Quality and shelf life assessment of ready to cook fish products during storage at different freezing temperature

A Thesis
By

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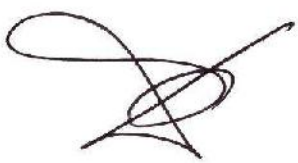

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Declaration

The results presented in the project thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.

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Dedicated To

My Beloved Parents

Acknowledgement

At first, all my admiration goes to the Almighty for his mercy to enable me to pursue my education in Fisheries and Marine Resource Technology Discipline. It is my great pleasure for being able to complete this thesis for the degree of Master of Science in Fisheries for which I am completely indebted to the Creator of this Universe, the Almighty.

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Abstract

It was aimed to investigate the sensory, bio-chemical, nutritional and microbiological quality related changes of some selected fish species namely tilapia (*Oreochromis mossambicus*), parse (*Liza parsia*) and rui (*Labeo rohita*), and it's processed Ready to cook (RTC) products during storage at variable freezing conditions. Sensory, biochemical, nutritional and bacteriological quality related changes of RTC products prepared from tilapia, parse and rui fish were determined during a storage period of 08 weeks (56 days) at two different freezing temperatures viz. -5°C and -18°C. Quality attributes determined in this study included sensory scores, pH, TMA-N, TVB-N, protein, lipid, moisture, ash, total viable count (TVC), sulfur producing bacteria (SPB) count and *Pseudomonas* bacteria count. All of the quality attributes were found to be very low in amount or its changes occurred slowly that were the indicative fresh product during the storage period. Therefore, tilapia, parse and rui can be kept in freezer at -5°C and -18°C for with little quality changes at least 56 days. Based on the quality changes during freezing at those temperatures, 1-3 years of shelf life can be attained.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and non-English Words

Abbreviation	Elaboration
CFU	Colony Form Unit
et al.	And Others
Fig.	Figure
FMRT	Fisheries and Marine Resource Technology
KU	Khulna University
Ml	Milliliter
MS	Microsoft
spp.	Species
UN	United Nations
USFDA	U.S. Food & Drug Administration
Viz	Namely/That is to Say/As Follows
RTC	Ready to Cook
TVB-N	Total volatile base-nitrogen
TMA	Trimethylamine
TVC	Total viable count
SPB	Sulfur producing bacteria

Chapter.....1

Introduction

1. Introduction

1.1 Background of the study

Fish is an excellent source of low-fat animal protein. It has many health benefits for various nutritional compositions that include 70-84 % water, 15-24 % protein, 0.1-22% fat and 1-2%, minerals, 0.5% calcium, 0.25% phosphorus and 0.1% vitamins A, D, B and C (Abraha *et. al.*, 2018). Due to nutritional importance and abundance in Bangladesh, fish provides the consumers with about 60% of their animal protein intake (DoF, 2018). Present fish production of the country is more than 04 million Metric Ton (MT) and the country has set a target of producing 4.5 million MT of fish by the year 2021 by increasing intensity of aquaculture technology as well as capture fisheries management (DoF, 2018).

Although avenues exist to increase the fish production, post-harvest losses of fish in the country is also enormous of about 20- 30% in different fish and fishery products during harvesting, transportation and processing (Nowsad, 2015). The losses are caused by poor handling practices, biochemical and microbiological changes, temperature abuse, poor processing and preservation, inadequate packaging and so on that ultimately result in reduced quality and short shelf life. As consequences, duration is elapsed for the recommended maximum time for which the product can be stored, during which the defined quality of a specified proportion of the goods remains acceptable under expected or specified conditions of distribution, storage and display (Martins *et al.*, 2008).

Fish is one of the most perishable food items. If special preservation techniques or any type of interventions are not apprehended, fish or fish products could be continually deteriorated in quality over time and spoiled straight (Oparaku, 2013). Quality assessment was based on microbiological and sensorial indices determination. In recent years, several socio-economic evolutions such as an increased female participation in the workforce, increasing time pressure brought about by job, a growing number of single-person and small households, lack of abilities and experience with preparing fish meals, have boosted the demand for convenience in Ready-to-Cook fish products. Food industry and retailers have readily reacted to this growing demand for convenience by considerably expanding their assortments of Ready-to-Cook fish products: fillets, burgers, cut into rings or entire and so on. The application of preservation methods that permit shelf life extension

of these products is of utmost importance. In addition, it is also to be considered that the lower consumption of some fish or shellfish species is often due to the poor knowledge on the optimal conditions to clean these products, and on the useful storage conditions to prolong their shelf life.

Various classical preservation procedures have allowed considerable extensions of shelf lives of fish to be obtained, but the interest in fresh, mildly preserved, and conveniently packed products has been increasing. In general, fish products have a limited shelf life in comparison with meat products, as a result of several factors such as the high post mortem pH in the flesh (usually >6), the presence of large amounts of nonprotein nitrogen, the high content of polyunsaturated fatty acids, the presence of autolytic enzymes, and so on (Gram and Huss 1996; Sivertsvik and others 2002). Spoilage begins as soon as the fish dies or is caught in consequence of a series of complicated changes brought about in the dead fish mainly by bacteria and enzymes (*Pastoriza et. al.*, 1996).

A variety of different methods exist for improving the quality and prolonging the shelf life of fresh fish and crustaceans following their harvest. Among the methods, fish storage at low temperatures or more precisely fish freezing is one of the most employed methods used for preserving fresh fish and other seafood products.

Frozen storage is an important preservation method for fish and it protects the initial sensory and chemical quality of fresh products. However, the quality and shelf life of fish would be deteriorated during the preservation because physical, chemical and enzymatic changes are still able to occur in the frozen state of fish (Magnussen, 2008). Quality deterioration of frozen fish can be caused by osmotic removal of water, oxidation, denaturation of proteins, sublimation and re-crystallization of ice crystals and mechanical damage (Thyholt and Isaksson, 1997). Extent to which the quality will be deteriorated depends on several factors such as storage temperature, temperature fluctuations, freezing rate and amount, thawing methods and freeze-thaw abuse, initial condition of fish, species and time elapsed between harvest and freezing and packaging type and materials (Magnussen, 2008; Turan *et. al.*, 2006).

Refrigeration is an important way of controlling microbial growth in perishable foods, and the time and temperature profile during storage of such food is critical to minimize the risk for development of microbial hazards leading to foodborne illness. Furthermore, proper refrigeration is vital for

maintaining the quality of the food by preventing spoilage before the claimed shelf life of the product (Gram *et al.* 2002; Sivertsvik *et al.* 2002). Storage temperature is the major factor implicated in the loss of quality and shelf life of frozen fish. The exact temperature of freezing varies among the different species. Icing (0±1°C), chilling (0.6 to -2.2°C), super chilling (-2 to -5°C) and refrigeration (4±1°C) can keep the fish for a few weeks only. If fish are properly frozen and stored at correct temperature below -5°C, it is possible to provide a product which closely resembles fresh fish; in many cases consumers are unable to distinguish between a piece of fresh fish and a piece of frozen fish.

During frozen storage of fish, packaging types and materials also plays critical roles to preserve product quality, protecting it from unwanted changes, providing containment, protection, communication and convenience (Han *et. al.*, 2005). In addition to the storage temperature and packaging materials, the number of freeze- thawing process has direct relation on the nutritional losses of fish and sea foods (Abraha *et. al.*, 2018). Freezing and thawing cycles are repeated several times especially at restaurant and fish markets where the excess amount of food may be put in the freezer again that results in quality changes and can affect consumer's health seriously.

Storage temperature fluctuations, low quality packaging and repeated freeze-thaw cycles during frozen storage have profound consequences on sensory, biochemical, nutritional and microbiological quality of the frozen fish. Some of the attributes of those quality related changes are- development of objectionable 'off odours' and unpleasant 'off flavours', loss of desirable visual characteristics, loss of juiciness and weight, drip loss and toughening, as well as microbial spoilage and autolysis, reduced crude protein and fat (Hallier *et. al.*, 2007; Foruzani *et. al.*, 2015). Alteration of those attributes makes the product less acceptable to consumer due to loss of the significant chemical compositions with tasteful constituents. Assessment of the quality related changes during frozen storage of fish products can be used as a tool for evaluating best suited temperature of a fish species for its preservation. Sensory, biochemical, nutritional and microbiological evaluation are also used to assess the shelf life of that species or its processed product, and can be employed at regular intervals during the storage period (Meilgaard *et. al.*, 2007).

With the increase in intensive aquaculture of different fish species in Bangladesh, it is important to provide proper storage facilities and conditions of the fish under refrigerated or frozen

conditions following harvest for keeping better quality. Proper frozen storage facilities are also important for fish processors and traders for minimizing quality loss of the product during marketing in local or international markets. Business that catch, process, buy, sell or trade fish product should have knowledge regarding the quality related changes during frozen storage and available shelf life of the product. Tilapia (*Oreochromis mossambicus*), Parse (*Liza parsia*) and Rui (*Labeo rohita*) has a reputation as a high quality commercial species, with premium eating qualities. They are important and valuable product in the Bangladesh fish processing industry. However, the fish may occasionally be subjected to inadequate storage conditions (temperature abuse) for a limited period during distribution from slaughter to consumer. The aim of the present experiment was to investigate the effects of different freezing temperatures (time-temperature index) on the quality and shelf life of that fish fillets. It is, therefore, necessary to conduct study on product quality and shelf life assessment of fish products during storage at low temperature.

1.2 Objectives

The objectives of this study were:

1. To assess sensory, biochemical, nutritional and bacteriological quality related changes of Tilapia (*Oreochromis mossambicus*), Parse (*Liza parsia*) and Rui (*Labeo rohita*) fish products (RTC products) during storage at different freezing temperatures.
2. To predict the shelf life of the products based on quality related changes in its muscle.

Chapter.....2

Review of Literature

2. Literature Review

2.1 Ready to cook fish products

Products that may not be fully cooked before they are eaten are often called Ready-to-Cook or RTC food products. RTC foods must be refrigerated properly to prevent the growth of harmful bacteria that could cause food borne illness and they must be handled properly during storage, preparation and serving. Globally, there is an increased demand for ready-made foods from fin fish, crustaceans and other fishery. Today, the main focus is on fresh fishery products, although, the fishing industry has seen a large increase in the production of frozen fish products, mainly due to short shelf-life of fresh fish (Magnussen *et al.*, 2008).This global mandate for fishery products has realized an increased demand for semi-frozen and frozen fish products which are thawed and processed before been sold on the markets (Magnussen *et al.*, 2008). To satisfy consumers changing demand in the developed countries, Bangladesh has started producing cooked and semi-cooked frozen foods, according to officials and exporters. Freezing minimizes microbial and enzymatic activity and hence preserves the flavour and the nutritional properties better than chilled storage. Protein aggregation in frozen fish depends on various factors such as the fish species, storage temperature, temperature fluctuation, storage time and enzymatic degradation (Badii and Howell, 2002). The susceptibility of fish species to changes induced by frozen storage is significantly different. In frozen fatty fish, oxidative changes in lipids and pigments affect the odour and colour as well as proteins, while in lean fish the main changes are reported to involve aggregation of proteins which alter muscle texture (Badii and Howell, 2002).

2.2 Fish Processing

Fish products processed and offered as fresh have a relatively short shelf-life. Depending on the quality of the fish and the conditions of storage, the shelf-life can be as short as a few days or as long as several weeks. Typical fresh products include whole drawn, headed, fillets, and other meat cuts. There is a direct correlation between the temperature of fresh fish products and the shelf-life. For maximum shelf-life, fish should be stored at temperatures near the freezing point of water (i.e., 0°C). At this point the flesh will not freeze, but bacterial and enzymatic activities will be minimized. As storage temperatures rise above 0°C, bacterial and enzymatic activities can increase

significantly, dramatically shortening the shelf-life. Where applicable, crushed ice is used to hold fresh fish. The ice maintains the desired temperature of the fish. Water from the melting ice 'washes' microorganisms from the product, thus extending shelf-life. Little or no sophisticated packaging is required for the product. Most fin fish can be processed for fresh markets.

2.3 Fish packaging

Fresh fish products are usually more perishable than most other foodstuffs due to their high aw, neutral pH, and presence of autolytic enzymes. The spoilage of fish and shellfish results from changes caused by oxidation of lipids, reactions due to activities of the fishes' own enzymes, and the metabolic activities of microorganisms (Ashie *et al.*, 1996). The rate of deterioration is highly temperature dependent and can be inhibited by the use of low storage temperature (e.g. fish stored on ice). The spoilage of fresh fish is usually dominated by microbial activities, however, in some cases chemical changes, such as autoxidation or enzymatic hydrolysis of the lipid fraction may result in off-odours and flavours and, in other cases, tissue enzyme activity can lead to unacceptable softening of the fish.

The term fish product covers a wide range, and includes fish that differ widely in composition, origin, shelf-life and applicability to novel packaging technologies. The range covers fish from temperate waters with a microbial flora adapted to psychrotrophic conditions to fish from tropical waters with different microflora, just as freshwater fish differ from sea fish.

There is also a wide distribution in the chemical composition of fish, for example, lean fish almost without fat, such as cod, and fatty fish, such as salmon; which often contains 20% fat. Some fish have a long natural shelf-life on ice (e.g. halibut) but others, like the pelagic species (e.g. mackerel and herring) have a very short shelf-life. Fresh fish is very different from the various processed fish products that need packaging: heat-treated fish products (ready meals, fish pudding/balls), smoked, dried, or salted fish. So the potential for an active packaging technology to be successful for a product would depend on the technology's ability to control and inhibit the shelf-life deteriorating spoilage reactions (e.g. bacterial growth of specific bacteria, oxidative rancidity, colour changes) in the specific product.

2.4 Freezing, frozen storage

Freezing followed by cold storage is an efficient method of fish preservation, nevertheless, the method does not improve product quality. The final quality depends on the quality of the fish at the time of freezing as well as other factors during freezing, cold storage and distribution (Johnston et al., 1994). The deterioration in fish quality during frozen storage is unavoidable. However, if the effects are to be reduced, it is essential that fish be of good quality prior to freezing.

The initial quality of the fishery product prior to freezing and cold storage is of utmost importance as well as the freezing time of the product is also fundamental. The freezing time is the time taken to lower the temperature of the product from its initial temperature to a given temperature at its thermal centre (Arason, 2014). The freezing time depends on the required final temperature, the physical properties of the product and the freezer specifications (Arason, 2014). Therefore, it is important to be aware of these parameters prior to an attempt of freezing a particular product. Moreover, the fish tissue fluid contains various salts and other compounds in solution, hence the fish muscle freezes in rather a different manner from water. Therefore, the initial temperature of fish has to be reduced to a much lower temperature than water before most of the heat is removed and the fish is completely frozen (Nicholson, 2001).

Freezing and frozen storage of fishery products may lead to denaturation and aggregation of myofibrillar proteins within the fish muscle. These changes within the fish muscle result in altered functional properties, changed textural attributes and reduced water holding capacity and juiciness (Burgaard, 2010). The result is a hard, dry and fibrous fish product which is not appealing to consumers. Moreover, during frozen storage, lipid oxidation occurs in lean as well as fatty species. Oxidation of the phospholipids in lean species results in cold-store flavour and oxidation of triglycerides in more fatty species results in a rancid taste and odour (Burgaard, 2010).

2.5 Thawing

Thawing is the process of changing a product from frozen to unfrozen state. It involves transferring heat to a frozen product to melt the ice that was formed within the flesh during the freezing process (Archer *et al.*, 2008). The thawing time is referred to as the time required to melt all the ice present in the frozen seafood and it occurs at the point where ice crystals are converted back to water

completely and the temperature throughout the seafood reaches -1°C (Archer *et al.*, 2008). Similar to freezing, thawing should be carried out as quickly as possible to maintain product quality, however, it should not be so quick that it adversely affects the product (Archer *et al.*, 2008).

Thawing generally occurs more slowly than freezing which can potentially cause further damage to frozen fishery product texture. The thawing rate during conventional thawing processes is controlled by two main parameters outside the product which are the surface heat transfer coefficient and the surrounding medium temperature (Alizadeh *et al.*, 2007).

2.6 Drip loss

Drip loss is defined to be a form of moisture migration. (Archer et al., 2008). The factors that influence thawing drip loss are many and complex. It is discerned by a number of factors intrinsic to the food product, the conditions of freezing and the thawing process and conditions. Thawing drip loss is visually unattractive, soluble nutrients are lost from the fishery product and it represents a significant economic loss to the processor (Archer *et al.*, 2008).

It is explained that the freezing process is generally much more important than thawing for drip loss of fishery products. The formation of large ice crystals is considered to be associated with slow freezing rates, high and fluctuating temperatures during frozen storage and long frozen storage times (Archer *et al.*, 2008). The role of thawing in minimizing drip loss is that very rapid thawing has generally been found to increase drip loss, possibly because of the reduced time for reabsorption of drip.

Long thawing times will be practically beneficial for the majority of fish species (Archer *et al.*, 2008). Consequently, the use of rapid freezing methods, well controlled frozen storage conditions and good temperature control throughout all stages of handling and processing is undoubtedly the best way of minimizing thawing drip loss in fishery products (Archer *et al.*, 2008). When muscle tissue is rapidly frozen, small ice crystals form both intra-and extra-cellularly, whereas ice crystals form first in the extracellular space when muscle is frozen slowly (Morkore and Lilleholt, 2007).

During further cooling, water inside the fish muscle fibers may migrate to ice crystals that already exist in extracellular areas, eventually resulting in very large extracellular ice crystals, compacted muscle fibers and extensive denaturation of the muscle proteins. Loss in liquid-holding capacity of the proteins along with mechanical damage to cells by ice crystals are proposed as the main

reasons for the higher amount of thaw exudates from fishery product frozen at higher temperatures (Morkore and Lilleholt, 2007).

2.7 Factors influencing shelf life of frozen seafood

Shelf life is defined as the period of time under defined conditions of storage for which a food product remains safe and fit for use (Guizani *et al.*, 2005). In other words, during that period of time the fishery product should retain its desired sensory, chemical, physical, functional or microbiological characteristics. The deterioration in quality of frozen seafood is caused by physical, enzymatic and chemical factors.

Quality loss in frozen fish has been attributed to protein denaturation which correlates strongly with loss of sensory quality such as taste, and texture characteristics over the frozen storage period (Martinsdottir and Magnusson, 1995). When the fish is thawed, its storage life will depend upon a large number of conditions such as species, biological condition, freezing method, storage temperature and time, thawing methods, and storage condition of thawed products (Martinsdottir and Magnusson, 1995). The main aims for post-freezing processes are to prevent fall in quality and protect the products during storage and distribution.

Correct post-freezing treatment will also reduce decolourisation of the fish flesh. Efficient packing is essential to reducing microbial and chemical contamination, dehydration and mechanical damage from the surroundings. Thus, the typical fish smell will be reduced by packaging and liquid loss from the product, especially after thawing, will also be reduced (Magnussen *et al.*, 2008). Gaping occurs in the flesh of fishery products when the collagenous microtubules connecting the myotomes and myocommata within the fish muscle breaks. Thus the formation of large ice crystals has been proposed to explain breakdown of the myocommata that is seen as a physical separation of the myotomes in fillets of fish (Morkore and Lilleholt, 2007).

The texture of fish is an important contributor to palatability and in turn can influence its purchase and wide use. Therefore, proper control of textural attributes will result in better quality products and will improve consumer acceptance of the fishery product (Morkore and Lilleholt, 2007). Fish muscle tissue that is frozen and stored in the frozen state inevitably loses some of its appeal to consumers, usually observed as a loss in juiciness and an increase in toughness after cooking. Furthermore, weight loss in terms of drip losses from fish products may represent direct economic

losses. The amount of liquid retained in fish is also important for the general appearance and moistness of the flesh (Morkore and Lilleholt, 2007)

2.8 Quality evaluation methods for fish quality

The quality of fish degrades because both microbial spoilage and biochemical reactions occur during handling and storage (Guizani *et al.*, 2005). Many methods have been used for the assessment of fish quality during storage. Such methods include changes in the microbial population, total volatile basic nitrogen (TVB-N), and sensory evaluation and in the case of frozen fishery products the changes in the protein and water content for the reasons that protein denatures during frozen storage and drip losses upon thawing. These analysis would give a good indication on the influence of freezing, thawing followed by chilled storage on the quality of the fishery product as it relates to its nutritional properties.

Among the chemical indices of spoilage assessed are trimethylamine (TMA), total volatile bases (TVB) and hypoxanthine contents of the flesh. TMA is the best known compound produced during fish spoilage and it is mainly derived from bacterial breakdown of trimethylamine oxide (TMAO) which is an osmolyte naturally found in marine fish (Lauzon *et al.*, 2010). TMA does not increase much during the early stages of spoilage. It is therefore not considered suitable for discriminating fish stored less than 6 days in ice or chill storage. TVB-N content is an alternative to measuring TMA, and includes ammonia, dimethylamine (DMA) and TMA (Lauzon *et al.*, 2010).

Chapter.....3

Materials and Methods

sensory, biochemical, microbiological and nutritional analysis (see the following sections). Each of the samples was analyzed in the laboratory with duplicate. A schematic diagram for the experiment-1 followed in the study is given below showing the activities of the research.

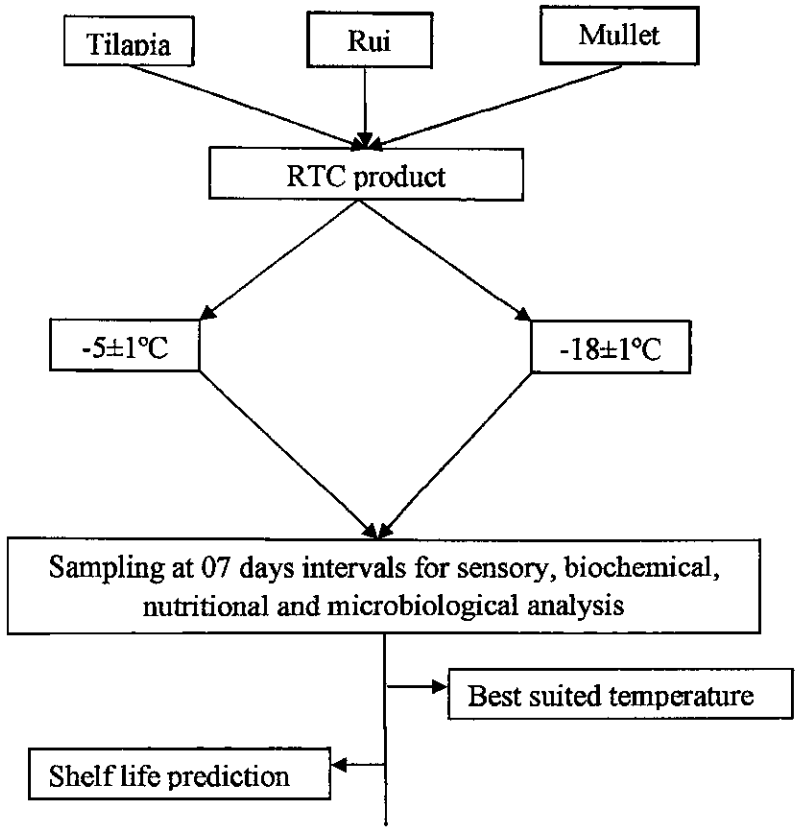


Figure 01: An example of step-wise activities and research design for the research

3.4 Analytical methods

3.4.1 Sensory Analysis

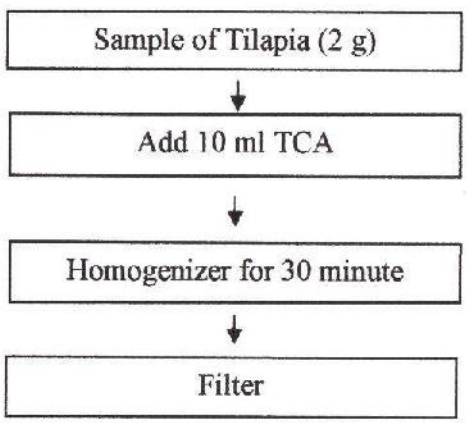
- Sensory analyses were conducted according to the modification of Aubourg (2012) by 6-10 panelists.
- Prior to the analysis, the panelists were assessed using recognition tests for odor (as for example; coffee, garlic, cheese, chocolate, orange, banana, lemon, honey etc), texture (hardness), basic tastes (salty, sweet, sour and bitter), and triangular test (salt test).

- Panelists who were successfully recognize the test were finally selected to test the sensory attributes of the experimental products.
- For the sensory tests, the experimental samples were served to the panelists using white ceramic plates coded with three random digits at every 7 days intervals (Meilgaard et al., 2007).
- The panelists rated the products for the attributes of: texture and odor, color, appearance, homogeneity and overall impression. The attributes were assessed by five different assigned scores ranging from 04 (worst) to 0 (best).

3.4.2 Chemical/Biochemical analysis

Biochemical analysis were performed in terms of pH value, total volatile basic nitrogen (TVB-N) content and trimethylamine nitrogen (TMA-N) content of the experimental samples.

- The pH were measured through a digital pH meter. 10 g of each of the fish samples weighed, diluted 1:1 and homogenized. The prob of the pH meter were dipped into the solution and the pH values were recorded according to the AOAC (1995).
- The TMA-N content and TVB-N content were determined by following process:
- TMA-N analysis was carried out according to the method proposed by AOAC, 1995 and TVB-N was determined according to the method of (Malle and Poumeyrol, 1989). The schematic steps of the methods are given below:-



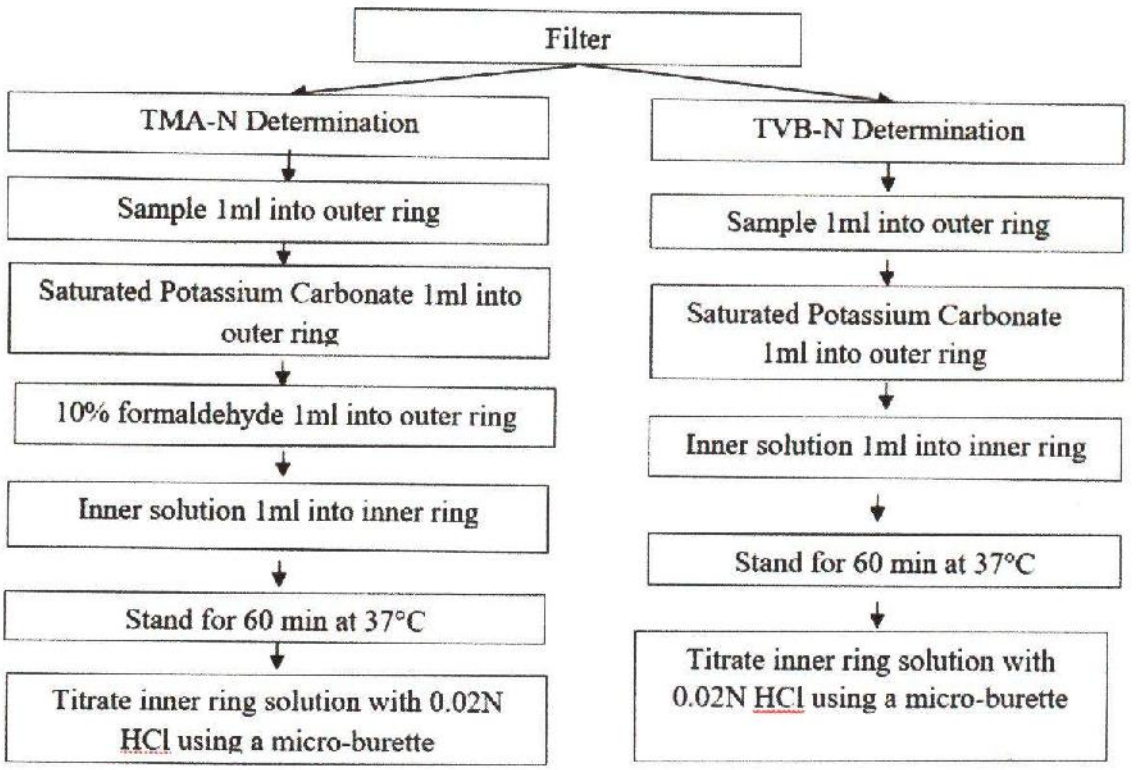


Figure 02: Procedures of determining TMA-N and TVB-N

TMA-N and TVB-N was calculated by using the following formula:-

TMA-N or TVB-N (mg/100g) = (Amount of HCl used in titration) × (Amount of available ammonium nitrogen equivalent to 1 ml of 0.02N HCl) × (Ratio of the amount of sample used to 100 g muscle)

$$\text{TMA-N or TVB-N} = = (V_S - V_B) \times (N_{HCl} \times A_N) \times \frac{[(Ws \times \frac{M}{100}) + Ve] \times 100}{Ws}$$

Here,

V_S = Titration volume of 0.02N HCl for sample extract (ml)

V_B = Titration volume for blank (ml)

N_{HCl} = Normality of HCl (=0.02N × f, factor of HCl)

A_N = Atomic weight of Nitrogen (× 14.00)

W_S = Weight of muscle sample (g)

M = percentage moisture of muscle sample.

V_e = Volume of 4% TCA used in extraction

Note: 1 ml of 0.02N HCI = 0.28 ammonium nitrogen

$$= (N_{HCl} \times f \times 14.00)$$

3.4.3 Nutritional analysis

3.4.3.1 Determination of moisture

The weighed quantity of sample (2-5 g) was dried in an oven at 105°C for 24 hours. Then the sample was kept in a dessicator until to get optimum room temperature. After cooling, the dry sample was weighed by an electric balance and the differences between the two weights gave the weight of the sample. The percentage of the moisture content was calculated by the following equation:

$$\% \text{ Moisture} = \frac{\text{Weight of sample} - \text{Weight of the dried sample} \times 100}{\text{Weight of the sample}}$$

3.4.3.2 Determination of protein

Protein was determination by using micro Kjeldahl method. Firstly 0.2-0.5 g of sample was weighed and inserted into a kjeldahl flask and the percentage of nitrogen was determined by the standard Kjeldahl method .Then the %N was multiplying by the conversion factor 6.25 to get the percentage of crude protein. Two steps were used to complete the total process. First step is digestion and second step is distillation. The distilled solution stored in the conical flask was taken and titrated against 0.102 (N) HCl solutions. The percentage of gross portentous nitrogen was calculated by the following formula:

$$\%N = \frac{\text{Volume of HCl} \times \text{normality of HCl} \times 0.014}{\text{weight of sample (gm)}} \times 100$$

%Protein= %N × 6.25 (conversion factor).

3.4.3.3 Determination of lipid

Crude lipid of the sample was determined by extracting a weighted quantity of sample (2-5g) with methanol and chloroform (1:2) solution which was mixed by using a mixer and filtered by a filter paper. Then 50 ml water was added with the sample solution to turn into milky color solution and it was kept in separation funnel for 8 hours. When three layers appear in the separation funnel a dried and weighted bicker was used to collect the solution only the bottom layer of the separation funnel. After collection of the separated solution from separation funnel was kept in the hot plate for drying. After evaporating all the chloroform a simple amount of sticky liquid left in the bottom of the bicker. Then it was taken out from the hot plate and allowed to cool down. After cooling down it was weighed and deducting the weight of the bicker that was measure quickly from the cooled bicker containing lipid to obtain lipid. The percentage of the lipid content was calculated by the following formula:

$$\% \text{ Lipid} = \frac{\text{Weight of lipid} \times 100}{\text{Weight of the sample}}$$

3.4.3.4 Determination of Ash

Ash levels were determined in a calcination process of organic matter in a muffle furnace at 550°C according to the AOAC Official Methods of Analysis 938.08—ash of seafood, 16th ed., 1995.

3.4.4 Bacteriological analyses

3.4.4.1 Aerobic plate counts

- Total aerobic bacterial counts were determined on the fish surface and in the fish flesh.
- Surface counts were obtained by plating a (5 by 5 cm) sterile template on the fish surface (at the middle region along the lateral line) and swabbing the area enclosed by the template.
- The swabs were transferred into 10 ml of sterile peptone (0.1% wt/vol) as diluting medium and shaken well before plating.
- Prior to taking the samples of flesh, the skin were removed aseptically, then underlying muscles were removed and homogenized in a stomacher for 1 min in 180 ml of sterile peptone (0.1% wt/vol).

- Tenfold serial dilutions of the same diluting medium were plated on the plate count agar (PCA, Difco, Detroit, Mich.).
- The plates were incubated at 35°C for 2 days when mesophilic bacteria were counted and at 20°C for 3 days when psychrotrophic bacteria were be counted.

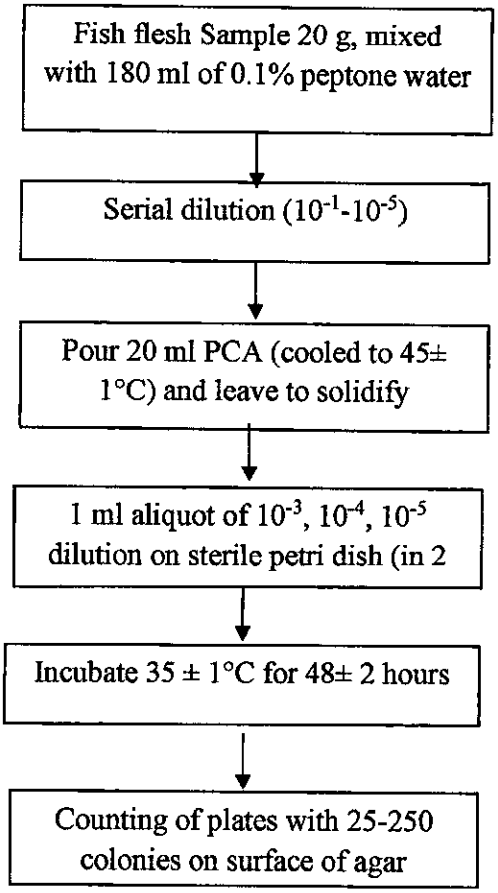


Figure 03: Flow chart for the enumeration of TVC

3.4.4.2 Hydrogen sulphide-producing bacteria/ Total volatile count

- Selective counts of hydrogen sulphide-producing bacteria were enumerated during the experiment.
- To enumerate the bacteria, Approximately 20 g of the underlying fish flesh sample were taken from the anterio-dorsal region of the fish.
- Prior to taking the samples of flesh, the skin were removed aseptically with 70% ethanol then underlying muscles were removed and homogenized in a stomacher for 1 min in 180 ml of sterile peptone (0.1% wt/vol).
- 1 ml aliquots were plated on the Iron Agar (Gram *et al.*, 1987) which were kept in an incubator at 37°C for 24 hour. Black colonies were recorded as sulphide-producing bacteria. Total volatile count were also be enumerated from the iron agar medium.

3.4.4.3 *Pseudomonas* bacteria

- Selective counts of *pseudomonas* bacteria were also be enumerated on the fish surface and in the fish flesh.
- Surface counts were obtained by plating a (5 by 5 cm) sterile template on the fish surface (at the middle region along the lateral line) and swabbing the area enclosed by the template.
- The swabs were transferred into 10 ml of sterile peptone (0.1% wt/vol) as diluting medium and shaken well before plating.
- To enumerate the bacteria, Approximately 20 g of the underlying fish flesh sample were taken.
- Prior to sampling the skin were rinsed with 70% ethanol and removed aseptically.
- Tenfold dilutions in 0.1% peptone water were prepared from the flesh samples and 1 ml aliquots were plated in *pseudomonas* agar. Which were kept in an incubator at 20°C for 03 days when *pseudomonas* bacteria were be counted.

3.5 Self-life prediction

All of the quality attributes were analyzed for predicting shelf life of the frozen product. Freshness period and shelf life was evaluated based on the rate of quality changes during icing in experiment 02. In experiment 02, the shelf life of the freezed samples were determined according to the modification of the Arrhenius equation (James and Olley, 1971):

$K = Ae^{-HIRT}$

where: K = rate of spoilage, A = orientation factor, e = mathematical constant, H = energy of activation, R = gas constant, and T = temperature (absolute).

3.6 Statistical analysis

SPSS 16 for Windows will be used to test the differences between mean values of the different analysed parameters. Differences between means were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test, when a significant difference were detected between the days of storage ($P<0.05$).

Chapter.....4

Results

4. Result

4.1 Sensory Analysis

The results of changes in sensory quality in the three experimented RTC fish products during freezing for 08 weeks (56 days) at -5°C and -18°C are presented in figure 04. The quality of fish was graded using the score from 0-4. The score points less than 1 were considered as excellent. The points from 1 to less than 2 were judged as good or acceptable, while 2 and above were considered as bad or rejected in terms of human consumption. On the basis of the quality scores, all the samples with their respective temperature were found in acceptable conditions of excellent quality.

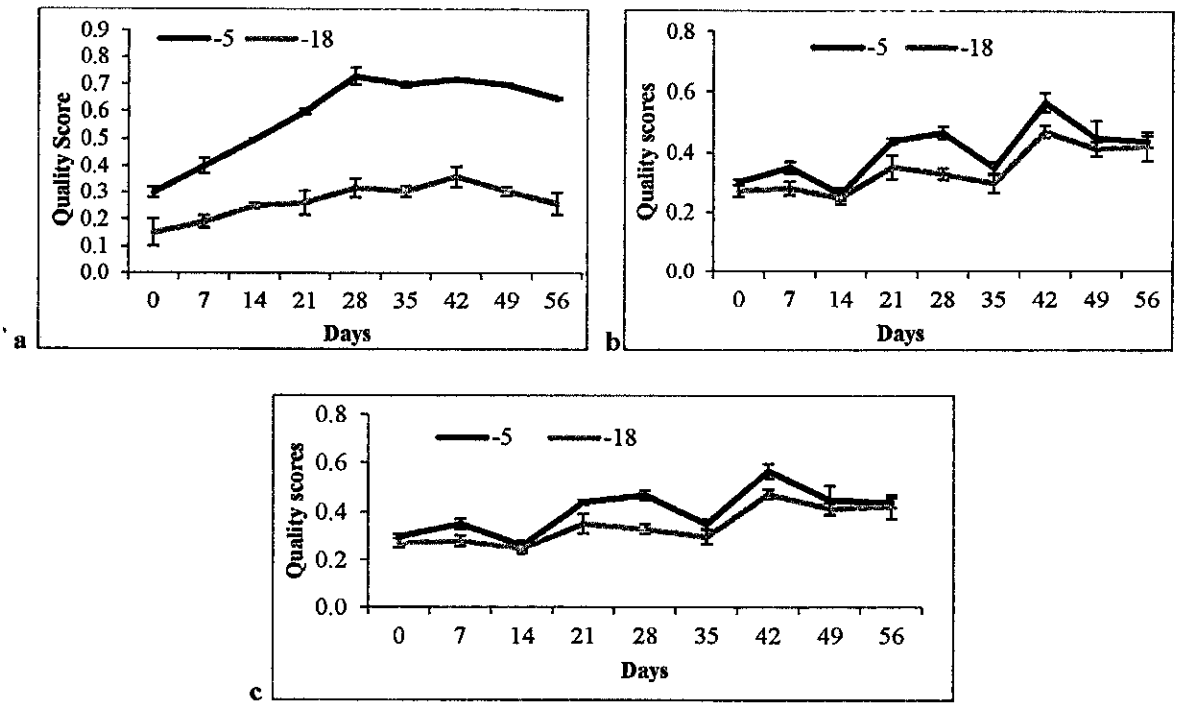


Figure 04: Changes of sensory quality scores in tilapia (a), parse (b) and rui (c) at -5°C and -18°C during the experiment period

Quality scores of less than 01 was found in every fishes during the storage period. Fishes at -5°C temperature storage showed higher value than the -18°C indicated that the storage temperature of -18°C kept the fishes better than other.

4.2 Chemical changes

4.2.1 pH

pH is an important intrinsic factor related to fish flesh and influence freshness because of its influence on bacterial growth (Gram and Huss, 1996). Changes of pH of in fish muscles during a certain time period of storage can determine the state of its freshness (Abbas *et al.*, 2008) as it start with low reading at the early stage of storage and increased when the fish had been stored for certain period of time.

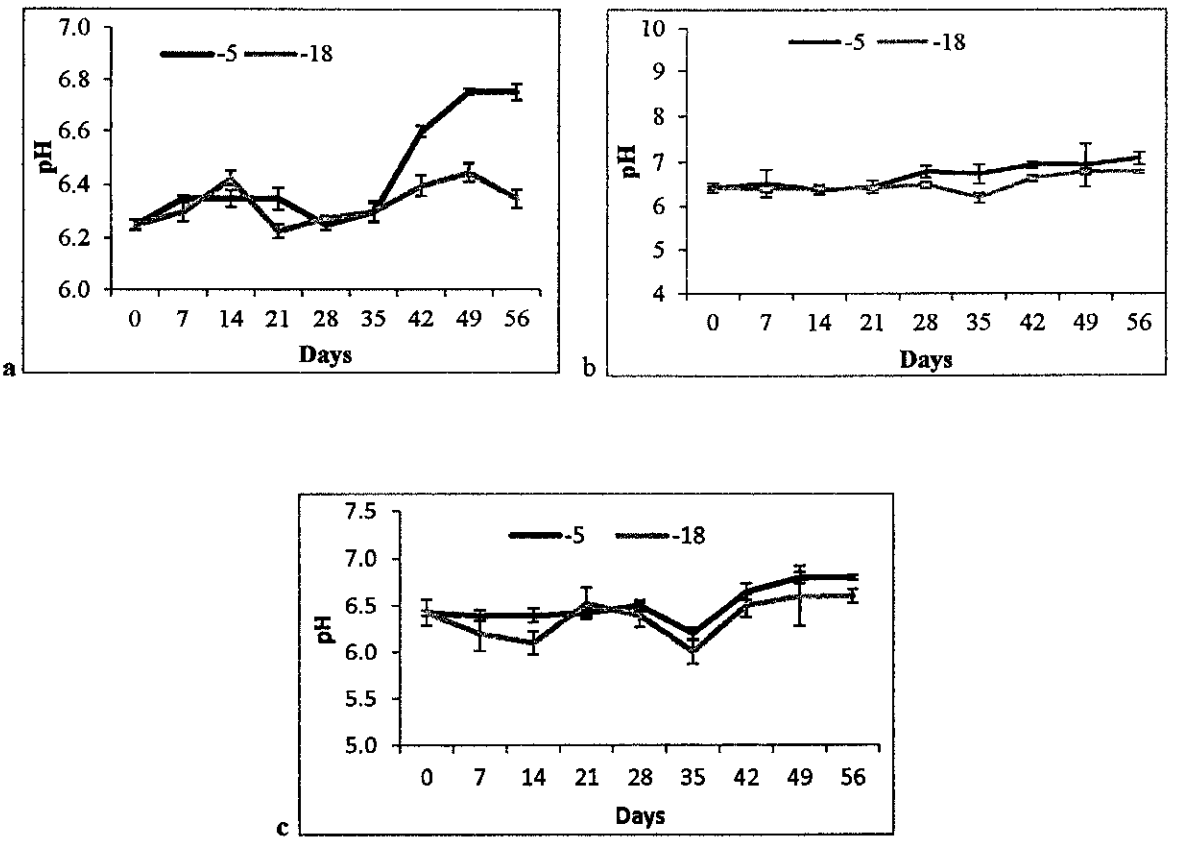


Figure 05: Changes of pH in tilapia (a), parse (b) and rui (c) at -5°C and -18°C during the experiment period

During the storage period of 56 days, little changes were observed or no changes in some sampling time. pH ranges were found to be higher than 6 to less than 7 (Figure 05). Few changes or pH of

less than 7 is an indicative of fresh freezing product. Such level of changes in pH indicates the low bacterial growth and possible spoilage (Sallam *et al.*, 2007).

4.2.2 TMA-N

TMA-N content has been one of the most extensively investigated chemical methods. Its content has been observed to be a useful measure of freshness quality. In this experiment, a TMA-N content of less than 2 mg/100g in tilapia and 1 mg/100g in parse were found during the sampling period following a low level of content. No content was found in rui or the content was lower than the detection limit of the process. In terms of quality, fish storage at -18°C showed better quality than -5°C because increasing rate of the value is the indicative of bacterial spoilage.

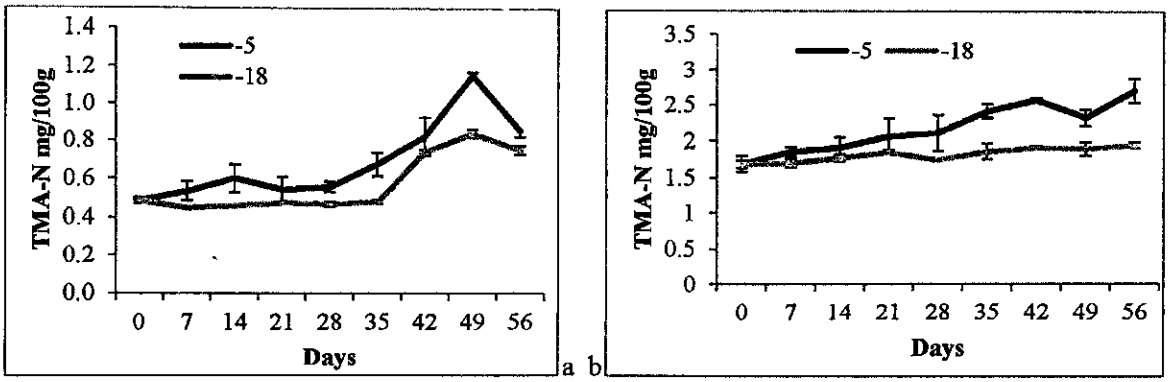


Figure 06: Changes of TMA-N mg/100g in tilapia (a) and parse (b) at -5°C and -18°C during the experiment period

These values are considered very low as compared to the upper value value of 10 mg/100 g flesh proposed by (Teskeredzic and Pfeifer, 1987). The small increase in TMA values for the preserved fish during the period reflects the low level of trimethylamine oxide (TMAO) in the flesh because TMA is produced by the decomposition of TMAO due to bacterial spoilage and enzymatic activity, (Hebard *et al.*, 1982).

4.2.3 TVB-N

Total volatile nitrogen has been widely used as an index for freshness of fish. For several fish species, TVB-N values were reported to increase linearly with the time. A level of 30 mg N/100 g of fish Muscle TVB-N has been considered as the upper limit above which some fishing products

are considered spoiled and unfit for human consumption as specified by European commission guidelines (Huss, 1995). In this experiment, TVB-N contents were found to be very low ranges of 4-10 with higher in parse species. At -5°C in tilapia and rui, the levels were higher than -18°C indicated that fish at -18°C was preserved in better in terms of freshness (Figure 07).

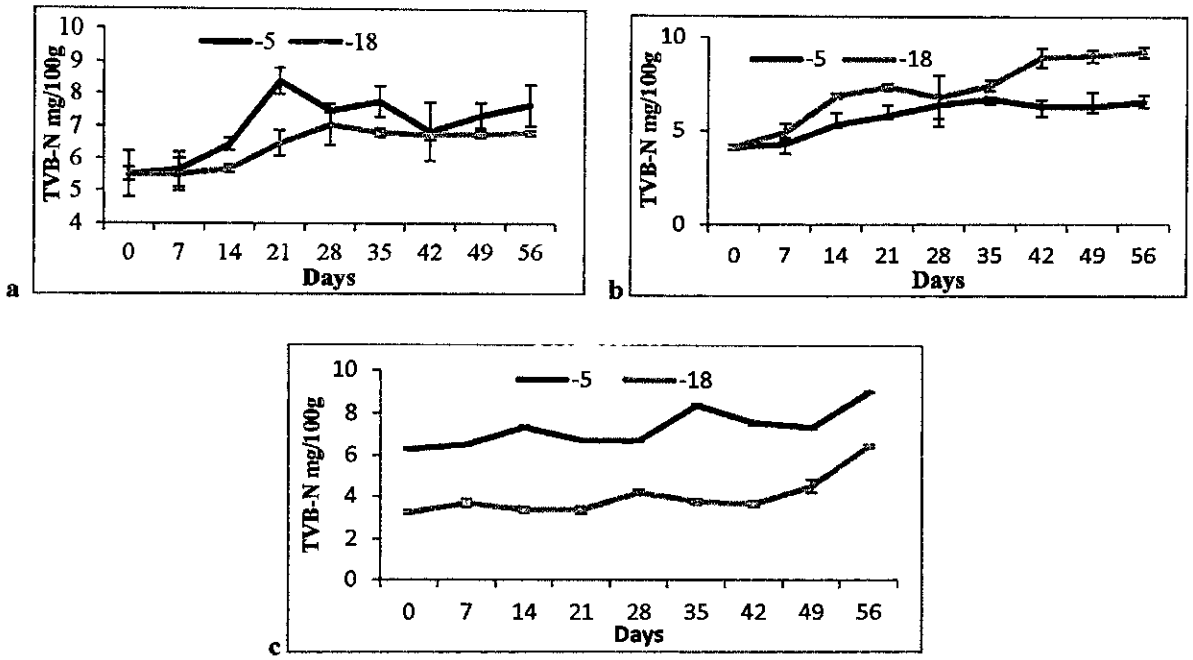
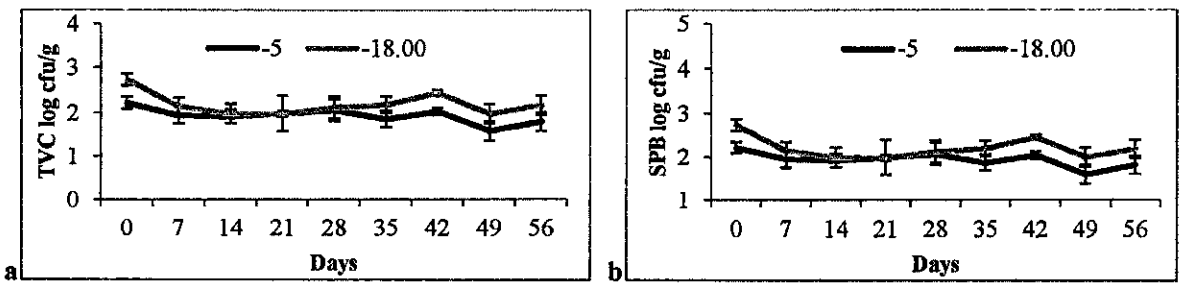


Figure 07: Changes of TVB-N mg/100g in tilapia (a) and parse (b) and rui (c) at -5°C and -18°C during the experiment period

4.3 Microbiological analysis

During the study period, TVC, SPB and *Pseudomonas* bacteria were found to be very low in number and none of the fish muscles showed the indication of spoilage. The changes of those bacteria in fish muscles are presented in the figure 08.



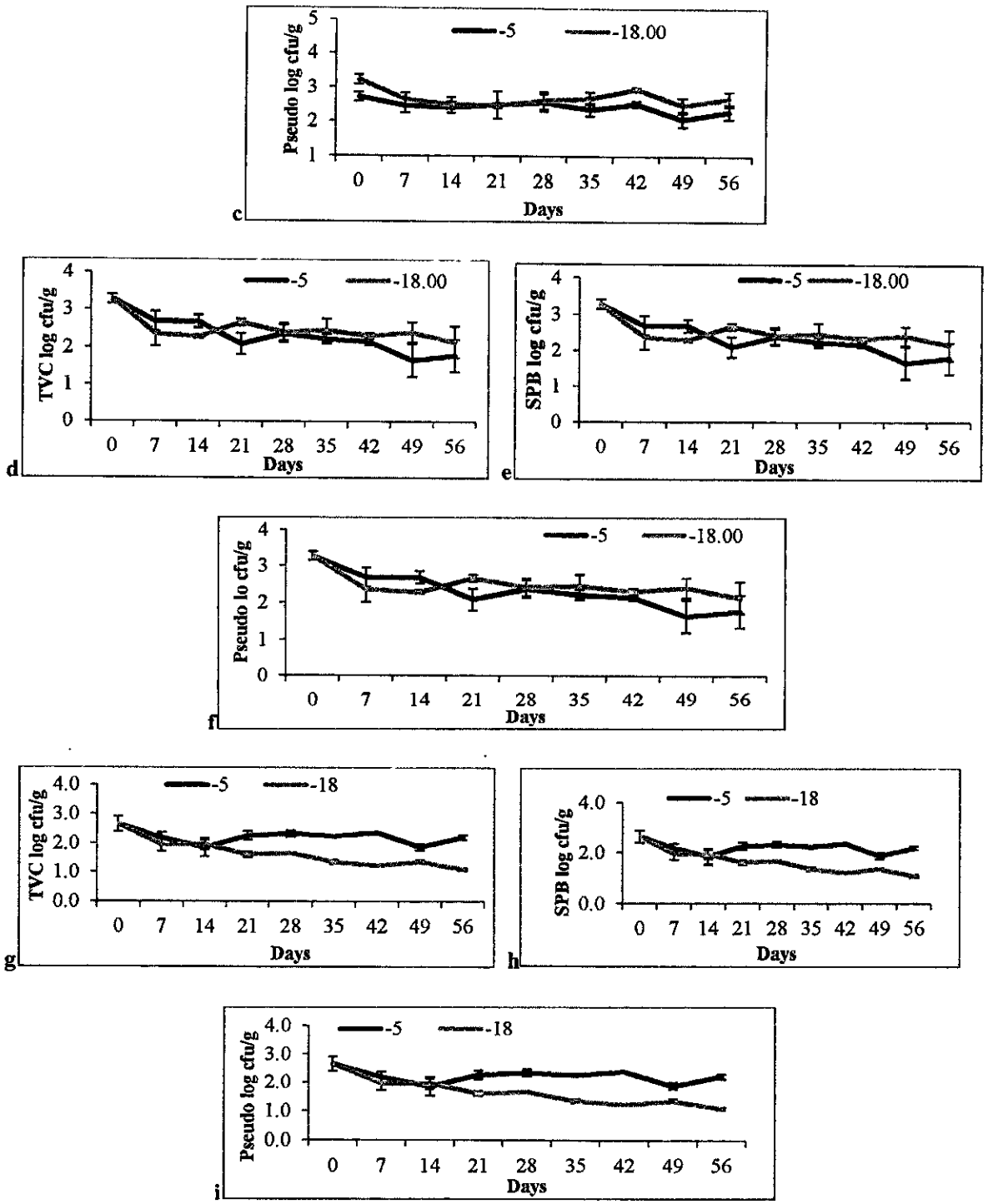


Figure 08: Changes of TVC (log cfu/g), SPB (log cfu/g) and *Pseudomonas* (log cfu/g) in tilapia (a-c) and parse (d-f) and rui (g-i) at -5°C and -18°C during the experiment period

Initial microbial load of freshwater fish varies depending on water conditions and temperature, most available literature on different freshwater fish species (tilapia, striped bass, rainbow trout, silver perch) reports bacterial counts of 10^2–10^6 cfu/g (2 log cfu/g to 6 log cfu/g) (Acuff *et al.*, 1984; Nedohula and Westhoff, 1997;). In this study, initial bacterial counts of 3.36 log cfu/g for tilapia, 4.1 log cfu/g for parse and 3.36 log cfu/g for rui are indicative of high fish quality at the initial period. During the storage period, the TVC were in decreasing pattern. Decreasing pattern might be the cause of shocking at freezing temperature.

4.4 Proximate Compositional Changes

In this experiment, higher proximate compositional changes were found in the products from -5°C storage comparing with initial composition. Proximate composition, particularly protein and lipid are the most concerning components. About 3-7% protein was denatured or decreased during the storage period. Lipid was decreased in higher rate than protein. The compositional rate of changes are shown in the following table (Table 01) and descriptive results are shown in table 5,6,7 of annexure section.

Table 01: Proximate compositional changes rate during the storage period in tilapia, parse and rui at -5°C and -18°C

Days	Protein (%)		Lipid (%)		Moisture (%)		Ash (%)	
	-5°C	-18°C	-5°C	-18°C	-5°C	-18°C	-5°C	-18°C
Tilapia								
Average	18.02±0.31	17.93±0.39	3.06±0.20	2.94±0.15	77.30±0.88	77.05±0.42	1.10±0.12	1.25±0.13
Change (%)	-3.85	-2.62	-19.50	-16.86	2.62	1.80	8.42	32.24
Parse								
Average	16.79±0.68	17.16±0.56	7.07±0.73	7.51±0.50	71.28±1.70	71.19±0.64	7.07±0.73	2.76±0.27
Change (%)	-7.40	-2.32	-20.67	-11.58	3.24	2.57	-20.67	-13.45
Rui								
Average	18.54±0.33	18.64±0.28	2.45±0.17	2.46±0.15	78.01±1.04	77.60±0.75	1.51±0.26	1.63±0.20
Change (%)	6.16	-5.71	-18.07	-15.86	1.63	2.13	41.31	50.67

4.5 Self Life Prediction

1. The calculated predicted shelf life was found to be 1 years and 08 months for Tilapia, 1 year and 01 months for parse and 02 years 02 months for rui species at -5°C.
2. However, the calculated predicted shelf life was found to be 2 years and 06 months for Tilapia, 1 year and 09 months for parse and 03 years 04 months for rui species at -18°C.

Chapter.....5

Discussion

5. Discussion

This study was aimed to determine the sensory, bio-chemical, nutritional and microbiological quality related changes of some selected fish species namely tilapia, parse and rui, and its finished products (RTC) storage at two variable freezing conditions viz. -5°C and -18°C and to predict their shelf life based on the quality related changes. Throughout this study, chemical, microbiological and nutritional analyses and sensory evaluation did not indicate any significant differences during a storage period of 08 weeks (56 days) at two different freezing temperatures viz. -5°C and -18°C. Quality attributes determined in this study included pH, TMA-N, TVB-N, protein, lipid, moisture, ash, total viable count (TVC), sulfur producing bacteria (SPB) count and *Pseudomonas* bacteria count.

For the sensory tests, the panelists rated the products for the attributes of: texture and odor, color, appearance, homogeneity and overall impression. The attributes were assessed by five different assigned scores ranging from 04 (worst) to 0 (best). Quality scores of less than 01 was found in every fishes during the storage period. Fishes at -5°C temperature storage showed higher value than the -18°C indicated that the storage temperature of -18°C kept the fishes better than other. According to sensory experts, the quality deterioration of fish is first characterized by the initial loss of the fresh fish odour and flavour (sweet, seaweedy) which is followed by the development of a neutral odour and flavour, leading to the detection of off odours and flavours. End of shelf life is usually determined when sensory attributes related to spoilage such as sour, pungent, TMA odour and/or flavour become evident (Lauzon *et al.*, 2010).

During the storage period of 56 days, little changes of pH were observed or no changes in some sampling time. pH ranges were found to be higher than 6 to less than 7 (Figure 05). Few changes or pH of less than 7 is an indicative of fresh freezing product. Such level of changes in pH indicates the low bacterial growth and possible spoilage (Sallam *et al.*, 2007). The higher final pH values were observed for control samples (ranging from 6.42 to 6.99), whereas the lowest pH values were recorded for samples packed under MAP (from 6.24 to 6.69). In fact, it is well documented that the dissolution of CO_2 in the fish muscle is associated with an increased carbonic acid production and a lower production of basic compounds (Sivertsvik *et al.*, 2002).

In this experiment, a TMA-N content of less than 2 mg/100g in tilapia and 1 mg/100g in parse were found during the sampling period following a low level of content. No content was found in rui or the content was lower than the detection limit of the process. In terms of quality, fish storage at -18°C showed better quality than -5°C because increasing rate of the value is the indicative of bacterial spoilage. In this experiment, TVB-N contents were found to be very low ranges of 4-10 with higher in parse species. At -5°C in tilapia and rui, the levels were higher than -18°C indicated that fish at -18°C was preserved in better in terms of freshness. The longer fishes have been kept frozen, the slower the TMA (trimethylamine) and TVB (total volatile bases) formation during chilled storage (Martinsdottir & Magnusson, 1995). Therefore, at frozen storage temperature of -18°C the breakdown of TMAO by the enzymatic action of trimethylamine demethylase would occur in both fast and slow frozen fishes and thus, lead to a low detection of TVB-N (Andrews, 2015).

In this study, initial bacterial counts of 3.36 log cfu/g for tilapia, 4.1 log cfu/g for parse and 3.36 log cfu/g for rui are indicative of high fish quality at the initial period. During the storage period, the TVC were in decreasing pattern. Decreasing pattern might be the cause of shocking at freezing temperature. Short term frozen storage (≤5weeks) has little effect on bacterial numbers (Martinsdottir & Magnusson, 1995). Thus, bacterial counts obtained from samples taken after thawing reflects bacterial numbers just prior to freezing, provide that the thawing processing was effectively controlled and did not allow for high temperatures which would aid in the proliferation of microbes. Low temperatures below 15°C are preferred when thawing at ambient temperatures to prevent the development of microbial flora which causes spoilage of the product (Alizadeh *et al.*, 2007)

In this experiment, higher proximate compositional changes were found in -5°C storage comparing with initial composition. Proximate composition, particularly protein and lipid are the most concerning components. About 3-7% protein was denatured or decreased during the storage period. Lipid was decreased in higher rate than protein. Chemical composition analysis of quenelle tilapia including moisture, lipids, proteins and carbohydrates did not vary during the 120 days storage (Angelini *et al.*, 2013). The literature reports lower moisture and lipid values than those found in the present study (Tokur et al., 2004; Kirschnik & MacedoViegas, 2009), as well as protein values (Bordignon et al., 2010). However, other authors found higher amounts of protein (Kirschnik & MacedoViegas, 2009). (Martinsdottir & Magnusson, 1995) have shown that these

properties will decrease over time (≥5weeks) in frozen storage. Rapid (fast) freezing of fishery products which has a more rapid freezing rate than slow freezing should produce a better quality product once thawed than slow freezing (Arason, 2014).

All of the quality attributes were found to be very low in amount or its changes occurred slowly that were the indicative fresh product during the storage period. Therefore, tilapia, parse and rui can be kept in freezer at -5°C and -18°C for with little quality changes at least 56 days. Based on the quality changes during freezing at those temperatures, 1-3 years of shelf life can be attained.

Chapter.....6

Conclusion

6. Conclusion

The broad objective of the research was to explore the best suited freezing conditions of tilapia, parse and rui fish products (RTC) storage at variable temperature regime for ensuring better quality and extended shelf life of the products for consumer's health and safety concern. Tilapia, parse and rui can be kept in freezer at -5°C and -18°C for with little quality changes at least 56 days. Based on the quality changes during freezing at those temperatures, 1~3 years of shelf life can be attained. From the findings, it is advisable to thaw those fish in refrigerator for better nutrient.

Chapter.....7

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Annex

Annexure-01

Table 2: Grading of fresh tilapia

Grade	Demerit Points	Degree of freshness
A	0	Excellent/Acceptable
B	>0-5.0	Very good/Acceptable
C	5.1-10.0	Moderately good/Acceptable
D	10.1-15.0	Bad/Rejected
E	15.1-22.0	Very bad/Rejected

Table 3: Grading of fresh fish

Grade	Points	Degree of freshness
A	0	Excellent/ Acceptable
B	>0-3.0	Good/ Acceptable
C	3.1-6.0	Moderate/ Acceptable
D	6.1-9.0	Bad/ Rejected
E	9.1-15	Very bad/ Rejected

Proximate compositional changes

Table 05: Proximate compositional changes rate during the storage period in tilapia at -5°C and -18°C

Days	Protein (%)		Lipid (%)		Moisture (%)		Ash (%)	
	-5°C	-18°C	-5°C	-18°C	-5°C	-18°C	-5°C	-18°C
0	18.32±0.26	18.32±0.26	3.25±0.14	3.25±0.14	76.38±0.70	76.38±0.70	1.03±0.21	1.03±0.21
7	18.36±0.42	18.05±0.07	3.22±0.30	3.04±0.31	76.5±01.37	77.13±0.18	1.13±0.10	1.18±0.12
14	18.29±0.09	17.72±0.75	3.24±0.08	3.06±0.07	77.23±0.29	76.47±0.29	1.13±0.03	1.17±0.30
21	18.28±0.18	17.94±0.23	3.20±0.48	2.97±0.10	77.01±1.15	76.62±0.60	1.12±0.17	1.31±0.15
28	18.14±0.23	17.89±0.32	3.21±0.28	2.93±0.02	77.42±0.59	77.04±0.05	1.12±0.10	1.35±0.12
35	17.91±0.50	17.88±0.88	3.00±0.27	2.88±0.30	77.30±1.21	77.45±0.34	1.05±0.09	1.26±0.11
42	17.67±0.11	17.87±0.25	3.02±0.10	2.86±0.00	77.73±0.39	76.76±0.66	1.13±0.14	1.27±0.04

49	17.64±0.44	17.85±0.07	2.77±0.03	2.78±0.18	77.75±1.19	77.90±0.14	1.04±0.09	1.29±0.07
56	17.61±0.57	17.84±0.64	2.62±0.10	2.70±0.26	78.38±1.03	77.75±0.81	1.11±0.14	1.36±0.06
Average	**18.02±0.31**	**17.93±0.39**	**3.06±0.20**	**2.940.15**	**77.30±0.88**	**77.05±0.42**	**1.10±0.12**	**1.25±0.13**
Difference	**0.70**	**0.48**	**0.63**	**0.55**	**-2.00**	**-1.37**	**-0.09**	**-0.33**
Change (%)	**3.85**	**2.62**	**19.50**	**16.86**	**-2.62**	**-1.80**	**-8.42**	**-32.24**

Table 06: Proximate compositional changes rate during the storage period in parse at -5°C and -18°C

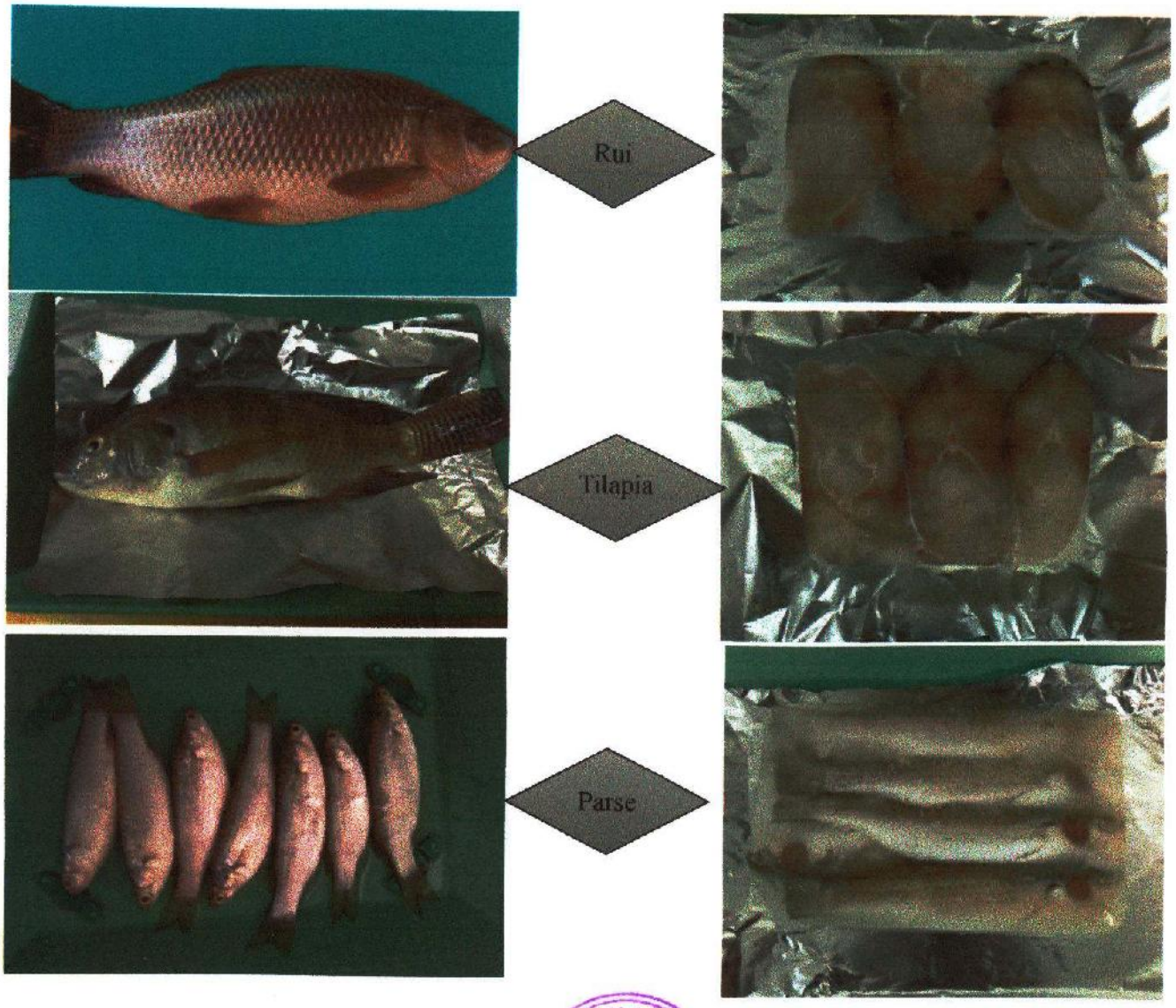
Days	Protein (%)		Lipid (%)		Moisture (%)		Ash (%)	
	-5°C	**-18°C**	**-5°C**	**-18°C**	**-5°C**	**-18°C**	**-5°C**	**-18°C**
0	17.40±0.50	17.40±0.50	8.05±0.78	8.05±0.78	70.28±1.55	70.28±1.55	8.05±0.78	2.60±0.11
7	17.33±1.03	17.26±0.90	7.36±0.80	7.77±0.31	70.64±1.91	70.84±0.53	7.36±0.80	2.61±0.15
14	17.10±0.99	17.28±0.99	7.35±0.62	7.55±0.84	70.73±1.35	71.11±0.81	7.35±0.62	2.66±0.20
21	17.09±0.55	17.27±0.22	7.34±1.27	7.66±0.36	70.87±2.15	71.16±0.39	7.34±1.27	2.64±0.37
28	16.78±0.43	17.14±0.43	7.15±1.34	7.68±0.54	71.26±2.68	70.71±1.36	7.15±1.34	2.75±0.40
35	16.83±0.73	17.10±0.35	6.84±0.48	7.40±0.35	71.48±0.60	71.03±0.17	6.84±0.48	2.82±0.25
42	16.34±0.72	17.04±0.52	6.64±0.27	7.21±0.60	71.19±3.07	71.57±0.36	6.64±0.27	2.83±0.33
49	16.15±0.78	16.93±0.42	6.51±0.44	7.19±0.36	72.52±0.48	71.93±0.34	6.51±0.44	2.97±0.45
56	16.11±0.39	17.00±0.67	6.39±0.57	7.12±0.34	72.56±1.50	72.09±0.25	6.39±0.57	2.95±0.18
Average	**16.79±0.68**	**17.160.56**	**7.07±0.73**	**7.51±0.50**	**71.28±1.70**	**71.19±0.64**	**7.07±0.73**	**2.76±0.27**
Difference	**1.29**	**0.40**	**1.66**	**0.93**	**-2.28**	**-1.81**	**1.66**	**-0.35**
Change (%)	**7.40**	**2.32**	**20.67**	**11.58**	**-3.24**	**-2.57**	**20.67**	**-13.45**

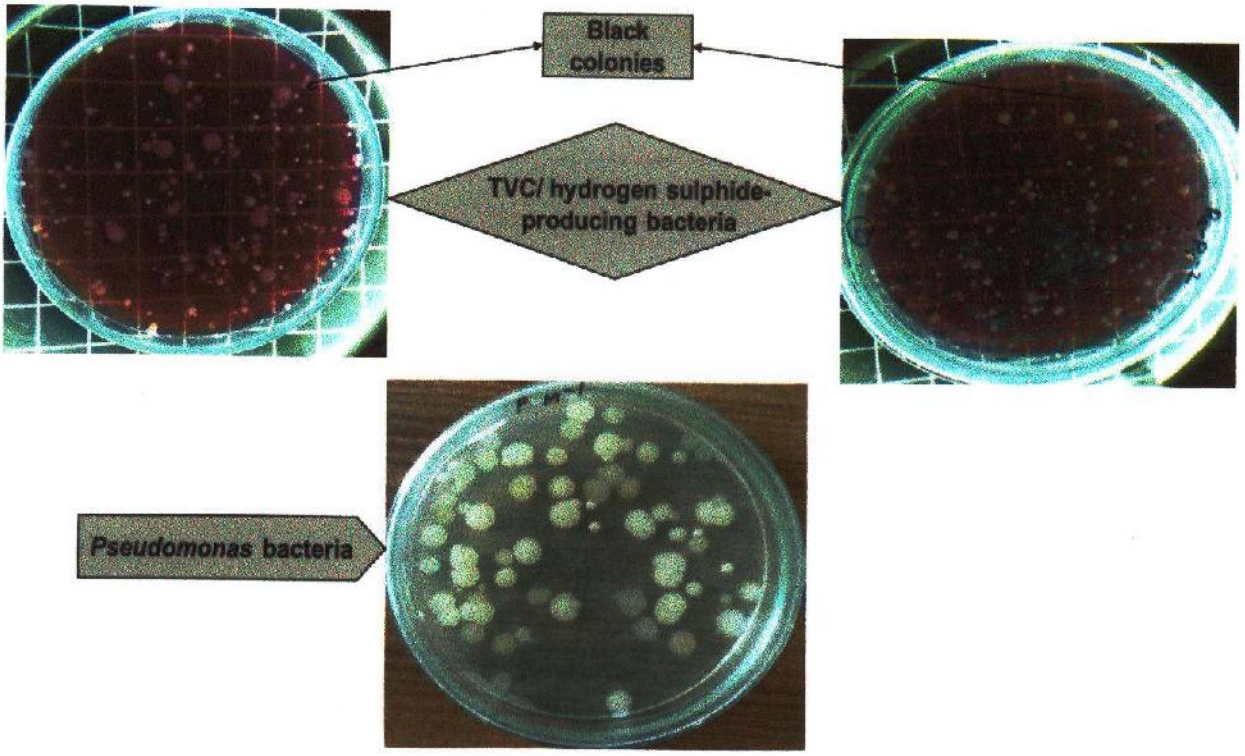
Table 07: Proximate compositional changes rate during the storage period in rui at -5°C and -18°C

Days	**Protein (%)**		**Lipid (%)**		**Moisture (%)**		**Ash (%)**	
	-5°C	**-18°C**	**-5°C**	**-18°C**	**-5°C**	**-18°C**	**-5°C**	**-18°C**
0	19.14±0.19	19.14±0.19	2.66±0.37	2.66±0.37	77.12±1.44	77.12±1.44	1.30±0.06	1.30±0.06
7	18.72±0.43	18.91±0.18	2.60±0.06	2.58±0.04	77.15±0.72	77.04±0.54	1.32±0.12	1.35±0.13
14	18.54±0.25	18.83±0.62	2.54±0.29	2.54±0.29	77.24±0.91	77.21±1.26	1.33±0.31	1.43±0.03
21	18.64±0.18	18.84±0.05	2.55±0.19	2.50±0.11	77.38±0.47	77.60±0.11	1.31±0.20	1.51±0.23
28	18.50±0.23	18.60±0.35	2.43±0.14	2.41±0.08	77.58±1.53	77.74±0.33	1.38±0.12	1.54±0.13
35	18.80±0.53	18.61±0.02	2.41±0.23	2.42±0.07	78.09±2.33	77.87±2.01	1.55±0.33	1.80±0.16
42	18.55±0.11	18.55±0.36	2.37±0.10	2.40±0.10	77.73±0.39	77.34±0.16	1.73±0.40	1.81±0.36
49	17.99±0.45	18.26±0.33	2.27±0.08	2.35±0.15	81.38±0.54	77.71±0.41	1.86±0.47	1.99±0.60
56	17.96±0.58	18.05±0.46	2.18±0.08	2.24±0.13	78.38±1.03	78.76±0.48	1.84±0.32	1.96±0.11
Average	**18.54±0.33**	**18.64±0.28**	**2.45±0.17**	**2.46±0.15**	**78.01±1.04**	**77.60±0.75**	**1.51±0.26**	**1.63±0.20**

Difference	**1.18**	**1.09**	**0.48**	**0.42**	**-1.26**	**-1.64**	**-0.54**	**-0.66**
Change (%)	**6.16**	**5.71**	**18.07**	**15.86**	**-1.63**	**-2.13**	**-41.31**	**-50.67**

Photo gallery




Black colonies
TVC/ hydrogen sulphide-producing bacteria
Pseudomonas bacteria