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Sterilization of bioreactor media on the basis of computer-calculated thermal input designated as F_0

LaVerne D. Boeck, Roger W. Wetzel, Stanton C. Burt, Floyd M. Huber, Gary L. Fowler and Joseph S. Alford, Jr.

Eli Lilly and Company, Indianapolis, IN, U.S.A.

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SUMMARY

Industrial fermentation media are normally sterilized with steam to destroy the indigenous microbial population prior to inoculation with a specific microorganism. Because biological validation of each sterilization cycle is impractical, an 'overkill' approach is commonly employed on the basis that alteration of heat-sensitive nutrients is less detrimental than survival of indigenous microbes. However, the heat destruction of microbes is known to be a probability function amenable to calculation. A computer has been programmed to calculate the on-line heat input as F_0 values during sterilization of media in stirred bioreactors. The accumulation of F_0 values is then announced verbally to bioreactor operators by a communications controller with voice synthesizer.

INTRODUCTION

Before inoculation with a desired microorganism, industrial fermentation media are normally sterilized to destroy the indigenous microbial population. Sterilization is commonly achieved by autoclaving, or heating with steam in a vessel pressurized to 15 lbs/in^2. If the air has been expelled and the atmosphere consists of saturated steam, this corresponds to a temperature of 121°C. An alternate method of sterilizing media employs removal of the contaminating microbes by filtration. This process additionally removes other particulate materials that may be present, and industrial media frequently contain large amounts of insoluble nutrients that quickly occlude filters. Further, removal of these particles alters the nutritional quality of the media. Autoclaving may also alter the concentration of heat-sensitive and chemically reactive media nutrients in a time- and temperature-dependent manner, either by destroying vital nutrients or by forming inhibitory compounds [2]. However, the availability and convenience of heating with steam contribute to the widespread use of thermal sterilization methods.

By accepted standards, the term 'sterile' signifies the absence of all viable microorganisms [7], and

Correspondence: L.D. Boeck, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, U.S.A.

sterilization is often misconstrued as an absolute process. In reality, heat destroys the viability of microorganisms at an approximately logarithmic rate. Plots of inactivation kinetics reveal that sterilization is a probability function [3]. The goal in autoclaving heat-sensitive materials [5], which include fermentation media, is to assure a low probability of microbial survival with minimum, and consistent, effects on the product. Although the minimal heat required to achieve sterility varies with different media and autoclaving conditions, biological validation of every sterilization cycle is impractical because of the effort involved and the time lag required for survivorship detection. However, entire lots of liquid media in which sterility has not been achieved are generally lost due to rapid outgrowth of the survivors. To avoid this well known consequence in the absence of validation data, an 'overkill' approach [5] to sterilization is widely employed on the basis that nutrient alteration is less detrimental than survival of indigenous microbes. An alternate validation method [6] involves representing the total heat applied during the cycle as an F value and calculating the probability of microbial survivorship. The temperature measurements and calculations for F values are manually tedious but are readily accomplished by computers. The F value method has numerous advantages. These include on-line availability, incorporation of altered heating effects during temperature fluctuations or deviations, ready adjustment for vessel volume, and the ability to evaluate each heating cycle prior to inoculation of the media with the desired microorganisms. In addition, F values assure uniform heat application to successive batches of media so that, if an adequate safety factor has been included in the target F value selected, sterility is achieved with minimum, and consistent, nutritional modification.

This report describes a procedure for sterilizing liquid media in stirred bioreactors on the basis of on-line F values, referred to as F_0.

MATERIALS AND METHODS

Bioreactors were fully baffled pressure vessels of conventional design with two open-center six-bladed turbine impellers that stirred the media continuously. Operating volume was approximately 115 liters. Vessels were equipped with permanently mounted dual-range Honeywell resistance bulbs housed in thermal wells that projected into the media. Sterilization cycles were monitored with the high range on the resistance bulbs, which was calibrated to a nominal accuracy of $\pm 0.03°$ at 121°C. All control loops were under the jurisdiction of Foxboro Interspec hardware interfaced with a Hewlett-Packard 1000 computer. An SLC II communications controller with voice synthesizer (Digital Pathways, Palo Alto, CA) transparently monitored data strings transmitted from the computer to an associated printer. In sterilization mode, the Hewlett-Packard 1000 continually measured temperatures at 1-s intervals for computation of the accumulating F_0 values.

All bioreactors were 'wet' pre-sterilized for 1 h prior to formulation of the fermentation media or installation of heat-sensitive sensors such as galvanic oxygen electrodes. This eliminated microbes which might survive a less intense sterilization cycle during residence in adjacent locations that did not receive lethal heat and could provide delayed access of the survivors to the media with ensuing recontamination.

Although fermentation media are not normally pasteurized prior to sterilization, the viable microbial population of bioreactor media was determined both before and after pasteurization in order to differentiate the heat-sensitive vegetative cells from the heat-resistant endospores present. Pasteurization was accomplished by a 30 min holding period at 62.5°C, with rapid pre-heating and cooling. The number of indigenous colony-forming units (cfu) in each broth was determined by plating on Trypticase Soy Agar medium with subsequent incubation at 30°C for 5 days.

RESULTS AND DISCUSSION

The microorganism whose spores have been shown to be most resistant to moist heat is *Bacillus stearothermophilus*. The average Z value of these spores (the temperature change required for a survivor decrease of 1 log unit) has been shown to be 10°C [1]. When this relationship exists, F_0 has been used to represent the equivalent lethality in minutes of any thermal process to a thermal process at exactly 121°C [6]. In this context, 1 min at 121°C equals F_1. Because the relative sterilizing effect of temperatures above or below 121°C is included in the calculation, deviation from that temperature is taken into account. The basic equation used to express this relationship is:

$$F_0 = \Delta t \Sigma 10^{(T - 121)/Z}$$

where Δt = the time interval between measurements, T = temperature at time t, and Z = 10°C. Σ indicates that F_0 is an additive term [1].

Since lethality is an exponential function of temperature, a linear temperature increase or decrease of as little as 1.67°C inversely changes the probability of microbial survival several-fold [4]. Consistent heating is thus essential for the achievement of consistently lethal effects. The effect of temperature changes on F_0 values is shown in Table 1.

Adequate assurance of sterility in Fermentation Pilot Plant (FPP) media had been achieved by autoclaving the media-containing bioreactors at the standard temperature of 121°C for an empirically derived period of 45 min. Concern regarding possible overkill sterilization and its potentially negative effects on heat-sensitive nutrients led to computer monitoring and F_0 calculation of thermal input. Time/temperature profiles of sterilization cycles revealed that temperatures oscillated by as much as 3° in response to the adjustment of steam-inlet and pressure-regulating valves during manually controlled autoclaving cycles. Bioreactor operators were also found to be acutely aware that inadequate sterilization could result in loss of fermentations due to bacterial contamination. To gain additional assurance of avoiding this problem, the operators tended to maintain the temperature above 121°C during sterilization. The extent of their individual biases varied, as did their skill in maintaining a precise temperature level (Fig. 1). This regularly resulted in the application of excess heat during autoclaving, usually producing 70 or more F_0 values. In addition, presumably uniform 45-min sterilizations under manual control did not result in uniform heat application during successive sterilization cycles, since F_0 values were found to range from 60 to 85. On the other hand, computer control

Table 1

Effect of temperature on accumulation rate of F_0 values

Temperature (°C)	F_0 values produced/min	Minutes required per F_1
101	0.01	100.0
111	0.1	10.0
115	0.25	4.0
121	1.0	1.0
125	2.5	0.4
131	10.0	0.1

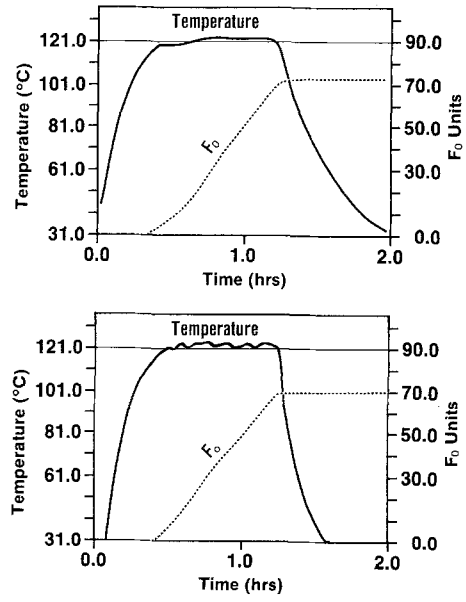

Fig. 1. Temperature and F_0 tracings of 45-min bioreactor sterilization cycles demonstrating two types of profiles typically generated during manual control.

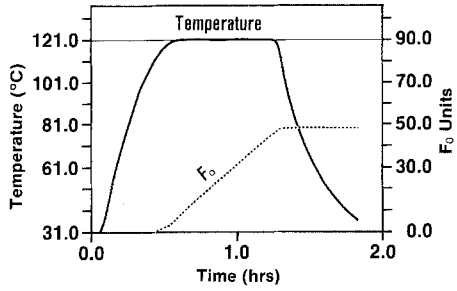

Fig. 2. Temperature and F_0 profiles of 45-min bioreactor sterilization cycle under computer control.

of a single valve to regulate temperature by controlling vessel pressure during the autoclaving cycle resulted in precise set-point control with consistently reproduced heating values of F_{47-49} (Fig. 2).

Computer-controlled sterilization on the basis of F_0 was therefore selected as a preferred method of standardizing heat input to successive lots of bioreactor media. In order to establish the number of F_0 values apparently required for acceptable assurance of sterility, the indigenous bioburden of various media was determined. These media consisted of typical formulations used in the FPP and included 'rich' media believed to be high in microbial content. Representative examples of the number of cfu present in various media initially, and those surviving pasteurization, are shown in Table 2. The A25822 medium contained the largest number of cfu observed in unpasteurized media, 4.1×10^5. The largest number of cfu encountered in pasteur-

Table 2

Indigenous microbial bioburden of various fermentation media

Medium component	Medium designation				
	actaplanin	A25822	A40104	A51568	narasin
Glucose	1.0[a]	4.0	1.5		
Dextrin	3.0	2.0	3.0	3.0	8.0
Soybean oil					0.5
Glycerol			0.5		
N-Z-Amine A (Sheffield)		0.4			0.2
Amber EHC (Marcor)					0.6
Cottonseed flour		1.5			
Bacto-peptone (Difco)				0.7	
Liquid meat peptone	1.5	1.0			
Dog Chow (Ralston Purina)			2.5		
Soybean flour	0.5				
Yeast				0.5	
Blackstrap molasses	1.5	1.5		2.0	1.5
Corn steep liquor	0.5				
$(NH_4)_2SO_4$					0.1
$MgSO_4 \cdot 7H_2O$		0.5	0.05		0.05
K_2HPO_4	0.05				
$CaCO_3$	0.2	0.2	0.1		0.2
H_2O					
tap	×				×
deionized		×	×	×	
cfu/ml					
unpasteurized	2.9×10^5	4.1×10^5	1.4×10^3	1.6×10^3	5.9×10^2
pasteurized	1.9×10^3	5.5×10^2	$<0.3 \times 10^2$	9.1×10^2	5.6×10^2

[a] Percent

ized media, 1.9×10^3, was present in the actaplanin medium.

Although cultures in the latter group were not classified taxonomically, microscopic examination of random colonies determined that all were spore-forming bacilli. They were presumed to be *B. stearothermophilus*, the bacterium most resistant to thermal inactivation, whose average Z value is 10. The number of F values selected as an acceptable sterility target must be an individual choice based on the goals and total working system involved. It should include an adequate safety factor to compensate for both normal and atypical fluctuations in various parameters, such as the initial bioburden. A generally accepted standard for probable sterility requires that 10^6 sterilized bioreactors shall contain no more than one bioreacter that remains unsterile [6]. The effect of bioreactor volume and initial microbial concentration on the number of F_0 values required to achieve this standard is shown in Table 3. If the indigenous bioburden does not exceed the 1.9×10^3 observed in the pasteurized actaplanin medium (Table 2), adequate assurance of sterility in a volume of 115 liters could be achieved by the application of F_{15} heating units. Even if the 4.1×10^5 cfu encountered in the unpasteurized A25822 medium were composed entirely of *B. stearothermophilus* spores, only F_{17} heating units would be required. On this basis, the manually controlled 45-min sterilization cycles previously employed by the FPP, producing about F_{75} heating units, represented approximately 350% 'overkill'. A large reduction in the quantity of heat applied during sterilization cycles was thus suggested. However, peripheral vessel hardware and associated piping may not attain the same temperature as the liquid contents during a given time period because of more rapid heat dissipation and/or entrapped air. Therefore, since each additional F_0 requires only one more minute at 121°C, an extra safety margin of heating was included in establishing target F_0 values. Approximately 700 lots of bioreactor media have now been sterilized by the application of F_{20-25} thermal units. Because the percentage of fermentations normally lost due to contamination by endospore-producing bacilli has not been increased by the application of this reduced-heat procedure, still further heat reduction may be appropriate.

Table 3

Effect of bioreactor volume and microbial bioburden on thermal exposure requirements[a]

Initial bioburden (cfu/ml)	Bioreactor volume (l)	Thermal exposure required (F_0)
10^4	1.0	F_{13}
10^4	10.0	F_{14}
10^4	100.0	F_{15}
10^6	100.0	F_{17}
10^8	100.0	F_{19}
10^4	1000.0	F_{16}
10^4	10000.0	F_{17}
10^4	100000.0	F_{18}

[a] To achieve a probability of no more than one bioreactor remaining unsterile per 10^6 bioreactors sterilized.

Inclusion of the on-line vocal reports of the SLC II represented an optional enhancement of the F_0 sterilization procedure. Without this unit, bioreactor operators were able to monitor the accumulation of F_0 values only through visual contact with the computer screen. In addition, the screen display included parameter data for single bioreactors. When multiple bioreactors were sterilized simultaneously, paging of displays at the terminal was necessary to maintain contact with each vessel. The SLC II relieved operators of the need to maintain either physical contact with the computer keyboard or visual contact with the computer screen. This permitted them to pursue normal duties in the vicinity so long as they remained within auditory range, which could be adjusted with the SLC II's variable volume control. The SLC II also tracked multiple bioreactor sterilizations simultaneously and reported F_0 values for each vessel immediately as they became available.

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