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Interaction of media components during bioreactor sterilization: definition and importance of R_0

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SUMMARY

Sterilization of bioreactor media, to destroy viability of the indigenous microbial population, is normally accomplished by autoclaving, or heating with pressurized steam. However, simultaneous chemical changes in media can also be expected to result from the high temperatures. A kinetic procedure involving on-line computer calculation of heat input, designated as F_0 values, was previously developed to estimate sterility achievement. A similar kinetic procedure, based on a general purpose Arrhenius 'pseudo' rate equation and designated as R_0 values, has now been designed to evaluate and control the effects of temperature and heating time on chemical reactions occurring in the media. Data are presented indicating that R_0 may be a useful parameter for reducing variability in culture metabolism and 'scale-up' when these variations result from different nutrient concentrations produced by non-standard heating during media sterilization in stirred bioreactors.

INTRODUCTION

After industrial fermentation media are formulated, they are normally sterilized to destroy viability of the indigenous microbial population prior to inoculation with the desired culture. The sterilization method most commonly employed is heating with pressurized steam. Although the application of heat is a simple technique, precise control of the total heating/cooling process to achieve consistently reproducible temperature profiles is difficult and has not previously been justified. Further, to avoid the manifestly ruinous consequences of incomplete sterilization, 'overkill' heating is frequently employed [4]. Heat input measurement and partial control has been justified and employed through on-line computer calculation of log reductions in the viability of contaminating organisms, known as F_0 [2].

Although eradication of the indigenous microbes is the primary goal of autoclaving, a less obvious, but potentially critical, secondary effect can be ex-

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pected to occur simultaneously. Many fermentation media are complex mixtures of poorly defined nutrients that may include carbohydrates, lipids, proteins, peptones, amino acids, vitamins, nucleosides, nucleotides and minerals. Autoclaving such heterogeneous mixtures can be expected to alter their composition through one or more of several mechanisms: degradation, hydrolysis, conjugation and the formation of insoluble compounds such as the calcium or magnesium salts of phosphate. Phosphates, for example, are essential for growth and may strongly impact the biosynthesis of secondary metabolites [3]. Insoluble salts may not be bioavailable, which may be unfavorable if their supply is limiting, or advantageous if ready availability of those quantities would provide excessive and detrimental levels. Inhibitory compounds may also be formed during heat sterilization [1].

The rate of a chemical reaction is dependent on the nature of the reaction, the concentration of the reactants, possible catalysts, and the temperature. The rate constant for a given reaction varies according to the collision frequency and activation energy, per Arrhenius' Law. Increasing the temperature has the same effect as decreasing the activation energy required. As a result, the rates of nearly all chemical reactions increase with temperature.

The numbers and types of chemical reactions that can occur in fermentation media during heat sterilization are too complex for definition. However, the time/temperatures profile of the sterilization process could theoretically affect the resulting component concentrations. Nutritionally variable media could, in turn, affect culture growth and/or metabolite synthesis. Inconsistent metabolic behavior of the organisms being cultured could be expected to generate capricious fermentation data that may foster erroneous conclusions and/or require additional experiments to confirm initial interpretations. Overshadowing these considerations, however, is the rigid, primary sterilization requirement for sufficient heating to destroy the viability of all indigenous microbes.

The equation employed for computer calculation of F_0 values [2] is as follows:

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

where Δt = the time interval between measurements (min), T = temperature at time t (°C), and Z = 10°C. This algorithm can be used effectively to regulate and standardize the process of eliminating viability of the inherent microbes. However, it may not produce consistent concentrations of media components after sterilization because the temperature dependency of F_0 is quite unlike that predicted by the standard Arrhenius model for chemical reactions. Thus, variation in sterilization time/temperature profiles could theoretically lead to a high degree of variability in media component concentrations after heat sterilization even though F_0 values were identical.

Kinetic studies have done much to advance our understanding of microbial killing processes [7]. A kinetic approach may also allow at least partial control of chemical reactions during sterilization. Therefore, the following general purpose Arrhenius 'pseudo' rate equation is proposed:

$$R_0 = \Delta t \sum_{t=0}^{t} k_o e^{-\Delta E/RT}$$

where t = time (min), R = gas constant, T = temperature, e = 2.817, and ΔE = activation energy. This equation would apply qualitatively to a large class of reactions, including zero-order reactions, and all first- and second-order reactions for which the reacting components were in significant excess. In order to determine the 'pseudo' values of constants 'k_o' and 'ΔE', two assumptions are necessary: (1) the reaction rate doubles for each temperature increase of 10°C; (2) an arbitrary reference is defined such that 'R_0' increases by 1.0 unit/min when the temperature is 121°C (which is the same for F_0). Solving the resulting two equations in two unknowns leads to the following values: $\ln k_o = 26.596$ and ΔE = 20 748 cal/gmol.

R_0, then, is proposed as a parameter for pseudo-quantifying the overall sterilization process with respect to the chemical interaction of media components. Concurrent use with F_0 would permit simultaneous sterility estimation.

This study was initiated to explore the hypothesis that different time/temperature sterilization profiles result in chemically and nutritionally different media. Evidence is based on both physical parameters and metabolic activity of the microorganism subsequently cultured in the sterilized media.

MATERIALS AND METHODS

Culture

The culture used in this study was *Streptomyces roseosporus* NRRL 11279. The procedures used for development of inoculum stages prior to the final culture have been described previously [5]. Fermentors were seeded with 3% inoculum (v/v).

Medium

Media sterilized by heat were prepared and subjected to steam-heating in stirred bioreactors, then cooled rapidly and transferred aseptically to sterile Erlenmeyer flasks. Media sterilized without heating were prepared by passage through 0.22-μ pore size filters. All fermentations were conducted in Erlenmeyer flasks with media volumes of 50 ml. The fermentation medium, containing no insoluble components, was composed of (w/v): 4.0% glucose, 0.4% Bacto-peptone (Difco Laboratories), 0.081% KH_2PO_4, 0.019% K_2HPO_4, 0.00096% $CoSO_4 \cdot 5H_2O$, 0.00043% $CuCl_2 \cdot 6H_2O$, 0.0464% $MgSO_4 \cdot 7H_2O$, 0.019% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.0038% $MnSO_4 \cdot H_2O$ and 0.0038% $ZnSO_4 \cdot 7H_2O$ in tap H_2O.

Sterilization procedure

Bioreactors employed for media sterilization were fully baffled pressure vessels of conventional design equipped with two open-center six-bladed turbine impellers. Media were stirred continuously at a constant rate of 250 rpm, which was identical in all vessels. Operating volume was approximately 115 liters. Media were heated rapidly, controlled precisely at the prescribed temperature for the desired 'R_0' value, and cooled quickly. 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. In sterilization mode, the Hewlett-Packard 1000 continually measured temperatures at 1-s intervals for computation of the accumulating R_0 and F_0 values.

Analytical measurements

Growth of *S. roseosporeus* was determined gravimetrically. Whole broth samples were centrifuged at 1600 $\times$ g. The cell pellet was resuspended in deionized water, centrifuged as before, transferred to a tared weigh boat and dried at 70°C to constant weight.

Glucose was estimated by the glucose oxidase method (Biodynamics/BMC, Indianapolis, IN). Automated assays were also employed for determination of ammonia nitrogen and residual inorganic phosphorus (Industrial Method No. 93-70w, Technicon Industrial Systems, Tarrytown, NY).

Coloration of sterilized, but uninoculated, media was determined by spectrophotometric measurement at 320 nm.

All data reported are mean values derived from five replicates.

RESULTS

Because fermentation media are commonly sterilized at 121°C, four media were autoclaved at 121°C for different time intervals expected to produce R_0 values of 25, 50, 75 and 100. The F_0 values concurrently achieved were simply monitored and recorded.

Filter-sterilized media were considered to be the 'controls' for autoclaved media. All media were nearly colorless when initially formulated prior to sterilization. Filtration did not affect coloration. However, noticeable color developed in all media during autoclaving, presumably at least partially due to caramelization of the glucose. Color devel-

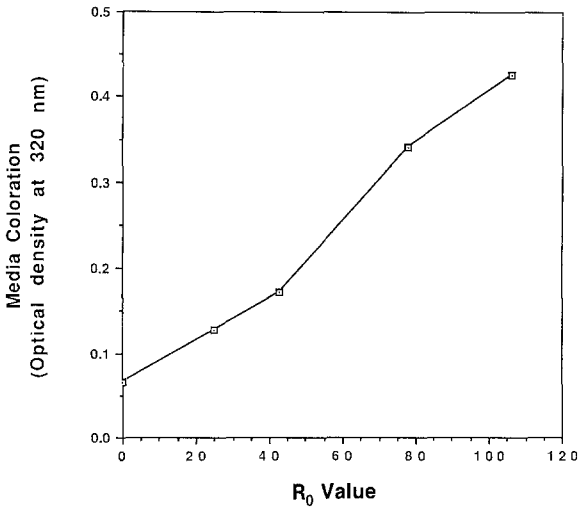

Fig. 1. Effect of R_0 value on media coloration. Data for $R_0 = 0$ were obtained from filter-sterilized media.

opment provided an immediate presumptive indication of increasingly greater chemical change within the media at higher R_0 values. This initial visual observation was confirmed by spectrophotometric measurement, confirming that pigment intensity was directly proportional to the R_0 value generated during heating (Fig. 1). The pH of all heat-treated media was significantly lower than that of filtered media, exhibiting an inverse relationship to R_0 (Fig. 2).

Flask fermentors were sampled at 0, 24 and 48 h

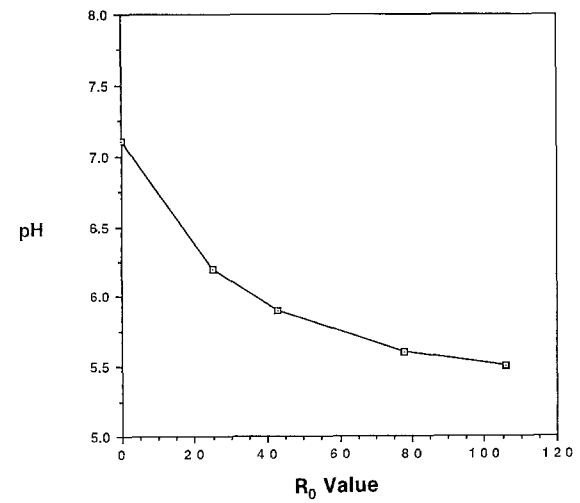

Fig. 2. Effect of R_0 value on post-sterilization media pH. Data for $R_0 = 0$ were obtained from filter-sterilized media.

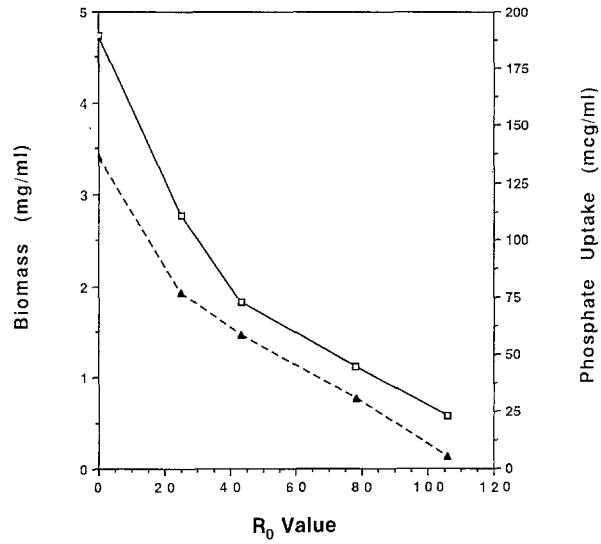

Fig. 3. Effect of various R_0 values on the metabolic response of *S. roseosporeus*. Total biomass and phosphate uptake were determined after an incubation period of 48 h. □——□ = biomass; ▲- - -▲ = phosphate uptake.

post-inoculation and several parameters were examined. These included biomass, residual phosphate, residual glucose and residual ammonia. The levels of residual glucose and ammonia were not

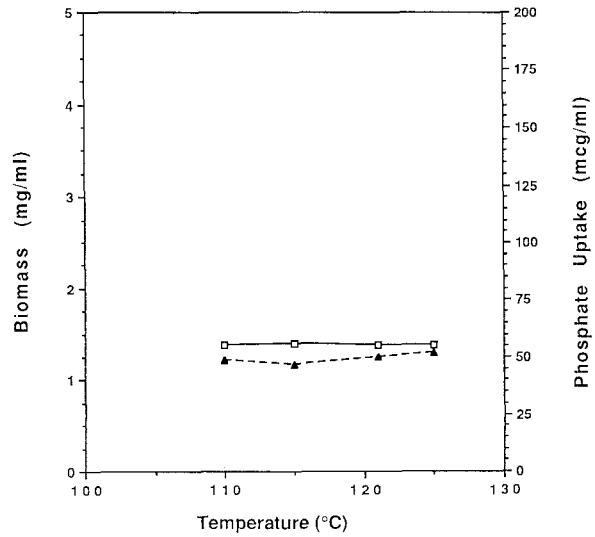

Fig. 4. Effect of different heating temperatures maintained to $R_0 = 50$ on the metabolic response of *S. roseosporeus*. Total biomass and phosphate uptake were determined after an incubation period of 48 h. □——□ = biomass; ▲- - -▲ = phosphate uptake.

affected significantly by R_0 values. Fig. 3 shows the parallel relationship of 48-h growth and phosphate uptake profiles as a function of R_0 values. Similar to a previous report [8], filter-sterilized media were superior, supporting the greatest phosphate uptake and the most rapid growth rate. Both parameters were significantly lower in all heat-sterilized media, being increasingly depressed at progressively higher R_0 values. These data indicated that color development during autoclaving appeared to reflect one or more chemical reactions that affected growth and phosphate uptake of *S. roseosporeus* adversely.

The specific effect of temperature was evaluated by steam-sterilizing media at 110°C, 115°C, 121°C, and 125°C until an R_0 value of 50 was attained at each temperature. Growth and phosphate uptake in these media are shown in Fig. 4. The straight-line relationships indicate that sterilization to constant R_0 values produced consistent results regardless of the sterilization temperature. Fig. 5 shows the lack of correlation between R_0 and F_0 at the different sterilization temperatures. Under these conditions, F_0 is important only for assurance that sufficient heat has been applied to accomplish sterilization.

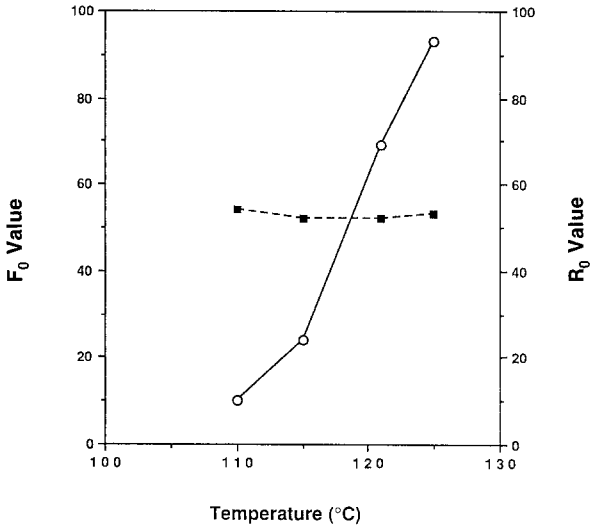

Fig. 5. Relationship of computer-calculated R_0 and F_0 values at different heating temperatures. ○——○ = F_0 value; ■- - -■ = R_0 value.

DISCUSSION

Variability has been a historical concern in fermentation processes. In some instances, large numbers of replicate fermentations have been required in order to establish statistically significant values during process development. In other cases, improvements unequivocally established in small bioreactors have not 'scaled-up' into larger vessels.

Use of the proposed form of R_0 does not unequivocally guarantee consistent concentrations of media components after heat sterilization. In this respect, R_0 is much like the F_0 equation, which cannot predict the kill rate of all contaminating organisms that may possibly be present, since different microbes are killed at different rates. For this reason, F_0 is based on a 'worst-case spore kill' scenario. R_0 is not proposed as relating similarly to a specific chemical reaction because such detailed kinetic studies of media reactions are not available, although one study on the regulation of starch gelatinization in rice by controlled steaming has been reported [6]. Uniform temperature distribution and mixing conditions have been assumed because of the uniform vessel geometry and agitation rate employed during all sterilizations. This assumption may not be valid for other vessels and/or conditions. However, the goal of this initial report on R_0 is introduction of a generalized concept, one intended for broad application without requiring detailed preliminary analyses. Therefore, the R_0 premise is based on general chemical information. The proposed form of R_0 offers a practical and inexpensive step toward standardizing media sterilization without the need for detailed kinetic studies on individual media or reactions.

Although fermentation media commonly contain insoluble components, practical considerations dictated use of a soluble medium for this study. Complete solubility was required in order that a comparison of the effects of heat sterilization with filter sterilization could be obtained. Unambiguous gravimetric determinations of culture growth could also be procured only with a soluble medium. However, there appears to be no theoretical reason to believe R_0 would be less applicable to complex

media. It may, in fact, be more important in those situations if complex media are associated with slower reactions whose state of completion is more strongly dependent on heating profiles.

Preferably, both F_0 and R_0 should be monitored during either batch or continuous heat sterilization of media. A minimum value for F_0 must be attained to assure sterility. Economy of labor and services is adversely affected by excessive heating, although negative contamination implications do not accrue from exceeding the minimum F_0 value. The data presented here indicate that the chemical/nutritional composition of media after steam sterilization is affected more significantly by the value of R_0 than F_0. The target R_0 value may be selected to duplicate values associated with 'worst-case' sterilization conditions, or it may be employed as an experimental variable in process optimization studies. On this basis, a suggested rationale for sterilizing fermentation media in computer-controlled stirred bioreactors would be: (1) Pre-select target F_0 and R_0 values. F_0 must attain, or exceed, the critical minimum number for assurance of sterility. (2) Heat the bioreactor as usual. Calculate accumulating F_0 and R_0 values. (3) Initiate and control cool-down to achieve the target R_0 value, provided the minimum F_0 value is fulfilled or exceeded.

An appropriate strategy for temperature control during cool-down can be determined for any vessel consistent with worst-case availability of cooling utilities. The strategy could also include other considerations, such as effects of the heating cycle on the useful life of in-line probes that may be adversely affected by high temperatures.

Where culture metabolism varies in response to the levels of critical nutrients present, and the latter reflects the effect of inconsistent heating profiles during sterilization, R_0 can be expected to aid in reducing process variability.

Finally, it is noted that calculation of R_0 depends only on measurement of time and temperature and certain assumptions, which include good mixing and uniform temperature distribution. Therefore, R_0 should be viable as a scale-up parameter for media sterilization processes and, providing the axial temperature profile of a continuous sterilizer is known or monitored, ought to be independent of continuous or stirred-batch methods.

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