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BIOMASS ANALYSIS OF COMMERCIALLY IMPORTANT *Euglena gracilis* CULTIVATED ON BBM, BG-11, AND CHU #10 GROWTH MEDIA

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ABSTRACT

Microalgae have been utilized as a potential biomass source for food and other products over the past few decades. *Euglena gracilis* is considered a promising candidate among commercially important microalgae. The commercialization of *E. gracilis* production is centered around the "5Fs of Biomass" approach, which includes the development and manufacturing of commercial products such as food, fiber, feed, fertilizer, and fuel derived from biomass. The chemical composition of culture media affects the yield of algae therefore; media optimization is important for the commercialization of microalgae-based products. This study aims to identify the most suitable culture medium for *E. gracilis* by evaluating biomass production to optimize its cultivation for biotechnological applications. The *E. gracilis* was collected from Nasir Bagh, Lahore, Pakistan (31°57′11.6″N, 74°30′62.9″E). The impact of the three selected media (BBM, BG-11, and Chu #10) on the biomass of the isolated strain was evaluated on day 3, 6, 9, 12, and 15 intervals. The experiment was laid out in a Completely Randomized Design (CRD) with two factors, replicated three times. Statistical analysis was performed using two-way ANOVA to test the significance of treatments and their interaction, and Fisher's Least Significant Difference (LSD) test at $p < 0.01$ was applied to compare treatment means. The maximum biomass (2.18 g) was obtained in the BG-11 medium followed by BBM (0.85 g) and Chu #10 media (0.34 g) under a light intensity of 120 µmol m^{-2} sec^{-1} and a 16:8 light: dark cycle at 25 ºC. BG-11 medium proved to be the most suitable among these media for the growth of *E. gracilis*, both in terms of maximum biomass production (2.18 g) and average growth rate (0.084 g/day). This study highlights the superior biomass yield of *E. gracilis* on BG-11 medium, providing a basis for its optimized large-scale cultivation, and enhancing its potential applications in biotechnology, biofuels, and sustainable nutrition.

Key words: *Euglena gracilis*, microalgae cultivation, biomass yield, commercial application, BG-11 medium

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INTRODUCTION

Microalgae are gaining attention for their role in nutraceuticals, pharmaceuticals, cosmetics, and biofuels, owing to their capacity to synthesize proteins, lipids, pigments, vitamins, and polysaccharides (Dolganyuk *et al.,* 2020). Among them, *E. gracilis* is considered as particularly valuable species because of its unique physiology, flexible metabolism, and ability to produce high value compounds under a range of cultivation modes (Gissibl *et al.,* 2019). Unlike many other microalgae, *E. gracilis* lacks a rigid cell wall and instead has a flexible pellicle, enabling it to tolerate diverse growth conditions and environmental stresses. Its ability to grow photoautotrophically, heterotrophically, and mixotrophically further enhances its commercial potential (Zhang *et al.,* 2023).

Commercial interest in *E. gracilis* is mainly due to its accumulation of paramylon, a β-1,3-glucan polysaccharide that may constitute more than 80% of its dry weight (Kim *et al.,* 2020), along with high levels of proteins (39–61%) and lipids suitable for conversion to biofuels (Gissibl *et al.,* 2019). The biochemical profile make it attractive both for health related products and renewable energy development (Nurafifah *et al.,* 2023). Its tolerance to heavy metals and other pollutants further supports applications in bioremediation (Khatiwada *et al.,* 2020). Despite this versatility, achieving high productivity and metabolite accumulation is highly dependent on the choice of culture medium.

Culture media supply essential macro and micronutrients that regulate cell growth and metabolism. Nitrogen, for example, plays a central role in protein synthesis and directly influences both biomass accumulation and biochemical composition (Aslam *et al.,* 2021). Therefore, selecting an appropriate medium is a critical step for optimizing growth efficiency and

ensuring cost effective large scale production (Novoveská *et al.,* 2023).

BBM, BG-11, and Chu #10 are among the most widely applied synthetic freshwater media used for laboratory cultivation of microalgae. Each differs in formulation and thereby produces distinct physiological responses. BBM, enriched with nitrate as its primary nitrogen source, is widely used for freshwater green algae and supports robust photoautotrophic growth (Ram *et al.,* 2019). BG-11, originally designed for cyanobacteria, has been shown to enhance chlorophyll and carotenoid synthesis in euglenoids under optimized conditions (Kishore *et al.,* 2018). Chu #10, in contrast, contains lower nitrate concentrations and is often used in nutrient limitation studies, favoring the accumulation of starch and lipids (Ram *et al.,* 2019). Studies comparing these media highlight significant differences in growth outcomes. Yadav *et al*. 2023 reported variation in biomass yield, lipid accumulation, and pigment content when *E. gracilis* was cultivated in BBM, BG-11, and Chu #10. Likewise, optimized nitrogen sources similar to those in BBM and Chu #10 have been linked to higher paramylon productivity (Ivušić *et al.,* 2022), while BG-11 have supported exceptionally high biomass yields (>38 g/L) with protein content exceeding 50% dry weight (Pandey *et al.,* 2020; Yan *et al.,* 2023). Together, these findings indicate that no single medium maximizes all growth parameters, reinforcing the need for comparative evaluation. A comparative evaluation is particularly important for *E. gracilis*, as its commercial applications require both high biomass and targeted metabolite production (Khan *et al.,* 2018; Kim *et al.,* 2020). Optimizing media not only improves yield but also lowers the cost, which is essential for scaling from laboratory studies to industrial biotechnology (Novoveská *et al.,* 2023).

This study evaluated the growth performance of *E. gracilis* in BBM, BG-11, and Chu #10 media under controlled conditions. This comparative analysis provides insights into how media composition influences biomass production and supports the broader goal of developing cost effective strategies for industrial scale cultivation and exploitation of *E. gracilis* in biofuels, nutraceuticals, and environmental applications.

MATERIALS AND METHODS

Study site and sample collection: Water samples were taken from the freshwater pond of Nasir Bagh, Lahore, Pakistan (31°57′11.6″N, 74°30′62.9″E) in spring season 2022, and brought to the Phycology Laboratory, Department of Botany, Government College University, Lahore for the cultivation.

Separation of *E. gracilis* from collected samples: *E. gracilis* was isolated from pond samples using filtration, centrifugation, and serial dilution, followed by single cell micropipette isolation on the sterilized petri plates. The species was identified morphologically with authentic literature (Prescott, 1962; Masud-ul-Hasan, 1980; Shameel, 2012). Single cell micropipette isolation was repeated multiple times to reach the appropriate number of cells for considerable growth on agar plates.

Sterilization of the Glassware: All glassware, including petri plates, beakers, pipettes, and slides, was washed with warm water and detergent, disinfected with 70% ethanol, dried in a dust free environment, and sterilized by dry heat.

Culture Media and Preparation: BBM, BG-11, and Chu #10 were selected as they are standard freshwater algal media, commonly used for culturing green algae because of their balanced nutrient composition (Bajwa *et al.,* 2017). The chemical composition of these three media is presented in Table 1. The pH was adjusted to 6.6 for BBM, 7.5 for BG-11, and 7.6 for Chu #10 using a calibrated pH meter. Agar was dissolved in each medium, sterilized by autoclaving at 121 °C and 15 psi for 15–20 minutes, poured into oven-dried sterile petri plates, and allowed to solidify at room temperature (Andersen, 2005).

Experimental Setup and Conditions: After confirming sterility, *E. gracilis* cells were aseptically inoculated into each medium and incubated at 25 ± 2 °C under a 16:8 light: dark cycle with a light intensity of 120 µmol m^{-2} s^{-1} (Kishore *et al.,* 2017). The experiment lasted for 15 days and was arranged in a Completely Randomized Design (CRD) with two factor and three replicates per treatment to ensure reliability.

Observations and Measurements: Biomass and specific growth rate were recorded as wet weight at each sampling interval (days 3, 6, 9, 12, and 15) to monitor the growth stages of *E. gracilis*.

Specific growth rate (µ) was calculated using the formula (Levasseur *et al.,* 1993):

$$\mu = \frac{\ln(g_2/g_1)}{T_2 - T_1}$$

Here, µ = specific growth rate per day; ln = natural logarithm; g_2 = specific final biomass concentration; g_1 = specific initial biomass concentration; T_2 = final time; T_1 = initial time

Statistical Analysis: The experiment was conducted in triplicate, and all values of growth are presented as mean ± standard deviation (n = 3). Statistical analysis was performed using two-way ANOVA to test the significance of treatments and their interaction, and Fisher's Least Significant Difference (LSD) test at $p < 0.01$ was applied to compare treatment means by using Statistix 8.1.

Table 1. Composition of BBM, BG-11, and Chu #10 media (Andersen, 2005).

Chemical components of BBM	Stock Solution (g L^{-1} dH_2O)	Quantity Used	Concentration in Final Medium (M)
Macronutrients			
$NaNO_3$	25.00	10 mL	2.94×10^{-3}
$CaCl_2 \cdot 2H_2O$	2.50	10 mL	1.70×10^{-4}
$MgSO_4 \cdot 7H_2O$	7.50	10 mL	3.04×10^{-4}
K_2HPO_4	7.50	10 mL	4.31×10^{-4}
KH_2PO_4	17.50	10 mL	1.29×10^{-3}
NaCl	2.50	10 mL	4.28×10^{-4}
Alkaline EDTA Solution		1 mL	
EDTA	50.00		1.71×10^{-4}
KOH	31.00		5.53×10^{-4}
Acidified Iron Solution		1 mL	
$FeSO_4 \cdot 7H_2O$	4.98		1.79×10^{-5}
H_2SO_4		1 mL	
Boron Solution		1 mL	
H_3BO_3	11.93		1.85×10^{-4}
Trace metal solution		1 mL	
$ZnSO_4 \cdot 7H_2O$	8.82		3.07×10^{-5}
$MnCl_2 \cdot 4H_2O$	1.44		7.28×10^{-6}
MoO_3	0.71		4.93×10^{-6}
$CuSO_4 \cdot 5H_2O$	1.57		6.29×10^{-6}
$Co(NO_3)_2 \cdot 6H_2O$	0.49		1.68×10^{-6}
Chemical components of BG-11			
Fe Citrate solution		1 mL	
Citric acid	6.00	1 mL	3.12×10^{-5}
Ferric ammonium citrate	6.00	1 mL	$\sim 3 \times 10^{-5}$
$NaNO_3$	-	1.5 g	1.76×10^{-2}
$K_2HPO_4 \cdot 3H_2O$	40	1 mL	1.75×10^{-4}
$MgSO_4 \cdot 7H_2O$	75	1 mL	3.04×10^{-4}
$CaCl_2 \cdot 2H_2O$	36	I mL	2.45×10^{-4}
Na_2CO_3	20	1 mL	1.89×10^{-4}
$MgNa_2EDTA \cdot H_2O$	1.0	1 mL	2.79×10^{-6}
Trace metal solution		I mL	
H_3BO_3	-	2.860 g	4.63×10^{-5}
$MnCl_2 \cdot 4H_2O$	-	1.810 g	9.15×10^{-6}
$ZnSO_4 \cdot 7H_2O$	-	0.220 g	7.65×10^{-7}
$CuSO_4 \cdot 5H_2O$	79.0	1 mL	3.16×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	-	0.391 g	1.61×10^{-6}
$Co(NO_3)_2 \cdot 6H_2O$	49.4	1 mL	1.70×10^{-7}
Chemical components of Chu #10			
$Ca(NO_3)_2$	40	1 mL	2.44×10^{-4}
K_2HPO_4	5.0	1 mL	2.87×10^{-5}
$MgSO_4 \cdot 7H_2O$	25.0	1 mL	1.01×10^{-4}
Na_2CO_3	20.0	1 mL	1.89×10^{-4}
Na_2SiO_3	25.0	1 mL	2.05×10^{-4}
$FeCl_3$	0.8	1 mL	4.93×10^{-6}

RESULTS

E. gracilis exhibited distinct and significant biomass differences across the three media (Figure 1). Among the tested media, BG-11 supported the highest biomass accumulation with constant increase and reaching 2.18 g on day 15, which was significantly greater ($p < 0.01$) than the values recorded in BBM and Chu #10. BBM promoted moderate growth and exhibited the gradual and consistent increase in biomass (0.85 g) till the day 15. Surprisingly, Chu #10 reached its maximum biomass on

day 12 (0.34 g), followed by a decline to 0.32 g by day 15. (Table 3). Similarly, BG-11 showed highest average specific growth rate (0.084 g/day) than BBM (0.062 g/day), and Chu #10 (0.022 g/day) (Figure 2). Analysis of variance revealed that all the media (f-value 1999.49) had highly significant effect on biomass of *E. gracilis*. Similarly, the time intervals and interactions between media and the time intervals (M×T) also shown the highly significant (412.10 and 137.97 respectively) effect on biomass of *E. gracilis* (Table 2).

a) BBM **b) BG-11** **c) Chu #10**

Figure 1. Petri plates showing the growth differences of *E. gracilis* (a) BBM, (b) BG-11, and (c) Chu #10

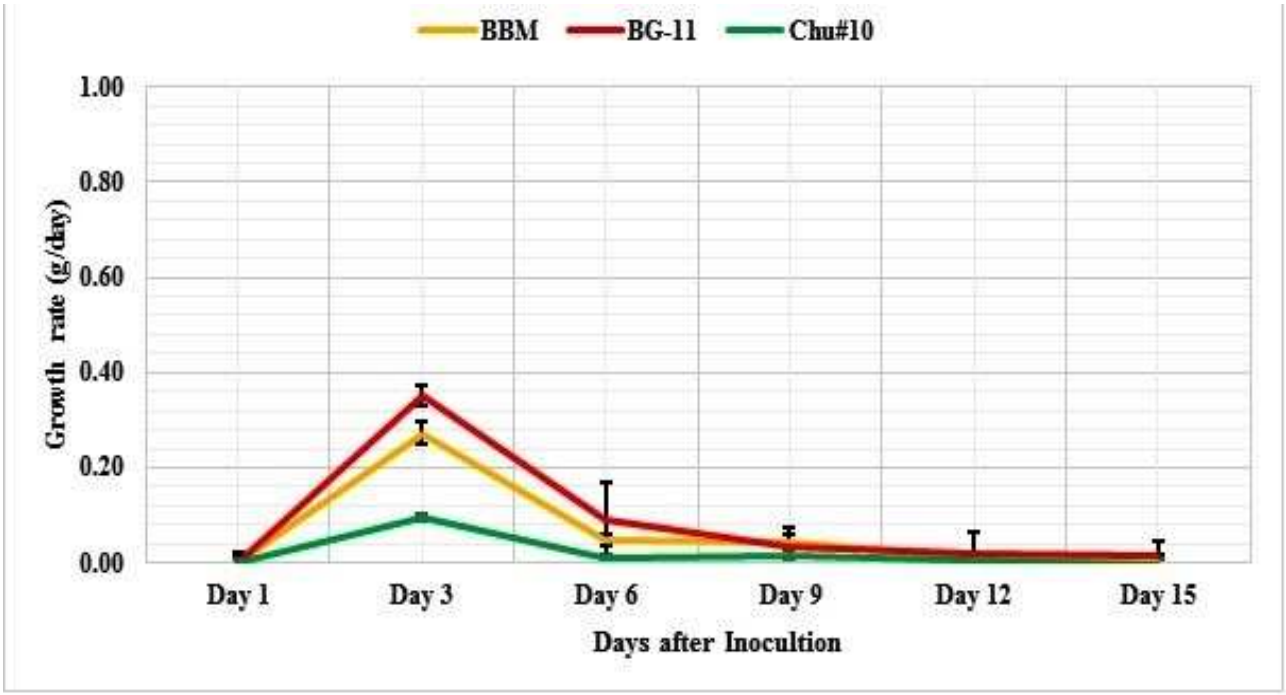

Figure 2. Effect of BBM, BG-11, and Chu #10 on specific growth rate of *E. gracilis*.

Table 2. Effect of different media and time intervals on biomass of *E. gracilis*

Source	Degree of freedom	Sum of squares	Mean squares	F-value	P-value
M	2	9.21	4.62	1993.49	0.0000
T	5	4.76	0.95	412.10	0.0000
M×T	10	3.19	0.32	137.97	0.0000
Error	36	0.08	0.002		
Total	53	17.23			

Here, M = Media, T = Time interval, M×T = Interaction between media and time interval

Table 3. Biomass (g) of *E. gracilis* cultivated on BBM, BG-11, and Chu #10 media

Media Type	Day 1	Day 3	Day 6	Day 9	Day 12	Day 15
BBM	0.23 kl	0.40 hi	0.51 h	0.72 fg	0.83 ef	0.85 e
BG-11	0.35 ij	0.71 g	1.13 d	1.45 c	1.81 b	2.18 a
Chu #10	0.21 l	0.25 j-l	0.28 j-l	0.31 i-l	0.34 i-k	0.32 i-k
LSD valut at 1%			0.11			

Means not sharing the same letters, with a column or row, differ significantly at p 0.01 according to LSD test at p<0.01
BBM = Bold's basal medium, BG-11 = Blue green medium, Chu #10 = Chu #10 medium, LSD = Least significant differences

DISCUSSION

The interaction between media type and time intervals was highly significant ($p < 0.01$), as confirmed by the LSD test (Table 3), indicating that biomass yield varied across days and was not uniform over time. BG-11 consistently outperformed the other media, with the magnitude of differences becoming more pronounced at later intervals. Overall, the growth response of *E. gracilis* differed significantly among the three media, reflecting the influence of nutrient composition on biomass production. BG-11 supported a prolonged exponential phase and achieved the highest biomass accumulation, while BBM showed moderate growth and Chu #10 the lowest. The significant interaction between media and time further emphasizes that the superiority of BG-11 increased with culture duration, whereas BBM and Chu #10 showed comparatively limited improvement over time. These results demonstrate that medium composition plays a critical role in determining biomass yield. Kishore *et al.* (2018) found similar results, showing that *E. gracilis* cultivated in BG-11 medium produced the highest biomass compared to BBM, while growth in Chu # 10 was limited. They suggested that the reduced biomass in BBM could be attributed to the presence of NaCl (0.025 g/L) and a lower concentration of $NaNO_3$ (0.25 g/L) in the medium. The trend in specific growth rates likely reflects a prolonged exponential phase in BG-11 relative to BBM and Chu #10, followed by a decline after nutrient depletion, consistent with typical microalgal culture dynamics where nutrient depletion leads to growth check and cell death (Munir *et al.,* 2015).

The choice of carbon source plays a crucial role in the growth and biomass production of *E. gracilis*. BG-11 medium, which contains Na_2CO_3 as a carbon source, resulted in the highest biomass accumulation (2.18 g) compared to BBM and Chu #10. This suggests that the availability of carbonate ions in BG-11 may enhance photosynthesis and support higher growth rates. The presence of Na_2CO_3 in BG-11 likely provides a more efficient carbon source under slightly alkaline conditions, which facilitates better carbon assimilation by *E. gracilis* (Abraham *et al.,* 2023). In contrast, BBM lacks carbonate ions, which may limit the algae's ability to achieve optimal growth under phototrophic conditions. Chu #10 similarly lacks carbonate supplementation, which, coupled with its lower overall inorganic carbon content, may further restrict CO_2 fixation and photosynthetic efficiency. This observation aligns with previous studies that indicated superior growth of microalgae in media containing carbonate sources (Liu *et al.,* 2013; Li *et al.,* 2022). Additionally, the role of organic carbon sources has been demonstrated to enhance algal growth and lipid production (Jonynaite *et al.,* 2024).

Iron is a critical nutrient for microalgae, acting as a cofactor in photosynthetic processes and cellular respiration (Qiu *et al.,* 2021). The high bioavailability of iron in BG-11 likely contributed to the enhanced growth and biomass production observed in this medium. Iron is essential for the electron transport chain during photosynthesis, and its availability directly impacts the efficiency of carbon fixation. In the present study, BG-11 exhibited significantly higher biomass productivity as compared to BBM and Chu #10, possibly due to the higher concentration of chelated iron in BG-11. BBM contains iron, but in lower chelated concentrations, which may reduce uptake efficiency. Chu #10 contains even less total iron and no chelated form, further limiting bioavailability. Previous research also supports the role of iron in boosting algal growth, with studies showing enhanced biomass and lipid accumulation in cultures supplemented with chelated iron (Schwarzhans *et al.,* 2015; Gissibl *et al.,* 2019). Furthermore, iron has been found to be critical in increasing lipid content in microalgal cultures, thus improving biofuel yield (Islami and Assareh, 2019).

The influence of nutrients such as $NaNO_3$, phosphorus, and other micronutrients on algal growth was evident in this study. BG-11 contains higher concentrations of $NaNO_3$ and phosphorus, which are essential for protein synthesis and cellular structure. These nutrients are efficiently assimilated in slightly alkaline conditions, promoting faster and more efficient growth of *E. gracilis* (Shekh *et al.,* 2022). BBM has lower $NaNO_3$ levels (0.25 g/L), which restricts nitrogen availability, slows protein synthesis, and limits growth potential. Chu #10, which lacks $NaNO_3$ entirely, exhibited the lowest biomass yield (0.34 g), indicating severe nitrogen limitation. Furthermore, Chu #10 also contains lower phosphorus levels compared to BG-11, which may have further contributed to restricted growth (Xie *et al.,* 2023). The importance of nitrogen in

enhancing protein synthesis and supporting growth is widely recognized (Bedard *et al.,* 2024). These findings are consistent with previous studies that highlight the importance of nitrogen and phosphorus for the optimal growth of *E. gracilis* (Yaakob *et al.,* 2021; Maltsev *et al.,* 2023).

E. gracilis can adapt to phototrophic, mixotrophic, and heterotrophic cultivation modes (Gissibl *et al.,* 2019; Barsanti *et al.,* 2021). While mixotrophy can enhance lipid, carotenoid, and unsaturated fatty acid yields (Ghosh *et al.,* 2019; Kim *et al.,* 2020), this study focused solely on phototrophic growth in solid media, making such mode comparisons beyond its scope.

This study confirmed that BG-11 medium is the most effective for cultivating *E. gracilis*, yielding the highest biomass production. The superior performance of BG-11 can be attributed to its nutrient profile, particularly the presence of Na_2CO_3 as a carbon source, chelated iron, and higher concentrations of $NaNO_3$ and phosphorus. In comparison, BBM and Chu #10 media exhibited lower growth and biomass yields, with Chu #10 showing the least favorable results due to combined nitrogen, phosphorus, and iron deficiencies. These findings suggest that BG-11 is the optimal medium for the cultivation of *E. gracilis*, particularly for applications in biofuel production and other industrial uses.

While this research was conducted using solid media (BBM, BG-11, and Chu #10), it is important to recognize that solid media may limit nutrient uptake due to nutrient gradients formed across the agar. This could explain the lower biomass yields observed in BBM and Chu #10 compared to BG-11. Future research should consider exploring both solid and liquid media to determine whether liquid cultures, with their more uniform nutrient distribution, could enhance biomass yield and improve nutrient uptake efficiency.

Conclusion: *E. gracilis* achieved the highest biomass yield and growth rate on BG-11 medium, outperforming BBM and Chu #10. These findings confirm BG-11 as the most effective medium for biomass yield and a promising choice for large scale production. Further investigations under varied conditions are needed to optimize its application in biofuel, nutraceutical, and pharmaceutical industries.

Author's contribution: SAB performed the research, write the original draft and applied the statistical analysis. GYB supervised the study and proof read the manuscript and approved the final version.

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