ADVANCES IN APPLIED MICROBIOLOGY

Vol. 39

PT. 1

ADVANCES IN

Applied Microbiology

Edited by

SAUL NEIDLEMAN
Vacaville, California

ALLEN I. LASKIN
Somerset, New Jersey

VOLUME 39

Academic Press, Inc.
A Division of Harcourt Brace & Company
San Diego New York Boston
London Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Academic Press, Inc.
1250 Sixth Avenue, San Diego, California 92101-4311

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

International Standard Serial Number: 0065-2164

International Standard Book Number: 0-12-002639-2

PRINTED IN THE UNITED STATES OF AMERICA
93 94 95 96 97 98 BB 9 8 7 6 5 4 3 2 1

ADVANCES IN
APPLIED MICROBIOLOGY

ADVANCES IN APPLIED MICROBIOLOGY

VOLUME 39

39
1993

Medicinal and Therapeutic Value of the Shiitake Mushroom

S. C. JONG AND J. M. BIRMINGHAM

Yeast Lipid Biotechnology

Z. JACOB

Pectin, Pectinase, and Protopectinase: Production, Properties, and Applications

TAKUO SAKAI, TATSUJI SAKAMOTO, JOHAN HALLAERT, AND ERICK J. VANDAMME

Physicochemical and Biol[illegible]l Treatments for Enzymatic/ Microbial Conversion [illegible]gnocellulosic Biomass

PURNENDU GHOSH AND AJAY SINGH

Physicochemical and Biol[illegible]l Treatments for Enzymatic/ Microbial Conversion [illegible]gnocellulosic Biomass

PURNENDU GHOSH AND AJAY SINGH

practice. A low-level, contaminated batch in a bioreactor, with normal product biosynthesis, achieves the empirical sense of "sterility," though not the absolute one (Reisman, 1988).

There are many industrial fermentations, e.g., ethanol, baker's and fodder yeasts, and vinegar, where no serious attempt is made to maintain asepsis either in the fermentor medium or in the subsequent conduct of the process. In fact, such attempts are not warranted because of rapid culture proliferation, rapid metabolic transformations, and the resultant environment being generally nonconducive to contaminant growth (Herold and Necasek, 1959; Bailey and Ollis, 1986). It was during the first world war that Weizmann made the pioneering efforts to establish the first truly aseptic acetone–butanol fermentation (Hastings, 1978). However, the inactivation of penicillin by penicillinase-producing microbes necessitated engineering developments aimed at carrying out biotechnological processes with absolute exclusion of foreign microbes.

The fermentation industry does not publish figures on the rate of nonsterile operations, yet a figure of 5–30% nonsterility of bioreactors is realistic (Saudek, 1956; K. Gerlach, unpublished communications, 1986). At times the rise in figures warrants being called a "wave" of contamination. Economic considerations indicate that a contamination probability of 1 in a 100 is acceptable for batch fermentations, considering the norm of 1 in 1000 as the probability of contamination commonly employed in design calculations for a sterilization process (Banks, 1979). A nonsterility rate of 1% or less is often regarded as a commendable performance (Soderberg, 1983). Artificially selected industrial microbes generally used in biotechnology endeavours are at risk of being overwhelmed by competing wild organisms. Mammalian/animal/plant cell cultures are especially prone to microbial contamination because of a long process cycle (20 or more days) and a relatively slow growth rate (doubling time as long as 100 hr). These cell cultures can be compared to an artificial organ without autoimmune protection, lacking any defense system, and therefore, extremely vulnerable to a breach of sterility (Knight, 1989). Yet the industry now operates large-scale animal cell cultures routinely with contamination rates of just 2% (Spier, 1988), even with culture lengths of several months and intermittent additions of fresh medium.

The physicochemical environment generally maintained in a bioreactor is optimal for a host of microorganisms. Very rarely is the medium "protected", i.e., selectively utilizable by a limited range of microbes (Stanbury and Whitaker, 1984) or else has an antimicrobial added to it. The intended metabolite, even if an antimicrobial, is normally produced late (in idiophase). Very often, the bioreactor in trophophase

would therefore be an ideal incubator for numerous microorganisms, if provided an opportunity of entry.

II. "Invaders"

The microorganisms that invade the sterile fermentation process are bacteriophages, mycoplasma, bacteria, or fungi. Phages normally infect through air and the development of phage-resistant strains has resulted in the rarity of such infections, particularly in view of the host specificity of phages. Mycoplasma and viral infections are more common in animal cell cultures through serum and can be eradicated easily (Arathoon and Birch, 1986). The microsize, omnipresence, and faculty of utilizing widely varying substrates for nutrition make the bacteria capable of causing widespread infections and maximum damage to the fermentation process. While gram-positive bacteria have air as their main source, gram-negative bacteria are transmitted through liquids, particularly water. Among the fungi, yeasts may originate mainly from insufficient sterilization of substrates. Filamentous fungi have air as their main source. Fungal infections occur rarely (Herold and Necasek, 1959).

III. Consequences

The invasion of a fermentation process in a bioreactor by a foreign microorganism can result in a variety of consequences:

1. A fast growing, wild contaminant may outcompete the normally slow growing desired strain, deplete the nutrients, and fatally interfere with the chemistry of the process and the final product.
2. The invader may not outgrow the desired strain, but may cause minor to appreciable alterations in the physicochemical characteristics of ongoing fermentation. The contaminant may also produce undesirable and possibly toxic metabolites that may lead to lower yields and productivity.
3. The contaminant may grow to a certain level and subsequently be inhibited by the metabolite(s) produced by the desired strain. The fermentation may continue to its logical end, as at times in the case of broad spectrum antibiotics.
4. Contaminants, i.e., phages, could result in the lysis of the desired microbe in a bacterial/actinomycete fermentation.
5. Mucilage/slime produced by the contaminant may choke the filter pores to varying degrees, which in a worst-case scenario would ruin the entire batch on hand.

6. Another consequence of nonsterility could be the degradation/racemization of the desired metabolite, e.g., the enzymatic degradation of β-lactam antibiotics by β-lactamase-producing bacteria, leaving behind a totally unproductive batch or producting DL- or D-amino acids in an L-amino acid fermentation.

7. Undesirable moieties produced by the contaminant may lead to interference in the downstream recovery of the product, resulting in not only lower yields but a substandard product as well. The processing of the product may cause increased production costs.

8. The contaminant may render the final product unusable, e.g., single cell proteins where the cells constitute the product.

9. Not every contaminant at the fermentation stage exerts detrimental effects. The contaminant has been reported to increase fermentation yields and to better downstream processing (Reisman, 1988) due to the presence of useful enzymes like proteases and lipases.

Irrespective of the consequences of contamination, preventing the entry of contaminants is necessary. The attempt, therefore, should be to identify the sources of contamination and to conduct fermentation processes in an aseptic manner.

IV. Sources

The sources of contamination in a fermentation process can be broadly ascribed to several factors.

A. Inoculum

Contamination at the inoculum development stages assumes greater significance in view of the consequent nonavailability of quality "starter" material for the bioprocess and therefore the opportunity loss. The presence of a contaminant in laboratory-grown inoculum, often in concentrations low enough to escape detection up to seed bioreactor maturity, can lead to the subsequent manifestation of nonsterility in the fermentation bioreactor. In view of the relatively small volumes used for sterility testing, sensitive methodologies are obligatory. The sources of laboratory inoculum nonsterility in turn could be autoclaving deficiencies, "lumpy" medium, inadequate maintenance of sterile chambers, ineffective ultraviolet irradiation from germicidal lamps, use of inefficient germicidal solutions, wetting of plugs, and insufficient/prolonged stocking of sterilized media/glasswares. Inadequately screened cell banks are potential sources of viral contamination in animal cell culture processes (Arathoon and Birch, 1986).

B. Nutrient Medium

Nonsterility of the nutrient medium used in the seed/fermentation bioreactor may culminate in a contaminated operation. An inadequate sterilization operation, "lumpy" medium, and the bioreactor itself may contribute to medium nonsterility. The choice between batch and continuous sterilization and their design depends on the scale of operations and the characteristics of the medium. In the case of separate medium sterilization, the tank/continuous sterilizer, piping, and receiving vessel could be sources of nonsterility. Animal sera used for cell culture processes may sometimes carry viral and other contaminants and render the process nonsterile (Arathoon and Birch, 1986; Maurer, 1986; Elander, 1989).

C. Bioreactor System

A continuous stirred tank reactor is the most commonly used bioreactor in view of its versatility and flexibility of operations therein. The sterility considerations of such a bioreactor satisfactorily cover most of the other types of bioreactors as well. The design, material, and fabrication of the bioreactor are important factors contributing to the sterility of operations. Apart from these factors, aberrations in fermentor sterilization, air supply, agitation system, sampling and monitoring ports, and uncontrolled foaming could lead to invasion of the bioreactor boundary by undesired microorganisms. The agitator shaft entry and the air exit are the most vulnerable points in a bioreactor. All types of seals, except a double mechanical seal, provide gaps as entry points for contaminants (Steel and Miller, 1970; Bull *et al.*, 1983; Aiba *et al.*, 1986; Reisman, 1988). Intermediate bearings on shafts, improper impeller hubs and keyways (Reisman, 1988), and pipe in pipe connections provide unhygienic places to harbor contaminants. Too many interior fittings create pockets likely to conceal microbes, are difficult to sterilize, and can result in contamination of the bioreactor. Stress corrosion cracking in the vessel and internal coil leads to repeated contaminations. Hammering caused by steam during batch sterilization causes stress on spargerflanged joint components and creates nonuniform steam distribution-related sterility hazards.

The numerous ports, pipes, and valves required in a fed-batch process are further sources of microbial entry if not properly selected or designed. Flanged/threaded joints, pervious sealing materials, defective slopes of pipes, dead ends, pockets, indentations, crevices, solid depositions, stagnant layers, rising stem valves, leaking or "weeping" pipes/flanges/valves, and the absence of steam seals/crosses are potential

causes of contaminations. The continuous presence of humidity/condensed moisture and entrained medium in the air exhaust area provides an environment conducive to microbial growth at this boundary point of direct contact between sterile and nonsterile zones. Uncontrolled foam generation during fermentation causing "foaming out" through the air exhaust increases the chances of fermentor contamination (Solomons, 1967; Ghildyal *et al.*, 1988).

D. Air/Liquid Transfer

Contamination through the depth filter is possible because of inadequate filter packing, channeling, free moisture, or fiber fragmentation. Individually or collectively, these factors contribute to filter inefficiency. In the case of the membrane filter, damaged O rings/membrane cause losses in filter integrity. The air filter, soaked in nutrient media because of backflow/overflow, can act as an incubator for contaminating microbes. The liquid transfer systems, including those for the inoculum, feeds, and supplements, may also contaminate the process because of system deficiencies described earlier and/or inadequate sterilization. A common system for transfers to several fermentors, if having such deficiencies, would be a catastrophe.

E. The "Rogue"

For all practical purposes even the very rare appearance of a nonproducing wild "rogue," which occurs due to reversion mutation of the production culture during the fermentation process, leads to contamination of the bioreactor. There are no precautionary or remedial measures against such mutant appearances and early downstream processing may be warranted for salvage, if any.

V. Approaches

Ever since the aseptic submerged culture technique for penicillin production was introduced, there have been attempts to perfect fermentation techniques, design, and systems in achieving asepsis. Coordinated efforts of microbiologists, technologists, engineers, and biochemists are required to evolve various bioreactor contamination control strategies. The successful commercial scale production of numerous fermentation products is a measure of the magnitude of such achievements. Exhaustive reviews on such techniques have been made by Rhodes and Fletcher (1966), Solomons (1969, 1971), Augurt (1983), Wallhausser (1985), and Bailey and Ollis (1986).

Aseptic design development is based on several norms by which the intrusion of foreign microbes into a defined fermentation system can be precluded. A closer look at the behavior of a microbe at different types of boundary layers reveals the following criteria (Lundell and Laiho, 1976) relevant to asepsis:

1. Nonpenetration of homogeneous solids by a microbe.
2. No movement or growth of a microbe through holes smaller than its own dimensions.
3. No propulsion of a microbe against the flow of carrier medium.
4. No movement of a microbe on dry surfaces without external forces.
5. No growth on a surface with temperatures exceeding a microbe's maximum growth temperature.
6. No growth on nonmetabolizable/hydrophobic/toxic materials.
7. Inactivation of a microbe by high temperature, toxic chemicals, and irradiations.
8. A characteristic doubling (reproduction) period of each microbe.

Most of the aseptic design considerations involve any or all of the combinations of these criteria. The sterile design of a bioreactor normally adds 15–25% to the cost toward its design, purchase, and installation (Reisman, 1988). The following approaches constitute some of the techniques essential for achieving aseptic bioprocesses:

A. Sensitive Sterility Assessment Methodology

The absence of a contaminant(s) in the culture inoculum, sterilized seed/fermentation media, and equipment needs to be ascertained at every stage in order to avoid nonsterility and to detect the stage at which the contaminant invaded the process. Often the results of conventional sterility checks may not be available before the culture has reached the production bioreactor or before the contaminant has reached a growth level capable of disturbing the desired fermentation. Speed of detection is germane to the salvage of a batch. A sensitive sterility assessment methodology with accelerated results would be useful for such applications. The use of a variety of nutrient media in tubes (broth, slants, and stabs) and flasks incubated at varying incubation temperatures under static/shaken conditions would be ideal in covering a range of growth conditions required by different contaminants (Soderberg, 1983). The use of thioglycolate, with a small amount of agar (0.05%), and oxido-reduction dyes like methylene blue/resazurin have been recommended for the fast detection of aerobes as well as anaerobes in a single medium (U.S. Pharmacopoeia, 1980).

Membrane filter discs could prove useful for sterility checks of broths containing antimicrobials. Penase (a potent lactamase preparation) is recommended for the inactivation of penicillin/β-lactams during sterility testings (Difco Laboratories, 1985). Sterilized filter assemblies with membrane filter discs/glass wool pads find application in sterility checks of air/liquids passed into the fermentation equipment. Trial runs with uninoculated lean nutrient media are used to ascertain the sterility status of fermentation equipment, transfer manifolds, and air systems during validation. The use of selected radioactive carbon sources in sterility check media has been recommended under the "Bactect" system for a fast detection of contaminants (McLaughlin *et al.*, 1983). The mass spectrophotometric identification of 3-hydroxymyristic acid, a characteristic of gram-negative bacteria, has been applied for a rapid sterility assessment by Elmroth *et al.* (1990). The techniques of sampling for sterility checks were described by Elsworth (1960), and newer techniques (Charton, 1990) involving the use of thermoplastic elastomer tubings for improving sterile access to bioreactors have been described. A mobile flexible film containment cabinet attached to the bioreactor is used in sampling recombinant DNA-based fermentation processes (Hambleton *et al.*, 1991). Elander (1989) described the use of a sterile stainless steel container with a small sterilizing filter on its vent that was attached to the fermenter, union sterilized, sample drawn, and the union finally resterilized before disconnection and further processing of the sample drawn. For animal cell cultures, the establishment and testing of suitable cell banks with screening for freedom from viruses and other adventitious agents is a major exercise (Lubiniecki and May, 1985). Further work on accelerated sterility assessment methodology in the fermentation industry is needed.

B. Certified Aseptic Laboratory Inoculum

The inoculum preparation room needs to have a clean room design and scheduled validation/checks of this sterile room through exposure of plates. Schedules for area fumigation and *in situ* integrity testing of HEPA filters by aerosols/dioctyl phthalate/dioctyl sebacate ensure sterility in these rooms. The area used for inoculum preparation needs to be isolated from that used for in-process sterility assessment. Only validated disinfectant dilutions should be permitted in sterile rooms. The rotation of disinfectants to avoid buildup of resistant microflora is necessary and must be practiced rigidly. Principles and practices of laboratory management related to facilities, design, decontamination, access to work place, personal hygiene, apparel, sanitation, ventilation,

and safety are amply described (Rhodes and Fletcher, 1966; Soderberg, 1983; Wallhausser, 1985; Scheirer, 1987). The careful planning of premises, air circulation, and pressure differentials has recently become important, particularly in culturing animal cells and genetically engineered microbes (Scheirer, 1987; Knight, 1989). Finch (1958), Sykes (1958), Borick (1968), Benarde (1970), and Wallhauser (1985) have discussed the utility of various disinfectants and chemical sterilants for such purposes. "Biosafety in Microbiological and Biomedical Laboratories" published by the U.S. Department of Health and Human Services (1984) describes the standard and special microbiological practice guidelines and designs for aseptic laboratory operations.

The proper storage of biodegradable raw materials like corn-steep liquor, soya flour, corn flour, and seed meals under hygienic conditions and preferably at reduced temperatures and humidity minimizes the further increase of microbial load. Fine powders, presoaking and preboiling, proper batching sequence, and straining help prevent the "lumpy" medium threat to aseptic processing (Soderberg, 1983).

Validation and proper operation of autoclaves used for media/glassware sterilization is mandatory for pure inoculum propagation. The proper venting of air and steam ensures the absence of air pockets necessary for attainment and sensing of correct uniform temperature in the autoclave. Air, being heavier than steam, must also be expelled from the lowest level in the autoclave. Using a jacket steam ejector to remove leftover steam in the autoclave chamber after sterilization is advisable in order to minimize moisture condensation on sterilized wares (Wilkinson and Baker, 1964). Autoclave performance may be checked using "biological indicators" (Banks, 1979) or sterilizing temperature indication stickers/tapes, like those supplied by the 3M Corporation, to confirm proper autoclave operation. Such indicators have been reviewed by Augurt (1983) and Wallhausser (1985). Procedures obligatory for efficient performance of autoclaves have been described by Rhodes and Fletcher (1966), Stumbo (1976), Augurt (1983), and Wallhausser (1985).

To avoid dirt accumulation, it is advisable to use rimless glassware and presterilized cotton/synthetic hydrophobic plugs. Needless to say, wrapping with waterproof papers/aluminum foil, vacuum drying through steam ejection, and hot air oven drying of autoclaved materials is very useful. Incubation of sterilized media at different temperatures for 72 hr and subsequent checks ensure their sterility before use. A need-based planning of sterile glassware and media is necessary to avoid the "emergency" use of freshly autoclaved material or prolonged stocking of autoclaved material that could be a "sterility risk," particu-

larly in a humid climate. Longer necks and optimum degree of filling of containers prevent the plugs from getting wet during incubation on shaker. Attention to these details results in significant improvements in sterility at the laboratory stage.

C. Autoclavable Bioreactor

Smaller glass or stainless steel bioreactors (1–5 liter capacity) containing medium can be sterilized by autoclaving and later connected aseptically to various nutrients, air, pH, and pressure maintenance lines for operations. Bioreactors with capacities higher than 5 liters are difficult to sterilize in an autoclave, and the possibility of sepsis is increased if connections of larger diameters are used (Solomons, 1969).

D. Sterile Medium/Feed

The precautions mentioned earlier for laboratory medium preparation are necessary for bioreactor medium preparation as well. Medium mixing facilities are often neglected in the fermentation industry. Tank design for total drain out, smooth internal finish, high-pressure water jet cleaning after each operation, and chemical cleaning at regular intervals eliminate the formation of dried medium crusts and discourage microbial buildup in the tank. Sterilization of industrial media is not possible through filtration (which in fact is not recommended because suspended matter is a desirable ingredient of the medium) and is unreliable through irradiation or chemical sterilants. Steam sterilization is, therefore, the only choice and can be carried out in the bioreactor itself or in a separate pressure vessel/continuous sterilizer. Aseptic considerations warrant a preference for steam sterilization over other alternatives.

1. *Batch Sterilization*

Indirect heating through a jacket, external or internal coils, hollow baffles, and steam sparging through an air delivery system and dip pipes or any combination of the aforementioned are used for batch sterilization. Efficient mixing and circulation of the medium promote the efficiency of heat transfer and ensures the uniform heating essential for proper sterilization. In such a case the vessel also gets sterilized along with the medium. It is imperative that steam is supplied to the bioreactor through all dip pipes or ports in direct contact with the medium (Bull *et al.*, 1983; Stanbury and Whitaker, 1984). In fact, it is desirable to continue regulated steam supply at these points throughout the sterilization cycle since the stagnation of improperly heated medium

inside the pipe section, external to the bioreactor, is a serious sterility hazard. Relative merits of *in situ* sterilization and the use of separate pressure vessel have been discussed in detail by Richards (1966, 1968). More often it is the *in situ* sterilization that is preferred.

2. *External Continuous Sterilization*

The external continuous sterilizer involving high temperature short time treatment (Baily and Ollis, 1986) is the method of choice when large volume bioreactors are used. The advantages of continuous sterilization of initial medium as well as nutrient feeds (Aunstrup *et al.*, 1979) over batch sterilization have been comprehensively described by Solomons (1969, 1971), Augurt (1983), Banks (1979), Cooney (1985), Wallhausser (1985), and Aiba *et al.* (1986). A shorter process time, better heat recovery, better medium quality, and cost effectiveness are ensured. The design of a continuous sterilizer is extremely important. Plate heat exchangers are suitable for media containing low levels of suspended solids. For media containing substantial solids or for viscous media, tubular heat exchangers with high flow rates and turbulent flow are used (Bull *et al.*, 1983; Cooney, 1985). The sterilization of oils and viscous antifoams has to be planned carefully (Bader *et al.*, 1984). Oils free of moisture are often difficult to sterilize. It is preferable to mix water in oils/antifoams before sterilization and to use a tubular continuous sterilizer.

A spiral heat exchanger and an injector-flash cooler sterilizer (Banks, 1979; Stanbury and Whitaker, 1984) are useful versions of continuous sterilizers. The replacement of process water, held up inside the heat exchanger, with demineralized water prior to system sterilization with steam or superheated water (125°C) is necessary for eliminating stress corrosion cracking (Soderberg, 1983). Designing the sterilizer with near plug flow, proper retention, automatic control of the sterilization temperature, monitoring of inlet as well as outlet temperature at the holding section, and automatic switch over to the recirculation mode with a simultaneous shut off of the delivery line to the bioreactor when the sterilization temperature drops are mandatory features for aseptic performance of the system. Figure 1 represents a standard external continuous sterilization system for medium and feeds for bioreactors. Installation of a conductivity probe in the cooling water exit enables instant detection of a leak in the system.

3. *Sterilization by Filtration*

Sterilization through filtration by "depth" or membrane filters is a choice for clear media, especially where volumes are relatively low (Bull