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Influence of spore and surface hydrophobicity on decontamination with hydrogen peroxide vapor

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„Es kommt nicht darauf an wie es lang ist,
sondern der Weg ist das was sein muss“

Adam Grabowski

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Abbreviations

Latin symbols

A	absorption, surface	-
AFM	atomic force microscopy	
ATCC	American Type Culture Collection	
<i>B.</i>	<i>Bacillus</i>	
BI	Biological indicator	
BIER	Biological indicator evaluation resistometer	
CFM	chemical force microscopy	
cfu	colony forming units	
CM	carrier material	
CotA, CotY, CotZ	spore coat proteins	
DF	dilution factor	
DPA	dipicolinic acid	
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen	
D-value	decimal reduction time	s
exp	exponential function	
FDA	Food and Drug Administration	
<i>g</i>	gravity	m·s ²
<i>G.</i>	<i>Geobacillus</i>	
<i>H</i>	degree of hydrophobicity	%
H ₂ O, W	water	
H ₂ O ₂ , H	hydrogen peroxide	
HPLC	high-performance liquid chromatography	
KatA, KatB, KatX	spore enzymes	
LDPE	low density polyethylene	
log	logarithm to the basis of 10	
n	sum of spores in four big squares	
OH•	hydroxyl radical	
P	saturated equilibration pressure	
PE	polyethylene	

Abbreviations

PET	polyethylene therephthalate	
ppm	parts per million	
R	universal gas constant	
r, R	radius	
R ²	correlation coefficient	
ROS	reactive oxygen species	
S	survival rate	
SAL	sterility assurance level	
SASP	small acid soluble spore proteins	
SEM	scanning electron microscopy	
Spo0A	phosphorelay regulator	
t	time	s
T	absolute temperature	K
UV	ultraviolet radiation	
x	amount of spores	cfu mL ⁻¹
z-value	increase of temperature to cut the D-value to 10%	K

Greek symbols

α	level of significance
γ	activity coefficient
χ	mole fraction
π	Ludolph's constant

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1 General introduction

Packaging technology has to fulfill numerous requirements such as partitioning the product in small quantities, protecting it from sunlight, oxygen, water vapor, insects and microorganisms, inform the consumer and provide convenience. One of the most challenging requirements is microbiological safety. As microorganisms are ubiquitous, they occur in the product and on the packaging material surface but also in the air and on every wall and floor in the processing plant outside of cleanrooms. To obtain a microbiologically safe product, the microbial load has to be reduced in an extent so that no growth of microorganisms can modify the product and no risk for human health arises (Buchner 1999; Heiss and Eichner 1995; Sun 2011).

For foods with short storage life, pasteurization at moderate temperatures is sufficient. Pasteurization means the inactivation of all pathogenic vegetative microorganisms. Sterilization in contrast means inactivation of all microorganisms including bacterial spores. Statistics shows that complete inactivation of all microorganisms is impossible, however, reduction of the microbial load to 1 microorganism in 10.000 packed products is feasible and commonly referred to as sterilization (Kessler 2006).

In food and pharma industry, there are basically two different ways of sterilization:

- Terminal sterilization means sterilization of the completely packed product. The product is filled into the packaging material whereat both may contain microorganisms. After the filling process, the whole unit is sterilized, usually by thermal methods as batch or continuous process. However, due to the problem of irregular heat distribution in the end product, over-processing and loss of valuable components can occur. The most popular example for this packaging and sterilization process is food packed in tins (Heiss and Eichner 1995).
- For aseptic processing, the filling and sterilization step are decoupled. The inactivation of microorganisms in the product and on the packaging material takes place prior to the packaging process. The packaging process itself has to be carried out without recontamination of either product or packaging material under aseptic conditions. The advantage of this process lies in the direct processing of food and material which results in milder inactivation conditions. Thus, sensitive ingredients are less affected. Besides, the packaging material can be treated by means of physical or chemical methods not suitable for the product (Heiss and Eichner 1995). This process is of major importance for food and pharma industry.

In 1961, Alpura (today Nestlé) developed an aseptic process for Tetra Pak applying a packaging material consisting of cardboard covered with polyethylene (PE) on both sides. This packaging material was decontaminated with hydrogen peroxide (H₂O₂) without

alteration of the PE surface. Approval of hydrogen peroxide by the FDA (Food and Drug Administration) as disinfection agent in the USA led to an increase of aseptic processes. H₂O₂ is a cost-efficient disinfectant with highly inactivating character, which is nowadays applied in many decontamination plants. Probably the most fascinating aspect of the colorless oxidation agent is its decomposition to water and oxygen (Buchner 1999).

Numerous studies concerning the influence of H₂O₂ concentration, temperature and stabilizers are available for the application in its liquid form as H₂O₂ bath. However, vaporized H₂O₂ with its application in industry only dating back some 10 to 15 years is a rather new process. Compared to liquid H₂O₂, the number of influencing factors is higher when vapor is applied. Studies considering most of the influencing factors in a coherent way and thus providing deeper insights into this decontamination process are scarce, especially for high H₂O₂ concentrations of 3000 to 6000 ppm.

There are two processing options for hydrogen peroxide vapor: It can be applied either in its gas form without condensate formation, which is mostly used for the decontamination of clean rooms and isolators. The second option is to steer the process in a way that condensate will form on the surface. Therefore, either the amount of H₂O₂ or water in the gas mixture has to be elevated or the temperature of the decontamination surface has to drop below the dew point temperature of the respectable gas concentration.

Pruss (2013) studied the influence of condensate formation of H₂O₂ and compared the inactivation effect of both processes, with and without condensate formation, on different bacterial spores. She formulated the hypothesis that surface hydrophobicity of bacterial spores does have a major impact on the decontamination with gaseous and condensing hydrogen peroxide. To understand this assumption, it is important to know how condensate formation takes place. According to Beysens (2006), condensate forms preferentially at condensation nuclei. The phase transition from gas to liquid is an energy-consuming process. The required energy depends on the surface hydrophobicity of the condensation nuclei, whereat the least amount of energy is needed for the most hydrophilic surface. Thus, condensation nuclei with hydrophilic surface will be engulfed by the condensate first. According to the hydrophobicity theory, the specific surface hydrophobicity of bacterial spores might be the deciding characteristic for survival.

Efficacy of vaporized H₂O₂ decontamination is measured by the use of Biological Indicators (BI). BI are microorganisms that show a high resistance towards the specific decontamination process. For H₂O₂, spores of *Bacillus subtilis* (*B. subtilis*), *B. atrophaeus* and *Geobacillus stearothermophilus* (*G. stearothermophilus*) are commonly applied. So far, surface hydrophobicity of BI has not been taken into account as an influencing factor on inactivation results. Several studies show that by means of cultivation conditions such as temperature and cultivation media, specific characteristics of bacterial spores such as heat

resistance can be modified. If the spore resistance which depends on interior spore structures can be influenced, surface characteristics such as hydrophobicity of bacterial spores might be influenced as well. However, little is known about the influence of culturing conditions on the surface hydrophobicity of bacterial spores.

In order to examine the surface hydrophobicity of bacterial spores, a consistent method to measure surface hydrophobicity is needed. With this knowledge, manipulation of bacterial spores by means of culturing conditions can be investigated to obtain spores of different surface hydrophobicity. With those spores, the influence of surface hydrophobicity on the decontamination with gaseous and condensing hydrogen peroxide can be investigated.

1.1 Bacterial spores

Certain microorganisms possess the ability to form endospores when they encounter depletion of nutrients and high cell density (Grossman 1995). Those metabolically inactive endospores (in this thesis simply referred to as spores) have several resistance mechanisms towards harsh environmental conditions such as extreme temperatures, dryness or depletion of nutrients (Madigan and Martinko 2013; Eitinger et al. 2014). Therefore, they can survive for several years until environmental conditions are suitable for germination and subsequent growth before returning to a vegetative cell. Some controversially discussed studies indicate that spores are able to survive in their dormant state for more than 25 million years (Cano and Borucki 1995) or even 250 million years (Vreeland et al. 2000).

The most prominent spore formers are the gram-positive species *Bacillus* and *Clostridia*. The latter ones are anaerobic whilst the ubiquitous *Bacilli* are aerobic. Under a light microscope, the small spherical spores can easily be distinguished from their rod-shaped vegetative form. Instead of cytoplasm, the spore core contains a complex consisting of dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) and calcium (Ca^{2+}) that changes the refraction of light and accounts for their bright appearance (Antranikian 2006). Phase bright spores of *B. subtilis* are depicted in Figure 1-1.

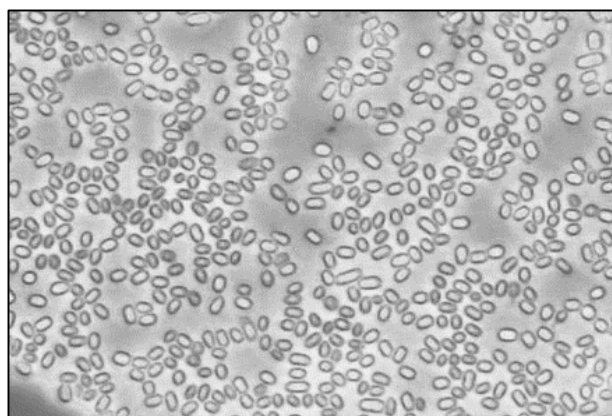


Figure 1-1: Light microscopic picture of bright *B. subtilis* spores.

1.1.1 Formation of spores

The vegetative cell cycle and binary fission of vegetative spore formers are interrupted when depletion of nutrients or other environmental stresses occur. In lab-scale, sporulation is usually initiated when the lag-phase/stationary phase begins and the carbon, nitrogen or phosphorous source present at the beginning have been metabolized (Nicholson and Setlow 1990).

Many studies classify the act of sporulation according to their morphological features as well as biochemical and genetic criteria in 8 different stages of sporulation. However, as during step 1 only anchoring of the chromosome at the two cell poles, ensuring of the proper chromosome copy number and replication of the chromosome takes place (Ben-Yehuda et al. 2003), stage 1 is not explicitly shown and discussed anymore. The schematic sporulation process has been changed to basically six steps of spore formation (Nicholson and Setlow 1990; McKenney et al. 2012).

The spore former about which most research was published is probably *B. subtilis*. Even its genome sequence was completely revealed by Kunst et al. (1997). Therefore, the process of sporulation and the spore structure will be explained using the example *B. subtilis*. However, many other spore formers exhibit similar sporulation pathways.

The transition from a vegetative cell to a spore is depicted in Figure 1-2.

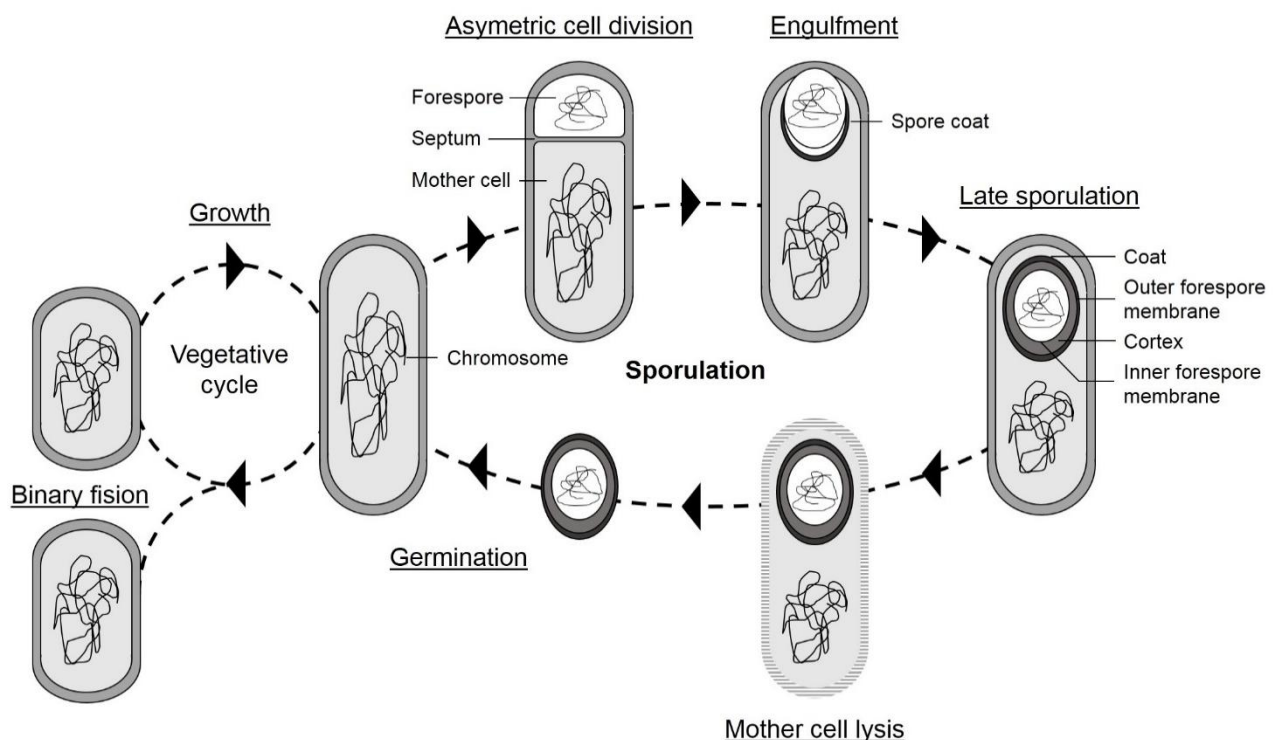


Figure 1-2: Cycle of sporulation and germination, modified after McKenney et al. (2012)

Sporulation is directed by a genetic program and results in complete reorganization of the cellular structure. The activation of histidine sensor kinases which activate the transcription factor Spo0A by phosphorylation induces the sporulation process. This factor is the master regulator of sporulation controlling a huge amount of genes and initiating many spore formation mechanisms (Stephenson and Hoch 2002; Fujita and Losick 2002). After activation of Spo0A, axial filamentation and asymmetric cell division lead to a bigger mother cell and a smaller forespore, which will be the spore later on. Each of the two cell compartments has its own complete chromosome (McKenney et al. 2012). During the next step, the engulfment, the mother cell “swallows” the forespore. The result is a double membrane around the forespore which lies in the cytoplasm of the mother cell (Tan and Ramamurthi 2014). After the engulfment, several layers begin to form around the spore simultaneously. Formation of spore cortex and coat are essential for spore resistance mechanisms. At the stage of late sporulation, those layers are completed and the spores’ resistance towards various environmental stresses such as heat, radiation, mechanical disruption and chemicals increases. The last step is the lysis of the mother cell and release of the fully developed spore in its dormant state. When the environmental conditions are suitable, germination takes place and the vegetative cell cycle will begin again. The complete sporulation process takes approximately 8 hours (Nicholson and Setlow 1990; Kay and Warren 1968; Driks 2002). Without further maturation time, spore resistance against wet heat and hypochlorite is low. Sanchez-Salas et al. (2011) showed that spores acquire their full heat resistance after a maturation time of 24 h. They assume changes in the coat structure to be responsible for the increased resistance.

1.1.2 Bacterial spore structure and its relevance for resistance

Bacterial spores can be seen as dormant encapsulated DNA surrounded by several protection mechanisms just waiting for environmental conditions to become favorable for germination. Structure and chemical composition of the spore play the key role in spore resistance properties (Setlow 2006). Beginning with the inside of the spore, the complex spore structure and the distinct protection mechanisms will be explained in the following section. The schematic structure of a *B. subtilis* spore is depicted in Figure 1-3.

Most of the spore’s enzymes, the DNA, the ribosomes and tRNAs are located within the spore core. Protection of the spore’s DNA is essential for successful germination and the subsequent vegetative cell cycle. Therefore, the innermost part of the spore has three different protection mechanisms (Setlow 2007). Besides providing 90 % of the protein needed for spore germination by their own degradation, small, acid- soluble spore proteins (SASPs) are important for DNA protection of the dormant spore. They make up 5 – 10 % of the total spore core protein content which is a sufficient amount to saturate the spore DNA. The biggest part of SASPs consists of alpha/beta type SASPs. Those proteins bind to spore DNA, alter the structure significantly and increase the resistance of the spore against many

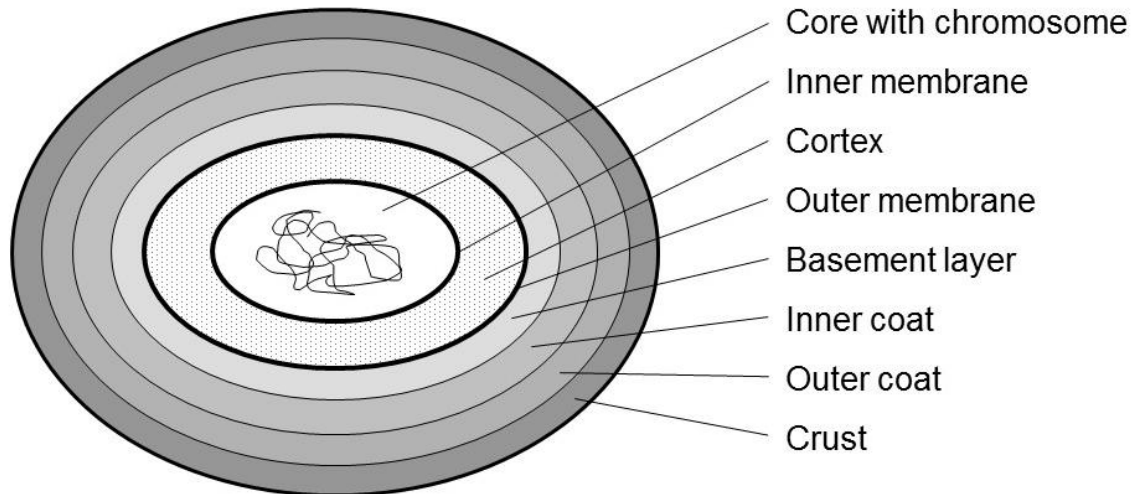


Figure 1-3: Schematic structure of a *Bacillus subtilis* spore, modified after McKenney et al. (2012)

DNA-damaging agents. They slow down DNA depurination due to wet or dry heat, protect DNA against cleavage by enzymes, damage by nitrous acid, hydroxyl radicals and hydrogen peroxide, prevent cytosine deamination by uracil and lower the UV sensitivity of DNA (Mason and Setlow 1986; Setlow 1988; Setlow and Setlow 1979, 1993; Tennen et al. 2000; Setlow 2006)

In vegetative cells, the water content is about 75 to 80 % of the wet weight, whereat in spores it's only 28 – 57 %. Due to DPA which is assumed to chelate with divalent cations, mostly Ca^{2+} , most of the water in the spore core is immobilized (Gerhardt and Marquis 1989). Kaieda et al. (2013) strengthen the gel scenario due to NMR measurements. As the amount of free water in the spore is extremely low, macromolecular movement is almost impossible. By means of Ca-DPA, the spore resistance to wet and dry heat, desiccation and hydrogen peroxide increases however spore DNA becomes more sensitive to UV radiation (Setlow et al. 2006; Douki et al. 2005). In case of damaged spore DNA, spores possess the ability to repair it. However, some of the specific repair mechanisms only begin with spore germination (Setlow and Setlow 1996; Setlow 1992).

The inner membrane was formerly the membrane of the forespore and later becomes part of the future plasma membrane of the vegetative cell (Tocheva et al. 2011). It surrounds the spore core and acts as strong permeability barrier to chemicals otherwise damaging DNA such as formaldehyde, nitrous acid and hydrogen peroxide (Swerdlow et al. 1981; Cortezzo and Setlow 2005). Between the inner and outer spore membrane lies a thick peptidoglycan rich layer. The spore cortex accounts for most of the peptidoglycan layer, the germ cell wall (not shown in Figure 1-3) accounts only for a very small part. The whole peptidoglycan layer contains muramic lactam which during germination allows the spore to degrade the cortex but not the germ cell wall. The germ cell wall in turn serves as a basis for vegetative cell wall

formation during germination (Popham et al. 1996). The cortex is important to maintain the spores' dehydrated condition during dormancy, the exact process remains unknown (Popham 2002; Popham et al. 1996). The outer spore membrane results from the mother cell during the engulfment process and encloses the cortex from the other side. The outer spore membrane is essential for the formation of the spore coat during sporulation (Piggot and Hilbert 2004). Its precise function in the dormant spore is not clear though (Ramamurthi et al. 2006; Setlow 2006).

The outermost section of the spore is the spore coat. Its structure and biochemical composition vary depending on spore species and strain (Melly et al. 2002b). For *B. subtilis* spores, this outermost section consists of a basement layer, inner coat, outer coat and a structure detected relatively recently called the crust (Imamura et al. 2010; McKenney et al. 2010). The coat with its distinct layers is comprised of at least 70 different proteins produced by the mother cell and put into their place during engulfment and spore maturation (McKenney and Eichenberger 2012). It is responsible for resistance against chemicals such as H₂O₂, hypochlorite or chlorine dioxide as well as lysozyme. One possible explanation is that the coat acts as a diffusion-barrier for those molecules. Another explanation, especially for H₂O₂, is that coat proteins serve as oxidation target and hence decrease the effective concentration of H₂O₂ (Riesenman and Nicholson 2000; Young and Setlow 2003). Hullo et al. (2001) state that the spore coat protein CotA generates a pigment structurally similar to melanin which increases the resistance of spores to H₂O₂ and UV light. Above that, the spore coat protects the spore against predatory eucaryotic microbes (Klobutcher et al. 2006).

Some vegetative microorganisms possess the enzyme catalase that protects them against low amounts of H₂O₂ by decomposition of the oxidizing agent (Ma and Eaton 1992). There are microorganisms with high catalase activity like *Exiguobacterium oxidotolerans* that are able to survive, depending on cell density, concentrations of up to 15 mg·mL⁻¹ (Pruss 2013). Bacterial spores exhibit catalases called KatA and KatB in their vegetative state (Loewen and Switala 1987). Bol and Yasbin (1994) found out that vegetative *B. subtilis* lacking those enzymes are more sensitive towards liquid H₂O₂. However, in their spore form, KatA and KatB are not present whilst another enzyme, KatX, is exhibited (Singh et al. 1977). However, this enzyme does not contribute to the spore's resistance against H₂O₂ (Casillas-Martinez and Setlow 1997).

Concluding, bacterial spores possess numerous factors that contribute to their extremely high resistance, but catalase is none of those factors.

1.1.3 Biological indicators

According to the FDA, every sterilization procedure should be monitored to verify the sterilizing conditions and draw conclusions concerning the microbiological status of the product. Therefore, mechanical, chemical and biological indicators (BI) are used. BI are test microorganisms which are commercially available as suspensions in distilled water or 40 % ethanol, as paper-strips with defined microorganism load or as ampoules with microorganisms in combination with cultivation media and a pH indicator that changes the color when microbiological growth occurs (Rieth and Krämer 2016). The BI resistance described as the D-value of the lot against the specific decontamination process and the microbiological count define the BI quality (Leitfaden). Mechanical and chemical indicators can be applied to monitor the sterilization parameters, however, BI are the only option to obtain knowledge about the direct decontamination impact on the microbial load.

BI are applied when new devices are brought into service, to survey periodically the efficacy of aseptic processes or to survey the efficacy of clean room decontaminations. The microorganism species should be chosen depending on the inactivation method. The VDMA (Verband Deutscher Maschinen- und Anlagenbauer) provides three essential demands for microorganisms to act as biological indicators (VDMA, 2000). The test organisms should:

- Have a high, defined resistance against the decontamination process
- Be easy to detect
- Be nonhazardous for health

In order to obtain reliable results, microorganisms that are very resistant against the decontamination process should be applied. Different types of microorganisms display different defense mechanisms. Chemicals for example are kept out of bacterial spores by means of the cortex and spore coat, mycobacteria have a waxern cell wall that prevents chemicals from entering whereat the outer membrane of gram-negative microorganisms provides protection from chemicals otherwise entering the cell. Bacterial spores have the highest resistance against most inactivation processes, next are mycobacteria, fungi and gram-negative microorganisms (Rutala and Weber 2008). The only exception is UV-C treatment, where *Aspergillus brasiliensis* conidiospores are most resistant due to their melanin rich cell wall (Esbelin et al. 2013). Table 1-1 shows some commonly applied BI for specific decontamination processes.

Table 1-1: Microorganism species often applied for specific decontamination procedures

Decontamination procedure	Species
Wet heat / Steam	<i>Geobacillus stearothermophilus</i> (Rutala and Weber 2008; DIN EN ISO 14161)
Dry heat	<i>Bacillus atrophaeus</i> (Rieth and Krämer 2016)
Ethylene oxide	<i>Bacillus subtilis</i> / <i>atrophaeus</i> (DIN EN ISO 14161)
Ionizing radiation	<i>Bacillus pumilis</i> (Rieth and Krämer 2016)
Hydrogen peroxide	<i>Bacillus subtilis</i> / <i>atrophaeus</i> (Rutala and Weber 2008)
UVC energy	<i>Aspergillus brasiliensis</i> (Esbelin et al. 2013)

The DIN ISO norm 14161 entitled „sterilization of health care products – biological indicators“ serves as guideline for the development, validation and monitoring of sterilization procedures. This guideline, along with the VDMA and the FDA, suggests some spore species suitable for the particular decontamination process. Strict regulations concerning the choice of microorganism species for BI application do not exist. The use of microorganism species other than shown in Table 1-1 might also be purposeful, depending on the aim of the test (DIN EN ISO 14161; VDMA 2003).

Since bacterial spores acting as BI are commonly more resistant to most inactivation methods than other microorganisms and above that being present in larger numbers, successful inactivation of the BI strongly suggests that other potentially contained pathogens are inactivated as well (Leitfaden).

The inactivation results of different decontamination devices working with the same inactivation process (e.g. gaseous H₂O₂) should be comparable. Therefore, a constant and consistent resistance of BI is essential. Agalloco et al. (2007) even propose to verify the BI resistance of every spore lot with a biological indicator evaluation resistometer (BIER) vessel prior to BI implementation. False positive results due to a more resistant indicator lead to intensification of decontamination parameters. False negative results due to less resistant indicators even lead to the conclusion that the decontamination device is working properly which isn't the truth. Both situations induce conflicts, insecurity and avoidable costs. Therefore, BIs need to be of consistent high quality. To examine the efficacy of inactivation prior to the onset of production, several test methods can be applied. Two of the most commonly applied methods will be explained in detail.

Count reduction test: BI are brought into the decontamination device, e.g. an aseptic filling machine or a clean room. The number of viable spores is detected before and after passing the decontamination device. The difference between those two viable spore counts provides the rate of inactivation (Merkblatt 6). This test is suitable if the exact amount of inactivated microorganisms is of interest.

End point test: For this test, special BI are produced from spore suspensions in combination with an adequate carrier material. The microorganisms are brought onto the carrier material in three different concentrations with a difference of 1 log each. After decontamination, BI are put in rich medium and incubated. The number of sterile and unsterile BI is detected later on (Merkblatt 6).

The effectiveness of the decontamination can be determined with both test methods. The difference compared to the count reduction test is that on the one hand, BI with different amounts of microorganisms have to be produced prior to the test which is time-consuming. On the other hand, for the end point test, it is possible to apply the relevant packaging material as carrier material for BI, conduct the decontamination step and subsequently fill in the correspondent product. Therefore, the end point test provides more realistic results and moreover, even allows a conclusion about the degree of efficiency from product infeed system to sterile sealing of the packed product (Merkblatt 6).

The goal of the BI application and subsequently examination of the decontamination performance is to achieve a certain sterility assurance level (SAL). SAL means the probability that one single microorganism occurs on the item after the inactivation process. This quantitative value generally ranges between 10^{-3} to 10^{-6} whereat 10^{-3} means that one package in 1.000 packages is contaminated, 10^{-6} stands for 1 contaminated package in 1.000.000 units. Hence, an SAL of 10^{-6} has a lower value but provides a greater assurance of sterility than does a value of 10^{-3} (Gillis et al. 2010).

1.1.4 Influence of cultivation conditions on spore properties

The characteristics of microorganisms can be influenced by means of cultivation conditions (Baweja et al. 2008). Hence, microorganism properties can be modified without genetic modification, microorganisms of the same genetic setup but with different characteristics can be obtained. Besides, vegetative cells form spores of the same resistance independent of the previous cell history or physiological state (Baril et al. 2012a). This study shows that vegetative cells that have been modified by means of cultivation conditions run through a retrogression with ongoing cell cycle under normal cultivation conditions. Hence they lose their special characteristics again. Bacterial spores in contrast can be harvested and stored as they are in a dormant state and thus keep their characteristics at least for a certain time.

There are several studies dealing with the influence of cultivation temperature, level of pH and oxygen, cultivation media and time. The most obvious cultivation condition having influence on cell characteristics is the sporulation medium. Ramirez-Peralta et al. (2012) cultivated *B. subtilis* in rich and poor medium, whereat the spores formed in rich medium showed faster germination.

Mineral supplements in the cultivation medium influence the spore number and size as shown by Buhr et al. (2008) who examined *B. atrophaeus* and *B. cereus* spores. With rising amount of phosphate in sporulation medium, resistance of spores of *B. cereus* to heat increases as well. However, the addition of sodium chloride (NaCl) and potassium chloride (KCl) had no significant impact on heat resistance (Mazas et al. 1997).

The influence of antifoam agents on sporulation intensity, spore recovery and cell debris was investigated by Vidyarthi et al. (2000) and Buhr et al. (2008). They showed that the resulting amount and purity of spores, meaning spores without vegetative cells, depends on sporulation salts, glutamate and antifoam agent whereat the latter one had a positive effect on the resulting amount of spores.

There is an influence on spore characteristics whether they are cultivated on agar plates or in liquid. According to Rose et al. (2007), spores prepared in liquid are threefold less resistant to heat and germinate faster whereat spores cultivated on agar plates are darker. Investigation of coat protein levels and cross linking, the amount of DPA, core water content and SASPs resulted in no major difference. The only difference was the higher ratio of anti-iso to iso fatty acids in spores prepared on agar plates which means the spores differ concerning their inner membrane composition.

Abbas et al. (2014) cultivated different *B. cereus* strains under aerobic and anaerobic conditions. Some strains showed sporulation under both conditions, whereat anaerobic cultivation led to less spores which are more heat resistant at 90 °C. However, with rising temperature, the difference was not significant anymore. Anaerobic spores are also more resistant against sodium hydroxide and nitrous acid but not against 5 % H₂O₂, UVC (ultraviolet radiation) and formaldehyde.

Most studies concerning the impact of culture conditions focus on the influence of cultivation temperature. Lindsay et al. (1990) produced spores of *B. subtilis* at temperatures of 23 to 49 °C. Heat resistance continuously increased with increasing cultivation temperature until 45 °C. Those spores displayed the highest amount of magnesium and calcium. Spore surface was also changed, at 23 °C the surface was mostly rough and at 49 °C spores showed smooth surfaces for the biggest part. Aouadhi et al. (2016) received similar results for spores of *B. sporothermodurans* cultivated at temperatures between 4 and 50 °C. With increasing cultivation temperature, resistance to heat combined with nisin increased to a peak at 37 °C cultivation temperature.

Baril et al. (2012b) examined the influence of various pH levels and cultivation temperatures on spores of *B. weihenstephanensis* and *B. licheniformis*. Their results are similar to the results of Lindsay et al. and Aouadhi et al. (2016), very high and very low cultivation temperature cause low spore heat resistance. Acidic pH-values although lead to low spore

heat resistance. For the test spores, cultivation boundaries roughly correspond to sporulation boundaries in terms of pH and temperature whereat a pH-value of 7,00 led to optimal growth (Baril et al. 2012a).

Craven (1990) was among the first to investigate the influence of cultivation pH on bacterial spores. He modified the pH on agar plates from 7.00 to 8.50 and examined the heat resistance of the resulting *Clostridium perfringens* spores. An elevated pH led to increased heat resistance. Depending on the buffer to keep the pH constant during sporulation, heat resistance and amount of spores also changed. Similar results were obtained by Mazas et al. (1997) for *B. cereus* spores cultivated on nutrient agar. They observed good spore production between pH 6.5 and 8.3. Higher cultivation pH led to increased heat resistance here as well. Spore resistance against a combination of nisin and heat was investigated by Aouadhi et al. (2016). The results displayed higher spore resistance at elevated cultivation pH values.

Interestingly, the pH value in the dormant spore is independent of the pH value of the surrounding medium (Setlow and Setlow 1980).

1.2 Hydrophobicity

The International Union of Pure and Applied Chemistry (IUPAC) defines hydrophobicity as “the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules” (van de Waterbeemd et al. 1997).

At first sight, this definition seems to be quite simple. However, the understanding of hydrophobicity on a chemical basis is rather complex and will therefore be explained in the following paragraphs.

The term “hydrophobicity” arises from the ancient Greek word “hydor” which means water, and the word phobia. Freely translated hydrophobicity is the urge to avoid water. Looking closer at the water molecule, it is a polar molecule consisting of two hydrogen atoms with positive charge and one oxygen atom with negative charge as depicted in Figure 1-4. The core areas of the negative and positive charges are not the same but from the outside the molecule is neutral, which makes water a polar molecule. It’s dipole moment is 1,84 Debye (Mortimer and Müller 2010; Law 2014).

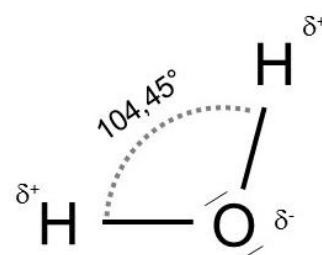


Figure 1-4: Schematic water molecule modified according to Belitz et al. (2008)

Unpolar molecules, for example hexadecane in an aqueous solution, want to reach the lowest possible energy level. Due to the polar character of water, this can only be achieved by forming clusters of hexadecane to keep the overall interface between unpolar and polar liquids as small as possible and the state of energy as well. However, water does not reject unpolar molecules. Water attracts other polar water molecules whereat unpolar molecules do not have attractive interactions with water. Due to the mutual attraction of water molecules, an exclusion of unpolar molecules takes place and associations appear (Dörfler 2002).

According to van Oss (2006), there are different non-covalent interaction energies. The three most important interaction energies will be described in short: The Lifshitz-van-der-Waals forces, the polar Lewis-acid-base interactions and the electron forces. All three of them are depicted in Figure 1-5. Another interaction energy which will not be considered in this work is the Brownian molecule movement.

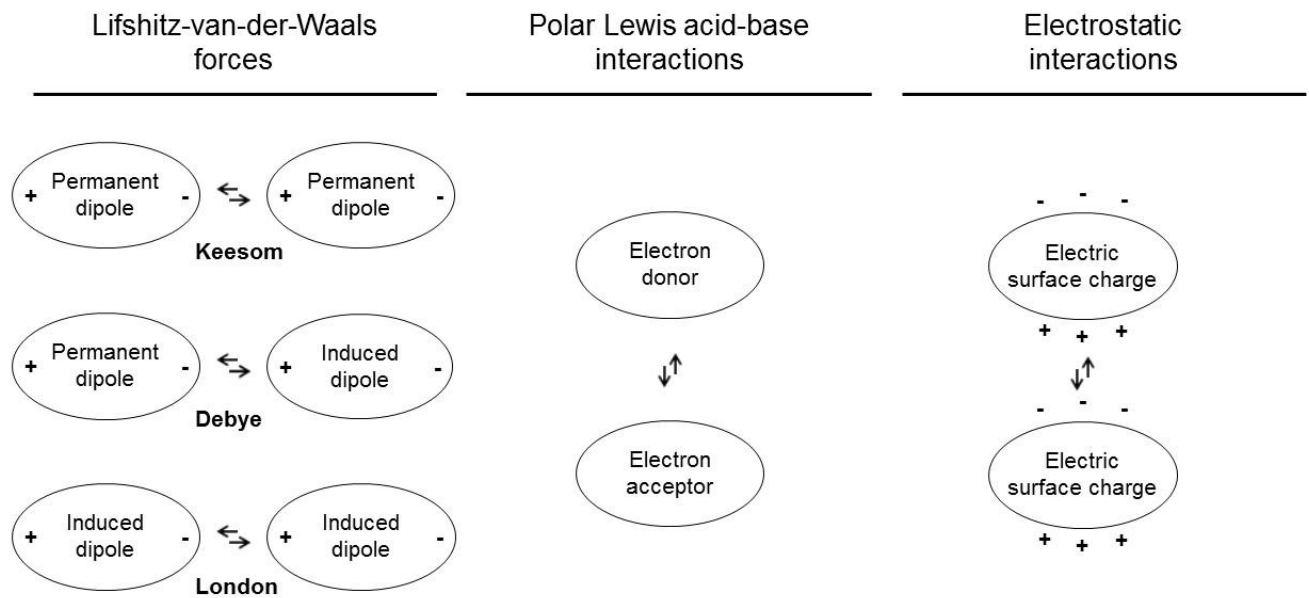


Figure 1-5: Non-covalent interaction energies as described by van Oss (2006)

Lifshitz-van-der-Waals forces are weak non-covalent interactions between atoms or molecules and can be divided into three parts: The Keesom energy exists between two permanent dipoles whereat the Debye energy involves one permanent dipole and a dipole induced by it. As even in neutral atoms quickly fluctuating dipoles occur, they can induce dipoles in other molecules and thus attract them. This energy is called London energy (van Oss 2006).

Lewis-acid-base-interactions are also called hydrophobic interactions (Franks, 1975). A Lewis-acid means an electron acceptor, so electrons can add to it. A Lewis-base in contrast is an electron donator which provides electrons. According to van Oss (2006), hydrophobic attraction and hydrostatic repulsion are both based on polar Lewis-acid-base-interactions.

Electrostatic interactions arise from electric surface charge of particles and molecules. Polymers or particles with the same sign of charge will repel each other when immersed in a polar liquid such as water.

With few exceptions, none of these forces appears in its pure form alone. In reality, an interaction of the three forces adds up to what we describe as the exclusion of unpolarmolecules from aqueous environment, the hydrophobicity (Dörfler 2002; van Oss 2006).

1.2.1 Surface hydrophobicity of microorganisms

In this thesis, the detached term hydrophobicity has no meaning. However, the classification in high and low hydrophobicity will be applied whereat low hydrophobicity describes hydrophilic behavior and high hydrophobicity stands for hydrophobic characteristics.

Microbial adhesion is an important factor in biofilm formation (Abdallah et al. 2014). As the outer cell surface is responsible for adhesive behavior (Wilson et al. 2001), it is also responsible for cell surface hydrophobicity. The molecular composition of the outermost layer of the spore is the crucial factor for the specific hydrophobic characteristics.

Although Imamura et al. (2010) and McKenney et al. (2010) detected the outermost layer of *B. subtilis* spores, the crust, little is known about its exact chemical composition except that it consists mostly of glycoproteins (McKenney et al. 2010; Imamura et al. 2010).

The tendency to avoid water results directly from the presence of unpolar or not polarizable macromolecules on the surface of microorganisms. However, oxygen and nitrogen residues on the surface promote interactions with water molecules via hydrogen bonds and thus reduce bacterial surface hydrophobicity. According to Mozes et al. (1988), hydrophobicity relates directly with the amount of carbon in hydrocarbon form and indirectly with the concentration of oxygen or with the ratio of nitrogen to phosphate on the surface.

Chen et al. (2010) applied infrared spectroscopy analysis to examine the chemical surface composition of spores of *B. subtilis* and *B. anthracis*. The spore surface of *B. subtilis* has more functional and polarizable groups of aldehydes and carboxylic acids than does *B. anthracis*. A decrease in those functional groups increases the tendency to adhesion. *B. anthracis* spores are surrounded by an exosporium which is generally considered to be hydrophobic (Koshikawa et al. 1989).

1.2.2 Methods to measure surface hydrophobicity

There are several studies proposing suitable measurement methods to evaluate surface hydrophobicity of bacterial spores.

MATH-Test

The MATH-test (Microbial Adhesion to Hydrocarbons) can be conducted with a simple experimental setup which might be one reason for its prevalent application. It was first described by Rosenberg et al. (1980). This optical density measurement is based on the adhesion of microorganisms to hydrocarbons.

A microorganism suspension consisting of microorganisms in water is layered with a hydrocarbon, for example hexadecane. The absorption of the aqueous phase is measured prior to the test at 400 nm. The resulting two phase system is mixed for a defined time so that spores can adhere to the unpolar hydrocarbon droplets. As the density of hydrocarbons is generally lower than the density of water, it will subsequently rise to the top of the test tube and remove the microorganisms adhering to hydrocarbon droplets from the suspension. The absorption of the aqueous phase is measured again. The degree of hydrophobicity (H) in percent can be calculated as shown in (1-1), the basic principle is displayed in Figure 1-6.

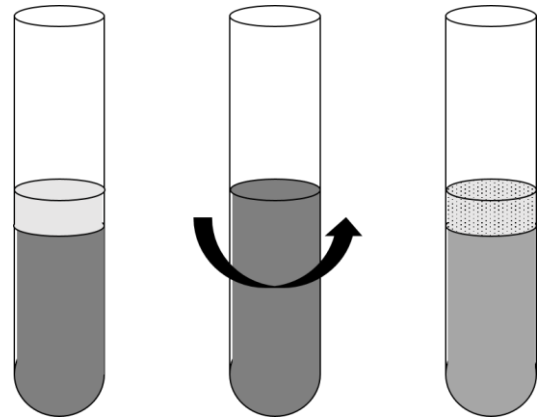


Figure 1-6: Left test tube with spore suspension and hydrocarbon layer, middle test tube with mix, right test tube with spore suspension and hexadecane with spores

In eq. (1-1), H is the degree of hydrophobicity, A_0 is the absorption of the microorganism suspension prior to the experiment and A_1 is the absorption of the microorganism suspension after the experiment.

$$H = \frac{(A_0 - A_1)}{A_0} \cdot 100\% \quad (1-1)$$

Adhesion tests

Rönner et al. (1990) identified a correlation between bacterial adhesion and bacterial hydrophobicity. Adhesion is often measured by means of adhesion tests to specific surfaces. The test surfaces, mostly beads or slides, are put in contact with a microorganism suspension of selected cell density for a defined time. Many studies describe stirring of the suspension with a low number of revolutions per minute (rpm) to prevent spores from settling on the ground. During treatment time, spores adhere depending on their distinct surface characteristics. A subsequent rinsing step removes cells that adhere only slightly. The number of adhering spores is quantified microscopically. (Bos et al. 1999; Dillon et al. 1986; Rönner et al. 1990).

Salt aggregation test

Lindahl et al. (1981) proposed the salt aggregation test (SAT) whereat the precipitation of cells depends on the salt concentration of the surrounding medium. For this test, serial dilutions of ammonium sulfate with different molar concentrations are mixed in equal parts with a microorganism suspension on glass beads. Lindahl et al. (1981) proposed a volume

of 25 μL for each part. The test is regarded as positive when aggregation of the spores results in a clear solution with small aggregates.

Hydrophobic Interaction Chromatography

Another method to study surface hydrophobicity is the Hydrophobic Interaction Chromatography (HIC) (Smyth et al. 1978). A hydrophobic gel of a certain pH and ionic strength is loaded with cell suspension and equilibrating solution. Depending on their surface characteristics, spores adhere to the hydrophobic gel. The absorbance of this fraction (A_1) is measured and compared to the initial absorbance (A_0), which results from mixing the same amount of spores and equilibrating solution as in the chromatography experiment. The degree of hydrophobicity is calculated as shown in eq. (1-2).

$$H = \frac{(A_0 - A_1)}{A_0} \cdot 100\% \quad (1-2)$$

A_0 is the initial absorbance, A_1 is the absorbance after treatment and H the resulting degree of hydrophobicity in percent.

Chemical force microscopy (CFM)

This method is a modified version of atomic force microscopy (AFM) suitable to measure the strength of different chemical bonds and thus provides information about the chemical composition of a specific surface. CFM applies a functionalized tip, mostly gold-coated, that screens the surface and maps the chemical surface structure (Dufrêne 2008).

A similar method was applied by Bowen et al. (2002) who immobilized one single spore at the tip of the atomic force microscope and investigated the resulting adhesive forces to a hydrophilic glass surface and a hydrophobic-coated glass surface.

Contact angle measurement

Young (1805) stated that “for every combination of a solid and a fluid, there is an appropriate angle of contact between the surfaces of the fluid, exposed to the air, and to the solid.” More than 200 years later, several studies apply contact angle measurement, of course with advanced technical equipment. A schematic water contact angle measuring device is depicted in Figure 1-8.

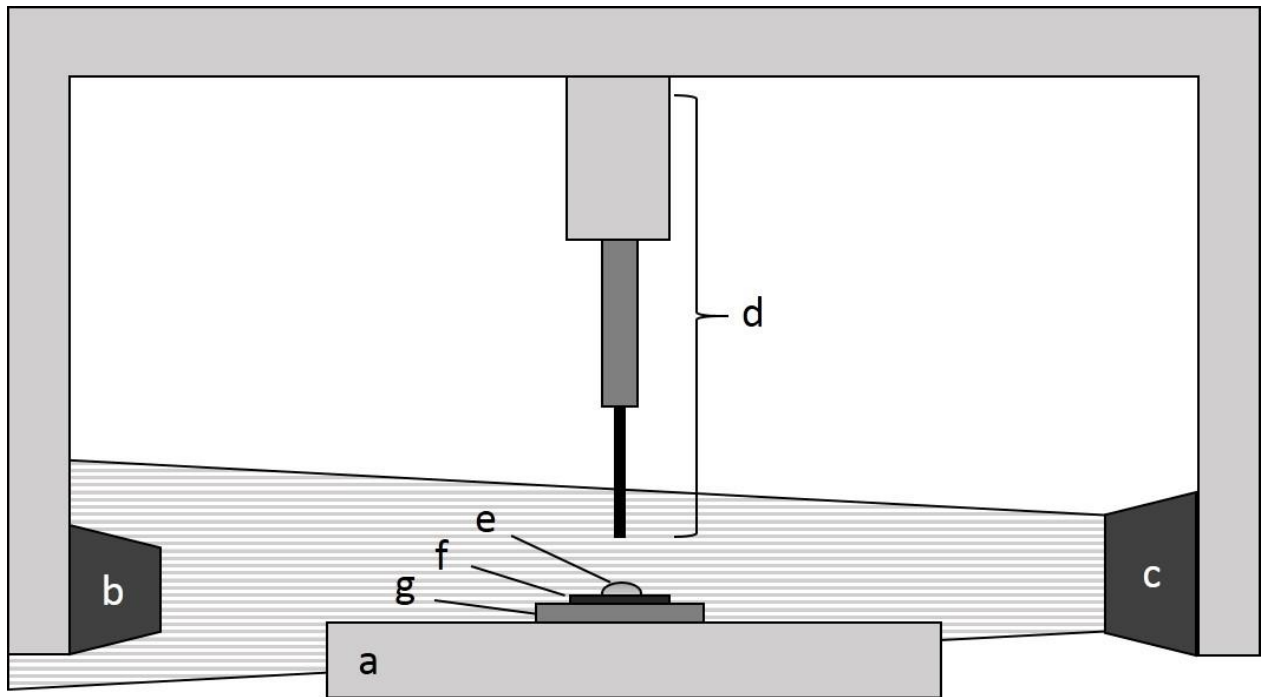


Figure 1-8: Schematic setting of the contact angle measuring instrument. a) table, b) camera, c) lamp, d) mobile syringe to deposit the drop of liquid, e) drop of liquid, f) strip of the filter with microorganisms, g) glass slide.

For water contact angle measurement, a certain amount of bacterial spores is filtrated to form a smooth surface. The resulting spore lawn is subsequently dried. Some studies apply a further equilibration step prior to the drying step. A small drop of water is deposited on the spore lawn whereat the drop volume increases until the final volume is reached. This drop deposition is filmed, video analysis provides several single pictures of the advancing drop of water and thus the advancing water contact angle. Figure 1-7 gives an example of the contact angle (blue dotted line) on a bacterial spore lawn, meaning the angles formed by the tangents along the liquid (red) and the baseline (green).

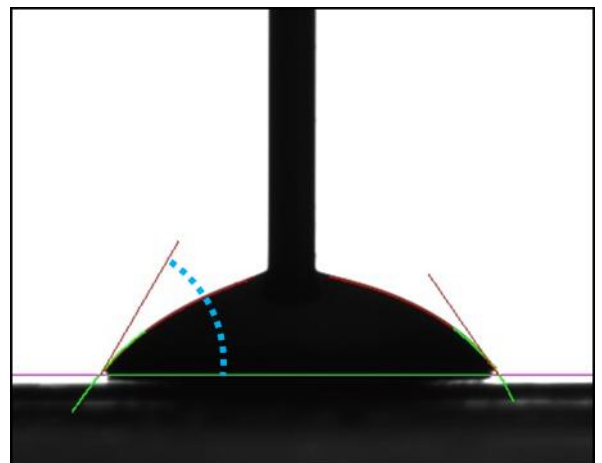


Figure 1-7: Drop of water on microorganism lawn, tangents along the drop of water compose the contact angles together with the baseline.

Concluding, there are numerous methods to evaluate cell surface hydrophobicity. However, the advantages and disadvantages need to be considered carefully.

On the one hand, the MATH-test has easy experimental setup and is widely applied. On the other hand, several authors state that the MATH test shows an interplay of all physico-

chemical and structural adhesion factors and allows no differentiation of the specific forces (van der Mei, H.C., et al. 1998; van der Mei et al. 1995; Busscher et al. 1995). Besides, there are several influencing factors that make a reasonable comparison of published MATH-results almost impossible. The amount and type of hydrocarbon as well as the temperature, pH and ionic strength of the microorganism suspension influence the amount of spores adhering to the hydrocarbon phase (Dillon et al. 1986; Rosenberg 2006; Bunt et al. 1993).

Adhesion tests and the SAT test exhibit a similar disadvantage as they too depend on hydrophobic and electrostatic interactions (Marshall et al. 1971). The pH and the ionic strength of the surrounding medium as well as the temperature strongly influence the results. van der Mei et al. (1987) and Dillon et al. (1986) investigated the correlation of different adhesion tests as well as HIC and SAT and found out that there is only little correlation. They stated that those tests should not be used as the only method to investigate cell surface hydrophobicity. Physico-chemical and structural adhesion factors of biological material are too numerous and the resulting interplay of forces too complex to be able to characterize the sole quality hydrophobicity.

Each of those tests has its meaningfulness depending on the objective of the specific research topic. However, to quantify cell surface hydrophobicity, water contact angle measurement is the only method that yields direct results (Mozes and Rouxhet 1987) without enormous experimental effort and data analysis compared to CFM.

Above that, by using two polar and one unpolar liquids as proposed by Busscher et al. (1984), the surface free energy of bacterial cell surfaces can be calculated and going further, the adhesional interfacial free energy as well (Busscher et al. 1984). For the surface free energy, different approaches exist. In short, the two options are the thermodynamic approach, in which the interfacial free energies of the interacting surfaces are compared and the extended DLVO theory (Balance of Lifshitz-van-der-Waals attractive force, electrostatic forces and acid/base interaction forces (Sharma and Rao 2002; van Oss 2006)). Both of those approaches have been developed from several authors over the years, each of them pointing out another important facet. However, to conduct those calculations, precise and accurate contact angle results need to be obtained.

1.3 Hydrogen peroxide

The chemist Louis-Jaques Thenard discovered H_2O_2 in 1818 during his work with barium peroxide (Thénard 1818). By treating barium peroxide with nitric acid, later on with hypochloric acid he obtained barium chloride and H_2O_2 whereat the latter one can be precipitated with sulfuric acid. However, with this method it was only possible to produce aqueous solutions of low concentrations (3 to 6 %). Besides, this basic principle of H_2O_2 production referred to as wet chemical process was expensive and the resulting H_2O_2 unstable due to impurities.

Since 1908, electrochemical processes were industrially applied for the production of H_2O_2 . The principle is based on the electrolysis of sulfuric acid with peroxidisulfuric acid as its product. After hydrolysis with water, sulfuric acid and hydrogen peroxide are the products. The production of highly concentrated solutions was possible and increased steadily until 1950 (Goor et al. 2010; Degussa, 1976).

From 1935 to 1945, the Riedel-Pfleiderer Process (Antrachinone Process) was developed based on the reaction of hydroquinones with oxygen to form quinones and hydrogen peroxide. Until today, this third option to produce hydrogen peroxide is widely applied. Since the establishment of the Antraquinone process, H_2O_2 is widely used as a disinfectant in low concentrations in ultrasonic disinfection cleaning baths for dental and medical instruments, in swimming pools and for contact lenses. As sterilization agent, it's commonly applied for aseptic packaging and clean room technology (Degussa, 1976; Goor et al. 2010).

1.3.1 Properties of H_2O_2

Liquid hydrogen peroxide looks clear without color or odor. It can be mixed with water in all concentrations. Mixtures of H_2O_2 with water of at least 65 % H_2O_2 are soluble with a variety of organic solvents. Mixed with water, H_2O_2 is weakly acidic (Goor et al. 2010).

The H_2O_2 molecule was investigated in several studies (Koput 1986). However, due to the fact that pure H_2O_2 is difficult to handle, its properties as a liquid were studied by only few authors. Yu and Yang (2011) and Martins-Costa and Ruiz-López (2007) present the most recent results.

The chemical structure of H_2O_2 in its open-book configuration is depicted in Figure 1-9. To describe the properties of H_2O_2 , a differentiation in H_2O_2 as a monomer and as a cluster of several H_2O_2 molecules has to be made. The most stable structure of the hydrogen peroxide monomer is the open book structure with a dihedral angle of $112,0^\circ$. Dihedral angle means the angle enclosed by H-O-O-H. The angle between O-O-H is $99,3^\circ$ and the bond length of H-O is $0,968 \text{ \AA}$ whereat the bond length of the O-O bond is longer with $1,463 \text{ \AA}$. The dipole moment is $2,21 \text{ Debye}$ (Yu and Yang 2011).

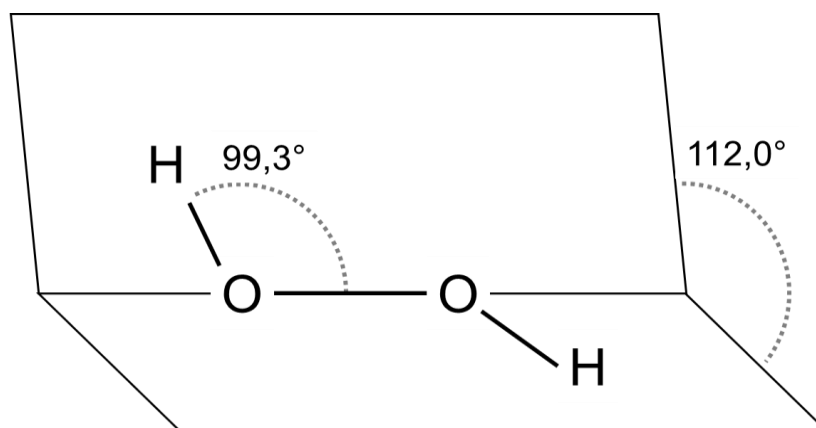


Figure 1-9: Hydrogen peroxide molecule in open-book structure

The given values account only for the monomer. However, H_2O_2 is only present as a monomer in an ideal gas. Martins-Costa and Ruiz-López (2007) describe the molecule properties for liquid aqueous H_2O_2 solutions. As the H_2O_2 is solved in water, the solvent effects modify the OH bonds. They are weakened and elongated by $0,02 \text{ \AA}$. Above that, by means of solvent effects, the OH bonds are polarized and the tendency as proton donor is increased. In aqueous solutions, H_2O_2 is a better proton donor than H_2O but a weaker proton acceptor. The dihedral angle decreases by 11° and the dipole moment increases by $0,8$ Debye. The O-O bond length and order are largely unaffected and do not change much. Martins-Costa and Ruiz-López (2007) suppose that by solving H_2O_2 , molecules tend to proton donation, but not dissociation in OH radicals.

The most important physical properties of H_2O_2 as aqueous solution in different concentrations are summarized in Table 1-2.

Table 1-2: Physical properties of water aqueous hydrogen peroxide solutions of different concentrations (according to Goor et al. (2010))

	Properties	Water	Hydrogen peroxide				
			35 %	50 %	70 %	90 %	100 %
Phase transition temperatures [°C]	Melting point	0	-33	-52,2	-40,3	-11,9	-0,43
	Boiling point (101,3 kPa)	100	107,9	113,8	125,5	141,3	150,2
Density [g·cm⁻³]	0	0,9998	1,1441	1,2110	1,3071	1,4136	1,4700
	20	0,9980	1,1312	1,1953	1,2886	1,3920	1,4500
	25	0,9971	1,1282	1,1914	1,2839	1,3867	1,4425
Viscosity [mP·s]	0	1,792	1,820	1,870	1,930	1,880	1,819
	20	1,002	1,110	1,170	1,230	1,260	1,249

The properties of H_2O_2 and water are different. Those differences are employed to measure the concentration of liquid H_2O_2 in aqueous solutions. One method based on optical density uses the absorption difference between water and H_2O_2 at 240 nm. H_2O_2 absorbs UV-light at 240 nm, whereas water does not. As this measurement is temperature dependent, a

calibration with defined concentrations at the relevant temperature needs to be established prior to the test. Beers and Sizer (1952) applied this method to measure the rapid breakdown of H_2O_2 upon addition of catalase. this method is suited for low concentrations of 0.35 % and smaller. Due to the low wavelength, quartz glass cuvettes which are completely penetrable by those wavelengths have to be applied.

Easton et al. (1952) were the first to investigate the density of H_2O_2 solutions at different temperatures. They investigated the density by means of a simple displacement method which is based on weighing a glass sinker in air, water and H_2O_2 solutions. Nowadays completely automated density measurements are possible by means of flexural vibration. Concentrations of 5 to 100 % can be measured accurately with this method.

The iodometric method as described by Autenrieth and Keller (1951) is often applied as reference method to quantify the amount of liquid H_2O_2 . The basic mechanism is that hydrogen peroxide dissolves molecular iodine (I_2) from potassium iodide (KI) in an acidic solution. Molecular iodine shows a yellow-brownish color. By titration with sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), molecular iodine is reduced to colorless iodine ions. The amount of sodium thiosulfate that is needed depends on the amount of molecular iodine which results from a certain amount of H_2O_2 in the solution.

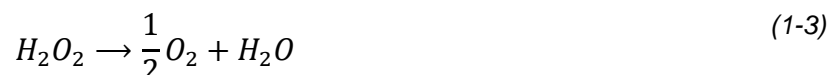
Although gaseous hydrogen peroxide is already applied since several years, few methods are available to monitor the concentration of active hydrogen peroxide in its gas form. One method to measure the amount of gaseous hydrogen peroxide is to transform it in its liquid form again by means of washing bottles. Engelhard (2006) describes this method where a defined gas volume is channeled through washing bottles filled with a certain amount of cold distilled water. The gas flows from the bottom of one bottle throughout the contained liquid to the bottom of the next bottle to guarantee complete solving of H_2O_2 . The content of the bottles is subsequently united and the amount of H_2O_2 quantified by titration as described above with the method of Autenrieth and Keller (1951).

Other methods discussed in literature include UV sensors, near-infrared H_2O_2 gas monitoring devices, semiconductor sensors and catalytic sensors. The latter ones are based on the exothermic reaction of H_2O_2 with a catalytic material to oxygen and water. Manganese oxide can serve as such a catalytic material. During this decomposition, energy is released and the temperature rises. This additional heat is measured by thermometers. One of those thermometers serves as a reference and is not coated, the other one provides catalytic material at the surface, favors H_2O_2 decomposition and detects the resulting temperature. The temperature difference serves as measure for the active H_2O_2 concentration (Näther et al. 2009; Näther et al. 2006; Pruss 2013)

Within this thesis, the concentration of liquid H_2O_2 was quantified with the iodometric method (Autenrieth and Keller 1951), gaseous H_2O_2 concentration was determined by the catalytic method established by Pruss (2013).

Decomposition of H_2O_2

The measurement of gaseous H_2O_2 is based on the decomposition of H_2O_2 to water (H_2O) and oxygen (O_2). This decomposition is depicted in eq. (1-3) (Goor et al. 2010).



During the decomposition of gaseous H_2O_2 , $105.8 \text{ kJ}\cdot\text{mol}^{-1}$ are released. The decomposition of liquid H_2O_2 provides $98.3 \text{ kJ}\cdot\text{mol}^{-1}$ which makes both reactions exothermic (Hultman et al. 2007).

Decomposition is triggered by either high temperatures or catalyzers. Even very low amounts of catalyzers are enough to start this reaction. Depending on the kind of catalyzer, decomposition can be homogenous (dissolved ions of the metals iron, copper, manganese, chromium) or heterogenous (suspended oxides and hydroxides such as manganese, iron, copper) (Goor et al. 2010; Degussa, 1976). Due to this predisposition, transport and storage of H_2O_2 have to be carefully controlled. Neither must H_2O_2 be contaminated nor heated. Usually, low amounts of stabilizers such as sodium pyrophosphate or sodium stannate are obstructed to the H_2O_2 solution. Besides, the containers are made of polyethylene which is on the one hand resistant against the oxidizing potential and on the other hand provides no reacting agent (Hoare et al. 1959; Goor et al. 2010).

1.3.2 Formation of condensate

For many pure substances such as water, the parameters leading to the formation of condensate are known for a long time, well investigated and documented. However, for aqueous hydrogen peroxide vapors, some peculiarities have to be considered. Gaseous H_2O_2 is the most important disinfection agent for the decontamination of clean rooms. For decontamination purpose, a mixture of 35 % H_2O_2 and water with small amounts of stabilizers is mostly applied. When aqueous H_2O_2 is evaporated, the amount of H_2O_2 in the air of the decontamination room rises. However, the amount of water in the vapor mixture increases as well. Quite often, electronic devices are fixed in clean rooms and H_2O_2 condensate has corrosive properties. Therefore, condensate formation has to be avoided. Application of gaseous H_2O_2 without condensate formation is possible with strict monitoring of the process if the process of condensate formation is well understood.

The formation of dew begins with nucleation, which means the formation of the smallest liquid drop that is stable and not evaporating again. Basically, two different forms of dew

formation are known: The homogenous dew formation that happens in a bulk and the heterogeneous formation of dew appearing on surfaces. Thus, condensation is the transmission of vapor into liquid droplets on a substrate. After formation of a sufficient amount of condensate droplets covering the surface, the droplets will grow by means of droplet fusion or coalescence (Beysens 1995; Marcos-Martin, et al. 1996; Beysens et al. 2006).

The driving force for condensate formation is the ambition to reach the lowest possible state of energy. In its liquid form, the energy state is lower than in the gaseous form. However, an energy barrier called phase transition has to be crossed as formation of two new interfaces takes place: The liquid-gas and the liquid-substrate interface. This energy barrier is influenced by the wettability of the surface (Marcos-Martin, et al. 1996). Different wettabilities are displayed in Figure 1-10.

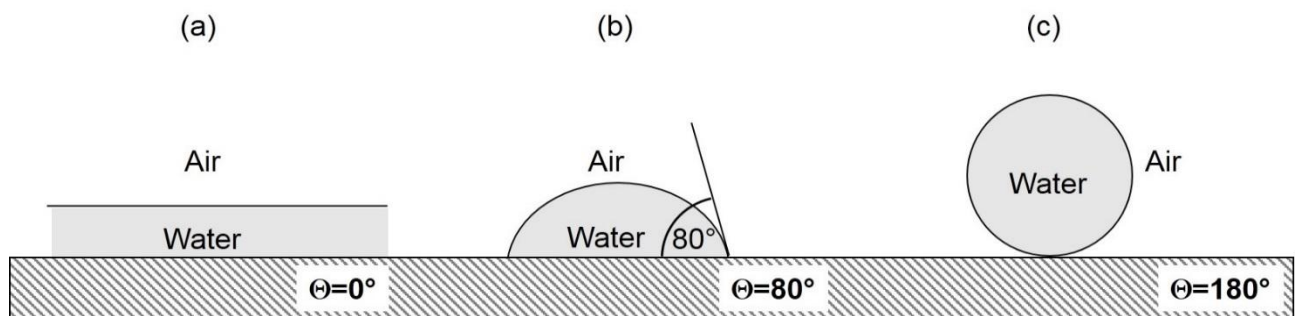


Figure 1-10: Condensate formation on substrates of different wettability, modified according to Marcos-Martin et al. (1996). (a): Substrate with optimal wettability, resulting condensate forms a film of water on the surface. (b): Substrate with middle wettability, drop formation of the condensate occurs. (c) Ideal substrate with no wettability and thus high hydrophobicity, drop formation occurs as sphere to keep the contact with the substrate as small as possible.

The formation of condensate is induced if either the actual pressure is above the saturation pressure or the temperature of the substrate is below the dew point temperature (Beysens 1995).

Imagining a closed vessel with an aqueous H_2O_2 solution at a defined temperature, an equilibrium will adjust after some time. The formation of condensate begins at that point when an equilibrium between the liquid phase and the vapor phase is accomplished. The task is to find those equilibrium parameters. For isobaric pressure, there is one dew point temperature below which condensate begins to form and for an isothermic temperature, one dew point pressure exists above which the formation of condensate begins (Parks and Watling 2004). The authors published a collection of formula that provide the option to calculate the dew point temperature depending on the amount of H_2O_2 and H_2O in the gas. These formula are summarized and explained in the following section.

The fundamental equation for saturated water equilibration pressure (P_W) was established by Keyes (1947), eq. (1-4).

$$\log P_W(T) = G + \frac{A}{T} + B \cdot \log T + C \cdot T + D \cdot T^2 + E \cdot T^3 + F \cdot T^4 \quad (1-4)$$

With A, B, C, D, E, F and G being constants, T is the absolute Temperature.

$$A = -2892.3693, B = -2.892736, C = -4.9369728 \cdot 10^{-3}, D = 5.606905 \cdot 10^{-6}, E = -4.645869 \cdot 10^{-9}, \\ F = 3.7874 \cdot 10^{-12}, G = 19.3011421$$

Equation (1-5) was developed by Scatchard et al. (1952). This equation is similar to eq. (1-4) however for the saturated pressure of hydrogen peroxide (P_H).

$$\log P_H(T) = D + \frac{A}{T} + B \cdot \log T + C \cdot T \quad (1-5)$$

With A, B, C and D being constants and T is the absolute Temperature. $A = -4025.3, B = -12.996, C = 4.6005 \cdot 10^{-3}, D = 44.5760$

As expressed in Raoult's law, it is possible to calculate the equilibrium vapor pressure of an ideal multicomponent liquid. However, there are bonds between molecules of water and H_2O_2 . Thus, an activity coefficient (γ) is needed to modify the law of Raoul as shown in eq. (1-6).

$$P_A = \gamma_A \cdot \chi_A \cdot P_A^0 \quad (1-6)$$

The saturated vapor pressure P_A of component A at mole fraction χ_A is calculated with the activity coefficient γ_A and the vapor pressure of pure substance A at that temperature P_A^0 .

The activity coefficient γ has to be calculated for water (eq. (1-7)) and H_2O_2 (eq. (1-8)).

$$\gamma_W = \exp \left\{ \frac{(1 - \chi_W)^2}{R \cdot T} \cdot [B_0 + B_1(1 - 4\chi_W) + B_2(1 - 2\chi_W) \cdot (1 - 6\chi_W)] \right\} \quad (1-7)$$

$$\gamma_H = \exp \left\{ \frac{\chi_W^2}{R \cdot T} \cdot [B_0 + B_1(3 - 4\chi_W) + B_2(1 - 2\chi_W) \cdot (5 - 6\chi_W)] \right\} \quad (1-8)$$

However, eq. (1-7) and eq. (1-8) both contain the molar fraction of water which is unknown. The sum of the molar fractions of water and H₂O₂ has to be 1 as shown in eq. (1-9).

$$\chi_H + \chi_W = 1 \quad (1-9)$$

Reorganization of eq. (1-6) with focus on the mole fraction results in eq. (1-10) and eq. (1-11) respectively for water and H₂O₂.

$$\chi_W = \frac{P_W}{\gamma_W \cdot P_W^0} \quad (1-10)$$

$$\chi_H = \frac{P_H}{\gamma_H \cdot P_H^0} \quad (1-11)$$

Replacing the molar fraction of eq. (1-9) with the term established in eq. (1-10) and eq. (1-11) results in eq. (1-12).

$$\frac{P_W}{\gamma_W \cdot P_W^0} + \frac{P_H}{\gamma_H \cdot P_H^0} = 1 \quad (1-12)$$

Solving eq. (1-12) after the actual vapor pressure P_W results in eq. (1-13).

$$P_W = \gamma_W \cdot P_W^0 \cdot \left(1 - \frac{P_H}{\gamma_H \cdot P_H^0} \right) \quad (1-13)$$

This equation expresses the vapor equilibrium pressure of water and H₂O₂ vapor layered above an aqueous phase. With this equation, the relative humidity necessary for condensate formation can be calculated. However, the temperature and the H₂O₂-concentration have to be known.

The molar fraction has to be calculated iteratively. The letter E stands for “calculated with estimated molar fraction”. Equation (1-14) is a modified version of eq. (1-13) which includes the iteratively calculated activity factor, P_{HM} and γ_{HG} .

$$P_W = \gamma_{WE} \cdot P_W^0 \cdot \left(1 - \frac{P_{HM}}{\gamma_{HG} \cdot P_H^0} \right) \quad (1-14)$$

The vapor pressure of the condensate has to be equal to the vapor pressure of the vapor. Therefore, the vapor pressure P_w resulting from eq. (1-14) has to be compared to the vapor pressure calculated with the estimated molar fraction from eq. (1-6). Those two results need to coincide. If they are different, the temperature has to be changed and the calculations repeated until the results are conform or at least very close to each other.

The relative humidity that is necessary for condensate formation can be calculated according to eq. (1-15).

$$\frac{P_w}{P_w^0} \cdot 100\% = \text{Relative humidity} \quad (1-15)$$

With those equations, the dew point temperature below which condensate begins to form can be calculated. For complete vaporization of an aqueous solution of 35 % H_2O_2 , this dew point temperature was calculated. With rising H_2O_2 concentration in the gas, the amount of water in the gas rises as well, however is not depicted in Figure 1-11.

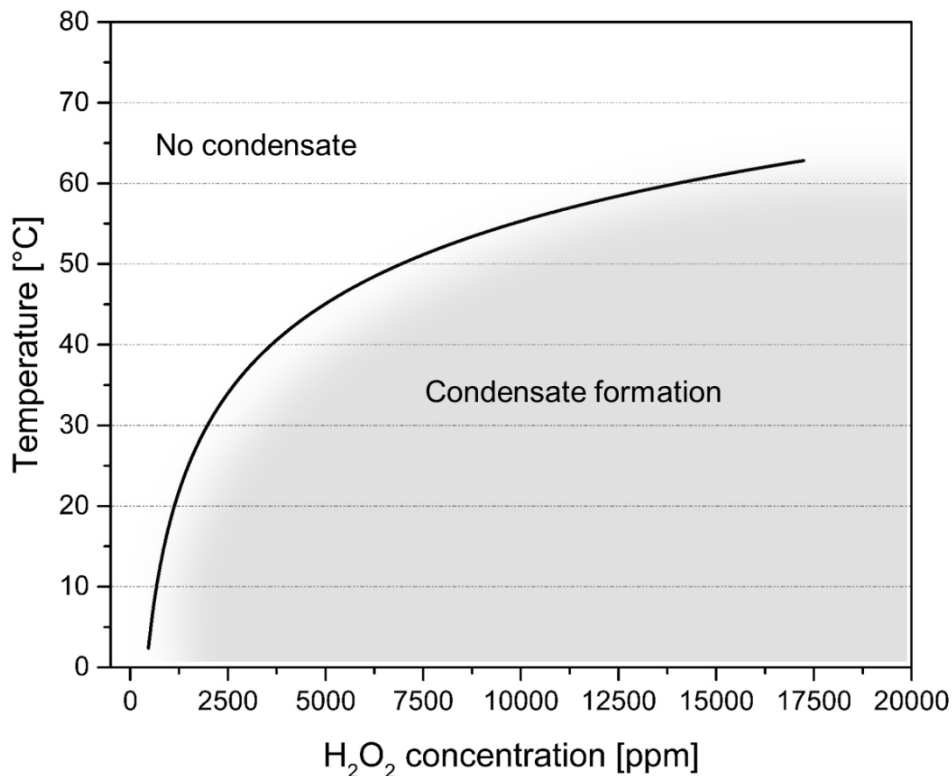


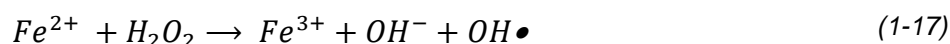
Figure 1-11: Dew point temperature depending on the amount of completely vaporized 35% H_2O_2 , modified after Radl et al. (2009) and Pruss (2013)

1.3.3 H₂O₂: Mode of action

H₂O₂ is an oxidizing agent, therefore the basic mechanism of action is chemical oxidation of organic components. The redox potential of H₂O₂ is 1,76 V (Jones 1999). The higher the redox potential, the more increases the urge to take an electron from another molecule. Removing this electron is called oxidation. At the same time H₂O₂ is reduced due to this process (Finnegan et al. 2010).

According to Haber and Weiss (1934), this reaction is thermodynamically unfavorable in biological systems, because it has a second order rate constant of zero in aqueous solutions. However, a catalyst would solve this problem.

Metal ions can act as catalyst, especially Fe²⁺. First, Fe³⁺ is oxidized by O₂^{-•} creating Fe²⁺ and molecular oxygen (O₂) as shown in eq. (1-16). In combination with Fe²⁺ as catalyst, H₂O₂ is decomposed to OH⁻ and OH• (eq. (1-17)). At the same time, Fe³⁺ is generated again. Other transition metal ions than Fe²⁺ can also catalyze this reaction.



The resulting netto-reaction is known as Haber-Weiss-reaction and depicted in (1-18). The superoxide-radical reacts with H₂O₂ resulting in a very reactive hydroxyl radical (OH•) which is a reactive oxygen species (ROS).



Melly et al. (2002a) investigated the mechanism of bacterial spore inactivation by liquid hydrogen peroxide. Upon H₂O₂ treatment, the inner membrane permeability barrier remains intact, which seems to suggest that the spore DNA is not the major target for H₂O₂.

The majority of spores that are inactivated by H₂O₂ is still able to initiate germination. This means the spores release DPA and begin to degrade the cortex. The next step would be swelling of the spore core due to water uptake. This swelling leads to enzyme activation in the spore core due to an increased amount of water and enlargement of the spore. However, the swelling and enlargement of the spore do not happen in spores inactivated by liquid H₂O₂. The onset of metabolism is prevented.

Melly et al. (2002a) suppose that one or more components essential for the swelling of the spore core are damaged by H₂O₂. Those components could be the peptidoglycan of the

germ cell wall that must expand to encompass the swelling cell. Another possibility is damage of a protein needed for remodeling of the germ cell wall to enable its expansion. Finally, it could also be damage of a protein that is needed in some way for core expansion itself.

Finnegan et al. (2010) investigated the mode of action of liquid and gaseous H_2O_2 in vitro. Their results show that liquid H_2O_2 acts as strong oxidizing agent against amino acids with tryptophan being the only amino acid that is not oxidized. Gaseous H_2O_2 oxidized all amino-acids except for tryptophan when the amino acids were in a filter paper, however did not have any effect on amino acids dried on stainless steel. The enzyme aldolase was decomposed from liquid and gaseous H_2O_2 . On bovine serum albumin (BSA), liquid H_2O_2 did not have any effect. In contrast, gaseous H_2O_2 oxidized BSA.

Finnegan et al. (2010) concluded that due to the higher kinetic energy of gaseous H_2O_2 compared to liquid H_2O_2 , the gas irrupts deeper in the protein structure. There, it oxidizes cysteine residues and destroys bonds between subunits. Thus, at least one difference in the mode of action between liquid and gaseous H_2O_2 is the better penetration ability of the gas.

However, a final and consistent study concerning the inactivation of bacterial spores with gaseous H_2O_2 has not been conducted so far.

1.3.4 Inactivation by H_2O_2 : Influencing factors

Most inactivation mechanisms are influenced by numerous factors, this is also the case for H_2O_2 . The following section summarizes the influencing factors in a chronological way, as far as possible.

Liquid H_2O_2

Swartling and Lindgren (1968) investigated the influence of H_2O_2 concentration in decontamination processes based on liquid H_2O_2 . They tested aqueous solutions of 10, 15 and 20 % H_2O_2 for the inactivation of *B. subtilis* spores at a constant temperature whereat each increase in concentration resulted in 50 % enhanced inactivation. They also investigated the influence of temperature in a range of 25 to 80 °C and detected linear inactivation kinetics at 60 to 80 °C. For lower temperatures, a lag-phase at the beginning of the inactivation was observed. In general, higher treatment temperatures led to faster spore inactivation.

These relations were also observed by Toledo et al. (1973). Above that, they investigated the impact of spore application prior to the inactivation whereat spores that were dried on a surface showed less resistance than spores immersed in the decontamination agent.

Cerny (1976) inactivated different bacterial spores, fungi and vegetative microorganisms whereat bacterial spores proved to be most resistant against liquid H₂O₂. Especially spores of *B. stearothermophilus* and spores of *B. subtilis* survived the treatment with 30% H₂O₂ of 23 °C. Both spores exhibited less than one log inactivation after 6 minutes of treatment.

Gaseous H₂O₂

The effectiveness of gaseous H₂O₂ was assumed by Huber (1979) who inactivated microorganisms present on the packaging surface by injection of nebulized H₂O₂. Although only a share of 24 to 35 % of the surface was wetted by the decontamination agent under optimal conditions, the whole surface was decontaminated. The authors concluded that during the following drying step with sterile hot air, the evaporated H₂O₂ acts bactericidal as well. This was the beginning of decontamination processes applying vaporized H₂O₂.

Wang and Toledo (1986) applied H₂O₂ in its gaseous form and investigated the inactivation kinetics of *B. atrophaeus* spores. Inactivation was enhanced with rising temperature. They evaporated 35 % aqueous H₂O₂ by bubbling dry filtered air through two liquid traps containing the H₂O₂ solution. Due to constant, non-automated evaporation, the amount of H₂O₂ in the air increased with rising temperature of the surrounding gas. Therefore, no clear distinction can be made between the effect of temperature and H₂O₂ concentration. However, this is the first study examining the effect of inactivation with gaseous H₂O₂ systematically obtaining scientifically reliable results.

According to Wilke (1992), each influencing factor has to be investigated with all the other influencing factors remaining unchanged. He solved the problem of the temperature-dependent rise in concentration by completely evaporating a small amount of H₂O₂. In his thesis, he showed that rising treatment temperature leads to enhanced inactivation of bacterial spores. This applies for increasing H₂O₂ concentration as well.

Another influencing factor was postulated by Engelhard (2006) and Unger-Bimczok et al. (2008). They investigated the influence of different H₂O₂ concentrations with unchanged amounts of water in the vapor. Their results coincide with the previously identified trend that rising H₂O₂ concentration leads to increased inactivation velocity. For different amounts of H₂O₂ and temperatures, Chung et al. (2008), Reisert et al. (2011) and Pruss (2013) obtained a similar trend. Engelhard (2006) investigated the influence of temperature with a maximum of 180 °C, Reisert et al. (2011) applied a maximum temperature of 340 °C and observed that above 300 °C, a further increase of temperature does not lead to further improvement of the inactivation. For the purpose of aseptic filling and clean room decontamination, temperatures of several 100 °C are not applicable. Therefore it can be concluded that for the relevant temperatures between 20 and 100 °C, a rise in treatment temperature leads to an increasing inactivation effect.

The amount of water in the H₂O₂-water-air-mixture was investigated by Pruss (2013) for different amounts of H₂O₂ in a range of 1700 ppm to 10900 ppm. For all experiments where condensate formation was certainly prevented, a higher amount of water in the gas mixture led to decreasing inactivation success. Pruss (2013) and Engelhard (2006) suppose the similarity between water and H₂O₂ molecules to be responsible for this effect. Water molecules could occupy the binding site which otherwise H₂O₂ would bind to. By presence of water, less H₂O₂ can oxidize the bacterial spores which in turn leads to less inactivation effect.

All of the mentioned influencing factors depend on the treatment time. With elongated treatment time, the intensity of the other influencing factors rises. Therefore, time has to be counted as an influencing factor as well.

Apparently, decontamination with gaseous H₂O₂ is a multifactorial process. Applied in its pure gas form, the basic influencing factors are concentration of H₂O₂, temperature of the decontamination agent, treatment time and amount of water in the gas. In its liquid form, influencing factors are the same however resistance of microorganisms against liquid and gaseous H₂O₂ differs (Finnegan et al. 2010). Therefore, microorganisms that are resistant to the gas can be inactivated quickly by liquid H₂O₂ and vice versa. A process combining those two modes of action is the formation of condensate.

Inactivation with gaseous and condensing H₂O₂

The formation of condensate during treatment with vaporized H₂O₂ basically depends on three parameters:

- The amount of water in the vapor
- The amount of H₂O₂ in the vapor
- The dew point depending on pressure and temperature. For the sake of simplicity, this section will refer to ambient pressure and only consider the dew point temperature.

Those three parameters are also depicted in Figure 1-12. Besides, this figure shows two further major influencing areas, the microorganisms and the carrier material on which the inactivation takes place. As inactivation with gaseous and condensing H₂O₂ is a multifactorial process, the focus of this chapter lies on the influence of the variables concerning the vapor, the microorganisms and the carrier material.

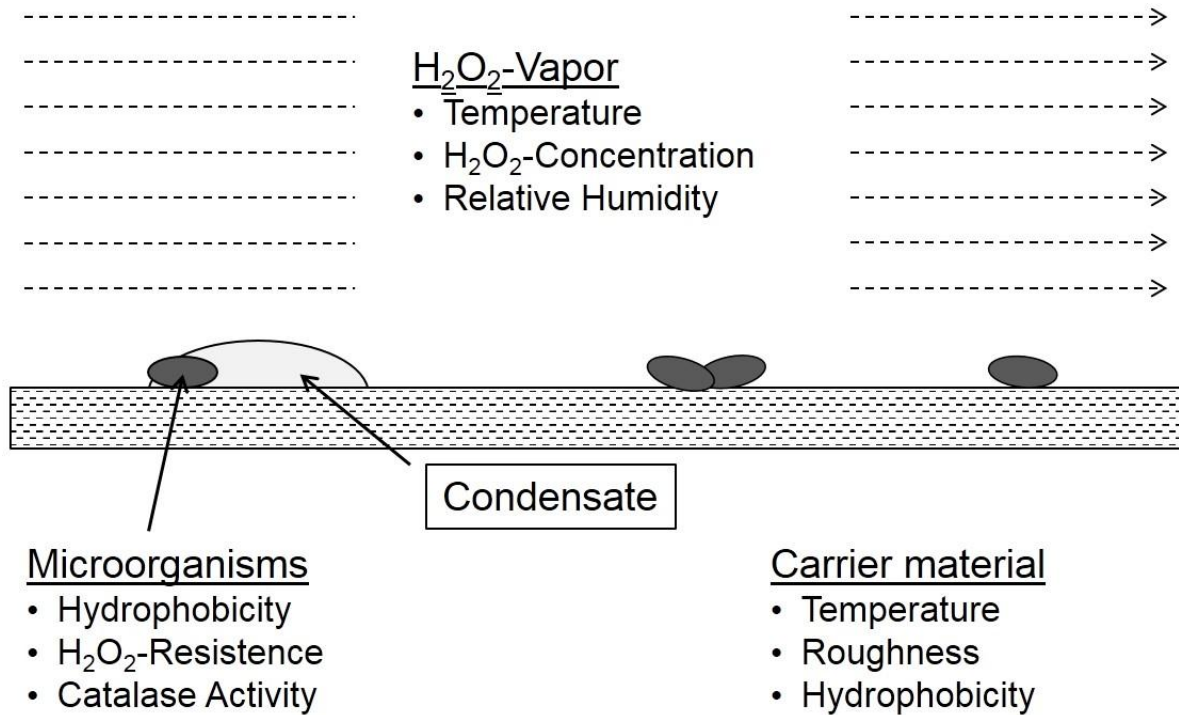


Figure 1-12: Influencing factors on the inactivation with gaseous H₂O₂, modified after Eschlbeck and Kulozik (2016)

Influence of the vapor variables

The effect of condensate formation together with a measure of the resulting condensate was investigated by Unger-Bimczok et al. (2008). They induced condensate formation at relatively low temperatures of 22 °C by increasing the amount of H₂O₂ on the one hand and by increasing the amount of H₂O on the other hand. For low H₂O₂ concentrations of 400 and 600 ppm, an increase of humidity at constant H₂O₂-level led to a strongly enhanced inactivation rate. For 800 ppm H₂O₂ however, increase in H₂O did not result in significant changes of the resulting D-values.

Increasing the amount of H₂O₂ on the other hand enhanced the inactivation with gaseous H₂O₂ only to a certain amount as well. At very high concentrations of water of about 20 000 ppm, inactivation was very fast and differences in inactivation kinetics can hardly be seen.

The authors concluded that for low amounts of condensate (2.9 µg·mm⁻²) which includes 400 and 600 ppm H₂O₂, inactivation is enhanced. This condensate which is invisible to the eye is called microcondensate. Increasing the amount of H₂O₂ or the amount of water so that visible condensate formation occurs does not result in enhanced inactivation kinetics.

Pruß et al. (2012) induced condensate formation by an undercut of the dew point temperature. Practically speaking, the test materials had a temperature below the dew point temperature. On the surface of the comparably cold packaging material condensate

developed. This condensation led to increased inactivation compared to inactivation at the same parameters, meaning without condensate formation. Same parameters means same chamber temperature, gas concentration but different, higher test material temperature above the dew point temperature. However, inactivation with condensate formation resulted in inactivation kinetics that were not log-linear. Pruß et al. (2012) came up with the hypothesis, that efficacy of condensate formation might be influenced by and therefore depend on the surface hydrophobicity of microorganisms. This influencing factor will be discussed later on.

Influence of microorganisms

Microorganisms can exist either singularly or in pairs so that the decontamination agent in its liquid or gaseous form is able to reach and inactivate them (Figure 1-12). However, if microorganisms are present in several layers on the surface meaning if they form clumps, tailing of the inactivation process can occur (Wilke 1992). Tailing describes the phenomenon, that after an initial log-linear inactivation, the inactivation rate slows down. Engelhard (2006) investigated different ways of microorganism application whereat some resulted in single organisms whilst others led to microorganism clusters. He showed that inactivation with gaseous and condensing H₂O₂ is strongly influenced by this factor, as most probably only the upper layers are inactivated and the undermost spores survive. Therefore, the inactivation kinetics of several layers of microorganisms show a tailing.

The specific factors responsible for spore resistance against gaseous and liquid H₂O₂ were already described in chapter 1.1.2. However, spore resistance can be influenced by cultivation conditions (Melly et al. 2002b; Rose et al. 2007) and thus should be checked by means of a simple resistance test prior to each inactivation test.

Marcos-Martin et al. (1996) were the first who stated that “microorganisms, which are hygroscopic and so are completely hydrated by water are better nucleation sites than surrounding fatty surfaces”. Above that, they supposed that every irregularity on the surface can act as preferred nucleation site due to capillary effects. Microorganisms can be seen as such irregularities and are thus preferred nucleation sites.

The statement that microorganisms are completely hydrated due to their hygroscopic characteristics can be argued however the implication that they are better nucleation sites than the surrounding fatty surfaces makes sense and implies that the nucleation sites are hydrophilic.

Hall et al. (2007) state exactly the opposite. They argue that microorganisms are protected from vaporous hydrogen peroxide (vhp) because of their high surface hydrophobicity. Microcondensate does not engulf the microorganisms, thus the hydrophobic germs are not

affected by the oxidizing agent and have a better chance to survive. Both statements were made without scientific results that could confirm or strengthen their claim.

The relationship between surface properties of microorganisms and decontamination with gaseous and condensing was first investigated by Pruß et al. (2012). Different bacterial spores with water contact angles between 10.4 and 20.9 ° and thus defined surface hydrophobicity were applied. Inactivation was carried out with exactly the same parameters for the decontamination gas, which were 5200 ppm H₂O₂ resulting from vaporization of 35% H₂O₂ and treatment temperature of 70 °C. The only variable was the initial surface temperature of the packaging material inoculated with bacterial spores which was set to 63°C and 22°C. For the first test material temperature, pure gas without condensation occurred because the temperature was above the dew point temperature. The latter one was below the dew point temperature so that condensate formation was induced. The inactivation kinetics over a time of 10 s differed significantly, pure gas showed similar inactivation results for all spore species whereat *B. atrophaeus* was most resistant. *B. atrophaeus* had a water contact angle of 13.1 °. The inactivation results obtained with condensate formation depicted that the most hydrophobic bacterial spore survived best after 10 s. Together with the resistance test with *B. atrophaeus* against liquid H₂O₂, Pruß et al. (2012) concluded that high surface hydrophobicity of spores might be an advantage for the hydrophobic spores which leads to less condensate formation around the spores and thus less inactivation.

Influence of carrier materials

Temperature of the carrier material is relevant for the formation of condensate which was already discussed above.

Rogers et al. (2005) studied the inactivation of spores of *B. anthracis*, *B. subtilis* und *G. stearothermophilus* with gaseous H₂O₂ on different surfaces. They observed that spores of *B. anthracis* on industrial carpet and bare pine wood were less inactivated. They concluded that porosities and cavities of the surfaces in which spores lay might shelter them. Unger et al. (2007) also investigated the effect of cavities on the inactivation with gaseous H₂O₂ and had similar results.

Sigwarth and Stärk (2003) investigated the influence of different carrier materials used in isolators on the resistance of *G. stearothermophilus* spores against gaseous H₂O₂. The experiments were conducted without condensate formation. Their results show that surface roughness influences the inactivation results and extremely rough surfaces increase bacterial spore resistance. However, no clear correlation could be found between surface roughness and spore resistance.

The influence of surface wettability for room decontamination with low amounts of H₂O₂ was investigated by Unger et al. (2007), however, they measured wettability by means of contact angle measurement with 40% ethanol as the application of microorganisms later on was also carried out as a droplet of aqueous suspension with 40% ethanol. Therefore, their results show the influence of microorganism application as carrier material with good wettability allows the drop to spread and thus microorganisms are scattered on the carrier material. For bad wettability, microorganisms form layers whereat only the upper layers are affected by H₂O₂ and the lower layers survive (Engelhard 2006). Grand et al. (2010) also investigated the influence of surface wettability. However, their results might be explained with the application of microorganisms as well as they too applied drop deposition.

In conclusion, several aspects respective H₂O₂ inactivation of spores with and without condensate formation remain unclear, contradictive or unexplained. In particular, the effects of spore and carrier material surface hydrophobicity as influencing factors have not been sufficiently investigated.

2 Objective and outline

In aseptic packaging technology, H_2O_2 is the decontamination agent of choice and can be applied with or without the formation of condensate. Bacterial spores are very resistant against H_2O_2 and therefore commonly applied as BI to monitor inactivation. When condensate occurs, a phase transition from gas to liquid takes place whereat an energy barrier has to be crossed. Microorganisms can act as condensation nuclei and thereby lower this energy barrier. Some authors state that microorganisms with more hydrophilic characteristics will be engulfed by the condensate first. Microorganisms with a more hydrophobic surface thus have a better chance to survive the inactivation process with H_2O_2 condensate.

It is the aim of this thesis to investigate the influence of surface hydrophobicity on the inactivation with gaseous H_2O_2 with and without condensate formation. The general approach includes establishing a measurement method to quantify surface hydrophobicity. Water contact angle measurement is well suited. However, several influencing factors are not clear as different measurement procedures are described in literature. Detection of the influencing factors on water contact angle measurement is the first step. The impact of the two most striking discrepancies, in particular the moisturizing medium and the equilibration time, are studied to define a measurement method facilitating reproducible, reliable and unaffected results.

One of the leading ideas during the experimental part of this thesis was the generation of bacterial spores of the same species, thus the same genetic setup, however with significantly different surface hydrophobicity. Keeping the pH-value during cultivation at a constant level without the application of buffers is not possible on agar plates. Cultivation of bacterial spores in a bioreactor as relatively new practice enables the generation of spores of *B. subtilis* at different static pH-values and might provide an option to modify surface hydrophobicity. The generated spores will be applied to obtain inactivation kinetics with gaseous hydrogen peroxide. The influence of surface hydrophobicity of the carrier material on which the spores are placed prior to the treatment will be investigated with different materials of varying water contact angles.

In reality, not only one spore species is to be expected on the decontamination surface. The hypothesis that condensate engulfs the most hydrophilic spore and thus enables the more hydrophobic spore to survive will be investigated with a mixed spore population. By combining two relatively similar spore species however with different growth patterns on agar plates allows the detection of both spore species after treatment with gaseous and condensing H_2O_2 . The resulting inactivation kinetics provide insight if bacterial surface hydrophobicity influences the inactivation with gaseous H_2O_2 for both modes of action: With and without condensate formation.

3 Results

3.1 Effect of moisture equilibration time and medium on contact angles of bacterial spores

Summary and contribution of the doctoral candidate

Microbial adhesion is of major importance in food and bioprocess industry. Contact angle measurement is one of the most often applied method to do so. The basic principle starts with the filtration of a certain amount of microorganisms or bacterial spores followed by an equilibration time on a certain media to remove the excess moisture from the spore layer. Removing the filter from the media and drying it for the time-dependent measurement is the last step prior to the measurement during which a small drop of water is put on the spores and the video analyzed. Many different work groups conducted contact angle measurement of vegetative microorganisms and bacterial spores. However, the exact procedure of the method differs. Therefore, it was the aim of the study to critically review the existing experimental procedures for water contact angle measurement and to point out two factors influencing the results of the water contact angle measurement. Those two influencing factors are the moisture equilibration time and medium. For the experiments, spores of *Bacillus subtilis*, *Bacillus atrophaeus* and *Geobacillus stearothermophilus* were cultivated, purified and characterized concerning their cell size. Complete covering of the filter was checked by means of scanning electron microscopy. Filters were stored on different moisturizing media for 120 minutes each. To investigate the influence of moisture equilibration time, filters with spores of *Bacillus subtilis* were stored for different equilibration times on moisturizing media. For both influencing factors, a drying step for 50 minutes followed by time-dependent water contact angle measurement was carried out. The influence of the moisturizing media on the spore film was investigated by means of HPLC. Influence of the moisturizing media: The hygroscopic glycerol containing media resulted in spore layers with lower water contact angles. HPLC results showed that glycerol is present in those spore layers. An amount of 1 or 2 % Agar-Agar only shows little difference in the resulting water contact angles however still allows an equilibration time. Influence of the equilibration time: The resulting water contact angles do not show any significant differences, however, the lowest standard deviations are obtained after 2 h. Therefore, 2 % agar-agar and an equilibration time of 2 h are proposed in order to obtain results that are not influenced by one of those factors and comparable among each other. The doctoral candidate cultivated and purified the bacterial spores, planned and conducted the major part of the contact angle measurements, prepared the filters for the scanning electron microscopy, discussed and supervised the HPLC experiments and carried out thoma chamber experiments. The analysis of the resulting data, interpretation of datasets and plotting of data were also conducted by the doctoral candidate. The manuscript was essentially written by the doctoral candidate.

Adapted original manuscript¹

Effect of moisture equilibration time and medium on contact angles of bacterial spores²

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Abstract

Contact angle measurement of microorganisms is often described in literature, either to investigate their hydrophobic characteristic or the adhesion behavior of cells. However, in some key aspects the preparation methods differ. Thus, it is difficult to compare results and to choose a procedure for repetition of measurements. The aim of this paper is to point out some critical points during microorganism film preparation that can alter the resulting contact angles. Depending on the moisturizing medium and equilibration time, contact angles differ significantly.

Keywords:

Contact angle, hydrophobicity, bacterial spores, adhesion, *Bacillus*

3.1.1 Introduction

Microbial adhesion is of major importance for processes in the environmental and medical area, but also in the food processing sector. Settlement of microorganisms in the early phase of biofilm formation can lead to biofouling in pipes of water supply and in food industry as foulants in filtration membranes (Characklis 1981; Zottola and Sasahara 1994). However, the ability to adhere provides an advantage for probiotics during the colonization of the gut (Amund 2016) and is an important criteria for the immobilization of biofilms in bioreactor systems (Characklis 1981). To estimate the predisposition for adhesion of bacterial spores, it is crucial to study their surface characteristics.

van Oss et al. (1975) were the first authors to describe the measurement of contact angles on biological material. Their target was to investigate the opsonization behavior of phagocytes. The hypothesis was that phagocytosis depends on the surface hydrophobicity of the phagocytes and the particles or bacteria to be opsonized. To characterize the cells, they chose the contact angle measurement due to the fact that this method is suitable for fragile cells such as leucocytes (van Oss et al. 1975). Since then, many authors applied and modified the contact angle measurement to characterize the surface of cells and microorganisms, as shown in Table 3-1. Depending on the work group, the exact procedure for this method is different, but the basic approach, consisting of four steps, remains. Figure 3-1 gives an overview on steps and variables of the procedure.

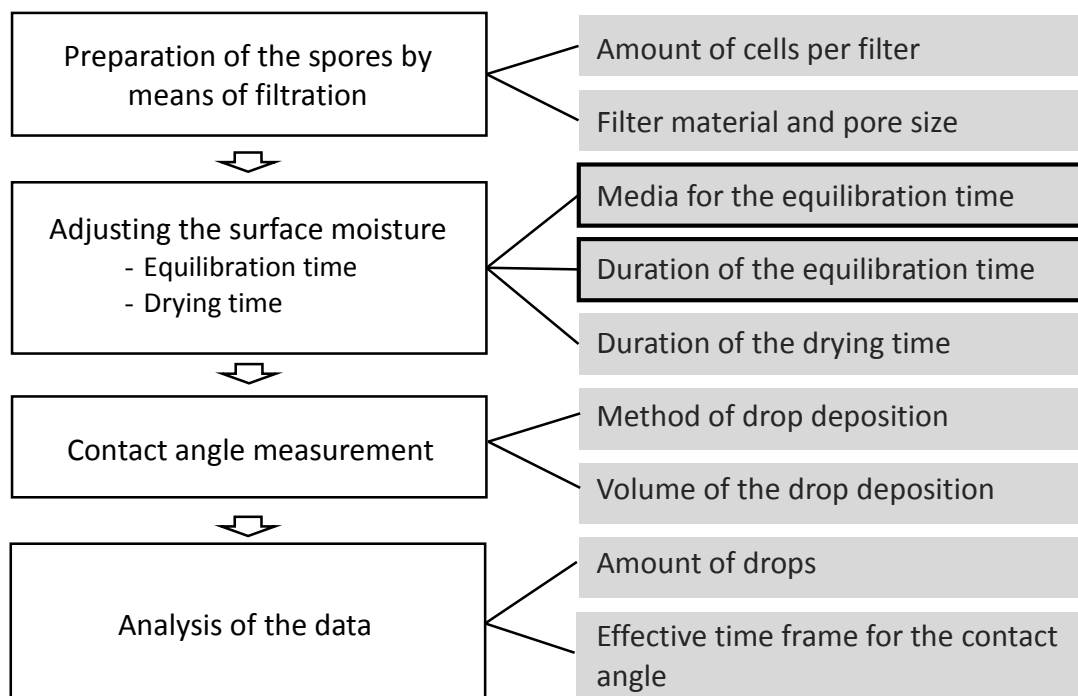


Figure 3-1: General procedure of the contact angle measurement, focus of this study on the framed steps

To prepare the microorganisms as smooth, homogeneous layer, the cells are filtered and the moisture content on top of the filter is subsequently adjusted by two steps: First, the

equilibration time, where the cell covered filter is put on a moist medium to unify the moisture content throughout the filtercake. Second, the drying time, during which the filter is placed on a firm, dry surface to desiccate until a plateau in moisture is reached. The measurement takes place during the plateau time, whereat a drop of measurement liquid is deposited on the surface of the filter. By drawing a tangent along the drop contour through the three phase point, the contact angle can be determined.

Table 3-1 shows water contact angles of different vegetative microorganisms and bacterial spores. Clearly, the results differ depending on the species, but also depending on the strains (Chen et al. 2010; Ahimou et al. 2001). *Bacillus subtilis* spores show contact angles of 10,3 ° (Pruß et al. 2012) to 59 ° (Ahimou et al. 2001). Grasso et al. (1996) showed that contact angles also depend on the physiological state of the cells. However, there are some key aspects in the procedure that differ depending on the method applied by each of the work groups.

Table 3-1: Summary of different references for contact angle measurements of microorganisms and their differences

Authors	Cells / microorganisms and water contact angle	Purpose of the study	Amount of cells per filter, moisturizing medium, equilibration time and drying time
van Oss et al. (1975)	Phagocytes <i>S. aureus</i> (17 °) <i>E. coli</i> (19 °)	Investigate opsonization behavior of phagocytes	- Layer cells on thin, flat glycerin agar (2 % agar, 10 vol% glycerin) - Dry at least 3 hours till plateau
Absolom et al. (1983)	<i>E. coli</i> 055 (16,7 °) <i>S. aureus</i> 049 (18,5 °) <i>E. coli</i> 2627 (21,2 °) <i>S. epidermidis</i> (23,4 °) <i>L. monocytogenes</i> (26,1 °C)	Study the adhesion of five strains to polymeric surfaces	- Drying suspension on 1 cm ² of glycerin agar (1 % agar, 10 vol% glycerin) - Drying time not specified
Busscher et al. (1984)	Streptococci: <i>S. salivarius</i> (20 °) <i>S. sanguis</i> (42 °) <i>S. mitior</i> (26 °) <i>Veillonella alcalescens</i> (55 °)	Describe a technique to measure contact angles on bacterial layers	- Equilibration time on glycerin agar (1 % agar, 10 vol% glycerin) - "... water contact angles were measured as a function of drying time."
(Mozes and Rouxhet 1987)	<i>Enterobacter aerogenes</i> (62 °) <i>Klebsiella fragilis</i> (53 °) <i>Acetobacter acetii</i> (28 °) <i>Moniliella pollinis</i> (Fungi, >90 °) <i>Saccharomyces cerevisiae</i> (yeast, 26 °) <i>Kluyveromyces oxytoca</i> (yeast, 50 °)	Comparison of five methods to measure hydrophobicity of microorganisms, one being contact angle measurement	- Equilibration time on glycerin agar (1 % agar, 10 vol% glycerin) - "... and contact angles were measured as a function of time."
(van Loosdrecht et al. 1987)	<i>Pseudomonas fluorescens</i> (21,2 °) <i>Pseudomonas aeruginosa</i> (25,7 °) <i>Pseudomonas putida</i> (38,5 °) <i>Pseudomonas sp. strain 26-3</i> (20,1 °) <i>Pseudomonas sp. strain 52</i> (19,0 °) <i>Pseudomonas sp. strain 80</i> (29,5 °) <i>Escherichia coli</i> NCTC 9002 (15,7 °) <i>Escherichia coli</i> K-12 (24,7 °) <i>Arthrobacter globiformis</i> (23,1 °) <i>Arthrobacter simplex</i> (37,0 °) <i>Arthrobacter sp. strain 177</i> (60,0 °) <i>Arthrobacter sp. strain 127</i> (38,0 °)	Investigate the role of bacterial cell wall hydrophobicity in adhesion	- No equilibration time - Drying for 0,5 – 3 h

	<p><i>Micrococcus luteus</i> (44,7 °) <i>Acinetobacter</i> sp strain 210 A (32,6 °) <i>Thiobacillus versutus</i> (26,8 °) <i>Alcaligenes</i> sp. strain 175 (24,4 °) <i>Agrobacterium radiobacter</i> (44,1 °) <i>Bacillus licheniformis</i> (32,4 °) <i>Azotobacter vinelandii</i> (43,8 °) <i>Corynebacter</i> sp. strain 125 (70,0 °) <i>Mycobacterium phlei</i> (70,0 °) <i>Rhizobium leguminosarum</i> (31,0 °) <i>Rodopseudomonas palustris</i> (34,3 °)</p>		
(Rijnaarts et al. 1993)	<p><i>Arthrobacter</i> sp. strain DSM 6687 (15 °) <i>Coryneform</i> strain DSM 6685 (29 °) <i>Rhodococcus</i> sp. strain C125 (70 °) <i>Rhodococcus erythropolis</i> A177 (87 °) <i>Corynebacterium</i> sp. strain DSM 6688 (89 °) <i>Corynebacterium</i> sp. strain DSM 44016 (103 °) <i>Gordon</i> asp. strain 1775/15 (115 °) <i>Gordona</i> sp. strain DSM 44015 (117 °) <i>Pseudomonas oleovorans</i> ATCC 29347 (17 °) <i>Pseudomonas fluorescens</i> p62 (25 °) <i>Pseudomonas</i> sp. strain B13 (32 °) <i>Pseudomonas putida</i> mt2 (40 °)</p>	Study the deposition of microorganisms with different hydrophobicities on Teflon and glass surfaces	- Not specified, refers to van Loosdrecht et al. (1987)
(Grasso et al. 1996)	<p><i>Pseudomonas aeruginosa</i> - Logarithmic state (33,5 °) - Stationary state (49,8 °) - Decay state (37,7 °)</p>	Impact of physiological state on adhesion of <i>Pseudomonas aeruginosa</i>	- No equilibration time - Drying for 0,5 h
(Garry et al. 1998)	<p><i>Bacillus subtilis</i> - Vegetative form (20 to 24,3 °, depending on growth temperature) - Spores (34 °) <i>Bacillus cereus</i> ATCC 14579 - Vegetative form (20,1 to 31,1 °, depending on growth temperature) - Spores (36 °)</p>	Investigation of growth temperature on adhesion of <i>Bacillus cereus</i> and <i>Bacillus subtilis</i> vegetative bacteria and spores	- Equilibration time and conditions not specified - Drying time not specified
(Ahimou et al. 2001)	<p><i>Bacillus subtilis</i> ATCC 7058 - Vegetative form (31 °) - Spores (59 °) <i>Bacillus subtilis</i> ATCC 12432 - Vegetative form (31 °) - Spores (55 °) <i>Bacillus subtilis</i> ATCC 12695 - Vegetative form (34 °) - Spores (33 °) <i>Bacillus subtilis</i> ATCC 15129 - Vegetative form (32 °) - Spores (42 °) <i>Bacillus subtilis</i> ATCC 15476 - Vegetative form (20 °) - Spores (44 °) <i>Bacillus subtilis</i> ATCC 15561 - Vegetative form (28 °) - Spores (52 °) <i>Bacillus subtilis</i> ATCC 15811 - Vegetative form (47 °)</p>	Comparison of hydrophobicity of <i>Bacillus subtilis</i> strains (vegetative cells, spores) with different methods including contact angle measurement	- Equilibration time on glycerin agar (1 % agar, 10 vol% glycerin) for 2 h - Drying on air for 60 min

Results

	<ul style="list-style-type: none"> - Spores (49 °) <i>Bacillus subtilis B 213</i> <ul style="list-style-type: none"> - Vegetative form (27 °) <ul style="list-style-type: none"> - Spores (48 °) <i>Bacillus subtilis S 499</i> <ul style="list-style-type: none"> - Vegetative form (34 °) - Spores (46 °) 		
(Seale et al. 2008)	Spores of <i>Geobacillus</i> isolates from milk samples <ul style="list-style-type: none"> - D4 (13 °) - E7 (18 °) - E 11 (25 °) - CGT-8 (20 °) 	Impact of surface characteristic of <i>Geobacillus</i> spores on attachment to different surfaces	<ul style="list-style-type: none"> - Equilibration time on glycerin agar (2 % agar, 10 vol% glycerin) - Drying for 45 min on air
(Chen et al. 2010)	Spores of <ul style="list-style-type: none"> <i>Bacillus subtilis</i> wild type (24,1 °) <i>Bacillus subtilis</i> cotO (33,2 °) <i>Bacillus subtilis</i> cotB cotG (27,2 °) <i>Bacillus anthracis</i> wild type (23,1 °) <i>Bacillus anthracis</i> cotO (32,9 °) <i>Bacillus anthracis</i> bclA (25,6 °) 	Investigate adhesive properties of <i>Bacillus subtilis</i> and <i>Bacillus anthracis</i> spores	<ul style="list-style-type: none"> - No equilibration time - Drying for 0,5 h
(Gallardo-Moreno et al. 2011)	<i>Staphylococcus</i> strains, results of water contact angles shown graphically	Show particularities of contact angle measurement, especially the influence of different pH values prior to measurement and time dependence of data during drop deposition	<ul style="list-style-type: none"> - No equilibration time - Drying for 60 min on air
(Pruß et al. 2012)	Spores of <ul style="list-style-type: none"> <i>Bacillus subtilis</i> SA 22 (10,4 °) <i>Bacillus subtilis</i> DSM 347 (20,9 °) <i>Bacillus atrophaeus</i> (13,1 °) 	Influence of temperature dependent condensation of gaseous H ₂ O ₂ on inactivation of spores with different surface properties	<ul style="list-style-type: none"> - Equilibration time on glycerin agar (1 % agar, 10 vol% glycerin) for 2 h - Drying on air for 45 min

Regarding the equilibration time, some authors do not apply an equilibration time at all (van Loosdrecht et al. 1987; Chen et al. 2010) whereas other authors use glycerin-agar to unify the moisture content throughout the microorganism lawn (Ahimou et al. 2001; Seale et al. 2008; Pruß et al. 2012). In the majority of articles, no duration of the equilibration time is mentioned. To our knowledge, there is no literature available concerning the background of the equilibration media. Most likely the equilibration time is applied to attain a uniform moisture content throughout all the microorganism layers on the filter. Hence a mixture of glycerol and agar-agar is applied as glycerol acts hygroscopic and the gel forming agar-agar provides defined moisture content.

Those differences prior to the measurement induce the question whether different sample preparation methods lead to consistent results. Thus, the purpose of this study is to review the conditions used in the various works and to provide an insight into some critical points of contact angle measurement of bacterial spores. To investigate the influence of moisturizing media and equilibration time on the water contact angle, those two command variables were varied in certain ranges.

3.1.2 Materials and Methods

Microorganisms:

As cell surface hydrophobicity might be a leading factor for decontamination with condensing hydrogen peroxide vapor, we used three different species of bacterial spore formers that are relevant for decontamination processes as biological indicators (Pruß et al. 2012): *Bacillus subtilis* (*B. subtilis*, DSM 4181, SA 22), *Bacillus atrophaeus* (*B. atrophaeus*, DSM 675) and *Geobacillus stearothermophilus* (*G. stearothermophilus*, DSM 22). The microorganisms were obtained from the DSMZ (German collection of microorganism and cell cultures, Braunschweig, DE) as freeze-dried cultures. They were revitalized following the manufacturer's instructions and stored on Nutrient Agar (for 1000 mL distilled water: 5,0 g peptone (Merck, Darmstadt, DE), 3,0 g beef extract (Gerbu, Heidelberg, DE), 15 g agar-agar, (Fisher Scientific, Schwerte, DE) with 20 mg of manganese sulfate (Merck, Darmstadt, DE).

Cultivation: To obtain the bacilli as spores, they were cultivated with a sporulation media (for 1000 mL of distilled water: 5,0 g peptone from casein (Gerbu, Heidelberg, DE), 3,0 g beef extract (Gerbu, Heidelberg, DE), 3,5 g potassium chloride (Merck, Darmstadt, DE), 250 mg magnesium sulphate (Roth, Karlsruhe, DE), after autoclaving 10 mL of 10 % glucose in water (Merck, Darmstadt, DE) and 1 mL of the following micronutrients were added: 1 M calcium nitrate (Sigma Aldrich, Darmstadt, DE) 0,01 M manganese chloride (Merck, Darmstadt, DE), 1 mM iron sulphate (Fluka, Seelze, DE) either in a bioreactor (*B. subtilis* and *G. stearothermophilus*) or on agar plates (*B. atrophaeus*). For cultivation on agar plates, agar-agar (15 g/L, Fisher Scientific, Schwerte, DE) was added to the composition.

The sporulation conditions were 37 °C at a constant pH-value of 7,5 for 48 h. The level of oxygen was adjusted to 30 %. For *G. stearothermophilus*, the temperature was adjusted to 60 °C. *B. atrophaeus* was grown under the same conditions on agar plates for 10 days, the pH and oxygen level could thus not be monitored. The spores were subsequently removed from the agar plates by pouring 10 mL of cold, sterile distilled water on the plates, suspending the spores with a spatula and collecting the suspension.

Purification: The washing steps were the same for all three spore species. To obtain the spores and separate the vegetative bacteria, each batch was washed at least 4 times with sterile, distilled water and centrifuged with 4000g for 10 min at 4 °C. The spores were heat activated with a temperature of 80 °C for 20 min (*G. stearothermophilus*: 90 °C for 20 min) and subsequently cooled down in ice water. The purity of the spores i.e. the absence of vegetative cells was checked with a light microscope (Axioskop, Carl Zeiss, Oberkochen, DE) and was higher than 95 % for every spore suspension. Storage was carried out in distilled water at 4 °C.

Cell size:

The cell size was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, DE). The measurement was conducted at a fixed angle of 173 ° and a wavelength of 632,8 nm. The measurement was done in triplicate, each measurement consisted of ten individual runs of 60 s duration.

Scanning electron microscopy (SEM):

The samples were required to dry for 24 h at room temperature prior to the gold sputtering process. Samples were sputtered for 20 min. Electron microscopy pictures were taken under vacuum conditions with a scanning electron microscope (Jeol JSM 5900 LV, Tokio, Japan) at the department of electron microscopy (TU Munich, Garching, DE).

Thoma cell counting chamber:

The amount of spores was directly counted with a Thoma Brightline counting chamber (depth: 0,100 mm, Labor Optic, Friedrichsdorf, DE) and a light microscope (Axioskop, Carl Zeiss, Oberkochen, DE). The spores were diluted with distilled water at the ratio 1:10 until an amount of approximately 1 to 20 spores was counted per small square. The Thoma counting chamber consists of 16 big squares, each containing 16 small squares. The spores of 4 big squares were counted and the amount of cells per mL calculated using (3-1)

$$x\left(\frac{cfu}{mL}\right) = \frac{n}{64} \cdot 4 \cdot 10^6 \cdot DF \quad (3-1)$$

where n is the sum of the spores of all four big squares, and DF is the dilution factor.

Contact angle measurement:

Preparation of the spore film: For the characterization of the spore surface, the filter surface has to be completely covered with cells. The circular filter (Sartorius Stedim, Göttingen, DE) possesses a pore size of 0,22 µm and an active diameter of 44 mm in the vacuum filtration device. By calculating the surface of a circle with radius 22 mm, a surface area of 1520 mm² has to be covered.

The size of the spores was investigated with Zeta sizer measurements. The amount of spores was directly counted. Considering the spore size, the required quantity of spores for the smallest spores to fully cover the surface can be calculated as shown in (3-2). According to Kepler's assumption, the highest possible packing density of spheres with r (radius of the filter) and R (radius of the spores) is 74 %, which was included in (3-2).

$$x = \frac{(r^2 \cdot \pi)}{(R^2 \cdot \pi)} \cdot 0,74 \quad (3-2)$$

The required amount of spores to fully cover the filter with a monolayer was calculated. An amount of spores to produce 4 – 5 layers was filtered on the surface of each filter. The even distribution i.e. the complete cover of the filter was controlled by SEM. For each spore suspension, the required aliquot per filter was diluted with 250 mL of distilled water and vacuum filtrated. By means of negative pressure filtration (vacuum pump, Sartorius Stedim, Göttingen, DE), a smooth layer of cells was achieved.

Adjusting the surface moisture: Filters with homogenous spore layers were subsequently transferred to the equilibration media to achieve a uniform moisture content throughout all the microorganism film. Different equilibration media were used, their composition consisting of 1 % agar, 2 % agar or 2 % agar with 10 vol% glycerol

After 2 h, the filter was cut into four stripes of approximately the same size, removed from the equilibration media and fixed on glass slides (VWR, Darmstadt, DE) by means of double-sided adhesive tape. The fixed filter strips were dried on air until a plateau in contact angles was obtained.

Prior to reaching the plateau, excess water on the filter surface disturbs the measurement and reduces the contact angles to a smaller value. The increase in contact angle means that the excess water is removed by evaporation into the air until a constant value of surface hydrophobicity is reached. This is when the spore surface is exposed to air and the microorganisms film still one smooth surface. After exceeding the plateau, cracks and fissures in the surface can occur and values are not stable anymore. Therefore, time dependent measurements were done after the equilibration time to determine the plateau. All measurements were carried out during the plateau time.

Measurement of the contact angle: The glass slides with filter strips were mounted on the table of the contact angle measuring instrument DSA 100 (Krüss, Hamburg, DE). All measurements were carried out at 20 °C. As the surface consists of microorganisms, the drop will begin to sink into the surface immediately after deposition. Therefore, the sessile drop method was applied. After 50 min of drying time, a drop of water was placed on a dried piece of spore covered filter every 2 min. The first 3 μL were dispensed at a rate of 200 $\mu\text{L min}^{-1}$, the following 5 μL were dispensed at a rate of 100 $\mu\text{L min}^{-1}$ to obtain a constant

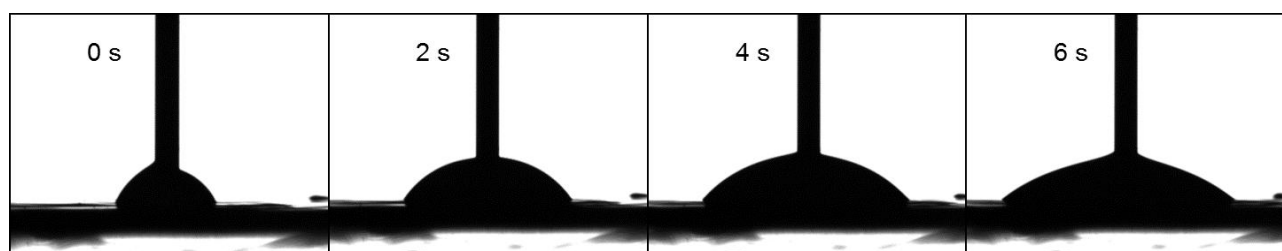


Figure 3-2: Advancing contact angle after deposition of the drop (0 s) and during extension of the drop (2 and 4 s) until the drop starts to sink in (6 s)

advancing contact without influence of capillary effects. A video was taken over the drop deposition time of 6 s.

Analysis of the data: The DSA 4 software (Krüss, Hamburg, DE) was utilized to analyze the video data. The contact angle is very high at first (0 to 2 s) as the drop formation is taking place until the drop finds its final advancing form and provides constant contact angles during the time frame between 2 and 4 s (Figure 3-2). Therefore, the average of the data from this time frame was calculated, resulting in the value for one drop. Afterwards whole volume of the drop is deposited (4 to 6 s) and the drop starts to sink into the microorganism film due to capillary effects.

For each filter, the average of at least 5 individual drops deposited within the plateau time was calculated. For each spore suspension, at least three filters were used for the measurement. Between 2 and 4 seconds 26 individual pictures were taken and the contact angle was measured on the left and on the right side. This yields at least 780 single values of contact angles of each spore suspension ($5 \cdot 3 \cdot 26 \cdot 2$). Mean values and standard deviations were calculated from the contact angles of three individually prepared filters of one spore suspension.

HPLC analysis of the spore film:

The amount of glycerol that diffuses through the filter into the film was determined by HPLC (Agilent 1100, Agilent Technologies, Santa Clara, USA) using an Aminex HPX-87H (300 x 7.8 mm) column with a precolumn (Bio-Rad Laboratories, Hercules, USA) isocratically at 50 °C. Sample preparation was done by filtering spores according to the protocol for the contact angle measurement and placing them on different equilibration media. After an equilibration time of 2 h, the spores were completely removed from the filters with glass spatula and mixed with 2 mL of Milli-Q water. An aliquot of 500 µL was mixed with 50 µL of 60 % perchloric acid to precipitate proteins and diluted with 1 mL of deionized water. The resulting solution was subsequently filtered (pore size 0,22 µm, Minisart NML, Sartorius Stedim, Göttingen, DE). The amount of glycerol was quantified using 0,005 M sulfuric acid as an eluent, a flow rate of 0,6 mL min⁻¹ and a refraction index detector.

Statistical methods:

To assess the significance between resulting contact angles, a t-Test for independent samples with a level of significance (α) of 0,05 was applied.

3.1.3 Results and discussion

Adjusting the surface moisture: Influence of the equilibration media:

Zeta sizer measurements resulted in a $d_{50,3}$ of 1,307 to 1,417 μm , assuming a shape of round globules. The amount of spores sufficient for 4 to 5 spore layers was calculated (3-2) and checked with scanning electron pictures as shown in Figure 3-3. Due to the complete drying that is necessary for electron microscopy, the layers have fissures and cracks that show there are several layers of microorganisms.

To compare various moisture media in their effects on the contact angle measurement, the duration of the drying time to reach the plateau has to be investigated for every spore species and every equilibration medium as each of them can show its distinct hygroscopic characteristic. Therefore, advancing contact angles were measured every 5 min over a time span of 80 min and the time frame after evaporation of the excess water during which a constant contact angle was achieved was identified as plateau time. For reasons of

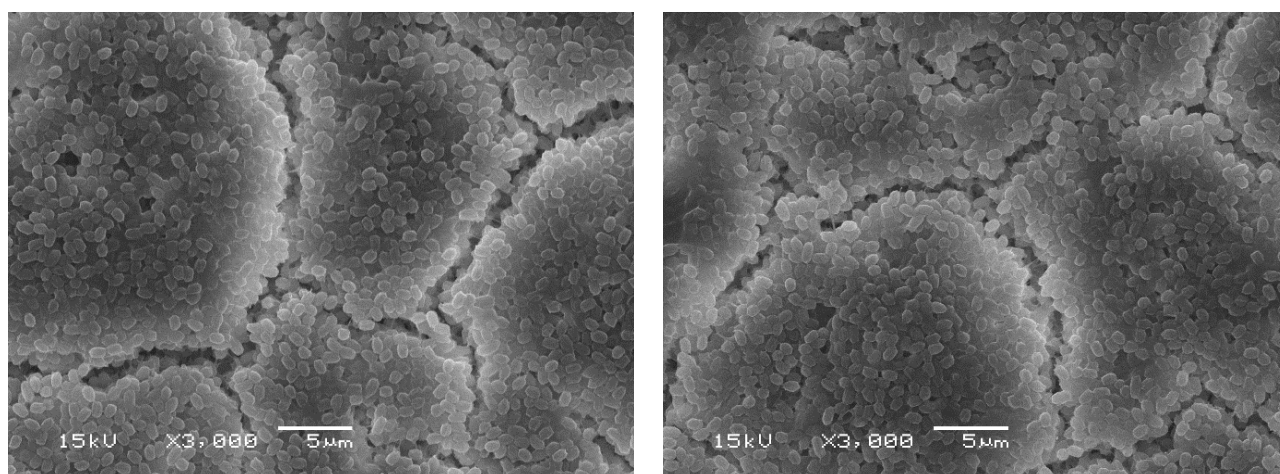


Figure 3-3: Electron microscopy pictures of a filter covered with *B. subtilis* spores, 3000 x augmentation

comparability the equilibration time prior to the drying time was set to a duration of 2 h, which is in accordance with (Ahimou et al. 2001) and (Seale et al. 2008). The effect of the equilibration medium on the drying time is negligible. For all spores and equilibration media the plateau was reached between 50 and 70 min. Therefore, all measurements were carried out in this time frame.

For the equilibration medium, (Busscher et al. 1984), (Ahimou et al. 2001) and (Pruß et al. 2012) used glycerol-agar containing 1 % agar-agar, (Seale et al. 2008) used glycerol-agar containing 2 % agar-agar, whereas (van Loosdrecht et al. 1987) and (Chen et al. 2010) applied the direct drying of the cell covered filter on air without equilibration time. To investigate the influence of the glycerol and different amounts of agar-agar in the equilibration medium, contact angle measurements with water were carried out, the results are shown in Figure 3-4.

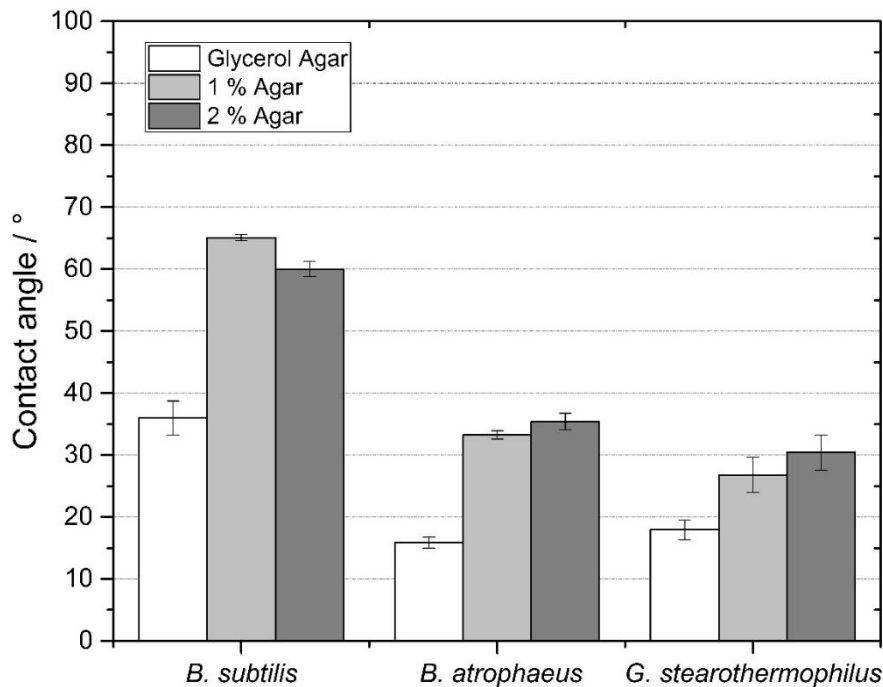


Figure 3-4: Water contact angle of bacterial spores, stored on different equilibration media. Each spore-substratum combination was measured in triplicate, error bars represent standard deviations. White: Stored on Glycerol-agar, bright grey: Stored on 1 % agar, dark grey: Stored on 2 % agar

The spores of *B. subtilis* show a higher water contact angle and thus a higher surface hydrophobicity on every equilibration medium. Comparing the resulting contact angles on 1 % Agar and 2 % Agar, only a small difference for *B. subtilis* and no significant difference for *B. atrophaeus* and *G. stearothermophilus* could be detected. Both concentrations of the gelling polysaccharide were able to form a firm, moist gel suitable for the equilibration time. In accordance with our expectations, no significant influence on the cell surface and the resulting contact angle was detected.

For every spore suspension, the contact angles obtained on glycerol-agar are significantly lower compared to those obtained without glycerol contact. T-test results ($\alpha=0,05$) reveal that stored on glycerol agar, there is no significant difference between the contact angles of *B. atrophaeus* and *G. stearothermophilus*. However, stored on 1 % Agar or on 2 % Agar, contact angles show a significant difference.

As glycerol (IUPAC-name: Propane-1,2,3-triol) is a sugar alcohol that is completely soluble in water and has a hygroscopic nature, we suspected the glycerol to diffuse because of a concentration gradient through the filter and the spore layers to the surface and thus alter the results. Hence the water contact angle is expected to be lower if glycerol diffuses to the surface of the spores. The contact angles of all three spore species are significantly higher when equilibration time took place on a media without glycerol. To verify the hypothesis of glycerol diffusion, the amount of glycerol on the filter was checked with an HPLC analysis. For all three strains, an amount of glycerol of 1 to 2,5 mg was detected when deposited on

glycerol agar for 2 h. The HPLC results of the spores stored on media without glycerol did not show any detectable glycerol quantity. The amount of glycerol should not be discussed here but the results show that glycerol did diffuse into the microorganism film and thus had an impact on the resulting water contact angles. However, many studies do not consider this factor and measure contact angles on microorganism films with an equilibration time on glycerol-agar. Most of those authors refer to (Busscher et al. 1984), who worked with vegetative cells and applied enough cells to generate a filtercake with 10^{10} cfu cm^{-2} . For a round filter with a diameter of 45 mm, this adds up to an amount of $1,52 \cdot 10^{11}$ cfu per filter, which is enough for multiple layers of microorganisms. In these articles, the applied number of cells was significantly higher than in our study ($3,5 \cdot 10^9$) which results in more cell layers and a higher filtercake, where glycerol might or might not be able to diffuse to the surface. However, it is important to keep in mind that the results might show the contact angle of the cells mixed with glycerol and that therefore the contact angles might be lower. This is of special interest for a comparison of existing literature data amongst different works.

Adjusting the surface moisture: Influence of the equilibration time

The purpose of the equilibration time is an even moisture distribution after the filtration to achieve an optimal initial moisture content for the next step, the drying on air. Some authors apply this equilibration time (Busscher et al. 1984; Ahimou et al. 2001; Seale et al. 2010; Seale et al. 2008), whereas other authors skip this step completely and start the drying on air immediately after the filtration (Gallardo-Moreno et al. 2011; Chen et al. 2010). The applied equilibration time in literature ranges from 0 to 3 h. To assess the influence of the time, we used the 2 % Agar sample. For this medium, no time-dependent diffusion was

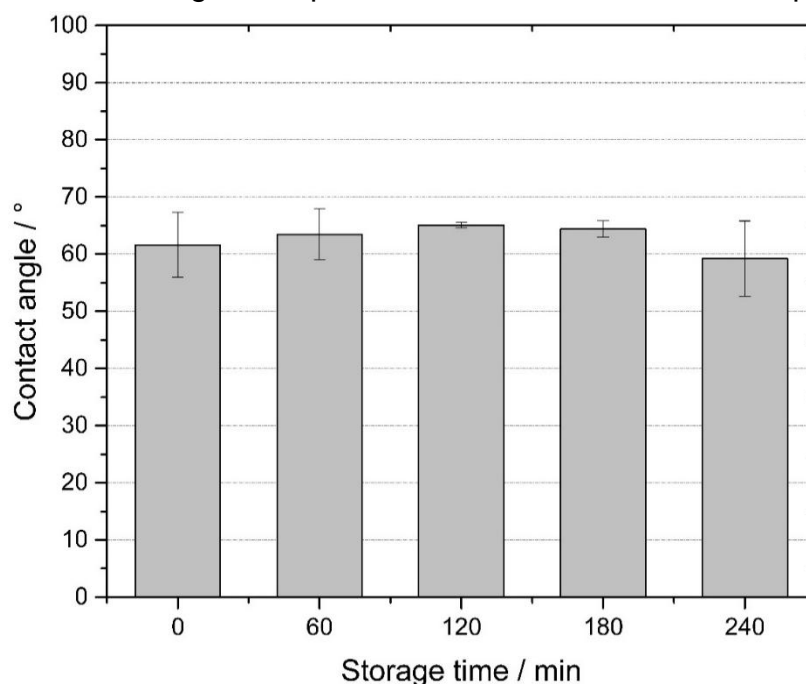


Figure 3-5: Water contact angles of *B. subtilis* spores, measured after different equilibration times (0 to 240 minutes) with standard deviations

expected. The tests were done with *B. subtilis* spores. The same amount of bacterial spores was applied as before. The equilibration time varied between 0 - 240 min, the subsequent drying step to the plateau was carried out for 50 min for all equilibration times. The resulting water contact angles are shown in **Fehler! Verweisquelle konnte nicht gefunden werden..**

The water contact angle of *B. subtilis* spores does not show any significant differences with changing equilibration time. However, the standard deviations are considerably smaller after an equilibration time of 2 h. We therefore conclude that the moisture distribution in the filtercake is not unified directly after filtration. However, after 4 h on the 2 % Agar increased standard deviations were present. Although the 2 % Agar provides moisture for the filter, a partial drying of the surface of the filtercake might have occurred and result in less accurate contact angles. In order to obtain clear results with the contact angle measurement which is known to provide substantial standard deviations on biological material, we propose to keep the equilibration time on 2 % Agar for 2 h.

3.1.4 Conclusion

We compared water contact angles on spores prepared on different equilibration media. The significance of our findings is that glycerol from glycerol-agar as substratum diffuses through the filter and spore film and has a major effect on the resulting contact angles. However, the investigation of different equilibration times on the same media showed that equilibration time has little impact on the results but a duration of 2 h provides the lowest standard deviation.

This study is of importance for everyone dealing with water contact angle measurement and might as well be crucial for contact angle measurement with other liquids as glycerol diffuses throughout the spore layers independent of the liquid applied lateron.

Our results show that a standardized method for the contact angle measurement of microorganisms considering the critical points such as number of cells, equilibration medium and time is required on the one hand to provide a method for reproducible contact angle results and on the other hand to obtain results that are comparable among each other.

3.2 Effect of cultivation pH on the surface hydrophobicity of *Bacillus subtilis* spores

Summary and contribution of the doctoral candidate

For aseptic packaging and clean room decontamination, H₂O₂ is applied in its liquid or gaseous form. To evaluate the success of the decontamination step, biological indicators with very high resistance are applied. For H₂O₂, spores of *B. subtilis* are appropriate. Several studies showed that cultivation conditions have an impact on spore resistance, however no study investigated the influence of cultivation conditions on surface hydrophobicity. Commercially available BI are classified concerning their resistance towards the respective decontamination process, the number of cfu/mL and the expiring date. Surface hydrophobicity is not classified. Pruß et al. (2012) erected the theory that bacterial surface hydrophobicity influences the decontamination with gaseous H₂O₂. If surface hydrophobicity has an impact on inactivation with gaseous H₂O₂, it is crucial to understand how cultivation conditions influence the surface of bacterial spores to be able to produce reliable and reproducible inactivation results. The aim of this study was therefore to understand the influence of cultivation pH on spore surface hydrophobicity and resistance against liquid H₂O₂. For this purpose, spores were cultivated in a bioreactor with controlled air supply and static levels of cultivation pH. Sporulation was induced by exhaustion of the carbon source. Surface hydrophobicity of the resulting spores was analyzed by water contact angle measurement. Resistance of the BI was assessed in liquid H₂O₂ using a standard protocol also applied under practical conditions. The obtained amount of spores varied between $1.28 \cdot 10^7$ to $3.40 \cdot 10^8$ cfu/mL. The amount of spores slightly depends on cultivation pH, however is still quite similar for microbiological dimensions. Surface hydrophobicity is highest at pH 8.5 and shows a water contact angle of 80 °. With decreasing pH, water contact angles decrease as well. The resistance against liquid H₂O₂ shows a minimum resistance for spores cultivated at pH 8.00. The resulting Pearson correlation factor of -0.78 indicates a negative correlation between surface hydrophobicity and H₂O₂ resistance which are both influenced by cultivation pH. The doctoral candidate planned and conducted the major part of cultivation and purification of the *B. subtilis* spores. She also planned and supervised the water contact angle measurements and the resistance tests against liquid H₂O₂. The analysis of the resulting data, interpretation of datasets and plotting of data were also conducted by the doctoral candidate. The manuscript was essentially written by the doctoral candidate.

*Adapted original manuscript*¹

Effect of cultivation pH on the surface hydrophobicity of *Bacillus subtilis* spores

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Abstract:

Bacillus subtilis spores are often used as biological indicators (BI) to monitor decontamination processes with gaseous hydrogen peroxide. Results in practical inactivation validation tests, however, vary considerably with no available explanation so far. This study reports on the effect of cultivation pH on spore surface hydrophobicity. Surface hydrophobicity is suspected to have an impact on the decontamination of technical surfaces such as packaging material when gaseous, condensing hydrogen peroxide is applied. It is the aim of this study to examine the impact of different cultivation pH levels on surface hydrophobicity and resistance of *B. subtilis* spores. Submersed cultivation of *B. subtilis* in bioreactors at controlled conditions with different static pH levels led to contact angles ranged between 50 ° and 80 °, which was analyzed with water on a homogeneous layer of spores on a filter sheet. Resistance of spores was also affected by the cultivation pH. The results show that the culturing conditions during BI production should be controlled to obtain BI with specified characteristics in inactivation validation tests.

Keywords:

Bacillus subtilis, submersed spore production, bioindicator, hydrogen peroxide, resistance, surface hydrophobicity

¹ Adaptions refer to formatting issues: e.g. numbering of sections, figures, tables and equations, abbreviations, manufacturer specifications, axis labeling, figure captions and style citation.

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3.2.1 Introduction

In food and pharma industries sterile surfaces for aseptic packaging and clean rooms are often indispensable. The processes to obtain surface sterility are either based on physical or on chemical action. Hydrogen peroxide (H_2O_2) is among the most often used disinfectants. H_2O_2 , mostly a mixture of 35 % H_2O_2 in water and low amounts of stabilizers, can be applied in its liquid form as immersion bath, as spray or as vapor (Engelhard and Kulozik 2006; Pruß et al. 2012).

To validate decontamination processes, resistant, non-pathogenic test organisms are applied as biological indicators (BI) as the detection of survivors is the only direct method to assess the inactivation result (Block 2001; Sella et al. 2013). Spores of *Bacillus subtilis* (*B. subtilis*) and *Bacillus atrophaeus* (*B. atrophaeus*) meet the requirements for BI in inactivation processes with high concentrations of H_2O_2 (Sella et al. 2012; VDMA, 2006).

The mode of action of vaporized H_2O_2 depends on time, temperature of the vapor and surface as well as concentration of H_2O_2 and H_2O in the gas with each of those variables affecting each other. For a decontamination process with vaporized H_2O_2 and the intentional formation of condensate, even more influencing factors arise (Agalloco and Akers 2013). Therefore, an easy test method to estimate the resistance of BI towards H_2O_2 is required. Resistance tests applying liquid H_2O_2 with controlled temperature are used as standard tests at practical level to test the resistance of BI (Muranyi et al. 2006; Pruß et al. 2012; Deinhard et al. 2016).

Pruß et al. (2012) investigated the influence of the surface temperature of packaging specimens on the inactivation effect of *Bacillus* spores with gaseous, condensing H_2O_2 . From their results they postulated that variable inactivation effects may depend on the surface hydrophobicity of the spores applied as BI. This means that, if condensate formation takes place, spores with a low surface hydrophobicity, i. e. good wettability, would preferably induce condensation and would thus be covered by extremely concentrated H_2O_2 condensate. In contrast, spores with a lower wettability would be less affected by the condensate formation. Therefore, inactivation was suspected to be less pronounced for hydrophobic BI.

Surface hydrophobicity of microorganisms is often indirectly measured by the water contact angle (Busscher et al. 1984; Mozes and Rouxhet 1987; van Loosdrecht et al. 1987; Seale et al. 2008). Water contact angle of *B. subtilis* in its vegetative and spore form depends on the strain and growth phase and can vary between 10 ° and 59 ° (Garry et al. 1998; Ahimou et al. 2001; Mozes and Rouxhet 1987). Microorganisms are classified as hydrophilic if their water contact angle is below 20 ° and as hydrophobic if they show contact angles above 50 ° (Rijnaarts et al. 1993).

Several authors report that spore resistance to heat or hydrogen peroxide is significantly influenced by the culturing conditions (Leaper 1987; Melly et al. 2002b; Rose et al. 2007; Minh et al. 2008; Baril et al. 2012a). Results are inconsistent and the mechanism of inactivation of spores is still incompletely understood. Melly et al. (2002a) state that liquid H₂O₂ leads to damage of the peptidoglycan layer or proteinaceous components, which play a key role in spore core expansion. Therefore, water uptake of the spore and germination are impaired. The resistance of the spores cultivated at different temperatures varied, a high cultivation temperature of 48 °C led to increased resistance compared to a cultivation temperature of 22 °C or 30 °C. Several spore components known to have spore protecting properties such as SASP (small, acid soluble spore proteins), the amount of dipicolinic acid and the composition of spore coat and spore cortex were analyzed. Significant differences were only observed in the coat protein composition. As the coat protein composition changes with sporulation temperature (Melly et al. 2002b), it is likely that the composition of the outermost layer varies as well. For *B. subtilis* spores, this outer layer consists of glycoproteins which tightly surround the outer spore coat. This so-called “crust” is responsible for the surface characteristics of *B. subtilis* spores (Imamura et al. 2010; McKenney et al. 2010).

Apart from temperature, cultivation pH also influences spore characteristics. However, those studies investigating the influence of cultivation pH conducted cultivation of spores on agar plates where the pH cannot be controlled (Craven 1990; Mazas et al. 1997). Some years ago Rose et al. (2007) performed a study showing the differences in spore characteristics between spores prepared on agar plates and in liquid medium. Before that, due to simplicity lab-scale studies have often been carried out on agar plates. The relatively new concept of submerged cultivation in bioreactors provides options to control influencing factors throughout fermentation such as maintaining a defined static pH throughout the procedure.

Some authors investigated the influence of pH on spore yield (Monteiro et al. 2005; Baril et al. 2012a), but its impact on surface hydrophobicity has not been studied so far. Mazas et al. (1997) investigated the influence of culturing pH on the resistance of *B. subtilis* spores against heat. They observed a decreasing D_{100°C} value the lower the cultivation pH was. Minh et al. (2011) studied the impact of cultivation pH on *B. subtilis* spore resistance to heat and high pressure. They applied pH values of pH 6,0 and pH 10,0. Most of the resulting spores were more resistant than the reference spores produced at optimum conditions. What remains open, however, is whether a correlation exists between spore surface hydrophobicity and spore resistance.

Commercially available BI from various suppliers can vary considerably regarding resistance against the respective chemical or physical inactivation process, where they are used to validate the efficiency of inactivation processes. Surface hydrophobicity or the culturing conditions, however, are not among the specified characteristics of commercial

spore BI. The hypothesis of Pruß et al. (2012) was that surface hydrophobicity of bacterial spores might possibly influence decontamination with gaseous H₂O₂, especially when conditions allow for condensation or micro-condensation. If this was the case, validation of decontamination processes using BI would be affected as well. For hydrophilic BI, inactivation kinetics could lead to false positive results. The decontamination process might be too mild and decontamination not successful. Therefore, it is important to understand the potential impact of cultivation conditions on surface hydrophobicity in order to achieve reproducible validation results.

It is therefore the aim of this study to examine the impact of cultivation pH on surface hydrophobicity and resistance of *B. subtilis* spores. Spores were cultivated in a bioreactor with controlled air supply and static cultivation pH levels. Sporulation was induced by exhaustion of the carbon source. The surface of the resulting spores was analyzed by water contact angle measurement. In a first approach the BI resistance was assessed in liquid H₂O₂ using a standard protocol also applied under practical conditions and circumstances to evaluate whether an effect can be determined, even when gaseous H₂O₂ is finally applied.

3.2.2 Materials and Methods

Microorganisms

The experiments were carried out with *Bacillus subtilis* (*B. subtilis*, DSM 4181) spores. A freeze dried culture of *B. subtilis* was obtained from the DSMZ (German collection of microorganisms and cell cultures, Braunschweig, DE). The microorganisms were at first revitalized following the manufacturer's instructions and cultivated on Nutrient Agar (for 1000 mL distilled water: 5,0 g peptone (Merck, Darmstadt, DE), 3,0 g beef extract (Gerbu, Heidelberg, DE), 15 g agar-agar, (Fisher Scientific, Schwerte, DE) with 20 mg of manganese sulfate (Merck, Darmstadt, DE) for 10 d at 37 °C. The spores were subsequently removed from the agar plates by pouring 10 mL of cold, sterile distilled water on the plates, suspending the spores with a spatula and collecting the suspension as starting material for the submerged production of spores in a stirred bioreactor.

Washing steps

To obtain only the spores the vegetative bacteria were separated by centrifugation at 4000g (10 min, 4°C) applying a fourfold washing by sterile, distilled water. The spores were heat activated with a temperature of 80 °C for 20 min and subsequently cooled down in ice water. Thereby all remaining vegetative cells were inactivated and simultaneously spores were activated to germinate faster under suitable conditions (Keynan et al. 1964). The targeted purity of the spores was more than 95 %, the absence of vegetative cells was checked with a light microscope (Axioskop, Carl Zeiss, Oberkochen, DE). Storage was in distilled water at 4 °C.

Preparation of inoculum for submersed cultivation

The inoculum was prepared using 100 mL of a sporulation medium (for 1000 mL of distilled water: 5,0 g peptone from casein (Gerbu, Heidelberg, DE), 3,0 g beef extract (Gerbu, Heidelberg, DE), 3,5 g potassium chloride (Merck, Darmstadt, DE), 250 mg magnesium sulphate (Roth, Karlsruhe, DE). After autoclaving 10 mL of 10 % glucose in water (Merck, Darmstadt, DE) and 1 mL of the following sterile filtrated micronutrients were added: 1 M calcium nitrate (Sigma Aldrich, Darmstadt, DE) 0,01 M manganese chloride (Merck, Darmstadt, DE), 1 mM iron sulphate (Fluka, Seelze, DE). The medium of pH 7,5 was filled in a 250 mL baffled flask (Duran, Wertheim/Main, DE) and 100 μ L of the original spore suspension ($5 \cdot 10^8$ cfu/mL) were added. Cultivation took place at 37 °C for 12 h before transferring the bacilli into the bioreactor. The procedure of inoculum preparation was the same for each fermentation.

Cultivation in the bioreactor

By inoculation of the preculture in 1500 mL of the same medium in a bioreactor (Biostat A plus, Sartorius AG, Göttingen, DE) cultivation was started at 37 °C and constant oxygen supply of 2 L/min filtered air. The pH was kept constant by automatic addition of 0,5 M NaOH (AppliChem, Darmstadt, DE) or 0,5 M HCl (Sigma Aldrich, Darmstadt, DE) with a control unit (Biostat A plus DCU, Sartorius AG, Göttingen, DE). The pH levels were varied between 7,00 and 9,00 in steps of pH 0,50. After 48 h the fermentation was stopped, microorganisms harvested and washed as described above. All spores were subsequently heat activated at 80 °C for 20 min. The viable amount of spores was examined by serial decimal dilutions in Ringer's solution (Merck, Darmstadt, DE). 100 μ L of the appropriate dilutions were plated on plate count agar (for 1000 mL: 5,0 g peptone from caseine (Gerbu, Heidelberg, DE), 2,5 g yeast extract (Sigma Aldrich, Darmstadt, DE), 1,0 g glucose (Merck, Darmstadt, DE), 15 g agar-agar (Fisher Scientific, Schwerte, DE)) and incubated at 30 ° for 24 h.

Contact angle measurement

The surface hydrophobicity was investigated by water contact angle measurement as described in Eschlbeck and Kulozik (2017). In short, filtration of cells was applied (approx. $4 \cdot 10^9$ cells per filter) on a cellulose acetate filter (pore size 0,22 μ m, Sartorius Stedim, Göttingen, DE), followed by adjusting the surface water content by storing the filters with spore layers on petri dishes consisting of 2 % agar-agar for 2 h to equilibrate the moisture content throughout the spore layer. The filter sheet was cut in stripes and fixed on glass slides by means of double-sided adhesive tape and dried for further 50 min on air to remove excess water and to reach a defined state in moisture content without crack formation of the spore layer. The measurement was carried out at 20 °C with a DSA 100 (Krüss, Hamburg, DE) and the software DSA 4 (Krüss, Hamburg, DE). A drop of water was deposited on the

filter surface and the cell hydrophobicity was determined by video analysis of the advancing contact angle.

Resistance against liquid H₂O₂

The test procedure is a modified version of the method described by Muranyi et al. (2006) and Deinhard et al. (2016). Spore suspensions (approx. 10⁸ cfu/mL) were mixed with 35 % H₂O₂ (Evonik, Essen, DE) at 25 °C at a ratio of 1:99. A magnetic stirrer (200 rpm) was applied for homogeneous spore distribution. Aliquots of 0,1 mL were taken after certain inactivation times and dilution series were generated. The contained H₂O₂ was immediately decomposed by preparing the first test tube with 9,8 mL of Ringer's solution and 0,1 mL of 10 % catalase (Catalase from *Micrococcus lysodeikticus*, Sigma Aldrich, Darmstadt, DE). Preparing serial decimal dilutions and plating every dilution on two independent plate count agar plates (for 1000 mL: 5,0 g peptone from caseine (Gerbu, Heidelberg, DE), 2,5 g yeast extract (Sigma Aldrich, Darmstadt, DE), 1,0 g glucose (Merck, Darmstadt, DE), 15 g agar-agar (Fisher Scientific, Schwerte, DE)) was followed by incubation for 48 h at 30 °C and detection of the number of survivors.

The aim was to compare the resistance of the spores, therefore a value for resistance is necessary. We chose 1st order reaction kinetic as model for the inactivation curve. With Eq. (3-3), the resulting decimal reduction time can be calculated for the resulting spores from every cultivation pH. The D-value is the time required to inactivate 90 % of spores at the given parameters and thus is a measure of resistance of spores against H₂O₂ that offers the possibility to compare the resistance properties. In this study, the D-value achieved at 25 °C with 35% H₂O₂ will be referred to as D_{H₂O₂}. Resistance test was done in triplicate for every spore suspension.

Calculation of the D_{H₂O₂} was done as shown in (3-3).

$$\log_{10}S(t) = -\frac{t}{D} \quad (3-3)$$

with S being the survival rate at a certain exposition time (t).

3.2.3 Results

Concentration of spores

Submerged bioreactor cultivation resulted in an amount of spores of 1.28·10⁷ - 3,40·10⁸ cfu per mL after heat activation as shown in Figure 3-7. The highest concentration of spores was obtained at a cultivation pH of 8.00. However, all cultivation pH values yielded amounts of spores only differing slightly more than one log. Cultivation at a static pH of 7.00 and 9.00 can thus be considered successful even with a lower spore yield compared to the other

cultivation pH values. The resulting amount of spores were sufficient for water contact angle measurement in triplicate. Higher cultivation pH levels could not be investigated as cultivation at pH 9.50 and pH 6.50 did not result in a sufficient amount of spores (data not shown).

Surface hydrophobicity of the spores

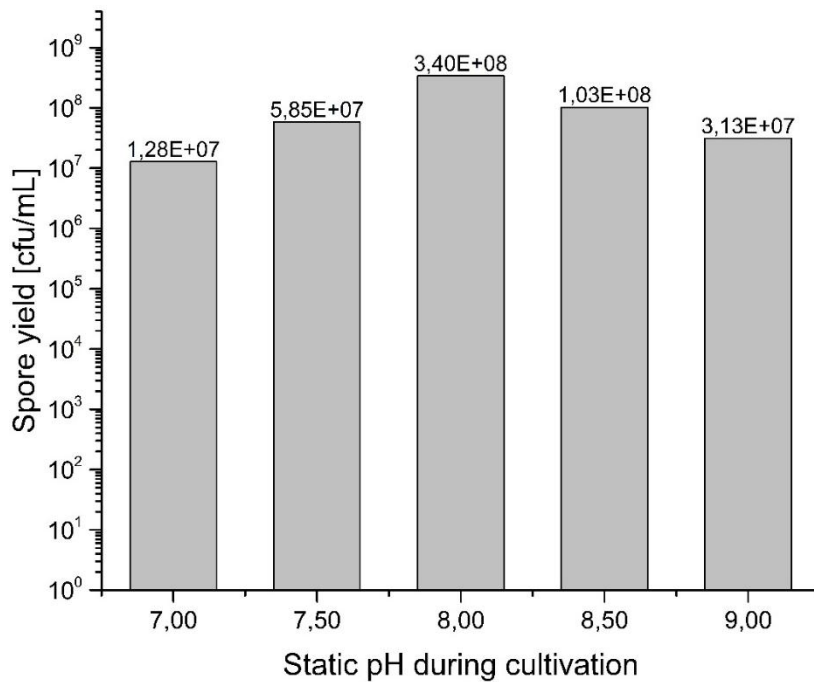


Figure 3-7: Spore yield shown as colony forming units (cfu) per mL

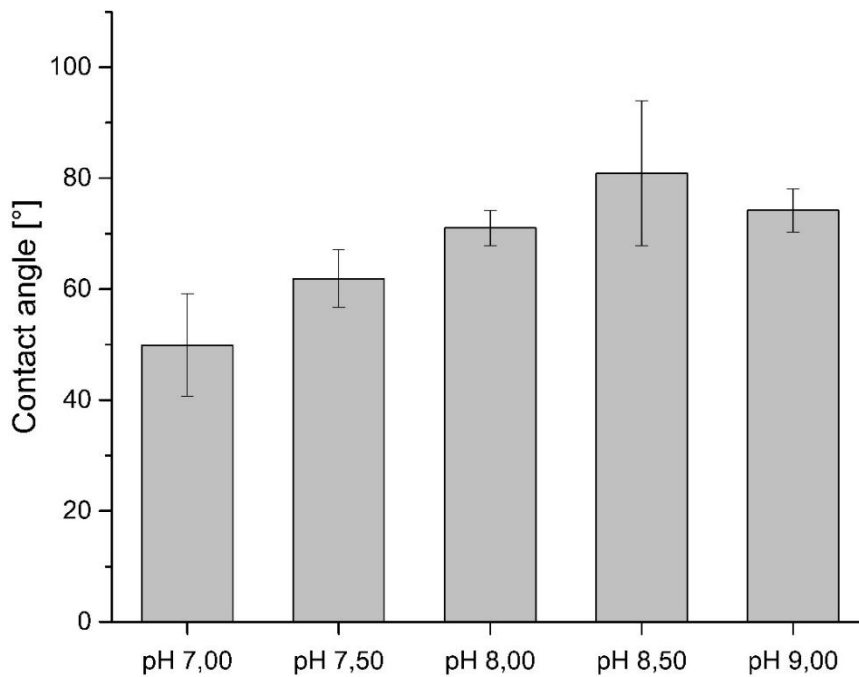


Figure 3-6: Resulting water contact angles of *B. subtilis* spores cultivated at different pH-values

Figure 3-6 depicts the resulting water contact angles (CA). A trend of increasing contact angles can be seen beginning at pH 7.00 (CA 50 °) to a maximum of 80 ° at pH 8.50. This means that spores become more hydrophobic with increasing pH-value. At a pH of 9.00 a tendency to lower contact angles was found (Figure 3-6).

Resistance towards liquid H₂O₂

A higher decimal reduction time means higher resistance towards H₂O₂. The resistance towards liquid, 35 % H₂O₂ is shown as logarithmic survival rate over treatment time in **Fehler! Verweisquelle konnte nicht gefunden werden..** During the first 15 s of inactivation time, the resulting number of survivors decreases rapidly for spores cultivated at

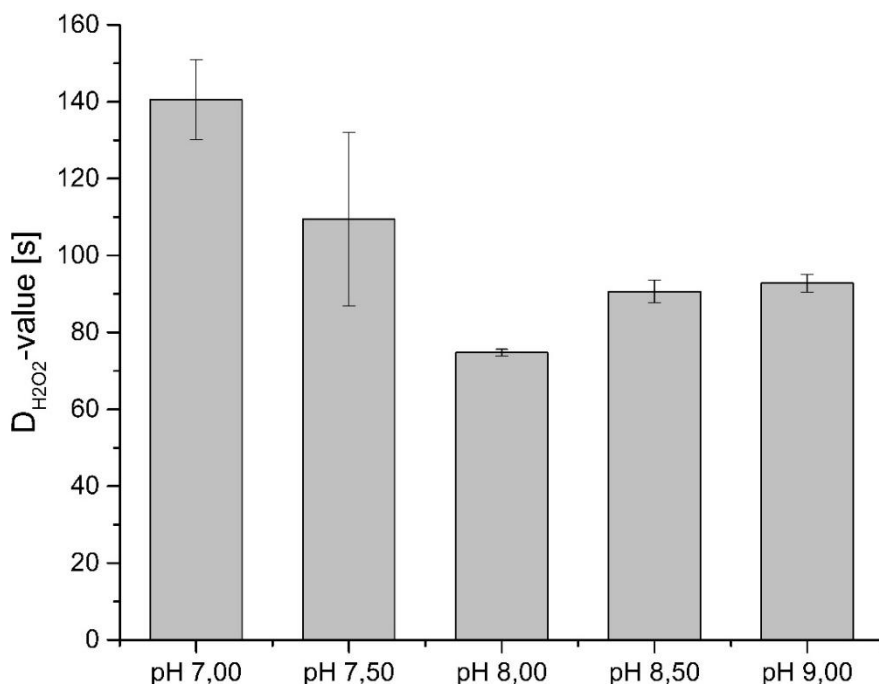


Figure 3-8: Resulting $D_{H_2O_2}$ -values of the different static pH cultivations, $D_{H_2O_2}$ -values in seconds

pH 7.50, 8.50 and 9.00. This might be due to an equilibration of cultivation conditions meaning that the least resistant spores of the spore population are quickly inactivated. After the first 15 s log-linear characteristics are present for all spore suspensions.

The correlation coefficient (R^2) was calculated for all inactivation curves. For spores cultivated at pH 7.50 R^2 was 0.89, for all other inactivation curves R^2 was higher than 0.94. $D_{H_2O_2}$ -values were calculated for each cultivation pH. The $D_{H_2O_2}$ -values are depicted in Figure 3-8.

Resistance towards liquid H₂O₂ clearly differs depending on cultivation pH. Spores cultivated at pH 8.00 are least resistant whereat a cultivation pH of 7.00 provides spores with the highest resistance.

Correlation of surface hydrophobicity and resistance

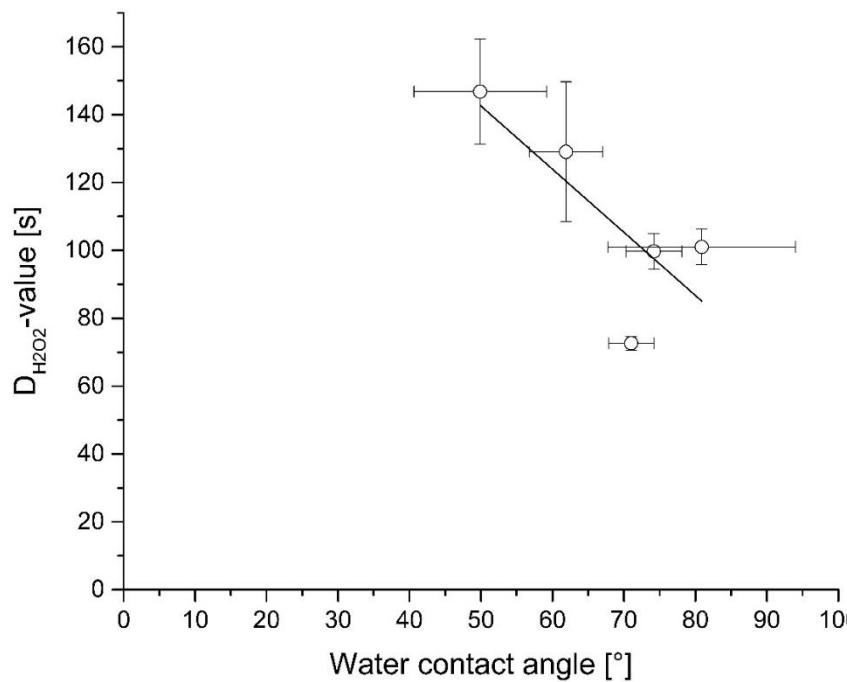


Figure 3-9: Resistance against liquid H₂O₂, shown as D_{H₂O₂}-value in seconds, as a function of the water contact angle with linear regression

To investigate a possible correlation of surface hydrophobicity and resistance as shown in Figure 3-9, the resistance against liquid H₂O₂ expressed as D_{H₂O₂}-value is plotted as a function of the water contact angle. A linear regression was carried out and the Pearson correlation coefficient was calculated. The correlation coefficient is -0.78 which indicates a negative linear relationship meaning that rising D_{H₂O₂}-values result in lower water contact angles and vice versa.

3.2.4 Discussion

The obtained amount of spores are in a range of $1,28 \cdot 10^7$ to $3,40 \cdot 10^8$ cfu per mL and thus slightly dependent on cultivation pH. For the applied cultivation conditions including aeration, media composition and cultivation time, a pH level of 8,00 resulted in a maximum amount of spores. pH-values from 7,00 to 9,00 resulted in similarly high spore yields and provided enough spores for all further experiments. Further optimization of the cultivation conditions will presumably result in higher spore yields, however this was not the aim of the study.

The water contact angles of *B. subtilis* spores differ significantly depending on cultivation pH. According to the classification of Rijnaarts et al. (1993), all of the resulting *B. subtilis* spores are hydrophobic, but the degree of hydrophobicity varies. *B. subtilis* spores are surrounded by the outer spore coat consisting of four distinct layers whereat the crust is the outermost one (Imamura et al. 2010; McKenney et al. 2010). Therefore, the crust is the interface between spore and environment and responsible for surface characteristics such

as hydrophobicity and adhesion. Some of the proteins localized on the crust surface, e.g. CgeA, CotY and CotZ, have already been identified (Imamura et al. 2011). Chen et al. (2010) applied infrared spectroscopy to identify various functional groups apart from proteins and considered aldehyde and carboxylic acid groups to be responsible for spore adhesion properties. McKenney et al. (2012) state that diversity of the coat structure including the crust is driven by adaptation to different niches in nature to survive. Therefore, we suppose that spores tend to adapt to different cultivation pH and that presumably the composition of the crust changes depending on cultivation conditions. This assumption has to be clarified in further studies, however, this study has shown that surface hydrophobicity can be modified by means of various static pH levels.

Our results show that not only surface characteristics but also resistance of *B. subtilis* spores against liquid, 35% H₂O₂ varies with the cultivation pH. The D_{H₂O₂}-values of 75 s to 141 s are similar to the D_{H₂O₂}-value of 168 s obtained by Pruß et al. (2012) with a similar method. As Pruß et al. (2012) applied a lower concentration of liquid H₂O₂, their time to inactivate 90 % of the spore population is slightly longer but in a similar dimension.

Hydrogen peroxide inactivates spores as it damages some component that is needed for the core to expand due to hydration. One reported explanation for variable resistance properties is a change in peptidoglycan composition so that the spore does not expand sufficiently. Another possibility is a quantitative change or altered sensitivity of one or more proteins either needed for remodeling of the expanding cell wall or essential for core expansion itself (Melly et al. 2002a). However, it is not the purpose of this study to clarify the molecular mechanisms, but to approach an aspect of BI application.

A Pearson correlation factor of -0,78 indicates a strong negative correlation between water contact angle and resistance towards liquid hydrogen peroxide. However, surface hydrophobicity measured by contact angle measurement depends on the molecular structure of the outermost layer, the crust, whereat resistance towards liquid hydrogen peroxide presumably depends on some components inside the spore. Therefore, this correlation factor seems quite high and a possible correlation between cell surface hydrophobicity and resistance has to be verified in further experiments.

Surface hydrophobicity is suspected to have an impact on the decontamination of surfaces such as packaging material when gaseous, condensing hydrogen peroxide is applied. Surface hydrophobicity as well as resistance towards liquid H₂O₂ depend on cultivation pH. At the moment, no standardized protocol for the cultivation of BI exists. Our results show clearly that a standardization is very important as cultivation pH does not only influence spore resistance but also spore characteristics that are not screened on a regular base such as surface hydrophobicity. The development of a standardized production and application of BI should result in more consistent inactivation test results.

3.3 Influence of spore and carrier material surface hydrophobicity on decontamination efficacy with condensing hydrogen peroxide vapor

Summary and contribution of the doctoral candidate

For decontamination in aseptic packaging technology, gaseous H₂O₂ can be purposefully applied with or without the formation of condensate. Spores of *B. subtilis* and *B. atrophaeus* are often applied as BI to validate the inactivation success. The process of condensate formation is induced if the temperature of the carrier material is below the dew point temperature of the H₂O₂ vapor. Due to an energy barrier that has to be crossed to perform phase transition, condensation nuclei lowering this energy barrier are needed to start condensate formation. Bacterial spores can act as condensation nuclei. However, the influence of surface hydrophobicity of the spores is not known, neither is the impact of the surface wettability of the carrier material on the resulting inactivation success.

The aim of this study was to investigate the influence of surface hydrophobicity of carrier material and bacterial spores of *Bacillus subtilis* SA 22 and *Bacillus atrophaeus* (DSM 675) on the inactivation with condensing hydrogen peroxide gas.

Bacterial spore surface hydrophobicity and carrier material wettability was determined by means of contact angle measurement resulting in water contact angles of 90 ° and 42 ° for *B. subtilis* and *B. atrophaeus* respectively. The resistance of the microorganisms against liquid H₂O₂ was investigated at room temperature whereat spores of *Bacillus subtilis* showed a D_{H₂O₂}-value of 101 s and *Bacillus atrophaeus* spores 906 s. The spores were deposited on carrier materials of different wettability with water contact angles of 115 ° to 30 °, either just one spore species or an equal mixture of both spore suspensions. The exposure of biological indicators with 5200 ppm of gaseous H₂O₂ at 70 °C treatment temperature for defined times up to 10 seconds led to inactivation kinetics. Surfaces with high hydrophobicity showed faster spore inactivation than surfaces with lower hydrophobicity. Regarding the mixed biological indicator, better survival of hydrophobic spores was expected. However, this effect can only be seen as a slight trend and is not significant after 10 s.

The doctoral candidate supervised and planned the cultivation, purification and characterization of both spore species regarding contact angle measurement and resistance against liquid H₂O₂. The inactivation of the spores by means of gaseous H₂O₂ was coordinated by the doctoral candidate. The analysis of the resulting data, interpretation of datasets and plotting of data were also conducted by the doctoral candidate. The manuscript was essentially written by the doctoral candidate.

Adapted original manuscript¹

Influence of spore and carrier material surface hydrophobicity on decontamination efficacy with condensing hydrogen peroxide vapor²

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Abstract:

Aims: To investigate the influence of surface hydrophobicity of carrier material and bacterial spores of *Bacillus subtilis* SA 22 and *Bacillus atrophaeus* (DSM 675) on spore inactivation with condensing hydrogen peroxide gas.

Methods and Results: Surface hydrophobicity of bacterial spores and carrier material was determined by means of contact angle measurement. Spores of *Bacillus subtilis* showed water contact angles of 90 °, spores of *Bacillus atrophaeus* showed water contact angles of 42 °. Above that, a resistance test against liquid H₂O₂ at room temperature was conducted with resulting D_{H₂O₂}-values of 101 s (*Bacillus subtilis*) and 906 s (*Bacillus atrophaeus*). The spores were deposited on carrier materials of different wettability (water contact angles of 115 ° to 30 °). The spores were applied either individually or as an 1:1 mixture of both spore species. Exposure of biological indicators with 5200 ppm of gaseous H₂O₂ at 70 °C treatment temperature for defined times up to 10 s led to inactivation kinetics. Surfaces with high hydrophobicity showed faster spore inactivation than surfaces with lower hydrophobicity for single spore species. Regarding the mixed biological indicator, better survival of hydrophobic spores was expected. However, this effect can only be seen as a slight trend and is not significant after 10 s.

Conclusions: Surface hydrophobicity of carrier materials does influence the decontamination with gaseous, condensing hydrogen peroxide. However, surface hydrophobicity of spores in a mixed population does only have a small influence on inactivation results.

Significance and Impact of the Study: The intensity of spore inactivation depends more on other factors than on the wettability of the bacterial spores. However, hydrophobic surfaces lead to faster inactivation effects and should thus be preferred for aseptic packaging technology.

Keywords: *Bacillus* spores, condensation, hydrogen peroxide vapor, inactivation, surface hydrophobicity

3.3.1 Introduction

Beverages like milk and juices with extended shelf life are often filled using aseptic packaging technology. Product and packaging materials are decontaminated separately prior to the filling process. Cardboard packaging materials with synthetic surfaces are often sterilized by a combination of hydrogen peroxide (H₂O₂) and mild thermal inactivation (Toledo 1988). For H₂O₂ based inactivation, spores of *Bacillus subtilis* (*B. subtilis*) and *Bacillus atrophaeus* (*B. atrophaeus*) are used as biological indicators (BI) to validate the inactivation effect (VDMA, 2006; Sella et al. 2014). To achieve short inactivation times allowing fast filling processes, vaporized H₂O₂ at high concentrations is applied. Depending on the ratio of H₂O₂ and water in the gas as well as on the surface temperature of the carrier material, H₂O₂ can be applied with or without the formation of condensate (Toledo et al. 1973; Bockelmann and Bockelmann 1998; Pruß et al. 2012). However, the influence of surface hydrophobicity of spores and carrier material on the inactivation with condensing H₂O₂ is widely unknown.

For a long time, liquid H₂O₂ was applied for the decontamination of packaging materials. It has been found out that the gas form is very effective and nowadays completely vaporized H₂O₂ is an often used method for surface decontamination. The resulting mixtures of vaporized H₂O₂, water vapor and hot air form condensates if the temperature is below dew point temperature or the partial pressure is above saturation conditions. There are controversial opinions about the effect and necessity of condensate formation. Engelhard (2006), Hultman et al. (2007) and Chung et al. (2008) state that condensate formation should be avoided to prevent material corrosion, to eliminate the need for residue removal and to achieve more consistent and less variable inactivation effects. However, according to Unger-Bimczok et al. (2008) and Pruß et al. (2012), the formation of condensate leads to increased inactivation effects due to high H₂O₂ concentration in the condensate and should therefore be actively induced. Concentration and dew point temperature can be calculated as shown by Parks and Watling (2004). However, these studies differ concerning the concentration of H₂O₂, relative humidity, surface temperature and microorganism strain. Both H₂O₂ gas applications, with and without condensate formation, are used in industry for decontamination of packaging material, clean rooms and isolators (Fisher and Caputo 2004; McDonnell 2002).

Condensation is induced if the prevailing partial pressure exceeds the saturation pressure or the respective surface temperature is lower than the saturation temperature. A metastable state occurs during which phase transition to the liquid phase takes place, as it is the state of minimal energy. However, a minimum energy level has to be exceeded as a new barrier at the interfaces of condensate-surface and condensate-gas is (has to be) formed. Condensation can be classified as homogeneous or heterogeneous condensation depending on the conditions. Heterogeneous condensation occurs at surfaces. The energy level for the gas to form condensate is influenced by the wetting behavior of the surface. To assess the wettability, water contact angle measurement is often applied. The better the wettability and the lower the water contact angle, the lower is the energy level that has to be exceeded and the more readily does condensate formation take place (Beysens et al. 2006; Marcos-Martin, et al. 1996). An uneven surface or chemical gradient leads to higher probability of condensate formation. Marcos-Martin, et al. (1996) argue that microorganisms on the surface contribute to roughness and therefore act as condensation nuclei.

Hall et al. (2007) state that hydrophobic microorganisms are less likely to induce condensation. They should therefore be less affected by high H₂O₂ concentration in the condensate and should thus be better protected against the inactivation effect. Pruß et al. (2012) investigated the influence of the packaging surface temperature, which directly influences condensate formation on different bacterial spores. Inactivation of spores of *B. subtilis* DSM 347 was enhanced compared to spores of *B. subtilis* DSM 4181 and *B. atrophaeus* DSM 625, whereas inactivation with H₂O₂ gas without condensate formation was similar for all spores. They postulated that this finding was related to the low surface hydrophobicity of *B. subtilis* DSM 347 spores. However, the authors applied spores of different species with relatively low water contact angles and thus surface hydrophobicity (10.4 ° to 20.9 °). This might have introduced other influencing factors that just surface hydrophobicity.

Sigwarth and Stärk (2003) and Unger et al. (2007) investigated the inactivation of *Geobacillus stearothermophilus* spores on several carrier materials and showed that carrier materials have a major impact on the resulting D_{H₂O₂}-values. Surface hydrophobicity of the different materials examined by water contact angle measurement was not investigated.

Therefore, the first hypothesis of this study is that the surface hydrophobicity of carrier material, measured as water contact angle, does influence the condensate formation and, thereby, the inactivation of bacterial spores. Condensate should preferably form around the spores if they are more hydrophilic than the carrier material and vice versa. It is thought that high surface hydrophobicity of the carrier material will enhance the inactivation effect as condensate at first forms on the microorganisms' surfaces. If the packaging material is more hydrophilic than the microorganisms, condensate will first form on the carrier material,

spores will not be preferably covered by the condensate. Therefore, inactivation should be less effective.

In reality, mixed microorganism populations with different surface hydrophobicity are located on surfaces. Assuming surface hydrophobicity to play a significant role, spores with high surface hydrophobicity might be protected from condensate formation and thus inactivation will be less pronounced. Condensate will form instead on the more hydrophilic spores and their inactivation might be enhanced. Therefore, the second hypothesis is that for a mixed microorganism population, hydrophobic microorganisms are protected from condensate formation as the condensate will preferably form on the more hydrophilic microorganisms and inactivation of the more hydrophobic microorganisms will thus be less intense.

To our knowledge, there is no systematic study considering the influence of surface hydrophobicity of the spores as mixed population in combination with carrier materials of different wettability.

For the investigation we chose two microorganisms differing in their surface hydrophobicity and appearance on agar plates for the experiments. They were deposited on carrier materials with different water contact angles. They were then inactivated with condensing hydrogen peroxide under conditions typical for integrated industrial filling operations with very high filling speed. To investigate the influence of a mixed population, the spores were applied as single species and as a spore mixture consisting of an equal amount of both spore species. The inactivation kinetics were compared.

3.3.2 Material and Methods

Bacterial spores

The bacterial spore formers *B. subtilis* SA 22 (DSM 4181) and *B. atrophaeus* (DSM 675) were obtained from the DSMZ (German collection of microorganism and cell cultures, Braunschweig, DE) as freeze-dried cultures. After rehydration according to the manufacturer's instructions, storage was carried out on Nutrient Agar (for 1000 mL distilled water: 5.0 g peptone (Merck, Darmstadt, DE), 3.0 g beef extract (Gerbu, Heidelberg, DE), 15 g agar-agar, (Fisher Scientific, Schwerte, DE) with 20 mg of manganese sulfate (Merck, Darmstadt, DE)).

Cultivation: Both bacilli were cultivated with a sporulation media (for 1000 mL of distilled water: 5.0 g peptone from casein (Gerbu, Heidelberg, DE), 3.0 g beef extract (Gerbu, Heidelberg, DE), 3.5 g potassium chloride (Merck, Darmstadt, DE), 250 mg magnesium sulfate (Roth, Karlsruhe, DE), after autoclaving 10 mL of 10 % glucose in water (Merck, Darmstadt, DE) and 1 mL of the following micronutrients were added: 1 M calcium nitrate (Sigma Aldrich, Darmstadt, DE) 0.01 M manganese chloride (Merck, Darmstadt, DE), 1 mM iron sulphate (Fluka, Seelze, DE)). *B. subtilis* was cultivated in a bioreactor as described in

Eschlbeck et al. (2017), *B. atrophaeus* was cultivated on agar plates. To obtain the sporulation media as agar plates, agar-agar (15 g/L, Fisher Scientific, Schwerte, DE) was added to the composition.

Sporulation conditions: *B. subtilis* was cultivated at a constant pH value of 8.5 and a temperature of 37 °C with constant oxygen supply (2 L/min sterile filtered air). *B. atrophaeus* spores were incubated at 37 °C for 10 days. The spores were subsequently removed from the agar plates by pouring 10 mL of sterile distilled water on the plates, suspending the spores with a spatula and collecting the suspension.

Purification: The process of purification was the same for both spore species. Heat activation of the spores was carried out at 80 °C for 20 min to inactivate all vegetative bacilli. Each spore suspension was washed at least 4 times with sterile, distilled water and centrifuged at 4000 g for 10 min at 4 °C. Inactivated vegetative cells or their cell debris were removed in the centrifugation step together with the supernatant. The spores were subsequently cooled down in ice water. The purity of the spore suspension and the absence of vegetative cells was checked with a light microscope (Axioskop, Carl Zeiss, Oberkochen, DE) and washing steps were repeated until at least 95 % pure spores were in the suspension. Spores were stored in distilled water at 4 °C.

Contact angle measurement

Water contact angle measurement (DSA 100, Krüss, Hamburg, DE) at 20 °C was applied to evaluate spore surface hydrophobicity. The method was carried out as described by Eschlbeck and Kulozik (2017). In short, an aliquot of spore suspension sufficient for several layers of spores was filtered on a cellulose acetate filter (pore size: 0.22 µm, Sartorius Stedim, Göttingen, DE). Subsequently, an equilibration time of 2 h on 2% agar-agar (Fisher Scientific, Schwerte, DE) was applied, afterwards filters were dried for 50 min on air. For the measurement, a drop of 8 µL Milli-Q water was set on the filter surface with the sessile drop method during the plateau time. For each drop, a video of the first 6 s was taken. The pictures from the resulting video were analyzed with DSA 4 (Krüss, Hamburg, DE) to evaluate water contact angles. Measurements between 2 and 4 s after the onset of drop deposition showed consistent contact angles. For each spore suspension, three independent filters were prepared. On each filter, a minimum of 5 drops was deposited and evaluated.

Resistance of spores against liquid H₂O₂

Resistance of the spores towards liquid, 35 % (11.63 mol/L) hydrogen peroxide was examined as described in Eschlbeck et al. (2017). The whole experiment was conducted in a sterile glass beaker containing H₂O₂ that was placed in a water bath of controlled temperature. An equilibration time of 5 min was applied to allow for the 9.9 mL of H₂O₂-

solution to adjust to the respective temperature. Spores were mixed with the hydrogen peroxide (Oxteril 350 Spray, Evonik Industries, Essen, DE) at controlled temperatures of 25, 35, 45 and 55 °C at a ratio of 1 part to 99 parts resulting in 0.1 mL of spore suspension and 9.9 mL of H₂O₂. Homogeneous spore distribution was achieved by magnetic stirring (200 rpm, Arex Digital Heating Magnetic Stirrer, VELP Scientifica, Usmate, Italy). After certain inactivation times, aliquots of 0.1 mL were taken and dilution series generated. The contained H₂O₂ was immediately neutralized by preparing the first test tube with 9.8 mL of Ringer's solution and 0.1 mL of a catalase (Catalase from *Micrococcus lysodeikticus*, Sigma Aldrich, Darmstadt, DE) in water mixture consisting of 1 part catalase and 9 parts distilled sterile water. Survivors were detected with the plating method on plate count agar (for 1000 mL: 5.0 g peptone from caseine (Gerbu, Heidelberg, DE), 2.5 g yeast extract (Sigma Aldrich, Darmstadt, DE), 1.0 g glucose (Merck, Darmstadt, DE), 15 g agar-agar (Fisher Scientific, Schwerte, DE)) and incubated at 30 °C for 48 h. Except for the first 15 s, the inactivation was log-linear. Thus, it can be concluded that the resulting D-values apply for most spores of the population. The resulting D_{H₂O₂}-values provide an estimation of the resistance towards liquid H₂O₂ at certain temperatures.

Calculation of the D-value was done as shown in (3-4).

$$\log_{10}S(t) = -\frac{t}{D} \quad (3-4)$$

S is the degree of survival at a certain time *t*. For each spore suspension and temperature, the resistance test was carried out in triplicate.

To describe dependence of the D_{H₂O₂}-value of the temperature, z-values were calculated as depicted in (3-5). The z-value is the required temperature increase to reduce the D-value by 90 %.

$$z_{H_2O_2} = \frac{T_1 - T_2}{\log D_2 - \log D_1} \quad (3-5)$$

T is the treatment temperature and D is the resulting D_{H₂O₂}-value at a certain treatment temperature.

Carrier materials

The surface characteristics of different carrier materials (CM) were assessed by contact angle measurement as described above for the bacterial spores. For each CM, at least 5 drops of Milli-Q water were deposited at different spots and evaluated. Based on those results, different CM were chosen to investigate the influence of hydrophobic and hydrophilic surfaces on spore inactivation kinetics. Cardboard packaging material pieces with a surface consisting of standard LDPE (low density polyethylene, provided by Tetra Pak, Modena,

Italy) and a size of 4 by 5.5 cm were applied as basic material. To ensure similar heating behavior, thin films of the chosen materials were mounted on the basic LDPE cardboard material by means of double-sided adhesive tape. Sterilization of the cardboard packaging material was carried out by means of gamma radiation (25 kGy, Synergy Health, Allershausen, DE). Sterilization of the films mounted on the cardboard packaging material was done by means of gaseous hydrogen peroxide (9000 ppm, 20 s, 70 °C treatment temperature) with the hydrogen peroxide decontamination device.

Preparation of Biological Indicators

An aliquot of 300 µL of spore suspension was mixed with 700 µL ethanol (Merck, Darmstadt, DE). 10 µL of the resulting mixture (approximately 10^8 cfu/mL) were spread on carrier materials by means of Drigalsky spatula as described by Pruss *et al.* (2012). The resulting amount of spores on each carrier material was 10^6 . For the experiments with mixed spore populations, 150 µL of each spore suspension were applied for the ethanol mixture. Even distribution of spores was checked by electron microscopy, data not shown. Prior to measurements, drying of the BI was carried out for at least 1 h under sterile conditions.

Hydrogen peroxide treatment

Hydrogen peroxide decontamination device: Liquid, 35 % (11.63 mol/L) hydrogen peroxide (Evonik Industries, Essen, DE) was completely evaporated (Controlled Evaporator Mixer, Bronkhorst NL), the vapor tempered to 70 °C and directed into the treatment chamber. The temperature of the vapor was measured continuously with two temperature sensors (Angled Thermocouple, pentronic, SWE) that were integrated into the treatment chamber. One of the sensors is coated with catalytic material so that hydrogen peroxide dissolves in an exothermic reaction as described by Pruss (2013). The resulting temperature difference goes up with increasing H₂O₂ gas concentration. This catalytic sensor system was applied to monitor the active hydrogen peroxide concentration in the chamber. For all experiments, the H₂O₂ concentration was set to 5200 ppm and continuously monitored for the whole experimental time (LabVIEW, National Instruments, USA). The H₂O₂ concentration remained constant except for partially occurring peaks of high H₂O₂ concentration probably resulting from small oxygen bubbles in the H₂O₂ transport tubes. Experiments showing such a deviation were discarded.

Inactivation of BI: By opening the treatment chamber for less than one second, a perfectly fitting specimen holder with the prepared BI was inserted into the treatment chamber for the respective treatment time. Directly after the treatment, the BI was removed from the treatment chamber, the treatment chamber was closed again and the BI was completely covered with 10 mL of a tween-catalase solution consisting of 9.9 mL of sterile, 0.1 % tween 80 (Gerbu Biotechnik GmbH, Heidelberg, DE) and 0.1 mL of catalase (Catalase from

Micrococcus lysodeikticus, Sigma Aldrich, Darmstadt, DE). Remaining active hydrogen peroxide in the condensate was immediately dissolved by catalase to stop the inactivation effect. Spores were detached from the carrier material by magnetic stirring (200 rpm, Arex Digital Heating Magnetic Stirrer, VELP Scientifica, Usmate, ITA) in a solution consisting of 9.9 mL water with 0.1% Tween 80 (Sigma Aldrich, Darmstadt, DE) and 0.1 mL of a catalase mixture consisting of 10% catalase (Catalase from *Micrococcus lysodeikticus*, Sigma Aldrich, Darmstadt, DE) with water. Dilution series were generated and survivors detected with the plating method on plate count agar (as described above). Incubation was carried out at 30 °C for 48 h. Spores of *B. subtilis* form white, opal colonies whereas *B. atrophaeus* colonies show smaller, glossy yellow colonies. Therefore, for the mixed spore population experiments, surviving spores can be distinguished and individual inactivation kinetics can be generated for *B. subtilis* and *B. atrophaeus* respectively.

To obtain a reference value of 0 s treatment time, BI were prepared and detached as described above however without being put into the treatment chamber which means without any contact with gaseous H₂O₂. For each carrier material, experiments were conducted with *B. subtilis* spores, *B. atrophaeus* spores and the mixed spore population. For each of those combinations, treatment times of 0, 2, 4, 6, 8 and 10 s (as typical for industrial inactivation procedures) were set and all experiments were carried out in triplicate as shown in Table 3-2. The same experiments were carried out for all chosen carrier materials.

Table 3-2: Test matrix for carrier material 1. Triplicate means that the experiments were carried out three times.

Carrier material 1	0 s	2 s	4 s	6 s	8 s	10 s
<i>B. subtilis</i> spores	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate
<i>B. atrophaeus</i> spores	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate
Mixed spore population	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate	Triplicate

Formation of condensate

Condensate formation is a temperature dependent process. If the surface temperature is lower than the dew point temperature, condensate formation takes place. For a H₂O₂ gas concentration of 5200 ppm, the dew point temperature is 43.6 °C. The dew point of H₂O₂ in the condensate was calculated according to Parks and Watling (2004). To ensure condensate formation, the initial surface temperature of the carrier materials was always 26 °C.

Surface temperature of carrier materials during treatment was determined with an infrared thermometer (FLIR i3, FLIR Systems GmbH, Frankfurt am Main, DE). However, during 10 s of treatment time the surface temperature of the CM did not exceed the dew point temperature.

Statistical methods

All experiments were carried out in triplicate and the standard deviation was calculated. To assess if results differ significantly, a t-test for independent samples with a level of significance (α) of 0,05 was applied.

3.3.3 Results

Surface hydrophobicity

To investigate the influence of surface hydrophobicity on the inactivation with gaseous, condensing H₂O₂, surface hydrophobicity of spores and carrier materials was determined by means of water contact angle measurement.

B. subtilis spores showed a water contact angle of 90 °, whereas *B. atrophaeus* spores were more hydrophilic with a water contact angle of 42 °. The aim was to obtain a broad range of wettability to test the hypothesis that the difference in hydrophobicity between spores and surface influences the inactivation results. Several prototype films were examined by water contact angle measurement. Three prototype films with different water contact angles and thus different surface hydrophobicity were chosen as depicted in Table 3-3. A cardboard packaging material with LDPE-surface was chosen as basic carrier material.

Table 3-3: Abbreviations and water contact angles of chosen carrier materials

Abbreviation	Materials	Water contact angle
CM 1	LDPE cardboard packaging material (Tetra Pak, Modena, Italy)	115 °
CM 2	PET film, high roughness (23 µm thick, Huthamaki, Ronsberg, DE)	101 °
CM 3	PET film, smooth surface (23 µm thick, Huthamaki, Ronsberg, DE)	81 °
CM 4	PET film covered with silicium oxide (23 µm thick, Huthamaki, Ronsberg, DE)	30 °

Surface hydrophobicity of the chosen materials varied between 115 ° and 30° water contact angle. 30 ° stands for a relatively low water contact angle and means good wettability. 115 ° results from a rather round drop, which means low wettability. The surface of the *B. subtilis* spores is more hydrophobic than the surface of CM 3 and CM 4, but more hydrophilic than the surface of CM 1 and CM 2. The spores of *B. atrophaeus* (42 °) are slightly more hydrophobic than the surface of CM 4 (30 °). Water contact angles of the CM and the

bacterial spores are depicted in Figure 3-10. The y-axis on both sides of the figure show the water contact angles of CM and bacterial spores. They represent the same results, however for easier understanding the right side will be depicted next to each figure showing inactivation results in an adapted version to visualize the difference of the contact angles of the utilized spores and carrier materials.

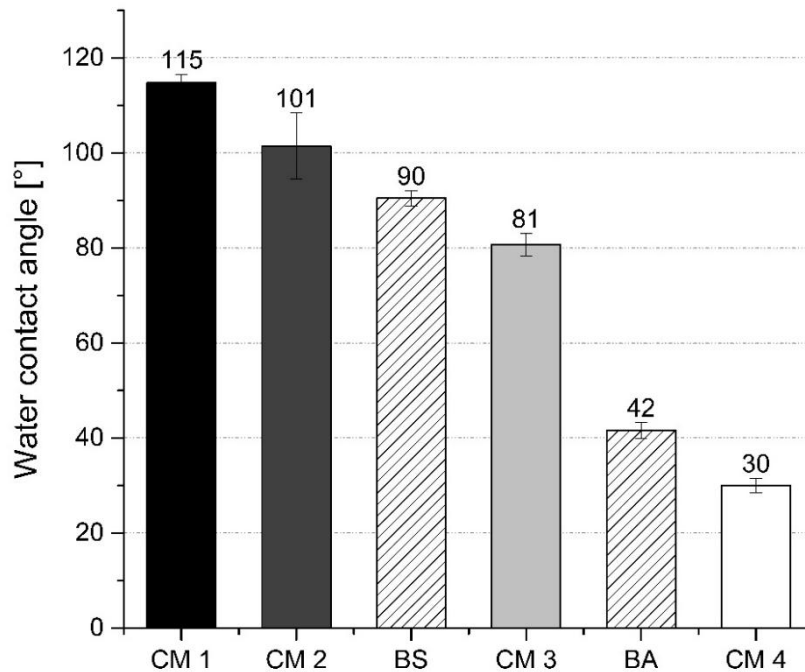


Figure 3-10: Water contact angles of carrier materials (dark grey) and bacterial spores (bright grey).
 CM 1: Polyethylene, CM 2: Rough PET, CM 3: Smooth PET, CM 4: Silicium oxide surface, BS: *B. subtilis* spores, BA: *B. atrophaeus* spores

Resistance of the spores

For both spore species the treatment with liquid, 35% hydrogen peroxide at 25 °C resulted in log-linear inactivation kinetics, depicted in. Inactivation of *B. subtilis* spores is much faster than inactivation of *B. atrophaeus* spores. The latter ones are clearly more resistant to treatment with liquid H₂O₂ at 25 °C. D_{H₂O₂}-values are 101 s for *B. subtilis* spores and 906 s for *B. atrophaeus* spores. All other treatment temperatures also resulted in log-linear inactivation kinetics, as shown in Figure 3-12.

Figure 3-11 shows the sensitivity towards temperature of both spore species. The higher the logarithmic D_{H₂O₂}-value, the higher is the spore resistance towards liquid H₂O₂. Spores of *B. atrophaeus* are very resistant at 25 °C, whereas *B. subtilis* spores are less resistant. For the higher temperatures, the resistance of *B. atrophaeus* spores decreases. The resulting z-values are 37.5 °C for *B. subtilis* spores and 15.5 °C for *B. atrophaeus* spores, which characterizes the latter ones as more sensitive against H₂O₂ at higher temperatures.

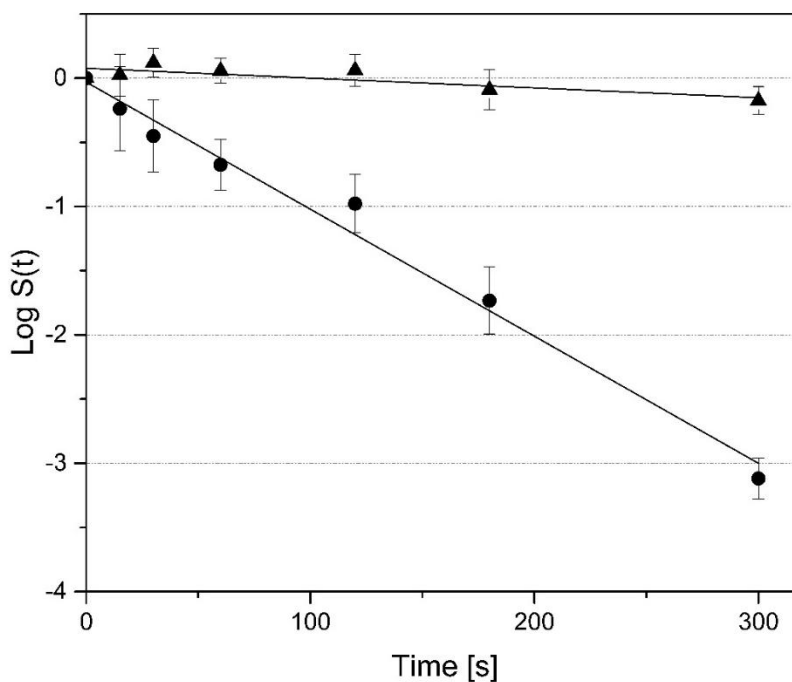


Figure 3-12: Log-linear inactivation of *B. subtilis* spores (●) and *B. atrophaeus* spores (▲) with liquid H_2O_2 at 25 °C

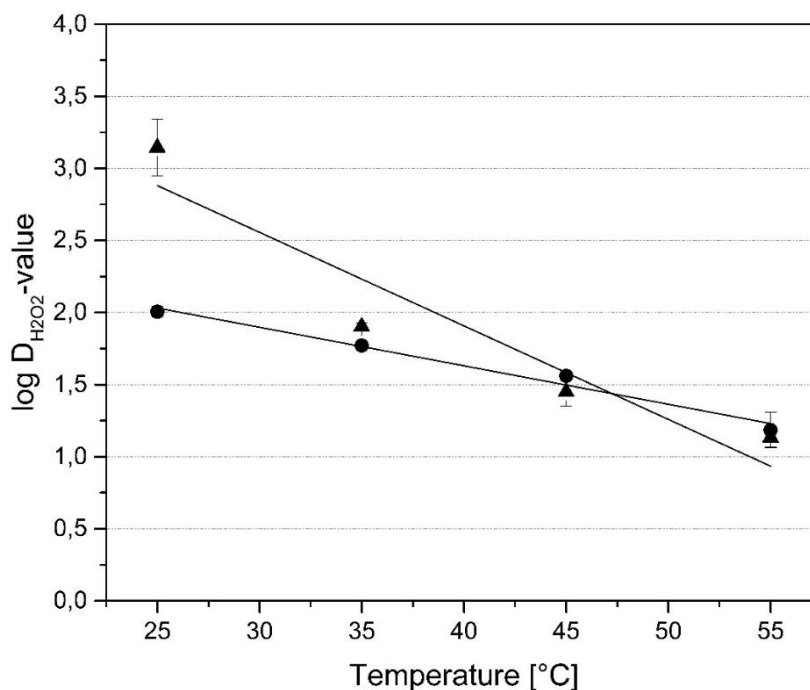


Figure 3-11: Logarithmic $D_{H_2O_2}$ -values of *B. subtilis* spores (●) and *B. atrophaeus* spores (▲) as function of treatment temperature

Influence of the surface hydrophobicity of the carrier materials

Inactivation results show that there is a distinct influence of the carrier material on the inactivation of *B. subtilis* spores as shown in Figure 3-14. Inactivation on the most hydrophobic CM 1 is almost 2 log after 10 s, whereas inactivation of *B. subtilis* spores on

more hydrophilic carrier materials is slower. On the most hydrophilic CM 4, only 1 log of spore inactivation was achieved within 10 s treatment time. The difference in inactivation after 10 s between CM 1 and CM 4 was found to be significant at a level of significance of 0.05.

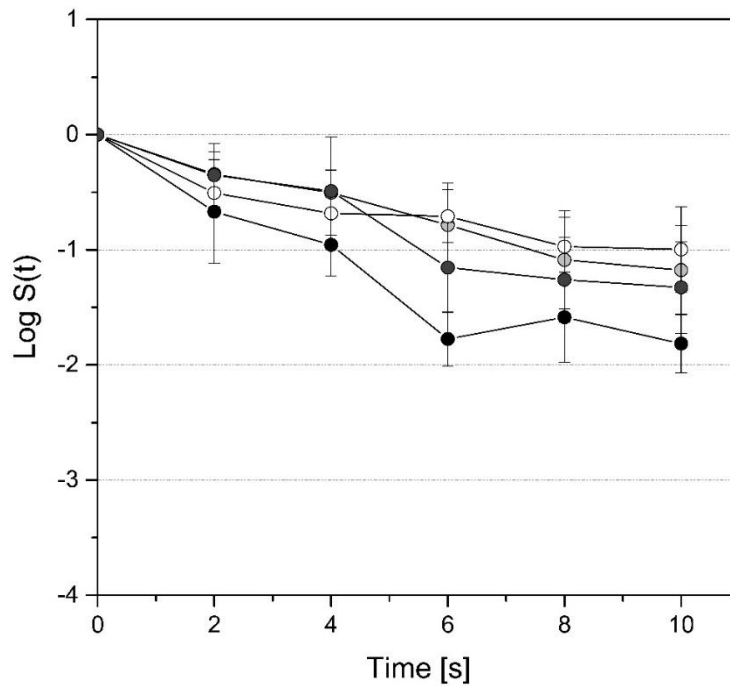


Figure 3-14: Inactivation of *B. subtilis* spores on different carrier materials (Black: CM 1, dark grey: CM 2, bright grey with dashed line: CM 3, white with dashed line: CM 4). Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm.

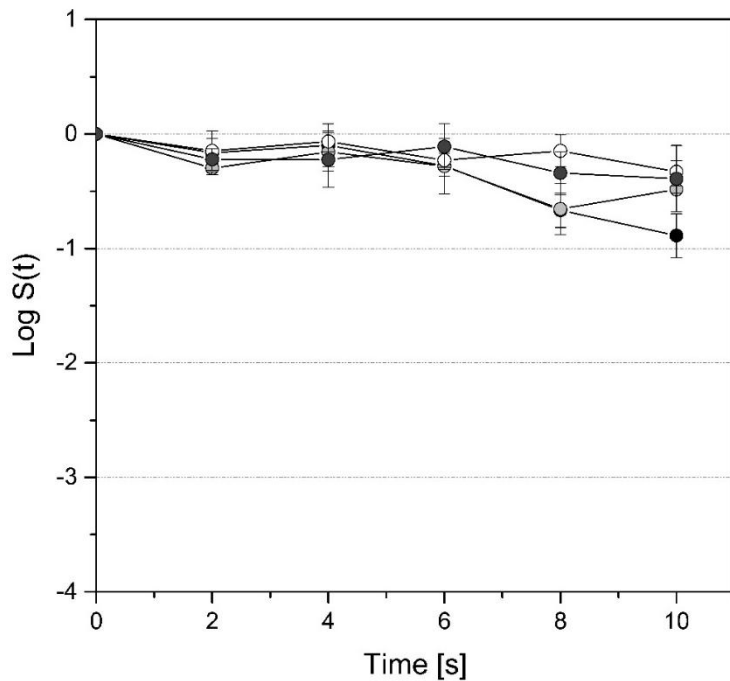


Figure 3-13: Inactivation of *B. atrophaeus* spores on different carrier materials (Black: CM 1, dark grey: CM 2, bright grey with dashed line: CM 3, white with dashed line: CM 4). Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm.

Figure 3-13 shows the inactivation of *B. atrophaeus* spores on different carrier materials. Compared to Figure 3-14, spores of *B. atrophaeus* are clearly more resistant to hydrogen peroxide condensate than spores of *B. subtilis*. The trend of the graph is very flat compared to the one of the *B. subtilis* spores. Only about 0.5 log of spores are inactivated within 10 s. However, the same trend concerning surface hydrophobicity of the carrier materials becomes apparent at the end of treatment time. Inactivation on carrier materials with high surface hydrophobicity is enhanced compared to more hydrophilic materials. However, the difference between CM 1 and CM 4 after 10 s was not found to be significant.

Influence of the surface hydrophobicity of a mixed spore population

Spores of *B. subtilis* and *B. atrophaeus* were inactivated singly and as an 1:1 mixture on different carrier materials. Inactivation was carried out at a concentration of 5200 ppm H₂O₂ and a constant treatment chamber temperature of 70 °C. For the inactivation results shown in Figure 3-15 and Figure 3-17, both carrier materials are more hydrophobic than the spores. *B. atrophaeus* is clearly more resistant towards inactivation with gaseous, condensing hydrogen peroxide. Within 10 s, reduction is less than 1 log. Inactivation of *B. subtilis* spores occurs faster, approximately 1.5 log within 10 s. Comparing the inactivation as single culture and as mixed population results in very similar inactivation kinetics. A t-test with a level of significance of 0.05 did not result in significant differences after 10 s, where the inactivation kinetics differ the most, but the trend might nevertheless be of interest regarding variations observed in industrial practice.

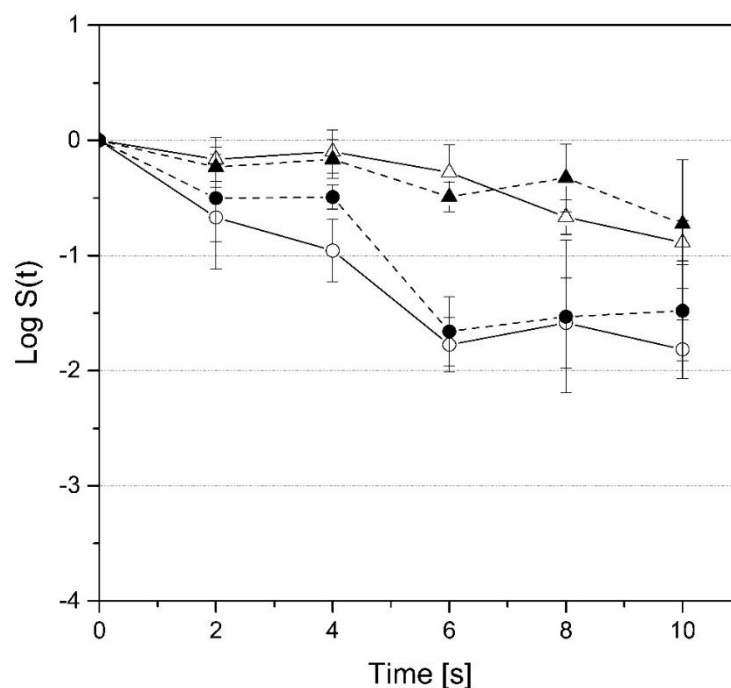


Figure 3-15: Comparison of *B. subtilis* and *B. atrophaeus* on CM 1 (115 °) as single and as combined culture. ● *B. subtilis* single, ○ *B. subtilis* combined, ▲ *B. atrophaeus* single, △ *B. atrophaeus* combined, dashed lines are single spore populations). Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm

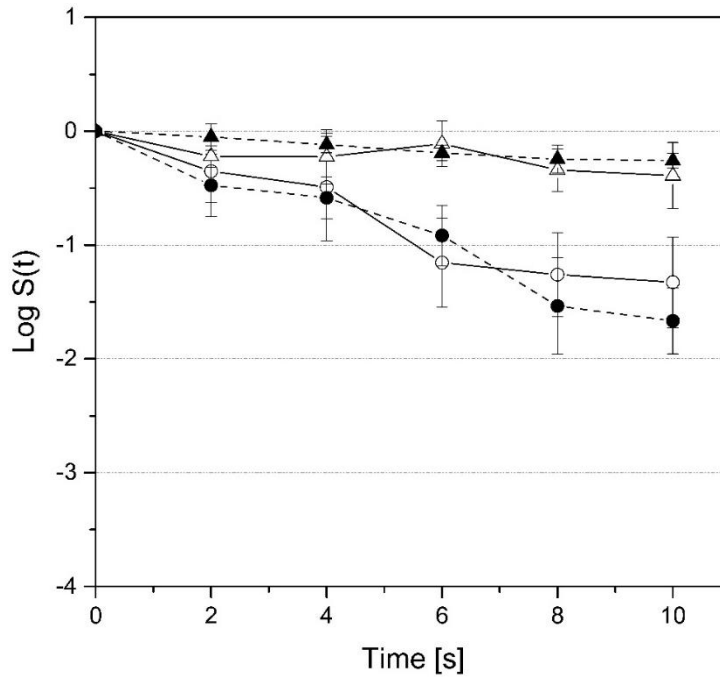


Figure 3-17: Comparison of *B. subtilis* and *B. atrophaeus* on CM 2 (101 °) as single and as combined culture. ● *B. subtilis* single, ○ *B. subtilis* combined, ▲ *B. atrophaeus* single, △ *B. atrophaeus* combined, dashed lines are single spore populations). Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm

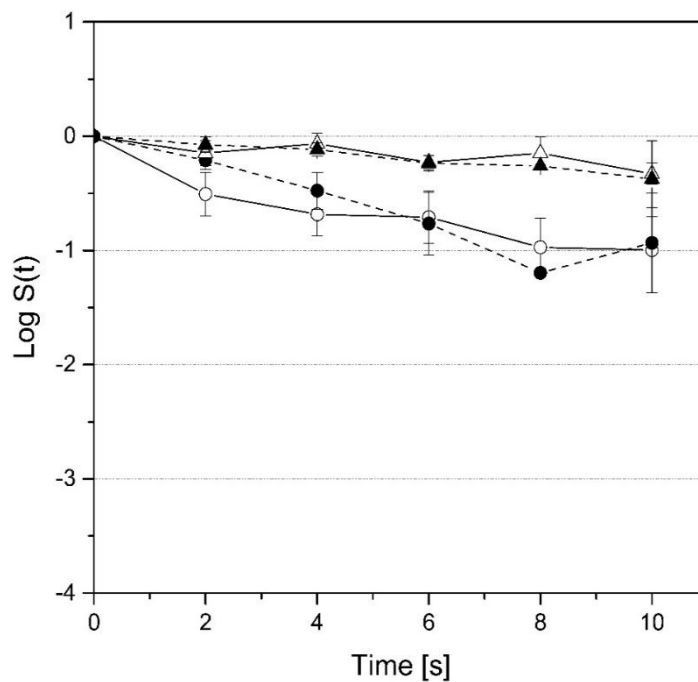


Figure 3-16: Comparison of *B. subtilis* and *B. atrophaeus* on CM 4 (30 °) as single and as combined culture. ● *B. subtilis* single, ○ *B. subtilis* combined, ▲ *B. atrophaeus* single, △ *B. atrophaeus* combined, dashed lines point to single spore populations). Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm

For CM 4, both microorganisms are more hydrophobic than the carrier material. However, Figure 3-16 depicts that for most of the inactivation data, no difference can be observed in the trend of the inactivation between mixed population and the single cultures. However, after 2 s, a significant difference occurs between the inactivation of *B. subtilis* spores as single spore suspension and mixed spore suspension. After several seconds of inactivation, this difference cannot be detected anymore.

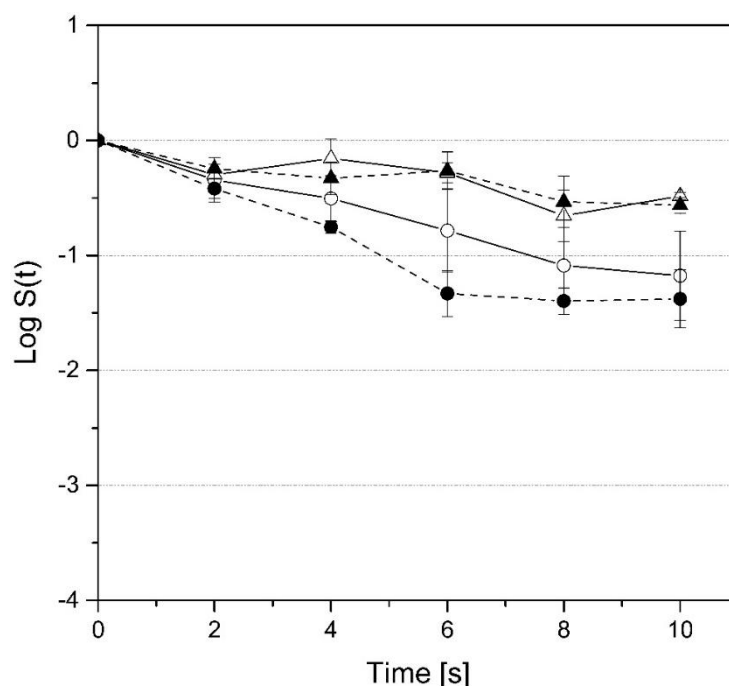


Figure 3-18: Comparison of *B. subtilis* and *B. atrophaeus* on CM 3 (81 °) as single and as combined culture. ● *B. subtilis* single, ○ *B. subtilis* combined, ▲ *B. atrophaeus* single, △ *B. atrophaeus* combined, dashed lines point to single spore populations) Temperature of treatment chamber: 70 °C, H₂O₂ gas concentration: 5200 ppm

B. subtilis spores are more hydrophobic than CM 3, whereas *B. atrophaeus* spores are more hydrophilic. As shown in Figure 3-18, the inactivation results does not differ significantly for the first 2 s, but then they start to differ. For the time of 4 to 8 s, inactivation of the pure *B. subtilis* spores is faster than for the mixed *B. subtilis* spores, but significant only at 6 s. However, after 10 s, the difference becomes very small and not significant again and both BI show an inactivation result of almost 1.5 log.

3.3.4 Discussion

Influence of the surface hydrophobicity of the carrier materials

The first hypothesis of this study was that surface hydrophobicity of carrier materials does influence the condensate formation and thereby the inactivation of bacterial spores. For the *B. atrophaeus* spores, inactivation kinetics with gaseous H₂O₂ on carrier materials with different surface hydrophobicity showed little difference (Figure 3-13). The resistance test against liquid H₂O₂ at 25 °C already resulted in high spore resistance (Figure 3-12).

Condensed H₂O₂ is liquid as well, however with a much higher concentration of H₂O₂. The resistance of *B. atrophaeus* spores against liquid H₂O₂ was very temperature dependent (Figure 3-11). Higher temperatures resulted in faster spore inactivation. Therefore, the inactivation of *B. atrophaeus* spores with condensed H₂O₂ was expected to be smaller than the inactivation of *B. subtilis* spores. This expectation was confirmed. The inactivation kinetics of *B. atrophaeus* spores showed a slow inactivation of spores at the beginning when surface temperature of the CM was close to 26 °C and a more pronounced inactivation after 10 s, when the surface temperature of the CM was higher but still below the dew point temperature of 43.6 °C. At this point, inactivation results differ slightly, but not significant. Nevertheless, a trend can be seen: Inactivation on a more hydrophobic CM is faster than on more hydrophilic CM. For *B. subtilis* spores, which are less resistant to liquid H₂O₂ at 25 °C than *B. atrophaeus* spores (Figure 3-12), inactivation with condensing H₂O₂ was more intense for each surface temperature. Inactivation of *B. subtilis* spores was significantly enhanced on the most hydrophobic CM compared to the most hydrophilic CM as shown in figure 4. We suppose that condensate formation took place on the most hydrophilic condensation nuclei. For the case of a very hydrophobic CM, most of the condensate covers the spores, which are inactivated by a high concentration of liquid H₂O₂. In the case of a more hydrophilic CM, condensate formation takes place on the spores, but also on the CM. As the CM show a certain surface roughness, they can also provide condensation nuclei. Accordingly, less condensate formation takes place at the bacterial spores, which results in less inactivation of the spores. Inactivation thus depends on the surface hydrophobicity of the CM.

Sigwarth and Stärk (2003) investigated the influence of different carrier materials on the inactivation of *Geobacillus stearothermophilus* spores. Hydrophobicity of the carrier materials was not examined in their study. However, they applied glass and polyethylene. Of course, surface hydrophobicity also depends on the surface morphology, but glass in general is very hydrophilic, while polyethylene was quantified as hydrophobic (Table 3-3). The results of Sigwarth and Stärk (2003) are contradictory to our results as glass with rather low hydrophobicity results in small D-values and PE with high hydrophobic characteristic leads to higher D-values. However, their experiments were carried out with low concentrations of H₂O₂ (approximately 330 ppm, this study in contrast applies 5200 ppm) and at room temperature, which is above the dew point temperature for 330 ppm H₂O₂. Thus, condensate formation did not take place, spores were inactivated by pure H₂O₂ gas. The mode of action of H₂O₂ gas compared to liquid H₂O₂ such as condensate is different (Finnegan et al. 2010). Unger et al. (2007) conducted a similar study with different materials. However, they also applied lower concentrations of H₂O₂ (850 ppm) at room temperature, which is still below the dew point. Therefore, the contradiction to our results appears only

apparent and results difficult to be compared head-to-head, because of the quite different experimental conditions.

Our results show significant differences for the inactivation of *B. subtilis* spores; however, not for *B. atrophaeus* spores depending on the surface hydrophobicity of carrier materials. Therefore, we suppose that surface hydrophobicity of carrier materials influences the inactivation with gaseous and condensing H₂O₂. However, further investigations need to be carried out to substantiate this trend.

Influence of the surface hydrophobicity of a mixed spore population

The second hypothesis was that there is an influence of spore surface hydrophobicity on the inactivation with gaseous and condensing H₂O₂. As described earlier, Marcos-Martin, et al. (1996) and Hall et al. (2007) stated that high microbial surface hydrophobicity protects microorganisms from condensate formation and thus leads to a lower inactivation effect. However, they conducted no experiments to prove this putative statement. Pruß et al. (2012) performed experiments with bacterial spores of different surface hydrophobicity. They concluded that the differing inactivation results are based on differing spore surface hydrophobicity with the most hydrophobic spore being the most resistant towards condensing H₂O₂. However, they applied different spore species resulting in different genetic setup and therefore possibly with different intrinsic resistance factors such as composition of the inner spore membrane. Melly et al. (2002a) suppose that the inner spore membrane is the target for inactivation with liquid H₂O₂. Inactivation with gaseous H₂O₂ is based on a different inactivation mechanism, which is not completely understood so far (Finnegan et al. 2010). If spores are treated with gaseous, condensing H₂O₂, they are in contact both with the gas and the liquid H₂O₂. Depending on the spore species, they can be resistant against the gas, but not against the liquid H₂O₂ and vice versa. This makes it difficult to investigate the influence of spore surface hydrophobicity.

Cultivation conditions are able to modify the surface hydrophobicity of bacterial spores. However, resistance against liquid H₂O₂ changes as well (Eschlbeck et al. 2017). Therefore, a mixed spore population was applied. If surface hydrophobicity influences the inactivation with condensing H₂O₂, there should be a lower inactivation effect for the hydrophobic spores (*B. subtilis*) in a mixed spore population compared to the inactivation of the single hydrophobic spores.

The first part of the discussion already showed that surface hydrophobicity of CM probably influences the inactivation of bacterial spores. The influence of spore surface hydrophobicity examined by applying a mixed spore population will be discussed in three parts relative to the respective surface hydrophobicity of the CM.

1. CM more hydrophobic than both spore species (Figure 3-15 and Figure 3-17): According to the assumption of Beysens et al. (2006), hydrophilic surfaces will be the first to be wetted. For hydrophobic CM, condensate will form close to or at the spores and fully cover them. *B. subtilis* spores are more hydrophobic than *B. atrophaeus* spores. However, no significant difference was observed between the inactivation kinetics of the single spore species and the mixed spore population. One possible explanation is that spores were mounted on the CM together and probably reside side by side. In that case, even if the condensate covers the hydrophilic spores, the neighboring hydrophobic ones will be inactivated by the condensate as well. Thus, the influence of the hydrophobic carrier material is stronger than the influence of spore surface hydrophobicity for this constellation.
2. CM more hydrophilic than both spore species (Figure 3-16): Inactivation results of the single and mixed spore population are very similar. After 10 s of inactivation, no significant difference was found. According to the expectation that condensate forms at the most hydrophilic condensation nuclei, which in this case is the CM, condensate will form at the CM. Thus, the inactivation of the spores partly takes place due to the remaining H₂O₂ in the gas and partly due to minor condensate formation at the spores which explains the lower inactivation intensity.
3. One spore species more hydrophobic and one spore species more hydrophilic than the CM (Figure 3-18): The most interesting inactivation results were obtained for the inactivation on CM 3. Inactivation of the *B. subtilis* spores in a mixed spore population is slightly slower than the inactivation of *B. subtilis* spores as single spore population. After 6 s of inactivation, the difference becomes significant. After 10 s, no significant difference can be detected anymore. We suppose that condensate formation took place around the most hydrophilic condensation nucleus, which are the *B. atrophaeus* spores. However, due to their high resistance against H₂O₂ at low temperatures, no enhanced inactivation was detected. Further condensate forms on the CM, which is more hydrophilic than the *B. subtilis* spores. Those hydrophobic spores are the last target for condensate formation, which results in better survival compared to the single spore species. However, this effect is not significant for most of the inactivation time. However, when spores of *B. subtilis* and *B. atrophaeus* are present next to each other in close distance on the CM the condensate will primarily affect the hydrophilic spores, but will also inactivate the hydrophobic ones covered by the same droplets of condensate.

Concluding from our results, there was no significant difference after 10 s of inactivation time between inactivation kinetics of single spore species and mixed spore populations. Therefore, the influence of spore surface hydrophobicity can be neglected as an effect of practical relevance. For packaging decontamination, several influencing factors have to be considered. Based on the presented results, the surface hydrophobicity of the respective contaminant microorganism only plays an insignificant role. In contrast, the hydrophobic property of the carrier material, in industry the packaging material or other surfaces to be sterilized, does influence the inactivation with gaseous and condensing H₂O₂. Therefore, the surface hydrophobicity of the respective material should be carefully considered and well specified when choosing a packaging material for aseptic filling technology or when changing to another supplier of the nominally same material.

3.4 Influence of carrier material surface hydrophobicity and roughness on a dry hydrogen peroxide vapor inactivation process

Summary and contribution of the doctoral candidate

For aseptic packaging technology, short inactivation times allowing fast filling processes are essential. Vaporized hydrogen peroxide (H_2O_2) at high concentrations provides an effective decontamination solution. The decontamination agent can be applied with or without the formation of condensate. In the previous publication, we already discussed the influence of spore and carrier material surface hydrophobicity during a process with condensate formation. However, little is known about those influencing factors during a dry H_2O_2 process without condensate formation.

The purpose of this study was to investigate the influence of surface hydrophobicity of carrier materials and bacterial spores of *Bacillus subtilis* SA 22 and *Bacillus atrophaeus* (DSM 675) on the inactivation during a dry H_2O_2 inactivation process.

Our results show that there is an influence of carrier material surface hydrophobicity. However, due to the complexity of the process, no clear correlation can be observed. To our knowledge, the influence of surface hydrophobicity of carrier materials and mixed populations on the inactivation with gaseous hydrogen peroxide without condensate formation has not been investigated so far. Concluding from this publication, clearly it is essential to consider the specific characteristic of the surface when it comes to dimensioning of sterilization cycles of clean rooms or production of biological indicators as there is an influence of the surface even during a dry H_2O_2 process.

The doctoral candidate planned and supervised the cultivation, purification and characterization of the bacterial spores regarding contact angle. Measurement of carrier material hydrophobicity and roughness was planned and supervised by the doctoral candidate. The doctoral candidate also calculated the dew point temperature to avoid condensate formation and coordinated the inactivation of the spores by means of gaseous H_2O_2 and. Analysis of the resulting data, interpretation of datasets and plotting of data were conducted by the doctoral candidate. The manuscript was essentially written by the doctoral candidate.

Adapted original manuscript¹

Influence of carrier material surface hydrophobicity and roughness on a dry hydrogen peroxide vapor inactivation process²

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Abstract

The aim of this study was to investigate the influence of surface hydrophobicity and roughness of carrier materials on the inactivation of bacterial spores with gaseous hydrogen peroxide whereas condensate formation is prevented. Surface hydrophobicity of the carrier materials was measured by water contact angle measurement. The water contact angles of the carrier materials varied from 30 ° to 115 °. Roughness was characterized by means of atomic force microscopy. Spores of *Bacillus subtilis* and *Bacillus atrophaeus* were applied either as single spore culture or as a mixed spore population to simulate contamination in reality. Inactivation with gaseous hydrogen peroxide was carried out at 5200 ppm hydrogen peroxide with a carrier material surface temperature above the dew point to prevent condensate formation. The inactivation results of the two spore species on carrier materials with varying surface hydrophobicity differed significantly which might be due to the higher adsorption of gas molecules on hydrophilic materials. However, inactivation of the mixed spore populations resulted in similar resistance compared to the single spore batches which means, that there is no significant influence of spore surface hydrophobicity alone. Surface hydrophobicity most probably has an impact on the inactivation with gaseous hydrogen peroxide. Though there is no condensate formation, there is adsorption of water vapor and hydrogen peroxide gas molecules. However, due to the complexity of the process on the one hand and the unclarified inactivation process of bacterial spores with gaseous hydrogen peroxide, no clear correlation could be found. This study is important for the validation of inactivation processes with hydrogen peroxide as there is an enormous influence of the surface.

To obtain reliable validation results, the surface material of interest has to be applied as carrier material for the bioindicator.

Key words:

Bacterial spores, hydrogen peroxide, water contact angle, bioindicator, inactivation

3.4.1 Introduction

For aseptic packaging processes, hydrogen peroxide (H_2O_2) in its vaporized form is an often applied decontamination agent. Many of the influencing factors such as temperature, concentration, amount of water in the gas e.g. have been investigated and published (Pruß et al. 2012; Wang and Toledo 1986; Unger-Bimczok et al. 2008). The influence of surface hydrophobicity of the carrier material and the bacterial spores, respectively, during a process with condensate formation have also been studied (Eschlbeck et al. 2018a). However, little is known about the influence of surface hydrophobicity and surface roughness on the inactivation by vaporized H_2O_2 without condensate formation. Due to the absence of condensate, this process can be seen as a dry process, but still an effect of the surface properties might play a role and help to explain often unexpected and variable inactivation results.

When vaporized H_2O_2 is applied, the formation of condensate depends on the amount of water and H_2O_2 in the gas as well as saturation pressure and temperature (Beysens 1995). According to Parks and Watling (2004), the dew point temperature can be calculated as a function of constant saturation pressure and gas concentration. The dew point temperature is the temperature below which condensate begins to form. If the temperature of the gas is constantly above the dew point temperature, the process is free of condensate. This includes the gas close to the surface. Therefore, the surface temperature has to be above the dew point temperature as well to prevent condensate formation.

However, a certain reversible occupancy of the surface with gas molecules takes place which is called adsorption (Dörfler 2002). Adsorption is influenced by the surface hydrophobicity and polarity of the adsorbent on the one hand and on the polarity of the gas molecules on the other hand. The degree of polarity of a single molecule is indicated by its dipole moment. Water molecules have a dipole moment of 1.84 Debye (Mortimer and Müller 2010; Law 2014), whereas H_2O_2 molecules have a dipole moment of 2.21 Debye (Yu und Yang 2011). As the dipole moment of H_2O_2 is higher, the adsorption on hydrophilic surfaces might be enhanced compared to more hydrophobic surfaces. Besides, the overall adsorption of H_2O_2 and water is higher on hydrophilic carrier material compared to hydrophobic material. Therefore, on

hydrophilic carrier materials, inactivation of bacterial spores is expected to be less intense as the H₂O₂ molecules adsorb on the surface, are not present in the gas anymore and thus can't inactivate microorganisms. At the same time, microorganisms which are more hydrophilic than the carrier material would be preferred adsorption spots and thus inactivation would be enhanced.

At the same time, high surface roughness leads to a bigger surface on which adsorption can take place. In addition, surface roughness might induce microcondensation resulting in locally variable but high H₂O₂ concentration. Besides, surface roughness provides the opportunity for microorganisms to be covered and therefore be protected from the inactivation effect (Unger et al. 2007).

The Merkblatt 6" (Merkblatt 6), recommends spores of *Bacillus subtilis* (*B. subtilis*) or *Bacillus atrophaeus* (*B. atrophaeus*) as test cultures or "bioindicators" (BI) to monitor H₂O₂ inactivation processes. However, no carrier materials are recommended. Commercially available BI consist of one spore species which is either already applied on a carrier material e. g. a paper strip, or dispersed in water or ethanol. The BI is brought into the decontamination device or filling machine and subsequently inactivated. Together with a reference BI which is not treated, the inactivation intensity can be assessed. However, surface contamination in reality displays a mix of different microorganisms with different surface characteristics. Assuming that surface hydrophobicity of the carrier material influences the adsorption of vaporized H₂O₂, surface hydrophobicity of bacterial spores should also have an impact on the inactivation results as well.

The mechanism of bacterial spore inactivation with H₂O₂ gas is quite complex and not yet fully understood. Finnegan et al. (2010) investigated the inactivation with gaseous H₂O₂ compared to liquid H₂O₂ and concluded that the gas irrupts deeper into the spore structure than liquid H₂O₂ and most probably oxidizes amino acids essential for the spore germination.

Pruß et al. (2012) investigated the influence of different surface temperatures on the inactivation with gaseous H₂O₂. They performed inactivation experiments with the same gas concentration and temperature, while varying the surface temperature of the carrier material. This h resulted in a dry process for a high carrier material temperature and condensate formation for a low carrier material temperature. Their results clearly show that the resulting inactivation kinetics differ depending on the process.

Sigwarth and Stärk (2003) applied different carrier materials to investigate their influence on the resistance of *Geobacillus stearothermophilus* spores against gaseous H₂O₂. Their results show that surface roughness influences the inactivation results.

Extremely rough surfaces increase bacterial spore resistance. However, no clear correlation could be found between surface roughness and spore resistance.

Rogers et al. (2005) studied the inactivation of spores of *Bacillus anthracis*, *Bacillus subtilis* und *Geobacillus stearothermophilus* with gaseous H₂O₂ on different surfaces. The authors concluded that due to porosities and cavities of the surfaces, spores might be sheltered and thus show higher resistance on those materials. However, the conditions applied by these authors are far from real conditions of packaging material properties.

The influence of different carrier materials was also examined by Unger et al. (2007). They applied *Geobacillus stearothermophilus* spores to evaluate the influence of roughness during a clean room decontamination cycle. However, they applied relatively low amounts of H₂O₂ for a long time. No correlation could be observed. The authors, however, assume that there is some kind of relationship between roughness and resistance of spores against gaseous H₂O₂.

Grand et al. (2010) investigated the influence of different carrier materials. However, they applied two different methods for carrier material contamination which are both not suitable to generate a monolayer of bacterial spores. Therefore, spores can be protected by several layers in spore clusters or cavities and thus show different inactivation kinetics compared to spore monolayers. A conclusion about the sole influence of surface hydrophobicity or roughness of the carrier material from their results is thus not possible.

Concluding, recent literature indicates that there might be an influence of carrier material roughness and surface hydrophobicity on the inactivation with gaseous H₂O₂. However, those characteristics have not been sufficiently investigated yet.

3.4.2 Materials and methods

Bacterial spores

Both *B. atrophaeus* (DSM 675) and *B. subtilis* SA 22 (DSM 4181) were obtained from the DSMZ (German collection of microorganism and cell cultures, Braunschweig, DE) as freeze-dried cultures. Rehydration was carried out according to the manufacturer's instructions. Cultivation of both bacilli was carried out with a sporulation media (for 1000 mL of distilled water: 5.0 g peptone from casein (Gerbu, Heidelberg, DE), 3.0 g beef extract (Gerbu, Heidelberg, DE), 3.5 g potassium chloride (Merck, Darmstadt, DE), 250 mg magnesium sulphate (Roth, Karlsruhe, DE), after autoclaving 10 mL of 10 % glucose in water (Merck, Darmstadt, DE) and 1 mL of the following micronutrients were added: 1 M calcium nitrate (Sigma Aldrich, Darmstadt, DE) 0.01 M manganese

chloride (Merck, Darmstadt, DE), 1 mM iron sulphate (Fluka, Seelze, DE)). *B. subtilis* was cultivated in a bioreactor as described in Eschlbeck et al. (2017), *B. atrophaeus* was cultivated on agar plates. Agar-agar (15 g/L, Fisher Scientific, Schwerte, DE) was added to the composition to obtain the sporulation media as agar plates.

B. atrophaeus spores were incubated at 37 °C for 10 days. Spores were removed from the agar plates by pouring 10 mL of sterile distilled water on the plates, suspending the spores with a spatula and collecting the suspension. *B. subtilis* was cultivated at a constant pH value of 8.5 and a temperature of 37 °C with constant oxygen supply (2 L/min sterile filtered air).

The process of purification was identical for both spore species. Spores were heat activated at 80 °C for 20 min to inactivate all vegetative bacilli. Each spore suspension was washed at least 4 times with sterile, distilled water and centrifuged at 4000 g for 10 min at 4 °C. Inactivated vegetative cells or their cell debris were removed in the centrifugation step together with the supernatant. Spores were subsequently cooled down in ice water. The absence of vegetative cells and the purity of the spore suspension was verified with a light microscope (Axioskop, Carl Zeiss, Oberkochen, DE) and washing steps were repeated until at least 95 % pure spores were in the suspension. Spores were stored in distilled water at 4 °C.

Contact angle measurement

To evaluate spore surface hydrophobicity, water contact angle measurement (DSA 100, Krüss, Hamburg, DE) at 20 °C was applied. The method was carried out as described by Eschlbeck and Kulozik (2017). In short, an aliquot of spore suspension sufficient for several layers of spores was filtered on a cellulose acetate filter (pore size: 0.22 µm, Sartorius Stedim, Göttingen, DE). A subsequent equilibration time of 2 h on 2% agar-agar (Fisher Scientific, Schwerte, DE) was applied, then filters were dried for 50 min on air. For the measurement, a drop of 8 µL Milli-Q water was set on the filter surface with the sessile drop method during the plateau time. For each drop, a video of the first 6 s was taken. To evaluate water contact angles, the pictures from the resulting video were analyzed with DSA 4 (Krüss, Hamburg, DE). Measurements between 2 and 4 s after the onset of drop deposition showed consistent water contact angles. Three independent filters were prepared for each spore suspension. On each filter, a minimum of 5 drops was deposited and evaluated.

Carrier materials

The surface characteristics of different carrier materials (CM) were assessed by contact angle measurement as described above for the bacterial spores and as already

described in Eschlbeck et al. (2018a). The chosen materials together with their surface hydrophobicity are described in Table 3-4.

Table 3-4: Carrier materials and water contact angles (Eschlbeck et al. 2018a)

Abbreviation	Materials	Water contact angle	Standard deviation
CM 1	LDPE cardboard packaging material (Tetra Pak, Modena, Italy)	115 °	2,08 °
CM 2	PET film, optically rough (23 µm thick, Huthamaki, Ronsberg, DE)	101 °	2,69 °
CM 3	PET film, optically smooth (23 µm thick, Huthamaki, Ronsberg, DE)	81 °	1,40 °
CM 4	PET film covered with silicium oxide (23 µm thick, Huthamaki, Ronsberg, DE)	30 °	0,64 °

Cardboard packaging material pieces with a surface consisting of standard LDPE (low density polyethylene, provided by Tetra Pak, Modena, Italy) and a size of 4 by 5.5 cm were applied as basic material. Thin films of the chosen materials were mounted on the basic LDPE cardboard material by means of double-sided adhesive tape. Sterilization of the cardboard packaging material was carried out by means of gamma radiation (25 kGy, Synergy Health, Allershausen, DE). Sterilization was done by means of gaseous hydrogen peroxide (9000 ppm, 20 s, 70 °C) with the hydrogen peroxide decontamination device.

Surface roughness

Surface roughness was investigated by means of an atomic force microscope (Alpha-500, WITec, Ulm, DE) at the Fraunhofer Institut für Verfahrenstechnik und Verpackung IVV (Freising, DE). Dry carrier materials were cut in small pieces and fixed on specimen holders by means of double-sided adhesive tape. Therefore, concavities in the test materials were avoided. The size of the examined surfaces was 50 µm by 50 µm for CM 1, CM 3 and CM 4 and 50 µm by 100 µm for CM 2. For each carrier material, three independent spots were measured and the average surface roughness R_z was calculated (3-4).

$$R_z = \frac{1}{3} \sum_{i=1}^3 R_z(i) \tag{3-6}$$

$R_z(i)$ is the distance between the highest and lowest detected peak of the respective sample. In this manuscript, the difference between those peaks specifies the surface roughness.

Application of Biological Indicators to the test material surface

An aliquot of 300 μL of spore suspension was mixed with 700 μL ethanol (Merck, Darmstadt, DE). 10 μL of the resulting mixture (approximately 10^8 cfu/mL) were spread on carrier materials by means of Drigalsky spatula as described by Pruss *et al.* (2012). The resulting amount of spores on each carrier material was 10^6 . For the experiments with mixed spore populations, 150 μL of each spore suspension were applied to produce the BI mixture. Even distribution of spores was checked by electron microscopy, data not shown. Prior to measurements, drying of the BI was carried out under sterile conditions for at least 1 h.

Hydrogen peroxide treatment

Liquid, 35 % (11.63 mol/L) hydrogen peroxide (Evonik Industries, Essen, DE) was completely evaporated (Controlled Evaporator Mixer, Bronkhorst NL), the vapor tempered to 70 °C and directed into the treatment chamber. The temperature of the vapor was measured continuously with two temperature sensors (Angled Thermocouple, pentronic, SWE) integrated into the treatment chamber. One of the sensors is coated with catalytic material so that hydrogen peroxide dissolves in an exothermic reaction as described by Pruss (2013). The resulting temperature difference rises and correlates with increasing H_2O_2 gas concentration. This catalytic sensor system was applied to monitor the active hydrogen peroxide concentration in the chamber. H_2O_2 concentration was 5200 ppm for all experiments. The concentration was continuously monitored (LabVIEW, National Instruments, USA) and remained constant for the majority of experiments. Exceptions with peaks of high H_2O_2 concentrations were discarded.

The prepared BI was inserted into the treatment chamber for the respective treatment time. Directly after the treatment, the BI was removed and completely covered with 10 mL of a tween-catalase solution consisting of 9.9 mL of sterile, 0.1 % tween 80 (Gerbu Biotechnik GmbH, Heidelberg, DE) and 0.1 mL of catalase (Catalase from *Micrococcus lysodeikticus*, Sigma Aldrich, Darmstadt, DE). Potentially remaining active hydrogen peroxide on the surface was immediately dissolved by catalase to stop the inactivation effect. Spores were detached from the carrier material by magnetic stirring (200 rpm, Arex Digital Heating Magnetic Stirrer, VELP Scientifica, Usmate, ITA) in a solution consisting of 9.9 mL water with 0.1% Tween 80 (Sigma Aldrich, Darmstadt, DE) and 0.1 mL of a catalase mixture consisting of 10% catalase (Catalase from

Micrococcus lysodeikticus, Sigma Aldrich, Darmstadt, DE) with water. Dilution series were generated and survivors detected with the plating method on plate count agar (as described above). Incubation was carried out at 30 °C for 48 h. Spores of *B. subtilis* form white, opal colonies whereas *B. atrophaeus* colonies show smaller, glossy yellow colonies. Therefore, for the mixed spore population experiments, surviving spores can be distinguished and individual inactivation kinetics can be generated for *B. subtilis* and *B. atrophaeus* respectively.

For each carrier material, experiments were conducted with *B. subtilis* spores, *B. atrophaeus* spores and the mixed spore population. For each of those combinations, treatment times of 0, 2, 4, 6, 8 and 10 s (as typical for industrial inactivation procedures) were set and all experiments were carried out in triplicate for all chosen carrier materials.

Condensate formation is a temperature dependent process. If the surface temperature is lower than the dew point temperature, condensate formation takes place. For a H₂O₂ gas concentration of 5200 ppm, the dew point temperature is 43.6 °C. The dew point of H₂O₂ in the condensate was calculated according to Parks and Watling (2004). To ensure that the inactivation is without condensate formation, the initial surface temperature of the carrier materials was always raised to 65 °C (Arex Digital Heating Magnetic Stirrer, VELP Scientifica, Usmate, ITA).

Surface temperature of carrier materials during treatment was determined with an infrared thermometer (FLIR i3, FLIR Systems GmbH, Frankfurt am Main, DE). During 10 s of treatment time, the surface temperature of the CM did not vary significantly.

Statistical methods

All experiments were carried out in triplicate and the standard deviation was calculated. To assess if results differ significantly, a t-test for independent samples with a level of significance (α) of 0.05 was applied.

3.4.3 Results and Discussion

Surface hydrophobicity of carrier materials

Data of water contact angles of the applied carrier materials and bacterial spores were already published in Eschlbeck et al. (2018a). For better understanding of the influence of surface hydrophobicity on the inactivation with gaseous H₂O₂, the results are depicted in Figure 3-19.

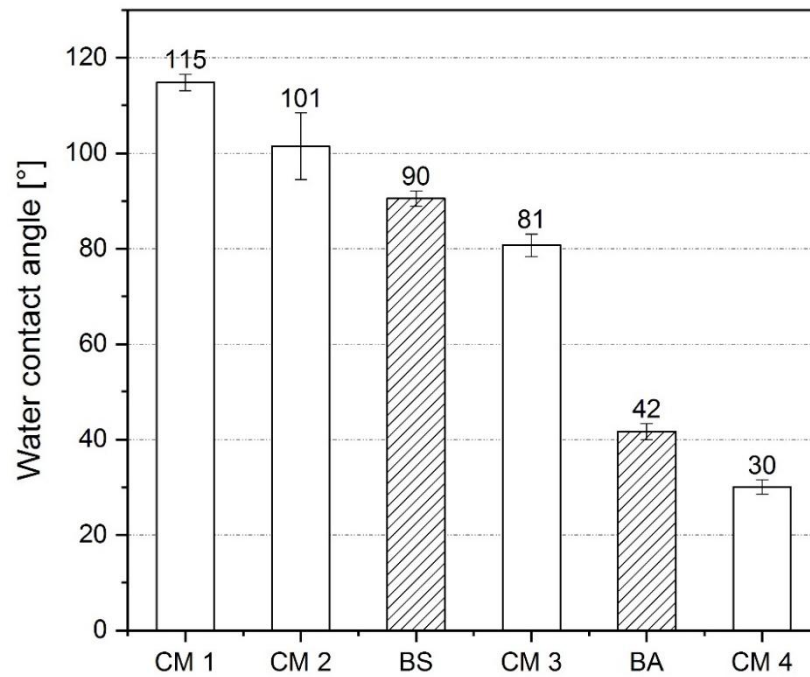


Figure 3-19: Water contact angles of the carrier materials (CM 1 to CM 4) and *B. subtilis* spores (BS) as well as *B. atrophaeus* spores (BA) (Eschlbeck et al. 2018a)

The two carrier materials CM 1 and CM 2 are more hydrophobic than the spores of *B. subtilis*. Surface hydrophobicity of CM 3 is between the two bacterial spores whereas CM 4 is more hydrophilic than both spore formers and with a water contact angle of 30 ° the most hydrophilic carrier material under investigation.

Surface roughness of carrier materials

The resulting average surface roughness values are displayed in Table 3-5. Clearly, CM 2 has the highest roughness whereas CM 1 is smoother and CM 3 exhibits only little roughness.

However, atomic force microscopy of CM 4 was not possible as the cantilever of the AFM could not produce any results on this surface. We expect CM 4 to be the most smooth surface as Vasko (2006) also examined the surface roughness of silicium oxide with an arithmetic mean roughness R_a of 0.86 nm.

Table 3-5: Average surface roughness R_z of the different carrier materials

	CM 1	CM 2	CM 3	CM 4
R_z (nm +/-)	3800 (+/- 477)	16038 (+/- 895)	304 (+/- 22)	Not measurable

Inactivation on different carrier materials

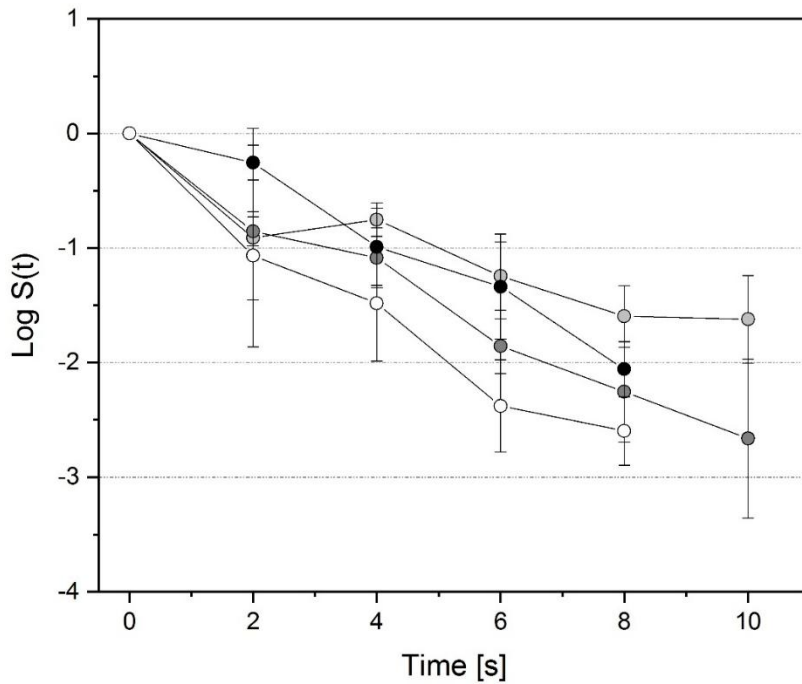


Figure 3-20: Inactivation results of *B. subtilis* spores on CM 1 (black), CM 2 (dark grey), CM 3 (bright grey) and CM 4 (white) with gaseous H_2O_2 (5200 ppm, 70 °C)

The inactivation kinetics of *B. subtilis* spores on different carrier materials are depicted in Figure 3-20. After the first 2 s, the inactivation kinetics can be seen as log-linear with CM 3 as carrier material which provides the best survival and CM 4 as carrier material

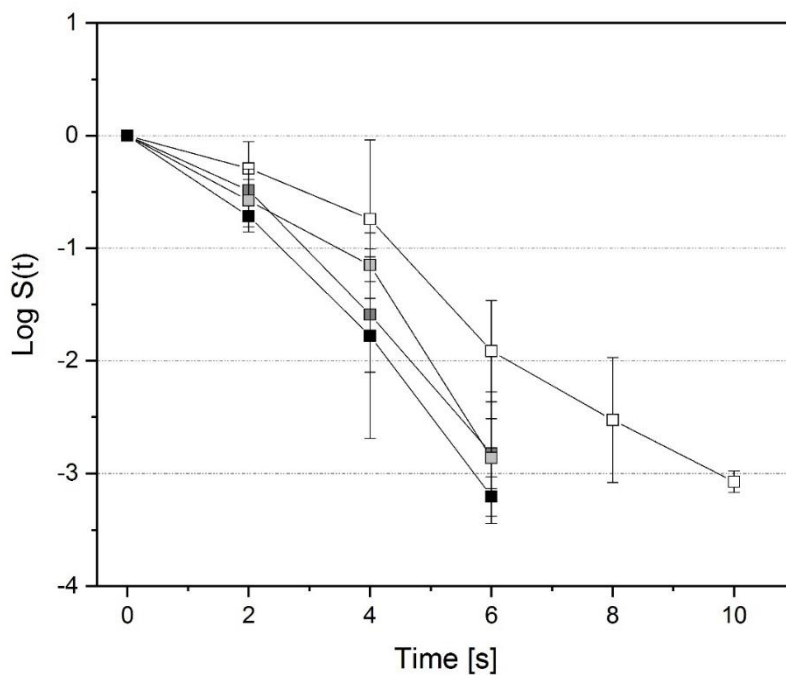


Figure 3-21: Inactivation results of *B. atropheus* spores on CM 1 (black), CM 2 (dark grey), CM 3 (bright grey) and CM 4 (white) with gaseous H_2O_2 (5200 ppm, 70 °C)

with the fastest inactivation. CM 1 and CM 2 show an inactivation of approximately 2 log after 8 s.

The inactivation of spores of *B. subtilis* seems to be independent of the surface hydrophobicity of the carrier materials as the slowest inactivation and thus highest resistance results from the combination with CM 3. CM 3 has a similar water contact angle (81 °) as the spores of *B. subtilis* (90 °). The inactivation results on CM 1 and CM 2, which are more hydrophobic, are similar and show a lower resistance than CM 3. CM 4, which is most hydrophilic, leads to the fastest inactivation of *B. subtilis* spores.

The inactivation kinetics of *B. atrophaeus* spores are displayed in Figure 3-21. Except for CM 4, the spores of *B. atrophaeus* are less resistant on all carrier materials against dry gaseous H₂O₂ than the spores of *B. subtilis*. After a treatment time of 6 s, almost 3 log of inactivation were achieved on CM 1, CM 2 and CM 3. Inactivation of the spores on the most hydrophilic carrier material, CM 4, results in an inactivation time of 10 s for 3 log. This is contrary to the inactivation of *B. subtilis* spores (Figure 3-20) as they are less resistant on CM 4.

Inactivation with gaseous H₂O₂ compared to liquid H₂O₂ was investigated by Finnegan et al. (2010). The authors suppose that due to the higher kinetic energy of the gaseous H₂O₂, molecules erupt deeper into the spore structure and inactivate the spore by oxidation of inner structures. If inner spore structures are responsible for spore resistance against gaseous H₂O₂, it is supposedly their hydrophobic characteristic that leads to more or less adsorption of the gas molecules. Literature does to our knowledge provide no option to measure the inner surface characteristics but water contact angle measurement only depicts the surface characteristics of the outermost spore layer which might one explanation why no correlation occurs.

Assuming that H₂O₂ gas molecules adsorb on the most hydrophilic surface available, the H₂O₂ molecules would adsorb on CM 4 and therefore be not available in the gas anymore. Therefore, the inactivation is expected to be less pronounced. For *B. atrophaeus*, this effect can be monitored, however, not for *B. subtilis* spores.

Comparing the inactivation kinetics on CM 4 of the two spore species, their kinetics are similar. For both bacterial spores, inactivation of 3 log occurred after 8 s of treatment time. Therefore, we suppose that adsorption on hydrophilic CM 4 results in uniformity of the inactivation. On the more hydrophobic surfaces, less molecules adsorb on the surface and inner spore structures and specific spore resistance to gaseous H₂O₂ are decisive for spore survival.

One possible explanation consists in the abrupt ending of the decontamination. This leads to a concentration gradient between the environment without H₂O₂ and the surface with adsorbed H₂O₂ molecule. By means of this gradient, molecules desorb and for a very short time, a high concentration of H₂O₂ molecules close to the surface develops. This might lead to a similar inactivation effects of spores on hydrophilic surfaces.

Influence of surface roughness

Interestingly, the surface roughness only seems to play a minor role, at least in the range investigated in this study. For *B. subtilis* spores as single spore species (Figure 3-20), the inactivation on smooth CM 3 results in the highest spore resistance. The

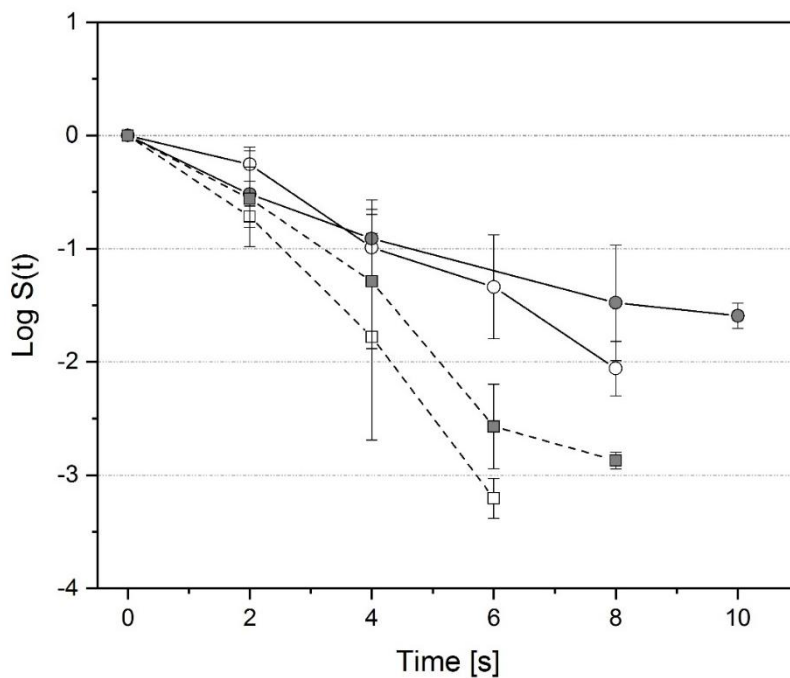


Figure 3-22: Inactivation with gaseous H₂O₂ (5200 ppmn, 70 °C) on CM 1. White circle: *B. subtilis* spores single culture, grey circle: *B. subtilis* spores mixed culture, white square: *B. atrophaeus* spores single culture, grey square: *B. atrophaeus* spores mixed culture.

fastest inactivation takes place with CM 4, which is assumed to be even smoother. Besides, the highest average surface roughness of 16 µm might not provide enough space for microorganisms to hide or be sheltered from the inactivating gas.

Effect of gaseous H₂O₂ on a mixed spore population

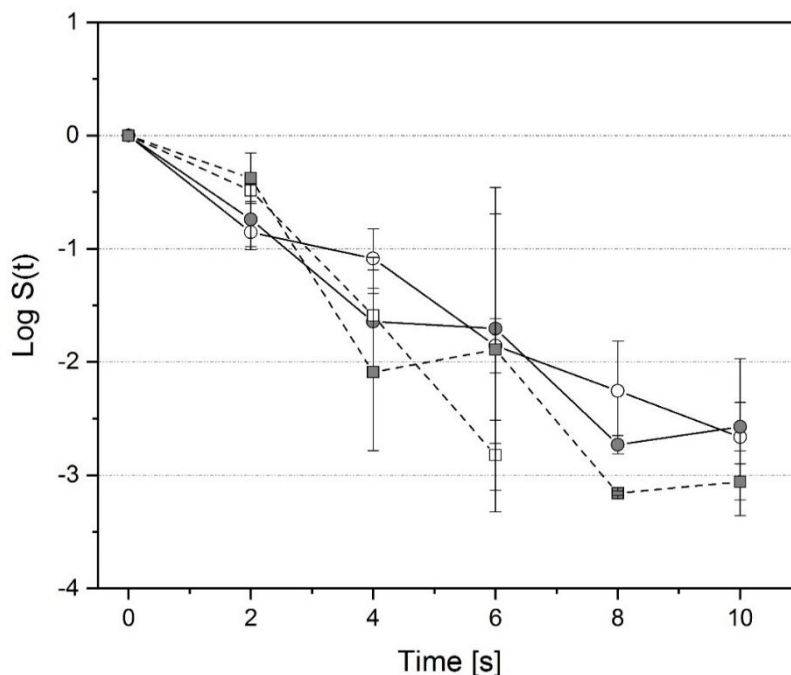


Figure 3-23: Inactivation with gaseous H₂O₂ (5200 ppmn, 70 °C) on CM 2. White circle: *B. subtilis* spores single culture, grey circle: *B. subtilis* spores mixed culture, white square: *B. atrophaeus* spores single culture, grey square: *B. atrophaeus* spores mixed culture.

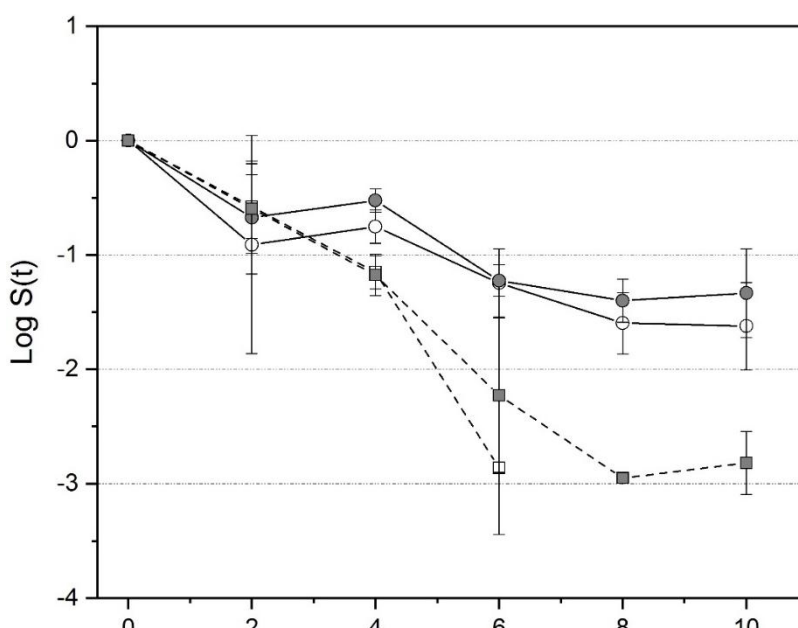


Figure 3-24: Inactivation with gaseous H₂O₂ (5200 ppmn, 70 °C) on CM 3. White circle: *B. subtilis* spores single culture, grey circle: *B. subtilis* spores mixed culture, white square: *B. atrophaeus* spores single culture, grey square: *B. atrophaeus* spores mixed culture.

A comparison of the inactivation kinetics of the single spore batches and the mixed spore cultures (Figure 3-22 to Figure 3-25) reveals that within the standard deviations, there are no significant differences in the inactivation.

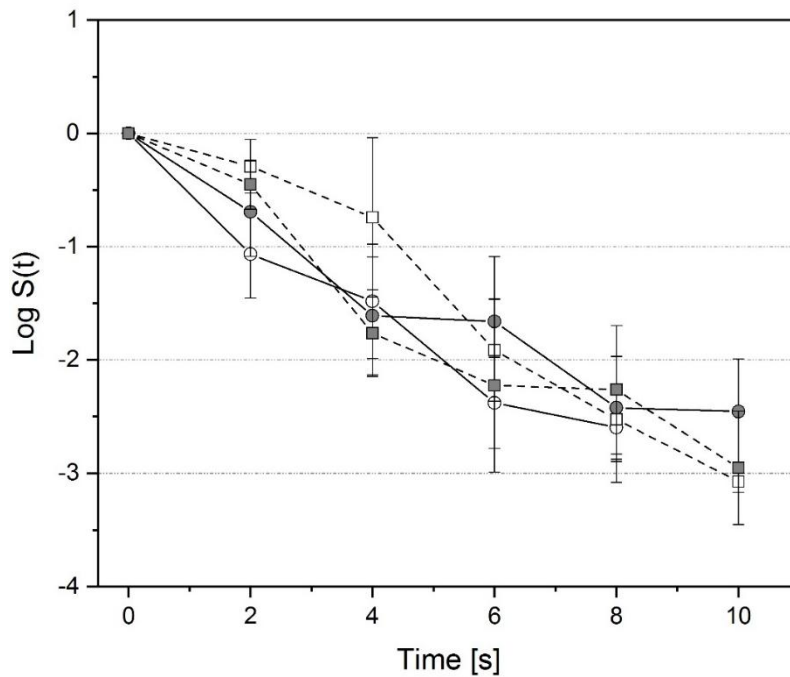


Figure 3-25: Inactivation with gaseous H_2O_2 (5200 ppmn, 70 °C) on CM 4. White circle: *B. subtilis* spores single culture, grey circle: *B. subtilis* spores mixed culture, white square: *B. atrophaeus* spores single culture, grey square: *B. atrophaeus* spores mixed culture.

In Figure 3-25, the inactivation kinetics of both spore species on CM 4 are displayed. Their inactivation results as single spore species are quite similar with an inactivation of 2.5 log after 8 s of treatment. If there is an intense adsorption of H_2O_2 molecules on CM 4, the molecules adsorb also on the surface of the carrier material, which is very close to the spores. As adsorption is a reversible process, the desorption during the short time between removing of the BI from the decontamination device and decomposition of adsorbing H_2O_2 molecules which takes approximately one second might increase the concentration of H_2O_2 in close surrounding of the spores and lead to a similar inactivation of both spore species.

If there was an influence of spore surface hydrophobicity, the inactivation of *B. subtilis* spores would be less intense in a mixed spore population as the H_2O_2 molecules would prefer the more hydrophilic *B. atrophaeus* spores for adsorption. Due to the fact that there is no significant difference in the inactivation of single spore species and mixed spore populations, we conclude that the surface hydrophobicity of bacterial spores measured by water contact angle measurement does not influence the inactivation with dry gaseous H_2O_2 .

Our results are in accordance with the results obtained by Unger et al. (2007) although the applied H_2O_2 concentration of this study, the resulting dew point temperature and the carrier material temperature to prevent condensate formation are much higher. Concluding, surface hydrophobicity of the carrier material does most probably have an

impact on inactivation during a dry H₂O₂ gas process. However, the mode of action of gaseous spore inactivation is complex and not fully understood. However, it is essential to consider the specific characteristic of the surface when it comes to dimensioning of sterilization cycles of clean rooms or production of biological indicators as there is an influence of the surface even during a dry H₂O₂ process.

4 Overall discussion

As described above, hydrogen peroxide is often applied to decontaminate packaging materials for aseptic processes and can be deployed with or without condensate formation. When condensation occurs, a phase transition from gaseous H_2O_2 to liquid H_2O_2 droplets has to take place. This phase transition requires energy. The required level of energy is lower if suitable condensation nuclei are present. Microorganisms on the surface of the packaging materials are condensation nuclei. However, no investigations exist so far concerning the influence of microbial surface hydrophobicity on the formation of H_2O_2 . In theory, microorganisms with a more hydrophilic surface will be engulfed by the condensate first whereas hydrophobic microorganisms have a better chance to survive the treatment.

Above that, reversible adsorption of gas molecules takes place during an inactivation process without condensate formation. As adsorption is influenced by the surface hydrophobicity and polarity of the adsorbent on the one hand and the polarity on the other hand, adsorption of H_2O_2 and water molecules might be enhanced on hydrophilic material compared to hydrophobic surfaces.

Therefore, it was the aim of this thesis to investigate the influence of surface hydrophobicity on the inactivation with gaseous H_2O_2 .

The general approach consisted of four steps:

- Establishing of a measurement method to quantify bacterial spore surface hydrophobicity
- Generation of spores of the same genetic setup but with different surface hydrophobicity
- Investigation of the influence of carrier material surface hydrophobicity on the inactivation of bacterial spores
- Investigation of the impact of bacterial spore surface hydrophobicity on the inactivation with gaseous H_2O_2

Establishing a measurement method to quantify bacterial spore surface hydrophobicity

It is essential for the purpose of this thesis, to choose a measurement method that quantifies spore surface hydrophobicity. Literature provides several methods including MATH (Rosenberg et al. 1980), SAT (Lindahl et al. 1981) and various adhesion tests. However, water contact angle measurement is the only method to obtain results that allow a fast and direct measure of the wettability of bacterial spores.

The results obtained by Eschlbeck and Kulozik (2017) show that there are at least two factors that influence water contact angle measurement. One of these factors is the equilibration media, on which the filter containing several layers of bacterial spores is stored to achieve an equally distributed water content throughout the filter. The other factor under investigation was the time that is needed for moisture equilibration. In literature, those two steps in the measurement procedure are either described differently or not described at all. Based on the results by Eschlbeck and Kulozik (2017), a media containing 2 % agar-agar and an equilibration time of 2 h are proposed. Furthermore, a standard procedure for water contact angle measurement of spores is proposed that allows the reproducible and reliable quantification of surface hydrophobicity. This standard procedure is depicted in Figure 4-1.

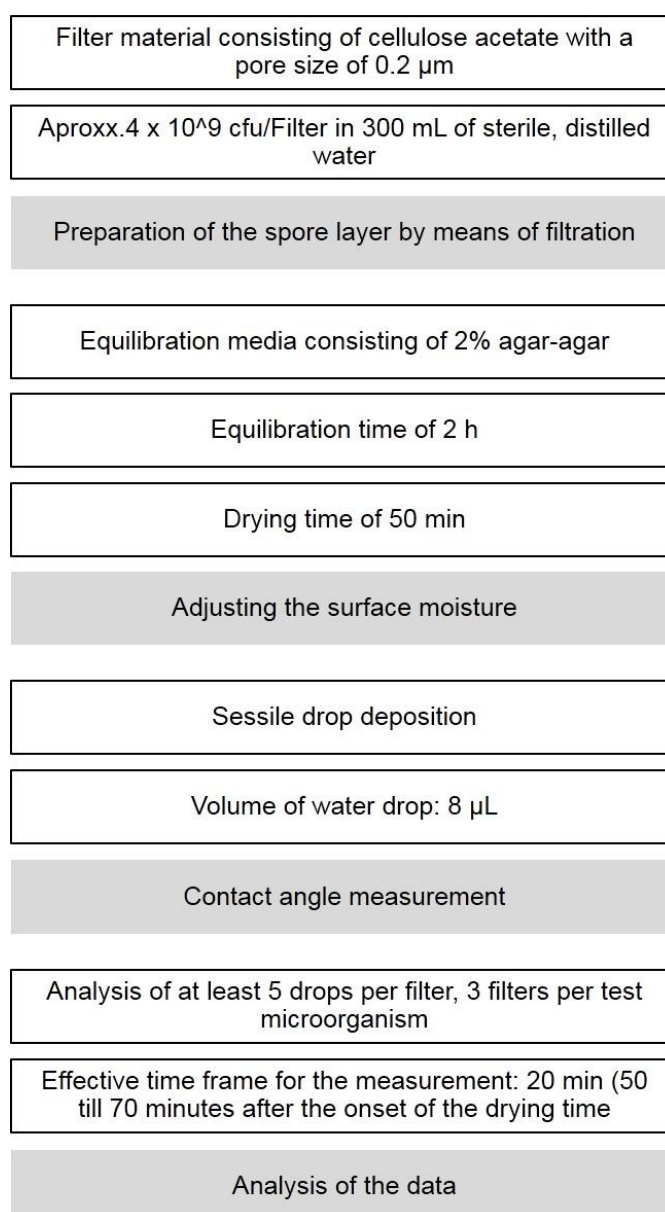


Figure 4-1: Standard procedure for water contact angle measurement of bacterial spores. Grey fields: Working step. White fields: Detailed operating procedure.

This standard measurement procedure was consequently applied for all further water contact angle measurements throughout this thesis.

Generation of spores with the same genetic setup however with different surface hydrophobicity

To eliminate further influencing factors such as different composition of the spore coat e. g., the ideal are microorganisms of the same species but with different surface hydrophobicity. Eschlbeck et al. (2017) showed that it is possible to modify the surface hydrophobicity of *B. subtilis* spores of the same strain and species by means of cultivation conditions. Resulting water contact angles of *B. subtilis* spores varied between 50 ° and 95 °.

As such high water contact angles of 95 ° were only achieved in one spore batch and the average was 80 °, supposedly further influencing factors such as the amount of oxygen which is influenced by the number of microorganisms in the bioreactor, but also the amount and distribution of antifoam agent have an influence on surface hydrophobicity.

However, there is a major influencing factor prohibiting the application of the obtained spores to examine the influence of surface hydrophobicity. In accordance with the results of Melly et al. (2002b), the results show that cultivation conditions influence the resistance towards liquid H₂O₂ as well. Spores cultivated at different pH-levels show varying resistance against liquid H₂O₂.

Inactivation with gaseous and condensing H₂O₂ is a process including inactivation with pure gas but also inactivation with liquid H₂O₂ during the condensation phase. Independent of the inactivation behavior with gaseous H₂O₂, the inactivation results depend on the inactivation with the condensate as well. With spores of varying resistance properties, it is not possible to investigate the influence of surface hydrophobicity without the influence of spore resistance properties.

Concluding this part of the thesis, the results show that it is possible to cultivate bacterial spores that usually show rather small water contact angles (Garry et al. 1998) in a way, that high water contact angles and thus a high surface hydrophobicity can be achieved. However, it is not possible to evaluate the influence of surface hydrophobicity by comparing the inactivation kinetics of spores of the same species but different surface hydrophobicity as their resistance is different as well.

Investigation of the influence of carrier material surface hydrophobicity on the inactivation of bacterial spores

With the investigation of the influence of surface hydrophobicity as stated in the title of this thesis, not only the influence of spore surface hydrophobicity is included but also the influence of the material surface hydrophobicity. First, the inactivation with condensate formation will be discussed.

In theory, the inactivation of a hydrophilic spore on a more hydrophobic surface begins with condensate formation on the hydrophilic spore. On the one hand, microorganisms as irregularities on the surface serve as condensation nuclei, on the other hand, the spore is more hydrophilic than the carrier material. The test materials that were chosen in Eschlbeck et al. (2018a) differ concerning their water contact angle between 30 ° and 115 °. The results show that the most hydrophobic surface leads to the fastest inactivation of *B. subtilis* spores. For spores of *B. atrophaeus* which are more resistant towards H₂O₂, this effect can only be seen as a slight insignificant trend. However, for both spores it is very likely that condensate engulfed the spores first, then deposited on the hydrophobic test material surface. As the condensate has a high H₂O₂ concentration of approximately 60% (calculation according to Parks and Watling (2004)), the inactivation effect of the condensate is intense.

On the more hydrophilic surface, however, spores are still condensation nuclei as they represent surface irregularities but the surface is more hydrophilic than both spore species. Therefore, condensation takes place on the test surface as well as the spores which results in less condensate around the spores and thus slower inactivation.

The inactivation without condensate formation starts theoretically with reversible adsorption of the gas molecules with the highest polarity on the most hydrophilic surface which can be either the spore or the respective carrier material. If the carrier material is hydrophilic, the amount of adsorbing H₂O₂ molecules is higher compared to less hydrophilic carrier materials and as the adsorbed molecules are not available in the gas anymore, inactivation should be less intense. However, the results obtained by Eschlbeck et al. (2018b) show no clear correlation between surface hydrophobicity of the carrier materials and bacterial spore inactivation kinetics.

One explanation might be the abrupt ending of the decontamination which results in a concentration gradient between the environment without H₂O₂ and the surface with adsorbed H₂O₂ molecules. This leads to desorption of the molecules and, for a very short time, to a high concentration of H₂O₂ molecules close to the surface. Therefore, the observed similar inactivation effect of both spores on the most hydrophilic surface can be explained, independent of their specific resistance against gaseous H₂O₂.

Rogers et al. (2005) also investigated the influence of the surface on the inactivation kinetics of bacterial spores with hydrogen peroxide gas. They observed severe differences in spore inactivation depending on the porosity of the surface whereat more porous surfaces resulted in less inactivation success. Grand et al. (2010) found an influence of surface wettability in their studies. However, they attributed the differing inactivation results to the different adhesion of the spores to the surface. Spores that adhere better might expose less surface to the condensate is the explanation they anticipated for the least inactivation of spores on glass which is hydrophilic and non-porous.

The influence of the test material surface hydrophobicity was also investigated by Sigwarth and Stärk (2003). For their study, they applied contact angle measurement with 40% alcohol. Therefore, the results are not consistent with the results of Eschlbeck et al. (2018a) and Eschlbeck et al. (2018b). Besides, they pipetted the microorganism suspensions on the surface, which is another difference. Sigwarth and Stärk (2003) observed severe differences in the inactivation results and stated, consistent with Eschlbeck et al. (2018a) and Eschlbeck et al. (2018b), that the influence of surface hydrophobicity has a major importance on the inactivation with gaseous and condensing H₂O₂.

Concluding, there is an influence of test material surface hydrophobicity that has to be considered for BI production. The intensity of an inactivation depends on the surface on which the microorganisms are present. For validation purposes, this factor has to be critically considered and a relevant test material has to be chosen.

Investigation of the impact of bacterial spore surface hydrophobicity on the inactivation with gaseous H₂O₂

On the surface of packaging materials, there is not only one species of microorganisms but usually a mixed population with varying cell structure and thus different surface characteristics. For a dry H₂O₂ vapor process, the results show, that surface hydrophobicity of the microorganisms measured by water contact angle measurement does not correlate with the inactivation kinetics. According to Finnegan et al. (2010), gaseous H₂O₂ has a higher kinetic energy compared to liquid H₂O₂, therefore erupts deeper into the spore structure to affect the spore by oxidation. As water contact angle measurement only depicts the surface hydrophobicity of the outermost spore layer, this might be the explanation why no correlation occurs for gaseous H₂O₂.

This is in contrast to the gaseous H₂O₂ process with condensate formation, where condensate as liquid H₂O₂ affects the spores. As described by Marcos-Martin et al. (1996), Hall et al. (2007) and Pruss (2013), condensate starts to form at the most hydrophilic condensation nuclei. For a mixed microorganism population with different surface

hydrophobicity, the more hydrophilic ones should be inactivated faster compared to the more hydrophobic organisms.

In contrast to the results obtained by Pruß et al. (2012), the results of this thesis were obtained by application of a mixed spore population. Simultaneous detection of two different microorganisms during one inactivation cycle was done by choosing two spore formers that depict completely different colony growth on agar plates.

The results of the mixed population inactivation in comparison to the inactivation of only one species on the same material show only insignificant differences independent of the test material. These results seem odd at first as there is an influence of the test material surface hydrophobicity. However, the surface of the test material compared to the surface that the applied spores represent is a lot bigger. For an amount of $1 \cdot 10^6$ cfu/test material, an average spore size of $1.417 \mu\text{m}$ (Eschlbeck and Kulozik 2017) and assuming spores are spheres, the surface of all spores together adds up to 6.305 mm^2 , calculated as shown in (4-1).

$$A = 10^6 \cdot 4\pi r^2 \quad (4-1)$$

With A as surface of all spores and r as spore radius.

In contrast, the surface of the test material adds up to 2200 mm^2 (size of $40 \text{ mm} \cdot 55 \text{ mm}$) which is more than 300 times the size of the spore surface. This difference in dimensions might be an explanation for the influence of test material surface hydrophobicity but not spore surface hydrophobicity.

5 Conclusion

Hydrogen peroxide in its gaseous form is often applied in clean rooms and for the decontamination of packaging materials. For clean rooms, formation of condensate is unfavorable due to possibly present electric devices. Therefore, condensate formation is avoided. However, for packaging decontamination, gaseous H_2O_2 is deployed with and without condensate formation.

Most of the applied packaging material surfaces such as carbon materials with polyethylene surface depict hydrophobic characteristics. The obtained results show that surface hydrophobicity of carrier materials has a major influence on the decontamination with gaseous H_2O_2 with and without condensate formation but bacterial surface hydrophobicity has not. From an industrial point of view, this is a favourable outcome as the surface of packaging materials can be influenced or varied, the contamination of the surface consisting of microorganisms with different surface characteristics however has to be dealt with.

Prior to the implementation of a new decontamination device, the inactivation efficiency has to be verified by means of BI. Of course, the surface of the carrier material has to be very similar to the actual product in terms of hydrophobicity. A test material surface hydrophobicity that is higher than the hydrophobicity of the actual material might result in an unrealistically fast inactivation for condensing H_2O_2 . Vice versa, the inactivation results might be less intense than required. Both incorrect findings lead to cost-intensive consequences, either because the inactivation conditions are intensified or because the actual inactivation is not successful and, in the worst case, a recall has to be executed.

Surface hydrophobicity of the test organisms only plays an insignificant role but still, the cultivation conditions of the BI are of major importance. The test organisms' resistance changes depending on cultivation conditions. A standardization of BI resistance respectively the establishment of a standard resistance for the particular inactivation method is of major importance to create reliable and reproducible inactivation results.

6 Summary / Zusammenfassung

6.1 Summary

For aseptic packaging processes, decontamination with gaseous H_2O_2 with or without condensate formation is a commonly applied process. For condensate formation, an energy barrier has to be crossed for the phase transition from gas to liquid condensate. Condensation nuclei lower this energy barrier. Microorganisms are irregularities of the surface and can thus act as condensation nuclei. Condensate begins to form at the most hydrophilic condensation nuclei, therefore microorganisms with high surface hydrophobicity might be less inactivated by condensate formation than microorganisms with low surface hydrophobicity. Similarly, gaseous H_2O_2 molecules adsorb preferably on a hydrophilic surface.

It was the aim of this thesis to investigate the influence of surface hydrophobicity of bacterial spores and test material on the inactivation with gaseous H_2O_2 with and without condensate formation. The approach consisted of establishing a measurement method to quantify bacterial spore surface hydrophobicity followed by the generation of spores of the same genetic setup but with different surface hydrophobicity. With those results, the investigation of the influence of carrier material and spore surface hydrophobicity on the inactivation with gaseous H_2O_2 was carried out.

Two important critical points for water contact angle measurement were investigated and a measurement procedure was established. Cultivation of *B. subtilis* spores with different static pH-valued resulted in spores of different surface hydrophobicity however also with different resistance against liquid H_2O_2 . The results clearly showed that test material surface hydrophobicity influences the inactivation of bacterial spores. For a process with condensate formation, higher the surface hydrophobicity of the test material leads to increased condensate formation around the spores and an intensified inactivation. Carrier material surface hydrophobicity during a dry H_2O_2 gas process also has a significant impact on the inactivation of bacterial spores as H_2O_2 molecules tend to adsorb to the most hydrophilic surface. Nevertheless, no clear correlation could be observed for this mode of action. Surface hydrophobicity of bacterial spores however only has an insignificant influence on the inactivation results which might be due to the difference in test material surface size compared to the spore surface size. Concluding from this thesis, surface hydrophobicity of the carrier material is of major importance in packaging decontamination but also for BI production with gaseous H_2O_2 . Surface hydrophobicity of bacterial spores can be modified by means of cultivation conditions, however, resistance of bacterial spores also changes. This is of major importance of production of BI as they are applied to verify inactivation processes. Therefore, further research should be done in the field of standardized BI production.

6.2 Zusammenfassung

Aseptische Verpackungsprozesse werden häufig mit gasförmigem H_2O_2 mit oder ohne Kondensatbildung realisiert. Der Vorgang der Kondensatbildung ist mit einem Phasenübergang verbunden, für den Energie benötigt wird. Kondensationskeime setzen die benötigte Menge an Energie herab und Mikroorganismen, die als Unebenheiten auf der Oberfläche vorliegen, können als Kondensationskeime fungieren. Das Kondensat bildet sich zuerst am hydrophilsten Kondensationskeim, dadurch werden Mikroorganismen mit vergleichsweise hydrophober Oberfläche möglicherweise weniger stark vom Kondensat betroffen und weisen infolgedessen ein höheres Überleben auf als hydrophile Keime. Ähnlich verhält es sich für einen H_2O_2 Prozess ohne Kondensatbildung, die H_2O_2 Gasmoleküle adsorbieren bevorzugt an der hydrophilsten Oberfläche.

Ziel dieser Arbeit war es, den Einfluss der Oberflächenhydrophobie auf die Inaktivierung mit gasförmigem H_2O_2 zu untersuchen. Der Ansatz hierfür beinhaltete die Etablierung einer verlässlichen Messmethode, um die Oberflächenhydrophobie der Sporen zu quantifizieren. Weiterhin sollten Sporen des gleichen genetischen Setups bei unterschiedlicher Oberflächenhydrophobie hergestellt werden, um anschließend den Einfluss der Oberflächenhydrophobie von Trägermaterial und bakteriellen Sporen auf die Inaktivierung zu eruieren.

Bei der Wasserkontaktwinkelmessung wurde der Einfluss von zwei Arbeitsschritten kritisch untersucht und eine Messmethode für das weitere Vorgehen festgelegt. Durch Kultivierung bei verschiedenen, statischen pH-Werten konnten *B. subtilis* Sporen des gleichen genetischen Setups mit zugleich verschiedener Oberflächenhydrophobie hergestellt werden. Die Resistenz gegenüber flüssigem H_2O_2 wurde ebenfalls durch die Kultivierung beeinflusst. Die Inaktivierungsergebnisse zeigen deutlich den Einfluss der Oberflächenhydrophobie des Trägermaterials. Je höher die Hydrophobie des Materials, um so schneller bildet sich Kondensat an bzw. um die Sporen und um so intensiver verläuft die Inaktivierung. Auch bei der rein gasförmigen Inaktivierung gibt es einen signifikanten Einfluss der Oberflächenhydrophobie des Trägermaterials, jedoch keine klare Korrelation. Die Oberflächenhydrophobie der Sporen hat hingegen lediglich einen marginalen Einfluss auf die Inaktivierung. Dieses Ergebnis ist möglicherweise durch den Flächenunterschied zwischen der Fläche des Testmaterials und der Sporenoberfläche begründet. Zusammenfassend wurde im Rahmen dieser Arbeit gezeigt, dass die Oberflächenhydrophobie des Trägermaterials einen wichtigen Einflussfaktor bei der Packstoffentkeimung mit gasförmigem und kondensierendem H_2O_2 darstellt. Gleiches gilt für die Herstellung von Bioindikatoren. Die Oberflächenhydrophobie von bakteriellen Sporen kann durch die Kultivierungsbedingungen beeinflusst werden, zugleich verändert sich jedoch auch die Resistenz der Mikroorganismen. Dieser Punkt ist von äußerster Wichtigkeit für die Herstellung von verlässlichen BI, da sie den prüfenden Schritt darstellen, bevor eine neue

Anlage in Betrieb genommen wird. Entsprechend ist die weiterführende Forschungstätigkeit im Bereich der Standardisierung von Bioindikatoren von essentieller Bedeutung.

7 References

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8 Appendix

8.1 Peer reviewed publications

Eschlbeck, E. and Kulozik, U. (2017) Effect of moisture equilibration time and medium on contact angles of bacterial spores. In: *Journal of Microbiological Methods* 135, 1–7.

Eschlbeck, E., Bauer, S.A.W. and Kulozik, U. (2017) Effect of cultivation pH on the surface hydrophobicity of *Bacillus subtilis* spores. In: *AMB Express* 7, 157.

Eschlbeck, E., Seeburger, C. and Kulozik, U. (2018) Influence of spore and carrier material surface hydrophobicity on decontamination with condensing hydrogen peroxide vapor. In: *Journal of Applied Microbiology* 124 (5), 1071 – 1081.

Eschlbeck, E., Seeburger, E. and Kulozik, U. (2018): Influence of carrier material surface hydrophobicity and roughness on a dry hydrogen peroxide vapor inactivation process. In: *Journal of Food Science* **submitted**.

8.2 Non reviewed publications

Eschlbeck, E.; Kulozik, U. (2013): Beeinflussung der Oberflächenhydrophobizität von bakteriellen Sporen durch verschiedene Anzuchtbedingungen, Jahresbericht zur Milchwissenschaftlichen Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL).

Eschlbeck, E.; Kulozik, U. (2014): Erhöhung der Wasserstoffperoxidkonzentration im Entkeimungsgas bewirkt bei Kondensatbildung keine verbesserte Inaktivierung bakterieller Sporen, Jahresbericht zur Milchwissenschaftlichen Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL).

Eschlbeck, E.; Kulozik, U. (2015): Einfluss des Fermentations-pH-Wertes auf die Resistenz von *Bacillus subtilis* Sporen gegenüber flüssigem Wasserstoffperoxid, Jahresbericht zur Milchwissenschaftlichen Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL).

Eschlbeck, E., Kulozik, U. (2016) Konzentrationsabhängiger Vergleich der Inaktivierung von *Bacillus subtilis* Sporen mit gasförmigem und kondensierendem Wasserstoffperoxid, Jahresbericht zur Milchwissenschaftlichen Forschung am Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL).

8.3 Oral presentations

- Eschlbeck, E, Pruss, K, Kulozik, U: Einfluss der Oberflächenhydrophobizität biologischer Indikatoren auf die Inaktivierung mit kondensierendem Wasserstoffperoxid, Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Freising, 26. – 28. Februar 2014
- Eschlbeck, E, Kulozik, U: Effect of microbial surface properties on adhesion properties to solid and physiological surfaces, Technology Seminar Weihenstephan – Food Bioprocessing: New functionalities through production, concentration and stabilization of biologically active components, Freising, 17. – 19. September 2014
- Eschlbeck, E, Kulozik, U: Einflussfaktoren auf die Inaktivierung von Mikroorganismen auf Packstoffoberflächen mittels gasförmigem Wasserstoffperoxid - Erzeugung bakterieller Sporen im Submersverfahren, Treffen des Arbeitskreises „Schnittstellenproblematik bei Aseptikanlagen“ des VDMA, Frankfurt am Main, 17. Oktober 2014
- Eschlbeck, E, Kulozik, U: Decontamination with gaseous H₂O₂ depends on bacterial surface hydrophobicity, 8th International Congress of Food Technologists, Biotechnologists and Nutritionists, Opatija / Kroatien, 21.-24. Oktober 2014
- Eschlbeck, E, Pruß, K, Kulozik, U: Decontamination with gaseous H₂O₂ depends on bacterial surface hydrophobicity, 28th EFFoST International Conference | 7th International Food Factory for the Future Conference, Uppsala / Schweden, 25.-28. November 2014
- Eschlbeck, E., Bauer, S.A.W., Kulozik, U.: Einfluss der Oberflächenhydrophobizität des Trägermaterials auf die Inaktivierung mit gasförmigem, kondensierendem H₂O₂, Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Magdeburg, 16.-18. März 2015
- Eschlbeck, E., Bauer, S.A.W., Kulozik, U.: Influence of bacterial surface hydrophobicity on the decontamination process with gaseous and condensing H₂O₂., ICEF 12 – International Congress on Engineering and Food, Québec City, Kanada, 14.-18. Juni 2015
- Eschlbeck, E., Bauer, S.A.W., Kulozik, U.: Influence of bacterial surface hydrophobicity on the decontamination process with gaseous and condensing H₂O₂. Innovations in Food Packaging, Shelf Life and Food Safety, Erding, 15.–17. September 2015
- Eschlbeck, E., Bauer, S.A.W., Kulozik, U.: Influence of surface hydrophobicity of the carrier material on the inactivation of bacterial spores with gaseous, condensing hydrogen peroxide, 29th EFFoST International Conference, Athen, Griechenland, 10.-12. November 2015
- Eschlbeck, E., Kulozik, U. Methods to manipulate the adhesion ability of the probiotic bacterial spore former *Bacillus subtilis*. Technology Seminar Weihenstephan –

Seminar on Emerging Dairy Technologies: Sustainability, Novel Nutritional Concepts, New functionalities and Structure, Freising, 14. – 16. September 2016

8.4 Poster presentations

Eschlbeck, E, Bauer S.A.W., Kulozik, U: Manipulation der Oberflächenbeschaffenheit bakterieller Sporen im Bioreaktor, Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Erlangen, Germany, 10 - 11. März 2016

Eschlbeck, E., Kulozik, U.: Decontamination with gaseous H₂O₂: Influence of surface hydrophobicity, 18th World Congress of Food Science and Technology (IUFoST), Dublin, Ireland, 21. – 25. August 2016