MEASURING AND MONITORING CONTAINMENT IN BIOPROCESS EQUIPMENT

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The current regulatory requirements concerning biological safety in industrial biotechnology are described with particular emphasis upon the need to test and monitor equipment and the airborne environment. Techniques for measuring and monitoring equipment containment are described together with the need to coordinate standards development in biotechnology.

Aerosols, Biosafety Regulations, Bioprocessing, Containment, Leak-Testing

INTRODUCTION

The safe use of biotechnology, has in recent years, received greater attention due principally to the introduction of powerful new techniques such as genetic modification of micro-organisms. The vast majority of industrially important modified micro-organisms are of intrinsically low risk to both the workplace and natural environments. Attention to containment during processing is still justified however for several reasons. For example:

- Many of the processes produce biochemicals which can cause occupational diseases or environmental difficulties,
- An environmental risk assessment may be required to satisfy regulatory authorities that releases or discharges will not harm the environment,
- Public and process operator perceptions and concerns exist about possible effects by release of modified micro-organisms.
- Companies may not wish competitors to obtain their genetically modified micro-organism by sampling outside the factory,

Within this paper, the regulatory requirements are reviewed in relation to safe biotechnology. The paper then focuses upon validation and monitoring techniques which form an important part of the regulatory requirement.

REGULATIONS AND GUIDELINES

National and international biosafety activity has largely focused on defining hazard groups of micro-organisms and the corresponding containment levels which need to be used.

The European Federation of Biotechnology (EFB), produced a four part classification scheme summarised on table 1.

TABLE 1 - EFB proposed classification scheme for micro-organisms according to pathogenicity (adapted from Vranch, (1) and Frommer and Krämer, (2))

EFB Class	RISK	DESCRIPTION
I	*Harmless*	Not identified as causative agents of disease in man. Offer no threat to environment.
II	*Low Risk*	May cause disease in man and therefore might offer a hazard to laboratory workers. Unlikely to spread in the environment. Immunoprophylactics are available and treatment is effective.
III	*Medium Risk*	Offer a severe risk to the health of laboratory workers but a comparatively small risk to the population at large. Immunoprophylactics are available and treatment is effective.
IV	*High Risk*	Cause severe illness in man and offer a serious hazard to laboratory workers and to the population at large. In general, effective immunoprophylactics are not available and no effective treatment is known.
A special category Group E was also introduced by EFB to classify micro-organisms that offer a more severe threat to the environment rather than to man. They may be responsible for heavy economic loss.		

TABLE 2 - Comparison by authors of containment levels for micro-organisms.

EFB	OECD (V,F)	ACGM		NIH		NIH (K)	ACDP (C)	CDC (K)
		Lab (C)	Scale up (C)	Lab (F)	Scale up (V)			
I	GILSP	1	GLSP	P1		BSL-1	1	1
II	1	2	LS 1	P2	BL1-LS	BSL-2	2	2
III	2	3	LS 2	P3	BL2-LS	BSL-3	3	3
IV	3	4	LS 3	P4	BL3-LS	BSL-4	4	4

C - A. Cottam (HSE, private communication, 1990)
F - Frommer and Krämer (2)
K - Kearns (3)
V - Vranch (1)

ACDP - Advisory Committee on Dangerous Pathogens (UK)
ACGM - Advisory Committee on Genetic Manipulation (UK)
CDC - Centres for Disease Control (USA)
GILSP - Good Industrial Large Scale Practice
NIH - National Institution of Health (USA)
OECD - Organisation for Economic Cooperation and Development (Paris)

The EFB classification covers all micro-organisms including those which have been genetically modified. Confusion often arises when corresponding national and international containment levels are compared. For example, table 2 compares containment levels using the EFB categorisation as the organism hazard class.

The Industrial Biosafety Project commissioned a questionnaire on fermenter integrity. It was clear from the industrial respondents that the variety of containment codes and levels did cause confusion and there was a need for an accepted standard nomenclature for containment levels.

For Genetically Modified Micro-organisms (GMMOs), the OECD guidelines (4) provided the initial basis for large scale operations. In the UK for example, the UK ACGM note 6 (5) contains many of the concepts in the OECD study.

At the European level, the recent EC directive on contained use (6), also has recognisable sections of the OECD study. Micro-organisms are divided into two broad hazard categories, Group I and Group II together with two types of operation, type A and type B. Type A operations are any operations used for teaching, research, development or non-industrial or non-commercial purposes and of a small scale (10 litre culture volume or less). A type B operation is anything else. In terms of the broad hazard categories, Group I micro-organisms correspond to the Good Industrial Large Scale Practice (GILSP) of the OECD and ACGM note 6 (GLSP). Group II micro-organisms are higher risk than group I and encompass the containment categories relating to OECD levels 1-3. Thus group II micro-organisms are those which require containment measures such as minimising release at OECD level 1 or preventing release at category levels 2 and 3. For group II micro-organisms, effluents also have to be treated and tested by validated means. The directive specifically states that containment measures shall be reviewed by the user to take into account new scientific or technical knowledge relative to risk management, and treatment and disposal of wastes.

Since the vast majority of industrially important GMMOs are of *intrinsically low risk and subject to the lowest containment category* (GILSP), there is obviously great interest in the definition and implementation of GILSP. Unfortunately, GILSP is the subject of wide debate on its interpretation and implementation. For this reason, the elaboration and illustration of GILSP scientific criteria and requirements was a priority area in OECD's follow up activity (Ager, (7)). OECD asked member countries via their regulatory agencies, to provide examples of approved GILSP operations. The results of this OECD initiative should be published during 1991.

The key requirement for all containment levels including GILSP is to apply the fundamental principles of *good safety and occupational hygiene*. This was recommended by OECD in 1986 whilst the recent EC contained use directive (6) stated the principles *should* be applied for both group I and II micro-organisms.

The principles of good safety and occupational hygiene include *inter alia* keeping workplace and environmental exposure to any physical, chemical or biological agent to the lowest practicable level, using engineering control measures at source and ensuring that the measures and equipment are tested adequately and maintained. The principles also include testing for the presence of micro-organisms outside the physical containment where

necessary. Environmental considerations should also be taken into account.

Within the UK, the good safety and occupational hygiene principles have similarities with the Control of Substances Hazardous to Health (COSHH) regulations. Indeed, Kearns (3) commented that it was debateable whether any micro-organism can be classified as completely harmless since prolonged exposure could lead to allergenic reactions in sensitised persons. Such considerations are covered in the UK by the COSHH regulations and Kearns argued that these regulations could mean GILSP ceased to be relevant.

A further EC directive is due to be implemented concerning the protection of workers from the risks related to exposure to biological agents in the workplace. The scope of this directive is broader including GMMOs and naturally occurring biological agents which are either known to be harmful or suspected of being so (Tachmintzis, (8)).

It is evident therefore that increasing emphasis is being placed on reducing or minimising material loss during bioprocessing even for "harmless" micro-organisms. The principles of good occupational hygiene and safety imply that equipment design and operation are important. It follows that methods need to be developed for measuring and monitoring containment to demonstrate to regulatory bodies that equipment is suitable for operation without risk to the operator and the environment.

CURRENT PRACTICE.

A serious problem in developing quantitative monitoring in the biotechnology industry is a lack of information on either occupational or environmental exposure limits. For micro-organisms, only one investigator has recommended an exposure limit. Clark (9) suggested, following an epidemiological study around a sewage works, that the work environment concentration of viable gram negative bacteria should not exceed $1000/m^3$. This figure was derived from an endotoxin (cell wall component of all gram negative bacteria) limit of $0.1\ \mu g/m^3$. Likewise, only one exposure limit is available for biological products. For proteases (detergent enzymes) exposure limits of $0.4\ \mu g/m^3$ have been set (Behizad *et al* (10)). For comparison, the limits on an 8 hour personal exposure to total inhalable dusts is $10\ mg/m^3$ (HSE, (11)), ie 25,000 times higher than protease.

In contrast to designing to comply with set exposure limits, the approach to date has been to implement containment principles to correspond to the risk of the micro-organism and its product. For example, at OECD category level 1, equipment should be designed to *minimise release* (without any specific figures on release of material). At level 2, *prevent release* is required and it might be inferred that this corresponds to an exposure level tending towards zero. At level 3, the containment requirements to *prevent release* are higher. The mechanical design interpretation of levels 1, 2 and 3 is shown schematically in figure 1. For static seal arrangements, Chapman, (12) suggested the single seal arrangement was suitable for level 1, a double seal for level 2 and a double seal with steam flush for level 3. Besides mechanical design, the key to maintaining the integrity of the systems is by regular planned/preventive maintenance with testing and monitoring as appropriate in accordance with the fundamental principles of good safety and occupational hygiene. Thus having designed or installed the equipment, it should be tested before use and then monitored to ensure it is operating to the required level.

Elliot *et al* (13) also recommended that environmental monitoring can prove

a cost effective alternative to monitoring of worker body fluids.

At GILSP levels, operators are principally concerned with prevention of product contamination. With fermentation processes for example, the Industrial Biosafety Project carried out a survey on current practice for monitoring and measuring fermenter integrity. No company recommended or used integrity testing with containment measurement as a primary objective but rather as a complementary consideration alongside the validation of sterile operation.

The nature of tests performed for both sterile operation and containment is summarised in table 3.

TABLE 3 - Nature of fermenter integrity tests performed routinely on surveyed industrial sites.

Containment	%	Sterility	%
Pressure test	52	Pressure test	20
Air monitoring	28	Air monitoring	4
Swabs	12	Culture methods	48
Nothing	8	Nothing	8
		Sterile hold and control	16
		pH, dissolved oxygen	4

The results presented in table 3 show that pressure testing is popular for both containment and sterility validation. Air monitoring is also a significantly used means of containment testing.

TYPES OF VALIDATION AND MONITORING METHODS.

Tests can be useful for prechecking equipment prior to operation, or monitoring during operation, or sometimes both. Both physical and biological methods are available, many of which have been evaluated and developed by the Industrial Biosafety Project.

Pressure hold test

As indicated above, pressure testing is widely practised in the biotechnology industry. The criteria for passing or failing a test is somewhat arbitrary and often depends upon operator's experience and the practicalities of the test. The test is relatively simple and involves pressurising the vessel to a given pressure, for example with air, then noting any change in pressure in a specified time period. Surprisingly very few fermenter manufacturers provide details of a pressure test. An exception is LH fermentation who recommend that a pressure test is carried out prior to every fermentation. The handbook for a LH Fermentation 140 litre cell culture vessel installed at the author's laboratory states a test pressure of 20 psig (1.36 bar) should be maintained for at least 3 hours. Should the pressure loss be greater than 0.5 psi per hour (34 mbar/hour), LH recommend checking for leaks using 0.5% Savlon solution to check for bubble emission, then making good any identified leaks.

At Warren Spring Laboratory, we have investigated leak testing techniques applied to fermentation equipment. The pressure loss of a vessel depends upon many factors and to work towards a common test criterion, these factors need to be combined. The initial approach taken was to reduce the data from a pressure test to an equivalent orifice size leak.

Pressure loss through an orifice as a function of time can be represented by equation (1).

$$P = P_0 \exp\left(\frac{-B\, C_D\, d^2\, t}{V}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

The constant B is calculated from equation (2).

$$B = \frac{\pi\, R\, T}{4\, m}\left(\frac{\gamma}{P_a\, v_a}\left(\frac{2}{\gamma+1}\right)^{\frac{\gamma+1}{\gamma-1}}\right)^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The value of the approach is that the equivalent orifice diameter d can be obtained for all fermenter vessel sizes to provide an overall index of its leak tightness. The magnitude of the diameter could provide a criterion for passing a pressure test. Using d as a criterion, the sensitivity of the pressure transducer and the duration of the pressure test can also be determined.

The loss of pressure with time follows an exponential decay in accordance with equation 1. It is possible to fit a best line of the test data to calculate d. This has been undertaken at Warren Spring Laboratory with the aid of a simple computer programme. The coefficient of discharge values were determined experimentally using orifice plates of various diameters. Alternatively the initial and final pressures can be used. Inserting the physical values for the test gas in equation (2) and rearranging equation (1) gives

$$d = \left(\frac{V \ln\left(\frac{P_0}{P}\right)}{343\, t}\right)^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

for helium test gas (SI units)

$$d = \left(\frac{V \ln\left(\frac{P_0}{P}\right)}{120\, t}\right)^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

for air or nitrogen test gas (SI units).

Thus using the criterion of LH fermentation of 0.5 psi per hour (34 mbar/hour), the equivalent orifice leak size is approximately 70 μm based on a 140 litre vessel. If 70 μm were taken as the pass/fail criterion for example, then for a 4 m^3 vessel and rearranging equation (4), a pressure

test of one hour would need pressure transducers to indicate a change in pressure to 99.94 % of the initial pressure eg 2.000 bar to 1.998 bar, 20.000 psi to 19.989 psi. This illustrates that pressure tests of large vessels may be of limited value unless the pressure transducer is sufficiently sensitive. Specialised leak testing monitors based on accurate pressure decay measurement are available (such as the Qualicheck® 160, Analytical Instruments, Cambridge) and their use should be considered. Many biotechnology and pharmaceutical companies are likely to have membrane filter validation instruments such as the Sartorius Sartocheck® or the Pall Forward Flow Tester. These could also be used for pressure testing but their limited sensitivity would restrict their application to smaller sized vessels.

The equivalent orifice leak represents, in general, the worst case in terms of breach of containment. Leaver and Stewart (14) summarised their studies to date on the release of liquids containing micro-organisms through orifices of diameters 35 to 100 μm and 0.11 mm length. The studies have continued with an investigation of fermenter head space aerosols through similar leaks. The ultimate aim of the work is to set a criterion for the pressure test related to likely aerosol release which in turn must be dependent on set exposure limits. In terms of liquid discharge, the 70 μm criterion currently seems a reasonable figure to *minimise release* since many leaks of 50 μm and below tend to become sealed relatively quickly by the microbial suspension. However, this criterion needs to be used with extreme caution since more investigation is required.

Leak Location

Should a pressure test reveal a significant leakage, then the leaks can be traced using a suitable technique. In the survey of fermenter users, soapy water appeared to be a popular choice. Helium detectors were reported to be occasionally used by one company. The latter technique is a highly sensitive means of leak location and in conjunction with a helium pressure test was demonstrated by Leaver and Stewart (14) on behalf of the Industrial Biosafety Project to locate misshapen and worn fermenter couplings. Sulphur hexafluoride (SF_6) leak detectors offer a less expensive and less bulky option to helium detectors. These are used routinely on high containment fermenters at *PHLS-CAMR* Porton Down (Hambleton *et al*, (15)). Another potentially useful technique, which practitioners should consider, is the ultrasonic detector. This can be used to complement the gas pressure hold test. Leakage is readily detected by the ultrasonic sounds set up by gas discharge. The detector was recently evaluated for the Industrial Biosafety Project. The advantages of the detector include relatively low cost (£500-£1000) and it can be used to monitor leakage during operation, in addition to testing before equipment operation.

Air Monitoring

The most likely route of occupational exposure in biotechnology is via aerosols generated by breaches of containment during cell growth in bioreactors and subsequent processing to separate, concentrate, and purify the desired product (Topping, *et al* (16). Hence aerosol measurement is particularly relevant to both assess and monitor containment. It is impossible to describe, in this paper, all the devices and developments in aerosol measurement but some of the devices that have been applied to biosafety are mentioned here.

Aerosol samplers can be divided into those which use an electronic counting

principle such as light scattering and those which collect the aerosols for further analytical determination such as microbial culture.

Electronic devices give near instantaneous readings of aerosol. They are commonly used for monitoring clean room environments and provide total particle counts often with size fractionation. They cannot discriminate particle types so results of monitoring have to be treated with caution to ascertain whether the aerosol is the result of a release of aerosol from the equipment being monitored or tested. Stewart and Deans (17) used a TSI laser light scattering monitor to measure the containment of a cell disruptor. The equipment was surrounded by a cabinet supplying HEPA filtered air, so that any aerosol detected was the result of a breach of containment.

Many bioprocess streams consist of significant quantities of dissolved salts which are highly electrically conductive. Measurement of electrical conductivity is a simple yet effective means of measuring release. A range of air samplers are available which collect the aerosol into liquid media. When coupled to a conductivity probe, an on-line device can be operated. Stewart and Deans (17) also used this technique for measuring the cell disruptor containment and were then able to report spray factors as a means of characterising aerosol release. Conductivity measurement for monitoring (bio)aerosol release has an additional advantage since the natural workplace in general is relatively low of salt aerosols and hence containment breach can be readily detected under workplace operating conditions.

Aerobiological samplers have been used for many years to record the level of micro-organisms in the air. Martinez *et al* (18) used a two stage Andersen Microbial Sampler to monitor the environmental air quality from "walk through" surveys undertaken at six fermentation plants. The strategy was to record background levels of micro-organisms which were of the order of 50-120 Colony Forming Units (CFU)/m^3. These were compared with counts taken near processing operations with micro-organism levels of the order of 200-1600 CFU/m^3. From the surveys, the authors were able to make suggestions to improve the operating practice. Bennett *et al* (19) used slit samplers and Andersen Microbial Samplers to record aerosol release from bioprocessing equipment. Tubular bowl centrifuges were found to be particularly troublesome. High levels of bioaerosols between 50,000-90,000 CFU/m^3 were recorded, with 90% being below 3 μm which are capable of being inhaled into the gas exchange regions of the lung.

Aerobiological samplers, using conventional culture techniques, require up to 48 hours for the microbes to be enumerated after incubation. This may be a problem if corrective action to a process needs to be implemented. Salusbury *et al* (20) demonstrated the value of rapid microbial techniques with an aerobiological sampler. Measuring the bacterial cell ATP (adenosine triphosphate) enabled results to be obtained within 2 to 30 minutes depending upon the microbial concentration.

Environmental monitoring for biochemicals has also received attention. Behizad *et al* (10) developed a prototype on-line sensing technique for protease and other biochemicals. The monitor was tested in a detergent factory environment and demonstrated to be sensitive and rapid. Thus it could be a useful development enabling enzyme airborne concentrations, well below the 0.4 $\mu g/m^3$ exposure standard described above, to be detected.

FUTURE DEVELOPMENT.

Standardisation

It is clear that current methods and practice of containment design, measurement, and monitoring are far from being standardised. CEN, the European Standards Committee has recently set up a technical committee (TC 233) to develop good practice guides in biotechnology. Four working groups have been established including large scale biotechnology and bioprocess equipment. Part of the brief of the equipment working group is concerned with equipment performance standards including sterilisation and leak tightness. Thus the containment aspects fall within the remit of this working group. In the USA, both the ASTM (American Society for Testing and Materials) and the ASME (American Society of Mechanical Engineers) have formed working groups to develop standards in the biotechnology industry.

The exact form of the emerging standards are currently hard to foresee. It is appropriate, however, that workable standards are developed and participation by liaison with the UK representatives is important to achieve this. Currently, the BioIndustry Association (BIA) represents the UK bio equipment interest and it is through this route that the author is liaising.

Personal Monitoring

A different CEN committee (TC137, working group 3) is concerned both with airborne particle size fraction definitions with respect to human health problems and also methods of how these particles should be monitored. The focus is on personal monitoring techniques. Epidemiological studies can only be effectively undertaken by sampling the operator's personal exposure. Thus in the context of biotechnology, personal air monitors suitable for sampling bioaerosols may need to be developed. Current technology, widely used for dust monitoring eg HSE (21), is not suitable for monitoring personal exposure to micro-organisms for example. Further developments are therefore needed to develop the personal sampler technology to obtain representative monitoring of personal bioaerosol exposure.

CONCLUSIONS

Biotechnology safety considerations have received greater attention in recent years due to the introduction of powerful new techniques. Currently, exposure limits for biochemical products and micro-organisms, have little application for compliance with occupational and environmental safety. Equipment design and monitoring principles are the current means of compliance. The principles of good safety and occupational hygiene apply to all processes. This includes the need to test equipment and monitor the environment.

With respect to fermentation processes, pressure testing and air monitoring are widely carried out. The criterion for passing a test is somewhat arbitrary. It is suggested that the equivalent orifice diameter could form the basis for a standard pressure test. This would dictate how the test is designed particularly when large fermenter vessels are involved.

A range of techniques based on aerosol measurement are available. Personal monitoring for micro-organisms is currently not available however. Standards development in biotechnology are currently baing debated in

Europe and the USA and workable standards depend upon the active participation of the various biotechnology sectors.

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SYMBOLS USED

B = constant dependent on gas used (m/s).

C_D = coefficient of discharge (-).

d = orifice leak diameter (m).

m = gas molecular weight (kg/kmol).

P = absolute pressure in vessel (Pa).

P_a = Ambient pressure, 10^5 (Pa)

P_0 = initial pressure at t=0 (Pa).

R = gas constant, 8314 (J/kmol K)

T = absolute temperature (K).

t = time (s).

V = vessel volume (m^3).

v_a = gas specific volume at ambient temperature and pressure (m^3/kg)

γ = gas specific heat ratio, 1.67 for helium, 1.4 for air, (-).

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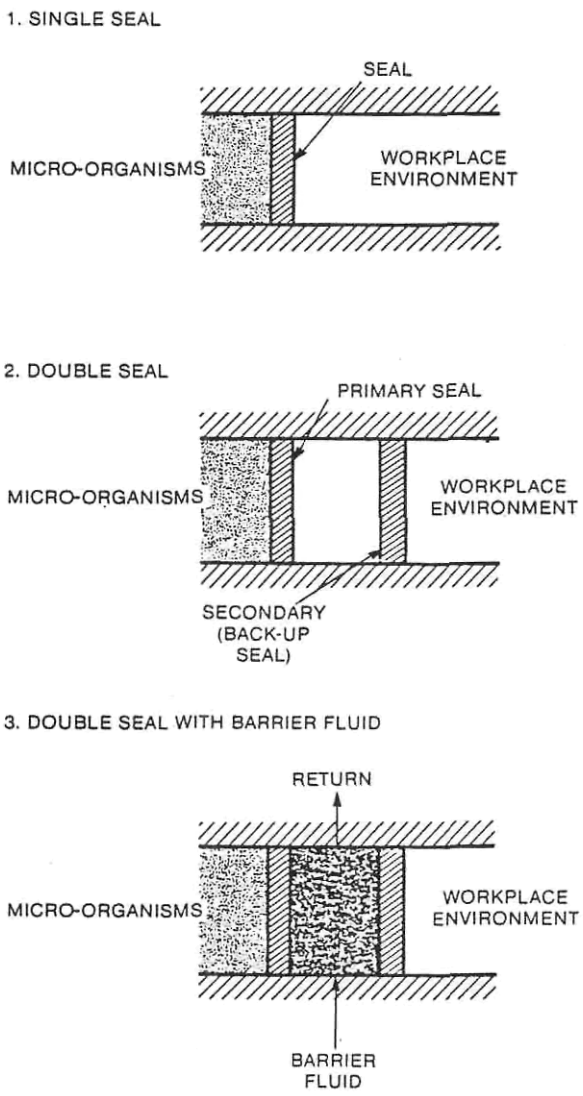

Figure 1 Mechanical design interpretation of containment levels 1, 2 and 3