EFFECT OF HIGH PRESSURE TREATMENT OF MILK ON CHEESE MAKING PROCESS

By

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ABSTRACT

Raw milk cheese has unique flavor and textural characteristics not obtainable in cheese from pasteurized milk. Several specialty cheeses made from raw milk are marketed worldwide, especially in Europe. However, because of safety concerns, many countries have imposed stringent restrictions on production and sale of raw milk cheeses. The purpose of this thesis research was to use high pressure (HP) treatment as a novel alternative for conventional pasteurization so that raw milk quality cheese could be produced without compromising food safety. The specific objectives of this research were to evaluate i) the effect of HP treatment of milk on its coagulation and gelation characteristics, ii) the destruction kinetics of microorganism and enzymes in milk, iii) cheese making characteristics of HP treated milk as compared to the raw, pasteurized and micro-filtered milk (controls) and, finally iv) to evaluate ripening characteristics of cheddar cheese made from HP treated milk in comparison with the controls.

Three coagulation parameters of milk - lag time, mean coagulation rate, and inflexion time (time for reaching the point of maximum coagulation rate) were evaluated as a function of pressure (200-400 MPa), temperature (3-21°C) and holding time (10-110 min) using a response surface methodology. In general, the lag time and inflexion time decreased while the mean coagulation rate increased with an increase in pressure, holding time or a decrease in temperature. The rennet gel characteristics were evaluated as gel strength (GS) and water-holding capacity (WHC). With a decrease in pressure level, temperature and holding time, there was a decrease in water-holding capacity and an increase in the gel-strength of the rennet curd.

Raw milk with a high count of indigenous micro-flora and raw milk inoculated with a high concentration of *E. coli* K-12 were subjected to HP treatment (250-400 MPa, 0-80 min) at two selected temperatures (3 and 21°C). Results confirmed the dual effect pressure destruction of microorganisms (characterized by an instantaneous pressure kill value during a pressure pulse and a first order reaction rate during the pressure hold). The kinetics parameters indicated a greater pressure resistance for natural micro-flora than *E. coli*. Since previous studies showed *Listeria monocytogenes* to be more resistant that the natural micro-flora in milk, the pressure process was designed based on destruction

kinetics of *Listeria monocytogenes*. Pressure inactivation kinetics of lipoprotein lipase and γ-glutamyl transferase were also evaluated. Lipase activity was enhanced by the application of pressure. With γ-glutamyl transferase the initial activation was followed by a first order rate of inactivation. However, within the treatment limits, the net extent of inactivation was only small.

The pH change kinetics during Cheddar cheese making from raw milk, heat pasteurized milk, micro-filtered milk and high pressure (HP) treated milk were evaluated. The pH decreased linearly with time in the ripening and cooking stages, and logarithmically in the cheddaring phase. With respect to raw milk (control), all treatments delayed the ripening of milk with micro-filtration influencing the most. During the cooking, pasteurized and micro-filtered milk slightly enhanced the rate of pH change while the HP treated milks continued to delay the pH change. During cheddaring, the rate of change in pH with the HP treated milk was about the same as in micro-filtered milk; however, both of these delayed the rate relative to raw and heat treated milks. The composition of cheese, yield characteristics and milk solid recoveries of Cheddar cheese were monitored. In general, cheeses from HP treated milk and pasteurized milk showed better fat, protein and total solid recoveries compared to raw and cheese from micro-filtered milk. Yield was higher in all pressure treated samples and was comparable from pasteurized milk cheese. Cheese from raw milk and MF milk showed lower yield and solid recoveries.

The ripening characteristics of the prepared cheese were evaluated by monitoring microbial changes (Lactococci and Lactobacilli) for 90 days, and proteolysis (water soluble nitrogen, 12% TCA soluble nitrogen and 5% PTA soluble nitrogen) for up to 300 days. Results indicated the presence of smaller peptides and amino acids at comparable levels in the pressure treated samples and raw milk cheese. The presence of lactobacilli in numbers comparable with raw milk cheese and the lower levels of lactococci, add weight to the suggestion that HP treatment of milk prior to cheese making could be a desirable alternative to heat treatment to produce high quality cheese similar to raw milk cheese.

RÉSUMÉ

Le fromage de lait cru a une saveur unique et des caractéristiques de texture qui ne peuvent pas être obtenues du fromage de lait pasteurisé. Plusieurs fromages fins faits de lait cru sont commercialisés dans le monde, particulièrement en Europe. Toutefois, pour des raisons de salubrité alimentaire, de nombreux pays ont imposé des restrictions sévères pour la production et la vente de fromages à base de lait cru.

Le but de cette thèse était d'utiliser le traitement à haute pression (HP) comme nouvelle alternative à la méthode de pasteurisation conventionnelle afin que des fromages à base de lait cru soient produits sans en mettre en péril la sécurité d'alimentation. Les objectifs spécifiques de cette recherche étaient d'évaluer i) l'effet du traitement HP du lait sur ses caractéristiques de coagulation et de gélification, ii) la cinétique de destruction des micro-organismes et enzymes du lait, iii) les caractéristiques de production du lait traité par la méthode HP comparativement aux laits cru, pasteurisé et micro-filtré (contrôles) et, finalement iv) d'évaluer les caractéristiques du fromage cheddar produit à partir de lait traité par la méthode HP comparativement aux laits contrôles.

Trois paramètres de coagulation du lait – le temps de, le taux de coagulation moyen ainsi que le temps d'inflexion (temps requis pour atteindre le point maximal du taux de coagulation) ont été évalué en fonction de la pression (200-400 MPa), de la température (3-21°C) et du temps de retenue (10 –110 min) en utilisant une méthodologie de surface de réponse. En général, le temps de délai et le temps d'inflexion ont dimininué alors que le taux de coagulation moyen a augmenté lors d'une hausse de la pression, du temps de retenue ou d'une diminution de la température. Les caractéristiques du gel de ont été évalués pour leur force de gel (GS) et pour leur capacité de rétention d'eau (WHC). Avec une diminution du niveau de pression, de température et du temps de retenue, il y a eu une diminution dans la capacité de teneur d'eau et une augmentation de la force de gel du lait caillé de présure.

Du lait cru comprenant une quantité importante de microbes indigènes et du lait cru inoculé avec une forte concentration de *E.coli* K-12 ont été soumis à des traitements de haute pression (250-400MPa) à deux températures (3 et 21°C). Les résultats confirment l'effet combiné de destruction des microorganismes par la pression

(caractérisé par une valeur instantanée de pression destructrice pendant l'impulsion de pression et un taux de réaction de premier ordre lors du maintien de la pression). Les paramètres de cinétique ont indiqué que les microorganismes naturels ont une meilleure résistance à la pression que le *E. coli*. Comme plusieurs études ont démontré que *Listeria monocytogenes* a une meilleure résistance à la pression que la flore naturelle du lait, le procédé de traitement de haute pression a été élaboré en se basant sur les paramètres de cinétique de déstruction du *Listeria monocytogenes*. Les paramètres de cinétique d'inactivation par la pression de la lipoprotéine lipase et de la γ-glutamyl transférase ont aussi été étudiées. L'activité de la lipase a été augmentée suite à l'application de la pression. Pour ce qui est de la γ-glutamyl transférase, l'activation initiale a été suivie par un un taux de réaction d'inactivation de premier ordre. Toutefois, considérant les limites de la méthode, l'inactivation globale nette fut minime.

La cinétique du changement de pH pendant la fabrication du fromage Cheddar à base de lait cru, de lait pasteurisé réchauffé, de lait micro-filtré et à base de lait traité à haute pression (HP) ont été évalués. Le pH a diminué de manière linéaire en fonction du temps lors des étapes d'affinage et de cuisson, et de manière logarithmique lors de la phase de cheddarisation. Comparativement au lait cru (contrôle), tous les traitements ont ralenti l'affinage du lait, plus particulièrement la micro-filtration. Durant la cuisson, les laits pasteurisé et micro-filtré ont augmenté légèrement le taux de variation du pH alors que les laits traités HP ont continué à ralentir les changements de pH. Lors de la cheddarisation, le taux de changement de pH du lait HP était approximativement le même que celui du lait micro-filtré. Toutefois, ces deux laits ont démontré des taux plus faibles que les laits cru et pasteurisé. La composition des différents fromages cheddar produits, leur caractéristiques de rendement ainsi que les solides du lait obtenus ont été évalués. En général, les fromages produits à partir des laits traité HP et pasteurisé ont démontré une meilleure rétention de gras, protéines et solides totaux comparativement aux fromages produits à partir du lait cru ou du lait micro-filtré. Le rendement fut amélioré pour tous les échantillons traités HP et comparable à celui du fromage à base de lait pasteurisé. Les fromages à base de lait cru et de lait micro-filtré on démontré un rendement et une quantité de solides plus faibles.

Les caractéristiques d'affinage du fromage produit ont été évaluées par un suivi des changements micro-biologiques (Lactocoques et Lactobacilles) pendant 90 jours, et par une protéolyse (azote hydrosoluble, azote à 12% soluble dans TCA et azote à 5% soluble dans PTA) jusqu'à 300 jours. Les résultats démontrent la présence de peptides plus petits et des acides aminés à des taux comparables aux échantillons de fromage de lait HP et de lait cru. La présence de lactobacilles retrouvés en quantité similaire au contenu du fromage de lait cru et des quantités faibles de lactocoques, appuient la proposition que le traitement à haute pression du lait avant la production de fromage pourrait être une alternative au traitement traditionnel à la chaleur afin de produire un fromage de qualité supérieure semblable aux fromages de lait cru.

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GENERAL CONNECTING STATEMENTS

The thesis has been prepared in the manuscript style with different concepts around the main theme of thesis presented as chapters rather the traditional Introduction-Methodology-Results & Discussion-References style thesis.

After the abstracts, acknowledgements and listing of table of contents, tables and figures, the thesis begins with a chapter on "Introduction (Chapter I)" focusing on the current status and objectives of research. This is followed by a concise literature review to give the background information in the area of the proposed research. Since relevant literature background is also included in individual chapters, the "Literature Review Chapter II" chapter is kept relatively short. Chapters III – VII describe the different aspects of the research in a systematic manner with a connecting statement provided at the beginning as a link.

Parts of the thesis research have been published/submitted as research papers as well as presentated at various conferences. The following is a detailed list of publications/presentations arising from the thesis. All experimental work was carried out by the candidate, Pramod Pandey, under the supervision of Dr. H.S. Ramaswamy. Part of the work has also be co-supervised by Dr. Daniel St.Gelais, Scientist, CRDA, St. Hyacinthe and Dr. E. Idziak, Professor of Microbiology, Natural Resource Sciences Department , McGill University.

List of Publications

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Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 2002. Evaluation of pH change kinetics during cheddaring of raw, pasteurized, micro-filtered and HP treated milk. Manuscript in preparation for submission to Lebenm.Wissen.-u-Technology (Food Science+Technology)

List of Presentations

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Pandey, P.K., Ramaswamy, H.S. and St-Gelais, D. 1999. Water-holding capacity and gel strength of rennet curd as affected by high-pressure treatment of milk. Paper presented at the CIFST Conference, Kelowna, BC, Canada, June 6-9, 1999.

Ramaswamy, H.S. and Pandey, P.K. 2001. Application of High Pressure processing in Dairy Industry. Paper presented at the Symposium on High Pressure Solutions, Agriculture and Agri-Food Canada, Food Research and Development Center, St. Hyacinthe, PQ. March 7, 2001.

Pandey, P., H.S. Ramaswamy and D. St-Gelais. 2001. Evaluation of manufacturing process and quality of Cheddar cheese made from high-pressure treated milk, microfiltered milk, raw milk and pasteurized milk during ripening. Paper presented at the 2001 Non-thermal Division Workshop of IFT, Newark, DL, March 26-28, 2001.

Pandey, P.K.; Ramaswamy, H.S. and St-Gelais, D. 2001. Proteolysis of Cheddar cheese manufactured from high-pressure treated milk, microfiltred milk, raw milk, and pasteurized milk. IFT New Orleans, June 23-27, 2001.

Pandey, P., H.S. Ramaswamy, and D. St.-Gelais. 2001. Effect of High Pressure Treatment of Milk on Cheddar cheese Ripening. Paper presented at the Agricultural and Food Engineering Conference, St. Hyacinthe, PQ. March 21, 2001.

Pandey, P.K., Ramaswamy, H.S. and St-Gelais, D. 2001. Textural changes in Cheddar cheese from high-pressure treated milk, micro-filtered milk, raw milk and pasteurized milk during ripening. CSAE, Guelph, ON, July 8-11, 2001

Pandey, P.K., Ramaswamy, H.S. and Idziak, E. 2002. Kinetics of high-pressure destruction of *E. coli* and indigenous microorganism in milk at two temperatures. Presented at IFT annual meeting at Anaheim, California June 15-19, 2002

CHAPTER-I

INTRODUCTION

Cheese-making began about 8000 years ago and now there are in excess of 1000 cheese varieties world wide (Sandine and Elliker. 1970; Fox, 1993), each unique with respect to its flavor and form. Manufacture of most cheese varieties involves combining four ingredients: milk, rennet, microbial culture and salt, which are processed through a number of common steps such as gel formation, whey expulsion, acid production and salt additions followed by a period of ripening. Variations in ingredient blends, the use of different microorganisms (lactic acid bacteria) and subsequent processing have led to the evolution of all these cheese varieties. While variations in processing parameters such as cooking temperature and curd handling techniques play a major role in determining the characteristics of each cheese type, the cheese microflora play a critical and pivotal role in the development of the unique characteristics of each cheese variety. The primary objective of cheese manufacture originally was to extend the shelf life and conserve the nutritious components of milk. This is achieved either by acid production and/or dehydration. Production of lactic acid by the starter flora during cheese manufacture results in a decrease in the pH of the milk and this, in combination with coagulation, cooking and stirring, promotes development of the cheese curd and expulsion of whey (Walstra, 1993). While all acid coagulated cheeses are consumed fresh, most rennet coagulated cheese undergo a period of ripening that can range from about three weeks for Mozzarella to two years or more for Parmesan and extra-mature Cheddar. Cheese ripening is a complex process involving a range of biochemical reactions. High densities of microorganisms are present in cheese throughout ripening and they play a significant role in the maturation process (Cogan, 2000)

Raw milk cheeses have been found to possess unique flavor and textural characteristics not obtainable in cheese from pasteurized milk. The conventional heat treatment is known to affect the quality of some cheeses as a result of destruction of thermolabile indigenous microflora, heat sensitive native milk enzymes like lipase, and denaturation of whey protein followed by interaction with casein. In many countries, around the world, raw milk is used for making some varieties of specialty cheeses. Considering the large number of outbreaks throughout the world food and health

regulatory agencies of many countries have imposed strict conditions on production and sale of raw milk and milk products.

With increased safety concern, the industry and food scientists are seriously looking for solutions that satisfy the safety concern without compromising the quality of cheese. High pressure (HP) processing, a novel alternative food preservation technique derived from material sciences could possibly provide the answer for this challenge. HP concept is not new; Hite (1899) of West Virginia University was the first to explore the possibilities of applying HP to enhance the shelf life of milk. However, it is surprising that this technology did not get momentum until a century later due to the lack of development of equipment suitable for food processing. The consumer trend towards natural taste and flavor of food has given incentive to the food scientists and industries to explore alternative processing possibilities in this area. High pressure offers potential for inactivating microorganisms and enzymes, denaturing proteins, and modifying functional properties of selected food ingredients. The successful application of high-pressure technology promises the industry to develop new and innovative processes for manufacturing food products with improved physical and sensory properties. While the process can reduce microbial population and inactivate pathogens, the other valuable constituents are mildly affected (Hayashi, 1989). In recent years research on high pressure processing of dairy food has increased many fold (Datta, and Deeth, 1999). Its cheese related application has drawn significant attention due to the fact that HP process has little or no influence on flavor change. Based on this unique characteristics of HP processing it is opined that "the biggest application of high pressure is processing of fruits and vegetable but the second largest application of high pressure would be processing of milk prior to cheese-making"(Hill, 1997).

There have been several studies on application of HP processing of milk and evaluation of its effect on microbial destruction kinetics, enzyme inactivation and effect on functional properties of milk (Mussa, et al., 1998; Rademacher, Kessler, 1997; Trujillo, et al., 2000). There is clear evidence of destruction of pathogens and spoilage microorganisms in milk by HP processing. It is evident from several studies that milk enzymes responsible for the delicate flavor of raw milk cheese are more pressure resistant than the pathogens and spoilage bacteria in milk. This differential effect of pressure on microorganisms and milk enzymes could provide a basis for pressure treatment which

results in retaining adequate activity of enzymes responsible for the desired flavor in cheese, while destroying the pathogens that cause public health concern. Cheese making is a long process involving several steps and each step would influence the final quality of cheese. Any process that modifies or changes the functional properties of milk would influence the cheese making properties that consequently affect the final cheese quality. It is possible through a detailed study, to elucidate conditions for optimal quality cheese making by using HP processing which ensures inactivation of the pathogens and leaves the other constituents and enzyme unaffected. To test this hypothesis a detailed experimental study involving testing the effect of HP processing of milk on cheese making properties was laid out. The objectives of this research were:

(i) To characterize the effect of high pressure processing conditions (pressure level, temperature, and holding time) on cheese making properties of milk. The study would involve pre-standardization of the following:

a. Development of experimental procedures for evaluating cheese-making properties.

b. Evaluation of effect of pressure processing on coagulation properties of milk (coagulation time, rate constant)

c. Study the rennet gel characteristics of milk

d. Development of an appropriate model for relating the different processing parameters.

(ii) To characterize the effect of UHP processing conditions on microorganisms and enzymes in milk

(iii) To study and compare the characteristics of different cheese making stages of cheddar cheese manufactured from raw-milk, heated-milk, micro-filtered milk and pressure treated milk.

(iv) Ripening characteristics of cheddar cheese made from raw, pasteurized, micro-filtered and HP treated milk.

a. Proteolysis during ripening period

b. Microbial changes during ripening

CHAPTER-II
LITERATURE REVIEW

Raw milk cheeses are known for its unique flavor and taste characteristics through out the world. Traditionally cheeses made from raw milk tend to develop a strong flavor and generally ripens faster than cheeses made from pasteurized milk (Wilson *et al.* 1945; Franklin and Sharpe, 1962; Scarpallino and Kosikowski, 1962; Price and Call, 1969; Melachouris and Tuckey, 1966; Kristoffersen, 1985; Banks *et al.*, 1986). Raw milk cheeses (Swiss, Cheddar, Manchego, Raclette and St. Paulin) developed characteristic flavor sooner and the flavor was stronger, richer and more diverse than the same cheeses made from pasteurized milk. Grappin and Beuvier, (1997) concluded that pasteurization modifies the biochemistry and microbiology of ripening, and subsequently affect the flavor and texture of the cheese. However emergence of cheese related outbreaks have limited the growth of raw milk cheese industry. The effort to preserve raw milk cheese production in certain countries of the European Union began in the early 1990's. From 1990-92, the European Union debated the safety of raw milk cheese and the Codex Alimentarius, which provides standards for the international trade of cheese, was considering the mandatory pasteurization of all dairy products (Dixon, 2000).

In contrast to thermal processing, the application of HP to foods causes negligible impairment of nutritional value, taste color flavor or vitamin content (Hayashi, 1989). There are three main aspects of high pressure in food application: (i) HP capable of inactivating microorganisms (Hoover *et al.*, 1989), hence substitute for thermal process, (ii) HP acts on non covalent interactions which stabilize the structure of biopolymers such as proteins and polysaccharides (Heremans, 1992) leading to modification of food texture and product development and, (iii) HP affect enzyme activity by modifying structure or changes in conformation of enzyme substrate (Kunugi, 1993).

Heat treatment of milk prior to cheese making

Enormous growth of cheese industry during middle of twentieth century, the importance of pasteurization of milk prior to cheese making was quickly recognized. In order to maintain the uniform quality and to minimize the risk of cheese borne illnesses,

heat treatment became mandatory in many countries during 1950's. Traditional cheeses made from raw milk tend to develop a strong flavor and generally ripen faster than cheeses made from pasteurized milk.

Under pasteurization conditions four major known important changes in milk have been reported and these changes can be very well linked to the effect on final characteristics of cheese.

Denaturation of whey protein and their interactions with casein

Significant changes occurs upon heating milk above 60°C include the denaturation of whey proteins interactions between denatured whey proteins and the casein micelles and the conversion of soluble calcium, magnesium and phosphate to the colloidal state. Casein micelles are very stable at high temperatures although changes in zeta potential, size, hydration of micelles, as well as some association-dissociation reactions do occur at severe heating temperatures (Fox, 1981; Singh *et al.*, 1988; Singh and Creamer, 1993). Thermal denaturation and aggregation of whey proteins have been extensively researched. Upon heating milk above 65°C, whey proteins are denatured by the unfolding of their polypeptides, thus exposing the side chain groups originally buried within the native structure. The unfolded proteins then interact with casein micelles or simply aggregate with themselves, involving thiol-disulphide interchange reactions, hydrophobic interactions and ionic linkages.

Denatured whey proteins have been shown to associate with κ-casein on the surface of the casein micelles, giving the appearance under an electron microscope of threadlike appendages, protruding from the micelles (Creamer and Matheson, 1980; Mohammad and Fox, 1987). The principal interaction is considered to be between β-lactoglobulin and κ-casein and involves both disulphide and hydrophobic interactions (Smits and van Brouwershaven, 1980, Singh and Fox, 1987a, 1987b).

Renneting properties

Heat treatment impairs the renneting properties of milk (Morrissey, 1969; van Hooydonk *et al.*, 1987; Singh *et al.*, 1988; Lucey, 1992; Lucey *et al.*, 1993). It has been established that when heated, β-lactoglobulin and κ-casein form a complex by sulfydryl-

disulfide interchange (Dalgleish, 1990a), although hydrophobic interactions may play a role in the initial stages of complex formation (Haque and Kinsella, 1988). Complex formation is the most important factor affecting the coagulability of milk (van Hooydonk *et al.*, 1987; Dalgeish, 1990b).

The rennet coagulation time (RCT) of milk increases with the severity of heat treatment (Morrissey, 1969; van Hooydonk *et al.*, 1987; Singh *et al.*, 1988; Dalgleish, 1990b; Lucey, 1992; Lucey *et al.*, 1993). Marshall (1986) reported that the enzymatic stage is hardly affected by heating, while van Hooydonk *et al.* (1987) reported that complex formation reduced both initial rate of κ-casein hydrolysis and the amount of hydrolysable κ-casein in severely heated milk. Reddy and Kinsella (1990) reported that severe heat treatment (85°C for 15 min) of milk inhibited chymosin hydrolysis, resulting in a reduced initial rate of hydrolysis (37%) and reduction (26%) in the amount of glycomacropeptide released. Heating of casein micelles alone did not affect subsequent hydrolysis by chymosin. Reddy and Kinsella (1990) suggested that conformation of the chymosin susceptible bond of κ-casein might be somewhat different and probably less readily accessible to the enzyme after complex formation with β-lactoglobulin. It is generally agreed (Pyne, 1945; Morrissey, 1969; Marshall, 1986; van Hooydonk *et al.*, 1987; Singh *et al.*, 1988) that the secondary phase of rennet coagulation is more adversely affected by heating than the enzymatic phase. Denatured whey proteins on the surface of casein micelles sterically hinder the aggregation of rennet-altered micelles, resulting in prolonged RCT (van Hooydonk *et al.*, 1987). Other factors may also affect the rate of aggregation of renneted micelles in heated milk, including the formation of heat-induced colloidal calcium phosphate (CCP) which changes the properties of the micelles, although heat treatment appears to have little effect on the ζ-potential of casein micelles (Darling and Dickson, 1979; Schmidt and Poll, 1986). Heating causes the precipitation of calcium phosphate with a concomitant reduction in soluble calcium (Mattick and Hallett, 1929; Pyne, 1945; Hilgeman and Jennes, 1951; Verma and Sommer, 1958), which inhibit the aggregation reaction (Pyne, 1945), which is very sensitive to changes in Ca^{2+} concentration

The rennet gel firmness is adversely affected in heated milk (Lucey, 1992; Lucey *et al.*, 1993; McMohan *et al.*, 1993). The reduction in gel firmness is presumably caused by the disruption of the continuity of the gel network caused by attachment of denatured whey proteins to casein micelles. The denatured whey proteins may sterically hinder the close approach and contact between casein micelles, resulting in a weaker, looser network due to reduced crosslinking (McMohan *et al.*, 1993). The reduced aggregation rate of heated milk means that its gel strength increases at a slower rate than in unheated milk. Thus, the strength of rennet-induced gels from heated milk, determined at a particular time after renneting, will be lower than from unheated milk.

Effect on indigenous milk enzymes

Milk contains about 60 indigenous enzymes, some of which have been isolated and well characterized (Andrew *et al.*, 1991, 1992). Fox and Stepaniak (1993) pointed out that among the several milk enzymes which have the potential to contribute to cheese ripening, only few of them have been investigated carefully. The enzymes activities affected by pasteurization include: the plasmin/plasminogen complex, the acid proteinase (cathepsin/procathepsin) complex, and enzymes from leucocytes, alkaline phosphatase and lipoprotein lipase and to some extent xanthin oxidase which is slightly affected by pasteurization. The indigenous enzymes most likely to contribute to cheese ripening are plasmin, lipoprotein lipase, and acid phosphatase and xanthine oxidase. It is interesting that all these enzymes except lipase are fairly heat stable and survive the high-temperature short-time (HTST) pasteurization almost unaffected.

Plasmin primarily cleaves β- and α_{s2}-caseins in cheese, and is believed also to possess some activity towards α_{s1}-caseins (Fox, 1989). The plasmin as well as proenzyme plasminogen, is quite stable at elevated temperatures, the activity of the enzyme is decreased by heat treatment of milk (Benefeldt *et al.*, 1997). Beuvier *et al.*, 1997 examined the ripening of Swiss type cheese manufactured from raw, pasteurized or micro-filtered milk. The inhibitor of plasmin is a heat sensitive enzyme which leads to elevate the plasmin activity after pasteurization of milk (Richardson, 1983). They found that cheese manufactured from pasteurized milk displayed a more pronounced proteolytic digestion of β-casein and an analogous increase in the amount of γ-casein compared to

cheese manufactured from raw or micro-filtered milk, indicating an increased plasmin activity in cheese manufactured from pasteurized milk. However lipoprotein lipase, γ-glutamyl transferase, alkaline phosphatase activities are completely reduced and several others have slight to considerable negative influence on activity due to pasteurization of milk.

In extra-mature cheeses, fatty acids probably make a positive contribution to flavor when properly balanced by products of proteolysis and other reactions. Reiter *et al.* (1966) suggested that volatile fatty acids may contribute to the background flavor of Cheddar but felt that longer chain fatty acids ($>C_{4:0}$) may be undesirable. Milk contains a very potent lipoprotein lipase which probably causes significant lipolysis in raw milk cheese which is not found in cheese made from heated milk. Milk heating to 78°C for 10s completely inactivates milk lipases as the D value for inactivation of milk lipoprotein lipase at 72°C is only 11.8s (Driessen, 1989), thus removing a potential advantage.

Elimination of microorganisms

The basis of milk pasteurization prior to cheese making is to destroy the target pathogenic microorganism in milk. In general, the microbial count in milk, expressed as standard plate count is reduced by al least 90% (1 log) by pasteurization. Along with all vegetative pathogens, all coliforms and most, if not all, psychrotrophs are destroyed (Burton, 1986, Grappin and Beuvier, 1997) in pasteurization. Among lactic acid bacteria (LAB), heat sensitivity varies according to the genus and the species, as shown by Turner *et al.*, (1986) who reported survival of 21 strains of *Leuconostoc, Pediococcus* and mesophilic lactobacilli. Most of the strains underwent a reduction of poulation of 6 logs or more except one strain of *Lactobacillus casei* var *casei* which was reduced by only 3.5 logs. Thermophilic LAB seems to be more heat resistant which is relevant in Swiss cheeses (not important in cheddar cheese).

Role of Microflora in cheese

Broadly there are two groups of microflora influences the overall cheese manufacturing and its final quality: starter lactic acid bacteria and secondary

microorganisms. Starter lactic acid bacteria are involved in acid production during manufacture and contribute to the ripening process.

Starter bacteria

The primary function of starter bacteria is to produce acid during the fermentation process; however, they also contribute to cheese ripening where their enzymes are involved in proteolysis and conversion of amino acids into favor compounds (Fox and Wallace, 1997). Mesophilic starter bacteria could be defined as isolates which produce sufficient acid to reduce the pH of milk to < 5.4 in 6 h at 30-37°C. Starter bacteria are either added deliberately at the beginning of manufacture or may be natural contaminants of the milk, as is the case in many artisanal cheese varieties made from raw milk. They grow during manufacture and typically attain densities of 10^8 cfu/g within hours of the beginning of manufacture. Either mesophilic or thermophilic starter cultures are used, depending on the cheese being manufactured; mesophilic cultures are used in the production of Cheddar, Gouda, Edam, Blue and Camembert, while thermophilic cultures are used for high temperature (50-55°C) cooked hard cheeses such as Emmental, Gruyère, Parmesan and Grana. Starter bacteria encountered most often are members of the genera *Lactococcus, Lactobacillus, Streptococcus Leuconostoc* and *Enterococcus.* Further both mesophilic and thermophilic cultures can be subdivided into mixed (undefined) cultures in which the number of strains is unknown, and defined cultures, which are composed of a known number of strains. Undefined or mixed-strain mesophilic cultures are mainly composed of *L. lactic* ssp. *cremoris* and *L. lactis* ssp. *lactis,* which may include citrate metabolizing strains, for favor production. A survey of 113 isolates from a commonly used undefined culture indicated that a range of strains having different plasmic profiles, acid producing capabilities and phage sensitive ranges were present, confirming that such cultures are indeed composed of mixed strains (Lodics and Steenson, 1993).

Non-starter lactic acid bacteria (NSLAB)

Secondary microorganisms do not contribute to acid production during manufacture, but generally play a significant role during ripening. The secondary microform comprise of non-starter lactic acid bacteria (NSLAB) which grow internally in

most cheese varieties and other bacteria, yeasts and or moulds, which grow internally or externally and are usually unique to specific cheese varieties or closely related types.

NSLAB are thermophilic lactobacilli and pediococci. which form a significant portion of the microbial flora of most cheese varieties during ripening. They are not part of the normal starter flora; they generally do not grow well in milk (Cogan *et al*. 1997), and do not contribute to acid production in the cheese vat. Presence of NSLAB is cheese depends on the initial content of NSLAB in raw milk, technological treatment given to milk prior to cheese making and post contamination through out cheese making. Starter cells are present at initial densities of irately 10^8-10^9cfu/g in most cheese varieties approximately but levels decrease during ripening due to the hydrolytic activity of their own autolysins enzymes (Thomas and Batt, 1969). It was demonstrated in a model buffer system (Thomas, 1987) that many strains of NSLAB could grow on the products released from starter cells under- going autolysis and this could be true vise versa.

The microbial flora of cheese, particularly those made from raw milk, is very complex. It is thus likely that many interactions occur between strains of NSLAB and other bacteria in the cheese. The study of these interactions is difficult because of the complex microbiological ecosystem found in cheese (Martley and Crow, 1993). The role of NSLAB in Cheddar cheese flavor development has been a contentious issue for many years. In the last decade, there have been a number of studies on the effect of adjunct lactobacilli on Cheddar favor development, with most authors reporting increased levels of proteolysis and enhanced flavor intensity (El Soda *et al*., 1981, Puchades *et al.*,1989, Lemieux *et al.*, 1989, Broome *et al.*, 1990, Trépanier *et al.*, 1991; McSweeney *et al.*, 1994; Johnson *et al.*, 1995; Lane and Fox, 1996; Lynch *et al*., 1996).

Raw milk cheese: Safety concern and cheese-borne illnesses

Raw milk cheese is made similar to any commercial cheese manufacturing process by coagulating raw milk in vats by coagulating enzymes and/or acids. After whey is drained, the large cheese clumps are removed and milled into curds, salted, and packaged and ripened. Raw milk consumption has been associated with campylobacteriosis, salmonellosis, *E. coli* O157:H7, yersiniosis, listeriosis, tuberculosis, brucellosis, cryptosporidiosis, and staphylococcal enterotoxin poisoning (Potter *et*

al., 1984). In 1950, the U.S. Food and Drug Administration (FDA) required manufacturers of soft and fresh cheeses to use pasteurized milk and allowed raw milk to be used only for certain aged cheeses (US-FDA, 1950). In 1986, *E. coli* O157:H7 illness was associated with consuming raw milk (Martin *et al.*, 1986). In 1987, FDA banned the interstate sale of raw milk in retail packages. During 1973-1992, 40 (87%) of 46 raw milk-associated outbreaks occurred in the 28 states that permitted the intrastate sale of raw milk (Hendrickx *et al.*, 1998). During the same period, 11 of 32 cheese-associated outbreaks were attributed to contamination before distribution (Altekruse *et al.*, 1998).

This outbreak investigation illustrates the hazards of using raw milk to produce commercial products that may lead to mislabeling or contaminating pasteurized product by equipment or ingredients. This practice can result in pasteurized products contaminated by equipment or ingredients and in product mislabeling. States that allow the sale of unpasteurized milk or dairy products made from unpasteurized milk should take appropriate steps to reduce the risk for contamination and mislabeling to prevent similar outbreaks.

Most of the food-borne diseases that one can think of, and a few that most of us would never think of, have at some time been transmitted by raw milk or by cheese and other dairy foods made from it. A group of infectious pathogens that have been of concern recently are *Listeria monocytogenes*, Salmonella, *E. coli* O157:H7, *Campylobacter jejuni* and *Yersinia entercolitica*. Some recent outbreaks of serious infections caused by *Listeria monocytogenes* that were transmitted by milk and cheese have been widely publicized. The source of Listeria in these outbreaks has generally been raw or inadequately pasteurized milk. Infected cows, including cows that show no clinical signs of infection, can shed *L. monocytogenes* intermittently in milk for long periods. Milk-borne Salmonella infections are common in some parts of the world where milk is not pasteurized, and cheese prepared from raw or inadequately pasteurized milk has been implicated in large outbreaks in developed countries.

Table 2.1. Pathogens transmitted by raw or inadequately pasteurized milk

Pathogen	Disease	Example
Listeria monocytogenes	Listeriosis	Cheese-300 cases, California, 1985 Cheese related 20 cases, France 2000 Mexican style cheese, 3 cases N. Carolina 2001 Abbott's cheese, 42 cases B.C. Canada 2002
Salmonella	Salmonellosis	Milk- 500 cases, Australia 1978; Cheddar cheese >2700 cases, Canada 1984
Escherichia coli	Diarrhoea	Milk, 30 cases Canada 1988
Campylobacter jejuni	Enteritis	Milk drink 500 cases, Switzerland 1981
Yersinia enterocolotica	Gastroenteritis	Milk, 52 cases, Canada 1975

(Source: Eyles, 1992; and updated through CDC)

Despite intense hygienic efforts, contamination of raw milk by pathogenic microorganisms cannot be completely excluded (Table 2.1). Infectious diseases in dairy cows or contamination of milk during milking, storage, transport, or processing present potential hazards. Cheese cannot be made safely from raw milk unless other processes that inactivate pathogens reliably applied during cheese making (Eyles, 1992). Pasteurization of raw milk is particularly effective because it controls an important pathway for pathogen transmission. On the contrary heat pasteurization adversely affects the flavor and final quality of cheese, therefore, exploration of alternative ways of ensuring pathogen destruction while at the same time keeping quality unchanged, are desirable.

The pathogens that are probably of greatest contemporary to raw milk cheese are Salmonella, *E. coli* O157:H7 and *Listeria monocytogenes*. Many of the microbiological issues related to production of raw milk cheese can be illustrated by concentrating on

these three pathogens. The degree of hazard presented by a particular pathogen in cheese prepared from raw milk depends on a number of things and most importantly does the pathogen survive or grow during the cheese making process and how does it behave during ripening.

The survival and growth of salmonellae has been studied most in relation to cheddar cheese production. *Salmonellae* can grow during cheddar cheese fermentation, and then usually die off during later stages of manufacturing and ripening. A very small number of cells of *Salmonella* can cause illness particularly if they are present in fatty food (Eyles, 1992) which provides protection against harsh conditions. A notable example of survival of Salmonella in cheese has been reported in Canada during 1980 to 1982. The outbreak occurred in Ontario, in raw milk Cheddar contaminated with *Salmonella muenster*. The source of contamination was traced back to a farm where one cow was shedding approximately 200 cfu *Salmonella*/ml in her milk. The survival of *Salmonella* was determined during cheddar manufacturing. Curd during manufacturing tested positive in 11 of 181 vats. Two of these lots still positive after pressing. During pressing one lot was negative after 30 days but one was positive after 125 days (Johnson *et al.*, 1990a, 1990b, 1990c). *Listeria monocytogenes* another notorious pathogen survive the cheese making process, the survival and growth of these organism during ripening varied

Regulations and Canadian prospective

The regulations controlling raw milk cheese production differs from country to country. Government of Canada regulations require that all cheese made from raw milk must be stored (aged) for at least 60 days before sale or consumption to eliminate pathogenic organisms. The large number of outbreaks, cheese borne diseases and several research studies conducted by Health Canada which reveal that the 60 days aging is insufficient to eliminate bacterial pathogens (Brodsky, 1984) provide enough reasons to evaluate the current regulatory status on raw milk cheese. After long consultation and review, the Health Canada proposed to amend the current regulation on raw milk cheese, which was published in Canada Gazette on March 30, 1996 (Food and Drug Act, 1996). This gave rise to virulent reactions from special interest groups and the issue was

discussed widely at different levels from consumer forum to House of Commons. Media also gave enough coverage to this issue. The Macleans magazine in its April 29, 1996 issue described a detail story on raw milk cheese and its impact with a title "Mightily cheesed off". The New York Times wrote in its April 28, 1996 issue with a title "Plan to regulate cheese has Quebec up in arms" calling it a confrontation between English and French speaking Canada over cheese. Due to political pressure, media coverage, and strong protest from consumers, Health Canada withdrew the proposed ban temporarily and is expected to come up with some amicable solutions.

Australia, Denmark, Germany and New-Zealand have banned the production of raw milk cheeses whereas the European Union allows the making, distribution and sale of raw milk cheese; however, strict conditions apply regarding farm practices handling of milk. Codex Alimentaries committee is currently working on international standards on production of raw milk cheese. In the United States, the regulation varies from state to state.

As a result of more number of raw milk cheese related outbreak and consequently the proposed ban on production of raw milk cheeses has provided new challenges and opportunity to research community and cheese industry to come up with a effective solution which can minimize the safety concern at the same time obtain the raw milk cheese quality. High pressure processing is an emerging non-thermal alternative process technology has potential to satisfy the above criteria that require extending safety against pathogen at the same time protecting the flavor of raw milk cheese.

Ultra high pressure processing

High pressure processing has emerged as the most innovative alternative non-thermal food process technology during past decades. The first report of high hydrostatic pressure killing bacteria was by H. Roger in late seventeen century. However, in food science and technology, the most important work involving microbial inactivation was that by Bert Hite, published in June of 1899 (Hite, 1899). Hite originally experimented with the application of high hydrostatic pressure on foods and food microorganisms. He showed that the shelf-life of raw milk could be extended by about 4 d after pressure treatment at 600 MPa for 1 h at room temperature.

Principles of High pressure

The following general principles describe the effect of high pressure:

(i) The isostatic principle indicates that pressure is transmitted in a uniform and quasi-instantaneous manner throughout the biological sample. The pressurization process time is therefore independent of sample volume, in contrast to thermal processing. Keeping the sample for extended period of time does not require any additional energy. Raising the pressure up to 400 MPa necessiates approximately the same amount of energy as that is needed to heat it to 30°C. When an aqueous solution is compressed, the compression energy E (Joule) is approximately equal to: $E = 2/5 \times P \times C \times V_0$, where P is the pressure (Pa), C is the compressibility of the solution, and V_0 initial volume (m^3). Thus the compression energy of one liter of water at 400 MPa = 19.2 kJ, as compared to 20.9 kJ for heating one liter of water from 20 to 25°C. The low energy levels involved in pressure processing may explain why covalent bonds of food constituents are usually less affected than weak interactions (Cheftel and Culioli, 1997).

(ii) Le Chatelier's Principles which describes the effect of pressure based on absolute reaction rate theory, states that any phenomenon (phase transition, change in molecular configuration or chemical reaction) accompanied by a decrease in volume is enhanced by pressure and vice versa. When the activation volume is negative, that is, when the volume of activated complex is smaller than that of initial reactants, a pressure increase and a temperature decrease will increase the reaction rate constant.

Most biochemical reactions result in a change in volume. Consequently, biological processes are influenced by pressure application. The overall volume change favors the dissociation of ionic interactions; disrupt hydrophobic interaction and Van der wall linkage under pressure. Hydrogen bonding formation is favored while covalent bonds are not disrupted by high pressure.

Thermodynamic interpretation of pressure effect

A convenient rationale for the action of pressure is to be found in the theory of absolute reaction rates (Johnson *et al.*, 1954). The theory of absolute reaction rates states that every rate process is in fact an unstable equilibrium between an activated complex and the reactants and the symbol K* is used to represent the equilibrium constant between the reactants and the activated complex. The initial states and activation states are separated by a free energy of activation, ΔF^*. For a process at constant temperature and pressure, the following thermodynamic relationship is applied:

$$F^* = H^* - TS^* + PV^* \ldots\ldots\ldots\ldots \quad (2.1)$$

where H is enthalpy, P is pressure, V is volume, T is absolute temperature and S is entropy. F* contains a volume term indicating that the active state will involve a swelling or shrinking; thereby pressure affects the reaction rate.

All pressure effects are due to the volume change, which occurs, in chemical processes (Erying and Magee, 1942; Johnson and Campbell, 1945; Marquis, 1976). Van't Hoff (1901) was one of the first to consider the effect of pressure on reaction rates in terms of a volume change. Van't Hoff's equations were developed by use of arguments that are similar to those used to develop the Arrhenius equation. He proposed a relationship between the reaction rate constant and pressure at constant temperature:

$$\ln k = \ln k_0 - \Delta V^* \frac{P}{RT} \ldots\ldots\ldots\ldots \quad (2.2)$$

where k is the reaction rate kinetics at pressure P and temperature T, ΔV^* is the volume of activation defined as the difference in volume of the transition state and the volume of the reactants and R is the gas constant (8.2 $cm^3\ K^{-1}\ mol^{-1}$). The plot of ln k versus pressure should yield a straight line with slope $-\Delta V^*/RT$. The value of ΔV^* can be measured directly from exponential measurements of k as a function of pressure P (ΔV^* =slope * RT).

Effect of high pressure

On food constituents

Water is the one of the major constituents of most foods and is highly affected by high pressure. Water is regarded as incompressible at normal pressures, but it is

compressible at high pressures. The decrease in water volume is close to 4% at 100 MPa and reaches about 15% at 600 MPa at 22°C. Foods that contain a high percentage of water and little gas have compressibility similar to that of water. Several physiochemical properties of water are reversibly modified under pressure (Bridgman, 1912; Heremans *et al.*, 1997). Adiabatic compression of water (or of aqueous solutions) causes a temperature increase 2-3°C per 100 MPa, depending on the rate of pressure increase (Heremans *et al.*, 1997)

Pressurization also increases the ionic product $[H^+]\times[OH^-]$ of water, by 10 to 100 fold at 100 MPa (depending on the temperature). The separation of positive and negative charges under pressure is driven by a water 'electrostriction' phenomenon: water molecules rearrange in a more compact manner with a smaller total volume around electric charges, due to dipole-dipole interactions and hydrogen bonding. The reaction: $H_2O = H^+ + OH^-$ is accompanied by a volume decrease of 21.3 ml per mole at 25°C (Bodanszky and Kauzmann, 1962). Thus, the pH of water, of weak acids and several buffers decrease by 0.2-0.5 pH units per 100 MPa. Phase transition of water especially melting and crystallization are influenced by pressure. The phase diagram indicates that water may remain in a liquid state down to -22°C at a pressure of 210 MPa (Bridgman, 1912).

The melting temperature of lipids (triglycerides) increase, in a reversible manner, by more than 10°C per 100 MPa. Thus, lipids present in a liquid state at room temperature will crystallize under pressure. Pressure enhances the formation of denser and most stable crystals. It is likely that pressure inactivation of microorganisms is partly due to changes in the structure and permeability of the cell membrane resulting from the crystallization of phospholipids.

The effect of pressure on the structure and activity of various proteins and enzymes have been studied extensively and are relatively complex (Gross and Jaenicke, 1994; Mozhaev *et al.* 1994). Conformational changes in proteins resulting from pressure application often imply small volume changes, below 1% v/v. These changes affect interatomic distances in weak intra- and intermolecular interactions, including protein

bound water. Covalent bonds and the primary structure of proteins are not much affected. It is generally admitted that high pressure induces the breakdown of (i) salt bonds, due to electrostriction, (ii) at least a part of hydrophobic interactions (alignment and volume reduction of water molecules close to hydrophobic groups), and (iii) hydrogen bonds are apparently reinforced (reversibly) under pressure. This is attributed to a decrease in the O--H interatomic distance and to resulting smaller molecular volumes.

The most common characteristics of hydrogen bonds suggests that the secondary structure of proteins (α-helices and β-sheets), in contrast to the tertiary or quaternary structure, may be stabilized under pressure. Various biochemical studies indicate that pressure above 100-200 MPa often causes: (i) the dissociation of oligomeric structure into their sub units (ii) partial unfolding and denaturation of monomeric structures (in most cases irreversibly) (iii) protein aggregation (probably as a consequences of unfolding), and (iv) protein gelation, whenever pressure and protein concentration are high enough (Gross and Jaenicke, 1994; Mozhaev *et al*, 1994; Mozhaev *et al*, 1996; Cheftel and Dumay, 1997; Hermans *et al.* 1997). The formation of intermolecular disulfide bonds through SH/SS interchange reactions appears to be enhanced under pressure (Funtenberger *et al.*, 1997). Changes in the hydration volume of proteins probably play a major role in pressure induced unfolding, dissociation, aggregation and gelation of food proteins. Recent results appear to indicate that there is no general pattern of protein denaturation by pressure, unfolding requiring, for example, very different pressure levels for distinct proteins.

Effect on microorganism

It is well established that the application of high pressure will result in the death of microorganisms and that this could have applications in the sterilization or pasteurization of foods (Zobell, 1970; Butz and Ludwig, 1991;). The mechanism of microbial destruction is not fully understood however the high-pressure effect on microorganisms can be explained in the following ways:

- Genetic mechanisms: Nucleic acids are much more resistant to hydrostatic pressure than proteins. Since the structure of the DNA helix is largely a result of hydrogen bond formation, the negative volume change inherent to hydrogen bond formation would be favored by an increase in pressure (Suzuki *et al.* 1972). *Bacillus subtilis* DNA solution (0.002-0.04%, pH 4.8-9.9) subjected to pressure up to 10,000 atm at room temperature also do not denature (Heden, 1964). The difference in pressure tolerance between DNA and protein may be a result of this high degree of intramolecular hydrogen binding. However despite the stability of DNA under pressure, the enzyme-mediated steps of DNA replication and transcription are disrupted by pressure. Pollard and Weller (1966) studied the induction of β-galactosidase and the incorporation of valine and proline into protein, uracil into RNA, and thymine into DNA in *E. coli*. The formation of β-galactosidase was reported cease at 450 atm.

- Metabolism: A hypothesis explaining the effect of high pressure on cell metabolism by Smelt *et al.* (1994) suggest that to maintain homeostasis an active transport mechanism is required to keep the intracellular pH in actively metabolizing cells close to 6.5. If pressure is applied several sites of bacterial cell can be damaged and membrane bound ATPase can no longer perform its function either as a result of direct denaturation or dislocation in the membrane. ATP is no longer hydrolyzed and hence is no longer available to perform efflux of protons causing internal pH to drop resulting in the death of cells.

- Cell membrane and wall: The cell membrane separates the intracellular constituents from the environment and, as a result, plays a very important role in cellular transport; it also functions in respiration. If the membrane is extensively permeabilized, cell death results. The main components of the membrane are phospholipids and proteins, with the structure maintained by hydrogen and hydrophobic bonds. Pressure-induced membrane malfunctions cause inhibition of amino acid uptake probably because of protein denaturation in the membrane (Paul and Morita, 1971). It is generally felt that for microorganisms the primary site of pressure damage is the cell membrane (Morita, 1975). This may be a primary factor for fungi. Eucaryotic microorganisms are generally more sensitive to pressure than procaryotic microorganism.

HP Effect on milk

On milk constituents

Effect of high pressure processing on milk has been examined by several researchers. Hite (1899) was the first to use pressure processing on milk. He reported a considerable increase in the shelf life of milk due to the application of high-pressure treatment. Schmidt and Bucheim (1970) reported irreversible disintegration of casein micelles to smaller fractions. There are two possibilities for this phenomenon either disruption of hydrophobic bonds that hold submicelles together or disruption of the colloidal calcium phosphate micellar framework. Shibauchi *et al.* (1992) reported that high pressure processing results in a disintegration of the casein micelles into chains or cluster with wide ranged size distribution, which is accompanied by an increase in Ca-ion activity attributed to solubilization of casein micelle-bound Ca. Therefore it is attributed that the functional properties of milk can be modified by pressure treatment due to change in colloidal calcium.

Change in the colloidal calcium phosphate of casein micelles would result in the significant alteration in the textural properties of rennet gel. Pressure processing of skim milk also increases the number of exposed hydrophobic groups on the surface of the proteins with increasing severity and duration of high pressure, which shows that considerable irreversible protein unfolding, is being caused (Johnston *et al.*, 1992). Gaucheron *et al.*, (1997) subjected milk to high-pressure (250, 450 and 600 MPa) treatment for 30 min at 4°C, 20°C and 40°C. All the treatment except 250 MPa led an increase in protein hydrophobicity, decrease in lightness, a decrease in average diameter of particle and slight solubilization of calcium and phosphorous from the colloidal to the aqueous phase of the milk. At 40°C and 250 MPa, the effects were very different because protein hydrophobicity remained unchanged. The changes to the conformational structure of the proteins indicated by the increase in the exposure of hydrophobic groups would be expected to alter functional properties of the system. Hence the functional properties of milk protein like foaming, emulsifying, gelling and water binding capacities of the milk

protein may all potentially be influenced. This could lead to development of a functional food ingredient prepared from milk proteins by controlled unfolding of their structure.

β-lactoglobulin is more pressure sensitive than serum albumin, the denaturation of β-lactoglobulin is partly reversible as a function of storage time after depressurization (Camp *et al.*,1997). The β-lactoglobulin is more pressure sensitive than α- lactoalbumin (Hayashi *et al.*, 1987) therefore can be selectively removed from milk. The difference in pressure sensitivity between β-lactoglobulin and α- lactoalbumin can be explain due to the fact that α- lactoalbumin contains four disulfide bonds as against two for β-lactoglobulin and also relatively more non covalent interactive forces. The more literature related with pressure effect on milk and cheese making properties have been discussed in relevant chapters.

On indigenous milk enzyme

During cheese ripening the caseins are cleaved by rennet, plasmin and bacterial proteinases into phosphorous rich peptides. The phosphate residues exert a protective effect against further proteolytic hydrolysis of the peptides (Schormuller, 1968). Complete casein degradation during cheese ripening can be achieved only by the combined action of proteinases and phosphatases (Larsen and Parada, 1988). The origin of active acid phosphatases in ripening cheese is controversial. The enzyme is a phosphomonoesterase and could be derived from a number of sources including bovine milk. This enzyme is very active on phospho proteins such as casein. γ-Glutamyl trasferase is another important proteolytic enzyme responsible for catalysis of the transfer of γ-Glutamyl residue from γ-Glutamyl-containing peptides to other acceptor amino acids or peptides. The activity of γ-Glutamyl transferase losses its >90% activity on pasteurization (Patel and Wilbey, 1994). Rademacher and Kessler (1997) reported that the inactivation of γ-Glutamyl transferase and alkaline phosphatase is possible only at very high pressure (>400 MPa) and concluded that these enzymes seem to be not pressure sensitive. A similar result was also reported by Mussa and Ramaswamy (1997) in which the D and z values of the alkaline phosphatase were found to be higher than the microorganism in raw milk. In general, several studies have concluded that the enzymes are more resistant to pressure than microorganisms.

Application of HP processing in cheese making

Rennet coagulation properties and curd formation

The rennet coagulation of milk is the most important and first step in cheese making process. It is a complex process comprising several sequential stages. However the rennet coagulation time (RCT) which is time from rennet addition until the curd is ready to cut (Kosikowski and Mistry, 1997). The coagulation process distinctly divided into two stages process: the first stage is the enzymatic (chymosin) hydrolysis of κ-casein (Phe_{105}-Met_{106} bond), which destabilizes the casein micelles and leads to aggregation and coagulation under second stage once the critical stages of hydrolysis of κ-casein has reached.

HP treatment of milk affects its coagulation process and cheese-making properties because of number of changes takes place on milk proteins, including reduction in the size of casein micelles and denaturation of β-lactoglobulin, probably followed by interaction with micellar κ-casein. Several studies have clearly demonstrated that the RCT of milk is affected by HP treatment, with the nature and magnitude of the effect depending on the pressure applied. Although an early study reported that RCT was unchanged following treatment at <150 MPa, and decreased following treatment at pressures 200-670 MPa (Desobry-Banon *et al.*, 1994). Most studies agree that milk RCT decreases on treatment at pressures up to 200 MPa (Needs, *et al.*, 2000; López-Fandiño *et al.*, 1996;1997;1998; and Shibauchi *et al.*, 1992). The RCT of milk treated at 400 MPa is generally reported to be close to that of untreated milk (Fandiño, *et al.*, 1996; López-Fandiño *et al.*, 1997; López-Fandiño *et al.*, 1998; Needs *et al.*, 2000; López-Fondino et al., 1997; Molina *et al.*, 2000) and treatment at 500-600 MPa increased RCT relative to untreated milk (Needs *et al.*, 2000). In agreement, Trujillo *et al.*, 1999a compared the coagulation properties of pasteurized (72°C for 15 s) and HP-treated (500 MPa for 15 min at 20°C) goat milk and found that RCT was significantly higher in HP-treated milk than in pasteurized milk.

A number of studies have examined the separate effects of HP treatment on the two stages of rennet coagulation of milk. Ohmiya *et al.* (1987) found that the primary (enzymatic) stage of coagulation was not affected by pressure up to 130 MPa. However, later studies (López-Fandiño et al; 1997 López-Fandiño and Olano 1998) reported that HP treatment reduced the rate of release of caseinomacropeptide (CMP) from κ-casein at 200-300 MPa, by rendering κ-casein less susceptible to the action of chymosin as a result of interaction with β-lactoglobulin indicating that the primary and secondary stages were affected differently by HP treatment. A second factor that may affect the secondary stage of rennet coagulation of milk is reduced casein micelle size post-HP treatment (Desobry-Banon *et al.*, 1994 and Needs *et al.*, 2000). Needs, *et al.*, 2000 suggested that pressure-induced reduction in the diameter of the casein micelles, accompanied by conformational changes of spherical particles into chains or clusters of submicelles, may accelerate aggregation, and hence reduce milk RCT in spite of reduced rate of release of CMP. Overall, two opposing effects of HP treatment on milk may contribute to the effect of HP on the secondary stage of rennet coagulation: (a) a direct effect of pressure on the properties of the micelles which enhances aggregation and (b) increasing denaturation of β-lactoglobulin which progressively reduces the rate of aggregation, probably through interference with the process of gel assembly (Needs *et al.*, 2000)

An early study of the effect of HP on the rate of curd formation or curd firming of renneted milk indicated that this process was accelerated by HP treatment, particularly at ~400 MPa (Ohmiya *et al.* (1987). But study of López-Fandiño, *et al.*, 1996 and 1997 reported that curd firming rate of milk was increased after treatment at pressures <200 MPa, but decreased following treatment at 200-400 MPa, although in all cases the rates were higher than in untreated milk, suggesting that the effect of HP on rate of curd firming is thus analogous to its effect on RCT of milk.

Effect of HP processing on Cheese yield

Cheese yield may be increased by the use of heat to induce interactions between β-lactoglobulin and κ-casein and thus reduce the rate of syneresis from the curd and increase protein incorporation (Pearse *et al.*, 1985), when the rate of syneresis from the

curd of heat-treated cheese milk is reduced there is a concomitant increase in the moisture content of the cheese. The higher the moisture content of the cheese the faster the cheese will ripen but the quality of the resultant cheese will be significantly reduced (Fox and McSweeney, 1998). In a somewhat analogous manner, cheese yield may also be increased by treatment of cheese milk at high pressures, through denaturation of whey proteins and increased moisture retention. Brooker *et al.*, 1998 reported that a decrease in the total protein content of whey obtained following rennet coagulation of milk treated at increasing pressures indicating that whey proteins are incorporated into rennet curds, and hence cheese, made from HP-treated milk. Incorporation of denatured whey proteins may affect cheese yield both directly, through recovery of additional protein, and indirectly, through interference with syneresis and moisture expulsion from curd during cheese making. However, incorporation of whey proteins and increased yield may have negative impact on the quality of the cheese. Sensory analysis of cheese made from HP-treated milk at 3 months of age indicated that Cheddar cheese made from pressurized milk had low texture scores with a pasty, weak texture and a whey taint flavor probably due to the increased moisture content (Drake *et al.*, 1997).

High pressure treatment of cheese

The use of HP to control microbial growth and extend the refrigerated shelf life of pasteurized goats' milk cheese was studied by Capellas *et al.*, (1996). Cheese milk was inoculated with *E. coli* CECT 405 to obtain 10^8 cfu/g cheese, with the resultant cheese being treated using combinations of pressure (400-500 MPa), temperature (2, 10 or 25°C) and time (5-15 min) and subsequently stored at 2-4°C. No survival of *E. coli* was detected 1 day after pressurization, except in samples treated for 5 min at 25°C at pressures of 400-450 MPa, while *E. coli* in the control cheese remained at ~10^8 cfu/g cheese. No surviving *E. coli* were detected in cheese treated at 400-500 MPa for 5-15 min at 2, 10 or 25°C, after 15, 30 or 60 days of storage.

The pressure treatment at the end of cheese manufacturing (or at early days of ripening) would reduce the starter bacteria counts however these bacteria would lyse and release the enzyme helpful in ripening. The potential use of HP to accelerate the ripening of cheese was first described in a patent by Yokoyama *et al.*, 1992. Cheddar cheese was exposed to HP from 5 to 300 MPa for 3 days at 25°C and proteolysis and flavour

development were studied and compared to untreated cheese and 6-month old commercial Cheddar. Free amino acid levels in cheese treated at 50 MPa were reported as 26.5 mg/g, while those in the 6-month old control cheese, which had not been, HP treated were 21.3 mg/g. The taste of both the cheese treated at 50MPa and the 6-month old control cheese were described as "excellent". Addition of lipase and protease to the cheese curd at salting, followed by HP treatment at 50 MPa for 3 days at 25°C, resulted in a Parmesan-type cheese equivalent to a commercial control in terms of levels of FAA and flavor scores (Yokoyama *et al.*, 1992). These data suggest that a considerable reduction in the ripening times of these two hard cheese varieties can be attained through the application of HP. However, it should be noted that the method of Cheddar manufacture reported by (Yokoyama *et al.*, 1992) would appear to be substantially different from conventional Cheddar manufacture. In particular, the level of starter bacteria added to the cheese milk was at least 10-fold higher than conventional inoculation rates, which may account for the very high levels of FAA reported in the untreated cheese.

The possibility for accelerating the ripening of commercial Cheddar cheese by exposure to a treatment at 50 MPa for 3 days at 25°C at different stages of ripening was further investigated by O'Reilly *et al* 2000. These authors found that there was an immediate increase in pH 4.6 soluble nitrogen, expressed as % total nitrogen, and FAA in cheese HP-treated at 2 days of age, although this effect decreased with cheese age. Urea polyacrylamide gel electrophoresis (PAGE) analysis of cheese samples indicated that HP treatment accelerated degradation of α_{s1}-casein and accumulation of α_{s1}-I-casein. O'Reilly *et al.*, 2000 concluded that the enhancement of proteolysis observed may be attributed to a combination of the temperature and pressure used in the treatment and that the increases in rates of proteolysis obtained after treatment of commercial Irish Cheddar cheese at 50 MPa at 25°C for 72 h were not as significant as suggested by Yokoyama *et al.*, 1992 for experimental cheese. O'Reilly *et al.*, 2000 also suggested that the level of increased proteolysis found post pressurization at 50 MPa for 3 days at 25°C would not warrant the industrial utilization of HP as a technology for acceleration of Cheddar cheese ripening.

While the results of the above study suggested that one of the principal effects of HP on cheese ripening concerned the action of chymosin on α_{s1}-casein, some studies have directly examined the effect of HP on chymosin. Goats' milk cheese HP-treated at 50

MPa for 3 days or 400 MPa for 5 min has been shown to exhibit faster proteolysis and higher pH values (0.5 units greater) than untreated cheese, possibly due either to a primary effect of pressure on cheese enzymes or a secondary effect due to changes in pH (Saldo *et al.*, 2000). One day-old goat's milk cheese was also treated at 14°C for 3 days at ambient pressure, 25°C for 3 days at ambient pressure or 50 MPa at 25°C for 3 days. From quantitative indices of proteolysis, it appeared that elevated storage temperature had the most significant proportionate effect on proteolysis (Sendra *et al.*, 1999). Kolakowski *et al.*, 1998 subjected Camembert cheese to pressures up to 500 MPa for 4 h and found an influence of HP on proteolysis proportional to the applied pressure and the maturity of the cheese. For 10-day old Camembert, the highest degree of proteolysis was observed post-pressurization at 50 MPa for 4 h.

HP treatment at 50 MPa for 8 h at 20°C also markedly affected pH and proteolysis in Père Joseph, a semi-hard smear-ripened cheese Messens *et al.*, 2000. The increased pH of the cheese post-pressurization possibly resulted in a higher amount and/or activity of the proteolytic enzymes of *Brevibacterium linens* and in a higher activity of peptidases of starter bacteria, leading to accelerated proteolysis near the rind of the cheese. SDS-PAGE analysis of cheese post-pressurization showed increased production of a polypeptide of ~20 kDa in HP-treated cheese. Increased levels of proteolysis have also been found for surface-mould ripened cheese (Paillardin cheese, containing the secondary inoculum *Penicillium camemberti*) that was HP-treated at 50 MPa for 8 h at 20°C (Messens *et al.*, 2001).

Some ripening characteristics of different cheese varieties may be unaffected by HP treatment. For example, lipolysis in Camembert cheese was only slightly affected by HP treatment of up to 500 MPa for 4 h, while lipolysis in Gouda cheese treated under the same conditions remained unaffected post-pressurization (Kolakowski *et al.*, 1998). None of these studies highlight the consequences of presence toxin producing pathogens presence and growth during cheese making although the cheese may have been pressure treated to destroy the pathogen in cheese. The residual effect of toxin produced by pathogen may be of serious concern however the concept of HP treatment after cheese making will have good potential in accelerated ripening of cheese.

Although there have been several studies of application of HP processing indicating influencing the cheese making parameters, there is not a single studies characterizing the effect of pressure process variables such as pressure, holding time and temperature on the cheese making properties For a successful process design it is important to model such parameters as a function of process variables.

CHAPTER III

EFFECT OF HIGH PRESSURE PROCESSING OF MILK ON CHEESE MAKING PROPERTIES

CONNECTING STATEMENT

This chapter covers the studies on effect of HP treatment on the cheese making properties of milk. HP processing is known to affect the functional properties of milk, and therefore, it is important to characterize its effect on cheese making properties. The chapter is divided in to two parts:

Part I. Effect of high pressure treatment on the rennet coagulation properties of milk

Part II. Effect of high pressure treatment of milk on the water holding capacity and gels strength rennet curd.

Some parts of this chapter have been published/presented:

Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 2000. Water-holding capacity and gel strength of rennet curd as affected by high-pressure treatment of milk. *Food Research International* 33: 655-663.

Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 2002. Effect of HP treatment on rennet coagulation properties of milk. Submitted for publication in Journal of Food Process Engineering.

Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 1999. Coagulative and gel characteristics of milk as affected by high-pressure treatment. Paper presented at IFT Annual Meeting, Chicago, IL USA, July 24-24, 1999.

Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 1999. Water-holding capacity and gel strength of rennet curd as affected by high-pressure treatment of milk. Paper presented at the CIFST Conference, Kelowna, BC, Canada, June 6-9, 1999.

All the experimental work and data analysis were carried out by the candidate under the supervision of Dr. Ramaswamy and Dr. St-Gelais. The experimental setup and protocol for the measurement of coagulation properties and curd characteristics were provided by CRDA, St-Hyacinthe, Quebec (St. Gelais).

PART I

EFFECT OF HIGH PRESSURE PROCESSING OF RENNET COAGULATION PROPERTIES OF MILK

ABSTRACT

The effect of high-pressure (HP) treatment of milk on rennet coagulation properties was evaluated. Three coagulation parameters - lag time, mean coagulation rate and inflexion time (time for reaching the point of maximum coagulation rate) were evaluated as a function of pressure level (200-400 MPa), temperature (3-21°C) and holding time (10-110 min) using a response surface method. A central composite rotatable design with a second order multiple response model was used to investigate the effect of process variables (pressure, temperature and holding time) and relate them to coagulation parameters. In general, with an increase in pressure or holding time, or a decrease in temperature, the lag time decreased, the mean coagulation rate increased and inflexion time decreased. Canonical analyses indicated that the stationary point was a saddle point for lag time and mean coagulation rate whereas a minimum for inflexion time. At the stationary point, the predicted values were: lag time, 7.7 min (with 85 min pressure treatment at 296 MPa and 5°C); inflexion time, 11.3 min (50 min treatment at 370 MPa and 6°C) and mean coagulation rate, 0.0034 min^{-1} (72 min treatment at 275 MPa and 14°C).

INTRODUCTION

High pressure (HP) processing in food applications is getting momentum and the process is being evaluated widely as an alternative to conventional thermal processing of foods. The unique characteristics of HP processing such as selective destruction of deleterious and pathogenic micro-flora while preserving the nutritional and sensory attributes of food samples have attracted considerable interest in industry and academia (Lopez-Fandino and Olano, 1998). High-pressure treatment is being widely explored as a preservation technique and, although in developing stage, it demonstrates a great potential for making products with better functional properties and microbiological stability (Cheftel, 1992; Knorr, 1999). High-pressure treatments have been found to improve the microbiological quality and technological properties for cheese making such as coagulation time, cheese yield, gel strength and effect on syneresis (Johnston *et al.*, 1993; De-sobry-Banon *et al.*, 1994; Patterson *et al.*, 1995; Lopez-Fandino *et al.*, 1996; Pandey *et al.*, 2000).

One of the very important cheese making steps is the rennet-induced coagulation of milk. In cheese industry, the optimum cutting time of milk coagulation is determined by subjective judgment of the cheese maker (Buffa *et al.*, 2001b). Milk protein systems are delicate structures and are maintained by a subtle balance between inter- and intra-molecular interactions between amino acids and the surrounding solvent. External factors such as pressure and temperature can influence these interactions and thereby modify the functional properties of milk (Schrader *et al.*, 1997), which in turn will affect the cheese making properties (Hermier and Cerf, 1986; van Hooydonk *et al.*, 1987; Johnson *et al.*, 1990a; Lopez-Fandino *et al.*, 1997).

Ohmiya *et al.* (1987) studied milk curdling by rennet under pressure and reported negligible pressure influence on casein hydrolysis by chymosin (primary phase), the delay of casein aggregation (secondary phase) or the enhancement of milk coagulation (tertiary phase). Many authors have reported that the coagulation properties of milk to be improved after pressure treatment (López-Fandiño *et al.*, 1997; Buffa *et al.*, 2001b); however there is little information available in the literature characterizing the pressure processing conditions (pressure, temperature and holding time) on milk coagulation properties. There

is no detailed study in the published literature that quantifies the development kinetics of rennet coagulation of milk as affected by HP processing. The objective of the present study was to evaluate the effect of different high-pressure process variables (pressure level, temperature and treatment time) on the development behavior and kinetics of rennet coagulation of milk.

MATERIALS AND METHODS

Milk sample preparation

Raw milk was obtained from the Macdonald Farm of McGill University (Ste. Anne de Bellevue, QC) and the milk samples were analyzed for proximate composition at the Programme d'Analyse des Troupeaux Laitiers du Quebec (milk laboratory associated with the university). Raw milk was filled into dual-peel sterilization pouches (400 mL/pouch) (Nasco Plastic, New Hamburg, ON) and sealed using a heat sealer. The samples were then stored at 3°C, removed and equilibrated to the test conditions just prior to pressure treatment as detailed in the next section. The milk pouches were also kept at 3°C after the pressure treatments for about 2 h prior to subjecting them to rennet coagulation tests.

Pressure treatment

An ABB Isostatic Press (Model # CIP 42260, ABB Autoclave System, Columbus, OH) was used in this study for subjecting milk to various pressure treatments. The high-pressure equipment consisted of a pressure chamber (10 cm internal diameter and 55 cm height) with an enclosed metal jacket that allowed temperature control in the pressure chamber by circulating water at the desired temperature. The pressure chamber was filled with a mixture of distilled water and a 2% water-soluble oil (part #5019, ABB Autoclave Engineers, OH), which was used as the hydrostatic fluid. The test pouches were submerged in the fluid and subjected to pressure treatment at 250-400 MPa for various time (0 to 80 min) as detailed in the experimental design. In order to achieve and maintain an approximate average temperature during test run, the test samples were equilibrated to about 3-5°C below the desired temperature prior to test run and the circulation water

temperature was kept slightly below the desired level. Temperatures monitored during selected test runs indicated average bath temperatures to be within $\pm 2^{\circ}C$ of the set point (except for the first five minutes when adiabatic heating due to pressure buildup resulted in an initial increase in temperature of about 6-10°C, which dissipated quickly to the surrounding shell). Following the treatment, test pouches were removed from the chamber and transferred to a cold room (3°C).

Coagulation test

Coagulation of pressure treated milk was measured by using the turbidity method described by St-Gelais and Savoie (1993), which was a modified method of McMahon *et al.* (1984). Pressure treated milk samples were weighed (50 g) into a 100 mL capacity beaker and placed in a water bath at 30°C and allowed to equilibrate. An active mesophilic lactic acid Group B starter culture, prepared from freeze dried culture of mixed strain (Institute Rossel Inc., St-Laurent, PQ) was added at 1% level to the milk. Incubation in the water bath was continued until the pH of the milk reached 6.55. A single strength rennet (Chymostar, Rhone-Poulenc, Marshall Products, Madison, WI) was added at this time at the rate of 200μL per 1L of milk. Immediately after addition of rennet to milk samples, 1 mL samples of rennet treated milk were poured into three disposable 10 mm polystyrene micro-cuvettes (Fisher Scientific, Pittsburg, PA) and transferred to a Beckman DU® 65 spectrophotometer (Beckman Instrument, Inc., Fullerton, CA) equipped with a Beckman auto 6-sampler accessory, a six sample cell holder. Three of the six cells were used to simultaneously follow coagulation in three replicate samples. The temperature was maintained at 30 ± 0.2 °C with a water circulator. Optical density (OD) was recorded at 700 nm at 13 second intervals for 50 min by using a Beckman DU®Data leader™ software.

Coagulation parameters

The optical density ratio (ODR) was calculated by dividing the measured optical density value by the average of initial optical density values for the first 7 data points (1.5 min) after rennet addition (St-Gelais and Savoie, 1993). Since the optical density increases with time, the ODR vs time represents a normalized transient optical density curve during

the coagulation process, increasing from the base value of 1.0. A typical ODR curve is essentially a logistic sigmoid type curve of optical density ratio vs. time (Figure 3.I.1), and can be modeled as an asymmetrical sigmoid equation (Caron *et al.*, 1997) as shown in Equation (3.I.1):

$$ODR = \left[\frac{a-d}{\left[1+\left(\frac{t}{c}\right)^{b}\right]^{e}}\right]+d \qquad (3.I.1)$$

where ODR = the optical density ratio, a = minimum ODR, which is 1.0 in this case, b= slope coefficient, which is indicative of the rate of coagulation, c= inflexion point coefficient, d= maximum ODR at infinite time (ODR_{∞}), e= a symmetry parameter, and t = time (min). These parameters (a, b, c, d, and e) were obtained by curve fitting to the above model using Sigma Plot software version 6.0 (SPSS Inc. Chicago, IL). Equation (3.I.1) and its first and second derivatives (obtained by Mathcad, 5.0 software-from MathSoft Inc, Cambridge, MA) allow the determination of the lag time (t_{lag}) which is defined as the time when ODR indicates an increase by 0.001 units from the initial value of 1.0, the inflexion time (t_{max}) is time when the rate coagulation is maximum, and D_{max} the coagulation rate (slope) at the inflexion point which is the maximum. The parametric calculations are shown below:

$$t_{lag} = \left[-1+\left[\frac{(a-d)}{((a+0.001)-d)}\right]^{\left(\frac{1}{e}\right)}\right]^{\left(\frac{1}{b}\right)}\cdot c \qquad (3.I.2)$$

$$t_{\max} = \left[\frac{(b-1)}{(e\cdot b+1)}\right]^{\left(\frac{1}{b}\right)}\cdot c \qquad (3.I.3)$$

$$D_{\max} = \frac{(-a+d)}{\left[1+\left[\left[\frac{(b-1)}{(e\cdot b+1)}\right]^{\left(\frac{1}{b}\right)}\right]^{b}\right]^{e}} \cdot e \cdot \left[\left[\frac{(b-1)}{(e\cdot b+1)}\right]^{\left(\frac{1}{b}\right)}\right]^{b} \cdot \frac{b}{\left[\left[\left[\frac{(b-1)}{(e\cdot b+1)}\right]^{\left(\frac{1}{b}\right)}\right] \cdot \left[c \cdot \left[1+\left[\left[\frac{(b-1)}{(e\cdot b+1)}\right]^{\left(\frac{1}{b}\right)}\right]^{b}\right]\right]\right]}$$

(3.I.4)

The rate at inflexion point does not represent an average rate, but the maximum rate at the inflexion point. In order to obtain a more representative average value, coagulation rates around the peak value (± 2.5 min around the inflection point, ~15-30 data points) were averaged. This value represents the slope of the linear region of the ODR curve around the inflexion point. The rate of ODR increases up to and diminishes beyond the inflexion point when visible clotting / coagulation begins to appear.

Experimental design

Five levels of pressure (200-400 MPa), temperature (3-21°C) and holding time (10-110 min) were selected using a central composite rotatable design, CCRD, (Box *et al.*, 1978) as shown in Table 3.I.1. This design was used in order to permit measurement of changes in coagulation properties, such as coagulation rate, lag time and inflexion time as a result of changes in the process variables from the asymmetrical sigmoid equation and an average rate of coagulation. Response surface methodology (RSM) is currently one of the most popular optimization techniques in the field of food science because of its comprehensive theory, reasonably high efficiency and simplicity (Arteaga *et al.*, 1994). The most common experimental design used in RSM is the central composite rotatable design (CCRD) that has equal predictability in all directions from the center. In addition, CCRDs are optimized designs for fitting quadratic models. The number of experimental points in the CCRD is sufficient to test statistical validity of the fitted model and lack-of-fit

of the model (Arteaga *et al.*, 1994). The central point in CCRD is replicated several times to estimate the error due to experimental or random variability.

Statistical analyses

The results obtained from the experiments were analyzed using the Statistical Analysis System (SAS, 1999) software. The RSREG procedures of the statistical analysis software system were used for testing significance and develop appropriate models for prediction of each response. Further, the model was refined using a stepwise regression procedure of SAS (1999) to eliminate the non-significant process variables. A general second-order polynomial given below was used to correlate the coagulation properties, to the process variables (pressure, temperature and time):

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} x_i x_j \qquad (i=1\sim3, j=1\sim3) \qquad (3.I.5)$$

The experimental factors and levels are shown in Table 3.I.1. The relationship between coded variables and real variables (Table 3.I.1) were as follows:

$$X_1=(P-300)*1.682/100; \quad X_2=(T-12)*1.682/9; \quad X_3=(t-60)*1.682/50 \qquad (3.I.6)$$

RESULTS AND DISCUSSION

Raw milk composition

In order to minimize the variation due to change in the composition of milk, the whole set of experiments was conducted within a short period of time (one week). Since test samples used in this experiment were from the pooled milk from the farm herd, day-to-day variation in the chemical composition of test samples was nominal as shown in Table 3.I.2 as means and standard deviations.

Regression models of responses

Figure 3.I.1 shows the typical coagulation curve (pressure treated sample), with points representing experimental data and the line representing the model (Equation 3.I.1)

prediction. The excellent overlap of model prediction with experimental data and smoothness of the curve demonstrates the model to be adequate in describing the coagulation behavior. The logistic sigmoid type model thus appears to be a good fit for the data. The model fitted curve had an R^2 value of 0.99. Figure 3.I.2 shows the first derivative curve of Equation 3.I.1 from which the inflexion time could be obtained. After fitting the coagulation model to experimental data, the different coefficients that characterize the model were obtained and the related coagulation parameters were computed. The model curve parameters, a-e (Equation 3.I.1) are summarized in Table 3.I.3 along with the associated R^2 values demonstrating a good fit in most cases.

As shown in Figure 3.I.1, the coagulation process started at about 6 min (lag time, t_{lag}, Equation 3.I.2) after rennet addition and reached the peak rate (slope) at the inflexion point (t_{max}, Equation 3.I.3; Figure 3.I.2) around 15 min. The maximum ODR (d) appears to be an equilibrium value of about 1.15 (ODR at infinite time). An average coagulation rate was computed from observed rates around the inflexion point ($\pm$2.5 min) to be 0.0036 min^{-1} while the peak rate was 0.0043 min^{-1}. The dynamics of aggregation of casein micelles (coagulation process) were evaluated by these three parameters, t_{lag}, t_{max} and the coagulation rate.

Values of the three coagulation process responses obtained under different experimental conditions are summarized in Table 3.I.4. The central point was replicated 5 times to obtain the associated standard error (coefficient of variation). A second order polynomial equation (Equation 3.I.5) was fitted to relate these parameters to experimental variables using RSREG and PROC REG procedures (stepwise) of SAS software. It was possible to develop a quadratic polynomial model for describing the effects of independent variables [pressure level (X_1), temperature (X_2) and holding time (X_3)] on each of the three milk coagulation parameters: lag time (Y1), time at inflexion point (Y2) and coagulation rate (Y3). The following were the polynomials showing the fitted response surfaces:

$$Y_1 = 8.32 - 0.27X_1 + 0.29X_2 - 0.17X_3 + 0.16X_1X_2 + 0.42X_1X_3 - 0.35X_1X_2X_3 \quad (R^2 = 0.88) \qquad \textbf{(3.I.7)}$$

$$Y_2 = 12.9 - 0.55X_1 + 0.65X_2 - 0.39X_3 + 0.23X_2^2 + 0.41X_3^2 + 0.44X_1X_3 \quad (3.I.8)$$

$$(R^2 = 0.84)$$

$$Y_3 = 34.6\times10^{-4} + 1.96\times10^{-4}X_1 - 1.75\times10^{-4}X_2 - 1.53\times10^{-4}X_1^2 - 4.66\times10^{-4}X_1X_2 \quad (R^2 = 0.79) \quad (3.I.9)$$

The associated R^2 values were reasonably high. The analysis of variance results indicating the model performance is summarized in Table 3.I.5 for each coagulation parameter. In each case the model was found to be significant ($p<0.01$). Further, the models for coagulation parameters were significantly ($p<0.05$) affected by combinations of linear, quadratic and cross product terms of process variables.

Effect of process variables on lag time and time at inflexion point

From the statistical analysis of variance (ANOVA) (Table 3.I.6), it was found that pressure and temperature had a significant ($p<0.05$) effect on lag time. In the original model, the holding time effect was not significant; however, in the stepwise PROCREG procedure of SAS, which eliminates non-significant parameters in a stepwise fashion, holding time was included as a significant process variable (Table 3.I.6 and Equation 3.I.8). It is also interesting to note that in the RSREG procedure (Table 3.I.6) although holding time alone was not significant, the interaction of time and pressure was highly significant ($p<0.01$). Similarly pressure and temperature were highly significant for time at inflexion point ($p<0.01$) whereas holding time was less significant ($p<0.05$). The quadratic term of holding time was highly significant ($p<0.01$) suggesting non-linear relationship. Surface and counter plot showing effect of different process variables on lag time taken two variables at a time by keeping third at middle level are shown in Figure 3.I.3. The lag time decreased as treatment temperature was lowered at any given pressure level (with a greater effect at higher pressure level), and also it decreased with an increase in treatment pressure (especially at the lower temperature). Thus, in general, higher pressure and lower temperatures had the potential to decrease the coagulation lag time. Overall, the lag time decreased from 8.7 min at 200 MPa and 21°C to 7.0 min at 400 MPa and 3°C, with a 60 min hold time (Figure 3.I.3a). The effect of pressure and holding time (Figure 3.I.3b) on

min hold time (Figure 3.I.3a). The effect of pressure and holding time (Figure 3.I.3b) on lag time showed some clear interaction effects. Combinations of low treatment pressures (200-250 MPa) and shorter holding times (up to 40 min), as well as higher pressure (400 MPa) - longer holding time (80-100 min) resulted in relatively longer (8.75- 9.25 min) lag times. The lag times were moderate at intermediate pressures and holding times. Combinations of lower pressure (200 MPa) - longer holding time (100 min) and higher pressure (400 MPa) - shorter holding time (20 min) yielded lower lag times. The effect of holding time and temperature while keeping pressure at the middle level (300 MPa) is shown in Figure 3.I.3c. The surface plot demonstrates only minor, but consistent, changes in lag time as influenced by temperature and hold time at the mid pressure level of 300 MPa. There was a small decrease in lag time with an increase in holding time as well as a decrease in temperature. Shortest lag time occurred following longest treatment at the lowest temperature. Interaction between holding time and temperature was not significant. The effect of process variables on the time at inflexion point is shown in Figure 3.I.4, again as before, taking two variables at one time while keeping the third one at the mid level. With holding time fixed at 60 min, an increase in treatment pressure and a decrease in temperature resulted in a reduction in the inflexion time from 16 min to about 12 min (Figure 3.I.4a). A low pressure - high temperature combination had a relatively longer inflexion time as compared with a high pressure - low temperature combination treatment. Effect of holding time and pressure at the middle temperature of at 12°C is shown in Figure 3.I.4b demonstrating a wide range of treatment combinations resulting in low inflexion times. Longer inflexion times were apparent at low-pressure levels and short holding times. As the pressure levels and treatment times increased, there was a progressive reduction in the inflexion time. At 400 MPa, the inflexion time was relatively low even up to a relatively long holding time of 80 min. A short treatment time of 20 min decreased the inflexion time from 17 min at 200 MPa to 13 min at 400 MPa. Figure 3.I.4c shows the effect of holding time and temperature by keeping pressure at middle level. A short treatment time (20 min) at 20°C (and 300 MPa) resulted in an inflexion time of 16 min. Treatment at 12°C for 60-80 min resulted in lowering the inflexion time to 12 min. The treatment effect on inflexion time appears to be more sensitive at the higher

temperature. The temperature effects were more pronounced when changed from high temperature-low holding time to low temperature moderate holding time (50-60 min).

Effect of process variables on coagulation rate

From the statistical analysis of variance (ANOVA) of RSREG procedure, it was found that all process variables (pressure, temperature and holding time) and their interactions (except time and temp) had significant effects on the coagulation rate (Table 3.I.6). Figure 3.I.5 shows the response surface and contour plots of the combined effect of different processing variables taken two at a time by keeping the third at the middle level (level 0, Table 3.I.1). Figure 3.I.5a shows the effect of pressure and temperature on coagulation rate by keeping holding time constant at the middle level (60 min). It is evident from the figure that the changes in coagulation rate are antagonistic at the two extreme ends of pressure level (lower and high end). The change in temperature at lower pressure level (200 MPa) from 3 to 21°C increased the coagulation rate from 0.0020 to 0.0035 min^{-1}; however, the effect was reversed at higher pressure level, i.e., the rate dropped from 0.0045 min^{-1} to 0.0020 min^{-1}. Changing pressure from 200 to 400 MPa at lower temperature (3°C) increased the rate from 0.0020–0.0045 min^{-1}. The effect of pressure and holding time is shown in Figure 3.I.5b. The effect of these two variables by keeping temperature at the middle level resulted in a marginal change in the coagulation rate (Figure 3.I.5a). Further, Figure 3.I.5a demonstrates that between 12 and 15°C, the effect of pressure is very marginal. By changing pressure from 200 to 400 MPa at the lower holding time changed coagulation rate from 0.0026 to 0.0032 min^{-1} at 3°C. Holding time alone was significant at $p<0.1$ level; however, its square term was more significant ($p<0.05$). Figure 3.I.5c shows the effect of holding time and temperature on coagulation rate. The effect of temperature at lower holding time was less pronounced than at a higher holding time. The coagulation rate changed from 0.0028 to 0.0032 min^{-1} by changing the temperature from 21 to 3°C at 10 min holding time, and from 0.0030 to 0.0036 min^{-1} at 100 min hold time (Figure 3.I.5c contour plot).

The coagulation of milk by rennet is an essential step during cheese-making and is the result of two processes, the proteolysis of κ-casein by the rennet enzyme leading to

destabilization of the casein micelles, and aggregation of the destabilized micelles leading to the formation of a gel. After coagulation, the firmness of the gel increases for several hours (Zoon *et al.*, 1988; Lopez, *et al.*, 1998). Milk coagulation process is governed by the stability of casein, mineral balances (especially calcium) as well as the pH. Calcium in milk occurs is in a variety of forms; of the 32 mM calcium in skim milk, 22 mM is in colloidal state and 10 mM is soluble. Only 3 mM of this soluble calcium is actually free Ca^{++}. The rest is complexed with citrate, phosphate or serum proteins (Bloomfield *et al.*, 1973,1975; McMohan *et al.*, 1984). In case of the phosphate ions, 19 mM are in the colloidal state, 5 mM exist as free inorganic phosphate and the remaining 6 mM are bound with calcium in the milk serum (Bloomfield *et al.*, 1975). The pressure-induced dissociation of the colloidal calcium phosphate in milk and milk products may change their technological properties (Schrader *et al.*, 1997). Alteration of Ca^{++} activity affects the enzymatic coagulation of milk. Acidification of milk or pressure treatment of milk causes reduction in colloidal calcium phosphate concentration and reduction of enzymic coagulation time (reduction in lag time and inflexion time, and an increase in coagulation rate). In both cases, it is due to an increase in Ca^{++} activity (Shalabi and Fox, 1982; Schrader *et al.*, 1997). High pressure processing is also known to have an effect on casein micelles. Further, it has been found that the disintegration of micellar casein due to pressure treatment at lower temperature is much stronger and quicker (Schrader *et al.*, 1997) as the low temperature weakens the hydrophobic interactions that maintain the casein in the colloidal state. Due to increase in the surface area, not enough κ-casein may be available to surround the micelles and protect them from further aggregation and hence the rennet action may coagulate the milk faster. This argument is supported by López-Fandiño, *et al.* (1997) suggesting that pressure treatment of milk favored the coagulation process not by enhancing κ-casein hydrolysis, but by promoting the aggregation. The enhancement in the enzymatic coagulation characteristics by HP treatment of milk can be explained by its above-mentioned effect on mineral balance and modification of casein micelles (Schrader *et al.*, 1997). In general HP treatment of milk enhanced the coagulation characteristics of milk.

Canonical analysis

The stationary points were obtained by canonical analysis (SAS, 1999) and response surface around these stationary points were evaluated. It was found that the stationary point was a saddle point for lag time and coagulation rate whereas minimum for the inflexion time. The predicted value at stationary point for lag time was 7.7 min (with associated inflexion time 12.1 min and coagulation rate 0.0037 min^{-1}); for inflexion time, 11.7 min (with an associated lag time 7.3 min and coagulation rate 0.0043 min^{-1}) and for mean coagulation rate, 0.0034 min^{-1} (with associated lag time 8.4 min and inflexion time 13.0 min).

Localization of conditions

The coordinates of local minima in terms of the processing variables were determined by differentiating Equation 3.I.7 for lag time, Equation 3.I.8 for inflexion time and Equation 3.I.9 for coagulation to X_1, X_2 and X_3, and setting the result thus obtained equal to zero, according to the following equations:

$$\left(\frac{\partial t_{tlag}}{\partial X_1}\right)_{X_2,X_3} = 0 \qquad (3.I.10)$$

$$\left(\frac{\partial t_{t\max}}{\partial X_2}\right)_{X_1,X_3} = 0 \qquad (3.I.11)$$

$$\left(\frac{\partial t_{coag.rate}}{\partial X_3}\right)_{X_1,X_2} = 0 \qquad (3.I.12)$$

The conditions obtained at saddle point for lag time were X1=296 MPa, X2=5°C and X3= 85 min; the conditions obtained for a minimum inflexion time were X1=370 MPa, X2= 6°C and X3= 50 min, and the conditions at saddle point for coagulation rate were X1=275 MPa, X2=14°C and X3=72 min. These points were located within the experimental range implying that the analytical techniques could be used to identify their minimal conditions. The principal effect of processing variables appears to be unidirectional; however, at

certain conditions the coagulation characteristics demonstrated conflicting interactions. In general, higher pressures and lower temperatures favorably improved the coagulation characteristics of milk. It may be necessary to make an acceptable compromise in all three coagulation characteristics for optimization of processing conditions. However, it should also be noted that the above general observations are only true for the region bound by the stationary point and the longer end of the process variables.

CONCLUSIONS

High pressure processing of milk significantly affected the coagulation characteristics of rennet treated milk. The coagulation parameters (coagulation rate, lag time and time at inflexion time) were influenced by the HP processing parameters. The parameters obtained from turbidity curve showed that higher-pressure levels and lower temperatures significantly decreased the lag time and inflexion time, and increased the coagulation rate. Processing at lower temperature may be promising in terms of improving cheese-making properties. The inflexion point of sigmoidal phase of coagulation curve can be correlated with actual cutting time. A multiple correlation model was developed for each coagulation parameter to relate them to the process variables (pressure, temperature and holding time). It is important to understand the effect of pressure processing of milk on coagulation characteristics in order to obtain good quality cheddar cheese. Improper coagulation and cutting time would lead to poor quality cheese and there will be higher economical loss due to drainage of curd fines with whey if curd is cut at inappropriate time. This would also further affect the final quality of cheese.

Table 3.I.1. Values of Independent variables and their levels

Independent variables	Symbol		Levels				
	Coded	Uncoded	-1.682	-1	0	1	1.682
Pressure (MPa)	X1	P	200	240.55	300	359.45	400
Temperature (°C)	X2	T	3	6.65	12	17.35	21
Holding time (min)	X3	t	10	30.27	60	89.73	110

Table 3.I.2. Proximate Composition of milk

Milk Constituents	% (w/w)*
Fat	3.36±0.065
Protein	3.15±0.036
Lactose	4.73±0.012
Total Solid	12.24±0.15
Water content	87.76±0.15

* Mean ±standard deviation

Table 3.I.3. Coagulation curve fit model parameters and associated correlation coefficient

Exp. no	Independent Variables			Coefficients of coagulation curve					Model
	X1	X2	X3	a	b	c	d	e	R^2
1	-1	-1	-1	1.00	10.04	12.12	1.19	0.067	0.999
2	-1	-1	1	1.00	7.21	10.41	1.17	0.055	1.000
3	-1	1	-1	1.00	7.23	12.55	1.18	0.071	0.990
4	-1	1	1	1.00	8.90	11.33	1.18	0.067	0.996
5	1	-1	-1	1.00	6.84	10.29	1.16	0.077	0.993
6	1	-1	1	1.00	9.56	11.27	1.17	0.091	0.998
7	1	1	-1	1.00	8.28	11.71	1.21	0.062	0.997
8	1	1	1	1.00	8.46	12.32	1.09	0.228	0.999
9	-1.682	0	0	1.00	7.04	11.46	1.40	0.017	0.994
10	1.682	0	0	1.00	9.94	9.60	1.19	0.055	0.993
11	0	1.682	0	1.00	9.35	9.82	1.21	0.049	0.995
12	0	1.682	0	1.00	7.40	12.83	1.11	0.153	0.996
13	0	0	1.682	1.00	7.63	11.94	1.19	0.058	0.998
14	0	0	1.682	1.00	7.58	10.81	1.19	0.065	0.998
15	0	0	0	1.00	9.39	10.67	1.19	0.068	0.998
16	0	0	0	1.00	10.11	10.56	1.20	0.065	0.991
17	0	0	0	1.00	9.96	10.70	1.19	0.066	0.985
18	0	0	0	1.00	8.86	10.72	1.19	0.066	0.997
19	0	0	0	1.00	8.50	10.89	1.19	0.065	0.998
20	0	0	0	1.00	8.46	10.97	1.18	0.076	0.995

Table 3.I.4. Rotatable central composite design arrangement and computed coagulation parameters.

Experiment	Variables			Responses		
	X1	X2	X3	Y1	Y2	Y3
1	-1	-1	-1	0.0026	9.44	14.34
2	-1	-1	1	0.0030	7.68	12.80
3	-1	1	-1	0.0034	8.90	15.27
4	-1	1	1	0.0034	8.64	13.56
5	1	-1	-1	0.0043	7.19	12.52
6	1	-1	1	0.0039	8.51	13.22
7	1	1	-1	0.0029	8.67	14.16
8	1	1	1	0.0028	8.72	13.76
9	-1.682	0	0	0.0027	8.85	14.56
10	1.682	0	0	0.0034	7.63	11.45
11	0	1.682	0	0.0036	7.72	11.84
12	0	1.682	0	0.0029	8.81	14.89
13	0	0	1.682	0.0029	8.81	14.58
14	0	0	1.682	0.0036	7.82	13.15
15	0	0	0	0.0035	8.17	12.69
16	0	0	0	0.0036	8.24	12.50
17	0	0	0	0.0038	8.36	12.68
18	0	0	0	0.0036	8.12	12.85
19	0	0	0	0.0036	8.16	13.10
20	0	0	0	0.0035	8.10	13.12

Coefficient of variation of coagulation rate (Y1) =5.08%

Coefficient of variation of lag time (Y2) =4.52%

Coefficient of variation of time at inflexion point (Y3) =4.69%

Table 3.I.5. Analysis of variance of regression models for Coagulation rate, Lag time and time at inflexion point

Source of Variance	DF	Coagulation rate			Lag time			Time at inflexion point		
		SS	MS	F-ratio	SS	MS	F-ratio	SS	MS	F-ratio
Model	9	3.5×10^{-6}	3.9×10^{-7}	12.25[a]	4.35	0.48	3.43[b]	21.43	2.38	6.25[a]
Linear	3	1.0×10^{-6}	3.4×10^{-7}	10.80[a]	2.47	0.83	5.84[b]	14.73	4.91	21.60[a]
Quadratic	3	6.1×10^{-7}	2.0×10^{-7}	8.80[a]	0.23	0.08	0.55	5.39	1.79	4.72[b]
Cross Product	3	1.86×10^{-6}	2.0×10^{-7}	19.52[a]	1.65	0.55	3.89	1.30	0.43	0.38
Residual	10	3.2×10^{-7}	3.1×10^{-8}		1.41	0.14		3.81	0.38	
Pure error	5	3.7×10^{-8}	7.53×10^{-9}		0.07	0.01		0.46	0.09	

[a]: $p<0.01$; [b]: $p<0.05$

Table 3.I.6. Analysis of variance of regression of process variables and their interactions for coagulation rate, lag time and time at inflexion point

Process Variables	DF	t-value		
		Coagulation rate	Lag time	Time at inflexion point
Pressure (X1)	1	4.06[a]	-2.61[b]	-3.82[a]
Temperature (X2)	1	-3.64[a]	2.83[b]	4.16[a]
Holding time (X3)	1	1.64[c]	-1.65	-2.60[b]
X1*X1	1	-4.64[a]	0.69	1.49
X2*X1	1	-7.39[a]	1.21	0.49
X2*X2	1	-1.98[c]	0.76	1.82[c]
X3*X1	1	-1.96[c]	3.19[a]	1.68
X3*X2	1	-0.36	0.22	-0.58
X3*X3	1	-2.33[b]	0.95	3.29[a]

a: $p<0.01$; b: $p<0.05$; c: $p<0.1$

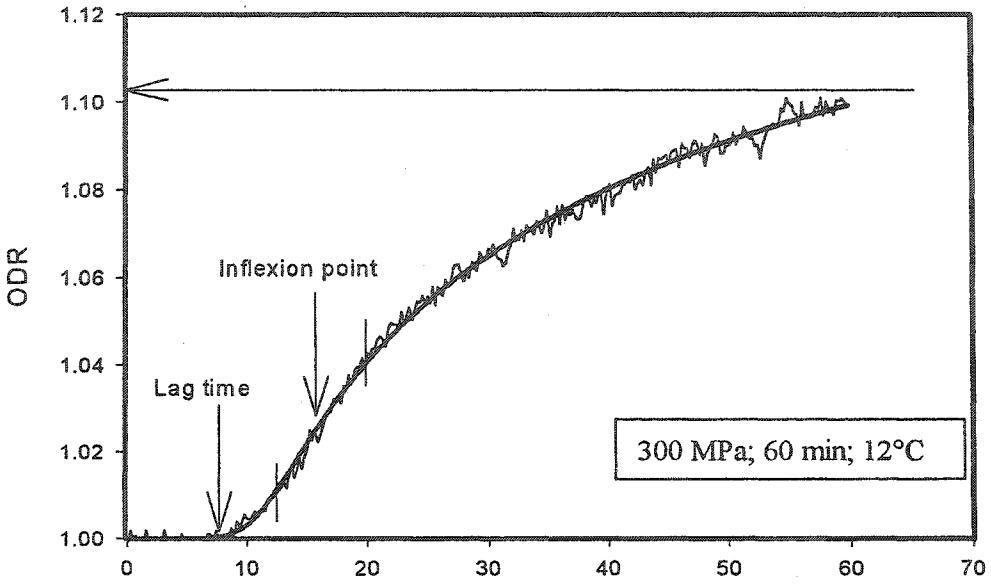

Figure 3.I.1. Typical coagulation curve of optical density ratio vs. time showing a good fit of selected model.

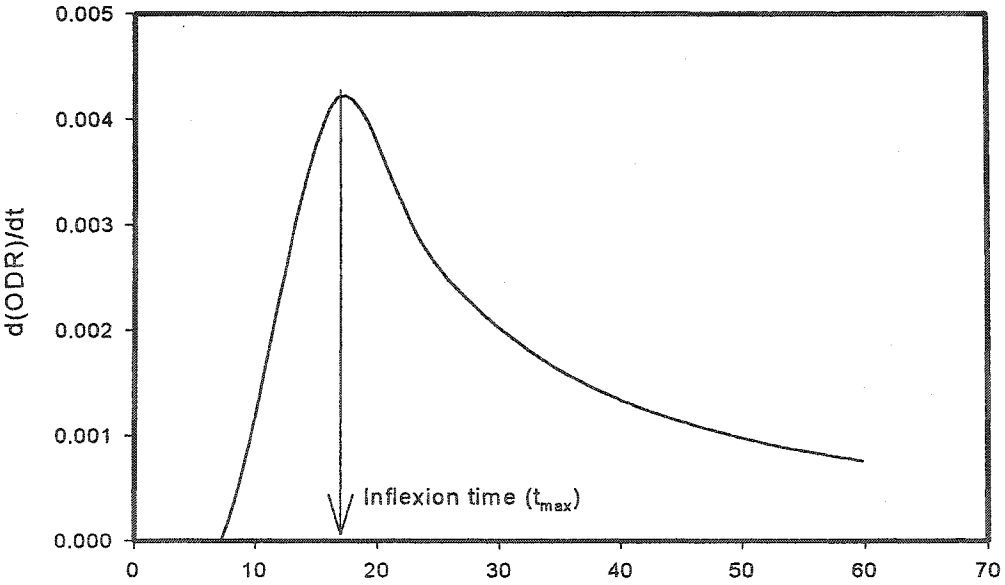

Figure 3.I.2. First derivative curve of coagulation data showing the inflexion time.

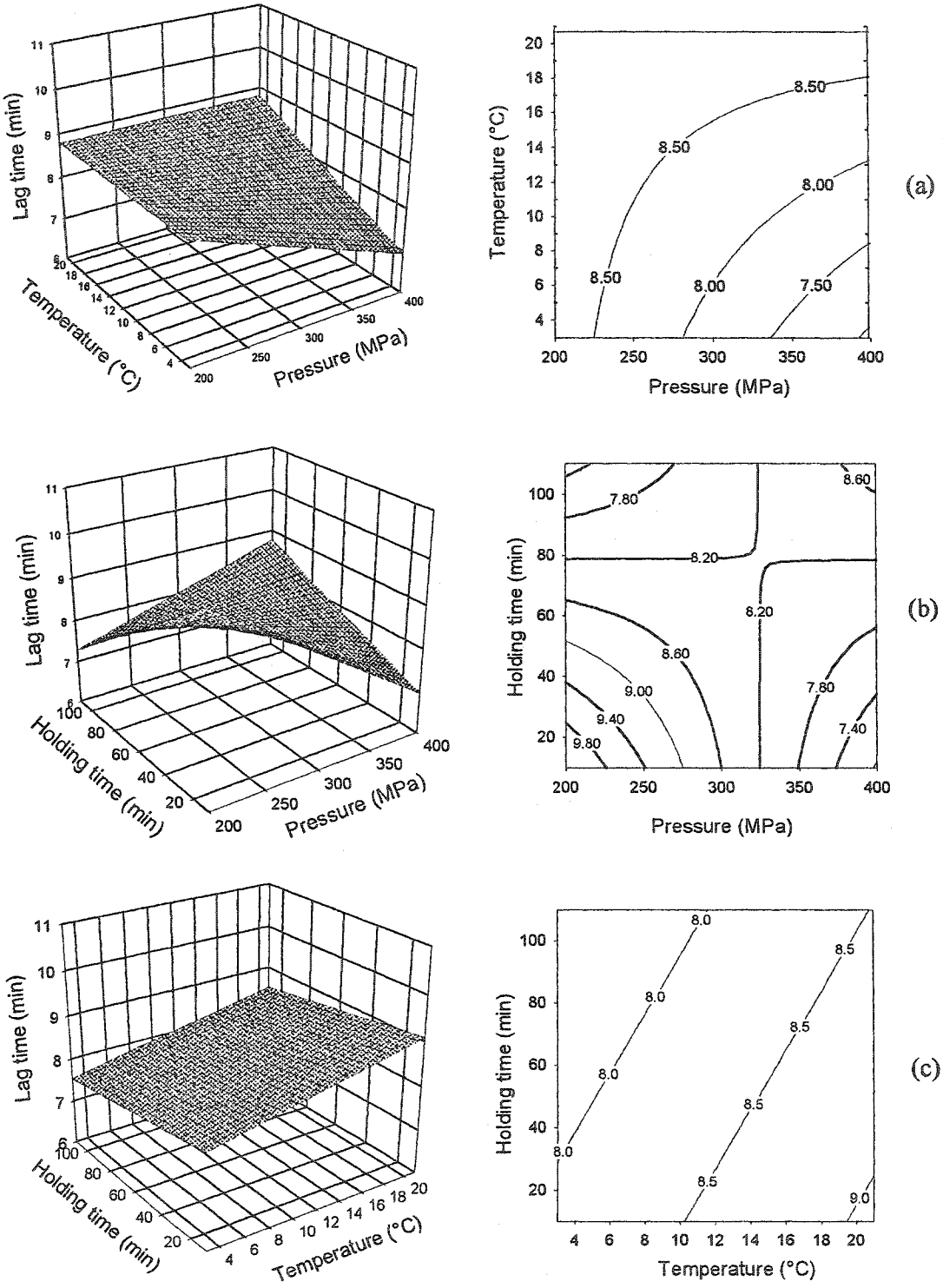

Figure 3.I.3. Surface and contour plots showing the effect of pressure process variables on Lag time (a) pressure and temperature (b) pressure and holding time (c) holding time and temperature

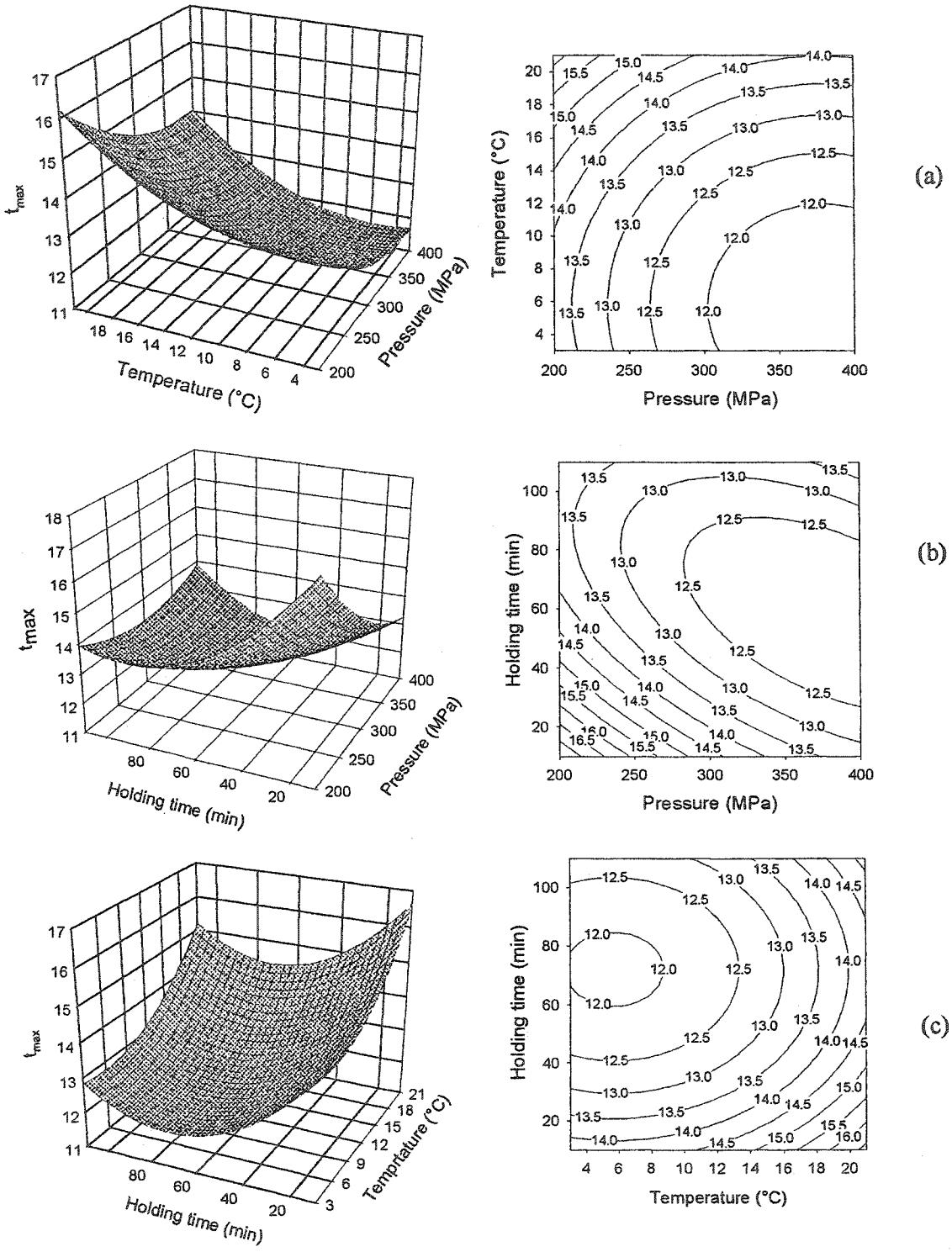

Figure 3.I.4. Surface and contour plots showing the effect of pressure processing on inflexion time (a) pressure and temperature (b) pressure and holding time (c) holding time and temperature.

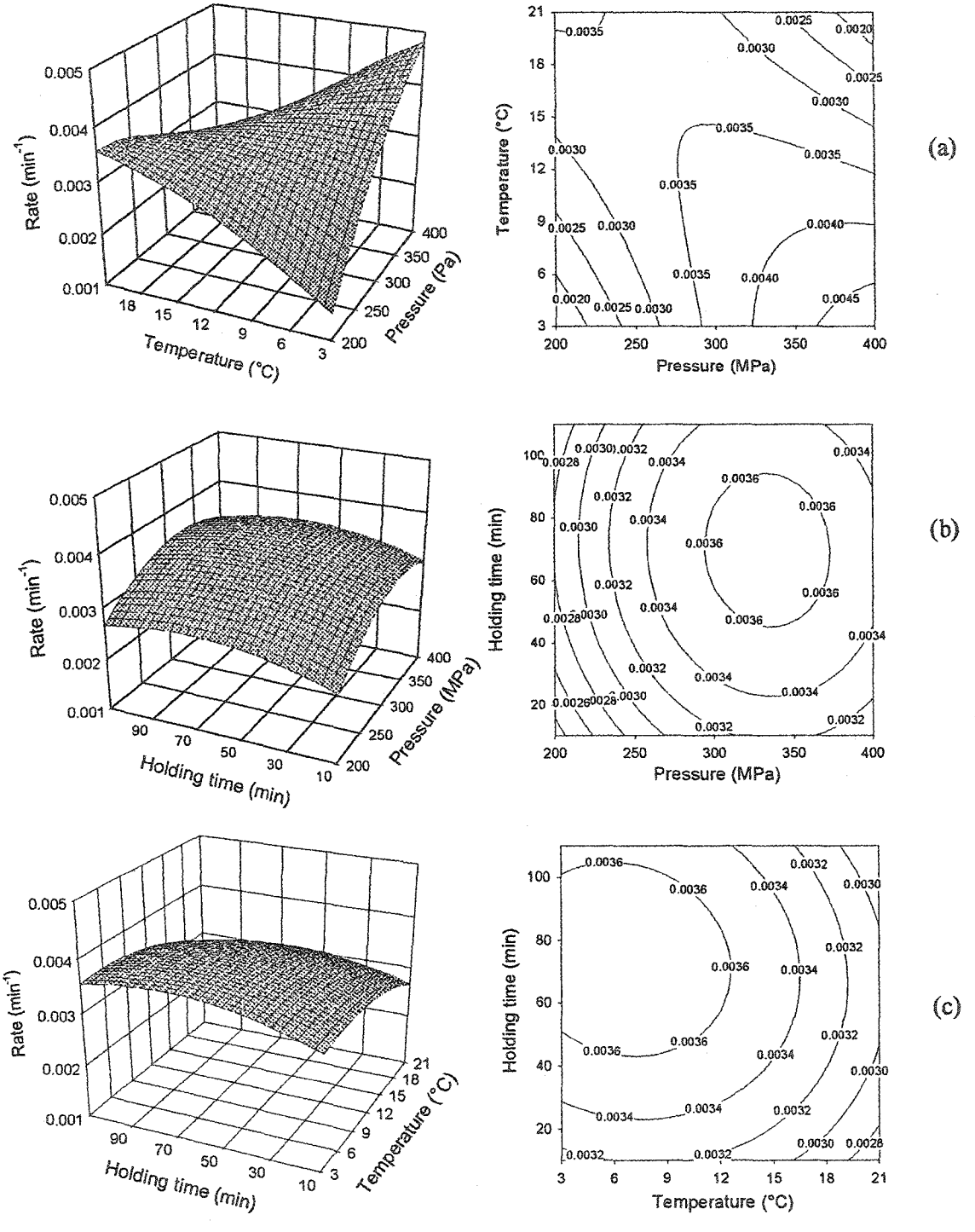

Figure 3.I.5. Surface and contour plots showing the effect of pressure variables on coagulation rate (a) pressure and temperature (b) pressure and holding time (c) holding time and temperature

PART-II

WATER-HOLDING CAPACITY AND GEL STRENGTH OF RENNET CURD AS AFFECTED BY HIGH-PRESSURE TREATMENT OF MILK

ABSTRACT

The effect of hydrostatic pressure (HP) treatment on two gel characteristics, water-holding capacity (WHC) and gel-strength (GS), of rennet curd was evaluated as a function of pressure level (200-400 MPa), temperature (3-21°C) and holding time (10-110 min) using a response surface methodology. A central composite design was used to investigate the effect of process variables and a second order multiple response model was used to relate the pressure, temperature or holding time to WHC and GS. In general, with a decrease in pressure level, temperature and holding time, there was a decrease in water-holding capacity and an increase in the gel-strength of the rennet curds. The conditions for minimum of WHC (40%) were: pressure, 280 MPa; temperature, 9°C, and holding time, 42 min which also resulted in a high gel strength of 0.47N slightly below the maximum of 0.52N.

INTRODUCTION

There is a considerable interest in high pressure processing of foods in recent years because of the progress in the engineering aspects of high pressure processing equipment allowing the technology to be adapted to the needs of food industry (Knorr, 1999). High-pressure treatment is being widely explored as a preservation technique and, although in developing stage, it demonstrates a great potential for making products with better functional properties and microbiological stability (Cheftel, 1992). High-pressure treatments have been found to have beneficial effects on milk, ranging from destruction of microorganisms (Hite, 1899 and Johnston, 1995) to desirable changes in functional properties (de la Fuente, 1998; Datta and Deeth, 1999). However, not all functional changes would be desirable in case of cheese making (Grappin and Beuvier, 1997; Lopez-Fandino *et al.*, 1996, 1997).

Milk proteins (β-lactoglobulin, and some casein fractions) are delicate structures, maintained by interactions within the protein chain determined by the amino acid sequence, and by interactions with the surrounding solvent. Changes in external factors, such as pressure and temperature, can perturb the subtle balance between inter-molecular and solvent-protein interactions, which can lead to unfolding / denaturation of the polypeptide chain. Further, the pressure-induced dissociation of the colloidal calcium phosphate in milk and milk products may change their technological properties (Schrader *et al.*, 1997). As a result, the functional properties of milk are altered, which in turn, affect the cheese making properties of milk (Lopez-Fandino *et al.*, 1997; Johnson *et al.*, 1990c; van Hooydonk *et al.*, 1987; Hermier and Cerf, 1986).

The gel characteristics of rennet curd, such as water-holding capacity and gel-strength, are important parameters in the cheese-making process and affect parameters such as yield, moisture content and textural attributes. Careful control of moisture content is needed to obtain desired characteristics in cheese. On the other hand, improper gel-strength of rennet curd could result in a higher loss of fat and curd fines. It is important in cheese-making to cut the curd at an appropriate firmness so that the whey drains properly while the loss of milk solids is minimized (Green, 1977). The actual rate of curd firming may not be important for determining the properties of the curd (Green, 1977), but its

control and contributing factors are important in cheese-making (McMahon and Brown, 1984). Variations in curd firmness at the time of cutting may result in losses of milk solids and reduce the cheese yield (McMahon and Brown, 1984). Monitoring of curd firmness during cheese-making thus offers a potential for reducing such losses by cutting it at a consistent curd-firmness to optimize cheese manufacturing conditions (McMahon and Brown, 1984). Similarly, the water-holding capacity of rennet curd plays a decisive role in the final moisture content of the cheese. Improper moisture content would result in poor body and textural properties, and at the same time influence the ripening characteristics of cheese (Vries, 1979).

The objectives of this research were, therefore, to investigate the effect of high pressure processing of milk on rennet curd characteristics, and to develop a response surface model for predicting optimized processing conditions for the cheese-making process.

MATERIALS AND METHODS

Details of the milk sample preparation and high pressure treatment are described in the previous section (Part I of Chapter III).

Water-holding capacity and gel strength measurement

Pressure treated milk samples were weighed (600 gms) into a beaker and placed in a water bath at 30°C and allowed to equilibrate. An active mesophilic lactic acid Group B starter culture, prepared from freeze dried culture of mixed strain (Institute Rossel Inc., St-Laurent, PQ) was added at 1% level to the milk. Incubation in the water bath was continued until the pH of the milk reached 6.55. A single strength rennet (Chymostar, Rhone-Poulenc, Marshall Products, Madison, WI) was added at this time at the rate of 200μL per 1L of milk. Immediately after addition of rennet, the test milk was distributed into 6 centrifuge bottles (Fisher Scientific Co., Pittsburg, PA), 30 g in each bottle, for measuring water-holding capacity. The remaining milk was poured into six flat bottom culture tubes (25mm diameter and 100 mm length, Fisher Scientific Co., Pittsburgh, PA)

for measurement of firmness of the curd. All samples (centrifuge bottles and culture tubes) were further incubated in the water bath at 30°C for 30 min to obtain the curd.

The water-holding capacity was determined by centrifuging the curd at 4500G for 10 min and measuring the exudate, and expressed as:

$$WHC = \left(\frac{M \times m_0}{100} - E\right)\left(\frac{100}{M - E}\right) \qquad \textbf{(3.II.1)}$$

where M = mass of curd before centrifugation (30g); E= mass of exudate and m_0 = percent initial moisture content of the curd (wet basis).

A Universal Testing Machine (Lloyd Model LRX, Fareham, Hans, U.K.) fitted with a 10 N load cell was used to evaluate the gel-strength (GS) of pressure treated rennet curd. The load cell was attached with a 20 mm diameter cylindrical flat bottom plunger. The instrument was programmed to move at 100 mm/min and the force required to break the gel was recorded. The gel strength was taken as the maximum force required to break the curd. Statistical analyses were carried out as detailed in the previous section.

RESULTS AND DISCUSSION

Regression models of responses

The mean values (n= 6) of the two responses (water holding capacity and gel strength) obtained under the different experimental conditions are summarized in Table 3.II.1. The variability associated with test samples was small as indicated by the coefficients of variation given in the parenthesis. A second order polynomial equation (3.II.2) was fitted to the experimental data (Table 3.II.1) using RSREG procedure of SAS (SAS, 1996). It was possible to develop a quadratic polynomial model for describing the effects of independent variables (pressure, temperature and holding time) on the two responses: water-holding capacity and gel strength. The following were the polynomials showing the fitted response surfaces:

$$\begin{aligned} WHC = {} & 44.193 + 4.097X_1 + 5.503X_2 + 3.519X_3 + 4.903X_1^2 + 4.379X_2^2 \\ & + 2.536X_3^2 + 1.062X_1X_3 \end{aligned} \qquad \textbf{(3.II.2)}$$

$$(R^2 = 0.98)$$

$$GS = 0.4135 - 0.03888X_1 - 0.024522X_2 - 0.0253X_3 - 0.0073X_1^2 + 0.00965X_2X_1 + 0.013X_2^2 + 0.0155X_3X_1 - 0.0088X_3X_2 + 0.0181X_3^2 \quad (R^2 = 0.98) \qquad \textbf{(3.II.3)}$$

Effect of Process variables on Water-holding capacity

The analysis of variance for the model for water-holding capacity (Table 3.II.2) indicated that the model was statistically significant ($p<0.01$) with an R^2=0.98. From the statistical analysis of variance (ANOVA), it was found that all process variables (pressure, temperature and holding time) had an effect on the water-holding capacity of rennet curd (Table 3.II.3). Figure 3.II.1 shows the effect of different processing variables taken two at a time by keeping the third at the middle level (level 0, Table 3.I.1). From Figure 3.II.1a, it can be seen that the WHC decreased as the treatment temperature decreases at each pressure level, and also it decreased as the treatment pressure decreases at each temperature, although a reverse trend was observed at the low ends of both temperature and pressure. Thus, in general, lower temperatures and lower pressures had the potential to decrease the water-holding capacity of the rennet gel. From the angle at which the 3-D figure is presented, the effect of pressure on lowering WHC appears to be considerably different at the two ends of the temperature range. At 20°C, the WHC decreased from about 82% to 70% (about 15% reduction), while at 3°C, the decrease was from 68% to 54% (about 14% reduction) as pressure changed from 400-200 MPa. Similarly, looking at the temperature effect, a decrease in temperature from 20°C to 3°C at 400 MPa resulted in decreasing of WHC from 82% to 68% (a 16% decrease) while that at 200 MPa range from 70% to 54% (a 22% decrease). Thus, lower temperature and lower pressures acted synergistically to reduce the WHC. Figure 3.II.1b shows the effect of pressure level and holding time at the middle temperature (12°C). Again, WHC decreased with a decrease in holding time and a decrease in pressure (with exception at the lower ends). The effect of pressure on lowering of WHC at the pressure holding time of 110 min varied from 78% to 65% (a 17% reduction) while after a short holding time of 10 min, it varied from 66% to 52% (a 21% decrease) as the pressure decreased from 400 to 200 MPa. With respect to a

given pressure, as the hold time decreased from 110 to 10 min, WHC decreased from 64% to 52% (a 19% decrease) at 200 MPa and 78% to 65% (a 17% decrease) at 400 MPa. The cumulative effect of low pressure and short pressure hold time on WHC reduction again is apparent. The effect of holding time and temperature is shown in Figure 3.II.1c indicating minimal values of WHC about the middle region of both temperature and holding time. With reference to individual effects, the temperature effect was similar to what was observed earlier, resulting in lowering of WHC from 68% to 50% (a 27% reduction) at 10 min hold time, and 80% to 61% (24% decrease) at 110 min hold time. At 20°C, varying hold time from 110 to 10 min decreased WHC from 80% to 68% (a 15% decrease) while a similar change at 3°C resulted in WHC to change from 62 to 50% (19% decrease). In all cases, a progressive decrease in WHC was observed due to combined action of parameters.

High pressure is known to destabilize non-covalent interactions, negative volume change associated with chemical bond breakage is enhanced by pressure (Pollard and Weller, 1966). As a consequence of pressure treatment, the globular protein is unfolded and may exhibit an increase in water-holding capacity. Globular proteins such as β-lactoglobulin display varying degrees of hydration, depending on denaturation, aggregation, and interaction with other proteins (De Wit, 1984; Kinsella, 1984). The tertiary structure in milk protein is a three-dimensional configuration as a consequence of non-covalent interactions between side chains of amino acids. The surface hydrophobicity has been found to increase following pressure release. The higher the pressure and the greater the treatment time, the higher the exposure (Johnston *et al.*, 1992a) of hydrophobic groups. The additional hydrophobic surface causes more water to assume a tightly packed structure. In addition, quaternary structure of multimeric globular proteins (such as β-lactoglobulin) held together by non-covalent bonds, are dissociated by the application of comparatively low pressures (>150 MPa) resulting in partial denaturation of protein (Rademacher *et al.*, 1997). The exposure of protein surface to solvent result in binding of water molecules (Hendrickx *et al.*, 1998). Changes in solvation volume are mainly caused by pressure-induced ionization changes in solvent, exposure of amino acid side-chains and peptide bonds available for interaction with water molecules, and diffusion

of water into cavities located in the hydrophobic core of the protein (Hendrickx *et al.*, 1998). Not much information is available regarding the effect of pressure treatment at low temperatures on milk proteins. However, in a study on the effect of cold pressure processing on proteins and enzymes, Howley (1971) reported higher degree of denaturation when the samples were pressure treated at lower temperature. It has been found that as a consequence of pressure treatment the casein micelles get further fragmented due to loss of calcium phosphate (Johnston *et al.*, 1992). Further, it has been found that the disintegration of micellar casein due to pressure treatment at lower temperature is much stronger and quicker (Schrader and Buchheim, 1997) as the hydrophobic interactions that maintain the casein in the colloidal state are weakened. The hydrophobic interaction was reported to be lower as result of decrease in the temperature (Privalov, 1990), thus exposing more hydrophobic groups that may result in lowering water-holding capacity. This is contrary to the above general statement explaining that the exposure of hydrophobic group enhances the water-binding at higher pressure; however, Low and Somero (1975) stated that the exposure of hydrophobic groups to water can lead to volume increase or decrease, depending on the types and concentrations of adjacent hydrophobic groups. Localized unfolding and sub-unit dissociation can result in the unmasking of buried groups that are able to pair with newly exposed groups. This process can lead to aggregation (Masson, 1992). Therefore, it can concluded that as a consequence of pressure treatment, a decrease in volume results from water-binding around charged groups, water structuring around newly solvent-exposed apolar groups (hydrophobic hydration), and solvation of polar groups through hydrogen bonding. At lower temperatures excessive exposure of hydrophobic group lead to further aggregation by pairing with another exposed group forming cluster resulting in lower water-holding capacity.

Effect of Process variables on Gel-Strength

The analysis of variance indicated the developed regression model (Table 3.II.2) was highly significant ($p<0.01$) with an $R^2=0.98$. Figure 3.II.2 shows the effect of different processing parameters on the gel-strength of rennet curd taking two at a time by keeping

the third parameter at the mid level. Figure 3.II.2a shows effect of pressure and temperature while keeping the holding time at 60 min. As pressure level and temperature increased, the gel strength of rennet curd decreased. The effect of pressure was more pronounced at lower temperatures and the effect of temperature was more pronounced at lower pressure. Interaction effect of pressure and temperature is also evident from Figure 3.II.2a. Temperature and pressure thus have a synergistic effect on the gel strength of the rennet curd. Figure 3.II.2b shows the effect of pressure and holding time at the mid temperature (12°C). The curd firmness was reduced with an increase in pressure. Increase in holding time decreased the gel-strength as well. The effect of pressure on change of gel-strength was more evident at lower holding time. Similarly, Figure 3.II.2c shows the effect of temperature and holding time at the mid level of pressure and demonstrated a more pronounced effect. With an increase in holding time or temperature the gel-strength showed a decreasing trend. The temperature effect was highly prominent at longest holding time. The combination effect of high temperature and long holding time was detrimental to the gel strength. An almost comparable high gel-strength of 0.52N was obtained by combination of 10 or 110 min hold at 3°C and 10 min hold at 21°C at an operating pressure of 300 MPa.

In general, with a decrease in pressure level, temperature or holding time, there was an increase in gel-strength of rennet curds and a decrease in water-holding capacity. As pressure level or holding time increases, the hydration capacity of the gel network increases resulting in increased binding of water, effectively increasing the WHC and decreasing the GS. Also higher degree of whey protein denaturation and exposure of more hydrophobic groups at lower temperatures (Privalov, 1990) may increase the severity of pressure effect resulting in association and aggregation (with the conversion of protein bound water into highly compressed free water) within the protein molecules (Cheftel and Dumay, 1998) than binding with water. These conditions thus favor lower WHC and GS.

Localization of minimal conditions

The stationary points were obtained by canonical analysis (SAS, 1985) and response surface around these stationary points were evaluated. It was found that

stationary point was a minimum for WHC and a saddle point for GS. The predicted value at stationary point for WHC is 40% (with an associated GS of 0.47N) and for GS is 0.39N (with an associated WHC of 69%).

The coordinates of local minima in terms of the processing variables were determined by differentiating equation (3.II.3) for WHC and equation (3.II.4) for GS independently with respect to X_1, X_2 and X_3, and setting the result thus obtained equal to zero, according to the following equations:

$$\left(\frac{\partial WHC}{\partial X_1}\right)_{X_2,X_3} = 0 \qquad \textbf{(3.II.4)}$$

$$\left(\frac{\partial WHC}{\partial X_2}\right)_{X_1,X_3} = 0 \qquad \textbf{(3.II.5)}$$

$$\left(\frac{\partial WHC}{\partial X_3}\right)_{X_1,X_2} = 0 \qquad \textbf{(3.II.6)}$$

For a minimum WHC, the conditions obtained were: X_1=280 MPa, X_2=9°C, and X_3=40 min (Eqs. 6, 7 and 8) and the values of processing variables at saddle point for GS were X_1=280 MPa, X_2=20°C and X_3=100 min. These points were located within the experimental range implying that the analytical techniques could be used to identify their minimal conditions. The principal effect of processing variables appear to be opposite with respect to the two parameters studied- WHC increasing and GS decreasing at higher pressures, holding times and temperatures. Interestingly, however, the lower value of WHC and higher value in GS would be desirable in cheese-making, therefore, lower values of process variables would be more desirable. It may be necessary to make an acceptable compromise in WHC and GS for optimization of processing conditions. However, it should also be noted that the above general observations are only true for the region bound by the stationary point and the longer end of the process variables. Within the interval between the stationary point and the other end levels of these variables, the specific trends were obviously opposite to the trends described above. For example, within temperature

region 3 to 9°C, WHC decreases as temperature increases, between 200 and 280 MPa, decreases with an increase in pressure, and for the holding time within range of 10-40 min, decreases with an increase in time. Similarly, within the pressure range 200-280 MPa, the gel strength increases with an increase in pressure. Interestingly though, with respect to gel strength, the stationary point with respect to holding time was 100 min, close to the maximum of 110 min used, and with respect to temperature, it was indeed the highest temperature used. Hence, these effects were almost unidirectional.

CONCLUSIONS

A response surface method was used to evaluate the effects of HP treatment variables-pressure level, pressurization temperature and holding time- of milk on waterholding capacity and gel strength of rennet curds. Predictive models for WHC, and GS were developed as functions of pressure temperature and holding time. In general, with a decrease in pressure level, temperature and holding time, there was a decrease in waterholding capacity and an increase in gel strength of rennet curds. The pressure processing conditions yielding an optimum (minimum) WHC were: pressure, 280 MPa; temperature 9°C, and holding time 40 min and for GS the stationary point was a saddle point at pressure 280 MPa; temperature, 20°C, and holding time 100 min. The predicted values for WHC and GS under the optimal conditions were 40% and 0.47N respectively. The resulting GS was slightly lower than the maximum 0.52N obtained in the study.

The coagulation properties affected by HP treatment of milk was discussed in chapter-III part I. Parameters obtained from turbidity curve showed that higher-pressure levels and lower temperatures significantly decreased the lag and inflexion times, and increased the coagulation rate. Processing at lower temperature may be promising in terms of improving cheese-making properties. The gel characteristics of rennet curd affected by HP treatment of milk presented in this part suggest that a compromise of some cheese making properties may be required, as all parameters can't be optimized simultaneously. It should also be recognized that in addition to optimizing cheese making properties, the design of the process for safe cheese manufacturing should consider microbial destruction kinetics of pathogen concerned.

Table 3.II.1 Rotatable Central Composite Design arrangement and responses

Experiment	Variables			Responses	
	X1	X2	X3	Y1*	Y2*
1	-1	-1	-1	45.9(1.8)	0.55(5.9)
2	-1	-1	1	49.4(2.2)	0.49(1.4)
3	-1	1	-1	54.8(3.4)	0.50(3.2)
4	-1	1	1	60.4(2.3)	0.40(3.4)
5	1	-1	-1	50.8(2.3)	0.41(2.7)
6	1	-1	1	60.4(2.6)	0.41(2.7)
7	1	1	-1	61.3(2.2)	0.40(3.2)
8	1	1	1	69.3(1.8)	0.36(7.5)
9	-1.682	0	0	49.9(0.7)	0.44(3.9)
10	1.682	0	0	64.7(2.8)	0.34(5.7)
11	0	-1.682	0	45.2(1.6)	0.49(2.7)
12	0	1.682	0	66.5(1.1)	0.40(4.1)
13	0	0	-1.682	44.3(3.5)	0.50(2.5)
14	0	0	1.682	56.9(1.5)	0.42(5.1)
15	0	0	0	44.8(3.5)	0.42(9.6)
16	0	0	0	44.4(4.7)	0.41(3.2)
17	0	0	0	43.7(2.8)	0.40(8.0)
18	0	0	0	44.8(1.5)	0.42(4.7)
19	0	0	0	44.3(1.6)	0.41(3.3)
20	0	0	0	43.6(1.5)	0.42(5.3)

*Mean and (coefficient of variation, %); Y1 = WHC; Y2 = GS

Table 3.II.2. Analysis of variance of regression models for Water-holding capacity (WHC) and Gel strength (GS)

Source of Variance	DF	WHC			GS		
		SS	MS	F Ratio	SS	MS	F Ratio
Model	9	1430.56	158.951	94.22[a]	0.0490	0.00545	47.169[a]
Linear	3	812.00	270.668	160.40[a]	0.0376	0.01255	108.600[a]
Quadratic	3	609.45	203.150	120.40[a]	0.0081	0.00271	23.469[a]
Cross Product	3	9.11	3.036	1.8	0.0032	0.00109	9.453
Residual	10	16.87	1.686		0.0011	0.00012	
Pure error	5	1.43	0.286		0.0003	0.00006	

a: $p<0.01$; b: $p<0.05$

Table 3.II.3 Analysis of variance for the overall effect of the Independent variables and their interaction on Water-holding capacity and Gel strength of rennet curd

Process Variables	DF	WHC			GS		
		SS	MS	F Ratio	SS	MS	F Ratio
Pressure (X1)	4	585.01	146.25	86.96[a]	0.02400	0.00601	52.06[a]
Temperature (X2)	4	690.19	172.54	102.30[a]	0.01200	0.00302	26.14[a]
Holding time (X3)	4	270.97	67.74	40.16[a]	0.01610	0.00401	34.75[a]
X1*X2	1	1.16	1.16	0.69	0.00070	0.00070	6.45[b]
X1*X3	1	9.03	9.03	6.39[b]	0.00019	0.00019	16.52[a]
X2*X3	1	1.76	1.76	1.05	0.00062	0.00062	5.39[b]

a: $p<0.01$; b: $p<0.05$

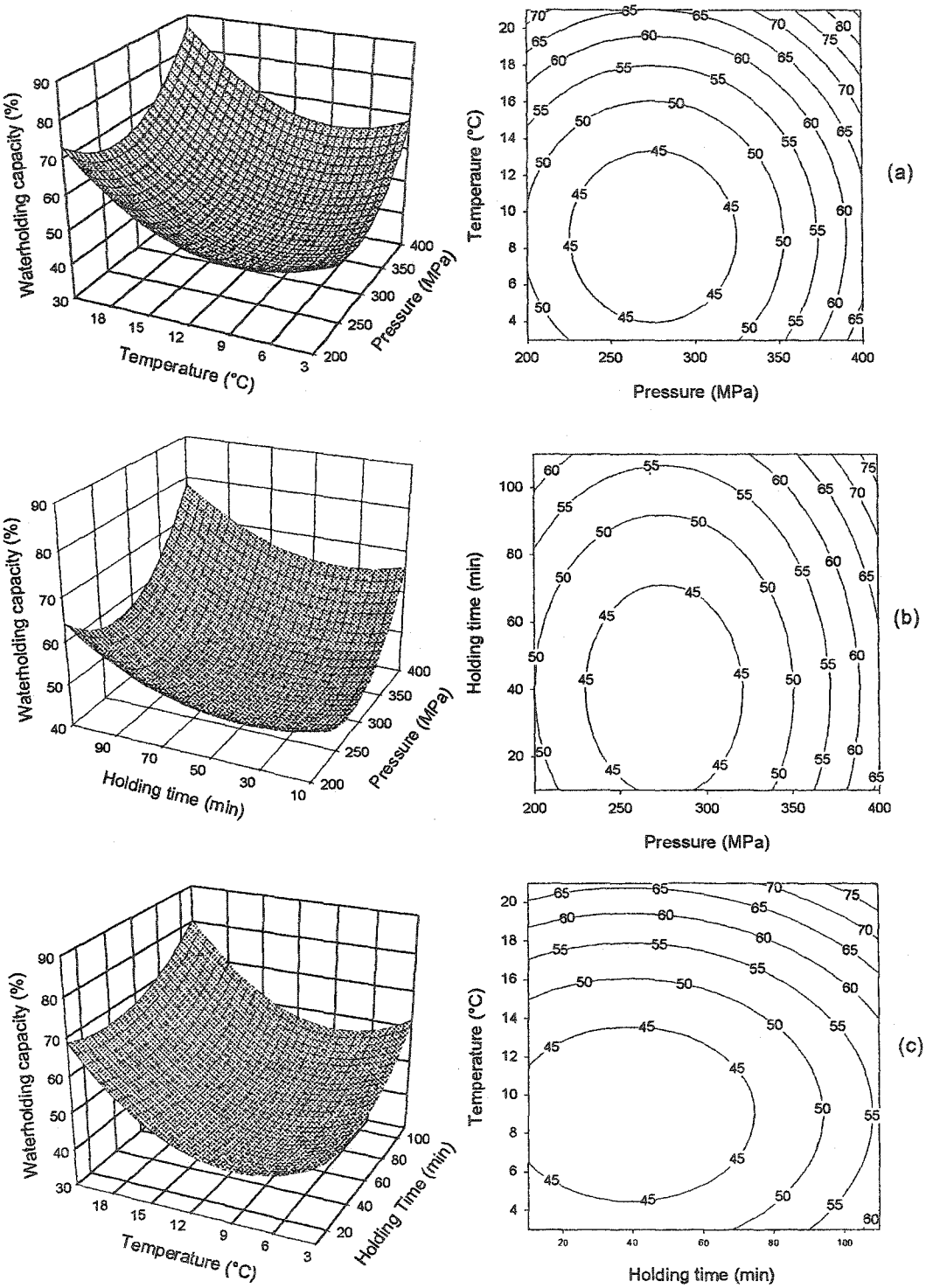

Figure 3.II.1. Surface and contour plots showing the effect of pressure process variables on waterholding capacity (a) Pressure and temperature (b) Pressure and holding time (c) Holding time and temperature

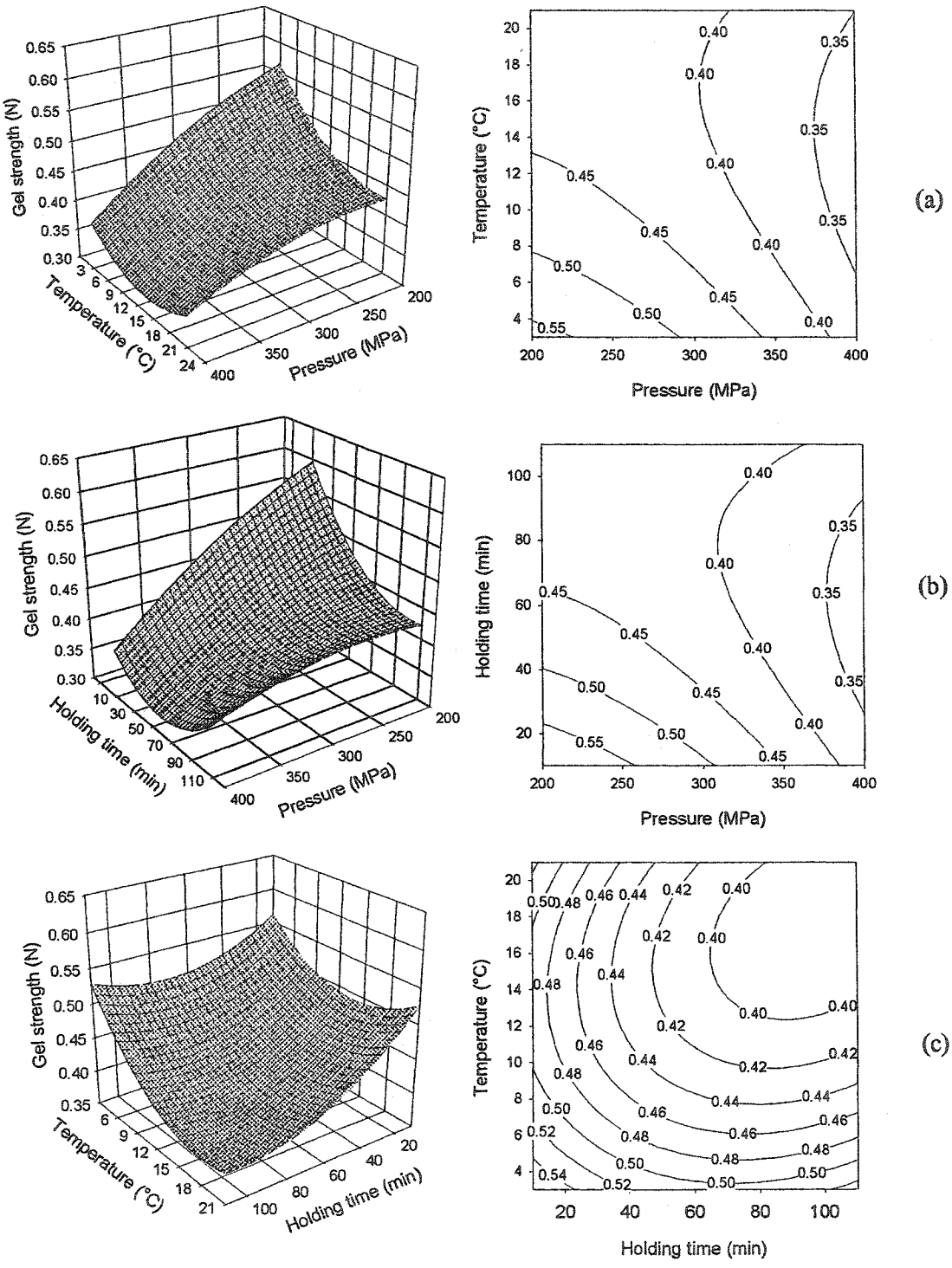

Figure 3.II.2 Surface and contour plots showing the effect of pressure variables on Gel strength of rennet curd (a) Pressure and temperature (b) Pressure and holding time (c) Holding time and temperature

CHAPTER IV

HIGH PRESSURE DESTRUCTION KINETICS OF ESCHERICHIA COLI AND NATURAL MICRO-FLORA IN MILK

CONNECTING STATEMENT

This chapter highlights the effect of high pressure on the destruction kinetics of *Escherichia coli* K-12 (ATCC-29055) and natural microflora of milk at selected operating temperature and pressure holding time. This was evaluated to see if *E. coli*, a contaminant bacteria is more pressure resistant than the natural spoilage bacteria or the common pathogen *Listeria monocytogenes* (HP destruction kinetics of *Listeria monocytogenes* in milk work has been reported from this lab [**Mussa**, D.M. Ramaswamy, H.S. Smith, J.P. *1998* High pressure (HP) destruction kinetics of Listeria monocytogenes Scott A in raw milk *Food Research International.* 31 (5): 343-350].

Some parts of this chapter have been prepared for publication/presented:

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Pandey, P.K., H.S. Ramaswamy and E. Idziak. Kinetics of high-pressure destruction of *E. coli* and indigenous microorganism in milk at two temperatures. Presented at IFT annual meeting at Anaheim, California 2002 June 15-19.

All the experimental work and data analysis were carried out by the candidate under the supervision of Dr. Ramaswamy and Dr. E. Idziak (Professor of Microbiology, Department of Natural Resource Sciences, McGill University).

HIGH PRESSURE DESTRUCTION KINETICS OF ESCHERICHIA COLI AND NATURAL MICRO-FLORA IN MILK

ABSTRACT

Raw milk with a high count of indigenous micro-flora, with and without inoculated *Escherichia coli* K-12 (ATCC-29055), was subjected to high pressure treatment (250-400 MPa) for various holding times (0-80 min) at two temperatures (3 and 21°C). From the microbial survival data, an instantaneous pressure kill value (IPK) that represented the effect of a pressure pulse, and kinetic parameters (rate constant, D-value, z- value and activation volume) were evaluated based on pressure hold-time first order kinetics. Both IPK and D values were dependent on pressure level and temperature. Higher pressures, longer holding times and lower temperatures resulted in larger destruction of microorganisms, and *E. coli* was more pressure sensitive than indigenous micro-flora. Pressure dependence of kinetic parameters was well described by both pressure death time and Arrhenius models. At 3 and 21°C, the z_p values were 227 and 240 MPa and $\Delta V^{\neq}$ values were –2.32 and –2.34 ($\times 10^{-5}$ m^3 $mole^{-1}$), respectively for indigenous micro-flora, and 179 and 205 MPa (z_p) and –2.95 and –2.75 ($\times 10^{-5}$ m^3 $mole^{-1}$) ($\Delta V^{\neq}$), respectively, for *E. coli*.

INTRODUCTION

High pressure (HP) treatment is a physical process that is believed to cause selective destruction of microorganisms (including pathogens) in milk while protecting several key enzymes that are important in cheese ripening (Cheftel, 1992; Earnshaw, 1992; Hayashi, 1989; Rademacher and Kesseler, 1997). The applied pressure is uniform and instantaneous throughout the food sample without any gradients (Hoover *et al.*, 1989). The high-pressure process is expected to accelerate reactions that result in a volume decrease, while those reactions involving a volume increase are inhibited (Erying, 1942). High pressure processing has certain advantages over traditional food processing methods: they require no preservative, can lead to development of novel texture, can be used to treat solid (cheese) or very viscous (honey) foods and can be effective at low temperatures (freezing and thawing applications) or moderately high temperatures without damaging the flavor (Mertens, 1993). Hite (1899) explored the possibility of using high pressure to extend the shelf life of milk. Until recently, this technology had not gained momentum due to non-availability of equipment for commercial production. With an increase in consumer awareness to use fresh and minimally processed foods, there has been increased interest in industrial processing of foods using high pressure.

A considerable amount of cheese is produced from non-pasteurized milk in many countries. The production of safe raw milk cheese depends on the successful inactivation of pathogens in milk without affecting other quality parameters, which are responsible for developing the strong characteristic flavor in raw milk cheeses. High-pressure treatment of milk prior to its use in the production of raw milk cheese will have the potential to eliminate pathogens without extensive inactivation of milk enzymes responsible for development of the characteristic cheese flavor. In a previous study (Pandey *et al.*, 2000), the effect of high pressure treatment of milk on water holding capacity and gel strength of the rennet curd was evaluated for identifying pressure processing conditions which provide better conditions for cheese making. Similar studies have also been carried out by López-Fandiño *et al.* (1997) and Buffa, *et al.* (2001a, b).

Successful application of HP processing of milk for cheese making relies on the effective destruction of naturally occurring pathogens and contaminant microorganisms.

Several studies have been carried out on high-pressure destruction kinetics of microorganisms in milk (Rademacher and Kessler, 1997; Mussa *et al.*, 1998; Patterson and Kilpatrick, 1998; Gervilla *et al.*, 1999). In the context of high pressure processing, Mussa *et al.* (1998) studied destruction of *Listeria monocytogenes* and indigenous microorganisms present in milk. They found the pathogen to be more pressure resistant than indigenous microorganisms and recommended using the destruction of *Listeria monocytogenes* as the criterion for milk pasteurization. The present study deals with the kinetics of pressure destruction of a contaminant microorganism (*Escherichia coli*) in raw milk and compares its pressure resistance with that of the indigenous microorganisms.

The objective was to study the effect of different treatment conditions (pressure time and temperature) on the destruction of *E. coli* and indigenous micro-flora in milk and develop a high-pressure process for milk to produce a microbiologically safe cheese.

MATERIALS AND METHODS

Inoculum preparation

Freeze dried culture of *E. coli* K-12 (ATCC-29055) in thermosealed vials obtained from the American Type Culture Collection (ATTC, Rockville, MD) was used in this study. The culture was maintained at 4°C until used. The dried culture was rehydrated in 10 mL brain heart infusion (BHI) broth (Difco laboratories Inc., Detroit MI) at 37°C for 24h. Subsequently, 1 mL of this rehydrated culture was transferred into fresh 50 mL BHI broth and incubated at 37°C for 24 h. Three such transfers were made in succession to obtain the stock culture with viable counts of 10^8 cfu mL^{-1}.

Inoculation, milk sample preparation and packaging

Raw milk was obtained from the Macdonald campus (McGill University) dairy farm. One portion of the milk was transferred into a sterile glass bottle and incubated at 30°C for 8 h to increase the population of natural micro-flora in milk from about 10^2-10^3 cfu mL^{-1} to about 10^6 cfu mL^{-1}. The other portion of raw milk was stored at 4°C until next day and inoculated with 1 mL *E. coli* cfu mL^{-1} stock culture per 99 mL milk in order to obtain approximately 10^6 cfu mL^{-1} *E. coli* in raw milk. All milk samples were transferred to

dual peel sterilization pouches (Nasco Plastic, New Hamburg, ON) and sealed. Each pouch contained about 10 mL test sample of raw milk (with indigenous micro-flora with and without *E coli* K-12 of about 10^6 cfu mL^{-1}) before pressure treatment. An untreated control was kept in each case to evaluate the initial count.

Pressure Treatment

The experimental setup used to carry out the high-pressure treatment is described earlier (Part I of Chapter III). The samples were subjected to pressure treatments at 250-400 MPa for various times (0 to 80 min) as detailed in Table 4.1. The destruction kinetics of natural micro-flora and *E. coli* were studied at two temperatures 21 and 3°C. After pressure treatment, the samples were stored at 4°C until the enrichment stage was started. In order to achieve and maintain the target processing temperatures during test run, the samples were equilibrated to about 3-5°C below the desired processing temperature prior to test run and the temperature of the water circulated was also kept slightly below this level. For the lower temperature test run, some crushed ice was added to the medium to provide the heat sink. Temperatures monitored during selected test runs indicated average bath temperatures to be within $\pm$2°C of the set point. Following treatment, test pouches were removed from the chamber and processed for microbial enumeration.

Recovery of injured cells

The treated samples were subjected to two methods of enrichment for *E. coli*. The first method involved holding treated sample at room temperature for 4 h followed by a pour plate method using Violet Red Bile (VRB) Agar (Difco Laboratories Inc., Detroit MI). The second method involved spreading on BHI agar to allow injured cells to recover (4 h at room temperature), followed by overlaying with VRB agar There was not much difference between the counts from these two methods. Considering the ease of handling, the pour plate method was subsequently adopted. Control samples were always stored at 4°C until used for microbial assay. Enrichment process for the natural micro-flora were not required as the media employed - plate count agar (PCA; Difco Laboratories Inc., Detroit

MI) – provided the enrichment nutrition. However, the 4 h holding at room temperature was also adopted to these samples to allow for recovery of any injured cells.

Microbiological assay

Subsequent to pressure treatment and enrichment process, each sample was enumerated. A serial dilution technique using sterile 0.1% peptone water was employed and VRB agar was used as the selective medium for *E. coli* in both treated and control samples (Gervilla *et al.*, 1999). The plates were incubated at 37°C for 24 h. Purple colonies were counted as those representing *E. coli*. Plate count agar was used to enumerate the surviving natural micro-flora. These plates were incubated at 30°C for 48h.

Kinetic data analysis

There were small day-to-day variations in initial counts (control) of test microorganisms. For easy comparison of treatment effects, a normalization procedure was used to express the surviving micro-flora as a fraction of their initial counts. Each survival count was divided by the initial count (untreated control ~10^6 cfu mL^{-1}) and the result was multiplied by 10^6 to get the 10^6 cfu mL^{-1} as the nominal initial count (control). Such normalization would not affect any of the evaluated kinetic parameters; however makes the survivor plots easier to compare between the treatments (Mussa, 1999)

Pressure destruction of microorganisms was analyzed as a dual effect: an instantaneous change in concentration due to pressurization/depressurization (constituting a pressure pulse), followed by a first order rate of destruction during the pressure hold time. The change in activity due to the pressure pulse was defined as an instantaneous pressure kill value (IPK) and was obtained by subtracting the survival count after one pulse from the initial count (Basak and Ramaswamy, 1996).

The pressure destruction of microorganisms during pressure-hold time was assumed to follow a first order kinetic process expressed as:

$$Log_e\left(\frac{N}{N_0}\times 10^6\right) = -kt \qquad (4.1)$$

where, N = number of surviving microorganism in treated sample at time t; N_0 = initial number of microorganisms (untreated sample); and k= reaction rate constant (min^{-1}). The reaction rate constant was obtained from the linear regression of $\log_e (N/N_0 \times 10^6)$ vs. t as the negative slope.

$$k = -(\text{slope}) \qquad (4.2)$$

The IPK value can also be obtained from the regression intercept of first order rate model (Basak and Ramaswamy, 1996). The D-value or decimal reduction time (time required for 90% destruction of microorganisms from initial population was expressed as:

$$D = 2.303/k \qquad (4.3)$$

The pressure sensitivity of the kinetic parameters was analyzed by an Arrhenius approach and a pressure death time (PDT) similar to TDT (thermal death time). In the Arrhenius type the activation volume ($\Delta V^{\neq}$) which is measure of net pressure effect at constant temperature was obtained by plotting $\log_e$ k values against pressure:

$$\Delta V^{\neq} = -RT\left[\frac{\Delta(\log_e k)}{\Delta P}\right]_T \text{ or } -RT\,(\text{slope}) \qquad (4.4)$$

where ($\Delta V^{\neq}$) is the activation volume in (m^3 $mole^{-1}$), P is the pressure in MPa, k is the rate constant (min^{-1}), T is the absolute temperature (°K), R is the gas constant (8.314×10^{-6} m^3 $mole^{-1}$ MPa $°K^{-1}$) (Erying and Magee, 1942).

The other pressure sensitivity parameter relates the D-values to pressure. From the regression of $\log_{10}$ (D) vs. pressure data, the pressure z-value (z_p) was determined as the negative reciprocal of the slope:

$$z_p = \left(\frac{\Delta P}{\Delta Log(D)}\right) \text{ or } z_p = -\left(\frac{1}{\text{slope}}\right) \qquad (4.5)$$

The z_p is defined as the pressure range between which the decimal reduction time is changed by a factor of 10.

Statistical analysis

All experiments (Table 4.1) were replicated three times and each dilution was plated in duplicate. An analysis of variance (ANOVA) was performed using GLM procedure of Statistical Analysis System (SAS; SAS Institute, NC). Duncan grouping test was performed to compare significance among group means. The evaluation of differences between variables was tested at a 5% significance level.

RESULTS AND DISCUSSION

Indigenous micro-flora

Instantaneous pressure kill (IPK) value

Survivor curves of indigenous micro-flora as affected by pressure and holding time at two temperatures (21°C and 3°C) are shown in Figure 4.1. A careful analysis of survivor curves shows that the destruction of microorganisms by high-pressure treatment did follow the first order rate kinetic model; however, only after an initial instantaneous drop (at time zero, due to a pulse effect of pressure, a phenomenon that was more apparent at higher pressures). Thus, the pressure destruction of microorganism followed a bi-phasic, dual destruction behavior. This concept was initially observed by Hayakawa *et al.* (1994), but mostly quantified in later studies (Basak and Ramaswamy, 1996; Mussa *et al.*, 1998). The instantaneous pressure kill (IPK) value can be experimentally measured by subjecting the sample to a quick pressure pulse or obtained from the intercept of regression of logarithmic survivor vs. pressure hold time (Table 4.2). It is evident that the experimental and regression values of IPK parameters are comparable. In a similar study by Mussa *et al.* (1998) with *Listeria monocytogenes* and indigenous micro-flora, the IPK value for indigenous micro-flora in milk was reported to be slightly higher at the higher pressure levels (300-400 MPa), but close to the observed values at 250 MPa in this study. In general, the IPK value increased with an increase in pressure level. At a given pressure level, there were small differences in the IPK values between the two temperatures. Figure 4.2 shows IPK as a function of pressure and temperature.

Pressure Destruction Kinetics

Pressure destruction of indigenous micro-flora in milk (Figure 4.1) essentially demonstrated a first order rate of destruction following IPK. The lower temperature (3°C) was included because cheese-making properties of milk were found to be improved when the milk was treated at low temperature in previous studies (Pandey *et al.*, 2000).

Pressure destruction of indigenous micro-flora was highly dependent on the magnitude of pressure and holding time. Table 4.3 shows destruction kinetics parameters (D and k values) at 3 and 21°C, and at different pressure levels. The linearity of the curves (Figure 4.1) and the associated high R^2 values (Table 4.3) indicate the appropriateness of the first order model for the pressure hold destruction of microorganisms. As expected, D values decreased (and k values increased) with an increase in pressure level. The associated D values at 21°C were consistently higher than the corresponding D values at 3°C at each pressure level indicating a slightly improved destruction at the lower temperature. While the difference in D values at the two temperatures might be small, when process establishments involving multiple decimal reductions are required, the use of lower temperature will offer some time savings. For example, a 6D reduction at 400 MPa requires about 50 min treatment time at 21°C as compared with 40 min at 3°C.

Figure 4.3 shows the decimal reduction time curve for the indigenous microorganisms as a function of pressure at the two temperatures. The computed pressure sensitivity values (z_p) at the two temperatures are also shown in Table 4.3 indicating small differences in the associated z_p (214 and 227 MPa at 21°C and 3°C, respectively). The associated activation volumes were -2.34 and -2.32 x 10^{-5} m^3 $mole^{-1}$, respectively. Statistically, the D values associated with different pressure levels were significantly different ($p<0.05$) (decreasing with an increase in pressure) from each other at both temperatures. Further, D values at 250 and 400 MPa between the two temperatures were significantly different ($p<0.05$) while those at 300 and 350 MPa were not significantly different ($p>0.05$). Overall, the temperature effect was significant ($p<0.05$) with the lower temperature demonstrating a lower D value.

Two parameters can be derived to compare the pressure pulse with pressure hold results. IPK describes the pressure pulse effect while the D value indicates a measure of the pressure hold effect. Since IPK represents a logarithmic reduction in microbial population due to a single pulse, its equivalence of D value can be established in terms of the number of pressure pulses (N_D) required to result in one decimal reduction in microbial population. Alternately, the D value equivalence of an IPK can be computed as number (or fraction) of decimal reductions (D_{IPK}) achieved by one pressure pulse, which is simply the reciprocal of IPK. These values are also shown in Table 4.2. These two numbers can be meaningful in assessing the importance of pressure pulse vs. pressure hold approach for microbial destruction. For example, N_D at 400 MPa and 21°C is about 1, meaning a single pulse is adequate to achieve one decimal reduction. Six pulses would result in a 6D process, and assuming a pressure pulse cycle to last 2-3 min, this would require 12-18 min. Based on the D value of 8.26 min at 400 MPa, this would require a considerably longer (50 min) process demonstrating that the pressure pulse technique would be time-effective. The D_{IPK} under this condition is about 9 min indicating that a single pulse will do an equivalent job of 9 min holding time, better than the D value. Thus, if the D value is smaller than D_{IPK}, the pulse approach would be favorable while the opposite will favor a hold approach. However, the number of pulses required to do the job also should be taken in to consideration. If more than a couple of pulses are required, there could be significant cost factor resulting from the rapid wear and tear of the HP equipment.

E. coli

In the present study the effect of high-pressure treatment on inactivation kinetics of *E. coli* K-12 (ATCC-29055) at two temperatures was evaluated in context of safe cheese making. Although the presence of *E. coli* (non pathogenic) in milk and milk products is not necessarily life threatening, it is indicative of inadequate processing. The pressure resistance of *E. coli* was evaluated in this study to test whether it can be used as indicator microorganism. Although the presence or absence of *E. coli* in milk may only show the effectiveness of plant hygiene, if it can be used as indicator against a pathogen, the evaluation process could be lot easier to handle. A secondary objective of this study was to

compare the pressure destruction kinetics of *E. coli* with that of Listeria *monocytogenes* that has been reported previously (Mussa *et al.*, 1998).

Instantaneous Pressure Kill (IPK)

The instantaneous pressure kill value (IPK), calculated as described earlier, for *E. coli* is also included in Table 4.2. The IPK values at different pressures (250-400 MPa) and two temperatures (3 and 21°C) were significantly ($p<0.01$) affected by pressure level, but temperature effect was not significant. Figure 4.4 shows a response surface plot of the effect of pressure and temperature on IPK demonstrating the greater dependence of IPK on pressure level than temperature. Some interaction effect is also evident showing an increase in temperature effect on IPK at lower pressures and a decreasing temperature effect at higher pressures. The IPK values for *E. coli* were comparable to the IPK for the indigenous micro-flora (Figure 4.2)

Pressure Destruction Kinetics

As with the indigenous microorganisms, the pressure destruction of *E. coli* followed first order kinetics, after the IPK (Figure 4.5), both at 3°C and 21°C. The kinetic data computed from the survivor curves also included in Table 4.3 indicated the D-value of *E. coli* to vary from 4 min to 32 min in the pressure range 400-250 MPa. The associated D values were lower at the lower temperature, more significantly so ($p<0.05$) at the higher pressure of 400 MPa. Figure 4.6 shows the decimal reduction time as a function of pressure for *E. coli* at two temperatures. As with the indigenous microorganisms (Figure 4.3), the D value curve at 21°C was above that at 3°C. The associated pressure-z values (z_p) computed from the kinetic data (Table 4.3) for both *E coli* and indigenous micro-flora were lower at the lower temperatures indicating a slightly higher pressure sensitivity. Likewise, the z_p values for the *E. coli* were lower than those associated with the indigenous micro-flora, indicating the destruction of *E. coli* to be more pressure sensitive. The other parameter, which also indicates the pressure sensitivity, is activation volume change ($\Delta V^{\#}$) and is also computed from the same kinetic data (ln k vs. pressure) (Table 3). As can be expected, the $\Delta V^{\#}$ values were more negative at for *E. coli* and at lower

temperatures, again indicative of greater pressure effect. Gerville *et al.* (1999) reported the inactivation of *E. coli* (405 CECT) in milk by high pressure (250 and 300 MPa) at two temperatures (at 2°C and 25°C). They reported much lower D-values, 5.2-6.8 min, compared to this study. Further, they reported the temperature effect to be opposite with slightly lower D values at the higher temperature. The difference can be ascribed to the differences in the strain, as well as the fact that they did not account for IPK. However, the same authors reported a higher rate of destruction at lower temperature for *P. fluorescens.* Patterson and Kilpatrick (1998) studied the effect of pressure and temperature on pathogens in milk and reported enhancement of lethal effect when the treatment was carried out at sub ambient temperatures. Takahashi (1992) reported that pressure treatment at –20°C was more lethal to bacteria and yeast than at 20°C. Eza (1989) and Privalov (1990) postulated that the lethal effect at low temperature can be due to the enhancement of denaturation of different proteins at sub-ambient temperatures. It can also be argued that higher pressures and lower temperatures act to push toward reducing the reaction volume and therefore should be expected to have enhanced effects as the pressure is increased and temperature is lowered. Exceptions may occur when temperatures are in the lethal region (i.e. at elevated temperatures).

In some previous studies (Mussa, 1999; Mussa *et al.*, 1998), destruction kinetics of *Listeria monocytogenes* was extensively studied in milk and pork. At 350 MPa, a D-value of 10 min was reported for *L. monocytogenes* and 8 min for indigenous micro-flora indicating *L. monocytogenes* to be more pressure resistant than indigenous micro-flora in milk. The D values for indigenous micro-flora observed in this study were slightly higher (Table 4.3), probably due to differences in the chemical composition and type of micro-flora present in milk; however, the overall pressure sensitivity of the microorganisms were in the same range (z_p value: 244 vs. 240 MPa).

Process Development

The process development for safe and high quality cheese requires careful consideration of several changes taking place in cheese milk as a result of high-pressure treatment. Over processing may render the milk to be safe (free from pathogens) but may

lead to drastic changes in the functional properties of milk, ultimately affecting the cheese making properties and overall quality of cheese. Based on the available kinetic data, the process should be based on the destruction of *L. monocytogenes,* as it appears to be the most resistant pathogen among those studied. Levels of security might dictate the use of a 4-6D process with respect to the above pathogen.

Previous studies (Pandey *et al.*, 2000) on the effect of high-pressure treatment on cheese making properties of milk indicated 280 MPa for 40 min to be optimal for developing favourable water holding capacity and gel strength characteristics. This study also revealed that the principal process variables had somewhat opposite effects with respect to water holding capacity and gel strength of the rennet curd, with water holding capacity increasing and gel strength decreasing at higher pressures, longer holding times and higher temperatures. Interestingly, for cheese making, lower water holding capacity and higher gel strength is desirable. Hence, lower pressures and lower temperatures would be more desirable. However, for microbial destruction, lower pressures would necessitate longer holding times. This calls for a compromise between the different influential factors.

Since the process conditions can have considerable effects on residual enzyme activity and hence cheese ripening behavior, it is suggested that such a study on cheese be evaluated at two different levels of pressure and application time. To study the kinetics of enzyme inactivation, it was considered desirable to use a 4 and 6 D process with respect to *L. monocytogenes* and carry out the pressure treatment at 300 MPa and 400 MPa. Treatment times of 60 and 90 min were chosen at 300 MPa (D = 15 min) to provide 4 D and 6D processes and at 400 MPa, the treatment times were set at 32 and 48 min (D = 8 min). A 2-3°C treatment temperature was recommended.

CONCLUSIONS

Pressure destruction kinetics of indigenous micro-flora and *E. coli* in raw milk were studied for 250-400 MPa pressure treatments with holding times of 0 to 80 min at two temperatures, 3 and 21°C. Results demonstrated and confirmed the dual effect pressure destruction of microorganisms with an instantaneous destruction effect attributable to the pressure pulse and a subsequent first order rate destruction (during pressure hold) with characteristic kinetic parameters such as D and k values. Pressure dependence of kinetic parameters was well described by both pressure death time and Arrhenius models. Higher pressures, longer holding times and lower temperatures resulted in larger destruction of microorganisms, and *E. coli* was more pressure sensitive than indigenous micro-flora. It should be noted that a pressure hold process includes the effect of a pressure pulse effect, although multiple pulses can be given without any holding time. It is desirable to quantify the pressure pulse effect when its contribution is significant.

Table 4.1 Pressure levels and holding times used for HP treatment of milk containing indigenous micro-flora and *E. coli*.

Pressure (MPa)	Processing time (min)				
250	0	20	40	60	80
300	0	15	30	45	60
350	0	10	20	30	45
400	0	5	10	15	20

Table 4.2. Instantaneous pressure kill (IPK) and related parameters of pressure destruction indigenous micro-flora and *E. coli* in milk.

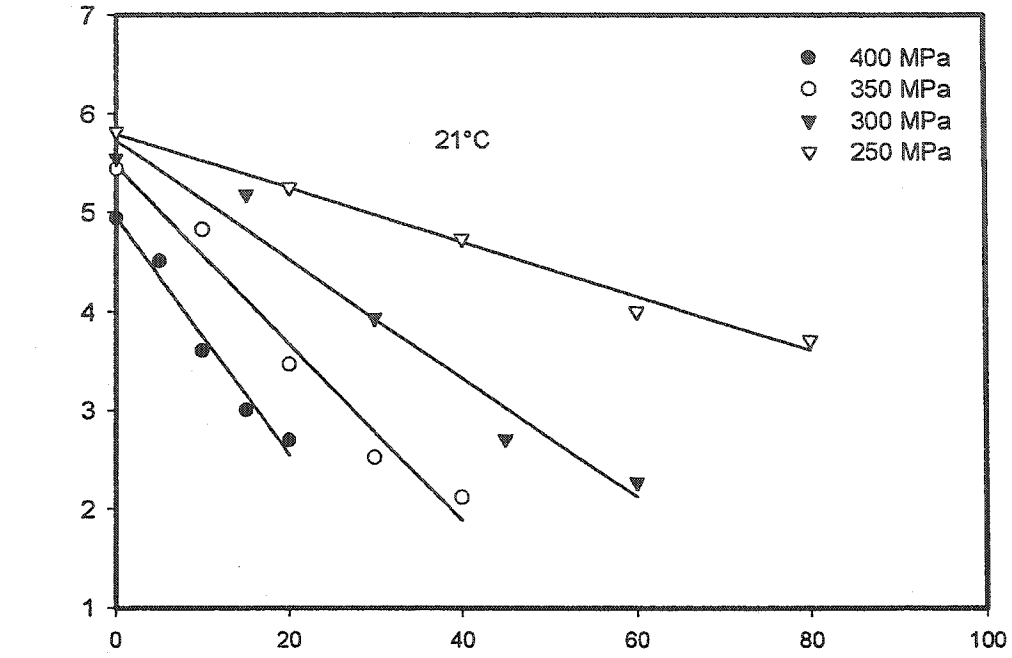
Microflora in milk	Temp. of treatment	Pressure (MPa)	IPK (log)		N_D		D_{IPK}		R^2
			Exp.	Reg.	Exp.	Reg	Exp	Reg	
Natural microflora (total count)	21°C	250	0.18^{d}	0.20^{d}	5.56^{a}	5.00^{a}	6.48^{b}	7.20^{b}	0.99
		300	0.30^{c}	0.25^{c}	3.33^{b}	4.00^{b}	4.89^{d}	4.08^{d}	0.98
		350	0.56^{b}	0.53^{b}	1.80^{c}	1.89^{c}	6.31^{c}	6.01^{c}	0.98
		400	1.05^{a}	1.02^{a}	0.95^{d}	0.96^{d}	8.68^{a}	8.60^{a}	0.99
	3°C	250	0.18^{d}	0.16^{d}	5.56^{a}	6.25^{a}	5.63^{b}	5.01^{d}	0.99
		300	0.35^{c}	0.42^{c}	2.86^{b}	2.35^{b}	5.10^{c}	6.21^{b}	0.97
		350	0.68^{b}	0.64^{b}	1.47^{c}	1.56^{c}	6.75^{b}	6.36^{a}	0.99
		400	0.90^{a}	0.81^{a}	1.11^{d}	1.23^{d}	5.96^{a}	5.36^{c}	0.97
E. coli	21°C	250	0.32^{d}	0.29^{d}	3.13^{a}	3.45^{a}	10.41^{a}	9.43^{a}	0.99
		300	0.44^{c}	0.42^{c}	2.27^{b}	2.38^{b}	6.98^{b}	6.66^{b}	0.99
		350	0.66^{b}	0.62^{b}	1.52^{c}	1.61^{c}	6.18^{c}	5.81^{c}	0.98
		400	0.81^{a}	0.69^{a}	1.23^{d}	1.45^{d}	4.83^{d}	4.11^{d}	0.98
	3°C	250	0.19^{d}	0.24^{d}	5.26^{a}	4.17^{a}	5.75^{c}	3.94^{c}	0.99
		300	0.44^{c}	0.30^{c}	2.27^{b}	3.33^{b}	6.45^{a}	4.40^{b}	0.99
		350	0.75^{b}	0.62^{b}	1.33^{c}	1.61^{c}	6.26^{b}	4.51^{a}	0.99
		400	1.00^{a}	0.93^{a}	1.00^{d}	1.08^{d}	4.29^{d}	3.99^{c}	0.99

Superscript letters a, b, c, d showing significant difference of IPK parameters within different pressure at a given temperature and microorganisms

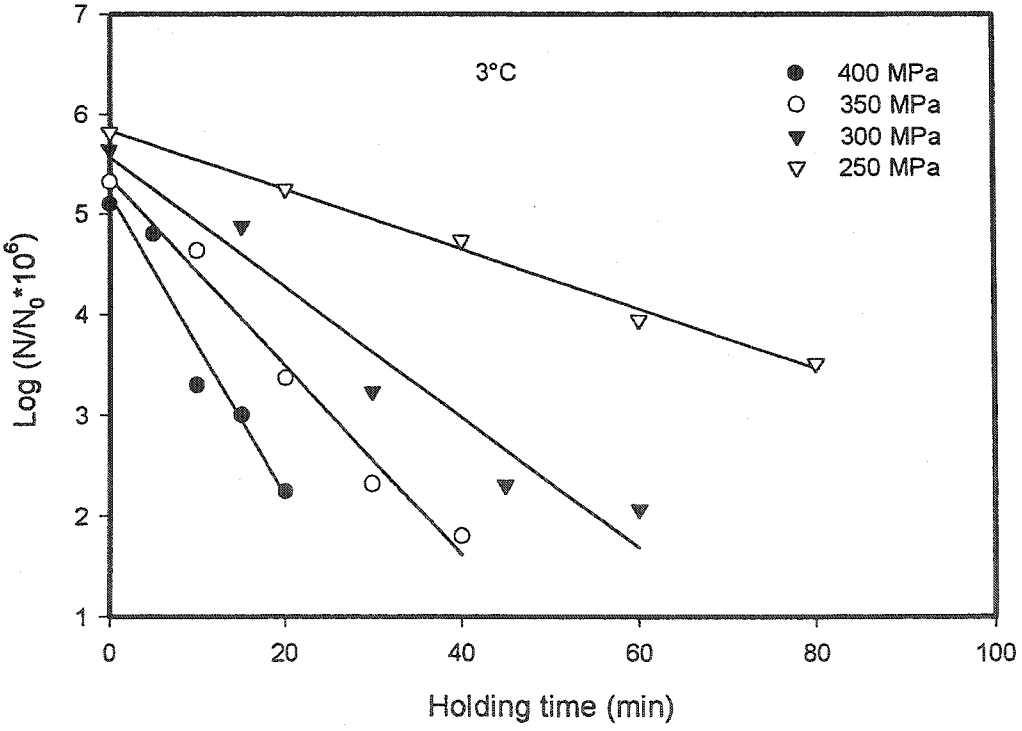
Table 4.3. The high-pressure destruction kinetic data for indigenous micro-flora and *E. coli* in milk

Microflora in milk	Temp. of treatment	Pressure (MPa)	D-value (min)	k- value (min^{-1})	R^2	z_p (MPa)	$\Delta V^{\neq}$ ($\times 10^{-5}$ m^3 $mole^{-1}$)
Natural microflora (total count)	21°C	250	36.00^a	0.06^d	0.96	240	-2.34
		300	16.32^b	0.14^c	0.97		
		350	11.34^c	0.20^b	0.99		
		400	8.26^d	0.28^a	0.96		
	3°C	250	31.33^a	0.11^d	0.99	227	-2.32
		300	14.58^b	0.23^c	0.99		
		350	9.95^c	0.33^b	0.97		
		400	6.60^d	0.50^a	0.97		
E. coli	21°C	250	32.50^a	0.07^d	0.98	205	-2.75
		300	15.89^b	0.15^c	0.99		
		350	9.37^c	0.25^b	0.97		
		400	5.96^d	0.39^a	0.96		
	3°C	250	30.28^a	0.08^d	0.98	179	-2.95
		300	14.68^b	0.16^c	0.99		
		350	8.35^c	0.28^b	0.97		
		400	4.29^d	0.54^a	0.94		

Superscript letters a, b, c, d showing significant difference of kinetic parameters (D and K-value) within different pressure at a given temperature and microorganisms



(a)



(b)

Figure 4.1. Survival curve of natural microflora at (a) 21°C and (b) 3°C and at different pressure

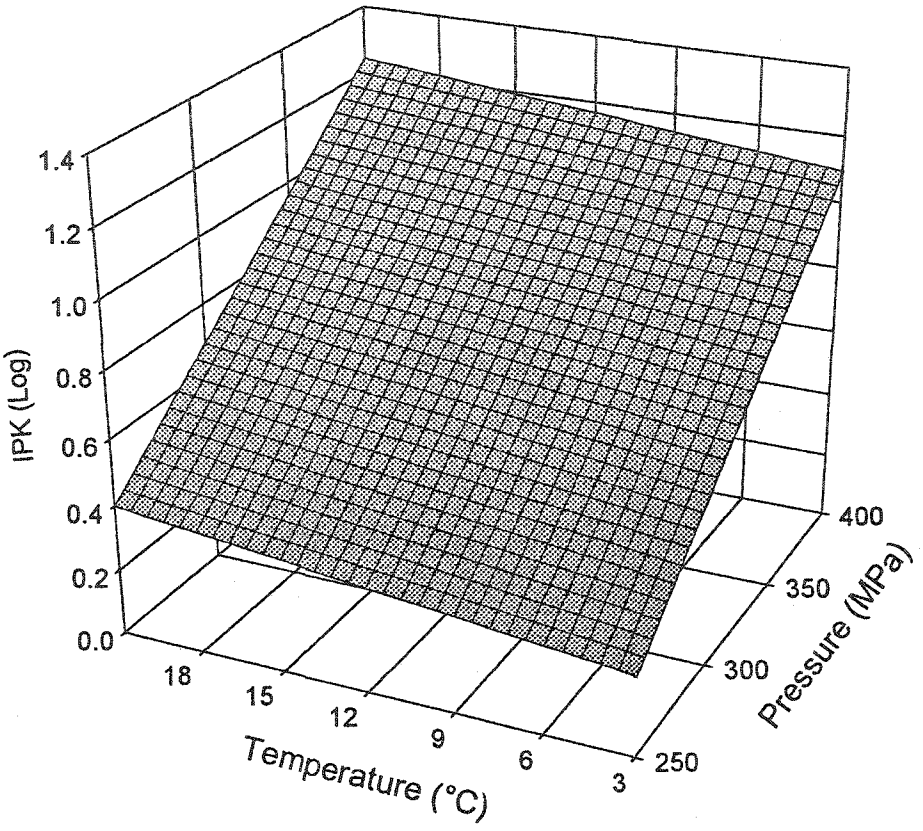


Figure 4.2 Effect of pressure and temperature on IPK value of indigenous microflora.

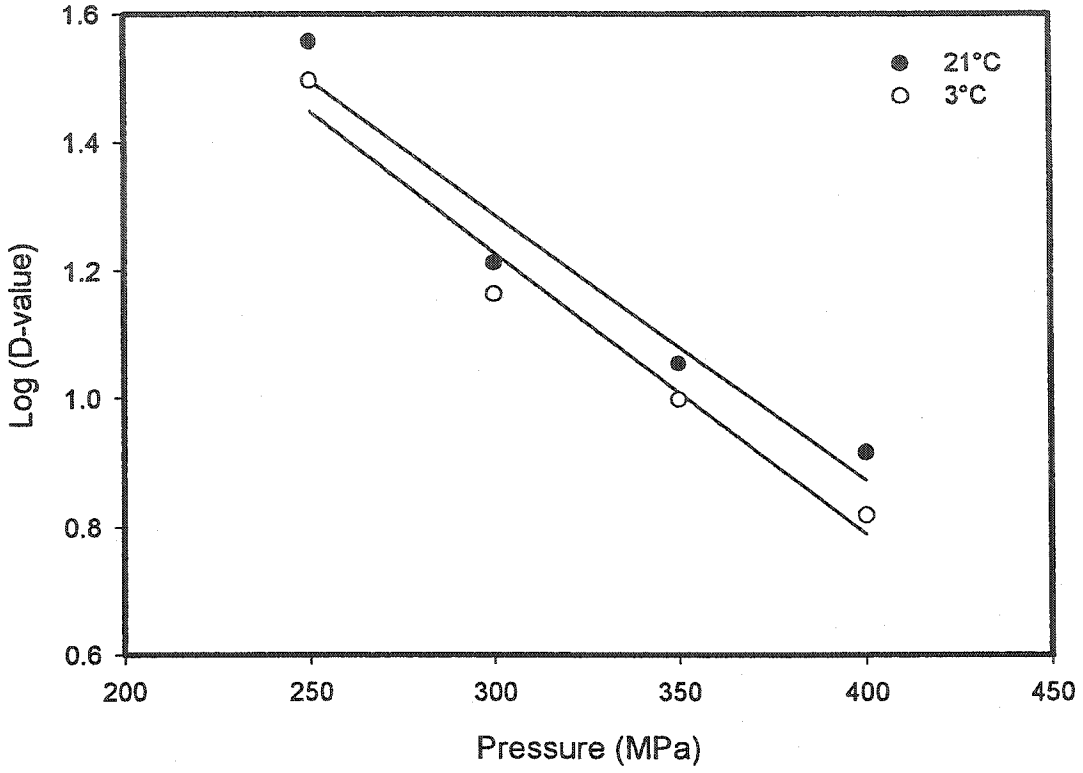


Figure 4.3 Pressure sensitivity of natural microflora in milk affected by two different temperature.

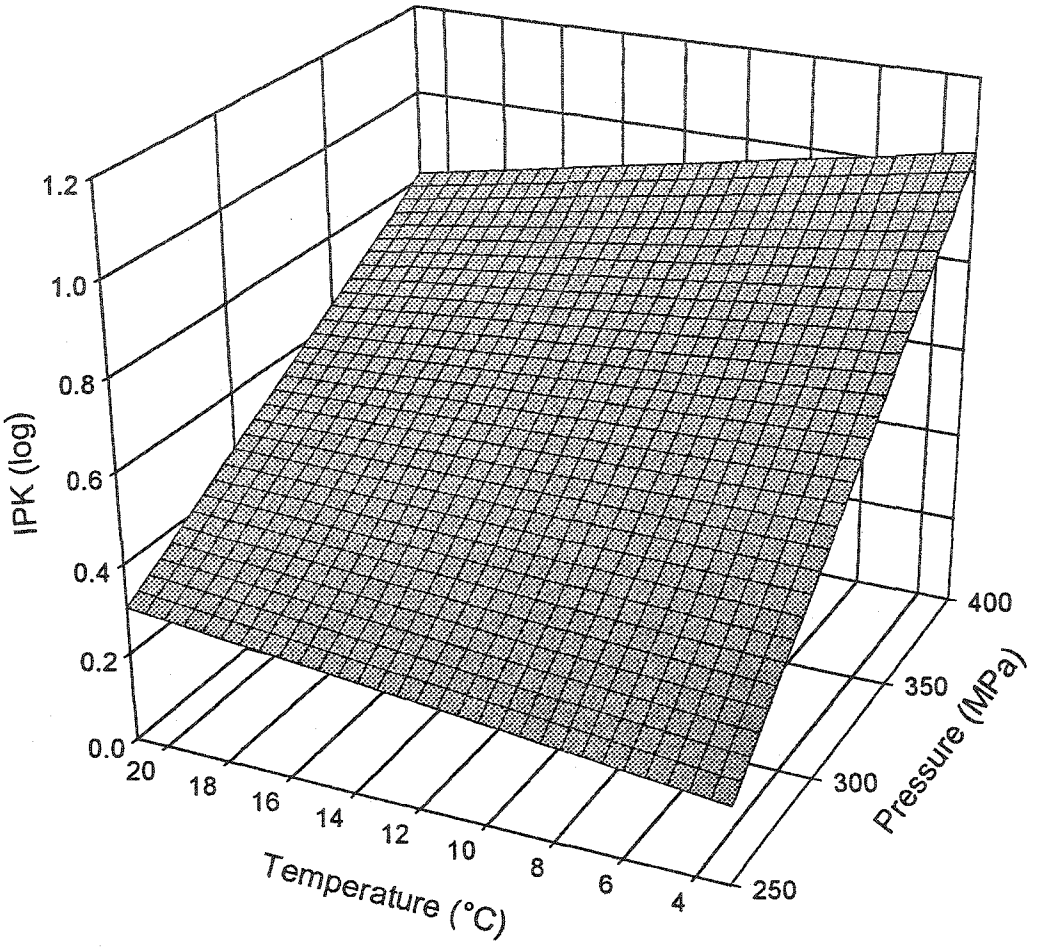


Figure 4.4 Effect of pressure and temperature on IPK value of *E. coli*

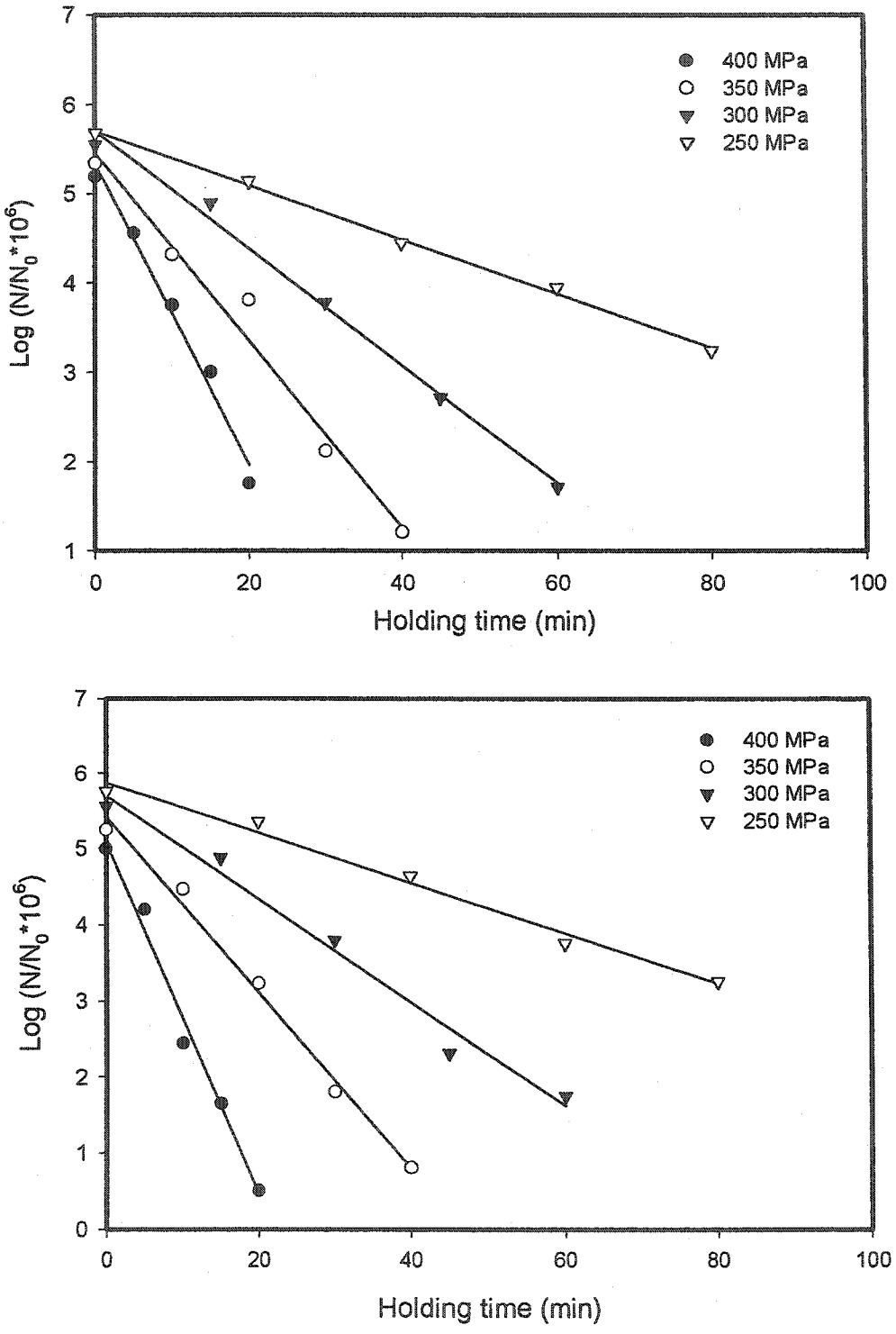


Figure 4.5 Survival of E. coli at 21°C (top) and 3°C (bottom), and at different pressure

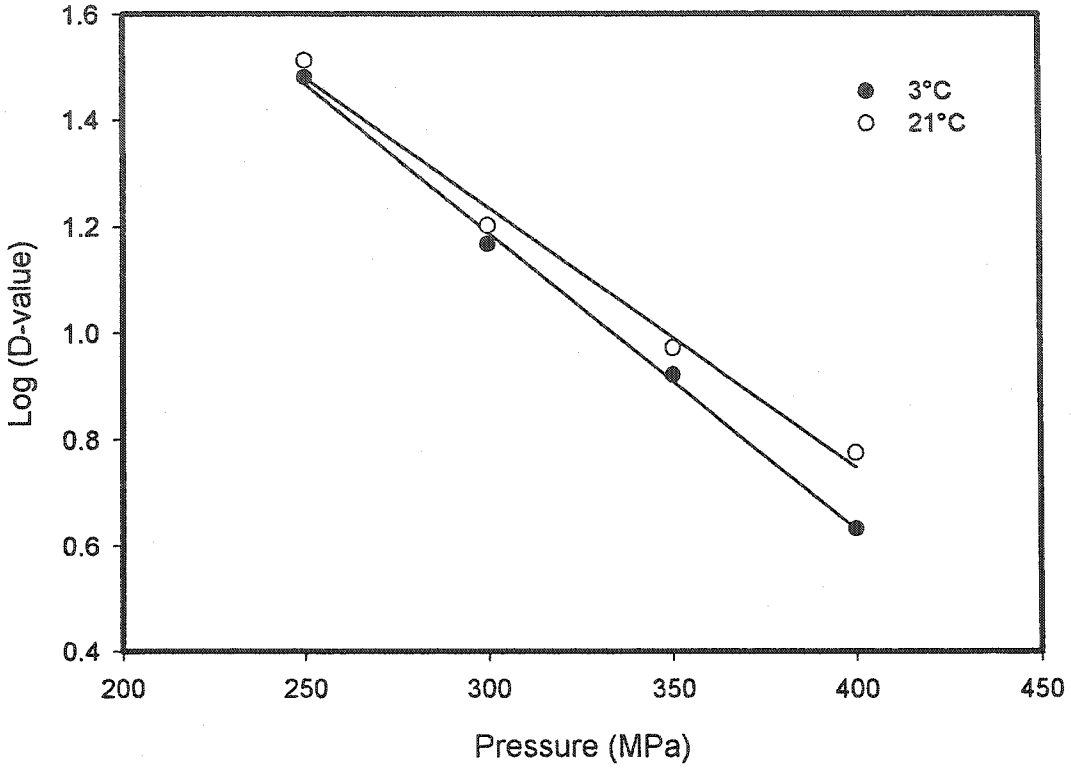


Figure 4.6 Pressure sensitivity of *E. coli* in milk affected by two different temperature.

CHAPTER V

EFFECT OF HIGH PRESSURE TREATMENT OF MILK ON LIPASE AND γ-GLUTAMYL TRANSFERASE ACTIVITY

CONNECTING STATEMENT

This chapter tests a part of the hypothesis of this research verifying the effect of high pressure processing on selected milk enzymes. The detailed study on enzyme kinetics was also one of the basis for selecting process parameters. Most literature available on activity of milk enzymes as affected by pressure processing has been carried out at room temperature. As low temperature processing was recognized in this study to yield better cheese making properties, it was also important to verify the sensitivity of milk enzymes to such processing conditions.

Some parts of this chapter have been prepared for publication/presented:

Pandey, P.K., H.S. Ramaswamy and D. St-Gelais. 2002. Effect of HP treatment of milk on lipase and γ-glutamyl transferase activity in milk. Paper submitted for publication in J. Food Biochemistry.

Ramaswamy, H.S. and P.K. Pandey. 2001. Application of High Pressure processing in Dairy Industry. Paper presented at the Symposium on High Pressure Solutions, Agriculture and Agri-Food Canada, Food Research and Development Center, St. Hyacinthe, PQ. March 7, 2001).

All the experimental work and data analysis were carried out by the candidate under the supervision of Dr. Ramaswamy and Dr. St-Gelais.

EFFECT OF HIGH PRESSURE TREATMENT OF MILK ON LIPASE AND γ-GLUTAMYL TRANSFERASE ACTIVITY

ABSTRACT

High-pressure treatment (0 to 180 min at 300 to 400 MPa) was applied to milk to investigate the pressure inactivation kinetics of lipoprotein lipase and γ-glutamyl transferase. Pressure treatment resulted in some enhancement in the activity of both enzymes. The extent of enhancement in activity depended on the pressure level with lower pressure resulting in a greater enhancement. In case of lipase, there was no inactivation during the entire pressure hold time (up to 100 min). However, with γ-glutamyl transferase, following the initial activation, continued pressure treatment resulted in inactivation of the enzyme typically following a first order kinetic model. The pressure sensitivity of the inactivation parameters (k and D values) was adequately described by the pressure death time and Arrhenius models with a z_p of 543 MPa and an associated volume of activation, $\Delta V^{\neq}$, of -3.28×10^{-8} m^3 $mole^{-1}$.

INTRODUCTION

The manufacture and ripening of rennet-coagulated cheeses are essentially enzymatic processes. Most bioconversions in Cheddar, Dutch and similar cheeses are catalyzed by enzymes. Although, in Swiss-type cheeses, ripening is achieved by mold and bacterial smears, living microorganisms play key roles only at certain stages, e.g. in fermentation of lactate and de-acidification processes. Enzymes of microbial origin are believed to play a more important role in ripening (Fox and Stepaniak, 1993).

Milk contains about 60 indigenous enzymes, most of which have been isolated and well characterized (Andrews *et al.*, 1991, 1992). Many of the indigenous enzymes have the potential to contribute to various aspects of cheese ripening, but their specific contribution is unclear and in fact, the possible contribution of most has not been fully assessed. Perhaps this lack of interest arises from the fact that activities of most indigenous enzymes are relatively constant in the bulk milk supply, many of them are inactivated by pasteurization and the activity of many is low in comparison with similar enzymes from other sources, i.e. coagulant, starter and non starter bacteria (Fox and Stepaniak, 1993).

The indigenous enzymes most likely to contribute to cheese ripening are plasmin, lipoprotein lipase, acid phosphatase and xanthine oxidase. It is interesting that all these enzymes except lipase are fairly heat stable and survive the high-temperature short-time (HTST) pasteurization almost unaffected; pasteurization, however, completely inactivates indigenous lipase.

For many years, the lipoprotein lipase was considered the most technologically important indigenous enzyme in milk. The level of lipase in milk is such that if it was fully active it would release sufficient fatty acids to cause hydrolytic rancidity within a short time. The extra mature cheeses show considerable lipolysis and the flavor impact is balanced by the products of proteolysis and possible glycolysis. Lipolysis makes a significant contribution in raw-milk cheese, about twice that in pasteurized milk cheese (McSweeney *et al.*, 1993). Most of the free fatty acids (FFAs) generated from lipolysis are precursors of volatile compounds such as methyl ketones, alkanones, lactones etc., which play an important role in the development of cheese flavor (Nájera *et al.*, 1993). Buffa *et al.* (2001a, b) reported an increase in lipolysis level in high-pressure (HP) treated goat milk

cheese compared to cheese made from heat pasteurized goat milk. Further, they concluded that the level of lipolysis was similar to that in raw milk cheese. Thus, from the point of cheese making, it is important to evaluate the effect of high pressure processing on milk lipoprotein lipase.

γ-glutamyl transferase (GGTP; EC. 2.3.2.2) is a milk fat-globule, membrane-bound enzyme (Kitchen, 1985). GGTP in milk has been used as an indicator of the severity of heat treatment (McKellar and Emmons, 1991). This enzyme plays a significant role during the synthesis of milk as an amino acid transporter. Its function in cheese ripening is not well understood (Patel and Wilbey, 1989; Carter *et al.*, 1990; McKellar and Emmons, 1991).

The objective of this study was to study the effect of high pressure (HP) treatment on the activity of native enzymes in milk in the context of cheese making, i.e., to evaluate their relative resistance to pressure inactivation as compared to those of pathogens that may be present in milk. Two key enzymes, namely, the lipoprotein lipase and γ-glutamyl transferase, were evaluated in this study. Higher pressure resistance of enzymes relative to pathogens offers a considerable advantage for HP treatment of milk prior to cheese making. Such an HP treatment would render the milk safe from pathogens, but will not adversely affect the activity of native enzymes that are important in the development of cheese character during ripening.

MATERIALS AND METHODS

Milk sample collection and preparation as well as the high-pressure treatment are described in Part I of Chapter III.

Test samples were subjected to high-pressure treatment for 0 to 180 minutes at 300-400 MPa (Table 5.1). After pressure treatment, the samples were stored at 3°C (2h) prior to enzyme assay. All the pressure treatments were carried out at 3°C. Details of pressure treatment are also given in Chapter III.

Lipase assay

Titrimetric determination of lipase activity in milk was carried out using a procedure recommended by Sigma (Sigma Chemical Co., St-Louis, MO), which was based on the method of Tietz and Fiereck (1966) involving hydrolysis of triglycerides in olive oil into fatty acids, diglycerides and to some small extent, into monoglycerides and glycerol. The rate of formation of fatty acids, under the specific conditions of the test was used as a measure of lipase activity in the sample. The pool of fatty acids formed were determined by titration with sodium hydroxide standard. Most chemicals and supplies required were purchased from Sigma (Sigma Chemical Co., St-Louis, MO): olive oil, 50% (v/v) and sodium azide, 0.1%, added as preservative; Trizma ® buffer contains Tris aminomethane (0.2 mol/L, pH 8.0 at 25°C). Thymolphathalien indicator solution was prepared as 0.9% (w/v) solution in ethanol. Ethanol (95%) and standard sodium hydroxide (0.05N) were obtained from Fisher Scientific (Fisher Scientific Co., Pittsburgh, PA).

The test sample was prepared by adding 2.5 mL water, 3.0 mL Sigma lipase substrate, 1.0 ml of Trizma® buffer to 1.0 mL milk. A blank was similarly prepared without milk (Table 5.2). Both tubes (test and blank) were placed in a water bath at 37°C for 6 h (incubation). After incubation, 1.0 mL milk was added to the blank, and 3.0 mL 95% ethanol added to both the blank and the test sample, and mixed thoroughly. The contents of each tube were then transferred to a separate flask, 4 drops of thymolphthalein indicator solution was added and then titrated against 0.05N NaOH solution until end point (slight but definite blue color end point). The lipase activity expressed as Sigma-Titz Units/mL, which is the number of mL of 0.05(N) NaOH needed to neutralize fatty acids liberated during incubation period (6 h) of the test.

γ-glutamyl transferase assay

This assay was based on the effect of the enzyme on a specific substrate, L-γ-glutamyl-p-nitroanilide, transferring the glutamyl group to the receptor, glycylglycine. The p-nitroaniline released was measured using a spectrophotometer. All substrates and reagents were purchased from Sigma (Sigma Chemicals, St-Louis, MO): substrate: γ-glutamyl-p-nitroanilide (51 μmol), glycylglycine (1.1 mmol); TRIZMA® buffer solution

(0.1 mol/L, pH 9.0); sodium nitrite tablets: ammonium sulfamate (1% (w/v), with chloroform added as preservative); N-1-naphthylethylenediamine DiHCL vial; γ-*glutamyl transferase* calibration solution; p-nitroaniline (0.126 μmol/L).

γ-glutamyl transferase substrate solution was prepared by adding 11.0 mL TRIZMA® buffer solution to one vial of γ-*glutamyl transferase* substrate and shaken vigorously for few seconds, with the vigorous shaking repeated one more time after 2-3 min. Naphthylethylenediamine solution was prepared by adding 105 ml water to contents of bottle and mixed vigorously. Acetic acid solution [1.7 N, 10%(v/v)] was prepared from glacial acetic acid obtained from Fisher (Fisher Scientific Co. Pittsburgh, PA)

Calibration: The amount of calibration reagents indicated in Table 5.3 were pipetted into 5 cuvets labeled 1-5. To each cuvet 2.0 mL acetic acid solution was added and mixed by gentle swirling. Subsequently, 1.0 mL sodium nitrite solution was added and after mixing, the contents were allowed to stand at room temperature for 3 min. 1.0 mL ammonium sulfamate solution was added to each cuvet next, mixed quickly and allowed to remain at room temperature for another 3 min. Finally, to each cuvet, 1.0 mL naphthylethylenediamine solution was added and mixed vigorously so that all the bubbles rose to the top. The absorbance of the solution was then measured at 540 nm using a Beckman spectrophotometer in cuvets 2-5 with cuvet 1 as the reference. A calibration curve of absorbance vs. corresponding serum γ-*glutamyl transferase* activity was established, from which the enzyme activity in test samples were estimated.

Data analysis

When inactivation was applicable, the transient change in the residual activity of the enzyme was analyzed using a conventional first order kinetic model as:

$$\log_e\left[\frac{A}{A_0}\right] = -kt \tag{5.1}$$

where, A is the residual enzyme activity after a pressure treatment time t (min); A_0 is the initial enzyme activity; k is the reaction rate constant (min^{-1}). The reaction rate constant was obtained from the linear regression of $\log_e(A/A_0)$ vs. time as the negative slope:

$$k=-(\text{slope}) \tag{5.2}$$

The D-value or decimal reduction time (time required for 90% inactivation of enzyme activity) is expressed as

$$D=2.303/k \tag{5.3}$$

The pressure sensitivity of the kinetic parameters was analyzed by both Arrhenius and a pressure destruction time (PDT) approach [similar to TDT (thermal destruction time) model]. In the Arrhenius approach, the activation volume was obtained by plotting $\log_e(k)$ values against pressure and the activation volume ($\Delta V^{\neq}$) was evaluated from the slope (a measure of net pressure effect on reaction rate constant at constant temperature):

$$\Delta V^{\neq} = -RT\left[\frac{\Delta(\log_e k)}{\Delta P}\right]_T \text{ or } -RT\,(\text{slope}) \tag{5.4}$$

where ($\Delta V^{\neq}$) is the activation volume in (m^3 $mole^{-1}$), P is the pressure in MPa, k is the rate constant (min^{-1}), T is the absolute temperature (°K), R is the gas constant (8.314×10^{-6} m^3 $mole^{-1}$ MPa $°K^{-1}$) (Erying and Magee, 1942).

For the pressure destruction time (PDT) model, $\log_{10}(D)$ vs pressure data were used and the pressure z-value (z_p) was determined as the negative reciprocal of the slope:

$$z_p = \left(\frac{\Delta P}{\Delta Log(D)}\right) \text{ or } z_p = -\left(\frac{1}{\text{slope}}\right) \tag{5.5}$$

The z_p of the process is defined as the pressure range between which the decimal reduction time is changed by 10 fold.

RESULTS AND DISCUSSION

Lipase activity as affected by pressure treatment

The activity of lipase after various pressure treatments is shown in Figure 5.1. All pressure treatments resulted in an increase in the lipase activity. The pressure pulse treatment (treatment with zero holding time, i.e., pressurization to the desired level and

immediate depressurization) had no effect at 300 MPa while showed a large enhancing effect at 350 MPa (133%) and at 400 MPa (100%). From the enzyme activity plot (Figure 5.1), kinetic changes were not obvious. The activity following the pressure-pulse treatment at 300 MPa continued to increase up to 80-100 min treatment time (holding time). With pressure treatment at 350 and 400 MPa, generally no further increase in enzyme activity was noticeable beyond that caused by the pressure pulse. From the data, it was not possible to characterize the pressure effect as belonging to any kinetic model (activation or inactivation). There is not much information in the literature on the activity of lipoprotein lipase in raw milk as affected by HP treatment of milk. Seyderhelm *et al.* (1996) reported that the lipase activity could be reduced by 40% in Tris-buffer at pH 7.0 subjected to HP treatment of 600 MPa for 10 min at ambient temperature. The authors also reported that a further increase in pressure would reduce the time required for similar inactivation. It was not possible to achieve pressure levels higher than 400 Mpa with the equipment available during this study.

Lipoprotein lipase is the main lipolytic enzyme in milk that is mostly inactivated (<3% residual activity) by the pasteurization process (Andrew *et al.*, 1987). Driessen (1989) reported that heat treatment of milk to 78°C for 10 s totally inactivated the lipase in milk. A number of nonstarter lactic acid bacteria (NSLAB) have been shown to possess lipolytic (El-Soda *et al.*, 1986) or esterolytic activity (Piakietwiecz, 1987). These lipases contribute to the release of fatty acids during ripening of Cheddar cheese. The present study indicated that the pressure levels selected to process raw milk would not have any negative effect on the activity of native lipoprotein lipase.

High pressure processing is known to affect the conformation of protein structure. Lipase being a monomer protein, the pressure sensitivity seems to be higher as it do not possess quaternary structure and the influence of HP treatment on the tertiary structure may be responsible for the resulting conformational changes and enhancement of activity of enzyme.

γ-glutamyl transferase activity as affected by HP treatment of milk

Effect of pressure on the activation and inactivation of γ-*glutamyl transferase* at various pressure levels is shown in Figure 5.2. The effect of pressure pulse was quite obvious in this case clearly showing an enhancement effect, the magnitude of which depended on the pressure level. The lower pressures caused a larger enhancement effect. Upon continued application of pressure, there was a clear inactivation effect following a semi-logarithmic trend with treatment time (first order reaction). The enhancement of activity ranged from 45% at 300 MPa and 19% at 350 MPa to just 3% at 400 MPa. The γ-*glutamyl transferase* activity was same as in raw milk even after 90 min pressure treatment at 300 MPa, 30 min at 350 MPa and a pressure pulse treatment (zero holding time) at 400 MPa (Figure 5.2). Further, an increase in treatment time at each pressure level caused a decrease in activity that followed a first order kinetic model. Rademacher and Kessler (1997) who studied the effect of high pressure on γ-*glutamyl transferase* reported that only 15% activity was lost by 8 min treatment at 400 MPa; however, when pressure was increased from 400 to 600 MPa the loss in activity increased from 15 to 90%. In this study, a 15% loss in activity of γ-*glutamyl transferase* was observed at 400 MPa when treated for about 30 min while a similar treatment at 350 MPa resulted in almost no change and at 300 MPa there was about 35% activation indicating somewhat similar results. The kinetic parameters, k and D-values were obtained from the regression of log (A/A_0*100) vs. time plots (Figure 5.2) are shown in Table 5.4 indicating a good fit. Rate constant (k) increased with an increase in pressure level (D values decreased). Mussa and Ramaswamy (1997) reported kinetic data on alkaline phosphatase with D-value and k-value of 493 min and 0.00467 min^{-1}, respectively at 350 MPa, somewhat lower than the value found in this study for γ-*glutamyl transferase*. In that study, the pressure destruction of microorganisms in milk including the pathogen *Listeria monocytogenes* was reported to be much more effective than the inactivation of phosphatase enzyme.

The pressure sensitivity of kinetic parameters can be obtained as z_p and $\Delta V^{\neq}$. Figure 5.3 shows the log (D) vs. P curve from which z_p value can be obtained as the negative reciprocal slope. The associated z value (z_p = 543 MPa) and activation volume ($\Delta V^{\neq}$ = -3.28 x 10^{-8} m^3 $mole^{-1}$, obtained using Equation 5.4) are listed in Table 5.4

indicating a relatively low sensitivity of inactivation kinetic parameters of *γ-glutamyl transferase* to changes in pressure. Other studies have also shown a high resistance for enzyme inactivation in milk or in other media (Thakur and Nelson, 1998). Plasmin and a pro-enzyme plasminogen is a principal proteolytic milk enzyme and plays significant role in cheese ripening. This was found to posses a neutral effect on pressure treatment (Trujillo *et al.*, 1997).

Implications of HP treatment on cheese making process

Pressure treatment of milk for cheese making could be advantageous only if it can selectively destroy the milk pathogens such as *L. monocytogenes* while sparing the native enzymes that would be desirable for the ripening process. Mussa *et al.* (1998) found the D values for pressure inactivation of *Listeria monocytogenes* in milk to range from 15 min at 300 to 10 min at 350 MPa. Considering a 5D reduction, the treatment times will range from 75 min at 300 to 50 at 350 MPa. Under these conditions, the lipase activity will be largely unaffected while the *γ-glutamyl transferase* activity will be enhanced by 20% at 300 MPa and reduced by less than 5% at 400 MPa, thus preserving their original activity. Hence the HP treatment is deemed appropriate and beneficial for the cheese making process.

CONCLUSIONS

In the study, the effect of high pressure (300-400 MPa, 0-180 min) on two milk enzymes lipoprotein lipase and γ-*glutamyl transferase* was evaluated. In general, the enzyme activities were enhanced by the high-pressure treatment. Lipoprotein lipase resisted inactivation during the entire range of pressure treatment. The γ-*glutamyl transferase* also demonstrated an activity enhancement after a short pressure pulse, but followed a first order inactivation rate kinetics thereafter. Enhancement in the activity was higher at the lower applied pressure. At the pressure treatment levels required for pathogen control, considerable retention of native enzyme activity was possible, thereby demonstrating a beneficial effect of HP treatment of milk in cheese making.

Table 5.1. Pressure and holding time used for HP processing of milk for Lipase and γ-*glutamyl transferase* kinetic study.

Pressure (MPa)	Treatment time (min): Lipase						
300	0	25	50	75	100		
350	0	20	40	60	80		
400	0	15	30	45	60		
Pressure (MPa)	Processing time (min): γ-glutamyl transferase						
300	0	15	30	60	90	120	180
350	0	15	30	60	90	120	
400	0	15	30	60	90	120	

Table 5.2 Measurement of Lipase activities

Reagents and sample	Test	Blank
Water	2.5	2.5
Sigma Lipase substrate	3.0	3.0
TRIZMA Buffer	1.0	1.0
Milk	1.0	-

Table 5.3. Calibration data for GGTP activity estimation

Cuvet No	GGTP Calibration solution (mL)	Water (mL)	Serum GGTP Activity (units/mL)	Absorbance
1	0.0	0.5	0	0.000
2	0.1	0.4	20	0.128
3	0.3	0.2	60	0.372
4	0.4	0.1	80	0.491
5	0.5	0.0	100	0.606

Table 5.4. Kinetic parameters for the pressure inactivation of *γ-glutamyl transferase* in milk at different pressures.

Pressure (MPa)	Kinetics parameters			Pressure sensitivity		
	D-value (min)	k-value	R^2	z_p MPa	$\Delta V^{\neq}$ ($\times 10^{-8}$ m^3 $mole^{-1}$)	R^2
300	848.5^A	0.0027^A	0.98			
350	711.6^B	0.0032^B	0.99	543.5	-3.28	0.99
400	555.5^C	0.0041^C	0.99			

A, B, C: Significantly different ($p<0.05$) at each pressure level

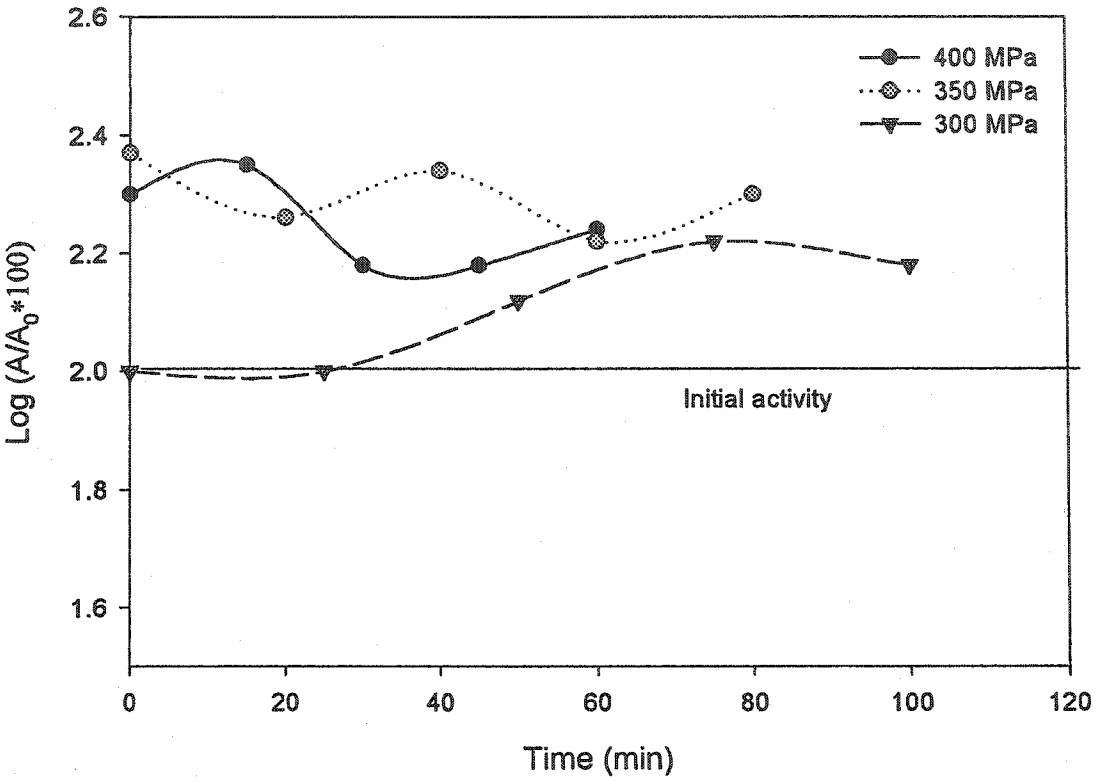


Figure 5.1 Pressure inactivation curve of lipase at various pressures.

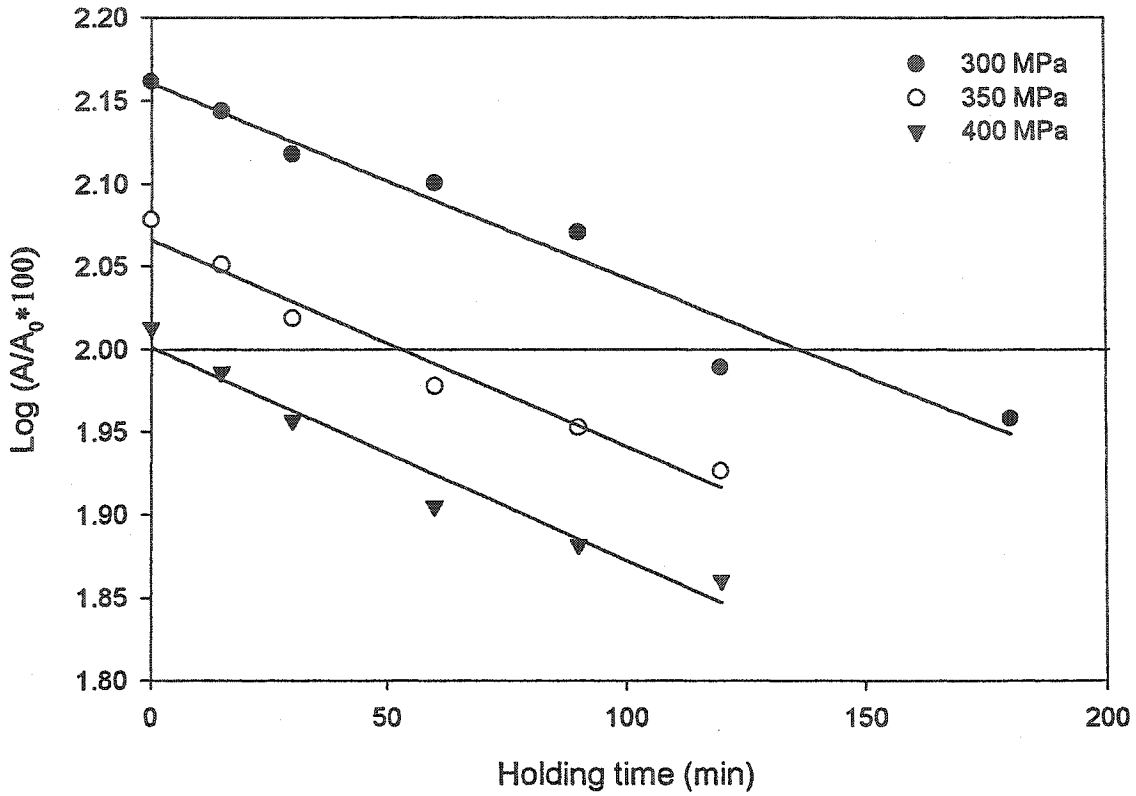


Figure 5.2 Inactivation curve of γ-glutamyltransferase at different pressures.

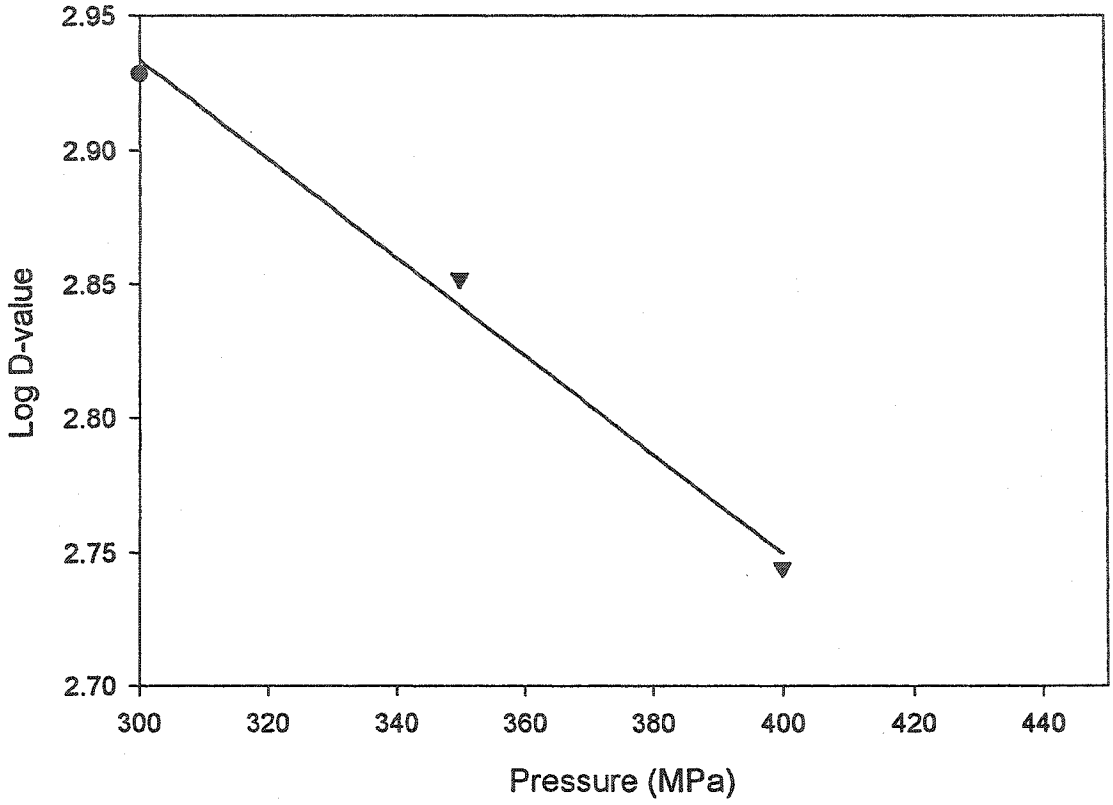


Figure 5.3 Pressure sensitivity of γ-glutamyltransferase

CHAPTER-VI

CHEESE MAKING PROCESS AND QUALITY CHARACTERISTICS OF CHEDDAR CHEESE MADE FROM RAW, PASTEURIZED, MICRO-FILTERED AND HIGH PRESSURE TREATED MILK

CONNECTING STATEMENT

This chapter highlights and compares the characteristics of Cheddar cheese made from raw milk, pasteurized milk, micro-filtered milk and high-pressure treated milk. It is divided into two parts:

Part I: Evaluation of pH change kinetics during cheddaring of raw, pasteurized, micro-filtered and HP treated milks

Part II: Evaluation of composition, yield, milk solid recovery and cutting time of rennet curd during cheddaring of raw, pasteurized, micro-filtered and HP treated milks

Some parts of this chapter have been prepared for publication/presented at a conference:

Pandey, P.K., Ramaswamy, H.S and St-Gelais, D. 2002. Evaluation of pH change kinetics during cheddaring of raw, pasteurized, micro-filtered and HP treated milk. Manuscript in preparation for submission to Lebenm.Wissen.-u-Technology (Food Science+Technology)

Pandey, P., Ramaswamy, H.S. and St-Gelais, D. 2001. Evaluation of manufacturing process and quality of Cheddar cheese made from high-pressure treated milk, microfiltered milk, raw milk and pasteurized milk during ripening. Paper presented at the 2001 Non-thermal Division Workshop of IFT, Newark, DL, March 26-28, 2001.

The pilot scale Cheddar cheese manufacturing, experimental work and analysis were carried out at Food Research and Development Center (FRDA), Saint-Hyacinthe, Quebec under supervision of Dr. St-Gelais and Mr. Gaétan Bélanger. Research was carried out by the candidate under the supervision of Dr. H.S. Ramaswamy and Dr. D. St. Gelais.

PART-I

EVALUATION OF PH CHANGE KINETICS WHILE MAKING CHEDDAR CHEESE FROM RAW MILK, HIGH PRESSURE TREATED MILK, MICROFILTERED AND PASTEURIZED MILK

ABSTRACT

The pH change kinetics during Cheddar cheese making from raw milk, heat treated milk, microfiltred milk and high pressure (HP) treated milk were evaluated. Heat treatment and micro-filtration were equivalent of achieving commercial pasteurization of milk. High pressure treatment included two pressure levels and two treatment times (300 MPa for 60 and 90 min and 400 MPa for 32 and 40 min) calculated to be equivalent to achieve 4 and 6 decimal reduction of *Listeria monocytogenes*. The pH change kinetics was assessed in the three phases – milk ripening, cooking/holding and cheddaring – of the Cheddar cheese making process. The influence of different treatments on pH change were somewhat different in the three phases studied. The changes were in general linearly decreasing in the first two stages while logarithmically with time in the final cheddaring phase. With respect to raw milk (control), all treatments delayed the ripening of milk with micro-filtration influencing the most. During the cooking/holding, pasteurized and micro-filtered milk slightly enhanced the rate of pH change while the HP treated milks continued to delay the pH change. During the final phase (cheddaring process), the rate of change in pH with the high pressure treated milk was about the same as in micro-filtered milk; however, both of these delayed the rate relative to raw and heat treated milks.

INTRODUCTION

Cheese is the most diverse group of dairy products and is, arguably, the one that has been studied to be most academically interesting and challenging. While many dairy/food products, if properly manufactured and stored, are biologically, biochemically and chemically very stable (low moisture: milk powder; and high temperature treated products: UHT treated milk); cheeses are, in contrast, biologically and biochemically dynamic, and, consequently, inherently unstable (Fox, 1993). Throughout the manufacture and ripening, cheese production represents a finely orchestrated series of consecutive and concomitant biochemical events, which, if synchronized and balanced, lead to products with highly desirable aroma and flavours. There has been a lot of work done to improve the flavour and sensory characteristics of several varieties of the cheese. One of the important parameters that may affect the final sensory quality of cheese is the treatment of the milk such as pasteurization, irradiation, and micro-filtration prior to cheese making and, among all; the most common pre-treatment of milk prior to cheese making is the pasteurization. As a result of massive industrialization of cheese industry following the 2nd World War, pasteurization became mandatory prior to cheese making in order to maintain uniform quality and better production control. Also, in the recent past, the pasteurization has shown to improve the safety aspects and minimize the cheese related outbreaks. However, there is still a large demand for raw milk cheeses which possess unique and strong flavour which are derived from the native enzymes and bacteria present in raw milk and the heat treatment of milk affects these flavour characteristics. These products are popularly sold in the many parts of the world including Canada (Grappin and Beuvir, 1997; McSweeney *et al.*, 1993; Rehman *et al.*, 2000). However, in spite of strict sanitary farm conditions and clean manufacturing practices, raw-milk cheeses have been associated with the several cheese borne outbreaks in the recent past (De Buyser *et al.*, 2001). Food scientists have been looking for an alternatives that ensure the safety of the cheese, but at the same time yields desirable taste and flavour in cheese.

. High pressure (HP) treatments are increasingly being studied as alternatives to thermal treatments for food preservation. High-pressure treatment of milk was found to improve its microbiological quality but have little effect on the native milk enzymes

(Rademacher *et al.*, 1997) while no effect on flavour or vitamins. This unique characteristic of high pressure enables selectively to destroy the target microorganism at the same time keep the enzymatic activity and flavour attributes intact at a desired level in the milk may be valuable in obtaining good flavour raw milk cheese (Trujillo *et al.*, 1997). However, in addition to microbial destruction, milk constituents undergo physicochemical changes that lead to modification in functionality which may affect the technological properties of cheese such as changes in the colloidal calcium phosphate and soluble calcium phosphate equilibrium that affect the buffering capacity of the milk (Shibauchi *et al.*, 1992, Lopez-Fandino *et al.*, 1996, Schrader *et al.*, 1997, Lopez-Fandino *et al.*, 1998, de la Fuente, 1998).

Acid development during different stages of cheese making is one of the essential requirements in cheese making. Three compositional parameters have been identified as being important in determining the quality of Cheddar cheese (Gilles and Lawrence, 1974; Pearce and Gilles, 1979) are the moisture in the non-fatty substance (MNFS), salt-in-moisture (S/M) and attaining pH to desire level. A correlation between the pH of the young Cheddar cheese and its quality at maturity has long been established (Price 1936; Dolby *et al.*, 1940). The single most important factor in the control of cheddar cheese quality is the extent of acid production in the vat since this largely determines its final pH (Lawrence *et al.*, 1993; Creamer *et al.*, 1988). The pH content of the cheese results mainly from the amount of lactic acid (produced from lactose by lactic starter added at the beginning of the cheese manufacturing) which is moderated by the buffering capacity of the milk. Each cheese variety has a characteristics pH range, within which the quality of cheese is dependent on its composition and the way in which it is manufactured (Laurence and Gilles, 1982).

Acid development at a desired rate is important in the cheese making process. Three stages have been recognized with respect to the acid development (pH change): a) milk ripening, b) cooking/holding and c) cheddaring. There has been little or no information reported on the kinetic behavior of change in pH during cheese making from milk treated with high pressure as compared to no treatment (raw milk), or other alternative treatments (pasteurization, micro-filtration). The objectives of this study were therefore to evaluate and compare the pH change kinetics during the cheese making

process (milk ripening, cooking/holding and cheddaring) with raw, pasteurized, micro-filtered and HP treated milk.

MATERIALS AND METHODS

Milk preparation

Raw milk was obtained from Laiterie Mont-St-Hilaire (St-Hyacinthe, QC, Canada) one day prior to cheese making and stored at 3°C until used. A portion of milk was used for micro-filtration, pasteurization and high-pressure treatment. From same bulk of milk, separated into the four parts, each approximately 18 liters, four batches of cheese were made on each production day using 20-L vats. The production schedule was randomized within 7 type of cheese (four HP treated milk, raw milk, pasteurized milk and micro-filtered milk) as detailed in Table 1. Two HP treatments were included with each production schedule combined with the other two so as to get approximately same number of lots for each group. Each type of cheese making was thus replicated for three times on different days.

Milk Treatments

(i) *High pressure treatment*

High-pressure treatment of milk was performed after packaging the milk in polyethylene bottles (2 L capacity). Approximately 36 L of milk was pressure treated each day enabling to make two batches of cheese. The bottles were double sealed in polyethylene bags before being pressurized to prevent any exchange with the pressurization fluid (water). The pressure treatment conditions were 400 MPa (32 and 48 min) and 300 MPa (60 and 90 min). These treatments were predetermined based on earlier studies of milk on pressure destruction of *Listeria monocytogenes* (Mussa *et al.*, 1998). The calculated times were equivalent of achieving 4 and 6 logarithmic reductions in the population of *L. monocytogenes* at each pressure level. Before and after pressure treatment, milk was stored refrigerated. The pressure treatment of milk was carried at 3°C as this temperature found to have better cheese making properties (Pandey *et al.*, 2000). The pressure treatment was given using a jacketed 30 L pressure vessel (ACB Pressure

Systems, Nantes, France) located at the Food Research and Development Center at St. Hyacinthe, Quebec, Canada.

(ii) Heat Pasteurization

The milk was pasteurized at 72°C using a high temperature short time technique in a milk pasteurizer (plate heat exchanger: Alfa Laval, Tumba, Sweden).

(iii) Microfiltration

Raw milk was skimmed and micro-filtered using an cross-flow micro-filtration unit (Alfa –Laval, MFSI, Tumba, Sweden) with a ceramic membrane pore size of 1.4 μm; membrane area 0.2 m^2; flow rate 600 Lh^{-1} m^{-2} and temperature, 50°C. After micro-filtration of skim milk, the milk was standardized using pasteurized cream to its initial level of milk fat.

Cheese making

Four 20-L cheese vats were used for preparing the Cheddar cheese. Cheeses were made from four high-pressure treated milks: HP-I (400 MPa/32 min), HP-II (400 MPa/48 min), HP-III (300 MPa/60 min), HP-IV (300 MPa/90 min), Raw milk, Pasteurized milk (Past) and Micro-filtered milk (MF). The four vats and these treatments were rotated for each replication to reduce systemic errors. Calcium chloride solution (Marshall Products, Madison, WI, USA) was added at a rate of 0.2g/kg of milk just before adding the starter culture and milk was heated to 30°C. The mixed strain of mesophilic cheese type B starter culture was added at the rate of 1.5% of milk. Ripening period of milk (stage 1, milk ripening) varied for each treatment and day-to-day production to attain the pH of 6.45. Single strength rennet extract (Rhone-Poulene, Marshall Products, Madison, WI, USA) was added at a rate of 0.2g/kg of milk. When the coagulum was set to the desired consistency (approximately 20-30 min), the curd was cut with a specially designed knife (approximately $1cm^3$). Cutting time was determined by taking into account of flocculation time of renneted milk and multiplying by 2.5. Curd was allowed to heat at 38°C in 30 minutes with gentle stirring and then held for sufficient time to attain the pH 6.00 (stage 2, cooking/holding). Whey was drained and curd was matted in the mould and turned periodically at 32°C. After the curd reached the pH of 5.4 (stage 3, cheddaring), the curd

was milled and salted at a rate of 2.0% and subsequently standard cheese making procedures were observed.

Monitoring of pH

The pH and acidity was measured at each of the three stages of cheese making at a predetermined interval as detailed below:

(a) Milk ripening stage: Milk ripening is the first stage in the cheese making. Milk received for the cheese making is added with 1.5% starter culture at 30°C. This resulted in a small but quick in pH of milk followed by a gradual change due to development of lactic culture. During the milk ripening period there is a gradual drop in the pH from 6.55 to 6.45. At least three measurements were made during milk ripening stage.

(b) Cooking / Holding stage: Once the curd is cut into cubes, the cooking of curd is started with gentle stirring, with the temperature allowed to raise from 30 to 38°C in 30 min. The curd is held and stirred gently at this temperature until the pH of the whey is dropped to 6.0. Further, further this stage will be referred as cooking stage although it is cooking and holding at 38°C.

(c) Cheddaring stage: At this stage the curd fused together and forms a chicken breast like texture. The pH at this stages helps in further expulsion of water and allowing curd particle to fuse together. The final pH at the end of this stage required having 5.40 before curd is ready for milling and salting stages.

Different times were required to attain the pH during milk ripening (drop in pH by 0.10 point, i.e., pH 6.5 to 6.4), cooking (change in pH from 6.4 to 6.0) and cheddaring (change in pH from 6.0 to 5.40) processes depending up on the treatments. These were evaluated and modeled during the cheese making process.

Statistical analysis

A GLM procedure was performed to see the significance of different treatments on the pH change kinetics during cheese making. Duncan grouping test was also performed to differentiate between the treatments. Simple regression analysis was performed to develop the models and determine the rate constants. The SAS® System software version 8 (1999) was used in all the statistical analysis.

RESULTS AND DISCUSSION

Change in pH throughout Cheddar cheese making

Figure 6.I.1 shows a typical plot of pH change in raw milk and one HP treated milk during the cheese making process. Figure 6.I.1 has been grouped in to the three main stages (milk ripening, cooking and cheddaring), within each stage the control of pH very important. From the figure, it can be recognized that the first two stages (milk ripening and cooking) show a linear change in pH with time, while in the final phase (cheddaring), the drop in pH is more rapid and the behavior is clearly non-linear with time. An exponential change was more obvious in this phase. To model the kinetic changes during the cheddaring stage, the pH data were converted to a logarithmic ratio form as follows (a fraction conversion model - Levenspiel, 1974):

$$Log\left(\frac{pH_t - pH_\infty}{pH_i - pH_\infty}\right) = -kt \qquad (6.I.1)$$

where pH_t is the pH at time t, pH_i is the initial pH at Cheddaring, which is 6.0, and pH_∞ is the pH at infinite time and k is the rate constant of change of pH. It was noticed that the pH after few days of all cheese converged near to 5.2.

Table 6.I.2, 6.I.3 and 6.I.4 show the Duncan grouping of rate of change of pH during three different stages and associated R^2 of regression analysis. Same letters in Duncan grouping showing no difference within the treatments.

Changes in the pH during milk ripening stage

Changes in pH during milk ripening is important for rennet action and further development of acidity in the curd throughout cheese making process and ripening. This stage allows starter culture to begin growing and produce some amount of lactic acid, which will eventually help in better rennet action. Production of lactic acid is important at this stage in many respects such as better rennet action, firmer curd and most importantly the activity of starter culture. Any deviation from normal condition such as higher competition with other microorganisms, presence of inhibitory substance or occurrence of bacterial phage would lead to slowdown of culture and eventually not enough acid will be

produced at these stages. The milk ripening duration varies from 30 min to more than an hour depending on the starter activity and its adaptation to the vat milk. During the milk ripening period, it is expected that the pH will drop by 0.10 unit.

The initial pH of cheese milk varied from 6.5 to 6.7 on a day-to-day basis. The drop in pH was noticed in two stages, one instantly after starter culture addition which is due to mixing of starter culture (1.5%) in the cheese milk that accounted for a quick drop in pH (by 0.1-0.15 unit) and the ripening stage which followed a slow zero order kinetics (by 0.1 unit). The rate of change of pH (rate constant) during milk ripening was monitored during the second stage and regression analysis was performed in order to obtain the rate constant.

The change kinetics for different treatments was shown in Figure 6.I.2a. Table 6.I.2 shows the Duncan grouping of mean value of rate constants, the associated R^2 and coefficient of variance (%). The same letter indicating no significance difference within the treatment. The rate of change during milk ripening showing higher rate for raw milk followed pasteurized and HP-II milk. All other three different pressure treated milk showed no difference. The Micro-filtered milk showed the least pH change rate, but not different from most pressure treated samples. There was a large difference in the rate constant in pH from pressure treated and MF milk to raw milk and pasteurized milk. MF milk showed a slightly lower rate constant even lower than HP treated milk. This was somewhat expected since all non-starter microorganisms were filtered out (99.99%) during MF process and the pH kinetics were solely influenced by the starter culture. It is interesting to note here that lower pressure and longer holding time (300 MPa, 90 min) caused further delay and lower rate in the milk ripening as compared with other treatments. Among pressure treated milk samples, it is also evident from the Table 2 that the equivalent lethal effect can't be generalized in terms of other effects on milk in cheese making characteristics.

There was a large difference between the rate constants for HP treated milks and pasteurized milk. The first explanation for the difference in the rate constant could be due to some irreversible change in the milk constituents which leads to inhibit or delay the culture activity to some extent. Green and Jezeski (1957) studied the effect of heat treatment of milk on simulation and inhibitory action of starter in milk. They reported

both simulation and inhibition depending on the severity of heat treatment and demonstrated the transition from one phase to another as a result of release of denatured serum protein nitrogen or cysteine. The balance of amino-nitrogen level is the important part of the growth factor for starter culture. HP processing is known to affect the serum protein and change in the free nitrogen content (Johnston *et al.*, 1993). The other explanation of delay in the change in pH would be the shift in colloidal and soluble Ca phosphate equilibrium which enhances the buffering capacity of the pressure treated milk thus may result in delayed ripening. The major buffering components of the cheese curd are the casein and to lesser extent phosphate but any ion present (Ca^{++} or Mg^{++}), which is able to associate with other ions involved, will move the balance of ionic equilibrium and may cause a change in the buffering capacity (Lawrence and Gilles 1982).

Change in pH during cooking and holding stage

A major change, which occurs in the conversion of milk into cheese, is the removal of water from coagulated milk solids. In manufacture of Cheddar cheese, the removal of water from the gel and the establishment of desirable levels of lactose, lactic acid and minerals in the cheese must largely be achieved while the curd is in the whey. The process of syneresis is accompanied by the development of lactic acid with the consequent solubilization of mineral constituents of the casein complex and by diffusion of substances such as lactose and lactic acid into or out of the curd. The curd after cutting is very fragile and easily broken. Therefore, after the coagulum was cut, some healing time (~10 min) is essential to minimize the breaking and formation of fine curd particles before cooking commenced. The temperature of curd is generally increased at 2°C/6 min from 30°C until it reaches 39°C (30 min). Cooking stage is very important in cheddar cheese making. At this stage most of the whey is removed from the curd. The starter culture activity is reduced to a great extent due to the higher temperature (and acid). Increase in temperature and increase in surface area (due to cutting of the curd) increases the syneresis action. The final moisture, textural quality and many related characteristics of the cheese are influenced by this stage. At the end of this stage, the whey is required to attain a pH 6.00. Many factor controls the change of pH during this stage such as buffering capacity of whey and activity of starter culture.

The pH change kinetics associated with different treatments was shown in Figure 6.I.2b. Table 6.I.3 shows the effect of different treatment on the rate constants of pH during this stage through Duncan grouping. Statistically, there were no differences in the rate constants between raw milk, MF, pasteurized milk. The higher rate at this stage for MF compared to previous stage could be due to the fact that MF milk must have attained the critical number of lactic bacteria during longer milk ripening period (Table 6.I.2). Also there is no other competitor for lactic culture bacteria in this case. Also among raw, pasteurized and HP-II there was no significant difference. However, the other HP treated samples had significantly lower rates than the rest. There were no differences between HP-I, II and IV or HP-I, IV and III. Again HP-II ranked higher among pressure treated samples, numerically. This indicated that at cooking stage all three controls (raw, micro-filtered and pasteurized milk) behaved similarly, and the pressure treated samples in general showed a lower rate constant for pH change. Again delay in achieving required pH at this stage among the HP treated samples could be due to enhanced buffering capacity of whey released due to syneresis of the curd.

Change of pH during cheddaring stage

The series of operations consisting of packing, turning, piling and repiling the slabs of matted curd is known as cheddaring. It has been established by many researchers (Lowrie *et al.*, 1982 and Harkness *et al.*, 1968) that Cheddaring is one of the steps in cheese making that helps to attain the necessary degree of acidity and further whey can be released from the curd. This loss of whey is controlled by temperature and acidity. The pH at this stage continued change until it reaches 5.4 from 6.0, which is the pH of the whey at the end of cooking.

It is obvious from Figure 6.I.1 that cheddaring stage do not behave similar to those of milk ripening and cooking stages, the curve showing non linear behavior and it looks like after a long time, eventually all samples would reach to common pH. The change in the pH rate during this period may be due to the fact that most of the whey, which is drained, carried away lactose with it. The shortage of lactose could reduce the activity of lactic acid bacteria and the acid production. Hence the decreasing slope with time in this stage that is apparent from Figure 6.I.1. Due to non-linear behaviour of this stage the data

were treated modeled using a fractional conversion model (semi-logarithmic model approaching a constant at infinite time). The equilibrium pH was close to 5.2 and hence this was employed in the model.

The change in pH kinetics in the cheddaring stage are shown in Figure 6.I.2c. Rate of constants for pH change are summarized in Table 6.I.4. Duncan's multiple range test again indicated that there was no significant difference in the rate constants between any of the pressure treated samples or between these samples and the micro-filtered milk. Further, there was no difference in rates between the raw and pasteurized milk. Statistically, however, the rate constants associated with HP treated and micro-filtered milk were lower than raw and pasteurized milks. Slightly faster achievement of pH at cheddaring stage by most pressure treated samples indicated that delay during cooking and milk ripening must have out grown the starter bacteria numbers that has resulted in the faster development of acidity also at this stage after removing the whey which contains most of chelated soluble calcium as a result of pressure treatment has no bearing on the buffering capacity of the curd at Cheddaring and pressure level or holding time have no influence on buffering capacity (release of ca-phosphate in serum by pressure treatment).

Segment Times in Cheddar Cheese Making

Table 6.I.5 shows the model calculated times for individual segments as well as the total required for cheese making which will have a composite effect on the cheese making process. Since the influence of different treatments on rate constants associated with pH change in the three stages were different, their influence on the overall process could be expected to be some what different.

As can be expected, the milk ripening times for the HP treated milk and micro-filtered milks were significantly higher than for raw and pasteurized milk. HP-II showed higher rate of pH development therefore required shortest time among pressure treated samples. During the cooking stage, differences were somewhat more moderate, but again the HP treated samples required the longer times (with HP II again requiring a slightly shorter time). In the cheddaring stage, raw and pasteurized milk had the shortest time, while the others required somewhat similar times. The combination of all these three, which finally

affects cheese making process (and perhaps the quality) (Table 6.I.5), shows the order of time - shortest for raw and pasteurized milk, followed by micro-filtered milk and then the high pressure treated milk. Relatively the HP treated milk required about 30% longer time for the making of cheese as compared to raw and pasteurized milks. It is also evident from Table 6.I.6 that at the end of cooking most of soluble calcium from pressure treated sample must have leached out as a result showing higher acidity compared to their controls to attain similar pH value. Table-6.I.6 indicates acidity at milk ripening which has no much influence on pH (showing no difference) and acidity of whey at beginning of cooking and at whey off. As the cooking proceeded, syneresis process progressed until the time of whey off the effect of enhanced buffering capacity is more evident.

Time required to decrease the pH during curd making, and ensuing loss of Ca and phosphate into the whey (de la Fuente 1998, Schrader *et al.*,1997) plays an important part in delaying total time required in cheese making or delayed attainment of pH at each stages by pressure treated samples.

CONCLUSIONS

The pressure treatment of milk significantly retarded the rate of pH change during the course of Cheddar cheese making. Except for the HP-II which showed a slightly lower retarding effect, the other three HP treatments had similar results. Milk ripening rate with raw milk was fastest among all treatments, to attain desired pH. Raw milk attain similar pH in half time than MF milk.

The change in the pH value during the course of cheese making process provides an indication of the extent of acid production by starter bacteria. In general, the curd acidity at salting is a key factor in determining the pH of the dry-salted cheese. The pH of the cheese results mainly from the amount of lactic acid on the one hand and that of the buffering compounds on the other. The main buffering substance in curd and cheese is the Ca-paracasein Ca-phosphate complex, of which calcium phosphate contributes roughly one third of the buffering capacity. By measuring acidity at each stage of cheese making revealed that the buffering capacity of milk is enhanced by high pressure treatment of milk. In order to obtain the desired level of acid development during different stages of the cheese making, variables such as the amount of starter added, the cooking temperature, the length of time the curd is in the whey and the level of salt, must be manipulated.

Table 6.I.1. Experimental Plan for Cheddar cheese making

Batch	RA	PA	MF	HP-I	HP-II	HP-III	HP-IV
#1	X	X		X	X		
#2	X	X		X	X		
#3		X	X			X	X
#4	X		X	X		X	
#5	X	X			X		X
#6			X			X	X

Table 6.I.2 Duncan groupings of the treatments for milk ripening rate

Duncan grouping of mean value of milk ripening rate constants from three replicates				
Treatments	Rate constant min^{-1}	R^2	Coeff. Variance (%)	DUNCAN grouping*
Raw	2.09×10^{-3}	0.93	4.29	A
Past	1.83×10^{-3}	0.98	14.6	B
HP-II	1.44×10^{-3}	0.96	10.4	C
HP-III	1.07×10^{-3}	0.98	8.91	D
HP-IV	1.04×10^{-3}	0.99	13.0	D
HP-I	1.01×10^{-3}	0.96	9.29	D
MF	0.99×10^{-3}	0.98	5.86	D

*Same letter indicating not significantly different ($p>0.05$)

Table 6.I.3. Duncan groupings of the treatments for rate of change of pH during cooking/scolding

Duncan grouping of mean value of rate of change of pH during cooking rate constants from three replicates				
Treatments	Rate constant min^{-1}	R^2	Coeff. Variance (%)	DUNCAN grouping*
MF	3.37×10^{-3}	0.99	6.05	A
Past	3.04×10^{-3}	0.99	10.36	AB
Raw	2.98×10^{-3}	0.99	7.89	AB
HP-II	2.85×10^{-3}	0.98	4.98	BC
HP-I	2.56×10^{-3}	0.97	6.53	DC
HP-IV	2.51×10^{-3}	0.99	4.25	DC
HP-III	2.37×10^{-3}	0.99	8.44	D

Table 6.I.4. Duncan groupings of the treatments for rate of change of pH during cheddaring

Treatments	Rate constant min^{-1}	R^2	Coeff. Variance (%)	DUNCAN grouping*
Raw	5.8×10^{-3}	1.00	10.53	A
Past	5.7×10^{-3}	0.97	11.99	A
HP-II	4.4×10^{-3}	1.00	2.70	B
MF	4.4×10^{-3}	0.98	7.31	B
HP-IV	4.4×10^{-3}	0.99	12.67	B
HP-I	4.2×10^{-3}	0.99	10.92	B
HP-III	4.1×10^{-3}	0.99	8.68	B

\

Table 6.I.5 Duncan grouping for pH change times during different stages of cheese making

Treatments	Ripening time	Cooking time	Cheddaring Time	Total Time
Raw	47.6^{D}	129.5^{CD}	101.1^{B}	278.3^{D}
Past	55.3^{C}	124.9^{CD}	103.0^{B}	283.3^{D}
MF	101.3^{A}	117.7^{D}	139.2^{A}	358.3^{C}
HP-I	99.8^{A}	163.7^{AB}	138.8^{A}	402.4^{B}
HP-II	69.6^{B}	150.5^{BC}	132.7^{A}	352.9^{C}
HP-III	94.1^{A}	187.6^{A}	149.4^{A}	431.2^{A}
HP-IV	96.3^{A}	164.9^{AB}	140.7^{A}	402.0^{B}

Table 6.I.6. Acidity (% of Lactic acid) of different treatment during milk ripening (milk pH 6.55), beginning of cooking (whey pH 6.35) and end of cooking (whey pH 6.00)

	Milk ripening	Whey: Cooking	
Treatment/pH	6.55	6.35	6.00
Raw	0.188	0.145^{B}	0.191^{A}
Past	0.185	0.148^{A}	0.190^{A}
MF	0.175	0.148^{A}	0.186^{A}
HP-I	0.188	0.153^{A}	0.207^{B}
HP-II	0.190	0.157^{A}	0.202^{B}
HP-III	0.183	0.150^{A}	0.203^{B}
HP-IV	0.187	0.157^{A}	0.210^{B}

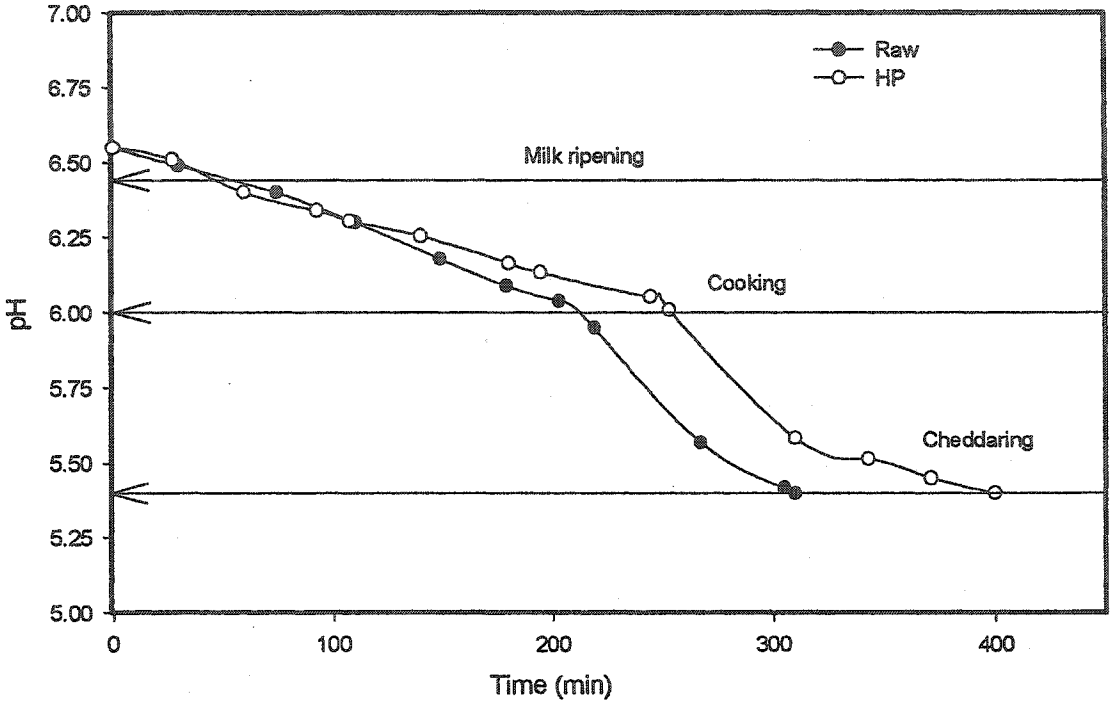


Figure 6.I.1. Typical curve of pH change during cheddar cheese making showing for raw and HP treated milk

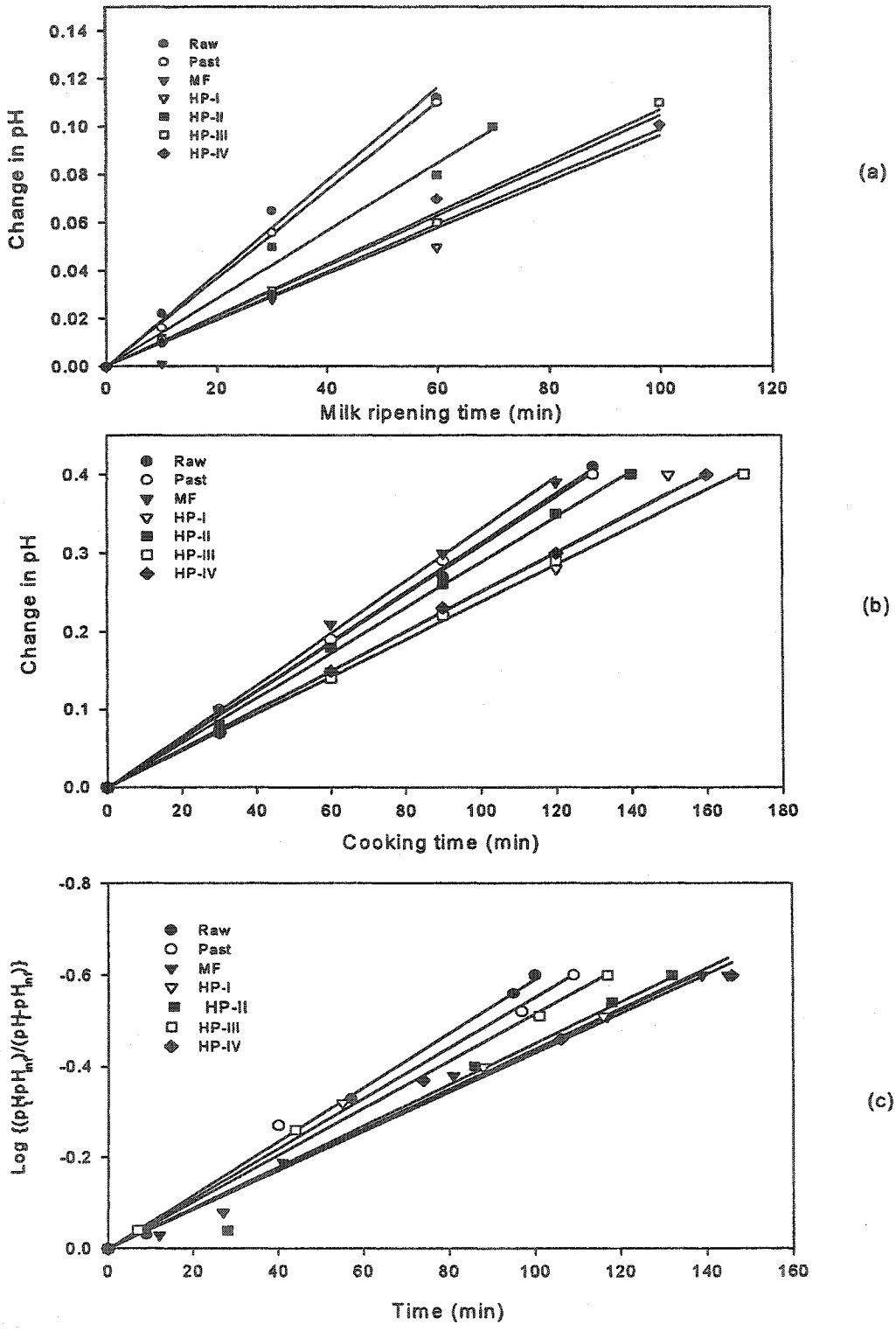


Figure 6.I.2. Change of pH (a) Milk ripening (b) Cooking and (c) Cheddaring process of cheese making affected by treatment of milk by HP, raw, pasteurization and microfiltration

PART-II

COMPOSITION, YIELD CHARACTERISTICS, MILK SOLID RECOVERY AND CUTTING TIME OF RENNET CURD DURING CHEDDAR CHEESE MAKING FROM RAW, PASTEURIZED, MICROFILTRED AND HIGH PRESSURE TREATED MILK

ABSTRACT

The composition of cheese, yield characteristics and milk solids recovery of Cheddar cheese made from raw, pasteurized, microfiltred, and four different high pressure (HP) treated milks (HP-I: 400MPa/32 min; HP-II: 400 MPa/48 min; HP-III: 300 MPa/60 min; HP-IV: 300 MPa/90 min) were monitored. In general cheeses from high pressure treated milk and pasteurized milk showed better fat, protein and total solids recovery compared to cheese from raw and microfiltred milk. Yield was higher in all pressure treated milks and comparable with pasteurized milk. Raw milk and MF milk cheese showed lower yield and solids recovery. Cheese from HP treated milk would have a potential in producing special functional cheeses such as low moisture cheese intended for slow ripening. The rennet curd cutting time reduced significantly ($p<0.05$) by HP treatment of milk.

INTRODUCTION

The moisture, composition and rheological characteristics play an important role in the final physical and sensory quality of cheddar cheese. Yield of cheese has economical significance and can be controlled to a great extent by varying manufacturing parameters.

High-pressure treatment of milk causes several protein modifications, such as micelle fragmentation and denaturation of β-lactoglobulin (López-Fandino *et al.*, 1998). These change the functional properties of milk affecting the cheese-making eventually leading to reduction in rennet coagulation time and to increased cheese yield, gel strength and resistance to syneresis (Johnston *et al.*, 1993; López-Fandino *et al.*, 1996). Cheddar cheese made from pressure treated milk was reported to have higher moisture content, which results in higher yields than from raw or pasteurized milk (Drake *et al.*, 1997). Trujillo *et al.* (2000) reported HP treated- milk cheeses showed higher yield, lower fat recovery and slightly higher nitrogen recovery than pasteurized cheeses. It has been also reported that pressure treatment of milk enhanced the water holding capacity of rennet curd (Trujillo *et al.*, 1997; Pandey *et al.*, 2000) which implicate higher moisture in cheese. However these studies provide no insight into the desired development of pH during the different stages of cheese making which may eventually affect many characteristics of cheese. The main objective of this part of the study was to investigate the effect of pressure processing of milk on moisture, yield and solids recovery of Cheddar cheese and compare them with cheese made from raw, pasteurized and microfiltred milk

MATERIALS AND METHODS

Milk preparation and analysis of cheese

The preparation of milk samples, high-pressure treatment and Cheddar cheese making process are described in Part I of this section. The moisture content of cheddar cheese was determined as prescribed by the AOAC method. The yield and solids recovery were obtained by the method described by St-Gelais *et al.* (1998) and Anderson *et al.* (1993). Cheese yield (Y_{act}) was expressed as kg of cheddar cheese per 100 kg of cheese milk. Cheese yield (Y_{act}) was also adjusted for any variation in the composition of milk on day-

to-day basis. Cheese yield (Y_{adj}) was also adjusted to 34% moisture content and was calculated as follows:

$$Y_{adj} = Y_{act} \times \frac{(100 - M_{act})}{(100 - M_{adj})} \qquad (6.II.1)$$

where M_{act} is the moisture (%) in actual cheese, and M_{adj} is the adjusted moisture (%) content (34%).

Fat (K_f) and protein (K_p) recovery were calculated as follows:

$$K_f = \left(\frac{F_c \times Y_{act}}{F_m} \right) \qquad (6.II.2)$$

$$K_p = \left(\frac{P_c \times Y_{act}}{P_m} \right) \qquad (6.II.3)$$

where F_c and P_c are the fat and protein (%) in cheese respectively, and F_m and P_m are the fat and protein (%) in cheese milk.

The composition (fat, protein, lactose and dry matter) of raw, pasteurized, microfiltred and high-pressure treated milk and whey obtained from the cheese made from these milks was determined using an infrared analyzer (Dairylab 2, Multispec Limited, Wheldrake, York, England). The dry matter of cheese was determined by air-drying at 100°C for 16 hrs. The evaluation of fat content in cheese samples was obtained by Mojonnier extraction procedure (Atherton and Newlander, 1977). The total protein was measured using the macro-Kjeldahl method (AOAC, 1995).

Statistical analysis

GLM procedure was performed to test the significance of the treatments. Duncan grouping test was also performed to differentiate between the treatments. The SAS® System software version 8.0 (1999) was used in all the statistical analysis.

RESULT AND DISCUSSION

Composition of cheese milk

Table 6.II.1 shows the average composition of milk used in cheese making after different technological treatments. The pressure treated milk sample showed no

significant difference except for the HP-III ($p<0.05$) which had slightly lower fat and total solids content than other pressure treated milk samples. Although statistically the composition of milk varied from one treatment to another, raw milk and high-pressure treated milk composition were hardly comparable. Major changes in the composition of cheese milk may influence several quality parameters of cheese (Guinee *et al.*, 1994; St-Gelais *et al.*, 1998).

Rennet curd cutting time

The effect of pressure processing of milk on coagulation characteristics has been discussed in Chapter III; however the actual cutting time was also monitored during the course of pilot scale Cheddar cheese making with milk treated with different technological methods (Figure 6.II.1). The reduction in cutting time was evident in the high-pressure treated samples compared to other controls such as raw, pasteurized and micro-filtered milk. The cutting time of different pressure treated samples were not significantly different. In addition, Raw, MF and Past were not significantly different ($p>0.05$) with respect to cutting time; however, the pressure treated samples were significantly different ($p<0.05$) from these three. Lowering of cutting time in pressure treated samples was due to enhancing of coagulation properties due to pressure treatment (Chapter III; Johnston, 1995; Buffa *et al.*, 2001a, b).

Composition of cheese

The composition of cheddar cheese made from raw, pasteurized, microfiltred and high-pressure treated milk are shown in table 6.II.1. Fat content of cheddar cheese varied from 32.06-35.84%, the highest fat percentage was reflected in microfiltred milk whereas the lowest one was with raw milk. Over all MF and Pasteurized milk cheese showed fairly better performance than raw milk in solid recovery. In the MF milk cheese, fat is separated before MF process and cream is added after MF process, which may have changed the state and size of fat globules affecting the fat retention in the cheese. Pressure treated samples have similar fat content except for HP-II which was slightly higher in fat content. Higher fat content of HP treated milk cheese is obvious due to the crystallization of milk fat enhanced by HP treatment. Pressure affects the

crystallization properties of fats since the specific volume of liquid fat decreases during solidification, the melting points of fats increasing approximately by 0.2°C per MPa (Trujillo, *et al.*, 1997; Datta and Deeth, 1999).

It has been recently been reported that under pressure of 100 to 500 MPa at 23°C for 1 to 15 min the fat phase within the natural milk fat globules can be forced to crystallize at temperatures at which the fat phase remains normally liquid because of the supercooling properties of emulsified fat phase (Buchhein, and EI-Nour, 1992). Fat crystallization increases with the length of HP treatment and crystal formation further increases during storage after pressure release (Yasuda and Mochizuki, 1992). Therefore solid milk fat is retained in the cheese rather than liquid fat lost in whey. In our studies there was no significance difference among the pressure treated milk cheese ($p>0.05$) except of HP-II. The effect of holding time in crystallization effect which should eventually reflect in fat content of cheese is not evident here as the holding time effect probably diminishes after few minutes of holding (holding time of milk processing varied from 32 minutes to 90 minutes at two pressure level).

Moisture content of cheese and moisture in non-fat substance differed considerably (Table 6.II.1) as the fat content varied between different treatments. It is important to deal with the amount of water in the fat free cheese, rather than with the absolute water content of the cheese (Pearce and Gilles, 1979). Higher moisture content in pasteurized milk cheese (MFFS) shows that the denatured whey proteins are responsible for enhanced water holding capacity. HP treated milk cheese clearly showed lower moisture content in contrast with previous reports (Lopez-Fandino *et al.*, 1996). As previously reported, pressure treatment enhanced the water-holding capacity of cheese (Pandey *et al.*, 2000). However the study quoted earlier (Lopez-Fandino *et al.*, 1996) either were conducted only up to the curd or did not consider the pH development at different stages of cheese making. The present detailed study on cheddar cheese making with the treated milks demonstrate that HP treated milk require higher cooking and holding time (prior to whey off) to attain the desired pH, which explains the low moisture content in the cheese. Higher moisture content of MF treated milk cheese can be explained by the Table 6.I.2, which demonstrates shorter cooking and holding (faster rate).

Protein content of cheeses did not differ significantly with different HP treatments, but if there was a significant difference in the initial protein in cheese milk, it was reflected in the cheese (MF cheese, Table 6.II.1). However, the total protein recovery has a higher score in pressure treated and pasteurized milk cheeses probably due to the pressure and heat denaturation of whey protein as compared to controls samples. This is also evident from the lower loss of protein in whey for pressure treated samples than other controls. The degree and extent of denaturation always depend upon the pressure level and duration of treatment (Lopez-Fandino *et al.*, 1996; Hinrichs *et al.*, 1996). The pH of the cheese on day-1 is also indicated in Table 6.II.1. Pressure treated milk cheese showed same pH ($p<0.05$) except for HP-I, which is minimum treatment at 400 MPa. The increase in pH on day-1 showed a trend with increase in process severity, especially with increase in holding time at the same pressure level. Although these numbers do not show a significant difference between the HP process treatments (HP-II, III, IV), numerically there is an increasing trend with increase in severity. This can be explained by the fact that the HP treatment releases calcium from colloidal calcium phosphate and convert it into soluble calcium. Apart from going into solution, some calcium and magnesium stick with the curd causing higher pH with increased pressure level.

Whey composition

The composition of the whey obtained after cheese making from different technologically treated milk is given in Table 6.II.1. The composition of whey is an indirect measure of the cheese making properties of milk. The fat loss in whey was higher in raw milk cheese followed by whey of microfiltred milk and high pressure treated samples. Pasteurized milk showed similar value as of HP-I. Although these values changed considerably from one treatment to another, they do not necessarily lead to a meaningful conclusion because of the fact that the starting fat content of milk fat also slightly varied. However HP-I, HP-II and HP-IV can be compared as their respective initial fat percentage did not show significant difference ($p>0.05$). The loss of fat in whey of HP treated samples showed no difference ($p<0.05$). Protein loss in whey of three pressure treated samples were same (HP-I, II, III) but HP-IV was significantly different which could be explained as this treatment lasted for 90 minutes and eventually may have

resulted in slightly higher level of denaturation. Lower level of protein loss in whey (Table 6.II.1) reflects the extent of whey protein denaturation. Higher protein content of in whey of raw milk (0.94%) indicates lack of association of whey protein in cheese.

Yield and solids recovery

The analysis of variance for yields and milk solids recovery is shown in table 6.II.2. The treatment means are compared and the same letter indicates that the treatment had no effect on yield parameters. The actual yield, which is kilograms of cheese obtained per 100kg of milk irrespective of their composition. Adjusted yield was obtained as the kilogram of cheese per 100 kg of milk for 34% final cheese moisture content. The adjusted yield was higher for all pressure treated samples and was comparable with pasteurized milk. The microfiltred milk and raw milk showed poor yield due to loss of milk solids in whey. Table 6.II.2 also indicates different milk solids recovery in cheese. Fat recovery was higher in cheeses made from pressure treated milk. However HP-III showed highest level of fat recovery followed by pasteurized milk. All HP treated milk cheeses and pasteurized milk cheese showed similar protein recovery, and raw and MF milk cheese showed lower recovery due to higher loss of whey proteins. In case of total solids recovery, pasteurized milk was rated highest followed by HP treated samples. There was no significant difference between the different pressure treated milk cheeses ($p>0.005$). Slightly lower TS recovery score in HP treated milk cheese can be explained due to fact that some minerals released from colloidal state to soluble state may have been lost through the whey (Schrader *et al.*, 1997).

CONCLUSION

Attempt was made to evaluate the cheese making process of high-pressure treated milk and compare them with cheese made from raw, pasteurized and microfiltred milk. HP treatment of milk has demonstrated that pressure-induced changes in the functional properties of milk cause several changes in cheese making properties. The pressure treated and pasteurized samples demonstrated better recovery of fat and protein compared with the cheese from raw and microfiltred milk. High-pressure treatment shows potential for pròducing low moisture cheese intended for slow ripening. A careful consideration would require modification of the manufacturing process in order to get the desired quality of cheese. Cheese from HP treated milk also showed better milk solids recovery and lower moisture.

Table 6.II.1. Composition of cheese milk, Cheddar cheese and whey

	Treatments						
	Raw	Past	MF	HP-I	HP-II	HP-III	HP-IV
Cheese milk							
Fat, %	4.02^{B}	3.71^{E}	4.06^{A}	3.98^{C}	3.99^{C}	3.81^{D}	3.97^{C}
Protein, %	3.32^{A}	3.30^{A}	3.11^{C}	3.30^{A}	3.30^{A}	3.32^{A}	3.31^{A}
Total solid, %	12.88^{A}	12.38^{D}	12.46^{DC}	12.76^{AB}	12.75^{AB}	12.63^{BC}	12.77^{AB}
Cheddar cheese							
Fat, %	32.33^{D}	32.06^{D}	35.84^{A}	34.37^{C}	35.00^{B}	34.38^{C}	34.31^{C}
Protein, %	23.34^{B}	23.71^{B}	22.44^{C}	24.54^{A}	24.67^{A}	24.75^{A}	24.95^{A}
Moisture, %	35.31^{A}	35.92^{A}	34.68^{B}	33.67^{C}	32.87^{D}	33.47^{C}	33.21^{C}
FDM[1]	49.97^{D}	50.03^{D}	54.86^{A}	51.52^{BC}	52.14^{B}	51.68^{BC}	51.37^{C}
MFFS[2]	52.18^{C}	52.87^{B}	54.05^{A}	51.30^{D}	50.57^{E}	51.02^{D}	50.56^{E}
pH[3]	5.13^{BC}	5.10^{C}	5.23^{AB}	5.17^{BC}	5.23^{AB}	5.22^{AB}	5.32^{A}
Whey							
Fat, %	0.46^{A}	0.30^{C}	0.43^{B}	0.31^{C}	0.30^{C}	0.26^{D}	0.32^{C}
Protein, %	0.94^{A}	0.81^{C}	0.86^{B}	0.75^{D}	0.74^{D}	0.75^{D}	0.69^{E}
Total solid, %	7.28^{A}	7.02^{B}	6.97^{C}	7.01^{B}	7.00^{B}	6.99^{B}	6.89^{D}

Table 6.II.2 Yield and component recovery of cheddar cheese made with raw, pasteurized, microfiltred and high-pressure treated milks.

	Treatments						
	Raw	Past	MF	HP-I	HP-II	HP-III	HP-IV
Yield							
Actual yield, %	9.94^{A}	9.63^{B}	9.45^{C}	8.95^{E}	8.96^{E}	9.12^{D}	9.53^{C}
Adjusted yield,%	9.44^{B}	9.66^{A}	9.35^{B}	9.75^{A}	9.77^{A}	9.67^{A}	9.70^{A}
Solid recovery							
Fat recovery, %	77.21^{D}	85.84^{AB}	83.65^{C}	83.91^{C}	84.12^{BC}	86.38^{A}	82.86^{C}
Protein recovery, %	67.39^{B}	71.36^{A}	68.32^{B}	71.89^{A}	72.46^{A}	71.38^{A}	72.29^{A}
TS recovery, %	48.24^{D}	51.42^{A}	49.66^{C}	50.25^{BC}	50.49^{B}	50.42^{BC}	50.10^{BC}

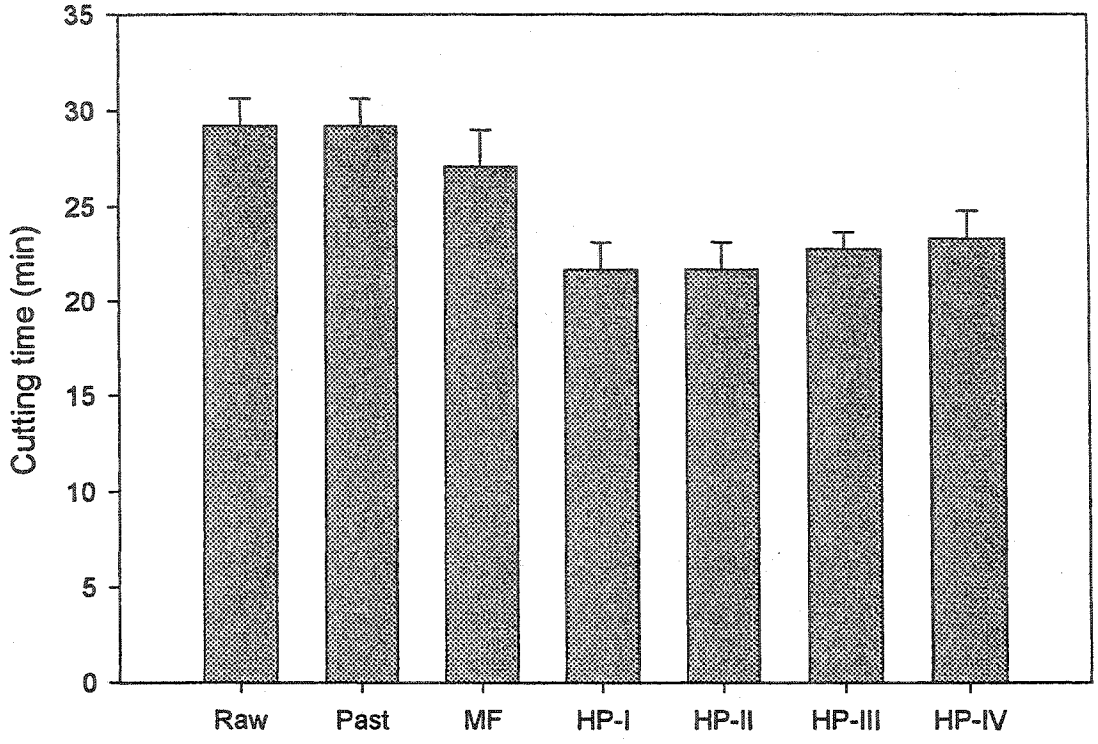


Figure 6.II.1 Cutting time required for raw, pasteurized, microfiltered and HP treated milks.

CHAPTER-VII

RIPENING CHARACTERISTICS OF CHEDDAR CHEESE MADE FROM RAW, PASTEURIZED, MICRO-FILTERED AND HIGH-PRESSURE TREATED MILK

CONNECTING STATEMENT

This chapter highlights the ripening characteristics of cheddar cheese made from raw, pasteurized, micro-filtered and HP treated milk. Proteolysis and microbial changes were monitored throughout ripening period.

Part of results of this chapter were presented at two scientific conferences:

Pandey, P.K.; Ramaswamy, H.S. and St-Gelais, D. (2001) Proteolysis of Cheddar cheese manufactured from high-pressure treated milk, microfiltred milk, raw milk, and pasteurized milk. IFT IFT New Orleans, June 23-27, 2001.

Pandey, P., H.S. Ramaswamy, and D. St. Gelais. (2001) Effect of High Pressure Treatment of Milk on Cheddar cheese Ripening. Paper presented at the Agricultural and Food Engineering Conference, St. Hyacinthe, PQ. March 21, 2001.

Research was carried out by the candidate under the supervision of Dr. H.S. Ramaswamy and Dr. D. St. Gelais. The pilot scale Cheddar cheese manufacturing, experimental work and analysis were carried out at Food Research and Development Center (FRDA), Saint-Hyacinthe, Quebec under supervision of Dr. St-Gelais and Mr. Gaétan Bélanger.

RIPENING CHARACTERISTICS OF CHEDDAR CHEESE MADE FROM RAW, PASTEURIZED, MICRO-FILTERED AND HIGH-PRESSURE TREATED MILK

ABSTRACT

Cheddar cheese was made from four pressure treated milk (400 MPa, 32 min and 48 min; 300 MPa, 60 min and 90 min) along with three controls (raw, pasteurized and microfiltred milk). Microbial analysis (coliform, PCA and psycrotroph) of treated and untreated milk samples prior to cheese making was carried. Microbial changes (lactococci and lactobacilli) during ripening period (1 to 90 days) and proteolysis (fractions of water, TCA and PTA-soluble nitrogen) were also monitored (up to 300 days). The pressure treated milk showed absence of coliform and psycrotrophs indicating that these organisms were pressure sensitive. The lactococci count in HP treated milk cheese was lower than controls and the lactobacilli count was higher compared to pasteurized and microfiltered milk cheese, but comparable with raw milk cheese. The WSN:TN and TCA:TN content in HP treated milk was significantly lower than controls in the early stages of ripening, but gradually increased during later stages. The PTA:TN was significantly higher in pressure treated sample throughout ripening and was comparable with raw milk cheese at the end of ripening period, indicating better flavor characteristic in pressure treated milk cheese. The PTA:TN content in HP treated sample was influenced by pressure level and holding time. Holding time influence was more pronounced than pressure level.

INTRODUCTION

Traditionally cheeses made from raw milk tend to develop a strong flavor and generally ripen faster than cheeses made from pasteurized milk (Wilson *et al.* 1945; Scarpallino and Kosikowski, 1962; Franklin and Sharpe, 1962; Melachouris and Tuckey, 1966; Price and Call, 1969; Kristoffersen, 1985; Banks *et al.*, 1986). On the other hand, changes in cheese milk associated with pasteurization such as denaturation of indigenous enzymes, partial denaturation of whey proteins and their interaction with casein, as well as destruction of thermolabile indigenous microflora, including nonstarter lactic bacteria (Lau *et al.*, 1991; McSweeney *et al.*, 1993) lead to a product with a different quality. Raw milk cheeses have been found to possess unique flavor and textural characteristics not obtainable in cheese from pasteurized milk. However, in spite of strict control of conditions on farm and handling of milk, there have been several cases of cheese-borne illnesses worldwide. High pressure (HP) processing, an emerging alternative food preservation technique, holds out a promising technology for this conundrum. High pressure processing has good potential in the production of cheese, however it affects the cheese making properties (Lopez-Fandino and Olano, 1998; Pandey *et al.*, 2000) affecting the cheese quality, (Messens *et al.*, 1999; Molina *et al.*, 2000; Trujillo *et al.*, 2000).

It has been shown that non-starter lactic acid bacteria (NSLAB) in raw milk cheese are responsible for intense flavor (McSweeney *et al.*, 1993). Proteolysis is one of the principal biochemical events during ripening of cheese and plays a vital role in the development of texture as well as the flavor. The index of proteolysis of cheese is generally measured as the fraction of water soluble nitrogen (WSN), 12% trichloroacetic acid (TCA) soluble nitrogen and 5% phosphotungstic acid (PTA) soluble nitrogen, as percentage of total nitrogen. These indices can be very well related to the flavor development and ripening in cheese (Aston *et al.*, 1983; Pham and Nakai, 1984). In addition to biochemical analysis microbial analysis provides a good basis for understanding the biochemical changes during ripening.

The main objective of this study was to investigate the effect of high pressure processing of milk on proteolytic characteristics of cheddar cheese during ripening and compare these attributes with cheese made from raw, pasteurized and microfiltred milk, as well as evaluate the survival of microorganisms (Lactococci and Lactobacilli)

MATERIALS AND METHODS

Details of milk selection, treatments and cheese-making are described in the previous chapters.

Assessment of Proteolysis

Water-soluble nitrogen (WSN) and 12% TCA soluble nitrogen (TCAN) from cheese samples were estimated by following the method of Kuchroo and Fox (1982). 5% PTA soluble nitrogen (PTAN) estimated as per the method suggested by Jarret *et al.* (1982). All the fractions were analyzed for nitrogen content in duplicate by macro-Kjeldahl method (AOAC, 1982). Samples were taken at day 14, 30, 60, 90 and 300 for proteolysis.

Microbiological analysis

Cheese samples were analyzed for lactobacilli count on selective acidified MRS agar (Difco Laboratories, Detroit, MI) and for Lactococci count on M17 agar (Difco Laboratories, Detroit, MI). Both selective enumerations were done anaerobically and plates were incubated at 30°C for 48 hours. Samples were drawn for microbiological enumeration at the same interval as of proteolysis analysis only up to day 90. The total microbiological count of milk was enumerated on plate count agar (PCA) incubated at 30°C for 48 hours and coliform count on Violet red bile agar (VRBA Difco) with aerobic incubation at 37°C for 24 hrs.

RESULTS AND DISCUSSION

Microbiological analysis of cheese milk

Table 7.1 shows total bacterial and coliform counts for the different cheese milk. The total count was lower in MF than pasteurized and pressure treated milk samples. No sample contained coliform and psychrophilic bacteria except raw milk. However the residual count or microbial destruction in HP treated milk depended on the severity of the treatment. Table-7.1 indicated that the *E. coli* and psychrophilic organisms tested negative in all pressure treated samples. Gervilla *et al.* (1999) studied the kinetics of

destruction of *E. coli* and *Pseudomonas fluorescences* in ewe's milk by high-pressure treatment and reported that these microflora are pressure sensitive. Therefore it can be concluded that psychrophilic and coliform bacteria are in general pressure sensitive, as *Pseudomonas fluorescences* represents most of the Pseudomonas spp as well as psychrotrophic bacteria, suggesting psychrotrophic presence in milk as indicator organism (Gervilla *et al.*, 1997; Gervilla *et al.*, 2000) for efficiency of a given process.

Microbial changes during ripening

The changes in the lactococci numbers throughout ripening (90 days) are shown in figure 7.1. Figure 7.1a shows lactococci count in raw, pasteurized and microfiltred milk cheeses, and Figure 7.1b shows lactococci count for all four HP treated milk cheeses. Similarly, Figure 7.2 depicts the numbers of lactobacilli.

It is evident that lactococcal count at the beginning of ripening was higher in control cheeses (raw, past and MF) compared to the HP treated samples, and continued to persist throughout the period studied. The lower count in HP treated milk cheese compared to controls is probably due to longer holding of HP treated samples during cooking period (lower pH and higher temperature for longer time) that restricted the growth or caused the death of starter bacteria. The starter cells are the major source of extracellular proteinases, and intracellular peptidases (Law and Kolstd, 1983; Fox *et al.*, 1996). Growth of lactococci ceases shortly after the end of curd production (Martley and Lawrence, 1972, Visser, 1977) and their intracellular enzymes are ineffective until the cells die and lyse. The primary role of lactococcal proteinases in cheese appear to be the hydrolysis of large and intermediate-sized peptides produced from casein by chymosin or plasmin. The prolonged cooking of HP-treated milk curd could lead to lysis of a larger number of starter bacterial cells, releasing the enzymes .

In general, the mesophilic lactobacilli count in Cheddar cheese has been reported to be around 10^1-10^3 cfu/g. HP treated milk cheese showed higher count of lactobacilli, which may be due to fact that the higher aminopeptidase activity in HP treated milk cheese (discussed later in PTA soluble fraction in proteolysis) caused further breakdown of peptides resulting in higher quantities of amino acid and small peptides that are required for the growth of Lactobacilli. Many mesophilic lactobacilli grow very poorly in

milk and they require supplementation with a source of small peptides and amino acids such as, yeast extract (Fox and McSweeney 1996).

Proteolysis

Water soluble nitrogen (WSN)

The WSN as percentage of total nitrogen of all HP treated and control cheese sampes is shown in Table 7.2. The WSN fraction increased from approximately 4.7% to 24.8% for all cheeses during ripening period. The principal agents for liberation of WSN in Cheddar cheese are plasmin and the coagulating agent (Fox *et al.*, 1994). The difference in the extent of their hydrolyzing action depends on the access to the substrates (different casein fractions). Different processing treatments lead to varying degrees of interaction between the whey proteins and casein fractions, causing physical changes that impede the casein-specific proteolytic activity of chymosin and plasmin (Hill *et al.*, 1974; Grufferty and Fox, 1988; Fox, 1989; Singh, 1992; Fox and Stepaniak, 1993; Fox *et al.*, 1994; Beuvier *et al.*, 1997). Hence, it is reasonable to expect that the amount of WSN fraction varies between raw and treated milks. Besides, heat treatment is found to enhance plasmin activity (Richardson, 1983; Benefeldt *et al.*, 1997), suggesting higher WSN content in cheese from pasteurized milk.

Among pressure treated samples, initially HP-IV showed lower WSN compared to HP-III, II and I. Dissociation of caseins, particularly κ- and β-caseins, also occurs under high pressure (Lopez-Fandino *et al.*, 1998). It has been demonstrated by these authors that higher the pressure, lower the casein particle size (up to 700 MPa), with modification of the micellar structure from spherical to chains or clusters. On the other hand, HP treatment of milk for 400-600 MPa for shorter periods accelerated proteolysis in milk during subsequent incubation, due to apparent barostability of plasmin activity combined with extensive pressure-induced casein micellar disruption (Scollard *et al.*, 2000). Therefore, higher amount of WSN from the beginning of the ripening period is expected in cheese made from pressure treated milk.

However, the results show an interesting tendency for the HP-IV to exhibit the highest level of proteolysis with increasing ripening. It is also very clear that the holding time has a significant influence on the rate of WSN production. It was not within the

scope of this work to investigate the molecular dynamics in detail. However, it can be suggested that the holding time in high pressure processing significantly influences the spectra of flavour compounds that result during ripening and the phenomenon deserves a detailed study.

TCA (12%) soluble fraction

Table 7.3 shows the effect of different treatments on TCA (tri-chloro acetic acid) soluble nitrogen (TCAN) of cheese during different stages of ripening. The TCAN (expressed as percentage of total nitrogen) increased between 2.91% up to 15.35% during ripening in all cases.

The trend in the results indicate the importance of microflora as well as the treatment-induced changes in milk for development of cheese flavour in form of small peptides and amino acids. MF milk, from which the bacterial cells are physically removed, shows distinctly lower amount of TCAN. The difference between the pressure-treated samples and raw milk samples (as well as pasteurized samples) indicates the favourable effect of high pressure processing in flavour development. Even though the bacterial cells (NSLAB) are affected by high pressure processing, the enzymes contained in their cells are released to the cheese matrix for utilization in proteolysis during ripening. It is also clear that the duration of pressure treatment (HP-IV) has a significant influence on the ripening process.

PTA(5%) soluble fraction

The PTA (phopho tungustic acid) soluble nitrogen fraction (PTAN) expressed as percentage of total nitrogen is presented in table 7.4. HP-IV samples showed a distinct trend with the highest PTAN content among all samples. However, the cheese from raw milk developed progressively increasing amounts of PTAN mid way during the ripening compared to other samples. It is also clear that longer holding time favored higher PTAN development in cheese in case of the pressure-treated samples. The higher amount of PTAN in pressure-treated cheese is in agreement with the results of Trujillo *et al.*, (1999)

who reported higher free amino acids in pressure-treated goat milk cheese than pasteurized milk cheese.

The PTAN mostly consists of free amino acids and very small peptides, and the presence of amino acids in cheese clearly indicates aminopeptidase activity. Since these enzymes are intracellular, their action indicates bacterial cell lysis (Fox and McSweeney, 1996). Proteolysis during ripening is a complex process controlled by many factors such as the amount of enzymes and the availability of their respective substrates. The proteolytic system of starter and non starter bacteria can be roughly divided into extra-cellular and intra-cellular proteinases and peptidases: extra-cellular ones may be bound either to the cell wall or to the cell membrane. Because of this relatively fixed location in the bacterial cell, starter enzymes can in some way be considered to be immobilized enzymes, at least as long as the cell remains intact. But even if the cells have undergone lysis, the cell content do not necessarily leak into the medium as they are embedded in the medium and surrounding medium is restricted by the compact casein matrix (Visser, 1993, Thomas and Pritchard, 1987). Therefore, induced lysis takes long time to release the intracellular enzyme in cheese because of casein network. In this case the pasteurization would have destroyed many NSLAB and the thermally dead cells also lose ability to produce enzymes like naturally dead cells. High pressure treated milk cheeses showed higher PTA soluble may be due to the release of bacterial cell fluid cytoplasm (from NSLAB) during pressure treatment (Smelt *et al.*, 1994) which contains intracellular amino peptidase enzymes. The release of cytoplasmic fluid due to pressure treatment may have resulted in the association with aminopeptidase and casein micelles resulting faster conversion of peptides to amino acids It is clearly agreed that high pressure processing leads to conditions favorable for proteolytic activity. However, the details of the relationship at the molecular level needs a closer study for better understanding.

CONCLUSIONS

The results of the present study indicate that maturation indices of cheddar cheese can be enhanced by using HP treatment of milk. A combination of influential factors lead to a product that matures slowly in comparison with cheese made from raw or pasteurized milk, but resulting in higher content of proteolysis products. The ability of the HP process to modify the proteinaceous substrates and destroy bacteria without losing their beneficial compounds, as well as retain (and in some cases, even enhance) the enzymes in cheese milk, can be effectively harnessed by the cheese industry. The elucidation of the chemical changes and processes at the molecular level is required for effective utilization of the results obtained during this study for commercial application.

Table 7.1 Profile of microflora in the cheese milk (samples drawn from the vat).

Milk	Total count Log (cfu/mL)	Psychrophilic count Log (cfu/mL)	Coliform count Log (cfu/mL)
Raw milk	5.15	2.94	2.85
Pasteurized	2.66	ND	ND
Microfiltred	2.10	ND	ND
HP-I	2.97	ND	ND
HP-II	2.78	ND	ND
HP-III	3.13	ND	ND
HP-IV	2.95	ND	ND

ND: Not detected

Table 7.2 Effect of HP processing of milk on proteolysis of cheddar cheese – Water soluble nitrogen expressed as percent fraction of total nitrogen for different treatments.

HP treatments and Controls	14 Days	30 Days	60 Days	90 Days	300 Days
Raw	7.80 ^{B}	9.78 ^{B}	13.50 ^{B}	17.11 ^{C}	22.26 ^{C}
Past	8.43 ^{A}	10.72 ^{A}	13.72 ^{A}	17.99 ^{B}	23.36 ^{B}
MF	7.21 ^{B}	8.18 ^{C}	12.42 ^{C}	15.54 ^{D}	19.92 ^{D}
HP-I	7.45 ^{B}	8.21 ^{C}	13.31 ^{B}	15.22 ^{D}	21.91 ^{C}
HP-II	7.36 ^{B}	8.01 ^{C}	13.22 ^{B}	15.27 ^{D}	22.39 ^{C}
HP-III	5.27 ^{C}	7.86 ^{CD}	12.33 ^{C}	17.60 ^{B}	22.89 ^{C}
HP-IV	4.67 ^{D}	7.08 ^{D}	12.24 ^{C}	19.97 ^{A}	24.85 ^{A}

Table 7.3 Effect of HP processing of milk on proteolysis of cheddar cheese - 12% TCA soluble nitrogen expressed as percent fraction of total nitrogen for different treatments.

HP treatments and Controls	14 Days	30 Days	60 Days	90 Days	300 Days
Raw	3.91 ^{A}	4.38 ^{B}	7.60 ^{A}	10.50 ^{A}	14.12 ^{BC}
Past	4.08 ^{A}	5.27 ^{A}	6.64 ^{B}	9.28 ^{B}	14.06 ^{BC}
MF	3.61 ^{B}	4.25 ^{B}	5.69 ^{C}	6.08 ^{D}	11.78 ^{D}
HP-I	3.47 ^{BC}	4.12 ^{BC}	6.29 ^{B}	7.17 ^{C}	14.43 ^{B}
HP-II	3.31 ^{C}	3.87 ^{CD}	6.25 ^{B}	7.22 ^{C}	14.04 ^{BC}
HP-III	3.08 ^{D}	3.77 ^{D}	6.38 ^{C}	7.23 ^{C}	13.68 ^{B}
HP-IV	2.91 ^{D}	3.80 ^{D}	6.14 ^{BC}	9.21 ^{B}	15.35 ^{A}

Table 7.4 Effect of HP processing of milk on proteolysis of cheddar cheese – 5% phosphotungstic acid soluble nitrogen expressed as percent fraction of total nitrogen for different treatments.

HP treatments and Controls	14 Days	30 Days	60 Days	90 Days	300 Days
Raw	0.37 ^{E}	0.57 ^{E}	1.67 ^{B}	2.08 ^{B}	4.27 ^{B}
Past	0.31 ^{E}	0.55 ^{E}	0.93 ^{E}	1.68 ^{D}	2.92 ^{E}
MF	0.33 ^{E}	0.84 ^{C}	0.91 ^{E}	1.25 ^{E}	3.07 ^{D}
HP-I	0.63 ^{D}	0.71 ^{D}	1.67 ^{B}	1.88 ^{C}	3.47 ^{C}
HP-II	0.71 ^{C}	0.87 ^{C}	1.44 ^{C}	1.86 ^{C}	3.56 ^{C}
HP-III	0.87 ^{B}	0.96 ^{B}	1.40 ^{D}	1.96 ^{C}	4.35 ^{B}
HP-IV	1.32 ^{A}	1.61 ^{A}	2.20 ^{A}	3.39 ^{A}	5.45 ^{A}

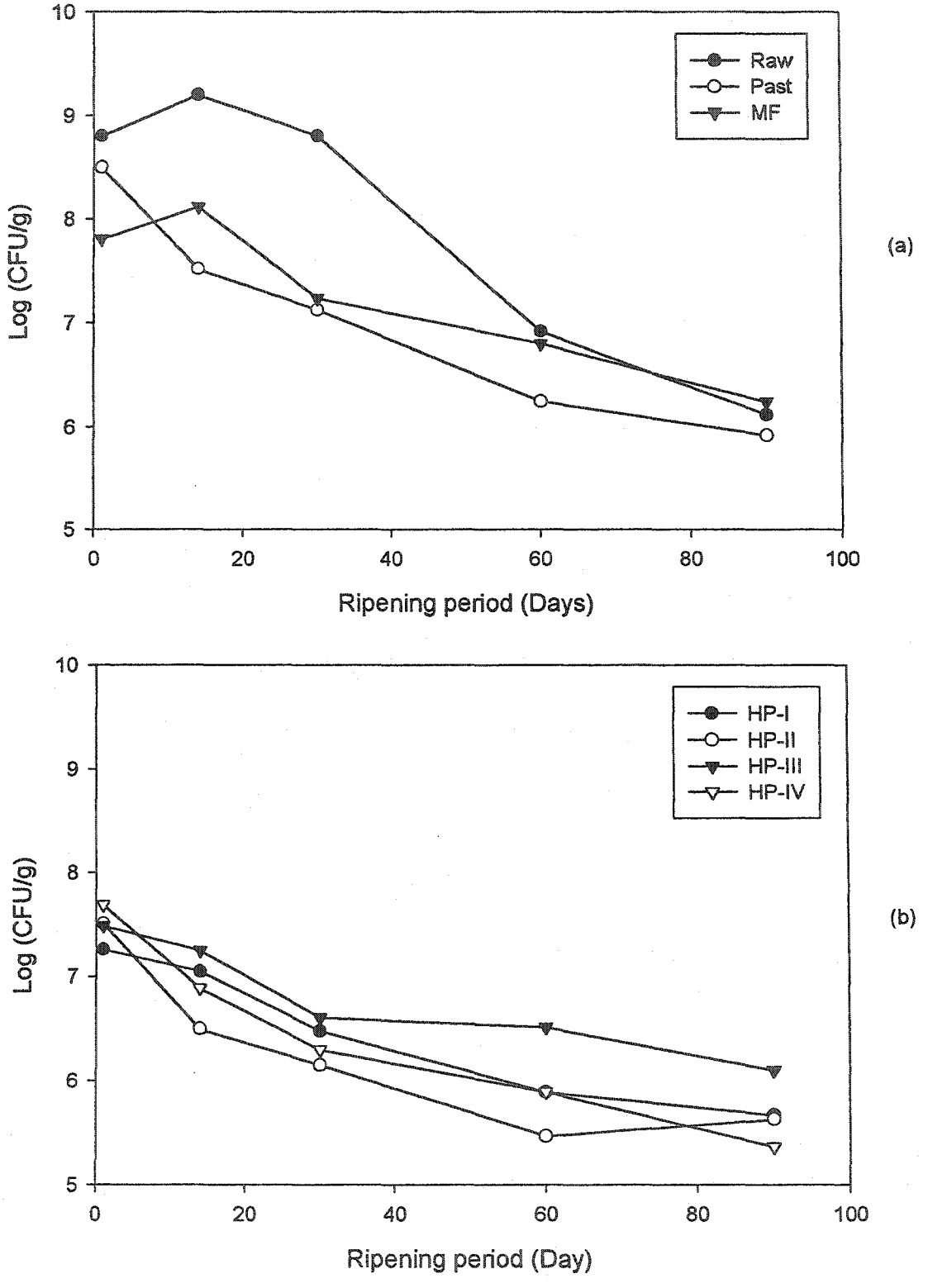


Figure 7.1 Lactococci counts during ripening of Cheddar cheese made from (a) Raw, Pasteurized and Microfiltered milks and (b) different high pressure treated milks.

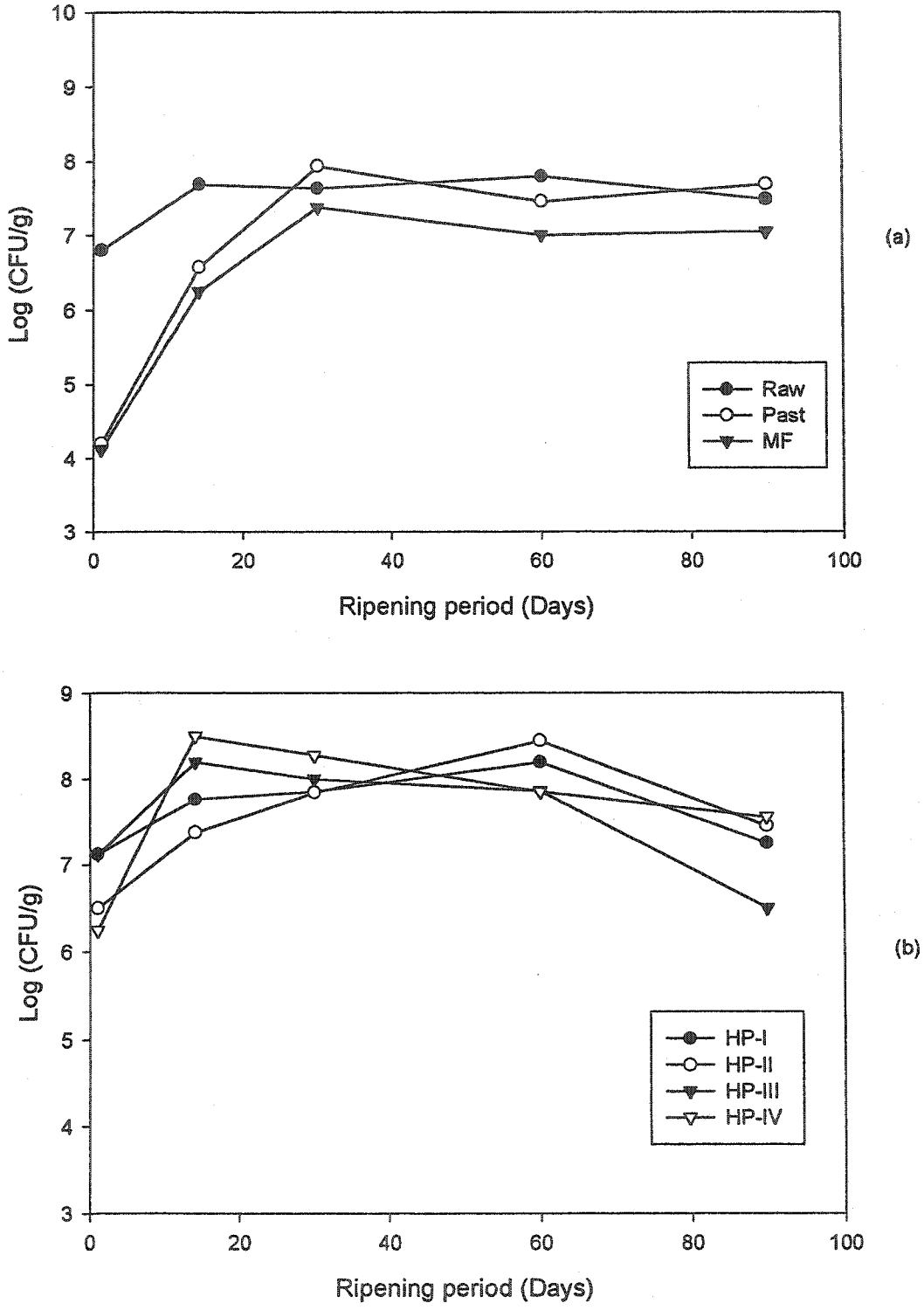


Figure 7.2 Lactobacilli counts during ripening of Cheddar cheese made from (a) Raw, Pasteurized and Microfiltered milks and (b) different high pressure treated milks.

CHAPTER VIII

GENERAL CONCLUSIONS

The main objective of this research project was to evaluate the potential for the application of high pressure (200-400 MPa) processing of milk for production of consumer safe cheddar cheese and evaluate if HP processing can produce raw milk like characteristics. This research was carried out in two stages- the first stage focused on the evaluation of effect of high pressure processing on the cheese making properties of milk, kinetics of HP destruction of micro-organisms and inactivation of enzymes, and the development of the process (pressure, holding time and temperature) for production of cheddar cheese from HP treated milk to produce safe and better quality cheese. The second part of the research highlights the manufacturing characteristics of cheese made from raw, pasteurized, micro-filtered and HP treated milks, and evaluation of changes associated with the cheese through the ripening period.

Coagulation properties of milk affected by pressure treatment

The coagulation of milk is the first and important step in cheese making. The effect of high pressure (HP) treatment of milk on rennet coagulation properties were evaluated. Three coagulation parameters - lag time, mean coagulation rate, and inflexion time (time for reaching the point of maximum coagulation rate) were evaluated as a function of pressure (200-400 MPa), temperature (3-21°C) and holding time (10-110 min) using a response surface methodology. Higher pressure levels and lower temperatures significantly decreased the lag time and time at inflexion point, and increased the coagulation rate. The pressure treatment, in contrast to heat treatment, improved the rennet coagulation characteristics of milk, although a similar phenomenon (probably not the same mechanism) occurs during pressure treatment of milk i.e. denaturation of β-lactoglobulin and interaction with casein micelles. High pressure treatment of milk causes disintegration of casein micelle resulting in reduced micelle size. In general, with an increase in pressure, holding time and a decrease in temperature, the lag time decreased, the mean coagulation rate increased and inflexion time decreased

Once the milk is coagulated by rennet the curd characteristics (gel strength and water-holding capacity) play an important role in further manufacturing of cheese. The second part of the study in cheese making properties involved evaluation of HP treatment effect on water-holding capacity (WHC) and gel strength of rennet curd. The water holding capacity of rennet curd increased with increase in pressure and holding time however decrease with decrease in pressure treatment temperature.

In general, with a decrease in pressure level, temperature or holding time, there was an increase in gel-strength of rennet curds. As pressure level or holding time increased, the hydration capacity of the gel network increased resulting in increased binding of water, effectively increasing the WHC and decreasing the GS. Also higher degree of whey protein denaturation and exposure of more hydrophobic groups at lower temperatures may increase the severity of pressure effect resulting in association and aggregation (with the conversion of protein bound water into highly compressed free water) within the protein molecules than binding with water resulting higher gel strength at lower temperature.

Pressure destruction kinetics of indigenous micro-flora and E. coli

In order to insure the safety of cheese manufactured from HP treated milk, the HP destruction of kinetics of E. coli and natural microflora in milk were studied. In the range of 250-400 MPa, 0-80 min holding time between 3 and 21°C, the study demonstrated and confirmed the dual effect pressure destruction of microorganisms with an instantaneous destruction effect attributable to the pressure pulse and a subsequent first order rate destruction (during pressure hold) with characteristic kinetic parameters such as D and k values. Pressure dependence of kinetic parameters was well described by both pressure death time and Arrhenius models. Higher pressures, longer holding times and lower temperatures resulted in larger destruction of microorganisms, and *E. coli* was more pressure sensitive than indigenous micro-flora. It should be noted that a pressure hold process includes the effect of a pressure pulse effect, although multiple pulses can be given without any holding time. It is desirable to quantify the pressure pulse effect when its contribution is significant.

Two parameters were derived to compare the pressure pulse with pressure hold results. IPK describes the pressure pulse effect while the D value indicates a measure of the pressure hold effect. Since IPK represents a logarithmic reduction in microbial population due to a single pulse, its equivalence of D value can be established in terms of the number of pressure pulses (N_D) required to result in one decimal reduction in microbial population. These two numbers (IPK and D) are meaningful in assessing the importance of pressure pulse vs pressure hold approach for microbial destruction.

Results confirmed an enhanced pressure destruction effect at lower temperature which can be due to the enhancement of denaturation of different proteins at sub-ambient temperatures. It can also be argued that higher pressures and lower temperatures act to push toward reducing the reaction volume and therefore should be expected to have enhanced effects as the pressure is increased and temperature is lowered. Exceptions may occur when temperatures are in the lethal region (i.e., at elevated temperatures).

Process Development

The process development for safe and high quality cheese requires a careful consideration of several changes taking place in cheese milk related as a result of pressure processing. Over processing may render the milk to be safe (free from pathogens) but may lead to drastic changes in the functional properties of milk ultimately affecting the cheese making properties and overall quality of cheese. The pressure treatment required is dependent on the target organisms to be eliminated. Based on the available kinetic data of *L. monocytogenes* the process was based since they appear to be most resistant than the *E. coli* and natural microorganisms in milk.

Results indicated that desirable process conditions for optimal water holding capacity and gel strength to be pressure treatment of about 40 min at 280 MPa. This study also revealed that the principal effect process variables were somewhat opposite with respect to water holding capacity and gel strength of the rennet curd with water holding capacity increasing and gel strength decreasing at higher pressures, longer holding times and higher temperatures. Interestingly, for cheese making, lower water holding capacity and higher gel strength would be desirable. Hence, lower pressures and lower temperatures would be more desirable. However, microbial destruction at lower pressures

would necessitate longer holding times, which may not be favorable for good quality cheese. Hence there appears to be a need for some compromising scenario. Since the process conditions can have considerable effects on residual enzyme activity and effect on milk protein affecting the ripening characteristics, it is suggested that such a study on cheese be evaluated at different two levels of pressure severity at different pressure levels. For such follow up studies based on these kinetics, it was considered desirable to use a 4 and 6 D process with respect to *L. monocytogens* and carry out the pressure treatment at 300 MPa and 400 MPa.

Kinetics of milk enzyme inactivation by HP

The hypothesis that microorganism destroyed faster than enzyme by HP process was tested. It is obvious that heat pasteurization of milk destroy completely the lipoprotein lipase and several other native milk enzymes. Therefore it was important to study the extent to which enzymes were affected by HP treatment as compared to heat pasteurization. In the study, the effect of high pressure (300-400MPa, 0-180 min) on two milk enzymes lipoprotein lipase and *γ-glutamyl transferase* were evaluated. In general, the enzyme activities were enhanced by the high-pressure treatment. Lipoprotein lipase resisted inactivation during the entire range of pressure treatment. *γ-glutamyl transferase* also demonstrated an activity enhancement after a short pressure pulse, but followed a first order inactivation thereafter. Enhancement in the activity was higher at the applied pressure. At the pressure treatment levels required for pathogen control, considerable retention of native enzyme activity was possible, thereby demonstrating a beneficial effect of HP treatment of milk in cheese making.

Effects HP treatment on cheese making process and quality

The most important factor in the control of cheddar cheese quality is the extent of acid production in the vat since this largely determines its final pH. The pH content of the cheese results mainly from the amount of lactic acid (produced from lactose by lactic starter added at the beginning of the cheese manufacturing) which is moderated by the buffering capacity of milk. Each cheese variety has a characteristics pH range, within

which the quality of cheese is dependent on its composition and the way in which it is manufactured.

The pH change kinetics were assessed in the three stages - milk ripening, cooking/holding and cheddaring – of the Cheddar cheese making process. The influence of different treatments on pH change were somewhat different in the three phases studied. The pH linearly decreased with time in the first two stages and logarithmically in the final cheddaring phase. With respect to raw milk (control), all treatments delayed the ripening of milk with micro-filtration influencing the most probably no contribution by NSLAB in lactic acid production (99% bacteria removed during the micro-filtration). During the cooking/holding, pasteurized and micro-filtered milk slightly enhanced the rate of pH change while the HP treated milks continued to delay the pH change. During the final phase (cheddaring process), the rate of change in pH with the high pressure treated milk was about the same as in micro-filtered milk; however, both of these delayed the rate relative to raw and heat treated milks.

The effect of different technological treatments (raw, pasteurized, microfiltered, HP treated) of milk on yield, moisture and different solid recoveries in cheddar cheese. In general high pressure treated milk and pasteurized milk cheeses showed better fat, protein and total solids recovery compared to raw and microfiltred milk cheese. Yield was higher in all pressure treated samples and comparable with pasteurized milk cheese. Raw milk and MF milk cheese showed lower yield and solid recoveries probably solid loss in whey. High pressure treated cheese may have potential in producing low moisture cheese intended for slow ripening. A careful consideration would require to modify the manufacturing process in order to get the desired quality of cheese.

Ripening characteristics of Cheddar cheese prepared from treated milks

Microbial analysis of treated and untreated milk samples demonstrated an absence of coliform and psychrotrophic bacteria in HP treated milk samples indicating these organisms were pressure sensitive. In general, microbial changes (lactococci and lactobacilli) during ripening period (1 to 90 days) indicated higher count of lactobacilli in HP treated milk and higher count of lactococci in raw, pasteurized and MF milk cheese from beginning of ripening. The lactococci count in HP treated milk cheese was lower

than controls and lactobacilli count was higher than pasteurized and microfiltred milk cheese but comparable with raw milk cheese. Higher lactobacilli in raw milk cheese is attributed to the presence of NSLAB and high numbers in HP treated samples could be due to the fact that longer cooking time would have restricted growth of lactococci and favored growth of lactobacilli. The lactococci proteinases and peptidases help in breaking down larger peptides produced by rennin and plasmin through breakdown of casein. The results indicated that maturation indices of cheddar cheese can be enhanced by using HP treatment of milk. Higher PTA:TN ratio in pressure treated milk cheese indicated higher peptidase and aminopeptidase activity from the beginning.

Since NSLAB (mostly Lactobacilli) do not appear to lyse in Cheddar cheese normally the question arises as to how much these intercellular peptidases contribute to proteolysis in cheese made from HP treated milk. Higher number of lactobacilli in HP treated cheese samples was evident; however, since the NSLAB cells are alive, even if not multiplying, they may transport small peptides into the cells. Certainly, *Lactobacillus* contributes to the formation of free amino acids in cheeses, e.g., the concentration of free amino acids in raw milk cheeses. A slow ripening rate in HP treated milk cheese was observed in the beginning which might be due to interaction of casein micelles and whey protein resulting poor accessibility of enzymes to casein fractions; however, once the barrier was broken the ripening rate were comparable to the controls. This was evident around day-60 and after indicating short ripening period may not yield desirable proteolysis in cheese resulting mild flavor cheese.

HP processing possesses a good potential in cheese making; however, a more detail study with respect to change in protein functionality, enzyme and effect on microorganisms is needed to provide insight into micro-level understanding of changes due to pressure treatment. This will be helpful in optimizing the design of the HP treatment process. The showed that HP treatment of milk prior to cheese making influenced cheese ripening characteristics. The presence of higher amounts of low molecular weight peptides and free amino acids may contribute to improved flavor. However, it is important to evaluate specific microbial changes during ripening and characterize and quantify the flavor profile of HP treated milk cheese.

CHAPTER IX

CONTRIBUTION TO KNOWLDGE AND SUGGESTIONS FOR FUTURE STUDIES

This is first time a comprehensive study on application of high pressure processing of milk for cheese making was carried out. Cheese manufacturing involves several factors which affect the product quality. While there is abundance of literature on the evaluation of HP treated milk and properties of HP treated milk cheese, this is the first time a complete study involving the development kinetics, application and ripening aspects. The following are the specific claims of this research:

1) HP effect on cheese making properties were characterized and quantified. The relationship between HP process variables and cheese making parameters such as coagulation properties, water holding and capacity gel strength were developed by building an appropriate model.
2) The effectiveness of the process was verified by determining the kinetic parameters of microorganisms.
3) HP destruction kinetics of indigenous milk enzymes were modeled in relation to cheese making and criteria of processing of milk for cheese making was established.
4) For the first time, the effect of different treatment of milk (including HP) on pH change kinetics in different stages of cheese making were modeled.
5) The study involved a systematic approach to monitor each component during cheese making process affected by HP treatment of milk.
6) For the first time, in a single study, all aspects from cheese making properties to ripening characteristic of cheddar cheese were combined.
7) The study accounts for the longest ripening study involving HP treated milk.
8) First time along with HP treated milk cheese with three controls: raw, pasteurized and micro-filtered milk cheese were compared for their cheese making and ripening characteristics.

SUGGESTED FUTURE WORK

In a global prospective, this thesis contains exhaustive work connecting the cheese making process from cheese making steps through cheese ripening. During the course of study many interesting phenomena were observed; but not all were explored fully due to limitations with respect to the time frame and scope of the study. The following can be explored further to provide interesting results

(i) The effect of HP on interaction with β-lactoglobulin and casein at micro level to explain the effect of coagulation and gel characteristics.

(ii) Exploring the effect of lower temperature (below 0°C) processing of milk protein on molecular level and microbial destruction and further relating the coagulation characteristics and gel properties.

(iii) Above study at moderately higher temperature (30-50°C).

(iv) Evaluation of kinetics as a function of pH during cheese making by varying quantity of starter and strain.

(v) Additional of pressure shocked starter culture at the end of cheese making to enhance the ripening process.

(vi) Establishment of kinetics parameters on the HP destruction of pathogens in cheese.

(vii) Influence of HP processing of raw cheese at various stages of ripening.

(viii) Isolation and identification of microflora present in cheese during ripening to understand precisely the ripening and flavor development.

CHAPTER-X

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