Establishment of Hepatocyte Cultures of *Rastrelliger kanagurta* and their Lipid Analyses

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In Partial Fulfilment of the Requirement for The Degree Of

Master Of Science in Zoology

By

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled "**Establishment of Hepatocyte Cultures of *Rastrelliger kanagurta* and their Lipid Analyses**" is based on the results of investigations carried out by me in the Zoology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision / Mentorship of Dr. Shanti N. Dessai and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the project work entitled "**Establishment of Hepatocyte Cultures of *Rastrelliger kanagurta* and their Lipid Analyses**" is a bonafide and authentic work carried out by **Ms.** Jochebed M. Fernandes under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in Zoology in the Zoology Discipline at the School of Biological Sciences and Biotechnology Goa University.

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Research has shown over and over again that the more you acknowledge your past successes, the more confident you become in taking on and successfully accomplishing new ones.

-Jack Canfield

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CHAPTER I

INTRODUCTION

Introduction

The Indian mackerel is a type of inshore pelagic fish species that can be found in the coastal bays, lagoons, and harbours of warm shallow waters along the coasts of the Indian and Western Pacific oceans. Its habitat ranges from the Red Sea and East Africa in the west, all the way to Indonesia in the east, and from China and the Ryukyu Islands in the north, to Australia, Melanesia, and Samoa in the south. This fish species has a moderately deep body and a long head, with thin dark stripes on the upper sides, and a black spot near the lower margin of its pectoral fin. It can grow up to 42.1 cm in length and 3.8 kg in weight and feeds mainly on plankton, such as larval crustaceans and fishes. The Indian mackerel spawns in batches, and the timing of its two seasons depends on the region. It reaches sexual maturity at around 17-21 cm in length and 1.1 years in age (Hossain *et al.,* 2018).

The liver is a vital organ in all vertebrates, including aquatic species. In fish, the liver has a significant role in producing yolk for eggs, which is not the case in mammals as they do not lay eggs. Fish mainly utilize lipids as an energy source, so the liver of fish has a minor role in carbohydrate metabolism. Conversely, mammals rely on the liver for regulating glucose levels in the blood, making carbohydrate metabolism a major function. Fish liver is characterized by its homogeneity and polygonal-shaped, weakly basophilic hepatocytes. On the other hand, the liver of mammals is more heterogeneous with cuboidal-shaped, strongly basophilic hepatocytes. The fish liver has many bile ducts that connect to the gallbladder, pancreatic duct, and biliary duct, while mammals have fewer bile ducts that connect to the common bile duct and cystic duct. The hepatic parenchyma of fish is very homogeneous, and hepatocytes are typically weakly basophilic compared to mammals. Hepatocytes are the main functional cells of the liver. They are polygonal-shaped cells that constitute roughly up to 80% of the liver mass. The liver is of key importance for the maintenance of internal homeostasis in vertebrates. It

adapts to fluctuating environmental conditions by continuously readjusting hepatocellular structures and functions, such as (a) metabolism of nutrients; (b) storage of energy (glycogen, lipid); (c) synthesis and secretion of proteins (e.g. albumin, vitellogenin, lipoproteins); (d) maintenance of plasma glucose levels; (e) elimination of nitrogen components after urea or ammonia formation; (f) metabolism of hormones; (g) metabolism of xenobiotics; and (h) bile formation. The highly dynamic nature of the liver and its regulation in many metabolic and physiological processes makes this organ a valuable model to study mechanisms and processes of environmental acclimation (Hardy and Farrell, 2002; Farrell, 2016; Terwinghe and Kestemont, 2017; Hernández-López and García-Carreño, 2017).

1.1. Literature review on Fish hepatocyte culture

Overall, the literature on the culture of hepatocytes from fish liver is extensive and continues to grow. Fish hepatocytes have been used for various applications, including toxicology, nutrition, and drug discovery. More recently, there has been growing interest in using fish hepatocytes for the production of *in vitro* meat, which has the potential to provide a sustainable source of protein and reduce the environmental impact of meat production.

The culture of hepatocytes from the fish liver has been extensively studied over the past few decades, and the literature on this topic is extensive. Fish hepatocytes have been used for various applications, including toxicology, nutrition, and drug discovery. Here is a brief literature review on the culture of hepatocytes from fish liver.

One of the earliest studies on fish hepatocyte culture was published by Segner *et al.*(*1992)* The authors used a two-step collagenase perfusion method to isolate hepatocytes from the liver of rainbow trout (*Oncorhynchus mykiss*). The isolated hepatocytes were then cultured in Leibovitz's L15 medium supplemented with fetal bovine serum, insulin, and dexamethasone. The authors reported that the cultured hepatocytes retained their morphology and function for up to 48 hours, as assessed by phase-contrast microscopy and assays for liver-specific enzymes.

Another study published by Kajimura *et al.,* in 2001 reported the successful culture of hepatocytes from the liver of Japanese Eel (*Anguilla japonica*). The authors used a modified two-step collagenase perfusion method to isolate hepatocytes, which were then cultured in Leibovitz's L15 medium supplemented with fetal bovine serum, insulin, and dexamethasone. The authors reported that the cultured hepatocytes retained their morphology and function for up to five days, as assessed by phase-contrast microscopy and assays for liver-specific enzymes.

In recent years, there has been growing interest in using fish hepatocytes for the production of *in vitro* meat. One study published by Shimizu *et al.* (2020) reported the successful production of cell-based meat using hepatocytes from the Japanese eel. The authors used a cell culture technique called "spheroid culture" to produce eel liver spheroids, which were then used as raw material for meat production. The authors reported that the cell-based meat had a texture and flavour similar to that of real eel meat.

Research studies demonstrate the successful isolation and culture of hepatocytes from a variety of fish species, including large yellow croaker, zebrafish, striped catfish, and sea bass. The studies also highlight the importance of optimizing culture conditions, including media composition and culture duration, to maintain the viability and functionality of the cultured hepatocytes. Additionally, the studies demonstrate the usefulness of fish hepatocytes for applications in fish health management, nutrition, and toxicology (Wang *et al.,* 2019; Das *et al.,* 2021; Yao *et al.,* 2020; Sae-Lim and Tunkijjanukij, 2017). Literature also shows the evidence of successful isolation and culture of hepatocytes from various fish species, including *Labeo rohita*, Asian seabass, Indian catfish, Red drum, and Olive flounder (Chakraborty *et al.,* 2019; Sibin *et al.,* 2020; Agarwal *et al.,* 2018; Cui *et al.,* 2017; Bong *et al.,* 2021). They also demonstrate the use of fish hepatocytes for applications in toxicology and environmental risk assessment. These studies highlight the need for optimization of culture conditions for maintaining the viability and functionality of fish hepatocytes in culture.

Fish cell lines have been widely used in toxicology and ecotoxicology applications (Bols *et al.,* 2005). Goldfish hepatocytes were the first fish cells used in research studies (Birnbaum *et al.,* 1976), and since then, a large number of studies have been conducted using fish hepatocytes (Segner, 1994). Epithelial cells and hepatocytes derived from fish are useful for various ichthyological research purposes (Hayashi*, 2002;* Lee *et al.,* 1993). Fish primary hepatocytes have been widely used to study xenobiotic metabolism, the formation of toxic products, chemically-induced DNA damage, enzyme induction, peroxisome proliferation, and effects on hormone-signaling systems, among others. The isolation and culture of teleost hepatocytes are reviewed by Segner (1992), and the cultivation conditions such as temperature, media, and sera, are species-dependent (Braunbeck & Segner*,* 2000). *In vitro* systems offer a great advantage for understanding liver basic properties and environmental adaptive responses. The isolated hepatocyte provides the benefits of an intact cell, such as functional organelles, enzyme interactions, physiological co-factor, and metabolite concentrations, without the complexity of the intact animal. Cultured hepatocytes are widely used in various biomedical applications to study liver metabolism, drug toxicity, and disease pathogenesis, among others. Lipid profiles of cultured hepatocytes are of great interest to researchers as lipid metabolism plays a crucial role in liver function (Etteldorf *et al.,* 1990). Wang *et al.* (2019) have provided a review of a comprehensive evaluation of the potential applications of fish hepatocyte culture in the environmental monitoring of aquatic pollutants. This review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish responses to environmental pollutants.

A review by Wang *et al.* (2021), provides an overview of the current status of fish hepatocyte culture, including the isolation and culture methods, culture conditions, and the maintenance of hepatocyte function *in vitro*. The review also highlights the potential applications of fish hepatocyte culture in toxicology, drug metabolism, and nutrition studies. A research review by

(Cerezo *et al.,* 2015), provides a critical evaluation of the current status of fish hepatocyte culture, including the isolation and culture methods, culture conditions, and the maintenance of hepatocyte function *in vitro*. The review also highlights the importance of optimizing culture conditions to maintain the viability and functionality of the hepatocytes in culture. Lee *et al.* (2020), in their review article, provide an overview of the current status of fish hepatocyte culture from marine fish, including the isolation and culture methods, culture conditions, and the maintenance of hepatocyte function *in vitro*. It also highlights the potential applications of fish hepatocyte culture in toxicology, drug metabolism, and nutrition studies, as well as the potential of fish hepatocytes as an alternative to mammalian hepatocytes for *in vitro* studies. Overall, these reviews provide a comprehensive evaluation of the current status of fish hepatocyte culture and highlight the potential applications of fish hepatocyte culture in advancing our understanding of fish physiology, health, and nutrition.

A review by (Chakraborty *et al.,* 2021), provides a comprehensive evaluation of the current status of fish hepatocyte culture and its applications in aquatic toxicology. The review covers the isolation and culture methods for fish hepatocytes, the characterization of hepatocytes in culture, and the potential of fish hepatocyte culture as an alternative to animal testing for toxicology studies. Kim *et al.* (2019), have provided a comprehensive evaluation of the current status of fish hepatocyte culture and its applications in toxicology and hepatotoxicity studies. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture as a model for *in vitro* toxicology and hepatotoxicity studies. Zha *et al*. (2019), have reviewed a comprehensive evaluation of the current status of the primary culture of fish hepatocytes, including the isolation and culture methods, culture conditions, and the maintenance of hepatocyte function *in vitro*. The review also covers the potential applications of fish hepatocyte culture in toxicology, drug metabolism, and nutrition studies, as well as the challenges and future prospects of fish hepatocyte culture. Overall, these reviews provide a comprehensive evaluation of the current

status of fish hepatocyte culture and highlight the potential applications of fish hepatocyte culture in advancing our understanding of fish physiology, health, and nutrition, as well as in the field of toxicology and hepatotoxicity.

A review by Isani *et al*. (2018), provides an overview of the current status of fish hepatocyte culture and its applications in studying various aspects of fish physiology, including toxicology and metabolism. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish physiology. Mahapatra *et al*. (2018) have provided a comprehensive evaluation of the current status of hepatocyte culture from freshwater fish and its applications in studying various aspects of fish physiology and toxicology. The review covers the isolation and culture methods for hepatocytes from different species of freshwater fish, the maintenance of hepatocyte function in culture, and the potential of freshwater fish hepatocyte culture in advancing our understanding of fish physiology and toxicology. Yu *et al.* (2016) have provided a comprehensive evaluation of the current status of hepatocyte culture from marine fish and its applications in studying various aspects of fish physiology and toxicology. The review covers the isolation and culture methods for hepatocytes from different species of marine fish, the maintenance of hepatocyte function in culture, and the potential of marine fish hepatocyte culture in advancing our understanding of fish physiology and toxicology. Overall, these reviews provide a comprehensive evaluation of the current status of fish hepatocyte culture and highlight the potential applications of fish hepatocyte culture in advancing our understanding of fish physiology, health, and nutrition, as well as in the field of toxicology and hepatotoxicity.

A review by Zou *et al*. (2017) has provided a detailed evaluation of the current status of fish hepatocyte culture and its potential applications in studying various aspects of fish physiology, nutrition, and toxicology. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish

hepatocyte culture in advancing our understanding of fish physiology and health. Sánchez-Muros *et al*. (2021) highlight the potential of fish hepatocyte culture as an ideal model for studying fish health and nutrition. Their review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish health, nutrition, and metabolism. A comprehensive evaluation of the current status of fish hepatocyte culture and its potential applications in ecotoxicology and nutrition is provided by Zhang *et al*. (2019). This review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish responses to environmental contaminants and nutritional requirements. These reviews highlight the potential of fish hepatocyte culture in advancing our understanding of fish physiology, health, and nutrition, as well as in the field of toxicology and ecotoxicology.

The research review of Zou *et al*. (2017), provides a comprehensive evaluation of the current status of fish hepatocyte culture and its potential applications in studying various aspects of fish physiology, nutrition, and toxicology. It also covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish physiology and health. Research compilation by Zhang *et al.* (2019), highlights the potential of fish hepatocyte culture as an ideal model for studying fish health and nutrition. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish health, nutrition, and metabolism. A comprehensive evaluation of the current status of fish hepatocyte culture and its potential applications in ecotoxicology and nutrition is provided in the review by Zhang *et al*. (2019). Their review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish responses to environmental contaminants and nutritional

requirements. Detailed review of the evaluation of *in vitro* hepatic models for drug toxicity evaluations, including fish hepatocyte culture is provided by Zhang *et al*. (2019). This review covers the advantages and limitations of using fish hepatocytes as an alternative model for drug toxicity evaluations, as well as the challenges and future directions of fish hepatocyte culture. A research review by Zhang *et al*. (2019), provides a comprehensive evaluation of the potential applications of fish hepatocyte culture in marine environmental toxicology. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish responses to environmental contaminants. Research compilation by Zhang *et al*. (2019) provides a evaluation of the potential applications of fish hepatocyte culture in ecotoxicology. The review covers the isolation and culture methods for fish hepatocytes, the maintenance of hepatocyte function in culture, and the potential of fish hepatocyte culture in advancing our understanding of fish responses to environmental contaminants. These reviews highlight the potential of fish hepatocyte culture in advancing our understanding of fish physiology, health, and nutrition, as well as in the field of toxicology and ecotoxicology.

1.2. Literature review on Hepatocyte cultures of *Rastrelliger kanagurta*

Rastrelliger kanagurta, commonly known as the Indian mackerel, is an important fish species in the Indian subcontinent. It is widely consumed and is an important source of protein for the local population. Culturing hepatocytes from the liver of *Rastrelliger kanagurta* can provide a valuable tool for studying the metabolism, toxicity, and nutritional requirements of this fish species. However, a literature review suggests that there are limited studies on the culture of *Rastrelliger kanagurta* hepatocytes, particularly *in vitro*.

There is a growing interest in the culture of fish hepatocytes *in vitro* due to their potential applications in various fields, including toxicology, nutrition, and aquaculture. Among the fish

species being studied, mackerel (*Scomber scombrus*) has emerged as a commercially important species, given its high nutritional value and worldwide consumption.

Researchers have been studying mackerel hepatocytes *in vitro* to gain insight into the metabolic pathways involved in the elimination of toxins, drugs, and other xenobiotics by this species (González *et al*., 2017). *In vitro* cultures of mackerel hepatocytes have also been used to investigate the effects of environmental contaminants, such as heavy metals, pesticides, and industrial chemicals, on mackerel health (Raldúa and Piña, 2012). These studies can contribute to the development of sustainable aquaculture practices and the protection of marine ecosystems.

Furthermore, the culture of mackerel fish hepatocytes *in vitro* can be used to study the nutritional requirements of this species. Mackerel is known for its high levels of omega-3 fatty acids, which are essential for human health. *In vitro* cultures can be used to investigate the effects of different diets and feeding regimes on the metabolism and nutritional quality of mackerel (Montero *et al*., 1999).

One study published by Hussain *et al*. (2019) reported the successful isolation and culture of *Rastrelliger kanagurta* hepatocytes. The authors used a two-step collagenase perfusion method to isolate hepatocytes from the liver tissue. The isolated hepatocytes were then cultured in Williams' medium E supplemented with fetal bovine serum, insulin, and dexamethasone. The authors reported that the cultured hepatocytes retained their morphology and function for up to five days, as assessed by phase-contrast microscopy, transmission electron microscopy, and assays for liver-specific enzymes.

Another study published by Parween *et al*. (2020) investigated the hepatoprotective activity of methanol extract of *Rastrelliger kanagurta* against carbon tetrachloride-induced hepatotoxicity in rats. The authors used hepatocytes isolated from the liver of *Rastrelliger kanagurta* to study the effects of the extract on cell viability, oxidative stress, and liver-specific enzymes. However,

the study did not report on the methods used for the isolation and culture of *Rastrelliger kanagurta* hepatocytes.

Culturing mackerel fish hepatocytes *in vitro* is an important tool for understanding the biology, toxicology, and nutritional requirements of this commercially important fish species. It can also contribute to the development of sustainable aquaculture practices and the protection of marine ecosystems. Overall, the literature on the culture of *Rastrelliger kanagurta* hepatocytes is limited, but the studies available suggest that it is possible to isolate and culture hepatocytes from this fish species *in vitro*. Further studies are needed to optimize the culture conditions and to explore the applications of *Rastrelliger kanagurta* hepatocytes in toxicology, nutrition, and other fields.

Mackerel hepatocyte culture can be used for the production of spheroids and organoids as well as for the production of *in vitro* liver meat.

Spheroids and organoids are 3D cell culture models that mimic the architecture and function of tissues *in vivo*. They are used in drug screening, toxicology testing, and regenerative medicine. Mackerel hepatocytes can be used to produce spheroids and organoids, which can then be used to study the effects of environmental contaminants, drugs, and other xenobiotics on liver function and to develop new therapies for liver diseases.

In vitro liver meat, also known as cultured meat or cell-based meat, is produced by culturing animal cells *in vitro* and then using them as a raw material for meat production. Mackerel hepatocytes can be used to produce *in vitro* liver meat, which has the potential to reduce the environmental impact of meat production and provide a sustainable source of protein.

The use of mackerel hepatocytes for spheroid and organoid production and *in vitro* liver meat is still in the early stages of development, but it shows promise as a tool for advancing the fields of toxicology, regenerative medicine, and sustainable food production.

1.3. Reasons for choosing fish liver for cell culture

Fish liver is an essential part of the fish that provides numerous health benefits. Here are some of the reasons why fish liver is important:

Rich source of nutrients: Fish liver is a rich source of nutrients, including vitamin A, vitamin D, and omega-3 fatty acids. These nutrients are essential for maintaining healthy skin, vision, immune system, and bone health.

Boosts brain function: The omega-3 fatty acids present in fish liver have been shown to improve cognitive function, memory, and overall brain health. Regular consumption of fish liver can help prevent cognitive decline and reduce the risk of Alzheimer's disease.

Supports heart health: Fish liver contains omega-3 fatty acids, which are known to lower cholesterol levels, reduce inflammation, and decrease the risk of heart disease. The vitamin D present in fish liver also helps regulate blood pressure and support heart health.

Enhances immune function: Vitamin A is essential for maintaining a healthy immune system, and fish liver is an excellent source of this important nutrient. Regular consumption of fish liver can help boost immune function and protect against infections.

Supports overall health: The nutrients in fish liver work together to support overall health and well-being. Regular consumption of fish liver can help prevent nutrient deficiencies, support healthy aging, and promote a healthy lifestyle.

In conclusion, the fish liver is a valuable source of nutrients that provide numerous health benefits. Regular consumption of fish liver can support overall health and well-being, making

it an important addition to a healthy diet (Bjarnason and Moody, 1991; Abbott *et al.,* 2006; Farzaneh-Far *et al.,* 2012; Semba, 2016; Hibbeln *et al.,* 2007).

1.4. Importance of Fish Hepatocyte Culture

Culturing fish hepatocytes *in vitro* is important for several reasons. Fish hepatocytes are responsible for important metabolic functions such as detoxification, energy metabolism, and protein synthesis. By studying fish hepatocytes *in vitro*, scientists can gain insight into how fish metabolize drugs, toxins, and other xenobiotics.

Moreover, fish hepatocytes can be used as an alternative to whole fish in toxicity testing. *In vitro* testing of fish hepatocytes can be more cost-effective and humane than animal testing, and can also provide more accurate results due to the controlled experimental conditions.

Furthermore, the ability to culture fish hepatocytes *in vitro* provides a tool for studying the effects of environmental contaminants on fish health. Fish are exposed to a wide range of contaminants in their natural habitats, and *in vitro* cultures can be used to study the mechanisms by which these contaminants affect fish health.

Overall, culturing fish hepatocytes *in vitro* is an important tool for understanding fish biology, toxicology, and environmental health.

1.5. Uses of Fish Hepatocyte culture

Fish hepatocyte culture can be a useful tool in various areas of research and applications. Here are some ways in which fish hepatocyte culture can help:

- Toxicology studies: Fish hepatocyte culture can be used to study the toxicity of chemicals and drugs. Researchers can expose the hepatocytes to different concentrations of a substance and observe the effects on the cells. This can provide valuable information on the potential toxicity of the substance and help in developing safer products.
- Disease modelling: Fish hepatocyte culture can be used to model liver diseases, such as hepatitis and cirrhosis. Researchers can induce liver damage in the hepatocytes and observe the cellular and molecular changes that occur. This can provide insights into the underlying mechanisms of the disease and help in developing new treatments.
- Drug metabolism studies: Fish hepatocyte culture can be used to study the metabolism of drugs in the liver. Researchers can expose the hepatocytes to different drugs and observe how they are metabolized. This can provide valuable information on the pharmacokinetics of drugs and help in optimizing dosing regimens.
- Aquatic toxicology: Fish hepatocyte culture can be used to study the effects of pollutants and contaminants on aquatic organisms. Researchers can expose the hepatocytes to water samples collected from contaminated environments and observe the effects on the cells. This can provide insights into the potential risks of exposure to these contaminants and help in developing strategies for environmental monitoring and management.

In conclusion, fish hepatocyte culture is a valuable technique that has several applications in research and industry. It can be used for toxicology studies, disease modelling, drug metabolism studies, and aquatic toxicology, among other applications. This technique can provide valuable insights into the mechanisms of diseases and the effects of drugs and environmental contaminants (Hultman *et al.,* 2019; Paulos *et al.,* 2019; Chen *et al.,* 2019; Lemaire and Chim, 2020).

1.6. Hypothesis of this research work

The establishment of hepatocyte cultures of *Rastrelliger kanagurta* under optimized culture conditions is expected to result in viable and functional hepatocytes that retain similar lipid contents to freshly isolated liver tissues from Indian mackerel. Since liver tissue has high regeneration capacity, it is expected to establish the culture without supplementation of growth factors in the culture.

1.7. Objectives of the work

The objective of this study is to establish and optimize a culture system for hepatocytes isolated from the liver of Indian mackerel (*Rastrelliger kanagurta*) and to perform lipid analyses on the cultured cells.

The specific objectives are as follows:

1. To isolate and culture hepatocytes from the liver of Indian mackerel using explant and free cell culture.
2. To compare cell cultures under different culture conditions to promote cell growth, proliferation, and retention of metabolic activity.
3. To perform lipid analyses on the cultured hepatocytes.

CHAPTER II

MATERIALS AND METHODS

Materials and Methods

2.1. Materials used to fulfill the objectives

All the required materials for this study were washed with distilled water and sterilized prior to commencing the experiment work. The material and equipment of the Animal Cell Culture laboratory used to carry out the research (Plate 1& 2) are described as follows:

Autoclave: Autoclave instrument was used for sterilizer, glass wares (glass petri dishes, media bottles, filter sterilizer, absorbent cotton, Millipore filter paper, media bottles, glass pipettes, distilled water, and glass beaker). This was done to kill contamination and sterilize the glassware.

Oven: Hot air oven was used to sterilize materials mostly Petri plates, glass pipettes, and beakers. The oven was heated for at least 60 minutes at 150°C prior to performing the experiment.

Ethanol: 70% Ethanol solution was used as a disinfectant to clean the laminar air flow before performing the experiment. 70% ethanol was used to sterilize all the materials that were not autoclaved which mainly included plastic material such as centrifuging tubes, Scissors pointed and blunt forceps.

Laminar air flow: All the procedures for cell culture experiment were carried out under the horizontal laminar air flow. This device was used to protect media and other materials from contamination by providing a sterile environment during the culture initiation. Media filtration was also performed in the lamina air flow.

Filtration unit and pump: Filtration unit and pump was used to sterilize and filter the media. The filtration unit was autoclaved before performing the filtration.

Magnetic stirrer: Magnetic stirrer was used for the trypsinization method to dissociate the cells from the matrix. Trypsin and tissue pieces were placed in the 100ml beaker along with the magnetic bead on the magnetic stirrer.

Inverted Microscope: Inverted microscope with image analyzer (MICAPS-MicroView image analyzer software on iOX 105S Inverted Microscope) was used for observing live cells or organisms at the bottom of a inoculating plates. This microscope was also used to take live images and videos of the cells and also study the proliferation of the cells.

TLC chamber: These chambers were used while performing the experiment on lipid profiles.

Weighing machine: Weighing machine was used to weigh the media powders and some of the chemical compounds which were used to perform protein tests.

Cell incubator chamber: The desiccator chamber without desiccation function was modified and sterilized to be used as an incubator chamber for incubating the cell cultures at room temperature.

Centrifuging machine: Benchtop laboratory was used to pellet out the cells /centrifuge the cells after trypsinization and mechanical dissociation or to wash the cell pellets.

Vacuum rotatory evaporator: Vacuum rotatory evaporator was used to evaporate the sample to prepare homogenate.

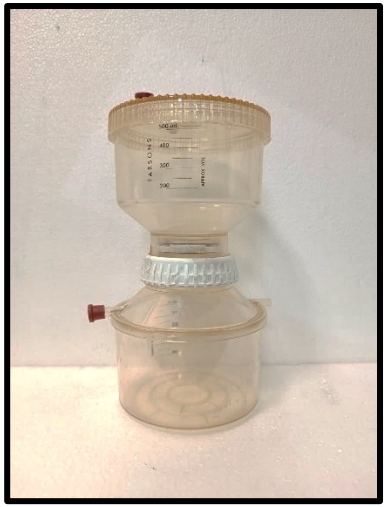
***Other Materials*:** Sterilized petri plates (small, medium, and large), sterile beakers,10ml pipettes, centrifuging tubes, micropipette with sterile tips, inoculating Petri plates,1M HEPES buffer, Filter sterilizer, test tubes, test tube stand, autoclave, MillQ water, rubber bands, sterilized dissection kit, a nylon filter, sterile cotton,70% alcohol, trypsin, PBS, L15 media , MEM media, magnetic stirrer, magnetic beads, antibiotics, Laminar airflow, microscope, spectrophotometer and ALT and AST kits (AST Product number HTBCOO8 and ALT Product number HTBCOO8).

PLATE 1- Materials and equipment used for establishing primary cell culture.

Autoclave

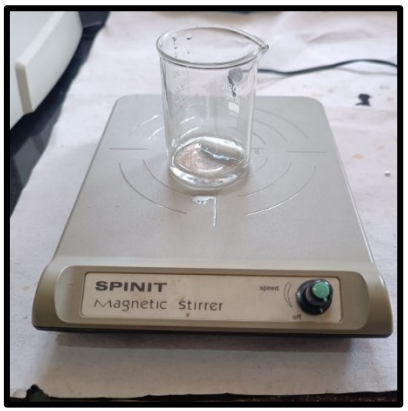
Hot Air Oven

Laminar Air

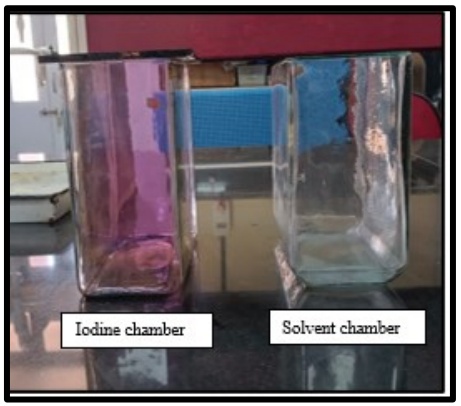


Filtration Unit

Filtration Pump



Magnetic



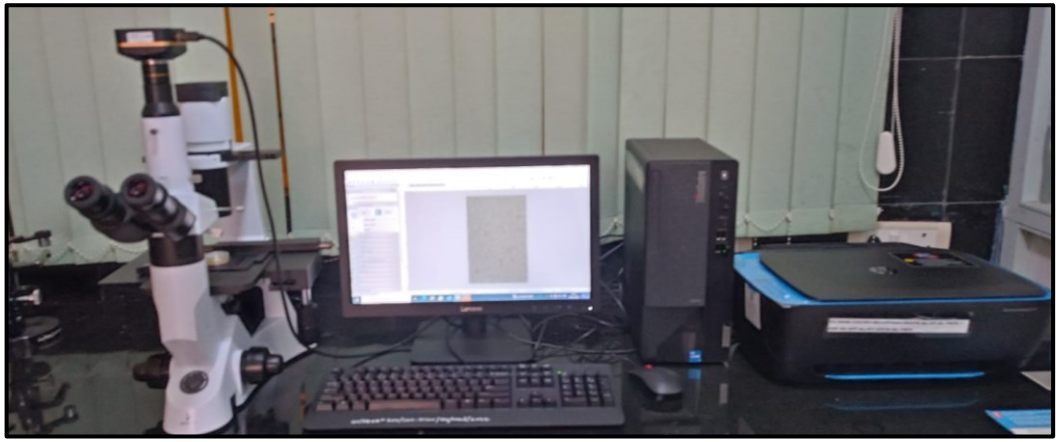
TLC Chambers

PLATE 2- Materials and equipment used for establishing primary cell culture.

Cell desiccator

Centrifuge

Vacuum rotatory evaporator



Inverted microscope with image analyzer-iox 105s

2.2. Preparation of laminar airflow for cell culture

All the internal surfaces of the laminar airflow chamber were wiped with 75% of ethanol followed by sterilization of the chamber by closing the glass shield and switching on the UV light for 15 minutes. Then, the blower was switched on for 15 minutes before performing the experiment. All necessary articles needed for the culture were wiped with 75% alcohol before placing them inside the laminar airflow (Plate 3).

2.3. Preparation of Culture media

Leibovitz's L15 medium (1.5x): 2.74 grams of L15 media powder was dissolved in 200 ml of distilled water as per the instruction provided on instructions accompanied with media powder bottle. After this pH was adjusted and the medium was filter sterilized under laminar air flow.

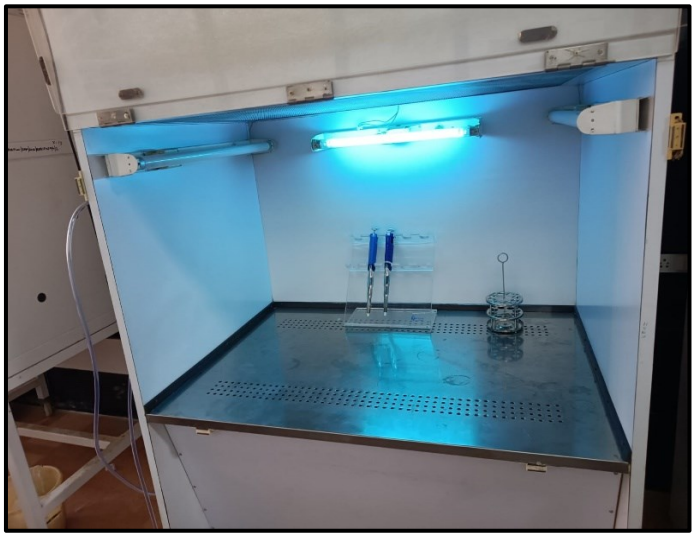
Minimum Essential Medium (MEM medium) (1.5x): 2.34 grams of MEM medium powder was dissolved in 200 ml of distilled water as per the instruction provided on the instructions accompanied by a media powder bottle. This media was supplemented with 5 mM of HEPES. After this pH was adjusted and the medium was filter sterilized under laminar airflow.

2.4. Hepatocyte isolation and establishment of primary cultures

The study was done on the liver of *Rastrelliger kanagurta* from which the hepatocytes were isolated. The fish sample was freshly collected on the day of experimenting and the cells were isolated for cell culture. The work surface and the instruments were sterilized with 75% alcohol after which it was left under UV rays for at least 15 minutes. The liver tissues of the fish sample were removed under aseptic conditions and were transferred to a Petri plate containing phosphate buffer saline (PBS) having 0.5% of the antibiotic solution, then the tissue was cut into small fragments of ~2.0 mm^3 (Plate 4). For this study, tissue pieces were subjected to various methods for the establishment of primary cell cultures such as explant and free cell

PLATE 3- Preparation of laminar air flow for establishing primary cell culture.

Wipe the laminar air hood with 75% ethanol



Close the glass window and Turn on the UV light for 15 minutes and

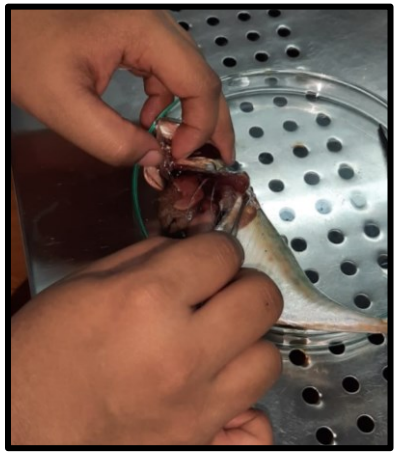
Turn off the UV light and place the sterile chemicals and glassware inside the laminar air flow

PLATE 4 -Dissection of the fish and isolation of the liver for the establishment of primary cell culture and other analyses

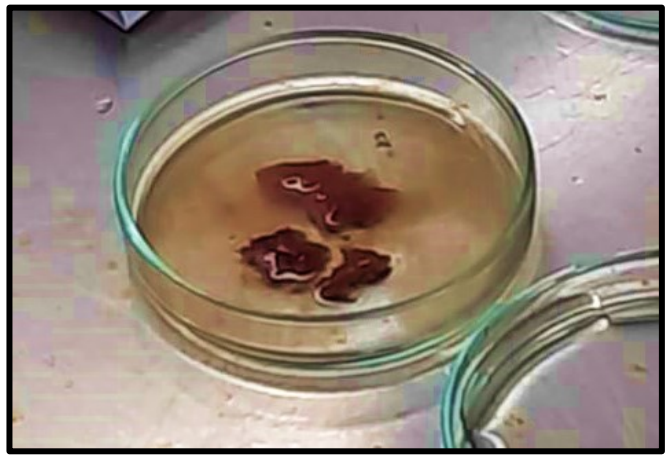
STEP 1: Place the fish in the petri plate

STEP 2: Make an incision near the anal region up to the gill region

STEP 3: Expose the peritoneal and pericardial cavities in the cranial region



STEP 3: Take out the organs obstructing the liver



STEP 4: Remove the exposed liver with the help of blunt forceps

culture (using mechanical, and enzymatic dissociation techniques). All these procedures were performed in a sterile environment using Laminar flow hoods.

Primary explant culture: All the tissue pieces were placed on the sterile slide, and with the help of a sharp sterile surgical blade, the tissue was cut into pieces with dimensions of 2 mm. These fine pieces were then rinsed with phosphate-buffered saline (PBS) supplemented with 0.5% antibiotic-antimycotic solution. For inoculation, the working media along with antibiotics was added. Petri dishes were then covered and sealed with parafilm, labelled, and then viewed under a microscope to check the cell's condition and contamination if any. Only non-contaminated Petri dishes were transferred to a cell incubator chamber at room temperature (Plate 5).

Primary free cell culture using mechanical dissociation: Tissue fragments were kept in 3ml of PBS for 15 minutes and they were then triturated with 10 Strokes using a 10 mL pipette to accomplish dissociation of cells from tissue pieces. The dissociated cell suspension thus obtained was filtered through a nylon mesh filters (100 µm pore size) and centrifuged at 1000 rpm for 10 minutes. After the first centrifugation, the cell suspension was washed with streptomycin antibiotics and was centrifuged once more for 15 minutes. The cell suspension was resuspended in PBS and finally in basal media. This step was repeated twice for mechanical dissociation. The pelleted cells were inoculated in two different prepared media, L15 (Leibovitz's) and MEM with 6 mM of HEPES buffer. Both media were supplemented with 0.5% antibiotic-antimycotic solution and the pH was adjusted to 7 to 7.8. The cell count of the cell suspension was checked using a Neubauer chamber/ hemocytometer slide. Seeding/inoculating cell density was adjusted to 3 x 10^5 cells/ml. Cultured cells were examined daily with an inverted microscope to observe the condition of the culture. All the cultures for experimentation were performed in triplicates (Plate 6).

Plate 5- Flowchart for setting up of Primary Explant culture

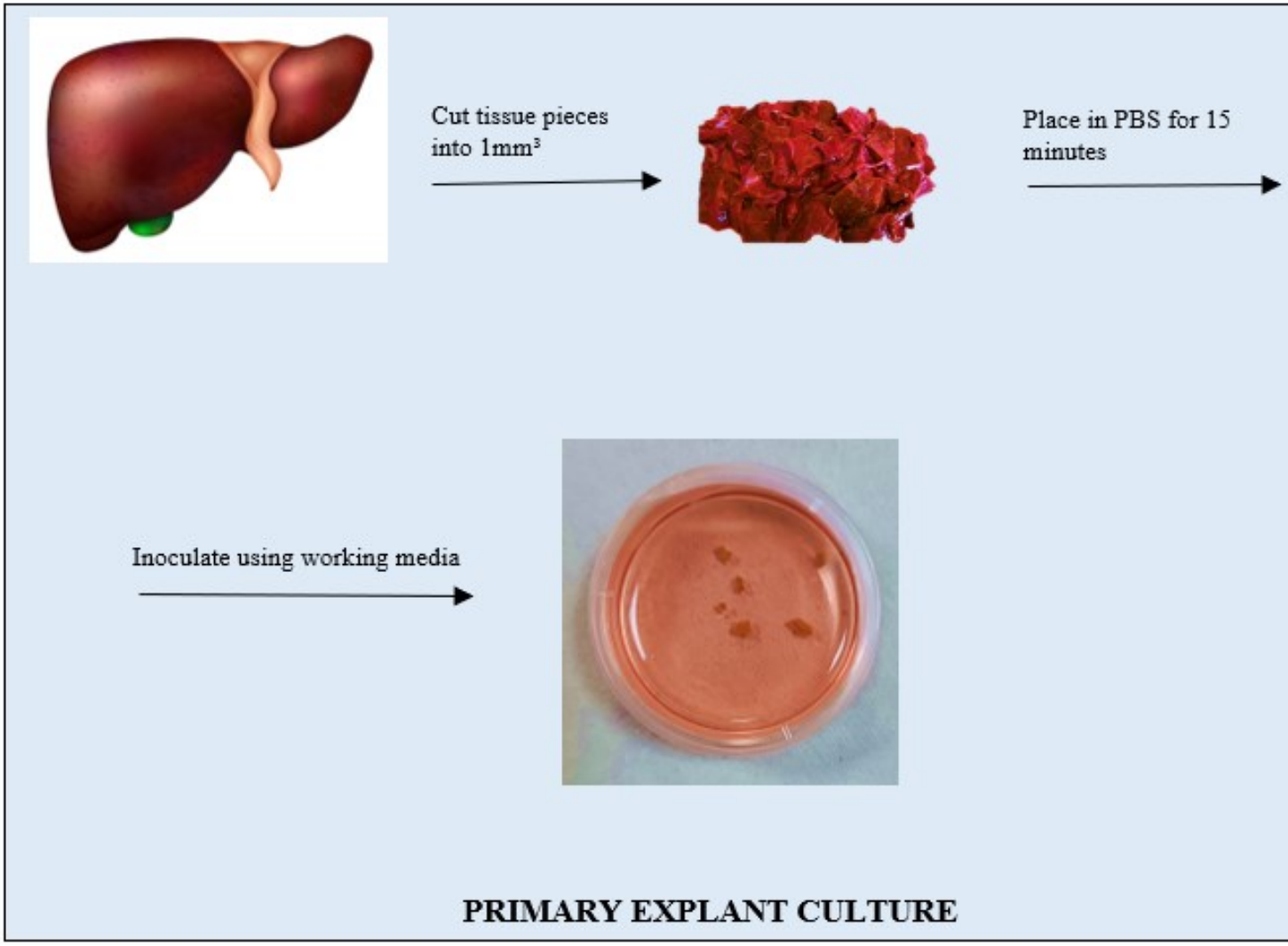
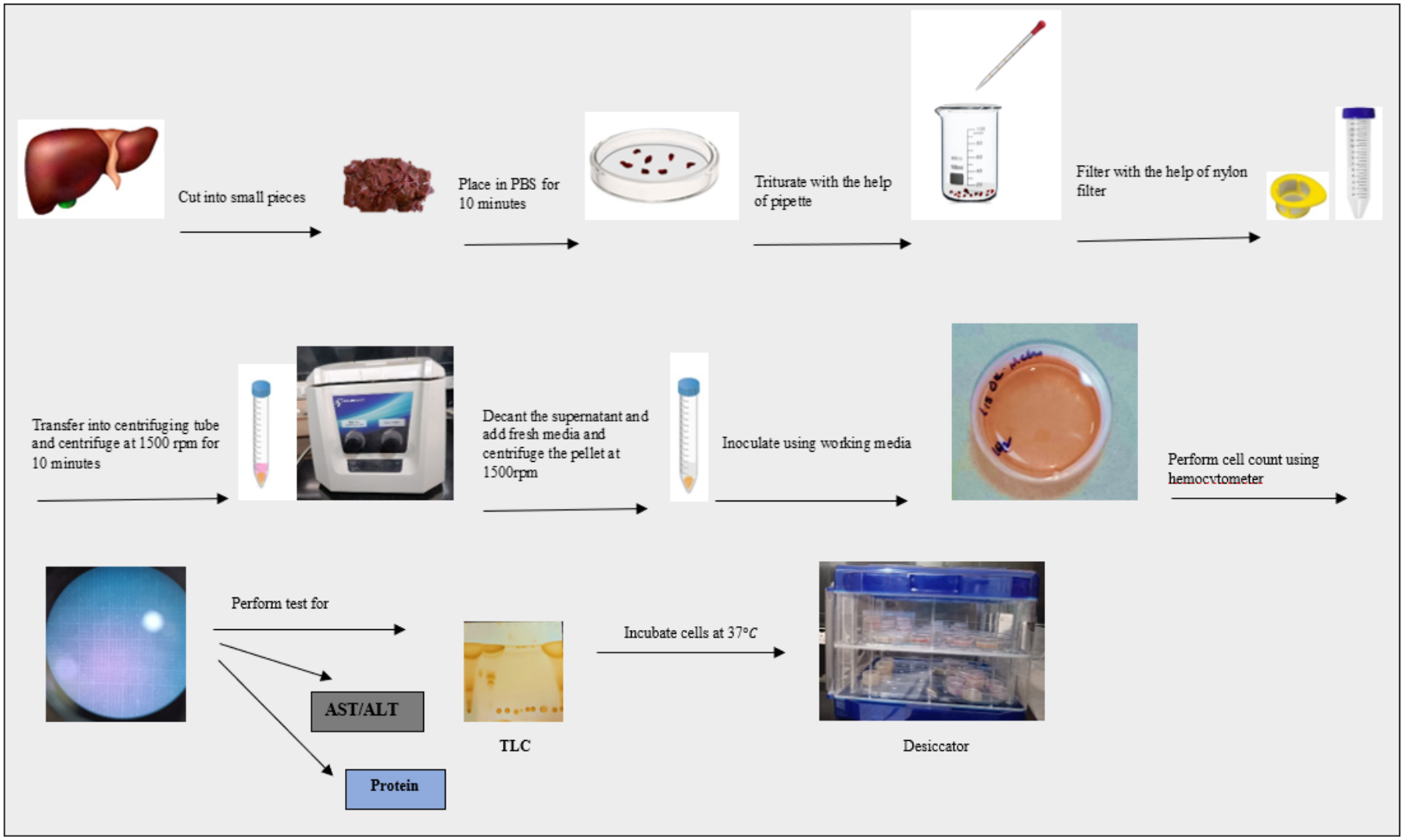


Plate 6- Flowchart for setting up Primary Free cell culture using Mechanical dissociation method.

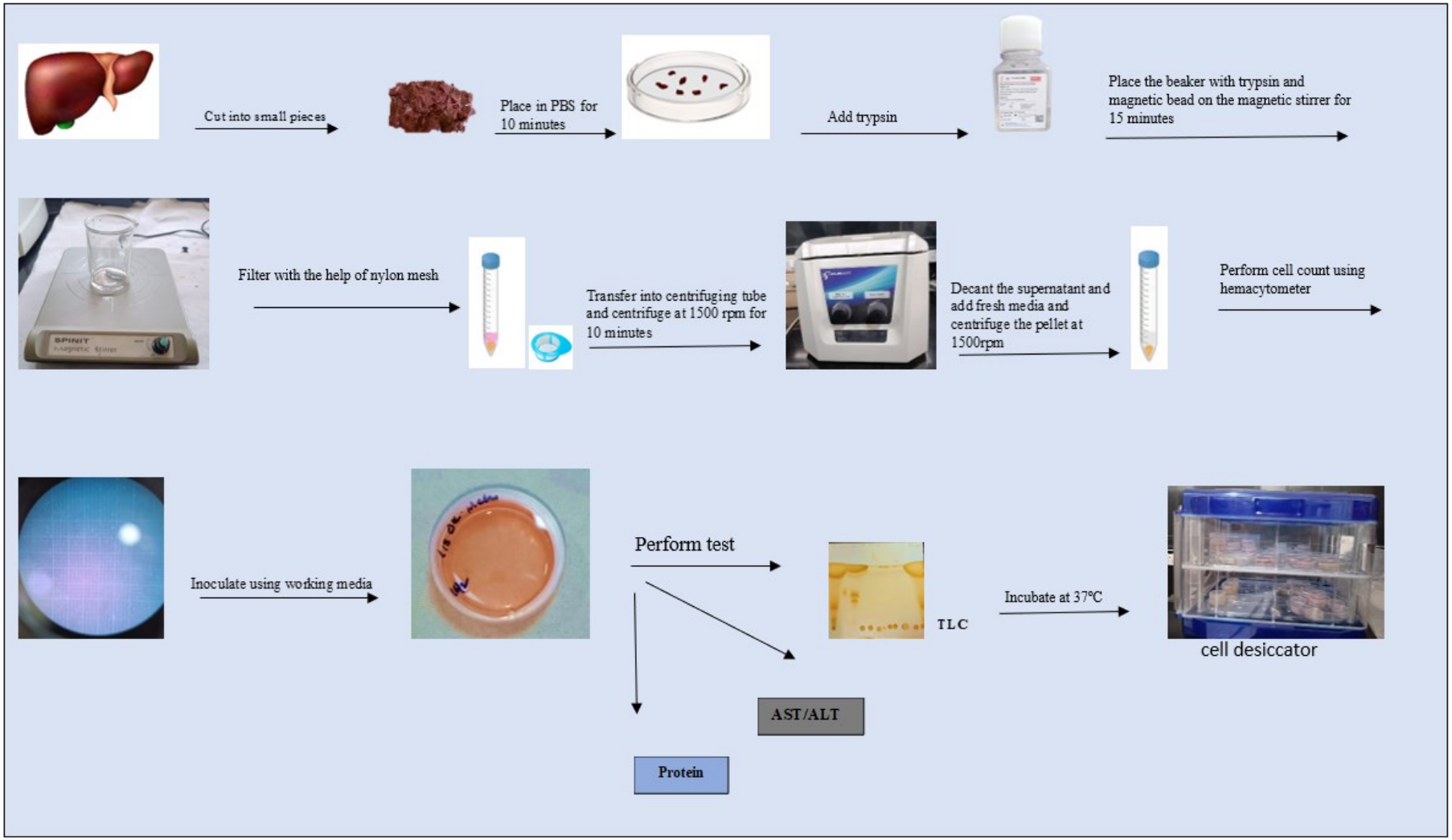


Primary free cell culture using enzymatic dissociation (trypsinization): The tissue pieces were first placed in PBS for 15 minutes and were then placed in trypsin to dissociate the cells from the cell matrix and to digest the unwanted tissue. The beaker containing trypsin was placed on the magnetic stirrer along with the sterilized magnetic bead. The dissociated cell suspension thus obtained was filtered through a nylon mesh (100 µm) and centrifuged at 1000 rpm for 15 minutes. After the first centrifugation, the cell suspension was washed with streptomycin antibiotics and was centrifuged once more for 15 minutes. The cell suspension was resuspended in PBS and finally in basal medium. The pelleted cells were inoculated in two different prepared media, L15 and MEM with 6 mM of HEPES buffer. Both media were supplemented with 0.5% antibiotic-antimycotic solution and the pH was adjusted to 7 to 7.8. The cell count of the cell suspension was checked using a Neubauer chamber/ hemocytometer slide using the trypan blue dye exclusion technique. Seeding/inoculating cell density was adjusted to 3 x 10^5 cells/ml. Cultured cells were examined daily with an inverted microscope to observe the condition of the culture. All the cultures for experimentation were performed in triplicates (Plate 7).

2.5. Cell Morphometry

Petri dishes inoculated with cells were viewed under inverted microscopes. Various cells in the primary culture at the time of inoculation were noted down with the help of image analyzer. Hundreds of each cell types were screened for their measurements for either diameters or their lengths. Hepatocytes were also processed for Giemsa staining**.** To stain the cells, cells were harvested and suspended in a culture medium by pipetting. Cell suspension was then centrifuged at 100 rpm for 10 minutes. Cell pellet was now dispersed in small amount of fixative and drop of the cell suspension was placed in the centre of glass slide. Drop spread instantaneously. Slides were then air-dried and labelled before staining. Slides were then stained in Giemsa stain for 10 minutes. Slides were then rinsed in distilled water and air dried to examine the cells under light microscope. Still images of the stained cells were photographed.

Plate 7- Flowchart for setting up Primary Free cell using Enzymatic dissociation method.



To stain the monolayers Petri dishes were directly processed for staining by skipping the cell pelleting steps.

2.6. Growth rate

The growth rate (GR) of cells cultures is expressed by growth curves and population doubling time. Three independent primary free cell cultures were propagated up to 14 days to study the growth curve of cultured hepatocytes. Viable cell count was measured when the cells were initially placed in suspension at the time of primary culture and at the time of passage of the culture. The viability of cells in suspension was tested using the trypan blue exclusion test whereby a sample of media containing the cells in suspension was added to the trypan blue dye (50:50). The number of healthy cells excluding the dye per square on a 1 mm^3 was counted using an inverted microscope and a hemocytometer. This experiment was performed only once due to time constraints and only up to 14^{th} day therefore, growth curve that was generated remained in log phase only.

2.7. Total Protein assay

Total protein assay was performed using Lowry's method using Bovine serum albumin as standard. Assay consisted of Lowry's reagent along with sample/standard that was incubated for 10 min followed by addition of Folin phenol reagent. After 10 minutes of incubation at room temperature, optical density was read at 606 nm against blank.

2.8. Aspartate aminotransferase (AST) and Alanine aminotransferase (AST) assays.

AST and ALT enzyme assay was performed using kits procured from HiGenomeMB company (AST Product number HTBCOO8 and ALT Product number HTBCOO8). AST and ALT enzyme assays were measured according to 2,4-dinitrophenyl hydrazine (2,4-DNPH) method. The incubation mixture for ALT contained 0.25 ml of alanine-a-ketoglutarate, 0.05ml of enzyme extract, 0.25 ml of 2,4- DNPH and 5 ml of 0.4 N NaOH. The incubation mixture for

AST contained 0.25 of aspartate-a-ketoglutarate, 0.05 ml of enzyme extract, 0.25 ml of 2,4-DNPH and 5 ml of 0.4 N NaOH. The optical density of corresponding brown-colored hydrazone formed in alkaline medium was read at 505 nm.

2.9. Lipid profiling of liver tissue, from isolated hepatocytes and cultured cells

Lipid profiles were performed for liver tissue, isolated cells, and primary, secondary & tertiary cells.

Sample preparation for lipid analysis: To prepare the sample for lipid analysis, 1g of tissue/ or cell pellet was homogenized with a mixture of chloroform and methanol (1:2 v/v). The homogenate was then filtered, and the filtrate was washed with water. The resulting liquid was either filtered using filter paper or centrifuged. Next, 4 ml of distilled water was added to the filtrate to wash the solvent. The mixture was vortexed for 2 minutes and then centrifuged at 2000 RPM. This process yielded a two-phase mixture, where the upper phase was siphoned off and the lower chloroform phase was evaporated using a rotary evaporator under vacuum. The residue left after evaporation was dissolved with 5 ml of chloroform.

Lipid analyses of liver tissue using Thin layer Chromatography (TLC): Liver tissue ~2g was weighed and homogenized with chloroform: methanol (2:1) followed by a wash of the extract with aqueous sodium to remove non-lipid contaminants. Ready-made TLC plates were used which are chemically inert and stable and coated with silica of thickness 0.2 mm. A small amount of the lipid extract solution processed from the sample was spotted at the origin line on the TLC plate, using a micropipette. Multiple spots were loaded along with standards with proper spacing to avoid overlapping spots. In the Thin Layer Chromatography Chamber/ developing chamber, 80ml of Solvent hexane: diethyl ether: glacial acetic acid (80; 20:1) (Søfteland & Olsvik, 2022) was added. TLC plates were then placed in developing chambers and then sealed with the glass lid to prevent solvent evaporation and to keep the entire process

dust-free. After 2 hours, the TLC plates were removed and kept for drying. After drying the plates were placed in the iodine-containing chamber for 30 minutes. The separated lipid components can be identified and quantified by measuring the distance travelled by each component relative to the origin line and comparing it to the distances travelled by known lipid standards run on the same TLC plate. The following formula was used to calculate the R_f value,

$$R_f = \frac{\text{Distance travelled by the substance from reference line (cm)}}{\text{Distance travelled by the solvent front from reference line (cm)}}$$

2.10. Statistical Analysis.

All experiments were performed in triplicates. Representation of the data in table and graph are mean ± standard deviation that was calculated using Microsoft Excel software.

CHAPTER III

RESULTS

Results

3.1 Cell Morphometry

In the fish liver, the cell size can vary depending on the species, age, and health of the fish, as well as the specific culture conditions used. However, here are the different types of cells found in the primary cultures that we established from the fish liver and they include abundant Hepatocytes, few Kupffer cells, rarely Stellate cells, Endothelial cells of blood vessels, and Epithelial cells of the bile duct. Explant primary culture showed all these cell types but primary free cell cultures using mechanical and trypsinization procedures lacked the presence of endothelial cells of blood vessels and epithelial cells of the bile duct and marked rare citing of Kupffer cells and Stellate cells.

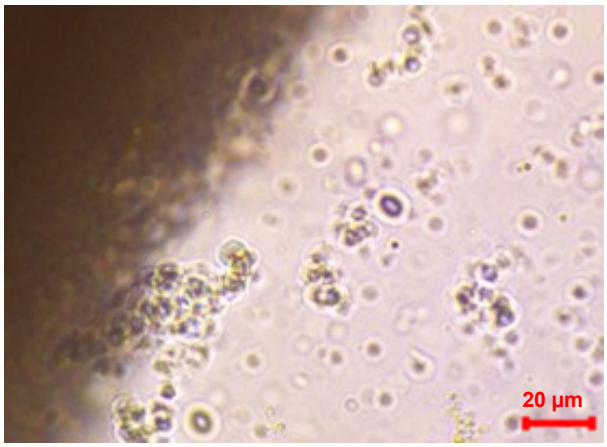
Cells that were migrating from the explant showed various cell shapes such as cuboidal, oval and rounded. Whereas the hepatocytes observed in primary free cell cultures were either rounded or oval and cell diameters ranged from 1.3 to 3.9 µm. The hepatocytes in cultures that were nearing monolayer formation were rounded and their diameter range was 3.5 -5µm. The cultures after the formation of monolayer showed the polygonal cells with large nuclei and peripheral cytoplasm with dense contents and ranged in their diameter as 3.5-5µm and this was also evident from Giemsa-stained Petri dishes with established monolayers (Plate 8).

Overall, it was observed in the primary culture soon after seeding or inoculation and the migrated hepatocytes from cultures showed an increase in cell size when the cells were nearing the monolayer formation and when the monolayer was formed.

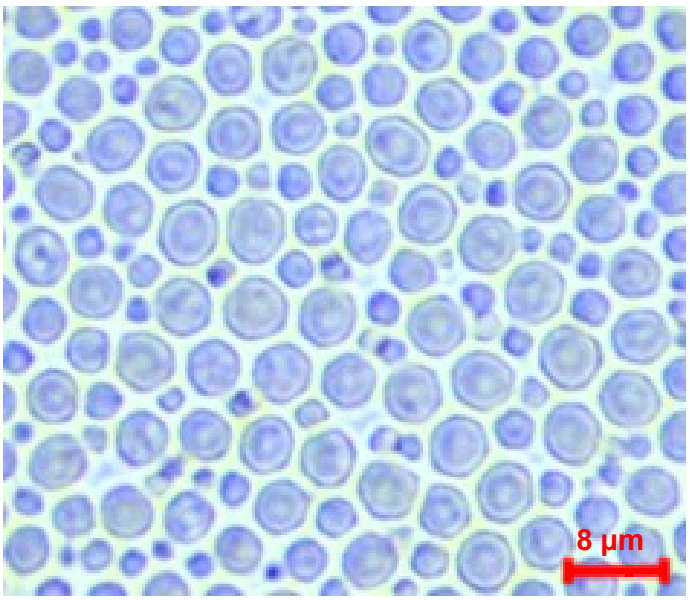
3.2. Primary culture establishment

Explant cultures showed repeated contaminations by the end of weeks' time after the establishment of culture, suggesting that primary explant culture cannot be considered as the method for establishment of hepatocyte culture. It also showed presence of other types of cells

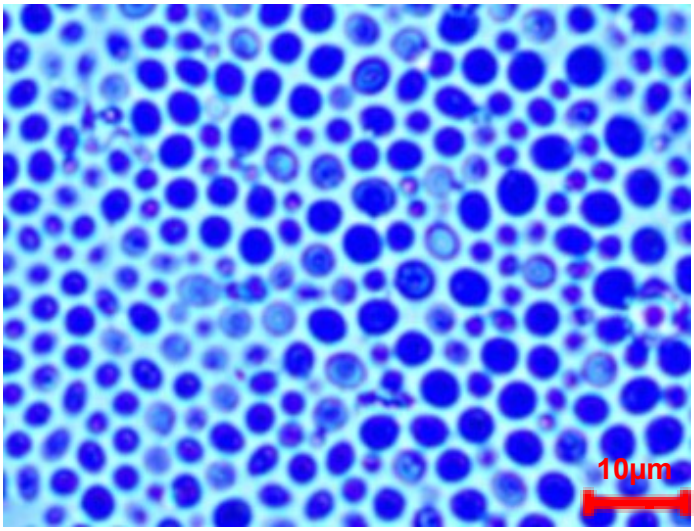
PLATE 8 **(A) Primary explant culture on day 0 (40x)**



(B) Unstained hepatocyte monolayer formed through free cell cultures



(C) Giemsa-stained hepatocyte monolayer formed through free cell cultures



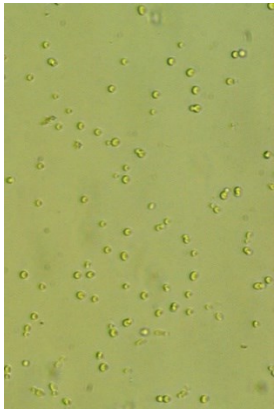
along with hepatocytes. Thus, predominance of the hepatocyte cell density will not be achieved to allow the formation of a monolayer in due course.

On day 0 (initiation of primary culture), Day 1(24 hours), day 2 (48hours) of primary cultures no proliferation of cells was seen. It was also observed that the explant cultures of both L15 and MEM mechanical and enzymatic got contaminated during the first week. Cell proliferation was observed for MEM mechanical culture on day four which was indicated by the increase in cell density from 3.9×10^5 to 8.2×10^5 3. While for Mem enzymatic (Trypsin) cultures no cell proliferation was seen at day 3. For L15 enzymatic (trypsin) cultures proliferation of cells was seen on day one, while for L15 mechanical cultures the cell proliferation was seen on day 3. It was also observed that L15 mechanical and enzymatic media cultures showed proliferation after which formation of monolayer was seen faster as compared to MEM mechanical and enzymatic media cultures (Plate 9-12). After the second passage especially L15 enzymatic cultures showed more contamination as compared to L15 mechanical. Better and faster growth was observed for MEM mechanical as compared to MEM enzymatic, L15 mechanical and, L15 enzymatic. After day 3 formation of Monolayer was observed for MEM mechanical and enzymatic cultures, whereas for L15 enzymatic monolayer formation was seen after Day 2 and for mechanical monolayer formation was observed after day 3. Major contamination was seen in L15 enzymatic cultures.

3.3 Growth rate

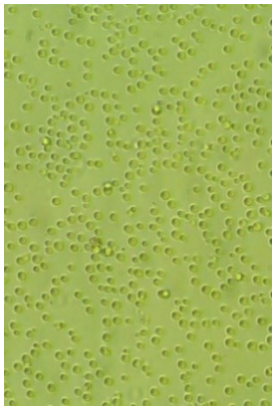
Growth curves (Figure 1.) were derived from the respective cell cultures such as cultures derived from the mechanical dissociation method and MEM culture medium (MECH-MEM), the trypsinization dissociation method and MEM culture medium (ENZ-MEM), the mechanical dissociation method and L-15 culture medium (MECH-L-15), and the trypsinization dissociation method and L-15 culture medium (ENZ-L-15). Growth curve thus derived shows different lag phases for different types of culturing systems and methods used as mentioned

PLATE 9 – Day-wise Progression of hepatocyte culture established with Primary Free cell culture using Mechanical dissociation method and MEM as a basal culture medium (40x).

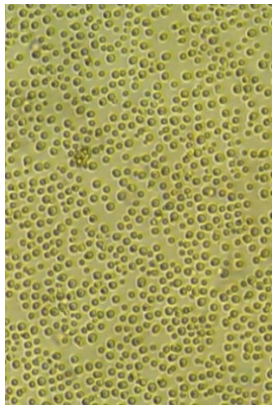


Day 1

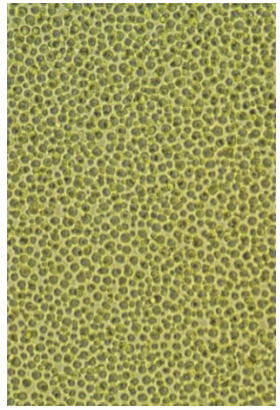
Day 2.



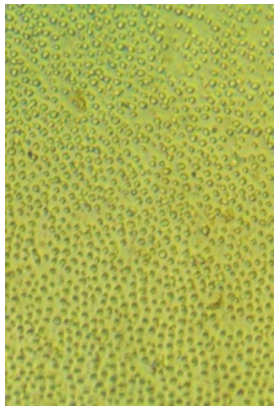
Day 3.



Day 4.

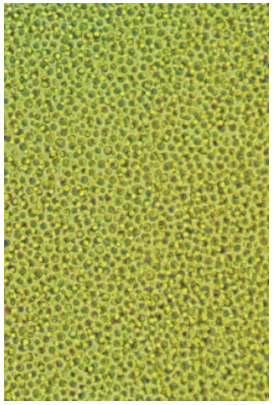


Day 8.



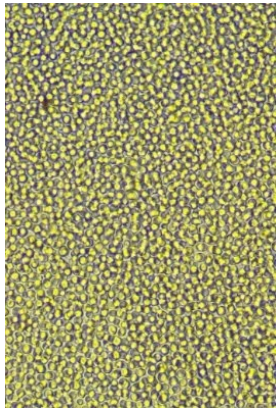
Day 9

Day 10

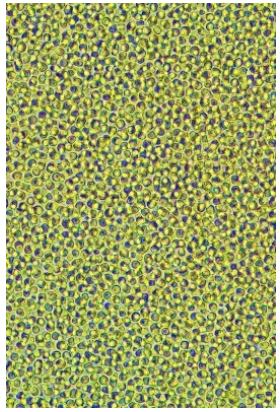


Day 11

Day 12



Day 13



Day 14

PLATE 10 – Day-wise Progression of hepatocyte culture established with Primary Free cell culture using trypsinization dissociation method and MEM as a basal culture medium (40x).

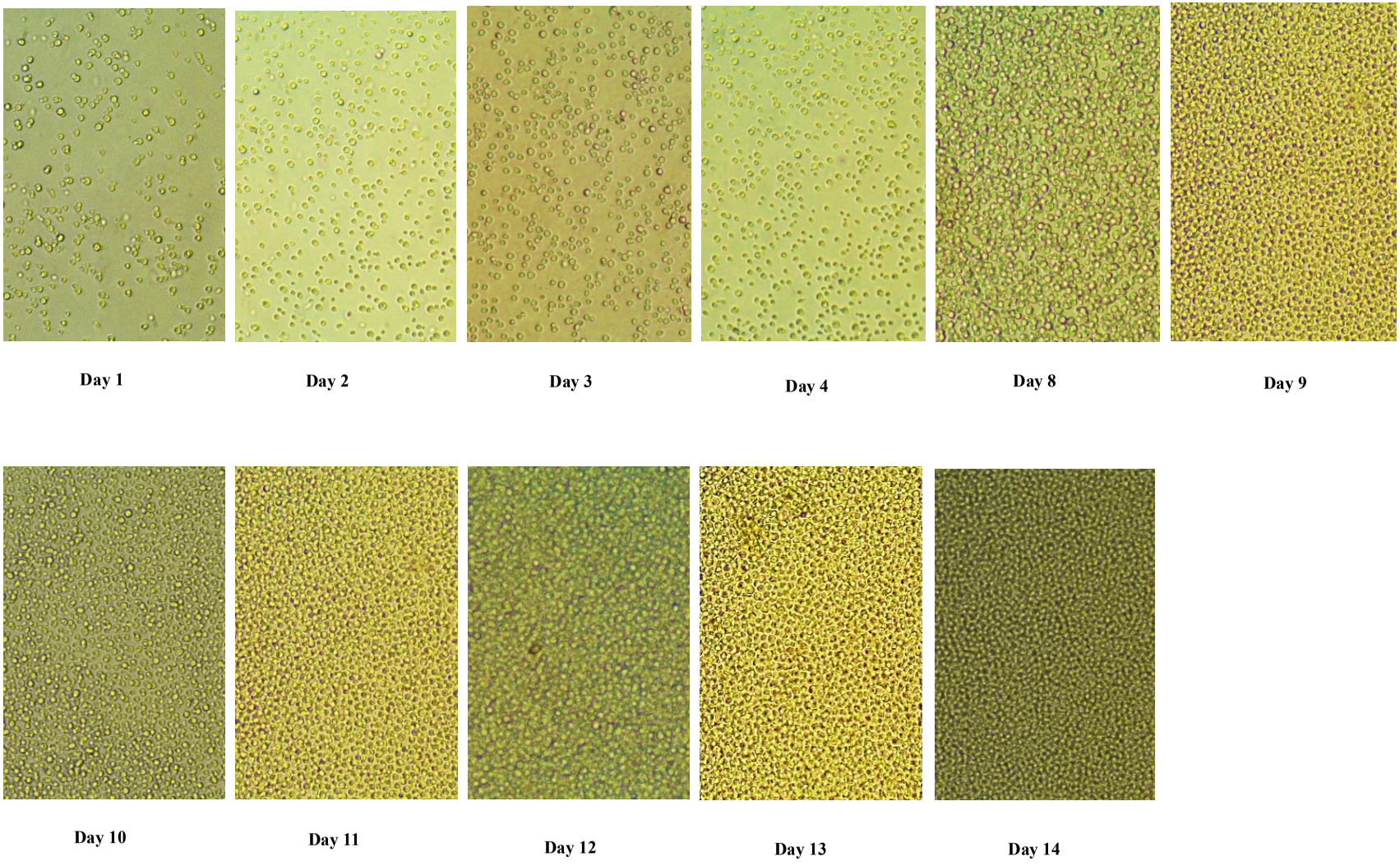
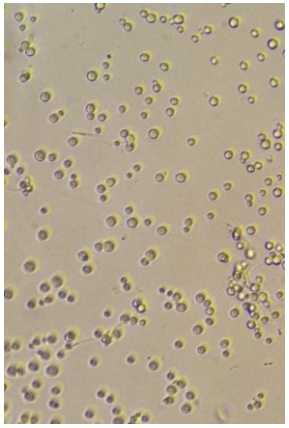
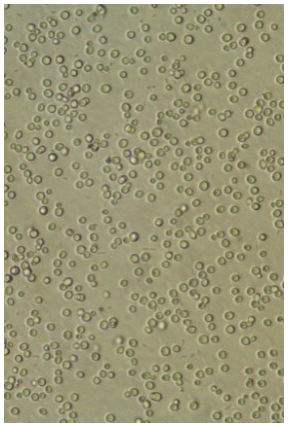


PLATE 11 – Day-wise Progression of hepatocyte culture established with Primary Free cell culture using Mechanical dissociation method and L15 as a basal culture medium (40x).

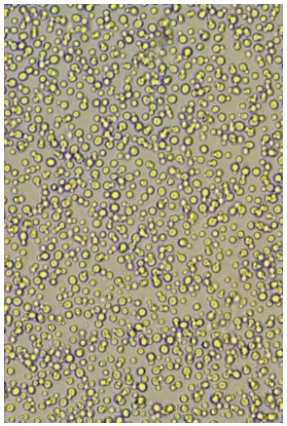


Day 1

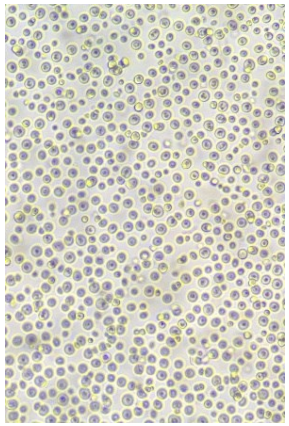
Day 2



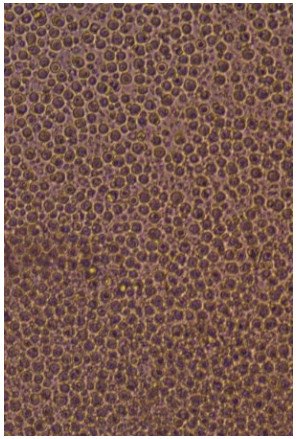
Day 3



Day 4



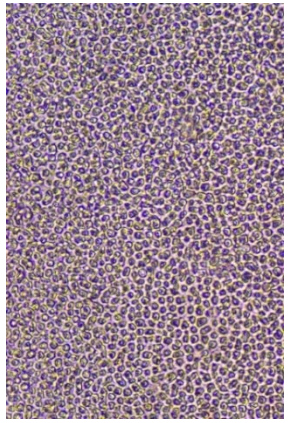
Day 8



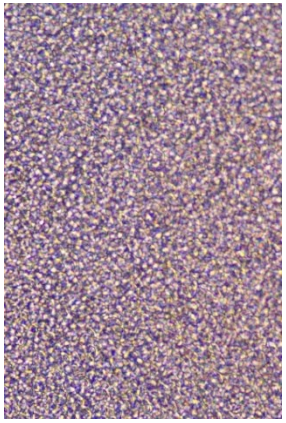
Day 9

Day 10

Day 11

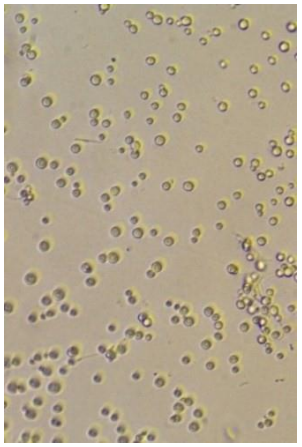


Day 12



Day 13

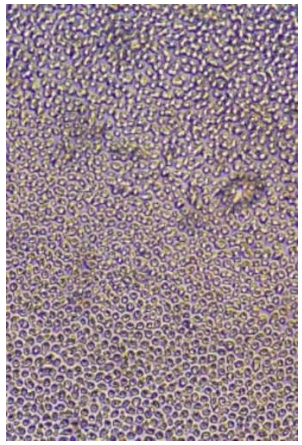
PLATE 12 – Day-wise Progression of hepatocyte culture established with Primary Free cell culture using Trypsinization dissociation method and L15 as a basal culture medium (40x).



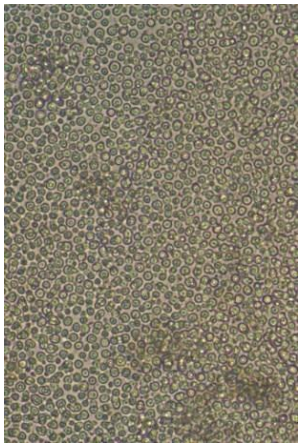
Day 1

Day 2

Day 3



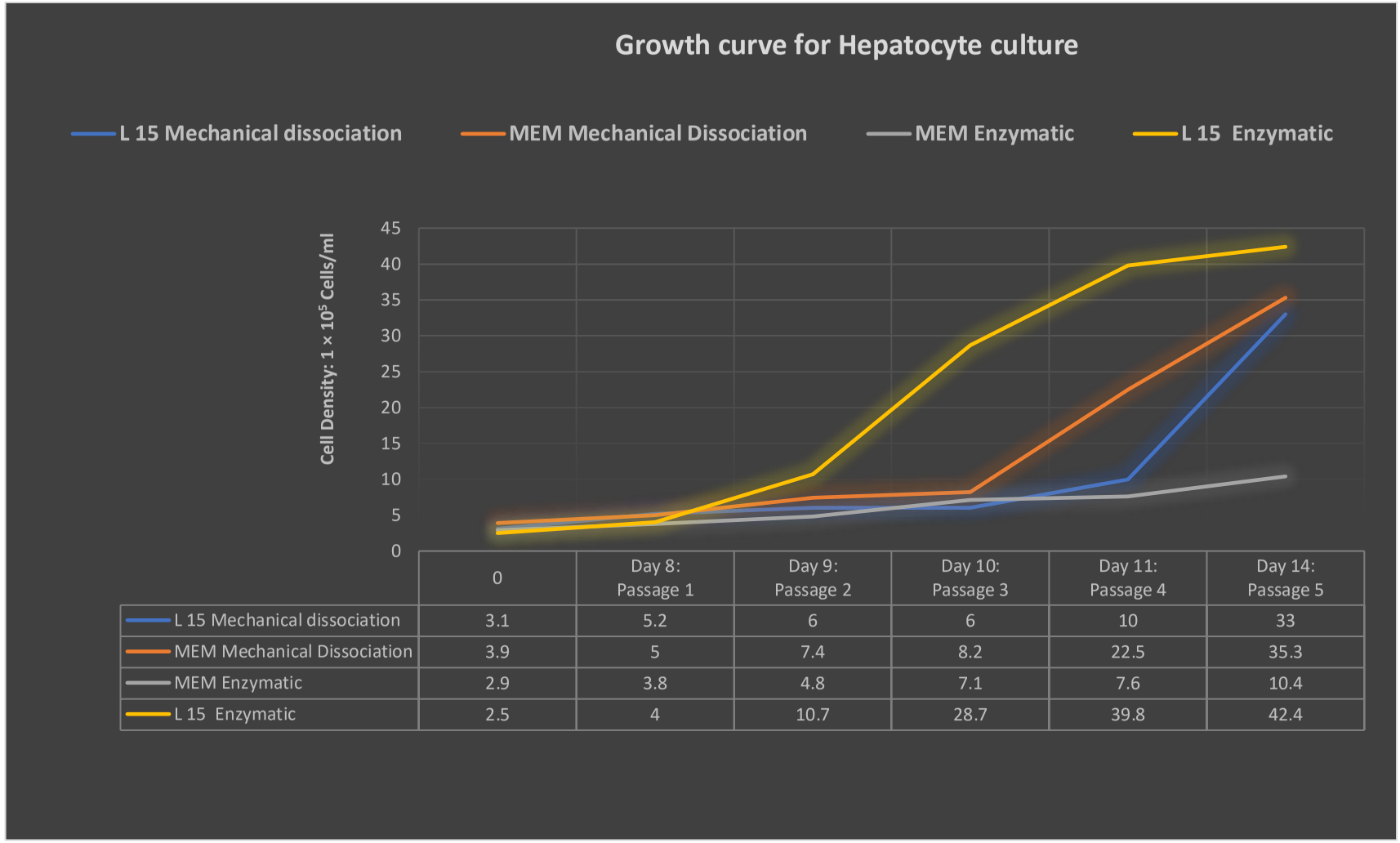
Day 4



Day 8

Day 9

Figure 1. Growth curve of cultured hepatocytes derived from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media.



	0	Day 8: Passage 1	Day 9: Passage 2	Day 10: Passage 3	Day 11: Passage 4	Day 14: Passage 5
L 15 Mechanical dissociation	3.1	5.2	6	6	10	33
MEM Mechanical Dissociation	3.9	5	7.4	8.2	22.5	35.3
MEM Enzymatic	2.9	3.8	4.8	7.1	7.6	10.4
L 15 Enzymatic	2.5	4	10.7	28.7	39.8	42.4

above. Lag phase for MECH-MEM was from day 0 to day 10, for MECH- L-15 it was from day 0 to day 11 , for ENZ-MEM lag phase was from day 0 to 11 day and for ENZ-L-15 it was from day 0 to day 8. Since the culture was studied after 14 for growth curve accomplishment, collected data focuses only the lag and log phase and plateau phase is yet to be attained after Day 14.

3.4. Total Protein assay

Total protein was assayed for its content in the homogenates of cells that were cultured through various methods. The total protein remained almost the same for all culture types undertaken for this study from the day of initiation to day 22 without passaging the cells and only media replacement was done (Table 1 and Figure 2).

3.5. Aspartate aminotransferase (AST) and Alanine aminotransferase (AST) assays.

Aspartate and alanine aminotransferase assays were performed for homogenates derived from hepatocytes of all types of cultures. These cultures were not passaged but were given the media change on alternate days. ALT enzyme activities remained almost in the similar range till seventh day but later showed significant increments at 15 days and 22 days. AST enzyme activities remained almost in a similar range till third-day but later showed significant increments at 7^{th} 15^{th} and 22^{nd} day (ALT: Table 3 and Figure 4; AST: Table 4 and Figure 5).

3.6. Lipid profiling of liver tissue, from isolated hepatocytes and cultured cell

Lipid profiling of liver tissue, from isolated hepatocytes and cultured cells was carried out. Thin layer chromatographic separation was performed to quantify PUFA, MUFA and cholesterol. Hepatocytes derived for the analyses were fours (0 hours (Day 0), 24 hours (Day 1) , 48 hours (Day 2), Day 7, Day 15, and Day 22. Spots for only PUFA were seen for day zero hepatocyte culture samples on its TLC Plate (Table 5 and Plate 13-15). Spots for MUFFA were seen on day 15^{th}. While spots for cholesterol were never observed.

Table 1. The total Protein content of cultured hepatocytes from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media. MECH-MEM denotes the mechanical dissociation method and MEM culture medium, ENZ-MEM denotes the trypsinization dissociation method and MEM culture medium, MECH-L15 denotes the mechanical dissociation method and L15 culture medium, ENZ-MEM denotes the trypsinization dissociation method and L15 culture medium.

Culture period	Cell Dissociation Method-Culture Medium			
	MECH-MEM	ENZ-MEM	MECH-L15	ENZ-L15
0 Day	285.86 ±15.88	279.09 ±11.05	272.63 ±14.57	242.63 ±27.42
1 Day	262.15 ±50.56	266.56 ±5.42	285.86 ±9.95	255.48 ±36.57
2 Days	272.53 ±14.77	273.23 ±6.62	289.68 ±12.71	268.23 ±19.39
3 Days	269.14 ±14.33	272.85 ±4.06	294.41 ±18.54	288.17 ±21.19
7 Days	283.82 ±19.24	280.59 ±4.00	300.86 ±17.20	276.61 ±4.55
15 Days	284.19 ±18.45	278.49 ±5.90	296.13 ±9.16	295.27 ±6.76
22 Days	289.57 ±25.02	285.97 ±13.72	291.24 ±3.54	305.22 ±10.70
All values represented are Mean ± Standard deviation				

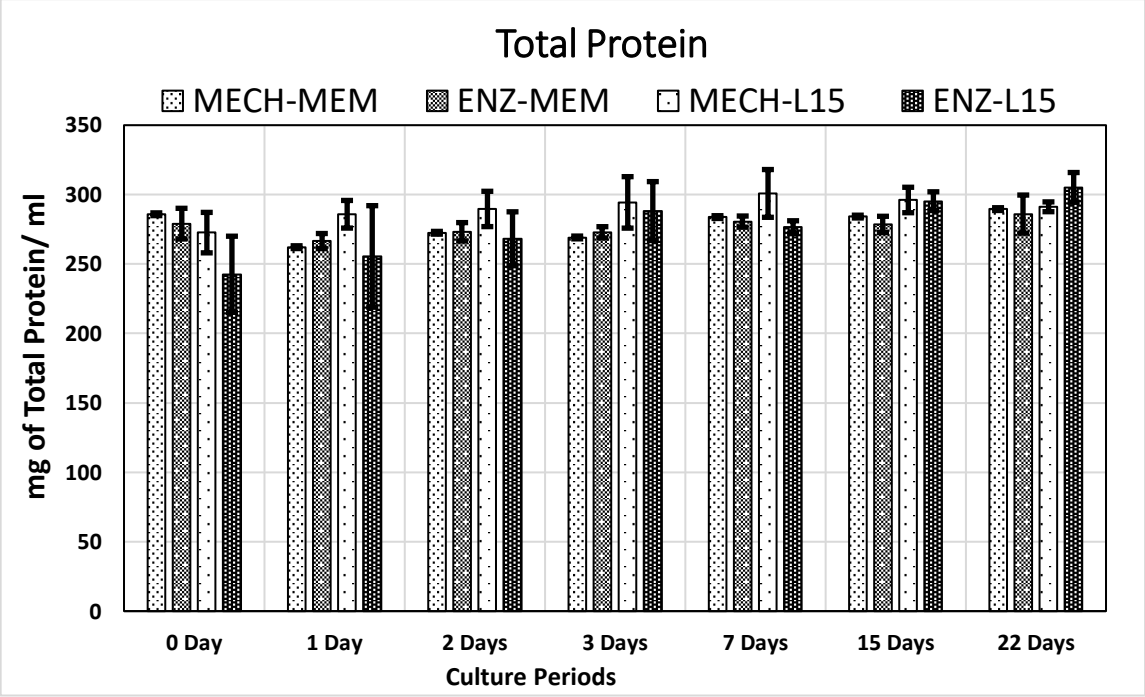


Figure 2. The total Protein content of cultured hepatocytes from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media. All values represented are Mean ± Standard deviation.

Table 2. Alanine aminotransferase activity of cultured hepatocytes from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media. MECH-MEM denotes the mechanical dissociation method and MEM culture medium, ENZ-MEM denotes the trypsinization dissociation method and MEM culture medium, MECH-L15 denotes the mechanical dissociation method and L15 culture medium, ENZ-MEM denotes the trypsinization dissociation method and L15 culture medium.

Culture period	Cell Dissociation Method-Culture Medium			
	MECH-MEM	ENZ-MEM	MECH-L15	ENZ-L15
0 Day	106.67 ±7.22	144.17 ±11.57	145.00 ±4.33	189.17 ±7.22
1 Day	145.83 ±27.42	150.83 ±5.77	169.17 ±5.77	217.50 ±25.98
2 Days	170.00 ±28.17	168.33 ±12.33	175.00 ±11.46	195.83 ±18.76
3 Days	154.17 ±13.77	179.17 ±7.64	184.17 ±12.83	198.33 ±14.65
7 Days	195.83 ±18.93	184.17 ±14.65	206.67 ±13.77	210.83 ±12.83
15 Days	490.00 ±11.46	466.67 ±24.66	315.00 ±19.53	310.83 ±14.65
22 Days	537.50 ± 8.66	555.83 ±28.10	521.67 ±23.23	607.50 ±43.80
All values represented are Mean ± Standard deviation				

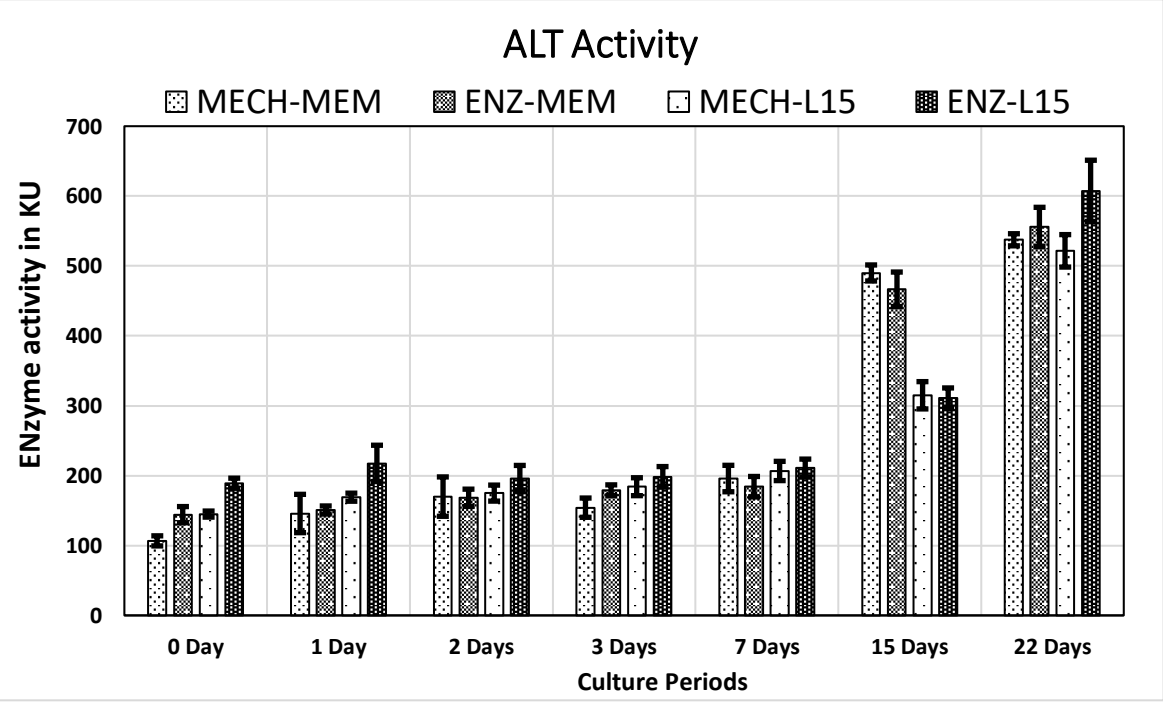


Figure 3. Alanine aminotransferase activity of cultured hepatocytes from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media. All values represented are Mean ± Standard deviation.

Table 3. Aspartate aminotransferase activity of cultured hepatocytes from *Rastregiller kangurta* for culture periods using cell dissociation methods and culture media. MECH-MEM denotes the mechanical dissociation method and MEM culture medium, ENZ-MEM denotes the trypsinization dissociation method and MEM culture medium, MECH-L15 denotes the mechanical dissociation method and L15 culture medium, ENZ-MEM denotes the trypsinization dissociation method and L15 culture medium.

Culture period	Cell Dissociation Method-Culture Medium			
	MECH-MEM	ENZ-MEM	MECH-L15	ENZ-L15
0 Day	101.67 ±18.43	130.42 ±23.20	146.67 ±16.07	169.17 ±31.75
1 Day	122.00 ±17.20	149.17 ±29.83	160.00 ±22.29	150.00 ±40.23
2 Days	140.83 ±10.10	168.08 ±14.11	175.83 ±13.77	162.50 ±31.32
3 Days	160.00 ±32.69	177.33 ±15.42	190.50 ±14.83	194.42 ±8.39
7 Days	151.77 ±31.66	230.83 ±60.23	178.33 ±18.09	247.50 ±51.05
15 Days	182.75 ±23.24	106.67 ±23.23	170.67 ±25.00	220.00 ±22.22
22 Days	200.00 ±19.84	153.33 ±23.76	198.33 ±27.65	286.67 ±27.54
All values represented are Mean ± Standard deviation				

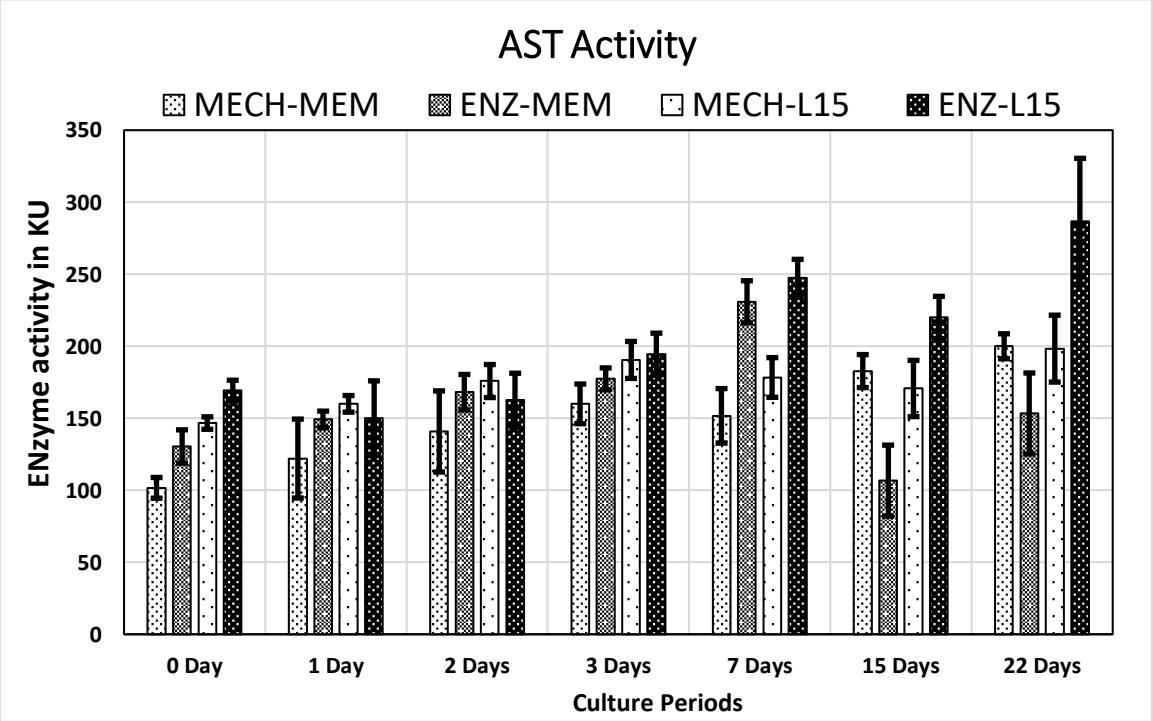


Figure 4. Aspartate aminotransferase activity of cultured hepatocytes from *Rastregiller kangurta* for different culture periods using cell dissociation methods and culture media. All values represented are Mean ± Standard deviation.

Table 4. Hepatocyte Lipid analyses data with Rf values for separated spots developed on TLC plates.

No. of DAYS	PUFFA (TLC) MEM enzymatic	PUFFA (TLC) MEM enzymatic (media)	PUFFA (TLC) MEM mechanical	PUFFA (TLC) MEM mechanical (Media)	MUFFA (TLC) MEM enzymatic (media)	MUFFA (TLC) MEM enzymatic	MUFFA (TLC) MEM mechanical	MUFFA (TLC) MEM mechanical (media)	CHLOESTROL (TLC) for MEM mechanical and enzymatic
0 hours	No spots	No spots	No spots	No spots	No spots	No spots	0.514	No spots	No spots
DAY 1	No spots	No spots	No spots	No spots	No spots	No spots	No spots	No spots	No spots
DAY 2	No spots	No spots	No spots	No spots	No spots	No spots	No spots	No spots	No spots
DAY 3	No spots	No spots	0.843	No spots	No spots	No spots	0.514	No spots	No spots
DAY 7	0.864	0.9332	0.8645	0.9548	0.8645	No spots	No spots	0.8580	No spots
DAY 15	0.8926	0.9982	0.929	0.9614	0.8926	No spots	0.518	0.876	0.7560
DAY 22	0.871	0.9871	0.872	0.9756	No spots	No spots	No spots	No spots	No spots

Plate 13- Developed TLC plates with lipid spots of hepatocyte samples for o hr, 24 hrs and 48 hrs cell cultures.

A) O Hours

L- Linoleic acid

C-Cholesterol

G-Gee

S-Sample

B) 24 Hours

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

ESL-Explant L15

ESM-Explant MEM

ML-Mechanical L15

MM-Mechanical MEM

EL-Enzymatic L15

C) 48 Hours

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

EM-Enzymatic MEM

ESL-Explant L15

ESM-Explant MEM

ML-Mechanical L15

MM-Mechanical MEM

EL-Enzymatic L15

Plate 14- Developed TLC plates with lipid spots of hepatocyte samples of 3 days and 7 days cultures.

3 days

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

EM-Enzymatic MEM

ESL-Explant L15

ESM-Explant MEM

ML-Mechanical L15

MM-Mechanical MEM

EL-Enzymatic L15

7 Days

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

EM-Enzymatic MEM

ESL-Explant L15

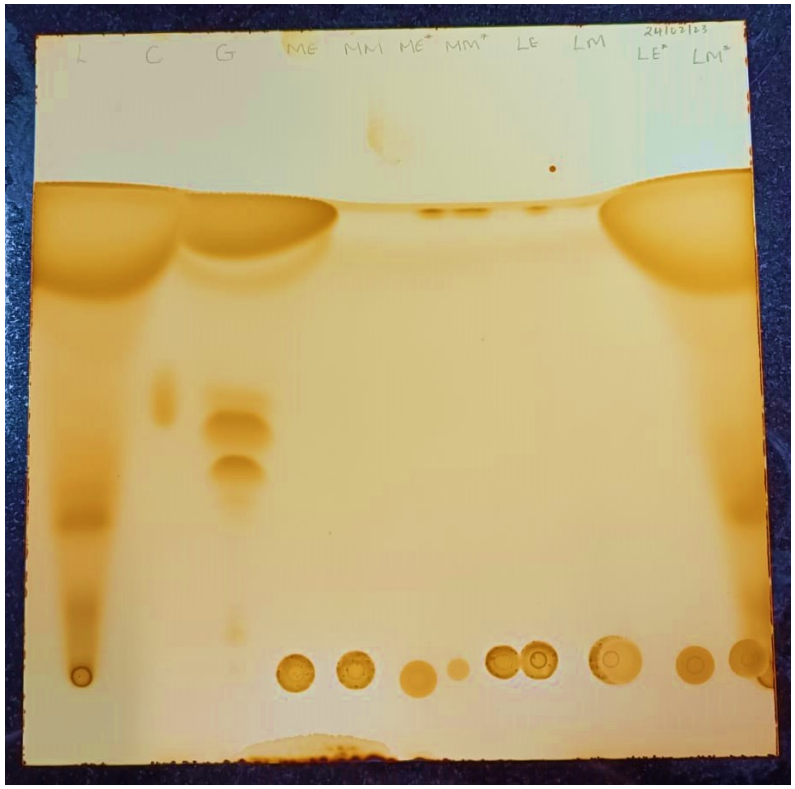
ESM-Explant MEM

ML-Mechanical L15

MM-Mechanical l MEM

EL-Enzymatic L15

Plate 15- Developed TLC plates with lipid spots of hepatocyte samples of 15 days and 22 days cultures.



15 Days

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

EM-Enzymatic MEM

LM*-L15 mechanical media

MM*-MEM mechanical media

ME*-MEM enzymatic media

LM- L15Mechanica

MM- MEM Mechanical

LE-Enzymatic L15

LE*-L15 enzymatic media

22 Days

L- Linoleic acid

C-Cholesterol

CP- Prepared cholesterol

G-Gee

S-Sample

EM-Enzymatic MEM

ESL-Explant L15

ESM-Explant MEM

ML-Mechanical L15

MM-Mechanical MEM

EL-Enzymatic L15

CHAPTER IV

Discussion and conclusion

Discussion

The need for a primary culture system for hepatocytes includes optimized isolation procedures and the facility of culture conditions such as medium and cellular microenvironment that are ideal for cell viability and metabolic activity (Braunbeck & Segner 2000) the help any growth factor cell culture established for this the medium will enhance the viability and function of the cultured hepatocytes, resulting in a higher total cell yield and improved lipid synthesis and metabolism. The lipid analysis of the cultured hepatocytes will provide valuable insights into the lipid metabolism of Indian mackerel, and the established culture system could serve as a useful tool for future studies investigating the effects of diet, environment, and disease on lipid metabolism in this species. The establishment of a culture system for hepatocytes from Indian mackerel will allow for the *in vitro* study of hepatocyte functions and metabolic processes, which can provide insights into the physiological and pathological conditions of the fish species. The lipid analyses will provide valuable information on the lipid metabolism of Indian mackerel and the potential use of its liver as a source of lipids for various applications, such as the production of omega-3 fatty acid supplements.

The variation in cell size in fish liver primary cultures is a well-known phenomenon, and it depends on several factors such as the species, age, and culture conditions. The increase in cell size was observed in the primary culture soon after seeding or inoculation, and the migrated hepatocytes from cultures showed an increase in cell size when they were nearing monolayer formation, and once the monolayer was formed. This observation indicates that cell size may play a role in the formation and maintenance of monolayer culture. However, further studies are necessary to confirm this hypothesis. Similar observations stating different types of cells that migrate from explants and were also reviewed by Alikunhi *et al.* (2013). Their findings mention variations in cell size and shape in fish liver cultures that are based on species, age,

and culture conditions. Different cell types found in primary cultures include hepatocytes, Kupffer cells, stellate cells, endothelial cells of blood vessels, and epithelial cells of the bile duct. It is also important to note that some variability in size and shape is to be expected, as cells in culture can respond to a number of factors, such as changes in nutrient availability, oxygen levels, or extracellular matrix composition. Overall, the shape and size of cultured hepatocytes can vary, but they generally retain some of the characteristics of hepatocytes *in vivo.*

The measurement of total protein content in the homogenates of cells cultured is an important indicator of cell growth and proliferation. The result that total protein content remained almost in the similar range for all culture types from the day of initiation to day 22 without passaging the cells. If the total protein content of cultured hepatocytes remains the same until day 22 from the day of establishment of culture, it could indicate that the hepatocytes are maintaining their metabolic activity and protein synthesis over time. This is important for the functional integrity of hepatocytes, as they play a critical role in many metabolic processes including protein synthesis, lipid metabolism, and drug metabolism (Ramaiah, 2007; Tuschl *et al.,* 2009). However, it is important to note that protein content is only one measure of hepatocyte function, and other measures such as enzyme activity, gene expression, and drug metabolism should also be considered when assessing the viability and functionality of cultured hepatocytes. In addition to this, the maintenance of protein content may depend on specific culture conditions and factors such as the origin of the hepatocytes, the composition of the culture media, and the presence of growth factors or other supplements. The study by Hsu *et al.* (2018) found that the total protein content of human hepatocytes remained relatively stable over a period of 10 days in culture without passaging. Similarly, a study by Arroyo *et al.* (2019) reported that the total protein content of primary human keratinocytes remained constant for up to 14 days in culture without passaging. Our findings also claim this reason as these cultures were not passaged till the 22nd day after the establishment of the culture. Therefore, the finding that total protein

content remained stable in the present study suggests that the cells were not actively dividing or proliferating during the culture period. This is consistent with the lack of significant changes observed in the lipid profile and enzyme activities of the cells. In this study the culturing systems were successful without the supplementation of any growth factors.

AST, also known as serum glutamic-oxaloacetic transaminase (SGOT), is an enzyme found in high levels in the liver, as well as in other organs such as the heart, muscles, and kidneys. In hepatocytes, AST plays an important role in the metabolism of amino acids, particularly in the breakdown of aspartate. AST catalyzes the transfer of an amino group from aspartate to alpha-ketoglutarate, forming oxaloacetate and glutamate as products. The resulting oxaloacetate can then be further metabolized through the citric acid cycle to produce energy. ALT also known as serum glutamic-pyruvic transaminase (SGPT), is an enzyme found predominantly in the liver, although it is also present in other organs such as the kidneys and muscles. In hepatocytes, ALT plays an important role in the metabolism of amino acids, particularly in the breakdown of alanine. ALT catalyses the transfer of an amino group from alanine to alpha-ketoglutarate, thus forming products such as pyruvate and glutamate. The resulting pyruvate can then be further metabolized through the glycolytic pathway to produce energy. The measurement of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) is commonly used to assess liver function and liver damage. The results suggest that the activity of both enzymes in the liver cells culture vary over as time period of the culture increases, indicating changes in hepatocyte metabolism after maintaining the culture for many days without passaging the cells. Past research literature (Hassan *et al.,* 2002, Chopra *et al.,* 2012) discusses the importance of measuring AST and ALT enzyme activities as biomarkers for liver injury and disease. The results obtained from the experiments described in the question suggest that the activity of these enzymes varies over time in cultured liver cells, which could reflect changes in liver metabolism and function during different stages of cell culture. Elevated levels of ALT in liver tissue or in the blood can indicate liver damage or disease, as the release of ALT from hepatocytes into the

bloodstream is a sign of hepatocyte injury or destruction. An increase in ALT activity in primary cultures of hepatocytes after 19 days of culture could indicate that the hepatocytes are undergoing some degree of stress or damage, which is causing an increased release of ALT into the culture medium. It is also possible that the increase in ALT and AST activity is a normal part of the aging or differentiation process of the hepatocytes in culture (Liu *et al.,* 2017, Ong *et al.,* 2014). To determine the specific cause of the increase in ALT and AST activities, further investigation would be necessary. This could include assessing the levels of other liver enzymes and markers of liver function, as well as examining the morphology and viability of the hepatocytes in specific.

The liver plays a key role in lipid metabolism and is responsible for the synthesis, storage, and breakdown of fats. PUFAs (polyunsaturated fatty acids) are important for cultured hepatocytes, which are liver cells grown in a laboratory setting. PUFAs play a crucial role in maintaining the structure and function of cell membranes, which are composed of a lipid bilayer that surrounds and protects cells. Studies have shown that PUFAs, especially omega-3 PUFAs, can improve the viability and function of cultured hepatocytes. Omega-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to reduce inflammation, enhance insulin sensitivity, and decrease oxidative stress in liver cells. These effects can help to protect the liver from damage and improve liver function. In addition, PUFAs have been shown to regulate gene expression in cultured hepatocytes, which can have important implications for liver health. For example, PUFAs can regulate the expression of genes involved in lipid metabolism, inflammation, and oxidative stress, which are all important processes in the liver (MUFA (monounsaturated fatty acids) are also important for cultured hepatocytes, which are liver cells grown in a laboratory setting. Like PUFAs, MUFAs play a critical role in maintaining the structure and function of cell membranes. MUFAs have been shown to have a number of beneficial effects on liver health. Studies have shown that MUFAs can improve liver function and reduce inflammation in the liver. MUFAs can also improve insulin sensitivity. In

addition, MUFAs can help to reduce the levels of harmful fats, such as triglycerides and LDL cholesterol, in the liver. This can help to prevent the development of non-alcoholic fatty liver disease (NAFLD), a condition characterized by the accumulation of fat in the liver.

Furthermore, MUFAs have been shown to have antioxidant properties, which can help to protect liver cells from damage caused by oxidative stress. Conditions such as oxidative stress result in an imbalance between the generation of reactive oxygen species (ROS) and the body's ability to detoxify them. Oxidative stress can damage liver cells and lead to liver disease. Therefore, MUFAs are important for maintaining the health and function of cultured hepatocytes and can have beneficial effects on liver health by reducing inflammation, improving insulin sensitivity, and reducing harmful fats and oxidative stress in the liver. MUFA-rich foods include olive oil, avocados, nuts, and seeds.

Cholesterol is an important component of the cell membrane and is involved in a variety of physiological processes in the liver. In cultured hepatocytes, cholesterol plays a role in regulating the expression of genes involved in lipid metabolism, bile acid synthesis, and cholesterol transport. Cholesterol is also involved in the synthesis of hormones and vitamin D, which are important for a variety of physiological processes in the body. Overall, while cholesterol is important for normal liver function and physiological processes, excessive cholesterol accumulation in cultured hepatocytes can be harmful and contribute to the development of liver diseases and cardiovascular problems. It is important to maintain healthy levels of cholesterol in the liver through a healthy diet and lifestyle.

PUFA and MUFA are both essential fatty acids that the body cannot produce on its own and must be obtained through the diet. They play an important role in maintaining cardiovascular health, brain function, and inflammation regulation. Fish liver is a rich source of omega-3 fatty acids, specifically EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). These are long-chain PUFA that have been shown to have numerous health benefits, including reducing

the risk of heart disease, improving brain function and mental health, and reducing inflammation. MUFA, such as oleic acid, have been shown to have cholesterol-lowering effects and can also help reduce the risk of heart disease. Cholesterol is a necessary component of cell membranes and is used in the production of certain hormones.

The results of the study show that only PUFA spots were observed on the TLC plate for the Day 0 hepatocyte culture samples, indicating the presence of PUFA in the cells at the beginning of the culture. However, spots for MUFA were seen on Day 15, indicating that the cells had undergone some changes in their lipid composition over time. No spots for cholesterol were observed in any of the samples. The results of the study suggest that fish hepatocytes have the potential to synthesize and accumulate PUFA and MUFA over time in culture, but not cholesterol. The presence of PUFA at the beginning of the culture indicates that fish hepatocytes naturally produce these essential fatty acids. The appearance of MUFA spots on Day 15 suggests that the cells underwent changes in their lipid metabolism, which may be influenced by the culture conditions. The absence of cholesterol spots in all samples suggests that fish hepatocytes may not be a good source of cholesterol for industrial applications. Overall, the study highlights the potential of fish hepatocytes for the production of PUFA and MUFA, which have numerous health benefits and industrial applications.

The fish liver is a rich source of cholesterol, but the cholesterol content varies depending on the species and the diet of the fish. Hepatocytes are the principal cells of the liver and play a critical role in lipid metabolism. *In vitro* culture of hepatocytes from fish liver is an effective method to study the lipid metabolism of fish and to investigate the effects of various factors on the lipid composition of fish. Fish hepatocytes are a good source of essential fatty acids, such as PUFA and MUFA, as well as cholesterol. The content and composition of these lipids can be manipulated through the use of specific diets and culture conditions, allowing for the production of fish hepatocytes enriched in specific fatty acids and cholesterol (Park & Kim, 2017, Chiarello *et al.,* 2017, Hu *et al.,* 2016, Lu *et al.,* 2020). Moreover, the production of PUFA, MUFA, and

cholesterol through cultured fish hepatocytes has several advantages over traditional methods. The production process is more efficient, as the culture of hepatocytes provides a controlled environment for the synthesis and accumulation of these lipids. Additionally, the production is more sustainable and environmentally friendly, as it does not require the use of live fish or other marine resources. Furthermore, the production can be scaled up to industrial levels, making it possible to meet the increasing demand for these lipids in the food and pharmaceutical industries. Overall, the results suggest that cultured fish hepatocytes have the potential to be used as cell factories for the production of PUFA and potentially MUFA, but further research is needed to determine the optimal conditions for lipid synthesis and modification in these cells. Therefore, it can be summarized that the culture of fish hepatocytes is a promising approach for the production of PUFA, MUFA, and cholesterol. It offers a sustainable and efficient method for the production of these lipids on a large scale and has the potential to meet the increasing demand for these essential nutrients in the food and pharmaceutical industries.

Conclusion

Hepatocyte cultures of *Rastrelliger kanagurta* under optimized culture conditions were established by comparing explant and free cell primary cultures techniques in combination with MEM and L15 culture media and cell dissociation methods. The results of this research do not recommend the establishment of the hepatocyte cultures through primary explant culture as they showed persistent contamination. Hepatocyte cultures established through this study showed protein synthesis as well as AST and ALT enzyme activities indicating their metabolic fitness. Metabolic activity of the cultured cells was also observed through the synthesis of PUFA and MUFA justifying its potential to synthesize liver-specific lipids. As hypothesized, cell cultures were established and propagated without the supplementation of hepatocyte growth factors in culture media thus encashing the high regeneration capacity of liver tissue.

Summary

The Indian mackerel is an important fish species in the Indian subcontinent, characterized by its homogeneity and polygonal-shaped, weakly basophilic hepatocytes. Culturing hepatocytes from the liver of *Rastrelliger kanagurta* can provide a valuable tool for studying the metabolism, toxicity, and nutritional requirements of this species. Mackerel hepatocyte culture is an important tool for understanding the biology, toxicology, and nutritional requirements of this commercially important fish species**.** The present study was designed to establish hepatocyte culture from Indian Mackerel to study its growth and metabolic health and to determine the lipid profiles of cultured fish hepatocytes. Returning to the hypothesis posed at the beginning of the study, it is now possible to state that the lipid profiles of the culture fish hepatocytes are approximately similar to that of the Zero Hours. This study has also revealed that cultures can be prepared by avoiding the use of growth factors. Results obtained also revealed that MEM media was the best for the monolayer and proliferation of the cells as compared to L15 media. In addition, the MEM mechanical dissociation demonstrated better growth as compared to l15 enzymatic and l15 mechanical media. Experiments performed revealed that the AST, ALT activities and total protein of the cultured fish hepatocytes were maintained in established cultures. The results of the study suggest that fish hepatocytes have the potential to synthesize and accumulate PUFA and MUFA over time in culture, but not cholesterol. Overall, the study highlights the potential of fish hepatocytes for the production of PUFA and MUFA, which have numerous health benefits and industrial applications but further research is needed to determine the optimal conditions for lipid synthesis and modification in these cells.

Recommendations for future studies

Future studies on the current topic are therefore recommended by including the following parameters:

- Complete characterization of the developed hepatocyte cell line.
- Development of the mackerel hepatocytes for spheroid and organoid production for a step towards the *in vitro* liver meat.
- Development of cryopreservation methods for the developed cell line.

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