Bioprocessing and Biomass Technology

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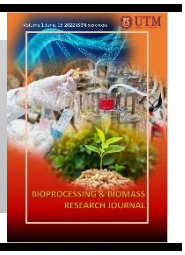

Research Article

Three-dimensional Culture System of Fibroblast Cells in the Fabricated Mini Bioreactor

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ARTICLE INFO

Article History:
Received 26 May 2025
Received in revised form 18 July 2025
Accepted 20 July 2025
Available online 31 July 2025

Keywords:
3D cell culture,
Fabricated mini bioreactors,
Cell viability,
Optimization operational parameters

ABSTRACT

Optimizing 3D culture is vital for developing *in vitro* models that mimic *in vivo* environments. This study evaluated static and dynamic 3D cultures of fibroblast cells encapsulated in alginate beads and examined the performance of a fabricated mini bioreactor system. Among three types of fabricated mini bioreactors – airlift, magnetic stirrer, and stirred tank mini bioreactors – the stirred tank mini bioreactor with a Rushton blade at 250 rpm and a CO_2 flow of 8 mL/min showed the best conditions for cell viability (81.14%) compared to static culture (71%). These findings, supported by both trypan blue and MTT assays, highlight the benefits of dynamic agitation in enhancing nutrient distribution while preserving cell integrity. Despite promising outcomes, contamination due to inadequate sterilization of the bioreactor lid calls for design improvements. Still, this study supports the potential of optimized fabricated mini bioreactors as practical solutions for scalable and efficient 3D cell culture.

INTRODUCTION

The two-dimensional (2D) cell culture has become the standard *in vitro* cell culture technique since the early 1990s. It has made a major contribution to the understanding of cell behavior and is essential to many research studies. However, 2D models deviate from real cellular responses because they incorrectly depict tissue cells *in vivo*. In contrast, three-dimensional (3D) cell culture is an *in vitro* cell growth and proliferation technique that mimics the *in vivo* microenvironment conditions (Bodgi et al., 2019; Jensen & Teng, 2020; Sośniak & Opiela, 2021). Although 3D cultures have been around since the 1980s, it was not until the past decade that they gained popularity due to advances in knowledge, the creation of biomaterials, and the use of technology to replicate the morphological, functional, and microenvironmental features of tissues and organs (Simian & Bissell, 2017; Sośniak & Opiela, 2021; Temple et al., 2022).

The 3D cell culture models are increasingly recognized as valuable *in vitro* platforms for therapeutic and anticancer research. Hence, researchers have been working to scale up the 3D systems from the laboratory to the industrial level to meet the rising demand (Chaicharoenaudomrung et al., 2019). Besides, culturing encapsulated cells under static conditions has some limitations, including homogeneity of the environment, nutrient distribution, waste generation, and gaseous exchange (Huh et al., 2010; Temple et al., 2022; Urzì et al., 2023). These constraints can be minimized by cultivating encapsulated cells in a dynamic bioreactor (Alsobaie et al., 2023; Hwang et al., 2009).

The bioreactor is a dynamic 3D platform for cell cultivation. Various bioreactor designs have been established to provide a microenvironment for cell growth, differentiation, and tissue development, such as stirred tank, airlift, and perfusion bioreactors (Mutaf & Oncel, 2023; Stephenson & Grayson, 2018). Nevertheless, using a large-scale bioreactor is neither feasible nor efficient in the optimization process of 3D cultivation.

Moreover, the biotechnology industry is keen on reducing the cost of bioreactors, which has led to the development and fabrication of mini bioreactors by

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DOI address: 10.11113/bioprocessing.v4n1.70
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researchers, as they are more reliable and reflect how the manufacturing process is operating (Achinas et al., 2020; Bareither & Pollard, 2011; Li et al., 2006). Hence, this study aims to optimize the fabrication of mini bioreactors for the cultivation of encapsulated fibroblast cells.

MATERIALS AND METHOD

Materials

The experiment was conducted using sodium alginate, 0.4% trypan blue, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Missouri, USA). Calcium chloride ($CaCl_2$; Merck, Darmstadt, Germany) was used as a crosslinking agent. The high-glucose Dulbecco's Modified Eagle Medium (DMEM; Pricella, Houston, Texas), fetal bovine serum (FBS), antibiotic-antimycotic (anti-anti), and 0.25% trypsin-EDTA (Capricorn Scientific, Ebsdorfergrund, Germany) were used for cell culture. The CO_2 gas was supplied by Universiti Teknologi Malaysia (UTM, Johor, Malaysia). Other chemicals/reagents used were trisodium citrate (R&M Chemicals, Essex, UK), phosphate-buffered saline (PBS) tablet (pH 7.4, Takara Bio Inc, Shiga, Japan), and dimethyl sulfoxide (DMSO; QReC, Auckland, New Zealand).

Preparation of Alginate Beads for Testing of the Fabricated Mini Bioreactor

Sodium alginate solution (2% w/v) was prepared by mixing with preheated distilled water (Raditya Iswandana, 2018). The solution was constantly stirred until it dissolved completely. Then, 2 mL of alginate solution was loaded into a 3-mL syringe and dropped into the 3% (w/v) $CaCl_2$ solution (Raditya Iswandana, 2018). The beads were allowed to solidify in the solution for 10 minutes before being used in the bioreactor.

Evaluation of the Performance of Fabricated Mini Bioreactors to Distribute the Alginate Beads

To determine the suitable bioreactor for cell cultivation, all three types of fabricated mini bioreactors underwent performance evaluation to assess their potential to distribute the beads throughout the bioreactor uniformly. The assessment was done to ensure that the nutrients and microenvironment for cell culture are well-mixed in the culture medium. Hence, the fabricated mini bioreactor, which can circulate the beads evenly and maintain steady conditions for long-term operation, was chosen for subsequent experiments.

A total of 30 alginate beads were used in this experiment to evaluate the internal mixing dynamics. The beads were used as model particles to allow visual assessment of the mixing pattern and hydrodynamic behavior within the reactor. The quantity of 30 beads was determined as it provided an optimal distribution throughout the reactor volume without causing excessive crowding, allowing easy visual assessment.

Fabricated Airlift Mini Bioreactor

The airlift mini bioreactor (Figure 1) used in this study had a total volume of 100 mL, with a working volume of 70 mL. A total volume of 70 mL distilled water was poured into the bioreactor, along with 30 alginate beads. The air sparger was automatically activated once the bioreactor was switched on, as no speed level was installed. The bioreactor was bubbled with air and ran for 3 minutes (Särkelä et al., 2019). Then, the distribution of alginate beads was observed.

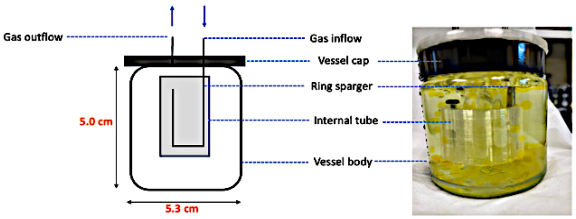


Figure 1 A detailed schematic diagram of fabricated airlift mini bioreactor.

Fabricated Magnetic Stirrer Mini Bioreactor

The experiment was set up according to Arriafdi et al. (2021) with slight modifications using alginate beads. The magnetic stirrer mini bioreactor (**Figure 2**) had a total vessel volume of 120 mL, with a working volume maintained at 70 mL. A magnetic stir bar (35 × 8 mm) was used to generate mixing at the base of the vessel. A total volume of 70 mL of distilled water and 30 alginate beads were added to the fabricated magnetic stirrer mini bioreactor. The bioreactor was run for 3 minutes, and the distribution of alginate beads was observed. The initial speed of the magnetic stirrer was set at 180 rpm because it was the minimum speed required to agitate the solution. However, the system was not stable due to inconsistent speed.

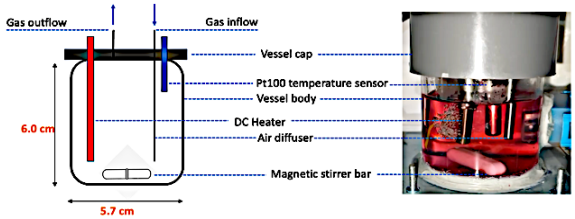


Figure 2 A detailed schematic diagram of fabricated magnetic stirrer mini bioreactor.

Fabricated Stirred Tank Mini Bioreactor

The stirred tank mini bioreactor (**Figure 3**) was fabricated with a total volume of 100 mL and operated at a working volume of 70 mL. The fabricated stirred tank mini bioreactor does not come with baffles to control vortex formation during mixing. No vortex of the solution was observed due to the small size of the bioreactor and controlled mixing. The fabricated stirred tank mini bioreactor was initially filled with 70 mL of distilled water, followed by the addition of 30 alginate beads. The bioreactor was run for 3 minutes, and the distribution of alginate beads was observed. The experiment was conducted with two impellers: the Rushton blades and the marine propellers.

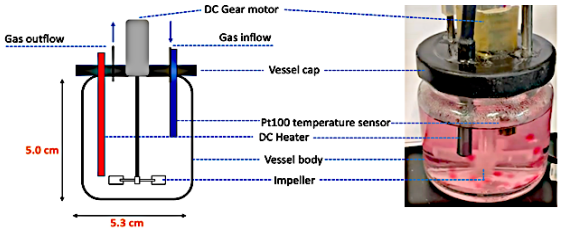


Figure 3 A detailed schematic diagram of fabricated stirred tank mini bioreactor.

Optimizing the Speed of Impellers for Fabricated Stirred Tank Mini Bioreactor

Both impellers (Rushton blades and marine propellers) enabled the well distribution of the beads in the fabricated stirred tank mini bioreactor. Hence, the impeller type and optimum speed were determined to ensure that the beads were still in good condition after a prolonged cell cultivation run. The speed was set at 210 and 300 rpm for both impellers, with an additional speed of 250 rpm applied to only the Rushton blade. The conditions of the alginate beads were observed after 10 days.

Optimizing CO_2 Flow Rate

The stirred mini bioreactor was aseptically filled with 70 mL of high-glucose DMEM. The CO_2 was supplied to the stirred mini bioreactor at three randomly chosen flow rates: 5, 8, and 10 mL/min. The flow rates were selected according to the corresponding volume of gas per volume of liquid per minute (VVM) value of approximately 0.71, 1.14, and 1.43 min^{-1} for a 70 mL working volume. These values were chosen to cover a range around the typical VVM values reported in other studies involving CHO cell cultures (0.00–1.43 min^{-1}), in order to determine the optimal gas flow rate for pH stability (Betts et al., 2014; Hemmerich et al., 2018). The flow rate of CO_2 was set up using an air-glass rotameter with a measuring range from 4 to 40 mL/min. The optimum CO_2 flow was determined by observing color changes of the DMEM medium due to altered pH (Dubey et al., 2021). The experiment was conducted for up to 6 hours.

Encapsulation of Fibroblasts

The fibroblast cells were seeded at 5×10^5 cells/mL in a sterilized sodium alginate solution (Suzuki et al., 2023). The cell-alginate solution, containing 95% sodium alginate solution and 5% cell media, was loaded into a 10 mL sterilized syringe and dropped into a sterilized 5% (w/v) $CaCl_2$ solution at a rate of one drop per second (Soo & Hii, 2021; Suzuki et al., 2023). The beads were allowed to be hardened in the $CaCl_2$ solution for 1 hour. Then, the beads were washed several times with PBS to remove excess $CaCl_2$ (Suzuki et al., 2023). The cell density was approximately 2.5×10^4 cells/bead.

Cultivation of Fibroblasts to Evaluate the Performance of Fabricated Stirred Tank Mini Bioreactor

A total of 60 alginate beads containing cells were placed into a stirred mini bioreactor with 70 mL of complete DMEM. Agitation was provided by a Rushton blade at 250 rpm. A 0.22 µm air filter was installed on both the CO_2 inlet and exhaust. The culture was maintained at 37 °C (Ersahan et al., 2020), and CO_2 was supplied at 11 psig through a humidifier into the bioreactor at a flow rate of 8 mL/min (Chopda et al., 2020). The beads were cultured in the bioreactor for 2 hours, then transferred to a 6-well plate (10 beads per well) with 10 mL of complete medium per well and incubated for another 22 hours in a CO_2 incubator. For comparison, another set of 60 beads was cultured under static conditions in a 6-well plate (10 beads/well) with 10 mL of complete DMEM at 37 °C and 5% CO_2. This setup was used to evaluate the effectiveness of the stirred mini bioreactor in supporting fibroblast cell growth. The entire cultivation lasted 1 day, and cell viability was assessed on day 0.

Dissolving Alginate Beads to Release the Fibroblasts

Ten beads were dissolved using 0.2 M trisodium citrate, a calcium chelator (Mun et al., 2021; Oyeagu et al., 2018). The process was conducted in a 37 °C water bath (dos Santos et al., 2023). The dissolved bead solution containing fibroblasts was then used for cell viability tests using trypan blue exclusion and MTT assay.

Trypan Blue Exclusion Assay

A 1:1 mixture of 100 µL cell suspension and 100 µL of 0.4% trypan blue was prepared. The mixture was loaded onto a hemacytometer and observed under an inverted microscope (Leica DMIL LED, Germany) to count the viable cells (Grässer et al., 2018; Heo et al., 2022). Live cells appeared rounded and bright, while dead cells were stained blue. Cell counts were performed in triplicate to obtain an average value.

MTT Assay

The bioactivity of the encapsulated cells was assessed on day 0 using the MTT assay. MTT was prepared at 5 mg/mL in PBS and sterilized using a 0.22 µm syringe filter. A total of 100 µL of cell suspension was added to a 96-well plate, followed by 20 µL of MTT solution (Yagiz Aghayarov et al., 2023). The plate was incubated at 37 °C with 5% CO_2 for 4 hours (Abdul Latif et al., 2019; Nirwana et al., 2021). After incubation, the solution was removed, and 200 µL of DMSO was added to each well to dissolve the purple-blue formazan crystals (Yagiz Aghayarov et al., 2023). The plate was then incubated at room temperature for 30 minutes. Absorbance was read at 570 nm using an ELISA microplate reader (SPECTRO star Nano, Ortenberg, Germany; (Abdul Latif et al., 2019; Vajrabhaya & Korsuwannawong, 2018).

RESULTS AND DISCUSSION

Evaluation of Fabricated Mini Bioreactor for the Cultivation of Encapsulated Fibroblast Cells

Fabricated Airlift Mini Bioreactor

Figure 4 shows the mixing performance of the fabricated airlift mini bioreactor tested with alginate beads. During operation, the airlift mini bioreactor facilitated bead circulation through the riser and downcomer via air sparging. As observed in **Figure 4b** and **Figure 4c**, most beads circulated effectively, with a few remaining stagnant at the base. The central positioning of the sparger (**Figure 4a**) may have contributed to the inefficient circulation, as optimal fluid movement typically requires air injection near the base of the column (Peh et al., 2022; Schonewill et al., 2015).

A continuous 24-hour test revealed performance issues, notably water loss, leading to system shutdown. This may be attributed to the absence of a humidifier, which is essential to prevent evaporation in bioreactors exposed to dry gases (Hoyle et al., 2022). Additionally, no air regulator was installed, resulting in strong mechanical vibrations, which could potentially lead to cell damage (Kanie et al., 2019). Overall, the fabricated airlift mini bioreactor lacked critical components required for stable performance and was therefore deemed unsuitable for animal cell culture applications.

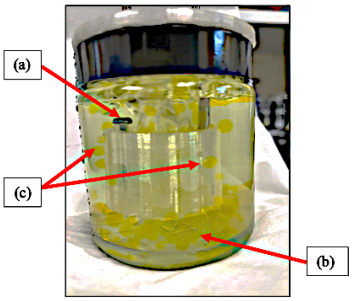


Figure 4 Fabricated airlift mini bioreactor tested with alginate beads. (a) The location of the air sparger, (b) stagnant beads at the bottom of the bioreactor, and (c) floating beads surrounding the column.

Fabricated Magnetic Stirrer Mini Bioreactor

Figure 5 shows the mixing performance of the fabricated magnetic stirrer mini bioreactor tested with alginate beads. The fabricated magnetic stirrer mini bioreactor was tested at a speed of 180 rpm, which represented the lowest effective setting for full bead circulation. At this speed, bead suspension was uniform, with no sign of stagnation, as illustrated in **Figure 5 (a)**. The magnetic stirrer successfully maintained the beads in suspension without physical contact with the bioreactor base.

However, after 30 seconds of operation, the speed became unstable. The beads moved roughly, causing bead fragmentation, as illustrated in **Figure 5 (b)**. The fragmented beads were difficult to capture, as they kept moving throughout the whole experiment. This behavior may be explained by a spinout event, where the magnetic stir bar shifts off-center and spins unevenly or stops (O'Driscoll, 2019). These results indicate that the fabricated magnetic stirred mini bioreactor lacks speed control stability and may cause mechanical stress to encapsulated cells. Consequently, it was deemed unsuitable for cell culture applications.

Figure 5 Mixing performance by fabricated magnetic stirrer mini bioreactor showing (a) beads distribution, and (b) beads fragment impact from the uncontrollable stirring.

Fabricated Stirred Tank Mini Bioreactor

A fabricated stirred tank mini bioreactor was developed with an interchangeable impeller system, enabling the use of either a Rushton blade or a marine propeller. At this stage, speed control has not been configured, so preliminary tests were conducted at maximum agitation speed to assess mixing performance. The results showed that both impeller types could circulate all the beads evenly throughout the bioreactor, as shown in **Figure 6**. According to Rotondi et al. (2021), impeller performance is influenced by flow pattern and power input. Axial impellers, such as marine propellers, require lower speeds for suspension, while radial impellers, like Rushton blades, rely on higher agitation to maintain the circulation (Aydin et al., 2019). The use of maximum speed likely enabled both impellers to perform effectively.

As a result, both impeller types were selected for further evaluation in continuous operations. Although all fabricated mini bioreactors achieved homogenous mixing, only the stirred mini bioreactor demonstrated consistent mixing performance, making it the most promising option for continuous operation and cell culture work.

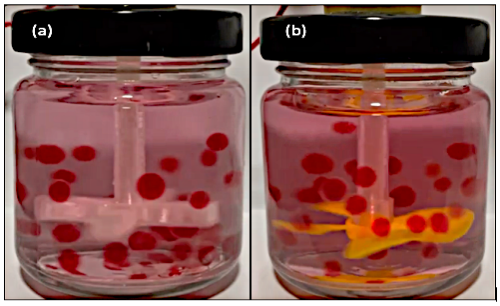
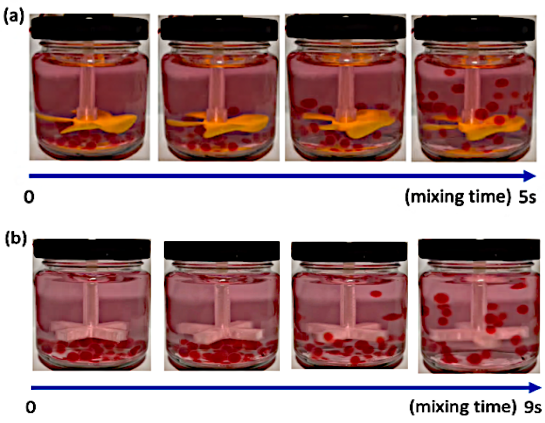


Figure 6 Mixing performance by the fabricated stirred tank mini bioreactor. (a) Using Rushton blade, and (b) using a marine propeller

Analysis of Impeller Type and its Impact on the Operating Speed of the Fabricated Stirred Tank Mini Bioreactor

In bioreactor systems, faster and more uniform mixing helps avoid uneven conditions that could affect cell growth and productivity (Bisgaard et al., 2021). Hence, mixing performance and bead distribution assessment were analyzed using alginate beads as tracers at 210 and 300 rpm for both the marine propeller and the Rushton blade. At 300 rpm, the marine propeller achieved a mixing time of 5 seconds, while the Rushton blade took 9 seconds. At 210 rpm, the mixing times were 9 seconds and 15 seconds, respectively (**Figure 7**).

These findings highlight the superior performance of the marine propeller across both speeds. Its axial flow design promotes downward and recirculating flow, leading to more efficient mixing, as previously reported by Abang Zaidel (2017). Conversely, the Rushton blade generates radial flow, which generally requires higher speeds to match the performance of axial impellers (Aydin et al., 2019).



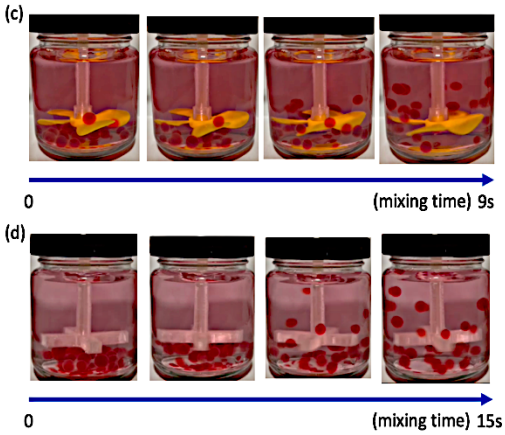


Figure 7 Time taken for beads to completely suspend in liquid with (a) Marine propeller at 300 rpm, (b) Rushton blade at 300 rpm, (c) Marine propeller at 210 rpm, and (d) Rushton blade at 210 rpm.

The bead distribution in the fabricated stirred tank mini bioreactor reflects the impeller's ability to evenly mix culture media, nutrients, cells, and temperature. At 210 rpm, the Rushton blade showed poor distribution, with most beads accumulating below the impeller (**Figure 8a**). This stagnant region may limit nutrient availability to cells. Aydin et al. (2019) attributed this to the radial flow of the Rushton blade, which directs fluid sideways and splits it into upward and downward streams, resulting in ineffective mixing at lower speeds.

At 300 rpm, the bead movement improved, achieving a more uniform distribution, as shown in **Figure 8b**. However, the increased agitation may be too intense. Khodabakhshaghdam et al. (2021) noted that high-speed stirring can damage encapsulated cells due to excessive shear stress, particularly affecting cells near the surface of the bead.

On the other hand, the marine propeller performed better at both speeds, with beads moving smoothly up and down (**Figure 8c and 8d**). This is due to its axial flow design, which helps circulate the contents evenly (Afedzi et al., 2023). Hence, the pattern supports better homogeneity. From this study, the Rushton blade at 210 rpm was excluded for further assessment.

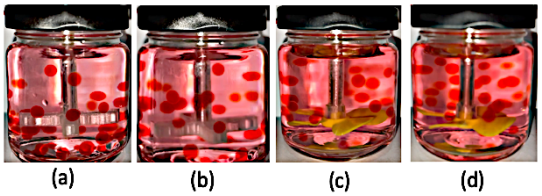


Figure 8 Distribution of the beads in the bioreactor with different impellers and agitation speed. (a) Rushton blade at 210 rpm, (b) Rushton blade at 300 rpm, (c) Marine propeller at 210 rpm, and (d) Marine propeller at 300 rpm.

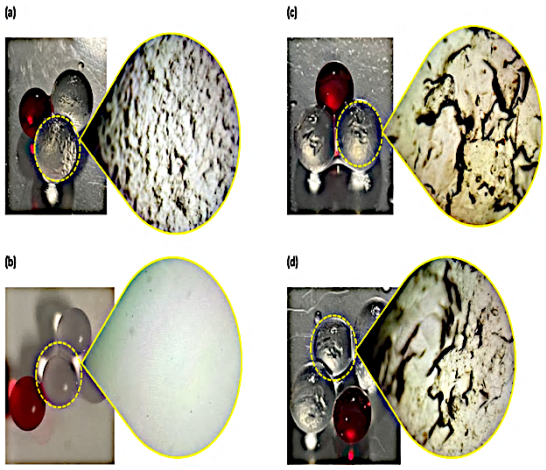


Figure 9 Comparison between control bead (untreated) and treated beads. The red color represents an untreated bead. (a) Rushton blade at 300 rpm, (b) Rushton blade at 250 rpm, (c) Marine propeller at 210 rpm, and (d) Marine propeller at 300 rpm.

To determine the optimal agitation speed for encapsulated fibroblast cells, the Rushton blade at 300 rpm and the marine propeller at both 210 and 300 rpm were tested. After 10 days, all tested speeds using the marine propeller resulted in surface damage to the beads (**Figure 9c and 9d**), while the Rushton blade at 300 rpm caused uneven bead surfaces (**Figure 9a**). These findings indicate that high agitation rates can be detrimental to bead integrity.

To address this, a moderate speed of 250 rpm using the Rushton blade was tested. The beads remained in good condition after 10 days (**Figure 9b**), similar to the untreated control. Thus, 250 rpm is considered the optimal speed, balancing sufficient mixing and minimal shear stress. This is because effective encapsulated cell cultivation requires maintaining the bead suspension, improving nutrient transport, and minimizing shear-induced damage (Bellani et al., 2020; Collignon et al., 2010).

Optimization of CO_2 Flow Rate for an Efficiently Fabricated Stirred Tank Mini Bioreactor

The mini bioreactor used in this study did not have a CO_2 flow control system. As a result, the standard 5% CO_2 condition for cell culture could not be applied. To determine the correct flow rate, a rotameter was installed, and CO_2 was introduced at 5, 8, and 10 mL/min for 6 hours. At 10 mL/min, the medium color changed from red to yellowish-orange (**Figure 10a**), indicating acidification. Conversely, at 5 mL/min (**Figure 10c**), the medium turned pink, indicating alkalinity. Only at 8 mL/min (**Figure 10b**) did the medium maintain a stable red color, suggesting pH stability suitable for cell growth.

This outcome reflects the importance of balancing CO_2 delivery with bicarbonate buffering in the medium (Dubey et al., 2021). Inappropriate CO_2 levels can disrupt this balance, leading to unfavorable pH shifts. Therefore, the flow rate of 8 mL/min was identified as optimal for maintaining physiological pH (7.0-7.7) in the fabricated stirred tank mini bioreactor.

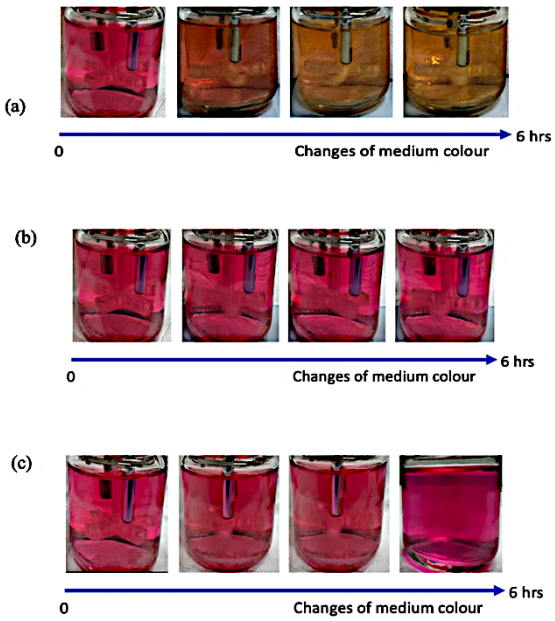


Figure 10 The transition of medium color at different CO_2 flow rates. Starting from the top with the rate of (a) 10 mL/min, (b) 8 mL/min, and (c) 5 mL/min

Optimization of Alginate Beads Preparation

In the initial stage of encapsulating fibroblast cells, 2% (w/v) sodium alginate from Acros Organic was crosslinked with 3% (w/v) $CaCl_2$ for 30 minutes. Although both control and bioreactor cultures used 70 mL DMEM, bead cracking occurred within 3 hours of agitation, whereas in the static control, cracks appeared after 24 hours. The observation, as shown in **Figures 11a** and **11b**, revealed internal structural degradation followed by surface disruption, respectively.

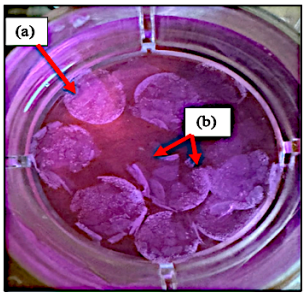


Figure 11 The alginate beads were (a) cracked from the middle of the beads, and (b) the outer layer was almost completely disrupted

This degradation was likely caused by Na^+ ions in DMEM disrupting the Ca^{2+}-alginate matrix, accelerated under agitation due to uniform ion diffusion (Palladino et al., 2024; Somo et al., 2020). To enhance structural integrity, immersion time in $CaCl_2$ was increased to 1 hour. However, this had no significant effect, as the beads were still degraded after 4 hours, possibly due to the low viscosity (350–550 cPs) of the alginate used (Kalogeropoulou et al., 2023).

Subsequent trials employed a higher viscosity alginate (>2000 cPs, Sigma) and reduced bioreactor run time to 2 hours/day. This combination preserved the bead structure up to Day 2. For further improvement, $CaCl_2$ was increased to 5% (w/v) to enhance cross-linking, as highlighted by Bennacef et al. (2023). Nevertheless, it was still insufficient, likely due to the persistent presence of Na^+ in DMEM, which interfered with Ca^{2+} bonds (Chui et al., 2019).

Hence, the final optimization involved reducing DMEM volume to 10 mL, thereby limiting Na^+ exposure. The overall experimental condition was summarized in **Figure 12**. This approach successfully maintained bead integrity for up to five days, validating that the media composition, particularly Na^+, is a critical factor in long-term bead stability.

Parameter Conditions	(a)	(b)	(c)	(d)
Number of beads	30 beads	30 beads	30 beads	30 beads
Volume of DMEM/ Distilled Water (DW)	10 mL of DMEM	70 mL of DMEM	70 mL of Distilled Water (DW)	70 mL of DMEM (2 hours) & 10 mL of DMEM (22 hours)
Observation	Maintain its shape	Start cracked at Day 1	Maintained its shape	Maintain its shape
Picture of Finding				

Figure 12 The observation of alginate beads under one different variable after 5 days of the experiment

Assessment of Fabricated Stirred Tank Mini Bioreactor Function in 3D Cell Culture

The experiment evaluated the performance of a stirred mini bioreactor in culturing alginate-encapsulated fibroblast cells under optimized conditions: 2% (w/v) alginate, 5% (w/v) $CaCl_2$, 1-hour crosslinking, and 2-hour bioreactor operation with 70 mL of DMEM. Due to contamination, only Day 0 results were analyzed. Approximately, beads (n = 480) were formed with an average volume of 0.05 mL/bead, and cell seeding was estimated at ~ 2.5×10^4 cells/bead. It was consistent with the hemacytometer seeding counts (~2.1×10^4 cells/bead).

After 4 hours, viable cell counts dropped to 1.5×10^4 cells/bead (static) and 1.705×10^4 cells/bead (dynamic), corresponding to 71% and 81.14% viability (**Figure 13**). This is because the encapsulation process can cause cell damage. Thus, cell viability in both conditions may be due to cell stress during seeding into the alginate beads. A dynamic culture yielded higher viability, likely due to better nutrient diffusion, despite potential shear stress from bead collisions with the impeller (Khodabakhshaghdam et al., 2021). This aligns with the findings of Sikavitsas et al. (2002), who reported that dynamic systems enhanced cell growth.

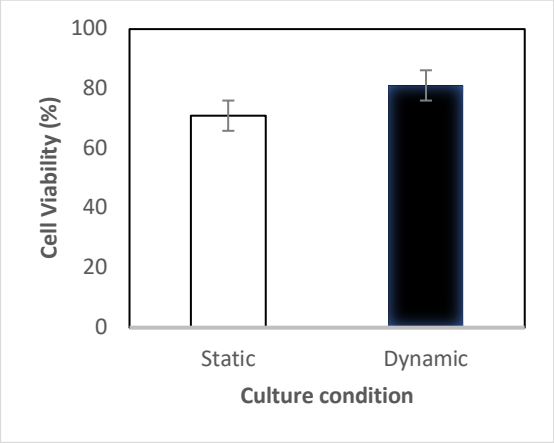


Figure 13 The percentage of cell viability of both static (control) and dynamic conditions after 2 hours of the experiment conducted by using Trypan blue exclusion assay

Interestingly, bead swelling was observed under dynamic conditions, with an increase in size to 5 mm (**Figure 14c**) compared to the initial size of 4 mm, as shown in **Figure 14a**. This might have resulted from Na^+ ions in DMEM replacing Ca^{2+} in the alginate, weakening the gel network and enhancing permeability (Chui et al., 2019). The improved nutrient delivery to the cells may further support cell survival under dynamic conditions. Meanwhile, the bead size in the static condition (**Figure 14b**) was maintained at 4 mm.

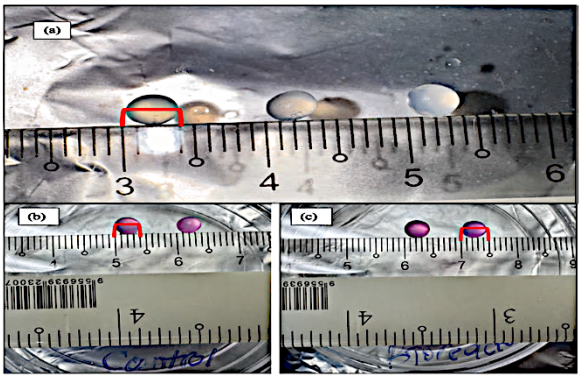


Figure 14 The size of the beads was measured (a) before being used for the experiment (4 mm), (b) after an experiment for the static condition (4 mm), and (c) after an experiment for the dynamic condition (5 mm)

The MTT assay was performed to confirm the trypan blue exclusion assay results. As shown in **Figure 15**, the optical density (OD) readings demonstrated a similar trend, verifying higher cell viability under dynamic conditions. This aligns with previous studies, where formazan dye formation is proportional to viable cell count and metabolic activity (Choi et al., 2023; Twentyman & Luscombe, 1987).

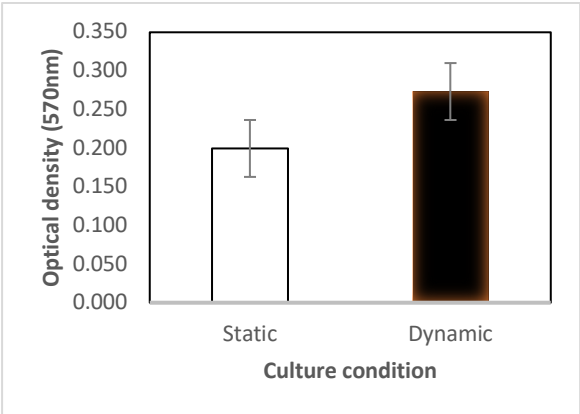


Figure 15 Optical Density (OD_{570}) reading from the MTT assay for both the static and the dynamic conditions

Despite the positive results, contamination was detected at Day 1. This was probably due to improper fabrication of the lid material, which led to an unsterilized lid. The lid appeared to be constructed from non-thermally resistant materials, thereby restricting the use of high-temperature sterilization methods. **Figure 16a** shows the lid structure, which includes CO_2 inlet and exhaust ports, a temperature sensor, and probe joints sealed using the Elephant Brand and epoxy glues. Unlike the bioreactor bottle, which was autoclaved, the lid was sterilized only with ethanol and UV (**Figure 16c**). However, UV sterilization is limited to clear and flat surfaces (Rudhart et al., 2022), whereas hidden areas, as shown in **Figure 16b,** may have retained contaminants. As a fabricated stirred tank mini bioreactor was set up with non-standard materials, the system could not achieve full sterilization, leading to a high risk of contamination.

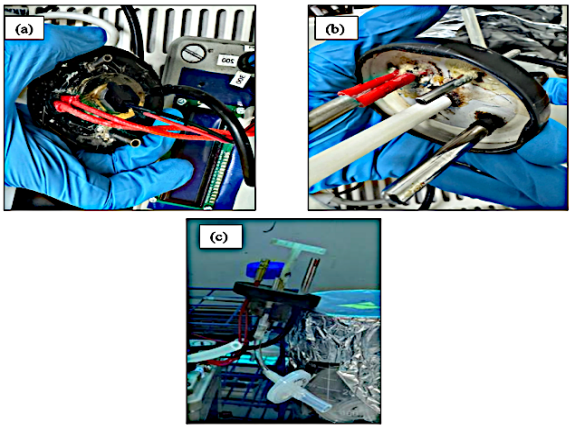


Figure 16 Components that are attached to the lid of the fabricated stirred tank mini bioreactor (a) top view, (b) bottom view, and (c) position of the lid during UV sterilization.

CONCLUSION

This study successfully optimized a fabricated stirred mini bioreactor for 3D fibroblast cell culture, focusing on the mixing speed, impeller design, and CO_2 flow rate. The best results were achieved using a Rushton blade at 250 rpm and a CO_2 flow of 8 mL/min. Despite these successes, bead cracking and contamination occurred. Adjustments to the $CaCl_2$ concentration and DMEM volume improved bead stability, while contamination was mainly due to the poor sterilization of bioreactor parts, which impaired the final part of this research. However, future work can be focused on redesigning the bioreactor lid using better materials and sealing methods to allow proper sterilization (autoclave machine). This can reduce the risk of contamination to the encapsulated cells in the fabricated bioreactor. Moreover, continuing the viability and proliferative studies of culturing cell lines in a 3D carrier such as alginate beads. Overall, these results provide a good starting point for developing better bioreactors for future cell research.

Acknowledgement

The authors acknowledge Universiti Teknologi Malaysia for funding this research (Q.J130000.3854.23H50).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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