

Sterilization of Objects, Products, and Packaging Surfaces and Their Characterization in Different Fields of Industry: The Status in 2020

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The treatment method to deactivate viable microorganisms from objects or products is termed sterilization. There are multiple forms of sterilization, each intended to be applied for a specific target, which depends on—but not limited to—the thermal, physical, and chemical stability of that target. Herein, an overview on the currently used sterilization processes in the global market is provided. Different sterilization techniques are grouped under a category that describes the method of treatment: radiation (gamma, electron beam, X-ray, and ultraviolet), thermal (dry and moist heat), and chemical (ethylene oxide, ozone, chlorine dioxide, and hydrogen peroxide). For each sterilization process, the typical process parameters as defined by regulations and the mode of antimicrobial activity are summarized. Finally, the recommended microorganisms that are used as biological indicators to validate sterilization processes in accordance with the rules that are established by various regulatory agencies are summarized.

or active deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).^[1,3] Disinfection is used to inactivate viable microorganisms to a level previously specified as being appropriate for a defined purpose.^[1] The validated processes that are used to render the product free from viable microorganisms are termed sterilization.^[1] In other words, disinfection is a process that reduces microbial load, whereas sterilization completely removes or deactivates all present microorganisms, and upon process success the product is called sterile.^[1,4] In this context, the viability of a microorganism is defined by its ability to reproduce under favorable conditions.^[5,6] Both disinfection and sterilization methods are a part of the biodecontamination process.^[7]

1. Introduction


Biological contaminants (biocontaminants) are either cells or biological entities other than the intended components present in the product.^[1] These contaminants can be present on a packaging surface or in a product prior to filling, which lead to potential consumer health issues or product damage. Cells or microbial life include viruses, bacteria, bacterial spores, fungi, protozoa, multicellular parasites, and contaminating eukaryotic cells.^[2] Biological entities include aberrant proteins (prions), endotoxins,

Generally, sterilization methods are used for a variety of purposes in different industrial sectors. With relation to consumer products, the goal of sterilization differs from one industrial sector to another, as follows: 1) “medical and healthcare”: It is used to prevent the (re-)transmission of microbes and diseases to patients and assure the optimal therapeutic activity of a product. In addition, it is to produce critical products that may come in direct contact with sterile tissues without the risk of infection. These include, for example, all sorts of medical implants, syringes, catheters, medical probes, wound dressings, and band-aids; 2) “pharmaceutics”: It is used to produce critical products that are free of biocontaminants, which can cause health issues to the patient. Here, critical products are defined as being those that come in direct or indirect contact with the sterile tissues of patients. These include, for example, fluids for inhalation (asthma patients), instillation into the eyes (glaucoma patients), and all medical injections; 3) “food and beverages”: It is used to produce biologically stable products with a long shelf-life without the addition of conservatives or the requirement of a cool chain. Also, it is to maintain the organoleptic properties of the product and prevent harmful biocontaminants, which cause health issues when they find their way to the end consumer; 4) “cosmetics”: It is used for the production of biologically sensitive products free of biocontaminants and the maintenance of hygienic standards set for critical nonsingle-use instruments such as lipsticks, creams, and eyeliners.

Hence, the overall goal of a typical sterilization process is the elimination of undesired contaminants that can cause damage (of any type) to a product or can cause health issues to a consumer.

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Contaminants can be “intrinsic,” meaning that they are already present in the untreated (raw) product or in/on packaging materials prior to processing, or “extrinsic,” such as those introduced by contamination during handling or manufacturing process.^[1,8] In terms of packaging of biologically sensitive products such as pharmaceuticals or food and beverages, or the handling of nonsingle-use surgical equipment, the target object is either terminally sterilized in its final package or aseptically filled.^[9,10] **Figure 1** shows the differences between these two packaging principles. Here, the choice of the principle depends on the type of the processed product and the packaging material and the thermal, physical, and chemical compatibility with the applied sterilization process.^[11] For example, during the production of canned food, such as soups, the filled cans (product and package) are terminally sterilized by heat (see **Figure 1a**). This heat is also used to cook-in the food inside the package.^[12–15] Terminal sterilization methods are also used in the consumer and medical fields, for example, for the sterilization of contact lenses and implants. Moreover, consumer or medical products such as milk or vaccines that contain essential proteins in their formulation, or those that are packaged in a heat- and radiation-sensitive material, cannot be sterilized using terminal sterilization processes. Here, aseptic filling techniques are used instead,^[11,12,16–18] see **Figure 1b**.

The concept of aseptic filling machines is to sterilize the package and the product separately and later combine them in a separate step under aseptic conditions.^[19] For this, the machine components in contact with products and packaging materials are sterilized by a validated sterilization process, e.g., using heat or chemicals.^[20–22] The raw product, such as milk or juice, is sterilized by heat or decontaminated by sterile (ultra-)filtration or bactofugation process.^[23–26]

In closed-system bactofugation, the product is filled into a rotating system where microorganisms heavier than the product are separated to the system’s outer rim by the acting centripetal

force.^[27] The sterile product is then filled into the sterile container followed by sealing under controlled and regulated aseptic conditions to prevent extrinsic recontamination.^[1,21,28,29] The aseptic region where the aseptic filling takes place is called “aseptic zone,” as shown in **Figure 1b**.

We can deduce from the previous examples that different products and packaging material require different sterilization techniques. As such, there is no standard, universal sterilization procedure in the market.^[11,30,31] Furthermore, the choice of the most appropriate sterilization process is additionally challenged by the inherent resistivity of present biocontaminants.^[32,33] These facts give rise to different sterilization processes that differ in terms of application, choice of the sterilizing agent (sterilant), and operation conditions.

In this article, we will give an overview of preferred surface sterilization processes for packaging materials and products applied in healthcare, food, and beverage industries. A list of these sterilization processes is shown in **Figure 2**; each process is placed into a describing category: radiation based, thermal, and chemical.^[19,34] Generally, these sterilization principles are also valid for the pharmaceuticals and cosmetics industry. The Review later focuses on the chemical sterilization process that uses hydrogen peroxide as the active sterilant and describes different technologies that are available in the market. At the end of this article, we will explain typical validation test methods for determining and quantifying the effectiveness of the sterilization process and development efforts in this perspective.

1.1. Factors Affecting the Sterilization Efficacy and Considerations on the Design of Sterilization Processes

The purpose of every sterilization system is to inactivate all viable microorganisms, which can cause health risks to patients (healthcare) or consumers (food and beverage) or can lead to a decrease in product quality and stability.^[11,35,36] To achieve

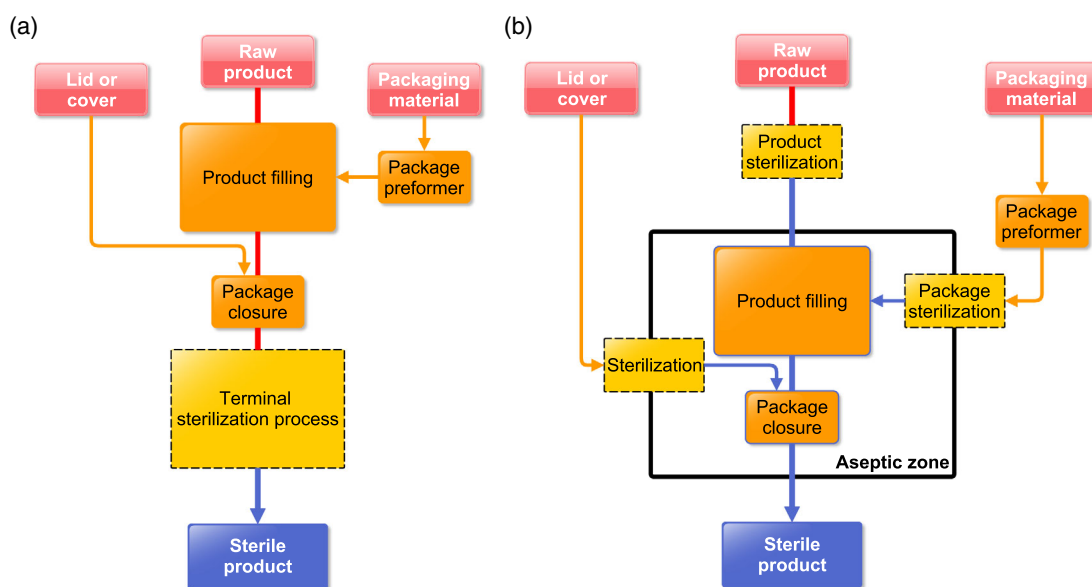


Figure 1. Flowchart portraying the design of a filling machine with a) terminal sterilization concept and b) setup of an aseptic filling machine.

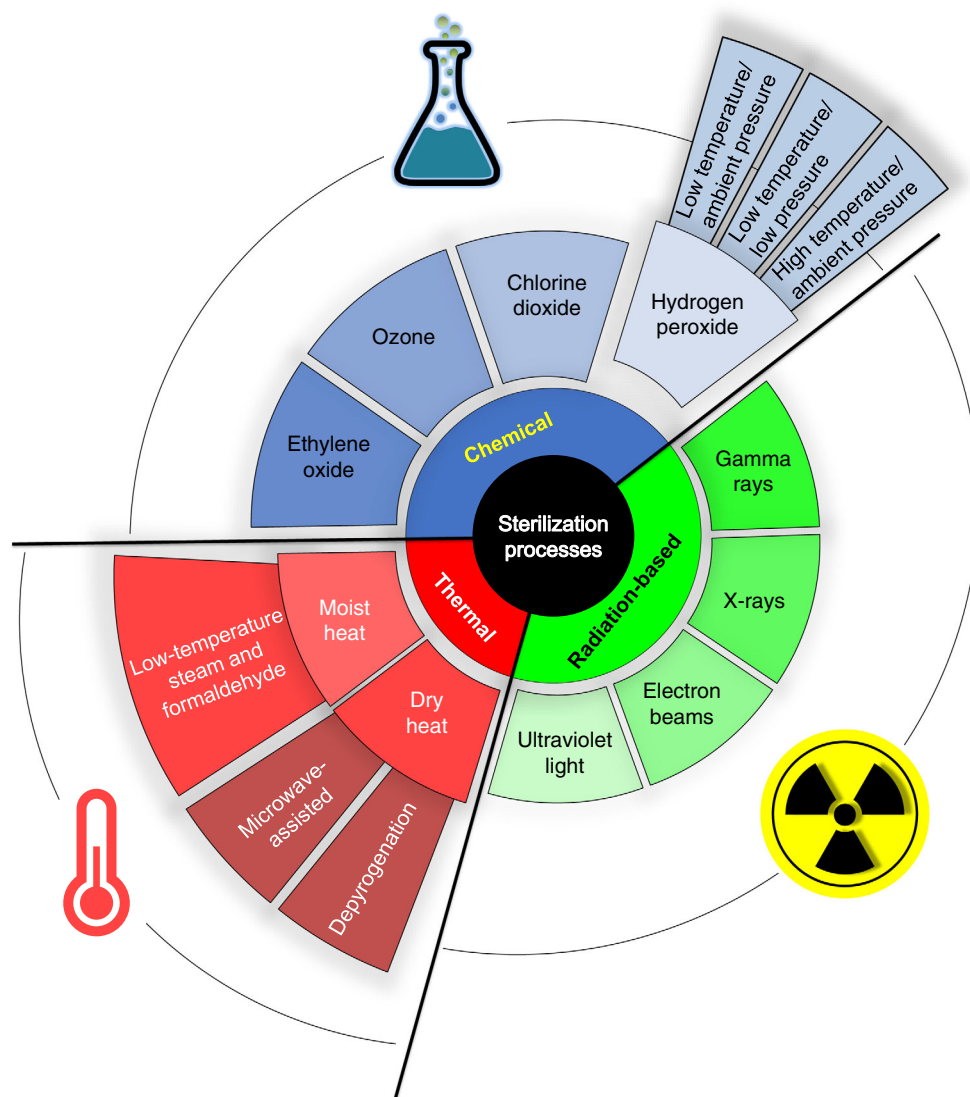


Figure 2. Overview of typical sterilization processes used in various fields of industry that will be described in this article. The outer shell of the graph is a derivative application technology of the sterilant.

these goals, the sterilization process must be correctly chosen and set up for the intended target(s) of sterilization.^[37–40] There are multiple factors that impact the design of the process and its efficacy, which are the type of biocontaminants, the location of bioburden, the surrounding conditions, and the exposure time to the sterilant.^[11,23,24,30,41–44]

In this respect, the types of microorganisms that are present in a product, or on the surface, and environmental factors that influence their proliferation have to be taken into account when defining the running parameters of a sterilization process.^[16,26,30,32,45] Typically, a higher bioburden level requires a higher sterilization dose by applying the sterilant at a higher concentration, or by increasing the dwell time, or a combination of both.^[40,46] Also, the distribution of microorganisms on the surface and the geometry of the sterilization target affect the sterilization efficacy. For example, the sterilization of packages that contain

crevices, joints, and channels is more challenging than flat surfaces.^[31,47] In addition, surfaces that are covered with a biofilm, e.g., postsurgical instruments with visible organic residue of blood and tissue, require presterilization cycles such as washing to clean the soiled surface and decrease the microbial load to achieve the intended sterilization goal.^[31,40,48] When it comes to microorganisms, having a prior knowledge of the expected type is advantageous to determine the most effective sterilization procedure and sterilant. For example, if the bioburden releases and leaves traces of endotoxins after a certain sterilization procedure, an additional depyrogenation cycle is required, as described in Section 3.1.^[30,45,49] Additional factors that must be considered include the operational and the surrounding conditions of the sterilization process. For instance, in gaseous sterilization processes, a high level of humidity and a low sterilant gas temperature can decrease the sterilization efficacy.^[23,43]

Besides the characteristics of the predicted type of bioburden, there are multiple concerns that affect the choice of the sterilization process or the applied sterilant. An increase in the dwell time of the sterilant increases the inactivation probability of the microorganism and with it the efficacy of sterilization.^[41,50] Nevertheless, assessment of the final sterilized product has to be conducted following a change in the treatment process.^[16,51] This includes organoleptic and nutritional changes to consumable products (food and beverages), chemical alterations to injectable pharmaceuticals (medical and healthcare), or physical and chemical alterations to the packaging material due to package-sterilant compatibility issues.^[46,52–54] Moreover, the operational and occupational safety of the applied sterilant must be considered in relation to its toxicological and environmental risks.^[7,41,55] These include 1) the time-weighted average (TWA) of dangerous gaseous chemical sterilants such as ethylene oxide, 2) the maximum occupational radiation dose limit, as well as 3) fire and explosive hazards related to the stability of chemical sterilants such as hydrogen peroxide. Consequently, the ability to monitor the sterilization process and sterilant is essential to assure a safe working environment and the production of safe final products. Finally, the removal or reduction of the level of residual sterilant from surrounding equipment and package or target surfaces to the limits set by regulations and standards is crucial before the commissioning of the sterilization process and system.^[41]

To call a product “sterile” does not mean absolute sterility as it is impossible to prove the absence of all microorganisms.^[30,56] Consequently, two terms and their values are defined in regulations and industries: spore log reduction (SLR) and sterility assurance level (SAL). The SLR provides a measure of the effectiveness of the sterilization process and is expressed by a logarithm with a base of 10.^[1] Each 1-log reduction represents a 90% decrease in the number of spores in the culture. SLR value is a predefined target by regulations and standards that describes the efficacy of a sterilization process and is validated from microbiological challenge tests (explained in Section 5). Here, a 6-log reduction for indicator microorganisms is typically assumed sufficient to achieve sterility.^[21,41,45] Other regulatory bodies define a 4-log reduction as the minimum requirement for an aseptic filling machine.^[57] SAL describes the probability that one microorganism is present on an item after sterilization (i.e., the probability of unsterility) and is expressed as a negative exponent to the base of 10.^[1] SAL is a target defined by industry that provides an overall assurance that the produced product is safe for the consumer. An SAL target better than 10^{-6} means that a chance for a contaminated product is 1 in 10^6 , which is a well-accepted overall target in industry.^[21,36,58,59]

Therefore, for a sterilization system to show an n -log SLR, it must be able to reduce a microbiological culture from an initial of 10^x colony-forming units (CFU) to 10^{x-n} CFU (e.g., 4-log SLR decreases a 10^6 CFU culture to a 10^2 CFU). In addition, let us assume that throughout the lifetime of the sterilization system, 10^0 products will be produced with the probability that a maximum of 10^z products is nonsterile. Here, the SAL target for this sterilization system and packaging machine is 10^{z-y} . Altogether, for a microbial load of 10^4 per product and an SAL of 10^{-6} , the sterilization system must demonstrate a 10-log SLR to assure a

maximum of 1 product that is nonsterile throughout the lifetime of the system.

1.2. Establishing an Effective Decontamination Process

For an effective decontamination process, the sterilant must be in contact with the target for a specific dwell time. Therefore, besides the factors affecting the sterilization efficacy, the following points must be considered for an effective decontamination process:

- 1) a homogeneous and complete distribution of the sterilant on the target surface or in a product; 2) a constant concentration of the sterilant throughout the decontamination cycle; 3) a good penetration strength when porous materials such as cloths or foams are used; and 4) an adequate and constant contact time of the sterilant. The constant time is required to guarantee qualitative similarity in the sterilization efficacy between cycles; and 5) optimal operational conditions such as process and target temperature, partial pressure of the sterilant, and relative humidity.

Depending on the intended application, note that there are multiple factors which play a role in establishing a decision tree of the most appropriate sterilization process or sterilant.^[24,40,60–63] In addition, system and machine manufacturers aim to comply with all applicable regulatory requirements regarding the goal of the sterilization system and occupational safety standards. This is completed in the countries or jurisdictions, where the system or the product is intended to be distributed and the specifications are typically defined by the producer of the end product. Different organizations and agencies may have different standards, regulations, or recommendations depending on the type of packaged product. **Figure 3** shows examples of these agencies and organizations at the global scale.

2. Radiation Sterilization

Following the discovery of X-rays by Wilhelm Conrad Röntgen in 1895, the influence of X-rays on bacterial activity of *Vibrio cholerae* was recorded by Hermann Rieder in 1898.^[64–66] This was the same year that Marie and Pierre Curie discovered several radioactive elements including polonium and radium.^[67–69] These discoveries advanced the field of research on sterilization using radiation. The result was the introduction of electron beam sterilization on a commercial basis for medical products in 1956 by Ethicon, Inc. (a Johnson and Johnson affiliate).^[30,70] After that, the first gamma (γ) sterilization facility for plastic medical products was installed in 1960 in the United Kingdom.^[70] More than 60 years were required since the discovery of X-rays by Röntgen for radiation sterilization to advance to a point where it is used in industrial scale. The commercial application of gamma radiation for sterilization began in 1964 by Johnson and Johnson.^[71] Further advances hence followed in the field of radiation sterilization by gamma, electron beams, X-rays, ultraviolet (UV) light, and microwave-assisted pasteurization processes.^[54,72–74]

Even though the public acceptance for radiation-based processes decreased over the years due to safety concerns of ionizing radiation and increased public awareness, many conventional and advanced sterilization technologies are still based on it. A complete phasing out of gamma irradiation comes with possible

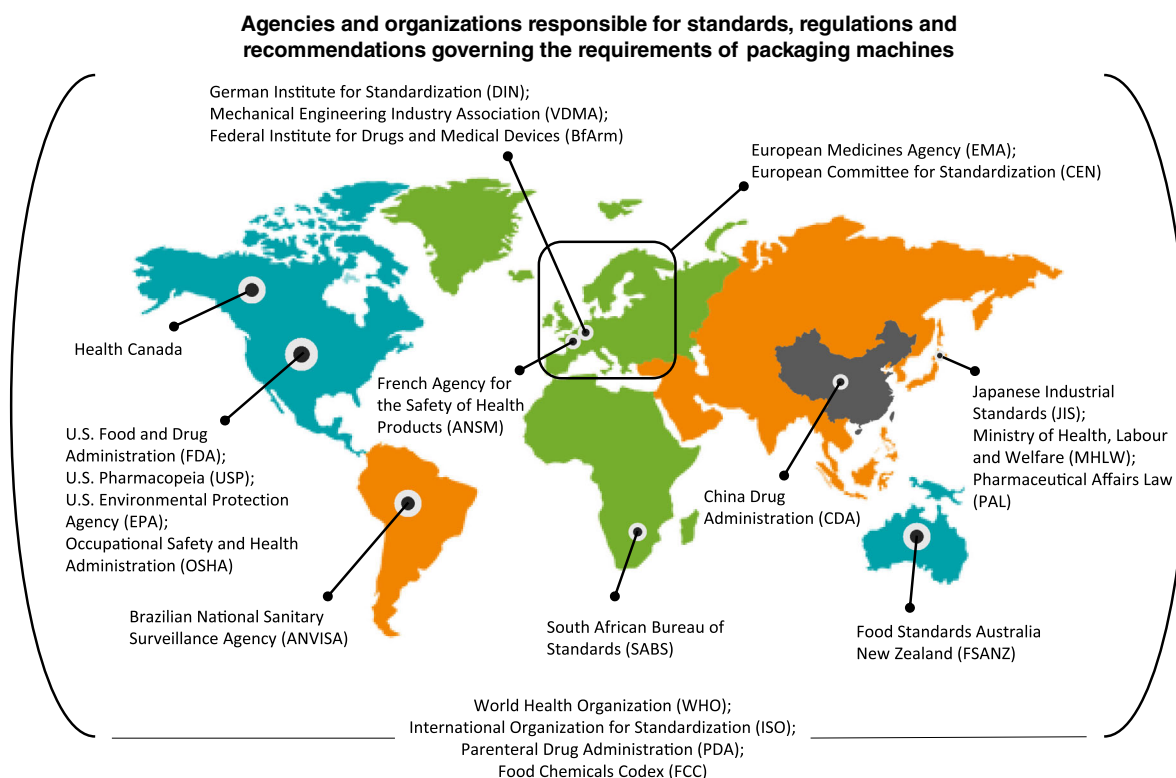


Figure 3. Examples of national and international agencies and organizations that are responsible for releasing standards, regulations, or recommendations governing the design and operation of packaging machines. Note that there are additional agencies, not mentioned here, that are responsible when it comes to certain, specialized types of products or sterilization methods.

consequences; a very recent study on this topic was published in 2018.^[75] In general, radiation sterilization can be applied to products before packaging such as dairy products or after packaging such as meat.^[76–78]

Gamma ray sterilization has been the conventional terminal sterilization method before the introduction of chemical sterilants or the development of hygienic filling standards. It is difficult to determine the number of facilities that currently apply gamma radiation for sterilization, but a 2004 report by the International Atomic Energy Agency (IAEA) placed this number close to 200 facilities worldwide.^[79]

Radiation sterilization facilities are still used in hospitals for medical purposes and as a pathogen control strategy in the food industry. A list of applications for radiation other than sterilization can be found in Encyclopaedia of Food Sciences and Nutrition.^[35] Compared with other sterilization methods such as heat, sterilization by radiation is characterized by short process time, its penetration capability through a wide range of substances, and simplicity of routine operation.^[77] Nevertheless, disadvantages of this type of sterilization include its initial setup and maintenance costs, incompatibility with some packaging materials, and safety concerns related to the disposal of radioactive waste.^[11,30,80–83]

The measurement of the amount of radiation absorbed by a material is expressed in kilo Grays (kGy), an equivalent unit to kJ kg^{-1} . This unit describes a calculated radiation dose per mass (kg) of the object for successful sterilization. In the health-care sector, a commonly applied radiation dosage is at least

25 kGy, which is achieved by a sterilization cycle that takes about 6–9 h at a normal dose rate of 4 kGy h^{-1} .^[12,72] The dose of 25 kGy is equivalent to the heat required to increase the temperature of 1 kg of water by about 6°C , which will mostly dissipate to the environment. Therefore, sterilization by radiation is considered a nonthermal sterilization process.^[84] Other radiation doses can be used depending on the sterilization target, for example, sterilization of frozen, packaged meats for National Aeronautics and Space Administration space flight programs requires a minimum dose of 44 kGy as stated in FDA title 21 of the Code of Federal Regulation part 179.26 (FDA 21 CFR 179.26).^[85] An extensive list of the recommended treatment dosage, dependent on the type of packaging materials, is provided by the FDA 21 CFR 179.45.^[85] By maintaining the recommended radiation dosage, material degradation caused by radiation can be controlled.^[77,86]

In this section, we will provide an overview on the currently used radiation-based sterilization processes in the market. These methods include the application of gamma rays, electron beams, X-rays, and UV light. The choice of the most effective radiation-based sterilization technique depends on multiple factors related to the properties of the product or package, as shown in **Figure 4**.

2.1. Gamma Rays

Gamma radiation, a form of ionizing radiation, became an established method for sterilizing single-use pharmaceutical, food,

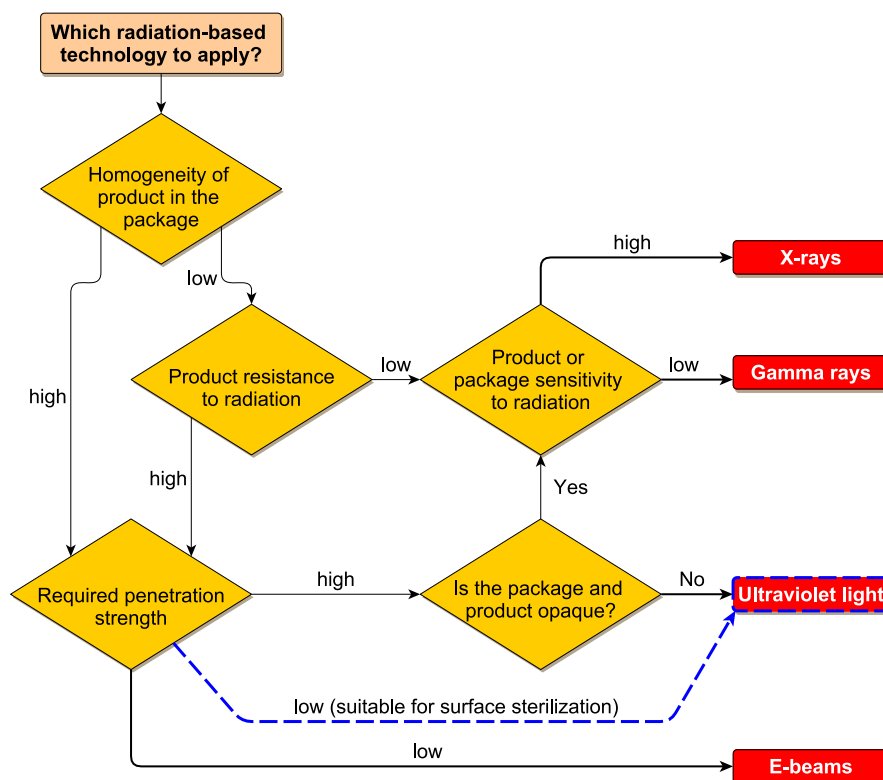


Figure 4. Flowchart that suggests the best-suited radiation-based sterilization technology depending on the properties of the sterilization target. UV light is a special case as it can be used for sterilizing clear fluids and opaque surfaces. Further details is available in the online resource by I.B.A Industrial and Sterilization Solutions.^[223]

and cosmetic products. It is characterized by its ease of use and compatibility with diverse packaging materials. The sterilization process occurs by exposing a product or package to a radioactive substance that releases high-energy photons. The interaction of the photons with the molecules of microorganisms leads to the destruction of cellular nucleic acid, breakage in DNA strands, and the inhibition of protein synthesis.^[12,30,87] The direct damage caused by the dissociation of DNA molecules from the high-energy radiation is further augmented with the formation of free radicals by irradiating water and other organic molecules.^[31,80] Studies also showed that gamma radiation results in a decrease in the level of endotoxins.^[72,88]

The most common radioactive nuclides used as a gamma radiation source for sterilization are cobalt-60, with cesium-137 on the second place. The latter is atypically applied as it is a fission byproduct of uranium in nuclear power plants.^[12,30] Cobalt-60 is industrially produced by converting cobalt-59, a nonradioactive metal, to cobalt-60 through neutron irradiation in a nuclear reactor.^[77] Cobalt-60 undergoes a beta decay with subsequent double-gamma decay of 1.17 and 1.33 MeV photon energy. Gamma radiation takes place at ambient temperature and is characterized by the highest penetration depth among the different radiation-based sterilization processes with an insignificant change to the object's temperature ($\leq 5^\circ\text{C}$).^[30,77] The penetration strength of gamma rays from a cobalt-60 source is about 30 cm for water substance, which decreases with an increase in the density of the target.^[80] **Figure 5** shows the penetration depth of all

types of radiation. Moreover, the advantage of gamma sterilization is the absence of toxic residues and relatively low operational costs of the facility.^[30,77]

One of the disadvantages of this type of sterilization is the requirement of a large technical facility that comes with a high capital investment.^[12] In addition, even if there is no restriction to the thermal and moisture stability of the packaging material, the use of gamma radiation comes with the risk of degradation of certain polymer-based packaging materials when the sterilization process is not correctly calibrated for the target material in use.^[12,81,82] The degradation of polymers is due to the formation of free chemical radicals inside the material that induces an alteration to the cross-linking, leading to, for instance, embrittlement and discoloration.^[11,30,52,81] Therefore, a list of recommended packaging materials and radiation dosages is given in FDA 21 CFR 179.45.^[85] Another possible side effect of gamma radiation includes the release of volatile chemicals that affect the organoleptic properties and shelf-life of the product.^[52,77] Consequently, a degradation assessment must be done after the sterilization and throughout the shelf-life of a product.

2.2. Electron Beams

Electron beam or e-beam sterilization uses a stream of accelerated electrons to deactivate surface microorganisms. To produce the high-energy e-beams, a particle accelerator is used where electrons are released from a heated filament (typically tungsten

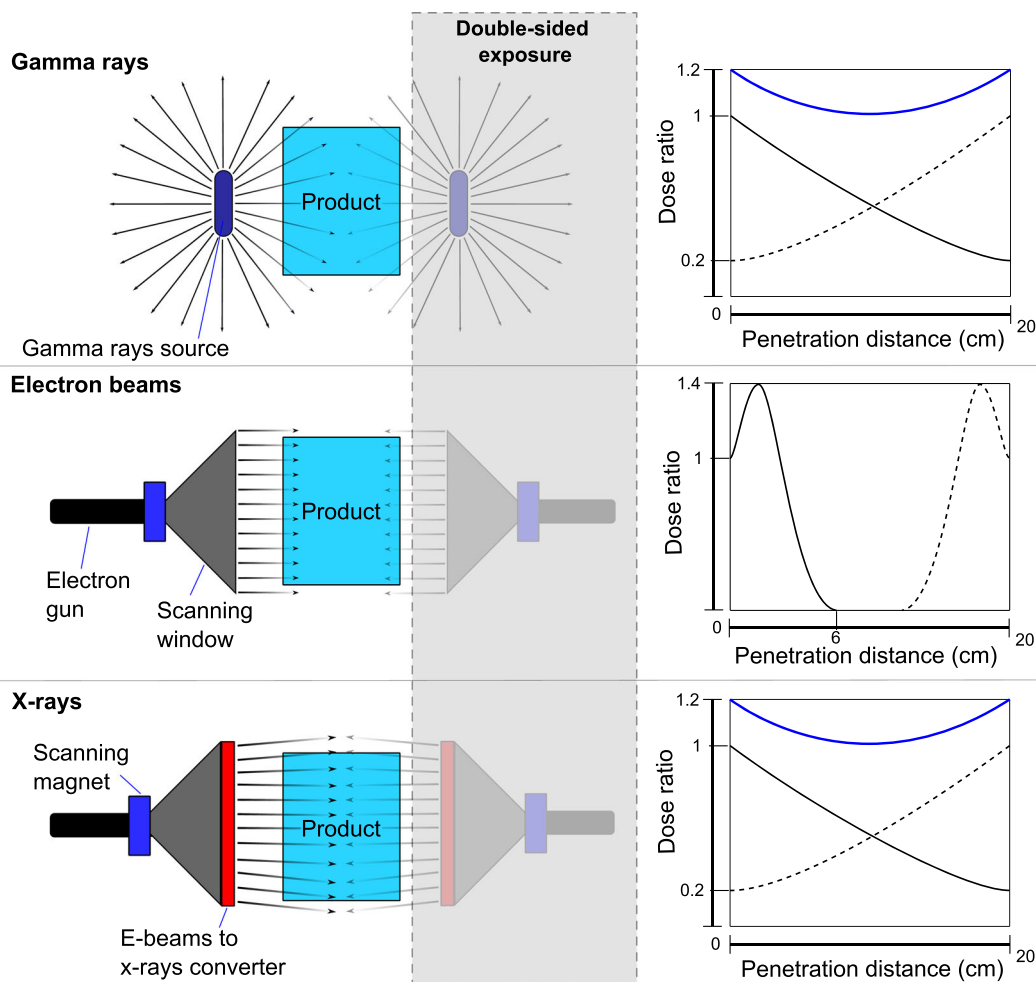


Figure 5. Schematic diagram of radiation-based sterilization methods depicting the radiation uniformity and the dose ratio in relation to its penetration depth. The dose ratio (y-axes) is the absorbed dose as a percentage of the irradiated value at the surface. The black curve represents the irradiation from the left side, the black dotted line is the irradiation from the right side (if double-sided exposure is applied), and the blue line is the sum effect of both sides. The penetration depth is inversely proportional to the density of the target and the figure shows the penetration through a water-like substance. Gamma and X-rays (7.5 MeV) show similar penetration behavior, whereas e-beams (10 MeV) have a weaker penetration effect. The e-beam dose decreases much faster through the material and concentrates within a few centimeters from the surface. Due to the density of the object, there is no sum effect in this case but may be available for a lower-density object. In general, the penetration depth of irradiation increases with a decrease in the object's density. The penetration depth data are available in various studies.^[35,80]

or tantalum) by a high-voltage source under vacuum. The electrons are collimated and accelerated near to the speed of light by passing them through a series of oscillating electric potentials.^[77,89,90]

These high-energy e-beams with particle energies up to 10 MeV react with the electrons found at the targeted surface (e.g., molecules of microorganisms).^[12,91] A higher energy level than 10 MeV (defined by FDA 21 CFR 179.26^[85]) can induce radioactivity in the target of sterilization. The collisions generate a cascade (branching) effect of ions and reactive radicals.^[91] The concentration of these particles is the highest at the surface of the target and decreases further in the material. Therefore, e-beams are characterized by a lower penetration depth than gamma or X-rays radiation,^[76] which is schematically shown in Figure 5. A single-sided treatment with a 10 MeV beam can penetrate

an about 5 cm-thick material with a density of 1 g cm^{-3} and about 10 cm when double-sided treatment is applied (see Figure 5).^[80,92] The penetration depth for 0.15 g cm^{-3} can reach 50 cm when the target object is irradiated from both sides.^[80]

A high dosage rate concentrated on the targeted surface means higher sterilization effects. Hence, e-beam sterilization has become a focus field in food and beverage industry for the surface sterilization of packages.^[78,93–96] Nevertheless, beam sterilization comes with high costs of maintenance, operation, and setting up of the required equipment. Furthermore, due to the high-voltage source required to generate the e-beams, ozone is formed around the sterilization system and suitable aeration cycles are required to maintain safe operational conditions.^[80,97]

2.3. X-Rays

X-rays are electromagnetic radiation with a wavelength between 0.5 and 2.5 Å. The simplest X-ray generator, an X-ray tube, consists of a heated filament (cathode) made of an element such as tungsten and a target anode made of a metal with a high atomic weight such as tantalum. The cathode and anode are placed under vacuum and connected to a high-voltage source. The voltage difference between the electrodes determines the energy of the produced e-beam released from the cathode. When the high-energy e-beam hits the metal target, most of its kinetic energy is converted to heat and a low portion of the electron's energy produces X-rays. The anode is tilted with a 45° angle so that the produced X-rays are almost perpendicular to the source. To prevent the damage of the anode, the metal is mounted on an element with a high thermal conductivity such as copper. The copper block is then connected to a cooling instrument to remove the generated heat.

There are various targets that can be used to produce X-rays: tantalum, tungsten, and gold are some of the common targets. Tantalum and tungsten have a melting temperature exceeding 3000 °C and gold poses a higher threshold for nuclear reaction than the others. Based on the energy of the bombarding electrons, a photonuclear reaction can occur in the element and the target becomes radioactive. Tantalum (Ta), a more workable metal than tungsten, has a threshold for nuclear reaction at 7.58 MeV for its main isotope Ta-181. The lowest threshold for the tungsten isotope (W-183) is 6.19 MeV and gold (Au-197) has its threshold set at 8.07 MeV.^[98,99] Therefore, the maximum X-ray energy is limited by regulations (FDA 21 CFR 179.26) to 5 MeV when a tungsten target is used and 7.5 MeV when tantalum or gold is installed.^[85]

The nature of source of e-beams means that X-rays can be supplied on-demand, which is a favorable solution for sterilization facilities and operators.^[100,101] However, the drawback of X-rays is the low yield of production.^[12] Nonetheless, the production efficiency of X-rays increases with an increase in the kinetic energy of the incident electrons and the atomic number of the target material.^[98] For example, the conversion efficiency of tantalum is 8–9% at 5 MeV and 12–13% at 7.5 MeV.^[98]

X-rays are a good alternative to both e-beams and gamma radiation. It has similar antimicrobial properties of both types and a higher penetration depth than e-beams. Unlike gamma radiation, X-rays are unidirectional, which results in the highest dose uniformity ratio between maximum and minimum required doses of radiation.^[100]

2.4. UV Light

UV is a type of electromagnetic radiation with a wavelength range of 10–400 nm. This spectral range is subdivided into three distinct UV types according to their wavelength and photon energy: UV-A (315–400 nm and 3.10–3.94 eV), UV-B (280–315 nm and 3.94–4.43 eV), and UV-C (100–280 nm and 4.43–12.4 eV).^[102] The higher-energy-level UV-C, in the wavelength range of 250–270 nm, is strongly absorbed by the nucleic acids of a microorganism with a peak at 262 nm.^[30,103] Hence, UV-C is typically used for sterilization and is produced by electrical or optical

excitation of a semiconductor material or lamps that contain xenon gas or low-pressure mercury. One mode of UV-C germicidal action is the production of peroxy radicals (ROO•) that induce chemical changes to the cellular structure and cell membrane, leading to deactivation.^[104] In addition, photons of UV-C are absorbed by the DNA of the microorganisms, forming thymine dimers that prevent further replication of the DNA strains.^[105] The properties of the UV radiation source used for the surface microorganisms' control of food and food products, or sterilization of water used in food production, are defined in FDA 21 CFR 179.38.^[85] Here, low-pressure mercury lamps are ideal for these purposes because 90% of their emission is at a wavelength of 253.7 nm.

High-intensity, low-wavelength pulsed light sources have also been studied for sterilization purposes: The antimicrobial properties of pulsed light treatment are a combination of photochemical and photothermal effects.^[30,106] The light pulse duration is less than 2 ms; within that time period, the UV part of the light damages the DNA of microorganisms. In addition, the high intensity and energy of the pulse cause a sudden increase in temperature that leads to thermal damage to microorganisms.^[106] Pulsed light sterilization technology, manufactured by the French company Claranor, is industrially applied for sterilization of food contact surfaces.^[107] The FDA cleared pulsed light for the treatment of food under a list of conditions under FDA 21 CFR 179.41.^[85] For instance, the radiation source is a xenon flash lamp that emits radiation at a wide spectrum (200–1100 nm) for a maximum of 2 ms.

Studies showed that UV-C shows poor penetration in opaque substances independent of the light dose.^[108] Therefore, UV-C is typically used for the sterilization of transparent fluids.^[105] For the surface sterilization process of packaging material, the UV-C permeability in a 52 µm polyethylene terephthalate (PET)/polyethylene (PE) composite is 0% and in a 40 µm polyamide (PA)/PE composite is 80%. For a list of UV-C permeability through other materials with varying thickness, see the study by Manzocco et al.^[108]

3. Thermal Sterilization

Sterilization by heat is the oldest, most readily available, and used sterilization method worldwide. Every substance or organism on the planet is influenced by heat. All biological cells and entities are made of chemical molecules that are arranged in a special 3D structure to serve a certain purpose. Heat causes an increase in the kinetic energy of atoms and molecules, which disrupts their bonds and their function within a biological entity such as a bacterium. The two main thermal sterilization principles are dry and moist heat as described in this section.

3.1. Dry Heat

This form of sterilization is achieved by increasing the temperature of an object (a package, a bulk product, or a packaged product) through heat transfer from a heat source. Given enough time, the bulk temperature of the object will reach a validated temperature value that is proved lethal for resilient microorganisms. Hence, the two main parameters for a successful

sterilization process are temperature and time, where 180 °C held for 30 min, or 170 °C for 1 h, or 160 °C for 3 h are typically applied.^[109] These recommended process parameters depend on the type and size of the object to be sterilized and on the presence of wrapping (used in the medical sector).^[110] The mode of microorganism deactivation is the coagulation of cellular proteins in the microorganism.^[41] There are three system types for dry heat sterilization, each is based on a heat-transfer principle.^[30,111]

1) Static oven is the simplest design where heat is mainly transferred by conduction; 2) unidirectional-airflow sterilizing tunnels increase the temperature of the object by forced convection of heated, sterile air, which is supplied through a high-efficiency particulate air (HEPA) filter; and 3) radiant heat tunnels that supply heat by infrared radiation from heating elements, which surround the conveyor belt that transports the goods to be sterilized.

The main advantage of sterilization by dry heat is that chemical sterilants are not required to achieve sterility, which means that there are no sterilant residues at the end of the sterilization process. Compared with moist heat sterilization in an autoclave, sterilization by dry heat requires a higher temperature and longer process time due to the poor thermal conductivity of air. Therefore, materials such as nonaqueous products, metals, glass, powders, oils, and oil-based injectable pharmaceuticals and some polymers that do not lose their integrity under high temperatures can be sterilized with this method.^[11,110,112] In addition, heat transfer is a slow process that requires a long time to reach a steady-state value, which reflects on the overall costs of the product. Nevertheless, due to the long period required to sterilize the object, thermal sterilization processes are often used in the food sector as a cook-in method, for instance, in canning such as soup.^[113]

3.1.1. Microwaves

Microwaves are a type of nonionizing electromagnetic radiation with a frequency that ranges between 300 MHz and 300 GHz. To prevent interference with telecommunication frequencies, a reserved band for industrial, scientific, and medical applications (ISM band) is used. Among the different ISM frequencies, 2.45 GHz is globally approved for domestic applications such as wireless networks and microwave ovens.

When microwaves propagate through a material with low electric conductance such as water, molecular heating is induced. The electromagnetic radiation interacts with the water molecules present in the microorganisms. The vibrating water molecules release heat into the microorganisms, resulting in coagulation of proteins and finally the inactivation of these organisms. Nonthermal antimicrobial properties of microwaves are unknown and the inactivation of microorganisms is therefore assumed to be thermal.^[114]

Microwave-assisted sterilization processes using multiple 2.45 GHz radiation sources are already applied worldwide; for an overview of this technology, we refer to the study by Stanley et al.^[74] Moreover, microwave sterilization systems operating within a bandwidth of 902–928 MHz are currently under development, where studies showed that these frequencies have a better penetration depth in food.^[115]

3.1.2. Depyrogenation

Unlike bactofugation and ultrafiltration techniques that physically remove microorganisms from a product, surface sterilization processes aim mostly to inactivate microorganisms. Nevertheless, an inactivated microorganism does not necessarily mean that the product is safe for the consumer. Depending on the type of bioburden (see Section 1.1), some microorganisms discharge toxins prior to or after the sterilization process.

Certain species of Gram-positive and Gram-negative bacteria release heat-labile toxins (exotoxins) to the surrounding medium, which lead to serious diseases. For example, *Clostridium tetani* and *Clostridium botulinum* bacteria have neurotoxin proteins; their 3D structure is shown in **Figure 6a,b**, respectively, that leads to tetanus or botulism. These toxins are destroyed by a validated thermal sterilization process. However, Gram-negative bacteria have lipopolysaccharide (LPS) molecules on their outer cell membrane.^[116,117] The lipid part of the LPS (termed endotoxin) is a heat-stable toxin that is released to the surrounding when the bacteria die or the cell wall ruptures. Some of these toxins are used in a medical context such as Botox; however, an unintended ingestion is likely to cause health issues ranging from fever to death.^[1,45,117]

Pyrogen is a name given to toxins and other biocontaminants such as active DNA and RNA molecules which result in a fever. The process in which these pyrogens are deactivated is termed depyrogenation. Here, the thermostability of endotoxins allows them to survive the temperature conditions of moist heat sterilization. Hence, dry heat sterilization has become the method of choice for depyrogenation.^[117]

The *D*-value describes the resistance level of a microorganism or a substance to reduce its level by 1 logarithmic order (or by 90%). The *D*-value for a pure endotoxin at 170 °C is 20 min and, to achieve a 3-log reduction (99.9%), a 60 min depyrogenation cycle at this temperature is required. Nonetheless, a typical depyrogenation oven operates at 250 °C for 30 min or more to achieve at least a 3-log reduction of the endotoxin level as defined by regulations.^[45,116,118,119] Due to the required high temperature of dry heat depyrogenation, it is mainly used in the medical sector for the overkill sterilization and depyrogenation of glassware, glass syringes, and ceramics that hold critical parenteral products.^[110] Other sterilization methods may be also used for depyrogenation only after validating the sterilization system.^[120]

Due to the target of sterilization (the endotoxin), the validation of the depyrogenation process is different from the biological indicator method (refer to Section 5). The validation of endotoxin reduction is assessed using a preparation of 10 000 endotoxin units (EU) derived from *Escherichia coli* (strain O113:H10); the final goal of validation is that 10 EU must be reached at the end of the depyrogenation cycle. For further details of these tests, see various studies.^[49,116–118]

3.2. Moist Heat

Moist heat or steam sterilization is a nontoxic and inexpensive process that has proven its microbicidal and sporicidal properties. It involves the sterilization of objects in an autoclave apparatus by a heated, saturated steam applied at high-pressure

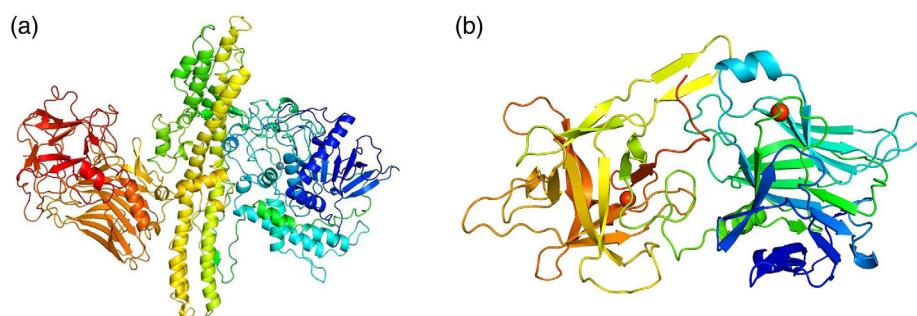


Figure 6. 3D structure of a) botulinum neurotoxin type A that is marketed as Botox and b) tetanus neurotoxin. Images generated using PyMol software and Protein Data Bank (PDB) file numbers 3BTA and 1A8D.

conditions. This sterilization method is widely used in the medical and pharmaceutical fields to sterilize nonsingle-use equipment and as a terminal sterilization process for glass ampoules, vials, syringes, and plastic containers.^[40,109]

The type of sterilization target reflects on the choice of exposure temperature and cycle length. Typical steam sterilization cycles use a temperature that ranges from 121 °C (30 min) to 135 °C (10 min) held at 2 bar to achieve a 6-log reduction value.^[40] Other recommended operational parameters are 121–124 °C at 2 bar for 15 min, 126–129 °C at 2.5 bar for 10 min, or 134–138 °C at 3 bar for 5 min.^[109] An exemplary moist heat sterilization cycle using the latter parameters is shown in **Figure 7**. The factors that define the operational conditions of such process include the type of steam (saturated or unsaturated), its temperature, the pressure in the autoclave, and the exposure time.^[121]

The minimum sterilization time depends on the type of object and its size to guarantee that all material in the autoclave has

reached the required temperature. Therefore, temperature probes are used to monitor the sterilization process and cycle conditions.^[109,118,122] The lower temperature compared with dry heat sterilization is related to the released latent heat during condensation of the steam when it hits colder surfaces. The mode of bactericidal and sporicidal action of this process is due to the denaturation and coagulation of the proteins of the microorganisms by the high process temperature.^[41]

There are two types of autoclaves, the first is based on gravity displacement, whereas the second type is a high-speed prevacuum sterilizer.^[41,53] In the gravity displacement sterilizer, the high-temperature steam is injected from the sides or from the top of the sterilizer. The lower density of the injected steam compared with the colder air in the closed chamber means that it will flow to the top of the sterilizer. Further steam injection will push the colder air out through the lower part of the sterilizer.^[110] For the high-speed prevacuum sterilizer, the unit is equipped with

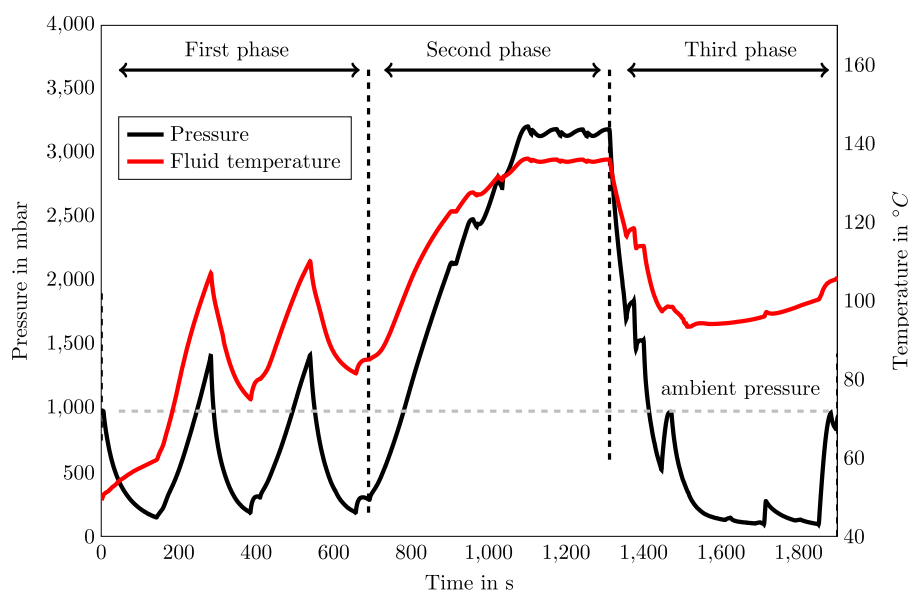


Figure 7. Typical moist heat sterilization cycle depicting the three phases of sterilization. In this example, the first phase starts by the evacuation of the sterilization chamber with a vacuum pump. In this phase, steam is injected (see fluid temperature) and evacuated multiple times to ensure the removal of all noncondensable gases from the steam sterilizer. In the second phase, steam is introduced to the sterilizer, which increases the temperature and pressure to a value of 135 °C and around 3 bar, respectively. These conditions are then maintained for 5 min following a pressure release, drying by evacuation, and cool down time of the load. Reproduced with permission.^[121] Copyright 2019, Elsevier.

vacuum pumps, such as membrane- or liquid-ring pumps, that evacuate the air and decrease the humidity of the chamber prior to steam injection.

3.2.1. Low-Temperature Steam and Formaldehyde Sterilization

Often it is necessary to sterilize materials and equipment that are heat-sensitive and cannot withstand the normal conditions of an autoclave system. This means the autoclave unit has to operate at lower cycle temperatures, which can adversely affect its sterilization efficacy. Therefore, to maintain the sterility of the target object, formaldehyde (a strong microbicidal agent) is introduced to the sterilization cycle. This sterilization method is known as low-temperature steam formaldehyde (LTSF) sterilization.^[123,124]

At room temperature, formaldehyde (CH_2O) is a highly flammable, colorless gas with a boiling point of -19°C .^[125] A steam-formaldehyde mixture is used to sterilize objects at a temperature range of $48\text{--}80^\circ\text{C}$.^[123,126] Formaldehyde is a small molecule that can easily penetrate the membrane of the microorganism and react with cellular proteins, DNA, and RNA structures.^[125]

The overall use of formaldehyde has decreased over the years and has been substituted by safer sterilization systems such as hydrogen peroxide plasma sterilizers (detailed in the article). This is related to its pungent odor at low concentrations down to 0.06 mg m^{-3} , its potential allergy-triggering effects, and its explosive properties. At the end of the sterilization process, formaldehyde residues might be present on the surface of the equipment or product, which requires a poststerilization cleanup. Formaldehyde is globally acknowledged as carcinogenic as stated by the United State Department of Health and Human Services (HHS), the Occupational Safety and Health Administration (OSHA), the European Chemicals Agency (ECHA), and other regulatory bodies.^[125]

4. Chemical Sterilization

A chemical sterilant refers to a substance which is characterized by microbicidal properties. These properties include the ability of the chemical agent, or one of its derivatives, to interfere with genetic material (DNA or RNA) that deactivates one or multiple cellular functions, causing the death of the microorganism. Other microbicidal courses of action are the inhibition of protein synthesis or enzymatic activity and direct damage to the cell membrane and cell wall.

There are a variety of emerging, new sterilization technologies such as supercritical carbon dioxide, and nitrogen dioxide sterilization, which are undergoing development and consideration.^[127–130] However, with status of 2020, the most widely used sterilants for the purpose of surface sterilization are ethylene oxide ($\text{C}_2\text{H}_4\text{O}$), ozone (O_3), chlorine dioxide gas (ClO_2), peracetic acid ($\text{CH}_3\text{CO}_3\text{H}$), and hydrogen peroxide (H_2O_2). These sterilants can be applied in different forms depending on the thermophysical properties of the particular chemical and the design of the sterilization apparatus. These application methods for surface sterilization are: 1) “liquid phase” through spraying nozzles and immersion baths (e.g., in case of peracetic acid and aqueous hydrogen peroxide solutions); 2) “gas phase” from gas generators working at atmospheric or low-pressure conditions

(e.g., hydrogen peroxide, chlorine dioxide gas, ethylene oxide, and ozone); and 3) a “mixed phase” (vapor) where surface condensation is during the sterilization cycle due to the thermophysical properties of the chemical sterilant, the lower surface temperature of the object, or due to the reduction of the surrounding pressure (e.g., hydrogen peroxide).

Gas or vapor sterilization is the common sterilization practice in healthcare, medical, and food industries. Typically, and independent of process parameters, the sterilization process is a combination of three consecutive steps: conditioning, sterilization, and post-treatment. These steps are schematically shown in **Figure 8** as part of the aseptic zone of an aseptic filling machine.

The conditioning step assures the optimal temperature of the object to be sterilized and its (surface) relative humidity. Here, the object is prepared to receive the sterilization process so that a reproducible sterility level is achieved. The next step is the treatment of the object's surfaces by the sterilant, where a validated process is used to render the surface free of viable microorganisms. At the end of the sterilization cycle, traces of the sterilant are still present on the surface of the object. Therefore, post-treatment and multiple aeration cycles are required to remove these residues and guarantee an acceptable residual level according to regulations.^[1,39,131]

There is a difference between gas and vapor sterilization: In gas sterilization, no condensation is possible due to the state of the sterilant at normal temperature and pressure such as chlorine dioxide and ethylene oxide gases. Based on the sterilant's thermophysical characteristics, it may condense on a surface, resulting in a vapor sterilization process which might be unfavorable to the sterilization process. The following might be done to suppress the condensation of gases:

1) decreasing the pressure in the sterilization chamber (for a closed system); 2) increasing the gas or surface temperature of the object and maintaining it above the dew point of the sterilant gas; or 3) keeping the concentration of the sterilant below saturation levels.

On the one hand, due to the small collision diameter of gas molecules, gas sterilization shows a stronger penetration behavior than vapor sterilization. Hence, these systems are used to sterilize packages that contain tight regions such as crevices or joints and even porous materials.^[43,132] Moreover, the concentration of the gas in a gas sterilization system is more uniform due to molecular diffusion. On the other hand, the sterilization efficacy of vapor sterilization—referring to condensable gas sterilants—is affected by the sterilant concentration in the condensate. This is a property best explained by the thermodynamic properties of a binary mixture of hydrogen peroxide and water vapor, which is presented in Section 4.4.

Gas sterilization is widely used for surface treatment of pharmaceutical equipment and packaging surfaces. By controlling the operational parameters of these systems, it is possible to sterilize heat-sensitive plastics. Multiple factors affect the sterilization efficacy of gas-based sterilization systems that include the gas temperature and system's pressure, relative humidity, exposure time, and gas concentration. A tight control over these parameters is required to ensure successful and reproducible sterilization, particularly while controlling the amount of residual at the end of the cycle. In the following sections, we will provide an

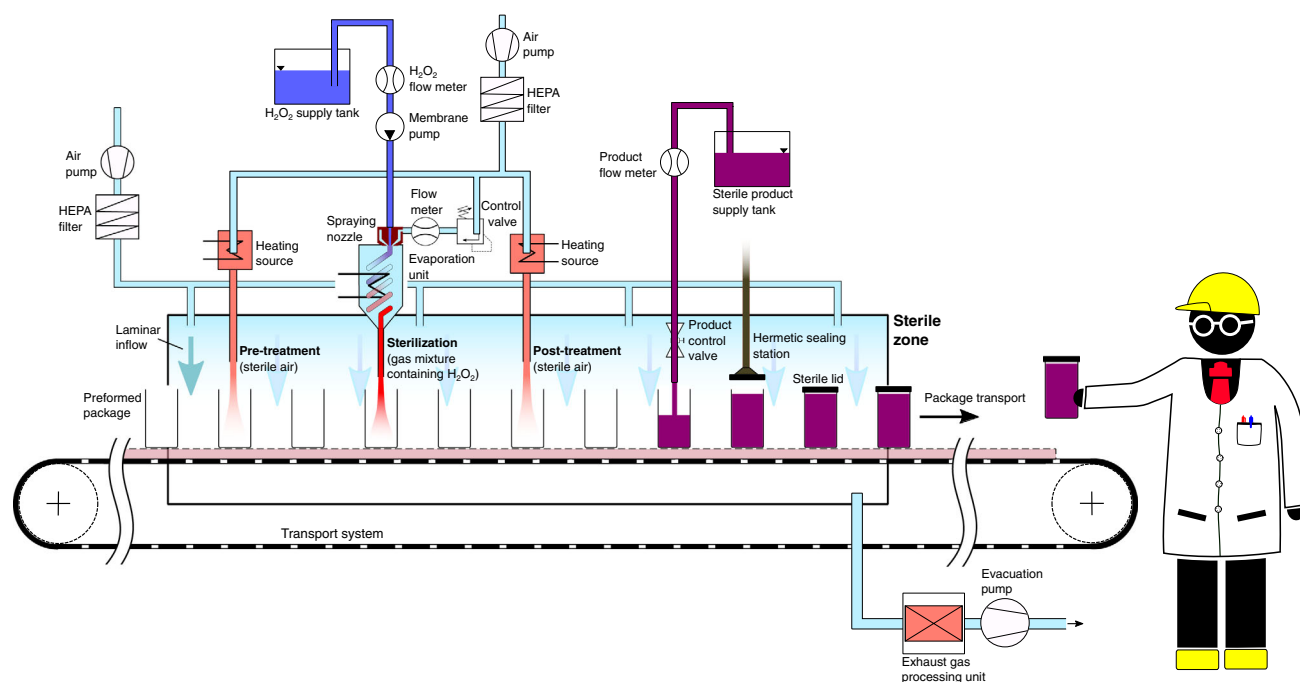


Figure 8. Scheme of the sterile zone of an aseptic filling machine, based on the description provided in the patent by Weiler et al.^[224] In this example, we depict a three-step sterilization process with H_2O_2 , which includes a pre-treatment of packages with heated, sterile air; sterilization with a gas mixture that contains H_2O_2 ; and a post-treatment step with heated, sterile air. Also, the figure shows the aseptic filling process of a sterile product into the sterile package followed by hermetic sealing occurring in the sterile zone. This three-step sterilization process with H_2O_2 can be substituted by other sterilants, or the pre- or post-treatment steps can be neglected as long as the process is validated. This means that the sterility of the object is guaranteed without adverse affects to the product or the consumer.

overview of the different types of gas and vapor sterilization systems.

4.1. Ethylene Oxide

Ethylene oxide (EtO, $\text{C}_2\text{H}_4\text{O}$) is an organic agent that is produced by direct oxidation of ethylene with oxygen on a silver catalyst.^[133] It is the simplest form of a cyclic ether and it is colorless in its liquid and gas state. It has a boiling point of 10.8°C at atmospheric pressure and is characterized by a high penetration strength in paper, cloth, and plastics.^[134] In addition, EtO is explosive with a lower explosive limit in air of 2.4% v/v that can be initiated by a spark or by contact with a catalytic substance.^[109,133]

EtO is a chemical that has multiple applications from the production of consumer goods such as antifreeze, adhesives, and detergents to its use as a fumigant for sterilization purposes. According to ECHA, CDC, US-EPA, and WHO, EtO is a carcinogenic and mutagenic substance that may lead to infertility. Conducted mortality studies by the CDC of individuals exposed to high levels of occupational EtO, either inhaled or skin contact, correlate with an increased risk of developing blood cancers among men and breast cancer among women.^[135,136] Therefore, since 1985, the TWA threshold for exposure set by the OSHA is 1 ppm.^[135]

The microbicidal properties of EtO come from the alkylation reaction with the DNA of microorganisms, clotting of proteins, and deactivation of enzymes and other components required for

a healthy cell. Due to its gaseous nature at room temperature, its strong microbicidal properties, and high penetration strength in paper and cloth, it is classified as a cold sterilization technique. Hence, it is used in the healthcare sector for sterilizing heat- and radiation-sensitive polymers, as well as surgical instruments.

In the USA, EtO is used as a pesticide and as a sterilizing agent in the food and medical sector. For instance, it is used to reduce *Salmonella* and *E. coli* levels in spices and herbs such as black peppercorns, with an estimate of 40–85% of spices in the USA being treated with EtO each year.^[134,135,137–140] Also, unlike sterilization by radiation, food products that are sterilized by EtO do not require a label on their package. Therefore, it is difficult to assert the current usage of EtO in the USA as a food sterilant. However, sterilization with EtO in the medical sector is tightly monitored by FDA in their facility updates website.^[141] The EU has banned the use of EtO as a pesticide and as a fumigant for food products.^[142] As a result, foods treated with EtO are not allowed to enter the European Union.^[143] Nevertheless, the use of EtO as a sterilant for the production of medical products is still accepted.^[136]

The main concern of using EtO is finding and defining the operational parameters of sterilization in a way that prevents toxic residues.^[134,138] EtO forms ethylene chlorohydrin when it hits chlorine-containing plastics and ethylene glycol when it reacts with water.^[135,144,145] Both substances have harmful effects to health, which include irritation, organ damage, mutagenesis, and carcinogenesis in humans and animals.^[146] Also, due to the health risks related to the emissions of EtO to the

surrounding, the FDA released their second innovation challenge that aims to reduce, capture, and process EtO emissions.^[147,148] This challenge attracted worldwide attention from national and international companies.

A typical EtO sterilization cycle is conducted for 1–6 h at 37–63 °C and a relative humidity of 40–80%. The recommended concentration of EtO under these conditions is between 450 and 1200 mg L⁻¹ mixed with an inert gas.^[40] This wide- and low-operational-temperature window is what makes EtO an attractive sterilant in the healthcare sector, where different heat-sensitive materials are used. At the end of the sterilization cycle, a post-treatment step is required to remove EtO residues from the chamber and the sterilized object.^[146] Evacuations using vacuum pumps and heating are examples of such post-treatment processes that increase the run time of the sterilization system.

4.2. Ozone

Ozone (O₃) gas is formed when an oxygen-rich atmosphere is subject to a high-energy electrical field or ionizing radiation such as a UV source. The dissociation of ozone releases an oxygen molecule and an oxygen radical. The oxygen radical has high reactivity and oxidation potential, which exhibits strong microbicidal properties by damaging the membrane structure of microorganisms, leading to cell death.^[149]

Ozone is highly unstable and cannot be stored or transported. For applications that require its use, a special equipment termed “ozone generator” is used. There are three types of ozone generators in industry based on electrolysis, corona discharge, and vacuum-ultraviolet (VUV) ozone generation.^[149]

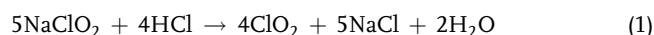
The electrolysis method works by supplying a high current density through an electrolysis bath that contains 68% w/w sulfuric acid, which can produce between 18% and 25% w/w ozone in oxygen.^[149] The corona discharge system works by passing an oxygen-rich gas stream between two highly charged parallel plate electrodes (≈10 kV). The electric discharge splits the O=O bond and forms two reactive oxygen atoms. Each oxygen atom then binds with another oxygen molecule forming ozone. The VUV system releases high-energy UV radiation with a wavelength below 200 nm that also splits the O=O bond and forms ozone from two reactive oxygen atoms reacting with oxygen molecules. The sterilization efficacy of ozone increases with an increase in the relative humidity of the environment and a level of 80–95% is required so that ozone penetrates the protective shell of microorganisms. At the same time, the unstable properties of ozone lead to a limited usage in industry. Nonetheless, ozone generation systems found their way to industry as part of hydrogen peroxide sterilization systems as a method to increase the efficacy of the decontamination cycle and shorten the aeration process.^[4] For example, VUV systems are used to augment the microbicidal properties of a hydrogen peroxide vapor sterilization system in the aseptic filling line TR/G7 by Tetra Pak, a machine manufacturing company for filling and packaging biologically sensitive products. In addition, corona-discharge ozone generators are coupled to low-temperature hydrogen peroxide sterilization systems for the medical and healthcare sector such as Sterrad systems by Advanced Sterilization Products (ASP), that are presented in Section 4.4.

4.3. Chlorine-Releasing Compounds

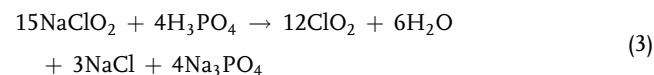
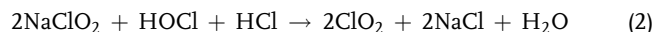
Chlorine is widely used for the disinfection of water or water-based substances and is a common acting agent for many household disinfection products. The biocidal properties of chlorine are due to its reaction with water, producing hypochlorous (HOCl) and hydrochlorous acids (HCl). This decreases the pH level of the target medium from the formation of hydrogen ions (H⁺) and hypochlorite ions (OCl⁻), which render the surrounding inhabitable for microorganisms. Moreover, hypochlorous acid reacts with and deactivates enzymes that are responsible for cell metabolism leading to cell death.^[150,151]

Chlorine dioxide (ClO₂) is a highly reactive oxidizing agent that exhibits antimicrobial properties.^[152] It is nonmutagenic or carcinogenic to humans and can be applied in gas phase or solution. In situ preparation of ClO₂ is required due to the explosive nature of the gas. To this end, there are different methods for preparation:

1) electrochemical generation from a chlorite-based substance such as sodium chlorite (NaClO₂)^[153]; 2) reaction between chlorine-containing solution with NaClO₂, as shown in Equation (1)



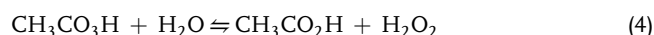
3) and reaction between NaClO₂ solution and concentrated acids, as shown in Equation (2) and (3).^[152,154]



Commercial ClO₂ gas sterilization systems are available in the market such as the Steridox-VP CD sterilizers from the USA-based company Consolidated Sterilizer Systems.^[155] Still, these systems require further research before ensuring their reliable use in the medical sector.^[129,156] For the treatment of water or water-based substances, companies similar to Dioxide Pacific (an Australian-based company) provide turnkey ClO₂ systems.^[153]

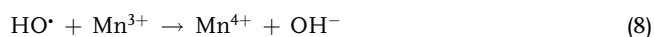
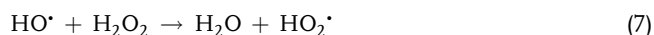
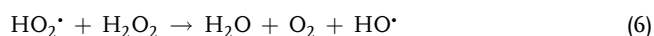
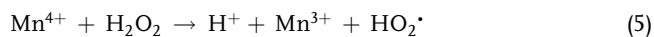
4.4. Hydrogen Peroxide

Hydrogen peroxide (H₂O₂) is known for its strong and fast acting microbicidal properties.^[41] Gaseous mixtures with varying concentrations of H₂O₂ are widely applied for sterilization of surfaces in the pharmaceutical, food, and beverage sectors.^[23,138] H₂O₂ and its solutions are also used for sterilizing heat-sensitive surfaces. For example, Tetra Pak applies H₂O₂ baths together with UV lamps to sterilize packaging material.^[157] In addition, for the sterilization of PET bottles in the beverage industry, peracetic acid (PAA, CH₃CO₃H) is typically applied. It is an equilibrium mixture of acetic acid (CH₃COOH), H₂O₂, and water in the form shown in Equation (4).



The dissociation of H₂O₂ with catalytic or organic substances follows a long reaction chain that produces a number of chemical

radicals.^[158,159] For example, in contact with manganese (IV) oxide, H_2O_2 dissociates, as shown in Equation (5)–(8), to produce radicals such as hydroxyl (HO^\bullet) and perhydroxyl (HO_2^\bullet). These radicals are strong oxidants that can damage the cell wall of microorganisms, leading to the loss of cellular matter and their death.^[160] This is demonstrated by transmission electron micrographs (TEM), shown in **Figure 9**, of *Bacillus atrophaeus* spores before and after the exposure to H_2O_2 gas.^[161]



The advantages of this sterilant are the ease of application and the nontoxic, odorless, and environment-friendly end products of water and oxygen, as shown in the overall dissociation reaction shown in Equation (9) and (10). Even though sterilization methods that apply H_2O_2 as a sterilant are approved by the FDA, they are disregarded as validated methods by the Pharmaceutical Inspection Co-operation Scheme (PIC/S), as observed by their absence in their official document (Annex 1) in PIC/S.^[162] As stated in the statement by Hopkins,^[163] the reason for discounting H_2O_2 sterilization systems by PIC/S is the lower penetration depth of the sterilant and its fragility compared with other methods such as EtO and radiation sterilization. Here, process fragility refers to the weak penetration depth of H_2O_2 due to occlusion from, for example, fatty acids of fingerprints, under which microorganisms are still viable.^[163]



H_2O_2 is commercially available and supplied in the form of aqueous solutions. Solutions of 35% w/w H_2O_2 and 65% w/w water (H_2O) or 59% w/w H_2O_2 and 41% w/w H_2O are established concentrations in food and pharmaceutical industries.^[7,34] In its pure form, H_2O_2 is relatively stable.^[164,165] The unstable nature of H_2O_2 solutions is from the dissolved or suspended catalytic impurities (metal ions) during the production process or from filling in contaminated containers. The decomposition

reaction of H_2O_2 solutions is highly exothermic and releases about 105 kJ mol^{-1} of heat.^[166] Therefore, to assure the transport safety of the solution, a variety of stabilizing agents that inhibit decomposition are added during production.^[42,160]

The chemical formulation to stabilize a H_2O_2 solution product intended for a certain application is not disclosed by the producing companies. This leads to product diversity, as it is evident in the product portfolio by Solvay Chemicals.^[167] In general, stabilizing agents that inhibit catalytic decomposition of H_2O_2 are characterized as

1) “complexing agents” such as inorganic phosphates that bind with metal ions^[164,168,169]; 2) “protective colloids” such as sodium stannate trihydrate or silicate ions that adsorb metal ions^[164]; and 3) “chelating agents,” which are soluble organic compounds that bind metal ions such as citric acid, cyclodextrines, and ethylenediaminetetraacetic acid (EDTA).^[160]

At the end of the sterilization process, impurities from H_2O_2 solution and added stabilizing agents can leave residues on contact surfaces. Therefore, the FDA defined in their regulation number 21 CFR 178.1005 the maximum allowed inorganic impurities in H_2O_2 solutions in accordance with the Food Chemical Codex.^[39,131] This comes down to a total solid residue after evaporation of less than 0.006% w/w from the H_2O_2 solution. In these residues phosphates must be less than 0.005% w/w and metal residues are limited to 0.5 mg kg^{-1} for iron, 10 mg kg^{-1} for tin, and 4 mg kg^{-1} for lead.

As described at the beginning of this section, H_2O_2 is used for sterilization in two forms, liquid and gaseous. For application of gaseous H_2O_2 , there are three techniques that depend on the temperature sensitivity of the target and the sterilization system’s design. Gas-phase H_2O_2 sterilization systems are hence grouped to: 1) low-temperature H_2O_2 vapor sterilization at ambient pressure; 2) low-temperature and low-pressure H_2O_2 gas sterilization; and 3) high-temperature H_2O_2 vapor or gas sterilization at ambient pressure.

4.4.1. Low-Temperature H_2O_2 Vapor Sterilization at Ambient Pressure

Vapor-phase H_2O_2 mostly refers to a low-temperature sterilization process. One method to ensure that H_2O_2 remains in the gas phase at ambient pressure is by maintaining the concentration below saturation level at the prescribed system conditions.

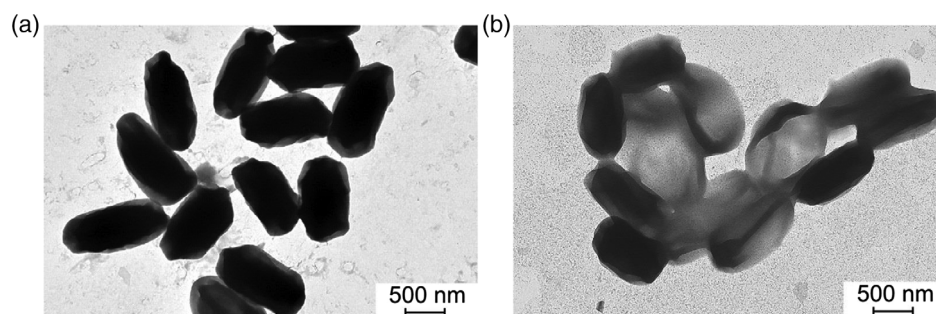


Figure 9. TEM of *Bacillus atrophaeus* spores (DSM 675) before and after a sterilization procedure for 0.3 s with 8.3% v/v H_2O_2 gas heated to 240°C . a) The nontreated spores show a uniform cell structure with a sharp contrast and b) spores after sterilization process, indicating a lighter contrast in the cell. The change in the contrast indicates a loss of cellular matter due to the rupture of cell wall. Reproduced with permission.^[161] Copyright 2018, Elsevier.

Therefore, knowledge of the vapor pressure equilibrium diagram for H_2O_2 – H_2O –air mixtures is necessary.

For facility sterilization in the healthcare sector, H_2O_2 solution (35% w/w or 59% w/w) is evaporated on a heated plate and mixed with a stream of hot, sterile air to achieve concentration levels of 200–400 ppm.^[4,170] This low gas concentration means that the typical cycle takes multiple hours to assure sterility. The length of the cycle is based on the size of the facility, the surrounding temperature, and relative humidity conditions.^[170]

In the medical industry, aseptic processing technology is in the form of isolators and restricted access barrier systems.^[55,171] The main difference between the two systems is the level of separation from the surrounding. Isolators are completely sealed units that have a physical separation coupled with unidirectional airflow. Here, the supplied air and sterilant flow to these machines are generated and maintained within the same sterilization system. A restricted access barrier system minimizes external contact with the product during packaging, yet it is not completely sealed from the environment. This type of system requires an overpressure to prevent incoming airflow from the surroundings to the sterilization zone. For both types of systems, the target H_2O_2 concentration is 400–1300 ppm.^[21]

Sterilization processes where isolators or restricted access barrier systems are used are typically conducted in batch operations. First, the object of sterilization is cleaned from outer contaminants, followed by a surface dehumidification process. The dehumidification occurs by circulating a flow of sterile air, while continuously monitoring its humidity content. Once a certain humidity level is reached, the surface sterilization with H_2O_2 begins and H_2O_2 vapor is introduced by injecting H_2O_2 solution through a heat exchanger mixed with air.^[172,173] The injection continues until a predetermined quantity of H_2O_2 solution is vaporized and introduced into the chamber. The H_2O_2 -containing air flow is then circulated to maintain a constant gas concentration for a preset exposure time. The final step is the aeration of the chamber by recirculation through catalytic units containing a H_2O_2 -decomposing catalyst such as platinum/palladium on alumina.^[174]

4.4.2. Low-Temperature and Low-Pressure H_2O_2 Gas Sterilization

To sterilize the surface of heat-sensitive objects with a temperature not exceeding 80 °C while maintaining a 6-log spore reduction, low-temperature sterilization systems are used. As explained in Section 3.2, the low-temperature autoclaving has its disadvantages so H_2O_2 gas sterilizers became the preferred alternative. The most widely used low-temperature sterilizers are the vaporized hydrogen peroxide (VHP) sterilizer series by Steris (previously American Sterilizer Company) and the Sterrad system by ASP. Both systems were developed in late 1980s and are still dominating the market since the start of 1990s.^[175,176] Nowadays, various companies design and manufacture sterilizers such as the 3M Company group (USA), MMM Group (Germany), and Tuttnauer (the Netherlands). These systems are still based on the operation principles of Steris and Sterrad that usually incorporate the three-step sterilization process (pretreatment, sterilization, and post-treatment) to completely sterilize a target.^[174]

A sterilization cycle takes about 15–60 min and depends on the type of load.^[177] The cycle begins with the evacuation of a closed chamber containing the object. The decrease in pressure evaporates condensed or residual water that might be present on the surface or inside a porous object. The next step is the evaporation or flash evaporation of a predetermined quantity of 35% w/w or 59% w/w H_2O_2 in a separate chamber using a specially designed heat exchanger.^[178] This is followed by the release of the H_2O_2 vapor to the chamber. The gas phase of H_2O_2 is maintained by the reduced pressure, which also decreases the H_2O_2 solution's bubble point and the gas-phase dew-point temperature, respectively. The dew point of H_2O_2 depends on the pressure inside the sterilization chamber and whether a supporting gas such as air is introduced to the chamber. The bubble- and dew-point curves for 35% v/v and 59% v/v H_2O_2 are shown in **Figure 10**. To prevent loss of the sterilant by condensation, the walls of the sterilization unit are kept at a temperature above the dew point of the H_2O_2 gas, typically at 30–60 °C.^[179] To ensure the sterility of the product, more than a single sterilization cycle, each with an injection of H_2O_2 gas, can take place.^[4] As a final step, multiple aeration and dehumidification cycles with sterile air are conducted to remove H_2O_2 residues. Another method for residue removal is by plasma discharge in the sterilization chamber.^[174,176] The high reactivity of the plasma will dissociate H_2O_2 to its nontoxic end products and, in addition, might increase the SAL parameter of the final product.^[7,180]

4.4.3. High-Temperature H_2O_2 Vapor Sterilization at Ambient Pressure

The throughput of packaging machines has increased over the years, thanks to technological advancements that aim for covering the growing market demand for packaged products. Some of the currently fastest machines in the market are the wet aseptic line of the Sidel Group with a packaging rate up to 40 000 bottles per hour for bottle formats of 250–500 mL,^[181] Tetra Pak A3/Speed with up to 24 000 packages per hour for formats of 100–1000 mL,^[157] and Kronos PET-Asep D packaging line with up to 48 000 bottles (500 mL format) per hour.^[182] These aseptic filling machines, with minor differences, share a similar sterilization system design as the one shown in **Figure 8**. A widely used method for fast surface sterilization of packaging material is using H_2O_2 gas at a much higher concentration than in low-temperature sterilization systems. To guarantee product sterility in a short time window, the concentration of H_2O_2 can reach levels that may exceed 50 000 ppm or 5% v/v. This gas mixture generated from a 35% w/w H_2O_2 solution contains 17.6% v/v H_2O and 77.4% v/v air. In general, condensation is more likely the higher the H_2O_2 and H_2O content in the gas mixture. To estimate the evaporation dynamics of the H_2O_2 solution, weighted vapor–liquid equilibrium (VLE) diagrams derived from vapor-pressure formulation of the binary H_2O_2 – H_2O system are used.^[183] These diagrams can also be used to predict the condensation dynamics of the H_2O_2 – H_2O gas mixture. The weight factor for the VLE diagram is the molar ratio of air in the gas mixture.^[184] For further details on the vapor-pressure formulation and VLE diagrams, we refer to various studies.^[183,185,186]

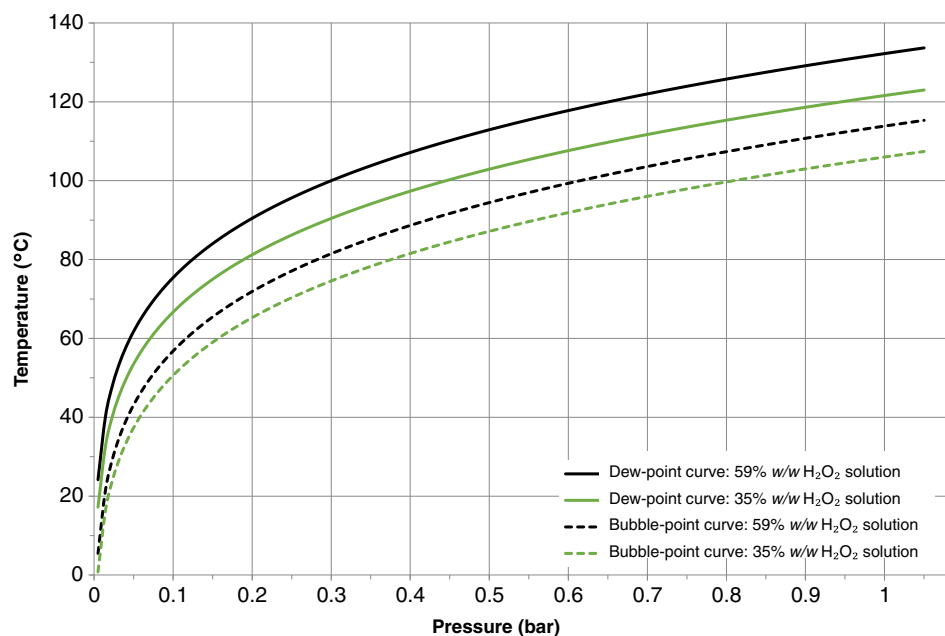


Figure 10. Dew- and bubble-point curves for a 59% w/w H_2O_2 solution (black) and 35% w/w H_2O_2 solution (green) as a function of pressure and temperature. Due to the binary nature of the H_2O_2 solution, evaporation and condensation are found in a distributed temperature range. The bubble-point temperature describes the onset of boiling or the end of vapor condensation. The dew-point temperature represents the end of boiling or the start of condensation. For a closed system, where H_2O_2 is injected directly into the sterilization chamber, the pressure value refers to the pressure inside the chamber. In sterilization processes, where H_2O_2 solution is mixed with another gas, the pressure in the figure depicts the partial pressure of the H_2O_2 – H_2O vapor. The diagram is generated from vapor pressure equations of a H_2O_2 – H_2O binary mixture as explained in the study by Jildeh et al.^[183]

To control the condensation behavior of H_2O_2 , the outlet temperature of the gas out of the H_2O_2 gas generator is regulated. If condensation is unfavorable, a gas temperature may be set to 300 °C. Above this temperature, the stability of H_2O_2 decreases and autothermal decomposition becomes prominent at 400 °C.^[187,188] H_2O_2 generators are heat exchangers that transfer thermal energy from an ohmic or inductive heating source to evaporate or vaporize H_2O_2 solution or its aerosol and maintain the generated gas at a set temperature.^[173,189–194] Heat from a primary heating circuit can be also used as the thermal energy source.^[195] Examples of these generators are shown in **Figure 11**.

Based on the surface temperature of the fluid channels inside the gas generator, some of the H_2O_2 -stabilizing agents are deposited on internal surfaces of the evaporation unit.^[183,196] These deposits will impede the heat-transfer process and lead to a depletion of stabilizers in the H_2O_2 gas. This might cause the decomposition of H_2O_2 and a decrease in the present H_2O_2 concentration that reaches the target.

H_2O_2 gas is transported from the gas generator to the target along a series of thermally isolated and passivated stainless steel or aluminum pipes. An example of an industrial passivation procedure is described in the recommendation by Solvay Chemicals.^[197] The transport pipe is best kept as short as possible to prevent temperature decrease and the loss of H_2O_2 concentration in the gas due to condensation or H_2O_2 decomposition. Another transport method from the H_2O_2 gas generator to the sterilization target is by gas nozzles or pipe manifolds. Schemes for such transport methods are shown in **Figure 12** and other examples can be found in various studies.^[198,199]

The design of the three steps for gas sterilization (conditioning, sterilization, and post-treatment) and their operational parameters depend on the thermal stability of the packaging material. For example, the exposure to heat can cause degradation of typical packaging polymers, leading to a change in their mechanical properties.^[11] PE-molded packages melt at an average temperature (T_m) of about 120 °C and start to deform under a pressure of 4.5 bar at a lower average temperature (deflection temperature, T_{dp}) of ≈ 45 °C. Packages molded by polypropylene (PP) are able to withstand more heat ($T_m = 145$ °C and $T_{dp} = 90$ °C) and PET is able to withstand higher temperatures ($T_m = 245$ °C and $T_{dp} = 90$ °C). All of the three mentioned polymers are used either alone or as a part of a multilayer package.

By regulating the temperature of the gas mixture and the concentration of H_2O_2 during the sterilization process, two sterilization principles can be distinguished.^[7,200–202] The first is gas-phase sterilization, where the temperature of the packaging material is increased in the conditioning step to a value above the dew-point temperature of the sterilant gas in the sterilization step. The second principle is sterilization by condensation, where the sterilant gas mixture condenses on the package surface. This occurs when:^[202] 1) the bulk temperature of the gas mixture decreases below the dew-point temperature; 2) the content of H_2O_2 and H_2O -vapor in the atmosphere reaches saturation level; or 3) when the temperature of the target surface is lower than the dew-point temperature of the gas mixture.

Each sterilization principle has its advantages and disadvantages: The main advantage of sterilization by condensation is the higher H_2O_2 concentration in the condensate, which is

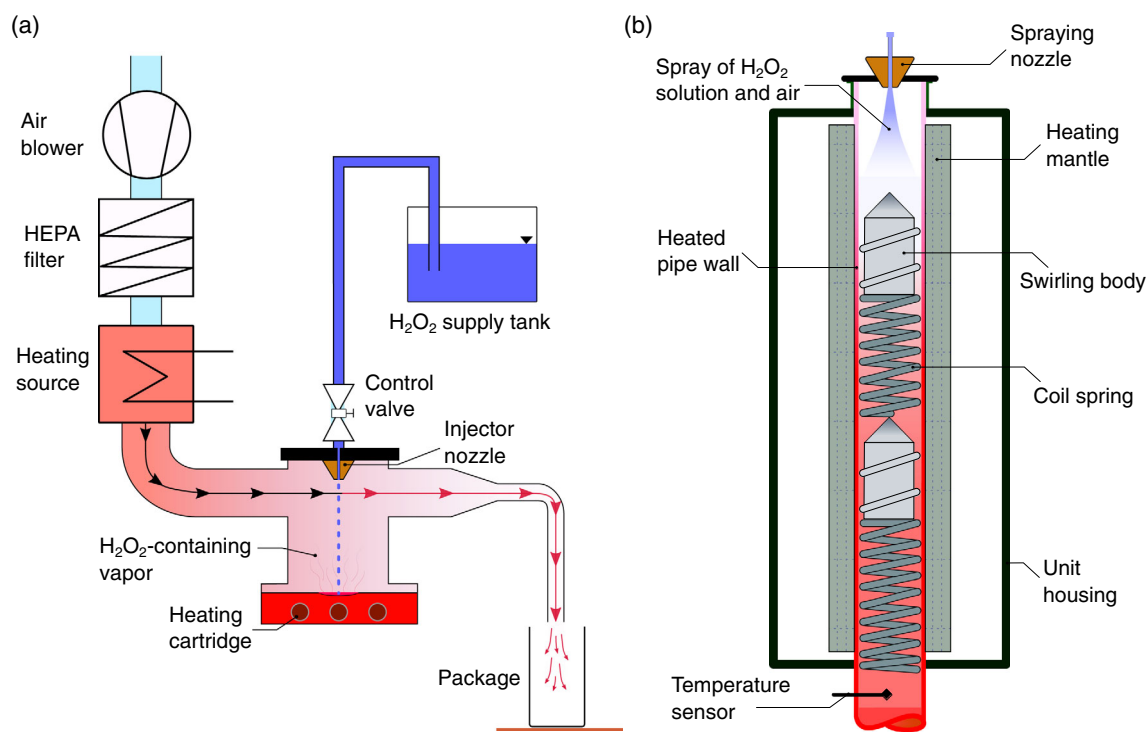


Figure 11. Cross-sectional schematics of evaporation units that generate a heated gas mixture that contains H₂O₂. a) A design based on a patent by Kronos AG.^[225] Here, the temperature and H₂O₂ concentration are controlled by regulating the electric power of heating cartridges. b) A design patented by SIG Combibloc and commercialized by Thermocoax.^[194,226] Both schematic illustrations are adapted from the original publications.

due to the lower vapor pressure of H₂O₂ compared with H₂O.^[185,202,203] The formation of a condensate means that more thermal energy is required in the post-treatment step to reduce the residual H₂O₂ from the packaging surface. The condensed thin film with a higher concentration of H₂O₂ than in the gas phase may have an even stronger microbicidal effect than gas-phase sterilization.^[7,43] A study in 2018 showed that the sterilization efficacy of H₂O₂ is affected by the hydrophobicity of the packaging material. The findings indicated that surface tension of the condensate can prevent the homogeneous wetting of the surface.^[132]

In the gas-phase sterilization principle, the process has a higher kill rate per unit volume of H₂O₂ solution due to the absence of condensation and the constant gas concentration all over the surface of the package. This decreases the required amount of H₂O₂ to achieve the required sterilization efficacy. The lower consumption of H₂O₂ and the absence of condensation mean that the H₂O₂ residual levels are lower and easier to handle with a lower net-energy consumption in the post-treatment step. A recent study has shown that gas-phase sterilization has the advantage of not being affected by the type or the roughness of either packaging material or the present microorganisms.^[132] The gas-phase sterilization principle requires more thermal energy in the sterilization process. Therefore, the temperatures are higher compared with sterilization by condensation. The materials constituting the sterilization system have to withstand the high temperatures

associated with the gas-phase sterilization process. Here, heat losses to the environment have to be minimized to decrease thermal loads and protect other parts and the operator of the packaging machine.

The post-treatment of packaging surfaces consists usually of aeration with heated, sterile air to remove H₂O₂ residues prior to product filling.^[43] As a result, the overall design of the sterilization process aims not only to inactivate all present microorganisms but also not to exceed a residual H₂O₂ concentration of 0.5 ppm in accordance with the FDA regulation 21 CFR 178.1005.^[39]

From an industrial point of view, the sterilization principle of H₂O₂ gas is defined by the manufacturing companies. For instance, the portfolio of Tetra Pak aseptic filling machines indicates a preference to sterilize packaging material sheets (prior to forming) by a combination of a heated H₂O₂ bath (35–59% w/w at ≈70 °C), hot air, and UV light source. In addition, the company applies a 3% H₂O₂ vapor (it is not mentioned if weight or volume percentage), without a preconditioning step, to sterilize preformed packages followed with a post-treatment step by UV light. Aseptic filling machines built by SIG Combibloc typically use the three-step sterilization process shown in Figure 8 for the sterilization of bottom-sealed packaging sleeves (preformed packages) or the concept shown in Figure 12a for the sterilization of packaging sleeves (open from both ends). The volumetric concentration of H₂O₂ in these sterilization systems can exceed 5% v/v H₂O₂ vapor.

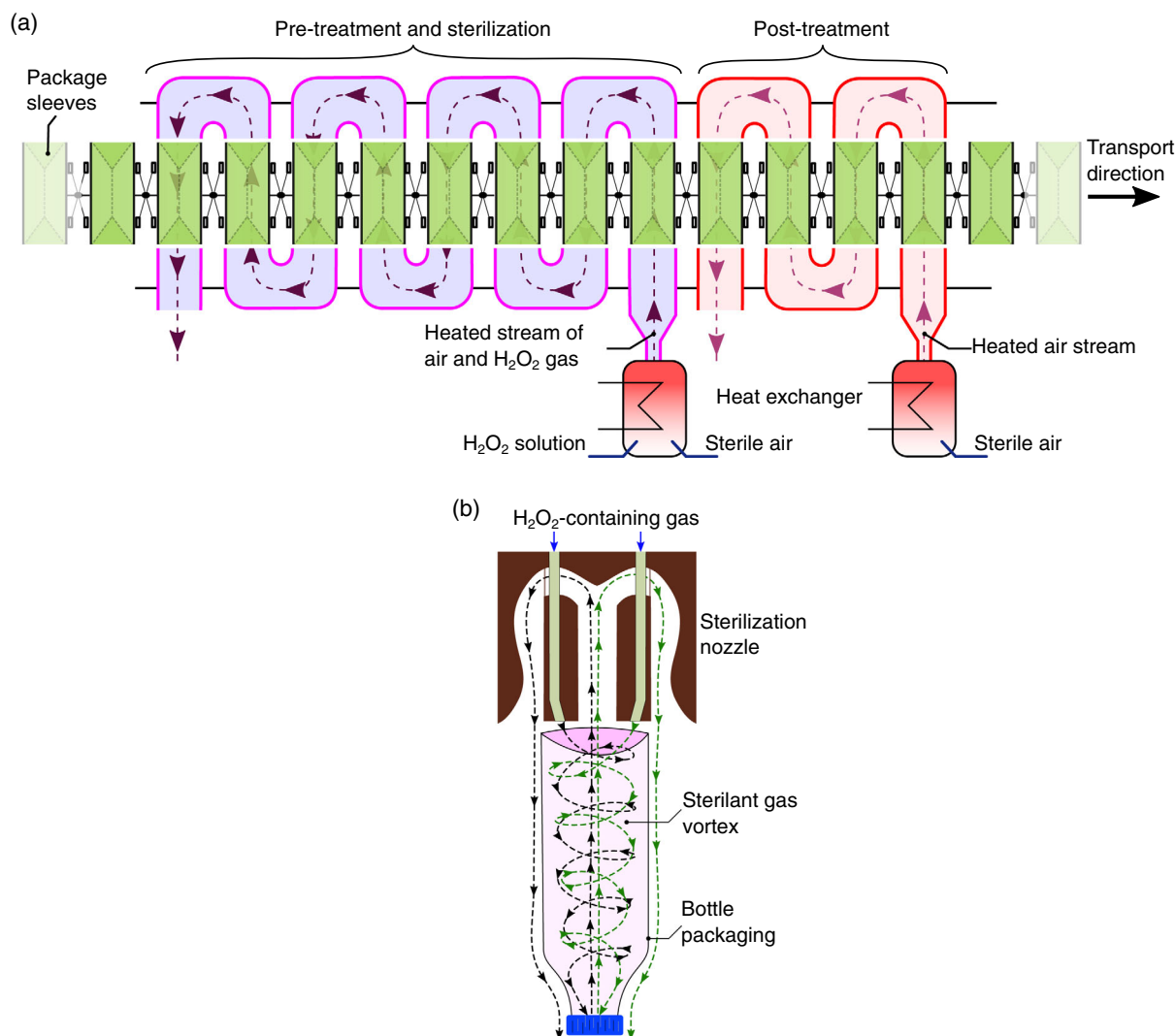


Figure 12. Scheme of exemplary H₂O₂ gas distribution systems. a) Sterilization tunnel for continuous sterilization of package sleeves which are open from both ends. The design is adapted from a patent by SIG Combibloc.^[227] b) Vortex generation (active) nozzle for bottle sterilization based on a patent by Tetra Pak.^[228] In this design, the bottle top is closed and sterilization takes place from the open bottle bottom prior to filling and sealing.

5. Biological Indicators

To validate the effectiveness of a sterilization process, standardized and regulated preparations of a selected group of resilient microorganisms are used.^[1,204] These tests are termed “challenge tests” or “validation tests” and the used microorganisms are called “biological indicators.” What is being challenged is the ability of the sterilization process to deactivate these test microorganisms. Therefore, the choice of a specific microorganism is based on multiple criteria^[33,118,205]:

- 1) the strain shows high resistance to the specified sterilization process; 2) it is nonpathogenic; 3) it can be easily cultured and is easy to handle; and 4) the microorganism exhibits a long shelf-life and can be commercially distributed.

Bacterial spores fit the requirements as a biological indicator and are applied as standard microorganisms to validate sterilization processes.^[4] Spores are a highly resilient, dormant form of

bacteria that form through sporulation when the surrounding conditions are not favorable for survival. Each sterilization process has a known and pure population of a specific spore genus and strain. Nonetheless, it is possible to apply biological indicators that consist of a mixture of spores.^[118] Recommended biological indicators for the validation of a specific sterilization process are shown in **Table 1**. Exemplary spores for the validation of H₂O₂ sterilization processes before and after sterilization are shown in the scanning electron micrographs (SEM) shown in **Figure 13**.

Geobacillus stearothermophilus, shown in Figure 13a, is a recurring microorganism in sterility tests. These bacteria are Gram-positive microorganisms from the genus *Geobacillus*. The rod-shaped bacteria can be found in hot and temperate environments such as hot springs, oilfields, and deep-sea sediments.^[206] They can survive at an environment with a temperature of 37–75 °C and an optimal at 55–65 °C.^[207] Spores of the *Geobacillus* genus

Table 1. Microorganisms that are approved as biological indicators to validate sterilization processes.

Sterilization method	Recommended test microorganisms ^{a)}	Strain	Literature reference
Radiation (Gamma, e-beams, X-rays)	<i>Bacillus pumilus</i> ^{b)}	ATCC 27142, CIP 77.25, NCTC 10327, NCIMB 10692	[109,118]
	<i>Bacillus cereus</i> , <i>Lysinibacillus sphaericus</i> ^{c)}	Not defined	[109]
UV light	<i>Cryptosporidium</i> , <i>Giardia lamblia</i> , viruses ^{d)}	Not defined	[229]
Moist heat	<i>Geobacillus stearothermophilus</i> ^{e)}	ATCC 7953, ATCC 12980, CIP 52.81, DSM 22, NCTC 10007, NCIMB 8157	[41,109,118,208,210,230]
Low-temperature moist heat ^{f)}	<i>Bacillus subtilis</i>	ATCC 35021	[230]
Low-temperature steam formaldehyde	<i>Geobacillus stearothermophilus</i>	ATCC 7953, ATCC 12980, ATCC 10149, DSM 6790, NCIB 8224	[123,231]
Dry heat	<i>Bacillus atrophaeus</i> ^{g)}	ATCC 9372, CIP 77.18, DSM 675, NCIMB 8058, NRRL B-4418	[109,118,210,232]
	<i>Bacillus subtilis</i>	DSM 13019	[232]
Ethylene oxide	<i>Bacillus atrophaeus</i> ^{h)}	ATCC 9372, CIP 77.18, DSM 2277, NCTC 10073, NCIMB 8058, NRRL B-4418	[41,109,210,233]
	<i>Geobacillus stearothermophilus</i> ⁱ⁾	ATCC 7953, CIP 52.81	[109]
Hydrogen peroxide ^{j)}	<i>Bacillus atrophaeus</i>	ATCC 9372, DSM 675	[208]
	<i>Bacillus subtilis</i> SA 22	NCA 72-52, DSM 4181	
	—		
	<i>Geobacillus stearothermophilus</i>	Not defined	[210]

^{a)}Unless otherwise stated bacterial spores are the main types of test microorganisms due to the reasons explained in Section 5; ^{b)}The *D*-value of these spores is about 3 kGy using 10^7 – 10^8 spores per carrier.^[109] Spores of *Streptococcus faecium* and *Micrococcus radiodurans* also show resistance to gamma radiation^[234]; ^{c)}These spores are used to validate radiation sterilization systems operating at higher doses than 25 kGy^[109]; ^{d)}Validation of UV sterilization systems for water treatment at a wavelength of 254 nm. A 4-log reduction is achieved by a UV dose of 22 mJ cm⁻² for *Cryptosporidium* and *Giardia lamblia* and a dose of 186 mJ cm⁻² for viruses^[229]; ^{e)}The *D*-value of these spores is 1.5–2 min at 121 °C using 10^6 spores per carrier.^[109] European Pharmacopeia recommended the use of viable spores that exceeds 5×10^5 spores per carrier. It is shown that the exposure to steam at 121 ± 1 °C for 15 min does not leave any revivable spores^[118]; ^{f)}For moist heat sterilization processes operating under 121 °C (not formaldehyde assisted). ^{g)}The *D*-value of these spores is 5–10 min at 160 °C using 10^6 spores per carrier^[109]; ^{h)}The *D*-value for a 10^6 spores per carrier is 2.5 min for a cycle with 600 mg L⁻¹ EtO at 54 °C and 60% relative humidity^[118,233]; ⁱ⁾Recommended is 10^6 spores per carrier^[109]; ^{j)}As of date, there is no clear regulation toward the validation of low- and high-temperature hydrogen peroxide systems. There are regulations currently under development, such as DIN EN 17180 and ISO/NP 22441. Catalase-positive spores that can counter the effect of sterilization with H₂O₂ are good candidates.^[235,236]

are common in food industries such as canned food and milk with reported levels in powdered milk that can exceed 10^3 spores g⁻¹.^[207]

The germination process reverts the spores back to bacteria that multiply when feeding on the nutrients in a product, releasing acids and other wastes, which can lead to product spoilage. Spore species other than *Geobacillus stearothermophilus* may also be used to validate the sterilization process, as long as they have been mentioned by the testing institute and their resilience is provided prior to the validation process.^[109,118,205]

Biological indicators are commercially available in different forms including frozen, freeze dried, spore suspension solution or inert carrier material inoculated with a spore suspension, and dried. Here, the used carrier material is resilient and inert to the conditions of the sterilization process such as glass or a temperature-stable polymer (PET). These carriers are inoculated with a calibrated quantity of spores from a prepared spore suspension. To test the effectiveness of an aseptic filling machine, i.e., the key processes as shown in Figure 8, sterility tests are conducted by inoculating packages at the start of the machine with a biological indicator. Following an incubation period, analysis on

the condition of the product in the package is conducted. This type of testing is also used for determining the effectiveness of a terminal sterilization process. Nonetheless, before conducting these sorts of tests, a compatibility analysis between the biological indicator and the product is required. This is done to assure that the growth of the microorganisms is not affected by the properties of the product such as its pH level.^[118] An environment (in this case a product) with an incompatible pH level can prevent spore germination or the survival of the bacteria. In this case, biological indicators will give wrong indication of the efficiency of the sterilization process parameters. For example, the optimal pH range for *Geobacillus stearothermophilus* spores is between 6.2 and 7.5.^[207]

The standard technique to quantify the sterilization efficacy is by applying statistical methods such as the end-point and count-reduction method, respectively, as described in various resources.^[208,209] Following the sterilization process, the indicator strips are transferred to an agar surface with a suitable culture medium, where the spores are incubated and allowed to germinate. The incubation temperature and time depend on the type and strain of the microorganism. Thermophilic spores require

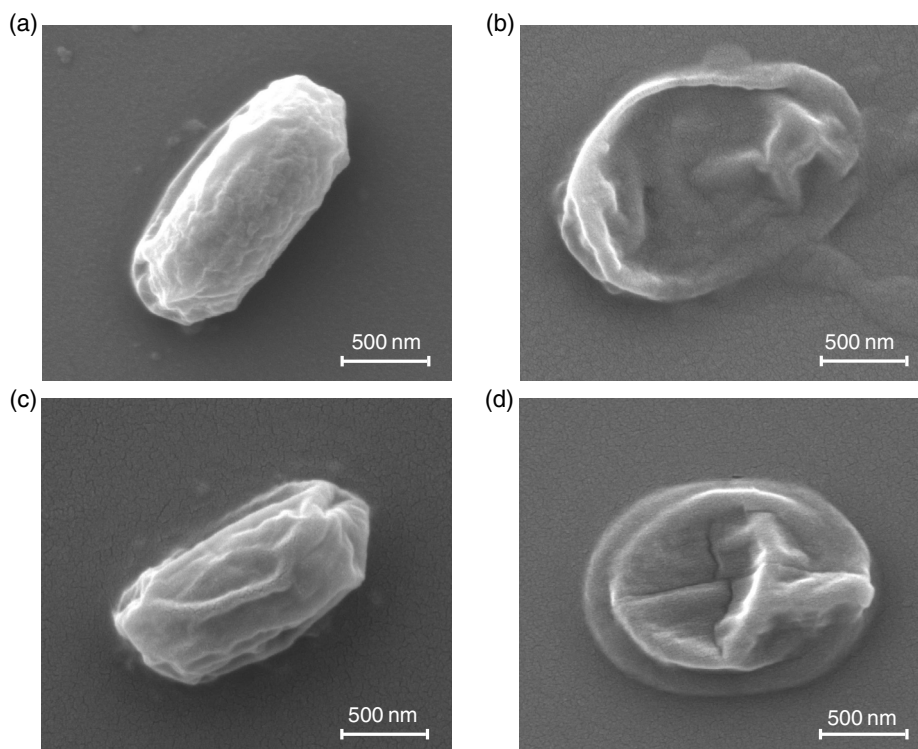


Figure 13. SEM (magnification $\times 40\,000$) taken by Joel JSM-7800F of a *Geobacillus stearothermophilus* (ATCC 7953) spore a) before and b) after a sterilization process and of a *Bacillus atrophaeus* (DSM 675) spore c) before and d) after the same sterilization process. In this example, a sterilization process by a 7.6% v/v H_2O_2 -containing gas at 240°C and 2 s is applied. The rupture of cell wall and loss of cellular matter are typical sporicidal actions of H_2O_2 . These are both observed here and shown in Figure 9. These spore types are applied to validate various sterilization processes and scenarios that are shown in Table 1.

an incubation temperature of $55\text{--}60^\circ\text{C}$ for at least 48 h and mesophilic spores require $30\text{--}37^\circ\text{C}$ for at least 48 h.^[205] The survived bacterial colonies are then counted under a microscope. Consequently, these methods are time-consuming and require the use of specialized labs with trained personnel. Because cell culturing is an essential part of this analysis, the total time-lag can span up to 7 days between the sterility tests and the results.

A qualitative method to validate a sterilization process is by combining the cell-culture medium used for incubating spore strips with a pH-sensitive color indicator. The color change of the medium signifies the presence of viable microorganisms indicating the failure of the sterilization process. This color change is due to the metabolic activity of the germinated spores that leads to an acidification of the culture medium. This validation technique is a conventional and convenient method that is used in industry.^[210,211] A combination of quantitative and qualitative methods is possible by end-point analysis using a pH-sensitive culture medium (with color indicator) for incubating test strips inoculated with different known concentrations of viable spores. To determine the SLR of the sterilization process, the statistical method of the most-probable number (MPN) can be applied. It depicts the logarithmic reduction in the number of spores from an original concentration value.^[36] For a full description of the MPN method, we refer to various studies.^[212,213]

The disadvantages of all microorganism-based validation methods are the possible false-negative and false-positive results

in sterility. In context to biological indicators, a false-negative result is an error that wrongly indicates a sterile sample. This can occur from residual sterilant on the biological indicator and the continued sporicidal effect in the culture medium. In contrast, a false-positive result is an error that wrongly indicates a nonsterile sample. This can occur due to cross contamination of the microbiological sample during handling and transferring to the culture medium.^[41] To decrease the impact of both errors, validation tests are conducted more than once to increase the reliability of the results, which extends the time period until the product is available to the consumer. It must be noted that a typical bioburden on a surface is not as pure and resilient as the applied microorganisms for the validation test. Following the sterilization process and upon successful deactivation of spores on indicator strips, the sterility of the product from packaging machine or sterilization system is guaranteed.

In heat- and radiation-based sterilization techniques, where the biological indicator does not require direct contact with the sterilant, a self-contained biological indicator (SCBI) can be used. These SCBIs are commonly applied for continuous monitoring of autoclaves in healthcare facilities as required by regulation.^[40] Here, a spore disk is placed in a plastic container along with a glass ampule that contains a nutrient medium mixed with a pH-sensitive color indicator. After the sterilization process, these SCBIs are activated by crushing the glass ampule. The color change in the nutrient medium indicates the presence

of active microorganisms.^[214] These systems prevent false-positive readings due to possible contamination during handling; the typical readout time for these systems is within 24–48 h.

Rapid readout SCBIs are also available in the market to validate product sterility from a steam sterilization process within 3 h (3M Attest 1492 V, 3M Company, the USA) and up to 10 h (NRI SCBI for steam, Etigam bv, the Netherlands).^[215,216] The 3M Attest validation system is unique as it is based on fluorometric detection of an enzyme (α -D-glucosidase) located on the outer coat of *Geobacillus stearothermophilus* spores. Studies show that the deactivation of this enzyme, e.g., due to heat from a sterilization system, correlates with the deactivation of the spores.^[217,218]

We briefly described the transition of microbiological challenge tests from quantification of lab-grown colonies from spore samples, to qualitative analysis by pH-sensitive culture medium, up to SCBIs for validating terminal sterilization processes capable of producing results within hours. The aim of these transitions was to simplify the routine operation of validating a sterilization process and assure optimal product quality without slowing down a production. The issue remains that validating surface sterilization processes found in the aseptic filling industry, where a surface sterilant is applied, cannot take advantage of SCBIs. In this case, there is still a time-lag of up to 7 days between the production and lab results that can lead (in the worst case) to product recall and serious financial losses to the producer. Online validation techniques might present a long-term solution in the future using label-free and biological sensors. A recent development in such sensor-based methods allows the quantification of viable bacteria down to low concentrations (limit of detection: 100 CFU mL^{-1}) within 1 h by modified heat-transfer method.^[219]

Another developed sensor based on interdigitated electrode structures measures the impedimetric change of an indicator spore sample before and after a sterilization procedure.^[220–222] These sensor chips have been tested to survive the harsh environment of in situ H_2O_2 gas sterilization of, e.g., up to 240°C and 8% v/v H_2O_2 concentration. The change in impedance is due to morphological changes in the spore and loss of cell elements, as shown in Figure 13. This impedance variation is later correlated with (calibrated by) microbiological challenge tests to determine the efficacy of the sterilization system, depending on the applied H_2O_2 concentration.^[161] The described sensor-based technique has been validated under laboratory conditions but still requires industrial tests before being commercially available. These sensors have the potential to become a useful tool for validation in the near future due to their readout speed (almost instantaneously), their accuracy, and reliability.

6. Concluding Remarks

Sterilization describes a treatment process that eliminates viable microorganisms from a surface or a product, here called a target. This treatment process comes in different forms and application methods depending on factors such as the thermal and chemical stability of the sterilization target. In this article, we have presented the different sterilization methods that are approved or under consideration by various regulatory bodies worldwide.

These methods are grouped under a specific category that describes the overall sterilization principle. Different industrial sectors have their favored sterilization method: 1) “Healthcare and medical sectors” have strict regulations that prohibit the use of unapproved sterilization techniques. This is apparent when we examine the working principle of autoclave units and closed-chamber sterilization systems that use hydrogen peroxide gas as the main sterilant, which did not see much change since their conception. Therefore, the main treatment processes are by heat (moist or dry), radiation, and ethylene oxide. Sterilization using hydrogen peroxide has been applied to some degree in the medical sector and is considered as an alternative to moist heat sterilization of heat-sensitive objects. In addition, for sterilizing healthcare facilities, fumigation of rooms with hydrogen peroxide is typically applied. Due to the carcinogenic properties of ethylene oxide and its possible residues, we observe a gradual decrease in its use. Alternative processes such as chlorine dioxide gas and supercritical carbon dioxide are being developed and might become a possible standard in future. 2) The “pharmaceutical industry” typically applies dry heat to treat glass containers before filling in sterile atmosphere. The dry heat will deactivate both microbial entities and any toxins present on the surface of the container. This is the preferred depyrogenation process. For noncritical products, isolators and restricted access barrier systems use hydrogen peroxide or ethylene oxide to sterilize containers and equipment. 3) The main sterilant to sterilize product-contact surfaces in “food and beverage” industries is either hydrogen peroxide solution/gas or a derivative thereof (e.g., peracetic acid). These industries are less reluctant to experiment and implement new technologies to make their machines faster, more efficient, and reliable. The main condition of the sterilization system in this case is that it does not leave residues in the final product that are either harmful to the consumer or might cause a change in the organoleptic properties of the product. If these conditions are proven and the new sterilization system is validated using a suitable procedure such as biological indicators, then it may be applied to the machine.

It has been about 40 years since the approval of hydrogen peroxide as a sterilant. Considerable research efforts were done on its microbicidal properties and it has become a standard surface sterilant of packaging materials prior to product filling. Filling machine manufacturers (pharmaceutical, food, or beverages) still favor hydrogen peroxide-based sterilization systems over the more experimental technologies. Nonetheless, we are observing nowadays a market shift toward the use of e-beam sterilization technology. There are good reasons for this trend: e-beam technology offers a higher control over the sterilization process than with the use of chemicals, it is supplied on-demand, and unlike hydrogen peroxide it offers minimum handling risks for the machine operator. Nonetheless, this technology has higher investment and maintenance costs. Once e-beam technology becomes economically more favorable to chemical sterilization methods, we might observe a gradual phase out for the use of chemical sterilants.

In conclusion, there are several opinions, which, among the described sterilization methods, represent the ultimate sterilization technique. However, while different sterilization processes can be applied to the same target, we have shown throughout this work that each sterilization method has its advantages and

disadvantages. Moreover, many of these methods are still under development and require technological breakthroughs before being called a standard technique. Consequently, there is still room for research and development on each sterilization method to create a more ecological and economical alternative or adaptation.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

Z.B.J. collected data from literature and industry, prepared the figures, and drafted the manuscript. P.H.W. and M.J.S. substantially contributed to the concept and design of the manuscript, revised it, and provided critical advices throughout the drafting process.

Keywords

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