Microbial test organisms for sterilization processes in food industry

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Abstract

The importance of sterilization processes has been a major topic in the food manufacturing industry since it preserves the quality and provides longer shelf-life by preventing microbiological recontamination of products. Aseptic packaging is an alternative technique to pack commercially sterile products into containers with a very low recontamination rate, thus, the entire production chain must be designed according to the aseptic condition, including the packaging material, the filling machines, and the surrounding area where the process takes place.

To evaluate a sterilization process, there is a validation test in which the process is challenged with certain test organisms. VDMA or *Verband Deutscher Maschinen- und Anlagenbau* (German Engineering Federation) has published a guideline (FS NuV No. 10/2005 Appendix A) containing the recommendation of the required outcomes for the validation test of the filling machines depending on the sterilization processes and test organisms.

Furthermore, the guideline has motivated us to obtain further confidence data on the performance of the test organisms. The present study aimed to investigate and compare the resistance of spore-producing organisms, *A. brasiliensis*, *B. atrophaeus*, and *B. subtilis*, against different disinfection/sterilization processes relevant to the food industry as indicated by VDMA, i.e. chemical (hydrogen peroxide and peracetic acid products of Oxonia), thermal (steam, hot water, and dry heat), as well as UV irradiation. A review of existing research was then compiled with the laboratory results to suggest suitable test organisms depending on the sterilization processes applied.

Based on the observations, *B. subtilis* spores are the test organism that showed highest resistance against H_2O_2 and Oxonia sterilization process with $D_{60\ °C}$ = 6,17 s and $D_{ambient}$ = 13,89 s respectively. This result was in accordance with the guidelines of VDMA. For the thermal processes, VDMA suggested *A. brasiliensis* spores as the recommended test organisms. However, our findings resulted that for disinfection and sterilization processes that employ hot water and dry heat, *B. atrophaeus* spores were found as the ideal test organism exhibiting the desired resistance towards the given processes with a $D_{90\ °C}$ and $D_{121\ °C}$ of 8,1 min and 71,94 min. Our review study on steam sterilization suggested *G. stearothermophilus* as the suitable test organisms for steam sterilization, although VDMA suggested *A. brasiliensis* spores. Throughout the study, *A. brasiliensis* spores have demonstrated rapid reductions for all the sterilization process evaluated, indicating low resistance and therefore were not considered as an ideal test organism to use. However, when looking into the available literature on UV irradiation, it was found that *A. brasiliensis* could be a possible test organism to assess its effectiveness considering its black spore pigment, aspergillin, which is responsible to the high UV resistance as it absorbs high amounts of the UV radiation to protect the cell.

Keywords: Aseptic processing and packaging, validation, test organisms, *Aspergillus brasiliensis*, *Bacillus atrophaeus*, *Bacillus subtilis*, sterilization, disinfection.

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Popular science summary

Food spoilage is the process leading to a product becoming either undesirable or unacceptable for human consumption, involving alterations in appearance, taste, texture or smell. This condition can be caused by the activity of specific groups of microorganisms, including spoilage bacteria, yeasts or mould. To make sure the food is easy to consume, the food is sterilized using a controlled heating process used to eliminate microorganisms that can reproduce inside the product under a nonrefrigerated state.

However, some microorganisms can form protective structures called spores when they are triggered by the stressful environment such as lack of nutrient. These spores are in a dormant state, which means their growth is temporarily stopped and helped them to conserve energy. Compared to the living cell, spores are more challenging to kill by heat or even by chemical agents and irradiation. Thus, spores are known to be resistant organisms.

Nowadays, aseptic technology is gaining popularity. The aseptic process allows food to be shelf stable for an extended period and eliminates the need for preservatives. The word ‘aseptic’ itself refers to a condition free of harmful microorganisms. For example, after UHT milk is sterilized for certain temperature and time, the milk is filled into a pre-sterilized container and the package is sealed under a sterilized environment. The aseptic technique is aimed to ensure that no recontamination occurs throughout the sterilization process.

Filling machines for aseptic processing and packaging (Tetra Pak, 2018a)

To make sure the sterilization process is working correctly, the aseptic process can be monitored using a validation or challenge test. This method is performed using resistant organisms, such as spore-producing organisms, to measure the effectiveness of certain sterilization processes. The challenge test is carried by firstly applying a defined load of the test organisms on the filling machines, subjecting the organisms to the sterilization process, and finally counting the number of spores that are recovered after the treatment. These values are known as logarithmic cycle reduction (LCR), expressing the number of spores that are killed throughout the sterilization whereas reflecting the effectiveness of the sterilization. Generally, killing the spores up to 4-5 LCR is needed to achieve a trustworthy sterilization process.

Illustration of the recovery of bacterial spores after treatment with sterilization process (Tetra Pak, 2018a)

In relation to that, VDMA or *Verband Deutscher Maschinen- und Anlagenbau* (German Engineering Federation) has published a guideline containing the required outcomes for the challenge test based on different sterilization processes and test organisms. In this guideline, VDMA suggests different test organisms for certain sterilization processes relevant to the food industry; chemical processes of hydrogen peroxide and peracetic acid product (e.g. Oxonia), heat processes of steam, dry heat, and hot water; as well as Ultraviolet (UV) irradiation process. Furthermore, the VDMA guidelines have motivated the present study to obtain further confidence data on the performance of the test organisms. Therefore, the aim of the study is to investigate and compare the level of resistance of three different test organisms of *A. brasiliensis, B. atrophaeus,* and *B. subtilis* against different sterilization process as listed by VDMA. After that, the results of the experiments will be compiled with a review of existing studies to finally propose suitable combinations of test organisms and sterilization processes.

Based on the research outcomes, both *B. atrophaeus* and *B. subtilis* spores are recommended as suitable test organisms to challenge the chemical sterilization processes. Meanwhile, for hot water and dry heat processes, *B. atrophaeus* is more suitable. Comparing with the guidelines from VDMA, *B. subtilis* is also recommended to test the chemical processes. However, for hot

water and dry heat, VDMA guidelines reported that *A. brasiliensis* is more suitable. In spite of the recommendation from VDMA, our test results implied that *A. brasiliensis* spores are highly sensitive to the sterilization processes, meaning that the spores are easier to kill, thus, not reliable to be an indicator of a successful sterilization process. However, when looking into existing researches on UV irradiation, it was known that *A. brasiliensis* could be suitable to evaluate a UV irradiation process, as the spores have a specific mechanism of a spore pigment that could absorb the UV to protect themselves. Additionally, *Geobacillus stearothermophilus* are the test organisms that are recommended for the steam sterilization based on literature reviews since many studies have shown the spores' resistance towards the steam process.

Table of contents

Abstract **1**
Acknowledgments **2**
Popular science summary **3**
Table of contents **6**
List of tables **8**
List of figures **9**
1. Introduction **11**
1.1 Aseptic processing and packaging **11**
1.2 VDMA guidelines **12**
1.3 Sterilization efficiency **15**
1.3 Microbiological fundamentals **15**
1.3.1 Vegetative cells and spores 16
1.3.2 Structure and function of the vegetative cell and spores 17
1.4 Microbiological test organisms **18**
1.5 Different sterilization processes **19**
1.5.1 Heat sterilization 19
1.5.2 Chemical sterilization 20
1.6 Aim **21**
2. Material and methods **23**
2.1 Test organisms **23**
2.2 Spore solutions and inoculation of carriers **23**
2.3 The EWA test rig **24**
2.3.1 Titration 25
2.3.2 Exposure of inoculated carriers and recovery of survivors 26
2.3.2.1 Exposure to hydrogen peroxide solution 27
2.3.2.2 Exposure to Oxonia solution 27
2.4 Hot water disinfection **28**
2.4.1 Preparation of the equipment 28
2.4.2 Exposure of inoculated carriers and recovery of survivors 28
2.5 Dry heat sterilization **29**
2.6 Enumeration of survivors and statistical analysis **29**
3. Results **31**

3.1 Resistance of the test organisms against hydrogen peroxide 31
3.1.1 Resistance of *A. brasiliensis* spores against hydrogen peroxide 31
3.1.1.1 Screening tests for selection of exposure parameters 31
3.1.1.2 Resistance tests of *A. brasiliensis* spores against hydrogen peroxide 33
3.1.2 Resistance of *B. atrophaeus* spores against hydrogen peroxide 34
3.1.2.1 Screening tests for selection of exposure parameters 34
3.1.2.2 Resistance tests of *B. atrophaeus* spores against hydrogen peroxide 35
3.1.3 Resistance of *B. subtilis* spores against hydrogen peroxide 35
3.2 Resistance of the test organisms against Oxonia 36
3.2.1 Screening tests for selection of exposure parameters 36
3.2.2 Resistance tests of *A. brasiliensis* spores against Oxonia 37
3.2.2.2 Resistance tests of *B. atrophaeus* spores against Oxonia 38
3.2.2.3 Resistance tests of *B. subtilis* spores against Oxonia 38
3.3 Resistance of the test organisms against hot water disinfection 39
3.3.1 Resistance tests of *A. brasiliensis* spores against hot water 39
3.3.2 Resistance tests of *B. atrophaeus* spores against hot water 40
3.3.3 Resistance tests of *B. subtilis* spores against hot water disinfection 41
3.4 Resistance of the test organisms against dry heat sterilization 41
3.4.1 Resistance tests of *A. brasiliensis* spores against dry heat sterilization 41
3.4.2 Resistance tests of *B. atrophaeus* spores against dry heat sterilization 42
3.4.3 Resistance tests of *B. subtilis* spores against dry heat sterilization 43
4. Discussion 44
5. Summary 48
6. Conclusion 50
7. Future work 51
8. References 52
Appendices 58
Appendix 1 Chemicals used in phosphate buffer 58
Appendix 2 Titration method provided by Ecolab Inc. for hydrogen peroxide and Oxonia 58

List of tables

Table 1 Hygienic filling machines for liquid and viscous products - classification and typical fields of application (FS-NuV No. 2/2007).....13

Table 2 Minimum microbiological requirements for hygienic filling machines of Class IV of different sterilization processes (FS NuV No. 10/2005 Appendix A). 14

Table 3 Experimental design for hydrogen peroxide treatment. 27

Table 4 Experimental design for hydrogen peroxide treatment. 27

Table 5 Impact of small amounts of H_2O_2 concentration on *A. brasiliensis* spores at ambient temperature. This data originates from previous investigations performed at Tetra Pak.....31

Table 6 First screening test of *A. brasiliensis* spores against different concentrations of H_2O_2 at ambient temperature. 32

Table 7 Second screening test of *A. brasiliensis* spores against H_2O_2 (3 ± 0,5 %) at 40 ± 0,2 ˚C. 32

Table 8 Third screening test of *A. brasiliensis* spores against H_2O_2 (3 ± 0,5 %) at 60 ± 0,2 ˚C...33

Table 9 Screening test of *B. atrophaeus* spores against H_2O_2 (30 ± 0,5 %) at 60 ± 0,2 ˚C.....34

Table 10 Second screening test of different test organisms against Oxonia (0,9 ± 0,5 %) at ambient temperature. 37

Table 11 Comparison of test organisms for different sterilization processes as proposed by this study and VDMA 48

List of figures

Figure 1 Principle of aseptic packaging (Tetra Pak, 2018a). 11

Figure 2 Packaging materials based on paper-based laminates (Tetra Pak, 2018b). 12

Figure 3 Concept of D-value (Chandrapati & Young, 2008). 15

Figure 4 Cell-cycle of Bacillus: sporulation and vegetative growth (Sella et al., 2015; modified from de Hoon et al., 2010). 16

Figure 5 Spore structure. The labeled spore layers are not drawn to scale. The sizes of the various layers, particularly the exosporium, vary significantly between spores of different species (Setlow, 2006). 17

Figure 6 Scanning electron micrographs of *B. atrophaeus* spores. (A) Vegetative cells, (B) vegetative cells and sporangia, and (C) spores and release of the mature spores from its mother cell compartment (Sella et al., 2015). 18

Figure 7 The electromagnetic spectrum (Calado et al., 2014, adapted from Satin, 1996) 21

Figure 8 Test carriers used in the study. A) Packaging carrier, B) stainless-steel carrier (Photograph by Y. Nabila). 23

Figure 9 The EWA test rig composed of (a) chemical bath, (b) sample holder, (c) pump, (d) heater and stirrer, ... 24

Figure 10 Titration of hydrogen peroxide (Photograph by Y. Nabila). 25

Figure 11 Titration of peracetic acid (Photograph by Y. Nabila). 26

Figure 12 Mini Block Heater used in the hot water experiment (VWR International, 2018). 28

Figure 13 Examples of plates containing CFU of *B. subtilis* (a), *B. atrophaeus* (b), *A. brasiliensis* (c) that were subjected to enumeration are provided (Photograph by Y. Nabila). 30

Figure 14 Inactivation curve of *A. brasiliensis* spores against 3 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed. 34

Figure 15 Inactivation curve of *B. atrophaeus* spores against 30 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed. 35

Figure 16 Inactivation curve of *B. subtilis* spores against 30 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed. 36

Figure 17 Inactivation curve of *A. brasiliensis* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed. 37

Figure 18 Inactivation curve of *B. atrophaeus* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed. 38

Figure 19 Inactivation curve of *B. subtilis* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed.....39

Figure 20 Inactivation curve of *A. brasiliensis* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 40

Figure 21 Inactivation curve of *B. atrophaeus* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 40

Figure 22 Inactivation curve of *B. subtilis* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 41

Figure 23 Inactivation curve of *A. brasiliensis* spores against dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 42

Figure 24 Inactivation curve of *B. atrophaeus* spores exposed to dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 43

Figure 25 Inactivation curve of *B. subtilis* spores exposed to dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed. 43

1. Introduction

Commercial sterility of thermally processed food is defined as "the condition achieved by the application of heat which renders the food free of (1) microorganisms capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution; and (2) viable microorganisms (including spores) of public health significance" (Food and Drug Administration, 2017). This state of commercial sterility can be achieved through inactivation of microorganisms in the product (e.g. pasteurization, irradiation, high heat treatment) or prevention/inhibition of growth of microorganisms in the product (e.g. chilling, drying, an addition of preservatives) (Gould, 1996). To preserve the high microbiological quality of a commercially sterile product, the food industry is employing several preservation techniques (e.g., heating, chilling, drying, curing, acidification, oxygen-removal, fermenting, etc) (Singh and Shalini, 2016).

1.1 Aseptic processing and packaging

Aseptic processing and packaging is a sophisticated food preservation method where the product is commercially sterilized outside the container, filled into pre-sterilized containers, followed by sealing in an aseptic environment (Sancho-Madriz, 2003). The term "aseptic" indicates that unwanted organisms are excluded from the product, container, or other specific areas within the packaging operation (Carlson and Burrell, 1980). Aseptically packaged products are intended for long periods of non-refrigerated storage. As a consequence, the performance level of an aseptic plant must be regarded entirely rather than that of a single component of the processing line. This could mean that existing link within the processing and packaging chain must be equally strong (Lindell, 2017) (see Figure 1).

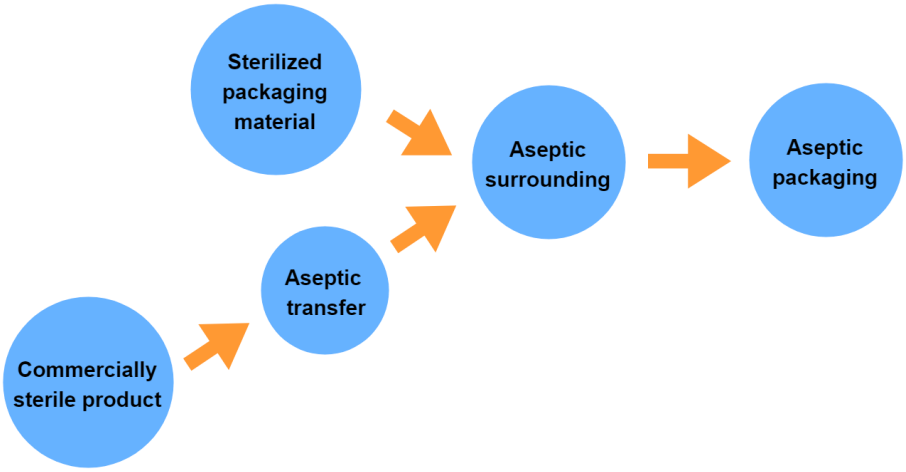


Figure 1 Principle of aseptic packaging (Tetra Pak, 2018b).

Aseptic packaging technology has been around for over 70 years. Food processors and retailers interested in shelf-stable food products that can withstand ambient storage conditions are particularly interested (Pillai and Shayanfar, 2015). Nowadays, the aseptic packaging technology is driven by the increased use in dairy- and non-dairy-based products for the ready-to-drink (RTD) market, such as teas, iced coffees, yogurt, smoothies, and cocktail concentrates. The growing demand for aseptic packaging and processing also motivates the industry to conduct extensive research and development activities covering the different aspects of aseptic packaging (David et al., 2013; Lindell, 2017). Furthermore, a large number of different aseptic filling machines are being developed to fill the products into various types of materials and packages:

(1) glass bottles, (2) cans, (3) plastic bottles, (4) plastic pouches, (5) plastic cups, and (6) paper-based laminates (David et al., 2013). For an example of paper-based laminates, see Figure 2.

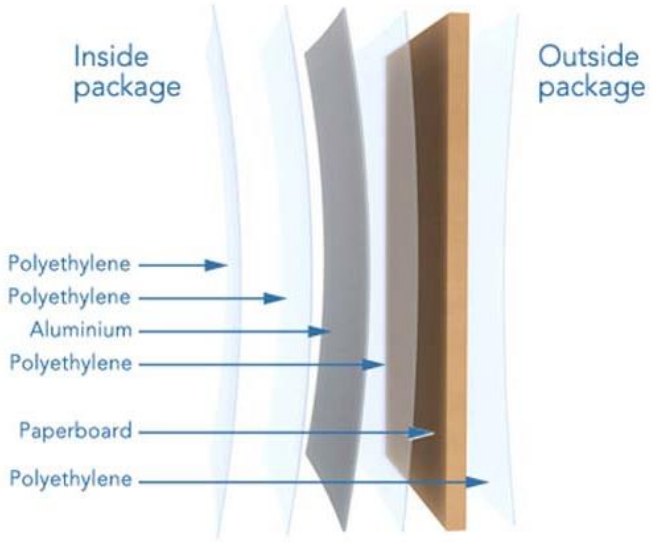


Figure 2 Packaging materials based on paper-based laminates (Tetra Pak, 2018c).

The principle of aseptic packaging is to pack commercially sterile product into sterilized containers without compromising the commercial sterility of the product. In order to do so, the entire production chain must be designed according to the aseptic condition (Ansari and Datta, 2003). Filling machines are designed to fill a commercially sterile product without recontamination into a sterile pack. This comes with high requirements not only on the filling machine themselves and their surroundings but also on the packaging to be filled and on the packaging aids (closures, lids, sealing foils, and possibly some applications such as pouring aids) which could infiltrate the sterile area in the interior of these filling machines (VDMA, 2011).

1.2 VDMA guidelines

VDMA or *Verband Deutscher Maschinen- und Anlagenbau* (German Engineering Federation) is a German nonprofit organization representing the local machinery and industrial equipment manufacturer. The VDMA has divided filling machines into 5 different categories on the basis of the features that the machines are equipped, as described in *Fachverbandsschriften Nahrungsmittelmaschinen und Verpackungsmaschinen* or VDMA-Documents Food Processing Machinery and Packaging Machinery (FS-NuV) No. 2/2007 (VDMA, 2007) (see Table 1).

Table 1 Hygienic filling machines for liquid and viscous products - classification and typical fields of application (FS-NuV No. 2/2007).

Machine fittings	**I**	**II**	**III**	**IV**	**V**[1]
Cleaning in place (CIP) of parts in contact with a product	Optional	x	x	x	x
Disinfection/sterilization of the filler	Optional	Disinfection or sterilization[2]	Disinfection or sterilization[2]	Disinfection or sterilization according to FS NuV 10/2005 Appendix A	Sterilization according to FS NuV 11/2005 Appendix A.1
Recontamination protection of parts in contact with a product	-	x	x	Maintenance of sterile condition[3]	Maintenance of sterile condition
Requirements to be met by the control system	-	Monitoring parameters relevant to • CIP • Disinfection/ sterilization of filler	Automatic control of parameters relevant to • CIP • Disinfection/ sterilization of filler • Packaging sterilization[4]	Automatic control of parameters relevant to • CIP • Disinfection/sterilization of filler • Packaging sterilization[5]	According to FS NuV 11/2005 Appendix A.2
Packaging sterilization[6] and recontamination protection of sterilized packaging until filling	-	-	x	According to FS NuV 11/2005 Appendix A	According to FS NuV 11/2005 Appendix A.1
Cleaning & sterilization plus recontamination protection of the sterile zone of the machine interior[7]	-	-	-	According to FS NuV 11/2005 Appendix A	According to FS NuV 11/2005 Appendix A.1
Product example	Fruit juice, hot filling (pH ≤ 4,5; shelf life > 6 months)	Natural yogurt with (pH ≤ 4,5; shelf life 12-15 days)	Natural yogurt with (pH ≤ 4,5; shelf life 2-4 weeks)	Natural yogurt with (pH ≤ 4,5; shelf life 30 days)	UHT milk with (pH 6,6; shelf life > 3 months)

1 Aseptic packaging machines for the food industry as indicated with VDMA FS NuV No. 11/2005.
2 Insofar as sterilization of the filling machine is necessary, the requirements are written in VDMA FS NuV No. 11/2005.
3 In the case of filling a sterile product.
4 The CIP process may be monitored and controlled by the central CIP facility. The monitoring of parameters of a concentration of cleaning and disinfection agents are usually not included in the automatic monitoring and control system.
5 The CIP process may be monitored and controlled by the central CIP facility. The monitoring of parameters of a concentration of cleaning and disinfection agents are usually not included in the automatic monitoring and control system.
6 Alternatively, the packaging sterilized outside the packaging machine may also be fed into the filling machine without recontamination. The requirements for packaging sterilization, in this case, are the same as for packaging sterilization inside the packaging machine.
7 Including parts in contact with the product.

By specifying the typical field of application of the different machines, it may help the manufacturers in comparing the machine category suitable for a particular product to be packaged. However, taking into account the diversity and varieties of the product characteristics, assigning the products to machine categories may be a difficult task. Accordingly, the hygienic

requirements of the filling machines should always be established for the specific application in question (VDMA, 2007).

Furthermore, to ensure the microbiological safety of these hygienic filling machines, there are minimum requirements that must be fulfilled. For instance, the filling machines must be technically competent to reliably reducing the microbial load as well as maintaining the achieved microbial state during operation. In order to achieve this condition, the filling machines can be adequately validated through a microbiological challenge test where specific test organisms are tested towards sterilization processes (FDA, 2004; VDMA, 2005).

According to the International Organization for Standardization-ISO 11139 (2006), a test organism is defined as "a test system containing viable organisms of a pure, specified strain providing a defined resistance to a specified sterilization process". The test organism is generally recognized and accepted as microorganisms to be used during validation to represent the prevailing microorganism of the specific sterilization process being employed (target organism) (IFTPS, 2011), Although physical monitors and chemical indicators have been used to provide valuable information regarding the sterility of a product, microbiological test organisms are still recognized by most authorities as being closest to the ideal monitor of the sterilization process because they can carry out critical measurement of the direct lethality, i.e. the inactivation rate of that process (Schneider, 2011). Typically, the test organisms selected for microbial challenge tests should carry a resistance against the inactivation process that is higher than the resistance of the target organism (Gomez and Moldenhauer, 2009).

In relation to the validation of the sterilization process, VDMA has published a list of the recommendation of minimum microbiological requirements that must be fulfilled by the filling machines, especially of Class IV (see Table 2).

Table 2 Minimum microbiological requirements for hygienic filling machines of Class IV of different sterilization processes (FS NuV No. 10/2005 Appendix A).

Sterilization processes	Test organisms; required log cycle reduction (LCR) values
Hydrogen peroxide (H_2O_2)	*B. subtilis* SA 22; LCR ≥ log 3 *A. niger* (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404); LCR ≥ log 4
Steam and hot water	*A. niger* (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404); LCR ≥ log 4
Peracetic acid products	*B. subtilis* SA 22; LCR ≥ log 3 *A. niger* (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404); LCR ≥ log 4
UV	*A. niger* (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404); LCR ≥ log 3
Infrared and dry heat	*A. niger* (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404); LCR ≥ log 4

From Table 2, recommendations about the required outcomes on validation test depending on the sterilization processes and test organisms are shown. In general, microbiological validation provides evidence that the processes applied for the machine and package sterilization deliver a logarithmic cycle reduction (LCR) higher than a stated target value for a suitable test organism. The choice of test organisms and the establishments of required LCR values for the selected test organisms are done as a function of the sterilization processes of interest, considering the distinct sensitivity of test microorganisms to the different sterilization processes (VDMA, 2005).

To perform a validation test, firstly, a defined load of the test organism is applied either on the filling machine or packaging material. After being subjected to the sterilization process, or other

similar treatment, the test organism is recovered to determine the efficiency of the sterilization process and the reduction level of the test organism under this process (Moruzzi et al., 2000; Sella et al., 2015). The number of viable spores recovered after treatment and enumerated after sterilization will be regarded as 'recovered spores' hereafter.

1.3 Sterilization efficiency

Aseptic packaging machines must reliably reduce microorganisms including bacterial spores. LCR is a commonly used measure of the efficacy of a sterilization process. It is expressed as the decimal logarithm of the ratio of the initial count (N_0) of the test organism and the count of the same organism (N) after a given time of contact with the sterilant (IFTPS, 2011).

Each sterilization process is characterized by a sterilization effect or efficiency. It is expressed by the number of LCR in the count of resistant spores, which are suitable for the respective sterilization procedure concerned (test organisms). The D-value is normally used to indicate the killing rate, i.e. the time required at a certain temperature and condition to kill 90% of the spores, which is the same as reducing their number by one logarithm unit (von Bockelmann, 1930) (see Figure 3).

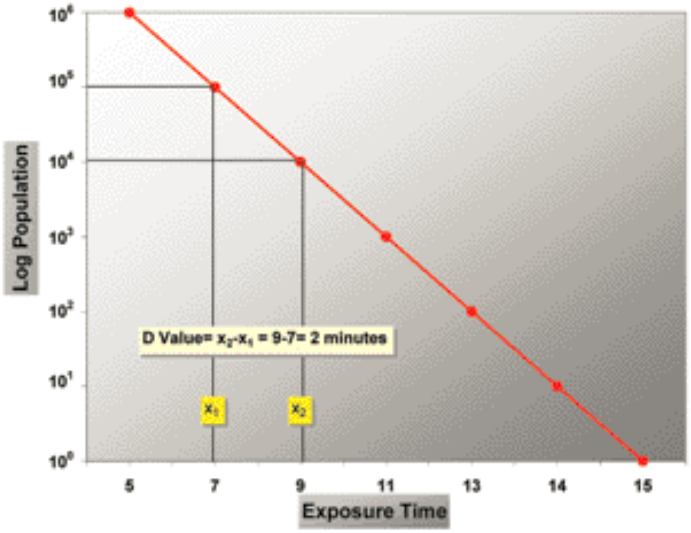


Figure 3 Concept of D-value (Chandrapati & Young, 2008).

The Food Processing and Packaging Machinery Association sector in VDMA has published test procedures for the determination of the count reduction rates (FS NuV No. 11/2006 Annex A). The sterilization for aseptic packaging operation has been defined as a reduction of the microbial load from 10^4 to 10^0, i.e. four-decimal reduction cycles or LCR ≥ 4 (VDMA, 2010).

1.3 Microbiological fundamentals

To evaluate a sterilization process and to select adequate microorganisms to test the efficiency of a process, basic knowledge towards vegetative cells and spores is required.

1.3.1 Vegetative cells and spores

Microorganisms vary in resistance towards harsh conditions with vegetative forms of bacteria and fungi being the least resistant, followed by large viruses and small viruses, while bacterial spores are those exhibiting the highest resistance (Chandrapati and Young, 2008). A vegetative microorganism is a cell with an active metabolism. If the circumstances provide beneficial habitat (temperature, humidity, nutrients) for the cell then it can live without changes. A virus needs a host to live in every case. The virus can not survive outside the host, e.g. on drugs or medical implants. For the evaluation of a sterilization process viruses are not taken into account (Halfmann, 2008).

The vegetative cells must be divided into two groups: the eukaryote and prokaryote cells. Eukaryote cells, which compose the human body, are much more complicated in means of metabolism and structure than the simpler prokaryote cells such as bacteria. In related to the sterilization purpose, the prokaryotes are haploid, meaning that there is only one copy of the gene. Additionally, prokaryotes often contain several plasmids as important parts in bacteria. A plasmid is a circular and double-stranded DNA molecule separated from chromosomal DNA. Plasmids can give a bacterium some specific capabilities, e.g. the ability to make the bacterium antibiotic resistant (Griswold, 2008).

Various bacteria (e.g. *Bacillus atrophaeus* and *Geobacillus stearothermophilus*) generate spores as a self-preservation strategy against harsh conditions. When the bacteria endure environmental stresses, caused by fewer nutrients, low or high temperatures, and UV irradiation, sporulation is stimulated and the formation of the spore occurs (see Figure 4). The state of a spore enables the bacteria to survive over many years while in addition, it becomes harder to kill/sterilize compared to the vegetative form (Leggett et al., n.d.; Setlow, 2006). To evaluate the efficiency of a sterilization process endospores are chosen (Halfmann, 2008).

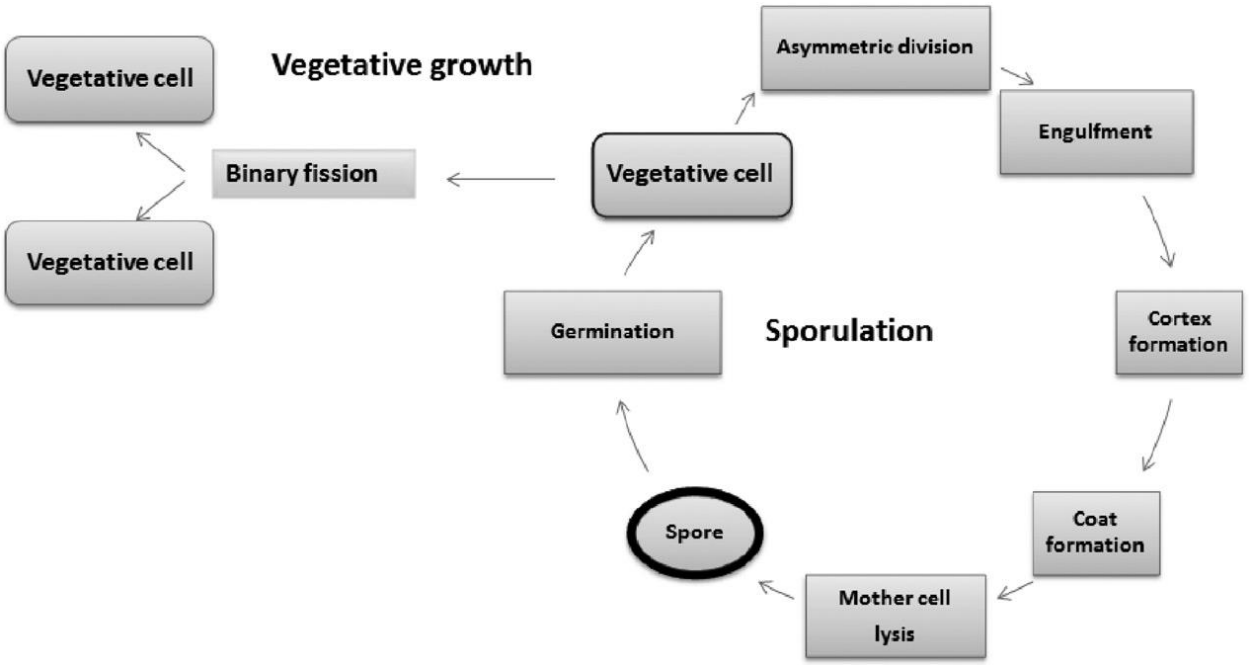


Figure 4 Cell-cycle of *Bacillus*: sporulation and vegetative growth (Sella et al., 2015; modified from de Hoon et al., 2010).

1.3.2 Structure and function of the vegetative cell and spores

The wall of a vegetative cell stabilizes the cell and is the interface to exchange nutrients, waste, and messengers. Peptidoglycan is the backbone of the cell wall, consisting of different structures. It must be emphasized that the important parts of the cell wall comprise mainly of hydrogen, carbon, oxygen, and nitrogen. The peptidoglycan builds one big molecule that encloses the cell, in which one cell can be enclosed by many peptidoglycan layers. If the cell grows, its wall has to grow. However, a lack in the structure can lead to cell death while growing (Atrih et al., 1999).

The endospore coat of a spore is different from the vegetative cell wall (see Figures 5 and 6). The spore coat differs in thickness, components, and function. The main function is to protect the microorganism from external stress and enable germination after a long time. Additionally, the spore coat differs from one bacterium to another. *Bacillus cereus* spores have a simple coat consisting of proteins. *B. atrophaeus* spores have two layers differing in thickness (70 nm and 70 - 200 nm) and *Bacillus anthracis* spores have three coats. Behind the coats is the peptidoglycan layer with a thickness around 8 nm for Gram-negative and 20 – 80 nm for Gram-positive bacteria (Halfmann, 2008).

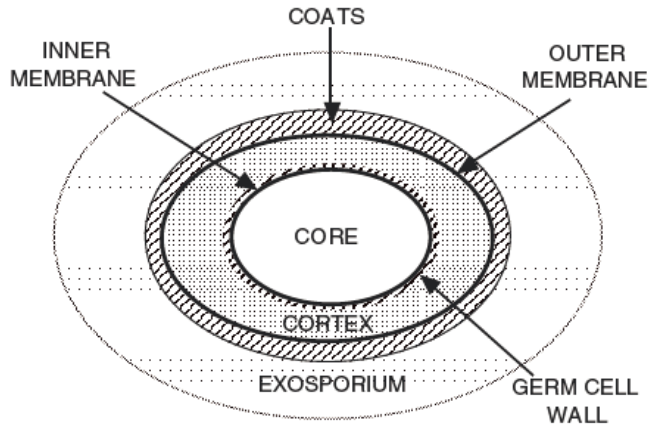


Figure 5 Spore structure. The labeled spore layers are not drawn to scale. The sizes of the various layers, particularly the exosporium, vary significantly between spores of different species (Setlow, 2006).

The spore coat is mainly made up of proteins with additions of carbohydrate and lipids. Proteins are organic compounds made of amino acids in different structures. They are arranged in linear chains and joined by peptide bonds between the carboxyl and amino groups. Lipids are mainly fatty-acids and their amphiphilic nature is needed for the coat. Driks (1999) describes in detail the structure and function of the coat, meanwhile, Aronson and Fitz-James (1976) discover a correlation between the structure and components of the spore coat and the ability to germinate and to grow.

The spore core is protected by organic compounds (Milhaud and Balassa, 1973). The outer coat protects the spore against lysozyme that is able to dissolve the spore coat (Zheng et al., 1988). Peptidoglycan behind the outer and inner coat must be protected by the spore coat. If the spore germinates, the peptidoglycan layer develops to the later cell coat (Aronson and Fitz-James, 1976).

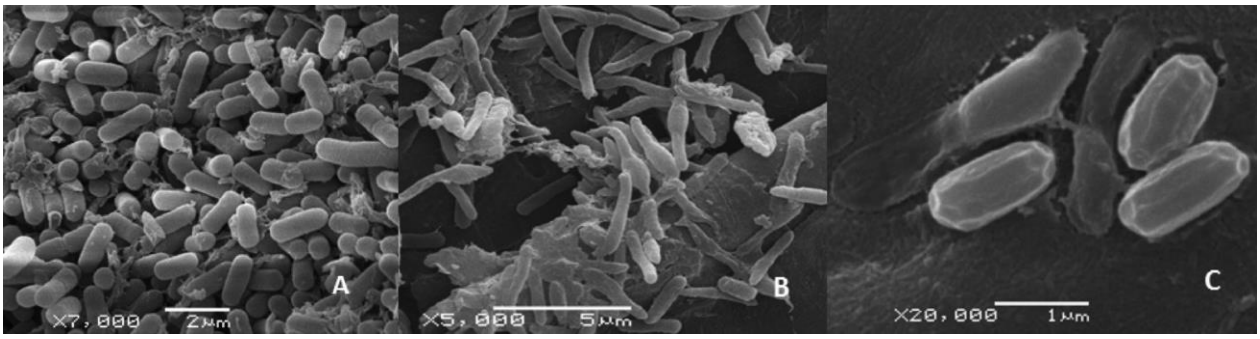


Figure 6 Scanning electron micrographs of B. atrophaeus spores. (A) Vegetative cells, (B) vegetative cells and sporangia, and (C) spores and release of the mature spores from its mother cell compartment (Sella et al., 2015).

1.4 Microbiological test organisms

Most widely used species as the test organisms in industry belong to the genus of *Bacillus, Geobacillus,* and *Clostridium*. These organisms are all Gram-positive bacteria with rod-shaped cells. *Bacillus* and *Geobacillus* being aerobic species while *Clostridium* anaerobic (Adams and Moss, 2008; Sella et al., 2015). *G. stearothermophilus, B. atrophaeus* and *B. subtilis* are some of the most prevalent species used as test organisms for validation of sterilization processes such as hydrogen peroxide, dry heat and moist heat (USP 29, 2005).

Within the *Bacillus* groups are many species and each species has different tolerances to sterilization processes. *B. atrophaeus* (also identified as *B. globigii, B. niger,* and *B. subtilis* var *niger*) and *B. subtilis* are two of the most recognized species for test organism. Nakamura (1989) stated that except for the color of the soluble pigment, all strains of *B. atrophaeus* showed the traits typical of *B. subtilis.*

B. atrophaeus has been established as industrial bacteria to produce test organisms for validation of many different sterilization processes including dry heat, ethylene oxide, hydrogen peroxide, as well as plasma sterilization (FDA, 2007; USP 29, 2005). It is a mesophilic organism with an optimum growth temperature ranged from 30 - 39˚C (Ridgeway, 2018).

B. subtilis is a mesophilic organism with an optimum growth temperature range between 30˚C - 39˚C (Dowben and Weidenmuller, 1968; Eschlbeck et al., 2018). Strains of *Bacillus subtilis* species produce spores of specific resistance to, for example, ethylene oxide, hydrogen peroxide-based sterilization (Eschlbeck et al., 2018; Fritze and Pukall, 2001). Additionally, Ridgeway (2018) added that this organism is often employed in low-temperature steam sterilization processes and is a good option to validate heat sensitive products that may be compromised by the time and temperature of the standard 121˚C steam sterilization cycles.

Besides the *Bacillus* genus, there is *Geobacillus,* a recognized test organism employed for hydrogen peroxide, steam, propylene oxide, formaldehyde and ozone sterilization processes. *Geobacillus* is a thermophilic, aerobic or facultatively anaerobic organism. Depending on the strain, its growth temperature can be as low as 35˚C to as high as 80˚C. *Geobacillus stearothermophilus* has an optimum growth temperature between 55˚C - 60 ˚C (Ridgeway, 2018; Zeigler, 2014). It is notably known for its resistance against heat and used to verify the sterilization efficiency in an autoclave process (Halfmann, 2008; Huesca-Espitia et al., 2016).

Apart from bacterial spores, mold spores can also exhibit resistance to sterilization processes and can, therefore, be used as test organisms in challenge tests. *Aspergillus niger* (renamed as *Aspergillus brasiliensis*) spores, commonly referred to as black mold, are darkly pigmented due to their high melanin content. Cited by Taylor-Edmonds et al. (2015), the study of *A. brasiliensis* spores is attractive due to their high UV-resistance and non-pathogenic nature. From the mentioned study, it was also concluded that the spores are not dormant and can undergo morphological changes when exposed to UV. In another study, up to 5 log spore reduction can be achieved after spores of the organism are exposed to UV light with a wavelength (λ) of 308, 222, and 172 nm (Trompeter et al., 2002). Halfmann (2008) reported that irradiation below λ values of 200 nm towards spores of *A. niger* is found to be highly efficient.

1.5 Different sterilization processes

Various types of sterilization/disinfection processes are currently used in the aseptic packaging system. The process employed should be established in terms of numbers of LCR of the most resistant organisms. In the current study, the effect of chemical sterilization, heat sterilization/disinfection, as well as irradiation towards microbial test organisms was evaluated.

1.5.1 Heat sterilization

Sterilization using heat is mainly categorized into moist heat and dry heat. Resistance to moist heat is probably the spore's most well-known function. Generally, spores are resistant to approximately 45 °C higher temperatures than vegetative cells, and spores of some thermophilic species can survive 100 °C for certain minutes (Scherrer et al., 1971; Warth, 1980). The optimum growth temperature of the organism contributes to the spore resistance to moist heat in the form of steam sterilization, in which thermophilic organisms (i.e. *Geobacillus stearothermophilus*) has the most heat-resistant spores than mesophiles. Spores of *G. stearothermophilus* are generally used for sterilization assurance of this method since this organism is a thermophile and the deriving spores have very low water content and high α/β-type SASP levels (Huesca-Espitia et al., 2016). Although the mechanism is still unknown, low water content property is probably related to the immobility of core proteins while high α/β-type SASP can protect spore DNA intensively from wet heat damage (Gomez and Moldenhauer, 2009). The other contributing factor is the DPA level. As previously mentioned, DPA has a major role in reducing core water content so in consequence, DPA-less spores have higher core water content and are much heat sensitive (i.e. *B. subtilis*) (Paidhungat et al., 2002).

Dry heat sterilization is known as a slow process that requires high temperature and longer times than wet heat sterilization (Joslyn (2001) in Kempf et al., (2008)). It kills spores largely by the accumulation of damage to spore DNA, specifically single-strand breaks (He et al., 2018). Although the precise mechanism of this damage has not been determined thoroughly, Alderton and Snell (1969) reported that spores of thermophiles are no more dry heat resistant than are spores of mesophiles. However, it is believed that the ability for DNA repair becomes important for dry heat resistance of spores (Setlow, 2006). The precise nature has not been known but the damage is mutagenic.

Another thermal inactivation is the hot water disinfection. Some studies describe hot water as thermal pasteurization, which marks the difference with the moist heat method that may involve higher exposure temperature and pressure. Inactivation of spores with hot water often occurs by

inactivation of core enzymes and breakage of the spore's inner membrane permeability barrier (Setlow et al., 2002). Based on a comparative analysis of different test organisms, it was reported that *B. atrophaeus* spores were inactivated more efficiently by hot water disinfection compared to *B. cereus*, while *G. stearothermophilus* spores were considered the most resistant spores (Veen et al., 2015).

1.5.2 Chemical sterilization

Chemical sterilization of food contact surfaces is the most frequently applied procedure in aseptic packaging, with chlorine-based disinfectants finding particularly widespread use (von Bockelmann, 1930). Two such oxidizing agents, peracetic acid (PAA) and hydrogen peroxide (H_2O_2), are also sporicidal and are used for industrial process decontamination, food decontamination, and medical device disinfection/sterilization and, increasingly, as environmental disinfectants.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (1.1)$$

H_2O_2 is the chemical compound mostly used in the process of sterilization of filling machines and packaging material. The hydrogen peroxide dissociates in the sterilization device and the oxidation capability of hydrogen and oxygen as OH and O inactivate organisms (Halfmann, 2008). This reaction depends on temperature, i.e. yields a faster reaction time. As an oxidizing agent, hydrogen peroxide has strong oxidizing potential and will oxidize many organic compounds, since it generates highly reactive hydroxyl radicals. This will create a sterile environment since the organic compounds will be destroyed. The spore coat has been identified as critical mechanisms against chemical sterilization, especially H_2O_2 (Riesenman and Nicholson, 2000). In addition, the killing of spores by this process can also be affected by core water content, wherein spores with higher core water levels are correlated with greater sensitivity to peroxide, although the mechanism is unclear (Popham et al., 1995).

The most common way to use hydrogen peroxide in aseptic packaging is to use a mixture of 35% w/w hydrogen peroxide and 65% w/w water at a temperature of 60-130˚C. The efficiency of the sterilization process is also concentration dependent and increases when the hydrogen peroxide concentration increases (Larsson, 2011). Depending on the design of the aseptic packaging equipment, different means of applying the sterilant are used: spray; vapor; roller systems; immersion bath, etc (von Bockelmann and von Bockelmann, 1998). For processes that involve plastic polyethylene sheet coming from the roll, the sterilization is usually employed by immersion in an H_2O_2 solution bath ranging from 10 to 35% for a few seconds at either room or higher temperatures. *B. subtilis* and *B. atrophaeus* are among the choices of test organisms to challenge this method (Ansari and Datta, 2003).

Peracetic acid (PAA) is another antimicrobial agent that is also an effective sporicide at low temperatures and shows continuous action in the presence of organic materials (Baldry, 1983). In various commercially available products used in the sterilization and sanitization of industrial equipment, PAA is the active compound. Similar to H_2O_2, the sporicidal activity of PAA is increased by raising its concentration and temperature (Cardoso et al., 2011).

$$CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH + H_2O_2 \rightarrow CH_3\overset{\overset{O}{\|}}{C}\text{-}O\text{-}OH + H_2O \quad (1.2)$$

In addition, it is well cited that PAA and H_2O_2 in combination (P/H) act synergistically to improve their bactericidal and sporicidal activities relative to those of either agent used alone. Several products exploiting this combination are currently approved by the U.S. Food and Drug Administration for use as a sterilant and/or high-level disinfectants (Leggett et al., 2016). Oxonia Active (hereafter referred to as Oxonia) is one of the examples, produced by a company called Ecolab. It is an equilibrium mixture of hydrogen peroxide, peracetic and acetic acids, and 1-hydroxyethylidene-1,1-diphosphonic acid, which is currently approved for use as a sanitizer (Blakistone et al., 1999).

1.5.3 Irradiation

When neither heat nor chemicals alone can be used to sterilize a given material, irradiation is considered as an option. Irradiation has been recommended as a suitable sterilant for packaging material food-contact surfaces (von Bockelmann, 1930). Electromagnetic irradiation (see Figure 7) such as infrared, ultraviolet and ɣ rays which are characterized by a frequency, a wavelength, penetrating power and an energy range are already employed either alone or in combination with chemicals for sterilization procedures, etc.

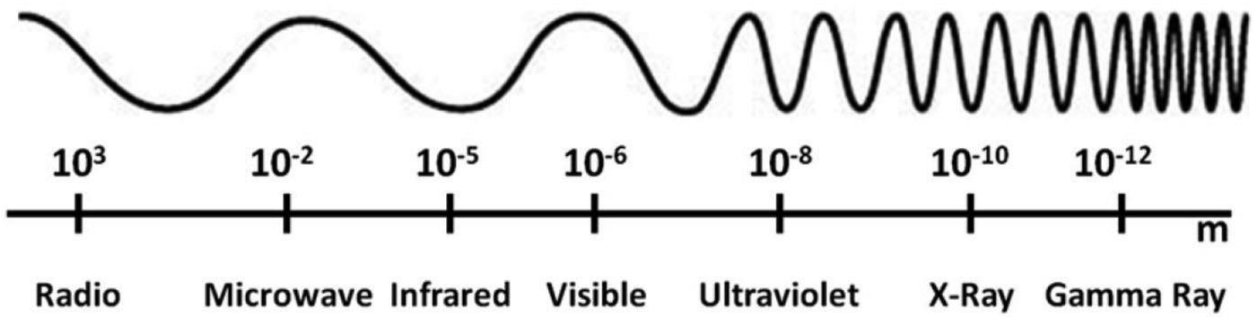


Figure 7 The electromagnetic spectrum (Calado et al., 2014, adapted from Satin, 1996)

The dosage and type of irradiation sterilization are decided according to the type and amount of the microbial load that has to be removed from a given material and equipment. As irradiation does not leave any residue on the treated surface nor affect the immediate environment, it appears at first sight to be a perfect solution for sterilization of packaging material. However, irradiation may indirectly affect chemical or biological properties, leading to interaction with the packaged product, changing its efficacy or usability (Ansari and Datta, 2003).

1.6 Aim

The VDMA recommendation of required outcomes of the validation test depending on the sterilization processes and test organisms (FS NuV No. 10/2005, Appendix A) has motivated us to obtain further confidence data on the performance of the test organisms.

This degree project is aimed to investigate and compare the resistance of *A. brasiliensis, B. atrophaeus, and B. subtilis* towards sterilization processes relevant to the food industry as indicated by VDMA, including hydrogen peroxide, Oxonia, hot water and dry heat

sterilization/disinfection processes. Furthermore, a review of the existing literature will be included and discussed together with the obtained laboratory data to suggest suitable test organisms depending on the sterilization processes applied.

2. Material and methods

This chapter describes the determination of the resistance of test organisms (*A. brasiliensis, B. atrophaeus*, and *B. subtilis*) towards different sterilization/disinfection processes (hydrogen peroxide, Oxonia, hot water, and dry heat). The experiments were conducted in Tetra Pak facility, Lund, Sweden.

2.1 Test organisms

Three spore-producing organisms, *Aspergillus brasiliensis* (*niger)* (ATCC 2275), *Bacillus atrophaeus* (ATCC 9372), and *Bacillus subtilis* (SA 22) were used in this study.

A. brasiliensis spores were obtained from Biomerieux (Sydney, Australia) as freeze-dried cultures, with each spore ball containing 10^8 spores/mL when re-hydrated in de-ionized water. *B. atrophaeus* and *B. subtilis* spores were previously produced in Tetra Pak, Sweden, as suspensions in 40% ethanol (Solveco AB, Rosenberg, Sweden) and de-ionized water respectively. The spore stock of *B. atrophaeus* contained 10^{10} spores/mL, while *B. subtilis* contained $10^{8,5}$ spores/mL. The spores were kept in the Ultra freezer (-80 °C) for *Bacillus* spores or freezer (-25 ˚C) for *A. brasiliensis* spores until use.

2.2 Spore solutions and inoculation of carriers

Spore solutions diluted to give a concentration of $10^{5.6}$ - $10^{6.0}$ spores/mL were prepared for the three test organisms used in the present work. For *A. brasiliensis*, the spore ball was re-hydrated according to the manufacturer's instructions in 900 µL of de-ionized water to reach a concentration of 10^8 spores/mL. An aliquot of 500 µL of *B. atrophaeus* stock spores was mixed with 4,5 mL of 40% ethanol. A similar method was applied to dilute the *B. subtilis* spores but instead, de-ionized water was used. This was done because previously the spores were suspended in 40 % ethanol for *B. atrophaeus* and de-ionized water for *B. subtilis.* Dilution was performed until the desired concentrations of spores were achieved.

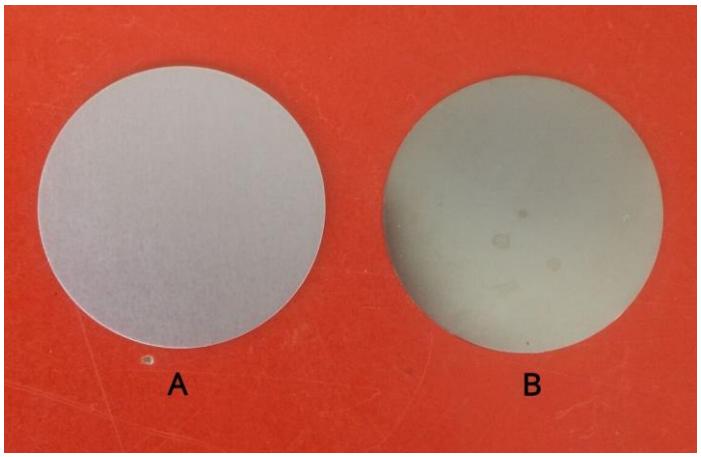


Figure 8 Test carriers used in the study. A) Packaging carrier, B) stainless-steel carrier (Photograph by Y. Nabila).

To evaluate the sterilization efficiency in this study, the spores were either sprayed or drop inoculated on the carriers but also used in the form of a spore solution. Especially for the hot water disinfection experiment, only the spore solution was used, while the rest of the experiments were performed using the carriers. There were two types of carriers used throughout the study.

The first carriers were round pieces (45 mm diameter and approximately 0,7 mm thick) of Tetra Pak packaging material (made of different layers of polyethylene, aluminum, and paperboard), while the second was stainless-steel carriers with the same shape and size (see Figure 8).

For experiments where chemical agents were used (hydrogen peroxide and Oxonia), the spores were inoculated using a device designed to spray carriers with defined spore loads of test organisms ($10^{5.6}$ - $10^{6.0}$ spores/carrier). The spores were sprayed onto the food-contact surface side of the carriers. After spraying, the carriers were left in a covered tray to dry for about 12 hours (20 - 23 ˚C) before used in the experiments.

For dry heat experiments, the stainless-steel carriers were used. The stainless-steel carriers were inoculated in a laminar flow hood using a micropipette and applying 10 droplets of 10 µL, from a total spore volume of 100 µL, evenly on the surface of the round pieces (final concentration of $10^{5.6}$ - $10^{6.0}$ spores/carrier). Also here, the inoculated carriers were left in a covered tray to dry for 12 hours (20 - 23 ˚C).

2.3 The EWA test rig

The EWA test rig is a specially designed equipment with a bath and regulators for temperature and concentration, where inoculated carriers can be submerged in a chemical solution for specified time intervals (see Figure 9).

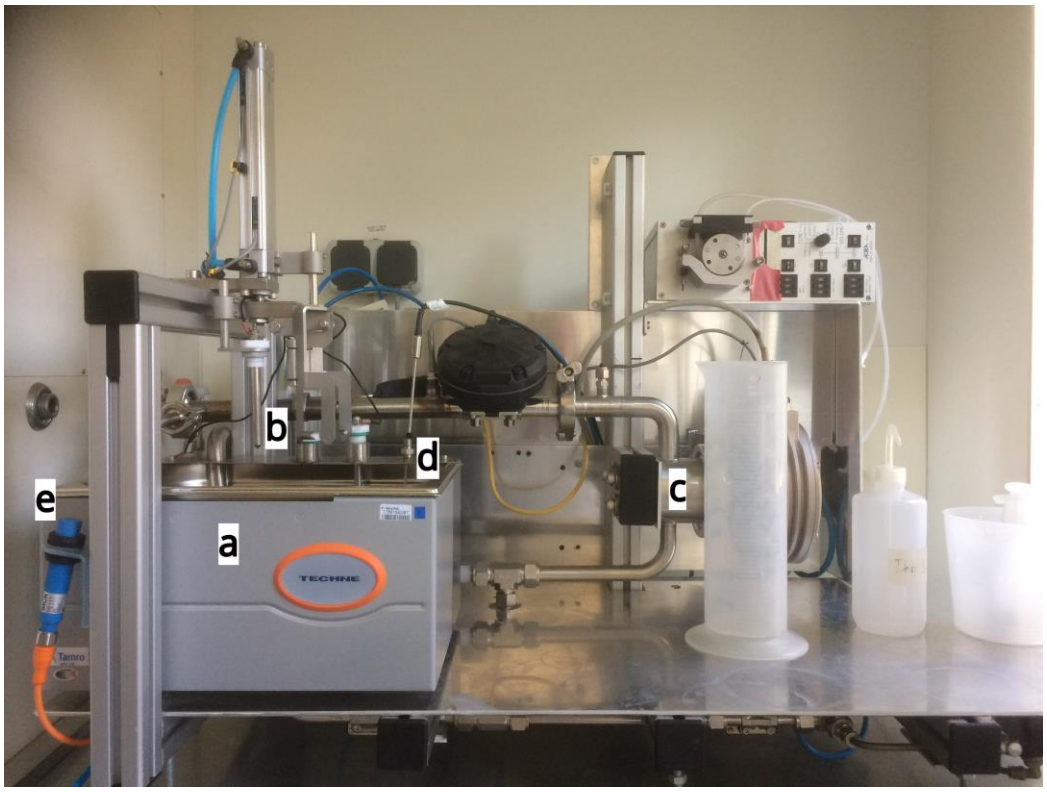


Figure 9 The EWA test rig composed of (a) chemical bath, (b) sample holder, (c) pump, (d) heater and stirrer, (e) sample exposing button, and additionally a computer (Photograph by Y. Nabila).

The chemical agent is automatically pumped into the bath and the desired concentration is reached by diluting the agent with de-ionized water. Temperature can be adjusted through the computer unit connected to the rig. The concentration of the solution in the bath is monitored by a refractometer and was confirmed by titration before each experiment. All the parameters can be monitored through the screen display of the computer unit.

2.3.1 Titration

The hydrogen peroxide concentration was determined by titration with 0,1 N potassium permanganate ($KMnO_4$; 0,02M). Firstly, a defined concentration of a solution was prepared from the concentrated peroxide (Ecolab Inc., St. Paul, Minnesota). After that, 1000 µL of diluted H_2O_2 was transferred to an Erlenmeyer flask and 20 mL of sulphuric acid (H_2SO_4; 10%) (Merck KGaA, Darmstadt, Germany) were added. The Erlenmeyer flask was then placed on the titration unit and drops of $KMnO_4$ was added carefully until a faint pink color persists for approximately 2-3 minutes (see Figure 10).

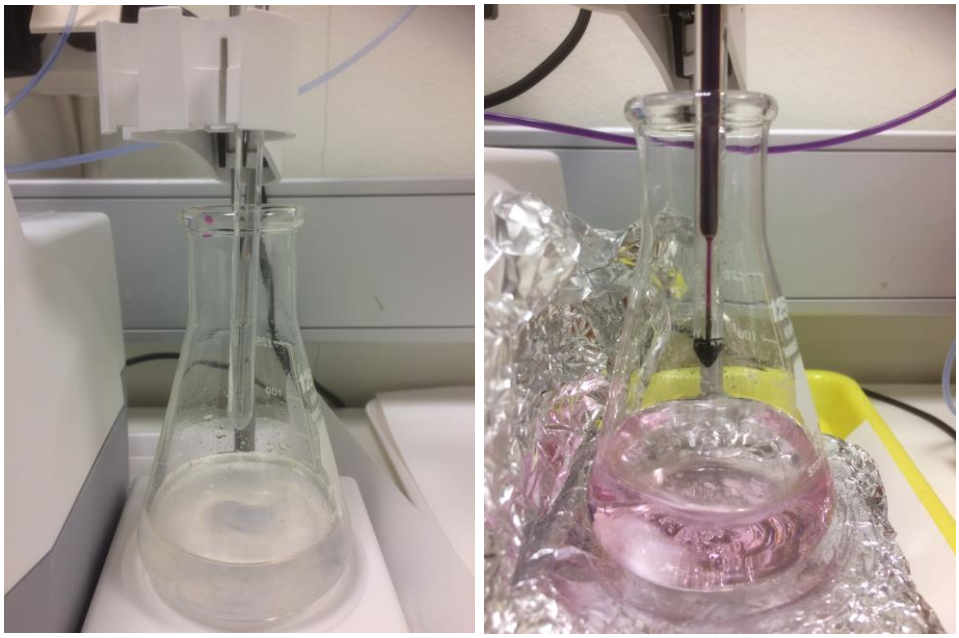


Figure 10 Titration of hydrogen peroxide (Photograph by Y. Nabila).

For Oxonia (Ecolab Inc., St. Paul, Minnesota), the peracetic acid concentration was determined by back titration. After the diluted solution was prepared from concentrated Oxonia, firstly the hydrogen peroxide was titrated. After that, 1 g of potassium iodide (KI; 166 g/mol) (VWR BDH Chemicals) was added. Depending on the amount of peracetic acid (PAA), the solution would turn yellow to brown. After that, 2 mL of starch solution was added with a subsequent change in colour to dark brown. The Erlenmeyer flask was then placed on the titration unit and drops of 0,1 N sodium thiosulfate (Na2SO3; 0,1 mol/L) (Merck KGaA, Darmstadt, Germany) were added until solution lessens in color and finally turns colorless (transparent) (see Figure 11).

ppm H_2O_2 = mL of $KMnO_4$ x 100 x 17	(*Equation 1*. Determination of H_2O_2 by titration)
ppm PAA = ml of $Na_2S_2O_3$ x 100 x 38	(*Equation 2*. Determination of PAA by titration)

The concentration of H_2O_2 and PAA was calculated using the procedures supplied by Ecolab Inc. (see Equation 1 and 2), where 1 ppm equals to 0,0001%. Complete titration procedure provided by Ecolab Inc. can be seen in Appendix 2.

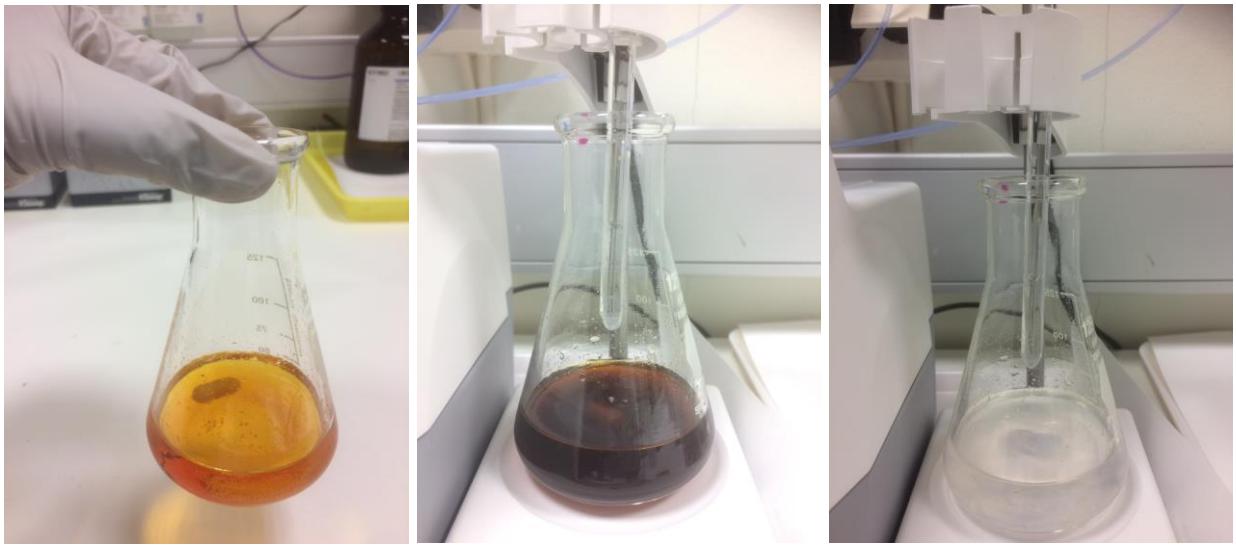


Figure 11 Titration of peracetic acid (Photograph by Y. Nabila).

2.3.2 Exposure of inoculated carriers and recovery of survivors

A minimum of five exposures towards sterilization process shall be used and shall include the following conditions:

a) One exposure in which the sample is not subjected to the sterilant (e.g. 0 time exposure or reference)
b) At least one exposure in which the viable population is reduced to 0,01 % of the spores (4 LCR), and
c) A minimum of three exposures covering the intervals between exposure a) and exposure b) above

In addition, not less than four test samples shall be used for each exposure in each determination. The same number of replicates shall be used for each exposure.

The inoculated carriers were exposed to the chemical agent through automatic dipping into the EWA rig bath for a certain exposure time (see Table 3 and Table 4). Reference carriers were not exposed to any chemical agent but otherwise treated similarly as the exposed carriers.

Recovery solution was prepared to neutralize the effect of the chemical agents right after the exposure. The desired amount of catalase (Sigma-Aldrich) (0,043 g/400 mL phosphate buffer) was weighed up, mixed initially with 10 mL of phosphate buffer (0,05M, pH = 7,0) (for the list of chemicals used, see Appendix 1), and vortexed for approximately five minutes. After that, the catalase was transferred and filtered by microfiltration into the remaining amount of phosphate buffer before transferring 10 mL to plastic beakers. For the Oxonia tests, 4 mL of sodium thiosulfate was added before adding the catalase.

After exposure, the test carrier was transferred immediately to the beaker containing 10 mL of recovery solution with the inoculated side (food contact surface) facing upwards. A magnetic stirring bar was added into the beaker and the beaker was then placed on a magnetic stirring plate (rotating at a speed of 700 rpm for at least five minutes) to release any remaining spores from the test carriers to the solution.

The sample solution was diluted to different concentrations in saline (Labrobot Products AB, Stenungsund, Sweden) and mixed carefully with the mixing plate, Dilushaker (Labrobot Products AB, Stenungsund, Sweden). 1 mL was transferred into a petri dish (double plates per dilution) and pour plated with Plate Count Agar (PCA) (50 °C). The Petri dishes were incubated at 30 - 32 °C for 48 h.

2.3.2.1 Exposure to hydrogen peroxide solution

The methods for chemical experiments used in this study were performed according to the method applied in Tetra Pak Microbiology Laboratories, Lund, Sweden. Packaging carriers were used for these experiments and the inoculation of the carriers was done as described previously in Section 2.2.

Food-grade hydrogen peroxide standardized to 35 % was supplied by Ecolab Inc. For these experiments, the packaging carriers were used. *B. subtilis* and *B. atrophaeus* spores were challenged against 30 ± 0.5 % H_2O_2 while *A. brasiliensis* spores were against 3 ± 0.5 % H_2O_2. Each test organism was tested three times and duplicates were made for every exposure time point, resulting in a total of six replicates per test organism (see Table 3). A temperature of 60 °C was used for all spores based on the practical usage when sterilizing filling machines in Tetra Pak.

Table 3 Experimental design for hydrogen peroxide treatment.

Spores	Exposure time	Peroxide concentration	Bath temperature
A. brasiliensis	1, 3, 5, 7, and 9 seconds	3 ± 0.5 %	60 ± 0.2 ˚C
B. atrophaeus	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 seconds	30 ± 0.5 %	60 ± 0.2 ˚C
B. subtilis	1, 3, 5, 8, 10, 12, and 15 seconds	30 ± 0.5 %	60 ± 0.2 ˚C

2.3.2.2 Exposure to Oxonia solution

P3-Oxonia Active was obtained from Ecolab Inc. the solution is based on 20-60 wt% of H_2O_2 and 2-10 wt% of $C_2H_4O_3$ (ethaneperoxoic acid) or also known as peracetic acid. The pH of the solution was 3 – 3,4. The inoculated packaging carriers were exposed to 1 ± 0,5 % of Oxonia at ambient temperature (20 – 25 °C) for different time points (see Table 4). One replicate was performed for each test organism.

Table 4 Experimental design for hydrogen peroxide treatment.

Spores	Exposure time
A. brasiliensis	1, 3, 5, 8, 10, 12, 15, and 18 seconds
B. atrophaeus	15, 30, 45, 60, 75, 80, and 90 seconds
B. subtilis	15, 30, 45, 60, 75, and 120 seconds

2.4 Hot water disinfection

The following section will describe the method of hot water disinfection, including the preparation of the equipment, the exposure of inoculated carriers, and recovery of survivors.

2.4.1 Preparation of the equipment

This test was performed based mainly on the previous experiment by Veen et al., (2015). Changes were made to the test method by replacing the usage of capillary tubes into a Mini Block Heater (VWR, U.S.A.) (see Figure 12). The hot water experiments were carried out three times for all spores.

Prior to the experiment, the Mini Block Heater was heated up to 90 °C while simultaneously a thermometer was used to check if the correct temperature had been achieved. The thermometer sensor was infused in an Eppendorf tube with water and the Eppendorf tube was placed in one of the block's wells also containing water to mimic the conditions to which the spores would be exposed to. At the same time, 150 µL Eppendorf tubes were pre-heated to a temperature of 60-70 °C in an incubator. These Eppendorf tubes would then be used for the spore solution to be exposed to the heat treatment. Ice water was prepared to cool down the Eppendorf tubes containing treated spores immediately after the exposure.

2.4.2 Exposure of inoculated carriers and recovery of survivors

1 mL spore solution was diluted to a concentration around $10^{5.6}$ to $10^{6.0}$ spores/mL and then exposed to 90 °C for different time intervals, using the Mini Block Heater. Spores of *B. atrophaeus* and *B. subtilis* were exposed for 5, 10, 15, 20 and 30 minutes, while spores of *A. brasiliensis* were exposed for 3, 5, 8, 10, 12 and 15 seconds. Three replicates were done for each test organisms.

Figure 12 Mini Block Heater used in the hot water experiment (VWR International, 2018).

The wells inside the block heater were filled with water to achieve a better heat transfer. Each experiment included at least five-time points and three references. The test Eppendorf tube was placed in the wells, and for each time point, 100 µL of the spore solution was transferred into another tube and immediately cooled on ice to stop the heat effect on the spores.

The 100 µL sample solution was diluted to different concentrations in 900 µL saline and mixed carefully using a vortex for 15 seconds. 100 µL of spores were then transferred into a petri dish (double plates per dilution) and pour plated with Plate Count Agar (PCA) (50 °C). The Petri dishes were incubated at 30 - 32 °C for 48 h and the growing colonies were counted.

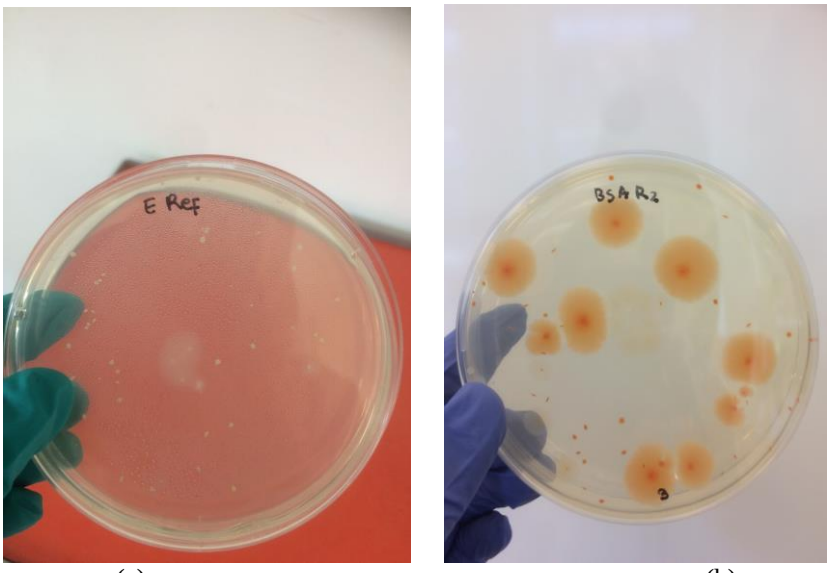
2.5 Dry heat sterilization

To investigate the effect of dry-heat, tests were performed according to the previous work by Kempf et al. (2008) with some modifications in which in the present experiment stainless-steel carriers were used instead of thermal spore exposure vessels (TSEVs). Moreover, the oven used controlled only the temperature but not the humidity.

Inoculation of the carriers was performed as described in Section 2.2. Approximately 24 h after inoculation, the stainless-steel carriers were placed in a temperature-controlled oven at 125 °C. Samples were taken every 5, 10, 15, 20, 40, 60, 75, and 90 minutes and moved to a refrigerated phosphate buffer (0,05M, pH = 7,0) solution to stop the heat reaction. After that, spore recovery was performed as described before. Assays were repeated three times for all spores (three replicates).

2.6 Enumeration of survivors and statistical analysis

The resistance determination of the test organisms were perform based on the procedure from SS-EN ISO 1138-3 (2017). This method determines the number of surviving test organisms by direct counting of colony forming units (CFUs), expressed in logarithmic unit or LCR. This method is also referred to as the “direct enumeration method.” (see Figure 13).



(a) (b)

(c)

Figure 13 Examples of plates containing CFU of *B. subtilis* (a), *B. atrophaeus* (b), *A. brasiliensis* (c) that were subjected to enumeration are provided (Photograph by Y. Nabila).

Agar plates showing between 30-300 colonies were included in the calculation of LCR:

$$LCR = (N_0 - N) + dilution \qquad \textit{(Equation 3)}$$

in which N_0 is the initial (reference) spores while N is the recovered spores. After that, the LCR values from all the three tests were plotted against the time points of exposure to a specific sterilization process in an inactivation curve. As stated in 2.3.2, a minimum value of 4 LCR is required to be able to have a linear inactivation trend. A linear trendline was added to the graph, showing the regression slope equation and R^2 value. The R^2 value is used to show whether the regression-line fits the data. A value of R^2 close to 1 indicates a strong correlation between x and y and while a value close to zero indicates no correlation between x and y values.

To calculate the D-value, the reciprocal (i.e. inverse) of the regression slope was used. The D-value is used to indicate the killing rate or. the time required at a certain temperature and condition to kill 90% of the spores. However, for methods that are not yet establised (i.e. Oxonia, hot water, and dry heat), only indicative D-value are calculated. This means that the D-values gained for these respective processes are yet to be considered as the established killing rate of certain test organisms, but only to demonstrate the overall resistance.

3. Results

This result chapter is divided based on the resistance test of *A. brasiliensis, B. atrophaeus,* and *B. subtilis* spores towards different sterilization/disinfection processes in the following order; hydrogen peroxide, Oxonia, hot water, and dry heat. Specifically for the hydrogen peroxide and Oxonia sterilization, the screening tests for selection of exposure parameters are presented beforehand.

3.1 Resistance of the test organisms against hydrogen peroxide

To efficiently design and optimize the experiments for the resistance evaluation of the selected test organisms against H_2O_2, a series of screening tests were performed to identify the processing parameters, i.e. exposure time, temperature, and H_2O_2 concentration. Screening tests were performed for *A. brasiliensis* and *B. atrophaeus* spores. For *B. subtilis* spores, test parameters were selected based on existing knowledge from previous research within Tetra Pak.

3.1.1 Resistance of *A. brasiliensis* spores against hydrogen peroxide

The impact of small amounts of H_2O_2 and exposure time on the LCR of *A. brasiliensis* spores have previously been investigated within Tetra Pak. H_2O_2 concentrations ranging from 0,01 % to 0,8% and ambient temperature (20 – 25 °C) were included and the exposure times varied from 15 min to 6 hr (see Table 5). No coherent conclusion could be drawn on the best combination of time/H_2O_2 concentration based on this data. Additional screening experiments were therefore performed in this project and presented below.

Table 5 Impact of small amounts of H_2O_2 concentration on *A. brasiliensis* spores at ambient temperature. This data originates from previous investigations performed at Tetra Pak.

Contact time	LCR of *A. brasiliensis* spores				
	0,01 % H_2O_2	**0,05 % H_2O_2**	**0,09 % H_2O_2**	**0,4 % H_2O_2**	**0,8 % H_2O_2**
0 min	0 LCR*	0,1 LCR	0,1 LCR	0,2 LCR	0,3 LCR
15 min	0 LCR*	0 LCR	0,1 LCR	0,3 LCR	N = 0**
1 h	0,3 LCR	0,2 LCR	0,3 LCR	N = 0**	N = 0**
4 h	0,4 LCR	1,8 LCR	2,4 LCR	N = 0**	N = 0**
6 h	0,7 LCR	2,3 LCR	2,9 LCR	N = 0**	N = 0**

* 0 LCR, meaning there was no log cycle reduction

** N (recovered spores) = 0, meaning there was no growth detected on plates

3.1.1.1 Screening tests for selection of exposure parameters

In this project, three screening tests were carried out for the resistance of *A. brasiliensis* spores against H_2O_2.

The first screening was developed based on the use of H_2O_2 during industrial application for filling machine and packaging material disinfection. Considering the later, the lowest H_2O_2 concentration evaluated was 3% with exposure times shown in Table 6 and ambient temperature. Additionally, 5 % and 10 % H_2O_2 were also used to evaluate the effect of an increased concentration on the inactivation of the spores. The test was performed by manually dipping the test carriers into a beaker containing the desired H_2O_2 concentration.

Table 6 First screening test of *A. brasiliensis* spores against different concentrations of H_2O_2 at ambient temperature.

Contact time (s)	LCR of *A. brasiliensis* spores		
	3 % H_2O_2	5% H_2O_2	10% H_2O_2
5	0,66	0,76	1,42
10	0,33	0,96	1,78
15	0,42	1,15	2,56
30	1,24	1,42	2,56
45	0,86	1,39	2,09

As shown in Table 6, the effectiveness of sterilization appeared H_2O_2 concentration-dependent. An increase in the obtained LCR was observed with higher H_2O_2 concentration and mostly with increasing contact times. However, a drop of LCR was attained after a contact time of 45 s for all three concentrations. The reason for this was unclear since even longer contact time points were not included in the experiment. This test series provided an indication about the influence of H_2O_2 concentration on the LCR of *A. brasiliensis* spores. The second screening test was designed to further understand the effect of a higher exposure temperature of 40 °C. The screening was divided into three tests (A, B, C), each consisting of two replicas, producing in total six replicas. The concentration of H_2O_2 was standardized at 3%, taking into account the previous results and the aforementioned practical application. Three different sets of time points were explored in duplicates to be able to pinpoint the most efficient combination (see Table 7). These screening tests were performed in the EWA Rig for more controlled processing parameters. Time points and final LCR are shown in Table 7.

Table 7 Second screening test of *A. brasiliensis* spores against H_2O_2 (3 ± 0,5 %) at 40 ± 0,2 ˚C.

Test A		Test B		Test C	
t	**LCR**	**t**	**LCR**	**t**	**LCR**
3 s	0,21 ± 0,06	1 min	0,06 ± 0,22	5 min	N = 0[b]
6 s	0,18 ± 0,02	1 min 30 s	0,96 ± 0,20	8 min	N = 0[b]
9 s	0,28 ± 0,23	2 min	1,65 ± 0,40	10 min	N = 0[b]
12 s	0,28 ± 0,10	2 min 30 s	2,17 ± 0,01	12 min	N = 0[b]
15 s	TFTC[a]	3 min	TFTC[a]	15 min	N = 0[b]
18 s	TFTC[a]	3 min 30 s	TFTC[a]	18 min	N = 0[b]
21 s	0,15 ± 0,05	4 min	TFTC[a]	20 min	N = 0[b]
25 s	0,26 ± 0,01	4 min 30 s	N = 0[b]		
30 s	0,21	5 min	N = 0[b]		
45 s	TFTC[a]				

[a] Too few to count (CFU ≤ 30)
[**]N (recovered spores) = 0, meaning there was no growth detected on plates

Unfortunately, the results obtained did not provide a coherent inactivation curve to understand the resistance level of *A. brasiliensis* spores. The first set of time points selected (test A) gave very low LCR values, implying a low killing effect of the selected parameters. On the other hand, the two next sets of exposure times appeared too long, and the recovery of survivors, i.e. spores surviving the treatment and forming a countable colony on plates, was low. Contact times in minutes are however considered unpractical for industrial applications, and therefore it was decided that the third screening test would be performed with the same H_2O_2 concentrations (3 %) but at a higher exposure temperature of 60 °C. With a higher temperature, it was expected that higher values of LCR would be obtained within a shorter exposure time.

Based on the results obtained so far, the third screening test was designed with an exposure temperature of 60 °C and time points from 5 to 180 s (Table 8). The screening was divided into three identical tests, each consisting of two replicas, producing in total six replicas. From the results, it was observed that the increase of temperature influenced the sensitivity of *A. brasiliensis* spores against H_2O_2 sterilization. The test results from all replicas followed the same behavior. After 5 s LCR reductions of 1,56; 0,93; and 1,01 for A, B and C respectively were achieved, while no survivors were observed for longer contact times. The lack of survivor is mainly attributed to dilution restrictions during the spore recovery.

In summary, from the screening tests performed, it was demonstrated that H_2O_2 concentration, contact time, and temperature have a strong impact on the resistance of *A. brasiliensis* spores against H_2O_2. From the different combinations explored, that of 3 % H_2O_2 concentration, 60 ˚C exposure temperature and exposure times between 1 to 18 s, was the one selected to continue within the following resistance tests.

Table 8 Third screening test of *A. brasiliensis* spores against H_2O_2 (3 ± 0,5 %) at 60 ± 0,2 ˚C.

	Test A	**Test B**	**Test C**
t	**LCR**		
5 s	1,56	0,93	1,01
20 s	N = 0*	N = 0*	N = 0*
40 s	N = 0*	N = 0*	N = 0*
60 s	N = 0*	N = 0*	N = 0*
80 s	N = 0*	N = 0*	N = 0*
100 s	N = 0*	N = 0*	N = 0*
120 s	N = 0*	N = 0*	N = 0*
130 s	N = 0*	N = 0*	N = 0*
150 s	N = 0*	N = 0*	N = 0*
180 s	N = 0*	N = 0*	N = 0*

* N (recovered spores) = 0, meaning there was no growth detected on plates

3.1.1.2 Resistance tests of *A. brasiliensis* spores against hydrogen peroxide

The inactivation curve obtained for *A. brasiliensis* spores after exposure to 3% H_2O_2 and 60 ˚C for 1 to 18 s is shown in Figure 14. The tests were made of 6 replicas. From the graph, a rapid LCR can be observed with increased exposure time. Already after 3 s, a reduction of more than 1 LCR was obtained. This indicates that the spores were sensitive to the selected parameters, as the recovered survivors followed a linear inactivation curve. A maximum LCR of 3,27 was obtained after exposure for 9 s. For time points after 9 s, there were no recovered spores due to the limitation of the initial maximum concentration of spore population that could be inoculated on the test carriers. The $D_{60\ °C}$-value obtained from this process was 3,15 s, showing that the spores were sensitive to this process. The maximum LCR value obtained was however still below the industry standard of 4 LCR for sterilization processes.

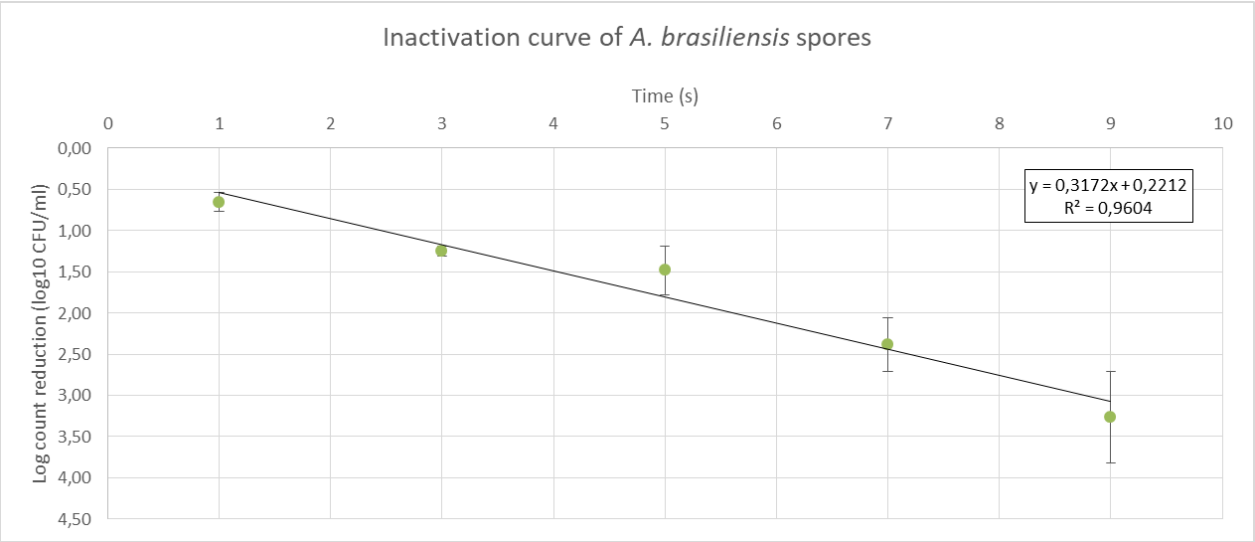


Figure 14 Inactivation curve of *A. brasiliensis* spores against 3 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed.

3.1.2 Resistance of *B. atrophaeus* spores against hydrogen peroxide

Previous research conducted within Tetra Pak was used as a guideline in selecting the exposure time for the resistance test of *B. atrophaeus* spores against H_2O_2. The exposure temperature and H_2O_2 concentration were standardized at the same levels as when performing microbial challenge tests on filling machines, i. e. 60 °C and 30 % respectively.

3.1.2.1 Screening tests for selection of exposure parameters

One set of screening tests (A, B and C) were performed for *B. atrophaeus*. Each test consisted of two replicas, resulting in six replicas in total. In test A, exposure times up to 27 s were evaluated, based on the guidelines used for the resistance test in Tetra Pak, SS-EN ISO 1138-3 (2017) (see Table 9). However, after 12 s, no surviving spores could be detected and thus, for B and C, exposure times were adjusted to a maximum of 13 s (Table 9). As observed from the final test results, a reduction in the number of survivors was highest after an exposure time of 12 s to 13 s. Based on these outcomes; the resistance test for *B. atrophaeus* was designed using exposure times ranging from 1 to 13 s under the temperature of 60 °C and 30 % H_2O_2 concentration.

Table 9 Screening test of *B. atrophaeus* spores against H_2O_2 (30 ± 0,5 %) at 60 ± 0,2 ˚C.

Test A		Test B		Test C	
t (s)	**LCR**	**t (s)**	**LCR**	**t (s)**	**LCR**
1	0,05	1	0,14 ± 0,04	1	0,10 ± 0,08
3	0,24	3	-0,03 ± 0,12	3	0,21
6	0,54	5	0,40 ± 0,04	5	0,42 ± 0,08
9	1,56	7	0,85 ± 0,23	7	0,79
12	3,59	9	1,36 ± 0,01	8	1,05 ± 0,29
15	N = 0*	11	2,20 ± 0,18	9	1,31 ± 0,12
18	N = 0*	12	3,06 ± 0,15	10	1,78 ± 0,01
21	N = 0*	13	3,92 ± 0,06	11	2,06 ± 0,23
24	N = 0*			12	3,40 ± 0,02
27	N = 0*			13	N = 0*

* N (recovered spores) = 0, meaning there was no growth detected on plates

3.1.2.2 Resistance tests of *B. atrophaeus* spores against hydrogen peroxide

The inactivation curve of *B. atrophaeus* spores when exposed to 30 % H_2O_2 and 60 °C for 1 – 13 s is shown in Figure 15 (made in six replicas). For the first 4 s, no distinct difference on the LCR could be observed. At longer exposure times, particularly after 8 s, a faster reduction of the spore population was detected. Finally, a maximum LCR value of 2,48 was achieved after 13 s. This value was however below the corresponding practical standard for sterilization of 4 LCR, and would need further investigation to understand the time needed to achieve this level of reduction. However, due to time constraints of the study, no further tests could be performed. The $D_{60°C}$ for this sterilization process was established at 5,56 s.

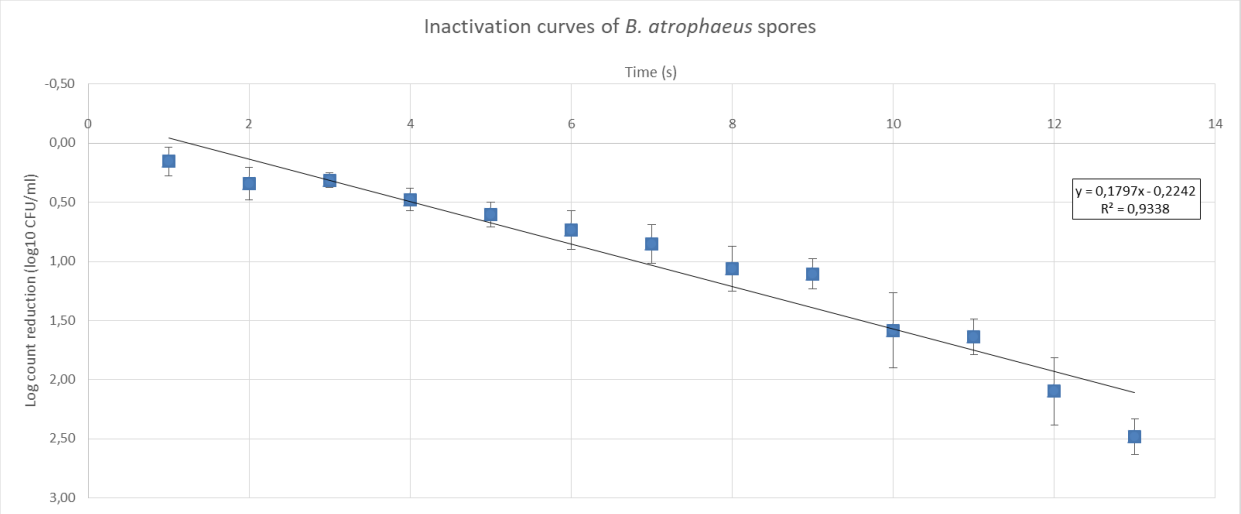


Figure 15 Inactivation curve of *B. atrophaeus* spores against 30 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed.

3.1.3 Resistance of *B. subtilis* spores against hydrogen peroxide

The parameters of H_2O_2 concentration and exposure time for *B. subtilis* spores were selected based on existing experimental data within Tetra Pak. As such, time points between 1 to 18 s were evaluated as shown in Figure 16. For these experiments, exposure temperature of 60 °C and concentration of 30 % H_2O_2 were used.

The obtained inactivation curve shows a distinct reduction in the spore population until 8 s. After this point, the inactivation continues in a linear pattern, however, the difference in the level of LCR obtained with time is smaller compared to that before 8 s. At sterilization time of 18 s, LCR of 3,83 was achieved, to which it almost passed the recommended standard value of spore reductions in practical application. $D_{60°C}$ value of this process was 6,17 s.

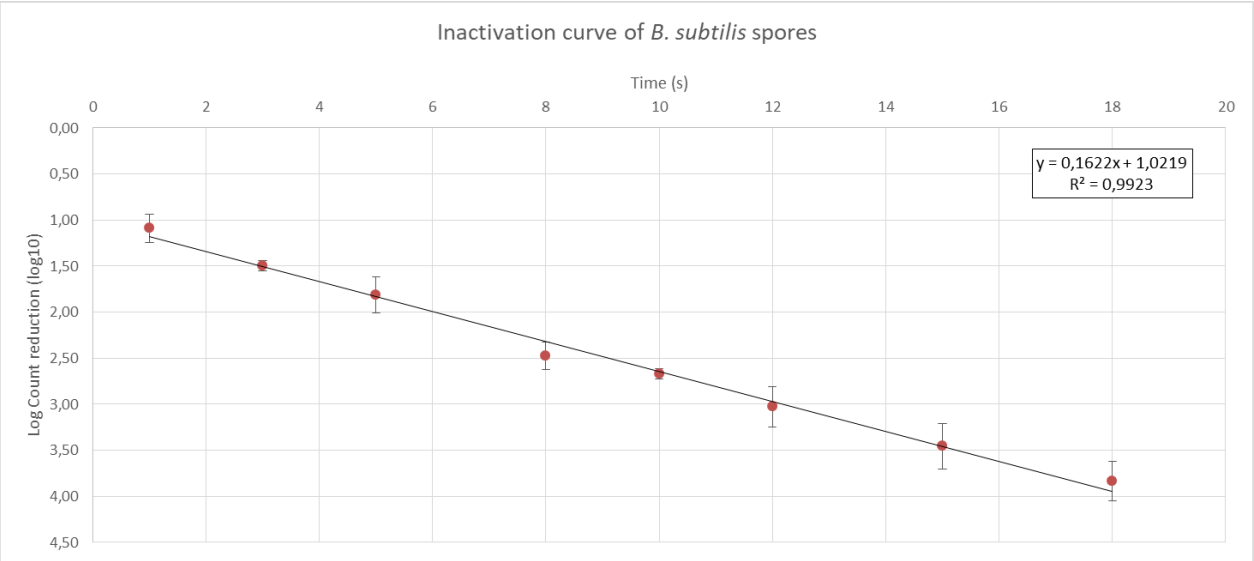


Figure 16 Inactivation curve of *B. subtilis* spores against 30 ± 0,5% H_2O_2 at 60 ± 0,2 ˚C. The average and standard deviation of six replicas are shown. Linear regression equation and R^2 value are displayed.

3.2 Resistance of the test organisms against Oxonia

Similar to H_2O_2, a series of screening tests were performed using the EWA test rig to develop the experimental design for the resistance tests of the selected test organisms against Oxonia. Screening tests were performed for all three test organisms, *A. brasiliensis*, *B. atrophaeus* and *B. subtilis*. The parameters to evaluate were selected based on industrial application practices and scientific references. As such, Oxonia concentration between 1 – 3 % and ambient temperature were applied. A study by Blakistone et al. (1999) was used to select the exposure time points for spores of *B. subtilis* and *B. atrophaeus*. In that study, it was found that the time to reach end-point (no survivors) against 2 % Oxonia at 40 °C for *B. subtilis* was within 150 and 240 s, while for *B. atrophaeus* it was between 90 and 120 s.

3.2.1 Screening tests for selection of exposure parameters

In the first screening test, 1.5 % of Oxonia was used to expose the test organism carriers at ambient temperature taking into consideration that this concentration was still within the practical range but not too low or too high. The exposure times were 30 s, 1, 3, 6, 10, 15 and 20 min. Nevertheless, almost no survivors were recovered from this experiment. Hence, a second screening test was developed to obtain a better combination of exposure parameters (see Table 10).

Table 10 Second screening test of different test organisms against Oxonia (0,9 ± 0,5 %) at ambient temperature.

t (s)	*A. brasiliensis*	*B. atrophaeus*	*B. subtilis*
15	1,93 LCR	-0,10 LCR	0,57 LCR
30	N = 0*	0,45 LCR	0,75 LCR
45	N = 0*	1,16 LCR	1,82 LCR
60	N = 0*	2,36 LCR	3,75 LCR
90	N = 0*	N = 0*	3,67 LCR
120	N = 0*	N = 0*	4,77 LCR
150	N = 0*	N = 0*	N = 0*
180	N = 0*	N = 0*	N = 0*

* N (recovered spores) = 0, meaning there was no growth detected on plates

Table 10 shows the results obtained after exposure of the spores at 0,9 % Oxonia at ambient temperature. It can be clearly seen that *A. brasiliensis* spores were more sensitive to the given concentration of Oxonia compared to the other two spores since after achieving 1,93 LCR at 15 s, no more survivors were recovered. The critical range between 15 and 30 s that was not evaluated in the screening was taken into account in developing the parameters for the final resistance test. For *B. atrophaeus*, an increased reduction could be observed with increasing exposure time up to 60 s (LCR of 2,36). After that, no survivors could be observed. Inactivation of *B. subtilis* spores was more straightforward compared to the other spores, with a constant reduction of the population up to 120 s of exposure where 4.77 LCR could be achieved. To conclude, from the screening tests it was obvious that the reduction of Oxonia concentration from 1,5 to 0,9 % had a substantial impact on the resistance level of the spores. With the lower concertation of 0,9 %, an inactivation curve could be obtained at least for the *Bacillus* spores. To continue with the resistance tests, 0,9 % Oxonia concentration at ambient temperature was selected and exposure time points were chosen according to the attained end-point, i.e. the time to reach the condition where no growth detected on plates.

3.2.2 Resistance tests of *A. brasiliensis* spores against Oxonia

Spores of *A. brasiliensis* were exposed to 0,9 % Oxonia for 1, 3, 5, 8, 10, 12, 15, and 18 s at ambient temperature (see Figure 17)

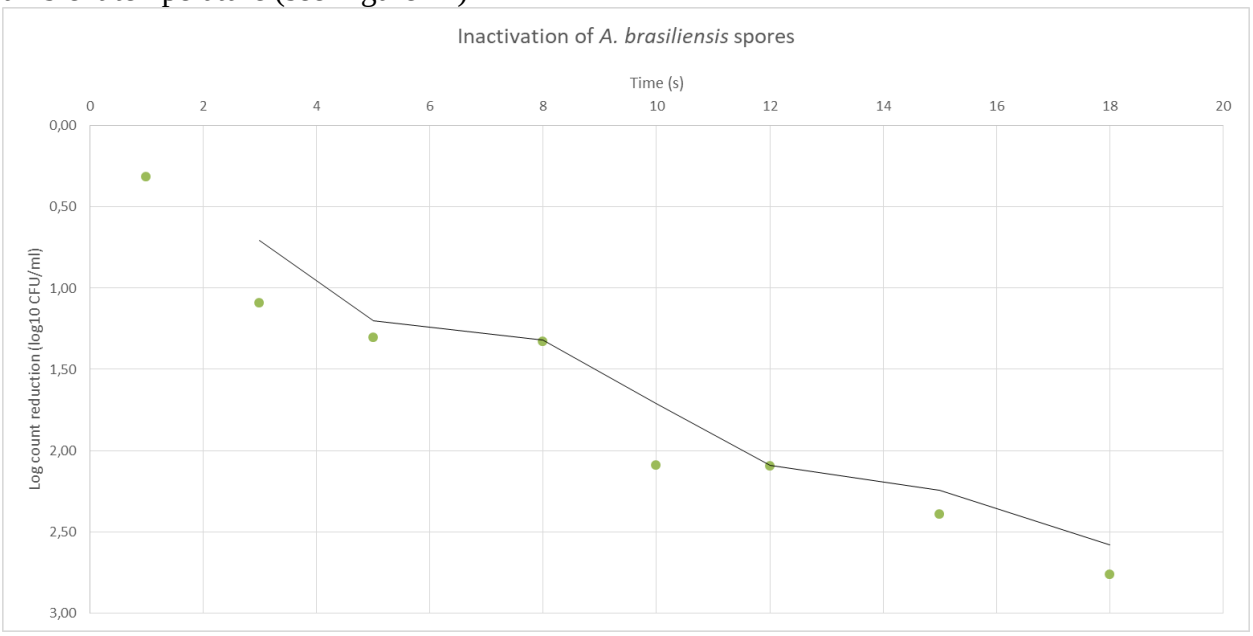


Figure 17 Inactivation curve of *A. brasiliensis* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed.

As observed in Figure 17, the inactivation effect of Oxonia on *A. brasiliensis* spores was rapid. After 3 s, LCR of 1,09 s was achieved and the spore population continued to drop throughout the process. Finally, LCR value of 2,76 was achieved at 18 s. Further investigation at longer time points could be done to see at what time 4 LCR would be accomplished. $D_{ambient}$-values was established at 7,63 s.

3.2.2.2 Resistance tests of *B. atrophaeus* spores against Oxonia

Figure 18 shows the resistance of *B. atrophaeus* spores towards 0,9 % Oxonia after exposure for 15, 45, 60, 75, 80 and 90 s at ambient temperature. Initially, negative LCR value was observed after 15 s exposure time. Since the results presented here have derived from one experiment, more replicas would be needed to understand whether this is a true negative value or not. After exposure for 60 s, the inactivation speed of the spores increased, reaching a maximum LCR of 5,27 at 90 s of exposure to Oxonia. This could imply a major impact of the sterilization process with increasing exposure time. Established $D_{ambient}$-value was 13,89 s.

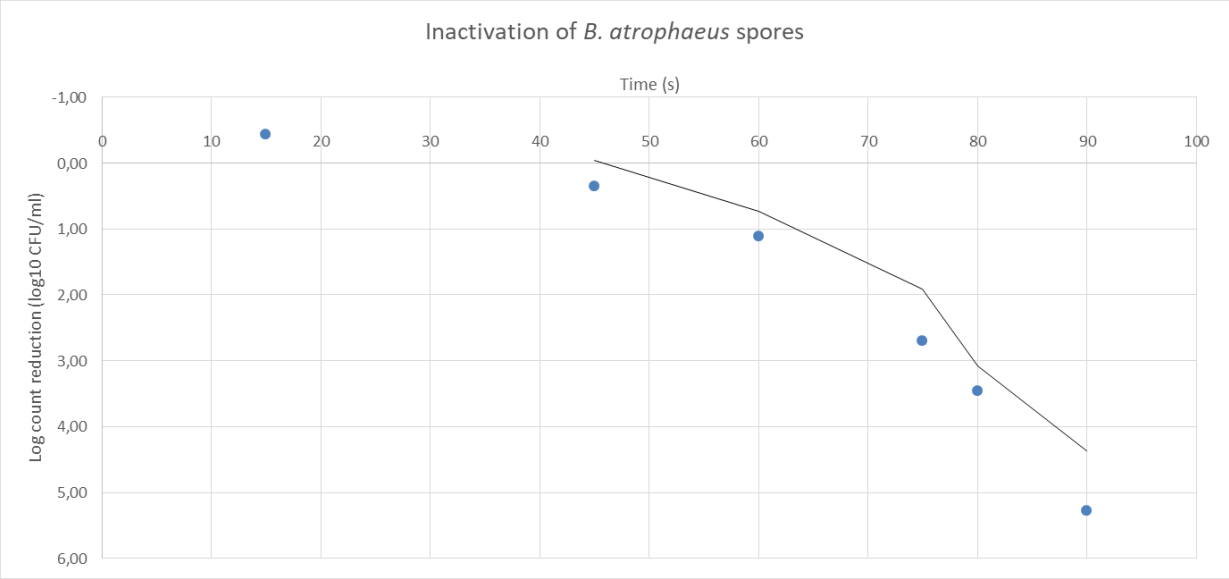


Figure 18 Inactivation curve of *B. atrophaeus* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed.

3.2.2.3 Resistance tests of *B. subtilis* spores against Oxonia

B. subtilis spores were sterilized against 0,9 % Oxonia at ambient temperature for 15, 45, 60, 75, and 120 s. From Figure 19, it can be seen that the inactivation curve started with initially 0,46 LCR before it reached 1,11 LCR at 45 s. With longer exposure to Oxonia, highly distinct differences on the LCR values could be clearly observed. From 60 to 75 s, the LCR values increased rapidly from 1,99 to 4,30, reaching a final LCR of 5,30 after 120 s of exposure. This sterilization process resulted in a $D_{ambient}$ value of 19,76 s.

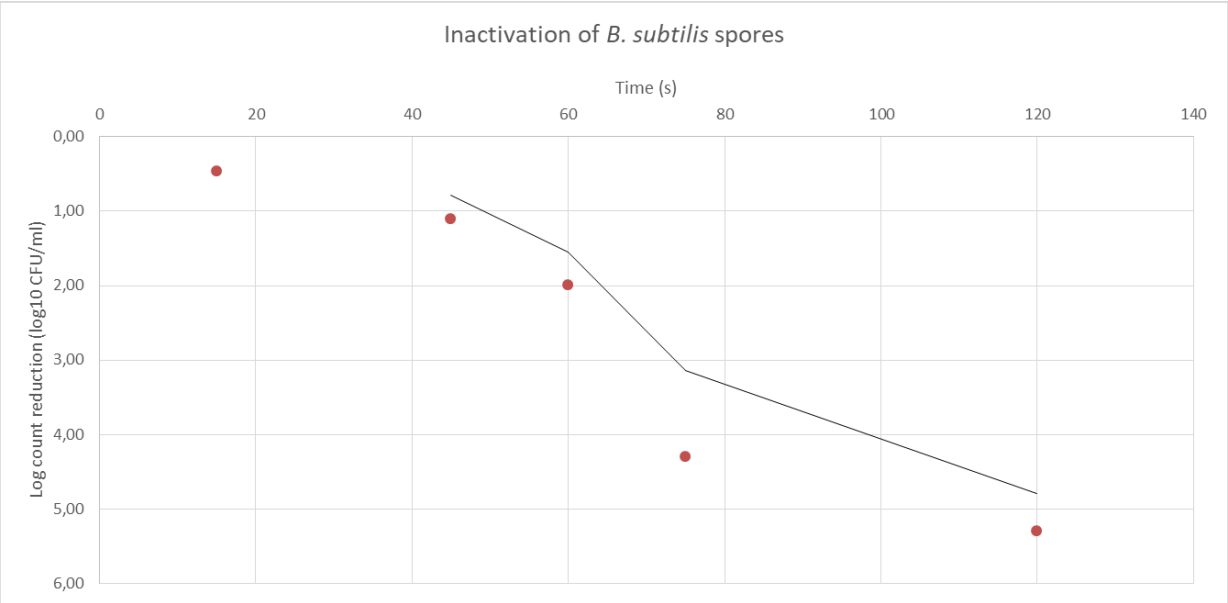


Figure 19 Inactivation curve of *B. subtilis* spores against 0,9 ± 0,5% Oxonia at ambient temperature. The LCR of one replica is shown. Linear regression equation and R^2 value are displayed.

3.3 Resistance of the test organisms against hot water disinfection

The impact of hot water disinfection on the three different test organisms, *A. brasiliensis, B. atrophaeus*, and *B. subtilis* was investigated by exposing the spore solution to hot water of 90 ± 0,2 ˚C using a Mini Block Heater (made in three replicas). Initially, all spores were subjected to identical exposure times based on the method by Veen et al. (2015), i.e. 5 to 30 min. However, for *A. brasiliensis* spores, no recovered spores were found on the plates so less exposure time was applied. Finally, the resistance tests were performed over a time range of 3 to 15 s for *A. brasiliensis* spores and 5 to 30 min for both *B. atrophaeus* and *B. subtilis* spores.

3.3.1 Resistance tests of *A. brasiliensis* spores against hot water

Spores of *A. brasiliensis* were challenged against hot water disinfection for exposure times of 3, 5, 8, 10, 12, and 15 s (see Figure 20). It can be seen that most of the inactivation occured after exposure time of 15 s resulting in a 3,85 LCR. This behavior together with the obtained $D_{90^{\circ}C}$ of 3.84 s reviled the notable sensitivity of the *A. brasiliensis* spores towards hot water disinfection.

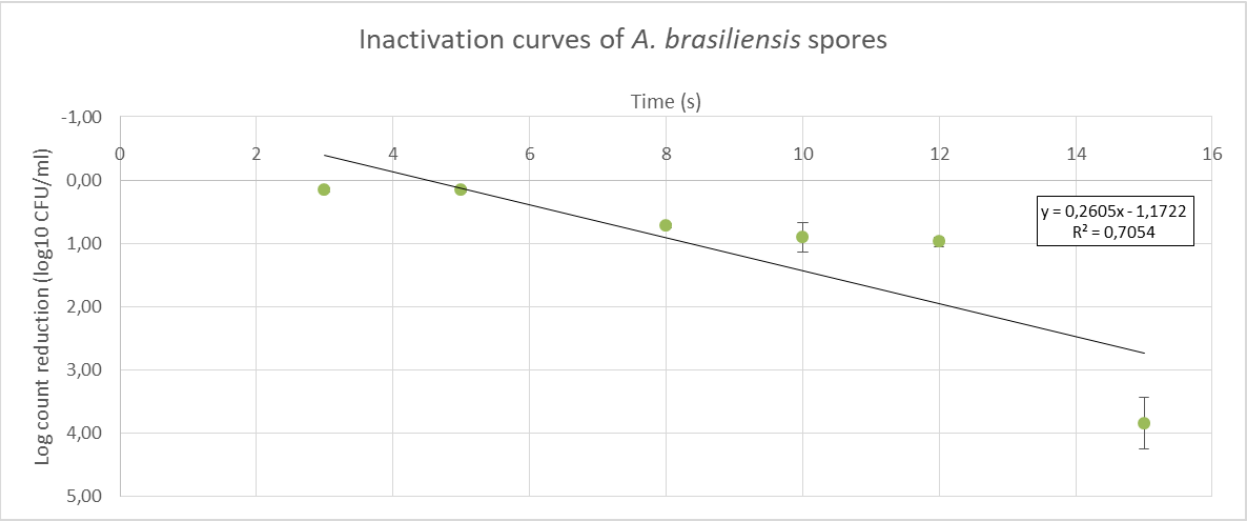


Figure 20 Inactivation curve of *A. brasiliensis* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

3.3.2 Resistance tests of *B. atrophaeus* spores against hot water

B. atrophaeus spores were exposed to hot water for 5, 10, 15, 20, and 30 min. The inactivation rates of the spores are represented in Figure 21, where a rapid reduction of the spore population could be observed throughout the process. Initially, the LCR value was 1,33 at the exposure time of 5 min and afterward, it increased up to 4,77 LCR after 20 minutes. The obtained $D_{90\ ^\circ C}$ value was 8,10 min for this process.

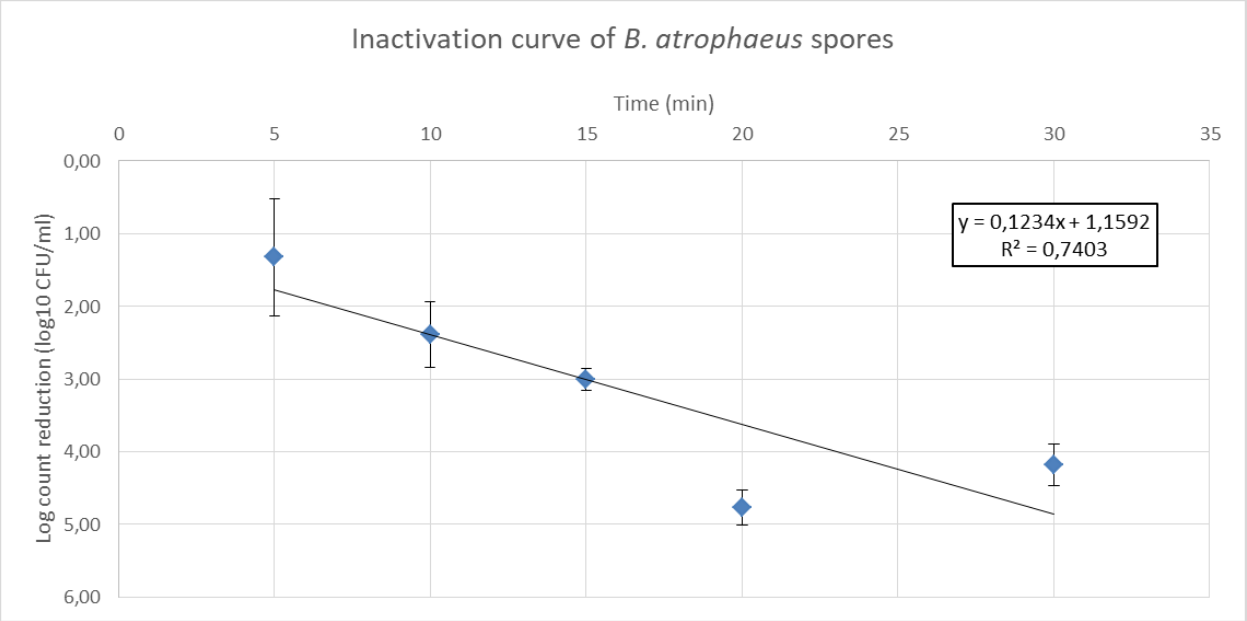


Figure 21 Inactivation curve of *B. atrophaeus* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

3.3.3 Resistance tests of *B. subtilis* spores against hot water disinfection

The effect of hot water disinfection at 90 ˚C towards *B. subtilis* spores was evaluated for 5, 10, 15, 20, and 30 min. The obtained inactivation curve revealed that instead of killing the spores and reducing their population, increased spore levels were recovered giving negative LCR values (see Figure 22). Initially, after 5 min of exposure to hot water, the LCR value was -1,04 and at the end of the experiment, the LCR value was -1,40. This could indicate a reverse effect of hot water disinfection on *B. subtilis* spores where instead of inactivation, germination occurs.

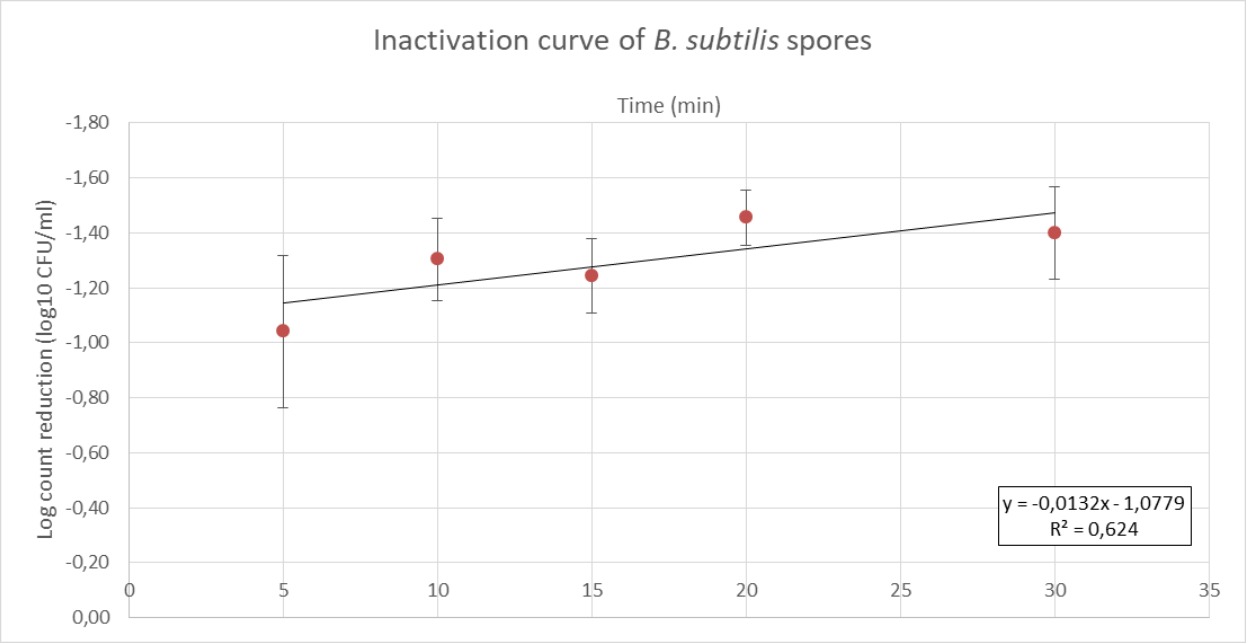


Figure 22 Inactivation curve of *B. subtilis* spores against hot water disinfection at 90 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

3.4 Resistance of the test organisms against dry heat sterilization

The resistance of *A. brasiliensis*, *B. subtilis*, and *B. atrophaeus* spores towards dry heat sterilization was investigated, made in three replicas. A temperature-controlled oven at 125 ˚C was used to challenge the inoculated carriers for 5, 10, 15, 20, 40, 60, 75, and 90 min. The LCR versus time plots for each spore are given in Fig 23 (*A. brasiliensis*), Fig 24 (*B. atrophaeus*), and Fig 25 (*B. subtilis*). No screening tests were performed since the tests were intended to check the overall resistance of the spores as well as the indicative D-values, not to optimize the resistance. In addition, according to the writer's knowledge, there has not been any similar challenge test performed using a packaging carrier. These tests were done according to an experiment of dry heat for spaceflight hardware by (Kempf et al., 2008).

3.4.1 Resistance tests of *A. brasiliensis* spores against dry heat sterilization

Figure 23 displays the dry heat inactivation curve of *A. brasiliensis* spores. These results strongly indicate that dry heat has a lethality enhancing effect at 125 °C on the spores, showing a rapid spore reduction with longer exposure times. Maximum reduction of 3,83 logs was obtained after 15 minutes, however, no detectable survivors were found afterward, which might due to the low initial spore concentration ($10^{5.5} – 10^6$/mL). This process delivered a $D_{125\ ^\circ C}$ value of 8,45 min.

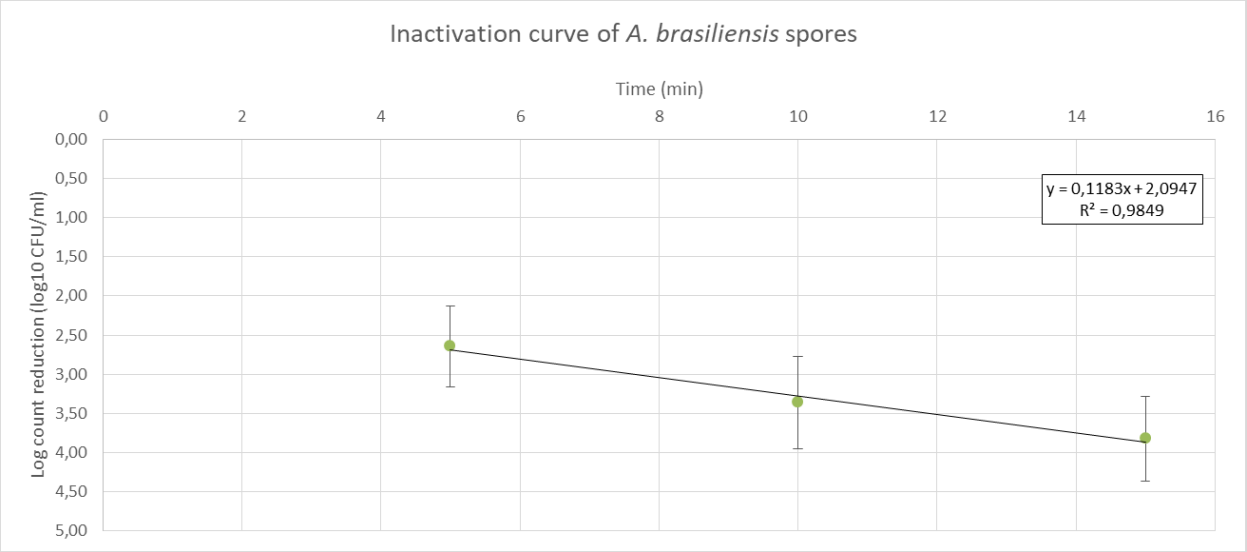


Figure 23 Inactivation curve of *A. brasiliensis* spores against dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

3.4.2 Resistance tests of *B. atrophaeus* spores against dry heat sterilization

B. atrophaeus spores were exposed to dry heat (125 ˚C) for up to 90 minutes. The time kinetics of *B. atrophaeus* presented in Figure 24 reveals that the curve comprises of two different growth phases. The first phase displayed almost no decrease in the spore population with LCR values ranging from 0,38 – 0,10 within the first 20 minutes. After this phase, an almost linear decrease in the population could be observed, with LCR of 0,77 after 40 minutes of exposure. Following this, the inactivation almost showed no change before it dropped to LCR of 1,47 at 90 minutes. However, a big standard deviation was noted for this value. The deviation on the LCR value after 90 minutes of exposure would need further investigation to understand the possible reasons behind it. All of these outcomes could mean that in the beginning, the effect of dry heat was not very strong, hence the unstable amount of reduction, but as the spores were exposed for longer times, the lethality rate also increased. This sterilization process demonstrated a $D_{125\ ^\circ C}$ value of 71,94 min.

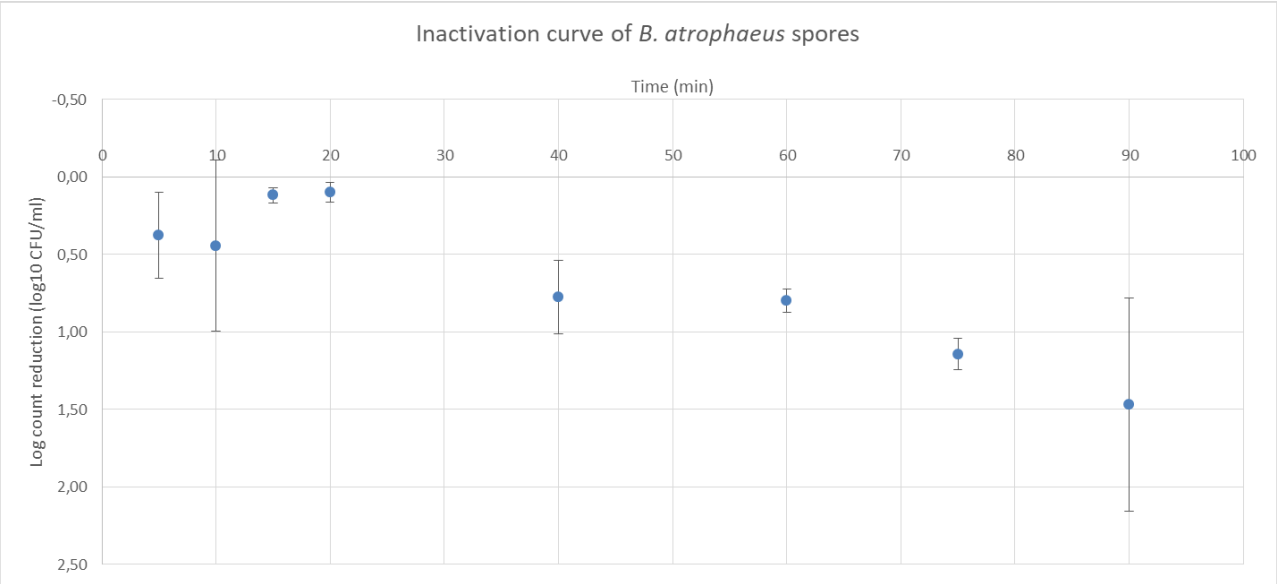


Figure 24 Inactivation curve of *B. atrophaeus* spores exposed to dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

3.4.3 Resistance tests of *B. subtilis* spores against dry heat sterilization

B. subtilis spores were exposed to dry heat at 125 ˚C for 5, 10, 15, 20, 40, 60, 75, and 90 minutes. Figure 25 presents the inactivation curve where three different phases can be observed. The first phase displayed the very low reduction occurring within the exposure time of 20 min where indistinct differences on LCR values were noted. After reaching 0,07 LCR, the values shifted into the second phase, showing a rapid reduction and reaching 2,02 LCR after 60 minutes. The third phase was characterized by a decreasing trend on the achieved LCR, with 1,66 LCR at the end of the experiments. However, as seen from the standard deviation, these three LCR were still around the same values. The dry heat sterilization process gave a $D_{125\,°C}$ value of 37,45 min.

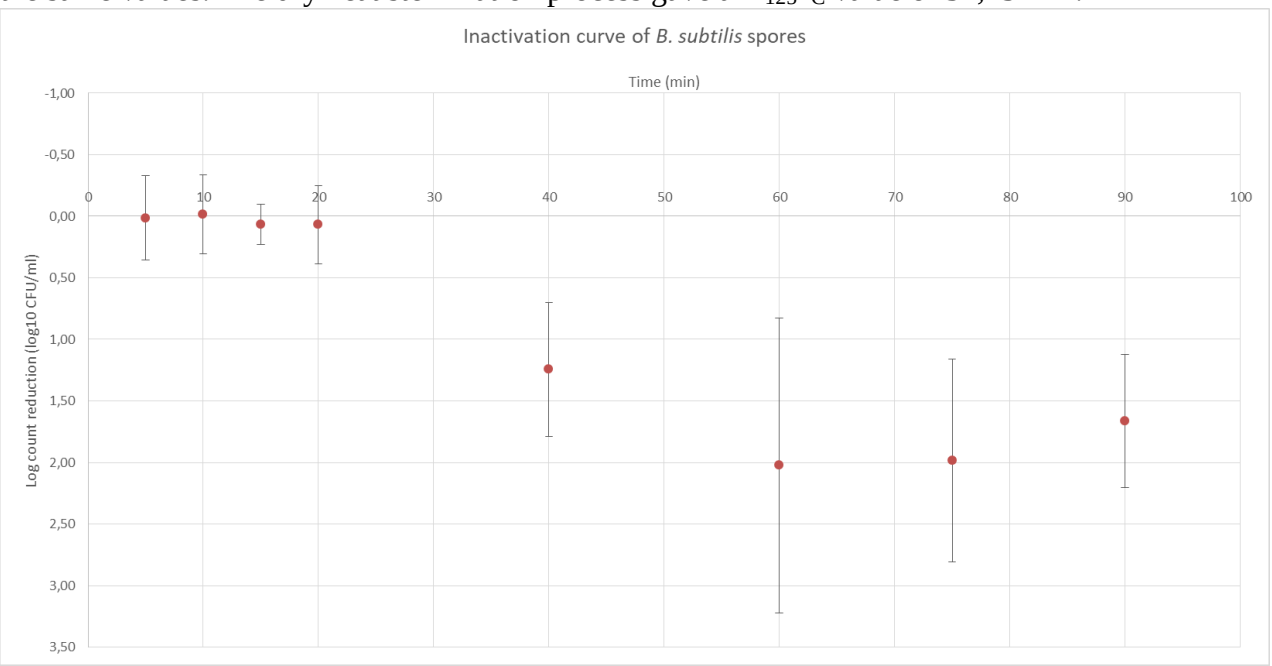


Figure 25 Inactivation curve of *B. subtilis* spores exposed to dry heat sterilization at 125 ± 0,2 ˚C. The average and standard deviation of three replicas are shown. Linear regression equation and R^2 value are displayed.

4. Discussion

In the present study, the efficacy of microbial test organisms was investigated in a comparative analysis against sterilization and disinfection processes, including H_2O_2, Oxonia, hot water and dry heat. Additionally, the steam and UV sterilization processes will also be included in this discussion as a literature review study. The spore-producing microorganisms, *A. brasiliensis*, *B. atrophaeus*, and *B. subtilis* were selected as the test organisms of interests as intended by VDMA. VDMA's Food Processing and Packaging Machinery Association sector published a recommendation of minimum microbiological requirements for packaging and hygienic filling machines (FS NuV No. 10/2005 Appendix A), consisting of a list of sterilization processes with the selected test organisms and minimum LCR. To provide evidence of the sterilization effectiveness for an industrial application, a validation test has to be performed to ensure microbial reduction of at least 4 LCR for a suitable test organism towards the respective sterilization processes of concern. These recommendations were used as a guideline in designing the resistance tests in order to create data to help select test organisms depending on the sterilization process applied.

The D-values obtained for the H_2O_2 (30 %) exposure tests at 60 °C revealed that *B. subtilis* has a higher resistance compared to *B. atrophaeus* under the same exposure conditions. Additionally, 4 LCR was achieved by *B. subtilis*, but not with *B. atrophaeus* due to the time constraints during the study. The trendlines of these two organisms imply the difference inactivation kinetics towards H_2O_2, where the latter organisms did not demonstrate a linear trendline. Meanwhile, *B. subtilis* spores showed a more linear inactivation curve, meaning that if the process was extended to longer exposure time, the killing effect might conform to the same consistency. Similar observations were found in the study by Eschlbeck et al. (2018), testing the spore inactivation of *B. subtilis* SA 22 and *B. atrophaeus* DSM 675 against the liquid 35 % H_2O_2 at 25, 35, 45, and 55 °C for 300 s. Although at 25 °C *B. atrophaeus* spores seemed more resistant than *B. subtilis* with $D_{25\ °C}$ of 101 s and 906 s respectively, it was found that *B. atrophaeus* became more sensitive towards H_2O_2 at higher temperatures. Toledo (1974) also stated that at ambient temperatures H_2O_2 gives very slow sporicidal activity but decreases D-values rapidly at elevated temperatures. It may be speculated that both *B. subtilis* and *B. atrophaeus* may comprise of different organic compounds, such as spore coat, as well as core water contents that may affect their capability of maintaining their protection against oxidizing potential of H_2O_2 sterilization process, as reported by Riesenman and Nicholson (2000) and Popham et al. (1995). Nonetheless, the existing literature has not thoroughly described the specific structural differences between these two spores.

Spores of *A. brasiliensis* were sterilized using a lower concentration of H_2O_2 (3 %) at the same temperature. This process established a D-value of 3,15 s, implying the sensitivity of the spores. The inactivation curve further implies that the LCR values were escalated rapidly with longer exposure times. Moreover, as the spores followed the linear trendline, the reduction may continue with more reduction as the processing time is extended. Unlike bacterial spores, fungal spores are part of the normal life cycle of fungi, and, therefore, they are less resistant to chemicals and adverse environmental conditions (Clontz, 2008). To my knowledge, this is the first work reporting the effects of H_2O_2 on *A. brasiliensis* spores, therefore it is only possible to compare the study with another species of mold spores, *A. fumigatus* D52-B1 which was studied by Oliveira et al. (2016). In this study, the mold spores were exposed to 10 - 20 mmol/L (1-2 %) H_2O_2 for 1 h at

37 °C, in which nearly a loss of 50% viability was achieved at 14 mmol/L (1,4 %) H_2O_2. This study concluded that H_2O_2 induces the spores in a dose-dependent manner, which is relevant to the present study as in the screening test similar outcomes were found.

From the three types of spore, it can clearly be said that *A. brasiliensis* spores are the least resistant among the others, although direct comparison cannot be made as different concentrations of H_2O_2 were used. However, the 3% H_2O_2 was shown to eliminate most of the survivors after 9 s, which implies the sensitivity of the spores towards the process. Meanwhile, *B. subtilis* and *B. atrophaeus* can both be options of test organisms for the validation of this type of sterilization although *B. subtilis* possess more advantages with the linear inactivation kinetic. Flat-like inactivation curve was observed in the first few seconds of *B. atrophaeus* spores, which may mean there was only a few reduction. Therefore, to gain the necessary LCR of 4, it would be crucial to use elevated processing parameters, i.e. temperature and concentration of H_2O_2 in the filling machine, since this sterilant is efficacious at a higher temperature. This may economically cost the industry as well as an energy waste.

In an aseptic packaging system, H_2O_2 is often used with the concentration between 15 – 35 %. The toxicity of H_2O_2 itself has been studied extensively, where the breakdown products of water and oxygen are known as harmless substances. Furthermore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had evaluated the safety of H_2O_2 in 1965, 1973 and 1980 respectively. JECFA considered that ingestion of small amount of H_2O_2 would produce no toxicological effects due to rapid decomposition of the chemical by the enzyme catalase of the intestinal cells (Joint FAO/WHO, 2014). US FDA Regulation (21 CFR 177.1520) on residual H_2O_2, stated that its application in the sterilization of plastic surfaces of packaging material is the most complete and best, provided that; only those plastic surfaces listed in the regulations are treated, all additives used are permitted by a regulation, the concentration is within the regulated limit, and immediately after packaging, the residual must not exceed a regulated level (at present 0,5 ppm) (FDA, 2018).

With the knowledge that H_2O_2 works more effective at high temperature, it could be possible that the suitable temperature can reach higher than some of the packaging materials can tolerate. The use of chemical substance in cleaning the filling machines using high temperature can also cost a lot of money. In its first era of utilization, peracetic acid was known to be sporicidal, but not widely used because of handling, toxicity, and stability problems. Peracetic acid is very reactive and quickly decomposes to acetic acid (acid in vinegar), oxygen and water. This sterilant has been approved by the FDA as a sanitizer on food contact surfaces (21 CFR 178.1010) and for direct food contact with fruits, vegetables (21 CFR 173.315) and meat, poultry and seafood (21 CFR 173.370) (FDA, 2018). Furthermore, sanitizer suppliers have developed a stabilized combination of H_2O_2, acetic acid, and peracetic acid, in which Oxonia is one of the marketed product as an alternative sterilant other than H_2O_2.

From the exposure of the selected test organisms to 0,9 % Oxonia at ambient temperature, it was known that *B. subtilis* was the one exhibiting the highest resistance followed by *B. atrophaeus* and *A. brasiliensis*. Existing literature by Blakistone et al. (1999) using 2% Oxonia at 40 °C shows a similar inactivation trend for *B. atrophaeus* and *B. subtilis*, wherein the latter spores were more resistant than the first, as the time to reach end-point occurred between 150 and 180 s compared to 90 and 120 s respectively. Meanwhile, for the mold spores, no comparison from

existing studies could be found and when compared to the other two *Bacillus* spores, it displayed much rapid inactivation of spores even at shorter exposure times. This implies the *A. brasiliensis* spores as very sensitive and therefore not considered a suitable test organism for this sterilization process. Leggett et al. (2016) stated that the spore coat plays a vital role in protecting the spores from the sporicidal effect of synergistic sporicidal activities of PAA and H_2O_2 combination in the Oxonia solution. Therefore, the differences in spore coat composition between *B. subtilis* and *B. atrophaeus* may affect the resistance.

For hot water disinfection, it was known from the resistance test that *B. atrophaeus* was the most resistant among the spores, with $D_{90\,°C}$-value of 8,1 min. *A. brasiliensis* spores were reduced with almost 4 LCR after just 15 s, while for *B. subtilis* spores, there was an indication that the spores germinated instead. When the dormant spore is exposed to an appropriate environmental stimulus, in this case, the high temperature, it initiates the process of germination (Sella et al., 2015). The inactivation curve also shows a large standard deviation, which means the number of survivors throughout the exposure time may not differ significantly. Based on these results, it can be stated that this type of disinfection is not suitable for all the tested organisms. For *A. brasiliensis* and *B. subtilis*, the reason is clearly that of the low resistance. Meanwhile, for *B. atrophaeus* spores, although inactivation of 4 LCR was achieved the exposure time of 30 minutes was needed. To have less processing time, a higher temperature can be used, although it might be costly and contribute to the waste of energy. It was mentioned that hot water can be used to sterilize/disinfect the filling machine. The surface walls of the product pump and piping are heated to a temperature usually in excess of 121 °C, held for a specified amount of time, typically for at least 45 minutes. However, it was not investigated whether this is an effective treatment to inactivate bacterial spores.

When the spores of the test organisms were subjected to dry heat sterilization *B. atrophaeus* was found to be the least sensitive one with a $D_{125\,°C}$ value of 71,94 min and followed by *B. subtilis* with a $D_{125\,°C}$ value of 37,45 min and finally *A. brasiliensis* with a $D_{125\,°C}$ value of 8,45 min. *A. brasiliensis* clearly demonstrated a sensitive behavior towards this sterilization process with such rapid reduction along the exposure time. Although differences in the D-values were observed between the two *Bacillus* spores, the non-linear inactivation curve that was observed from the experiments performed in this study as well as the big variability between the replicas could indicate that the selected test organisms are not the most efficient ones to be used as biological indicators for this type of sterilization process. In addition, this method is generally not used in the food industry. Previous work by Kempf et al. (2008) reported that *B. atrophaeus* ATCC 9372 was the most resistant organism and generally used as a monitoring standard for dry heat process used mostly in spaceflight hardware. The method involves heating the spaceflight hardware to temperatures between 104 °C and 125 °C for up to 50 hr while controlling the humidity to very low values.

Apart from the sterilization and disinfection processes that have already been discussed above, there are also steam and irradiation processes that may be a suitable sterilant for aseptic operation. Spores of *G. stearothermophilus*, previously *Bacillus stearothermophilus*, are recognized as the test organisms for assessing the efficacy of steam sterilization because of their high wet heat resistance (Albert et al., 1998; Alfa et al., 2002; McCormick et al., 2003; Schneider, 2014). Treatment of *G. stearothermophilus* at 116 °C for 9 minutes resulted in a loss of spore viability for less than 0,1 %, which gives us insight into the high resistance of the spores

(Huesca-Espitia et al., 2016). The resistance can be explained by the significant amount of α-glucosidase activity in the spore core, as well as perhaps even the capacity to synthesize at least some of these enzymes, which persist even after spores have lost viability due to autoclave treatment (Setlow et al., 2016). In addition, it should be noted that *B. subtilis* is not generally recommended for validation of steam sterilization (Larousse and Brown, 1997). Ansari and Datta (2003) added that to gain adequate inactivation in the short time for the packaging units, the surface temperature of the packaging material must reach 135 °C. Meanwhile, obtaining a sufficiently high surface temperature becomes a technological problem in relation with time, high production rates, and to avoid softening and deformation of the material. For equipment sterilization, the process can be attained by exposure of the surface to an appropriate time and temperature sequence, e.g. 30 min at a surface temperature of at least 121 °C

After understanding further about steam sterilization, the following review study will discuss irradiation technology. In recent years, ultraviolet (hereafter regarded as UV) 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).

Spores of *A. brasiliensis* were proven to have high UV-resistance and non-pathogenic nature, thus being recommended as the test organisms for UV sterilization. UV dose of 250 mJ/cm^2 at a wavelength of 253,7 nm has shown to give 2 LCR of the spores (Taylor-Edmonds et al., 2015). Meanwhile, a compiled study on *B. subtilis* spores treated using UV dose ranged from 5-78 mJ/cm^2 resulted in 4 LCR. These spore reduction values might give the conclusion that even with higher UV-dose, *A. brasiliensis* spores still give higher resistance than the *B. subtilis* spores. Clauß (2006) added that *A. brasiliensis* are most resistant at UV radiation wavelength of a 222 nm. The spores' black spore pigment, aspergillin, is reported to be of high UV resistance as it absorbs high amounts of the UV radiation to protect the cell.

When comparing mold and bacterial spores from the aforementioned findings, it can be implied that mold spores of *A. brasiliensis* can tolerate more UV dose than *Bacillus* spores. The killing efficiency of UV irradiation is affected by several factors, e.g. dust particles, the shape of the container, agglomerates of microorganisms, as well as humidity. von Bockelmann (1930) reported that only 90-99 % reduction of the microbial load was achieved after UV irradiation exposure with up to 1500 mW/m^2, wherein dust particles and microorganism aggregation was known to be the source of limitation.

5. Summary

To decide the ideal test organisms for sterilization/disinfection processes, there are several points to be considered. Firstly, the test organisms must be sufficiently resistant towards the given sterilization process to reach the industrial standard of reduction of 4 LCR. If less resistant spores are chosen as the monitoring test organisms, it may be possible that the design of the process might imbalance the sterilization requirement, i.e. too low temperature or sterilant concentration, leading to an ineffective sterilization process. In contrast, if the test organism is too strong, although the more the spore reduction the better, it has to be balanced in relation to processing parameters, safety requirement, as well as energy and cost consumption.

The outcomes of the study are summarized in Table 11, which states which test organism is considered suitable to the respective design, set-up, optimization, validation, as well as control of the sterilization/disinfection processes.

Table 11 Comparison of test organisms for respective sterilization process between this study and VDMA

Sterilization processes	Test organisms for respective sterilization process	
	Suitable test organisms according to this study	**VDMA**
Hydrogen peroxide (H_2O_2)	*B. subtilis* (SA 22) *B. atrophaeus* (ATCC 9372)	*B. subtilis* (SA 22) *A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)
Oxonia (PAA product)	*B. subtilis* (SA 22) *B. atrophaeus* (ATCC 9372)	*B. subtilis* (SA 22) *A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)
Hot water	*B. atrophaeus* (ATCC 9372)	*A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)
Dry heat	*B. atrophaeus* (ATCC 9372)	*A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)
Steam	*G. stearothermophilus*	*A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)
UV	*A. brasiliensis*	*A. niger* (*brasiliensis*) (DSM 1957/ATCC 6275 or DSM 1988/ATCC 16404)

The VDMA guidelines stated that *B. subtilis* is the optimal test organism for H_2O_2 as well as Oxonia (i.e. PAA product). *A. brasiliensis* is also listed as test organism for both H_2O_2 and Oxonia, followed by hot water, dry heat, steam, and UV processes. In comparison with VDMA, the present study comes to a similar conclusion, meaning that *B. subtilis* can be regarded as a suitable test organism to challenge the process for both H_2O_2 and Oxonia sterilization. However, *B. atrophaeus* spores are also regarded as suitable test organisms for the respective chemical sterilization processes since the spores have shown similar resistance with *B. subtilis*. In contrast, the present study concludes that *A. brasiliensis* is not suitable to be used for H_2O_2 and Oxonia validation, because it was demonstrated here that the spores were very sensitive towards the exposure of both H_2O_2 and Oxonia.

For the thermal processes, VDMA recommends *A. brasiliensis* spores as the suitable test organism, while results of the present study question the suitability of *A. brasiliensis* here. Hot water sterilization outcome implied that *B. atrophaeus* can give good sterilization effect by

reaching 4 LCR, although the time needed was 30 minutes. For the dry heat process, from all three spores, *B. atrophaeus* was recognized to be the least sensitive, thus considered as the most suitable test organism.

Next is the results of the literature review study for the choice of appropriate test organisms for both steam and UV sterilization. Literature search results suggested that *G. stearothermophilus* was optimal for steam sterilization. This conclusion is in contradiction with the VDMA recommendation of *A. brasiliensis* as best test organism for steam sterilization. Literature search results suggested *A. brasiliensis* was optimal for UV sterilization. This finding is in line with the VDMA recommendation.

6. Conclusion

Based on the outcomes of the present study, the following conclusions can be drawn:

- Microbial test organisms act differently towards different sterilization processes
- Not all test organisms are similarly effective to validate certain sterilization process
- *B. subtilis* spores are the test organism that is most resistant against H_2O_2 and Oxonia sterilization process with $D_{60\ °C}$ = 6,17 s and $D_{ambient}$ = 13,89 s respectively. This result is in accordance with the guidelines of VDMA.
- For the hot water and dry heat processes, our study recommends *B. atrophaeus* as the most suitable test organism with $D_{90\ °C}$ of 8,1 min and $D_{125\ °C}$ of 71,94 min. This result is nonconformed towards the VDMA guidelines which reported that *A. brasiliensis* is more suitable for hot water and dry heat.
- Steam sterilization review study suggested *G. stearothermophilus* as the test organism, meanwhile, VDMA suggested *A. brasiliensis* spores.
- *A. brasiliensis* spores demonstrated rapid reductions for all the sterilization processes evaluated, indicating very low resistance and therefore were not considered as an ideal test organism to use. However, when looking into the available literature on UV irradiation, *A. brasiliensis* could be a possible test organism to assess its effectiveness considering its black spore pigment, aspergillin, which is responsible to the high UV resistance as it absorbs high amounts of the UV radiation to protect the cell.

7. Future work

Further investigations could be carried out to improve the knowledge about resistance test of test organisms towards sterilization processes in the food industry, which are suggested as follows:

- Investigation of resistance in vaporized hydrogen peroxide, since this method is also being used in aseptic processing and packaging.
- Investigation of *B. atrophaeus* spores' resistance towards 30% liquid hydrogen peroxide with 60 °C at longer exposure times to see whether the spores would demonstrate a linear inactivation curve.
- Investigation of resistance of *A. brasiliensis, B. atrophaeus,* and *B. subtilis* spores towards 0,9% Oxonia sterilization under ambient temperature with more replicates

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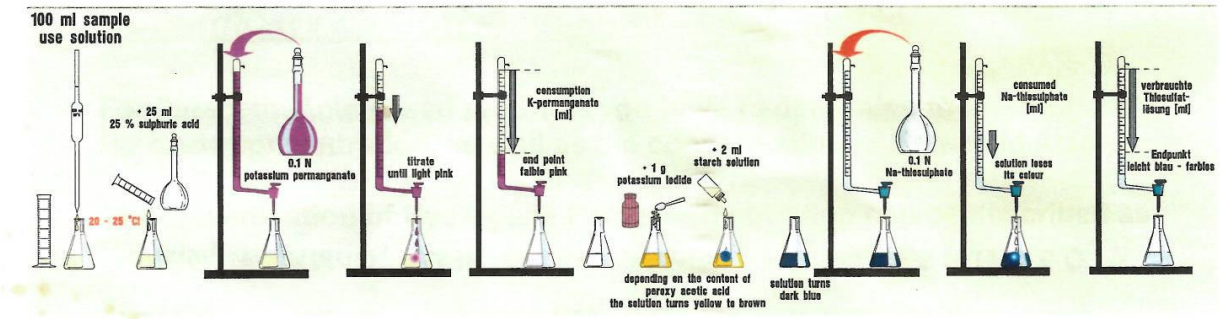
Appendices

Appendix 1 Chemicals used in phosphate buffer

Chemicals	Amount per 1 L
Tween 80	0,05 g
Sodium dihydrogen phosphate ($NaH_2PO_4*H_2O$)	4 g
di-Potassium hydrogen phosphate (K_2HPO_4)	6 g

Appendix 2 Titration method provided by Ecolab Inc. for hydrogen peroxide and Oxonia



Often solution turns pink after Ading the first drops. Then wait For 2 – 3 minutes!

The change of colour to a stable, faint pink should just happen with one drop. Adding Permanganate in excess will spoil the following titration! Samples with Too much Permanganate must be discarded.

Consumption ml K-Permanganate x 17 = ppm H_2O_2

Consumption ml Na-Thiosulphate x 38 = ppm POAA