A Microwave-Powered Sterilizable Interface for Aseptic Access to Bioreactors That Are Vulnerable to Microbial Contamination

James E. Atwater,*,† William F. Michalek,† Richard R. Wheeler, Jr.,† Roger Dahl,† Teddie D. Lunsford,† Frank C. Garmon,† and Richard L. Sauer‡

UMPQUA Research Company, P.O. Box 609, Myrtle Creek, Oregon 97457, and Lyndon B. Johnson Space Center, National Aeronautics and Space Administration, Houston, Texas 77058

Novel methods and apparatus that employ the rapid heating characteristics of microwave irradiation to facilitate the aseptic transfer of nutrients, products, and other materials between microbially sensitive systems and the external environment are described. The microwave-sterilizable access port (MSAP) consists of a 600-W magnetron emitting at a frequency of 2.45 GHz, a sterilization chamber with inlet and outlet flow lines, and a specimen transfer interface. Energy is routed to the sterilization chamber via a coaxial transmission line where small quantities of water couple strongly with the incident radiation to produce a superheated vapor phase. The efficiency of energy transfer is enhanced through the use of microwave susceptors within the sterilization chamber. Mating surfaces are thermally sterilized through direct contact with the hot gas. Efficacy has been demonstrated using the thermophile *Bacillus stearothermophilus*.

Introduction

The onset of microbial contamination is frequently a key factor in determining the end point for a variety of bioreactor production runs (*1*). The primary opportunities for contamination occur during inoculation, nutrient delivery, harvesting of reaction products, or collection of samples for analysis. More effective means to prevent the introduction of unwanted microbial strains are clearly needed. Conversely, preventing contamination of the external environment while investigators study pathogenic microbes or genetically modified organisms of unknown virulence can be equally problematic. For these reasons, the microwave sterilizable access port (MSAP) was developed as a means for the prevention of migration of unwanted organisms either into or away from biological containment systems during those times in which materials are transferred between the system and the exterior realm.

The prototype MSAP system (*2*) is illustrated in Figure 1. The device is composed of three subsystems: the microwave power source, the sterilization chamber with integral in-line valve-port, and the materials transfer interface. The in-line valve-port assembly is normally closed. To access the bioreactor, the materials transfer interface is inserted into the sterilization chamber and all mating fixtures are thermally sterilized. This is achieved by the introduction of a small volume of water into the chamber, followed by microwave irradiation of the closed system. Microwave energy is applied via a shielded coaxial transmission line that terminates at an antenna located inside the sterilization chamber. The incident radiation is absorbed directly by water molecules within the system and also by a ceramic block composed of α-silicon carbide (SiC). The energy flux results in a rapid phase change, producing steam, which then superheats, increases internal pressure within the chamber, and destroys microorganisms in the same manner as in an autoclave but much more rapidly. This rapid localized heating minimizes the potential for damage to thermally labile materials within the bioreactor. Entry into the system for the introduction or removal of materials is then achieved by the penetration of the sterile septum by the sterile needle and by rotation of the integral in-line valve-port to the "access" position. For the prototype testing reported herein materials were transferred via a syringe-like device.

To validate the concept, a prototype unit was fabricated and thoroughly tested. A series of microbial challenges was conducted using *Bacillus stearothermophilus*, a thermophilic Gram-positive rod, which is commonly used to evaluate the efficacy of thermal sterilization procedures and equipment. Variable factors that were investigated included incident microwave power, composition of the liquid phase, volume of the liquid phase, and sterilization time.

Materials and Methods

Microwave-Sterilizable Access Port (MSAP) Design and Operation. The prototype MSAP design is the culmination of a series of developments that began with elementary sterilization experiments using microwave irradiation in relatively inefficient multimode cavities (*3*) and progressed through intermediate stages using rectangular waveguide based irradiation systems (*4, 5*). On the basis of this initial work, coaxial transmission of microwaves to an antenna located within a miniaturized steam sterilization chamber was selected as the most appropriate means to achieve extremely rapid thermal sterilization via a portable microwave power source.

The system consists of two primary components: the microwave power controller and the sterile access port

* Email: jatwater@urcmail.net.
† UMPQUA Research Company.
‡ NASA Johnson Space Center.

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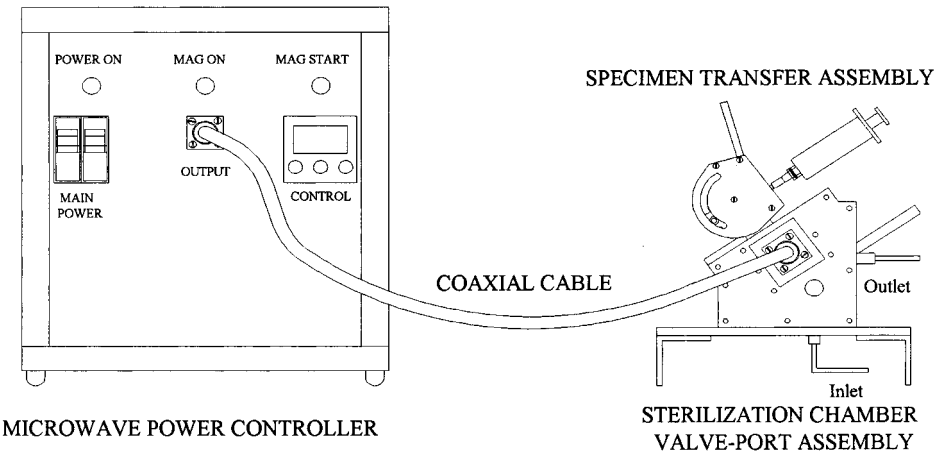

Figure 1. Components of the prototype microwave sterilizable access port.

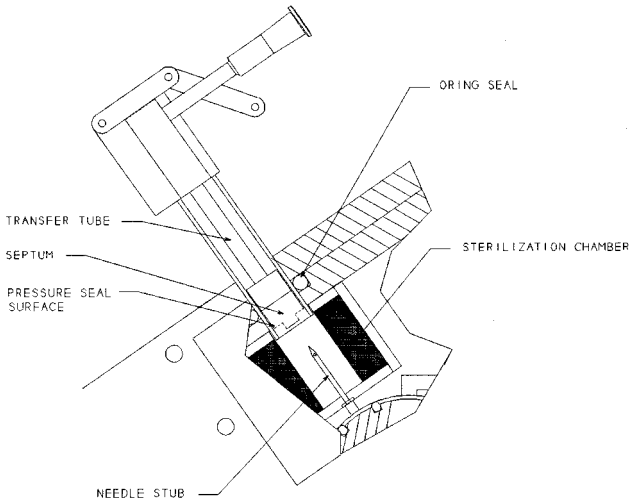

Figure 2. Materials transfer interface mated with the miniaturized sterilization chamber. Prior to accessing the microbially vulnerable system, the exposed surface of the septum and the needle stub are sterilized. Following sterilization, the rotating cam forces the needle to penetrate the septum, and aseptic access to the system is achieved.

assembly, which houses the sterilization chamber. These are linked via coaxial cable. The access port is fitted with inflow and outflow lines that are connected to the bioreactor system. Access occurs through the aseptic interface consisting of a sterile needle residing within the sterilization chamber, which penetrates a sterile septum. Microwave radiation is generated using a 600-W magnetron emitting at 2.45 GHz into a WR 430 rectangular waveguide based launcher section fitted with waveguide to coaxial transmission line transition elements. Power is transferred to the sterilization chamber via shielded coaxial cable. The design of the sterilization chamber has been described in a previous publication (*6*). It consists of a small hollow aluminum block with an internal volume containing a SiC ceramic monolith in which the antenna is mounted. Microwaves are emitted directly into the monolith. An approximately 1 cm^3 cavity within the ceramic contains a stainless steel hypodermic-like needle stub that provides the channel of communication for the passage of materials between the bioreactor and the external environment. The sterilization chamber is also fitted with a solenoid-controlled steam vent and thermocouples to monitor operating temperatures.

In operation, the presterilized L-shaped materials transfer interface (MTI) (Figure 2), consisting of stainless steel tubing with a rubber septum attached to one end and a Luer-Lock at the other, is mated with the sterilization chamber. The Luer-Lock may be attached to a sterile syringe or other suitable vessel. The MTI is secured in place via the three-position rotating cam shown in Figure 3. The three positions are [1] closed, [2] sterilization, and [3] access. The system is normally in the [1] closed configuration. Access for the aseptic transfer of materials is achieved by first rotating the cam to the [2] sterilization position and after sterilization to the [3] access position. To achieve sterilization, while in position [1] a small quantity (<1 mL) of water is introduced into the sterilization chamber using a microsyringe. The cam is then rotated to position [2], which establishes a pressure-tight seal of the chamber. Microwave power is applied until a preset temperature is reached. This produces superheated vapors, which contact all surfaces and destroy any microbes that may be present, if system temperatures and exposure times are sufficient. After a predetermined end-point temperature is reached, the sterilization event is terminated by the activation of the steam vent solenoid, which relieves pressure within the chamber. The cam is then rotated to the [3] access position. This forces the penetration of the MTI rubber septum by the needle stub within the sterilization chamber. Materials may now flow, free from potential sources of contamination, either into or away from the bioreactor. To reclose the system, the cam is rotated back to the [1] closed position. Alternatively, if contamination of the environment by the contents of the bioreactor is a concern, the cam may be rotated first to position [2] for a second sterilization, prior to closure of the system. Here, the terminology may be somewhat confusing; in the [1] position the bioreactor system is closed but the sterilization chamber is open. Thus, after the aseptic transfer is completed, harmful microorganisms can be destroyed with a second sterilization event prior to opening the MSAP to the environment.

Microbiological Methods. A variety of microbial challenges were conducted to determine appropriate operating conditions and to establish proof of efficacy once these were established. Pure cultures of the thermophilic spore former *Bacillus stearothermophilus* (ATCC 7953) were obtained from the American Type Culture Collection (ATCC). All bacterial cultures were incubated at 55 °C for periods between 18 and 24 h. *B. stearothermophilus* spores were grown on soil yeast extract agar (SYE) spore enhancement media, and R2A agar (DIFCO 1826-17) was used as recovery media. Serial dilutions were prepared by the introduction of 5 mL of sterile water onto the surface of an agar slant and the formation of an aqueous

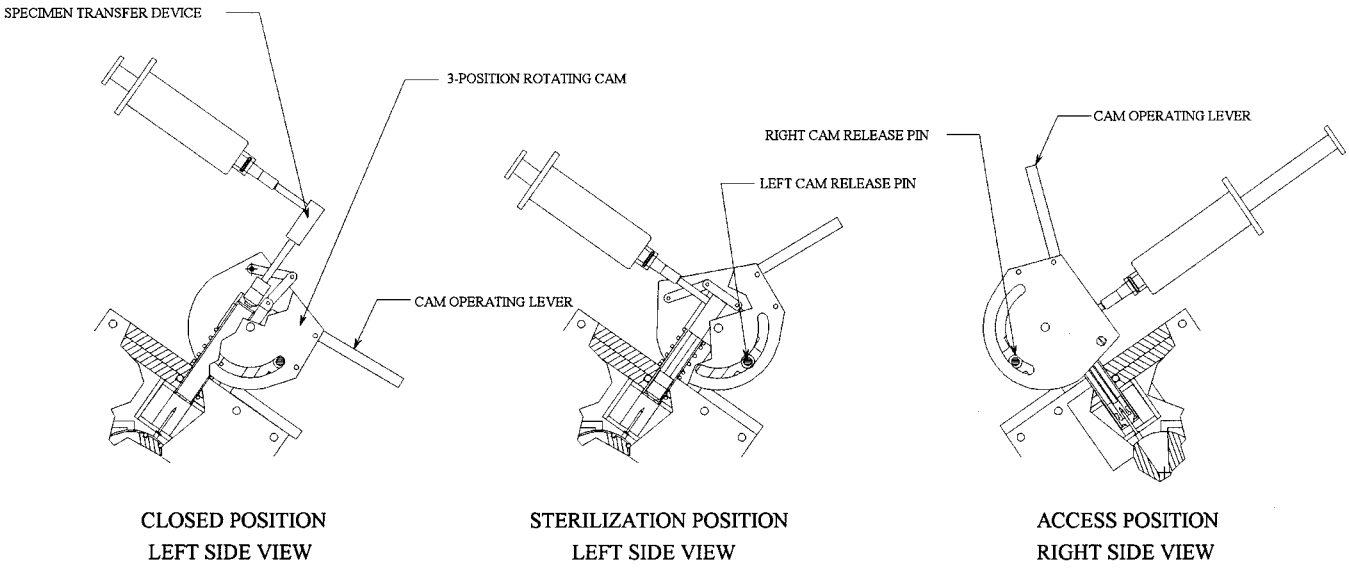

Figure 3. Prototype microwave sterilizable access port.

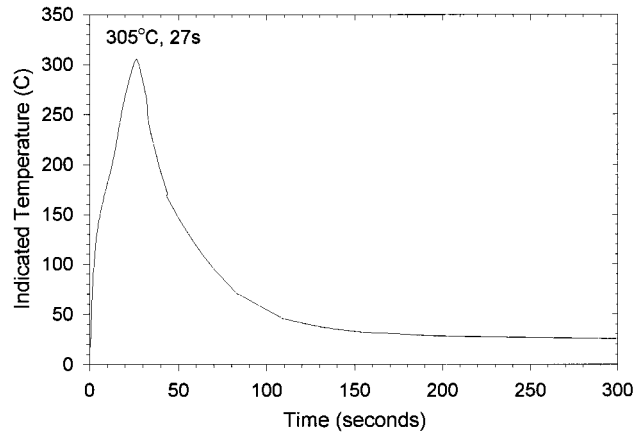

Figure 4. MSAP sterilization chamber temperature profile.

bacterial suspension by removal of surface microbes with a sterile loop. After agitation, 1 mL of the bacterial suspension was transferred to a sterile dilution bottle containing 99 mL of sterile water and thoroughly mixed. Further dilutions were made from this 10^{-2} stock solution.

Microbial challenges were conducted in two primary variations. These included the intentional contamination of the presterilized rubber septa attached to the L-shaped MTI by direct contact with dense microbial populations at the surface of agar plates, and insertion of contaminated PTFE coupons directly into the sterilization chamber.

Results and Discussion

Preliminary experiments indicated that 500 μL of deionized water provided sufficient sterilant to achieve rapid microbial kill. This volume was used in all subsequent experiments. To avoid the need for feedback circuitry and more complex and expensive means for regulating the microwave power levels incident to the sterilization chamber, the apparatus was operated by the application of power until a predetermined temperature was reached, after which microwave irradiation was terminated. This method of operation resulted in rapid heating of the sterilization chamber and also relatively rapid cooling. The temperature profile for a typical sterilization event is shown in Figure 4. Using a set-point temperature of 305 °C, the maximum temperature was reached after 27 s of irradiation. While this temperature does not necessarily correspond to the average system temperature, because of the excellent thermal conductivity of both the aluminum and SiC materials of construction, relatively uniform temperature distributions result.

The rapid system heat-up can be attributed to the excellent microwave absorbing properties of both SiC and water. In susceptible solids, heat is produced via oscillating induced interfacial (Maxwell-Wegner) and space-charge polarizations. In water, absorbed microwave energy produces high-frequency rotation of the dipolar molecules. Microwave susceptibility is governed by the complex permittivity (ϵ^*) of the medium (*7*−*10*):

$$\epsilon^* = \epsilon' - i\epsilon'' \tag{1}$$

Attenuation of a microwave beam directed along the *x*-axis by an absorbing material is described by

$$P(x) = P_o \exp(-2\alpha x) \tag{2}$$

where the attenuation coefficient (α) is a function of the angular frequency (ω), complex permittivity, and complex permeability (μ^*):

$$\alpha = \omega \sqrt{\sqrt{\epsilon'^2 + \epsilon''^2}\sqrt{\mu'^2 + \mu''^2}} \times \sin\left[\frac{\arctan\left(\frac{\epsilon''}{\epsilon'}\right) + \arctan\left(\frac{\mu''}{\mu'}\right)}{2}\right] \tag{3}$$

In nonmagnetic materials, values of μ^* are extremely low, and hence, microwave absorption is dominated by the complex permittivity. In the complex plane, the imaginary component, ϵ'', represents dielectric losses such as those that result in heating. The loss factor or dissipation factor ($\tan \delta$) is defined as

$$\tan \delta = \frac{\omega\epsilon'' E_o^2}{\omega\epsilon' E_o^2} = \frac{\epsilon''}{\epsilon'} \tag{4}$$

where *E* is electric field strength. The average power per

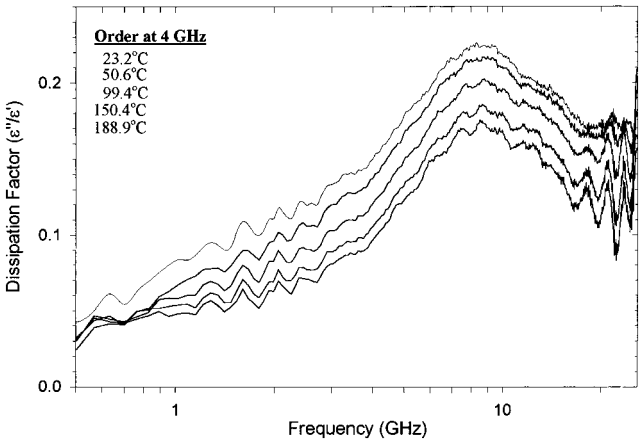

Figure 5. Dissipation factor versus frequency for α-SiC between 23 and 189 °C.

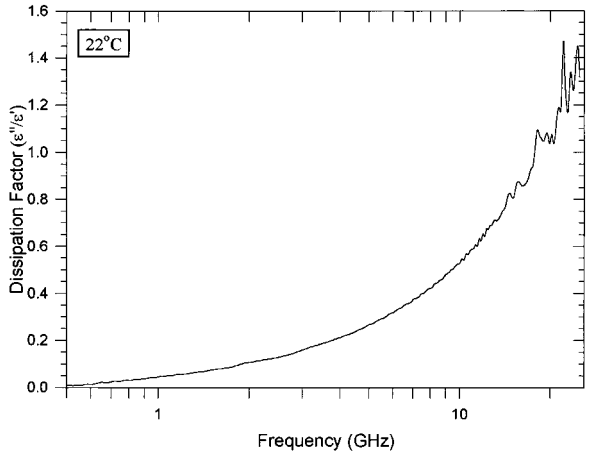

Figure 6. Dissipation factor versus frequency for pure water at 22 °C.

unit volume (P) consumed by loss mechanisms can then be calculated as

$$P = \frac{1}{2}\omega\epsilon'' E_o^2 \tag{5}$$

Measurements made in our laboratory using a vector network analyzer based measurement system (HP 8722D) derived values of tan δ for α-SiC as a function of frequencies between 0.5 and 26 GHz and temperatures (23−189 °C) as shown in Figure 5. The results for similar measurements of deionized water samples at 22 °C are shown in Figure 6. While 2.45 GHz is not the most efficient irradiation frequency for these materials, it is clearly adequate. This frequency was selected for employment in the MSAP because of the relatively low cost and widespread availability of suitable magnetrons.

While many microorganisms can be destroyed under relatively mild conditions, bacterial spores, particularly those of thermophiles such as *B. stearothermophilus*, can be quite resistant (*11*−*14*). Two factors are of paramount importance to achieve wet thermal sterilization: temperature and contact time. Reaction rates, in general, are exponential functions of temperature. This is the case for thermally induced microbial destruction; however, this relationship is often expressed in terms of lethality factor (F_o). An F_o of 16 is equivalent to the level of sterilization achieved using routine autoclave procedures. For this lethality factor, the relationship between contact time (τ) in minutes and temperature (T) in °C (*15*) can be expressed as

$$\ln \tau \approx 33.90 - 0.2535T \tag{6}$$

The maximum transient state temperature of 305 °C that is measured within the SiC monolith does not correspond to a system-wide temperature. A much better approximation of an average temperature within the confines of the gas-filled internal volume can be gained from the internal system pressures during sterilization. Assuming the system is pressurized with superheated steam originating from an initial 500 μL injection of water, a maximum temperature of ∼142 °C is indicated. At this temperature, eq 4 suggests that a sterilization time of 7.4 s is required to achieve an F_o of 16. Thus, the transient state temperature regime resulting from the microwave irradiation appears more than adequate.

To confirm the efficacy of sterilization under these conditions, a variety of microbial challenges was conducted. Prior to integration of the sterilization chamber into the MSAP device a series of tests was performed using PTFE coupons mounted directly inside the chamber (*6*). The coupons were contaminated with $\sim 10^6$ colony forming units (CFU) of *B. stearothermophilus*. Volumes of deionized (DI) water ranging between 50 and 1000 μL were introduced into the system, and the maximum indicated chamber temperatures were varied. Thermal sterilization was achieved using 500 μL of DI water and maximum indicated temperatures ≥ 255 °C. For these experiments, the chamber was irradiated until a preset temperature was reached. An elapsed time of 26 s was required for the indicated temperature to reach 255 °C. Under these conditions, pressures within the chamber rose to 3.3 atm. Ninety-six similarly contaminated PTFE coupons were subsequently irradiated in separate tests using the 255 °C end point. No bacterial survivors were identified during these experiments.

The fully integrated prototype MSAP was repetitively challenged by contamination of the silicone rubber septa. Dense populations of spore enhanced *B. stearothermophilus* were cultured on SYE agar. Presterilized septa were contaminated by touching the exposed surfaces to the agar plates. After injection of 500 μL of DI water into the sterilization chamber, materials transfer interfaces bearing contaminated septa were locked into position on the prototype MSAP. The duration of microwave irradiation was controlled using predetermined end point temperatures. The initial experiments were performed over a range of temperatures between 250 and 310 °C. Following the sterilization procedure, the septa were used to streak R2A agar plates and then were immersed in SYE broth. Both agar plates and broth were incubated 24 h to determine if survivors were present. These relatively nonspecific recovery methods were capable of detecting the presence of a wide variety of aerobic bacterial species in addition to *B. stearothermophilus*. Initial surface bacterial populations were determined using non-microwave-irradiated control septa, otherwise treated in an identical manner. Sterilization was evident for end point temperatures equal to or greater than 305 °C. At lower temperatures surviving bacilli were encountered sporadically.

Further experiments were then conducted using 500 μL injections of water and 305 °C as the end point. In the integrated MSAP, this end point temperature was reached after 27 s of irradiation (Figure 4) and resulted in maximum sterilization pressures of approximately 3.8 atm. To investigate the aseptic transfer of materials from

within a microbially vulnerable system, a closed sterile recirculating loop containing 20 mL of SYE broth was attached to the MSAP. The recirculating loop was prepared using a 25 mm × 150 mm glass culture tube connected to MSAP inlet and outlet ports via 0.32 cm i.d. plastic tubing. A peristaltic pump was used to establish the recirculating flow at 5 mL/min. All materials used in the recirulation loop were presterilized. Material transfer interfaces bearing septa contaminated with $\sim 10^6$ CFU of spore enhanced *B. stearothermophilus* were locked into place and sterilized using the MSAP, after which replicate 3 mL samples were withdrawn from the recirculation loop through the MSAP using a sterile syringe. Septa were again examined for bacterial survivors. All samples and the remnant recirculating growth media were incubated at 55 °C for 48 h. This experiment was repeated 48 times using fresh broth and presterilized recirculating loops. No bacterial survivors were found in any of these experiments. A similar protocol was followed to examine the transfer of materials into the sterile system. In this variation, MTIs bearing contaminated septa were mated to the MSAP and sterilized, after which 3 mL samples were first withdrawn and then reinjected into the recirculating flow of sterile SYE growth media. After each experiment, recirculating media, septa, and the interior surfaces of the syringe were examined for microbial contamination. A series of 32 replicate transfers were mediated by the MSAP. Again, no microbial survivors were found.

These results indicate the validity of the MSAP hardware and methodology for the aseptic transfer of materials between a biologically sensitive system and the external environment. Aseptic transfers of materials into and out from the system have been demonstrated using spore enhanced cultures of *B. stearothermophilus*, the standard challenge organism for determining efficacy of thermal sterilization techniques. This has been accomplished using 500 μL injections of deionized water and sterilization times, including system cool-down, of under 5 min.

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