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The Study of Enumeration of Live Cells of Microalgae Growth by Using Different Fertilizer Compositions

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Abstract

This study investigates the growth performance of *Botryococcus sp*., a promising microalga for biofuel production, under different nitrogen, phosphorus, and potassium (NPK) fertilizer compositions. The microalgae were cultured with three types of fertilizers, namely NPK Blue (16:16:16), NPK Pink (13:13:21), and NPK White (15:15:15), over a period of 28 days. All-important parameters, like cell density, growth phases were monitored to study the different nutrient compositions that affect growth dynamics in *Botryococcus sp*. The results indicated that NPK White (15:15:15) elicited the quickest and strongest growth, where cell density was highest and active colony formation was observed from Day 26. Due to the balanced nutrient supply, there was sustained energy and metabolic support, thus making it ideal for promoting fast and steady growth. On the other hand, NPK Blue (16:16:16) showed a regular pattern of growth, marked by consistent proliferation of cells with structural integrity. This fertilizer proved suitable for long-term cultivation, offering a stable nutrient supply that avoided rapid depletion. In contrast, NPK Pink (13:13:21) stimulated strong early growth due to its higher potassium content but showed a decline in later stages, probably because of nutrient depletion or imbalance, showing the importance of a balanced nutrient composition. A balanced composition of NPK can give optimal growth and biomass production, obtained in this study.

1. Introduction

Botryococcus braunii is a colonial green microalga commonly found in freshwater and brackish environments. Its buoyancy is due to the accumulation of long-chain hydrocarbons and ether lipids in its extracellular matrix, making it a promising candidate for biofuel production. However, challenges such as its slow growth rate and the need for optimized bioreactor systems limit its commercial viability [1]. Key factors affecting its growth include temperature (25–27°C), light intensity (50–100 μmol m^{-2} s^{-1}), and pH (6.5–8.5), all of which influence metabolic and photosynthetic efficiency [2] [3]. Nutrients such as nitrogen, phosphorus, and potassium (NPK) play critical roles, with nitrogen and phosphorus promoting protein synthesis and photosynthesis, while potassium regulates enzyme activity and osmotic balance [4] [5]. The study investigates the impact of different NPK fertilizer compositions on *B. braunii's* growth, focusing on optimizing nutrient levels to balance biomass production and lipid accumulation. Excess or insufficient NPK can cause oxidative stress or nutrient deficiency, affecting growth and hydrocarbon synthesis [6]. Figure 1 shows an image of *Botryococcus braunii*.

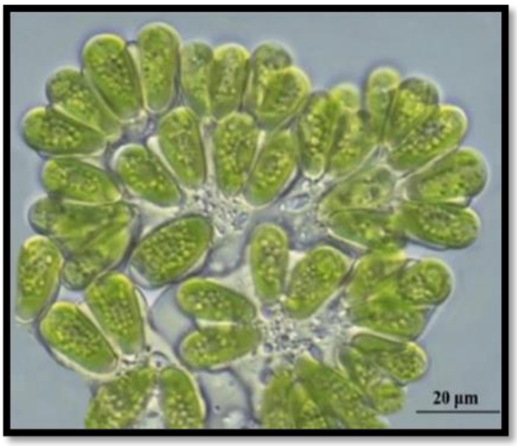

Fig. 1 Botryococcus braunii

Cell density, a key metric for assessing growth, highlights the effects of environmental and nutrient factors on biomass accumulation, with controlled adjustments necessary for optimal cultivation [4]. This research explores the use of a hemocytometer to monitor the growth dynamics of *B. braunii* cultured under different NPK compositions. The aim is to identify optimal conditions for live cell growth and enhance productivity. The experiments analyze cell density and proliferation using microscopy, contributing to sustainable biomass harvesting for industrial applications. Given the increasing global demand for liquid fuels and the depletion of fossil fuels, *B. braunii* keeps promise for biodiesel production due to its high photosynthetic rate and lipid storage capacity [7]. The objectives of this study are to analyze the growth of microalgae cells using a hemocytometer for different fertilizer compositions and evaluate live cells in various fertilizer media. Optimizing NPK levels is critical to supporting microalgae growth, addressing metabolic needs, and maintaining balance for biomass production and biofuel applications [6].

2. Literature Review

Macroalgae are widely used in food, especially in countries like Korea and Japan. Microalgae, though less utilized, have great potential due to their bioactive compounds, ability to grow in water without using land, fast growth, and role in improving soil fertility. They can also clean wastewater by removing nutrients, heavy metals, and pollutants (e.g., *Botryococcus*) [8][9]. Microalgae can be grown in open systems (ponds, tanks) or closed systems (tubes, panels) [10]. Successful cultivation depends on nutrients like nitrogen (for growth), phosphorus (for energy transfer), and potassium (for stability), along with proper light, pH, and temperature [11][12]. Fertilizers like NPK can boost growth, making them cost-effective for large-scale uses like biofuel, aquaculture feed, and medicine. Monitoring growth is key to optimizing nutrients and harvest timing, supporting sustainable applications like carbon capture and water management.

2.1 Botryococcus braunii

Botryococcus braunii is a green microalga found in various water bodies worldwide [13]. It forms colonies surrounded by extracellular biopolymers, which help it float, absorb light, and resist environmental changes [14]. This alga produces large amounts of hydrocarbons like fossil fuels, making it ideal for biofuel production. Unlike other microalgae, it secretes these hydrocarbons, simplifying extraction [15]. Its lipids are also used in bioplastics, cosmetics, and medicine [16]. Growing *B. braunii* requires nutrients like nitrogen, phosphorus, and potassium, as well as suitable light, temperature (20–30°C), and pH [11] [17]. It can be cultivated in open ponds or closed systems, with the latter offering better control but at a higher cost [10]. This alga thrives in wastewater, using nutrients to reduce contaminants while producing biomass for biofuels, fertilizers, and pigments [18][19]. Challenges include slow growth, achieving high biomass output, and efficient harvesting. Open-pond systems are cheaper but harder to manage, while photobioreactors are costly but more effective [20] [21]. Despite these issues, *B. braunii* holds great potential for sustainable biofuel and environmental benefits.

2.2 Role of NPK Nutrients

Optimal cultivation of *Botryococcus braunii* requires nitrogen (N), phosphorus (P), and potassium (K). Nitrogen supports growth, protein synthesis, and lipid production, but imbalances can disrupt metabolism or reduce biomass [22][23]. Phosphorus aids energy transfer (ATP), photosynthesis, and hydrocarbon production, with excess potentially harming ecosystems through [24][25]. Potassium regulates water balance, enzyme activity, and photosynthesis, improving growth and stress resistance. A deficiency reduces biomass and metabolic efficiency [26][27]. Balanced NPK nutrients enhance sustainable production and biofuel yield while minimizing environmental impact.

2.3 Influence of NPK Composition

Botryococcus braunii cultivation benefits significantly from NPK (nitrogen, phosphorus, potassium) fertilizers, which are essential for growth and metabolism. Nitrogen supports protein synthesis, phosphorus drives energy transfer (ATP) and photosynthesis, and potassium aids enzyme activity and stress resistance. Research shows that NPK fertilizers enhance growth, biomass production, and hydrocarbon yield, offering a cost-effective method for biofuel production [28]. Excess nitrogen can lead to overgrowth at the cost of hydrocarbon production, while phosphorus or potassium deficiencies reduce photosynthesis and growth. Optimizing these nutrients ensures sustainable growth and industrial viability [29]. Proper NPK management also prevents ecological harm, such as harmful algal blooms caused by nutrient imbalances. Maintaining balanced levels supports both algal productivity and environmental stability [30][31]. Though growth is slow, *B. braunii* is promising for biofuels, bioplastics, and valuable products like pigments and fertilizers. Optimizing cultivation and nutrient strategies enhances its commercial potential [32].

2.4 Methods for Quantifying Cell Counts

The hemocytometer, invented in the late 19th century, is widely used for cell counting due to its affordability and versatility. Louis Charles Malassez developed the first version in 1874, with later improvements by Karl Burker, who added gridded glass slides for better accuracy [33]. Modern hemocytometers use laser-etched grids for precision and feature 1×1 mm squares for easy cell density calculations. Viability analysis is often done alongside counting, using stains like Trypan Blue for dead cells and fluorescein diacetate for live cells [34]. Manual counting with a hemocytometer is common for tracking microalgae growth but is labor-intensive and prone to errors caused by user experience, cell clumping, and sample dilution. Repeated counts are often needed to ensure reliability [33][35]. Despite its limitations, the hemocytometer remains a valuable tool for small-scale analysis and diverse cell counting when handled by skilled users [36].

3. Methodology

The methodology is a critical component of any research, providing a structured and systematic approach to achieve the study's objectives. This section presents the research methodology using a flow chart, visually detailing each step of the process. Each component is thoroughly explained, including the rationale for the steps and how they address potential challenges and limitations. This ensures the research is well-organized, reliable, and aligned with the study's goals. The methodology emphasizes efficient project management by identifying potential issues early and defining clear objectives. Detailed planning and scheduling allocate sufficient time for tasks and milestones, keeping the project on track and within the specified timeframe. Regular monitoring and adjustments ensure timely resolution of challenges, minimizing delays and maintaining progress. This structured approach not only mitigates risks but also ensures the project's successful completion, delivering accurate and reliable results.

3.1 Microalgae, *Botryococcus sp.* Preparation

The *Botryococcus* sp. culture, provided by Universiti Tun Hussein Onn Malaysia, was mixed with dechlorinated tap water (prepared by leaving tap water to stand for 48 hours to remove chlorine). Samples were divided into a blank group (no fertilizer) and treatment groups with different fertilizers: NPK pink (13:13:21), NPK blue (16:16:16), NPK white (15:15:15), and a customized NPK solution (20:20:10). The customized solution included NPK, urea with boron, and enhanced rock phosphate, dissolved in dechlorinated water. Aeration was applied to all samples to maintain oxygenation and prevent sedimentation, ensuring consistent nutrient availability. This setup allowed comparison of growth between the blank and treated groups.

3.2 Cultivation of *Botryococcus sp.* via NPK

The *Botryococcus sp.* culture process began with inoculating microalgae into tanks treated with aerated NPK nutrient solutions on Day 0. To promote photosynthesis, constant illumination was provided, and preliminary testing was conducted to establish baseline growth and nutrient absorption. The experiment ran for 28 days, with regular monitoring to assess nutrient usage, growth, biomass buildup, and lipid synthesis. Microalgae development is influenced by environmental factors such as light, temperature, and pH. Optimal photosynthesis for most microalgae occurs at light levels between 60–200 μmol photons/m^2/s, but excessive light can cause photoinhibition [37] [38]. Ideal temperature ranges are 20–30°C, with growth inhibited below 15°C or above 35°C [39]. Additionally, pH levels of 7–9 are preferred for most species, as extreme acidity or alkalinity hinders photosynthesis and enzyme activity. Neutral to slightly alkaline conditions generally support optimal growth.

3.3 Microalgae Growth Enumeration

The growth of *Botryococcus sp.* was monitored until it reached its peak. Samples were collected from each tank, including the blank and three NPK treatment groups and one blank, on each testing day. Cell density was the primary metric for assessing growth. Four samples were collected daily, using a pipette to transfer the samples into test tubes for laboratory analysis. This systematic collection and evaluation ensured accurate tracking of growth dynamics across different fertilizer treatments.

3.3.1 Monitor growth of microalgae cells by using hemocytometer.

Cell density in each *Botryococcus sp.* sample was measured using a compound microscope and a hemocytometer. The hemocytometer, with its precision-etched grid, allowed for accurate counting and calculation of cell concentration within a defined volume. Before use, the hemocytometer was cleaned with alcohol and dried to ensure precision. The sample tube was gently shaken to evenly distribute cells, and a pipette was used to place the sample onto the hemocytometer chamber. A cover glass was then placed to spread the sample evenly. The hemocytometer was positioned under the compound microscope, and cell images were captured at 10X, 20X, and 40X magnifications using a mobile camera. Cells were counted within specific grid squares, and the counts were averaged to calculate the overall cell density. For example, see Equation 1, Fig. 2 and Table 2. This method provided reliable data on growth rates and culture health, enabling effective monitoring of *Botryococcus sp.* growth dynamics.

Equation 1 Equation of cell density.

Cell density = (*Average number of cell per square x Dilution factor (if any))/ Volume of the square*)

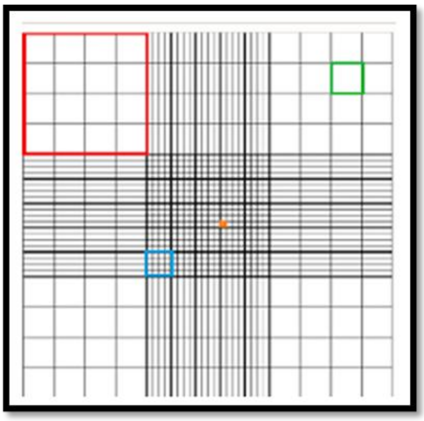

Fig. 2 Schematic representation of a hemocytometer grid

Table 1 Classification of the square chamber of hemocytometer [40]

Unit	Width (mm)	Area (mm^2)	Volume (mm^3)	Volume (ml)	No	
Chamber	3	9	0.9	0.0009	2	Per hemocytometer
Square (red)	1	1	0.1	0.0001	9	Per chamber
Small square (green)	0.25	0.0625	0.00625	0.00000625	16	Per corner square

Smaller square (blue)	0.2	0.04	0.004	0.000004	25	Per central square
Smallest square (orange)	0.005	0.0025	0.00025	0.00000025	16	Per smaller square

3.3.2 Live Cell Count Monitoring

To monitor live cell counts in *Botryococcus sp*., a plate-based method was used, ensuring even cell distribution and contamination-free handling. The plate surface was cleaned with alcohol, and the microalgae sample was evenly spread using a pipette. The plate was then placed on a compound microscope, and cell images were captured at 10X, 20X, and 40X magnifications using a mobile camera. This process allowed detailed observation of cell size, health, and growth rate. Live cell counts are critical for evaluating microalgae viability, growth rates, and culture health. This technique helps differentiate living cells from dead ones, providing insight into cell division, biomass accumulation, and overall productivity. Regular monitoring also identifies the effects of environmental factors such as temperature, light intensity, and nutrient availability on cell viability. Maintaining a high percentage of healthy, active cells is essential for applications like biofuel production, where viable cells contribute to efficient lipid accumulation and metabolic activity. By tracking live cell density, researchers can optimize culture conditions, enhancing the sustainability and productivity of microalgal systems [41].

4. Result and Discussion

This chapter provides a detailed analysis of the experimental data collected to evaluate the growth performance of *Botryococcus sp*. in response to different NPK fertilizers. The study aimed to optimize the growth of this microalgae species by testing three fertilizers: NPK Pink (13:13:21 + 28% phosphorus), NPK Blue (16:16:16 + 28% phosphorus), and NPK White (15:15:15 + 28% phosphorus). The growth of *Botryococcus sp.* was tracked over 28 days by measuring live cell counts using a compound microscope, ensuring accurate and consistent monitoring. Daily observations allowed the identification of growth trends, peak periods, and the effectiveness of each fertilizer in promoting cell proliferation. This analysis highlights the impact of different fertilizer compositions on microalgal growth and identifies the optimal nutrient balance for maximizing growth. The findings offer valuable insights into further applications of *Botryococcus sp.* in biofuel production and other biotechnological fields.

4.1 Analysis for cells

The growth of *Botryococcus sp*., a microalga with significant potential for biofuel production, depends heavily on nutrient availability. NPK fertilizers provide essential macronutrients: nitrogen for protein synthesis and photosynthesis, phosphorus for energy transfer and nucleic acid production, and potassium for stress tolerance and osmotic regulation. The effectiveness of these fertilizers varies based on their nutrient composition and ratios, which influence microalgal growth dynamics. This study evaluated the growth of *Botryococcus sp.* over 28 days using three NPK fertilizers: NPK White (15:15:15 + 28% phosphorus), NPK Pink (13:13:21 + 28% phosphorus), and NPK Blue (16:16:16 + 28% phosphorus). The results highlighted how nutrient balance affects key growth phases, including the lag, exponential, peak, and stationary stages. By comparing growth patterns, the analysis aims to identify the most effective fertilizer formulation for promoting sustained growth and biomass production in *Botryococcus sp.*

4.1.1 Comparison of fertilizer

The growth of *Botryococcus sp.* treated with NPK White, NPK Pink, and NPK Blue fertilizers over 28 days showed different patterns due to nutrient composition. During the lag phase, all treatments started similarly at 6.1 Log10 Cells/mL as the microalgae adapted to the environment. In the exponential phase, NPK Blue grew fastest, reaching 6.5 Log10 Cells/mL by Day 8, thanks to its balanced 16:16:16 formula. NPK White followed closely, with steady growth supported by its 15:15:15 composition and high phosphorus content. NPK Pink grew slower due to its lower nitrogen content (13:13:21), which limited early protein and chlorophyll production. At the peak, NPK White achieved the highest cell density (6.8 Log10 Cells/mL) with consistent growth. NPK Blue reached a similar peak but showed some variability, while NPK Pink peaked at 6.7 Log10 Cells/mL, performing less effectively overall. These results highlight that balanced nutrients are key for maximizing growth, with NPK White being the most effective, NPK Blue excelling early, and NPK Pink showing reduced efficiency. For example, see Fig. 3.

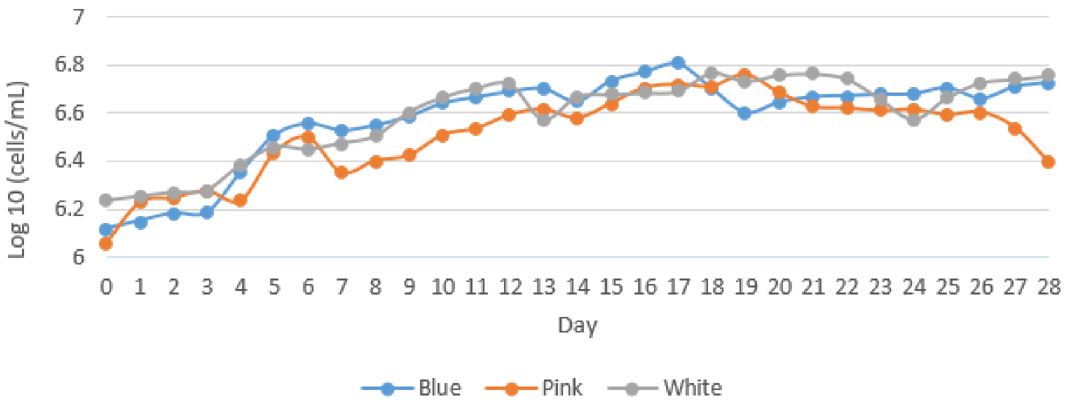

Fig.3 Cell density of Botryococcus sp. samples with 3 type of fertilizer.

4.2 Analysis of Live Cell

Live cell analysis is a key tool for studying living cells in real time, providing insights into processes like growth, division, and death, as well as how cells respond to changes in their environment, such as pH, temperature, or nutrients. In this study, it is used to evaluate how NPK fertilizers affect microalgae by tracking cell health, viability, and activity. This technique is widely used in fields like pharmacology and biotechnology to test the effects of drugs or nutrients. It also helps in studying the cell cycle, developmental biology, and cancer. Unlike fixed-cell methods, live cell analysis captures real-time changes, offering more accurate results without disturbing the cells. Applications range from agriculture, where it studies microorganisms and algae, to regenerative medicine, where it tracks stem cell growth and differentiation, making it an essential tool across many scientific fields [42].

4.2.1 Summary

The impact of Blue NPK (16:16:16), Pink NPK (13:13:21), White NPK (15:15:15), and a blank sample on microalgal growth were compared on Days 17, 21, and 26. The blank sample showed minimal growth, with cells remaining spherical and showing no clustering due to the absence of enhanced nutrients, emphasizing the importance of NPK fertilizers for metabolic activity and development. By Day 26, Blue NPK (16:16:16) supported the strongest growth, with dense clustering and consistent development due to its balanced nutrients and high phosphorus content, which promotes protein synthesis, energy metabolism, and cell health. Pink NPK (13:13:21), with slightly lower nitrogen and higher potassium, also fostered significant growth, forming tight clusters and colonies. The increased potassium likely improved structural integrity and osmotic regulation, complementing phosphorus-driven energy production. White NPK (15:15:15) also showed steady growth, forming large, densely packed colonies by Day 26 due to its balanced nutrient ratios, which supported consistent metabolic activity. The varying nutrient levels influenced specific growth patterns, with high phosphorus being a key driver across all fertilizers. These findings highlight the effectiveness of NPK fertilizers, particularly those with balanced nutrients and high phosphorus, in supporting robust microalgal growth and metabolism.

5. Conclusion

This study analyzed the growth of *Botryococcus sp*. using a hemocytometer under different NPK fertilizer treatments, NPK Blue (16:16:16), NPK Pink (13:13:21), and NPK White (15:15:15) over 28 days to optimize microalgal growth for biofuel production. It also evaluated live cell performance to determine how varying nutrient compositions influence growth and metabolism. The results showed that nutrient availability, especially nitrogen, phosphorus, and potassium, significantly impacted growth. NPK Blue supported robust and consistent early growth, peaking at 6.8 Log^{10} Cells/mL on Day 16, due to its balanced composition and high phosphorus content. NPK White maintained steady growth throughout, proving most effective for long-term cultivation. NPK Pink exhibited good early growth but declined after Day 25, likely due to nutrient imbalance or environmental stress. Minimal growth in control highlighted the essential role of fertilizers in sustaining cellular development. The study successfully met its objectives by using the hemocytometer to analyze cell growth and evaluate live cell performance across different fertilizers. It concluded that NPK White is ideal for sustained growth, while NPK Blue excels in early growth. NPK Pink has potential but requires further optimization for long-term use. These findings reinforce the importance of balanced NPK fertilization in maximizing *Botryococcus sp*. productivity and provide a basis for improving nutrient strategies in future biofuel production research.

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Conflict of Interest

Authors declare that there is no conflict of interest regarding the publication of the paper.

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