Project ID and Title: APPLICATION OF WATER BUBBLES FOR TISSUE CULTURE

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Project Title: Application of water bubbles for Tissue culture

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a. Introduction:

Fish cell lines serve as vital *in vitro* models and are used for virus isolation, toxicology, biomedical research and the production of cell-based fish meat. Fish cells were first cultured *in vitro* in the early 1960s (Wolf and Quimby 1962). Cell lines have been established from a variety of fish, which reduces the need to sacrifice live animals and allows for extremely accurate results in controlled artificial environments (Burden et al. 2020). Cell culture is an important part of ongoing research and fish cells are a valuable source of animal cell models. Cell lines of higher animals are used to produce cultivated meat and an attempt was made to produce cell cultured fish meat.

Cellular aquaculture is a novel as well as growing area that tries to generate aquatic foods from cultivating cells rather than the entire fish. The generation of cell-cultured fish meat has the potential to be a significant source of animal protein, enhancing global food security and improving the welfare of people, the environment, and animals (Mithra et al., 2025b). Cell lines may be acquired from primary cell cultures or commercial cell lines and are cultivated using 2-Dimensional or 3-Dimensional culture techniques (Nguyen et al., 2024). In 2D cell culture, the cells adhere to a flat surface, usually a glass or polystyrene petri dish. Monolayer cultures lack the 3D complexity of tissues within their biological milieu; and have several drawbacks (Dessauge et al., 2021). Since 1961, global fish consumption has increased at an annual rate nearly double that of growing populations (FAO 2022), making it essential to increase seafood production to meet rising consumption demands. In this context, cell-based fish meat production is a modern method of food production supplement current fish production. The aim of this work was to develop scaffold-free, tissue culture. Creating these technologies for cell-based fish flesh would help in the progress of science while at the same time also lowering costs for biomedical and therapeutic purposes. As contrasted to seafood derived from animals, cell-based seafood may integrate contemporary aquaculture methods with advancements in biomedical engineering (Goswami et al., 2022). This approach not only addresses critical challenges in marine conservation and food security but

also contributes to the advancement of cellular aquaculture technologies (Mithra et al., 2025a).

b. Selection of problem and Background Information:

Overfishing and Marine Depletion: Unsustainable fishing practices have led to the depletion of wild fish populations and high value fishes, threatening marine biodiversity and ecosystem balance (Naylor et al., 2000).

Rising Demand for Seafood: With global population growth and increasing protein consumption, the demand for seafood is projected to surge, putting further pressure on natural resources (Clavelle et al., 2019). There's a growing need for alternative protein sources that are safe, ethical, and environmentally friendly. Cell-based fish meat offers a cruelty-free solution without sacrificing nutritional value.

c. Objective: To develop tissue from fish muscle cells through an innovative technique using water bubbles.

Statement of the problem:

Asian seabass is an economically important fish cultured in marine and brackish water. It is a popular fish among consumers due to its taste and the quality of its flesh. Fish muscle mass makes about 50-70% of their total weight, which is quite significant economically. Likewise, overfishing is becoming more common across the world, inflicting tremendous damage to the marine environment. There is a critical need for protecting sustainable fishing resources to fulfil the increasing demand for seafood. Our research demonstrated that it is possible to develop tissue culture using water bubble as a scaffold.

Plan for the experimental design:

The experimental design begins with isolating muscle cells from healthy Asian seabass specimens under sterile conditions. These cells are cultured in optimized growth media to establish stable cell lines and promote proliferation. A novel water bubble-based scaffold is then prepared to support three-dimensional tissue formation. Muscle cells are seeded onto this scaffold and cultivated in bioreactors or orbital rockers to simulate natural growth conditions. Over several weeks, the cells are monitored for differentiation into structured tissue. Finally, the engineered tissue is assessed for texture, composition, and safety to evaluate its potential as a cell-based seafood product.

c. Hypothesis

Low-cost water bubble approach proves to be the best scaffold for producing tissue among all other scaffolds.

d. Experimental procedure

Design of study:

Independent variable – Water bubbles

Dependent variable – Cell viability

Constant variable – Medium and temperature

Materials:

- Muscle cell line
- Water bubbles
- Cell culture flasks and cell culture plates
- Trypsin
- Cell culture medium
- Incubator
- Microscope
- Log note book

Procedure:

Scaffold:

In this project, commercially available low-cost water bubbles will use for tissue culture.

Preparation of bubbles for cell culture

The water bubbles were sterilized by soaking in 70% ethanol for 24 h and washing thoroughly 5 times using ultrapure water (Milli-Q). After washing, the bubbles were soaked again overnight in ultrapure water followed by soaking in L-15 medium (serum-free) overnight and then used for cell seeding.

Toxicity evaluation of Bubbles

The cytotoxicity of water bubbles was evaluated by direct and indirect extract methods following ISO standard 10993-parts 5 and 12 using fish cell lines. In the indirect method, the bubbles were incubated separately in containers containing Leibovitz's L-15 medium for 24 h on a shaker at speed of 20 rpm. Once the incubation period was complete, the medium was collected and filtered using a 0.22 μm syringe filter, and the resulting filtrate was used for the cytotoxicity assay. Cells were seeded in 96-well plates at a concentration of 10^4 cells per well and maintained at 28°C for 24 h. After incubation, the medium was removed. The cells were exposed to various concentrations of medium extracted from scaffolds (20, 40, 60, 80 and 100%) for 24 h. The cytotoxicity of cells due to media extracts collected from bubbles was assessed by MTT and AB assays as described by Mithra et al.

(2024a). Cytotoxicity evaluation was carried out with control groups including cells with regular medium and blank medium. The cytotoxicity tests were carried out with eight replicates for each given dose. The results were presented as the mean ± standard error.

In the direct method, sterilized bubbles (single bead per well) were placed into a 96 well plate. The cell culture medium at the volume of 100 µl per well was added into each well containing bubbles. Then, the cells were seeded on each well at the density of 5, 10, 20 and 40 x 10^3 cells/µl (n = 8) and incubated at 28°C. Control groups such as bubbles without cells and blank medium were included and analyzed along with experimental groups. After incubation, the MTT and Alamar Blue assays were carried out at various intervals of 24, 48, 72, and 96 hours.

Production of micro-tissue using water bubbles

The sterilized and treated water bubbles (10 beads per 5 ml tube) were placed into a 5 ml sterile vial. Fish cells were seeded at a concentration of 5 × 10^6 cells per vial (n = 3) using Leibovitz's L-15 medium and incubated at 28°C for three days. Following this incubation period, the cell-laden bubbles were transferred to a 6-well tissue culture plate and incubated at 28°C. The medium was changed at intervals of 3 days for a period of 30 days.

Viability of cells from micro-tissue obtained from bubbles

After 30 days, tissue fragments were removed from the culture micro-tissue and cells were separated by trypsinization method. The cells released from tissue fragments were counted and seeded on 6 well tissue culture plate along with culture medium, and maintained at 28°C. Following three days of incubation, the morphology and attachment of the cells were assessed.

Results

MTT and Alamar blue colorimetric analyses were done to determine the biological compatibility of the water bubbles by means of quantitative evaluation of toxicity for production of micro-tissue using SBM cells by direct contact to the water bubbles and indirect extract method. Before investigating the application of the water bubbles for the production of micro-tissue using SBM cell line, to assess the safety of these water bubbles and their biocompatibility with Asian seabass cell lines, cytotoxicity tests were conducted. The results of MTT and AB assays showed that indirect media extract was found to be nontoxic to SBM cells up to concentrations of 100% at different time intervals **(Table 1 & 2).** The MTT and AB assays carried out on cells grown on water bubbles revealed of multi-layered cells on water bubbles, some cells were shed from the bubbles and formed multi-layered microtissue in the culture plate. After 30 days, the tissue fragments grown on water

bubbles were removed and treated with trypsin. The cells after trypsinization were seeded into 6 well tissue culture plate without water bubbles. The cells grew well and formed a monolayer.

The SBM cells grew well on water bubbles and formed colonies over both the water bubbles. On the 4th day, the water bubbles with cells were transferred to 6 well tissue culture plate from the tubes. After transfer, the cells on scaffolds proliferated rapidly and covered the entire surface of water bubbles, and developed multilayers of cells on day 10 after seeding (**Fig. 1**). After formation of multi-layered cells on water bubbles, some cells were shed from the water bubbles and formed multi-layered micro-tissue in the culture plate **(Fig. 2)**. After 30 days, the tissue fragments grown on water bubbles were removed and treated with trypsin. The cells after trypsinization were seeded into 6 well tissue culture plate without water bubbles. The cells grew well and formed a monolayer. The micro-tissue which formed on water bubbles was processed for Rhodamine 123 staining. For Rhodamine 123 staining, cells derived from SBM were visualized using Rhodamine 123 staining. Our results showed that the muscle tissues growth on the water bubbles were confirmed **(Fig. 3).**

Table 1. Viability of fish cells exposed to direct and indirect media extracts of water bubbles

S. No.		**Concentrations**	**Percentage of Cell viability (mean ± standard error)**	
			MTT	**AB**
1		Direct methods	94.6	95.03
2		Indirect extract methods		
	a	20%	100	99.98
	b	40%	100	98.15
	c	60%	98.13	97.56
	d	80%	96.52	96.33
	e	100%	95.04	95.34

Table 2. Number of cells and viability of tissue after 30 days of culture at different cells density.

Cell density	**Number of cells**	**%. of viability**
5×10^5 cells / ml	43,24,135	82.34±2.22
10×10^5 cells / ml	57,56,147	86.22±1.85
15×10^5 cells / ml	69,84,692	93.56±1.39
20×10^5 cells / ml	88,86,454	98.84±0.25

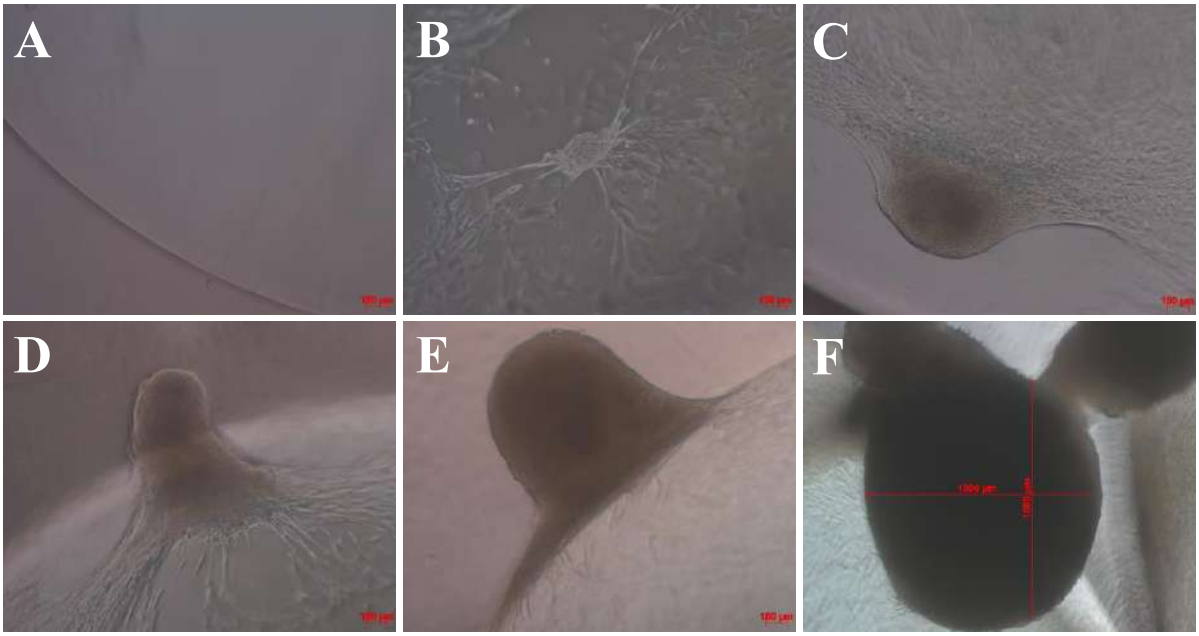

Fig. 1. Cells attachment and proliferated over the water bubble on different days (A) Water bubble without cells, (B) SBM cell growth on 10th day, (C) SBM cell growth on 15th day, (D) SBM cell growth on 20th day, (E) SBM cell growth on 25th day and (F) SBM cell growth on 30th day. Images were captured at 40 × magnification

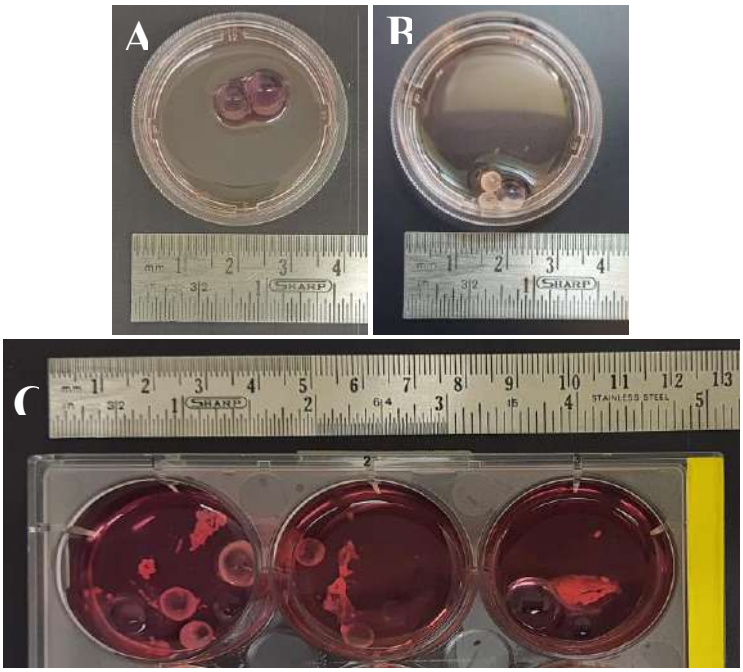

Fig. 2. Cells attachment and proliferated over the water bubble on the cell growth and cover the surface of the water bubbles. (A) Water bubble without cells, (B) SBM cells growth on water bubble on 15th day and (C) SBM cells growth on water bubble on 30th day.

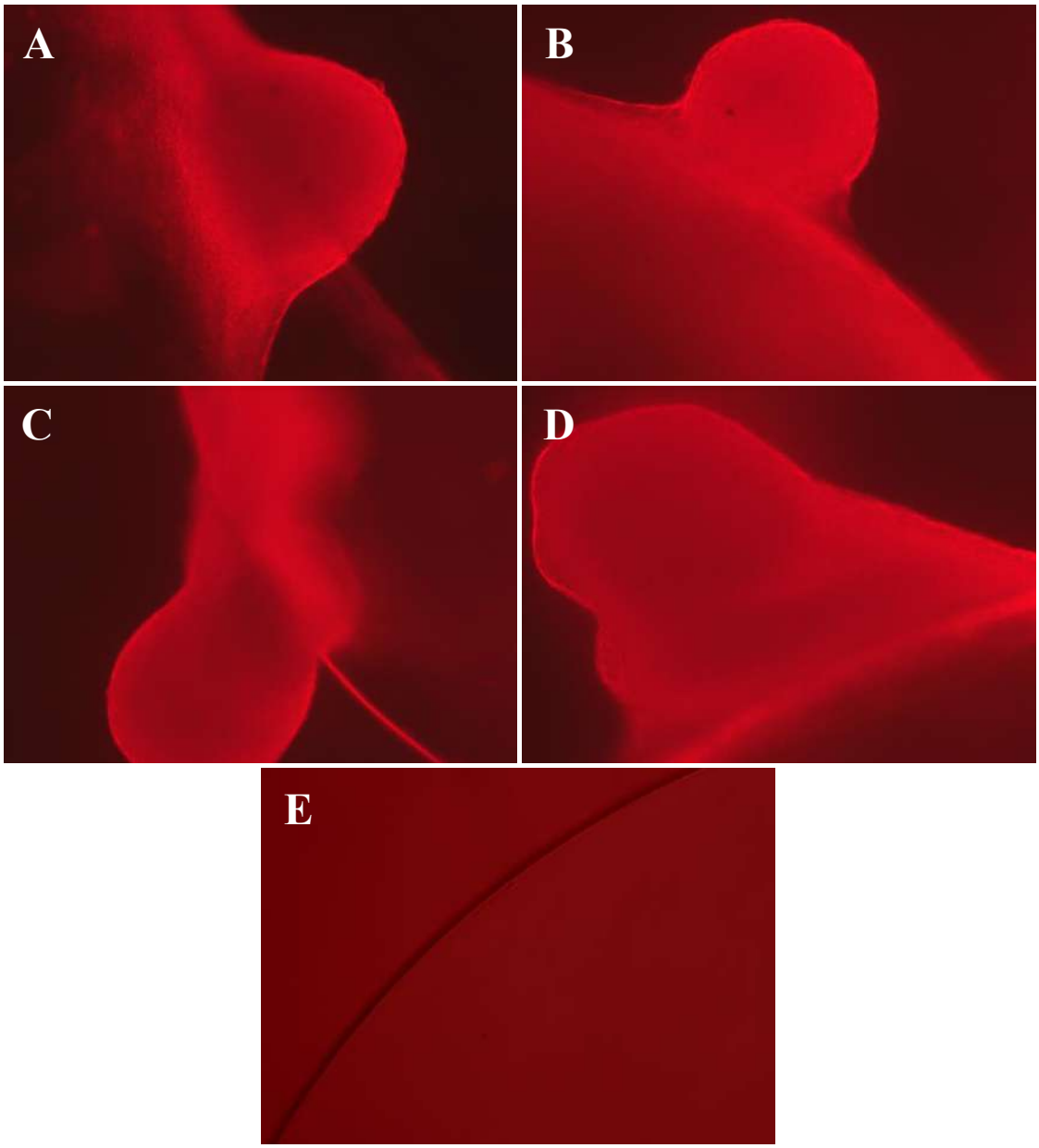

Fig. 3. Cells attachment and proliferated over the water bubble stained with Rhodamine 123 (A-D) SBM cell growth on 30th day and (B) Water bubble without cells. Images were captured at 40 × magnification.

Discussion

Natural biomaterials from renewable resources, including plants, animals, and microbes, have diverse components, microstructures, and physiological characteristics. These materials provide biological support for cell adhesion and proliferation, and also have several

roles in their natural environment (Huang et al. 2017; Ullah and Chen 2020). The scaffolds utilized in this investigation have been found to be non-toxic and extremely compatible to the muscle cells of seabass. Chen et al. (2015) reported that Rat L6 myoblasts cultured on 3D micro-grooved scaffolds for 14 days showed the formation of organized multiple layers of muscle cells as observed in the present study. The multi-layered cells of SBM grown on water bubbles have prominent nuclei with clear cytoplasmic texture in stained histological sections as observed by Xu et al. (2023) in cultured fish fiber. BarShai et al. (2021) used two marine seaweeds (Cladophora sp. and Ulva sp.) to produce fabricated cellulose scaffolds which were found to be nontoxic to mammalian fibroblasts when cultured for a period of 40 days. The results of the present study showed that SBM cell line of Asian seabass were found to be suitable cell lines for production of cell culture-based fish meat using low-cost biocompatible scaffolds.

Conclusion

In the present study, Asian seabass muscle cell line was used for production of microtissue and cell cultured fish meat. These cell lines have been characterized and applied for production of micro-tissue using biocompatible scaffolds. Scaffolds such as water bubbles were found to non-toxic and highly biocompatible for the cells of muscle of seabass, and useful for production of fish meat. The established muscle cells of Asian seabass, and biocompatible scaffolds could be valuable for producing cell culture-based fish meat in future.

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