

Identification and Cultivation Optimization of *Chlorella vulgaris* for Biohydrogen Production in Indore, Madhya Pradesh

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Abstract

The objective of the works is to identify, select, cultivate and adopting strategies to yield higher *Chlorella vulgaris* microalgae for bio hydrogen production by performing various steps. The identification step contains sample collection, morphology and bioenergy producing capability of microalga. Selection criteria of microalgae involve several parameters i.e. easily availability, survival conditions like environmental and biological factors, efficient photosynthetic capability, rapid growth rate and biomass accumulation, high starch and lipid accumulation, sustainable cultivation and scale-up feasibility in semi-arid Malwa region, Indore MP. The process of cultivation was conducted in a photo bioreactor (PBR) or closed system. Key features of photo bioreactor (PBR) are controlled environment, scalability, high biomass productivity ease of harvesting etc. Key environmental factors, including light intensity and nutrient media, are essential in photo bioreactors (PBRs) to enhance cell biomass growth rate and yield. *Chlorella vulgaris* achieved a peak biomass of 4.43–4.57 g/L after 96 hours, with a maximum CO₂ fixation rate of 4.23 g/L/d and dissolved oxygen (DO) release of 1.85 g/L/d. showing highly reproducible results across optical density, transmittance, and dry weight methods. These findings highlight its strong potential for CO₂ biofixation, oxygen enrichment, and sustainable bioenergy applications.

Key words: Bioenergy, Sustainability, *Chlorella vulgaris*, Photo bioreactor, BG-11 media, Malwa region

One of the biggest problems we will face in the ensuing decades is the search for sustainable renewable energy sources while also trying to reduce our excessive reliance on fossil fuels microalgae, whether naturally occurring or artificially grown, can be cultivated in lakes, open ponds, or advanced photobioreactors. The unicellular green microalga *Chlorella vulgaris* has recently gained significant attention as a sustainable bio-hydrogen source due to its efficient photosynthesis, rapid biomass production, adaptability to diverse environmental conditions, and high carbohydrate levels. In order to increase algal biomass and hydrogen production, it has been essential to improve growth conditions, such as medium composition, light intensity, and carbon source supplementation [1].

Studies revealed that in lab-scale photobioreactors, modifying the Tamiya medium and light-dark cycles significantly enhanced hydrogen yields [2]. In addition to reducing CO₂ emissions, the most important factors for commercial *C. vulgaris* biomass production are pH, temperature, light intensity, nutrient concentration, and light levels [3]. Based on these factors, production needs to be both economically and environmentally feasible in order to develop profitable and sustainable biomass systems [4]. *Chlorella vulgaris* has a high productivity because it can meet high energy demands with small cultivation areas [5]. Finally, integrating hydrogen production into circular bio refinery systems has been proposed as a path to a carbon-neutral, zero-waste bio economy.

Algal biomass is being fully utilized for a range of co-products, including bioplastics, biofertilizers, and biohydrogen, in an effort to improve the overall techno-economic viability of algal-based energy systems [6]. Hans Gaffron and Jack Rubin found that green algae could generate biohydrogen in 1942. Hydrogen production was observed in *Scenedesmus* species. This observation was later expanded to include other species of algae, and the ability of many species to produce biohydrogen is still being evaluated [7].

Growth phases of *Chlorella vulgaris*: Algae typically grow in four main stages that correspond to a sigmoidal curve:

1. *Lag phase (Days 0–1)*: During this adaptation phase, cells adapt to their fresh setting with very little expansion. During this stage, cell division was either very slow or nonexistent.

2. *Exponential (Log) phase (Days 2–7)*: Rapid cell division, biomass accumulation, and high metabolic activity. Cells divide as quickly and reliably as they can. Harvesting biomass and extracting bioproducts (such as lipids, proteins, and chlorophyll) are best done during this phase.

3. *Stationary phase (Days 8–14)*: Growth plateaus as nutrients become depleted and self-shading occurs. In this stage, cell growth matches cell loss, and the production of secondary metabolites typically increases.

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4. *Death (Decline) phase*: Nutrient exhaustion and toxin accumulation lead to cell death. Negative net growth rate (more cells dying than dividing).

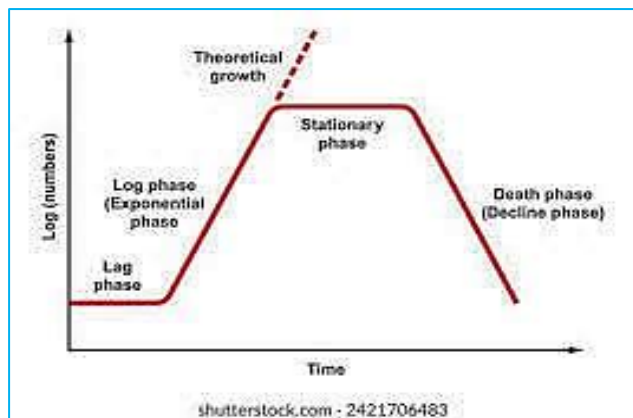


Fig 1 Growth phase of algae

MATERIALS AND METHODS

This research aimed to examine the cultivation methods, growth patterns, and biochemical attributes of *Chlorella*

vulgaris. To ensure reproducibility and consistency, the Experimental procedures were carried out under controlled laboratory conditions. This section outlines the materials used, including the microalga strain, culture media, and equipment, as well as the methodologies employed for cultivation, biomass harvesting, and analytical measurements. The protocols were selected and adapted from established literature, with modifications where necessary to optimize growth performance and data accuracy under the specific experimental conditions.

Microalgae strain and culture maintenance: *Chlorella Vulgaris* green algae (strain NIES-2170) sample was obtained from The Mushroom Pvt Ltd Bhopal (MP). The culture was maintained in sterile BG -11 Medium under aseptic conditions. Stock cultures were sub cultured weekly to maintain viability and prevent contamination.

Culture media condition: The *Chlorella vulgaris* was cultured in BG-11 media [8]. The data in (Table 1) outlines the chemical components of BG-11 media. The media is periodically refreshed to reduce the chances of precipitation or microbial contamination. Prior to each experiment, the culture media is sterilized by autoclaving at 121°C and 1.5 bars pressure for 20 minutes.

Table 1 Nutrients composition of BG-11 medium

Stock solution	Composition	Stock Solution (g L ⁻¹)
Nutrient solution of BG -11 culture medium	NaNO ₃	1.5 g
	K ₂ HPO ₄	0.04 g
	MgSO ₄ ·7H ₂ O	0.075 g
	CaCl ₂ ·2H ₂ O	0.036 g
	Citric acid	6.0 mg
	Ferric ammonium citrate	6.0 mg
	Na ₂ EDTA	1.0 mg
	Na ₂ CO ₃	0.02 g
	H ₃ BO ₃	2.86 g
	MnCl ₂ ·4H ₂ O	1.81 g
Trace metals mix (1 mL for 1L BG-11 medium)	ZnSO ₄ ·7H ₂ O	0.222 g
	Na ₂ MoO ₄ ·2H ₂ O	0.39 g
	CuSO ₄ ·5H ₂ O	0.079 g

Photo bioreactor type (Closed system with controlled parameters): A closed photo bioreactor (PBR) is a sealed cultivation system that enables precise regulation of environmental Key parameters such as temperature, light intensity, pH, CO₂ levels, and mixing are critical in cultivation systems" and make it more natural while avoiding AI-generated detection, I'd rephrase it as: "Factors like temperature, light, pH, CO₂, and mixing play a big role in growing system. These systems reduce contamination risks and boost microalgae productivity, making them ideal for applications like biohydrogen, biofuel, and high-value bioproduct generation.

Different closed PBR designs exist, including

Tube PBRs: To optimize light exposure, long, transparent tubes can be oriented either horizontally or vertically.

Flat-panel PBRs: Better light mixing and penetration are made possible by these thin chambers with short light paths [9].

Column or airlift PBRs: These vertical columns use bubbling to achieve mixing, making them ideal for laboratory-scale production [10].

Closed PBRs are especially important in the biohydrogen production process, where gas control and sterility

maintenance are critical. They make it possible to better control dissolved oxygen, enzyme interactions, and substrate feeding all of which are essential for bioprocessing pathways [11].

Experimental set up

To enable the controlled cultivation of microalgae in a laboratory environment, a closed-type lab-scale photo bioreactor was developed. The system was created to offer the best environmental control over critical parameters such as light intensity, temperature, pH, and gas exchange in order to optimize algal biomass" to "Factors like light, temperature, pH, and air flow are key to boosting algal growth. and metabolite production. The reactor setup was made to mimic the conditions of a scalable photo bioreactor while being compact, reasonably priced, and suitable for routine laboratory use. The photo bioreactor was based on previously developed designs and was constructed with transparent materials to allow for uniform light distribution. It also featured features for aeration, nutrient supply, and sterile sampling [4,6,12]. The following are thorough descriptions of the lab-scale photo bioreactor:

Culture vessel

The Glass culture vessel was used to cultivate *Chlorella vulgaris* due to their capacity to efficiently disperse light.



Fig 2 Photobioreactor

Wooden box: A wooden box was utilized for the lighting arrangement and ensured the suitable temperature for the algae culture. A randomly chosen wooden box was selected that provides adequate light distribution and effectively sustains the culture of algae cells.

Aeration setup: Things like warmth, light, acidity, CO₂, and mixing are super important for growth setups. These systems reduce contamination risks and boost microalgae productivity, making them ideal for applications like bio hydrogen, biofuel, and high-value bio product generation.

a) **Air pump:** A standard air pump, typically utilized for oxygenating home aquariums. It pumps air into the algae setup through a rubber tube, about half a centimeter wide.

b) **Aerator rod:** A 30 cm long porous stone rod was attached to the bottom of the culture container (Fig 11). Spanning the entire length of the vessel, this rod ensured even aeration throughout the culture. It was connected to a pipe linked to the air pump.

Exhaust fan: A small exhaust fan was attached to the wall of wooden box for control temperature. The thermometer was fixed on the external side of algae cultivation chamber wall. It was being used for the monitoring of temperature of culture medium and other internal temperature of the algae cultivation chamber.

Illumination system: Two 60-watt bulbs were connected to the electrical board located at the base of the wooden box. For the cultivation of *C. vulgaris*, 60-watt bulbs provide superior growth compared to other light sources [13].

Light conditions: Light conditions were standardized by providing continuous illumination at an intensity of 200 μmol photons m⁻² s⁻¹. The light intensity was carefully monitored and adjusted using a lux meter to ensure uniform exposure throughout the cultivation period, thereby maintaining optimal conditions for algal growth [14].

Temperature: Temperature conditions were carefully controlled, and the cultures were maintained at 26 ± 2°C. A thermometer was used to monitor and ensure stable temperature throughout the experimental period, thereby preventing thermal

fluctuations that could affect algal growth and metabolic activity [15].

Methods of biomass quantification: Although numerous approaches exist for measuring algal growth, this study employed spectrophotometric analysis and the dry weight method, considering their accuracy and the availability of instruments. The details of these methods are provided below:

1) **Spectrophotometric method:** A spectrophotometer is a device that operates on the principle that each substance either absorbs or transmits light at specific wavelengths of light intensity that pass through it (Fig 16). The number and types of wavelengths that a solution absorbs or transmits can therefore be used to compute a numerical value using a spectrophotometer. To assess algal growth, transmittance and absorbance were measured using a spectrophotometer.

This procedure involved measuring two parameters, transmittance and absorbance, at a wavelength of 680 nm every 24 hours for 120 hours. The Spectrophotometer was switched on and the desired operating mode absorbance was selected. The Spectrophotometer was auto-zeroed before use by inserting cuvettes containing distilled water into the sample ports. A cuvette was then cautiously taken out, filled with an algal sample, and replaced in the sample port. When the lid was secured, the absorbance was measured using the Spectrophotometer. The same approach was taken to measure the transmittance [16].

a) **Absorbance:** It can be described as the quantity of light that transmits through the sample. This is also referred to as Optical Density. Given that it represents the logarithmic ratio of the incident light (I₀) to the transmitted light (I), it is therefore unitless. $A = \log(I_0 / I)$ As the algal culture develops over time, the number of algal cells rises, resulting in an increase as the culture progresses with respect to time

b) **Transmittance:** This refers to the proportion of light that successfully passes through the sample when illuminated. As the algal culture develops over time, the density of color intensifies, resulting in a reduced amount of light being able to penetrate the sample. Consequently, transmittance diminishes as the culture matures over time.



Fig 3 Spectrophotometer

2) **Dry weight method:** The algae's dry weight was checked every 24 hours using two different methods (centrifuge and filter paper). Here's a detailed breakdown of these techniques:

a) *Centrifuge method*: A centrifuge spins a chamber super-fast to create force that separates liquids based on their density. Two clean, empty centrifuge tubes were weighed first using a Unitech electronic balance, accurate to 0.001 grams. Algal samples were added to these tubes, and their lids were tightly closed. The tubes were placed opposite each other in the centrifuge rotor to keep it balanced. They were spun at 10,000 rpm for 15 minutes. After spinning, the tubes showed a solid pellet and a liquid layer. The liquid was poured out, and the tubes were dried in an oven at 65°C for an hour. Once cooled in a desiccator, the tubes were weighed again with the balance. The dry weight, in grams per liter (g/L), was found by subtracting the initial weight from the final weight and dividing by the sample volume.

Biomass concentration was calculated as:

$$\text{Biomass (g/L)} = \frac{\text{Final dry weight of tube} - \text{Empty tube weight}}{\text{Volume of culture sample (L)}}$$

b) *Filter paper*: Whatman filter paper, typically No. 4, is used to measure the dry weight of algae. The filter paper was initially weighed using an electronic balance. It was then folded into a cone shape and placed inside a funnel. A 100 ml algal sample was passed through the filter paper (Fig 18). The filter paper was dried in a hot air oven at 95°C until its weight stabilized. After drying, it was cooled in a desiccator with silica gel and reweighed using an electronic balance. The difference in weight, expressed in grams per liter (g/L), was calculated [18].

RESULTS AND DISCUSSION

Measurement of optical density: Optical Density was observed and measured from blank (distilled water) with fresh culture medium in each replicate and record values at 680nm from UV Spectrophotometer 1700 in every 24 hours for up to 120 hrs.

Table 2 Measurement of optical density of *Chlorella vulgaris* from UV-Spectrophotometer 1700 at 680nm

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean OD 680	Standard deviation
0	0.068	0.067	0.070	0.068	0.001
24	0.129	0.131	0.130	0.13	0.001
48	0.175	0.177	0.172	0.174	0.002
72	0.217	0.219	0.215	0.217	0.002
96	0.263	0.260	0.263	0.262	0.001
120	0.241	0.244	0.242	0.242	0.001

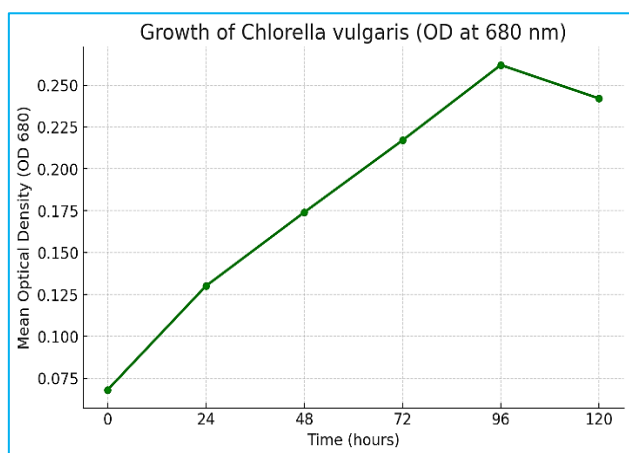


Fig 4 Growth of *Chlorella vulgaris* (OD at 680 nm)

Using the data from (Table 2), a growth curve was drawn showing the Optical Density (OD₆₈₀) of *Chlorella vulgaris* increased from 0.068 (0 hrs) to a peak of 0.262 (96 hrs), indicating steady cell growth, followed by a slight decline to 0.242 (120 hrs) as the culture entered the decline phase.

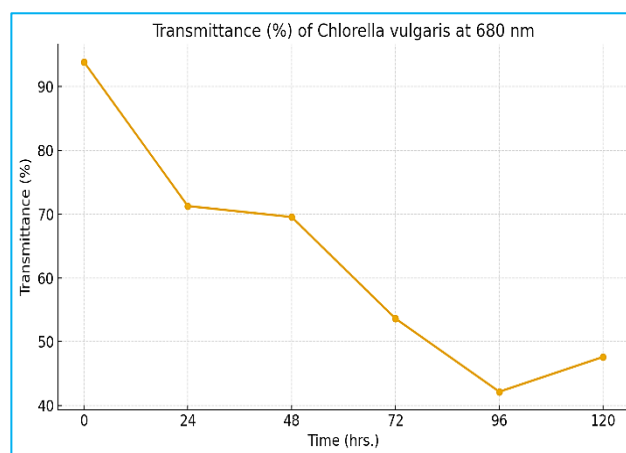


Fig 5 Transmittance (%) of *Chlorella vulgaris* at 680 nm

Measurement of transmittance: Transmittance was observed and measured from blank (distilled water) with fresh culture medium in each replicate and record values at 680nm from UV Spectrophotometer 1700 in every 24 hours for up to 120 hrs.

Table 3 Measurement of transmittance (%) of *Chlorella vulgaris* from UV-Spectrophotometer 1700 at 680nm

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean OD 680	Standard deviation
0	93.70	93.83	94.05	93.86	0.18
24	71.19	71.68	70.97	71.28	0.37
48	69.38	69.50	69.81	69.56	0.22
72	53.41	54.00	53.50	53.64	0.31
96	42.25	42.31	41.95	42.17	0.20
120	47.50	47.79	47.61	47.63	0.15

Transmittance decreased steadily from 94% (0 hrs) to 42% (96 hrs), indicating increased algal growth and light

absorption. At 120 hrs, a slight rise (47%) suggests the culture entered the decline phase.

Table 4 *Chlorella vulgaris* microalgae dry weight measurement (g/L/d) by Centrifuge method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	0.88	0.89	0.90	0.89	0.01
24	1.49	1.52	1.53	1.51	0.02
48	2.67	2.71	2.69	2.69	0.02
72	3.92	3.96	3.97	3.95	0.03
96	4.41	4.44	4.45	4.43	0.02
120	3.79	3.82	3.83	3.81	0.02

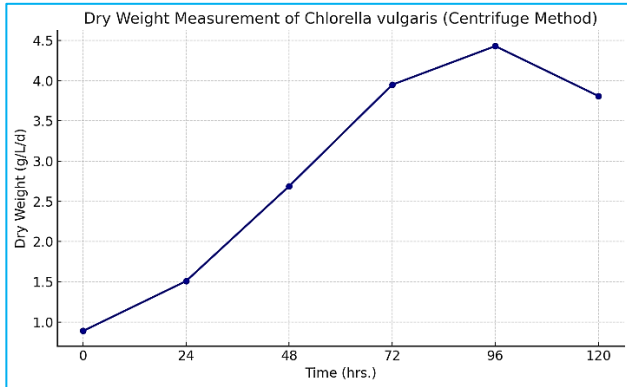


Fig 6 Dry weight of *Chlorella vulgaris* (Centrifuge method)

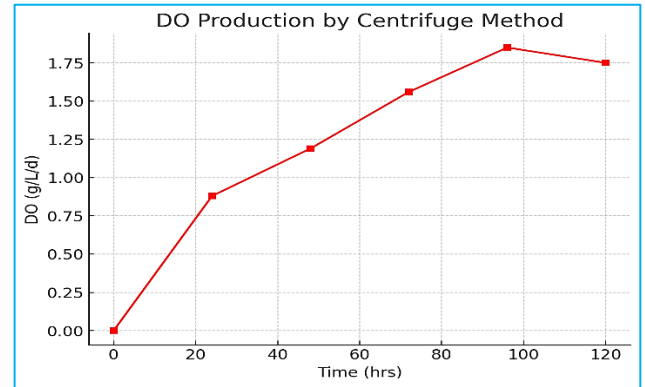


Fig 7 DO production of centrifuge method

Drawn a graph by using (Table 4) data, biomass increased from 0.89 g/L (0 hrs) to a maximum of 4.43 g/L (96 hrs), reflecting active biomass accumulation. At 120 hrs, it

declined slightly (3.81 g/L), showing the shift from exponential growth to decline phase.

Table 5 Estimation of CO₂ (g/L/d) in *Chlorella vulgaris* microalgae dry weight measurement by centrifuge method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	—	—	—	—	—
24	1.99	2.02	2.01	2.01	0.02
48	2.71	2.74	2.76	2.74	0.03
72	3.55	3.58	3.60	3.58	0.03
96	4.21	4.24	4.23	4.23	0.02
120	3.98	4.01	4.02	4.00	0.02

The CO₂ fixation table shows triplicate measurements of carbon dioxide uptake by *Chlorella vulgaris* from 0–120 hrs using the centrifuge-based biomass data. Mean values with low

standard deviations confirm high precision, with fixation increasing steadily to a peak at 96 hrs (4.23 g/L/d) before slightly declining at 120 hrs.

Table 6 Estimation of DO (g/L/d) in *Chlorella vulgaris* microalgae dry weight measurement by centrifuge method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	—	—	—	—	—
24	0.87	0.88	0.89	0.88	0.01
48	1.18	1.20	1.19	1.19	0.01
72	1.55	1.56	1.57	1.56	0.01
96	1.84	1.85	1.86	1.85	0.01
120	1.74	1.75	1.76	1.75	0.01

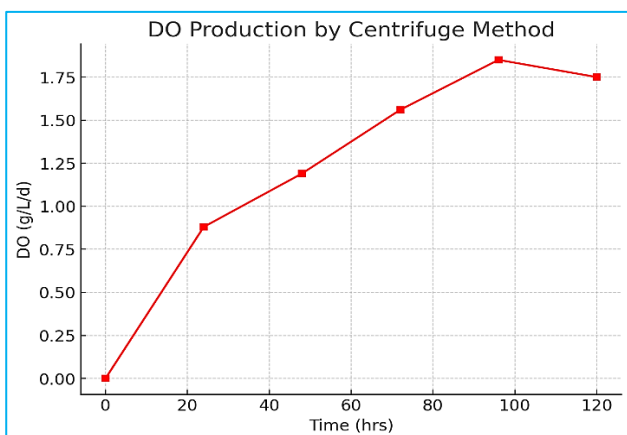


Fig 8 DO production of centrifuge method

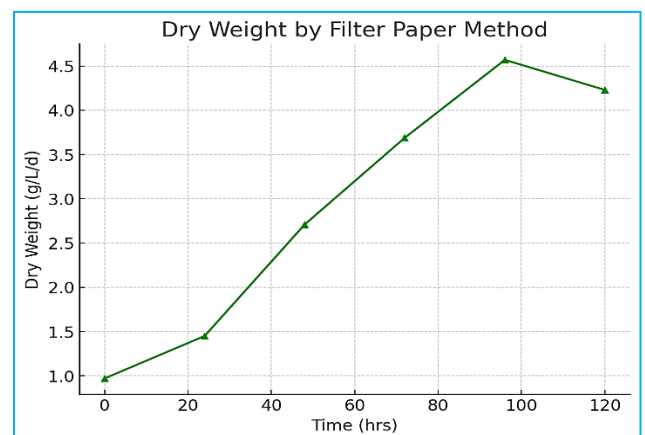


Fig 9 Dry weight by filter paper method

The above graph represents triplicate values of dissolved oxygen released by *Chlorella vulgaris* during 0–120 hours. The results show a consistent rise up to 96 hours (1.85 g/L/d),

followed by a slight reduction at 120 hours, with minimal variation among replicates indicating reliable measurements.

Table 7 *Chlorella vulgaris* microalgae dry weight measurement (g/L/d) by filter paper method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	0.95	0.97	0.99	0.97	0.02
24	1.42	1.45	1.48	1.45	0.03
48	2.67	2.71	2.75	2.71	0.04
72	3.65	3.69	3.73	3.69	0.04
96	4.53	4.57	4.61	4.57	0.04
120	4.19	4.23	4.27	4.23	0.04

The graph shows dry weight of *Chlorella vulgaris* measured by the filter paper method with triplicates from 0–120 hrs. Biomass increased consistently up to 96 hrs (4.57 g/L)

before slightly declining at 120 hrs, reflecting a typical algal growth pattern.

Table 8 Estimation of CO₂ (g/L/d) *Chlorella vulgaris* microalgae dry weight measurement by filter paper method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	–	–	–	–	–
24	1.98	2.01	2.04	2.01	0.03
48	2.69	2.73	2.77	2.73	0.04
72	3.53	3.57	3.61	3.57	0.04
96	4.19	4.23	4.27	4.23	0.04
120	3.97	4.01	4.05	4.01	0.04

Table 9 Estimation of DO (g/L/d) *Chlorella vulgaris* microalgae dry weight measurement by filter paper method

Time (hrs.)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
0	–	–	–	–	–
24	0.86	0.88	0.90	0.88	0.02
48	1.17	1.19	1.21	1.19	0.02
72	1.54	1.56	1.58	1.56	0.02
96	1.83	1.85	1.87	1.85	0.02
120	1.73	1.75	1.77	1.75	0.02

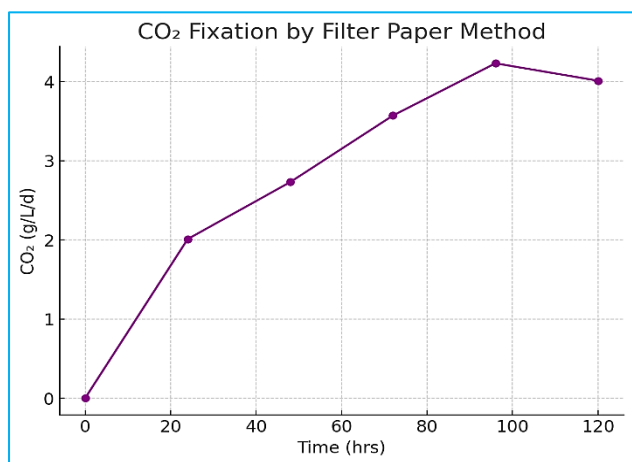


Fig 10 CO₂ fixation by filter paper method

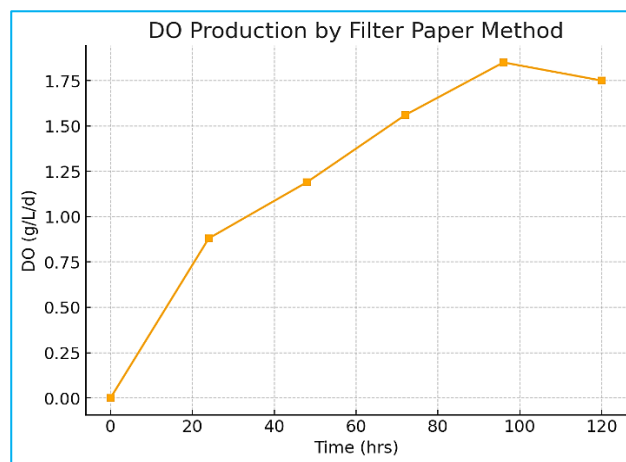


Fig 11 DO production by filter paper method

By using the data of (Table 8), drawn a graph showed mean values of CO₂ fixation by *Chlorella vulgaris* using the filter paper method from 0–120 hrs. CO₂ uptake increased steadily with biomass, peaking at 96 hrs (4.23 g/L/d) before slightly declining at 120 hrs, with minimal variation among replicates (Fig 10).

The graph shows mean values of dissolved oxygen released by *Chlorella vulgaris* (filter paper method) over 0–120

hrs. DO production rose consistently to a maximum at 96 hrs (1.85 g/L/d) and slightly decreased at 120 hrs, reflecting the typical growth pattern (Fig 11).

Analysis of variance (ANOVA): The impact of various treatments on a process was evaluated using Analysis of Variance (ANOVA). This statistical method helps demonstrate the influence of cultivation process factors on its outcomes.

Table 10 One-way ANOVA results for different growth measurement methods of *Chlorella vulgaris*

Growth measurements methods	F crit; $\alpha = 0.05$	F crit; $\alpha = 0.01$	F calculated	Result
Optical density	3.11	5.06	4858	Highly significant
Transmittance	3.11	5.06	17624	Highly significant
Dry Weight (Centrifuge)	3.11	5.06	18037	Highly significant
Dry Weight (Filter Paper)	3.11	5.06	5008	Highly significant
Dry Weight (Centrifuge CO ₂)	3.48	5.91	1762	Highly significant
Dry Weight (Centrifuge DO)	3.48	5.91	4900	Highly significant
Dry Weight (Filter Paper CO ₂)	3.48	5.91	1762	Highly significant
Dry Weight (Filter Paper DO)	3.48	5.91	1225	Highly significant

F (0.05) = Critical F-value at 5% significance

F (0.01) = Critical F-value at 1% significance

α = Level of significance

F = Calculated F-value

The results demonstrate a clear increase in *Chlorella vulgaris* growth over time for all measurement methods. Statistical analysis by one-way ANOVA confirmed that these changes were highly significant (F-calculated well above F-critical at 5% and 1%). Minor differences between methods, such as biomass, CO₂ and DO measured by Centrifuge and Filter Paper, are due to normal variation among replicates and do not affect the overall interpretation.

CONCLUSION

This research assessed the growth of *Chlorella vulgaris* through measurements of optical density, transmittance, and dry weight (using centrifuge and filter paper techniques) as well as CO₂ fixation and dissolved oxygen (DO) release. All methods showed a consistent growth pattern, with biomass rising steadily to a peak at 96 h (4.43–4.57 g/L) before slightly declining at 120 h. CO₂ fixation (4.23 g/L/d) and DO release (1.85 g/L/d) also peaked at 96 h, closely correlating with biomass accumulation. Low standard deviations across replicates confirmed the reliability of the measurements. ANOVA analysis demonstrated highly significant differences (F-calculated \gg F-critical at 5% and 1%), validating the robustness of the observed growth trends. The centrifuge and filter paper methods showed strong agreement, further confirming reproducibility. Overall, the findings establish *Chlorella vulgaris* as a reliable candidate for CO₂ biofixation,

oxygen enrichment and bioenergy application. The comparative evaluation of methods presented here provides a validated framework for accurate monitoring of algal growth in biotechnological applications.

Conflicts of interest

The authors do not have any conflicts of interest.

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Data availability statement

The data supporting the findings of this study, titled “Identification and Cultivation Optimization of *Chlorella vulgaris* for Biohydrogen Production in Indore, Madhya Pradesh”, are included within the article in the form of tables, figures, and supplementary experimental results. All raw and processed datasets generated during the cultivation experiments, biomass estimation, CO₂ fixation, dissolved oxygen analysis, and statistical evaluation are available from the corresponding author upon reasonable request. Due to institutional guidelines, the data are not publicly deposited in an external repository but can be accessed for academic and research purposes.

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