# EFFECTS OF VACUUM AND MODIFIED ATMOSPHERE PACKAGING ON SHELF LIFE OF ROHU FISH (*Labeo rohita*) STORED AT REFRIGERATED TEMPERATURE (4°C)

A Thesis

Submitted to

Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (MS)

IN

FISHERIES TECHNOLOGY

By

**Krishno Chandra Das**

**Examination Roll No. 1705509**

**Session: 2017-2018**

**Semester: July-December, 2018**

DEPARTMENT OF FISHERIES TECHNOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY,

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# EFFECTS OF VACUUM AND MODIFIED ATMOSPHERE PACKAGING ON SHELF LIFE OF ROHU FISH (*Labeo rohita*) STORED AT REFRIGERATED TEMPERATURE (4°C)

## ABSTRACT

The study was conducted to evaluate the effects of vacuum and modified atmosphere packaging on the quality and shelf-life of sliced Rohu fish (*Labeo rohita*) stored at refrigerated temperature (4°C). The study was conducted at quality control laboratory of the Department of Fisheries in the University of Rajshahi from September 2018 to December 2018. Quality and shelf-life of sliced Rohu fishes were evaluated by biochemical and microbiological analysis by using four types of packaging: (i) without sealed pack (control); (ii) vacuum pack as treatment-1; (iii) MAP-1 (50% $CO_2$ & 50% $N_2$) as treatment-2 and (iv) MAP-2 (50% $CO_2$ & 50% $O_2$) as treatment-3. Samples were analyzed at every 2 days interval during 18 days of refrigerated storage (4°C). Different biochemical parameters such as pH, total volatile base nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS) and total viable count (TVC) of bacteria as a microbiological parameter were evaluated and analyzed throughout the storage period. In all packaging conditions, pH values (were in the range 6.8~7.0) throughout the storage period. No significant ($p<0.05$) differences were observed on the changes in pH values in all four packaging conditions during the storage period. Changes in TVB-N values were observed significantly lower ($p<0.05$) at $9^{th}$ day of storage in samples MAP-1 (2.94±0.59 mg/100g) and MAP-2 (2.66±0.20 mg/100g) pack samples as compared to control (3.90±0.76 mg/100g) samples. In case of PV, MAP1 showed better result compared to MAP2 during the storage period. However, the TBARS value in MAP-2 sample exceeded the acceptable limit (2 mg malonaldehyde/kg) on $9^{th}$ day of storage. The initial total viable count (TVC) was 4.29 log CFU/g in all packaging conditions during the storage period. When compared with control, significant ($p<0.05$) lower TVC values were observed on $9^{th}$ and $12^{th}$ day of storage in MAP samples. Shelf-life of sliced Rohu fish (*Labeo rohita*) in refrigerated temperature was in acceptable conditions for 8 days in control samples, 11 days for vacuum pack, 13 days for MAP-2 and 16 days for MAP-1 samples. Among the various packaging techniques, MAP1 showed extended shelf life of Rohu fish stored at refrigerated temperature (4°C).

# ABBREVIATIONS

APHA = American Public Health Association

AOAC = Association of Official Analytical Chemists

ATP = Adinosine Tri Phosphate

BFRI = Bangladesh Fisheries Research Institute

CFU = Colony Forming Unit

$CO_2$ = Carbon di oxide

°C = Degree Celsius

DD = De-ionized Distilled

DoF = Department of Fisheries

EGTA = Ethylene Glycol Tetraacetic Acid

FAO = Food and Agriculture Organization

GDP = Gross Domestic Product

g = Gram

ha = Hectare

hr = Hour

IDF = International Dairy Federation

kg = Kilogram

kPa = Kilopascal

lb = Pound

MA = Modified Atmosphere

MAP = Modified Atmosphere Packaging

mEq = Milliequivalents

mg = Milligram

ml = Milliliter

MT = Metric Ton

N =Normal

$N_2$ = Nitrogen

NaOH = Sodium Hydroxide

nm = Nano meter

$O_2$ = Oxygen

PPS = Peptone Physiological Saline

PUFA = Poly Unsaturated Fatty Acid

PV = Peroxide Value

RTC = Ready To Cook

rpm = Rotation per minute

sq = Square

TBA = Thiobarbituric Acid

TBARS = Thiobarbituric Acid Reactive Substance

TVC = Total Viable Count

TVB-N = Total Volatile Base Nitrogen

U.K. = United Kingdom

UV = Ultra Violet

VP = Vacuum Packaging

w/v = Weight Per Volume

v/v = Volume Per Volume

µm = Micrometer

µL = Microlitre

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# CHAPTER 1

# INTRODUCTION

Bangladesh is encompassed with a vast area of inland fresh water body such as rivers, canals, ponds, lakes, marshes, estuaries, and floodplains (Hossain, 2014). This country has been recognized for its largest flooded wetland and the third largest aquatic biodiversity in Asia (Shamsuzzaman *et al.*, 2017). Huge scope for aquaculture was facilitated by these vast water bodies. Therefore, Bangladesh is famous for its diversified and potential fisheries resources that are one of the richest fisheries resources of the world due to the fish habitats created by the Bengal Delta wetlands and the confluence of the Brahmaputra, Ganges and Jamuna rivers flowing from the Himalayan Mountains into the Bay of Bengal (Hossain, 2014). Thus, aquaculture is dominating with a lion share for the total fish production in Bangladesh. According to a recent report of FAO, Bangladesh is ranked 5th in the world aquaculture production (FAO, 2018).

According to Department of Fisheries (DoF), it was revealed that in 2016-17 Bangladesh secured a surplus fish production with an astonishing annual production of 41.34 lakh Metric Ton (MT), whereas the demand was 40.50 lakh MT (DoF, 2017). Therefore, the daily intake of fish consumption has been increased to 62.58 g of fish per person in Bangladesh (DoF, 2017). The fisheries sector is providing a big role towards country's national GDP (3.61%) growth (DoF, 2017).

Inland aquaculture includes pond/ditch, baor, shrimp/prawn farm, seasonal cultured water-body, pen and cage culture etc. covering an area of about 8.33 lakh hectare and contributing about 56.44%of the total fish production (DoF, 2017).

The inland aquaculture is growing rapidly due to the establishment of new technologies, quality fish seed, intensification and improvement of fish farming over the country (Planning Commission, 2016). Freshwater aquaculture includes farming of carps (indigenous and exotic), pangasid catfish, tilapia, climbing perch, and a number of other indigenous fish species. Semi-intensive carp culture covers a vast area of 110,000 ha, and intensive forms of entrepreneurial pond culture cover only

15,000 ha (Belton *et al.*, 2011). Among the carps, Indian major carps and some minor carps are very popular for culture purposes across the country.

Rohu (*Labeo rohita*) fish which is also known as Rui (Indian major carp) is a cyprinid fish available in the riverine system of Bangladesh. *Labeo rohita* has been introduced into many countries due to the success of breeding in Sri Lanka, Japan, China, Philippines, Malaysia, Nepal and few countries of Africa (Jhingran, 1982). Rohu fish is available allover Bangladesh and preferred as an aquaculture species for their higher environmental tolerances, disease resistance, faster growth and high flesh content. Being a fast growing omnivorous fish, it mainly thrives on the filamentous algae, aquatic plant leaves, phytoplankton, zooplanktons and minorly on small insects (Bairagi *et al.*, 2002). In contrast, Rohu does not grow well below 14 °C and the optimum temperature for growth ranges between 16.8-37.0 °C, while the optimum temperature for spawning is 22-31 °C (FAO, 2017).

Polyculture involves culturing of more than one species of aquatic organisms in the same water body such as pond. In this system, Rohu used to culture along with Katla (*Catla catla*) and Mrigal (*Cirrhinus mrigala*) (Reddy, 1999). Rohu moves from column to bottom and possesses a wider feeding niche, therefore, stocking of fingerling used to practice at higher levels in the rearing ponds compared to the other two species . Its higher growth rate and greater consumer preference makes it very popular in many countries including Bangladesh. In 2016-17 fiscal years, the total production of Rohu fishes in Bangladesh were 3,70,627 Metric Ton (MT) (DoF, 2017).

Fish and fishery products are a good source of protein as well as vitamins and minerals. Fish protein are rich in omega-3 fatty acids and vitamins such as vitamin D and vitamin $B_2$ (riboflavin) and also act as a great source of minerals particularly calcium, phosphorous and some other micro minerals (Falls, 2012). Fatty fishes contain fat-soluble vitamins (A, D, E and K) and essential fatty acids. These elements are vital for the healthy function of the body (Fellows and Hampton, 1992). As an important source of proteins, vitamins and minerals, *Labeo rohita* is one of the most preferred major carp. The fat content of *Labeo rohita* contains vitamins like A, D, E, K and C as well as essential fatty acids like PUFA, Omega-3 fatty acids like alpha linolenic acid, eicosapentaenoic acid, docosahexaenoic acid. Minerals like calcium,

zinc, iron and thallium are also available in *Labeo rohita* (De Silva *et al.*, 1995). Moreover, Omega-3 fatty acids have beneficial effect on cardiovascular disease (CVD) (Krauss *et al.*, 2000). Natural sources of polyunsaturated fatty acids having two important ω-3 PUFAs in fish oil includes EPA (Eicosapentaenoic Acid) and DHA (Docosahexaenoic Acid) that have been proven useful effects on human body (Imad *et al.*, 2008). Therefore, Rohu being a slightly fatty to fatty fish species contains considerable amount of essential fatty acids, amino acids, vitamins and minerals.

Fresh fish generally spoils quickly after harvesting due to the enzymatic autolysis, oxidation and microbial growth which are the three main basic mechanisms responsible for fish spoilage (Ghaly *et al*., 2010). In the tropical regions due to high ambient temperatures, the spoilage process commonly known as 'Rigor mortis' starts rapidly within 12 hrs of the catch (Berkel *et al*., 2004). Rigor mortis is a process through which stiffening of fish muscle occurs just after the few hours of death of the fish that leads to loss of flexibility of fish flesh (Adebowale *et al*., 2008). Breakdown of numerous components and formation of new compounds basically occur during fish spoilage. Thereby, these newly formed compounds are mainly responsible for the changes in texture, odor and flavor of the fish meat.

Enzymatic breakdown of body compounds in fishes occur shortly after capture that results in chemical and biological changes mainly in dead fish (FAO, 2005). The autolytic reactions are controlled mainly by endogenous enzymes present in the fish muscle tissue as well as in the gut (Ashie *et al.*, 1996). A number of proteolytic enzymes are found in muscle and viscera of the fish and these enzymes contribute considerably to the post mortem degradation in fish muscle and products during storage and processing. Degradation of proteins commonly known as proteolysis is followed by a process of solubilization that mainly occurs during the improper storage of whole fish (Lin and Park, 1996). Autolysis of fish muscle proteins also produce peptides and free amino acids that contributes to spoilage of fish as a result of microbial growth and production of biogenic amines (Fraser and Sumar, 1998). The leakage of proteolytic enzymes from pyloric caeca and intestine to the ventral muscle also leads to considerable spoilage commonly known as 'belly bursting'.

Lipid oxidation is a major cause of deterioration and spoilage of fatty fish. Lipid oxidation includes a three stage free radical mechanism, named as: initiation, propagation and termination (Frankel, 1985). Fish lipids of cultured Rohu contain higher levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) while Rohu from wild sources contain higher levels of polyunsaturated fatty acids (PUFA) (Sharma *et al*., 2010). Therefore, Rohu is extremely susceptible to oxidation. In fish, lipid oxidation takes place not only enzymatically but also non-enzymatically.

The microflora composition of the newly caught fish depends on the microbial loads of the water in which the fish live. The common microflora in fish includes bacterial species such as *Pseudomonas, Alcaligenes, Vibrio, Serratia* and *Micrococcus* (Gram and Huss, 2000). Fresh fish caught from warm water normally contains microbial population such as *Micrococcus,* coryneforms, and *Bacillus* while cold-water fish species are dominated by psychrophilic gram-negative microbes including *Moraxella/Acinetobacter, Pseudomonas, Flavobacterium,* and *Vibrio* genera (Cann 1977 and Liston 1980). The microflora of *Labeo rohita* is dominated by strains of *Bacilli, Pseudomonas, Aeromonas, Enterobacter* (Ghosh *et al*., 2010). However, strains of *Flavobacterium, Micrococcus, Achromobacter, and Vibrio* are also visible in the gastrointestinal tract of *Labeo rohita* (Hossain *et al*., 1999). The major causes of fish spoilage are microbial growth and metabolism that produces various compounds like amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with obnoxious and improper odors and off-flavors (Dalgaard *et al*., 2006; Emborg *et al*., 2005; Gram and Dalgaard, 2002). Gram-negative, fermentative bacteria (such as *Vibrionaceae*) are mainly responsible for spoilage related to unpreserved fish, whereas in case of chilled fish the psychrotolerant gram-negative bacteria (such as *Pseudomonas* spp. and *Shewanella* spp.) are the predominant ones (Gram and Huss, 2000). The microbial compositions of fishes are also changing due to adoption of new preservation techniques such as modern packaging.

The introduction of new preservation techniques is mainly due to the changes in food preference and increasing demand for fresh fish and fishery products with extended shelf life. The changes in food preference of people over the last few years may have occurred due to the considerable social and economic development of Bangladesh. At

present the meaning of food has been changed. Foods are no longer intended only to satisfy the hunger and provide necessary nutrients but also prevent nutritional diseases, and at the same time to improve physical and mental well-being of the consumers (Menrad, 2003). There are several factors affecting the demand function of fish and fishery products that includes price, income, income distribution, substitutes, tastes and fashion, advertising and expectations of the consumers, etc. (De Silva, 2011). Nowadays city and mega-city residents particularly busy women's, mothers and housewives are asking for ready-to-cook (RTC) food products instead of the raw one's that take away their considerable amount of precious time during cooking or preparation. As a result, the fish-processing industry and retail superstores are actively seeking different methods for shelf life extension and marketability of fresh, refrigerated fish. Ice storage or refrigeration in combination with modern packaging techniques can be fruitful to delay spoilage, extend the shelf life, maintain a high quality of fish and fishery products and may assure food safety even though at a higher price.

Consumers concern on price and quality of the product are mainly affected by the food consumption and food habits. Acceptance of the new product along with food safety stays the main concern. If a product is lower in quality, its appeal will not be there, not even health benefit promises can do justice to accept it by the consumers (Sosa *et al.*, 2008). But if a product has high sensory acceptability, the additional issues for instance, packaging, price and convenience have to be resolved to ensure overall acceptability. According to a study by Rahman *et al.*, (2017) it was revealed that the superstore outlets in Dhaka city sell superior quality wet fish at a rate of 20-25% elevated price compared to those sold through general fish markets. This eventually proves a strong point that if the quality is ensured in a product; people are ready to pay irrespective of the high price. At present, there is lack of or no RTC or MAP type packaged fish and fishery products in the markets of Bangladesh.

An efficient fish marketing system is a key to enable the customers to get the fresh fish. The domestic fish market in Bangladesh is quite big in relation to volume, value and employment. The basic marketing infrastructures including cold storage, ice, insulated transport facilities, fish landing centers and wholesale markets is basically improper, unhygienic and in poor state. In Bangladesh, fish are generally landed in

road-side shore of natural watershed or farm house (Nowsad, 2010). The harvested fish through different channels are transferred from the landing centers to the consumer markets (Ali *et al*., 2004). The intermediaries take their benefits during the handover of the fish in the retailing system. The fish producers or farmers most of the times do not get the proper or expected price of their product due to the involvement of so many brokers in the marketing channel. Although during marketing almost all intermediaries use ice but their usage of ice in fish preservation is not scientifically correct due to which considerable quality loss of fish occurs. During retail selling, some of the sellers use ice and some do not. As a result, amount of raw fishes that undergoes quality deterioration during retailing is quite significant (Hossain *et al*., 2013). Under this scenario, some fraud traders use formalin in fishes which is a very hazardous chemical and a direct threat for human health (BFRI, 2006). In order to change the scenario, modernization of transportation, handling, preserving, packaging, and storing facilities are very important (Islam and Habib, 2013).

As with time, the people in Bangladesh due to their immense business and consciousness about time are heading towards departmental store instead of going to separate markets for their groceries including fish. Thus, they can save their time, money and energy. By ensuring hygiene and food safety, the superstores or super shops may display the raw (whole fish), dressed or processed (fillets/slice) fish under chilling for marketing purpose, even though at a higher prices than the traditional fish markets. Under current icing and refrigerated storage conditions the shelf-life of fish and fishery products typically ranges from 2 to 14 days (Stammen *et al.*, 1990). In order to delay oxidation, control food borne pathogens, and meet the growing demand of consumers for safe and high-quality products as well as to improve antimicrobial properties, active food packaging is very significant (Choi *et al.*, 2016). So it is obvious that there is room for MAP packaged fish and fishery products (fillets/slice) under refrigerated storage to take an edge over the other traditional fish products particularly in superstore outlets in cities and megacities.

According to Gupta and Dudeja (2017), packaging acts as a physical barrier to protect food from external factors, so the stability of packaging materials is absolutely vital for enhancing food quality and safety and increasing shelf-life. Packaging also ensures sturdy, attractive, economical, and convenient products to consumers.

The evolution of novel and innovative packaging techniques that maintain and used to monitor food safety and quality, extend shelf-life, and reduce the environmental burden of food packaging have been mainly stimulated by frequent changes in consumer demand, industrial production trends, retailing practices, and customer lifestyles (Dainelli *et al.*, 2008).

Placement of a product inside a packaging material with low permeability to oxygen followed by air exhaustion and sealing is termed as vacuum packaging (Smith *et al.,* 1990). Though in vacuum packaging the gaseous atmosphere is completely reduced, but changes in gas composition is very obvious particularly during storage days. It is mainly due to microbial activity that results in about 10 to 20% increase in the $CO_2$ amount that may suppress the growth of undesirable microorganisms (Silliker and Wolfe, 1980). Vacuum packaging has been extensively used to remove oxygen in the package prior to sealing but it is unable to remove the oxygen that permeates from the external environment into the package. The presence of $O_2$ in food packages occurs mainly as a result of permeability of packaging material, failures in the packaging process and ineffective vacuum (Mohan *et al.,* 2019).

Oxygen is responsible for oxidative rancidity in fatty fish and stimulates the growth of aerobic microorganisms (Arashisar *et al.*, 2004). Complete removal of oxygen is very essential particularly for oxygen-sensitive foods as the presence of $O_2$ ultimately leads to the growth of aerobic bacteria, moulds and oxidation. VP inhibits the growth of aerobic bacteria that grow under aerobic storage conditions dominating the spoilage of freshwater and marine fish thereby, extend the shelf life of fish fillets (Gram and Huss, 1996).

Modified atmosphere packaging (MAP) is basically a preservation technique that involves alteration of atmospheric environment around a food that is perishable by the replacement of single or a mixture protective gases (Arashisar *et al*., 2004; Del Nobile *et al*., 2009). Stammen *et al.* (1990) defined MAP as a system where the air within a package is instantly replaced by a mixture of different gases at the time of sealing. MAP has been reported to considerably inhibit the spoilage as well as to elongate the shelf life of fresh fish products (Torrieri *et al.,* 2006).

The principle of MAP involves replacing the air in the package with an altered gas mixture. After the incorporation of gas mixture in the package, no further control of the gas composition is done and the gas composition will certainly change with time (Sivertsvik *et al.*, 2002). Modification of the headspace during food packaging with different gas mixtures to delay bacterial activity and chemical reactions is the main aim of MAP (Tsironi and Taoukis, 2018).

In terms of MAP applications, $O_2$, $CO_2$ and $N_2$ are the most commonly used gases and their concentrations mainly depend on the food product and the mechanism of spoilage that limits the shelf life (Kirtil *et al.*, 2016).

The exclusion of oxygen is generally desirable mainly due to aerobic bacteria and oxidative reactions as its absence will inhibit spoilage and prolong the quality with storage life. However, in the absence of $O_2$ anaerobic respiration occurs, that accelerates spoilage of the concerned product. Mainly to counter the effects of anaerobic/micro-aerophilic organisms and non-oxidative reactions, $O_2$ is used in various concentrations in modified atmosphere packages. Pantazi *et al*., (2008) found that oxygen hinder the growth of anaerobic bacteria as well as accumulation of toxin by *Clostridium botulinum* type E when it is introduced in modified atmosphere packages. $O_2$ maintains the colouring pigment myoglobin intact in the bright red, oxygenated form (oxymyoglobin) that consumers prefer (Hood and Riordan, 1973).

The most widely used gas for MAP of fish products is $CO_2$ that generally inhibits microbial growth. The inhibition of microbial growth mainly depends on the $CO_2$ concentration as growth of respiratory organisms such as *Pseudomonas* spp. and *Shewanella putrefaciens* can be delayed by $CO_2$ (Sivertsvik *et al.*, 2002). The effectiveness of $CO_2$ depends upon the growth phase of any microorganisms present. $CO_2$ increases the duration of lag phase of microbes while decreases the growth rate during the logarithmic phase (Farber, 1991). Under this consideration, the shelf life of refrigerated fish products can be prolonged effectively by MAP.

$N_2$ is an inert and tasteless gas and it is comparatively less prone to pass either into the product or out through the packaging material than the other gases normally used. It is commonly used as a balance or filler gas replacing $O_2$ in the packages, either as a

substitute to vacuum packaging when the product is delicate, or to stop pack collapse caused by the $CO_2$ absorption (Church and Parsons, 1995).

The optimum packaging system design for any particular product includes a number of factors, the most important being amounts of $O_2$ and $CO_2$ present. It is mainly determined by the volume of these respective gases within the pack, the headspace volume as well as the permeability of the packaging material. Proper optimization of the packaging system will result in enhancement of sensory quality and minimization of public health risks.

The use of Modified atmosphere packaging (MAP) is relatively new in respect of Bangladesh and such packaging system has not been developed and introduced yet for preservation of fishes. MAP provides several advantages like high quality fish fillets with an extended shelf-life, good hygienic standards, and most importantly food safety. With continuous and consistent demand for high quality food with extended shelf life that most of the present techniques fail to do, there is no choice but to go for alternative modern techniques like vacuum packaging and modified atmosphere packaging. MAP promises good keeping quality of fish with elongated shelf-life. Therefore, it is critically important to develop appropriate vacuum and MAP technology particularly for fish fillets and/or slice in order to lower the qualitative and quantitative losses of raw fishes that will ultimately ensure the supply of good quality fishes to the consumer in a convenient way.

The objectives of this study are-

- To evaluate the quality changes of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).
- To determine the safety aspects of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).
- To determine the overall shelf-life of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).

# CHAPTER 2

# REVIEW OF LITERATURE

As modified atmosphere packaging (MAP) is a newly introduced technology in Bangladesh, therefore, there is literally no information available regarding MAP of fish and fishery products in Bangladesh. Modified atmosphere packaging is a comprehensive and extensively used technology for increasing shelf life of fish and fishery products in many developed countries. Thus, information regarding vacuum packaging and particularly MAP were collected from those countries where these types of techniques are available. The present literature reviews highlighting the most relevent studies related to this study.

## 2.1 Vacuum and Modified atmosphere packaging

Smith *et al.* (1990) defined vacuum packaging as placement of a product in a film of low oxygen permeability, followed by the removal of air from the package and the hermetic sealing.

Banks *et al.* (1980) and Arias (2009) reported that in vacuum packaging the gas proportions are modified by evacuating air from the packages, the absence of $O_2$ results in altering bacterial composition from gram-negative to predominantly gram-positive lactic bacteria. Due to partial sugar fermentation, the pH also decreases thereby inhibiting the growth of Gram-negative bacteria.

White and Roberts (1992) stated that to reduce oxidative rancidity considerably, $O_2$ content of less than 2% v/v is required in vacuum packaging.

DeWitt and Oliveira (2016) found that for seafood products, an alternative to vacuum packaging is done by the simple flushing with nitrogen, which is mainly used to replace $O_2$ in packages to postpone oxidative rancidity and inhibit growth of aerobic microorganisms.

Del Nobile *et al*. (2009) defined modified atmosphere packaging (MAP) as a preservation technique by changing atmospheric environment around a perishable food commodity by incorporating single or a mixture of protective gases.

Stammen *et al.* (1990) defined MAP as a system where the air within a package is instantly replaced by a mixture of different gases at the time of sealing.

MAP was also defined as the enclosure of food products in gas-barrier materials, in which the gaseous environment changes in order to inhibit spoilage agents and therefore either maintain a higher quality or extended shelf-life (Young *et a1.*, 1988).

In order to extend shelf-life of fresh fish products, MAP packaging was first introduced in 1930's (Killefer, 1930; Coyne, 1932, 1933; Stansby and Griffiths, 1935; Smith *et al.,* 1988) and was used in U.K. during the last quarter century with substantial success (Cann, 1988; Lioutas, 1988).

Bouletis *et al.* (2017) stated that to offer the safer distribution of quality aquaculture fishery products MAP can certainly play a vital role.

During the last two decades, MAP technique has extensively modernized. The positive effect of MAP on fish and shellfish quality during preservation was reported.

## 2.2 Role of gases in MAP

According to Church and Parsons (1995), three gases are generally used for MAP packaging which are oxygen ($O_2$), nitrogen ($N_2$) and carbon dioxide ($CO_2$). All of these gases have a specific function.

Alfaro *et al.* (2013) concluded that $CO_2$ is a bacteriostatic agent which is considered as the principal gas having a significant effect on fish microflora.

Campus *et al.* (2011) and Arashisar *et al.* (2004) reported that the shelf-life of fish products kept at high $CO_2$ levels enhance the shelf-life of the products due to microbial growth inhibition.

Davis (2009) reported that in order to preserve the red color of haem pigments, high oxygen concentrations in combination with $CO_2$ was used. The main reason to include oxygen in the gas mixture of MAP fish and fishery products was mainly not to intensify the potential risk of botulism from products in sealed packs.

Sun Lee *et al.* (2008) suggested that for fatty fishes, gas mixtures containing $CO_2$ (40–60%) with balanced level of Nitrogen ($N_2$) followed by 0–2$^oC$ storage is recommended.

Sivertsvik *et al.* (2002) noted that $O_2$ is mostly used in MAP gas mixtures as a filler (e.g., $CO_2$:$N_2$) mainly due to its low solubility in water and fat, which prevents packaging deterioration. They also found that nitrogen as an inert gas replaced oxygen in the pack which ultimately reduced oxidative rancidity as well as inhibited growth of aerobic microorganisms. They also reported about the relationship between the higher concentration of $CO_2$ and higher inhibitory effect. They concluded that there is a positive correlation between these two factors.

Reddy *et al.* (1992) concluded that the total removal of $O_2$ was necessary due to greater chances of high oxidative rancidity that may lead to altering color and texture of the flesh particularly for fatty fish such as common carp.

According to Smith *et a1.* (1990) white fish was typically packed in 30% $O_2$/30% $N_2$/40% CO, where $O_2$ being excluded in the latter stages to minimize rancidity.

Statham (1984) and Yambrach (1987) concluded that $CO_2$ and $N_2$ alone or in combination were most abundantly used gases in MA packaging particularly of fresh seafood products, along with the occasional use of $O_2$ in low levels.

Genigeorgis (1985) reported that $O_2$ enhanced odor and color shelf-life and keeps the oxygenated form of myoglobin intact.

Ogrydziak and Brown (1982) and Statham (1984) found that the growth of anaerobic spoilage bacteria was hindered by $O_2$.

## 2.3 Vacuum and Modified atmosphere packaging (MAP) of different fish species

Sterniša *et al.* (2016) in their study reported that the safety and quality of the common carp products can be enhanced by adopting novel smart packaging systems.

Mohan *et al.* (2019) studied the effects of vacuum packaging and oxygen scavenger packaging on the shelf life and quality of Indian oil sardine (*Sardinella longiceps*) kept under under chilled storage (1–2$^{o}$C). They found that the oxygen scavenger was efficient in reducing the oxygen level in the pack within 24 hrs and the growth of total mesophilic bacteria were hindered by low oxygen level compared to control air-packed samples.

Pattarapon *et al.* (2018) investigated the freshness of grass carp (*Ctenopharyngodon idella*) fillets under vacuum packaging with vacuum (50 and 30 kPa) and without vacuum were stored at 4$^{o}$C for 2 weeks. From their results they concluded that statistically significant difference between the grass carp fillet samples stored under vacuum and without vacuum conditions was found.

Rodrigues *et al.* (2016) conducted a study on Rainbow trout (*Oncorhynchus mykiss*) fillets quality for a period of 22 days by the action of UV-C radiation, modified atmosphere packaging (MAP) and their combination followed by storage at 4$^{o}$C. They found that the total production of ammonia, TVB-N and putrescine was reduced under MAP, whereas total production of TVB-N and cadaverine during the entire storage period was reduced under MAP + UV-C. They concluded that MAP retarded microbial growth and delayed chemical changes by doubling the shelf life of rainbow trout fillets.

Lunda *et al.* (2016) studied the effect of vacuum packaging on the quality of Common carp (*Cyprinus carpio* L.) fillets descaled by four different methods followed by refrigerated storage. By analyzing the growth of microbial communities they concluded that fillets without skin showed the lowest TVC.

Babic´ *et al.* (2015) in their study compared the effects of two different MAPs, MAP1 (60 % $N_2$ and 40 % $CO_2$) and MAP2 (100 % $CO_2$) on carp steak quality stored at 3.0 ± 0.5$^{o}$C over 15 days. They found that in terms of TVC values, MAP2 showed greater shelf life as compared to MAP1. They also found that MAP2 resulted in lower pH compared to MAP1 in terms of decreasing the growth of Enterobacteriaceae that mainly occurred due to $CO_2$ absorption as well as acidification of the fish muscle.

Cyprian *et al.* (2013) concluded that air storage proved to be more beneficial for tilapia fillets compared to MAP(50% $CO_2$/50% $N_2$) under chilled (1$^{o}$C) and super chilled (-1$^{o}$C) storage due to unwanted coloration of the samples. On the other hand, microbial parameters were better preserved on MAP samples compared to control.

Ali (2012) determined the shelf life of brined golden mullet (*Liza aurata*) during vacuum refrigerated storage at 4°C. He observed that the shelf life of golden mullet enhanced to a certain extent when kept under a combination of brining, vacuum packaging followed by storage at refrigerated temperature. His results also revealed

that the longest shelf life for vacuum packed brined golden mullet stored at 4°C was 30 days.

Hudecova *et al.* (2010) conducted a study on carp samples to compare the effects of MAP comprsing (30% $CO_2$/70% $N_2$) for MAP1 and (80% $O_2$/20% $CO_2$) for MAP2 stored at 4 ± 0.5°C. They found that TVC values were considerably lower on MAP samples compared to that of control carp samples even on the 10th day of storage. They concluded that a shelf life elongation of three and five days was achieved with MAP 1 and MAP 2, respectively, compared to control carp samples (three days).

Jezek and Buchtova (2010) in their study observed the effect of an altering atmosphere with (5% $O_2$/25% $CO_2$/69%$N_2$/1% CO) gas composition for the shelf life extension of carp followed by storage at 2 ± 2°C for 18 days. They found that MAP samples showed considerably lower TVBN levels compared to that of control samples even at 11th day of storage.

Grzegorz *et al.* (2010) studied the influence of method of packing (air packaging and vacuum packaging) on physicochemical and textural changes in Atlantic herring during frozen storage. They observed that fluctuations in lipid content were evident in case of frozen storage of air-packed material, while vacuum packing reduced lipid extractability from fish muscle along with storage time. They concluded that textural parameters of fillets changed to the smaller extent in case of vacuum-packed fish.

Sevik and Korkut (2009) worked on the effect of package types and storage period on characteristics of *Tinca tinca.* They indicated that as per TBV-N and TBA values, vacuum packed *T. tinca* were safely stored up to 30 days and 60 days respectively. They concluded that the shelf-life of vacuum packed *T. tinca* was 30 days.

Schirmer *et al.* (2009) in their research investigated the effect of MAP (100% $CO_2$ with gas/product ratio 0.2/1.0 v/v) and brine solution contained several contents of citric acid, acetic acid and cinnamaldehyde on salmon samples stored at 4°C. They concluded that pretreatment in combination with MAP showed controlled microbial populations of both inoculated and natural strains for 14 days.

Choubert *et al.* (2008) from their study reported that under Argon (Ar) atmosphere, TBARS concentration was lower in samples stored under MAP2 (60% Ar/40% $CO_2$) compared to MAP1 (40% $CO_2$/60% $N_2$) when Rainbow trouts fed with two types of

food astaxanthin and canthaxanthin, followed by slaughtering and stored under for 26 days at 2$^{o}$C. From the results it was concluded that the investigated samples shelf life was doubled due to the inclusion of Argon.

Cakli *et al.* (2006) compared the effects of modified atmosphere packaging and vacuum packaging on the shelf life of hot smoked rainbow trout (*Onchorincus mykiss*). Based on the results of microbiological and sensory analysis they concluded that the shelf life of smoked trout under MAP (60% $CO_2$:40% $N_2$) was 14 days more than vacuum packs.

Ozogul and Ozogul (2006) worked on the effects of modified atmosphere packaging (MAP) and vacuum packaging (VP) on biogenic amine formation during storage of sardines (*Sardina pilchardus*) at 4$^{o}$C. They concluded that Sardines were organoleptically acceptable for up to 3, 9 and 12 days under air, VP and MAP respectively. They also reported that comparatively lower amine contents of sardine were found under MAP and VP.

Masniyom *et al.* (2004) conducted their study to evaluate the effect on chemical parameters of seabass kept at 4$^{o}$C under a MAP (10% $O_2$/80% $CO_2$/10% $N_2$). They found that unaltered levels of $Ca^{2+}$, $Mg^{2+}$, and $Mg^{2+}$–$Ca^{1+}$C ATPase activity of natural actomyosin was noticed on the 21st day on samples stored under MAP, while the Mg-EGTA ATPase activity was indicating a slight increase. They also found that as compared to the control sample, the sulfhydrilcontent dropped at a lesser rate under MAP packaging.

An investigation was done by Ozogula *et al.* (2004) on sardines (*Sardina pilchardus*) to know the total viable count and histamine forming bacterial count under MAP comprised of ($CO_2$/$N_2$: 60/40) followed by storage at 4$^{o}$C. The results showed comparatively lowest total viable count (TVC) under MAP compared to air and vacuum packaging.

Tryfinopoulou *et al.* (2002) in their study found that the microbial flora of sea-bream stored under MAP (40% $CO_2$/30% $N_2$/30% $O_2$) at 0, 10, and 20$^{o}$C were dominated by *Pseudomonas lundensis* and *P. fluorescens*. Among the 106 isolates, MAP samples were dominated by the proteolytic and less lipolytic strains and microbial parameters were better preserved on MAP samples compared to control.

Reddy *et al.* (1996) in their study found that as hurdle techniques to avoid toxin production, vacuum packaging and MAP (75% $CO_2$/25% $N_2$) were tested on tilapia fillets inoculated with *C. botulinum* type E and stored at refrigeration(4$^oC$) and altered temperatures (8$^oC$ and 16$^oC$). They concluded that toxin detection occurred after more than 10 days at 4$^oC$ when sensory spoilage on both vacuum and MAP stored samples were evident.

Silva and White (1994) studied the effects of packaged catfish fillets in which $CO_2$ concentrations were (25%/75% $CO_2$/air for MAP A) and (80%/20% $CO_2$/air for MAP B) stored at 2$^oC$ and 8$^oC$. The lowest microbial numbers were recorded from MAP A samples kept at 2$^oC$ which ultimately proved to be the best studied treatment.

Drosinos *et al.* (1996) in their study experimented with Gilthead seabream (*Sparus aurata*) fillets kept under MAP (30% $O_2$/40% $CO_2$/30% $N_2$) followed by storage at 0 ± 1$^oC$. From the results it was evident that the size of bacterial population was always 2 log CFU/g lower in MAP and MAP samples also showed lower TMA-N levels compared to air packed samples.

## 2.4 Effect of MAP and Vacuum packaging on microorganisms

Ibrahim *et al*. (2008) from their study reported increase in halophilic bacterial count under vacuum conditions and MAP after two weeks of storage and then a drop on the third week of storage at 4$^oC$ for smoked mullet samples. However irrespective of the storage period, bacterial count of smoked mullet samples showed an upward growth mainly for the vacuum stored ones rather than those under MAP.

Chen and Xiong (2008) in their study observed positives of packaging effectiveness for coliform counts as well as for aerobic plate count for samples of precooked and peeled red claw crayfish. Under all packaging treated samples, the coliform count was low until 7th day but severe uplift in coliform count was evident in both treated samples on day 21 of storage. On the contrary MAP treated samples showed lowest coliform count at the end phase of storage (21 day).

Pantazi *et al.* (2008) observed that Mediterranean swordfish stored under MAP $CO_2$/$O_2$/$N_2$: 40/30/30 exhibited Enterobacteriaceae count as low as 4.2-5.6 logs CFU/g on the 16$^{th}$ day of the storage.

Stamatis and Arkoudelos (2007) in their study found that fresh chub mackerel (*Scombercolias japonica*s) fillets under MAP $CO_2/N_2$: 50/50 showed *Pseudomonas* counts below 7 log CFU/g when stored for 15 days at 3 and 6$^oC$ respectively.

A study conducted by Arkoudelos *et al.* (2007) showed that farmed eel (*Anguilla anguilla*) under modified atmosphere packaging ($CO_2/N_2/O_2$:40/30/30) followed by storage at 0$^oC$ exhibited *Pseudomonas* counts of 4.2 log CFU/g on day 37 of storage. Results also illustrated that *Pseudomonas* count was considerably lower in farmed eel treated with MAP than with air condition (7.3 logs CFU/g at day 18) and vacuum packaging (5.9 log CFU/g at day 31).

Goulas *et al*. (2005) carried out his research on microbial changes under modified atmosphere packaging of ($CO_2/N_2$: 50/50), ($CO_2/N_2$:80/20) and ($CO_2/N_2/O_2$:40/30/30) refrigerated stored mussels. Results illustrated that lowest count for total viable count 7.0 logs CFU/g on 15 days of storage was found for MAP with composition of ($CO_2/N_2$:80/20) which was also same for vacuum packaging. On the other hand, control and MAP with composition of $CO_2/N_2/O_2$: 40/30/30 attained the microbiological acceptable limit (7 log CFU/g) on 8th and 11th day of storage respectively.

Ozogul *et al.* (2004) investigated that histamine forming bacteria enhanced in sardines samples (*Sardina pilchardus*) throughout the storage period at 4$^oC$ from all treatments (MAP $CO_2/N_2$: 60/40, vacuum packaging and air). They concluded that under MAP, the histamine forming bacterial count was lower compared to other treatments.

Arashisar *et al*. (2004) observed that Enterobacterial growth inhibition was evident in rainbow trout fillets treated with MAP of both (100% $CO_2$) and ($O_2/N_2/CO_2$: 2.5/7.5/90) after 6 days of storage. Enterobacterial growth was inhibited by treating the rainbow trout fillets with MAP of (100% $CO_2$) and ($O_2/N_2/CO_2$: 2.5/7.5/90) respectively after storage of 6 days.

Lopez-Caballero *et al*. (2002) from their studies reported that the growth of $H_2S$ producing microorganisms in shrimps was inhibited by MAP. Under MAP comprising ($CO_2/O_2/N_2$: 40/30/30) and ($CO_2/O_2/N_2$: 45/5/50) respectively, $H_2S$ producing microorganisms count was about 1.1 and 1.6 log CFU/g respectively, after nine days of storage period.

Cai *et al.* (1997) experimented with inoculation of a mixture of 4 E-type *C. botulinum* strains which were introduced into packages of catfish (*Ictalurus punctatus*) packed in $O_2$-permeable film, in 80% $CO_2$ and 20% $N_2$ modified atmosphere and stored at 4$^oC$. The toxin production was evident in $O_2$-permeable film and modified atmosphere packaging after 9 and 18 days, respectively. Deterioration process further accelerated the toxin production under all packaging methods.

Hintlian and Hotchkiss (1986) suggested that increase in the growth of potential *Clostridium* under MAPs was an alarming issue and many researchers were aware of it. *Clostridium perfringens* was basically responsible for gastrointestinal diseases while *Clostridium botulinum* produces a neurotoxin that causes facial paralysis. *Clostridium botulinum* is classified into types A, B, C, D, E, F and G where the types A, B and F are important to humans.

## 2.5 MAP and shelf life extension of fishery products

Arvanitoyannis and Stratakos (2012) concluded that MAP can certainly lead to the supply of high quality fishery products due to significant shelf life extension by reducing potential economic losses and thereby, ensures product stability.

Therefore, it can be concluded that the vacuum and more importantly the modified atmosphere packaging (MAP) of fresh fish will ensure an extended shelf life of fish and fishery products under refrigerated temperature. Therefore, this new technology can be facilitated by retailers, superstores and most importantly the fish processors having refrigeration or icing facilities. In addition, it will also ensure the supply of fresh fish to the consumer in a convenient way by ensuring food safety.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Sample Collection and Preparation

Live Rohu fishes (*Labeo rohita*) with an average size of 1.5±0.3 kg were collected from Shaheb Bazaar, Rajshahi. The experiment was carried out in the quality control laboratory under Department of Fisheries, University of Rajshahi from September 2018 to December 2018. The samples were transported in an insulated box in properly iced condition to the Quality Control laboratory of Department of Fisheries, University of Rajshahi. Then the whole fishes were washed using tap water, be-headed, de-scaled, gutted and cut into small slices with an average weight of approximately 100g each piece (shown in Plate 1). Then the sliced Rohu fishes were washed with tap water and the finally washed using distilled water.

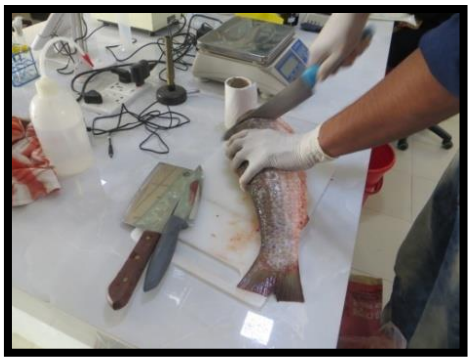

Plate: (A) Rohu fish was cut for sample preparation

Plate: (B) Sliced Rohu fishes were further cut into small pieces

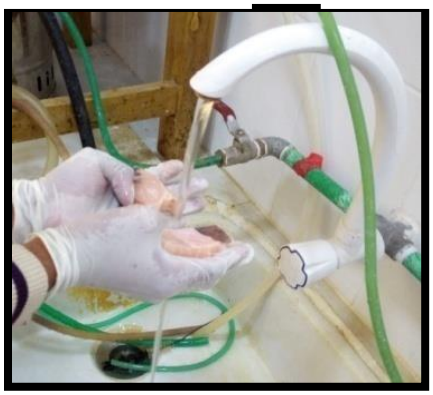

Plate: (C) Sliced Rohu fishes were washed by running tap water

**Plate 1: Preparation of fish samples (*Labeo rohita*) before inserting pieces of fish into the various packaging conditions**

## 3.2 Packaging and Storage of Samples

The required quantity of Rohu slices were packed under vacuum and modified atmosphere packaging by using low gas and moisture permeable plastic pouch (shown in Plate 2). Multi-layer transparent pouch having 100 µm density was used as packaging material. Four types of packaging were applied with different gas ratio using the method described by Noseda and others (Noseda *et al.*, 2012). These four types packaging were used as treatments, namely: (i) aerobic, (without sealed pack) as Treatment-0 (control); (ii) vacuum as treatment-1; (iii) MAP 1 (50% $CO_2$ & 50% $N_2$) as treatment-2 and (iv) MAP 2 (50% $CO_2$ & 50% $O_2$) as treatment-3. Vacuum and MA packaging were done using a packaging machine (C100 Multivac, Haggenmuller, Germany) attached with Gas Mixer (KM100-3MEM, WITT, Germany) by following the standard instruction of the manufacturer. Monitoring and analysis of the $O_2$, $N_2$ and $CO_2$ levels in the headspace of the packaged samples were regularly performed with a gas analyzer (Oxybaby M+, WITT, Germany) before and after storage at 4°C. All samples were stored at 4°C in a laboratory refrigerator. Three samples from each packaging condition were analyzed at every two days interval for 18 days at 4°C (0day, 3day, 6day, 9day, 12day, 15day and 18day).

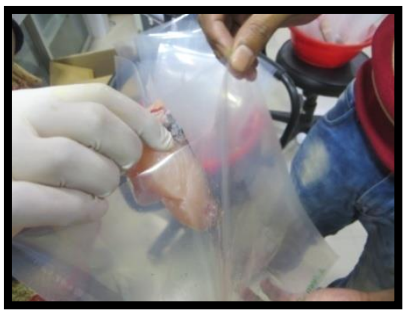

Plate: (A) Samples were placed in plastic pouches

Plate: (B) Packaging was done by Multivac packaging machine

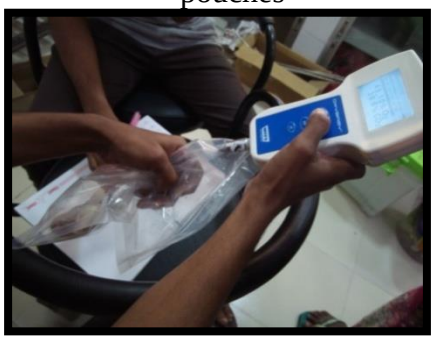

Plate: (C) Gas ratios of sealed packages were checked

Plate: (D) All packed samples were stored in refrigerator at 4°C

**Plate 2: Packaging of sliced Rohu fish (*Labeo rohita*) by Multivac packaging machine and gas composition analysis by Oxybaby gas analyzer**

## 3.3 Biochemical Analysis

Biochemical parameters such as (pH, TVB-N, PV and TBARS) were analyzed in order to know the quality of packaged sliced Rohu fishes and to determine the shelf-life of sliced fishes at refrigerated storage.

### 3.3.1 Analysis of pH Value

The flesh of the sliced Rohu fish was cut into small pieces from each pack stored in the refrigerator. Ten (10) grams of cut flesh was homogenized with 50 mL of distilled water and the pH value of the homogenate was measured by a glass electrode pH meter (HI2002-Edge, Hanna Inst, USA) (shown in Plate 3).

Plate: (A) 50ml of distilled water was added with 10 grams of sample

Plate: (B) Homogenized the sample in a blender

Plate: (C) Determined the sample pH

**Plate 3: pH determination of sliced Rohu (*Labeo rohita*) by HANNA pH meter**

### 3.3.2 Estimation of Total Volatile Base Nitrogen (TVB-N) Value

Total volatile base nitrogen (TVB-N) value was estimated according to the EC (2005) method. The flesh of the sliced rohu fishes were cut into small pieces from each of the pack and carefully ground using a blender. Ten (10) g of the ground fish sample was weighed into a suitable container and mixed with 90 mL of 6% perchloric acid solution, homogenized for two minutes with a blender (Bajaj, India), and then filtered to obtain extract. Fifty (50) mL of extract was taken in a Kjeldahl flask and 8-10 drops of phenolphthalein indicator was added in the flask. After adding some glass-beads, 6.5 mL of 20% NaOH or required amount was added to the flask after placing on the Kjeldahl distillation unit (GSGW, India). Immediately the steam distillation was started.

Plate: (A) Homogenized sample was filtered after blending

Plate: (B) Added 6.5 mL of 20% NaOH to the Kjeldahl flask

Plate: (C) Kjeldahl flasks were placed on the Kjeldahl apparatus

**Plate 4: Estimation of TVB-N value of sliced Rohu (*Labeo rohita*) by Kjeldahl apparatus**

The steam distillation was regulated in order to collect 100 mL of distillate in ten minutes. The distillation outflow tube was submerged in a receiver with 100 mL with 3% boric acid solution, to which 3-5 drops of the mixed indicator solution (2 g methyl-red and 1 g methylene-blue were dissolved in 1000 mL 95 % ethanol) were added. After 10 minutes, distillation process was ended. Then distillation outflow tube was removed from the receiver and washed out with water. The volatile bases contained in the receiver solution were determined by titration with 0.01 (N) HCl solution. The pH of the end point should be 5.0 ±0.1. A blank test was conducted by the same procedure without using sample.

The TVB-N concentration was calculated using the following equation:

$$\text{TVB} - \text{N}\ (\text{mg}/100\text{g sample}) = \frac{(V_1 - V_0) \times 0.14 \times 2 \times 100}{M}$$

Where,

$V_1$ = Volume of 0.01 (N) HCl in mL for sample

$V_0$ = Volume of 0.01 (N) HCl in mL for blank

M = Weight of sample in g

### 3.3.3 Determination of Peroxide Value (PV)

**Preparation of Iron (II) Chloride Stock Solution:** At first, 0.4 g barium chloride dehydrate was dissolved in 50 mL deionized water. This solution was added slowly and with constant stirring to an iron (II) sulfate solution (0.5 grams of $FeSO_4.7H_2O$ in 50 mL deionized water). 2 mL of 10N HCl was added to the resulting solution. The barium sulfate precipitated was filtered off to give clear iron (II) solution, which was stored in a brown bottle and kept in the dark.

**Preparation of Ammonium Thiocyanate Solution:** Thirty (30) g of ammonium thiocyanate was dissolved in distilled water, and the volume was made up to 100 mL.

After preparation of those above mentioned solutions, Peroxide value was determined by IDF standards (1991) described by Shantha and Decker (1994) using fish oil. For this purpose, firstly the oil was extracted by means of Soxhlet apparatus (JSGW, Haryana, India) by following standard method of AOAC (AOAC 1995). About 10–15 g of chopped fish sample was taken in thimble paper and kept in an oven at 105°C for 5 hours for drying. Then oil was extracted with 200 mL of diethyl ether in the soxhlet apparatus, followed by solvent removal under reduced pressure at 70°C using rotary evaporator (HAHNVAPOR, HS- 2005V, Korea).

Plate: (A) Fish oil was extracted through soxhlet apparatus

Plate: (B) Evaporation of fish oil was done in a rotary evaporator

**Plate 5: Peroxide value determination by fish oil extraction through soxhlet apparatus followed by evaporation in rotary evaporator**

To determine the peroxide value, 0.01-0.30 g sample fish oil was taken in a test tube and added 9.8 mL chloroform: methanol (7:3) mixture and then mixed on a vortex for 2-4 seconds. Ammonium thiocyanate solution (50 µL) was added and mixed on a vortex for 2-4 seconds. Then 50 µL iron (II) solution was added and mixed again on a vortex for 2-4 seconds. After 5 minutes of incubation at room temperature, the absorbance of the sample was determined at 500 nm against a blank that contained all the reagents except the sample by using an UV-visible spectrophotometer (UV- 1601 PC, Shimadzu, Japan). The entire procedure was conducted in subdued light. The peroxide value, expressed as milliequivalents of peroxide per kilogram of sample.

Peroxide value was calculated by using the following formula:

$$\text{Peroxide value (mili Eq/kg fish oil)} = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2}$$

Where,

$A_s$ = absorbance of the sample

$A_b$ = absorbance of the blank

m = slope, obtained from calibration curve (m used here was 41.52 for IDF method conducted by Shantha and Decker, 1994)

$m_0$ = mass in grams of the sample

55.84 = atomic weight of iron.

### 3.3.4 Estimation of Thiobarbituric Acid Reactive Substance (TBARS) value

TBARS value was measured according to the procedure of (Witte *et al.*, 1970). Twenty (20) g of sliced Rohu flesh was homogenized with 50 mL of 20% trichloroacetic acid (in 2 M phosphoric acid) at 1000 rpm for two minutes using a homogenizer (IKA T18 digital ULTRA TURRAX, Staufen, Germany). The resulting slurry was then transferred into a 100 mL mass cylinder. The slurry was diluted to 100 mL with de-ionized distilled water and homogenized again. After approximately 50 mL was filtered through filter paper (Whatman No. 1, 100 nm), 5 mL of filtrate was transferred into a test tube and 5 mL of 2- thiobarbituric acid (0.005 M in DD water) was added. The test tube was shaken well and kept in the dark for 15 hours at room temperature. The reactive substances were measured at 530 nm using a spectrophotometer UV-Visible Spectrophotometer (UV-1601 PC, Shimadzu, Japan). Two replicates of 20 g sample were taken for the measurement.

TBARS value was calculated as follows:

TBARS value (mg malonaldehyde/kg) = optical density (O.D) ×5.2

Plate: (A) Homogenized the fish sample in a homogenizer

Plate: (B) Homogenized sample was filtered with a filter paper

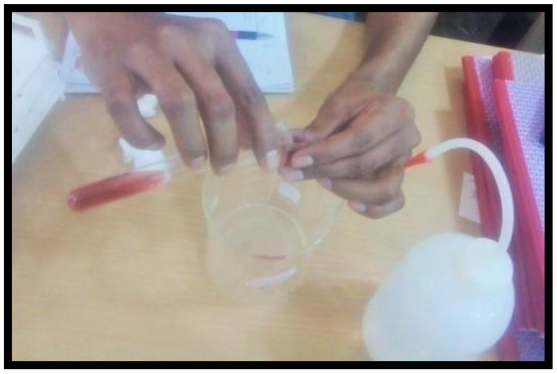

Plate: (C) After keeping the samples in the dark for 15 hours they were placed in small tubes

Plate: (D) Reactive substances absorbance were measured in spectrophotometer

**Plate 6: Estimation of TBARS value of sliced Rohu (*Labeo rohita*) by spectrophotometer**

## 3.4 Microbiological Analysis

### 3.4.1 Preparation of Peptone Physiological Saline (PPS) Solution

For the preparation of each 1000 mL of peptone physiological saline (PPS) solution, one (1) g of peptone and 8.5 g of NaCl (sigma-Aldrich, USA) were weighed on an electric balance and taken in a glass bottle containing required amount of distilled water to dissolve and make the volume 1000 mL (shown in Table-1).Then the mixture was mixed properly on magnetic stirrer. Nine (9) mL of PPS was transferred to each of the test tubes. The PPS tubes were then sterilized in an autoclave (Tomy Digital Biology, Japan) for 15 minutes at 121$^{o}$C under 15 lbs/sq inch pressure.

(A) Peptone Physiological Saline (PPS) was prepared.

(B) PPS was placed in an autoclave for sterilization

Plate: (C) Autoclaving was done for 15 min at 121$^{0}$C

**Plate 7: Preparation of Peptone Physiological Saline (PPS) solution**

**Table 1**: **The composition of the PPS solution and plate count agar (1 L)**

| Ingredients | PPS | Plate count agar |
|---|---|---|
| Peptone | 1 g | - |
| NaCl | 8.5 g | - |
| Plate count agar | - | 23.5 g |
| Distilled water | Up to 1000 mL | Up to 1000 mL |

### 3.4.2 Media Preparation

For bacterial count, plate count agar (Sigma-Aldrich, USA) was used. For this purpose, 23.5 g of agar was weighed by an electric balance and taken in prescribed amount of distilled water to dissolve and make the volume 1000 mL (shown in Table 1). Then the mixture was mixed properly with magnetic stirrer. In case of plate count agar, the mixture was boiled and stirring with a stick so that the ingredients mix thoroughly. Then the media was sterilized for 15 minutes at 121$^{o}$C under 15 lbs/sq inch pressure.

**Plate 8: Preparation of Media (Agar)**

### 3.4.3. Preparation of Sample for Microbial Analysis

The sliced Rohu fishes were cut into small pieces by using knife from each of the packages which were stored at refrigerator (4°C) on the sampling day. Twenty five (25) g of sample from each pack was aseptically collected in a sterile stomacher bag

with 225 mL of peptone physiological salt solution (PPS) (up to 10 times of the sample) to obtain decimal dilution. Blending was done for 30 seconds in a stomacher blender (SJIA-04C, Led Techno, China) (shown in Plate 9).

Thus, a sample of 1:10 dilution was obtained. One (1) mL sample was then transferred by micropipette to a test tube containing 9mL of PPS and the test tube was properly shaken on a vortex mixture thoroughly. Using similar process, several ten folds dilutions were made up to desired level.

Plate: (A) Fish sample was diluted by adding PPS

Plate: (B) Diluted sample was blended in a stomacher blender

**Plate 9: Preparation of sliced Rohu (*Labeo rohita*) sample for microbial study**

### 3.4.4. Total Viable Count (TVC)

Total viable count (TVC) expressed as colony forming units (CFU/g) of the representative samples were determined by standard plate count method on plate count agar following the serial dilution technique described by (APHA, 1992). Microbiological data were transformed into logarithms of the number of colony forming units (log CFU/g).

At first, 1 mL of prepared, well shaken diluted sample was transferred to empty plates using micropipette (Fig. 9). Samples were pipette out and transferred aseptically to the plates by raising the upper lids sufficiently enough to admit the tip of the pipette. Then the required amount of prepared plate count agar (cooled to 45°C± 1°C using water bath) was poured to the plates (shown in Plate 10). All of these activities were performed inside a laminar air flow cabinet (BBS-V1300, Biobase). At least three appropriate dilutions were enumerated for all cases. All the plates were inoculated in

duplicate. After solidifying the agar, the plates were incubated at 35°C in inverted position in an incubator (Binder, Germany). After 48±2 hours of incubation, colonies were developed and only the plates having 30-300 colonies were counted by using colony counter (Labotronics, India) (shown in Plate 11). All the plates were examined visually for typical colony types and morphological characteristics associated with each growth medium.

Plate: (A) Samples were taken from stomacher bag

Plate: (B) Samples were transferred in PPS tubes

Plate: (C) Samples were transferred in plates

Plate: (D) Plates were prepared by following pour plate technique

**Plate 10: Bacterial sample transfer in PPS tubes for dilution followed by bacterial culture in agar medium by pour plate method**

The result was performed from following formula-

$$N(CFU/g) = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2)] \times (d)}$$

Where,

N= Number of colonies per mL or g of product (CFU/g)

∑C= Sum of all colonies on all plates counted

$n_1$= Number of plates in first dilution counted

$n_2$=Number of plates in second dilution counted

d= Dilution from which the first counts were obtained

Plate: (A) Prepared plates were incubated at $35^0$C for 48 hrs in dry air oven


Plate: (B) Bacterial colony was counted in a digital colony counter

**Plate 11: Incubation and counting of bacterial colonies**

## 3.5 Statistical Analysis

All the trials were replicated three times. The values were expressed as mean ± standard deviation. Differences among treatments were estimated by using one-way ANOVA with the application of Tukey test using SPSS Version 20. Average values were considered significantly different when $p<0.05$.

## 3.6 Instruments and Appliances

Various instruments and appliances were used in the laboratory during the completion of this experiment. Some of the key instruments photographs are as follows:

**Plate 12: Showing drying oven (on left) and incubator (on right) respectively**

**Plate 13: Showing autoclave (on left) and stomacher blender (on right) respectively**

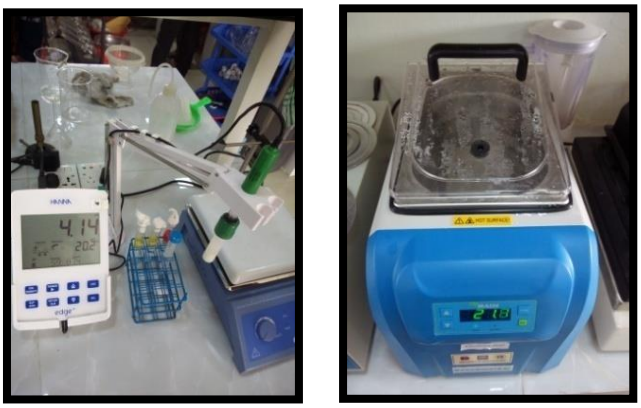

**Plate 14: Showing $p^H$ meter (on left) and water bath (on right) respectively**


**Plate 15: Showing vortex machine (on left) and digital colony counter (on right) respectively**

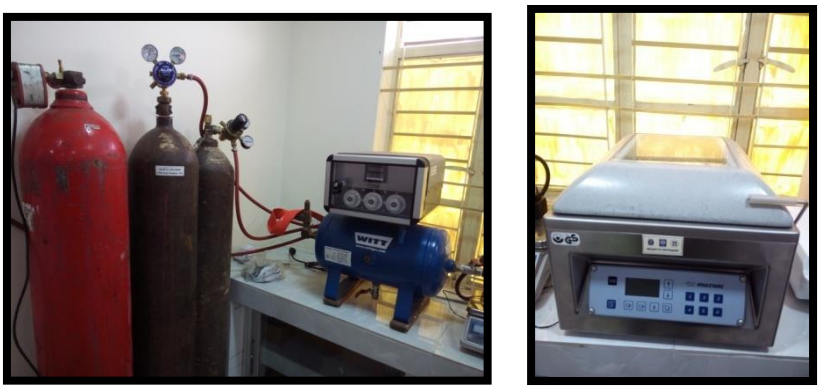

**Plate 16: Showing gas mixture machine (on left) and packaging machine (on right) respectively**

**Plate 17: Showing PC connected spectrophotometer (on left) and soxhlet apparatus (on right) respectively**


**Plate 18: Showing plate count agar bacterial culture medium used in the study**

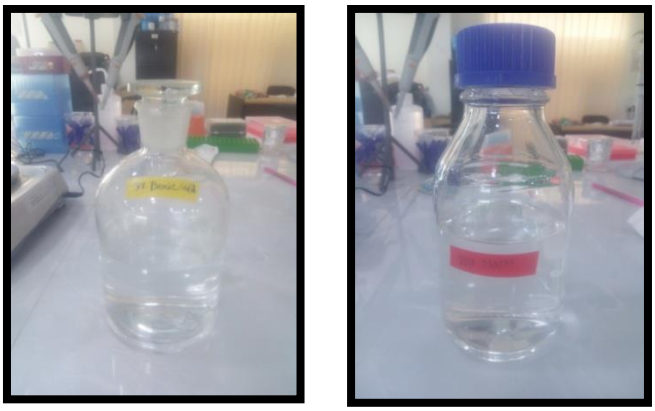

**Plate 19: Some key chemicals used in the study, 3% Boric acid (on left) and 20% NaOH (on right) respectively**

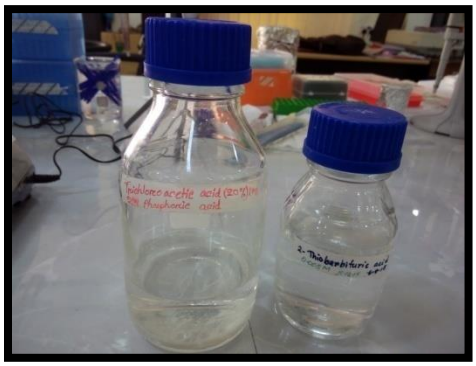

**Plate 20: Some key chemicals used in the study, 20% trichloroacetic acid (in 2 M phosphoric acid) (on left) and 2- thiobarbituric acid (0.005 M in DD water) (on right) respectively**

# CHAPTER 4
# RESULTS AND DISCUSSION

The present study was conducted to identify the optimum condition for preserving fresh Rohu fish at refrigerated storage using vacuum and modified atmosphere packaging techniques. For this purpose, different quality parameters were evaluated such as pH, TVB-N, Peroxide and TBARS value as well as the growth of bacteria in fresh Rohu fish stored at refrigeration temperature (4°C) using different packaging conditions. As a result, all of these evaluations determined the optimum condition of preservation as well as the shelf life for fresh Rohu fish at different packaging conditions.

## 4.1 pH Value

The initial pH value of sliced Rohu fish was 6.50 immediately after death in sliced samples. The pH value was gradually decreased until 3$^{rd}$ days of storage for without sealed pack samples which was treated as control, 6$^{th}$ day for vacuum and MAP-2 sample and 9$^{th}$ day of storage for MAP-1 samples, and then pH values showed an increasing trend with some fluctuations (shown in Table 2). pH values in all storage conditions were within the range of 6.8~7.0 (Metin *et al.*, 2001).

There were no significant ($p<0.05$) differences in changes in pH values among four packaging conditions during the storage period. The lower pH value of fish packaged with MAP condition at a higher concentration of $CO_2$ was reported in other studies (Stamatis and Arkoudelos, 2007; Masniyom *et al.*, 2002; Provincial *et al.*, 2010). The results obtained from the present study is more or less similar phenomenon those reported in previous studies. In vacuum packaging and MAP, further acidification perhaps caused by lactacidogenic bacteria. This is connected with the inhibition of gram-negative aerobic bacteria (mainly pseudomonads), and they become predominant during the course of the storage period as their number increases (Leroi, 2010).

In contrary, under both MAP-1 and MAP-2 pack sample, gradual decrease of pH value was evident until 9$^{th}$ day of storage, followed by a gradual increase until the end of the storage period. In both cases of MAP, the initial drop of pH value may be (by 6$^{th}$ day of storage) occurred due to the dissolution of $CO_2$ in muscle tissues.

Moreover, surface of fish muscle absorbs $CO_2$, thus acidifying it with the formation of carbonic acid (Banks *et al*, 1980). Previous studies found that the consequent increase of pH at later stages was usually coupled with the production of basic components such as ammonia, dimethylamine, trimethylamine, other biogenic amines, and also due to the results of microbial spoilage (Goulas and Kontominas, 2007, Fan *et al.*, 2008). When the pH value compared with different MAP system, relatively lower $p^H$ values were found in MAP 1 than MAP-2 throughout the entire storage period. The lower pH value of muscle tissue in MAP1 (shown in Table 2) perhaps reflected by $CO_2$ increase during the course of storage**.**

**Table 2: pH value of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 day | 3 days | 6 days | 9 days | 12 days | 15 days | 18 days |
| Control | 6.50±0.18$^{a}$ | 6.14±0.04$^{a}$ | 6.48±0.04$^{a}$ | 6.22±0.04$^{a}$ | | | |
| Vacuum pack | 6.50±0.18$^{a}$ | 6.25±0.05$^{ab}$ | 6.24±0.01$^{a}$ | 6.36±0.09$^{a}$ | 6.60±0.06 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 6.50±0.18$^{a}$ | 6.35±0.07$^{ab}$ | 6.16±0.10$^{a}$ | 6.12±0.06$^{a}$ | 6.39±0.19 | 6.37±0.18 | 6.44±0.06 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 6.50±0.18$^{a}$ | 6.41±0.04$^{b}$ | 6.25±0.16$^{a}$ | 6.27±0.07$^{a}$ | 6.58±0.02 | 6.38±0.04 | 6.77±0.11 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

## 4.2 Total Volatile Base Nitrogen (TVB-N) Value

The total amount of ammonia ($NH_3$), dimethylamine (DMA) and trimethylamine (TMA) in fish as a whole is termed as total volatile base nitrogen (TVB-N) content of fish and is commonly used to estimate bacterial spoilage of fish (Wu and Bechtel, 2008). TVB-N also used as an indicator to assess the quality and shelf-life of fish and fishery products.

In the present study, the initial TVB-N value was 1.18 mg/100g in sliced Rohu fish. The gradual increase in TVB-N value was evident during the storage period (shown in Table 3). The maximum TVB-N value at the end of the experiment was found 6.04 mg on $18^{th}$ day of storage for MAP-1 pack sample.

However, in the present study, the TVB-N values were found within the acceptable limit (shown in Table 3) in all packaging conditions (30-35 mg/100g) as suggested by Huss, (1988). According to past study, it was stated that TVB-N value less than 20 mg/100g is considered as safe for human consumption (Jezek and Buchtova, 2010). In the present study the TVB-N values ranged from 1.18-5.44 mg/100g, 1.18-6.04 mg/100g, 1.18-5.46 mg/100g while preserved in vacuum packaging, MAP1 and MAP2 respectively. Therefore, this result indicates that all three preservation system can ensure safe limit of TVB-N value for human consumption.

There were no significant differences ($p<0.05$) in relation to TVB-N values among four packaging conditions until the $9^{th}$ day of the storage (shown in Table 3). Significant lower ($p < 0.05$) TVB-N values were recorded at $9^{th}$ day of the storage for MAP-1 and MAP-2 pack samples as compared to control samples. It was evident that the samples which were packed in MAP showed slower increase of TVB-N value which is similar to previous study result in order to preserve silver carp fillets at $4^0$C (Rahmatipoor *et al.,* 2017).

Among the MAP samples used in the present study, MAP1 (50% $CO_2$ & 50% $N_2$) showed better performance than MAP2 (50% $CO_2$ & 50% $O_2$) from start to end of the storage period. Similar results were reported by Jezˇek and Buchtova´ (2012) while preserved silver carp fillets where the MAP-2 (70% $N_2$, 30% $CO_2$) with higher $CO_2$ and $N_2$ concentrations showed better TVBN performance than MAP-1 (69% $N_2$, 25% $CO_2$, 5%$O_2$, 1% CO).

Better performance of MAP1 in present study may be due to the bacteriostatic properties of $CO_2$. The presence of carbon dioxide gas is responsible for partial prevention and delay of the growth of spoilage bacteria (Farber, 1991). The presence of $O_2$ in MAP2 package may accelerate the growth of aerobic bacteria and eventually speeds up the spoilage process. TVB-N value depends on a number of factors such as feeding, season, size and others environment parameters (Binsi *et al*., 2015).

**Table 3: TVBN value of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 1.18±0.48[a] | 2.38±0.59[a] | 3.36±0.79[a] | 3.90±0.76[ab] | | | |
| Vacuum pack | 1.18±0.48[a] | 2.66±0.99[a] | 3.92±0.79[a] | 5.05±0.4[b] | 5.44±0.15 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 1.18±0.48[a] | 1.82±0.59[a] | 2.50±1.16[a] | 2.94±0.59[ab] | 3.30±0.31 | 4.20±0.57 | 6.04±0.57 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 1.18±0.48[a] | 2.10±0.59[a] | 2.64±0.17[a] | 2.66±0.20[a] | 3.30±0.71 | 4.62±0.59 | 5.46±0.59 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

## 4.3 Peroxide Value (PV)

Lipid oxidation is one of the prime causes of fish spoilage. It affects fatty acids, particularly polyunsaturated fatty acids and produce off-odours and off-flavours which is unpleasant for the consumer (Ferna´ndez *et al.,* 1997). Fish lipid is susceptible to oxidation. Peroxide value is a measure of hydro peroxides formation. It indicates products that are formed during primary lipid oxidation. Peroxide value is widely used as an indicator for the assessment of degree of primary lipid oxidation and the peroxide values expressed as mill moles or mill equivalents of active oxygen per kg of fat (Masoud *et al.,* 2008).

In the present study, the initial peroxide value was 4.93 mEq/Kg fish oil in sliced Rohu fish. However, the values varied between 1.24-8.18 mEq/kg fish oil during the storage period. The peroxide values of sliced Rohu fish were within the recommended values of 10-20 mEq/kg of fish oil which is the acceptable limit as suggested by (Connell, 1995). Significant lower ($p<0.05$) PV values were observed in vacuum samples on $9^{th}$ day of storage compared to that of control samples (shown in Table 4**).** In vacuum packaging, lipid oxidation can be delayed by limiting access to oxygen thus preserving the quality of muscle foods (Etemadianet al., 2012).

The PV showed significant fluctuations from the beginning till the end of the storage period in all packaging conditions. Similar fluctuations of PV were reported by some other authors (Ozyurt *et al.,* 2009; Bahmani *et al.,* 2011; Jezˇek and Buchtova´, 2011). The initial PV indicates that the oxidation process already started during the period of handling and processing, and a consequent decline in PV is then caused by competing reactions and increases in thiobarbituric acid values (Chaijan 2011).

Lower PV was recorded for MAP-1 compared to that of MAP-2 pack samples during the end of the storage period. This may be due to the presence of $O_2$ in MAP-2 pack (50% $CO_2$ & 50% $O_2$) that triggered the oxidation process and formed hydroxides, hydro peroxides during the end of the storage period. Similar results were found by Jezˇek and Buchtova´, (2012) in silver carp fillets and the progress of primary lipid oxidation was rather erratic as in this study.

**Table 4: Peroxide value (PV) of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 4.93±2.08$^{a}$ | 2.87±0.04$^{a}$ | 4.93±0.40$^{a}$ | 5.54±0.40$^{bc}$ | | | |
| Vacuum pack | 4.93±2.08$^{a}$ | 5.37±2.03$^{ab}$ | 7.04±1.08$^{a}$ | 1.33±0.42$^{a}$ | 1.45±0.16 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 4.93±2.08$^{a}$ | 8.18±1.26$^{b}$ | 5.43±0.95$^{a}$ | 7.04±2.39$^{c}$ | 3.94±0.21 | 1.24±0.23 | 1.33±0.13 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 4.93±2.08$^{a}$ | 5.37±2.40$^{ab}$ | 6.83±0.88$^{a}$ | 2.59±0.99$^{ab}$ | 3.40±1.64 | 3.75±0.36 | 3.13±3.12 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

Lipid oxidation limits the shelf life of oily fish as suggested by (Hossain *et al.*, 2005). The rate and extent of oxidative deterioration depends on the factors such as the as fish species, storage period and temperature, saturation degree of fatty acids, antioxidants or prooxidants and availability of oxygen (Bahmani *et al.*, 2011). However, PV alone cannot be considered as a suitable fish muscle freshness indicator as suggested by (Jezˇek and Buchtova´, 2007).

## 4.4 Thiobarbituric Acid Reactive Substances (TBARS)

TBARS is a procedure which evaluates degree of secondary lipid oxidation and thus quality of food. TBARS index is used to measure the amount of malonaldehyde which is the secondary product of the oxidation of polyunsaturated fatty acids (Bremner, 2002). The second stage of auto-oxidation in which modification of peroxide occurs, results in production of materials such as aldehydes and ketones (Feliciano *et al.*, 2010).The acceptable limit of TBARS value is 2 mg malonaldehyde/kg fish sample and beyond this limit, an objectionable odor and taste develops in fish (Connell, 1990).

In the present study, the initial TBARS value was 0.23 mg malonaldehyde/kg in sliced Rohu fish. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. However, significant ($p<0.05$) lower TBARS values were observed on 3$^{rd}$ and 6$^{th}$ day of storage in vacuum, MAP1 and MAP2 pack samples in comparison to control samples. Significant ($p<0.05$) higher TBARS values were also observed on 9$^{th}$ day of storage in case of MAP-2 sample as compared to other samples. In the present study, the TBARS values were within the acceptable limit (2 mg malonaldehyde/kg) in all samples except MAP-2 sample.

The TBARS value of MAP-2 pack sample exceeded the acceptable limit of 2 mg on and after 9$^{th}$ day of the storage (shown in Table 5). This may be the results of higher rate of secondary lipid oxidation due to the presence of high $O_2$ concentration in MAP-2 (50% $CO_2$ & 50% $O_2$) packed samples. Arashisar *et al.* (2004) also found similar results in rainbow trout fillets packaged with 30% $O_2$ as compared to the MAP with no oxygen. $O_2$ not alone but also some bacterial enzymes also participate in oxidation process (Herna´ndez *et al.*, 2009).

**Table 5: TBARS value of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 0.23±0.11[a] | 0.56±0.01[b] | 1.08±0.10[c] | 1.17±0.07[b] | | | |
| Vacuum pack | 0.23±0.11[a] | 0.41±0.03[a] | 0.17±0.06[a] | 0.46±0.05[a] | 0.74±0.07 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 0.23±0.11[a] | 0.45±0.04[a] | 0.53±0.17[b] | 1.09±0.02[b] | 0.78±0.11 | 1.40±0.05 | 2.70±0.12 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 0.23±0.11[a] | 0.56±0.04[b] | 0.90±0.12[c] | 2.38±0.17[c] | 3.69±0.47 | 3.27±0.54 | 5.40±0.17 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

Substances of foul odor or taste are often evident during secondary oxidation of lipids. Some such substances may be responsible for the textural changes particularly when they form covalent bonds with muscle proteins (Huss, 1995). Thiobarbituric acid is a more suitable indicator of the degree of fish muscle oxidation than PV as malonaldehyde may interact with other components such as nucleosides, nucleic acids, proteins and other aldehydes. Moreover, Thiobarbituric acid value need not always accurately reflect the current level of lipid oxidation (Ozyurt *et al.,* 2009).

## 4.5 Total Viable Count (TVC)

In the present study, the initial total viable count (TVC) of sliced Rohu fish was found 4.29 log CFU/g which indicated an acceptable quality of fish. It was evident in the literature that freshly caught fresh water fishes (sea bass, tilapia, rainbow trout, and silver perch) contain bacterial counts of 2-6 log CFU/g (Gelman *et al.,* 2001).

The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. However, significant ($p<0.05$) lower TVC values were recorded on $9^{th}$ and $12^{th}$ day of storage in vacuum, MAP1 and MAP2 packaged samples compared to that of control. Similar results were observed in the studies of Babic *et al.* (2015) on common carp steaks kept under MAP comprising (MAP- 1: 40% $CO_2$/60% $N_2$ and MAP- 2: 100% $CO_2$) followed by storage at +3±0.5°C, although in our study the MAP2 composition was (50% $CO_2$ & 50% $O_2$). Hudecová *et al.* (2010) also reported similar results on fresh common carp under two different modified atmosphere packaging (MAP-1: 70% $N_2$/30% $CO_2$; MAP-2: 80% $O_2$/20% $CO_2$) and air (control samples) stored at 4 ± 0.5°C where MAP1 and MAP-2 showed better performance than the control sample which is in relevance to our present study.

Higher shelf life was observed in both MAP samples in the present study. This may be due to the bacteriostatic effect of $CO_2$ in MAP packaging. In a number of studies, the effect of various $CO_2$ concentrations showed delayed microbial growth as concluded by various researchers such as in Swordfish (Pantazi *et al.,* 2008), Cod (Sivertsvik, 2007), Chub Mackerel (Stamatis and Arkoudelos, 2007), sea bass (Poli *et al.,* 2006), Sardines (Özogul *et al.,* 2004), rainbow trout (Arashisar *et al.,* 2004; Randell *et al.,* 1997), herring (Özogul *et al.,* 2000; Randell *et al.,* 1997), and salmon (De la Hoz *et al.,* 2000). The bacteriostatic effect of $CO_2$ in modified atmospheres

results in a decrease in the growth rate of bacteria during the logarithmic stage (Farber, 1991).

Overall, best quality was observed under MAP1 (50% $CO_2$ & 50% $N_2$) in comparison to other samples. MAP-1 packaging creates anaerobic condition inside the pack; as a result aerobic bacterial growth is inhibited and the shelf-life is prolonged. Additionally, $CO_2$ has bacteriostatic effect. It also retards the oxidative rancidity and inhibits the growth of aerobic microorganisms by displacing oxygen from packaging (Farber, 1991).

TVC values exceeded the 7 log CFU/g, which is considered as the upper acceptable limit (ICMSF, 1986) for fresh and frozen fish, about $8^{th}$ day for control, $11^{th}$ day for vacuum, $16^{th}$ day for MAP-1 and $13^{th}$ day of storage for MAP-2 sample (shown in Figure.1). Considering the bacterial counts, the shelf-life of sliced Rohu (*Labeo rohita*) in refrigerated temperature was in acceptable conditions for 8 days in control, 11 days in vacuum pack, 16 days in MAP-1, and 13 days in MAP-2 sample.

**Table 6: Total viable count of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C)**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Not sealed (Control) | $4.29\pm0.09^{a}$ | $4.31\pm0.08^{a}$ | $4.67\pm0.10^{a}$ | $7.33\pm0.45^{b}$ | $8.23\pm0.13^{c}$ | | |
| Vacuum pack | $4.29\pm0.09^{a}$ | $4.18\pm0.10^{a}$ | $4.70\pm0.25^{a}$ | $6.38\pm0.09^{ab}$ | $7.33\pm0.14^{b}$ | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | $4.29\pm0.09^{a}$ | $5.00\pm0.73^{a}$ | $5.44\pm0.02^{b}$ | $5.70\pm0.12^{a}$ | $6.14\pm0.07^{a}$ | 6.77±0.41 | 7.67±0.16 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | $4.29\pm0.09^{a}$ | $5.24\pm0.01^{a}$ | $5.32\pm0.06^{b}$ | $5.31\pm0.32^{a}$ | $6.64\pm0.27^{a}$ | 7.65±0.04 | 7.49±0.11 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

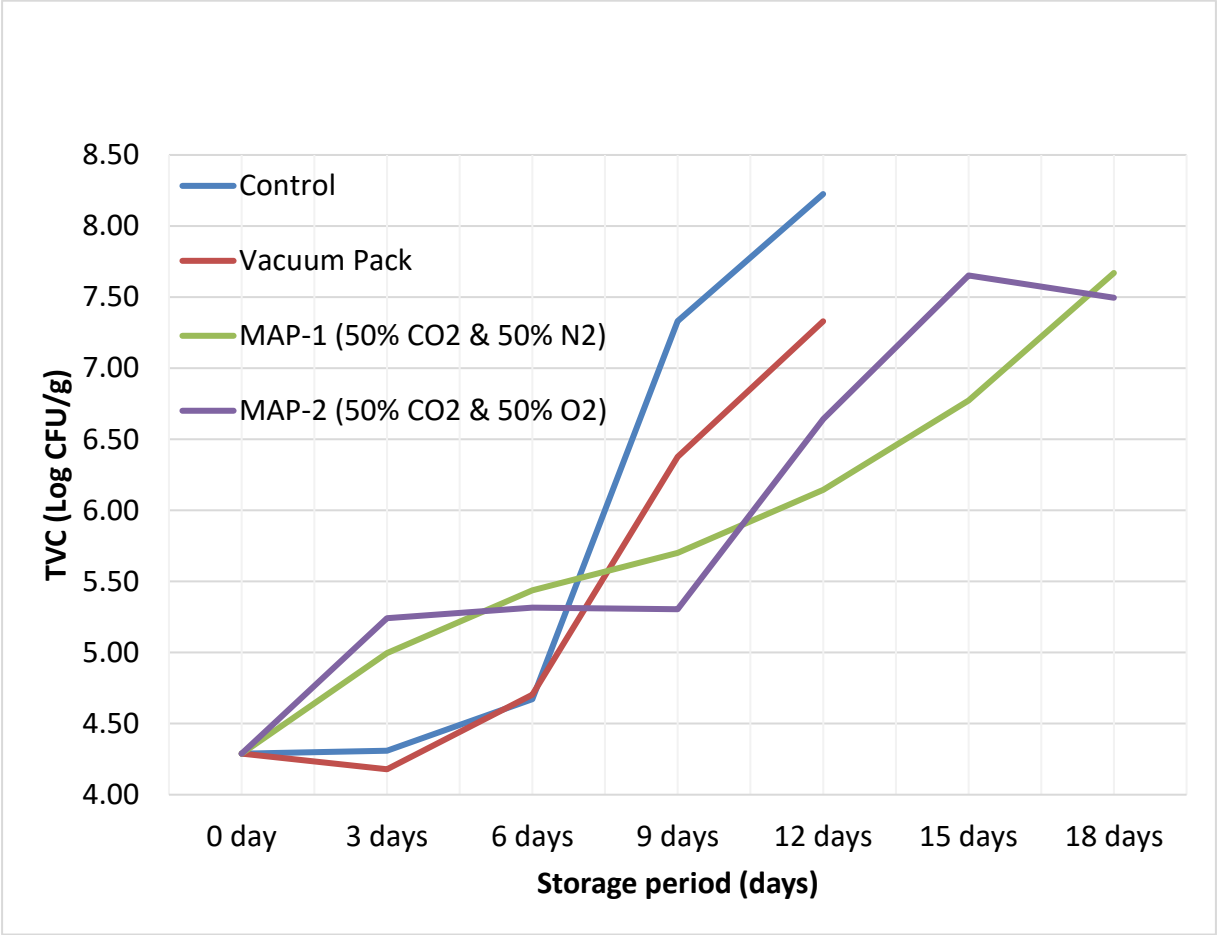


**Figure 1: Total viable count of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C)**

# CHAPTER 5

# SUMMARY AND CONCLUSION

The present study was conducted to identify the optimum condition for preserving fresh Rohu fish at refrigerated storage using vacuum and modified atmosphere packaging techniques. The initial pH value of sliced Rohu fish was 6.50 immediately after death in sliced samples. The pH value was gradually decreased until 3$^{rd}$ days of storage for without sealed pack samples which was treated as control, 6$^{th}$ day for vacuum and MAP-2 sample and 9$^{th}$ day of storage for MAP-1 samples, and then pH values showed an increasing trend with some fluctuations. There were no significant ($p<0.05$) differences in changes in pH values among four packaging conditions during the storage period.

In the present study, the initial TVB-N value was 1.18 mg/100g in sliced Rohu fish. The gradual increase in TVB-N value was evident during the storage period (shown in Table 3). The maximum TVB-N value at the end of the experiment was found 6.04 mg on 18$^{th}$ day of storage for MAP-1 pack sample. There were no significant differences ($p<0.05$) in relation to TVB-N values among four packaging conditions until the 9$^{th}$ day of the storage.

In the present study, the initial TBARS value was 0.23 mg malonaldehyde/kg in sliced Rohu fish. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. However, significant ($p<0.05$) lower TBARS values were observed on 3$^{rd}$ and 6$^{th}$ day of storage in vacuum, MAP1 and MAP2 pack samples in comparison to without sealed (control) samples. Significant ($p<0.05$) higher TBARS values were also observed on 9$^{th}$day of storage in case of MAP-2 sample as compared to other samples. In the present study, the TBARS values were within the acceptable limit (2 mg malonaldehyde/kg) in all samples except MAP-2 sample. The TBARS value of MAP-2 pack sample exceeded the acceptable limit of 2 mg on and after 9$^{th}$ day of the storage.

In the present study, the initial total viable count (TVC) of sliced Rohu fish was found 4.29 log CFU/g which indicated an acceptable quality of fish. The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. However, significant ($p<0.05$) lower TVC values were recorded on 9$^{th}$ and

$12^{th}$ day of storage in vacuum and MAP packaged samples compared to that of control.

The results of the present study revealed that pH, TVB-N and PV under all packaging conditions never crossed the prescribed acceptance limit during the entire storage period. There was decreasing and increasing trend in pH value in the samples in all storage conditions but it was in the range of 6.8~7.0. The gradual increase in TVB-N value was evident in the samples in all treatments during the storage period. The PV values showed siginificant fluctuating manner from the beginning till the end of the storage period under all packaging conditions. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. Based on bacterial counts and 7 log CFU/g value as the upper acceptable limit the shelf life of sliced Rohu fish (*Labeo rohita*) was determined about 8 days for without sealed pack, 11 days for vacuum pack, 13 days for MAP-2, and 16 days for MAP-1 pack samples.

By analyzing all biochemical and microbiological parameters it was evident that the best performance was observed in MAP-1 (50% $CO_2$ & 50% $N_2$) package sliced Rohu fish sample compared to without sealed pack (control), vacuum pack and MAP-2 pack fish samples. Moreover, MAP technology proved to be effective to extend the shelf life and improve the overall quality of packaged sliced Rohu fish, which can be utilized by the fish processors.

# RECOMMENDATIONS

There is a great potential for VP and more importantly MAP system in Bangladesh in order to preserve fish and fishery products to extend the shelf-life as well as to add value and convenience. High production cost, requirement of skilled staff, drip loss and chemical and microbiological hazard in the end product are some of the drawbacks of MAP which can be overcome. MAP technology can be very handy at this period of time when food safety has become one of the most burning issues. These packaging technologies can be utilized by the processors or superstores to display and preserve fish and fishery products under refrigeration condition which will increase the quality, value and shelf-life of the product as well as will ensure considerable levels of food safety to the consumers. Vacuum packaging and more importantly MAP was found effective on the extension of shelf-life of Rohu fish in a refrigerated condition. Similar studies should be continued using other commercially important fishes using different gas compositions to analyze the effects and justify the use of this modern technology in our country.

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# EFFECTS OF VACUUM AND MODIFIED ATMOSPHERE PACKAGING ON SHELF LIFE OF ROHU FISH (*Labeo rohita*) STORED AT REFRIGERATED TEMPERATURE (4°C)

## ABSTRACT

The study was conducted to evaluate the effects of vacuum and modified atmosphere packaging on the quality and shelf-life of sliced Rohu fish (*Labeo rohita*) stored at refrigerated temperature (4°C). The study was conducted at quality control laboratory of the Department of Fisheries in the University of Rajshahi from September 2018 to December 2018. Quality and shelf-life of sliced Rohu fishes were evaluated by biochemical and microbiological analysis by using four types of packaging: (i) without sealed pack (control); (ii) vacuum pack as treatment-1; (iii) MAP-1 (50% $CO_2$ & 50% $N_2$) as treatment-2 and (iv) MAP-2 (50% $CO_2$ & 50% $O_2$) as treatment-3. Samples were analyzed at every 2 days interval during 18 days of refrigerated storage (4°C). Different biochemical parameters such as pH, total volatile base nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS) and total viable count (TVC) of bacteria as a microbiological parameter were evaluated and analyzed throughout the storage period. In all packaging conditions, pH values (were in the range 6.8~7.0) throughout the storage period. No significant ($p<0.05$) differences were observed on the changes in pH values in all four packaging conditions during the storage period. Changes in TVB-N values were observed significantly lower ($p<0.05$) at $9^{th}$ day of storage in samples MAP-1 (2.94±0.59 mg/100g) and MAP-2 (2.66±0.20 mg/100g) pack samples as compared to control (3.90±0.76 mg/100g) samples. In case of PV, MAP1 showed better result compared to MAP2 during the storage period. However, the TBARS value in MAP-2 sample exceeded the acceptable limit (2 mg malonaldehyde/kg) on $9^{th}$ day of storage. The initial total viable count (TVC) was 4.29 log CFU/g in all packaging conditions during the storage period. When compared with control, significant ($p<0.05$) lower TVC values were observed on $9^{th}$ and $12^{th}$ day of storage in MAP samples. Shelf-life of sliced Rohu fish (*Labeo rohita*) in refrigerated temperature was in acceptable conditions for 8 days in control samples, 11 days for vacuum pack, 13 days for MAP-2 and 16 days for MAP-1 samples. Among the various packaging techniques, MAP1 showed extended shelf life of Rohu fish stored at refrigerated temperature (4°C).

# ABBREVIATIONS

APHA = American Public Health Association

AOAC = Association of Official Analytical Chemists

ATP = Adinosine Tri Phosphate

BFRI = Bangladesh Fisheries Research Institute

CFU = Colony Forming Unit

$CO_2$ = Carbon di oxide

°C = Degree Celsius

DD = De-ionized Distilled

DoF = Department of Fisheries

EGTA = Ethylene Glycol Tetraacetic Acid

FAO = Food and Agriculture Organization

GDP = Gross Domestic Product

g = Gram

ha = Hectare

hr = Hour

IDF = International Dairy Federation

kg = Kilogram

kPa = Kilopascal

lb = Pound

MA = Modified Atmosphere

MAP = Modified Atmosphere Packaging

mEq = Milliequivalents

mg = Milligram

ml = Milliliter

MT = Metric Ton

N =Normal

$N_2$ = Nitrogen

NaOH = Sodium Hydroxide

nm = Nano meter

$O_2$ = Oxygen

PPS = Peptone Physiological Saline

PUFA = Poly Unsaturated Fatty Acid

PV = Peroxide Value

RTC = Ready To Cook

rpm = Rotation per minute

sq = Square

TBA = Thiobarbituric Acid

TBARS = Thiobarbituric Acid Reactive Substance

TVC = Total Viable Count

TVB-N = Total Volatile Base Nitrogen

U.K. = United Kingdom

UV = Ultra Violet

VP = Vacuum Packaging

w/v = Weight Per Volume

v/v = Volume Per Volume

µm = Micrometer

µL = Microlitre

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# CHAPTER 1

# INTRODUCTION

Bangladesh is encompassed with a vast area of inland fresh water body such as rivers, canals, ponds, lakes, marshes, estuaries, and floodplains (Hossain, 2014). This country has been recognized for its largest flooded wetland and the third largest aquatic biodiversity in Asia (Shamsuzzaman *et al.,* 2017). Huge scope for aquaculture was facilitated by these vast water bodies. Therefore, Bangladesh is famous for its diversified and potential fisheries resources that are one of the richest fisheries resources of the world due to the fish habitats created by the Bengal Delta wetlands and the confluence of the Brahmaputra, Ganges and Jamuna rivers flowing from the Himalayan Mountains into the Bay of Bengal (Hossain, 2014). Thus, aquaculture is dominating with a lion share for the total fish production in Bangladesh. According to a recent report of FAO, Bangladesh is ranked 5th in the world aquaculture production (FAO, 2018).

According to Department of Fisheries (DoF), it was revealed that in 2016-17 Bangladesh secured a surplus fish production with an astonishing annual production of 41.34 lakh Metric Ton (MT), whereas the demand was 40.50 lakh MT (DoF, 2017). Therefore, the daily intake of fish consumption has been increased to 62.58 g of fish per person in Bangladesh (DoF, 2017). The fisheries sector is providing a big role towards country's national GDP (3.61%) growth (DoF, 2017).

Inland aquaculture includes pond/ditch, baor, shrimp/prawn farm, seasonal cultured water-body, pen and cage culture etc. covering an area of about 8.33 lakh hectare and contributing about 56.44%of the total fish production (DoF, 2017).

The inland aquaculture is growing rapidly due to the establishment of new technologies, quality fish seed, intensification and improvement of fish farming over the country (Planning Commission, 2016). Freshwater aquaculture includes farming of carps (indigenous and exotic), pangasid catfish, tilapia, climbing perch, and a number of other indigenous fish species. Semi-intensive carp culture covers a vast area of 110,000 ha, and intensive forms of entrepreneurial pond culture cover only

15,000 ha (Belton *et al.*, 2011). Among the carps, Indian major carps and some minor carps are very popular for culture purposes across the country.

Rohu (*Labeo rohita*) fish which is also known as Rui (Indian major carp) is a cyprinid fish available in the riverine system of Bangladesh. *Labeo rohita* has been introduced into many countries due to the success of breeding in Sri Lanka, Japan, China, Philippines, Malaysia, Nepal and few countries of Africa (Jhingran, 1982). Rohu fish is available allover Bangladesh and preferred as an aquaculture species for their higher environmental tolerances, disease resistance, faster growth and high flesh content. Being a fast growing omnivorous fish, it mainly thrives on the filamentous algae, aquatic plant leaves, phytoplankton, zooplanktons and minorly on small insects (Bairagi *et al.*, 2002). In contrast, Rohu does not grow well below 14 °C and the optimum temperature for growth ranges between 16.8-37.0 °C, while the optimum temperature for spawning is 22-31 °C (FAO, 2017).

Polyculture involves culturing of more than one species of aquatic organisms in the same water body such as pond. In this system, Rohu used to culture along with Katla (*Catla catla*) and Mrigal (*Cirrhinus mrigala*) (Reddy, 1999). Rohu moves from column to bottom and possesses a wider feeding niche, therefore, stocking of fingerling used to practice at higher levels in the rearing ponds compared to the other two species . Its higher growth rate and greater consumer preference makes it very popular in many countries including Bangladesh. In 2016-17 fiscal years, the total production of Rohu fishes in Bangladesh were 3,70,627 Metric Ton (MT) (DoF, 2017).

Fish and fishery products are a good source of protein as well as vitamins and minerals. Fish protein are rich in omega-3 fatty acids and vitamins such as vitamin D and vitamin $B_2$ (riboflavin) and also act as a great source of minerals particularly calcium, phosphorous and some other micro minerals (Falls, 2012). Fatty fishes contain fat-soluble vitamins (A, D, E and K) and essential fatty acids. These elements are vital for the healthy function of the body (Fellows and Hampton, 1992). As an important source of proteins, vitamins and minerals, *Labeo rohita* is one of the most preferred major carp. The fat content of *Labeo rohita* contains vitamins like A, D, E, K and C as well as essential fatty acids like PUFA, Omega-3 fatty acids like alpha linolenic acid, eicosapentaenoic acid, docosahexaenoic acid. Minerals like calcium,

zinc, iron and thallium are also available in *Labeo rohita* (De Silva *et al.*, 1995). Moreover, Omega-3 fatty acids have beneficial effect on cardiovascular disease (CVD) (Krauss *et al.*, 2000). Natural sources of polyunsaturated fatty acids having two important ω-3 PUFAs in fish oil includes EPA (Eicosapentaenoic Acid) and DHA (Docosahexaenoic Acid) that have been proven useful effects on human body (Imad *et al.*, 2008). Therefore, Rohu being a slightly fatty to fatty fish species contains considerable amount of essential fatty acids, amino acids, vitamins and minerals.

Fresh fish generally spoils quickly after harvesting due to the enzymatic autolysis, oxidation and microbial growth which are the three main basic mechanisms responsible for fish spoilage (Ghaly *et al*., 2010). In the tropical regions due to high ambient temperatures, the spoilage process commonly known as 'Rigor mortis' starts rapidly within 12 hrs of the catch (Berkel *et al*., 2004). Rigor mortis is a process through which stiffening of fish muscle occurs just after the few hours of death of the fish that leads to loss of flexibility of fish flesh (Adebowale *et al*., 2008). Breakdown of numerous components and formation of new compounds basically occur during fish spoilage. Thereby, these newly formed compounds are mainly responsible for the changes in texture, odor and flavor of the fish meat.

Enzymatic breakdown of body compounds in fishes occur shortly after capture that results in chemical and biological changes mainly in dead fish (FAO, 2005). The autolytic reactions are controlled mainly by endogenous enzymes present in the fish muscle tissue as well as in the gut (Ashie *et al.*, 1996). A number of proteolytic enzymes are found in muscle and viscera of the fish and these enzymes contribute considerably to the post mortem degradation in fish muscle and products during storage and processing. Degradation of proteins commonly known as proteolysis is followed by a process of solubilization that mainly occurs during the improper storage of whole fish (Lin and Park, 1996). Autolysis of fish muscle proteins also produce peptides and free amino acids that contributes to spoilage of fish as a result of microbial growth and production of biogenic amines (Fraser and Sumar, 1998). The leakage of proteolytic enzymes from pyloric caeca and intestine to the ventral muscle also leads to considerable spoilage commonly known as 'belly bursting'.

Lipid oxidation is a major cause of deterioration and spoilage of fatty fish. Lipid oxidation includes a three stage free radical mechanism, named as: initiation, propagation and termination (Frankel, 1985). Fish lipids of cultured Rohu contain higher levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) while Rohu from wild sources contain higher levels of polyunsaturated fatty acids (PUFA) (Sharma *et al*., 2010). Therefore, Rohu is extremely susceptible to oxidation. In fish, lipid oxidation takes place not only enzymatically but also non-enzymatically.

The microflora composition of the newly caught fish depends on the microbial loads of the water in which the fish live. The common microflora in fish includes bacterial species such as *Pseudomonas, Alcaligenes, Vibrio, Serratia* and *Micrococcus* (Gram and Huss, 2000). Fresh fish caught from warm water normally contains microbial population such as *Micrococcus,* coryneforms, and *Bacillus* while cold-water fish species are dominated by psychrophilic gram-negative microbes including *Moraxella/Acinetobacter, Pseudomonas, Flavobacterium,* and *Vibrio* genera (Cann 1977 and Liston 1980). The microflora of *Labeo rohita* is dominated by strains of *Bacilli, Pseudomonas, Aeromonas, Enterobacter* (Ghosh *et al*., 2010). However, strains of *Flavobacterium, Micrococcus, Achromobacter, and Vibrio* are also visible in the gastrointestinal tract of *Labeo rohita* (Hossain *et al*., 1999). The major causes of fish spoilage are microbial growth and metabolism that produces various compounds like amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with obnoxious and improper odors and off-flavors (Dalgaard *et al*., 2006; Emborg *et al*., 2005; Gram and Dalgaard, 2002). Gram-negative, fermentative bacteria (such as *Vibrionaceae*) are mainly responsible for spoilage related to unpreserved fish, whereas in case of chilled fish the psychrotolerant gram-negative bacteria (such as *Pseudomonas* spp. and *Shewanella* spp.) are the predominant ones (Gram and Huss, 2000). The microbial compositions of fishes are also changing due to adoption of new preservation techniques such as modern packaging.

The introduction of new preservation techniques is mainly due to the changes in food preference and increasing demand for fresh fish and fishery products with extended shelf life. The changes in food preference of people over the last few years may have occurred due to the considerable social and economic development of Bangladesh. At

present the meaning of food has been changed. Foods are no longer intended only to satisfy the hunger and provide necessary nutrients but also prevent nutritional diseases, and at the same time to improve physical and mental well-being of the consumers (Menrad, 2003). There are several factors affecting the demand function of fish and fishery products that includes price, income, income distribution, substitutes, tastes and fashion, advertising and expectations of the consumers, etc. (De Silva, 2011). Nowadays city and mega-city residents particularly busy women's, mothers and housewives are asking for ready-to-cook (RTC) food products instead of the raw one's that take away their considerable amount of precious time during cooking or preparation. As a result, the fish-processing industry and retail superstores are actively seeking different methods for shelf life extension and marketability of fresh, refrigerated fish. Ice storage or refrigeration in combination with modern packaging techniques can be fruitful to delay spoilage, extend the shelf life, maintain a high quality of fish and fishery products and may assure food safety even though at a higher price.

Consumers concern on price and quality of the product are mainly affected by the food consumption and food habits. Acceptance of the new product along with food safety stays the main concern. If a product is lower in quality, its appeal will not be there, not even health benefit promises can do justice to accept it by the consumers (Sosa *et al.,* 2008). But if a product has high sensory acceptability, the additional issues for instance, packaging, price and convenience have to be resolved to ensure overall acceptability. According to a study by Rahman *et al.,* (2017) it was revealed that the superstore outlets in Dhaka city sell superior quality wet fish at a rate of 20-25% elevated price compared to those sold through general fish markets. This eventually proves a strong point that if the quality is ensured in a product; people are ready to pay irrespective of the high price. At present, there is lack of or no RTC or MAP type packaged fish and fishery products in the markets of Bangladesh.

An efficient fish marketing system is a key to enable the customers to get the fresh fish. The domestic fish market in Bangladesh is quite big in relation to volume, value and employment. The basic marketing infrastructures including cold storage, ice, insulated transport facilities, fish landing centers and wholesale markets is basically improper, unhygienic and in poor state. In Bangladesh, fish are generally landed in

road-side shore of natural watershed or farm house (Nowsad, 2010). The harvested fish through different channels are transferred from the landing centers to the consumer markets (Ali *et al*., 2004). The intermediaries take their benefits during the handover of the fish in the retailing system. The fish producers or farmers most of the times do not get the proper or expected price of their product due to the involvement of so many brokers in the marketing channel. Although during marketing almost all intermediaries use ice but their usage of ice in fish preservation is not scientifically correct due to which considerable quality loss of fish occurs. During retail selling, some of the sellers use ice and some do not. As a result, amount of raw fishes that undergoes quality deterioration during retailing is quite significant (Hossain *et al*., 2013). Under this scenario, some fraud traders use formalin in fishes which is a very hazardous chemical and a direct threat for human health (BFRI, 2006). In order to change the scenario, modernization of transportation, handling, preserving, packaging, and storing facilities are very important (Islam and Habib, 2013).

As with time, the people in Bangladesh due to their immense business and consciousness about time are heading towards departmental store instead of going to separate markets for their groceries including fish. Thus, they can save their time, money and energy. By ensuring hygiene and food safety, the superstores or super shops may display the raw (whole fish), dressed or processed (fillets/slice) fish under chilling for marketing purpose, even though at a higher prices than the traditional fish markets. Under current icing and refrigerated storage conditions the shelf-life of fish and fishery products typically ranges from 2 to 14 days (Stammen *et al.*, 1990). In order to delay oxidation, control food borne pathogens, and meet the growing demand of consumers for safe and high-quality products as well as to improve antimicrobial properties, active food packaging is very significant (Choi *et al.*, 2016). So it is obvious that there is room for MAP packaged fish and fishery products (fillets/slice) under refrigerated storage to take an edge over the other traditional fish products particularly in superstore outlets in cities and megacities.

According to Gupta and Dudeja (2017), packaging acts as a physical barrier to protect food from external factors, so the stability of packaging materials is absolutely vital for enhancing food quality and safety and increasing shelf-life. Packaging also ensures sturdy, attractive, economical, and convenient products to consumers.

The evolution of novel and innovative packaging techniques that maintain and used to monitor food safety and quality, extend shelf-life, and reduce the environmental burden of food packaging have been mainly stimulated by frequent changes in consumer demand, industrial production trends, retailing practices, and customer lifestyles (Dainelli *et al.*, 2008).

Placement of a product inside a packaging material with low permeability to oxygen followed by air exhaustion and sealing is termed as vacuum packaging (Smith *et al.*, 1990). Though in vacuum packaging the gaseous atmosphere is completely reduced, but changes in gas composition is very obvious particularly during storage days. It is mainly due to microbial activity that results in about 10 to 20% increase in the $CO_2$ amount that may suppress the growth of undesirable microorganisms (Silliker and Wolfe, 1980). Vacuum packaging has been extensively used to remove oxygen in the package prior to sealing but it is unable to remove the oxygen that permeates from the external environment into the package. The presence of $O_2$ in food packages occurs mainly as a result of permeability of packaging material, failures in the packaging process and ineffective vacuum (Mohan *et al.*, 2019).

Oxygen is responsible for oxidative rancidity in fatty fish and stimulates the growth of aerobic microorganisms (Arashisar *et al.*, 2004). Complete removal of oxygen is very essential particularly for oxygen-sensitive foods as the presence of $O_2$ ultimately leads to the growth of aerobic bacteria, moulds and oxidation. VP inhibits the growth of aerobic bacteria that grow under aerobic storage conditions dominating the spoilage of freshwater and marine fish thereby, extend the shelf life of fish fillets (Gram and Huss, 1996).

Modified atmosphere packaging (MAP) is basically a preservation technique that involves alteration of atmospheric environment around a food that is perishable by the replacement of single or a mixture protective gases (Arashisar *et al*., 2004; Del Nobile *et al*., 2009). Stammen *et al.* (1990) defined MAP as a system where the air within a package is instantly replaced by a mixture of different gases at the time of sealing. MAP has been reported to considerably inhibit the spoilage as well as to elongate the shelf life of fresh fish products (Torrieri *et al.*, 2006).

The principle of MAP involves replacing the air in the package with an altered gas mixture. After the incorporation of gas mixture in the package, no further control of the gas composition is done and the gas composition will certainly change with time (Sivertsvik *et al.*, 2002). Modification of the headspace during food packaging with different gas mixtures to delay bacterial activity and chemical reactions is the main aim of MAP (Tsironi and Taoukis, 2018).

In terms of MAP applications, $O_2$, $CO_2$ and $N_2$ are the most commonly used gases and their concentrations mainly depend on the food product and the mechanism of spoilage that limits the shelf life (Kirtil *et al.*, 2016).

The exclusion of oxygen is generally desirable mainly due to aerobic bacteria and oxidative reactions as its absence will inhibit spoilage and prolong the quality with storage life. However, in the absence of $O_2$ anaerobic respiration occurs, that accelerates spoilage of the concerned product. Mainly to counter the effects of anaerobic/micro-aerophilic organisms and non-oxidative reactions, $O_2$ is used in various concentrations in modified atmosphere packages. Pantazi *et al*., (2008) found that oxygen hinder the growth of anaerobic bacteria as well as accumulation of toxin by *Clostridium botulinum* type E when it is introduced in modified atmosphere packages. $O_2$ maintains the colouring pigment myoglobin intact in the bright red, oxygenated form (oxymyoglobin) that consumers prefer (Hood and Riordan, 1973).

The most widely used gas for MAP of fish products is $CO_2$ that generally inhibits microbial growth. The inhibition of microbial growth mainly depends on the $CO_2$ concentration as growth of respiratory organisms such as *Pseudomonas* spp. and *Shewanella putrefaciens* can be delayed by $CO_2$ (Sivertsvik *et al.*, 2002). The effectiveness of $CO_2$ depends upon the growth phase of any microorganisms present. $CO_2$ increases the duration of lag phase of microbes while decreases the growth rate during the logarithmic phase (Farber, 1991). Under this consideration, the shelf life of refrigerated fish products can be prolonged effectively by MAP.

$N_2$ is an inert and tasteless gas and it is comparatively less prone to pass either into the product or out through the packaging material than the other gases normally used. It is commonly used as a balance or filler gas replacing $O_2$ in the packages, either as a

substitute to vacuum packaging when the product is delicate, or to stop pack collapse caused by the $CO_2$ absorption (Church and Parsons, 1995).

The optimum packaging system design for any particular product includes a number of factors, the most important being amounts of $O_2$ and $CO_2$ present. It is mainly determined by the volume of these respective gases within the pack, the headspace volume as well as the permeability of the packaging material. Proper optimization of the packaging system will result in enhancement of sensory quality and minimization of public health risks.

The use of Modified atmosphere packaging (MAP) is relatively new in respect of Bangladesh and such packaging system has not been developed and introduced yet for preservation of fishes. MAP provides several advantages like high quality fish fillets with an extended shelf-life, good hygienic standards, and most importantly food safety. With continuous and consistent demand for high quality food with extended shelf life that most of the present techniques fail to do, there is no choice but to go for alternative modern techniques like vacuum packaging and modified atmosphere packaging. MAP promises good keeping quality of fish with elongated shelf-life. Therefore, it is critically important to develop appropriate vacuum and MAP technology particularly for fish fillets and/or slice in order to lower the qualitative and quantitative losses of raw fishes that will ultimately ensure the supply of good quality fishes to the consumer in a convenient way.

The objectives of this study are-

- To evaluate the quality changes of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).
- To determine the safety aspects of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).
- To determine the overall shelf-life of packaged sliced Rohu fish under different packaging conditions stored at refrigerated temperature ($4^oC$).

# CHAPTER 2

# REVIEW OF LITERATURE

As modified atmosphere packaging (MAP) is a newly introduced technology in Bangladesh, therefore, there is literally no information available regarding MAP of fish and fishery products in Bangladesh. Modified atmosphere packaging is a comprehensive and extensively used technology for increasing shelf life of fish and fishery products in many developed countries. Thus, information regarding vacuum packaging and particularly MAP were collected from those countries where these types of techniques are available. The present literature reviews highlighting the most relevent studies related to this study.

## 2.1 Vacuum and Modified atmosphere packaging

Smith *et al.* (1990) defined vacuum packaging as placement of a product in a film of low oxygen permeability, followed by the removal of air from the package and the hermetic sealing.

Banks *et al.* (1980) and Arias (2009) reported that in vacuum packaging the gas proportions are modified by evacuating air from the packages, the absence of $O_2$ results in altering bacterial composition from gram-negative to predominantly gram-positive lactic bacteria. Due to partial sugar fermentation, the pH also decreases thereby inhibiting the growth of Gram-negative bacteria.

White and Roberts (1992) stated that to reduce oxidative rancidity considerably, $O_2$ content of less than 2% v/v is required in vacuum packaging.

DeWitt and Oliveira (2016) found that for seafood products, an alternative to vacuum packaging is done by the simple flushing with nitrogen, which is mainly used to replace $O_2$ in packages to postpone oxidative rancidity and inhibit growth of aerobic microorganisms.

Del Nobile *et al*. (2009) defined modified atmosphere packaging (MAP) as a preservation technique by changing atmospheric environment around a perishable food commodity by incorporating single or a mixture of protective gases.

Stammen *et al.* (1990) defined MAP as a system where the air within a package is instantly replaced by a mixture of different gases at the time of sealing.

MAP was also defined as the enclosure of food products in gas-barrier materials, in which the gaseous environment changes in order to inhibit spoilage agents and therefore either maintain a higher quality or extended shelf-life (Young *et a1.*, 1988).

In order to extend shelf-life of fresh fish products, MAP packaging was first introduced in 1930's (Killefer, 1930; Coyne, 1932, 1933; Stansby and Griffiths, 1935; Smith *et al.,* 1988) and was used in U.K. during the last quarter century with substantial success (Cann, 1988; Lioutas, 1988).

Bouletis *et al.* (2017) stated that to offer the safer distribution of quality aquaculture fishery products MAP can certainly play a vital role.

During the last two decades, MAP technique has extensively modernized. The positive effect of MAP on fish and shellfish quality during preservation was reported.

## 2.2 Role of gases in MAP

According to Church and Parsons (1995), three gases are generally used for MAP packaging which are oxygen ($O_2$), nitrogen ($N_2$) and carbon dioxide ($CO_2$). All of these gases have a specific function.

Alfaro *et al.* (2013) concluded that $CO_2$ is a bacteriostatic agent which is considered as the principal gas having a significant effect on fish microflora.

Campus *et al.* (2011) and Arashisar *et al.* (2004) reported that the shelf-life of fish products kept at high $CO_2$ levels enhance the shelf-life of the products due to microbial growth inhibition.

Davis (2009) reported that in order to preserve the red color of haem pigments, high oxygen concentrations in combination with $CO_2$ was used. The main reason to include oxygen in the gas mixture of MAP fish and fishery products was mainly not to intensify the potential risk of botulism from products in sealed packs.

Sun Lee *et al.* (2008) suggested that for fatty fishes, gas mixtures containing $CO_2$ (40–60%) with balanced level of Nitrogen ($N_2$) followed by 0–2$^oC$ storage is recommended.

Sivertsvik *et al.* (2002) noted that $O_2$ is mostly used in MAP gas mixtures as a filler (e.g., $CO_2$:$N_2$) mainly due to its low solubility in water and fat, which prevents packaging deterioration. They also found that nitrogen as an inert gas replaced oxygen in the pack which ultimately reduced oxidative rancidity as well as inhibited growth of aerobic microorganisms. They also reported about the relationship between the higher concentration of $CO_2$ and higher inhibitory effect. They concluded that there is a positive correlation between these two factors.

Reddy *et al.* (1992) concluded that the total removal of $O_2$ was necessary due to greater chances of high oxidative rancidity that may lead to altering color and texture of the flesh particularly for fatty fish such as common carp.

According to Smith *et a1.* (1990) white fish was typically packed in 30% $O_2$/30% $N_2$/40% CO, where $O_2$ being excluded in the latter stages to minimize rancidity.

Statham (1984) and Yambrach (1987) concluded that $CO_2$ and $N_2$ alone or in combination were most abundantly used gases in MA packaging particularly of fresh seafood products, along with the occasional use of $O_2$ in low levels.

Genigeorgis (1985) reported that $O_2$ enhanced odor and color shelf-life and keeps the oxygenated form of myoglobin intact.

Ogrydziak and Brown (1982) and Statham (1984) found that the growth of anaerobic spoilage bacteria was hindered by $O_2$.

## 2.3 Vacuum and Modified atmosphere packaging (MAP) of different fish species

Sterniša *et al.* (2016) in their study reported that the safety and quality of the common carp products can be enhanced by adopting novel smart packaging systems.

Mohan *et al.* (2019) studied the effects of vacuum packaging and oxygen scavenger packaging on the shelf life and quality of Indian oil sardine (*Sardinella longiceps*) kept under under chilled storage (1–2$^{o}$C). They found that the oxygen scavenger was efficient in reducing the oxygen level in the pack within 24 hrs and the growth of total mesophilic bacteria were hindered by low oxygen level compared to control air-packed samples.

Pattarapon *et al.* (2018) investigated the freshness of grass carp (*Ctenopharyngodon idella*) fillets under vacuum packaging with vacuum (50 and 30 kPa) and without vacuum were stored at 4$^{o}$C for 2 weeks. From their results they concluded that statistically significant difference between the grass carp fillet samples stored under vacuum and without vacuum conditions was found.

Rodrigues *et al.* (2016) conducted a study on Rainbow trout (*Oncorhynchus mykiss*) fillets quality for a period of 22 days by the action of UV-C radiation, modified atmosphere packaging (MAP) and their combination followed by storage at 4$^{o}$C. They found that the total production of ammonia, TVB-N and putrescine was reduced under MAP, whereas total production of TVB-N and cadaverine during the entire storage period was reduced under MAP + UV-C. They concluded that MAP retarded microbial growth and delayed chemical changes by doubling the shelf life of rainbow trout fillets.

Lunda *et al.* (2016) studied the effect of vacuum packaging on the quality of Common carp (*Cyprinus carpio* L.) fillets descaled by four different methods followed by refrigerated storage. By analyzing the growth of microbial communities they concluded that fillets without skin showed the lowest TVC.

Babic´ *et al.* (2015) in their study compared the effects of two different MAPs, MAP1 (60 % $N_2$ and 40 % $CO_2$) and MAP2 (100 % $CO_2$) on carp steak quality stored at 3.0 ± 0.5$^{o}$C over 15 days. They found that in terms of TVC values, MAP2 showed greater shelf life as compared to MAP1. They also found that MAP2 resulted in lower pH compared to MAP1 in terms of decreasing the growth of Enterobacteriaceae that mainly occurred due to $CO_2$ absorption as well as acidification of the fish muscle.

Cyprian *et al.* (2013) concluded that air storage proved to be more beneficial for tilapia fillets compared to MAP(50% $CO_2$/50% $N_2$) under chilled (1$^{o}$C) and super chilled (-1$^{o}$C) storage due to unwanted coloration of the samples. On the other hand, microbial parameters were better preserved on MAP samples compared to control.

Ali (2012) determined the shelf life of brined golden mullet (*Liza aurata*) during vacuum refrigerated storage at 4°C. He observed that the shelf life of golden mullet enhanced to a certain extent when kept under a combination of brining, vacuum packaging followed by storage at refrigerated temperature. His results also revealed

that the longest shelf life for vacuum packed brined golden mullet stored at 4°C was 30 days.

Hudecova *et al.* (2010) conducted a study on carp samples to compare the effects of MAP comprsing (30% $CO_2$/70% $N_2$) for MAP1 and (80% $O_2$/20% $CO_2$) for MAP2 stored at 4 ± 0.5$^oC$. They found that TVC values were considerably lower on MAP samples compared to that of control carp samples even on the 10th day of storage. They concluded that a shelf life elongation of three and five days was achieved with MAP 1 and MAP 2, respectively, compared to control carp samples (three days).

Jezek and Buchtova (2010) in their study observed the effect of an altering atmosphere with (5% $O_2$/25% $CO_2$/69%$N_2$/1% CO) gas composition for the shelf life extension of carp followed by storage at 2 ± 2$^oC$ for 18 days. They found that MAP samples showed considerably lower TVBN levels compared to that of control samples even at 11th day of storage.

Grzegorz *et al.* (2010) studied the influence of method of packing (air packaging and vacuum packaging) on physicochemical and textural changes in Atlantic herring during frozen storage. They observed that fluctuations in lipid content were evident in case of frozen storage of air-packed material, while vacuum packing reduced lipid extractability from fish muscle along with storage time. They concluded that textural parameters of fillets changed to the smaller extent in case of vacuum-packed fish.

Sevik and Korkut (2009) worked on the effect of package types and storage period on characteristics of *Tinca tinca.* They indicated that as per TBV-N and TBA values, vacuum packed *T. tinca* were safely stored up to 30 days and 60 days respectively. They concluded that the shelf-life of vacuum packed *T. tinca* was 30 days.

Schirmer *et al.* (2009) in their research investigated the effect of MAP (100% $CO_2$ with gas/product ratio 0.2/1.0 v/v) and brine solution contained several contents of citric acid, acetic acid and cinnamaldehyde on salmon samples stored at 4$^oC$. They concluded that pretreatment in combination with MAP showed controlled microbial populations of both inoculated and natural strains for 14 days.

Choubert *et al.* (2008) from their study reported that under Argon (Ar) atmosphere, TBARS concentration was lower in samples stored under MAP2 (60% Ar/40% $CO_2$) compared to MAP1 (40% $CO_2$/60% $N_2$) when Rainbow trouts fed with two types of

food astaxanthin and canthaxanthin, followed by slaughtering and stored under for 26 days at 2$^{o}$C. From the results it was concluded that the investigated samples shelf life was doubled due to the inclusion of Argon.

Cakli *et al.* (2006) compared the effects of modified atmosphere packaging and vacuum packaging on the shelf life of hot smoked rainbow trout (*Onchorincus mykiss*). Based on the results of microbiological and sensory analysis they concluded that the shelf life of smoked trout under MAP (60% $CO_2$:40% $N_2$) was 14 days more than vacuum packs.

Ozogul and Ozogul (2006) worked on the effects of modified atmosphere packaging (MAP) and vacuum packaging (VP) on biogenic amine formation during storage of sardines (*Sardina pilchardus*) at 4$^{o}$C. They concluded that Sardines were organoleptically acceptable for up to 3, 9 and 12 days under air, VP and MAP respectively. They also reported that comparatively lower amine contents of sardine were found under MAP and VP.

Masniyom *et al.* (2004) conducted their study to evaluate the effect on chemical parameters of seabass kept at 4$^{o}$C under a MAP (10% $O_2$/80% $CO_2$/10% $N_2$). They found that unaltered levels of $Ca^{2+}$, $Mg^{2+}$, and $Mg^{2+}$–$Ca^{1+}$C ATPase activity of natural actomyosin was noticed on the 21st day on samples stored under MAP, while the Mg-EGTA ATPase activity was indicating a slight increase. They also found that as compared to the control sample, the sulfhydrilcontent dropped at a lesser rate under MAP packaging.

An investigation was done by Ozogula *et al.* (2004) on sardines (*Sardina pilchardus*) to know the total viable count and histamine forming bacterial count under MAP comprised of ($CO_2$/$N_2$: 60/40) followed by storage at 4$^{o}$C. The results showed comparatively lowest total viable count (TVC) under MAP compared to air and vacuum packaging.

Tryfinopoulou *et al.* (2002) in their study found that the microbial flora of sea-bream stored under MAP (40% $CO_2$/30% $N_2$/30% $O_2$) at 0, 10, and 20$^{o}$C were dominated by *Pseudomonas lundensis* and *P. fluorescens*. Among the 106 isolates, MAP samples were dominated by the proteolytic and less lipolytic strains and microbial parameters were better preserved on MAP samples compared to control.

Reddy *et al.* (1996) in their study found that as hurdle techniques to avoid toxin production, vacuum packaging and MAP (75% $CO_2$/25% $N_2$) were tested on tilapia fillets inoculated with *C. botulinum* type E and stored at refrigeration($4^oC$) and altered temperatures ($8^oC$ and $16^oC$). They concluded that toxin detection occurred after more than 10 days at $4^oC$ when sensory spoilage on both vacuum and MAP stored samples were evident.

Silva and White (1994) studied the effects of packaged catfish fillets in which $CO_2$ concentrations were (25%/75% $CO_2$/air for MAP A) and (80%/20% $CO_2$/air for MAP B) stored at $2^oC$ and $8^oC$. The lowest microbial numbers were recorded from MAP A samples kept at $2^oC$ which ultimately proved to be the best studied treatment.

Drosinos *et al.* (1996) in their study experimented with Gilthead seabream (*Sparus aurata*) fillets kept under MAP (30% $O_2$/40% $CO_2$/30% $N_2$) followed by storage at 0 ± $1^oC$. From the results it was evident that the size of bacterial population was always 2 log CFU/g lower in MAP and MAP samples also showed lower TMA-N levels compared to air packed samples.

## 2.4 Effect of MAP and Vacuum packaging on microorganisms

Ibrahim *et al*. (2008) from their study reported increase in halophilic bacterial count under vacuum conditions and MAP after two weeks of storage and then a drop on the third week of storage at $4^oC$ for smoked mullet samples. However irrespective of the storage period, bacterial count of smoked mullet samples showed an upward growth mainly for the vacuum stored ones rather than those under MAP.

Chen and Xiong (2008) in their study observed positives of packaging effectiveness for coliform counts as well as for aerobic plate count for samples of precooked and peeled red claw crayfish. Under all packaging treated samples, the coliform count was low until 7th day but severe uplift in coliform count was evident in both treated samples on day 21 of storage. On the contrary MAP treated samples showed lowest coliform count at the end phase of storage (21 day).

Pantazi *et al.* (2008) observed that Mediterranean swordfish stored under MAP $CO_2/O_2/N_2$: 40/30/30 exhibited Enterobacteriaceae count as low as 4.2-5.6 logs CFU/g on the $16^{th}$ day of the storage.

Stamatis and Arkoudelos (2007) in their study found that fresh chub mackerel (*Scombercolias japonica*s) fillets under MAP $CO_2/N_2$: 50/50 showed *Pseudomonas* counts below 7 log CFU/g when stored for 15 days at 3 and 6$^oC$ respectively.

A study conducted by Arkoudelos *et al.* (2007) showed that farmed eel (*Anguilla anguilla*) under modified atmosphere packaging ($CO_2/N_2/O_2$:40/30/30) followed by storage at 0$^oC$ exhibited *Pseudomonas* counts of 4.2 log CFU/g on day 37 of storage. Results also illustrated that *Pseudomonas* count was considerably lower in farmed eel treated with MAP than with air condition (7.3 logs CFU/g at day 18) and vacuum packaging (5.9 log CFU/g at day 31).

Goulas *et al.* (2005) carried out his research on microbial changes under modified atmosphere packaging of ($CO_2/N_2$: 50/50), ($CO_2/N_2$:80/20) and ($CO_2/N_2/O_2$:40/30/30) refrigerated stored mussels. Results illustrated that lowest count for total viable count 7.0 logs CFU/g on 15 days of storage was found for MAP with composition of ($CO_2/N_2$:80/20) which was also same for vacuum packaging. On the other hand, control and MAP with composition of $CO_2/N_2/O_2$: 40/30/30 attained the microbiological acceptable limit (7 log CFU/g) on 8th and 11th day of storage respectively.

Ozogul *et al.* (2004) investigated that histamine forming bacteria enhanced in sardines samples (*Sardina pilchardus*) throughout the storage period at 4$^oC$ from all treatments (MAP $CO_2/N_2$: 60/40, vacuum packaging and air). They concluded that under MAP, the histamine forming bacterial count was lower compared to other treatments.

Arashisar *et al.* (2004) observed that Enterobacterial growth inhibition was evident in rainbow trout fillets treated with MAP of both (100% $CO_2$) and ($O_2/N_2/CO_2$: 2.5/7.5/90) after 6 days of storage. Enterobacterial growth was inhibited by treating the rainbow trout fillets with MAP of (100% $CO_2$) and ($O_2/N_2/CO_2$: 2.5/7.5/90) respectively after storage of 6 days.

Lopez-Caballero *et al.* (2002) from their studies reported that the growth of $H_2S$ producing microorganisms in shrimps was inhibited by MAP. Under MAP comprising ($CO_2/O_2/N_2$: 40/30/30) and ($CO_2/O_2/N_2$: 45/5/50) respectively, $H_2S$ producing microorganisms count was about 1.1 and 1.6 log CFU/g respectively, after nine days of storage period.

Cai *et al.* (1997) experimented with inoculation of a mixture of 4 E-type *C. botulinum* strains which were introduced into packages of catfish (*Ictalurus punctatus*) packed in $O_2$-permeable film, in 80% $CO_2$ and 20% $N_2$ modified atmosphere and stored at $4^oC$. The toxin production was evident in $O_2$-permeable film and modified atmosphere packaging after 9 and 18 days, respectively. Deterioration process further accelerated the toxin production under all packaging methods.

Hintlian and Hotchkiss (1986) suggested that increase in the growth of potential *Clostridium* under MAPs was an alarming issue and many researchers were aware of it. *Clostridium perfringens* was basically responsible for gastrointestinal diseases while *Clostridium botulinum* produces a neurotoxin that causes facial paralysis. *Clostridium botulinum* is classified into types A, B, C, D, E, F and G where the types A, B and F are important to humans.

## 2.5 MAP and shelf life extension of fishery products

Arvanitoyannis and Stratakos (2012) concluded that MAP can certainly lead to the supply of high quality fishery products due to significant shelf life extension by reducing potential economic losses and thereby, ensures product stability.

Therefore, it can be concluded that the vacuum and more importantly the modified atmosphere packaging (MAP) of fresh fish will ensure an extended shelf life of fish and fishery products under refrigerated temperature. Therefore, this new technology can be facilitated by retailers, superstores and most importantly the fish processors having refrigeration or icing facilities. In addition, it will also ensure the supply of fresh fish to the consumer in a convenient way by ensuring food safety.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Sample Collection and Preparation

Live Rohu fishes (*Labeo rohita*) with an average size of 1.5±0.3 kg were collected from Shaheb Bazaar, Rajshahi. The experiment was carried out in the quality control laboratory under Department of Fisheries, University of Rajshahi from September 2018 to December 2018. The samples were transported in an insulated box in properly iced condition to the Quality Control laboratory of Department of Fisheries, University of Rajshahi. Then the whole fishes were washed using tap water, be-headed, de-scaled, gutted and cut into small slices with an average weight of approximately 100g each piece (shown in Plate 1). Then the sliced Rohu fishes were washed with tap water and the finally washed using distilled water.

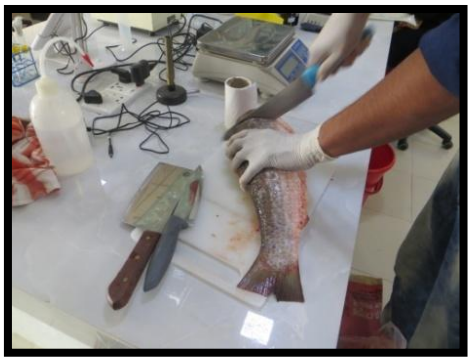

Plate: (A) Rohu fish was cut for sample preparation

Plate: (B) Sliced Rohu fishes were further cut into small pieces

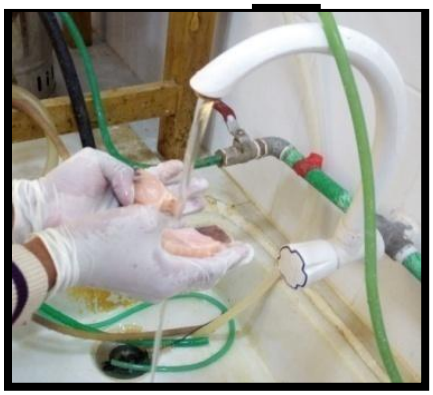

Plate: (C) Sliced Rohu fishes were washed by running tap water

**Plate 1: Preparation of fish samples (*Labeo rohita*) before inserting pieces of fish into the various packaging conditions**

## 3.2 Packaging and Storage of Samples

The required quantity of Rohu slices were packed under vacuum and modified atmosphere packaging by using low gas and moisture permeable plastic pouch (shown in Plate 2). Multi-layer transparent pouch having 100 µm density was used as packaging material. Four types of packaging were applied with different gas ratio using the method described by Noseda and others (Noseda *et al.*, 2012). These four types packaging were used as treatments, namely: (i) aerobic, (without sealed pack) as Treatment-0 (control); (ii) vacuum as treatment-1; (iii) MAP 1 (50% $CO_2$ & 50% $N_2$) as treatment-2 and (iv) MAP 2 (50% $CO_2$ & 50% $O_2$) as treatment-3. Vacuum and MA packaging were done using a packaging machine (C100 Multivac, Haggenmuller, Germany) attached with Gas Mixer (KM100-3MEM, WITT, Germany) by following the standard instruction of the manufacturer. Monitoring and analysis of the $O_2$, $N_2$ and $CO_2$ levels in the headspace of the packaged samples were regularly performed with a gas analyzer (Oxybaby M+, WITT, Germany) before and after storage at 4°C. All samples were stored at 4°C in a laboratory refrigerator. Three samples from each packaging condition were analyzed at every two days interval for 18 days at 4°C (0day, 3day, 6day, 9day, 12day, 15day and 18day).

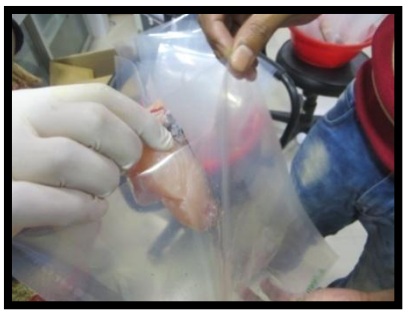

Plate: (A) Samples were placed in plastic pouches

Plate: (B) Packaging was done by Multivac packaging machine

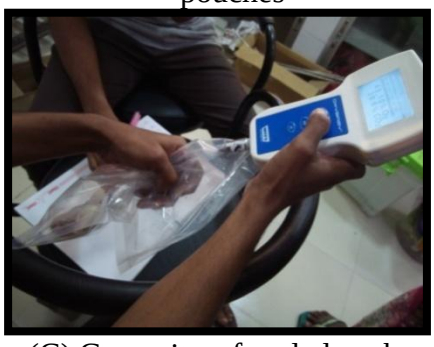

Plate: (C) Gas ratios of sealed packages were checked

Plate: (D) All packed samples were stored in refrigerator at 4°C

**Plate 2: Packaging of sliced Rohu fish (*Labeo rohita*) by Multivac packaging machine and gas composition analysis by Oxybaby gas analyzer**

## 3.3 Biochemical Analysis

Biochemical parameters such as (pH, TVB-N, PV and TBARS) were analyzed in order to know the quality of packaged sliced Rohu fishes and to determine the shelf-life of sliced fishes at refrigerated storage.

### 3.3.1 Analysis of pH Value

The flesh of the sliced Rohu fish was cut into small pieces from each pack stored in the refrigerator. Ten (10) grams of cut flesh was homogenized with 50 mL of distilled water and the pH value of the homogenate was measured by a glass electrode pH meter (HI2002-Edge, Hanna Inst, USA) (shown in Plate 3).

Plate: (A) 50ml of distilled water was added with 10 grams of sample

Plate: (B) Homogenized the sample in a blender

Plate: (C) Determined the sample pH

**Plate 3: pH determination of sliced Rohu (*Labeo rohita*) by HANNA pH meter**

### 3.3.2 Estimation of Total Volatile Base Nitrogen (TVB-N) Value

Total volatile base nitrogen (TVB-N) value was estimated according to the EC (2005) method. The flesh of the sliced rohu fishes were cut into small pieces from each of the pack and carefully ground using a blender. Ten (10) g of the ground fish sample was weighed into a suitable container and mixed with 90 mL of 6% perchloric acid solution, homogenized for two minutes with a blender (Bajaj, India), and then filtered to obtain extract. Fifty (50) mL of extract was taken in a Kjeldahl flask and 8-10 drops of phenolphthalein indicator was added in the flask. After adding some glass-beads, 6.5 mL of 20% NaOH or required amount was added to the flask after placing on the Kjeldahl distillation unit (GSGW, India). Immediately the steam distillation was started.

Plate: (A) Homogenized sample was filtered after blending

Plate: (B) Added 6.5 mL of 20% NaOH to the Kjeldahl flask

Plate: (C) Kjeldahl flasks were placed on the Kjeldahl apparatus

**Plate 4: Estimation of TVB-N value of sliced Rohu (*Labeo rohita*) by Kjeldahl apparatus**

The steam distillation was regulated in order to collect 100 mL of distillate in ten minutes. The distillation outflow tube was submerged in a receiver with 100 mL with 3% boric acid solution, to which 3-5 drops of the mixed indicator solution (2 g methyl-red and 1 g methylene-blue were dissolved in 1000 mL 95 % ethanol) were added. After 10 minutes, distillation process was ended. Then distillation outflow tube was removed from the receiver and washed out with water. The volatile bases contained in the receiver solution were determined by titration with 0.01 (N) HCl solution. The pH of the end point should be 5.0 ±0.1. A blank test was conducted by the same procedure without using sample.

The TVB-N concentration was calculated using the following equation:

$$\text{TVB} - \text{N (mg/100g sample)} = \frac{(V_1 - V_0) \times 0.14 \times 2 \times 100}{M}$$

Where,

$V_1$ = Volume of 0.01 (N) HCl in mL for sample

$V_0$ = Volume of 0.01 (N) HCl in mL for blank

M = Weight of sample in g

### 3.3.3 Determination of Peroxide Value (PV)

**Preparation of Iron (II) Chloride Stock Solution:** At first, 0.4 g barium chloride dehydrate was dissolved in 50 mL deionized water. This solution was added slowly and with constant stirring to an iron (II) sulfate solution (0.5 grams of $FeSO_4.7H_2O$ in 50 mL deionized water). 2 mL of 10N HCl was added to the resulting solution. The barium sulfate precipitated was filtered off to give clear iron (II) solution, which was stored in a brown bottle and kept in the dark.

**Preparation of Ammonium Thiocyanate Solution:** Thirty (30) g of ammonium thiocyanate was dissolved in distilled water, and the volume was made up to 100 mL.

After preparation of those above mentioned solutions, Peroxide value was determined by IDF standards (1991) described by Shantha and Decker (1994) using fish oil. For this purpose, firstly the oil was extracted by means of Soxhlet apparatus (JSGW, Haryana, India) by following standard method of AOAC (AOAC 1995). About 10–15 g of chopped fish sample was taken in thimble paper and kept in an oven at 105°C for 5 hours for drying. Then oil was extracted with 200 mL of diethyl ether in the soxhlet apparatus, followed by solvent removal under reduced pressure at 70°C using rotary evaporator (HAHNVAPOR, HS- 2005V, Korea).

Plate: (A) Fish oil was extracted through soxhlet apparatus

Plate: (B) Evaporation of fish oil was done in a rotary evaporator

**Plate 5: Peroxide value determination by fish oil extraction through soxhlet apparatus followed by evaporation in rotary evaporator**

To determine the peroxide value, 0.01-0.30 g sample fish oil was taken in a test tube and added 9.8 mL chloroform: methanol (7:3) mixture and then mixed on a vortex for 2-4 seconds. Ammonium thiocyanate solution (50 µL) was added and mixed on a vortex for 2-4 seconds. Then 50 µL iron (II) solution was added and mixed again on a vortex for 2-4 seconds. After 5 minutes of incubation at room temperature, the absorbance of the sample was determined at 500 nm against a blank that contained all the reagents except the sample by using an UV-visible spectrophotometer (UV- 1601 PC, Shimadzu, Japan). The entire procedure was conducted in subdued light. The peroxide value, expressed as milliequivalents of peroxide per kilogram of sample.

Peroxide value was calculated by using the following formula:

$$\text{Peroxide value (mili Eq/kg fish oil)} = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2}$$

Where,

$A_s$ = absorbance of the sample

$A_b$ = absorbance of the blank

m = slope, obtained from calibration curve (m used here was 41.52 for IDF method conducted by Shantha and Decker, 1994)

$m_0$ = mass in grams of the sample

55.84 = atomic weight of iron.

### 3.3.4 Estimation of Thiobarbituric Acid Reactive Substance (TBARS) value

TBARS value was measured according to the procedure of (Witte *et al.*, 1970). Twenty (20) g of sliced Rohu flesh was homogenized with 50 mL of 20% trichloroacetic acid (in 2 M phosphoric acid) at 1000 rpm for two minutes using a homogenizer (IKA T18 digital ULTRA TURRAX, Staufen, Germany). The resulting slurry was then transferred into a 100 mL mass cylinder. The slurry was diluted to 100 mL with de-ionized distilled water and homogenized again. After approximately 50 mL was filtered through filter paper (Whatman No. 1, 100 nm), 5 mL of filtrate was transferred into a test tube and 5 mL of 2- thiobarbituric acid (0.005 M in DD water) was added. The test tube was shaken well and kept in the dark for 15 hours at room temperature. The reactive substances were measured at 530 nm using a spectrophotometer UV-Visible Spectrophotometer (UV-1601 PC, Shimadzu, Japan). Two replicates of 20 g sample were taken for the measurement.

TBARS value was calculated as follows:

TBARS value (mg malonaldehyde/kg) = optical density (O.D) ×5.2

Plate: (A) Homogenized the fish sample in a homogenizer

Plate: (B) Homogenized sample was filtered with a filter paper

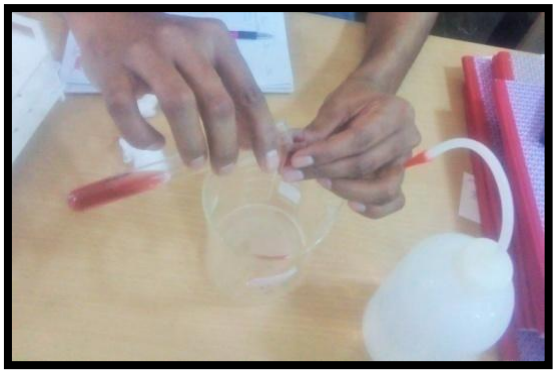

Plate: (C) After keeping the samples in the dark for 15 hours they were placed in small tubes

Plate: (D) Reactive substances absorbance were measured in spectrophotometer

**Plate 6: Estimation of TBARS value of sliced Rohu (*Labeo rohita*) by spectrophotometer**

## 3.4 Microbiological Analysis

### 3.4.1 Preparation of Peptone Physiological Saline (PPS) Solution

For the preparation of each 1000 mL of peptone physiological saline (PPS) solution, one (1) g of peptone and 8.5 g of NaCl (sigma-Aldrich, USA) were weighed on an electric balance and taken in a glass bottle containing required amount of distilled water to dissolve and make the volume 1000 mL (shown in Table-1).Then the mixture was mixed properly on magnetic stirrer. Nine (9) mL of PPS was transferred to each of the test tubes. The PPS tubes were then sterilized in an autoclave (Tomy Digital Biology, Japan) for 15 minutes at 121$^{o}$C under 15 lbs/sq inch pressure.

(A) Peptone Physiological Saline (PPS) was prepared.

(B) PPS was placed in an autoclave for sterilization

Plate: (C) Autoclaving was done for 15 min at 121$^{0}$C

**Plate 7: Preparation of Peptone Physiological Saline (PPS) solution**

**Table 1**: **The composition of the PPS solution and plate count agar (1 L)**

| Ingredients | PPS | Plate count agar |
|---|---|---|
| Peptone | 1 g | - |
| NaCl | 8.5 g | - |
| Plate count agar | - | 23.5 g |
| Distilled water | Up to 1000 mL | Up to 1000 mL |

### 3.4.2 Media Preparation

For bacterial count, plate count agar (Sigma-Aldrich, USA) was used. For this purpose, 23.5 g of agar was weighed by an electric balance and taken in prescribed amount of distilled water to dissolve and make the volume 1000 mL (shown in Table 1). Then the mixture was mixed properly with magnetic stirrer. In case of plate count agar, the mixture was boiled and stirring with a stick so that the ingredients mix thoroughly. Then the media was sterilized for 15 minutes at 121$^{o}$C under 15 lbs/sq inch pressure.

**Plate 8: Preparation of Media (Agar)**

### 3.4.3. Preparation of Sample for Microbial Analysis

The sliced Rohu fishes were cut into small pieces by using knife from each of the packages which were stored at refrigerator (4°C) on the sampling day. Twenty five (25) g of sample from each pack was aseptically collected in a sterile stomacher bag

with 225 mL of peptone physiological salt solution (PPS) (up to 10 times of the sample) to obtain decimal dilution. Blending was done for 30 seconds in a stomacher blender (SJIA-04C, Led Techno, China) (shown in Plate 9).

Thus, a sample of 1:10 dilution was obtained. One (1) mL sample was then transferred by micropipette to a test tube containing 9mL of PPS and the test tube was properly shaken on a vortex mixture thoroughly. Using similar process, several ten folds dilutions were made up to desired level.

Plate: (A) Fish sample was diluted by adding PPS

Plate: (B) Diluted sample was blended in a stomacher blender

**Plate 9: Preparation of sliced Rohu (*Labeo rohita*) sample for microbial study**

### 3.4.4. Total Viable Count (TVC)

Total viable count (TVC) expressed as colony forming units (CFU/g) of the representative samples were determined by standard plate count method on plate count agar following the serial dilution technique described by (APHA, 1992). Microbiological data were transformed into logarithms of the number of colony forming units (log CFU/g).

At first, 1 mL of prepared, well shaken diluted sample was transferred to empty plates using micropipette (Fig. 9). Samples were pipette out and transferred aseptically to the plates by raising the upper lids sufficiently enough to admit the tip of the pipette. Then the required amount of prepared plate count agar (cooled to 45°C± 1°C using water bath) was poured to the plates (shown in Plate 10). All of these activities were performed inside a laminar air flow cabinet (BBS-V1300, Biobase). At least three appropriate dilutions were enumerated for all cases. All the plates were inoculated in

duplicate. After solidifying the agar, the plates were incubated at 35°C in inverted position in an incubator (Binder, Germany). After 48±2 hours of incubation, colonies were developed and only the plates having 30-300 colonies were counted by using colony counter (Labotronics, India) (shown in Plate 11). All the plates were examined visually for typical colony types and morphological characteristics associated with each growth medium.

Plate: (A) Samples were taken from stomacher bag

Plate: (B) Samples were transferred in PPS tubes

Plate: (C) Samples were transferred in plates

Plate: (D) Plates were prepared by following pour plate technique

**Plate 10: Bacterial sample transfer in PPS tubes for dilution followed by bacterial culture in agar medium by pour plate method**

The result was performed from following formula-

$$N(CFU/g) = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2)] \times (d)}$$

Where,

N= Number of colonies per mL or g of product (CFU/g)

∑C= Sum of all colonies on all plates counted

$n_1$= Number of plates in first dilution counted

$n_2$=Number of plates in second dilution counted

d= Dilution from which the first counts were obtained

Plate: (A) Prepared plates were incubated at $35^0$C for 48 hrs in dry air oven

Plate: (B) Bacterial colony was counted in a digital colony counter

**Plate 11: Incubation and counting of bacterial colonies**

## 3.5 Statistical Analysis

All the trials were replicated three times. The values were expressed as mean ± standard deviation. Differences among treatments were estimated by using one-way ANOVA with the application of Tukey test using SPSS Version 20. Average values were considered significantly different when $p<0.05$.

## 3.6 Instruments and Appliances

Various instruments and appliances were used in the laboratory during the completion of this experiment. Some of the key instruments photographs are as follows:

**Plate 12: Showing drying oven (on left) and incubator (on right) respectively**

**Plate 13: Showing autoclave (on left) and stomacher blender (on right) respectively**

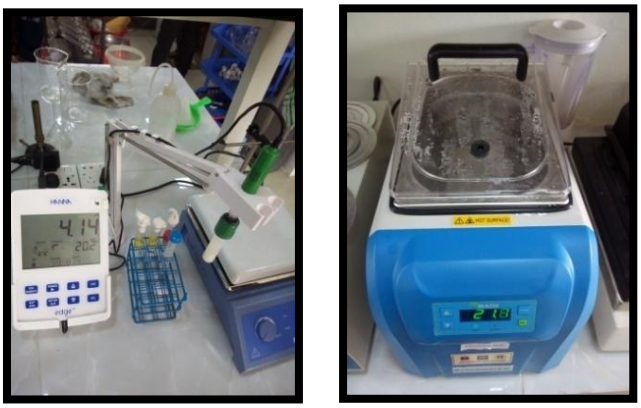

**Plate 14: Showing $p^H$ meter (on left) and water bath (on right) respectively**


**Plate 15: Showing vortex machine (on left) and digital colony counter (on right) respectively**

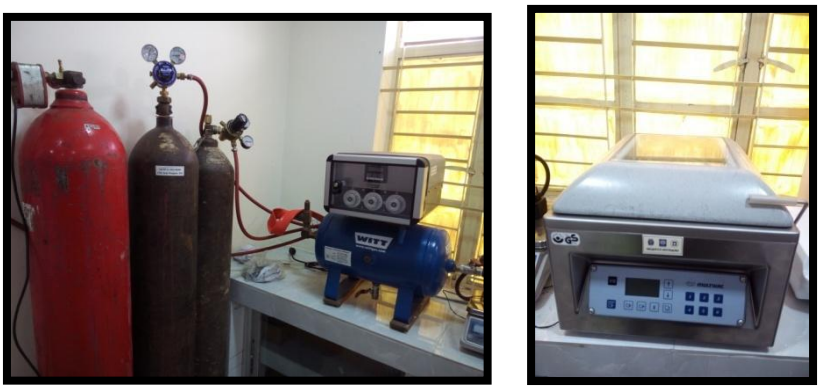

**Plate 16: Showing gas mixture machine (on left) and packaging machine (on right) respectively**

**Plate 17: Showing PC connected spectrophotometer (on left) and soxhlet apparatus (on right) respectively**


**Plate 18: Showing plate count agar bacterial culture medium used in the study**

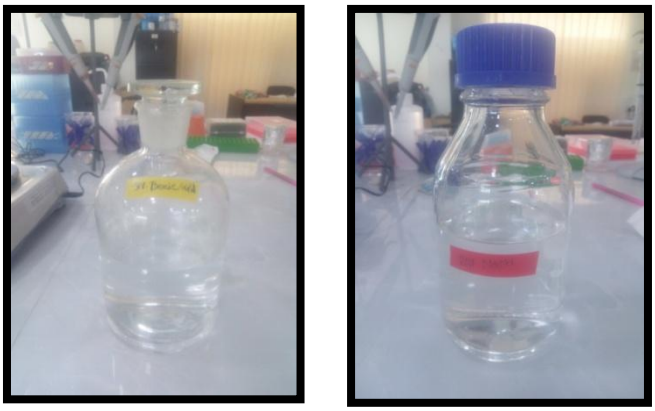

**Plate 19: Some key chemicals used in the study, 3% Boric acid (on left) and 20% NaOH (on right) respectively**

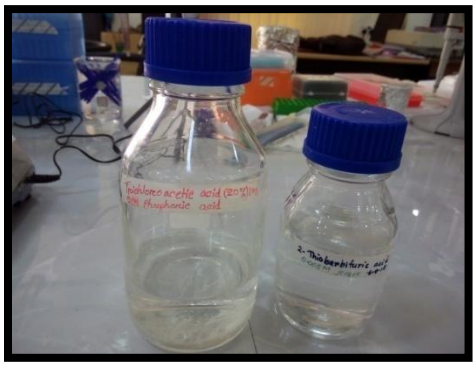

**Plate 20: Some key chemicals used in the study, 20% trichloroacetic acid (in 2 M phosphoric acid) (on left) and 2- thiobarbituric acid (0.005 M in DD water) (on right) respectively**

# CHAPTER 4
# RESULTS AND DISCUSSION

The present study was conducted to identify the optimum condition for preserving fresh Rohu fish at refrigerated storage using vacuum and modified atmosphere packaging techniques. For this purpose, different quality parameters were evaluated such as pH, TVB-N, Peroxide and TBARS value as well as the growth of bacteria in fresh Rohu fish stored at refrigeration temperature (4°C) using different packaging conditions. As a result, all of these evaluations determined the optimum condition of preservation as well as the shelf life for fresh Rohu fish at different packaging conditions.

## 4.1 pH Value

The initial pH value of sliced Rohu fish was 6.50 immediately after death in sliced samples. The pH value was gradually decreased until 3$^{rd}$ days of storage for without sealed pack samples which was treated as control, 6$^{th}$ day for vacuum and MAP-2 sample and 9$^{th}$ day of storage for MAP-1 samples, and then pH values showed an increasing trend with some fluctuations (shown in Table 2). pH values in all storage conditions were within the range of 6.8~7.0 (Metin *et al.*, 2001).

There were no significant ($p<0.05$) differences in changes in pH values among four packaging conditions during the storage period. The lower pH value of fish packaged with MAP condition at a higher concentration of $CO_2$ was reported in other studies (Stamatis and Arkoudelos, 2007; Masniyom *et al.*, 2002; Provincial *et al.*, 2010). The results obtained from the present study is more or less similar phenomenon those reported in previous studies. In vacuum packaging and MAP, further acidification perhaps caused by lactacidogenic bacteria. This is connected with the inhibition of gram-negative aerobic bacteria (mainly pseudomonads), and they become predominant during the course of the storage period as their number increases (Leroi, 2010).

In contrary, under both MAP-1 and MAP-2 pack sample, gradual decrease of pH value was evident until 9$^{th}$ day of storage, followed by a gradual increase until the end of the storage period. In both cases of MAP, the initial drop of pH value may be (by 6$^{th}$ day of storage) occurred due to the dissolution of $CO_2$ in muscle tissues.

Moreover, surface of fish muscle absorbs $CO_{2,}$ thus acidifying it with the formation of carbonic acid (Banks *et al*, 1980). Previous studies found that the consequent increase of pH at later stages was usually coupled with the production of basic components such as ammonia, dimethylamine, trimethylamine, other biogenic amines, and also due to the results of microbial spoilage (Goulas and Kontominas, 2007, Fan *et al.*, 2008). When the pH value compared with different MAP system, relatively lower $p^H$ values were found in MAP 1 than MAP-2 throughout the entire storage period. The lower pH value of muscle tissue in MAP1 (shown in Table 2) perhaps reflected by $CO_2$ increase during the course of storage**.**

**Table 2: pH value of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 6.50±0.18$^{a}$ | 6.14±0.04$^{a}$ | 6.48±0.04$^{a}$ | 6.22±0.04$^{a}$ | | | |
| Vacuum pack | 6.50±0.18$^{a}$ | 6.25±0.05$^{ab}$ | 6.24±0.01$^{a}$ | 6.36±0.09$^{a}$ | 6.60±0.06 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 6.50±0.18$^{a}$ | 6.35±0.07$^{ab}$ | 6.16±0.10$^{a}$ | 6.12±0.06$^{a}$ | 6.39±0.19 | 6.37±0.18 | 6.44±0.06 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 6.50±0.18$^{a}$ | 6.41±0.04$^{b}$ | 6.25±0.16$^{a}$ | 6.27±0.07$^{a}$ | 6.58±0.02 | 6.38±0.04 | 6.77±0.11 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

## 4.2 Total Volatile Base Nitrogen (TVB-N) Value

The total amount of ammonia ($NH_3$), dimethylamine (DMA) and trimethylamine (TMA) in fish as a whole is termed as total volatile base nitrogen (TVB-N) content of fish and is commonly used to estimate bacterial spoilage of fish (Wu and Bechtel, 2008). TVB-N also used as an indicator to assess the quality and shelf-life of fish and fishery products.

In the present study, the initial TVB-N value was 1.18 mg/100g in sliced Rohu fish. The gradual increase in TVB-N value was evident during the storage period (shown in Table 3). The maximum TVB-N value at the end of the experiment was found 6.04 mg on $18^{th}$ day of storage for MAP-1 pack sample.

However, in the present study, the TVB-N values were found within the acceptable limit (shown in Table 3) in all packaging conditions (30-35 mg/100g) as suggested by Huss, (1988). According to past study, it was stated that TVB-N value less than 20 mg/100g is considered as safe for human consumption (Jezek and Buchtova, 2010). In the present study the TVB-N values ranged from 1.18-5.44 mg/100g, 1.18-6.04 mg/100g, 1.18-5.46 mg/100g while preserved in vacuum packaging, MAP1 and MAP2 respectively. Therefore, this result indicates that all three preservation system can ensure safe limit of TVB-N value for human consumption.

There were no significant differences ($p<0.05$) in relation to TVB-N values among four packaging conditions until the $9^{th}$ day of the storage (shown in Table 3). Significant lower ($p < 0.05$) TVB-N values were recorded at $9^{th}$ day of the storage for MAP-1 and MAP-2 pack samples as compared to control samples. It was evident that the samples which were packed in MAP showed slower increase of TVB-N value which is similar to previous study result in order to preserve silver carp fillets at $4^0$C (Rahmatipoor *et al.,* 2017).

Among the MAP samples used in the present study, MAP1 (50% $CO_2$ & 50% $N_2$) showed better performance than MAP2 (50% $CO_2$ & 50% $O_2$) from start to end of the storage period. Similar results were reported by Jezˇek and Buchtova´ (2012) while preserved silver carp fillets where the MAP-2 (70% $N_2$, 30% $CO_2$) with higher $CO_2$ and $N_2$ concentrations showed better TVBN performance than MAP-1 (69% $N_2$, 25% $CO_2$, 5%$O_2$, 1% CO).

Better performance of MAP1 in present study may be due to the bacteriostatic properties of $CO_2$. The presence of carbon dioxide gas is responsible for partial prevention and delay of the growth of spoilage bacteria (Farber, 1991). The presence of $O_2$ in MAP2 package may accelerate the growth of aerobic bacteria and eventually speeds up the spoilage process. TVB-N value depends on a number of factors such as feeding, season, size and others environment parameters (Binsi *et al*., 2015).

**Table 3: TVBN value of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 1.18±0.48[a] | 2.38±0.59[a] | 3.36±0.79[a] | 3.90±0.76[ab] | | | |
| Vacuum pack | 1.18±0.48[a] | 2.66±0.99[a] | 3.92±0.79[a] | 5.05±0.4[b] | 5.44±0.15 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 1.18±0.48[a] | 1.82±0.59[a] | 2.50±1.16[a] | 2.94±0.59[ab] | 3.30±0.31 | 4.20±0.57 | 6.04±0.57 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 1.18±0.48[a] | 2.10±0.59[a] | 2.64±0.17[a] | 2.66±0.20[a] | 3.30±0.71 | 4.62±0.59 | 5.46±0.59 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

## 4.3 Peroxide Value (PV)

Lipid oxidation is one of the prime causes of fish spoilage. It affects fatty acids, particularly polyunsaturated fatty acids and produce off-odours and off-flavours which is unpleasant for the consumer (Ferna´ndez *et al.,* 1997). Fish lipid is susceptible to oxidation. Peroxide value is a measure of hydro peroxides formation. It indicates products that are formed during primary lipid oxidation. Peroxide value is widely used as an indicator for the assessment of degree of primary lipid oxidation and the peroxide values expressed as mill moles or mill equivalents of active oxygen per kg of fat (Masoud *et al.,* 2008).

In the present study, the initial peroxide value was 4.93 mEq/Kg fish oil in sliced Rohu fish. However, the values varied between 1.24-8.18 mEq/kg fish oil during the storage period. The peroxide values of sliced Rohu fish were within the recommended values of 10-20 mEq/kg of fish oil which is the acceptable limit as suggested by (Connell, 1995). Significant lower ($p<0.05$) PV values were observed in vacuum samples on $9^{th}$ day of storage compared to that of control samples (shown in Table 4**).** In vacuum packaging, lipid oxidation can be delayed by limiting access to oxygen thus preserving the quality of muscle foods (Etemadianet al., 2012).

The PV showed significant fluctuations from the beginning till the end of the storage period in all packaging conditions. Similar fluctuations of PV were reported by some other authors (Ozyurt *et al.,* 2009; Bahmani *et al.,* 2011; Jezˇek and Buchtova´, 2011). The initial PV indicates that the oxidation process already started during the period of handling and processing, and a consequent decline in PV is then caused by competing reactions and increases in thiobarbituric acid values (Chaijan 2011).

Lower PV was recorded for MAP-1 compared to that of MAP-2 pack samples during the end of the storage period. This may be due to the presence of $O_2$ in MAP-2 pack (50% $CO_2$ & 50% $O_2$) that triggered the oxidation process and formed hydroxides, hydro peroxides during the end of the storage period. Similar results were found by Jezˇek and Buchtova´, (2012) in silver carp fillets and the progress of primary lipid oxidation was rather erratic as in this study.

**Table 4: Peroxide value (PV) of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | $4.93\pm2.08^{a}$ | $2.87\pm0.04^{a}$ | $4.93\pm0.40^{a}$ | $5.54\pm0.40^{bc}$ | | | |
| Vacuum pack | $4.93\pm2.08^{a}$ | $5.37\pm2.03^{ab}$ | $7.04\pm1.08^{a}$ | $1.33\pm0.42^{a}$ | $1.45\pm0.16$ | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | $4.93\pm2.08^{a}$ | $8.18\pm1.26^{b}$ | $5.43\pm0.95^{a}$ | $7.04\pm2.39^{c}$ | $3.94\pm0.21$ | $1.24\pm0.23$ | $1.33\pm0.13$ |
| MAP-2(50% $CO_2$ & 50% $O_2$) | $4.93\pm2.08^{a}$ | $5.37\pm2.40^{ab}$ | $6.83\pm0.88^{a}$ | $2.59\pm0.99^{ab}$ | $3.40\pm1.64$ | $3.75\pm0.36$ | $3.13\pm3.12$ |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

Lipid oxidation limits the shelf life of oily fish as suggested by (Hossain *et al.*, 2005). The rate and extent of oxidative deterioration depends on the factors such as the as fish species, storage period and temperature, saturation degree of fatty acids, antioxidants or prooxidants and availability of oxygen (Bahmani *et al.*, 2011). However, PV alone cannot be considered as a suitable fish muscle freshness indicator as suggested by (Jezˇek and Buchtova´, 2007).

## 4.4 Thiobarbituric Acid Reactive Substances (TBARS)

TBARS is a procedure which evaluates degree of secondary lipid oxidation and thus quality of food. TBARS index is used to measure the amount of malonaldehyde which is the secondary product of the oxidation of polyunsaturated fatty acids (Bremner, 2002). The second stage of auto-oxidation in which modification of peroxide occurs, results in production of materials such as aldehydes and ketones (Feliciano *et al.*, 2010).The acceptable limit of TBARS value is 2 mg malonaldehyde/kg fish sample and beyond this limit, an objectionable odor and taste develops in fish (Connell, 1990).

In the present study, the initial TBARS value was 0.23 mg malonaldehyde/kg in sliced Rohu fish. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. However, significant ($p<0.05$) lower TBARS values were observed on 3$^{rd}$ and 6$^{th}$ day of storage in vacuum, MAP1 and MAP2 pack samples in comparison to control samples. Significant ($p<0.05$) higher TBARS values were also observed on 9$^{th}$ day of storage in case of MAP-2 sample as compared to other samples. In the present study, the TBARS values were within the acceptable limit (2 mg malonaldehyde/kg) in all samples except MAP-2 sample.

The TBARS value of MAP-2 pack sample exceeded the acceptable limit of 2 mg on and after 9$^{th}$ day of the storage (shown in Table 5). This may be the results of higher rate of secondary lipid oxidation due to the presence of high $O_2$ concentration in MAP-2 (50% $CO_2$ & 50% $O_2$) packed samples. Arashisar *et al.* (2004) also found similar results in rainbow trout fillets packaged with 30% $O_2$ as compared to the MAP with no oxygen. $O_2$ not alone but also some bacterial enzymes also participate in oxidation process (Herna´ndez *et al.*, 2009).

**Table 5: TBARS value of Rohu fish (*Labeo rohita*) under vacuum and MAP at refrigerated storage (4°C).**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Control | 0.23±0.11[a] | 0.56±0.01[b] | 1.08±0.10[c] | 1.17±0.07[b] | | | |
| Vacuum pack | 0.23±0.11[a] | 0.41±0.03[a] | 0.17±0.06[a] | 0.46±0.05[a] | 0.74±0.07 | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | 0.23±0.11[a] | 0.45±0.04[a] | 0.53±0.17[b] | 1.09±0.02[b] | 0.78±0.11 | 1.40±0.05 | 2.70±0.12 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | 0.23±0.11[a] | 0.56±0.04[b] | 0.90±0.12[c] | 2.38±0.17[c] | 3.69±0.47 | 3.27±0.54 | 5.40±0.17 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

Substances of foul odor or taste are often evident during secondary oxidation of lipids. Some such substances may be responsible for the textural changes particularly when they form covalent bonds with muscle proteins (Huss, 1995). Thiobarbituric acid is a more suitable indicator of the degree of fish muscle oxidation than PV as malonaldehyde may interact with other components such as nucleosides, nucleic acids, proteins and other aldehydes. Moreover, Thiobarbituric acid value need not always accurately reflect the current level of lipid oxidation (Ozyurt *et al.,* 2009).

## 4.5 Total Viable Count (TVC)

In the present study, the initial total viable count (TVC) of sliced Rohu fish was found 4.29 log CFU/g which indicated an acceptable quality of fish. It was evident in the literature that freshly caught fresh water fishes (sea bass, tilapia, rainbow trout, and silver perch) contain bacterial counts of 2-6 log CFU/g (Gelman *et al.*, 2001).

The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. However, significant ($p<0.05$) lower TVC values were recorded on $9^{th}$ and $12^{th}$ day of storage in vacuum, MAP1 and MAP2 packaged samples compared to that of control. Similar results were observed in the studies of Babic *et al.* (2015) on common carp steaks kept under MAP comprising (MAP- 1: 40% $CO_2$/60% $N_2$ and MAP- 2: 100% $CO_2$) followed by storage at +3±0.5°C, although in our study the MAP2 composition was (50% $CO_2$ & 50% $O_2$). Hudecová *et al.* (2010) also reported similar results on fresh common carp under two different modified atmosphere packaging (MAP-1: 70% $N_2$/30% $CO_2$; MAP-2: 80% $O_2$/20% $CO_2$) and air (control samples) stored at 4 ± 0.5°C where MAP1 and MAP-2 showed better performance than the control sample which is in relevance to our present study.

Higher shelf life was observed in both MAP samples in the present study. This may be due to the bacteriostatic effect of $CO_2$ in MAP packaging. In a number of studies, the effect of various $CO_2$ concentrations showed delayed microbial growth as concluded by various researchers such as in Swordfish (Pantazi *et al.,* 2008), Cod (Sivertsvik, 2007), Chub Mackerel (Stamatis and Arkoudelos, 2007), sea bass (Poli *et al.,* 2006), Sardines (Özogul *et al.,* 2004), rainbow trout (Arashisar *et al.,* 2004; Randell *et al.,* 1997), herring (Özogul *et al.,* 2000; Randell *et al.,* 1997), and salmon (De la Hoz *et al.,* 2000). The bacteriostatic effect of $CO_2$ in modified atmospheres

results in a decrease in the growth rate of bacteria during the logarithmic stage (Farber, 1991).

Overall, best quality was observed under MAP1 (50% $CO_2$ & 50% $N_2$) in comparison to other samples. MAP-1 packaging creates anaerobic condition inside the pack; as a result aerobic bacterial growth is inhibited and the shelf-life is prolonged. Additionally, $CO_2$ has bacteriostatic effect. It also retards the oxidative rancidity and inhibits the growth of aerobic microorganisms by displacing oxygen from packaging (Farber, 1991).

TVC values exceeded the 7 log CFU/g, which is considered as the upper acceptable limit (ICMSF, 1986) for fresh and frozen fish, about $8^{th}$ day for control, $11^{th}$ day for vacuum, $16^{th}$ day for MAP-1 and $13^{th}$ day of storage for MAP-2 sample (shown in Figure.1). Considering the bacterial counts, the shelf-life of sliced Rohu (*Labeo rohita*) in refrigerated temperature was in acceptable conditions for 8 days in control, 11 days in vacuum pack, 16 days in MAP-1, and 13 days in MAP-2 sample.

**Table 6: Total viable count of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C)**

| Treatments | Storage Period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 day** | **3 days** | **6 days** | **9 days** | **12 days** | **15 days** | **18 days** |
| Not sealed (Control) | $4.29\pm0.09^{a}$ | $4.31\pm0.08^{a}$ | $4.67\pm0.10^{a}$ | $7.33\pm0.45^{b}$ | $8.23\pm0.13^{c}$ | | |
| Vacuum pack | $4.29\pm0.09^{a}$ | $4.18\pm0.10^{a}$ | $4.70\pm0.25^{a}$ | $6.38\pm0.09^{ab}$ | $7.33\pm0.14^{b}$ | | |
| MAP-1(50% $CO_2$ & 50% $N_2$) | $4.29\pm0.09^{a}$ | $5.00\pm0.73^{a}$ | $5.44\pm0.02^{b}$ | $5.70\pm0.12^{a}$ | $6.14\pm0.07^{a}$ | 6.77±0.41 | 7.67±0.16 |
| MAP-2(50% $CO_2$ & 50% $O_2$) | $4.29\pm0.09^{a}$ | $5.24\pm0.01^{a}$ | $5.32\pm0.06^{b}$ | $5.31\pm0.32^{a}$ | $6.64\pm0.27^{a}$ | 7.65±0.04 | 7.49±0.11 |

Superscript letters in the same column represent significant difference (mean values) among the treatments ($p < 0.05$)

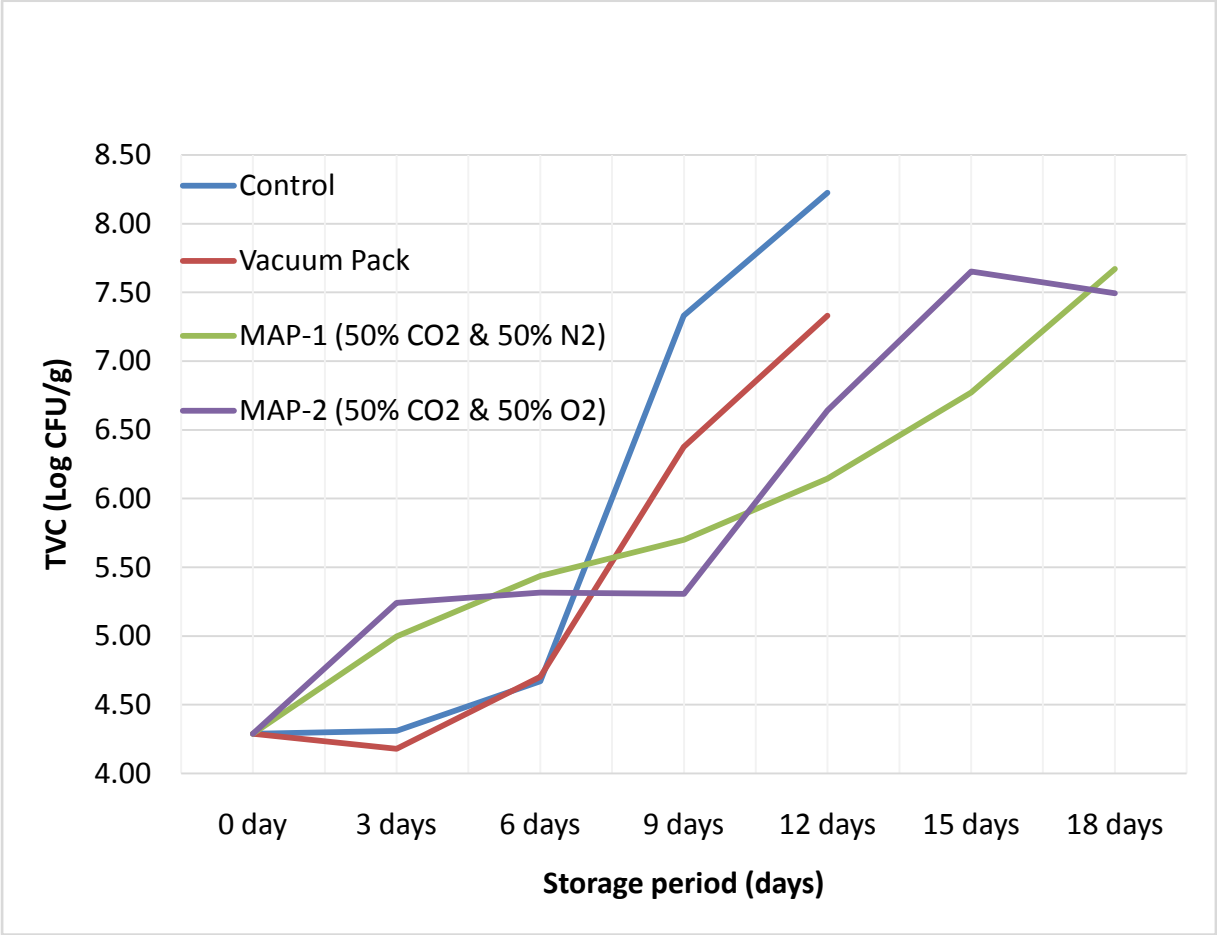


**Figure 1: Total viable count of Rohu fish (*Labeo rohita*) under vacuum and MAP condition at refrigerated storage (4°C)**

# CHAPTER 5

# SUMMARY AND CONCLUSION

The present study was conducted to identify the optimum condition for preserving fresh Rohu fish at refrigerated storage using vacuum and modified atmosphere packaging techniques. The initial pH value of sliced Rohu fish was 6.50 immediately after death in sliced samples. The pH value was gradually decreased until 3$^{rd}$ days of storage for without sealed pack samples which was treated as control, 6$^{th}$ day for vacuum and MAP-2 sample and 9$^{th}$ day of storage for MAP-1 samples, and then pH values showed an increasing trend with some fluctuations. There were no significant ($p<0.05$) differences in changes in pH values among four packaging conditions during the storage period.

In the present study, the initial TVB-N value was 1.18 mg/100g in sliced Rohu fish. The gradual increase in TVB-N value was evident during the storage period (shown in Table 3). The maximum TVB-N value at the end of the experiment was found 6.04 mg on 18$^{th}$ day of storage for MAP-1 pack sample. There were no significant differences ($p<0.05$) in relation to TVB-N values among four packaging conditions until the 9$^{th}$ day of the storage.

In the present study, the initial TBARS value was 0.23 mg malonaldehyde/kg in sliced Rohu fish. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. However, significant ($p<0.05$) lower TBARS values were observed on 3$^{rd}$ and 6$^{th}$ day of storage in vacuum, MAP1 and MAP2 pack samples in comparison to without sealed (control) samples. Significant ($p<0.05$) higher TBARS values were also observed on 9$^{th}$day of storage in case of MAP-2 sample as compared to other samples. In the present study, the TBARS values were within the acceptable limit (2 mg malonaldehyde/kg) in all samples except MAP-2 sample. The TBARS value of MAP-2 pack sample exceeded the acceptable limit of 2 mg on and after 9$^{th}$ day of the storage.

In the present study, the initial total viable count (TVC) of sliced Rohu fish was found 4.29 log CFU/g which indicated an acceptable quality of fish. The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. However, significant ($p<0.05$) lower TVC values were recorded on 9$^{th}$ and

$12^{th}$ day of storage in vacuum and MAP packaged samples compared to that of control.

The results of the present study revealed that pH, TVB-N and PV under all packaging conditions never crossed the prescribed acceptance limit during the entire storage period. There was decreasing and increasing trend in pH value in the samples in all storage conditions but it was in the range of 6.8~7.0. The gradual increase in TVB-N value was evident in the samples in all treatments during the storage period. The PV values showed siginificant fluctuating manner from the beginning till the end of the storage period under all packaging conditions. The TBARS value slowly increased with the progression of time in all packaging conditions during the storage period. The TVC values of sliced Rohu fish gradually increased with the extension of time under all packaging conditions. Based on bacterial counts and 7 log CFU/g value as the upper acceptable limit the shelf life of sliced Rohu fish (*Labeo rohita*) was determined about 8 days for without sealed pack, 11 days for vacuum pack, 13 days for MAP-2, and 16 days for MAP-1 pack samples.

By analyzing all biochemical and microbiological parameters it was evident that the best performance was observed in MAP-1 (50% $CO_2$ & 50% $N_2$) package sliced Rohu fish sample compared to without sealed pack (control), vacuum pack and MAP-2 pack fish samples. Moreover, MAP technology proved to be effective to extend the shelf life and improve the overall quality of packaged sliced Rohu fish, which can be utilized by the fish processors.

# RECOMMENDATIONS

There is a great potential for VP and more importantly MAP system in Bangladesh in order to preserve fish and fishery products to extend the shelf-life as well as to add value and convenience. High production cost, requirement of skilled staff, drip loss and chemical and microbiological hazard in the end product are some of the drawbacks of MAP which can be overcome. MAP technology can be very handy at this period of time when food safety has become one of the most burning issues. These packaging technologies can be utilized by the processors or superstores to display and preserve fish and fishery products under refrigeration condition which will increase the quality, value and shelf-life of the product as well as will ensure considerable levels of food safety to the consumers. Vacuum packaging and more importantly MAP was found effective on the extension of shelf-life of Rohu fish in a refrigerated condition. Similar studies should be continued using other commercially important fishes using different gas compositions to analyze the effects and justify the use of this modern technology in our country.

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