Packaging Sterilization: Aseptic Filling Technology

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ABSTRACT

Xenos Ltd. is a technology driven food company, that specializes in aseptic processing and packaging beverage products in bottles. Their aseptic filling technology is based on packaging sterilization with combined treatments of oxidizing agents and Ultraviolet radiation. Recent research studies have suggested that there is a synergistic effect of hydrogen peroxide (0.5 – 1 %) plus UV on inactivation of microorganisms including spores. Advantages of the combined treatment include rapid inactivation, minimum hydrogen peroxide residue in products, with the method being applicable to a wide range of packaging types. Based on this principle, a unique aseptic packaging technique has been developed by Xenos Ltd., which utilizes the combination of vaporized Perform (a commercial sterilizing agent manufactured by Orica Chemnet containing 25% hydrogen peroxide and 5% peracetic acid) and UV radiation at 7.5 – 12.5 W/m^2.

The aim of the project was to improve and validate the effectiveness of the packaging sterilization process through challenge tests. Challenge tests were conducted using *Bacillus subtilis* spores as the test microorganism to determine the log reductions delivered by the packaging sterilization system. The tests were firstly carried out on a pilot plant scale aseptic filling machine, in order to test the sterility of the small scale system, and investigate processing parameters (operational conditions) which could

affect and improve sterility. The established operational conditions for achieving target sterility were used for designing and modifying an upgrade aseptic packaging system. Finally validation of the upgrade packaging sterilization system was conducted through challenge tests to prove sterility.

It is highly recommended that in order to ensure sterility, the packaging sterilization system with vaporized Perform plus UV treatment must meet the requirements listed below during the sterilization process:

- Hydrogen peroxide concentration of Perform condensate on bottles (after steaming) is best within 0.5 – 1 %;
- Perform loading level should be minimum 300 mg/bottle after vaporized Perform treatment;
- UV treatment time applied is greater than 2 seconds during UV treatment;
- At least 20 seconds of penetration time (time between Perform treatment and UV treatment) should be allowed.

The upgrade sterilization system used by Xenos Ltd. has been improved to meet the above operational conditions. With spore loading level of 10^6 per bottle and 10^5 per cap, the system is able to deliver at least a 6 log reduction of *B. subtilis* spores on PET or glass bottles and a 5 log reduction on bottle caps. Moruzzi et al. (2000) stated that at least a 4 log reduction is commercially required for an aseptic packaging process. Therefore, the system's sterility would meet the commercial acceptable sterility.

1. INTRODUCTION

This project was sponsored by Xenos Ltd based in Palmerston North, New Zealand, an innovative food manufacturer for aseptic processing and packaging of beverages into plastic or glass bottles. Their patent pending technology is based on the synergistic effect of oxidising agents plus UV light on the destruction of micro-organisms. With the technology, the company has developed a unique technique for sterilizing bottles and caps to ensure the sterility and shelf life of the products. The design of the aseptic processing and packaging system enables the company to produce a wide range of products including dairy & soy drinks, vegetable & fruit juices, smoothies, cream etc. in various packaging styles.

Most common packaging sterilization techniques used in the food industry are thermal treatment, chemical treatment, and radiation. However, there are limitations when using these treatments to sterilize food packaging materials. For example, thermal treatments only apply to heat resistant packaging materials, chemical treatments and ultraviolet radiation have limited effectiveness on certain microorganisms such as bacterial spores, and also difficulties raised on minimizing chemical residues in food products after chemical sterilization. Under these circumstances, recent research groups such as Bayliss & Waites (1979a&b) and Gardner & Shama (1998) demonstrated significant improvements on sterilization

effectiveness when using combinations of these treatments. From their reports, the combination of oxidizing agents (chemical treatment) and UV light (radiation) has shown the advantage of rapid inactivation of microorganisms (including spores) with minimum chemical residue in food products. In addition, this technology is applicable to a wide range of packaging types.

The aim of this project was to study, improve and validate the packaging sterilization system used by Xenos Ltd, which involves the combination of Perform (manufactured by Orica Chemnet - a solution containing 25% hydrogen peroxide and 5% peracetic acid) and UV radiation of wavelength 200-280 nm. The aseptic packaging process applied by this company involves 4 main steps:

- Steaming - bottles are steamed with vaporized Perform containing 0.5 – 1% hydrogen peroxide;
- UV treatment – UV lamps are inserted into bottles;
- Drying – Condensates on bottles from steaming are dried by micro filtered air at 40 – 50 °C.
- Filling – Sterilized bottles are filled with products.

To validate the packaging sterilization process described above, challenge tests would be required to ensure acceptable commercial sterility can be achieved using a pilot plant scale aseptic filler and a newly installed upgraded aseptic filler.

2. AIM AND OBJECTIVES

2.1 Aim

To improve and validate the effectiveness of the packaging sterilization process used by Xenos Limited through challenge tests.

2.2 Objectives

The objectives of the project were:

- To review the sterilization effectiveness of UV radiation, hydrogen peroxide, peracetic acid, and their synergistic effects on bacterial spores;
- To select a suitable test microorganism and establish a methodology for challenge tests;
- To challenge the sterilization effectiveness of the pilot plant scale sterilization system;
- To investigate factors influencing the effectiveness of the packaging sterilization system, and improve the sterilization process;
- To validate the upgrade packaging sterilization system via challenge tests.

3. LITERATURE REVIEW

3.1 Introduction

Packaging is playing a very important role in food and beverage industry. It provides consumer convenience and safety from physical, microbiological and chemical hazards. It also meets the demand of processed food & beverages with prolonged shelf life. Aseptic packaging is one of the new methods used in food & beverage industries that, allows the storage of long shelf life products under ambient temperature. According to Robertson (1993), aseptic packaging can be defined as the filling of product into sterile containers under aseptic conditions and sealing of the containers to avoid recontamination or infection of the product. The complete process is often called “aseptic processing” or “aseptic technology”.

Packaging sterilization methods used most commonly include thermal treatment (including saturated steam and superheated steam); radiation (such as UV light, infrared light, ionizing light, light pulse); chemical methods (hydrogen peroxide, ethylene oxide, peracetic acid, propiolactone, ethanol, ozone, chlorine and it’s oxide) (Ansari & Datta, 2003).

Hydrogen peroxide is one of the most popular chemical sterilants for packaging sterilization in food industry, because it has both bactericidal and sporicidal properties and leaves no long term toxic residues (Rutherford et al., 2000). Another strong chemical sterilant used in food packaging sterilization is peracetic acid. Its lethal effectiveness against bacterial spores even at lower temperatures has been proven by past scientific studies (Leaper, 1984; Binet & Gutter, 1994). UV radiation is known to be capable of inactivating a wide range of microorganisms by causing the formation of thymine dimers, such dimer formation, unless repaired could cause cell death. UV radiation is commonly used for water treatment and surface sterilization. However, the sterilizing effectiveness of these three sterilizing agents is limited by factors such as temperature, process efficiency, packaging material conditions, health and safety, maximum residue level permitted by food safety standards, and impact on product quality. Such limitations can be overcome by combined treatments e.g. combinations of hydrogen peroxide and peracetic acid, or combination of hydrogen peroxide and UV radiation. This is also known as hurdle technology, which employs the intelligent combination of different sterilization factors or techniques to achieve synergistic, multi-target and reliable sterilization effects. Because low concentrations or mild treatments can be used by either component of the synergistic system, this technology enables food manufacturers to produce safe, stable, nutritious, tasty, and economical food products.

The objectives of the literature review are to understand the concepts of aseptic

packaging process; study the sterilization techniques using hydrogen peroxide, peracetic acid and UV radiation, including their advantages, limits and synergistic effects; and compare the resistances of common microorganisms against UV plus hydrogen peroxide sterilization. The review is organized into subheadings:

- Aseptic Packaging in Beverage Manufactures;
- Sterilization of Packaging Materials with hydrogen peroxide, Peracetic Acid, UV, and their Combinations;
- Resistances to UV plus hydrogen peroxide Treatment of Common Spoilage Causing Microorganisms in Beverage Manufacturing**.**

3.2 Aseptic Packaging in Beverages Manufacturing

Aseptic packaging has advantages of having low distribution and storage costs, extended product shelf life, relief of pressure on chilled cabinet, and freedom from additives, which are of benefit to both consumers and distribution channels (Ansari & Datta, 2003). Aseptic packaging can be used in the packaging of a wide range of products e.g. milk, juice, concentrates, wine, tea, mineral water, nutritional beverages, etc. In the current beverage industry, aseptically packed products can be grouped as two categories: (a) sterile products, e.g. milk and dairy products, fruit and vegetable juices; (b) non-sterile products such as fermented dairy products e.g. drinking yogurt. The aseptic system must be carefully designed to meet the commercial acceptable

sterility, especially for low acid beverages (Ansari & Datta, 2003).

The main purposes of using aseptic packaging are (Robertson, 1993):

- To enable the use of containers which are not suitable for in-pack sterilization;
- To incorporate with the high-temperature-short-time (HTST) sterilization process, which leads to products with a superior sensory and nutritional quality;
- To extend product shelf life under normal temperature storage conditions.

Typically the form of the process can be outlined as below (Holdsworth, 1992):

1) Sterilization or pasteurization of the food product such as UHT process;
2) Sterilization of the packaging material. For metallic containers, superheated steam is commonly used; and chemical sterilants or irradiation treatments are often applied for plastic based materials;
3) Filling the product into the pre-sterilized packaging under aseptic conditions and finally sealing.

In addition, there are four criteria suggested by Robertson (1993) that any aseptic system should meet:

- Able to ensure an effective sterilization of the line before and after use;
- Able to aseptically transfer the product through the processing system;
- Able to be operated during the filling, sealing and critical transfer processes in

a sterile environment.

3.3 Sterilization of Packaging Materials with Hydrogen Peroxide, Peracetic Acid, UV, and their Combinations

Sterilization of packaging materials is a crucial step in any aseptic packaging process, because it directly affects the shelf life, safety, and quality of the products. According to Ansari and Datta (2003), sterilization of packaging materials should meet the following requirements:

- Rapid lethal activity;
- Compatible with packaging materials and equipment;
- Chemical sterilants used must be easily removed with minimum residues;
- No health hazard to the consumer;
- No adverse effect on product quality;
- No health hazard to operation personnel;
- Environmental friendly;
- Non-corrosive to the treated surface and equipment;
- Reliable and economical.

3.3.1 Hydrogen Peroxide

Hydrogen peroxide is commonly used as a sterilant for packaging materials in food industry. Typical hydrogen peroxide treatments in aseptic packaging processes involve applying 30 to 33% of hydrogen peroxide on packaging materials by dipping, spraying or rinsing, and followed by removing excess hydrogen peroxide via rollers or air blasts and hot air drying (Reuter, 1988). Wetting of the packaging material by hydrogen peroxide solution and forming a uniform film of liquid on the material surface are essential factors for good sterility (Ansari & Datta, 2003). Recent studies have shown some benefit in the decontamination of surface contaminants by hydrogen peroxide vapour (HPV). One benefit of HPV is potential to decontaminate hard-to-reach surfaces as well as complex surfaces, giving good surface coverage of the materials (Johnston et al., 2005).

Rutherford et al. (2000) suggested the inactivation mechanisms of hydrogen peroxide are different between spores and vegetative cells. For spores the targets of killing could be peroxide sensitive enzymes, since the DNA of spores could be protected by small, acid soluble, spore proteins (SASP) attached to DNA, whereas the killing of vegetative cells by hydrogen peroxide could be due to their DNA damage (Marquis and Baldeck, 2007).

According to Nelson et al. (1987), effectiveness of spore inactivation depends on the

temperature, concentration of hydrogen peroxide, and exposure time. At room temperature and low concentration hydrogen peroxide is very weak in terms of killing spores, but as the temperature increases its potency increases dramatically (Rutherford et al., 2000). It had been reported that at 25ºC 0.1% v/v hydrogen peroxide was not lethal to bacterial spores over 3 hours, whereas it was lethal at 50ºC, and at 125ºC, 15-20% v/v peroxide could produce adequate decontamination of packaging board in 10 seconds (Swartling and Lindgren, 1962). Marquis and Baldeck (2007) indicated that at constant temperatures the peroxide killing was dependent on dose, which is exposure time × [concentration]. Thus, without elevated temperature a high concentration and long exposure time are crucial for a good lethal effect. For example, at room temperature 15% peroxide and 20 min exposure time were required to achieve a 3 log reduction of *Bacillus subtilis* var. *globigii* (Smith and Brown, 1980). However, in the literature, there appears to be no published model for the inactivation efficiency as a function of three variables concentration, temperature and time.

Problems arise with packaging sterilization using hydrogen peroxide alone. Due to the relatively high concentration of hydrogen peroxide used, it becomes impractical because of the difficulties in removing the excess in order to meet certain food safety standards. FDA permits only 0.5 ppm of hydrogen peroxide residue in a container (David et al., 1996). According to Food Standards Australia New Zealand (2008), the maximum permitted level of hydrogen peroxide is 5 ppm in a container. Stannard & Wood (1983) found that by drying 0.1 ml 30% hydrogen peroxide with hot air at

100ºC for 10 seconds, the hydrogen peroxide residue was still 100 times higher than the FDA standard. Moreover, residue of hydrogen peroxide, including that trapped in the packaging headspace at the time of sealing, can cause a major impact on the nutritional quality of food products. When the concentration of hydrogen peroxide exceeded 0.1ppm in a container, significant ascorbic acid degradation occurred in bottled orange juice and orange juice concentrates (Toledo, 1975). Therefore, it is not desirable to use hydrogen peroxide to sterilize the packaging of food products containing ascorbic acid, unless the hydrogen peroxide vapour in packaging headspace can be removed and the packaging material is completely free from hydrogen peroxide residues. In addition, hydrogen peroxide is classified as a hazardous material. According to Nelson et al. (1987), concentrated hydrogen peroxide can be a fire hazard by generating sufficient oxygen. Hydrogen peroxide is also a strong oxidizing agent, which causes skin irritation and eye damage. Therefore, handling and storage of this material become difficult, and require the use of corrosion resistant materials such as glass, stainless steel, polyethylene and alloys of aluminum.

3.3.2 Peracetic Acid

Peracetic acid (CH_3COOOH) is a strong oxidizing agent and particularly effective against bacterial spores. According to Leaper (1984), peracetic acid is more effective

than hydrogen peroxide at room temperatures. The research demonstrated a concentration of 0.1% peracetic acid was able to achieve a similar lethal effect as 23.6% hydrogen peroxide at 20ºC, and the effectiveness was significantly improved by increasing the temperature from 20ºC to 40ºC. Binet & Gutter (1994) found that 0.5 – 1% peracetic acid at 20 – 45ºC could reduce the number of various microorganisms on PVC or PET bottles by 5 to 6 log reduction within 30 s.

However, peracetic acid vapour is very pungent and irritating. Therefore, when using peracetic acid in an aseptic packaging system, environmental release of this chemical must be prevented. During the packaging sterilization process, it is very important to ensure the residues of peracetic acid on packaging materials are very low, because the presence of peracetic acid can cause unpleasant off flavours in food products (Ansari & Datta, 2003).

3.3.3 Combination of Hydrogen Peroxide and Peracetic Acid

Hydrogen peroxide is normally used in combination with peracetic acid as a sanitizer. It is effective for using on a wide range of materials and surfaces including stainless steel and polyurethane (Lindsay & von Holy, 1999). Alasri et al. (1993) suggested that the sporocidal activity of the combination of peracetic acid and hydrogen peroxide was synergistic. The combination reduced the minimum sporocidal concentration by

2-8 times than that required for the single biocides. Most of the killings by peracetic acid / hydrogen peroxide happened during the first minute of exposure (Lindsay, 1997).

Leaper (1984) was able to demonstrate the synergistic effect of hydrogen peroxide and peracetic acid against *B. subtilis* spores. This study showed that a solution containing 5.9% hydrogen peroxide and 0.2% peracetic acid at 20ºC would achieve a 4 log reduction within 1.4 min. Abreu & Faria (2004) also found that up to 7 log reduction of *B. subtilis* var. *globigii* spores could be achieved by treating the inoculated PET bottles with a combination of 1.25% peracetic acid and 1.9% hydrogen peroxide at 46ºC within 16 seconds. Moreover, increasing the temperature of the hydrogen peroxide and peracetic acid mix improved the sporicidal effect with every 10ºC temperature rise causing a halving of the D value.

Perform is a stabilized formulation of peracetic acid (5%) in hydrogen peroxide (25%). It is commercially provided by Orica Chemical Company. It has microbicidal effect against a wide range of contaminants including bacteria and their spores, yeasts, moulds, and viruses. Perform is an approved sanitizer by AgriQuality NZ for use in all food and beverages, and by NZSFA in all animal products except dairy (Orica Chemnet, 2004). Parkar et al. (2004) reported a 7 log reduction of 18 hr old biofilms of a thermophilic *Bacillus* spp on stainless steel was achieved by the treatment using 0.2% Perform solution for 5 min at 22ºC.

3.3.4 Ultra Violet Radiation

In recent years, Ultra Violet radiation has been used for water treatment and surface disinfection of a wide range of materials in food and pharmaceutical industrials. UV at 200 – 280 nm (also called UV-C) is capable of altering a living microorganism's DNA and keeping it from reproducing. UV with 264 nanometers is the peak antimicrobial wavelength, which is known as the germicidal spectrum. When the UV intensity was kept constant, there was a linear relationship between the log kill of a number of bacterial spores and the UV exposure time (Hirose et al., 1989).

Reuter (1988) summarized a number of factors affecting the effectiveness of UV irradiation:

1) Presence of dust particles which causes a shadow-effect on the surface.
2) The contamination level (number of microorganisms/cm^2) on the surface. A high contamination level (greater than $10^6/cm^2$) can cause a shadow-effect due to cell aggregations.
3) Distance from the radiation source. The closer to the radiation source, the higher the intensity of UV light hitting on the surface.
4) The geometry of the package and the radiator surface.
5) Existing air humidity. The death rate decreases when the humidity of air is over 80%. This is probably because of the formation of a protective water layer around the cell

Past studies have shown that conventional UV lamps are effective in inactivating vegetative bacteria, but they are relatively inefficient in reducing the contamination of bacterial spores as a long exposure time is required to achieve desired sterility. For example, Narasimhan et al. (1989) studied the disinfection effectiveness of UV radiation on various food packaging materials, and found that with an irradiation intensity of 50 W/m^2, mesophilic vegetative cells from milk products were eliminated by treatment for 10 min. However, bacterial spores required treatment for at least 20 min. On the other hand, high intensity UV light has been proved to be more effective. Maunder (1977) mentioned that compared with conventional UV light, high intensity UV light (1,000 – 10,000 W/m^2) could increase the rate of inactivation of certain vegetative cells, bacterial spores and molds by up to 10 times. Conversely, the costs of high intensity UV lamps are relatively high compared with conventional lamps.

Finally, the side effect of UV radiation including promoting lipid oxidation in food products and also safety issues needs to be considered for designing a sterilization process in realistic situations.

3.3.5 Combination of Hydrogen Peroxide and Ultraviolet Radiation

3.3.5.1 The Synergistic Effect of Hydrogen Peroxide and Ultraviolet Radiation

Work by Bayliss and Waites (1979 & 1980) suggested that the combination of hydrogen peroxide and ultraviolet radiation was capable of producing a 2000-fold greater kill than either hydrogen peroxide or ultraviolet radiation used alone. In addition, this synergistic effect of hydrogen peroxide or ultraviolet radiation was observed on both bacterial spores and vegetative cells.

The synergistic effect of hydrogen peroxide plus UV against bacterial spores was also reported by McDonald et al. (2000) and Rutherford et al. (2000). McDonald et al. (2000) concluded that 1% hydrogen peroxide alone could only reduce about 10% of the *B. subtilis* spores in 24 hours, and UV alone with fluence of 80 J/m2 only led to a 2 log reduction of spores. However, when UV and 1% hydrogen peroxide treatment were used concurrently, a 4 log reduction of spores dried on Petri dish surface was achieved using UV fluence of 40 J/m^2. At the same time, Rutherford et al. (2000) reported that the combination of 0.1% hydrogen peroxide and 270 J/m^2 of UV radiation led to a 7.5 log kill of *Bacillus megaterium* spores, where as UV or Hydrogen Peroxide alone only caused a 0.5 log kill or 3 log kill respectively.

Studies have also been carried out on various packaging materials and similar synergistic effects were also reported. Stannard et al. (1983) studied the combined treatment of hydrogen peroxide and ultra-violet irradiation to inactivate a variety of microorganisms on preformed food packaging cartons. It's been found that a 3.7 log reduction in *Bacillus subtilis* spores was achieved with 0.5% wt/vol hydrogen peroxide at room temperature and 10 s of UV irradiation (same UV lamp as used by Baliss and Waites), whereas UV alone had caused a 1.9 log reduction, and peroxide alone only had very little lethal effect.

This observed synergistic effect could be due to the wetting of spores by liquid hydrogen peroxide, because the resistances to UV treatment are different between wet and dry spores. Gardner and Shama (1998) reported that the rate of inactivation of wet *B. subtilis* spores using UV radiation was at least 6.7 times that of dry spores. Keller and Horneck (1992) suggested that the differential rates of inactivation could be due to the difference in the nature of the lethal photoproduct produced following irradiation for wet and dry spores. Secondly, the UV irradiation aids the breakdown of the peroxide into hydroxyl radicals, so the overall lethal effect is greater than the sum of the effects of the two agents alone (Robertson, 1993).

According to Marquis and Baldeck (2007), the inactivation mechanism is not simply due to the radicalization of hydrogen peroxide in the environment, which produces lethal hydroxyl radicals. They indicated that UV radiation could be absorbed by

hydrogen peroxide, and provide localized radicals which would inactivate some UV-hydrogen peroxide sensitive enzymes. The damage may also involve direct oxidation without production of the OH radical. Marquis and Baldeck (2007) also found that a set of enzymes within the spore core, such as glucose 6-phosphate dehydrogenase, were very sensitive to UV-hydrogen peroxide inactivation. Unlike the general protein damage caused by the ionizing radiation, the damage only occurs at certain sites on certain enzymes with the most likely being the histidine residues with sensitive sulfhydryl groups. Reidmiller et al. (2003) investigated the targets for damage of bacterial spores by the combined treatment of hydrogen peroxide + UV inactivation. Their experimental data showed that using the combined treatment, there were no signs of DNA damage in the bacterial spores. Therefore, proteins are very likely to be the targets for spore damage. The inactivation of enzymes e.g. glucose 6-phosphate dehydrogenase could affect the viability of the spore. The synergistic killing effects of UV combined with hydrogen peroxide could arise from cumulative damage to multiple enzyme targets.

The kinetics of UV plus hydrogen peroxide inactivation on bacterial spores was studied by Gardner and Shama (1998). Their study was carried out on filter paper and using *Bacillus subtilis* ATCC 6633 spores. Based on the assumption that the inactivation of *B. subtilis* spores by UV plus 1% v/v hydrogen peroxide follows first order kinetics, Gardner and Shama (1998) reported that when the test was done on Grade 2 filter paper which had low density of micro-fibrils, the kinetics model could

be described as:

$$Log_{10}(N/N_0) = -0.0452 \times Fluence$$

whereas N = number of survivors

N_0 = initial inoculums

Fluence (J/m2) = UV intensity × time

When the test was done on Grade 6 filter paper which had higher density of micro-fibrils, the model was described as:

$$Log_{10}(N/N_0) = -0.022 \times Fluence$$

The model for Grade 6 filter paper had a lower inactivation rate coefficient compared with that for Grade 2 filter paper. This is most likely due to the higher density of the micro-fibrils presented on the filter paper, shielding the spores from the UV light. Furthermore, their experimental data suggested that the rate of disinfection was independent of the UV intensity and only depend on UV fluence.

Work by Marquis and Baldeck (2007) determined the lethal effects of various lengths of time between hydrogen peroxide treatment and UV exposure. Marquis and Baldeck (2007) discovered that the synergistic effect was still evident when the *B. cereus* spores were UV irradiated 24 hours after they were treated with hydrogen peroxide and dried. One explanation is that bacterial spores have very high capacity to take up and retain hydrogen peroxide (Marquis and Baldeck, 2007). Another explanation is that spores are able to retard the losses of hydrogen peroxide (Rutherford et al., 2000).

This could be because most of the peroxides form combinations with spore components (called adducts), and such adduct formation slows down the decomposition of hydrogen peroxide and also evaporation (Marquis and Baldeck, 2007). Hence, spores might be able to retain hydrogen peroxide inside the cells at some levels, and thus peroxide activity could be remained for a longer period. Therefore, it was possible to separate the hydrogen peroxide exposure and UV radiation steps for up to 24 hours. This would allow easier manipulation of the sterilization technology in terms of experimental procedures, machine design and scheduling of sterilization processes.

In addition, minimization of hydrogen peroxide residues after the treatment is required in aseptic packaging process design. Stannard & Wood (1983) studied the residual hydrogen peroxide in preformed food cartons decontaminated with hydrogen peroxide and ultraviolet radiation. It was found that without hot air drying, the hydrogen peroxide residue level after 0.2 ml 1% hydrogen peroxide plus UV treatment met the Food Standards Australia New Zealand requirement (5ppm per container) but not the FDA standards (0.5ppm per container). A hot air drying step after the sterilization treatment would help reduce hydrogen peroxide residues, because the breakdown of hydrogen peroxide is faster under higher temperatures. However, the time for drying will still need to be investigated for different process conditions in terms of packaging type, packaging surface area, hydrogen peroxide droplets temperature and size on packaging surface, etc.

The combined UV plus hydrogen peroxide sterilization technique has a number of advantages which help improve the effectiveness and efficiency of an aseptic packaging system:

- Rapid inactivation which allows a faster packaging process;
- Excellent alternative for high temperature sterilization;
- Enabling cold filling of a commercially sterile food in a previously sterilized package under sterile conditions, which helps maintain the nutritional value of the products and allows the storage of the product under ambient temperature;
- Allowing the use of low heat resistance packaging types such as PET packaging;
- Being able to deliver commercially acceptable sterilities, and meanwhile minimize chemical residues which affect the products quality;
- Low cost compared with other standard aseptic packaging systems.

3.3.5.2 Factors Affecting the Lethal Effect of UV plus Hydrogen Peroxide Treatment

The lethal effect of the UV plus hydrogen peroxide treatment can be influenced by a few factors listed below, thus considerations need to be taken for validation and optimization of an aseptic packaging system.

- Concentration of Hydrogen Peroxide

Bayliss and Waites (1979a) established that the optimal hydrogen peroxide concentration for spore killing was 0.3 M (1%). This is in agreement with the finding from Gardner and Shama (1998) that the optimum killing effect by the combination of UV and hydrogen peroxide occurred when the concentration of hydrogen peroxide was in the range of 0.5 – 1% v/v. On the other hand, Bayliss and Waites (1979a) found that when the peroxide concentration increased above 0.6 M (2%), the synergistic effect started to decrease and totally disappeared at 1.5 M hydrogen peroxide (5.1%). This loss of synergism may be caused by the absorption or shielding of UV by the peroxide molecules (Symons, 1960).

- Temperature of the hydrogen peroxide applied on packaging surfaces

Reidmiller et al. (2003) showed that at 50ºC UV radiation (4.2 J/m^2) combined with 0.15% hydrogen peroxide produced a 6.7 log reduction of *B. megaterium* spores, whereas at 25ºC only a 1.5 log reduction was achieved even with a much higher UV fluence (up to 518 J/m^2). Therefore, at higher temperature, a sufficient log reduction of spores could be delivered using low concentrations of hydrogen peroxide, which then would have less shielding effect. In other words, the lethal potency can be increased and the shielding effect can be reduced by raising the temperature (Reidmiller et al., 2003).

- Presence of Scavenger

The presence of scavenger can reduce the UV-hydrogen peroxide killing (Reidmiller et al., 2003). For example, when 167 mM of pyruvate was added, the achieved log reduction on spores could be reduced by 6.4 compared with the absence of pyruvate (Reidmiller et al., 2003). Presence of transition metal cations such as $Cu^{+/2+}$ and $Fe^{2+/3+}$ could also protect spores against UV-hydrogen peroxide treatment. Data from Reidmiller et al. (2003) showed that the lethal effect of the combined treatment was reduced by about 2 log reduction with the presences of these cations. The protection only occurred when the metal cations were added before or at the same time as the addition of hydrogen peroxide. The protective effect was totally eliminated by adding the cations after the addition of hydrogen peroxide. One possible explanation for this observation was that spores rapidly absorbed the hydrogen peroxide, and the peroxide within the cells could not readily react with the metal cations outside the spores.

- Wavelength of UV radiation

Waites et al. (1988) suggested that the optimal wavelength of irradiation was approximately 270 nm in the presence of hydrogen peroxide, and the most effective UV wavelength range was between 240 to 290 nm.

- Inoculated Spore Density

Clumping, which prevents UV photons from penetrating through spores, would occur

with increasing spore density. Consequently, log reduction of the inoculated spores would be reduced. It was observed by McDonald et al. (2000) that shielding of the microbes from UV light happened when spore density was reaching 10^6 per 4 cm^2. In addition, such "shielding effect" could be different depending on the type of test surface or packaging materials. Warriner et al. (2000) also found that spore clumping only appeared to occur on aluminum and polyethylene coated packaging surfaces when spore density was greater than 10^7 per 4 cm^2. This could possibly be due to the hydrophobic nature of these surfaces, as they may have enhanced spore clumping as the deposited spores dried onto the surface.

- Smoothness, Reflectivity and Geometry of the Surface being Irradiated

The rate of inactivation is also dependent on the smoothness of the surface where the spores where inoculated on. Surface irregularities can cause shielding effects, which protect spores or cells from UV incident (Gardner and Shama, 1998). Similarly, Huang and Toledo (1982) reported that UV radiation was more effective in inactivating microorganisms on smooth surface, whereas rough surface could create "shadow" for the microorganism to survive.

Reflectivity is another factor, and it can be altered by lamination. For example, Stannard et al. (1985) established that the lethal effect against *B. subtilis* spores was reduced on aluminum laminated cartons by about 2 log reduction compared with

cartons without aluminum in the laminate. This was because that the aluminum layer may have reflected more UV light of wavelengths between 325 and 550 nm. These wavelengths are known to be capable of causing inactivation of bacterial cells previously exposed to UV irradiation (Rupert & Harm, 1966).

Furthermore, the geometry of the surface to be irradiated is also an important factor. Stannard et al. (1985) found that on aluminum laminated boards, the lethal effects of UV plus 1% hydrogen peroxide treatment increased with the angle of UV incidence as follows: 60º, 90º, and 30º. However, no clear explanation was made in the study.

3.4 Resistances to UV plus Hydrogen Peroxide Treatment of Common Spoilage Causing Microorganisms in Beverage Manufacturing

3.4.1 Common Spoilage Causing Microorganisms in Beverages

Beverage spoilage is commonly caused by a wide range of microorganisms. According to Doyle et al. (1997), common spoilage causing bacteria in beverage products include:

- Spore-forming bacteria include *Bacillus* spp. such as *B. licheniformis*, *B. cereus*, *B. subtilis*, *B. megaterium*, *B. circulans*, *B. mycoides*, *B. Stearothermophilus*, and *Alicyclobacillus* spp. which are one of the most heat resistant contaminants of fruit juices. Over 90% of contamination on packaging materials was associated with aerobic spore forming bacteria (Pirttijärvi et al., 1996). Most of the aerobic strains found on packaging materials are predominantly members of the genus *Bacillus* (Väisänen et al., 1991). Defects produced in dairy beverages are described as sweet curdling, because it first appears as coagulation without significant acid or off flavour being formed.

- Non-sporing acid-producing fermentative bacteria: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus*. Gas production, off flavour, appearance defects (ropy texture) are normally observed in the failed products.

- Coliforms: *Enterobacter*, *Klebsiella*, *Escherichia* spp.

- Molds and yeast, most commonly *Penicillium* spp. *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Candida famata*, and *C. kefyr*. Such spoilage usually happens in low acid beverage and fermented milk product. Defects include fruity or yeasty odour and/or gas formation.

3.4.2 Comparison of Resistance among Species

In terms of UV resistance, Defigueiredo and Splittstoesser (1976) indicated that spores are relatively more resistant than most of non-sporing vegetative bacteria. Bayliss and Waites (1980) showed that for the non-sporing bacteria *E. coli* and *S. faecalis*, a 4.3 log reduction could be achieved by 78 J/m^2 UV irradiation at 254nm in the presence of 1% hydrogen peroxide. Various bacterial spores were showing higher resistances as the log reductions achieved were between 2 to 4 using the same treatment (Bayliss and Waites, 1979b). Furthermore, from the research done by Bayliss and Waites (1979b) on two types of spore forming bacteria including 14 strains of *Bacillus* and 1 strain of *Clostridium*, it was found that the 14 strains of *Bacillus* spp. seemed to be more resistant than the one *Clostridium* strain against UV plus hydrogen peroxide treatment. Under the same UV plus 2.5% hydrogen peroxide treatment for 30 s followed by heating at 85ºC for 60 s, the log reduction for *Bacillus* spp. was from 4 up to 6, whereas for *Clostridium* at least a 6 log reduction was achieved. Most *Bacillus* spores can be easily handled in the laboratory, therefore, Nelson et al. (1987) indicated that *B. subtilis* spores were one of the most useful test microorganisms for UV plus hydrogen peroxide treatment.

3.4.3 Comparisons of Resistances among Strains

From literature review, the log kills under certain UV fluence for some *Bacillus* spores are summarized in Table 3.1, and plots of fluence vs. log kills for these microorganisms are shown as Figure 3.1.

Table 3.1: Literature data of lethal kill by U.V. plus 0.5 – 1 % hydrogen peroxide for different microorganisms

Strains	Fluence (J/m2)	Log Kill	Literature
B. subtilis (ATCC 6633)	0	0	Gardner & Shama (1997)
	25	-1.1	
	50	-2.3	
	75	-3.4	
	100	-4.5	
B. subtilis 706	0	0	Bayliss & Waites (1979b)
	39	-2	
	78	-4	
	117	-4.7	
	156	-5	
B. subtilis var. niger	78	-4	Bayliss & Waites (1979b)
B. subtilis 738 (ATCC 9372)	78	-4	Bayliss & Waites (1979b)
B. subtilis 713	90	-2.8	Peel & Waites (1979)
B. pumilus 312	90	-4	Peel & Waites (1979)

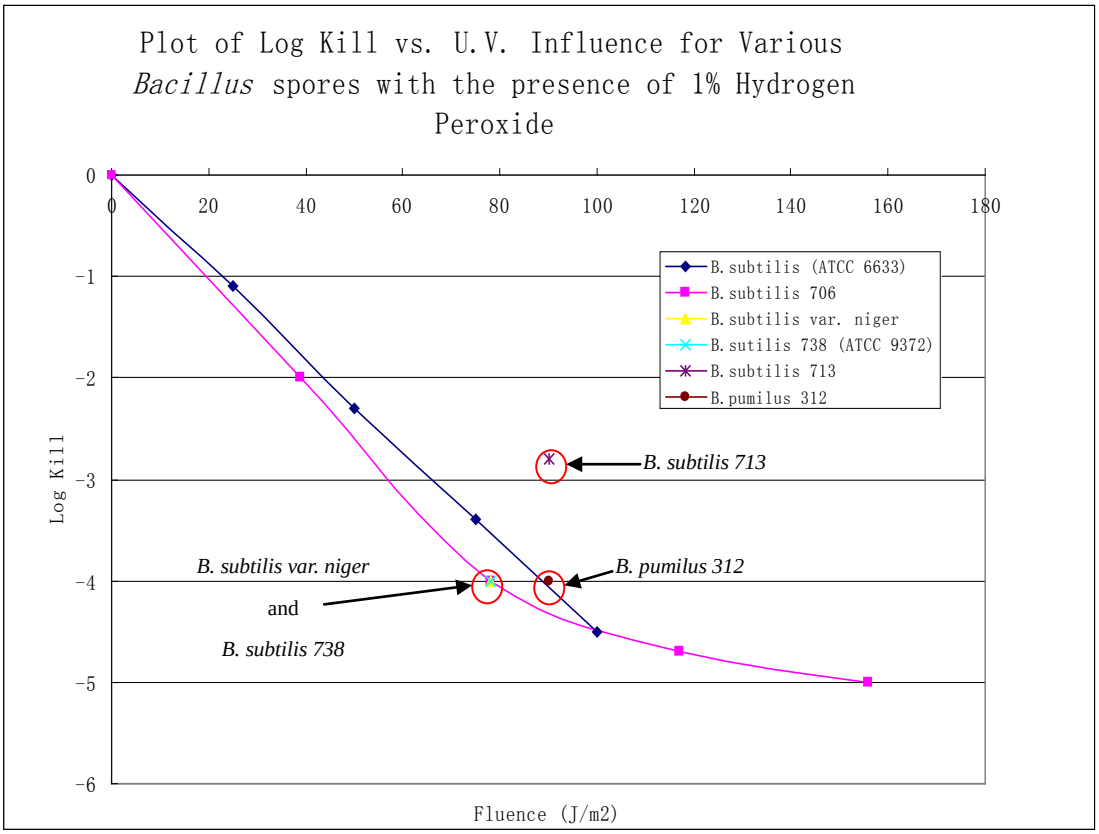

Figure 3.1: Comparison of resistance of various *Bacillus* spp. to hydrogen peroxide and UV treatment (data taken from Table 3.1)

From Figure 3.1, *B. subtilis* 713 showed the least log reduction (greatest resistance) under the same U.V. fluence among the 6 microorganisms. Under 90 J/m2 UV fluence, a log 4 reduction is achieved for most of the *Bacillus* spp., whereas only a 2.8 log reduction is reached for *B. subtilis 713*. *Bacillus subtilis* (ATCC 6633) was also relatively resistant, and there was more work published with this test microorganism (see references above for more information), including spore inactivation models using UV plus hydrogen peroxide treatment and spore preparation method. Thus, *Bacillus subtilis* (ATCC 6633) was considered the most appropriate test microorganism for this project.

3.5 Conclusions

Aseptic packaging plays an essential role in aseptic processing of long shelf life food and beverages. From the literature, common aseptic packaging methods include thermal treatments, chemical methods, and radiation. However, limitations exist when a single treatment is used in packaging sterilization. Thermal treatment is not suitable to be used for non heat resistant materials e.g. PET bottles; hence the variety of packaging type is limited. Hydrogen peroxide alone shows no lethal effect against bacterial spores at lower temperatures, and long treatment time is still needed even at some higher temperatures. Peracetic acid can only be used at very low levels since it has impacts on product quality. Conventional UV radiation alone is only suitable for sterilizing regular and smooth packaging surfaces. It also has limited lethal effect and requires long exposure time. Under these circumstances, combined treatments have been researched and practiced, and have demonstrated significant improvements in sterilization effectiveness and efficiency. The lethal effect of hydrogen peroxide at lower temperatures can be enhanced by combining with a low level of peracetic acid. Most importantly, UV plus low concentrations of hydrogen peroxide has synergistic effect on the inactivation of resistant bacteria spores. These combined treatments enable the minimization of chemical residues in the products, and achieving desirable sterilities of an aseptic packaging system at the same time. *Bacillus* spores have been recognized as common spoilage microorganisms, and they are relatively resistant to UV plus hydrogen peroxide sterilization. Among various strains of this species, *B.*

subtilis 713 appears to be one of the most resistant strains. Meanwhile, *B. subtilis* ATCC 6633 is also fairly resistant and has been used most commonly by a lot of research groups as a test microorganism for UV plus hydrogen peroxide inactivation.

From the literature review, most research work has been done on sterilization techniques using UV radiation, or hydrogen peroxide, or peracetic acid, or combinations of two of these agents. There is limited research on sterilization techniques with the combination of the three. Current project would be focusing on the utilization of vaporized Perform solution (a combination of hydrogen peroxide and peracetic acid), followed by UV treatment to sterilize commonly used packaging materials in beverage manufacturing. Finally, the outcome from this project will be compared with literature data in terms of improvement on packaging sterilization technology.

4. CHALLENGE TESTS

4.1 Introduction

The industrial acceptable sterility is less than 1/10,000 failure rate (Moruzzi et al., 2000), which theoretically means achieving a 4 log reduction by the applied sterilization process (see APPENDIX 1 for more information). In order to assess the sterilization effectiveness of a packaging sterilization system, challenge tests are required to be designed and carried out under defined operational conditions. A challenge test is a method to validate processes that are intended to deliver some degree of lethality against a target organism delivered by a specific packaging system. The method includes loading the food packaging with an unnaturally high level of a selected microorganism which is highly resistant to the applied sterilization treatment, determination of the number of survivals after the sterilization process, and calculation of the achieved log reduction. The challenge test used in this study is precisely described in Appendix 1. Based on the studies on common spoilage causing microorganisms and their resistances against UV plus hydrogen peroxide treatment (refer to Section 3.4), spores of *Bacillus subtilis* ATCC 6633 was selected to be the test microorganism.

The aim of the challenge test was to assess the sterility that could be delivered by the pilot plant scale aseptic packaging system, and then optimize the packaging sterilization technique. Hence, there are 5 main objectives to achieve the aim:

1) To conduct a challenge test and determine the sterility delivered by the pilot plant scale aseptic filler;
2) To investigate potential factors affecting the sterilization effectiveness;
3) To carry out experiments via challenge tests to optimize the sterilization technique;
4) To validate the upgrade aseptic filler under optimized sterilization conditions;
5) To make conclusions and recommendations from the validation of the upgrade aseptic system.

4.2 Experimental

4.2.1 Spore Generation and Spore Suspension Preparation

Materials

- *Bacillus subtilis* (ATCC 6633) culture (provided by New Zealand Culture Collection);
- Nutrient Agar Slop;

- Nutrient Broth (Oxoid);
- Sporulation Agar Plates (formulation referring to Bayliss & Waites (1979a));
- Sterile distilled water.

Equipment

- Rotary Incubator;
- Centrifugal machine;
- Vibratory Mixer;
- Water bath;
- Erlenmeyer flask;
- Glass Spreader and Loops;
- Petri dishes;
- Autoclave.

Method for Spore Production

The method used for spore generation was similar to that used by Gardner and Shame (1998). One loopful of *B. subtilis* culture was inoculated into Oxoid Nutrient Broth in an Erlenmeyer flask and incubated under 30ºC for 24 hours on a rotary incubator at 200 rev/min. 0.2 ml of this *B. subtilis* culture was inoculated onto each sporulation agar plate via spreading over the plates using a flamed glass spreader. The plates were incubated at 30ºC for 14 days. To harvest the spores, 5 ml of sterile distilled water was

pippetted onto the surface of each plate, and the spores were detached and suspended by scraping the plate surface with a flamed wire loop. The spore suspension from each plate was then immediately pippetted into sterile centrifuge tubes and centrifuged at 8000g for 20 min. Then, the supernatants were replaced with 10 ml fresh sterile distilled water, and the pellets were re-suspended with a vibratory mixer. This is called the washing step in order to separate the spores from undesirable agar and cell fragments from spore harvest. The washing steps were repeated for 3 times for achieve sufficient cleanness. To inactivate vegetative cells, the pellets were re-suspended with 10ml distilled water and heated treated in water bath at 70ºC for 30 min. Finally, the prepared spore suspension was stored in sterile universal bottles at 4ºC until required. Spore concentrations of the prepared suspension were tested to be $10^7 \sim 10^8$ spores/g by Standard Plate Count Method.

4.2.2 Inoculation of Spore Suspension on Packaging Materials

Materials

- Spore suspension as prepared in Section 4.2.1;
- Packaging materials from Xenos Ltd. including:
    - PET bottles and corresponding caps (Figure 4.1)
    - Glass bottles and corresponding caps (Figure 4.2)
- Sterile distilled water;

- Ethanol solution.

Figure 4.1: 250ml PET bottle & cap

Figure 4.2: 175 ml plastic bottle & cap

Equipment

- Automatic spray system (Figure 4.3);
- Scale with 3 decimal places accuracy.

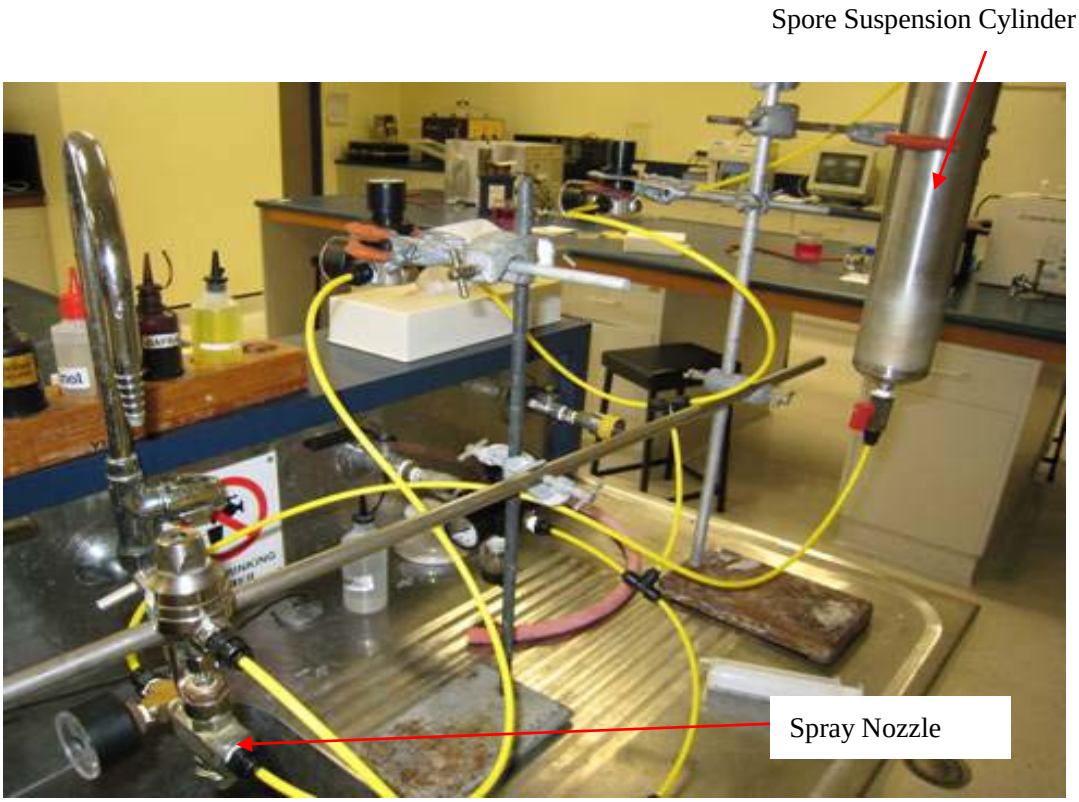

Figure 4.3: picture of the automatic spray system

The spray system was constructed using the available sources from Massey University and Xenos Ltd. A schematic diagram of the spray system is displayed in Figure 4.4. As shown by the diagram, spore suspension was mixed with compressed air in the mixing chamber of the spray nozzle, by which fine droplets of the spore suspension were formed. The spore suspension droplets were injected by compressed air in a controlled manner through the pressure adjusting valve. The pressures on the liquid side and air side of the spray nozzle were kept at 5 psi. A solenoid valve was used to switch on/off the compressed air which was responsible for activating the spray nozzle. The solenoid valve was controlled by a computer through a program called “Lab View”. Such set up enabled the number and frequency of spraying to be controlled automatically.

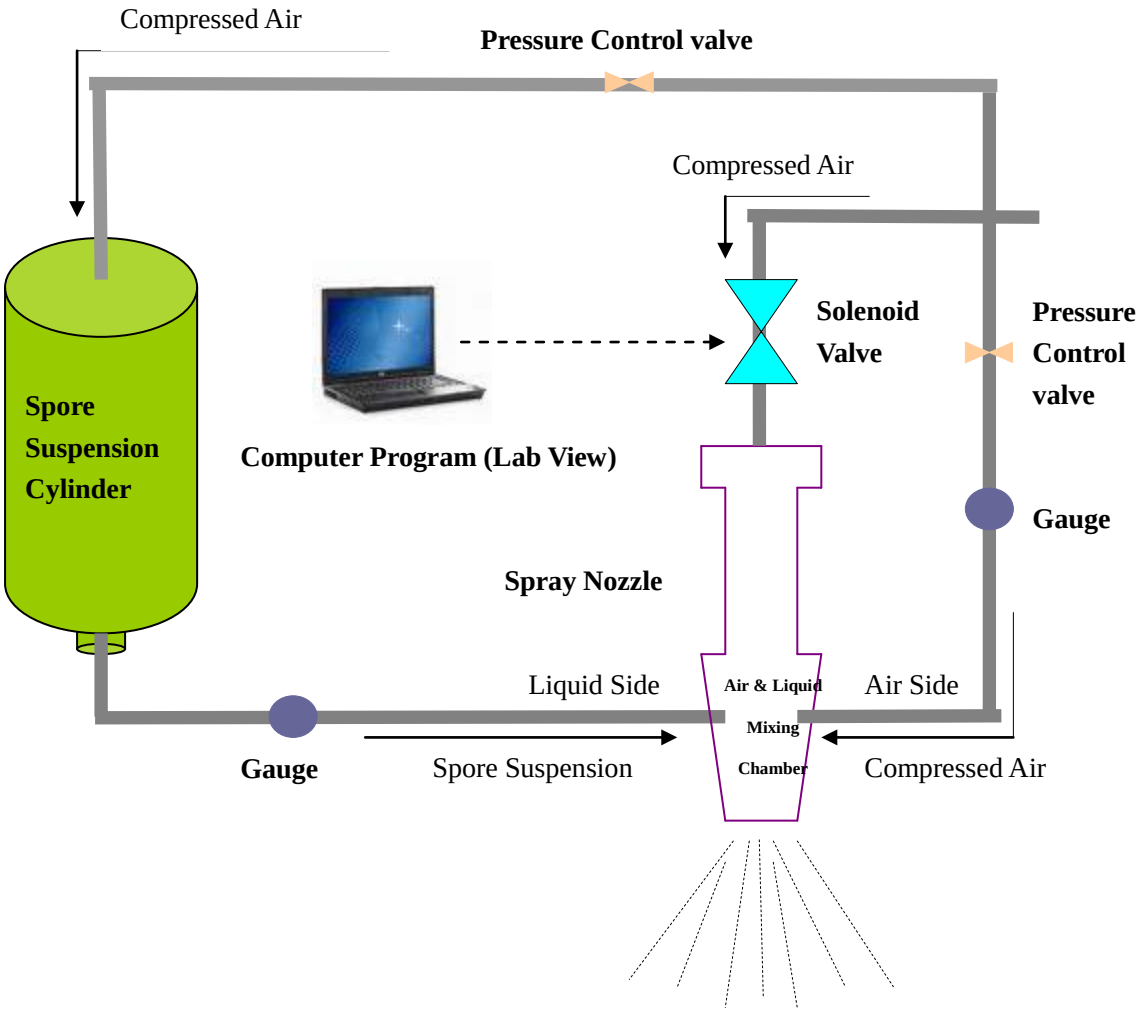

Figure 4.4: A schematic diagram of the automatic spray system

Method for Spore Inoculation of Packaging Materials

Due to the fact that the surface area of caps were approximately 1/10 of the surface area of bottles, the spore inoculation levels were designed to be on average 10^6 spores per bottle and 10^5 spores per cap, therefore theoretically spore coverage per unit area was the same on either bottles or caps. To achieve these inoculation levels, 0.1 g of the spore suspension was sprayed into each bottle, and 0.01 g was loaded onto each

cap. The quantity of spore suspension spray was monitored using a scale to measure the weights of bottles before and after spraying. The results were also analyzed to examine the performance of the spray system in terms of the repeatability for the quantity of spray. In addition, before and after using the sprayer, the entire spray system was sterilized by flushing alcohol and then sterile distilled water through the system. Finally, the inoculated bottles were air dried over night.

4.2.3 Decontamination of Spore Inoculated Bottles by UV and Perform Treatment

Materials

- Inoculated PET and glass bottles;
- 8% Perform solution.

Equipment

- The aseptic packaging equipments used by Xenos Ltd.;
- A scale with 3 decimal points accuracy;
- Peroxide test strips – Macherey Nagel Quantofix Peroxide 25;
- UV light meter (Lux meter);
- A timer.

Figure 4.5: UV lamp used in the bottle sterilization system.

Methods for Decontamination of Spore Inoculated Bottles by UV Light and Perform

1) Test of the Pilot Plant Scale Aseptic Packaging System

In order to determine the log reductions of *B. subtilis* spores that could be delivered by the pilot plant scale packaging sterilization system following variation in the time of exposure to UV. The UV lamp installed in this system was Philips Model PL-C 11 Watt as shown by Figure 4.5, which emitted UV light of wavelengths within 200-280 nm. UV light intensity was measured at various distances (r) from the UV source at different locations. For example, the light intensities measured at 50 mm were 12.5 W/m^2 at two-leg side, 7.5 W/m^2 at one-leg side, and 4.5 W/m^2 at the bottom of the lamp. Since light intensity (I) is proportional to $1/r^2$, the approximate UV intensities received by most bottles used at Xenos are: 43 W/m^2 facing the two-leg side, 35 W/m^2 facing the one-leg side, and 25 W/m^2 facing the bottom. Before the decontamination process, the concentration of hydrogen peroxide in the condensates,

which formed on the inside surface of bottles after the bottles were steamed, were tested using peroxide test strips to ensure 0.5 – 1% peroxide concentration was reached. The inoculated bottles were then decontaminated in a random order by the pilot plant scale filler. For each type of bottles, the UV insertion time was varied from 2 to 30 seconds. Tests were carried for both PET and glass bottles. Triplicates were conducted for each treatment. Three controls that did not undergo treatment were also included in the test for each type of bottle. During the test, the quantity of Perform condensate or weight gain of each bottle after steaming was measured with a 3 decimal places scale. After the treatment, the bottles were aseptically capped with caps that were previously soaked in 0.5% Perform (0.125% hydrogen peroxide).

2) Decontamination Test with Varying Perform Loading Quantity per Bottle and Penetration Time

This test was designed to find out influences of Perform loading level and penetration time on sterilization effectiveness of the system. Therefore, two variables were included in this test: Perform loading quantity which was the weight of Perform condensates gained by each bottle after steaming on the inside bottle; penetration time which was the holding time after Perform steaming and before UV insertion. PET bottles were used for the test. There were four levels of Perform loading: 0 mg, 100 mg, 200 mg, and 300 mg. For each Perform loading level except 0 mg, there were four penetration times tested: 10 s, 25 s, 40 s, 50 s, which would be the applicable

penetration time during real production. UV insertion time was kept at 12 seconds. In addition, 3 controls were involved receiving no sterilization treatment, and triplicates were carried for each experimental treatment. Before the test, the peroxide concentration in the condensate on the bottles from steaming was checked to be within the 0.5 – 1% requirements. During the experiment, Perform loading quantity was measured using a scale for each bottle, and the penetration time was monitored by a timer. Finally, the controls and treated bottles were capped with caps that were previously soaked in 0.5% Perform (0.125% hydrogen peroxide).

3) Decontamination Test with Varying UV Insertion Time

The aim of this test was to determine the effect of various UV insertion time on the log reduction of *B. subtilis* spores, for which Perform loading level (mg) and penetration time (s) were kept constant. PET bottles were used for conducting the test. Different UV insertion time tested was: 0 s, 2 s, 4 s, 6 s, 8 s, 10 s, 20 s, and 30 s. Similarly, peroxide concentration in the condensates after steaming of the bottle inside surface was checked to meet the requirement (0.5 – 1 % hydrogen peroxide). Three controls were included which received no sterilization treatment, and triplicates were carried out for each treatment. The Perform loading level for each bottle was inspected using a 3 decimal scale, and the penetration time and UV insertion time were monitored using a timer. Finally, the controls and treated bottles were capped with caps that were previously soaked in 0.5% Perform (0.125% hydrogen peroxide).

4.2.4 Enumeration of Survivals

Materials

- Decontaminated samples from the packaging sterilization process at Xenos;
- Peptone water for dilutions;
- Solution containing 0.1% peptone and 0.1% Tween-80;
- Standard Plate Count Agar.

Equipment

- Vibratory mixer;
- Membrane filtration unit and filter paper;
- Petri dishes;
- Universal bottles for dilution;
- Automatic pipette and pipette tips;
- Burner and forceps;
- Autoclave;
- Incubator at 30ºC.

Method to Enumerate Surviving Spores

After the decontamination process, the treated samples and controls were washed with 100 ml solution containing 0.1% peptone and 0.1% Tween-80. The Tween-80 acted as a surfactant which helped washing off the spores from the packaging surfaces. To enumerate the spore counts from controls, a standard plate count method was carried out which was suitable for enumerating high contamination counts. For the decontaminated bottles, membrane filtration method was conducted to estimate the number of surviving spores. The log reduction was calculated for each sample using the formula below:

$$\text{Log Reduction} = \log (N/N_0)$$

N = count of survived microorganisms

N_0 = inoculation counts

The log reductions were calculated, analyzed, and plotted using Excel. Spore recovery using this method was about 77% as described in Appendix 2.

4.3 Results and Discussions

4.3.1 Decontamination Test of the Pilot Plant Scale Aseptic Packaging System

A decontamination test was conducted on the pilot plant scale packaging sterilization equipment to determine its sterilization effectiveness (log reduction of *B. subtilis* spores) under conditions described in section 4.2.3. As there were two types of bottles most commonly used at Xenos Ltd.: 250 ml PET bottles and 175 ml glass bottles (photos are displayed in Section 4.2.2), decontamination tests were conducted for both types of bottles. From the decontamination experiment on the pilot scale aseptic packaging system, log reductions at different UV insertion time were calculated and plotted in Figure 4.6.

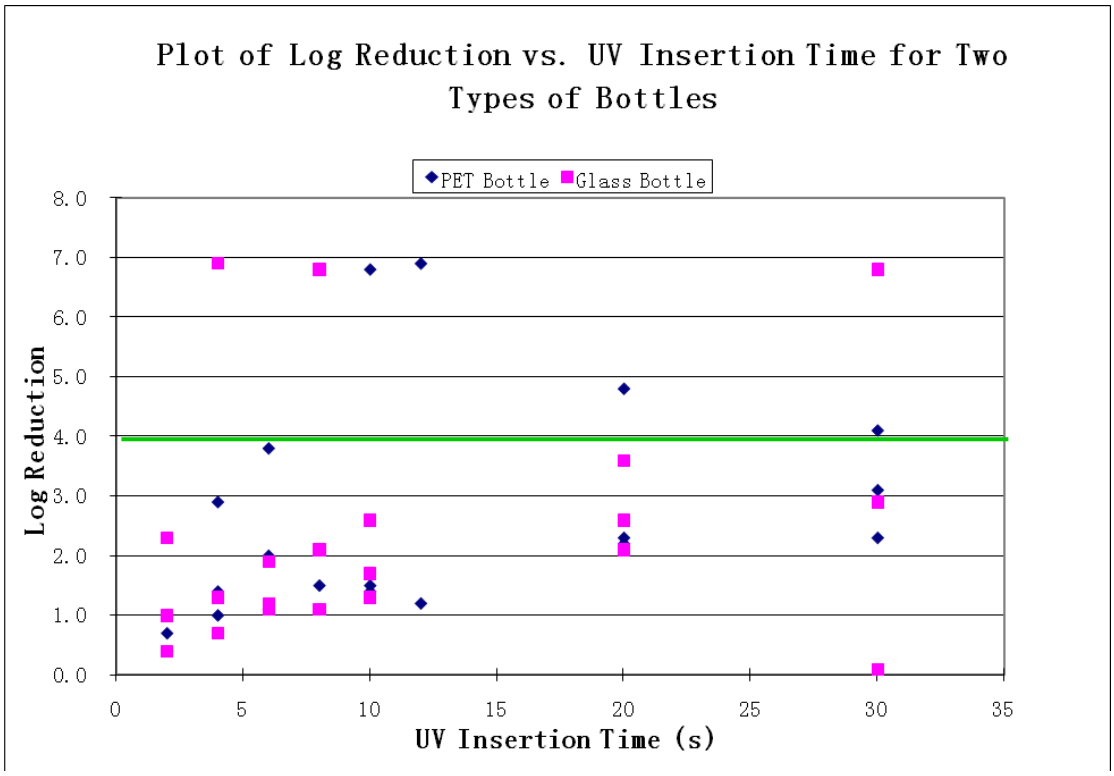

Figure 4.6: Log reductions of *B. subtilis* ATCC 6633 spores delivered by the pilot plant scale packaging sterilization system with various UV insertion time tested on PET bottles and glass bottles (R^2 = 0.082 for PET bottles, R^2 = 0.050 for Glass bottles). Perform loading level varies between 70mg/bottle and 340 mg/bottle.

Figure 4.6 shows that for both the PET and glass bottles, it was found that the obtained experimental data was too randomly scattered to determine an appropriate relationship between log reduction and UV insertion time. Only 16% of the data were showing a greater than 4 log reduction.

Random scattering of experimental data could be due to the quantities of Perform loading per bottle was not well controlled during the decontamination test. It was measured that the quantities of Perform loaded varied from 70 mg to 340 mg per

bottle. Therefore, the same log reduction data was re-plotted but vs. Perform loading quantity (mg/bottle) (Figure 4.7) for both PET and glass bottles. As shown in this figure, low log kills (less than 4 log reduction) were mainly observed at the Perform loading quantity less than 200 mg/bottle. More studies were carried out to discuss the relationship between log reduction and Perform loading quantity per bottle in the next section.

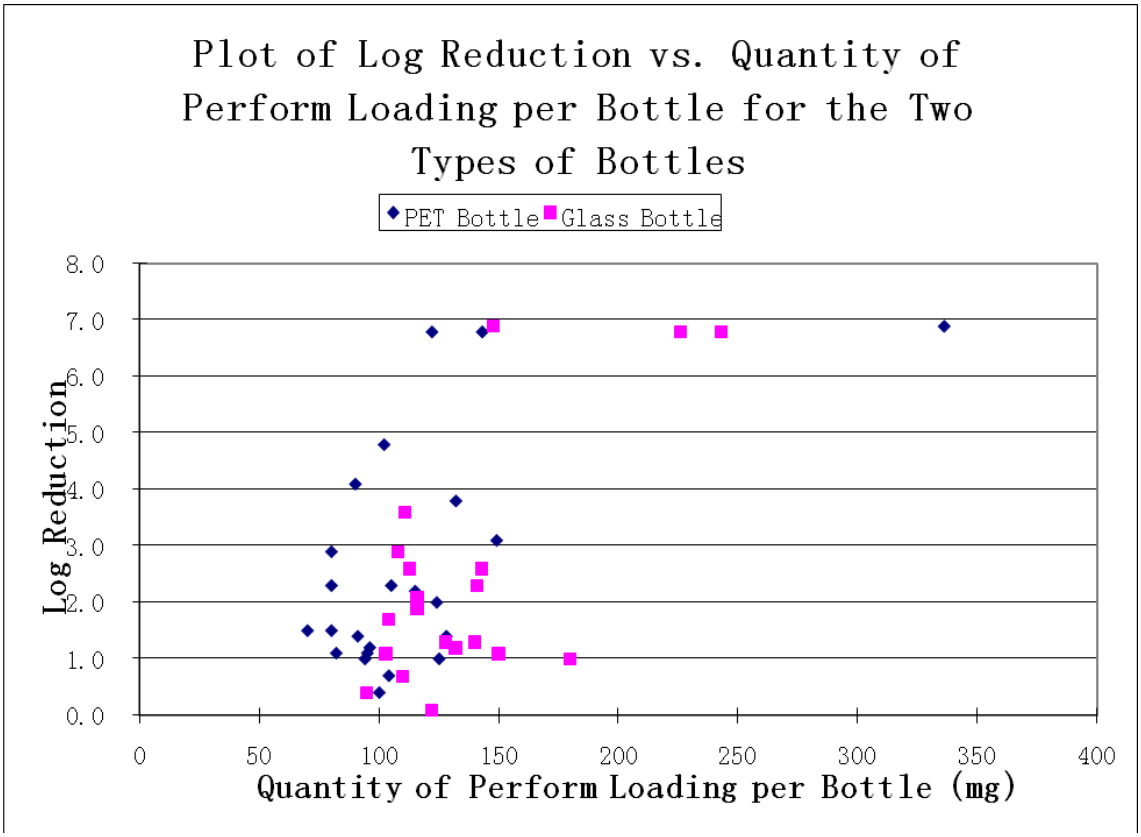

Figure 4.7: Log reductions of *B. subtilis* ATCC 6633 spores on PET bottles and glass bottles delivered by the pilot plant scale packaging sterilization system at various Perform loading quantities/bottle (R^2 = 0.333 for PET bottles, R^2 = 0.444 for Glass bottles).

4.3.2 Relationship between Log Reduction with Perform Loading Quantity per Bottle and Penetration Time

Section 4.3.1 (Figure 4.7) showed that there was some correlation between log reduction and Perform loading quantity ($R^2 = 0.333$). Therefore, in order to study their relationship, an experiment was carried out to determine the log reduction of *B. subtilis* spores achieved at three levels of Perform loading quantity: 100 mg/bottle, 200 mg/bottle, and 300 mg/bottle. In addition, in order to find out if log reduction could be affected by penetration time, an experiment was also conducted to determine log reduction delivered by the pilot plant scale system when penetration time was varied. 12 seconds of UV insertion time was chosen for the tests due to the layout of the filling machine and normal speed that the machine operates at. This experiment was conducted using method described in section 4.2.3.

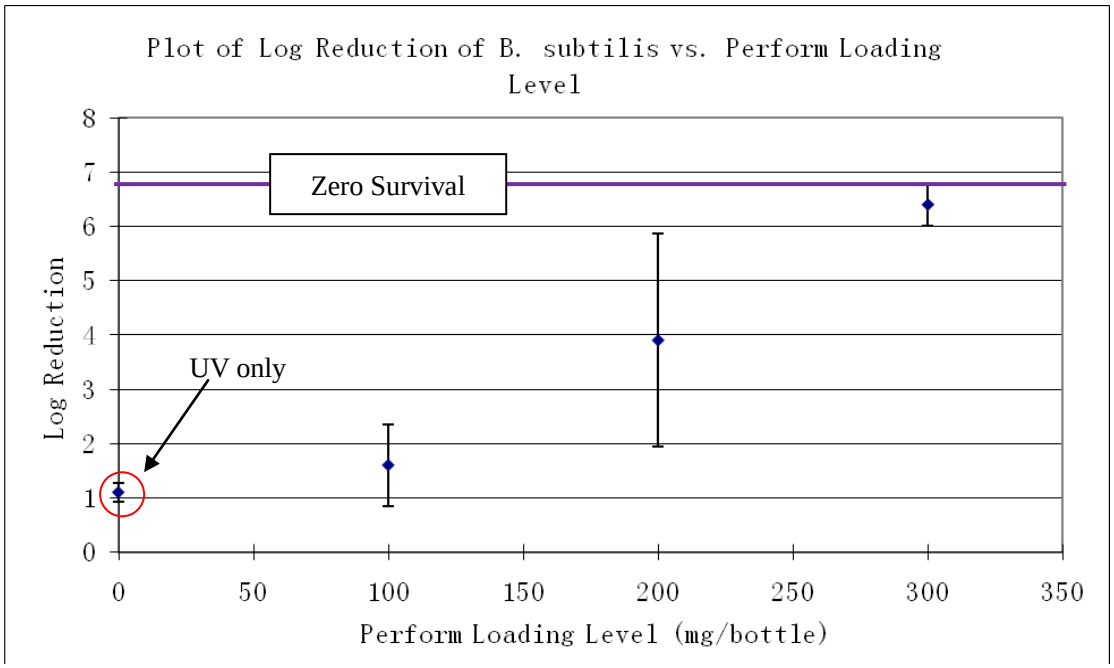

Figure 4.8: Log reductions (with 1 SD of 3 replicates)) of *B. subtilis* ATCC 6633 spores achieved at three levels of Perform loading/bottle on PET bottles (UV time = 12 seconds; Penetration time = 25 s)

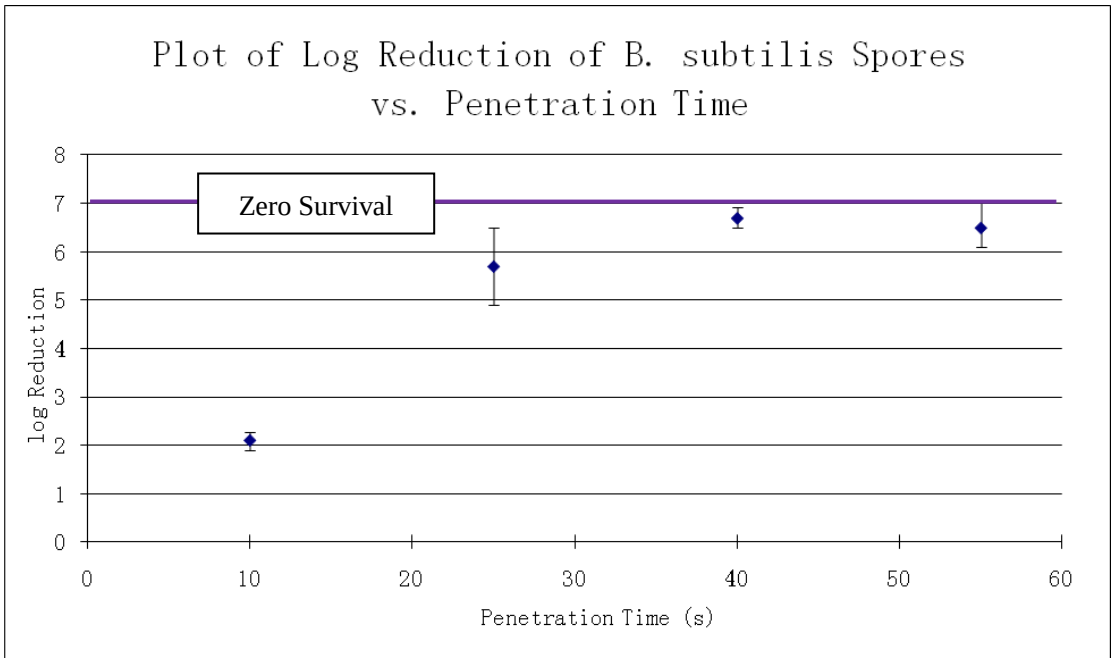

Figure 4.9 Log reductions (with 1 SD of 3 replicates) of *B. subtilis* ATCC 6633 spores achieved on PET bottles at various penetration time, 300mg Perform loading level and UV time = 12 seconds.

Figure 4.8 is showing that Perform loading level (i.e 100, 200 or 300mg/bottle of 1% hydrogen peroxide) is an important factor affecting the log reduction delivered by the packaging sterilization system. When there is no Perform loaded onto the packaging material (UV only), only a 1 log reduction in bacterial spores was recorded. At Perform loading level of 100 mg/bottle, only an average log reduction of 1.5 was achieved. When the Perform loading levels reached 200 mg and 300 mg, the average log reductions achieved were 4 and 6.4 respectively. However, triplicate log reductions at 200 mg/bottle of Perform loading level showed a large variation. This indicated that when Perform loading level was 200 mg/bottle, the sterilization effectiveness could not consistently achieve log 4 reduction, whereas at 300 mg/bottle the performance of the sterilization system could be improved not only in sterility but also system repeatability.

Figure 4.9 showed that penetration time could also affect the log reduction of *B. subtilis* spores at Perform loading level of 300 mg/bottle. Only a 2 log reduction was achieved when the penetration time was 10 seconds. However, when the penetration time increased to 25 seconds, the sterilization effectiveness was improved dramatically to around a 5.7 log reduction.

These results suggest that to achieve the targeted >4 log reduction, the minimum Perform loading level would be 300 mg/bottle, and required penetration time would be 20 to 25 seconds. Apart from Perform loading level and penetration time, UV

insertion time could be another important factor influencing sterilization effectiveness. Hence, the relationship between log reduction and UV insertion time was also studied and discussed in the following section.

4.3.3 Relationship between UV Insertion Time and Log Reduction of *B. subtilis* Spores

An experiment was designed and carried out to determine log reduction achieved by increasing UV insertion time when Perform loading quantity and penetration time were kept constant (refer to methodology (3) in section 4.2.3). From the literature review, UV fluence equals UV intensity multiplied by time, therefore as UV treatment time increases, the greater UV fluence.

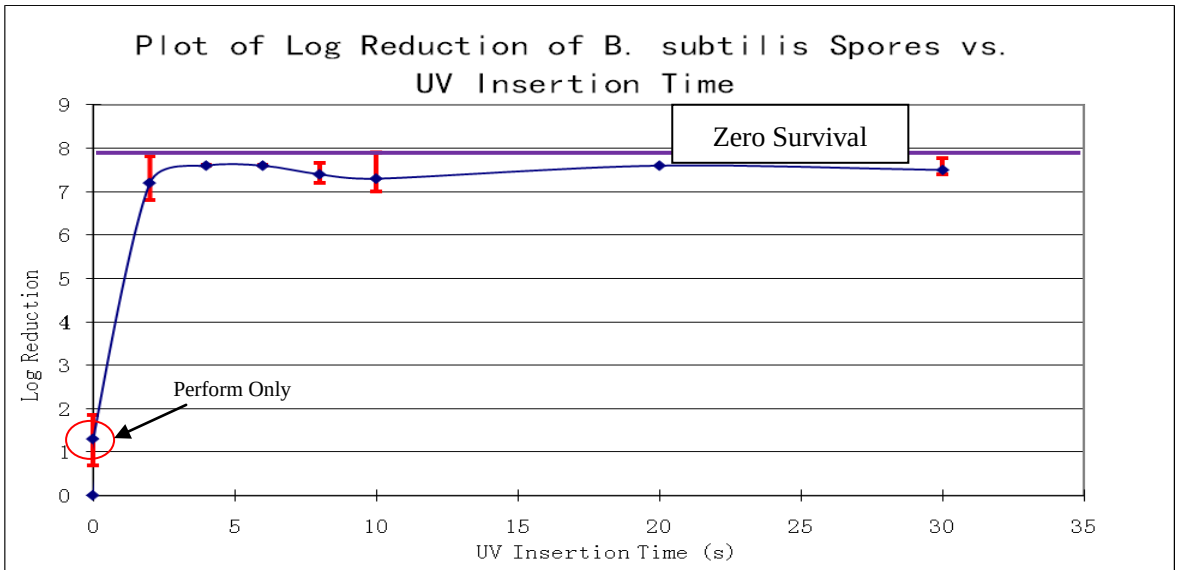

Figure 4.10: Log reduction (1 SD of 3 replicates) of *B. subtilis* spores at various UV insertion time

at constant Perform loading level (**300 mg/bottle**) and penetration time (**20 s**).

Figure 4.10 showed that only a 1.4 log reduction was achieved when using Perform alone at 300 mg/bottle without UV. With the addition of UV radiation, greater than 6 log reductions would be achieved when UV insertion time was more than 2 seconds. This suggested that 2 seconds UV insertion at 300 mg/bottle Perform loading level (containing 0.5 – 1% peroxide) and 20 seconds penetration time, would be sufficient for this sterilization technique to deliver a desirable sterility. In contrast, the time required to produce a 6 log reduction on *B.subtilis* spores would be 29 seconds using the model developed by Gardner and Shama (1998): $Log_{10}(N/N_0) = -0.0452 \times$ UV intensity $\times$ Time, when the UV intensity was 4.5 W/m^2 and hydrogen peroxide concentration was 1%. Therefore, the sterilization technique studied in this project had shown a more efficient inactivation of *Bacillus* spores compared with Gardner and Shama (1998). This may be because in this project hydrogen peroxide applied was in the form of steam, whereas Gardner and Shama used hydrogen peroxide solution at room temperature.

4.4 Conclusion and Recommendations

In order to ensure the packaging sterilization system is able to achieve commercially acceptable sterility, Perform loading level, hydrogen peroxide concentration in

vaporized Perform, time length of UV treatment, and penetration time are critical. The desirable requirements were: 300 mg/bottle Perform loading level, 0.5 – 1% hydrogen peroxide concentration in Perform condensates on bottles by vaporized Perform treatment, at least 2 seconds UV treatment time, and 20 seconds penetration time. A log 6 reduction of *B. subtilis* spores could be achieved on bottles with the above sterilization conditions.

5. VALIDATION OF THE UPGRADE PACKAGING STERILIZATION SYSTEM

5.1 Introduction

From Section 4, it was known that a 6 log reduction of *B. subtilis* (ATCC 6633) spores could be achieved on the pilot plant scale packaging sterilization system by the treatment of UV plus Perform steam. The installation of an upgrade sterilization system at Xenos Ltd, was also required to be validated by the use of a challenge test. The upgrade sterilization system used the same UV source as the pilot plant scale filler. However, other differences from the pilot plant scale system were: higher throughput capacity (increased from 17 bottles/min to 70 bottles/min) with compact structure; improved automation therefore labour cost can be reduced; user-friendly with easy screen operation; better monitoring of the process, for which operational data could be saved and transferred to a computer system; a cap sterilization system, which applied approximate UV fluence of 2580 J/m^2 plus Perform steam treatment. Therefore, a challenge test was carried out on the upgrade system to determine its sterilization effectiveness.

5.2 Experimental

Prior to the decontamination test, Perform steaming time was selected to ensure the minimum Perform loading level (300 mg/bottle) was reached (discussed in Section 4.3.2). The selected applicable UV insertion time was 10 seconds, which was greater than the minimum requirement of 2 seconds as determined in Section 4.3.3 to ensure a sufficient margin of safety in the real production system. Penetration time was varied according to the location of the UV section and speed of the sterilization process. Operation set up allowed at least 20 seconds between bottles being steamed and being UV treated. During this challenge test, steamer performance was monitored by measuring the concentration of hydrogen peroxide in the Perform condensates loaded on bottles. Measurements were conducted using Peroxide Test Strips (Macherey Nagel Quantofix Peroxide 25), and the test was carried out every 30 min for a period of 7 hours. After decontamination, enumeration of survivals after treatment was conducted using the same method described in Section 4.2.4.

5.3 Results and Discussions

Table 5.1 and Figure 5.1 showed the calculated log reductions determined by the challenge test on the upgrade packaging sterilization system. Even through the minimum requirement of Perform loading after steaming (300 mg/bottle) was

achieved, the log reduction was less than 4. This was most likely due to the fact that the peroxide concentration applied on the bottles did not meet the requirements of 0.5 – 1%. As shown in Figure 5.2, even though the original hydrogen peroxide concentration in the liquid Perform solution was 2%, the highest peroxide concentration was only 0.06%, which happened 30 min after the start up. Then the concentration reduced to less than 0.02% after 2.5 hours.

Table 5.1: Log reduction achieved by the upgrade packaging sterilization system

Samples	**Quantity of Spore Suspension Loading (g)**	**Quantity of Perform Steam (mg)**	**Survival Counts**	**Log Reduction**
Control	0.121	N/A	1.10E+06	0
Control	0.1		5.00E+05	0
Control	0.095		4.00E+05	0
1	0.11	350	250	3.4
2	0.103	337	220	3.5
3	0.104	330	100	3.8
4	0.096	355	100	3.8
5	0.098	341	100	3.8

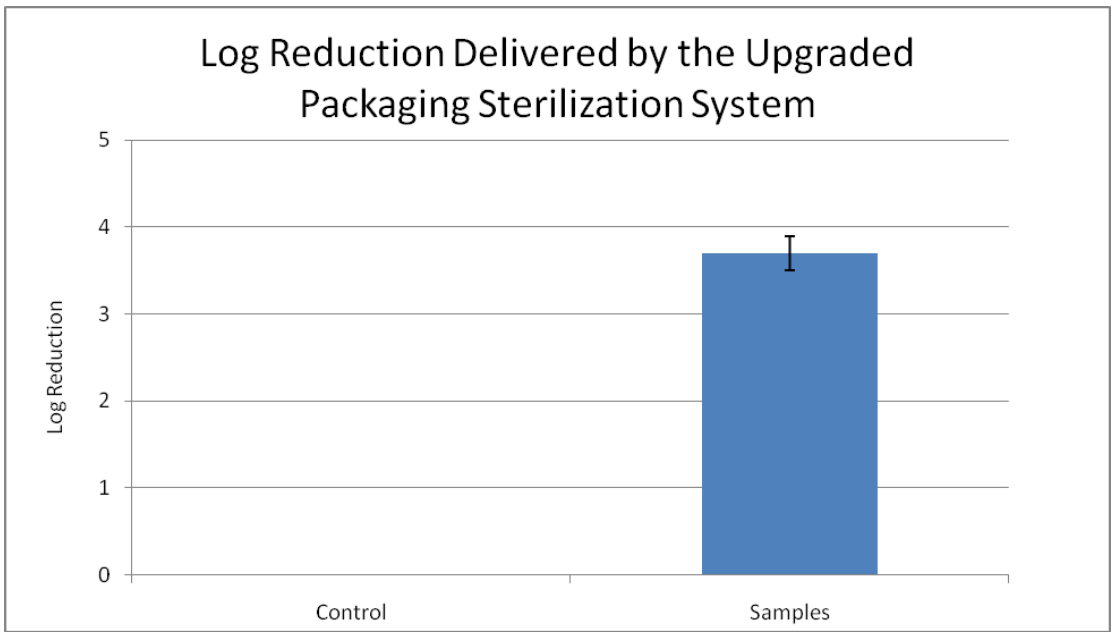

Figure 5.1: Average log reduction (with 1 SD of 5 replicates) of *B.subtilis* spores achieved by the upgrated packaging sterilization system, using penetration time >20 seconds and UV insertion time of 10 seconds.

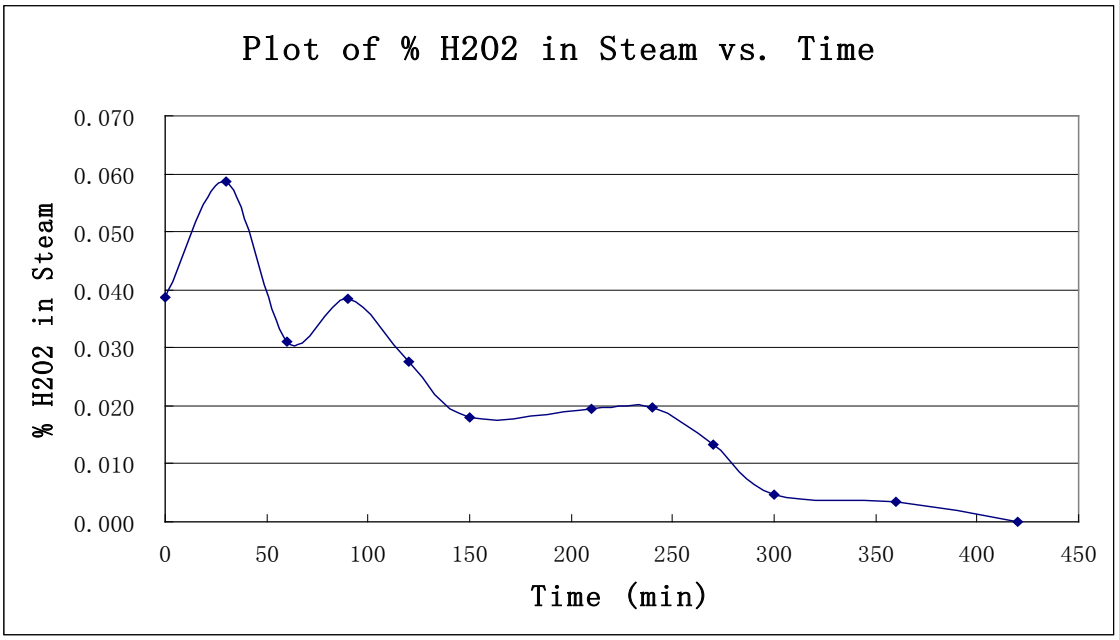

Figure 5.2: Peroxide concentrations of the Perform condensates on PET bottles measured by steaming a bottle every 30min for 7 hours.

The reason why the pilot plant scale system could deliver Perform steam with sufficient peroxide concentration but not the upgrade system may be due to the change in steamer design. Schematic diagrams of the steamers for the pilot plant scale and upgrade fillers are shown as Figure 5.3 and 5.4. In the pilot plant scale steamer, a small amount of liquid is passed through the heating device at 140ºC via the column. Therefore, the heat is sufficient to rapidly evaporate all the liquid if there is no heat loss, thus the hydrogen peroxide concentration in the gas phase is equal to that of the liquid phase. The disadvantage is that the steam generation capacity of this steaming system is fairly low; hence only one bottle can be steamed at a time. However, in the steaming unit of the upgrade filler, a 2% Perform solution is boiled at 120ºC in the vessel to generate steam. Under constant temperature, the liquid phase starts to evaporate until phase equilibrium is reached. Assuming the solution is a two component liquid composed of water and hydrogen peroxide (the amounts of peracetic acid and acetic acid were negligible), since water is more volatile than hydrogen peroxide, the concentration of hydrogen peroxide in gas phase must be less than that of the liquid phase at the equilibrium point. According to Manatt and Manatt (2004), at 120ºC the equilibrium mole fraction of hydrogen peroxide in vapour phase is less than 0.01 when the hydrogen peroxide mole fraction in liquid phase is 0.1.

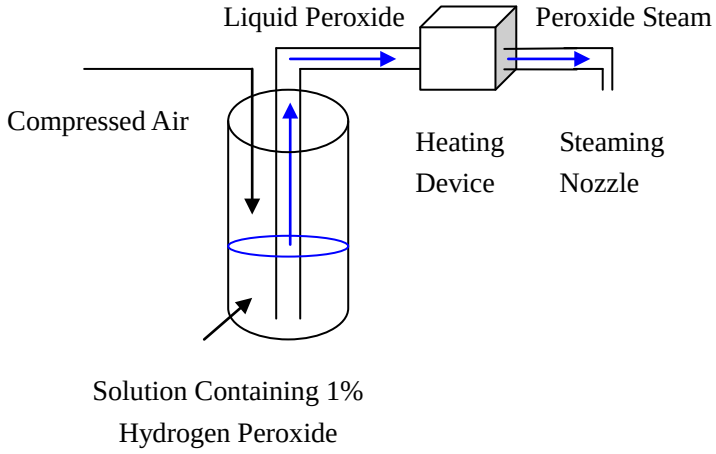

Figure 5.3: Steamer Configuration in the pilot plant scale packaging sterilization system

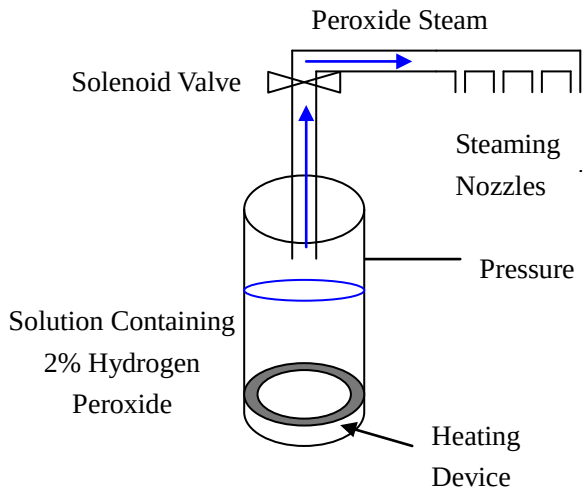

Figure 5.4: Steamer Configuration in the upgrade packaging sterilization system

Secondly, hydrogen peroxide is unstable at high temperature. It is stable at ambient temperature, but the rate of decomposition is doubled by every 10ºC increase in temperature (Technical Data Sheet, 2006). Therefore, as shown in Figure 5.2 the peroxide concentration in the steamer would decrease dramatically during the

sterilization process.

5.4 Conclusions and Recommendations

The log reduction delivered by the upgrade packaging sterilization system was less than 4 log values, thus target sterility of log 4 reduction was not achieved. This was largely due to that the steamer was not able to produce steam with required peroxide concentration of 0.5 – 1%. Therefore, it was recommended to re-modify the steamer to improve the effectiveness of the steaming process in delivering the required 0.5-1% hydrogen peroxide concentration in the steam.

6. MODIFICATION AND RE-VALIDATION OF THE UPGRADE ASEPTIC PACKAGING (PACKAGING STERILISATION) SYSTEM

6.1 Modification of the Steaming Unit

The steamer was re-modified as displayed in Figure 6.1. Instead of boiling the Perform solution 120ºC and vaporize the Peform solution, an automatic sprayer was built into the steaming system. This spray was designed to inject a small amount of Perform solution (containing 12.5% hydrogen peroxide), in the form of a fine mist, into a super heated steam stream. The super heated steam was produced by the steam generator at 120ºC. The energy released from the super heated steam vaporizes the injected mist of Perform solution, and produces vapour containing 0.5 – 1% hydrogen peroxide. The quantities of steam and Perform injections were controlled by solenoid valves, which were operated by an installed computer program. The pipes were insulated to prevent heat losses.

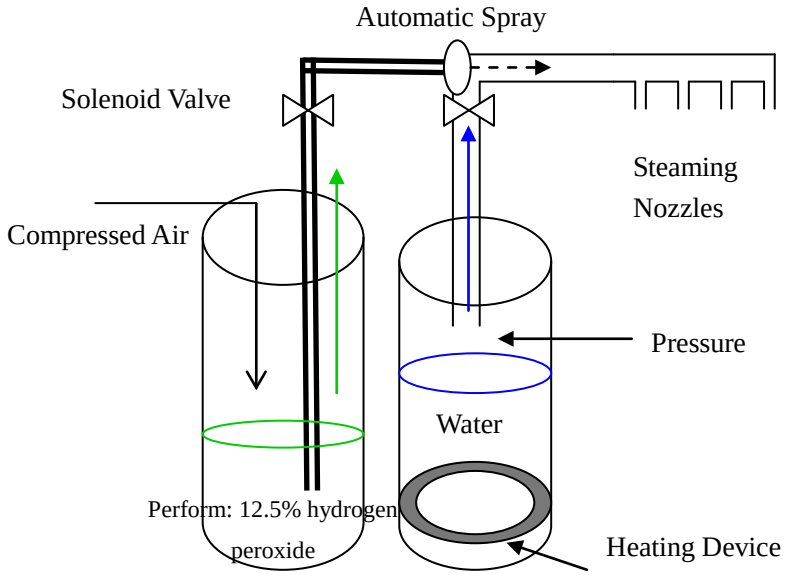

Figure 6.1: Re-modified steaming unit of the upgrade packaging sterilization system

In order to determine the steamer operational conditions which meet the requirements of Perform loading level and hydrogen peroxide concentration, tests were conducted by steaming sets of bottles under various operational conditions, and measuring the Perform loading level per bottle and hydrogen peroxide concentration. Detailed description of the test is shown in Appendix 3. Through the trial, the settings of the steamer for optimum performance were:

- Steam opening time: 2.1 s
- Perform opening time: 0.5 s
- Height from the steaming outlets to the top of bottles: 2 cm

Test results (refer to Appendix 3) showed that with the above steamer setting, 300 mg/bottle of Perform loading level was consistently achieved, and Perform

condensates on the bottles all reached 0.5 – 1% hydrogen peroxide concentration. Therefore, the functionality of the steamer had been effectively improved.

6.2 Re-validation of the Upgrade Packaging Sterilization System

6.2.1 Introduction

In order to test the sterilization effectiveness of the upgrade packaging sterilization system on a larger scale under the established operational conditions, final challenge tests on both bottles and caps were conducted. There were also the confirmation tests to exam sterility of the whole aseptic packaging system. To assess the reliability of the system performance, a large sample size was involved in the challenge tests and 99% confidence intervals were obtained for the delivered log reduction. The challenge tests were carried out for PET bottles, glass bottles, and also glass bottle caps.

6.2.2 Method

Samples and sample size included in the test were:

- 84 PET bottles inoculated with 10^6 spore per bottle;

- 119 glass bottles inoculated with 10^6 spore per bottle;
- 60 glass bottle caps inoculated with 10^5 spore per cap;

Before the decontamination process, the steamer performance was tested to ensure that 300 mg/bottle of Perform loading level and their 0.5 – 1% hydrogen peroxide concentration were achieved under the established steamer settings described in section 6.1. Inoculated bottles were sterilized by the upgrade packaging sterilization system, filled with UHT water, and capped with non-inoculated sterile cap. 30 spore inoculated caps were soaked in 0.5% Perform solution for 20 min before feeding through the sterilization system. The other 30 caps were sterilized without soaking. The decontaminated caps were caped onto sterile non-inoculated glass bottles filled with heat treated water by the aseptic packaging system. Three positive controls (no treatment) were used in each challenge test. Finally, the number of survivors was enumerated for each sample by the method described in Section 4.2.4.

6.2.3 Results and Discussions

Re-validation results from the challenge tests on both PET and glass bottles are shown by Figure 6.2. Detailed experimental results are displayed in Appendix 4. An average 6 log reduction was achieved (no survivals) for both PET and glass bottles. The 99% confidence interval was ±0.1 on log scale for both PET and glass bottles. In another

words, it would be 99% sure that the upgrade packaging sterilization system could deliver a >6 log reduction.

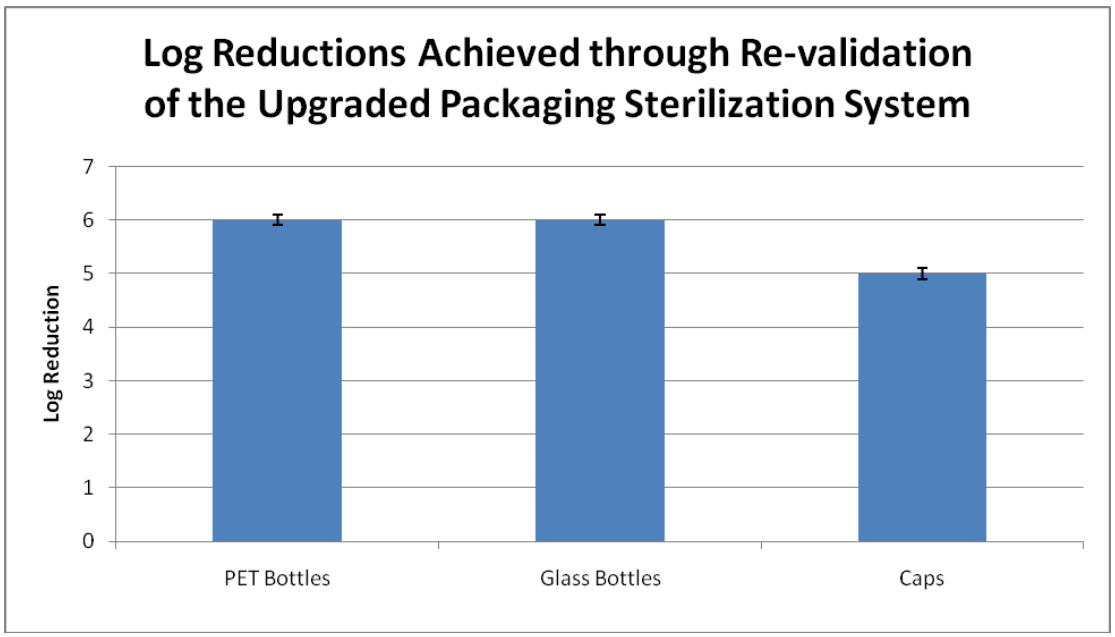

Figure 6.2: Log reduction of *B.subtilis* spores delivered on PET bottles, glass bottles, and glass bottle caps by the upgraded packaging sterilization system after steamer modification

Results for the challenge test on caps of glass bottles were also displayed in Figure 6.2 and Appendix 4. The results suggested that a >5 log reduction (±0.1 99% confidence interval on log scale) of spores of *B. subtilis* ATCC 6633 (no survivals) could be achieved on the glass caps by the upgrade packaging sterilization system with and without the soaking step. This finding also means that the system allows the elimination of the cap soaking step. It enables a dryer condition for the caps to easily travel through the cap shoot, and also helps reduce the peroxide residues in the final products.

6.2.4 Conclusions and Recommendations

From the challenge tests conducted for the upgrade packaging sterilization system, >6 log reduction and >5 log reduction could be achieved on the tested bottles and caps respectively by the upgrade and modified system. Hence, the commercial sterility requirement of 1/10,000 failure rate would be satisfied with the upgrade packaging sterilization system used at Xenos Ltd.

7. OVERALL SUMMARY

From the challenge tests conducted on the pilot plant scale packaging sterilization system, it was found that UV and Perform had synergistic effect on the inactivation of *B. subtilis* spores. Log reduction results showed that a greater than 6 log reduction could be achieved using the combined treatment, whereas only less than a 2 log reduction had been achieved when using UV or Perform alone. With the combined treatment, a log 6 reduction could be achieved with 2 seconds UV treatment time, whereas in the study by Gardner and Shama (1998) the estimated time to produce a 6 log reduction on *B.subtilis* spores would be 29 seconds using UV plus 1% hydrogen peroxide solution. Therefore, UV plus vaporized Perform steam showed a more efficient inactivation of *Bacillus* spores.

To ensure sterility (> 5 log reduction) delivered by the UV plus Perform sterilization system applied at Xenos Ltd., the following conditions would be very critical:

- Hydrogen peroxide concentration of Perform condensate on bottles (after steaming) is best within 0.5 – 1 %;
- Perform loading level should be minimum 300 mg/bottle after vaporized Perform treatment;
- UV treatment time applied is greater than 2 seconds during UV treatment;
- At least 20 seconds of penetration time (time between Perform treatment and UV

treatment) should be allowed.

Challenge tests carried out on the upgrade sterilization system before steamer modification showed an inadequate sterility with a less than 4 log reduction. This was most likely due to that the steamer was not able to consistently deliver steam with adequate hydrogen peroxide concentration, thus 0.5 – 1% hydrogen peroxide in Perform condensate could not be achieved on bottles after steaming. Under this circumstance, the steaming component was modified in order to generate Perform steam with required hydrogen peroxide concentration. Finally, the upgrade packaging system was validated also by challenge test on both bottles and caps, and results showed that at least a 5 log reduction could be delivered by the upgrade packaging sterilization system.

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APPENDIX

Appendix 1 - Challenge Test of Aseptic Packaging System

In order to understand and optimize an aseptic packaging system, the sterility of the system needs to be determined microbiologically. This can be assessed by a microbiological technique called “Challenge Test”. This method is designed to determine the log reduction of a selected microorganism, which is delivered by the aseptic packaging system on the applied packaging materials under operational conditions, and hence correspond to the sterility that the system should be able to achieve under those conditions. Moreover, selection of an appropriate microorganism for the challenge test is very important. Generally, the test microorganism should be relatively resistant to the applied sterilization treatments.

1. Definition, Purpose and Parameters of Challenge Test

Non-sterile package should rarely occur during a packaging process. Less than 1 faulty package per 10,000 is commercially required (Moruzzi et al., 2000). The effectiveness of a sterilization process must be verified to ensure commercial acceptable sterility. However, because ordinary microbial loads on packaging materials and equipment surfaces are low, and the targeted sterility is high, it becomes

impossible to verify the effectiveness under ordinary conditions (Moruzzi et al., 2000). Hence, the system needs to be verified by challenge tests. According to (Moruzzi et al., 2000), a challenge test can be defined as a method to determine the log reduction delivered by a packaging system. The method includes loading the food packaging with an unnaturally high level of a selected microorganism which is highly resistant to the applied sterilization treatment, determination the number of survival after the sterilization process, and calculation of the achieved log reduction. Theoretically, if a 4 log reduction is achieved, the sterilization system is proved to be able to reduce 10,000 organisms to 1, whether they exist on one single package or 10,000 packages. Therefore, assuming a “natural” load of 1 microorganism per package, no more than 1 survived organism every 10,000 packages or a failure rate of 1 per 10,000 can be expected for the sterilization system.

The parameters investigated in the challenge tests are depending on machine design. For packaging sterilization involving UV radiation and sterilizing chemicals, typical parameters include: UV output, wave band frequency, age of lamp, distance, exposure time, type of chemicals, concentration, quantity and application temperature, coverage and time (Rose, 1987).

2. Procedures of Conducting a Challenge Test

A challenge test usually involves the following step (Nelson et al., 1987):

1. Selection of test microorganisms. To choose an appropriate microorganism for a low acid aseptic system, the resistances of bacterial spores or other microorganisms of public health significance to the sterilization method should be established and compared. A number of strains should also be compared to select a resistant organism for a challenge test.

2. Inoculation of the test microorganism(s) to the aseptic packaging system. In this step, appropriate inoculum levels are introduced and dried onto the food contact packaging surfaces including both the containers and the lids. The inoculation levels are set corresponding to the target log reduction required, usually 10^5 to 10^6 per package related to the commercial acceptable sterility for aseptic packaging system. This concept takes into account that in reality the contamination level is much lower than the inoculation level.

3. Challenge the packaging system with the test microorganism(s). The inoculated containers or lids are fed through the sterilization cycle under various operating conditions including time, temperature or concentration of sterilizing agents. Then, the packaging is filled with a growth medium, incubated and monitored for growth.

4. Enumeration of survivals and calculation of achieved log reduction. The number

of survived microorganisms are counted and the log reductions delivered by the aseptic packaging system are calculated according to the equation:

$$\text{Log Reduction} = \log (N/N_0)$$

N = count of survived microorganisms

N_0 = initial count of contaminations

Appendix 2 - Spore Recovery Using the Current Spore Preparation and Enumeration Method

The objective of this test was to determine the number of spores could be recovered from bottles inoculated with prepared spore suspension. This was determined by comparing the spore counts recovered from spore inoculated PET bottles with that of the original spore suspension.

Materials

- Prepared Spore Suspension;
- PET bottles inoculated with the spore suspension;
- Peptone water for dilution;
- Solution containing 0.1% peptone and 0.1% Tween-80;
- Standard Plate Count Agar.

Equipment

- Automatic Spray System (see Figure 4.3);
- Petri dishes;
- Universal bottles for dilution;
- Automatic pipette and pipette tips;
- Vibratory mixer;

- Autoclave;
- Incubator at 30ºC.

Methodology

0.1 g prepared spore suspension was sprayed on each PET bottle. After drying, 100ml solution containing 0.1% peptone & 0.1% Tween-80 was used to wash off the spores from each inoculated bottle. Enumeration of spore counts for the freshly prepared spore suspension, and the washing solution from each bottle were conducted by Dilutions & Plate Count method. Triplicates were carried out. All plates were incubated under 30ºC for 48 hours before counting. Spore recovery was calculated for each replicate according to the equation below:

Spore Recovery (%) = (no. spores recovered from the inoculated bottles / spore counts in 0.1 ml prepared spore suspension) × 100

Results and Discussions

Table A1: Spore counts in spore suspension and that recovered from inoculated PET bottles.

Sample	**Spore Concentration**		**Spore Recovered from Inoculated Bottles**
	Per 1 g Spore Suspension	Per 0.1 g Spore Suspension*	
1	7.7 × 10^7	7.7 × 10^6	6 × 10^6
2	6.2 × 10^7	6.2 × 10^6	4 × 10^6
3	8.3 × 10^7	8.3 × 10^6	7 × 10^6
Average	7.4 × 10^7	7.4 × 10^6	5.7 × 10^6

* Calculated from spore counts per 1 g of spore suspension.

Spore Recovery (%) = 5.7 × 10^6 / 7.4 × 10^6 * 100 = 77%

Results suggested that the current spore preparation method could produce spore suspension of 10^7 spores per g. Spores recovery by the current enumeration method would be 77%. Since spore recovery was not 100%, the spore concentration of prepared suspension should not be used as initial counts (N_0) to calculate log reduction. Instead, spore counts from controls (inoculated bottles without sterilization) would be more accurate used to obtain the log reduction value.

Appendix 3 – Test on the Performance of the Modified Steamer for the Upgrade Aseptic Packaging System

Since improvement works had been done on the steamer of the upgrade packaging sterilization system, tests were carried out to verify if the requirements for steaming the bottles (300 mg/bottle Perform loading level and hydrogen peroxide concentration 0.5 – 1%) could be achieved consistently by the modified steamer.

Materials

- Perform solution (diluted to 12.5% hydrogen peroxide concentration)
- PET bottles and glass bottles
- Peroxide test strips – Macherey Nagel Quantofix Peroxide 25

Equipment

- the re-modified steaming system
- a 3 decimal places scale
- a timer

Methodology

2 liters of 50% v/v Perform solution containing 12.5% hydrogen peroxide was

prepared and filled into the Perform tank. The steamer was filled with water and heated until the temperature reached 120ºC. First part of the experiment was to trial different steamer settings to achieve the requirements of the steaming process, which would be 300 mg/bottle Perform Loading level and 0.5 – 1% peroxide concentration on each bottle. The test was conducted by adjusting the steam and Perform opening times. The Perform valve opening time should also be adjusted so that no condensates could come out of the steaming nozzles due to too much Perform injection. Through the trial, the settings of the steamer for optimum performance were found to be:

- Steam opening time: 2.1 s
- Perform opening time: 0.5 s
- Height from the steaming outlets to the top of bottles: 2 cm

The second objective of the experiment was to steam the bottles with the settings as mentioned above, and verify the outcome and consistency of the steamer. Before steaming the bottles, the steamer nozzles were warmed up by blasting the steamer for 10 to 15 times to minimize condensation occurring in the pipes works. Bottles were weighed before and after steaming, thus the weight gains were calculated to determine the quantity of Perform condensates gained by each bottle after the steaming process. To determine the peroxide concentration in the condensates, the steamed bottles were filled with 250 ml water, and tested with Peroxide test strips. This gave the peroxide levels (ppm) in the 250ml water solution. By knowing the weights of Perform condensates on the bottles, the peroxide concentration of the condensates were

calculated.

The steaming system contained 2 sets of steaming nozzles (6 nozzles per set). Duplicated tests were carried out for one of the sets to determine the repeatability of the steaming performance. There was a 16 seconds time interval between the duplicated tests, which represented the time interval between teaming two sets of bottles during real production. After the duplicated test, same test was conducted for the other set in order to check if the same steaming results could be achieved.

Results and Discussions

Table A2: Steamer validation tests results for one set of steamer nozzles

Bottle no.	Weight of Bottle Before Steaming (g)	Weight of Bottle After Steaming (g)	Quantity of Perform Condensate Loaded on the Bottle (mg)	Tested Perform Level in 250ml Water Solution(ppm)	Calculated H_2O_2 Concentration of Condensate (%)
Replicate One					
1	152.555	152.872	317	9	0.75
2	152.487	152.83	343	9	0.75
3	152.386	152.752	366	15	1
4	152.532	152.875	343	9	0.75
5	152.544	152.9	356	9	0.75
6	152.426	152.751	325	9	0.75
Replicate Two					
1	152.364	152.674	310	8	0.7
2	152.795	153.095	300	8	0.7
3	152.662	152.986	324	12	1
4	152.651	152.975	324	8	0.7
5	152.404	152.741	337	8	0.7
6	152.29	152.59	300	8	0.7

Table A3: Steamer validation tests results for the other set of steamer nozzles

Bottle no.	Weight of Bottle Before Steaming (g)	Weight of Bottle After Steaming (g)	Quantity of Perform Condensate Loaded on the Bottle (mg)	Tested Perform Level in 250ml Water Solution(ppm)	Calculated H_2O_2 Concentration of Condensate (%)
1	152.268	152.602	334	10	0.8
2	152.298	152.614	316	10	0.8
3	152.235	152.585	350	10	0.8
4	152.305	152.605	300	10	0.8
5	152.144	152.445	301	10	0.8
6	152.277	152.596	319	10	0.8

As shown in Table 5.2, the quantity of Perform loading level after steaming had met the minimum requirement of 300 mg/bottle. The peroxide concentration in the steam had achieved the requirements of 0.5-1%, which was significantly improved compared with the unmodified steamer (see Section 4.3.4). From the duplicated test, the steaming results were fairly consistent along the set of bottles, except bottle no. 3 which showed a slightly higher concentration. This might be due to a small amount of strips coming out of this nozzle. Test results for the second set of nozzles were showing similar results with better reliability.

In addition, the bottles retained their integrity during the steaming process. No shrinkage or deformation of bottles was observed. The condensates form on the bottle inside surface was uniform, and no big droplets were found inside the bottles.

Appendix 4 – Final Challenge Tests Results (Re-validation of the Upgrade Aseptic Packaging System)

Table A4: Final challenge test results on PET bottles

Sample No.	Quantity of Spore Suspension per Bottle (g)	No. of Survivals	Log Reduction
Control 7	0.102	2.50E+06	0
Control 6	0.099	2.50E+06	0
Control 3	0.129	1.00E+06	0
131	0.097	None found	>6
140	0.109	None found	>6
143	0.117	None found	>6
134	0.105	None found	>6
137	0.093	None found	>6
136	0.096	None found	>6
124	0.096	None found	>6
135	0.108	None found	>6
127	0.112	1	>6
138	0.093	None found	>6
128	0.116	None found	>6
132	0.132	1	>6
118	0.102	None found	>6
121	0.104	None found	>6
119	0.097	3	5.8
112	0.099	None found	>6
130	0.101	None found	>6
50	0.092	1	>6
133	0.103	None found	>6
139	0.098	None found	>6
115	0.105	None found	>6
109	0.117	None found	>6
123	0.109	None found	>6
122	0.108	None found	>6
117	0.114	None found	>6
111	0.095	None found	>6
106	0.105	None found	>6
113	0.112	None found	>6

54	0.103	None found	>6
125	0.099	None found	>6
48	0.101	None found	>6
142	0.115	None found	>6
57	0.094	None found	>6
47	0.128	None found	>6
52	0.095	None found	>6
43	0.106	None found	>6
97	0.095	None found	>6
101	0.105	None found	>6
98	0.11	None found	>6
85	0.109	None found	>6
51	0.099	None found	>6
59	0.09	None found	>6
46	0.095	None found	>6
126	0.116	None found	>6
55	0.103	None found	>6
45	0.099	None found	>6
90	0.104	None found	>6
42	0.104	None found	>6
141	0.119	None found	>6
60	0.109	None found	>6
129	0.097	None found	>6
56	0.104	None found	>6
99	0.098	None found	>6
104	0.105	None found	>6
116	0.105	None found	>6
92	0.109	None found	>6
121	0.104	None found	>6
110	0.103	None found	>6
108	0.094	None found	>6
114	0.121	None found	>6
93	0.101	None found	>6
58	0.105	None found	>6
44	0.091	None found	>6
53	0.095	None found	>6
88	0.113	None found	>6
41	0.091	None found	>6
2	0.13	None found	>6
49	0.093	None found	>6
107	0.097	None found	>6
86	0.102	None found	>6
103	0.106	None found	>6

105	0.11	None found	>6
5	0.095	None found	>6
16	0.107	None found	>6
96	0.093	None found	>6
4	0.107	None found	>6
8	0.111	None found	>6
87	0.112	None found	>6
89	0.101	None found	>6
1	0.107	None found	>6
95	0.126	None found	>6
91	0.119	None found	>6
102	0.095	None found	>6
100	0.096	None found	>6

Table A5: Final challenge test results on glass bottles

Sample No.	Quantity of Spore Suspension per Bottle (g)	No. of Survivals	Log Reduction
Control 1	0.106	2.00E+06	0
Control 2	0.114	1.00E+06	0
Control 3	0.108	2.50E+06	0
125	0.123	None found	>6
123	0.09	None found	>6
126	0.093	None found	>6
124	0.136	None found	>6
122	0.112	None found	>6
121	0.112	None found	>6
94	0.092	None found	>6
67	0.107	None found	>6
69	0.107	None found	>6
92	0.101	None found	>6
93	0.115	None found	>6
64	0.102	None found	>6
65	0.128	1	>6
95	0.128	None found	>6
96	0.105	None found	>6
97	0.103	None found	>6
39	0.12	None found	>6
84	0.102	None found	>6
37	0.114	None found	>6

35	0.107	None found	>6
36	0.112	None found	>6
39	0.12	None found	>6
79	0.097	None found	>6
80	0.093	None found	>6
81	0.128	None found	>6
38	0.096	None found	>6
83	0.101	None found	>6
82	0.095	None found	>6
7	0.098	None found	>6
11	0.094	None found	>6
70	0.103	None found	>6
78	0.136	None found	>6
72	0.114	None found	>6
77	0.097	None found	>6
71	0.109	None found	>6
61	0.131	None found	>6
47	0.114	None found	>6
76	0.104	None found	>6
8	0.113	None found	>6
12	0.104	None found	>6
10	0.106	None found	>6
9	0.099	1	>6
128	0.087	None found	>6
127	0.103	None found	>6
129	0.104	None found	>6
131	0.086	None found	>6
132	0.116	None found	>6
130	0.109	None found	>6
85	0.116	None found	>6
87	0.103	None found	>6
86	0.119	None found	>6
88	0.099	None found	>6
90	0.096	1	>6
89	0.1	None found	>6
3	0.097	None found	>6
6	0.106	None found	>6
5	0.11	None found	>6
1	0.103	None found	>6
153	0.098	None found	>6
17	0.105	None found	>6
13	0.117	None found	>6
16	0.123	None found	>6

18	0.097	None found	>6
15	0.11	None found	>6
21	0.109	None found	>6
20	0.1	None found	>6
137	0.112	None found	>6
19	0.094	None found	>6
146	0.115	None found	>6
150	0.094	None found	>6
53	0.091	None found	>6
141	0.118	None found	>6
148	0.104	None found	>6
119	0.096	None found	>6
114	0.102	None found	>6
138	0.101	None found	>6
140	0.099	None found	>6
142	0.11	None found	>6
139	0.109	None found	>6
135	0.117	None found	>6
136	0.102	None found	>6
145	0.105	None found	>6
108	0.107	None found	>6
103	0.098	None found	>6
101	0.102	None found	>6
115	0.086	None found	>6
118	0.089	None found	>6
113	0.095	None found	>6
100	0.118	None found	>6
143	0.101	None found	>6
134	0.095	None found	>6
149	0.091	None found	>6
147	0.137	None found	>6
99	0.124	None found	>6
104	0.103	None found	>6
49	0.106	None found	>6
144	0.1	None found	>6
151	0.114	None found	>6
154	0.099	None found	>6
156	0.126	None found	>6
58	0.135	None found	>6
152	0.098	None found	>6
14	0.097	None found	>6
24	0.121	None found	>6
59	0.118	None found	>6

23	0.1	None found	>6
16	0.123	None found	>6
51	0.123	None found	>6
55	0.115	None found	>6
50	0.113	None found	>6
57	0.112	None found	>6
133	0.082	None found	>6
22	0.102	None found	>6
60	0.131	None found	>6
117	0.1	None found	>6
54	0.11	None found	>6
52	0.099	None found	>6
56	0.099	None found	>6
155	0.09	None found	>6

Table A6: Final challenge test results on caps of glass bottles

Sample No.	Quantity of Spore Suspension per Bottle (g)	No. of Survivals	Log Reduction
Control	0.009	1.00E+05	0
Control	0.018	2.00E+05	0
Control	0.019	3.00E+05	0
3*	0.013	None found	at least 5
8*	0.022	None found	at least 5
11*	0.011	None found	at least 5
12*	0.018	None found	at least 5
14*	0.023	None found	at least 5
16*	0.018	None found	at least 5
18*	0.024	None found	at least 5
19*	0.019	3	4.9
20*	0.017	None found	at least 5
21*	0.016	None found	at least 5
22*	0.02	None found	at least 5
24*	0.016	None found	at least 5
27*	0.026	None found	at least 5
33	0.015	None found	at least 5
34	0.03	None found	at least 5
36	0.02	None found	at least 5
37	0.014	None found	at least 5
38	0.018	None found	at least 5
39	0.017	None found	at least 5

40	0.024	None found	at least 5
41	0.024	None found	at least 5
42	0.019	None found	at least 5
43	0.016	None found	at least 5
44	0.022	None found	at least 5
45	0.026	None found	at least 5
46	0.021	None found	at least 5
47	0.017	None found	at least 5
49	0.015	None found	at least 5
50	0.017	None found	at least 5
51	0.015	None found	at least 5
52	0.012	None found	at least 5
53	0.011	None found	at least 5
54	0.021	None found	at least 5
57	0.018	None found	at least 5
58	0.015	None found	at least 5
59	0.012	None found	at least 5

Note: '*' – sample being soaked in 0.5% Perform solution prior to treatment.

Some samples were lost during experimentation due to cross-contamination.