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Factors influencing the production of bacterial spores as biological indicators with standardized resistance to inactivation with hydrogen peroxide

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And in the end, it’s not the years in your life that count.
It’s the life in your years.

Abraham Lincoln

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Abbreviations

Abbreviation	Explanation	Unit
%	Percent	-
°	Degree	-
°C	Degrees Celsius	-
× g	Relative centrifugal force	-
Å	Angstrom	-
AMP	Adenosinmonophosphate	-
ATCC	American Type Culture Collection	-
ATP	Adenosindiphosphate	-
Bar	Pressure	-
BI	Biological indicator	-
BSM	*Bacillus* sporulation medium	-
CASO	Casein peptone soymeal peptone	-
CDC	Centers for Disease Control and Prevention	-
CFU	Colony forming units	1/mL
d	Day	-
D-value	Decimal reduction time	s; min;
DIN	Deutsche Norm / German standard	-
DNA	Deoxiribonucleic acid	-
DoE	Design of experiments	-
DSM	Difco sporulation medium	-
EN	Europäische Norm / European standard	-
EtOH	Ethanol	-
Exp.	Experiment	-
f(x,y)	Function of x and y	-
FDA	Food and Drug Administration	-
Fig.	Figure	-
g	Gram	-
GM9	*Geobacillus* Medium	-
GSLE	Germination-specific lytic enzyme	-
h	Hour	-
ISO	Internationale Norm / International standard	-
kJ	Kilojoule	-
L	Liter	-
LB	Lysogeny Broth-Lennox	-
LEEB	Low energy electron beam	-
LHP	Liquid hydrogen peroxide	-
Log	Logarithm	-

Abbreviations

Abbreviation	Explanation	Unit
LTSF	Low-temperature-steam-formaldehyde	-
M	Molar mass	g/mol
mg	Milligram	-
MilliQ-Water	Type 1 water according to ISO 3696	-
min	Minute	-
mL	Milliliter	-
mm	Millimeter	-
mM	Millimolar	-
mol	Mole	-
MSM	Minimal sporulation medium	-
NAD	Nicotinamid adenine dinucleotide	-
NADH	Reduced nicotinamid adenine dinucleotide	-
nm	Nanometer	-
OD600	Optical density at 600 nm	-
p-value	Probability value	-
PET	Polyethylene	-
pH	Potential of hydrogen	-
pO_2	Oxygen saturation	-
PP	Polypropylene	-
ppm	Parts per million	-
r^2 value	Coefficient of determination	-
rpm	Revolutions per minute	-
RSME	Root-mean-squared error	-
s	Second	-
SASP	Small acid-soluble protein	-
SB	Super broth	-
SCBI	Self-contained biological indicator	-
SEM	Scanning electron microscopy	-
SED	Secondary electron detector	-
Sol.	Solution	-
SSM	Submerged sporulation medium	-
t	Time	s; min; h; d;
T	Temperature	°C
Tab.	Table	-
TB	Terrific broth	-
Temp.	Temperature	°C
U	Unit	-
U.S.	United States	-
UHT	Ultra-high-temperature processing	-

Abbreviations

Abbreviation	Explanation	Unit
USA	United States of America	-
UV	Ultraviolet	-
VDMA	Verband Deutscher Maschinen- und Anlagenbau / German Engineering Federation	-
VHP	Vaporized hydrogen peroxide	-
α	Alpha	-
β	Beta	-
γ	Gamma	-
Δ	Delta	-
µL	Microliter	-

Content

1 General introduction

Product safety is paramount in the food and pharmaceutical industries to protect public health and prevent harm from contaminated or unsafe products. In both industries, products that are unsafe for consumption can have severe consequences for individuals, leading to illness or even death.

In the food industry, the Centers for Disease Control and Prevention (CDC) estimates that each year, approximately 48 million people get sick from a foodborne illness, leading to 128,000 hospitalizations and 3,000 deaths. This highlights the critical importance of ensuring food safety through appropriate handling, processing, packaging, storage and effective quality control measures (CDC, 2023).

Similarly, in the pharmaceutical industry, the safety of drugs and medical devices is essential to ensure that they are effective and do not cause harm to patients. The U.S. Food and Drug Administration (FDA) regulates the safety of drugs and medical devices and requires manufacturers to demonstrate the safety and efficacy of their products through extensive testing.

Ensuring product safety in these industries requires comprehensive quality control measures, adherence to rigorous safety standards, ongoing monitoring of manufacturing processes to identify and address potential hazards, and the sufficient sterilization of products and packaging materials.

This work focuses on the production of bacterial spores of *Bacillus* and *Geobacillus*, which are used as bioindicators for the validation of sterilization processes with hydrogen peroxide. Currently, there is no standardized production method of bacterial spores for this purpose. There is also currently no standardized test method for determining the resistance of bacterial spores. The research work starts at this point and develops methods for the production of bacterial spores and equally for determining their resistance to hydrogen peroxide. In the following introduction, a basic understanding is created to be able to classify and understand the methods developed and research results obtained from the research work.

1.1 Sterilization with hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a sterilization agent widely used in the food and pharmaceutical industries to reduce or eliminate biological contaminants and, therefore, to ensure product safety. It has already been used in medicine for over 100 years (Urban et al., 2019). Due to its broad-spectrum activity, it can sterilize various bacteria, fungi, viruses, and other biological contaminants. It is non-toxic and lacks carcinogenic or mutagenic effects, thus making it environmentally safe. Because of its rapid action, it acts quickly and reliably. Additionally, it is compatible with a variety of materials and

surfaces, making it an effective method for sterilizing both products and packaging materials (CDC, 2023; Eschlbeck et al., 2017; Finnegan et al., 2010; Krämer, 2007; Martins-Costa and Ruiz-López, 2007; Pruß et al., 2012; Rhodes et al., 2022; Scaramuzza et al., 2020; Wood et al., 2016).

Due to its advantages over other methods, hydrogen peroxide as a sterilization agent has become increasingly popular. In the food industry, hydrogen peroxide is often used to disinfect food contact surfaces and equipment and treat food products to reduce microbial contamination. In the pharmaceutical industry, hydrogen peroxide is commonly used to sterilize equipment and containers, such as vials, ampoules, and syringes.

In its liquid form, hydrogen peroxide is an odorless, transparent, slightly bluish liquid (Binnewies, 2011; Goor et al., 2007). It has a slightly acidic character, as it partially dissociates to a hydroperoxide anion (Martins-Costa and Ruiz-López, 2007; Goor et al., 2007). In its pure form, hydrogen peroxide is unstable and difficult to handle, so it is diluted with water for sterilization purposes. For example, for sterilizing packaging materials prior to the aseptic filling of UHT milk, it is used at concentrations between 30-40% (Krämer, 2007). In addition to applying hydrogen peroxide in liquid form (LHP, liquid hydrogen peroxide), sterilization by spraying or exposure to hydrogen peroxide in the gaseous state (VHP, vaporized hydrogen peroxide) is possible. This makes it possible to sterilize simple surfaces and entire rooms, including furnishings and electrical equipment, if non-condensing conditions are maintained during sterilization.

Due to its chemical composition, an oxygen-oxygen single bond, hydrogen peroxide is the simplest peroxide among the oxidizing peroxides. It leaves no residues during sterilization and decomposes into water and oxygen due to its thermodynamic instability (Krämer, 2007). The dissociation reaction is shown in equation (1). This dissociation also slowly occurs during storage, so stabilizers are usually added as a commercial sterilization reagent to increase storage stability (Binnewies, 2011). Equation (1) describes the stoichiometric situation (Goor et al., 2007):

$$2H_2O_2\ (l) \rightarrow 2H_2O\ (l) + O_2\ (g) \qquad \textbf{(1)}$$

$(l) = liquid; (g) = gaseous$

The dissociation of hydrogen peroxide is an exothermic reaction in which 98.3 kJ/mol is released in the case of liquid hydrogen peroxide. In the case of gaseous hydrogen peroxide, more energy, 105.8 kJ/mol, is released (Hultman et al., 2007).

The chemical structure of hydrogen peroxide as a monomeric molecule is a so-called "open-book" configuration (Yu and Yang, 2011). Depending on the reference, the molecule has a bond angle (dihedral angle) of 111.0°, respectively 112.0°, between the hydrogen atoms and an angle of 94.5°, respectively 99.3°, between the respective hydrogen and oxygen atoms (Binnewies, 2011; Yu and Yang, 2011) (Figure 1).

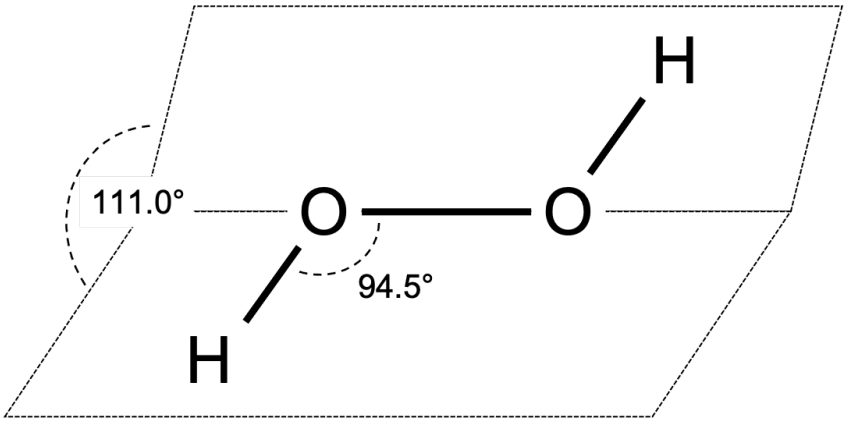


Figure 1. Chemical structure of a hydrogen peroxide monomer in its open-book configuration. Angle values according to Binnewies (2011).

In the liquid, non-monomeric state, the structure changes due to the solvent effects acting on the oxygen-hydrogen bond, which, for example, decreases the dihedral angle between the hydrogen atoms by approx. 11°. Additionally, the length of the oxygen-hydrogen bond increases by about 0.02 Å to 0.988 Å. However, the length of the oxygen-oxygen bond remains at its length of 1.463 Å (Martins-Costa and Ruiz-López, 2007; Yu and Yang, 2011).

The sterilizing effect of hydrogen peroxide on organisms is due to its strong oxidizing properties. During dissociation, hydrogen peroxide splits off oxidative oxygen (Equation (2), according to Kehrer, 2000), which irreversibly damages or kills microorganisms by destroying essential cell components, such as proteins, lipids, and nucleic acids (Fritsche, 2016). Higher hydrogen peroxide concentrations increase the sterilizing effect, as well as increasing sterilization temperature / hydrogen peroxide temperature at constant hydrogen peroxide concentration (Swartling and Lindgren, 1968; Toledo et al., 1973).

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet} \quad \textbf{(2)}$$

With the help of the superoxide radical, a hydroxyl radical is formed from hydrogen peroxide (Kehrer, 2000). This hydroxyl radical damages the genetic material, proteins, and lipids of microorganisms. In the process, the highly reactive molecules oxidize the bases of DNA, leading to DNA strand breaks, or they link proteins to DNA (Cadet et al., 1999).

Because iron ions catalyze the reaction (Equations (3) and (4), according to Kehrer, 2000), cells with a high ion concentration are more sensitive to hydrogen peroxide (Linley et al., 2012; Repine et al., 1981).

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad \textbf{(3)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad \textbf{(4)}$$

However, this is primarily true for vegetative cell forms since they are inactivated by hydrogen peroxide much more rapidly than dormant forms of some bacteria, so-called spores, which show the greatest resistance to hydrogen peroxide due to specific resistance mechanisms (Setlow, 2014). Chapter 1.3 discusses the resistance mechanisms of spores in more detail.

1.2 Sterilization process validation

Since sterilization is not directly measurable, validation tests are essential to confirm the effectiveness of sterilization processes to ensure that sterilization is adequate. In general, validation tests are carried out during plant or sterilization device qualification and requalification for the validation of sterilization methods, as well as regularly at regular intervals.

The primary purpose of validation tests is to verify that the sterilization process can achieve a predetermined level of microbial reduction. In other words, the validation tests demonstrate that the sterilization process can kill a certain number of microorganisms. Validation tests provide confidence that the sterilization process is effective, which is critical in ensuring that products are safe.

There are several reasons why validation tests are necessary for sterilization processes. One of the main reasons is that sterilization processes are typically complex, and many variables can influence their effectiveness. For example, factors such as temperature, pressure, humidity, and exposure time can all impact the effectiveness of the sterilization process. By conducting validation tests, it is possible to identify the optimal conditions for sterilization, which can help ensure that the method effectively kills microorganisms.

Another reason why validation tests are necessary is that microorganisms can be highly resistant to sterilization processes. Some microorganisms, such as the earlier mentioned bacterial spores, are tough to kill. The conditions required to sterilize them may differ from those needed to kill other microorganisms. Using biological indicators and conducting validation tests makes it possible to determine and ensure the most effective sterilization conditions for the specific microorganisms of interest.

Thus, depending on the sterilization result, the sterilization conditions can be adjusted. If the sterilization result is insufficient, it is possible, for example, to increase the concentration of the sterilizing agent or the sterilization temperature or time, depending on the sterilization process. If the sterilization result is sufficient, it is also possible to shorten the sterilization time or reduce the concentration of the sterilant or the sterilization temperature to save costs and time and thus make the sterilization process more economical.

Validation tests are also necessary because of regulatory requirements. Regulatory bodies like the FDA require that sterilization processes be validated to ensure safe products. Failure to validate sterilization processes can result in the rejection of products and significant financial losses for companies.

The validation process for sterilization with hydrogen peroxide typically involves microbial challenge testing, in which a known number of microorganisms are exposed to the sterilization process and then evaluated for viability.

These microbial challenge tests are typically performed with biological indicators made from bacterial spores of *Bacillus atrophaeus* (ATCC 9372) and *Geobacillus stearothermophilus* (ATCC 7953), which are highly resistant to hydrogen peroxide, making them ideal for use in hydrogen peroxide validation tests (FDA, 2007; McLeod et al., 2017; VDMA, 2021).

Therefore, the specifics of bacterial spores will be discussed in the next chapter.

1.3 Bacterial spores

Bacterial spores are specialized, dormant structures produced by various gram-positive *Bacillus* and *Clostridium* species to survive under adverse conditions (Setlow, 2014).

They carry the cell's genetic material but are otherwise entirely different from the vegetative cell form. Spores are characterized by their metabolic inactivity, i.e., the spore does not engage in cell growth or cell division. Additionally, spores have a completely different morphology from vegetative cells. This unique morphology also gives spores

extraordinary resistance to many environmental influences, including high temperatures, UV radiation, dryness, and many chemical substances that would otherwise damage or kill cells.

These resistances also give bacterial spores a special role in sterilization processes. On the one hand, they are difficult to inactivate and thus usually provide the minimum necessary sterilization parameters for sterilization processes. On the other hand, *Bacillus* and *Geobacillus* spores, in particular, also serve to check sterilization processes with hydrogen peroxide due to their resistance. Since this is the core topic of the present work, only the genus *Bacillus* and *Geobacillus* of the family *Bacillaceae* will be discussed in the following.

During the vegetative phase, the cells engage in aerobic or facultative aerobic growth and aerobic energy metabolism. When environmental conditions no longer permit this vegetative growth, the cells enter so-called sporulation and form endospores (Figure 2).

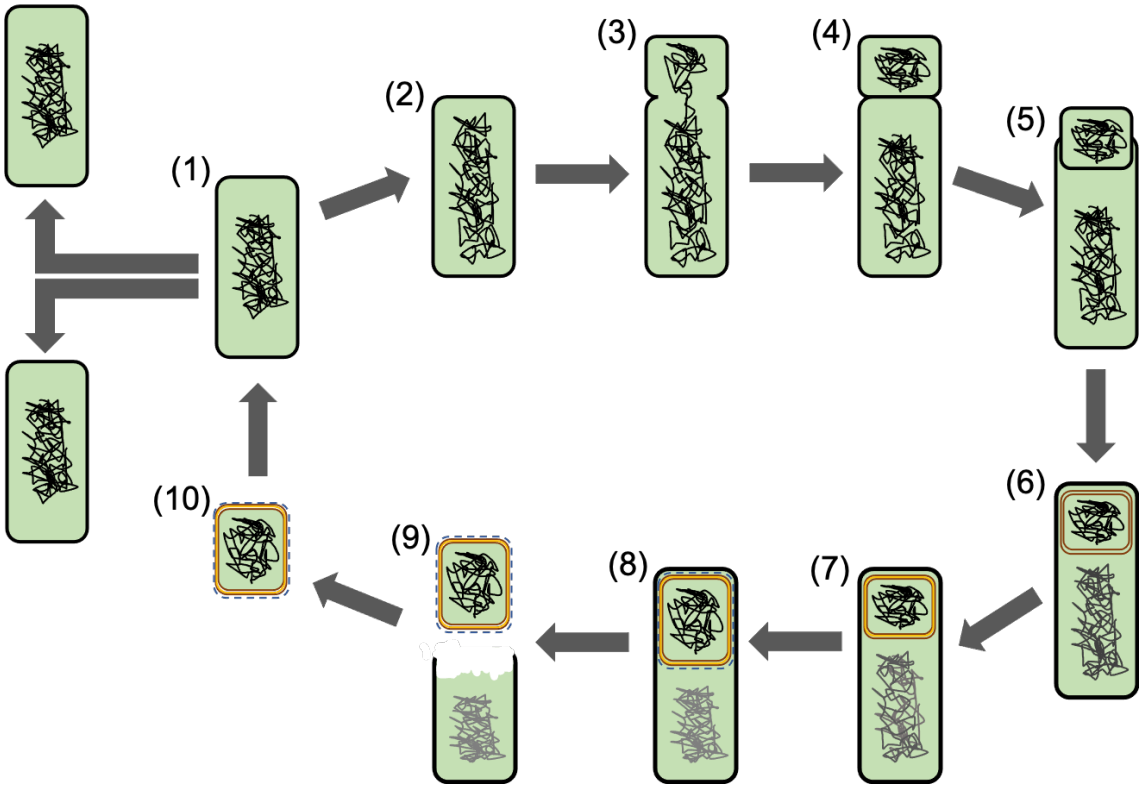


Figure 2. Transition of the binary fission to the sporulation. Step-by-step representation of the sporulation phases. Modified from Fritsche (2016) and Slonczewski and Foster (2012).

Under normal environmental conditions and with sufficient nutrient supply, cells proliferate by binary fission (Figure 2, (1)). As soon as the conditions (environmental conditions or nutrient supply) deteriorate, stress is caused to the cell population, and a transition from the exponential to the stationary growth phase occurs. If these conditions persist, the transition to sporulation occurs. The first step of sporulation is the replication of the chromosome in the vegetative cell. After replication, the chromosomes extend as an axial filament along the long cell axis (Figure 2, (2)). Subsequently, an asymmetric cell division of the cell occurs at one of the poles (Figure 2, (3)). The septum formed in this process traps the chromosome. Via the DNA translocase, the chromosome is translocated from the mother cell into the forming forespore. The resulting spore protoplast (forespore) contains one copy of the chromosome (Figure 2, (4)). The forespore is then enveloped by the mother cell (engulfment) (Figure 2, (5)). A double membrane surrounding the spore is formed (Figure 2, (6)). To form the spore cortex, a layer of peptidoglycan is incorporated between the membrane of the forespore and the membrane given by the mother cell in the phase of engulfment (Figure 2, (7)). The chromosome is being degraded. Coat proteins are positioned on the outside of the cortex (Figure 2, (8)), and dipicolinic acid is incorporated inside the forespore. The forespore partially dehydrates. The mature spore is released by lysis of the mother cell (Figure 2, (9, 10)). The resulting spores exhibit almost no metabolism and can survive decades. Germination turns a spore back into a vegetative cell. Various triggers, such as nutrients, water, or temperature, cause the spore shell to become permeable to certain signaling substances. These triggers react inside the spore, and the germination begins, ultimately ending in a vegetative cell's emergence. (Driks, 1999; Engelhard, 2006; Fritsche, 2016; McKenney and Eichenberger, 2012; McKenney et al., 2013; Pruß, 2013; Riley 2021; Sella et al., 2015; Slonczewski and Foster, 2012;).

In contrast to the vegetative cell form, the spore's content of energy-rich molecules such as NADH or ATP is largely reduced. However, the low-energy variants of these molecules, NAD and AMP, are present. As a result, metabolism and, thus, enzyme activity come to a complete standstill. Typical of spores is a high content of dipicolinic acid, which forms complexes with divalent ions (Setlow, 1994). Predominantly, these ions are magnesium, calcium, and manganese ions and are thought to be involved in maintaining the spore in a quiescent state (Atrih and Foster, 2002; Paidhungat et al., 2000). Small acid-soluble proteins (SASP) bind to the DNA and protect it. The structure of the spore's inner membrane is much more compressed and viscous than the vegetative cell. However, the difference between spore and cell is even greater in the water content, which is significantly lower in spores. This is 0.5 to 1 g of water per gram of dry weight in the spore, whereas in the vegetative cell, this value is between 3 and 4 g of water per gram of dry weight, depending on the species (Atrih and Foster, 2002; Setlow, 1994; Russell, 1990).

Due to these structural changes of the spore compared to the vegetative cell, i.e., due to the stagnant metabolism and the reduced enzyme activity, damage to macromolecules, the DNA or proteins cannot be repaired in case of damage. This damage can be repaired when the cell germinates, but only to a certain extent. Larger, more severe, or too much damage cannot be repaired by the existing repair mechanisms. Therefore, the question arises as to why the spores then manage to survive and why they are resistant to various sterilants. The reasons can be found in multiple protective mechanisms that enable them to survive short, very harsh environmental conditions and moderately poor conditions over a more extended period. The high resistance is primarily due to the unique structure of the spores (Setlow, 2006).

These protective mechanisms lie in the complex structure of the spores (Figure 3).

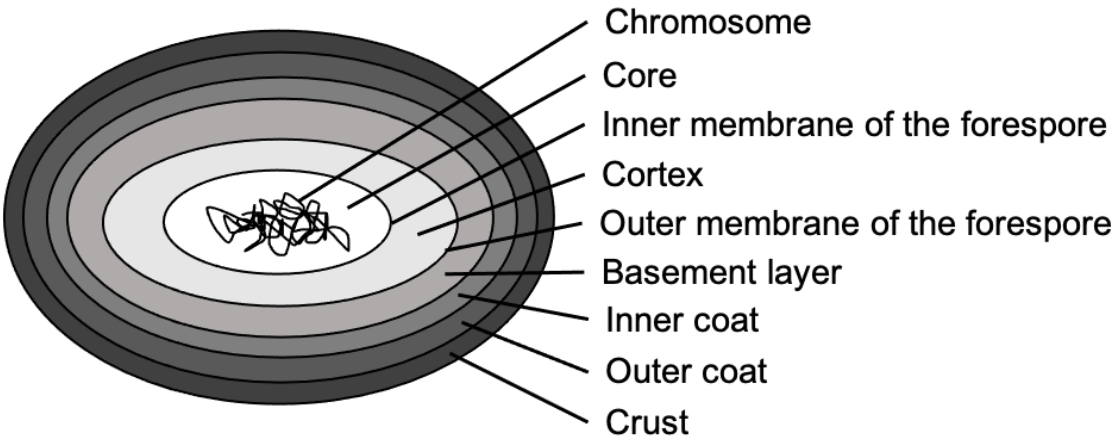


Figure 3. Schematic structure of a typical *Bacillus subtilis* spore, modified from McKenney et al., 2013.

The spore is built up like an onion from different layers. Each layer, in turn, consists of different cross-links, proteins, and lipids, described in more detail below.

Spore crust and coat

The spore coat appears as a series of concentric layers in the electron microscope. In *B. subtilis* spores, three layers can be observed: a lamellar inner coat, a coarsely layered outer coat, and the crust. The coat consists of layers of highly cross-linked polypeptides – it is known for *B. subtilis*, that the coat contains at least 70 proteins – as well as some enzymes that can detoxify potential biocidal chemicals such as peroxides. Some spore coats also contain pigments that can absorb UV radiation. However, despite the high resistance level, the coat allows the spore to respond to the presence

of nutrients that might arise when the environmental conditions improve for vegetative cell growth. It works as a molecular sieve that excludes large molecules but allows the passage of small molecules that can trigger germination. (Driks, 1999; McKenney et al., 2010; McKenney et al., 2013; Setlow, 2006; Setlow, 2014)

Outer membrane

Underlying the coat is the outer membrane. It also can contain pigments that may cause resistance against UV radiation. However, while it is crucial in sporulation, its role in spore resistance is not fully understood. (Setlow, 2014).

Cortex

The cortex lies directly underneath the outer membrane. It consists of peptide-glycan. An intact cortex is essential for spore survival, but no active involvement in resistance formation is known (Setlow, 2014). However, the thickness of the cortex is related to the heat resistance of the spores. The degree of cross-linking of the peptidoglycans must be high enough to maintain the reduced water content inside the spore and, simultaneously, low enough that their degradation is not affected during germination. The cortex also contains so-called GSLEs (germination-specific lytic enzymes – consisting of glucosaminidase and lytic transglycosylase), which are involved in cortex hydrolysis and become active during germination (Atrih and Foster, 2002; Engelhard, 2006).

Inner membrane

The inner membrane is the most significant permeability barrier for small molecules. The structure of this membrane contributes significantly to the protection of macromolecules inside the spore. Its fatty acid composition is not extraordinary, but it gives unique properties: The lipids in the membrane are largely immobilized. The spore's inner membrane is much more viscous than in the vegetative cell. The passive permeability of the inner membrane to small molecules is very low, even for water molecules and methylamine (Setlow, 2014). As sporulation temperature increases, permeability decreases further as the fatty acid composition changes. This helps to explain why spores produced at higher temperatures are more resistant to chemicals that damage the genetic material (Cortezzo and Setlow, 2005). The inner membrane is thought to be the target for many sporicidal oxidants. But the mechanism leading to inner membrane damage is currently unclear (Setlow, 2014). In addition, the membrane prevents leakage of nuclear contents, especially dipicolinic acid, and contributes to the osmotic stability of the spore (Driks, 1999; Cortezzo and Setlow, 2005). Concerning DNA-damaging chemicals, the inner membrane is the key protector. For example, the inner membrane of *B. subtilis* spores is known to withstand even treatment with 5% hydrogen peroxide at room temperature for up to 40 minutes (Melly et al., 2002).

Water content in the spore nucleus

Spores are highly dehydrated compared to vegetative cells. Dehydration occurs during sporulation when mineralization of the cell occurs and complexes of divalent ions and dipicolinic acid form, resulting in very little free water inside the spore core. The spore cortex maintains and regulates the spore's water content (Setlow, 1994). Especially against heat resistance, low water content plays an important role (Atrih and Foster, 2002). Although it is not known precisely what inactivation mechanisms are triggered by heat, the denaturation of some proteins located in the core is thought to play a major role in this process (Leggett et al., 2012). Studies showed that spore water content is affected by sporulation temperature, with higher sporulation temperature leading to a decrease in water content (Cortezzo and Setlow, 2005).

Mineralization in the spore core

The spore core contains a variety of ions that accumulate during sporulation. Not only the content but also the composition of these ions influences the properties of the spore and can be controlled by the ion content of the sporulation medium (Atrih and Foster, 2002). Calcium ions appear to contribute significantly to heat resistance (Leggett et al., 2012). Manganese ions increase the spore yield and stability. They also promote proper cortex structure by affecting enzyme activity and gene expression during cortex synthesis. Overall, spores with higher levels of divalent ions show greater resistance than spores containing more monovalent ions (Setlow, 2014). Additionally, dipicolinic acid forms complexes with the ions and is synthesized during sporulation, along with increased uptake of calcium ions (Fritsche, 2002). A decreased concentration of this molecule leads to an increase in free water, as described above, in the spore core and consequently leads to reduced resistance of the spores to hydrogen peroxide and heat. This, in turn, leads to increased enzyme activity, which results in an unstable spore state (Atrih and Foster, 2002). However, a high concentration of dipicolinic acid causes spores to become more sensitive to UV radiation (Leggett et al., 2012).

SASPs and DNA protection

DNA protection follows two principles: Repair of any damage during spore germination and prevention of DNA damage in the first place (Leggett et al., 2012; Setlow, 1995). For the latter, the protection of DNA, the spore contains so-called SASPs (small acid-soluble proteins, which are exclusively found in spores, and their formation occurs in the late stage of sporulation. SASPs occur in two forms, the α/β-form and the γ-form, with the γ-form not affecting resistance. The α/β-form binds to DNA and has a high proportion of hydrophobic amino acids. The low water content also aids the binding of SASPs to DNA in the core of the spore, as the binding affinity is not very high. Particularly by shielding the DNA backbone, the proteins protect DNA from, for example,

hydroxyl radicals and other chemicals that damage genetic material. They also increase resistance to restriction enzymes, endonucleases, and some physical influences such as heat (Leggett et al., 2012; Setlow, 1995). During germination, SASPs detach from DNA with increasing water content (Setlow, 2014). They are degraded again and serve as a source of amino acids for the germinating cell (Setlow, 1995).

Table 1 summarizes the most important protective factors against sporicidal influences.

Table 1. Sporicidal influences and protective factors of the spore (adapted from Setlow, 2014).

Type of sporicidal influence	Protective factor
Oxidizing agents	α/β-SASPs; detoxifying enzymes in the outer layers of the spore; spore coat protein; low permeability of the inner membrane;
UV radiation	α/β-SASPs; low water content in the spores' core; carotenoids in the outer layers of the spore; DNA repair during germination;
Desiccation	α/β-SASPs; dipicolinic acid;
Dry heat	α/β-SASPs; DNA repair during germination; dipicolinic acid;
Wet heat	α/β-SASPs; dipicolinic acid; low water content in the spores' core; divalent metal ion content;

1.4 Biological indicators

Using biological indicators such as *Bacillus atrophaeus* and *Geobacillus stearothermophilus* provides a direct measure of the effectiveness of the sterilization process. These microorganisms are exposed to the sterilization process in the same manner as the product or packaging. Any surviving microorganisms indicate that the method was ineffective in eliminating all microorganisms. This information is critical in determining whether the sterilization process is effective and whether it requires any modifications.

The standard DIN EN ISO 11138 (parts 1-5) describes bioindicators for hot air, ethylene oxide, steam, and LTSF (low-temperature-steam-formaldehyde) sterilization pro-

cesses. However, the standard does not include bioindicator specifications for sterilization with hydrogen peroxide. DIN EN ISO 11138-1 sets the following requirements for bioindicators:

- Bioindicators must be packaged in an envelope that prevents recontamination by the ingress of germs but needs to be permeable to the sterilant.

- The type of indicator with batch number must be indicated on the bioindicator wrapping. In addition, the bioindicator must be accompanied by a certificate stating the shelf life, germ concentration, and resistance (D-value) to the sterilizing agent.

For the specification of the resistance of bioindicators, usually, the D-value (decimal reduction time) is used. It indicates the time at a defined sterilization temperature of the respective sterilization process required to reduce the test germ load by one power of ten, i.e., by 90%. This means the D-value is specific for the respective test germ, considering the sterilization process and conditions. The killing kinetics follows a 1st-order exponential function. However, contrary to DIN EN ISO 11138-1, no standardized resistance values are given for sterilization validation with hydrogen peroxide for the bioindicators that have been commercially available to date. Additionally, no standard is yet available for D-value determination with hydrogen peroxide, i.e., there is no standardized test procedure for resistance determination.

Bioindicators are commercially available in different variants depending on the sterilization process (according to product research at GKE-GmbH, Waldems, Germany; Mesa Laboratories Inc., Lakewood, USA; STERIS Deutschland GmbH, Köln, Germany):

Self-contained bioindicators

So-called self-contained biological indicators (SCBI) contain spores immobilized on paper carriers, glass fiber carriers, Tyvek carriers, or stainless-steel carriers with nutrient medium and pH indicators. They are subjected to the sterilization process as a closed ampoule and then cultured at the optimal temperature for the test germ. If surviving spores germinate during this period, the color of the pH indicator contained in the vial changes due to the metabolites produced by the vegetative cell form. If there is no color change, the sterilization test is considered to have been passed. Depending on the sterilization process, e.g., with formaldehyde, a neutralizing agent is also added to the nutrient medium to enable incubation of the bioindicator immediately after the

sterilization process without the need for prior neutralization with Na_2SO_4, according to DIN EN ISO 11138-5.

Instant SCBIs

Instant SCBIs are a special form of SCBIs. They can be used in steam sterilization and contain nutrient medium and pH indicator in addition to the spores. This chemical indicator shows the test result directly at the end of the sterilization process. This means waiting for the spores to incubate before releasing them is no longer necessary.

Suspensions

In addition to the self-contained bioindicators, there are test germ suspensions, mostly in ethanol or water. With these suspensions, quantities of the test germs can be transferred directly to packaging materials or carrier materials.

Direct inoculation syringes

For sterilization testing of complex instruments or items, it is sometimes necessary to deposit test germs at undercuts or hard-to-reach positions in items or instruments to check whether sterilization also occurs reliably at these positions. For this purpose, bioindicator suspensions are available in ready-to-use syringes with a long cannula for direct injection.

Ampoules

For applications where SCBIs cannot be used, e.g., in highly humid environments such as steam sterilization or in liquids, sealed glass ampoules are available. They contain a suspension of test germ and nutrient medium with a pH indicator. The evaluation is similar to that of SCBIs.

Spore discs

These bioindicators are small round carrier materials made of glass fiber, Tyvek, PET, or stainless-steel on which a defined quantity of spores has been immobilized. They are positioned at a site to be tested and then subjected to the sterilization process. Subsequently, the carriers are transferred to a nutrient medium with a pH indicator and incubated. They are used, for example, for monitoring containers or in particular test specimens without loss of sensitivity.

Spore strips

Similar to the spore discs, the test germs in this type of bioindicator are immobilized on carrier strips made of filter paper, PET, glass fiber, stainless-steel, or Tyvek. The application and evaluation are analogous to the spore discs. These spore strips are also called "ribbons", depending on the manufacturer.

1.5 Current challenges in the validation

One of the challenges with validating sterilization processes using hydrogen peroxide is the lack of standardization of biological indicators (BIs). As described above, DIN EN ISO 11138 lacks a standard for sterilization with hydrogen peroxide. Additionally, there is no standardized test procedure for determining the resistance (D-value) of the biological indicators, meaning that the commercially available BIs against hydrogen peroxide have an undefined resistance to the sterilization agent.

The undefined resistance of biological indicators used in hydrogen peroxide sterilization validation tests can have significant implications for the results of the tests. Because the resistance of BIs to hydrogen peroxide can vary based on a number of factors, including sterilant concentration, exposure time, spore carrier material, and other factors, it can be difficult to determine the efficacy of the sterilization process. This can lead to inconsistencies in validation results and make comparing results between different laboratories and facilities challenging.

For example, false-positive validations results would lead to non-sterile products in regular production, reduced shelf-live and, as the worst possible consequence, the risk for the consumer or patient. In contrast, false-negative test results would lead to the wrong assumption that sterilization was insufficient. This, however, is not as dangerous as false-positive results but would trigger harsher sterilization conditions, such as increased sterilant concentration or sterilization temperature, inducing over-processing, higher environmental impact, and lower economic efficiency.

As mentioned, there have long been standards for sterilization processes such as ethylene oxide, steam sterilization, or formaldehyde, but not for hydrogen peroxide sterilization processes. The reason for the lack of such standards for a sterilization process that has been in widespread use for decades is a mystery, but it is high time standards were established here as well.

To standardize the production of BI, it is necessary to identify which factors influence the resistance or "inactivability" of BI. In my opinion, it is helpful to distinguish between the terms "resistance" and "inactivability" in this context. Although they mean the same thing in the first sense, they differ on closer examination. By resistance is meant the resistance of the spores themselves, of which the BI consists of the main part. However, the spores provide the resistance of the BI to some extent but do not account for it in full. The term "inactivability" completes this gap. In addition to the resistance of the spores themselves, other factors also influence the BI's overall resistance (= inactivability). On the one hand, the medium in which the spores are suspended or, depending on the BI type, the medium with which the spores were immobilized on a carrier must be mentioned. My experiments (Stier and Kulozik, 2020b) have shown that, depending on the immobilization medium, residues such as salts or surfactants remain on the

spores, which cause agglomerates and could physically protect the spores from contact with sterilization agents. Agglomeration also poses a problem for inactivability, as internal spores of these "spore clumps" are protected from the sterilant by the outer spore layers.

Figure 4 shows *B. atrophaeus* spores immobilized with Ringer's solution on cardboard composite material, which, e.g., is used as packaging for UHT milk. Due to the lower magnification compared to Figure 5, the individual spores can only be poorly recognized. This figure shows a spore cluster (agglomerate) consisting of several hundred spores. Salt crystals can be seen lying on top.

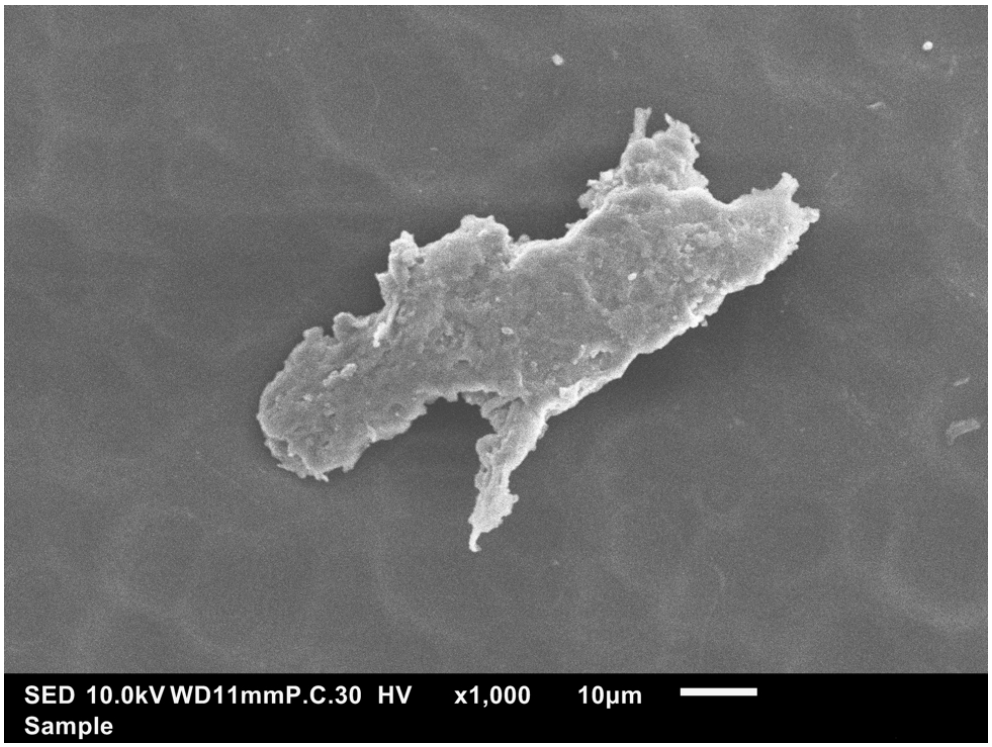


Figure 4. SEM (scanning electron microscope, SED = secondary electron detector) image (1,000X) of a spore agglomerate consisting of several hundred immobilized spores of *Bacillus atrophaeus* on cardboard composite with Ringer's solution as application medium (from Stier and Kulozik, 2020b).

Figure 5 also shows *B. atrophaeus* spores immobilized on cardboard composite material with 0.1% Tween 80 solution as immobilization medium. Spore agglomerates can also be seen here. In between are surfactant residues that hold the agglomerates together and partially overlay the spores.

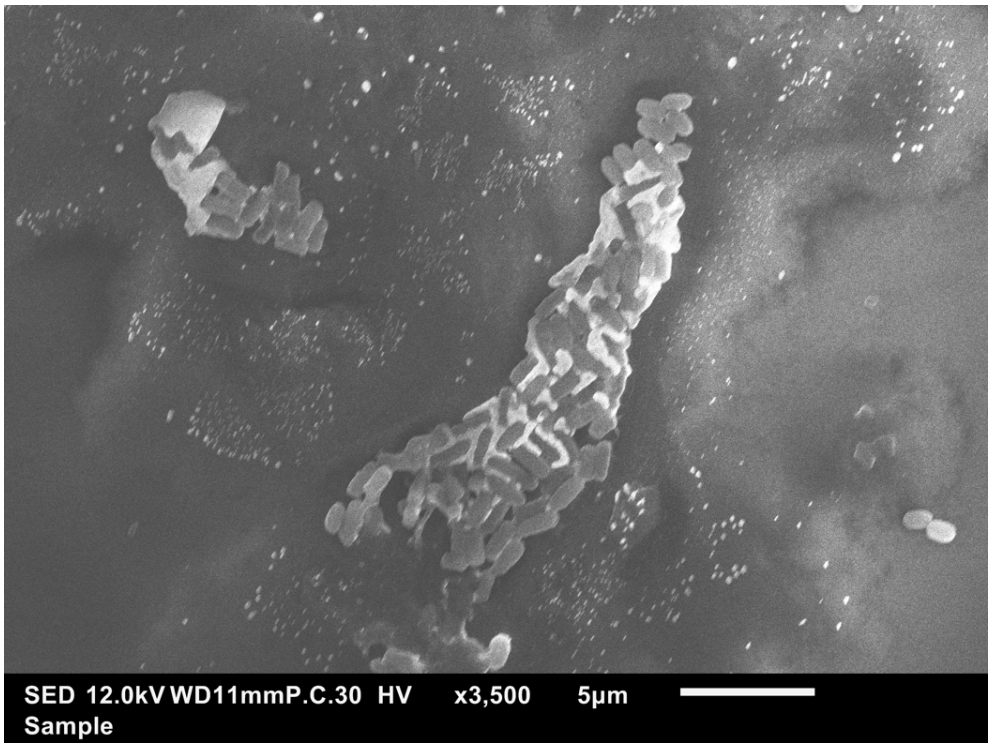


Figure 5. SEM (scanning electron microscope, SED = secondary electron detector) image (3,500X) of two spore agglomerates consisting of immobilized spores of *Bacillus atrophaeus* on cardboard composite with 0.1% Tween 80 / water suspension as application medium (from Stier and Kulozik, 2020b).

The carrier material, as my experiments have shown (Stier and Kulozik, 2020b), also significantly influences the inactivability of the applied spores. The tests showed that spores immobilized on cardboard composite material could be inactivated more poorly than spores of the same batch and resistance to polypropylene (PP) as a carrier.

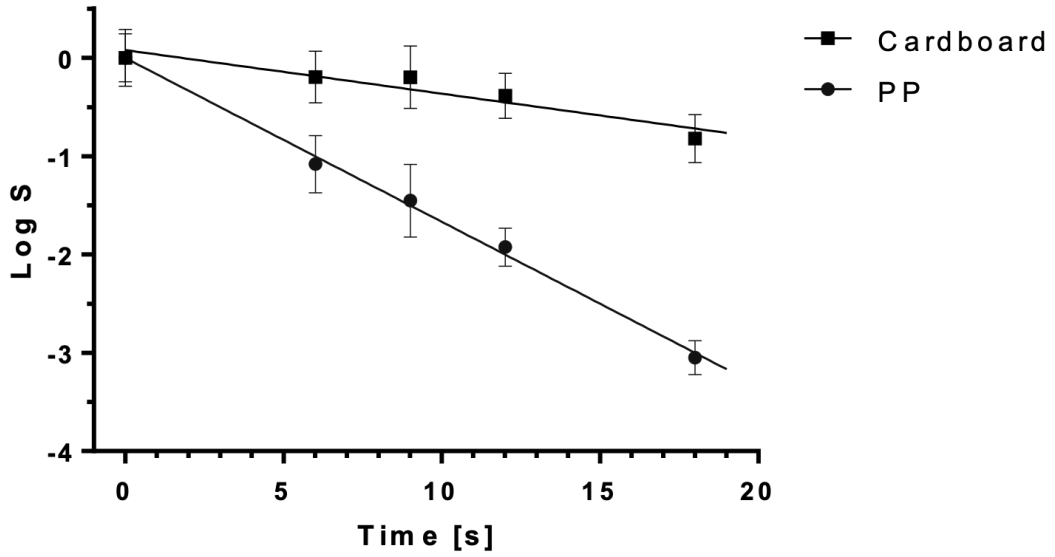


Figure 6. Comparison of the inactivation curves of *Bacillus atrophaeus* spores with gaseous hydrogen peroxide on cardboard composite material and polypropylene (PP) under the same sterilization conditions (70 °C, 4152 ppm gaseous H_2O_2) (modified from Stier and Kulozik, 2020b).

Nevertheless, above all, the resistance of the spores is crucial. It is widely known that sporulation conditions, in particular, exert a strong influence on the resistance of the spores. Nevertheless, there are also other influencing factors here, as explained in the next chapter.

1.6 Resistance influencing factors of bacterial spores

The resistance of spores is subject to many influencing factors. Especially the sporulation conditions exert a significant influence on the resistance of the spores. These include the sporulation temperature, the sporulation pH, the composition of the sporulation medium, and the method used for spore production (submerged production in shake flasks or bioreactors or solid-state production on agar plates). In addition, there are other possible influencing factors, such as the storage conditions or the purification method of the spores after their sporulation. Figure 7 shows the relationships between the factors influencing resistance. Since the previous chapter already discussed some factors influencing BI resistance (inactivability), this section addresses the factors influencing spore resistance since this was the focus of this research.

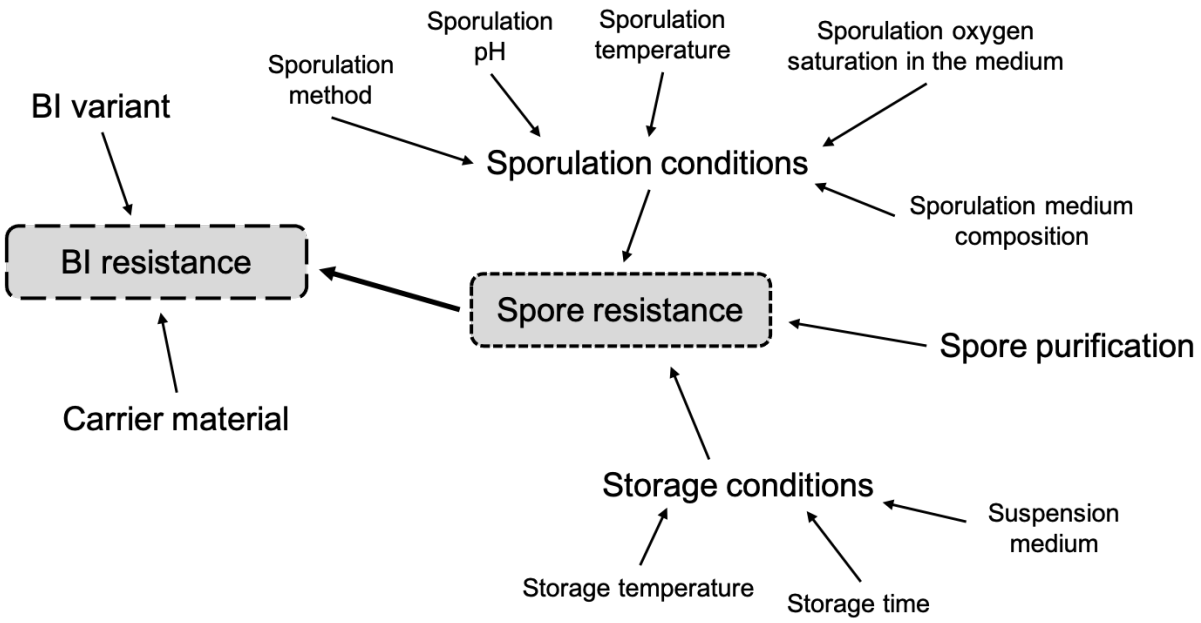


Figure 7. Possible factors influencing spore resistance and bioindicator resistance.

Sporulation conditions

Sporulation conditions directly influence the resistance of spores. They also represent the main influencing factors. Much of the research on spore resistance has focused on the influence of sporulation temperature on spore resistance to heat. Several publications found that heat resistance increased with increasing sporulation temperature (Beaman and Gerhardt, 1986; Condon et al., 1992; Lechowich and Ordal, 1962; Leguérinel et al., 2007; Palop et al., 1999). Cortezzo and Setlow (2005) and Melly et al. (2002) studied sporulation temperature's effect on spores' chemical resistance. They found that higher sporulation temperature also increased chemical resistance.

Sporulation pH also significantly affects resistance, according to Nguyen Thi Minh et al. (2011). For *B. subtilis* spores, they found that a pH of 10 produced higher resistance to heat than spores produced at a pH of 6. These findings were complemented by Guizelini et al. (2012), who found a quadratic dependence of spore resistance of *G. stearothermophilus* to heat on sporulation pH. The spores' resistance was greatest at a pH of 8.5 and decreased to pH values of 7.8 and 9.2.

The effect of oxygen saturation in the medium on spore resistance has not been previously studied (except for this present work) after a thorough literature search. In the case of solid-state production on agar plates, this concerns the oxygen content in the surrounding air. Since controlling this value is complex and cost-intensive in this manufacturing process, this influencing factor is not a useful control variable in commercial BI manufacturing. However, in the submerged production of spores for use as BI, it is

a crucial variable in the cultivation of the cells and, therefore, also represents a potential influencing factor on the resistance of the spores. In general, these two different production methods also impact spore resistance. In the submerged method, the cells are present individually or in very short cell strands due to the vigorous mixing of the medium. As a result, the sporulation conditions uniformly affect the cells. With this method, the sporulation conditions can also be controlled in a more targeted manner; as already mentioned, the oxygen saturation can be controlled, as can the sporulation temperature and the sporulation pH. In contrast, the sporulation pH cannot be actively controlled in the solid-state process. It can be initially adjusted during the preparation of the agar, but during the growth of the cells, the pH can change due to cell metabolism and cannot be actively adjusted. For this reason, the exact sporulation pH, i.e., the pH present when cells begin to sporulate, is unknown. In addition, in the solid-state procedure, the cells differentiate. For *B. subtilis*, it is known for agar colonies that the cells differentiate into so-called matrix-formers as well as motile cells, among others (Vlamakis et al., 2008). Due to this heterogeneity of the cell colony, part of the cells is already sporulating while another part of the population is still in vegetative cell growth. Thus, the cells have different statuses and may sporulate under other sporulation conditions.

The media composition also plays a role in the formation of spore resistance. Here, the presence of different ions is particularly important. Manganese, iron, and zinc accelerate and enhance sporulation, and manganese is even essential for the artificial generation of spores. Calcium is known to increase the heat resistance of spores and is used by cells in the formation of dipicolinic acid (Charney et al., 1951; Purohit et al., 2010; Scribner et al., 1974; Yamazaki et al., 1997).

Spore purification

Spore purification primarily aims to remove media residues, cell debris from the mother cells and mother cells containing endospores. Media residues and cell debris could still contain nutrients or triggers after the sporulation of the cells, which could lead to the re-germination of the spores during storage. This way, the resistance would be lost. In addition, the protein and lipid residues of the cell debris as well as the mother cells containing endospores, could affect the sterilization process. Figure 8 shows an example of purification of submerged produced *B. atrophaeus* spores. After centrifugation of the spore suspension, the components arranged in three phases in the pellet become visible.

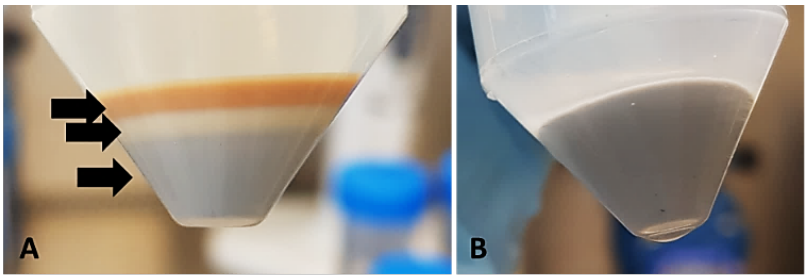


Figure 8. *B. atrophaeus* spore suspension after centrifugation. **A:** Three phases become visible in the pellet (arrows). The top phase contains cell debris, the middle phase contains mother cells containing endospores, and the bottom phase contains free spores. **B:** The sporulation medium and the top two phases were removed by several purification steps. The remaining free spores can be resuspended in the storage medium (from Stier and Kulozik, 2020b).

Figure 9 illustrates the importance of efficient purification of the spores. Under the phase-contrast microscope, the cell debris removed by purification is clearly visible.

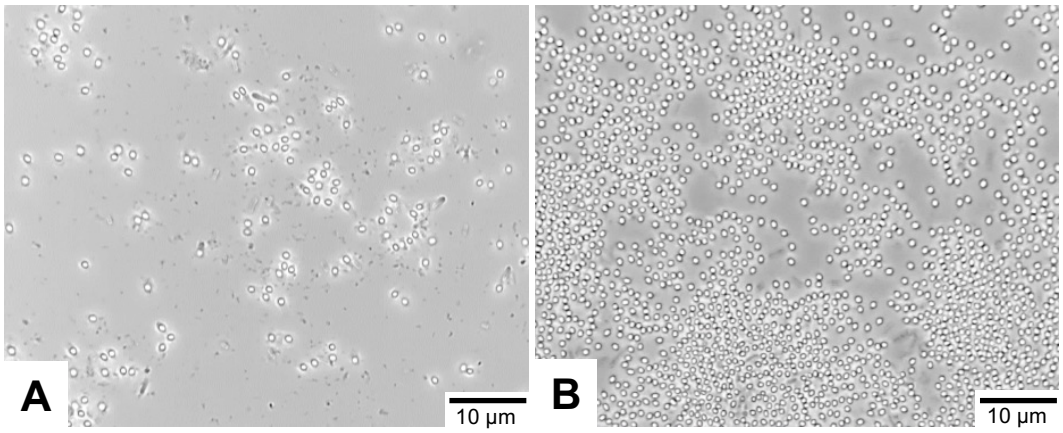


Figure 9. Phase contrast microscopy (100X) of *B. atrophaeus* spores. **A:** Spore suspension before purification. **B:** Spore suspension after purification (from Stier and Kulozik, 2020b).

Storage conditions

So far, there are no publications dealing with the storage conditions of spores and their influence on resistance. However, in my experiments (data in Stier and Kulozik, 2020b), I could find that the resistance of spores changes depending on the storage medium and storage time. Saline storage media such as Ringer's solution or phosphate buffer significantly increase resistance over the storage period. In water and

ethanolic solution, however, the resistance remains stable. Nevertheless, storage of the spores in ethanolic solution leads to increased agglomerate formation, which in turn can influence the inactivability of the spores, as already explained.

1.7 Resistance determination

As mentioned, the D-value (Decimal Reduction Time) serves as a measure of the resistance of bacterial spores. It indicates the time in seconds, or sometimes in minutes, in which the spore population is reduced by 1 log, i.e., by 90%, by inactivation at a defined temperature and concentration of the sterilizing agent.

To determine the D-value, the spore suspension to be measured must therefore be inactivated under defined conditions (this is referred to as a count reduction test). However, there is no standardized norm for determining resistance with hydrogen peroxide to date. In addition, it makes a difference whether the spores are treated with liquid hydrogen peroxide or inactivated with gaseous hydrogen peroxide. A secondary objective of this work was, therefore, also to provide a basis for standardizing the determination of resistance with hydrogen peroxide. Thus, special requirements were placed on the resistance test to ensure uniform measurement conditions. On the one hand, the D-value is only valid for the respective inactivation conditions, so the conditions must be kept reliably stable. Secondly, the spore suspensions to be measured exhibit varying degrees of resistance. Due to the possibly significant differences in the resistance of the spore suspensions, test conditions were necessary with which very resistant but also more sensitive spores could be measured simultaneously. This is particularly necessary for standardization, as spore resistances of different manufacturers and batches would otherwise not be comparable.

In the course of this research, spores were treated with both gaseous and liquid hydrogen peroxide. This was necessary to determine the resistance of the spores produced under different sporulation conditions and, thus, to conclude their influence on the resistance of the spores. Since the publications described here exclusively contain the determination of resistance with liquid hydrogen peroxide, this chapter concentrates on this measuring method. Nevertheless, the basics of determining gaseous hydrogen peroxide are also presented in the following to illustrate the differences between the two measurement methods.

Determination of resistance with liquid hydrogen peroxide

In the course of this research work, several trials of a test set-up were carried out to be able to detect a broad spectrum of spore resistances with the same test for the desired comparability (Figure 10).

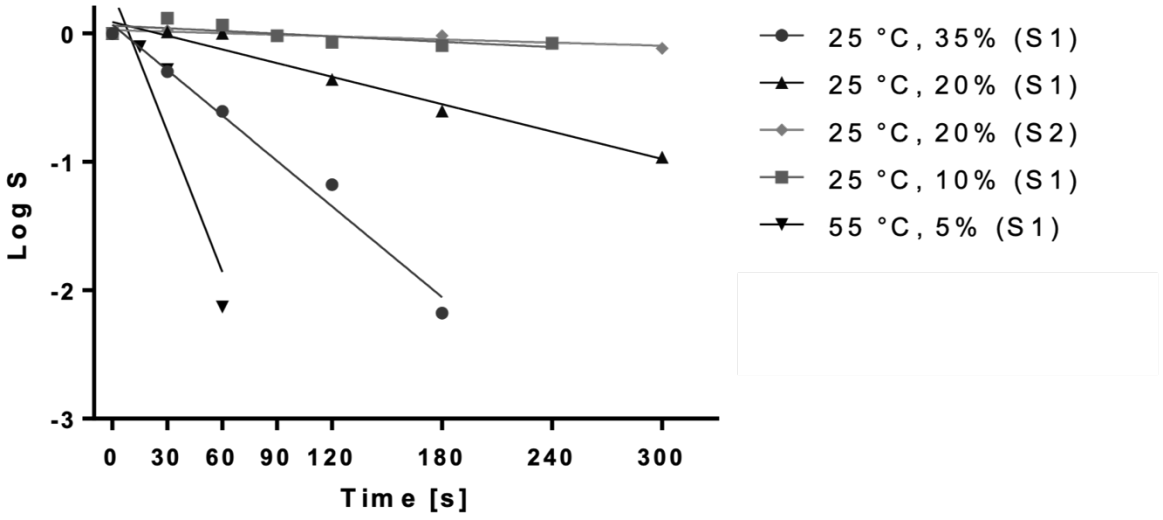


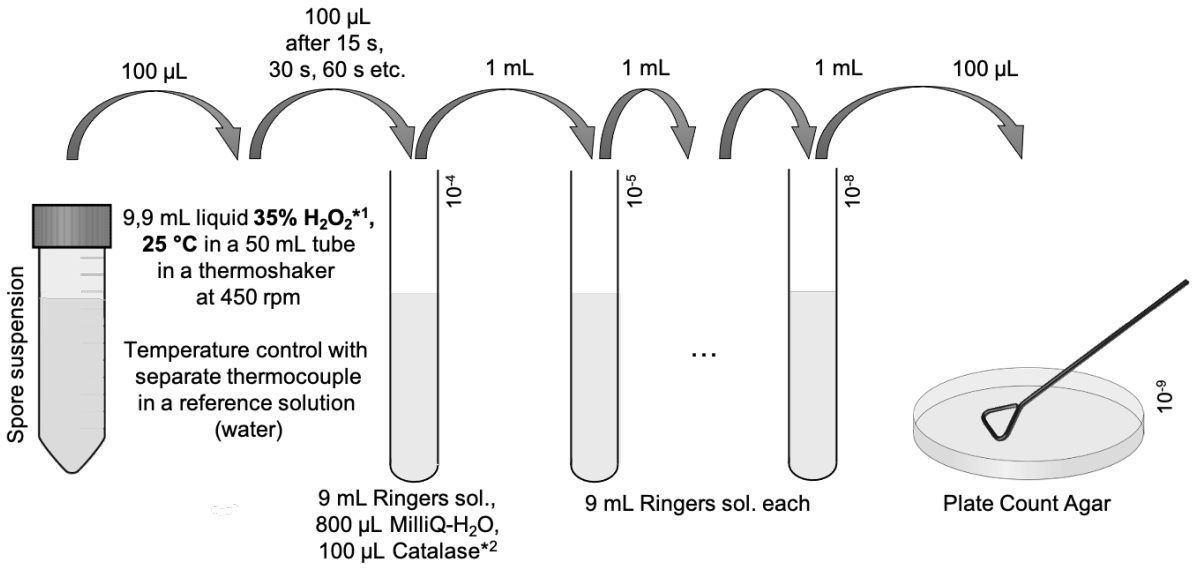
Figure 10. Inactivation of two spore suspensions with different resistances under different sterilization conditions (hydrogen peroxide concentration and temperature). S1 = spore suspension 1, S2 = spore suspension 2 (modified from Stier and Kulozik, 2020b).

Figure 10 shows two spore suspensions with different resistance (S1, less resistant, and S2, more resistant). Spore suspension 1 was inactivated at different hydrogen peroxide concentrations and temperatures. It was found that the inactivation processes differed considerably at the same temperature but at different hydrogen peroxide concentrations. The rate of inactivation increased with increasing hydrogen peroxide concentration and temperature. At 55 °C and a hydrogen peroxide concentration of 5%, no more spores could be detected in the suspension after only 90 s of measurement. Furthermore, measurements were also carried out at 55 °C with a hydrogen peroxide concentration of 10, 20, and 25%. In these measurements, however, the inactivation processes were so rapid that the spore concentrations were below the detection limit, and thus no resistance determination was possible. In general, the measurement of a longer inactivation course should be aimed at, as this makes the measurement method more robust and the result more repeatable, since the spore concentration remains above the detection limit for longer and therefore, the D-value is based on the data of as many measurement points as possible.

For this reason, further resistance measurements were performed at room temperature, 25 °C. However, at a hydrogen peroxide concentration of 10% at this inactivation temperature, spore suspension 1 was not significantly inactivated. Increasing the hydrogen peroxide concentration to 20% provided a targeted inactivation curve over

300 s with a population reduction of approximately 1 log, the minimum targeted population reduction during the resistance assay. However, a comparison measurement with the more resistant spore suspension 2 showed that more resistant spore suspensions were not significantly inactivated under these conditions. Finally, a hydrogen peroxide concentration of 35% and a temperature of 25 °C proved suitable.

Figure 11 shows the experimental setup of the established resistance test.



*1 OXTERIL® 350 Spray, Food Grade, Evonik Industries AG, Essen, Germany
*2 1:5 Catalase from *Micrococcus lysodeiktikus*, solution, activity 65,000−150,000 U/mL, Merck KGaA, Darmstadt, Germany

Figure 11. Experimental setup of the established resistance determination of bacterial spores in liquid hydrogen peroxide (35%) at 25 °C (modified from Stier and Kulozik, 2020b).

For resistance determination, 100 µL of spore suspension was transferred to 9.9 mL liquid 35% hydrogen peroxide at 25 °C. At defined times, a sample was taken and transferred to a solution of Ringer's solution, MilliQ-water, and catalase. Catalase stops hydrogen peroxide inactivation by enzymatic degradation. The catalase concentration was selected so that the hydrogen peroxide was inactivated as quickly as possible, but the solution did not yet foam over due to the enzymatic reaction. A dilution series was subsequently prepared, and the spores were plated on Plate Count Agar. The agar plates were incubated for 48 h at 30 °C for *B. atrophaeus* and at 57 °C for *G. stearothermophilus*. The colony-forming units were determined after 24 and 48 h in each case (counting after 24 h already took place to be able to determine colonies growing

into each other). Finally, the inactivation curve could be derived from the residual bacterial count of the respective exposure time, and the D-value was determined from the negative reciprocal slope of a best-fit straight line through the data points.

Resistance determination with gaseous hydrogen peroxide

In contrast to the resistance determination of bacterial spores in liquid hydrogen peroxide, the resistance determination with gaseous hydrogen peroxide was not carried out with a spore suspension but with spores immobilized on a carrier.

The carrier was cardboard composite material, as used for packaging UHT milk. The carrier thus represented a typical industrial packaging material. As mentioned, the carrier material can significantly influence the inactivability of the spores, respectively, the BI. For this reason, the carrier should represent the material also sterilized in the real sterilization process to have the most tremendous significance. Figure 12 schematically shows the structure of the carrier material.

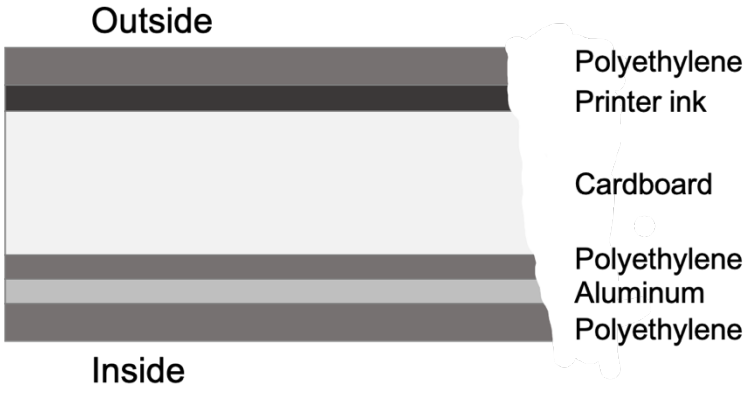


Figure 12. Schematical structure of cardboard composite material (modified from Krämer, 2007).

The cardboard composite material is composed of several layers. The aluminum layer forms a barrier against gas permeation. It is bonded to the cardboard via a polyethylene adhesion layer. The cardboard content is about 70% and is responsible for the necessary stability. Inside and outside, the packaging material is coated with polyethylene. This provides good durability against chemicals and also serves as a barrier against the penetration of microorganisms (Krämer, 2007).

On this carrier of 47 x 37 mm, 10 µL of a 70% ethanolic spore suspension was applied with a Drigalski spatula according to the spatula method established by Pruß (2013), following the scheme shown in Figure 13. The drying time was 1 h at room temperature under a laminar flow.

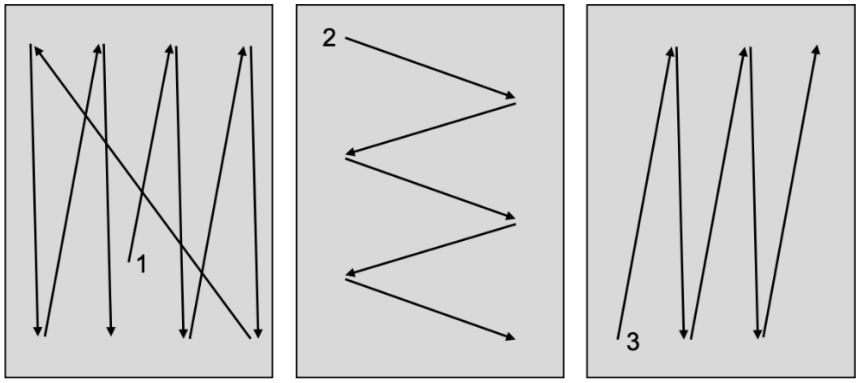


Figure 13. Application scheme according to the spatula method established by Pruß (2013). With the Drigaslki spatula, the spore suspension is spread over the carrier in three sections (1, 2, 3) according to the scheme (modified from Pruß, 2013).

The experimental plant, which was used to gasify the carrier with hydrogen peroxide, is shown schematically in Figure 14. The aim was to make gaseous hydrogen peroxide available and achieve condensate-free carrier loading (VHP).

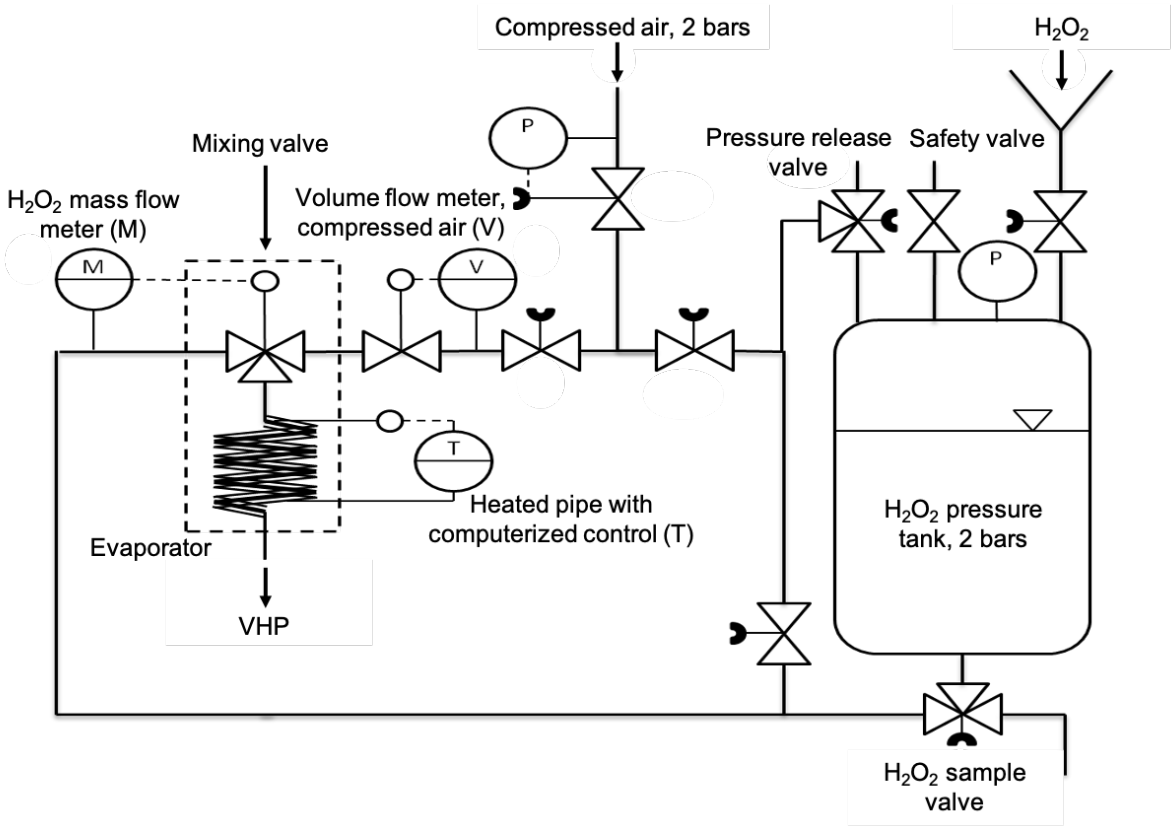


Figure 14. Schematic diagram of the experimental plant for the generation of VHP (modified from Pruß, 2013).

Hydrogen peroxide was fed into a pressure tank with an applied pressure of 2 bars. The mixing valve of the evaporation unit ensured fine spraying of the hydrogen peroxide together with oil-free, dry compressed air, resulting in complete evaporation of the hydrogen peroxide in the heated pipe section. A computerized control unit adjusted the evaporation temperature. The gas mixture flowed to the treatment chamber shown in Figure 15. The treatment chamber was surrounded by a stainless-steel tube in which hot air flowed to prevent condensation of the gas mixture and to keep the temperature of the treatment chamber constant (Pruß, 2013).

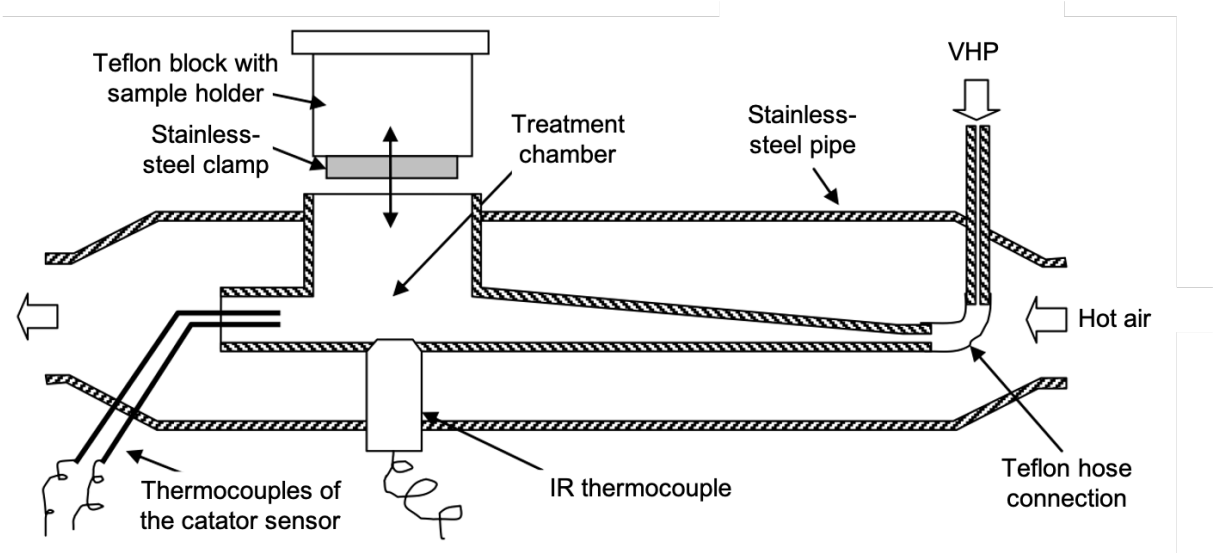


Figure 15. Schematic diagram of the treatment chamber (side view) (modified from Pruß, 2013).

An infrared thermocouple was located in the treatment chamber. At the top of the chamber was an opening through which the contaminated carrier was inserted. For this purpose, the carrier was attached to a Teflon block which only touched the long sides of the carrier so that no contaminated areas were covered. By inserting the Teflon block, the treatment chamber was closed entirely. Behind the treatment chamber, two thermocouples of the catator sensor were mounted to measure the hydrogen peroxide concentration. One of these sensors was a stainless-steel sheathed thermocouple that measured the gas temperature. The other was coated with a special catalytically active layer, on the surface of which hydrogen peroxide decomposed and released energy. Thus, the temperature of the second thermocouple was slightly higher than that of the first in the presence of hydrogen peroxide (Pruß, 2013).

The resistance determination procedure was similar to that in liquid hydrogen peroxide (Figure 16).

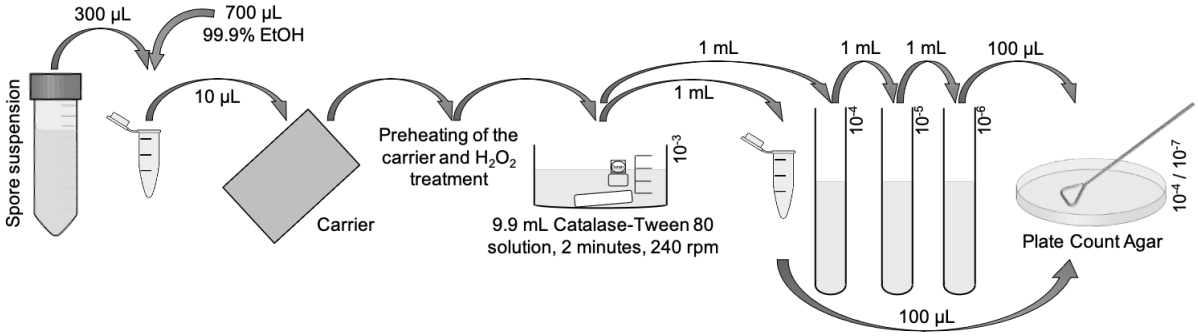


Figure 16. Experimental setup of the resistance determination of bacterial spores in gaseous hydrogen peroxide (modified from Stier and Kulozik, 2020b).

The contaminated carrier was dry preheated to 85 °C under sterile conditions to prevent condensate formation on the carrier when treated with gaseous hydrogen peroxide. It was then transferred to the treatment chamber and gassed with industry-standard hydrogen peroxide concentrations of 4152, 5505, or 6843 ppm at 40, 50, or 65 °C. Lower temperatures would have promoted undesirable condensate formation. The odd hydrogen peroxide concentrations in ppm were obtained by converting the adjustable set point in percent (9%, 12%, 15%; percentage of power input) to the gas concentration. The carrier was then removed from the treatment chamber and transferred to a Catalase-Tween 80 solution. The catalase led to an enzymatic decomposition of the hydrogen peroxide and, thus, stopped the inactivation reaction. With the surface-active polysorbates of Tween 80 and the mechanical stress of stirring in the solution, spores were detached from the sample. Finally, a sample was taken from the solution and diluted in the same way as for the resistance determination with liquid hydrogen peroxide, plated out on Plate Count Agar, and the D-value was determined.

Both methods can be used to determine the resistance of the spores reliably. However, it should be noted that the former method, resistance determination with liquid hydrogen peroxide, measures pure spore resistance. In the resistance determination with gaseous hydrogen peroxide, on the other hand, the carrier material influences the inactivability of the spores. The inactivation kinetics of the two methods are also different, which means that the resistance between the two methods cannot be directly compared or weighted. In addition, the concentrations of gaseous and liquid hydrogen peroxide (20% liquid hydrogen peroxide corresponds to 20,000 ppm) cannot be equated because this type of concentration indication cannot be correlated across different aggregate states. Apart from this, there is even the suspicion that resistance series of

differently resistant spore suspensions can be reversed, i.e., spores sensitive to liquid hydrogen peroxide suddenly show a higher resistance than other spore suspensions. However, this was not investigated in this work.

For the reasons mentioned above, the determination of resistance with liquid hydrogen peroxide was used in this research work to exclude any influences of a carrier material and limitations from the methodology of gaseous inactivation. Determining the resistance with gaseous hydrogen peroxide only makes sense if the real sterilization method is also carried out accordingly, and one wants to measure the resistance of the spores specifically to this method. Otherwise, the advantages of resistance determination with liquid hydrogen peroxide outweigh.

1.8 Resistance variability

In addition to the standardization of spore production, the lowest possible resistance variability is crucial for consistent and reliable validation results. Commercial BI differ in their resistance depending on the manufacturer. This also applies to available spore suspensions so that the mentioned influencing factors on BI resistance, such as the influence of the carrier material in the case of immobilized spores, do not come into play, but rather the spore resistance differs directly. The spore resistance varies in part, even with spores from the same manufacturer of different batches.

This leads to the fact that validation results are no longer only dependent on sufficient sterilization conditions but also the test germs. BI that are too resistant lead to the validation not being passed and the sterilization conditions being tightened, although they would be sufficient if less resistant BI were used, i.e., in general production sterilization. BI with low resistance, on the other hand, lead to the validation being passed, although the sterilization conditions are inadequate for the real case. Therefore, the lowest possible resistance variability should be aimed for by standardizing the manufacturing conditions.

2 Objective and outline

As explained in the introduction, BI produced from spores of *B. atrophaeus* and *G. stearothermophilus* play an essential role in validating sterilization processes with hydrogen peroxide. The resistance of BI affects the sterilization result and leads to different validation results under uniform sterilization conditions for BI with different resistance. This can lead to validation runs not passing and, thus, the sterilization conditions being questioned. In conclusion, this leads to unnecessary uncertainty and associated tightening sterilization conditions with increased cost, material, time, and additional environmental impact.

Sterilization validation is crucial for the reliability of sterilization processes and the safety of patients and consumers. Any uncertainties must therefore be avoided. As there is currently no standardized production of BI and no standardized resistance of BI, there is still a gap to be filled, despite the knowledge of the current difficulties on this topic of all stakeholders.

Based on this situation, the main objective of the work presented was to generate knowledge based on which standardization of the BI manufacturing process can be derived. On the one hand, this included determining which factors significantly influence the resistance of bacterial spores and how these can be controlled to achieve consistent spore resistance to hydrogen peroxide. The aim was to investigate which resistances are achieved under which production conditions, the interdependencies between the factors influencing spore resistance during spore production, i.e., whether these factors influence each other, and how resistance variability can be reduced as far as possible with the focus on industrial BI production. On the other hand, the aim was to establish a production method attractive to industry, with which spores can be produced with as little effort as possible and simultaneously have a resistance after production within the range of currently available and established BI. This is important not only for BI manufacturers, who would otherwise have to fear a loss of customers if the resistance of the spores produced by a new method deviates greatly from the previous resistance, but also for the end customers, who would otherwise have to fear a failure of validation runs.

In addition to imparting knowledge to BI manufacturers, the work shown here is also intended to impart knowledge to BI-using end customers. This should enable customers better to interpret the reasons for deviations in validation results and, secondly, point out the current situation. With the data conveyed here, customers could make informed claims to BI vendors and thereby accelerate the standardization of BI.

Finally, this work should lay the necessary foundation for a rethinking of the manufacturing industry and users of BI to permanently reduce future uncertainties in the validation of sterilization processes using hydrogen peroxide with *B. atrophaeus* and *G. stearothermophilus* BI and thereby make validation results more reliable.

Therefore, the effect of sporulation conditions of submerged spores of *B. atrophaeus* on resistance to hydrogen peroxide was first investigated. The aim was to examine the influence of the main factors during submerged production (sporulation temperature, sporulation pH, and minimum oxygen saturation of the medium) on the resistance of generated spores. With these results, it could be shown how and to what extent the spore resistance depends on the influencing factors and how the resistance can be specifically controlled during production to obtain consistent resistance.

When it was known from the data of the first part how the spore resistance of *B. atrophaeus* to hydrogen peroxide depends on the sporulation conditions, the influence of the sporulation conditions on the spore resistance to hydrogen peroxide of *G. stearothermophilus* was investigated in the second part. In this part, spores were not produced by the submerged method but by the solid-state method on agar plates because, first, the BI-producing industry uses both production methods (submerged and solid-state) and, second, a method for submerged production of spores was not yet available for the production of *G. stearothermophilus* spores.

Since spores from solid-state production were reasonably suspected of exhibiting more significant resistance variability due to less controllable sporulation conditions, a method for submerged production of *G. stearothermophilus* spores for use as BI against hydrogen peroxide was subsequently developed in the third part.

Finally, in the last part, the possibility of method optimization, i.e., simplification, of submerged production was investigated using the spore production of *B. atrophaeus* as an example. An even further simplified manufacturing process should promote further reasons for adopting the method in industrial BI production, thus leading to a forcing of standardized BI production.

The following chapters are organized chronologically according to the published works. Redundant content, such as references cited more than once, has been restructured. However, the content and results shown here correspond without change to the peer-reviewed publications.

3 Results

3.1 Effect of Sporulation Conditions Following Submerged Cultivation on the Resistance of *Bacillus atrophaeus* Spores against Inactivation by H_2O_2

Summary and contribution of the doctoral candidate

The effect of sporulation conditions on the resistance to hydrogen peroxide of submerged produced spores of *B. atrophaeus* was investigated.

For this purpose, a full factorial design of experiments was developed, based on which the spores were produced in a bioreactor at different sporulation conditions (sporulation temperature of 28-40 °C, sporulation pH of 6.0-8.5 and minimum oxygen saturation in the medium of 10-60%). The produced spores were then analyzed for their resistance (D-value) in liquid 35% hydrogen peroxide at 25 °C. The obtained $D_{25°C,35\%H2O2}$-values lay in between 40 and 197 s. Based on the measured data, a model was developed that depicts the influence of sporulation conditions on resistance, i.e., the dependence from the combination of the different influencing factors on the spores' resistance. With the equation derived from the model, the resistance of the spores could be calculated as a function of the sporulation conditions.

The resistance of the spores depended significantly on the sporulation temperature and the sporulation pH. The oxygen saturation of the medium had no significant effect on the resistance to hydrogen peroxide. The resistance to hydrogen peroxide increased linearly with increasing sporulation temperature. The pH had a quadratic effect on resistance, with resistance being highest at a slightly acidic pH. Sporulation temperature and pH also mutually influence their effect on resistance expression. Thus, sporulation temperature has a strong influence on resistance at acidic pH, but has little to no influence on resistance at a basic pH. With regard to the derived model, the highest theoretically possible resistance with a $D_{25°C,35\%H2O2}$-value of 200 s could be achieved at a sporulation pH of 6.9 and a sporulation temperature of 40 °C.

The results show the extent of the influence of sporulation conditions on resistance. Depending on the constellation or combination of influencing factors and due to their interaction with each other, even small deviations in the sporulation conditions can have a supposedly unintentional, significant influence on the resistance of the spores to hydrogen peroxide.

The doctoral candidate developed the concept of the experiments and established the methods. He critically reviewed the existing literature. Furthermore, he analyzed the data and interpreted the results. Additionally, the doctoral candidate wrote the manuscript and revised it. The co-author contributed to the experimental part and/or to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript [1]

Effect of Sporulation Conditions Following Submerged Cultivation on the Resistance of *Bacillus atrophaeus* Spores against Inactivation by H_2O_2 [2]

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Abstract

The resistance formation of spores in general and of *Bacillus atrophaeus* in particular has long been the focus of science in the bio-defense, pharmaceutical and food industries. In the food industry, it is used as a biological indicator (BI) for the evaluation of the inactivation effects of hydrogen peroxide in processing and end packaging lines' sterilization. Defined BI resistances are critical to avoid false positive and negative tests, which are salient problems due to the variable resistance of currently available commercial BIs. Although spores for use as BIs have been produced for years, little is known about the influence of sporulation conditions on the resistance as a potential source of random variability. This study therefore examines the dependence of spore resistance on the temperature, pH and partial oxygen saturation during submerged production in a bioreactor. For this purpose, spores were produced under different sporulation conditions and their resistance, defined by the D-value, was determined using a count reduction test in tempered 35% liquid hydrogen peroxide. The statistical analysis of the test results shows a quadratic dependence of the resistance on the pH, with the highest D-values at neutral pH. The sporulation temperature has a linear influence on the resistance. The higher the temperature, the higher the D-value. However, these factors interact with each other, which means that the temperature only influences the resistance when the pH is within a certain range. The oxygen partial pressure during sporulation has no significant influence. Based on the data obtained, a model could be developed enabling the resistance of BIs to be calculated, predicted and standardized depending on the sporulation conditions. BI manufacturers could thus produce BIs with defined resistances for the validation of sterilization effects in aseptic packaging/filling lines for the reliable manufacture of shelf-stable and safe food products.

[1] Adaptions refer to formatting, e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units and spelling.

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Keywords

aseptic packaging, *Bacillus atrophaeus*, bioindicators, hydrogen peroxide, product safety, resistance, spores, sporulation, sterilization, shelf life;

3.1.1 Introduction

In the food and pharmaceutical industry, sterility in production and packaging is extremely important in many instances. Pathogenic and spoilage-inducing spore formers can be particularly dangerous, since the spores, because of their high resistance, can survive pasteurization and inadequate sterilization processes and then germinate, thus contaminating the sterile product filled into nonsterile containers. As a result, they either significantly reduce the shelf life of the food or can even be harmful to consumers.

In order to prevent this, the sterility of the production facilities and all the sterilization methods within the aseptic production unit are initially validated and then regularly checked by challenge tests with so-called bioindicators (BI), since natural contaminations are variable and often not high enough to measure the target 5 log reduction effect. These BIs are test organisms, which should have a defined resistance against the respective sterilization process. Certain standard BI strains have been established, and these are routinely applied at the industrial level in validation test runs of filling machines, for instance. These BIs have a high level of resistance against certain inactivation methods using, e.g., H_2O_2, which covers the requirements in specific situations, e.g., in the food industry, filling neutral liquid products, or in the pharmaceutical industry. However, the resistance of commercially available BIs varies considerably.

In the case of sterilization with hydrogen peroxide, a process widely used in the food and pharmaceutical industries, *Bacillus atrophaeus* is used as a standard BI and is recognized as appropriate to cover all sorts of natural contamination. To be used as a BI, the spores of this bacterium are either immobilized on a carrier material or are used suspended in carrier liquids and exposed to the sterilization process. The performance of the sterilization can be assessed via the degree of inactivation of the BI. It is important in such applications that the resistance of the BI is defined and stable. However, in reality, depending on the manufacturer and sometimes differing batch to batch, the resistance of the BI varies. The reason for this is suspected to be that the resistance of spores is influenced by the sporulation conditions - i.e., the conditions during spore production. If the resistance differences of the BI are too large, this can lead to false positive or false negative test results when validating aseptic systems and sterilization processes.

False positive test results falsely indicate a sufficient sterilization effect when in fact this was not the case. The result then is that in regular production, nonsterile products occur at a high level. This can lead to a reduction in shelf life and, in the worst case, a

risk to consumers. False negative test results, on the other hand, indicate that the sterilization was apparently not sufficient. In response to that, the sterilization conditions are tightened, which leads to over-processing, the increased use of sterilization agents, the pollution of the environment and increased costs.

The origin of variable BI resistances has not satisfactorily been addressed in research so far. The reasons are suspected to be related to the conditions during cultivation and especially during sporulation following the growth phase of the BI strain's vegetative cells. A standardization of the production of BI of *B. atrophaeus* could solve this problem, whereby the same resistances would be obtained and uncertainties in the evaluation of challenge tests could thus be avoided.

A standardization of the production requires that the determining factors of influence which possibly affect the resistance of the spores have to be identified. Although *Bacillus* spp. and the formation of spore resistance has long been the focus of the science, bio-defense, pharmaceutical and food industries, little is known about the factors influencing its resistance against inactivation. We noticed that only a few studies in this field stem from the last few years, and some are in fact relatively old. Additionally, most studies deal with factors influencing the heat resistance of the spores. Lechowich and Ordal (1962) produced spores of *B. subtilis* at 30 and 45 °C and *B. coagulans* at 30, 45 and 52 °C. They found that the spores' heat resistance increased with an increasing sporulation temperature. Leguérinel et al. (2007) and Palop et al. (1999) confirm this phenomenon. However, Palop et al. (1999) also state that the magnitude of the effect of the sporulation temperature is not constant - i.e., it is not well enough understood. The dependence of resistance on temperature is not linear. This contradicts Beaman and Gerhardt (1986), who found that the influence of the sporulation temperature for *Bacillus* spp. spores is linear. Condon et al. (1992) found that the resistance of *B. subtilis* spores shows only a partially linear dependence on the sporulation temperature and that the resistance no longer increases above a certain temperature. The pH also influences the heat resistance of spores. Nguyen Thi Minh et al. (2011) produced *B. subtilis* spores at pH 6 and pH 10. They found that the spores produced at pH 10 were more resistant to heat. However, the cell growth was very poor at this pH and only a few spores could be obtained. Guizelini et al. (2012) examined *Geobacillus stearothermophilus* spores for their heat resistance. They found that the resistance follows the quadratic dependence of the pH. Resistance was greatest at a sporulation pH of 8.5 and decreased towards pH 9.2 and 7.8.

Much less, however, is known about the influencing factors during sporulation on the chemical resistance of the spores. Cortezzo and Setlow (2005) found that high sporulation temperatures could increase the chemical resistance of *B. subtilis* spores. However, this depends on the particular chemical. In line with Melly et al. (2002), they found that the spores' resistance to formaldehyde increases with an increasing sporulation temperature. They also obtained these effects regarding the resistance to nitrous acid

and hydrogen peroxide, but the latter only for $\alpha^-\beta^-$ small acid-soluble spore protein (SASP) *B. subtilis* spores. These spores are mutants that lack the genes encoding the two major α/β-type small acid-soluble spore proteins, which are relevant for DNA protection, and therefore they do not have the same protective mechanisms as wild-type spores. With regard to the resistance against ethylmethanesulphonate and methylmethanesulphonate, however, they found no influence of the sporulation temperature.

Since the mechanisms of resistance of the spores to different environmental influences are different (Setlow, 2014), it is not possible to transfer the relationships found for heat resistance and the resistance of other chemicals to the resistance of *B. atrophaeus* wild-type spores against hydrogen peroxide, for which no such study has been reported so far. For this reason, the factors influencing the resistance of *B. atrophaeus* and their effects on resistance to hydrogen peroxide were the subject of research in this study.

The hypothesis was that the influencing factors - pH, temperature and oxygen saturation - interfere, which has not been investigated so far. In the submerged production of spores, these factors, in addition to the medium, have the greatest influence on the cells during cultivation or sporulation. For this purpose, a design of experiments was created in order to be able to make statistically significant statements and strategically examine the factors influencing the resistance. According to this experimental plan, spores of *B. atrophaeus* (ATCC 9372) were produced submerged in a bioreactor under variation in the sporulation conditions (pH, temperature and oxygen saturation), and the resistance was determined as the D-value against 35% liquid hydrogen peroxide at 25 °C. By changing the resistance of the spores depending on the sporulation conditions, conclusions can be drawn about the degree of influence and the type of dependence.

3.1.2 Materials and Methods

Strain

Spores of *Bacillus atrophaeus* (ATCC 9372/DSM 675) with a concentration of 10^7 colony forming units (CFU)/mL, suspended in 70% ethanol were stored at -80 °C. The storage conditions ensured that the properties of the spores did not change over the experimental period. The spores were aliquoted at 1000 µL each. This enabled the spores to be taken without the need to thaw the remaining spore suspension.

Bioreactor

A 2 L bioreactor (Biostat A, Sartorius AG, Goettingen, Germany) was used for the experiments. It enabled the fully automatic control of the pH value, the temperature and the oxygen saturation. The temperature and pH were kept constant at the set value during the spore production. The pH was adjusted with 0.5 M of NaCl and 0.5 M of

NaOH. The set oxygen saturation corresponded to the minimum permissible oxygen saturation. If this value was undershot, the gas intake automatically increased first and then, if necessary, the stirring speed. These adjustments kept the oxygen saturation at the set value. Korasilon FG 30 (Kurt Obermeier GmbH & Co. KG, Bad-Berleburg, Germany) served as an anti-foaming agent. The anti-foaming agent was added automatically when needed.

Media

Terrific Broth (casein peptone 12 g/L (for microbiology, Gerbu Biotechnik GmbH, Heidelberg, Germany), yeast extract 24 g/L (for microbiology, Merck KGaA, Darmstadt, Germany), K_2HPO_4 9.4 g/L (ACS reagent, ≥98%, Merck KGaA), KH_2PO_4 2.2 g/L (ACS reagent, ≥99%, Merck KGaA), glycerol 8 g/L (Rotipuran ≥99.5%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany)) was used as the pre-culture medium. This medium enabled a rapid increase in the cell density of *B. atrophaeus* without showing any effect on the vitality of the cells.

Modified Difco Sporulation Medium (modified from Harwood and Cutting (1990)); casein peptone 5 g/L (for microbiology, Gerbu Biotechnik GmbH), beef extract 3 g/L (for cell biology, Gerbu Biotechnik GmbH), KCl 3.5 g/L (ACS reagent, 99.0-100.5%, Merck KGaA), $MgSO_4 \cdot 7H_2O$ 0.25 g/L (ACS ≥99%, Carl Roth GmbH & Co. KG), 30% glucose 10 mL/L (for biochemistry, Reag. Ph Eur., 97.5-102.0%, Merck KGaA), 1 M $Ca(NO_3)_2 \cdot 4H_2O$ 1 mL/L (ACS reagent, 99%, Merck KGaA), 10 mM $MnCl_2 \cdot 4H_2O$ 1 mL/L (ACS reagent, ≥98%, Merck KGaA), 1 mM $FeSO_4 \cdot 7H_2O$ 1 mL/L (ACS reagent, ≥99%, Merck KGaA)) was used for sporulation in the bioreactor. In this medium, *B. atrophaeus* showed a high sporulation rate with a very low cell mortality.

Pre-Culture

An aliquot of 1000 µL of the stored spore suspension was inoculated in 400 mL of Terrific Broth in a 1 L baffled shaking flask. Incubation took place at 30 °C, 135 rpm for 17 h. The cell suspension was then pelleted by centrifugation at 4000× g, 4 °C for 10 min. The supernatant was discarded and the cell pellet was resuspended with 25 mL of ultrapure water.

Spore Production in the Bioreactor

The bioreactor was inoculated with an OD_{600} of 1 with the cells of the pre-culture. Incubation took place for 48 h. The suspension was then harvested and checked for the presence of spores by light microscopy.

Spore Purification

The harvested spore suspension was purified prior to the determination of resistance to remove metabolites and residual media. For this purpose, the spore suspension was

washed three to five times with dist. water (4000× g, 10 min, 4 °C) and suspended. Subsequently, the suspension was shaken overnight at 480 rpm in order to dissolve the agglomerates of remaining cell debris and to release spores. This was followed by three more washing steps and subsequent centrifugation for 90 min at 6000× g and 4 °C. This step caused the formation of a very solid three-phase pellet. The upper two phases had to be removed. They included cell debris and maternal cells with endospores. The lower phase contained free spores. For cleaning, the supernatant was discarded and fresh dist. water was added carefully to the pellet. The pellet was now mixed rigorously. Since the lower spore phase was very firm due to centrifugation, only the upper phases (slimy consistency) dissolved. By replacing the supernatant, this step was repeated until the upper phases were removed. Finally, the remaining pellet could be dissolved with dist. water and stored at 4 °C.

Determination of the D-Value

In order to be able to compare the resistance of the spores due to the variation in the sporulation conditions, the D-value had to be determined. For this purpose, 100 µL of the purified spore suspension was exposed to 9.9 mL of a liquid solution of 35% hydrogen peroxide (Oxteril 350 Spray, Food Grade, Evonik Industries AG, Essen, Germany) at 25 °C and stirred at 480 rpm. In the course of the inactivation reaction, 100 µL of the samples were taken in defined time intervals and the reaction was stopped in a 1:10 dilution of dist. water with catalase (Catalase from *Micrococcus lysodeiktikus*, solution, activity 65,000−150,000 U/mL, Merck KGaA, Darmstadt, Germany). Then, appropriate dilutions were plated on a Plate Count Agar and incubated for 48 h at 30 °C. The decrease in CFU/mL over the course of treatment with hydrogen peroxide could be used to visualize the inactivation course and determine the D-value. The D-value is the time required, at a given set of conditions, to achieve a 1-log reduction.

3.1.3 Results

In order to be able to investigate the influence of the sporulation conditions (pH, temperature, oxygen saturation in the medium) on the resistance of the spores, a model had to be developed with which it is possible to make statistically significant statements about the influences on the resistance by varying the sporulation conditions. For this purpose, a full factorial experimental design (Design of Experiments, DoE) was developed. With factorial test plans, the influences of several factors on one (or more) target variables can be examined. The influencing variables are measurable variables, in this case the temperature, the pH and the oxygen saturation of the medium. With 2-3 influencing factors, a two-stage test plan is usually used - i.e., each influencing variable is examined at two levels (minimum and maximum value).

Therefore, these minimum and maximum values must first be determined. This step of modeling is extremely critical in microbiology. If these extremes are too far from the

growth optimum of the bacterium, spores could possibly no longer be formed or growth inhibition could occur and falsify the result. However, if these values are too close to each other - i.e., too close to the growth optimum of the bacterium - the measured data may be very similar to the target (D-value), so that no significant dependencies can be determined. In addition, when building the model, it must be taken into account that not only linear dependencies (1st order effects) of the influencing variables on the target variable are possible, but also non-linear dependencies (2nd order effects). If a factor was only examined in two stages, there is a risk of missing a quadratic relationship. To exclude this, so-called “center points” are included in the model, which allows us to assess whether the resistance of the spores has a linear or non-linear dependence on the influencing factors. The center points are to be understood as mean settings - i.e., as the mean between the respective extremes of the influencing variable.

For the modeling, preliminary tests were therefore carried out in the bioreactor to determine the extremes. The extremes were set to pH 6.0 and 8.5, with a center point of 7.25; for temperature, they were set to 28 °C and 40 °C, with a center point of 34 °C; and for the minimum oxygen saturation, they were set to 10%, 60% and 35%. An experimental plan was determined in accordance with this range of values, and spores were produced in a bioreactor under the specified sporulation conditions. Therefore, the cells were enriched in a culture medium and inoculated with an optical density (OD_{600}) of 1 into the bioreactor. The sporulation medium provides the cells with enough nutrients for exponential growth for about 4 h. Then, the sporulation of the cells began due to starvation - i.e., when the carbohydrate source was used up - and after 48 h, the spores were harvested. At this point, it is important to allow the spores to fully mature, which is why it is essential to avoid harvesting the spores too early. The temperature and pH were kept constant over the entire time in the bioreactor. The oxygen saturation in the medium decreased from saturation (100%) to the set value with the beginning of the exponential growth phase due to cell metabolism. The oxygen level was kept at the set value during the growth phase until the beginning of sporulation and then increased again, because no oxygen was consumed anymore. After the produced spores were harvested and purified, the resistance of the spores was determined as the D-value against 35% liquid hydrogen peroxide at 25 °C.

When the D-values of the test series were determined, a model alignment was carried out. In this alignment, the influences of the variables on the target (D-value) were analyzed and the model adjusted accordingly. In this case, it was found that the dependencies are not purely linear, but that the pH has a quadratic influence on the D-value. Table 1-1 shows the full factorial DoE with the sporulation conditions and the resulting measured and calculated D-values. It can be seen that the measured D-values of the test series match the calculated D-values of the adapted model very well. Experiment No. 9 is an exception, which is why it is not included in the calculations. The spores of this experiment had an atypically dark color after harvesting and did not show the light

refraction typical for spores under a light microscope. This is most likely an error during sporulation in the bioreactor. It is therefore not surprising that the D-value differs from experiment No. 2, which was carried out under the same conditions.

Table 1-1. Experimental plan and results of the full factorial Design of Experiments (DoE) to investigate the influence of sporulation conditions (pH, temperature and oxygen saturation) on the resistance (D-value) of the spores of *B. atrophaeus* in the bioreactor.

No.	pH [-]	Temp. [°C]	pO_2 [%]	D-Value, Measured [s]	D-Value, Calculated [s]
1	8.5	28	10	40	60.04
2	6.0	28	60	77	70.88
3	8.5	28	10	65	60.04
4	6.0	40	10	134	161.17
5	6.0	40	60	185	161.17
6	8.5	28	60	84	60.04
7	7.25	34	35	158	171.14
8	7.25	34	35	188	171.14
9*	6.0	28	60	171	70.88
10	8.5	40	60	58	56.84
11	8.5	40	10	59	56.84
12	6.5	28	10	125	122.41
13	8.5	40	10	52	56.84
14	8.5	28	60	52	60.04
15	6.5	40	60	197	194.00
16	6.5	28	10	111	122.41

* The measured D-value of the 9th test run was rated as an outlier and was therefore not taken into account in the evaluation.

The measured D-values of the 16-test series are between 40 and 197 s and fit very well with the calculated D-values of the model. This is confirmed by plotting the measured D-values against the calculated D-values in Figure 1-1, with an r^2 value of 0.93. The model alignment also shows that the oxygen saturation in the medium has no significant influence on the D-value. The oxygen saturation is therefore not taken into account in further model calculations.

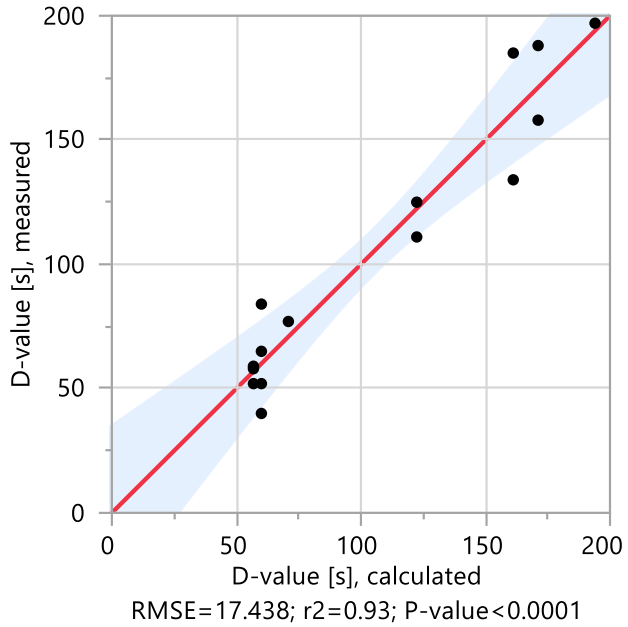

Figure 1-1. Observed values versus prediction. The diagram shows the actual measured D-values compared to the predicted calculated D-values. For a good approximation, the points on the scatter diagram must move to the red line. The points are all very close to the line, which means that the model maps the dependencies very well. Another indicator of model accuracy is the r^2 value. This measures the percentage of variability in the D-value that is explained by the model. A value closer to 1 means that the model gives a good forecast (in this case, 0.93).

Based on the D-values, the model yields an equation (Equation (1-1)) by which it is possible to predict all the D-values within the model range - i.e., within the extremes of the influencing factors temperature and pH:

$$\begin{aligned}\text{D-value [s]} = {} & 171.13781504 + (-28.78900087) * \left(\frac{(pH-7.25)}{1.25}\right) \\ & + 21.772689936 \\ & * \left(\frac{(Temp.[°C]-34)}{6}\right) + \left(\frac{(pH-7.25)}{1.25}\right) \\ & * \left(\left(\frac{(Temp.[°C]-34)}{6}\right) * (-23.37179094)\right) \\ & + \left(\frac{(pH-7.25)}{1.25}\right) \\ & * \left(\left(\frac{(pH-7.25)}{1.25}\right) * (-83.90606869)\right)\end{aligned} \quad \textbf{(1-1)}$$

Using this equation, a three-dimensional prediction model of the D-value on the temperature and the pH can be established (Figure 1-2):

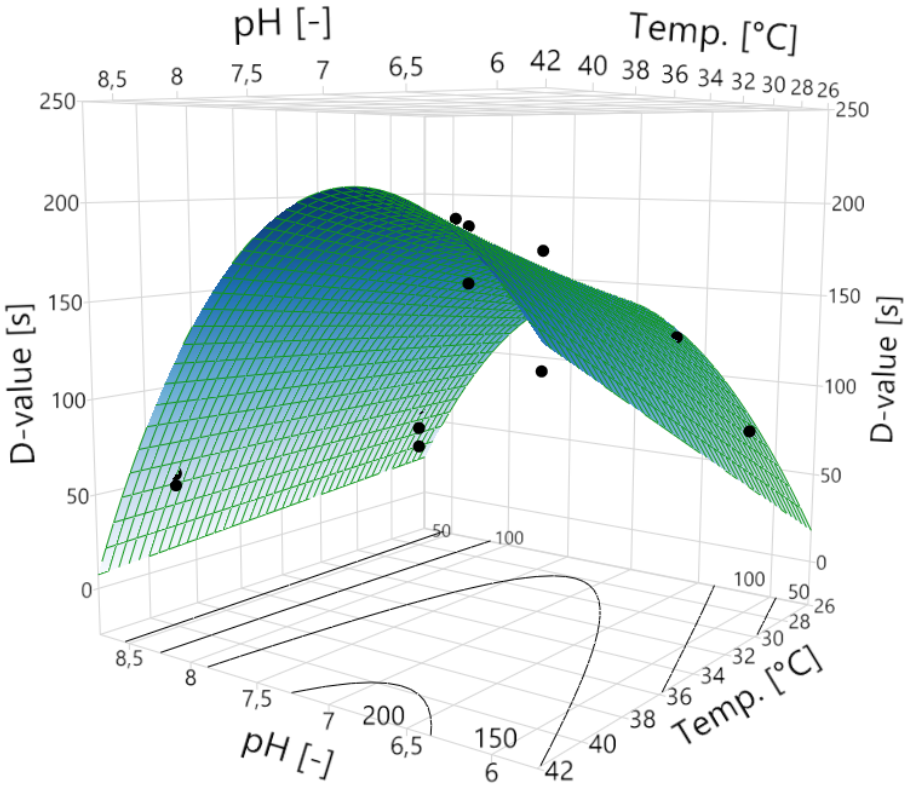

Figure 1-2. Dependence of the D-value on the temperature and pH. The surface plot shows the expected D-values for the respective sporulation conditions. The black data points are the actually measured D-values on which the model is based. Some of the values are hidden by the surface. The D-value is greatest at a high temperature of 40 °C and a pH of 6.9. If the temperature drops or the pH changes to acidic or basic, the D-value also decreases.

The highest D-value of 200 s is obtained at pH 6.9 and 40 °C. The expected D-value drops towards lower and higher pH values. Depending on the selected pH value, the temperature has a different effect on the D-value. At a low pH such as 6.0, the D-value shows a very strong linear dependence on the temperature, which means that with increasing temperature, the D-value also increases. At the pH of 8.5, increasing the temperature does not increase the D-value. This interaction of the two influencing variables becomes obvious in Figure 1-3.

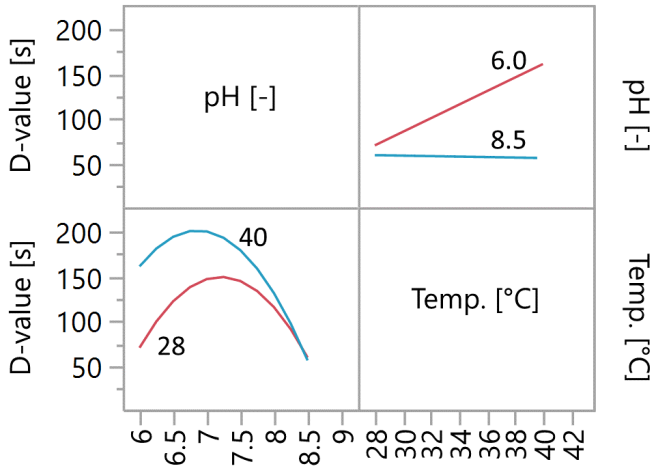

Figure 1-3. Interaction profiles of pH and temperature. The influence of temperature on the D-value increases with decreasing pH. The pH value at which the greatest D-value is expected shifts from 7.2 to 6.9 when the temperature rises from 28 to 40 °C.

With increasing pH, the influence of the temperature decreases to such an extent that from pH 7.8 to 8.5, the temperature no longer has a significant influence on the D-value. Thus, at pH 8.0 D-values from ~115 s to ~131 s are predicted at temperatures between 28 and 40 °C, but it can no longer be said that there is a significant difference. If the pH is further increased to 8.5, the D-value in the entire temperature range is predicted to be ~59 s. There is no longer a difference in the D-value between 28 °C and 40 °C. This makes the pH the only variable that influences the resistance of the spores. The greatest effect of the temperature on the D-value is at the slightly acidic pH of 6.0. Here, D-values from ~70 s to ~160 s are to be expected (increasing from 28 °C to 40 °C). Towards pH 7.0, the influence of temperature on the D-value softens but is still significant. Thus, across the range of investigated temperatures, D-values of ~150 s to ~200 s are predicted at pH 7.0.

In addition, the influence of the pH on the D-value changes as well, depending on the temperature. At 28 °C, the highest D-value is expected at a pH of 7.2. The D-value decreases towards lower and higher pH values. At a temperature of 40 °C, the highest D-value is expected at a pH of 6.9. This means that the pH at which the greatest D-value is expected shifts with rising temperature. Overall, the prediction model shows D-values in the range of ~59 s to ~200 s.

3.1.4 Discussion

In this study, it could be shown that the resistance of submerged produced spores to hydrogen peroxide depends largely on the pH and temperature. The pH has a quadratic influence on the resistance, which means that there is an optimum at which the

resistance reaches its maximum. If the pH changes further to acidic or basic, the resistance of the spores also decreases. The resistance, however, shows a linear dependence on temperature. With increasing temperature, the resistance of the spores to hydrogen peroxide also increases. It should be noted that there is an interaction between pH and temperature which means that the factors influence each other. This has a significant impact on the degree of the effect of temperature on the resistance of the spores. The influence of temperature is strongest in the neutral and acidic pH range. When the pH becomes more alkaline, the effect of the temperature is reduced until the temperature finally has no influence on the resistance. The oxygen saturation of the medium has no significant influence on the resistance of the spores.

It should be noted that the results apply to *B. atrophaeus* only in the examined area of the DoE with the media and methods used. It is generally known that the sporulation medium also has an influence on the resistance of bacterial spores. However, it can be assumed that the dependencies found qualitatively also apply to other media in the submerged production of *Bacillus* spores, possibly even their quantity. Whether and to what extent there is an interaction of the temperature and the pH with the medium or its composition (main ingredients, concentration and presence of manganese, iron, zinc, calcium, etc.) and whether this affects the resistance of the spores to hydrogen peroxide was not examined in this study.

The effect of the sporulation temperature on the resistance of *Bacillus* spores has already been investigated in many publications (Baril et al., 2012a; Baweja et al., 2008; Beaman and Gerhardt, 1986; Condon et al., 1992; Garcia et al., 2010; González et al., 1999; Lechowich and Ordal, 1962; Leguérinel et al., 2007; Melly et al., 2002; Palop et al., 1999; Planchon et al., 2011; Raso et al., 1995). The spore structure was found to be decisive for their resistance. It is believed that treating the spores with hydrogen peroxide primarily damages the germination apparatus and the inner membrane. Core proteins may also be affected by the damage (Storz and Hengge, 2011). The spores are probably protected by superoxide dismutases in the coat and catalase activity, which are also associated with the outer layers of the spore (Cybulski et al., 2009; Henriques and Moran, 2007;). Furthermore, polycyclic terpenoids (sporulenes) contribute to the resistance of the spores to hydrogen peroxide (Bosak et al., 2008). α/β-type small acid-soluble spore proteins (SASPs) protect the DNA in the spore core (Storz and Hengge, 2011). The low water content of the spores also contributes to their resistance and is influenced by the sporulation temperature (Setlow, 2006). The higher the sporulation temperature, the lower the water content of the spores will be. The result is that of an increase in the resistance of the spores to heat (Beaman and Gerhardt, 1986; Melly et al., 2002). Above a certain temperature, however, the resistance decreases again, so it is not linear—e.g., without limit Baril et al. (2012a). An increase in resistance to chemicals such as formaldehyde could also be shown by increasing the sporulation temperature (Cortezzo and Setlow, 2005; Melly et al., 2002) and can

be confirmed in this study in the case of hydrogen peroxide depending on the pH value during sporulation. A reduction in the water content presumably slows down chemical reactions and thus protects the spores (Storz and Hengge, 2011).

It is not mechanistically clear what influence the sporulation pH has on the resistance of the spores, but it is known that the pH influences the structure of the spores during sporulation. Depending on the pH, the surface hydrophobicity (Eschlbeck et al., 2017) or the size and shape of spores can change (Lowe et al., 1989). It is not known, however, whether and how this affects the resistance of the spores to liquid hydrogen peroxide.

The spores have the greatest resistance when the pH is in a range that corresponds approximately to the optimum growth of the spore formers. This is in line with the results of Guizelini et al. (2012), who found a quadratic influence of the pH on the resistance of submerged produced spores of *Geobacillus stearothermophilus*, the highest resistance being at a sporulation pH of 8.5 and decreasing at 7.8 and 9.2. A sub-optimal pH value may have a negative influence on the bacteria even in a vegetative stage and/or when sporulation and spore maturation are inhibited, which means that the resistance of the spores cannot be fully developed.

The interaction of pH and temperature during sporulation and their mutual influence on resistance is striking. Apparently, the temperature has only a minor influence, since its effect on the resistance only comes into play in a certain pH range. From this, it can be concluded that the influence of the pH on the resistance interferes more fundamentally with the structural effects influencing the resistance of the spores than the temperature does. A reduction in the water content of the spores can be neglected if a sub-optimal pH value has a fundamental influence on the structure of the spores.

3.1.5 Conclusion

In this study, it could be shown that the resistance of the submerged produced spores of *B. atrophaeus* is dependent on the pH as well as the incubation temperature. The key finding is that these influencing factors interact with each other, and therefore the temperature only influences the resistance in a certain pH range and is subordinate to the pH as the main influencing factor. These results help us to understand the effects of sporulation conditions on the variability of resistance of BIs better. It could be shown how important the understanding of the formation of resistance of spores is in order to reduce or prevent resistance fluctuations in BIs. In terms of scientific progress, this outcome shows that the formation of resistance by spores is influenced by many factors that mesh with one another, can influence one another, or are dependent on one another. Therefore, such influencing factors should no longer be examined individually in the future or only with the greatest caution. Regarding practical applications, it can be stated that making use of our results in the production of commercial BIs could be

a significant step forward towards more reliable results in sterilization test runs - e.g., as part of the commissioning of newly installed production or filling lines. However, another practical implication would be that all stakeholders in this context would have to agree on implementing these findings in producing BIs with standardized resistance. Additionally, it would be required to agree on the target resistance level. Standardizing the production of BIs based on these findings on the formation of spore resistance could help to increase the product shelf life and safety in the food and pharmaceutical industries and to counteract uncertainties in the validation of sterilization systems and methods. Further work in this field is required to address other factors such as the sporulation medium composition in relation to sporulation-influencing components (e.g., manganese, zinc, iron and calcium) and to transfer this kind of approach also to other established Bis - e.g., those of *G. stearothermophilus* applied as BI in pharmaceutical applications.

3.1.6 Acknowledgments

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We gratefully acknowledge Christian Ederer, Franz Fraunhofer and Erich Schneider for technical support.

3.1.7 Author contributions

Conceptualization, P.S. and U.K.; methodology, P.S.; validation, P.S.; formal analysis, P.S.; investigation, P.S.; resources, P.S.; data curation, P.S.; writing—original draft preparation, P.S.; writing—review and editing, P.S., U.K.; visualization, P.S.; supervision, U.K.; project administration, P.S., U.K.; funding acquisition, U.K. All the authors have read, edited and agreed to the published version of the manuscript.

3.1.8 References

The references were combined into a joint list of all publications at the end of the thesis.

3.2 Effect of sporulation conditions following solid-state cultivation on the resistance of *Geobacillus stearothermophilus* spores for use as bioindicators testing inactivation by H_2O_2

Summary and contribution of the doctoral candidate

The effect of sporulation conditions on the resistance to hydrogen peroxide of solid-state produced spores of *G. stearothermophilus* was investigated.

Therefore, a full factorial design of experiments was developed, based on which the spores were produced on agar-plates (Minimal Sporulation Medium Agar, MSM Agar) at different sporulation conditions (sporulation temperature of 51-63 °C and sporulation pH of 6.0-8.0). The produced spores were then analyzed for their resistance (D-value) in liquid 35% hydrogen peroxide at 25 °C. The obtained $D_{25°C,35\%H2O2}$-values lay in between 131 and 306 s. Based on the data, a model was developed that depicts the influence of sporulation conditions on resistance. With the equation derived from the model, the resistance of the spores could be calculated as a function of the sporulation conditions.

The resistance of the spores depended significantly on the sporulation temperature and the sporulation pH. The resistance to hydrogen peroxide increased linearly with decreasing sporulation pH, with resistance being highest at pH 6.0. The sporulation temperature had a quadratic effect on resistance, with resistance being highest at 57 °C. Sporulation temperature and pH did not interact with each other. The highest $D_{25°C,35\%H2O2}$-value of 306 s could be obtained at a sporulation temperature of 57 °C at pH 6.0.

The results show that the resistance can be adjusted precisely by specific manipulation of sporulation pH and temperature. The adjustments can be done independently of the other factor in each case, since the factors do not interact with each other.

The doctoral candidate developed the concept of the experiments and established the methods. He critically reviewed the existing literature. Furthermore, he analyzed the data and interpreted the results. Additionally, the doctoral candidate wrote the manuscript and revised it. The co-authors contributed to the experimental part and/or to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript [1]

Effect of sporulation conditions following solid-state cultivation on the resistance of *Geobacillus stearothermophilus* spores for use as bioindicators testing inactivation by H_2O_2 [2]

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Abstract

Geobacillus stearothermophilus spores play an essential role in the pharmaceutical and food industries as bioindicators (BI) to evaluate inactivation effects of hydrogen peroxide in processing and packaging lines' sterilization. Unstandardized, inconsistent BI production, as is the case for currently available commercial BI, can lead to variable resistances and incorrect test results. The solution to this problem could be the standardization of BI spore production. This study assessed the effects of sporulation temperature and pH on spore resistance in the production of *G. stearothermophilus* ATCC 7953 spores on solid-state agar. For this purpose, spores were produced under different sporulation conditions (51-63 °C, pH 6-8), and their resistance was determined by the D-value in 35% liquid hydrogen peroxide at 25 °C. Based on these data, a mathematical equation describing the resistance as a function of the sporulation conditions was derived. The analysis showed a quadratic effect of the sporulation temperature and a linear effect of the examined domain's pH on the resistance, without interactions between these factors. We observed the highest $D_{25°C,35\%H2O2}$-value of 309 s at sporulation conditions of 57 °C and pH 6.0.

Keywords

aseptic packaging, D-value, hydrogen peroxide, product safety, sterilization;

[1] Adaptions refer to formatting, e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units and spelling.

[2] Originally published in: LWT (2021), Vol. 151, 112078; doi:10.1016/j.lwt.2021.112078

3.2.1 Introduction

Sterility is of great importance in the food and pharmaceutical industries. Consequently, validations for the inactivation performance assessment have to be carried out with so-called bioindicators (BI) initially upon installing new sterilization devices and regularly for revalidation of inactivation effects induced, for instance, by H_2O_2. With these BI, it is possible to measure the inactivation performance under defined conditions since natural contamination is highly variable and mostly not high enough to measure the desired target of 5-log reduction. In sterilization with hydrogen peroxide, BI made from spores of *Bacillus atrophaeus* ATCC 9372 and *Geobacillus stearothermophilus* ATCC 7953 prevail (FDA, 2007; McLeod et al., 2017; VDMA, 2021). Nevertheless, the currently commercially available BI resistance varies greatly, leading to uncertainties during validation due to false-positive and false-negative results. A false-positive validation result incorrectly indicates sterility, which in regular production leads to a high proportion of non-sterile products, a reduction in shelf life, and, in the worst case, to risk for the consumer. A false-negative result, instead, leads to the assumption that the sterilization was insufficient. As a result, the sterilization conditions are unnecessarily intensified, which leads to increased consumption of the sterilant and pollution of the environment, overprocessing and increased costs.

On the one hand, the origin of resistance variability can presumably be found in the manufacturing process because, most likely, every manufacturer produces BI using other media and sporulation conditions, resulting in spores with different resistance. On the other hand, however, BI users associated with us, and we could regularly observe that the resistance of spores from the same manufacturer can also differ depending on the batch. The reasons have not been adequately investigated so far. However, we assume that, besides the natural variability of spore resistance, slight variations in the sporulation conditions significantly influence BI's resistance. The solution to this problem could be the standardization of BI spore production. Standardized BI production requires knowledge of the factors and the degree of their influence on spore resistance during sporulation. Many studies have already shown that the sporulation conditions have a significant influence on the structure of the spores (Abhyankar et al., 2016; Bressuire-Isoard et al., 2016; Eschlbeck et al., 2017; Isticato et al., 2020; Nguyen Thi Minh et al., 2011; van der Voort and Abee, 2013; Wells-Bennik et al., 2019; Xu Zhou et al., 2017). Since changes in the spore structure directly influence the resistance to various environmental influences (Setlow, 2014), it is comprehensible that even small changes in the sporulation conditions can have major effects on the resistance. It is known, for example, that the medium, but also the spore preparation, can significantly influence the resistance against heat or low energy electron beam (LEEB) treatment (Bressuire-Isoard et al., 2018; Wells-Bennik et al., 2019; Zhang et al., 2018). However, in commercial BI production, sporulation media and spore preparation are usually optimized for spore-rich yields and cost reduction, so changing them

to influence spore resistance could counteract BI production goals. In contrast, changing sporulation conditions like the sporulation temperature or the medium's pH in BI production is easily feasible, controllable, and less time-consuming. The sporulation temperature, in particular, has often been the focus of studies that investigated its influence on the heat resistance of spores. It was found that the sporulation temperature had a significant impact on heat resistance, regardless of the medium or the species used. Depending on the sporulation temperature's examined domain, different effects on the spores' resistance could be shown. Some studies have found that the resistance to heat, hydrogen peroxide, or LEEB linearly increases with increasing sporulation temperature (Lechowich and Ordal, 1962; Leguérinel et al., 2007; Raso et al. 1995; Sala et al., 1995; Stier and Kulozik, 2020a; Wells-Bennik et al., 2019; Zhang et al., 2018). Other studies, however, have also shown a decrease in heat resistance with increasing sporulation temperature, i.e., quadratically effects of sporulation temperature on the spores' resistance (Baril et al., 2012b; Condon et al., 1992; Mtimet et al., 2015). The same phenomenon was observed for the sporulation medium's pH (Baril et al., 2012a,b; Guizelini et al., 2012; Mtimet et al., 2015; Nguyen Thi Minh et al., 2011; Stier and Kulozik, 2020a). This phenomenon occurs, because the sporulation conditions have an optimum where the spores' resistance is at its highest. With the focus on BI production, it is therefore important to know when the hydrogen peroxide resistance does not increase any further and to what extent changes in the sporulation conditions affect the resistance. However, the resistance optimum depends on the strain, the resistance mechanism, and other factors influencing the resistance. Additionally, chemical resistance strongly depends on the particular inactivating chemical (Cortezzo and Setlow, 2005; Melly et al., 2002). Therefore, none of these findings provide information about the effects of the sporulation temperature and pH on hydrogen peroxide resistance of *G. stearothermophilus* ATCC 7953 spores, and to the best of our knowledge, there are no other studies published about this. This fact leaves a significant knowledge gap, given that BI produced from this microorganism are established and widely used as test BI in industries where sterility is of paramount importance.

The spores of *G. stearothermophilus* for use as BI are mostly produced on agar solid-state, which is suspected to be one of the primary sources of inadvertent resistance variability. Submerged production of spores in bioreactors would possibly help produce BI with less variability since the sporulation conditions can be better controlled, but attempts to establish conditions to aim high spore yield for this strain failed so far. However, knowing the influences of sporulation conditions on hydrogen peroxide resistance of solid-state produced BI could reduce resistance variability and ease this unsatisfying situation. Therefore, more insights from studying the effects of sporulation conditions must be gained.

As mentioned, sporulation temperature and pH significantly impact spore resistance and can easily be changed in BI production, which means manipulating the resistance

towards a targeted level can practically be well achieved. For this reason, we focused on the effects of sporulation temperature and pH on hydrogen peroxide resistance and set the sporulation medium and the spore preparation as constant parameters.

The sporulation boundaries in which spores are formed are similar to or included within the range of temperature and pH supporting growth (Baril et al., 2012a,b). For the related strain *G. stearothermophilus* ATCC 12980, Mtimet et al. (2015) obtained the highest spore concentration and the highest heat resistance values from spore suspensions produced at a temperature and pH close to the growth optimum of the vegetative cells (57 °C, pH between 5.83 and 7.10). In order to meet the requirements of BI production, we wanted to investigate the effect of the sporulation temperature and pH on the hydrogen peroxide resistance in the rough range of the optimal growth conditions since this is where the best sporulation and the highest resistance are assumed (Baril et al., 2012a; Mtimet et al., 2015). Additionally, sporulation conditions too far away from the optimal growth conditions could markedly impair sporulation. This, in turn, would cause low spore yield or spontaneous germination of the spores because of low spore stability and, therefore, would counteract with BI production goals.

There are several options for examining the effects of the sporulation conditions on the resistance. On the one hand, Penna et al. (2003), e.g., investigated media effects on spore yield and thermal resistance using a “one-variable-at-a-time” approach. Even though this method is well suitable for small numbers of trials, this procedure is very time-consuming, and, more importantly, no interactions between variables could be determined. Since it is known that sporulation temperature and pH can interact in their influence on hydrogen peroxide resistance (Stier and Kulozik, 2020a), it is imperative to consider this option here as well. On the other hand, mathematical models, e.g., the gamma-concept (Zwietering et al., 1992) and the cardinal growth model (Rosso et al., 1995), could be used to describe the effects of sporulation conditions on the resistance (Baril et al., 2012a). However, in addition to the natural variability of spores and the effects of sporulation conditions, we also aimed to consider special features of BI production, such as the presence of agglomerates in solid-state-produced spore suspensions. These agglomerates could often be observed by BI users associated with us and us, leading to shoulder formation in the inactivation curve or delaying the inactivation in challenge tests, thus influencing BI’s resistance. For this reason, we decided to use a Design of Experiments (DoE) as the basis of our experiments that provides statistically significant data, enables the observation of interactions between variables, considers the presence of agglomerates, and at the same time reduces the experimental effort.

The aim of this study was, therefore, to examine the effect and possible interactions of the sporulation temperature and pH on the hydrogen peroxide resistance of *G. stearothermophilus* ATCC 7953 spores with a mathematical equation based on the meas-

ured data in a domain suitable for BI production. For this purpose, spores were produced under different combinations of the sporulation temperature (51-63 °C) and pH (6.0-8.0), based on a randomly generated experimental plan, with *Geobacillus* medium as the parameter, determined in preceding screening experiments. The resulting BI resistance was determined as the D-value (decimal reduction time; time required, at a given set of conditions, to achieve a 1-log reduction) in a liquid solution of 35% hydrogen peroxide at 25 °C.

3.2.2 Materials and Methods

Strain

Spores of *Geobacillus stearothermophilus* ATCC 7953 with a concentration of 10^7 colony forming units (CFU)/mL were suspended in 70% ethanol and stored at -80 °C. To enable the spores to be taken without the need to thaw the remaining suspension, the spores were aliquoted at 500 µL each. Also, this ensured that the spores' properties did not change over the experimental period.

Media

Geobacillus Medium 9 (GM9) was used as the pre-culturing medium, comprised of casein peptone 16 g/L (for microbiology, Gerbu Biotechnik GmbH, Heidelberg, Germany), yeast extract 10 g/L (for microbiology, Merck KGaA, Darmstadt, Germany), glycerol 13 g/L (Rotipuran® ≥99.5%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), KCl 2.5 g/L (ACS reagent, 99.0-100.5%, Merck KGaA), NaCl 5 g/L (ACS reagent, ≥99.0%, Merck KGaA), NH_4Cl 2 g/L (for molecular biology, ≥99.5%, Merck KGaA), $MgSO_4$ x $7H_2O$ 0.8 g/L (ACS ≥99%, Carl Roth GmbH & Co. KG), $MnCl_2$ x $4H_2O$ 0.003 g/L (ACS reagent, ≥98%, Merck KGaA), and sodium pyruvate 5 g/L (for biochemistry, ≥99.0%, Merck KGaA); pH 7.0. This medium is a custom-designed medium established in pre-work for vegetative growth of *G. stearothermophilus* ATCC 7953 (data not shown).

Minimal Sporulation Medium Agar (MSM Agar) (modified from Pruß et al. (2012)) was used for sporulation. It comprised casein peptone 5 g/L (for microbiology, Gerbu Biotechnik GmbH), beef extract 3 g/L (for cell biology, Gerbu Biotechnik GmbH), $MnSO_4$ x H_2O 10 mg/L (≥99%, p.a., ACS, Carl Roth GmbH & Co. KG), and agar-agar 15 g/L (for microbiology, Carl Roth GmbH & Co. KG). The pH was initially set to 6.0, 7.0, 8.0 with 0.1-2 M HCl (ACS reagent, 37%, Merck KGaA) and 0.1-2 M NaOH (≥98%, pellets (anhydrous), Merck KGaA), depending on the experiment.

Pre-culture

An aliquot of 500 µL of the stored spore suspension was inoculated in 400 mL of GM9 in a 1 L baffled shaking flask. Incubation took place at 57 °C, 150 rpm for 16 h. The

cell suspension with an OD_{600} of 3.8 was subsequently pelleted by centrifugation at 4000× g, 25 °C for 10 min. The supernatant was discarded, and the cell pellet was resuspended with 100 mL of Milli-Q water.

Spore production on agar plates according to a Design of Experiments (DoE)

According to the experimental plan of the DoE described below, spores were produced under different combinations of sporulation temperature and pH. In order to obtain a sufficient number of spores for the subsequent measurement of the resistance and to average out the natural variability in spore resistance, which could be stronger or weaker on individual agar plates, spores were produced on 20 agar plates in total for each sporulation condition, i.e., for each experimental run. For this purpose, 100 µL of the cleaned and well-mixed cell suspension from the pre-culture were plated on each MSM agar plate with a Drigalski spatula. The pH of the medium was adjusted before the agar plates were poured. According to the DoE, the agar plates were then incubated at the respective sporulation temperature for 10 d. Since the incubation temperatures were relatively high (51-63 °C), the stacks of 20 agar plates were packed in sealed plastic bags to protect them from drying out.

Spore harvesting and purification

The spores were harvested using a cell scraper and adding 3 mL of sterile Milli-Q water to each agar plate. The harvest of the 20 agar plates of each experimental run was collected in two 50 mL centrifuge tubes, checked for spores by phase-contrast microscopy, and subsequently centrifuged at 4000× g for 10 min at 4 °C. The supernatant was discarded, and the pellets were resuspended with 15 mL of Milli-Q water each and put together into one 50 mL centrifuge tube. The suspension was further purified, according to Stier and Kulozik (2020a). For this purpose, the spore suspensions were washed three to five times with Milli-Q water (4000× g, 10 min, 4 °C) until the supernatant became clear. After that, the suspension was shaken overnight at 480 rpm to help the remaining agglomerates of cell debris and spores disintegrate. Then, the suspensions were washed three more times with Milli-Q water (4000× g, 10 min, 4 °C) and ultimately centrifuged at 6000× g for 90 min at 4 °C. This strong centrifugation caused the formation of a solid three-phase pellet. There were two upper phases containing cell debris and mother cells with endospores that had to be removed, and the sediment containing free spores. For purification, the supernatant was discarded, and the pellet was carefully layered with small amounts of Milli-Q water. Since the sediment phase was very solid, the pellet could be mixed vigorously to dissolve the upper phases (firm but slimy consistency) without dissolving the sediment phase. This step was repeated until the upper phases were removed entirely by replacing the supernatant with fresh Milli-Q water. In the end, the pellets were dissolved in Milli-Q water, and the successful purification was confirmed by phase-contrast microscopy. The spore suspensions with

concentrations between 3 x 10^7 and 1 x 10^8 CFU/mL were stored at 4 °C under the exclusion of light for about a week until their resistance was determined.

Determination of the spore resistance

The spores' resistance was defined by the $D_{25°C,35\%H2O2}$-value, and was determined by a count reduction test in 35% liquid hydrogen peroxide at 25 °C in analogy to Stier and Kulozik (2020a). The $D_{25°C,35\%H2O2}$-values ensured to determine effects of the sporulation conditions on the spores' resistance. For this purpose, the purified spore suspension was mixed well for 1 min, treated in an ultrasonic bath for 20 min, and mixed again for 1 min to dissolve agglomerates as much as possible. As mentioned, these agglomerates often occur in spores that have been produced on solid-state agar. Then, 100 µL of the spore suspension were added to 9.9 mL of a liquid solution of 35% hydrogen peroxide (OXTERIL® 350 Spray, Food Grade, Evonik Industries AG, Essen, Germany) in a 50 mL centrifuge tube at a constant temperature of 25.0 ± 0.1 °C and stirred at 450 rpm in a thermomixer. At defined time intervals of 30 s, 60 s, 120 s, 180 s, 300 s and 420 s, samples of 100 µL each were taken exact to the second, added to a freshly prepared dilution of 9 mL Ringer's solution, 800 µL Milli-Q water and 100 µL 1:5 diluted catalase (Catalase from *Micrococcus lysodeiktikus*, solution, activity 65,000-150, 000 U/mL, Merck KGaA) and mixed right after. Due to the substantial dilution of the sample in the catalase solution and its enzyme concentration, the effect of hydrogen peroxide on the spores was immediately significantly reduced and stopped due to the enzyme reaction. The catalase concentration was chosen so that the addition of the treated spore suspension in hydrogen peroxide did not result in foaming over the test tube due to oxygen formation, but that there were bubbles and foam formation in order to inactivate the hydrogen peroxide as fast as possible. To determine the starting value of the inactivation curve at 0 s of treatment, 100 µL of the spore suspension were added directly to a dilution of 9 mL Ringer's solution, 800 µL Milli-Q water, and 100 µL 1:5 diluted catalase without hydrogen peroxide treatment. Apart from that, these spores were treated the same way as the others. After that, dilutions in Ringer's solution were performed and plated on Plate-Count Agar (pH 7.0). The incubation took place at the optimal growth temperature of 57 °C for 48 h to avoid negative influences on the spores' germination. The colonies were counted after 24 h and 48 h. The spore suspension of each experimental run was subjected to the inactivation test three times (technical triplicate). Then, the mean value was formed from the bacterial counts determined and taken logarithmically as a function of the inactivation curve. The negative reciprocal slope of a best-fit straight line through these data points corresponded to the $D_{25°C,35\%H2O2}$-value. Any inhibitory effects due to suboptimal recovery conditions, as described by Trunet et al. (2015), for example, were not considered in the determination of the $D_{25°C,35\%H2O2}$-value since all spore suspensions were incubated at optimal growth conditions and treated equally. Figure 2-1 shows the inactivation curves of the technical triplicates of the spore suspensions while treated with hydrogen peroxide. In

the inactivation curves of Exp. 4 (from 180 s) and Exp. 5 (from 300 s), the number of spores fell below the detection limit of 1 x 10^5 CFU/mL, which is why no more data points are displayed. The detection limit results from the substantial dilution of the treated spores in the catalase solution, which is, as mentioned, necessary to stop the inactivation reaction.

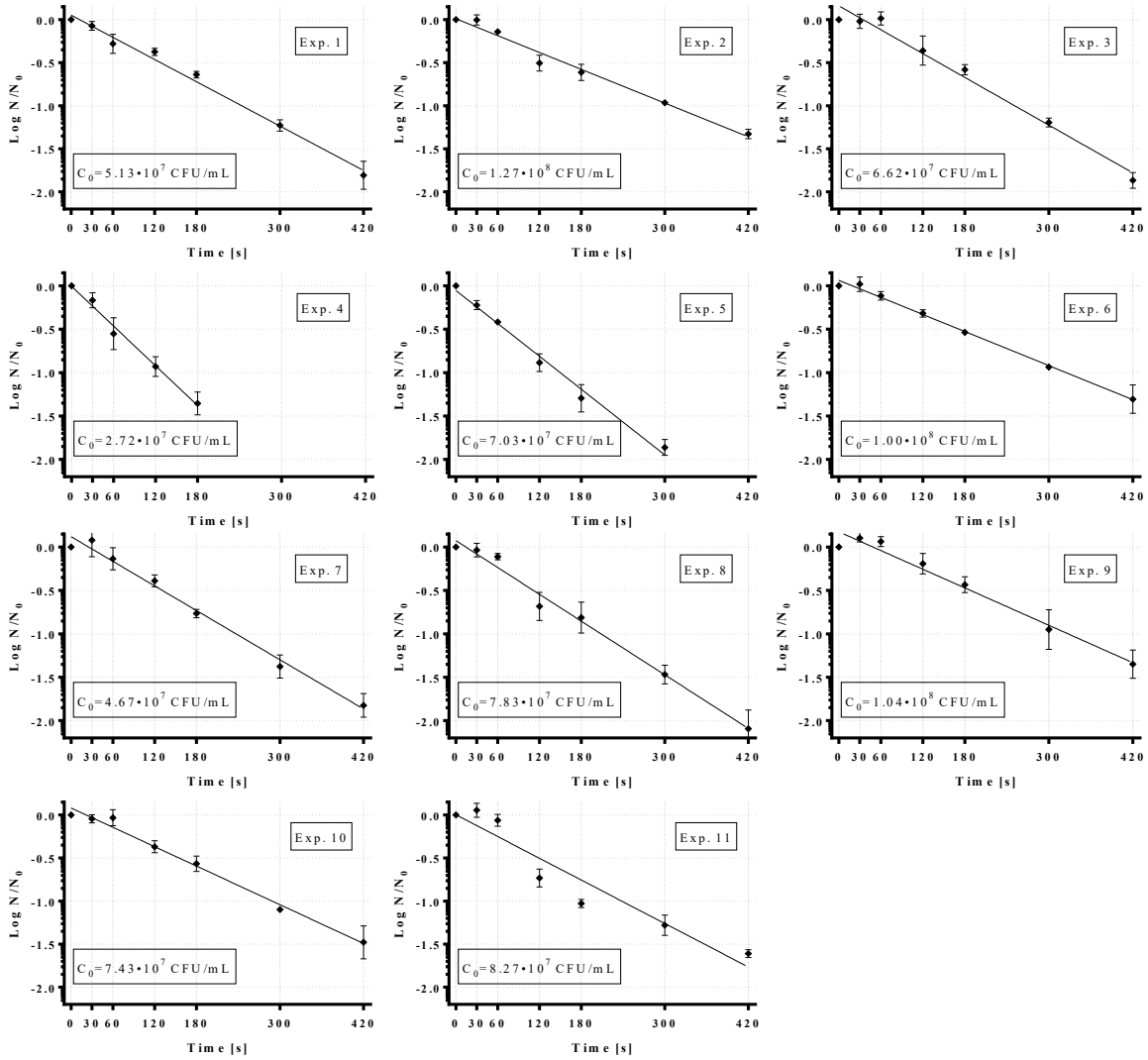

Figure 2-1. Inactivation courses of *G. stearothermophilus* ATCC 7953 spore suspensions during the treatment with 35% liquid hydrogen peroxide at 25 °C. The numbers correspond to the experiments shown in Table 2-1.

The numbering corresponds to the numbers of the experiments in Table 2-1. In some inactivation curves, the number of germs initially increased (shoulder formation in the inactivation curve within the first 30-60 s). The reason for this is the disintegration of remaining agglomerates due to the harsh inactivation conditions. As mentioned, these

shoulders also occur in commercial spore suspensions and influence BI's resistance in challenge tests. For this reason, we integrated the shoulders into the $D_{25°C,35\%H2O2}$-value calculation to take them into account when describing the effects of the sporulation conditions on the hydrogen peroxide resistance. Calculating the $D_{25°C,35\%H2O2}$-value just on the linear section of the inactivation curve would have changed the results only with an average of 21 s.

Table 2-1. D-values of *G. stearothermophilus* ATCC 7953 spores after exposure to 35% liquid hydrogen peroxide at 25 °C as a function of sporulation pH and temperature.

Exp.	pH [-]	Temp. [°C]	$D_{25°C,35\%H2O2}$-value [s], measured	$D_{25°C,35\%H2O2}$-value [s], calculated
1	7	63	233	192
2	7	57	306	279
3	6	51	217	220
4	8	63	131	162
5	8	51	158	160
6	7	57	305	279
7	6	63	212	222
8	7	51	194	190
9	6	57	279	309
10	7	57	267	279
11	8	57	238	249

Design of Experiments (DoE)

To investigate the influence of the sporulation pH and temperature on the spores' resistance against hydrogen peroxide and to obtain statistically significant results with the experiments, a DoE was created with the software JMP® Pro in advance of the spore production. The DoE enabled the investigation of the influencing factors (initial pH of the agar and the incubation temperature during cultivation and sporulation) on the target variable (resistance of the spores formed, measured as $D_{25°C,35\%H2O2}$-value) as well as the investigation of possible interactions of those variables. Since the experiments carried out in this study have two influencing variables, a two-level experimental plan was used in which each variable was examined in a minimum and maximum value. Additionally, so-called "center-points" were included in order to be able to determine not only linear but also quadratic effects. With the focus on BI production, the aim was for the center-points to be in optimal growth conditions since this is where the best sporulation was assumed in addition to the highest resistance (Baril et al.,

2012a; Mtimet et al., 2015). Since the bacteria examined are, of course, biological organisms, the levels (extremes for sporulation temperature and pH) needed to be chosen so that the spore-formers could still grow reliably. Otherwise, sporulation could have been markedly impaired what would cause low spore yield or spontaneous germination of the spores because of low spore stability. However, the levels must not be too close to the vegetative cells' optimal growth conditions, not to cause too small differences in the target value, i.e., the spores' resistance, which would cause an insignificant influence on the outcome variables. The levels and center-points we established on this basis were 51, 57, and 63 °C for the incubation temperature and pH 6.0, 7.0, and 8.0 for the initial medium's pH. Based on this experimental domain, the software JMP® Pro created a randomly generated experimental plan to obtain statistically significant results while reducing test runs. According to this plan, the spores were produced under various combinations of sporulation temperature and pH, and the inactivation curves were obtained (Table 2-1 and Figure 2-1). Then, the data were fitted by the method of least squares (using the software JMP® Pro), resulting in the calculated $D_{25°C,35\%H2O2}$-values also shown in Table 2-1.

The goodness of fit was checked in an Actual by Predicted plot (Figure 2-2). The plot provides a visual assessment of the goodness of fit and reflects variation due to random effects.

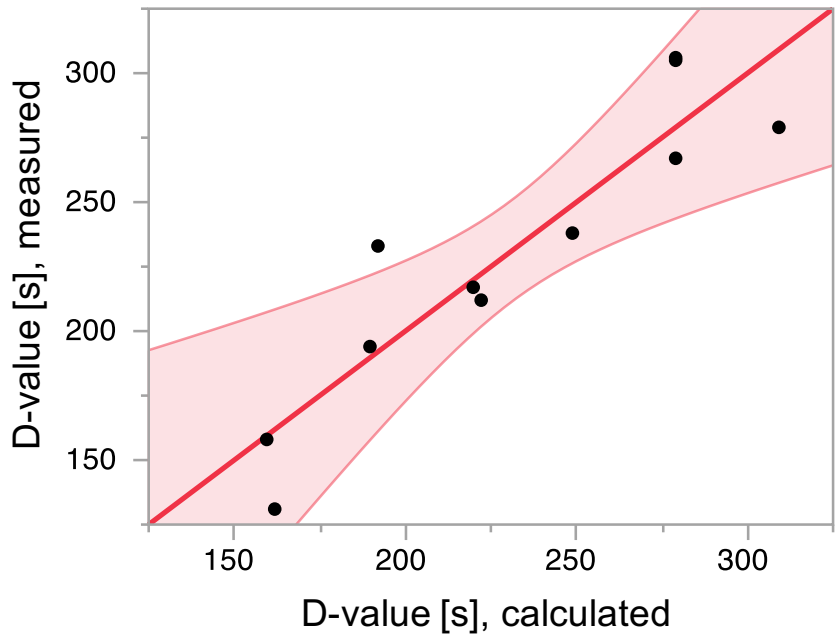

Figure 2-2. Actual by Predicted plot. RSME = 27.621, r^2 = 0.83, p-value = 0.0041. Significance level (α, area around the red angle bisector) = 0.05. The plot confirms the good approximation of the mathematical equation (Equation (2-1)) to the measured D-

values. Since the p-value is less than the significance level of 0.05, the data is statistically significant.

For a good approximation, the data points must be on or near the angle bisector (Figure 2-2, red line). The coefficient of determination (r^2) was 0.83, meaning that 83% of the dependent variable variance is predict- able from the independent variables. The root-mean-squared error (RMSE) indicates how well the function curve is adapted to the data. It states by how much, on average, the estimate deviates from the measurement and may only be viewed in connection with the data itself. The RMSE, in this case, was 27.621, which means an average deviation of the $D_{25°C,35\%H2O2}$-values of 28 s. Since the p-value is 0.0041, the data is statistically significant.

3.2.3 Results and Discussion

The impact of the sporulation conditions on the hydrogen peroxide resistance can be displayed graphically as a Response Surface plot. In Figure 2-3, the $D_{25°C,35\%H2O2}$-value is plotted as a function of the sporulation medium's initial pH and the sporulation temperature.

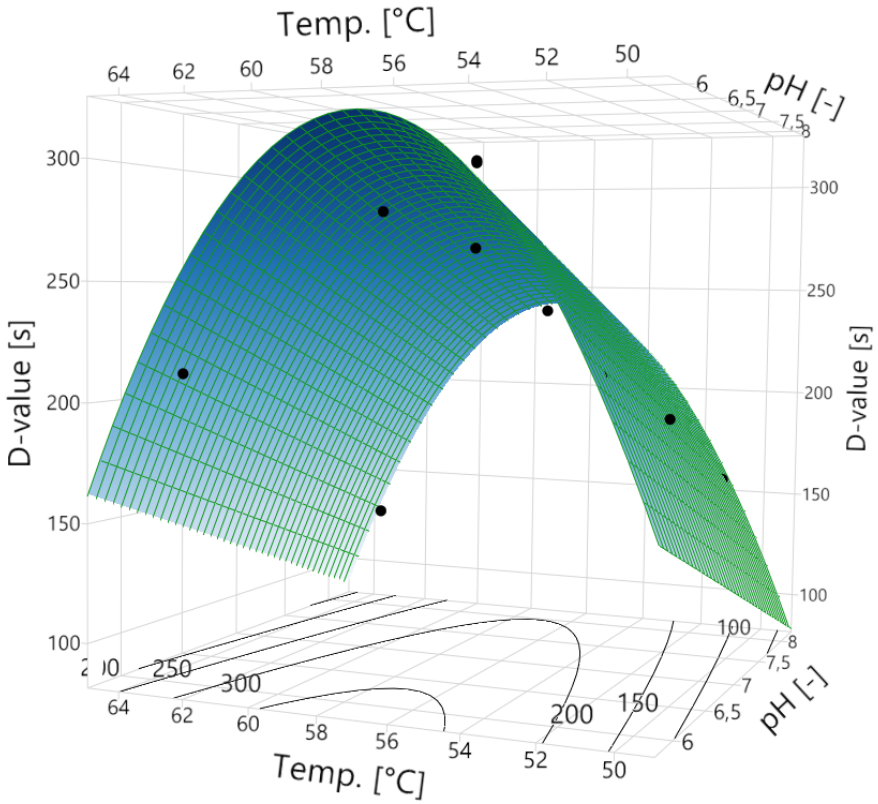

Figure 2-3. Response Surface plot. $D_{25°C,35\%H2O2}$-value of *G. stearothermophilus* ATCC 7953 spores as a function of the initial pH of the sporulation medium and the sporulation temperature. The response surface represents the calculated $D_{25°C,35\%H2O2}$-

values for an infinite number of combinations of the sporulation temperature and pH within the domain examined. The black points represent the measured $D_{25°C,35\%H2O2}$-values of the experimental plan (some are covered by the response surface).

Equation (2-1) describes the function on which the Response Surface plot is based, where $f_{(x,y)}$ describes the $D_{25°C,35\%H2O2}$-value [s], x represents the sporulation temperature [°C], and y represents the initial pH [-] of the sporulation medium:

$$f(x,y) = 279 + (-30.17) * (y-7) + 1.17 * \left(\frac{(x-57)}{6}\right) + \left(\frac{(x-57)}{6}\right) * \left(\frac{(x-57)}{6} * (-88.17)\right) \quad \textbf{(2-1)}$$

In the domain examined (51-63 °C, pH 6.0-8.0) $D_{25°C,35\%H2O2}$-values of 160 s-309 s with an RMSE of 28 s could be achieved by different combinations of sporulation temperature and pH. The highest $D_{25°C,35\%H2O2}$-value of 309 s was obtained at an initial medium's pH of 6.0 and a sporulation temperature of 57 °C. The $D_{25°C,35\%H2O2}$-value decreased when temperature deviated from this optimum as well as when the pH increased from 6.0 to 8.0. At sporulation temperatures of 51 °C and 63 °C and a pH of 8.0, the resistance was lowest with a $D_{25°C,35\%H2O2}$- value of 160 s.

The influences of the sporulation conditions on the spores' resistance were also assessed for possible interaction. The analysis showed that the sporulation conditions did not interact with one another. So, in the case of solid-state produced *G. stearothermophilus* ATCC 7953 spores, the sporulation temperature and the pH in the domain examined affect the resistance independently of each other.

The fact that the sporulation pH and temperature did not interact is in contrast to a study based on observations made when assessing the resistance as a function of sporulation temperature and pH in submerged produced spores of *B. atrophaeus* (Stier and Kulozik, 2020a). In this study, sporulation pH and temperature interacted and therefore had a distinct influence on the spores' resistance depending on the combined values of pH and temperature. The approach to describe the results was the same in this study, but the strain and spore production methods differ. For this reason, it must be clarified through further tests whether interactions may occur in submerged spore production of *G. stearothermophilus* ATCC 7953.

In Figure 2-2, we were able to show that the data are statistically significant. On the one hand, this means that the calculated $D_{25°C,35\%H2O2}$-values reproduce the actual resistances with a high probability. On the other hand, it also means that the resistance of spores of *G. stearothermophilus* produced by the solid-state method to hydrogen

peroxide can be manipulated explicitly by the defined influence of sporulation pH and sporulation temperature, compared to random variations of resistance often observed in practical applications.

Based on this fact, it was possible to generate a response surface plot (Figure 2-3) describing the influence of the sporulation conditions investigated in this study on hydrogen peroxide resistance. It is striking that the resistance decreased symmetrically from the optimal conditions depending on the sporulation temperature. Thus, the influence of hydrogen peroxide on spores was equally for spores formed at sporulation temperatures of, e.g., 60 °C and 54 °C (at the same sporulation pH). The highest hydrogen peroxide resistance was found at a sporulation temperature of 57 °C. This temperature also represents the vegetative cells' optimum growth temperature and is in line with Mtimet et al. (2015), who also observed the highest resistance at this sporulation temperature for *G. stearothermophilus* ATCC 12980 against heat.

Regarding the sporulation pH, the highest hydrogen peroxide resistance in the domain examined could be achieved at pH 6.0. Since the resistance increased linearly from pH 8.0 to 6.0, no statements can be made about the actual pH optimum and the then achievable $D_{25°C,35\%H2O2}$-value. Nonetheless, this shows that the effects of sporulation pH on sensitivity to hydrogen peroxide were independent of sporulation temperature. However, it must be said that the pH only represents the initial pH of the sporulation medium, and we cannot make any statements about the actual pH at which the spores formed, as the pH will have changed as the cells have grown. Mtimet et al. (2015) found for *G. stearothermophilus* ATCC 12980 that the pH of nutrient sporulation agar within the range of 5.60 and 7.30 changed only by 0.1-0.2 units to the initial medium's pH during incubation. Therefore, it can be assumed that the respective initial pH of the sporulation medium we used in this study has not significantly changed. Comparing our results with other studies is difficult as most address different resistance mechanisms, and there are no studies on the hydrogen peroxide resistance of *G. stearothermophilus*. However, Guizelini et al. (2012) investigated the effect of the sporulation pH on the heat resistance of *G. stearothermophilus* ATCC 7953 spores produced on sporulation agar. They found the highest heat resistance at a sporulation pH of 8.5 that decreased towards pH 7.8 and pH 9.2. In contrast, Mtimet et al. (2015) found the highest heat resistance for *G. stearothermophilus* ATCC 12980 at a sporulation pH between 5.83 and 7.10 (no significant difference between these pH-values) with a significantly lower heat resistance at a sporulation pH of 7.95. The highest resistances were thus achieved in the area of optimal growth conditions. Nevertheless, it has been shown that even slight deviations from the optimal conditions lead to a significant change in the achievable resistance. This is confirmed by our data describing the effects of sporulation temperature and pH on hydrogen peroxide resistance of *G. stearothermophilus* ATCC 7953.

Our results provide information about the impact of sporulation temperature and pH on hydrogen peroxide resistance, as well as the degree of their effects. It has been shown that these influencing factors can be used independently of one another as control variables for manipulating the resistance and that no side effects due to interactions have to be feared. The results can be quickly transferred to practical application using the mathematical equation derived and enable a targeted adjustment of the hydrogen peroxide resistance in BI production. Commercial BI producers can now be aware of the effects of changes or fluctuations in sporulation temperature and pH on hydrogen peroxide resistance. This will help avoid the variability of commercial BI and uncertainties related to this issue and, thus, enable spores' production with standardized resistance. For a cross-company standardization of BI resistance, however, the effects of additional factors, such as the sporulation medium's composition or the spore preparation method, must be examined in further studies to take into account that commercial BI producers use different media and methods for market-relevant reasons.

3.2.4 Conclusion

This study examined the effects of the sporulation temperature (51-63 °C) and pH (6.0-8.0) on the hydrogen peroxide resistance of solid-state produced *G. stearothermophilus* ATCC 7953 spores. The key findings are that the sporulation temperature and pH highly impact the hydrogen peroxide resistance. In the domain examined, the sporulation temperature had a quadratic effect, and the sporulation pH had a linear effect on the resistance, with no interaction between these factors. Resistance was shown to decrease equally whether sporulation temperature increased or decreased from optimum, and the effect of sporulation pH on the resistance did not change when changing the sporulation temperature. In terms of novel insights, this means that the sporulation temperature and pH can be used independently of one another as control variables for manipulating the resistance of solid-state-produced *G. stearothermophilus* spores. In terms of the practical meaning of the results, it could be shown that resistance can be very precisely adjusted by specific manipulation of sporulation pH and sporulation temperature during solid-state production of *G. stearothermophilus* spores. This is supported by the statistical significance of the data and makes it possible to produce bioindicators to validate sterilization processes using hydrogen peroxide with specific resistance, thus making validation procedures more reliable and avoiding unexpected false-positive or false-negative test results. Therefore, it can be stated that the use of the results can be a decisive step towards standardized resistance and greater reliability of BI when performing validation and sterilization challenge tests. At which level the resistance should be defined would require agreement between all stakeholders, i.e., BI producers and their clients applying BI. Additionally, there must be a consensus on the sporulation medium's choice and the spore preparation method for cross-com-

pany standardization. Further progress in standardizing *G. stearothermophilus* BI resistance could be achieved by submerged spore production with better control of pH and other milieu conditions.

3.2.5 Acknowledgments

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3.2.6 Author contributions

Philipp Stier: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. Sabrina Maul: Investigation, Writing – review & editing. Ulrich Kulozik: Writing – review & editing, Supervision, Funding acquisition.

3.2.7 References

The references were combined into a joint list of all publications at the end of the thesis.

3.3 Submerged Bioreactor Production of *Geobacillus stearothermophilus* ATCC 7953 Spores for Use as Bioindicators to Validate Hydrogen Peroxide Inactivation Processes

Summary and contribution of the doctoral candidate

A method for submerged production of *G. stearothermophilus* spores for use as BI against hydrogen peroxide was developed.

For this purpose, the vegetative cells of *G. stearothermophilus* were grown submersed in a cultivation medium (*Geobacillus* Medium 9, GM9; developed in-house) at 57 °C, 150 rpm for 16 h. Subsequently the cells were transferred to a modified sporulation medium (Submerged Sporulation Medium, SSM) in a bioreactor at 57 °C, pH 7.0 with 30% minimal oxygen saturation for 48 h. In this regard, two methodological approaches for the transfer of the cells into the sporulation medium were used and compared. On the one hand, the cells were washed before transfer to remove used cultivation medium including metabolites. On the other hand, the cells were transferred into the sporulation medium without a washing step.

The data showed that no free spores formed in the method in which the cells were washed before the transfer to the sporulation medium. In contrast, free spores were formed with the latter method, in which the cells were not washed before the transfer. The total yield of germinable spores was determined. The yield of germinable spores was (1.0-1.1) x 10^6 CFU/mL with the first approach and (3.9-4.1) x 10^6 CFU/mL with the second. In total, calculated back to the unpurified suspension in the bioreactor, the yield of liberated spores was 1.6 x 10^7 CFU/mL.

Since in the first methodological approach no free spores were produced, only the spores of the second approach could be tested for their resistance against hydrogen peroxide (35%, 25 °C). The results were compared with the resistance of commercially available spores. The resistance of the spores produced by the developed method ($D_{25°C,35\%H2O2}$ = 73 s) was within the range of commercially available BI of *G. stearothermophilus* ($D_{25°C,35\%H2O2}$ = 54-154 s).

The results show the importance of the methodological approach for the submerged production of *G. stearothermophilus* spores. The production of the *G. stearothermophilus* spore requires a specific procedure. The resistance of the spores produced in this study correspond to the resistance of commercially available spores, which means that the developed method could be directly transferred to industrial production without a change in the established BI resistance.

The doctoral candidate developed the concept of the experiments and established the methods. He critically reviewed the existing literature. Furthermore, he analyzed the

data and interpreted the results. Additionally, the doctoral candidate wrote the manuscript and revised it. The co-author contributed to the experimental part and/or to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript [1]

Submerged Bioreactor Production of *Geobacillus stearothermophilus* ATCC 7953 Spores for Use as Bioindicators to Validate Hydrogen Peroxide Inactivation Processes [2]

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Abstract

In the food and pharmaceutical industries, evaluating the sterilization performance preceding aseptic production processes is of central importance. In the case of hydrogen peroxide sterilization of solid surfaces, bioindicators (BI) consisting of spores of *Bacillus atrophaeus* or *Geobacillus stearothermophilus* are used to validate the effectiveness and efficiency of the inactivation procedure. Commercial production of *G. stearothermophilus* is commonly performed on agar plates, where cultivation and sporulation conditions are not well-defined. Therefore, the produced BI can vary in their resistance, which in turn creates unacceptable uncertainties in the evaluation of aseptic processes. Submerged production in the bioreactor would allow more control over sporulation conditions, while reducing production time, resistance variability, and avoidance of false-positive or false-negative test results. In addition, submerged production of *G. stearothermophilus* so far was a challenge to achieve sufficiently high spore concentrations for BI production. This study reports on the development of a method for submerged production of *G. stearothermophilus* spores (pH 7.0, 57 °C, 30% pO_2) that can achieve 1.6×10^7 spores/mL with a resistance against 35% H_2O_2 at 25 °C of $D_{25°C,35\%H2O2}$ = 73 s. This resistance ranks within the range of commercially available BI, making the results directly transferable to industrial applications.

Keywords

aseptic production, product safety, sterilization, validation;

[1] Adaptions refer to formatting, e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units and spelling.

[2] Originally published in: Methods Protoc. (2021), 4, 63; doi:10.3390/mps4030063

3.3.1 Introduction

In the pharmaceutical and food industries, many production processes are operated under aseptic conditions, where the sterility of all materials and equipment in contact with the product is of central importance for the safety and shelf-life of the products. To ensure aseptic conditions, sterilization performance must be validated regularly under defined conditions. Bioindicators (BI), which consist of specific microorganisms highly resistant to the sterilization process, are used for this purpose. Employing such BI, it is possible to assess whether the conditions necessary to achieve sterility are applied to inactivate a specific number of germs. A commonly applied inactivation target level is a reduction by 10^5 colony forming units (CFU). For validation of sterilization processes using hydrogen peroxide as the sterilant, BI made of spores from *Bacillus atrophaeus* (ATCC 9372) and *Geobacillus stearothermophilus* (ATCC 7953) have become widely accepted as test BI, representing high resistance, thus covering the potential worst-case scenario of natural contamination situations (FDA, 2007; McLeod et al., 2017; VDMA, 2021). When validating sterilization performance with BI, it is essential that the spores used have a defined resistance to the sterilization process. Otherwise, false-positive or false-negative validation results may occur. False-positive validation results would lead to the sterilization being incorrectly considered successful. This, in turn, would lead to a high proportion of non-sterile products in regular production, a reduction in product shelf-life, and, in the worst case, a health risk for the consumer. On the other hand, false-negative validation results would result in the wrong assumption that the sterilization was ineffective. While this does not impair product safety or shelf stability, this result would trigger harsher sterilization conditions and would thus induce higher environmental impact, over-processing, and increased costs.

However, as reported by BI users associated with us and according to oral information from member companies of the ‘German Association of Machinery and Processing Equipment Manufacturers’ (VDMA e.V.), these scenarios are a real threat, as the resistance of commercial BI varies irregularly and is mostly even unknown to the user. It can be assumed that the variation in BI resistance stems from the fact that manufacturers use different cultivation methods or inconsistent, variable sporulation conditions to produce the spores, as there are no uniform standards for BI production to date. For example, the medium or spore preparation used for spore production has not been standardized, even though it is known to significantly impact the resistance to heat or low-energy electron beam (LEEB) (Bressuire-Isoard et al., 2018; Wells-Bennik et al., 2018; Zhang et al., 2018). On the other hand, BI users report, based on practical experience, that even the resistance of spores from the same manufacturer can differ from batch to batch, which is a particular problem when neither supplier nor client are aware of it. The potential reasons for these variations have not yet been sufficiently investigated. It can be assumed that, in addition to a certain natural variability of spore

resistance, this is caused by even slight variations of the conditions during spore production. In particular, the production of spores by the solid-state method on agar plates is suspected of causing resistance variations because the sporulation conditions can only be poorly controlled and hardly influenced. For instance, it is possible to affect the sporulation temperature, but it is not possible to directly influence the pH during sporulation since the pH of the agar plates is only set initially and changes due to cell metabolism during incubation (Mtimet et al., 2015).

However, the control of these influencing factors is of decisive importance because it was already shown in some studies that sporulation conditions, temperature and pH in particular, have a significant influence on the resistance of spores, e.g., against heat (Baril et al., 2012a; Guizelini et al., 2012; Mtimet et al., 2015), LEEB (Zhang et al., 2018), hydrogen peroxide (Eschlbeck et al., 2017), or other chemical sterilizing agents (Cortezzo and Setlow, 2005), which is commonly explained by the influence of the sporulation conditions on the spore structure (Abhyankar et al., 2016; Bressuire-Isoard et al., 2016; Isticato et al., 2019; Setlow, 2014). These findings clearly show that even minor variations in sporulation conditions can significantly impact resistance, making control and precise management of influencing factors all the more critical. This fact has been known for some time but is still part of ongoing research.

Submerged BI production would allow better and more precise control of the sporulation conditions. With this method, not only the sporulation temperature and sporulation pH could be controlled and influenced in real-time, but also the oxygen saturation of the medium. Moreover, on agar plates, the cells within a colony can differ strongly, depending on the position at the surface or in the center and depending on the dynamically changing population development. For *Bacillus subtilis*, motile cells and matrix formers are known to form while another portion of the cells is already sporulating (Vlamakis et al., 2008). This means that not all cells have the same status, and sporulation is not uniform when BI are produced on agar plates until all nutrients are eventually depleted. Compared to that, in submerged spore production, the cells are constantly mixed and are evenly supplied with nutrients and oxygen. As a result, they do not form a biofilm with highly differentiated cells and thus have a more uniform status. This means that in submerged spore production, sporulation can be induced in a more targeted manner since nutrient shortage, which is usually used as a sporulation trigger, arrives more uniformly and at the same time for all cells. Therefore, the cell population in the bioreactor sporulates within a much smaller time interval between cells. For this reason, a lower resistance variability can be expected from submerged BI production conditions.

Another advantage relevant to the BI manufacturing industry is that spore production in the submerged process in the bioreactor takes significantly less time. Spores are usually ready for harvesting after 48 h of incubation in the bioreactor, whereas spores are incubated on agar plates for approximately 10 days and have to be manually

scraped off and purified from the individual, usually hundreds of agar plates. For these reasons, in some instances, BI from spores of *B. atrophaeus* are already produced in the bioreactor. Thus, submerged production of *G. stearothermophilus* spores could be a way to reduce resistance variability of commercial BI and, therefore, to eliminate the uncertainties in validation described above. However, according to practical experience from commercial BI producers (verbal information), this has not yet been satisfactorily achieved for the production of *G. stearothermophilus* spores for unspecified reasons.

In the production of *B. atrophaeus* spores, we found in preliminary experiments that media suitable for solid-state production are often qualified for the submerged spore production as well. However, in the case of *G. stearothermophilus*, this was not even remotely the case in any of the media we tested (data not shown). Instead of sporulating, the cells died in the sporulation media. Thus, unlike *B. atrophaeus*, *G. stearothermophilus* seems to have distinctly different requirements for liquid media compared to solid media. Additionally, we also found in preliminary experiments that vegetative cells of *G. stearothermophilus* had to be handled differently. In contrast to the mesophilic *B. atrophaeus*, *G. stearothermophilus* is thermophilic with no significant growth at temperatures around 37 °C and below (Wells-Bennik et al., 2018). Accordingly, the handling of the cells and the methodological procedure must be adapted and cannot simply be adopted from the production of *B. atrophaeus* spores.

We hypothesized that submerged spore production of *G. stearothermophilus* contributes to a reduction in resistance variability because of the more defined and uniform cultivation and sporulation conditions, as described above. Therefore, we considered submerged production of *G. stearothermophilus* spores as the logical development in BI production.

However, the appropriate frame of conditions must first be identified to produce *G. stearothermophilus* spores with a suitably high spore concentration. In addition, the methodology must be robust and suitable for use in commercial spore production. Commercial BI of *G. stearothermophilus* often have a concentration of 10^7 CFU/mL with a sales volume per vial of 10 mL. In our experience and according to industry reports, commercial BI are produced at a scale of 20-30 L. Thus, if our results for the production of *G. stearothermophilus* were applied to this scale, a large spore amount with suitable concentrations could be expected, which would be highly relevant for industrial application. To achieve this goal, we developed a methodological approach for submerged *G. stearothermophilus* spore production. For this purpose, we use different media for cultivation and sporulation because sporulation is an emergency mechanism of cells, and sporulation media that reproduce these conditions are unsuitable for cultivating vital cells and increasing cell mass.

For submerged production of *B. atrophaeus* spores in a bioreactor, it is common practice to wash the inoculum before transferring the vegetative cells into the sporulation medium to remove metabolites and residues of the spent cultivation medium. On the one hand, this complex step introduces a source of variation and does not meet the demands of thermophilic *G. stearothermophilus* since the cells cool down very quickly due to washing and media changes outside the incubator. On the other hand, we suspected that there are specific triggers and metabolites in the spent medium that promote sporulation of the cells and that the washing step loses these. We therefore assumed that omitting the washing step, in addition to suitable cultivation and sporulation medium, would have a significant effect on spore yield, even though it transfers nutrients or media residues to the sporulation medium.

Thompson and Thames (1967), in 1967, already produced submerged spores of *G. stearothermophilus* (ATCC 7953). According to the current view, their cultivation of the cells was relatively rudimentary in milk dilution bottles without gassing or shaking. Sporulation was then performed in a hypering flask with a stainless-steel gas (air) dispersion tube in a water bath at 60 °C. Despite this relatively simple set-up, it was possible to produce *G. stearothermophilus* spores at concentrations up to (1-2) × 10^7 spores/mL. However, liberated spores were rarely encountered and the cells clumped together during growth. Thus, these spores would not be suitable for use as BI because cell debris, the presence of endospore mother cells remnants, and of spore agglomerates may have a significant impact on BI resistance and storability.

Nevertheless, this study showed that submerged production of *G. stearothermophilus* was possible in principle. Therefore, we developed a new method to produce spores of *G. stearothermophilus* submerged in the bioreactor according to current standards while omitting the washing step of the inoculum. Our goal was to obtain as many liberated spores as possible, suitable for use as BI.

To investigate the influence of the washing step on spore yield, *G. stearothermophilus* spores were produced using both methods (with and without the washing step of the inoculum), but with otherwise identical sporulation conditions (pH 7.0, 57 °C, minimum oxygen saturation of 30%), and spore yield was compared. To determine the resulting spore resistance compared to spores of commercial BI, a resistance test was performed in liquid 35% hydrogen peroxide at 25 °C, in a similar and standardized procedure as commonly applied in industrial BI production.

3.3.2 Materials and Methods

Strain

Spores of *Geobacillus stearothermophilus* (ATCC 7953) with a concentration of 10^7 colony forming units (CFU)/mL were suspended in aliquots of 500 µL each in 70%

ethanol and stored at -80 °C. This enabled the spores to be taken without thawing the remaining spore suspension to ensure that the spores' properties did not change over the experimental period.

Media

Geobacillus Medium 9 (GM9) was used as the pre-culturing medium, comprised of casein peptone 16 g/L (for microbiology, Gerbu Biotechnik GmbH, Heidelberg, Germany), yeast extract 10 g/L (for microbiology, Merck KGaA, Darmstadt, Germany), glycerol 13 g/L (Rotipuran® ≥ 99.5%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), KCl 2.5 g/L (ACS reagent, 99.0-100.5%, Merck KGaA), NaCl 5 g/L (ACS reagent, ≥99.0%, Merck KGaA), NH_4Cl 2 g/L (for molecular biology, ≥99.5%, Merck KGaA), $MgSO_4$ $7H_2O$ 0.8 g/L (ACS, ≥99%, Carl Roth GmbH & Co. KG), $MnCl_2$ $4H_2O$ 0.003 g/L (ACS reagent, ≥98%, Merck KGaA), and sodium pyruvate 5 g/L (for biochemistry, ≥99.0%, Merck KGaA), pH 7.0. This medium is a custom-designed medium established in pre-work for vegetative growth of *G. stearothermophilus* ATCC 7953 (data not shown).

Submerged Sporulation Medium (SSM) modified from Thompson and Thames (1967) was used for spore production in the bioreactor, comprised of casein peptone 30 g/L (for microbiology, Gerbu Biotechnik GmbH), K_2HPO_4 0.3545 g/L (ACS reagent, ≥98%, Merck KGaA), KH_2PO_4 0.177 g/L (ACS reagent, ≥99%, Merck KGaA), and $MnSO_4$ H_2O 0.015 g/L (≥99%, p.a., ACS, Carl Roth GmbH & Co. KG).

Pre-Culture

An aliquot of 500 µL of the stored *G. stearothermophilus* ATCC 7953 spore suspension was inoculated in 400 mL of GM9 in a 1 L baffled shaking flask (Schott Duran® baffled culture flask, Erlenmeyer shape, straight neck for metal caps; Duran Group GmbH, Mainz, Germany). Incubation took place at 57 °C, 150 rpm, for 16 h. After incubation, the optical density at 600 nm (OD_{600}) was 3.9. Following the methodological approach from Thompson and Thames (1967), the pre-culture was not washed but only homogenized and inoculated into the bioreactor, as described in the next section. To compare this method with standard practice, in a second experiment, we washed the cells before transferring them into the sporulation medium. This washing step was carried out by centrifugation (4000× g, 10 min, 25 °C) of the pre-culture, followed by discarding the supernatant and resuspending the vegetative cells with Milli-Q water (25 °C).

Submerged Spore Production

One goal was to make sporulation conditions in submerged production as controllable as possible. This was achieved by automatically controlling and influencing the sporulation pH, sporulation temperature, and oxygen saturation in the sporulation medium. Second, our methodological approach should reflect the state-of-the-art and industrial

BI production. This is particularly important for ensuring that the experimental results can be transferred to industrial applications without any hurdles. Additionally, it will allow us to compare the resistance of the obtained spores with commercial BI.

For this reason, we used a 2 L laboratory bioreactor (Sartorius Stedim Biostat® A, Sartorius AG, Göttingen, Germany) for spore production. The bioreactor was equipped with two 6-blade disk stirrers, a flow breaker, a sparger, a heating jacket, a cooling finger, an anti-foam probe, a pH probe, a pO_2 probe (measuring oxygen saturation), exhaust air cooling, and real-time control of oxygen saturation, pH, and temperature. The stirring speed and the gassing rate (gassing with air) were controlled automatically depending on the preset minimum oxygen saturation in the medium. If the oxygen saturation in the medium fell below the minimum value, the gassing rate increased. An increase in the stirrer speed was not necessary for the experiments carried out. The anti-foam agent Korasilon FG 30 (concentration of 3 mL/500 mL Milli-Q water, Kurt Obermeier GmbH & Co. KG, Bad-Berleburg, Germany) was added, fully automatically, as needed. The pH was adjusted fully automatically with 0.5 M HCl (ACS reagent, 37%, Merck KGaA) and 0.5 M NaOH (≥98%, pellets (anhydrous), Merck KGaA).

For spore production without the inoculum washing step, the pre-culture was inoculated directly at a ratio of 1:3 into the SSM provided in the sterilized bioreactor (total volume 1 L), and incubation was started. The OD_{600} at the beginning of the spore production in the bioreactor was 1. For the spore production method that includes the inoculum washing step, the washed pre-culture was also inoculated to an OD_{600} of 1 (total volume 1 L). The minimum oxygen saturation was set at 30%, pH at 7.0, and temperature at 57 °C. The incubation time was 48 h, sufficient for sporulation and maturation of spores. These optimal sporulation conditions were determined in preliminary experiments. Subsequently, the spores were harvested and purified. The spore production was performed as a biological duplicate.

Spore Harvesting and Purification

After incubation and sporulation of the cells in the bioreactor, the suspensions were entirely harvested. The obtained spore suspensions were further purified, largely according to Stier and Kulozik (2020a), by separating the cell debris, endospore-containing mother cells, and liberated spores by several washing, mixing, and centrifugation steps. For this purpose, the suspension of each spore production run was divided equally into two 1 L centrifuge bottles and centrifuged at 4000× g for 10 min at 4 °C. After discarding the supernatants, the pellets were resuspended with 70 mL of 4 °C cold Milli-Q water each and equally divided into four 50 mL centrifuge tubes, resulting in 35 mL suspension in each centrifuge tube. After another centrifugation at 4000× g for 10 min at 4 °C, the supernatants were again discarded, and the pellets were resuspended with 35 mL each of fresh, 4 °C cold Milli-Q water. This washing step was re-

peated three to five times until the supernatant was clear. Then, after another centrifugation at 4000× g for 10 min at 4 °C, the pellets of the four centrifuge tubes were transferred in equal amounts into two 50 mL centrifuge tubes by discarding the supernatant and resuspending and combining the pellets with 5-10 mL of Milli-Q water. The centrifuge tubes were then made up to 40 mL with 4 °C cold Milli-Q water. The suspensions were shaken overnight at 480 rpm at 4 °C to help break down any remaining agglomerates consisting of cell debris and spores. The next day, the suspensions were centrifuged one more time (4000× g for 10 min at 4 °C), and the supernatant was discarded and resuspended with 35 mL of 4 °C cold Milli-Q water. In a final centrifugation step, the suspensions were centrifuged at 6000× g for 90 min at 4 °C, resulting in the formation of solid pellets. These pellets consisted of three phases: the top phase containing cell debris, the middle phase containing endospore-containing mother cells, and the bottom phase having pure spores. Since the sedimented pellet was very firm and the upper two phases less firm with a rather slimy consistency, the phases could be well-separated after centrifugation. For this purpose, first, the supernatant was discarded, and carefully, 3-5 mL of 4 °C cold Milli-Q water was added to the pellet and mixed vigorously. The vigorous mixing caused the two upper phases to separate slowly from the lower phase. Unlike the purification of *B. atrophaeus* by the same method, the upper phase of *G. stearothermophilus* was slightly more solid. If, in this case, the vigorous mixing was not sufficient, the phases were scraped off very carefully with a pipette tip and then washed further with some fresh, 4 °C cold Milli-Q water and vigorous mixing. The pure spore pellet was then resuspended with 45 mL of 4 °C cold Milli-Q water. The successful purification was confirmed using phase-contrast microscopy to verify that the spore suspension contained only liberated spores (Figure 3-1). The purified spore suspension was stored at 4 °C under the exclusion of light for further tests.

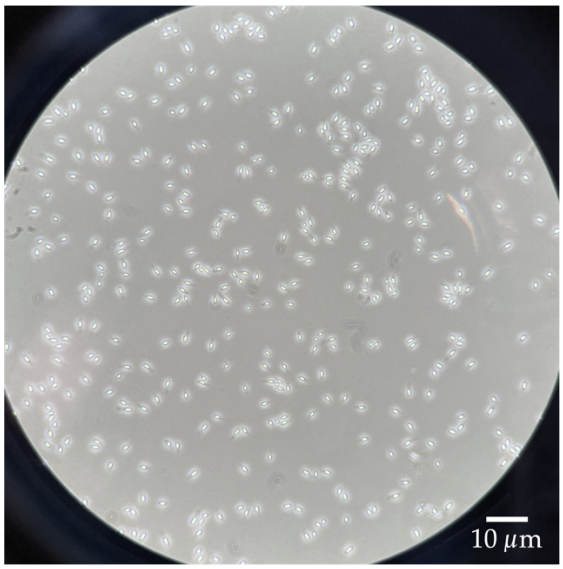

Figure 3-1. Purified spores of *G. stearothermophilus* (ATCC 7953) under the phase-contrast microscope (100x).

Determination of the Spore Resistance

To measure the resistance of spores, we determined the D-value, which describes the time required to inactivate 1-log of the spore population under constant inactivation conditions. We determined the $D_{25°C,35\%H2O2}$-value in liquid 35% hydrogen peroxide (OXTERIL® 350 Spray, Food Grade, Evonik Industries AG, Essen, Germany) at 25 °C, in analogy to Stier and Kulozik (2020a). For this purpose, after approximately one week of storage, the purified spore suspension was first homogenized by vigorous mixing for 1 min, treatment in an ultrasonic bath for 20 min, and vigorous mixing for another minute. Then, 100 µL of this suspension was transferred into 9.9 mL of liquid 35% hydrogen peroxide, which was previously adjusted to a constant temperature of 25 ± 0.1 °C in a thermomixer. Inactivation in the hydrogen peroxide took place at 450 rpm to ensure uniform distribution of spores in the sterilant. At defined time intervals, after 30, 60, 90, 120, 180, and 300 s, 100 µL of the sample was drawn and transferred to a test tube filled with 9 mL of Ringer's solution, 800 µL of Milli-Q water, and 100 µL of 1:5 diluted catalase (Catalase from *Micrococcus lysodeiktikus*, solution, activity 65,000-150,000 U/mL, Merck KGaA). The substantial dilution of the sample and the presence of catalase, which enzymatically degrades the hydrogen peroxide, instantaneously stopped the inactivation reaction. The catalase concentration was chosen to stop the inactivation reaction as quickly as possible without causing the test tube to foam over due to the release of oxygen from the degradation reaction. Dilutions were prepared from the samples in Ringer's solution and plated out on Plate Count Agar (casein peptone 0.5%, for microbiology, Gerbu Biotechnik GmbH; yeast extract 0.25%, for microbiology, Merck KGaA; glucose 0.1%, for biochemistry, Reag. Ph Eur., 97.5-102.0%, Merck KGaA; agar-agar 1.5%, for microbiology, Carl Roth GmbH & Co. KG; pH 7.0) with a

Drigalski spatula to determine the number of spores still capable of germination. To determine the spore concentration at time 0 s, i.e., before inactivation, the untreated suspension was also diluted and plated out. These cells were treated in the same way as the other samples, except for treatment with hydrogen peroxide. Incubation was performed at 57 °C for a total of 48 h, and colonies were counted after 24 and 48 h. The total cell count after 48 h was used for the determination of the inactivation curve. The agar plates were packed in plastic bags during incubation to prevent the agar plates from drying out due to the relatively high incubation temperature. The optimal incubation conditions of 57 °C and pH 7.0 were intended to ensure no inhibitory effects due to suboptimal recovery conditions, described in Trunet et al. (2015). The resistance test was performed in a technical triplicate. From the obtained colony counts of the dilutions, the mean value was formed and taken logarithmically as a function of the inactivation curve. The $D_{25°C,35\%H2O2}$-value corresponds to the negative reciprocal slope of a best-fit straight line through the measurement data points.

3.3.3 Results

The spore suspensions from the two different methodological approaches, with and without washing the inoculum before transfer in the sporulation medium, already differed by an extreme color difference. The spore suspension in which the inoculum was washed before inoculation into the sporulation medium, according to the classical approach, was significantly lighter in color than the spore suspension inoculated without the washing step. In the purification process, the normally three-phased pellet, obtained before the final step of purification, showed only two phases. The bottom phase, which usually contains free spores, was utterly absent in the classical approach. Examination under the phase-contrast microscope confirmed that the suspension had only cell debris and endospore-containing mother cells. In contrast, the suspension in which the inoculum was not washed showed three phases in the pellet, as expected. Examination under a phase-contrast microscope showed that the bottom phase consisted of liberated spores. In addition, the pellet was almost twice as large as the pellet of the classical approach, which explains the darker color of the suspension due to the higher spore concentration overall (endospores and free spores).

Since there were no liberated spores in the pellet of the classical methodological approach, the number of germinable spores could only be determined with the endospore-containing mother cells, and was (1.0-1.1) × 10^6 CFU/mL on Plate Count Agar. To compare this value with the suspension obtained by omitting the washing step of the inoculum, the number of germs from the two bottom phases, i.e., endospore-containing mother cells and liberated spores, was determined for this suspension, as well. The number of germinable spores was about four times higher, at (3.9-4.1) × 10^6 CFU/mL. The determination of the spore concentration of liberated spores, calculated

back to the unpurified suspension, resulted in a yield of liberated spores of approximately 1.6×10^7 CFU/mL in the bioreactor. This result clearly shows the superiority of the developed production method in terms of spore yield, in which the inoculum was not washed before transfer into the sporulation medium.

Sporulation of cells represents an emergency mechanism for surviving adverse environmental conditions. Thus, one might think that the cells should have a higher number of spores, separated from the remaining nutrients of the cultivation medium due to the washing step and inoculated into a medium optimized for sporulation. Moreover, the washing step inevitably cools the suspension, representing an additional deterioration of the environmental conditions.

However, it cannot be conclusively stated why it is that the method leads to the formation of larger quantities of spores without this washing step of the inoculum. It can be assumed that, as mentioned above, metabolites and triggers are contained in the cultivation medium, which promote sporulation and are thus carried over into the sporulation medium. Possibly, the cooling of the suspension by the washing step also leads to the fact that the cells are wakened and lyse instead of sporulating in the sporulation medium, which is not suitable for the pure cultivation of vegetative and vital cells by its nature. Thus, it appears that the otherwise uncommon transfer of spent cultivation medium results in significantly improved sporulation. Nevertheless, it must be mentioned that this changes the composition of the sporulation medium in an undefined way. In the case of BI production, however, this circumstance is of minor importance if the same methodical sequence is always followed from batch to batch, and is an acceptable disadvantage, in view of the possibility of submerged production of liberated spores.

The next step was to investigate whether the spores obtained had a resistance to hydrogen peroxide that was suitable for use as BI in the validation of sterilization processes. For this purpose, the resistance of the self-produced spores was compared with the resistance of suspended commercial spores. Since only liberated spores can be used to determine resistance, as the cell debris and residues of the mother cells could influence the resistance test, only the resistance of the spores from the modified production method could be subjected to a resistance test. For this purpose, the purified liberated spores (total amount of 90 mL per batch with a concentration of (1.7-1.8) x 10^8 CFU/mL) were exposed to liquid 35% hydrogen peroxide at 25 °C, and the resistance was determined as $D_{25°C,35\%H2O2}$-value from the inactivation curve. Figure 3-2 shows the inactivation curve of the two spore production runs in which the inoculum was not washed before inoculation into the sporulation medium.

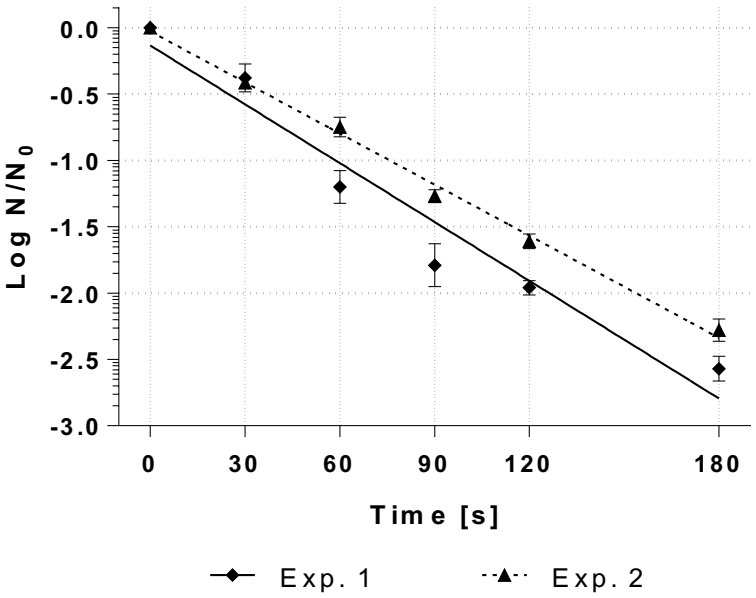

Figure 3-2. Inactivation curves with treatment with 35% hydrogen peroxide at 25 °C (technical triplicate) of *G. stearothermophilus* (ATCC 7953) spores produced submerged in the bioreactor at a sporulation temperature of 57 °C, sporulation pH of 7.0, and 30% minimal oxygen saturation. These spore suspensions were prepared without washing the inoculum before transferring into the sporulation medium. Some error bars are smaller than the symbols and thus not visible.

The treatment with hydrogen peroxide caused a linear inactivation of the spore suspensions of *G. stearothermophilus* (ATCC 7953). The average resistance of the technical triplicates of the spore suspensions was $D_{25°C,35\%H202}$-value = 68 s for experiment 1 and 78 s for experiment 2 of the double determination, which can be considered very close to each other.

For comparison with the resistance of commercial spores, the resistance of two BI spore suspensions of *G. stearothermophilus* (ATCC 7953) from an established BI manufacturer, without a declaration of a $D_{25°C,35\%H202}$-value, was determined in the same way. Figure 3-3 shows the inactivation curves of both BI as an average of the technical triplicates.

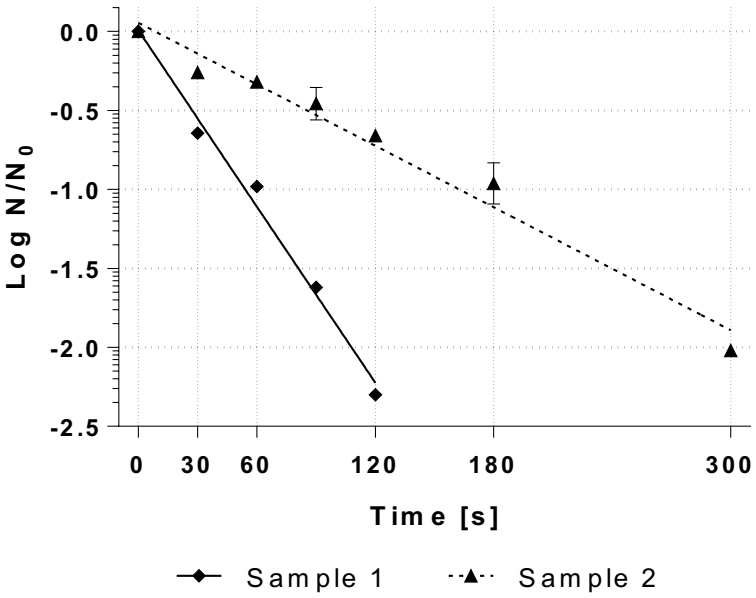

Figure 3-3. Inactivation curves with treatment with 35% hydrogen peroxide at 25 °C (technical triplicate) of BI samples of *G. stearothermophilus* (ATCC 7953) spores in suspension from two different batches of the same BI manufacturer. Some error bars are smaller than the symbols and thus not visible.

The average resistance of the commercial BI was $D_{25°C,35\%H202}$-value = 54 s for sample 1 and 154 s for sample 2. These resistance values are very far apart, assuming that the BI manufacturer probably did not change the method of spore production between these two batches. As described at the beginning, this showed that the resistance of commercial spores can vary greatly not only from manufacturer to manufacturer but also from batch to batch.

3.3.4 Discussion

Considering that the commercial BI shown in Figure 3-3 are regularly used for the validation of sterilization processes, this result also showed that the spores produced submerged in this study according to the developed method are in the resistance range against hydrogen peroxide that the industry expects for BI, but with lower variability within the batches. However, it is not known under which sporulation conditions and sporulation medium the commercial spores were produced. Therefore, no deeper comparison is possible.

In a recently published study (Stier et al., 2021), we produced spores of *G. stearothermophilus* (ATCC 7953) on agar plates. For spores produced under equal sporulation conditions (57 °C, initial pH of the sporulation agar of 7.0), but with the solid-state

method on agar plates, we obtained a $D_{25°C,35\%H2O2}$-value of 293 s on average, determined with the same resistance test described in this study. The resistance showed a difference in the $D_{25°C,35\%H2O2}$-value of up to 39 s within the experiments, although meticulous care was taken to avoid unwanted influencing factors during preparation causing resistance variability. For example, even the smallest differences in the pH of the medium were avoided, and the methodological sequence, media preparation, sporulation conditions, the purification process, and storage conditions where strictly adhered to. However, the resistance was significantly higher than that of the spores produced submerged. This can be justified by the fact that the spores were produced with a different medium as sporulation agar and, of course, with another production method for the reasons mentioned above. However, this shows that the variability even under experimental conditions is significantly greater for solid-state produced spores than for submerged produced spores, where the difference in $D_{25°C,35\%H2O2}$-value was only 10 s. This confirms the superiority of submerged spore production over solid-state production in terms of reducing resistance variability.

3.3.5 Conclusion

By the newly developed method for submerged production of *G. stearothermophilus* spores in the study presented, suitable spore concentrations and resistances could be obtained for use as BI against hydrogen peroxide. The key findings are that the proposed method obtained spores with concentrations of 10^7 CFU/mL, making the method directly transferable to industrial application. The resistance of the produced spores lies precisely in the resistance range with which BI users are already working, which means that there are no uncertainties when BI producers would have to switch to other resistances. Additionally, this method made it possible to significantly reduce resistance variability compared to solid-state production on agar plates, increasing the safety of using the spores as BI and reducing the occurrence of false-positive and false-negative validation results. The results obtained are thus of great interest both for industrial application and in terms of scientific aspects. In future studies, it has to be clarified to what extent resistance variabilities can be further reduced by submerged spore production. The first cornerstone in this direction has been laid with the described method. To further reduce uncertainties in the validation of sterilization processes, the standardization of BI production, i.e., the definition of methods, sporulation conditions in spore production, or establishing a standard resistance of BI for sterilization with hydrogen peroxide, is necessary.

3.3.6 Acknowledgments

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3.3.7 Author contributions

Conceptualization, P.S. and U.K.; methodology, P.S.; validation, P.S.; formal analysis, P.S.; investigation, P.S.; resources, P.S.; data curation, P.S.; writing—original draft preparation, P.S.; writing—review and editing, P.S. and U.K.; visualization, P.S.; supervision, U.K.; project administration, P.S. and U.K.; funding acquisition, U.K. All authors have read and agreed to the published version of the manuscript.

3.3.8 References

The references were combined into a joint list of all publications at the end of the thesis.

3.4 Comparison of one-step with two-step production of *Bacillus atrophaeus* spores for use as bioindicators

Summary and contribution of the doctoral candidate

A method for the optimization, i.e., simplification, of the submerged production of *B. atrophaeus* spores for use as BI against hydrogen peroxide was investigated. Therefore, the previously established two-step approach (cultivation of the vegetative cells of *B. atrophaeus* in Terrific Broth (TB) and sporulation in Difco Sporulation Medium (DSM)) was compared with the one-step approach in which the cells were cultivated and sporulated in the same medium (DSM).

Since one vegetative cell can only form one spore, the cell yield before sporulation is essential to gain the needed spore amount for industrial spore production. To compare the yield of cells in a cultivation medium with the yield of cells in a sporulation medium and to examine a broader spectrum of results regarding the suitability of media, several cultivation and sporulation media (Lysogeny Broth-Lennox (LB), Casein Peptone Soymeal Peptone Medium (CASO), Super Broth (SB), Terrific Broth (TB), Difco Sporulation Medium (DSM) and *Bacillus* Sporulation Medium (BSM)) were compared for vegetative cell growth of *B. atrophaeus* (cultivation at 30 °C, 120 rpm for 22 h). The results showed that cell yield in pure cultivation media was far superior to that in sporulation media.

To examine whether the sporulation media were also suitable for the one-step production process and if they could lead to similar results as the two-step approach, cells of *B. atrophaeus* were inoculated in three different sporulation media (DSM, BSM and Minimal Sporulation Medium (MSM)) until the end of sporulation (30 °C, 120 rpm, 96 h).

In BSM, an average spore concentration of 4.8×10^7 CFU/mL could be observed, in MSM 8.4×10^7 CFU/mL and in DSM 1.6×10^8 CFU/mL. However, in the two-step production approach an average spore concentration in DSM of 1.1×10^9 CFU/mL could be achieved.

The results show that the one-step production process is, in principle, suitable for obtaining spores with reduced effort and costs. Nevertheless, since the spore yield in the two-step approach is far superior, the data underline the suitability of the two-step manufacturing process for industrial BI production.

Adapted original manuscript [1]

Comparison of one-step with two-step production of *Bacillus atrophaeus* spores for use as bioindicators [2]

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Abstract

The production method of spores significantly influences the resistance of spores used as bioindicators (BI) in the validation of sterilization of packaging material surfaces in aseptic food manufacturing. Therefore, the standardization of the spore production method represents an important and desirable goal in industrial BI production to ensure reliable validation test results. Previously, we recommended a two-step production approach for submerged spore production, in which the cultivation phase to obtain high cell mass was separate from the sporulation phase. In this work, a one-step manufacturing process was investigated to reduce production complexity and facilitate standardization of spore production. It was found that one-step BI production is technically possible but at the expense of spore yield. The two-step manufacturing process can realize almost 10-fold higher spore yields.

Keywords

Bacillus, bioindicator, spore production, sterilization validation;

[1] Adaptions refer to formatting, e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units and spelling.

[2] Originally published in: Microbiology Open (2022), 5, 1332; doi:10.1002/mbo3.1332

3.4.1 Introduction

In the food and pharmaceutical industries, bioindicators (BI) consisting of spores of *Bacillus atrophaeus* or *Geobacillus stearothermophilus* are used to assess the inactivation performance of sterilization processes, for example, of packaging material treatment with liquid or vaporized hydrogen peroxide (FDA, 2007; McLeod et al., 2017; VDMA, 2021) before aseptic filling. It is known that the sporulation conditions can cause a significant change in the spore structure and, thus, in the resistance of the spores (Abhyankar et al., 2016; Bressuire-Isoard et al., 2016, 2018; Isticato et al., 2020; Setlow, 2014). This means that the manufacturing method significantly influences the resistance of the BI, which may result in differences in the inactivation result and, thus, in uncertainties in the validity of sterilization process test results when using BI with variable and, most times, unknown resistances against the applied sterilization method. Therefore, standardization of BI production would be essential and a great step forward to ensure consistent quality and resistance of spores. Currently, state-of-the-art is that there are no agreed standardized BI production protocols, including the selection of nutrient contents, cell growth, and sporulation conditions.

We already showed that spore resistance can be influenced and predicted in submerged production or solid-state production on agar plates as a function of sporulation temperature and sporulation pH (Stier and Kulozik, 2020a; Stier et al., 2021). Compared to the solid-state method, which typically results in a high degree of cell differentiation due to inconsistent conditions across and through the agar plate (Vlamakis et al., 2008), submerged production offers more consistent fermentation conditions. In our experiments on the submerged production of *B. atrophaeus* spores in the bioreactor (Stier and Kulozik, 2020a), we observed during fermentation that sporulation occurred almost simultaneously for all vegetative cells and thus under uniform sporulation conditions. In addition, sporulation is faster (2 days in submerged bioreactor production vs. approximately 10 days on agar plates), and spore harvesting and purification from fermentation broth residues were reported to be easier and more consistent (Eschlbeck et al., 2017; Eschlbeck and Kulozik, 2017; Stier and Kulozik, 2020a; Stier et al., 2021).

Furthermore, more parameters influencing spore resistance can be controlled in the bioreactor than in the solid-state process on agar plates, for instance, pH and oxygen conditions, which can vary considerably in solid-state production. The BI manufacturing industry has recognized these advantages, and companies have partially switched production to the submerged process (anonymous, oral source from our industry partners). However, standardization of BI production conditions has still not been achieved; therefore, the issue of different BI resistances remains. In our previous work (Stier and Kulozik, 2020a), submerged spore production was investigated in a two-step procedure, where the cultivation phase of vegetative cells and the sporulation phase to form

spores were separated. As a cultivation medium for *B. atrophaeus*, Terrific Broth (TB) and a modified version of Difco Sporulation Medium (DSM) as sporulation medium were used. As the results demonstrated, this has the advantage of generating vital vegetative cells with the desired high cell numbers before exposing them to the sporulation medium, which in its composition, was optimized to induce sporulation rapidly and simultaneously for all cells. This differs from other works that reported a one-step approach combining cultivation and sporulation. For example, in the production of *B. subtilis* spores, Nguyen Thi Minh et al. (2011) used the sporulation medium already to culture the vegetative cells. Upon finalization of limited cell growth, the authors continued sporulation in the same medium and had to over inoculate the volume of the preculture for spore production into the same sporulation medium to achieve the desired high number of spores. Since there was no washing step between cultivation and sporulation in the same medium required, this procedure can be described as a one-step method, although technically, there were steps transferring the cells at the end of cultivation into another bioreactor for sporulation. Eschlbeck and Kulozik (2017) did not over inoculate but combined the phase of cultivation and sporulation in a single production step simply by continuing the cultivation step to achieve sporulation in the same bioreactor using the same medium. This, however, resulted in a low spore yield.

Avoiding two separate production steps (i.e., the elimination of cultivation and sporulation in separate media) represents a substantial simplification of spore production. This way, it could have corresponding advantages in industrial BI production and facilitate standardization. The purpose of this work, therefore, was to test the one-step in a head-to-head comparison versus the two-step method.

To address the potential for optimization of spore production, we investigate the possibility of the one-step production process for its application in BI production. We combined this approach with testing the suitability of different established sporulation media types for different *Bacillus* species, modified, however, in their glucose contents to allow for more cell growth, on cultivating vegetative cells before the onset of sporulation and compared these results with cell growth in several cultivation media. The reason for increasing the glucose concentration in classical sporulation media with typically low glucose contents to induce starvation was that the vegetative cells should not sporulate immediately after cell growth when inoculated into the bioreactor to adapt to the sporulation conditions.

Finally, we reviewed the sporulation yield of *B. atrophaeus* in a one-step process in different sporulation media and compared the results with those obtained in our previous work (Stier and Kulozik, 2020a), applying the two-step method with separated cultivation and sporulation steps.

3.4.2 Materials and Methods

Strain

Spores of the strain *B. atrophaeus* ATCC 9372, which has been established as a hydrogen peroxide BI (FDA, 2007; McLeod et al., 2017; VDMA, 2021), was used as starting material. The spores were of the same production batch and stored at aliquots of 500 µL with a concentration of 10^7 colony-forming units (CFU)/mL at -80 °C to ensure the initial characteristics of the spores did not change. The aliquots enabled the spores to be taken without thawing the rest of the spore suspension.

Media

The following media were used for cultivation or, depending on the experiment, for the sporulation of *B. atrophaeus*. These media are typical growth media used in microbiology (Lysogeny Broth- Lennox [LB], Casein Peptone Soymeal Peptone Medium [CASO], Super Broth [SB], and TB) and established sporulation media for *Bacillus* spp. (*Bacillus* Sporulation Medium [BSM], DSM, and Minimal Sporulation Medium [MSM]). Most of the single ingredients used for formulating the media were from Merck KGaA, unless mentioned otherwise.

LB comprised of casein peptone 10 g/L (for microbiology, Gerbu Biotechnik GmbH), yeast extract 5 g/L (for microbiology), and NaCl 5 g/L (ACS reagent, ≥99.0%).

CASO was made of casein peptone 17g/L (for microbiology, Gerbu Biotechnik GmbH), K_2HPO_4 2.5g/L (ACS reagent, ≥99%), glucose 2.5g/L (for biochemistry, Reag. Ph. Eur., 97.5%-102.0%), NaCl 5 g/L (ACS reagent, ≥99.0%), and soy peptone 3 g/L (for microbiology, Gerbu Biotechnik GmbH).

SB consisted of casein peptone 35 g/L (for microbiology, Gerbu Biotechnik GmbH), yeast extract 20 g/L (for microbiology), and NaCl 5 g/L (ACS reagent, ≥99.0%).

TB contained casein peptone 12g/L (for microbiology, Gerbu Biotechnik GmbH), yeast extract 24 g/L (for microbiology), K_2HPO_4 9.4 g/L (ACS reagent, ≥98%), KH_2PO_4 2.2 g/L (ACS reagent, ≥99%), and glycerol 8 g/L (Rotipuran ≥ 99.5%, Carl Roth GmbH & Co. KG).

BSM (modified in glucose content, based on Posada-Uribe et al. (2015) comprised of glucose 3g/L (for biochemistry, Reag. Ph. Eur., 97.5%-102.0%), $MgSO_4 \cdot 7H_2O$ 0.59g/L (ACS ≥99%, Carl Roth GmbH & Co. KG), KH_2PO_4 6 g/L (ACS reagent, ≥98%, Merck KGaA), beef extract 5 g/L (for cell biology, Gerbu Biotechnik GmbH), casein peptone 3g/L (for microbiology, Gerbu Biotechnik GmbH), NaCl 0.01 g/L (ACS reagent, ≥99.0%), and stock salt solution (0.1 M $FeSO_4 \cdot 7H_2O$ 1.136 mL/L (ACS reagent, ≥99%), 0.1 M $ZnSO_4 \cdot 7H_2O$ 300 µL/L (puriss. p.a., ACS reagent, reag. ISO, Reag. Ph. Eur., ≥99.5%), 0.1 M $CaCl_2$ 9.9 mL/L (anhydrous, Supelco), and 0.1 M $MnCl_2x4H_2O$ 30 mL/L

(ACS reagent, ≥98%). For BSM, the glucose content was adjusted to 3 g/L, analogous to the subsequent DSM. This ensures better comparability of the media with regard to the growth of *B. atrophaeus*, regardless of the glucose content present.

DSM (modified in glucose content, based on Harwood and Cutting (1990)) comprised of casein peptone 5g/L (for microbiology, Gerbu Biotechnik GmbH), beef extract 3g/L (for cell biology, Gerbu Biotechnik GmbH), KCl 3.5g/L (ACS reagent, 99.0%-100.5%), $MgSO_4 \cdot 7H_2O$ 0.25g/L (ACS ≥99%, Carl Roth GmbH & Co. KG), 30% glucose 10ml/L (for biochemistry, Reag. Ph. Eur., 97.5%-102.0%), 1 M $Ca(NO_3)_2 \cdot 4H_2O$ 1 mL/L (ACS reagent, 99%), 10 mM $MnCl_2 \cdot 4H_2O$ 1 mL/L (ACS reagent, ≥98%) and 1 mM $FeSO_4 \cdot 7H_2O$ 1ml/L (ACS reagent, ≥99%). The glucose content was increased to 3 g/L during media optimization based on our previous work (Stier and Kulozik, 2020a), which was retained in this work for comparability.

MSM (modified for submerged use, based on Pruß et al. (2012)) comprised of casein peptone 5g/L (for microbiology, Gerbu Biotechnik GmbH), beef extract 3g/L (for cell biology, Gerbu Biotechnik GmbH) and $MnSO_4 \cdot H_2O$ 10 mg/L (≥99%, p.a., ACS, Carl Roth GmbH & Co. KG). This medium was not initially designed for the submerged growth of *Bacillus* spp. and was, therefore, not included in the submerged cultivation experiments in the beginning. However, the medium was applied in the sporulation experiments because, as described in more detail in the discussion, BSM was unsuitable for sporulation. Thus another sporulation medium had to be used for comparison. To adapt the medium for submerged sporulation, agar-agar was not added in the media preparation.

Cultivation of vegetative cells of *B. atrophaeus*

The aim was to compare the results from our previous work (Stier and Kulozik, 2020a) applying two-step spore production (cultivation in TB and sporulation in DSM) with the one-step procedure according to Eschlbeck and Kulozik (2017) (cultivation and sporulation in one step in DSM). For this purpose, cells of *B. atrophaeus* were cultivated in both TB and DSM. Since other media could be more suitable for *B. atrophaeus* cultivation and to examine a broader spectrum of results regarding the suitability of media, the media SB, LB, CASO, and BSM were also included in the comparison.

To use the same starting material in all cultures, 100 mL of each medium (pH 7.2) was inoculated with 500 µL each of *B. atrophaeus* spores from the same production batch in a 250 mL baffled shake flask (Schott Duran® baffled culture flask, Erlenmeyer shape, straight neck for metal caps; Duran Group GmbH) as a biological triplicate. Incubation was performed at 30 °C and 120 rpm for 22 h.

From 8 to 22 h of incubation, the optical density at 600 nm (OD_{600}) was determined several times per hour. This time window was chosen because the most significant differences were expected here due to the progressive growth of the cells. In addition,

the pH was checked every 4 h to exclude a negative influence of pH on cell growth since the pH was not controlled actively during the incubation.

Sporulation of *B. atrophaeus*

To examine the sporulation success in the one-step manufacturing process, cells of *B. atrophaeus* were incubated in the sporulation media DSM and BSM until sporulation occurred. This means that no preliminary cultivation in a cultivation medium took place. Since it could already be concluded from the first part of the experiment that BSM appears unsuitable for the sporulation of *B. atrophaeus*, the additional medium MSM was used for a more comprehensive comparison.

Each 500 µL of *B. atrophaeus* spores from the same production batch were inoculated into 400 mL of the respective sporulation medium (pH 7.2) in a 1 L baffled shake flask (Schott Duran® baffled culture flask, Erlenmeyer shape, straight neck for metal caps; Duran Group GmbH) as a biological duplicate and incubated at 30 °C and 120 rpm for 96 h. The cultivation volume was increased in this experiment compared to the experiments on the cultivation of vegetative cells still to identify enough spores in case of low spore yields. After 96 h of incubation, the total suspension volume was centrifuged (4000× g, 4 °C, 10 min), and the supernatant was discarded. The pellet resulting from centrifugation was resuspended with Milli- Q water.

To determine the spore concentration, a portion of this washed suspension was diluted with Ringer's solution (Ringer tablets for the production of sterile Ringer's solution, 1⁄4 Ringer's solution, item no. MC1155250001, Merck KGaA) in duplicate and plated out on Plate Count Agar (casein peptone 0.5%, for microbiology, Gerbu Biotechnik GmbH; yeast extract 0.25%, for microbiology; glucose 0.1%, for biochemistry, Reag. Ph Eur., 97.5%-102.0%; agar-agar 1.5%, for microbiology, Carl Roth GmbH & Co. KG; pH 7.0). After incubation for 48 h at 30 °C, the number of CFU was determined. Because it was important for the rest of the experiment not to separate the free spores from the cell debris and the forespores and because a proportion of the free spores is always lost during purification (Stier and Kulozik, 2020a; Stier et al., 2021), the spore concentration was determined with the non-purified suspension immediately after the end of incubation to minimize the risk of regermination. The presence of living cells of the suspensions, which would falsify the spore concentration determination, was tested by phase-contrast microscopy. Vital cells were observed as motile unicellular or bicellular cells (Kearns and Losick, 2005) in the cultivation phase, whereas the remaining cells floated motionless in the suspension after the sporulation. Additionally, this observation is supported by the fact that the cells were cultured for 96 h in a sporulation medium that no longer offered adequate living conditions at a certain point in time, which is why the cells sporulated. Therefore, the presence of living cells is highly unlikely even without phase microscopic evidence.

For phase-contrast microscopic assessment of sporulation, the suspensions were concentrated by a factor of 10 to ease visual inspection since the spore density in the original suspension was too low for a meaningful comparison. The concentration was done by another centrifugation step (4000× g, 4 °C, 10 min), discarding the supernatant and suspending the pellet with 1/10 Milli-Q water of the original volume. The pellet resulting from centrifugation was comprised of three phases, with only the lowest phase at the bottom containing free spores, followed by forespores and cell debris (according to Stier and Kulozik (2020a) and Stier et al. (2021)). Therefore, to study sporulation success, it was necessary to keep all three phases intact when discarding the supernatant not to distort the result, respectively the ratio of spores to non-sporulated cells by discarding one of the upper phases and not to lose spores, which is to some extent unavoidable with this type of purification.

3.4.3 Results and Discussion

First, the question was clarified whether the one-step process could lead to similar results as the two-step production. Since each vegetative cell can form only one spore, the cell density in the culture could already indicate the final maximally obtainable number of spores, provided that the sporulation medium allows the highest possible sporulation rate. For this reason, *B. atrophaeus* ATCC 9372 was cultivated in triplicate at 30 °C, 120 rpm for 22 h in four cultivation media (SB, TB, LB, and CASO) and two sporulation media (DSM and BSM). The results are shown in Figure 4-1. The OD_{600} was determined at intervals between 8 and 22 h of cultivation. Growth progression from 0 to 8 h was not recorded because no major differences were expected in this time window.

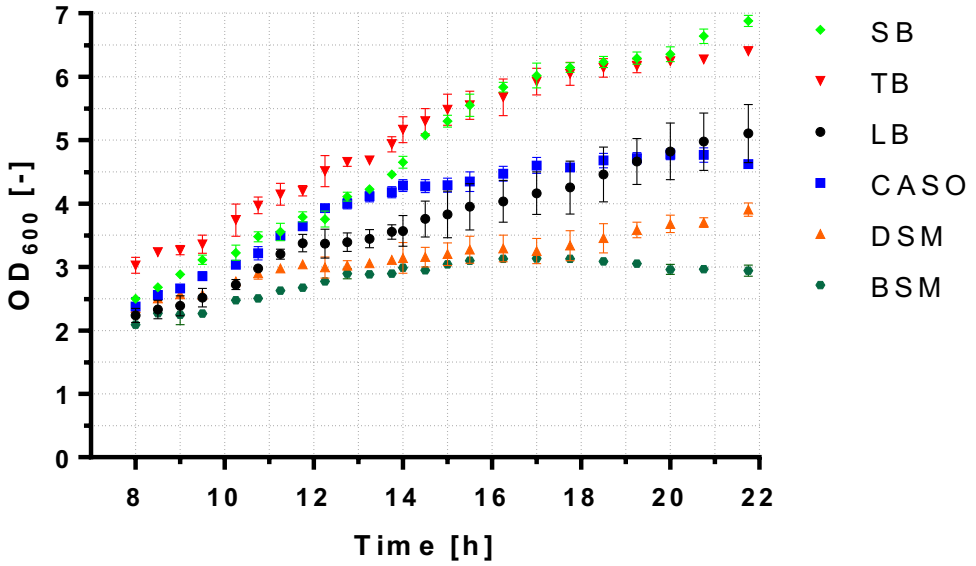

Figure 4-1. Optical densities (OD_{600}) of cultures of *Bacillus atrophaeus* in the media SB, TB, LB, CASO, DSM, and BSM. BSM, *Bacillus* Sporulation Medium; CASO, Casein Peptone Soymeal Peptone Medium; DSM, Difco Sporulation Medium; LB, Lysogeny Broth-Lennox; SB, Super Broth; TB, Terrific Broth.

After 8 h of incubation, slight differences in the cell densities of the cultures were already apparent. As expected, these differences increased during incubation. TB, as used in our last publication (Stier and Kulozik, 2020a) as a cultivation medium before inoculation into the sporulation medium, showed the highest cell density after 8 h with an OD_{600} of ~3. The other cultures were in the range of OD_{600} ~2-2.5. As cell growth progressed, the differences in cell densities became increasingly pronounced. After approximately 14 h of incubation, the cell densities of the cultures in TB and SB converged and continued to show strong cell growth. In contrast, the cell density in CASO started to level off after 14 h. In LB, the cultures showed the lowest cell density among the pure cultivation media in the first 19 h of cultivation but finally caught up with CASO by the end of the incubation. After 22 h, cell densities of OD_{600} ~6.5 in TB, ~6.9 in SB, ~5.1 in LB, and ~4.5 (i.e., maximum of ~4.6 after 21 h) in CASO were finally reached in the cultivation media.

The sporulation media BSM and DSM produced the lowest cell densities over the entire growth course. In addition, the cell density in BSM decreased after approximately 17 h of incubation, while the cell density in DSM continued to increase. After 22 h of incubation, a cell density of OD_{600} ~3.9 was reached in DSM and ~2.9 in BSM (with a maximum OD_{600} of ~3.1 after 17 h). This shows that the sporulation media have limited suitability for cell cultivation since large cell densities could not be achieved. Based on

the decrease in cell density in BSM, it can also be assumed that sporulation had already started.

To assess the state of sporulation in the sporulation media, the cell suspensions were checked by phase-contrast microscopy at the end of the 22 h incubation. Surprisingly, no spores were found in either of the sporulation media. Therefore, the decrease in cell density in BSM cannot be explained by the formation of spores but probably by the beginning lysis of cells, triggered either by starvation or other milieu conditions. This is notable because BSM and DSM have relatively similar nutrient compositions. Despite their rather similar composition of the nutrient components, the cell density in DSM, in contrast to BSM, continued to increase in the course of growth. This could either be because casein peptone is preferred to beef extract, which is why the composition of DSM would be slightly better, or that the milieu conditions, that is, concentration and presence of ions in BSM compared to DSM, are of adverse effect for *B. atrophaeus*. The decrease in cell density in BSM could also mean that BSM is not well suited for the sporulation of *B. atrophaeus* ATCC 9372. Otherwise, the formation of spores would have to be expected in parallel with the decrease in cell density. However, the cells in this medium seem to have died before sporulation.

The data showed that cultivation is possible in all the media listed, although cell yields are significantly higher in the media dedicated to cell growth. This may be partly due to the significantly higher nutrient contents in the cultivation media compared to the sporulation media but also due to lower levels of specific ions. The pH value of the media (Table 4-1) is not expected to be a reason for growth stagnation since the changes were moderate within the incubation time.

Table 4-1. pH in the course of the incubation period in the media SB, TB, CASO, LB, DSM, and BSM.

	pH (-)					
Incubation (h)	**SB**	**TB**	**CASO**	**LB**	**DSM**	**BSM**
10	7.03	6.75	7.20	7.41	7.44	7.34
14	6.90	6.67	7.26	7.58	7.69	7.51
18	6.71	6.61	7.41	7.83	8.05	7.63
21	6.50	6.60	7.60	7.99	8.23	7.90

Note: The initial pH was set to 7.2 in all media before inoculation. Abbreviations: BSM, *Bacillus* Sporulation Medium; CASO, Casein Peptone Soymeal Peptone Medium;

DSM, Difco Sporulation Medium; LB, Lysogeny Broth-Lennox; SB, Super Broth; TB, Terrific Broth.

The next step was to examine whether the sporulation media were also suitable for the one-step production process or could lead to similar results as the two-step production process. Since cell growth in our past study (Stier and Kulozik, 2020a) also took place in a shake flask and the vegetative cells were only transferred to a bioreactor for sporulation, this experiment was performed exclusively in a shake flask. This provided better comparability between the experiments because vegetative cell growth to sporulation in the bioreactor would have had a significantly different effect on the cultures.

As described above, the cell density in BSM decreased at a certain time of incubation without spores forming. Due to this, we suspected that this medium could not be suitable for producing *B. atrophaeus* ATCC 9372 spores. We, therefore, extended the comparison and included MSM as another sporulation medium. In the previous cultivation media experiments, this medium was initially not included because it is a medium for obtaining spores using the solid-state method on agar plates (Pruß et al., 2012). To allow submerged cultivation with this medium, the recipe was adjusted by omitting agar-agar. The media were inoculated with 500 µL of *B. atrophaeus* spores from the same production batch and incubated for 96 h. Within this period, cultivation and sporulation occurred in the same medium without additional intervention. Figure 4-2 shows representative results of the double determination of spore formation in the respective sporulation media after 96 h of incubation.

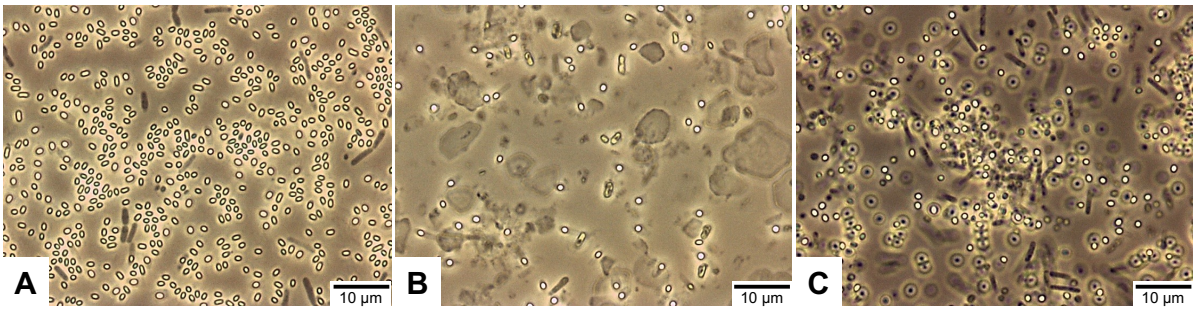

Figure 4-2. Phase-contrast microscopy (x100) of the sporulation results of *Bacillus atrophaeus* after cultivation and sporulation after 96 h in the media (A) Difco Sporulation Medium (DSM), (B) *Bacillus* Sporulation Medium (BSM), and (C) Minimal Sporulation Medium (MSM). Spores can be observed as bright spots and dead cells as gray rods.

As suspected, the spore yield in BSM (Figure 4-2B) was comparatively low. Only a few free spores could be found in this medium. In addition, a few dead cells (gray rods) were sighted, suggesting that the majority of the cells lysed, as suspected already in the first experiment. Significantly more spores were formed in MSM (Figure 4-2C). Although this medium was not intended for submerged spore production in its original recipe, it appears suitable for *B. atrophaeus* ATCC 9372. Nevertheless, not all cells sporulated in this medium, and a large proportion died. The medium DSM (Figure 4-2A) produced the best sporulation efficiency in this comparison. In this medium, the majority of cells appeared to be sporulated, and only a few dead cells were found. The absence of vegetative cells was tested by microscopy of several duplicate samples.

The spore suspensions of these sporulation media were also plated on Plate Count Agar to determine the spore concentration. Since there were no longer any vegetative cells in the suspensions, the results provided information about the pure number of germinable spores in the respective medium (1 CFU was set equivalent to 1 germinable spore). The spores were not heat-activated before plating. In DSM, an average spore concentration of 1.6×10^8 CFU/mL could be observed, in BSM 4.8×10^7 CFU/mL, and in MSM 8.4×10^7 CFU/mL (these spore concentrations correspond to the original spore suspension before concentration by a factor of 10 for phase-contrast microscopic evaluation). These results confirm the results of the phase-contrast microscopic evaluation that the highest number of spores was formed in DSM. Since the initial cell concentration applied for inoculation was identical in all media, this result shows that DSM produces the best sporulation efficiency among the tested sporulation media.

The results also show that the one-step production process is, in principle, suitable for obtaining spores, depending on the sporulation medium selected. It is notable, though, that an OD_{600} ~3.9 was achieved in DSM during cultivation, and no decrease in growth or beginning sporulation was observed up to this point. In our previous procedure to produce *B. atrophaeus* spores (Stier and Kulozik, 2020a), we inoculated the bioreactor with an OD_{600} of 1.0. Sporulation, therefore, started about 3 h later at a comparatively low cell density. Nevertheless, we achieved spore yields of 1.1×10^9 CFU/mL in this two-step production approach under similar sporulation conditions (30 °C, pH 7.2, 30% pO_2). Accordingly, overinoculation of vegetative cells from a cultivation medium into the sporulation medium seems to result in a more significant proportion of cells sporulating than in the one-step manufacturing process. In our opinion, this is the only way to explain why, despite a lower cell density in the sporulation medium, we obtained a spore yield increased by a factor of almost 10 in the two-step production process. This difference can be attributed to the best conditions during the cell growth phase in the two-step method achieving high cell numbers at first before entering the sporulation phase, which was also performed under optimal conditions yielding high spore num-

bers and avoiding losses due to cell death. These results underline our previous recommendations for submerged production of *B. atrophaeus* spores for use as BI. Although the one-step manufacturing procedure could reduce effort and cost, this is accompanied by significantly lower spore yields.

3.4.4 Conclusion

Standardization of BI production is essential to ensure consistent quality and resistance of spores. We have previously recommended a two-step manufacturing process for producing spores for use as BI. However, a one-step manufacturing process would have potential advantages in terms of effort and cost, which would potentially also enable standardization to be uniformly established by and between BI manufacturers. However, since the one-step manufacturing process could obtain only low spore yields, we restate our previous recommendation to prefer the two-step manufacturing process with separated cultivation and sporulation phases. Practical experience in aseptic food and pharmaceutical production shows that standardization of BI manufacturing is essential to reduce uncertainties of sterilization validation tests. An aspect to be addressed in future works is to test the resistance variability between the one-step and the two-step BI production methods. It has already been shown in our previous work (Stier and Kulozik, 2020a) that resistance variability can be reduced by a better understanding of the relationship between sporulation conditions and spore resistance. Since suitable media and methods are now available as a result of this work, this will be tested in future experiments.

3.4.5 Acknowledgments

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3.4.6 Author contributions

Philipp Stier: Conceptualization (lead); data curation (lead); investigation (lead); methodology (lead); project administration (equal); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). Ulrich Kulozik: Conceptualization (supporting); funding acquisition (lead); project administration (equal); writing – review and editing (equal).

3.4.7 References

The references were combined into a joint list of all publications at the end of the thesis.

4 Overall discussion, conclusion and outlook

The validation results of sterilization processes with hydrogen peroxide depend significantly on the resistance of the BI, i.e., the spores used to produce the BI. Practice shows that nowadays, there are still considerable differences in resistance between manufacturers and between production batches of the same manufacturers. These differences lead to uncertainties in the evaluation of validation results. According to the latest information that I have received verbally from BI users, this problem is also known to the users. This leads to the fact that users are confronted with great difficulties and fears when the BI of the favored manufacturer is unavailable due to supply difficulties. Especially if the validation results show an insufficient sterilization result, the question arises why the validation was not passed, whether it was due to the too-high resistance of the BI or whether the sterilization conditions are insufficient for the standard application.

Unification or standardization of BI production could counteract this. Since two test germs, *B. atrophaeus* and *G. stearothermophilus*, are used for sterilization with hydrogen peroxide, and BI are produced industrially both submerged in the bioreactor and the solid-state process on agar plates, standardization, therefore, had to be investigated at several levels.

Therefore, the main objectives of this work were:

- To investigate the main factors influencing resistance to hydrogen peroxide during submerged spore production.
- Investigation of the most important factors influencing the resistance to hydrogen peroxide during solid-state spore production.
- Method development to also enable submerged spore production of *G. stearothermophilus* with a focus on industrial BI production in bioreactors with the lowest possible resistance variability.
- Investigation of optimization possibilities of the established two-step production method.

<u>Investigation of the most important factors influencing the resistance to hydrogen peroxide during submerged spore production</u>

Standardization of spore production conditions to obtain uniform resistance to hydrogen peroxide in each production batch can only succeed based on a good understanding of the factors influencing resistance during production.

The study by Stier and Kulozik (2020a) showed that during submerged production of spores of *B. atrophaeus* in the bioreactor, the pH prevailing during sporulation and the

sporulation temperature were the main factors influencing resistance. In contrast, oxygen saturation in the medium had no significant effect on the developed resistance. Sporulation temperature influenced resistance linearly, with resistance increasing with increasing sporulation temperature. Sporulation pH had a quadratic effect on resistance. Resistance was greatest at a pH of approximately 6.9 and decreased when the pH deviated from this value. Using the experimental setup via a design of experiments, it was also shown that the factors sporulation pH and sporulation temperature influenced each other. Thus, the influence of temperature on resistance increased with decreasing sporulation pH and decreased with increasing sporulation pH. Finally, when the pH became too basic, a change in sporulation temperature did not influence resistance at all, which means that the sporulation temperature could no longer control resistance.

With these findings, it must be mentioned that the obtained results can be interpreted exclusively for *B. atrophaeus* under the investigated conditions and with the used media. It can be assumed that the degree of influence of the mentioned factors changes in the production of other spore species, e.g., *G. stearothermophilus*, when using a different medium. However, this is contradicted by the work of Guizelini et al. (2012), who also found a quadratic influence of sporulation pH on the resistance of submerged produced spores of *G. stearothermophilus*. Nevertheless, the results show that in submerged production of spores, the focus should not be placed on only one influencing factor to control resistance, but that only by considering both influencing factors (sporulation pH and temperature) is it possible to control resistance specifically.

From the controllability of the resistance due to the known and targeted influence on the resistance-influencing factors also arises the possibility of tracking occurring resistance variability between production batches or preventing it in the long term.

It has been known for some time from the literature that sporulation temperature can influence spore resistance (Baril et al., 2012a; Baweja et al., 2008; Beaman and Gerhardt, 1986; Condon et al., 1992; Garcia et al., 2010; González et al., 1999; Lechowich and Ordal, 1962; Leguérinel et al., 2007; Melly et al., 2002; Palop et al., 1999; Planchon et al., 2011; Raso et al., 1995;). However, no direct influence of sporulation pH on this effect has been shown so far. As mentioned, sporulation temperature does not influence resistance above a certain pH, so the results published in the literature depend significantly on the sporulation pH used in each case.

It is not clear by which mechanistic effects the influencing factors sporulation temperature and pH influence each other, even if it is known that pH influences spore structure (Eschlbeck et al., 2017; Lowe et al., 1989). However, of primary importance with a focus on standardization of spore production or industrial BI production is the fact that and to what extent the factors influence each other and affect spore resistance.

Since spores for use as BI are not only produced submerged but also in the solid-state process on agar plates, the next step was to identify and characterize the influencing factors for this production process as well. In addition, no suitable procedure for the submerged production of spores of *G. stearothermophilus* was available. For this reason, the influencing factors were carried out during solid-state spore production with *G. stearothermophilus*.

Investigation of the most important factors influencing the resistance to hydrogen peroxide during solid-state spore production

In analogy to the previous investigations of the most important influencing factors during submerged spore production, the investigations of the influencing factors during solid-state spore production were carried out.

The study by Stier et al. (2021) showed that the resistance of solid-state-produced spores also depends on several factors. In this regard, the sporulation temperature and the initial sporulation pH of the agar medium could be identified as decisive influencing factors. In contrast to the linear effect of sporulation temperature on resistance known from submerged production, sporulation temperature had a quadratic effect on the resistance of spores to hydrogen peroxide of solid-state output. Resistance was greatest at a sporulation temperature of 57 °C and decreased with a temperature change. Also, contrary to previous findings for submerged spore production, sporulation pH in the solid-state process did not take a quadratic influence on resistance but a linear one. That means resistance was greatest at an acidic pH, and resistance decreased linearly with increasing pH. Furthermore, in the solid-state procedure, the influencing factors sporulation temperature and pH did not interact with each other, meaning that under the influence of one factor, the resistance of the spores could be controlled independently of the other factor.

Regarding the sporulation pH, it must be mentioned that this is the initial pH, as already mentioned. Thus, it is the pH at which the agar medium was adjusted prior to inoculation with vegetative cells. Natural cell metabolism during the cultivation phase, before the cells sporulate due to nutrient deficiency, then causes the pH to change, albeit minimally. Since it is impossible to determine the actual pH at the time of cell sporulation, the real pH that led to developing the respective resistance is, therefore, unknown. However, since the methodological procedure as well as the cell and media preparation were strictly identical, a robust data basis for the control of resistance via pH can still be derived. Particularly with the focus on industrial BI production, in which only the initial pH is used, the results shown represent a reasonable basis for the resistance control of solid-state produced spores.

Nevertheless, even for these results, the effects can only be mapped with the media and sporulation conditions used here. For example, Guizelini et al. (2012) for *G. stearothermophilus* ATCC 7953 and Mtimet et al. (2015) for *G. stearothermophilus* ATCC 12980 found resistance optima at other pH values.

As addressed at the beginning, and this was also shown in the study by Stier et al. (2021), the resistance variability is more significant in the solid-state process than in the submerged production process. This means there is greater variability in the resistance of the manufactured spores under the same sporulation conditions. For this reason, the next goal was to develop a method for submerged production of *G. stearothermophilus* that would allow future BI producers to dispense entirely with the solid-state production method for BI.

Method development to also enable submerged spore production of *G. stearothermophilus* with a focus on industrial BI production in bioreactors with the lowest possible resistance variability

The study by Stier and Kulozik (2021) compared two different methodological approaches to submerged spore production of *G. stearothermophilus*. This was to develop a method in advance that could produce free spores on the one hand and a sufficient number of spores for industrial BI production on the other hand. The submerged BI production would allow better and more precise control of sporulation conditions and, thus, according to the hypothesis, a reduction of resistance variability. On the other hand, the obtained spores from suspensions can be purified more efficiently and in a cost- and time-saving manner, which shows further advantages of the submerged process over the solid-state process.

The procedures used in the study differed in the methodological approach of inoculating the vegetative cells from the cultivation medium into the sporulation medium but otherwise involved the same procedure. In one scenario, the inoculum was washed before over-inoculation into the sporulation medium, whereas in the other method, the washing step was omitted.

The study results show that no free spores were formed in the former method, where the inoculum was washed. Spores were formed, but they were in the form of mother cells containing endospores. This type of spores cannot be used in industrial BI production because, on the one hand, the spores are not yet fully mature in this status. On the other hand, the cell debris or the surrounding mother cells represent nutrients that could lead to the re-germination of the spores during prolonged storage. This means that, on the one hand, the storability of the spores is not given; on the other hand, no uniform or defined resistance can be achieved with these spores due to their

immaturity. The second methodological approach, therefore, yielded free spores in an amount (1.6 x 10^7 CFU/mL) that would be sufficient for industrial BI production.

At this point, the study could already show which method was suitable for industrial BI production. Subsequently, it had to be determined whether the process could also be used to produce spores with a resistance ideal for sterilization validation and whether the spores had a sufficiently low resistance variability.

Therefore, the free spores obtained were tested for their resistance to liquid hydrogen peroxide (35%, 25 °C). As a result, these spores exhibited a resistance ($D_{25°C,35\%H2O2}$ = 68-78 s) within the range of resistance of comparable commercial spores ($D_{25°C,35\%H2O2}$ = 54-154 s). Thus, these spores could be directly used in sterilization validation without adjusting to new resistances of BI. The resistance comparison also showed that the commercially available BI of *G. stearothermophilus* from the same BI manufacturer exhibited a wide spread, namely with a resistance difference between production batches of $\Delta D_{25°C,35\%H2O2}$ = 100 s. It can be assumed that the BI manufacturer probably did not change the manufacturing method between the two batches, illustrating the extent to which resistance variability occurs in practice. In contrast, the spores produced using the developed submerged spore production method had very similar resistances (resistance difference of only $\Delta D_{25°C,35\%H2O2}$ = 10 s), so the method seems suitable for low resistance variability.

Comparing the resistance results of the submerged-produced spores of this study with the work of Stier et al. (2021), in which the spores of *G. stearothermophilus* were produced by the solid-state method, we find that the submerged method reduced resistance variability under otherwise identical production conditions (pH 7.0, 57 °C). In the work of Stier et al. (2021), the observed resistances of the prepared spores were at $D_{25°C,35\%H2O2}$ = 267-306 s, whereas in the work of Stier and Kulozik (2021), as mentioned above, the observed resistances were at $D_{25°C,35\%H2O2}$ = 68-78 s. This makes a resistance difference of $\Delta D_{25°C,35\%H2O2}$ = 39 s for the solid-state produced spores. Thus, the difference in resistance based on the D-value is 290% greater in the solid-state procedure than in the submerged approach under otherwise identical sporulation conditions.

These results support the hypothesis that submerged-produced spores have an advantage over solid-state-produced spores in terms of lower resistance variability. In submerged production, sporulation conditions can be more precisely controlled, meaning that not only can temperature be manipulated throughout, but also sporulation pH. On agar plates, pH can only be adjusted initially, but this pH changes by the time of sporulation due to natural cell metabolism. The vegetative cells in submerged cultures present in a more uniform way. Due to the mixing in the culture, the cells differentiate less and therefore have a more uniform status at the time of sporulation. On agar plates, the cells differentiate strongly. It is known that the cells differentiate into motile

cells and so-called matrix formers, among others, and the sporulation of the cells depends on their individual position in the colony (closer to the agar or resting on top of the population) (Vlamakis et al., 2008). This means that the cells sporulate at a different time point and thus at supposedly different sporulation conditions

Now that a submerged production method for spores of *G. stearothermophilus* suitable for BI production had been developed, the final step was to determine whether the production method of the established two-step procedure could be further simplified.

Investigation of optimization possibilities of the established two-step production method

In the study by Stier and Kulozik (2020a), a two-step method of spore production was established, in which vegetative cells were grown in a cultivation medium in the first step and inoculated into a sporulation medium in the second step.

In the study of Stier and Kulozik (2022), we investigated whether this method could be further optimized or simplified using *B. atrophaeus*. An even further simplification should create even more reasons and advantages for the BI manufacturing industry to change currently established manufacturing processes and thus lead to a self-developing standardization of BI manufacturing not by coercion but by obvious advantages.

For this purpose, the two-step method was compared to the so-called one-step method, in which the cells are not transferred to a sporulation medium after cultivation in a cultivation medium but are already cultivated in the sporulation medium until cell sporulation. This could save medium, as well as time and costs, and simplify the sporulation process overall. Therefore, in the first part of the study, several cultivation and sporulation media were investigated for their ability to generate large cell masses. Since one cell can only form one spore, this already allows conclusions to be drawn about the performance of the one-step method.

The results showed that the pure sporulation media are strongly inferior to the cultivation media in terms of cell growth. Thus, optical densities of only about OD_{600} = 3.4 could be achieved on average for all sporulation media. In contrast, cell densities of up to OD_{600} = 6.9 were achieved with the cultivation media. However, since in the two-step procedure, the vegetative cells are transferred to the sporulation medium with an OD_{600} of 1.0, these numbers did not yet have much significance regarding the suitability or non-suitability of the one-step procedure.

Therefore, in the second part of the study, the cells of *B. atrophaeus* were incubated in three different sporulation media in one step, i.e., without separating the cultivation from the sporulation. The recovered spores from the three sporulation media were then examined by phase-contrast microscopy for the amount of spores formed.

The results showed that free spores could be formed in general using the one-step method. However, the proportion of formed, i.e., free spores, differed greatly depending on the sporulation medium used. In the most promising sporulation medium (DSM), spores with a concentration of 1.6 x 10^8 CFU/mL could be produced. This value is basically suitable for industrial BI production but did not come close to the spore concentration that could be achieved with the two-step method (1.1 x 10^9 CFU/mL). This means that although the sporulation medium is inoculated with an OD_{600} of 1.0 in the two-step method, 10-fold more spores are formed overall than in the one-step method.

The data thereby showed that simplifying the established two-step method is generally possible but does not seem reasonable. Furthermore, the results support the suitability of the two-step method for industrial BI production.

Overall conclusion and perspectives

Standardized BI production is essential to ensure consistent spore resistance and quality. Standardizing BI manufacturing processes would reduce uncertainties in validating sterilization processes with hydrogen peroxide. In addition, the market would be opened for BI users, as BI from different manufacturers would no longer have different resistances, making it easier to switch BI manufacturers from a consumer perspective, e.g., in case of supply difficulties.

Overall, however, and this is the most crucial point concerning the standardization of BI manufacturing, the safety for end users, i.e., for consumers of the products sterilized by the sterilization process to be validated, would increase. This is because resistance variations of BI do not only move upwards, meaning that sterilization validations are not passed due to supposedly too weak sterilization conditions but also downwards. Thus, it happens that BI have too low resistance, which means that actually ineffective sterilization procedures pass validation due to unresistant BI.

The research presented in this work provides knowledge of the main factors influencing spore resistance during manufacturing. The data presented can be used to target spore resistance in both the solid-state and the recommended submerged processes. Furthermore, with the developed production methods for spores, this work provides procedures for BI production that could be directly adopted in industrial BI production based on the spore concentrations and resistances presented. The data show that the submerged process, in particular, is ideal for reducing resistance variability and is suitable for both *B. atrophaeus* and *G. stearothermophilus* spore production.

The current results will be followed by further research. First, it needs to be clarified whether the one-step process, despite its lower spore yield, has advantages in terms of further reducing resistance variability due to its lower methodological complexity. Second, it is unclear whether the results of the work of Stier and Kulozik (2020a) for *B. atrophaeus* are directly transferable to the submerged production of *G. stearothermophilus* (Stier and Kulozik, 2021) or whether the factors influencing resistance present themselves with other dependencies and effects.

5 Summary / Zusammenfassung

5.1 Summary

Validation of sterilization processes is essential to maintain the safety and quality of products sterilized with the sterilization process. Biological indicators (BI) of spores of *Bacillus atrophaeus* or *Geobacillus stearothermophilus* are used for validation for sterilization processes with hydrogen peroxide. These spores exhibit a natural resistance to hydrogen peroxide. The extent of their inactivation by the sterilization process provides information on the functionality and performance of the sterilization process. A uniform and consistent resistance of the BI used is of great importance to prevent false-positive or false-negative validation results. In practice, however, the resistance of commercial BI sometimes varies greatly - not only from BI manufacturer to BI manufacturer but also from batch to batch of the same BI manufacturer. The reason for this is the lack of a generally accepted and standardized method of BI production since the resistance of the spores depends largely on the manufacturing, i.e., sporulation conditions. This fact leads to significant uncertainties in the validation of sterilization processes.

Therefore, this work aimed to advance the standardization of spore production for use as BI against hydrogen peroxide. As a basis for this, the most critical factors influencing spore resistance during sporulation were identified and characterized. The degree of influence of these factors on resistance was determined, as well as any interactions between the influencing factors. Since commercial BI are produced both submerged and by the solid-state method, the influencing factors were analyzed for both production methods.

Furthermore, a method was developed with which it will also be possible to produce spores of *G. stearothermophilus* submerged in the future. Submerged spore production is superior to solid-state production in terms of lower resistance variability, process control, efficiency, cost, and labor requirements. Submerged production of *G. stearothermophilus* spores, analogous to submerged production of *B. atrophaeus* spores, would simplify commercial BI production, laying another foundation for long-term standardization of the manufacturing process.

Finally, the established two-step manufacturing method was investigated for the possibility of further optimization and simplification. It could be proven that the manufacturing methods established and developed in this thesis meet all requirements for industrial BI manufacturing. On the one hand, spore concentrations relevant to industrial BI production can be obtained with the methods presented here. On the other hand, the resistances of the spores produced with these methods to hydrogen peroxide lie in

a range in which current commercial BI also lie. Thus, a method transfer of the shown procedures is possible directly and without restrictions for BI users.

In summary, this work provides fundamental and directly transferable knowledge for the standardization of commercial BI production while reducing the resistance variability currently found in practice.

5.2 Zusammenfassung

Die Validierung von Sterilisationsprozessen ist essentiell zur Aufrechterhaltung der Sicherheit und Qualität der mit dem Sterilisationsverfahren sterilisierten Produkte. Zur Validierung für Sterilisationsprozesse mit Wasserstoffperoxid werden biologische Indikatoren (BI) von Sporen von *Bacillus atrophaeus* oder *Geobacillus stearothermophilus* verwendet. Diese Sporen weisen eine natürliche Resistenz gegen Wasserstoffperoxid auf. Das Maß ihrer Inaktivierung durch dem Sterilisationsprozess gibt Rückschlüsse auf die Funktionsfähigkeit und Leistungsfähigkeit der Sterilisation. Eine einheitliche und gleichbleibende Resistenz der verwendeten BI ist dabei von großer Wichtigkeit, um falsch-positive oder falsch-negative Validierungsergebnisse zu verhindern. In der Praxis variiert die Resistenz kommerzieller BI allerdings teilweise stark – nicht nur von BI Hersteller zu BI Hersteller, sondern auch von Charge zu Charge desselben BI Herstellers. Grund dafür ist das Fehlen einer allgemein anerkannten und standardisierten Herstellungsweise von BI, da die Resistenz der Sporen maßgeblich von den Herstellungs- bzw. Sporulationsbedingungen abhängt. Diese Tatsache führt dazu, dass große Unsicherheiten in der Validierung von Sterilisationsprozessen entstehen können.

Ziel dieser Arbeit war es deshalb, die Standardisierung der Sporenherstellung für die Verwendung als BI gegen Wasserstoffperoxid voranzutreiben. Als Grundlage hierfür wurden die wichtigsten Einflussfaktoren während der Sporulation auf die Sporenresistenz identifiziert und charakterisiert. Dabei wurde das Maß des Einflusses dieser Faktoren auf die Resistenz bestimmt, sowie etwaige Interaktionen zwischen den Einflussfaktoren. Da kommerzielle BI sowohl submers wie auch mit dem „solid-state“ Verfahren hergestellt werden, wurden die Einflussfaktoren für beide Herstellungsmethoden analysiert.

Des weiteren wurde eine Methode entwickelt, mit der es zukünftig möglich ist, auch Sporen von *G. stearothermophilus* submers herzustellen. Die submerse Sporenherstellung ist der „solid-state“ Herstellung in Bezug auf geringere Resistenzvariabilität, Prozesskontrolle sowie Effizienz-, Kosten- und Arbeitsaufwand überlegen. Eine submerse Herstellung von *G. stearothermophilus* Sporen würde deshalb, analog zur submersen Herstellung von *B. atrophaeus* Sporen, die kommerzielle BI Herstellung vereinfachen, was wiederum einen weiteren Grundstein für eine langfristige Standardisierung des Herstellungsprozesses legt.

Die etablierte Zwei-Schritt Herstellungsmethode wurde schließlich auf die Möglichkeit weiterer Optimierung und Vereinfachung untersucht. Dabei konnte bewiesen werden, dass die in dieser Arbeit etablierten und entwickelten Herstellungsmethoden allen Ansprüchen für eine industrielle BI Herstellung gerecht werden. Zum einen können mit den hier dargelegten Methoden Sporenmengen mit Relevanz für die industrielle BI-Herstellung gewonnen werden, zum anderen liegen die Resistenzen der mit diesen Methoden hergestellten Sporen gegen Wasserstoffperoxid in einem Bereich, in dem

auch aktuelle kommerzielle BI liegen. So ist ein Methodentransfer der gezeigten Verfahren direkt und ohne Einschränkungen für die BI Anwender möglich.

Zusammenfassend liefert diese Arbeit grundlegendes und direkt übertragbares Wissen für eine Standardisierung kommerzieller BI Herstellung bei gleichzeitiger Reduktion der aktuell in der Praxis auffindbaren Resistenzvariabilität.

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7 Appendix

7.1 Peer reviewed publications

Stier, P., Kulozik, U., 2020. Effect of Sporulation Conditions Following Submerged Cultivation on the Resistance of *Bacillus atrophaeus* Spores against Inactivation by H2O2. Molecules 25, 2985. https://doi.org/10.3390/molecules25132985

Stier, P., Maul, S., Kulozik, U., 2021. Effect of sporulation conditions following solid-state cultivation on the resistance of *Geobacillus stearothermophilus* spores for use as bioindicators testing inactivation by H2O2. LWT 151, 112078. https://doi.org/10.1016/j.lwt.2021.112078

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7.2 Non reviewed publications

Stier, P., Kulozik, U., 2016. Standardisierte Herstellung von biologischen Indikatoren für die Beurteilung der Oberflächenentkeimung mit Wasserstoffperoxid und Minimierung ihrer Varianz, Jahresbericht 2016 über die milchwissenschaftliche Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TUM, 44-45, ISBN 978-3-939182-93-1

Stier, P., Kulozik, U., 2017. Standardisierte Herstellung von biologischen Indikatoren für die Beurteilung der Oberflächenentkeimung mit Wasserstoffperoxid und Minimierung ihrer Varianz, Jahresbericht 2017 über die milchwissenschaftliche Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TUM, 51-53, ISBN 978-3-947492-00-8

Stier, P., Kulozik, U., 2018. Einfluss der Produktionsbedingungen bakterieller Sporen auf die Eignung zur Verwendung als biologische Indikatoren für die Beurteilung der Oberflächenentkeimung mit Wasserstoffperoxid, Jahresbericht 2018 über die milchwissenschaftliche Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TUM, 55-57, SBN 978-3-947492-10-7

Stier, P., Kulozik, U., 2019. Einfluss der Sporulationsbedingungen auf die Resistenz bakterieller Sporen für die Verwendung als Bioindikatoren, Jahresbericht 2019 über

die milchwissenschaftliche Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TUM, 63-65, ISBN 978-3-947492-16-9

Stier, P., Kulozik, U., 2020. Auswirkungen des Lagermediums und der Lagerdauer auf die Resistenz bakterieller Sporen für die Verwendung als Bioindikatoren gegenüber Wasserstoffperoxid, Jahresbericht 2020 über die milchwissenschaftliche Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TUM, 40-42, ISBN 978-3-947492-20-6

Stier, P., Kulozik, U., 2020. Standardisierte Herstellung von biologischen Indikatoren für die Beurteilung der Oberflächenentkeimung mit Wasserstoffperoxid und Minimierung ihrer Varianz. Abschlussbericht zu AiF 19358 N. FEI Forschungskreis der Ernährungsindustrie.

7.3 Oral presentations

Stier, P., Kulozik, U., 2017. Standardized production of biological indicators for the evaluation of surface decontamination with hydrogen peroxide and minimization of their variance, DrinkTec, München, 11.-15.09.2017

Stier, P., Kulozik, U., 2017. Standardized production of biological indicators for the evaluation of surface decontamination with hydrogen peroxide and minimization of their variance, VDMA Arbeitskreis Schnittstellenproblematik bei Aseptikanlagen, Frankfurt, 17.11.2017

Stier, P., Kulozik, U., 2019. Validierung von Sterilisationsprozessen durch Sporen mit standardisierter Resistenz als Bioindikatoren, Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising, 10.-11.10.2019

Stier, P., Kulozik, U., 2020. Standardisierte Herstellung von biologischen Indikatoren für die Beurteilung der Oberflächenentkeimung mit Wasserstoffperoxid und Minimierung ihrer Varianz, VDMA Arbeitskreis Schnittstellenproblematik bei Aseptikanlagen, Frankfurt, 09.10.2020