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FEASIBILITY OF MICROALGAE IMMOBILISATION IN ALGINATE BEAD FOR KITCHEN WASTEWATER TREATMENT

Microalgae harvesting after wastewater treatment is a critical challenge due to the free-floating nature of cells. This study evaluates the feasibility of immobilising *Botryococcus* sp. in alginate beads, focusing on bead size stability, compressive strength, and cell retention. Sodium alginate and calcium chloride ($CaCl_2$) were optimised to produce beads with improved mechanical properties and minimal cell leakage. Beads formed with 40 gL^{-1} alginate and 40 gL^{-1} $CaCl_2$ demonstrated superior compressive strength and stability. The immobilised microalgae exhibited no cell leakage at low and medium biomass stocking densities. Moreover, the alginate matrix supported microalgae growth, underscoring its potential for sustained wastewater treatment applications. These findings highlight the viability of alginate-immobilised microalgae systems for biomass recovery and reuse.

Keywords: Botryococcus sp.; immobilisation; sodium alginate; kitchen wastewater

1. Introduction

Microalgae have been extensively applied in sustainable wastewater treatment worldwide due to their rapid growth, ability to metabolise nutrient and versatility in various applications [1]. However, the primary challenges in microalgae-based systems lies in the harvesting of biomass after treatment. To address this issue, different method has been establish including membrane filtration, flocculation, and immobilisation, but they often involve high energy consumption and operational cost, which limit their scalability [2-4].

Immobilisation of microalgae cells into calcium alginate beads offers various notable advantages, especially in harvesting microalgae after treatment, while preserving their removal efficiency. Alginate, a natural polymer, forms a stable matrix that retains microalgae cells from outer environments yet still allows nutrients to flow into the cell to support proliferation [5,6]. Moreover, alginate are natural polymer that are nontoxic to algal cell and therefore offers greater operational flexibility and easier separation in wastewater treatment [7]. Previous studies reported that microalgae alginate bead was able to further enhance the wastewater treatment through physical absorption and chemical precipitation [8].

To apply immobilised microalgae for nutrient removal in wastewater, the stability of the alginate in the treatment system should be the greatest concern. The major limitations for the alginate entrapment of microalgae biomass were the instability of alginate beads upon in contact with chelating agent such as phosphate and citrate [9]. Therefore, this study focuses on optimising alginate bead formulation for immobilising *Botryococcus* sp., in kitchen wastewater. The mechanical properties, bead size stability and cell leaking analysis were evaluated to develop durable alginate-immobilised systems capable of efficient biomass recovery and reuse.

2. Material and methods

2.1. Wastewater collection

Wastewater was collected randomly from various restaurants at three different time sessions: 8:00 to 9:00 am, 12:00 to 1:00 pm, 3:00 to 4:00 pm. 10 litres of wastewater were collected from different discharge point and stored in a high-density polyethylene (HDPE) bottle. Wastewater was autoclaved at 121°C for 20 min and then left cooled to room temperature Wastewater

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was then transferred to laboratory within 1 hour and stored at 4°C, before being used in experiment. The following wastewater quality parameters were recorded: 2084±160.7 mg/L of Chemical Oxygen Demand (COD), 43.4±8.6 mg/L of Total Phosphate (TP), 53.5±6.6 mg/L of Total Nitrogen (TN) and 3.1±0.3 pH. These values were compared to the Malaysian Environmental Quality (Sewage and Industrial Effluents) Regulations 1979 to assess their compliance with wastewater discharge standards.

2.2. Microalgae culture

The native microalgae strain *Botryococcus* sp., was obtained from the Geo-environment laboratory at the University of Tun Hussein Onn Malaysia. The strain was inoculated using modified Chu 13 medium, as described by Dayananda, et al. [10]. The culture medium was autoclaved at 121°C for 20 min and then left cooled to room temperature before inoculation. Microalgae cultivation was conducted in a photobioreactor housed within the Geoenvironmental Laboratory, under a constant air supply of 3.6 L/min, maintained at a temperature of 28±2°C, with a light intensity of 94.5 µmol photons/m^2/s and a photoperiod of 12/12 hours synchronising light and dark cycle [11].

2.3. Microalgae immobilisation

Microalgae alginate beads was prepared as described in Soo, et al. [12] with modifications. Alginate solution was prepared by slowly dissolve sodium alginate into warm distilled water and then autoclaved at 121°C for 2 min. Microalgae culture was centrifuged at 3500 rpm for 10 min at 10°C and the biomass pellets were resuspended again in deionised water. To immobilise microalgae, concentrated microalgae biomass was mixed with a known concentration of sodium alginate (*w*/*v*) before transferred to a 5 mL dropper. The mixture was dropped into a known concentration of $CaCl_2$ (*w*/*v*) from a height of 2.5 cm above at a rate of one bead per second. The beads were washed with distilled water to remove excess $CaCl_2$. Approximately 2500 beads were produced from 100 mL of alginate solution. Blank beads were prepared by replacing the microalgae fraction with deionised water in the alginate mixture. Produced microalgae alginate beads were stabilised for 24 hours and washed with deionised water before being used for wastewater treatment. The content of alginate beads with varying microalgal biomass are as detailed in TABLE 1.

2.4. Experiment design

2.4.1. Determination of mechanical properties

The texture of the microalgae alginate beads was analysed using texture analyser (TA. XTplus, Stable Micro Systems, UK), equipped with a 5 kg load cell and a cylindrical stainless-steel probe. According to the methodology adapted from de Jesus, et al. [13] with slightly modification, the sample was compressed at a constant pre-test speed of 1 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 1 mm/s over a varied distance adjusted based on the dimension of the sample to achieve complete compression. The probe compressed each sample until a 50% deformation or until bead rupture occurred. The maximum force exerted on the bead before deformation or rupture was recorded as the compressive strength, providing a quantitative measure of the bead's mechanical durability [14]. Ten beads were taken in each sampling time, and the testing was performed in triplicate.

2.4.2. Effect of alginate and $CaCl_2$ concentration on beads size stability

To study the effect of alginate concentration on beads stability, six concentration of alginate solution which are 20 gL^{-1}, 30 gL^{-1}, 40 gL^{-1}, 50 gL^{-1} and 60 gL^{-1} were used to prepare microalgae alginate beads and 20 gL^{-1} as hardening agent. For sodium alginate hardening, six concentrations of $CaCl_2$ which are 20 gL^{-1}, 30 gL^{-1}, 40 gL^{-1}, 50 gL^{-1} and 60 gL^{-1} were used to study the effect of $CaCl_2$ on beads stability. The beads were positioned randomly on an orbital shaker under the abovementioned culture conditions for 16 days. At each sampling time, ten beads were taken to test the stability of beads. The diameter of the beads before and after treatment were measured using a digital calliper (GERE), with an accuracy of ±0.1 mm.

2.4.3. Effect of biomass volume on cell leaking

A cell leakage test was conducted to evaluate the ability of alginate beads to retain immobilised microalgae cells during wastewater treatment. This test aimed to quantify the extent of cell leakage over time, providing insights into the beads' containment efficiency and structural integrity under simulated operational conditions. The test was conducted according to Soo, et al. [12] with slight modification. During each experiment, 100 g

TABLE 1

Alginate to microalgae biomass ratio of different samples

Sample	Content of microalgae beads	Microalgae biomass volume
Blank	Alginate + deionised water	100 mL deionised water
Low stocking beads	Alginate + microalgae biomass	25 mL microalgal suspension + 75 mL deionised water
Medium stocking beads	Alginate + microalgae biomass	50 mL microalgal suspension + 50 mL deionised water
75% stocking beads	Alginate + microalgae biomass	75 mL microalgal suspension + 25 mL deionised water
High stocking beads	Alginate + microalgae biomass	100 mL microalgal suspension

of the prepared microalgae alginate beads were introduced in Erlenmeyer flask containing 2000 mL of wastewater. All flasks were incubated on an orbital shaker set at 250 rpm to provide consistent agitation. The temperature was maintained at 27±1°C to reflect typical ambient temperatures conducive to microalgae growth. The incubation period lasted for 16 days. Samples were withdrawn from each flask every 4 days for 16 days to monitor cell leakage over time. At each sampling point, a 10 mL of the wastewater was collected using sterile pipettes to check for any cell leakage. The presence of microalgae cells in the wastewater was counted by a Neubauer improved hemacytometer, indicating cell leakage of alginate beads. Cell density within the beads was measured after dissolving 10 beads in a tri-sodium citrate solution, allowing quantification of leakage as the percentage of cells lost from the cell density within the beads. Samples were also visually inspected for any green colouration, which would indicate the presence of free microalgae cells due to leakage from the beads.

2.6. Data and statistical analysis

Analysis of variance (ANOVA) was used in all tests. The ANOVA procedure was first used to evaluate any significant differences between compression resistance of alginate beads produced from different alginate and $CaCl_2$ concentration. The initial and final diameters of the alginate beads produced from different concentration of alginate and $CaCl_2$ were compared using ANOVA, followed by the Tukey's test when ANOVA test showed a significant difference between result. The statistical analysis was conducted with $\alpha = 0.05$ using SPSS V29.

3. Result and discussion

3.1. Mechanical properties

The mechanical compression of the alginate beads is an indicator of a durable bead. Alginate beads used in wastewater treatment should be able to withstand vigorous handling and extended incubation during treatment. Therefore, the compression resistance of the alginate beads was evaluated through a texture analyser. The compression strength of the beads with various alginate and $CaCl_2$ concentration provided the insight of the relationship between alginate and $CaCl_2$ concentration on bead strength.

As illustrated in Fig. 1, the increase in alginate concentration from 10 gL^{-1} to 60 gL^{-1} leading to significant rise in bead compression strength ($p < 0.05$). This statement is supported by the findings of de Jesus, et al. [13], suggesting that compressive strength of alginate beads increase with increasing biopolymer concentration, which refers to sodium alginate. Beads produced with 60 gL^{-1} alginate and 50 gL^{-1} $CaCl_2$ exhibited a compression resistance of 39564±1805 g, which was the highest across all sample tested. In contrast, alginate beads produced with 10 gL^{-1} alginate and 40 gL^{-1} $CaCl_2$ had the lowest compression resistance, at 3213 ± 217 g. This trend underscores that higher alginate concentrations enhance the mechanical durability of the beads, which enable them to withstand greater compressive forces.

Interestingly, when the alginate concentration was maintained at 10 gL^{-1}, 20 gL^{-1}, 30 gL^{-1} and 40 gL^{-1}, no significant differences in compression resistance were observed ($p \geq 0.05$) across varying $CaCl_2$ concentration from 10 gL^{-1} to 60 gL^{-1}. However, at higher alginate concentration, specifically, 50 gL^{-1} and 60 gL^{-1}, there was a noticeable increase in compression strength when $CaCl_2$ concentrations increased from 10 gL^{-1} to 60 gL^{-1}. These results align with the findings from Stößlein, et al. [15], which suggested that the concentration of $CaCl_2$ alone does not substantially impact the compression strength. In fact, mechanical properties of alginate beads were more dependent on their gelling time, which in here refers to the time allowed for the sodium alginate to transition fully into calcium alginate. As sodium alginate droplets are introduced into the $CaCl_2$ solution, a hydrogel formed almost immediately, creating a core shell structure on the surface of beads, which means that the outer shell part of bead is composed of calcium alginate, while the inner core of the beads remained as sodium alginate. Over time, Ca^{2+} will further diffusion into the inner core of beads, gradually transform the inner core sodium alginate into calcium alginate gel, thereby strengthening the bead structure [16,17]. According to Kim [17], even the $CaCl_2$ concentration as low as 3mM is sufficient to form alginate beads, provided that enough gelling time is allowed for the transition. However, if the $CaCl_2$ concentration is too low, there is a risk of Ca^{2+} leaching from alginate matrix over time, which could weaken the gel structure, resulting in water absorption into the beads and potentially causing them to rupture [18].

Stößlein, et al. [15] stated that mechanical properties of alginate beads were linearly dependent on the guluronic acid content of sodium alginate. Higher alginate concentration have more D-mannuronic and L-guluronic units available for cross linking with cations as they can form various bonds and linkages with calcium ions, resulting in a more rigid structure in calcium-alginate-gel [19]. The hypothesis was further supported by Chan, et al. [20], who suggested that by using a better gelling cation with higher chemical affinity toward alginate, the stiffness of alginate beads can be significantly enhanced.

Compression resistance testing revealed that beads produced with higher alginate concentration exhibited superior mechanical durability. Specifically, beads formulated with 60 gL^{-1} alginate and 50 gL^{-1} $CaCl_2$ demonstrated the highest compression resistance. This level of strength ensures that the beads can maintain their form during extended use, even under vigorous shaking conditions. However, it is essential to balance mechanical resistance with practicality. Beads formed with alginate concentration exceeding 40 gL^{-1} have excellent structural durability, but it exhibited excessive viscosity during production, leading to difficulties in immobilising the microalgae cells [13,20]. Based on these results, the optimal combination was identified as alginate beads formed with 40 gL^{-1} alginate and

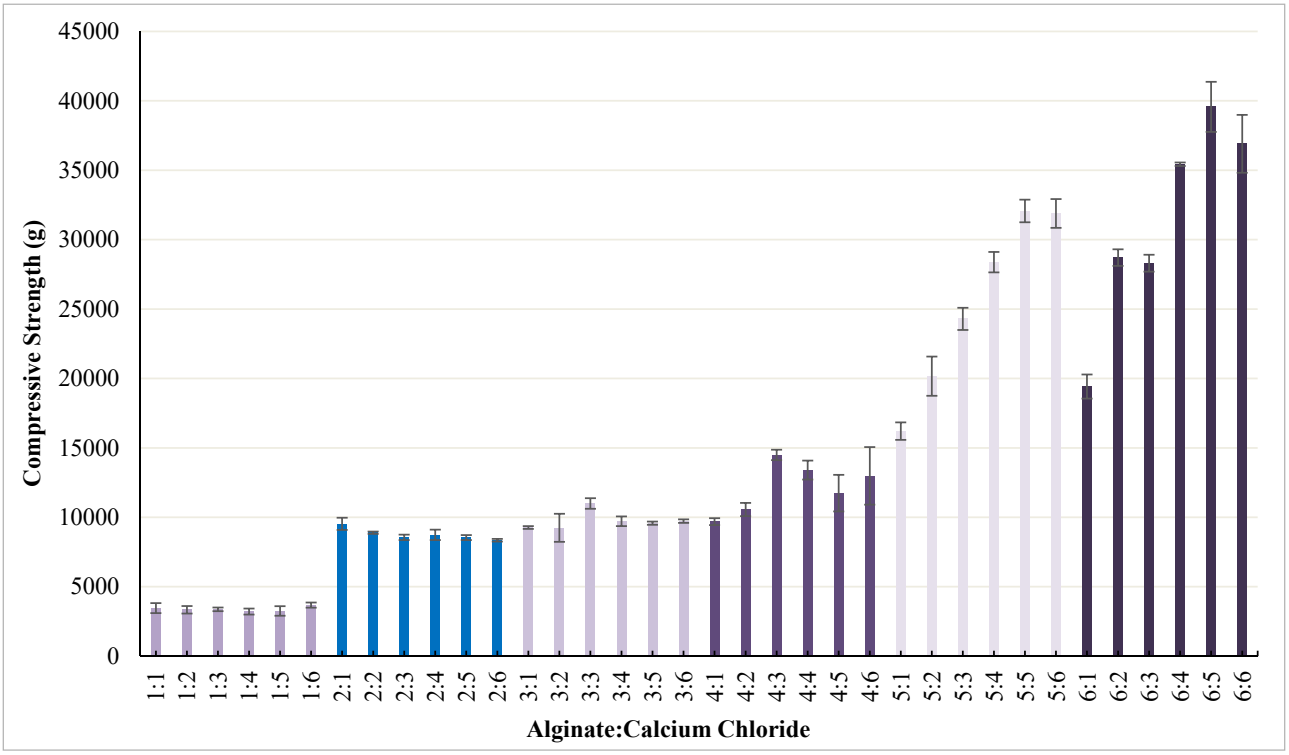

Fig. 1. The compressive strength of alginate beads with different alginate and $CaCl_2$ concentration with 1:1 as 10 gL^{-1} alginate to 10 gL^{-1} $CaCl_2$ and 6:6 as 60 gL^{-1} alginate to 60 gL^{-1} $CaCl_2$.

hardened with 40 gL^{-1} $CaCl_2$. Although they exhibited slightly lower compression resistance, the beads still offered substantial mechanical durability and thus were selected for subsequent experiments.

3.2. Beads size stability in wastewater

Other than mechanical compression, their ability to resist shrinkage in wastewater treatment are also equally important to serve as the guideline to produce quality beads. In this study, various $CaCl_2$ and sodium alginate concentration were used to produce alginate bead, and their size was measured before and after a 16-day incubation period. Fig. 2 illustrated the size variation of the beads during this period.

The initial diameters of the beads ranged from 2.45 mm, for those formed with 10 gL^{-1} alginate to 4.05 mm, for those produced with 60 gL^{-1} alginate. A significant increase in bead size was observed when the alginate concentrations increase ($p < 0.05$). This finding was aligned with Banerjee, et al. [8] and Lee, et al. [21] studies, who reported a bead size of 2 mm to 5 mm when 50 gL^{-1} or lower alginate concentration is used for bead formation.

In contrast, the impact of $CaCl_2$ concentration on initial bead size was minimal and was statistically insignificant ($p \geq 0.05$). This highlighted that alginate concentration played a more vital role than $CaCl_2$ in determine bead size during immobilisation. This is attributed to the fact that the mechanical and swelling properties of alginate beads are not primarily influenced by the concentration of cross-linking ions, but rather the type of cation used for cross-linking. For example, Fe^{3+}, Cu^{2+} and Sr^{2+} imparted stronger elastic modulus to alginate compared to Ca^{2+}, meaning that alginate hardened by these cations can resist higher compression and deformation. Moreover, due to higher elastic modulus, alginate beads hardened with these cations will resist swelling and shrinking more effectively than conventional Ca^{2+} [22].

All beads exhibited shrinkage in diameter after the 16 days incubation, with the extend of shrinkage depending on the concentration of alginate and $CaCl_2$ used during bead formation. Beads formed with 10 gL^{-1} alginates showed the most substantial reduction, with their diameter decreased by 7.4% from the initial size after incubation. Similarly, beads hardened with 10 gL^{-1} $CaCl_2$, exhibited a diameter reduction of 6.6%, which are the highest across all sample tested. In contrast, the most stable beads were those formed with 60 gL^{-1} alginates, which displayed only a 4.2% reduction in size after incubation. Beads hardened with 60 gL^{-1} $CaCl_2$ also exhibited minimal shrinkage, with a size reduction of just 5.2%, which was the lowest among all samples. Other satisfactory results were observed in beads formed with 40 gL^{-1} and 50 gL^{-1} alginates, where shrinkage was limited to 5.4% and 4.5% respectively. Likewise, beads hardened with 40 gL^{-1} and 50 gL^{-1} $CaCl_2$ also showed minimal changes in size, shrinking by 5.5% and 5.4% respectively.

These findings aligned with Soo, et al. [12], who reported that alginate beads formed with lower alginate concentration, 10 gL^{-1} alginate, exhibited the lowest stability, whereas stability improved with higher alginate concentrations. Similarly, Rajmohan and Bellmer [14] found that bead stability and size reduction

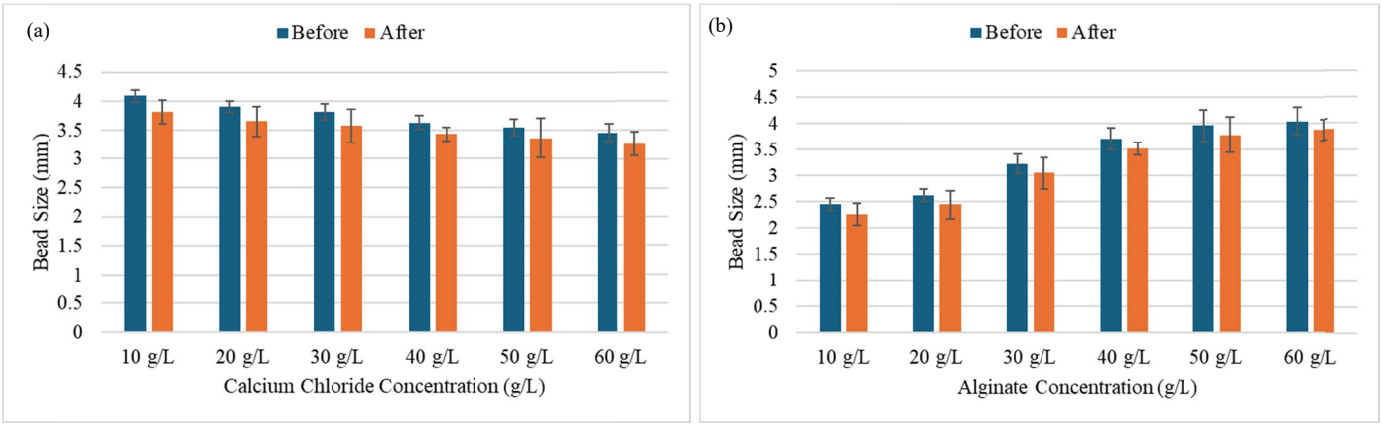

Fig. 2. The bead size of alginate beads with various (a) $CaCl_2$ and (b) alginate concentration

in *Spirulina* sp. alginate beads were primarily influenced by the alginate concentration, with beads diameter increase as alginate concentration increased. Moreover, the same study confirmed that $CaCl_2$ concentration had no significant effect on initial bead diameter, especially when alginate concentrations was constant, further supported the result of this study.

Hence, based on the mechanical and bead size analysis, the optimal combination was identified as 40 gL^{-1} alginate and 40 gL^{-1} $CaCl_2$, and this formulation will be used on the upcoming study. Beads produced with this formulation maintained their structural integrity throughout the 16-day incubation period, showing only minimal shrinkage of 5.4%. This formulation was also supported by Lee, et al. [21] who found that beads formed with these concentrations had a diameter of approximately 3.5 mm, which is ideal for nutrient uptake and pollutant removal in wastewater treatment.

Alginate concentrations exceeding 40 gL^{-1} were found to be impractical. Beads formed with higher alginate concentration tend to have larger bead size, which will negatively impact the nutrient uptake efficiency due to the increased in diffusion distance within the bead matrix. Banerjee, et al. [8] explained that larger beads have thicker biofilm layer that hindered the nutrient diffusion and reduce the efficiency of nutrient uptake from wastewater. Furthermore, beads formed with excessively high alginate concentrations are non-spherical in shape and have long tails, which will reduce their uniformity during production, as referred in Fig. 3 and potentially affect the performance in wastewater treatment [14,20]. Chan, et al. [20] further stated

Fig. 3. Shape of alginate beads from different alginate concentrations

that alginate concentration below 20 gL^{-1} form beads with sphericity exceeding 0.05, indicating that beads are not in ideal spherical shape.

Moreno-Garrido [6] emphasised the importance of balancing bead stability with cost effectiveness in bead production. Therefore, using 40 gL^{-1} alginate concentration are no doubt the best option as it provided an optimal balance, ensuring that durable beads is produced without incurring unnecessary expenses. In conclusion, beads formed with 40 gL^{-1} alginate and 40 gL^{-1} $CaCl_2$ exhibited the best combination of stability, performance, and cost-efficiency.

3.3. Effect of biomass volume on cell leaking

While mechanical strength and bead size are critical factors for operational durability, the ability of alginate beads to retain microalgae cells without leakage is equally important to ensure effective containment in wastewater treatment applications. Therefore, this study evaluated the cell containment efficiency of alginate beads with varying microalgae stocking densities incorporated in the immobilisation formulation optimised in previous studies. The samples were incubated in wastewater sample for 16 days and the result were illustrated in Fig. 4.

For low stocking alginate beads, with an initial cell concentration of 7.5×10^5 cells/mL, no leakage was detected throughout the entire 16-day observation period. The percentage of leakage remained consistently at 0%, suggesting that the alginate matrix effectively retained cells at this biomass level without structural compromise. By day 12 and 16, cell concentration within the beads increased to 3.17×10^6 ells/mL, showing that microalgae growth occurred within the immobilised structure. The consistency in cell containment, in combination with observed cell growth, indicated that the alginate structure was capable of supporting microalgae proliferation.

For medium stocking beads, the initial cell concentration was higher at 1.08×10^6 cells/mL. Similarly, no leakage was observed up to day 16, with the final cell concentration of 3.08×10^6 cells/mL. This result indicated that the alginate beads effectively retained a moderate amount of biomass without structural compromise, as it maintained 0% leakage rate throughout the observation period, suggesting that medium stocking density falls well within the retention capacity of the alginate matrix.

At 75% stocking density, the initial cell loading was 1.67×10^6 cells/mL. No cell leakage was observed at the initial incubation period. Only on day 16, there was a minor leakage observed, with 4.0×10^4 cells/mL escape into the wastewater, accounting for 1.04 % of the total cell population. By day 16, the cell density within the alginate matrix have reached up to 3.83×10^6 cells/mL. This small percentage of leakage at the end of the testing period indicated that the containment threshold of the alginate beads is close to its limit at 75% stocking density.

In the high stocking density beads, the initial cell concentration was 2.5×10^6 cells/mL. Leakage began on day 12, with 3.0×10^4 cells/mL escape into the wastewater sample, accounting for 0.78% of the total cell population, while immobilised matrix was still holding 3.83×10^6 cells/mL of cells. Leakage continued to rise by day 16, reaching up to 6.0×10^4 cells/mL, or 1.41% of the total cells, with the cell density inside the beads reaching 4.25×10^6 cells/mL. This trend of increasing leakage

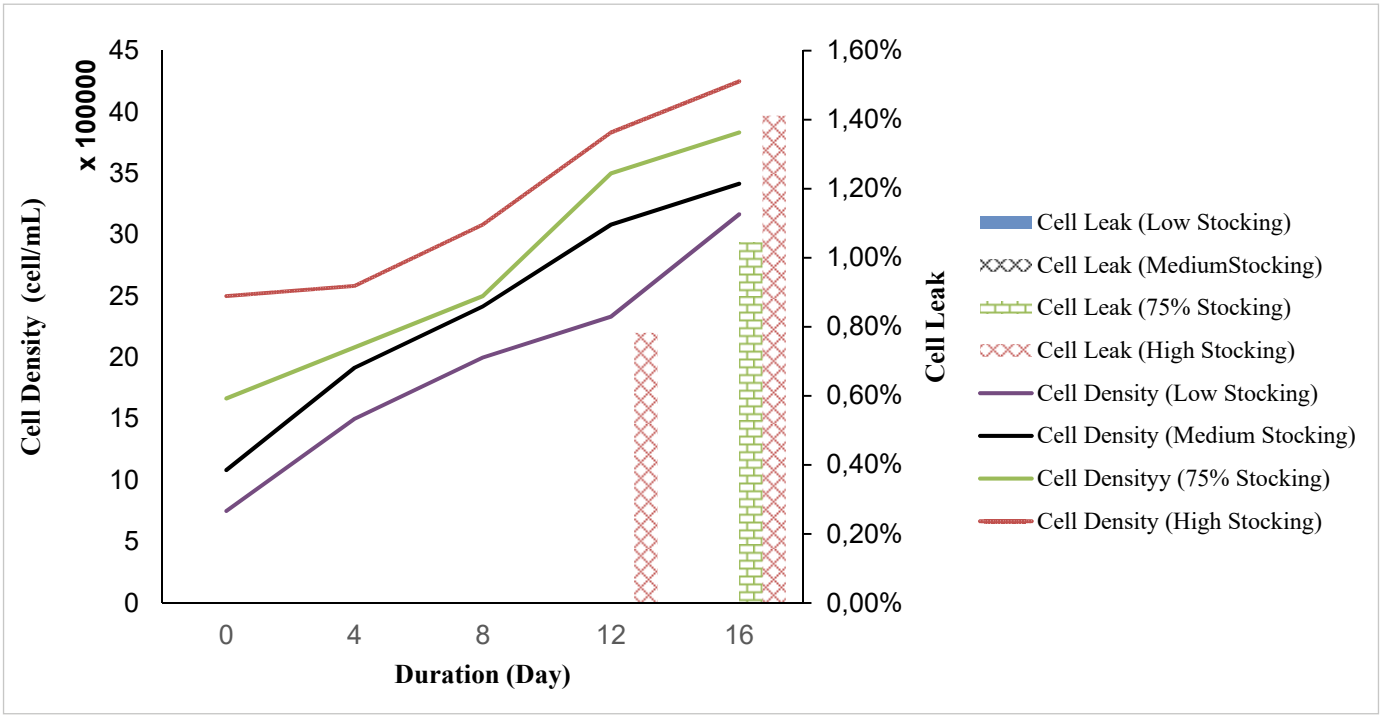

Fig. 4. Cell density of microalgae in alginate beads and cell leakage, expressed as the percentage of the cell density within the beads over 16 days. No leakage was observed in low and medium stocking samples., while leakage occurred at 75% and high stocking densities at the later stage of experiment.

at high stocking density suggested that, while alginate beads can initially contain a high concentration of microalgae, their retention capability diminished over time. The higher cell leakage observed in the wastewater on day 16 may be either due to the additional cell leakage from alginate beads or the growth of previously escaped cells in the wastewater and subsequently proliferated as free cells in the sample.

Interestingly, immobilisation did not inhibit microalgae growth, which can be shown by a steady increase in cell density within the beads across all stocking densities. Lam and Lee [23] reported that immobilisation with lower biomass resulted in higher growth due to the thinner alginate biofilm, which allowed better mass transfer of nutrients and CO_2 into the alginate matrix, thus continuous supported microalgae growth. Lee, et al. [21] suggested that immobilised microalgae exhibited better growth and nutrient removal than free cell culture in photobioreactors. Soo, et al. [12] also concluded that microalgae cell growth was unaffected by immobilisation, and the higher nutrient removal by immobilised cell make it even better in wastewater treatment.

In summary, this analysis indicated that low and medium stocking densities maintained complete containment with 0% leakage throughout the 16 days incubation period, underscoring their suitability for long-term microalgae immobilisation. The 75% stocking density showed minimal leakage only at day 16 (1.04%), while high stocking density experienced gradual leakage starting from day 12 and reached 1.41% by day 16. These findings suggested that, while low and medium densities fall within the optimal containment range, the 75% stocking density approaches the containment threshold, and high stocking density exceeds it, leading to compromised bead integrity. Nevertheless, the leakage rates observed (0.78% to 1.41%) in this study, were still within the acceptable limit for wastewater treatment applications, with Soo, et al. [12] who reported a low leakage rate of 0.22% for *Nannochloropsis* sp. alginate beads. However, low to medium stocking densities are recommended for reliable containment and enhanced nutrient removal due to their superior stability and long-term retention performance in wastewater treatment.

4. Conclusion

This study successfully demonstrated the feasibility and effectiveness of immobilising *Botryococcus* sp. in alginate beads for potential wastewater treatment and biomass recovery applications. The findings emphasised that optimising sodium alginate and $CaCl_2$ concentrations is essential to produce stable, uniform, and spherical beads. Beads formulated with 40 gL^{-1} alginate and 40 gL^{-1} $CaCl_2$ exhibited excellent mechanical resistance, consistent size stability and reliable structural integrity over 16 days of incubation. The alginate matrix effectively retained microalgae cells, with minimum cell leakage observed at low and medium biomass stocking levels. Additionally, the study highlighted the ability of immobilised microalgae to growth within the alginate matrix, ensuring continuous biomass proliferation in wastewater. These findings underscore the practicality and sustainability of alginate-immobilised microalgae system, which offer a scalable solution to address the challenges in microalgae harvesting and reuse. The optimised formulation not only simplifies biomass recovery but also integrates beads durability with biomass growth into a cohesive system. However, while higher alginate concentrations improved mechanical properties, the increased viscosity posed challenges in beads production. This study highlighted the importance of proposed alginate bead formulation in achieving balance between the durability and practicality production.

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