MICROBIAL INACTIVATION KINETICS IN SOYMILK DURING CONTINUOUS FLOW HIGH PRESSURE THROTTLING SYSTEM

by

VIJENDRA SHARMA

(Under the Direction of Rakesh Singh)

ABSTRACT

Effect of the high pressure throttling (HPT) process on the shelf life of soymilk and determination of injured microorganisms was investigated. Soymilk was pressurized at 207, and 276 MPa at four different exit temperatures (85, 121, 133 and 145^{o}C) and (102, 121, 133, 145^{o}C) respectively at three different flow rates (0.75, 1.0, and 1.5 L/min). Inactivation of *C. sporogenes* in soymilk was higher at 276 MPa as compared to that at 207 MPa when the exit temperature was 121^{o}C and hold time was 20.8 s. However, when temperature was increased to 145^{o}C more than a 5 log reduction occurred at both pressures and all the three hold times (20.8, 15.6, and 10.4 s). Pressure, time, and temperature were found to be significantly different in the inactivation of *C. sporogenes* in soymilk. There were more injured cells (0.5 log) at 207 MPa than at 276 MPa. When the temperature and time was increased there were fewer injured cells implicating that spores were completely inactivated rather than injured. The D_{121} value of *C. sporogenes* by heat alone was 3 folds more in soymilk than in 0.1% peptone water.

INDEX WORDS: high pressure throttling, microbicidal effects, soy milk, injured spores.

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DEDICATION

I would like to dedicate all my accomplishments to my mother Ramtapasya Sharma, brother and sister-in-law Devendra Kumar and Sheerin, respectively, my wife Tripti and my family back in India. Without their love and support, I would have never been able to accomplish this.

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CHAPTER 1

INTRODUCTION

Consumers' demand for high quality and convenient food products with natural flavor and taste is increasing day by day. They prefer fresh and minimally processed food of high nutritional and organoleptic quality, free from additives and preservatives. This has stimulated research for processes involving minimal heat exposure therefore, emerging technologies have gained attention in the scientific community (Hoover and others 1989). In order to harmonize such demands without compromising the safety of products, a number of nonthermal techniques have evolved. Examples are high intensity pulsed light, ozone treatment, pulse electric field pasteurization, high intensity pulsed magnetic field, gamma radiation and many more. High pressure processing also known as high hydrostatic pressure (HHP) is one of the techniques that has the potential to process safe and nutritionally rich food.

High pressure processing is gaining in popularity with the North American food industry as an alternative to conventional thermal processing of milk, fruit juices and other foods for eliminating pathogens and spoilage organisms that may have adverse effects on nutritional value, taste color or flavor (Smelt, 1998). Use of high hydrostatic pressure processing technique on the inactivation of microorganisms in milk was first reported by [(Hite, 1899) and is cited by (Hoover, 1993; Lechowich, 1993; Rastogi, 2007; Tewari, 2007)]. Hite (1899) treated milk to a pressure of 650 MPa for 10 min at room temperature and observed a 5 to 6 log reduction in viable numbers of natural microflora present in milk. Later Hite and others (1914) reported the effect of pressure on microorganism in fruits and vegetables. It was only after 1980s that

attempts were made by researchers to study the relationship between HHP and food quality and safety. In HHP, liquid or solid foods are subjected to a pressure between 100-1000 MPa. Specially designed vessels are used to withstand such high pressure safely for multiple cycles. Specified process temperature and time must be used to ensure safety of the treated product (Food and Drug Administration, 2000). The most important attribute of HHP processing is the instantaneous and uniform transfer of pressure throughout the mass of a packed food independent of shape, size, and food composition (Smelt, 1998; Rastogi, 2007). The application of such high pressure results in an increase in temperature of food through adiabatic heating which is approximately $3^{o}C$ per 100MPa depending upon the food composition (de Heij and others 2003).

HHP works on two principles basically Le Chatelier's principle and isostatic principle. HHP only affects noncovalent bonds and covalent bonds remain unaffected. Thus, low molecular weight food components responsible for sensory and nutritional characteristics are preserved by the treatment and therefore HHP provides a unique benefit over other food processing techniques (Tewari, 2007). The ability of HHP to inactivate a variety of microbial vegetative cells has been demonstrated but bacterial spores remain unaffected up to a pressure of 1200 MPa. This very high pressure can change the physicochemical properties of food. Therefore currently, combined HHP and some other treatment like heat is used to achieve commercial sterility of low acid foods (San Martín and others 2004).

For the past 15-20 years HHP has been extensively studied and in 1991 the first commercial pressure processed food product (jams and jellies) was launched in Japan (Hoover, 1997). Since then several HHP products have been introduced in the European Union and U.S. Some examples of commercially pressurized products are guacamole by Avomex Company in

U.S., orange and grapefruit juices by UltiFruit in France, sliced ham (both cured cooked and raw cooked) by Espuna Company in Spain, some other food products like oysters, fish, meat products, sliced ham, salad dressing, rice cakes, juices and yogurts are being marketed in Japan and Europe (Tewari, 2007; Sizer and others 2002; San Martín et al., 2004).

High pressure throttling (HPT) is an alternative technique to conventional heat treatment to achieve microbial inactivation and acceptable levels of physiochemical changes in food products. HPT was developed as a technique for microbial inactivation of fluid food at The University of Georgia, Athens (Toledo and Moorman JE, 2000; Sivanandan, 2007). In HPT forces of shear, cavitation, and turbulence are simultaneously applied to disrupt microbial cells. Continuous flow high pressure throttling (CFHPT) uses a relatively low pressure of 310 MPa relative to those used in HHP to continuously pressurize and throttle fluid food through a micrometering valve or the throttling valve to exit the system at atmospheric pressure. The process inactivate microbes and modify proteins which could result in improved desirable rheological properties of the food (Moorman 1997). The fluid temperature rises (depending on the pressure applied) as the fluid exits the throttling valve and it is held at that particular temperature in the holding tube, followed by quick cooling to minimize exposure of the food to heat. Advantages of the CFHPT system are: it does not require large volume pressure vessels, a lower pressure is used compared to high hydrostatic pressure system and therefore system first cost and operating cost is lower.

Soymilk is a beverage made from soybean. Soymilk is a widely used traditional beverage in the Far East for several centuries. Demand of soymilk has tremendously increased in Western countries due to its perceived health benefits. Soy milk is a rich source of protein, vitamin E, poly-unsaturated and mono-unsaturated fats, and isoflavones. It is safe for consumption by

lactose intolerant people (Lakshmanan and others 2006). Soymilk is an ideal medium for microbial growth, being high in moisture, nearly neutral in pH and rich in nitrogenous compounds, fat, sugar, minerals and vitamins. Therefore, its quality can easily deteriorate due to rapid growth of spoilage microorganisms (Kwok and Niranjan, 1995).

Clostridium sporogenes PA 3679 is a mesophillic spore forming bacteria. *C. sporogenes* spores have greater thermal resistance than *C. botulinum* and therefore have been widely used in the canned food processing studies as a surrogate microorganism because of its non- toxicity and similar physiological requirements as *C. botulinum* (Luechapattanaporn and others 2004).

Though many HHP food products are being commercialized in the market, there are no commercially sterile low acid foods produced commercially. There are also no CFHPT product available in the market. Therefore the goal of this research are:

- To investigate the effect of CFHPT system on inactivation kinetics of *C. sporogenes* spores in low acid food (soy milk).
- To study the extent of inactivation or injury to spores in low acid food (soy milk) subjected to the CFHPT treatment.

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CHAPTER 2

REVIEW OF LITERATURE

In past few decades, consumers' demand for foods with 'fresh like' quality has immensely increased. Consumers are becoming more concerned about the health benefits of the food they are consuming. Therefore, these consumer demands and awareness has increased pressure on the food industry to develop alternative techniques to thermal to produce safe, nutritional, and good tasting food products with increased shelf life (Lado and Yousef, 2002; Manas and Pagán, 2005). High pressure processing has emerged as a promising solution to the above stated scenario and has been shown to be effective in inactivating microbial vegetative cells and bacterial spores that are potential safety and spoilage threats in low acid foods.

Low acid canned foods

Low acid foods refer to any food product other than alcoholic beverages that have a pH > 4.6 and water activity > 0.85. The food products that come under this category are meat and marine products, milk, most vegetables, meat and vegetable mixtures. Low acid food products are spoiled by several types of bacteria, such as thermophillic flat sour group (e.g., *Bacillus stearothermophillus*, *Bacillus coagulans*), sulfide spoilers (e.g., *Clostridium nigrificians*), and gaseous spoilers (e.g., *Clostridium thermosaccharolyticum*). Mesophillic spoilage can occur through putrefactive anaerobes (e.g., *Clostridium sporogenes* PA 3679 spores) and spoilage and toxin production by *Clostridium botulinum* strains. The main concern with low acid canned

foods is to ensure safety from *Clostridium botulinum* that can grow in foods and produces a neurotoxin. Low acid foods provides a favorable environment for *Clostridium botulinum* growth and toxin production as they are packaged in absence of oxygen, contain high water content, favorable pH and temperature. Therefore, for pressure commercially sterilized low acid foods must fulfill the FDA requirements (21 CFR113, 1999) for low acid canned foods in order for a product to enter commercial production.

Common problems associated with low acid canned foods

Microbes of great importance associated with low acid canned foods other than toxin production by *Clostridium botulinum* are: Obligate aerobes (e.g., *Bacillus subtilus, Bacillus mycoides*), they require molecular oxygen for growth; in cans usually no swelling occurs except in cured meats when nitrite and sugar are present. These organisms are not a problem in low acid canned foods as very low levels of molecular oxygen is present and is insufficient to support growth of the organisms. Facultative anaerobes *(*e.g., *Bacillus coagulans, Bacillus stearothermophillus*) generally cause flat sour spoilage; that is they produce acid but little or no gas. In canned foods, there is possible loss of vacuum upon storage, the can swells and the pH of the product is markedly lowered and may have slightly abnormal odor. Thermophillic anaerobes (e.g., *Clostridium nigrificians*), produce hydrogen sulfide and are responsible for the "sulfur stinker" spoilage of canned foods. These organisms produce H_2S in great quantities and are proteolytic; the product usually absorbs the H_2S gas giving itself a rotten egg odor, the can usually remains flat and many of the products spoiled by these organisms become black because of the interaction of hydrogen sulfide with iron. Mesophillic anaerobes (e.g., *Clostridium sporogenes* PA 3679, *Clostridium botulinum*) cause the cans to swell, may burst and produce

typical putrid odor of the product. *Clostridium botulinum* is of great importance among organisms of this group. Seven types of botulism strains are known in which types A, B, E and F cause human botulism. Spores of type A and B are more heat resistant than spores of E and F, but are not nearly as heat resistant as the spores of *Clostridium sporogenes* PA 3679 and for this reason have a great importance as a test organism to check the adequacy of canning processes.

Research done on low acid foods

Numerous studies have been done on low acid foods. Due to microbial spoilage low acid foods with high water activity are considered as a possible threat to the food industry if improperly processed. Adequate inactivation of bacterial spores is a challenge for the food industry (Ananta and others 2001). Shelf stable low acid foods must not contain a viable spore population which may produce toxins in the product. Spores are highly resistant to inactivation and can withstand severe stress conditions hence their inactivation becomes difficult. High pressure processing was proposed as a promising technique in achieving sterile conditions from the turn of the 20^{th} century (Larson and others 1918). Recent studies however indicate that sterilization of low acid foods with high pressure must be combined with other forms of mild treatments (Rastogi, 2007) to produce foods with better quality than those obtained with thermal treatments..

Patterson and Kilpatrick (1998) observed a 5 log reduction of *E. coli* in milk and approximately 6 log reduction in poultry meat after 15 minutes treatment at 400 MPa and 50^oC whereas the treatment at atmospheric pressure and 50^oC for 15 minutes less than 1 log reduction was observed. Also 6 log reductions of *S. aureus* in milk and 5 log reductions of *S. aureus* in poultry meat were achieved with a treatment at 500 MPa and 50^oC for 15 minutes. Similarly,

Simpson and Gilmour (1997) observed 6 log reduction of *Listeria monocytogenes* in milk after the treatment of 200 MPa and 55^{o}C for 15 minutes. Crawford and others (1996) reported a 5 log reduction of *Clostridium sporogenes* in chicken breast treated at ambient temperature and 689 MPa for 60 minutes. Carlez and others (1994) studied the effect of high pressure on minced meat samples and observed total inhibition of microorganisms at 400-450 MPa and reported that gram negative bacteria were more sensitive to high pressure than gram positive bacteria. Wilson and Baker (2001) claimed to achieve sterility using a 10^{6}/g spores of *B. stearothermophillus*, *C. sporogenes* and *B. subtilis* in a meat emulsion where initial process conditions were 621 MPa and 85^{o}C for 30 min or 621 MPa and 98^{o}C for 5 min. Meyer (2001) reported achieving sterility in low acid foods using pulsed high pressure in conjunction with heat and an initial product temperature of atleast 70^{o}C. Due to pressure treatment the temperature increased up to 105^{o}C. By this treatment sterility was achieved in macaroni and cheese with a 10^{6} spore load of *C. sporogenes*. Capellas and others (1996) reported that no surviving *E. coli* were detected with an initial count of 10^{8}/g in goat milk cheese up to 60 days of storage after treatment with 400-500 MPa at 2, 10 or 25^{o}C for 5-15 min.

Soybean is a principal world field crop. It has been used in the Far East for centuries and still forms part of the indigenous diet. The relatively low cost of soybean protein as compared to animal proteins and the easy way in which it can be prepared into palatable high protein food makes it a common high protein source, particularly for developing countries. Soybeans are versatile to use and are noted for their health benefits. Soy consumption has proven to reduce breast, lung, stomach, rectal, colon and prostrate cancers. It has also been proven to reduce cholesterol levels, kidney disease, diabetes, high blood pressure and prevention of osteoporosis (N'Kouka and others 2004). As per the American Journal of Clinical Nutrition “It can be

concluded that soy protein can serve as a sole protein source in all human beings except premature infants" (Mindell, 1995). It is low in fat and contains no cholesterol. It is an excellent food for babies, children, elderly people and pregnant and lactating women. In a recent development (CFR 21:101.82, USFDA 1999) the U.S. Food and Drug Administration (USFDA) has authorized a health claim for cardiovascular benefits of soy consumption saying that "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (N'Kouka et al., 2004). This health claim stating the cardiovascular benefits of soy consumption has additionally heightened awareness and interest in soy. The soybean contains useful nutrients and those nutrients are available to people through the palatable foods that are made from soybeans. One of the most promising soybean foods is soymilk.

Soymilk is essentially the water extract of soybeans, and was first used in ancient China around 2,000 years ago. The protein content of soybean is about 40%, which is the highest among foods of plant origin. Nutritionally, soy protein is the most balanced plant protein for human consumption. It is a cholesterol free product and is rich in polyunsaturated fatty acids of phospholipids, especially lecithin and also linolenic acid. Besides being rich in protein, minerals and vitamins, soymilk is very economical, lactose free and a nutritious alternative of dairy and meat centered diet. The main problem of these alternatives is that their sensory characteristics do not match original products. Soymilk produced by traditional methods does not have bland flavor or smooth texture of cow's milk. The beany flavor and odor is quite objectionable. N'Kouka and others (2004) stated that soy aromatics and bitterness make soymilks flavor profile less balanced than dairy milks. Astringency has also been considered as negative quality of soymilks (Chien and Snyder, 1983). The off-flavor and odor characterized as 'beany' are due to volatile compounds formed by lipoxygenase catalyzed reactions which develop almost instantaneously

upon wet grinding (Wilkens, 1967). For producing a bland and palatable product, elimination of this enzymic off-flavor development and destruction of growth inhibitors in raw soymilk are important concerns. The growth inhibitory substances include soybean trypsin inhibitors, haemagglutinins, saponins and antivitamins. Trypsin inhibitor is one of the important components proposed as the antinutritional factor in soybean. Growth depression, pancreatic hypertrophy, hyperplasia, and adenoma in experimental animals have been reported due to the interference with the digestion and absorption of proteins and is partly or fully attributed to soy trypsin inhibitors (TI) (Rackis and Gumbmann, 1981). Proper heat treatment improves the nutritional value of soymilk by inactivation of TI and by increasing digestibility of soy proteins (Rackis, 1974), whereas overheating to completely remove proteolytic inhibitor activity may reduce nutritive value of soybeans (Liener, 1981).

Soymilk is an ideal medium for microbial growth, being high in moisture, nearly neutral in pH and rich in nitrogenous compounds, fat, sugar, minerals, and vitamins and therefore, its quality can easily deteriorate due to rapid growth of spoilage and pathogenic microorganisms. As nutrient contents are similar, microbial spoilage patterns in soymilk also resemble that for dairy milk. At room temperature after standing for 24 h; soymilk undergoes acid curdling with a rapid drop in pH accompanied by separation of curds and whey. Proteolytic spoilage can, however, take place within a week at refrigeration temperatures of 1^{o}C (Lo and others 1966). Organisms that grow in soymilk are acid formers, gas formers and putrifiers such as *Bacillus stearothermophillus*, *Clostridium sporogenes* (Kwok and Niranjan, 1995).

***Clostridium sporogenes* PA 3679**

Clostridium sporogenes PA 3679 ATCC 7955 has been used as a surrogate organism for testing adequacy of treatments to eliminate *C. botulinum* in low acid foods like soymilk. *C. sporogenes* is a typical heat resistant, gram positive, nonpathogenic, mesophillic spore forming and putrefactive anaerobe organism. Sporulation occurs in most media and spores of *Clostridium sporogenes* are oval, subterminal and distend the cell. Following sporulation, the vegetative material may disintegrate rapidly to leave free spores. Surface colonies on agar plates are 2-6 mm in diameter, irregularly circular, possess a coarse rhizoid edge, have a raised yellowish gray center and a flattened periphery composed of entangled filaments. The optimum temperature range for growth is 30-40^{o}C and good growth occurs in an atmosphere containing up to 100% CO_2 (Sneath and others 1986). The heat resistance of spores of *Clostridium sporogenes* PA 3679 has been found to be equal or greater than that of *Clostridium botulinum* (Koutchma and others 2005). Therefore, lower counts of *Clostridium sporogenes* can be used to validate a process equivalent to at least a 12 log reduction in C*lostridium botulinum*. Also these bacterial spores known for their resistances to heat and irradiation have also shown resistance to pressure (Maggi and others 1996).

Rovere and others (1996) reported that total destruction of *C. sporogenes* (strain PA 3679; ATCC 7955) spores could be obtained using a combined pressure (1,000 MPa) and temperature (50-60 C). Mills and others (1998) concluded that spores of *C. sporogenes* could not be inactivated by pressure alone. They found that treatments of 600 MPa for 30 min at 20^{o}C caused no significant inactivation of *C. sporogenes* spores. Pressure treatments of 900 MPa for 10 min at 30^{o}C were unable to destroy 8.4 x 10^2 *C. sporogenes* spores in truffle cream (Gola and others 1996). The minimum levels of water activity, pH, and temperature for Clostridia growth

range from 0.93-0.95, 4.6-9.0 and 3.3-50^{o}C (Doyle; Lund and others 2000; Doyle and others 2002). It is reported that germination of *C. botulinum* spores do not occur at low water activity (<0.95), and this may be the reason that Yetim and others (2006) found no increase in number of viable *C. sporogenes* spores in kavurma during storage. Also 3 log reductions in *C. sporogenes* spores were reported by these authors after cooking of kavurma.

Sporulation

Gram positive bacteria like *Bacillus* and *Clostridium* initiate sporulation as a response to unfavorable conditions like starvation, environmental stress and slow growth. These spore forming bacteria create a major problem for the food industry (Doyle and others 1997). The spores of *Bacillus* and *Clostridium* if present in foods not only cause detrimental effect on sensory characteristics of food but also cause foodborne illnesses due to the toxins produced by them which can range from mild emetic type illness to fatal illness. *Clostridium botulinum* is one of the major sporeforming food pathogen and is causative of foodborne botulism, which is caused by ingestion of neurotoxin produced by *C. botulinum* in food (Black and others 2007). At low pH sporeforming bacteria cannot germinate. Therefore they pose little threat to high acid food but are major concern for low acid foods (Splittostoesser and others 1994).

Sporogenesis in sporeforming bacteria starts when the nuclear material of the vegetative cell fuses to form axial thread. Then some of the chromatin material migrates to the sporangium, a septum starts growing and completely encloses the chromatin body. At maturation a cortical region, coat layers and an exosporium are formed around spore body inside the septum. This septum becomes the cell wall when spore germinates with the formation of spore coat and cortex spore becomes refractile (Murrell, 1961; Young and Fitz-James, 1959a; Young and Fitz-James,

1959b). Spores are metabolically dormant and contain very little ATP's. Spores are extremely resistant to lethal treatments like radiation, pressure and heat.

The spores have more than 10% dry weight accumulation of pyridine 2, 6 dicarboxylic acid (DPA) and divalent cations like calcium. DPA is usually present in 1:1 complex with divalent cations predominantly calcium. DPA is found only in spores and not in vegetative cells (Murrell, 1969). DPA and calcium are considered to attribute heat resistance of spore. Spores can readily germinate on getting proper and favorable environment (Setlow, 2003). As the spore germinates it looses all of its DPA by excretion and thus spore looses its high resistances and eventually a spore turns into a vegetative cell (Doyle et al., 1997).

Spore structure

Dormant spores have a very unique structure compared to that of a growing cell; and hence accounts for continued viability of spores under severe conditions (Setlow, 2007). The exosporium is the outermost layer of spore. The presence and size of the sporium depends on the species. The exosporium is composed of carbohydrates and proteins (Driks, 1999; Redmond and others 2004). (Setlow and others 2006) suggested the exosporium does not play a significant role in resistance. Beneath the exosporium is the spore coat which is composed of proteins (Kim and others 2006). The spore coat is important in imparting spore resistance to chemicals and lytic enzymes and can detoxify such chemicals (Setlow and others 2006). Spore coat do not play a significant role in high pressure resistance (Paidhungat and others 2002). The next layer under the spore coat is an outer membrane that plays an important role in spore formation. However, the outer membrane has no role in the high pressure resistance of spores or in the process of spore germination (Paidhungat et al., 2002). Further inside the outer membrane lies the spore

cortex. It is composed of peptidoglycans and the structure somewhat resembles that of a vegetative cell but specific modifications are inherent in spores (Popham, 2002). DPA is contained in the cortex (Hashimoto and others 1960). The cortex disintegrates immediately as spore germination starts and all the DPA is loss. Thus, spore resistance to extreme conditions is lost. Metabolism and growth is initiated. The germ cell wall is the next layer and is also composed of peptidoglycans. Its structure is identical to that of vegetative cell wall. Beneath the germ cell wall lays the inner membrane, which is composed of phospholipids and fatty acids. The membrane has low permeability and therefore very important in dormant spore resistance, as it does not let lethal agents cross the membrane to damage DNA in the spore core (Cortezzo and Setlow, 2005). The inner membrane is also important because it assembles the proteins that triggers spore germination as nutrients become available in the medium (Setlow, 2007). Then finally in the centre of spore lies the spore core. Spore core contains DNA, RNA, and many enzymes. Core has low water content and thus account for spore resistance to wet heat (Gerhardt and Marquis, 1989). The spore core contains high levels of small acid-soluble spore proteins (SASPs). The major function of SASP is in spore resistance to UV radiation and dry heat. SASP also acts as amino acid and energy reservoir during spore outgrowth (Setlow, 2007).

The process of spore germination starts with activation. Spores can be activated for germination by heat shock or high pressure. Second step is germination. Germination occurs due to “nutrient germination stimulants”. During germination, spores lose about 30% of spore dry weight, excretion of DPA occurs, SASPs break down and loss of spore resistance occurs (Adams, 1974). After germination, outgrowth occurs and is characterized by ordered synthesis of RNA, protein, cell wall and DNA for spore development to a mature vegetative cell. Outgrowth

also involves re-uptake of water and enlargement of the cell. Finally the spore coat breaks, cell releases and resumes normal growth pattern.

Alternative food processing technologies

Currently used preservation techniques act by preventing microorganisms from accessing food, inactivating those microbes that are already present in the food. But with the new trend now researchers are interested in modifying currently used techniques or developing new ones which are non-thermal and have reduced conditions detrimental to food quality such as reduced intensity of heat treatments (Lund et al., 2000). So in order to meet consumer demand of more fresh-like quality and good nutritional value in products perceived to be healthier than heat treated foods the industry is now focusing on nonthermal alternative food processing technologies (Lado and Yousef, 2002). Some examples of alternative techniques are high hydrostatic pressure, PEF, high intensity laser or non-coherent light pulses, oscillating magnetic fields, irradiation, microwave, radio frequency heating, ultrasound, cavitations, and mild chemical treatments.

Pulse electric field (PEF) treatment utilizes the high intensity electric field pulses for few milli-seconds, within the intensity range of 5-55KV/cm. It is generally used for fruit juices and liquid eggs. This technique has limited approval in the US (Qin and others 1996).

Microbial inactivation using high intensity laser or non-coherent light has been known for years (Mertens and Knorr, 1992). Laser has been known of being capable to inactivate microbes and spores present on food products surfaces, transparent liquid foods and packaging materials, in short duration high intensity pulses (Dunn and others 1991).

Ultraviolet radiation of wavelengths between 200-280 nm and intensity range between 0.5 – 20 J/m^2 has proved successful in inactivating microorganisms like *E. coli* O157:H7 and *Cryptosporidium parvum* in processed fruit juices (Vasavada, 2003).

Hydrodynamic cavitation is also a potential technique for inactivation of microbes. As cells are subjected to physical stress developed by cavitation, they get severely damaged and finally killed (Frizzell, 1988).

Ionizing radiations, more precisely gamma radiation and electron beam in between the intensity range of 2-10 kGy have proved useful in inactivation of microorganisms in raw poultry meat, raw red meat, sea food and liquid eggs (Farkas, 1998). Inactivation of E. coli in milk up to seven log reduction was observed using 10 kGy irradiation (Garin-Bastuji and others 1990).

High hydrostatic pressure

High pressure processing is gaining in popularity with the North American food industry because of the fact that consumers demand minimally processed high quality food products with natural flavor and taste, safe and without any preservatives (Tewari, 2007). High pressure processing is an emerging non-thermal technique in food processing that has been attracting food industries for past 15 years because of its immense advantages over thermal processing like superior flavor, inactivation of microorganisms, nutrient retention, causing novel functional properties, reduces processing times, processing at ambient temperatures, eliminates the need of preservatives (Rastogi, 2007).

Use of high hydrostatic pressure processing technique on the inactivation of microorganisms in milk was first reported by Hite in 1899 [cited by (Hoover, 1993; Lechowich, 1993; Rastogi, 2007; Tewari, 2007)] when he treated milk to a pressure of 650 MPa for 10 min at

room temperature and observed a 5-6 log reduction in viable numbers of natural microflora present in milk. Later Hite and others (1914) reported the effect of pressure on microorganisms in fruits and vegetables. It was only after 1980s that attempts were made by researchers to study the relationship between HHP and foods. For the past 15-20 years HHP has been extensively studied and in 1991 first commercial pressure processed food product (jams and jellies) was launched in Japan (Hoover, 1997).

In HHP processing pressure is applied on the pressure vessel containing pressure transmitting medium, usually water, for the duration of treatment depending on the food samples (San Martín and others 2004). There are basically two principles that govern HHP processing. First is Le Chatelier's principle, which states that, any phenomena in equilibrium (phase transition, chemical reaction, and change in molecular configuration) involving a volume reduction are enhanced by pressure and vice versa. The HHP affects non covalent bonds therefore high molecular weight components (responsible for functionality determination) may get altered. Covalent bonds are not affected by HHP because of the low energy level in processing and hence low molecular weight food components (responsible for nutritional and sensory characteristics) remain unaffected. Second is isostatic principle according to which pressure transmittance is instantaneous and uniform (independent of size and geometry of food) that is used to inactivate microorganism present in food (Tewari, 2007; Smelt, 1998).

High pressure has been widely studied for applications other than microbial inactivation in food. Yokoyama and others (1992) reported the reduction in ripening time of cheese by 3 days by a pressure treatment of 250 MPa. Soy protein isolates, β- 7S and A-11S polypeptide have shown better ability in holding at oil-water interface (Puppo and others 2005). High pressure treatment improves protein gelation properties and therefore has been used in producing various

types foods (Hoover, 1993). Olsen, Kristiansen, and Skibsted (2003) reported the possibility of reducing milk allergenicity by a specific treatment of HHP, 250 MPa to hydrolyze the β-lactoglobulin (Devlieghere and others 2004). Pressure treatment can also be used to inactivate or change the functionality of enzyme due to change in active site or protein denaturation caused by the pressure treatment (Rastogi, 2007). Pectin Methylesterase inactivation in orange juice greatly depends on pressure treatment, hold time, pH and total soluble solids (Basak and Ramaswamy, 1996). Pressure treatments of more than 600 MPa caused instantaneous inactivation of the heat labile form of pectin esterase; orange juice pressurized at 700 MPa for 1 min had no cloud loss for 50 days (Goodner and others 1998). High pressure can cause conformational changes in polygalactouronase and therefore reducing the affinity for substrate binding and also cause enzyme inactivation (Rastogi, 2007). Solubilization of alphas1- and beta-casein due to disruption of hydrophobic interaction and solubilization of colloidal calcium phosphate occurs as result of high pressure treatment of milk at 100-600MPa (Huppertz and others 2004). High pressure treated skim milk produced gels showed improved rigidity and gel breaking strength (Johnston and others 1992). High pressure treatment of milk caused an increase in percentage moisture and wet weight yield of cheese in comparison to the cheese made from pasteurized or raw milk. Prestamo and Penas (2004) reported the increase in antioxidant activity of soy whey protein, but decreased antioxidative activity of hydrolysates after high pressure processing at 100 MPa (Doblado and others 2007). Soy milk pressure treated at 500 MPa for 10 min has shown improved emulsifying activity and stability whereas, if treatment time increased to 30 min it changes from liquid form to solid state (Kajiyama and others 1995). High pressure treatment has shown to increase the formation of more hydrophobic region in soy protein, and forming insoluble aggregates after dissociation. The high pressure

treatment can cause increase in viscosity of soymilk and therefore has a potential of processing soymilk to form tofu gels (Zhang and others 2005).

High pressure homogenization

Homogenization has been extensively used in food industry and has a wide application wherever emulsions are involved like, dairy industry (Peck, 2004). Auguste Gaulin in 1899 developed conventional homogenization (Sivanandan, 2007). Standard industrial processes involving classical homogenization uses <100 MPa by forcing fluid through 100-300μm gap in homogenizer valve, to stabilize product against creaming and coalescence and to attain desires rheological properties with upstream pressure of about 20-60 MPa (Kessler, 1981). In homogenization turbulent condition persist and therefore adsorption of aggregates occurs due to the fact that dense material are displaced by convectional movement (Walstra, 1983). Nowdays, Ultra high pressure homogenization, also called high pressure homogenization or dynamic high pressure is used in food industry frequently to achieve desired physiochemical properties with reduction in microbial count, normally pressure used is more than 200 MPa.

Continuous flow high pressure throttling (CFHPT) system

The High pressure throttling system was developed at The University of Georgia, Athens as a means of continuous inactivation of microorganisms in fluid foods (Toledo and Moorman, 2000). Micrometering valve that is used, as the throttling valve is a fine restriction such as partially closed valve or porous plug to control the flow of fluid through a micrometering valve. Continuous flow high pressure throttling (CFHPT) system uses high pressure of 310 MPa that is used to continuously throttle the fluid food through a micrometering valve or the throttling valve

to atmospheric pressure for the purpose of inactivating microbes and modifying proteins which should change the rheological properties of the food (Moorman 1997). Due to the use of high pressure to throttle liquid foods through a micrometering valve to achieve microbial inactivation the name continuous flow high pressure throttling aptly suits the process. The CFHPT system consists of a positive displacement feed pump that maintains a constant pressure to the fluid feed to the intensifier and a restricted orifice discharge valve. The use of CFHPT is limited to fluid foods that contain very small size suspended solids. The fluid food at high pressure is forced through a throttling valve with a high velocity and increasing pressure increases the potential energy of the fluid food. The fluid contains a high energy density (energy/volume) at high pressure. Suddenly the pressure is reduced by passing the fluid through a small orifice and the potential energy gets converted into heat energy. Thus, instantaneous rise in the fluid temperature occurs upon discharge of the fluid through the throttling valve. At very high pressures entering the micrometering valve, reduction of pressure to atmospheric pressure requires very high rates of shear (Areekul, 2003). After the fluid food passes through the throttling valve, microbial cell wall disrupts killing the cell due to a combine action of instantaneous pressure reduction, shearing action on the fluid and suspended particles, and high turbulence causing cavitation. Temperature rise, increase in mechanical forces on the suspended particles and macromolecules will occur with an increase in the fluid pressure entering the throttling valve, thus increasing the microbicidal effect (Areekul, 2003).

The temperature rise occurred after the throttling valve can be explained by first law of thermodynamics (Amornsin 1999; Sivanadan 2007). Pressure difference between the inlet and outlet of the throttling valve represents the form of energy converted and is conserved in the form of heat energy at the exit of throttling valve and results in temperature rise of the product.

Thus, as the pressure is reduced across the throttling valve potential energy due to high pressure gets converted into heat energy. This adiabatic heat gain can be given by the following equations given below ((Toledo, 1991)

$$q = Cp\,(T_{out} - T_{in}) = \frac{(P_{in} - P_{out})}{\rho} \qquad (1)$$

$$(T_{out} - T_{in}) = \frac{(P_{in} - P_{out})}{\rho\, Cp} \qquad (2)$$

$$T_{out} = T_{in} + \frac{(P_{in} - P_{out})}{\rho\, Cp} \qquad (3)$$

Where

q = Energy per unit mass (J/kg)

Cp = Specific heat of the fluid at constant pressure (J/kg^{o}C)

T_{out} = Temperature of the fluid at the outlet to the throttling valve (^{o}C)

T_{in} = Temperature of the fluid at the inlet of the throttling valve (^{o}C)

P_{out} = Pressure of the fluid at the outlet of the throttling valve (Pa), assumed as atmospheric pressure

P_{in} = Pressure of the fluid at the inlet of the throttling valve (Pa)

ρ = density of the fluid (kg/m^{3})

The exit temperature is affected by the initial temperature of the fluid or the temperature to which fluid is heated in the tubular heat exchanger (T_{in}) and there by the temperature rises in all of the equations given above. The temperature variation due to volumetric flow rate difference is avoided if the flow rate is constant at the exit for product collection. However at the exit of throttling valve there is further reduce in the measured temperature (experimental

temperature) because of the heat loss due to convection (to the surroundings) and conduction (through the connecting pipe to cooling heat exchanger) (Amornsin 1999; Sivanandan 2007)

Moorman (1997) studied the effects of different holding times during pressurization (310 MPa) and after depressurization on ultra filtered skim milk and permeate. Milk was held for 0.3 s and 1s at the high pressure applied using the tube of length 7.6 cm or 176.5 cm. By changing the holding time (using different holding tubes) after the throttling valve at elevated temperature (80^0C) for 0 s and 10 s two more treatment combinations were studied. Moorman (1997) reported increase in viscosities of CFHPT treated milk concentrations and increase in apparent viscosities and water holding capacity of yogurt. The author also observed 2 to 4 log reductions in native microbial populations in skim milk through CFHPT process from 310 MPa to atmospheric pressure with a high pressure dwell of only 0.3 s and also inactivation of *P. putida* was reported from an initial population of approx 10^8 cfu/ml to undetectable levels.

Advantages of CFHPT system include that the system does not require large volume pressure vessels, a lower pressure is used as compared to high hydrostatic pressure systems, and therefore system first cost and operating cost is lower. CFHPT processing includes simultaneous homogenization and sterilization which can benefit physical properties, texture, and stability of sterilized product during storage.

Effect of Pressure on Microorganisms

Microbial resistance to high pressure depends on the intrinsic resistance as well as on their physiological state. The degree of inactivation depends on number of different factors, such as microorganism type, level of pressure, temperature, time, water activity and composition of food stuff (Arroyo and others 1999). The effect of pressure on microorganisms also differs

between species and even strains, and inactivation can also depend on pH, growth conditions and suspending media (Patterson, 1999). Bacteria in the stationary phase are less sensitive to pressure than they are during the early log phase of growth (ZoBell, 1970; Mackey and others 1995). Gram positive bacteria are more pressure resistant than Gram negative bacteria and cocci are more resistant than rod shaped bacteria. Gram negative microorganisms need the application of 300-400 MPa at 25^0C for 10 min to achieve inactivation, while gram positive microorganisms are inactivated with treatments of 500-600 MPa at 25^0C for 10 min. Vegetative forms of yeasts and molds are the most pressure sensitive (Smelt, 1998; Trujillo and others 2002). Bacterial cells, moulds and yeasts are relatively sensitive to pressure below 700 MPa but bacterial spores especially of *Clostridium* species are relatively resistant to it (Hoover and others 1989; Smelt, 1998). Arroyo and others (1997) reported the reduction of *Bacillus* species spores to less than 1 log unit after the pressure treatment of 400 MPa at 10^0C for 20 min whereas complete inactivation of moulds and yeasts was observed by a treatment of 300 and 350 MPa at 10^oC for 20 min. Bacterial cells are relatively less sensitive to hydrostatic pressure at 20-35^0C but become more sensitive to pressure above 35^0C, due to phase transition of membrane lipids (Kalchayanand and others 1998).

The pH of the food has an important role in the destruction of microorganisms. Decreasing pH causes microorganisms to be more susceptible to pressure treatment (Smelt, 1998). The effect of high pressure treatment is significantly influenced by the composition of substrate like acidity, sugar and salt concentration and antimicrobial agents in which microorganisms are present. The substrate composition substantially exerts a synergistic effect on pressure mediated inactivation of bacteria (Alpas and others 2000). Low pH, presence of nisin or the presence of sucrose laureate in the substrate has been reported to enhance the inactivation

of bacterial spores by pressure treatment (Roberts and Hoover, 1996). At low pH pressure treatment of spores increases the exchange of minerals from the spore core with the protons and decrease the spores heat resistance (Wuytack and Michiels, 2001). Margosch and others (2004) reported that pH shift from 6.0 to 5.15 did not significantly affect the inactivation kinetics of *Clostridium botulinum* in pressure stable tris buffer; though decreasing the pH to 4.0 aggravated the reduction in viable spore counts and the release of DPA from the spores. Stewart and others (1997) observed an additional 3 log reduction in *Listeria monocytogenes* cell counts after a pressure treatment at 353 MPa and 45^0C for 10 min in buffer at pH 4.0 as compared to that at pH 6.0.

Effect of pressure on cell membranes

In microorganisms, the cell membrane is supposed to be the first and foremost site of damage caused by pressure (Patterson, 2005). Physical damage to cell membrane occurs as a result of pressure, such as leakage of ATP from cell membrane (Patterson (2005) or increased uptake of fluorescent dyes which cannot penetrate healthy cell membrane as proposed by (Benito and others 1999). According to Singer and Nicholson (1972) cell membranes have fluid mosaic model in which a phospholipids bilayer structure has proteins embedded in it. A phase transition has been observed in lipid bilayer under pressure, indicating that phospholipids present in membrane are extremely sensitive to pressure (San Martín and others, 2004). During phase transition liquid crystalline phase changes to gel, accompanied by a decrease in cell membrane fluidity, that eventually results in cell membrane breakage. Furthermore, membrane protein denatures causing functionality loss that is in transporting ions and other substances across the membrane (San Martín and others, 2004). It has been reported by Casadei and others (2002) that

membrane fluidity affects pressure resistance of exponential and stationary phase cells. The effect of pressure on cell membrane causes leakage of intracellular substances from cytoplasm of yeast cells at high pressures, leakage of metallic ions and permeability of extracellular substance into cell and tissue (Kato and Hayashi, 1999). Manas and Mackey (2004) reported the inactivation of exponential phase cell because of irreversible damage to cell membrane due to high pressure. It is postulated that stationary phase cells are normally more pressure resistant than exponential phase cells (Patterson, 2005). Wouters and others (1998) also reported loss of membrane functionality as a result of high pressure treatment on *L. plantarum*.

Effect of pressure on cell wall

In comparison to cell membrane cell wall is less affected by high pressure treatment (Patterson, 2005). Any alteration in cell wall structure due to high pressure treatment leads to solubilization of intracellular substances (Kato and Hayashi, 1999). Lysis of large cells was also reported by Hoover and others (1989) due to mechanical disruption of a stressed cell wall after pressure treatment of 20-40 MPa. In *Lactococcus lactis* subsp. cremoris cell wall hydolase activity was increased by 100 MPa pressure treatment (Malone and others 2002).

Effect on cell morphology

Intracellular damage due to pressure treatment has been observed by researchers using a light microscope. Bud scars were observed on the cell surface of *Listeria monocytogenes* after a treatment of 400 MPa for 10 min using scanning electron microscopy (Ritz and others 2001). A variety of morphological changes like separation of cytoplasmic membrane from the cell wall, decreased number of ribosomes, clear zone of spongy structure in the cytoplasm and thickened

cell walls with no membrane were reported after a pressure treatment (Hoover and others, 1989). In *L. monocytogenes* and *L. lactis* subsp. cremoris MG 1363 using transmission electron microscopy a low density intracellular region was observed. These regions of low density are caused by transient membrane invaginations under pressure that are eventually reversed, leading the region with low density near cell membrane (Malone and others, 2002; Mackey and others 1994). No low density regions were observed in *Salmonella enterica* serovar Thompson treated with high pressure, indicating the association of this phenomenon only with Gram positive cell wall (Mackey and others, 1994).

Effect of pressure on biochemical reactions

It has been observed that high pressure treatment retards reaction that causes volume increase and catalyses reaction causing decrease in volume (Patterson, 2005). Different studies conducted on volume changes in proteins have showed that the hydrophobic and electrostatic interactions are highly affected by pressure (Patterson, 2005). Enzymes vary in great range in regard to ability of withstanding pressure. Enzymes are affected by high pressure in both ways structurally and functionally in a complex way by alteration of intra and intermolecular interaction involved in stability of protein (Dallet and Legoy, 1996). Some microbial enzymes can withstand large pressures also, like α amylase from *Bacillus subtilis* can resist pressure up to 500 MPa (Suzuki and Kitamura, 1963). Covalent bonds are generally unaffected by pressure range used in food industry and because of this reason sensory and nutritional value of food do not changes, there by giving food industry a very important advantage of using high pressure for food processing (Patterson, 2005).

Effect of pressure on genetic mechanism

As the DNA consists largely of hydrogen bonds, nucleic material of cell are very resistant to high pressures but if enzymes involved in replication or transcription are denatured the DNA disrupts (Patterson, 2005). However condensation of nuclear material was reported by Mackey and others (1994) in *L. monocytogenes* and *S. typhimurium*. The reason for observation of such phenomenon was postulated by Chilton and others (1997) that at increased pressure DNA comes in contact with endonuclease which breaks DNA. The phenomenon of condensation is reversible but if the high pressure enzyme deactivates, the cell will not be able to reproduce (Smelt, 1998). It has been studied that upon unfavorable conditions gene expression in microbes adjusts to adapt environmental changes by regulating several DNA binding protein (Ishii and others 2005).

Effect of pressure on spores

The inactivation of bacterial spores from food presents a major challenge for food industry. Bacterial spores are highly resistant to pressure and they can withstand pressures up to 800 MPa for several hours (Raso and Barbosa-Cánovas, 2003). Combination of high pressure with heat can be an effective approach to produce shelf stable low acid foods (Patterson, 2005). It has been proposed by several researchers that at low temperatures and pressures germination of spores occur and then germinated spores are subsequently inactivated by the pressure treatment (Wuytack and others 1998). Maggi and others (1996) suggested that at higher temperatures germination step is bypassed and direct spore inactivation mechanism is likely to happen. Rastogi (2007) suggested that inactivation of spores of *Clostridium botulinum* can be achieved by a pressure treatment of 500-800 MPa in addition to a temperature of 90-121^{0}C. Nakayama and others (1996) reported that at pressure treatment of 980 MPa at room temperature

for 40 min, there was no significant inactivation of six *Bacillus* species. Whereas significant inactivation can be achieved by a treatment of pressure more than 400 MPa at temperatures higher than 50^0C. Hayakawa and others (1998) reported a 6 decimal reduction in *Bacillus stearothermophillus* by a treatment of 500 MPa at 70^0C during six cycles of 5 min. *Bacillus subtilis* spore were inactivated to 8 decimal reductions by a treatment of 500 MPa at 70^oC during ten cycles of 1 min (Sojka and Ludwig, 1997). Stewart and others (2000) observed only 0.5 decimal reductions in *C. sporogenes* at pH 6.0 when treated at 404 MPa at 70^0C for 15 min. Combined effect of pressure and temperature on the inactivation of *Clostridium sporogenes* PA 3679 spores in liquid media at pH 7.0 in beef or carrot broth medium, and phosphate buffer was studied by Maggi and others (1996) and they reported that 1500 MPa at 20^0C for 5 min resulted in no spore inactivation, whereas 1500 MPa at 60^0C completely inactivated the spores. Rovere and others (1996) reported that combined treatment of pressure 1000 MPa and temperature 50-60^0C is required for complete bacterial spore inactivation. Reddy and others (1999) reported 5 log cycle reductions in *Clostridium botulinum* type E spores when temperature was increased from 35 to 55^0C at pressure of 827 MPa. Inactivation of bacterial spores was greatest combining pressure and temperature when the pH was near neutrality and was lowest when at extremes value of pH (Raso and others 1998; Fornari and others 1995). The difference in pH was assumed to affect membrane ATPase and intracellular functions of the spore, thus destabilizing the microorganisms (MacDonald, 1992). However Stewart and others (2000) reported that higher inactivation in *Clostridium sporogenes* was obtained when pH of the medium was reduced from 7 to 4.

Injured Cells:

A high pressure treatment may not always completely inactivate microorganisms but rather may injure a proportion of the population. Bacterial cells exposed to different chemical and physical treatments suffer injury that could be reversible in food materials during storage. Injured bacteria may be repaired which could affect the microbiological quality of foodstuffs with an important safety consideration especially in low acid canned foods (Bozoglu and others 2004). Where as high hydrostatic pressure induced injury can be advantageous for high acid foods, where lower pressure can be used to produce injured cells that cannot repair in acidic medium. Researchers have indicated that both gram positive and gram negative bacterial cells, endospores, yeasts and molds may be injured by stresses (Adams, 1978; Ray, 1979). Injury may result from many food processing techniques which include thermal treatment, refrigeration, freezing, drying, irradiation, high hydrostatic pressure, irradiation or from exposure to preservatives, acidity or low water activity. Injured cells have been observed by several authors after high pressure treatments (Metrick and others 1989; Patterson and Kilpatrick, 1998; Bozoglu et al., 2004; Boziaris and others 1998; Ariefdjohan and others (2004) that may be resuscitated or permitted to repair the damage if given suitable conditions such as incubated in an appropriate, nonselective environment. Ariefdjohan and others (2004) compared the number of *E. coli* O157: NM and *Listeria monocytogenes* cells recovered without repair step (surface-plating on MacConkey agar with Cefixime and modified Oxford agar for *E. coli* O157: NM and *Listeria monocytogenes* respectively) to number enumerated with repair step procedure (membrane transfer method for *E. coli* O157: NM and overlay method for *Listeria monocytogenes*) in alfalfa seeds after high hydrostatic pressure treatment (275, 375, 475, 575 MPa) for 2 min at 40^{o}C. More numbers were recovered for each treatment when repair step was incorporated than surface

plating, suggesting that surface plating on C-MAC and MOA did not allowed for resuscitation of injured cells. Ramaswamy and others (2003) observed that with increasing pressure and time the counts of *E.coli* 29055 in apple juice decreased more rapidly in VRBA (selective medium) than in BHIA (nonselective medium) indicating that more number of cells got injured then immediately killed with the application of pressure. Similarly Hauben and others (1996) also observed that increasing pressure from 180 to 270 MPa resulted in an increased percentage of sublethally injured cells. They studied the effect of 320 MPa for 15 min on E. coli K-12 in buffer, pH 7 at room temperature and found approximately a 4-log unit cycle reduction in numbers, of which 2 log units of organisms were sublethally injured. Subsequent recovery and growth of *Pseudomonas fluorescens* in milk was observed after storage from days 1 to 10 during refrigerated storage when treated at 250 MPa for 5 min at 20^{o}C (Black and others 2005). Patterson and Kilpatrick (1998) found that the combined pressure and temperature treatments cause sublethal injury, for E. coli O157:H7 in poultry meat and UHT milk at lower pressures (< 200Mpa) most of the sublethal injury was due to the effect of heat (> 55^{o}C) and as the pressure increased, injury was observed at lower temperatures suggesting that pressure became the main cause for injury.

Several researchers have used selective compounds such as surface active agents, salts, antibiotics, organic dyes, bile salts, acids in nutritive culture media to act as a selective media that inhibits the repair of injured cells of the target microorganisms (Vch and Fung, 2001). These agents however may inhibit the repair of injured cells of the target microorganisms. The metabolic processes during injury repair with the nature of stress and involve the synthesis of ATP, RNA, DNA and mycopeptides. Cell wall, cytoplasmic membrane, ribosomal RNA and DNA as well as some enzymes are the structural and functional components known to be

damaged by the sublethal stresses (Yuste and others 2003; Bozoglu and others 2004; Pagán and others 2001). The primary site of pressure damage is usually the cell membrane in which cell permeability and ion exchange are altered and are probably the main cause of sublethal injury (Yuste and others 2004; Hoover and others 1989). After high pressure treatment from a microbiological point of view one population of microorganisms will be killed (lethally injured), second population will survive (non injured), and a third population will be injured sublethally (Yuste and others 2004; Vch and Fung, 2001). Generally only healthy microbiota can develop on selective media as the media contain agents which may inhibit repair of sublethally injured cells, however selective media allow for differentiation and enumeration of the specific target microorganisms. Hence selective media may underestimate the microbial content of a particular food product. On the other hand nonselective agars will allow the growth of both sublethally injured cells and non injured cells but cannot differentiate target pathogens from a mixed population (Kang and Fung, 2000; Vchw and Fung). Bozoglu and others (2004) defined three states of cells just after pressure treatment as: active cells (these cells can form visible colonies on both selective and non-selective agar); primary injury (these cells can form colonies on non-selective agar but not on selective agar, but forms colonies on selective agar during prolonged storage) and secondary injury (these cells can form visible colonies on either non-selective or selective agar, but colonies were first formed on non-selective agar and later on selective agar during prolonged storage.

Ideally a method used to detect microorganisms in food should detect both normal and the injured microorganisms. Kang and Fung (1999) discovered the thin agar layer (TAL) method to recover the injured microorganisms. The TAL method is designed to improve recovery of sublethally injured cells and consists of selective medium overlaid with non selective medium.

During the first few hours of incubation of TAL plates, injured cells recover and start to grow on the non selective medium top layer, whereas the agents of the selective medium gradually diffuse to the top layer. Then, the target microorganism performs most reactions that it typically does on selective medium, and growth of most other microorganisms is inhibited by the lower concentration of selective agents. The TAL method is expected to combine the advantages of both selective and nonselective media by allowing differentiation and enumeration of target microorganisms and recovery of sublethally injured cells. The TAL method has proven to be effective to recover inoculated pathogens injured by treatments with heat, acid and chemicals (Kang and Fung; Kang and Fung, 2000; Yuste and others 2003).

Injured bacterial spores

Injured bacterial spores exhibit unique properties as a result of cellular damage during exposure to environmental stresses like injured vegetative cells. Spore injury is more complex than injury to vegetative cells as any of the several steps in the spore cycle (activation, germination, outgrowth, and growth) can be independently affected (Hurst, 1977). Bacterial spores present in foods are not considered as threats to future quality and safety in injured, dormant or superdormant states unless they germinate and become metabolically active. It is very important to be able to detect undamaged as well as injured, dormant spores in food as the repair and proliferation of injured or dormant spores may lead to food safety problems or product quality loss or both (Ray, 1989). Completion of various stages in their spore cycle is involved for the enumeration and recovery of normal, injured or dormant spores which are susceptible to injury and repair (Adams, 1978). The physical treatments involved in the damage to bacterial spores include wet, dry and ultrahigh temperature heating; chemical; ionizing and UV radiations;

hydrostatic pressure. Bacterial spore injury by heat has been observed by many researchers on both anaerobic and aerobic bacteria when elevated temperatures (50 to 170^{o}C) were used including *Clostridium sporogenes* (Futter and Richardson, 1970; Olsen and Scott, 1950; Duncan and Foster, 1968; Grischy and others 1983), *Bacillus stearothermophillus* (Pflug and others 1981), *Bacillus cereus* (Busta and others 1976), *Clostridium botulinum* (Alderton and others 1974).

Heat injured bacterial spores are unable to develop visible signs of growth under conditions that are optimal for unheated spores. Injury to bacterial spores is expressed in various forms as a need for nonnutrient germination stimulants by the injured spores, an altered nutritional requirement by the survivors, an increased sensitivity of the survivors to inhibitors and selective agents, and modified optimum incubation temperatures for the enumeration of survivors (Adams, 1978). To detect injured spore formers the kind of culture medium used influences their recovery and also culture media used for the recovery of injured bacterial spores depends on the type of stress and the organism. A wide variation in the recovery was obtained for heat injured *C. perfringenes* spores depending on the strain and the recovery media (Labbe and Norris, 1982). Heat injured *C. sporogenes* PA 3679 were better recovered in Anderson's pork pea infusion agar than in trypticase peptone agar, yeast extract agar, pork infusion agar, and T-Best agar (Polvino and Bernard, 1982). Similarly more heat stressed *C. sporogenes* PA 3679 spores were recovered on modified PA 3679 agar than on yeast extract agar and peptone trypticase agar (Grischy and others, 1983). When they incorporated lysozyme in MPA 3679 (0.1 µg/ml) agar they found 20-25% increase in the recovery of heat stressed spores and also did not inhibit the recovery of heat activated spores when compared to enumeration in the medium without lysozyme. Although recovery of heat stressed and heat activated spores was suppressed

when concentration of lysozyme was in excess of 0.1 µg/ml. Similarly sodium bicarbonate improved the recovery rate of heat activated and heat stressed when it was included in MPA 3679 agar. Like lysozyme recovery of heat stressed spores was suppressed when sodium bicarbonate was in excess of 0.1%. Recovery of heat stressed spores decreased by 35% when the concentration of sodium bicarbonate in the medium was increased from 0.1% to 0.25%. *C. sporogenes* injured spores treated with irradiation were recovered best in Eugon cystine agar (Wheaton and others 1959). Injured bacterial spore formers may need nutrient supplementation in the recovery media (Foegeding and Busta, 1981). Addition of glucose or blood to the nutrient agar improved the recovery of heated bacillus spores (Curran and Evans, 1937). In general media made from fresh infusions have proved better than those made from dehydrated ingredients for recovery of injured spores (Lund and others, 2000). Antibiotics with surface active properties (e.g., polymyxin, kanamycin, neomycin) and other surface active agents such as sodium lauryl sulfate, sodium deoxycholate, and quaternary ammonium compounds as well as sodium chloride, nitrite, nitrate, acids, alkali, fatty acids, and glycerol and sucrose esters of fatty acids acts as selective agents and are sensitive for injured spores (Sofos and Busta, 1980; Chumney and Adams, 1980; Tsuchido and others 1987; Ray, 1989). Optimum temperatures for injured spores are different than for uninjured spores. Grischy and others (1983) found that optimum recovery and enumeration of heat stressed spores required an incubation time of not less than 96 hr at 32^{o}C. Similarly (Adams, 1978) found that injured spores of C. botulinum were recovered best at 25^{o}C compared with 31 to 37^{o}C for uninjured controls.

Thermal Kinetics of Microbial Inactivation

The decrease in number of viable microorganisms when a suspension of microorganisms is heated at constant temperature follows a first order reaction. The first order reaction is governed by equation (1)

$$- dN/dt = kN \quad (1)$$

Where k = first order rate constant for microbial inactivation, t = time and N = Number of viable microorganisms.

Integrating equation 1 and using initial condition $N = N_0$ at t = 0 we get equation (2)

$$\ln N/N_0 = -kt \quad (2)$$

The slope of the natural logarithm of survivor plotted against time is the rate constant k. Equation (2) suggests a linear semilogarithmic plot of N against t. In common logarithms equation (2) can be expressed as equation (3):

$$2.303 \log N/N_0 = - kt \quad \text{or} \quad \log N/N_0 = - kt/2.303 \quad (3)$$

$$\text{Or:} \quad \log N/N_0 = -t/D \quad (4)$$

Equation (5) described below shows the relation between D and the rate constant k

$$D = 2.303/k \quad (5)$$

Equation (4) defines the decimal reduction time, D value, is defined as the time required to destroying 90% (one log cycle) of microorganisms (reducing viable population by a factor of 10) and is determined from a decimal reduction curve.

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CHAPTER 3

MICROBICIDAL EFFECTS OF CONTINUOUS FLOW HIGH-PRESSURE THROTTLING IN SOYMILK

Sharma V, Singh RK, Toledo RT. To be submitted to Journal of Food Science

Abstract

The thermal resistance of *Clostridium sporogenes* PA 3679 ATCC 7955 was determined in Soymilk (pH 7) and 0.1% peptone water (pH 7) by the capillary tube method. A significant increase in heat resistance was observed in *Clostridium sporogenes* spores when heated in soymilk in comparison to 0.1% peptone water. The D_{121} value for spores in soymilk was approximately 3 folds the values in peptone. The z value was also much higher in soymilk as compared to 0.1% peptone water. Continuous flow high-pressure throttling (HPT) from 207 and 276 MPa to atmospheric pressure reduced the microbial populations in inoculated soymilk up to 6 log cycles when the holding time was 10.4, 15.6 and 20.8 s and temperature was 85, 121, 133, and 145^0C. The sporicidal effect increased as the operating pressure, time and temperature were increased. Milk temperatures increased due to instantaneous pressure release. The soymilk was immediately cooled on line to below 40^0C. More injured spores were found at 207 MPa than at 276 MPa, indicating that lower pressure caused cell injury whereas high pressure caused cell death.

Keywords: high pressure throttling, soymilk, injured spores, sporicidal effects.

Introduction

High hydrostatic pressure is an emerging technique to satisfy consumer demand for more fresh-like, minimally processed and shelf stable food. Many other alternative non-thermal food technique are being studied and used, but high pressure has been most promising because of its simplicity and minimal processing. In 1899, the first experiment involving high pressure was reported by Hite, in which inactivation of microorganism in milk by high pressure was studied (Hoover, 1993). Specially designed vessels are used for high hydrostatic pressure processing and usually pressure between 100 and1000 MPa is used (Food and Drug Administration). The most important attribute of high hydrostatic pressure is uniform and instantaneous pressure distribution throughout the food system irrespective of size, shape and food composition (\Smelt 1998). The technique of HHP relies upon two principles Le Chatlier's and isostatic (Tewari, 2007). Le Chatelier's principle states that, any phenomena in equilibrium (phase transition, chemical reaction, and change in molecular configuration) involving a volume reduction are enhanced by pressure and vice versa. The HHP affects noncovalent bonds therefore high molecular weight components (responsible for functionality determination) may get altered. Covalent bonds are not affected by HHP because of the low energy level in processing and hence low molecular weight food components (responsible for nutritional and sensory characteristics) remain unaffected. The isostatic principle according to which pressure transmittance is instantaneous and uniform (independent of size and geometry of food) that is used to inactivate microorganism present in food (Tewari, 2007; Smelt, 1998).

Several studies have been done to investigate the various aspects of high pressure processing on food matrices. Ripening time of cheese can be reduced by 3 days by a pressure treatment of 250 MPa (Yokoyama and others 2002). Basak and Ramaswamy (1996) reported that

the inactivation of pectin methyl esterase depends on the pressure treatment, pressure-hold time, pH and total soluble solids. Soy protein isolates β –7S and A-11 S polypeptide have shown a better ability in holding at oil-water interface (Puppo and others 2005). Reduction in milk allergenicty was observed by Devlieghere in 2004 by breaking β-lactoglobulin at specific pressure treatments of 250 MPa.

Different species and strains of microorganism respond differently to pressure and thermal inactivation. In addition, the pH, growth conditions and suspending media also affect resistance to inactivation (Patterson, 1999). Gram positive bacteria are most resistant to pressure followed by gram negative and yeast and molds are least sensitive to pressure(Smelt, 1998). *Bacillus* species can be reduced by 1 log after pressure treatment of 300-350 MPa and 10 C for 20 min whereas complete inactivation of molds and yeast was observed at pressure treatment between 300-350 MPa and 10 C for 20 min (Arroyo and others 1997). Stewart and others (1997) observed an additional 3 log reduction in *Listeria monocytogenes* cell counts after a pressure treatment at 353 MPa at 45 C for 10 min. Chilton and others (1997) reported the breakage of DNA in microbial cells at high pressures. Lysis of large cells was reported by Hoover and others, (1989) due to mechanical disruption of stressed cell wall after pressure treatment of 20-40 MPa. Wouters and others (1998) reported loss of membrane functionality as a result of high pressure treatment in *L.plantarum.*

Continuous flow high pressure throttling (CFHPT) has been developed at The University of Georgia, Athens, Georgia (Toledo, 2000). The use of CFHPT is limited to fluid flood that contain very small size suspended solids. In CFHPT, the fluid food passes through a throttling valve, with high velocity and high pressure causing an pressure increase the potential energy. As the food passes through a small orifice, the potential energy changes to heat energy and

instantaneous rise in the temperature as the feed discharges from throttling valve. Thus, as a result of instantaneous pressure reduction and shearing on fluid the microbial cell wall disrupts thereby killing microbial cell. The conversion of pressure (potential) energy into heat across the throttling valve is given by the following equations given (Toledo, 1991):

$$q = Cp\,(T_{out} - T_{in}) = \frac{(P_{in} - P_{out})}{\rho} \qquad (1)$$

$$(T_{out} - T_{in}) = \frac{(P_{in} - P_{out})}{\rho\, Cp} \qquad (2)$$

$$T_{out} = T_{in} + \frac{(P_{in} - P_{out})}{\rho\, Cp} \qquad (3)$$

Where

q = Energy per unit mass (J/kg)

Cp = Specific heat of the fluid at constant pressure (J/kg^{o}C)

T_{out} = Temperature of the fluid at the outlet to the throttling valve (^{o}C)

T_{in} = Temperature of the fluid at the inlet of the throttling valve (^{o}C)

P_{out} = Pressure of the fluid at the outlet of the throttling valve (Pa), assumed as atmospheric pressure

P_{in} = Pressure of the fluid at the inlet of the throttling valve (Pa)

ρ = density of the fluid (kg/m^{3})

CFHPT processing includes simultaneous homogenization and sterilization which can benefit physical properties, texture, and stability of sterilized product during storage.

Microbial reduction of up to 2.14 logs in *Bacillus stearothermophillus* and 5.12 log reduction in *Bacillus megaterium* was observed after high pressure throttling treatment with hold time of 15 s at 135^{o}C (Areekul, 2003). Also Moorman (1997) reported 2 to 4 log reductions in

native microbial population in skim milk through CFHPT process from 310 MPa to atmospheric pressure with a high pressure dwell of only 0.3 s and he also reported inactivation of *P. putida* inoculated in skim milk from an initial population of approximately 10^8 cfu/ml to undetectable levels.

Traditional Chinese soymilk processing involves hydration of soybeans by soaking them in cold water, then grinding and filtering out the insoluble solid particles. Finally the filtered soymilk is boiled to get better flavor. It has been reported by Lo and others (1968) that this process yields only 65% of the soybean solids. Several researches have attempted to utilize whole soybean for soymilk without wasting any solid portion of the bean. The Illinois process recovers 88-90% of soybean solids but because of the low physical stability and chalkiness of the soymilk this process has not been widely accepted (Rosenthal and others 2003). In order to increase the stability of Soymilk and improve the incorporation of soybean solids in the milk, CFHPT or high pressure can be an effective approach but more research is required (Sivanandan, 2007). In this research the method described by Sivanandan (2007) which utilized all the solids in the soybean cotyledon was used to prepare the soymilk.

Clostridium sporogenes PA 3679 ATCC 7955 is a heat resistant, gram positive, spore forming organism. In the commercial sterilization process of low acid high moisture foods *Clostridium sporogenes* PA 3679 has been widely used as a surrogate organism for *Clostridium botulinum* since both have similar physiological properties and are associated with the spoilage of canned food products where the cans swell or explode. The heat resistance of spores of *Clostridium sporogenes* PA 3679 has been found to be equal or greater than that of *Clostridium botulinum* (Koutchma and others 2005). The heat resistance of *Clostridium sporogenes* is very well documented in literature. This study was conducted in soymilk which is a low acid food and

therefore very susceptible to pathogenic microbial growth like some *Bacillus* species and *Clostridium* species. No information is available on D value and z value of *Clostridium sporogenes* in soymilk Therefore information was needed on the thermal death kinetics of this organism in order to facilitate the adoption of processing parameters needed to ensure adequate spore inactivation in soymilk. We calculated D and z value of *Clostridium sporogenes* in both soymilk and 0.1% sterile peptone water.

The overall objective of this study was to determine heat resistance of *Clostridium sporogenes* in soymilk, to quantify the kinetic parameters for inactivation kinetic parameters as affected by various parameters like pressure, temperature and hold time in different combinations were determined. The extent of spore injury that results when processing the inoculated soymilk in the continuous flow high pressure throttling system was also evaluated

Materials and Methods

Soybeans were collected from the Georgia Seed Development Commission, 2420 South Milledge Avenue, Athens, GA 30605. The soybeans that were used for this study were Benning variety, Group VII cultivar soybean (Glycine max [L.] Merrill), harvested in 2005 from Davisboro, GA. To minimize the changes in composition, soybeans were stored in closed polyethylene bags at 4^{o}C and 20% RH in the dark throughout the experiments until it was processed into soymilk. Deionized water (DW) was used throughout the experiments to prepare soymilk.

Preparation of Soymilk

The equipment used for soymilk preparation was made of either stainless steel or plastic. Soymilk preparation involved series of steps which is shown in the figure 3.1. The details and working of the machine used in the process are as follows:

Soybeans were placed in a tray and dehulled in an impinger oven (Lincoln Impinger Model 1450, Lincoln Food Service Products, Inc., Fort Wayne, Indiana, USA.) that was set at 154.4^{o}C for 5 min. The tempered soybeans were coarse ground in a plate mill (Quaker City Mill Model 4-E, QCG Systems, LLC, Phoenixville, PA, USA), keeping in mind that the plates were spaced far enough to crack the hulls but not to break the cotyledons. The hulls and cotyledons were separated manually by air classification. Soymilk was prepared from whole dehulled soybeans by blanching in deionized water (1:5 w/w:: dehulled soybean: DW) at 60^{o}C for 2.5 hrs. The blanched cotyledons were drained and rinsed 3 times with DW. The mixture was suspended in DW (1:3 w/w:: blanched dehulled soybean: DW) followed by grinding in food processor (Robert Coupe Model RSI 10V, Robert Coupe USA, Inc., Joliet, IL, USA) at 2500 rpm for 2.5 minutes and at 3000 rpm for 2.5 minutes. The comminution was done in a Megatron (Model MTK 5000Q, Kinematica Inc., Cincinnati, OH, USA) at 13,000 rpm for 15 min that was described as the best process to further reduce the particle size (Sivanandan, 2007). The process included 2 levels of pressure treatment. The diagram of the CFHPT system is shown in figure 3.2. The comminuted suspension was pressurized at 207 and 276 MPa using Megatron.

Preparation of spores

Spores from *C. sporogenes* PA 3679 ATCC 7955 were tested in soymilk and processed with a continuous flow high pressure throttling system to determine the lethality. To ensure

healthy cultures, *C. sporogenes* was grown in reinforced clostridium medium (RCM, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.) and incubated for 24 h at 37^{o}C during three initial transfers, in anaerobic jars with an atmosphere containing 5-10% CO_2 obtained by the application of gas packs (BD BBL^{TM} GasPak^{TM} Anaerobic System Envelopes, Difco Laboratories, Div. of Becton, Dickinson and Co., Sparks, Md., U.S.A.). Following initial transfers *C. sporogenes* vegetative cells were grown in RCM for 24 h at 37°C before sporulation. Spores were prepared by distributing actively growing culture into a medium comprised of 3% trypticase soy broth (TSB, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.), 0.1% yeast extract (YE, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.) and 1% ammonium sulfate (J.T. Baker, Phillipsburg, NJ 08865) (Kalchayanand and others 2004). The medium was incubated for 14 days at 35˚C. Spore suspensions were centrifuged (Centrific™ Centrifuge Model 225, Fisher Scientific, U.S.A.) at 10,000 x g for 20 min, rinsed with steriled distilled water and centrifuged again as before. The process was repeated 3 times. Spores were collected and stored at 4˚C for further use. Microscopic examinations were performed to confirm that the suspension consisted primarily of *Clostridium sporogenes* spores. Prior to inoculating the spores in soymilk, spores were heat shocked at 80^{o}C for 15 min in 100 ml of soymilk immediately prior to sample inoculation. Enumeration of *C. sporogenes* spores were carried out in anaerobic jars as before, on brain heart infusion agar (BHIA, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, MD., U.S.A.) for 48 h at 37°C. Soymilk samples yielding zero colonies on enumeration i.e. samples with no detectable CFU (detection limit ≤1 CFU/ml) were subjected to enrichment (48 h at 37°C) in RCM and visually checked for turbidity.

CFHPT Treatment

Soymilk was prepared according to the method shown in Figure 3.1 containing all solids in the soybeans. The schematic diagram of the CFHPT system is shown in Figure 3.2. The CFHPT system consisted of a feed pump that is used to maintain a constant pressure to the fluid feed to the intensifier, a dual pressure intensifier pistons that works alternately to take in fluid food product where as the other discharges the high pressure fluid, a heat exchanger to increase the temperature of the pressurized fluid product before throttling, a throttling valve to immediately drop the pressure that further increases the temperature of the fluid product, a back pressure valve to avoid flashing of the fluid food, a holding tube to give desired residence time to the fluid food, and a tubular heat exchanger to immediately cool the product using ice water as the coolant after it leaves the holding tube .

Spores were heat shocked at 80^{o}C for 15 min in 500 ml of soymilk immediately before sample inoculation. The comminuted suspension of the soymilk was pressurized at 207and 276 MPa using two separate intensifier pistons acting alternately. Piston movement was synchronized by a microprocessor which also controls the opening and closing of the intake and discharge valves of fluid entering and leaving the intensifier (Stansted Fluid Power LTD., UK). The pistons were driven by a hydraulic pump (Model nG7900, Stansted Fluid Power LTD., Stansted, Essex, UK). The pressure generated were read from the pressure gauge located in the CFHPT system. The resulting fluid high pressure was generated when a pre-set hydraulic fluid pressure drives the piston while a small opening of the throttling valve restricts flow out of the system. The throttling valve was a micrometering valve (model 60VRMM4882, Autoclave Engineers, Fluid Components, Erie, PA 16506-2302).

The inlet temperature of the soymilk to the CFHPT system was kept at 30 ± 2^{o}C. For all the treatments, a tubular heat exchanger was installed between the pressure intensifier and the throttling valve to heat the soymilk after pressurization to (53, 64, and 76^{o}C) to get final exit temperature of the soymilk after depressurization of 121, 133, and 145^{o}C for 276 MPa and for (70, 82, and 94^{o}C) to get final exit temperature of 121, 133, and 145^{o}C for 207 MPa. All the exit temperatures fluctuated by ± 4^{o}C. When the temperature of the soymilk leaving the tubular heat exchanger was 32^{o}C ± 2C the final exit temperature was 85^{o}C for 207 MPa and 102^{o}C for 276 MPa. Thermocouples were connected at the outlet of steam heated tubular heat exchanger and at the end of holding tube that was located after the throttling valve to record the temperature of the soymilk after it leaves the tubular heat exchanger and holding tube, respectively. After throttling, a minimum back pressure of 0.4 MPa was applied to avoid flashing of vapors at the outlet by raising the boiling point of the fluid. The minimum back pressure varied for each applied pressure as the adiabatic temperature rise varied with applied pressure. The minimum back pressure was calculated from the saturated steam table using the saturated temperature of the water at applied pressure (Toledo, 1991). The soymilk remained at the elevated temperature after depressurization for desired residence time in the holding tube between the throttling valve and back pressure valve. Thus the adiabatic temperature rise as the fluid passes through the throttling valve is responsible for both microbial inactivation and desirable physical effects on the fluid product. A thermocouple was used to measure the elevated temperature at the end of holding tube. By using another heat exchanger coil completely immersed in ice bath the temperature of the sterilized soymilk was lowered to 4^{o}C to avoid additional heat. The soymilk samples were collected in pre sterilized glass bottles. Volumetric flow rates of 0.75, 1.0, and 1.5 L/min were used. (Hold time calculated at these three flow rates were 10.4, 15.6, 20.8 respectively).

Immediately after processing, samples were kept in a cooler at 4^{o}C. The stored soymilk samples were analyzed within 24 hrs of processing.

Determination of heat resistance of *Clostridium sporogenes*

The pasteurized soymilk was inoculated with *Clostridium sporogenes* spores to give an inoculum of approximately 10^{8} spores. The inoculated soymilk was heat shocked as described earlier, for determination of viable spore counts and to facilitate filling into capillary tubes. The thermal treatment was determined by capillary tube method (Stumbo, 1973).

Heating the inoculated soymilk (80^{o}C for 15 min) was done to activate the germination of spores and to kill the vegetative cells if present in soymilk. It is essential to heat shock the spore suspension in order to break the dormancy of spores and for rapid germination (Stumbo, 1973).

Fifty µl of inoculated soymilk was filled in the capillary tube (1.5 mm inside diameter, 1.8 outside diameter and 100 mm length, Kimax-51, USA) ensuring that there was no formation of bubbles in the capillary tube. The open ends were flame sealed. The capillary tubes were then completely immersed in a constant temperature oil bath (Isotemp Model 1013S, Fisher Scientific Inc., U.S.A.) at the desired temperatures ±0.5^{o}C. Antifreeze (Valuecraft™, Best parts, Inc., U.S.A.) was used as the heating medium. After a designated heating time duplicate tubes were removed and immediately immersed in cooling water. To determine the come up time duplicate controls were used. The capillary tubes were first washed in soap solution, rinsed with sterile deionized water and immersed in 70% ethanol and finally air dried. The controls (unheated capillary tubes) were cooled in ice water as the heated samples, but were heat shocked at 80^{o}C for 15 min to activate spore germination before enumeration of viable spores. Spores were also suspended in sterile 0.1% peptone and subjected to the following thermal treatment 98.9, 110.0,

121.0^{o}C for different time combinations. The viable counts were determined by duplicating plating on BHIA and incubating for 48 h at 37^{o}C.

Thermal resistance of *Clostridium sporogenes* spores was determined using thermal death time capillary tube method procedures described by (Stumbo, 1973) and kinetic data was further calculated as discussed by (Toledo, 1999). The destruction of *Clostridium sporogenes* spores was modeled following the first order reaction kinetics:

$$\text{Log } (N/N_o) = -t/D \quad (4)$$

Where, N is the viable number of spores after heating, N_o is the number of viable spores before heating, t is the heating time and D is the decimal death time (D value is the time needed to destroy 90% of the microorganisms). The D values were calculated as the negative reciprocal of the slopes of the survival curves against time. In addition to D value determinations, z values (z value is the number of degrees Celsius required to bring about a 10-fold change in D value) were then determined by calculating the negative inverse slope of the $\log_{10}$D compared with temperature plot.

Enumeration of microorganisms

Soymilk samples were stored at (4^{o}C) and enumerated within 5 h. Prior to enumeration, the soymilk samples in glass bottles were heat shocked at 80^{o}C for 15 min (to heat shock the ungerminated spores and to inactivate pressure induced germinated spores). Spores were enumerated by spread plating in duplicate onto brain heart infusion agar (BHIA, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.) for 48 h at 37°C). Further investigation was carried out to confirm if complete inactivation had been achieved. For this purpose 1 ml of each soymilk sample yielding zero enumeration results, i.e., samples with

less than detectable CFU (detection limit ≤1 CFU/ml) were subjected to enrichment (48 h at 37°C) in RCM. Then a loopful of RCM was streaked out on brain heart infusion agar, which was incubated at 37°C for 48 h. To enumerate the injured spores, the thin agar layer (TAL) method was used to recover the injured spores (Kang and Fung, 2000). To prepare TAL plates, selective medium (brain heart infusion agar) was poured in the plate and left to solidify, and then selective medium was overlaid with 14 ml of melted tryptic soy agar (7 ml and after solidification another 7 ml). Appropriate decimal dilutions were made in 0.1% peptone (Bacto™ peptone, Difco laboratories, division of Becton Dickinson and Co., Sparks, Md., U.S.A.). The colony forming units (cfu) in plates with 25 to 250 colonies were enumerated and the averages of duplicate plates in the samples were presented.

Statistical analysis

Analysis of variance of the GLM procedure of SAS® software (the SAS® System for Windows™ version 9.1 SAS Inst., Cary, N.C., U.S.A 2004.) was done on the log of the inactivation (N/N_o, where N is the number of surviving cells and N_o is the number of initial cells). Level of significance was set for $P < 0.05$. For each factor (temperature, time, pressure) differences among means were determined. Further Tukey test was conducted to find that which levels were significantly different at each factor.

Results and Discussion

Heat resistance of *Clostridium sporogenes*

We observed larger population of C. sporogenes (~2 log) when the spores were heat shocked as compared to when the heat shock was not done (data not presented). This was in

accordance with Alcock (1984) who observed a D value of 0.6 min when the spores were not heat shocked and the thermal resistance increased to D value of 1 min when the spores were heat shocked for 10 min at 100^{o}C.

Survivor curves were obtained by plotting the number of survivals against the heating time. The D value for *Clostridium sporogenes* in 0.1% peptone water was much less than the D value observed for *C. sporogenes* in soymilk for all the three different temperatures. The calculated D_{121} value for soy milk was 1.47 min and 0.53 min in 0.1% peptone. The D value for all the temperature in 0.1% peptone and soymilk are shown in Table 3.1**.** The z value for 0.1% peptone water was also much lower than soymilk Table 3.1. The z value for 0.1% peptone obtained is 14.02^{o}C and 16.73^{o}C in soymilk (Figure 3.6). The survival curves for *C. sporogenes* in both 0.1% peptone and soymilk are shown in Figure 3.5. The D_{121} value for *C. sporogenes* spores can be as high 3 min (Cameron and others 1980) but in general the D_{121} value range between 0.1-1.5 min (Stumbo, 1973). We calculated the D value in 0.1% peptone and in soymilk so direct comparison with published data is not sensible as various factors like bacterial strain, environmental conditions including media and temperature, the heating medium and properties and the composition of recovery medium, incubation conditions, influences the heat resistance and recovery of heat-treated bacterial spores (Cameron and others 1980; Stumbo, 1973). The D and z values that we calculated are similar to those found by some authors and are different from others. This could be due to the techniques used in the thermal resistance determination, sample heating, and recovery medium and different counting methods employed. Significant differences in D and z values were observed depending on the media, type of heat, and sporulation (Augustin and Pflug, 1967). The highest D values obtained by these authors were in beef infusion or pea infusion when moist heat was used. They observed a D value of 1.4 min when grown in beef

heart and the recovery medium was beef infusion. Our results were similar to the results obtained by these authors, however we used different media for sporulation, enumeration and calculated the D value in soymilk. The D_{121} and D_{110} values in phosphate buffer were reported as 1.3 min and 10.9 min, respectively, by (Da Vi and Zottola, 1978). The results in this study were slightly higher in soymilk and slightly lower in 0.1% peptone, which could be due to the reason that the components of soymilk had some protective effect on the thermal resistance of spores. The calculated D_{121} value of 1.46 min in soymilk and 0.50 min in 0.1% peptone could be due to the types of sugars, fatty acids present in soymilk as the pH and a_w was in the same range. The z value obtained for soymilk was also greater than the value obtained in peptone. The z value also depend on the nature of heat treatment, the z value obtained for *C. sporogenes* subjected to moist heat was approximately double to those obtained with dry heat (Augustin and Pflug, 1967). (Stumbo and others 1950) observed z value of *C. sporogenes* that ranged from 16.6 to 20.5^{o}F. Thermal resistance also varies with different buffer solutions at same pH and some authors have reported higher D value in buffer than in food substrate (Tsuji and others 1960). Cameroon and others (1980) observed a lower D value and higher z value of 14^{o}C in phosphate buffer than in pea puree with a higher D value and lower z value of 12.2^{o}C when the pH was 7 in both cases. We obtained a lower D and z value in 0.1% peptone than in soymilk.

Microbicidal effects of Continuous flow high pressure throttling on *Clostridium sporogenes* in soymilk

The log reduction of *C. sporogenes* in soymilk increased as the hold time, pressure, and temperature were increased. At 207 MPa, log reductions from 0.4 to 5.6 were observed at different exit temperatures (85, 121, 133, and 145^{o}C) and different residence times in the holding

tube (10.4, 15.6, and 20.8 s) (Figure 3.7). Similarly at 276 MPa and the residence time of 10.4, 15.6, and 20.8 s, log reduction increased from 0.85 to 5.8 at different exit temperatures (102, 121, 133, and 145^{o}C) (Figure 3.8). Mean log reduction achieved at different combinations of pressure, hold time, and exit temperatures are shown in (Table 3.2). Almost complete inactivation was achieved when the exit temperature was 145^{o}C for both 207 MPa and 276 MPa. It was observed that at 276 MPa increasing the temperature of the soymilk entering the throttling valve from 53^{o}C to 64^{o}C using a tubular heat exchanger, increased the microbial reduction from 2.05 log to 3.32 log and further increasing the temperature to 76^{o}C almost caused complete inactivation reducing the counts by 5.84 log. Similarly at 207 MPa increasing the temperature of the soymilk entering the throttling valve from 70^{o}C to 82^{o}C using a tubular heat exchanger increased the reduction from 1.04 to 3.14 log and further increasing the temperature to 94^{o}C caused 5.64 log reductions. Feijoo and others (1997) also observed that increasing inlet temperature and operating pressure had positive sporicidal effects in *Bacillus* spores. Similar results were observed by Areekul, (2003) who observed 2.37 to 5.26 log reductions in *B. megaterium* after increasing the temperature of the fluid entering the throttling valve from 75^{o}C to 85^{o}C. Not much research is available on HPT and most of the works on HPH were conducted on vegetative cells. These results were much more effective over microbicidal effects of pressure reported earlier (Thiebaud and others 2003; Feijoo and others 1997). We found that the D value decreased by increasing the pressure, and the D value calculated at 121^{o}C at 207 MPa was 12**.**39 s and D_{121} at 276 MPa was 9.50 s. The z value calculated at 207 MPa was also much higher than that at 276 MPa. The z value at 207 MPa was 71.3^{o}C and 60.9^{o}C for 276 MPa (Figure 3.9). The mean D value at each temperature and pressure and z value is shown in Table 3.4. Rovere and others (1996) observed a D value of 41.7 s at 800 MPa and 108^{o}C and similarly (Koutchma and

others (2005) observed a D value of 49 s at 108^{o}C and 800 MPa in *C. sporogenes* whereas we observed a D value of 14.28 s at 276 MPa and 102^{o}C. The lower D value observed in this study indicated that the multiple effects of shear and other mechanical forces were involved in increasing inactivation on *C. sporogenes* spores. In this study we observed that the effect of pressure and hold time were significantly different from each other in the inactivation of *C. sporogenes* spores. Temperature was the most effective in spore inactivation ($P< 0.0001$). The average mean in the inactivation of *C. sporogenes* inoculated in soymilk for each temperature and hold times were significantly different from each other (Appendix C).

No significant inactivation of *C. sporogenes* spores occurred when treated at 600 MPa for 30 min at 20^{o}C (Mills and others 1998). However, we observed a high reduction at lower pressures and high temperature due to the combination of instantaneous temperature rise, impacts of pressure, high shear, high turbulence, and exposure to hydrodynamic cavitations that usually contributes to the microbial inactivation by disrupting cell membrane integrity.

In general gram-positive bacteria are more pressure resistant than gram-negative microorganisms. Gram negative microorganisms are normally inactivated with 300-400 MPa at 25^{0}C for 10 min to achieve inactivation, while gram positive microorganisms are inactivated with treatments of 500-600 MPa at 25^{0}C for 10 min and the bacterial spores especially of *Clostridium* species, are relatively resistant to it (Hoover and others 1989; Smelt, 1998). In microorganisms, the cell membrane is supposed to be the first and foremost site of damage caused by pressure (Patterson, 2005). Physical damage to cell membrane occurs as a result of pressure, such as leakage of ATP from cell membrane (Patterson, 2005). The HPT process has an affect on the morphological characteristics of microbial cells (Kheadr and others 2002). In HPT

the death of a cell occurs as a result of sudden pressure drop that causes the shear, and instantaneous temperature rise and high turbulence after the throttling valve.

Injured Spores

This study was conducted to assure presence/absence of injured spores after the CFHPT treatment. Injured spores can repair which could affect the microbiological quality of foodstuffs and safety especially in low acid canned foods (Bozoglu and others 2004). In this study we observed that injured spores were present after the CFHPT treatment. We observed that CFHPT did not completely inactivate the microorganisms. It is very important to be able to detect undamaged as well as injured, dormant spores in food as the repair and proliferation of injured or dormant spores may lead to food safety problems or product quality loss or both (Ray, 1989). To enumerate the injured spores, we followed the method developed by Kang and Fung (2000) (TAL method), in which the nonselective (TSA) media was overlaid on selective media (BHIA). The number of injured spores was determined by subtracting cell counts enumerated on BHIA from cell counts observed in TAL. Shelf life of treated soymilk was studied up to 10 days and if injured spores were observed after plating on day 0, the study for shelf life was discontinued.

We observed more counts in TAL in comparison to BHIA, indicating the presence of both healthy and injured spores. The injured spore counts were more at 207 MPa than 276 MPa. The counts in TAL decreased as the treatment temperature or the holding time was increased. It was noticed that the TAL counts for both pressures were significantly different from each other. Hold times were also significantly different from each other in the inactivation of *C. sporogenes* spores inoculated in soymilk. Temperature was the most effective in spore inactivation ($P < 0.0001$). The average means for the inactivation of *C. sporogenes* spores for each temperature

and hold times were significantly different from each other (Appendix D). Mean log reduction achieved at different combinations of pressure, hold time, and exit temperatures are shown in (Figure 3.10). The results indicate that an increasing number of spores were destroyed as a result of pressure treatment and the destruction increased with both temperature and hold time. The BHIA counts (healthy spores) decreased more rapidly than TAL counts. This indicates that more spores get injured than are immediately killed. Finally, at higher pressure and temperature, the counts in both TAL and BHIA were undetectable indicating that no spores were present. In most of the treatments, injured spores were able to resuscitate after plating on day 0 (Table 3.2). Only at few treatments, for example at higher temperature (133, 145^{o}C) spores took longer time to repair indicating that at higher temperatures and at both pressures they were severely injured and showed counts on day 5 and day 10 (Table 3.3). These results were in accordance with the results presented by other researchers (Yuste and others 2003; Ariefdjohan and others 2004). The results also indicate that the CFHPT system caused sublethal injury to spores instead of completely eliminating them.

Temperature rise

The temperature rise in the CFHPT system after the throttling valve was calculated by Equation 3 and was directly proportional to the operating pressure. The exit temperature obtained was the sum of inlet temperature and the temperature rise in the system. Sivanandan (2007) showed that at a particular pressure in CFHPT system, the rise in temperature was not significantly affected by flow rates (Appendix E). The temperature rise must have contributed in the inactivation of spores at high turbulence, shear and cavitations occurred at the throttling valve. The calculation for the temperature rise is shown in Appendix B.

Conclusions

The thermal resistance of *C. sporogenes* was found to be greater in soymilk in comparison to 0.1% peptone water. When pressurized soymilk was passed through a steam heated tubular heat exchanger prior to depressurization significant 5.6 and 5.8 log reductions occurred as a result of pressure treatment at 207 and 276 MPa, respectively. The lethal effect increased as the operating pressure, temperature and the residence time in the holding tube was increased. The injured spores were also present (as the counts in TAL were greater in comparison to those in BHIA) after passing the soymilk through CFHPT system. The high pressure and temperature treatment cause the injured spores to undetectable level.

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Table 3.1- Decimal reduction time (D value, min) and z value and r^2 value for *C. sporogenes* in soymilk and peptone water.

Temperature (^oC)	Soymilk			Peptone water		
	D value (min)	r^2 value	z value (^oC)	D value (min)	r^2 value	z value (^oC)
99	30.9	0.97		20.0	0.89	
110	14.6	0.99	16.7	4.4	0.90	14.0
121	1.5	0.99		0.54	0.93	

Table 3.2 - Mean log reductions [$\log_{10}$ (N/No) (CFU/ml) for *C. sporogenes* in BHIA (brain heart infusion agar, selective medium) and TAL (thin agar layer, non selective medium+selective medium) after plating on day 0.

Pressure (MPa)	Temperature (^{o}C)	Time (s)	BHIA Mean Log N/No	TAL Mean Log N/No
207	85	10.4	-0.45	-0.25
207	85	15.6	-0.64	-0.47
207	85	20.8	-0.82	-0.68
207	121	10.4	-1.04	-0.88
207	121	15.6	-1.22	-1.07
207	121	20.8	-1.61	-1.41
207	133	10.4	-2.39	-2.19
207	133	15.6	-2.62	-2.50
207	133	20.8	-3.11	-2.95
207	145	10.4	-4.37	-4.37
207	145	15.6	-4.95	-4.95
207	145	20.8	-5.47	-5.47
276	102	10.4	-0.82	-0.58
276	102	15.6	-1.15	-0.93
276	102	20.8	-1.34	-1.11
276	121	10.4	-1.42	-1.24
276	121	15.6	-1.71	-1.47
276	121	20.8	-1.97	-1.82
276	133	10.4	-2.65	-2.49
276	133	15.6	-3.23	-3.02
276	133	20.8	-4.11	-4.05
276	145	10.4	-5.27	-5.27
276	145	15.6	-5.79	-5.79
276	145	20.8	-5.79	-5.79

Note – Shelf life for soymilk was discontinued after observing counts on day 0.

Table 3.3 – Mean log reductions [$\log_{10}$ (N/No) (CFU/ml) for *C. sporogenes* in BHIA (brain heart infusion agar, selective medium) and TAL (thin agar layer, non selective medium+selective medium) after plating on day 5 and day 10.

Pressure (MPa)	Temperature (^{o}C)	Time (s)	BHIA Mean Log N/No	TAL Day 5 Mean Log N/No	TAL Day 10 Mean Log N/No
207	133	15.6	-2.88	-2.81	NA
207	133	20.8	-3.09	-3.02	NA
207	145	10.4	-4.37	-4.16	NA
207	145	15.6	-4.95	-4.95	-4.88
207	145	20.8	-5.47	-5.47	-5.47
276	133	10.4	-2.57	-2.52	NA
276	133	15.6	-3.41	-3.31	-3.31
276	133	20.8	-4.11	-4.11	-4.06
276	145	10.4	-5.27	-5.27	-5.27
276	145	15.6	-5.79	-5.79	-5.79
276	145	20.8	-5.79	-5.79	-5.79

NA- Not Applicable

Table 3.4 - Mean D values calculated from SAS after plotting [$\log_{10}$ (N/No)] Vs time for *C. sporogenes* in soymilk.

Pressure (MPa)	Temperature (^{o}C)	D value (s)	z value (^{o}C)
207	85	24.7	
207	121	12.4	71
207	133	6.0	
207	145	3.3	
276	102	14.4	
276	121	9.5	61
276	133	4.8	
276	145	2.9	

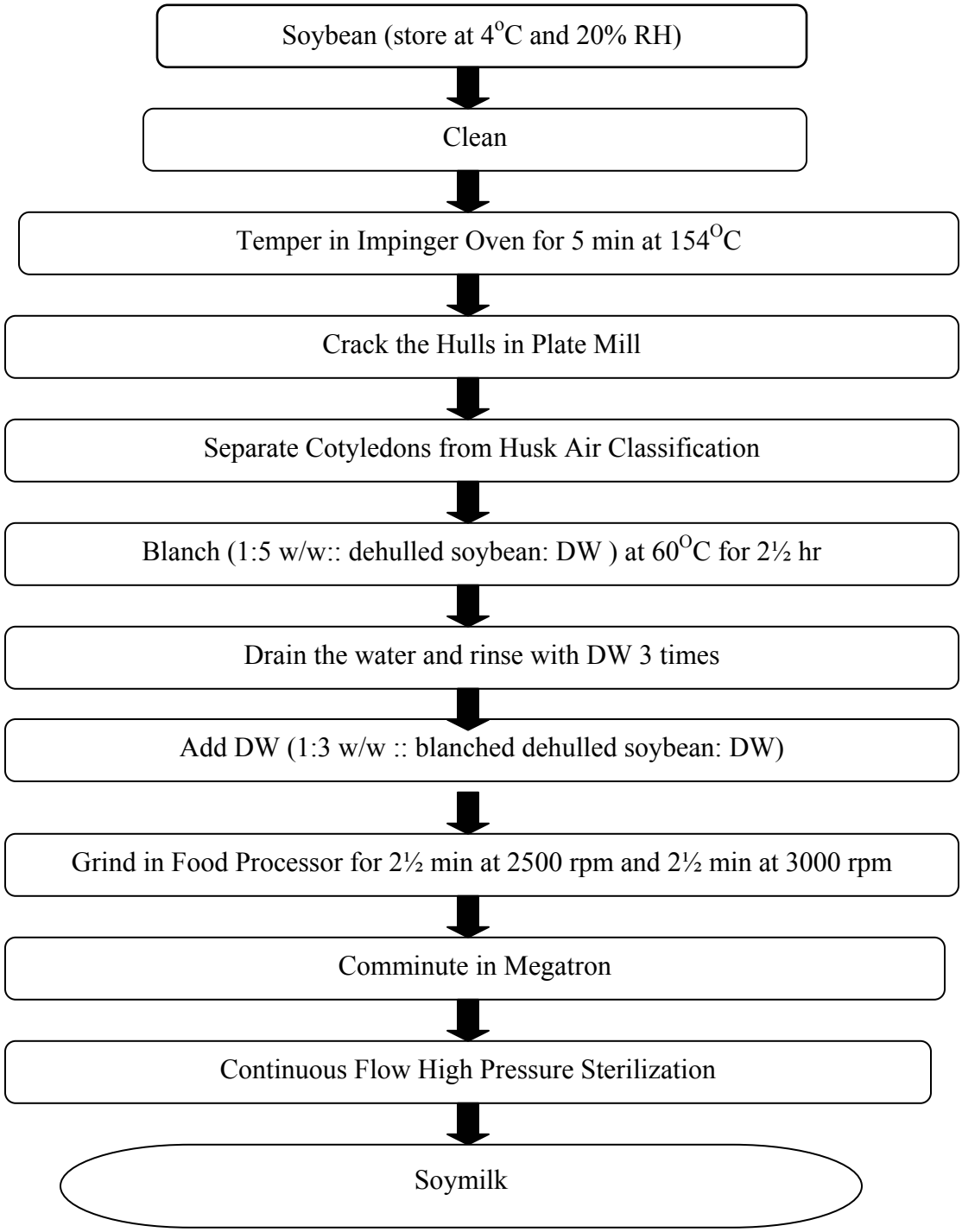


Figure 3.1- Method for preparation of soymilk.

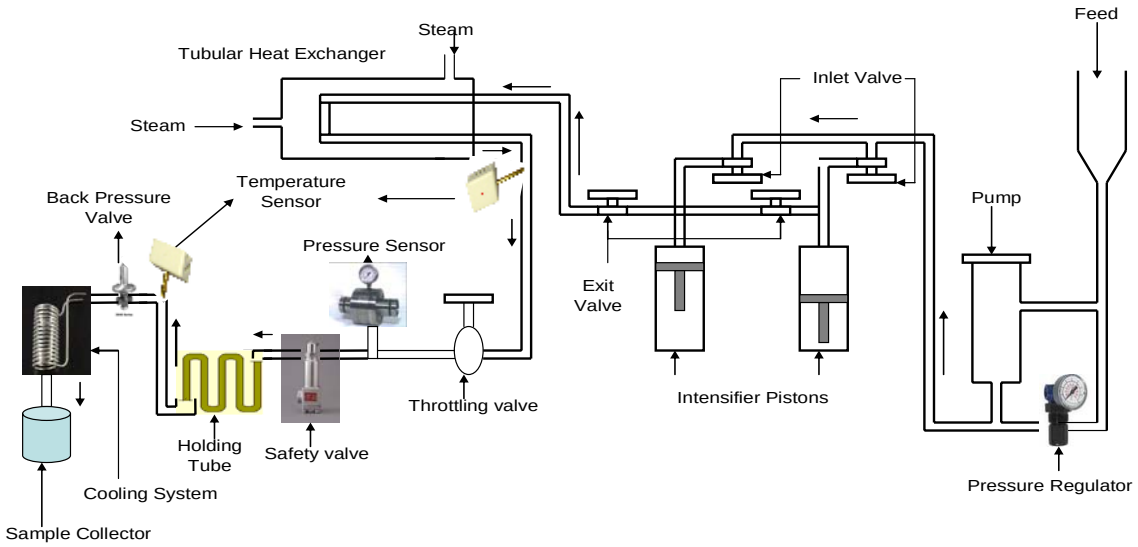


Figure 3.2- Schematic diagram of the CFHPT system showing the flow direction of fluid food.

Figure 3.3- Outside view of Stansted CFHPT system (Model nG7900, Stansted Fluid Power Ltd., Stansted, Essex, UK) showing different attachments. Holding tube (white arrow), Thermocouple (black arrow), back pressure controlling valve (grey arrow), Fluke Hydra Data Bucket (PO Box 9090, Everett, WA 98206-9090), (Black dashed arrow)

Figure 3.4 - Inside view of Stansted CFHPT system (Model nG7900, Stansted Fluid Power Ltd., Stansted, Essex, UK) with throttling valve (dashed white arrow), intensifiers (two head black arrow), inlet valves to intensifiers (black arrow), outlet vave from intensifiers (white arrow), feed inlet (dashed black arrow).

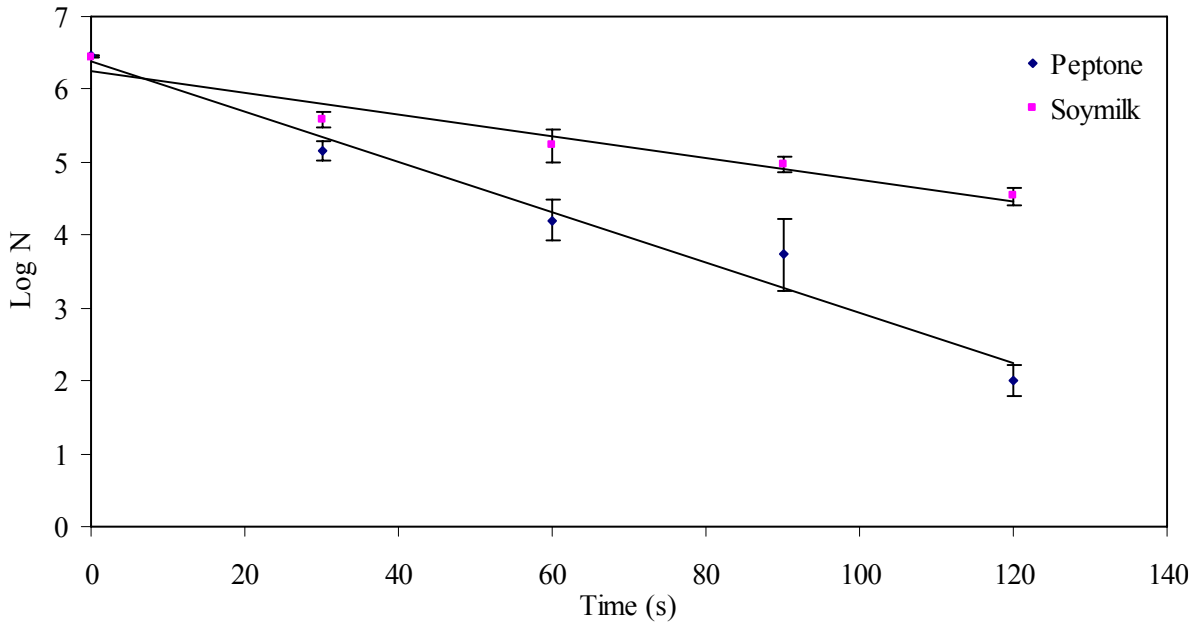


Figure 3.5 - Survival curve for *C. sporogenes* at 121°C in peptone and soymilk

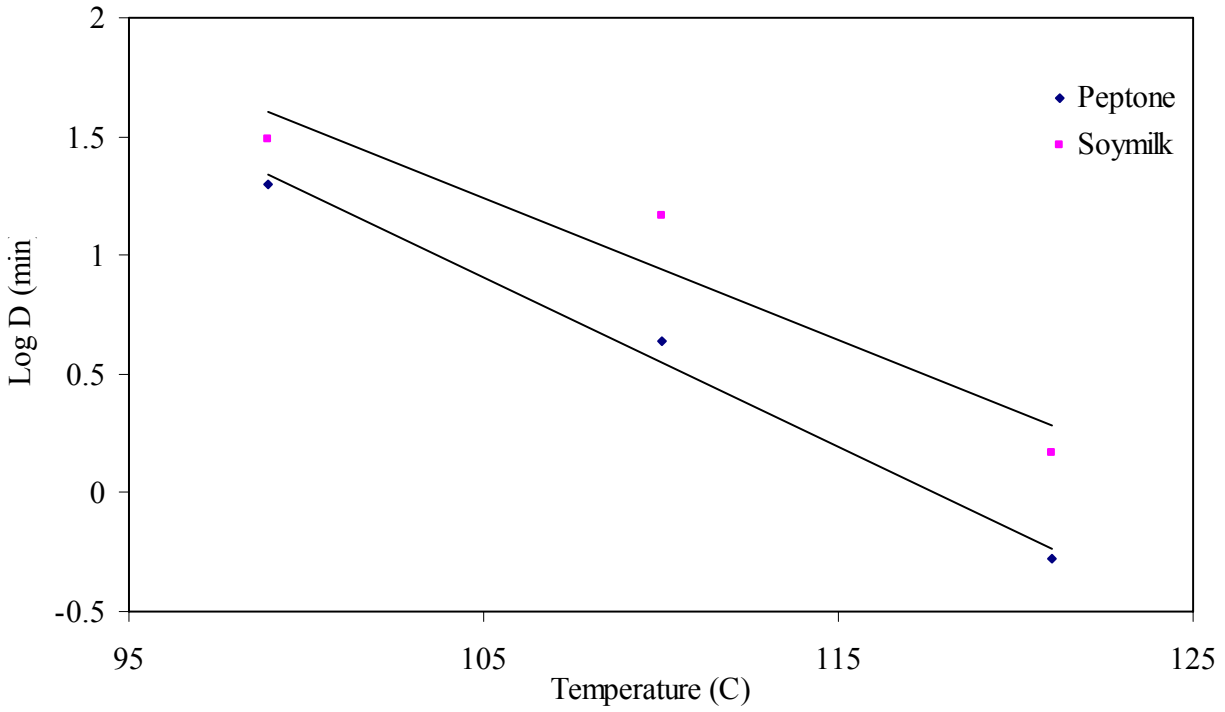


Figure 3.6 - Plot log D Vs temperature for *C. sporogenes* in peptone water and soymilk

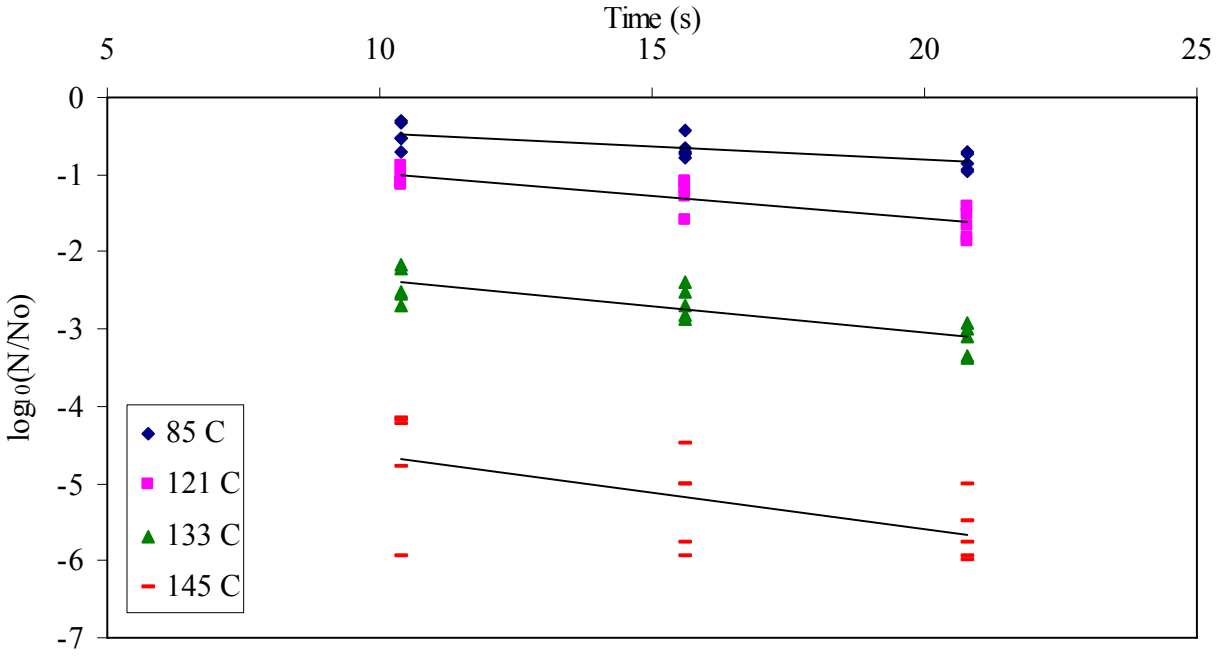


Figure 3.7 - Decimal logarithm of the survival fraction [$\log_{10}$ (N/No)] for *C. sporogenes* in soymilk at 207 MPa at (85, 121, 133, and 145°C).

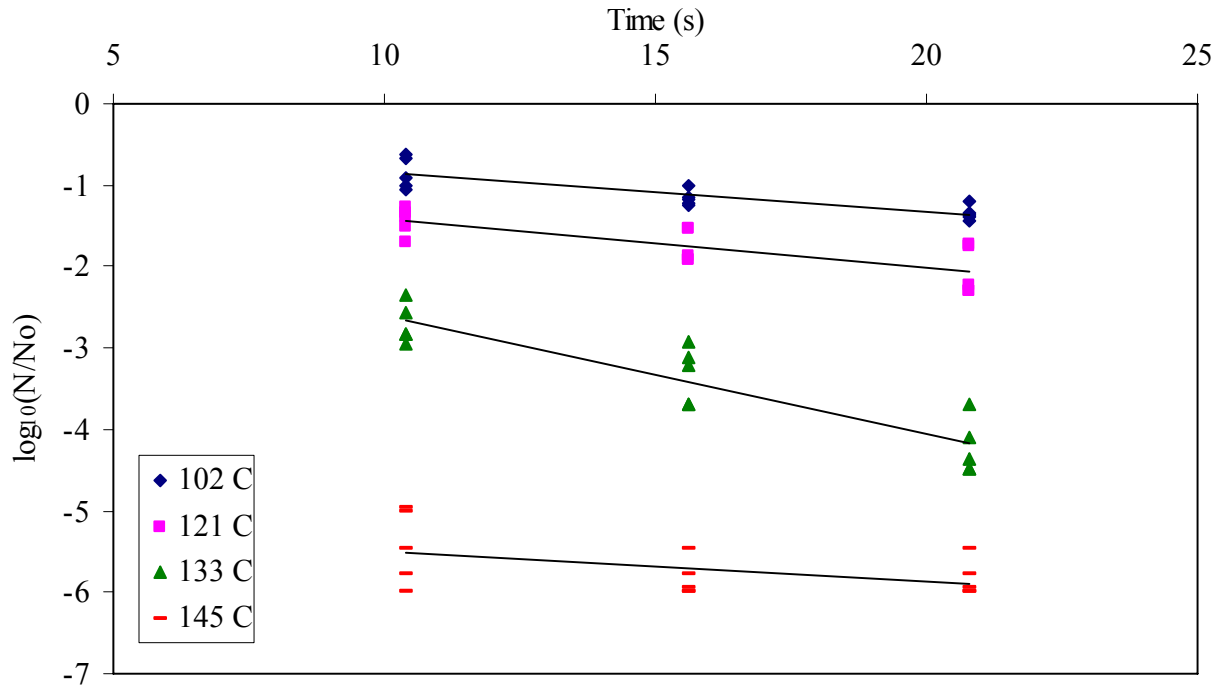


Figure 3.8 - Decimal logarithm of the survival fraction [$\log_{10}$ (N/N_0)] for *C. sporogenes* in soymilk at 276 MPa at (102, 121, 133, and 145°C).

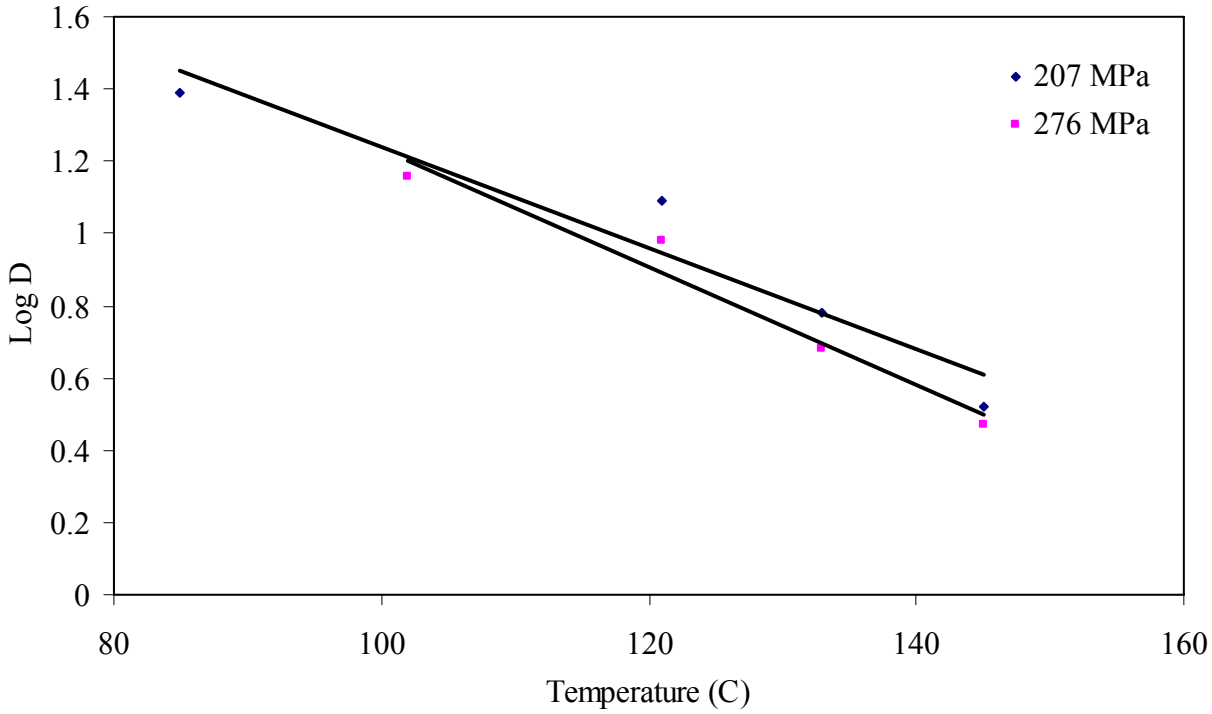
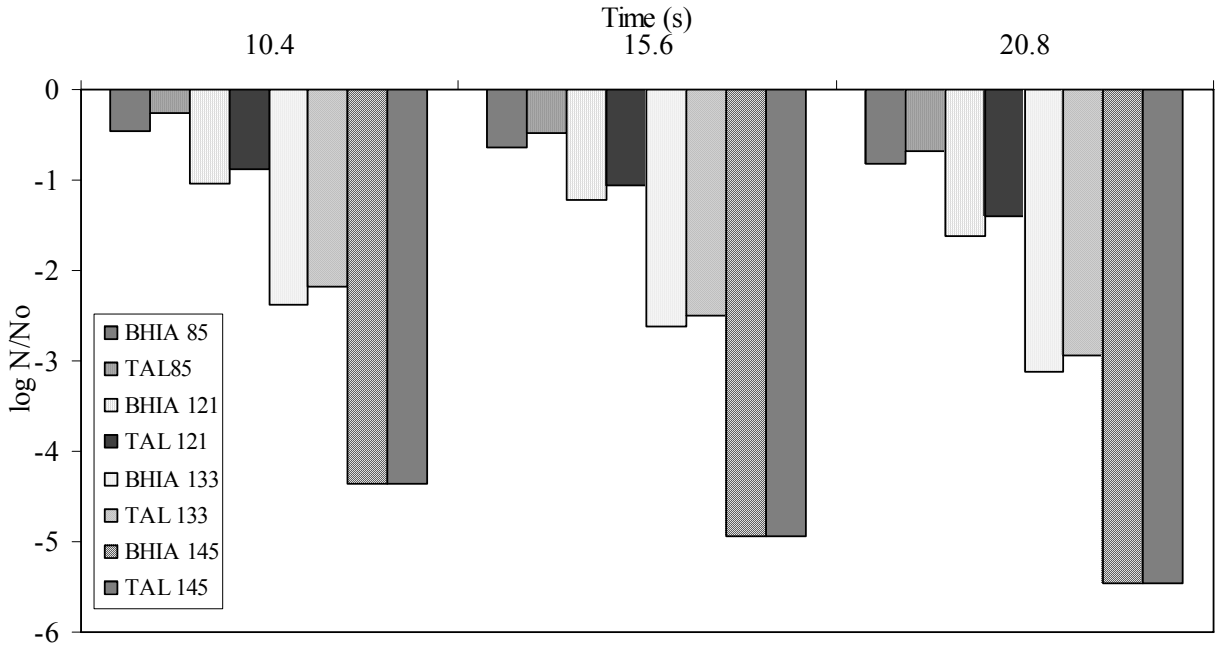
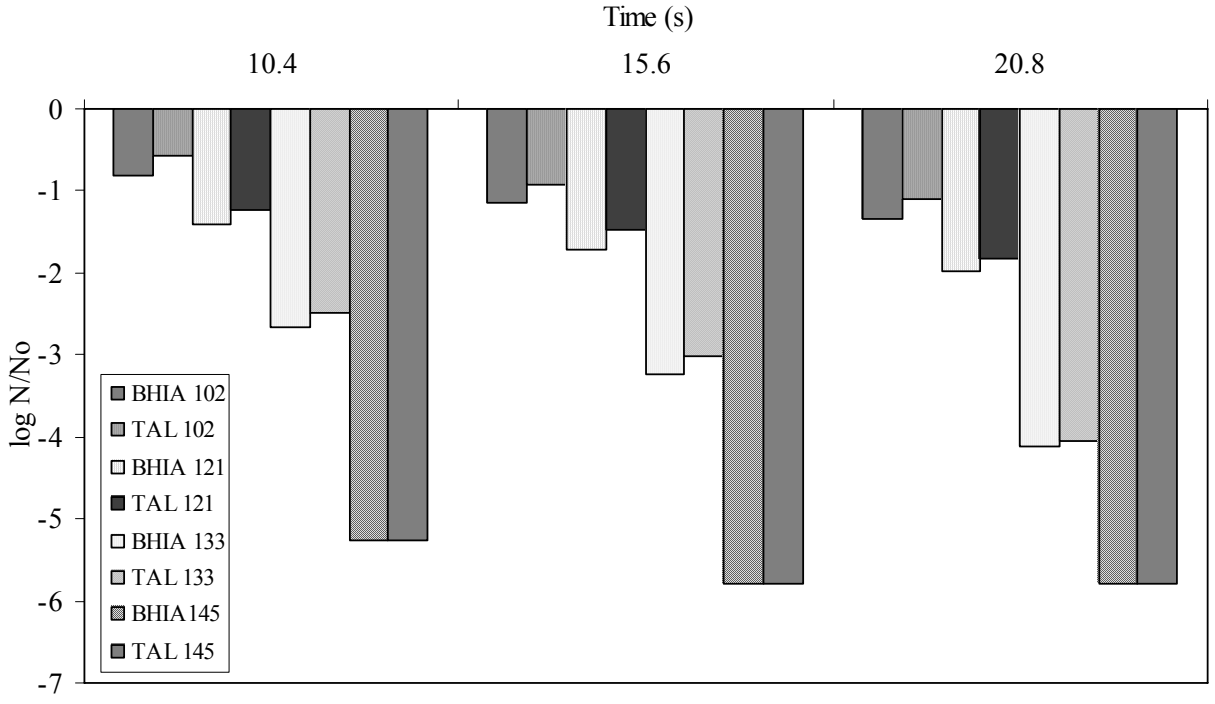


Figure 3.9 - Plot log D Vs temperature for *C. sporogenes* at 207 and 276 MPa.



(a)



(b)

Figure 3.10 – Bar graph showing the mean log reduction at (a) 207 MPa (b) 276 MPa at day 0 in both BHIA (brain heart infusion agar) and TAL (thin agar layer).

CHAPTER 4

SUMMARY AND CONCLUSIONS

Soymilk was processed successfully using the continuous flow high pressure throttling system. The heat resistance of *C. sporogenes* was determined by capillary tube method in soymilk and 0.1% peptone water. The D_{121} value for *C. sporogenes* in 0.1% peptone water was 3-folds less than that in soymilk. The z value observed was also lower in peptone than in soymilk. Similar effect in the microbial inactivation was observed for the two pressures used in this study when the temperature exceeded 145^{o}C. Complete inactivation of spores occurred when the hold time was increased to 20.8 s from 10.4 s. Statistical analysis also revealed that inactivation was significantly affected by the three main variables time, temperature and pressures. A 20.8 s hold time caused most significantly different effect on microbial counts from a 10.4 s hold time. The results showed that spore inactivation increased with increasing operating pressure and exit temperatures and increasing the residence time in the holding tube after the throttling valve. In HPT, the inactivation of microbes occurs as a result of sudden pressure drop that causes the shear, and instantaneous pressure rise and high turbulence after the throttling valve.

The presence of injured spores was determined after enumerating in two different media. Injury was observed more at 207 MPa as compared to 276 MPa suggesting that at lower pressure and temperature a large number of spores were injured while at high pressure and high

temperature no recoverable injured spores were observed. So 276 MPa was the most effective treatment with any of the time, temperature combinations in comparison to 207 MPa.

From our research it can be concluded that high hydrostatic pressure is an effective technique to satisfy consumer demand for more fresh-like, minimally processed and shelf stable product. Many other alternative non-thermal food techniques are being studied and used but among all, the high pressure has been the most promising because of its simplicity and minimal processing. CFHPT system commercially sterilizes the soymilk in seconds and no viable microorganisms remain. Time and temperature are key factors in inactivating microorganisms even under high pressure.

APPENDICES

APPENDIX – A

RESIDENCE TIME OF THE FLUID IN THE HOLDING TUBE

The residence time in the holding tube is calculated as cited by (Toledo, 2007)

The time of residence is calculated by the volume of the holding tube and the volumetric flow rate delivered by the positive displacement pump.

$$\text{Holding time} = \frac{\text{Volume (L)}}{\text{Volumetric flow rate (L/min)}}$$

Volume of the holding tube was calculated by filling the holding tube with soymilk by closing one end. Volume of the holding was found to be = 260ml

Three different flow rates were = 0.75, 1.0, and 1.5 L/min

So the holding time at each volumetric flow rate was determined

Holding time at these flow rates were

0.75 L/min = 20.8 s

1 L/min = 15.6 s

1.5 L/min = 10.4 s

Appendix B

CALCULATIONS FOR TEMPERATURE RISE AFTER THROTTLING VALVE IN CFHPT SYSTEM

For theoretical temperature rise calculations (equation 1) (Toledo, 2007; Amornsin, 1999), Choi and Okos' (1987) equation as cited by Toledo (2007), were used for calculating the soymilk's specific heat C_P, (J/kg.^{O}C) and density, ρ (kg/m^3) as used in equation 1. Inlet temperature (T_{in}) used was 80^{O}C, inlet pressure (P_{in}) was the applied pressure (Pa), and the outlet pressure (P_{out}) was the atmospheric (101325 Pa) pressure. The proximate composition of soymilk used in calculation was taken from (Sivanandan, 2007). [The proximate analysis of soymilk was 3.68% protein, 1.58% fat, 0.26% crude fiber, 92.5% moisture, 0.24% ash, and 1.73% carbohydrate (by difference)].

Table B.1 - Density and specific heat calculation for soymilk using Choi and Okos (1987) equation as cited by Toledo (2007).

Protein, P (kg)	3.68*10^-2	ρ_p = 1288.45	ρ_p*P = 47.41	C_{pp} = 2096.51	P* C_{pp} = 77.15
Fat, F (kg)	1.58*10^-2	ρ_f = 892.18	ρ_f*F =14.10	C_{pf} = 2071.34	F* C_{pf} = 32.73
Crude fiber, Fi (kg)	2.60*10^-3	ρ_{fi} =1282.23	ρ_{fi}*Fi = 3.33	C_{pfi}= 1970.58	Fi*C_{pfi} = 5.12
Water, W (kg)	9.25*10^-1	ρ_w = 973.38	ρ_w*W= 900.48	C_{pw}= 4211.22	W*C_{pw} = 3895.80
Ash, A (kg)	2.40*10^-3	ρ_a = 2401.35	ρ_a* A= 5.76	C_{pa} = 1220.21	A*C_{pa} =2.93
Carbohydrate, C (kg)	1.73*10^-2	ρ_c = 1574.26	ρ_c* C= 27.23	C_{pc} = 1667.79	C*C_{pc} = 28.85
			ρ_{avg} (kg/m^3)= 998.32		C_{avg} (J/kg.k)= 4042.58

Table B.2 - Temperature rise calculations for soymilk using equation (1) (Toledo 2007; Amornsin 1999; Sivanandan 2007).

P_1 (Pa)	Pressure after throttling, P_2 (Pa)	T_1 (^{O}C)	T_2 (^{O}C)=T_1 + (P_1-P_2)/(ρ_{avg}* $C_{avg)}$)	Temperature rise (^{O}C)= T_2-T_1
206842710	101325	80	131.23	51.2
275790280	101325	80	148.31	68.3

References

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Appendix C

SAS OUTPUTS FOR BHIA

The SAS System 00:27 Thursday, April 17, 2008 17

The GLM Procedure

Class Level Information

Class	Levels	Values
pressure	2	207 276
temp	3	121 133 145
time	3	10.4 15.6 20.8

Number of Observations Read 18
Number of Observations Used 18

The SAS System 00:27 Thursday, April 17, 2008 18

The GLM Procedure

Dependent Variable: lognn0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	47.10836662	3.62372051	64.39	0.0005
Error	4	0.22510241	0.05627560		
Corrected Total	17	47.33346902			

R-Square	Coeff Var	Root MSE	lognn0 Mean
0.995244	-7.274247	0.237225	-3.261159

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pressure	1	1.48291249	1.48291249	26.35	0.0068
temp	2	43.32676025	21.66338012	384.95	<.0001
time	2	2.02995186	1.01497593	18.04	0.0100
pressure*temp	2	0.06184890	0.03092445	0.55	0.6154
pressure*time	2	0.01299375	0.00649688	0.12	0.8938
temp*time	4	0.19389936	0.04847484	0.86	0.5557

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pressure	1	1.48291249	1.48291249	26.35	0.0068
temp	2	43.32676025	21.66338012	384.95	<.0001
time	2	2.02995186	1.01497593	18.04	0.0100
pressure*temp	2	0.06184890	0.03092445	0.55	0.6154
pressure*time	2	0.01299375	0.00649688	0.12	0.8938
temp*time	4	0.19389936	0.04847484	0.86	0.5557

The SAS System 00:27 Thursday, April 17, 2008 19

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

temp	lognn0 LSMEAN	LSMEAN Number
121	-1.49473359	1
133	-3.01726606	2
145	-5.27147843	3

Least Squares Means for effect temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lognn0

i/j	1	2	3
1		0.0008	<.0001
2	0.0008		0.0002
3	<.0001	0.0002	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

pressure	lognn0 LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
207	-2.97413318	0.0068
276	-3.54818554	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

time	lognn0 LSMEAN	LSMEAN Number
10.4	-2.85448416	1
15.6	-3.25207258	2
20.8	-3.67692133	3

Least Squares Means for effect time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lognn0

i/j	1	2	3
1		0.0915	0.0085
2	0.0915		0.0758
3	0.0085	0.0758	

Appendix D

SAS OUTPUTS FOR TAL

The SAS System 00:27 Thursday, April 17, 2008 37

The GLM Procedure

Class Level Information

Class	Levels	Values
pressure	2	207 276
temp	3	121 133 145
time	3	10.4 15.6 20.8

Number of Observations Read 18
Number of Observations Used 18

The SAS System 00:27 Thursday, April 17, 2008 38

The GLM Procedure

Dependent Variable: logtal

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	52.97005229	4.07461941	78.38	0.0004
Error	4	0.20793246	0.05198311		
Corrected Total	17	53.17798475			

R-Square	Coeff Var	Root MSE	logtal Mean
0.996090	-7.207165	0.227998	-3.163491

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pressure	1	1.62433809	1.62433809	31.25	0.0050
temp	2	48.68832644	24.34416322	468.31	<.0001
time	2	2.28680295	1.14340147	22.00	0.0069
pressure*temp	2	0.10664507	0.05332253	1.03	0.4369
pressure*time	2	0.01724331	0.00862166	0.17	0.8527
temp*time	4	0.24669643	0.06167411	1.19	0.4362

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pressure	1	1.62433809	1.62433809	31.25	0.0050
temp	2	48.68832644	24.34416322	468.31	<.0001
time	2	2.28680295	1.14340147	22.00	0.0069
pressure*temp	2	0.10664507	0.05332253	1.03	0.4369
pressure*time	2	0.01724331	0.00862166	0.17	0.8527
temp*time	4	0.24669643	0.06167411	1.19	0.4362

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The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

temp	logtal LSMEAN	LSMEAN Number
121	-1.31429498	1
133	-2.86632234	2
145	-5.30985676	3

Least Squares Means for effect temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logtal

i/j	1	2	3
1		0.0007	<.0001
2	0.0007		0.0001
3	<.0001	0.0001	

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The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

pressure	logtal LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
207	-2.86308995	0.0050
276	-3.46389277	

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The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

time	logtal LSMEAN	LSMEAN Number
10.4	-2.73775499	1
15.6	-3.14263253	2
20.8	-3.61008656	3

Least Squares Means for effect time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logtal

i/j	1	2	3
1		0.0777	0.0059
2	0.0777		0.0506
3	0.0059	0.0506	

Appendix E

Effect of flow rate on pressure fluctuation

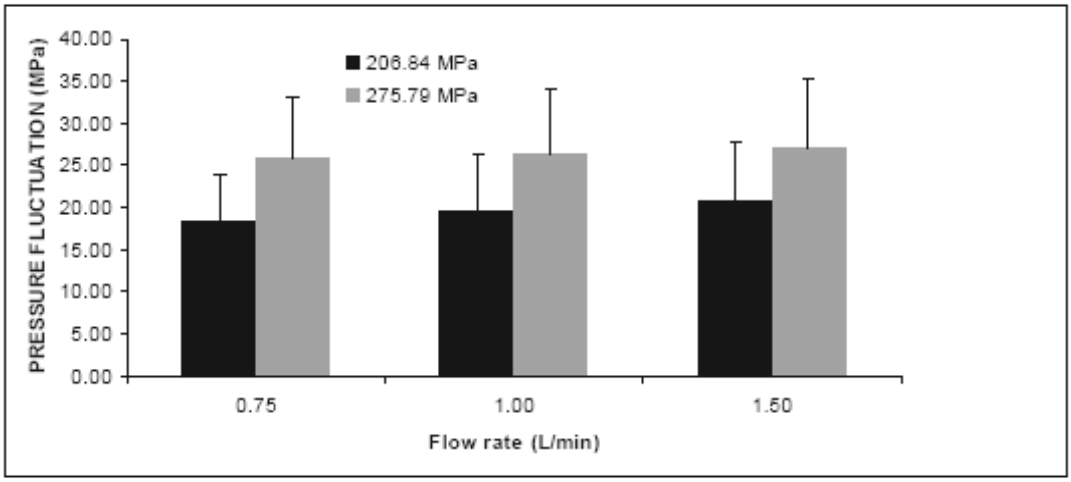

Figure E.1- Pressure fluctuation in CFHPT system at different flow rates. The values are means with SD from six replication. Source: Sivanandan (2007).