STUDIES ON MODIFIED ATMOSPHERE PACKAGING OF PEARL SPOT (*Etroplus suratensis*) WITH SPECIAL EMPHASIS TO BIOCHEMICAL AND MICROBIOLOGICAL PARAMETERS

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CERTIFICATE

This is to certify that this thesis entitled "**STUDIES ON MODIFIED ATMOSPHERE PACKAGING OF PEARL SPOT (*Etroplus suratensis*) WITH SPECIAL EMPHASIS TO BIOCHEMICAL AND MICROBIOLOGICAL PARAMETERS**" embodies the original work conducted by Mrs. LEEMA JOSE under my guidance from 31.10.2001 to 31-12-2004. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or institution. She has also passed the Ph.D qualifying examination of the COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY, Cochin held in May 2004.

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INTRODUCTION

1.0 INTRODUCTION

Fisheries play an important socio-economic role – it supplies cheap and nutritious food, generates employment and income, earns foreign exchange through export, and stimulates subsidiary industries. More than six million fishermen and fish farmers in the country depend on fisheries and aquaculture for their livelihood. India's inshore waters have been exploited almost to the sustainable level, but the contribution to production from the deep sea has been insignificant. Annual fish production in 1999-2000 was estimated at 5.66 mt — 2.834 mt from the marine sector (against a potential of 3.934 mt), and 2.823 mt from the inland sector against a potential of 4.5 mt. India exported marine fish and fish products worth Rs 51 167 million in 1999-2000 against Rs 17 674.3 million during 1992-1993. The exports go to some 70 countries, with Japan being the single largest market. Imports, so far, are negligible. A growth rate of 2.5 per cent has been proposed for marine fisheries during the Tenth Five- Year Plan (2002 – 2007), and a growth rate of 8 per cent for inland open water fisheries and aquaculture development. This will enable a total fish production of 8.09 million tonnes (3.26 million tonnes from the marine sector and 4.83 million tonnes from the inland sector) at the end of the Tenth Plan, according to estimates.

Fish plays a very vital role in human nutrition and is the primary source of animal protein for over one billion people of developing countries. While considering fish as a source of food the main emphasis has been put on the

protein. About 85-90% of fish protein are easily digestible and contain all essential amino acids. Fish represents about 14% of all animal protein and about 5% of total protein eaten on global basis. It is estimated that 60% of the people in developing countries obtain 40-100% of animal protein in their diet from fish (Clucas and Ward, 1996). Aquatic resources are finite unless man intervenes in some way to increase production from aquaculture, habitat improvement or by restocking. Therefore it is very essential to make best use of the harvested resources to give food security to the increasing population. Current fish utilization trends in India indicate that the major quantity of fish is marketed in fresh form i.e. about 65% of the total fish production.

Fresh fish is highly susceptible to spoilage from postmortem autolysis and microbial growth. The high ambient temperature of our country favours the rapid growth of microorganisms. Presently ice and mechanical refrigeration are the most common means of retarding microbial and biochemical spoilage in freshly caught seafoods during distribution and marketing. Fish is more susceptible for spoilage compared to land animals because of many factors hence it should be preserved and processed properly to reduce post harvest losses and to increase its demand. Presently 39.7% of world fish catch is consumed fresh, 8.7% is used for canning, 7.3 % for curing, 20.0 % for freezing and 24.2% is used for other purposes. (FAO, 2002).

The main objective of the preservation methods is to slow down or even stop the spoilage process until consumption. The efficiency of methods depends in its effect in reducing the spoilage rate and thus increasing the shelf life and

improving final quality. The selection of a method of preservation is based on the nature and type of fish, economic viability of the method and technical knowledge. There are several methods to preserve fish. Icing is a common method for short-term preservation. Studies have shown that Modified Atmosphere Packaging in combination with icing improves the shelf life considerably. So the present work deals with the studies on Modified Atmosphere Packaging of fresh pearl spot in ice.

1.1. Modified Atmosphere Packaging

The recent trend in seafood consumption worldwide has been toward various forms of fresh fish. Fresh forms of seafood's and other muscle foods are considered superior in taste and quality to their frozen counterparts. The need for long term fresh fish preservation techniques, such as freezing, is necessary due to the biological nature of fish and its inherent instability and the fact that consumer markets are far remote from coastal fishing ports. However, with increasing consumer demands for fresh fish and increasing energy costs associated with freezing and frozen storage, the fish processing industry is actively seeking for alternative methods of shelf life preservation and marketability of fresh fish, while at the same time economizing on energy costs. One method, which fulfills both objectives, is modified atmosphere packaging.

A modified atmosphere may be defined as packaging or storage of a perishable product in an atmosphere other than that of air. The normal composition of air is 20% oxygen (O_2), 79% nitrogen (N_2) and 1% CO_2. A modified atmosphere, as the name implies, is one in which the normal composition of air is changed or

modified. While some modified atmospheres are employed simply to inhibit oxidation, others are bactericidal or bacteriostatic. Terms used to describe this modification are Controlled atmosphere and Modified atmosphere. A controlled atmosphere (CA) involves maintaining a precisely defined atmosphere within a sealed storage chamber. This control is achieved by means of system, which continually compensates for atmospheric changes caused by product or microbial respiration or container permeability. Controlled atmosphere storage (CA) is generally applied for bulk storage of perishable products, specially fruits and vegetables.

A modified atmosphere (MA) applies to food packaged in small convenient retail units in which the gaseous atmosphere in the packaged products changes continuously throughout the storage period. Thus, while both CA and MA mean that the gaseous atmosphere around the product differs from air, CA has more precise gas compositional control than MA. Despite the increasing commercial interest in the use of MAP to extend the shelf life of fishery products, the concern about the potential growth of *Clostridium botulinum* in fish stored under MA remains the limiting factor for commercial use of the method. This concern stems from the fact that (a) non proteolytic *Clostridium botulinum* types B, E, and F, all of which occur in aquatic environments, are capable of growth and toxin production at atmospheres as low as 3.3°C, (b) *Clostridium botulinum* is a highly prevalent natural contaminant of seafood, and (c) the restricted growth of normal aerobic spoilage bacteria of fish flesh by MAP may enhance the growth

and toxin production before spoilage is detected by the processor or the consumer

1.2. Advantages of Modified Atmosphere Packaging of foods

Advantages of modified atmosphere packaging include increased shelf life, reduction in production and storage costs, reduction in use of inhibitors, improved presentation, fresh appearance, clear view of the product and easy separation of slices.

The pearl spot (*Etroplus suratensis*) is an important brackish water fish belonging to the family Cichlidae. It inhabits both freshwater and brackish water and is endemic to the peninsular India and Sri Lanka. Three species of Etroplus have been reported from Indian waters, namely *E. suratensis*, *E.maculates* and *E.canarensis*. While *E.canarensis* is confined only to the Uttara Kannada region, *E.suratensis* and *E.maculates* are known to occur widely in the backwaters, freshwater lakes, ponds and reservoirs of the peninsular India. *E.suratensis,* being the largest fish among them, is the most suitable species for aquaculture. It is considered as a good table fish, has wide salinity tolerance, breeds in confined waters and has highly adaptable feeding habits. It can be grown under mono and mixed culture systems, with fishes like carps or murrels in freshwater and mullet, milkfish and or peneaid prawns in brackish water. It is considered to be a delicacy in the state of Kerala with a good market demand. With the boom of backwater tourism, the demand for pearl spot, the high valued food fish in Kerala, is on the increase. Since the most valuable species are generally

exploited to the maximum, the fishery of this species is further subject to increasing pressures.

The capture fishery of this fish is a minor one and information on the catches is limited. Landing of all fishes from Inland sector was 40,365 tonnes whereas pearl spot contribute 3907 tonnes during 1991-1992. (CIBA Bulletin, 1995).

Fresh fish is a highly perishable commodity. Consumers of fish prefer to have them as fresh as possible so that the characteristic flavours, which they desire, are still retained. Bulk transportation of fresh fish in ice provides only a limited extension of storage life. Since the fish are not protected by over wrapping or other packaging, cross contamination and other forms of abuse cannot be avoided. Also flooding of fish with water from melting ice during transportation results in leaching of soluble nutrients and flavouring compounds. Modified atmosphere packaging, a technologically viable method has been developed in combination with mechanical refrigeration to reduce losses and to extend the storage life of fresh seafood products.

For perishable food like fish it is a promising technique as it gives better shelf life extension compared to icing. Many research workers have reported the extension of shelf life by adopting modified atmosphere packaging using thermoformed trays for temperate climate fishes. There is a great scope to use modified atmosphere packaging technology for tropical brackish water fishes like pearl spot to extend the shelf life. The present work was carried out to see the effect of modified atmosphere packaging on the shelf life of fresh pearl spot stored in ice to extend the shelf life.

1.3. Objectives

- To study the suitability of Thermoformed Trays for modified atmosphere packaging.
- To standardize the most suitable gas mixture for modified atmosphere packaging of pearl spot based on sensory evaluation.
- To find out the effect of modified atmosphere packaging (most suitable gas mixture) in comparison to air packaging.
- To study the biochemical, microbiological, sensory and textural characteristics during storage.
- To study the safety concern regarding the *Clostridium botulinum* during modified atmosphere packaging.
- To find out the most suitable chemical quality indices for modified atmosphere stored pearl spot.

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

2.1. Fish and the aquatic environment

Fresh food is normally considered a safe food however it can be an important source of bacterial food poisoning (Shewan, 1962). Oetterer (1991) described that, due to the presence of excellent quality proteins associated with higher water contents, as well as the presence of reasonable amounts of free nitrogen substances that favour deterioration, fish can support great microbial development. Germano *et al.* (1998) mentioned that fish may be a vehicle for spreading pathogenic microorganisms among human beings. As a direct consequence of improper handling, fish can become a favorable environment for the multiplication of *Streptococcus* spp. and *Staphylococcus aureus*. Other bacterial agents can contaminate fish, representing health hazard. Dodds (1993) states that waters are frequently contaminated with *Clostridium botulinum* spores and it is expected that fish will be also contaminated. Fish contamination may occur due to exposure to spores before fish capturing, during processing or storage. There is immense diversification of contaminated environments and great microbial variety, being the greatest concentration of microorganisms found in the fish's bowels, gills and skin. Ward (1994) states that a low rate of contaminants in the gills and skin are commonly associated to clean and cold waters, while higher contaminant levels are related to tropical and subtropical waters, as well as to polluted waters. The high contaminant level in the bowels is directly related to feeding, which is intense in artificially fed fish and low in extensively farmed fish.

Intensively farmed fish generally presents sanitary problems under control and are raised in clean and good quality water, as it is the case of salmon and trout in temperate zones. However, in clay bottom tanks with little water circulation, there may occur the structuring of non-proteolytic strains of *C. botulinum*, leading to undesirable levels of contamination .

2.2. Shelf life of Fish and deterioration

Fish shelf life is determined by the intensity of enzymatic reactions and by the number and species of microorganisms, affecting the products perishability. Other determining characteristics on shelf life are the storage temperature, which must be evaluated along the several production stages, the temperature during capture, delay in refrigeration, variation in the storage temperature and the retail temperature (Huss, 1971). According to Braga *et al.* (2000), the determining factor to improve fish quality is the early sanitation of the captured fish using treated water. The authors monitored the water and shrimps (*Xyphopeneaus kroyeri* and *Penaeus schmitti*), evaluating the micro biota associated to the surface of shrimps. The number of bacteria in fresh fish can be decreased and the shelf life prolonged through washing the product using high pressure water jets or using a 0.1% chlorine-cetypyridine solution (Mayer and ward, 1991). Sprayed chlorine, chlorine dioxide or potassium sorbate can also be used to reduce the bacterial count in fresh fish (Mayer and ward, 1991; Lin *et al.*, 1996). Several methods can be used to decrease or inhibit decaying changes, such as refrigerated storage, freezing, drying, heat processing, use of

additives and chemical preservatives, irradiation and packaging (Sharp *et al.*, 1986).

2.3. Methods of fish preservation

2.3.1. Fish curing

The moisture content of the fresh fish varies between 75-80%. Bacteria cannot survive if the moisture of the fish is reduced below 25%. Below 15% moulds ceases to grow. Drying can be carried out alone or in combination with salting and smoking.

2.3.1.1. Drying

Drying is used as a method of preservation for many foods, including fish. Drying as a method of preservation is based upon the fact that microorganisms and enzymes need water in order to be active. In preserving fish by this method, the moisture content of the fish is lowered to a point where the activities of the spoilage and food poisoning microorganisms are inhibited. Dried or low moisture foods are those that generally do not contain more than 25% moisture and have a water activity of 0.60 or less. Drying can be achieved either naturally or by mechanical means. Natural drying is by using the sun energy to drive the moisture out. The main advantage of sun drying is that the energy is free. But traditional sun drying methods are heavily depending upon the mercy of weather. Mechanical drying allows the temperature; humidity and air flow to be controlled.

2.3.1.2. Salting

Salting is often used in conjunction with drying and smoking. As most bacteria cannot grow in salt concentrations above 6% salting will reduce bacterial action. If the product is salted, there will be a loss of water and water-soluble nutrients during the salting process, and a further reduction during drying process.

2.3.1.3. Smoking

Smoking is a common practice for processing meat and fish. Smoking provides not only flavor components but also components that exert an antimicrobial action. Smoking of fish is a preservation procedure, which combines the processes of salting (brining), heating and drying and finally the addition of smoke itself. Sodium nitrite ($NaNO_2$) in combination with sodium chloride (NaCl) has been used for decades in processing meat and smoked fish products as a preservative that imparts color and enhances flavor. Sodium nitrite and Sodium chloride are also used to prevent the outgrowth and toxin production by *Clostridium botulinum* types A and E in the smoking process. However, the overall effect of the smoking process on fish proteins often results in amino acids being affected by any or all of these steps. Fishes are generally smoked over open fires or in simple kilns to accelerate the drying process. If the relative humidity is high and salt is scarce, hot smoking during which the fish are cooked is the common method. In cold smoked products the flesh is not cooked. Cold smoking is usually done for imparting the smoked flavour. Drying and smoking both results in a loss of weight mainly due to loss of water rather than nutrients.

2.3.2. Chilling

Short term preservation by chilling, is carried out using ordinary water and ice, although dry ice and chilled seawater are also used. The bacteria responsible for spoilage are psychrophilic, so even fish is chilled at 0°C under the best conditions of handling, can result in severe loss of quality approaching inedibility after a few days (Paine and Paine 1992). Objective of chilling is to cool the fish as quickly as possible to reach low temperature without freezing. Chilling cannot prevent the spoilage altogether, but in general, the cooler the fish, the greater the reduction in bacterial and enzymic activity (Clucas and Ward, 1996). Ice storage is relatively short-term method of preservation, with storage life varying between a few days to 4 weeks. Moreover proteins and some minerals and vitamins, are lost if the fish are washed or if they are stored in refrigerated or chilled seawater systems. The other disadvantages of this method of preservation are, it provides only a limited shelf life extension of fish, it is expensive, product can be contaminated through drip loss resulting in a further reduction in shelf life.

2.3.3. Freezing

This is used for long-term storage. The fishes are cooled below temperatures of -35°C and stored at -18°C. The much long shelf life is due to the almost complete halting of autolytic and bacterial action at these lower temperatures and also free water is effectively locked as ice (Clucas and Ward, 1996). Freezing may be useful for long-term storage and export through the cold chain. But freezing plants are expensive and costly to run. Another disadvantage during freezing, especially when its slow or if the storage temperature is allowed

to fluctuate considerably, the texture of fish can deteriorate because of cell damage and this increases the amount of drip when thawing. Other major disadvantages of freezing are high energy costs associated with freezing and storage, excessive drip loss when thawing, textural changes in fish muscle. During badly controlled freezing processes, denaturation of proteins with loss of amino acids, break down of fats with loss of fatty acids and vitamins, and production of unpleasant odours and chemical reaction between the major nutrients can also occur. Frozen stored products require cold chain throughout distribution.

2.3.4. Vacuum packaging

Vacuum packaging represents a static form of hypobaric storage that is widely applied in the food industry due to its effectiveness in reducing oxidative reaction in the product at relatively low cost. By vacuum packaging, the growth of bacteria in fish can be slowed down and the rate of development of rancidity can also be decreased, and because of these changes measurable extension in keeping time was observed. It is emphasized that the success of vacuum packaging is completely dependent upon the initial quality of fish and adequate temperature control through out storage. While oxygen depletion is effective in retarding the growth of typical spoilage bacteria, there is a possibility that if the product temperature is abused, it may become toxic.

2.3.5. Canning

In canning fishes are processed at high temperatures after enclosing in airtight containers to destroy microbial and enzyme activity. The product is protected

from further bacterial contamination by being hermetically sealed within the cans. Canning is a method of preservation in which selected food materials are prepared for the table, packed in containers capable of being sealed airtight, heated sufficiently to destroy the spoilage organisms within the container and cooled rapidly. The inside of the can must be resistant to its contents and the outside to ambient temperatures. Canned foods have a shelf life in excess of 1 year.

2.3.6. Irradiation

Another method of fish preservation, which has not been widely used but has been gaining in popularity, is the use of ionizing radiation. Radiation in suitable doses can kill the microorganisms, insects and parasites, which may be present in food and inhibit enzyme activity. Irradiation is not used commercially to any great extent because of the costs involved and consumer resistance.

2.3.7. High pressure processing

This is a method of hyperbaric storage. High pressure can stop microbial growth and reduce enzymic activity. Refrigerated storage of lean fish at high pressure extends the shelf life considerably. It is widely accepted that conformational changes of protein takes place at higher pressure, which may be responsible for the shelf life extension. High pressure processing destroys the bacteria without changing the nutritive value of the product. However, because of the technical difficulties in building a commercially feasible high-pressure storage unit, this method of preservation has not become popular.

2.3.8. Modified Atmosphere Packaging

However the recent consumer trend is toward refrigerated fresh foods. In an effort to expand the market of high quality fresh seafood, research has been directed at extending the shelf life of fresh seafood. Traditionally seafood is stored on ice or under refrigeration with an anticipated shelf life of 5 to 10 days. Modified Atmosphere Packaging (MAP) in conjunction with refrigeration, has been shown to double shelf life. MAP has been utilized for a number of years to extend the shelf life of red meats, poultry, but commercial seafood applications of MAP have received widespread research attention only in the last 15 years. According to the definition of Sacks and Gore (1987), MAP is replacement of air in a pack by a different mixture of gases, where the proportion of each component is fixed when the mixture is introduced, but no further control is exercised during storage. Controlled Atmosphere Packaging is packaging in an atmosphere where the composition of gases is continuously controlled throughout storage.

Controlled Atmosphere Packaging requires monitoring to maintain a specific balance of gases and is applicable to bulk storage of commodities such as fresh produce, which continue to respire post harvest. Modified Atmosphere Packaging includes Vacuum packaging since microbial action within the package produces CO_2 (10 to 30%) and a dynamic atmosphere results. Here the work is on MAP systems where CO_2 has been shown to be the most effective gas for shelf life extension. A gas flush or evacuation and backfill are used to replace the air in a package with a gas mixture specific to the product. Carbon dioxide,

oxygen, and nitrogen individually or in combination are the gases most frequently used in MAP. Nitrogen is used as an inert filler to counteract the pressure when a vacuum is drawn, and maintains package integrity to prevent the product from being crushed and/ or sticking together. Oxygen is added to meats to maintain colour, and to white fish to reduce drip, but may be omitted in white fish to reduce drip and in fatty fish to control the development of oxidative rancidity. Carbon dioxide acts as an anti microbial agent.

2.4. Packaging and combined processes

Most fish are caught from the wild in nets or with lines of baited hooks. Some die before being hauled from the water, some suffer physical damage, and all farmed or wild, are inevitably stressed before death. These, and other differences due to biological condition, structure, composition and post mortem change, present fish processors and distributors with a raw material very different from other food materials used in modern processing industries. As with other raw meats, fish at retail sale require some form of wrapping to protect the foods from contamination, and the purchaser from soiled hands and clothing. Wrapping in greaseproof (kraft) paper or, later, polyethylene film has long satisfied the need in small shops and markets. Demand from higher volume stores for conveniently pre packaged products led to some in shore production of cellophane over wrapped fish and shellfish products and, later, factory production of over wrapped, chilled portions on trays (Almaker, 1965) and frozen portions in bags (Anon., 1967). Whilst plastic films were appreciated as a necessary component in the growth of the market for frozen fish, Gibson and

Soulby (1970) found that the need for similarly convenient pre packaging of wet fish continued to be challenged through the 1960s. Limited shelf life was aggravated by problems of poor temperature control in storage, distribution and display systems. Instead, the products were often frozen for distribution and buffer storage, and then thawed for retail sale. Factory pre packaging of chilled retail fish products continued to develop but injection of preservative gas mixtures did not appear on a significant commercial scale for another decade. Interest in carbon dioxide as an aid in the preservation of fish has a longer history than that of the plastic films, which eventually made practical the modified atmosphere packaging (MAP) of retail products. Development started in the 1930s with several workers observing that CO_2 atmospheres prolonged the storage life of whole fish (Coyne, 1933; Stansby and Griffiths, 1935) and shell fish (Hjorth-Hansen, 1933). Shewan (1950), summarizing the earlier work, concluded that gas storage onboard fishing vessels was only worthwhile for fish which would be more than 14 days in ice before landing at the quayside. Subsequent interest in the use of CO_2 turned to refrigerated seawater systems as an alternative to the use of iced storage for small fish and shell fish on board catching vessels or for road transport (Nelson and Barnet, 1971; Barnett *et al.*, 1978; Hiltz *et al.*, 1976; Bullard and Collins, 1978). Some interest in the technically more difficult task of transporting chilled, whole fish in carbon dioxide atmospheres then reappeared. With diminishing supplies and higher prices of fish, studies have included cod (Villemure *et al.*, 1986) and cod fillets (Leblanc and Leblanc, 1992) as well as salmon (Sorensen *et al.*, 1990) under CO_2

atmospheres. Salmon was of most concern in earlier studies conducted by Nelson and Tretsven (1977), which were followed by commercial trials in 1977 (Barnett *et al.*, 1982). This was a period when all variables associated with manipulation of storage temperatures were keenly examined. Reductions in rates of deterioration of several species of fish have been recorded in tests using pressure chambers under both hyperbaric (Charm *et al.*, 1977) and hypobaric (Varga *et al.*, 1980; Haard and Lee, 1982) conditions. The earliest use of CO_2 in retail products exploited the solubility of CO_2 to produce a snugging down effect (Douglas, 1970), regarded as characteristic of CO_2 packs. The vacuum appearance and prevention of movement of the product was seen, ironically in view of the later developments in MAP, as being advantageous but there was only a small amount of kipper fillet packed in this way, with no application of white fish or other fish products (Abbey, 1970). Earlier vacuum packaging had been adopted more as a means of protecting frozen fish from dehydration (Anon, 1967). With unfrozen material, nitrogen flushing for oxygen sensitive products was recommended and applied to shrimps and prawns, but was not recommended for meat and fish which are susceptible to spoilage by anaerobic bacteria (Anon, 1967). As had occurred earlier with CO_2 preservation of whole fish, development and application of controlled gas mixtures for consumer packaging was concerned first with foods other than fish (Schweisfurth and Kalle Aktiengesellschaft, 1970; Anon, 1977). According to Kimber (1984), the technology of gas packaging was first perfected and patented in 1963 by Bohme and Kalle Films but it took until 1977 to produce gas flushed packs successfully.

UK manufacturers were slow to adopt the process but subsequently appear to have been more willing to widen the range of products and include fish. The first UK application to fish products was in 1979 in Northern Ireland (Kimber, 1984), with a few specialty products. The technique become more widespread as manufacturers of vacuum packaging equipment adapted their products, and super markets sought alternative ways of presenting fish. In some European countries, however, there has been little or no growth in prepackaging of chilled fish (Gormley and Zeuthen, 1990). With equipment capable of making more precisely designed and practicable products, research workers returned their attention to the demands of prepackaging fish products and the use of gases – mainly CO_2. The ways in which fish spoil, and the differences caused by their different composition greatly affect the consequences from storing them in modified atmospheres, and the choice of gas mixtures to be applied. Trials were conducted with a large range of species and products and many of the results have been published in a variety of symposia proceedings, recommendations and codes of practice (Martin, 1981; 1982; Cann, 1984; Anon., 1985) as well as individual research reports and thesis. Food preservation is based on combined methods, which can be used for the quality improvement of conventional products or the development of new products. They assure stability and safety, resulting in products presenting adequate sensory and nutritional properties (Leistner, 1992). Along the latest three decades there has been an increase in gas packaged food products in the market. This increase has brought improvements to the packaging industry, which has lead to the development of

high barrier polymers and thermo mold packaging equipment. Gas packaging is simply an extension of the vacuum packaging technology. Food packaging under modified atmosphere employs different gases, such as CO_2, N_2, and O_2, with CO_2 probably being the most common and effective gas, whether associated with other gases or not (Wolfe, 1980). CO_2, the most inhibitory gas has a strong anti microbial action, which varies between different microorganisms. It is responsible for extending the lag phase of bacterial growth and for decreasing growth rate during logarithmic phase (Farber, 1991). The bacteriostatic effect of CO_2 depends on the gas concentration, the initial bacterial count and the type of fish product (Finne, 1982; Gray *et al.*, 1983). The use of CO_2 to inhibit bacterial growth is not a new technology. In 1877 Pasteur and Joubert observed that Bacillus anthracis could be killed by CO_2 (Valley, 1928) and the first article on the preservative effect of carbon dioxide on food was published showing extended storage life for ox meat placed inside a cylinder filled with a carbon dioxide atmosphere. There are several techniques through which the atmosphere around a product can be modified. According to Silliker and Wolfe (1980), the most relevant techniques applied to fish and its related products are MAP and Vacuum packaging.

Modified Atmosphere Packaging- In MAP the air inside the packaging is replaced by a specific gas or a mixture of gases that differ from the air composition. The proportions of each gas are established, the mixture is introduced in to the packaging and no further control is carried out during storage (Silliker and Wolfe, 1980).

Vacuum packaging: the product is placed inside a type of packaging presenting low permeability to oxygen, the air is exhausted and the packaging is sealed. The gaseous atmosphere of the vacuum packaging is reduced, but it is probably altered during storage, thus considered modified due to a 10 to 20% increase in CO_2 amount produced by microbial activity. This CO_2 may inhibit the growth of undesirable microorganisms (Silliker and Wolfe, 1980).

The three main commercially used gases in modified atmosphere packaging are: carbon dioxide (CO_2), nitrogen (N_2) and oxygen (O_2). Examples of some meat and fish products currently gas packaged as well as the composition of gas mixtures used to extend the shelf life of each product are shown in table (1).

Table 1: Examples of gas mixtures for selected food products.

Product	Temp°C	%O_2	%CO_2	%N_2
Fresh meat	0-2	70	20	10
Cured meat	1-3	0	50	50
Poultry	0-2	60-80	20-40	0
Fatty fish	0-2	0	60	40
White fish	0-2	30	40	30
Cheese	1-3	0	60	40
Baked product	R.T	0	60	40
Pizza	R.T	0	60	40
Dry snacks	R.T	0	20-30	70-80

R.T – Room temperature

(Smith *et al.*, 1990)

CO_2 is soluble, not only in water, but also in lipids, being the main gas responsible for the bacteriostatic effect in modified atmospheres. Its general effects on microorganisms are an intensification of their latest growth stage and a decrease in the growth rate during the logarithmic stage (Farber, 1991). The bacteriostatic effect is influenced by the CO_2 concentration, the initial bacterial population, the storage temperature and the product being packaged (Reddy *et al.*, 1992). CO_2 is highly soluble in water, forming carbonic acid that may lower the pH (Smith *et al.*, 1990). The solubility in water at 0°C and 1 atmosphere is 3.38 g CO_2/kg H_2O (Knoche, 1980). The anti microbial efficacy of CO_2 is greatly enhanced as the storage temperature of the product is reduced due to its increasing solubility with decreasing temperature (Gould, 2000). Thus to guarantee maximum anti microbial effect, the storage temperature should be kept as low as possible (Finne, 1982; Farber, 1991). The solubility of CO_2 leads to dissolved CO_2 in the food product (Knoche, 1980)

The concentration of CO_2 in the food is dependent on the products water and fat content, and of the partial pressure of CO_2 in the atmosphere, according to Henrys law (Ho *et al.*, 1987). Devlieghere *et al.* (1998a, 1998b) have demonstrated that the growth inhibition of microorganisms in MAP is determined by the concentration of dissolved CO_2 in the product. After the packaging has been opened, the CO_2 is slowly released by the product and continues to exert a useful preservative effect for a certain period of time, referred to as CO_2's residual effect (Stammen *et al.*, 1990).

The action of CO_2 on the preservation of foods was originally thought to be caused by displacement of some or all of the O_2 available for bacterial metabolism, thus slowing growth (Daniels *et al.*, 1985). However, experiments with storage of bacon and pork showed a considerable increase in shelf life under 100% CO_2 compared with storage in normal air atmospheres (Callow, 1932), but the preservative effect was not because of the exclusion of O_2, as storage in 100% nitrogen (N_2) offered no advantage over normal air storage.

A drop in surface pH is observed in MA products because of the acidic effect of dissolved CO_2, but this could not entirely explain all of CO_2's bacteriostatic effect (Coyne, 1933). It was shown that CO_2 was more effective at lower temperatures and that the change in pH caused by the CO_2 did not account for the retardation of growth. In a study on several pure cultures of bacteria isolated from fish products, CO_2 atmospheres were found to inhibit the growth of bacteria markedly, whereas normal growth patterns were observed under air or N_2 atmospheres (Coyne, 1932). It was also observed that bacterial growth was inhibited even after the cultures were removed from the CO_2 atmosphere and transferred to an air environment, interpreted as residual effect of CO_2 treatment. Bacterial growth was distinctly inhibited under 25% CO_2 and almost no growth was observed under higher CO_2 concentrations for 4 days at 15°C. The results obtained could neither be explained by the lack of O_2 nor the pH effect. The effect of CO_2 on bacterial growth is complex and four activity mechanisms of CO_2 on microorganisms has been identified (Parkin and Brown, 1982; Daniels *et al.*, 1985; Farber, 1991);

1. Alteration of cell membrane function including effects on nutrient uptake and absorption;
2. Direct inhibition of enzymes or decreases in the rate of enzyme reaction;
3. Penetration of bacterial membranes, leading to intracellular pH changes;
4. Direct changes in physico – chemical properties of proteins

Probably a combination of all these activities account for the bacteriostatic effect. In food presenting high moisture and/ or fat amounts, such as fish, beef and poultry, the excessive absorption of CO_2 may lead to a phenomenon known as packaging collapse (Parry, 1993). A certain amount (depending on the foodstuff) of CO_2 will dissolve into the product to inhibit bacterial growth (Gill and Penney, 1988). The ratio between this volume of gas and volume of food product G/P ratio) should usually be 2:1 or 3:1 (The volume of gas is two to three times the volume of food). This high G/P ratio is also necessary to prevent package collapse because of the CO_2 solubility in wet products. Dissolved CO_2 takes up much less volume compared with CO_2 gas and after packaging a product in CO_2 atmosphere, a drop in pressure is developed within the package and package collapse may occur. Increase in dripping is caused by the dissolution of gases on the muscle surface in atmospheres containing high CO_2 levels (>60%), reduced pH and, consequently, low protein water retention ability (Parry, 1993; Randell *et al.*, 1995). As a consequence, high CO_2 concentrations promote organoleptic changes as, for example, texture alterations in meat. N_2

can be used as an inert gas in smaller proportions than CO_2. O_2 can also be employed, provided fish does not undergo color alterations (Cann *et al.*, 1983). N_2 is an insipid and inert gas, showing low solubility in water and lipids. It is used for displacing the oxygen from the packaging, decreasing oxidative rancidness and inhibiting the growth of aerobic microorganisms (Farber, 1991). Due to its solubility, it is used as a filling gas, preventing the possible packaging collapse caused by the accumulation of CO_2. O_2 generally stimulates the growth of aerobic bacteria and may inhibit the growth of exclusively anaerobic bacteria, although anaerobic microorganisms show different sensitivity levels to oxygen (Farber, 1991). The presence of oxygen may cause oxidative rancidity problems in fish presenting high lipid amounts, promoting the formation of low molecular weight aldehydes, ketones, alcohols and carboxylic acids. The use of oxygen in modified atmospheres is generally avoided with this kind of fish, in order to minimize such effects. The use of O_2 in modified atmosphere packaging for fish is supported by Davis and Slade (1995), who states that there are evidences showing that the use of O_2 reduces the exudation in fish during storage, and suggested that O_2 can be used in low fat fishes. Reddy *et al.* (1992) claim that the use of oxygen associated with N_2 or CO_2 gives a false idea of reducing botulinum risks in fresh packed fish and may lead to illusory safety. However for some products oxygen could or should be used. High levels of oxygen are used in red meat and red fish meat (tunas, yellowtails, etc.) to maintain the red colour of the meat, to reduce and retard the browning caused by formation of

metmyoglobin. Oxygen in MAP packages of fresh fish will also inhibit reduction of TMAO to TMA (Boskou and Debevere, 1997).

MAP associated to high CO_2 levels improves the stability of fresh fish, increasing its shelf life (Baker *et al.*, 1986). Gas mixtures presenting 40% CO_2, 30% O_2 and 30% N_2 have been recommended for low fat fish and a 40-60% CO_2 mixture, in equilibrium with N_2, has been recommended for fatty fish (Guidelines, 1985). Problems related to temperature abuses can occur with all manufactured foods, once the bactericidal and bacteriostatic effects of CO_2 vary with temperature (Wolfe, 1980; Church, 1998). Lack of refrigeration at any period throughout the products shelf life may allow the growth of microorganisms that had been inhibited by CO_2 during storage at low temperatures. Facultative anaerobic microorganisms and aerobic pathogens resistant to the anti microbial effects of CO_2, but which were unable to grow at low temperatures, can also thrive as the result of temperature abuses (Wolfe, 1980). Ogrydziak and Brown (1982) studied the temperature effects on the solubility and inhibition of CO_2 and concluded that, disregarding the synergetic mechanism between temperature and solubility, all evidences show that increases in temperature reduce the solubility and increase microbial growth, which is proportionally higher in MAP than under air. The use of high hygienic sanitary quality raw materials represents an important factor for the successful use of modified atmosphere packaging. Besides initially using high quality raw materials, the use of good hygiene practices during fishing, the selection of the right packaging material and a good temperature control are also necessary (Stammen *et al.*, 1990).

Revisions done by Wolfe (1980); Daniels *et al.* (1985); Pedrosa-Menabrito and Regenstein (1988); Stammen *et al.* (1990); Church and Parsons (1995); Alvarez *et al.*, (1998) and Sivertsvik *et al.* (2002) documented the MAPs ability to lengthen the shelf life of several perishable products, such as meat, poultry and fish. Prentice and Sainz (2000) developed a minimally processed product made from grass carp (*Ctenopharyngodon idella*) washing fish fillets using sodium hypochlorite and brine and vacuum packing, achieving shelf life periods of 30 and 60 days, when the product was kept under refrigeration temperatures of 8°C and 2°C, respectively. Kosak and Toledo (1981) studied the combination of a chlorine solution (1000 μg/mL free chlorine) with vacuum polyethylene packaging for mullet (*Mugil cephalus*) kept at –2°C. All treatments were organoleptically acceptable up to 14 days of storage. The effects of carbonic acid on cod (*Gadus morhua*) fillets packed in semi permeable film and kept at 1°C were tested by Daniels *et al.* (1985; 1986). The carbonic acid increased the shelf life from 7 to 21 days. However, the organoleptic quality was considered to be poor. Results indicated that the carbonic acid was as effective as the 98% CO_2 controlled atmosphere. Woyewoda *et al.* (1984) observed that bacterial growth and organoleptic deterioration in cod (*Gadus morhua*) fillets decreased slightly under 60% CO_2 atmosphere at 1°C. The small differences between samples kept in MAP and those kept in atmospheric air increased along with the storage period. Matches and Layrisse (1985) studied shrimp (*Pandalus platyceros*) kept under 100% CO_2 controlled atmosphere and observed moderate discoloration, not associated to undesirable smells, differing from the control

group, shrimp exposed to atmospheric air, which was not in an acceptable state. The CO_2 concentration delayed the appearance of black spots caused by enzymatic action. According to Villemure *et al.* (1986), gutted filleted cod (*Gadus morhua*) bulk stored at 0±1°C in 25% CO_2 /75% N_2 atmosphere maintained reasonable organoleptic quality up to 20 days, outlasting cod stored in atmospheric air. As to fish to fish surface pH, values of 6.6 and 7.5 were observed using MAP and atmospheric air respectively. The chemical, physical and microbiological alterations in raised catfish (*Silurus glanis*) were evaluated during storage period by Manthey *et al.* (1988). The acceptable storage time was considered to be 20 days. On the 27^{th} day of storage, fish fillets showed total anaerobic bacteria count of $10^8/cm^2$ of fish skin and only $10^5/g$ of muscle. Ammonia amounts increased from 11.5 to 18.7 mg/100 g of muscles, and Thio Barbituric Acid Reactive Substances (TBARS) amounts varied from 0.73 to 1.98 mg of malonaldehyde /Kg. Trimethyl amine amounts were low, while dimethyl amine was not detected. The bacterial count, as well as the surface pH of catfish (*Ictalurus punctatus*) fillets packaged in 90% CO_2/7.5% N_2/2.5% O_2 atmosphere and in atmospheric air, stored at 0 and 10°C were evaluated by temperature, but by storage time. The bacterial count was reduced by the MAP treatment. Huang *et al.* (1993) packed weakfish (*Cynoscion regalis*) in different packaging materials, storing the product in ice for 3 weeks. The results showed that vacuum skin packaging reduced fish rancidity and lipid hydrolysis when compared to the traditional over wrapping packing. Microbiologic acceptability was limited to 14 and 17 days for the over wrapping and vacuum skin packing,

respectively. Reddy *et al.* (1994; 1995) evaluated the effect of modified atmospheres (75% CO_2/ 25% N_2; 50% CO_2/50% N_2; 25% CO_2/75% N_2) on the shelf life of tilapia (*Tilapia* spp) fillets packed in high barrier film at 4°C. The authors observed that tilapia fillets packed in 75% CO_2/25% N_2 showed an increased shelf life of more than 25 days, presenting acceptable sensory characteristics. The shelf life of hake (*Merluccius merluccius*) and salmon (*Salmo salar*) slices stored in ice (2±1°C) under different atmospheres (40% CO_2/50% N_2/10% O_2; 60% CO_2/ 30% N_2/10% O_2; 40% CO_2/30% N_2/30% O_2; 60% CO_2/10% N_2/30% O_2), was evaluated by Pastoriza *et al.* (1996a; b). Hake and salmon slices could be ice stored in MAP for upto 21 and 18 days, respectively, with no quality loss.

The sensory, physical, biochemical and microbial qualities of Atlantic mackerel (*Scomber scombrus*) fillets stored and packed in modified atmosphere at –2°C was evaluated by Hong *et al.* (1996). MAP storage increased shelf life to 21 days, causing a slight increase in Total Volatile Base Nitrogen and trimethylamine contents. A number of coliforms, and molds (< 10 cfu/g) were also detected. Lopez- Galvez *et al.* (1995; 1998) evaluated tuna slices (*Thunnus alalunga*) and sole (*Solea solea*) fillets under different atmospheres (20% CO_2/80% atmospheric air; 40% CO_2/60% atmospheric air and 40% CO_2/ 60% O_2) stored at 2°C using physical – chemical and microbiological parameters. Sensory results showed that the shelf life of tuna slices increased from 4 to 8 days under 20% and 40% CO_2, respectively. The 40% CO_2/60% air atmosphere was the most effective as per microbiological and biochemical examinations for the tuna slices.

As to the sole fillets, the 40% CO_2/ 60% O_2 atmosphere was the most effective regarding microbiological and sensory aspects, increasing shelf life by one week. Randell *et al.* (1997; 1999) evaluated the quality of trout (*Salmo gairdneri*), herring (*Clupea harengus*) and salmon (*Salmo salar*) packaged in polystyrene films, under vacuum and MAP (35% CO_2/65% O_2; 40% CO_2/60% N_2) and stored at 2°C. They observed that mesophillic bacteria grew better in polystyrene packed fillets, while the number of coliforms was greater in vacuum packed fillets, which presented faster deterioration than MAP fillets. The sensory quality of trout and herring was similar for the three packaging types. The mixture of gases did not lengthen the fillets shelf life. Sivertsvik *et al.* (1999) studied the quality of refrigerated gutted salmon (*Salmo salar*) stored in plastic bags containing 50% and 100% CO_2 and 60% CO_2/40% O_2, as well as in conventional packaging material (polystyrene) during transport. They observed that the microbial growth was greater in the conventionally packed salmon. The MAP salmon presented better sensory quality than the conventionally packed one after 13 days of storage. Bak *et al.* (1999) studied the effect of MAP under 100% N_2 on shrimp (*Pandalus borealis*) stored at – 17°C. They observed that the MAP promoted a 9- month shelf life period; resulting in better color, lower oxidation and greater fish firmness than the storage in atmospheric air. Ozogul *et al.* (2000 b) compared the quality of Atlantic herring (*Clupea harengus*) stored in ice-free boxes under vacuum and MAP at 2 ±2°C, using ice-stored herring as control. Through chemical and microbiological analysis, the authors observed that the herring shelf life was extended by 10 and 8 days, for MAP and Vacuum

packaging, respectively, when compared to the ice stored herring. Low trimethylamine amounts and slight increase in drip were observed after 15 days of storage. Ordonez *et al.* (2000) stored hake (*Merluccius merluccius*) in atmospheres containing 20% and 40% CO_2, as well as in atmospheric air at 2±1°C. They observed that the shelf life increased to 4 and 11 days under 20% and 40% CO_2, respectively. The results showed that the 40% CO_2 atmosphere was more effective, with regard to biochemical and microbiological parameters, for refrigerated hake. Whole gutted hake (*Merluccius merluccius*) was ice stored in boxes under controlled atmospheres (CAP) presenting different gas mixtures (60% CO_2/15% O_2/25% N_2; 40 % CO_2/40% O_2/20% N_2; 60% CO_2/40% O_2 and 40% CO_2/60% O_2) and were examined during 33 days at 0±1°C. Through physical chemical and sensory analysis, Ruiz Capillas and Moral (2001) found that the 60% CO_2/40% O_2 CAP was more effective than other mixtures and promoted a better product with sensory acceptance, being more effective than the other mixtures.

2.5. Quality Changes of fish in ice and MAP storage.

2.5.1. Physical Changes

2.5.1.1. Composition of the headspace gas mixtures.

When compared to some packs containing meat products with similar concentrations of CO_2 (McMullen and Stiles, 1991), changes in the composition of gas mixtures within MAP fish packs appear to be rapid. The proportion of CO_2 in the headspace falls as CO_2 dissolves in fish tissues (Strasdine *et al.*, 1982 and

Davis, 1990). As a consequence, proportions of the diluent components increase and concentrations of oxygen, often above the atmospheric levels to start with, are even increased further. Later, as the CO_2 solubilisation rate is overtaken by the rate of release caused by bacterial respiration, the curve reverse. Davis (1990) calculated that, at the CO_2 minima, the amounts absorbed at three different storage temperatures were approximately 30% of the saturation solubility. However, that observation referred to a specific combination of gases and fish, and relevance to results of other workers is limited because of insufficient attention given to important details; particularly, the gas to fish ratio. Any chemical effects on the fish tissues will be affected by the amount of CO_2, which dissolves, but as the contaminant flora is limited to the fish surfaces, much of the bacteriostatic effect is likely to be more influenced by the residual atmosphere they have to face. Davis (1990), working with cod fillets packed in a gas mixture containing 40% CO_2 found that the Gas to Product ratio had to be at least 2:1 for maximal benefit. Most research reports, whilst often concerned with the composition of the gas mixtures, have ignored the concomitant matter of the amount of gas required (at any given storage temperature.) in relation to the mass of the product. For a given gas mixture, high gas to fish ratio will present a very different chemical balance from a low ratio. Similarly, any given mass ratio of CO_2 to product will not necessarily confer the same benefits (or harm) when applied via different gas mixtures. Thus, combined with the considerable variation which exists between fish products in terms of surface area to volume ratio as well as chemical differences, there can be little surprise at the extend of

the variation in reports of the benefits conferred by MAP on fish products. Silva and White (1994) in modified atmosphere packed refrigerated Channel Cat fish observed CO_2 concentration fluctuated between 63% and 87% for higher CO_2 concentration packs and 16 to 33% in low CO_2 concentration packs. Samples stored under air had increased CO_2. The increase in CO_2 in air packed fillets was the result of microbial respiration. Debevere and Boskou (1996) studied modified atmosphere packed cod fillets and observed a decrease in CO_2 until day 4 because of diffusion in to the fish muscle, again CO_2 increased due to bacterial and enzymatic activity. Reciprocally to the CO_2 content the proportion of O_2 increased up to day 4, and due to respiration of bacteria the proportion of O_2 decreased later on. Amanatidou *et al.* (2000), in fresh Atlantic salmon using 50% CO_2 + 50% O_2 observed increased O_2 concentration in the packs.

2.5.1.2. Changes in thaw drip during storage

Exudate or drip in increased amounts is another consequence of treating fish with CO_2. Normally, the small amount released from raw fillets is not a great problem but it becomes a limiting factor for some products in MAP. The problem may be solved by limiting the amount of CO_2 and by placing the fish on absorbent pads within the packs (Tiffiney and Mills, 1982; Cann, 1984). The lower water contents of smoked products and fatty fish leaves them less vulnerable to further losses under 60% CO_2 than occurs with raw white fish for which the concentration of CO_2 reduced to the initial level to 40% (Tiffiney and Mills, (1982). Some success in further reducing drip by inclusion of oxygen was implied. In most reports detailing release of drip and pack collapse, the

problems are aggravated at the lower storage temperatures, perhaps a reflection of the effect on the solubility of CO_2. Nevertheless, there are unexplained variations (Tiffiney and Mils, 1982; Cann *et al.*, 1983, 1984, 1985). Pastoriza *et al.* (1996) observed no marked effect of CO_2 on exudation of salmon slices stored in the ice state under CO_2 atmospheres. MAP stored fish showed significant increase of weight loss from the 14^{th} day storage onwards. Dalgaard *et al.* (1993) observed an increase in drip loss during modified atmosphere storage of cod fillets. The largest drip loss was observed for fillets stored in high CO_2 concentrations. Pastoriza *et al.* (1998) observed increased exudation in air and MAP stored fish showed exudates values higher than control after 7 days of storage. Fey and Regenstein (1982) found increased drip losses for red hake, Chinook salmon and to a lesser extent, Sockeye salmon stored in 60% CO_2, 21% O_2 and 19% N_2 compared to air packed product. Tiffiney and Mills (1982) found that packing in 100% CO_2 increased the rate and quantity of drip loss. In all instances the amount of drip loss of fish stored in high CO_2 concentrations was higher at 0°C than at 5°C.

2.5.1.3. Changes in pH during storage.

Apparent contradictions arise in considering almost every aspect of MAP fish including, inevitably, muscle pH. Fey and Regenstein (1982) reported little or no change whilst others have seen a decrease proportional to CO_2 concentration (Lannelongue *et al.*, 1982a, 1982b; Belleau and Simard, 1987). Fish muscle of relatively high post mortem pH can be expected to be more affected by a given amount of CO_2 than muscle of lower pH but this will be complicated by other

variations in the chemical composition affecting the buffering capacity of the tissue. Additional variation in reported measurement may also occur because of differences in method of measurements, mainly because of the gradients, which occur between the product surface and deeper tissue (Tiffiney and Mills, 1982). As initial dissolution of CO_2 (preceding formation of carbonic acid) and bacterial activity (which produces high pH waste products) is both surface phenomena, it is here that the most rapid and extreme fluctuations in pH occur. The general pattern seems to be that after any initial fall in pH, surface pH rises whilst internal pH changes lag behind.

Pastoriza *et al.* (1996) observed an increase in pH in air stored samples throughout the storage period, with a value of 8.03 after 21 days, MAP stored samples showed lower pH values throughout storage. Increase in pH is associated mainly to bacterial activity as a consequence of volatile amine production (Wang and Brown, 1983). Dalgaard *et al.* (1993) reported a pH increase from about 6.6 to 6.8 in experiments with low CO_2 concentrations, but remained almost constant at about 6.6 in experiments with high CO_2 concentrations. Debevere and Boskou (1996) observed counter effects of CO_2 on the pH increase by TVB production, resulting in a stabilization of the pH around 6.7. Pastoriza *et al.* (1996) observed slight increase of pH in the control during the low temperature storage of salmon slices whereas in MAP, an initial decrease of pH was observed followed by an increase after 6 days of storage. Layrisse and Matches (1984) indicated that CO_2 is rapidly dissolved or absorbed in MAP stored muscle and consequently, pH decreased. Parkin *et al.* (1981) and

Lannelongue *et al.* (1982) observed that the decrease of pH could be proportional to the CO_2 content of the package. Banks *et al.* (1980) and Parkin *et al.* (1981) attributed the pH change due to conversion of CO_2 to carbonic acid at the fish muscle surface. Pastoriza *et al.* (1998) observed a pH value of 7.74 in control samples of hake slices after two weeks iced storage. Fish stored under MAP conditions showed pH values lower than the control after ten days storage. Lopez- Galvez *et al.* (1998) observed a pH increase in refrigerated storage of air packaged sole and only a slight increase was observed in samples stored in 20% CO_2 and no change in pH were observed when samples were stored in CO_2/Air (40/60) (V/V). Therefore in general, the dissociation of carbonic acid in fish flesh results in a slight drop in pH. Both the buffering capacity of the fish proteins and the composition of the spoilage flora determine the magnitude of pH change (Cutting, 1953). However Barnett *et al.* (1978) found no significant change in the pH of the salmon flesh stored in 90% CO_2. The extent to which pH decrease is proportional to the concentration of CO_2 in the atmosphere (Lannelongue *et al.*, 1982a; Tiffiney and Mills, 1982). Studies have shown that CO_2 is absorbed rapidly and pH drops over the first two days of storage. Parkin *et al.* (1981) noted a drop in pH of rockfish from pH 6.7 to 6.3, which was maintained throughout the storage period. However Fey and Regenstein (1982) found that after an initial decrease, pH increased and after 27 days storage in a CO_2 enriched environment had reached a level similar to its initial pH.

2.5.2. Changes in Biochemical parameters

2.5.2.1. Changes in Trimethylamine Nitrogen Content (TMA-N content)

Dalgaard *et al.* (1993) observed that the level of TMA was typically around 10-15 mg TMA-N/100g in aerobically stored fresh fish when rejected by sensory panels. Ababouch *et al.* (1996) observed that the limit of acceptability for sardines was found to be 5-10 mg TMA-N/100g of samples according to comparison of sensory and chemical data. Pastoriza *et al.* (1996) observed very low TMA values for salmon slices. Such low values can be consequences of a very low bacterial load leading to a very much-reduced enzymic deterioration of fish muscle (Gerdes *et al.*, 1989). Ozogul *et al.* (2000b) reported a value of 13.5 mg in herring kept in MAP (CO_2:N_2 60/40) for 10 days at 2±2°C. Cann *et al.* (1983) reported a TMA value of 10.4 mg/100g in herring fillets stored in MAP with the same gas mixture for 11 days at 0°C. Ozogul *et al.* (2004) reported that significant differences were discovered between sardines held under air and Vaccum pack and MAP treatments after 4 days of storage. The concentration of Trimethyl amine in numerous fatty fishes never reached the limit of 5 mg TMA-N /100g although the rejection limit in flesh is usually 5-10 mg TMA-N/100g. Nevertheless, this limit cannot be applied to sardines stored under MAP and VP since this fish were spoiled before reaching this level. This could be because, sardine is a fatty fish.

2.5.2.2. Changes in Total Volatile Base Nitrogen content (TVB-N) during storage.

The more rapid increase of TVB-N at high microbial numbers indicated the stage of substantial spoilage of the fish. Ababouh *et al.* (1996) reported that the limit of acceptability for sardines was 25-35 mg /100g of flesh. Ozogul *et al.* (2004) reported TVB-N content of sardines stored in air, vacuum pack, and MAP at 4°C. Initial TVB-N value was 5 mg/100g for sardines stored under air. The release of total volatile bases increased up to 15 mg/100g for sardines in VP and 17 mg/100g in MAP at the last day of sensory acceptability for each storage conditions. Pastoriza *et al.* (1996) observed salmon slices stored under CO_2 showed a much slower increase, with values much lower than those considered as limit of acceptability after 20 days storage, and similar to those of control after 10 days storage. They considered 30 mg TVB-N/100g as limit of acceptability. Pastoriza *et al.* (1998) observed a reduction in TVB-N values during MAP storage of hake slices, assuming 35 mg TVB-N/100g as the limit of acceptability for consumption of fish. Fraser and Sumar (1998) indicated that bacterial catabolism of amino acids in fish muscle results in the accumulation of ammonia and other volatile bases.

2.5.2.3. Changes in Thio Barbituric Acid Value (TBA) during storage

Oxidative rancidity is an important organoleptic characteristic for rejection or approval of fish after prolonged storage. Thiobarbituric acid (TBA) value gives a good indication of secondary stage of lipid oxidation.

Boyd *et al.* (1992) reported very low TBA values in pond raised striped bass and Kyrana and Longevis, (2002) also reported very low TBA values for farmed sea bream stored under air in chilled conditions. Papadopoulos *et al.* (2003) also observed low values for sea bream stored under air in chilled conditions. Pastoriza *et al.* (1996) found increased TBA values in CO_2 modified atmospheres compared to air stored samples in salmon slices. Pastoriza *et al.* (1998) found the same results in modified atmosphere stored slices of hake. Amanatidou *et al.* (2000) found that salmon samples stored under modified atmospheres and kept under chilled conditions, with TBA value above 1.9 were characterized by unacceptable organoleptic characteristic (rancid taste).

2.5.2.4. Changes in K value during storage.

The initial quality loss in fish is primarily caused by autolytic changes and is unrelated to microbiological activity. Of particular importance in this respect is the degradation of nucleotides (ATP- related compounds), which is caused by autolytic enzymes. It is now widely accepted that the loss of the intermediate nucleotide, inosine monophosphate (IMP), is responsible for the loss of fresh fish flavour, but apart from this, the autolytic changes are contributing to spoilage mainly by making catabolites available for bacterial growth. The breakdown of IMP and inosine proceeds faster in naturally contaminated fish than in sterile samples and it has been shown repeatedly that several bacteria participate in the degradation.

Objective assessment of fish muscle quality is very important, even though ultimate rejection depends on sensory evaluation. In Japan, a term known as

the K value is used extensively as a commercial index for estimating fish freshness (Ehira and Uchiyama, 1986). Saito *et al.* (1959) were the first to estimate the freshness of fish muscle from the ratios of the sum of the inosine and hypoxanthine to the sum of all other ATP breakdown products. K value as an index of estimating fish freshness has come to be widely used in Japan. It is defined as (Saito *et al.*, 1959),

$$K = ((Hx+HxR)/(ATP+ADP+AMP+IMP+Hx+HxR)) \times 100$$

Uchiyama *et al.* (1970) suggested that K value could be reliably used as an index of evaluating the real freshness of fish. Ehira and Uchiyama (1973) suggested that K value could really and reliably distinguish the freshness in all the investigated fish species; Hypoxanthine (Hx) could not be used as an index in inosine forming species. Bremner *et al.* (1988) observed that shelf life and overall acceptability were more related to IMP degradation products than bacterial spoilage. Kiesvaara *et al.* (1990) could establish that K value served as a freshness indicator for several Finfish freshwater species. Ryder (1985) found that K value was a good objective index for the freshness of hoki and rainbow trout, especially during iced storage. Lakshmanan *et al.* (1996) suggested that K value increased linearly with storage time and appeared to be a good index of freshness for the two brackish water fish, Pearl spot and Mullet. Boyle *et al.* (1991) studied the adenine nucleotide degradation in modified atmosphere chill stored whitefish and rainbow trout. The results indicated CO_2 atmospheres did not alter the K values compared to those observed for aerobically held fish. However CO_2 atmospheres caused decrease in hypoxanthine concentrations

compared to aerobically held samples. Huynh *et al.* (1992) found the same results with sockeye salmon and herring. Randell *et al.* (1995) studied the effect of gas/product ratio and CO_2 conentration on the shelf life of MA packed fish. The results indicated CO_2 concentration did not affect the K values of Rainbow trout. Reddy *et al.* (1997) also found that at 4,8, and 16°C storage K values of MAP stored fillets of cat fish increased gradually during early and middle storage time and decreased towards end of storage period with sensory spoilage indicating no relationship between sensory spoilage and K value. Lopez-Galvez *et al.* (1998) with sole fillets reported no effect of the atmosphere on K values. Ozogul *et al.* (2000 c) studied the degradation products of adenine nucleotide in rainbow trout stored in ice and in modified atmosphere packaging. CO_2 concentration did not seem to affect the K values of trout, however CO_2 atmosphere affected hypoxanthine concentration. Ozogul *et al.* (2000a) studied the effects of modified atmospheres on K values of herring stored at 2°C. They observed 60% CO_2 atmospheres showed lower K values compared to those observed for aerobically stored fish, in addition CO_2 decreased the formation of Hypoxanthine compared to aerobically and vacuum held fish. Ozogul *et al.* (2004) studied the effects of modified atmospheres on K values of Sardines at 4°C. The lowest increase in K value was observed for sardines stored in MAP, which was possibly influenced by the presence of CO_2.

2.5.3. Changes in Colour during storage.

Discoloration can occur via a bleaching action of cut surfaces (Cann, 1984) probably due to low pH precipitation of sarcoplasmic proteins (Statham and

Bremner, 1989). On whole fish, eyes become opaque and skin pigments fade (Coyne, 1933; Stansby and Griffiths, 1935). There is also risk of discoloration of haem pigments (Przybyski *et al.*, 1989). Less important as a visual feature of white fish, having much less pigment than in meat, it is not usually a problem but some observed a brown discoloration in fish minces in MAP which was linked to elevated levels of O_2. Brown *et al.* (1980) demonstrated that inclusion of 1% carbon monoxide in the gas mix could help retain a red color, unlike Fey and Regenstein (1982) who observed a negative effect. Inclusion of O_2 as a means of maintaining more attractive red color of haem pigments is recommended for red meats but high concentrations are necessary. With fatty fish, Tiffiney and Mills (1982) found that the fresh appearance was retained for longer in O_2- free packs. High concentrations of CO_2 cause discoloration of meats, especially red meats. The natural pigment of meats is a dark, purple-red compound called myoglobin. This pigment, when combined with oxygen (O_2) forms oxymyoglobin, which gives fresh meat the blooming effect or red colour. The degree of redness depends upon the amount of pigment present in the meat and the availability of O_2. Myoglobin can be oxidized in an atmosphere rich in CO_2 to form metmyoglobin, thus reducing the amount of redness to a light brown discoloration. Since fish are low in myoglobin, the discoloration induced by elevated concentrations of CO_2 is not as noticeable as in red meats. Colour changes in fish include graying of the Cornea (Coyne, 1933), bleaching of the skin and damage to bloom at high CO_2 concentrations (>60%) (Stansby and

Griffiths, 1935). Goodfellow (1982) found it necessary to restrict CO_2 levels to 25% or less to prevent discoloration problems.

Silva and White (1994) in modified atmosphere packaged refrigerated channel cat fish observed increased Hunter L* values in all packaging environments over time. Hunter a* value had an initial decrease in fish held in high CO_2 concentration but increased as time progressed. Hunter b* values showed no differences among any treatments. Handumrongkul *et al.* (1994) observed MAP strips had more total colour difference than did air packed strips of refrigerated striped bass. Pastoriza *et al.* (1996) observed increased Hunter L* and b* values and decreased Hunter a* value in both air and MAP stored samples during the storage period. Amanatidou *et al.* (2000) in modified atmosphere packaging of Atlantic salmon observed a product with Lightness above the threshold value of 70 or red colour below 13 is unacceptable.

2.5.4. Changes in texture during storage

Texture of the fish is very important, since a poor texture can result in bad visual appearance of the product. Springiness can be used to simulate finger feel of the raw fillets/portions, and shear value as an index of tenderness/firmness of the raw fish. Many of the methods used for measuring the instrumental texture of fish are modified versions previously used for meat. The most common types of measurements are based upon rheological principles (1) shear strength (2) puncture and (3) compression. The recent trend is away from the point measurements that reflect either only one parameter or as overall value for a group of parameters, and towards a multiple point or curve method that can give

information on several parameters. Compression test can include 1 or 2 successive compressions. For measuring the hardness single compression is only required. Those with two successive compressions from the Texture Profile Analysis (TPA) result in curves from which several textural parameters can be obtained. Two compressions are said to be necessary, if parameter such as cohesiveness, elasticity, adhesiveness, chewiness and gumminess are to be measured.

Amanatidou e*t al.* (2000) observed a lower cutting strength in MA packed fresh atlantic salmon, indicating an increase in the softness upon storage. Textural characteristics were retarded in MAP compared to control. Fagan *et al.* (2004) tested effects of modified atmosphere packaging with freeze chilling on some quality parameters of raw whiting, mackerel and salmon portions. The results indicated that storage time had no effect on the springiness of Whiting, mackerel or salmon portions. MAP had no effect on the springiness of whiting or salmon portions. Mackerel fillets were less springy. MAP had no effect on the shear values of whiting fillets or salmon portions. However mackerel fillets stored in 100% CO_2 had higher shear values than samples from other treatments.

2.5.5. Microbiological spoilage in modified atmosphere packaging of fish stored under chilled conditions.

Microbiological spoilage of foods may take diverse forms, but all of them are a consequence of microbial growth and /or activity, which manifests itself as changes in the sensory characteristics. Raw foods are initially contaminated with a wide variety of microorganisms, but only a selection of these contaminants is

able to colonize the food and grow to high numbers. The term spoilage association has been coined for such a specific microbial community. The precise mechanism by which one group of bacteria predominates over another, closely related group is not always fully understood. It is well known that only minor changes in processing and packaging of fish products are causing a dramatic change in the development and composition of the spoilage association and a complete different type of product, spoilage may develop differently, depending on geographical origin and other unknown factors interacting with microbial development.

2.5.5.1. Fish as substrate for bacterial growth

All food commodities have their own distinctive microbiology. Important factors contributing to the microbiological complexity of seafood are:

- Specific as well as non specific contamination of the live animal from the environment and of products during processing;
- Growth conditions for microorganisms due to specific intrinsic and extrinsic factors (temperature, a_w, pH, Eh, microbial interactions etc.).

The wide range of environmental habitats (freshwater to saltwater, tropical waters to arctic waters, pelagic swimmers to bottom dwellers and degree of pollution) and the variety of processing practices (iced fish products to canned products) are all important factors in determining the initial contamination of fish and fish products. The part of the micro flora, which will ultimately grow on the products, will be determined by the intrinsic and extrinsic parameters. There are

several important specific intrinsic factors in fish, which greatly influence the microbiology and spoilage:

- The poikilotherm nature of the fish and its aquatic environment;
- A high post mortem pH in the flesh (usually>6.0);
- The presence of large amounts of non-protein nitrogen (NPN);
- The presence of trimethylamine oxide (TMAO) as part of the NPN fraction.

Bacteria establish themselves on the outer and inner surfaces of the live fish (gills, skin, gastro intestinal tract). The poikilotherm nature of fish allows bacteria with a broad temperature range to grow. Thus, the micro flora of temperate water fish is dominated by psychrotrophic Gram negative, rod shaped bacteria belonging to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Flavobacterium*, *Vibrionaceae* and *Aeroemonadaceae*, but Gram-positive organisms such as *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus* and *Corynebacterium* can also be found varying proportions. The flora on tropical fish often carries a slightly higher load of Gram positive and enteric bacteria, but is otherwise similar to the flora on temperate water fish (Liston, 1980).

An important intrinsic factor related to fish flesh is the very high post mortem pH (> 6.0). Most fish contain only very little carbohydrate (< 0.5%) in the muscle tissue and only small amounts of lactic acid are produced post mortem. This has important consequences for the microbiology of fish as amongst other factors it allows the pH sensitive spoilage bacteria *Shewanella putrefaciens* to grow.

The non-protein nitrogen (NPN) fraction of the fish flesh consists of low molecular weight water-soluble nitrogen containing compounds such as free amino acids and nucleotides and is a readily available bacterial growth substrate. The decomposition of the sulphur containing amino acids cysteine and methionine is particularly important in spoilage, as it causes off odours and flavours due to formation of hydrogen sulphides and methylmercaptane respectively (Herbert and Shewan, 1975; 1976)

Trimethylamine oxide (TMAO) is part of the NPN fraction and its presence in all marine (Hebard *et al.*, 1982) and some fresh water fish (Gram *et al.*, 1989) species is well established. TMAO is known to cause a high (positive) redox potential (Eh) in the fish flesh (Huss and Larsen, 1979; 1980), however, the significance of this is not clear. The spoilage of fresh fish is certainly influenced by the presence of TMAO, particularly under conditions where oxygen is excluded. A number of well defined spoilage bacteria (*Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Vibrionaceae*) are able to utilize TMAO as the terminal electron acceptor in an anaerobic respiration resulting in off odours and -flavours due to the formation of trimethylamine (TMA) (Gram *et al.*, 1987; 1990; Dalgaard *et al.*, 1993).

2.5.5.2. Principles of bacterial spoilage

In its simplest form, food spoilage is a result of microbiological growth per se and becomes evident as visible growth (moulds, pigmented or non pigmented, slimy bacterial colonies). In such cases, of course, there is a direct relationship between the total numbers of microorganisms and degree of spoilage.

More often spoilage is a result of the production of off odours and flavours caused by bacterial metabolism. In this case there is no correlation between total numbers of bacteria and spoilage (Castell *et al.*, 1948; Huss *et al.*, 1974), since only a fraction of the total flora participates in the spoilage. A clear distinction should be made between the terms spoilage association and spoilage organisms (bacteria) since the first describes merely the bacteria present on the fish when it spoils whereas the latter is the specific group that produces the off-odours and off-flavours associated with spoilage.

It is not an easy task to determine which of the bacteria isolated from the spoiled fish are those causing spoilage, and it requires extensive sensory, microbiological and chemical studies. First, the sensory, microbiological and chemical changes during storage must be studied and quantified, including a determination of the level of a given chemical compound that correlates with spoilage (the chemical spoilage indicator). Secondly the bacteria are isolated at the point of sensory rejection. Pure and mixed cultures of bacteria are screened in sterile fish substrates for their spoilage potential, i.e., their ability to produce sensory (off-odours) and chemical changes typical of the spoiling product (Castell and Anderson, 1948; Herbert *et al.*, 1971; Gram *et al.*, 1987). Spoilage potential can be assessed in substrates such as sterile, raw fish juice (Lerke *et al.*, 1963), heat sterilized fish juice (Castell and Greenough, 1958; Gram *et al.*, 1987; Dalgaard, 1995a) or on sterile muscle blocks (Herbert *et al.*, 1971). The latter is the most complicated, but is also the one, which is most comparable to the product.

Finally, the selected strains are tested to evaluate their spoilage activity, i.e., their growth kinetics and their qualitative and quantitative production of off-odours in the product of concern (Gram, 1989; Dalgaard, 1995a). The later step is important, as some bacteria may produce the chemical compounds associated with spoilage, but are unable to do so in significant amounts at the normal conditions prevailing in the product and they are thus not the specific spoilage bacteria.

The spoilage association developing in aerobically stored fish consists typically of Gram-negative psychrotrophic non-fermenting rods. Thus, under aerobic iced storage, the flora is composed almost exclusively of *Pseudomonas* sp. and *Shewanella putrefaciens*. This is true for all fish and shellfish whether caught or harvested in temperate (Levin, 1968; Gram *et al.*, 1987) or sub-tropical and tropical waters (Lannelongue *et al.*, 1982a; Gram *et al.*, 1990; Lima dos Santos, 1978). At ambient temperature (25°C), the micro flora is dominated by mesophilic *Vibrionaceae* (Gorezyca and Pek Poh Len, 1985; Gram *et al.*, 1990) and, particularly if the fish are caught in polluted waters, mesophilic *Enterobacteriaceae* (Gram, 1992). *Shewanella putrefaciens* is the specific spoilage bacteria of marine temperate-water fish stored aerobically in ice and the number of *Shewanella putrefaciens* is inversely linearly related to remaining shelf life of iced cod (Jorgensen *et al.*, 1988). *Shewanella putrefaciens* strains isolated from fish products have similar spoilage potential (Jorgensen and Huss, 1989; Dalgaard, 1995a). *Pseudomonas* species are the specific spoilers of iced stored tropical fresh water fish (Lima dos Santos, 1978; Gram *et al.*, 1990) and

are also together with *Shewanella putrefaciens*, spoilers of marine tropical fish stored in ice (Gillespie and MacRea, 1975; Gram, 1992). *Shewanella putrefaciens* has been isolated from tropical fresh waters, but does not appear to be important in the spoilage of iced fresh water fish from tropical waters (Lima dos Santos, 1978; Gram *et al.*, 1990). This may be due to occurrence of very low numbers and the inability of organism to compete with high numbers of antagonistic pseudomonads (Gram, 1993; Gram and Melchiorsen, 1996).

In vacuum-packed iced stored fish from temperate marine waters an increased development of TMA is seen while the shelf life is unaffected compared to aerobically stored fish. The number of *Pseudomonas* is reduced, but *Shewanella putrefaciens*, which is capable of anaerobic respiration using TMAO, grows to levels of 10^6-10^8 cfu/g (Gram *et al.*, 1987; Jorgensen *et al.*, 1988; Dalgaaard *et al.*, 1993). Numbers below 10^8 cfu/g are unlikely to be important in spoilage and consequently other organisms must be involved. Jorgensen *et al.* (1988) observed that vacuum-packed cod consisted some very large, almost yeast like cells and suggested that these were involved in spoilage. It was recently shown that these cells are heat sensitive *Photobacterium phosphoreum* (Dalgaard *et al.*, 1993). *Photobacterium phosphoreum* is a marine vibrio, which has escaped microbiologists, as it does not grow when, pour plating and incubation at high temperatures is used (Van Spreekens, 1974; Dalgaard *et al.*, 1993). It is easily isolated from intestines of various fish (Van Spreekens, 1974; Dalgaard, 1995a). The organism produces 10-100 fold more TMA per cell than *S. putrefaciens* (Dalgaard, 1995b) but does not cause off-odours as foul as *Shewanella*

putrefaciens (van Spreekens, 1977) probably because it does not produce volatile sulfides (van Spreekens, 1977; Dalgaard *et al.*, 1993; Dalgaard, 1995a). The spoilage of vacuum-packed fish from temperate marine waters is caused by these two bacteria and differences in initial numbers of *Shewanella putrefaciens* and *Photobacterium phosphoreum* probably decides which of the two becomes most important.

Carbon dioxide and vacuum packing of fish caught in fresh water or warmer waters where these particular heat sensitive, sodium requiring *P. phosphoreum* are probably not as common, logically result in decrease in TMA production. The micro flora becomes dominated by various Gram-positive organisms, mainly lactic acid bacteria (Banks *et al.*, 1980; Lannelongue *et al.*, 1982a,b; Oberlender *et al.*, 1983; Pedersen and Snabe, 1995). However TMA can be detected later in the storage (Reddy *et al.*, 1995; Oberlender *et al.*, 1983), which shows that TMAO reducing organisms must be present at some level.

Gram and Huss (1996) reported that *Shewanella putrefaciens* and *Pseudomonas* are the specific spoilage bacteria of iced fish regardless of the origin of the fish. Shewan (1977) reported that *Pseudomonas* species as the main spoilage bacteria in fish stored in ice mainly because of their short generation time. Koutsoumanis and Nychas (1999; 2000) and Gennari *et al.* (1999) identified *Pseudomonas* spp. as the dominant bacterial group in freshly harvested and spoiling Mediterranean fish stored aerobically at 0-15°C. Suphide producers often constitute a major proportion of the microbial flora of spoiling fish, the predominant sulphide producing bacteria being *Shewanella putrefaciens*. (Gram *et al.*, 1987; Gram,

1992). When the number of these bacteria exceeds 10^6 cfu/g, significant amounts of volatile, sulphur containing compounds are produced and spoilage become sensorilly evident, whilst levels of 10^8- 10^9 cfu/g are generally required to cause spoilage in ice stored fish (Gram and Huss, 1996). Few studies have been published on *B.thermosphacta* and its relation to spoilage of fresh water and marine fish. (Drosinos and Nychas, 1996; Koutsoumanis and Nychas, 1999; Savvaidis *et al.*, 2002). *B. thermosphacta* and *Enterobacteriacea* have been reported to be part of spoilage micro flora of Mediterranean fish (Drosinos and Nychas, 1996; Koutsoumanis and Nychas, G.J. E., 1999.)

Banks *et al.* (1980); Brown *et al.* (1980); Finne (1982); Layrisse and Matches (1984); Lannelongue *et al.* (1982); Stenstrom (1985) reported that CO_2 has been shown to delay spoilage of fresh seafood by inhibiting psychrotrophic aerobic Gram negative bacteria. Daniel *et al.* (1985) reported that to achieve microbiological benefits the storage temperature of MAP products should be as low as possible since the solubility of CO_2 decreased with increased temperature. Clark and Lentz (1969); Christopher *et al.* (1979) reported that *Pseudomonas* spp. were effectively inhibited by atmospheres enriched with 20% or more CO_2. Molin and Stenstrom (1984) reported that Shewanella is more resistant to CO_2. Lopez Galvez *et al.* (1995; 1998) noted that the growth of this microorganism is inhibited by higher CO_2 concentrations. Gill and Molin (1991) attributed the high growth rate of lactic acid bacteria and *Shewanella putrefaciens* under 40% CO_2+30% O_2+30% N_2 to the tolerance of these microorganism to CO_2. Brown *et al.* (1980) noted an atmosphere of CO_2 inhibited microbial growth and extended

sensory acceptance of salmon fillets. Pastoriza *et al.* (1996) reported that significant differences ($P<0.05$) were found between control and MAP stored samples in terms of bacterial counts. Ozogul *et al.* (2000a) reported lowest counts in MAP stored herring compared to those in air-stored herring. Ozogul *et al.* (2004) observed significant differences ($P<0.05$) between samples kept in air and in MAP. In recent European studies, Dalgaard (1995); Gram and Huss (1996); Drosinos and Nychas (1996) suggested that *Photobacterium phosphoreum*, *Shewanella putrefaciens*, lactic acid bacteria and *Brochothrix thermosphacta*, bacteria resistant to carbon dioxide are important for the spoilage of fresh fish stored under either VP or MAP. In similar studies in Australia and the U.S.A. Oberlender *et al.* (1983); Statham *et al.* (1985); Wang and Orgydziak (1986) reported that lactic acid bacteria and *Alteromonas* are the dominant organism in fish stored under MAP conditions. Statham *et al.* (1985); and Fletcher *et al.* (1988) reported that *B.thermosphacta* were the dominant organism in morwong fish and scallops when these were treated with polyphosphates or potassium sorbate and then stored under 100% CO_2.

Most bacteria identified as specific spoilage bacteria produce one or several volatile sulphides. *Shewanella putrefaciens* and some Vibrionaceae produce H_2S from the sulphur containing amino acid L- cysteine (Gram *et al.*, 1987; Stenstrom and Molin, 1990). In contrast, neither *Pseudomonas* nor *P.phosphoreum* produce significant amounts of H_2S. Thus, hydrogen sulphide, which is typical of spoiling iced cod stored aerobically, is not detected in spoiling CO_2 packed cod (Dalgaard *et al.*, 1993). Methylmercaptan (CH_3SH) and dimethylsulphide

[$(CH_3)_2S$] are both formed from methionine (Herbert and Shewan, 1975). Taurine, which is also sulphur containing, occurs as free amino acid in very high concentrations in fish muscle and disappears from the fish flesh during storage but this is because of leakage rather than because of bacterial attack (Shewan and Jones, 1957).

2.5.5.3. *Clostridium botulinum*

Modified atmosphere packaging poses the threat of failure to detect presence of pathogens in product. Modified atmosphere packaging conditions create an environment, which supports the growth of psychrotrophic, anaerobic bacterial pathogens, including non-proteolytic types of *C. botulinum*. It is an anaerobic, rod shaped spore former that produces a protein with characteristic neurotoxicity. Botulism, a severe food poisioning, results from ingestion of food containing botulinal toxin produced during the growth of these organisms in food. Although this food poisoning is rare, the mortality rate is high and the 962 botulism outbreaks in the United States from 1899 to 1990 were recorded involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks the food implicated contained both types A and B toxins. The limited number of reports of C and D toxins as the causative agent of human botulism have not been generally accepted. However, all types except F and G, which have not been as thoroughly studied, are important causes of animal botulism.

Antigenic types of *C. botulinum* are identified by complete neutralization of their toxins by the homologous antitoxin; cross neutralization by heterologus antitoxins does not occur or is minimal. There are seven recognized antigenic types: A, B, C, D, E, F and G. Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern, which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic, with carbohydrate metabolic patterns differing from the C and D nonproteolytic groups. Strains that produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other seafoods. Types A and B are most commonly encountered in foods subjected to soil contamination. In the United States, home canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of food borne illness caused by these types.

Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinal spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not

contain viable *C. botulinum*. Home canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners great awareness and better control of the required heat treatment.

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms don't grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of NaCl, an inhibitory concentration of $NaNO_2$ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by nonproteolytic strains unless the temperature is precisely controlled and kept below 3.3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for nonproteolytic strains it is 26-28°C. Nonproteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the nonproteolytics don't manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in

epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulinal toxin in the blood, feces, or vomitus of the patient. Identifying the causative food is most important in preventing additional cases of botulism.

Botulism in infants 6 weeks to 1 year of age was first recognized as a distinct clinical entity in 1976. This form of botulism results from growth and toxin production by *C. botulinum* within the intestinal tract of infants rather than from ingestion of preformed toxin. It is usually caused by *C. botulinum* types A or B, but a few cases have been caused by other types.

Genigeorgis (1985); Baker and Genigeorgis (1990); Stammen *et al.* (1990); Reddy *et al.* (1992) have reviewed the risks from botulism in MAP fish. Post *et al.* (1985); Garcia and Genigeorgis (1987); Taylor *et al.* (1990) reported that toxin has been detected in MAP fish prior to the product being considered spoiled. Cann *et al.* (1984); Reddy *et al.* (1996; 1997a; b; 1999) and Cai *et al.* (1997) reported that MA or vacuum packed fish spoiled prior to or in coincidence with toxin production. Gibson *et al.* (2000) demonstrated that 100% CO_2 could have an inhibitory effect on the growth of *C.botulinum* at chill temperatures, and an inhibitory effect was observed when combining 100% CO_2 with increased NaCl level and decreased pH level. Reddy *et al.* (1999) reported the fat content might influence the margin of safety of MA packaged aqua cultured fresh fillets during storage. The safety margin being less for fattier fish (salmon, catfish) when compared with leaner fishes (tilapia, cod). Lilly and Kautter (1990) reported in a study of 1074 test samples of commercial, vacuum packaged fresh

fish none of the marginally organoleptic acceptable samples was positive for *C. botulinum* after 12 days at 12°C. The authors concluded that either the fish did not contain *C.botulinum* spores, or the spores were unable to grow out and produce toxin before the spoilage made the product marginally unacceptable. However, an increased use of modified atmosphere and other minimal processing technologies combined with improper cold chains and abuse of temperature may represent an increased risk of botulism.

2.6. Sensory evaluation

Organoleptic responses to fish in very high concentrations of CO_2 are not all favorable. Longer shelf life is obtained when measured via microbiological growth and the direct spoilage consequences but other adverse changes occur. The reduction of bound water, which leads to excessive exudates, is accompanied by a coarsening of the texture, which is described by taste panelists as slight increase in toughness and dryness (Tiffiney and Mills, 1982), grainy (Wang and Brown, 1983) and powdery (Haard and Lee, 1982). When packs of fish are first opened, odours which otherwise would have slowly dissipated are suddenly released. They are generally inoffensive if the product has been properly stored, but less so if abused (Cohen, 1981). With packs handled correctly there are subtle changes in odour, which have been analyzed, chemically, by Lindsay *et al.* (1986). Acidic (Stier *et al.*, 1981; Tomlins *et al.*, 1981) or effervescent sensations (Tiffiney and Mills, 1982) may be detected, and flavour may be similarly affected (Jensen *et al.*, 1980). Not only can effervescent effects be detected in the taste of products such as crabmeat

(Cann, 1984), which are not usually reheated, but appear sometimes to persist through cooking. The carbonated taste of salmon, reported by Haard and Lee (1982) seems very similar and may be a consequence of higher solubility in the lipids of a high lipid content species. Tiffiney and Mills (1982) reported another flavour effect associated with cod under high levels of CO_2. It was described by panelists as cold store flavour similar to that which develops in frozen fish.

The shifts in overall patterns of chemical change which MAP gas mixtures must induce if they are to be effective, appear to be insufficient to cause expert panelists any significant additional problems. Published results make little mention of unusual effects other than rancidity in fatty fish, which led to a shorter shelf life for Herring in Modified Atmosphere Packaging (with O_2 included) than in Vacuum packaging. Tiffiney and Mills (1982) did not describe the flavour consequences of packing mackerel and trout products in MAP but found that exclusion of O_2 extended the times taken to reach specified flavour scores. Some change in the balance of oxidative rancidity and microbiological spoilage flavors might be expected by packing under enhanced levels of O_2, and Davis (1990) showed that, in minced fish, oxidation was, indeed promoted by the inclusion of 30% O_2.

Detailed patterns of changes in sensory score of MAP fish vary. In a comparison of fish packed fresh and not so fresh, both had a slightly slower, fairly uniform rate of spoilage over the main edible period (Tiffiney and Mills, 1982). For the fresh fish there followed a long plateau, which markedly delayed the onset of

gross spoilage; this contrasts with the inhibition seen in the earlier stages by Cann (1983).

The literature reveals a great variability within and between species which is to be expected from the differences between species, batches, treatments, experimental methods, attributes evaluated and, especially the end point criteria employed. The main source of variation between batches is probably the history of the fish between catching and packing. There is broad agreement that the use of MAP is only worthwhile when applied to fish, which is fresh. An exception was the suggestion by Regenstein and Regenstein (1981), that treatment with CO_2 and or/ sorbate may be more effective once spoilage organisms have reached early logarithmic growth phase. Aside from some discussion of the relevance to inhibition of botulism (Lindsay *et al.*, 1987), this delayed pack hypothesis has gained little credence. Nevertheless, some authors have acknowledged the use of relatively stale fish in their storage experiments, while many have not known, or have failed to define, the initial quality of their raw material.

Acceptance by consumers requires the appearance of a food product to inspire confidence that it will prove satisfactory when purchased and eaten. Thus, the important sensory properties fall into two categories. The physical effects (of both the package and the fish product) which can be assessed visually, including pack collapse and production of exudates and discoloration; and the organoleptic properties of odour, flavour and texture. Generally the quality deterioration of fresh fish is characterized organoleptically by an initial loss of fresh fish flavour

(sweet, seaweedy). After a period where the odour and flavour is described as neutral or non-specific, the first indications of off-odours and flavors are detectable. These will progressively become more pronounced and lead to rejection of the fish. The time to spoilage depends mainly on storage temperature and fish species.

The off odours and flavours developing in fish stored in air depend on the fish species and origin of fish. The spoilage of marine temperate water fish is characterized sensorically by development of offensive fishy, rotten, H_2S off odours and flavours. This sensory impression is distinctly different for some tropical fish and freshwater fish, where fruity, sulphydryl off odours and flavours are more typical (Lima dos Santos, 1978; Gram *et al.*, 1989).

2.7. Factors influencing shelf life of fish packaged under modified atmospheres

Several interrelated factors influence the shelf life and keeping quality of fish packaged under CO_2 enriched atmospheres. These include (a) concentration of CO_2 in gas mixture, (b) packaging film permeability, (c) storage temperature and (d) types and numbers of microbial contaminants.

2.7.1. Concentration of gases

Concentration of gases is a very important factor for shelf life of fish packaged under MAP conditions. The bacteriostatic effect MAP storage increases with increasing CO_2 concentrations (King and Nagel, 1967; Enfors and Molin, 1980; Gill and Tan, 1980). With cod fillets stored in different concentrations of CO_2 and O_2/N_2, Stenstrom (1985) showed that the shelf life increased as the CO_2

concentration increased. Haines (1932) reported that concentrations as low as 10 to 20% CO_2 were sufficient to inhibit the growth of *Pseudomonas* and *Achromobacter* species. High CO_2 concentrations might be used for many types of seafood with little risk of heme protein discoloration because of the low pigment concentration (Parkin and Brown, 1982). It has long been known that carbon dioxide has an inhibitory effect on many common spoilage organisms. Coyne (1933) conducted experiments with several spoilage organisms and showed that carbon dioxide inhibits some types of microorganisms but has no direct effect on others. Most non-sterile food products contain a variety of organisms, and those organisms favored by environmental conditions may grow. In MAP, the inhibition of gram negative, rod shaped spoilage organisms such as *Pseudomonas* coincides with the growth of Gram positive, rod shaped spoilage organisms such as lactic acid producing organisms such as Lactobacillus. Carbon dioxide in sufficient concentrations can inhibit the growth of spoilage molds (Brown, 1922; Moran *et al.,* 1932; Tomkins, 1932; Hintlian and Hotchkiss, 1986). Shewn (1949) recommended concentrations of between 30 and 40% CO_2 atmospheres and reported superior quality for fish stored in the higher concentration of CO_2. Tarr (1954) recommended a minimum of 40 to 50% CO_2 in the package headspace to derive maximum benefit of CO_2 storage of fresh fish. Coyne (1933), in one of the original studies on use of carbon dioxide atmospheres to prolong fish quality, recommended concentrations of CO_2 of 40 and 60% CO_2. Based on these studies, approximately 50-60% CO_2 in the package headspace is commonly used, with the remainder comprising of a

mixture of O_2 and N_2. Several studies have shown that carbon dioxide inhibits some types of microorganisms but has no direct effect on others. Most non-sterile food products contain a variety of organisms, and those organisms favored by environmental conditions grow. In MAP conditions, the inhibition of gram negative, rod shaped spoilage organisms such as *Pseudomonas* coincides with the growth of gram positive lactic acid producing organisms such as Lactobacillus. The pioneering work of Coyne (1932, 1933) demonstrated that high levels of CO_2 (20-100%) would inhibit microbial growth and extend the shelf life of fish. His findings substantiated earlier studies by Killefer (1930) who found that similar concentrations of CO_2 could be used to extend the shelf life of cod. Stansby and Griffiths (1935) demonstrated that whole Haddock stored in CO_2 enriched atmosphere kept approximately twice as long as fish stored in air. Later, based on these studies, Shewan (1949) suggested that the use of carbon dioxide showed sufficient promise to warrant further investigation for shelf life extension of fish. He suggested that the best percentage of CO_2, while not known with certainty, was probably 30-40%.

2.7.2. Packaging film permeability

Modified atmosphere packaging can only be successful if used in conjunction with packaging materials of correct O_2/CO_2 permeability characteristics. It is no use having the correct atmosphere if the film allows the atmosphere to change too rapidly. The properties required for a suitable packaging film are seldom formed in one polymer and individual polymers are laminated to produce films of superior barrier properties. Examples of polymers used in construction of barrier

films are: Polyethylene, Polypropylene, Polyvinylidene chloride (PVDC), Ethylene vinylacetate (EVA), Ethylene vinyl alcohol (EVOH), and Metallised polyesters. Examples of high, medium and low barrier films are shown in Table 2

Table 2. Oxygen permeation rates for packaging films expressed in different units*

Film	cc per 100 in^2 /day	cc per m^2 /day mm Hg	cc per m^2 /day
PP/EVOH/PP	<0.001	<0.00001	0.01-0.02
Foil laminate (mylar/AL/poly)	<0.01	<0.00001	<0.01-0.1
PVDC	1	0.02	15
Acrylonitrile Polymer	1	0.02	15
Brickpak flat	2-3	0.04-0.05	30-40
PET (polyester)	4-6	0.08-0.13	60-100
PVC	10	0.2	150
Brickpat(folded and scored)	100	2	1500
HDPE	130	2.6	1980
PP	150	3.0	2280
LDPE	400-500	8-10	6000-7000

*For 1 mil flat (unless composite laminate) at 30°C and 50% RH

2.7.3. Storage temperature

Modified atmosphere packaging (MAP) is not a substitute for proper storage temperature (Ogrydziak and Brown, 1982). The effectiveness of MAP is decreased as the storage temperature increases due to the fact that the solubility of CO_2 also decreases at higher temperatures. For respiring products, increasing the temperature also increases the rate of respiration, resulting in a decrease in shelf life. The effects of temperature abuse are particularly important from the standpoint of safety. Temperature abuse of MAP muscle foods may result in the rapid growth of both spoilage and pathogenic bacteria. The minimum reported temperature for *Salmonella* and *Escherichia coli* inoculated in ground meat grew equally well at 12.5 °C when the meat was packaged in low and high permeability film (Goepfert and Kim, 1975). *Staphylococcus aureus* can grow and produce enterotoxin under anaerobic conditions at 10°C or less. The minimun-recorded temperature for enterotoxin production under anaerobic conditions is 10°C, while *Yersinia enterocolitica* has been reported to grow at temperatures as low as –2°C. Of major concern, with respect to safety of MAP fish, is the growth and toxin production by *Clostridium botulinum* type E that is capable of growth at 3.3 °C (Palumbo, 1986). Proper refrigeration is therefore essential in order to assure the effectiveness of CO_2 as an anti microbial agent and to prevent potential growth of pathogenic organisms.

2.7.4. Levels of microbial contamination

The shelf life extension of fish under modified atmospheres is dependent on the initial microbial load and the types of organisms. Higher initial counts will accelerate spoilage and shorten the shelf life of fish products. Furthermore, high numbers of aerobic bacteria will also consume rapidly headspace O_2 and may change electronegative potential of product and enhance the growth of anaerobic organism such as *Clostridium botulinum* type E.

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

Fresh Pearl spot (*Etroplus suratensis Bloch)* procured from Fort Cochin market were brought to the laboratory in iced condition.

3.1.2. Thermoformed Trays

High Density Polyethylene thermoformed trays imported from Dynopack Industries, Norway, were used for the study. The tray has got a capacity of 260 ml. with dimension of 540 X 400 X 430 mm. and thickness of 0.5 mm. The tray has got good barrier properties towards water vapour, O_2 and CO_2. Physical properties of thermoformed tray used are presented in Table 4

3.1.3. Flexible packaging material for sealing the trays

12 μ polyester laminated with 300-gauge polyethylene was used for sealing the top of the tray. Physical properties of this packaging material used are presented in Table 5

3.1.4. Gases.

Food grade CO_2, O_2 and N_2 gases were used for the study. They were purchased from Manorama gas Ltd. Kizhakambalam. Kerala.

3.1.5. Chemicals

Chemicals used for the experiments were of Analar grade of Merck and Sigma brand.

3.2. Methods

3.2.1. Packaging material Testing

3.2.1.1. Determination of Tensile Strength and Elongation at break: (IS: 2508-1984)

Tensile strength has been defined as the force parallel to the plane of the specimen required to produce failure in a specimen of specified width and length under specified condition of loading.

Apparatus: Tensile Strength Machine:

The machine used should be able to maintain a constant rate of traverse of one grip. The load scale should be accurate to within 1% or 0.1 N whichever is less. The load range should be such that the breaking load of the test pieces should fall between 15% and 85% of the full scale reading.

Preparation of samples:

Gauge length of the Specimens:

Plastics - 50±1mm length x 15mm width

Traverse speed of machine: Plastics - 500 mm/min.

Samples were cut in lengthwise and crosswise direction, five numbers each. Total length was 50mm longer than the gauge length. The thickness was measured using a screw gauge.

The conditioned specimen was clamped between the grips of the machine. Machine was then switched on at the pre adjusted speed. The load and elongation at break were recorded.

Calculation: -

The tensile strength at break calculated in Kg/cm^2 from the original area of cross section.

i.e., Kgf/cross section area in cm^2. The mean of five results was expressed for the lengthwise and crosswise samples (MD and CD).

Cross Section area = width x thickness in cm.

Elongation at break was expressed as percentage of the original length between the reference lines. The mean value of the five results is expressed from MD & CD samples.

$$\% \text{ E-B} = \frac{(L_2 - L_1) \times 100}{L_1}$$

Usually Tensile Strength is more in MD and Elongation is less in MD.

3.2.1.2. Determination of Water Vapour Transmission Rate (ASTM E96-66):

This is an important property of the packaging material under 3 mm thickness, to be considered in the selection of barrier materials for hygroscopic foods. It is measured as the quantity of water vapour in gms that will transmit from one side to the other of the film of an area of one sq. meter in 24 hrs. when the

relative humidity difference between the two sides is maintained at 90±2% at 37°C.

Apparatus: Test Dishes.

Shallow aluminium dishes of as large a diameter as a can were used. A wax seal between the test piece and the dish was given so as to prevent the transmission of water vapour at or through the edges of the sheet.

Method:

WVTR was determined by sealing the open end of the dish containing the desiccant (fused Calcium Chloride) by the test specimen and exposing the dish to the desired RH and temperature conditions. For standard test this condition is 37°C and 92% RH, when the desiccant used exerts 2% RH. Increase in weight of the desiccant after a known period of time gives the amount of water vapour transmitted by the specimen.

$$WVTR = \frac{Qx24x90}{A\,t\,(H_1-H_2)} \quad g/m^2/24hrs. \text{ at } 90\pm2\% \text{ RH \& } 37°C.$$

Q - quantity of water vapour pass through the test material of area **A** sq.meter for **t** hours when the relative humidities on either side maintained at H_1 and H_2.

94% RH - Saturated solution of Potassium Nitrate.

Sealing Wax - Combination of microcrystalline wax and paraffin wax in 60:40 ratio.

2% RH - Fused Calcium Chloride or Magnesium perchlorate.

Area of test specimen - 50 cm^2.

3.2.1.3. Determination of Gas Transmission Rate: (ASTM (1975) D 1434 & BS 2782 method 514 A procedure 2)

The permeability of plastic films by gases is described as the volumetric rate of transmission of the gas, under known pressure differential, through a known area of film and is usually expressed as the transmission rate in ml per square meter per 24 hrs per atmosphere ($ml/m^2/24$ hrs. atmos). The permeability of plastic materials to different gases is of considerable significance in many applications. It can often be desirable to achieve a certain degree of permeability to certain gases, rather than to produce an entirely impermeable pack.

The phenomenon of gas permeability is dependent on the physical nature of the film, its density, degree of crystallinity and thickness and on the other the size and mobility of the gas molecules. The degree of polarity of both plastic materials and gas molecules as well as their tendency to be either hydrophobic or hydrophilic do influence the permeability of films with respect to particular gases.

Apparatus: Gas Permeability Apparatus (Davenport - designed in general accordance with B.S.2782, method 514A, Procedure 2 and ASTM D 1434)

Procedure:

Unscrewed the bolts holding down the upper half of the permeability cell and removed it. As supplied, the apparatus will have the 'X' volume controlling insert correctly fitted in the lower half of the cell. A dried circular filter paper

(Whatmann No.1) was placed on the top of the insert and the sample of film spread over the filter paper. The upper part of the film permeability cell was then replaced. The bolts were then reinserted and tightened up with the box spanner.

The test gas was now turned on and the cell 'flushed out ' with a brisk stream of gas for a few seconds, after the flow may be reduced to a slow rate, to ensure that no air can diffuse back in to the cell (1 bubble/second through liquid paraffin). The lower part of the cell was then evacuated (using vacuum pump capable of giving a vacuum at least as low as 0.2 mm Hg. A vacuum gauge also be connected between the apparatus and the vacuum pump- Tipping Mc Leod gauge) as rapid as possible and as soon as the gauge indicates that the pressure was 0.2 mm Hg or lower. The apparatus was tilted to the left until the mercury runs out of the reservoir into the manometer, partially filling it. Returned the apparatus to the normal position and immediately set the movable scale to a convenient starting point, started a stopwatch and began to take readings, at suitable time intervals. Repeated the test with another samples.

Calculation:

$$GTR = \frac{273xpVx24x10^4}{A \times T \times P}$$

where,

GTR = Gas transmission rate in $ml/m^2/24$ hrs at 1 atmosphere pressure difference.

P = Rate of pressure change in capillary in cm. Hg per hour.

V = Total volume in ml of the space between the lower

surface of the film and the top of the mercury column in the capillary.

This total volume expressed as,

(a) The volume of cell cavity (i.e. 5,10,15 or 20)

(b) The volume of capillary tube above the mercury level half way through test; as the area of cross section of the capillary is 0.018 cm^2, this volume will be 0.018 X, when X is the length of the capillary above the mercury at the half way point in cm.

(c) The 'free space' volume of filter paper - can be taken as 0.24 ml.

A = Area of the specimen - 23.77cm^2

T = Temp. in °K (273+°C)

P = Pressure difference (76cm Hg)

i.e. = $\frac{273 \times pV \times 24 \times 10^4}{23.77 \times 76\ (273+^{o}C)}$

3.2.1.4. Determination of Overall Migration Residue:

Migration is mass transfer of materials from plastics to foods under specified conditions. Migrants are materials thus transferred from plastics to food. In order to assess the toxic effects of the plastic packaging materials, the specification laid by various countries prescribe short-term extraction test called the global migration tests for quantifying the migrants. It is difficult to estimate quantitatively the amount of migrants in actual foodstuffs because of the complex nature of the food and diverse food categories. Accordingly, global migration tests, measure gravimetrically the amount of migrants from plastics to different food Simulating Solvents (FSS), which are based on the Categorisation of foods

Food Categorisation: - As per US FDA 176,170

I	Non-acid, aqueous products, may contain salt or sugar or both(above pH 5)
II	Acid, aqueous products, may contain salt or sugar or both including oil-in-water emulsions of low or high fat content (pH below 5)
III	Aqueous, acid at non-acid products containing free oil or fat, may contain salt and including water-in-oil emulsions of low or high fat content.
IV	Dairy products and modifications A. Water-in-oil emulsions, high or low fat B. Oil-in-water emulsions, high or low fat.
V	Low moisture fats and oils.
VI	Beverages: A. Containing upto 8 % alcohol B. Non-alcoholic C. Containing more than 8 % alcohol
VII	Bakery products A. Moist bakery products with surface containing free fat or oil B. Moist bakery products with surface containing no free fat or oil
VIII	Dry solids with the surface containing no free fat or oil (no end test required)
IX	Dry solids with the surface containing free fat or oil

Method :

Filled the tray to their filled capacity with distilled water at test temperature (40°C). Exposed to 21.1°C maintained for 48 h. After exposure for the specified duration, removed the tray and quickly transferred the distilled water into clean glass beaker with three washing with distileed water. Evaporated the extractant to about 50-60 ml and transferred into a clean tared stainless steel dish along with three washings and further evaporated to dryness in an oven at 100°C. Cooled this in a dessicator for 30 minutes and weighed. Calculate the extractives in milligrams/dm^2 and mg/litre.

$$\text{OMR} = \frac{\text{Mass of residue in mg X 100 mg/dm}^2}{\text{(A) Area exposed in cm}^2}$$

or

$$= \frac{\text{Mass of Residue in mg X 1000}}{\text{(V) Volume of simulant in ml.}}$$ mg/litre (ppm)

Maximum limit value = 60 mg/litre or 10 mg/dm^2

3.2.2. Modified Atmosphere Packaging (MAP) – Standardization using different gas mixtures.

Modified atmosphere packaging was done using a Dynopack 500VG machine imported from Norway. The two-line display incorporated in the front face of the DYNO 500 VG enabled the user to visualize the sequence of the machine cycle and to adjust its parameters. Machine cycle visualization and cycle parameters modification were possible with this instrument. Machine operation

included Vacuum adjusting, Gas setting, Gas injecting and sealing. Gas setting involves controlling the level of reinjection by the terminal, setting the contact vacuum gauge threshold to 0, setting the reinjection time to 0 on the terminal and setting the required reinjection value on the contact vacuum gauge by moving the transparent needle to the corresponding graduation, setting the gas inlet pressure between 1.5 and 3 bars and loading the top film. With this machine four trays can be gas filled at a time. Fresh Pearl spot (*Etroplus suratensis*) procured were dressed and gutted and brought to the laboratory in iced condition. They were washed thoroughly with chilled water and a dip treatment in 2 ppm chlorine water was given for 10 minutes, before packing into trays.

For standardization of different gases, Pearl spot were packed in thermoformed HDPE trays. Fish weighing approximately 100 g packed in trays were divided in to 6 lots and the following mixtures of gases were introduced. I = Control pack, II= 40% CO_2 + 60% O_2 , III = 50% CO_2 + 50% O_2 , IV = 60% CO_2 + 40% O_2 , V = 70% CO_2 + 30% O_2 , VI = 40% CO_2 + 30% O_2 + 30% N_2 mixture. In all these cases fish: gas ratio was 1:2 (w/v). These trays were immediately transferred to insulated boxes with proper icing, (fish: ice ratio 1:1). The box was kept in a chill room, where the temperature was maintained at 0-2°C, till the end of storage. Sampling was done in triplicate and the mean value was taken as the average. Ideal gas mixture was selected based on sensory evaluation.

Modified Atmosphere Packaging of fish in thermoformed trays were carried out using the ideal gas mixture i.e. 60% CO_2 +40% O_2 . Fish to gas ratio was 1:2 (weight: volume). Approximately 100 g of fish were packed in the tray using Modified Atmosphere Packaging Machine (Dynopack 500 VG, Norway). Top portion of the tray was heat-sealed using 12 μ polyester laminated with 300-gague polyethylene. The filled trays were immediately placed in insulated boxes with proper icing (fish: ice ratio 1:1). The boxes were kept in a chill room, where the temperature was maintained at 0-2°C, till the end of storage period. Sampling was done in triplicate and the average value was recorded. Control batches in air i.e. without gas mixture, were also evaluated for comparison purposes.

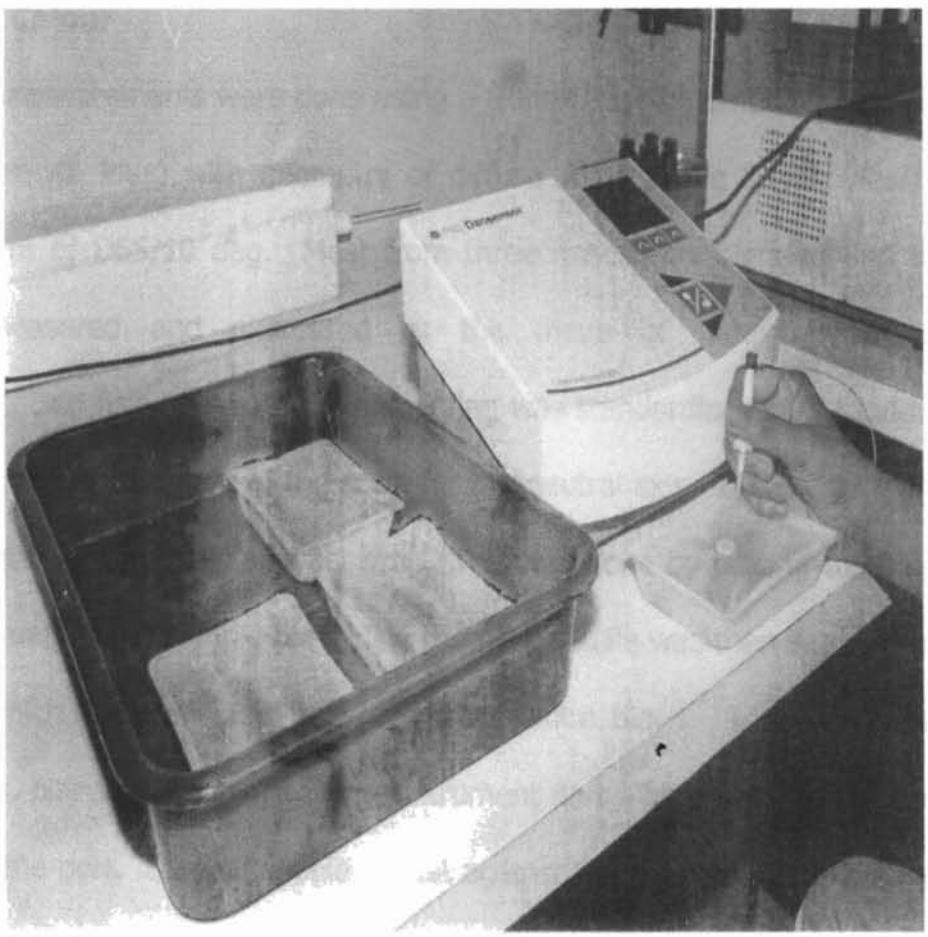
Plate 1: Modified Atmosphere Packaging Equipment

3.2.3. Gas Analysis.

Gas analysis was carried out in triplicate packs using a Gas analyzer (PBI Dansensor, Checkmate 9900 O_2 / CO_2, DK-4100, Ringsted, Denmark.) on each sampling day by inserting a gas tight pressure lock syringe inserted through an adhesive silicone septum attached to the outside of the package. The septum keeps the package airtight. First a calibration was done measuring on atmospheric air and a 20.9% O_2 calibration is necessary. After calibration, measurement was done on the sample packs as follows.

- Removed the protective film from the septum
- Affixed the septum to the package at a place with free space underneath.
- Pierced the needle through the septum and the packaging film.
- Pressed the button to start the spot test measuring. A time bar indicated the measuring time.
- The lowest or highest measured peak value, different from 20.9%, is displayed while measuring.
- When the O_2 time bar is filled, the O_2 and CO_2 measurements are finished and the needle can be put back into the needle retainer.
- The O_2 and CO_2 were displayed and the balance was considered as N_2.

Plate 2: Gas Analyzer



3.2.4. Thaw drip.

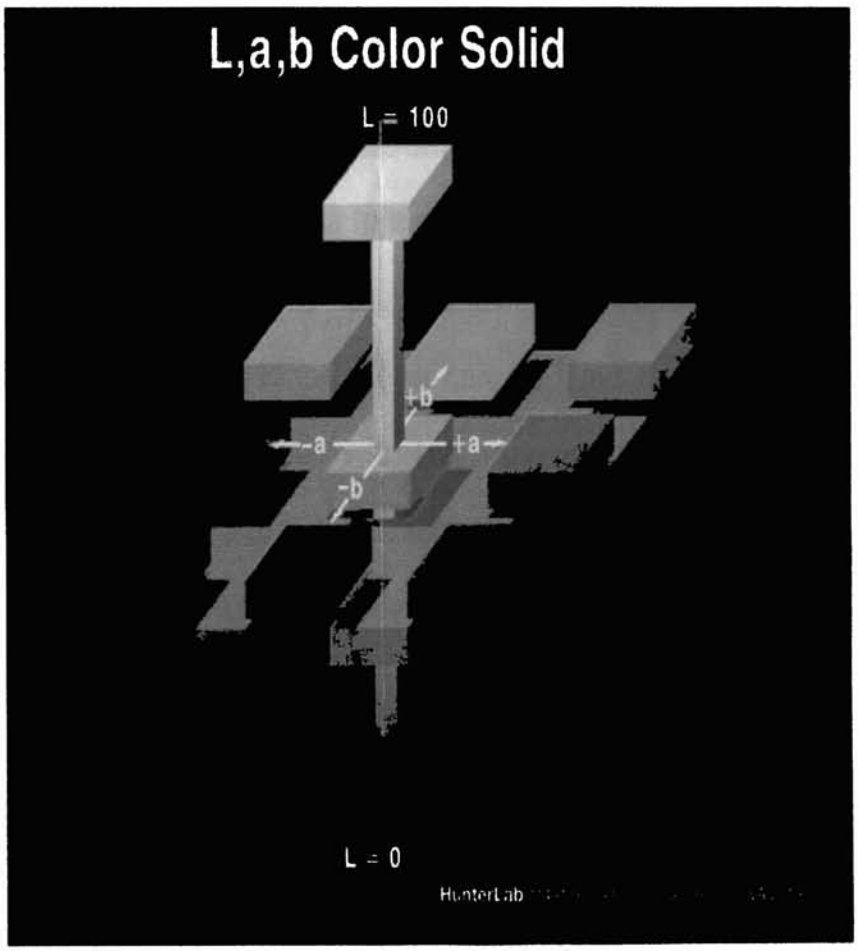
Drip loss was measured by transferring exudates from samples from each batch in to a 10 ml measuring cylinder. Fish were weighed and drip loss was calculated as percentage (w/w)

3.2.5. Colour

Colour measurements were done using a Hunter lab Colorimeter Model No D/8-S (Miniscan XE Plus) with geometry of diffuse /8° (sphere 8 mm view) and an illuminant of D65/10 deg. Meat from three fishes was blended and the color was measured and expressed as the mean of measurements of the homogenized fish. First step in measuring was standardization. Standardization sets the top and bottom of the scale for the neutral axis. During standardization the bottom of the scale was set first. This was done by placing the black glass or light trap at the sample port. The top of the scale was then set by scaling the light, which was reflected to a calibrated white tile. In order to measure samples, placed the sample at the instrument port with the side to be measured toward the port. Sample should be flat against the port and completely covered it. When the read key is pressed the sample will be measured and its values saved in the instrument. In the Hunter scale, L* measures lightness and varies from 100 for perfect white to zero for black, approximately as the eye would evaluate it. The chromacity dimensions (a and b) give understandable designations of color as follows: a* measures redness when positive, gray when zero, and greenness when negative. b* measures yellowness when positive, gray when zero, and blueness when negative.

Plate 3: Hunter lab Colorimeter (Mini Scan XE Plus)

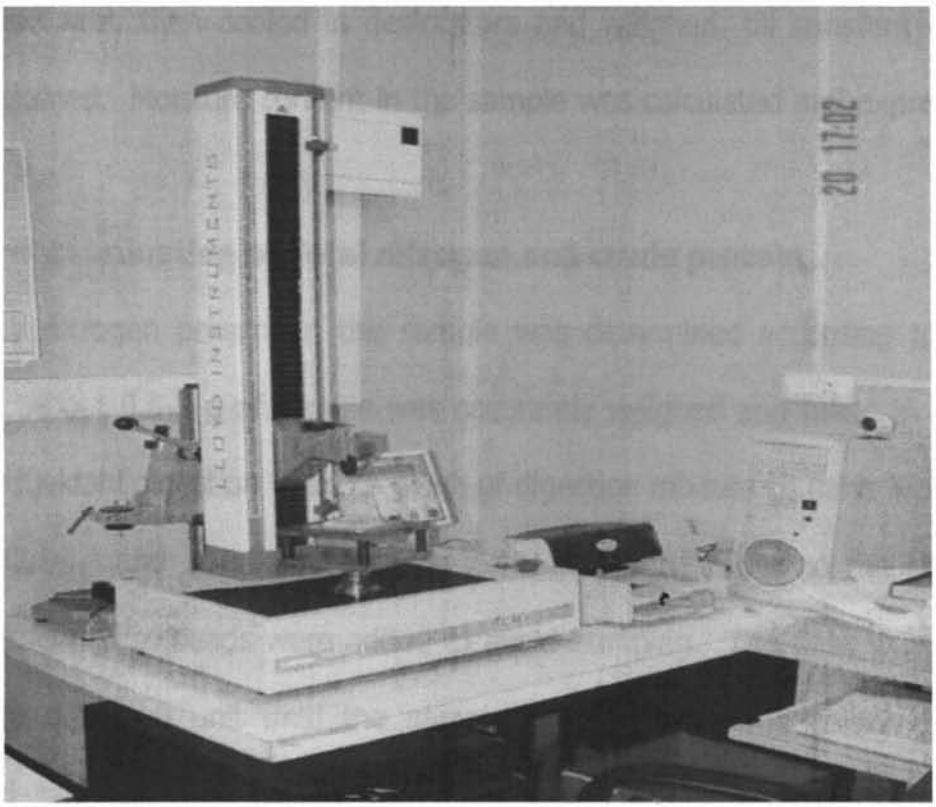
Hunter color lab scale



3.2.6. Texture.

Instrumental Texture Profile Analysis was carried out using a Food texture Analyzer (Lloyd Instruments, Model LRX Plus). It was a general-purpose material-testing machine manufactured by Lloyd instruments, UK (Model LRX plus). The software used in the instrument was Nexygen. When used with Nexygen software, data output was to a computer display and printer. The main part of the instrument was a load cell. Standard cells were there with values of 5000 N, 500 N and 50 N and each one can be used depending on the type. The LRX plus machine was fitted with two magnetically activating limit stops. Reaching magnetically activated limit stop will result in the machine stopping. The speed of the cross edge movement varies from 0.01-1016 mm/min. The unit has a liquid crystal display (LCD) to show set up information, load and extension values and a key pad to input information for operating the machine when under the control of the console. The display, which has 4 lines of forty characters, was used to show or request information. The test was done at a speed of 12 mm/min. using a 50 N load cell. The probe used for the experiment was a 50 mm diameter cylindrical probe. Uniform samples were allowed for a compression of 40% with a trigger force of 0.5 kg. From the double compressions, parameters such as hardness 1, hardness 2, Cohesiveness, Springiness, and Chewiness was determined. Texture Profile Analysis results was tabulated using Nexygen Software.

Plate 4: Texture Analyzer



3.3. Biochemical Analysis

Proximate Composition

3.3.1. Determination of moisture.

Moisture content of the meat was determined according to AOAC (2000). About 10 g of minced fish meat was weighed into a clean and dry, pre-weighed petridish. The dishes were kept open in hot air oven at 100±1°C for 16-18 h. The same were then cooled in desiccators and weighed, till constant weights were obtained. Moisture content in the sample was calculated and expressed in percentage.

3.3.2. Determination of Total nitrogen and crude protein

The total nitrogen present in the sample was determined according to AOAC (2000). About 0.5-1 g of sample was accurately weighed and taken in a clean and dry Kjeldahl digestion flask. A pinch of digestion mixture (8 parts K_2SO_4 and 1 part $CuSO_4$) and 10 ml of concentrated H_2SO_4 were added to the digestion flask. A few glass beads were added to avoid bumping. The flask was heated on micro digestion unit until the sample became clear and colorless. The digested samples were cooled and made upto 100 ml with distilled water. Distillation was done in a microkjeldahl distillation unit. The distillate was collected in 10 ml of 2% boric acid solution and the nitrogen content in the sample was estimated by titrating against 0.01 N standard sulphuric acid. The crude protein in the sample was obtained by multiplying total nitrogen content in the sample with the conversion factor 6.25

3.3.3. Determination of Ash

Determination of ash content of the fish meat was done according to AOAC (2000). About 2 g of the moisture free sample was weighed into a porcelain crucible and was heated over a low flame till all the materials got completely charred. The charred sample was heated in a muffle furnace for about 3-5 h at 600°C until the ash turned white or grayish white in colour. The sample was cooled, weighed and the ash content was calculated and expressed as percentage.

3.3.4. Estimation of crude fat

About 2-3 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and was extracted with petroleum ether (40-60°C boiling point) in a soxhlet apparatus for about 10 h at a condensation rate of 5-6 drops per sec. Excess solvent was evaporated and the fat was dried at 100°C to constant weight. The crude fat was calculated and expressed as percentage.

Shelf life Studies.

Fish stored at 0-2°C in air and MAP conditions were periodically analyzed for sensory, biochemical changes like pH, Thiobarbituric acid value (TBA value), Tri methylamine content (TMA), Total volatile nitrogen content (TVN), K value analysis and microbiological analysis.

3.3.5. TBA value

TBA value was determined according to the method of Tarladgis *et al* (1960). About 10g of fish meat was mixed with 100 ml. 0.2 N HCl and homogenized to make slurry. Slurry was poured into a round bottom flask and connected to the

TBA distillation apparatus. Distillation was done until 50 ml. of the distillate was collected within 10 minutes. 5 ml of the distillate was taken in a test tube; 5 ml TBA reagent was added and heated for 35 minutes. A blank was also done with distilled water. Colour developed was measured in a spectrophotometer at 538 nm and TBA value was calculated and expressed in mg malonaldehyde/kg of fish sample.

3.3.6. pH

About 10 g of the sample was homogenized with 20 ml of distilled water and the pH was recorded using a digital pH meter (Cyber scan 510 model).

3.3.7. Preparation of Trichloro acetic acid (TCA) extract

About 10 g of accurately weighed sample was extracted with 10% trichloroacetic acid (TCA) by grinding in a mortar and pestle, then the content was filtered quantitatively through Whatman Filter paper No.1. Filter paper was thoroughly washed with TCA and filtrate was made up to 100 ml. The TCA extract was used to measure Trimethyl amine and Total Volatile base nitrogen of fish.

3.3.8. Determination of Total Volatile Base Nitrogen (TVB-N)

Determination of total volatile base in the sample was determined as total volatile base nitrogen (TVB-N) by the micro diffusion method (Conway, 1962). 1 ml of standard N/100 sulphuric acid was taken in the inner chamber of the diffusion unit. To the outer chamber 1 ml of TCA extract was added followed by 1 ml of saturated potassium carbonate. The unit was then sealed with the glass lid and kept undisturbed overnight. The amount of unreacted acid in the inner chamber was determined by titrating against standard N/200 sodium hydroxide

with Tashiros indicator. Similarly a blank was also run. TVB-N was calculated and expressed in mg/ 100g of the sample.

3.3.9. Determination of Trimethyl amine (TMA-N)

TMA was determined as trimethyl amine nitrogen (TMA-N) by microdiffusion method (Conway, 1962). 1 ml of standard N/100 sulphuric acid was taken in the inner chamber of the diffusion unit. To the outer chamber 1 ml of TCA extract was taken followed by 1 ml neutralized formaldehyde. This was kept as such for 3 min. to ensure that formaldehyde had bound with all the primary and secondary amines and ammonia contained in the extract. The analysis was further proceeded as explained in TVB-N determination. TMA-N was calculated and expressed in mg/ 100g of the sample.

3.3.10. K value analysis

K values were determined from perchloric acid extract of fish meat by a method of Ryder (1985). Nucleotide analysis was performed with a high performance liquid chromatographic method similar to that reported by Ryder (1985). A Merck system was used, with a bonda packed C18 stainless steel column. Extraction of the nucleotide from muscle was done using 0.6 M perchloric acid at 0°C and neutralized using 1M KOH. It was then filtered through a Millipore (0.45 μm) syringe filter. Nucleotide standards and Potassium phosphate were obtained from Sigma Chemical Company. The mobile phase was comprised of 0.06 M Di Potassium hydrogen phosphate and 0.04 M Potassium di hydrogen phosphate at pH 6.5-6.8. The buffer solutions were prepared everyday in glass double distilled water and filtered through a Millipore filter (0.45 μm). The flow

rate was 1 ml/min. and the eluate was monitored at 254 nm. The detector response for each of the six nucleotides found in fish muscle was calibrated daily by injecting 20 μl of 0.166 mM solution of each reference compound. All solutions were passed through a 0.45 μm aqueous filter before injection on to the column. The K value was computed from the results as defined by Saito *et al.* (1959).

3.4. Microbiological Analysis.

3.4.1. Sample preparation

From each fish, a sample of 10 g was diluted 10 fold in saline (0.85% NaCl) and homogenized for 60 seconds in a stomacher (Lab Blender 400; Seward Medical, London, U.K.). The homogenates were further diluted in saline for microbiological analysis.

In all experiments, counts of total mesophilic bacteria, total psychrotrophic bacteria, *Pseudomonas*, H_2S producing bacteria, *B.thermosphacta*, were determined as spread plate counts in respective agar medium pre dried at 56°C for 1 hour.

In this experiment, *Enterobacteriacea*, *Staphylococcus aureus* and Feacal *streptococci* were determined initially and at the time of rejection. In all experiments, anaerobic bacteria, *Enterobacteriacea* and *Staphylococcus aureus* were determined as pour plate counts in respective agar medium.

3.4.2. Microbiological media and enumeration

3.4.2.1. Total viable mesophiliic bacteria

Total viable mesophiliic bacterial counts were determined using plate count agar (PCA, Merck). 0.5 ml of the sample was spread plated into 20 ml of pre dried (56°C for 1 hour) agar in sterile petri dish and incubated for 48 h at 37°C.

3.4.2.2. Total Psychrotrophic bacteria

Total Psychrotrophic bacterial counts were determined using plate count agar (PCA, Merck). 0.5 ml of the sample was spread plated into 20 ml of pre dried (56°C for 1 hour) agar in sterile petri dish and incubated for 48 h at 7°C.

3.4.2.3. *Pseudomonas*

Pseudomonas was enumerated on cetrimide fusidin cephaloridine agar (CFC, Oxoid code CM 559, supplemented with SR 103). The agar pH should be between 6.8 to 7.0. 0.5 ml of the sample was spread plated into 20 ml of pre dried (56°C for 1 hour) agar in sterile petri dish and incubated at 20°C for 2 days (Mead and Adams, 1977). To the incubated plates cytochrome oxidase reagent was added and the colonies, which retain the blue colour of the cytochrome oxidase, were counted as *pseudomonas*.

3.4.2.4. *Brochothrix thermosphacta*

Brochothrix thermosphacta was enumerated on streptomycin sulphate (500 mg/l) (Oxoid), thallous acetate (50 mg/l) (Oxoid), cycloheximide (actidione) (50 mg/l) (Oxoid) agar (STAA, Oxoid code CM 881 supplemented with SR 151). 0.5 ml of the sample was spread plated into 20 ml of pre dried (56°C for 1 hour) agar in sterile petri dish and incubated at 20°C for 3 days (Gardner, 1966). To the incubated plates cytochrome oxidase reagent was added and the pinpoint

white colonies, which were cytochrome oxidase negative, were counted as *Brochothrix thermosphacta.*

3.4.2.5. Lactic acid bacteria

Lactic acid bacteria were enumerated on MRS (De Man Rogosa and Sharpe Agar with sorbic acid) agar (Lactobacillus, MRS agar, Oxoid code CM 361) supplemented with 2% potassium sorbate. The agar pH should be 5.8 to 6.0. 0.5 ml of the sample was spread plated into 20 ml of pre dried (56°C for 1 hour) agar in sterile petri dish, after setting an overlay of the medium was added and incubated in an anaerobic jar at room temperature for 5 days. White colonies were counted as Lactic Acid Bacteria.

3.4.2.6. Hydrogen sulphide producing bacteria

Hydrogen sulphide producing bacteria was enumerated on Iron agar (IA, Oxoid code CM 867) supplemented with 2 ml of 2% L-cysteine hydrochloride and 2.0 ml of Ferric Ammonium Citrate and 0.2 ml of sterile sodium hydroxide solution to adjust the pH to 7.0 were added in each 100 ml Iron agar medium. 1.0 ml was inoculated into 10 ml of molten (45°C) iron agar. After setting, an overlay of molten medium was added. Iron agar plates were incubated at 20°C for 4 days (Gram *et al.,* 1987). Black colonies formed by the production of H_2S were enumerated after 2-3 days (Gennari and Campanini, 1991).

3.4.2.7. Sulphite reducing clostridia

Sulphite reducing clostridia was enumerated on TSC agar supplemented with D cycloserine (0.1 g in 10 ml) (each 100 ml agar supplemented with 4 ml of D cycloserine). 1 ml was pour plated into 20 ml of molten (45°C) TSC agar, after

setting an overlay of molten medium was added and incubated in an anaerobic jar at 30°C for 2 days. Black colonies developed were enumerated as sulphite reducing clostridia.

3.4.2.8. *Enterobacteriaceae*

Enterobacteriaceae, was enumerated on violet red bile glucose agar (VRBGA, Oxoid code CM 485). 1.0 ml was pour plated in to 10 ml of molten (45°C) agar and after setting, a 10 ml overlay of molten medium was added. The incubation was at 30°C for 24 h. The large colonies with purple halos were counted (Mossel *et al.*, 1979).

3.4.2.9 *Staphylococcus aureus*

0.5 ml of 10^2 dilution of sample was added in duplicate to the sterile Baird Parker agar (Oxoid, UK) plates added with 1 ml of 1% potassium tellurite solution and 5 ml egg yolk emulsion. Then it was spreaded using sterile bent glass rod. The plates were incubated at 37°C for 24-36 hours. The colonies of *Staphylococcus aureus* were black, convex, narrow white with entire margin surrounded by a clearing zone of 2-5 mm width. Suspected colonies were confirmed by plasma coagulase test and microscopic examination. Positive colonies were counted, calculated and the results were expressed as number of *Staphylococcus aureus* per gram of the sample.

3.4.2.10. *Feacal streptococci*

Feacal Streptococci was enumerated on KF (Kenner's Faecal Streptococci Agar) agar.1 ml was pour plated into 10 ml of molten (45°C) agar in sterile petridishes.

The incubation was at room temperature for 24 h. Red to pink colonies were counted as feacal Streptococci.

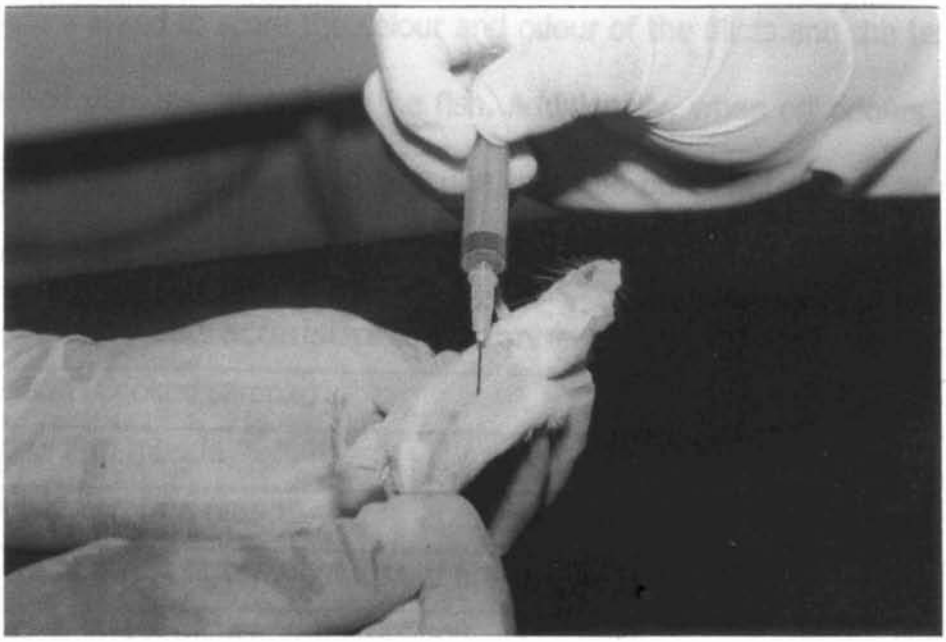
Three replicates of at least two appropriate dilutions were enumerated (Anon, 1978). All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. In addition, selectivity of each medium was checked routinely by Gram staining and microscopic examinations of smears prepared from randomly selected colonies from all media. Microbiological data were transformed into logarithms of the number of colony forming units (cfu g^{-1}).

3.4.2.11. *Clostridium botulinum* toxin testing procedure

The mouse bioassay procedure as described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA, 2001) was used for botulinal toxin testing. 25 g of the sample were aseptically transferred to the stomacher bag and add 2 times (50 ml) of sterile Gel-Phosphate buffer (0.05M). Homogenised for 60s at 10,000 rpm and then centrifuged (27,000 x g, 20 min, 4°C). From the supernantent, 0.9 ml was pipetted out into another small test tube and added 0.1 ml of trypsin (0.1 g in 1 ml GP buffer) and 0.1 ml of serum. Incubated at 37°C in a waterbath for 30 minutes. Injected 0.5 ml of each sample into the mice intraperitoneally (i.p) with using a 1 or 3 ml syringe with 5/8 inch, 25 gauge needle. Observed all mice periodically for 48 h for symptoms of botulism. Recorded symptoms and deaths. Typical botulism signs in mice begin usually in the first 24 h with ruffling of fur, followed in sequence by

labored breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure.

Plate 5: Mouse bioassay



3.5. Sensory Evaluation

Sensory evaluation was based on characterization and differentiation of the various sensory characters such as appearance texture, odour and flavour. Score was given based on 9-point hedonic scale (Table. 3), as described by Peryam and pilgrims (1957). A sensory score of 4 was taken as the borderline of acceptability.

Panelists were asked to score the colour and odour of the fillets and the texture of the cooked portions taken from the fish. Additionally, when off odours were detected, panelists were asked to describe them as pungent, sour, marinade, stale, cabbage and putrid.

Table: 3. Sensory characteristics and scores for taste panel studies

Observation (Cooked sample)	Score
Sweet and firm muscle	9
Sweet taste and firm muscle	8
Slight loss of sweet taste, slight loss of firmness of muscle	7
More loss of sweet taste and texture soft	6
Blank taste, texture slightly pasty	5
Dull colour, bland taste and pasty consistency	4
Pasty muscle, slight off odor	3

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

4.1. Packaging material

Physical properties of the Thermoformed tray used for packaging the fish were shown in the table 4.

Table 4: Physical properties of the HDPE Tray used.

1.	*Tensile strength	299 kg/cm^2
2.	*Elongation at break	80%
3.	Water Vapour Transmission rate As per IS: 1060-Part II, 1960	0.29 g/m^2/24 h at 90% RH at 37°C.
4.	Oxygen transmission rate (OTR) (As per ASTM 1975)	85 cc/m^2/atmosphere/24h/room temperature 28-32°C
5	CO_2 transmission rate (As per ASTM 1975)	255 cc/m^2/atmosphere/24h/room temperature 28-32°C

*As per IS: 2508, 1984

Packaging material used for the study has low water vapour trasmission rate, oxygen trasmission rate and carbon dioxide trasmission rate. It has got enough strength to withstand machine handling. The overall migration residue (Water extractives) at 21°C for 48 h of the sample was 5.5 mg/l, which is below the acceptable limit for food contact application.

Physical properties of the Laminate used for sealing the tray were shown in the table 5.

Table 5: Physical properties of the packaging material

1.	*Tensile strength - Machine Direction - Cross Direction	 363 kg/cm^2 349 kg/cm^2
2.	*Elongation at break (M.D) Elongation at break (C.D)	80% 80%
3.	*Heat Seal Strength (M.D) Heat Seal Strength (C.D)	249 kg/cm^2 194 kg/cm^2
4.	Water Vapour Transmission rate As per IS: 1060-Part II, 1960	3.62g/m^2/24 h at 37°C at 90±2% RH
5.	Oxygen transmission rate (OTR) (As per ASTM 1975)	65 cc/m^2/atmosphere/24h/room temperature 28-32°C
6.	CO_2 transmission rate (As per ASTM 1975)	195 cc/m^2/atmosphere/24h/room temperature 28-32°C

* As per IS: 2508, 1984

Packaging material used for the study has low water vapour trasmission rate, oxygen trasmission rate and carbon dioxide trasmission rate. It has got enough strength to withstand machine handling. The overall migration residue (Water extractives) at 21°C for 48 h of the sample was 3.35 mg/l, which is below the acceptable limit for food contact application.

4.2. Proximate composition

Proximate composition of fresh pearl spot analysed had 75.30% moisture, 22.50% protein, 2.40% fat and 0.90% ash. (Table 6)

Table 6: Proximate composition of fresh pearl spot

Component	%
Moisture	75.30
Fat	2.40
Protein	22.50
Ash	0.90

4.3. Standardization of different Gas mixtures used.

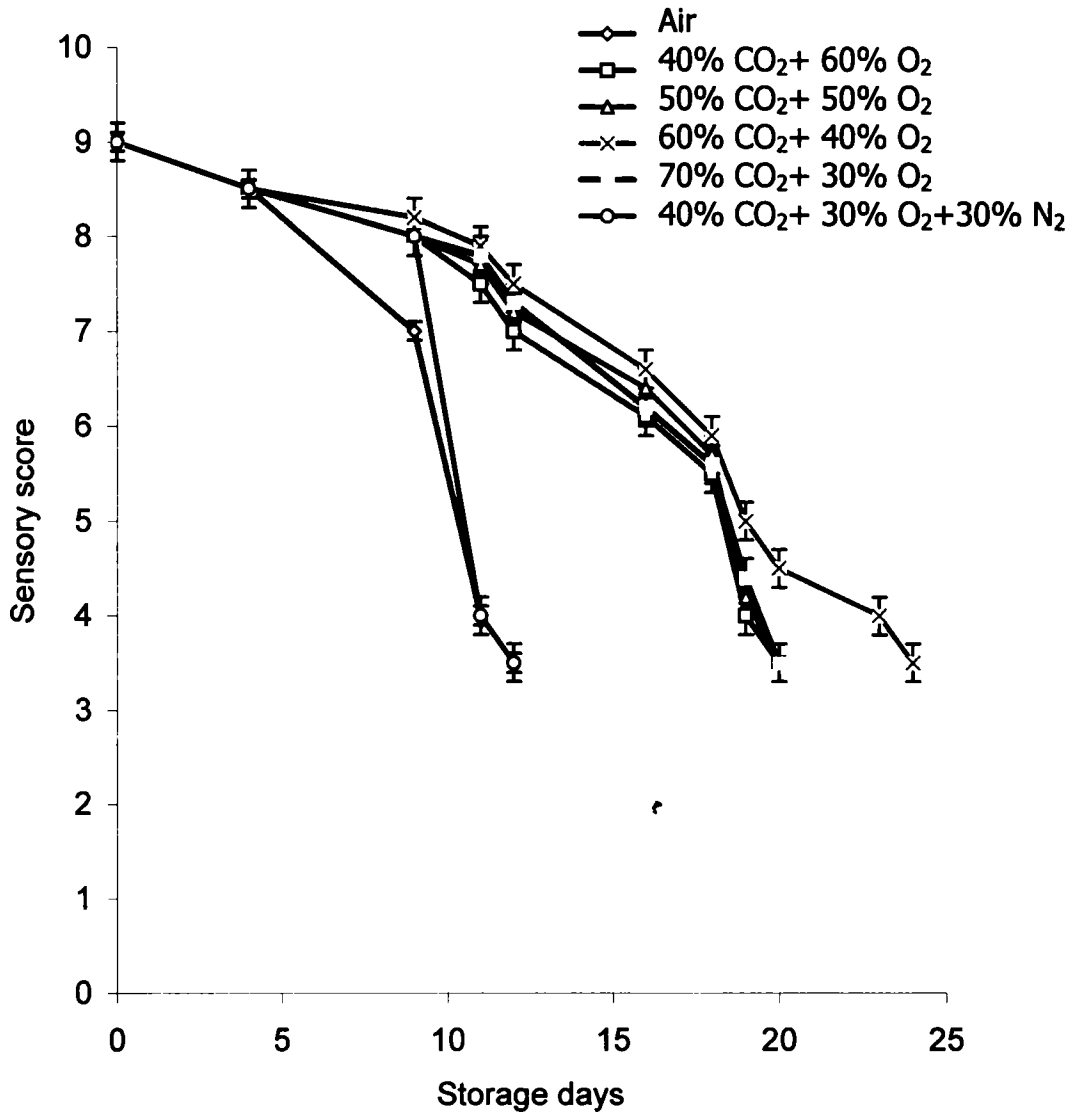
Standardization of gas mixtures was based on sensory analysis. Sensory analysis was done on a 9 point hedonic scale and 4 was the acceptability limit. Changes in the sensory score with various levels of CO_2, O_2 and N_2 are shown in Fig. 1. Based on the sensory score the shelf life is as follows.

Batch I	Air pack	11 days
Batch II	40% CO_2+60%O_2	19 days
Batch III	50%CO_2+ 50%O_2	19 days
Batch IV	60%CO_2+40%O_2	23 days
Batch V	70%CO_2+30%O_2	19 days
Batch VI	40%CO_2+30%O_2+30%N_2	11 days

The odour of fish (evaluated as odour when opening the package) changed as chill storage proceeded from neutral odour upto the appearance of off odours

once spoilage had taken place. Air stored fish were sensorially rejected after 11 days of storage. However MAP stored fish were acceptable for a longer period. The spoilage pattern of pearl spot packaged in CO_2 enriched atmosphere was very different from that packed under air. No slime was observed and a sour, light marinade odour indicative of spoilage was detected at the time of sensory rejection. Pearl spot packed under MAP showed a much slower development of off-odours than those stored under air. The limit of acceptability reached in samples stored under MAP after 19 days storage except in batch VI. Pearl spot stored under $40\%CO_2+30\%O_2+30\%N_2$ followed a similar pattern as that of control and rejection occured on 12^{th} day. In earlier reports gas mixtures containing high CO_2 levels were the most effective on preservation. But in the present trial pearl spot stored under 70% CO_2+ 30% O_2 mixture pearl spot scored less because of the color changes during storage. A shelf life of 19 days was observed for pearl spot stored under MAP of batches II, III, and V. Whereas in Batch IV, appearance of the pearl spot was very good and it gave a shelf life of 23 days. Therefore $60\%CO_2+40\%O_2$, was considered as optimum gas mixture and changes in biochemical, microbiological and textural characteristics of pearl spot were studied in detail.

Fig.1: Changes in Sensory score of pearl spot packed under different MAP and Air.



4.4. Changes in CO_2 concentration during MAP storage of pearl spot.

In air stored pearl spot, CO_2 concentration increased from 1.8% to 12.2% on rejection day i.e. on 12^{th} day of chill storage. In MAP stored pearl spot CO_2 concentration decreased from an initial value of 60% to 33% on 13^{th} day and then gradually increased to 40.6% on 24^{th} day of MAP storage (Fig 2). In air stored pearl spot, increase in microbial growth resulted in increased CO_2 concentration whereas in MAP stored pearl spot, the initial decrease was due to absorption of CO_2 in fish tissues and after that microbial growth resulted in increased CO_2 concentration. Silva and White (1994) in modified atmosphere packed channel cat fish observed that CO_2 concentration fluctuated between 63% and 87% for higher CO_2 concentration packs and between 16% to 33% in low CO_2 concentration packs. The increase in CO_2 in air packed fillets was the result of microbial respiration. Debevere and Boskou (1996) studied modified atmosphere packed cod fillets and observed a decrease in CO_2 until day 4 because of diffusion into the fish muscle, again CO_2 increased due to bacterial and enzymatic activity. Reciprocally to the CO_2 content the proportion of O_2 increased upto day 4, and due to respiration of bacteria the proportion of O_2 decreased later on.

4.5. Changes in O_2 concentration during chill storage of air and MAP stored pearl spot.

In pearl spot stored under air pack, O_2 concentration decreased from 21% to 1.5% on 12^{th} day of chill storage i.e. on rejection day. In pearl spot stored under MAP, O_2 concentration increased from 40% to 51% on rejection day i.e.

on 24th day of chill storage (Fig 3). Decrease in O_2 concentration was mainly due to respiration of bacteria. The proportion of CO_2 in the headspace falls as CO_2 dissolves in fish tissues (Strasdine *et al.*, 1982 and Davis, 1990). As a consequence, proportions of the diluent components increase and concentrations of oxygen, often above the atmospheric levels to start with, are even increased further. Later, as the CO_2 solubilisation rate is overtaken by the rate of release caused by bacterial respiration, the curve reverse. Amanatidou *et al.* (2000), in fresh Atlantic salmon using 50% CO_2+ 50% O_2 observed increased O_2 concentration in the packs. Debevere and Boskou (1996) studied modified atmosphere packed cod fillets and observed a decrease in CO_2 until day 4 because of diffusion into the fish muscle, again CO_2 increased due to bacterial and enzymatic activity. Reciprocally to the CO_2 content the proportion of O_2 increased upto day 4, and due to respiration of bacteria the proportion of O_2 decreased later on. The results of the present study agreed well with that of Debevere and Boskou (1996).

Fig.2: Changes in Carbon dioxide concentration in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

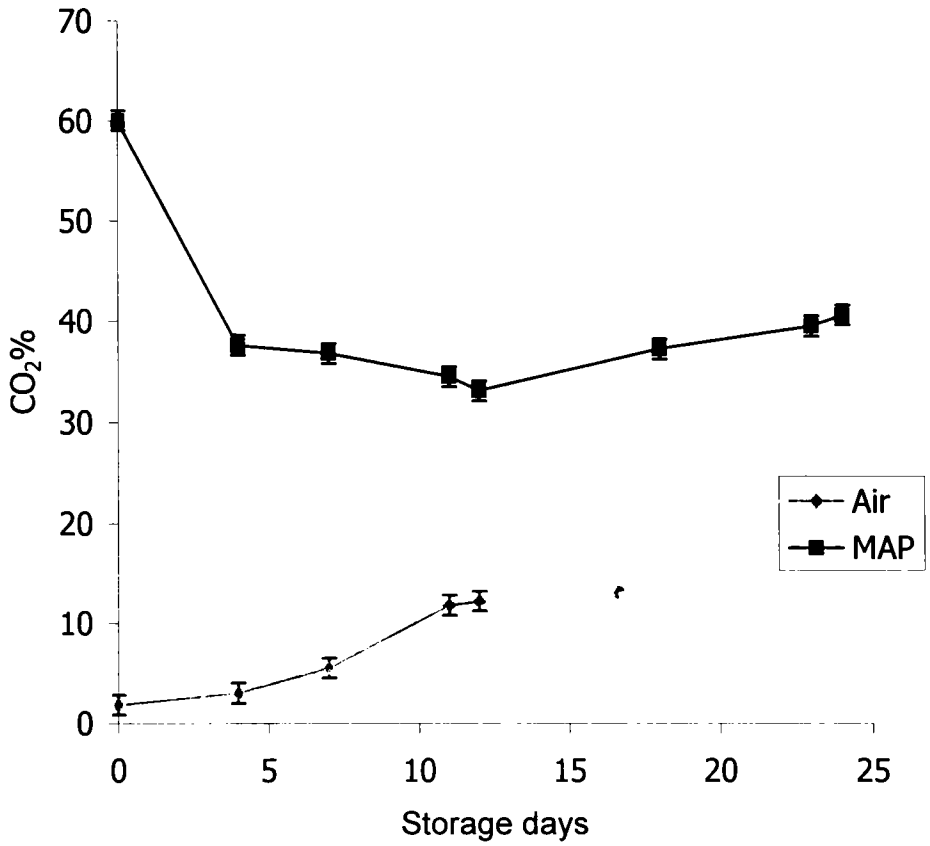
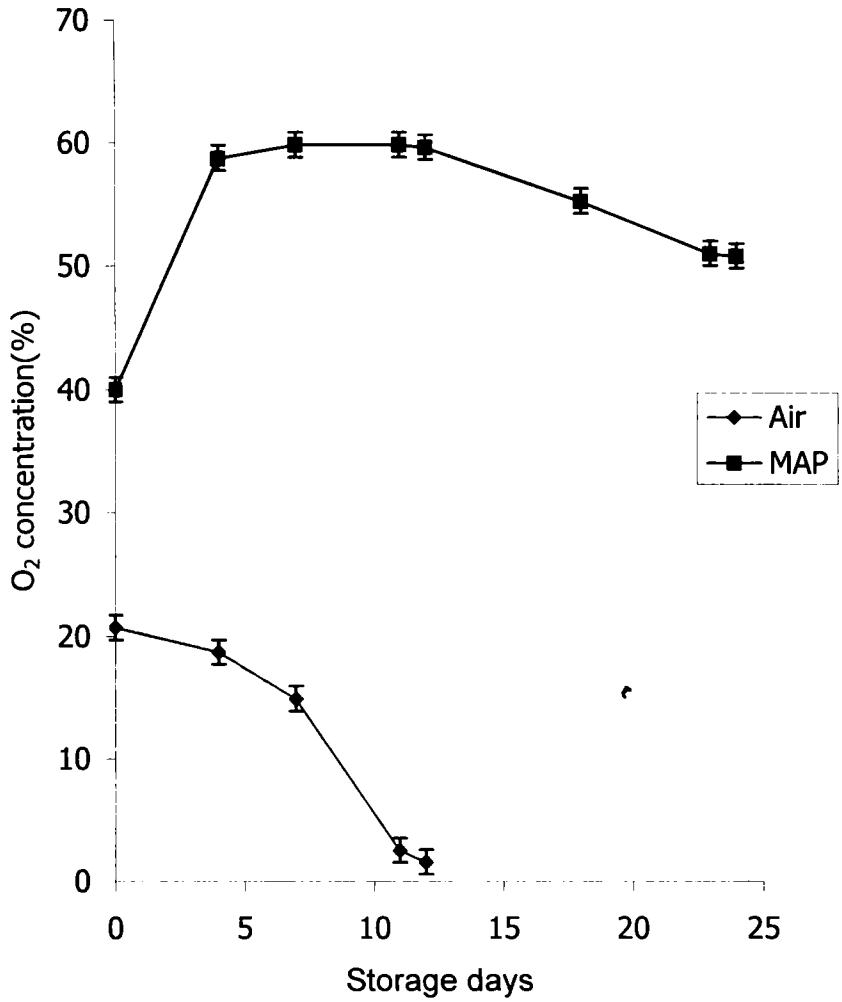


Fig 3: Changes in Oxygen concentration in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.6. Changes in Colour in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Changes in L* values during storage.

Pearl spot packed under MAP were brighter compared to pearl spot packed under air. For pearl spot packed under air, L* values decreased gradually from 62 to 58 on 12 th day of chill storage whereas in pearl spot packed under MAP. L* values remained constant up to 22 days and decreased to 56 on 24 th day. (Fig 4). Silva and White (1994) in modified atmosphere packaged refrigerated channel cat fish observed higher L* values for MAP stored samples compared to air stored samples. Trials on pearl spot showed similar trends in colour i.e. MAP stored samples were more bright compared to air stored samples. Przybylski *et al* (1989) concluded that CO_2 enriched atmospheres bleach the colour of fish. However in pearl spot stored under air and MAP, an increase in L* values were not noticed, instead a slight decrease in L* values were observed during storage.

Changes in b* values during storage

Samples stored under MAP were less yellow compared to pearl spot stored under air, because b* values were low in MAP compared to air stored samples. b* values decreased from an initial 7 to 6.2 in the case of pearl spot packed under air and decreased to 4 in the case of pearl spot packed under MAP. (Fig. 5). Pastoriza *et al* (1996) observed a* and b* values were higher in the control than in MAP stored samples. In pearl spot similar results were obtained. Pastoriza *et al* (1996) observed slight increase in b* value during modified

atmosphere packed storage of iced hake slices. In pearl spot stored under air, a slight increase in b* values were observed.

Changes in a* values during storage

Samples stored under air were more towards red compared to samples stored under MAP throughout storage. Pearl spot stored under air had higher a* values compared to samples stored under MAP. a* values of pearl spot packed under air increased throughout chill storage. For pearl spot packed under MAP, a constant a* values were observed up to 22 days, and on 24th day the value decreased to –2.81. (Fig. 6). Decreased a* values showed that the fish tended to become less red. The natural pigment of meat is a dark, purple red compound called myoglobin. This pigment when combined with oxygen (O_2) forms oxymyoglobin, which gives fresh meat the blooming effect or red colour. The degree of redness depends on the amount of pigment present in the meat and the availability of oxygen. Myoglobin can be oxidized in an atmosphere rich in CO_2 to form metmyoglobin, thus reducing the amount of redness to a light brown colouration. The increased absorption of CO_2 in to the fish flesh turns the fish less red because oxymyoglobin is converted in to metmyoglobin. Pastoriza *et al* (1996) observed a* and b* values were higher in the control than in MAP stored samples. In pearl spot similar results were obtained. In air stored pearl spot an increase in a* values and in MAP stored pearl spot, a slight decrease in a* values were observed. MAP storage gave rise to increases in b* values (A yellowish colour) and decrease in a* values as a result of haemoglobin oxidation in the presence of O_2 and CO_2 (Przybylski *et al* 1989).

Fig.4: Changes in L* values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

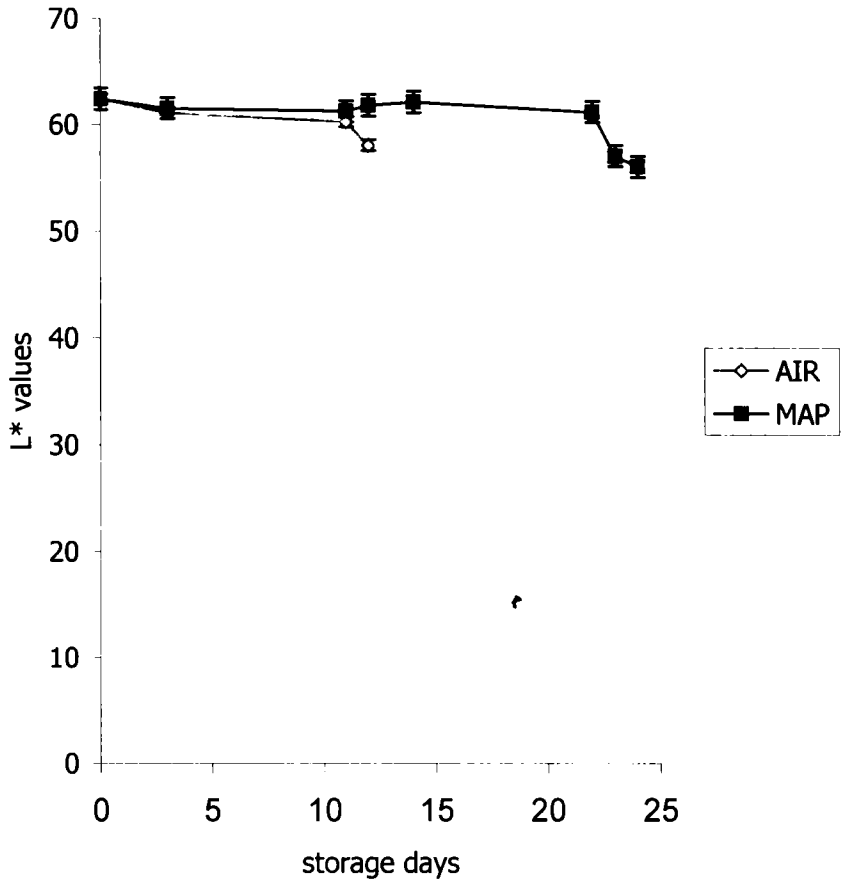


Fig.5: Changes in b* values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

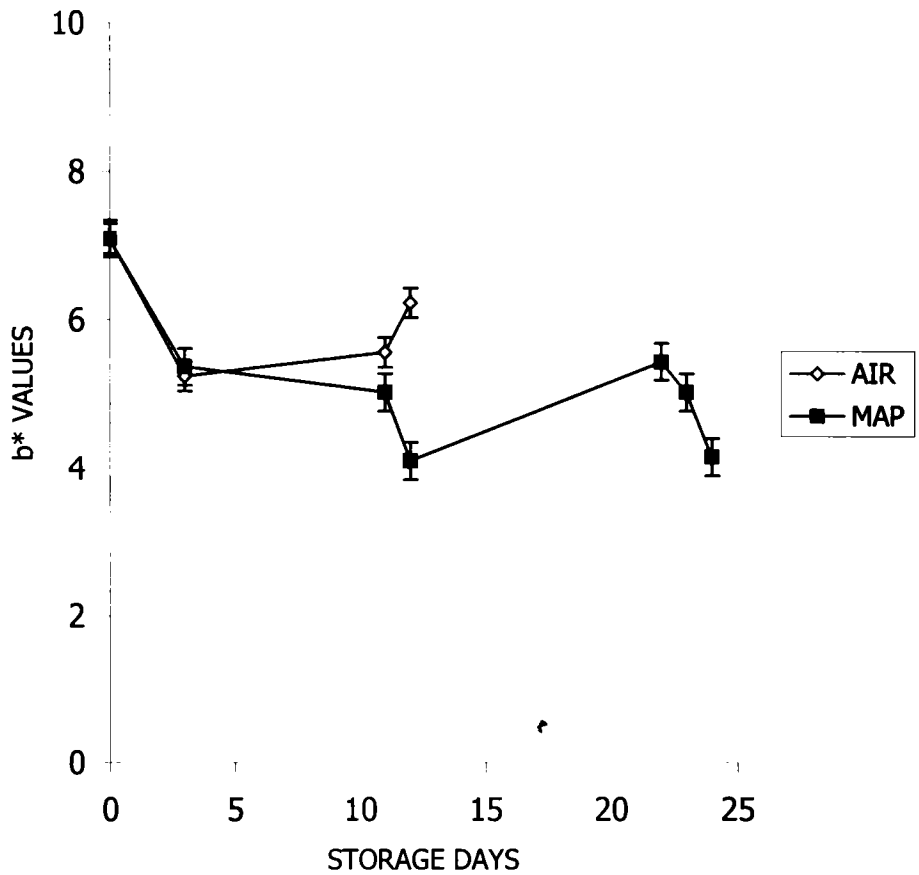
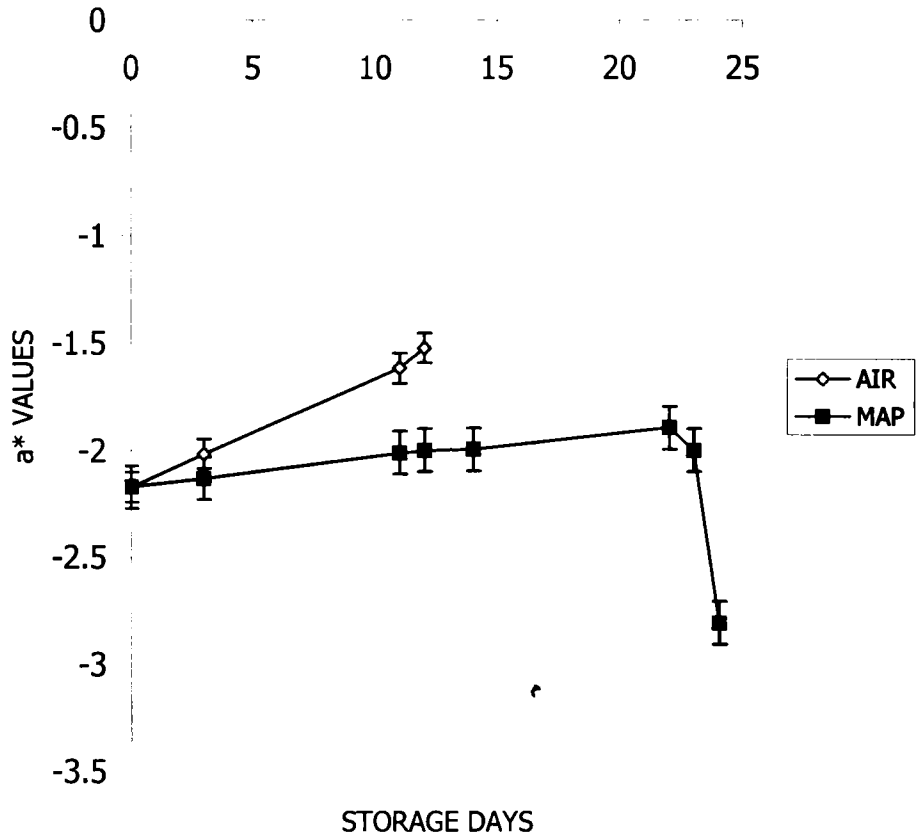


Fig.6: Changes in a* values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.7. Changes in pH in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Pearl spot stored under air, showed a pH change from 6.33 to 6.74 on 12th day of chill storage. In pearl spot stored under MAP, pH first decreased to 6.23 on 7th day and gradually increased to 6.80 on 24th day. Increase of only 0.47 unit was observed in the case of MAP stored Pearl spot (Fig 7). MAP stored pearl spot showed lower pH values compared to air stored samples throughout storage. Various workers in fish reported similar results. Pastoriza *et al.* (1996) observed an increase in pH in air stored samples throughout the storage period, with a value of 8.03 after 21 days, MAP stored samples showed lower pH values throughout storage. Increase in pH is associated mainly to volatile amine production as a consequence of bacterial activity. (Wang and Brown, 1983). Dalgaard *et al.* (1993) reported a pH increase from about 6.6 to 6.8 in experiments with low CO_2 concentrations, but remained almost constant at about 6.6 in experiments with high CO_2 concentrations. Debevere *et al.* (1996) observed counter effects of CO_2 on the pH increase by TVB production, resulting in a stabilization of the pH around 6.7. Pastoriza *et al.* (1996) observed slight increase of pH in the control during the low temperature storage of salmon slices whereas in MAP, an initial decrease of pH was observed followed by an increase after 6 days of storage. Layrisse and Matches (1984) indicated that CO_2 is rapidly dissolved or absorbed in MAP stored muscle and consequently, pH decreased. Parkin *et al.* (1981) and Lannelongue *et al.* (1982) observed that the decrease of pH could be proportional to the CO_2 content of the package.

Banks *et al.* (1980) and Parkin *et al.* (1981) attributed the pH change due to conversion of CO_2 to carbonic acid at the fish muscle surface. Pastoriza *et al.* (1998) observed a pH value of 7.74 in control samples of hake slices after two weeks iced storage. Fish stored under MAP conditions showed pH values lower than the control after ten days storage. Lopez- Galvez. *et al.* (1998) observed a pH increase in refrigerated storage of air packaged sole and only a slight increase was observed in samples stored in 20% CO_2 and no change in pH were observed when samples were stored in CO_2/Air (40/60) (V/V). Therefore in general, the dissociation of carbonic acid in fish flesh results in a slight drop in pH. Both the buffering capacity of the fish proteins and the composition of the spoilage flora determine the magnitude of pH change (Cutting, 1953). However Barnett *et al.* (1978) found no significant change in the pH of the salmon flesh stored in 90% CO_2. The extent to which pH decrease is proportional to the concentration of CO_2 in the atmosphere (Lannelongue *et al.*, 1982a; Tiffiney and Mills, 1982). Studies have shown that CO_2 is absorbed rapidly and pH drops over the first two days of storage. Parkin *et al.* (1981) noted a drop in pH of rockfish from pH 6.7 to 6.3, which was maintained throughout the storage period. However Fey and Regenstein (1982) found that after an initial decrease, pH increased and after 27 days storage in a CO_2 enriched environment had reached a level similar to its initial pH.

4.8. Changes in Thaw drip in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

It is a well-known factor that Carbon dioxide favors exudation by acidifying fish muscle, so that capacity of fish proteins to hold water is reduced. But in the present trial, thaw drip % was lower in the case of MAP stored pearl spot than in air stored pearl spot. Thaw drip follows a pattern similar to that of water holding capacity. In the case of MAP stored pearl spot, water holding capacity was higher in MAP compared to air stored pearl spot. At the time of sensory rejection i.e. on 12^{th} day of chill storage exudation of air packed samples were 4.5% whereas in MAP, exudation was 3.25% on 24^{th} day of chill storage (Fig. 8). In most reports detailing release of drip and pack collapse, the problems are aggravated at the lower storage temperatures, perhaps a reflection of the effect on the solubility of CO_2. Nevertheless, there are unexplained variations (Tiffiney and Mils, 1982; Cann *et al.*, 1983, 1984.). Pastoriza *et al.* (1996) observed no marked effect of CO_2 on exudation of salmon slices stored in the ice storage under CO_2 atmospheres. Dalgaard *et al.* (1993) observed an increase in drip loss during modified atmosphere storage of cod fillets. The largest drip loss was observed for fillets stored in high CO_2 concentrations. Pastoriza *et al.* (1998) observed increased exudation in air and MAP stored fish showed exudates values higher than control after 7 days of storage. Fey and Regenstein (1982) found increased drip losses for red hake, Chinook salmon and to a lesser extent, Sockeye salmon stored in 60% CO_2, 21% O_2 and 19% N_2 compared to air packed product. Tiffiney and Mills (1982) found that packing in 100% CO_2 increased the rate and quantity of drip loss. In all instances the amount of drip loss of fish stored in high CO_2 concentrations was higher at 0°C than at 5°C.

Fig.7: Changes in pH in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

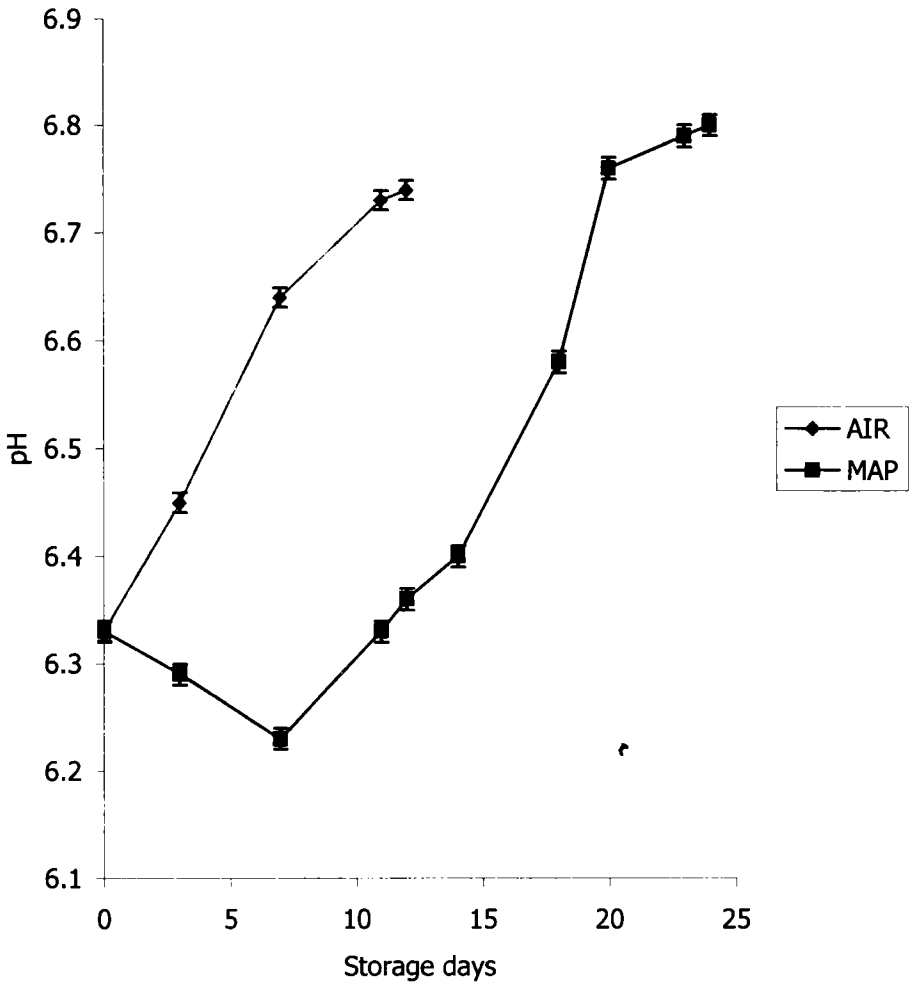
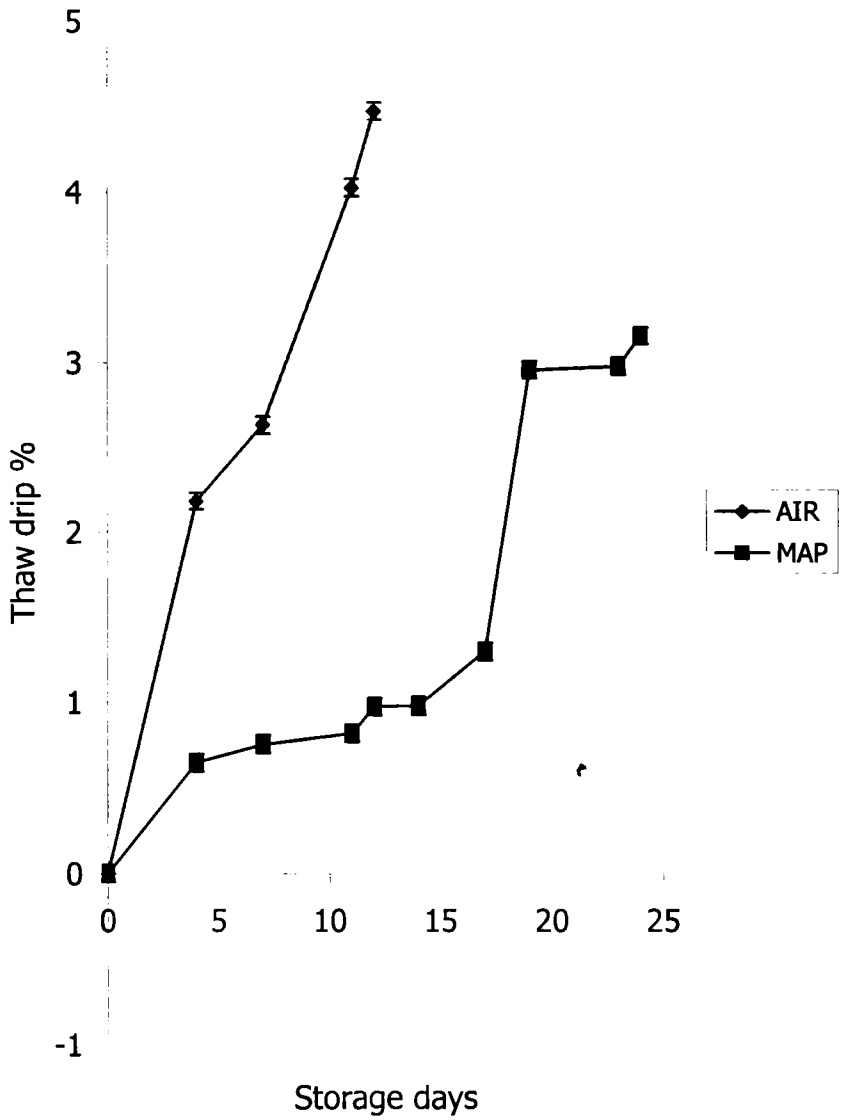


Fig.8: Changes in Thaw Drip % in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9. Chemical changes in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

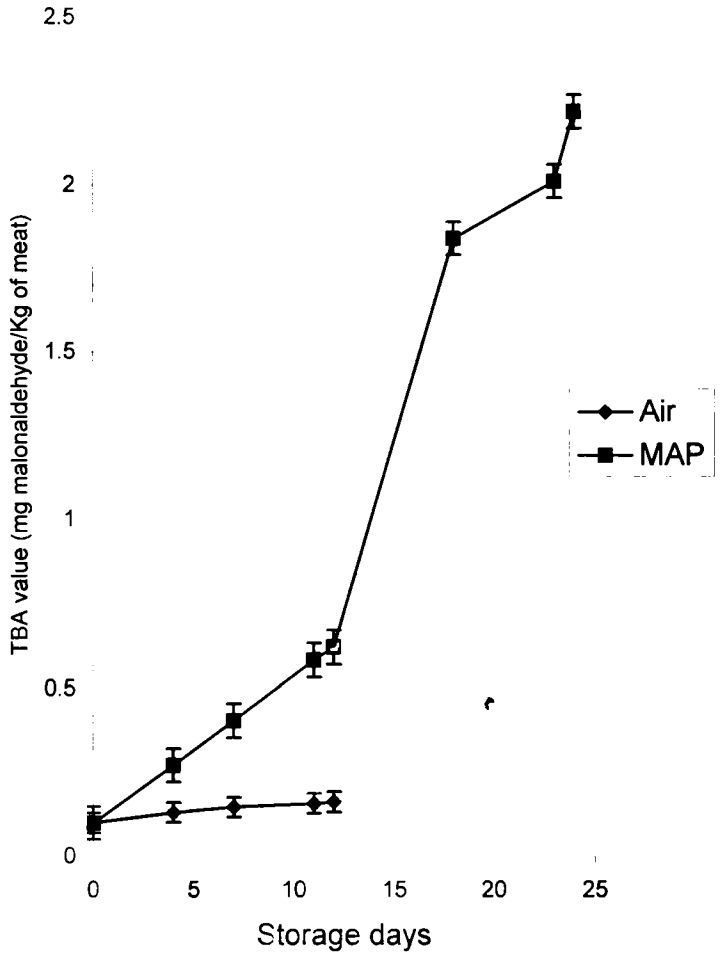
4.9.1. Changes in TBA values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

TBA value increased very slowly in pearl spot stored under air and reached a value of 0.16 mg malonaldehyde/kg of fish on 12 days of chill storage. Lipid oxidation was found to be insignificant in pearl spot stored under air. In pearl spot stored under 60% CO_2+ 40% O_2 atmosphere, the TBA value reached 2.2 mg malonaldehyde/kg of fish on 24th day of chill storage (Fig.9). Increased lipid oxidation observed in pearl spot stored under MAP may be due to increased oxygen concentration (40%) in the pack. Rancidity development is observed only from 24th day onwards when the fish was rejected sensorily. The results indicated that pearl spot stored under 60% CO_2+ 40% O_2 atmosphere were characterized by unacceptable organoleptic characteristic (rancid taste) at the time of sensory rejection i.e. on 24th day when TBA value was above 2.0. Therefore TBA value correlated well with sensory score and can be used as a chemical index in the case of pearl spot stored under MAP. In the case of pearl spot stored under air, the results obtained were comparable to the early reports on fresh water species. Boyd *et al.* (1992) reported very low TBA value in pond-raised striped bass. Kyrana *et al.* (1997), Kyrana and Longovis (2000), Papadopoulos *et al* (2002) also reported very low TBA values for farmed sea bream stored under air. Kyrana and Longovis (2002) reported a TBA value of <

0.2 µmol/g lipid during the edible storage period of sea bream stored under air. Similar TBA values have been reported for farmed rainbow trout stored under air (Dawood *et al.*, 1986). Thus in pearl spot stored under air, lipid oxidation appeared to be a minor spoilage process. Increased levels of dietary fish oil and digestible protein may increase the susceptibility of muscle to lipid peroxidation (Alvarez *et al.*, 1998). The percentage of total lipids in pearl spot was found to be 2.4%. This very low value also contributed to the very low TBA value of pearl spot in air storage. Kyrana and Longovis (2002) reported that all chemical tests (TBA, Free fatty acids, pH and trimethyl amine) other than K value could not be used to determine the loss of quality or end of storage life of sea bream stored under air.

Amanatidou *et al.*, (2000) reported similar results in salmon samples in which TBA value above 1.9 was characterized by unacceptable organoleptic characteristic. Pastoriza *et al.* (1996; 1998) reported increased TBA values in salmon slices and hake slices stored under modified atmospheres compared to samples stored under air.

Fig.9: Changes in TBA values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

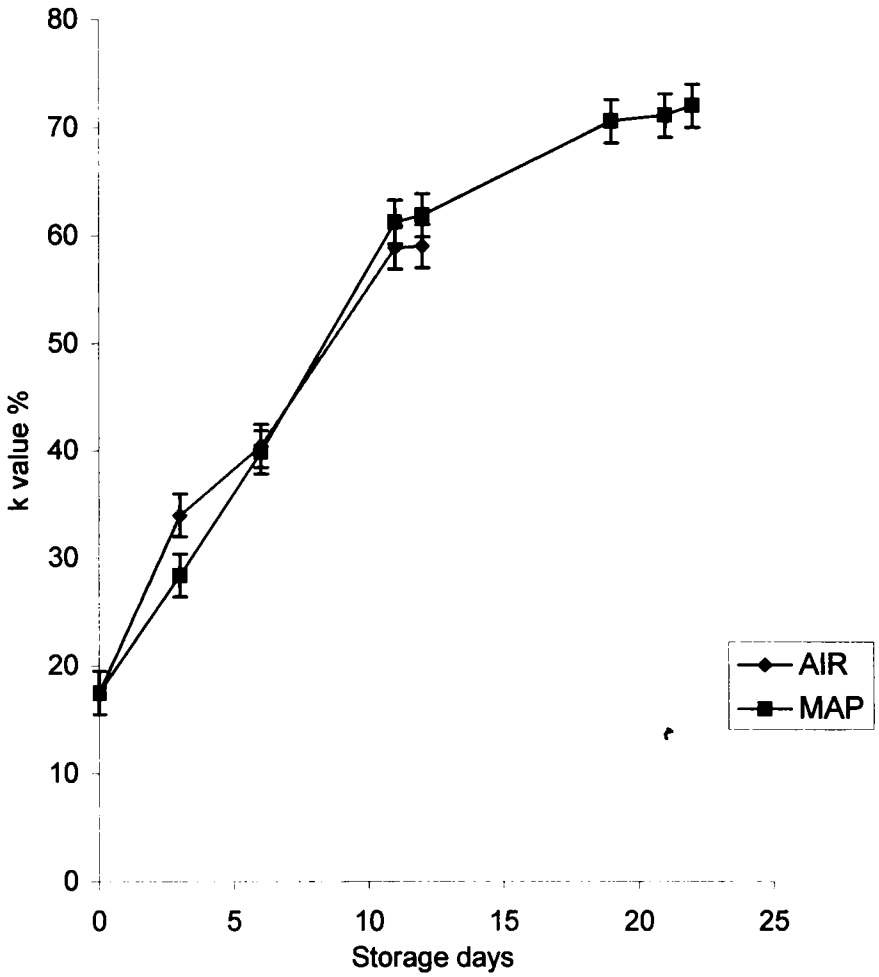


4.9.2. Changes in K value in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

The initial K value was 17.4%. In pearl spot stored under air, the K value reached a maximum of 59% on 12th day of chill storage (rejection day) whereas in pearl spot stored under 60% CO_2 + 40% O_2 atmosphere, the K value reached 72% on 24th day (Fig 10). Identical K values were obtained for Pearl spot packed in either aerobic or carbon dioxide modified atmosphere. This indicated that presence of CO_2 did not alter the rate of degradation of adenine nucleotides. K value of Pearl spot increased during storage of air and modified atmosphere packed Pearl spot, however modified atmosphere packed Pearl spot were still acceptable sensorily even at high K values. K values were independent of sensory spoilage and correlate only with the length of storage of MAP packed Pearl spot. In the present trial, the K value reached 59% at the time of sensory rejection in air stored refrigerated Pearl spot which is in good agreement with the value reported for Pearl spot by Lakshmanan *et al.*, 1996. This value of pearl spot stored under air was comparable to the 60% value set by Ehira and Uchiyama (1974) and Ehira (1976). No effect of CO_2 modified atmosphere on K value was reported by Boyle *et al.* (1991), Huynh *et al.* (1992), Randell *et al.* (1995), Reddy *et al.* (1997), Lopez- galvez *et al.* (1998), Ozogul *et al.* (2000b). Similar results were observed on K value of pearl spot stored under MAP. Reddy *et al* (1997) found that at 4°C, 8°C and 16°C storage, K value of MAP stored fillets of catfish increased gradually during early and middle storage time

and decreased towards the end of storage period with sensory spoilage, indicating no relationship between sensory spoilage and K value.

Fig.10: Changes in K values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.3. Changes in individual nucleotides in pearl spot packed under air and stored at 0-2°C

After the fish have been caught, the levels of adenine triphosphate decreased and most of the adenine nucleotides are degraded to inosine mono phosphate (IMP) in 1-3 days. As the degradation continues, inosine and Hypoxanthine will be produced. Hypoxanthine has a bitter flavor whereas IMP is desirable as a flavor component in fresh fish. In pearl spot stored under air, ATP, ADP, AMP amounts remained almost constant during storage and very low concentration of all these nucleotides were observed IMP was the most prominent nucleotide in the early period of iced storage. Initial IMP concentration was 11.6 µmol/g and it decreased to 2.7 µmol/g on 12th day of chill storage (on sensory rejection) (Fig.11). IMP concentration dropped rapidly, resulting in an increase in K value with increasing storage time. Ozogul *et al* (2000 b) reported IMP decrease was rapid in air packed samples compared to MAP packed samples. The present trial on pearl spot revealed almost constant rate of IMP degradation in both air and MAP packed pearl spot. Hypoxanthine concentration increased from an initial level of 0.45 to 2.5 µmol/g, on 12th day of storage (Fig.11). Rockfish and salmon showed increased Hx concentration over time (Brown *et al.*, 1980; Parkin *et al* 1981). Lindsay *et al.*, (1986) observed the same results for rockfish and whitefish. Inosine level increased from 2.1 to 3.5 µmol/g on 3rd day and again decreased to the initial level on 12th day (Fig 11). Similar results were reported for pearl spot by Lakshmanan *et al.*, (1996).

4.9.4. Changes in individual nucleotides in pearl spot packed under MAP and stored at 0-2°C

In MAP stored Pearl spot, ATP, ADP, and AMP levels remained almost constant during storage. IMP content dropped rapidly, resulting in an increase in K value with increasing storage time. Ozogul *et al* (2000) reported a decrease in rate of IMP concentration during MAP storage. In pearl spot stored under MAP no decrease in rate of degradation was noticed. Therefore identical K value resulted for both air and MAP stored pearl sot. Initial IMP concentration was 11.6 µmol/g and it decreased to 1.7 µmol/g on 24th day in MAP stored Pearl spot (Fig. 12). Brown *et al.*, (1980) and Parkin *et al.*, (1981) indicated that in rockfish and salmon, stored under MAP the hypoxanthine concentration was inconsistent. Lindsay *et al.*, (1986) observed the same results of hypoxanthine concentration in rockfish and whitefish. In pearl spot stored under MAP hypoxanthine concentration increased from an initial value of 0.45 µmol/g to 3.4 µmol/g on 14th day (reached a peak) and then decreased to 2.0 µmol/g on 24th day (Fig.12). Ozogul *et al* (2000a) observed herring held under 60% CO_2 showed Hx concentration increased steadily to 1.9 µmol/g after 16 days indicating that the presence of CO_2 influenced the accumulation of Hx. Dhananjaya and Stroud (1994) found that lower Hx contents were obtained in CO_2 packs than in iced herring. Inosine concentration was initially 2.1 µmol/g and it increased to 4.5 µmol/g on 19th day and then it decreased to 3.2 µmol/g (Fig .12).

Fig.11: Changes in the concentration of ATP degradation products in pearl spot stored under air at 0-2°C.

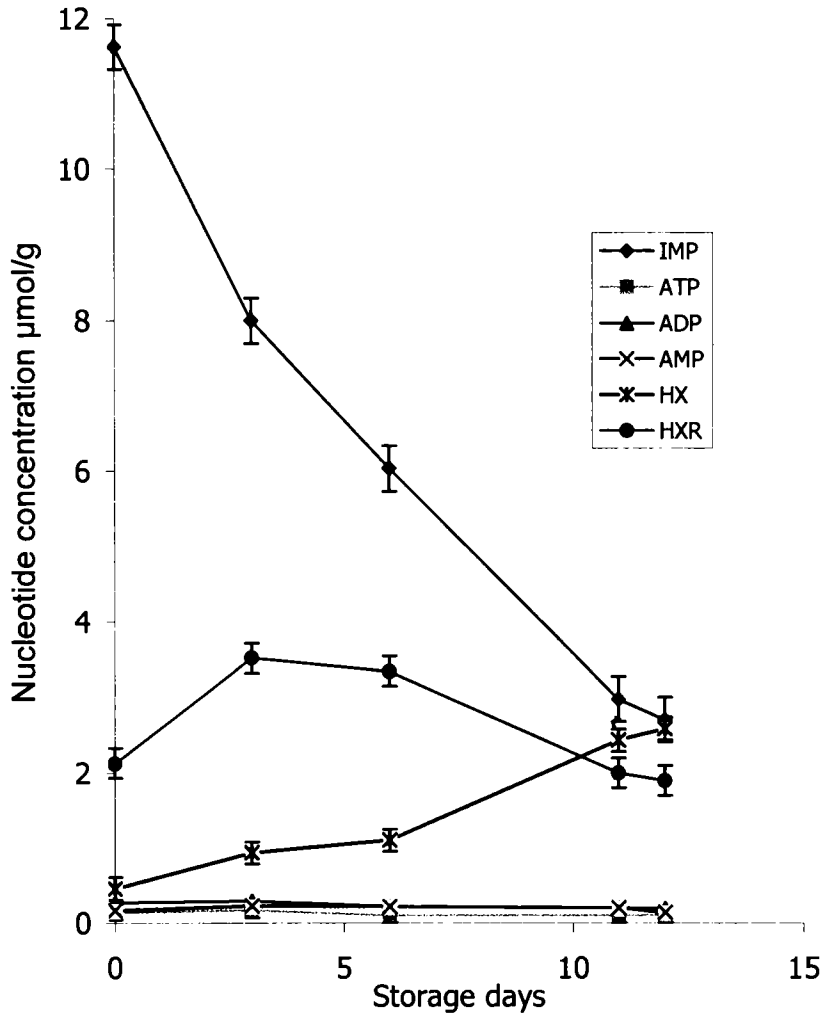
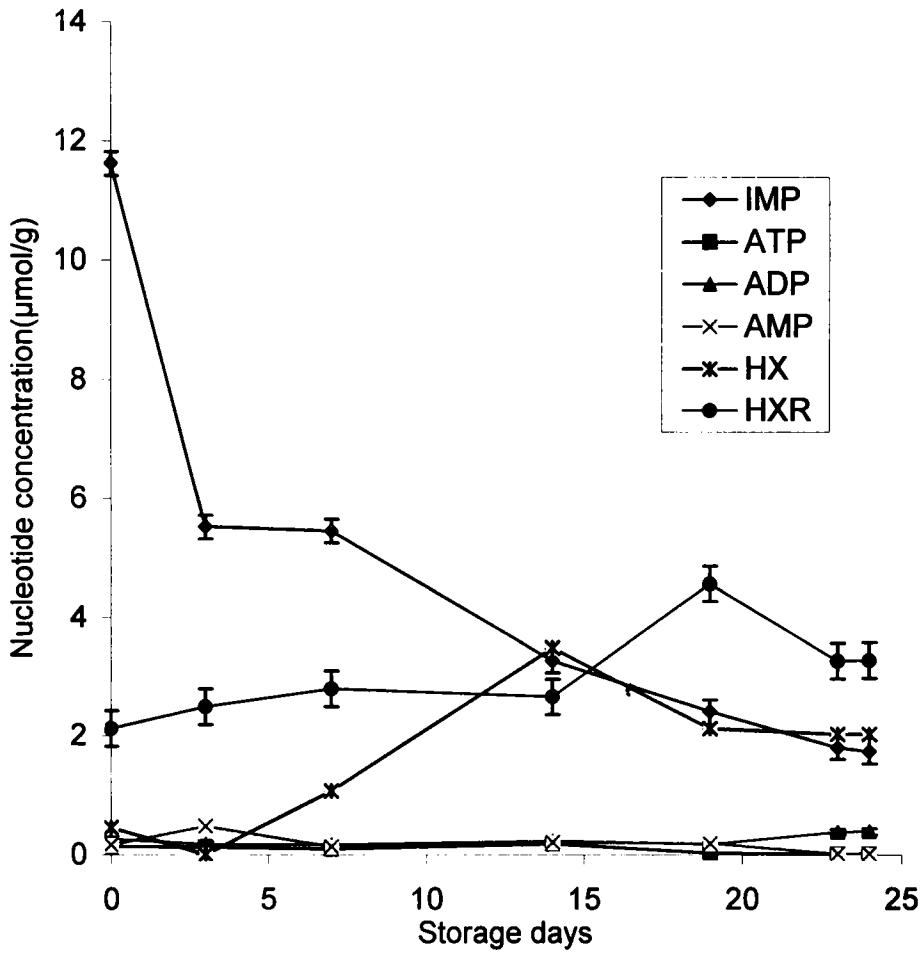


Fig.12: Changes in the concentration of ATP degradation products in pearl spot stored under MAP at 0-2°C



4.9.5. Changes in TMA content in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

TMA value remained constant throughout storage, i.e. 5.6 mg TMA/100 g of fish in both air and MAP stored packs. Lakshmanan *et al*., (1996) reported negligible TMA content in Pearl spot supporting our results. In the present trial the original TMAO present may be very low, therefore considerable TMA production was not noticed, further TMAO reducing bacteria, *Shewanella putrefaciens* never reached the 10^8 cfu/g in the case of pearl spot stored under both air and MAP at the time of sensory rejection.

Trimethylamine oxide (TMAO) is a major constituent of the non protein nitrogen fraction in marine teleosts with osmoregulatory fuction. (Agustsson and Strom, 1981; Huss, 1988; Gill, 1992). Trimethyl amine which is highly volatile and largely responsible for the fishy odour of spoiling seafood, arises from the bacterial reduction of Trimethylamine oxide (TMAO), a naturally occurring osmoregulatory substance whose content varies with fish species, season, size and age of fish. (Gill, 1992; Gram and Huss, 1996). The ability of bacteria to reduce TMAO to TMA is used as a taxonomic criterion for the identification of non fermentative *Shewanella putrefaciens*. (Lee *et al*., 1977). TMAO is found to promote microaerophilic or anaerobic growth (Easter *et al*., 1982). When oxygen levels are depleted, TMAO serves as a terminal electron acceptor for anaerobic respiration and is reduced to TMA (Easter *et al*., 1983). The higher oxygen availability in the atmosphere may lead to low utilization of TMAO as a secondary electron acceptor. So this particular atmosphere is effective for the

inhibition of TMA production. According to Jorgensen and Huss (1989), Dalgaard (1995a, b) and Dalgaard *et al.* (1993), a concentration of more than 10^8 cfu/g for *Shewanella putrefaciens* is required to produce TMA at a concentration of 30 mgN/100g and have perceptible spoilage. When CO_2 and O_2 were combined in the same modified atmosphere there was inhibition on both the growth and TMA production of the *Shewanella* like strains. These two gases were complementary to each other. Combination of the two gases resulted in lower growth rates and lower TMA production than a single application of each gas. Although CO_2 is mainly responsible for the growth rate restriction there is a joint effect of the two gases on TMA production.

The level of TMA was found to be different in different species. Dalgaard *et al.* (1993) reported TMA level around 10-15 mg TMA-N/100g in aerobically stored chilled fish. Ababouch *et al.* (1996) observed that the limit of acceptability for sardines was found to be 5-10 mg TMA-N/100g. Pastoriza *et al.* (1996) observed very low TMA values for salmon slices. A very low amount of TMA (0.05-1.60 mg /100g) has also been reported for other fish (tilapia, hake) fillets (Reddy *et al.*, 1994; 1996; Pastoriza *et al.*, 1996) after 30 days of storage at 2-4°C.

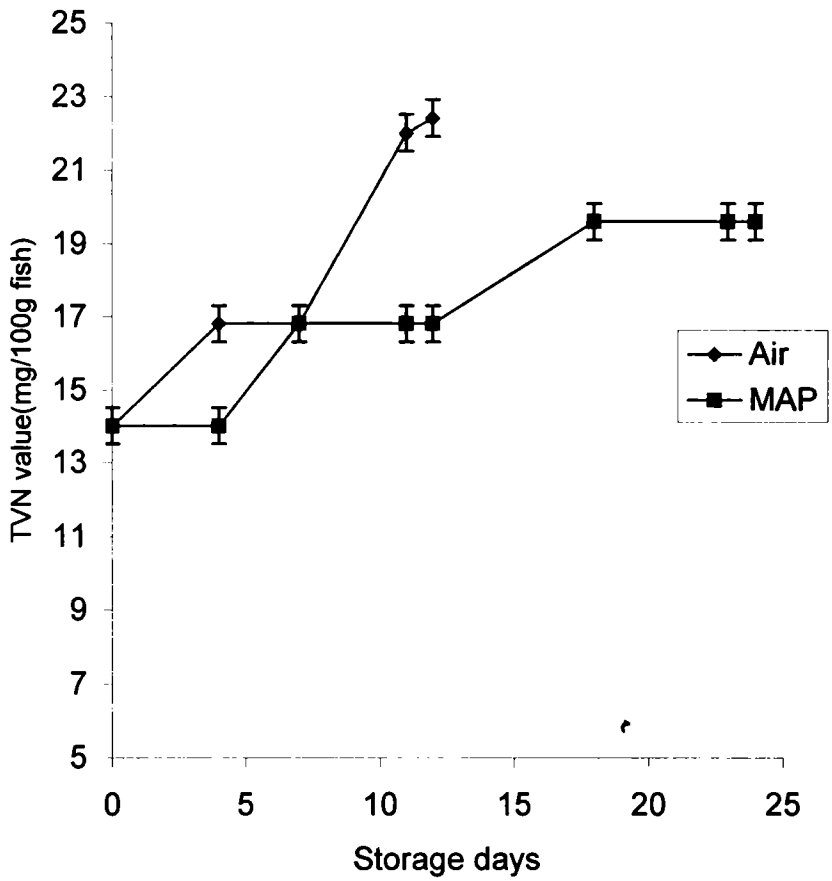
4.9.6. Changes in TVB N values in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Initial TVBN value was 14 mg/100g of fish. TVBN values increased gradually in pearl spot stored under air and MAP. TVBN value increased to 22.4 mg N/100g in pearl spot stored under air on 12th day of chill storage and the value reached

19.6 mg N/100g in pearl spot stored under MAP on 24th day i.e. on rejection day. But these values were well within the limit (Fig. 13). TVN did not reach 30mg/100g threshold of initial spoilage in Pearl spot during 12 days of air storage and 24 days of modified atmosphere storage. Lakshmnan *et al.*, (1996), Surendran and Iyer (1985) and Varma *et al.*, (1985) had also observed a very slow increase of TVN in Pearl spot during the 20 days of iced storage.

Chemical changes as potential diagnostic or quality indices of microbial spoilage of fish have not been studied to the same extent as microbiological changes. For example, only the concentration of Trimethylamine (TMA) and total volatile bases (TVB) in fish muscle are used in research and industry for a quality assessment or as potential indicators of the bacteriological status of fish products (Gram, 1992; Dainty, 1996). These can be used in fish spoilt by *Photobacterium phosphoreum* and *Shewanella putrefaciens* (Gram, 1992; Dalgaard, 1995) but not in those spoilt by pseudomonads (Gram, 1992), lactic acid bacteria or *Brochothrix thermosphacta* (Kakouri *et al.* 1997). In the latter case, acetic acid and lactate have been proposed as potential quality indices. This may be the reason in the present trial TMA and TVN were not found to be good index for quality assessment.

Fig.13: Changes in TVB-N content in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.7. Changes in textural properties in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

4.9.7.1. Changes in Hardness 1 in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

The peak force during the compressive part of the test is known as hardness. It is identified as Hardness 1 for the peak during the first compression and Hardness 2 for the peak during the second compression. Initial Hardness 1 was 1.48 kgf. In pearl spot stored under air, Hardness 1 decreased gradually and reached a value of 1.1 kgf. on 12th day of chill storage (Fig. 15). In the case of pearl spot stored under MAP, Hardness 1 decreased to 1.0 kgf on 3rd day and remained constant throughout storage up to 24 days of chill storage (Fig.14).

4.9.7.2. Changes in Hardness 2 in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Initial Hardness 2, was 1.16 kgf. In the case of pearl spot stored under air, hardness 2, decreased to 0.92 kgf. on 12th day of chill storage. In the case of pearl spot packed under MAP, hardness 2, reached 0.75 kgf. on 7th day and remained constant throughout storage up to 24 days (Fig.15). Amanatidou *et al.* (2000) reported a lower cutting strength of modified atmosphere packed samples of fresh Atlantic salmon compared to control, indicating an increase in the softness upon storage. The time course of the changes revealed that the textural characteristics were retarded compared to the control. In pearl spot similar trend was observed. Hardness was lower in MAP stored pearl spot compared to air stored pearl spot and in air stored pearl spot, hardness was

gradually decreasing while in MAP stored samples after an initial decrease it maintained almost constant value throughout.

4.9.7.3. Changes in Cohesiveness in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Cohesiveness is the ratio of work during the second compression divided by the work done during the first compression. This result is an indication of the visco elasticity of the material. A value of 1 indicates total elasticity and a value of 0 indicates that the sample did not recover at all. Initial cohesiveness was 0.2. In the case of pearl spot stored under air, as well as in MAP and the value remained almost constant through out chill storage (Fig.16).

4.9.7.4. Changes in Springiness in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

The height that the sample springs back between the end of first compression and the beginning of the second compression is known as springiness. This is sometimes referred to as elasticity. The initial springiness was 1.5 mm. In the case of pearl spot stored under air, the springiness decreased to 1.0 mm on 12^{th} day of chill storage, whereas in pearl spot stored under MAP, the value decreased to 1.1 on 3^{rd} day and remained constant throughout storage (Fig.17).

4.9.7.5. Changes in Chewiness in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

The product of Hardness, Cohesiveness and Springiness is defined as chewiness. Initial chewiness was 0.52 kgf.mm. In the case of pearl spot stored under air, chewiness decreased gradually and reached a value of 0.28 on 12^{th} day of chill

storage. In the case of pearl spot stored under MAP, the value reached 0.25 on 7^{th} day and remained constant throughout storage upto 24 days. (Fig.18)

Fig.14: Changes in Hardness 1 in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

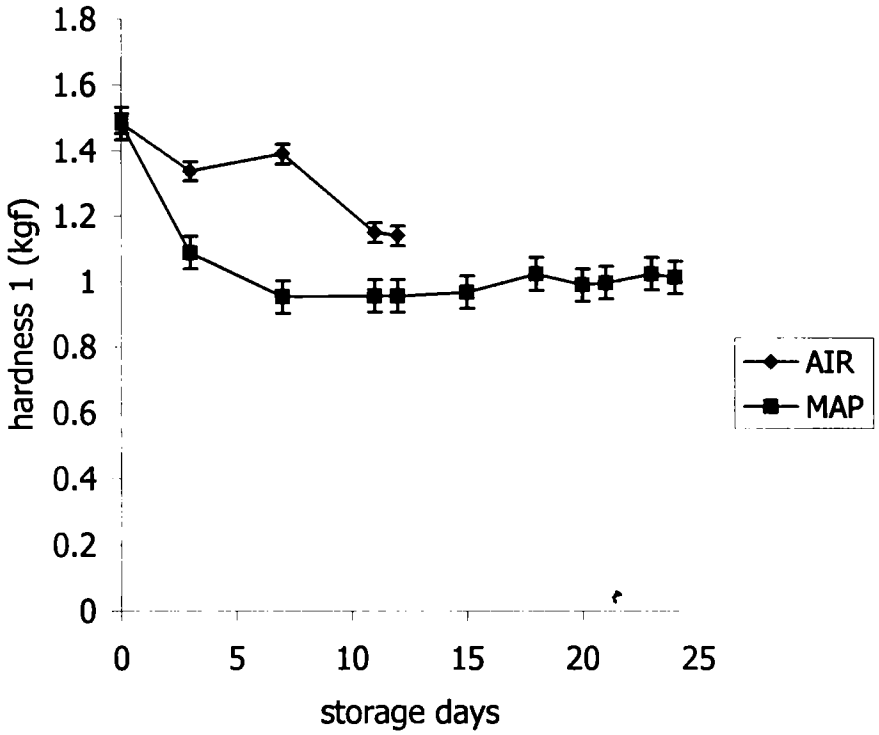


Fig.15: Changes in Hardness 2 in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

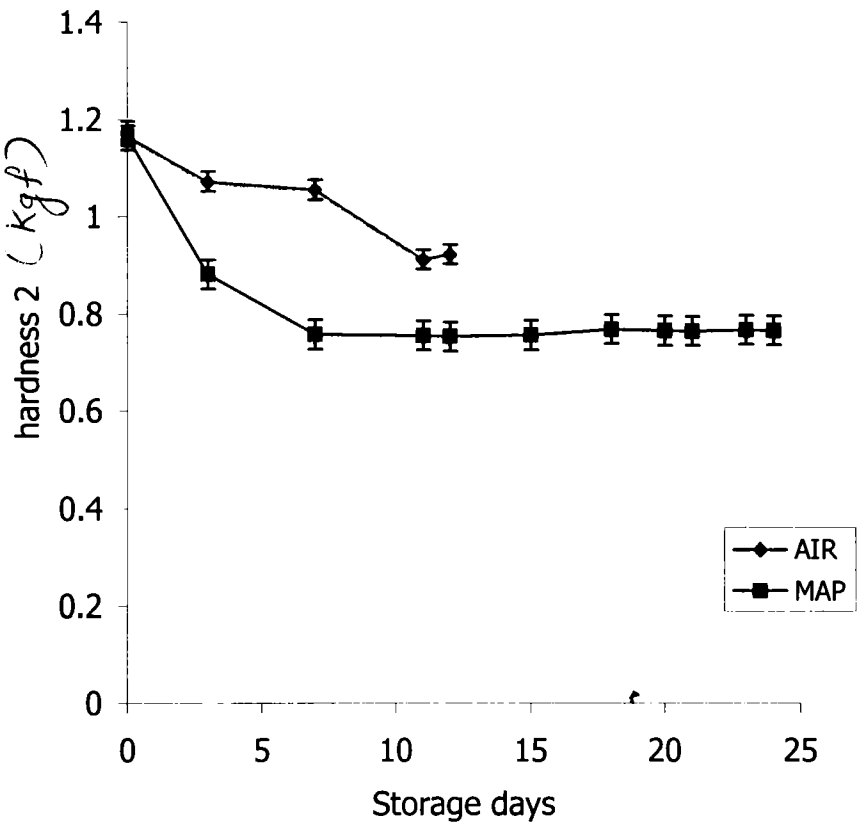


Fig.16: Changes in Cohesiveness in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

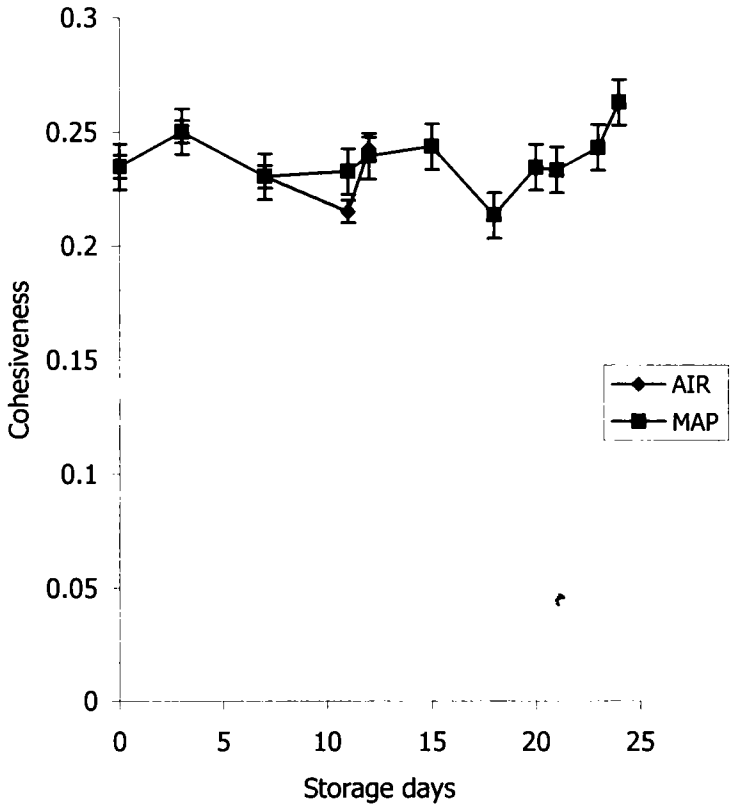


Fig. 17: Changes in Springiness in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

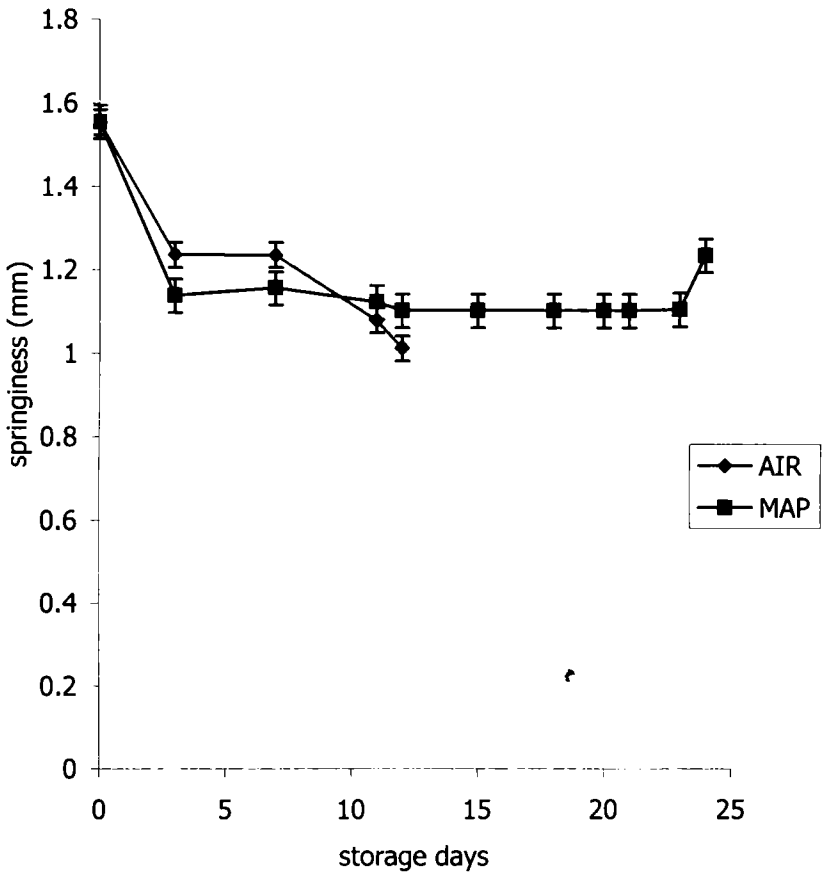
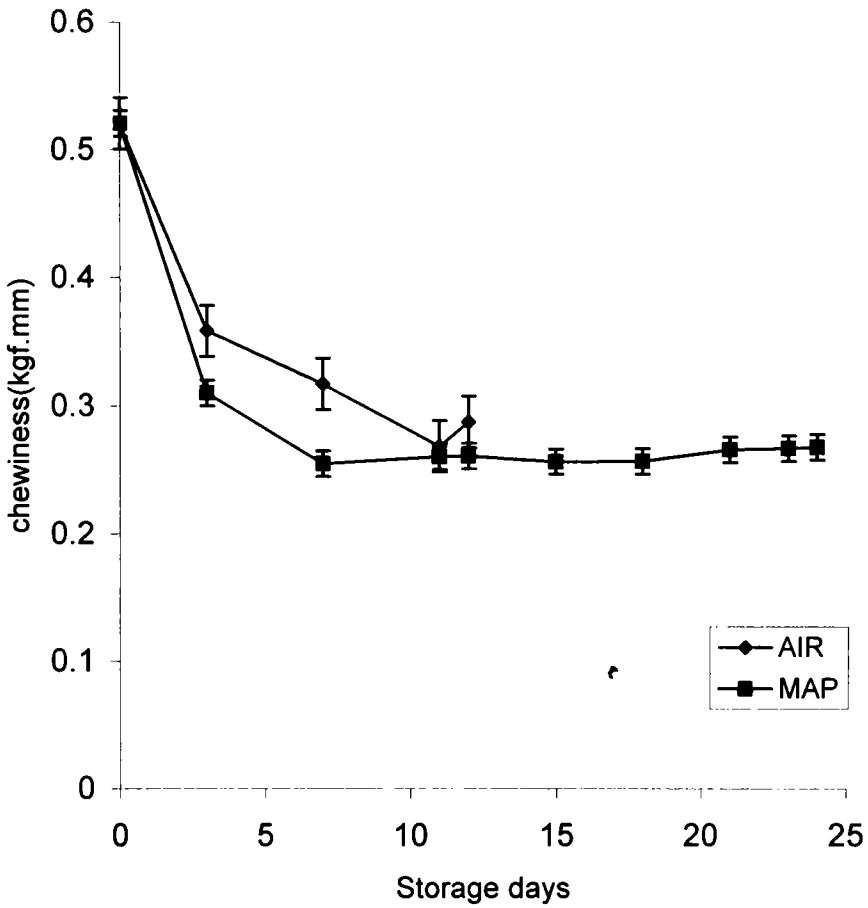


Fig.18: Changes in chewiness during storage



4.9.8. Microbiological changes in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

4.9.8.1. Quantitative changes in mesophilic and psychrotrophic bacterial counts in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Initial total mesophilic counts of Pearl spot were 5.4 log_{10} cfu g^{-1}. Mesophilic counts gradually increased during storage at 0-2°C and reached 6.7 log_{10} cfu g^{-1} and 7.0 log_{10} cfu g^{-1} respectively on 8th and 11th day. At the time of sensory rejection, the mesophilic counts exceeded the limit count of 10^7 cfu g^{-1} in pearl spot stored under air. (Fig.19). In pearl spot stored under MAP, the count reached 7.0 log_{10} cfu g^{-1} on 23rd day and the count reached 7.4 log_{10} cfu g^{-1} on 24th day (Fig.19). The mesophilic counts exceeded the limit count of 10^7 cfu g^{-1} in pearl spot packed under air and MAP respectively on 12th and 24th day of storage when the samples were rejected based on sensory scores.

Initial mesophilic counts of 5.4 log cfu/g indicate good fish quality, considering the proposed limit for aerobic plate count of $5x10^5$ cfu g^{-1} for fresh fish (ICMSF, 1986). Initial mesophilic counts between 4 and 6 log cfu/g were reported for freshwater fish species tilapia, rainbow trout, and silver perch by various workers (Accff *et al.*, 1984; Gonsalez *et al.*, 1999; Gelman *et al.*, 2001; Savvaidis *et al.*, 2002), and Mediterranean fish species (Koutsoumanis and Nychas, 1999; Tejada and Huidobro, 2002). Counts of 7 log_{10} cfu g^{-1} were reported for Mediterranean boque (*Boops boops*) after 4 days of storage aerobically.

Bacterial growth was delayed when pearl spot was packaged in 60% CO_2 + 40% O_2 mixture. Mesophilic counts in pearl spot packed in refrigerated atmospheres enriched with 60% CO_2 were ~ 1 log unit lower than that packed under air during the first 12 days of iced storage. An extension of shelf life of 12 days was noticed in pearl spot packed under MAP compared to that stored under air. In MAP, an extension of lag phase of 11 days was observed and the log phase was apparently extended. It has been reported that CO_2 has an inhibitory effect on microbial growth, exerting a selective inhibitory action (Huss, 1972). Aerobic microorganisms were generally sensitive to CO_2, therefore MAP delays the spoilage of fish and other seafood. It has also been reported that CO_2 has been shown to delay the spoilage of fresh seafood by inhibiting psychrotropic, aerobic and Gram-negative bacteria (Banks *et al.*, 1980; Brown *et al.*, 1980; Finne, 1982; Layrisse and Matches, 1984). The overall effect of modified atmosphere packaging is the suppression of bacterial growth and subsequent extension of shelf life. For example lag phase lasting for 6-10 days have been reported in brown shrimp (Lannelongue *et al.*, 1982a), swordfish (Lannelongue *et al.*, 1982b), fresh water crayfish packaged in CO_2 enriched atmosphere (Wang and Brown, 1983). The total mesophilic counts of 7.0 $\log_{10}$ cfu g^{-1} is considered as the upper limit of acceptability, shelf life of air stored Pearl spot was 11 days and 23 days when packed in 60% CO_2 + 40% O_2.

Total psychrotrophic viable counts increased in pearl spot stored under air and MAP during storage (Fig. 19). Apparently there was no lag phase in air stored and MAP stored Pearl spot. In pearl spot stored under air, the psychrotrophic

count reached 7.3 log_{10} cfu g^{-1} on 11th day and remained same on 12th day of storage. In pearl spot stored under MAP, the count reached 7.4 log_{10} cfu g^{-1} on 23rd day and the count reached 7.6 log_{10} cfu g^{-1} on 24th day of storage. Carbon dioxide delays bacterial growth, acting preferentially on Gram negative flora (Silliker and Wolfe, 1980; Stier *et al.*, 1981; Lannelongue *et al.*, 1982; Oberlender *et al.*, 1983; Dalgaard *et al.*, 1993).

Table 7: Quality changes in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Name	Initial count	Final count (Air)	Final count (MAP)
TPC at 37°C	5.4 log_{10} cfu g^{-1}	7.45 log_{10} cfu g^{-1}	7.4 log_{10} cfu g^{-1}
Enterobacteriacea	3 log_{10} cfu g^{-1}	6.18 log_{10} cfu g^{-1}	4.34 log_{10} cfu g^{-1}
Staphylococcus aureus	N.D	N.D	N.D
Faecal Streptococci	2.5 log_{10} cfu g^{-1}	3.1 log_{10} cfu g^{-1}	2.6 log_{10} cfu g^{-1}

4.9.8.2. Qualitative changes in bacteria in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

On 12th day of air storage (when samples were sensorily rejected), the identification of the colonies on the PCA plates revealed that 95% of these were Gram negative, oxidase positive organisms, which could indicate that the spoilage flora was dominated by Gram negative psychrotrophs, probably

Pseudomonas and *Shewanella putrefaciens*, *Aeromonas*, *Brochothrix thermosphacta* and *Enterobacteriacea* also contributed in final population. Eklund, (1982) and Van Spreekens (1977) reported that bacterial spoilage in refrigerated fish under aerobic storage condition resulted from Gram negative psychrotrophic organisms. Hobbs (1991) and Lindsay *et al.*, (1986) reported that among Gram negative psychrotrophic, the dominant being *Pseudomonas* and *Shewanella putrefaciens*. On 24th day of MAP storage (when samples were sensorily rejected), the identification of the colonies on the PCA plates revealed that *Brochothrix thermosphacta* and *Shewanella putrefaciens* were mainly contributed in final population.

Plate 6: Total Plate Count - control

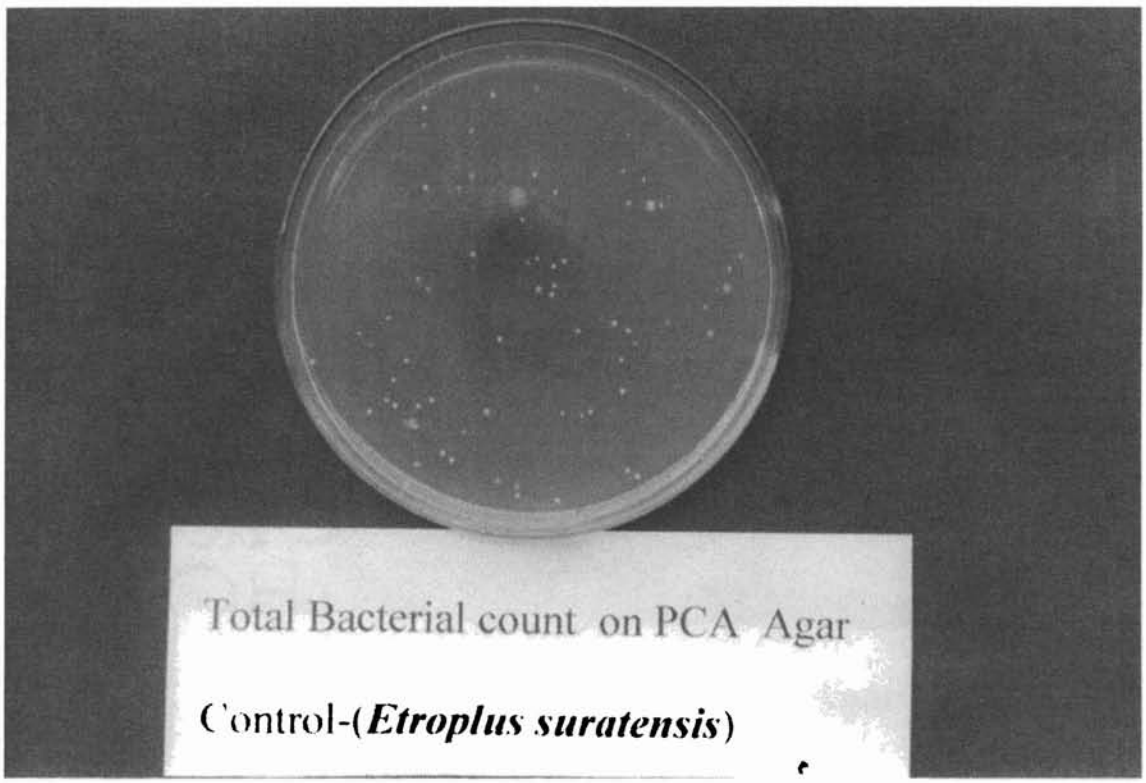


Plate 7: Total Plate Count MAP

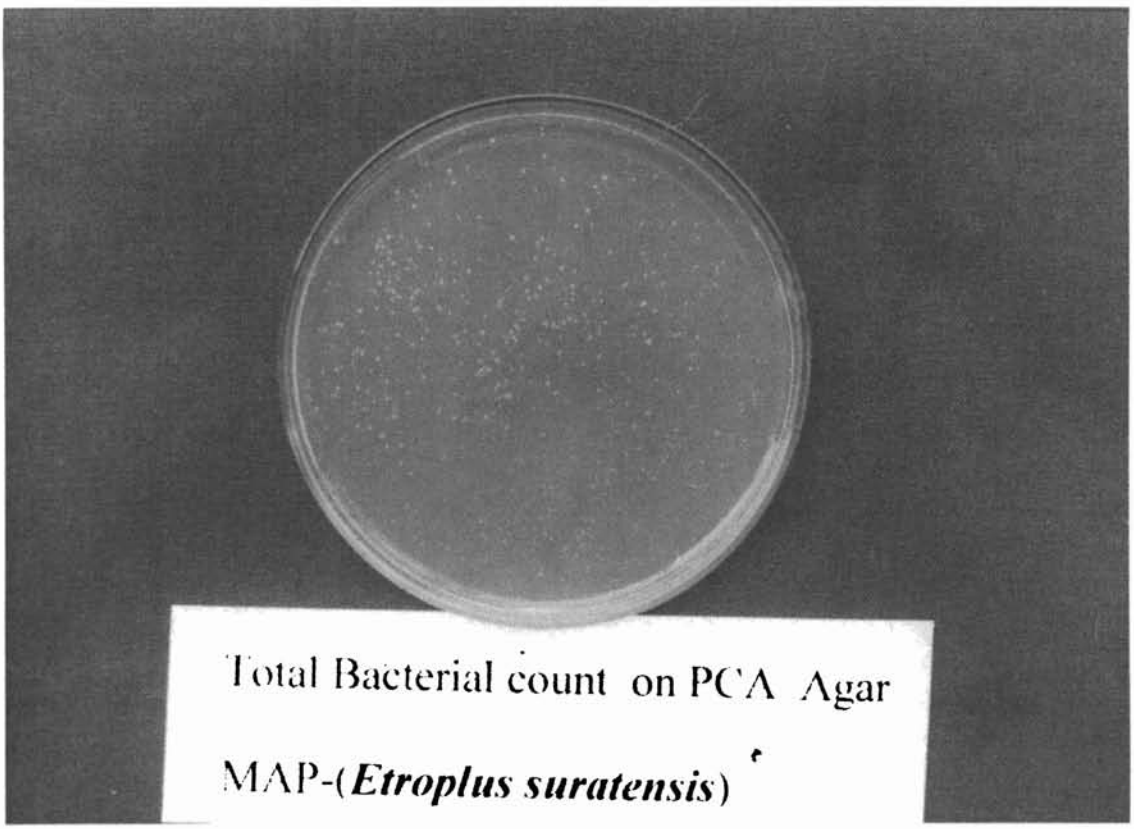
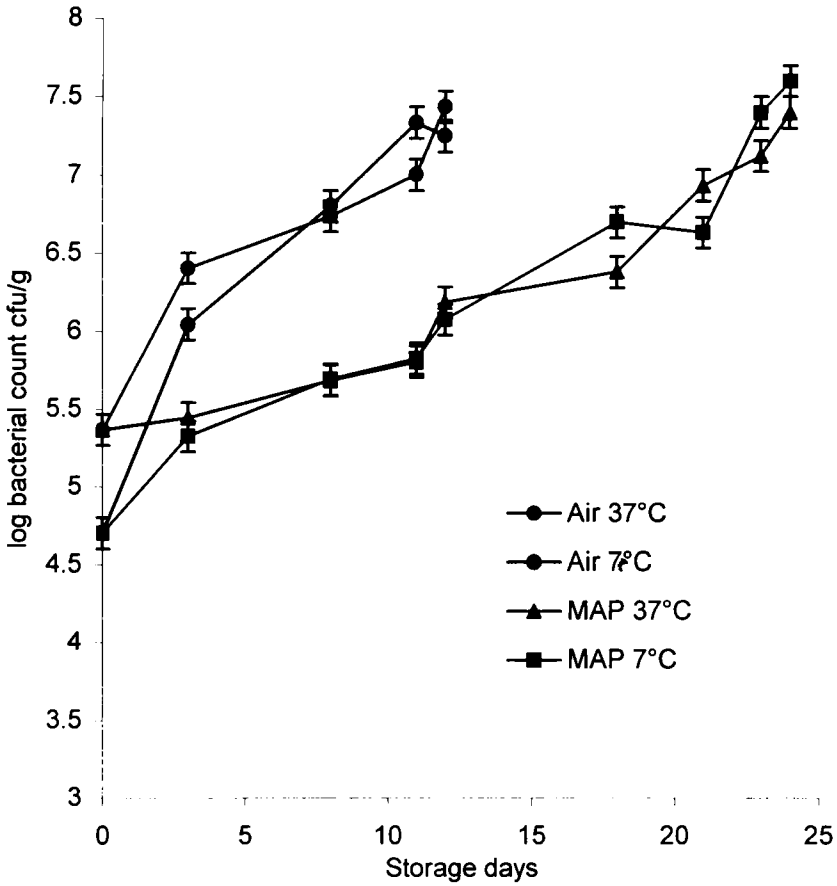


Fig.19: Changes in mesophilic and psychrotrophic counts in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.8.3. Changes in LACTIC ACID BACTERIA (LAB) in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Initial level of LAB was 3.5 log_{10} cfu g^{-1} in Pearl spot. The count remained constant up to 8 days in pearl spot packed under air and on 12^{th} day it increased to 4.1 log cfu g^{-1}. In the case of pearl spot packed under MAP, the count remained almost constant around 3.4 log cfu/g upto 21 days and on 24^{th} day, the count increased to 3.7 log_{10} cfu g^{-1}. (Fig.20)

The increase in LAB count were typical for muscle foods packaged under modified atmosphere conditions when LAB became the dominant spoilage flora (Banks *et al.*, 1980; Silliker and Wolfe 1980; Lannelongue *et al.*, 1982 a; 1982 b). But in the present trial the low numbers of LAB found in this study indicated that this flora are without any quantitative importance for the spoilage process. Lactic acid bacterial growth in pearl spot was limited. The results were similar to that reported in modified atmosphere packaged of cod fillets (Debevere and Boskou, 1996) and sole fillets (Lopez-Galvez *et al.*, 1998).

Plate 8: Lactic Acid Bacteria- Control

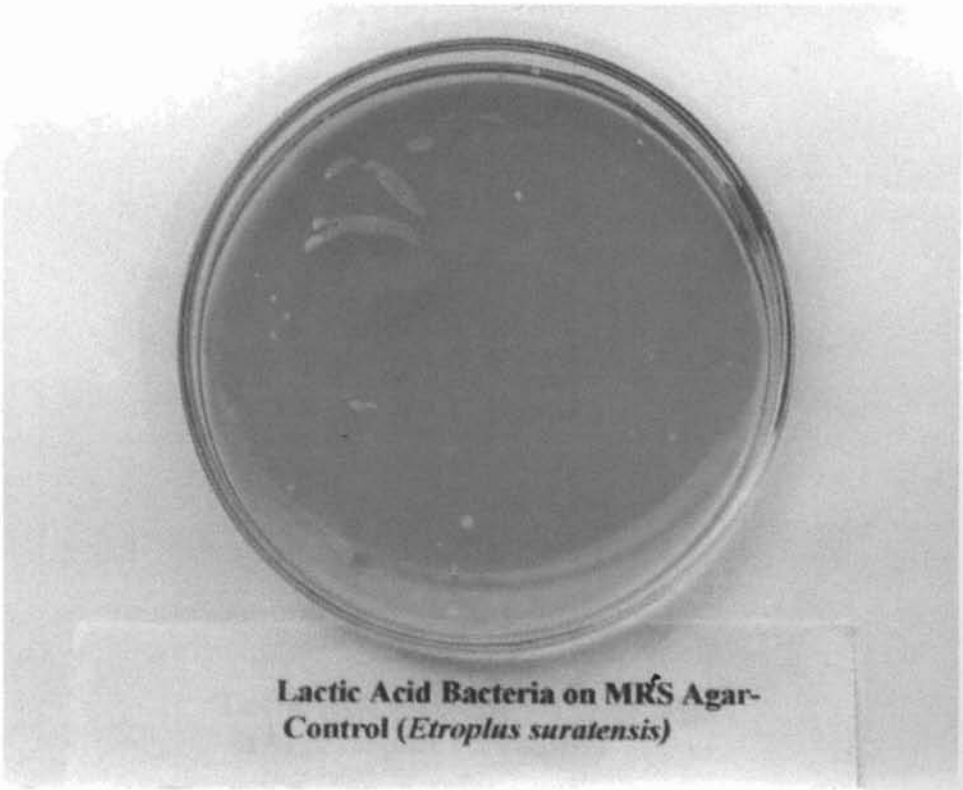
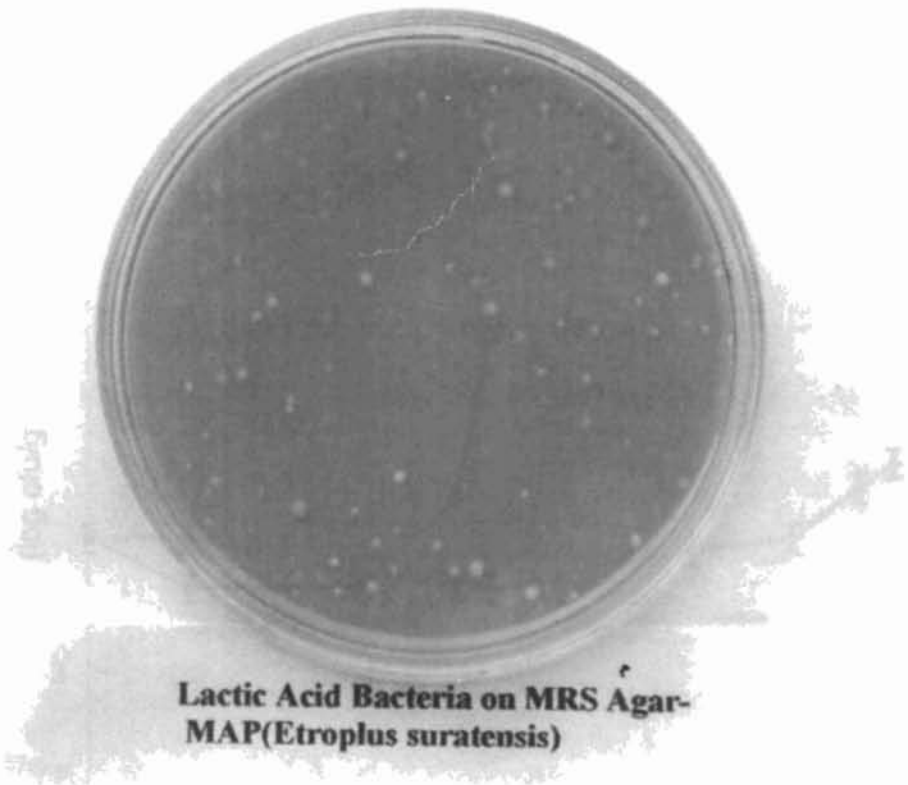
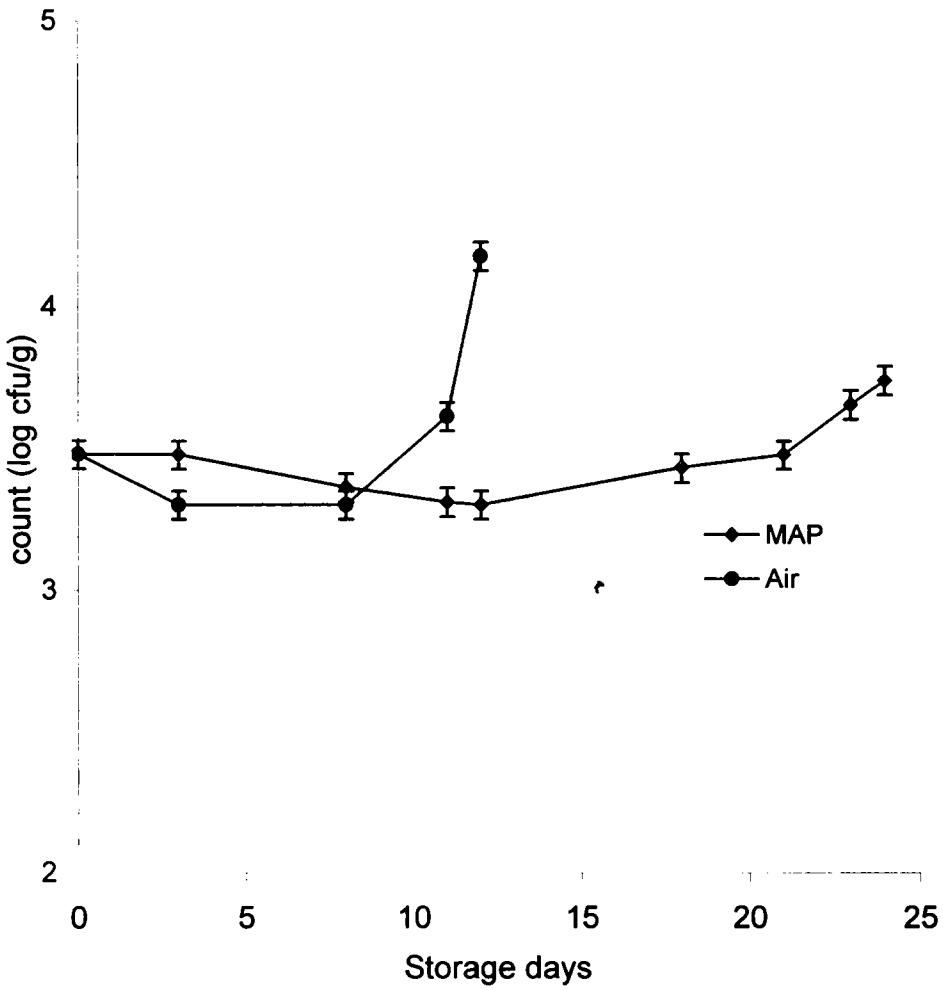


Plate 9: Lactic Acid Bacteria -MAP



Lactic Acid Bacteria on MRS Agar-
MAP(Etroplus suratensis)

Fig.20: Changes in Lactic Acid bacterial count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.8.4. Changes in H_2S producing bacterial count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Counts of H_2S producing bacteria gradually increased in pearl spot stored under air at 0-2°C and reached 6.8 log_{10} cfu g^{-1} at the time of sensory rejection (Fig.21). In pearl spot stored under MAP, the count increased slightly during the first week of storage, then decreased during the second week of storage and thereafter gradually increased and reached 6.8 log_{10} cfu g^{-1}. The result indicated the inhibition of H_2S producing bacteria caused by CO_2 enriched atmosphere. However at the time of sensory rejection H_2S producing bacteria mainly *Shewanella putrefaciens* count reached 10^7 cfu g^{-1} indicated the higher growth rate which could be attributed to the tolerance of these organisms to CO_2. *Shewanella putrefaciens* was noticed in pearl spot stored under MAP at 0-2°C. Growth rate and maximum concentration of H_2S producing bacteria was reduced when pearl spot was stored under MAP at 0-2°C. Similar results were reported earlier for cod stored under MAP (Jensen *et al.*, 1980; Cann *et al.*, 1983; Woyewoda *et al.*, 1984; Daniels *et al.*, 1985; Stenstrom,1985; Villemure *et al.*, 1986; Jorgensen *et al.*, 1988; Einarsson, 1992). *Shewanella putrefaciens* has been identified as the main spoilage bacteria of whole cod stored in ice and of chilled vacuum packed cod fillets (Gram *et al.*, 1987; Jorgensen and Huss 1989). 60% CO_2+ 40% O_2 mixture efficiently inhibited the Shewanella population. Lopez Galvez *et al.* 1995 and 1998 reported the same results in tuna and sole fillets stored under air and MAP.

Sulphide producing bacteria have been used as indicators of spoilage (Jorgensen *et al.*, 1988; Capell *et al.*, 1997). Sulphide producers often constitute a major proportion of the microbial flora of spoiling fish, the predominant sulphide-producing bacteria being *Shewanella putrefaciens* (Gram *et al.*, 1987; Gram, 1992). Gram and Huss, 1996 reported that when the number of these bacteria exceeds 10^6 cfu/g, significant amounts of volatile sulphur containing compounds are produced and spoilage becomes sensorily evident, while the levels of 10^8-10^9 cfu g^{-1} are generally required to cause spoilage in ice stored fish. CO_2 packing of marine fish from temperate waters inhibits the respiratory organisms like *Pseudomonas* and *Shewanella putrefaciens* and their number rarely exceed 10^5-10^6 cfu/g. In the present experiment H_2S producing bacteria mainly *Shewanella putrefaciens* count reached 10^7 cfu g^{-1}, indicating that the higher growth rate which could be attributed to the tolerance of these organism to CO_2. Similar observations were made by Gill and Molin (1991).

Plate 10: H_2S producing bacteria on Iron Agar: control

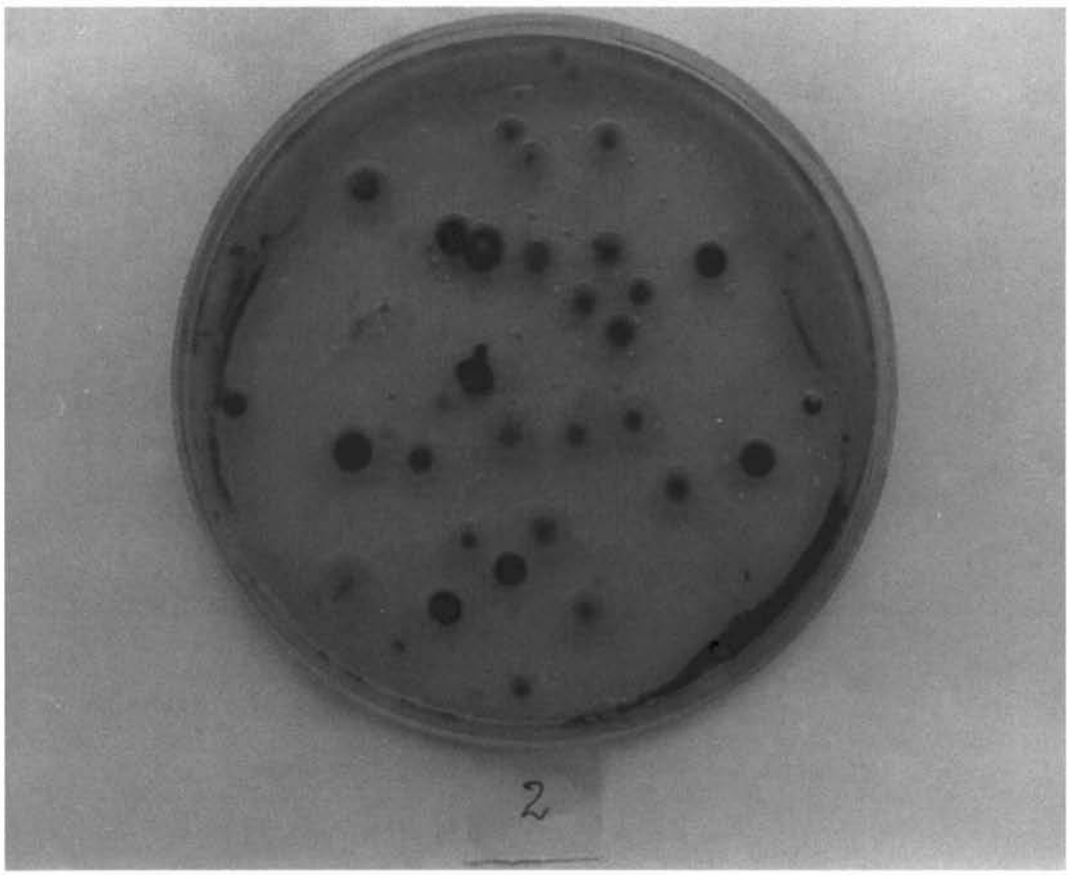


Plate 11: H_2S producing bacteria on Iron agar: MAP sample.

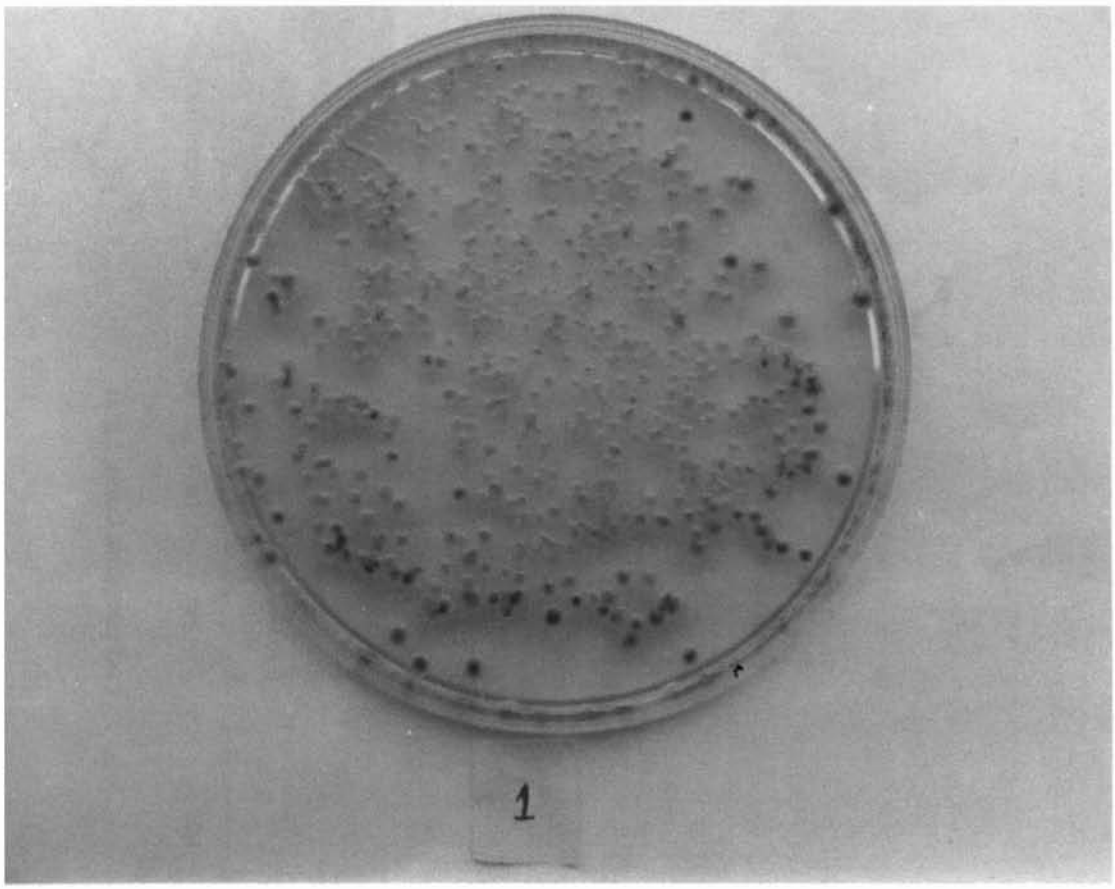
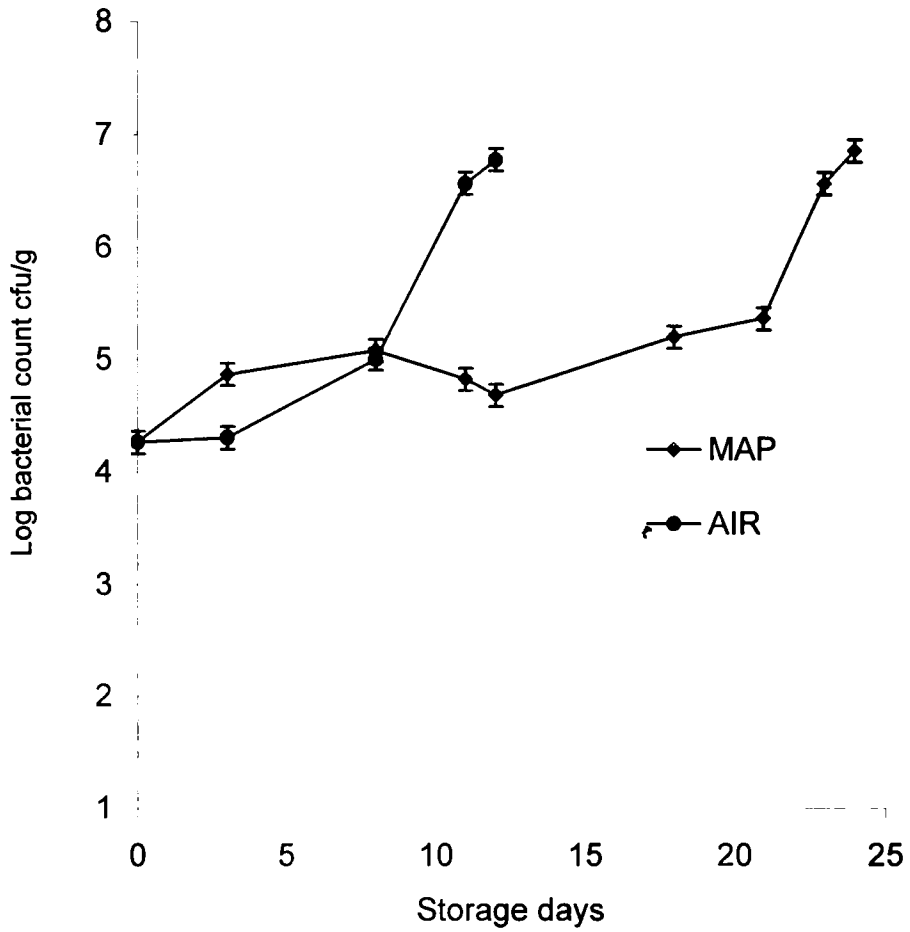


Fig.21: Changes in H_2S producing bacterial count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.8.5. Changes in *Pseudomonas* count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Pseudomonas spp. comprises only a small proportion of the initial micro flora of Pearl spot and the count was 2.9 log cfu/g. Their count gradually increased during storage under air and reached 7.2 log_{10} cfu g^{-1} at the time of sensory rejection (Fig 22). In pearl spot stored under MAP, the *Pseudomonas* count increased gradually after an initial decrease and the count reached 4.2 log_{10} cfu g^{-1} on 19th day of storage. Thereafter the count remained same during the entire storage period. The results indicated the inhibition of *Pseudomonas* caused by MAP (60% CO_2+ 40% O_2). The low level of Pseudomonas in MAP samples indicated that this flora was not important in the spoilage process of pearl spot stored under MAP whereas in pearl spot packed under air, this flora was one of the spoilage flora.

Plate 12: Pseudomonas on CFC agar

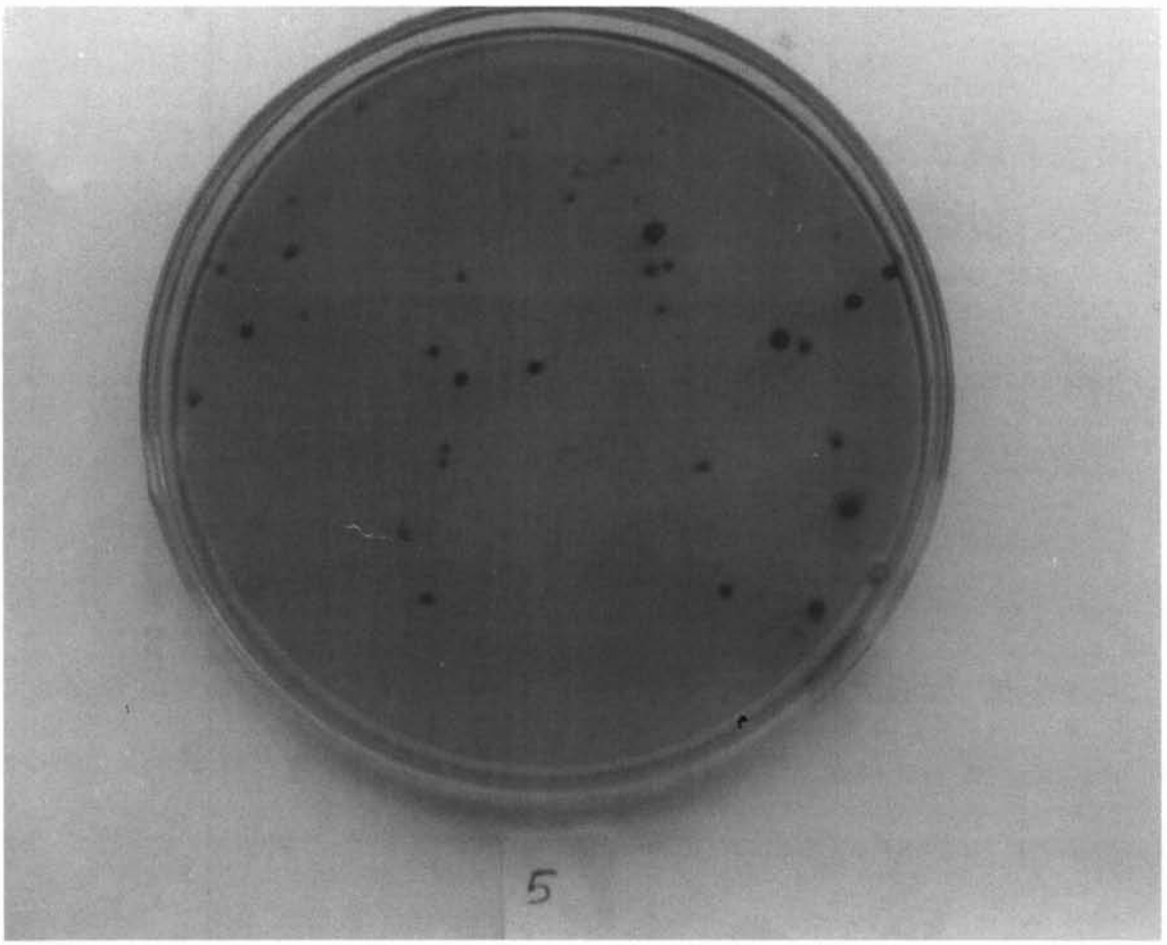
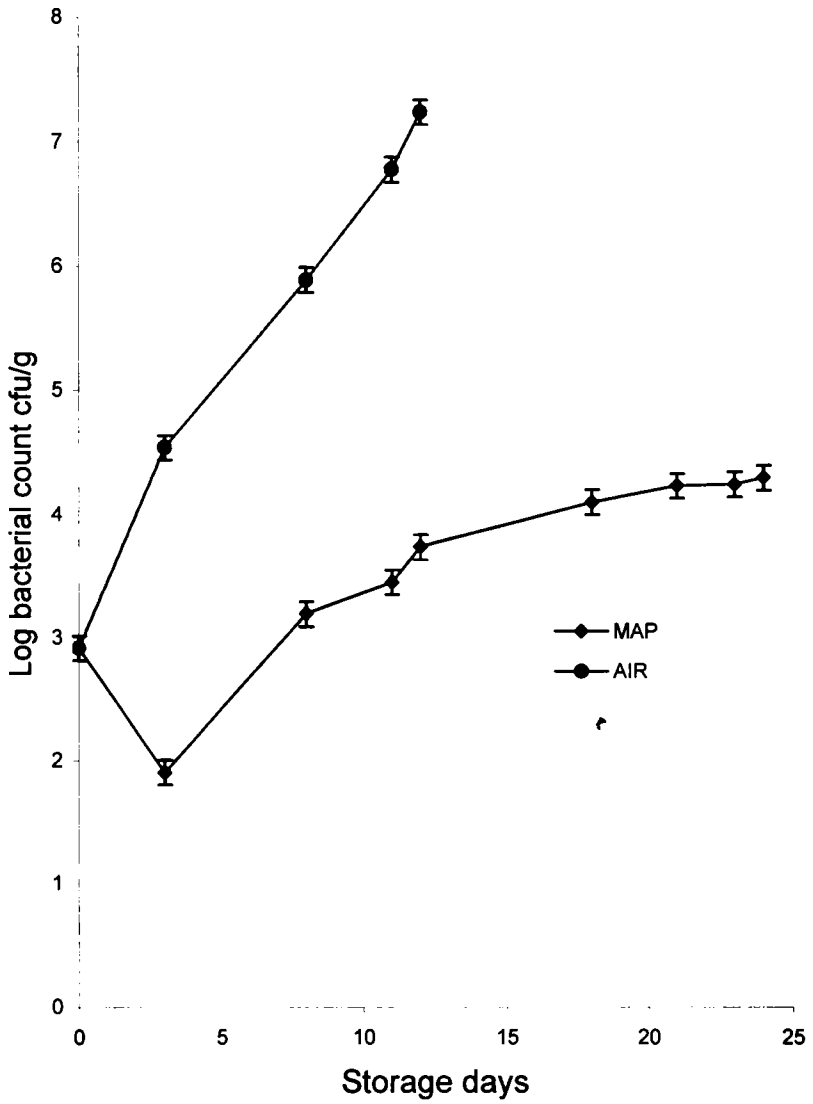


Fig.22: Changes in *Pseudomonas* count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



4.9.8.6. Changes in *Brochothrix thermosphacta* count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Brochothrix thermosphacta count (counts on STAA), was 2 log cfu/g initially, showed an almost convergent trend with that of Total Viable Count from the 18th day of storage in MAP packed Pearl spot and the count reached 7.2 log cfu/g on 23rd day of storage and 7.3 log cfu/g on 24th day of chill storage. Counts on STAA were ~ 3.3 log units lower than the TVC on 0 day of storage indicating that this flora is not dominant in the initial bacterial flora of pearl spot. However at the time of sensory rejection, *Brochothrix thermosphacta* accounted for ~ 90% of the TVC in samples stored in 60% CO_2+ 40% O_2. The count reached 6.9 log_{10} cfu g^{-1} in pearl spot stored under air at sensory rejection. (Fig 23). The results of the present study indicated that selection of Gram positive flora namely; *Brochothrix thermosphacta* by the synergy of CO_2 and low temperature and it is a well-known feature of the flora developed under modified atmosphere. This also indicated that the higher growth rate of *Brochothrix thermosphacta* than the remaining groups could be attributed to the tolerance of these organisms to CO_2. Similar observation were made earlier by Gill and Molin (1991). The high contribution of *Brochothrix thermosphacta* to the final climax population needs to be stressed. It suggests that this man made ecosystem favours the growth of *Brochothrix thermosphacta* over the other microorganisms. *Brochothrix thermosphacta* was reported to be the dominant

microorganism in Mediterranean fresh fish, gopa (*Boops boops*) (Drosinos and Nychas, 1996), white octopus (*Eledone cirrhosa*) and squid (*Toodaropsis eblanae*) (Paarup *et al.*, 1995) stored under vacuum or modified atmosphere conditions. Statham *et al.* (1985) and Fletcher *et al.* (1988) reported that *Brochothrix thermosphacta* was the dominant organism in morwong fish and scallops when these were treated with polyphosphates or potassium sorbate and then stored under 100% CO_2. In recent European studies, it has been suggested that *Photobacterium phosphoreum*, *Shewanella putrefaciens*, lactic acid bacteria and *Brochothrix thermosphacta* bacteria resistant to CO_2 are important for the spoilage of fresh fish stored either VP or MAP (Dalgaard, 1995; Gram and Huss, 1996; Drosinos and Nychas, 1996). In the climax population, there was a co-dominance of *B.thermosphacta* and *Shewanella putrefaciens* (only black colonies on iron agar medium). *Pseudomonas*, Lactic acid bacteria and *Enterobacteriacea* were also present in insignificant amounts at the end of storage.

Fig.23: *Brochothrix thermosphacta* count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

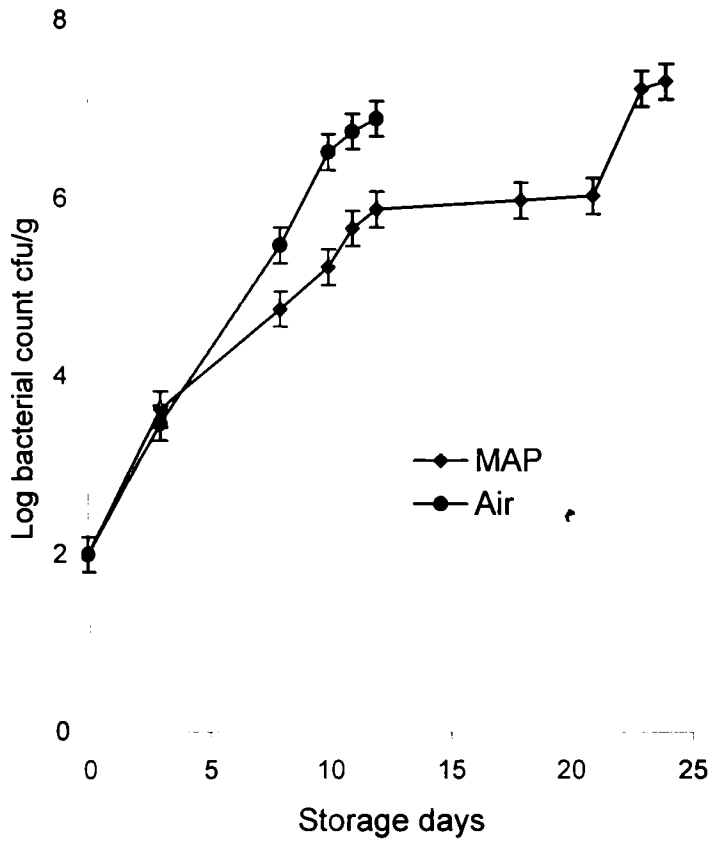


Plate 13*: Brochothrix thermosphacta* count on STAA agar control

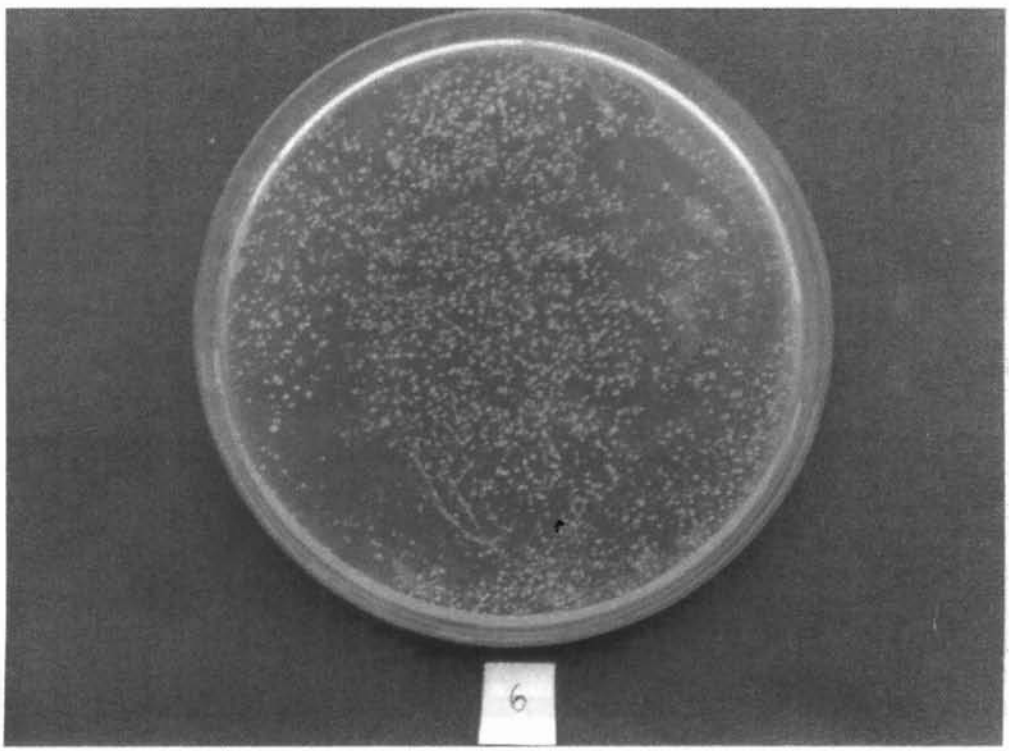
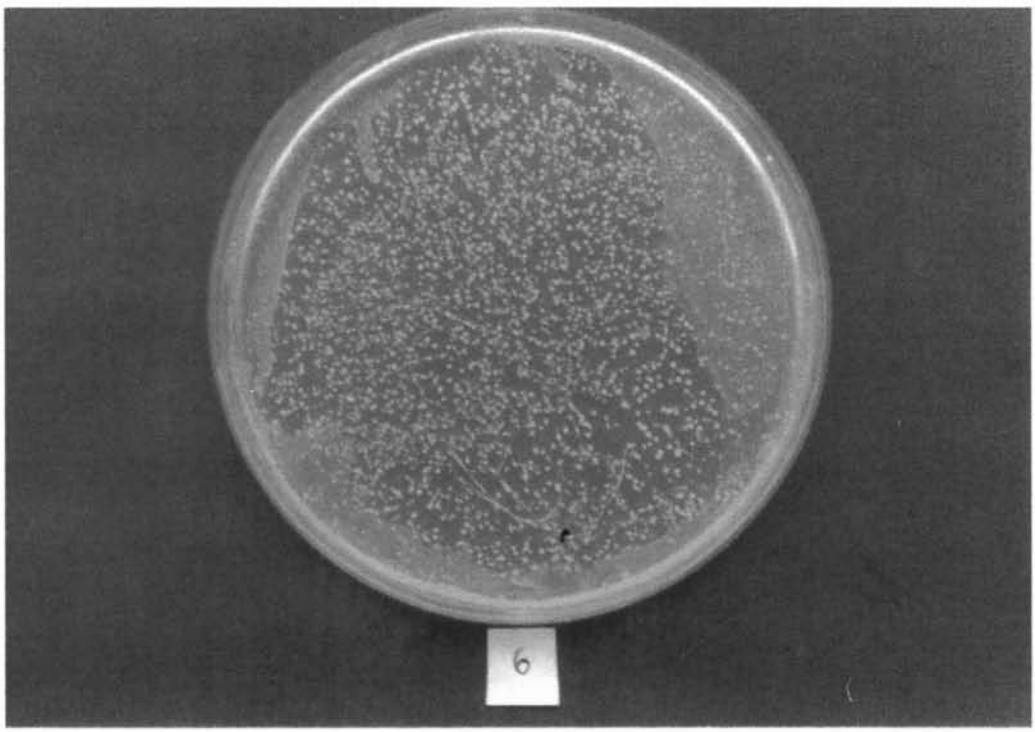
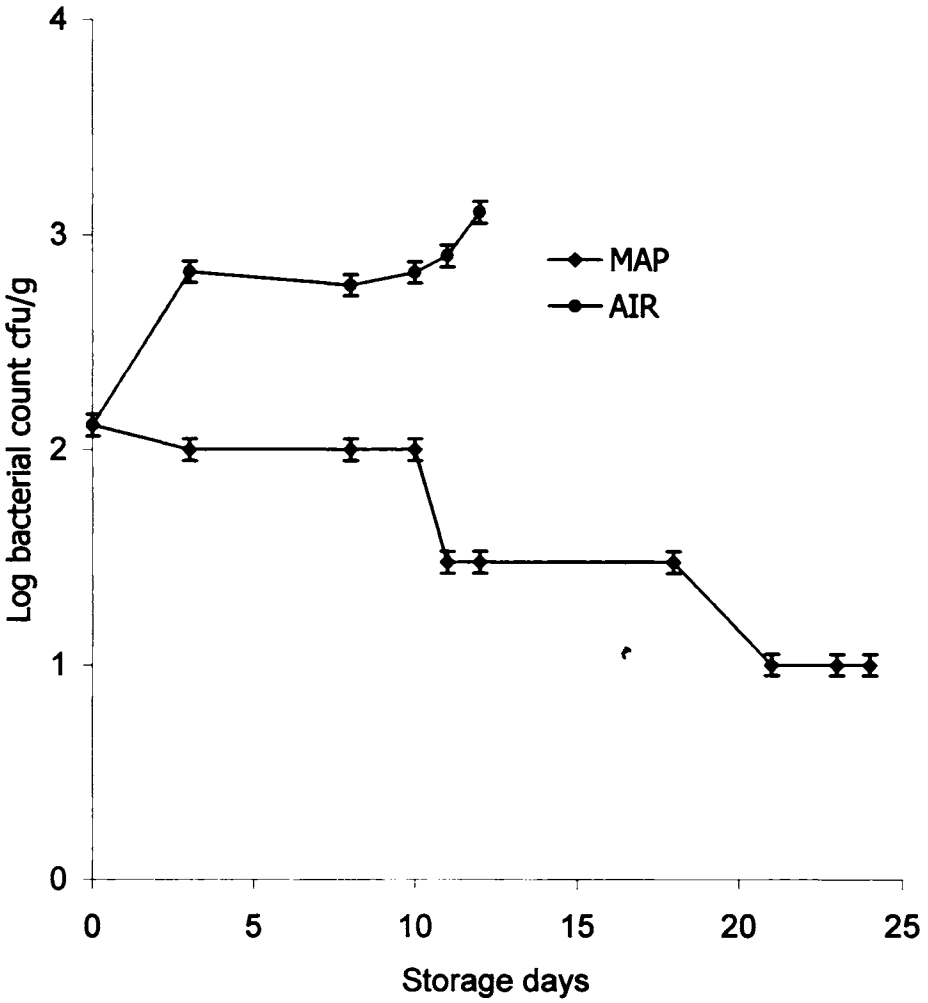


Plate 14: *Brochothrix thermosphacta* count on STAA agar MAP sample



4.9.8.7. Changes in Sulphite reducing Clostridial count in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

Sulphite reducing clostridial numbers were low initially. A gradual reduction was noticed during 3 weeks in MAP samples. The count remained < 10^1 cfu/g during the entire storage period. (Fig 24). This experiment proved that sulphite reducing clostridia was highly sensitive to chilling. Similar results were reported by Hobbs *et al.* (1965) and Barnes *et al.* (1963).


4
3
2
1
0
Log bacterial count cfu/g
MAP
AIR
0
5
10
15
20
25
Storage days

4.9.8.8. *Clostridium botulinum* toxin mouse bio assay

All the samples tested were found to be negative for *clostridium botulinum* toxin by mouse bioassay confirming that samples were safe throughout MAP storage.

4.9.8.9. Discussion

Bacterial counts of Pearl spot packaged in 60% CO_2+40% O_2 atmosphere showed a longer delay in bacterial growth than that of air packed Pearl spot . Counts remained at a relatively low level ($<10^7$ cfu/g) during 21 days, reaching value close to 10^7 cfu/g on 23rd day of storage.

Counts on MRS agar remained constant around 3.4 log cfu/g during 24 days of storage indicating the insignificance of these bacteria in MAP stored Pearl spot.

Counts on STAA were almost convergent with that of TVC from the 23 days of storage indicating the predominance of *B.thermosphacta* in 60% CO_2+40%O_2 enriched atmosphere.

Counts on Iron Agar were ~ 1 log unit lower than TVC throughout storage.

These results indicated that *B.thermosphacta* accounted for ~ 90% of the TVC in samples stored under modified atmosphere and the remaining 10% were *S. putrefaciens*.

Counts on VRBGA reached a value of 4.3 log cfu/g on 24th day of storage, which were 3.3 log cfu/g lower than TVC, whereas in air stored Pearl spot the count reached 6.1 log cfu/g on 12th day, indicating that this bacterial growth was efficiently inhibited by 60% CO_2.

The most important concern regarding MAP was potential toxin development in carbon dioxide enriched atmospheres, but mouse bio assay of all the samples

throughout the storage indicated that no toxin was developed during storage, confirming that if the temperature is properly controlled below 3.3°C, no toxin was developed and the most important precaution is the proper maintenance of temperature throughout storage

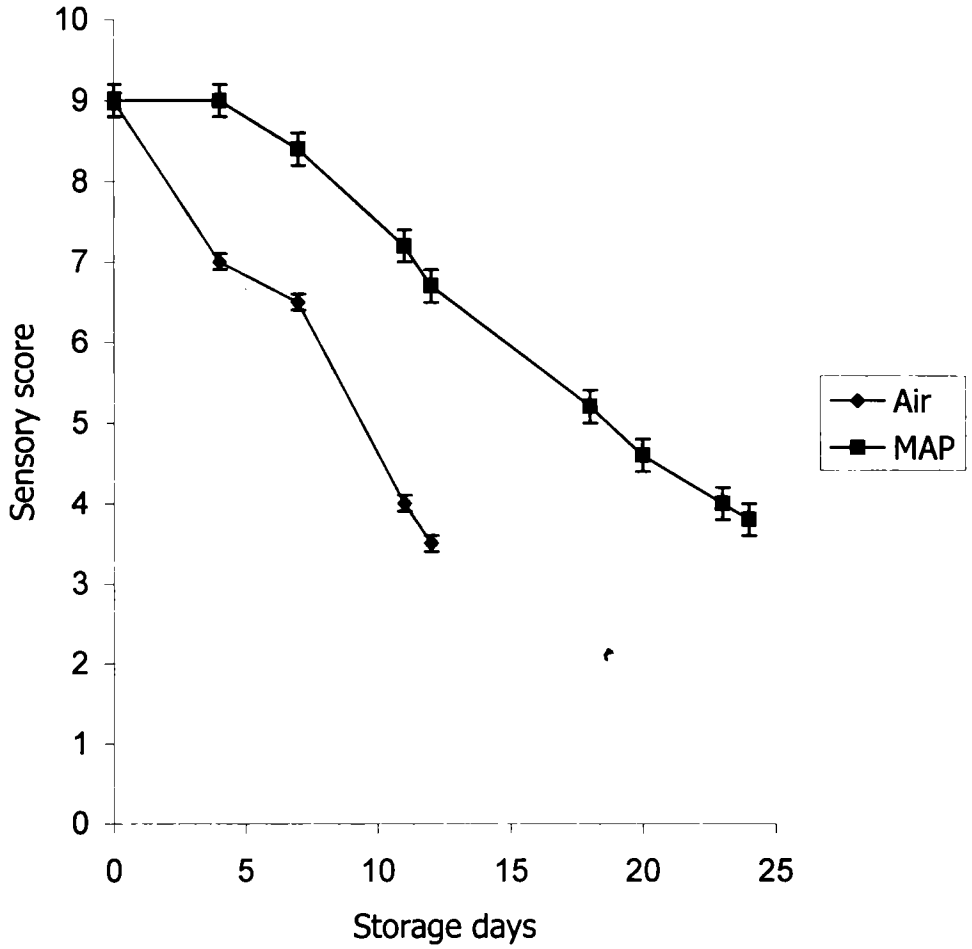
4.10. Sensory changes in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C

The changes in overall sensory scores of pearl spot stored under air and MAP packs and stored under chilled conditions are shown in Fig.25. There was significant decline in sensory score in control and MAP packs with storage period. From an initial sensory score of 9, the scores declined to 3.5 in control and 3.8 in the case of MAP packs on the day of rejection of 12 and 24 days respectively. A score of 4 was taken as the acceptable limit for determining the shelf life of pearl spot in ice. Control samples were acceptable upto 11 days whereas MAP samples remained in good condition upto 23 days. An extension of 12 days of shelf life was noticed due to packing under modified atmosphere. Based on the results it is observed that modified atmosphere packaging increases the shelf life of chilled pearl spot significantly. The samples are acceptable upto 11 days in control packs and 23 days in modified atmosphere packs. On 12^{th} day of air storage (when samples were sensory rejected), a noticeable off- odour (putrid, stale, pungent and strong marinade) was detected and the sensory score was 4.0 (Fig. 25). In the case of MAP stored Pearl spot, a sour odour indicative of spoilage was detected on 24^{th} day of storage. Samples were rejected by their odour after 23 days of storage indicating that the shelf life

of fish may be extended from 11 to 23 days. (Fig.25). Drosinos *et al.* (1996) reported that sensorial evaluation of the odour of raw fillets gave higher scores for samples stored under modified atmosphere than in control. Several literature reports indicate the use of modified atmosphere for preservation of salmon. Pastoriza *et al.* (1996) suggested that salmon slices stored under an atmosphere enriched with CO_2 are of high quality after 18 days at chilled storage although Haard and Lee (1982) observed a bland taste and powdery texture when fresh salmon was stored under pure CO_2. Brown *et al.* (1980) reported that an atmosphere of 40% CO_2 inhibited microbial growth and extended the sensory acceptance of salmon fillets. Since the modified atmosphere packaging of pearl spot revealed that *B.thermospacta* was the dominant organism in MAP stored pearl spot at the time of sensory rejection, the metabolism of *B.thermosphacta* is very important in the sensory rejection. Until recently, it was believed that Gram positive bacteria like Lactic acid bacteria and *B.thermosphacta* were of little importance for the sensory changes during storage of fish products and Leistner (1992) showed that no or very faint off odours were produced by Lactic acid bacteria compared to the very obnoxious off odours produced by Gram negative spoilers. However, Truelstrup Hansen (1995) found that several lactic acid bacteria were able to produce some of the off odours (sour, cabbage, sulphurous) associated with spoilage of cold smoked salmon. The metabolism of B.thermosphacta may be either aerobic or anaerobic. Under aerobic conditions, this bacterium generates acetoin and acetic, isobutyric and isovaleric acids (Dainty and Hibbard, 1980, 1983; Dainty and Hofman, 1983). On the other

hand under anaerobic conditions glucose is metabolized by B.thermosphacta yielding as the main metabolites L (+) Lactic acid and ethanol in a ratio of almost 3:1(Hitchener *et al.*, 1979). Hitchener *et al* (1979), Dainty *et al.*,(1979) reported that no acetoin and no or small amounts of short chain fatty acids are produces. Under 60% CO_2+40% O_2 mixture, the metabolism of *B.thermosphacta* followed an anaerobic path. The lower accumulation of short chain fatty acids (including acetic, isobutyric and isovaleric acids) and the higher content of lactic acid in the 60% CO_2 enriched atmosphere may have a beneficial sensory contribution for samples given that lactic acid has a less offensive flavors than that of the short chain fatty acids. Lopez Galvez *et al* (1998) reported similar microbiological association in sole fillets and therefore similar sensory rejection in sole fillets.

Fig. 25: Sensory changes in air packed and modified atmosphere (60% CO_2+ 40% O_2) packed pearl spot stored at 0-2°C



5.0 Summary and Conclusion

The shelf life was based on time for the total plate count to reach 10^7 cfu g-1, and sensorial changes upon opening of product as detected by a trained taste panel. Based on this data, the best gas mixture selected was 60% CO_2+ 40% O_2 mixture and the shelf life of pearl spot was terminated after 11 days for all air packaged samples. A two fold increase in shelf life was possible by packaging pearl spot under a modified atmosphere (60% CO_2+ 40% O_2).

Packaging material used for the study has low water vapour trasmission rate, oxygen trasmission rate and carbon dioxide trasmission rate. It has got enough strength to withstand machine handling. The overall migration residue (Water extractives) was below the acceptable limit for food contact application.

Headspace gas analysis proved an expected trend in gas concentrations. In pearl spot stored under air an increase in microbial growth resulted in increased CO_2 concentration whereas in pearl spot stored under MAP, the initial decrease was due to absorption of CO_2 in fish tissues and after that microbial growth resulted in increased CO_2 concentration. Decrease in O_2 concentration was mainly due to respiration of bacteria.

Colour measurements revealed not much difference in pearl spot stored under air and MAP. MAP stored samples were more bright compared to air stored samples and this may be due to the bleaching effect of carbon dioxide.

Measurements of pH revealed that pearl spot stored under MAP had lower pH values compared to pearl spot stored under air. Dissolution of CO_2 in muscle tissues resulted in lower pH values for pearl spot stored under MAP. Increase of

only 0.47 unit was observed in the case of pearl spot stored under MAP during storage. Increase in pH was associated mainly to volatile amine production as a consequence of bacterial activity

In modified atmosphere packaging thaw drip was considered as a major problem, but in the present trial, thaw drip % was lower in the case of MAP stored pearl spot than in air stored pearl spot.

Lipid oxidation was found to be insignificant in pearl spot stored under air. In pearl spot stored under 60% CO_2+ 40% O_2 atmosphere, the TBA value reached 2.2 mg malonaldehyde/kg of fish on 24th day of chill storage. Increased lipid oxidation observed in pearl spot stored under MAP may be due to increased oxygen concentration (40%) in the pack. Rancidity development is observed only from 24th day onwards when the fish was rejected sensorily. Therefore TBA value correlated well with sensory score and can be used as a chemical index in the case of pearl spot stored under MAP.

Identical K values were obtained for Pearl spot packed in either aerobic or carbon dioxide modified atmosphere. This indicated that presence of CO_2 did not alter the rate of degradation of adenine nucleotides. K value of Pearl spot increased during storage of air and modified atmosphere packed Pearl spot, however modified atmosphere packed Pearl spot were still acceptable sensorily even at high K values. K values were independent of sensory spoilage and correlate only with the length of storage of MAP packed Pearl spot.

TMA value remained constant throughout storage, i.e. 5.6 mg TMA/100 g of fish in both air and MAP stored packs. In the present trial the original TMAO present

may be very low, therefore considerable TMA production was not noticed, further TMAO reducing bacteria, *Shewanella putrefaciens* never reached the 10^8 cfu/g in the case of pearl spot stored under both air and MAP at the time of sensory rejection.

Initial TVBN value was 14 mg/100g of fish. TVBN values increased gradually in pearl spot stored under air and MAP. TVBN value increased to 22.4 mg N/100g in pearl spot stored under air on 12^{th} day of chill storage and the value reached 19.6 mg N/100g in pearl spot stored under MAP on 24^{th} day i.e. on rejection day. But these values were well within the limit. TVN did not reach 30mg/100g threshold of initial spoilage in Pearl spot during 12 days of air storage and 24 days of modified atmosphere storage.

The time course of the changes revealed that the textural characteristics were retarded in pearl spot stored under MAP compared to the control. Hardness was lower in MAP stored pearl spot compared to air stored pearl spot and in air stored pearl spot, hardness was gradually decreasing while in MAP stored samples after an initial decrease it maintained almost constant value throughout. Initial cohesiveness was 0.2. In the case of pearl spot stored under air, as well as in MAP and the value remained almost constant through out chill storage.

The initial springiness was 1.5 mm. In the case of pearl spot stored under air, the springiness decreased during storage, whereas in pearl spot stored under MAP, the value decreased to 1.1 on 3^{rd} day and remained constant throughout storage. In the case of pearl spot stored under air, chewiness decreased gradually and reached a value of 0.28 on 12^{th} day of chill storage. In the case

of pearl spot stored under MAP, the value reached 0.25 on 7^{th} day and remained constant throughout storage upto 24 days.

The mesophilic counts exceeded the limit count of 10^7 cfu g^{-1} in pearl spot packed under air and MAP respectively on 12^{th} and 24^{th} day of storage when the samples were rejected based on sensory scores. Bacterial growth was delayed when pearl spot was packaged in 60% CO_2 + 40% O_2 mixture. An extension of shelf life of 12 days was noticed in pearl spot packed under MAP compared to that stored under air.

In MAP, an extension of lag phase of 11 days was observed and the log phase was apparently extended. CO_2 has an inhibitory effect on microbial growth, exerting a selective inhibitory action. Aerobic microorganisms were generally sensitive to CO_2; therefore MAP delayed the spoilage of fish and other seafood. The overall effect of modified atmosphere packaging is the suppression of bacterial growth and subsequent extension of shelf life. The total mesophilic counts of 7.0 log_{10} cfu g^{-1} is considered as the upper limit of acceptability, shelf life of air stored Pearl spot was 11 days and 23 days when packed in 60% CO_2 + 40% O_2.

Total psychrotrophic viable counts increased in pearl spot stored under air and MAP during storage. In pearl spot stored under air, the psychrotrophic count reached 7.3 log_{10} cfu g^{-1} on 11^{th} day and remained same on 12^{th} day of storage. In pearl spot stored under MAP, the count reached 7.4 log_{10} cfu g^{-1} on 23^{rd} day and the count reached 7.6 log_{10} cfu g^{-1} on 24^{th} day of storage. Carbon dioxide delayed bacterial growth, acting preferentially on Gram negative flora.

The low numbers of LAB found in this study indicated that this flora was without any quantitative importance for the spoilage process. Lactic acid bacterial growth in pearl spot was limited.

Counts of H_2S producing bacteria gradually increased in pearl spot stored under air at 0-2°C and reached 6.8 $\log_{10}$ cfu g^{-1} at the time of sensory rejection. In pearl spot stored under MAP, the count increased slightly during the first week of storage, then decreased during the second week of storage and thereafter gradually increased and reached 6.8 $\log_{10}$ cfu g^{-1}. The result indicated the inhibition of H_2S producing bacteria caused by CO_2 enriched atmosphere. However at the time of sensory rejection H_2S producing bacteria mainly *Shewanella putrefaciens* count reached 10^7 cfu g^{-1} indicated the higher growth rate which could be attributed to the tolerance of these organisms to CO_2. *Shewanella putrefaciens* was noticed in pearl spot stored under MAP at 0-2°C. Growth rate of H_2S producing bacteria was reduced when pearl spot was stored under MAP at 0-2°C.

Pseudomonas spp. comprises only a small proportion of the initial micro flora of Pearl spot and the count was 2.9 log cfu/g. Their count gradually increased during storage under air and reached 7.2 $\log_{10}$ cfu g^{-1} at the time of sensory rejection. In pearl spot stored under MAP, the Pseudomonas count increased gradually after an initial decrease and the count reached 4.2 $\log_{10}$ cfu g^{-1} on 19th day of storage. Thereafter the count remained same during the entire storage period. The results indicated the inhibition of *Pseudomonas* caused by MAP (60% CO_2+ 40% O_2). The low level of *Pseudomonas* in MAP samples indicated

that this flora was not important in the spoilage process of pearl spot stored under MAP whereas in pearl spot packed under air, this flora was one of the spoilage flora.

Brochothrix thermosphacta count (counts on STAA), was 2 log cfu/g initially, showed an almost convergent trend with that of Total Viable Count from the 18th day of storage in MAP packed Pearl spot and the count reached 7.2 log cfu/g on 23rd day of storage and 7.3 log cfu/g on 24th day of chill storage. The count reached 6.9 log cfu g^{-1} in pearl spot stored under air at sensory rejection. The results of the present study indicated that selection of Gram positive flora namely; *Brochothrix thermosphacta* by the synergy of CO_2 and low temperature and it is a well-known feature of the flora developed under modified atmosphere. This also indicated that the higher growth rate of *Brochothrix thermosphacta* than the remaining groups could be attributed to the tolerance of these organisms to CO_2. The high contribution of *Brochothrix thermosphacta* to the final climax population needs to be stressed. It suggested that this man made ecosystem favours the growth of *Brochothrix thermosphacta* over the other microorganisms.

On 12th day of air storage (when samples were sensorily rejected), the identification of the colonies on the PCA plates revealed that 95% of these were Gram negative, oxidase positive organisms, which could indicate that the spoilage flora was dominated by Gram negative psychrotrophs, probably *Pseudomonas* and *Shewanella putrefaciens*, *Aeromonas*, *Brochothrix thermosphacta* and *Enterobacteriacea* also contributed in final population. On 24th day of MAP storage

(when samples were sensorily rejected), the identification of the colonies on the PCA plates revealed that *Brochothrix thermosphacta* and *Shewanella putrefaciens* were mainly contributed in the final population.

All the samples tested were found to be negative for *clostridium botulinum* toxin by mouse bioassay confirming that samples were safe throughout MAP storage.

In conclusion, the use of modified atmosphere packaging (MAP)(60% CO_2+ 40% O_2) can double the shelf life of fresh pearl spot in fresh form at refrigerated storage conditions. Such an extension in shelf life and keeping quality of fish and reduction in frozen storage costs made this method superior to other methods of fish preservation.

6.0 CODE OF PRACTICE FOR MAP FISH

National Advisory Committee on microbiological criteria for foods (NACMCF) report on Modified Atmosphere Packaging for Refrigerated Raw Fishery Products (1992)

NACMCF examined microbiological safety issues associated with modified atmosphere packaging of refrigerated raw fishery products. They were as follows.

1. Raw fish quality

Prior to packaging proper handling of raw fish must be assured from the point of harvest. MAP must not be used to extend the shelflife of fish whose quality has deteriorated.

2. Hazard Analysis Critical Control Point Plan.

A HACCP plan from point of harvest till it is marketed must be developed for MAP, recognizing that rigid temperature control is the primary preventive measure to ensure safety.

3. Hazard Analysis/ Risk Assessment.

The studies to support hazard analysis/risk assessment must be completed by food safety experts who are competent in HACCP systems, *Clostridium botulinum* methodology, sensory evaluations, and statistical procedures.

The evaluated system cannot be used for marketing fish unless both the experimental results and the statistical modeling demonstrate that odour rejection will always precede toxin production. If this is not demonstrated, i.e., if

toxin production precedes odour rejection, MAP technology cannot be used for the species of fish studied.

No new study to demonstrate the efficacy and safety of the process is necessary as long as the data from the basic study can be applied. A new study, including the inoculated pack and shelflife, however, is necessary when different species of fish are used, a new ingredient is added, a different gas composition is proposed, or the distribution systems or targeted users have substantially changed.

The studies to support hazard analysis/ risk assessment of MAP include the following:

A. Process Description

A HACCP – based description of the species of fish investigated, the handling procedures and the gas composition must be developed.

B. Inoculated Pack Studies

Inoculated pack studies must be performed for each type of fish and packaging technology to confirm the efficacy and safety and to guide the development of an appropriate distribution strategy. All studies must be conducted in a facility that has the safeguards in place to work with *Clostridium botulinum.*

C. Sensory Evaluation

A sensory evaluation method using specific odour rejection criteria to determine the onset of spoilage must be selected, standardized, and accepted.

D. Statistical Evaluation.

Predictive Statistical models must be based on the experimental data to evaluate the potential of toxin production before odour rejection.

4. System Design Validation

Use the results of the inoculated pack and sensory evaluation study to determine whether the system design is effective and safe. Modify the processing system where necessary. Perform additional inoculated pack and sensory evaluation studies if the system is modified. Validate the HACCP plan. The total processing system and associated HACCP system must be approved by food safety experts competent in assessing the potential food safety hazards.

5. Labeling Requirements

Labeling requirements must include:

A. Keep refrigerated at 3.3 °C or below.

B. Use by date.

C. Cook fully before serving.

The use-by date must be experimentally determined, and should be a date at which product quality is still maintained.

6. Time and Temperature Records.

Time/temperatures recorders should be enclosed in selected sealed master cartons during shipment of seafood products at the time each shipment is received, the individual time temperature charts should be examined and the shipment or carton rejected if the product temperature exceeds 3.3°C.

Technology, reliability, and costs of time/temperature integrators are to be developed to the point where their use becomes very practical.

Recommended practice for MAP products.

1. The unrestricted use of modified atmosphere packaging technology for refrigerated raw fishery products not to be permitted.
2. The restricted use of MAP for refrigerated raw fishery products can be considered only if sufficient safeguards detailed in an established HACCP plan can be implemented and verified to control the specific safety concerns regarding the product.
3. MAP technology be permitted only when it is assured that detectable spoilage and rejection by the consumer precedes the possibility of toxin production.
4. MAP should be used only with high quality raw fish. It must not be used to extend the shelflife of fish whose quality has deteriorated.
5. MAP products must be held at or below 3.3°C at all points from packaging through final preparation. This temperature requirement must be clearly indicated on shipping cartons and retail package labels. There should not be any abuse of temperature during storage and transportation.
6. Secondary measures in addition to refrigeration must be employed to increase assurance of product safety. These include additional labeling requirements and stringent processing controls.
7. MAP technology must ensure that the intended vacuum or intended MAP gas compositions are achieved with appropriate films.

8. The minimum conditions for MAP technology as described above must be followed.

9. In India it can be effectively applied for highly priced table fishes like seer, pomfret, pearl spot etc

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