging (Abdel-Karim *et. al*., 2019).

The isolated and purified DNA was amplified via PCR to target its 18S region, and the obtained sequence was subjected to BLAST analysis. The constructed phylogenetic tree confirmed the identification of the *Chlorella vulgaris* [AF350260] species (Fig. 5).

The PHB granules were identified by Sudan black dye test. The algal culture, subjected to Sudan Black B and safranin staining, revealed distinct characteristics under the microscope, appearing in a characteristic black colour. The staining process provided visual insights into the cellular structure and composition of *Chlorella vulgaris*, laying the groundwork for subsequent analyses.

For bioplastic production, the outlined methodology not only facilitated the successful extraction of PHB from *Chlorella vulgaris* but also provided a comprehensive approach to bioplastic production. The combination of extracted PHB with sorbitol, gelatin, and glycerol show cased the versatility of *Chlorella vulgaris* in producing sustainable materials with potential applications in eco-friendly product development.

Chlorella, a protein-rich green algae with dense cell walls, is commonly used in biomass-polymer blends for bioplastic production. Blending with additives and polymers is deemed essential for commercial applications, with studies indicating higher quality bioplastics from Chlorella compared to Spirulina (Onen Cinar *et al.,* 2020). Chlorella biomass can yield starch granules suitable for starch-based bioplastics with a high gelatinization temperature. Pre-treatments like ultra-sonic homogenization improve homogeneity in Chlorella-PVA blends, and modifications with maleic anhydride positively impact the tensile strength of Chlorella-PE composites (Sabathini *et al.,* 2018; Otsuki *et al.,* 2004). Agar, blended with plasticizers like glycerol and sorbitol via film-casting, produces eco-friendly bioplastics, where glycerol enhances elasticity, sorbitol boosts tensile strength, and higher agar concentrations affect film thickness and strength in the bioplastic samples (Asif *et al.,* 2021). Genetically engineered microalgae can produce high yields of PHB thus providing a source of biomaterial and biofuels from a single algal source (Kaparapu, 2018).

The results obtained from the microscopic examination and the extraction and production processes underscore the feasibility and efficacy of utilizing *Chlorella vulgaris* as a promising source for bioplastic production, contributing to the growing discourse on sustainable and environmentally friendly material alternatives. Further analyses and optimizations can enhance the scalability and commercial viability of this approach.

Fig. 1. Micro algal culture on first and tenth day of inoculation.

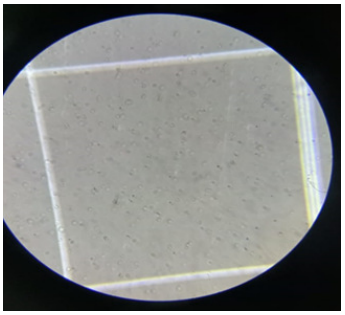

Fig. 2. Microscopic observation of *Chlorella vulgaris* under 45x magnification.

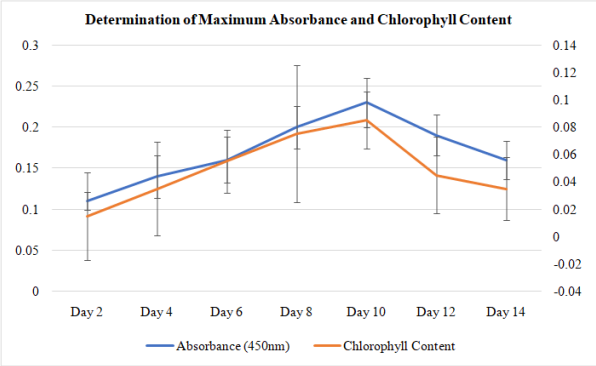

Fig. 3. Determination of Maximum Absorbance (Culture) and Chlorophyll content.

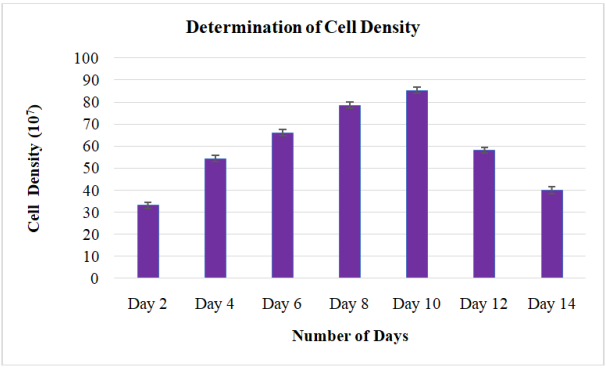

Fig. 4. Determination of Cell Density.

Fig. 5. Phylogenetic Tree.

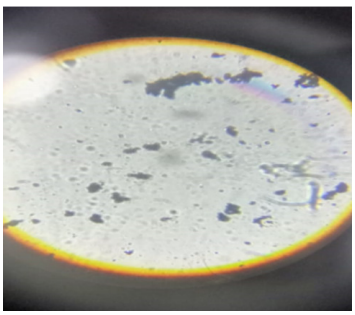

Fig. 6. Microscopic observation after Sudan black dye test.

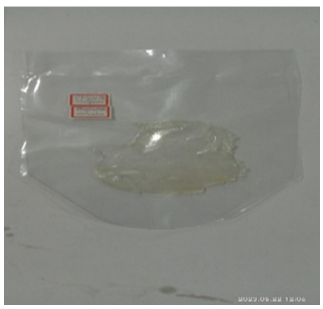

Fig. 7. Bioplastic produced from the *Chlorella vulgaris.*

CONCLUSIONS

The chosen microalgal species, a strain of *Chlorella vulgaris*, was selected for its rapid cell division, elevated biomass index, and commercial significance compared to other available strains. Cultures were prepared and incubated under optimal conditions. Microscopic observations under 40X magnification were conducted daily for five days to monitor cell growth and multiplication. The biomass growth efficiency was determined through optical absorbance at 700nm, establishing maximum absorbance using a spectrophotometer. Microalgal concentrations in the mixed culture were quantified using an improved Neubauerhemocytometer counting chamber. Chlorophyll content was assessed spectrophotometrically, measuring absorbance at 665nm and 750nm. Molecular sequencing of the 16S rRNA marker genes facilitated microalgae identification. DNA isolation, quantification, and purity assessment were performed using CTAB and spectrophotometry. Gel electrophoresis with a 250bp DNA marker aided in confirming DNA integrity. PCR amplification of the 16S rRNA region, gel purification, and sequencing were executed, followed by BLAST analysis. Additionally, PHB content analysis was conducted through the Sudan black dye test, and PHB extraction was performed. The comprehensive methodology employed ensures a robust and detailed investigation of the microalgal species.

The microalgal culture was confirmed as *Chlorella vulgaris* through DNA amplification and subsequent BLAST analysis. The PHB granules were then extracted and used for bioplastic production. This study successfully identified and confirmed *Chlorella vulgaris* through DNA amplification and BLAST analysis, with PHB granules extracted and utilized for bioplastic production, demonstrating the strain's commercial potential and efficiency.

FUTURE SCOPE

Further studies are needed for the pilot production of algal bioplastic. Additionally, it's necessary to assess the environmental benefits of PHB bioplastic. Evaluating its potential strength and applications in developing packaging materials, electronic gadgets, consumer goods and house hold appliances is also crucial.

Acknowledgement. The authors extend their gratitude to the Managing Director and the team at Scire Science Pvt. Ltd., Ernakulam, for their invaluable support for providing the facilities where this research was conducted.

Conflict of Interest. None.

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How to cite this article: Sona A., Bhagyalakshmi S. and Priya Senan V. (2024). Harnessing the Potential of *Chlorella vulgaris* for Sustainable Bioplastic Production. *Biological Forum – An International Journal, 16*(8): 312-317.