
Chisti, Y., in *Bioseparation and Bioprocessing: A Handbook*, vol. 2, (Subramanian, G., editor), Wiley-VCH, New York, 1998, pp. 379-415. Biosafety.

13 Biosafety

Yusuf Chisti

13.1 Introduction

The bioprocessing industry has an undeniable record of safe operation. Yet, equally undeniable is the continuing public concern regarding the safety of biotechnology [1], including bioprocessing. Many hazards are associated with industrial bioprocessing: genetically modified organisms with real or perceived risks may have to be handled; highly pathogenic bacteria, viruses, and potentially contaminated substances such as blood may need to be processed; or the bioproduct may be so active that minute amounts may cause allergenic, toxic, or other activity-associated reactions in personnel exposed to it [1]. To assure safe processing, bioprocess engineers, operators, and managers must be intimately aware of the nature of the biohazard, the containment and regulatory issues, and how design and operation must satisfy the biosafety demands. This chapter examines risk assessment, biohazard containment and inactivation practices, and other- biosafety issues relevant to industrial bioprocessing. Considerations relating to deliberate release of genetically modified organisms (GMOs) are not discussed as that subject is outside the scope of this chapter. Deliberate release has been reviewed elsewhere [2].

13.2 Risk Assessment

Most micro-organisms and animal cells used in industrial processes pose little or no risk to human health and the environment; nevertheless, some high-risk human, animal, and plant pathogens are used (Table 13-1). By definition, a pathogen is any micro-organism or virus that can cause disease in any other living organism, including other micro-organisms. Whenever a

Table 13-1. Some commercially used hazardous microorganisms.

<i>Bordetella pertussis</i>	Yellow fever virus
<i>Clostridium tetani</i>	Poliovinis
<i>Corynebacterium diphtheriae</i>	Rabies virus
<i>Mycobacterium tuberculosis</i>	Rubella
<i>Salmonella typhi</i>	Foot-and-mouth disease virus

pathogen is used, it must be contained. In the long run, the trend is to move to safer processes by replacing harmful microbes with non-pathogenic recombinant producers.

The nature of the viable agent — microorganisms, viruses, animal and plant cells — has the greatest impact on the containment needs. Whenever possible, ‘generally recognized as safe’ or

GRAS species should be used in commercial processes. Recombinant variants of GRAS organisms are preferable to non-GRAS microbes. When non-GRAS species must be used, known pathogens should be avoided, or variants not capable of producing disease should be preferred. For example, pathologically incompetent *Escherichia coli* K-12 strains are used in producing recombinant proteins. Many microorganisms have a long history of safe use in food [3], and at least one previously unused species has been successfully commercialized as human food after exhaustive safety testing [4].

Use of new species or those with unknown risks must be preceded by assessment of risk [5,6] including pathogenicity testing [7]. The internal institutional biosafety committee, in consultation with the published guidelines and the Recombinant Advisory Committee of the U.S. National Institutes of Health (NIH), establishes the appropriate containment level for a strain.

Understanding of how viable and bioactive substances spread and invade the body is essential to assessing risk of processing. Microbial entry into the body occurs through inhalation of aerosols and particles; transfer to mouth via contaminated hands; damaged skin; and eye-hand contact or splashes. In addition, bioactive substances may be absorbed through intact skin. Aerosols and airborne particles are particularly troublesome sources of contamination. Aerosols spread easily and widely. Particles smaller than 5 μm dry instantly and remain suspended in the environment for long periods while circulating with air currents [8]. Many operations generate aerosols, including centrifugation, homogenization, mixing, blending, aeration of liquids, leakage of liquids under pressure, and handling of solids. Laboratory procedures can contribute. For example, aerosols are generated by bursting bubbles, breakage of liquid film as in pipeting, drops falling on surfaces, splashes, ultrasonic vibrations, sampling with syringe and needle, and during pouring and siphoning [9,10]. Contaminated apparels, hands, equipment and process streams, and circulating air spread micro-organisms, as do insects, rodents, and other pests.

Although relatively few infection episodes have been associated with industrial activity – the majority having occurred in research and diagnostic laboratories – only about 20 % of the laboratory-acquired infections have been ascribed to specific causes [8]. Unknown or unrecognized causes for most of the events suggest a continuing insufficiency of knowledge on the links between operational practices and infection. Thus, continuing vigilance is advised. Potential sources of contamination include direct accidental inoculation (needles, sharps, cuts or abrasions, animal bites), inhalation of aerosols, ingestion, and contact of contaminated material (hands, spills, contaminated surfaces) with membranes [81].

In addition to microbes, most physiologically active fermentation products – antibiotics, mycotoxins, enzymes, steroids, hormones, vaccines, deactivated microorganisms, antibodies, and other proteins – can be disruptive to health, and certain products are highly toxic. Aflatoxins are potent carcinogens. The fermentation conditions – temperature, pH, type of substrate, agitation, metabolic energy source, dissolved oxygen and carbon dioxide, the nature and concentration of micronutrients, metal ions, and other chemicals — influence the spectrum of biochemicals synthesized by an organism [3]. Under certain environmental conditions organisms such as *Aspergillus flavus* and *Aspergillus oryzae* are known to produce lethal toxins [11]. Species of the genus *Claviceps* and some members of other genera produce toxic ergot alkaloids. Mycotoxin production is widespread among fungi [12].

Several types of micro-organisms are known to cause allergic reactions when inhaled in large amounts. Implicated organisms include Actinomycetes, *Aspergillus* sp., *Aspergillus niger*, *Aureobasidium pullulans*, *Bacillus subtilis*, Baculoviruses, *Candida tropicalis*, *Penicillium* sp., and *Penicillium citrinum* [11]. Allergic reactions may be rapid, or the response may not occur until several hours after exposure, making connecting to the allergen difficult. Reactions may be extremely serious and, occasionally, fatal. Severe allergic reactions to *Bacillus subtilis* proteases are well known [11].

The hazard posed by nonviable bioactive material such as cytotoxic agents or endotoxins may not be eliminated by sterilization. In such cases, additional chemical decontamination of work areas, equipment and waste streams would be necessary using validated processes [13].

For example, solutions of sodium hydroxide (0.1 M) readily inactivate the botulinum toxin and are recommended for surface decontamination [14].

Gram-negative bacteria produce thermostable endotoxins. Endotoxin-containing aerosols may be generated, for example, during cell disruption. Inhaled endotoxins are implicated in allergic response; parenteral administration causes a pyrogenic reaction and other symptoms. Adverse reactions to endotoxins have been observed with *Enterobacter agglomerans*, *Flavobacterium* sp., *Methylophilus methylotrophus*, *Methylomonas methanolica*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *E. coli* [11]. Up to 4% of the dry weight of *E. coli* K-12 has been estimated to be endotoxin [11]. An action threshold value of 30×10^{-12} kg m⁻³ for airborne endotoxin has been recommended [11].

Increasingly, industrial processes use recombinant micro-organisms and animal cells. Some specific issues relating to such use are discussed in Sections 13.2.1 and 13.2.2. Biosafety considerations for solid-state fermentation processes have been discussed by Chisti [3], and issues relating to composting have been treated by Stentiford and Dodds [15]. The commonly used bioprocessing schemes and individual unit operations have been detailed elsewhere [16–20].

13.2.1 Recombinant Microorganisms

Development of containment requirements for recombinant microbes must consider environmental and ecological consequences of inadvertent release. Important considerations include survivability and colonization potential of the organism in the environment, and the organism's ability to transfer any part of its genome to indigenous populations [21]. Survival and persistence studies are carried out in ecosystems such as activated sludge, mammalian gastro-intestinal tract, soil, and river water [21]. Gene transfer studies may be combined with those of persistence. Such work should be 'designed to show that the recombinant construct behaves similarly to the host in a representative ecosystem where the organism could be introduced inadvertently' [21]. Assessments of potential biohazard should take into account characteristics of the unaltered parent, the unaltered plasmid vector, and the transposable elements. The U.S. Food and Drug Administration (FDA) has discussed these aspects in some detail [21]. As a general guide, a recombinant production strain should not have any known combination of pathogenicity, high colonization ability, and high genetic transfer competency [21]. In addition to the genes of interest, selectable marker genes are introduced into the host during transformation. Safety of such markers and the proteins they encode remains a subject of debate [22]. Specifically, the effects of any antibiotic resistance markers should be considered: such markers may facilitate colonization of gastro-intestinal tracts of fermentation process workers receiving antibiotic therapy [21]. Indeed, evidence is emerging that antibiotics in animal feeds alter the intestinal microflora in farm animals, and similar altered ecosystems become established in farm-workers that routinely contact the animals.

Recombinant *E. coli* K-12 strains and their plasmidless hosts are unable to establish in environments consistent with various deliberate release scenarios [23]. Moreover, those strains are non conjugating and apparently incapable of transferring genes [23] to other organisms.

13.2.2 Animal Cells

Cell cultures may be contaminated with pathogenic viruses (e.g., HIV, hepatitis B) and mycoplasma (e.g., *Mycoplasma pneumoniae*). Again, immunosuppressed individuals are especially susceptible even to otherwise harmless viruses. Human cell lines are particularly high-risk and so are those derived from nonhuman primates; other mammalian cells are

somewhat less risky, but may harbor agents capable of producing disease in humans (e.g., rabies, bovine spongiform encephalopathy agent, Hantaan virus in rodents). Avian and fish cells, and those from invertebrates may be lower risk. Previously uncontaminated cells can become infected during processing through human contact, or use of contaminated sera.

Other than being contaminated, established animal cell lines are potentially tumorigenic. Immunocompromised individuals are particularly susceptible, but a healthy immune system may be effectively circumvented if the transformed cell culture is compatible with an individual [24]. In one case, an accidentally inoculated (needle puncture, human tumor cells) laboratory worker developed a tumor [24]. Clearly, as with any microbiological process, workers with temporarily damaged skin should not handle viable material.

Because cell lines can harbor undetected viruses, and the lability of therapeutic proteins rules out the use of severe treatments that are capable of destroying viruses, a contamination-free product cannot be guaranteed; however, the risk of contamination can be reduced to extremely low levels using a multifaceted approach including use of exhaustively characterized cells and in-process controls.

Cells used in production originate in a manufacturer's working cell bank (MWCB) that is derived from a master cell bank (MCB). Only well-characterized cell lines are used in production. Characterization must assess identity of the cell line, its microbial and viral contamination, genetic stability, and, for genetically modified cells, the genetic construct must be verified [24]. Guidelines for characterization of cell banks have been established by the FDA, and common practices have been described by Lubiniecki [25].

Use of a well-characterized cell in production is insufficient assurance of a safe product; additional in-process controls are necessary. Controls that need implementing include assessment of pre-harvest culture broth for relevant viruses and establishment of acceptance/rejection criteria for viral loads taking into account the validated capabilities of downstream virus removal/inactivation steps. Combinations of those approaches reduce risk to extremely low levels. In fact, so far not a single case of viral infection has been associated with the use of cell culture-derived biopharmaceuticals (see also [12]). Product purity and viral safety issues for cell culture-derived therapeutic proteins have been discussed further by others [12,25–29].

13.3 Containment Levels

Containment requirements are based on assessment of potential biohazard of an agent as reflected in its risk classification [5]. Conventional pathogens have been categorized into four risk groups with increasingly stringent containment needs. The U. S. Centers for Disease Control and Prevention/National Institutes of Health recognize biosafety containment levels BL1-4 for laboratory operations. A different biosafety level assignment is used for large-scale processing: GLSP, BL1-LS, BL2-LS, and BL3-LS. The BL1-LS (LS = large scale) corresponds to BL1 of the laboratory scale, and so forth. The GLSP is a lower level than the BL1 designation. The BL4 has no equivalent at the large scale, and agents requiring BL4 containment are not used in commercial production. BL4 organisms require high-level containment; they are pathogenic and hazardous to laboratory personnel; and they produce transmissible diseases for which no prophylaxis or treatment exists. The biosafety level assignment considers whether an agent is pathogenic, poses a hazard to laboratory and plant personnel, is transmissible to the community, and availability of prophylaxis or treatment. Pathogenicity, or the ability to produce disease, depends on factors such as virulence, invasiveness, and infectivity. The specific risk categories for various organisms have been noted by Richardson and Barkley [14]. Table 13-2 lists the BLx containment requirements for several pathogens.

In addition to the United States Public Health Service CDC-NIH biohazard classification system [14], other guidelines have been established by the World Health Organization (WHO), the European Federation of Biotechnology (EFB), and national agencies in the United Kingdom [18], Canada [30], as well as other countries. Here, the focus is on the U.S. practices that are similar to those of the other developed countries. Practices and regulatory frameworks for other jurisdictions have been noted by Collins and Beale [20] and by Hambleton et al. [19]. Table 13-3 lists the four risk categories used by the WHO in classifying hazardous micro-organisms.

Of the four biosafety levels that are relevant to large-scale work, the GLSP level is suitable for non-pathogenic and non-toxigenic strains (including genetically modified variants) that have an extended history of safe industrial use. The U.S. GLSP derives from the 'good industrial large-scale practice', or GILSP guidelines originally established by the Organization for Economic Cooperation and Development (OECD) in 1986 for use with suitable recombinant strains. Ideally, all industrial processes should comply with those minimal requirements. GLSP

Table 13-2. Biohazard level classification of some pathogens.

Microorganism	BL2	BL3	BL4
Bacteria			
<i>Bordetella pertussis</i>	●		
<i>Clostridium tetani</i>	●		
<i>Corynebacterium diphtheriae</i>	●		
<i>Coxiella burnetii</i>			●
<i>Mycobacterium tuberculosis</i>		●	
<i>Neisseria meningitidis</i>		●	
<i>Salmonella typhi</i>	●		
<i>Yersinia pestis</i>		●	
Fungi			
<i>Aspergillus flavus</i>	●		
Viruses			
Ebola			●
Hantaan			●
Hepatitis B		●	
HIV		●	
Influenza	●		
Lassa			●
Poliovirus	●		
Rabies virus		●	
Rubella	●		
Yellow fever		●	

The containment levels shown are for relatively small-scale operations. For large-scale work, use of the next higher containment level is recommended (except BL3). Consult Richardson and Barkley [14] for further guidance.

is appropriate to organisms satisfying the following criteria [31]:

1. The host organism is non-pathogenic and free of adventitious agents. The host has an extended record of safe industrial use, or it has built-in incompetencies that limit its survival in the environment, and it has no adverse environmental consequences.
2. The genetically modified version is non-pathogenic and safe in an industrial setting, and without adverse environmental consequences.

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3. The DNA vector is well-characterized and free from known harmful sequences. To the extent possible, the size of the insert is limited to that necessary for the intended function, and the insert does not increase the stability of the construct in the environment unless required by the intended function; the insert is poorly mobilizable, and it does not transfer resistance markers to micro-organisms not known to naturally acquire resistance if such acquisition would compromise use of a drug for controlling disease in man, veterinary
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Table 13-3. Biohazard risk classification used by the World Health Organization.

Risk group 1	(no or very low individual and community risk) A microorganism that is unlikely to cause human or animal disease.
Risk group 2	(moderate individual risk, low community risk) A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.
Risk group 3	(high individual risk, low community risk) A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one individual to another, directly or indirectly. Effective treatment and preventive measures are available.
Risk group 4	(high individual and community risk) A pathogen that causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

medicine, or agriculture.

GLSP requirements further include: (i) implementation of a health and safety program; (ii) suitably trained personnel and written operational procedures; (iii) facilities, equipment, protective clothing and practices appropriate to risk; (iv) regulatory compliance with regard to discharges to the environment; (v) minimization of aerosol generation to prevent adverse risk to employee health; and (vi) a spill control plan within the emergency response plan [31]. Often, bioprocesses must comply also with the Good Manufacturing Practices (GMP) regulations; hence, in certain areas an otherwise GLSP process may actually meet much higher standards. For example, GLSP does not require any special containment, but to meet product protection requirements, use of enclosed equipment is advisable for processing of biopharmaceuticals [32].

Genetically modified *Saccharomyces cerevisiae*, *E. coli* K-12, *B. subtilis*, *Aspergillus oryzae*, and Chinese hamster ovary (CHO) cells, for example, can be used under GLSP classification. However, it should be noted that the bioactivity and the nature of the specific recombinant product being produced could strongly affect the acceptable containment level. Thus, an otherwise GLSP species being used to make a toxic or unusually bioactive substance, may have to be contained not because of an intrinsic 'biohazard', but because of the nature of the product. GLSP-compliant processing aims to attain the lowest practicable exposure of workplace and the environment to any physical, chemical, or biological agent [18].

Processes requiring greater containment than GLSP should preferably be designed to one level higher than the minimum acceptable biosafety level. Typically, the cost of building to BL2-LS level is a minor increment over BL1-LS [33]. Indeed, many U.S. companies have

designed production facilities to BL2-LS containment requirements where the BL1-LS measures would have sufficed [34]. Occasionally, the containment requirements for a given micro-organism differ slightly between jurisdictions. Again, the preferred practice is to err toward caution. The GLSP and BLxLS containment requirements are summarized in Table 13-4.

As noted earlier, depending on the risk, process support laboratories may have to comply with BL1-3 laboratory standards. Even a minimum containment GLSP process support laboratory should comply with good microbiological practices (Table 13-5) that are intended to protect both the operator and the product. Table 13-5 lists minimal requirements. Detailed guidelines on laboratory practices appear elsewhere [10,14,30].

In view of the different guidelines for laboratory- and large-scale operations, the question of demarcation between the two scales is important. In the U.S., processes larger than 10 L are assessed as 'large scale'. In the U.K., there is no specific volume guideline to distinguish between large and small scale [18]. For otherwise identical circumstances, the risk can be reasonably assumed to increase with the scale of operation, although the contrary has been argued [18].

The EFB Working Party on Safety in Biotechnology continues to produce 'reports' [9,24,35-39] that provide a useful perspective on biosafety issues. These reports have discussed plant pathogens [37,38], handling of micro-organisms of various risk classifications [35], assessing the impact on human health [9], hazard-based classification of micro-organisms [39], work with human and animal cell cultures [24], and general biosafety issues [36]. The regulatory approaches to biosafety issues in the European Community and the United States have been compared [31]. Because the published guidelines specify only general requirements — not methods of compliance — ongoing consultations with experts and the internal biosafety committee [40] are essential. Future European standards are likely to be quite specific on 'technical specifications, codes, methods of analysis and lists of organisms' than the current U.S. practices.

In most countries, responsibility for biosafety issues is spread over several regulatory agencies. In the United States, biotechnology products and production facilities may come under the jurisdiction of the FDA, the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Occupational Safety and Health Administration (OSHA). The roles of the relevant agencies and the specific Acts under which they are empowered have been summarized elsewhere [34].

Table 13-4. The biohazard containment requirements

Specification	GLSP	BL1-LS	BL2-LS	BL3-LS
1. Only authorized personnel allowed		●	●	●
2. Written procedures and training for good housekeeping and safety	●	●	●	●
3. Implementation and enforcement of institutional codes for hygiene and safety	●	●	●	●
4. Protective work wear and changing facilities	●	●	●	● ^a
5. Hand washing facilities	●	●	●	●
6. No eating, drinking, smoking, mouth pipeting, or cosmetics application in work area	●	●	●	●
7. Institutional accident reporting system	●	●	●	●
8. Biosafety manual	●	●	●	●
9. Medical surveillance			●	●
10. Closed equipment or other primary containment for processing viable agent		●	●	●
11. Inactivation of culture by validated procedures before removal from closed systems		●	●	●
12. Enclosed sampling, material additions, and transfers to/from closed systems to prevent/minimize aerosols, surface contamination, etc.	● ^b	● ^c	● ^d	● ^d
13. Treatment of exhaust gases from closed equipment		● ^c	● ^d	● ^d
14. Inactivation of viable agent by validated means before opening of closed systems		●	●	●
15. Emergency plan, systems and procedures for handling large accidental spills	●	●	●	●
16. No leakage of viable agent from rotating seals or other penetrations and mechanical devices		● ^c	●	●
17. Evaluation and monitoring of integrity of containment in closed systems			●	●
18. Evaluation/validation of containment with host organism prior to using recombinant organism			●	●
19. Containment and treatment of effluent before discharge	● ^e	● ^f	● ^f	● ^f
20. Permanent identification of closed process equipment and use of identification on batch records			●	●
21. Display of universal biohazard sign on contained equipment and use of identification on batch records			●	●
22. Display of universal biohazard sign in doors to contained areas during operation			●	●
23. Low-pressure operation of process systems				●
24. Operations to be in a controlled area:				●
Separate specified entry				●
Air-locks at all entrances (including emergency exits)				●
Readily cleaned and decontaminated finishes ^g				●
Protection of utilities, services, process piping and wiring against contamination				●
Separate gowning and washing facilities at each entrance: shower facilities in close proximity				●
Personnel should shower before leaving controlled area				●
Areas sealable for fumigation				●
Sealed penetration into area				●
Ventilation (controlled negative pressure in area; HEPA filtered exhausts, once through ventilation)				●

^a Complete change. ^b Minimize release using procedural controls. ^c Minimize release using engineered controls. ^d Prevent release. ^e Comply with local environmental codes. ^f Inactivate by validated means. ^g Required in all GMP-compliant processing. Also consult Richardson and Barkley [14] and the Canadian Medical Research Council laboratory biosafety guidelines [30].

Table 13-5. Good microbiological practices for safe handling of potentially risky microorganisms[35].

1. Operators should have a basic knowledge of microbiology. All personnel should be aware of the risks of cultivated pathogens. Only essential personnel with the necessary training should be allowed into the work area. Practices that prevent spread of pathogens should be routinely followed. Suitable full-front laboratory apparels should be used. Work wear should remain within the work area. Hands must be washed with suitable disinfectant soap after removing latex gloves.
 2. No eating, drinking, smoking, mouth pipeting, and application of cosmetics in the work area. No contact between work area materials or tools and the mouth of operators. Use of good aseptic technique.
 3. Aerosol generating activities (e.g., filling of bottles and tubes, centrifugation) should be confined to biological safety cabinets. Any washing activities require special care.
 4. Infected waste is sealed in containers the outside of which is disinfected prior to transfer to autoclave or incinerator.
 5. Use of validated thermal or chemical sterilization processes that assure the requisite kill.
 6. Use of reliable equipment.
 7. Disinfection of all work surfaces and hands after normal work.
 8. Disinfection of work surfaces, floors and hands after spill of infectious material.
 9. An emergency action plan with details of first aid, cleaning and disinfection. Staff trained to deal with emergencies.
 10. Decontamination of laboratory clothing.
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13.4 Risk Management

Design and operation of a bioprocessing facility must assure safety of personnel within the facility and those in the surrounding community [41]. Protection is achieved through a combination of engineered facilities, processes, and equipment; worker training and education; use of personnel protective equipment; operational practices; validation of machinery and methodologies; controlled access to facilities; biosafety committee or subcommittee; and medical and environmental surveillance [8]. Typically, enclosed process equipment or biological safety cabinets provide primary containment to the viable material. In the event of inadvertent release from process equipment, further secondary containment is provided by the building. Environmental monitoring seeks to ascertain the satisfactory functioning of primary and secondary containment [42].

This section details the various aspects of risk management, including selection and use of biological safety cabinets which are invariably encountered at numerous stages of bioprocessing. Also included is a section on handling of biohazardous spills.

13.4.1 Biological Safety Cabinets

Biological safety cabinets are the primary means of containment in process support laboratories and during early stages of culture development. Based on design and the protection afforded, biological safety cabinets are designated as Class I, II, and III. Capabilities of the various classes are summarized in Table 13-6 [43]. Note that laminar flow 'clean benches' are not biological safety cabinets and should not be used to handle potentially hazardous material.

Class I cabinets (Fig. 13-1) do not protect the work area against microbial or particulate contamination. The operator is protected so long as a minimum linear air velocity of 0.4 m s^{-1} is maintained through the front opening [43]. The cabinet is hard-ducted to the building exhaust system (Fig. 13-1).

Table 13-6. Protection capability and biohazard suitability of various classes of biological safety cabinets.

Biohazard level	Protection provided			Cabinet class
	Personnel	Product	Environment	
BL 1—3	Yes	No	Yes	I
BL 1—3	Yes	Yes	Yes	II (A, B1—3)
BL4	Yes	Yes	Yes	III(B1,B2)

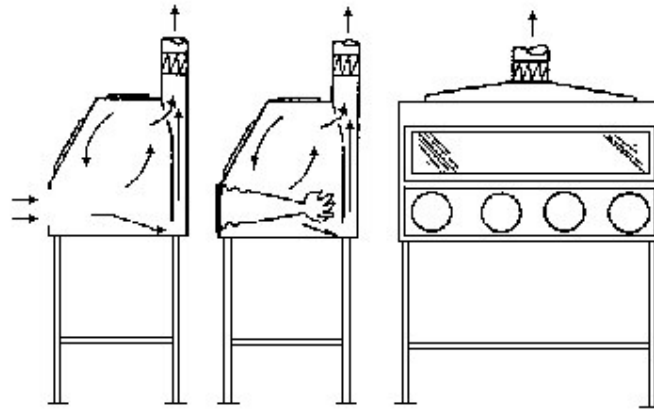


Fig. 13-1. Class I biological safety cabinet.

Class II biosafety cabinets (Fig. 13-2) protect the operator, the product, and the environment. The work area is bathed in downward laminar flow of particle-free, recirculated air. In addition, air from the room is drawn in through the front opening to prevent leakage of aerosols and contaminated air. The linear air flow rate at the opening should be 0.4 m s^{-1} or greater. HEPA filtered air may be exhausted into the laboratory as in Class II Type A cabinets (Fig. 13-2), or the discharge may be hard-piped to the building exhaust system as in Class II Types B 1-3. Type B1 cabinets recirculate part of the air over the work area, hence they may be used to process only minute amounts of volatiles. Type B2 cabinets are total exhaust devices that may be used also for some chemical containment, so long as the fumes are not susceptible to electrical ignition. As a general rule, no class of biological safety cabinets is suited to handling volatile toxic substances, but nonvolatile toxic chemicals can be handled in all classes of cabinets

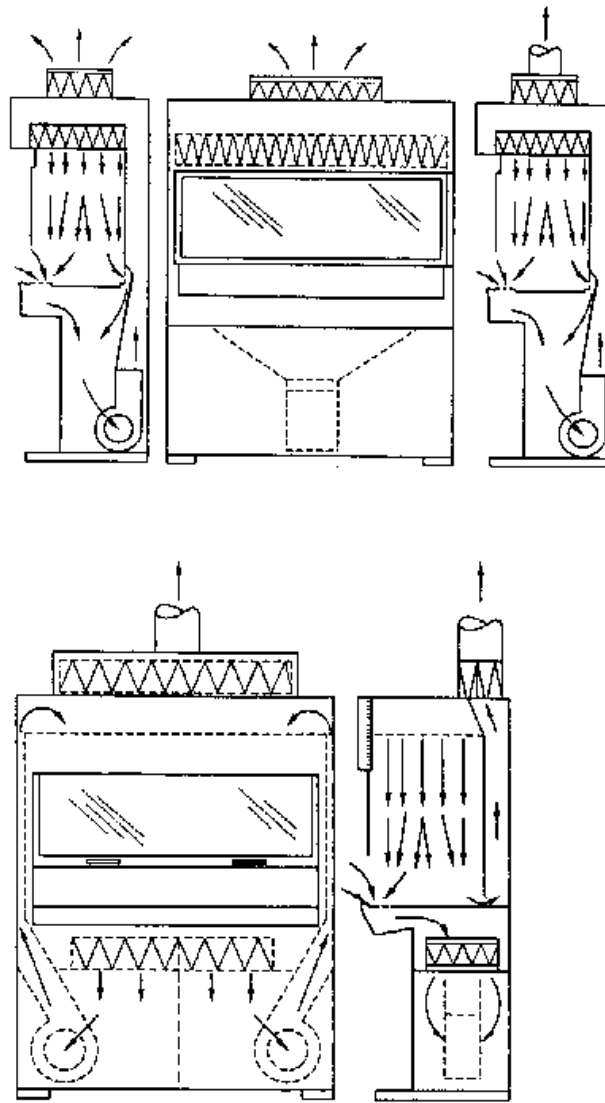


Fig.13-2. Class II biological safety cabinets Types A (top) and B.

Class II Type B3 cabinets are ducted Type A devices that like other Type B cabinets provide a minimum linear air velocity of 0.51 m s^{-1} at the opening. All positive-pressure contaminated plenums within a Type B3 cabinet are surrounded by negative-pressure chambers to prevent leakage to the environment.

Class III biosafety cabinets (Fig. 13-3) are designed for handling BL4 biohazard agents. The cabinet is a fully sealed chamber with HEPA filtered air inlet and exhaust. The front end is provided with a sealed window and ports with heavy-duty, arm-length rubber gloves. Access to the chamber is through a side-mounted, disinfectant-filled dunk tank or through a sterilizable double-door pass-through such as an autoclave. The operator, the environment and the work area are protected. Air from Class III cabinets must be exhausted through two HEPA filters in series, or one HEPA filter and an incinerator. A dedicated, independent exhaust system exterior to the cabinet is used to maintain air flow [43]. The enclosed work chamber is kept at a lower pressure than the laboratory. Usually a 0.5 inch (1.3 cm) water gage pressure differential is maintained [43]. Class III cabinets are usually installed only in maximum containment laboratories having other suitable safeguards.

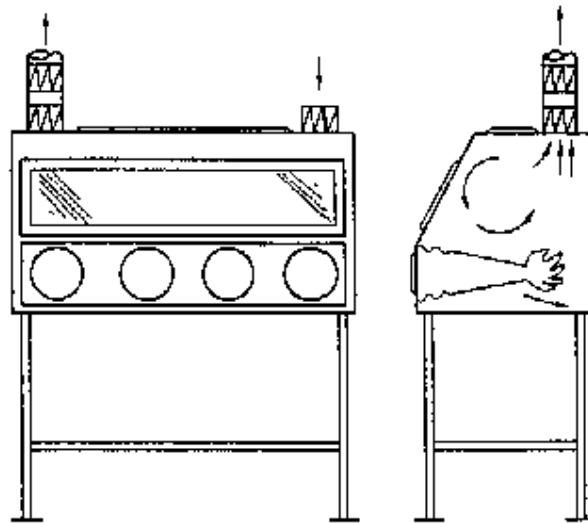


Fig 13-3. Class III biological safety cabinet. Side-mounted dunk tank not shown.

Biological safety cabinets rely on HEPA filters at air exhaust and/or intake to provide requisite protection to personnel, product and the environment [43,44]. Generally, HEPA filters are rated to remove particles down to $0.3 \mu\text{m}$ with an efficiency of 99.97 %, but more expensive higher efficiency (99.99 % or higher) filters are available. The $0.3 \mu\text{m}$ particles are least easily filtered compared with larger or smaller particles; hence that size is used for HEPA filter performance specifications [44]. Filters are susceptible to shock-induced mechanical damage; therefore, performance and integrity of a biosafety cabinet must be certified after initial installation, after relocation, after repair, and at yearly intervals. The specific certification tests depend on the type of cabinet. Some essential tests include the downflow velocity and volume testing (Class I and II); the inflow velocity test (Class I and II); the negative-pressure testing (Class II and III); air flow smoke patterns tests (Class I and II); the HEPA filters leak tests (Class I-III); the cabinet leak test (Class II and III); and testing of alarms and interlocks (Class III). HEPA filters should be decontaminated prior to replacement. Decontamination is usually done with formaldehyde or hydrogen peroxide vapor, and decontamination provisions should be

provided during installation. Class II cabinets usually have sensors for monitoring the pressure drop across the HEPA filter, and a low exhaust flow alarm is provided.

Proper technique is essential for safe use of biological safety cabinets. The containment air curtain at the opening of Class II cabinets is easily disrupted by rapid sweeping arm movements into and out of the cabinet [43]. Other activities such as rapid movement of personnel around the cabinet and opening/closing of room doors also disrupt the air barrier [43]. Arm movements into/out of the cabinet should be slow and perpendicular to the front face of the cabinet [43]. The number of arm entries should be minimized by preparing a checklist of the required materials and placing them in the cabinet. The seating height should be adjusted so that the face of the operator is above the front opening [43]. Manipulations should be delayed for about 1 minute after arms/hands are placed inside the cabinet [43]. Arms, hands or other objects should not rest across the front grill or the room air may flow into the sterile work area. All manipulations inside the cabinet should be at least 10 cm (4 inches) from the front grill [43]. Any aerosol-generating equipment should be operated in the rear of the cabinet [43]. Used pipets should be discarded into a horizontal, disinfectant-containing discard tray kept within the cabinet [43]. Potentially contaminated material should not be brought out until after surface decontamination with a suitable disinfectant [43]. The cabinet air blower should be switched on at least 3-5 minutes before commencing work [43]. The work surfaces and the interior walls should be disinfected before and after use by wiping with 70 % ethanol or other suitable disinfectant. If chlorine bleach is used, a second wiping with sterile water is needed to remove residual chlorine, which is corrosive to stainless steel [43]. Also, any material and containers placed in the cabinet should be wiped with 70 % ethanol to reduce risk of contamination. Consult Richmond and McKinney [43] for additional guidance on selection, installation, and proper use of biological safety cabinets. Management of spills within a biosafety cabinet is discussed in Section 13.4.2.

13.4.2 Spill Management

Primary containment is provided by fully closed process equipment such as fermenters, centrifuges, heat exchangers, and pumps. However, even the best designed primary containment can fail; therefore, emergency response procedures must be in-place. Accidental release from process equipment poses a potential risk to employees and the local environment [45]. The extent of risk depends on the pathogenicity, virulence, invasiveness, and infectivity of the agent, and the volume being handled [45]. Release or spill may occur within the confines of a biological safety cabinet, in unconfined areas within a facility, or more broadly due to catastrophic failure of a process equipment [45].

For spills within a biosafety cabinet, immediate decontamination should be effected by a trained, suitably equipped (latex gloves, laboratory coat, safety glasses) technician while the cabinet air circulation system continues to operate [45]. Decontamination requires flooding the work tray with a disinfectant while minimizing aerosol generation. Thorough contact of the spill and the disinfectant is necessary for a preferred minimum of 30 minutes. The spill is then absorbed into disposable cloth or paper towels and discarded into autoclavable bags. The work surface, the cabinet walls, and any equipment inside is wiped with a disinfectant-soaked cloth. If the spill extends to the exhaust grills, the catch basin should be flooded (30 minutes) with disinfectant which is then drained into an autoclavable bag. A disinfectant-soaked cloth is used to wipe the grill and the catch basin [45]. The outside of the autoclavable container and bag should be wiped with a disinfectant-soaked cloth. Upon completion of cleanup, all solid material (including gloves, wiping cloth, lab coat, and any contaminated garments) that came into contact with the viable agent should be placed into an autoclavable bag. This material should be autoclaved at 121°C for a minimum of 1 h or other suitable period that has been

previously established and validated for a particular load size and distribution [45]. Once contaminated gloves and clothing have been removed, germicidal soap should be used to wash arms, hands, and face.

Disinfectants suitable for most purposes are chlorine bleach (500 ppm available chlorine), iodine solution (25–1600 ppm available iodine), formaldehyde (0.2–8.0 %), and 2 % glutaraldehyde. Only chlorine bleach is satisfactory for treating liquids; other agents noted are suited for wiping surfaces, glassware, etc. Disinfectants such as quaternary ammonium compounds, ethyl alcohol, and isopropyl alcohol are not broadly effective. Chlorine bleach should not be used on stainless steel process equipment, and care should be taken to ascertain that bleach is compatible with the fluid being disinfected. The disinfection capability of chlorine bleaches declines with increasing pH.

Readily accessible spill carts should be provided to deal with small unconfined spills [45]. A spill cart should have supplies of chlorine- and iodine-based disinfectants (e.g., 5 % Wescodyne, and 5 % Clorox), spill control supplies (autoclavable squeegee, autoclavable dust pan, autoclavable forceps, autoclavable biohazard bags, bucket, disposable wipes, and spill pillows), as well as protective clothing and equipment (disposable lab coats and jump suits, disposable latex gloves, disposable safety glasses, autoclavable boots, and half-face or full-face respirator with HEPA filter cartridges). The following treatment procedure, adapted from Van Houten [45] is recommended: Warn others of spill and leave the area holding your breath to avoid inhaling potentially hazardous aerosols; remove contaminated clothing, folding contaminated areas inwards and discard into an autoclavable bag; wash potentially contaminated body areas as well as face, arms, and hands with germicidal soap; shower if necessary; wear protective clothing (disposable lab coat, latex gloves, safety glasses, autoclavable boots) and, if necessary, HEPA filter-equipped half or full-face mask; enter the area with the spill cart; use spill pillows to isolate floor drains if present and connected directly to sewer (i.e., not connected to a biokill treatment facility); encircle the spill with disinfectant, ensuring adequate spill–disinfectant contact while minimizing aerosolization; allow a 30-minute contact time; pick up broken glass and other sharp objects with the forceps, dust pan, squeegee, and place them in a leak-proof autoclavable container; use disposable wipes or spill pillows to mop up the liquid and discard it into an autoclavable bag; wipe the outside of the autoclavable bags with a disinfectant-soaked cloth. Use an uncontaminated, disinfectant-soaked cloth to wipe the area of the spill. Upon completing clean-up, all solid material that came in contact with the viable agent should be placed into autoclavable bags. Bags, containers, and contaminated clothing should be decontaminated in an autoclave at 121°C for 1 hour or other prevalidated period. Arms, hands, and face should be washed with germicidal soap. Shower if necessary. The spill cart should also be disinfected. Exposed personnel may have to undergo prophylactic or other treatments and medical surveillance in accordance with preestablished policies. Larger, unconfined spills are usually handled by especially trained spill response personnel using procedures similar to the ones noted for smaller spills.

13.4.3 Buildings and Facilities

The design of a facility determines its ability to provide secondary containment. Facilities processing especially hazardous material should preferably be located away from heavily built-up areas. In extreme cases, dispersal patterns for any inadvertently released material should be considered for all the meteorological scenarios relevant to the location. Access to the facility should be restricted to authorized personnel, with certain areas being ‘off limit’ to all but the relevant personnel [41]. Access control may require security fencing, electronic card controlled entry, vandal-proof exterior windows, etc. In addition to containment, the design of the facility needs to assure protection of the product. Products need to be protected against contamination,

particularly microbial contamination to which they are highly susceptible [32]. Protection must be provided throughout manufacturing and storage [32]. All facilities need an effective insect and rodent control program.

Certain bioprocess facilities may require holding or handling of infected or potentially infected or suspect animals. Design requirements for such facilities are beyond the scope of this chapter; consult Richardson and Barkley [14] for guidance.

Table 13-7. Design concepts for biohazard containment^a

1. Controlled access
 2. Work areas at negative pressure relative to surroundings
 3. HEPA filtered air exhaust
 4. Additional containment of aerosol-generating activities
 5. Personnel training
 6. Personnel protective equipment
 7. Decontamination of bioactive process wastes
 8. Medical surveillance of ‘at-risk’ personnel
 9. Environmental monitoring
-

^aAdapted from Flickinger and Sansone [13].

The principal concepts for design and operation of biohazard containment facilities are summarized in Table 13-7. Specific features are discussed in the following sections. The guidelines given comply with the U.S. FDA recommendations [32].

13.4.3.1 Layout

The layout of the facility affects efficiency of operations, the potential for containment, and prevention of cross-contamination. Attention to layout is required by the GMPs [46]. Because the flow of personnel, equipment, materials and air in the facility must be controlled, the building and the process must be closely integrated by design [47]. ‘Contained’, ‘clean’, and ‘dirty’ areas should be identified on the process flow sheets and the building layout drawings. Movement of personnel, equipment, process streams, and air across containment boundary must assure integrity of containment through a combination of engineered systems and operational protocols. In general, flows must be unidirectional [32], from clean to dirty areas, and not vice versa. The dirty and clean paths should not cross [32], and there should be no back-tracking. The biohazard containment areas and the aseptic product filling areas should be located in different wings of the facility, with no sharing of common hallways or direct access [32]. Adequate space must be provided for various uses. There should be no overcrowding in work areas, especially the contained areas.

13.4.3.2 Air Handling

Quality of the ventilation air and its flow in a facility are crucial to containment, biosafety, as well as protection of the product. Air in a facility is handled by the heating, ventilation and air conditioning (HVAC) system. Designing the HVAC system requires classification of process areas as ‘clean’, ‘dirty’, or ‘contained’. Air-locks are used to isolate the contained areas and

those with critical cleanliness requirements, from other zones.

Effective containment depends critically on management of air flow and pressure differentials [32]. Area pressure differentials help to prevent airborne contaminants from intruding into contamination-free parts of a facility. Pressure differentials of 1.3 mm (0.05 inches) of water are typically used between adjacent areas. Areas containing infectious agents (e.g., viral vaccine) must be maintained under negative pressure relative to the surroundings [41,48]. Otherwise, the air flow is generally from 'clean' to 'dirty' areas. Access to a contained process area should be through an air-lock that is maintained at a lower pressure than the contained area and the outer access corridor [41]. Access should be restricted (e.g., card-controlled entry).

The HVAC system design should assure that unwanted air pressure differentials do not develop in the event of mechanical failure. Visual and audible indication of ventilation failure should be provided. When feasible, once-through ventilation is preferred to prevent spread of contamination or the likelihood of cross-contamination [41]. Air from the facility is exhausted usually on the roof, away from any air intake. All air from contained areas in a BL3 -LS facility should be exhausted through HEPA filters, and the area should be under negative pressure with respect to the surroundings (Table 13-4).

The BL2-LS facility design guidelines do not specify secondary containment through air flow management, or HEPA filtering of the area's air supply and exhaust. Primary containment must be achieved by using closed systems or appropriate biosafety cabinets (Table 13-4). The requirements notwithstanding, HEPA filtered air supply is recommended for minimizing the potential for contaminating the product [33]. Pipework should be minimized in contained areas, and wall penetrations and light fixtures should be sealed.

Aerosol build-up in work areas can be reduced by HEPA filtered ventilation with a sufficient number of air changes per hour – 20–30 are not unusual in BL2-LS processing areas [33]. The mandated minimum number of air changes in various areas may have to be exceeded to account for factors such as humidity and heat rejection in the area, its typical function, personnel capacity, production of vapors and fumes, and generation of aerosols. The relative humidity in most processing areas is controlled at 40–50 ± 5 %. Relative humidities exceeding 50 % promote corrosion, whereas values lower than 40 % lead to problems with static electricity. Ventilation is discussed further by First [44], del Valle [48], and Lee [49].

Air quality is of particular concern in the processing environment of the sterile final dosage forms of pharmaceuticals. Areas where the sterile product, containers, and closures are exposed to the environment are designated as 'critical' areas. Examples include 'fill rooms' and other aseptic processing areas. For a long time, the air quality in critical areas was required to be at least Class 100, that is, no more than 100 particles of $\geq 0.5 \mu\text{m}$ per cubic foot of air. Higher standards are now in demand. In critical areas, the number of colony forming units (CFU) should not exceed one per cubic foot (0.03 m^3) of space, and a 0.05-inch (1.3 mm) water gage positive pressure differential must be maintained relative to adjacent areas. Class 100 areas are typically contained within Class 100,000 areas in which the particle count of $\geq 0.5 \mu\text{m}$ particles does not exceed 10^5 per cubic foot (0.03 m^3). In addition, the CFUs do not exceed 25 per 10 cubic feet (0.3 m^3) of air. Moreover, the Class 100,000 surrounding space must equal or exceed 20 air changes per hour. A higher number, generally 60–75 air changes per hour, must be provided in critical aseptic fill rooms [48]. The Class 100 area itself should be designed for at least 600 air changes per hour. HEPA filtered air is almost always supplied at the ceiling, and low wall-mounted returns are preferred. Aseptic filling areas need to be maintained at positive pressure [32]. Additional issues relating to HVAC system design for protection of the product have been discussed by del Valle [48] and Dobie [50].

13.4.3.3 Construction, Finishes and Practices

How easily and well a facility may be cleaned, sanitized or decontaminated depends on its construction, and finish. The building and room finishes are subject to GMP and containment guidelines [46,47,30,51]. Processing areas should have non-shedding, smooth, impervious, splash-resistant finishes that are capable of being cleaned and disinfected. Even a GLSP-level fermentation area should be capable of being hosed down. Contained areas should be capable of being sealed and decontaminated by disinfectant spray and by fumigation. Decontamination procedures should be established beforehand [41] and validated. Formaldehyde vapor is a commonly used fumigant, but it is carcinogenic. In addition, mixing of formaldehyde with chlorine-containing disinfectants can produce potent lung carcinogens. Paraformaldehyde gas may be used to surface-sterilize heat-sensitive equipment.

Floors must slope to drains. Drains should have a slope of at least 2 cm per linear meter to assure complete drainage. Floor drains in areas that are susceptible to spills should not be connected directly to the municipal sewer [33]; instead, the drains should be piped to the biokill system. Drains should be provided with 20 cm water trap seals to prevent back-escape of vapor, gases, and aerosols from the containment sump into the work area. Sometimes, in addition to water traps, the contained drains are provided with check valves that prevent back-flow in case of a pressure build-up in the containment sump [33]; but usually suitable venting of sumps and tanks is sufficient to preclude pressure build-up. All traps should be decontaminated after a spill, and on a regular basis by pouring several trap volumes of a disinfectant solution down the drain.

Catastrophic failure of pressure vessel fermenters is unlikely, but large leakages of fluid may occur from failed valves, ports, or gaskets. All material released up to the full volume of the largest fermenter should be contained within a diked area emptying into a sump, and treated through the biokill system. After the spill has been treated, the diked area should also be disinfected (see Section 13.4.2).

The spill containment and treatment system should be capable of functioning on demand [32], and it should have sufficient capacity to handle the entire process volume. A minimum containment capacity of twice the production capacity has been recommended [32] which is quite reasonable considering that the process fluid as well as the subsequent wash effluent must be contained.

Generally, concrete floors with troweled-on epoxy finish are preferred in processing areas, but in some low-traffic laboratory areas welded PVC flooring (not tiles) may be used. The epoxy finish (or vinyl) should extend at least 10 cm up the walls, and the edges should be coved. Walls are generally concrete masonry units with epoxy paint. Ceilings are suspended dry wall, painted, or epoxy finished. In aseptic areas and those processing BL1-LS or higher agents, all internal corners (including wall-to-ceiling) should be preferably coved and all finishes should be flush (base flush with wall, door and window frames flush with walls). Sealed windows are the norm. In addition, any exterior windows should be break-resistant whenever high-level containment is required. Light fixtures should be flush mounted, and, in high-level containment areas, they should be of a type that can be serviced from outside the contained area.

The area furnishings should be of a sanitary design, resistant to water, process chemicals, and disinfectants. Usually, work surfaces are stainless steel or epoxy tops with baked epoxy painted casework [33]. Placement of equipment and furnishings should not interfere with cleaning and disinfection [33]. Equipment may be placed on housekeeping pads with radiused edges, or raised on legs that comply with hygienic design standards. Floor penetrations should be minimized and penetrations should be fully sealed to prevent seepage [33]. Supporting equipment from wall-mounted brackets, or from overhead supports is preferable [33].

Hand-washing facilities should be provided near exits in the contained areas. Sink faucets (taps) should be automatic, or elbow- or foot-operated. Suitable disinfectant soap should be provided. Although hand air dryers have been recommended [33], paper towels are preferable. Electric dryers recirculate particles and aerosols that are deposited on hands. The BL2-LS facility design has been discussed by Miller and Bergmann [33] and the essential requirements

are noted in Table 13-4.

A BL3-LS contained area should have separate facilities for gowning and washing at each entrance and showering facilities should be provided in close proximity (Table 13-4). Air-locks should be provided at all entrances and exits (including emergency exits) in a BL3 -LS contained area. Only a minimal number of essential personnel should be allowed into contained areas. Monitoring should be from outside the contained area, through sealed windows, intercoms, and closed-circuit television.

Containment features for pilot-scale fermentation facilities for producing cytotoxic agents and oncogenic viruses have been described [13]. Some of the methods noted are no longer state-of-the-art, but are effective nonetheless. For example, more elegant and reliable contained sampling methods are now available [52]. Specific construction details of animal cell culture facilities have been noted by Donnelly [53] and Lubiniecki [25].

The GMP regulations require provision of separate facilities for handling of spore-forming microorganisms. In addition, dedicated, segregated facilities are needed for processing penicillins (and other β -lactams) because cross-contamination with penicillins and penicillin-containing substances cannot be reasonably prevented in a multiproduct facility [12]. Separate air handling systems are necessary if a building processes penicillins as well as non-penicillin products. Similarly, in facilities producing several viral vaccines, Damm [411] recommends complete isolation of areas dealing with different viruses.

Containment and decontamination capabilities must function under normal conditions as well as during emergencies [54]. Provisions and practices must be in-place for evacuation during emergencies such as fire. Consequences of loss of power on containment should be evaluated during design, and emergency power should be provided to prevent loss of containment. In addition, some essential process equipment may require standby power and steam supply.

13.4.4 Process Equipment

The design of process machinery determines its primary containment capability. Moreover, specific equipment may have specific hazards associated with its use. For example, high-pressure equipment such as cell disruptors may generate sprays of contaminated fluid in the event that a gasket fails [55]. Similarly, aerosols are produced during centrifugation and submerged aeration of culture broth in fermenters.

Design and evaluation of process equipment require identifying areas where potential leakage of contained material could occur (Table 13-8). Points of possible leakage should be minimized, better contained, or, when feasible, eliminated. Containment capabilities should be assessed for all equipment, including fermenters, centrifuges, filters, solvent extraction units, cell disruption devices, spray and freeze dryers, sterilizers and autoclaves, pumps, valves, pipes, and heat exchangers, bottling and vialing machinery, downstream purification units, and waste treatment systems. In addition, the HVAC system and the utilities should be assessed for potential of becoming contaminated. Potentially contaminated lubricating fluids, steam condensate, wash fluids, etc., should be treated through the contaminated waste system.

Process equipment, control cabinets, electrical housings, switches, etc., should be splash-resistant. Alternatively, control cabinets and instrumentation may be located outside the contained area and serviced through sealed cables [40]. Utility lines (e.g., steam, air, water, vacuum) that are connected directly to process equipment are at risk of becoming contaminated [40]. Protective measures include maintaining a positive pressure in the delivery lines relative to contaminated equipment, use of microbial-grade filters and back-flow preventers.

To the extent feasible, equipment entering and leaving the contained area should be decontaminated by thermal sterilization in double-door autoclaves connecting the inside and the outside of the work area. Waste should be sterilized in a separate autoclave that is not used for process sterilizations [33]. Autoclaves should have interlocked doors, and fail-safe devices that prevent opening the autoclave until a complete sterilization cycle has been implemented. Pre-

vacuum type autoclaves are preferred for sterilization of solid waste and other general process uses. Sterilization cycles and load configurations should be pre-validated. Principles of thermal inactivation have been described elsewhere [16].

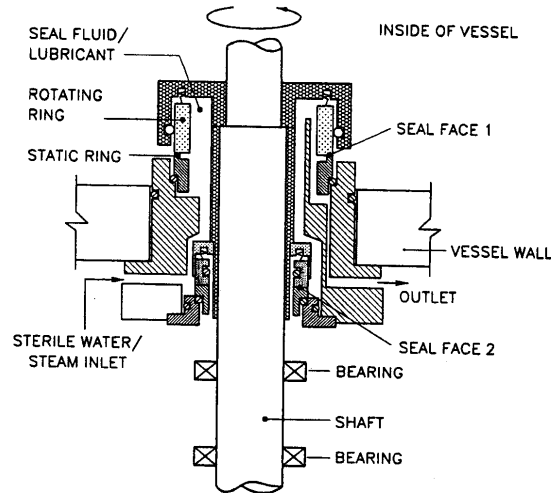


Fig. 13-4. Double mechanical seal with water lubrication. A mechanical seal consists of two seal rings made of materials such as silicon carbide, tungsten carbide, or carbon. The flat faces of the rings press against each other to form the seal. The small gap between the faces is lubricated by a film of liquid (either culture broth or the sterile sealing fluid) or a film of gas (in dry-running seals). One of the seal rings forms a static seal with the shaft and rotates with the shaft. The other ring makes a static seal with the vessel or the stationary seal housing, and remains stationary. Two such seals in close proximity on the same shaft constitute a double mechanical seal. A film of fluid between the running and the static faces is essential to the sealing action, and any damage (e.g. scratching) to the seal faces would produce leakage. Seals must be periodically replaced as a part of the preventive maintenance program.

Table 13-8. Points of potential release of contained material.

- Shaft seals (vessels, pumps)	- Perforated pipes, vessels, equipment
- Flanges (pipes, valves, vessels)	- Leakage into cooling water
- Pumps	- Tubings and hoses
- Points of entry of probes and sensors	- Sanitary connections
- Valve packings	- Rupture discs
- Ruptured diaphragms in valves	- Pressure relief valves
- Sample points	- Exhaust gases
- Leaking seals and gaskets anywhere	- Waste collection and treatment system

13.4.4.1 Fermentation Plant

Fermenters usually contain large amounts of potentially hazardous viable material. Frequently, in addition to containing the viable agent used in production, entry of any other viable material into the fermenter must be prevented. Leakages from fermenters are not uncommon and considerations relating to containment and treatment of spills have already been discussed (Sections 13.4.2 and 13.4.3). In addition, all through operation, most fermenters need to be aerated, and, the fermenter exhaust gases must also be sterilized. Usually, two 0.2 μm rated absolute hydrophobic filters in series are used to filter sterilize the exhaust air. Those filters are often heated to above dew point to prevent condensation, and, on larger or highly aerated vessels, filters are preceded by condensers [40]. In addition, cyclonic separators installed before the filters may be used to protect the filter against contamination by foam and spray. Mechanical foam breakers may also be used [56]. In some cases, one exhaust gas filter may be followed by an incinerator. Integrity of the exhaust filters should be checked in-place using the forward flow diffusion method after the filter is sterilized, but before use.

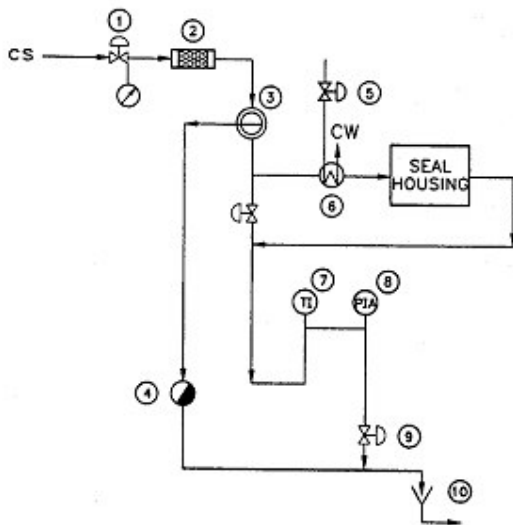


Fig. 13-5. Pipework for sterilization and sterile lubrication of mechanical seals. The inlet and outlet shown on the seal housing correspond to those shown on the seal assembly in Fig. 13-4. For sterilization, the clean steam (CS) regulating valve ① is set to the desired pressure. The valve below the sight glass ③ and valve ⑨ are opened to drain the system. Steam enters the system through strainer ② and the sterilization setpoint temperature is attained on temperature indicator ⑦. Valve ⑨ is now pulsed to allow drainage of condensate. Steam trap ④ also serves to drain condensate. After predetermined sterilization time, valve ⑨ is closed and the cooling water supply valve ⑤ is opened. Clean steam is condensed and the condensate is cooled to $\sim 25^{\circ}\text{C}$ in the condenser ⑥. The pipe section above valve ⑨, the seal housing and the sight glass assembly fill up with the cooled sterile water needed to lubricate the seal. Steam pressure on top of the water assures that the seal housing remains under positive pressure with respect to the fermenter. Pressure is monitored at indicator ⑧ that is equipped with the low-pressure alarm.

Fermenters are protected against overpressurization with a rupture disc [57] that should be piped to a HEPA-filter-vented containment/treatment system. Sometimes, the rupture disc is followed by a pressure relief valve so that the vessel returns to a contained state once the pressure is released. This arrangement should have an attained pressure indicator for detecting disc rupture. In addition, use of overpressure sensors to shut off the sparger air supply in case of overpressurization has been recommended [13,40], but this practice is unusual: normally, the air supply regulator is set to a pressure significantly lower than the value required to open the rupture disc; thus, in case of a blocked exhaust filter, the vessel would attain a pressure equal to the air supply pressure that is still well within the vessel's capabilities.

Double mechanical seals (Fig. 13-4) with sterile lubricating water between them are used to seal agitator shafts in fermenters requiring high-level containment. The pipework required for

sterilizing and lubricating the seal with sterile clean steam condensate is shown in Fig. 13-5. (Fig. 13-4) is kept higher than that in the fermenter, hence any leakage is into the vessel [40,57]. Low-pressure alarms are recommended for the sealing fluid chamber. Miller and Bergmann [33] recommend using a 'collection tube' in the seal fluid chamber drain for detecting seal failure. Presumably, debris or colored matter would accumulate in the tube if the seal on the culture side failed. A conductivity sensor in the water chamber should provide a better method of detecting leakage of culture fluid into the chamber. Most fermentation media are relatively conductive, whereas uncontaminated sterile water produced by condensing clean steam is a poor conductor of electricity. Rotating seals can be eliminated altogether by using magnetically coupled agitators, but torque consideration limit the vessel size to about 800 L with animal cell culture bioreactors [57], and only to about 80 L with microbial fermenters. Large airlift bioreactors that do not require mechanical agitation are particularly suited to containment [16,58]. In addition, airlift reactors are more reliable than the mechanically stirred ones.

Added protection against microleaks of viable agents can be provided by specifying double O-ring seals on all fermenter entry ports. *In situ* sterilizable probes that can be removed from a fermenter during cultivation, and decontaminated in-place before exposing the surroundings are available. The fermenter flanges may have double O-ring gaskets with a zone of live steam in between. Similarly, a live steam barrier can be maintained on the outside of valves that connect directly to the fermenter (Fig. 13-6) [52]. All contaminated or potentially contaminated condensate should drain to the biokill system.

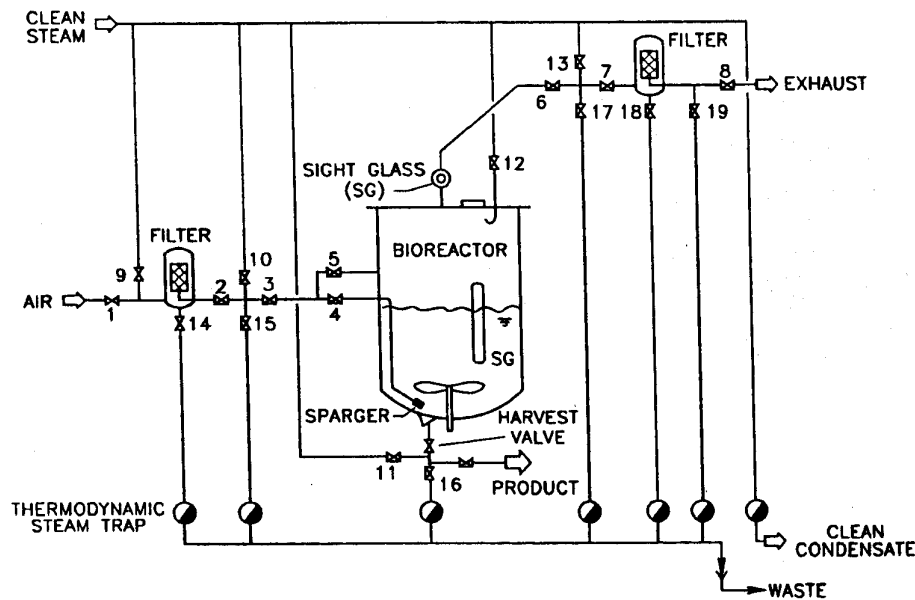


Fig. 13-6. Steam barrier at the harvest valve on a fermenter [52]. During culture, steam is supplied to the outlet side of the closed harvest valve through valve 11. The condensate drains through valve 16 and the steam trap to the biokill system. Inlet and exhaust air is sterilized through submicron absolute filters. A double mechanical seal at the point of entry of the agitator shaft (Fig. 13-4) assures leak-free operation.

Closed systems should be sampled such that exposed surfaces are not contaminated, and no aerosols are released [40]. This requirement must be met at the BL2-LS and higher. Needle-and-syringe sampling through rubber septa does not meet containment requirements. Moreover,

hypodermic needles have been frequently implicated in accidental inoculation of handling personnel. Contained sampling that releases no viable aerosols is detailed in Fig. 13-7 [57]. In contrast, an uncontained sampling system is depicted in Fig. 13-8. The contained sampling principles detailed in Fig. 13-7 can be incorporated in automated sampling devices one of which, available from Bioengineering AG, is shown in Fig. 13-9. The sample container should be opened within a suitable biosafety cabinet.

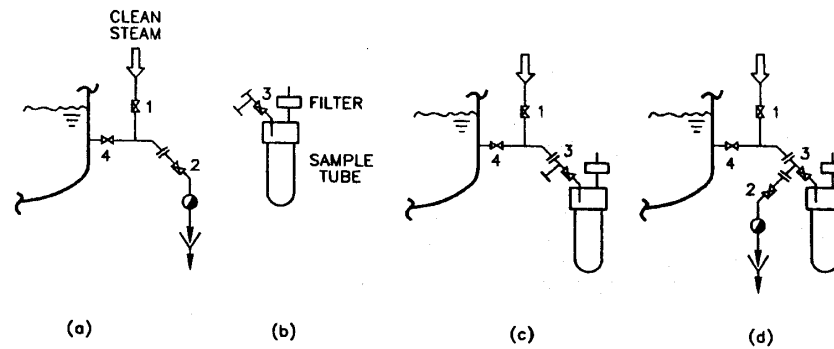


Fig. 13-7. Sterile, contained, aerosol-free sampling [52]. (a) Prior to sampling, the fermenter sampling valve 4 is closed and a clean steam barrier is maintained on the outside of the valve (steam supply valve 1) and condensate is removed (valve 2) to the contained drain. For sampling, valves 2 and 1 are closed, and the system is allowed to cool. The steam trap and valve 2 assembly is disconnected at the sanitary quick coupling. A presterilized (autoclave) sample container (b) having a 0.2 µm breathing filter is attached to the fermenter as in (c). Valve 3 of the sampling device remains closed. The steam trap assembly is reconnected as shown in (d). Valves 1 and 2 are opened in sequence to sterilize the connection using steam at 121 °C for 25 minutes. Valves 2 and 1 are now closed in sequence. After the assembly has cooled, the sample is withdrawn by opening valves 3 and 4. After the sample has been collected, valves 4 and 3 are closed, and the connection is resterilized by opening valves 1 and 2. Upon cooling, the connection is returned to state (a), and the contained sample container is opened in a biological safety cabinet. The system shown is suitable for biohazard containment as well as sampling of bioactive substances that are inactivated by thermal sterilization.

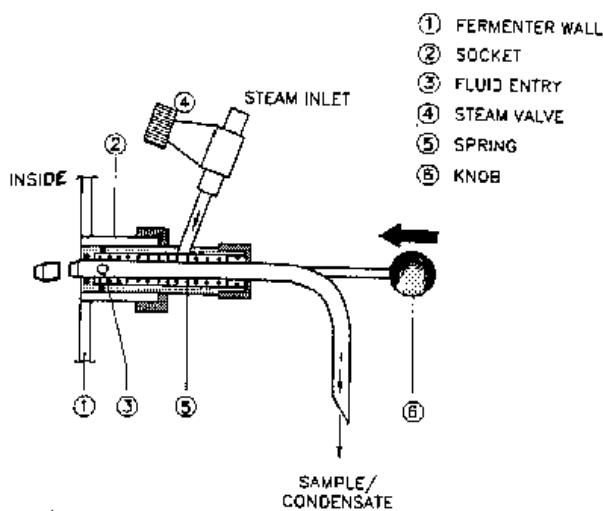


Fig. 13-8. Assembly for uncontained sampling (shown closed). The sampling assembly is mounted in port ② on the wall ① of the fermenter. For sterilization, steam supplied through valve ④ surrounds and enters the sample pipe at ③; condensate issues from the sample outlet. Once the assembly has sterilized, the steam supply is shut off and the system is allowed to cool. The sample is withdrawn by pushing the knob ⑥ to move the fluid inlet ③ into the fermenter. Releasing the knob causes the spring ⑤ to push the valve into closed position. (Diagram courtesy of Bioengineering AG.)

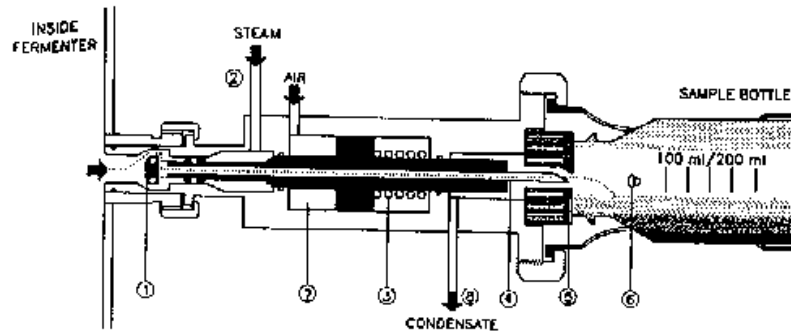


Fig. 13-9. Contained sampling. Normally, valve ① is closed by the action of spring ③ and the needle ④ is retracted into the housing. For sampling, a closed sampling bottle with a rubber diaphragm cap ⑤ and a breathing filter ⑥ is attached to the sampling device. Steam is now run through ② to sterilize the sample path, including the outside of the rubber diaphragm on the sampling bottle. The condensate is withdrawn at ⑧. Once sterilization is complete and the assembly has cooled, valve ① is opened by pneumatic action of compressed air supplied to chamber ⑦. The spring is compressed, the needle moves through the rubber diaphragm into the bottle, and sample flows in. Releasing the air pressure in chamber ⑦ closes the valve by the action of spring ③. The assembly must be re-sterilized and cooled before the sample bottle is removed. The entire sampling operation, including installation and removal of bottles can be automated, and sampling can be done at pre-programmed intervals. (Diagram courtesy of Bioengineering AG.)

The acid and alkali reservoirs in BL3 -LS fermentation plants should be stainless steel pressure vessels that are hardpiped to the fermenter [40]. For lesser level containment, glass reservoirs connected using silicone rubber or other similar tubing and peristaltic pumps are acceptable for pH control. Hardpiping, as opposed to using rapid connection couplings, is preferred for minimizing aerosol generation when high-level containment is necessary [13]. Culture transfer between fermenters should be through permanent hardpiped transfer lines in BL3 -LS facilities [40]. Pertinent transfer practices have been described by Chisti [52]. A transfer system meeting the BL2-LS criteria is shown in Fig. 13-10 [52]. Although the needle-and-diaphragm type of connections are still frequently used, particularly during inoculation of fermenters, latest designs of safety connection devices (Fig. 13-11) have virtually eliminated the need for needle-type connectors.

Fully contained processing within closed equipment is feasible and has indeed been implemented, for example, in production of recombinant human interferon using *E. coli* K-12 [591]. Facilities manufacturing vaccines such as mumps, measles, rubella, varicella, or hepatitis need particular attention to containment [41]. Fermenter design practices noted by Chisti [52,57] are generally sufficient for GLSP and BL1-LS operations, and can be easily extended to BL3-LS level (e.g., use of double mechanical seals; two exhaust filters in series). More general design issues relating to equipment for submerged culture [16,52,57] and solid-state culture [3] have been addressed elsewhere.

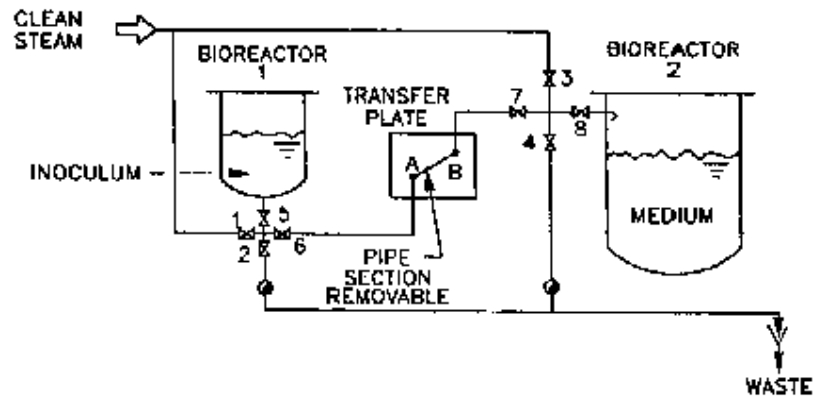


Fig. 13-10. A transfer system meeting the BL2-LS standards [52]. For transferring inoculum from bioreactor 1 to bioreactor 2, a pipe section is connected (sanitary couplings) between points A and B on the transfer plate. The entire transfer pipe between the two fermenters is now sterilized by supplying steam through valves 1 and 3 while valves 6 and 7 are open. The condensate drains through valves 2 and 4. Valves 5 and 8 remain closed. Once the system has sterilized and cooled, valves 5 and 8 are opened, and contents of fermenter 1 are transferred to fermenter 2 by pressurizing (sterile air) vessel 1 relative to vessel 2. Upon completion of the transfer, valves 5 and 8 are closed. The entire transfer line is steam sterilized and cooled prior to removing pipe section A-B. As noted by Chisti [52], the correct sequencing of the various valves is important during sterilization and transfer. All contaminated condensate is piped to the biokill system.

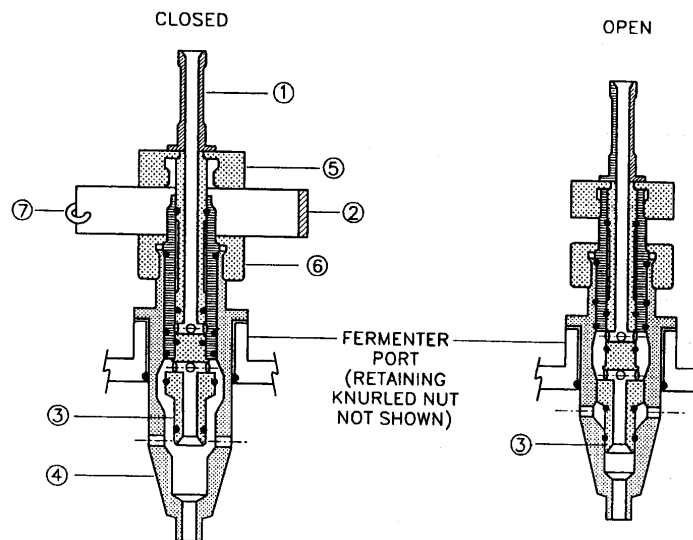


Fig. 13-11. A safety connection valve for coupling inoculum, acid, alkali, and antifoam reservoirs to the fermenter. The nipple ① on the closed valve assembly is connected to transfer tubing attached to an empty reservoir equipped with a breathing filter. The entire set-up (safety connection valve, transfer tube, and reservoir with filter) is autoclaved and cooled. The reservoir is filled with culture fluid inside a biological safety cabinet. The valve body ④ is now installed in a port on top of the fermenter. The fermenter is sterilized after installation of all reservoirs (e.g., inoculum bottle, acid and alkali containers), and cooled. For inoculation, the protective clamp ② is removed and the valve ③ is pushed in to allow pumping of the fluid into the fermenter. The system shown is satisfactory for GLSP processing, but not for higher-level containment.

13.4.4.2 Downstream Processing

During processing, the viable agent should be removed or inactivated as early as feasible in the process sequence. This usually means inactivation upon completion of fermentation, prior to downstream processing. Inactivation of *E. coli* K-12 using sulfuric acid in commercial processing has been noted [59]. Alternatively, cells may be removed by microfiltration or ultrafiltration; however, operations such as centrifugation and macroporous filtration (e.g., filter presses) do not remove all viable particles. Once the viable agent has been deactivated or removed, and the bioproduct poses no special risk, subsequent processing may proceed in open systems [18] with due regard to the GMP-dictated requirements for protecting the product. When inactivation or removal are not feasible (e.g., live vaccines), downstream processing must be contained. Containment of some downstream processing machinery – particularly centrifuges and cell-disruption devices – has been discussed by Deans and Stewart [60], and other commercially relevant mechanical cell-disruption equipment has been described [16,55].

In addition to the viable agent, the physiological and toxicity profiles of products and by-products constrain the choice of process equipment. Certain process schemes may be ruled out by the extent of containment needs and the amount and types of waste streams that would need to be handled. In one case, a difficult-to-contain rotary drum filter that also generated difficult-to-dispose filter aid-mixed solids was replaced with fully contained ultrafiltration for recovery of cefoxitin [61].

Sometimes, primary containment within the process equipment may not be feasible, or contaminated machinery may have to be dismantled – for example, during harvesting of solids from tubular bowl batch centrifuges – and processing within enclosures would be necessary for primary containment.

Isolation of aerosol-generating process equipment in HEPA-filter-exhausted, negative-pressure enclosures is a suitable means of containment [13,41]. The containment room doors should be interlocked with the equipment so that the doors can be opened only when the equipment has stopped running, and sufficient time has elapsed for several space volumes of air to be exhausted from the contained area. Ideally, the equipment itself should be designed for primary containment, and isolation within enclosures should be an added safety measure to contain accidental release from high-pressure devices such as centrifuges and cell disruptors. Performance of the primary and the secondary containment should be validated and monitored regularly.

Multiproduct facilities may require product-dedicated process equipment to eliminate the likelihood of cross-contamination. This is especially so for equipment that cannot be reasonably freed of all traces of a product. For example, chromatography media and membrane filters may have to be dedicated to specific products. The use status – clean, in use, dirty, washed, sterile, etc. – of equipment should be clearly identified at all times. Downstream purification and formulation areas should process only one product at a time. A label control program must be in place.

Handling of a concentrated, bioactive product can be the most dangerous part of bioprocessing [11]. Fine powders are easily aerosolized; hence, handling of freeze-dried cultures or toxins can be particularly hazardous [62]. Because of their aerosol-generating potential, spray-dryers and freeze-dryers require special attention to containment [11]. Freeze-drying of biohazardous substances has been discussed by Adams [62]. As a general rule, automation and fully enclosed mechanized operation can significantly enhance containment while reducing the need for human-process contact.

13.4.4.3 Other Systems

Heat exchange devices are frequently encountered in bioprocessing plants. Corroded heat exchangers (vessel jackets, plate heat exchangers, condensers, shell-and-tube exchangers, etc.) or those with leaking gaskets can contaminate the cooling fluid with the viable agent. Heat exchange equipment should be selected with regard to containment requirements, and cooling water may have to be monitored for contamination.

Clean-in-place (CIP) systems are another common feature of bioprocessing facilities. Automated CIP systems reduce exposure of personnel to hazardous material and assure consistent cleaning. The CIP system design has been treated by Chisti and Moo-Young [56]. For contained facilities, fully closed CIP systems should be used. Attention should be given to cleanability and sterilizability of the CIP system itself, and generation of aerosols should be prevented. In view of the containment and cross-contamination considerations, certain process areas may require dedicated CIP systems.

13.4.5 Personnel Protective Equipment

Certain process operations are difficult to contain, and even the best designed primary containment can fail. Therefore, use of personnel protective clothing appropriate to risk is essential. Lab coats over street clothing are satisfactory for BL2-LS [33] and lower-rated containment areas. Correct gowning room practice is essential to preventing spread of contamination. Protective clothing – gowns, shoe coverings, head covering, face masks, and gloves — should be removed in the proper fashion before leaving the work area [41].

BL3-LS operations, for example, in hepatitis vaccine production, require that personnel remove all street garments down to underwear and don ‘bunny suits’, face masks, and gloves, prior to entering the work area [41]. Protective clothing that completely isolates an individual from the environment is sometimes used [131]. Depending on the characteristics of the product, such protective suites may be required even for BL2-LS or lower-rated processes. This type of isolation is provided by positive-pressure suits with battery-operated, forced air supply drawn from the surrounding environment through HEPA filters. Head-and-shoulder half suits with HEPA filtered air supply can also be used over other disposable protective clothing. The suits are equipped with low-battery alarms.

Use of showers is necessary prior to leaving the degowning area of a BL3-LS biohazard containment zone [323]. Lower level containment areas should be equipped with hand washing facilities near the exit from the area. Sinks with automatic or hand- or foot-operated faucets (taps) are used. Suitable germicidal soap should be provided.

Awareness of the biohazard is important to risk reduction. Consequently, BL2-LS and higher containment areas must display the universal biohazard sign (Fig. 13-12) on the entrance to the contained area. Additional information should include the containment level, the specific biohazard agent, any special entry requirements, and emergency contact details of responsible personnel.



Fig. 13-12. Universal biohazard sign.

13.4.6 Personnel Training

Personnel training is an essential part of safe bioprocessing. Even the best designed facilities, equipment, and practices will fail to provide the intended protection if the operators do not have the knowledge, the training, and the right attitude to personal safety, that of colleagues, the product, and the community. Training should be provided in specific processing methods, operation of equipment, use of personnel protective equipment, gowning practices, aseptic and good microbiological technique (see Table 13-5), containment and biosafety measures consistent with the hazard, emergency procedures, and authorized practices. Written operational protocols should identify the specific, actual or potential, hazards. Established practices should be strictly followed [32]. Personnel should be supervised to assure consistent use of prescribed practices [32,40]. Training in Good Manufacturing Practices is also required, and should be a continual process.

13.4.7 Medical Surveillance

Routine medical surveillance appropriate to risk is recommended. For example, electrocardiograms monitoring of individuals working with cardiotoxic substances [13], and seroconversion of individuals handling antigens. Although medical surveillance by itself does not protect against exposure, surveillance is useful in early detection and treatment. Surveillance also helps in identifying procedural or mechanical lapses. Medical surveillance is especially necessary when pathogenic, potentially pathogenic, or new micro-organisms are being investigated, or when the nature of the hazard is unknown. In addition to the viable agent, the bioactivity of the product or any contaminants may pose a health hazard. Thus, for example, a nonpathogenic recombinant species may pose no risk beyond that associated with the corresponding wild strain, but the product of the inserted foreign gene may be highly bioactive, allergenic, or otherwise toxic.

When suitable vaccines are available, the process personnel as well as those providing support services but not directly working in the process areas (e.g., management, administration), should be immunized [41]. A 'wait time' is necessary for development of immunity. Process workers should show resistance to the infective agent before being allowed into the work area [41]. Furthermore, it should be recognized that vaccination may not guarantee protection against a high dose of the etiologic agent [8].

The workplace should be receptive to reporting of illnesses and accidents. Even apparently minor incidents — for example, being scratched while cleaning a process vessel — should be reported and recorded. Personnel who are ill, and those with open sores and cuts, should not be allowed into critical work areas. Immunocompromised individuals, those with diseases such as cancer and diabetes, those undergoing antimicrobial, steroid or immunosuppressive therapy [8] are especially at risk.

Attention to protection of peripheral support staff, for example the maintenance and cleaning personnel, is especially important because they may not have the knowledge or training for the potential risks [3].

13.4.8 Biowaste

All contaminated liquid, solid, and gaseous waste from a facility must be decontaminated prior to release. Solid waste is generally autoclaved or incinerated. Gases are filter sterilized and/or

incinerated. Liquid effluent is collected into a containment sump and treated through the biokill system. All decontamination procedures should be validated, and the treated material should be examined for sufficiency of kill before being released. The regulations (OSHA, NIH/CDC, EPA, U.S. Postal Service, etc.) relating to disposal and shipping of biohazardous wastes in the United States have been discussed by Tumberg [63].

Effluent from the containment sump may be chemically disinfected or heat sterilized. Sterilization may be batchwise or continuous using direct steam injection or indirect heating. Good mixing of chemical additives and uniform sterilization temperature must be achieved for defined periods. Waste decontamination areas are generally held at negative pressure which is mandatory when decontaminating BL3-LS effluent. There should be provisions for preventing accidental release of untreated material to sewer. For example, a locked effluent drain valve may be employed; the valve is opened only when a treated batch is released. Use of chemical disinfection prior to thermal sterilization is recommended to reduce the hazard in case of inadvertent effluent release (e.g., rupture disc failure). The decontamination process may be automated.

The sump and the sterilization tanks should be vented only through HEPA filters that may have to be heated to prevent condensation. The filtered exhaust from the waste decontamination tank may have to be treated for odor control if odor is a nuisance [33]. Odor may be controlled by incineration, scrubbing, or absorption.

Effluent decontamination has been discussed further by Wirt et al. [54]. Other relevant effluent management issues have been examined by Court [64]. Miller and Bergmann [33] have discussed treatment of BL2-LS effluent. Design features of batch thermal biokill systems have been described by Kossik and Miller [65]. In addition, the hygienic design practices noted by Chisti [52,57] for fermentation plant apply also to biokill machinery.

After decontamination, the biohazardous waste is generally disposed of using the same practices that apply to other nonbiological waste. Sometimes special treatment is necessary to destroy nonviable bioactive or otherwise environmentally harmful substances [63,66,67].

Abbreviations

BLx	Biosafety level x (x = 1—4)
BLx-LS	Biosafety level x (x = 1—3) large scale
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CHO	Chinese hamster ovary
CIP	Clean-in-place
DNA	Deoxyribonucleic acid
EFB	European Federation of Biotechnology
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GILSP	Good industrial large-scale practice
GLSP	Good large-scale practice
GMO	Genetically modified organisms
GMP	Good manufacturing practices
GRAS	Generally recognized as safe
HEPA	High efficiency particulate air
HIV	Human immunodeficiency virus
HVAC	Heating, ventilation and air conditioning
MCB	Master cell bank

MWCB	Manufacturer's working cell bank
NIH	National Institutes of Health
OECD	Organization for Economic Cooperation and Development
OSHA	Occupational Safety and Health Administration
PVC	Poly(vinyl chloride)
USDA	United States Department of Agriculture
WHO	World Health Organization

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