Table of Content

List of Figures

List of Tables

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. CHAPTER 2 Thermal resistance of Saccharomyces yeast ascospores in beers. Milani, E. A., Gardner, R., Silva, F. V. M., 2015. Thermal resistance of Saccharomyces yeast ascospores in beers. International Journal of Food Microbiology, 206, 75-80.

Nature of contribution by PhD candidate	Idea, experimental works, writing of the paper
Extent of contribution by PhD candidate (%)	80

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, main supervision of the work and editing of the paper
Prof. Richard Gardner	Supervision on microbiology, including the production of spores

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
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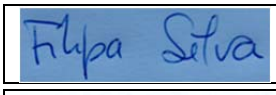
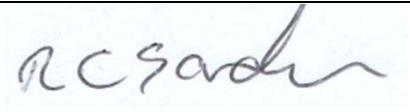
Dr Filipa Silva		1/12/2015
Prof. Richard Gardner		4/12/2015

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. CHAPTER 3 Pulsed Electric Field continuous pasteurization of different types of beers Milani, E. A., Alkhafaji, S., Silva, F. V. M., 2015. Pulsed Electric Fields continuous pasteurization of beer. Food Control, 50, 223-229

Nature of contribution by PhD candidate	Idea, experimental works, writing of the paper
Extent of contribution by PhD candidate (%)	80

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, supervision of the work and editing of the paper
Dr. Sally Alkhafaji	Supervision on PEF technology

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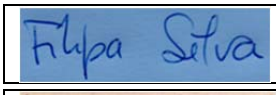
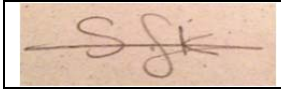
Dr Filipa Silva		1/12/2015
Dr. Sally Alkhafaji		10/12/2015

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Nature of contribution by PhD candidate	Idea, experimental works, writing of the paper
Extent of contribution by PhD candidate (%)	85

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, supervision of the work and editing of the paper

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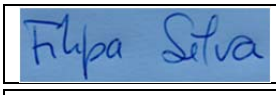
Dr Filipa Silva		1/12/2015

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. CHAPTER 5 Ultrasound pasteurization of beers with different alcohol levels: Modelling the inactivation kinetics of Saccharomyces cerevisiae ascospores Content submitted to Ultrasonics Sonochemistry (Elham A. Milani & Filipa V.M. Silva, 2015)

Nature of contribution by PhD candidate	Idea, experimental works, writing of the paper
Extent of contribution by PhD candidate (%)	85

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, supervision of the work and editing of the paper

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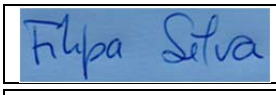
Dr Filipa Silva		1/12/2015

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. CHAPTER 6 Inactivation of Saccharomyces cerevisiae ascospores in beer by high pressure processing, thermosonication, and thermal processing: comparing processes and modelling Content submitted to Journal of Food Engineering (Elham A. Milani, John G. Ramsey, Filipa V.M. Silva, 2015)

Nature of contribution by PhD candidate	Idea, experimental works, writing of the paper
Extent of contribution by PhD candidate (%)	65

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, supervision of the work and editing of the paper
John Ramsey	Part of the experimental work and writing

Certification by Co-Authors

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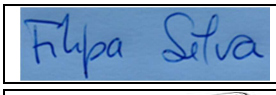
Dr Filipa Silva		16/12/2015
John Ramsey		16/12/2015

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. CHAPTER 7 Studies on the mechanisms of Saccharomyces cerevisiae spores inactivation by scanning electron microscope observations	
Nature of contribution by PhD candidate	Idea,experimental works, writing
Extent of contribution by PhD candidate (%)	65

CO-AUTHORS

Name	Nature of Contribution
Dr Filipa Silva	Idea, supervision of the work and editing of the paper
Siti Nadjiha Mohd Rozali	Part of the experimental work and writing

Certification by Co-Authors

The undersigned hereby certify that:

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Siti Nadjiha Mohd Rozali		16/12/2015

Introduction and thesis framework

Introduction

Beer is an alcoholic beverage, which is produced by yeast fermentation of the starch from malted cereal grains (e.g. barley, wheat) to sugar. *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are yeasts used to produce top and bottom fermenting beers, respectively. The industrial production of bottled beer ends with a process of thermal pasteurization. The aim of this heat process is to inactivate the fermenting yeast and other potential spoilage microorganisms/enzymes. Pasteurization enables the stabilization of the beverage for a longer shelf-life. The pasteurization measure for beer is the PU (pasteurization unit). 1 PU is equivalent to treatment for 1 min at 60ºC, with z=7ºC (Del Vecchio *et al*., 1951). Fifteen PU is the minimum pasteurization required for beer (Baselt 1954; 1958), based on the heat-resistant spoilage yeasts and bacteria in beer (Del Vecchio *et al*., 1951; Portno, 1968; European Brewery Convention, 1995). Beer also contains carbon dioxide and alcohol, and is bittered with hops, all of which are natural antimicrobials, so a mild thermal pasteurization is effective for its stabilization at room temperature (e.g. 20 to 120 PU) (Silva & Gibbs, 2009). However, safety concerns have been expressed about mild pasteurization, especially for alcohol-free and less bitter beers, which are becoming increasingly popular with consumers. Presently, the beer industry applies a more severe pasteurization process (e.g. 120 to 300 PU) to cope with on-going modifications in the traditional beer composition (Silva *et al*., 2014).

Since the conventional thermal process can negatively affect the beer flavour, this research investigated nonthermal techniques for beer pasteurization. High Pressure Processing (HPP) is a commercial nonthermal technology that was also used in this research. There are different types of HPP technologies, which can be used in a semi continuous mode for liquid beverages or in batch mode for solid and liquid foods. Since HPP does not affect the covalent bonds of the food

constituents, treated food retains its freshness, which is an important advantage to food processors (Farkas & Hoover, 2000). Although some researchers have investigated the impact of HPP on *S. cerevisiae* and other yeast inactivation in juices, there are no studies of beer pasteurization by HPP so far.

Power ultrasound is another innovative technology used for microbial and enzyme inactivation (Evelyn & Silva, 2015a; Evelyn & Silva 2015b; Evelyn *et al*., 2016; Sulaiman *et al*, 2015). It relies on the application of ultrasonic waves at intensities higher than 1 W/cm^2 (typically in the range 10 to 1000 W/cm^2) and frequencies between 18 and 100 kHz (McClements, 1995). The beer industry has used ultrasound at the beginning of the mashing process to improve beer yield as well as during fermentation to maintain the nutritional value of alcoholic beverages by enhancing oxidation, which leads to early maturation (Knorr, Zenker, Heinz, & Lee, 2004; Mason, Paniwnyk, & Lorimer, 1996). Except for a few studies on *S. cerevisiae* vegetative cell inactivation in different beverages, no research has been carried on ultrasound microbial inactivation of yeast ascospores in beer.

Pulsed Electric Field (PEF) has been investigated to pasteurize different types of beers (Evrendilek *et al.,* 2004; Levesley & Kennedy, 1999; Ulmer *et al.,* 2002; Walking-Rebeiro *et al.,* 2011). Foods are electrical conductors because they contain large concentrations of ions as electrical charge carriers (Barbosa-Canovas, Pothakamury, Gongora-Nieto, & Swanson, 1999). In PEF, the food contained in the treatment chamber between two electrodes is exposed to high voltage electric short pulses, causing significant microbicidal effect (Ho & Mittal, 1996). A few studies on PEF yeast inactivation in beer were found in the literature: Levesley and Kennedy (1999) registered PEF inactivation of *S. cerevisiae* up to 4 log (16.7 kV/cm and 1280 pulses) at 12- 22°C in India pale ale beer; MacGregor, Farish, Fouracre, Rowan, and Anderson (2000)

reported up to 4.6 log reduction in *S. cerevisiae* cells using 3000 pulses and ~30 kV/cm electric field intensity at 25-30°C; Evrendilek, Li, Dantzer, and Zhang (2004) got a 4.1 log reduction ion vegetative cells of *Saccharomyces uvarum* in a standard keg beer treated with 22 kV/cm, 10.5 mL/s flow rate and 14 µs pulse duration time for 216 µs treatment time; Walkling-Ribeiro, Rodríguez-González, Jayaram, and Griffiths (2011) measured up to 5.1 log inactivation of *S. cerevisiae* vegetative cells in a standard lager beer (45 kV/cm, 536 and 819 µs, 47.1°C).

In conclusion, few studies have investigated the use of nonthermal and thermal pasteurization techniques to inactivate microorganisms in beer. Therefore, in this research, alternative methods like PEF, HPP and power ultrasound processing were used to pasteurize different types of beers.

A detailed literature review has been carried out and is presented in Chapter 1. The results obtained in the research are presented in six chapters. In Chapter 2 the thermal resistance of four *Saccharomyces cerevisiae* strains related to brewing were studied. The modelling of yeast ascospore survivors and comparison of the inactivation level of yeast ascospores by HPP, thermosonication (combined ultrasound and heat), and thermal processing is shown in Chapter 3. For this study, *S. cerevisiae* ATCC 9080 strain was used. This strain is generally found in brewery wastes. For Chapters 4, 5, 6 and 7 DSMZ 1848, the most heat resistant strain of *S. cerevisiae,* was selected for further studies with HPP, TS, and PEF technologies. This strain was isolated from a bottom fermenting ale beer. In Chapter 4, nonthermal pasteurization of beer using HPP was investigated, and the inactivation of *S. cerevisiae* ascospores in different alcohol beers was modelled. In the Chapter 5, the ultrasound pasteurization of beers with different alcohol levels and modelling the inactivation kinetics of *S. cerevisiae* ascospores was carried out. Chapter 6 presents data on Pulsed Electric Field continuous pasteurization of different types of beers. The comparison of energy requirements for equivalent inactivation of yeast ascospores

by HPP, thermosonication, PEF and thermal processing is presented in Chapters 3 and 6. Taste assessment of beer pasteurized by PEF, HPP, and power ultrasound was also carried out and the results are shown in Chapters 4-6.

The last study presented in Chapter 7 refers to the microscopic observation of the morphology of *S. cerevisiae* live and dead spores, which were treated by non thermal HPP and thermal processes and to describe the possible mechanism for inactivation.

Thesis Objectives

The foundation of this study was built on previous investigations on the nonthermal pasteurization of nonalcoholic and alcoholic drinks and aims to investigate the effectiveness of novel technologies such as HPP, ultrasound, and PEF to pasteurize beers with different alcohol content, while trying to retain the freshness of the beer taste. The main focus was the inactivation of the most heat-resistant *S. cerevisiae* ascospores as the most abundant microorganism in beer after fermentation, responsible for changes in the beer characteristics during storage. The specific objectives of this research are listed below:

1- To study the thermal resistance of four strains of *S. cerevisiae* yeast ascospores in different alcohol content beers

2- To model the inactivation kinetics of ATCC 9080 *S. cerevisiae* ascospores by HPP and thermosonication in beer

3- To study the inactivation of DSMZ 1848 *S. cerevisiae* ascospores by HPP, power ultrasound, and PEF in different alcohol content beers

4- To compare the energy requirements of the inactivation of *S. cerevisiae* ascospores by HPP, thermosonication, and PEF with thermal processing

5- To assess the sensory of HPP, TS, PEF and thermally treated beers and compare the overall flavour with untreated beer.

6- To study the mechanisms of *S. cerevisiae* spores inactivation by scanning electron microscope observations

Thesis framework

Introduction and thesis framework	
Chapter 1- Literature review	
Chapter 2- Thermal resistance of *Saccharomyces* yeast ascospores in beers	Objectives 1
Chapter 3- High pressure processing and thermosonication of beer: comparing the energy requirements and *Saccharomyces cerevisiae* ascospores inactivation with thermal processing and modelling	Objectives 2, 4
Chapter 4- Nonthermal pasteurization of beer by high pressure processing: Modelling the inactivation of *Saccharomyces cerevisiae* ascospores in different alcohol beers	Objectives 3, 5
Chapter 5- Ultrasound pasteurization of beers with different alcohol levels: Modelling the inactivation kinetics of *Saccharomyces cerevisiae* ascospores	Objectives 3, 5
Chapter 6- Pulsed Electric Field continuous pasteurization of different types of beers	Objectives 3, 4, 5
Chapter 7- Studies on the mechanisms of *Saccharomyces cerevisiae* spores inactivation by scanning electron microscope observations	Objective 6
Conclusion and future work recommendations	

Chapter 1. Literature review

1.1. Beer

1.1.1. History

Fermented extract of malted barley is called beer which is a world-known alcoholic drink used for refreshment. Chemical investigation of antique pottery jars has revealed that Sumerians (now known as Iranians) started beer production in 7000 BC. Fermentation and brewing were recognized as the primary biological engineering tasks, which used the fermentation process. Ale is one of the oldest man-made drinks, which based on the history of Ancient Egypt and Mesopotamia, initiated in 5000BC. A Sumerian tablet from 6000 years ago that was found in Mesopotamia is the oldest proof of beer. The tablet depicts people drinking a liquid through reed straws from a shared bowl. Also, there is evidence of a 3900 year-old poem attributed to the Sumerian Goddess of brewing, Ninkasi. This poem explains how to produce beer from barley. It is acknowledged that it is possible to use wild yeasts in the air to ferment any kind of cereal holding definite sugars. As such, it can be concluded that cereal culturing by tribes around the world has preceded beer production. Around 3500-3100 BC in the central Zagros Mountains of Iran, the first barely beer was produced chemically based on evidence revealed at Godin Tepe (El-Mansi *et al*., 2011; McGoven *et al*., 2004; Mirsky, 2007; Protz, 2004).

1.1.2. Beer production, yeasts in beer and beer styles

This statistic presents the worldwide trend of beer production from 1998 to 2014. In 2011, global beer production amounted to about 200 hectolitres, up from 130 hectolitres in 1998. Beer is a widely consumed beverage around the world, which is produced by water, malt, hops, and yeast as basic ingredients. China, the United States, and Brazil are the global leading countries in beer production.

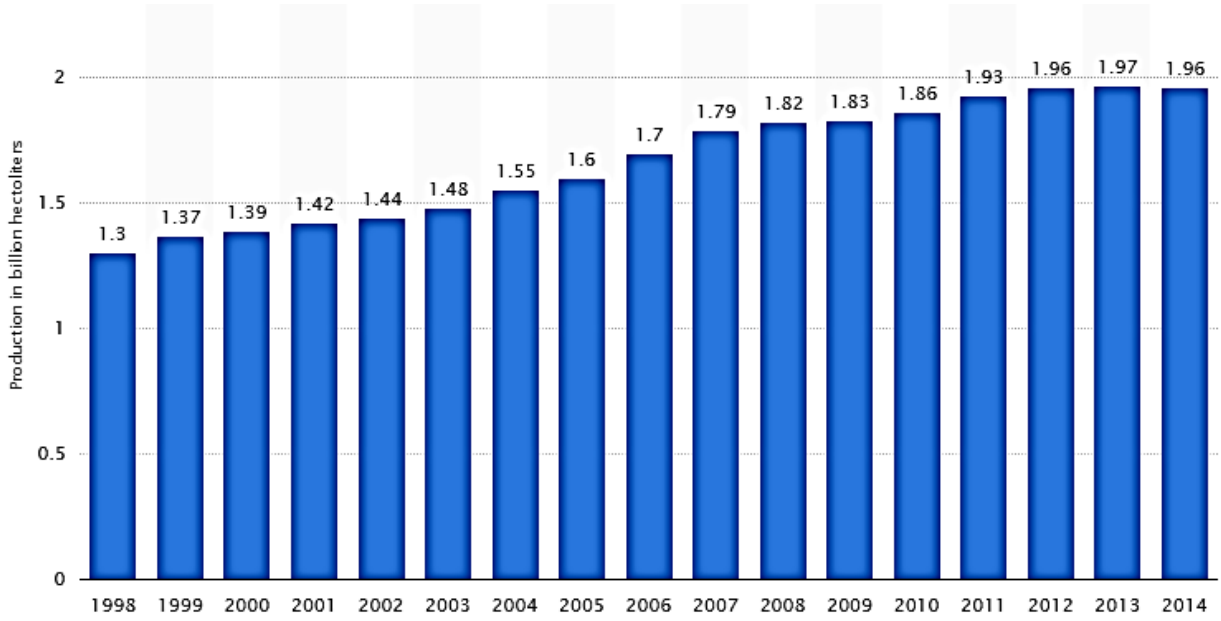

Figure 1.1. World beer production (1998-2014) (Statista, 2015).

The basic types of beers are ale and lager that will be discussed in this section. Lager and ale beers have similar basic ingredients and brewing method; yet they differ in the yeasts strains that are used for fermentation. The following diagram (Figure 1.2) briefly presents the brewing process. Brewers employ different *Saccharomyces* strains based on the type of produced beer.

S.cerevisiae is recognized as top fermenting beer and is used to produce ale beer. *S. pastorianus* known as bottom fermenting beer is mostly applied for lager beer.

Yeasts require temperatures of 18 to 25°C for top fermenting beer. In this method, the yeast-produced biomass floats over the fermented wort until the end of the fermentation. Ale yeasts have less flocculation in comparison to lager yeasts. Bubbles of carbon dioxide absorb the cells that were taken to the wort surface. In the top fermenting method, the yeasts need to become accustomed to the wort, in which they are inoculated during the first step of fermentation. This will take 12 to 18 hours. Fine white bubbles are observed on the vessel or barrel surface as the result of the yeasts' growth. The bubbles lead to "break" and brownish colour flakes are seen on the top. By the time fermentation begins in the wort, the specific gravity decreases. The temperature rises at this stage, called top heat of fermentation, in order to get to the appropriate heat. The yeasts should remain in this state for 36 to 48 hours in order to achieve the peak point of fermentation. To maintain the temperature at a suitable point, some cooling is also accomplished. The number of yeast cells reaches the maximum range at this level; nevertheless, the activation of yeasts discontinues slowly and the colour of wort head changes to creamy as the nutrients in the wort decline. This might be due to the rate of sugar uptake, which depends on the gravity or density reduction; yet the fermentation progress is mainly managed by temperature.

Conversely, fermentation of lager yeasts, known as bottom fermenting yeasts, happens in lower temperatures between 8 to 12°C. In comparison to the ale yeasts, they are completely different in their capability to consume raffinose and melibiose. Consequently, using the sucrose, glucose, fructose, maltose, and malatotriose they flocculate underneath the wort. The yeasts settle to the barrel bottom or cone while the fermentation is done. It is suggested to take out a small portion of the yeasts from the bottom 12 hours after the start of fermentation in order to retain a good

lager beer. Typically, after 7 days of fermentation, decline in the wort nutrients slows down the yeast activity. The process is normally longer due to cooling of the vessels, as this type of beer requires a lower temperature. Yeast should be gathered 24 to 48 hours following the shrinking point to get to the best outcome. Measuring the gravity decrease is the most popular method to verify the progress of a lager beer. In comparison to ale beers, lager beers have a lower collection, and the highest temperature of bottom fermenting method is lower than the maximum temperature of top fermenting method (Comi & Manzano; 2008; El-Mansi *et al*., 2006; Priest & Yeasts; 2006; Romano *et al*. 2006; Tamang, 2010).

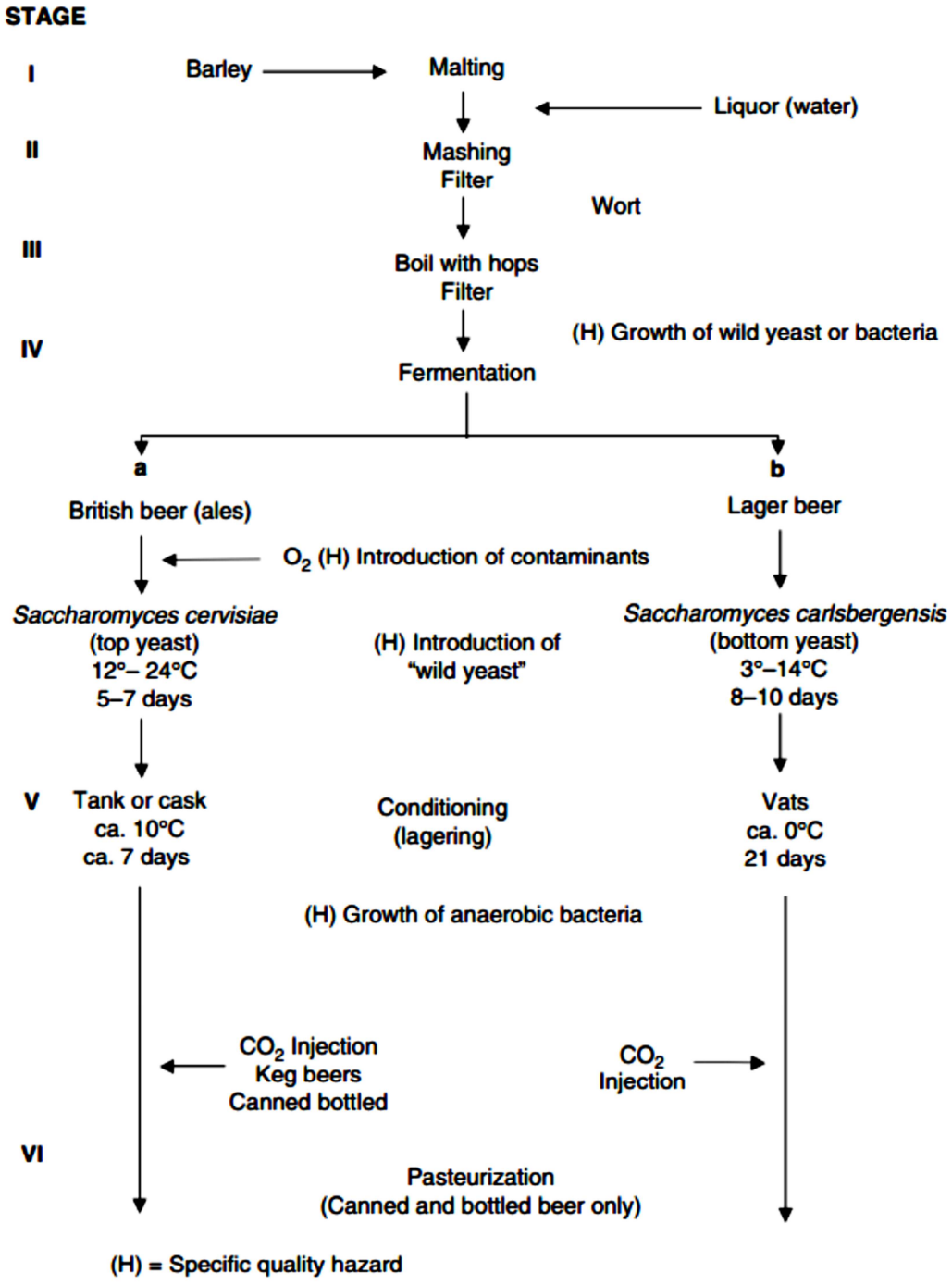

Figure 1.2. Schematic flow chart of beer making process. (Modified from Odhav, (2004). Copy right permission from Taylor & Francis Group LLC).

Over time, the names of brewing yeasts and their taxonomic position have been altered many times; yet it is noticeable that non-brewing or laboratory yeasts are completely different from brewing yeasts. *Saccharomyces sensu stricto* is the source of brewing yeasts, which is produced to different species of *S. cerevisiae.* Over centuries, these species have been categorized by particular beer production conditions (Codon *et al*, 1998).

Industrially produced beer contains several microorganisms such as yeasts and microorganisms moulds. Regardless of different preservation, methods such as thermal pasteurization during processing, yeasts occasionally spoil the processed beer partially or fully. Even though the majority of microorganisms are killed by the boiling wort, throughout the fermentation a few redundant yeast types might be still observed.

As opposed to pitching yeasts, wild yeasts are recognized as redundant yeasts throughout fermentation in beer production. Two major groups of wild yeasts belonging genera of non-*Saccharomyces* and *Saccharomyces* can spoil the beer.

Overall, excluding the brewing picked yeasts, other *S. cerevisiae* strains in the beer are viewed as pollutants. For instance, ale yeast strains in wine distillery or lager beer can be the most hazardous pollutants as they have similar characteristics to brewer's yeasts and similarly it might be harder to distinguish them. Species of *Saccharomyces* are known as the majority of spoilage yeasts that are particularly found in draft beers, which retail without pasteurization (Lawrence, 1988).

1.1.3. Beer sensory

Carbonyl compounds (particularly aldehydes), furfuryl derivatives, and other types of organic chemicals are thought to play the most important role in the development of off-flavors in aged

beer samples. Beer photooxidation (lightstruck reaction) creates the well-known, intensely flavor-active compound 3-methyl-2-butene-1-thiol (MBT) (Marsili *et al*., 2007). The sensory threshold in beer for this malodorous compound is 2–7 ng/L. Two previously unidentified compounds with aromas were also discovered by Vesely *et al*. (2003) using solid-phase microextraction and multidimensional GC-olfactometry, which were indistinguishable from the "skunky" or "foxy" aroma used to describe MBT. These additional skunky aroma compounds undoubtedly contribute to the overall lightstruck character in beer. Additionally, they found that MBT and one of the two other compounds slowly formed during beer thermal oxidation in the absence of light. These off-flavours were mainly formed in thermal treated canned beer during aging.

The possibility of formation of these off-flavors is increased during thermal pasteurization. The use of nonthermal novel technologies can ensure no noticeable changes in overall sensory of beer. A number of studies have been carried out on the sensory of processed beer by nonthermal technologies which are summarized in Table 1.2. One of the objectives in this thesis is to validate and explain the beer sensory after achieving the minimum pasteurization requirements of beer by nonthermal pasteurization technologies such as Pulsed Electric Fields, High Pressure Processing, and power ultrasound and to test if there were significant changes in flavour and aroma during beer processing

Table 1.1. Summary of sensory assessment on nonthermally treated beer.

Beer processed technology	Processing conditions	Sensory assessment outcome	Reference
HPP	300, 500, 700 MPa 5 min	no changes in the spectrum flavour of the beer	Fischer *et al.* (1999)
HPP	200, 300, 350 MPa 3,5 min	No effect on the main attributes of the beer	Buzrul *et al*. (2005a)
PEF	41 kV/cm 175 μs	differences in the beer flavour and mouth feeling detected	Evrendilek *et al.* (2004)
Ultraviolet light irradiation (UV-C)	254 nm	lightstruck flavour formation detected	Mezui & Swart (2010)

Fischer *et al.* (1999) ran HPP at 300, 500 and 700 MPa for 5 min on a bright lager beer and comparison untreated and pasteurized (60 °C for 20 min) beer samples presented no significant change in the colour, foam durability and the spectrum of flavor materials. However, turbidity increased at 500 and 700 MPa. The effect of HHP (200, 250, 300, 350 MPa for 3 and 5 min at 20 °C) on the quality parameters of lager beer by Buzrul *et al.* (2005a) and the results were compared with conventional thermal pasteurization (60 °C for 15 min). According to the results, the colour, protein sensitivity and chill haze values increased as the pressure and pressurization time increased and changes in bitterness was higher in conventional heat pasteurization. Beers sensory can be classified in different categories in terms of analysis. Brief descriptions of each factor are provided below (Ogle, 2007).

1.1.3.1. Appearance

The visual characteristics that can be seen in beer are clarity, nature of the head, and colour. The beer colour mainly depends on the used malt, and particularly in darker beers the adjuncts added. Systems known as Lovibond, EBS and SRM are used to measure the colour intensity of the beers. Some beers like Hefeweizen or Chimay are considered to be cloudy beers as the beer contains yeasts, but most of the beers are transparent. Opaque beers are another group of beers including porters, stouts, and other extremely dark beers (this chapter explains all types of beers). Other factors of appearance after leaving in the glass are the thickness of the head and maintenance.

1.1.3.2. Aroma and flavour

The aroma of beer is formed by the alcohol content in the fermented malt, the types and strength of hops, and a variety of other components from yeasts, which were mentioned earlier, and other components derived from the brewing process and the water. The taste characteristics of the beers are influenced by the amount and type of used malt, the bitterness strength and flavour passed on by the yeasts. International Bitterness Units Scale measures the bitterness of the beers as IBUs.

1.1.3.3. Mouth feel

The feeling of the beer in the mouth is another factor of the beer style that is gained from the carbonation of the liquid and the thickness. The carbonation level is so important in mouth feel that can give a thick and creamy feeling or a grumpy sensation to the beer.

1.1.3.4. Strength

The strength factor in the beer is alcohol content. Either specific gravity is used to determine the strength as an indirect method or through a more direct method of the overall alcohol percentage determination.

1.1.3.5. Gravity

In 1880, beer gravity was introduced in both Ireland and UK. The density of the beer estimates the exact gravity. The measuring scales including Brix, Balling, and Baume depend on the beer source. For example, Plato is the source in Europe. Beer gravity is dependent on the melting amounts of alcohol and sugars and is one of core features of the beer style. During fermentation, sugars transform into alcohol that affects the beer gravity. Moreover, the wort gravity and original gravity are different prior to fermentation. This difference determines the quantity of sugar transformed to alcohol, which in turn defines another factor of beer style i.e. the amount of beer strength (Eckhardt, 1989; Harrington *et al*., 2006; Ogle, 2007).

1.1.3.6. Alcohol concentration

Alcoholic drinks are categorized based on the percentage of alcohol by volume (abv). In addition, the alcohol by weight (abw) is used by some domestic breweries to categorize beers based on alcohol content (Varnam & Sutherland, 1994).

1.1.4. Beer pasteurization

The main aim of pasteurization is to extend beer shelf life through the inactivation of undesirable enzymes or microorganisms that will otherwise promote unwanted chemical reactions.

1. Literature review

Louis Pasteur first developed the theory of pasteurization around 1865 in which heating was carried out under a limited temperature to inactivate microorganisms. Since beer's spoilage organisms are not pathogenic, partial inactivation is allowed. A commercial rule of thumb that recorded the most suitable relation between temperature and time is 15 min at 60 °C that leads to 15 pasteurization units (PU), where 1 PU is defined as exposure to 60 °C for 1 min (Baselt, 1952; Portno 1968; Reveron *et al*, 2005; Tshang & Ingledew, 1982). Although laboratory tests have indicated that values from 1 to 5 PU are effective for microbial inactivation, 8–30 PU are generally used, perhaps to have a built-in safety factor in case of possible resistant organisms (Del Vecchio *et al*., 1951; Tshang & Ingledew, 1982). It is essential to consider some key factors such as beer type and microorganism type to reach an efficient level of pasteurization. The main goal is to develop balance between the effects of heat on the beer's sensory quality and inactivation of harmful organisms. Moreover, the level of pasteurization temperature is determined based on the heat resistance of the microorganisms. The z-value and D-value are the key factors to realize this. Moreover, other factors like the constituents such as alcohol level of the beer play a great role on the pasteurization level, which is one of the main objectives of this study. The time necessary to inactivate 90% of the viable microorganism population is called D-value, which directly relies on the microorganism type. z-value indicates increase of temperature by which D-value is decreased by 90% and this reduction differs for each type of microorganism. However, this has to be done by many analyses in practice, because the type and quantity of microorganisms in the beer change gradually. The typical information in this regard is presented in Table1.2.

Table 1.2.Typical PU levels for different types of beers and microorganisms (The institute of brewing and distilling, 2005).

Product type	Typical PU level
Alcoholic beer	15-25
Low alcoholic beer	50-100
Juices or cider	>1000
Microorganism type	**Typical PU level**
Brewer's yeasts	1-5
Wild yeasts	10-15
Pediococcus sporidium and *Lactobacillus sporidium*	1

1.1.4.1. Tunnel thermal pasteurization

In tunnel pasteurization beer is first filled into sterile glass bottles and then pasteurized through tunnel pasteurizers (Buzrul, 2007). According to European Brewery Convention manual of good practice, the typical temperature used in tunnel pasteurization is 60-66°C based on the beer type. In tunnel pasteurization, hot water is sprayed on the beer bottles or cans while they pass through the tunnel.

Tunnel or in-package pasteurization is comparable to flash pasteurization in goal and target.

1.1.4.2. Flash thermal pasteurization

In flash pasteurization beer is first pasteurized and then packaged aseptically usually into metal kegs (Buzrul, 2007). The typical temperature used in flash pasteurization is 71-74°C for 15-30 seconds. However, over-pasteurisation can adversely affect flavour and the control of temperature and holding time is necessary to minimise such effects (Fricker, 1984).

1.1.4.3. Filtration

One of the most universal nonthermal methods is sterile filtration, which is used in many breweries to discard the beer's spoilage microorganisms. The packaged beers attain the required microbiological stability using this method. An adequate amount of spoilage microbes are separated from the product while flavour is minimally affected during this process. To perform this, certain quantities of filters are located prior to packaging the beer. The beer's flavour or colour compounds stay fresh in this method (Dilay *et al.,* 2006; Vaughan *et al.,* 2005). However, the beer shelf-life is shorter in comparison to pasteurized beers (Curtis, 1968).

During beer thermal pasteurization off-flavors are easily formed, as beer is a delicate beverage. With respect to flavour as the top priority, using a method of pasteurization with no or less heat would be of great help to the brewing industry (Folkes, 2004). Furthermore, due to the existence of alcohol, hops, and acidity (CO_2), spoilage yeasts tend to be more resistant by changing to ascospore form. In this study, using emerging technologies in the food industry including Pulsed Electric Field, high pressure process, and high intensity ultra sound are taken into consideration as novel methods to improve the desired rate of inactivation as well as the overall sensory of beer. Table 1.4 summarizes the first order thermal resistance parameters of *S. cerevisiae* in beer.

Table 1.3.Thermal Inactivation of *S. cerevisiae* in alcoholic drinks.

Media	Ascospores/ vegetative cells	Processing temperature (°C)	z-value (°C)	D-value (min)	References
Beer	Vegetative cells	43 45 47 49 51	4.1	26.3 11.2 3.5 1.0 0.4	Tsang & Ingledew (1982)
Chenin Blanc Wine	Ascospores	55	-	106.0	Splittoesser *et al.* (1986)
Beer	Vegetative cells	47 48 49 50 60	4.4	3.1 2.6 1.7 0.7 0.01	Reveron *et al.* (2003)

Table 1.4. Thermal Inactivation of *S. cerevisiae* in non-alcoholic liquid foods.

Media	Ascospores/ vegetative cells	Processing temperature (°C)	D-value of vegetative cells (min)	D-value of ascospores (min)	References
Orange juice	Vegetative	60	8.0	-	Juven *et al.* (1978)
Sucrose solution	Vegetative	65	1.7	-	
Soft drinks	Ascospores	60	-	19.2	
fruits in sugar syrup	Ascospores	60	-	17.5	
Orange juice	Both types		0.2	19.2	
Black cherry juice	Both types		0.2	8.1	
Black currant juice	Both types		0.2	8.2	
Cherry juice	Both types		0.2	17.0	Put & De Jong (1982)
Cherry juice	Both types	60	0.1	10.8	
Black cherry	Both types		0.1	5.1	
Apple sauce	Both types		0.2	12.0	
Cherry juice	Both types		0.1	7.5	
Cherry juice	Both types		0.1	8.5	
Strawberry juice	Both types		0.3	17.5	
Apple juice	Both types	55	0.9	106	Splittoesser *et al.* (1986)
Calcium added apple juice	Ascospores	57	-	32.0	Shearer *et al.* (2002)

1.2. High pressure processing (HPP)

High-pressure processing (HPP) is one of the most significant innovations in food processing in 50 years (Dunne, 2005). This technique retains the food's natural freshness and often the quality parameters such as aroma, colour, and important food components (Farkas & Hoover, 2000). HPP is also referred to as "ultra-high-pressure" (UHP) or "high-hydrostatic pressure" (HHP) processing. The pressures in HPP, in the range of 200 to 600 MPa, normally achieve microbial inactivation. Pressure can be used at ambient temperature, thus avoiding induced cooked off-flavors. Consequently, such a technology is a breakthrough in processing solid or liquid foods.

Nowadays, HPP food processing is being applied on ever-increasing commercial basis. Clearly, there are opportunities for innovative use and new food products promotion. However, the major disadvantages of pressure treatment are the capital cost of the technology and not being a continuous process (Ferrentino *et al*, 2015).

1.2.1. Historical background of HPP technology

The history of studies on the impacts of high pressures on food products dates back to more than one century ago. In the late 19th century, Bert Hite made a high-pressure unit for pasteurizing foods such as milk (Hite, 1899). He designed a machine that was able to achieve pressures in excess of about 6800 atmospheres (about 700MPa). He also investigated the potential application of HPP processing for various food products. In that era, it was a very sophisticated process in relation to packaging materials and processing systems (Hoover, 1993). Furthermore, Hite observed that pressures of 450 MPa could improve the preserving quality of milk (Hite, 1899). In the early twentieth century, Hite found lactic acid bacteria and yeasts of sweet, ripe fruit are

more susceptible to pressure compared to other organisms, particularly spore-forming bacteria of vegetables (Patterson *et al*., 1995).

Hite's prototype system was very primitive in comparison with recent HPP systems. Nowadays, along with developments in areas such as new materials and computational stress analysis, high capacity pressure systems permit valid HPP processing of food at even higher pressures (Hoover, 1993). Since the late nineteenth century, the potential for HPP processing of food products has been known, but the wide application of this system has only recently begun. Recently, the application of HPP as a method for food preservation has achieved momentum worldwide and several studies have considered the impacts of HPP on food products. For a couple of reasons mentioned earlier in this chapter, this technique is an alternative to traditional heat-based approaches. The majority of studies on the application of HPP for food preservation have investigated the inactivation of microorganisms (Ashie and Simpson, 1996; Krebbers *et al*., 2003).

1.2.2. HPP process and the key principles

Packaging is an essential step before HPP. The elimination of air from the food is followed by vacuum packaging of the foods in flexible and high-barrier films or containers. Air removal is necessary to avoid bursting the packs during each cycle. In addition, it ensures that compression work will not be wasted on air in the system. The containers are put into a carrier basket or loaded directly into the pressure vessel. The operation of loading is like a batch steam retort. Commercial batch vessel volumes range from 30 to 600 L. A common process cycle first loads the vessel with the pre-packaged product and then fills the rest of the vessel void space with water, acting as the pressure-transmitting fluid. Then the vessel closes and the expected process

pressure is obtained through adding water that is delivered via an intensifier. After keeping the product at the target pressure for the expected time, the vessel is decompressed by releasing the water (Balasubramaniam *et al*., 2008). It is also possible to process liquids in two modes: batch or semi-continuous. In the first mode, the liquid is pre-packaged and pressure-treated as explained for packaged food products. In the latter mode two or more pressure vessels with free-floating pistons are employed for compressing the liquid. Furthermore, the pressure vessel is filled with liquid through a low-pressure transfer pump. The pressure vessel inlet valve becomes closed after filling. Furthermore, the pressure-transmitting fluid (usually water) is used behind the free piston for compressing the liquid food. After processing at suitable holding time, the system is decompressed by releasing the pressure on the pressure-transmitting fluid. The free piston moves towards the discharge port through a pump. Then the treated liquid, held in a sterile tank, can be filled aseptically into sterile containers. In a semi-continuous mode, three batch vessels can be linked: The first vessel discharges the product, the second one is compressed, and the last one is loaded. Therefore, the output can be maintained steadily and continuously (Balasubramaniam *et al*., 2008).

1.2.2.1. Pressure come up time

The required time for increasing the pressure of the sample from atmospheric to the target process is commonly determined as “pressure come-up time” (Farkas & Hoover, 2000) (Figure 1.3). The come uptime is a function of the expected target pressure, the horsepower of the pump intensifier and the volume of the pressure vessel used. Typical commercial scale high-pressure equipment is constructed to achieve a come up time in the range of 2–3 minutes reaching 600 MPa (87,000 psi). Decreasing the hourly cycling rate causes longer come up times to be added to the total process time. This influences product output. Consistency as well as awareness of such

times seems to be significant in the development of HPP (Farkas & Hoover, 2000; Ting *et al*., 2002; Balasubramaniam *et al*., 2008). During HPP, the temperature of food materials increases, (T_1 toT_2) as an unavoidable thermodynamic effect of compression (Ting *et al*., 2002) as shown in Figure 1.4., which is not desirable in most of the commercial applications.

1.2.2.2. Pressure holding time

No more additional energy is added to the process, when the expected pressure is achieved, and it is assumed that there is no important pressure drop in the system as a consequence of heat exchange with the surroundings.

Therefore, pressure-holding time is determined as the interval between the end of compression and the outset of decompression (t_2 to t_3). In order to achieve the expected microbial inactivation and/or quality, the products are maintained at the target pressure and temperature (if specified) for a predetermined holding time. The shortest processing time (<10 minutes) is typically expected since process time has an important impact on output (Balasubramaniam *et al*., 2008). Possibly, during the holding time, the stability of product temperature at pressure depends on the insulation traits of the pressure vessel. If the equipment is improperly insulated, the temperature of the product reduces from T_2 to T_3 (Figure 1.34) during pressure holding time because of the thermal exchange through the pressure vessel walls.

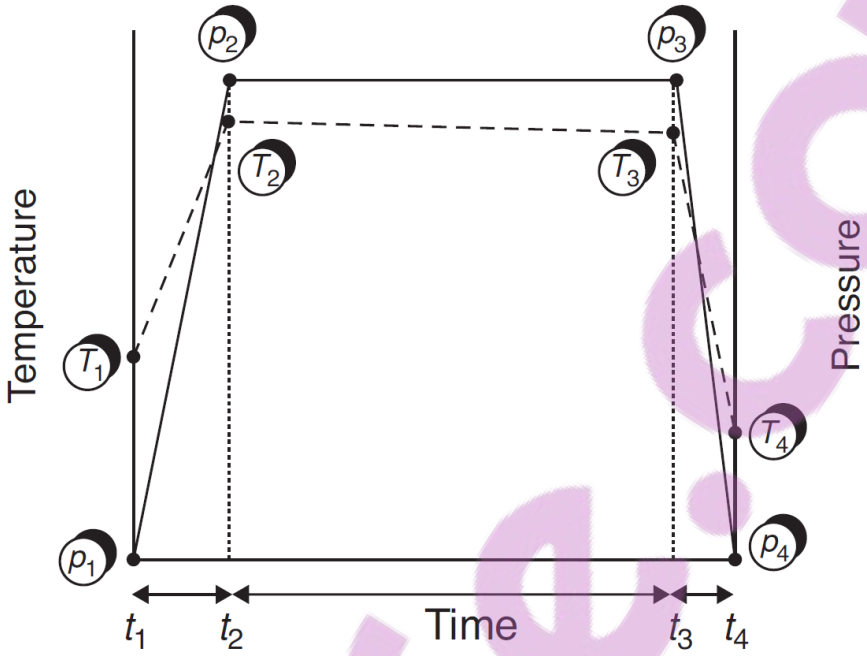

Figure 1.3. Typical pressure–temperature response of a water-based food material undergoing high-pressure processing. Come-up time, t_1 –t_2; holding time, t_2 –t_3; decompression, t_3 –t_4. (Nguyen & Balasubram, (2011). Copyright permission from John Wiley and Sons).

1.2.2.3. Decompression time

Decompression time" is the time (Figure 1.3) when a food sample is brought from the process pressure to near atmospheric pressure. Most pressure equipment permits product decompression in a few seconds. Particular foods may alter their structure during decompression because of fast extension of occluded or dissolved gas. If structural changes seem unpleasant, decompression can occur at a slower rate, which can be controlled via inserting a smaller venting line or via other throttling tools. However, this can increase the cycle time. Decompression drops the product temperature towards T_4, which can be lower than its primary temperature value (T_1). The

difference between the primary temperature of the sample and its final temperature after decompression (T_1–T_4) shows the extent of heat loss from the product to the surroundings (Ting *et al*., 2002).

1.2.2.4. Cycle time

"Cycle time" is generally regarded as the total time for loading, closing the vessel, compression, holding, and decompression and unloading. The cycle time along with the volumetric efficiency determines the system output and the cost of the HPP process.

1.2.2.5. Process pressure

"Process pressure" (Figure 1.3) is the holding pressure during the sample treatment. To measure pressure, strain gauges on the pressure vessel and displacement transducers on the external frame are common and accurate approaches. At least two methods are suggested to be employed to measure pressure. In addition, an appropriate suitable periodic calibration program is required (Balasubramaniam *et al*., 2004; Farkas & Hoover, 2000). Furthermore, a reference sensor or gauge is required for periodic calibration of process instrumentation.

1.2.2.6. Product initial temperature

The primary temperatures (T_1) of the product, the process vessel and the pressure-transmitting fluid should be recorded if the temperature is at a specified set point for microbial inactivation during the high pressure process. For heterogeneous food samples, additional time is possibly required to reach temperature equilibrium within the sample. The high pressures utilized in food

processing have no effect on the type K thermocouple readings at temperatures below 500°C (Bundy, 1965). It is necessary that the reference thermocouple sensor is placed at a cold point or in an equivalent region within the pressure vessel and calibrated to an accuracy of 0.5°C (Farkas & Hoover, 2000).

1.2.3. Effect of HPP on beer properties

Some studies were carried out on the application of HPP in the process of brewing and beer properties, which are summarized in Table 1.5. For example, Fischer *et al.* (1999) applied HPP at 300, 500, and 700 MPa for 5 min on mash, wort, and beer. Compared to an untreated mash sample, the content of dissolved protein in HHP treated mash increased as the pressure increased. Moreover, the fermentation degree dropped with the increasing pressure and no changes were determined for pH value. For the treatment of wort by Fischer *et al*. (1999), the results revealed HHP treatment could increase the bitterness and the amount of iso-a -acids more than thermal treatment. The results on bright lager beer samples showed that HHP treatment did not significantly change the color, foam durability, and the spectrum of flavor materials. In a second trial a pale ale and a mild ale were used by Castellari *et al.* (2000). Samples were treated by HHP (600 MPa for 5 min) and heat (60 °C for 10 min) and then stored in the dark at 20 °C for 1, 8, 14, 26, and 49 days. The HHP and heat pasteurization similarly affected the pH, bitterness and phenol content of beers up to 49 days of storage. Under these conditions, the storage time had no significant influence on these parameters. The results show that hydroxymethylfurfural (HMF) increased significantly with heat pasteurization. The HHP beers retained a significantly higher permanent haze throughout the storage period. The heat pasteurized beers showed a sharp decrease of Nephelos Turbidity Unit (NTU) values in the first days of storage, with an increase

of chill haze values at the same time. Permanent haze was more influenced by the stabilizing process in pale ale than in mild ale.

Overall, HPP processing of beer seemed to have no effects on the quality of beer. However, referring to the literature, there is no study on the taste assessment of HHP treated beer, which is one of objectives of this thesis.

Table 1.5. Effect of HHP on beer characteristics.

Processing pressure (MPa)	Processing time (min)	Achievements	Reference
300, 500, and 700	5	No change of colour, and foam durability. Increase in turbidity. Decrease in potential for the arising of turbidity.	Fischer *et al*. (1999)
600	5	No effect on main chemical constituents of the beer. No significant changes on colour. Reduction of total aerobic, yeast and moulds counts. No lactic acid bacteria were detected.	Castellari *et al*. (2000)
300, 500, and 700	5	No change was observed for the turbidity. Potential for arising the turbidity decreased at 300 and 500 MPa and increased at 700 MPa. Pilsner type beer was more stable.	Fischer *et al*. (2002)
300, 400, 500, and 600	20	Increase in the foaming and haze characteristics of the beer. Improvement of saturated ammonium sulfate precipitation limits value.	Pérez-Lamela *et al*. (2004)
300	120	More effective inactivation at lower pH values. Sublethal injury at short holding times.	Fischer *et al*. (2006)
100, 150, 170, 190, and 200	-	A pressure of 250MPA can be used to inactivate common beer spoilage mechanisms	Franchi *et al*. (2013)

1.2.4. HPP inactivation of yeasts

Yeasts such as *S. cerevisiae* are of significance in the beer industry, as they can cause spoilage in the brewing process and cause off-odours. The formation of ascospore in *S. cerevisiae* can be induced in beer where there are high concentrations of ethanol, carbon dioxide, and hops. Pasteurization treatment often uses pressures with a maximum 600 MPa (87,000 psi) for a particular holding time (Anon 2006; Cheftel, 1995; Farkas & Hoover, 2000). Some studies have been carried out on the inactivation of *S. cerevisiae* vegetative cells and ascospores in the past, which have been summarized in Table 1.6 and Table 1.7. No studies have been carried out on the HPP pasteurization of beer, which is one of the main objectives of this research.

The scope of microbial inactivation studies is based on different factors such as yeast type, yeast age, pH, food composition, and water activity. Generally, vegetative cells of yeasts are sensitive to HPP, but the ascospores of yeasts seem to be analogous bacterial spores exhibiting higher resistance. Gram-negatives are less resistant than gram-positive organisms and spores are more resistant than vegetative cells (Cheftel, 1995; Dunne, 2005). For example, Ogawa *et al*. (1990) observed more than 5 log reduction of each of nine species of yeasts and molds in fruit juice when treated at 350 MPa for 30 minutes or 400 MPa for 5 minutes. She demonstrated higher pressure resistances for ascospores than vegetative cells. Ascospores were found to be 5 to 8 times more heat resistant than the vegetative cells. Moreover, older spores seemed to be more resistant to HPP (Knorr*,* 1995). Younger ascospores may succumb to HPP because of a weaker underdeveloped cell wall, whereas mature ascospores have a denser cell wall which may protect them from HPP.

Some studies have identified that a lower pH (~ 3.5) will eliminate yeasts (Palou *et al.,* 1998). Although very few studies on beer with HHP are available today, the potential of HHP

technology is huge in the beer industry. Studies have shown that HHP treatment not only inactivates the undesirable microorganisms but also improves the organoleptic properties of beer (Section 1.2.3). The pressure levels used to treat beer and wine are similar to the commercial applications used in the fruit juice industry i.e., 400–600 MPa. It should be noted that installation of an HHP equipment in a brewery would definitely incur an extra cost. However, the HHP-treated beer would have a "fresh-like" taste, which would most likely attract the attention of consumers.

Table 1.6. HPP inactivation of *S. cerevisiae* ascospores in different beverages.

Media	Processing pressure (MPa/Bar)	Processing temperature (°C)	Processing time (min)	Log reduction	References
Orange juice	100-600	23	5-30	≥6.0	Ogawa *et al.* (1990)
Orange juice	350-459	25	-	≤4.5	Parish (1998)
Orange juice & Apple juice	300-500	34-43	0-30	≥6.0	Zook *et al.* (1999)
Apple juice	100-300	20	-	5 log	McKay (2009)

Table 1.7. HPP inactivation of vegetative yeasts in beverages.

Strain	Media	Processing pressure (MPa)	Processing temperature (°C)	Processing time (min)	Log reduction	References
Not mentioned	Water	0-500	Ambient	-	7 to 10 log	Hamada *et al.* (1992)
ATCC 2373	Citrate buffer	150, 300	25, 45	10, 20	≥7.0	Pandya (1995)
2407	Pineapple juice	0-270	-	6.7	≥5.1	Aleman *et al.* (1996)
IAM 4274	Deionized water	60-140	0 to -30	-	4-5 log at 103 Mpa	Hayakawa *et al.* (1998)
VWk43	Phosphate buffer	0.1-300	25	15	≤6.0	Brul *et al.* (2000)
Not mentioned	-	50-250	45	10	≤8.0	Donsi *et al.* (2003)
Not mentioned	Orange juice Pineapple juices	100-250	25-45		4 (pine apple juice) 7 (orange juice)	Donsi *et al.* (2007)
NCFB 3191	Phosphate buffer Beetroot juice	300	20	10	5 (buffer) 3.5 (beetroot juice)	Sokołowska *et al.* (2013b)
CBS 1171	Malt Wickerham (MW) medium	100-350	-20 to25		1 to 8	Perrier-Cornet *et al.*

Strain	Media	Processing pressure (MPa)	Processing temperature (°C)	Processing time (min)	Log reduction	References
Lab strain	Orange juice	300	30	-	5.6	(2005) Compos and Christianini (2006)
Not mentioned	Broccoli-apple juice	500	-	10	Total inactivation	Houska *et al.* (2006)
CCRC20271 Wine yeast	YM broth	300	-	20	6.0	Chen and Tseng (1997)
Not mentioned	Mandarin juice	300	21	5-10	≤4.0	Takahashi *et al.* (1993)
ATCC4113	Apple juice	600	21	7	Total inactivation	Marx *et al.* (2011)
KCCM12224	Red wine	1000-3500	-	0-30	Total inactivation	Mok *et al.* (2006)

1.2.5. Mechanism of microbial inactivation by HPP

There are several studies on the mechanism of microbial inactivation via HPP. These studies have concluded that this microbial inactivation is the result of a combination of factors. The cell membrane is the primary site for pressure-induced microbial inactivation. Microorganisms are known as resistant to selective chemical inhibitors because of their capability to eliminate such agents from the cell, through the action of the cell membrane. However, once the membrane is injured, this tolerance becomes lost. Furthermore, HPP leads to some alterations in cell morphology, protein denaturation, biochemical reactions, and inhibition of genetic mechanisms. Other mechanisms of action, which might be in charge of microbial inactivation, denaturalize major enzymes and disrupt ribosomes (Linton & Patterson, 2000). Various microorganisms have reactions towards high pressure treatment in different ranges of resistance. Larger injury to the cell membrane from quick alterations in intracellular-extracellular differences at the membrane interface leads to the higher inactivation rate (Palou *et al*., 1998).

The patterns of high-hydrostatic-pressure inactivation kinetics with various microorganisms seem to be very varied. Several studies showed first-order kinetics in the case of many bacteria and yeasts (Chen & Hoover, 2003; Farkas & Hoover, 2000; Gervilla *et al.,* 1999; Ghani & Farid, 2007. Some other studies indicated a shift in the slope and a two-phase inactivation phenomenon (Evelyn & Silva, 2015c; Evelyn & Silva, 2016). They showed the first fraction of the population was rapidly inactivated, while the second fraction was more resistant. In addition, the pattern of inactivation kinetics is affected via factors such as temperature, pressure, and composition of the medium.

There are few reports on the kinetics of microbial inactivation by HPP, particularly for alcoholic beverages like beer. Kinetic data for yeast spores would be necessary regarding beer

pasteurization. One of the main objectives of this study is to model the kinetics of the inactivation of *S. cerevisiae* ascospores in beer.

1.3. Power ultrasound

Ultrasound is vibrations and sonic waves of the same physical nature as sound but with frequencies above the range of human hearing. Based on frequency difference and sound intensity, ultrasound waves are classified into two categories. High frequency ultrasound (diagnostic ultrasound) operates at frequencies of 2-20 MHz with sound intensities in the range of 0.1-1 W/cm^2. Food quality assessment, medical imaging, and non-devastating inspection are examples in which high frequency ultrasound is used. Lower frequencies like 20-100 kilo Hertz (kHz), with a sound intensity of 10 to 1,000 W/cm^2 is employed in power ultrasound (also called high intensity ultrasound), the technique used in this thesis. Because of high energy level, power ultrasound is considered appropriate to be used in food industry for microbial destruction (Baumann *et al*., 2009; Baumann *et al*., 2005a; Ugarte *et al*., 2006; Ugarte *et al*., 2007). Power ultrasound processing, often called sonication, operates through a liquid medium which can be the food or water containing a solid food.

Application of ultrasound as one of the recent nonthermal technologies in food storage has been studied in the last ten years (Mason *et al*., 2003a; Mason *et al*., 2003b). For example, in the beer industry the application of ultrasound at the beginning of the mashing process causes the beer yield to improve (Knorr *et al*., 2004) and accelerates the fermentation time to 36-50%. This process increases the oxidation in fermented products, which leads to better flavour and early maturation. Ultrasound of 1 MHz changes the alcohol/ester balance creating evident aging in the

product. It has been applied for wines, whiskey, and spirits (Mason, 1996). Furthermore, this technology improves the hygiene of the defobing and defoaming of the beer before bottling (Chemat *et al*., 2011). However, the application of power ultrasound in food processing at a commercial scale is limited to emulsification, size reduction, crystallization, and solvent extraction (Feng & Yang, 2011).

1.3.1. Ultrasound historical background

It has been more than 50 years since acoustic energy was first used to assist processing in different industrial sectors but food processing has begun to use acoustic energy more recently. The possibility of employing a more intensive form of ultrasound (5 W/cm^2) at a lower frequency (generally around 40 kHz) was discovered by food technologists. This discovery of ultrasound dates back to 1927 when a paper entitled "The chemical effects of high frequency sound waves: a preliminary survey" was published. This paper investigated the development of power ultrasound across a span of food processes like emulsification and surface cleaning (Richards & Loomis, 1927). The first work of Harvey and Loomis (1929) concentrated on the success of the destruction of micro structures with power ultrasound in the 1920s. Their work studied sonication at 375 kHz under temperature-controlled conditions, causing a decrease of light emission from a seawater suspension of rod shaped *Bacillus fisheri.* Processing industries properly admitted the uses of power ultrasound in 1960s and this technology is still developing (Abramov, 1998; Mason & Lorimer, 1999).

1.3.2. Ultrasound process and the key principles

Cavitation, which is an indirect phenomenon, causes the occurrence of the chemical and most of the mechanical effects of ultrasound. It is the production of bubbles in the solvent when the energy wave spreads in the medium (Crum, 1995a; Crum, 1995b; Leighton, 1995). In order for the cavities to be created, nucleation sites must exist in the liquid. This step is one of the most influential processes in food processing. The composition of the system, which is determined by the existence of the suspended atoms, liquefied ions, surface active agents and other components, will greatly affect the generation of the cavities. If the pressure change is big enough and it is above the "cavitation threshold", then bubbles will be created (Leighton, 1995). It may take a few microseconds for the bubbles to grow and crumple at 20 kHz (Hardcastle *et al*., 2000). The monitored behaviour of cavitation that is under the effect of high intensity ultrasound reveals that a critical bubble size R_{crit} has to exist in a state where pressure balance across the phase boundary can no longer be retained and explosive growth must follow, accordingly (Blake *et al*., 1997; Blake *et al*., 1999). If the size of bubble nuclei is R_0, the hydrostatic pressure is p_0, the vapour pressure of the liquid is p_v and the surface tension is σ, then the sudden increase happens if $p_0 \geq p_v - 4\sigma/3R_{crit}$ (Minnaert, 1933).

Ultrasound measurement in food processing is a result of two main values. The first value is P_{in}, the actual power consumed by the generator that can be determined using a high-precision wattmeter (W) on the electrical input line. The second important power value is P_{diss}, the power dissipated in the treatment vessel. The value of P_{diss} can be measured by calorimetry as the ultrasonic waves in the process vessel dissipate as heat. P_{diss} can be estimated using Equation 1.1.

$$P_{diss} = mc_p \frac{dT}{dt} \quad (1.1)$$

where m is mass of liquid (kg), c_p is the specific heat capacity of the liquid (J/(kg°K)), and (dT/dt) is the initial slope (K/s) of the temperature versus time curve measured for the first 30 seconds of sonication.

Therefore, by getting assistance from the medium particles, the sound wave can move from one part of the medium to another with assessable speed. This movement makes the medium particles oscillate about their steady and balanced positions. The medium particles steadily move close or far from each other all the time, so this movement creates zones of alternate intensification and reduction in the intensity and pressure of the medium. As a result, the ultrasound imposes only mechanical energy into the medium and is the reason for the movements of the medium particles (Feng & Yang, 2011).

1.3.2.1. Intensity of the wave

The intensity of the ultrasonic wave is one of the key processing parameters that influence the sonochemical effects of high-intensity ultrasound. Minimum intensity is required to induce cavitation. This minimum is dependent on the frequency and the physicochemical properties of the medium that is treated. As the intensity increases, larger numbers of cavitation bubbles are generated, thus increasing the observed sono chemical effects. However, if the number of cavities is very high, the bubbles coalesce to form larger, longer lasting bubbles (Mason & Lorminer, 1999).

1.3.2.2. Frequency of the wave

The frequency of the high-intensity ultrasound waves has a major influence on the size of the cavities as has been discussed. Because the time available for expansion and collapse of bubbles shortens with increasing frequencies, it becomes more difficult to maintain the extent of the cavitation at higher frequencies. At frequencies in the megahertz region, cavitation completely ceases because a finite amount of time is required for the molecules to physically separate and form the cavity. Production of transducers with high-power output at high frequencies has proven to be difficult. Most of the power ultrasound transducers, available for commercial application, are in the range of 20–100 kHz (Feng & Yang, 2011).

1.3.2.3. Presence of gases

The presence of dissolved or occluded gases has a positive effect on the efficiency of ultrasound due to improved generation and collapse of cavities. The introduction of gas cavities (bubbles) into a system increases the number of nucleation sites. This results in a more uniform energy distribution throughout the system (Mason & Lorminer, 1999).

1.3.2.4. Temperature

The use of ultrasound in combination with heat is called thermosonication. Mason and co-authors investigated the effect of temperature on the efficiency of particle size reduction using power ultrasonication (Mason *et al*., 2003b). They observed a decrease in the delivered intensity of ultrasonic power to the solution from79 to 23 W/cm as the temperature increased from 0 to 90°C.

This inverse relation between temperature and ultrasonic power has been explained by the increase in vapour pressure of the solvent resulting in a delayed time of collapse of gas bubbles and decoupling. Simultaneously, both viscosity and surface tension, properties that influence generation of cavities, decrease with increasing temperature. As the solvent reaches the boiling point, vapour bubbles interfere with the cavitation bubbles, effectively dampening all sonochemical effects. In some processes though, the increase in temperature can lead to synergistic effects possibly due to temperature-induced structural changes that may increase the susceptibility of the system to ultrasound (Bermúdez-Aguirre *et al*., 2011; Feng & Yang, 2011).

1.3.2.5. Probe system

The components of a probe system are as follows: a generator to convert electrical energy to high-frequency shifting current, a transducer to change the shifting current to mechanical vibrations and a distribution probe to transfer the sound vibrations to a loading medium, which pairs ultrasonic vibrations with the processed material (Figure 3.1). The material used to make the probe is usually titanium, aluminium, or steel and the shape of it can be chosen among shapes like rod, plate, bar, or sphere based on the shape of the load and the required gain. The ultrasound probe can directly be in touch with the foods or can be placed into a processing room or flow cell of determined geometry to transfer energy to the food system with more efficient energy. The varying volume of the processing chamber strongly affects the design of a high sound intensity (W/m^2) system or a high volumetric sound power density (APD) (W/mL) system. The application of a probe system will have some potential disadvantages. Entering the ions and powders into the food is one of them; this happens by the pitting and corrosion of the probe, especially the blades of the metal or even the metal processing chamber. The quality of food is

lowered by such contaminants. One should care a lot about the temperature control of the loading medium in a probe system. The creation of more bubbles near the tip of a probe may produce free radicals which can oxidize the food. Therefore, those areas which have more bubbles will have more energy and the zones with intense reaction will have an irregular energy distribution in an active reactor. It is better to set an obstacle among the ultrasonic generator, the transducer, and the loading medium in order to optimize energy pairing with food (Feng & Yang, 2011; Mason *et al.,* 2003a).

1.3.2.6. Batch systems vs. continuous systems

The base of a batch system is upon the ultrasonic bath in which the whole treatment is carried out in a bath as a reactor and the transducers are in direct connection with the treatment chamber (Quartly-Watson, 1998). In a continuous system, the liquid flows through pipes in which the walls can vibrate with ultrasonic waves. So the energy of sound produced from the transducers attached to the other side of the tube is emitted into the liquid inside the pipe. The material used to make the commercial tube reactors is stainless steel and their shapes can be rectangular, pentagonal, hexagonal, or circular.

The medium attenuates the sound wave and thus the distance from the transducer has a large impact. The wavelength (λ) is applied to the distance between two consecutive amplitude tops. The relation between the wavelength and frequency (*f*) is based on the speed (*c)* at which the wave travels, as shown in Equation (1.2) (McClements, 1997; McClement, 1999).

$$\lambda = \frac{c}{f} \qquad (1.2)$$

1.3.3. Ultrasound inactivation of yeasts

Much research has been done to understand the mechanism played by ultrasound on the disruption of microorganisms, which has been explained by acoustic cavitation and its physical, mechanical, and chemical effects that inactivate microbes (Joyce *et al*, 2003). Nowadays, ultrasound technology could be used more widely in food pasteurization applications. Although some studies have been carried out on ultrasound microbial inactivation (Table 1.8), the inactivation of yeasts as the main target of beer pasteurization by power ultrasound has not been studied.

Table 1.8. Power ultrasound inactivation of vegetative *S. cerevisiae* in beverages.

Media	Strain	Amplitude (μm)	Processing power (W)	Processing frequency (kHz)	Processing temperature (°C)	Log reduction	References
Water	VL1	95.2	50-180	20	55	2.0-6.0	Ciccolini *et al.* (1997)
Sabourad broth	KE 162	71-110	-	20	35, 45, 55	6.0 at 55°C	Guerrero *et al.* (2001)
Milk	USFSCC 462	124	750	20	-	Total inactivation	Cameron *et al.* (2008)
Tomato juice	DSMZ 70090	24.4-61	-	20	-	5.0	Adekunte *et al.* (2010)
Pineapple, grape and cranberry juice	ATCC 4113	120	400	24	40, 50, 60	7.0 (pine apple juice) 7.0 (cranberry juice) 6.0 (grape juice)	Bermudez-Aguirre and Barbosa-Canovas (2012)

Lepeschkin and Goldman, 1952 and Kinsloe *et al*., 1954 found that when no cavitation exists, there could still be microbial inactivation because of ultrasound. This means that younger cells are stronger than the older ones while the vegetative cells are weaker than the spores. Generally,

frequency around 20 kHz is required for inactivation of yeasts. However, to improve the microbial inactivation in liquid foods, ultrasound in combination of heat is needed to inactivate the yeasts ascospores. Moreover, the shape and size of the vegetative yeasts are bigger than ascospores (~ 6 microns). The bigger cells are more sensitive to ultrasound than smaller cells.

1.3.4. Mechanism of the microbial inactivation by ultrasound

The inactivation mechanism of ultrasound can be explained through the effect of cavitation on microbial cell walls. Water jets of liquid, generated by the asymmetric implosion of transient cavitating bubbles, may cause severe cell envelope damage and cleavage of the texture of the polymeric materials of the cell walls. In terms of the chemical effects, transient cavitation can create OH- and H radicals and hydrogen peroxide. Also, stable cavitating bubbles can generate microstreaming alongside the bubble and create high hydrodynamic shear stresses, which cause cell membrane damages (Wu *et al*., 2001).

There are few reports on the kinetics of microbial inactivation by ultrasound, particularly for alcoholic beverages like beer. The kinetic data for yeast spores would be necessary regarding beer pasteurization. One of the main objectives of this study is to model the kinetics of the inactivation of *S. cerevisiae* ascospores in beer.

According to the literature, ultrasound inactivation can be characterized by log-linear kinetic parameters at ambient temperatures. At higher temperatures the inactivation kinetics does not appear to follow a log linear relationship (D'amico *et al*., 2006; Lee *et al*., 2009). Shoulders may relate to cell disaggregation, while tailing has been attributed to a progressive loss in cavitation intensity during sonication. This may be the case for an open system where degassing can occur, with the subsequent loss of cavitation intensity. In addition, Evelyn and Silva (2015b) also

observed that the inactivation of *Clostridium perfringens* spores by TS was not linear and described by the Weibull model.

A good understanding of the microbial destruction kinetics and the food product quality degradation kinetics is indispensable. This is needed for the substantiation and optimization of a practical ultrasound food preservation operation. To achieve this, the control parameters, sonication protocols, and ultrasonic equipment used must be well defined and reported (Mason *et al*., 2003a; Miller *et al*., 1996).

1.4. Pulsed Electric Fields (PEF)

Knowledge about microorganisms helped in the scientific improvement of food pasteurisation. Since the flavour and colour of food was greatly influenced by heating and consumers increasingly asked for high quality food, different pasteurisation methods without heat were demanded to maintain food taste and freshness (Barbosa-Caovas *et al*, 1999).

The application of Pulsed Electric Fields (PEF) received significant attention during the last few decades because of its potential to improve or to create alternatives to conventional methods in food processing. The application of PEF is one of the most innovative processing methods having low processing temperature and short residence time, which leads to effective microbial inactivation while retaining product quality. The cellular tissue permeabilization ability of PEF in microseconds can be developed to replace conventional thermal pasteurization techniques.

1.4.1. PEF historical background

The use of electric fields to preserve food was started as early as the twentieth century. A number of studies investigated the fatal consequence of non pulsed irregular currents applied to microorganisms that in turn resulted in high heating to create grape must (Tracy, 1932) or pasteurize milk (Moses, 1938). As this technology was very energy consuming, in the 1940s novel technologies were replaced (Palaniappan *et al.,* 1990). Sales, Heinz Doevenpeck, and Hamilton first established research on PEF in the 1960s. Krupp Maschinentechnik developed Doevenpecks experimentations on fish slurry in the 1980s. From 1990 to date, Pulsed Electric Fields (PEF) have been investigated broadly in studies that mainly focused on the PEF effect on pasteurisation of water and liquid food (Toepfl *et al.*, 2006).

PEF technology is still in the early stages of commercialization and scaling up to cost effective industrial operations is highly dependent on further research of the engineering principles behind this technology to fully understand the mechanisms in practice (Chauvin, 2004; Dutreux *et al*., 2000).

1.4.2. PEF process and the key principles

A typical PEF system is based on a high voltage pulse generator with a treatment chamber and suitable fluid handling system as well as monitoring and controlling devices. Liquid food product is pumped through the treatment chamber, mainly in continuous mode, where two electrodes are connected together with nonconductive material to avoid the flow of electric current from one to the other. Generated high voltage electrical pulses are applied to the electrodes and high intensity electrical pulses are conducted to the product placed between the two electrodes. The food product experiences a force per unit charge, while the dose of the application is adjusted by

means of electric field intensity (peak voltage and the gap between electrodes) and the number of pulses (treatment time. The main process parameters that determine PEF treatments are electric field strength, shape and width of the pulse, treatment time, frequency, specific energy density, and temperature. The intensity of these parameters determines the final lethal effect on the microbial population while width and frequency of the pulses contribute to define the process time (Altunakar & Barbosa-Canovas, 2011; Abram *et al*., 2001)

In order to generate quick Pulsed Electric Fields, electrical energy should be rapidly discharged. To this end, a pulse forming network (PFN) should be developed that acts as an electrical circuit with capacitors (0.1-10 *μF),* resistors (2-10MΩ), switches (semiconductors, tetrode, ignitron, spark gap, and thyratron), inductors (30μH), treatment chambers and one or more power supplies for voltage charge (equal to 60kV) (Barbosa-Canovas *et al.,* 1999).

1.4.2.1. Power supply

The essential duration, shape and intensity of pulses are supplied by a pulse generator with high voltage that is very important in the technology of PEF. Zhang *et al.,* 1995 stated that it is possible to use this power supply like a direct current (DC) resource or one that uses alternating current (AC) to charge a capacitor with an elevated frequency input that creates elevated cyclical rates of charge in comparison to DC power supply.

Electric field intensity has been identified as the most applicable factor during microbial inactivation by PEF. Electric field intensity in combination with treatment time is mainly effective on the extent of membrane cell disruption (Hamilton and Sale, 1967). Understanding the electrical principles behind PEF technology is essential for a comprehensive analysis of the PEF system. The electrical field concept, introduced by Faraday, explains the electrical field

force acting between two charges. E_r or electric field is a factor of the force (F) applied to a known point location r undergoing q or a positive charge (Altunakar & Barbosa-Canovas, 2011; Barbosa-Canovas & Sepulveda, 2005; Zhang *et al*., 1995). Equation (1.3) shows the definition of E:

$$E_r = F_{qr} / \text{q} \quad (1.3)$$

Also given that the

$$E_r = \text{Newton / coulomb} \quad (1.4)$$

And

$$\text{Volt} = \text{Newton} \times \text{metre / coulomb} \quad (1.5)$$

The following analysis is achieved:

$$E_r = \text{volt / metre} \quad (1.6)$$

Bearing in mind that an electric field is generated by creating voltage between two spots, the E (intensity) should obviously be relative in a linear relation to the probable discrepancy (V) and conversely to the distance amid the two spots *(D)*. Thus, the following equation shows that:

$$E = V / D \quad (1.7)$$

1.4.2.2. Treatment chamber

An additional essential element is the treatment chamber, which affects PEF considerably. Characteristics of the food product define the design of treatment chambers, yet generally key

groups of designs are classified as continuous and batch. Normally, the chamber connecting the electrodes contains the passage of electric pulses with high-voltage. Voltage application creates an electric field with high intensity in the space between the two electrodes where the food target is located.

Overall, treatment chambers for PEF are composed of polymers such as polyacetylene or polysulfur nitride that are electrically conductive, and two electrodes of a conductive material including graphite, gold or platinum. Nevertheless, stainless steel is generally used to make them as it provides a cleaner situation. The container linking the two electrodes has a non-conductive property that unites the electrodes. The non-conductive material is a plastic polymer such as polycarbonate that holds high electrical resistance and dielectric power.

1.4.2.3. System of fluid transfer

A sequence of piping and pumps are used to transfer fluid through the PEF system. In a PEF system with a continuous design, the most widespread pumps used are peristaltic or positive dislocation pumps. The effectiveness of pipes can be increased by eliminating air from the stream; continuous or pulseless pumps are suggested to achieve homogenous flow. As mentioned previously, stainless steel is most commonly employed in PEF technology due to its high levels of sanitation. The same applies to food-degraded products, as stainless steel is excellent for a PEF piping system, yet a number of components are made of plastic tubes for electrical insulation.

Usually temperature swappers and chilling coils are mounted at entry as well as outlet of the treatment chamber in order to reach to a wanted temperature throughout the treatment (Barbosa-Canovas & Sepulveda 2005).

1.4.2.4. Monitoring and controlling the system

In PEF, it is critical to regulate current, temperature, flow rate, voltage, and applied pulses' power curves throughout the treatment. For control and checking of the system performance, temperature is examined by PID controllers, and other data is precisely measured by microprocessor units linked to the main computer and subunits of Programmable Logic Controllers (PLC). Afterwards, software such as HP V Lab® or Lab View® is used to process the obtained information; this software computes electric factors by an oscilloscope card using a high-intensity voltage or current probe (Barbosa-Canovas & Sepulveda, 2005; Zhang *et al.,* 1995).

1.4.3. PEF inactivation of microorganisms in beer and other beverages

In general, gram-negative bacteria, yeasts, and moulds are the most susceptible microorganism in beer when subjected to PEF. Moreover, the most resistant microorganisms are spore formers such as *Bacillus cereus, Bacillus subtilis,* and *S. cerevisiae.* Since the PEF technique has the possibility to be used in a continuous mode of operation, it has high potential to be implemented at commercial scale in the beer industry as a promising food preservation technology (Walkling-Ribeiro *et al*., 2011).

Many studies have investigated the effect of combined PEF (Knorr *et al*., 1994) or temperature-assisted PEF (Amiali *et al*., 2007; Walkling-Ribeiro *et al*., 2008; Walkling-Ribeiro *et al*., 2011) on the inactivation of microbial populations in liquid foods, while less research has been done on a PEF treated alcoholic beverage like beer. Table 1.9 is a summary of studies that have been carried out on the inactivation of different microbes in beer by PEF and Table 1.10 presents the *S. cerevisiae* inactivation by PEF in various beverages

Table 1.9. PEF inactivation of *S. cerevisiae* in beer and other beverages.

Media	Strain	Vegetative/ ascospore	PEF electrical intensity (kV/cm)	Processing time (μs)	Processing temperature (°C)	Log reduction	References
NaCl solution	-	Vegetative	3.5	20	21	1.3	Jacob *et al*. (1981)
Apple juice	ATCC16664	Vegetative	1.2	20	≤30	≤4.2	Qin *et al*. (1994)
Apple juice	ATCC16664	Vegetative	1.2	60-90	4-10	3.5-4.0	Zhang *et al*. (1994a)
Beer	-	Vegetative	16.7	1280	-	4.0	Levesley & Kennedy (1999)
Orange juice	-	Ascospore	50	-	50	≤2.5	McDonald *et al.* (2000)
Beer	ATCC36026	Vegetative	35-45	402-2296	-	≥6.8	Walking-Ribeiro *et al*. (2011)
Red wine	-	Vegetative	31	-	-	≥6.0	Abca & Everendilek (2014)

Table 1.10. PEF inactivation of other microorganisms in beer.

Microorganism Type	Strain	Vegetative/ spore	Electrical intensity (kV/cm)	Processing time (μs)	Log reduction	References
Lactobacillus plantarum	TMW 1.460	Vegetative	13	-	2.0	Ulmer *et al*. (2002)
Saccharomyces uvarum	-	Vegetative	22	216	4.1	Evrendilek *et al*. (2004)
Rhodotorula rubra		Vegetative			4.3	
Lactobacillus Plantarum		Vegetative			4.7	
Bacillus subtilis		spore			4.8	
Pediococcus damnosus		Vegetative			5.8	
Salmonella choleraesuis	ATCC51741	Vegetative	35-45	402-2296	≤1.5	Walking-Ribeiro *et al*. (2011)
B. subtilis	ATCC6051	spore			≤2.5	
L. plantarum	ATCC14917	Vegetative			≤4.0	

The process of microorganism inactivation through PEF is influenced by numerous parameters. Process parameters, microbial parameters, and product factors are the most significant factors which can alter the rate of microbial inactivation by PEF. Furthermore, polarity of pulse, treatment temperature, strength of electric field, number of pulses or duration of treatment, and pulse shape and width are also significant factors with a crucial part in microorganisms' inactivation of by PEF (Martín *et al*., 1997).

According to the literature, the increase of treatment time, electric field or both causes a rise in microbial inactivation (Abram *et al*., 2003; Cserhalmi *et al*., 2002; Elez-Martínez *et al*., 2006; Evrendilek *et al*., 1999; Hülsheger *et al*., 1981; Martin *et al*., 1997; Qin *et al*., 1998; Zhang *et al*., 1994; 1997). Functional pulse number increases as time of treatment rises. As the pulse width increases, the microbial inactivation rate for the comparable number of pulses and/or electric density rises. Similarly, the pulse number augments with stable pulse width (Elez-Martínez *et al*., 2006; Jayaram *et al*., 1992). Moreover, a higher rate of microbial inactivation has been revealed with the use of a continuous process than a batch process, mainly for beverages and liquid food (Martín *et al*., 1997). Lastly, the temperature during PEF treatment greatly affects microbial inactivation as well. Higher microbial inactivation is observed with increased temperature (Jayaram *et al*., 1992; Pothakamury *et al*., 1996; Reina *et al*., 1998; Vega-Mercado, 1997; Zhang *et al*., 1995).

According to microorganism type, lower inactivation has been identified in cells at the stationary phase in comparison to logarithmic stage (Alvarez *et al.,* 2003; Saldaña *et al*., 2009; Hülsheger *et al*., 1983; Pothakamury *et al*., 1996; Rodrigo *et al*., 2003). The size and shape of the microbes plays a great role on the rate of inactivation by PEF. Since yeasts are bigger in shape and size (4-6 μm) compared to bacteria, they have been reported to be more vulnerable to PEF in comparison to vegetative bacteria. In addition, bacteria considered gram positive are less sensitive than gram negative bacteria (Aronsson *et al*., 2001; Castro *et al*., 1993; Qin *et al*., 1998; Sale & Hamilton, 1967; Wouters & Smelt, 1997; Zhang *et al*., 1994a). Additionally, higher resistance to PEF is observed in bacterial spores (Barbosa-Cánovas *et al*., 1999; Barsotti & Cheftel, 1999; Marquez *et al*., 1997). Current study aims to explore how PEF affects yeast spores.

With respect to the food product exposed to PEF, the electrical conductivity of food plays a great role in the PEF treatment. Foods with lower electrical conductivity show higher efficiency due to less dissipated energy and keeping PEF a nonthermal treatment (Alvarez, Condon, & Raso, 2006).

1.4.4. Mechanism of microbial inactivation by PEF

With respect to the mechanism of microbial inactivation by PEF, it can cause electroporation, the permeabilization of the membranes of cells and organelles, or electrofusion, the connection of two separate membranes into one (Qin *et al*., 1996). There are several theories to explain how pores are formed but it is still unclear whether it occurs in the lipid or in the protein matrices (Barbosa-Cánovas *et al*., 1999). The electric field induces structural changes in the microbial cells and membranes of microorganisms as shown in Figure 1.4 (Barbosa-Canovas, *et al*., 1999). Some studies postulate the cell membrane as the site of critical effect of reversible or irreversible loss of membrane function as the semi permeable barrier between the cell and its environment. An external electric field of short duration was assumed to induce an imposed transmembrane potential above a critical electric field intensity, which may produce a dramatic increase of membrane permeability. Exchange or loss of cell contents, cell lysis, and irreversible destruction may occur as secondary mechanisms. This will limit the ability of cells to repair themselves, which adversely affect permeable cells through osmotic pressure differences between the medium and the interior of the cell.

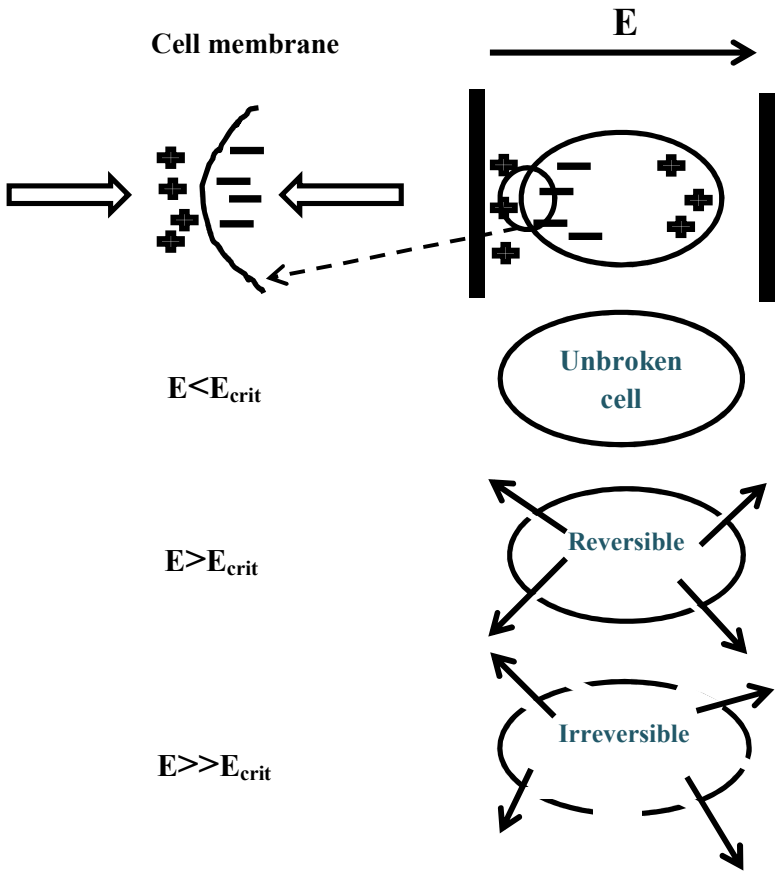

Figure 1.4. Electroporation of the cell membrane and membrane permeabilization when exposed to high-intensity electric fields (Modified from Toepfl *et al*., (2005)).

Chapter 2. Thermal resistance of *Saccharomyces* yeast ascospores in beers

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Chapter Abstract

The industrial production of beer ends with a process of thermal pasteurization. *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are yeasts used to produce top and bottom fermenting beers, respectively. In this research, first the percentage of sporulation of 12 *Saccharomyces* strains was studied. Then, the thermal resistance of ascospores of four *S. cerevisiae* strains (DSMZ 1848, DSMZ 70487, ATCC 9080, Ethanol Red®) was determined in 4% (v/v) ethanol lager beer. $D_{60°C}$-values of 11.2, 7.5, 4.6, and 6.0 min and z-values of 11.7, 14.3, 12.4, and 12.7°C were determined for DSMZ 1848, DSMZ 70487, ATCC 9080, and Ethanol Red®, respectively. Lastly, experiments with 0 and 7% (v/v) beers were carried out to investigate the effect of ethanol content on the thermal resistance of *S. cerevisiae* (DSMZ 1848). $D_{55°C}$-values of 34.2 and 15.3 min were obtained for 0 and 7% beers, respectively, indicating lower thermal resistance in the more alcoholic beer.

These results demonstrate similar spore thermal resistance for different *Saccharomyces* strains and will assist in the design of appropriate thermal pasteurization conditions for preserving beers with different alcohol contents.

Keywords: pasteurization; ethanol; heat resistance; strain; sporulation method; percentage of sporulation

2.1. Introduction

A Sumerian tablet found in Mesopotamia dated 6000 years ago is the oldest evidence of beer production (Mirsky, 2007; Nelson, 2014). Beer is an alcoholic beverage obtained by yeast fermentation of the sugar from malted cereal grains (e.g. barley, wheat). The production of beer consists of several stages: the transformation of barley water extract to malt (malting), the conversion of malt to wort (mashing), yeast pitching, fermentation of sugars to ethanol and post-fermentation operations. The main post-fermentation operations are beer clarification/filtration, packaging, and pasteurization. The hops added during production are responsible for the bitter flavour and contribute to its natural preservation. The beer ingredients (e.g. water, cereal, hops, and yeast) can be combined in different ways to create different styles of beers such as ale, lager, stout, pilsner, etc. A few regions such as Senne Valley in Belgium still use wild yeasts for spontaneous fermentation. Ale and lager are the two major classes of beers, obtained with top and bottom fermentation yeasts, respectively. An ale beer ferments with top-cropping *Saccharomyces cerevisiae* at temperatures around 15 to 20°C. A lager beer is fermented by bottom-cropping yeasts such as *Saccharomyces carlsbergensis (pastorianus)* or *Saccharomyces uvarum* at temperatures ranging between 8 and 13°C (Hardwick *et al*., 1995; Hornsey, 2003). Brewer's yeast has been the focus of several studies (Hammond, 1993; Linko et al., 1998; Priest and Yeasts, 2006; Stewart and Russell, 1986). Dengis and Rouxhet (1997) studied the surface properties of top- and bottom-fermenting yeast and Fleet (1998) reviewed the microbiology of alcoholic beverages.

The production of industrial bottled beer ends with a process of thermal pasteurization. This thermal process aims to inactivate the fermenting yeast used as starter along with potential spoilage microorganisms such as wild yeasts, *Lactobacillus, Pediococcus*, *Leuconostoc* and other bacteria that can contaminate the beer during the fermentation (Priest, 2003; Priest and Yeasts, 2006). The pasteurization enables the stabilization of the beverage for a longer

period, increasing the beer shelf-life. The thermal pasteurization measure for the beer is the pasteurization unit (PU): 1 PU is equivalent to 1 min at 60ºC. The minimum thermal pasteurization applied by breweries is 15 PU = 15 min at 60°C, which was established based on the thermal resistance of the brewing yeast in the vegetative form. The processing time for 15 PU at other temperatures can be estimated based on the yeast z-value of 7.0°C (Del Vecchio *et al*., 1951; Portno, 1968). Beer contains carbon dioxide, alcohol, and hops, all of which are natural antimicrobials, so a mild pasteurization is effective for its stabilization at room temperature (Silva & Gibbs, 2009; Silva et al., 2014). Higher *S. cerevisiae* percentage of sporulation was registered when beer, barley, and malt extracts were added to the sporulation agar (Lin 1978; 1979). This suggests it is possible to find yeast ascospores during brewing, especially due to the adverse conditions created by the ethanol, hops and carbon dioxide, all antimicrobial beer components. Ascospores are more resistant to thermal processing than vegetative cells, so inactivation of the ascospores will also inactivate the vegetative cells (Milani *et al.* 2015a).

King *et al*. (1978) found that flash pasteurization at 71°C for 30 s did not fully inactivate the beer spoilage organisms such as *Lactobacillus brevis*, *Pediococcus cerevisiae*, and a wild yeast *Saccharomyces diastaticus.* Normally, bottled beer is processed at 65-68°C for 20 min or 72-75°C for 1-4 min, equivalent to 10-20 PU (Fricker, 1984), since beer is carbonated, contains ethanol, has a low pH from 3 to 4.2 (Horn et al., 1997) and is bittered with hops, which are all natural antimicrobials. Therefore, thermal pasteurization is effective for its stabilization at room temperature (Silva & Gibbs, 2009). However, concerns have been expressed, especially in ethanol-free and in less bitter beers, the last being a trend in consumer preference. L'Anthoen and Ingledew (1996) reported that the D-value of lactic acid bacteria was four- to seven-fold higher in ethanol-free beer compared to 5% (v/v) ethanol beer. In addition, pathogens such *Escherichia coli* O:157:H7 and *Salmonella typhimurium*

were also more heat resistant by three to seventeen times in alcohol-free beer. Presently, the beer industry applies a more severe pasteurization process (e.g. 120 to 300 PU), to cope with on-going modifications in the traditional beer composition (Silva et al., 2014).

The thermal inactivation of microorganisms is often described by first order kinetics, with D- and z-values being the parameters estimated. Buzrul (2007) used first order kinetics for modelling *S. carlsbergensis* vegetative cell survivors in beer. D-value is the time required at a given temperature to inactivate 90% of the studied microorganisms and z-value is the temperature required for a one-log reduction in the D-value (Bigelow and Esty, 1920; Silva and Gibbs, 2009). The D- and z- values are used to define beer pasteurization times at different temperatures.

Although some researchers have determined *S. cerevisiae* thermal resistance parameters, only one performed tests in beer and these experiments were carried out with vegetative cells (Tsang and Ingledew, 1982). Past work with *S. cerevisiae* in fruit juices (Put *et al*., 1976; Put and Jong, 1982) demonstrated that the ascospores are 25 to 350 times more heat resistant than vegetative cells, and the highest $D_{60°C}$-value for ascospores (among the 21 strains tested) was 19.2 min. Considering the huge difference between the thermal resistance of ascospore and vegetative cells, one can assume that if spores are destroyed, all the vegetative cells will also be. Lin (1979) obtained higher percentage of sporulation of *S. cerevisiae* when beer, barley, and malt extracts were added to the sporulation agar. This suggests it is possible to find yeast ascospores during brewing, especially due to the adverse conditions created by the ethanol, hops and carbon dioxide, all natural antimicrobial beer components. In another study using Pulsed Electric Fields, we have observed that the inactivation of *S. cerevisiae* ascospores was easier in high-alcohol beers (Milani *et al*., 2015b; Chapter 6). Hence, the study of the effect

of beer alcohol content on the thermal inactivation of yeast ascospores is also important to investigate.

Therefore, the objectives of this work were to determine: (i) the percentage of sporulation of different brewing and non-brewing *Saccharomyces* yeast strains; (ii) the thermal resistance (D- and z-values) of ascospores of four *S. cerevisiae* strains in beer; (iii) the effect of beer alcohol content on the thermal resistance of *S. cerevisiae* DSMZ 1848 ascospores.

2.2. Material and methods

2.2.1. Yeast strains

The eight strains of *S. cerevisiae* and four strains of *S. pastorianus* used in this investigation were obtained from different culture collection described in Table 2.1. ATCC 9080, CBS 1171 (top fermenting yeast, neo type strain isolated from beer), CBS 1503 (type strain bottom fermenting), CBS 1538 (neo type strain isolated from beer), DSMZ 1848 (hybrid isolated from bottom fermenting beer), DSMZ 70487 (isolated from super attenuated beer), Wyeast 1469 (commercial bottom fermenting brewing yeast) and Wyeast 2278 (commercial top fermenting brewing yeast). In addition the following strains from the School of Biology Sciences of the University of Auckland were used because of their good sporulation: BC186 (natural isolate from oak trees), SK1 (=NCYC 3265, lab strain isolated from soil; Liti *et al*., 2009), Zymaflore F15 (commercial wine yeast; Harsch & Gardner, 2013), and Lesaffre Ethanol Red® (industrial fermentation).

All the strains were tested for sporulation while for the thermal inactivation experiments the strains DSMZ 1848, DSMZ 70487, ATCC 9080, and Ethanol Red® were used.

2.2.2. Yeast enumeration

Colony formation was used for yeast enumeration. Yeast Extract Peptone Glucose (YEPG) medium was prepared by mixing 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 2.0% (w/v) glucose, 2.0% (w/v) agar. The agar medium was autoclaved at 121°C for 10 min. A volume of 100 µL of appropriately diluted beer samples containing the yeast was spread into duplicate agar plates and colonies were counted after 2 days of incubation at 28°C.

2.2.3. Ascospores production

The culture stored at -80°C was streaked on YEPG agar and after growth a fresh single colony was inoculated into 50 mL of presporulation sterilised liquid (121°C, 10 min) composed of 0.8% yeast extract, 0.3% peptone, 10% glucose, and zinc sulphate 25 mg/L. After inoculation, the presporulation flasks (500 mL) were incubated overnight in incubators (with rotary shaking at 168 rpm) at 28°C. When optical density (PG Instrument T60 set at 600 nm) reached around 0.2 to 0.8, an appropriate portion of the presporulation broth (ca. 1.5 mL) was inoculated into sterile sporulation broth (10 mL) to yield 10^7 cfu/mL. Sporulation broth consisted of potassium acetate 1% (w/v), bacto yeast extract 0.1% (w/v), glucose 0.05% (w/v), zinc sulphate 25 mg/L. The mixture was incubated at 18°C for 14 days (with rotary shaking at 230 rpm) in 1-L Erlenmeyer flasks. The solution was split in 1-mL Eppendorf tubes and the spores were extracted from the vegetative (parental cells) by adding 100 µL Zymolyase solution (5 mg/mL solid Zymolase in pH 7.2 buffer containing 1.2 M sorbitol and 0.1 M KH_2PO_4), 900 µL spheroblasting buffer (2.2 M sorbitol), and 800 µL softening buffer (100 mM Tris-SO_4, pH 9.4, 10 mM dithiothreitol (DTT) solution). Then, the mixture was incubated at 30°C in a water bath for 2 h and the Eppendorfs were gently inverted every 20 min to accelerate the break-up of tetrads into single ascospores. The spores were harvested by

centrifuging three times at 9700 *g* (rotor F-45-12-11) for 1 min and resuspending in 200 µL of 0.5% Triton X-100 to ensure total removal of the enzyme. After the last resuspension, 4 µL DTT was added to the Eppendorfs containing the spore solution. Then, the Eppendorfs were sonicated three times at 6 Hz for 2 min, both to break up tetrads into single ascospores and to kill any vegetative cells remaining in the medium. Finally, 1 mL of salt triton dithiothreitol (STD) solution (0.1 g NaCl in 10 mL of 0.05% Triton X-100) was added to the spore solution to avoid spore aggregation (Xiao, 2006).

2.2.4. Determination of percentage of sporulation

The percentage of sporulation was determined after 7 days of incubation and reassessed after 10 and 14 days. Strains showed different behaviours during sporulation. Some strains changed into tetrads, some triads, some dyads, and others stayed as vegetative cells. In order to measure the percentage of sporulation, a portion of 50 µL of the spores was diluted into 950 µL of a 1:1 mixture of sterile water and methylene blue (ca 10^7 cfu/ml) and the spores were counted under a microscope using a haemocytometer. Adding the methylene blue to the spore suspension allowed differentiating the live from dead cells, due to permeation of the methylene blue through the cell walls of dead cells. Blue-staining (dead) cells were not counted. Percentage of sporulation was calculated as the percentage of tetrads and/or triads divided by the total cell counts (tetrads, triads, dyads, and vegetative cells). Four replicate counts were carried out for each strain and the percentage of sporulation average ± standard deviation was determined. ANOVA was used to investigate significant differences between yeast strains (Statistica version 8, USA), and when differences were detected ($p<0.05$), Tukey's Honest Significant Difference (HSD) test was carried out to separate the average values.

2.2.5. Saccharomyces thermal inactivation experiments

Ethanol is the major alcohol of beer fermentation by yeast. Alcohol by volume abbreviated as ABV, abv, or alc/vol is a standard measure of how much alcohol (ethanol) is contained in a given volume of an alcoholic beverage. It is expressed as a volume percent and defined as the number of millilitres of pure ethanol present in 100 mL of beer at 20°C, (% v/v ethanol). Commercial beers with 0, 4 and 7% ethanol were selected for the thermal inactivation studies, since they represent the minimum, standard and maximum alcohol concentrations found in commercial beers (Turner, 1990; Priest & Stewart, 2006). The alcohol content was read from the beer bottle label. For the comparison of the thermal resistance of the four strains' ascospores, 4% alc/vol beer was used. With respect to the effect of alcohol content on the thermal resistance, the strain DSMZ 1848 was used in 0 and 7% alc/vol beers.

A preliminary experiment was initially carried out to investigate the degree of difference in thermal resistance between ascospores and vegetative cells of DSMZ 1848 *S. cerevisiae* in 4% alc/vol beer, and the $D_{55°C}$-value was determined for vegetative and ascospore cells. Then, the main experiments were carried out at 50, 55, 60 and 65°C with ascospores of *S. cerevisiae* DSMZ 1848, DSMZ 70487, and Ethanol Red® and *S. pastorianus* ATCC 9080 (also named *S. cerevisiae*) using 4% alc/vol beer. In the last set of experiments, spores of the most thermal resistant yeast, *S. cerevisiae* DSMZ 1848, were used in 0 and 7% alc/vol beers to investigate the effect of beer ethanol content on the ascospores D-value at 50 and 55°C.

Each yeast ascospore solution was centrifuged to remove the STD solution. Filter-sterilized beer was mixed with the spore pellet to yield a final ascospore concentration of ca. 10^6-10^7 cfu/mL. The clustering and the large size of ascospores did not allow the use of higher initial spore concentration. Five millilitres of beer samples containing the yeast spore were vacuum packed in 5×5 cm heat-resistant pouches that had been previously sterilized (Caspak, New

Zealand). The removal of the air inside the bag increased the heat transfer and produced more reliable results, with less variation. The 154-μm thick film can withstand temperatures up to 110°C and was composed of linear low density poly ethylene (LLDPE) and poly ethylene therephthalate (PET). A thermostatic water bath (W28 Grant Instruments, Cambridge, Ltd, England) equipped with stirring ensured uniform temperature throughout the bath during thermal experiments. After setting the water bath temperature to the desired treatment temperature, the packed beer samples were fully submerged in the water bath for pre-specified times between 3 and 90 min. For each time point, two replicates of beer samples were removed and placed immediately into an ice container to avoid more spore killing. The yeast spore survivors were enumerated before and after thermal processing for different times as described in Section 2.2.2.

2.2.6. Estimation of the first order kinetic parameters

Generally, the inactivation of microorganisms in foods follows the first-order/Bigelow model pattern (Bigelow & Esty, 1920; Silva & Gibbs, 2009):

$$\frac{N}{N_o} = 10^{-\frac{t}{D_T}} \quad (2.1) \qquad \text{or} \qquad Log\left(\frac{N}{N_o}\right) = -\frac{1}{D_T} \times t \quad (2.2)$$

Where N is the number of microbial spores, N_0 is the initial number of microbial spores, D_T - decimal reduction time (min) at temperature T, and t is time (min).

$$\frac{D_T}{D_{T_{ref}}} = 10^{\left(\frac{T_{ref}-T}{z}\right)} \quad (2.3) \qquad \text{or} \qquad Log\left(\frac{D_T}{D_{T_{ref}}}\right) = -\frac{1}{z}\left(T - T_{ref}\right) \quad (2.4)$$

D_{Tref} is the decimal reduction time at a reference temperature and z is the number of degrees Celsius required to reduce D by a factor of 10.

First, the linearity of survival was confirmed by plotting log (N/N_0) *vs*. time (Equation 2.2). For each temperature and strain, D-value±SE was estimated by linear regression from Equation 2.2 using Table Curve 2D software (version 5.01, Systat software Inc., USA). Then, for each strain z-value±SE was estimated using Equation 2.4. The goodness of fit was also assessed by the adjusted R^2 (Adj R^2) and the mean square error (MSE) associated with each parameter estimation:

$$Adj\,R^2 = 1-(1-R^2)*\left(n-1/n-p-1\right) \quad (2.5)$$

Where *n* is the number of data points and *p* is the number of explanatory variables.

$$MSE = \frac{\sum_{i=1}^{i=n}(Oi-Pi)^2}{n-P} \quad (2.6)$$

Where O_i is the observed value and P_i is the i^{th} predicted value. To assess the effect of ethanol on D-values of the DSMZ 1848 strain at 50 and 55°C, ANOVA was used to investigate any significant differences (Statistica version 8, USA). When differences were detected (p<0.05), Tukey's Honest Significant Difference (HSD) test was carried out to separate the average D-values and different letters were used for strains with significantly different D-values.

2.3. Results and discussion

2.3.1. Percentage of sporulation

The percentage of sporulation is presented in Table 2.1. *S. cerevisiae* CBS 1171, Wyeast 1469 and *S. pastorianus* Wyeast 2278 presented no live spores after 14 days of incubation at 18°C. *S. pastorianus* CBS 1538 and CBS 1503 had ≤ 1% sporulation. All the other yeasts sporulated after 10 d and their percentage of sporulation remained constant between 10 and 14 days. ATCC 9080, ER® and SK1 were similar in terms of percentage of sporulation (49.5 to 58.7%). DSMZ presented 45% sporulation and DSMZ 70487 8%. It is known from the literature that sporulation of *Saccharomyces* yeast is highly dependent on the yeast strain, culture phase, medium, and environmental factors (Lin, 1978). The percentage of sporulation of *S. cerevisiae, S. diastaticus*, and *S. willianus* on Kleyn's medium sporulation media was significantly higher (up to 40%) for *S. cerevisiae* spores (Bilinski *et al*., 1986). The same author demonstrated that 21°C was better than 27°C for sporulation of an ale brewing yeast, and that acetate medium gave a higher yield of sporulation than Kleyn's medium.

Based on their percentage of sporulation and relation with brewing industry, strains DSMZ 1848 (bottom fermenting brewing yeast), DSMZ 70478, ATCC 9080, and the industrial strain Ethanol Red® were chosen for determining ascospore thermal resistance, as described in the following section.

Table 2.1. Percentage of sporulation of different *Saccharomyces* strains after 14 days incubation at 18°C.

Species	Microbial Strain	Sporulation ±SD (%)
S. cerevisiae	BC 186*	96.2±0.01[a]
S. cerevisiae	Zymaflore F15*	65.2±0.05[b]
S. cerevisiae	ATCC 9080	58.7±0.04[bc]
S. cerevisiae	Ethanol Red®*	50.5±0.02[cd]
S. cerevisiae	SK1* *(=NCYC 3265=ATCC 204722)*	49.5±0.04[cd]
S. cerevisiae	DSMZ 1848	45.0±0.07[d]
S. cerevisiae	DSMZ 70487	8.0±0.03[e]
S. pastorianus	CBS 1503[T]	< 1
S. pastorianus	CBS 1538[NT]	< 1
S. cerevisiae	CBS 1171[T]	No live spores
S. pastorianus	Wyeast 2278	No live spores
S. cerevisiae	Wyeast 1469	No live spores

*Strains BC 186, Zymaflore F15, Ethanol Red®, SK1 were supplied from the School of Biological Sciences yeast collection, University of Auckland.
NCYC- National Collection of Yeast Cultures, UK; ATCC- American Type Culture Collection; DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; CBS- Centraalbureau voor Schimmelcultures, The Netherlands ; Wyeast- supplier of commercial brewing yeast strains.
[T:] Type strain; [NT]: neotype strain; Percentage of sporulations followed by different letters are significantly different ($p<0.05$) according to Tukey test. "No live spores" indicates that although some "ascus-like" shapes were seen under the microscope, all were non-viable as judged by methylene blue staining.

2.3.2. Thermal resistance of Saccharomyces ascospores in 4% beer

The *S. cerevisiae* ascospore survival lines for the four *Saccharomyces* strains (DSMZ 1848, DSMZ 70487, ATCC 9080, and Ethanol Red®) studied at 50, 55, 60 and 65°C are presented in Figure 2.1 (A-D). At 65°C, after 20 and 30 min no growth was observed in the plates, and

those points could not be plotted on Figure 2.1. The first order kinetic model fitted well to the survival of yeast ascospores in beer. The D-values, which are proportional to the inverse of the slope, were estimated by regression for each temperature and strain and are presented on Table 2.2. Splittstoesser *et al*. (1986) determined the thermal resistance of *S. cerevisiae* ascospores in wine and apple juice, and also observed log linear behaviour in both beverages.

Before running the survival experiments with the yeast ascospores, the thermal resistance of the vegetative cells of *S. cerevisiae* DSMZ 1848 was determined at 50°C. The vegetative cells presented a $D_{50°C}$-value=14.6 min as opposed to 62.0 min obtained with ascospores in 4% alc/vol beer, confirming at least 4-fold more heat resistance of ascospores in comparison to the corresponding vegetative cells. Research carried out with juice spoilage yeasts concluded the $D_{60°C}$-values of ascospores were 25 to 350 times higher than those of the corresponding vegetative cells (Put and De Jong, 1980). The thermal resistance of the ascospores may also be affected by the sporulation medium and the technique used to produce spores.

2. Thermal resistance of *Saccharomyces* ascospores in beer

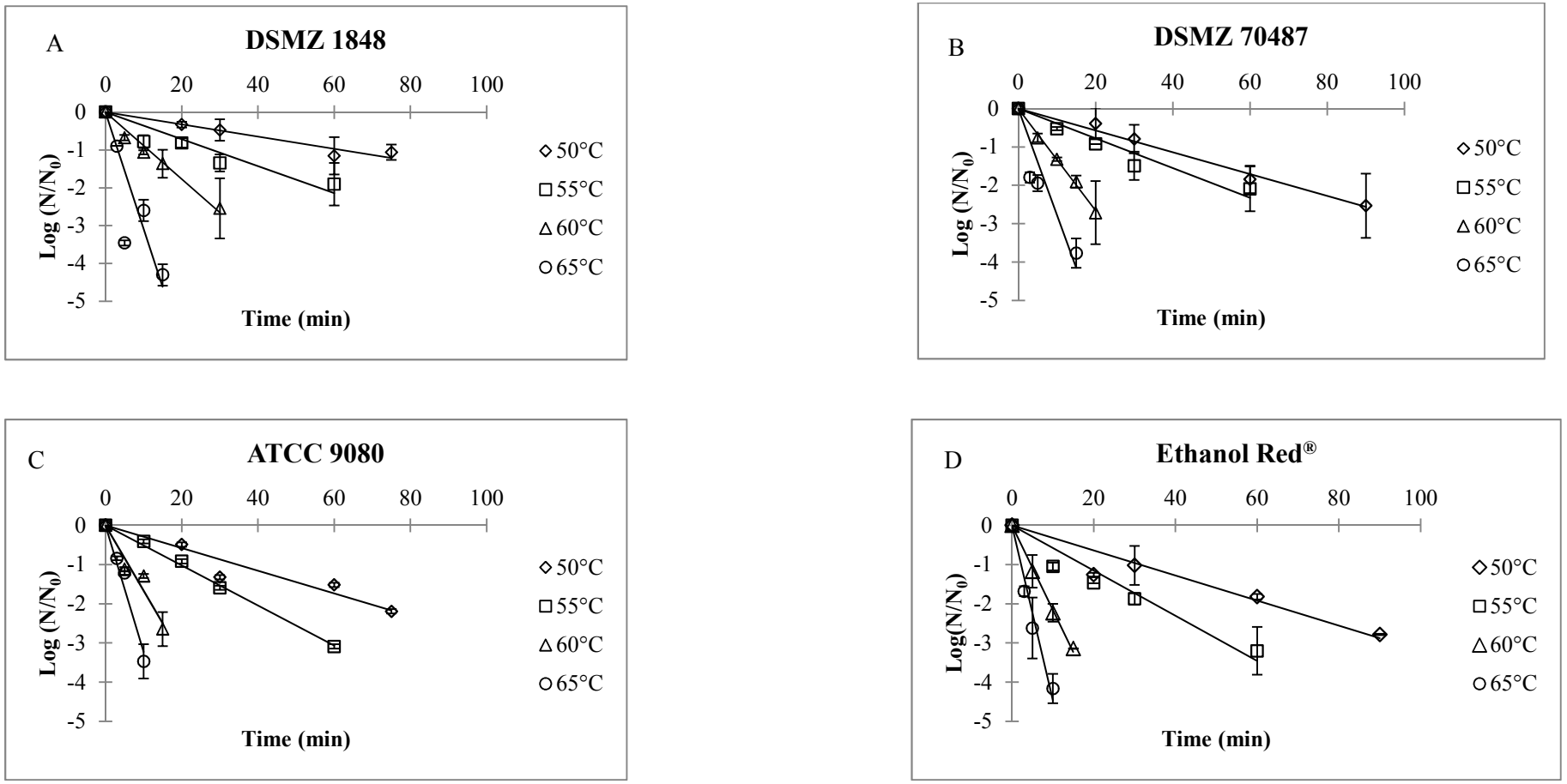

Figure 2.1. Ascospores survival of *S. cerevisiae* DSMZ 1848 (A), DSMZ 70487 (B), ATCC 9080 (C), and Ethanol Red® (D) in 4% alc/vol beer after thermal processing (solid lines represent the first order model fitting. The error bars are standard deviations).

Figure 2.2 shows the thermal death time lines for the four strains, with the z-value inversely proportional to the slope. While DSMZ 1848 presented higher D-values for 50, 55 and 60°C, the other three strains presented similar D-values (Figure 2.1, Table 2.2), which can indicate close thermal resistances. The z-values were similar for the four strains as shown by similar slopes in Figure 2.2. DSMZ 1848 was selected for subsequent study with different ethanol content beers.

Past research on *Saccharomyces* thermal resistance in beer was carried out with vegetative cells and not ascospores, so D-values were much lower and only comparable to the $D_{50°C}$-value of 14.6 min that we determined for vegetative *S. cerevisiae* DSMZ 1848. Tsang and Ingledew (1982) studied the heat resistance of two wild yeasts in beer and obtained $D_{51°C}$-values of 0.46 min for vegetative *S. carlsbergensis* and a z-value of 4.4°C. The *S. willianus* was not well modelled by first order kinetics, and Buzrul (2007) suggested a Weibull model. Watier *et al.* (1996) determined $D_{60°C}$-values of 0.2 min for *Megasphaera cerevisiae* in beer. Reveron *et al.* (2005) determined the thermal resistance of *Lactobacillus paracasei* ($D_{60°C}$-value = 0.02 min, z-value = 6.5°C) and *Aspergillus niger* ($D_{60°C}$-value = 0.04 min, z-value = 3.7°C) in Pilsner beer, which seem of much lower magnitude than *Saccharomyces* yeasts.

Table 2.2**.** First order thermal inactivation parameters (D- and z-values) of ascospores of four strains of *Saccharomyces cerevisiae* in a 4% alc/vol beer.*

D-value±SE (min)	DSMZ 1848	DSMZ 70487	ATCC 9080	Ethanol Red®
55°C	28.0±3.14	25.7±2.11	17.3±1.36	19.5±0.43
Adj R^2	*0.785*	*0.898*	*0.994*	*0.900*
MSE	*0.080*	*0.051*	*0.006*	*0.102*
60°C	11.2±0.57	7.5±0.14	4.6±0.10	6.0±0.54
Adj R^2	*0.961*	*0.993*	*0.896*	*0.993*
MSE	*0.025*	*0.005*	*0.080*	*0.007*
65°C	3.2±0.55	3.6±0.55	2.2±0.15	2.5±0.07
Adj R^2	*0.577*	*0.706*	*0.946*	*0.941*
MSE	*0.025*	*0.463*	*0.077*	*0.118*
z-value± SE (°C)	11.7±1.25	14.3±3.01	12.4±1.81	12.7±1.59
Adj R^2	*0.966*	*0.878*	*0.937*	*0.953*
MSE	*0.005*	*0.013*	*0.008*	*0.006*

* Values in italic are model performance indices for the parameter estimates. Adjusted R^2 close to 1.0 and low Mean Square Errors (MSE) indicates the goodness of fit.

2.3.3. Effect of beer ethanol content on thermal resistance of S. cerevisiae DSMZ 1848 ascospores

Figure 2.3 presents the average D-values from two survival experiments. The standard deviations could be lower if more survival experiments were carried out. The $D_{55°C}$-value of yeast ascospores decreased significantly with the alcohol content, from 34.2 min for 0% alc/vol beer to 15.4 min for 7% alc/vol beer. At 50°C, the $D_{50°C}$-values were 61.2 min for 0% alc/vol and 29.1 min for 7% alc/vol. There is a good indication from these data that the higher the ethanol content, the more effective is the inactivation of the yeast ascospores, with lower D-values. Although there has not been any direct comparison of the ethanol effect in a controlled background medium, some published D-values with in other beverages support an important role for ethanol content. Since no thermal data has been published with 0% (alc/vol) beer and alcoholic beers, thermal results with juices and wines are the closest found and will be compared. For example, Splittstoesser *et al.* (1986) determined a $D_{55°C}$-value for

S. cerevisiae ascospores of 106 min vs 0.90 min for vegetative cells in apple juice. The same study reported a much lower $D_{55°C}$-value of 0.57 min for the ascospores in Chenin Blanc wine (11% alc/vol). In a pH 4.5 buffer without ethanol, *S. cerevisiae, Saccharomyces chevalieri* and *Saccharomyces bailii* ascospores exhibited $D_{60°C}$-values of 22.5, 13 and 10 min, respectively (Put *et al*., 1976). Previous investigations showed that temperatures between 48-51°C were sufficient to fully inactivate all vegetative yeasts in sweet fruit juices, as their D-value can vary from 10 min at 51°C to 30 min at 48°C (Beuchat, 1982). Finally, Couto *et al.* (2005) determined D-values of wine yeasts *Dekkera bruxellensis* ($D_{50°C}$-value =3.8 and $D_{55°C}$-value=0.3 min) and *Dekkera anomala* ($D_{50°C}$-value=2.0 min and $D_{55°C}$-value=0.2 min) vegetative cells in the standard 12% wine.

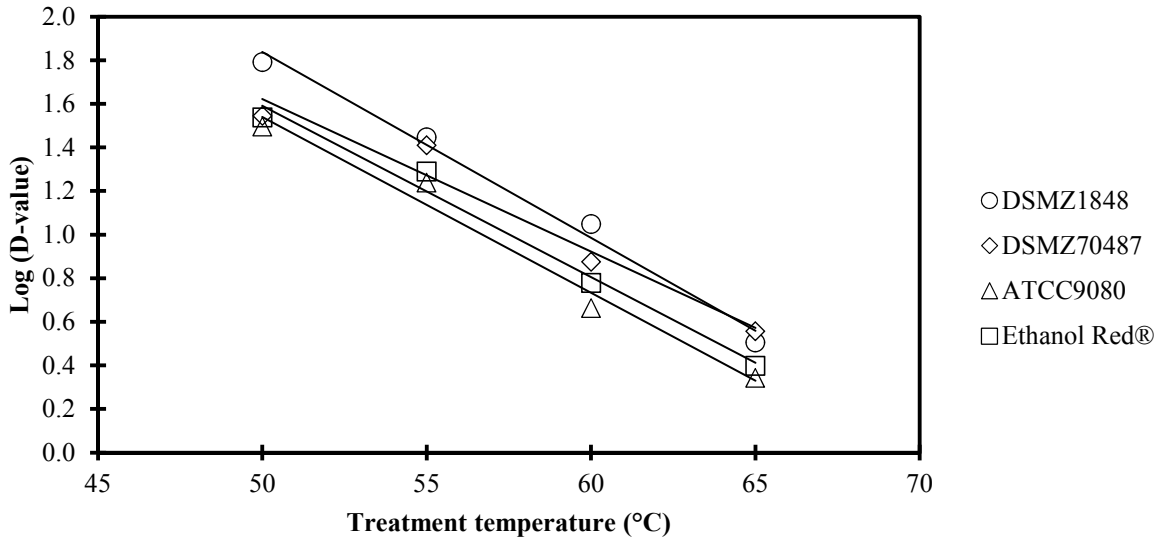

Figure 2.2. Thermal death time lines for *S. cerevisiae* ascospores (similar slopes indicate similar z-values between strains) in 4% alc/vol beer.

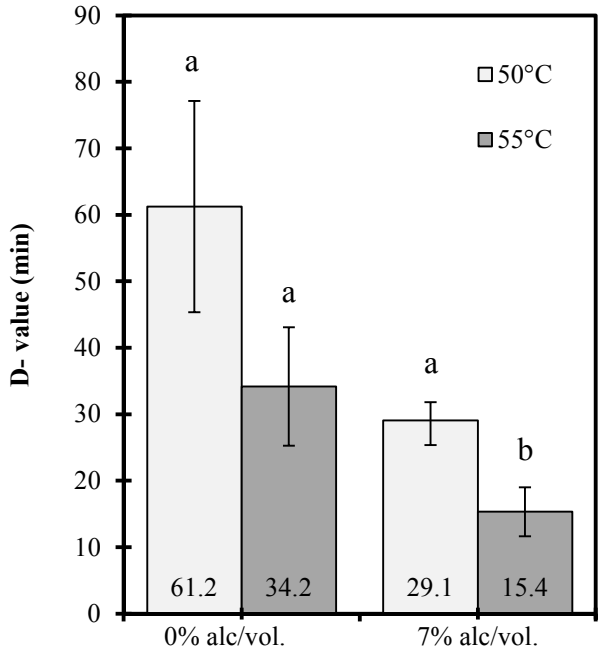

Figure 2.3. D-value of ascospores of *S. cerevisiae* DSMZ 1848 in two different alcohol content beers (results are average±SD from two survival experiments; different letters indicate values that are significantly different in each experiment).

According to Belmans *et al*. (1983), Eilers and Sussman (1970) and Sussman (1976), chemical compounds such as ethanol were able to break the dormancy of ascospores. It was hypothesized that these compounds may act by causing an alteration in lipid moieties of the spore. Studies on the survival of yeast vegetative cells during exposure to ethanol have shown a clear influence of elevated temperature (Balakumar and Arasaratnam, 2012; D'Amore *et al*., 1989; Shi *et al*., 2012), which has been attributed to their combined effects on membrane composition and fluidity.

2.4. Conclusions

The results of this study demonstrate that certain strains did not sporulate and the others sporulate between 8 and 96%. Furthermore, first order kinetic model was fitted well to the inactivation kinetics of the yeast ascospores in beer. The ascospores thermal resistance for different strains of *Saccharomyces* was very close. These results would be helpful in the design of appropriate thermal pasteurization conditions for beer preservation with different alcohol contents.

Chapter 3. High pressure processing and thermosonication of beer: comparing the energy requirements and *Saccharomyces cerevisiae* ascospores inactivation with thermal processing and modelling

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Chapter Abstract

In this research, pasteurization of beer by nonthermal high pressure processing (HPP) and thermosonication (TS) were compared with thermal pasteurization. The inactivation of *Saccharomyces cerevisiae* ascospores in beer was studied and modelled for HPP at 200, 300 and 400 MPa, and for TS at 50, 55 and 60°C with an acoustic energy density of 16.15 W/mL. The energy requirements for equivalent ascospore inactivation by HPP, TS, and thermal processes were compared. For the same processing time, ascospore inactivation was greatest with HPP, followed by 60°C TS, then 60°C thermal processing. Nonlinear survival curves, which could be described by the Weibull model, were observed for both HPP and TS. To achieve a 2.5 log reduction in ascospores, HPP required 77.4 kJ/L compared with 188.8 kJ/L for thermal processing and 2612.1 kJ/L for TS. HPP and thermosonication may be alternatives to thermal beer pasteurization, achieving greater log reductions in *S. cerevisiae* ascospores with shorter processing times (TS and HPP) or less energy (HPP).

Keywords: *HPP, ultrasound, heat, spore, inactivation kinetics, energy*

3.1. Introduction

Beer is a beverage of low alcohol content, commonly around 4-5%. A prolonged shelf-life is vital but consumers are also becoming more discerning about the quality of beer as a result of the craft and speciality beer movement. Because thermal pasteurization can have negative effects on the beer's organoleptic properties, a method of pasteurization that does not affect the beer's sensory characteristics is of great interest to the brewing industry. *Saccharomyces cerevisiae* is a yeast used for brewing and is often the most abundant microorganism detected in the beer after fermentation and before pasteurization (Reveron *et al*., 2012). The activity of *S. cerevisiae* can cause changes in the beer by releasing ethanol and carbon dioxide

High pressure processing (HPP), also known as high hydrostatic pressure processing (HHP) and thermosonication are two alternative methods of pasteurisation that have been suggested for the treatment of foodstuffs (Evelyn & Silva, 2015a; 2015b; 2015c; 2016a; Evelyn *et al*., 2016b; Farkas & Hoover, 2000; Hoover *et al*., 1989; Silva *et al*., 2012; Silva *et al*., 2015; Sulaiman *et al*., 2015a; 2015b), which could also have potential for use in the brewing industry (Buzrul *et al*., 2005a; 2005b; Castellari *et al*., 2000; Fischer *et al*., 2002; Gazle *et al*., 2001; Perez-Lamela *et al*., 2004). HPP is already used commercially for the treatment of fruit juices, meat and seafood but as yet, not for beer, although some studies have been carried out to determine the effect of HPP on microorganisms and flavour properties in beer (Buzrul, 2012; Silva *et al*., 2015). Sensory tests by Silva *et al.* (2015) revealed no significant difference in the overall flavour of untreated and HPP beers. Information about how microorganisms in beer respond to these treatments is needed in order to identify if they are valid techniques for industrial application and also how to optimize the industrial process. HPP subjects food products to pressures of between 100 and 800 MPa through a pressure-transmitting medium, usually distilled water. High pressure inactivates the microorganisms in several ways, including

denaturing of enzymes, cell membrane damage and ribosome disintegration (Farkas & Hoover, 2000; Hoover *et al*., 1989). Yeasts and moulds are mostly very susceptible to inactivation by high pressure but can be very resistant in ascosporic form (Evelyn & Silva, 2015b; Evelyn *et al*., 2016; Georget *et al*., 2015). HPP has been found to inactivate *S. cerevisiae* ascospores in orange and apple juice at pressures between 300 and 600 MPa (Zook *et al.*, 1999; Parish, 1998). These studies found that spore inactivation fitted a first-order kinetic model. In contrast, vegetative *S. cerevisiae* inactivation was nonlinear in wine (Mok *et al.,* 2006). We have carried out other studies to determine the HPP inactivation kinetics in non-alcoholic fruit mediums of two moulds' ascospores and bacterial spores, which were nonlinear and suitably modelled by the Weibull equation (Evelyn & Silva, 2015a, 2015a; 2015b; Evelyn *et al*., 2016; Silva *et al*., 2012).

Power ultrasound is classified as ultrasonic waves with a frequency of between 20 and 100 kHz and a sound intensity ranging from 10 to 1000 W/cm^2 (Feng *et al*., 2008; Feng & Yang, 2011). Power ultrasound alone can be used for the inactivation of microorganisms. Thermosonication, the process of combining power ultrasound treatment with heat has been found to greatly improve the death rate of microorganisms compared with power ultrasound alone (Evelyn & Silva, 2015b; 2015c; 2016a; Evelyn *et al*., 2016). Although thermosonic pasteurization still requires heat, it may reduce the time and temperature needed to achieve the same reduction in spoilage microorganisms as thermal processing alone, which would be advantageous for maintaining the beer's organoleptic properties. Ultrasound waves cause the cavitation of the liquid through which they propagate. The collapse of the bubbles caused by the ultrasound waves results in shock waves that rapidly change the pressure and temperature. This phenomenon causes the inactivation of bacteria, moulds and yeasts by damaging their cell membrane. The rapid change in pressure is the main mechanism of microbial inactivation (Condon *et al*., 2004; Feng & Yang, 2011; Piyasena *et al*. 2003). Bermúdez-Aguirre and

Barbosa-Cánovas (2012) showed that *S. cerevisiae* in its vegetative state could be inactivated by thermosonication using 200 W ultrasound at 24 kHz and 120 μm amplitude in combination with temperatures of between 40 and 60ºC. The modified Gompertz equation suited the inactivation kinetics best. No studies have been published to date modelling the inactivation kinetics of *S. cerevisiae* in its ascosporic form by thermosonication in alcoholic or non-alcoholic beverages.

The aim of this study was to describe the inactivation of ATCC 9080 *S. cerevisiae* ascospores in a lager beer (4% alcohol by volume) by a suitable model using HPP processing at varying pressures and thermosonication at varying temperatures, and compare these processes with conventional thermal processing. These ascospores were chosen as they are more resistant to temperature and pressure than yeast cells in their vegetative state and therefore represent more of a challenge to industry (Milani *et al*., 2015a). This strain of *S. cerevisiae* is also known as *Saccharomyces pastorianus* or *Saccharomyces carlsbergensis*, which is the commonly found in lager type of the beers.

Therefore, the main objectives were: (i) to model the HPP inactivation of *S. cerevisiae* ascospores in beer; (ii) to model the thermosonication inactivation of *S. cerevisiae* ascospores in beer; (iii) to compare HPP, thermosonication, and conventional thermal inactivation of ascospores in beer; (iv) to compare the energy requirements for equivalent pasteurizations using different technologies.

3.2. Material and methods

3.2.1. Microbiology

3.2.1.1. Yeast strain, production of ascospores, and inoculation

The production of the *S. pastorianus* ATCC 9080 (also named *S. cerevisiae*) ascospores followed the method outlined by Xiao (2006) and updated by Milani *et al*. (2015a) which produced the ascospores suspended in a salt triton dithiothreitol (STD) solution to avoid spore aggregation. For the inoculation of the DB Export Gold lager (4.0% alc/vol Dominion Breweries, Auckland, New Zealand), the spore samples were centrifuged and washed with sterile water to remove the STD solution, centrifuged again and the water removed. The spores were then added to the desired amount of beer that had previously been filtered using a sterile syringe filter with a pore size of 0.2 µm (Sartorius AG, Germany) to ensure that the *S. pastorianus* ascospores were the only microorganisms present in the sample. The initial concentration of ascospores was between 10^6 and 10^7 colony forming units per millilitre (CFU/mL). For more details please consult Section 2.2.3 of previous chapter.

3.2.1.2. Enumeration of ascospores

Colony formation was used for yeast enumeration. Once the beer samples had been processed by the various pasteurization techniques, the surviving *S. pastorianus* ascospores in each beer sample were enumerated using the serial dilution method. A volume of 100 µL of appropriately diluted beer samples were streaked upon yeast extract peptone dextrose (YPD) agar medium consisting of 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 2.0% (w/v) dextrose and 2.0% (w/v) agar that had been autoclaved at 121°C for 15 min. Each plate of colony sample was enumerated in duplicate and counted after 2 days of incubation at 28°C. Then the number of colonies were

counted in the dilutions with plates presenting a number of colonies between 30 and 300 and averaged for each tube dilution. The concentration of ascospores was calculated and the result was expressed in colony forming units per millilitre of beer (cfu/mL). For each pressure-time processing condition, the mean ± SD of two processed beer samples was calculated and plotted in the charts

3.2.2. High pressure processing

Five mL of filter-sterilized beer samples containing the yeast spore were sealed in 5×5 cm 154 μm thick pouches that had been previously sterilized (Caspak, New Zealand). The plastic film was composed of linear lowdensity polyethylene and polyethylene therephthalate. The pouches containing the beer samples were then packed twice with the same plastic film, and the second bag was vacuum sealed, to avoid bursting during the depressurization phase of the HPP cycle. The pouches were placed inside a 2 L 700 Laboratory Food Processing System (Avure Technologies, Columbus, Ohio, USA) for varying processing times and pressures. The pressures applied were 200, 300, and 400 MPa and more samples were processed at early processing times when changes in the log reductions were higher. The system uses distilled water to pressurize the samples. The compression and decompression times, pressure, and temperature of the chamber throughout the processing were recorded. The compression times were 15, 26, and 45 s at 200, 300, and 400 MPa, respectively, and the decompression time was ≤8 s for the three HPP pressures tested. The initial temperature of the beer samples was 23˚C (pressure transmitting fluid was 24.6˚C) so that the temperature within the pressure chamber was never above 30ºC, ensuring a non-thermal HPP process. Once processed, the two pouches were immediately placed in ice water and refrigerated before enumeration of surviving *S. cerevisiae* ascospores. Two replicates for each HPP pressure-time conditions were carried out.

3.2.3. Thermosonication

The thermosonication experiments were conducted on the apparatus shown in Figure 3.1 at 50, 55, and 60˚C. A UP200S ultrasonic processor (Hielsher Ultrasound Technology Gmbh, Germany) was used to pass longitudinal mechanical vibrations with a frequency of 24 kHz, an amplitude of 125 μm, and an acoustic power density of 105 W/cm^2 through the sample via a 14 mm diameter sonotrode. A power of 161.6 W is calculated by multiplying the cross-sectional area of the sonotrode (1.539 cm^2) with the acoustic power density of the 14 mm probe (105 W/cm^2, according to the manufacturer's manual). The ultrasonic processor was set on continuous energy supply and no pulses were used. A water jacket was used to maintain the desired processing temperature inside the chamber. Before starting the TS of beer, the water bath was set to the desired temperature and circulated through the chamber prior to beer addition. This procedure minimized the temperature come-up time of the beer, which was negligible (≤5 s). The temperature measurements were recorded in the water inlet and outlet. The chamber has a maximum volume of 15 mL but only 10 mL of beer was used for each test. Thus, the acoustic energy density supplied to the beer sample was equal to 16.15 W/mL (161.6 W/10 mL of beer).

The system was designed to be used in continuous flow mode of beer through the processing chamber. However, in order to achieve higher residence time and microbial inactivation, batch operation was used by filling the chamber with beer and closing the beer inlet and outlet valves. The apparatus was sterilized by passing a solution of disinfectant Vircon^{TM} diluted in distilled water (1% w/v) through the chamber using a pump. After this, the system was purged with sterile water to remove any remaining disinfectant solution and emptied. The beer was added to the chamber for processing by removing the ultrasonic processor and pipetting the sample into the top of the chamber. The ultrasonic processor was then replaced and the sample treated for

the desired treatment time. The beer was removed from the chamber using a sterile pipette and kept refrigerated before enumeration of the surviving *S. cerevisiae* ascospores. Two repetitions of each processing time and temperature were carried out.

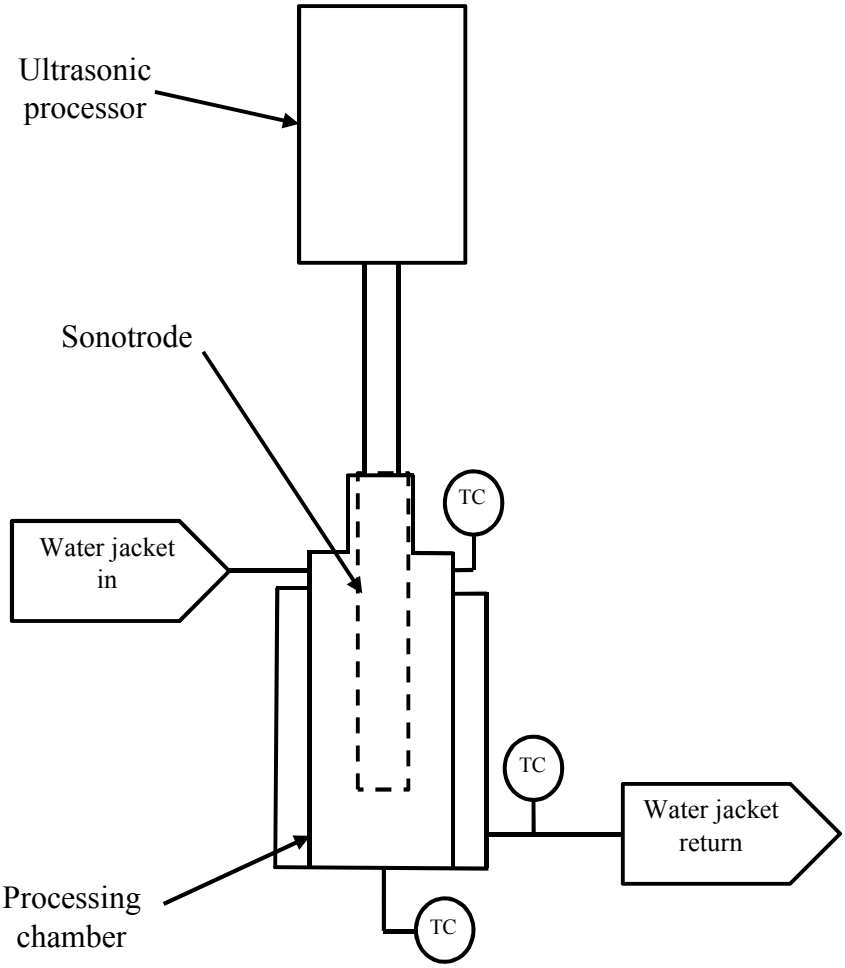


Figure 3.1. Schematic diagram of the power ultrasound machine set up in batch mode at the University of Auckland. TC refers to the thermocouples mounted on the machine.

3.2.4. Specific energy calculation for equivalent HPP, thermosonication, and thermal processes

The following procedure (Sulaiman, 2015) was used to estimate the energy requirements. Eq. 3.1 was used to determine the sensible heat to warm up the temperature of the beer before thermal, thermosonication and HPP processes:

$$Q = mc_p\Delta T \quad (3.1)$$

where Q is the heat energy needed to raise the beer temperature (J); m is the mass of the beer sample (kg); c_p is the beer specific heat capacity (4070 J/(kg.°C)); ΔT is the increase of beer temperature (°C) up to 60°C for thermal and thermosonication processing. The beer pasteurization occurs at final stages of beer production, after the beer fermentation, and therefore the beer fermentation temperature (14°C) was considered the initial beer temperature in the calculations of sensible heat. With respect to HPP, the sensible heat to raise the temperature to 23°C (the initial temperature of beer before HPP cycle) was also accounted.

Then, Equation 3.2 was used to estimate the compression work during the nonthermal (T≤36°C) HPP pressurization (Smith *et al.*, 2005; Rodriguez-Gonzalez *et al*., 2015):

$$W_{compression} = \frac{1}{2} \times \beta \times V \times P^2 \quad (3.2)$$

Where $W_{compression}$ is the compression work of incompressible fluid by high pressure (J); V is the volume of the HPP unit chamber (=0.002 m^3), P is the applied pressure (300×10^6 Pa), and β is the isothermal compressibility coefficient of water (1/Pa).

With respect to thermosonication, first Eq. 3.1 was used to estimate the heat required to warm up 10 mL of beer before ultrasound processing. Next, the ultrasound power of 161.6 W (mentioned in section 3.2.3) was multiplied by the TS treatment processing time. Then the total energy was divided by 10 mL to obtain the specific energy in J/L.

3.2.5. Modelling

Table curve 2D version 5.01 software (Systat Inc., USA) was used to find an appropriate model for the HPP and thermosonication survival curves. The software calculated the parameters of models as well as performance indices. The mean square error (MSE) and adjusted coefficient of determination (Adj R^2) were used to compare how well a model fitted the data. Low MSE values and values of Adj R^2 close to unity indicate a good level of fit. The log survivors were non linear and three models were attempted.

3.2.5.1. Weibull model

Weibull model is given by Eq. 3.3:

$$log\frac{N}{N_o} = -bt^n \quad (3.3)$$

where N is the concentration of surviving ascospores (CFU/mL) after processing time t (min). N_0 is the initial concentration of ascospores (CFU/mL); b and n are rate and shape parameters, respectively. When n=1, the model becomes the first order kinetics. A shape factor a shape factor less than 1 gives upwardly concave survival curves, while n>1 gives downwardly concave survival curves. The Weibull model, unlike first-order kinetics, does not assume that the whole microbial population have an equal time independent probability of inactivation.

3.2.5.2. Log-logistic model

The log-logistic equation has been suggested for the modelling of nonlinear survival curves by Chen (2007) and Cole *et al*. (1993) and is given by Eq. 3.4 as follows:

$$log\frac{N}{N_0} = \frac{A}{1+e^{4\sigma(\tau-logt)/A}} - \frac{A}{1+e^{4\sigma(\tau+6)/A}} \quad (3.4)$$

where σ is the maximum rate of inactivation (log (CFU/mL)/log min), τ is the log time to the maximum rate of inactivation (log min) and A is the difference between the upper and lower asymptote of the survival curve (log CFU/mL) (Chen and Hoover, 2003).

3.2.5.3. Modified Gompertz model

Bermúdez-Aguirre and Barbosa-Cánovas (2012), Chen and Hoover (2003), and Gil *et al.* (2006) have suggested the modified Gompertz equation for modelling microbial survival curves. The modified Gompertz equation is given by Eq. 3.5 as follows:

$$log\frac{N}{N_0} = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}} \quad (3.5)$$

Where C is the difference between the upper and lower asymptote of the survival curve (log CFU/mL), M is the time at which the death rate is at its highest (min) and B is the death rate (log (CFU/mL)/min) at time M (Xiong, 1999).

3.3. Results and discussion

3.3.1. Modelling the HPP inactivation of S. cerevisiae ascospores

HPP tests were carried out at 200 MPa with processing times (holding times) up to 1 h, 300 MPa with times up to 5 min and 400 MPa with times up to 30 s (Figure 3.2). This difference in the range of processing times was needed in order to model the inactivation at 200, 300 and 400 MPa and meant that the HPP pressure had a huge effect in the spore inactivation Spore reduction of ≥2.5 logs were obtained after 30 min, 27 s, and 12 s for 200, 300, and 400 MPa, respectively. Inactivation might occur during the compression phase of the HPP cycle, which could affect the initial shape of the survival curve, especially at 400 MPa. The log survivor as a

function of time data collected was clearly nonlinear. The quality of adjustments of Weibull, log-logistic and modified Gompertz models can be compared through the Adj R^2 and MSE (Table 3.1). The Weibull model fitted well the HPP inactivation of *S. cerevisiae* ascospores at different pressures as confirmed by Adj R^2 values, which ranged between 0.975 and 0.999, and MSE was between 0.010 and 0.030 (Table 3.2). The log-logistic was also attempted and showed good performance indexes, but is a more complex model characterized by 3 parameters and thus Weibull was a better option. Table 3.2 also displays the estimated Weibull model parameters for each HPP pressure. The nonlinear nature of the HPP survival curves for *S. cerevisiae* ascospores suggest that a resistant subpopulation of ascospores exists, which causes the nonlinearities (Fig. 3.2). The shape factor *n* of the Weibull model is less than 1 for all pressures, confirming the upward concavity of the survival curves (Fig. 3.2). This feature of the survival curve shows that sensitive members of the populations are destroyed at a relatively fast rate leaving behind resistant survivors. The *n* parameter was approximately constant (0.32-0.36) for the three pressures. Cunha *et al*. (1998) suggested *n* should indicate the kinetic pattern of the model, be constant and independent of the HPP pressure. As expected, the scale factor *b*, increased with the HPP pressure from 0.78 at 200 MPa to 4.46 at 400 MPa, meaning that higher pressure causes a more rapid inactivation of ascospores (Table 3.2).

No modelling studies were found for the inactivation of yeast ascospores in beer by HPP. However, the Weibull model has previously proved to be useful for fitting the survival curves of various microbial spores inactivated by HPP (Evelyn &Silva 2015a; 2015b; 2016b; Evelyn et al. 2016). Mok *et al.* (2006) found a biphasic model for vegetative yeast inactivation in red wine, also suggesting two patterns of resistance to pressure. As opposed to our results with *S. cerevisiae* ascospores in beer, Parish (1998) and Zook *et al.* (1999) found that the effect of HPP on yeast ascospore inactivation in fruit juice followed first-order kinetics. For modelling the

inactivation kinetics, it is also possible to use N_0', the initial number of microorganisms after the compression phase of the HPP cycle. This approach will be explained later in Chapter 4 (Section 4.3.2.1).

Table 3.1. Performance of non-linear models used to describe HPP inactivation of *S. cerevisiae* ascospores in beer.*

Model	Pressure (MPa)	*Adj* R^2	MSE
Weibull	200	0.999	0.013
	300	0.999	0.010
	400	0.975	0.030
Log-logistic	200	0.988	0.015
	300	NA	NA
	400	0.976	0.038
Modified Gompertz	200	0.845	0.208
	300	0.991	0.059
	400	0.826	0.281

**Adj* R^2 and MSE are the adjusted coefficient of determination and Mean Square Error, respectively. NA- not applicable.

Table 3.2. Parameters of Weibull model used to describe HPP inactivation of *S. cerevisiae* ascospores in beer.*

Pressure (MPa)	*b*±SE	*n*±SE
200	0.78±0.06	0.34±0.02
300	3.30±0.07	0.32±0.02
400	4.46±0.09	0.36±0.03

* *b* and *n* are the scale and shape factors from the Weibull model (Equation 3.3), respectively. In addition, the residual plots were random.

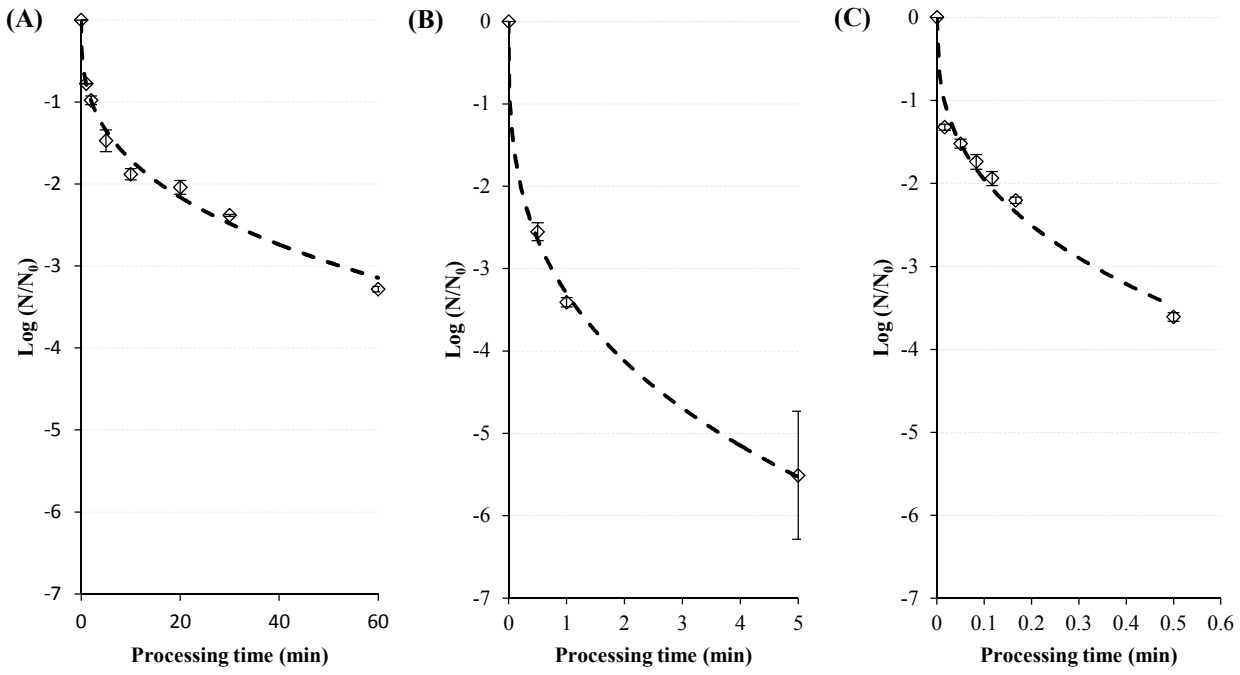


Figure 3.2. Weibull model fitted to ATCC 9080 *S. cerevisiae* ascospores survivors in beer after HPP processing at (A) 200 MPa, (B) 300 MPa and (C) 400 MPa (values are average of two processed samples and error bars are standard deviation).

3.3.2. Modelling the thermosonication inactivation of S. cerevisiae ascospores

Thermosonication experiments were carried out at 50, 55, and 60°C. Similar to HPP, the thermosonication *S. cerevisiae* ascospores survivors were strongly nonlinear (Fig. 3.3). A spore reduction of 2.5 logs was readily achieved after 2.5 min at 60°C TS, whereas 50 and 55°C TS required more than 40 min. The nonlinear nature of the inactivation kinetics with upward concavity suggests that the *S. cerevisiae* ascospores in the beer sample had a range of resistances to treatment. Like HPP, as processing continued, the rate of inactivation decreased, suggesting some ascospores developed resistance to the ultrasonication. The resistance of microorganisms to thermosonication is analogous to microorganisms' resistance to pressure. This could be due to the dormant state of the spores.

The Weibull, log-logistic and modified Gompertz models were fitted to the data and performance indices are shown in Table 3.3. The Weibull model presented good performance fittings ($0.942 \leq Adj\ R^2 \leq 0.986$; $0.009 \leq MSE \leq 0.055$) (Table 3.4) for the TS inactivation of *S. cerevisiae* ascospores in beer. Once again, the log-logistic model was suitable but a more complex model with 3 parameters and therefore Weibull was selected. Figure 3.3 shows the thermosonication survival curves for *S. cerevisiae* ascospores in beer at 50, 55 and 60°C fitted to the Weibull model and Table 3.4 presents the Weibull model parameters. Similar to HPP, the *n* value was approximately constant (0.34–0.37) and the *b* value increased with the TS temperature from 0.57 at 50°C to 1.81 at 60°C. Evelyn and Silva (2015d) also observed that the TS inactivation of *Clostridium perfringens* spores in beef slurry was not linear and described by the Weibull model. Regarding the inactivation of vegetative cells of *S. cerevisiae,* although Ciccolini *et al.* (1997) and Guerrero *et al.* (2001) have reported first-order kinetic, Bermudez-Aguirre and Barbosa-Canovas (2012) observed shoulders, which were modelled by modified Gompertz equation. The same authors found that a 7 log reduction of *S. cerevisiae* was achieved after 10 min at 60°C-TS with similar ultrasound conditions (24 kHz, 400 W, 120 μm), whereas in our study only a 4 log reduction was registered, confirming the higher resistance of *S. cerevisiae* in its ascosporic form.

Table 3.3. Performance of non-linear models used to describe thermosonication (16.16 W/mL) inactivation of ATCC 9080 *S. cerevisiae* ascospores in beer.*

Model	TS temperature (°C)	*Adj* R^2	MSE
Weibull	50	0.986	0.009
	55	0.942	0.048
	60	0.976	0.055
Log-logistic	50	0.987	0.010
	55	0.946	0.058
	60	N/A	N/A
Modified Gompertz	50	0.924	0.059
	55	0.791	0.223
	60	0.962	0.134

**Adj* R^2 and MSE are the adjusted coefficient of determination and Mean Square Error, respectively. NA- not applicable.

Table 3.4. Parameters of Weibull model used to describe thermosonication (16.16 W/mL) inactivation of ATCC 9080 *S. cerevisiae* ascospores in beer.*

TS temperature (°C)	*b*±SE	*n*±SE
50	0.57±0.05	0.34±0.03
55	0.58±0.12	0.37±0.06
60	1.81±0.04	0.36±0.04

**b* and *n* are the scale and shape factors from the Weibull model (Equation 3.3), respectively. In addition, the residual plots were random.

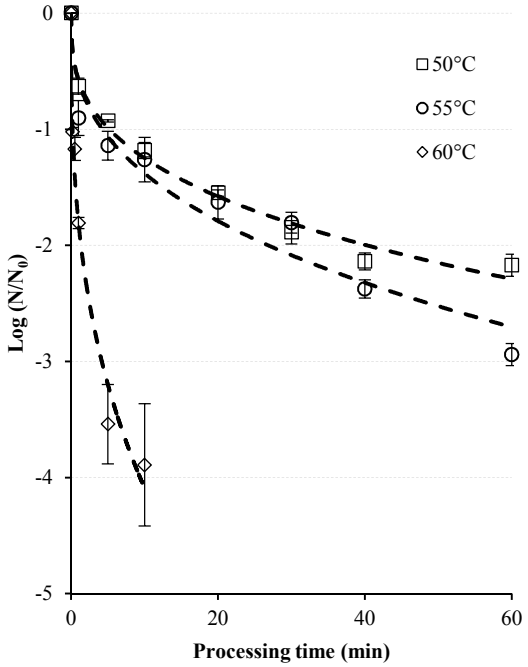


Figure 3.3. Weibull model fitted to ATCC 9080 *S. cerevisiae* ascospores survivors in beer after thermosonication at 16.16 W/mL (values are average of two processed samples and error bars are standard deviation).

3.3.3. Comparison of HPP, thermosonication, and thermal inactivation of ascospores

Figure 3.4 compares the first-order 60°C thermal inactivation of ATCC 9080 strain of *S. cerevisiae* ascospores taken from Milani *et al*. (2015a), the same strain used in this study, 60°C thermosonication and nonthermal HPP processing at 300 MPa. A 2.5 log reduction of ascospores was achieved after 15 min, 2.5 min, and 27 s processing of beer by thermal, TS, and HPP, respectively. Although no heating was used for HPP, a lower treatment time was required for the same log reduction of ascospores, which demonstrates that this technology is highly efficient for yeast spore inactivation and beer pasteurization. Referring to TS, although the heating of beer may cause negative effects on the beer quality, the ultrasound process can offer a reduction in the processing time from 15 to 2.5 min to achieve the same inactivation of

S. cerevisiae ascospores compared with thermal processing alone. For example, a 1.8 log reduction of ascospores in beer was obtained after only 1 min of thermosonication. In contrast, for thermal processing of beer, approximately 10 min were needed for the same log reduction. This reduction in processing time with thermosonication may offer potential advantages in the brewing industry and productivity gains.

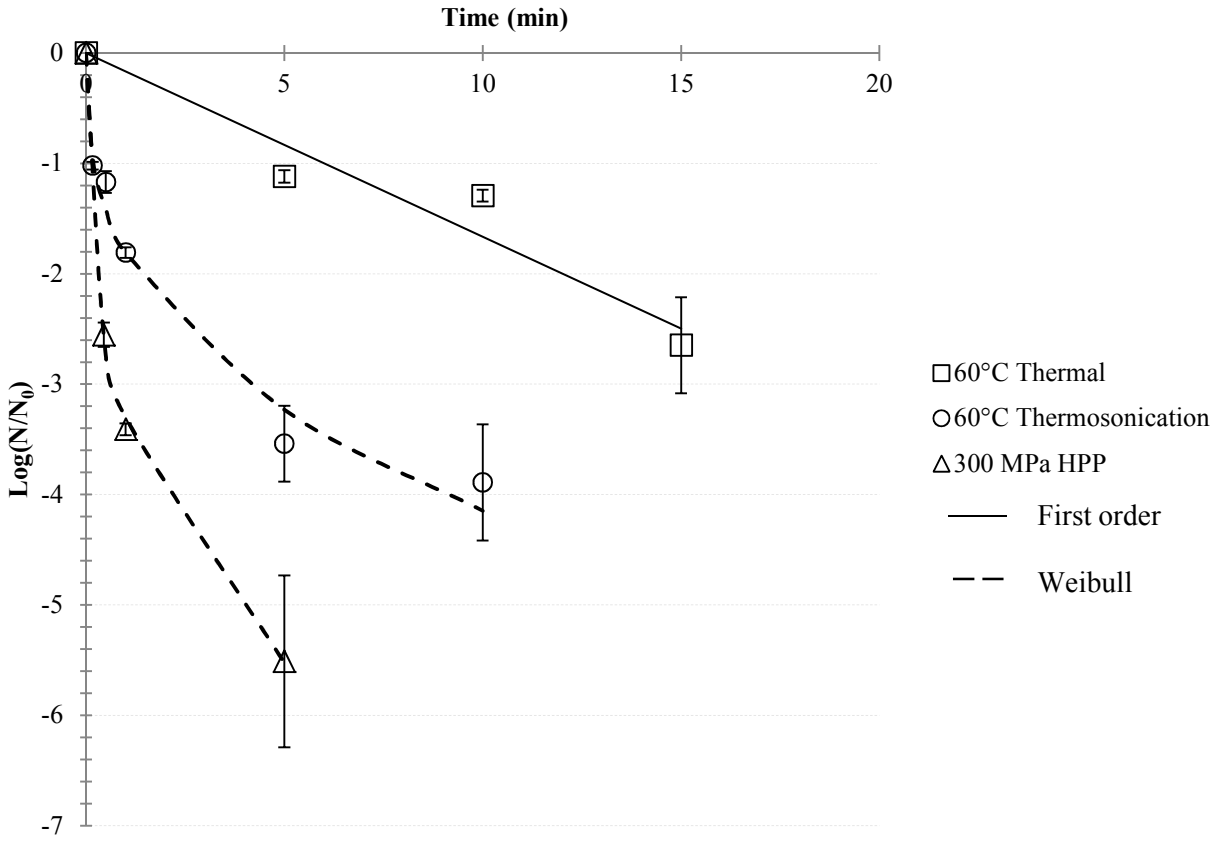


Figure 3.4. Nonthermal HPP at 300 MPa and 60°C-thermosonication (16.16 W/mL) compared with 60°C-thermal inactivation of ATCC 9080 *S. cerevisiae* ascospores in beer (thermal line data was taken from Chapter 2; values are average of two processed samples and error bars are standard deviation).

3.3.4. Specific energy requirements for equivalent pasteurization processes

Based on the minimum pasteurization of 15 PU or 15 min at 60°C established for commercial thermal processes, Milani *et al* (2015a) found that this achieved 2.5 log reductions in ATCC 9080 ascospores, the strain used in the current study. The following 15 PU pasteurization processes were selected for comparison in terms of specific energy requirements: thermal processing at 60°C for 15 min, nonthermal HPP at 300 MPa for 27.0 s (T≤30°C), and thermosonication at 60°C for 2.5 min. For the thermal and TS processes at 60°C, 188.8 kJ/L were required to heat up the beer to 60°C (Eq. 3.1). Then for TS the ultrasound power of 161.6 W (mentioned in Section 6.2.3) was multiplied by the processing time of 150 s to give 24,233 J, and divided by 10 mL, the volume of beer processed to give 2423.3 kJ/L. The final specific energy result for TS adds to 2612 kJ/L. With respect to HPP, first Eq. 3.1 was used to calculate a sensible heat of 36.9 kJ/L to raise the temperature to 23°C (the initial temperature of beer before HPP cycle), and then a compression work of 40.5 kJ/L during the HPP pressurization was calculated with Eq. 3.2 ($\beta_{32.9°C} \sim 4.5 \times 10^{-10}$ 1/Pa), giving a total of 77 kJ/L. The results indicate that lower energy is required for HPP (77 kJ/L) than thermal processing (189 kJ/L). The difference in the energy is much higher when comparing both processes to TS process (2612 kJ/L). Moreover, to achieve 4 log reductions a 10 min 60°C-TS process required much more energy (9885 kJ/L) than the energy estimated for a 5.5 log reduction by HPP (300 MPa, 5 min, 102.63 kJ/L). Most of the HPP energy is compression work and not much energy was spent to maintain the high pressure for a longer holding time. No other study has been carried out using yeast ascospore inactivation as the basis for comparing the energy requirements of different technologies. Sulaiman (2015) also estimated much higher energy needs for 15 min ultrasound at 33°C (1233 kJ/kg), compared to 65°C thermal for 15 min (291 kJ/kg) and HPP at 600 MPa, 48°C for 15 min (240 kJ/kg) of strawberry puree, all processes resulting in the same

polyphenoloxidase inactivation. Regarding apple juice processed by HPP, Jordan *et al.* (2001) estimated 483 kJ/kg for HPP processing at 500 MPa-42°C for 300 s and Bayındırlı *et al*. (2006) determined 338 kJ/kg for HPP at 350 MPa-40°C for 300 s. Sampedro *et al*. (2014) compared the energy consumption for pasteurization of orange juice by thermal (85°C, 5s) and HPP (550 MPa, 90s) processes using commercial size units and estimated higher energy consumption for thermal processing (38.1×10^3 kWh/year) in comparison to HPP (1.02×10^6 kWh/year).

3.4. Conclusions

The HPP and thermosonication processes generated accentuated nonlinear survival curves for *S. cerevisiae* ascospore inactivation in beer, which fitted a Weibull model. Both HPP and thermosonication are capable of achieving greater inactivation of *S. cerevisiae* ascospores in a shorter amount of time than traditional thermal processing, making them techniques that the brewing industry can consider as alternatives to thermal treatment. However, HPP processing appears to offer several potential advantages if implemented in the beer industry. First, HPP uses no heat during processing, which is likely to preserve the organoleptic properties of the beer. Moreover, nonthermal HPP requires less energy to achieve 15 PU in a shorter time, compared with TS and thermal processing at 60°C. This study can help industry and other researchers to design HPP and thermosonication processes for a targeted reduction in *S. cerevisiae* ascospores.

Chapter 4. Nonthermal pasteurization of beer by high pressure processing: Modelling the inactivation of *Saccharomyces cerevisiae* ascospores in different alcohol beers

Chapter Abstract

In this research, the nonthermal pasteurization of beer by high pressure processing (HPP) was carried out. First, the effect of alcohol content on *Saccharomyces cerevisiae* ascospore inactivation at 400 MPa was studied. The number of ascospores in 0.0, 4.8, and 7.0% alc/vol beers for 10 min processing time decreased by 3.1, 4.9, and ≥6.0 log, respectively. The Weibull model fitted the ascospore inactivation by HPP in 0.0, 4.8, and 7.0% alc/vol beers. At 400 MPa, 7.2 seconds could ensure the minimum pasteurization of beers and for 600 MPa 5 s were enough for ≥7 log reductions. The overall flavour of HPP *vs*. untreated beers was evaluated for a lager and an ale, with no significant differences between the untreated and HPP beers. Thus, nonthermal HPP is a feasible technology to pasteurize beer with different alcohol contents without heat.

Keywords: *alcoholic beverages; high hydrostatic pressure; sensory; yeast; spores; stability*

4.1. Introduction

Thermal pasteurization of beer, which is commonly used in the industry, alters the flavour of the beer. Pasteurization was re-defined by the United States Department of Agriculture as: "any process, treatment, or combination thereof, that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage" (NACMCF, 2006; Silva *et al*., 2014). This definition therefore includes nonthermal pasteurization processes such as high pressure processing (HPP), Pulsed Electric Fields (PEF), power ultrasound, dense phase CO_2, ultraviolet light irradiation and filtration, which have all been researched with beer (Fischer *et al*. 1999; Evrendilek *et al*. 2004; Levesley and Kennedy, 1999; Milani *et al.* 2015b; Walkling Ribeiro *et al.* 2011; Dagan and Balaban, 2006; Lu *et al.* 2010; Mezui & Swart, 2010) and are known to maintain better the beer flavor and nutrients. The main sensory concern in beer is the lightstruck character off-flavour, which can limit the beer shelf-life (Marsili *et al*., 2007; Milani *et al*., 2015b). HPP is a commercial technology already applied to preserve other foods/beverages and has clear advantages in terms of better retention of the beer body, nutritive components and overall beer properties. Filtration is another nonthermal pasteurization method currently used by breweries. Together with the undesirable microorganisms removed from the beer, other important components of the beer can also be retained in the micro size filters, affecting the beer final quality.

From our previous thermal results with ascospores of DSMZ 1848 *S. cerevisiae* (the most heat-resistant brewing yeast among the four strains investigated), because yeast ascospores are 25 to 350 times more heat-resistant than vegetative cells (Put *et al*., 1976; Put & De Jong, 1982).

As briefly mentioned in the previous Chapter, HPP is a nonthermal pasteurization technique where food is subjected to elevated pressures from 100 to 800 MPa to inactivate microbes or enzymes, depending on the food (Evelyn & Silva, 2015a; 2015b; Evelyn *et al*., 2016; Farkas & Hoover, 2000; Hoover *et al*., 1989; Ludwig *et al*., 1992; Silva *et al.*, 2012; Sulaiman *et al*., 2015). Generally, pressurization is carried out for a desired time in a confined space (pressure vessel) through a pressure transmitting medium, which is usually distilled water. Thus, this technology is also referred as high hydrostatic pressure (HHP) (Cheftel, 1995; Hogan *et al.*, 2005; Norton & Sun, 2008; Patterson *et al.*, 2007; Takahashi *et al*., 1993). HPP can damage the microbial cell membrane, which affects permeability and ion exchange, denature, and inactivate proteins including enzymes involved in microbial replication (Linton & Patterson, 2000; Patterson *et al.*, 2007). The pressure sensitivity of different organisms is highly variable, depending on the suspending medium, species, strain, size and shape, and processing conditions (Black *et al.*, 2007; Torres & Velazquez, 2009). Microbial vegetative cells from bacteria, yeasts, and molds are more sensitive to HPP than spores (Arroyo *et al*., 1997; Black *et al*., 2011; Brul *et al.,* 2000; Chauvin *et al*., 2005; Chauvin *et al*, 2006; Donsi *et al*., 2003; Mc Kay, 2009; Ogawa *et al*., 1990; Oxen & Knorr, 1993; Parish, 1998; Perrier-Cornet *et al*., 2005; Sokolowska *et al*., 2013b; Zook *et al*., 1999).

Although there are no studies on the inactivation of *S. cerevisiae* ascospores by HPP in beer, some authors studied the HPP yeast ascospore inactivation in juices (Ogawa *et al.*, 1990; Parish, 1998; Takahashi *et al*., 1997; Zook *et al.,* 1999; Mc Kay, 2009). The modelling of *S. cerevisiae* survivors in alcoholic beverages treated by HPP is limited to Mok *et al.* (2006), which observed a fractional conversion kinetic model for *S. cerevisiae* vegetative cells in red wine and total inactivation after 300 MPa for 20 min. Other studies conducted with nonalcoholic beverages and *S. cerevisiae* vegetative cells, linearity was observed (Butz & Ludwig, 1986; Donsi *et al.,* 2007;

Hashizume *et al.*, 1995). No modelling with yeast ascospores in beverages treated by HPP has been carried out.

The effect of alcohol content on yeast inactivation by HPP was not studied. Nevertheless, a few studies with nonthermal PEF revealed that the inactivation of *S. cerevisiae* ascospores in beers increased with the alcohol content (Milani *et al.*, 2015b; Walkling-Ribeiro *et al.*, 2011). The increase of thermal inactivation of yeast ascospores and other microorganisms with alcohol is well known (Milani *et al.*, 2015a; Splittstoesser *et al.*, 1986). Hence, the study of the effect of beer alcohol content on the HPP inactivation of yeast ascospores is also important. Beverage carbonation is another parameter that can affect yeast inactivation.

Buzrul *et al.* (2005) concluded the main attributes of the beer were not affected by HPP (200-350 MPa, 3-5 min at 20°C). Therefore, it is important to investigate whether HPP and other nonthermal technologies affect the beer flavour. In our study, the most resistant strain of *S. cerevisiae* DSMZ 1848 was chosen. This is commonly found in ale type of beers. Ascospores are more resistant to thermal processing than vegetative cells, so inactivation of the ascospores will ensure the inactivation of the corresponding less resistant vegetative cells. HPP was used to pasteurize beers with different alcohol contents and the main objectives were: (i) to study the effect of carbonation and alcohol content on the HPP inactivation of ascospores; (ii) to estimate the kinetic parameters of the HPP ascospore inactivation in 0.0, 4.8, 7.0% alc/vol beers under different pressures; (iii) to assess the sensory impact of HPP on beer; and (iv) to recommend HPP minimum pasteurization conditions for beer.

4.2. Material and methods

4.2.1 Microbiology

The strain used for this study was the most heat resistant strain among the 4 investigated in Chapter 2, DSMZ 1848. The production and enumeration of spores were described in the two previous Chapters. The concentration of ascospores was calculated and the result was expressed in colony forming units per millilitre of beer (cfu/mL). For each pressure-time processing condition, the mean±SD of two processed beer samples was calculated and plotted in the charts.

4.2.1.1. Beer inoculation

A portion of the ascospore stock suspension (ca. 8 mL) was centrifuged to remove the STD solution. Filter-sterilized beer was mixed with the spore pellet to yield a final ascospore concentration of ca. 10^6-10^7 cfu/mL. The clustering and the large size of ascospores did not allow the use of higher spore concentrations.

4.2.2. High Pressure Processing

The HPP unit used in this research was the 2 L-700 Laboratory Food Processing System (Avure Technologies, Columbus, Ohio, USA). The equipment consists of a 2-L cylindrical pressure treatment chamber, water circulation, a pumping system and the control system operated through a computer with software supplied by the manufacturer. Distilled water was used as the pressure transmitting medium in the chamber where the beer samples were placed. Two internal thermocouples were used to monitor the temperature in the distilled water contained in the pressure chamber, which was ≤36°C in all HPP treatments, to ensure a nonthermal process. Pressure come-up (=compression) times were 15, 26, 45 and 60 seconds for at min for 200, 300, 400, and 600 MPa, respectively, and depressurization took ≤ 5 seconds. The processing time

was the time during the constant pressure phase of the HPP cycle. Although the machine had the capacity of working with HPP pressure up to 600 MPa, the yeast ascospore inactivation is too rapid at this pressure (total inactivation after 5 seconds) to be able to model the inactivation. Therefore although for sensory we processed the beers at 600 MPa, for yeast inactivation experiments we have worked with HPP pressures between 200 and 400 MPa to be able to detect a gradual reduction on the microbial spore numbers with processing time, and investigate the effect of pressure and alcohol.

4.2.3. Yeast ascospore inactivation experiments

4.2.3.1. Beers preparation

To study the effect of carbonation on the HPP microbial inactivation, 4.0% alc/vol Export Gold (supplied by DB breweries) lager beer was used. The alcohol content was read from the bottle label, and is defined as the number of milliliters of pure ethanol present in 100 mL of beer at 20°C, (% v/v ethanol). It is also referred to as alcohol by volume (ABV) or alc/vol. Degassing of beer was carried out in a sterile container using a deaeration unit under vacuum overnight. Both degassed and commercial carbonated beers were filter-sterilized prior to inoculation with the spore pellet. To minimize the CO_2 loss during the filtration, the beer was removed from the can immediately after opening with a sterile syringe. The CO_2 was measured using the titration method and the CO_2 loss was negligible (4.4 g/kg) compared with the unfiltered beer (5.2 g/kg). The degassed filtered sterile beer had almost no CO_2 (< 0.5 g/kg).

For the survivor experiments in the modelling study, 0.0 and 4.8% alcohol lagers of the same brand were purchased from a local supermarket in Auckland, and the third beer, was prepared by adding pure ethanol to the alcohol-free beer to reach to 7.0% alc/vol content. All 3 beers had

the same composition except the level of alcohol, to be able to investigate the effect of alcohol on yeast spore inactivation.

Beer packaging procedure was the same in both experiments. Five mL of filter-sterilized beer samples containing the yeast spore were sealed in 5×5 cm 154 μm thick pouches that had been previously sterilized (Caspak, New Zealand). The plastic film was composed of linear low density polyethylene (LLDPE) and polyethylene therephthalate (PET). The pouches were then double-bagged with the same plastic film and the second bag was vacuum sealed to avoid bursting during the pressurization. Although in this study the beer was processed in batch operation mode, semicontinuous systems are generally used in the industry for pumpable liquids, which are subsequently aseptically packaged (Balasubramaniam *et al*., 2004). Due to the carbonation factor of beer, HPP packaging materials must be able to accommodate the volume expansion of carbon dioxide, which occurs during the high pressure processing. The packaging should not lose the seal integrity or barrier properties. For this reason, metal cans are not suited for HPP.

4.2.3.2. Experimental design and data analysis

For the effect of carbonation on the inactivation of *S. cerevisiae* ascospores, two replicates of carbonated and degassed inoculated beer samples were processed by HPP at 200 MPa for 10 minutes without heat (≤26.5°C). T-test was carried out to compare the carbonation effect of the degassed and normal beers using Statistica Software (Version 8, USA).

For studying the effect of alcohol on the inactivation of *S. cerevisiae* ascospores, bags containing beer samples were HPP treated at 400 MPa for 1 and 10 minutes. Four replicates

were carried out for each processing condition. One-way analysis of variance (ANOVA) followed by Tukey's test, with a confidence level of 95% (p<0.05) were used to compare the microbial log reductions for beers with different alcohol content and processed for different times. The average log reduction ± standard deviation (SD) were calculated and plotted.

Regarding the survival experiments and modelling, HPP was carried out at 200, 300, and 400 MPa in 0.0, 4.8, and 7.0% alc/vol beers for times up to 40 minutes depending on the experiment.. Two replicates were carried out for each pressure and processing time.

4.2.3.3. Estimation of the kinetic parameters of the HPP ascospore survivors

Linear first-order, biphasic, fractional conversion, and Weibull models were attempted to model the HPP ascospore inactivation in beer. Table curve 2D software (version 5.01, Systat software, USA) was used to fit the models to the microbial survivors and to perform all statistical analysis of data. Low mean square error (MSE) and adjusted coefficient of determination (Adj R^2) close to 1.00 indicated the quality of the adjustments. The Weibull model shown in Equation 3.3 was suitable (Mafart *et al.*, 2002; Peleg & Cole, 1998; Weibull, 1951).

4.2.4. Sensory experiments

4.2.4.1. Beer preparation and processing

For sensory tests, two commercial beers (one lager and one ale) were used. Both beers had an alcohol content of 4.0% alc/vol and were stored in the refrigerator until use. These beers were micro-filtered from the factory and not thermally pasteurized. The beers were transferred from the glass bottle into 30 mL plastic pouches and packed as explained in section 2.3.1, and then

HPP processed. The same plastic film pouches were used to pack beer for sensory experiments. HPP processing at 600 MPa for 30 seconds (constant pressure phase) was performed to ensure 1.34 log reductions in the DSMZ 1848 resistant spores, which is equivalent to 15 PU, the minimum pasteurization for beer (Milani *et al.,* 2015a).

4.2.4.2. Triangular and preference tests

Difference (triangular) and preference tests were carried out with 18 panellists to compare HPP with untreated beer. For each test, 30 mL beer samples were provided to the panellists. Participants were students and staffs of the university and the sensory tests were approved by The University of Auckland Human Participants Ethics Committee (Ethical approval number 012014). For the triangular tests, three beer samples coded with digit different numbers were presented to the panellists: two identical and one different. The panellists were asked to assess the overall flavour of the beer by tasting the sample from left to right and select the odd sample. The analysis of the triangular test was carried out by X^2 test using Statistica Software (Version 8, USA).

With respect to preference testing, for each beer type (one lager and one ale), the panellists were given 2 beer samples, the untreated and HPP-processed, and asked to rate the taste on a 9 point scale ranging from –4 (dislike extremely) to +4 (like extremely). T-test was carried out to compare the flavor of the untreated and HPP beers using Statistica Software (Version 8, USA).

4.3. Results and discussion

Preliminary experiments showed no growth (≥ 7 log reductions) of yeast spores in the plates after 5, 15, 20, and 30 seconds HPP processing at 600 MPa. Therefore, lower pressures were used for the spore inactivation experiments, to be able to study the effect of pressure and alcohol and model the inactivation.

4.3.1. Effect of carbonation and alcohol content on the HPP inactivation of ascospores

For a 10 min 200 MPa HPP process, the beer carbon dioxide had no significant effect on the inactivation of *S. cerevisiae* ascospores ($p>0.05$). Standard carbonated (dissolved CO_2 ~5.16 g/kg at 28°C) and degassed beers (≤0.5 g/kg at 28°C) presented 2.3±0.10 and 2.1±0.01 log reductions after 200 MPa and 10 min in 4.0% alc/vol beers, respectively. Walkling-Ribeiro *et al*. (2011) could not detect differences on the PEF inactivation of *S. cerevisiae* vegetative cells in fully carbonated and degassed beer.

Figure 4.1 presents the effect of alcohol content for 1 and 10 min processing times and 400 MPa pressure on the inactivation of *S. cerevisiae* ascospores. Higher inactivation was observed after 10 min processing compared to 1 min in the three alcohol content beers tested ($p<0.05$). After 10 min processing, ≥6 log reductions in *S. cerevisiae* ascospores were registered in 7.0% alc/vol beer, while the lowest inactivation of 1.7±0.05 log reduction was registered in 0.0% alc/vol after 1 min processing. Significantly higher log reduction in 4.8 and 7.0% alc/vol beers compared to 0.0% alc/vol was registered after 1 min ($p<0.05$). Although no difference was observed for 4.8 and 7.0% alc/vol beers for 1 min HPP, after 10 min the inactivation was greater and the differences between the inactivation were more significant.

Gaunzle *et al.* (2001) also studied on the effect of alcohol content on the inactivation of *Lactobacillus plantarum* in a model beer using 5 and 10% alcohol and found out that ethanol enhanced the effect of pressure on the inactivation rate of *L. plantarum.* Although no other HPP studies on the effect of alcohol were found, it is known from the literature that thermal inactivation of microorganism increases in higher alcohol content beverages. For example, the $D_{55°C}$-value of *S. cerevisiae* ascospores in alcohol-free beer was 34.2 min compared with 15.3 min in 7.0% alc/vol beer (Milani *et al.*, 2015a). Moreover, thermal $D_{60°C}$-value of *S. cerevisiae* ascospores decreased from 6.1 min in apple juice to 1.2 min in apple juice with 6% ethanol (Splittstoesser *et al.*, 1986). Mok *et al.* (2006) demonstrated total inactivation of *S. cerevisiae* vegetative cells in red wine (9% alc/vol) after 300 MPa for 20 min.

Since the studies of HPP inactivation of yeast ascospores in beer or other alcoholic beverages are rare, the results of this study with 0.0% alc/vol beer can be compared with results obtained with juices that are alcohol-free. Ogawa *et al.* (1990) and Parish (1998) studied the inactivation of *S. cerevisiae* ascospores in orange juice and found that ascospore numbers decreased by 6 log with HPP at 350 MPa pressure for 30 min at room temperature. McKay (2009) got 5 log reductions of *S. cerevisiae* ascospores in apple juice at 300 MPa. The inactivation of *S. cerevisiae* DSMZ 1848 ascospore in 0.0% alc/vol beer in our study was 4.4 and 3.5 log after 30 min HPP at 400 and 300 MPa, respectively.

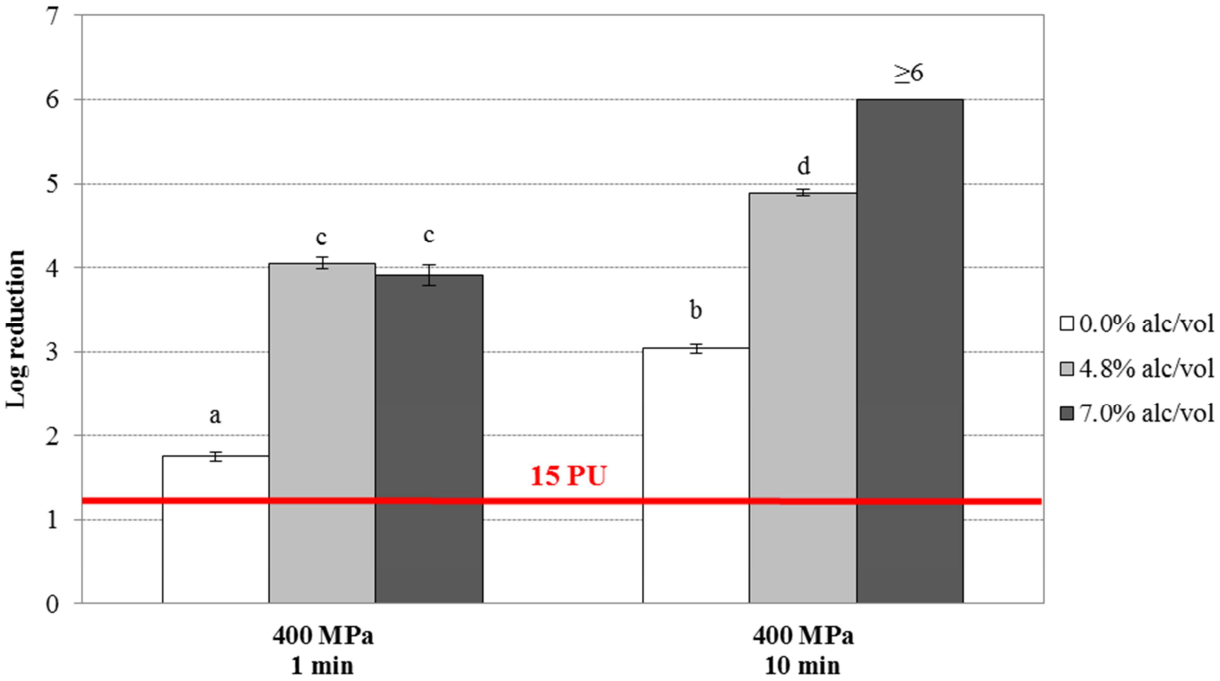


Figure 4.1. DSMZ 1848 *S. cerevisiae* ascospore log reduction after 400 MPa HPP for 1 and 10 min in different alcohol content beers (Error bars are standard deviation. Different letters indicate significantly different log reductions, $p<0.05$).

4.3.2. Weibull model parameters for HPP ascospore inactivation in different alcohol content beers

Figure 4.2 presents the survival curves of yeast ascospores in 0.0, 4.8, and 7.0% alc/vol beers. The survival curves displayed sharp reductions in the beginning of the HPP treatment, followed by a gradual slowing of the ascospore reduction rate. Given the nonlinearity of HPP survival curves, Weibull, biphasic, and fractional conversion models were attempted. Weibull or fractional conversion models were suitable for these data. The Weibull parameters which were estimated for 0.0, 4.8, and 7.0% alc/vol beers are shown on Table 4.1. The model goodness of fit was demonstrated by consistently lower MSE (≤0.13) and higher AdjR^2 (≥0.84). Furthermore, the residuals were random. The *b* value for 0.0, 4.8, and 7.0% alc/vol beers increased linearly from 0.596, 1.359, and 1.229 to 2.092, 3.731, and 3.963 with an increase in pressure from 200 to 400 MPa, respectively (Figure 4.3A, Table 4.1). This increase demonstrates *b* is pressure dependent in which the higher the pressure, the higher is the value of *b*, which translates in higher rate of microbial inactivation. The increase in the inactivation rate with pressure is also visible in Figure 4.2 for each beer tested. Moreover, the beer alcohol content also increases *b*, in particular for the higher pressures tested of 300 and 400 MPa (Figure 4.3B). The increase of alcohol from 0 to 4.8% increased *b*, but not so much effect on *b* was observed between 4.8 and 7% alc/vol.

The Weibull *n* shape factor was less than 1.0, indicating the concave upward shape of the curves observed in Figure 4.2, and seemed not to change a lot with pressure. This is expected as *n* is related with the kinetic order and should not be affected by external factors such as pressure.

This is in support with the results presented in Chapter 3 (Table 3.2) that were achieved for ATCC 9080 ascospores in 4.0% alc/vol beer. No modelling studies were found for the inactivation of yeast ascospores in beer by HPP. However, the Weibull model has previously

proved to be useful for fitting the survival curves of various microbial spores inactivated by HPP (Peleg, 2006; Evelyn & Silva, 2015a; 2015b; Evelyn *et al*., 2016). The results of our study demonstrated that the inactivation of *S. cerevisiae* ascospores by HPP is affected by pressure and is also dependent on the beer alcohol content. Mok *et al.* (2006) found a two-fraction model for ascospore inactivation in red wine. Other researchers such as Parish (1998) and Zook *et al.* (1999) found that the effect of HPP on yeast ascospore inactivation in fruit juice followed first-order kinetics, but this was not the case for the effect of HPP on ascospore inactivation in carbonated or alcoholic beverages. Hashizume *et al*. (1995) also observed first-order kinetics for yeast spore inactivation in different broths (NaCl, sucrose, trisodium citrate, and glycerol).

Table 4.1. Weibull model parameters for DSMZ 1848 *S. cerevisiae* ascospore inactivation by high pressure processing in 0.0, 4.8, and 7.0 % alc/vol beers.*

	0.0% alc/vol beer		4.8% alc/vol beer		7.0% alc/vol beer	
Weibull parameter	*b*±*SE*	*n*±*SE*	*b*±*SE*	*n*±*SE*	*b*±*SE*	*n*±*SE*
200 MPa	0.596±0.090	0.318±0.049	1.359±0.124	0.175±0.032	1.229±0.137	0.251±0.040
300 MPa	1.094±0.117	0.314±0.040	2.219±0.149	0.235±0.024	2.406±0.070	0.137±0.018
400 MPa	2.092±0.098	0.203±0.020	3.731±0.096	0.119±0.016	3.963±0.202	0.173±0.032

**b* and *n* are the scale and shape factors from the Weibull model (Equation 3.3), respectively. *SE* is the standard error of the estimated parameters. Mean Square Errors (MSE) of 0.052-0.13 and Adj R^2 of 0.84-0.95 obtained were indication of good model performance. In addition, the residual plots were random.

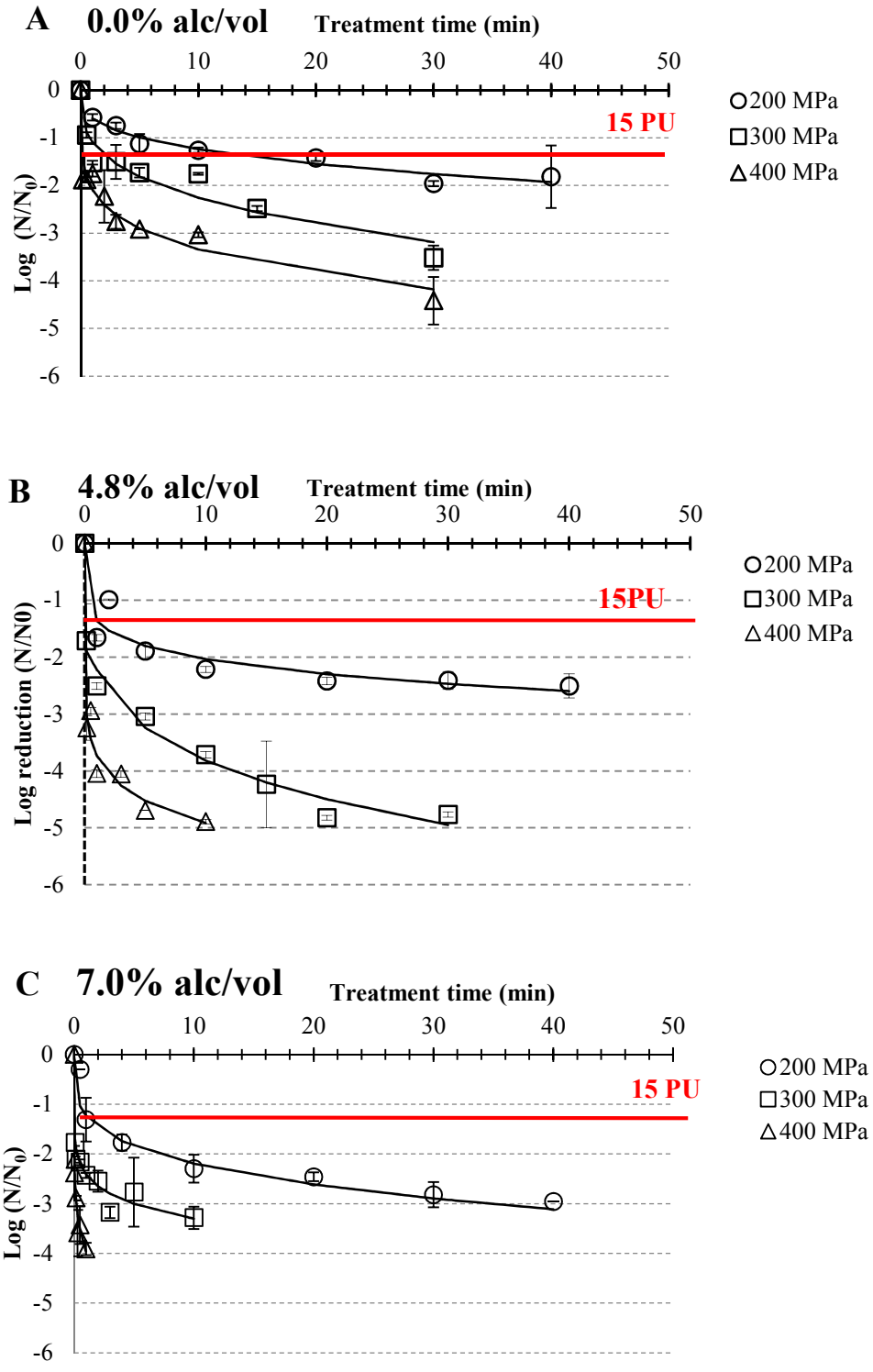
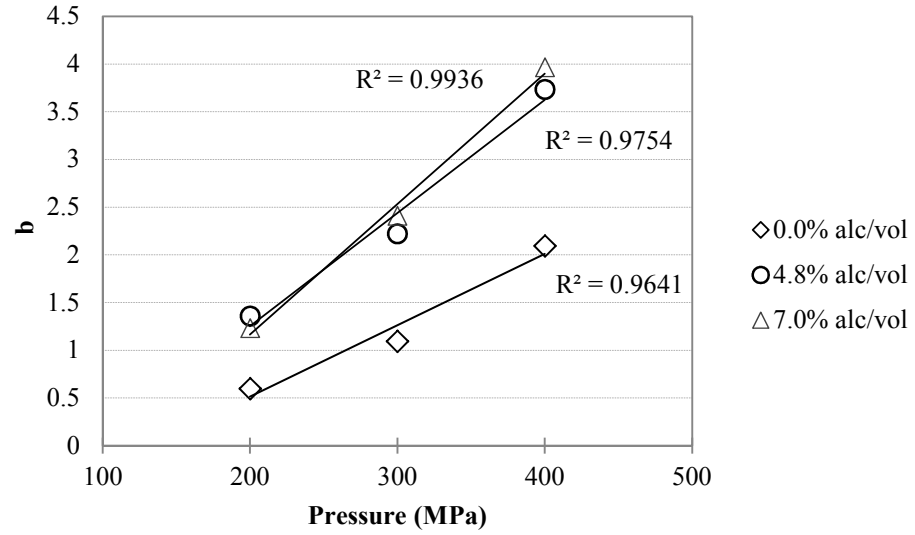


Figure 4.2. Weibull model fitting to DSMZ 1848 *S. cerevisiae* ascospore inactivation by HPP in 0.0 (A), 4.8 (B), and 7.0% alc/vol beers (C). (Error bars are standard deviation).

A



B

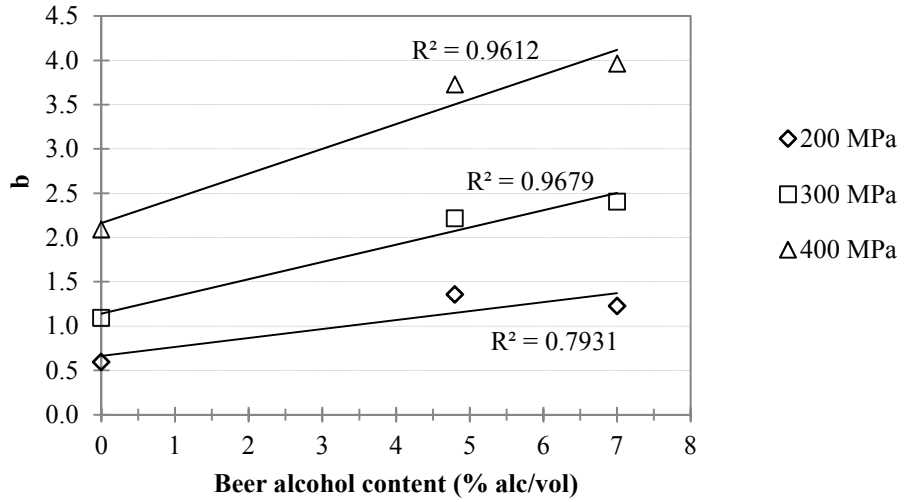


Figure 4.3. The proportional relation between and the Weibull model scale parameter *b* and the HPP pressure (A), and beer alcohol content (B).

4.3.2.1. Modelling the HPP inactivation of ascospores using N'_0 after compression as opposed to N_0 before compression

Inactivation of spores can occur during the compression phase of the cycle, therefore affecting the initial number of ascospores. Thus, in this subsection the modelling was revisited assuming N'_0, the initial number of ascospores right after the compression and before constant pressure phase of the cycle. One example was presented for 400 MPa, since maximum inactivation during compression is obtained at the maximum pressure tested.

Figure 4.4 presents the survivor curve of 4.8% alc/vol beer at 400 MPa using N'_0. The log reductions of the ascospores still exhibit nonlinearity, and Weibull Model fitted again the inactivation of ascospores, presenting *Adj* R^2 of 0.892 and MSE 0.090. However, compared with Table 4.1 (b=3.731), the new b value of 1.685 was smaller indicating lower inactivation rate. Moreover, the new n value is 0.246 is lower than 1.0, confirming the nonlinearity with the upward concavity for the inactivation of spores by HPP.

Although the estimated Weibull parameters are different, it is better to use N_0 (initiaol number of microbes in untreated food) since this approach will result in a more realistic prediction of the final microbial number N after HPP processing, reproducing better the manufacturing industry.

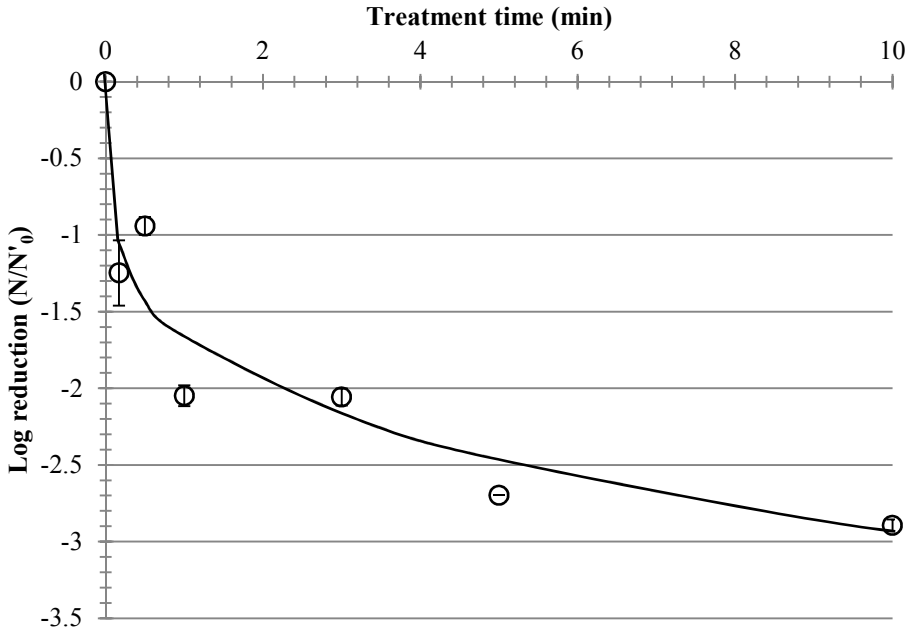


Figure 4.4. Weibull model fitting to DSMZ 1848 *S. cerevisiae* ascospore inactivation by HPP at 400 MPa in 4.8% alc/vol beer using N'_0 after compression (error bars are standard deviation).

4.3.3. Sensory assessment of HPP processed beers

The triangle test was carried out with one ale and one lager beers. In both cases no significant differences were registered between the overall taste of the untreated and HPP-treated beers. Likewise, preference testing confirmed no preference between the untreated and HPP-treated beers.

Mok *et al.* (2006) reported no differences in the aroma, taste, mouth-feel, and overall sensory quality between red wine treated with HPP at 350 MPa for 10 min and untreated red wine. Their results confirmed that HPP pasteurized the wine without affecting its sensory quality. Other sensory studies on nonalcoholic beverages had similar results (Baxter *et al.*, 2005; Deliza *et al.*,

2005; Laboissiere *et al.*, 2007; Oey *et al.*, 2008), thus demonstrating the benefit of HPP technology to pasteurize beverages while retaining the sensory quality.

4.3.4. Recommendation of minimum HPP pasteurization conditions for beer

The minimum thermal pasteurization applied by breweries is 15 PU (15 min at 60°C, z-value = 7.0°C) (Del Vecchio *et al*., 1951; Portno, 1968). As previously mentioned, 1.34 log reduction of DSMZ 1848 *S. cerevisiae* ascospores will deliver the required 15 PU pasteurization for beer (Milani *et al.,* 2015a). Therefore, from the Weibull model predictions, HPP processing at 300 MPa for 120, 7.2, and 1.0 seconds could provide the minimum pasteurization for 0.0, 4.8, and 7.0% alc/vol beers, respectively. At 400 MPa 7.2 seconds processing times are enough and can be easily applied commercially with high throughput yields. The models used for estimating minimum HPP processing times for different HPP pressures account for a possible mix of HPP-sensitive and -resistant populations of spores, which can be responsible for the nonlinearity observed. Furthermore, based on the sensory results, beer taste was not affected by HPP even at 600 MPa and 30 s processing time, which is more intense than the minimum pasteurization required. In addition, a study with another yeast strain estimated energy requirements for 15 PU beer pasteurization by HPP and thermal processing and concluded HPP is more energy efficient, requiring 77 kJ/L compared to 189 kJ/L for conventional thermal treatment (Milani *et al.*, 2016; Chapter 3).

4.4. Conclusion

The results of this study showed that a 5 seconds HPP process at 600 MPa resulted in ≥ 7 log reduction in the yeast ascospores, demonstrating the efficiency of HPP technology for beer pasteurization. The extent of inactivation of *S. cerevisiae* ascospores by HPP is related to alcohol content, with ≥6.0 log for 7.0% alc/vol, 4.9 log for 4.8% alc/vol, and 3.1 log for 0.0% alc/vol beers after 10 min process at 400 MPa. With respect to modelling, ascospore survival curves are nonlinear with nonthermal HPP treatments, and Weibull was better than biphasic or fractional conversion models to predict the inactivation of *S. cerevisiae* ascospores in beer. For 400 MPa, a processing time of 7.2 seconds or longer will ensure the required 15 PU for beer pasteurization. In addition, triangular and preference taste assessments revealed no significant difference between the HPP and untreated beer (for an ale and a lager), which demonstrates that nonthermal HPP technology is a suitable option for beer pasteurization. The results of this study are helpful for designing appropriate nonthermal HPP conditions to pasteurize beers with different alcohol contents.

Chapter 5. Ultrasound pasteurization of beers with different alcohol levels: Modelling the inactivation kinetics of *Saccharomyces cerevisiae* ascospores

Chapter Abstract

The industrial production of beer ends with a process of pasteurization. This study investigated the ultrasound assisted thermal pasteurization of beer or thermosonication (TS), aiming the inactivation of *Saccharomyces cerevisiae* ascospores, the most resistant form of the yeast. The efficacy of 30 s TS in batch and continuous operation mode at 60, 65, and 70°C was studied. After that the ascospore inactivation in beers was modelled and TS pasteurization conditions recommended. Lastly, the inactivation of *S. cerevisiae* ascospores in beer by TS *vs*. thermal processing at 55°C was compared. Ultrasound alone and continuous TS operation were not enough for *S. cerevisiae* spore inactivation. The TS survival curves were fitted with a Weibull model. TS at 50°C-1.9 min and TS at 55°C-26 s were enough for pasteurization, as opposed to 55°C-38 min thermal process. The results are helpful for designing appropriate thermosonication conditions to pasteurize beer with different alcohol contents.

Keywords: *beer; thermosonication; batch; continuous; yeast; spore; pasteurization; Saccharomyces cerevisiae*

5.1. Introduction

Yeasts as a key role of beer fermentation yields to by-products of the flavour of beer, which are advantageous to the quality of beer such as higher alcohols, organic acids, and esters, while others create undesirable off-flavours like diacetyl and other carbonyls, sulphur compounds (Deak & Beuchat, 1996). Increasing consumer demand for beverages with better nutritional and sensorial qualities has prompted research on novel nonthermal pasteurization alternatives such as power ultrasound, high-pressure processing (HPP), Pulsed Electric Fields (PEF), dense phase CO_2, and ultraviolet light irradiation (Dagan & Balaban, 2006; Fischer *et al*., 1999; Franchi *et al*., 2011; Mezui & Swart, 2010; Milani *et al*., 2015b).

Villamiel and De Jong (2000) found out that continuous-flow ultrasonic treatment could be a promising technique in the food industry. Power ultrasound (also known as high intensity ultrasound) operates at low frequencies, typically 20–100 kiloHertz (kHz), with a sound intensity ranging from 10 to 1000 W/cm^2 (Feng & Yang, 2011). The high energy level available in power ultrasound makes it suitable for use in the food industry for microbial inactivation (Feng & Yang, 2011; Weiss *et al*., 2011). Most power ultrasound applications are performed in a liquid medium and can be referred to as sonication or ultrasonication. The ultimate reason for microbial inactivation via ultrasound is believed to be the mechanical damage caused by cavitation. Application of high-frequency sound waves to liquids at sufficiently high intensities leads to mechanical and chemical effects. The inactivation mechanism of ultrasound can be explained through the effect of cavitation on microbial cell walls (Raso *et al*., 1998). The water jets of liquid generated by the asymmetric implosion of transient cavitating bubbles may cause severe cell envelope damage and cleavage of the polymeric materials of the cell walls. In addition, stable cavitating bubbles can generate micro streaming alongside the bubble and create high hydrodynamic shear stresses, which cause cell membrane damage and lead to the

inactivation of bacteria, moulds, and yeasts (Álvarez *et al*., 2000; Condón *et al*., 2004; Feng & Yang, 2011; Piyasena *et al*., 2003). Room temperature power ultrasound treatment generally results in low microbial and enzyme inactivation especially at low acoustic power densities (Char *et al*., 2010; Evelyn & Silva, 2015a; Bhardwaj *et al*., 2002; Sulaiman *et al*., 2015). However, when sonication is conducted with heat (thermosonication, TS), the microbial destruction rate is greatly improved (Ciccolini *et al*., 1997; Earnshaw *et al*., 1995; Evelyn & Silva, 2015d; Evelyn *et al*., 2016; Guerrero *et al*., 2001). Because of the increase in lethality of heat treatments when these are combined with ultrasonication, TS may offer the potential to substantially reduce the intensity of conventional heat treatments to achieve food safety, whilst improving the quality of foods preserved by traditional heat processes (Feng & Yang, 2011; Sala *et al*., 1995).

In general, vegetative cells are regarded as sensitive to power ultrasound, while spores are resistant and can be inactivated only through the use of combined treatments, like ultrasound+heat (TS) or ultrasound+pressure (mano-sonication) (Bevilacqua *et al*., 2014; Butz & Tauscher, 2002; Chemat & Khan, 2011). Although many studies have been carried out on the inactivation of vegetative yeasts (Ciccolini *et al*., 1997; Bevilacqua *et al*., 2014; Adekunte *et al*., 2010a; Bermúdez-Aguirre & Barbosa-Cánovas, 2012; Limaye & Coakley; 1998; Oyane *et al*., 2009; Tsukamoto *et al*., 2004), no investigation on the inactivation of yeast ascospores by power ultrasound in beers or other beverages has been published.

Ultrasound technology is currently used in the beer industry for improving the beer yield at the beginning of the mashing process, during fermentation to speed up the process by 36–50%, and for defogging the beer before bottling (Chemat & Khan, 2011; Bermúdez-Aguirre *et al*., 2011; D'Amico *et al*., 2006; Knorr *et al*., 2004). However, the application of this technology for beer pasteurization is an open area for research.

5. Ultrasound pasteurization of beers with different alcohol levels

The patterns of ultrasound inactivation kinetics observed with different microorganisms are quite variable. Generally, logarithmic inactivation of yeasts in juice follows biphasic behaviour presenting two inactivation rates (Gabriel, 2014). However, yeast inactivation may also be described by a Weibull model, four-parameter models, and modified Gompertz equation (Bermúdez-Aguirre & Barbosa-Cánovas, 2012; Adekunte *et al*., 2010b). First-order kinetics was used by Ciccolini *et al.* (1997) and Lopez-Malo *et al.* (2005) to describe *S. cerevisiae* cell inactivation by thermosonication.

Although some modelling works of *S. cerevisiae* vegetative cells survivors after ultrasound treatment have been published, the inactivation of *S. cerevisiae* ascospores has never been investigated. Therefore, the main objectives of this research were: (i) to investigate the ultrasound inactivation of *S. cerevisiae* ascospores at room temperature in 0.0, 4.8, 7.0% alc/vol beers; (ii) to compare TS spore inactivation by continuous and batch modes of operation; (iii) to estimate the Weibull model parameters for the TS inactivation of ascospores in 0, 4.8 and 7% alc/vol beers; (iv) to recommend ultrasound pasteurization conditions for beers with different alcohol contents; (v) to compare the spore inactivation by TS and thermal processing at 55°C; (vi) to assess the taste of TS treated beer with a sensory panel .

5.2. Material and methods

5.2.1. Microbiology

The strain used for this study was the most heat resistant strain among the 4 investigated in Chapter 2, DSMZ 1848. The production and enumeration of spores were described in previous Chapters. The concentration of ascospores was calculated and the result was expressed in colony forming units per millilitre of beer (cfu/mL). For each TS-time processing condition, the mean±SD of two processed beer samples was calculated and plotted in the charts.

5.2.1.1. Beer inoculation

For the continuous operation mode experiment, a portion of the ascospore stock suspension (ca 100 mL) was inoculated in approximately 3 L of filter-sterilized beer to yield a spore concentration of ~10^6 cfu/ml. For the batch mode experiments, 10-15 mL of beer was processed for each replicate at the same spore concentration of ~10^6 cfu/ml. The inoculated beer was well stirred and mixed before processing.

5.2.2. Ultrasound processing of beer

5.2.2.1. Set up of ultrasound in batch and continuous mode

A UP200S ultrasonic processor by Hielscher (Hielscher-Ultrasonic Gmbh, Germany) was used in this study. The processor generates longitudinal mechanical vibrations through electrical excitation with high frequency (24 kHz). All the inactivation experiments were done using a standard sonotrode with a 14 mm diameter tip at maximum amplitude. A power of 161.6 W is calculated by multiplying the cross-sectional area of the sonotrode (1.539 cm^2) with the acoustic

power density of the 14 mm probe (105 W/cm^2, according to the manufacturer's manual). The sonotrode was fixed in the Hielscher's stainless steel D14K temperature-controlled 15 mL flow vessel, which was tightly closed using 2 rubber O-rings (Figure 5.1). The closed vessel avoided beer splash, degas, and evaporation of ethanol and other volatile components during the sonication process. In both batch and continuous processing, the beer was contained in a closed vessel which seemed to prevent CO_2 loss during the sonication process (beer $CO_2 \sim 4.0$ g/kg).

For continuous operation of TS experiments, the flow chamber was connected to the beer inlet and outlet pipes as shown in Figure 1. The inlet pipe was connected to the feeding tank and the beer containing the yeast spores was pumped through the chamber. Treated samples were collected from the outlet pipe. The residence time in the sonication chamber was 30 s for a flow rate of 0.53 mL/s. 10.8 W/mL of acoustic energy was supplied continuously to the beer (161.6 W/15 mL of beer) and the residence time was the treatment time.

For the TS batch experiments, the chamber was closed by two valves coupled to the beer inlet and outlet. The beer was injected into the top of chamber before inserting the sonotrode. A maximum of 10 mL of beer was added to the treatment chamber to avoid CO_2 running out because of the pressure inside the chamber during sonication. The acoustic energy density was 16.2 W/mL (161.6 W/10 mL of beer). The treatment times for batch experiments varied from 0.5 to 60 minutes, to be able to model the kinetic microbial changes.

For both batch and continuous modes, beer temperature was controlled by flowing a jacket of water continuously through the outer wall of the chamber (Figure 5.1). Before starting the TS of beer, the water bath was set to the desired temperature (between 23 and 70°C, depending on the experiment) and circulated through the chamber prior to beer addition. This procedure minimized the temperature come up time of the beer, which was negligible (≤5 s). The temperature measurements were recorded by a picoscope (Pico Technology, England), with

fibre optic temperature sensors which carry a fast response time (<1 ms). The temperature sensors were mounted in the beer inlet and outlet as well as the water outlet, and the temperatures were digitally recorded. As expected, the beer temperature in the continuous operation oscillated up and down close to the average process temperature. With respect to TS batch process, Figure 2 shows one example of beer (4.8% alc/vol) temperature history for a 10 min process min which resulted in an average temperature of 50.3±1.7°C. The use of a thermostatic water jacket minimized the increase of temperature during the TS processes.

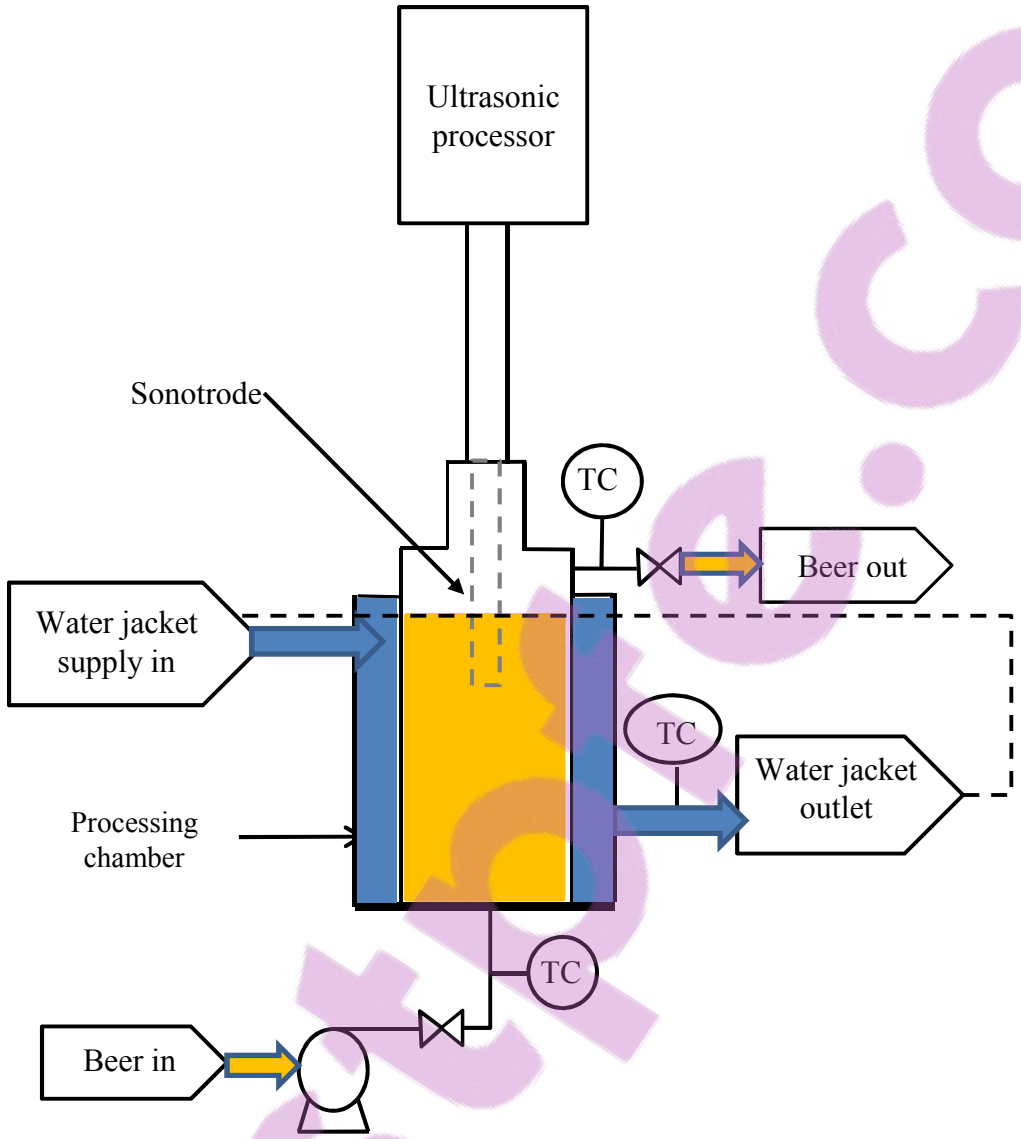


Figure 5.1. Scheme of continuous power ultrasound unit and cooling system set up at the University of Auckland. TC refers to the thermocouples mounted on the equipment.

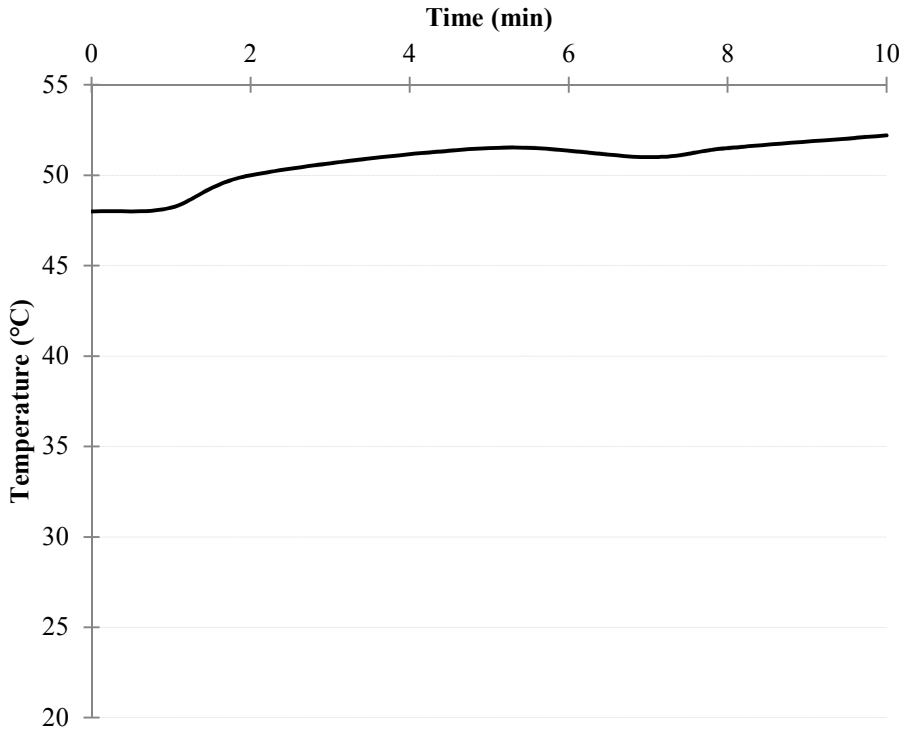


Figure 5.2. Example of 4.8% alc/vol beer temperature history during a 10 min thermosonication in batch mode (16.2 W/mL) which resulted in processing average temperature of 50.3±1.7°C.

5.2.2.2. Disinfection

Prior to running each experiment, the pipes of the ultrasound system were disinfected with Vircon^{TM} diluted in distilled water (1% w/v) and washed with 3 liters of sterilized distilled water. After each experiment, a detergent solution composed of 1% w/v caustic soda (NaOH) dissolved in distilled water at 65°C was circulated for 15 min. Then, the entire system was flushed with hot water.

5.2.3. Yeast ascospore inactivation experiments

5.2.3.1. Beer preparation

Commercial lager beers 0.0 and 4.8% alc/vol beers were purchased from a local supermarket in Auckland. Other beer containing 7.0% alc/vol was prepared by adding pure ethanol to the alcohol-free beer, so that the three beers had similar ingredients except the amount of ethanol. This range of alcohol content is representative of commercial beers and allowed us to study the effect of alcohol content on the spore inactivation rate. The alcohol content was read from the bottle label based on alcohol by volume (abbreviated as ABV or alc/vol). This is a standard measure of how much alcohol (ethanol) is contained in a given volume of an alcoholic beverage. It is expressed as a volume percent and defined as the number of milliliters of pure ethanol present in 100 mL of beer at 20°C, (% v/v ethanol). The amount of dissolved carbon dioxide in beer was ~ 5.2 g/kg using titration method. Prior to inoculation with spore solution all the beer samples were sterilized using a syringe filter with a pore size of 0.2 µm (Sartorius AG, Germany). The CO_2 of filtered beer was ~ 4.4 g/kg.

5.2.3.2. Experimental design

According to past results of Milani *et al*. (2015a), 15 PU are equivalent to 1.34 log reductions of *S. cerevisiae* DSMZ 1848 ascospores, the same strain used in the present study. First, the effect of room temperature ultrasound (≤23°C) at 125 µm amplitude equivalent to 16.2 W/mL (161.6 W, 10 mL of beer) on the inactivation of ascospores was studied using 0.0, 4.8, and 7.0% alc/vol beer. The inoculated beer samples containing the ascospores were processed up to 30 minutes in batch mode. Second, the effect of batch *vs.* continuous mode for 30 s TS on ascospore inactivation at different temperatures (60, 65, and 70°C) in 0.0 and 4.8% alc/vol was investigated. For survival experiments and modelling, each beer (0.0, 4.8, and 7% alc/vol) was

thermosonicated at 43, 50, and 55°C and processed up to 60 minutes at a maximum power of 16.2 W/mL in batch mode. Two replicates were carried out in these experiments for each TS temperature and processing time. At the completion of the sonication experiments, the collected samples were immediately transferred to an ice water bath (0–4°C) to avoid spore germination after treatment. The ascospores were enumerated using the method described previously.

5.2.3.3. Estimation of Weibull model parameters for HPP ascospore inactivation

The *S. cerevisiae* ascospore logarithmic reduction (log N/N_0) was calculated and plotted for each beer (0, 4.8, and 7% alc/vol) after ultrasound and thermosonication at 43, 50, and 55°C. Significant differences in the microbial log reductions among treatments and beers were investigated by one-way analysis of variance (ANOVA) followed by Tukey's test (Statistica 8.0, Statsoft, USA), with a confidence level of 95% ($p<0.05$).

Survival curves were tested using linear first-order, biphasic, fractional conversion, and Weibull models to find the model of best fit for the inactivation of ascospores. Table curve 2D software (version 5.01, Systat software, USA) was used to perform all statistical analysis of data. Least mean square error (MSE) and adjusted coefficient of regression (*Adj* R^2) close to 1.00 indicated the quality of the adjustments. The Weibull model was suitable to model the log survivors vs. time data in beer. The model is based on the assumption that cells in a population have different resistance and the resistance to a stress follows a Weibull distribution (Mafart *et al*., 2002; Weibull, 1951) and is presented on Equation 3.3.

5.2.4. Preliminary sensory assessment

5.2.4.1. Beer preparation and processing

Two commercial beers (one lager and one ale) were used for preliminary sensory assessment. Both beers had an alcohol content of 4.0% alc/vol and were stored in the refrigerator until use. These beers were filtered pasteurized from the factory and not thermally pasteurized. The beers were transferred from the glass bottle into the feeding tank. The beers were then pumped from the feeding tank to the cylinder in which the ultrasound machine was mounted. The thermosonication process was carried out at 0.73 mL/s flow rate, 24 kHz frequency, 10.8 W/mL power at 75°C for 20.5 s to ensure 1.34 log reductions in the DSMZ 1848 resistant spores, which is equivalent to 15 PU, the minimum pasteurization for beer (Milani et al., 2015a). The treated samples were then collected in to aseptic bottles.

5.2.4.2. Preference tests

Preference tests were carried out to compare the flavour of the TS treated beer and untreated beer. Tests were conducted with an ale and then repeated with a lager beer. The panellists were asked to rate the overall taste preference of the two beers (untreated and TS) by tasting the samples from left to right. The taste was rated on a 9 point scale ranging from –4 (dislike extremely) to +4 (like extremely). T-test was carried out to compare the flavour of the untreated and TS beers using Statistica Software (Version 8, USA).

5.3. Results and discussion

5.3.1. Room temperature ultrasound ascospore inactivation in beers

Beers with 0.0 and 7.0% alcohol content achieved the equivalent to 15 PU after 30 min of sonication at room temperature (≤23°C, 16.2 W/mL). However the same treatment did not deliver 15 PU (=1.34 log) in 4.8% alc/vol beer, the most common one (Figure 5.3). Char *et al.* (2010) were able to demonstrate only a 1 log reduction of *S. cerevisiae* vegetative cells in orange juice at 30-35°C sonication (20 KHz, 91.2 μm). Since room temperature ultrasound caused minor inactivation in the yeast ascospores, thermosonication was attempted and the results are shown in the following section.

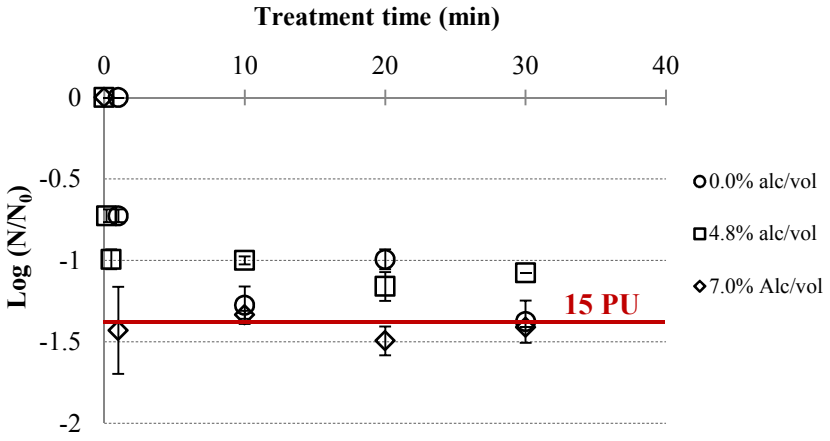


Figure 5.3. Inactivation of DSMZ 1848 *S. cerevisiae* ascospores by room temperature power ultrasound (16.2 W/mL) at 23°C in 0.0, 4.8, and 7.0% alc/vol beers (Error bars are standard deviation).

5.3.2. Yeast spore inactivation by continuous vs. batch thermosonication (TS) in beers 0.0 and 4.8 % alc/vol beers

Figure 5.4 presents the log reduction of *S. cerevisiae* ascospores in 0% and 4.8% alc/vol beers by batch and continuous thermosonication for 30 s. The inactivation of ascospore in both beers was significantly higher using the batch process than the continuous process ($p<0.05$). The maximum log reduction of batch thermosonication was 2.3 and 2.7 at 70°C for 0.0 and 4.8% alc/vol beers, respectively. However, only 0.2 and 1.0 log reduction in 0.0 and 4.8% alc/vol was obtained in continuous thermosonication. This could be due in part to the fact that less power (10.8 W/mL) was consumed in continuous operating mode compared with the batch operating mode (16.2 W/mL) for the same processing time. Thus, batch power ultrasound could achieve the minimum pasteurization of 15 PU (1.34 log reduction), but not the continuous mode. Likewise, D'Amico *et al.* (2006) reported lower TS inactivation of *Escherichia coli* in apple cider in continuous mode *vs* batch operating modes: 5.07 *vs*. 5.9 log reduction, respectively.

Increasing the TS temperature from 60 to 70°C had no significant effect on yeast inactivation after 30 s of continuous mode treatment. During batch processing, an increase in the temperature from 60 to 65°C did not affect the yeast inactivation, whereas an effect was observed when the temperature was increased from 65 to 70°C, especially for the 4.8% beer. The magnitude of the ascospore reduction increased by close to 1 log in the 4.8% beer when the temperature was increased from 65 to 70°C.

Since no studies of ultrasound inactivation of yeast ascospores in beer were found, the results of this study with 0.0% alc/vol beer can be compared with results obtained with other alcohol-free liquids. Guerrero *et al.* (2001) registered up to 5.0 log reduction of S. *cerevisiae* vegetative cells in Sabouraud broth at 55°C for 10 min (95.2 μm, 20 KHz). Bermudez-Aguirre and Barbosa-

Canovas (2012) got maximum reduction of 6.0 logs of yeast vegetative cells in grape juice after 10 min thermosonication at 60°C (24 KHz).

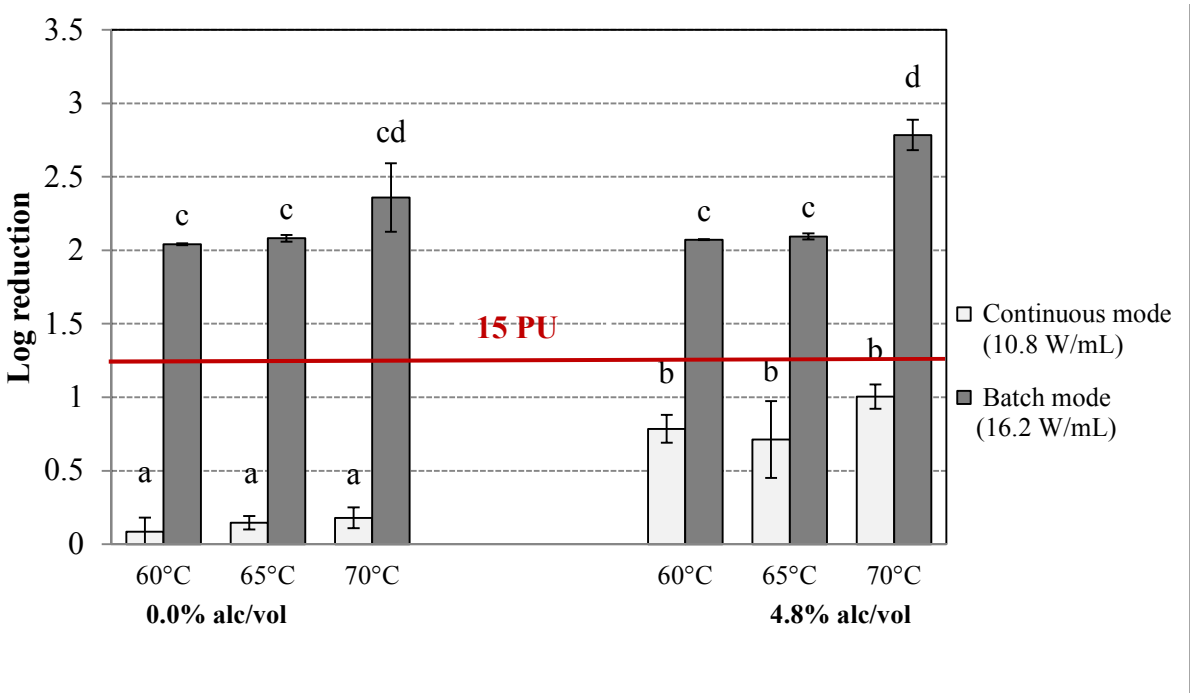


Figure 5.4. Thermosonication (TS) inactivation of DSMZ 1848 *S. cerevisiae* ascospore by continuous vs. batch operation for 30 seconds treatment (Error bars are standard deviation).

5.3.3. Estimation of the Weibull model parameters for S. cerevisiae ascospore inactivation after batch TS in 0, 4.8 and 7% alc/vol beers

Figure 5.5 shows the nonlinearity of the yeast ascospore survival curves of in 0.0, 4.8, and 7.0% alc/vol beers. Among the nonlinear models tested, Weibull was the best to describe the ascospore inactivation in beers with 0.0, 4.8, and 7% alc/vol (Table 5.1). The model goodness of fit was demonstrated by consistently lower MSE (≤0.191) and higher *Adj* R^2 (≥0.87) (Table 5.1). The *b* value increased with temperature from 0.645 to 2.659 in 0.0% beers, from 0.262 to 1.668 in 4.8% beers, and from 0.145 to 1.306 in 7.0% alc/vol beers. Log b increased linearly with

temperature as shown in Figure 6. The *n* values were all <1, confirming the concave upward curve of the modelling lines (Figure 5.6). The *n* values showed minimal variation with the temperature (Table 5.1). This is in support with the results presented in Chapter 3 (Table 3.4) for another strain in 4.0% alc/vol beer.

Evelyn and Silva (2015b) also observed that the TS inactivation of *Clostridium perfringens* spores in beef slurry was not linear and described by the Weibull model. The model was also very useful in fitting survival curves of *S. cerevisiae* ascospores after nonthermal HPP of beer (Milani & Silva, 2015). Although some researchers such as Ciccolini *et al.* (1997) and Guerrero *et al.* (2001) have reported that *S. cerevisiae* inactivation follows a first-order kinetic model, others like Adekunte *et al.* (2010b) and Bermudez-Aguirre and Barbosa-Canovas (2012) reported different nonlinear models for microbial inactivation such as Weibull, four-parameter model and modified Gompertz model.

The log reduction of ascospores in 0.0, 4.8, and 7.0% alc/vol beers seemed to be similar regardless of the alcohol content. The ascospore inactivation after 20 min thermosonication at 43°C was 1.9, 1.2, and 1.1 logs in 0.0, 4.8, and 7.0% alc/vol beers, respectively. At 50°C, the corresponding ascospore inactivation was 2.1, 2.8, and 2.9 logs. Lastly, at 55°C, ascospore inactivation was 3.6, 3.2, and 3.6 logs, respectively. As opposed to PEF (Milani *et al.,* 2015b; Chapter 6) and HPP (Chapters 3 and 4), the alcohol content in the beers seems to have less effect on the inactivation rate of *S. cerevisiae* ascospores by TS at the same treatment temperature and time.

Table 5.1. Weibull model parameters estimation for DSMZ 1848 *S. cerevisiae* ascospore inactivation by thermosonication (16.2 W/mL) processing in 0.0, 4.8, and 7.0% alc/vol beers.*

Temperature (°C)	0% alc/vol beer		4.8% alc/vol beer		7.0% alc/vol beer	
	b±*SE*	*n*±*SE*	*b*±*SE*	*n*±*SE*	*b*±*SE*	*n*±*SE*
43	0.645±0.075	0.361±0.033	0.262±0.065	0.589±0.069	0.145±0.056	0.663±0.010
50	0.918±0.117	0.345±0.042	1.093±0.106	0.322±0.037	0.713±0.089	0.417±0.040
55	2.659±0.096	0.111±0.018	1.668±0.121	0.263±0.016	1.306±0.176	0.341±0.047

**b* is a rate parameter and *n* is the shape factor from the Weibull model (Equation 5.1). Mean Square Errors (MSE) of 0.037-0.191 and *Adj* R^2 of 0.87-0.95 are indication of good model performance. In addition, the residual plots were random. The processing temperature is the average temperature during the treatment.

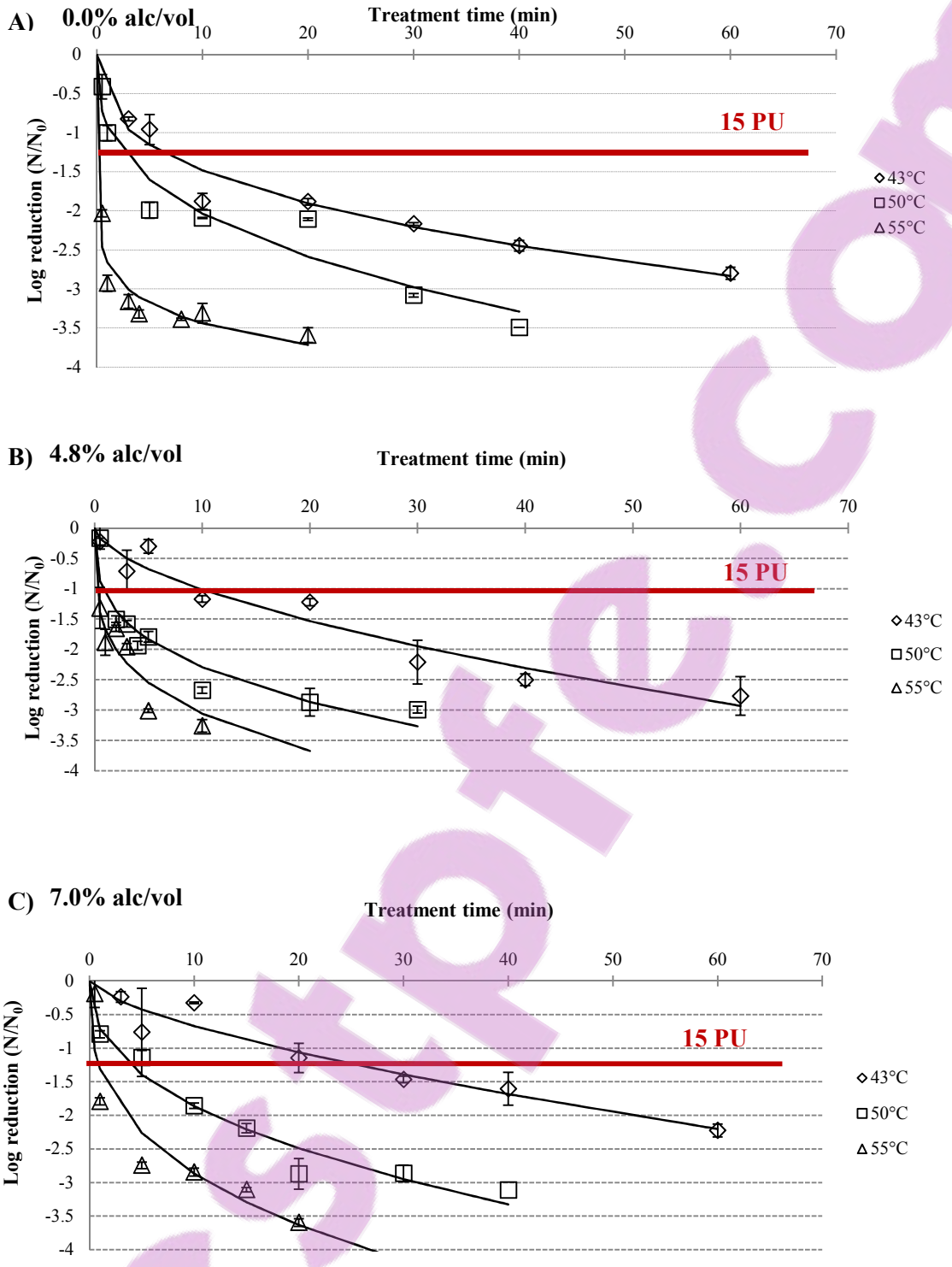


Figure 5.5. Weibull model fitting to DSMZ 1848 *S. cerevisiae* ascospore inactivation by TS (16.2 W/mL) in 0.0, 4.8, and 7.0% alc/vol beers (Error bars are standard deviation).

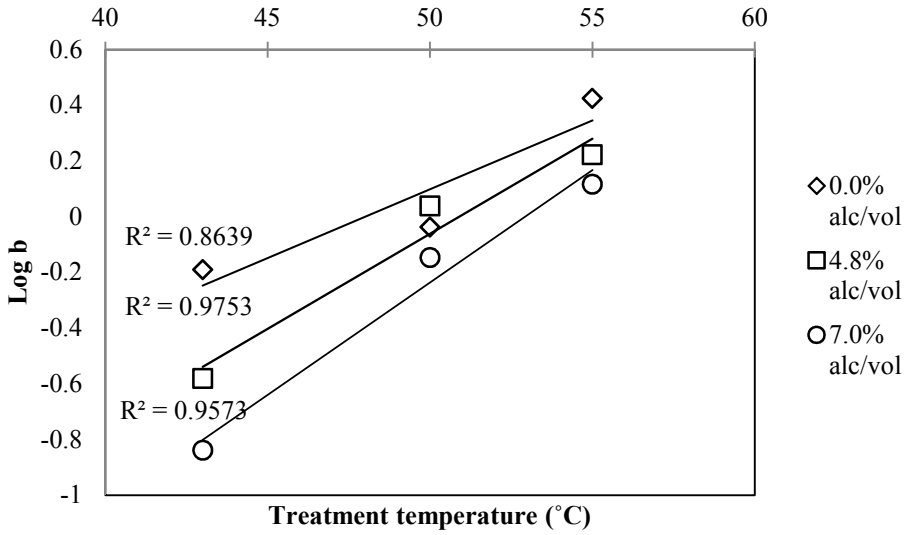


Figure 5.6. The proportional relation between TS (16.2 W/mL) temperature and the log of *b*, the Weibull model scale factor.

5.3.4. Recommendation of TS minimum pasteurization conditions for different alcohol beers

Since the common 15 PU thermal pasteurization applied by breweries (15 min at 60°C) results in 1.34 log reductions of DSMZ 1848 *S. cerevisiae* ascospores (Milani *et al*., 2015a), we can predict TS conditions (from the Weibull model) which deliver this level of pasteurization. Our data indicate that the minimum pasteurization time for TS at 50°C is 3.0, 1.9, and 4.5 min for beer with 0.0, 4.8, and 7.0% alc/vol, respectively. Regardless of the level of alcohol content, beers can be pasteurized using thermal-assisted power ultrasound within the range of 50-55°C.

5.3.5. TS vs. thermal processing at 55°C to inactivate S. cerevisiae ascospores in beer

Figure 5.7 presents the log reductions of *S. cerevisiae* ascospores by thermosonication (24 kHz, 10.8 W/mL) *vs.* conventional thermal processing at 55°C. The thermal inactivation line at 55°C was taken from our previous study with the same strain of *S. cerevisiae* ascospores (Milani *et*

al., 2015a, Chapter 2). The comparison revealed that TS processing of beer could deliver the minimum pasteurization of 15 PU (1.34 log reduction) after 26.4 s, while 37.7 min was required for achieving the same pasteurization with beer thermal processing alone. For example, the log reduction of ascospores in beer after 10 min of thermosonication was 3.2 logs while only 0.6 log reduction was registered for thermal processed beer. Evelyn and Silva (2015a) also recorded similar findings for inactivation of *B. cereus* spores in beef slurry, rice porridge, and cheese slurry. That study showed a more marked reduction in spore inactivation for 1.5 min TS at 70°C (24 kHz, 0.33 W/mL or W/g) *vs*. 70°C thermal inactivation: 4.2 *vs*. 0.7 log in beef slurry, 4.1 *vs*. 0.6 log in rice porridge, and 3.2 *vs*. 0.8 log in cheese slurry (Evelyn & Silva, 2015a). Another study of Evelyn and Silva (2016) determined a rate of *Alicyclobacillus acidoterrestris* spore inactivation six times higher for 78°C TS compared to 78°C thermal processing alone.

5.3.6. Preliminary taste assessment of TS beer

It was found that both ale and lager beers developed a haze with the TS process. TS beers presented less preference than untreated beers. Optimization of processing conditions that minimize the haze formation and impact on taste are recommended.

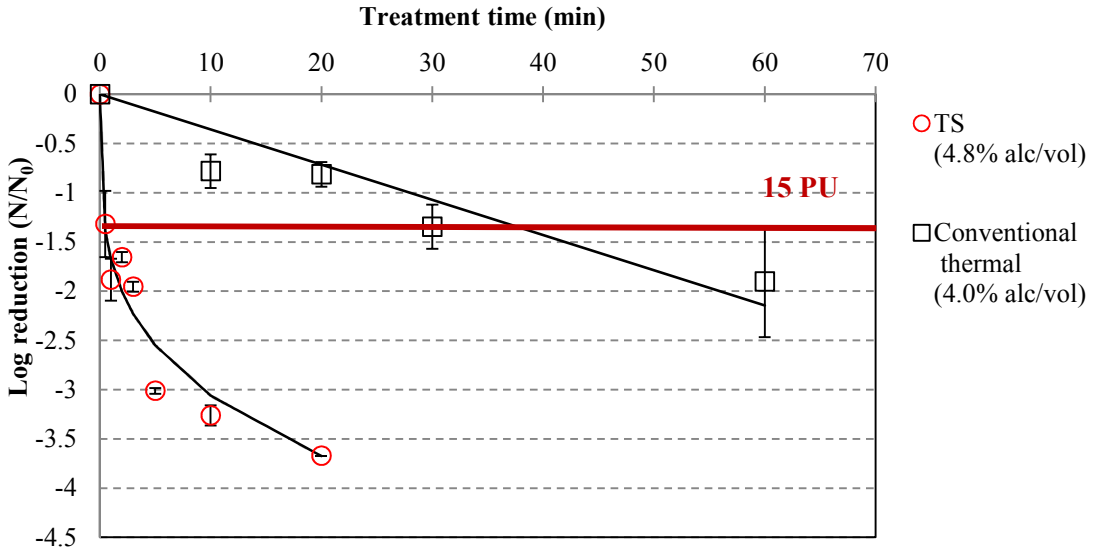


Figure 5.7. Thermal *vs*. TS inactivation of DSMZ 1848 *S. cerevisiae* ascospores at 55°C in 4.0 and 4.8% alc/vol beers (the thermal line was taken from previous results shown in Chapter 2 (Error bars are standard deviation).

5.4. Conclusion

The results of this study revealed that TS (43, 50, and 55°C) achieved higher *S. cerevisiae* yeast ascospore inactivation in beer compared with room temperature ultrasound, with a maximum of approximately 3.7 log reductions after 55°C and 20 min treatment regardless of the level of alcohol content. The survivorship patterns in beer were nonlinear, with the Weibull model being a better fit for the inactivation of *S. cerevisiae* ascospores than biphasic or fractional conversion models. Results of this study are helpful for designing appropriate conditions to pasteurize beers by thermosonication. TS at 50°C for 3.0, 1.9, and 4.5 min could deliver the minimum pasteurization of beer with 0.0, 4.8, and 7.0% alc/vol, respectively. However, the preliminary sensory assessments revealed that thermosonicated beer created haze in beer appearance and the processing conditions have to be optimized to avoid the haze formation. Moreover, a clear advantage in terms of microbial inactivation was obtained with TS compared with thermal processing alone.

Chapter 6. Pulsed Electric Field continuous pasteurization of different types of beers

Elham A. Milani, Sally Alkhafaji, Filipa V.M. Silva. 2015. Pulsed Electric Field continuous pasteurization of different types of beers. Food Control 50: 223–229.

Chapter Abstract

In this chapter, beer was processed using Pulsed Electric Field (PEF), a continuous preservation technology that has the potential to be implemented at a commercial scale by the brewing industry. The main goal of this work was to investigate the feasibility of PEF for yeast inactivation and its impact on beer sensory.

First, the effect of a PEF process (temperature below 43°C, 45 kV/cm electrical field intensity, 46 pulses, 70 μs) on *Saccharomyces cerevisiae* ascospores inactivation in nine different beers comprising ale, lager, dark, low alcohol, and no alcohol, was investigated. Log reductions of 0.2 and 2.2 were registered for 0.0 and 7.0% alc/vol beers, respectively, which indicates that the alcohol content is the major beer constituent driving the microbial inactivation. Then, 0.0, 4.0, and 7.0% alc/vol beers containing *S. cerevisiae* ascospores were submitted to PEF combined with thermal processing up to 53°C. An increase in the PEF treatment temperature from 43 to 53˚C caused at least an additional 0.7, 2.1 and 1.8 log reductions in the yeast spore population for 0.0%, 4.0%, and 7% alc/vol beers, respectively. Results of another experiment carried out with 4.0 and 7.0% alc/vol beers, showed the huge advantage of using PEF compared with thermal processing. Additionally, the lightstruck attribute sensory tests revealed six (aroma) and three (flavour) PEF beers did not develop the lightstruck character, being acceptable in terms of sensory. The results of this study can be helpful for designing appropriate PEF conditions to pasteurize beers with different alcohol contents.

Keywords: beer, alcohol, PEF, Saccharomyces cerevisiae, ascospore

6.1. Introduction

Increasing consumer demand for beverages with better nutritional and sensorial qualities has prompted research on novel nonthermal pasteurization alternatives such as Pulsed Electric Fields (PEF), High Pressure Processing (HPP), dense phase CO_2, and ultraviolet light irradiation (Dagan & Balaban, 2006; Fischer *et al.,* 2010). In this work, PEF was used to pasteurize different types of beers. Depending on PEF equipment, semi-solid or liquid foods can be processed. Zhang, Barbosa-Cánovas, and Swanson (1995) investigated the engineering aspects of PEF pasteurization and Barbosa-Canovas *et al.* (1999) studied food preservation by PEF. In PEF, the food contained in the treatment chamber, between two electrodes, is exposed to high voltage electric short pulses, which causes significant microbicidal effects (Ho & Mittal, 1996). Doevenspeck (1960) and Sale and Hamilton (1967) were the pioneers of Pulsed Electric Field technology. Since then, scientists such as Zimmermann *et al*. (1974) and Zimmermann (1986) have investigated the mechanisms of irreversible microbial electroporation by PEF. Using PEF technology, enzymes, pathogenic and spoilage microorganisms can be inactivated with minimum impact on the food colour, flavour, nutrients and overall quality. The electric field inactivates the microorganisms in foods by inducing a transmembrane potential in the cell membrane, which results in electroporation (the permeabilization of the cell and organelles membranes) and subsequent cell death (Heinz *et al.,* 2001; MacGregor, Farish *et al.,* 2000). Since the PEF pasteurization technique can be used in a continuous mode, it has the potential to be implemented in the beverages industries at a commercial scale to preserve drinks. The rate of microbial inactivation is dependent on the medium conductivity, which is hard to work with when liquid foods with higher electrical conductivities are used. This is because they generate smaller electric field peaks across the treatment chamber. Furthermore, foods with lower electrical conductivities are recommended, since they dissipate less energy, not affecting the

food temperature, and keeping PEF a nonthermal treatment (Alvarez *et al*., 2006). Additionally, the presence of gas bubbles in the beer causes the electric field magnitude to decrease significantly near the boundary of the bubbles, thus threatening the uniformity of the PEF treatment across the chamber (Alkhafaji & Farid, 2010).

Hülsheger *et al.* (1983) and Grahl and Märkl (1996) studied the effect of electric fields on yeast and bacteria cells. They found that the cell size and growth phase of the microorganism have a great effect on the microbial inactivation. Cells in the exponential growth phase and yeasts (which have a larger shape than bacteria) have higher inactivation rates. Knorr, *et al*. (1994) reported that *Bacillus cereus* spores and ascospores are more resistant to electric field pulses than vegetative cells. However, Raso & Heinz (2006) have been studying the effect of PEF on mould spores and conidiospores in fruit juices and concluded that they are very sensitive to PEF, being a few number of pulses with moderate electric field intensity enough for their inactivation.

Although, some works on PEF inactivation of vegetative *Saccharomyces* cells in beers have been published, the PEF inactivation of *Saccharomyces cerevisiae* ascospores, the most resistant microbial form of the yeast, has not been investigated in beer. Therefore, the main objectives of this research were: (i) To determine the Pulsed Electric Fields (PEF) inactivation of *Saccharomyces cerevisiae* ascospores in nine different commercial beers; (ii) To study the inactivation of *S. cerevisiae* ascospores by heat-assisted PEF in beers of three different alcohol concentrations; (iii) To compare PEF with conventional thermal pasteurization in terms of *S. cerevisiae* ascospore inactivation; and (iv)To assess the development of the undesirable lightstruck attribute in nine commercial beers processed by PEF.

6.2. Material and methods

6.2.1. Microbiology

DSMZ 1848 was used in this study and the method for production and enumeration of ascospores was previously described in other Chapters.

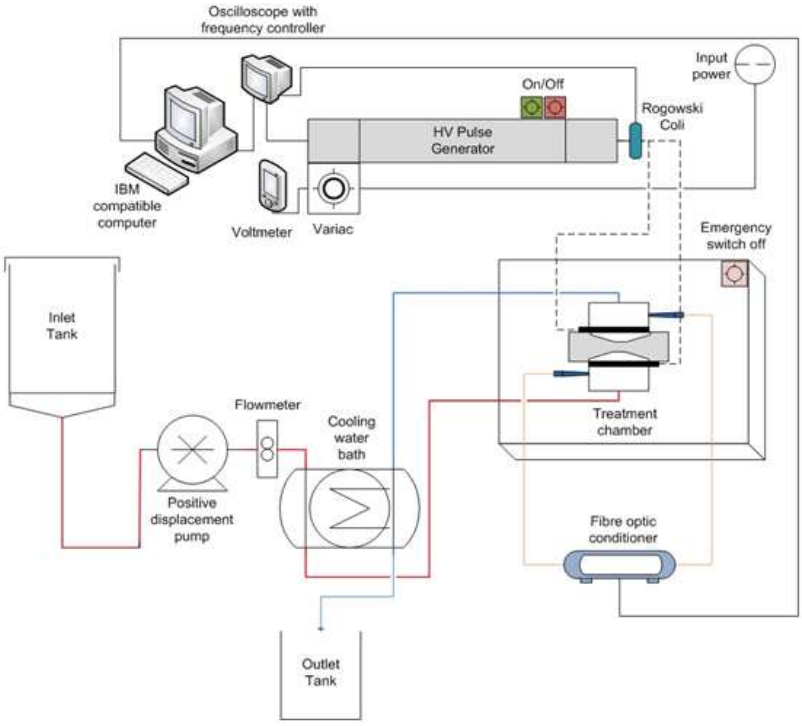
6.2.2. PEF components and disinfection

A Pulsed Electric Field (PEF) unit (Fig. 6.1a) was designed and constructed in the University of Auckland by (Alkhafaji &Farid, 2007). The main equipment consists of the high voltage pulse generator, and the treatment chamber. Depending on the application, this PEF system has the capability of using multiple treatment chambers for more effective treatment (Alkhafaji & Farid, 2010). The system was constructed to involve a high-voltage pulse generator and a treatment chamber, data collection and fluid managing system, voltage and current tools, degassing and cooling system. The setup of the unit creates high efficacy regarding microbial inactivation as well as energy saving (Alkhafaji & Farid, 2007). The pulse generator made by H. F. Power Ltd. (Auckland, New Zealand) can present high voltage up to 30 kV and square bipolar pulses with a pulse width of 1.5 μs and frequencies up to 1 kHz. The treatment chamber was made of a stable synthetic fluoropolymer of tetrafluoroethylene (polytetrafluoro ethylene) commercially recognized as Teflon. This structure can withstand high temperatures (up to 260°C) without being deformed. The treatment chamber was assembled in a vertical position to achieve accurate control of the fluid residence time and temperature distribution and hence, product local conductivity (which changes with temperature). The treatment chamber was designed to include two mesh electrodes (made of 316 food grade stainless steel) and an insulation part that shaped a narrow treatment field and assured a higher electric density area (Fig. 6.1b). The diameter of

each electrode was 50 mm and the distance between the two electrodes was 15 mm. The treatment zone depth and diameter were 5 and 8 mm, respectively. The total volume of the treatment chamber was 251.2 mm^3 and the residence time was 0.058 seconds. The system was equipped with a water bath with cooling capacity from Grant Instruments Ltd (Cambridge) that could be used to decrease or increase the beer inlet/outlet temperatures. Fibre optic temperature sensors (FISO Technologies, Canada) that carry a fast response time (<1 Ms), were connected to a fibre optic conditioner (UMO signal conditioner) which converted the signals to engineering units.

Prior to running each experiment, the pipes of PEF system were sterilised with Vircon™ diluted in distilled water (1% w/v) and washed with 6 litres of sterilized distilled water. After each PEF experiment, the detergent solution composed of 1% w/v caustic soda (NaOH) dissolved in distilled water at 65°C was circulated for 15 min. Then, the entire system was washed with hot water.

(A)



(B)

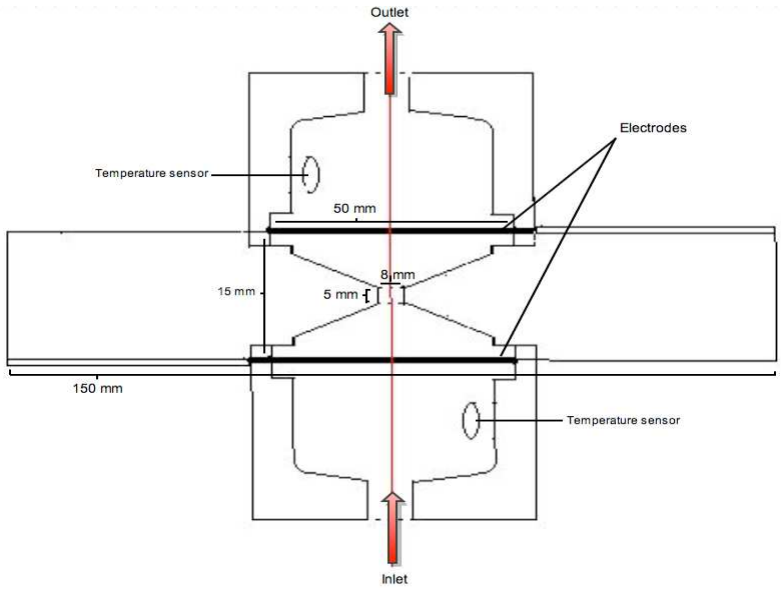


Figure 6.1. Pulsed Electric Field unit built at University of Auckland (A); Cross-sectional view of the treatment chamber (B).

6.2.3. Beer preparation and Pulsed Electric Field (PEF) treatment

Nine premium preservative free commercial beers sourced from several New Zealand breweries were processed by Pulsed Electric Fields (PEF). The beers tested comprised of pilsner lager, lager, ale and dark ale beers, with alcohol contents ranging from ≤0.05 to 7% alc/vol (alcohol by volume or ABV) (Table 6.1). The alcohol content was read from the bottle label. Different sources of sugar ingredients such as malt, honey, and potato were used in their production. The electrical conductivities of beers processed were measured twice before each experiment using a conductivity meter (Seven easy conductivity Mettler-Toledo, Switzerland) at room temperature (23°C) and average results are also shown on Table 1. As expected, the beers' electrical conductivities (between 1.4 and 2.8 mS/cm) were higher than values in drinking water (between 0.5 and 1 mS/cm).

Prior to PEF treatments degassing of the beers was carried out in a sterile container using a deaeration unit under vacuum, to avoid dielectric breakdown during processing. Walkling-Ribeiro *et al.* (2011) demonstrated no difference on the PEF inactivation of *S. cerevisiae* vegetative cells in fully carbonated and degassed beer. Additionally, for yeast inactivation experiments, the degassed beer was filter-sterilized prior to inoculation with the spore solution. Then the mixture was transferred to the feed tank, to be pumped at 4.34 ml/s through the system, exposed to 45 kV/cm electrical field intensity, and 800 Hz frequency, which is equivalent to 46.3 pulses and 70 μs treatment time. The process parameters were selected after some trials to ensure beer temperature was below 43°C, as required for the first set of experiments.

6.2.4. S. cerevisiae spore inactivation experiments

6.2.4.1. PEF inactivation in nine different beers

In this experiment, the beers listed on Table 6.1 were processed. Preliminary thermal inactivation experiments at 43°C (outlet temperature) revealed no spore inactivation and therefore the beers temperature during PEF was kept below 43°C. Just before the PEF treatment, the *S. cerevisiae* spores were inoculated into the beers and stirred to yield a final concentration of *ca* 10^5 cfu/ml. At the end of the PEF treatments (3 replicates), the samples were collected in sterilized tubes for analysis. The collected samples were immediately transferred to an ice water bath (0–4°C) to avoid spore germination after the PEF treatment.

Table 6.1. Characteristics of beers used in the Pulsed Electric Fields (PEF) experiments.

Brand of beer	**Type of beer**	**Alcohol (% volume)**	**Electrical conductivity* (mS/cm)**
A	Lager	≤ 0.05	2.20
B	Lager	2.5	1.48
C	Lager	4.0	1.37
D	Dark ale	4.5	1.98
E	Ale	5.0	1.96
F	Lager, Pilsner	5.0	2.13
G	Lager	5.0	2.09
H	Dark ale	5.2	1.97
I	Ale	7.0	2.76

*Average of two determinations.

6.2.4.2. Heat assisted PEF and thermal inactivation experiments

Beers A (0.0% alc/vol), C (4.0% alc/vol), and I (7.0% alc/vol) were used in heat assisted PEF and thermal inactivation experiments. The beer samples were heated slightly up to 33 °C before the PEF treatment in order to achieve the desired PEF processing temperatures, which were between 37.4 and 53.1 °C (outlet temperature). The same PEF conditions were used (45 kV/cm, 46.3 pulses, 70 μs). Thermal processing spore inactivation at 50 °C was carried out and compared with the PEF-thermal at 50 °C. Before the thermal processing, the beers were filter sterilized and inoculated with yeast ascospores. Heat resistant 5 × 5 cm bags of 154 μm thickness were used to pack 5 ml of inoculated beer samples (Cas-Pak, New Zealand). The high surface area of the bags compared to the volume of the beer packed, formed a thin layer, which enhanced the quick heat transfer (the come up time was less than 50 s) and had negligible come up and come down times. For each beer, two replicates were thermally processed at 50 °C for 20 min (=1200 s) using a water bath. Then, the pouches were immediately moved to an ice bucket to avoid spore germination after each treatment. *S. cerevisiae* spore numbers in the beers were counted before and after the heat treatment.

6.2.5. Lightstruck character sensory test

The main sensory concern in the breweries, is the "lightstruck character", which is an off-flavour developed in beer, especially after exposure to sunlight (Marsili, Laskonis, & Kenaan, 2007). 3-Methyl-2-butene-1-thiol and organic sulphur compounds contribute to the skunky off-notes. This undesirable off-flavour can limit the beer's shelf life. Therefore, it is important to investigate whether the PEF treatment generates the lightstruck character in the beer. Thus, for each of the nine different beers previously degassed, three samples were prepared and presented to the panellists for attribute scoring: control – beer kept in a dark place (not PEF treated); PEF – PEF processed beer kept in a dark place and not exposed to light; and

lightstruck – beer exposed to sunlight for 8 h, in order to develop the lightstruck character (not PEF treated). First the aroma was blindly evaluated by five trained panellists who smelled the three samples at room temperature, since the colder temperature could mask the lightstruck smell (Control, PEF, and Lightstruck). They then gave a score from 1 (indicating very bad smell) to 10 (excellent smell). After that, the flavour of each beer was also assessed by tasting the beer in 30 ml serving sizes. A scale ranging from 1 (indicating very bad flavour) to 5 (indicating excellent flavour) was used by the panellists to score the beer flavour. The control, lightstruck, and PEF-treated samples were served in randomly numbered disposable cups. Dips and snacks were served after each test to remove the taste of the previously tasted beer.

6.2.6. Statistical analysis of data

ANOVA was run to investigate any significant differences among the beers or treatments, depending on the experiment (Statistica version 8, USA). When differences were detected ($p<0.05$), the Tukey Honest Significant Difference (HSD) test was carried out to separate the average values for yeast log reductions.

6.3. Results and discussion

6.3.1. Pulsed Electric Fields (PEF) inactivation of S. cerevisiae spores in nine different beers

Figure 6.2 presents the average results of spore log reductions in nine different beers processed with PEF (45 kV/cm, 46.3 pulses, 70 μs). Although 0% ABV (beer A) and 7% ABV (beer I) beers were investigated, most of the commercial beers tested have alcohol

contents between 4 and 5% (beers C, D, E, F, G). The big deviation bars associated with the log reductions are related to the nature of yeast ascospores produced. The ANOVA could detect significant differences in the log reductions among some of the beers tested ($p<0.05$). Beer I - 7% ABV presented a higher log reduction (2.2) than beers A - 0% ABV (0.2) and H - 5.2% ABV (0.7), indicating a trend of higher inactivation for higher alcohol content. Except beer G, there were no significant differences between the 7 beers with alcohol content below 5.2% ABV (beers A, B, C, D, E, F, H). Thus, neither the beer production method (e.g. lager or ale), nor the different beer constituents seemed to affect the microbial reduction values. The effect of alcohol towards higher microbial inactivation is known from the literature. For example, thermal $D_{60°C}$-value of *S. cerevisiae* ascospores decreased from 6.1 min in apple juice to 1.2 min in apple juice with 6% ethanol (Splittstoesser *et al.*, 1986). Since studies of PEF inactivation of yeast ascospores in beer were not available, our results can only be compared with vegetative yeast PEF inactivation in beers and juices. Splittstoesser *et al.* (1986) concluded that the ascospores of *Saccharomyces cerevisiae* were over 100-fold more thermal resistant ($D_{55°C}$=106 min) than the vegetative cells ($D_{55°C}$=0.90 min) of the same strain. PEF (30 kV/cm, 10 pulses) of apple juice resulted in approximately 6 log reductions of *S. cerevsiae* vegetative cells (Qin *et al.,* 1998); Levesley and Kennedy (1999) registered close to 4 log inactivation of vegetative *S. cerevisiae* in India ale beer (16.7 kV/cm, 1280 pulses). MacGregor *et al*. (2000) determined 3.5 log reductions of *S. cerevisiae* cells in a test liquid (30 kV/cm, 3000 pulses). Similarly, Evrendilek, Li, Dantzer, and Zhang (2004) obtained 4.1 log reductions on vegetative cells of *Saccharomyces uvarum* in a keg beer (22 kV/cm, 216 μs). PEF (35 kV/cm, 4800 μs) inactivation of *S. cerevisiae* in apple juice was 4.2 log (Aguilar-Rosas *et al*., 2007). More recently, Walkling-Ribeiro *et al*. (2011) measured >6.8 log inactivation of *S. cerevisiae* vegetative cells in 3.5% alc/vol beer (45 kV/cm, 402 μs) and Abca and Evrendilek (2014) registered ≥6 log in 12% alc/vol red wine (31 kV/cm).

Other PEF studies with beer and juices revealed less than 1.0 log reduction of *Neosartorya fischeri* ascosopores (42 to 51 kV/cm, 40 pulses, pulse width 2.0 to 3.3 μs) in several fruit juices, while *Byssochlamys fulva* conidiospores resulted in 5.0 log reductions (Raso, Calderón, Góngora, Barbosa-Cánovas, & Swanson, 1998).

6.3.2. Thermal assisted PEF inactivation of S. cerevisiae spores in three different alcohol content beers

Being the alcohol content one of the most important factors in terms of spore inactivation, beers A (0% alc/vol), C (4.0% alc/vol), and I (7.0% alc/vol) were used in subsequent PEF microbial inactivation experiments. The results of heat-assisted PEF are shown in Figure 6.3. An increase from <43 to 53˚C in temperature while maintaining the same PEF conditions (45 kV/cm, 46.3 pulses, 70 μs treatment time) seems to cause further reduction on *S. cerevisiae* ascospores. At 53°C, approximately 0.9 (beer A – 0% ABV), 3.2 (beer C – 4% ABV) and 4.0 log reductions (beer I – 7% ABV) were obtained compared to 0.2, 1.1 and 2.2 at <43°C, respectively. McDonald *et al*. (2000) inactivated *S. cerevisiae* ascospores in orange juice to maximum of 2.5 log reductions at 50°C and 50 kV/cm.

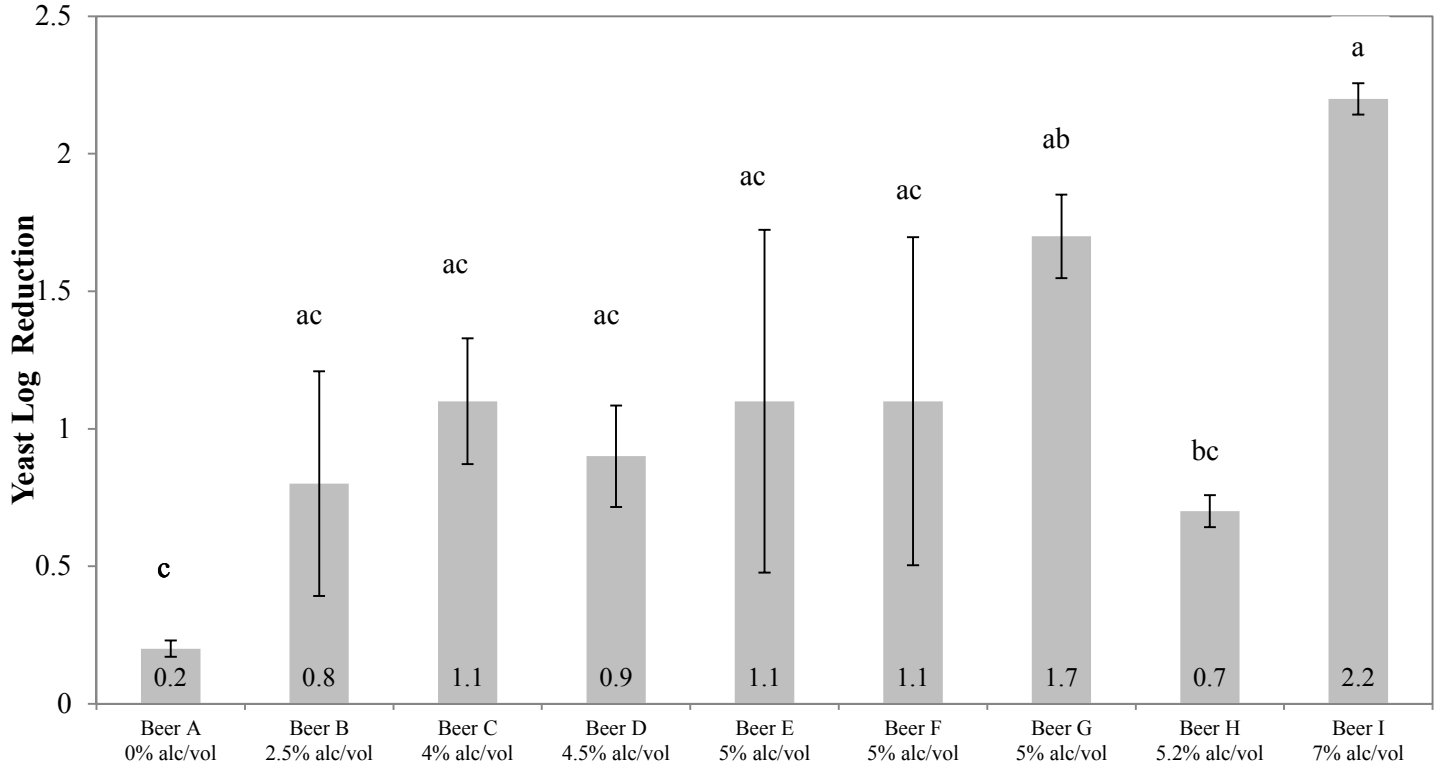


Figure 6.2. Pulsed Electric Fields (PEF) inactivation of DSMZ 1848 *S. cerevisiae* spores in nine different beers (T< 43°C, 45 kV/cm, 46.3 pulses, 70 µs). (Error bars are standard deviation

6.3.3. S. cerevisiae ascospore inactivation: Comparing PEF with conventional thermal processing

Beers A (0% alc/vol), C (4% alc/vol), and I (7% alc/vol) were once again used for these experiments (Fig. 6.4). The treatment time of PEF processed beers was 70×10^{-6} s, which was approximately 10^7 times smaller than the 20 min thermal treatment at the same temperature (50°C). This difference in time can represent huge gains in terms of beer productivity when PEF is used industrially to pasteurize beer. The ANOVA and Tukey test were run with log reduction data for all beers and processes. While beers C and I, containing alcohol presented higher log reductions for PEF-thermal (50°C, 45 kV/cm, 46.3 pulses; 3.2-3.5 log) than thermal (0.3-1.4 log) ($p<0.05$), beer A – 0% ABV didn't (0.5-0.6 log). In resume, the use of thermal assisted PEF at 50°C did not bring any extra microbial reduction to 0% ABV beer. On the contrary, higher inactivation was registered in beers with alcohol. The maximum log reduction of thermally treated (72°C, 15 s) *E. coli* in fruit smoothie was 6.3 while PEF treatment (34 kV/cm, 150 μs) was 5.4 log reductions (Walkling-Ribeiro *et al*., 2008). Azhuvalappil *et al*. (2010) obtained *ca* 6 log reductions of *E. coli* for both PEF (23 kV/cm, 150 μs, 49–51°C) and thermal processes (76°C, 1.3 s) in apple cider.

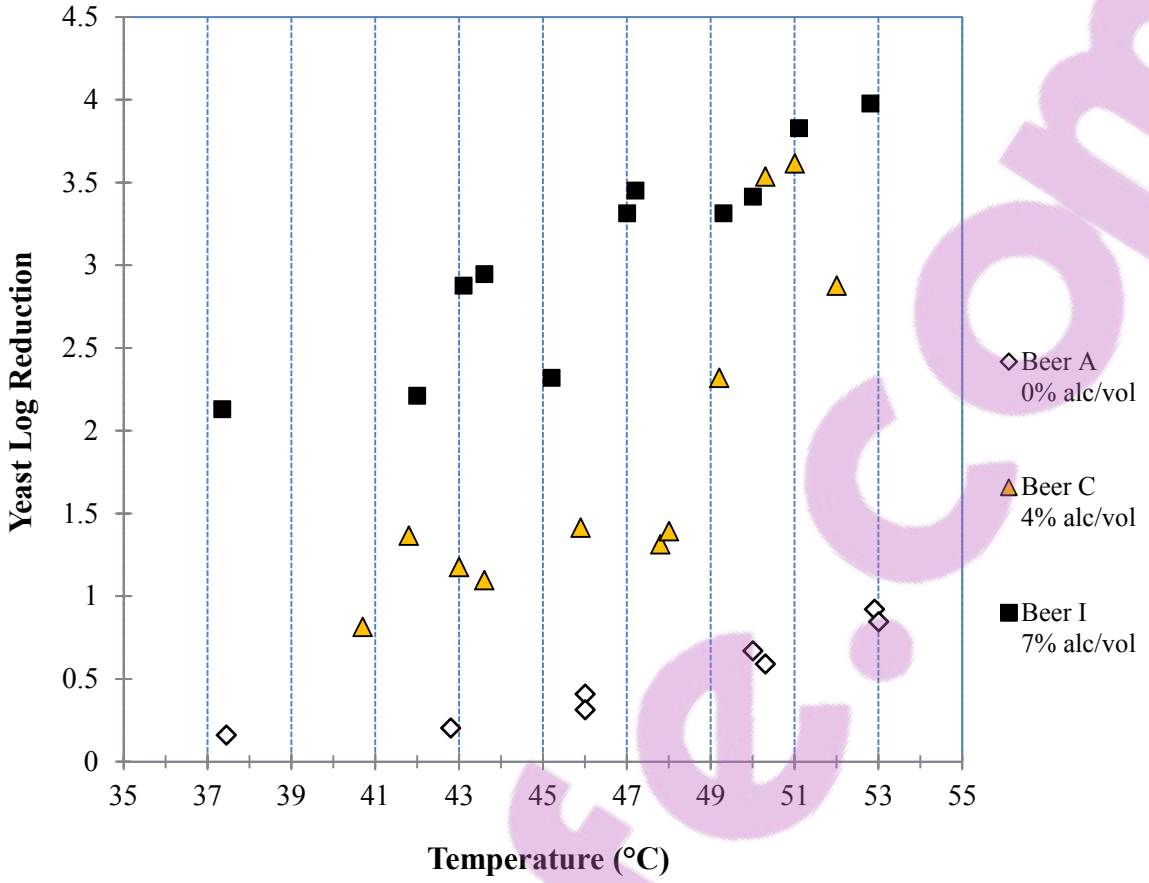


Figure 6.3. Inactivation of DSMZ 1848 *S. cerevisiae* ascospores in three different alcohol content beers using PEF in combination with moderate thermal processing (45 kV/cm, 46.3 pulses, 70 µs, 43°C≤T≤53°C).

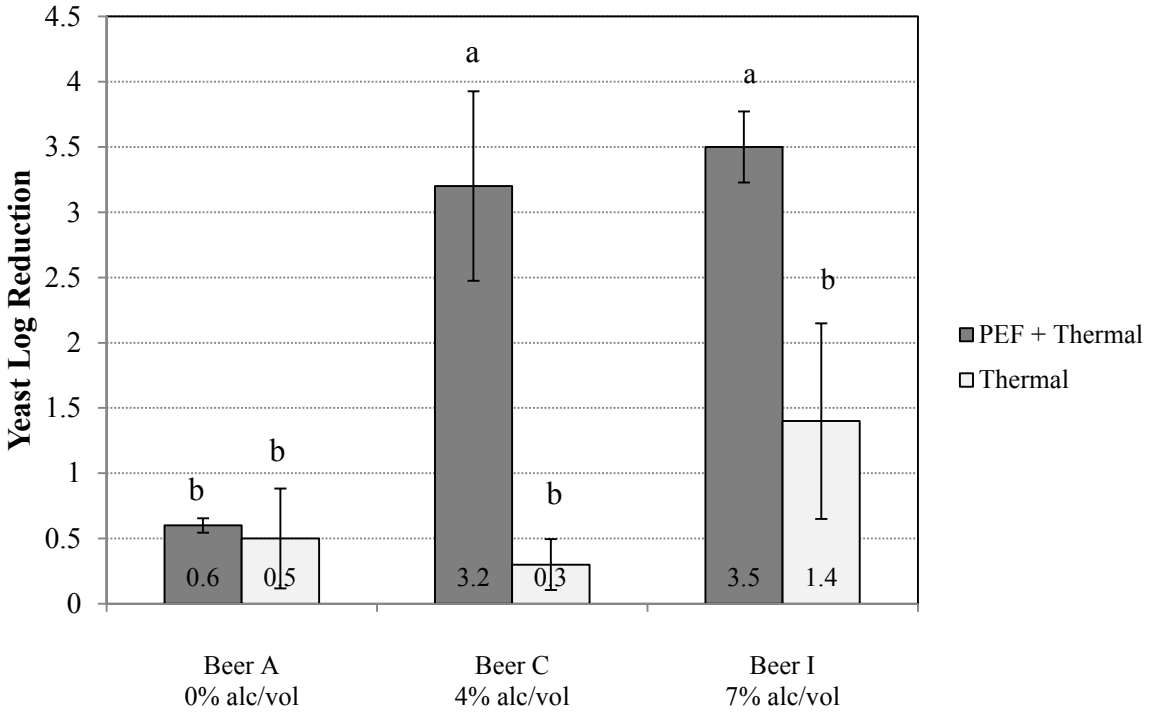


Figure 6.4. PEF-thermal (45 kV/cm, 46.3 pulses, 70×10-6 s) vs. thermal inactivation (20 min = 1200 s) of DSMZ 1848 *S. cerevisiae* spores at 50°C in three different alcohol content beers. (Error bars are standard deviation).

6.3.4. Lightstruck character sensory assessment in nine different beers

Although the main objective of this work was to inactivate yeast ascospores in beers by using the PEF process, the beer sensory was also investigated, since PEF processing can develop off-flavours in beers such as lightstruck, thus being commercially unacceptable. Therefore, in addition to the microbial inactivation shown on Figure 6.2 (section 6.1), the 9 beers were analysed for sensory. For each of the PEF treated beers, aroma and flavour lightstruck attribute tests were carried out (Control, PEF, and Lightstruck). Table 6.2 shows the averages of lightstruck aroma and flavour scores followed by a letter, which indicates if control, PEF and light exposed treatments belong to or do not belong to the same group (Tukey test). The ideal

result was that PEF beer would be similar to control and significantly different from lightstruck (8 h sunlight exposed beer). The sensory panel could not detect differences between aroma/flavour of control, PEF and lightstruck for beers F (Pilsner) and H (dark ale), indicating that these are not prone to developing the lightstruck character. Beers A, B, E, F, G and H had no detectable lightstruck effect on aroma since PEF and c ontrol beers belong to the same group (Tukey test). The panel could detect the lightstruck character in the other beers tested. The dark colour of beer H may have prevented the lightstruck character development. It is known the beers are packed in dark bottles or aluminium cans, not allowing the light to pass through, to avoid this undesirable reaction. Regarding the flavour test, with the exception of beers F, H and I, all the beers formed the undesirable lightstruck character with the PEF treatment.

Although overall acceptability of PEF (41 kV/cm, 175 µs) vs. untreated keg beer was the same, Evrendilek *et al*. (2004) detected differences in the beer flavour and mouthfeeling. Mezui and Swart (2010) sensory panel detected lightstruck flavour formation in beer processed by low ultraviolet light irradiation (UV-C). Walkling-Ribeiro *et al*. (2010) sensory panel found better colour, odour and flavour in untreated fruit smoothie compared to PEF smoothie (34 kV/cm, 60 µs), although overall acceptability was the same. Abca and Evrendilek (2014) concluded PEF (31 kV/cm) did not change the sensory properties of red wine.

Table 6.2. The effect of PEF treatment (45 kV/cm, 46.3 pulses, 70 μs) on lightstruck aroma and flavor character in different beers.*

Beer	**Aroma**			**Flavour**		
	Control	PEF	Lightstruck	Control	PEF	Lightstruck
A	8.0 ab	9.2 b	5.2 a	4.4 b	2.4 a	2.8 ab
B	7.2 c	6.0 c	4.6 c	4.2 d	2.6 c	2.8 cd
C	7.8 g	1.8 e	4.2 f	4.4 g	1.6 e	2.6 f
D	7.8 i	1.4 h	3.8 h	3.8 j	1.4 h	2.6 i
E	9.0 l	5.2 kl	4.0 k	5.0 l	2.6 k	2.4 k
F	8.4 m	6.8 m	5.2 m	4.2 m	3.0 m	2.8 m
G	8.0 o	6.0 no	3.8 n	4.0 o	2.6 n	2.4 n
H	8.4 p	9.0 p	6.0 p	4.4 pq	4.8 q	3.4 p
I	9.0 s	4.4 r	6.2 rs	4.8 s	3.8 rs	3.4 r

*For aroma lightstruck character 1 very bad to 10 excellent; For flavor lightstruck character 1 very bad to 5 excellent; Means for each beer aroma followed by the same letter are not significantly different (Tukey test, p=0.05); Means for each beer flavor followed by the same letter are not significantly different (Tukey test, p=0.05).

6.4. Conclusion

The results of this study showed that the inactivation of *S. cerevisiae* ascospores by PEF is higher in the beers with higher alcohol content, with a maximum of 2.2 log reduction for 7% alc/vol beer (45 kV/cm, 70 μs treatment, T<43°C). Thermal assisted PEF at 53°C led to 2 more log reduction in 4 and 7% alc/vol beers. Moreover, PEF treated beer at 50°C for 70 μs presented higher yeast ascospore inactivation than 50°C thermal treatment for a much higher treatment time of 12×10^8 μs.

Lightstruck character sensory assessment of nine PEF treated beers indicated that certain beers are more appropriate for this technology than others. Hence it is important to select the beer and optimize the PEF processing conditions to avoid the development of the undesirable lightstruck

character. To finalize, the only drawback of the PEF unit used in the experiments, was the beer degassing requirement prior to the PEF pasteurization treatment. This can add to the overall production costs, since CO_2 would have to be added to the beer after the PEF treatment. However, there are other PEF systems which do not require beer degassing.

The energy requirement for PEF to achieve the minimum pasteurization of 1.5 log reduction of *S. cerevisiae* ascospores was 192.23 kJ/L. By comparing this value with HPP (77.4 kJ/L), power ultrasound (2612.1 kJ/L) and thermal treatment (188.8 kJ/L) pasteurization of beer by PEF (192.23 kJ/L) seemed to be more energy saving than power ultrasound and thermal processing after HPP.

The results of this study can be helpful for designing appropriate PEF conditions to pasteurize beers with different alcohol contents.

Chapter 7. Studies on the mechanisms of *Saccharomyces cerevisiae* spores inactivation by scanning electron microscope observations

7. *S. cerevisiae* spores observations by microscope scanning electron observation

Chapter Abstract

This study aims to contribute for the explanation of the underlying mechanisms of *Saccharomyces cerevisiae* spore inactivation. The spores were inactivated using nonthermal HPP and thermal processing and the morphology of live and dead spores was assessed with scanned electron microscopy (SEM) and environmental scanning electron microscopy (eSEM).

First, the live and dead spores of *S. cerevisiae* in ascus format were observed under SEM using the air-drying method. Then, the live and dead free single spores of *S. cerevisiae* were observed after removing the ascus. HPP treatment of free spores attacked the cell membrane and formed openings, which induced leakage of intracellular components from the cytoplasm. The injured spores have undergone irreversible volume and shape changes. Thermally processed *S. cerevisiae* spores seemed to become totally deflated and shrunk after the treatment.

This study showed that SEM and eSEM observations were good but not sufficient for studying the mechanism of spore inactivation. The conclusions could be improved with the aid of other methods or equipment to get more details of the mechanism of the yeast ascospore inactivation by different processes.

7. *S. cerevisiae* spores observations by microscope scanning electron observation

7.1. Introduction

The beer pasteurization aims to inactivate the fermenting yeast used as starter, along with other beer spoilage microorganisms. As mentioned in previous chapters, it is possible for yeasts to sporulate at different stages of beer production. It is well known that spores are much more resistant than vegetative cells and therefore by inactivating the spores, other vegetative cells will surely be. Some studies have been conducted to investigate the performance and efficiency of different treatments such as thermal treatment, high pressure processing (HPP), Pulsed Electric Field (PEF) and ultrasound on the yeast vegetative cells and ascospore inactivation. However, the in depth mechanisms on how the ascospores are killed by different treatment processes is not well understood. Vegetative cell is a cell that reproduces by asexual means either through natural process such as budding or artificial process such as grafting. During the sexual stage under the right conditions, vegetative cell can form spores within it. Vegetative cell acts like a protective layer towards the spores. The inactivation of vegetative cell is much easier and simpler than spores. This is because the matured spores are difficult to be inactivated due to the concrete multilayer membranes, which can withstand multiple environmental conditions in their own way, and exhibit higher heat resistance (Put & Jong, 1982). Previous study proves that the thermal processing $D_{60°C}$ value of *Saccharomyces cerevisiae* spores is 30-350 folds higher than its vegetative cells (Put & Jong, 1982). Table 7.1 presents the $D_{60°C}$ value for different yeast species, showing that vegetative cell portrays less heat resistance than its corresponding spores (Put & Jong, 1982):

7. *S. cerevisiae* spores observations by microscope scanning electron observation

Table 7.1. $D_{60°C}$-value for various types of yeast species.

Yeast Species	$D_{60°C}$ (min)	
	Vegetative Cells	Spores
Saccharomyces cerevisiae	0.15	19.2
Saccharomyces chevalieri	0.1	16.4
Kluyveromyces bulgaricus	0.2	40.0

Considering the huge difference between the thermal resistance of vegetative cell and spores, one can ensure that inactivation of spores will surely destroy its corresponding vegetative cell.

S. cerevisiae is globular-shaped yeast and famously used in food production especially during fermentation process and ethanol production. *S. cerevisiae* is normally considered non-pathogenic and has less heat resistance compared to most of other microorganisms. *S. cerevisiae* spore wall consists of a multi-laminar coat that surrounds individual spore and protects spore from environmental stress with outermost layer is composed primarily of dityrosine (Briza *et al.,* 1994). Dityrosine is an amino acid inside the spore wall surface where it is part of highly cross-linked macromolecular network consists of glucosamine and few other amino acids (Briza *et al.,* 1996). The enhanced resistance of *S. cerevisiae* spores towards many stresses is attributed to this outermost layer of spore wall (Briza *et al*., 1990). The sporulation process ends with the completion of the spore wall, where the spores all fully formed and the original mother cell collapses around the four completed spores to give rise to the tetrahedral mature ascus. This event results in the existence of ascus, which consists of four spores enclosed together inside an ascal membrane and ascal wall, which are derived from the mother cell. This causes intact ascus to have similar surface texture to vegetative cells (Coluccio & Neiman, 2004). Extraction of single spore from ascus can be done using Zymolyase enzyme (Milani *et al*., 2015a).

7. *S. cerevisiae* spores observations by microscope scanning electron observation

Thermal inactivation is a well-known treatment to cause spores' death without any doubt. It has been suggested that the cell membrane is the primary site of thermal injury of spores (Flowers & Adams, 1976). Membrane damage causes an increase in the sensitivity of the spores towards environmental heat stress. Damage to the membrane then consequently affects the permeability barrier which then results in the release of the intracellular constituents. This indicates the death of the spores (Juneja, 2001). Heat effect also causes changes in the native structure of protein (Prokop & Humphrey, 1972).

Nonthermal processes are more energy efficient than conventional inactivation processes (Chen *et al*., 2010a). Among the nonthermal technologies studied in this research, HPP was found to be more suitable as no heating was needed for *S. cerevisiae* spore inactivation (see chapter 4). Pagán and Mackey (2014) reported that pressure treatment alters the cell membrane and gives impact on proteins and genetic mechanisms of microorganisms. For yeast cell inactivation, the primary inactivation mechanisms involved during HPP treatment are the cell membrane damage and organelle disruption with membrane damage, which are considered as one of the key events related to microbial (Harrison *et al.,* 2001).

SEM allows studying the microstructural characteristics of bulk materials with length scales ranging from nanometres to millimetres, which is suitable for observing spores due to its tiny size (Stokes *et al.,* 2013). The most straightforward specimen type that usually be analysed under SEM are metals. This is because metals are less susceptible to the effects of charging and damage under electron irradiation in high vacuum operated SEM (Stokes *et al.*, 2013). Materials which are not naturally solids tend to outgas in vacuum. Thus, there are methods for improving rigidity and preventing outgassing for these types of materials. These methods include critical point drying (CPD) and freeze drying (Stokes *et al*., 2013). Observation using SEM for non-solid materials thus is a bit complicated as it involves chemical fixation, drying and coating (Habold *et al*., 2003).

7. *S. cerevisiae* spores observations by microscope scanning electron observation

As oppose to SEM, eSEM is much easier and simpler to be used and handled, an adaptation of SEM for greater flexibility. The main difference includes the introduction of gases into the specimen area with purposes of mitigating charging effects in insulators and enabling hydrated or liquid specimens to be observed more easily. These advantages eliminate many of the specimen preparation steps that are required in SEM (Stokes *et al.,* 2013).

The research on the inactivation of vegetative cells by thermal and nonthermal HPP treatments has been well established since the past few years. However, there are currently very few in depth studies on the effects of thermal and nonthermal treatments such as HPP on microbial spores instead of vegetative cell. There are also fewer studies that compare the morphology of dead spores after the thermal and nonthermal treatments. Therefore, the purpose of this study was to progress in the understanding of spore inactivation by thermal and nonthermal HPP and to examine the morphology of live and dead *S. cerevisiae* spores under electron microscopes in order to explain the underlying mechanisms of spore inactivation.

7.2. Material and methods

7.2.1. Microscopes

Philips XL30S FEG (FEG = Field Emission Gun) unit manufactured in Netherlands was used for scanning electron microscopy (SEM) analysis while FEI Quanta 200 FEG unit manufactured in USA was used for environmental scanning electron microscopy (eSEM) analysis. Both units are located at Engineering of the University of Auckland owned by Research Centre for Surface and Material Science (RCSMS). The sputter coater used for standard electron microscopy samples is a Quorum Q150RS sputter coater. It is designed to

give a thin, minimal metal coating suitable for electron microscopy viewing. All the spore samples were sputter-coated with platinum, Pt. Both XL30 and Quanta can be used to analyse all spore samples prepared by any sample preparation method listed in the next sections. All spore images were collected at 5 and 10 kV.

7.2.2. Production of S. cerevisiae ascus and free spores

7.2.2.1. Production of S. cerevisiae ascus

The DSMZ 1848 *S. cerevisiae* culture stored at -80°C was streaked on YEPG agar and after growth a fresh single colony was inoculated into 50 mL of presporulation sterilised liquid (121°C, 10 min) composed of 0.8% yeast extract, 0.3% peptone, 10% glucose, and zinc sulphate 25 mg/L. After inoculation, the presporulation flasks (500 mL) were incubated overnight in incubators (with rotary shaking at 168 rpm) at 28°C. When optical density (PG Instrument T60 set at 600 nm) reached around 0.2 to 0.8, an appropriate portion of the presporulation broth (ca. 1.5 mL) was inoculated into sterile sporulation broth (10 mL) to yield 10^7 cfu/mL. Sporulation broth consisted of potassium acetate 1% (w/v), bacto yeast extract 0.1% (w/v), glucose 0.05% (w/v), zinc sulphate 25 mg/L. The mixture was incubated at 18°C for 14 d (with rotary shaking at 230 rpm) in 1-L Erlenmeyer flasks. The spore solution was centrifuged and washed 3 times. Then, the pellet was split in to 1 mL Eppendorf tubes and 1 mL of salt triton dithiothreitol (STD) solution (0.1 g NaCl in 10 mL of 0.05% Triton X-100) was added to the spore solution to avoid clustering of the ascus.

7.2.2.2. Production of S. cerevisiae free single spores

The spore solution (before the stage of STD addition), which was obtained from the previous section was split in 1-mL Eppendorf tubes. The spores were extracted from the vegetative

(parental cells and those that did not sporulate) by adding 100 µL Zymolyase solution (5 mg/mL solid Zymolase in pH 7.2 buffer containing 1.2 M sorbitol and 0.1 M KH_2PO_4), 900 µL spheroblasting buffer (2.2 M sorbitol), and 800 µL softening buffer (100 mM Tris-SO_4, pH 9.4, 10 mM dithiothreitol (DTT) solution). Then, the mixture was incubated at 30°C in a water bath for 2 h and the Eppendorfs were gently inverted every 20 min to accelerate the break-up of tetrads into single ascospores. The spores were harvested by centrifuging three times at 9700 *g* (rotor F-45-12-11) for 1 min and resuspending in 200 µL of 0.5% Triton X-100 to ensure total removal of the enzyme. After the last resuspension, 4 µL DTT was added to the Eppendorfs containing the spore solution. Then, the Eppendorfs were sonicated three times at 6 Hz for 2 min, both to break up tetrads into single ascospores and to kill any vegetative cells remaining in the medium. Finally, 1 mL of salt triton dithiothreitol (STD) solution (0.1 g NaCl in 10 mL of 0.05% Triton X-100) was added to the spore solution to avoid spore aggregation (Xiao, 2006).

7.2.3. S. cerevisiae thermal and HPP inactivation process conditions

For each spore solution, 10 µL of sample was inoculated inside 0.99 mL of sterile distilled water and was then packed into Cas-Pak pouches that were previously mentioned in Chapters 2 and 4. The thermal conditions used for this study were 15 min at 65°C and the HPP was 600 MPa for 5 min processing time at room temperature. These conditions ensured total ascospore inactivation according to our previous results (see chapters 2, 3 and 4). Two replicates were carried out and enumerated on YPD plates.

7.2.4. Spore sample preparation for electron microscopy observations

7.2.4.1. Short air-drying

For short air-drying, live and dead spores were directly adhered onto thin cover slips which were mounted on metal stubs. The attached spores were left to dry inside a desiccator for a maximum of 1 hour before they were sputter-coated with platinum.

7.2.4.2. Long air-drying

Regarding longer air drying, similar step performed during short air-drying was adapted but with longer duration of air-drying. The spores were allowed to dry at least 24 hours before they were sputter-coated with platinum.

7.3. Results and Discussion

7.3.1. Observation of ascus containing the spores after longer air-drying

The spores were in tetrad mode during inactivation treatment using HPP and heat treatment and both the live and treated spores containing the ascus were air-dried overnight. Longer duration of air-drying which took minimum of 24 hours was performed in order to improve the efficiency of air-drying method to produce sharper and clearer images under eSEM. The resulting images under XL30 are shown in Figures 7.1, 7.2 and 7.3. A clear and well-defined difference in the untreated ascus containing live spores (Fig. 7.1) versus ascus containing dead spores by HPP (Fig. 7.2) and thermal processing (Fig. 7.3) is observed. Live spores are visibly attached together in ascus form while the HPP dead spores can be seen to leak and escape from their ascus wall. Single spores are seen to spread over the thinning and destroyed ascal wall as shown in Figure 7.2. Figure 7.3 shows the heat treated tetrad being crumpled in

the ascus. That could be due the leakage of the intracellular components from the cytoplasm. However, being the spores in the ascus makes it difficult to compare the thermally dead spores with HPP ones. Moreover, due to the presence of the ascus around the tetrad, the spores might be not completely destructed.

The circled areas in Figure 7.1 are assumed to be the spores inside the ascus as they are in tetrad arrangement and their size is smaller compared to other bigger cells, which are considered to be vegetative cells left from presporulation process. The reason of the existence of vegetative cells in the untreated sample is that the Zymolyase enzyme treatment and sonication were skipped as previously mentioned. This is supported with the fact that the ascal wall is derived from the vegetative wall (Coluccio & Neiman, 2004). Besides that, the untreated spores in tetrad mode also show that they are not in a perfect round shape as shown in Figure 7.1 (Coluccio & Neiman, 2004).

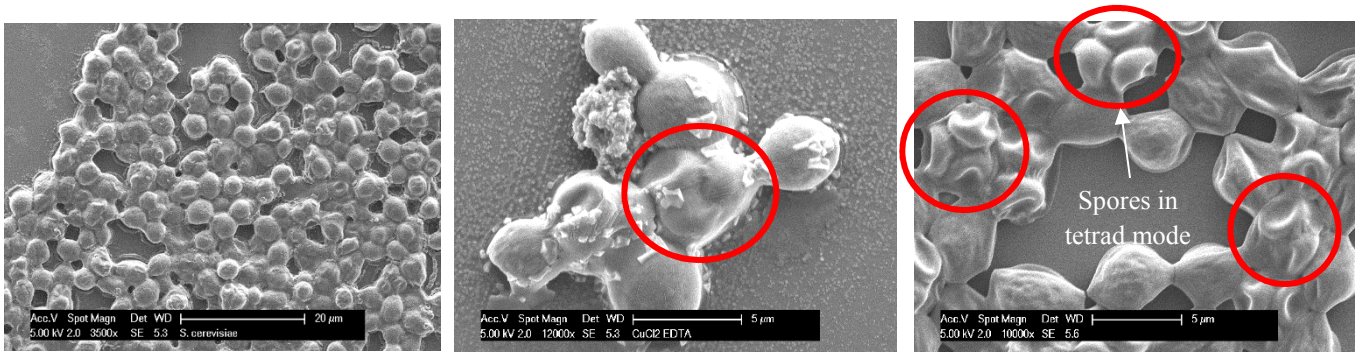


Figure 7.1. Observed live *S. cerevisiae* spores in tetrad mode or ascus form after air-drying (Images taken under eSEM).

7. *S. cerevisiae* spores observations by microscope scanning electron observation

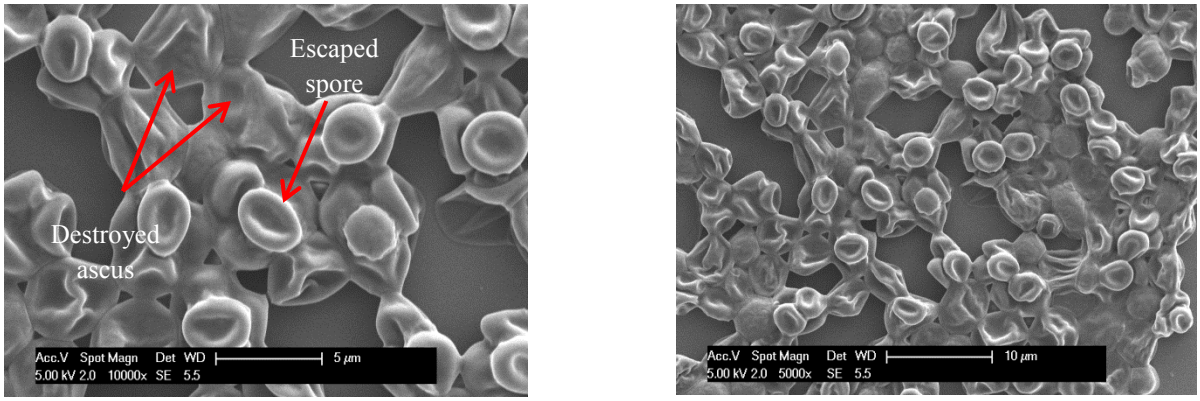


Figure 7.2. Observed dead *S. cerevisiae* spores in tetrad mode or ascus form after being treated by HPP and air-drying Images taken using eSEM).

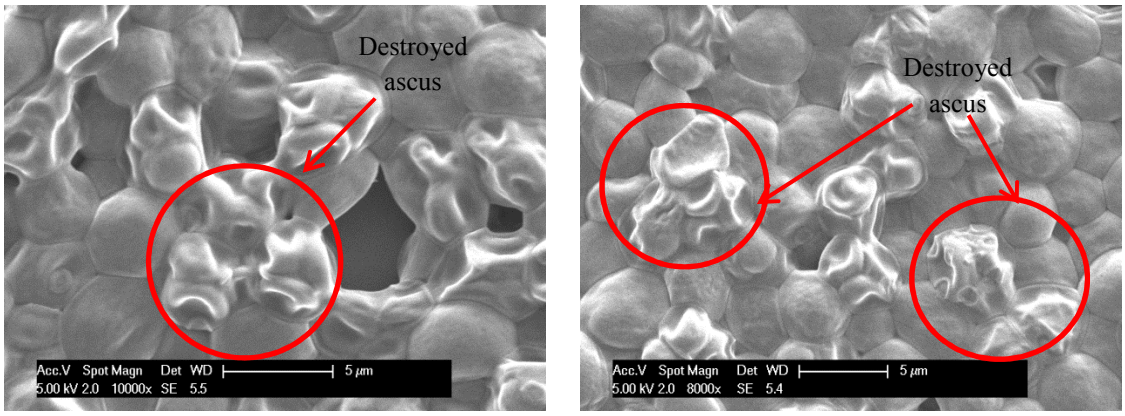


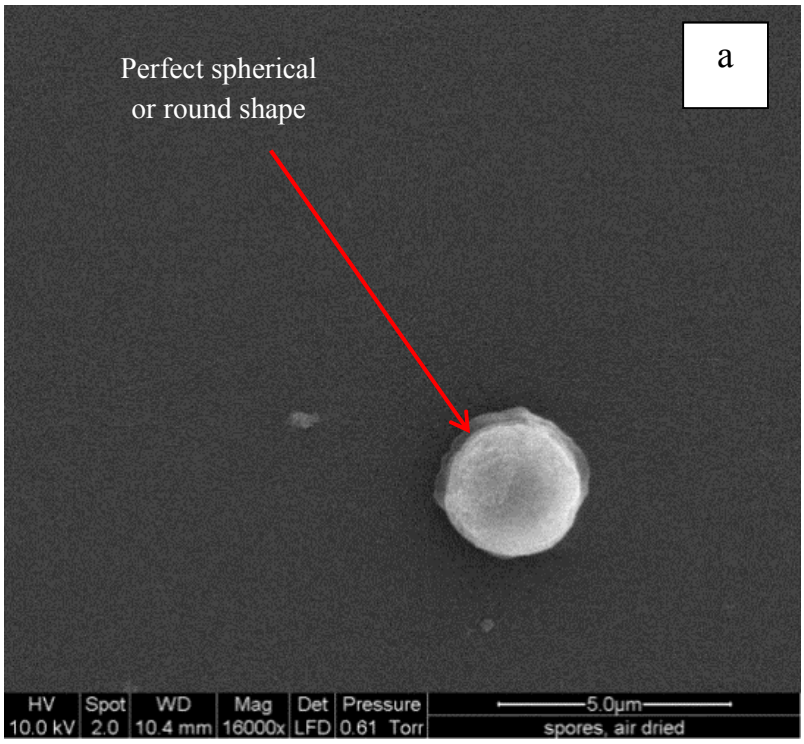
Figure 7.3. Observed dead *S. cerevisiae* spores in tetrad mode or ascus form after being treated by thermal processing and air-drying (Images taken under eSEM).

The morphology between live and dead spores was difficult to compare and analyse because of the mixing of vegetative cells and ascus, injury of live spores in tetrad form and difficulties to differentiate between intact asci and vegetative cells as they present similar surface texture (Coluccio & Neiman, 2004). Thus, the observation of live and dead single spores of *S. cerevisiae* was carried out in order to achieve more accurate results.

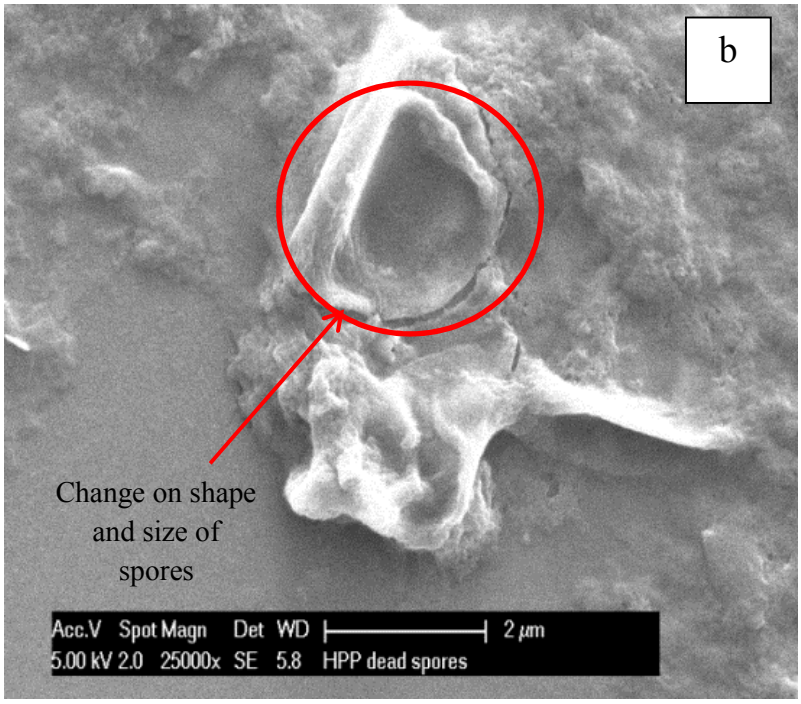
7. *S. cerevisiae* spores observations by microscope scanning electron observation

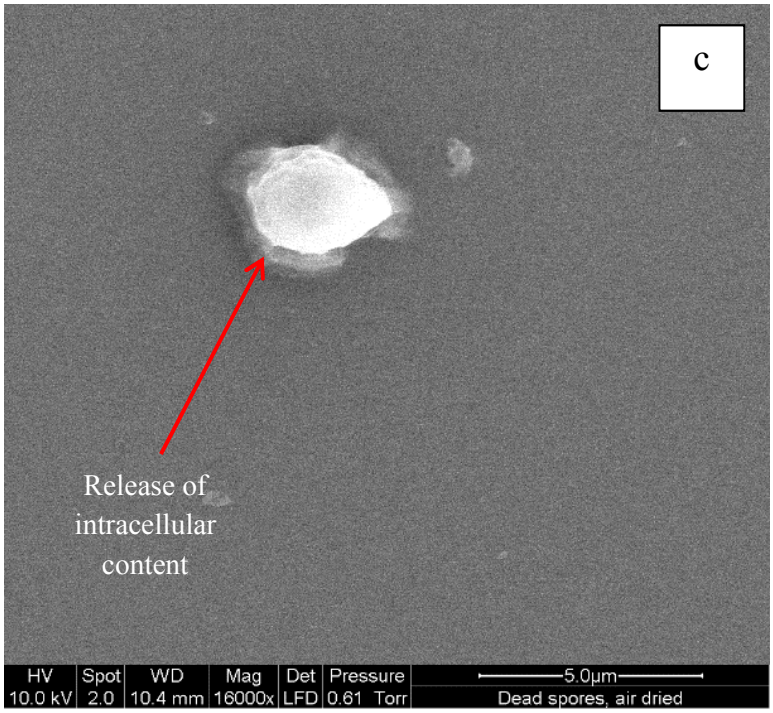
7.3.2. Observation of free spores after short air-drying

Figure 7.4. (a) shows a healthy untreated *S. cerevisiae* spore which possesses smooth surface and perfect spherical or round shape. However, Figure 7.4. (b and c) shows an imperfect round shape of a dead spore due to the applied HPP treatment. Figure 7.4(d) presents the thermally treated free spore of *S. cerevisiae* that seemed to become totally deflated and shrunk after the treatment.



7. *S. cerevisiae* spores observations by microscope scanning electron observation





7. *S. cerevisiae* spores observations by microscope scanning electron observation

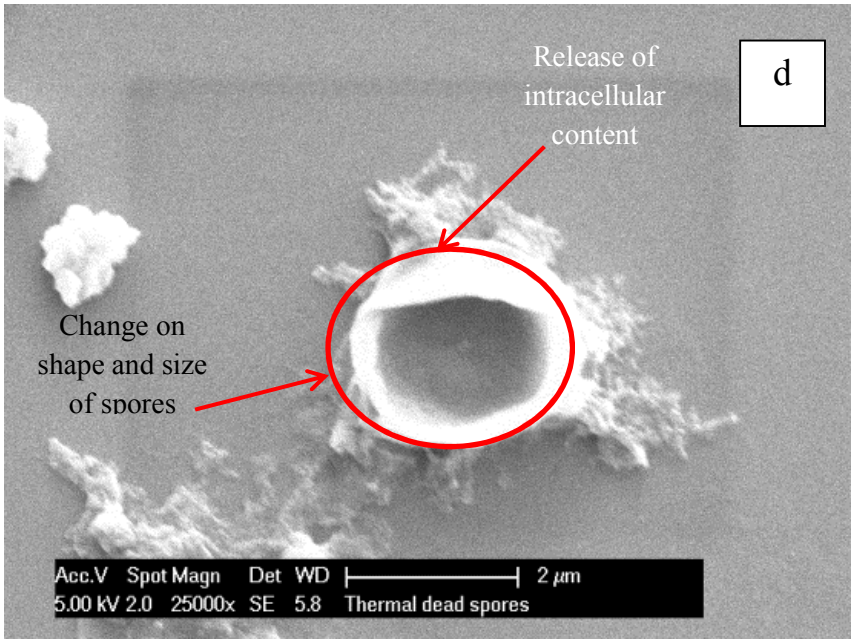


Figure 7.4. *S. cerevisiae* free spore images (a) untreated live under SEM (b, c) HPP-treated dead under SEM (d) thermally treated dead under eSEM.

According to (Marx *et al*., 2011), pressurized membrane alters the spore's permeability that allows changes in volume, which justifies the cause of imperfect round shape of the treated spores. The irreversible change in cell volume was suspected to be due to the mass transfer between the spores and the environment during the holding pressurization time, with water being the main component released from the cell. The presence of liquid-like component (the blurred area) around the dead spore was predicted due to the release of intracellular constituents that was pushed out from the cytoplasm through openings or holes present on the cell membrane. These openings or holes were formed due to the applied pressure on the spores. Hence, the damage on the cell membrane due to the applied pressure on the spores resulted in the loss of intracellular constituents from spore's cytoplasm to the outside area. A greater amount of leakages from the inner spores indicated a higher degree of injury towards the cell membrane, which then correlates to a greater extent of spore death (Hong & Pyun; 2001).

7. *S. cerevisiae* spores observations by microscope scanning electron observation

With respect to thermal processing, microbial inactivation in general includes wall damage, damage of membrane, ribosomes, chromosomal damage, and the active enzymes in the microbe (Hurst, 1977). For bacterial spores it is likely to have cell repair, but not for yeast spores. Furthermore, Sala *et al*., (1995) has mentioned that heat damages the cell structure including the cell membranes, ribosomes, DNA, RNA, and enzymes. DNA seemed to be the most likely reason of death target. However, the damage occurring in different structures of the cell may also result the heat inactivation (Gould, 1989). Figure 7.4d presents a dark hole and deflated-ball like shape of the ascospore, which could indicate the release of intracellular components that is in support with the previous studies. As oppose to HPP treated spores, the shape of the heat exposed cells did not deformed badly, but it is highly likely that the constituents of the ascospore was leaked out from the destructed cell membrane. The liquid-like shape around the spore also supports the idea of the intracellular constituent leakage of the spore.

As a conclusion, based on the image comparison of live and dead spores treated by HPP and thermal processing, it is safe to say that the mechanisms of *S. cerevisiae* spores inactivation due to HPP treatment started change on shape and size of spores with damage and irreversible injury on the cell membrane. For thermally treated spores, the intracellular components seemed to be discarded from the spore. In both thermal and HPP treated spores, the damage was characterised by the formation of openings or holes that cause leakage of intracellular component from the cytoplasm. This will then result in irreversible volume and shape changes of the spores. However, further evidences of spore inactivation to strengthen the identified mechanisms, cannot be seen clearly from the images. These include evidences such as the broken cell wall and its debris, cell compression or dent on the spore surface and the altered cytoplasmic content inside the spore. It is believed that the evidences were presented but could not be captured by the microscope. This might be due to the low

resolution images that were produced by the short period air-dried method. Furthermore, this method might also result in less sufficient drying of spores that made the detailed features on its surface vague or unclear.

Images captured by Coluccio & Neiman (2004) shown in Figure 7.5 demonstrate that *S. cerevisiae* live spore wall is composed predominantly of dityrosine while its vegetative cell wall primarily consists of mannoproteins (Coluccio & Neiman, 2004). These two different types of polymers can be distinguished by their surface appearances. While vegetative yeast cells have a smooth and velvety appearance, the spores have ridged or scalloped appearance with perfect round shape. The image of spores in Figure 7.5 (b) is contradicted with the smooth spore surface appearance captured in Figure 7.4. (a), which were thought due to the problems of low resolution image and insufficient drying. Both spores in Figure 7.4 (a) and Figure 7.5 (b) show similar perfect round shape.

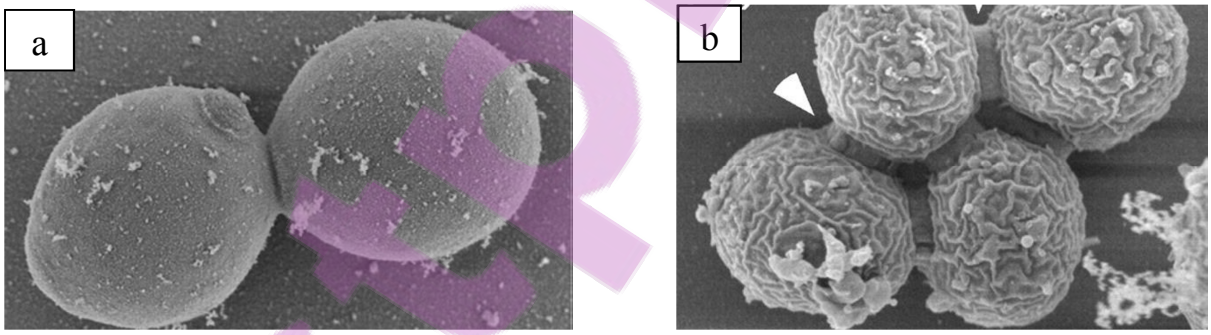


Figure 7.5. Surface morphology of *S. cerevisiae* spores is different from its vegetative cells (a) vegetative cell (b) spores (Coluccio & Neiman, 2004 copyright permission from Society for General Microbiology).

7.4. Conclusions

The results presented show that in both HPP and thermal processing the spore wall disruption resulted in the release of intracellular components from the spore core, which can be visible in certain images. However, the appearance of the cell wall in thermally treated spores was more crumpled. The suggested spore inactivation mechanisms can be further supported through the analysis of these intracellular components in the future. Meanwhile, SEM imaging combined with Focused Ion Beam (FIB) sectioning can be used to look at internal damage of spores to complement the outer appearances of the spore.

General Conclusions and future work recommendations

Conclusions

This thesis has demonstrated the advantage of PEF, HPP and thermosonication in terms of microbial inactivation compared to thermal processing, and these processes can be considered as alternatives to thermal pasteurization of beer. The alternative methods can achieve higher log reductions in ascospores, with shorter lower processing times or less energy compared with conventional thermal processing.

The results of thermal inactivation of *S. cerevisiae* ascospores in beer demonstrated first-order kinetics and almost similar thermal resistance across different strains of *Saccharomyces* yeast ascospores. Weibull model was suitable to predict the nonlinear concave upward inactivation curves of *S. cerevisiae* ascospores in beer by HPP and TS. The kinetic parameters will assist in the design of appropriate thermal pasteurization conditions for preserving beer.

S. cerevisiae ascospore inactivation by nonthermal HPP was not significantly different in carbonated and degassed beer. Higher alcohol content beer showed higher inactivation of ascospores in beer with ≥6.0 log for 7.0%, 4.8 log for 4.8% alc/vol, and 3.0 log for 0.0% alc/vol beers after 10 min process at 400 MPa. Regardless of the level of alcohol content, *S. cerevisiae* yeast ascospore inactivation was greater with TS than room temperature ultrasound, with a maximum of 3.7 log reductions after 55°C and 20 min of treatment. TS at 50°C for 3.0, 1.9, and 4.5 min could deliver the minimum pasteurization of beer with 0.0, 4.8, and 7.0% alc/vol, respectively. Thermal-assisted PEF (43°C≤T≤53°C) led to approximately 2 more log reduction in yeast ascospores compared with nonthermal PEF. Moreover, 50°C PEF-treated beer for 70 μs presented higher yeast ascospore inactivation than 50°C thermal treatment for 12×10^8 μs (=20 min), a much higher treatment time.

The inactivation of S. *cerevisiae* ascospores by HPP, PEF, and thermal processing was higher in the beers with higher alcohol content confirming that ethanol content is one of the drivers of microbial inactivation. However, beer alcohol content seemed to have less effect on spore inactivation by TS.

The taste assessments of beer treated by HPP, TS, and PEF revealed that HPP treated beer was not significantly differentfrom the untreated beer, which demonstrates that nonthermal HPP technology is a suitable option for beer pasteurization. Furthermore, ascospore inactivation in beer by HPP was the most efficient technology in terms of energy consumption among the emrging technologies investigated.

With respect to scanned electron microscope observations of the spores, the images of free dead spores of *S. cerevisiae* showed different damage and levels of destruction with particular characteristics. Both thermal and nonthermal HPP treatments initially affect the spore wall. The spore wall disruption resulted in the release of intracellular components from the spore core, which can be visible in certain images.

Recommendations for future work

The research on nonthermal pasteurization of alcoholic beverages and the effect on spoilage microorganisms is expanding over the years. Yet more studies of microbial inactivation in different beverages are needed to elucidate the industrial feasibility of these novel technologies. The mechanisms of inactivation of yeast ascospores and other spores of relevance in foodstuffs by nonthermal and thermal methods using scanned electron microscopy is still an open area of research. More studies or different methods/equipment (for example Focused Ion Beam) to investigate the conformational changes of spores before and after processing could provide further understanding of how spores are inactivated by different methods, including chemical methods.

Modelling *S. cerevisiae* ascospore inactivation as the most heat-resistant spoilage microorganism in beer is important for the basis of process design. However, other microbes important for the beer industry may present different kinetics. More studies are required on the inactivation kinetics of other beer spoilage microorganisms based on the processing technology employed. Work on other microbes would further supplement the inactivation kinetics database of beer pasteurization.

Future studies on spore inactivation mechanisms can be further supported through the analysis of the spore intracellular components.

More engineering research is required for optimal reactor design especially for PEF systems to process carbonated beverages, and also for continuous operation of PEF and ultrasound with higher throughputs. Furthermore, the optimization of ultrasound and PEF processing conditions to minimize any detrimental effects on the sensory quality of beer should be investigated. The capital investment on HPP, ultrasound, and PEF technologies at a commercial scale, together with the energy requirements for beer pasteurization, are also

significant areas of research that could be further explored in order to determine the cost effectiveness of these methods for the beer industry.

Publications and presentations based on thesis work

Publications and presentations based on thesis work

Refereed articles

Milani, E. A., Alkhafaji, S., Silva, F. V. M. (2015). Pulsed Electric Fields continuous pasteurization of beer. *Food Control,* 50, 223-229.

Milani, E. A., Gardner, R., Silva, F. V. M. (2015). Thermal resistance of *Saccharomyces* yeast ascospores in beers. *International Journal of Food Microbiology*, 206, 75-80.

Milani, E. A., Ramsey, J. G., & Silva, F.V.M. 2016. High pressure processing and thermosonication of beer: comparing the energy requirements and *Saccharomyces cerevisiae* ascospores inactivation with thermal processing and modelling. *Journal of Food Engineering.*

Submitted articles

Milani, E. A. & Silva, F.V.M. 2016. Ultrasound pasteurization of beers with different alcohol levels: Modelling the inactivation kinetics of *Saccharomyces cerevisiae* ascospores.

Milani, E. A. & Silva, F.V.M. 2016. High pressure processing nonthermal pasteurization of beer: Modelling the inactivation of *Saccharomyces cerevisiae* ascospores in different alcohol beers.

Conference presentations

Milani, E. A., Alkhafaji, S., Silva, F. V. M., (2013). Pulsed Electric Fields pasteurization of different types of beer. Poster presentation at Innovation Food Conference (iFOOD) 2013, Hannover, Germany.

Milani, E. A., Gardner, R., Silva, F. V. M., (2013). Thermal resistance of *Saccharomyces* yeast ascospores in beers. Poster presentation at CHEMECA 2013 conference, Brisbane, Australia.

Milani, E. A. & Silva, F. V. M., (2015). Pasteurization of beer by Pulsed Electric Fields, High Pressure Processing, and Power ultrasound. Oral presentation at ICEF12 conference 2015, Quebec City, Canada.

Silva, F. V. M., Milani, E. A, & Carr, K. (2015). Beer pasteurized by pulsed electric fields, high pressure processing and power ultrasound: taste assessments. Poster presentation at ICEF12 conference 2015, Quebec City, Canada.

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Appendices

Appendix A – Units specifications

HPP unit specifications

1. High voltage pulse generator
Output peak power 4.5 MW

Output peak voltage 30 kV (+15 kV – 15 kV relative to ground)

Output peak current 150 A

Nominal load impedance 200 Ω

Output waveform: positive pulse, pause, negative pulse

Pulse width 1.5 μs

Pause duration 2 μs

Peak energy per double pulse 9 J

Maximum repetition frequency 950 Hz

Peak average output power 1.8 kW

2. Variac
Input 400 V, 50 Hz, output 0-450 V 10 A C/B 10 A

TSL Transformer Specialties Ltd.

3. Piston pump
FMI lab pump, Model QV

240 V, 50 Hz

Maximum flow 4 L/min (60 gal/hr) in forward and reverse directions

Maximum pressure 100 psig (6.9 bar)

4. Signal conditioner
Multi-channel system (4 or 8)

Precision (0.025% FS)

Resolution (0.01% FS)

20 Hz sampling rate

RS-232 and voltage outputs

Data logging capabilities (50000 samples)

Large Vacuum Fluorescent Display (VFD)

½ DIN enclosure

150 ms switching rate

5. Fiber optic temperature sensors

Response time (< 1 ms)

Diameter size of 150 microns

A thickness of 100 microns

High accuracy (better than 0.3^{o}C)

6. Water bath
Grant LTD20G, -30 to 100^{o}C

1 kW/50-60 Hz

Grant Instruments (Cambridge) Ltd. England

7. Rogowski coil
C2G

400 W/50-60 Hz

8. Variable transformer
The Zenith Electronic Co. Ltd.

England

Type 608 M

Input 240 V

Output 0-270 V

No. 7896

Figure A1. PEF unit and treatment chamber at the University of Auckland

HPP unit specifications

1. QFP 2L technical specification

Maximum vessel pressure 100,000 psi (6900 bar)

Inner vessel diameter 4.0 in (100 mm)

Inner vessel height at maximum pressure 10.0 in (254 mm)

Maximum temperature 194°F (90°C)

Minimum temperature 50°F (10°C)

2. Electrical power

Choice of 3 ph, 60 Hz, 440-480 VAC, 50 amps, 415 KVA or

3 ph, 50 Hz, 380 VAC, 60 amps, 415 KVA

3 ph, 50 Hz, 220 VAC, 110 amps, 415 KVA

3 ph, 60 Hz, 220 VAC, 110 amps, 415 KVA

3. Process module

Dimensions 54.0 in wide x 72.0 in deep x 58.0 in high (1372 x 1830 x 1473 mm)

Weight 4,000 lbs (1800 kg)

4. Control module

Dimensions 21.0 in wide x 12.0 in deep x 6.0 in high (534 x 305 x 153 mm)

Weight 33 lbs (15 kg)

5. Compressed air 100 psi and 5 SCFM for value actuation and top closure air cylinder

6. Plant water 10 gpm (38 lpm); 86°F (30°C) maximum; 30 psi (2 bar) minimum

Figure A2. HPP Avure machine at University of Auckland.

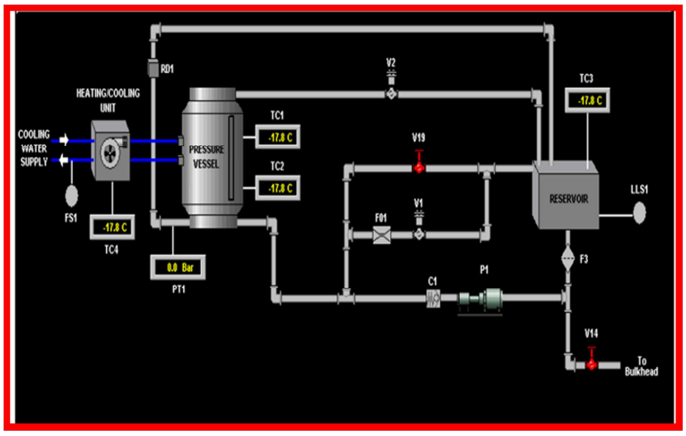

Figure A3. Flow schematic diagram of the machine.

Power ultrasound unit specifications (UP200S/UP400S)

1. Technical specification

Efficiency > 90%

Working frequency 24 kHz

Control range ±1 kHz

Output control 20%... 100%, steplessly adjusted

2. Electrical data

Connected load 200… 240 V AC, 48… 63 Hz or

100 -130 V AC, 48-63 Hz

Fuses primary internal 230 V: 2 A (for UP200S); 230 V: 4A (for UP400S)

Usable/ nominal output 110 V: 4 A 180-200 W depending on the probe (for UP200S); 110 V: 8A 300-400 W depending on the probe (for UP400S)

Maximum energy density 12…600 W/cm^2 depending on the probe

Maximum amplitude 12…260 µm depending on the probe

3. Device parameters

Dimensions 300 x 210 x 100 mm for UP200S

30 x 210 x 145 mm for UP400S

Mass 2.35 kg for UP200S

3.8 kg for UP400S

Figure A4. Thermosonication unit at University of Auckland

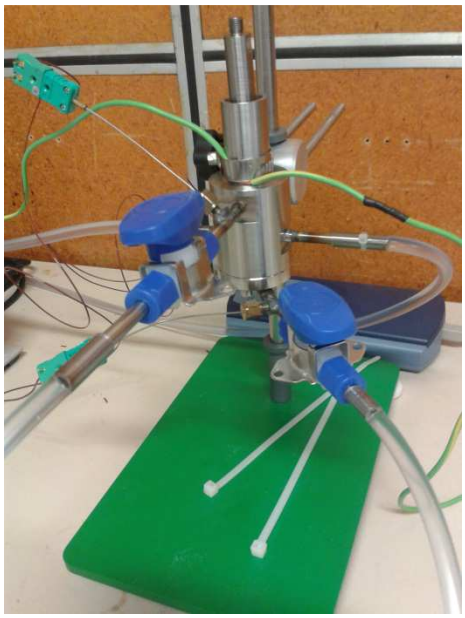

Figure A5. Flow chamber for ultrasound treatment.

Appendix B

This appendix summarizes an example of the observed data after thermal processing. Results, which belong to *S. cerevisiae* ascospores DSMZ 1848, DSMZ 70487, and ATCC 9080 at 55°C in 4.0% alc/vol beer in Chapter 2 (Figure 2.1 and Table 2.2) are presented below.

In order to plot the thermal treated lines of *S. cerevisiae* spores, results (log N/N_0) from two replicates were averaged ±SD (each replicate was the mean value of two processed samples). For example for DSMZ 1848 at 55°C and 10 min in Figure 2.1, average of -0.78 ((-0.66-0.90)/2) and SD of 0.17 were used.

DSMZ 1848			
Treatment time (min)	Mean log (N/N_0)	Predicted log (N/N_0)	SD
0	0	0	0
10	-0.781570963	-0.357371	0.1718539
20	-0.815044357	-0.714742	0.1245153
30	-1.346118311	-1.072113	0.2249047
60	-1.903089987	-2.144227	0.5627722
DSMZ 70478			
Treatment time (min)	Mean log (N/N_0)	Predicted log (N/N_0)	SD
0	0	0	0
10	-0.523845995	-0.388103	0.0410065
20	-0.920818754	-0.776205	0
30	-1.494037243	-1.164308	0.368332
60	-2.092922703	-2.328616	0.5839609
ATCC 9080			
Treatment time (min)	Mean log (N/N_0)	Predicted log (N/N_0)	SD
0	0	0	0
10	-0.411775722	-0.511231	0.0527739
20	-0.914651886	-1.022461	0.0559896
30	-1.594162858	-1.533692	0.0535426
60	-3.089660115	-3.067383	0.054458

Appendix C

This appendix summarizes an example of the observed data and Weibull modelling results after HPP processing. Results, which belong to *S. cerevisiae* ascospores DSMZ 1848 at 200, 300, and 400 MPa in 4.8% alc/vol beer in Chapter 4 (Figure 4.1 and Table 4.1) are presented below. The MSE values were calculated using the following equation:

$$MSE = \sum (\text{predicted values} - \text{observed values})^2 / (n - p)$$

In order to plot the HPP lines of *S. cerevisiae* spores, results (log N/N_0) from two replicates were averaged ±SD (each replicate was the mean value of two processed samples). For example for HPP at 400 MPa and 10 min in 4.8% alc/vol in Figure 4.1, average of -4.89 ((-4.92-4.86)/2) and SD of 0.04 were used.

Treatment pressure (MPa)	Treatment time (min)	Mean $\log(N/N_0)$	Predicted $\log(N/N_0)$	SD
200 MPa	0	0	0	0
	1	-1.659379381	-1.35943	0.055989596
	1		-1.35943	
	2	-0.99148333	-1.535397	0.01204439
	2		-1.535397	
	5	-1.893639923	-1.803443	0.138253384
	5		-1.803443	
	10	-2.216134019	-2.036883	0.050456247
	10		-2.036883	
	20	-2.422635896	-2.300539	0.064891453
	20		-2.300539	
	30	-2.41195437	-2.470318	0.156870755
	30		-2.470318	
	40	-2.507062321	-2.598324	0.212860351
	40		-2.598324	
300 MPa	0	0	0	0
	0.5	-1.716134019	-1.884562	0.656650534
	0.5		-1.884562	
	1	-2.510681526	-2.219271	0.05962387

Treatment pressure (MPa)	Treatment time (min)	Mean $\log(N/N_0)$	Predicted $\log(N/N_0)$	SD
	1		-2.219271	
	5	-3.043093074	-3.243899	0.060942809
	5		-3.243899	
	10	-3.718615728	-3.820035	0.057749444
	10		-3.820035	
	15	-4.236830361	-4.20339	0.760649411
	15		-4.20339	
	20	-4.824875991	-4.498496	0.041006499
	20		-4.498496	
	30	-4.768262545	-4.949937	0.046782705
	30		-4.949937	
400 MPa	0	0	0	0
	0.17	-3.247425011	-3.017516	0.212860351
	0.17		-3.017516	
	0.5	-2.941298058	-3.434091	0.058928303
	0.5		-3.434091	
	1	-4.048455007	-3.731613	0.068525727
	1		-3.731613	
	3	-4.05731939	-4.256863	0.055989596
	3		-4.256863	
	5	-4.698970004	-4.525671	0
	5		-4.525671	
	10	-4.893639923	-4.917766	0.038436672
	10		-4.917766	

Appendix D

This appendix summarizes an example of the observed data after thermosonication processing. Results, which belong to *S. cerevisiae* ascospores DSMZ 1848 at 43, 50, and 55°C in 4.8% alc/vol beer in Chapter 5 are presented below.

Treatment time (min)	Mean log(N/N_0)	Predicted log(N/N_0)	SD	T (°C)
0	0	0	0	43°C
0.5	-0.222368575	-0.174159	0.056855866	
0.5		-0.174159		
3	-0.70973227	-0.50107	0.345789696	
3		-0.50107		
5	-0.30036082	-0.677243	0.112925549	
5		-0.677243		
10	-1.168841361	-1.019275	0.054457959	
10		-1.019275		
20	-1.222368575	-1.534045	0.056855866	
20		-1.534045		
30	-2.20973227	-1.948481	0.361317085	
30		-1.948481		
40	-2.501194761	-2.308792	0.098755943	
40		-2.308792		
60	-2.767046566	-2.932533	0.318176137	
60		-2.932533		
0	0	0	0	50°C
0.5	-0.163242411	-0.874787	0.183591303	
0.5		-0.874787		
2	-1.503642047	-1.366899	0.054334106	
2		-1.366899		
3	-1.580256235	-1.557504	0.054014717	
3		-1.557504		
4	-1.939663395	-1.708651	0.074059077	
4		-1.708651		
5	-1.794863128	-1.83592	0.088345028	
5		-1.83592		
10	-2.673344104	-2.294937	0.05354263	
10		-2.294937		
20	-2.872314108	-2.868718	0.227843448	
20		-2.868718		

Treatment time (min)	Mean log(N/N_0)	Predicted log(N/N_0)	SD	T (°C)
30	-2.993833132	-3.268742	0.055989596	
30		-3.268742		
0	0	0	0	55°C
0.5	-1.317741873	-1.390003	0.337375675	
0.5		-1.390003		
1	-1.882908758	-1.668586	0.212860351	
1		-1.668586		
2	-1.654725741	-2.003001	0.051301069	
2		-2.003001		
3	-1.955755737	-2.228886	0.051301069	
3		-2.228886		
5	-3.012727413	-2.550077	0.029269048	
5		-2.550077		
10	-3.261389733	-3.06116	0.103328125	
10		-3.06116		
20	-	-3.674674	0	

Appendix D

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Fig. 1. The surface morphology of spores is distinct from that of vegetative cells. Vegetatively growing yeast cells (a), asci (b), isolated spores (c), and

Title or numeric reference of the portion(s)	germinating spores (d) were examined by SEM. Representative cells from each type are shown. Over 100 cells of each class were examined. Arrowheads in (c) indicate interspore bridges. Arrows in (d) indicate germinating cells. Scale bars, 1 mm, Fig. 2. Individual spores are connected by interspore bridges. (a) An interspore bridge as seen by SEM. (b) Higher magnification of spores in (a). (c, e) TEM images of interspore bridges. (d, f) Higher magnification of bridges in (c) and (e), respectively. Representative cells from over 200 cells examined are shown. Arrowheads in (d) and (f) mark the position of branches in the outer spore wall layers. Scale bars: (a) 1 mm; (bâf) 200 nm.
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	(f) mark the position of branches in the outer spore wall layers. Scale bars: (a) 1 mm; (bâf) 200 nm.
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