ISOLATION AND CHARACTERIZATION OF MYOSATELLITE CELLS FROM *CLARIAS MAGUR* (HAMILTON,1822)

Dissertation submitted in partial fulfilment
of the requirements
for the degree of

M.F.Sc. (Fish Biotechnology)

by

RINU FATHIMA, B.F.Sc.

(FBT-MB2-05)

ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(University Established Under Section 3 of UGC Act 1956)
Panch Marg, Off Yari Road, Versova,
Andheri (W), Mumbai – 400 061

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DEDICATED TO MY BELOVED FAMILY, AND FRIENDS...

भा.कृ.अनु.प.- केन्द्रीय मात्स्यिकी शिक्षा संस्थान
ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(A University Established Under Sec. 3 of UGC Act 1956)
Ministry of Agriculture & Farmers Welfare,
Govt. of India.

Dated: 01/10 / 2024

CERTIFICATE

Certified that the dissertation entitled **"ISOLATION AND CHARACTERIZATION OF MYOSATELLITE CELLS FROM *CLARIAS MAGUR* (HAMILTON,1822)"**is a bonafide record of independent research work carried out by **Ms. RINU FATHIMA** during the period of study from December 2023 to September 2024 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Biotechnology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Major Advisor/Chairman

(Mukunda Goswami)
Principal Scientist & Head
FGB Division,
ICAR-CIFE, Mumbai-61

Advisory Committee

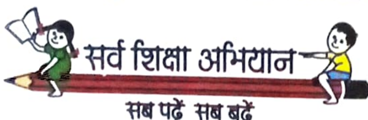
(Dhanjit Kumar Das)
HOD & Scientist E,
Stem Cell Biology,
NIRRCH, Mumbai-61

(Arvind A.Sonwane)
Principal Scientist,
FGB Division,
ICAR-CIFE, Mumbai-61

(Kiran D Rasal)
Scientist
FGB Division,
ICAR-CIFE, Mumbai-61

पंच मार्ग, ऑफ यारी रोड, वर्सोवा, अंधेरी (प), मुंबई - ४०० ०६१. (भारत)
Panch Marg, Off Yari Road, Versova, Andheri (W), Mumbai - 400 061. (India)

कार्यालय / Office : 022-26361446/7/8,
Fax : 022-26361573
Website : www.cife.edu.in



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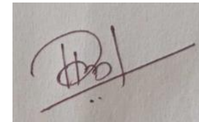
I hereby declare that the dissertation entitled "**ISOLATION AND CHARACTERIZATION OF MYOSATELLITE CELLS FROM *CLARIAS MAGUR* (HAMILTON,1822)**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.



Date: 01.10.2024
Place: Mumbai

(Rinu Fathima)
M.F.Sc. Student
ICAR - Central Institute
of Fisheries Education

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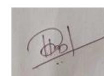

October 1, 2024

Place: Mumbai *Rinu Fathima*

सारांश

वयस्क कंकाल मांसपेशियों की मरम्मत मयोसैटेलाइट कोशिकाओं पर निर्भर करती है, जो कि मांसपेशी स्टेम कोशिकाएँ होती हैं और पूरी तरह से विकसित कंकाल मांसपेशी तंतुओं के सरकोलेमा और बेसल लैमिना के बीच स्थित होती हैं। उपग्रह कोशिकाओं को अलग करने और उनकी खेती के लिए मानकीकृत प्रक्रियाओं की स्थापना इन विट्रो मांस उत्पादन की रीढ़ होती है, और इन कोशिकाओं के कार्यों को नियंत्रित करने वाले आंतरिक और बाहरी कारकों को समझने के लिए भी महत्वपूर्ण है। इस प्रकार के शोध से मांसपेशियों की चोटों के बाद उनकी मरम्मत और मांसपेशी क्षय के मामलों में सुधार की रणनीतियाँ विकसित करने में मदद मिल सकती है। वर्तमान अध्ययन का उद्देश्य व्यापक रूप से इस्तेमाल होने वाले एंज़ाइमेटिक विधि और विभेदन आसंजन तकनीकों का उपयोग करके क्लारियस मैगुर से मयोसैटेलाइट कोशिकाओं की संस्कृति विकसित करना था। कोशिकाओं को L-15 मीडिया में 20% FBS और 1% PSA के साथ 28°C पर संवर्धित किया गया। स्थापित संस्कृति की पुष्टि जीन अभिव्यक्ति विश्लेषण और इम्यूनोहिस्टोकेमिकल परीक्षा के माध्यम से की गई, जिससे Pax7, Myod1 और Myog जैसे मांसपेशीय जीन की उपस्थिति प्रमाणित हुई। कोशिकाओं ने 56% जीवितता दिखाई और आठ दिनों में 70-80% कन्फ्लुएंस प्राप्त किया। इम्यूनोफ्लोरेसेंस विश्लेषण से पता चला कि 90% कोशिकाएँ मांसपेशीय थीं और उनकी आकारिकी स्पिंडल-आकार की थी। qPCR विश्लेषण में Pax7 और Myod1 का उल्लेखनीय अपरेगुलेशन देखा गया, जबकि Myog की अभिव्यक्ति तटस्थ या कम पाई गई, जिससे यह संकेत मिलता है कि अधिकांश कोशिकाएँ अविभाजित थीं और सीमित प्रारंभिक विभेदन हुआ था। ये निष्कर्ष कोशिका-आधारित मछली मांस अनुसंधान और विकास में उनके संभावित अनुप्रयोगों में योगदान देते हैं।

ABSTRACT

The restoration of adult skeletal muscle relies on myosatellite cells, which are myogenic stem cells positioned between the sarcolemma and the basal lamina of completely developed skeletal muscle fibres. Establishing standardized procedures for isolating and cultivating satellite cells forms the backbone of *in vitro* meat production and is crucial for comprehending the intrinsic and extrinsic factors that govern their function. Insights gained from such research can be highly useful for creating strategies to improve muscle recovery following injuries and in cases of muscle atrophy. The current study was aimed to develop a myosatellite culture from *Clarias magur* employing the widely used enzymatic method and differential adhesion techniques. The cells were cultured in L-15 media supplemented with 20% FBS and 1% PSA at 28 ^{0}C.The established culture was validated through gene expression analysis and immunohistochemical examination, confirming the presence of myogenic genes like *Pax7, Myod1* and *Myog*. The cells exhibited 56% viability and reached 70-80% confluence within eight days. Immunofluorescence analysis showed that 90% of the cells were myogenic, marked by spindle-shaped morphology. qPCR analysis indicated significant upregulation of *Pax7* and *Myod1*, while *Myog* expression remained neutral or downregulated, suggesting most cells were undifferentiated with limited early differentiation. These findings contribute to their potential applications in cell- based fish meat research and development.

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1.INTRODUCTION

Meat is a staple food for many people around the world, and meat production has tripled in the last 50 years, reflecting a growing global demand (Ritchie *et al.*, 2024). Meat contains omega-3 fatty acids, vitamin B_{12}, high protein content, readily absorbable iron and other essential nutrients (Bender, 1992; Verma and Banerjee, 2010), which are important for preventing nutritional deficiencies and supporting physical and mental development of children. The escalating demand for meat and animal-based products can be attributed to several factors like the rapid population growth, urbanization, economic growth, global market expansion and changes in food preferences among the consumers (Delgado, 2003; Steinfeld *et al.*, 2006; Bhat and Fayaz, 2011).

Global meat production has currently reached over 350 million tonnes per year (Ritchie *et al.*, 2024). However, this increased output boosts environmental degradation substantially through increased fresh water use, land consumption, and the resultant greenhouse gas emission. According to de Haan *et al.* (1997), the meat production is also accountable for the emissions of nitrogen and phosphorus, the contamination of water by pesticides, the contamination of soil with heavy metals, and the formation of acid rain due to ammonia emissions. Therefore, it is necessary to produce and consume meat, dairy, and other protein products in a way that reduces their environmental impact. Given the global scenario, a shift towards vegetarianism or abstention from meat is impractical as the percentage of vegetarians amongst the industrialized societies is low, and the market penetration for vegan meat substitutes is also limited (Post, 2014).

Stem cell-based *in vitro* meat production is an alternative method offering a number of advantages like improved animal welfare, control over the process, environmental sustainability, and resource efficiency. This method involves growing protein cells from animal stem cell cultures or fabricating whole muscles in a controlled environment in the laboratory (Sharma *et al.*, 2015). This would minimize the need for animals, reduce foodborne pathogens, and lower land use and greenhouse gas emissions (Langelaan *et al.*, 2010). Cultured meat offers the prospect of greatly reducing the number of animal victims and making the consumption of animals redundant while satisfying the nutritional and sensory

needs of meat consumers (Hopkins and Dacey, 2008). It has been proven that with the production of cultured meat, there is a reduction in global warming by 92 percent, air pollution by 93 percent, land use by 95% and water use by 78% compared to conventional meat production methods, especially when renewable energy is integrated into the process (Jain, 2023).

From the commercial point of view, relying on animals as the main source of meat may have several disadvantages that could affect the dependability and stability of the end product. Animals often suffer various adversities such as disease, stress, and irregular growth habits that could again impact the quality and quantity of the meat they provide (Bhat and Fayaz, 2011). This results in huge variability in the meat supply chain and may further lead to economic loss for producers. On the other hand cultured meat, which is grown inside a bioreactor under stringent control and sterility, ensures that meat is produced without animal health issues or stress factors. In addition, cultured meat production is not necessarily bound to special locations or climates; hence it allows for production in areas where traditional farming is unfeasible, potentially reusing lands for other purposes or nature conservation (Bhat and Fayaz, 2011). This decentralization enhances food security and reduces environmental impact compared to conventional methods.

The *in vitro* production of cultured or synthetic meat hinges on the isolation and cultivation of satellite skeletal muscle cells, which serve as progenitor cells driving skeletal muscle growth, regeneration, and repair (Mauro, 1961; Hawke and Garry, 2001; Yablonka-Reuveni, 2011). During embryogenesis, individual mononuclear cells referred explicitly to as myoblasts, undergo fusion to form myotubes. These myotubes subsequently differentiate to become multinucleated muscle fibres (myofibres), the functional components that control skeletal muscle contraction (Campion, 1984). However, the myonuclei (myofibre nuclei) are post-mitotic, signifying they cannot re-enter a proliferative stage to contribute new nuclei (Danoviz and Yablonka-Reuveni, 2012). Consequently, to sustain myofibre growth, homeostasis, and repair during post-natal life, specific cells known as satellite cells remain in a dormant state.

Mauro (1961) initially discovered satellite cells while examining myofibre bundles extracted from frog skeletal muscle tissue under an electron microscope. Morphologically, these are small spindle-shaped cells having a heterochromatic nucleus and few organelles and are positioned between the sarcolemma and the basal lamina of completely developed skeletal muscle fibres (Mauro, 1961). Subsequent research has firmly established their role as the primary source of myogenic cells, playing a crucial role in the postnatal growth and repair of skeletal muscle (Mauro, 1961, 1979; Campion, 1984; Grounds and Yablonka-Reuveni, 1993).

During the juvenile growth phase of muscle development, satellite cells undergo active proliferation, adding nuclei to growing myofibres. However, in adult skeletal muscle, these satellite cells are in a state of mitotic quiescence, only becoming active in response to specific stimuli such as injury, stress from activities like exercise, denervation, or mild compression. The extent of proliferation and differentiation of these cells is dependent on the extent of the injury or stress, with minor injuries resulting in minimal proliferation, while significant trauma can prompt a substantial recruitment and prolonged proliferation before differentiation (Grounds and Yablonka-Reuveni, 1993; Hawke and Garry, 2001; Danoviz and Yablonka-Reuveni, 2012).

Small myofibre injuries can occur on a regular basis during daily activities. Hence a repair mechanism is required for muscle maintenance throughout life (Danoviz and Yablonka-Reuveni, 2012). In the absence of such a mechanism, the cumulative results of such repeated microtraumas may induce a progressive failure of muscle function and could very well predispose muscles to chronic injuries. Satellite cells lie at the heart of the repair and regeneration process after a myofibre injury (Collins *et al*., 2005; Sacco *et al*., 2008). Their prompt and effective action on the common occasions of injury is crucial for the integrity and functionality of muscle tissue to be maintained over time.

In addition to their visible function of aiding muscle development, satellite cells have many other significant roles that highlight their relevance as tissue-specific stem cells. Their ability to contribute to or bolster existing myofibres ensures that muscle tissues remain resilient against future injuries. Also, satellite

cells are able to self-replenish and preserve a reservoir of dormant cells which can be triggered whenever they are needed (Collins *et al.*, 2005; Sacco *et al.*, 2008; Danoviz and Yablonka-Reuveni, 2012). Renewal capacity is paramount for the long-term sustenance of the muscles as it provides a continual supply of invulnerable cells in case another wound arises.

Besides *in vitro* meat production, myosatellite cells can also be utilized for muscle tissue engineering. However, cultivating only muscle cells in a construct does not produce a tissue structure comparable to *in vivo* muscle. The potential solution to this issue likely involves co-culturing them with other cell types like fibroblasts or adipocytes (Langelaan *et al.*, 2010). Trans-differentiation is a process in which a differentiated cell undergoes transformation into another type without reverting to a pluripotent stem cell stage. Myosatellite cells have the capacity to be reprogrammed into different cell types, including adipocytes (fat cells) or osteoblasts (bone cells) (Lin *et al.*,2017; Chen *et al.*, 2019). Progress in cellular reprogramming and gene editing techniques is expected to improve our ability to convert myosatellite cells into specific cell types, offering promising prospects for research and therapeutic applications.

Given the multi-faceted role of satellite cells, a detailed understanding of the regulation and behaviour of these myogenic stem cells is anticipated to shed valuable insights into muscular atrophy both during the aging process and under several disease conditions (Webster and Blau, 1990; Danoviz and Yablonka-Reuveni, 2012). Such knowledge, therefore, constitutes a core component of developing targeted interventions that may alleviate the impacts of such condition on muscle health.

Clarias magur, commonly called Walking catfish, is an important catfish species recognized for its medicinal and therapeutic properties. It is an amphibious air-breathing catfish, which can survive in both water and land habitats due to its innate characteristics and underlying molecular pathways. The species possesses notable nutritional value with high protein content (14.87 ± 1.19%) and is also identified as a rich source of polyunsaturated fatty acids (PUFA) (25.56% of total lipid) among freshwater cultivable fish species (Jakhar *et al.*, 2012; Borah, 2020). It has traditionally been prepared and offered to mothers during post-natal

care, to Pox patients for fast recovery and resistance to secondary infections, to traumatic patients and to address iron deficiency. The unique taste and nutritive value have led to an increase in demand for this fish, which eventually led to its over-exploitation. However, the complex captive breeding behaviour of the species hinders its aquaculture production (Kushwaha *et al*., 2021). According to the IUCN Red List, *C. magur* is considered endangered (A3cde + 4acde). In this scenario, one of the possible solutions could be cultured/synthetic/ *in vitro* meat production as an alternative to animal protein. This approach could meet the rising demand of the fish without further stressing wild populations or relying on aquaculture.

To facilitate cellular fish meat production, appropriate cell lines and cell types are dispensable. Several researchers have identified the presence of muscle stem cells / satellite cells *in situ* and detailed their morphologic properties from a variety of fish species (Powell *et al*., 1989*;* Koumans *et al*., 1990; Mulvaney *et al*., 1995; Bower and Johnston, 2009, 2010; Alexander *et al*., 2011; Miramontes *et al*., 2020; Duran *et al*., 2022; Long *et al*., 2023; Krishnan *et al*., 2023). There is no available report on isolation and culture of satellite cells from *C. magur.* Keeping all these in mind, the present study was aimed at achieving the following objectives.

Objectives

1. To isolate and culture myosatellite cells from *Clarias magur* muscle tissue.

2. To characterize the cultured myosatellite cells.

2. REVIEW OF LITERATURE

2.1 *In vitro* Meat: From Concept to Innovation

The concept of producing meat without using animals has been an iterative theme in science fiction literature for many years. In Winston Churchill's "Thoughts and Adventures" (1932) and H. Beam Piper's "Space Viking" (1963), the notion of creating meat synthetically, without the involvement of animals, is explored (Eschner, 2017; Swartz, 2017). This approach aims to bypass inefficiencies in traditional meat production methods. Authors like Isaac Asimov (*The caves of steel* -1954, *The naked sun* -1957), Simak (*Time is the Simplest Thing*-1961), Keller (*Unto us a child is born*-1969), Herbert (*Whipping Star* -1969), Gibson (*Neuromancer*- 1984), Brin (*Natulife*-1994), Arthur C. Clarke (*The Final Odyssey* -1997), Atwood (*Oryx and Crake*-2003) and Robert Heinlein (*Methuselah's Children* -1941, *Farmer in the Sky*- 1950) also depicted advanced food production technologies in their works, which resemble the modern idea of cultured meat, even if not explicitly stated as such (Buscemi, 2017; Bulleid, 2023). Their imaginative portrayals have contributed to the broader discussion on futuristic and sustainable food production methods. These visionary works foretold the development of cultured meat technology, intending to create a more efficient, ethical, and sustainable meat production method.

In 1912, Alexis Carrel successfully maintained the viability and contractility of a piece of chick heart muscle in a controlled environment of a Petri dish. This pivotal experiment substantiated the feasibility of preserving living muscle tissue extracorporeally, contingent upon appropriate nourishment (Carrel, 1912; Langelaan *et al*., 2010). In the early 1950s, van Eelen pioneered the concept of using tissue culture to produce meat products, which was later patented in 1999 (van Eelen *et al*., 1999). Extensive research into artificial meat involved the cultivation of muscle tissue from frogs and the utilization of a fungal medium to minimize infection risk (Langelaan *et al*., 2010).

The scientific pursuit of lab-grown or cultured meat began in the 20th century. In the 1990s, NASA supported research into cultured meat as a potential food source for long-term space missions (Benjaminson *et al*., 2002). In 1998, Jon

Vein secured a patent for cultured meat production, and four years later, scientists created the first edible lab-grown meat—a fish fillet derived from goldfish cells (Benjaminson *et al.*, 2002). It was Jason Matheny, the founder of New Harvest -an *in vitro* meat research organization, who popularized the concept of lab grown meat in the early 2000s (Edelman, 2005). In April 2008, the Norwegian Food Research Institute organized the world's first ever '*In-Vitro* Meat Symposium' to address and deliberate on the fundamental scientific hurdles that must be overcome for cultured meat to attain commercial feasibility, to unite a network of scientists striving for this common objective, consolidating their efforts, and facilitating the funding required for essential research and activities (New Harvest, 2014). Following this, in 2009, some Dutch Scientist successfully grew pork meat in their laboratory (Rogers, 2009; Chodkowska *et al.*, 2022).

In 2013, Mark Post revealed the world's first lab-grown burger in London (Post, 2014). Google co-founder Sergey Brin funded the project and the techno-hamburger was estimated to have cost $325,000 (Fountain, 2013; Buscemi, 2017). This event marked a significant milestone in cultured meat technology, demonstrating the feasibility of producing meat without relying on animals. In September 2019, a company named 'Aleph Farms' successfully produced the first cultivated meat in space using 3D bioprinting technology. Further, on April 8 2022, they sent animal cells and growth equipment to the International Space Station to study how reduced gravity affects the growth and maturation of cow cells, with the goal of developing muscle tissue for cultivated steaks (Clayton, 2022; Chodkowska *et al.*, 2022). This 'Aleph Zero' space program aims to grow high-quality, delicious animal products, providing astronauts with a self-sustaining food system for long-duration space missions (Aleph Farms, 2024). In 2020, Singapore made history by becoming the first country to grant regulatory approval for the commercial sale of cultured meat, paving the way for Eat Just to market its lab-grown chicken (Chodkowska *et al.*, 2022).

In 2022 November, UPSIDE Foods acquired the first-ever "No Questions" Letter from the U.S. FDA for its cell-based chicken meat products, indicating its safety for consumption (UPSIDE Foods, 2023; Madhusudana Rao *et al.*, 2024). Furthermore, in June 2023, they received USDA label approval as well as a USDA Grant of Inspection (GOI) enabling them to operate as a meat

establishment, and to process, package, and sell cultivated chicken in the United States under the inspection of the USDA's Food Safety and Inspection Service (UPSIDE Foods, 2023). The collaboration between an Israeli startup "Steakholder Foods", and the Singaporean cultivated seafood company "Umami Meats" successfully developed the world's first 3D-printed, cultivated fish fillet from grouper fish in April 2023 (Food Dive, 2023).

The Compound Annual Growth Rate (CAGR) of global *in vitro* meat market is predicted to be 15.7% between 2025 and 2032, with estimates ranging from $214 million to $593 million (Markets and Markets, 2023). Some analysts predict even faster development of the industry. The market for cultivated meat, as estimated by Allied Market Research, was valued at over $1.6 million in 2021 and is projected to reach approximately $2800.1 million by 2030 (Buchholz, 2018).

2.2 Cell- based Seafood

According to SOFIA 2024, global hunger affects around 735 million people, since the onset of COVID-19 pandemic, which marks an increase of 122 million. Moreover, about 3.1 billion people cannot afford a nutritious diet, and it's projected that 600 million will face chronic under-nutrition by 2030.

The significant reduction of ocean biomass, attributed largely to industrialized fisheries and marine capture practices, has led to a concerning decline in wild fish populations, with estimates suggesting a depletion rate of up to 80% (Myers and Worm, 2003). Although aquaculture is often viewed as an environmentally and economically viable solution, it may not effectively alleviate these pressures. Carnivorous farmed fish are frequently fed wild fish, leading to alterations in natural habitats (Naylor *et al*., 2000) and many other fish are fed fishmeal sourced from wild-caught fish (Merino *et al*., 2012; Rubio *et al*., 2019).

The concept of generating seafood from fish cell and tissue cultures is gaining momentum as a way to tackle problems with industrial aquaculture systems and marine capture. As an alternative to animal- based seafood, cell-based seafood combines contemporary aquaculture methods with advancements in biomedical engineering, such as the generation of animal cells in a closed system bioreactor (Rubio *et al*., 2019). According to Goswami *et al.* (2024), cultivated seafood has the

potential to be used as an alternative smart protein to feed the 10 billion world population by 2050.

Table 1. Companies involved in developing cell-based seafoods

Sl.no	Company	Fish species considered for cell-based seafood production
1	Another Fish (Montreal)	Whitefish
2	Avant Meats (Hong Kong)	Fish Maw, Sea Cucumber, Grouper
3	Blue Fin Foods (USA)	Bluefin tuna
4	Blue Nalu (USA)	Yellowtail, Bluefin tuna, Mahi mahi, Red Snapper
5	Bluu Seafood (Germany)	Atlantic Salmon, Rainbow trout
6	Cell Ag Tech (Canada)	Whitefish
7	Cultured Decadence (USA)	Lobster
8	Finless Foods (USA)	Bluefin tuna
9	Forsea Foods (Israel)	Freshwater eel
10	Magic Caviar (Amsterdam)	Caviar
11	Memphis Meats (USA)	Coho Salmon
12	Mermade Seafood (Israel)	Scallops
13	Shiok Meats (Singapore)	Shrimp, Lobster, Crab, Crayfish
14	Steakholder Foods (Israel)	Whitefish, Salmon, Eel
15	SoundEats (Washington)	Whitefish, Zebrafish
16	Umami- Meats (Singapore)	Japanese eel, Yellowfin tuna, Red snapper
17	Wildtype (USA)	Salmon

2.3 Indian Scenario

In India, the production of lab-grown meat is currently in its nascent stages, primarily driven by scientific research and entrepreneurial endeavors. While the technology exhibits substantial promise, its development remains largely confined to research laboratories and pilot-scale projects. The objective is to emulate the achievements witnessed in other regions, such as the United States

and Europe, where cultivated meat has made significant strides. India's robust biotech sector and its increasing emphasis on sustainable food production are propelling research efforts in this domain.

In 2018, the Humane Society International (HSI) and the Centre for Cellular and Molecular Biology (CCMB) in Hyderabad initiated a first-of-its-kind government-sponsored project in India, aimed at producing commercially viable lab-grown meat by 2025 (PTI, 2018; Stone, 2019). This collaboration seeks to unite regulators, startups, and industry stakeholders, aligning with global efforts to find sustainable food solutions and mitigate the environmental impact of conventional meat production. Similarly, ICAR-Central Institute of Fisheries Education (CIFE) in Mumbai has also initiated research on cultivated seafood as an integrated project (ICAR News, 2024).

Despite these advancements, the commercialization of *in vitro* meat in India encounters several challenges. These include elevated production costs, particularly concerning the growth media and bioreactors essential for large-scale production. Furthermore, consumer acceptance presents a noteworthy obstacle due to cultural and religious considerations, in addition to concerns regarding the sensory properties of lab-grown meat in comparison to traditional meat products. However, as investments in research and development increase, there is optimism that India could become a significant player in the global cultivated meat market, contributing to more sustainable food systems. India has recently allocated a substantial sum of money to improve the research and development facilities for cultivating meat. In the latest budget plan, the government has earmarked a substantial sum of US$ 162.7 million (Rs. 1,345 crore) with the aim of positioning the country as a prominent bio-manufacturing hub on a global scale (IBEF 2023).

2.4 Methods of Production

Two significant technologies have been devised to bring the concept of *in vitro* meat to fruition. At the core of this approach lies the application of biotechnological methods, encompassing cell culture, tissue culture, and tissue engineering techniques. These techniques are commonly known within the

biotechnology realm as "Scaffold-based" and "Self-organizing" methods (Sharma *et al.*, 2015).

The self-organizing technique involves the utilization of an explant from the muscle of a donor animal, which is subsequently cultured in a nutrient medium. This approach facilitates the creation of structured meat with a well-defined 3-D structure, akin to the natural conformation of meat, through the application of tissue engineering principles for muscle tissue synthesis (Edelman *et al.*, 2005; Sharma *et al.*, 2015). However, limitations exist in terms of proliferation potential, necessitating regular biopsies from donor animals.

The scaffold-based technique involves the utilization of suitable stem cells, such as embryonic myoblasts or adult skeletal muscle satellite cells. These cells are proliferated, affixed to a scaffold or carrier, and then circulated with a culture medium in an appropriate bioreactor. The introduction of various environmental cues induce cellular fusion into myotubes, and ultimately prompt their differentiation into myofibres, which can be harvested, prepared, and consumed as a source of meat (Kosnik *et al.*, 2003; Sharma *et al.*, 2015).

2.5 Cells

Skeletal muscle tissue, which constitutes the bulk of meat, is primarily made up of various specialized cell types. Among these, the most significant are the skeletal muscle fibres, which originate from the intricate processes of proliferation, differentiation, and fusion of embryonic myoblasts or satellite cells. These processes are crucial for the formation and repair of muscle tissue (Langelaan *et al.*, 2010). Myosatellite cells, embryonic stem cells, and adult stem cells have all been proposed as key players in *in vitro* meat production systems by various researchers.

Stem cells, including both embryonic and adult types, are highly versatile and can differentiate into a diverse range of cell types. Embryonic stem cells (ESCs) hold the theoretical promise of endless regenerative capacity for *in vitro* meat production systems. Yet, in practice, several significant obstacles limit their utility for sustained use. These include the accumulation of genetic mutations over time, complications in inducing differentiation into muscle cells (myoblasts), and challenges in preserving their limitless self-renewal capabilities (Sharma *et al.*, 2015). Epithelial stem cells are considered a viable option for culturing cells in *in*

vitro meat production systems, as they form the primary muscle component. Adipose tissue-derived adult stem cells are also multi-potent and less invasive to collect than myosatellite cells, but they carry the risk of malignant transformation (Sharma *et al.*, 2015).

Myosatellite cells, often referred to as satellite cells, are considered to be the most favourable cell source for culturing meat due to their unique characteristics. These cells, although rare within muscle tissue, possess a significant yet finite regenerative capacity, making them particularly well-suited for the controlled environment of *in vitro* meat production (Edelman *et al.*, 2005; Sharma *et al.*, 2015). Unlike embryonic stem cells, which require complex stimulation to differentiate and often face challenges in maintaining their regenerative properties, satellite cells naturally and efficiently replicate the process of myogenesis—the formation and development of muscle tissue. This inherent efficiency in initiating and carrying out muscle fibre formation makes muscle satellite cells an ideal candidate for producing cultured meat with consistency and reliability.

2.6 Culture Media, Serum and Growth Factor

The culture media, in conjunction with growth factors, promotes and supports *in vitro* cell growth. Several types of culture media originally designed for mammalian cells, such as Medium 199, RPMI-1640, L-15, Eagle's MEM, DMEM, DMEM/F12, and Ham’s F10, have also been proven effective for cultivating fish cells. These media are typically enhanced with serum, such as Fetal Bovine Serum (FBS), Fetal Calf Serum, or Horse Serum, to encourage cell proliferation and adhesion. FBS, commonly used at concentrations of 5-20%, is widely preferred; however, its use raises ethical concerns, prompting the development of serum-free alternatives. Several researchers have developed serum-free media to support the growth and proliferation of satellite cells *in vitro*. McFarland *et al.* (1991) formulated a serum-free medium (TSFM) for the growth and proliferation of turkey myogenic satellite cells, Dollenmeier *et al.* (1981) developed a medium to promote the proliferation and differentiation of chick skeletal muscle cells, while Ham *et al.* (1990) focused on the rapid growth of normal human skeletal muscle satellite cells. Benjaminson *et al.* (2002) successfully utilized a maitake mushroom extract-based serum-free medium, demonstrating superior growth rates compared to FBS.

It is essential to supply a sufficient quantity of growth factors to promote the development and proliferation of myogenic cells. Muscle cells synthesize and release growth factors themselves, while other cell types in tissues generate them both locally (paracrine effects) and non-locally (endocrine effects) (Edelman *et al.*, 2005). Growth factors like transforming growth factor beta (TGF- β), fibroblast growth factor (FGF), insulin, and insulin-like growth factor-I (IGF-I) have all been shown to promote proliferation and differentiation of myogenic cells (Allen *et al.*, 1985; Florini *et al.*, 1991; Kosnik *et al.*,2003). Hepatocyte growth factors also play a role in the activation, migration, and proliferation of muscle satellite cells (Sheehan *et al.*, 2000; Kosnik *et al.*, 2003).

2.7 Hypertrophy and Hyperplasia

In postembryonic vertebrates, myofibre hypertrophy and hyperplasia are thought to drive the skeletal muscle growth, both of which rely on the activity of satellite cells. Myofibre hypertrophy is achieved by the fusion of mononucleated satellite cells with pre-existing myofibres, resulting in an increase in the number of nuclei, whereas myofibre hyperplasia involves fusion of satellite cells with one another (Powell *et al.*, 1989).

In fish, hyperplasia continues to foster muscle growth long into the juvenile period, unlike in mammals and birds where it terminates shortly after birth. Hyperplasia has a direct correlation with growth rate and maximum size of the fish. The longer a species can undergo hyperplasia, the faster its growth rate and the larger its ultimate size (Valente *et al.*, 2002). Hypertrophy (outgrowth of existing fibres) accounts for the majority of additional muscle growth in birds and mammals after birth. One potential explanation for this disparity in muscle growth is alterations in myosatellite cell populations of these animals (Koumans *et al.*, 1990).

Fish undergo two hyperplasia stages: stratified hyperplasia (SH) and mosaic hyperplasia (MH). SH occurs at the end of embryogenesis, forming myotubes from lateral germinal areas, increasing fibre diameter. MH, on the other hand, results in a significant increase in total fibre number from all muscle layers, forming a mosaic shape. MH contributes up to 40% of fish total length and is likely

the main process contributing to muscle growth in aquaculture species (Velarde *et al.*, 2016).

2.8 Isolation and culture of fish myosatellite cells

Research into the isolation and cultivation of satellite cells from various fish species has significantly advanced our understanding of muscle cell differentiation and growth *in vitro*. Powell *et al.* (1989) demonstrated the ability to harvest satellite cells from the skeletal muscle tissue of rainbow trout (*Salmo gairdneri*), with these cells showing the potential to differentiate into multinucleated, striated myotubes, confirming their capability for *in vitro* muscle formation. Building on this foundation, Koumans *et al.* (1990) developed a refined method for the dissociation and purification of myosatellite cells from the white epaxial muscle of carp. These cells were identified based on their morphology, ultrastructure, and the formation of multinucleated myotubes containing myofibrils. Additionally, immunocytochemical markers such as desmin were used to identify terminally differentiated cells, while 5-bromo-2'-deoxyuridine (BrdU) served to mark proliferating myosatellite cells, offering a comprehensive approach to studying these muscle progenitors.

Further advancements were made by Alexander *et al.* (2010), who isolated myogenic progenitor cells from adult zebrafish using a transgenic fish line expressing red fluorescent protein (RFP) specifically in skeletal muscle. This study not only confirmed the successful expansion of these cells but also demonstrated their differentiation into multinucleated myotubes, providing a robust model for studying muscle cell biology in zebrafish. Similarly, Bruheim *et al.* (2022) established a method for isolating satellite cells from both adult and larval lobsters. Their research highlighted the importance of optimizing cultivation parameters, such as extracellular matrix coatings, temperature, and media supplements, to enhance the viability and proliferation of primary muscle cells.

The isolation of skeletal muscle cells for *in vitro* cultures has typically relied on methods such as enzymatic dissociation and explant techniques. Enzymatic dissociation, in particular, has been the method of choice for most primary skeletal muscle cultures due to its ability to produce cell suspensions that include fibroblasts, muscle satellite cells, myoblasts, and other precursor cells. As

Long *et al*. (2023) reported, myoblasts adhere more slowly than other cell types, allowing for the selective purification of muscle cells through differential adherence when using laminin-coated culture surfaces treated with poly-lysine. Furthermore, Krishnan *et al*. (2023) highlighted that cultures prepared using enzymatic methods reach confluence more rapidly than those prepared through explant methods, underscoring the efficiency of enzymatic dissociation in muscle cell culture preparation.

2.9 Methods for satellite cell purification

Popular methods for satellite cell isolation and purification are pre-plating method, fluorescence-activated cell sorting (FACS), and magnetic bead isolation method.

The pre-plating technique is based on the variable adhesive characteristics of muscle cells, notably with satellite cells (SCs) exhibiting the lowest adherence. This method involves plating the heterogeneous mix of skeletal muscle cells onto uncoated dishes, followed by short-term incubation for 1-24 h at a temperature appropriate for the species (e.g., 37^0C for humans and 28^0C for most fish) and finally transferring the floating cells to new matrix coated dishes (Yafee & Saxel, 1977; Koumans *et al*., 1990; Danoviz & Yablonka-Reuveni, 2012; Keire *et al*., 2013). The resulting cell culture consists of a variable mixture of both SCs and fibroblasts. To enhance the purity of SCs, the pre-plating process may be conducted repeatedly, every 24 hours, across a span of six days (Keire *et al*., 2013). However, this method is very time-consuming and give rise to cultures of variable purity (Syverud *et al*., 2014). In order to enhance the purity of SC cultures, a revised pre-plating method has been devised. Following a 2-day expansion period, the adherent cells are trypsinized and subsequently replated onto matrigel-coated plates (Yoshioka *et al*., 2020). This process helps remove non-myogenic cells (e.g., fibroblasts) that do not attach as efficiently after trypsinization, allowing for the enrichment of myogenic cells.

The FACS sorting technique involves the separation of muscle mononuclear cells that have been pre-labelled with antibodies specific to satellite cells, using a FACS sorting instrument (Pasut *et al*., 2012; Liu *et al*., 2015). The FACS sorting method is currently considered as the gold standard for isolating and

studying SCs (Benedetti *et al.*, 2021). However, its high cost, the need for a FACS sorter instrument, and the requirement for technical expertise limit its widespread use. Additionally, the cell labelling step can potentially stress or damage the cells, and the purity of the cells can also vary (Syverud *et al.*, 2014).

The magnetic bead method relies on magnetic cell separation (MACS) and involves the use of magnetic columns and specific magnetic bead kits for satellite cell isolation (Blanco-Bose *et al.*, 2001; Benedetti *et al.*, 2021). However, this method is less precise compared to the FACS sorting method and it is expensive, time-consuming, and stressful for the cells. Similar to the other methods, cell purity can vary, and there is a risk of fibroblast overgrowth in the SC cultures by day 7 (Syverud *et al.*, 2014; Benedetti *et al.*, 2021).

Benedetti *et al.* (2021) developed a cost-effective, efficient method for the isolation and expansion of pure mouse and human satellite cells using ice-cold treatment. The The ICT method functions as a mild passaging technique that enables SC expansion for prolonged time period without impacting their ability to proliferate and differentiate. This technique capitalizes on the varying adhesive characteristics of muscle cells and the capacity of stem cells to quickly react to stress signals. Therefore, the conjunction of a gentle cold-stress signal and a reduction in adhesion induced by cold causes only the detachment of stem cells. The process is rapid, easily replicable, involves minimal cell handling, and significantly decreases the need for a large number of animals or human biopsies to obtain an adequate number of SCs.

2.10 Effects of different parameters on the proliferation and differentiation of myosatellite cells

The cellular behaviour in vivo is modulated by a constellation of factors including, but not limited to, growth factors, extracellular matrix proteins, neurological stimulation, and mechanical attributes encompassing dynamic stretch and matrix elasticity (Boonen & Post, 2008). Therefore, to engender enhanced proliferation, differentiation, and maturation of muscle cells *in vitro*, it is imperative to address certain pivotal factors. These include substrate stiffness that helps progenitor cells to proliferate and myotubes to mature; electrical stimulation that boosts myotube maturation; mechanical stimulation that aligns and fuses myotubes

and contributes to their hypertrophy. In addition to these, the extracellular matrix proteins and growth factors are also essential for the differentiation and maturation of muscle progenitor cells (Langelaan *et al.*, 2010).

2.10.1 Temperature

Temperature plays a significant role in regulating various physiological processes in fish, including growth, muscle development, and gene expression. Research has shown that elevated temperatures can have a profound impact on these aspects, particularly by inhibiting overall growth while simultaneously stimulating specific cellular and genetic responses. For instance, higher rearing temperatures have been observed to suppress general growth in fish but promote muscle hyperplasia, which is the increase in the number of muscle fibres. This process is closely associated with the elevated expression of the *MyoD* gene, a key regulator of muscle development. An investigative study conducted by Brahmane *et al.* (2017) epitomizes the complex interrelation between thermal conditions and the expression of the *MyoD* gene, illustrating how elevated temperatures drive satellite cell activity, culminating in enhanced muscle fibre formation. Such a response is indicative of an adaptive mechanism employed by fish to cope with the environmental changes induced by temperature fluctuations.

The research undertaken by Matschak and Stickland (1995) offers a profound exploration into the cellular mechanisms behind these temperature effects. They specifically examined how temperature influences the proliferation and differentiation of myogenic cells, in a controlled culture environment. Their findings revealed that an increase in temperature not only accelerates the differentiation rate of these cells but also significantly promotes hypertrophic growth. This suggests that while higher temperatures may limit overall body growth, they simultaneously enhance specific muscle development processes, potentially as a compensatory mechanism to maintain muscle function and structure under thermal stress.

2.10.2 Substrata, Plating density and Culture media

Greenlee *et al.* (1995) conducted an extensive examination on how different substrates, plating densities, and tissue culture media influence myoblast proliferation and differentiation. The study highlighted the importance of substrate

choice, finding fibronectin or laminin boosted cell proliferation the most, whereas Matrigel significantly enhanced cell differentiation. Additionally, the research showed that variations in culture media, particularly regarding their serum supplementation, carbohydrates, and buffering systems, had diverse impacts on the cells' growth and development.

2.10.3 Age

Age-related declines in the proportion of myosatellite cells have been reported in several species, including *Cyprinus carpio* (Koumans *et al*., 1991), *Oncorhynchus mykiss* (Greenlee *et al*., 1995), and various mouse strains (Shefer *et al*., 2006; Day *et al*., 2010). Following a slight initial increase, the total number of myosatellite cells in the white axial muscle of carp appears to remain relatively constant during the fish's growth phase. However, these cells become progressively diluted among an increasing number of myonuclei with advancing age (Koumans *et al*., 1991). Additionally, the prevalence of non-myogenic cells within the preparations may rise with age, necessitating potential modifications to experimental conditions, such as the duration of enzymatic digestion, the extent of tissue trituration, cell straining methods to eliminate debris, and the centrifugation speed of the harvested suspension in order to decrease the presence of unwanted cell types (Danoviz & Yablonka-Reuveni, 2012).

2.10.4 Genetic origin and Nutritional Status

Both the genetic origin and the nutritional state of fish have a significant impact on the *in vitro* proliferation of myosatellite cells (Valente *et al*.,2002). Myosatellite cells are crucial for muscle growth and repair, and their activity is tightly regulated by various factors. For instance, myostatin, a key negative regulator of muscle growth, suppresses the proliferation of these cells, leading to their differentiation. In contrast, insulin-like growth factor 1 (IGF-1) promotes the proliferation of myosatellite cells but has a minimal and delayed effect on their differentiation. Interestingly, IGF-1’s proliferative effects are counteracted by myostatin, highlighting the complex interplay between these factors in muscle development (Garikipati & Rodgers, 2012).

Recent research by Duran *et al*. (2022) further emphasizes the role of nutrition in muscle development, demonstrating that amino acids are essential for the upregulation of muscle-specific microRNAs and genes involved in fish myogenesis and muscle growth. Additionally, IGF-1 is crucial for stimulating amino acid metabolism, which is necessary for effective muscle development.

Moreover, the differentiation process of myoblasts into myotubes is influenced by the culture conditions. Katayama *et al*. (2023) found that myoblasts rapidly differentiate into myotubes and lose their proliferative capacity when the culture medium is switched from a serum-rich to a serum-reduced medium. This shift in serum concentration is a critical factor in controlling the balance between proliferation and differentiation in muscle cells.

Zhang *et al*. (2023) made a significant contribution to the field of cell-culture-based meat production by identifying an optimized proliferation medium based on F10 basal medium. This medium was found to be highly effective in promoting the rapid proliferation of piscine satellite cells (PSCs) while preserving their stemness, or the ability to differentiate into various cell types. By shortening the culture time required to expand PSCs, this medium offers a promising solution for the large-scale production of cells necessary for lab-grown meat.

2.11 Myogenic Transcription Factors in fish

Satellite cell progeny can be differentiated from the quiescent progenitors by their unique gene expression patterns. In their quiescent or inactive state, satellite cells express specific surface markers such as CD34, *Pax7*, and *Myf5* (Rudnicki *et al*., 1993; Morgan & Partridge, 2003). Proliferating progeny (myoblasts) sustain the expression of *Pax7* while concurrently expressing *MyoD*, setting them apart from their quiescent counterparts. A decrease in *Pax7* expression, coupled with the induction of the muscle-specific transcription factor myogenin (*Myog*), signifies the onset of cell cycle withdrawal and the differentiation phase in myoblasts (Weintraub *et al*., 1991; Zammit *et al*., 2006). Concurrent with or shortly after the upregulation of myogenin, differentiating myoblasts initiate the expression of several structural protein genes, notably sarcomeric myosin, culminating in their fusion into myotubes (Halevy *et al*., 2004; Shefer *et al*., 2006; Danoviz & Yablonka-Reuveni, 2012). Myosin heavy chain (*MyHC*), the primary

structural component of myosin, plays an essential role in muscle contraction, and its expression provides insights into muscle specialization and the ongoing process of muscle remodelling (Sinha *et al.*, 2022). Desmin, an intermediate filament protein expressed by nearly all skeletal muscle cells, is utilized as a marker of the myogenic phenotype (Kaufman and Foster, 1988).

During myoblast development, a distinct group of mononucleated cells undergoes a reduction in *MyoD* expression and subsequently withdraws from the cell cycle, all the while preserving their expression of *Pax7*. This group of cells subsequently amalgamates into a reserve population, which is posited to be indicative of the self-renewal capabilities of satellite cells (Halevy *et al.*, 2004; Yablonka-Reuveni, 2004; Zammit *et al.*, 2004; Shefer *et al.*, 2006).

In examining the characteristics of muscle cell differentiation and development, it has been observed that the Myf5 protein is not detected in quiescent satellite cells. This is despite the active status of the *Myf5* promoter within these cells and the subsequent expression of *Myf5* in their proliferating progeny. It appears that the generation of Myf5 protein may not occur until the cells initiate proliferation. As differentiation progresses, *Myf5* expression diminishes, in contrast to *MyoD* levels which remain elevated throughout the differentiation phase (Danoviz & Yablonka-Reuveni, 2012). *Mrf4* represents the final transcription factor generated subsequent to myogenin activation during somitogenesis, marking the formation of secondary muscle and the onset of motor innervation (Velarde *et al.*, 2016).

The myogenic regulatory factors such as *MyoD, Myf5, Myog*, and *Mrf4* are highly conserved across fish species, underscoring their essential role in muscle development. However, certain fish lineages have experienced whole-genome duplications, resulting in the emergence of gene paralogues (Jailon *et al.*, 2004; Macqueen and Johnston, 2008; Codina *et al.*,2014). For instance, the gilthead sea bream possesses two *MyoD* paralogues, *Myod1* and *Myod2*, as a result of an early teleost whole-genome duplication event (Tan and Du, 2002). In contrast, Atlantic salmon has lost *Myod2* but has three paralogues of *Myod1* (*Myod1a, Myod1b*, and *Myod1c*) with distinct expression patterns in development and myogenic cell cultures (Macqueen *et al.*, 2007). The cichlid fish *Haplochromis burtoni* and the Northern Pike, *Esox Lucios* exhibits variations of *Myf5* (Velarde *et al.*, 2016). In

contrast, only single copies of *Myog* and *Mrf4* have been identified in teleosts, indicating limited duplication or undiscovered paralogues for these genes (Macqueen *et al.*, 2007, Codina *et al.*, 2008, Codina *et al.*,2014). These observations elucidate a species-specific complexity within the regulatory mechanisms of muscle development.

Further examination into carp (*Cyprinus carpio,* L) discloses a distinct sequence in the expression of these MRFs during the course of somite development (Velarde *et al.*, 2016). Initially, *Myf5* is expressed, signifying its foundational role in triggering the muscle development cascade. This early expression is subsequently followed by that of *MyoD*, heralding the onset of muscle differentiation. Progressing further into the development timeline, myogenin expression marks a pivotal transition towards muscle cell differentiation. Ultimately, the expression of MyHC (myosin heavy chains) and α-actin, both of which are integral structural proteins, culminates the muscle *fibre* formation and functional maturation. This sequential orchestration of MRF expression delineates a rigorously controlled myogenesis process within teleost fish, underscoring the distinct yet interconnected roles each MRF plays at various junctures of muscle development.

Temperature has a significant effect on fish myogenesis, resulting in variations in myogenic gene expression across culture conditions and expression patterns among fish species (Velarde *et al.*, 2016). Several other molecular entities like E proteins, Id proteins, Myocyte Enhancer Factor 2 (MEF2), Cysteine-rich proteins (CRPs), the Bone Morphogenetic Proteins (BMPs) and other related proteins within the Transforming Growth Factor (TGF-β) family, including myostatin collectively orchestrate the precise control of muscle development and differentiation, underscoring the complexity of the regulatory mechanisms involved in myogenesis (Velarde *et al.*, 2016).

2.12 Characterization

Skeletal muscle cell culture technology is a fundamental tool in biological research, particularly in the study of muscle physiology, disease modelling, and regenerative medicine. One of the most critical aspects of this technology is the accurate identification of the cells being cultured. The legitimacy of experimental outcomes heavily hinges on this accuracy, as it significantly affects

both the validity and the repeatability of the results. Throughout the culture process, cells might exhibit genetic and phenotypic alterations, underscoring the importance of continuous identity verification throughout the experiment.

2.12.1 Cytogenetic characterization

Karyotyping, an analytical method that assesses chromosomal numbers and structures, is an efficacious approach to identifying such alterations. Karyotype analysis can ascertain the genetic constancy of cells, thereby certifying that the cultured cells perpetually preserve their original traits (Long *et al.*,2023). Roberts (1970) successfully employed cell culture to ascertain the chromosome numbers of fishes. Cell culture presents several advantages for chromosome studies due to the arrest of chromosomes at the metaphase stage, allowing for the acquisition of a substantial number of cells during this phase.

2.12.2 Molecular Characterization

Identifying the species of origin becomes equally imperative in cell culture, especially when handling cells from various species or in instances necessitating the exclusion of cell line contamination. This can be achieved through protein or nucleic acid analysis. Protein analysis encompasses a variety of techniques such as isoelectric focusing (IEF) and immunological assays. IEF segregates proteins based on their isoelectric points, facilitating the recognition of species-specific protein configurations (Cuénod *et al.*,2021) . Conversely, immunological assays leverage antibodies to detect distinct proteins that are characteristic of particular species or cell types, offering a dependable mode of identification.

Nucleic acid analysis offers a range of techniques for species identification including random PCR amplification, restriction enzyme digestion, DNA hybridization, Species-specific PCR primers use, DNA sequencing, and mitochondrial DNA (mtDNA) sequencing (Collura and Stewart, 1995). Within the realm of nucleic acid analysis, mitochondrial DNA (mtDNA) sequencing stands out for its effectiveness. Mitochondrial genes, including the 12S and 16S rRNA genes, harbour considerably conserved areas that facilitate precise species identification.

These segments exhibit notable specificity, sensitivity, and reliability, rendering them exemplary for discerning between species (Yang *et al.*, 2014).

The mitochondrial CO1 gene sequence serves as a crucial genetic marker for the differentiation of animal species (Herbert *et al.*,2003). The utility of DNA barcoding in verifying the identity of animal cell lines has been further demonstrated through the works of Lorenz *et al.* (2005) and Cooper *et al.* (2007). Ivanova *et al.* (2007) expanded this application to the aquatic realm, employing DNA barcoding with the aid of the Barcode of Life Database (BOLD) and universal primers targeted at the mitochondrial CO1 gene to accurately identify fish species. This method has proven effective in confirming the species of origin for various fish cell lines, such as the caudal fin (PDF) and heart (PDH) cell lines derived from *Puntius denisonii* (Lakra *et al.*,2011), in addition to a caudal fin (PSCF) cell line from *P. sophore* (Lakra and Goswami, 2011), and a fin (RTF) cell line also from *P. denisonii* (Swaminathan *et al.*, 2012b).

2.12.3 Immunostaining

The identification of myosatellite cells *in vitro* is mainly based on their morphological characteristics and their ability to produce muscle-specific proteins. However, relying on cell morphology for myosatellite cell identification *in vitro* poses challenges due to potential alterations in cultured myosatellite cell morphology and ultrastructure caused by culture conditions, such as medium composition or substrate coating (Koumans *et al.*, 1990).

Immunostaining is a technique used to identify myosatellite cells by employing antibodies against cell-specific markers. Commonly utilized markers for identifying satellite cells on the myofibres or in cell culture include the paired box protein Pax7 (Feng *et al.*, 2006, 2018; Shefer *et al.*, 2004) and MyoD (Yablonka *et al.*, 1999; Zammit *et al.*, 2004). Zammit *et al.* (2004) noted that nearly all satellite cells initially coexpress Pax7 and MyoD before undergoing proliferation, with most subsequently committing to differentiation. However, some satellite cell progeny continue to express Pax7 without MyoD, suggesting they may remain in an undifferentiated or quiescent state. Additionally, co-immunostaining, with antibodies against MyoD and Myog aids in determining the number of cells in the earliest stages of the differentiation process.

Kaufman and Foster (1985) introduced 5-bromo 2-deoxyuridine (BrdUrd) into DNA and utilized anti-BrdUrd antibody staining to detect H36 and the muscle-specific intermediate filament protein desmin in replicating mammalian myoblasts.

2.13 Contamination in Cell Culture

Microbial contamination is a major challenge in cell culture, impacting various aspects such as cell genetics, physiological properties, nutrition, proliferation, and production. Therefore, early detection of microbial contamination is crucial to prevent adverse effects on experimental results (Ali and Mahmood, 2017). To mitigate contamination risks, antibiotics and antimycotics are routinely employed. Among the most prevalently utilized antibiotics in fish culture are streptomycin (100µg/mL-1), gentamicin (50 µg/mL-1), penicillin-G (100µg/mL-1), and kanamycin (100µg/mL-1); and commonly used fungizone is amphotericin B (2.5 µg/mL-1). Mycoplasma represents a common and extensive source of cell culture contamination, with the potential to infect cell cultures for extended periods without inflicting observable damage on the cells (McGarrity *et al.*,1985). Furthermore, viruses as microscopic infectious agents, pose significant detection and elimination challenges in cell culture laboratories due to their minuscule scale (Berthold *et al.*, 1996; Merten, 2002).

2.14 Cryopreservation

Cryopreservation represents a principal technique in cellular preservation, significantly contributing to the maintenance of cell seed integrity. This method involves the storage of cells in liquid nitrogen at sub-zero temperatures, effectively suspending their growth activities, thus preserving their intrinsic characteristics while preventing contamination-induced losses. Cryoprotectants such as dimethyl sulphoxide (DMSO) minimize ice formation and solute concentration during freezing, lowering ionic stress. It has been reported that the cell morphology is not affected by cryopreservation duration and conditions, but the cell viability decreases with the extension of cryopreservation time (Kumar *et al.*, 2016).

3. MATERIALS AND METHODS

3.1 Location

The present study was carried out in the Central Cell Culture facility and Fish Genetics and Biotechnology Laboratory, ICAR - CIFE, Mumbai.

3.2 Animals and Sample collection

Five healthy fingerlings of *C. magur* (3 months old) were collected from the Aquaculture facility of ICAR-CIFE for the current experiment. The fish were maintained in a tank with adequate water and were fed once daily with a commercial diet at a feeding rate of 1.5%. Fish were fasted for 24 hours prior to the experimental use.

3.3 Isolation of myosatellite cells

Briefly, the fish were subjected to treatments with sodium hypochlorite solution (500 ppm available chlorine), iodophore solution (0.5 w/v iodine) for 10 s, and swabbed with 70% ethanol to prevent bacterial or fungal contamination. The external body was washed with $KMnO_4$ solution, and the donor fish was sacrificed by euthanization. About 5 g of white epaxial muscle was aseptically removed using sterile scalpels and forceps and transferred to a petri dish containing DPBS (1X, Cell Clone™; Lot number: CP22-5468) to preserve the viability of the cells. The tissue was washed thrice with DPBS containing 1% penicillin/ streptomycin/ amphotericin (HiMedia; Lot number: 0000493510) and subsequently minced into 3 mm^3 pieces using a sterile scissor. The minced tissue was then transferred to a 50ml tube and incubated with 10 mL of 0.1% collagenase type IV (HiMedia; Lot number: 0000594550) solution in DPBS for 50 min at room temperature with gentle agitation. Following this, the mixture was centrifuged @300xg and the supernatant was collected into 40 mL of isolation medium comprising of L15 medium supplemented with 2 mM L- Glutamine (HiMedia; Lot number: 0000589273), 10% fetal bovine serum (FBS; Gibco; Lot number: 2800811), and 1% PSA. To increase the cell yield, the pellet was given another enzymatic treatment with 10 mL of 0.1% trypsin (1 X, HiMedia; Lot number: 0000590728) in DPBS, incubated for 15 minutes at room temperature and combined with the previous isolation medium.

Subsequently, the mixture was passed through nylon strainers of three different pore sizes (100, 70, and 40 µm from HiMedia). The filtrate was then subjected to centrifugation @ 300×g for 15 min, followed by removal of the supernatant. The resultant pellet was re-suspended in 10 mL of proliferation medium comprising L-15 with 2mM L- glutamine supplemented with 20% FBS and 1% PSA. The cell count was determined using the Biorad TC20™ Automated Cell Counter.

3.4 Gelatin and collagen coating on flasks

500 µL of 2% gelatin stock (Sigma Aldrich; Lot number: 1003483469) was mixed with 9.5ml of PBS (pH 7.4 1X; HiMedia; Lot number: 0000594339) to get 0.1% working solution. About 3 ml of the working solution was sufficient to coat each of the 25 cm^2 flasks. Similarly, collagen stock (IIT Powai) was also mixed with PBS to make 0.1% working solution and sufficient quantity was taken for coating the flasks. The coated flasks were then incubated at 28 °C for 1-2 hours. The excess gelatin and collagen solution were aspirated prior to cell seeding.

3.5 Purification of the isolated myosatellite cells

Pre-plating method was adopted for the purification of myosatellite cells from rest of the fibroblastic contaminants. Cells were seeded into 25-cm^2 uncoated cell culture flasks at a density of 2.21×10^6 cells/ ml and pre-incubated for 2 hours at 28 °C. The floating cells were then transferred to collagen-coated and gelatin-coated flasks and incubated for 16 hours at 28 °C. The next day, the supernatant was once again transferred to a new set of collagen-coated and gelatin-coated dishes and incubated for 24 h.

The ice-cold treatment method described by Benedetti *et al.* (2021) was also employed for myosatellite cell purification. Following seeding, the dishes containing a mixed population of adhered muscle cells were washed three times with PBS, and then 10 ml of ice-cold PBS was added to each dish. Subsequently, the dishes were placed on ice (0 °C) for 15–30 minutes with occasional gentle manual shaking. The detached cells were then collected, centrifuged, resuspended in growth medium, and plated into 0.1% gelatin-coated 35-mm dishes at a density of 10^3 cells/ dish.

3.6 Culture of myosatellite cells

Satellite cells were cultured in complete growth medium comprising of L-15 with 2mM L- glutamine supplemented with 20% FBS and 1% PSA. When the satellite cells began adhering to the flasks, the media was replaced every alternate days. In about 8 days, the cells attained 70-80% confluence. They were then subjected to trypsinization with 1 mL of 0.25% trypsin (1X, HiMedia; Lot number: 0000590728) for subsequent subculture.

3.7 Characterization

3.7.1 Species authentication using molecular markers

3.7.1.1 DNA Extraction

Genomic DNA was extracted from the myosatellite cells following the conventional Phenol- Chloroform- Isoamyl alcohol method. Media was removed from the culture flasks and cells were washed with PBS, followed by trypsinization. The detached cells were resuspended in 2ml PBS and transferred to a centrifuge tube. After centrifugation @8000 rpm for 8 min, the cells were treated with lysis buffer comprising 500μL of TEN buffer, 50 μL of 10% SDS, and 5 μL of proteinase K at a temperature of 55°C in a water bath for 1 hour. Subsequently, an equal volume of Tris-saturated phenol was added to the lysate, ensuring thorough mixing until an emulsion was formed. The emulsified tubes were then subjected to centrifugation @ 8000 rpm for 8 minute at 4°C, subsequent to which the aqueous layer was carefully transferred into new 1.5 mL tubes. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each sample prior to centrifugation under identical conditions. Post centrifugation, the clear aqueous layer was collected into new tubes; followed by the addition of a chloroform: isoamyl alcohol solution (24:1), thorough mixing, and further centrifugation @ 8000 rpm for 8 minutes at 4°C. The aqueous layer was once again transferred to pristine tubes, to which 0.6 volumes of isopropanol was added to precipitate the genomic DNA. The mixture was then centrifuged @ 8000 rpm for 8 minutes, after which the supernatant was carefully discarded. The resultant DNA pellet was washed twice using 600 μL of 70% ethanol, air-dried for a period of 15-20 minutes, and ultimately resuspended in 30 μL of TE buffer. Extracted DNA was treated with 1 μL of RNase A at 37 ^{0}C for one hour to prevent RNA contamination and then stored at -20°C for future analyses.

3.7.1.2 Amplification of COI gene

The Universal pair of primers F1 and R1 were used for the amplification of mitochondrial cytochrome C oxidase subunit I (COI) gene. Table 2, 3 and 4 contains the details of the primers used in the study, the reaction mixture and, the thermal regime and cycle parameters, respectively. PCR products were visualized in 1% agarose gel electrophoresis by staining with Ethidium Bromide (EtBr) and documented using the UV Transilluminator.

Table 2. List of Primers used for amplification of COI

Mt region	**Primer name**	**Primer sequence (5’-3’)**	**Length (bp)**
COI	Fish F1 Forward Primer	5’-TCAACCAACCACAAAGACATTGGCAC-3’	650
	Fish R1 Reverse primer	5’-TAGACTTCTGGGTGGCCAAAGAATCA-3’	

Table 3. Reaction Mixture for COI

Reaction mixture for COI	
Template (DNA)	1 µL
Forward Primer (F1)	0.5 µL
Reverse Primer (R1)	0.5 µL
PCR Mastermix	6.25 µL
NFW	4.25 µL
Reaction Volume	**12.5 µL**

Table 4. Thermal Regime for COI Gene amplification

Steps	Condition		Cycles
	Temperature	Time	
Initial denaturation	95 ^{0}C	3 min	1 cycle
Denaturation	95 ^{0}C	30 sec	35 cycles
Annealing	54 ^{0}C	30 sec	
Extension	72 ^{0}C	1 min	
Final Extension	72 ^{0}C	7 min	1 cycle
Hold	4 ^{0}C		

3.7.1.3 Qualitative & quantitative estimation of amplified DNA

The quantity of the amplified DNA was determined using a Nanodrop spectrophotometer (Thermo Scientific; USA), whereas the quality and integrity were estimated by running in 1% agarose gel electrophoresis. Only those DNA samples with A260/A280 value in the range of 1.8-2.0 were considered for subsequent steps.

3.7.1.4 Sequence analysis

Utilizing a gel extraction kit (Qiagen, USA), the amplified PCR products were eluted, and sent to the external sequencing facility to generate the sequence data. Multiple sequence alignment was used to align the forward and reverse sequences, after which they were aligned using BLASTn, or BLAST nucleotide for matches to the known sequence of *C. magur.*

3.8 Immunofluorescence

The cells were transferred to 12 well plates and kept overnight at 28^{0}C for the cells to adhere to the plate. The cells were then pre-incubated in 0.5mL of 4% paraformaldehyde (HiMedia; Lot Number: 0000591240) for 5 min, by direct addition to the culture media. Media was subsequently removed from the plate and cells were fixed in 0.5 mL 4% paraformaldehyde at room temperature for another 5 min. Following this, the fixed cells were thoroughly washed three times with DPBS (1X, Cell Clone™; Lot number: CP22-5468), each time for 5 minutes with gentle

shaking, and subsequently permeabilized with 0.1% Triton X-100 (Sisco Research Laboratories; Batch no: 4444105) for ten minutes at room temperature. The cells were then washed thrice with DPBS and incubated for 30 min in 1 mL of 1.5% BSA (PH 7.0; HiMedia; Lot number: 0000018584). Subsequently, they were incubated with primary antibodies against MyoD (Invitrogen; Lot Number: ZF4370878D) and Pax7 (NeoBiotechnologies; Lot number: ZF4374137) at a dilution of 1: 100 and kept in dark overnight at 4^0C. After washing thrice with DPBS, fluorescently labelled secondary antibodies (Alexa Fluor™ 568 Goat anti-rabbit IgG (H+L) Invitrogen; Lot Number: 2782620 and Alexa Fluor™ 488 Goat anti-mouse IgG (H+L) Invitrogen; Lot Number: 2833436) at a dilution of 1: 1000 were added and incubated at 37^0C for 2 hour in dark, and then nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole). Samples were examined using fluorescence microscopy.

3.9 Expression quantification of key myogenic genes

3.9.1 RNA Isolation

The total RNA of myosatellite cells were extracted from the initial stages of the culture according to the instructions of TRIzol™ reagent (Invitrogen, USA; Lot no: 506505). All the apparatus used for RNA isolation were treated with 0.1% DEPC water to prevent RNase contamination. The cells were washed in PBS, pelletized and re-suspended in 1 mL TRIzol™. After the cells were dissolved, they were incubated for 5 min at room temperature, followed by the addition of 200µL chloroform. The tubes were then shaken vigorously and centrifuged @ 12, 000 rpm for 15 min at 4^0C. Subsequently, the aqueous layer was carefully transferred to new 1.5 mL tubes and 0.6 volumes of isopropanol were added to each tube. The tubes were thoroughly mixed and left at room temperature for 10 min, followed by centrifugation @ 12, 000 rpm for 10 min at 4^0C. The supernatant was carefully decanted and the pellet was washed twice with 200 µL of 75% ethanol. Finally, the pellet was air-dried for 15 min, dissolved in 20 µL of 0.1% DEPC water and then stored at -20^0C.

3.9.2 Qualitative and quantitative estimation of RNA

The quantity and purity of the isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Scientific, USA) and those samples with A260/A280 values near to 2.0 were considered for subsequent steps. The quality and integrity of the isolated RNA were assessed by performing 1% agarose gel electrophoresis.

3.9.3 Agarose gel electrophoresis

To ensure proper results, the gel casting tray and electrophoresis unit were soaked in 1% SDS overnight, followed by washing with Milli-Q water and a final rinse with 0.1% DEPC treated water. For gel preparation, about 1 g of Low EEO agarose was mixed with 100 mL of TAE buffer made in 0.1% DEPC treated water. The solution was then boiled in a microwave oven for over a minute to dissolve the agarose. When it cools down to a hand bearable temperature, 5-6 μL of Ethidium Bromide was added, followed by a gentle stirring to disperse it. The solution was then poured to gel casting tray sealed from all the edges and then a comb was inserted. Once the gel had solidified, the seal and the comb were removed carefully from the gel without breaking the wells. The casting tray was then placed into electrophoresis tank containing 1X TAE buffer from the same batch used to prepare the gel. The tray was kept in a position such that the wells were near the cathode. Then, using an appropriate micropipette, the samples (2 μL RNA+ 2 μL dye) were loaded to each well. The electrophoresis chamber lid was closed, the current was applied, and RNA was run at a constant voltage of 80 V for 30 min. The RNA moved towards the anode due to the negative charges on its phosphate backbone. After 30 min run, the gel was visualized under UV transilluminator for specific bands.

3.9.4 Primer designing for key myogenic genes

In line with literatures, three important myogenic genes *Pax7, MyoD* and *Myog* and one house keeping gene *β-actin* were considered for the current study. Using the known sequences in NCBI database, specific primer pairs were designed for each target gene by the IDT PrimerQuest Tool. The table 5 shows the details of the primers used in the study.

Table 5. Primers for key myogenic genes

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	NCBI Reference for gene sequence
Myod1	CCATCAGTTACATCGAGTCCC	GCAGGGCCATTAAAATCCATC	QNUK01000066
Pax7	GCACTACCCCGACATATACAC	TCGTCTATTGCTGAACCACAC	QNUK01000053
Myog	AGTGATCTGATGCAGTGCTC	AGCAGGATCACGAGCAAG	QNUK01000166
β-actin	TGAACCCTAAAGCCAACAGG	CGGAGTCCATCACAATACCAG	NC_071102.1

3.9.5 Primer dilution

All the steps were performed inside a laminar hood to ensure a sterile environment. Initially, the primers in the lyophilized form were centrifuged @10,000 rpm for 10 seconds and then main stock solution was prepared by adding adequate amount of IDTE (1X).

Volume of IDTE (1X) required for dilution (µL) = nmols of primer X 10

Once diluted, the tubes were tapped gently at the bottom and stored at -20 ^{0}C until further use. For the preparation of working solution, the primers were initially kept in ice and 90 µL of IDTE was added to 10 µL of each stock solutions.

3.9.6 Primer optimization

For optimizing primers, RNA was extracted from the muscle tissue of *Clarias magur* following the instructions of TRIzol^{TM} reagent (Invitrogen, Lot no: 506505). Once extracted, the quantity and quality of the RNA were analysed by Nanodrop and 1 % agarose gel electrophoresis as described above. The RNA having A260/A280 value between 1.9 and 2.1 were then reverse transcribed into cDNA.

3.9.6.1 Reverse Transcriptase PCR (RT-PCR)

The Revert Aid First Strand cDNA synthesis Kit (Thermoscientific; Lot no: 2906890) was used for the reverse transcription of RNA template. Table 6 and 7 show the details of reaction mixture and thermal regime for cDNA synthesis, respectively. The synthesized cDNA was stored at -20 ^{0}C for subsequent use.

Table 6. Reaction mixture for cDNA synthesis

Reaction Mixture for cDNA synthesis	
RNA (template)	4 µL
5X reaction buffer	4 µL
dNTPs (10 mM)	2 µL
Oligo(dt)$_{18}$ (0.5 µg/ µL)	1 µL
Ribolock RNase Inhibitor (20U/ µL)	1 µL
Revert Aid Reverse transcriptase (200U/ µL)	1 µL
NFW	7 µL
Reaction Volume	**20 µL**

Table 7. Thermal Regime for cDNA synthesis

Condition		**Cycles**
Temperature	**Time**	
42 ^{0}C	60 min	1 cycle
72 ^{0}C	5 min	1 cycle
4 ^{0}C	infinity	

3.9.6.2 Gradient PCR

Gradient PCR was utilized to assess the optimal annealing temperature for the designed set of primers. This involved subjecting the primers to four different annealing temperatures in a single PCR run to identify the temperature at which all four primers yield the expected product while eliminating primer dimers or non-specific products. The reaction mixture and the thermal regime for Gradient PCR are detailed in the provided table.

Table 8. Reaction Mixture for Gradient PCR

Reaction Mixture for Gradient PCR	
PCR Master Mix	6.25 μL
Forward Primer	0.5μL
Reverse Primer	0.5μL
Template (cDNA)	0.5 μL
NFW	4.75 μL
Reaction Volume	**12.5 μL**

Table 9. Thermal regime for Gradient PCR

Steps	**Condition**					**Cycles**
	Temperature				**Time**	
Initial denaturation	95 ^{0}C				3 min	1 cycle
Denaturation	95 ^{0}C				30 sec	30 cycles
Annealing	Zone I	Zone II	Zone III	Zone IV	30 sec	
	52 ^{0}C	54 ^{0}C	56 ^{0}C	58 ^{0}C		
Extension	72 ^{0}C				1 min	
Final Extension	72 ^{0}C				5 min	1 cycle
Hold	4 ^{0}C					

Following amplification, the resultant products were evaluated for quality and integrity through 1.5% agarose gel electrophoresis. Subsequently, the gel was visualized under a UV transilluminator to determine the optimal annealing temperature for the primers.

3.9.7 PCR for myosatellite gene amplification

The complementary DNA (cDNA) derived from the RNA of myosatellites was subjected to amplification using the ProFlex PCR system under specified conditions.

Table 10. Reaction Mixture for PCR

Reaction Mixture for PCR	
PCR Mastermix	6.25 µL
Forward Primer (FP)	0.5 µL
Reverse Primer (RP)	0.5 µL
Template (cDNA)	0.5 µL
NFW	4.75 µL
Reaction Volume	**12.5 µL**

Table 11. Thermal Regime for PCR

Steps	**Condition**		**Cycles**
	Temperature	**Time**	
Initial denaturation	95 ^{0}C	3 min	1 cycle
Denaturation	95 ^{0}C	30 sec	30 cycles
Annealing	54 ^{0}C	30 sec	
Extension	72 ^{0}C	1 min	
Final Extension	72 ^{0}C	5 min	1 cycle
Hold	4 ^{0}C		

Upon completion of the PCR amplification, the resultant products were subjected to electrophoresis in a 1.5% agarose gel. Following this step, they were visualized under a UV transilluminator to assess the quality of the products.

3.9.8 Quantitative PCR (qPCR) or Real Time PCR

Real time PCR amplification was performed using Light Cycler® 480 II Real-Time PCR Detection System (Roche, Switzerland) with Bio-Rad iTaq™ Universal SYBR® Green Supermix (Lot no: L001751 A; USA). The table 12 and 13 shows the details of the reaction mixture and the thermal regime used for qPCR.

Table 12. Reaction Mixture for Real Time PCR

Reaction Mixture for Real Time PCR	
SYBR Green	5 μL
Forward Primer	0.5μL
Reverse Primer	0.5μL
Template	0.5 μL
NFW	3.5 μL
Reaction Volume	**10 μL**

Table 13. Thermal Regime for Real Time PCR

Steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 ^{0}C	3 min	1
Amplification	95 ^{0}C	30 sec	40
	54 ^{0}C	30 sec	
	72 ^{0}C	30 sec	
Melting Curve	95 ^{0}C	1 min	1
	55 ^{0}C	1 sec	
	95 ^{0}C		
Cooling	37 ^{0}C	2 min	1

Three biological replicates and two technical replicates were taken for each experiment to minimize the standard error. The relative expression levels of the target genes were normalized to *β-actin* employing the $2^{-\Delta\Delta Ct}$ value method. Specifically, the muscle tissue, which served as the control, was arbitrarily assigned a value of 1. To confirm the specificity of amplification and the formation of a single PCR product, melt curve analysis was performed at the end of each PCR run.

The comparative Ct (ΔΔCt) method was employed to quantify the relative expression levels of the mRNA. Initially, the ΔCt value was ascertained by deducting the Ct value of the *β-actin* gene, which acted as the internal control, from the Ct value of the target gene. Subsequently, the ΔΔCt was determined by subtracting the ΔCt value of the control group from the ΔCt value of the experimental group. The relative expression levels were then calculated using the $2^{-\Delta\Delta Ct}$ value method, as outlined by Livak & Schmittgen (2001). The fold changes in expression levels were determined by comparing these to those of the control group, providing a quantitative measure of gene expression alterations.

3.9.9 Statistical analysis

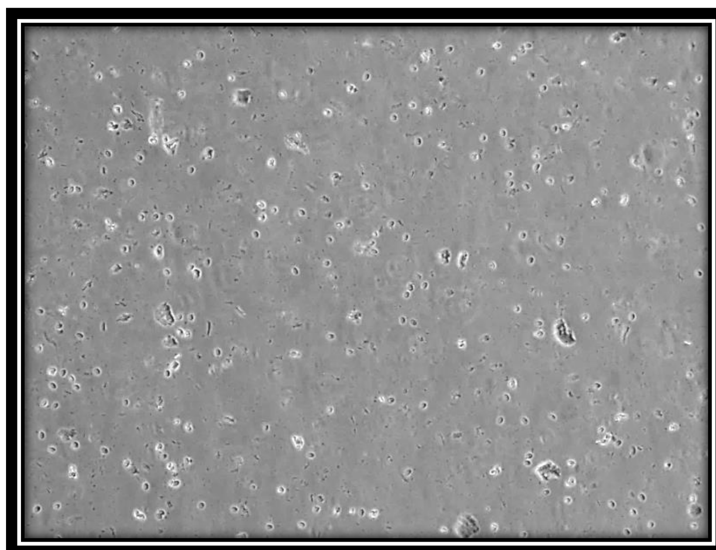
The data were subjected to One-way ANOVA (Analysis of Variance) followed by Duncan's multiple range test using the IBM SPSS Statistics Version 22 statistical package. The value $p < 0.05$ was considered statistically significant. All values were expressed as mean ± SD.

4. RESULTS

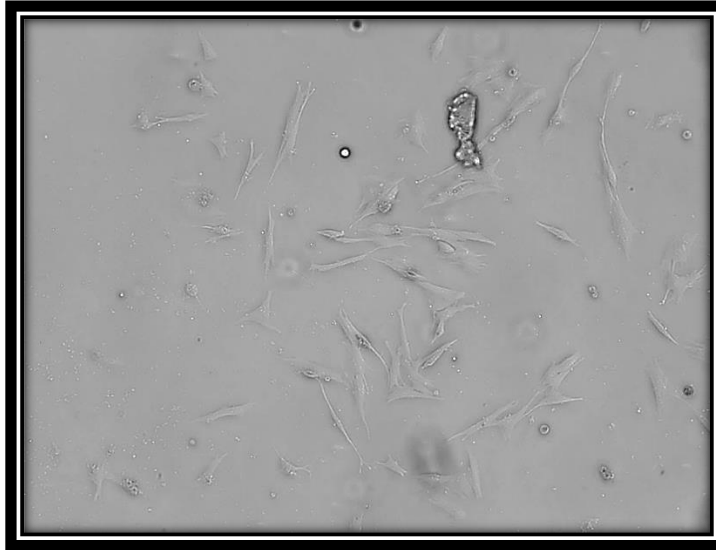
Several attempts were undertaken to isolate and culture myosatellite cells from *C. magur*. Despite replicating methodologies from various scientific studies, these approaches proved ineffective for this particular species, indicating the necessity for a standardized protocol. The developed culture of CM myosatellite cells underwent thorough characterization, encompassing species authentication, immunofluorescence, and analysis of myogenic gene expression.

4.1 Isolation and purification of myosatellite cells

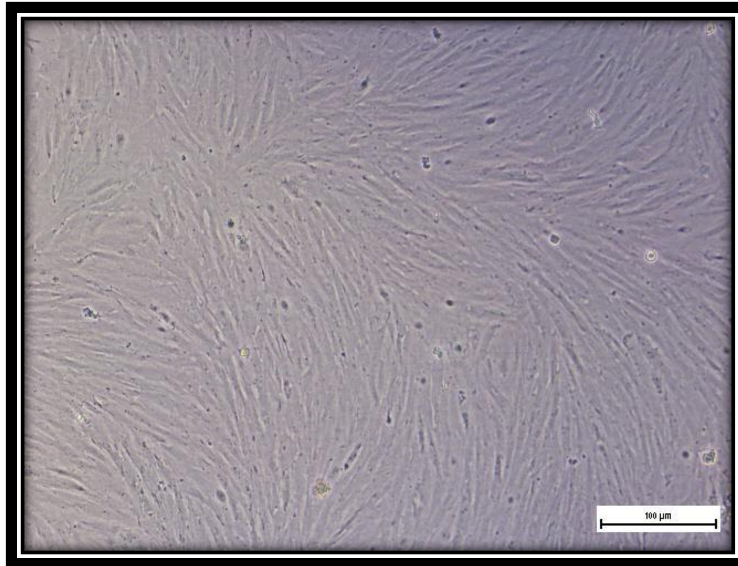
The enzymatic digestion of 5 g of white epaxial muscle yielded approximately 3.95 x 10^6 cells/mL, with 56% of the cells being viable (2.21 x 10^6 cells/mL) and the remaining 44% non-viable (1.74 x 10^6 cells/mL). In the experiments with ice cold treatment, no anticipated results were observed. However, positive outcomes were observed in the pre-plating experiments. Initial observations at day 0 highlighted the presence of scattered mononuclear cells, indicative of cell replication. Notably, from day 3 onwards, a morphological transformation was observed in myosatellite cells. By day 8, the culture has achieved a confluence level of approximately 70-80%. Subsequent to reaching this level of confluence, the cells were subjected to trypsinization. Regrettably, this procedure resulted in a marked decrease in cell attachment, ultimately leading to the loss of the culture.



Day 0



Day 3



Day 8

Fig. 1: Phase contrast photomicrographs of *Clarias magur* muscle satellite cells attachment and proliferation from days 0 to 8. Scale bar: 100 µm; Magnification: 10 X

4.2 Immunofluorescence

The isolated cells were characterized through immunofluorescence staining using specific myoblast markers, notably Pax7 and MyoD. The results revealed a notable uniformity, with approximately 90% of the cells exhibiting positivity for Pax7 and a similar percentage for MyoD, as illustrated in Figure 2 & 3.

These findings strongly indicate that the isolated cells possess distinct myogenic characteristics.

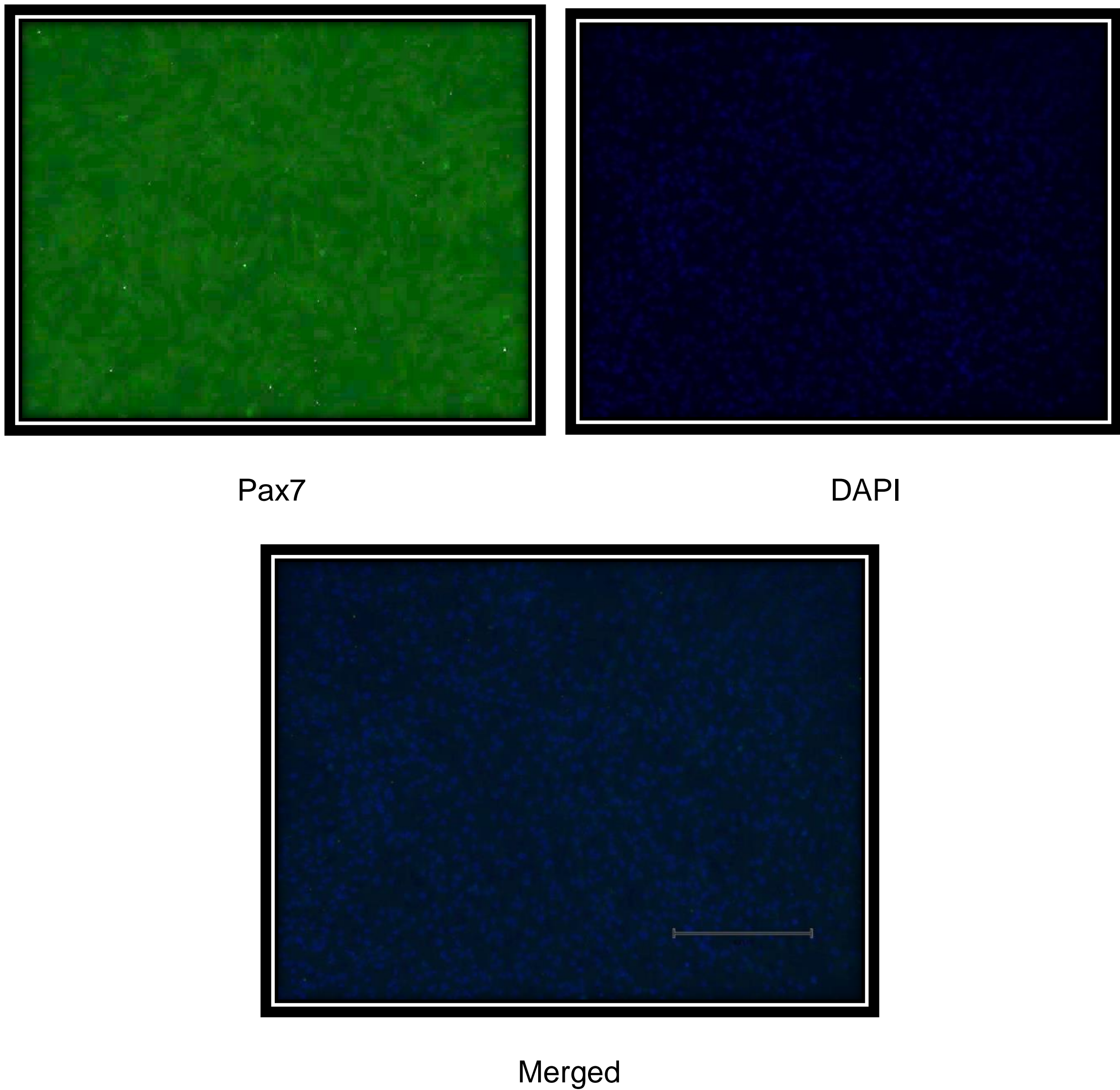


Fig.2: Identification of myosatellite cells in fluorescence staining: Green- Pax7; Blue- DAPI; scale bar = 100 μm

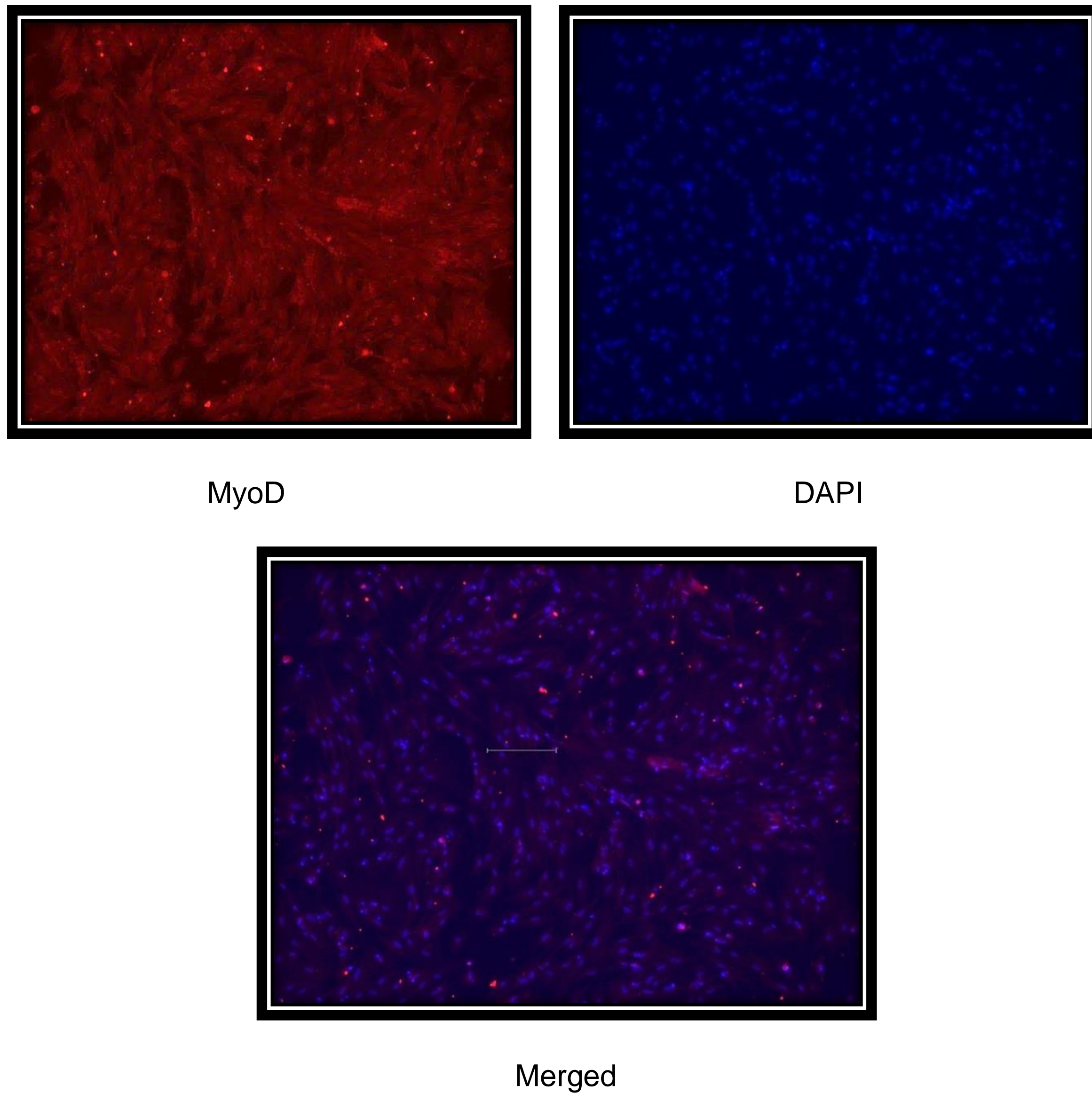


Fig. 3: Identification of myosatellite cells in fluorescence staining: Red- MyoD; Blue- DAPI; scale bar = 100 µm.

4.3 Species authentication using COI gene

The mitochondrial COI gene from the *Clarias magur* skeletal muscle cell culture system was amplified using a pair of universal primers (F1 & R1) which revealed the expected PCR products amplicon size of 650 bp. The sequence alignment of the COI gene obtained from the cells showed 99.69% similarity with the known mitochondrial gene sequence of *C. magur* voucher specimen (Accession no : KM259918).

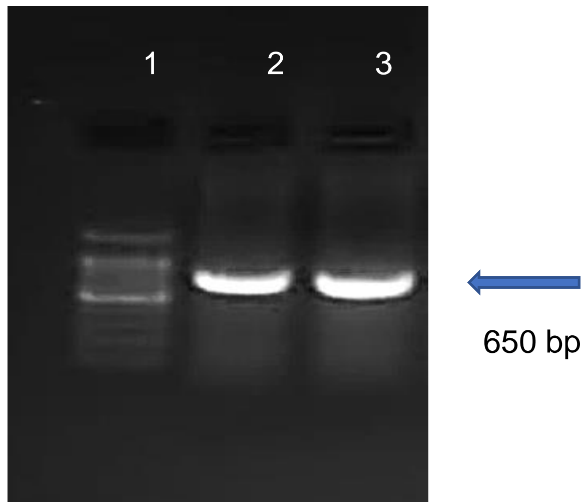
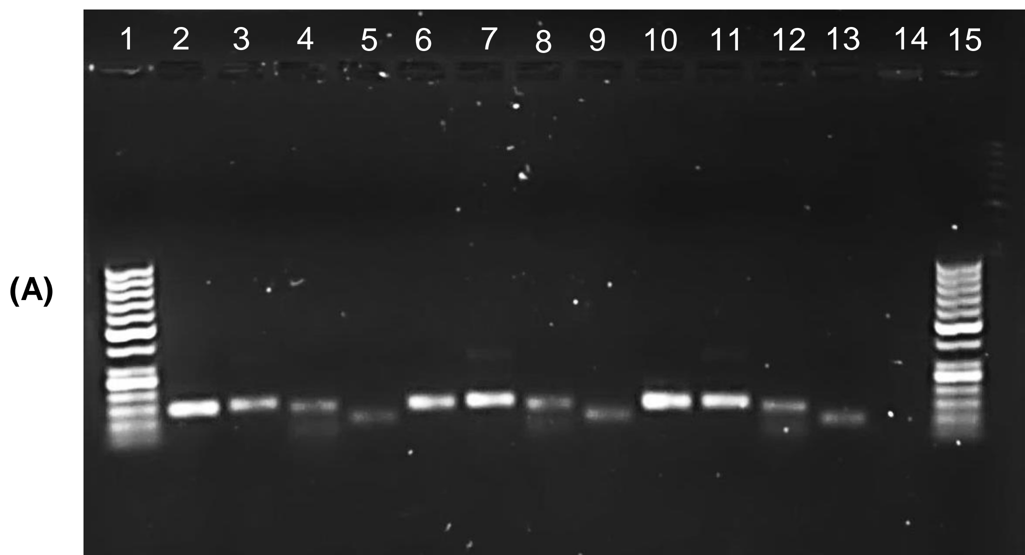


Fig.4: PCR amplification of 650 bp fragment of *C. magur* using oligonucleotide primers from the conserved portions of COI region Lane 1 – 100 bp DNA Ladder; Lane 2 –CMM Positive control; Lane 3 –CMM COI

4.4 Gel electrophoresis of Gradient PCR products

The gel images derived from electrophoresis run of Gradient PCR products were meticulously analyzed to ascertain the optimal annealing temperature for all four primer sets. It was observed that the bands from products annealed at temperatures of 52 ^{0}C, 54 ^{0}C, and 56 ^{0}C, were significantly more pronounced than those annealed at 58 ^{0}C. Consequently, an optimal annealing temperature of 54 ^{0}C was selected for subsequent PCR amplification of cDNA from myosatellite cells, as well as for Real-Time PCR applications.



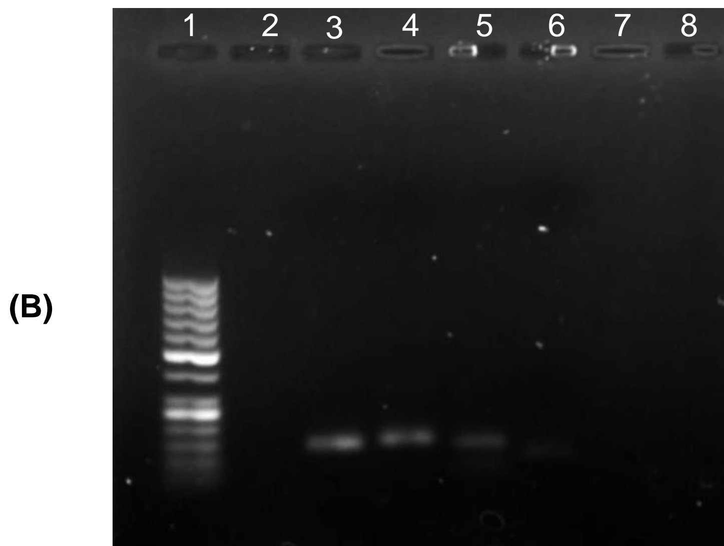


Fig.5: Gradient PCR Products in 1.5% agarose gel (A) Lane 1 &15: 50bp DNA ladder; Lane 2-5: *β-actin* (138 bp), *Myod1* (148 bp), *Myog* (129 bp) and *Pax7* (91 bp), respectively at 52 ^{0}C; Lane 6-9 : *β-actin*, *Myod1*, *Myog* and *Pax7*, respectively at 54 ^{0}C; Lane 10-13 : *β-actin*, *Myod1*, *Myog* and *Pax7*, respectively at 56 ^{0}C. (B) Lane 1 : 50bp DNA ladder ; Lane 3-6*: β-actin*, *Myod1*, *Myog* and *Pax7*, respectively at 58 ^{0}C.

4.5 RNA isolation from myosatellite cells

The RNA extracted from myosatellite cells was found to have a concentration of 695.4 ng/μL and exhibited a purity ratio of 2.03. This RNA was further subjected to quality and integrity assessment through 1% agarose gel electrophoresis. In the first two wells, 2 μL of RNA samples were loaded with 2 μL of 6X Loading dye, while in the following two wells, the samples were loaded with 1 μL of RNA and 2 μL of dye. The resultant electrophoresis gel image revealed two prominent bands, indicative of the 28S rRNA and 18S rRNA, with approximate sizes of 4.8 kb and 2.0 kb, respectively. Notably, a third band, which would typically represent the 5.8S or 5S RNA, was conspicuously absent from the gel image.

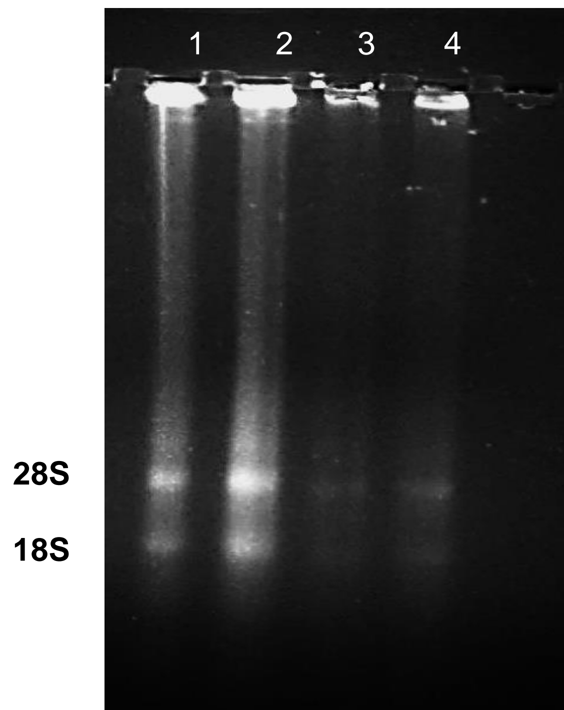


Fig. 6: Quality and Integrity test for RNA isolated from myosatellite cells. The two bands corresponds to 28S and 18S rRNA

4.6 Gel electrophoresis of PCR- amplified myosatellite cDNA products

After the reverse transcription of the isolated RNA into cDNA and its subsequent amplification at an annealing temperature of 54 ^{0}C, the resultant PCR products were analyzed using 1.5% agarose gel electrophoresis to assess their integrity. The gel image revealed distinct bands corresponding to the four Myogenic genes: *β-actin* (138 bp), *Myod1*(148 bp), *Myog* (129 bp) and *Pax7* (91 bp).

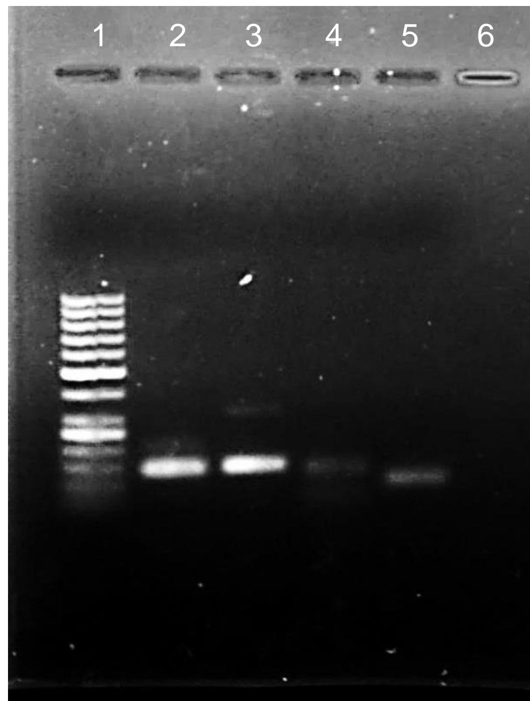
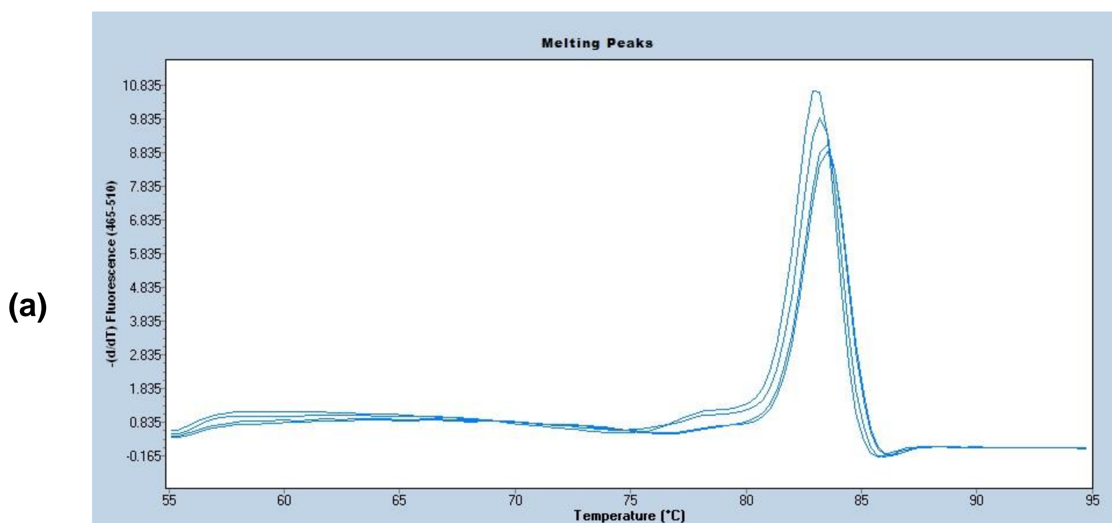


Fig 7: PCR Amplification of Myogenic genes from myosatellite cells; Lane 1: 50 bp DNA ladder, Lane 2: *β-actin*, Lane 3: *Myod1*, Lane 4: *Myog*, Lane 5: *Pax7*

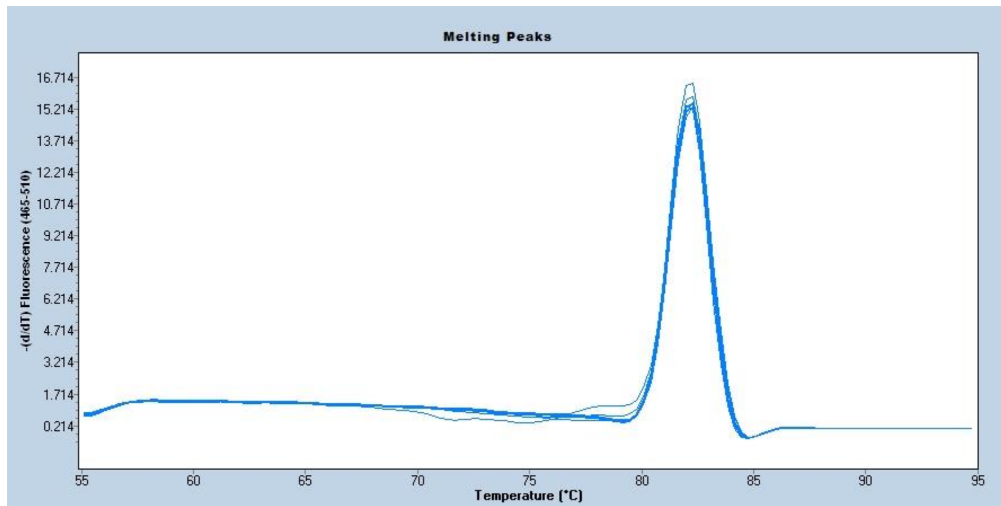
4.7 Analysis of myogenic gene expression

Three key genes associated with myogenesis were identified through quantitative PCR (qPCR). The expression levels of these genes were normalized against the internal control gene, *β-actin*. To ascertain the specificity and quality of the amplified product, the melting curve and peaks were meticulously analyzed. The results indicate the absence of primer dimers or non-specific products.

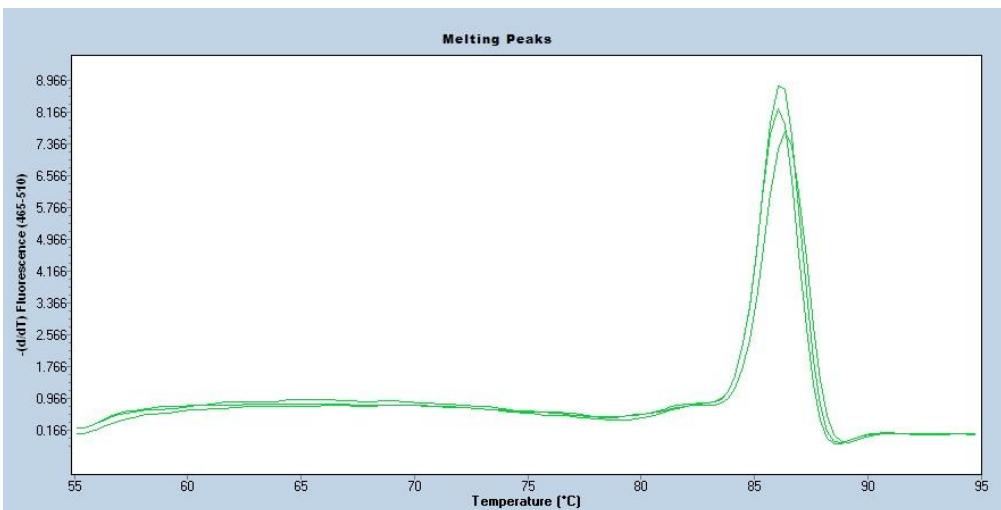
(a)



(b)



(c)



(d)

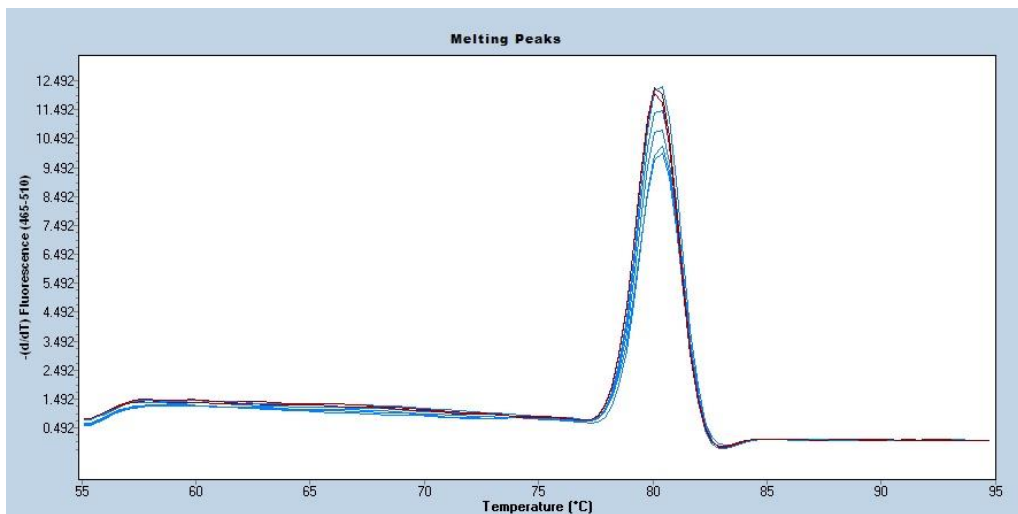


Fig.8: Melting peaks represent the T_m points belonging to the individual primer pairs

(a) *β-actin* (b) *Myod1* (c) *Myog* (d)*Pax7*

4.7.1 Expression quantification of key myogenic genes

In the study, the expression levels of *Myod1*, *Pax7*, and *Myog* within myosatellite cells were meticulously examined and juxtaposed with their levels in muscle tissue derived from *Clarias magur*. The findings, delineated in Figure 9, reveal that, with the exception of *Myog*, the expression levels of key myogenic regulators, specifically *Pax7* and *Myod1*, in the initial stages of myosatellite cell cultures were markedly elevated in comparison to those in muscle tissue, with statistical significance denoted by $p<0.05$. Notably, the expression of *Pax7* in myosatellite cells was found to be 68-fold greater than that in muscle tissue. Similarly, *Myod1* expression was elevated, being 15 times higher in myosatellite cells than in muscle tissue. In contrast, myogenin (*Myog*) exhibited the lowest expression levels in myosatellite cells when compared to its counterparts.

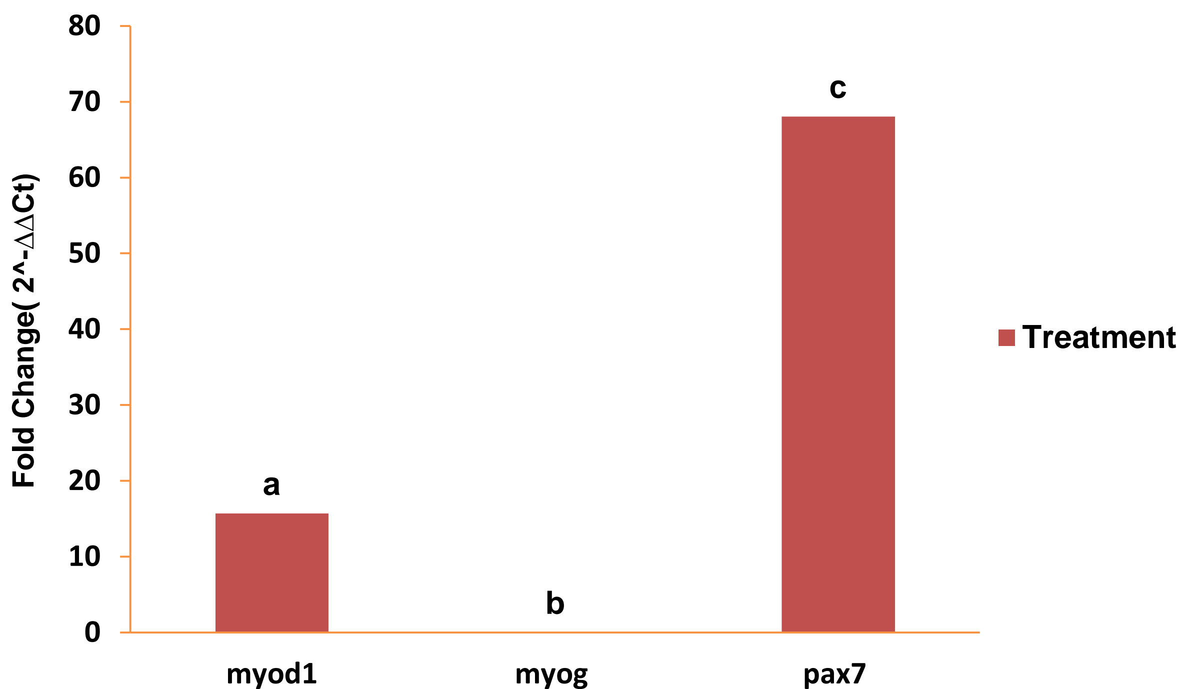


Fig.9: Relative expression levels of *Myod1, Myog* and *Pax7* in myosatellite cells. Different alphabetical superscripts (i.e. a,b,c) denotes the significant difference of the genes across the treatment (Sig. p<0.05).

5. DISCUSSION

Skeletal muscle cell cultures can be obtained *in vitro* through several methods, among which the commonly used are enzymatic dissociation and explant methods. Also, between enzymatic hydrolysis and explant, the former remains the preferred method because of its efficiency in isolating a variety of cell types from muscle tissue (Yafee and Saxel, 1977; Montserrat *et al*., 2007; Gabillard *et al*., 2010; Long *et al*., 2023). Cell suspensions obtained via enzymatic hydrolysis are known to contain fibroblasts, muscle satellite cells, myoblasts, and other precursor cell populations. However, the differential adhesive property of myoblast allows to separate it from the rest of the fibroblastic contaminants (Yafee and Saxel, 1977; Montarras *et al*., 2005; Tone *et al*., 2021; Long *et al*., 2023). In the present study, the myosatellite cells of *C. magur* were isolated and purified using the enzymatic method and subsequently cultured by exploiting their differential adhesive property. Despite replicating methodologies from various scientific studies, these approaches proved ineffective for this particular species, indicating the necessity for a tailored protocol.

5.1 Enzymatic digestion

The enzymes commonly used to dissociate satellite cells from muscle fibres include collagenase, trypsin, dispase, and pronase, either individually or in combination. Pronase, derived from *Streptomyces griseus*, exhibits broad activity due to its protease content (Danoviz & Yablonka-Reuveni, 2012), whereas collagenase, derived from *Clostridium histolyticum*, targets native collagen for digestion. Dispase, derived from *Bacillus polymyxa*, selectively cleaves fibronectin and type IV collagen (Stenn *et al.,* 1989).The duration of enzymatic digestion depends on the age and muscle complexity of the species. According to Danoviz & Yablonka-Reuveni (2012), pronase or trypsin digestion may not be ideal for enriching satellite cells because of the extended digestion of surface antigens, whereas collagenase and dispase cause less damage to surface antigens. However, the myogenic cell preparations isolated by pronase show lower non-myogenic cell levels compared to collagenase methods, possibly due to certain non-myogenic populations not surviving well after pronase digestion.

Successful isolation of satellite cells has been reported from common carp (Koumans *et al*., 1990), rainbow trout (Greenlee *et al*., 1995), Atlantic salmon (Bower and Johnston, 2009), tilapia (Miramontes *et al*., 2020) pacu (Duran *et al*., 2022) , yellow croaker (Zhang *et al*.,2023) and grass carp (Long *et al*., 2023) utilizing a specific combination of collagenase and trypsin. Similarly, Alexander *et al*. (2011) used a blend of collagenase and dispase for the isolation of myogenic muscle cells from Zebrafish. In addition, Powell *et al*. (1989) and Danoviz & Yablonka-Reuveni (2012) have focused attention on the employment of pronase for the isolation of muscle satellite cells. Mulvaney and Cyprino (1995) successfully established a channel catfish satellite cell culture system employing only trypsin digestion. Building upon these foundational studies, the current research used a combination of collagenase and trypsin to increase the cell yield from magur muscle tissue.

5.2 Effect of temperature

The optimal temperature maintained during the isolation process and subsequent culture significantly influences cell viability. Notably, species adapted to cold water or temperate environments, such as rainbow trout (Powell *et al*.,1989) and Atlantic salmon (Bower and Johnston, 2010) requires lower temperature conditions in comparison to species originating from tropical climates. Therefore, in the current study with *Clarias magur*, a temperature range of 25-28°C was consistently maintained throughout the duration of the experiment.

5.3 Purification of myosatellite cells

Several methods have been documented in the literature for purifying myosatellite cells from the crude cell suspension obtained through enzymatic hydrolysis. These methods include Pre-plating (Yaffe, 1968), Percoll gradient centrifugation (Yablonka-Reuveni *et al*., 1987), the use of a laminin coating on the substratum (Foster *et al*., 1987), FACS sorting (Pasut *et al*., 2012; Liu *et al*., 2015), magnetic bead separation (Blanco-Bose *et al*., 2001) and ice-cold treatment method (Benedetti *et al*., 2021). In the conducted study, the efficacy of ice-cold treatment and pre-plating methods for myosatellite cell purification from magur was evaluated. The findings demonstrated that the ice-cold treatment (ICT) method resulted in unsatisfactory outcomes, whereas the pre-plating method exhibited a moderately

positive efficacy in the purification process. This could potentially be attributed to the adverse effects on cell viability resulting from a sudden temperature shock experienced during ICT.

Density gradient centrifugation with Ficoll-paque has been demonstrated to be an effective method in purification of myosatellites (Alexander *et al*., 2011; Krishnan *et al*., 2023; Ulagesan *et al*., 2024). Despite the absence of published reports, the efficacy of Histopaque – a product commonly used for mononuclear cell separation-was also tested. However, it failed to yield the expected results.

5.4 Effect of culture media and matrix coating

Greenlee *et al*. (1995) and Zhang *et al*. (2023) have illuminated the significant role of matrix coating, plating densities, and culture media in the proliferation and differentiation of myosatellite cells. A range of media, including DMEM, DMEM/F12, Ham's F10, RPMI 1640, and L-15, when supplemented with FBS and growth factors, have been identified as optimal environments for the culture of myosatellite cells across various species. Notably, L-15 stands out as the only medium that negates the need for supplemental CO_2 to maintain an appropriate pH level. In a related study, Hua *et al*. (2022) pinpointed DMEM/F12 as the most conducive medium for lower-serum adherent culture, whereas Greenlee *et al*. (1995) showed that a combination of L-15 and 10% FBS yields the greatest extent of myoblast differentiation in rainbow trout. Considering the efficacy and cost-effectiveness of L-15 media, the present experiment has adopted L-15 supplemented with 20% FBS for the culturing of myosatellite cells.

Numerous studies have investigated the use of various matrix coatings to promote the proliferation and differentiation of myosatellite cells. Greenlee *et al*. (1995) reported that fibronectin or laminin boosted cell proliferation the most, whereas matrigel significantly enhanced cell differentiation. Foster *et al*. (1987) initially demonstrated that myogenic cells from newborn rat hindlimbs exhibited significantly enhanced myogenicity when cultured on surfaces coated with Poly-L-Lysine followed by laminin, as opposed to the collagens commonly used. Subsequently, several researchers have utilized a blend of Poly-L-Lysine and laminin for myosatellite purification and culture in fish (Koumans *et al*., 1990; Bower

and Johnston, 2009; Alexander *et al.*, 2011; Long *et al.*, 2023; Zhang *et al.*, 2023). Additionally, Powell *et al.* (1989), Koumans *et al.* (1990), and Alexander *et al.* (2011) have documented the use of gelatin coating for myosatellite purification in the context of pre-plating. So, the current study examined the impact of gelatin coating and collagen coating on myosatellite proliferation, and the results indicated a modest level of effectiveness.

5.5 Characterization

5.5.1 Myogenic gene expression analysis using qPCR

In the quiescent state, satellite cells are characterized by cell surface markers such as CD34, *Pax7*, and MRF-*Myf5* (Rudnicki et al., 1993; Morgan and Partridge 2003). Upon activation and subsequent proliferation, they upregulate genes specific to *MyoD* (Weintraub *et al.,* 1991). Bower and Johnston (2010) demonstrated that in Atlantic Salmon myogenic cell cultures, the expression of *Pax7* increased as cell proliferation increased and reached a maximum at 8 days in cultures grown in complete growth medium. The expression of other MRFs such as *Myf5, MyoD1b,* and *MyoD1c* also peaked at 8 days and was down regulated thereafter.

In vitro studies carried out in grass carp cell line found that both *Pax7* and *Myf5* were expressed in primary and passage cells, with higher expression in primary cells, suggesting a higher proportion of stationary satellite cells in primary culture compared to passage cells (Long *et al.*, 2023). However, the expression of *Pax7* in primary and passage cells was much lower than that of *MyoD*, suggesting that myoblasts were the predominant cell type in primary and passage cultures (Long *et al.*,2023).The cells in the current study harbours a larger proportion of quiescent satellite cells, as evidenced by the highest expression of *Pax7* and a significantly elevated expression of *Myod1*.

Myog plays an important role in mammalian muscle development as it drives myoblasts to differentiate into myotubes and is essential for the formation of muscle fibres (Zhang *et al.*, 2018). Krishnan *et al.* (2023) examined the levels of MRFs such as *MyoD, Myog,* and desmin in Olive flounder cells and found that *MyoD* showed pronounced expression in undifferentiated cells, with its expression

decreasing through the differentiation process. Conversly, the differentiated cells exhibited increased quantities of Myogenin and desmin, providing valuable insights into the mechanisms of muscle cell differentiation. The neutral expression of *Myog* in the present study, thus indicates that there is a lower proportion of differentiated cells within the isolated cells.

5.5.2 Immunostaining

To further confirm the cell type and myogenic characteristics of the myosatellite culture, the expression of myosatellite specific markers, Pax7 and MyoD were detected by immunohistochemistry, as reported in previous studies (Shefer *et al.*,2004; Zammit *et al.*, 2004; Starkey *et al.*,2011).

Shefer *et al.* (2004) reported that Pax7-expressing progenitors can commit to either myogenic or non-myogenic cell lineages. However this commitment occurs prior to cell proliferation, and thereafter no trans-differentiation occurs within descendants. Although both myogenic and non-myogenic clones can arise from individual myofibres, the proportion of each type of clone varies among fibres. This clonal dichotomy suggests that satellite cells possess plasticity and multipotency. Contrarily, research conducted by Starkey *et al.* (2011) revealed that satellite cells do not spontaneously differentiate into adipogenic lineages under conventional culture conditions. They suggested that the presence of non-myogenic phenotypes in single muscle fibre cultures might be attributed to the inadvertent co-purification of non-myogenic progenitors from the muscle interstitium.

The immunostaining results from Shefer *et al.* (2004) and Zammit *et al.* (2004) revealed the dynamic expression of Pax7 and MyoD in cultured cells. Initially, Pax7 expression was predominant over MyoD. However, within the first week, concurrent expression levels of Pax7 and MyoD were observed. Interestingly, by the fourth day, a downregulation of Pax7 was noted in non-myogenic (MyoD-) cells, while its expression persisted in myogenic (MyoD+) cells. Co-immunostaining with antibodies against MyoD and Myog revealed that only limited number cells were in early phase of differentiation during the same time period.

In light of the aforementioned studies, it becomes apparent that the Pax7+/MyoD+ cells within our culture system exhibits myogenic properties and remains predominantly undifferentiated. Furthermore, immunostaining techniques have, to a certain degree, illuminated the spindle-shaped morphology of the cells in our culture.

6. SUMMARY

Myosatellite cells, which are stem cells found within the skeletal muscle tissue, play a significant role in the postnatal growth and skeletal muscle regeneration. Beyond their critical function in muscle repair and regeneration, the exploration into the regulatory mechanisms governing these myogenic stem cells is expected to yield profound insights into muscular dystrophy and the intricacies of myogenesis. Furthermore, researchers are investigating their ability to undergo trans-differentiation, a process in which a differentiated cell transforms into another type without reverting to a pluripotent stem cell stage.

C. magur is an important catfish species recognized for its distinctive flavour, nutritional value, and medicinal properties. However, it faces challenges in aquaculture production due to its complex captive breeding behaviour. Furthermore, the International Union for Conservation of Nature (IUCN) has classified *C. magur* as endangered, noting a critical decline in its wild population. Against this backdrop, developing a myosatellite cell culture system for this species represents a foundational step towards understanding myogenesis in this endangered fish, thereby contributing to its conservation and the promotion of sustainable aquaculture practices. The establishment of a myosatellite cell culture from *C. magur* could significantly benefit the cultured meat industry, as *in vitro* meat production is contingent upon the availability of a sufficient quantity of stem cells. Additionally, this myosatellite cell culture system may be explored for its trans-differentiation potential.

In the present study, myosatellite cells from *C. magur* were successfully isolated and purified utilizing an enzymatic method coupled with differential adhesion techniques. The protocols employed for the isolation process were meticulously adapted from existing methodologies applicable to a variety of fish species, to devise an optimized protocol that meets the specific requirements of *C. magur*. The validity of the established culture was rigorously confirmed through a comprehensive approach that incorporated gene expression analyses and immunohistochemical examinations. These methods verified the expression of

critical myogenic genes, notably *Pax7, Myod1,* and *Myog*, which serve as indicators of satellite cell activity.

The viability of the isolated cells using the enzymatic method was determined to be 56%. Upon attachment, these cells exhibited a robust proliferative capacity, reaching 70-80% confluence within an eight-day period. However, it was noted that subsequent trypsinization led to a reduction in their ability to adhere effectively.

Immunofluorescence analysis revealed that a substantial majority of the cells displayed myogenic characteristics. This was demonstrated by positive reactions to Pax7 and MyoD antibodies, indicating the presence of muscle progenitor cells and early myogenic markers. Additionally, the cells exhibited a distinctive spindle-shaped morphology, further confirming their myogenic characteristics.

The authentication of cell cultures through DNA barcoding to ascertain the species of origin is a critical procedure. The CM myosatellite culture system underwent authentication using the mitochondrial Cytochrome C Oxidase subunit I (COI) gene. The analysis of the mitochondrial COI gene extracted from the culture system conclusively identified the cells as belonging to *C. magur*.

Furthermore, quantitative PCR (qPCR) analysis was employed to examine the relative expression of *Pax7, Myod1*, and *Myog* in the myosatellite cell culture, compared against the expression levels of these genes in the muscle tissue of the species. The findings indicated a significantly higher expression of *Pax7* and *Myod1* in the myosatellite cells, whereas *Myog* was neutrally expressed. This expression pattern suggests that the vast majority of cells within the established culture were in an undifferentiated state, with a very limited number of cells transitioning into the early differentiation stage.

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APPENDIX I

1. Standards

a) 1 kb of ss RNA	340000 Da
b) 1 kb of ds DNA	660000 Da
c) 1 kb of ss DNA	330000 Da
d) 1 Average MW of nucleotide	330 Da

1.1. Spectrophotometric Conversions

a) 1 A260 unit of ds DNA 50 μg/ml

b) 1 A260 unit of ss DNA 33 μg/ml

c) 1 A260 unit of ss RNA 40 μg/ml

APPENDIX II

2. Diethylpyrocarbonate (DEPC) water

DEPC	1ml
Sterile dH20	1 litre

Allowed to stand for 1hr at 37°C or overnight at room temperature and then autoclaved for 15 min at 15 psi (1.05 kg/cm).

2.1. EDTA (0.5 M; pH 8.0)

EDTA	18.61 g
DMW	80 ml

pH adjusted to 8.0 with NaOH. Make up the volume to 100 ml and sterilize by autoclaving.

2.2. Tris-Cl (1M; pH 8.0)

Tris base	12.11g
DMW	100 ml

pH adjusted to 8.0 with conc. HCl and sterilized by autoclaving.

2.3. TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M; pH 8.0)	100 ml
Final volume made to	1000 ml.

2.4. Ethidium bromide (10 mg/ml)

Ethidium bromide	0.1 g
DMW	10 ml

Covered with aluminium foil and stored at 4°C.

2.5. Gel loading buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	3 ml

Make the volume to 10ml using distilled water and stored at 4°C.

ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
μg	Microgram (10^{-6} gm)
μL	Microliter (10^{-6} L)
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
blastn	Nucleotide blast
BOLD	Barcode of Life Databases
BMP	Bone morphogenetic protein
bp	Basepair
cDNA	complementary DNA
CD34	Cluster of differentiation 34
cm	Centimetre
COI	Cytochrome C Oxidase subunit 1
Conc.	Concentration
CRP	Cysteine Rich Protein
DPBS	Dulbecco's Phosphate Buffered Saline
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
ICT	Ice cold treatment
IGF1	Insulin like growth factor 1
IUCN	International Union for Conservation of Nature
L-15	Leibovitz’s 15
mM	milli Molar
MRF4	Myogenic Regulatory Factor 4
Myf5	Myogenic Factor 5
MyHC	Myosin Heavy Chain
Myod1	Myogenic Differentiation 1
Myog	Myogenin
NASA	National Aeronautics and Space Administration
Pax7	Paired box7
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PSA	Penicllin/streptomycin/amphotericin

RNA	Ribonucleic acid
RNase	Ribonuclease
qPCR	Quantitative PCR
SC	Satellite cell
SOFIA	The State of World Fisheries and Aquaculture
β	Beta
Δ	Delta
ΔCt	Threshold cycle number