NOTE TO USERS

Safety and Quality of High Pressure (HP) Treated Fish: Evaluation of Pressure Destruction Kinetics of Pathogens and Associated Quality Changes During Storage

By

Shafi Ullah Zaman

Department of Food Science and Agricultural Chemistry
Macdonald Campus of McGill University, Montreal
Quebec, Canada

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DEDICATION

To my Loving Parents who helped and supported me to reach where I stand today...

ABSTRACT

High pressure processing (HPP) has been emerging as a novel technique for extending the shelf-life and safety of processed food. This study is aimed at evaluating the safety and quality of pressure treated fish. In order to assess safety of refrigerated fish, the sensitivity of key pathogens like *Escherichia coli O157:H7* and *Listeria monocytogenes* Scott A, which thrive under refrigerated storage conditions, needs to be assessed. Furthermore, pressure treatment should not adversely affect the sensory qualities of the treated fish.

In the first part of the study, the pressure destruction kinetics of *E. coli* and *L. monocytogenes* in fish were evaluated at 250 to 400 MPa with a holding time ranging from 0-60 min. A slurry was prepared by blending 20 g filleted fish and 80 ml sterile peptone water (0.1%) in a stomacher. To the slurry, stock cultures of *E. coli 0157:H7* and *L. monocytogenes* were added separately and final counts of 10^7 and 10^6 CFU/ml were achieved, respectively. The inoculated slurry was packaged in sterile pouches and subjected to selected pressure treatments, after which the surviving population were enumerated on strain selected media. Destruction kinetics were evaluated based on a dual behaviour with a combination of pulse effect due to pressurization/depressurization cycle without holding and a first order rate hold time (D-value approach) effect during the pressure treatment. The pressure sensitivity D-value was assessed based on a z-value approach with z_p indicating a pressure range between which the D-values change by an order of 10. *E. coli* was more sensitive to pressure pulse than *L. monocytogenes.* Based on D-values, *E. coli* was more resistant than *L. monocytogenes* at higher pressures ($\geq$350 MPa, pressures likely to more practical), while at lower pressures (<350 MPa) *L. monocytogenes* was more resistant. D-values at 400 MPa were 3.19 and 1.49 min for *E. coli* and *L. monocytogenes. E. coli* (z_p = 185 MPa) was less sensitive to changes in pressure than *L. monocytogenes* (z_p = 103 MPa). Due to the higher overall pressure resistance at 400 MPa, *E. coli* was selected as the target microorganism in this study for pressure destruction.

The second part of the study focused on storage studies. The first phase of the storage study was a repeat of previous set of experiments with fish slurry inoculated with only *E. coli O157:H7* which was more resistant to pressure destruction (challenge study).

The treatments were designated to result in a pressure lethality of 0D (control), 6D, 8D and 10D based on the D-value for *E. coli*. Test samples were evaluated for survivors immediately after the treatment as well as during refrigerated storage to ensure no regeneration during the storage. Results indicated that the *E. coli* counts were below detection levels immediately after treatment and during subsequent storage at 4°C. However, their recovery during storage at 20°C indicates, that even at 10D, some cells may not be completely dead but injured/shocked and fail to grow on the selected media.

The second phase of storage studies evaluated the quality changes associated with pressure treated fish. Freshly cut and filleted mackerel (*Scomber scombrus*) were packaged in sterile pouches and sealed. Pressure treatment was applied to achieve a pressure lethality of 0D (control), 6D, 8D and 10D based on the D-value of *E. coli O157:H7*. The pressure treated samples were analyzed for microbial survivors (aerobic plate counts), texture, color, drip loss and pH during storage. Estimated shelf-life of treated fish based on microbial counts (time required to regain the original microbial load) and quality changes during storage at 4, 12 and 20°C were analyzed for up to five weeks. Microbial growth during storage was well described by a first order growth model. Quality parameters drip loss, pH, color parameters - L*, a* and b* values, texture parameters - hardness and springiness were affected by pressure treatment, storage time and temperatures. The shelf-life of fish based on microbial growth basis was 29 days at 4°C storage temperature with a 10D pressure lethality treatment. The results demonstrated high pressure treatment could be successfully used to produce safe and high quality fish with an extended refrigerated shelf-life.

RÉSUMÉ

Un traitement par haute pression s'est révélé comme étant une nouvelle technique pour prolonger la durée de vie d'un produit et assurer l'innocuité des aliments transformés. Cette étude est destinée à l'évaluation de l'innocuité et de la qualité de poisson traité par haute pression. Pour évaluer l'innocuité du poisson réfrigéré, la sensibilité de pathogènes cibles comme *Escherichia coli O157:H7* et *Listeria monocytogenes* Scott A qui prolifèrent dans des conditions d'entreposage réfrigéré doit être évaluée. En outre, le traitement de pression ne devrait pas affecter défavorablement les qualités sensorielles du poisson.

Dans la première partie de l'étude, la cinétique de destruction par pression *d'E. coli* et *L. monocytogenes* dans le poisson a été évaluée à 250 jusqu'à 400 MPa avec un temps de retenue entre 0 et 60 min. Les échantillons ont été préparés en mélangeant 20 g poisson fileté et 80 g d'eau peptonée stérile (0.1 %) dans un stomacher. Les cultures *d'E. coli 0157:H7* et de *L. monocytogenes* ont été ajoutées séparément pour obtenir des comptes finaux de 10^7 et 10^6 UFC/ml respectivement. Le mélange inoculé a été placé dans des sacs stériles et soumis aux traitements de pression choisis. Ensuite, la population survivante a été dénombrée sur des milieux de croissance sélectifs. La cinétique de destruction a été évaluée en se basant sur un double comportement avec une combinaison d'impulsions due au cycle de pressurisation/dépressurisation sans rétention et un premier taux de retenue (approche de la valeur de D) pendant le traitement. La sensibilité à la pression la valeur de D a été évaluée en se basant sur une approche de la valeur de Z avec z_p comme indicateur d'une étendue de pression entre laquelle le changement de la valeur de D est de l'ordre de 10. *E. coli* étaient plus sensibles aux impulsions de pression *que L. monocytogenes*. En se basant sur les valeurs de D, *E. coli* étaient plus résistants *que L. monocytogenes* aux hautes pressions (des pressions de $\geq$350 MPa, sont plus pratiques), tandis qu'aux pressions inférieures (< 350 MPa), *L. monocytogenes* étaient plus résistants. Les valeurs de D à 400 MPa étaient 3.19 et 1.49 minutes pour *E. coli* et *L. monoctygenes*. *E. coli* (z_p = 185 MPa) étaient moins sensibles aux changements de pression que *L. monocytogenes* (z_p = 103 MPa). En raison de la résistance générale à la pression de 400 MPa, *E. coli* a été choisi comme le microorganisme cible dans cette étude pour la destruction par traitement de pression.

La deuxième partie de l'étude s'est concentrée sur des études d'entreposage. La première phase de l'étude d'entreposage était une répétition de précédentes expériences avec le mélange de poisson inoculé avec seulement *E. coli O157:H7* qui était plus résistant à la pression (défi de l'étude). Les traitements ont été désignés afin d'aboutir à une mortalité, due à la pression, de 0D (témoin), 6D, 8D et 10D en se basant sur la valeur de D de *E. coli*. Les échantillons ont été évalués afin de dénombrer les survivants immédiatement après le traitement et pendant la période d'entreposage réfrigéré pour assurer qu'il n'y ait aucune régénération. Les résultats ont indiqué que les comptes d'*E. coli* était au-dessous des niveaux de détection immédiatement après le traitement et pendant l'entreposage à 4°C. Cependant, leur rétablissement pendant le stockage à 20°C indique que même à 10D, quelques cellules ne peuvent pas être complètement détruites, mais peuvent être inhibées et ne peuvent se multiplier sur des milieux sélectifs.

La deuxième phase de l'étude de l'entreposage a été d'évaluer les changements de qualité associés au poisson traité par pression. Du maquereau (Scomber scombrus) fraîchement coupé et fileté a été placé dans des sacs stériles et scellés. Le traitement de pression a été appliqué pour parvenir à une destruction de 0D (témoin), 6D, 8D et 10D en se basant sur la valeur de D de *E. coli* O157:H7. Les échantillons ont été analysés en recherchant les survivants (compte aérobique). La texture, la couleur, la perte d'eau et le pH pendant l'entreposage ont aussi été analysés. La durée de vie estimée d'un produit de poisson traité, basée sur des comptes microbiens (temps nécessaire pour atteindre la charge microbienne initiale) et les changements de qualité pendant le stockage à 4, 12 et 20 °C ont été analysés sur une période de cinq semaines. Les augmentations de la microflore ont été évaluées sur la première base de cinétique. Les paramètres de qualité; la perte d'eau, le pH, les valeurs de L*, b* et de a*, la dureté et l'élasticité ont effectuer par traitement de haute pression, stockage, les temps et la température. La durée de vie d'un produit de poisson basé sur des comptes microbiens était de 29 jours à 4°C avec un traitement létal de10D. Les résultats ont démontré que le traitement par hautes pressions pourrait être utilisé avec succès pour produire un poisson sûr et de haute qualité avec la durée de vie d'un prolongée.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adinosine diphosphate
DMA	Dimethylamine
DSC	Differential Scanning Calorimetry
IMP	Inosine monophosphate
INO	Inosine
HP	High Pressure
HPP	High Pressure Processing
Hx	Hypoxanthine
LPS	Lipopolysaccharide
PEF	Pulsed Electric Field
POV	Peroxide value
PUFAs	Polyunsaturated Fatty Acids
TBA	Thiobarbituric acid
TMA	Trimethylamine
TMAO	Trimethylamine oxide

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Chapter 1

INTRODUCTION

Consumption of fish has been increasing tremendously around the globe because of its high nutritive value and consumers' awareness of health benefits associated with food prepared from fish. The world seafood capture showed a considerable increase from 91,708,499 metric tonnes in 1994 to 94,848,674 metric tonnes in 2000 (Food and Agricultural Organization (FAO), 2000). The high biological value proteins, unsaturated fatty acids, minerals and vitamins present in fish make them an excellent choice food for inclusion in the human diet (Sidhu, 2003). Fish contains enough quantity of essential amino acids and fatty acids required for human nutrition. The incidence of heart disease (Bang and Dyerberg, 1980), cancer (Rose, 1997), diabetes (Berry, 1997) and depression (Adams et al., 1996) are believed to be considerably lowered with consumption of fish due to presence of omega-3 polyunsaturated fatty acids (PUFAs). The PUFAs are directly associated with beneficial health effects in humans thereby causing high demand of fish and fish products (Sargent and Tacon, 1999).

Along with being a rich and healthy source of food, fish is also highly susceptible to spoilage from the time it is caught. The main causes of fish spoilage are believed to be enzymatic reactions, microbial growth and chemical changes which accelerate once the fish dies. Different methods have been used for processing and preservation of fish in order to prevent these undesirable changes in fish quality. Techniques used to preserve and extend the shelf-life of fish include refrigeration (ice storage), freezing, drying, salting, smoking and canning. Among these techniques, refrigerated storage provides

fresh fish with a limited short term storage-life. Salting as a preservation method considerably increases shelf-life although it is reported to be insufficient to stop the growth of spoilage and pathogenic microorganisms (Horner, 1992). Canning of fish using heat sterilization tremendously increases the shelf-life of fish even when stored at room temperatures. However a higher loss in sensory properties and nutritional value of the product occurs when fish is thermally processed into canned fish. Freezing gives a shelf-life of greater than six months, especially when fish is vacuum packaged to prevent oxidative changes. Frozen thawed fish is the closest alternative to fresh fish. The advantages of freezing include a relatively high energy cost associated with freezing and frozen storage. Some nutritional losses take place in frozen fish especially; proteins and fatty acids are susceptible to changes under the freezing/thawing conditions.

Refrigerated storage of fish on ice (0-4°C) is a common technique used to keep fish fresh and one can expect about 14-18 days shelf-life. Under these conditions a shelf-life of the 18 days can be expected. Although the quality and textural degradation are minimized with refrigeration, these changes continue to take place during refrigerated storage. The microbial and enzymatic spoilage are the main factors of spoilage of fish under these refrigerated storage conditions. The psychrotrophic microorganisms include *Pseudomonas, Alteromonas, Flavobacterium, Achromabacter, Shewanella, Acinetobacter* and *Vibrio* spp. have been implicated in the spoilage of fish during storage (Hubbs, 1991). Pathogens such as *Escherichia coli, Listeria monocytogenes, Campylobacter* and *Salmonella* species, introduced during handling and processing, can also cause serious foodborne illnesses (Ingham, 1991). These techniques do not assure safety of the product and only provide a short term extension of shelf-life under refrigerated storage conditions.

In order to improve the shelf-life and safety of these products, refrigerated storage needs to be supplemented with additional processing and/or preservation techniques which would help to prevent growth of spoilage and pathogenic microorganisms.

As we turn in to the new century, consumers will be more sophisticated and discretionary, demanding safer, low fat, high quality; additive free and convenience oriented foods. In order to be competitive in both the domestic and international market, the food processor not only needs the best from the existing technologies, but also needs to benefit from the latest ones offering state of the art technologies. Processing methods and/or novel combinations of existing methods are continually being investigated by the industry in the pursuit of producing better quality foods more economically. High pressure processing (HPP) is an innovative technological concept that has great potential for extending the shelf-life of foods with no heat treatment. Other major advantages include additive-free processing at low temperatures to maintain the food essentially fresh. Other techniques such as pulsed electric fields (PEF), high-intensity pulsed lights, high intensity pulsed-magnetic field, ozone treatment, irradiation and ultra-filtration have also been tried as potential alternatives in recent years (Leadley and Williams, 1997). Among new technologies, HPP is gaining popularity in food industry because of its ability to reduce spoilage caused by microbes and enzymes while maintaining nutritional quality of food (Zimmerman and Bergman, 1993).

HPP can be accomplished uniformly throughout the food independent of size, shape and food composition. The isostatic principle states that pressure transmission is uniform and instantaneous throughout the system and in to the biological samples (Heremans, 1992). The effect of HPP to destroy microorganisms, deactivate enzymes

while maintaining quality in food products has been reported by several authors (Metric et al., 1989; Farr, 1990; Hoover, 1993; Ohnishi and Shigehisa, 1994; Knorr, 1995; Cheftel, 1995; Basak and Ramaswamy, 1996; Cheftel and Culioli, 1997; Leadley and Williams, 1997; Autio, 1998; Thakur and Nelson, 1998).

To fulfill the demand for fresh-like fish as a good source of protein and unsaturated fatty acids, HPP can be used an alternative/adjunct technique to extend shelf-life and promote safety. HPP has ability to the destroy microorganisms (both spoilage and pathogenic types) and inactivate endogenous enzymes. They also affect the secondary and tertiary structures of protein molecules leading to changes in their functional properties. On the safe side, covalent bonds are not destroyed by HPP and hence most nutritional qualities largely remain unaffected.

Only limited studies have been carried out on HPP of seafood products and ones dealing with fish alone are rare. In order to successfully establish a high pressure process for a specific food, one needs to understand the effect of pressure on the food components, enzymes and microorganisms. The purpose of this study was to promote shelf-life extension of refrigerated fish by controlling spoilage bacteria and promote safety by destroying pathogenic bacteria through a combination of pressure treatments involving different pressure levels and holding times. Furthermore, it was necessary to minimize the pressure influence on product quality changes during refrigerated storage.

The objectives of this study were to:

1. Evaluate the high-pressure destruction kinetics of pathogenic bacteria (*Escherichia coli O157:H7* and *Listeria monocytogenes*) in fish.
2. To obtain data on microbial destruction kinetic parameter, decimal reduction time

(D-value), and evaluate its pressure dependence. This phase of the research was necessary for identification and characterization of the pathogen that is high resistance to pressure treatment.

3. To carryout challenge studies with pressure treated products challenged with the pressure resistant pathogen to verify the designated level of pathogen destruction immediately after the pressure treatment and during storage.
4. Finally, to evaluate the shelf-life and quality changes in pressure treated fish stored at different temperatures.

Chapter 2

LITERATURE REVIEW

2.1 High Pressure Processing (HPP)

High pressure (HP) treatment is rapidly gaining world-wide popularity as a non-thermal processing technology for food preservation and processing. It can inactivate pathogenic microorganisms without excessive thermal treatment or the use of chemical preservatives, resulting in the shelf-life extension of foods with maximum retention of nutritional and sensory characteristics.

HP treatment has being applied for decades in non-food industries to process ceramic products, carbon graphite, composite materials and plastics. High pressure processing has also been known for more than a century now as a food preservation method since it first suggested use for food at the beginning of last century (Hite, 1899). Hite et al. (1914) reported a reduction in the microbial load of milk with use of pressure treatment at 650 MPa. The use of HPP for improvement of functionality of different foods has also been recognized since 1914 (Bridgman, 1914). This technology is now gaining in popularity because of its ability to offer high quality foods compared to conventional thermal processing and other methods of preservation (Mertens and Knorr, 1992; Cheftel, 1995; Williams 1994;). The first commercial HPP product, a fruit jam, was introduced in Japan in 1990. In recent years HPP has been adopted by France, United States, United Kingdom and Canada as a new technique for a variety of products. The acceptability and commercial application of HPP products has been slow due to economic

and safety concerns. However, advancement in engineering technology and availability of appropriate HPP equipment has opened the door to new commercial HPP products (Mertens and Deplace 1993).

Serious hurdles to the commercial adaptation, consumer acceptance and regulatory clearance of HPP include the need to gather scientific data on destruction kinetics of spoilage and pathogenic microorganisms, standardization of methodology for its adaptation and providing appropriate means to monitor the process. This review describes some of the work related to various HPP applications, the effect of pressure treatment on microbial destruction and changes in quality of foods.

2.1.1 Principles of HPP

High pressure processing is the application of high hydrostatic pressure to materials by compression that transmits pressure throughout the product evenly and rapidly (Hayashi, 1989). High pressure processing is based on two main principles which emphasize the effects of high pressure on food materials. The first law is the Le Chatelier-Braun principle states that under equilibrium conditions, any phenomena (chemical, phase transformation, enzyme reaction, etc.), is accompanied by a decrease in volume and will be accelerated by the application of pressure. On the other hand, high pressure suppresses the rate of those reactions which tends to result in an increase in volume.

Thus, under pressure, reaction equilibrium is shifted towards the most compact state, and the reaction rate constant (k) is either increased or decreased, depending on whether the 'activation volume' (ΔV) of the reaction is negative or positive. Pressure primarily affects the volume of the system. The influence of pressure on the reaction rate

can be described by the transition-state theory, where the rate constant of a reaction in a liquid phase is proportional to the quasi equilibrium constant for the formation of active reactions. Based on this assumption, it was reported that at constant temperature, the pressure dependence of the reaction velocity constant (k) is due to the activation volume of the reaction (ΔV):

$$\left[\frac{\partial \ln(k)}{\partial p}\right]_T = -\frac{\Delta V^*}{R \cdot T} \qquad (1)$$

$$\ln k = \ln k_o - (\Delta V * P/RT) \qquad (2)$$

where P is the pressure, T is the gas constant (8.314 cm^3/MPa/K/mol) and T is the temperature (K). Hence, the acceleration and deceleration of reactions depend on sign and magnitude of the activation volume (Eyring and Magee, 1942; Johnson and Campbell, 1945; Marquis, 1976; Johnston, 1995; Cheftel, 1995).

The second principle is Pascal's law or isostatic principle, which states that pressure is transmitted in a uniform and quasi-instantaneous manner throughout the biological sample or solution (this may not hold when a large volume of gas is present). The time necessary for pressure processing is therefore independent of sample size, in contrast to the situation prevailing for thermal or mechanical processes. Thus, the entire product is subjected to an identical treatment and therefore over-processing could easily be avoided.

2.1.2 Advantages of HPP

HPP provides a number of advantages over other processes, specifically conventional thermal processing. HP penetrates into the material regardless of size, shape

and chemical composition of the food or packaging material compared to other processing techniques, such as thermal processing in which the slow heating and existing thermal gradients make the process less efficiently and inherently over destructive from quality standpoint. This is one of the main advantages of HPP. The process has the ability to destroy spoilage and pathogenic microorganisms while maintaining the nutritional quality of the product. In conventional thermal processing, the process depends on time and temperature relationships where lower temperatures decrease the treatment efficiency, and overheating causes changes in the functional, chemical and sensory properties of treated product (Banwart, 1989).

HP treatment can be applied at room temperature and requires less energy as water compression ranges from 4% at 100 MPa to 15% at 600 MPa at room temperature (22°C) (Farkas, 1993; Sawamura et al., 1989). The energy calculated for one litre of water at 400 MPa will be 19.2 kJ which is equivalent to that required to increase the temperature of water by 5°C (Cheftel and Culioli, 1997). Hence, relative to thermal sterilization in which temperature increases of almost 120°C are necessary, the energy savings become readily obvious. HPP is also an energy efficient process since pressure is generated with a pump and once the pressure is reached, the pump is stopped, valves are closed and the pressurized liquid is maintained in a steel cylinder of adequate thickness and resistance, and keeping the sample under pressure for extended period of time does not require any additional input of energy (Farr, 1990). It only requires electricity for pressure build-up. The potential for future omission of size reductions of foods prior to high-pressure processing could substantially reduce food-processing wastes and save time and labour, so it is environmentally friendly (Thakur and Nelson, 1998).

The low energy levels involved in pressure processing allow the preservation of covalent bonds in food constituents (83 and 50 kcal/mole bond energy for C-C and S-S respectively), and only non-covalent bonds are affected (Hahashi and Hayashida, 1989). Hence, small molecules such as amino acids, vitamins, pigments and flavor/fragrant components, that are responsible to sensory and nutritional characteristic and are mostly stabilized by covalent bonds, are not affected appreciably by HP treatments. Consequently, processed products retain its initial color, flavor /fragrant and nutritional qualities are mostly sacrificed when traditional treatments are used.

High pressure affects the non-covalent bonds (1-7 kcal /mole bond energy), and hence larger molecules, such as proteins, enzyme, polysaccharides and lipids, etc., (relatively large biopolymers) whose function depends on the quaternary, tertiary and secondary structures are denatured by high pressure. Similar to thermal processing, high pressure also induces a variety of modifications in food systems, such as, protein denaturation, inactivation or activation of enzymes, gel formation, tenderization, and texturization, etc. The appearances of pressure induced protein denatured products are more attractive and maintain their original color and flavor as well as produce cooked product texture. Texturization of most vegetables and fruits indicates that without any additive, pressure can improve the texture of the product.

2.1.3 High Pressure Production Units

High pressure can be generated by heating a pressure media in a confined volume. Since volume cannot increase, it will result in a pressure increase since pressure, volume and temperature are related: PV = RT. The other way to achieve pressure is indirect

compression using a high pressure intensifier to drive a piston to compress the pressure medium in a closed vessel to the required level of pressure. This will generate compression heat. A direct compression method can also be used in which the pressure level can be achieved by directly pressurizing the pressure medium (usually water or mineral oil mixer) by pushing the medium into a closed chamber with help of a piston driven high pressure pump.

The equipment used experiments was ABB Isostatic Press Model# CIP42260 (ABB Autoclave System, Autoclave Engineers, Columbus, Ohio, USA) with 10 cm diameter and 55 cm height making 8L capacity with maximum capability of 414 MPa pressure. The main component consists of stainless steel vessel filled with 2% mineral oil pressure medium (Autoclave Engineers, Part# 5019, Columbus, Ohio, USA) connected to a medium reservoir through a HP pump. The samples packaged in the flexible material pouches were placed inside the vessel. The vessel was closed with a lid followed by lid lock for further protection. The high pressure pump pushed the medium into the vessel to build-up the required pressure level. The pressure release and sacrificial valves were used for discharging the pressure. To check the temperature of the vessel, K-thermocouples were used which were attached to a data logger with computer system. A water pump circulating the water around the vessel was used to maintain the chamber temperature during treatment.

2.2 Factors Affecting HPP

High pressure treatment can be affected by number of factors related to the process, system and food. Process related factors include pressure level, holding time and

temperature. System related factors include the type and size of pressure source, nature of pressurization etc. Food related factors consist of product structure/composition and pH. These factors are discussed as bellow:

2.2.1 Pressure Level

The pressure level applied to the product is the major factor influencing HPP. The pressure level is directly proportional to the effects in destruction of microbes and enzymes. It has been reported that biochemical reactions can start at 100 MPa while at 300 MPa irreversible reactions begin to occur. Foods are processed in pressure ranges from 100-1000 MPa although lower pressures, such as 50-200 MPa, have also used for processing some foods (Cheftel, 1995). Lower pressures 100-250 MPa have been found useful to inactivate *Bacillus subtilis* than higher pressure of 11,800 MPa (Sale et al., 1970) by actually resulting in germination of spores and germinated spores can easily be destroyed.

Another way of pressurization is cyclic or oscillation which refers to successive series of pressurization and depressurization without necessarily involving a holding time. The effect of cyclic pressurization results from combination effect of each cycle. Furthermore, the cyclic pressurization found to be more effective to inactivate enzymes and destruction of microorganisms than that of static pressurization alone (Alemán et al., 1998; Basak and Ramaswamy, 1996).

2.2.2 Pulse Effect

Pulse effect refers to a single or successive pressurization and depressurization without any holding time at certain pressure level. Its effect also depends on the pressure

level achieved. It has also showed a great deal of promise in destruction of microorganisms, enzyme deactivation more effectively. It has been reported that the effect of pulse pressurization is more useful on microorganisms due to the fact that the sudden depressurization may cause pressure shock that results in bursting of cell wall resulting in microbial death (Foster et al., 1962; Zobell, 1970; Hayakawa et al., 1994; Basak and Ramaswamy, 1996; Palou et al., 1998a,b,c; Hayakawa et. al., 1998). It has been suggested that pulse pressurization can lower the cost of production in large units than smaller pressure units. This is because it costs less to have one large vessel rather than several small units with an overall same capacity (Olsson, 1995).

2.2.3 Holding Time

The pressure effect comes from the combination of pressure treatment (holding time) and pressure level as in the case of thermal treatment the effect of which is measured by the combination of temperature and time. The pulse effect is generally complementary to the pressure-hold effect. The pressure-time effect is non-linear and it would appear to have a synergetic effect on destruction of microorganisms and deactivation of enzymes. The pressure-hold application has advantages over the pulse effect in some instances as no extra energy is required to maintain the pressure once pressure has been established at the desired level. However, the pressure processing equipment is expensive and longer process times will a mean lower production capacity, and hence greater production costs. For example, it has been shown that in terms of production cost as function of time, processing at 400 MPa with a 10 min hold time can be twice as expensive as processing at 800 MPa with no hold time (Olsson, 1995).

Similarly, a holding time process coupled with a large production unit will require less extra cost compared to smaller units of similar capacity for high pressure processing (Olsson, 1995). Thus, the combination effect of equipment capacity, pressure level, pressure pulse, pressure hold-time and temperature on microbial inactivation and the associated product cost must be evaluated carefully for process optimization.

2.2.4 Temperature

Temperature is one of the most important factors influencing the effect of high pressure processing. Usually an increase in temperature at a constant pressure increases the efficiency of pressure destruction of microorganisms. Consequently, the higher temperature can disrupt covalent bonds which are stable under high pressure. Hydrophobic interactions were found to be stabilized with an increase in temperature while at above 60°C they were disrupted (Marquis, 1976). Therefore, temperature consideration with high pressure treatment is important and strict guidelines are necessary to have reproducible results. The adiabatic temperature increase induced due to pressure can be beneficial for destruction of microorganisms (Zobell, 1970). Spores of spore forming bacteria are resistant to high pressure, however high pressure coupled with a mild increase in temperature can be beneficial for destruction of these spores. The spores of *Bacillus stearothermophilus* evaluated under high pressure treatment were inactivated because water permeability of spore-cell-wall with temperature increase enhanced the explosion of spores (Hayakawa et al., 1994). Enzyme inactivation can also be improved using combined high-temperature HP treatment (Hite et al., 1914; Anese et al., 1995; Hayakawa, 1994; Hendrickx et al., 1998).

At the opposite, other application of HPP at low temperatures are pressure shift freezing and high pressure thawing which takes into advantage the pressure shift in the phase-change temperature (Kalichevsky et al., 1995). Pressure shift freezing can be achieved by quick depressurization of super-cooled food (to –20°C at 210 MPa) which results in the formation of a large amount of ice nuclei instantaneously. Freezing is often completed at atmospheric conditions in a cold room. Such a process has been shown to be beneficial in protecting the tissue structure and texture from freezing damages. HP thawing makes use of a reverse concept to thaw a frozen food at 200 MPa (allowing a larger temperature gradient between the food and thawing medium). This will enhance the thawing rate, facilitates microbial control and often reduces drip loss. Zhu et al. (2004b) examined the quality of pressure-shift frozen pork with that of conventional air frozen, plate frozen and liquid nitrogen frozen samples (Zhu et al., 2004b). They found noticeable color and texture (increase in hardness) change in the treated product up on thawing. Likewise they also showed that HP thawing of Atlantic salmon (*Salmo salar*) can be advantageous in terms of faster thawing rates, better microbial quality and decreased drip loss (Zhu et al., 2004a)

2.2.5 Food Composition

As far as the composition of food is concerned, covalent bonds are not affected by high pressure processing (Hayashi and Hayashida, 1989; Knorr, 1993). Proteins and other macromolecules are easily affected and cause changes in texture, while vitamins and flavor are unaffected by high pressure treatment. It has been reported that the reaction volume and energy required to disrupt covalent bonds is quite low, therefore primary

structures responsible for color, taste, flavor and nutritional qualities can be maintained with high pressure processing (Eshtiaghi et al., 1994).

Certain food constituents like proteins, lipids and carbohydrates can also provide pressure protection to some microorganisms. Non-nutrient salt has been reported to lower the pressure tolerance of microorganisms (Dring, 1976). The presence of sugar has been reported to protect enzyme inactivation and destruction of microorganisms under high pressure treatment (Ogawa et al., 1992; Horie et al., 1991). The water activity (a_w) is also an important factor in food which affects the pressure destruction of microorganisms. Vegetative cells have been found to be protective under pressure when the water activity is $\leq$ 0.9 (Knorr et al., 1992).

2.2.6 Food pH

The pH of food affects microbial growth, enzyme activity and protein denaturation under high pressure treatment (Hite et al., 1914). High acid foods exhibit high pressure sensitivity due to their low pH (Hite et al., 1914, Ogawa et al., 1990). Vegetative bacteria are therefore more resistant to pressure in higher pH products such as in meat, while bacterial spores are more resistant at neutral pH (Smelt, 1998).

A decrease of 0.2-0.5 pH units per 100 MPa has been observed under high pressure treatment which reverses back to normal values upon pressure release (Marquis, 1976). Therefore, under higher pressure lower pH favourable in destruction of microorganisms and enzyme inactivation. The ionic dissociation of water and weak acids substantially increase at higher pressures causing a decrease in pH which further results in biochemical changes, such as protein denaturation, enzyme inactivation and destruction

of microorganisms (Brandts et al., 1970; Zipp and Kauzmann, 1973). Marking of pH changes in food during and subsequent to pressure treatment is required to determine its role in the destruction of microorganisms or inactivation of enzyme inactivation.

2.3 Seafood Spoilage

Fish has been used as a source of food from very early stages of mankind development. There has been a tremendous increase in fish cultivation and harvesting to fulfil consumers high demand for freshly processed fish. Fish contains several high biological value proteins grouped as i) sarcoplasmic proteins from muscle, ii) myofibrillar contractile proteins and iii) storma or connective tissue proteins from the extra-cellular matrix. Fish protein contains all essential amino acids for good health making it an excellent source of protein. Fish is also a very good source of polyunsaturated fatty acids which reduces the risk of certain diseases (heart, cancer, depression, diabetes, arthritis) and help in improving the immune system (vision, reproduction, brain). Most of the health benefits are due to omega-3 polyunsaturated fatty acids present in fish. There are also large amounts of vitamins present in fish including A, D, E, K, thiamine, riboflavin, niacin, B6, pantothenic acid, biotin, folate, B12 and C which are essential for normal physiological functions. Fish also contains high amounts of macro minerals e.g. calcium, magnesium, phosphorous, sodium, potassium and chlorine and some trace minerals including chromium, cobalt, copper, iodine, iron, manganese, molybdenum, selenium, zinc and fluorine, which are considered essential for physiological functions in human and animals. However, fish after harvesting has an extremely short shelf-life and deteriorates very fast. Thus, effective processing and preservation method are essential to

extend its shelf-life.

Changes in seafood occur from the time the fish starts to struggle at catch by using reserve energy followed by rapid deterioration of fish post-mortem. The main cause of these deteriorations in fish are enzyme and bacterial activities. Fish autolysis starts at death when the freshness begins to be lost. In second phase of spoilage, the imbalance of metallic reactions occur followed by complete spoilage due to microbial activities. The following sections detail the main factors responsible for seafood spoilage:

2.3.1 Enzymatic Spoilage

The autolytic reaction starts post rigor-mortis where ATP (adinosine diphosphate) breakdown causes enzymatic spoilage in fish. The breakdown ATP causes loss in texture, degradation of odor and flavor, lipolysis of lipids, change in color and destruction of trimethylamine oxide (TMAO). Due to breakdown of ATP the enzymes responsible for muscle structure cease function and ultimately cause contraction of fish muscle. The imbalance in muscle cell causes activation of proteolytic and endogenous enzymes causing loss of muscle structure (Lawrie, 1992). Further degradation of adenosine diphosphate (ADP) to the adenosine monophosphate (AMP) followed by a rapid change to inosine monophosphate (IMP) causes accumulation of inosine (INO) and hypoxanthine (Hx) in fish muscle. The ratio of INO and Hx to the total amount of ATP, ADP, AMP, IMP, INO and Hx I referred as k-value and can be used as index of fish freshness (Oshima et al., 1993). A summary of the formation of these compounds and uric acid is shown in Figure 2.1 (Flick and Lovell, 1972).

The enzymes responsible for texture degradations are known as proteases causing

hydrolysis of muscle and connective tissues ultimately softening the fish (Anese et al., 1995). These enzymes are present in meat and fish tissue and are secreted by microorganisms (Hisano et al., 1989). Protease enzymes work on proteins, such as myofibrillar proteins and cause proteolytic changes which enhances the interaction between proteins and water leading to softening of fish and make it smeary and texture changes.

The smell of fresh fish is mainly due to the presence of alcohol and carbonyl compounds. The off-odor of fish results from lipoxygenase activity and hydroperoxidation of fatty acids (Josephson et al., 1984a; 1984b). The fishy smell arises from the synergism between IMP and free amino acids while the strong meaty flavor comes from the interaction of IMP with Hx contents (Fletcher et al., 1990).

In the case of lipids, degradation takes place in two ways: lipolysis and oxidation. Different free fatty acids, such as lysophospholipids, glycerophosphocholine and phosphoric acids, are produced by lipid hydrolysis. Lipid hydrolysis is mainly found in fatty fish and believed not to occur from bacterial spoilage. Autoxidation is also reduced due to nitrogenous compounds and formation of triaglycerols, cholesterol esters and wax ester by phospholipids hydrolysis (Sikorski, 1990; Ohshima et al., 1993).

Another enzymatic spoilage occurs due to degradation of trimethylamine oxide (TMAO) to trimethylamine (TMA) by autolytic process. It gives fishy odor and cause sponginess of fish flesh during frozen storage. Moreover, degradation takes place due to formation of dimethylamine (DMA) and formaldehyde by TMAO demethalase. TMA formation from TMAO is also caused by bacterial growth in fish (Davis, 1995).

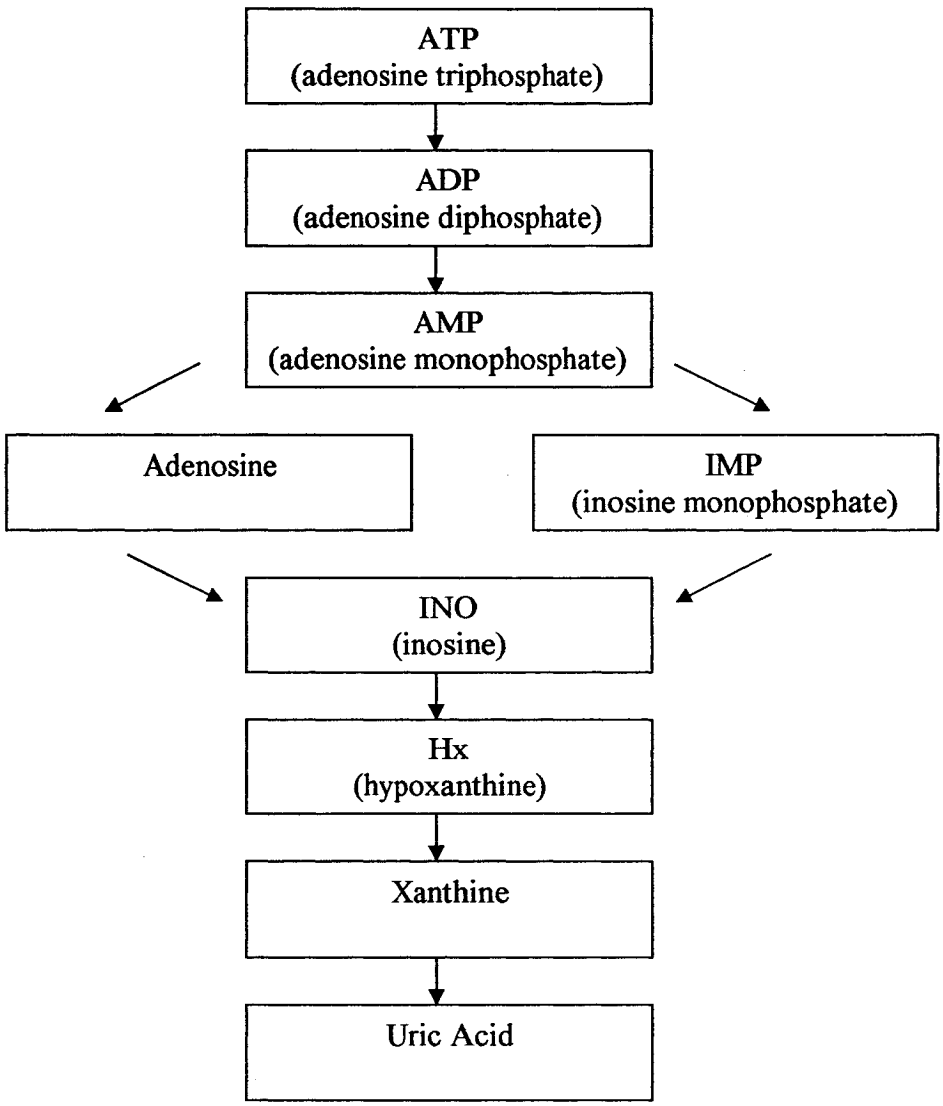

Figure 2.1 Enzymatic reactions involved in formation of uric Acid from ATP (Flick and Lovell, 1972)

Color is another quality factor affected by enzymatic and non-enzymatic oxidation of carotenoids present in skin, shells and exoskeletons of fish. The deterioration of this pigment forms a black appearance on skin and reduces the shiny appearance of fish. The translucent fish flesh also turns opaque due to oxidation and formation of phenolic compounds (Sikorski, 1990).

2.3.2 Microbiological Spoilage

The flesh of live fish is sterile because the immune system prevents growth of bacteria in fish flesh. After the death of fish, the immune system stops and leaves the flesh susceptible to microbial growth. Bacteria start growth from the fish surface and move inside the flesh through muscle fibre. Bacterial growth results in spoilage and enzymatic diffusion into the flesh (Huss and Gram, 1995).

Microbial spoilage is one of the main factors that causes rapid spoilage of fish post-mortems even at refrigerated storage (4°C). The endogenous microorganisms in fish include aerobic psychrotrophic *Pseudomonas, Alteromonas, Flavobacterium, Achromabacter, Acinetobacter* and *Vibrio* spp. which cause spoilage on storage. The genera list of different bacterial flora obtained from clear and unpolluted water is shown in Table 2.1 (Huss and Gram, 1995). The growth of microflora up to 10^7 CFU/g is used as indicator of fish spoilage (ICMFS, 1978).

The external contamination of fish from handling and processing can also cause fish spoilage. The compounds formed by bacteria includes TMA from TMAO; H_2S from cystein, methyl mercaptan (CH_3SH) and dimethly sulphide($(CH_3)_2S$) from methionine; acetate, CO_2 and H_2O from carbohydrates and lactate; Hx from inosine and IMP; esters,

Table 2.1 Bacterial flora of fish caught in clear, unpolluted water (Huss and Gram, 1995)

Gram-negative	Gram-positive	Comments
Pseudomonas	*Bacillus*	
Moraxella	*Clostridium*	
Acinetobacter	*Micrococcus*	
Shewanella putrefaciens	*Lactobacillus*	*S. putrefaciens* sodium requiring also isolated from fresh water
Flavobacterium	*Coryneforms*	
Cytophaga		
Vibrio		*Vibrio* is typical in marine water
Photobacterium		*Photobacterium* is typical in marine water
Aeromonas		*Aeromonas* is typical in fresh water

ketones, aldehydes from amino-acids (glycerine, serine, leucine); and NH_3 from amino acids and urea (FAO, 1995). The formation of Hx by bacteria will be at a higher rate compared to the autolytic process taking place in fish. The formation of these compounds causes off-odors and off-flavors in fish. TMA formation from TMAO, other than by enzymatic process, is due to bacteria such as *Alteromonas, Photobacterium, Vibrio, S. putrefaciens* and *Aeromonas* spp. present in fish (Kruk and Lee, 1982; Avery and Lamprecht, 1988; Hebert and Shewan, 1976; Smith et al., 1984; Watts and Brown, 1982).

2.3.3 Chemical Spoilage

Chemical spoilage is caused by number of factors which include moisture loss, oxidation, rancidity, loss of volatile flavors, loss of vitamins and change in odor and flavor. Color degradation, due to chemicals other than enzymatic browning, is the result of Maillard reaction and auto-oxidative lipid reactions. In the Maillard reaction, browning is caused due to a reaction between sugars and amino acids while oxidative lipids react with proteins (El-Zeany et al., 1975).

The rancidity of unsaturated fatty acids is caused by the formation of free radicals in three steps: initiation, propagation and termination. As the reaction initiates, free radicals start forming from unstable hydroperoxides and eventually increase the rate of auto-oxidation of fatty acids (Ashie and Simpson, 1996). The metal ions of Cu^{+2}, Fe^{+2} and Fe^{+3} present in fish work as catalysts to speed up the rancidity process. The overall deterioration process of fish is shown in Figure 2.2 (Jacober and Rand, 1982).

2.4 HPP Effects on Seafood

2.4.1 HPP Effects on Destruction of Vegetative Cells

HPP has gained considerable interest in the food industry as a method for microbial inactivation. However, the effectiveness of HPP on microbial inactivation has to be studied in more detail to ensure the safety of food treated by this method. Several studies have been carried out on the effect of HPP on yeast, moulds vegetative cells and spores of different pathogenic bacterial species. Few studied focusing on HP destruction of microorganisms in fish has been studied. Hence, more work is essential for establishing procedures for HPP of fish.

In a HPP study on tuna and squid at 450 MPa pressure for 15 minutes at 25°C, up to two log reduction was achieved and reported as inefficient (Shoji and Saeki, 1989). In another study on urchin eggs, the effective kill of *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio mimicus* were reported while maintaining their flavor and taste (Yukizaki et al., 1993). They also found that only 200 MPa pressure for 5 minutes at 0°C was required to destroy *V. parahaemalyticus* in a buffer solution but some compounds in urchin egg provide protection to this microorganism and thus requires higher pressures for inactivation (Yukizaki et al., 1994).

The destruction of most fungi, gram-positive and gram-negative bacteria were reported in fish paste (surimi) after treating between 300 to 400 MPa (Miyao et al., 1993). *Moraxella* spp., *Acinetobacter* spp., *Streptococcus faecalis* and *Corynebacterium* spp. were found to be resistant to 200, 300, 400 and 600 MPa respectively.

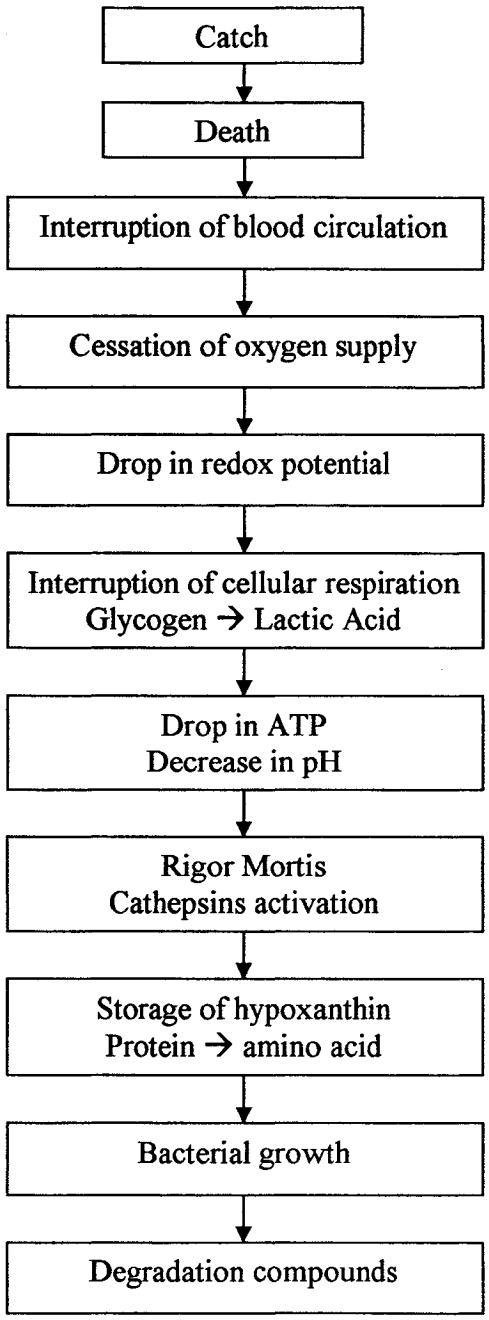

Figure 2.2 General schematic diagram of fish deterioration process (Jacober and Rand, 1982)

Complete destruction of *Bacillus, Moraxella, Pseudomonas* and *Flavobacterium* was achieved in minced mackerel after pressure treatment at 203 MPa for 60 min and shelf-life was extended to 4 days at 5°C by affecting bacterial growth (Fujii et al., 1994). *Coryneforms, Staphylococcus* and *Micrococcus* were found to grow during refrigerated storage and deterioration was enhanced by an increase in fat rancidity in pressurized mackerel.

The shelf-life of spreadable smoked salmon cream (pH 5.95) was extended to 60 and 180 days for 3 and 8°C storage respectively after the pressure treatment of 700 MPa for 3 minutes (Carpi et al., 1995). They found complete inactivation of *L. monocytogenes, S. aureus, S. typhimurium* and lactic acid bacteria in salmon cream. In another study on sea urchin eggs, a 5 log reduction was achieved with a 5 min treatment at 400 MPa (Lopez-Caballero et al., 2000a).

Pressure treatment combined with some other processing methods, showed a better effect and more retention of quality in some seafood products. The shelf-life of vacuum packaged prawns pressure treated at 400 MPa was extended by 2 weeks (Lopez-Caballero et al., 2000b).

2.4.2 HPP Effects on Destruction of Bacterial Spores

Spores are more resistant compared to vegetative cells. Some vegetative cells of bacteria can destroyed even at 100 MPa while spores may survive pressure treatments above 1200 MPa. The structure and thickness of the bacterial spore coat makes them resistant these higher pressure levels (Lechowich, 1993). Therefore, to inactivate bacterial spores, HPP is usually coupled with mild heat.

The heat used with HPP to inactivate spores has been found to help soften the spore-coat which ultimately burst under high pressure (Hayakawa et al., 1994), especially during depressurization. In smoked salmon cream, HP treatment at 700 MPa for 3 minutes was reported to be insufficient to inactivate sulfite-reducing *Clostridia* spores (Carpi et al., 1995).

2.4.3 HPP Effects on Protein

Protein denaturation under high pressure involves dissociation of oligomeric structures, unfolding of monomeric structure, protein aggregation and protein gelation (Balny and Masson, 1993; Gross and Jaenicke, 1994; Funtenberger et al., 1995; Cheftel, 1995). Protein denaturation can be reversible or irreversible depending on the level of high pressure, protein type and processing condition (Farr, 1990). Reversible denaturation of proteins has been reported within the pressure range of 100-300 MPa (Thakur and Nelson, 1998; Balny and Masson, 1993). Some monomeric proteins, however, did not show any changes with increase in pressure (Dickerson and Geis, 1969). The non-covalent bonds (such as hydrogen, hydrophobic interaction) and ionic bonds of quaternary and tertiary structures of proteins showed denaturation, coagulation or gelatinization under high pressure while covalent bonds were not effected (Okamoto et al., 1990; Cheftel, 1995). These changes can be useful where modification or elimination is required in some food materials (Hayashi and Hayashida, 1989). Therefore, this process can be used to improve functional properties of foods/ingredients, such as texture, emulsifying, whipping and dough formation (Hayashi et al., 1987).

The main proteins in fish are myofibrillar and sarcoplasmic proteins. Myofibrillar

proteins give structure to fish muscle while sarcoplasmic proteins are non-structural water soluble proteins. Ohshima et al. (1993) reported that HP treatment at 150 MPa for 30 minutes on crap myofibrils showed destruction and loss in pattern of myofibrillar proteins. Other proteins in gel, such as actin and heavy chain myosin, did not show any change up to 150 MPa or 38°C heat treatment (Shoji and Seaki, 1989). Cod and mackerel sarcoplasmic proteins became covalently linked after high pressure treatment (Ohshima et al., 1992).

2.4.4 HPP Effects on Lipids

Fish lipids are differentiated by high levels of polyunsaturated fatty acids (PUFAs) and are susceptible to auto-oxidation resulting in quality loss in products including loss in flavor, color, texture and nutritional value during processing (Eriksson, 1982). Oxidation of lipids present in muscles are reported to be accelerated by pressure whereas isolated marine lipids showed relative stability against auto-oxidation under high pressure (Oshima et al., 1993; Cheah and Ledward, 1995; Angsupanich and Ledward, 1998). The catalysis occurs due to changes in fat and tissue above 400 MPa pressure in the presence of metal ions from specific complexes which are responsible for catalyzing the oxidation reaction (Cheah and Ledward, 1997). It has shown that in muscle tissues haemosiderin, and other insoluble iron complexes, are responsible for these reactions (Cheah and Ledward, 1996; Ledward, 1998). Therefore, higher fat containing fish are more susceptible to lipid oxidation. A list of fish species according to their fat content being fatty, semi-fatty or lean are shown in Table 2.2 (Jacquot, 1961).

Extracted sardine oil, treated at pressures up to 506 MPa for 60 minute, did not

show any changes in peroxide value (POV) and thiobarbituric acid (TBA) (Ko et al., 1991). However, in cod muscle, pressure treatments at 202, 404 and 608 MPa for 15 and 30 minutes increased POV of extracted oil while an increase in holding time further enhanced POV in mackerel muscle lipids (Ohshima et al., 1992).

2.4.5 HPP Effects on Enzymes

Enzymes have been shown to be more resistant to HPP and require higher pressure levels for their inactivation (Ko et al., 1991). Furthermore, enzymes activity may be enhanced with an increase in pressure level instead of inactivation (Ohmori, et al., 1991; Knorr, 1993; Basak and Ramaswamy, 1996; Gomes and Ledward, 1996 and Castellari et al., 1997). Compared to microorganisms, enzymes show relatively greater resistance to pressure treatment (Jaenicke, 1987). Enzymes are responsible for breaking down of ATP into several compounds in fish muscle and, its accumulation during storage, is generally an indication of loss in freshness of fish (Saito et al., 1959; Sakaguchi and Koike, 1992). The resistance to pressure also depends on the type of enzymes. Pectin esterase, lipase, polyphenoloxidase, lipoxygenase, peroxidase, phosphatase and catalase are highly resistant to high pressure treatment (Syderhelm et al., 1996).

In crap muscles, HP treatment at 350 and 500 MPa and subsequent storage at 5°C resulted a decrease in the inosine 5'monophosphate (intermediate breakdown compound) level (Shoji and Saeki, 1989). Lipases are active even at lower temperatures and produce free fatty acids on storage. Therefore, high pressure treatment above 405 MPa are required before storage to decrease fatty acid production (Ohshima et al., 1993). Inactivation of ATPase due to heat treatment is higher compared to high pressure

treatment. The heat treatment shows a clear first order kinetics curve for its inactivation while, with pressure treatment, it is observed up to a break-point after which the inactivation progresses at a slower rate (Ohshima et al., 1993). Differential scanning calorimetry (DSC) thermograms of fish muscle also show different peaks for actin and myosin in comparison with heat and pressure denaturation of these proteins because of partial denaturation by pressure treatment (Iso et al., 1993).

In fish, trypsin, chymotrypsin, cathepsin and collagenase have been found susceptible to high pressure inactivation at 100-400 MPa with the inactivation being directly proportional to pressure holding time. Among these, trypisn was more susceptible to inactivation than chymotrypsin (Ashie and Simpson, 1996).

2.4.6 Effects on Texture

Pressure level less than 200 MPa are insufficient to markedly change fish muscle structure (Cheftel and Culioli, 1997). Pressure has a greater effect on myofibrillar proteins causing change in fish muscles (Ledward, 1998). The structural change in myosin of turkey and pork are more resistant to pressure treatment compared to fish myosin due to fish being a cold-blooded species (Cheah and Ledward, 1996; Angsupanich and Ledward, 1998; Angsupanich et al., 1999). The disappearance of the myosin peak in cod thermogram was observed after pressure treatments of 200-800 MPa and was reported to be due to denaturation of proteins and formation of new hydrogen bonds network (Angsupanich and Ledward, 1998). The texture of high pressure treated fish is different from the texture of heat treated fish.

Table 2.2 Categorization of fish species according to presence of fat (Jacquot, 1961)

Fatty	**Semi-fatty**	**Lean**
Herring	Barracuda	Coalfish
Mackerel	Bass	Cod
Pompano	Mullet	Haddock
Pike	Perch	Hake
Salmon	Shark	Plaice
Shad		Smelt
Trout		
Tuna		

In cod muscle, pressure treatment up to 400 MPa showed that the hardness in muscle was similar to the hardness in heat treated muscle (Angsupanich and Ledward, 1998; Angsupanich et al., 1999). Pressure treated bluefish at 101 MPa showed an increase in firmness of fish muscle on storage at 4-7°C while pressure treatment at 202 and 303 MPa was found to have a reverse effect (Ashie et al., 1997). Oyster treated with high pressure did not show much difference in hardness compared to untreated samples (Lopez-Caballero et al., 2000a). Similarly shrimp also did not show much change in hardness on high pressure treatment compared to untreated shrimp (Lopez-Caballero et al., 2000b).

2.4.7 HPP Effects on Color

Pressure treatment of about 300-400 MPa in meat causes a noticeable cooked appearance color in the form of translucency due to the denaturation of myofibrillar and sarcoplasmic proteins (Ohshima et al., 1992; Cheah and Ledward, 1996; Angsupanich et al., 1998). The brown color formation in fish due to high pressure treatment has been reported due to metmyoglobin formation (Carlez et al., 1995). In cod fish, sufficient changes in color have been found within 100-200 MPa due to myosin denaturation (Angsupanich and Ledward, 1998). Color changes have also been found to be due to oxidation of haemoprotein while at pressures up to 300 MPa the native pigment remained unchanged (Cheah and Ledward, 1996). It has been demonstrated that ferrous nitrosomyoglobin, responsible for the color of cured meat, could be stabilized by pressure treatment (Bruun-Jensen and Skibsted, 1996).

2.5 Pathogens of Concern in Seafood

Seafood, being a highly perishable commodity, is also susceptible to bacterial contamination during handling, processing and distribution. Food-borne human illnesses associated with fish ahs brought attention towards implementation of effective procedures to eliminate or reduce the risk from these pathogens. The main source of contamination of these pathogens is human handling where *Salmonella* spp. and *Shigella* spp. have been directly implicated in transmitting these pathogens through faecal-oral contacts.

Sewage water contaminates water and introduces pathogenic bacteria and viruses into food. The anaerobic spore forming *Clostridium perfringens* was found in fish caught near sewage out falls in Puget Sound, Washington (Matches et al., 1974). In another case of foodborne illness, *Salmonella* spp. was found in Flatfish caught at sewage discharge points in the Baltic Sea (Wuthe and Findel, 1972).

The pathogen *A. hydrophila* which can survive at cold temperatures and cannot grow well above 1.5% NaCl levels, is accompany contaminant of fresh water fish (Ingham and Potter, 1988; Eddy and Ketchell, 1959). Food-borne gastroenteritis caused by *A. hydrophila* has been isolated from oysters (Abeyta et al., 1986; Okrend et al., 1987). Other pathogens found in seafood include *V. parahaemolyticus* and *Vibrio vulnificus* which can grow up to 2-3% NaCl levels and have been found in raw and improperly cooked seafood. These halophilic bacteria grow well in high salt fish products, however their growth is reduced at refrigerated storage conditions (Ingham and Potter, 1988). The spore forming anaerobic pathogen *C. botulinum* type E has been found in raw, smoked, vacuum packaged and canned fish products (Ingham, 1991).

Other sources of seafood product contamination comes from ingredients such as flour, starch, spices, milk, eggs and water used in their preparation. Addition of spices in cooked food can also be a source of contamination. In spices, spores of foodborne pathogens *Clostridium perfringens* and *Bacillus cereus* have been reported (Powers et al., 1976). Ready-to-eat seafood meals can be contaminated during post-processing distribution and can cause foodborne illnesses. The pathogenic bacteria *Aeromonas hydrophila, S. aureus,* C. *botulinum, V. parahaemolyticus* and *L. monocytogenes* can be introduced during processing of such foods (Ingham and Potter, 1988).

The most common pathogens that have been found in marine and fresh water seafood include *Salmonella spp., Campylobacter spp., Listeria monocytogenes, E. coli* and *Yersinia enterocolitica* all of which can cause serious illnesses in humans (Ingham, 1991).

2.5.1 *Listeria monocytogenes*

2.5.1.1 Characteristics of Listeria monocytogenes

L. monocytogenes is a gram-positive, rod shaped, non-spore forming, motile and haemolytic pathogenic bacterium (Bahk and Marth, 1990). *L. monocytogenes* is psychrotrophic and capable of growing temperatures ranging from -0.4 to 50°C (Farber and Peterkin, 1991). It can also grow in both aerobic and anaerobic conditions (Pelroy et al., 1994). Its growth requires minimum of 0.92 water activity and 4.39 to 9.4 pH (ICMFS, 1996). Carbohydrates are essential for its growth, however, can survive for about four months in 25.5% NaCl solution (Bahk and Marth, 1990).

2.5.1.2 L. monocytogenes Foodborne Illnesses

L. monocytogenes is the main causative agent of listeriosis and is known as an entero-invasive pathogen (Ryser and Marth, 1991). The major risk of this disease is found in pregnant women, the elderly and immune-suppressed patients where it causes spontaneous abortion or serious unhealthy baby due to fetus infection during pregnancy (McLauchlin, 1993). Other than pregnant woman and immune-suppressive patients, people with diabetics, cancer, cardiovascular, renal collagen and neoplastic diseases are susceptible to *L. monocytogenes* infections (Nieman and Lorber, 1980). A zero tolerance has been imposed by Food and Drug Administration for *Listeria* spp. in food (Farber and Peterkin, 1991).

A high mortality rate of 29% was been found in New England after the outbreak of *L. monocytogenes* in milk (Fleming et al., 1985). In Finland, five persons were affected by consuming vacuum packaged cold-smoked rainbow trout containing *L. monocytogenes* (Miettinen et al., 1999). In another outbreak in New Zealand, it caused listeriosis on the consumption of raw fish and shellfish (Lennon et al., 1984). About 40-60 cases are annually reported in Canada (Farber and Harwig, 1996).

2.5.1.3 L. monocytogenes in Seafood

Sufficient evidence has been presented to show that contamination of *L. monocytogenes* occurs during seafood processing (Weagant et al., 1988). The contamination of *L. monocytogenes* in seafood can also be due to rivers flowing through agriculture land, rearing techniques, location of ponds, sewage system outfalls, slaughtering procedures and handling during processing of fish. Raw fish is more

susceptible to contamination; however processed fish products could be contaminated due to the presence of *L. monocytogenes* in processing plants (McCarthy, 1996). In ready-to-eat fish meal, contamination has been found due to hygienic defaults in the processing system (Jemmi and Keush, 1994; Pelroy et al., 1994). In eviscerated salmon, contamination of this pathogen has been found in slime, skin, head, tails and belly portions before further processing (Eklund et al., 1995).

2.5.2 *Escherichia coli*

2.5.2.1 Characteristics of Escherichia coli

A new class of diarrhoeagenic *Escherichia coli* was discovered in 1982 in USA after an outbreak identified as *Escherichia coli* O157:H7 causing hemorrhagic colitis (Riley et al., 1983; Wells et al., 1983). *Escherichia coli* is a gram-negative, rod shaped, non-spore forming, motile using flagella and facultative anaerobic pathogenic bacteria belonging to the family *Enterobacteriaceae* (Schaechter et al., 1998). The temperature range for *E. coli* growth is 10-46°C while pH requirement ranges 4.4 to 9 (Prescott et al., 1990). The outer membrane of *E. coli* is made up of lipopolysaccharide (LPS) which is endotoxic and capable of causing severe septic shock, intravascular coagulation and finally death (Schaechter et al., 1998). *E. coli* causes pathogenesis in one of three ways i) by producing cytotoxic Shiga-like toxins resulting in HUS (Haemolytic Uremic Syndrome) disease (Lansbury and Ludlam, 1997; Armstrong et al., 1996) ii) by production of haemolysin found in HUS patients serum (Schmidt et al., 1995) and iii) by its ability to colonize intestinal surfaces to enhance delivery of toxins to the cell surface (Paton et al., 1997). The main source of *E. coli* are cattle (Orskov et al., 1987; Wells et

al., 1991; Chapman et al., 1993; Hancock et al., 1994), but it has also been isolated from sheep (Kudva et al., 1996), deer (Keene et al., 1997), seagulls (Wallace et al., 1997), goats (Bielaszewska et al., 1997), dogs and horses (Trevena et al., 1996).

2.5.2.2 E. coli Foodborne Illnesses

E. coli infections include diarrhea, bloody diarrhea, abdominal pain, vomiting, fever, haemorrhagic colitis and HUS (Johnson et al., 1983; Karmali, 1989; Fang, 1993; Griffin, 1994; Willshaw et al., 1994). Shiga toxin-producing *E. coli* are considered a major cause of gastrointestinal disease in humans (Griffin and Tauxe, 1991). The infection has been seen mostly in young and elderly during warm weathers which permits the possibility of developing HUS after starting symptoms of diarrhoea, abdominal pain and fever (Thomas et al., 1993).

From December 1989 to January 1990 in USA (Cabool, Missouri) 243 cases of *E. coli* infections were reported with four deaths occurring due to the consumption of non-chlorinated drinking water (Swerdlow et al., 1992). The biggest outbreak in 1996 infected about 8000 children causing three deaths, due to eating white radish sprouts in a school of Sakai city, Osaka, Japan (Watanabe et al., 1996; Michino et al., 1998). About 500 confirmed cases and four deaths have been reported in 1993 in United States (Washington, Idaho, Nevada, and California) due to consumption of contaminated burgers (Kay at al., 1994). In Central Scotland in 1996; 20 people died while 501 were infected due to consumption of meat from a local butcher's shop (Cowden and Christie, 1997; Liddell, 1997; Ahmed and Donaghy, 1998). Another outbreak in 1995 in Australia (Adelaide) from *E. coli* serotype O111:H caused infections in 200 people due to eating

uncooked, semi-dry fermented sausages (Paton et al., 1996). In Canada (Northwest Territories) in the summer of 1991, *E. coli* O157:H7 infection cases reached 521 with two deaths due to the consumption of ground-beef and cross-contaminated foods (Orr et al., 1994).

2.5.2.3 E. coli in Seafood

The food types most commonly associated with *E. coli* outbreaks of food poisoning are of bovine origin, in particular minced beef and beef burgers (Davis et al., 1993; Willshaw, 1994). The fish microflora does not contain *E. coli* although it has been isolated from stomach and intestine of fish (Janssen, 1970; Hejkel et al., 1983). The higher bacterial pollution of water can increase the levels of pathogens in fish organs (Pal and Dasgupta, 1992). The sewage system, containing nitrogenous compounds, nutritive salts, bacteria and other pollutants on discharge in water can also introduce such pathogens in fish (Kakuta and Murachi, 1997). Inadequate sanitation can also result in *E. coli* in seafood product causing the foodborne illnesses (Smoot and Pierson, 1997). Seafood and meat bought from local market of Seattle (Washington) showed a higher proportion (17%) of *E. coli* VTEC type (Samadpour et al., 1994). An outbreak in last decade in Peru (Latin America) was reported due to use of contaminated water for ice to store fish (Ries et al., 1992).

2.6 High Pressure Application in Fish

The majority of research work on HPP of fish has focused on gel-making fish products like surimi (Farr, 1990; Ohshima et al., 1993). Other work includes enzymatic (Ashie and Simpson, 1996; Ohshima et al., 1992) and texture changes in seafood

(Ohshima et al., 1993). Some studies have been related to pathogen destruction in tuna meat (Shoji and Saeki, 1989). However there is a paucity of information on the effect of high pressure processing on destruction of pathogenic microorganisms and quality changes in fish and fish products. Noticeably very few studies have been carried out on whole fish, such as mackerel therefore in order to assure the safety of these products, detailed studies on HP destruction kinetics on pathogens and associated quality changes during storage need to be done.

Chapter 3

High Pressure Destruction Kinetics of *Escherichia coli* (O157:H7) and *Listeria monocytogenes* (Scott A) in Fish

3.1 Abstract

High Pressure (HP) destruction kinetics of *Escherichia coli* (O157:H7) and *Listeria monocytogenes* (Scott A) in mackerel (*Scomber scombrus*) were evaluated. Filleted fish was made into a slurry with sterile peptone water and inoculated with the respective microbial strain to prepare a stock culture containing 10^6-10^7 CFU/ml. Samples were prepared for pressure treatment by heat sealing 10 ml portions of the stock culture were packaged in sterile plastic bags. Pressure treatments (250 and 400 MPa for 0-60 min) were given at room temperature (20-25°C) in an isostatic press. Survival curves were established based on residual counts following treatment. Desturction kinetics were described as a dual effect: an initial destruction resulting due to a pressure pulse (pulse effect) followed by a first order rate of destruction. *E. coli* was found more sensitive to pulse pressure than *L. monocytogenes*. Substantial differences in high pressure resistance (D value) were found between the two microorganisms. D-values of *E. coli* were higher than for *L. monocytogenes* at pressure levels > 350 MPa, while a reverse trend was observed at lower pressures. The associated z_p values indicated that the destruction rate of *L. monocytogenes* (z_p = 103 MPa) was more sensitive than *E. coli* (z_p = 185 MPa) to changes in pressure. Challenge studies with the more resistant pathogen, *E. coli* (10^7/ml), showed that a 10D treatment, followed by refrigerated storage (4-12°C), prevented its

recovery/growth.

3.2 Introduction

Demand for fresh food products has increased dramatically in recent years due to consumer awareness and concerns towards health and healthy foods. Thermal processing (as a method) has been well recognized for preparing long term shelf-stable food products. However, its unavoidable adverse effect on some nutrients and overall quality of food has been the topic of discussion for optimization procedures. Reduction in the severity of thermal processing and introducing new concepts in food preservation which would have minimal effects on functional properties are continuously being explored to cope with such demands. Different non-thermal processes, such as high pressure processing (HPP), pulsed electric fields (PEF), irradiation and ultra-filtration have been emerging as alternative techniques to conventional thermal treatment. Among these new technologies, HPP promises to reduce spoilage caused by microorganisms and enzymes while maintaining nutritional quality of foods. Rupture of bacterial cells due to pressurization and depressurization is considered to be the main cause for the destruction of microorganisms under different pressure regimes (Yano et al., 1998).

HPP was carried out at pressures ranging from 100 to >1000 MPa at temperatures varying from 0-100°C with short holding times to process or pasteurize different foods. In HPP, the application of pressure can be accomplished uniformly throughout the food independent of the size, shape and food composition. Destruction of microorganisms and enzyme inactivation in food products without altering the nutritional value has been reported by several authors while using HPP (Ohnishi and Shigeshisa, 1994; Basak and

Ramaswamy, 1996; Cheftel and Culioli, 1997; Mussa et al., 1999; Ramaswamy et al., 2003).

High pressure processing has been known for more than a century as a food preservation method. HPP technology has been applied for years in industries to process ceramic products, carbon graphite, composite materials and plastics. In the food industry, the use of HPP was reported a century ago for functionality enhancement such as texture. This technology is gaining popularity because of its ability to offer superior quality products as compared to conventional thermal processing and other methods (Cheftel, 1995; Williams 1994; Zimmerman and Bergman, 1993). However, commercial applications have emerged only recently (Farr, 1990).

In the area of HPP of fish, some research has been carried out on different aspects. Much of them deal with application of HPP for gel formulations in fish products such as surimi and bluefish gels (Farr, 1990; Ohshima et al., 1993; Sareevoravitkul et al., 1996). HPP effects on endogenous enzymes related to texture properties of fish have also been studied by some researchers (Ashie and Simpson, 1996). HPP effects on fish muscle and myofibrillar proteins were investigated by Ohshima et al. (1993). The microbial destruction of pathogens by HPP was studied in tuna meat and squid mantle flesh by Shoji and Saeki (1989). Houjaij et al. (2004) studied HP destruction kinetics of indigenous microorganisms in shrimp and trout, and evaluated their quality changes during storage. More recently, Zare (2004) studied the HPP effects on biochemical and quality changes in tuna. The main focus of the studies so far have been evaluation of the effect of high pressure on endogenous enzymes, which results in post-harvest spoilage and texture deterioration. No major study has been carried out on the destruction kinetics

of pathogens in fish using HPP technique. Thus, the objective of this study was to evaluate the destruction kinetics of different pathogens in fish under different HPP conditions. Two pathogens, gram-negative bacterium *E. coli* (O157:H7), and gram-positive bacterium *L. monocytogenes* (Scott A), were selected for this study to compare their resistance to HPP under a range of processing conditions. These foodborne pathogens have been reported to be resistant to pressure treatments (Alpas et al., 1999).

3.3 Materials and Methods

3.3.1 Sample Preparation

Mackerel (*Scomber scombrus*) was obtained from a local market (Montreal, Quebec) pre-filleted and sealed in plastic bags. Fish were stored in a freezer below -18°C before processing. Fillets were thawed overnight at 4°C and cut into pieces weighing ~20 g and filled in to sterile stomacher bags. A fish slurry was made by adding 80 ml of 0.1 % sterile peptone water (Difco Laboratories, Detroit, MI, USA) to the bag and blending for 3 min in a stomacher (Model 400, A. J. Seeward, London, UK) to obtain 100 ml of sample slurry. The slurry approach was used in this study because previous enumeration results from fish fillets inoculated directly with the bacterium were too variable and unreliable due to non-homogeneity of test samples.

3.3.2 Preparation of Inoculum and Inoculation Procedure

Cultures of *E. coli* O157:H7 (ATCC# 43894)and *Listeria monocytogenes* Scott A strains, maintained in the frozen stock at the Food Microbiology Laboratory, Department of Food Science and Agricultural Chemistry, (McGill University, Montreal, Canada)

were used. *E. coli* culture was maintained on Violet Red Blue Agar (VRBA, Difco Becton Dickinson and Company, Sparks, Maryland, USA) and *L. monocytogenes* on Modified Oxford Agar (MOX, CM856, Oxide Ltd., Hampshire, England) with *Listeria* selective supplement (Oxide, SR140, Oxide Ltd., Hampshire, England). The cultures were transferred on a weekly basis to ensure their viability. Inocula were prepared by transferring isolated colonies of *E. coli* into 20 ml of sterile Brain Heart Infusion (BHI, Difco, Michigan, USA) broth while *L. monocytogenes* was transferred into Tryptic Soy Broth with Yeast Extract (TSBYE, Difco, Michigan, USA). *E. coli* sample, incubated at 37°C for 24 h, gave a stock suspension containing 10^9 CFU/ml while the *L. monocytogenes* sample, incubated at 37°C for 48 h, to give a stock suspension containing 10^8 CFU/ml. 100 ml of fish slurry, prepared as detailed in the previous section, was inoculated with 1 ml of the inoculum to give 10^7 CFU/ml for *E. coli* and 10^6 CFU/ml for *L. monocytogenes* for evaluating the HP destruction kinetics. Test pouches were prepared by taking 10 ml aliquotes of the slurry stock in Dual Peel sterile plastic pouches (Baxter Corp., Mississauga, ON) and hermetically sealing by avoiding air entrapment.

3.3.3 Microbial Safety Emphasis

Preparation of all cultures, dilutions and plates as well inoculation of all food samples were done under aseptic conditions in a biological safety cabinet (Labconco PurifierTM Class II, Labconco Corporation, Kansas City, MO). Lab coats and rubber gloves were used during all microbiological procedures. No transfers were done with mouth pipettes. Benches were routinely washed with a 1% hypochlorite solution at the end of each day while the safety cabinet was swabbed with 70% ethanol and UV light left

overnight. All contaminated materials were autoclaved and disposed off properly.

3.3.4 High Pressure Equipment

The equipment used for present experiments consisted of 8L capacity chamber for holding the HPP fluid and samples (Figure 3.1). The isostatic press (Model CIP 42260, ABB Autoclave System, Columbus, OH, USA) had a cylindrical pressure chamber, 10 cm diameter and 56 cm high, and rated for operation up to a maximum pressure of 414 MPa. The high pressure medium was water containing 2% mineral oil. The test product packaged in a flexible material, is placed in the chamber, the stainless steel lid is lowered and secured by lid lock for further protection.

A high pressure pump was used to push the medium inside vessel to build-up the required level of pressure. Pressure release and sacrificial valves were used for discharging the pressure. To check the temperature of vessel and product, thermocouples were attached to a data logger and computer system. An external water circulation system was used to circulate the water around the HPP chamber to maintain the chamber temperature during HPP. The pressure come up time was 0.5 to 3 min depending on the pressure level and the depressurization time was ~15 s.

3.3.5 High Pressure Treatment

3.3.5.1 Kinetic Studies

The inoculated hermetically sealed test samples were kept at 4°C before treatment. Sample pouches were subjected to pressure treatments in the range of 250-400 MPa and holding time ranging from 0-60 minutes for both *E. coli* and *L. monocytogenes*. The pressure level and treatment times are detailed in Table 3.1. All experiments were

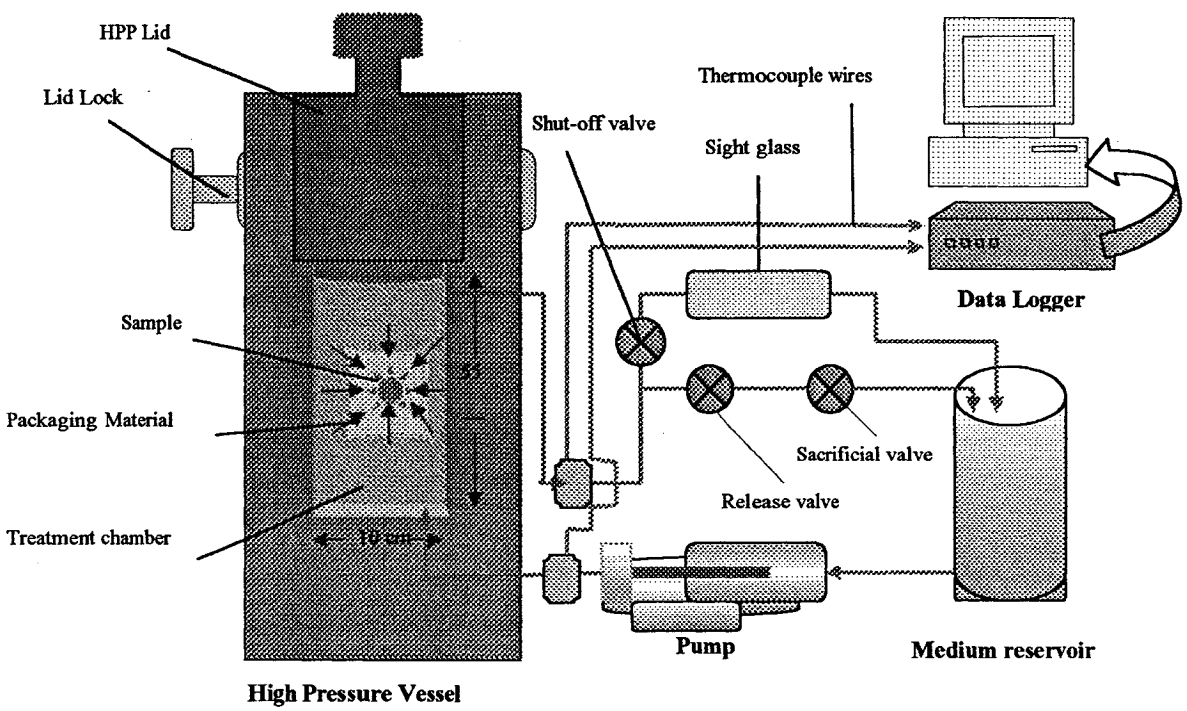

Figure 3.1 Schematic diagram of high pressure processing unit

performed in duplicate and the sample temperature was maintained within 20 to 25°C through water circulation around the chamber. All processed samples and control samples were kept at 4°C prior to enumeration.

3.3.5.2 Compensating for the adiabatic temperature rise

Adiabatic compression during pressurization results in the heating of the sample and liquid in the high pressure chamber. With water circulation maintained at 20°C, it was found that the initial temperature rise varied from about 10°C for treatment at 400 MPa to 5°C for 200 MPa. The temperature of the test sample and water (HPP medium) were kept below the ambient temperature by an appropriate margin (example the initial temperature was adjusted to 10°C for operating at 400 MPa) so that the medium temperature after pressurization would be around 20-25°C (which was maintained during the test by water circulation).

3.3.5.3 Challenge studies

Inoculated (with *E. coli*: 0157:H7) and sealed test samples, prepared in similar manner described earlier, were kept at 4°C before treatment. The sample pouches were subjected to pressure treatments 0, 18, 24 and 30 min (equivalent of 0D, 6D, 8D and 10D at 400 MPa calculated for *E. coli* from with a D value of 3 min determined from the kinetic studies). Again, experiments were performed in duplicate and temperature was maintained within 20 to 25°C throughout the process. All HP treated samples and control were stored at 4, 12 and 20°C and opened at selected intervals for enumeration.

3.3.6 Enumeration

The standard plate count method was used for the enumeration of microorganisms

Table 3.1 Treatment conditions for HPP processing of fish

Pressure (MPa)	Treatment Time (min)				
Escherichia coli O157:H7					
400	0	3	6	9	12
350	0	5	10	15	20
300	0	10	20	30	40
250	0	15	30	45	60
Listeria monocytogenes					
400	0	1.5	3	4.5	6
350	0	2.5	5	7.5	10
300	0	5	10	15	20
250	0	15	30	45	60

by using the standard plates. The treatment samples were transferred to disposable dilution tubes where all subsequent dilutions were made using 0.1% sterile peptone water. *E. coli* was enumerated on Violet Red Blue Agar (VRBA, Difco, Michigan, USA) and *L. monocytogenes* on Modified Oxford Agar (MOX, CM856, Oxide Ltd., Hampshire, England) with *Listeria* selective supplement (Oxide, SR140, Oxide Ltd., Hampshire, England). Plates were incubated 24 h for *E. coli* and 48 h for *L. monocytogenes* at 37°C. Pressure treated samples were enumerated to determine the survivor microbial populations. Initial counts were obtained from untreated control samples. Colonies were counted using a standard colony counter primarily considering plates with 20 to 300 colonies.

3.3.7 Data analysis

The pressure destruction of microorganisms was analyzed based on the dual effect of pressure inactivation with a pressure pulse effect (PE) and pressure-hold effect (Basak and Ramaswamy, 1996). The pulse effect represented the destruction achieved during a pressure pulse which was achieved by pressurization to the desired level followed by immediate release of pressure (with no holding time). In addition to the PE, pressure hold-time contributes significantly ($p<0.05$) to the destruction kinetics. The initial pressure pulse destruction of microorganism was generally followed by a first order destruction kinetics during the pressure-hold time.

3.3.7.1 Pulse effect

As part of experimental setup a zero holding time for pulse effect was included in the experimental model for different pressure levels. The PE values were calculated

experimentally by determining the logarithmic difference in the microbial counts between the control samples and pressure treated samples subjected to a pressure pulse. N_D is a parameter from PE which can be used to compare the pulse and hold effects of pressure destruction. N_D represents the number of pressure pulses required to achieve one decimal reduction in microbial population and was obtained from reciprocal of PE (Basak and Ramaswamy, 1996).

3.3.7.2 Decimal reduction time

The pressure destruction kinetics of microorganisms during the pressure-hold time phase were analyzed based on a first-order reaction indicating a logarithmic order of death, and expressed as:

$$\log_e(N/N_o) = -kt \qquad (1)$$

where N is number of surviving microorganisms following pressure treatment for time t (min), N_o is the initial number of microorganisms with no pressure treatment and k is reaction rate constant (min^{-1}). The treatment time at any given pressure resulting in 90% destruction of the existing microbial population, i.e. resulting in one decimal reduction of the surviving population, is referred to as the decimal reduction time or D-value. This was obtained as the negative reciprocal slope of the log (N/No) vs. time (excluding the untreated control) expressed as:

$$D = -[1/slope] \qquad (2)$$

D_{PE} is referred as decimal reduction time equivalent to destruction achieved by one pressure pulse, another parameter used to compare pulse vs hold approaches of pressure treatment. D_{PE} values were obtained as the product of PE and D-value at each pressure level (Basak and Ramaswamy, 1996).

3.3.7.3 Pressure sensitivity

The pressure dependence of the kinetic parameters was analyzed by pressure destruction time or PDT model. The pressure sensitivity of the D-values is determined by plotting the decimal logarithm of D-values vs. pressure. From the regression slope of log (D) vs. pressure data, the pressure z-value (z_p) was determined as negative reciprocal of the slope:

$$z_p = -(1/\text{slope}) \qquad (3)$$

The z_p of the process, also known as pressure sensitivity of destruction rate, represents the pressure range that results in a 10 fold change in D-value.

3.3.7.4 Microbial growth

The growth of *E. coli* in challenge studies was characterized by three main growth phases. The first phase was the lag phase where no growth was found. In the logarithmic growth phase, a steep increase was observed for the growth and in third phase, a reduction in the growth of *E. coli* could be observed as it moved towards the death phase. A line graph was used to show the different growth phases at different storage temperatures of high pressure treated fish.

3.4 Results and Discussion

3.4.1 Destruction Kinetics of Microorganisms

The survival curves of both microorganisms *E. coli* and *L. monocytogenes* are shown in Figures 3.2 and 3.3, respectively which indicated that the destruction was influenced by pressure level and holding time. For both microorganisms, the survivor curves at higher pressure levels were steeper than at lower pressures which illustrate that

the destruction rate is higher at higher pressures. The figures also demonstrated a good fit of data for the first order model to suggest that the pressure destruction of *L. monocytogenes* and *E. coli* followed the logarithmic destruction model during the pressure hold.

The computed D values at different pressures are tabulated for both microorganisms in Table 3.2. The associated high R^2 values (0.92-0.98) confirm the basis for the first order model for the pressure destruction. As expected, the associated D values were higher at lower pressures for both microorganisms. *L. monocytogenes* started with a higher value of D than for *E. coli* at 250 MPa indicating it to be more pressure resistant. However, as the pressure level was elevated, their differences started to diminish and beyond 350 MPa, the associated D values were higher for *E coli* than for *L. monocytogenes*. This indicates at higher pressures (≥350 MPa), *E. coli* was more resistant than *L. monocytogenes* to pressure destruction (while at lower pressures *L monocytogenes* could be more resistant). This is also evident from Figs. 3.2 and 3.3, but becomes obvious only when the extent of destruction at a given pressure level and time are carefully compared. Since the purpose of HPP is to achieve rapid destruction, data relevant to higher pressures are more useful. Equipment limitation restricted operation at a maximum pressure of 400 MPa, while commercial equipments are available for operation at much higher pressures. Hence, *E. coli* was selected from a process establishment point of view. At 400 MPa, the associated D values for *E. coli* and *L. monocytogenes* were 3.19 and 1.49 min, respectively.

Patterson et al. (1995) reported similar results for microbial destruction in milk and meat. The authors reported 5 log reductions following a 15 min treatment at 375 MPa

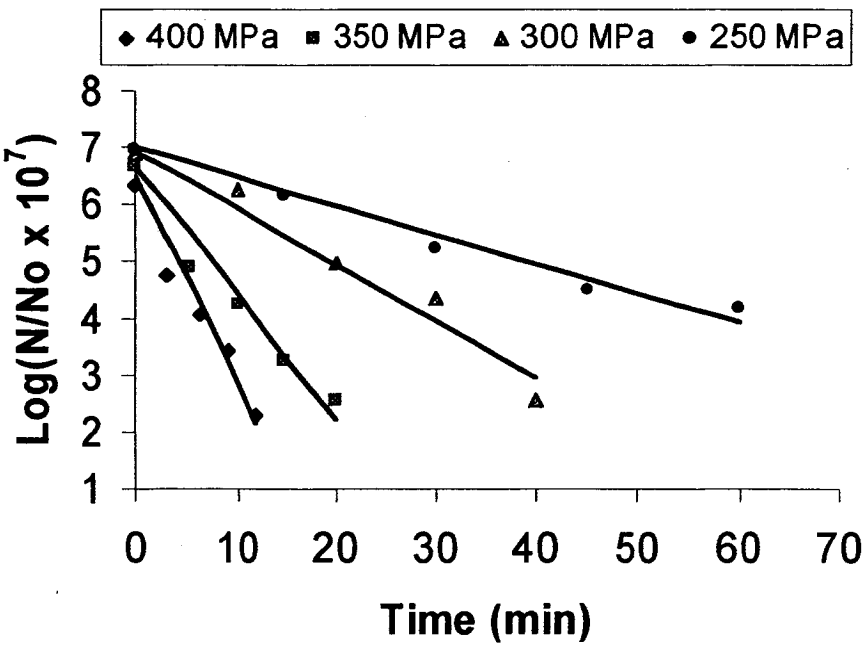

Figure 3.2 HPP survival curves of *E. coli* (O157:H7) in mackerel

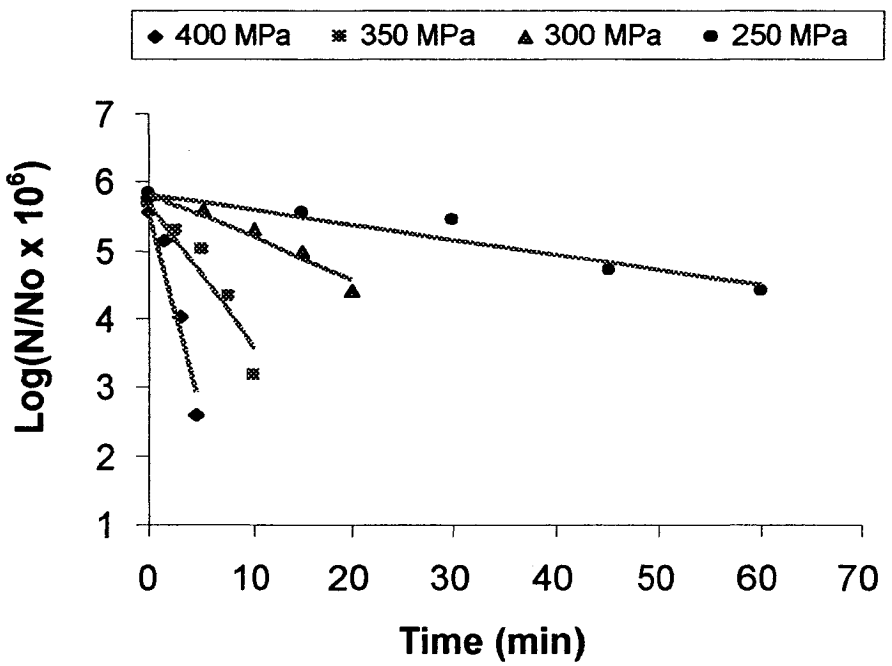

Figure 3.3 HPP survival curves of *L. monocytogenes* (Scott A) in mackerel

Table 3.2 Kinetic data (PE, D-value, R^2, D_{PE}, N_D) table for *E. coli* and *L. monocytogenes* in HP treated fish

Pressure (MPa)	PE (log)	D-Value (min)	R^2	D_{PE} (min)	N_D (cycles)
E. coli					
400	0.68	3.19	0.97	2.15	1.48
350	0.38	5.05	0.97	1.91	2.65
300	0.19	9.42	0.97	1.77	5.32
250	0.04	20.74	0.98	0.75	27.65
L. monocytogenes					
400	0.27	1.49	0.95	0.41	3.67
350	0.21	4.16	0.92	0.89	4.65
300	0.09	14.74	0.96	1.38	10.66
250	0.01	40.05	0.94	0.55	72.57

(20°C) for *L. monocytogenes* while *E. coli* required same treatment at 700 MPa for a similar reductions. A 6 log reduction in *E. coli* was reported by Gervilla et al. (1997) in 6% fat bovine milk after 5 min treatment at 450 MPa and 10°C, while 400 MPa pressure was adequate to achieve same reduction in *Pseudomonas fluorescens*. In this study, a D-value of ~1.5 min was achieved for *L. monocytogenes* at 400 MPa giving a treatment time of about 7.5 min for 5 log reductions, while *E. coli* would require a 15 min treatment. A slightly higher D-value of 2.17 min was reported for *L. monocytogenes* in fresh pork loin at 25°C at 414 MPa by other researchers (Ananth et al., 1998). Mussa and Ramaswamy (1999) also observed a much higher D-value of 3.5 min for *L. monocytogenes* in pork chops under similar conditions. Hence, it is clear that the pressure destruction kinetics of microorganisms depend not only on pressure parameters but also on the type and composition of food.

3.4.2 Pressure Death Time model (PDT)

Lower D-values are associated with higher pressures demonstrating a higher destruction rate for both microorganisms under these conditions (Table 3.2). D-value curves in Figure 3.4 prepared for computing the pressure sensitivity parameter (z_p value) also demonstrate this point. The cross-over of decimal reduction time curves for *E. coli* and *L. monocytogenes* indicated the pressure point of 320 MPa beyond which *E. coli* is more resistant to pressure than *L. monocytogenes*. This also demonstrates that in processes using lower pressures, *L. monocytogenes* would require longer holding times to achieve a similar level of destruction as *E. coli*. In some applications of HPP for fish, pressure levels below 300 MPa are intentionally used for facilitating other objectives

(pressure shift freezing, HP thawing, enzyme inactivation/activation etc). In such situations, if pathogen inactivation becomes a requirement, *L. monocytogenes* would become a target pathogen. For pressure processing intended for promoting safety and stability, the target pathogen has to be *E. coli* because it is more resistant at practical conditions of operating pressures (>400 MPa).

Further, the z_p value associated with *L. monocytogenes* was 103 MPa compared to 185 MPa for *E. coli*. This means that the destruction rate of *L. monocytogenes* is more sensitive to changes in pressure than *E. coli*. It also indicate that the target pathogen should be *E. coli* as the operating pressures get higher, it would be much easier to kill *L. monocytogenes* under those processing conditions. The z_p value can also be used to predict the D values at different pressures. With *E. coli*, with a z_p value of 185 MPa, it can expected that the D value at 600 MPa, could be as low as 15 seconds, making operating at such pressures to be attractive and time efficient. Generally gram-positive are considered to be more resistant to pressure than gram-negative bacteria (Hoover et al., 1989). However, this study showed it thus is not always the case as at higher pressure levels *E. coli* (gram-negative bacteria) was more resistant than *L. monocytogenes* (gram-positive bacteria). The previous studies also have demonstrated that the variability in resistance of microorganisms depends on a number of factors. Food constituents can protect microorganisms against high pressure (Yuste et al., 1998). Furthermore, the variation in pressure sensitivity can be due to numerous other interacting factors in the food itself (Patterson and Kilpatrick, 1998). Mackerel is a fat-rich fish which may provide extra protection to *E. coli* cells compared to *L. monocytogenes*. The other main consideration is the pH of the material that can result in variability of the resistance for different

microorganisms (Mackey et al. 1995; Stewart et al., 1997). Even different mutants of *E. coli* show a large difference in resistance as was found by Opstal et al. (2003) in buffer solution and in fruit juices by Garcia-Graells et al. (1998).

3.4.3 Pulse Effect, D_{PE} and N_D

In Figures 3.2 and 3.3, the first order rate curves were discussed and showed to be appropriate descriptors of HP destruction kinetics for both microorganisms. While they do confirm the first order kinetics, it is also obvious from these figures that the curves do not originate from the origin. There is a clear deviation of the first point from the counts associated with controls. The pulse effect can thus be observed from destruction kinetics curves (Figures 3.2 and 3.3) where a decrease in counts occurs at the beginning of the curves at zero holding time. This shift is the result of the pressure-pulse (considered time-zero for the hold-time kinetics). This effect of pulse without holding time was explained by the rapid pressurization and depressurization that leads to an adiabatic expansion of liquid system of cell and causes death of microorganisms (Ramaswamy et al., 2003). The first order rate holds good only during the pressure-hold period, i.e., after the pulse effect. In this study, the pulse effect ranged from almost an insignificant value to about 0.25 to 0.75 log reductions at 400 MPa. Still, it was relatively small compared to the pulse effects observed for other microorganisms in other foods (Basak et al., 2002; Ramaswamy et al., 2003).

In Figure 3.5, the pulse effect realized for the two microorganisms are compared. *E. coli* demonstrated a greater susceptibility to pulse pressure than *L. monocytogenes*. At lower pressures of 250 MPa, both microorganisms showed little or no effect to pressure

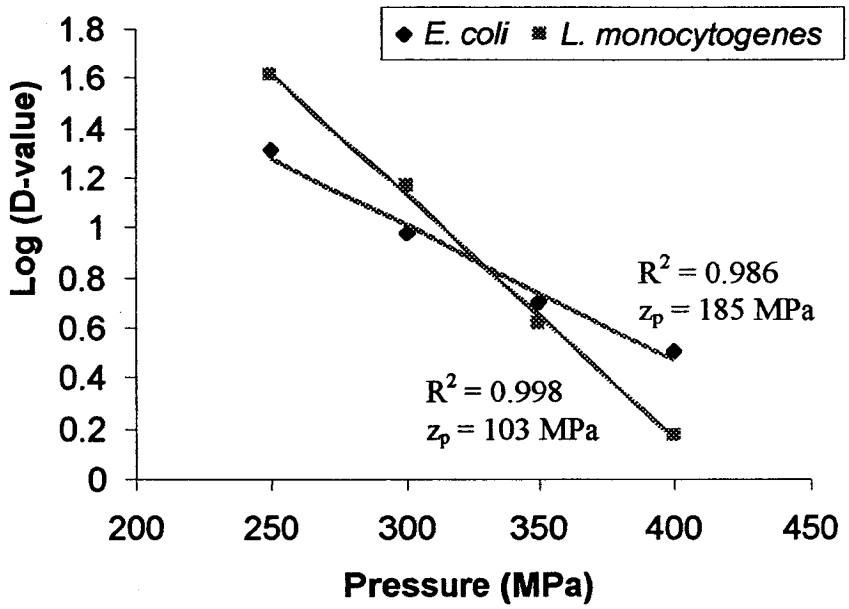

Figure 3.4 HP decimal reduction time curves for *E. coli* and *L. monocytogenes* in mackerel

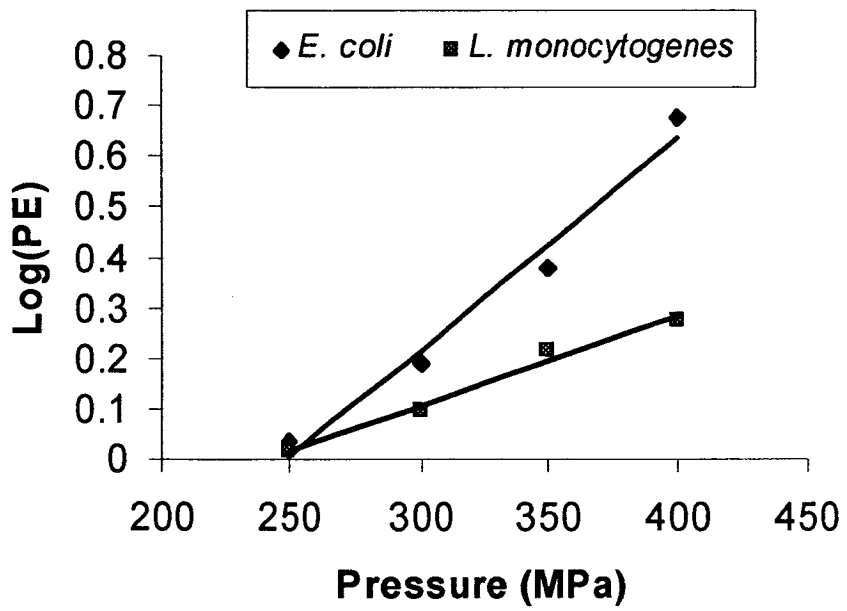

Figure 3.5 HP pulse effect on *E. coli* and *L. monocytogenes* in mackerel

pulse; however, as the pressure levels increased, the pulse effect became more obvious. Clearly, *E coli* is a better candidate to take advantage of pulse pressure than *L. monocytogenes.*

In Table 3.2, two other parameters are listed, D_{PE} and N_D, which can be used to further describe the impact of pulse effect in relation to pressure-hold effect. The D_{PE} values for *E. coli* decreased with a decrease in pressure indicating a smaller contribution of the pulse effect at the lower pressures. It is the magnitude of D_{PE} in relation to D that is important to recognize. At 400 MPa, the D_{PE} is in the same order of magnitude (70%) as D value (2.15 min as compared with 3.19 min) indicating a pressure pulse to be as effective as a treatment to achieve a decimal reduction in microbial population (a level that is quite adequate when enzyme inactivation is considered). In the case of *L. monocytogenes*, however, D_{PE} values were more variable, and never reached a level closer than 25% and hence, the pulse approach is therefore less practical for their destruction.

The N_D value is an indicator of the number of pulses required for achieving a decimal reduction in microbial population or activity, which is more easily recognizable. The N_D for both microorganisms dramatically decreased with an increase in pressure level. Therefore, less number of cycles are required to achieve a given level of destruction at higher pressures. At the highest pressure, 400 MPa, the N_D value of *E. coli* was 1.5 indicating that in three pressure pulses it was possible to reduce the counts by 2 log cycles. If for example it is intended to reduce the population of *E. coli* by 6 log cycles (6D), then it would require 9 pressure pulses, as opposed to a holding for 18 min. From the equipment maintenance point of view, so many cycles would result in excessive wear

and tear on pressure cylinders, gaskets etc, and is not necessarily desirable. In terms of time, a pressure pulse would require about 4 min to complete and hence 9 cycles would mean 36 min which is much higher than ~20 min required by the pressure-hold approach. Again in this case, the hold approach is better. The conclusion were confirmed in early studies on the inactivation *E. coli* in apple juice where a pulse approach was more desirable inactivation (Ramaswamy et al., 2003).

3.4.4 Challenge Studies with *E. coli O157:H7*

Figures 3.6, 3.7 and 3.8 show the growth curves of *E. coli O157:H7* in pressure treated fish stored at 20, 12 and 4°C, respectively. The three phases of the microbial growth curve - lag phase, logarithmic growth phase and stationary or the phase of death, were more noticeable as the storage temperature is decreased. The second most striking observation is that changes happen more rapidly at 20°C compared to 4°C which is clearly expected from a microbiological point of view. But there were also many temperatures specific and pressure treatment severity related differences which are discussed below. In each figure, the growth curves are displayed for *E. coli* in samples pressure treated for different holding times. The control (0D) samples were untreated. The 6D, 8D and 10D were computed based on a nominal D value of 3 min at 400 MPa (actual value from kinetic studies reported earlier was 3.19 min) giving treatment times ranging from 18 to 30 min.

At 20°C storage, immediately after the pressure treatment, the counts were reduced, as expected, to below 10 CFU/ml (demonstrating over 6 log cycle reduction in surviving population), while the control sample stayed at 10^7 CFU/ml. Since the control

was diluted from a maximum of around 10^9 CFU/ml, it showed short growth during storage at 20°C. However, *E. coli* in all samples pressure treated samples (6-10D) started recovering and growing during storage at 20°C. The lag phase was almost absent and the stationary stage of maximum growth was reached within 12 days. Control samples were completely spoiled by that time, reaching the stationary phase within 2 days and starting to decline after 6 days. However, the 6D, 8D and 10D showed a clear exponential growth phase followed by a stationary phase. Obviously, this means that even the 10D process did not kill all of the microbial population, but may have only injured the cells which subsequently recovered during storage at 20°C.

At 12°C, the inoculated samples without treatment reached the maximum exponential phase within six days of storage followed by a stationary phase and death phase. Samples pressure treated for 6D and 8D a similar trend in growth was observed, however, the exponential phase reached to maximum after 6 and 8 days respectively. The 10D treatment did not show any growth up to 18 days and indicating that the 10D was sufficient to inactivate *E. coli* to prevent its subsequent recovery and growth at 12°C. The growth behaviour of *E. coli* in samples at 4°C storage was somewhat different. The control sample, in which the initial population was 10^7 CFU/ml, started to show a decline in *E. coli* population indicating that these cells did not tolerate the refrigerated storage conditions. For the 6D and 8D treated samples, there was an apparent initial growth, but with continued storage, cells lost their viability. No lag-phase or exponential stage in *E. coli* growth was observed for any of the three pressure treated samples. Moreover, samples pressure treated to 10D level showed no viability during the entire 30 days, indicating again that the process was adequate for the inactivation of *E. coli*.

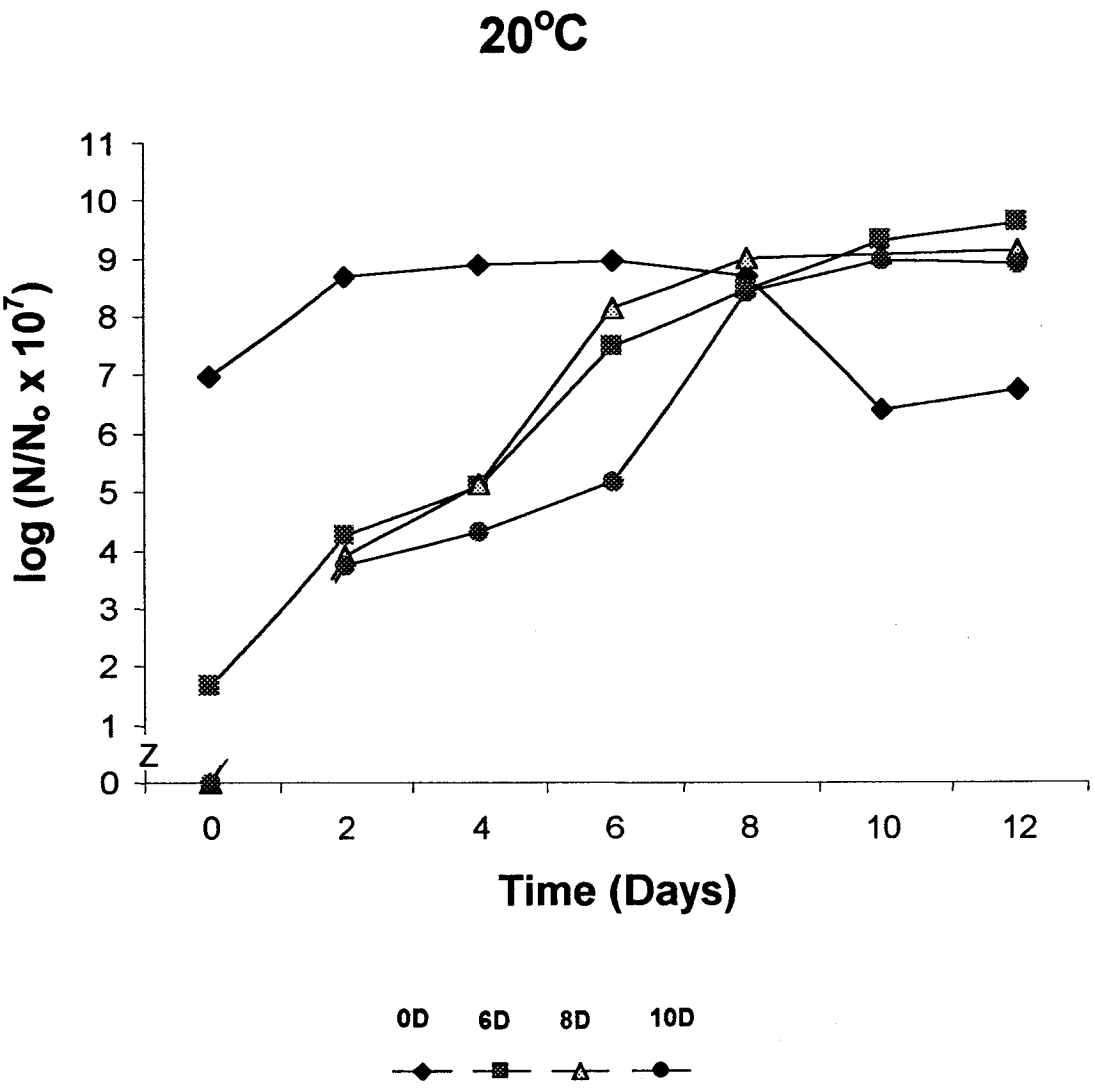

Figure 3.6 Changes in *E. coli* survivors in HP treated mackerel during storage at 20°C

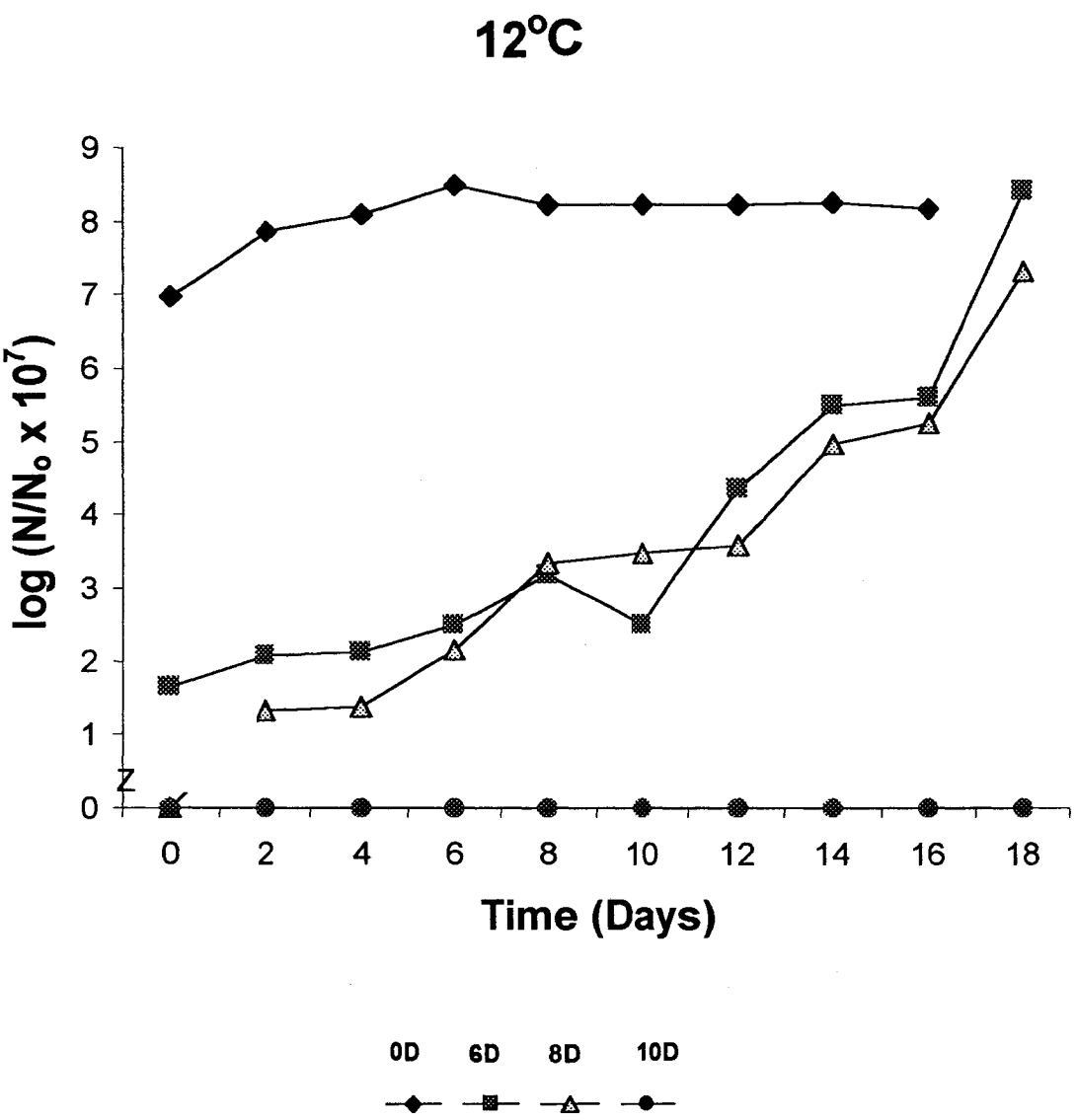

Figure 3.7 Changes in *E. coli* survivors in HP treated mackerel during storage at 12°C

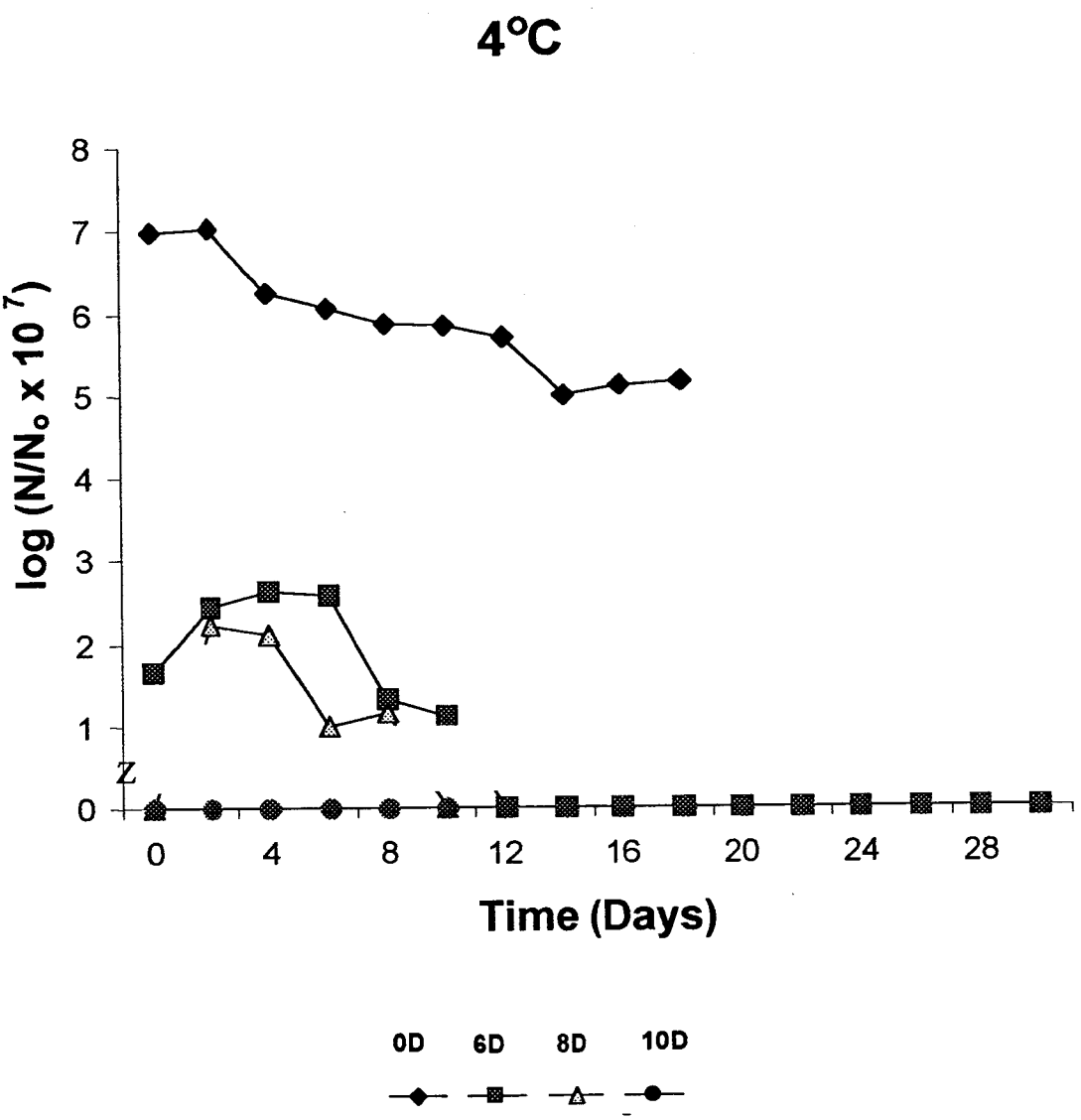

Figure 3.8 Changes in *E. coli* survivors in HP treated mackerel during storage at 4°C

Overall, the growth behaviour of *E. coli* at different temperatures showed that both pressure lethality and storage temperature had important roles on its survival and growth. At 4°C, a clear and different behaviour was observed possibly due to the fact that *E. coli* is not a psychrophilic microorganism. Hence, all treated samples (6-10D) were acceptable from safety point of view at this temperature showing no signs of recovery or growth. The10D treatment was also enough to fully suppress the recovery and growth of *E. coli* in the fish slurry stored at 12°C. However, the 6D and 8D treatments failed to provide an adequate level of inactivation for *E. coli,* where recovery and growth occurred at 12 and 20°C storage. The main reason for such growth differences at different temperatures was the fact that the minimum temperature requirement for the growth of *E. coli* has been recognized to be 10°C (Prescott et al., 1999).

A 10D process was considered adequate when used in combination with storage at refrigerated temperatures (4-12°C) to stop the growth and recovery of *E. coli* in a favourable fish medium.

3.5 Conclusions

Pressure destruction kinetics of both microorganisms (*E. coli* and *L. monocytogenes*) in fish followed a dual effect destruction behaviour that was characterized by a pulse effect (without any holding time) followed by a first order rate destruction in the number of survivors during the pressure-hold period. At pressure levels 350 MPa or higher, *E. coli* was more resistant than *L. monocytogenes,* although at lower pressures the reverse was true. Hence, pressure resistance may depend on the pressure level and type of microorganisms. Since higher pressures require shorter treatment times, high-pressure

short-time processes would be an attractive method for processing of fish. The pulse effect (PE) was more pronounced for *E. coli* destruction than *L. monocytogenes*. The lower z_p value associated with pressure destruction of *L. monocytogenes* indicated its pressure resistance was more sensitive to changes in pressure than those of *E. coli*. The pressure sensitivity can vary for gram-positive and gram-negative bacteria mainly depending on composition of food, pH and pressure level. In addition to evaluation of destruction kinetics, it is always useful to carry out challenge studies by storing pressure treated samples at typical conditions and follow the recovery/growth of pathogens. Test results showed that at temperatures of 12-20°C, there was a substantial recovery and growth of *E. coli* even though immediately following treatment the cells appeared inactivated. 6-8D treated samples stored at temperatures below 12°C resulted in no viable counts throughout 30 days storage of fish slurry.

Chapter 4

Microbial Shelf-life of and Quality Changes in High Pressure Treated Atlantic Mackerel

4.1 Abstract

The effect of high pressure treatment, designed to render the product pathogen-safe, on microbial and objective quality associated with Atlantic Mackerel (*Scomber scombrus*) during refrigerated storage was evaluated. Fish subjected to pressure treatments in an isostatic press at 400 MPa for times (18-30 min) equivalent of to 6, 8 and 10 log-cycle reductions in the pathogen *E. coli* (safety purpose) with untreated sample taken as control. All treatments were done at room temperature (20-25°C). Microbial growth, texture, color, drip loss and pH were evaluated in all test samples at selected time intervals after storage at 4, 12 and 20°C to assess the shelf-life of high pressure treated fish. Treatments decreased the population of indigenous microorganisms, but did not eliminate them since growth has observed during storage. Color was the major quality parameter affected by high pressure treatment and subsequent storage. Drip loss was not affected by treatment time, however lower temperature storage reduced the drip loss. HP treatment improved the tenderness, but storage contributed to texture loss. Changes in pH were small but consistent with microbial spoilage. Quality changes associated with HP treated fish during storage were caused by the growth and activity of indigenous microorganisms and enzymes. Lower storage temperature rendered longer lag phase for microbial growth and extended the shelf-life.

4.2 Introduction

Freshness and quality are two main factors receiving attention in fish marketing to fulfill consumer's expectations. Quality of a product not only depends on a set of attributes, but also depends on consumer's attitudes and consumption, methods of preservation and regional preferences (Connell, 1995). The main relevant factors, along with consumer acceptability, are safety, nutrition, flavor, texture, color and suitability of the raw material for further processing or storage (Haard, 1992).

Mackerel is an Atlantic fish which has been reported to have beneficial health effects in humans due to the presence of Ω-3 polyunsaturated fatty acids (De Deckere et al., 1998; Nettleton, 1991). The world production of Atlantic Mackerel was 666,964 tonnes in 1998 and reached 769,068 tonnes in 2002 thus showing a substantial increase in its use and consumption (FAO, 2002). Mackerel is available as fresh whole or filleted, frozen, canned and also served as smoked.

The quality parameters of fish include freshness, microbiological load and physical damage which are important factors to determine the shelf-life of fish (Fuselli et al., 1994). The maintenance of quality mainly depends on storage temperature like ice-storage (0°C) or refrigeration (4-6°C). The microbiological spoilage occurs even during refrigerated storage by psychrophilic microorganisms dominated by *Pseudomonas, Shweanella, Achromobacter, Acinetobacter, Flavobacterium, Alteromonas* species (Van Spreekens, 1977; Shewan and Murray, 1979; Eklund, 1982; Lindsay et al., 1986; Hubbs, 1991; Liston, 1992). The spoilage of fish results in the formation of off-odors when psychrotrophs reach a population around 10^7 to 10^8 CFU/cm^2 and slime appears when

counts reach 10^8 CFU/cm^2 on fish surface (Ayres, 1951). A very few studies have been carried out to inactivate microbes in seafood, specifically fatty fish, to extend its shelf-life. Most studies focussed mainly on controlling enzyme activity in fish.

The physical effects, in the form of loss in freshness are mainly related to structure and color changes, which be measured instrumentally (Olafsdottir et al., 1997). Pressure treated fresh cod muscle showed an increase in hardness which was similar to heat treated cod at 50°C as determined by texture profile analysis (TPA) and were reported due to changes in different protein structures (Angsupanich and Ledward, 1998; Angsupanich et al., 1999). Another study on blue fish revealed that pressures of 101 MPa increased the firmness of fish muscle during storage at 4-7°C while pressure treatment at 202 and 303 MPa had the opposite effect (Ashie et al., 1997). The noticeable color change in white fish, such as cod and mackerel, causing opacity after high pressure treatment has been found to be similar to that of cooked fish (Ohshima et al., 1992; Shoji et al., 1990; Cheah and Ledward, 1996; Angsupanich et al., 1999). In cod, such color changes have been reported at pressure treatments between 100 and 200 MPa (Angsupanich and Ledward, 1998). A slight increase in pH in HP treated cod fish on storage at 4°C was reported to be due to bacterial spoilage (Angsupanich and Ledward, 1998).

Different preservation methods have been used for fish to increase its shelf-life. Freezing has been traditionally used for long term preservation of fish, but that can bring some change in overall quality (modification of proteins) and loss in freshness upon thawing. Thermal processing yield shelf-stability, but results in degradation of nutrients and quality; and the product cooking during thermal processing pushes it far away from

the fresh. Other traditional methods also include rapid chilling and ice storage used to extend shelf-life of fish (Kyrana and Lougovois, 2002; Alasalvar et al., 2001; Tejada and Huidoboro, 2002). To increase shelf-life of a perishable commodity, such as, fish, there is an obvious need for the development of new processes, such as high pressure processing. As noted, HPP offers potential for extending the shelf-life of a variety food and has been investigated by several researchers (Cheftel and Culioli, 1997; Thakur and Nelson, 1998; Venugopal et al., 2001). In most studies dealing with HPP of fish, the pressure treatment has been limited to low pressures (<300 MPa) and relatively short times in order to minimize color changes. From the previous chapter, it is obvious that if the HPP were to be used to enhance safety, the pressure levels have to be at least as high as 400 MPa with treatment times ranging 18-30 min. In order to effectively use such a process, the effect of the given HP treatment (400 MPa 18-30 min) on product quality changes during storage need to be assessed. Although the process was developed to ensure safety, it is understood that such a process will not eliminate all indigenous bacterial, especially spores, which will cause spoilage. Hence, refrigerated storage is an essential addition to HPP.

4.3 Materials and Methods

4.3.1 Sample Preparation

Fresh mackerel was obtained from a local market (Montreal, Quebec) pre- filleted and sealed in bags. Samples of different weights (intended for different quality assessment: 20 g fillets for microbial growth, 20 g for drip loss and pH and about 60 g for color and texture) were packaged in sterile pouches and subjected to high pressure treatment at 400 MPa to achieve the pre-selected pressure lethality equivalent to reduce *E.*

coli population by 6, 8 and 10 log-cycle reduction (D) with untreated sample (0D) serving as control. The treated samples were stored at 4°C, 12°C and 20°C for different times and evaluated for microbial and other quality analysis to determine the shelf-life of fish. Analyses were carried out at 2-day intervals for samples stored at 4°C, and daily for those stored at 12 and 20°C. Pouches were aseptically opened on the pre-selected days for different parameter analyses. The analyses were discontinued when samples were rejected based on odor and/or sliminess of fish.

4.3.2 High Pressure Equipment

The equipment used for high pressure processing was ABB Isostatic Press Model #CIP42260 (ABB Autoclave System, Autoclave Engineers, Columbus, OH). The details are given in Chapter 3.

4.3.3 Microbial Enumeration

Pouches were aseptically opened on the day of evaluation and blended with 80 ml of 0.1% sterile peptone water in a stomacher. All subsequent dilutions were made from this slurry for plating. Total plate counts were evaluated on Tryptic Soy Agar (TSA) by incubating the petri-dishes at 37°C for 48 h. Microbial counts were multiplied by the number of the appropriate dilution factor to give counts in CFU/g.

4.3.4 Shelf-life Deduction on Microbial basis

A scale was set for maximum microbial growth to indicate the onset of microbial spoilage. Amanatidou et al. (2000) recommended microbial counts of 10^7 CFU/g to be considered critical counts for spoilage in their shelf-life study on HP treated Atlantic salmon. The acceptable microbiological limit for chilled stored fish is considered 10^6

CFU/g by Spanish law (Hurtado et al., 2001). Some agree on higher counts, such as 10^7 to 10^8 CFU/g for sensory rejection of fish in storage (Olasfsdottir et al., 1997). For this study the general microflora above 10^6 CFU/g was considered upper limit and once reached the sample was considered microbiologically unacceptable (by which time off-flavor was generally evident and the product would be rejected on a sensory basis).

4.3.5 Texture Evaluation

Texture analysis of fish samples was performed using a Universal Testing Machine (Lolyd Model LRX, Lloyd Instruments Ltd., Fareham, Hans, UK) to measure hardness (force necessary to attain a given deformation) and springiness (rate at which a deformed material returns back to its original form after removal of the force). A 50 N load cell was installed with a 50 mm diameter circular plate to perform a two stage compression test. The fish fillet was cut in 10 mm x 10 mm x 10 mm cube obtaining two such cubes from each duplicated sample. A 40% of compression rate at speed of 5 mm/s in stage 1 and 10 mm/s in stage 2 were applied to perform Texture Profile Analysis (TPA). The texture parameters were measured using following formulae:

Hardness (N) = maximum force (N) during the applied for deformation (1)

Springiness (%) = recoverable area (relaxation) / total area (compression) * 100 (2)

4.3.6 Color Measurement

The color of stored fish was measured using a Minolta spectrophotometer (Chroma Meter II, Minolta Corporation, Ramsay, NJ). The instrument was calibrated against Minolta standard white reflector plate. The color attributes L* (L = 0-100), a* (+a = red, -a = green), and b* (+b = yellow, -b = blue) were measured for lightness, redness

and yellowness, respectively. Three readings were taken at different locations on the inner surface of fish from each duplicate sample and readings were averaged.

4.3.7 Drip-loss measurement

To determine the drip loss a known weight of sample (about 20g) (in duplicate) was taken on specific sampling days. Samples were removed from the pouches and paper blotted to remove surface water and weighed. The difference between the initial weight and weight after water removal was considered as drip loss. It was expressed as percentage of initial weight and calculated as:

$$\text{Drip Loss \%} = (\text{loss in weight} / \text{initial weight of sample}) * 100 \qquad (3)$$

4.3.8 pH measurement

The pH of fish slurry was measured using a calibrated pH meter (Corning pH meter 220, Corning Science Products, Corning Glassworks, Corning, NY). The pH meter was calibrated with two buffer solutions at pH 7.0 and pH 4.0 before taking the readings. The slurry was made with 10 g of fish in 30 ml of distilled water and homogenized in a stomacher for 3 min. Readings from duplicate samples were averaged.

4.3.9 Data Analysis

The reaction rate for microbial growth during storage was analyzed assuming first order kinetic growth model (Mussa et al., 1999). Decimal multiplication time (D_m) (time required to increase the microbial survivors ten times) was obtained from the reciprocal of the slope obtained from semi-logarithmic curve of days versus $\log_{10}$ of microbial count as:

$$D_m = [1/\text{slope}] \qquad (4)$$

The z_m was also calculated from the slope of log D_m value versus pressure lethality to obtain the temperature sensitivity of treated samples.

The changes in pH, drip loss, color L*, color b* and decrease in color a*, hardness and springiness during storage were demonstrated using a bar graph of experimental values.

4.4 Results and Discussion

4.4.1 Microbial growth

The growth curves of microorganisms in the HP treated fish at 0D (control), 6D, 8D and 10D are shown in Figure 4.1 for storage at various temperatures. Microbial growth increased during storage and growth was highly influenced by pressure lethality and storage temperature in all samples. The initial counts of indigenous microflora was ~10^3 CFU/g. The microorganisms started multiplying immediately with no sign of a lag period in the control samples while growth being more rapid at higher storage temperatures (Figure 4.1).

The 6D treatment represented a pressure treatment of 18 min at 400 MPa. It resulted in the microbial counts to decrease below considerable counting levels (below 100 CFU/g). Again, storage temperatures showed marked differences in the growth rates. Comparing the 20°C storage to 4°C, a small lag period became noticeable. With 8D and 10D treatments (400 MPa 24-30 min), these changes became more obvious. Follow, the lag period increased with the degree of severity of the pressure treatment (longer treatments resulting in a longer lag period). Lower storage temperatures likewise resulted

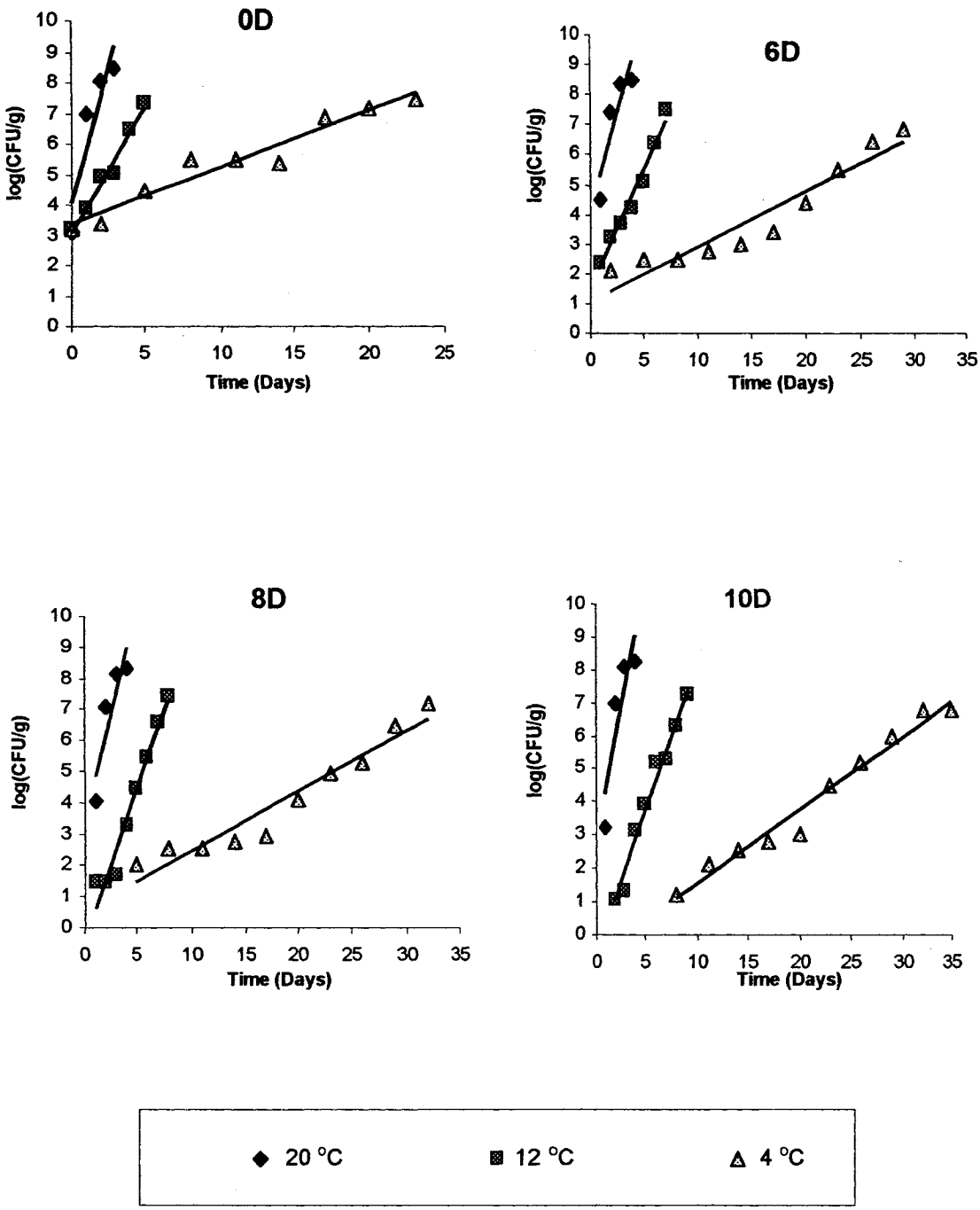

Figure 4.1 Effect of applied pressure lethality on microbial growth in fish at different storage temperatures

in an increase lag period which has as much as 7 days for 10D treated samples stored at 4°C.

Table 4.1 shows D_m values for microbial growth obtained from the semi-logarithmic regression analysis of growth versus storage time. High R^2 (>0.79) values in the table indicated that the microbial growth generally followed the first order kinetics growth model after the lag. The D_m values associated with lower storage temperature were generally higher indicating a slower rate of microbial growth (longer multiplication time). However, the pressure lethality (different treatment time at 400 MPa, expected to yield 6-10 log cycle reductions in *E coli* 0157:H7 population) did not have any effect on the decimal multiplication times. D_m values varied between 0.58-0.78 days at 20°C, 1.05-1.23 days at 12°C and 4.58-5.37 days at 4°C.

It should be noted, however, that although the pressure lethality did not show any effect on the growth rate, its role was nevertheless important, due to its effect on prolonging the lag period. The 10D pressure treated samples stored at 4°C were characterized by a lag phase of 7 days while 8D samples showed 2-3 days and 6D samples only 1 day lag phase at the similar storage temperature. Hence, this will off set overall growth. The rate of growth rate after the lag phase, however, dictated by the storage temperature with growth at 4°C being a considerably slower rate as compared to samples stored at 12 and 20°C. In fact, the multiplication rate at 20°C was 7-9 times the rate at 4°C resulting in rapid microbial spoilage at 20°C.

As noted earlier, the lower D_m values at higher temperatures indicate that storage temperature had the most dominating effect on microbial growth. The similarity of the

Table 4.1 D_m and regression coefficient values for microbial growth at different storage temperatures in HP treated fish

Pressure Lethality (#)	Temperature (°C)	D_m Values (Days)	R^2
0D			
	20	0.58	0.82
	12	1.23	0.97
	4	5.37	0.94
6D			
	20	0.78	0.80
	12	1.21	0.97
	4	5.42	0.91
8D			
	20	0.72	0.82
	12	1.05	0.96
	4	5.17	0.93
10D			
	20	0.62	0.79
	12	1.11	0.97
	4	4.58	0.97

growth rate for different HP treated samples at a given storage temperature is demonstrated in Figure 4.2 which shows horizontal lines for log of D_m values versus pressure lethality curves.

At lower storage temperature the horizontal lines move upward indicating slower rate of multiplication (i.e., higher D_m values). Table 4.2 lists the computed z_m values which are temperature sensitivity indicators of growth rates. They are in a similar range of all pressure treated products (18.46-19.05°C) with slightly lesser value (more sensitive) for the control (16.61°C). These results show that when storage temperature increasing to 16-19°C, the multiplication rate increased 10 times.

The lag phase after pressure treatment has been observed by other researchers in different fish and other meat products and has been credited to the pressure effect on cells which increases with the level of pressure and time (Carlez et al., 1994; Lopez-Caballero et al., 2000a; Hurtado, 2001). In these studies, an increase in shelf-life was observed by delaying microbial growth 2-6 days by pressure treatment at 400-450 MPa in minced meat followed by storage at 3°C. The pressure treatment was sufficient to reduce the total microflora by 3 to 5 log cycles while the growth of *Pseudomonas* species was reported to resume after the lag phase, depending on the pressure level. The processes were reported to extend the shelf-life of minced meat by 10-15 days at 3°C (Carlez et al., 1994). In another study, pike perch (*Lucioperca lucioperca*) fillets were reported to have a 28 day shelf-life at 4°C storage temperature after pressure treatment at 700 MPa for 5 min at 20°C (Myllymaki et al., 1997). These authors also observed similar critical level counts in control samples.

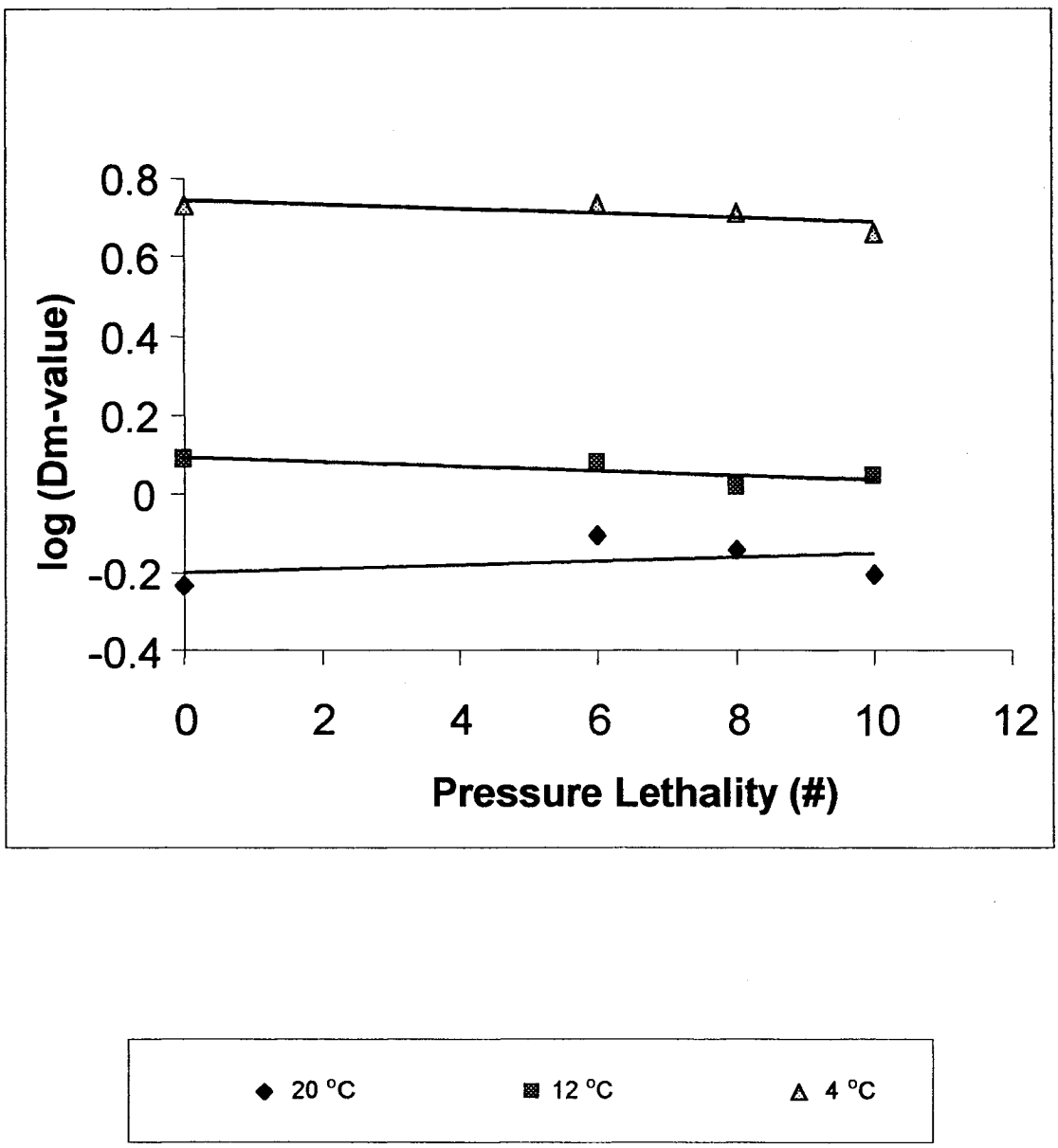

Figure 4.2 Pressure sensitivity curves of the D_m values for microorganisms in HP treated fish

Table 4.2 Temperature sensitivity (z_m) for microbial growth of fish treated with different pressure lethality

Pressure Lethality (#)	z_m (°C)	R^2
0	16.61	0.965
6	19.05	0.907
8	18.75	0.885
10	18.46	0.945

4.4.2 Changes in Texture

The changes in the hardness of fish are shown in Figure 4.3, 4.4 and 4.5 for 20°C, 12°C and 4°C, respectively. They all showed strikingly similar patterns, hardness of all samples decreasing with storage time at each temperature. Within each temperature, there was a slower decrease in hardness in HP treated fish compared to control which indicates an improvement in texture retention of fish with treatment. HP treatment considerably influenced the hardness value of fish. The hardness increased with pressure treatment at 400 MPa. The 6D treated samples (18 min holding time) resulted in the firmest samples, with 8D and 10D samples showing a slightly lower hardness compared to 6D. With storage the hardness value decreased for all samples. This decrease was much rapid with control samples which decreased below 0.6N within 3 days at 20°C and 20 days at 4°C. On the other hand, treated samples showing a comparable declining trend, maintained hardness values above 2N throughout storage. On a time scale, treated samples maintained good hardness for up to 4 days at 20°C, 7 days at 12°C and 29 days at 4°C, clearly showing that the lower temperature contributed the most in preventing tissue softening. Furthermore, there were only small differences in hardness values between the three pressure treatments (0.5-0.6N) at any given storage time/temperature combinations.

Changes in the springiness of the control and pressure treated fish are illustrated in Figures 4.6, 4.7 and 4.8, respectively, at 20°C, 12°C and 4°C storage conditions. These figures illustrate patterns similar to those observed for hardness. The associated changes however are somewhat steeper. They all demonstrated an increase in springiness with pressure treatment followed by a decrease in springiness with time at different storage

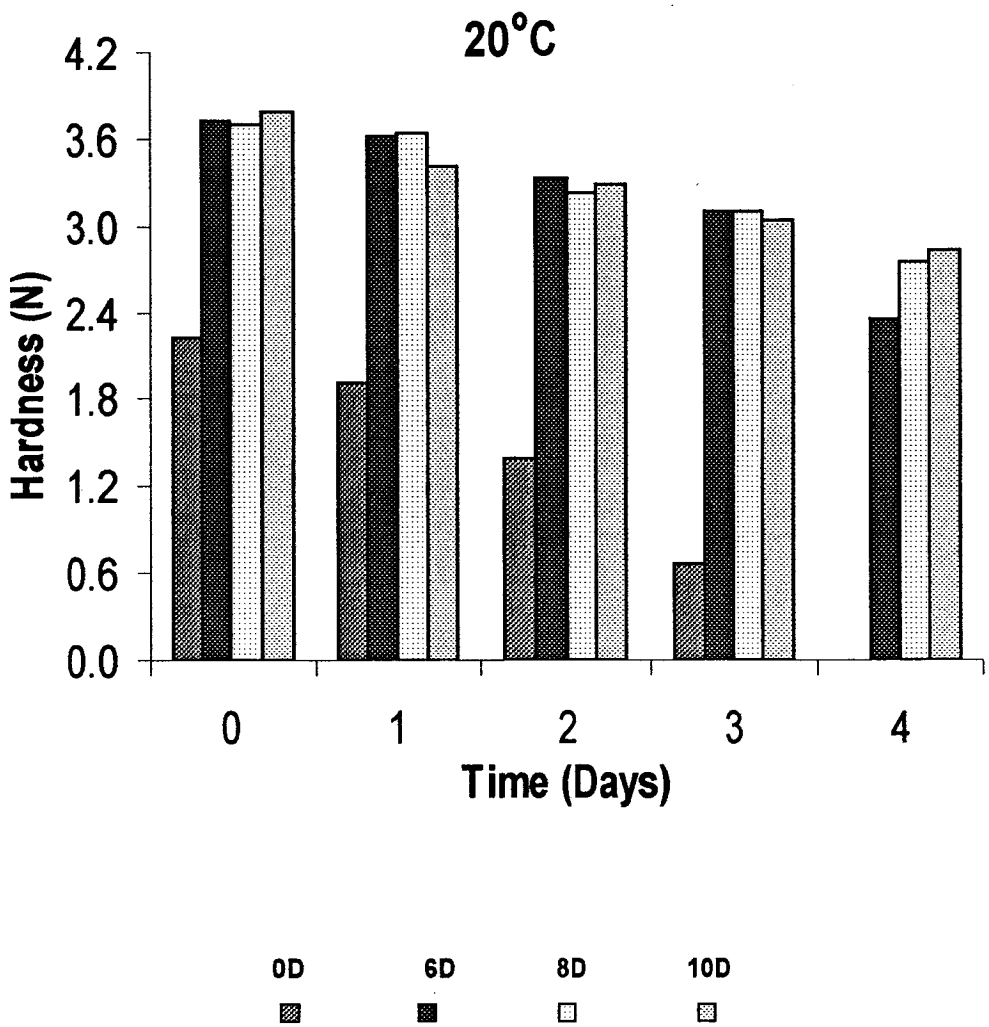

Figure 4.3 Effect of applied pressure lethality on hardness in fish at 20°C

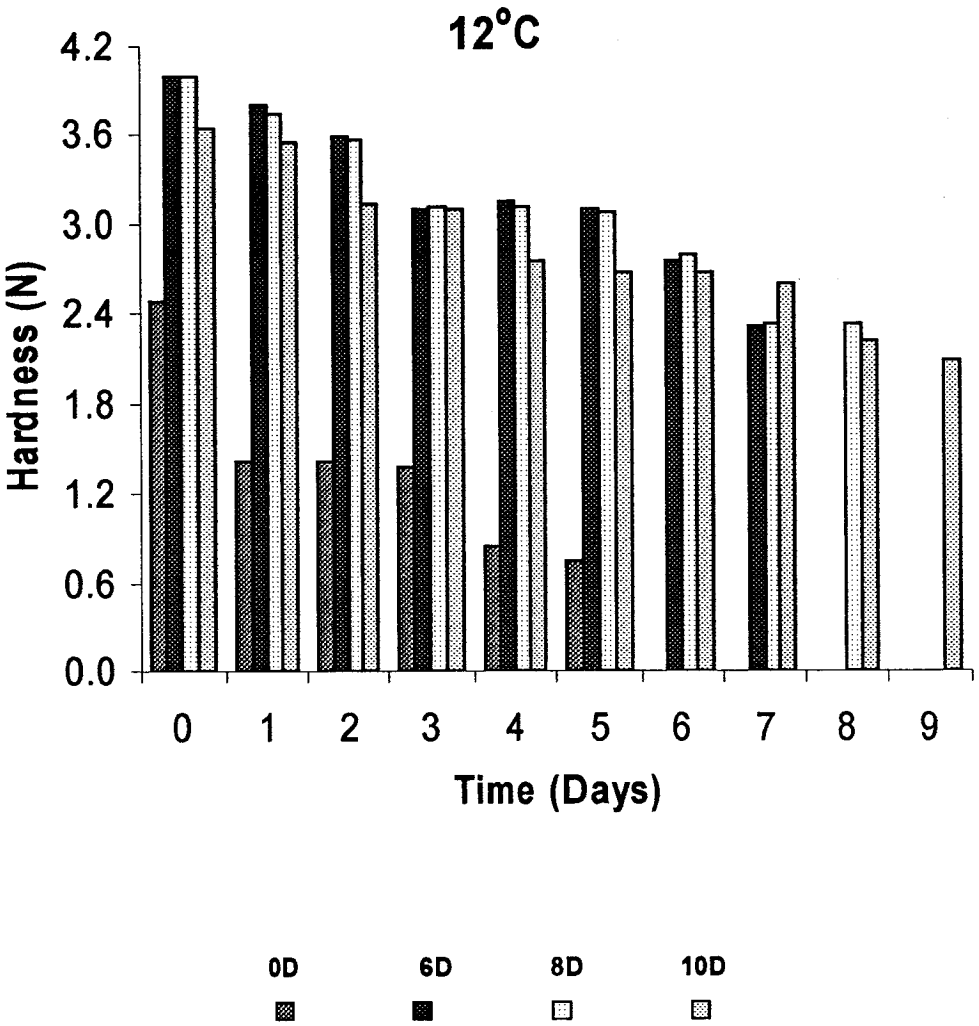

Figure 4.4 Effect of applied pressure lethality on hardness in fish at 12°C

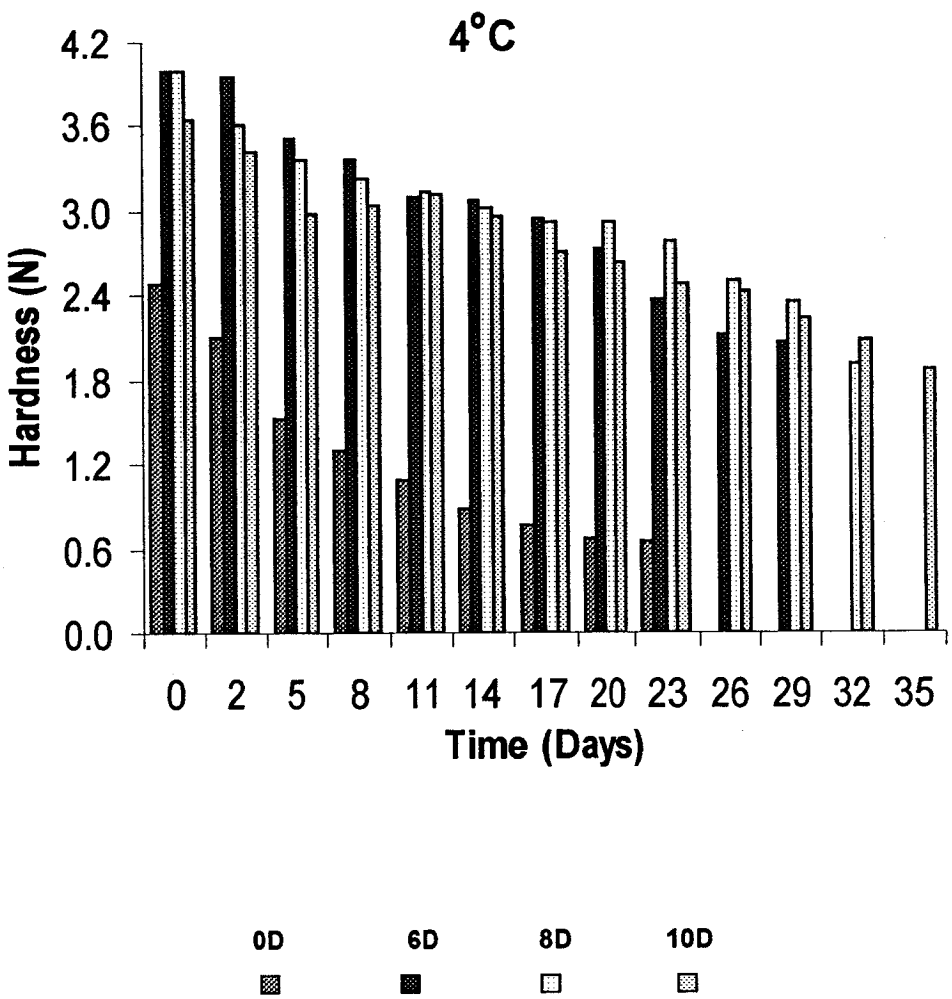

Figure 4.5 Effect of applied pressure lethality on hardness in fish at 4°C

temperatures. Storage at higher temperatures resulted in more rapid changes in springiness than lower temperatures all control samples resulted in faster changes than treated samples.

Control samples lost almost half of their springiness regardless of storage by day 3 at 20C and day 11 at 4C. Springiness (measure of chewiness) was about 25-30% higher in treated samples. Treated samples also maintained their springiness throughout storage (3 days at 20°C, 6 days at 12°C and 26 days at 4°C). The rate of change in springiness was directly related to storage temperature, i.e. higher the temperature faster the rate of change of springiness. At any given temperature, the change was semi-logarithmic.

Texture loss was explained as a loss in cohesive forces occurring within tissues as result of enzymatic activity in fish (Ashie and Simpson, 1996). In cod and mackerel, the decrease in volume, as a result of compaction, has been reported to enhance protein interactions under high pressure treatments (Ohshima et al., 1993). Some textural changes in fish have been due to degradation of myofibrillar proteins, degradation of collagen, formation of protein aggregates and ultra structural changes (Gill et al., 1979; Kanoh at al., 1988; Ando et al., 1991; Yamashita et al., 1996; Sato et al., 1997). Moreover, meat tenderization has been proposed using the high pressure technology to obtain firm and contracted raw meat (Macfarlane, 1973; Kennick et al., 1980).

4.4.3 Changes in Color

Figures 4.9, 4.10 and 4.11 shows changes in the color L*-value of HP treated fish stored at 20°C, 12°C and 4°C respectively. L values indicate the surface brightness or lightness on a scale of 0 to 100 with the zero value corresponding to a perfectly black

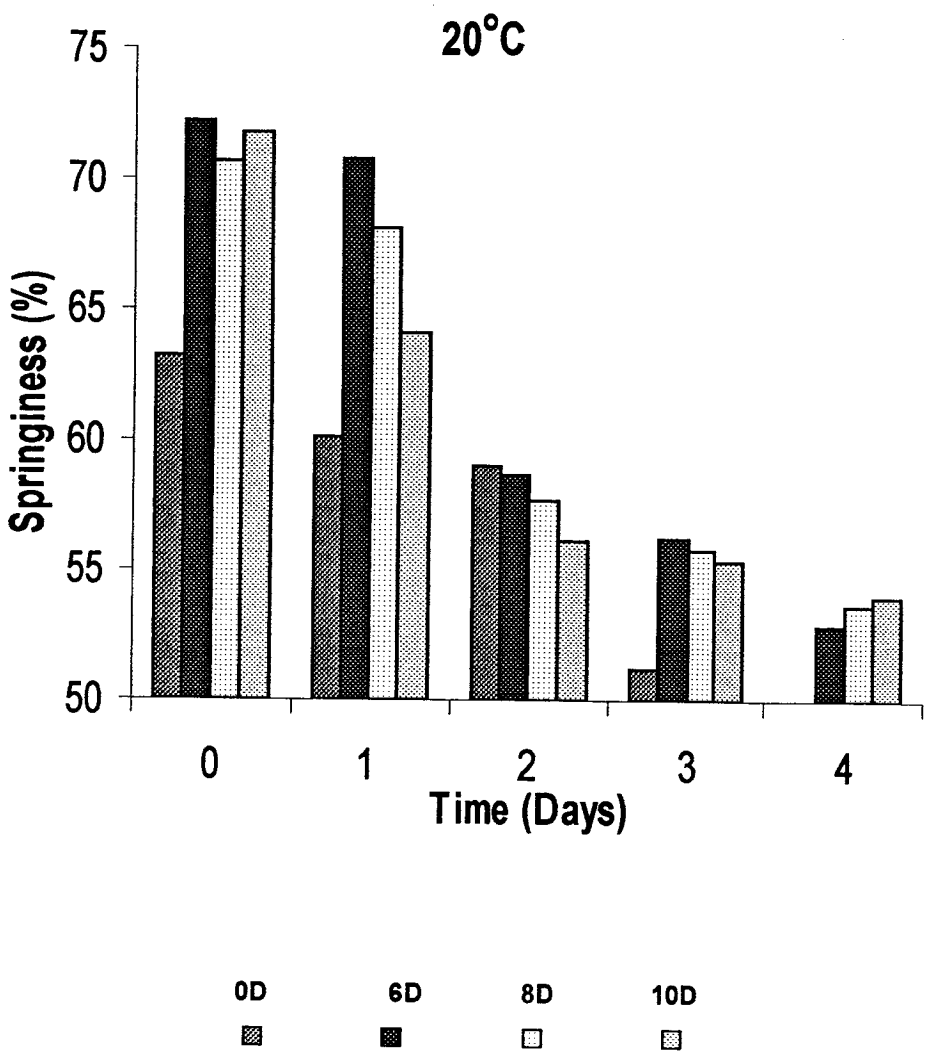

Figure 4.6 Effect of applied pressure lethality on springiness in fish at 20°C

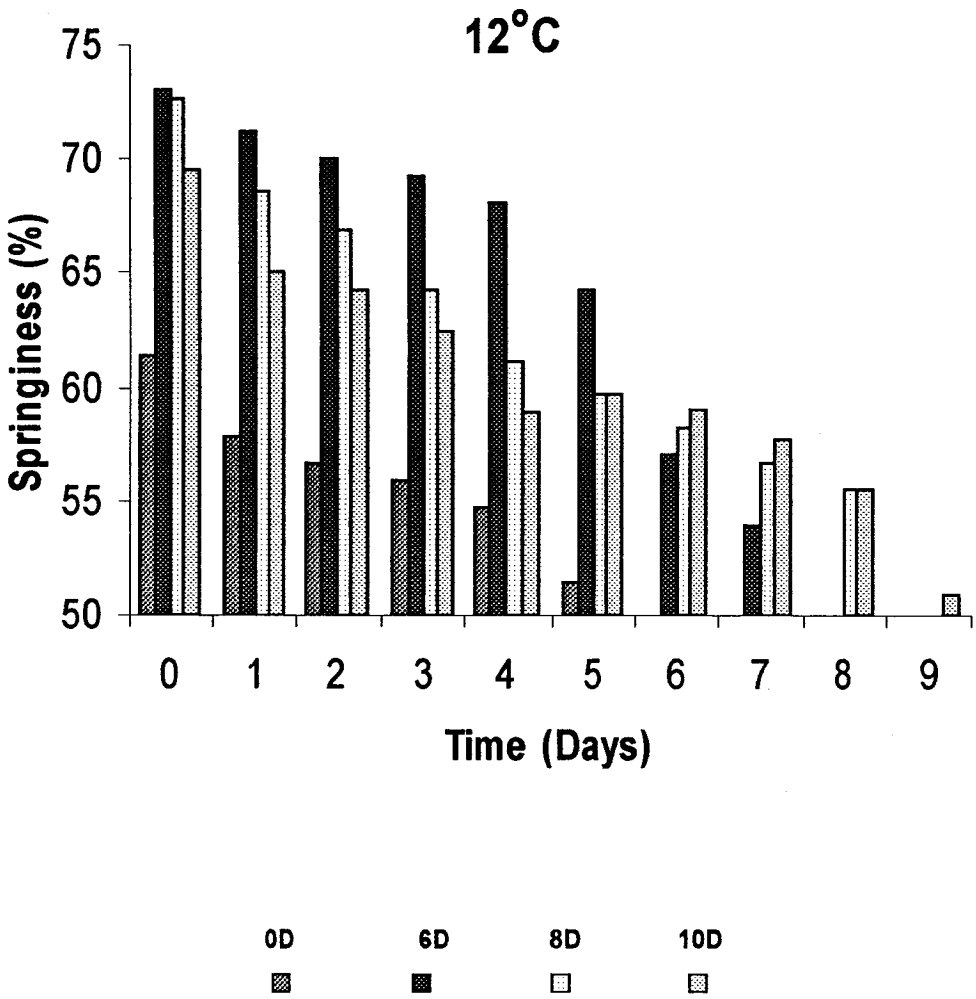

Figure 4.7 Effect of applied pressure lethality on springiness in fish at 12°C

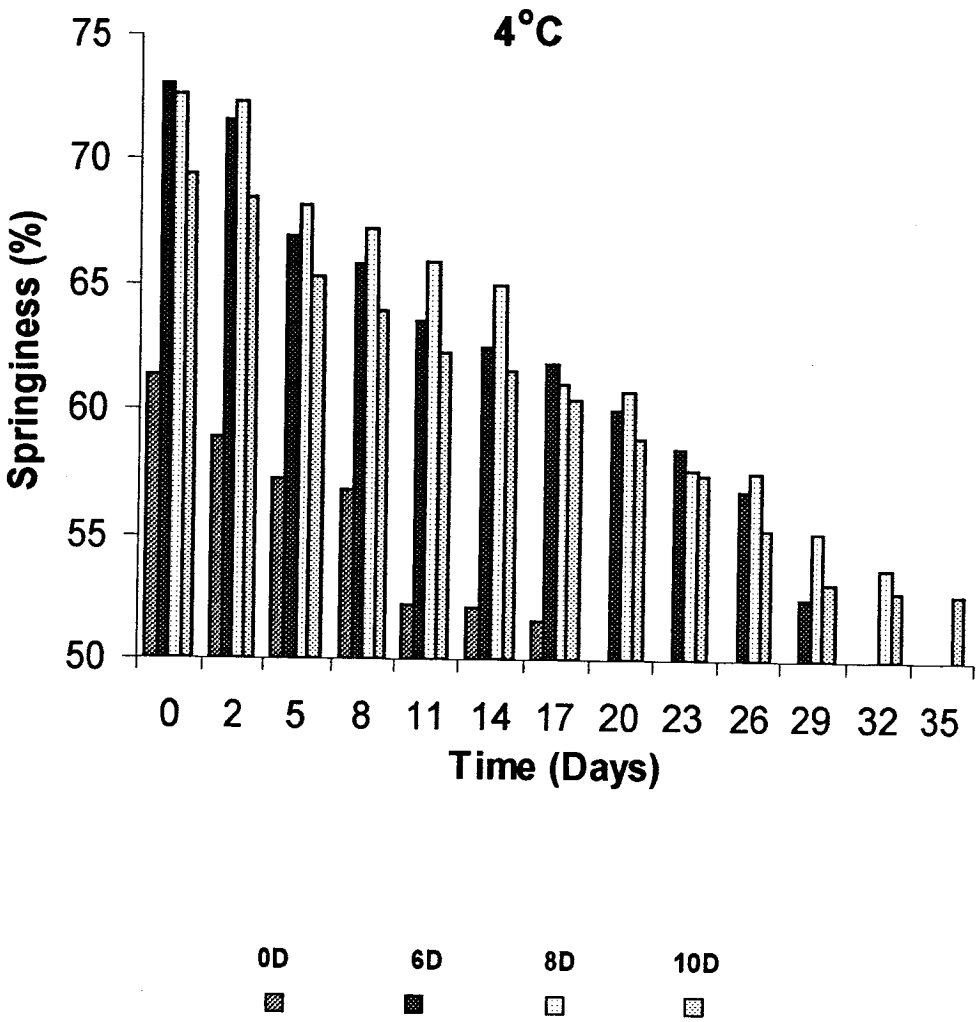

Figure 4.8 Effect of applied pressure lethality on springiness in fish at 12°C

sample (darkest) while 100 refers to a perfectly white (brightest) sample. Intermediate values provide the degree of brightness or lightness, higher values generally indicating a brighter or whiter sample.

There was increase in L* value immediately after high pressure treatment for all samples with the value for control samples being much lower than for the treated samples. Pressure treatment resulted in all samples to turn pale, resembling the color of a cooked product. It has been generally recognized that the shiny translucent appearance of fish is generally lost once pressure treated beyond 300 MPa. Hence this change was expected since higher pressures were used for longer duration enhance safety. The focus of the study was to evaluate the color, flavor and texture stability, so that once cooked result in a super quality product. The L* values for all samples including the control, increased with time during storage, changes being much lower at lower temperatures. The rate of increase in L* during storage was slower for pressure treated samples compared to control samples. The pressure severity levels 6D to 10D (18-30 min at 400 MPa) did not show much of a difference in L* values between test samples at any given storage time/temperature combinations. The best protection to changes in L* came from lowering of storage temperature, with the 4°C storage reaching equilibrium values of about 73 at the end of 35 days storage compared with 10 days at 12°C and 4 days at 20°C.

Figures 4.12, 4.13 and 4.14 show changes in the a* value of HP treated and control fish stored at 20°C, 12°C and 4°C, respectively. The a* value on the Hunter Lab color scale represents green to red transformations, a* changing from zero on the plus side indicating progressive increase in red shade and on the negative side a progression of green shade.

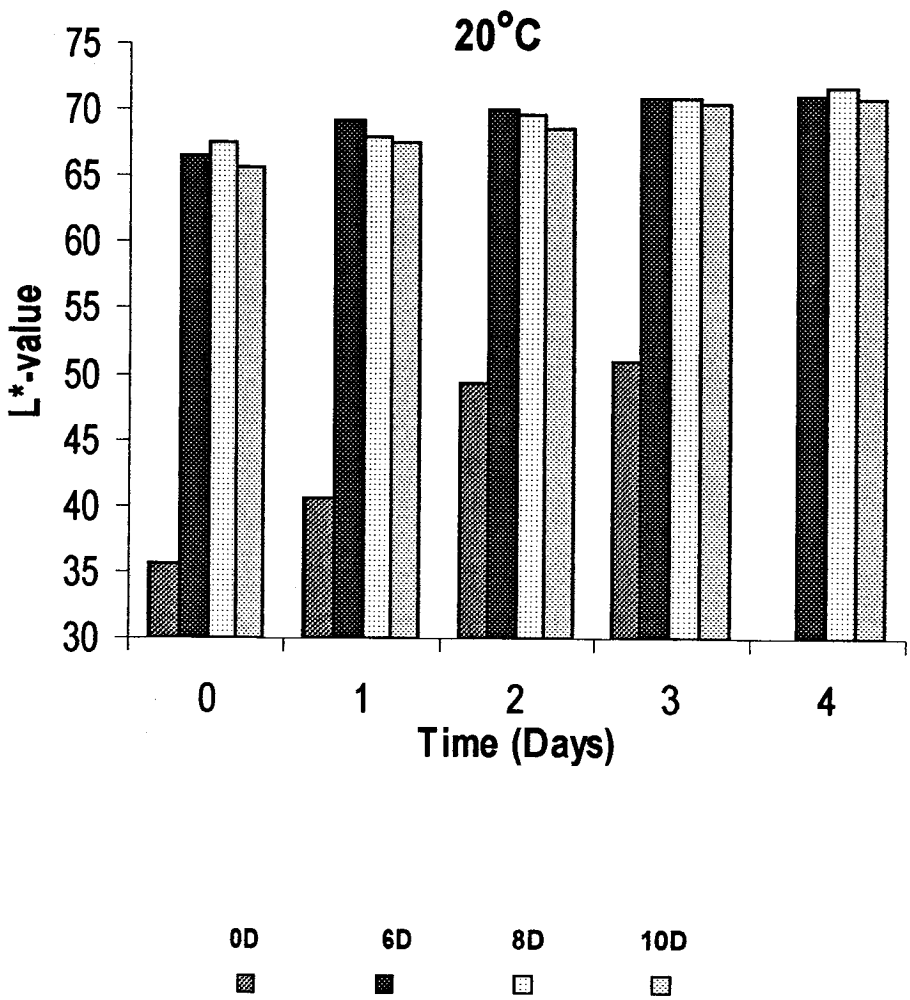

Figure 4.9 Effect of applied pressure lethality on L*-value in fish at 20°C

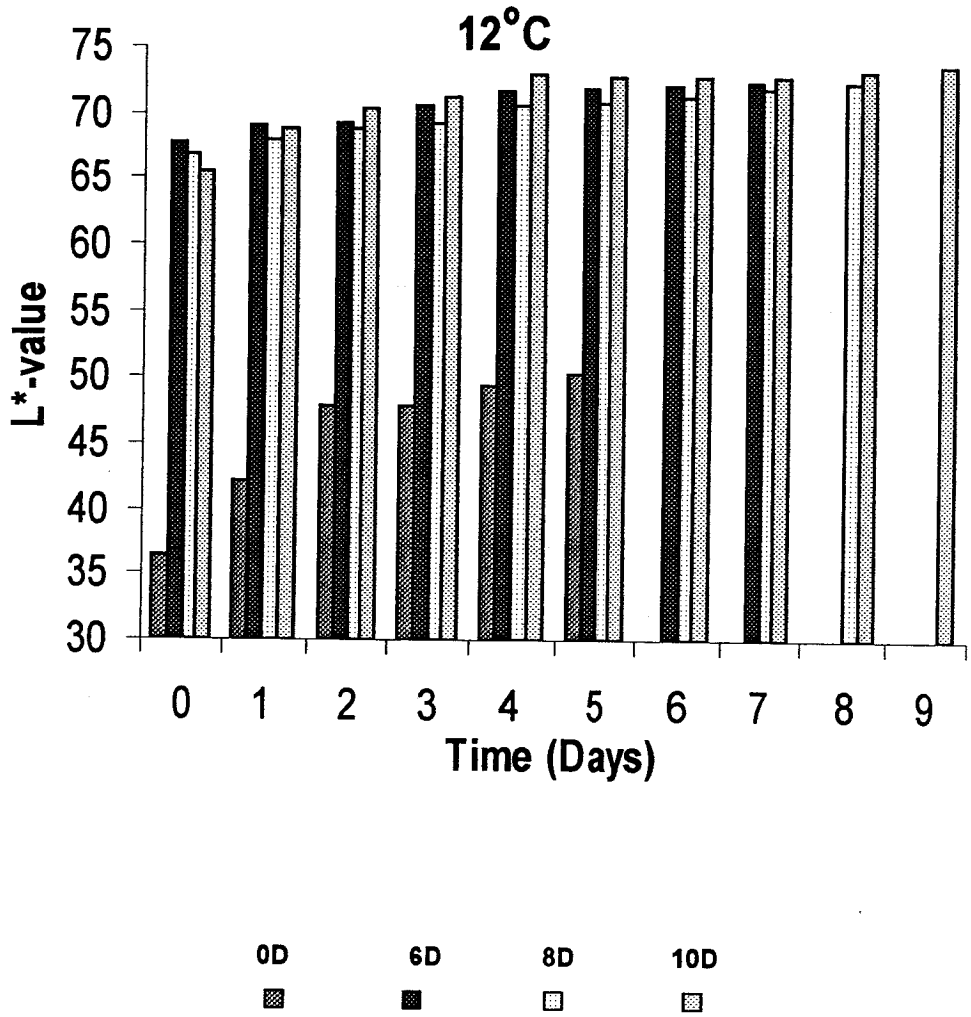

Figure 4.10 Effect of applied pressure lethality on L*-value in fish at 12°C

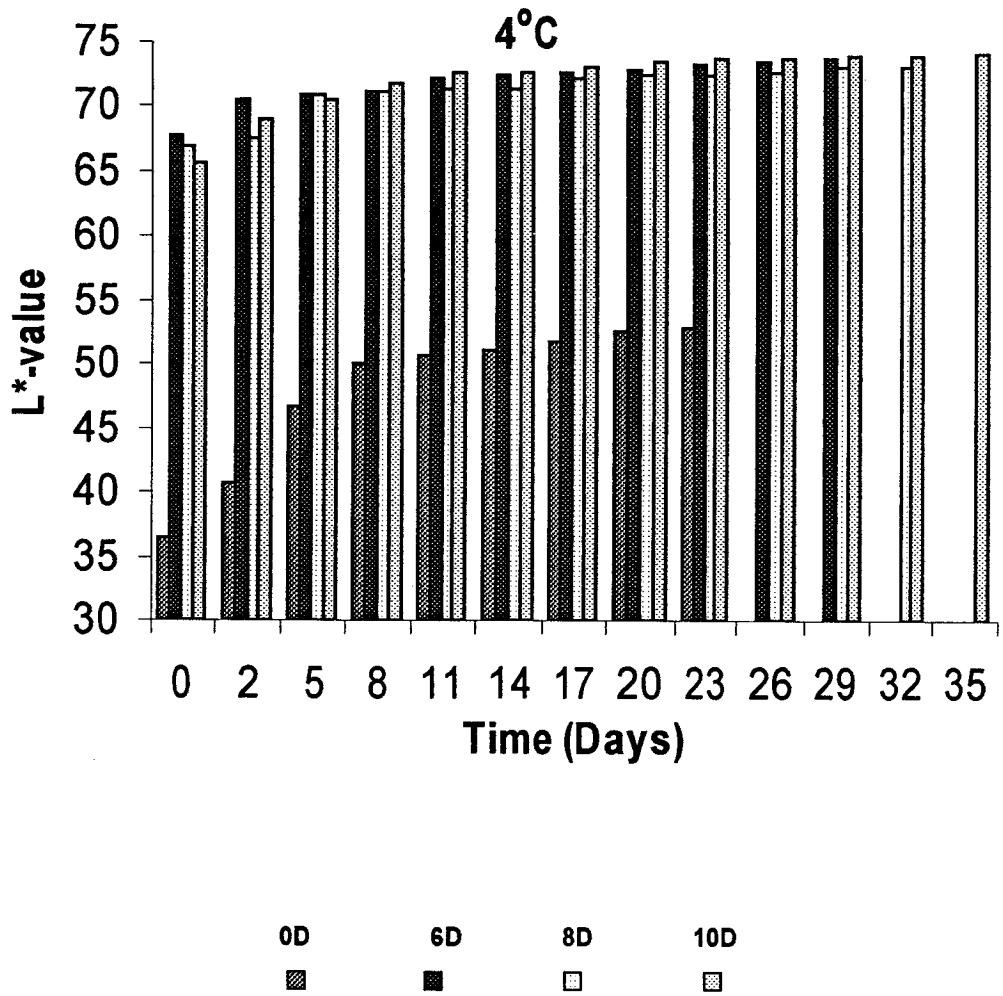

Figure 4.11 Effect of applied pressure lethality on L*-value in fish at 4°C

All samples had a positive a* value indicating some redness in the test samples. Pressure treatment had an immediate effect on a* values which decreased from around 5 to below 1. The considerable change of pressure induced decrease in a* can be observed with treated samples compared with control (Figures 4.12-4.14). The figures also demonstrate another important effect of the HP treatment which is the stability of color. Changes in a* associated with control samples during storage were much more dramatic than in treated samples. With reference to the initial value a* value for the control (~5), the margin was 5:1 for control versus treated samples. Control samples lost their color much more rapidly reducing this difference to 1.5:0.5 during storage. Overall, therefore, the color change in all control samples were about 4 units compared to about 0.5 units in treated samples.

Figures 4.15, 4.16 and 4.17 show changes in the color b*-value (yellowness) of HP treated fish stored at 20°C, 12°C and 4°C, respectively. On the color scale, b values represent blue-yellow changes. Positive b* indicates yellowness and negative values indicate blueness. As with the a* values, the pressure effect on the b* values was obvious. However, while the pressure treatment induced an increase in b* values the a* values decreased. Furthermore, b* values continued to increase during storage rather than decrease, as observed with a* values. Again, the rate of increase and the level of increase was much higher in control than in treated samples.

Changes in L* values were due to changes in light reflection which was related to the amount of water on the surface of fish muscle. The change in a* values on storage was higher than the initial decrease with applied pressure and may be due to microbial growth or oxidation on the fish surface during storage. Changes in b* values followed similar

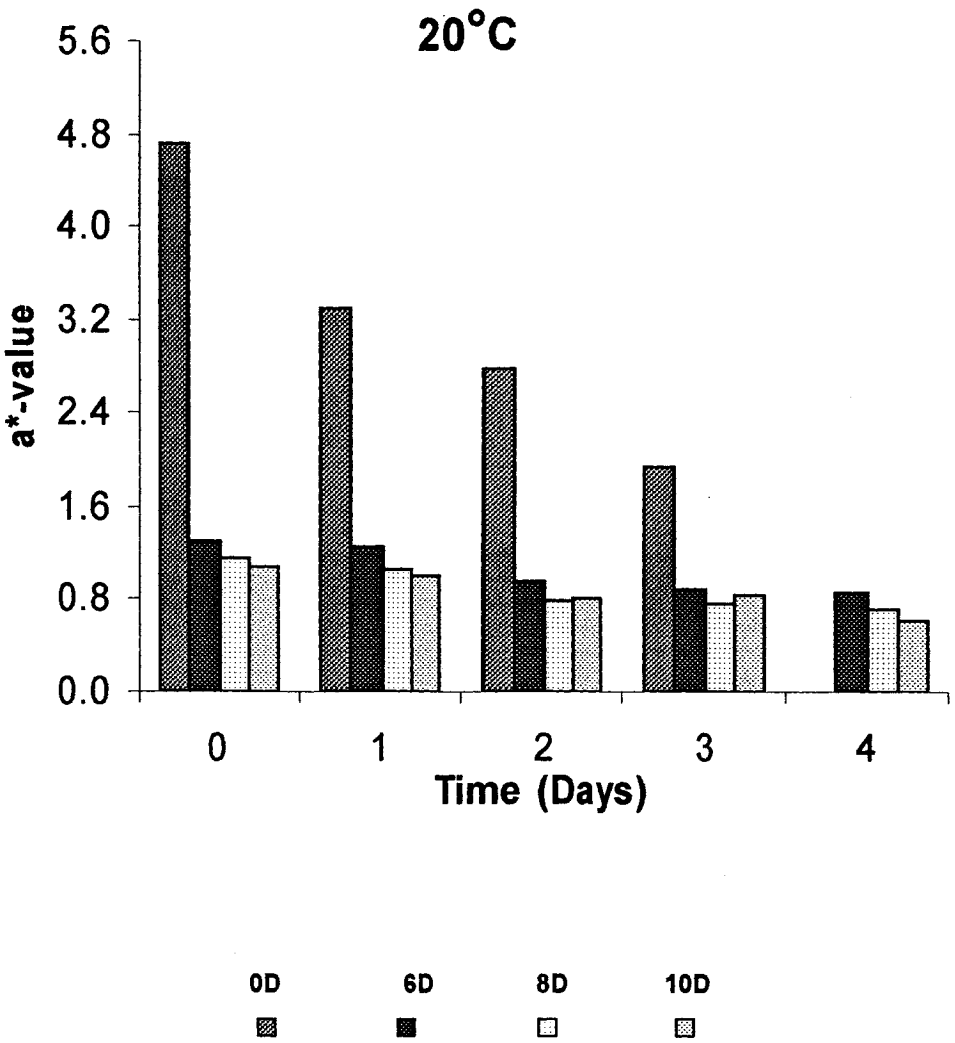

Figure 4.12 Effect of applied pressure lethality on a*-value in fish at 20°C

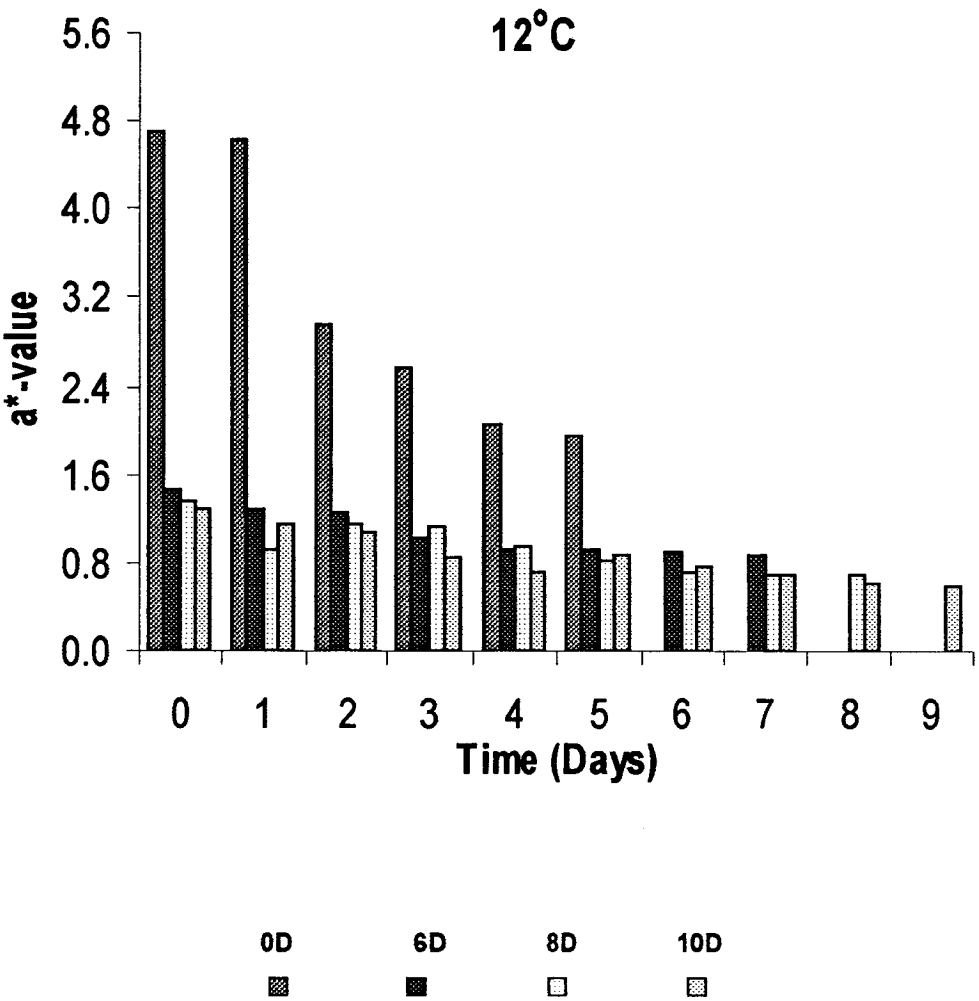

Figure 4.13 Effect of applied pressure lethality on a*-value in fish at 12°C

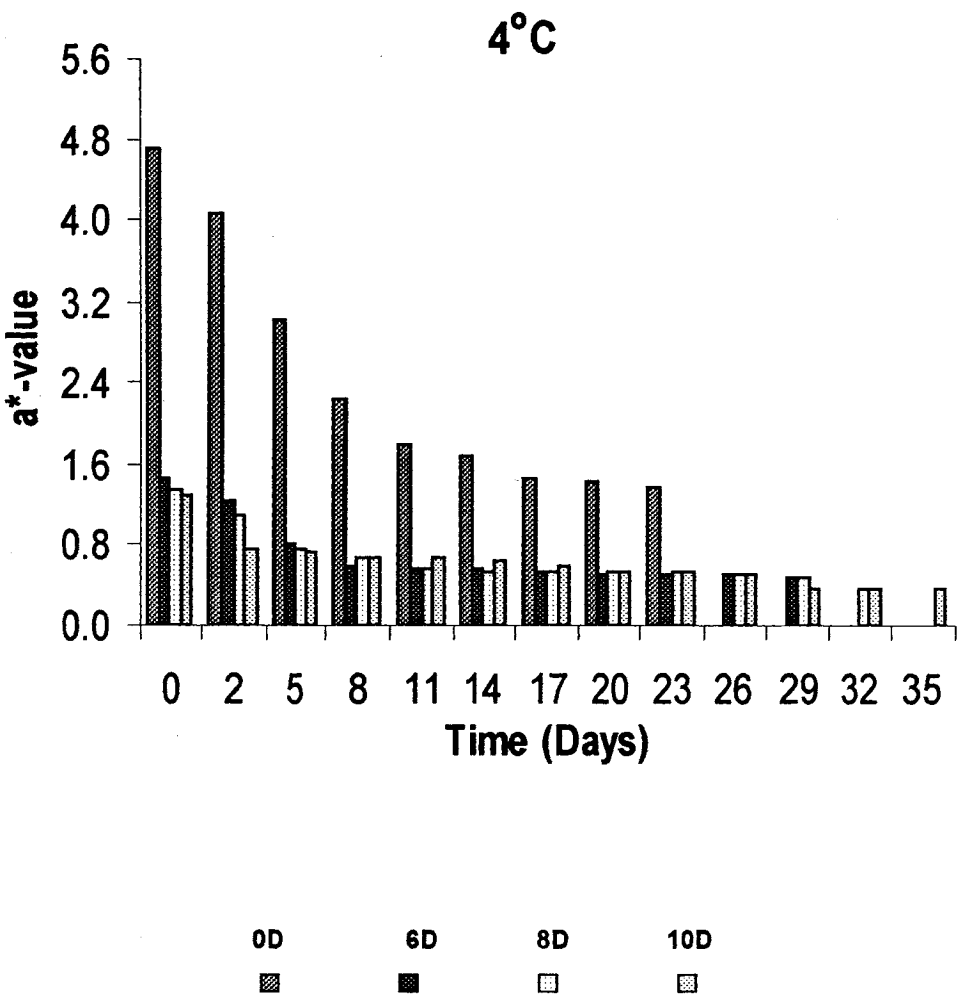

Figure 4.14 Effect of applied pressure lethality on a*-value in fish at 4°C

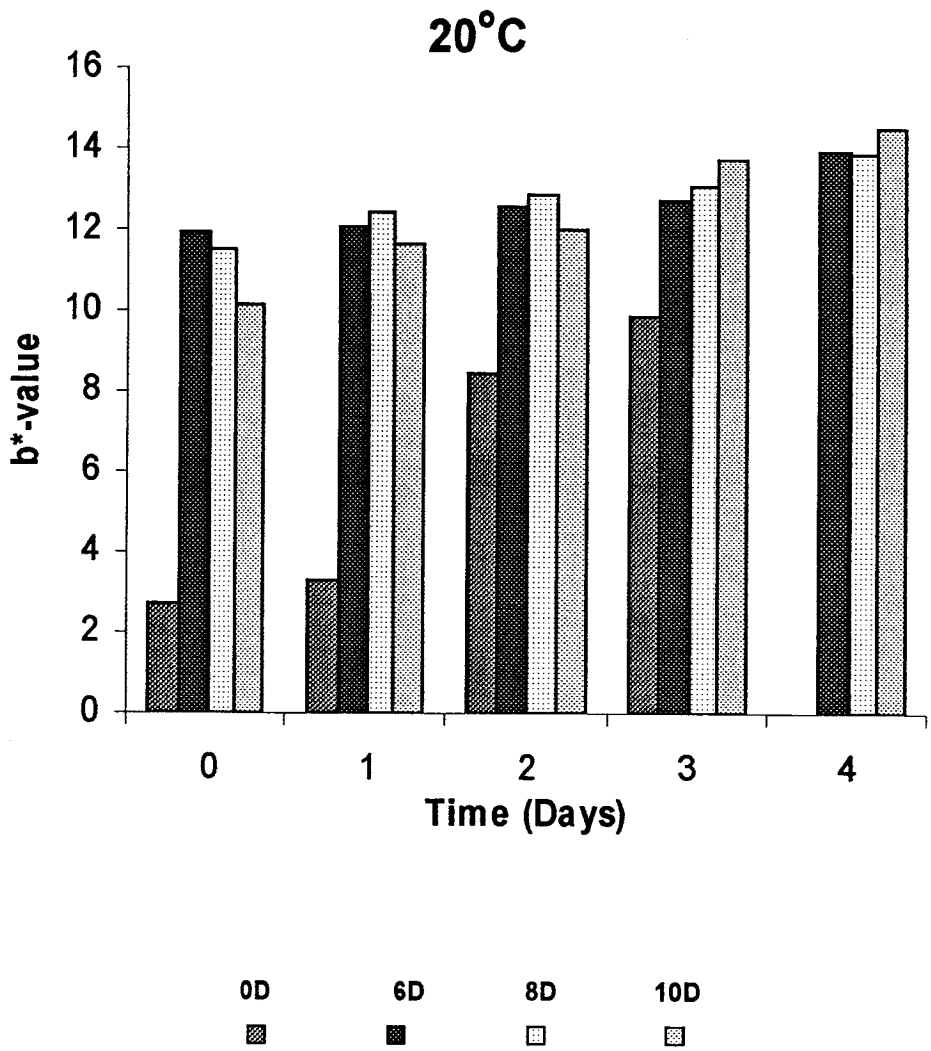

Figure 4.15 Effect of applied pressure lethality on b*-value in fish at 20°C

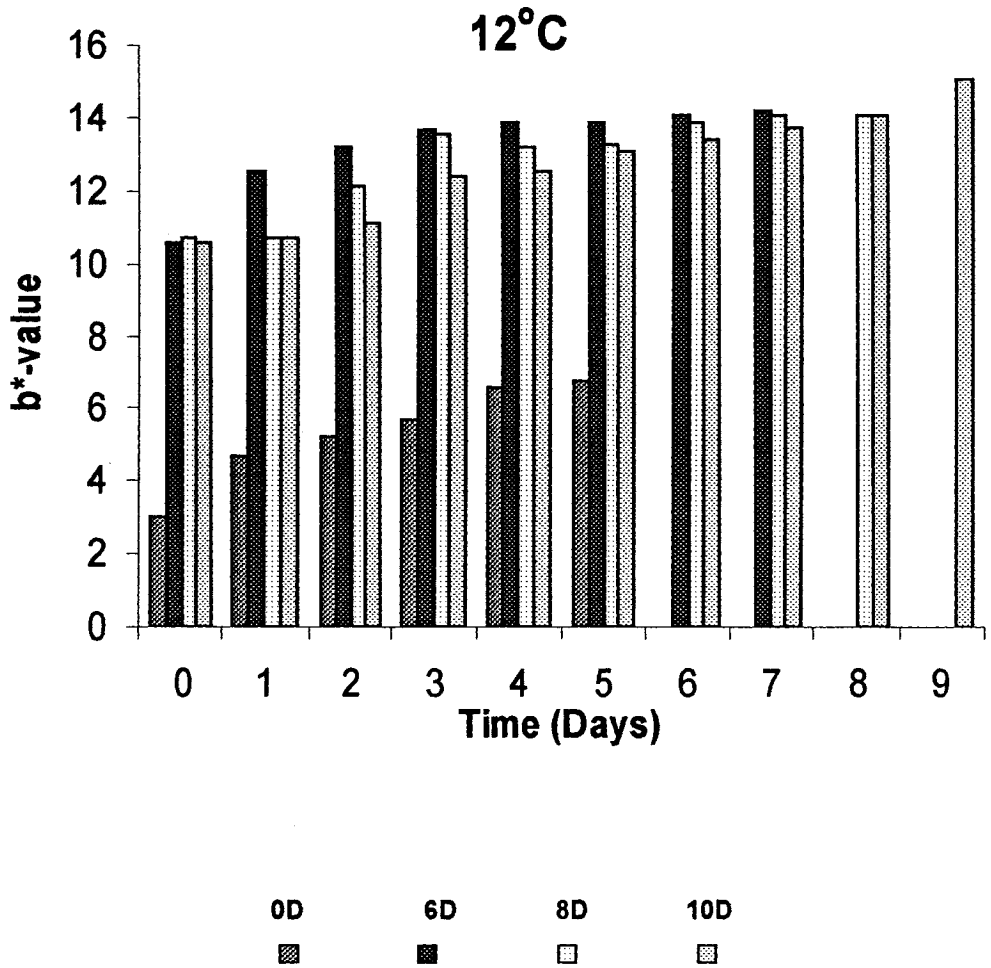

Figure 4.16 Effect of applied pressure lethality on b*-value in fish at 12°C

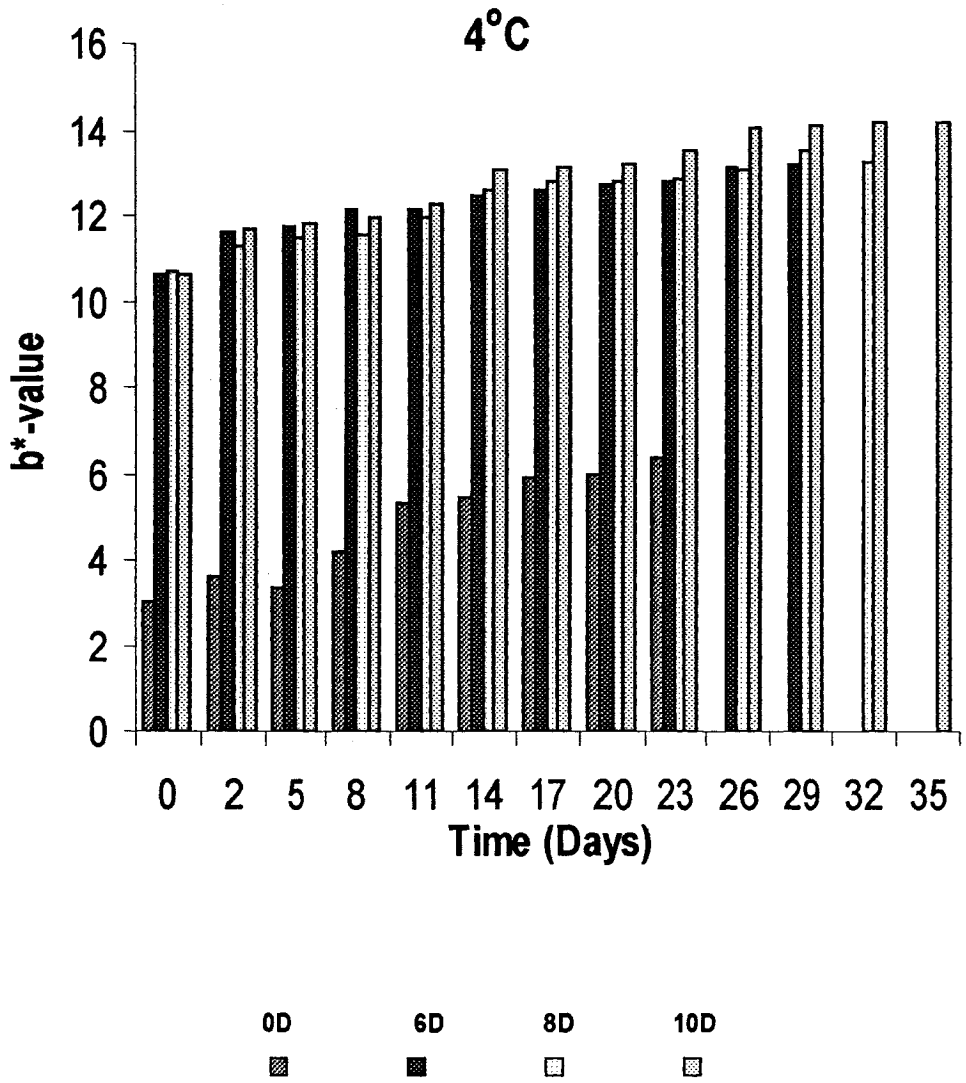

Figure 4.17 Effect of applied pressure lethality on b*-value in fish at 4°C

pattern as L* where the applied pressure increased the b* value and further increase was observed during storage at different temperatures. Similar color losses have been found in form of opacity and loss of redness in cod fish at 200 MPa pressure treatments (Oshima at al. 1993; Angsupanich and Ledward, 1998). Study on carp muscles showed a similar effect on L*, a* and b* after high pressure treatments between 100 to 200 MPa (Yoshioka et al., 1996). In another study on high pressure freezing and thawing of Atlantic salmon, the change in these colors were reported as induced due to pressure treatments rather than freezing (Zhu et al., 2004a). These color changes are necessarily favourable from consumer point of view. Some methods, such as pressurization in the absence of oxygen, may produce better color retention (Carlez et al., 1995). Alternately, the consumer should be informed of what to expect and the fact that following cooking these differences mostly disappear.

4.4.4 Changes in Drip Loss

Figures 4.18, 4.19 and 4.20 illustrate the drip loss in HP treated samples vs control samples with storage time at 20°C, 12°C and 4°C, respectively. The drip loss in fish was affected by pressure treatment, storage temperature and storage time. The initial loss as a result of HP treatment was about 3-5%, but it increased considerably for both control and treated samples, although storage temperature played considerable role in decreasing the amount of drip loss. During storage, samples showed different levels of drip loss for different pressure lethality and storage temperatures. The pressure compression resulted in a higher initial drip loss in fish fillets. Higher pressure lethality (10D, 30 min treatment) showed higher drip loss compare to 6D and 8D treatments.

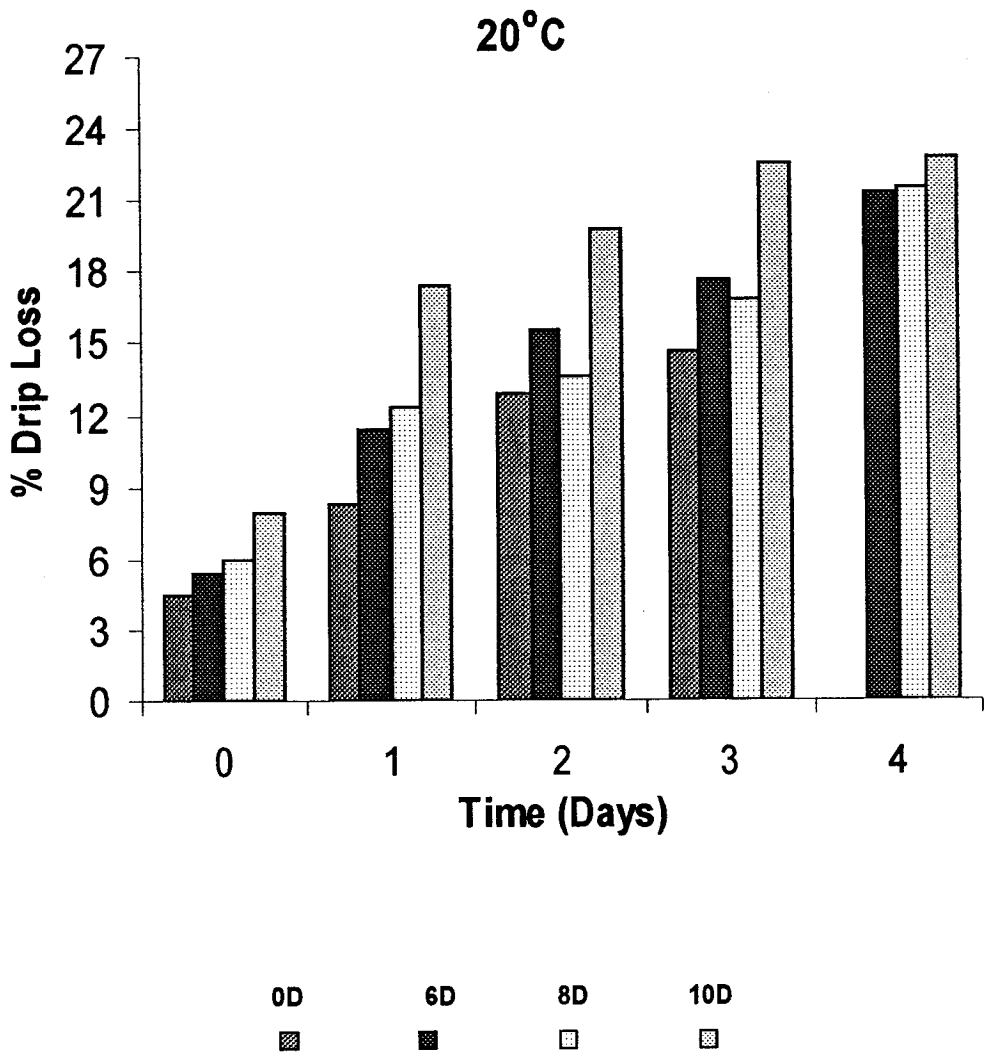

Figure 4.18 Effect of applied pressure lethality on drip loss in fish at 20°C

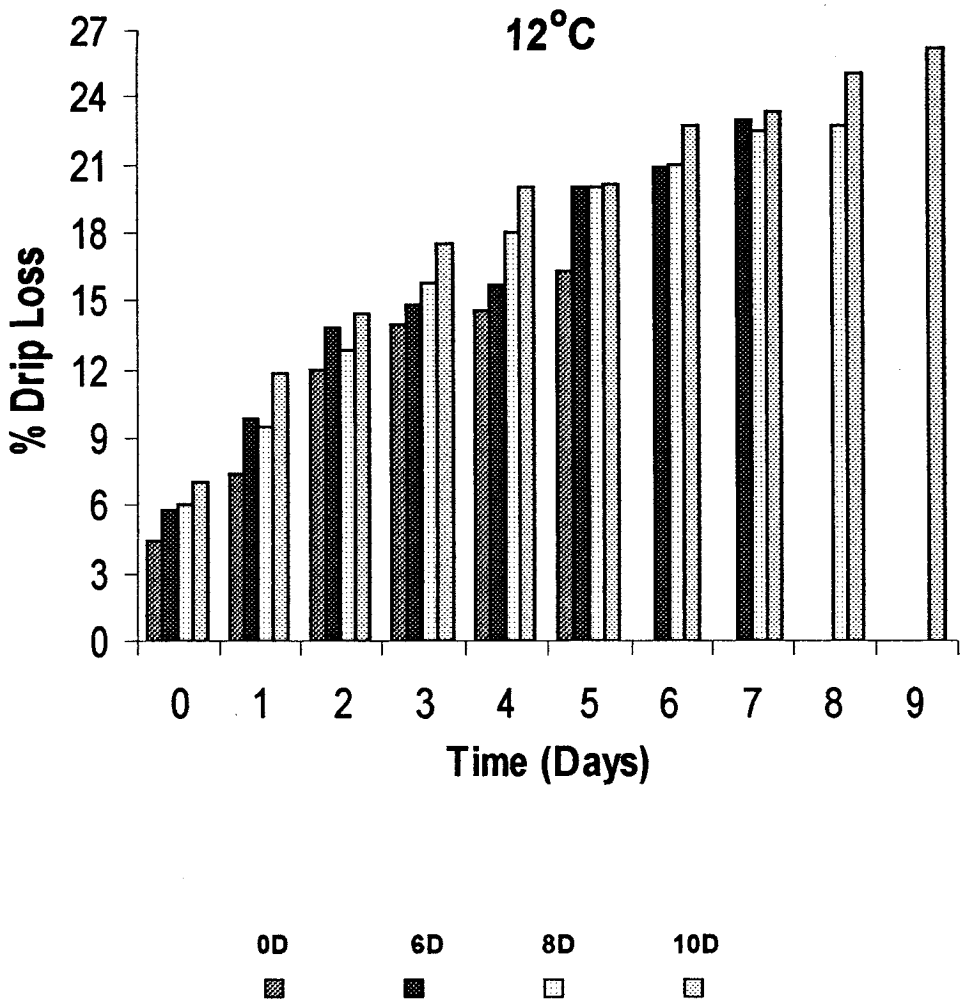

Figure 4.19 Effect of applied pressure lethality on drip loss in fish at 12°C

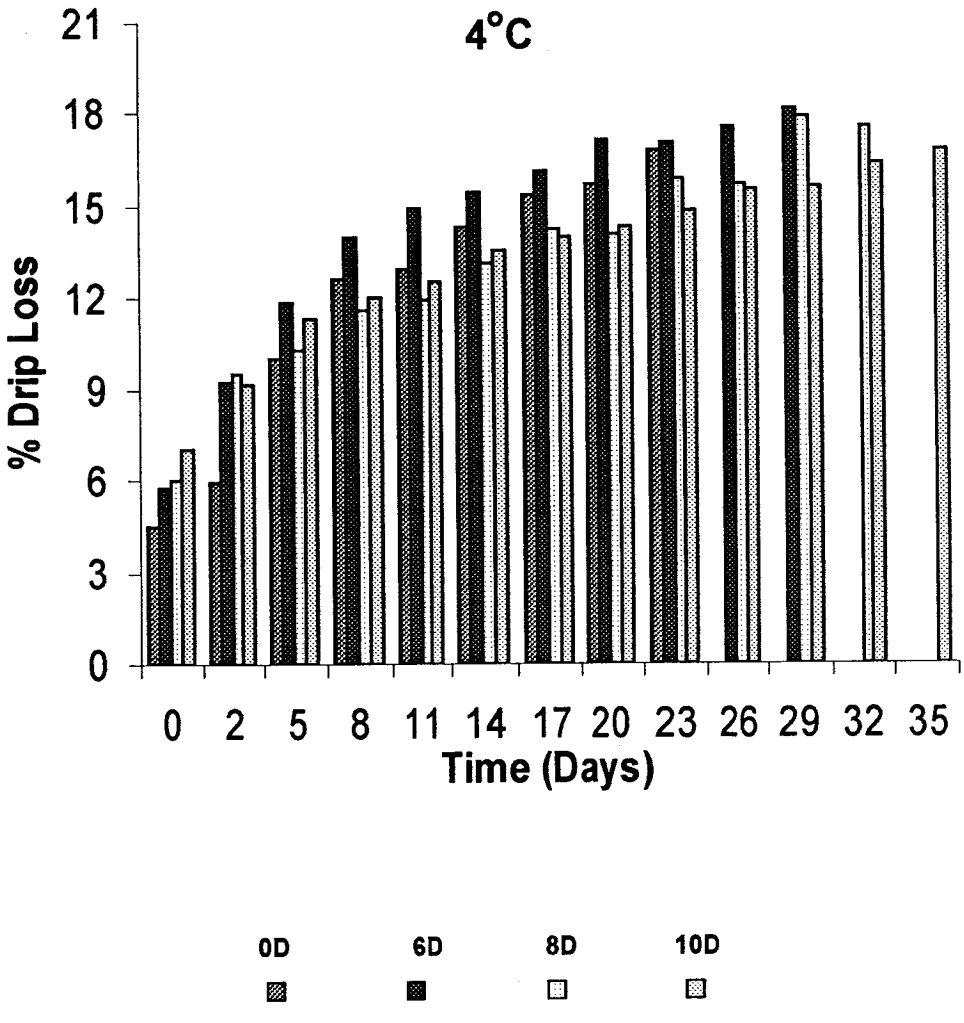

Figure 4.20 Effect of applied pressure lethality on drip loss in fish at 4°C

At the early stages of storage, a rapid increase in drip loss was observed in all samples which tapered off to an equilibrium value. Higher storage temperatures (20 and 12°C) resulted in a more rapid convergence to the equilibrium value than storage at the lower temperature of 4°C. About 24 % loss was observed as a result of drip (free moisture removed by blotting, not necessarily liquid oozing out of the sample within the package) at 20°C and 12°C storage within the short storage time while at 4°C storage, it took longer for a drip loss of 18%. The 10D treatment drip loss was relatively less than with 6D and 8D treatment at 4°C storage temperature at the end of storage period. The 10D treated stored at 4°C temperature showed lower drip loss rates comparable to other storage conditions and treatments which may have been due to the lower rate of spoilage of fish at this conditions. In the case of carp (*Cyprinus carpio*) fillets, 1.1 to 1.8% of drip loss was observed after pressurization at 100 to 500 MPa for 10 min at 20-22 °C (Yoshioka and Yamamoto, 1998). In another study, higher initial drip loss of pressure treated octopus was found to be reduced on chilled storage compare to control samples (Hurtado, 2001). In a study on drip loss in Atlantic salmon after high pressure thawing, Zhu et al. (2004a) reported similar losses (2-3%). In pressure-shift freezing of pork, Zhu et al. (2004b), reported that drip loss immediately after thawing was about 0.1g/g (dry matter) this is equivalent which work out to about 2%when the moisture content is about 80% increasing to 1g/g [equivalent to about 20%] when subsequent cooking is included.

4.4.5 Changes in pH

Changes in pH are shown in Figure 4.21, 4.22 and 4.23 for control and HP treated fish during storage at 20°C, 12°C and 4°C, respectively. There was very small but

consistent change in pH of fish after pressure treatment and during storage. As with other parameters, the changes associated at higher temperatures were greater. Pressure treatments had a consistent role in pH change with longer treatment resulting in a lower pH. Storage time and temperatures had major roles increasing the pH by about 0.3 to 0.6 units. Since the 10D treated samples had a lower initial pH at the beginning of storage, the relative increase in this sample was higher compared to other treatments. The overall pH was higher in the control than in treated samples, indicative of faster microbial deterioration in control samples.

The increase in pH in control samples can be explained by the fact that high pressure treatment kills several microorganisms which are responsible for the degradation of fish muscle (Shoji and Saeki, 1989; Ledward, 1995) and which normally produce volatile substances, such as acids, amines and bases (Spinelli et al., 1964; Shenderyuk and Bykowski, 1989). The increase in pH occurs due to the decomposition of nitrogenous compounds during post-mortem changes. The pH increase from an initial value of 6.3 to 7.9 was observed in ice stored fish muscle (Stroud et al., 1982). Furthermore, this increase in pH was reported as an indication of quality loss in fish (Gokoglu et al., 2004). Hence, the pH increase observed during storage represents a normal course of physiological activity. It was controlled by the HP treatment and storage temperature.

4.4.6 Shelf-life on Microbial Basis

Figures 4.24 and 4.25 show the effect of pressure lethality and storage temperature on shelf-life of mackerel fish based on microbial growth (time to reach the spoilage level count of 10^6CFU/g). In Fig 4.24, shelf-life is plotted against pressure-treatment lethality

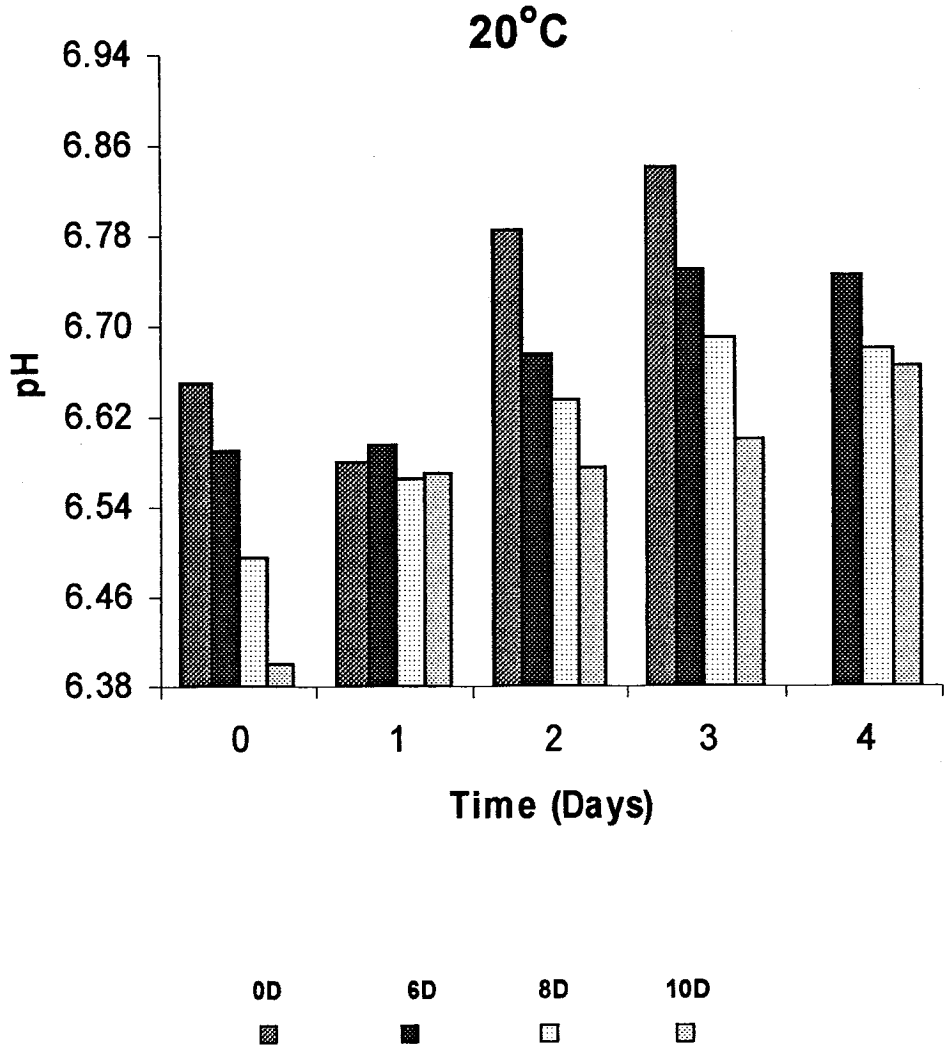

Figure 4.21 Effect of applied pressure lethality on pH in fish stored at 20°C

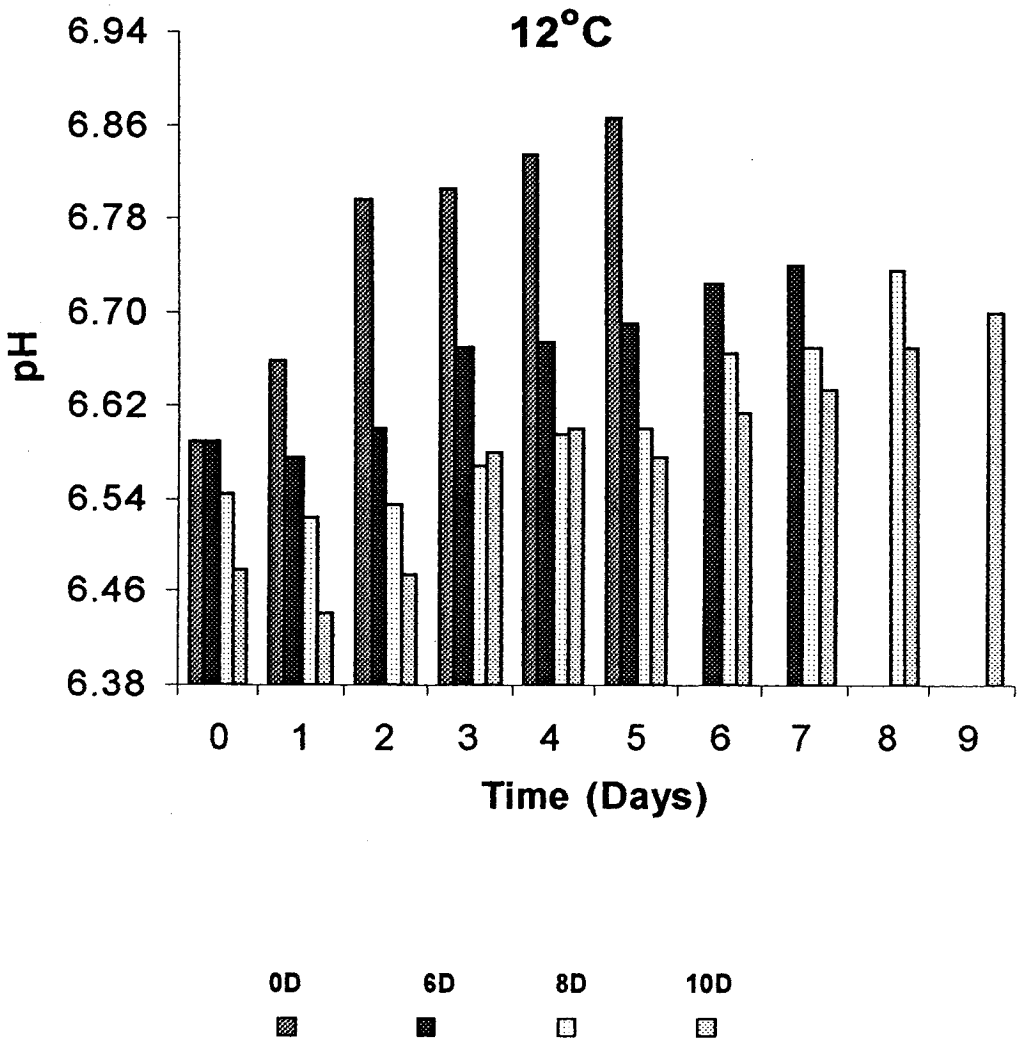

Figure 4.22 Effect of applied pressure lethality on pH in fish stored at 12°C

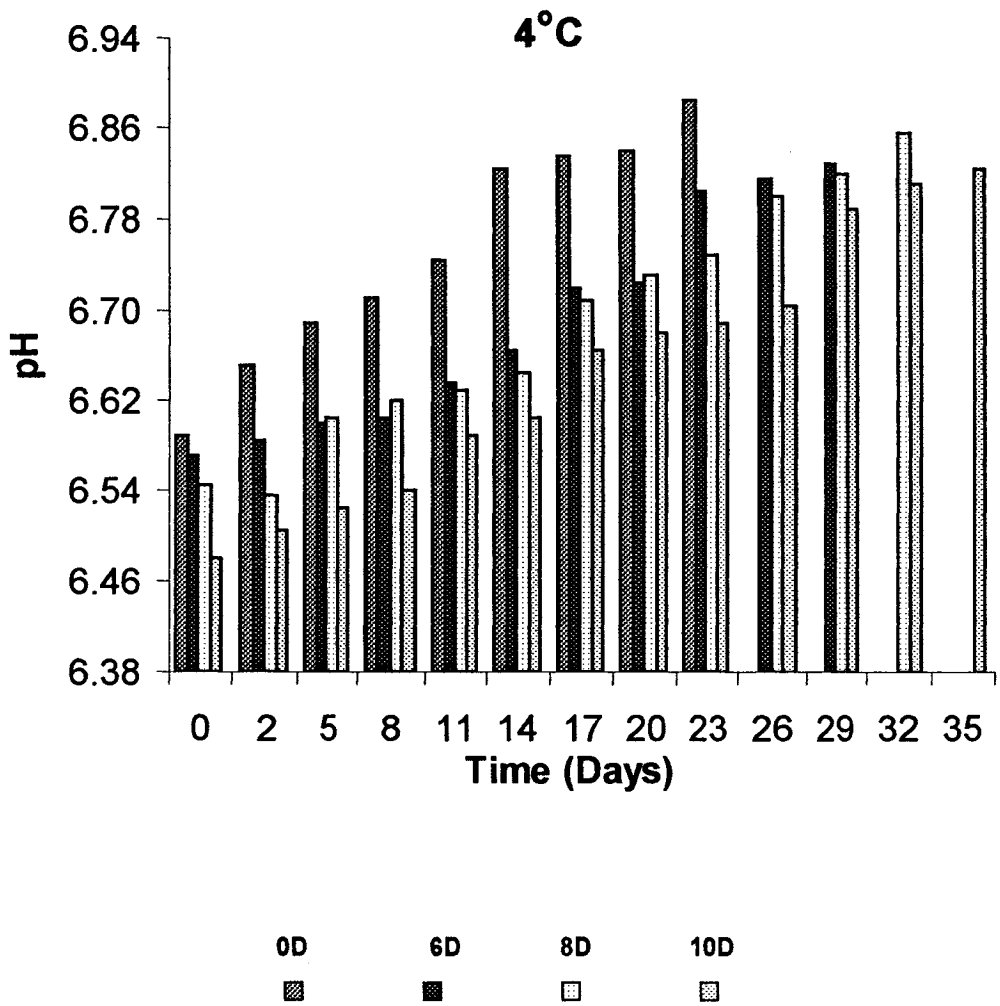

Figure 4.23 Effect of applied pressure lethality on pH in fish stored at 4°C

at the three storage temperatures. In Fig 4.25, the same data on shelf-life is plotted against storage temperature for the different treatments.

In general, at each storage temperature, the shelf-life of fish showed a progressive linear increase with the degree of pressure lethality achieved in samples (6-10D based on *E. coli* destruction, 18-30 min at 400 MPa). The increase was clearly more substantial with the higher pressure lethality and lower temperature. The other two storage conditions resulted in a most gain in shelf-life with increase in pressure lethality. In Figure 4.25, the temperature effects are more clearly shown. The shelf-life vs temperature relationship is clearly non-linear. Shelf-life decreased logarithmically with temperature. The different treatments showed a clear difference in shelf-life at 4°C ranging from about 14 days for control to 22-29 days for the 6-10D treated samples. These differences decreased to 2-3 days at 20°C. Overall, the combination of 10D treatment and refrigeration at 4°C increased the microbiological shelf-life of mackerel from 14 to 29 days.

4.5 Conclusions

High pressure treatment designed in the previous chapter to make the product pathogen safe was inadequate to stop the fish from spoiling. While pressure treatments decreased the population of indigenous microorganisms, they did not completely eliminate. The surviving and injured cells showed signs of recovery and growth during storage. The growth pattern and rate depended both on the pressure treatment and storage temperature. After a lag phase which depended again both on HP treatment and storage temperature, a first order growth behaviour was evident, with growth rates primarily dependent on storage temperature.

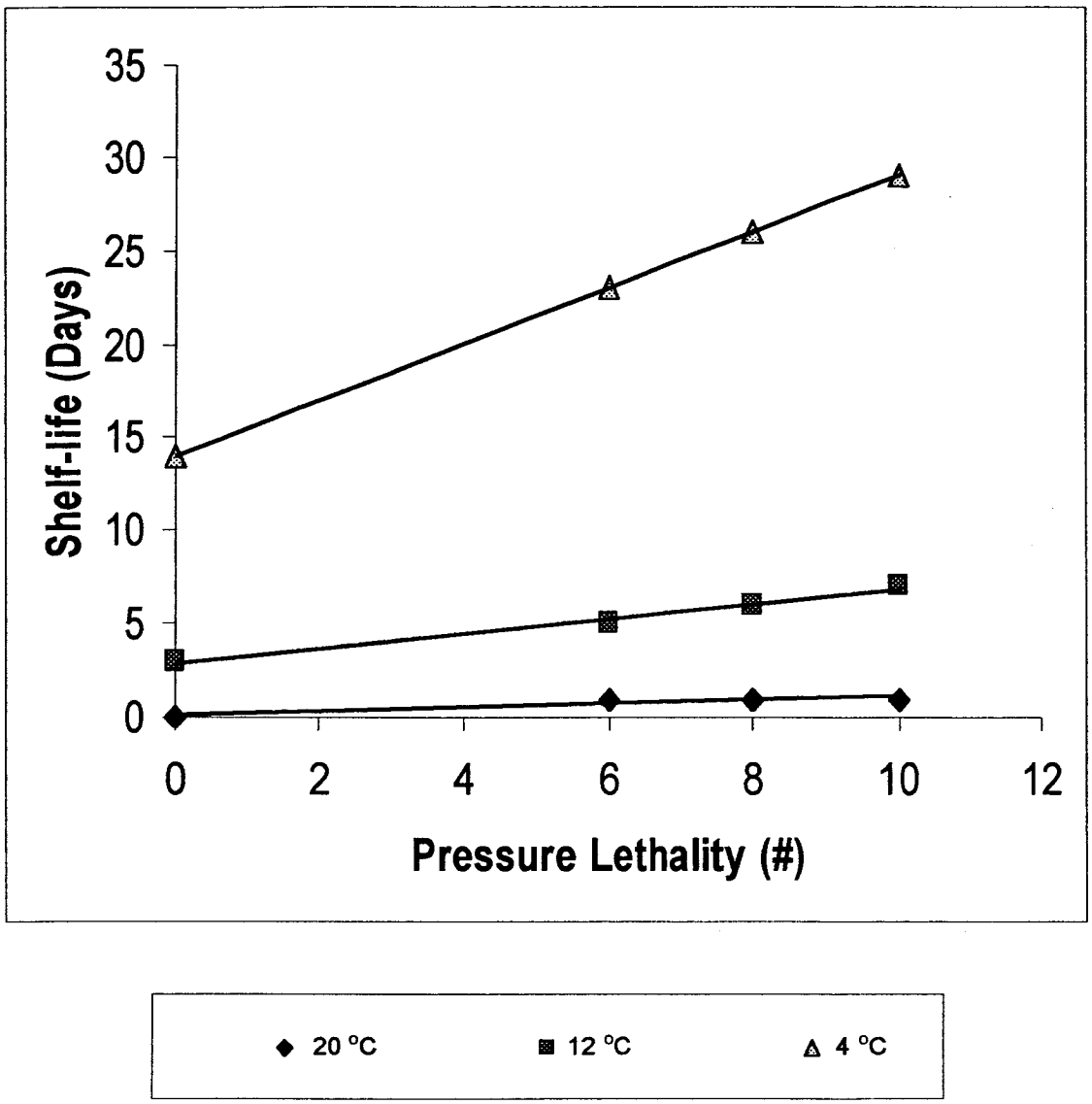

Figure 4.24 Effect of applied pressure lethality on the microbial shelf-life of fish

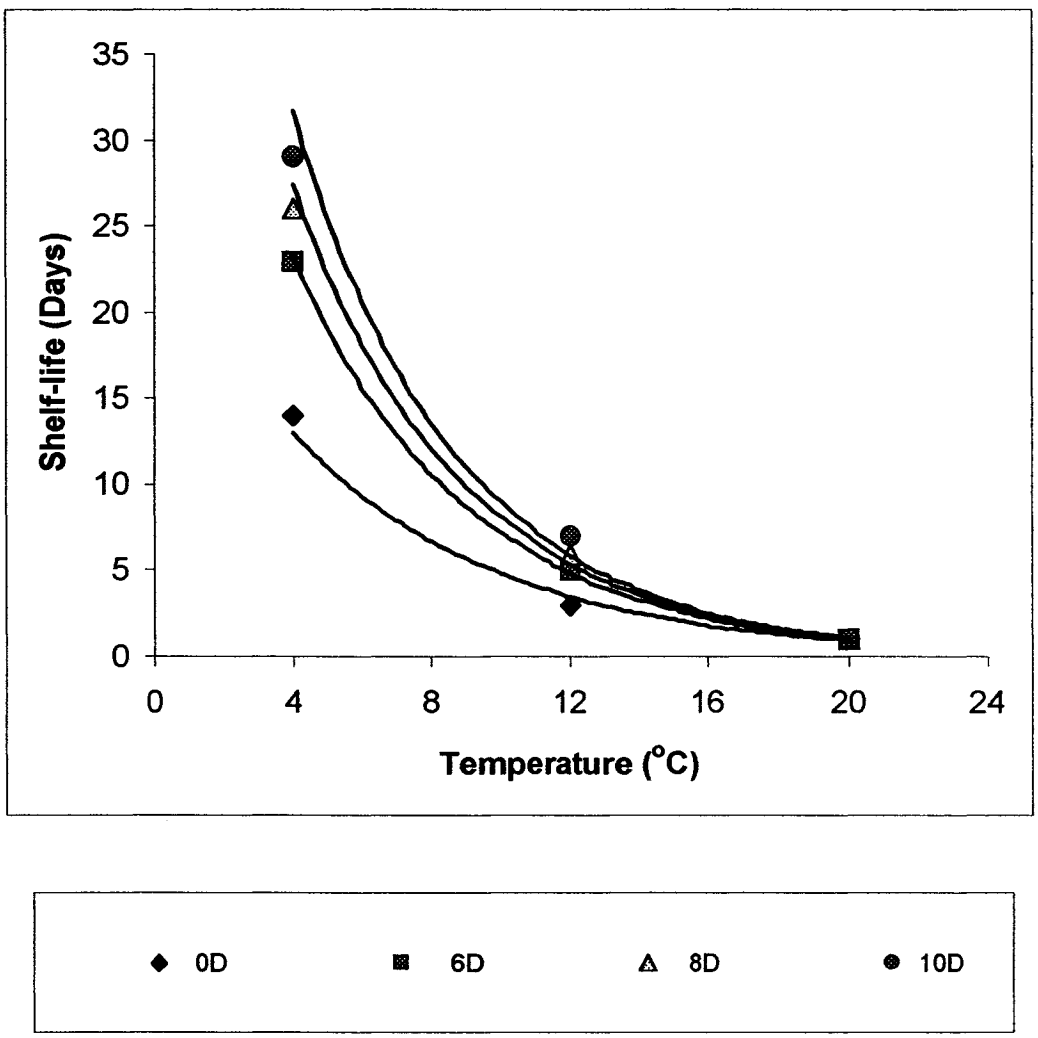

Figure 4.25 Effect of storage temperature on the microbial shelf-life of HP treated fish

Textural attributes were improved by HP treatment, however, with storage, both hardness and springiness of fish appear to decrease. Overall, the HP treatment of fish helped to stabilize texture parameters. As could be expected, based on the severity of the pressure treatment, color parameters were affected by high pressure treatment and subsequent storage. Again, the changes during storage, following HP treatment, were more severe in control than in treated samples. Drip loss was not greatly affected by pressure treatment time, but lower temperature storage, in combination with 10D treatment, helped to reduce the drip loss. Changes in pH were small, but consistent, with microbial spoilage. Quality changes associated with HP treated fish during storage were caused by the growth and activity of indigenous microorganisms. The lower storage temperatures resulted in longer lag phase for microbial growth and extended the shelf-life. HP treatment of fish together with low temperature storage, more than doubled the shelf-life of mackerel at 4°C.

Chapter 5

GENERAL CONCLUSION

This study was carried out to evaluate the safety and stability of high pressure treated fish. Under the conditions of the study, it was not possible to inactivate microbial spores (demonstrated by different researchers) and hence the study was only aimed at limited shelf-life extension under refrigerated storage conditions. Pathogens of concern were *E. coli* O157:H7 and *Listeria monocytogenes.* In the first phase high pressure destruction kinetics were carried out to determine which pathogen is more resistant to pressure. Pressure levels lower than 300 MPa were insufficient to destroy both pathogens. At 400 MPa, *E coli* was found to be more resistant than *L. monocytogenes,* and hence *E. coli* was used as the target microorganism for pressure processing. Its kinetics were characterized by a D value of 3.19 min at 400 MPa, with a z value of 185 MPa. Pulse pressure contributed to only a limited destruction, and hence however, in order to achieve 6-10D reductions in *E. coli* counts, the D value approach had to be used.

A challenge study was carried out with fish inoculated with *E. coli* and subjected to pressure treatments ranging from 6D to 10D based on kinetic data. It was noticed that the calculated levels of destruction were achieved immediately after the treatment; however, some recovery and growth were evident during subsequent storage of these samples at different temperatures. A faster recovery of injured *E. coli* cells was noticed at higher storage temperatures and growth rate was quite rapid. This recovery was true for all treated samples at 20°C and at 12°C for 6 and 8D treatments. The 10D samples had sufficient level of pressure lethality to prevent growth at 4 and 12°C throughout the 30

days storage at 4°C. It also demonstrated that the enumeration of survivors immediate after pressure treatment may not always indicate the real depiction since cells may show partial recovery under certain conditions of storage.

Quality evaluation studies indicated that the shelf-life of treated fish varied depending on the pressure lethality and storage temperature. The hardness and springiness increased with pressure treatment; however, on storage, fish muscle relaxed and hardness and springiness values decreased during storage. Fillets maintained their shape and structure during storage compared to untreated samples, which indicated an improvement in fish texture due to pressure treatment. All color parameters (lightness, redness and yellowness) were influenced by high pressure treatment and unavoidable from consumer point of view. High pressure treatment returned in cooked color to fillets despite the fact the fish was uncooked. The color parameters were more stable in treated samples compared to control samples during storage. Hence, quality preservation was possible and marketability could thus be initiated by consumer education of the nature of changes involved.

Drip loss was found to increase with higher pressure treatments. However at the end of storage, the drip losses in the treated samples were similar, At low temperature storage, a 10D treated product returned in the least drip loss. A small, but consistent change in pH of fish, on high pressure treatment and during storage was observed. The pressure treatments resulted in a slight decrease in pH. However, during storage there was gradual increase in pH, the increase being higher in control than in treated samples. The pH increase during storage could be due to the combination of enzyme and microbial activities (reported by researchers).

Microbiological activity was dependent on pressure treatment severity and storage temperature. Higher pressure lethality and lower storage temperatures contributed to extending the lag-phase and retarding the growth rate of microorganisms. Fish pressure treated to achieve a 10D count reduction in *E. coli* and storage at 4°C had a microbiologically established shelf-life of 29 days, which was more than double the shelf-life of control samples. Hence, HP treatment would have a positive impact on storage quality and shelf-life of mackerel fish.

5.1 Future Recommendations

The pressure treatment used in this thesis did increase the shelf-life to some extent; however, much higher levels of pressure are recommended to reduce treatment time. The current process time is 30 min at 400 MPa. This could be reduced below 3 min if the pressure employed could be one z_p value higher, i.e., ~600 MPa. Further studies are required for spoilage microorganisms along with enzyme inactivation, which can play an important role in fish spoilage during storage. To avoid oxidative color changes in the product, vacuum packaging can be used. The study to destroy spores at much high pressures in combination of mild heat will also be required.

Chapter 6

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McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

›roject should be commenced without prior approval of an application to use biohazardous materials. Submit this cation to the Chair, Biohazards Committee, and one month before starting new projects or expiry of a previously ›ved application.

PRINCIPAL INVESTIGATOR: Dr. H.S. Ramaswamy

ADDRESS: Macdonald Campus (McGill)

TELEPHONE: (514) 398-7919

FAX NUMBER: (514) 398-7977

DEPARTMENT: Food Science and Agricultural Chemistry

E-MAIL:ramaswamy@macdonald.mcgill.ca

PROJECT TITLE: ***Advanced value-added applications of high pressure processing: High pressure sterilization, Thermodynamic, molecular and functional property evalution/modeling***

FUNDING SOURCE: MRC ☐ NSERC ☒ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify)

Grant No.: G205694/NSERC NSERC STPGP 269840 **Beginning date: Nov 2003** **End date** Oct 2006

ıdicate if this is

☐ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

Approval End Date: ***Original Approval End Date Oct 2003***

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RESEARCH GRANTS OFFICE

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

RTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and ifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory safety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

ıtainment Level (circle 1): 1☐ 2☐ 3☐ 4☐

ncipal Investigator or course director (U.P. Smith) date: 16 Mar 2004 (day month year)

~~**airperson, Biohazards Committee:**~~ date: (day month year)

proved period: be 04 (year) ending 31 (day) 10 (month) 06 (year)

fined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. **RESEARCH PERSONNEL:** (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
. S. Ramaswamy	Food Sci. & Agri. Chemistry	✓				
m Smith	Food Sci. & Agri. Chemistry	✓				
ernard Cayouette	Food Sci. & Agri. Chemistry		✓			
ven Shao	Food Sci. & Agri. Chemistry				✓	
il Hiremath	Food Sci. & Agri. Chemistry				✓	
i Ullah Zaman	Food Sci. & Agri. Chemistry				✓	

EMERGENCY: Person(s) designated to handle emergencies

Name: Dr. Jim Smith Phone No: work: (514) 398-7923/8623 home: (514) 457-2262

Name: Mr. Bernard Cayouette Phone No: work: (514) 398-8623 home: (514) 254-2434

Briefly describe:

the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

cteria: *Listeria monocytogenes* Scott A (Risk Group 2)
Escherichia coli O157: H7 (Risk Group 2)
Bacillus cereus (Risk Group 2)
Leuconostoc mesenteroides (non pathogenic)

east: *Zygosaccharomyces baiili* (non pathogenic)
Pichia membranafaciens (non pathogenic)

) the procedures involving biohazards

ll stock cultures are prepared from frozen cultures in glycerol and grown overnight at 30 to 37°C by Mr. Bernard ayouette. Working cultures (~ 10 mL) are prepared the following day and food (milk, cheese, juice, fish ~ 20g) are oculated, in duplicate, in double lined plastic bags with various levels of pathogens to give final inoculum levels of 106-08 CFU/g. The inoculated samples are transferred to the Pilot plant in Styrofoam© container with ice where they are ubjected to various high pressure treatments. After each high pressure cycle, the bags are returned to the lab, again in a tyrofoam© container, for microbiological analysis. Plates are incubated under selected conditions and enumerated within 4-48 hours. All contaminated containers, plates, dilution bottles etc., are sterilized by autoclaving prior to discarding and re clearly identified as "Autoclaved and Sterilized" before pick-up by janitorial staff. Preparation of all cultures, dilutions nd plates, as well as inoculation of all food samples, are done under aseptic conditions in a biological safety cabinet. All staff/students wear face masks, lab coats and rubber gloves during all microbiological procedures. No pipetting is done by mouth. Benches are routinely washed with a 1% hypochlorite solution at the end of each day while the safety cabinet is swabbed with 70% ethanol and the UV light left overnight.

iii) The protocol for decontaminating spills

Spills seldom occur in our laboratory since good house keeping is encouraged and monitored on a regular basis to prevent events. However, in the event of a spill, the protocol for decontaminating spills of type 2 microorganisms is as tlined in both the McGill and Department laboratory safety manuals. If the spill occurs on the bench, it is absorbed by e white paper bench cloth routinely placed on each bench. The cloth is removed and the "contaminated" area is soaked ith 1% hypochlorite solution for ~ 30 minutes and then wiped dry. Then the area wiped up with paper towels soaked in)% ethanol. If spill occurs in the safety cabinet, the spill is wiped up and then the area is soaked with 70% ethanol for ~) minutes, dried with clean paper towels and the U.V light left on for ~ 1 hour prior to resumption of work. Students/ aff do not enter into the lab for at least 1 hour after a spill. All towels, contaminated paper clothes etc, lab coats, masks re placed in autoclavable bags and decontaminated by sterilization. All bags are clearly labeled "Autoclaved and terilized".

f a spill occurs on the body, the clothing is removed and sterilized. Splashes to the face are washed with germicidal soap nd hot water. All spills are reported to the laboratory supervisor and a follow up session is done with the students to go ver the cause(s) of the spill and to re-enforce preventive measures.

Copies of the McGill and departmental safety protocols are in the lab all the times for perusal by the students. Furthermore most of the work done in our laboratory is under the constant supervision of Mr. Bernard Cayouette.

---

es the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which ıld increase the hazards of the infectious agent(s)?

), Cultures are prepared in 10 ml amounts, in triplicate, for use in the inoculation studies.

) the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

'A

'hat precautions are being taken to reduce production of infectious droplets and aerosols?

ll inoculation/enumeration procedure are done under the strictest of aseptic conditions in a biological safety cabinet. All students/ staff wear appropriate protective clothing when handling cultures or inoculated foods.

ıist the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
onald Stewart	1-055	Labconco	36205-04	247196	Nov 21/03 11/09/01