Microbial Population Balancing for Containment Specification

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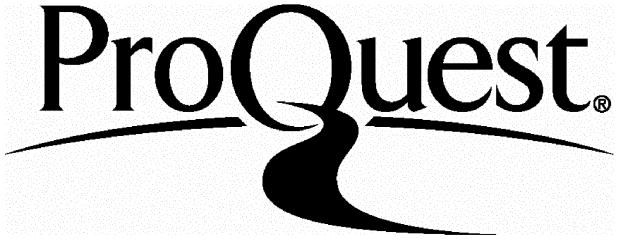

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Abstract

The aim of this project is to quantify the numbers of microorganisms released from bioprocessing equipment during its normal operation. This release can occur either in the form of a liquid or an aerosol. Experimental work has concentrated on sampling and detecting aerosol releases of microorganisms.

A population balancing system which allows the consistent quantitation of a microbial aerosol has been devised. This mass balancing system consists of the release and capture of a known aerosol in a contained environment. The aerosol was produced using an atomiser or a Collison Nebuliser. This aerosol was released into the Bassaire cabinet (volume 360L) and collected using an Aerojet-General cyclone sampler. The number of microorganisms that fell to the floor of the cabinet was also enumerated using Petri dishes containing a liquid. The total number of microorganisms collected from both the air in the cabinet and from the floor were enumerated under a microscope, using a counting chamber.

This mass balance was carried out using both *Saccharomyces cerevisiae* and *Escherichia coli*. The cell recovery in the cyclone, i.e. the % of those cells released that were captured, was found to depend upon the released cell concentration and the composition of the suspending medium. It was possible to consistently recover over 50% of the cells released into the cabinet.

The mass balance was then carried out exactly as before, but in a larger, soft film cabinet (volume $8.3m^3$). This cabinet was used because it was large enough to hold a piece of bioprocessing equipment, such as an APV 30CD high pressure homogeniser. Since the aim of this investigation was to quantify release from such a piece of equipment, the cabinet that it was located in should first be calibrated. Cell recoveries in the cyclone were found to be around 10-20%. Again, these recoveries were dependent upon the released cell concentration, but found to be consistent on repetition.

The Aerojet-General cyclone was then used to sample the air in the cabinet whilst the homogeniser processed a suspension of *S. cerevisiae*. It was not possible to detect released *S. cerevisiae* cells in the cyclone using the counting chamber. However, when the homogeniser was used to process a recombinant *E. coli* suspension, released cells could be detected in the cyclone. This was because the

Polymerase Chain Reaction (PCR) could be used to detect a DNA sequence present only in the recombinant *E. coli*. The microbial release was dependent upon operating time and pressure.

The difficulty experienced in aerosolising microorganisms from a liquid suspension was investigated further by using the cyclone to sample the headspace air above a fermenter. Released *S. cerevisiae* cells could not be detected using the counting chamber. However, using PCR, it was possible to detect *E. coli* cells in the fermenter headspace. It is suggested that *S. cerevisiae* cells are less easily aerosolised from a liquid surface than *E. coli* cells.

This study shows that during the normal operation of bioprocessing eqiupment such as a homogeniser and small scale fermenter, microbial release can be detected at a very low level only (approx. 10^6 *E. coli* cells.h^{-1}). These results have important implications for both equipment design for contained operation and GMO legislation.

Some suggestions for further work which could be carried out include: alterations to the cyclone design to improve cleanability, reduce liquid losses in the air stream and to achieve more efficient washing of collected cells from the cyclone walls; a more comprehesive analysis of the particle size distribution in the aerosols; an investigation of the off-gas from a recombinant *S. cerevisiae* fermentation; an investigation into the effects of operating pressure on microbial release from the 30CD homogeniser and finally the application of the microbial population balancing technique developed here together with Computational Fluid Dynamics to build up a picture describing the destination of release cells in a processing environment.

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Abbreviations

ACBE	Advanced Centre for Biochemical Engineering
ACGM	Advisory Committee on Genetic Manipulation
ACGIH	American Conference of Governmental and Industrial Hygienists
ADP	Adenosine DiPhosphate
AEC	Adenylate Energy Charge
AGI	All Glass Impinger
AMP	Adenosine MonoPhosphate
AMS	Anderson Multistage Sampler
APS	Aerodynamic Particle Sizer
ATP	Adenosine TriPhosphate
BSA	Bovine Serum Albumin
BTC	Bioaerosol Test Chamber
CD valve	Cell Disruption valve
cmtB1	A primer that binds to a site on the cmtB gene
CSS	Casella Slit Sampler
dNTP	deoxyNucleoside TriPhosphates
DOT	Dissolved Oxygen Tension
DSP	Downstream Processing room of the ACBE
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
G(I)LSP	Good (Industrial) Large Scale Practice
GMO	Genetically Modified Organism
HASAWA	Health and Safety at Work Act (1974)
HAZOP	Hazard and Operability Study
HEPA	High Efficiency Particulate Air Filter
HEPES	N-[2-hydroethyl] piperazine-N'-[2-ethanesulphonic acid]
ISO	International Standards Organisations
KM1 and KM2	Kanamycin primers
LF1	A primer
LPC	Laser Particle Sizer
LSCC1/2/3	Large Scale Containment Category 1/2/3
M13F and R	A primer set
MPN	Most Probable Number
nd	not done
OECD	Organisation for Economic Cooperation and Development

PCR	Polymerase Chain Reaction
RO	Reverse Osmosis
RODAC	Replicate Organism Direct Count
SAS	Surface Air System
SOP	Standard Operating Procedure/Practice
TBE	Tris-Borate-EDTA buffer
TK	Transketolase
tftc	too few to count
UM	Unmodified
YEP	Yeast Extract-Peptone-glucose broth

CHAPTER ONE: INTRODUCTION

1.1 Importance and Aims

1.1.1 The Significance of Microbial Population Balancing

It is widely recognised that although the majority of large scale bioprocesses are safe (WHO, (1984), ACGM Note 6, (1987)), some will need containment. This may be because the process involves a pathogenic organism or one that has been genetically manipulated. This containment is required because the release of the process organism may be hazardous to man or the environment and is a legal requirement (Health and Safety at Work Act, (1974) and Genetically Manipulated Organism (Contained Use) Regulations, (HSE, 1992)).

Current UK Legislation states that, depending upon the containment category that a process is operated at, a microbial release from the process must either be minimised or prevented. These two words "minimise" and "prevent" are subjective and would be easier to interpret if they were supported by numbers. Ideally, these numbers should take the form of a release limit. However, at present, there is little data available on the release of microorganisms from bioprocesses. Industry has reacted to this legislation (HSE, 1992) by applying more containment measures than are necessary, which inevitably will lead to higher plant costs and processes that are difficult to work with. Indeed, a recent report by the Select Committee on Science and Technology (1993) expressed concerns that the Contained Use Regulations would lead to a lack of competitiveness in the UK Biotechnology Industry.

Previous studies have monitored microbial release from bioprocesses (Dunnill, (1982), Lawrence and Barry, (1982), Ashcroft and Pomeroy, (1983), Cameron *et al.*, (1987), and Tinnes and Hoare (1992)). However, all of these, except that of Ashcroft and Pomeroy (1983), have been of a qualitative nature. These qualitative studies have not related the number of cells detected to the number of microorganisms that were initially released from the process. Since these two numbers often differ considerably, it is desirable to know the number of cells in the release itself.

A microbial release may occur either incidentally during normal operation of the process, or as part of an accident due to mechanical failure or operator error.

Dunnill (1982) and Lawrence and Barry (1982) describe release during the normal operation of centrifuges. Ashcroft and Pomeroy (1983) monitored aerosol release from a fermenter which they subjected to accidental damage. Cameron *et al.* (1987) monitored release from a fermenter during normal sampling and also when a sample pot was deliberately overfilled. A microbial release was only detected during overfill of the sampler. Tinnes and Hoare (1992) could not detect release from a disc-stack centrifuge during normal operation; however when a hose connection was loosened, microbial release was detected. In most of these studies, it is the concentration of organisms detected that is quoted, not the number of organisms released.

The above discussion highlights the need for a quantitative approach to release measurement during the normal operation of bioprocesses.

1.1.2 Aims of the Study

This study aims to achieve the following:

1. Relate the number of cells detected in an aerosol release to the number actually released from the process. This should be carried out as a microbial population balance in a contained environment and should be demonstrated to be reproducible. This procedure is also referred to as "mass balancing" throughout this thesis. However it is only microbial cells that are balanced, not the whole mass of the release (the release may also include suspended solids from broth etc.).

2. Carry out a microbial population balance on a release from a piece of bioprocessing equipment during normal operating conditions.

3. Relate the results of 2. above to equipment design for contained operation and regulatory requirements.

1.2 Release

1.2.1 Definition and Form

In the context of biosafety, release can be defined as the escape or setting free of microorganisms or their products, from containment, into the environment. The environment is either the processing plant or the external area surrounding the plant. Strauss's definition is different: "Release occurs when a specified number of microorganisms are no longer subject to containment.". Strauss (1987) took data from Lincoln *et al.,* (1985) and estimated that $2 \times 10^8 - 4 \times 10^9$ microorganisms a day are released from a BL1 laboratory (the lowest containment category for work with rDNA in the US). This value does not allow for day to day variation in emissions. Strauss would like to see this estimate used as an emissions standard, to determine whether an environmental release has occurred.

Lincoln *et al.,* (1985) simulated the release of microorganisms during different genetic engineering procedures. Using these simulations Strauss estimated the number of microorganisms released per day from laboratories. The computer simulations looked at 3 different operations:

1. Insertion of a rDNA molecule into a microorganism and selection of colonies for incorporated DNA.
2. Growth and harvest of 10L of each of 3 selected colonies.
3. Growth in a 250L fermenter.

Escape routes considered included aerosols, technician's hands and clothes, inhalation and ingestion by technicians, and liquid and solid waste. The dominant methods were thought to be by aerosolisation and liquid waste from large culture volumes. Strauss explained that because most labs work with many different genetically engineered strains, the total number released into the environment on any day is likely to be much higher than $2 \times 10^8 - 4 \times 10^9$. He suggests that this emissions limit be per strain.

1.2.2 Release of Microorganisms from Bioprocessing Equipment

Aerosols are produced when a force is exerted on a liquid. If sufficient force is exerted small droplet aerosols are formed, which may have diameters small enough to gain access to the lower the respiratory tract. Aerosols are generated from any

liquids involved in fermenter inoculation procedures, sampling and vessel overpressure situations. Centrifuges and homogenisers are two commonly used pieces of equipment with the potential to produce aerosols of allergenic biological material. Accidental spills often occur due to leaks in fermentation equipment (seals, head plates, quick disconnect fittings and probe penetrations) during the transfer of process liquids and in recovery operations such as filtration and crystallisation.

As explained in section 1.1.1, releases can be either incidental or accidental. Most of the literature published on this subject refers to simulated accidental releases. Some examples of this are discussed in this section although the design aspects are discussed in section 4.1.3.

Ashcroft and Pomeroy (1983) carried out experiments to simulate accidents which may occur during the large scale production of microorganisms. The accidents concerned fermenter operation and included:
1. Fermenter operation without the air outlet filter (At GILSP the outlet air would not normally be filtered).
2. Failure of antifoam supply.
3. Failure in pipework.
4. Vessel breakage.
The accidents resulted in the following contamination: Air outlet filter failure resulted in gross contamination of surfaces up to 1 m from the vessel. Failure in the antifoam supply resulted in the culture fluid being forced through the air outlet filter, settle plates showed widespread contamination of the floor with up to 40 particles.cm^{-2}. Pipework failure was simulated by removal of the fermenter drain plug, this caused the contents of the vessel to surge out under pressure from the agitator and compressed air supply, causing widespread visible contamination of the floors and walls. In the last experiment the glass vessel was struck by a missile which caused immediate collapse of the vessel and propulsion of the culture fluid onto the walls, floor and ceiling.

Ashcroft and Pomeroy (1983) found that most of the culture fluid expelled from the fermenter during these experiments was disseminated in large droplets which rapidly deposited on surfaces. They also concluded that the simulated processes selected were inefficient in creating the shear forces required to produce a stable aerosol.

Cremer and Warner (1982) calculated the probability of pipeline breakage in the chemical industry for a pipeline outdoors at 10^{-7}.year^{-1}. For pipeline sections of 10m with a diameter of 50mm or 50-150mm, the probability of failure is 2 x 10^{-8} and 3 x 10^{-9}.hour^{-1}. In bioprocessing, with watery fluids, without aggressive substances, at lower temperatures and pressures, the probability of failure is much lower. Leakage tends to be much more important than breakage because small leakages tend to happen first. The amount of aerosol produced from a liquid leak depends on the fermentation stage, i.e. the cell concentration in the liquid, the size of the opening, the velocity of the escaping liquid and obstacles that the liquid stream meets. A leak from a large opening will produce less aerosol than a small leak. If the escaping liquid hits the surface of other apparatus a secondary aerosol may be produced. Yeast and fungi occur much less in the inhalable aerosol because of their size.

According to Ashcroft and Pomeroy (1983) aerosols contain 10^{-4}% or 10^{-6} x the total number of bacteria escaping in a fluid. Therefore a release of 10L (10^{13} bacteria) would cause an aerosol of 10^{7} bacteria to spread through several m^3. The aerosol will quickly spread throughout the area available, especially if the area is ventilated. In one inhalation a person would inhale 500-600 cells. This will be 10-20 times higher near to the leak. The causes of gross damage to bioprocessing equipment are limited to bombs, falling aeroplanes, explosion and earthquakes. Cremer and Warner (1982) estimate the chance of this at 10^{-6}.fermenter^{-1}.year^{-1}. This is obviously independent of scale and fermenter contents.

Tinnes and Hoare (1992) monitored a high speed disc bowl centrifuge for the release of a mutant *E coli* strain. They used settle plates, a Casella Slit sampler and an air filter sampler to monitor a deliberate low pressure leak of 10 mL of supernatant from the centrifuge. The centrifuge was placed inside a soft-film cabinet to provide secondary containment and also to allow the air around the cabinet to be monitored. Inlet air was HEPA filtered and had a sufficiently high flow rate to provide a positive pressure inside the cabinet. The settle plates, both close to the leak, and further away showed viable cells present, therefore the release must have been at least part aerosol. If the release was accidental, the acceptability of such a release would depend on the organism involved and its effect on the environment and operator.

Flickinger and Sansone (1984) discuss containment of equipment for the large scale production and purification of microbially produced cytotoxic agents and oncogenic viruses. They state that the primary biohazard risk to workers and the environment are the generation of aerosols and accidental spills. Accidental spills often occur due to leaks in fermentation equipment, during transfer of process liquids and recovery operations such as filtration, centrifugation and crystallisation.

The physical containment of the equipment used in these processes is discussed in section 4.1. Flickinger and Sansone (1984) monitored environmental and worker exposure to a chemotherapeutic agent during material transfers and other aerosol producing operations when uncontained process equipment with few permanent lines was used. Samples were taken by swabbing workers, clothing, floors, walls, and equipment with pieces of filter paper wetted with n-butanol. The samples were analysed spectrophotofluorimetrically.

The results indicated that the most hazardous operations, in terms of worker and environmental exposure are material transfers, extraction and concentration. Substantial quantities of product were found on workers gloves, shoe covers, and suits. The degree of contamination of worker with product was 50-100 times higher during purification than during extraction and concentration. Flickinger and Sansone (1984) point out that the potential exposure hazard increases as the concentration of drug increases at each production stage.

1.2.2.1 Health Effects

Biotechnological processes that have given rise to illnesses include fungal fermentations, those involving Gram negative bacteria, including those involving endotoxin producing bacteria. The fungus *Aspergillus* produces a spore of 5 μm diameter which, if airborne, can invade the lower respiratory tract causing either infection or a hypersensitive response. During the production of single cell protein in 1972 some workers were exposed to aerosols of whole cells, which resulted in influenza-like symptoms, even though the organisms had previously been tested and found to be non-toxic and non-pathogenic.

Bennett *et al.,* (1990) describe various incidents where illness has resulted from the release of biological material from processing equipment. At Michigan State University the improper use of a tubular bowl centrifuge to process *Brucella*

abortus resulted in 1 death and 27 cases of illness. At University College London, 5 workers experienced severe stomach and kidney pains following the operation of a tubular bowl centrifuge to process *Pseudomonas aruginosa* cell debris.

Few exposure limits for aerosolised biological products have been suggested, let alone put into the regulations. Endotoxin is a rare example where exposure limits have been studied. Endotoxin has been estimated to be 3-4% of the dry weight of *E coli* K-12 by Kabir *et al.,* (1978) and is reported by Palchak *et al.,* (1990) to have clinically significant health effects. Endotoxins have a pyrogenic effect on mammals, they act on host cells to stimulate a large number of metabolic and immunological events simultaneously (Kabir *et al.,* 1978). Toxic effects of endotoxin inhalation include fever, obstructive pulmonary changes and inflammation of airways (Baseler *et al.,* 1983).

Palchak *et al.* (1990) found that operations such as culturing microbes, centrifugation, and homogenisation had associated endotoxin levels ranging from 0.07 - 12.8 $ng.m^{-3}$. Using a 10 fold safety factor under the threshold where clinically significant changes can be detected, an action level of 30 $ng.m^{-3}$ for large scale operations involving *E coli* was established. Palchak *et al.,* (1990) conclude that endotoxin generation and potential worker exposure is associated with large scale production of pharmaceuticals extracted from Gram negative bacteria. This may exist at a level associated with clinically significant changes in pulmonary function, if cultures become aerosolised.

1.2.3 Survival of Microorganisms in Aerosols

The effects of the release of (genetically modified) organisms into the environment either deliberately in agriculture to control plant pathogens, or unintentionally from bioprocessing equipment are not clear. Since the primary form of release is by aerosol, it is worth discussing the ability of microorganisms to survive in the aerosolised state.

The factors which influence aerosol survival and virulence are numerous and have been reviewed by Cox (1987). Much of the work carried out by Cox takes little account of the importance of growth conditions upon airborne survival and also involves cells that have been washed many times to remove all traces of spent media; which is not representative of the situation in biotechnological operations.

Data from previous studies indicates that aerosolisation can cause stress to bacterial populations (Marthi *et al.,* 1990). The survival of aerosolised bacteria is influenced by:

1. Growth conditions before aerosolisation.
2. Constitution of the aerosolised fluid.
3. The size of the aerosolised particles.
4. Method of aerosolisation and environmental conditions during aerosolisation (relative humidity, temperature, oxygen, sunlight, protective agents).
5. Methods of collection and enumeration.

Lindow *et al.,* (1988) carried out field trials to monitor the number of viable cells of a non-recombinant *Pseudomonas syringae,* following aerosolisation and deposited on plants and the soil following aerosolisation. An exponential decrease in the numbers of viable cells, deposited at increasing distances from the spray site was observed. The relative rate of survival of cells sprayed directly onto plants was more than 10 times higher than that of cells dispersed through the air to similar adjacent plants. The authors suggest that the results can be used to characterise a release in terms of containment and dispersal, and to develop sampling methodologies to evaluate survival and dispersal of GMOs released into the environment.

Marthi *et al.* (1990) investigated the effects of aerosolisation induced stress on bacterial survival. Non-recombinant, spontaneous antibiotic-resistant mutants of 4 organisms. *Enterobacter cloacae, Erwinia herbicola, Klebsiella planticola,* and *Ps. syringae* were sprayed in separate experiments in a greenhouse, and samples were collected up to 15m from the spray site. Spores of *Bacillus subtilis* were used as physical tracers to determine the extent of dilution of the aerosolised population, since their survival is not thought to be affected by most adverse conditions (Ehrlich *et al.* 1970, Miller *et al.* 1961). The target organism and the spores were sprayed together in each experiment. The results were expressed as a ratio of the number of viable target organism cells to the number of viable spores. Following the spraying of the cell suspensions, impingers (see section 3.2) were used to recapture the released cells. A comparison of the survival of the four bacteria and a study of the effects of droplet size, ambient temperature and relative humidity on survival were carried out.

Closer to the spray site (1 m), organisms survived better in the larger of two droplet diameters (450 μm and 130 μm). At distances of less than 1 m there was no significant difference in survival between the two sizes. As distance from the spray site increased, survival significantly decreased for both droplet sizes. Survival was also increased at 22°C compared to 12°C, this effect was enhanced further with a decreased droplet diameter.

At a higher ambient temperature the detrimental effects of aerosolisation are independent of droplet size. At a lower relative humidity (39%, 22°C) the rate of droplet evaporation is fast for both droplet diameters. On evaporation the droplets reach a constant diameter, when evaporated at 22°C, both initial droplet diameters reach the same final constant diameter. At 12°C the rate of evaporation is reduced because the relative humidity is 77%. Cox (1987) has shown that a relative humidity of 70-80% has a protective effect on aerosolised bacteria. Experiments showing that survival rate increases with increased distance from the spray site, for the smaller droplet diameter at 13°C can be explained (Marthi *et al.,* 1990) by spore clumping due to increased humidity. The number of spores counted and the relative ratio of microorganisms to spores would therefore be underestimated.

Marthi *et al.* (1990) also found that unwashed *Ps. syringae* sprayed in large droplets showed no change in survival with distance, whereas washed cells under the same conditions showed a significant reduction in survival. However, unwashed cells sprayed as small droplets showed significantly reduced survival. It is possible that organic materials from the growth medium may reduce evaporation and thus protect the bacteria, although this may be masked by the more rapid evaporation of smaller droplets. Further experiments were carried out to determine if the washing procedure caused any reduction in viability. The authors compared the viability of *Ps. syringae* washed in phosphate buffer with unwashed cells, and found no significant difference.

Benbough and Hood (1971) used micro threads (the cells are suspended on spider's webs) and ventilated spheres (a closed metal chamber, 22 ft diameter from which the aerosol is sampled, air is drawn in at 14 air changes/hour) to determine the viricidal activity of open air and compare it with bactericidal activity. It is thought that this bactericidal activity of open air is due to the combination of ozone and unsaturated hydrocarbons (Druett and Packman, 1968; Dark and Nash, 1970). The bactericidal activity of open air is rapidly lost when enclosed. The ventilated-

sphere technique was used by Benbough and Hood to determine the inactivation rates of viruses in the open air as a function of aerosol droplet diameter. The viruses used were Semiliki Forest Virus and Coliphages T1 and T7. *E. coli* MRE 162 was used for the comparison of viruses with bacteria and *B. subtilis* var *niger* spores were used as a tracer. Aerosols were generated by a Collison nebuliser and deposited on micro threads, they were exposed to open air and shaded from direct sunlight. A suspension of test virus and *B. subtilis* spores in a solute solution were aerosolised in the ventilated sphere and aerosol samples taken using a three stage liquid impinger (May, 1966).

The results showed large day to day differences in the decay rates of airborne *E. coli.* This could be accounted for by large variations in the concentration of an unknown bactericidal atmospheric pollutant. This concentration will depend on the distance between the pollutant source and test areas and wind direction. Since the viricidal activity of open air is constant this may be caused by another pollutant whose concentration is constant. Alternatively, airborne bacteria and viruses may be inactivated by the same component but in different concentrations. Benbough and Hood (1971) also discovered that, using the ventilated sphere method, although the infectivity of viruses in enclosed air is independent of particle size, in open air a particle size effect is seen. They postulate that the airborne component may attack through the particle surface containing the virus. If this is the case, the virus inactivation rate may be directly proportional to the surface to volume ratio of the particle.

Oxygen inactivates some bacteria such as *Serratia marcescens, E. coli,* and *Klebsiella pneumoniae,* in the airborne state. It is interesting to note that spores, phages, and viruses survive equally well in air and pure nitrogen (Bennett and Norris, 1989). Oxygen susceptibility usually increases with degree of desiccation, oxygen concentration and time, whether dehydration is through aerosolisation, freeze drying or drying on surfaces. For *E. coli* B it seems that cell division is the process that is inactivated by oxygen, which leads to loss of viability (Cox, 1987).

The sensitivity of aerosolised microorganisms to radiation is dependent upon the degree of desiccation, oxygen tension, particle size and the spray fluid used. Visible light can be lethal to bacteria but some viruses, e.g. Foot and Mouth disease virus and Polio virus are resistant. Dry disseminated bacteria are not affected by sunlight as much as wet disseminated bacteria (Cox, 1987).

Ozone as an air pollutant has shown antibacterial activity. Cox, (1987) describes several studies in which the toxic effects of ozone were evident when it chemically recombines with other photochemical oxidants commonly associated with car exhaust, petrol vapour and olefins. The toxic effects of ozone increase with relative humidity. After 30 minutes of exposure to 0.025 ppm ozone, 90 % of cells are killed if the relative humidity is 70 %. At 45 % relative humidity and 10000 ppm ozone, little bacterial death is reported (National Research Council Criteria Document, 1977).

1.2.4 Survival of Microorganisms in the External Environment

The primary escape route of microorganisms from bioprocessing plant is via exhaust gases. They are much diluted during transport and arrive at a niche as single organisms (Winkler, 1988). This means that the probability of growth in a niche is low. The risk to the environment depends on (Winkler, 1988): escape, transport and arrival in the niche, survival and adaptation, competitive growth, survival of famine, and colonisation of more niches. Winkler comments that one bacterium.m^{-2} is equivalent to a density of one man.million km^{-2}. At this density they cannot affect each other, they arrive as single organisms. Microorganisms can also enter aquatic environments via agricultural run-off, drainage of industrial effluent, or faecal contamination from birds or animals (Edmonds, 1976; White and Godfree, 1985). Much information concerning the survival and persistence of enteric bacteria is available since pollution by faecal bacteria is routinely monitored to maintain potable water quality standards. For example, *E. coli*, faecal streptococci and spore-forming bacteria (*Clostridium perfringens*) are often used as indicators of faecal pollution in water supplies. Beringer and Bale (1988) reviewed the factors affecting the survival of these and other organisms in the aquatic environment. The main influences can be summarised as follows:

1. Temperature: enhanced survival of microorganisms occurs at low temperatures, if other eukaryotic microorganisms are present.
2. Sunlight: most enteric microorganisms are inactivated by sunlight in freshwater and marine environments, the effect being greater in salt water compared to freshwater.
3. pH: the optimum pH for survival is 5.5-7.5, a low pH can be used by some bacteria as an electrochemical gradient to produce energy.

4. Suspended solids: the absorption to suspended solids often leads to the accumulation of introduced bacteria in sediments. Sediments enhance the survival of bacteria in laboratory systems containing sediment and sea water.

Beringer and Bale (1988) compared the release of GMOs into the environment with the well established release of *Rhizobium sp* for nodule formation in leguminous crops. Two concerns often raised about GMOs are that they might somehow become pathogenic and that they may colonise the environment and displace existing species. Millions of hectares of land are inoculated with *Rhizobium* each year to improve the growth of leguminous crops, and much work has concentrated on the survival and persistence of *Rhizobium,* due to its commercial importance. Strains of *Rhizobium* have been released into the environment since the turn of the century, with the emergence of legumes as major crops. In USA alone in 1980 almost 4x 10^6 kg of inoculant were produced for use on 15.5 x 10^6 hectares of land (Burton, 1982). The release of *Rhizobium* has been well documented in The Netherlands, Australia and the USA. The absence of disease linked to this practice might indicate that the release of microorganisms *per se* is not inherently dangerous or environmentally damaging. Beringer and Bale (1988) conclude by saying that experience shows that large numbers of microorganisms can be released into the environment without causing harm and that the release of GMOs into the environment should not be discouraged as long as the organisms are not pathogenic.

The survival of microorganisms will depend on interaction with already established organisms. It is influenced by competition, parasitism and predation. Indigenous bacteria prevent 'new' bacteria from establishing themselves by competition for nutrients. It is evident that bacteria can survive starvation stresses by a series of biochemical and morphological changes (Morita, 1982; Kjelleberg, 1987). It has been shown that starved populations of *E. coli, Salmonella enteritidis, Campylobacter jejuni,* and *Vibrio cholerae* enter a non-culturable resting stage, whilst maintaining their pathogenicity (Xu *et al.* ,1982; Baker *et al.,* 1983; Roszak *et al.,* 1984; Colwell *et al.,* 1985; Rollins and Caldwell, 1986).

Colwell *et al.* (1985) observed that over time, *Vibrio cholerae* and other related human pathogens enter a viable, but non-culturable state. Direct viable counts by epifluorescent microscopy are consistently higher than corresponding plate counts. The assumption that microorganisms die in the marine environment must therefore

be re-evaluated, since stressed or nutrient starved cells are unable to grow and be enumerated by standard plate count methods. These non-culturable cells continue to to be potentially infectious. The public health importance of these findings is obvious, but there is also relevance for the release of GMOs into the environment. It will not be possible to depend on plating methods for the recovery and monitoring of such organisms; direct detection methods of high specificity will be required.

The ability of microorganisms to survive in the natural environment for extended periods of time is illustrated by the following example. In the 1940's the MOD carried out some field trials using *Bacillus anthracis,* on Gruinard Island, off the NW coast of Scotland. These trials are worth discussing now because although they concern a pathogenic bacterium, not a GMO, the problems associated with the clean-up following release are the same. Manchee and Stewart (1988) describe the experiments and the decontamination procedure carried out almost 40 years later. In summary, an anthrax bomb of spores was exploded on the island and the movement of airborne spores monitored via strategically positioned sheep. In 1979 an improved detection method was used to determine that actually only 1.1 hectares (about 0.5% of the island) was contaminated, in most cases to a depth of 5 cm. Following this discovery formaldehyde was applied over the surface of the contaminated area. Formaldehyde was chosen because it is effective at killing bacterial spores, it is cheap and it does not leave toxic residues. Successful decontamination work was also carried out on the surrounding area, sheep now graze on Gruinard Island and no further deaths have been reported.

1.2.5 Exchange of rDNA in the External Environment

The possibility of large releases of GMOs into the external environment has triggered much research on the ability of microorganisms to transfer recombinant plasmids either to other microorganisms (released or indigenous) or to animals.

1.2.5.1 DNA Transfer to Other Microorganisms

There are three methods whereby DNA can be transferred from one bacterial cell to another. These are conjugation, transduction and transformation. During conjugation a plasmid from a donor cell integrates into the host's chromosome. In transduction bacterial DNA is transferred from one cell to another by a phage

particle containing the bacterial DNA. Transformation is the process by which cells acquire genes from free DNA molecules in the surrounding medium. This process of DNA transfer into a bacterial cell from the surrounding medium was first suggested by the experiments carried out by Griffiths (1928). Griffiths noted that non virulent (rough) cells of *Streptococcus pneumoniae* became virulent, killing the mice they were injected into, when mixed with either heat killed virulent (smooth) cells, or smooth cell extracts. Avery *et al.* (1944) mixed together purified DNA from smooth cells and growing rough cells, resulting in growing smooth cells, thus proving that DNA was the genetic material.

Bogosian and Kane (1991) have reviewed studies concerning the survival of *E. coli* K-12 strains containing recombinant plasmids derived from pBR322 and the potential to transfer such plasmids to other organisms in the environment (soil, water, sewage and the mammalian intestinal tract). These studies have indicated maximum survival times of approximately 15 days in water, 20 days in soil, 10 days in sewage, and 3-6 days in the intestinal tract of normal hosts. For the transfer of recombinant plasmids derived from pBR322, from these strains to indigenous microbial inhabitants, the following are required:

1. A conjugative plasmid to mediate conjugative pore formation.
2. A mobilisation plasmid to provide *trans*-acting components of the transfer complex.
3. The pBR322 plasmid to contain functional *bam* and *nic* sites.

The only known habitat for *E. coli* is the intestinal tract of warm blooded vertebrates (Brenner, 1984). The presence of *E. coli* in soil or water is taken as evidence of recent faecal contamination. As discussed by Bogosian and Kane (1991), *E. coli* K-12 is not a strain suited for survival in sewage or the mammalian intestinal tract, and therefore it is at a distinct disadvantage in environments outside the laboratory. Winkler and Parke (1992) point out that DNA is quickly degraded in the gut of animals by DNAases. Bearing in mind the stringent conditions required for gene transfer plus the limitations of pBR322 type plasmids, it is not surprising that the transfer of recombinant plasmids from *E. coli* K-12 to indigenous inhabitants of water, soil, sewage, or intestinal tracts, in their natural environments has never been demonstrated.

Winkler (1988) discusses the likelihood of genetic exchange occurring between released GMOs. He points out that DNA transfer cannot occur between organisms

without growth. This means that the probability of transfer is very low for single escaping organisms. DNA transfer by transformation or transduction in soil is limited and delayed by strong non-specific adsorption of DNA to clay and humus. On crowded surfaces, conjugation is possible, but is normally limited to closely related strains. If the host strain does not contain a transfer mechanism, two conjugations/transductions will be necessary for rDNA transfer, thus reducing the probability of transfer. If the rDNA product is of no use in the natural environment, the rDNA sequence will be lost. Constitutive enzymes that breakdown macromolecules are wasted in niches that do not contain that substrate, and will then hamper adaptation (Pardee, 1961).

Drahos *et al.* (1988) reported a field test of a recombinant *Pseudomonas aurifaciens* marked with the *E. coli lac ZY* tracking system. This was the first field test in the United States of a live recombinant microbe containing genes from two different strains. Drahos *et al.* (1988) discuss the rigorous pre-release testing procedures required, mainly to show that the organism intended for release is non-pathogenic to plants and animals, the stability of the introduced genes and the efficacy of the monitoring system to track the GMOs in the environment in the presence of native field microbes.

It is thought that the risk involved with the release of GMOs is small because rDNA techniques produce better characterised organisms than traditional techniques. A major concern regarding the use of GMOs is that recombinant techniques may convert non-pathogens into pathogens. However it is unlikely that single gene modifications with no pathogenic potential or history will result in pathogenicity. For a organism to cause disease, it requires recognition factors, adhesion ability, toxigenicity factors and resistance to the host defence system. These could not all be produced from single gene modifications.

Winkler and Parke (1992) suggest that by cloning DNA from a pathogen into a related harmless host, e.g. DNA from an enteric pathogen into *E. coli* K12, this question of the possibility of a non-pathogenic organism becoming pathogenic by gene transfer in a natural environment can be addressed. Guinee (1977) found that when the genes for adherence factor and enterotoxin were introduced into *E. coli* K12 cells they did not become pathogenic. Winkler and Parke point out that these arguments are also true for *S. cerevisiae*, *B. subtilis,* and other harmless

saprophytes that do not grow in the human body. The transfer of well defined DNA into harmless host organisms cannot accidentally produce pathogens.

1.2.5.2 DNA Transfer to Animal Cells

It has been suggested that microorganisms may transfer rDNA to animal cells, causing disease (Berg, 1974). Israel *et al.* (1979a) studied the effects of oncogenic polyoma virus on mice and hamsters. The complete viral genome was cloned into *E. coli* K12 cells, which were then injected into mice. They found that the PyDNA in bacteria was 10^9 times less dangerous than the viral particles. Injection into baby hamsters did not cause infection. Israel *et al.* then used wild type *E. coli* containing PyDNA administered orally. The mice did not become infected and did not produce antibodies against the polyoma virus. The probability of rDNA transfer from bacteria to a mammalian host was found to be less than 2 in 10^{13} .bacterium^{-1}. day^{-1}. Two other oncogenic viruses were also studied (including a retrovirus) and no transfer of DNA detected. The probability of transfer from *E. coli* K12 to animals is even lower because *E. coli* K12 does not colonise the gut.

In 1987 Bartels described how 5 molecular biologists working with tumour viruses, oncogenes and mutagens contracted cancer (mostly bone) at the same time. The following year, two of the researchers died, one of the deaths was ruled to be due to occupational disease. The 5 researchers worked in 2 adjacent laboratories. The chance of this happening is 1 in 10^6. The researchers worked on oncogene-containing tumour viruses.

Oncogenes are made of DNA and code for protein products and are able to transform normal cells into a cancerous state. In 1985, Yoakum showed that the "ras" oncogene on its own can turn normal human lung cells into cancerous ones. Obviously there must be occupational hazards for researchers involved in this kind of work. Without strict adherence to safety precautions there is little to prevent oncogenic agents accessing laboratory workers. Bartels (1987) recommends that a risk assessment is carried out on the techniques involved in the manipulation of oncogenic DNA.

1.3 Safety Legislation

Current regulations concerning the use of genetically modified organisms in the UK are summarised in table 1.1. They are numerous and often confusing to the uninitiated. The Health and Safety at Work Act (1974) was the first piece of legislation that included all places of work and most importantly, gave the employer responsibility for employee health and safety in the workplace. This act makes no mention of genetically modified organisms, however, all UK regulations concerning the contained use of GMOs are made under the power of this act. The regulations contained detailed information on risk assessment, organism classification and containment categories. They are supported by the ACGM guidance notes (ACGM, 1987, 1988a and 1988b). Both Guidance Note 6 (ACGM, 1987) and the Contained Use Regulations (HSE, 1992) themselves are currently under review.

1.3.1 The Robens' Report and the Health and Safety at Work Act (1974)

Current legislation concerning the use of GMOs and their effects on both workers and the environment originates in the Health and Safety at Work Act (1974). Although at this time GMOs were not used in industry and were not mentioned in the Act.

The Health and Safety at Work Act, 1974 (HASAWA) came into existence as a direct result of recommendations made in the Robens' Report (1972) . At the time the report was written there were no safety regulations which encompassed all places of work. The regulations that existed not only were different for almost every industry/workplace, but were also controlled by many different bodies. On average, one thousand people a year were being killed in accidents at work, in the UK. This accounted for 23 million lost working days. These statistics show that there are economic, not just humanitarian reasons for improvement.

The Robens' report highlights accidents that occurred because some workplaces were missed out of legislation altogether, and others that happened because it was not clear where the responsibility lay for a particular activity. The report found that the greatest single contributing factor to accidents at work was apathy, calling for the primary responsibility for safety and health at work to be placed with those who create the risks and those who work with them. The system relied too much

on state regulation. A more unified and integrated system was required to increase the effectiveness of the state's contribution, plus a large element of self regulation.

The report recognised the need to protect the public as well as workers from large scale hazards. If it is possible that an industrial establishment could put nearby residents at risk, a 'cordon of safety' should be provided between the hazardous activity and the residential area. The report discusses whether the Radioactive Substances Act (1960) and the Alkali etc. Works Regulation Act (1906) and their administration should be brought within the scope of the Robens' report. They were both administered by the Department of the Environment (DoE), and were concerned, not with the health and safety of work people but with the need to control certain emissions from workplaces in the interests of public health and amenity. They form part of a separate pattern of provisions for public health and control of environmental pollution which lie within the responsibility of the DoE and local health authorities. But, both Acts concern establishments subject to occupational safety and health provisions. The problem seems to be that of drawing the line between controls over the workplace environment and the general environment.

The report suggests inclusion of these two acts into the proposed unified arrangements for safety and health at work. This discussion is interesting in the context of GMOs, which may effect both workers and the external environment, if released from secondary containment. The Robens' Report made two major recommendations:

1. That a comprehensive act for safety and health at work should be passed. This act should be supported by a combination of regulations and non-statutory codes and standards. The regulations should be limited to general requirements only. Detailed specification on technical content should be undertaken by expert technical working parties.
2. That what is now known as the Health and Safety Executive should be set up to unify administrative arrangements and to provide a mechanism for linking voluntary and statutory activities in a more comprehensive way.

Table 1.1: Current Legislation Concerning the Safe Use of Genetically Modified Organisms

Title	Content
Health and Safety at Work Act (HASAWA) (1974)	Made it the legal responsibility of employers to ensure health, safety and welfare at work, of all employees.
OECD, rDNA Safety Considerations (1986)	Recommendations on use of rDNA technology in agriculture, industry and the environment. Risk assessment carried out on organisms and processes put into physical containment categories (GILSP, LSCC1, 2 and 3).
Containment of Substances Hazardous to Health (COSHH) Regulations (1988)	Protect employees from hazards such as allergens, toxic products, and carcinogens, associated with biological processes and products. Assessment of health risks, control of exposure, control measure testing, exposure testing, health surveillance, inform employees of the risks in their work.
Advisory Committee on Dangerous Pathogens (ACDP) (1989)	Produced a guidance document on the classification of pathogens according to the inherent hazard of the organism.
Advisory Committee on Genetic Manipulation (ACGM) (1987)	Guidance Notes to support Genetic Manipulation Regulations (1989). Require formation of a Genetic Manipulation Safety Committee at a local level. The safety committee is to assist in assessment of risks from proposed work and to decide on a containment level. Note 6 relates to the large scale use of GMOs Note 7 relates to the risk assessment of operations involving the contained use of GMOs. Both these notes are currently under review.
Environmental Protection Act (EPA) (1990)	Intended to regulate on internationally agreed principles, put the safety of people and the environment first and allow relaxation of control measures where indicated possible by experience.
Genetically Manipulated Organism (Deliberate Release) Regulations (1992)	Introduced in compliance with EC Directive 90/220/EEC (under the EPA) to deal with the deliberate release of genetically manipulated organisms into the environment.
Genetically Modified Organism (Contained Use) Regulations (1992) (replaced Genetic Manipulation Regulations (1989))	Introduced in compliance with EC Directive 90/219/EEC (under the HASAWA) to deal with the contained use of genetically modified organisms in teaching, research and industry. Nomenclature for organism (I or II), process (A or B), and containment level (B_1 to B_4). Risk assessment to consider risks to human health and environment. Emergency plans, notification and consent required.

The Health and Safety at Work Act (1974) places a general duty on the employer "requiring the provision and maintenance of a working environment for their employees that is, so far as is reasonably practicable, safe without risks to health and adequate as regards facilities and arrangements for their welfare at work". The employer is also charged with a duty to avoid exposure of those not in his employment, including the general public, to risks. These duties are qualified by the phase "so far as is reasonably practicable"; the employer must make a cost:risk analysis balancing the risk involved in carrying out the work against the difficulty and cost of avoiding that risk.

The Genetic Manipulation Regulations (1989) were made under the Health and Safety at Work Act (1974). They specify that no person shall carry out activities involving genetic manipulation unless notification of intention has been given to the Health and Safety Executive. The regulations classify organisms according to hazard involved.

1.3.2 The Gordon Research Conference

At this conference in the United States papers were presented that explained that DNA molecules from separate sources could be combined and transferred between living organisms. It was recognised that these techniques would probably involve unknown hazards and would need to be regulated. The National Academy of Science formed a committee on rDNA molecules and a voluntary embargo on some possible DNA manipulations was introduced. This prevented the introduction of antibiotic resistance into plasmids or bacterial strains not known to carry that trait. Experiments involving DNA coding for bacterial toxin synthesis, oncogene and animal viruses were included. This was the first attempt to classify rDNA experiments based on a perceived hazard (Turner, 1989). Two major problems were recognised:

1. The biological and ecological hazards or rDNA molecules were not known.
2. Procedures to minimise the spread within humans and other populations had not been developed.

1.3.3 The Asilomar Conference (1975)

This conference recommended for the first time, that biological and physical barriers be used to contain recombinant microorganisms. It was agreed that experiments with serious potential risks should not be carried out in the present containment facilities. Berg *et al.* (1975) point out that few, if any scientists believe that rDNA technology is free from risk. The potential risks should be dealt with by making containment an essential factor in experimental design and matching the level of containment used with the estimated risk. Berg *et al.* (1975) also claim that experiments requiring large scale operation seem to be riskier than the equivalent experiments on the small scale, therefore requiring more stringent containment procedures. It should be added that it is the increased volume of recombinant cells that could be released that makes large scale experiments more hazardous. The probability of failure or release (i.e. the risk) is independent of scale. The conference started an embargo on experiments over 10L in scale.

The conference recommended the use of vectors and bacterial hosts with restricted capacities to multiply out of the laboratory. The physical containment measures suggested included the use of hoods, limited access, negative pressure and adherence to good microbiological practices. It is suggested that experiments be classified into four different containment categories based on the risk involved.

1.3.4 The Organisation for Economic Co-operation and Development (OECD) (1986)

In 1986 the OECD published an extremely influential report entitled "rDNA Safety Considerations". It gave recommendations for the safe use of rDNA technology based on existing good practices. This was the first time the approach of GILSP (Good Industrial Large Scale Practice) was described. GILSP is now common practice throughout Europe and was the basis of the European Directive on the Contained Use of GMOs (90/219/EEC) introduced in 1990.

The containment levels for GMOs described by the report range from GILSP, to categories 1, 2, and 3 (see table 1.2). The main differences between these are explained below (OECD, 1986):

1. Viable organisms should be handled in a system which physically separates the process from the environment (closed system), this applies to all three categories.

2. The exhaust gases from the closed system should be treated so as to minimise release (category 1) or prevent release (categories 2 and 3).
3. Sample collection, addition of materials to a closed system, and transfer of viable organisms should be performed so as to: minimise release (category 1) or prevent release (categories 2 and 3).
4. Bulk culture fluids should not be removed from the closed system unless the viable organisms have been inactivated by validated means (category 1) or inactivated by validated chemical or physical means (categories 2 and 3).
5. Seals should be designed so as to minimise release (category 1) or prevent release (categories 2 and 3).
6. Closed systems should be located within a controlled area. This is optional for category 1 but compulsory for categories 2 and 3.

The OECD recognised that GILSP is not a containment category, but adopted it because many organisms used in traditional manufacture are regarded as safe since they have been used for many years and have not caused any problems. In the same way "modified organisms prepared by inserting segments of DNA that are well characterised and free from known harmful sequences into such organisms to improve their performance are unlikely to pose any risk" (OECD, 1986). Organisms in which DNA is inserted should have built in environmental limitations permitting optimal growth in an industrial setting but limited survival without adverse consequences in the environment. Only resistance markers that the organism is known to acquire naturally should be transferred. Therefore GILSP is not strictly a containment category because biological containment is being used. OECD containment category 1 specifies that the release should be minimised, and again, the organisms should have "built-in environmental limitations".

The Royal Commission on Environmental Pollution (Report, 1989) suggest that organisms with simple engineered gene deletions cannot be considered safe when released into the environment. They also accept that manufacture with recombinant organisms will produce microorganisms whose accidental release and disposal needs to be considered. They have not considered incidental releases from the normal operation of plant, which is unavoidable. Turner (1989) comments that this view seems to be almost universal, i.e. that physical containment is 100% efficient. However, absolute containment is almost impossible to achieve, and obviously, anything short of it constitutes release.

One question that could be asked of the OECD guidelines is, if containment can not be graded, how is it possible to have two upper levels, both preventing release? The answer (Turner, 1989) is that the OECD guidelines are not an increasingly stringent set of containment measures, but operational guidelines ensuring ever lower levels of release. The OECD regulations only attempt to control the scale of release; incidental release, although small can not be prevented, therefore these guidelines rely on biological containment to kill GMOs, once released. The regulations for the deliberate release of recombinants rely on the organisms surviving in a controlled and limited way once released. The difference between the two sets of regulations is therefore in the biological containment involved. Turner (1989) advocates a change from a hierarchy of increasing containment to one of decreasing release, so more effective guidelines can be written and different sets compared.

Table 1.2: OECD Containment Levels

Specification	Containment Category		
	1	2	3
1. Viable organisms should be handled in a system which physically separates the process from the environment (closed system).	Yes	Yes	Yes
2. Exhaust gases from the closed system should be treated so as to:	Minimise release	Prevent release	Prevent release
3. Sample collection, addition of materials to a closed system and transfer of viable organisms to another closed system, should be performed so as to:	Minimise release	Prevent release	Prevent release
4. Bulk culture fluids should not be removed from the closed system unless the viable organisms have been:	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means
5. Seals should be designed so as to:	Minimise release	Prevent release	Prevent release
6. Closed systems should be located within a controlled area.	Optional	Optional	Yes, and purpose built
a. Biohazards signs should be posted.	Optional	Yes	Yes
b. Access should be restricted to nominated personnel only.	Optional	Yes	Yes, via an airlock
c. Personnel should wear protective clothing.	Yes, work clothing	Yes	A complete change
d. Decontamination and washing facilities should be provided for personnel.	Yes	Yes	Yes
e. Personnel should shower before leaving the controlled area.	No	Optional	Yes
f. Effluent from sinks and showers should be collected and inactivated before release.	No	Optional	Yes
g. The controlled area should be adequately ventilated to minimise air contamination.	Optional	Optional	Yes
h. The controlled area should be maintained at an air pressure negative to atmosphere.	No	Optional	Yes
i. Input air and extract air to the controlled areas should be HEPA filtered.	No	Optional	Yes
j. The controlled area should be designed to contain spillage of the entire contents of the closed system.	No	Optional	Yes
k. the controlled area should be sealable to permit fumigation.	No	Optional	Yes
7. Effluent treatment before final discharge.	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means

1.3.5 The Control of Substances Hazardous to Health (1988)

It is recognised that biological processes can provide health risks due to the process organism and its products. These hazards include allergens, toxic products, and carcinogens. Microorganisms and their products are covered by the Control of Substances Hazardous to Health Regulations (COSHH, 1989). This legislation gives employers whose business involves the use or manufacture of potentially hazardous chemicals or microorganisms the following duties (Bennett and Norris, 1989):

1. Assessment of health risk: to enable a valid decision to be made about measures necessary to control potentially hazardous substances.2. Control of exposure: the employer must ensure that the exposure of employees to hazardous substances by any route is either prevented or, when this is not reasonably practical, adequately controlled.3. Control measure testing: to ensure that they continue the requirements of 2. i.e., ventilation and protective equipment must be regularly tested.4. Exposure testing :valid occupational hygiene techniques must be used to derive a quantitative estimate of exposure of employees to hazardous substances (not microorganisms). This involves periodic or continuous sampling of the atmosphere in the workplace to determine airborne concentrations of the contaminant and records of the monitoring must be maintained.5. Health surveillance: any employees subject to a hazardous substance must have a regular medical and these records must be kept for 30 years after the person ceases to be exposed.6. Provide information for person exposed. The company must inform its employees of the nature and degree of risk of exposure to the substance, the control measures adopted, the reasons for personal protective equipment and when to use them and the role of health surveillance.

1.3.6 Advisory Committee on Genetic Manipulation Guidance Notes

ACGM Note 6 (1987), on the large scale use of GMOs and Note 7 (1988a), on the risk assessment of operations involving the contained use of GMOs, were written to support the Genetic Manipulation Regulations (1989). Since these regulations have now been replaced by the GMO (Contained Use) Regulations (HSE, 1992), the guidance notes are currently under review.

As they stood when introduced in 1989, the Genetic Manipulation Regulations expressly required the formation of a genetic manipulation safety committee and gave guidelines on it's constitution. This local biological safety committee was to

assist in the assessment of risks from proposed work and to decide on it's containment level.

It was recognised that unit operations are discrete stages that require individual assessment. For example, cell separation methods like centrifugation have the potential for widespread contamination and aerosol generation unless precautions are taken. It was agreed that all work with GMOs to be carried out should be notified to the HSE and should be considered by a local safety committee.

Note 6 recognises that the majority of large scale applications of genetic manipulation will use low risk organisms. In accordance with the OECD this level is called Good (Industrial) Large Scale Practice (G(I)LSP). It includes measures such as keeping workplace and environmental exposure to physical, chemical and biological agents to the lowest practicable levels, and exercising engineering control measures at source. It realises a need for testing equipment and monitoring for the presence of viable process organisms outside the primary physical containment.

The following categories of engineered microorganisms are acceptable for working at G(I)LSP:

1. Non-pathogenic hosts, with no adventitious agents, and a long history of safe use or built in environmental limitations.
2. The vector-insert must be well characterised and include no harmful sequences. It should be limited to the DNA needed for the intended function only, and should not increase the stability of the recombinant organism in the environment. The insert should be poorly mobilisable and not transfer any resistance markers to microorganisms not known to acquire them naturally.
3. The resultant organism should also be non-pathogenic, and be as safe in the bioreactor as it is in the environment.

GMOs that are not included in G(I)LSP may need some physical containment measures to match the assessed risk. The level of physical measures and associated safety procedures must be matched to the conclusions of the risk assessment. The modified organism should be re-evaluated at the time of transfer to the large scale. As mentioned before, it is important to consider processes as many unit operations. It may be appropriate to select and combine containment on the basis of a unit

operation assessment rather than to implement a fixed category of containment to the whole process.

1.3.7 The Contained Use Regulations

The Genetically Modified Organisms (Contained Use) Regulations (HSE, 1992) replace the earlier Genetic Manipulation Regulations (1989) and implement the EC Directive 90/219/EEC which was adopted in 1990. The regulations came into force in 1993, were made under the powers of the HASAWA (1974) to cover both human health and environmental risks.

The Contained Use Regulations provide for:

1. Human health and environmental risk assessment.
2. Records of risk assessment.
3. The establishment of a local genetic modification safety committee to advise on risk assessment.
4. The categorisation of work on the basis of risks to human health and safety and of damage to the environment, taking into account the nature of the organism and the type of activity.
5. Advance notification to the Health and Safety Executive (HSE) of an intention to use premises for activities involving GMOs for the first time and in some cases consent from the HSE before work can start.
6. Notification to the HSE of individual activities involving genetic modification and for some activities, consent from the HSE before work can proceed.
7. Standards of occupational and environmental safety and levels of containment.
8. Notification of accidents, and where appropriate, drawing up of emergency plans.
9. Disclosure of information and public registers, with provision for confidentiality.
10. Fees for notification.

Activities covered by these regulations include laboratories, housing and breeding of modified animals, growth rooms and glasshouses, and fermenters. "Contained Use" refers to any operation in which organisms are genetically modified, or in which GMOs are cultured, stored, used, transported, destroyed or disposed of. Waste streams are therefore included.

Those planning experiments with GMOs must assign the organism involved to one of two hazard ranked groups. Either Group I, for low or zero risk microorganisms (equivalent to the GILSP), or Group II for microorganisms hazardous to man or the environment.

The operation being carried out is assigned to either type A, for research and teaching work (i.e. non-commercial work), or type B, for commercial work. The level of containment must also be determined for the experiment, these are B_1 (equivalent to GILSP), B_2, B_3 and B_4 (equivalent to OECD categories LSCC1, 2 and 3).

In addition, the system used to contain the organism should comply with the methods set out in ACGM Notes 6 (1987) and 8 (1988b). Note 8 is concerned with laboratory containment facilities. It should be possible to limit contact with man and the environment both in the event of accidental spillage and waste disposal. A figure of 10L is given in the EC Directive as an appropriate scale for this type of work, however it is recognised that this is not fixed. All operations that produce an industrial or commercial product are considered type B operations, even though they may be carried out at small volume.

A risk assessment is then performed, following guidance given in ACGM Notes 6 (1987) and 7 (1988a). Risks to both human health and to the environment must be considered. This encompasses categorisation of the microorganism and the operation for notification and the determination of appropriate control measures. For Group I microorganisms the principles of Good Occupational and Environmental Safety are applied. For Group II microorganisms the principles of the Brenner Scheme are applied. The containment measures used must provide a baseline level of protection for man. The containment required to protect the environment must also be re-evaluated.

Further information on organism classification and risk assessment is given in the Guide to the Genetically Modified Organism (Contained Use) Regulations (HSE, 1992).

1.4 Risks and Hazards in Bioprocessing

1.4.1 Definitions

In the literature there are two views of how risk should be defined, the simpler of the two is that risk is the probability of an event occurring (Royal Society Study Group, 1992 and Marshall, 1987). The alternative, suggested by Winkler and Parke (1992), is that risk is the probability of an event occurring multiplied by it's effect. However, a more logical approach is that the event is the chain of circumstances through which a latent hazard becomes active, with some detrimental effect (i.e. the consequence). Following this explanation, risk remains the probability of the event occurring.

Attempts have been made to divide risk into several different types. Marshall (1987) describes individual risk as the frequency at which an individual sustains harm from the realisation of a hazard (note that he now includes the effect in the definition of risk). As might be expected, societal risk, the risk to groups of individuals or society at large, is the frequency at which a number of people sustain harm from the realisation of a hazard. Neither of these types of risk take into account effects on the environment, which in the case of some industrial accidents, can be far more severe than the effect on people (e.g. the Exxon Valdez oil spill). In theory, individual risks should add up to the societal risk.

It would be useful to be able to define the "potential for disaster". However, this definition will be subjective, and depend upon the societal group which the definer belongs to. Winkler and Parke (1992) discuss factors affecting the "scope of the effect", i.e. the consequence. In the context of the large scale production of a pathogen, they include the gravity of the disease, availability of efficient cures and the chance of spread to other hosts. If environmental effects are included, the ability to spread and grow in other niches, the ability to disturb equilibria and to transfer rDNA to indigenous strains should also be considered. As an extreme example, few people would disagree that the production of a potent pathogen, for which there were no cures, which had a wide host range and could effectively transfer DNA would be a "risky" operation with a rather high "potential for disaster".

1.4.2 Public Perception of Risk

The public expect engineers and legislators to ensure that hazards are reduced to levels that are "tolerable", "acceptable" and "justifiable". These terms are subjective, they depend on the public perception of the risks posed by these hazards. The perception of risk will always be subjective, it will never be uniform throughout society. This makes the quantification of risk, i.e. it's numerical estimation, extremely important.

The control of hazards is carried out by a combination of self regulation and state regulation. Obviously the law can only concern itself with those hazards that are under the control of humans. This combination of self and state regulation was first envisaged by Robens (1972). He described a system where employers are free to make their own rules and safety codes, rather than relying on the state to make them. The Health and Safety at Work Act (1974) incorporates the spirit of self regulation although it does include much regulatory detail and many absolute requirements. Marshall states that it is the impossibility of reducing chemical processes to a common pattern that makes self regulation the only practical basis for control in the chemical and process industries. Recombinant DNA technology is regulated by a combination of prescription (Regulations enforced by the HSE) and self management (the interpretation of engineering practice). Employers tend to prefer the prescriptive approach which allows them to follow instructions rather than make decisions.

Often there is a discrepancy between the public's appraisal of the level of risk of injury to individuals/society from a hazard and the level assessed by risk analysis specialists. Lee (1981) suggests that the publics' perceptions are often irrational, although Marshall disagrees, stating that any discrepancy between the two views is more likely to be due to a lack of technical understanding on which to base an opinion. This discrepancy is quantitative not qualitative, both the public and scientists agree that a risk exists, it is just the size of the probability that does not match. There are often differences in the public's evaluation of a risk, depending on whether it is personal or public safety at risk.

The public seem unaware of the benefits that they get from some hazardous processes. For example, many hazardous processes produce household products, such as detergents. The public generally perceive the risk involved in using motor

cars to be lower than the actual risk. This is because they value the advantages afforded to them by road transport, and voluntarily accept the risk when they travel by car. The benefit that the public receives from the chemical industry, and for that matter biotechnology is unappreciated. Obviously different people find different levels of risk acceptable. For example, some people go hanggliding or rock climbing, both involving high levels of risk, but expect much lower levels of risk when travelling on public transport. There is a big difference between the size of the risk that the public will accept voluntarily and the size of those that are forced upon them. Kletz (1974) suggests that if an operation has an average failure rate of less than 10^{-7}.person^{-1}.year^{-1}, the risk should be accepted, and money should not be spent on its reduction. This is equivalent to the following situation: If all the sources for death are removed from the world, except that from a particular activity, all the people exposed to that risk would have an average lifetime of 10,000,000 years.

A Harris Poll conducted by the Office of Technology Assessment in the USA (1987) found that most Americans believed that the eventual benefits of new technology will outweigh the associated risks. However the poll found that there is substantial concern about the environmental risks of genetically manipulated organisms (GMOs), plants and animals. The public realises that these are unreasonable and exaggerated fears of biotechnology, but also real risks and a need for strict regulation.

In 1993 the European Commission published a "Eurobarometer" report which found that, of the people interviewed, 48% believe that biotechnology/genetic engineering "will improve our way of life in the next 20 years". The opposite was thought by 15% of the people. Interestingly, where a significant difference was found, the term "genetic engineering" was less well known and had a more negative connotation than the term "biotechnology".

The Eurobarometer survey found a massive demand for governmental control of the various applications of biotechnology/genetic engineering. Although the research itself was though to be worthwhile and should be encouraged. Since the last survey (1991) support for the different applications analysed has dropped slightly. In Germany this drop is particularly pronounced. The risk associated with these applications has remained stationary and the demand for control has dropped slightly.

The survey found that there is no clear link between cause (knowledge) and effect (optimism). The people questioned made a realistic evaluation of their own knowledge, however, he majority found the questions asked of them complicated and had, in fact, got the answers wrong.

Slovic, Fishoff and Lichtenstein (1985) created a psychometric factor called 'dread risk', which correlated with the degree to which people wish to see risks reduced and strict regulation enforced. The dread risk for biotechnology was less than that for nuclear disaster but well above that for toxic chemicals, air pollutants, smoking and motorbikes. Goldberg and Denison (1990) suggest two reasons for people perceiving the risks involved in biotechnology as having highly undesirable qualities. These are that scientists do not agree on the hazards involved in biotechnology and that most people do not understand genetics. The public does understand that microorganisms reproduce, disperse and are associated with disease.

1.4.3 Risk Assessment

The previous discussion demonstrates that the perception of risk is extremely subjective and necessitates a quantitative approach to risk assessment. The Robens' Report (1972) suggested approaching regulation by learning from hazardous events that have happened in the past. However, the one case when this does not work is when technology is advancing rapidly, as recombinant DNA technology is. So far there have not been any serious accidents involving rDNA. A method of detecting or predicting hazards as early as possible in the design of a new plant is therefore needed.

Some quantitative methods of risk assessment are outline below. A biochemical engineering unit operation has two components which can be assessed for risk. The first, which is dealt with in this section is the risk associated with the actual processing hardware, i.e. the equipment, piping, and it's operation. The second component which has an associated risk is the organism used in the process. This biological element includes the organism, the bioactive molecules it made contain, and any recombinant DNA that has been inserted into it. A risk assessment of the organism is required by the Genetically Modified Organism (Contained Use)

Regulations (HSE, 1992). At present both HAZOP studies and Fault Tree Analysis have been applied to the biotechnology industry.

1.4.3.1 Farmer Plots

Ballard (1993) describes the contribution that Farmer (1967) has made to industrial safety analysis. Farmer first suggested the use of a plot of probability (showing the time interval between events) against consequence (the equivalent ground level release of ^{131}I). He drew a line on the diagram to define a safety criterion of permissible probability for all fault consequences. This approach has lead to societal risk being a key issue for all potential hazardous industries.

The original "Farmer diagram" was by implication, a frequency-density plot with logarithmic scales on both axes. It has been updated since 1967, so that now a risk curve is more frequently used. This plots the frequency of events (F(C)) with consequences greater than or equal to C, against C. These are commonly referred to as F-N curves as they plot frequency against number of fatalities.

The use of I^{131} release as a consequence measure, as originally proposed by Farmer (1967), has the advantage of providing a measure of plant safety independent of the location of the plant. However, consequences are now calculated in terms of human or environmental harm. This approach causes a considerable increase in calculation load, but has the benefit of showing the effects of other factor such as weather conditions, population distribution and emergency planning.

Farmer's approach to risk assessment was used in the execution of the Canvey Island Report (HMSO, 1978). This was the first attempt by the Health and Safety Executive to calculate the risks to the public from a major industrial complex. The Control of Major Accident Hazards (CMAH) legislation, introduced in 1984, required major hazard plants to produce a safety case which discussed the risks posed by the plant. This was preferred to take the form of a quantified safety analysis.

1.4.3.2 Fault Tree Analysis

This is an algorithmic formal technique for setting out strings of sequential events leading to a top event. A probability is assigned to each event. The frequency of each outcome is given by the initial frequency multiplied by the probability at each event. Fault tree analysis (Marshall, 1987) also helps as a qualitative description of failure modes. The method has some disadvantages:

1. It contains no judgements regarding the relative importance of the events described.
2. It has limited applicability to the chemical and process industries in that process malfunctioning results from a deviation from a norm, rather than a simple on/off function. Departure from the norm could be treated in a binary sense, a value is either inside or outside a predetermined range, but this requires much selective judgement and may lead to consequences that are difficult to predict.
3. Crossland *et al.* (1992) point out that the method does not give adequate allowance for human error.

Jefferis and Schlager (1986) applied a fault tree analysis to containment loss in a model bioreactor. The generic bioreactor was designed for contained operation. The containment features were divided into six subsystems: air inlet, shaft seals, sampling, harvesting, inoculation and air exhaust. For each subsystem, a sequence of valves must be operated correctly for sterile operation under fully contained conditions. Reliability estimates were then calculated for each of the components, using an exponential probability distribution.

Using approximate data for component failure rates, the relative benefits of three different operating regimes were evaluated. The three regimes were: manual operation of valves, automatic operation of valves and in the last regime it was assumed that the diaphragm valves were changed every 6 months. A leak was defined as occurrence of an open pathway from the fermenter to a non controlled area. This fault tree covered the growth phase in the fermenter only. Another fault tree would be written for sterilisation and cleaning, or product loss through failure of control instruments. In this study, operator error was shown to be the dominant cause of possible failure in the system.

1.4.3.3 Hazard and Operability Studies

Hazard and operability studies (HAZOP) were devised by ICI Ltd and first described by Lawley (1974). They were defined by the Institution of Chemical Engineers (1984) as "a study carried out by the application of guide words to identify deviations from design intent (and) with undesirable effects for safety and operability". Guide words are defined as "lists of words applied to the system items or functions in a hazard study to identify undesired deviations".

HAZOP is based on the examination of individual process items on a flow sheet. The guide words are no (not), more, less, as well as, part of, reverse, other than, and in some instances: sooner, later, higher, lower. The consequences that would arise from these deviations are then assessed in relation to safety and plant operability. The analysis is both diagnostic and remedial. Marshall highlights one of its drawbacks as the considerable demands it makes upon manpower. Items that are not included on the list do not get checked in the study. HAZOP does not provide a method to quantify risks, it can only identify them, all that is claimed of it is that it has been successful in identifying hazards that had not been uncovered in the traditional course of design. However, there is no way to consider risks arising from unforeseen hazards, for example, those arising from a technique that has not been used previously.

Genhaz was devised as a system equivalent to HAZOP but for biological processes. It was described by the 14th Royal Commission on Environmental Pollution (Report, 1991). Genhaz aims to generate possible consequences and assess their acceptability. A multidisciplinary team reviews the individual elements of a planned release, to force an exploration of possible hazards and an evaluation of the associated risks. It takes the form of a series of questions about the release, a description of the rDNA involved and a set of guide words which are used to answer the questions.

Genhaz was devised to highlight the risks involved in the deliberate release of GMOs into the environment, and as such is not appropriate for assessing the risks involved in accidental release from bioprocessing operations. An approach based on HAZOP, with the possible addition of some extra guide words, as carried out by Gurnsey *et al.* (1990) seems a much more useful exercise.

Gurnsey *et al.* (1990) describe how a HAZOP study was carried out on a 1000L fermenter in a contained bioprocessing facility. The design team consisted of a scientist-in-charge, a project designer/engineer, a research microbiologist, and an independent chairman. They either looked at the fermenter directly or at photographs, although they conclude that the photographs were of limited use. It should be pointed out that HAZOP studies were intended to be carried out during the design stage of a new plant or piece of equipment, using the process flow diagrams. The inlet lines included in the study were: steam, water, compressed air, and inoculum. The outlet lines included were: outlet gases, fermenter broth, clean drain and dirty drain. Each line was studied with the guide words of Roach and Lees (1981). The situations where deviations could occur were noted, including the circumstances in which the deviations occur and their consequences.

The study showed many of the guide words to be inappropriate. For example, for the steam inlet to the fermenter, zero flow/empty was the only deviation thought to have potentially serious consequences. These included failure of sterilisation of equipment and possible failure of stirrer bearings (steam is used as a lubricant). Actions required were to re-sterilise the equipment and replace the bottom bearings. Although it is not possible to prevent this deviation occurring, the resulting damage could be limited by installation of an alarm.

A second example is the gas outlet line from the fermenter. High pressure in this line was thought to be hazardous. Blockage of the gas line with stable foam may result in increased pressure inside the vessel. If the pressure continued to rise the safety relief valve would operate, releasing the fermenter broth and gases into a dirty drain. If the safety valve failed the rupture disc would burst, causing a breach of primary containment. Some actions that would prevent this release are: installation of a foam detector in the gas outlet line and modification of the rupture disc deflector assembly to try to contain the broth when the disc ruptures. The deflector tube could also be connected to a vessel large enough to contain all possible escaped broth.

An overview was then carried out applying the guide words suggested by Tweedale, (1984) to the plant:

1. Toxicity - Gurnsey *et al.* (1990) suggest "pathogenicity" "biohazard" instead.
2. Breakdown - In the event of a power failure the boiler, air compressor, pumps, HEPA filters and computer shut down in a controlled and contained way. If one

HEPA filter fails, a second independent filter will operate. If the whole system fails fumigation is necessary.

3. Shutdown - Tweedale suggests the word "purging" but Gurnsey *et al.* (1990) find "sterilising" more appropriate. Heat sterilisation should be satisfactory for killing GMOs, although this should be validated. Fumigation may be required if primary containment is breached.

4. Effluent - The exit gas is sterile filtered. The cooling water from the fermenter jacket exits to a clean drain, all other liquid waste goes to a kill tank. All solid waste is autoclaved.

5. Emergency - Explosion may occur as a result of vessel overpressure. If the primary containment fails the floor is capable of holding the fermenter contents.

Gurnsey *et al.* suggest including "aerosol" and "containment" as extra guide words. They conclude that it is possible to successfully carry out a HAZOP study on a contained bioprocessing facility.

Microbial release from bioprocesses can occur either as an accident, due to equipment failure or operator error, or incidentally when the process is run under normal operating conditions. All of the risk assessment methods described in this section consider microbial release through accidents only. None of the methods include the quantification of the incidental release of microorganisms. However, in this study incidental release during the normal operating conditions is demonstrated to be very low, and it is suggested that the emphasis should remain on preventing accidental release.

CHAPTER TWO: METHODS AND MATERIALS

2.1 Addresses of Suppliers

Air Control Installations Ltd, Boden St, Chard, Somerset, TA20 2AE, UK.

Aldrich Chemical Company, New Rd, Gillingham, Dorset, SP8 4JL, UK.

Applied Biosystems, Kelvin Close, Birchwood Science Park, North Warrington, Cheshire, WA3 7PB, UK.

APV (UK) Ltd, Manor Royal, Crawley, West Sussex, RH10 2QB, UK.

Bassaire, Duncan Rd, Swanwick, Southampton, UK.

BDH, Merck Ltd, Merck House, Poole, Dorset, BH15 1TD, UK.

Bio-Orbit Oy, P.O. Box 36, 20521, Turku, Finland.

Bio-Rad Laboratories Ltd, Bio-Rad House, Marylands Avenue, Hemel Hempstead, Herts, HP2 7TD, UK.

Biral, PO Box 2, Portishead, Bristol, BS20 9JB, UK.

Casella London Ltd, Wolseley Rd, Kempston, Bedford, UK.

Difco Laboratories, Detroit, Michigan, USA.

Elwyn E Roberts Isolators Ltd, Ellerton House, Wistanwick, Market Drayton, Shropshire, TF9 2BT, UK.

Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leicestershire, LE11 0RG, UK.

FMC Bioproducts, 191 Thomaston St, Rockland, ME 04841, USA.

Hays Chemical Distribution Ltd, Rawdon House, Yeadon, Leeds, LS19 7XX, UK.

Hybaid Ltd, Waldegrave Rd, Teddington, Middlesex, TW11 8LL, UK.

IncelTech (UK) Ltd, 22A Horseshoe Park, Pangbourne, Reading, RG8 7JW, UK.

Molecular Medicine Unit, Kings' College School of Medicine and Dentistry, Rayne Institute, 123 Coldharbour Lane, London, SE5 9NU, UK.

Oxoid, Unipath Ltd, Wade Rd, Basingstoke, Hampshire, RG24 0PW, UK.

Pacific Scientific, Marlow, UK.

Pall Europe Ltd, Europa House, Havant Street, Portsmouth, PO1 3PD, UK.

Phillips Scientific, Cambridge, UK.

Pharmacia, Biotech Ltd, Davey Ave, Knowlhill, Milton Keynes, MK5 8PH, UK.

Pike and Sons, London, UK.

Sigma Chemical Company Ltd, Fancy Rd, Poole, Dorset, BH17 7BR, UK.

Sonco, Woodland Rd, Upper Batley, Batley, W. Yorkshire, WF17 0RE, UK.

Timart Precision, Dunstable, Beds, UK.

TSI, 500 Cardigan Rd, P.O. Box 64394, St. Paul, MN, 55 164, USA.

Warren Spring Laboratory, Gunnels Wood Rd, Stevenage, SG1 2BX, UK. (Now at AEA Technology, Biotechnology Services, 353 Harwell, Didcot, Oxfordshire, OX11 0RA, UK).

Watson-Marlow Ltd, Falmouth, TR11 4RU, UK.

Weber Scientific Ltd, Udney Park Rd, Teddington, Middlesex, TE11 9BG, UK.

2.2 Materials

2.2.1 Microorganisms

Saccharomyces cerevisiae was originally obtained in block form (Pike and Sons) and suspended in Ringers' solution. *Escherichia coli* strain NCIMB 86 was a gift from Warren Spring Laboratory. Transketolase *E. coli* strain JM107 containing plasmid pQR 701 was obtained from John Ward, Biochemistry Dept., UCL.

2.2.2 Media and Chemicals

i.) Agar

Tryptone soya agar (Oxoid)	$40g.L^{-1}$
Malt extract agar (Oxoid)	$50g.L^{-1}$
Nutrient agar, containing:	
Nutrient broth N^{o} 2 (Oxoid)	$25g.L^{-1}$
Bacto-Agar (Difco)	$20g.L^{-1}$

These were prepared using RO water and autoclaved at 121^{o}C for 15 minutes. Sterile filtered (0.2μm pore size) kanamycin (Sigma) was added to the autoclaved nutrient agar at a concentration of $20mg.L^{-1}$.

ii.) Broths

Tryptone soya broth (Oxoid)	$30g.L^{-1}$
Malt extract broth (Oxoid)	$20g.L^{-1}$
Nutrient broth N^{o}2 (Oxoid)	$25g.L^{-1}$

These were prepared using RO water and autoclaved at 121^{o}C for 15 minutes. Sterile filtered (0.2μm pore size) kanamycin was added to the autoclaved nutrient broth at a concentration of $10mg.L^{-1}$.

Luria Broth, containing:

Tryptone (Oxoid)	$10g.L^{-1}$
Yeast extract (Oxoid)	$5g.L^{-1}$
NaCl (AnalaR, BDH)	$5g.L^{-1}$
NaOH pellets, (GPR, BDH)	$1mL.L^{-1}$

This was prepared using RO water and autoclaved at 121°C for 20 minutes.

YEP Broth, containing:

Yeast extract (Oxoid)	$10g.L^{-1}$
Peptone (Oxoid)	$20g.L^{-1}$
Glucose (BDH)	$20g.L^{-1}$

The yeast extract and peptone were autoclaved together at 121°C for 20 minutes in the fermenter pot. The glucose solution was prepared and autoclaved separately at 121°C for 20 minutes.

iii.) Chemicals

Thiosulphate Ringers' solution (Oxoid) 2 tablets.L^{-1}.

This was prepared using RO water and sterilised at 121°C for 20 minutes.

3M Sodium hydroxide solution (prepared using pellets from BDH) was prepared using RO water and autoclaved at 121°C for 20 minutes. This alkali was used for fermentation pH control.

2.5M Sulphuric acid (BDH) was autoclaved at 121°C and also used for fermentation pH control.

iv.) Reagents for the Polymerase Chain Reaction (PCR)

The experiments carried out using the reagents listed here are described in sections 3.7, 4.2.3 and 4.3.2.

Deoxynucleotide triphosphates (dNTPs) (Pharmacia):

This is a ultra pure (pH 7.76) mixture of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate and deoxycytidine triphosphate. These were stored at -20°C as a stock solution of concentration 100 mM in sterile distilled water. They were used at a concentration of 1.25 μM of each in the reaction.

AmpliTaq DNA polymerase (Applied Biosystems):

This enzyme catalyses the extension of DNA molecules. It was stored at -20°C at a concentration of $5 \times 10^3.mL^{-1}$.

Primers (Molecular Medicine Unit, Kings' College School of Medicine and Dentistry).

These were diluted from the stock solutions to a final concentration of 20 μM using sterile distilled water and stored at -20°C.

The primers used include:

KM 1 and KM 2, a set of primers which bind to sequences in the kanamycin resistance gene.

M13 Forward and Reverse, a universal primer set which bind to sequences in the multiple cloning site in pUC plasmids.

LF1, the primer designed in this study to bind to the non-coding region following the cmtA gene in the pQR 700 and pQR 701 plasmids. It is designed to work in conjuction with either M13 F or R.

Primer cmtB1 bound to a site on the cmtB gene. The amplified sequence produced by cmtB1 and M13 Reverse was approximately 350 base pairs long and crossed the site of insertion of the transketolase encoded gene.

The structure of pQR 701 (the transketolase plasmid constructed by French and Ward (1992)), is shown in figure 3.10, in the next chapter.

Primer Sequences

KM1	510	5' TGA CTC ATA CCA GGC GTG AA 3'	529
KM2	1592	5' TAC AAG GGG TGT TAT GAG CC 3'	1573

M13 forward	5' G TAA AAC GAC GGC CAG 3'
M13 reverse	5' CAG GAA ACA GCT ATG AC 3'
LF1	5' CGA TCG GTA ATA CAG ATC 3'
cmtB1	5' CGTCAAAGAGTGTATTGAGG 3'

PCR Buffer I, containing:

KCl (Sigma)	500mM
Tris-HCl @ pH 8.3 (Sigma)	100mM
$MgCl_2$ (Aldrich)	15mM
Gelatin (Sigma)	0.01% (w/v)

This was sterilised by autoclaving at 121°C for 20 minutes and stored at -20°C. This stock of 10x buffer was diluted to 1x in the reaction mixture.

PCR Buffer II, containing:

KCl	500mM
Tris-HCl (pH 8.3)	100mM

This was sterilised by autoclaving at 121°C for 20 minutes and stored at -20°C. This stock of 10x buffer was diluted to 1x in the reaction mixture.

TBE Buffer, containing:

Trizma base (Sigma)	$108g.L^{-1}$
Boric acid (Sigma)	$55g.L^{-1}$
0.5 M EDTA @ pH 8.0 (Sigma)	$7.44g.L^{-1}$

This was prepared using RO water and titrated to pH 8.2 with NaOH pellets.

v.) Bio-Rad Protein Assay

The Bio-Rad protein assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The absorbance maxima for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Reisner *et al.*, 1975). The dye reagent was diluted 1 in 5 using deionised water, and filtered through Whatman No.1 paper. It can be stored at room temperature for up to 2 weeks.

The Bio-Rad protein standard consisted of lyophilised bovine albumin. It was reconstituted with 20 mL of distilled water, yielding a concentration of 1.4 $mg.mL^{-1}$.

2.2.3 Containment Cabinets

Bassaire Cabinet

This cabinet (manufactured by Bassaire) has a volume of 360L and is shown in figure 2.1. Air enters and leaves through opposite faces of the cabinet at a flow rate of $750L.min^{-1}$. Both the air inlet and outlet are fan assisted high efficiency particulate air (HEPA) filters. Flow through the cabinet is laminar. There is a fan

in the ceiling of the cabinet which can be used to mix the air, when the inlet and outlet fans are stopped.

Soft Film Cabinet

This cabinet (manufactured by A. C. and E. Isolation Systems, now trading as Elwyn E Roberts Isolators Ltd) has a volume of $8.37M^3$ and is shown in figure 2.2. It is constructed of heavy duty PVC film suspended from a metal frame. It has a fan assisted inlet only which draws air in at $40L.min^{-1}$, and is designed to operate under a positive pressure. Although the air inlet and outlet are on opposite faces of the cabinet the air flow is not laminar.

Bioaerosol Test Chamber (BTC)

This cabinet (figure 2.3) was designed by D. Griffiths at AEA Technology and was constructed by Timart Precision. It has a volume of 2.5 M^3 and has both temperature (15-25^oC) and humidity (30-90^oC) controls. Air enters at the top of the cabinet at 3.6 $M^3.min^{-1}$ and leaves from the bottom. The air is mixed in the centre of the cabinet by an impeller rotating at 2 RPM and is "straightened" by passing through a honeycomb layer.

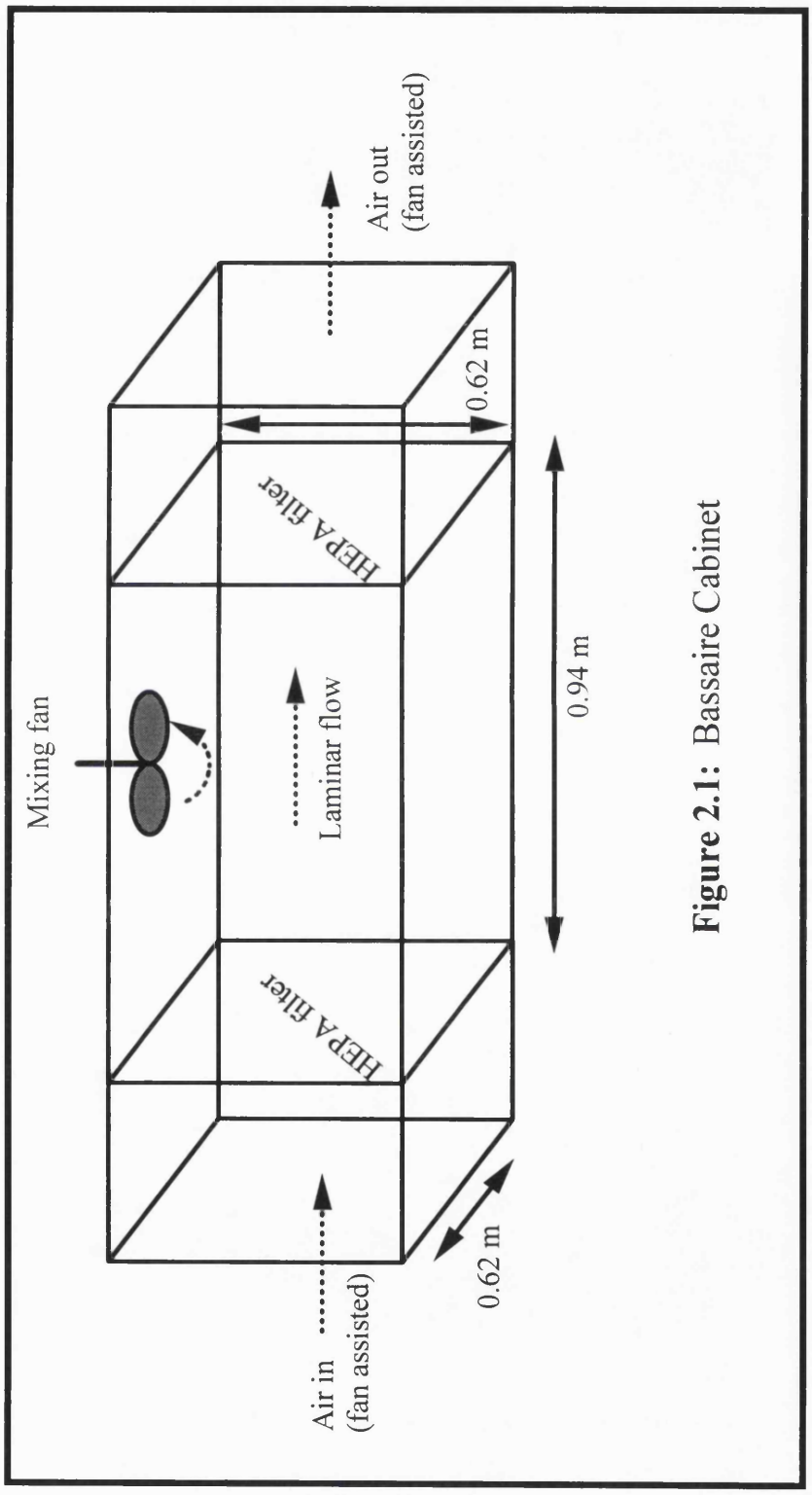


Figure 2.1: Bassaire Cabinet

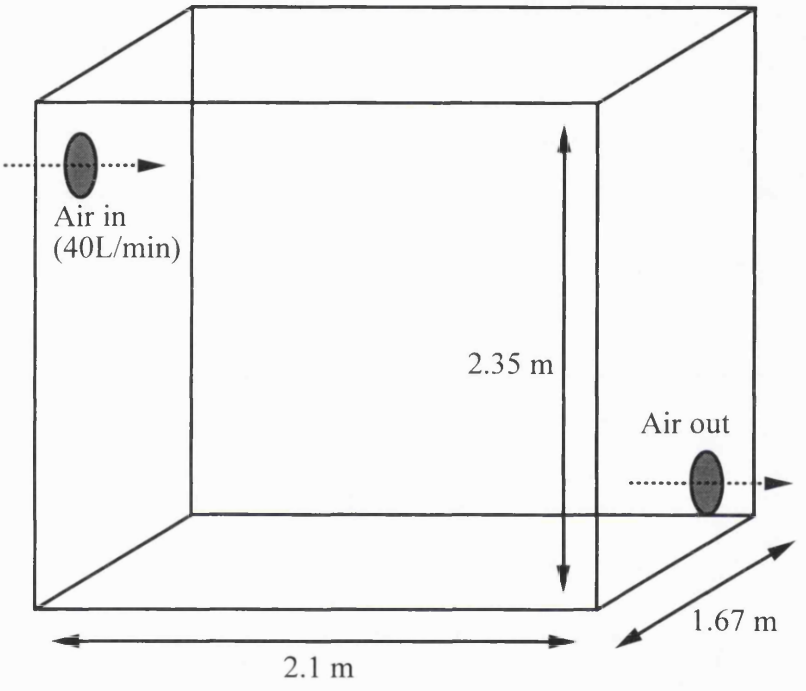


Figure 2.2: Soft Film Cabinet

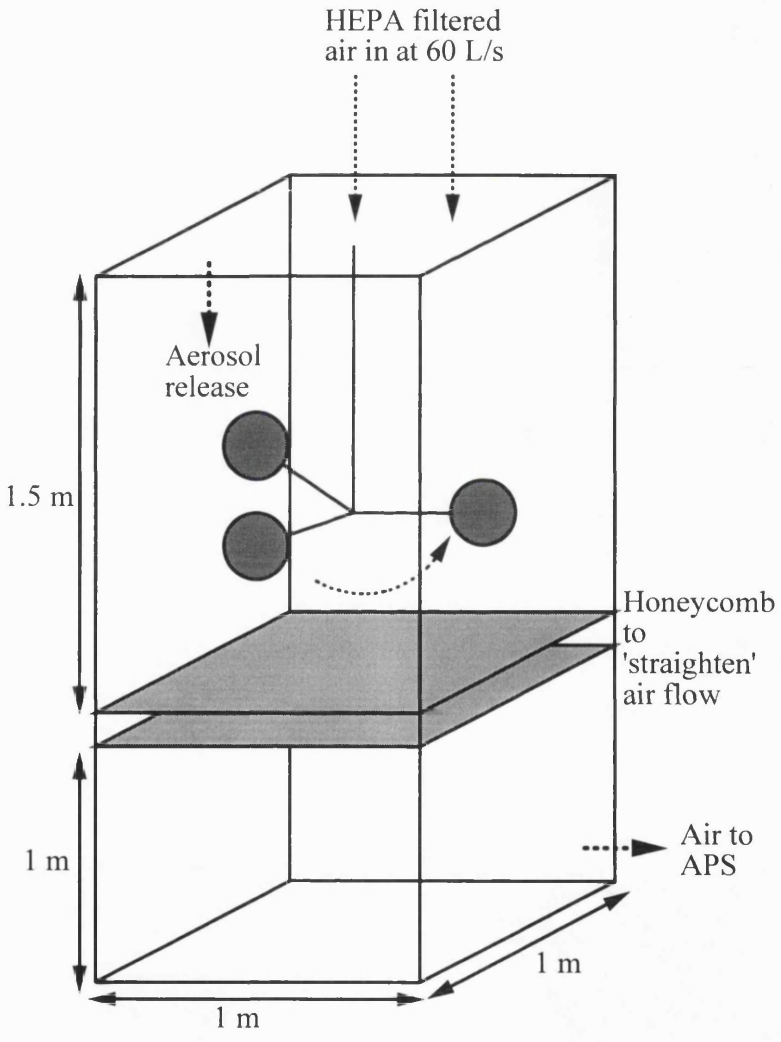


Figure 2.3: Bioaerosol Test Chamber

2.2.4 Atomisers

Collison Nebuliser

The Collison nebuliser (supplied by Biral) is an effective devise to generate aerosols from liquids. The modified MRE type (figure 2.4), used here, was specially designed for use with microorganisms and was described by May in 1972. It forces a liquid through 6 fine holes and causes it to hit the glass wall of the nebuliser. The resultant aerosol is forced out of the opening at the top of the nebuliser. Compressed air at a pressure of 20 psig is used to operate the nebuliser.

Glass Atomiser

This atomiser (Warren Spring Laboratory), shown in figure 2.5. consists of two concentric tubes, one for compressed air and the other for the liquid to be aerosolised. The aerosol is formed by the Bernoulli effect. As the gas leaves the atomiser it's velocity increases and pressure decreases. This draws the liquid from the tube and the resultant turbulence creates aerosol particles. A peristaltic pump (Watson-Marlow Ltd) is used to supply the cell suspension to the atomiser, at a flow rate of approx. 2mL.min^{-1}.

2.2.5 Cyclone

This is an Aerojet-General cyclone (Warren Spring Laboratory) and is shown in schematic form in figure 2.6. It operates at either 360L.min^{-1} using an air pump from Air Control Installations Ltd, or 750L.min^{-1} using an air pump from Casella London Ltd. The collection liquid, Ringers' solution, is recycled at a flow rate of approx. 20 mL.min^{-1} using a peristaltic pump (Watson-Marlow). The recycled collection liquid forms a jet across the air inlet of the cyclone. Airborne particles in the incoming air stick to the walls of the cyclone. These are then washed off by the liquid and thus the concentration of particles in the collection liquid increases with sampling time. The operation of the cyclone is discussed further in section 2.4.3.3. This cyclone was described by May *et al.* in 1976.

2.2.6 Fermenter Head-Space Sampler

This connection (figure 2.7) was designed by M. K. Turner and manufactured by the Glassblowers in the Chemistry dept. at University College London. Air from the fermenter head-space combines with room air and then enters the cyclone.

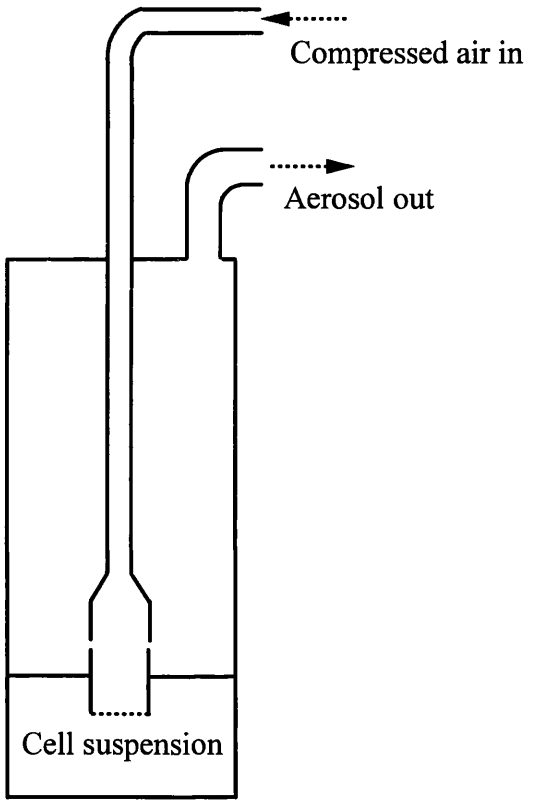


Figure 2.4: Collison Nebuliser

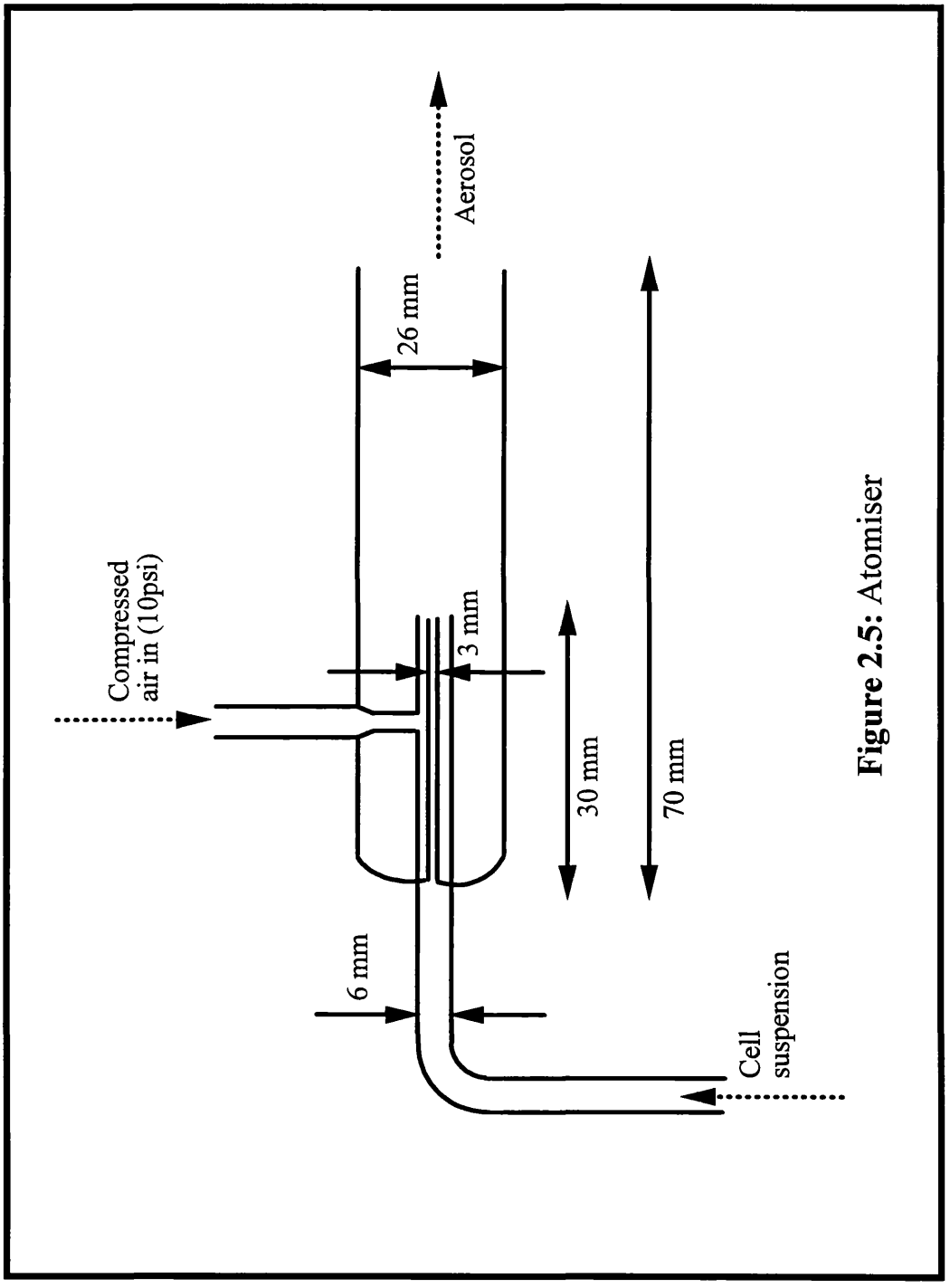


Figure 2.5: Atomiser

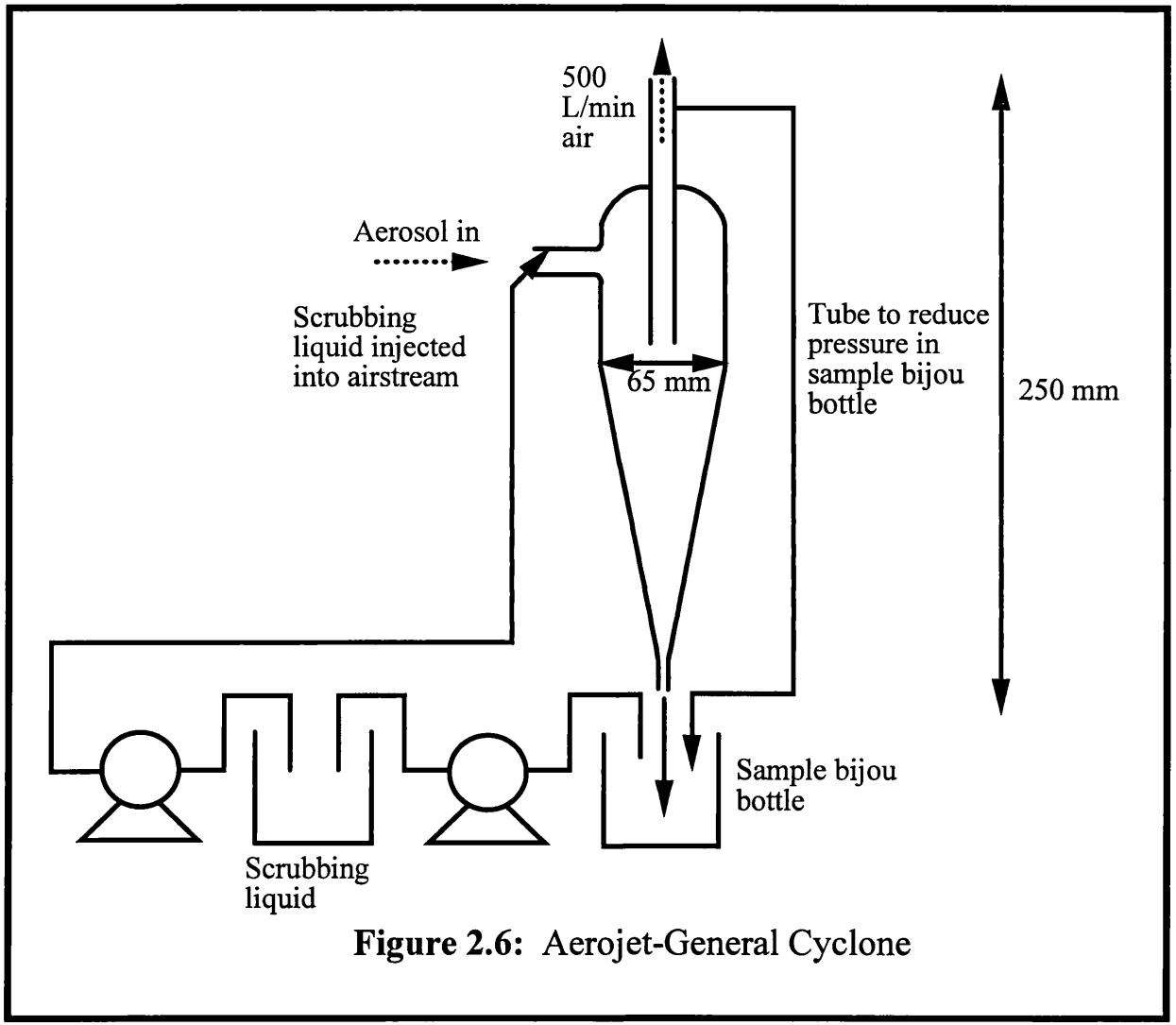


Figure 2.6: Aerojet-General Cyclone

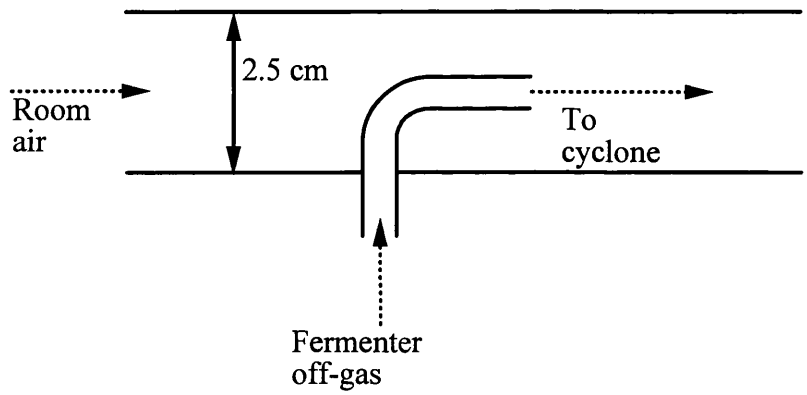


Figure 2.7: Connection for Fermenter Head-Space Sampling

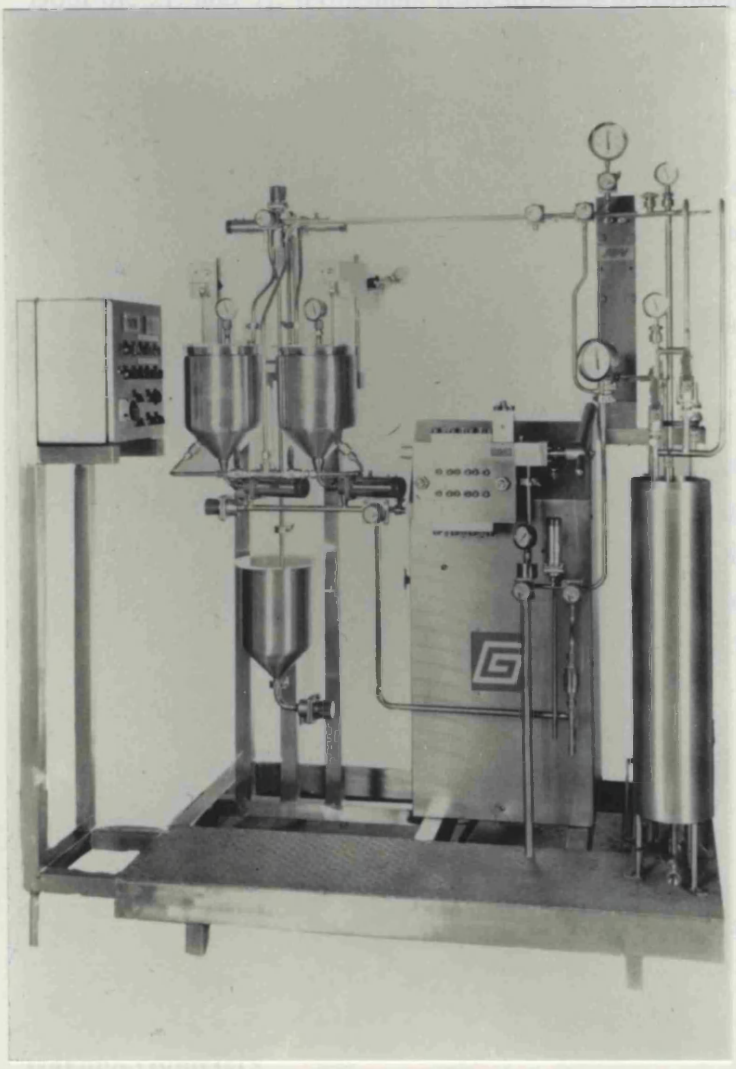
2.2.7 APV 30CD Homogeniser

The 30CD (APV (UK) Ltd) homogeniser contains a positive displacement, high pressure reciprocating pump (figure 2.8). When operating, the pressure in the pumping chambers varies from zero, during the suction stroke, to full pressure during the discharge stroke. The ceramic valve seat plugs into the cylinder and the valve body and is sealed by self-energising O-rings. The O-rings are backed by tough back-up rings to reduce extrusion. The valve is held by the valve guide and is pressed against the seat by a hand wheel, which is adjusted to vary a spring force acting on the valve rod.

This high pressure homogeniser has several features which make it suitable for contained bioprocessing: It has a triple action ram pump which allows pressures of 100MPa to be achieved. It is sealed with double self-energising elastomeric O-rings, to prevent aerosol production during a failure at high pressure. The O-rings and back-up rings are also counter bored, so that material leaking past the first must turn through 90^{o} to reach the second seal.

The 30CD has a simple system for detecting liquid leaks consisting of a pair of O-rings with a channel between them. Leakage past the first O-ring enters the channel, which connects to a length of transparent tubing through which the leaked material may be observed. The ram pump pistons (see figure 4.4) have secondary seals to prevent leakage from the primary packing. However if this packing deteriorates, the piston lubrication/cooling fluid will become contaminated, and it is for this reason the homogeniser has a lubrication/cooling fluid supply separate from that of any other equipment in the plant. The design of homogenisers for contained bioprocessing is discussed further in section 4.1.3.3.

Figure 2.8: APV 30CD Homogeniser



2.2.8 Fermenters

Both the 2L and 7L fermenters used in these experiments were manufactured by IncelTech (UK) Ltd. The 2L fermenter had a 6 blade Rushton turbine impeller and a working volume of 1.5L. During cell growth, the air entering the fermenter was prefiltered with a Gelman Acro50 filter (Fisons) with a pore size of 0.2μm. Air leaving the fermenter was passed through a condenser and then another Gelman Acro50 filter (0.2μm). The 2L fermenter had an airflow rate range of 20-500 $mL.min^{-1}$ and a stirrer speed range of 50-1500 rpm. The growth medium was sterilised in the fermenter pot by autoclaving at 121°C for 20 minutes.

The 7L fermenter had a 6 blade Rushton turbine impeller and a working volume of 5L. Air entering the fermenter was filtered through a Gelman Acro50 0.2μm filter. The air leaving the fermenter was passed through a condenser and then filtered with a Pall 0.2μm pore size, EMFLON II cartridge filter. The fermenter had an air flowrate range from 0.5-7.0 $L.min^{-1}$ and a stirrer speed range of 50-1200 rpm. Sterilisation of the growth media was achieved by steam heated internal coils.

2.2.9 Other Equipment

Conductivity meter

This is a temperature-compensating conductivity meter, model PW 9527 (Phillips Scientific).

Haemocytometers

A haemocytometer with Improved Neubauer ruling (figure 2.9) was used to count *Saccharomyces cerevisiae*. A Helber bacteria counting chamber with Thoma ruling (figure 2.10) was used to estimate *Escherichia coli* concentrations. Both were obtained from Weber Scientific Ltd.

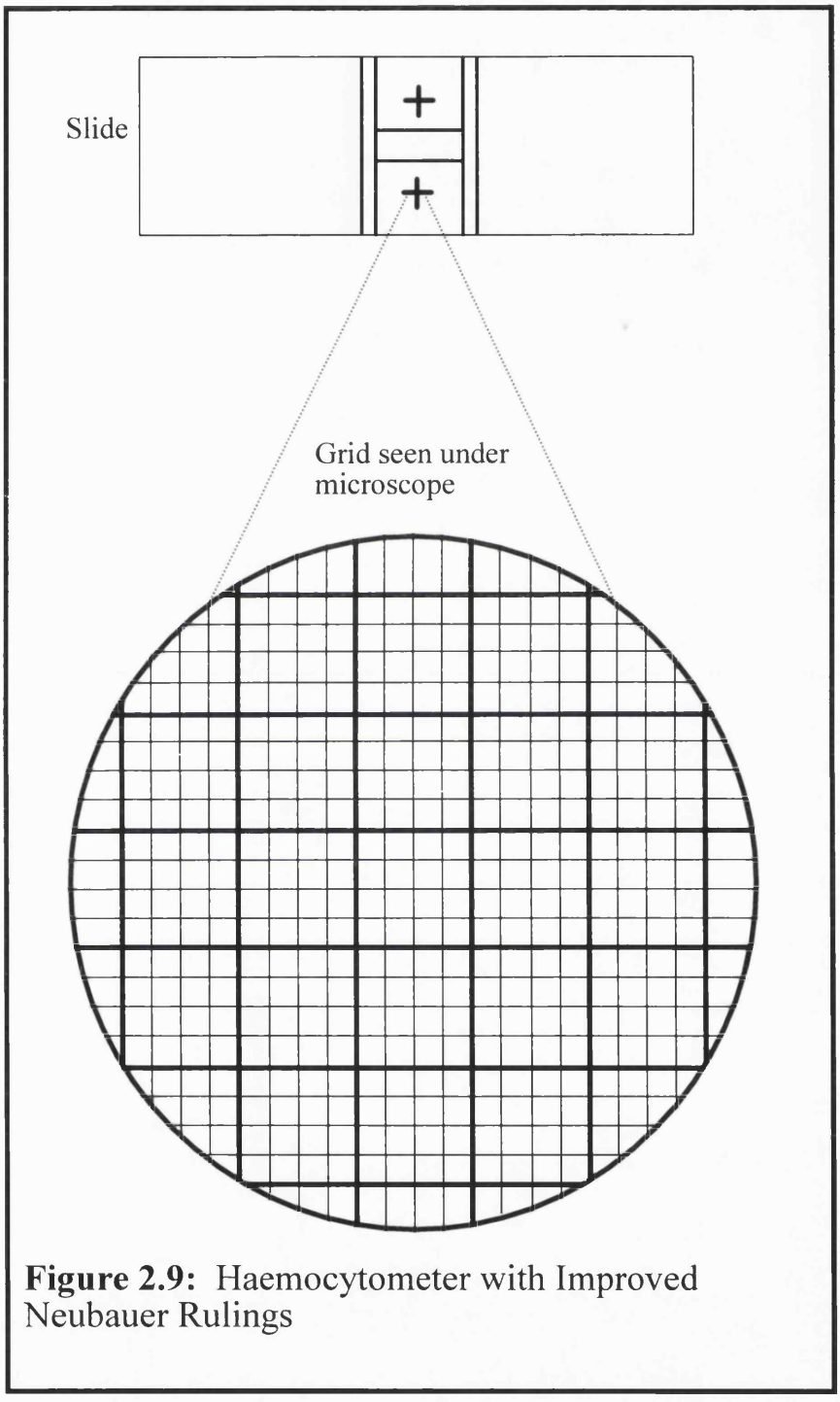


Figure 2.9: Haemocytometer with Improved Neubauer Rulings

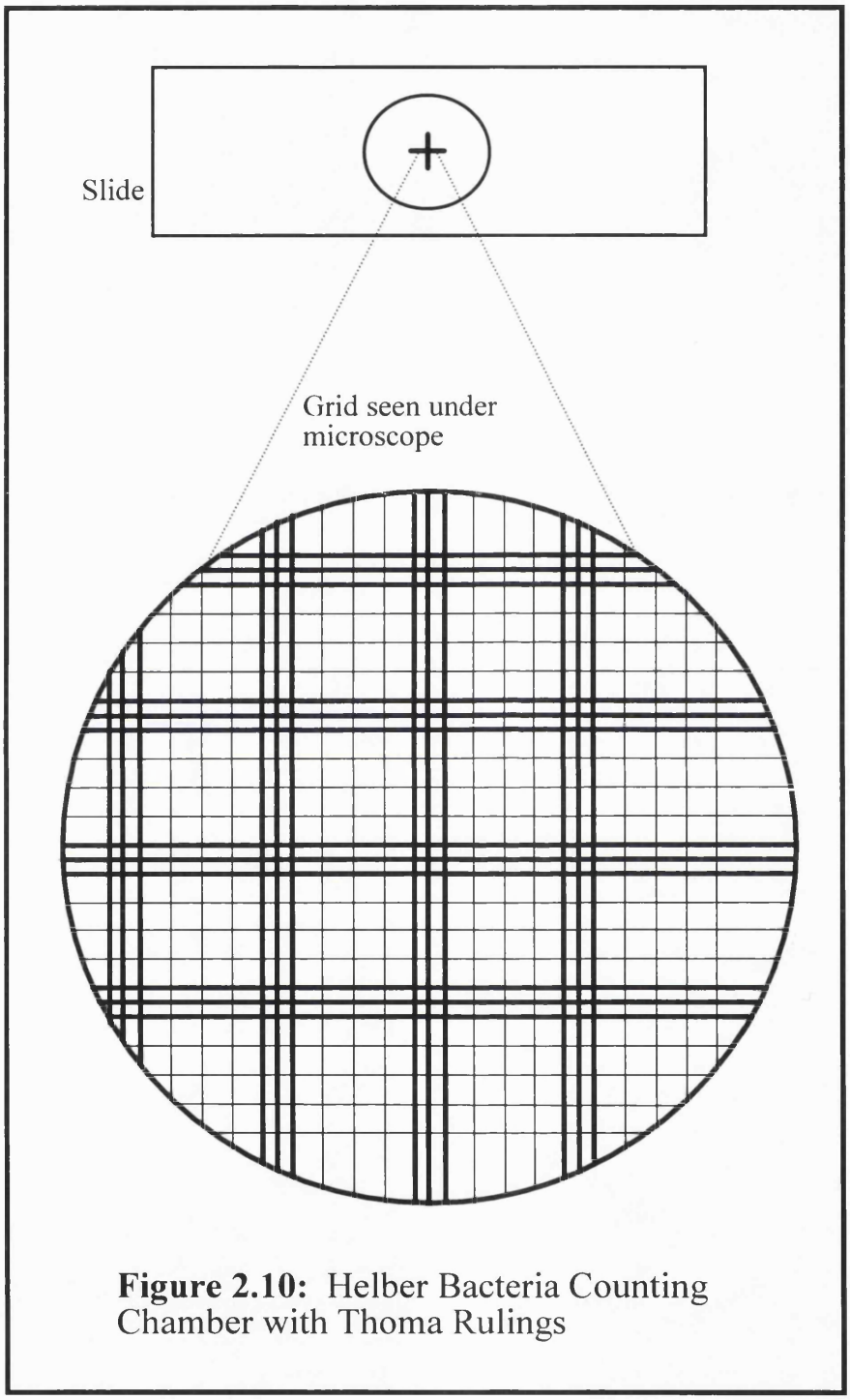


Figure 2.10: Helber Bacteria Counting Chamber with Thoma Rulings

HIAC Royco Particle Counter

The HIAC/Royco (Pacific Scientific) system 4102 counts airborne particles by an optical detection method. The flowrate of the sampled air is controlled by an orifice plate. The air flow is limited to the speed of sound by applying a low pressure. The particles pass through the detector beam singly. The amount of forward scattered light is sensed by a photo detector. The signal amplitude is proportional to particle size. Six size fractions are recognised: up to 1.5μm, 1.5-3.0μm, 3.0-5.0μm, 5.0-10.0μm, 10.0-15.0μm. The air sampling rate is 2.83 $L.min^{-1}$.

Aerodynamic Particle Sizer (APS)

The model 33B APS (manufactured by TSI) measures the aerodynamic diameter of particles. The measurement automatically takes into account particle shape, size and density. The APS takes in air at a flow rate of 5 $L.min^{-1}$ and particles in the size range 0.5 to 30μm are detected. The measurement is obtained by accelerating aerosols through a nozzle between two laser beams. The time taken for a particle to travel this distance is proportional to its size. All the output from the APS is sent to a PC. The APS is described further in Remiarz *et al.* (1983).

Laser Particle Counter (LPC)

The LPC (manufactured by TSI) sorts particles into two size ranges, >0.5μm and >5.0μm. Light from a laser diode is shaped by lenses and an aperture into a thin plane. Particles pass through the illuminated volume and scatter light. A proportion of the forward scatter light forms an image on a photo detector. The photo detector converts light into an electrical pulse, the amplitude of which is proportional to particle size.

OmniGene Thermal Cycler

This thermal cycler (manufactured by Hybaid Ltd) provides repeated heating and cooling of samples to programmed temperatures for DNA denaturation, primer annealing and DNA extension. Temperature control is via a thermistor in a dummy sample tube. The temperature ramping option precisely controls the rate of change of sample temperature in seconds.$^{o}C^{-1}$.

2.3 Methods

2.3.1 Preparation of Cells

A colony of *S. cerevisiae* cells grown on a malt extract agar plate was inoculated into 100mL of malt extract broth in a 1L baffled shake flask. This flask was then incubated at 28°C for 48 hours in a reciprocal shaker, set at 150 rpm. Cells were harvested in a Beckman J2-MI centrifuge at 2744g for 20 minutes, re suspended in either sterile Ringers' solution or the spent fermentation broth and stored in the fridge.

A colony of unmodified *E. coli* cells was removed from an agar plate and initially grown up in 5 mL of sterile Luria broth for approximately 6 hours. This was then used to inoculate 100mL of Luria broth. Baffled shake flasks (1L total volume) were incubated at 37°C for 24 hours in a reciprocal shaker set at 150 rpm. Cells were harvested in a Beckman J2-MI centrifuge at 2744g for 20 minutes, re suspended in either sterile Ringers' solution or the spent fermentation broth and stored in the fridge.

Transketolase *E. coli* cells were grown in nutrient broth supplemented with kanamycin, the same procedure described above for unmodified *E. coli* cells (NCIMB 86).

2.3.2 Cabinet Preparation

2.3.2.1 Bassaire Cabinet

Before an experiment was carried out, the inside of the cabinet was cleaned by swabbing with 70% ethanol. Petri dishes containing 15 mL of sterile Ringers' solution were left uncovered on the floor of the cabinet. The cyclone and atomiser (or nebuliser) were attached to the cabinet. The mixing fan in the ceiling of the cabinet was switched on. The inlet and outlet air fans were switched on until the HIAC Royco showed that there were no airborne particles in the cabinet. The outlet filter was then switched off before continuing with the experiment.

2.3.2.2 Soft Film Cabinet

Five Petri dishes containing 15 mL of sterile Ringers' solution were left uncovered on the floor of the cabinet. The cyclone was set up inside the cabinet. The inlet air fan was switched on and the outlet air filter unblocked. Air was flushed through the cabinet for 1 hour, and a positive pressure created inside the cabinet. The outlet air filter was then blocked.

2.3.3 Spraying and Collecting

2.3.3.1 Bassaire Cabinet

The mixing fan in the ceiling of the cabinet was switched on. An air pressure of 10 psi was used to aerosolise approximately 20 mL of suspension (*S. cerevisiae*, *E. coli* or 1M NaCl) from the atomiser into the cabinet. Cell suspension was then followed by 5 mL of Ringers' solution to wash residual cells from the atomiser and tubing. The cyclone was used to sample the cabinet air at a flow rate of 360 or 750 $L.min^{-1}$. Sampling was carried out at the same time as spraying and continued for an extra 10 minutes after spraying finished, in most cases. The aerosolised cells were collected into a final volume of approximately 50mL of Ringers' solution in the cyclone. Aerosolised NaCl was collected into distilled water. Figure 2.11 shows the positions of the atomiser and cyclone during the mass balancing experiments.

2.3.3.2 Soft Film Cabinet

An air pressure of 10 psi was applied to the atomiser to aerosolise approximately 20 mL of cell suspension (*S. cerevisiae* or *E. coli*) into the cabinet. This suspension was followed by 5 mL of Ringers' suspension. The cyclone sampled the cabinet air at a flow rate of 360 $L.min^{-1}$. Sampling continued for up to 10 minutes from the end of spraying. The air outlet of the cyclone was exhausted out of the cabinet. The sampled cells were collected into a 50 mL volume of Ringers' solution in the cyclone. Figure 2.12 shows the position of the atomiser and cyclone during these mass balancing experiments.

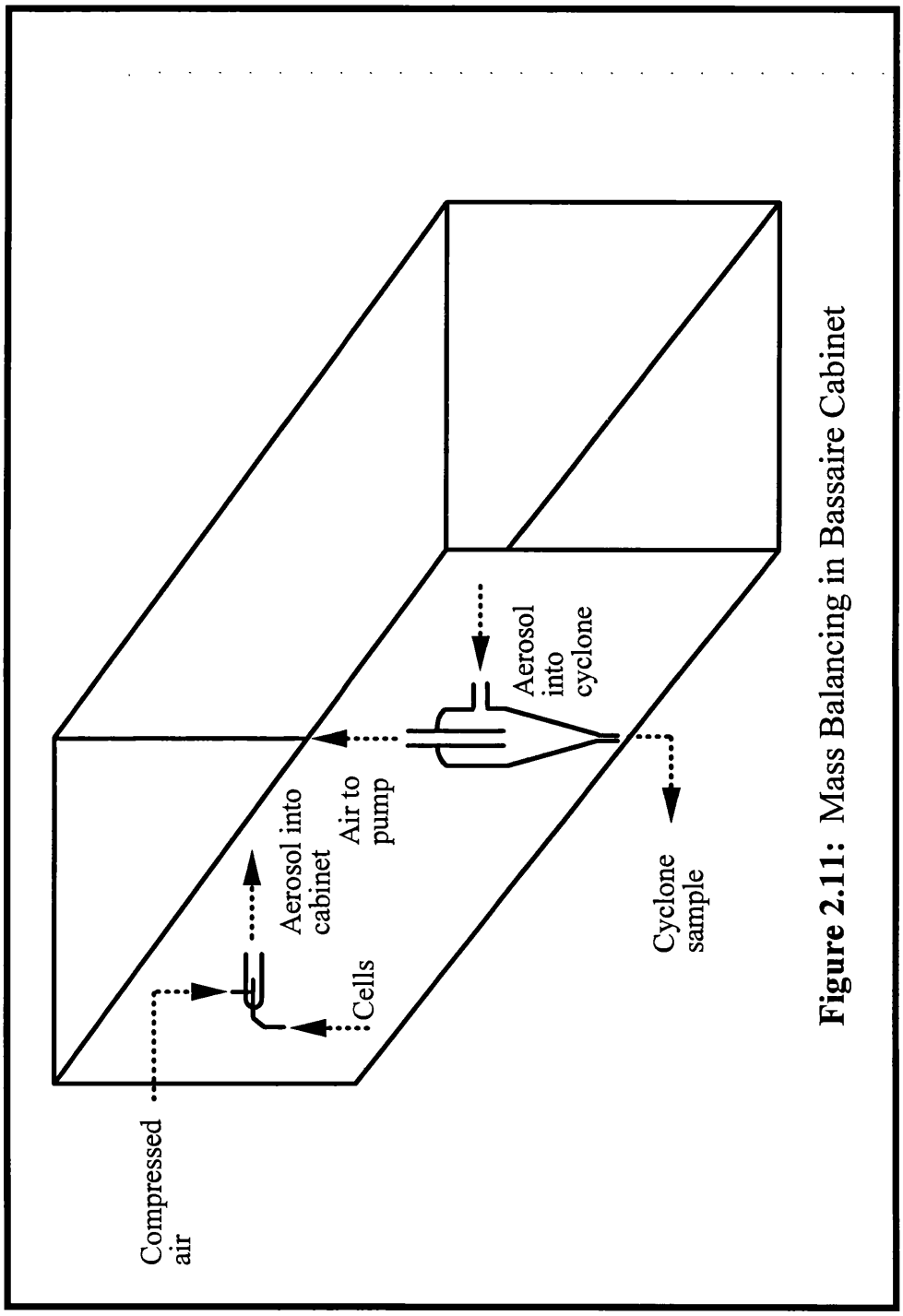


Figure 2.11: Mass Balancing in Bassaire Cabinet

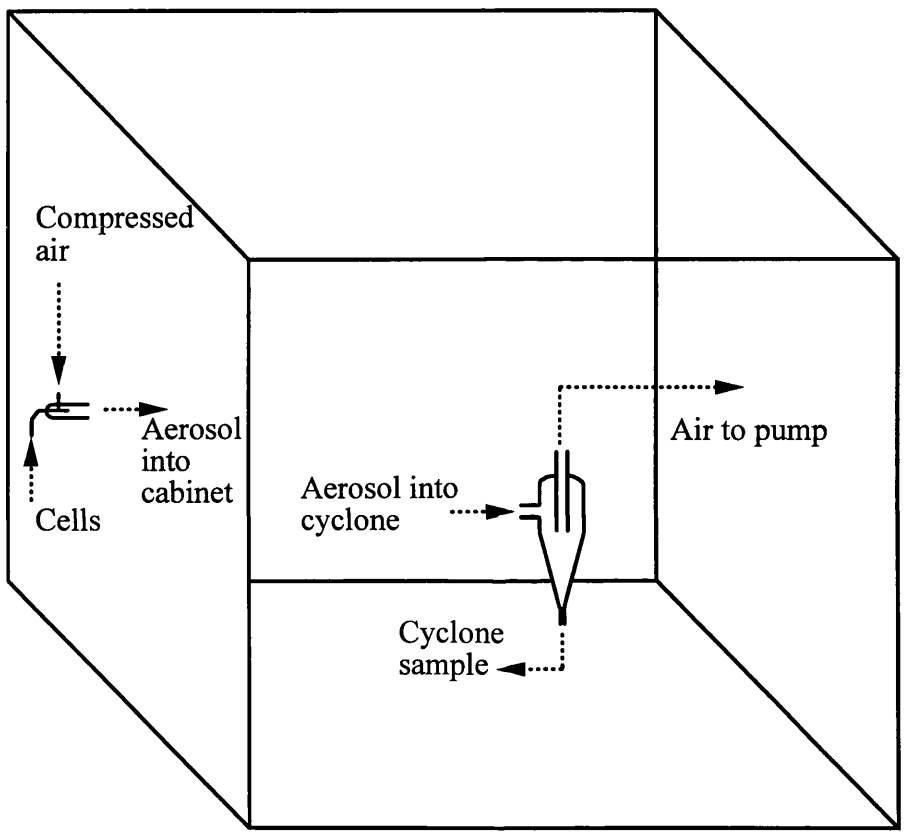


Figure 2.12: Mass Balancing in Soft Film Cabinet

2.3.3.3 Cyclone Operation

The cyclone is not an easy sampler to use. It's efficiency in collecting airborne particles is highly dependent upon the operating parameters used. It is therefore worth explaining these at this point. The design for this cyclone (Aerojet-General) was adapted from Errington and Powell (1969) and comparison of their findings with those from this work are discussed fully in section 5.1.1.

Errington and Powell (1969) have shown that when water is injected into the airstream, it forms an uneven film which completely covers the inner wall of the cyclone. The water moves helically towards the lower apex where rotation is very rapid and turbulent. Continuous scrubbing is achieved by inducing slight suction at the tail pipe, so that liquid is drawn into the sample receiver at the bottom. With the cyclone used here, this is achieved by means of flexible tubing between the air outlet at the top of the cyclone and the sample bijou bottle at the bottom.

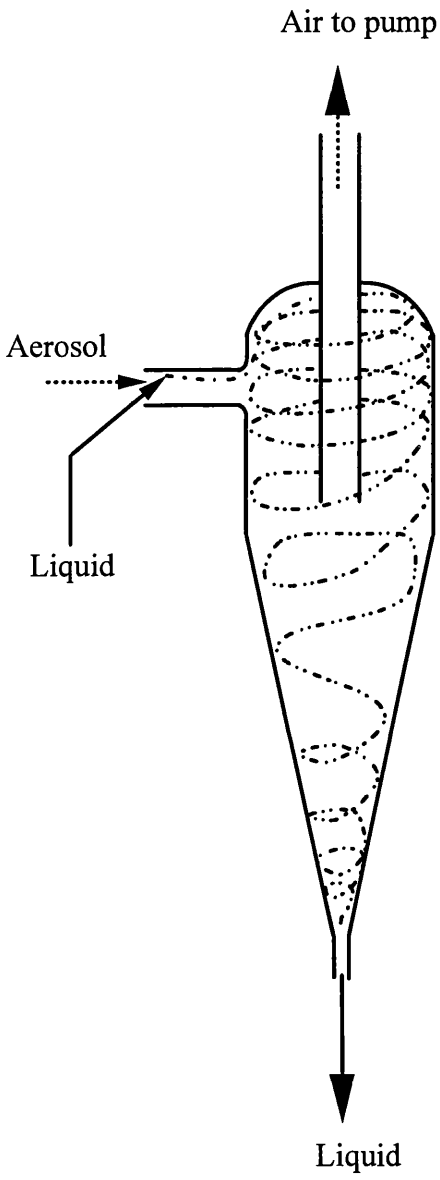
The flow of scrubbing liquid through the needle, along the air inlet and around the internal surface of the cyclone must be of a constant flow rate. This liquid washes any particles from the walls. To do this as efficiently as possible, the liquid stream must contact the cyclone walls from as high up as possible (see figure 2.13). Trial and error shows that an angle of 45^{o} between the needle and the vertical to be the most effective for washing particles from the cyclone walls.

Errington and Powell (1969) describe two causes of loss of scrubbing liquid that occur during operation of their cyclone:

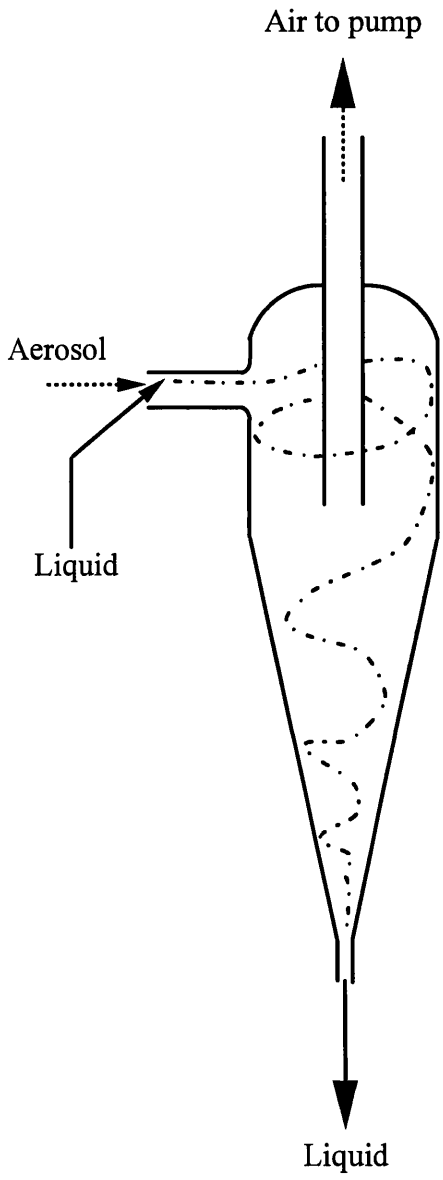
1. Some liquid impinges in the volute and reverse eddies in this region (van Tongeren, 1935) cause part of this liquid to creep over the roof of the cyclone and into the air outlet. Errington and Powell believe this loss to be negligible. The results in section 3.4.1.2 show that when two cyclones are operated in series, the recovery of particles in the second cyclone is less than 1% of that released.
2. The scrubbing liquid is brought into intimate and violent contact with the air, causing some of the liquid to evaporate. This loss will depend upon the ambient humidity.

The combined liquid lost from these two causes was found to be consistently around 25mL, i.e. approximately one third of the total initial volume. If the loss was in excess of this figure, this was seen as cause for concern.

Errington and Powell (1969) suggest that due to the reduced pressure at the lower apex of the cyclone compared with the air inlet, the sample container can be detached without liquid loss. The authors found that only little liquid accumulates during the few seconds taken to change sample container. In this way they obtained long and uninterrupted sampling. However, the cyclone used by Errington and Powell (1969) is considerably smaller (approx. 12 cm in length) than that used in this work (25 cm), and uses much less scrubbing liquid. The authors suggest a liquid flow rate of approx. $2mL.min^{-1}$, compared to 20 $mL.\ min^{-1}$ used here. Due to this greater liquid volume present in the cyclone, one liquid sample only is taken for each air sample, of 30 minutes duration.



The 'ideal' flow pattern in the cyclone washes cells from the walls.



Liquid flow like that shown above leaves cells stuck on the cyclone walls.

Figure 2.13: Fluid Flow in the Aerojet-General Cyclone

2.3.4 Cell Enumeration

2.3.4.1 Total Counts Using a Haemocytometer

The cell suspension to be counted was pipetted between the cover-slip and the counting chamber. The cover-slip should be attached to the chamber so that "Newton's rings" are visible. The slide was left for a minute to allow time for the cells to settle and then counted using the x40 magnification lens. For *E. coli* cells a two minute interval was set before the slide was counted to improve the consistency of the method. The haemocytometer with Improved Neubauer rulings (figure 2.9) was used for *S. cerevisiae* cells. The Helber bacteria counting chamber (figure 2.10) was used to count *E. coli* cells under phase contrast. The number of cells in the whole grid on the slide was counted.

Two counts were carried out using the above procedure for each sample (unless otherwise stated) and a mean concentration calculated. It is the mean that is quoted in the results in chapters 3 and 4. If the two counts differed by more than approximately 30% the count was carried out a third time.

The cell concentration, in cells.mL^{-1}, was obtained by multiplying the number of cells on the whole grid by 10^4 when using the haemocytometer with Improved Neubauer rulings, and by 7.8×10^4 for the Helber bacteria chamber. If there was less than one cell in each of the 25 portions of the grid (16 in the Helber chamber), the count is recorded as "tftc", i.e. too few to count. In effect this means that there were less than 2.5×10^5 cells.mL^{-1} (or 1.6×10^5 cell.mL^{-1} for the Helber chamber) in the sample. The total counts in the tables in chapter 3 are expressed to 2 significant figures.

2.3.4.2 Viable Counts

0.1 mL of cell suspension was pipetted onto the surface of an agar plate. The suspension is spread over the agar surface using a sterile glass rod. Plates were incubated for 2 days at 26°C if growing *S. cerevisiae* and for 1 day at 37°C if growing *E. coli*. Plates were counted at least in duplicate, and only included if there were between 30 and 300 colonies per plate.

The viable counts in the tables in chapter 3 are expressed to 1 significant figure if 30-100 colonies per sample were counted and to 2 significant figures if 100-300 colonies per plate were counted.

2.3.4.3 The Polymerase Chain Reaction

Section 3.7 (LGC method) and 4.2.3 & 4.3.2 (UCL method) describe experimentation in which PCR was used to detect cells.

LGC Method

Reaction Tube Preparation
A typical reaction mixture (total volume 25μL) included the following:

Primer 1	1.25μL
Primer 2	1. 25μL
dNTPs	4.0μL
Buffer	2.5μL
Amplitaq enzyme	0.125μL
Cell suspension	2.5μL

A bulk mixture of primers, dNTPs, buffer, sterile distilled water and taq (added to the tube last) was prepared in a 2mL Eppendorf tube. Sterile distilled water was added to make up the correct volume. This was mixed in a microfuge (for approximately 5 seconds at full speed) to ensure that the reagents were mixed and at the bottom of the eppendorf. Aliquots were then pipetted into 200μL eppendorf tubes. Finally cell suspension, or purified plasmid plus sterile distilled water were added and the contents of the tubes mixed again. The reaction tubes were then placed in the thermal cycler.

For every ten reaction tubes, a blank containing sterile distilled water instead of cell suspension was prepared. A positive control was also prepared. This included DNA which was known to contain the sequence to which the primers bound.

The volumes of primers, dNTPs, amplitaq and buffer did not change in any of the reaction mixtures prepared. *E. coli* cells were suspended in sterile distilled water for these reactions and the volume of cell suspension included in the reaction

mixture ranged from 2.5 - 10μL. Sterile distilled water was used to make the total reaction volume up to 25μL, in each case. Cells were suspended in water rather than buffer because an excess of magnesium ions in the buffer had a tendency to bind to the dNTPs. This is often counteracted by EDTA chelating Mg^{2+}, which has a stabilising effect on DNA. Primers were therefore always stored in water rather than buffer.

Thermal Cycler

The OmniGene thermal cycler was programmed to go through the following temperature cycles:

First cycle: 1 minute at 94°C to denature the DNA, then 30 seconds at 50°C for the primers to anneal to the separated DNA strands and 3 minutes at 72°C for extension of the new DNA strands by DNA polymerase.

Cycles 2 - 28: 30 seconds at 94°C, then 30 seconds at 50°C and 3 minutes at 72°C.

Final cycle: 30 seconds at 94°C, then 30 seconds at 50°C and 10 minutes at 72°C. Finally the reaction tubes are cooled to 30°C. The PCR products were then examined by gel electrophoresis.

Gel Electrophoresis

Gels were prepared by heating 100mL of TBE containing 1g of agarose (Seakem, Le., from FMC Bioproducts) in a microwave for 2 minutes. This was then cooled to approximately 50°C and 5μL of ethidium bromide added. The gels were run in tanks containing $2\mu g.mL^{-1}$ ethidium bromide (Sigma) in TBE buffer. 2.5μL of bromophenol blue (Sigma) was added to each reaction sample and then 15μL of this loaded into a well in the gel. A 100 base pair ladder (Pharmacia) was also run on each gel to aid size determination of the separated bands. Approximately 100 Volts were applied across the gel for separation to occur. When the bands had moved a significant distance down the gel, the gel was examined under UV light.

UCL Method

Reaction Tube Preparation

The PCR was carried out in 0.6 mL reaction tubes and consisted of a total reaction volume of 25 μL. It contained the following:

0.5 μL (1.25 units) Pfu polymerase (Stratagene).

2.5 μL 10x Pfu polymerase buffer (Stratagene), containing: 200mM tris HCl at pH 8.2, 100mM KCl, 60mM $(NH_4)_2SO_4$, 20mM $MgCl_2$, 1% triton x-100, 100ng.μL^{-1} nuclease-free bovine serum albumin.
4 μL dNTP solution, containing 1.25mM of each nucleotide (Pharmacia)
2.5 μL (2 pmoles.μL^{-1}) of each primer (M13R1 and cmtB1)
3 μL sterile RO water
10 μL sample.
Each reaction mixture was overlaid with 25 μL of light mineral oil to prevent liquid loss by evaporation and condensation.

Thermal Cycler
The tubes were then placed in an OmniGene thermal cycler which heated up to 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 3 minutes, and then one cycle of 10 minutes at 72°C.

Gel Electrophoresis
Electrophoretic separation of the PCR products was carried out using a 1.5% agarose gel made up with 0.5 x TBE. 5 μL of gel loading solution was added to 10 μL of each PCR product and then 10μL of each sample loaded on the gel. A molecular weight marker (Hinc II digest of ϕX-174-RF DNA obtained from Pharmacia) was also loaded. 5 μL of gel loading solution was added to 8 μL of marker and then 6 μL loaded on the gel. 100 volts were then applied to the gel for 2 hours.

2.3.4.4 Bio-Rad Protein Assay

Several dilutions of protein standard containing from 0 to 1.5 mg.mL^{-1} were prepared. A standard curve was carried out. 50 μL of standard or sample was placed in clean cuvettes. 1 mL of diluted dye reagent was added and the tubes mixed. After 5 minutes the tubes were read at OD_{595}. The standard curve is shown in Appendix C.

CHAPTER THREE: MASS BALANCING IN CONTAINED ENVIRONMENTS

3.1 Sampling Aerosols

3.1.1 Introduction

The process of detecting microorganisms in aerosols is a complex one. There are no direct reading instruments that indicate the presence of live microorganisms. The sample must first be collected, and then detection methods applied. The methods for both sampling and detection described in this section and the following one are equally well applied to naturally occurring microorganisms or to GMOs. There are many different types of sampler available (see table 3.1 for a summary), they fall into three broad categories: those collecting onto solid or semi-solid surfaces, those collecting into a liquid and those collecting onto a filter surface. The method chosen to collect airborne microorganisms seems to depend very much on personal preference. There are no standard devices for sampling microorganisms, or widely accepted guidelines for an allowable or desirable microbial burden in the air (Chatigny *et al.* 1989).

Juozaitis *et al.* (1994) compared three aerosol samplers that differed only in the medium that particles were collected on or in. The samplers collected cells onto either a glass slide, an agar surface or into a liquid. The three samplers were compared to examine their collection efficiencies independently from physical factors. Physical factors such as inlet and collection geometries and air flow rate were the same for all samplers.

The collection efficiency for the agar surface was found to depend upon the agar concentration and sampling time used. Collection efficiency is a measure of the number of cells present in the air that the sampler will collect. The liquid sampler was found to produce anomalous results with respect to air flow rate. At flow rates between 6 and 8 $L.min^{-1}$, it is thought that the higher flow rate may have removed the liquid from the collection surface so that cells escaped in the exit air stream. However, at 10 $L.min^{-1}$, 100% sampling efficiency was achieved for both sampler type. The obvious difference between these two sampler types is that cells collected onto a solid surface can only be detected by a plate count method. This will only give the number of cells able to form colonies on agar. Juozaitis *et al.*

(1994) recognise that aerosolised cells are often stressed so that their viable recoveries are low.

With respect to human exposure, the aerodynamic size range 0-10 μm is the most important (Hatch and Gross, 1964). Although pollen and spores (10-100 μm) are important allergens which are often inhaled at higher wind speeds (Decosemo, 1992). Most of the samplers described in this section were primarily designed for examining the health risks posed by aerosols in the workplace. Many were therefore intended to mimic the human nasal passages. The International Standards Organisations (ISO) and the American Conference of Governmental and Industrial Hygienists (ACGIH) have proposed new sets of criteria for health related aerosol sampling in the workplace (Vincent and Mark, 1990).

The idea that the only particles which present a risk to health are those in the inhalable fraction was first introduced in the 1970's. The inhalable fraction refers to those particles which enter the body through the nose and mouth during breathing (Vincent and Mark, 1990).

The aspiration efficiency of the human head is described by Vincent and Mark (1990), based on results from Ogden and Birkett, 1977, 1978; Vincent and Mark, 1982; Armbruster and Breuer, 1982 and Vincent *et al.* 1990. Results obtained by these workers from experiments using breathing mannequins in wind tunnels show that the aspiration efficiency is associated with inhalation (I) and particle aerodynamic diameter (d_{ae}). Vincent and Mark hypothesise that, in most industrial workplace situations, inhalability can be described by a single curve, with I initially falling as d_{ae} increases from zero, but levelling off at I=0.5 for d_{ae}>30 μm. This curve corresponds to the recommendations of the ACGIH.

In this project the aim is purely to quantify the number of microorganisms released from processing equipment. Particles in this range penetrate to varying depths in the respiratory tract, those with aerodynamic diameters below 5 μm are retained in the non-ciliated small lung passages and alveoli for periods of time significantly long enough to initiate infections. Particles less than 1 μm do not contain bacterial cells, although they may contain cell fragments, products or viruses. Those greater than 100 μm do not remain airborne for long indoors.

Samplers must take a representative sample from the air, and minimise stresses so that the biological activity of the sample is not impaired.
The efficiency of a sampler consists of two components:
1. The efficiency with which particles are collected.
2. The efficiency with which the viability of the microorganisms is preserved, i.e. the proportion of airborne cells capable of growth and multiplication in the sample.

It is important to characterise the overall efficiency (Chatigny *et al.* 1989) so that the observed concentration of airborne microorganisms can be adjusted to compensate for losses due to sampler inefficiency or damage to the microorganisms. Many efficiency tests reported in the literature (Decosemo, 1992) have been carried out either in static chambers or in open spaces, in both these situations the environmental conditions are not stable or reproducible.

Particles may lost by "slippage" within a sampler, by impacting with an obstacle, e.g. the inner surface of the sampler, rebounding and exiting with the airflow. Bubbling in the collection liquid may cause re aerosolisation of the microorganisms, reintroducing them into the airflow to exit the sampler. Samplers with low shear forces cause the least damage to microorganisms but usually have low physical efficiencies (Cox, 1987).

The physical efficiency of aerosol samplers is tested in a wind tunnel. This assesses the ability of the sampler to take a representative sample at varying wind speeds and particle size. Personal samplers are ideally tested while attached to a torso (Mark and Vincent, 1990). The microbial efficiencies however, cannot be tested in wind tunnel conditions, since temperature and humidity must be controlled.

Errors may arise if air is sampled from a cross-wind, for example when sampling the exhaust air from a fermenter (Benbough, 1994). The error arises because the air flow through the sampler does not match the air flow rate, or because the sampler is aligned so that the particles are deflected from the wind streamlines to the sampler streamlines. This effect of mismatched air velocities was first described by May (1967). To avoid this problem sampling should occur under isokinetic conditions, i.e. the velocity of the air in the sampler should equal that of the air being sampled and the air inlet (or probe) of the air sampler should be aligned with the flow direction of the air being sampled.

If the sampling velocity is less than the air velocity, the sampling efficiency will be greater than 100%. This is because larger particles will not be deflected from the sampler inlet, so the sample will be biased with these larger particles. If the sampling velocity is greater than the air velocity, the collection efficiency will be less than 100%, with the smaller particles influencing the sample. May (1967) first demonstrated this effect with a sampler inlet of 1-2 cm diameter, sampling at 5 $m.s^{-1}$. May found a maximum loss in sampling efficiency with an air velocity:sampler velocity ratio of 0.4. At ratios less than this figure the sampling efficiency increases until a situation of sampling from still air is reached. May (1967) also demonstrated that if the sampler is not aligned with the streamlines of the air it is sampling from, losses occur due to the inertia of particles which are required to follow the new direction of air flow.

Table 3.1: Sampling Methods for Aerosols

Method	Sample size or Rate	Sensitivity/ Efficiency	Cell estimation method	Portability and ease of use	Previous use
Casella Slit Sampler (CSS)	15, 60, 150, 375, 1500, and 3750L .	Large volumes of air sampled so sensitive to low [cell].	Growth of cells on agar plates. Gives time of collection.	Heavy. Easy to use.	Used by Tinnes and Hoare (1992) to monitor release from disc-stack.
Anderson Microbial Sampler (AMS)	Samples at 28.3L.min^{-1}.	If sample time too long, 1 colony on agar due to >1 cell impacting.	Growth of cells on agar plates. Give size distribution.	Reasonably portable. Easy to use.	Used by Kastelein and Logtenberg (TNO, 1989) to monitor centrifuge.
Surface Air System Sampler (SAS)	Samples at 180L.min^{-1}	Only 50% efficiency for particles of 2μm diam, therefore not good for single cell aerosols.	Growth of cells on agar.	Portable sampler. Easy to use.	Lach (1985) compared it with CSS.
Biotest RCS Sampler	Samplers at 40L.min^{-1}.	Only 50% efficient for particles 1μm diameter.	Growth of cells on agar.	Portable sampler. Easy to use.	Used by Houwink (1988) for daily monitoring in labs and plants.
Settle Plates		Agar plates become overcrowded very quickly	Not quantitative, growth of cells on agar.	Very portable and easy to use.	Used universally.
May Ultimate Impactor			Total cell numbers counted from microscope slides. Gives size distribution.	Reasonably portable.	Used by Lacey and Lacey (1987) in cotton mills.
Cyclone	Samples upto 750L.min^{-1}. 30min samples usual.	Liquid loss during operation helps increase [cell] during sample.	Viable and total cell numbers, or any assay.	Not very portable, fragile. Difficult to use.	Used by Brenner (1988) at waste water treatment site.
Porton All Glass impinger (AGI)	Samples at 12.5L.min^{-1}. 30min samples usual.		Viable and total cell numbers or any assay.	Not very portable, fragile. Easy to use.	Widely accepted as a standard sampler.

3.1.2 Samplers Collecting onto Solid or Semi-solid Surfaces

Casella Slit Sampler

This sampler collects air at either 30 or 750 $L.min^{-1}$, through a narrow slit onto a rotating agar plate, relying on the growth of the microorganisms for their detection. Samplers are supplied with either 1 or 4 slits arranged so the air impacts radially onto the agar surface. The distance between the slit and the agar is 2 mm. The agar plate is rotated by an electric motor which completes a single rotation in 30, 120, or 300 seconds, for a 4 slit sampler. No indication of the size distribution of the particles in the sample is given. The Casella slit sampler has not been fully characterised in terms of physical collection efficiencies. This sampler was used by Tinnes and Hoare (1992) to monitor release from a disc-stack centrifuge.

Anderson Microbial Sampler (AMS)

This is a cascade impactor, it collects airborne particles onto a series of nutrient agar plates, giving a size distribution. It has not been fully characterised. Detection is based on the cell's ability to grow following impaction onto the agar. The sampler consists of 6 sieve plates, each with 400 precision drilled holes, mounted over a 96 mm diameter agar plate containing a defined depth of agar.

Air is drawn at 28.3 $L.min^{-1}$ through the sampler, multiple jets of airborne particles are directed onto the collection plates, particles with high inertia cross the airstream lines and strike the agar surface. Smaller particles with less inertia remain in the airstream and pass onto the next stage. The smaller holes in the next stage cause the air velocity to increase, and therefore the inertia of the airborne particles. The largest of the remaining particles may have enough inertia to be collected here. Large particles are impacted onto the agar plates at the top of the sampler and the smaller particles at the bottom. The size of the particles collected at each stage can be related to deposition in the human respiratory tract.

After incubation of the agar plates, the number of colonies/plate and $CFU.L^{-1}$ of sampled air are calculated. Each colony corresponds to one hole in the sieve plate. A positive hole conversion (Anon, 1984) is used to calculate corrected $CFU.L^{-1}$ of sampled air. As the number of viable particles in the sampled air increases, so the probability of subsequent particles being captured through a vacant hole in the

sieve plate decreases, and the probability of more than one particle impacting through the same hole and forming a single colony. Barrett *et al.* (1984) found this sampler to have a collection efficiency >90% for particles with an aerodynamic diameter <2.5 μm, although this efficiency decreased dramatically with increased air movement. Zimmerman *et al.* (1987) used the AMS when monitoring airborne *E. coli* during simulated waste water spray irrigation dispersal studies. Kastelein and Logtenberg (TNO, 1989) used the AMS to monitor releases from a centrifuge as part of a risk assessment study.

Surface Air System Sampler (SAS)

This is a portable single stage sieve impactor which collects particles onto an agar filled RODAC (Replicate Organism Direct Contact) plate (Lach, 1985). There are two models which differ in the number of holes in their sieve plates, and the size of the RODAC plate. Air is sampled at a rate of 180 $L.min^{-1}$, each sampling run is timed by the sampler itself. Lach (1985) compared the performance of the SAS with the Casella Slit Sampler to collect *B. subtilis* var. *niger* spores. For particles <4 μm, the effective sampling rate of the SAS decreased, with 50% efficiency for particles of 2 μm diameter. Therefore it is not advisable to use this sampler where single cell aerosols may be present. The plate can also become overloaded quickly. This sampler has not been characterised in a wind tunnel.

Biotest RCS Sampler

Originally used by Macher and First (1983), this is a portable hand-held sampler which collects particles onto an agar strip, relying on the growth of the microorganisms for detection. No indication of the size distribution of the particles in the aerosol is given. The sampler consists of an open ended drum housing an impeller, an agar strip is inserted into the drum around the impeller blades. Air is drawn into the sampler at 40 $L.min^{-1}$, the impeller rotates at 4096 rpm. Airborne particles are subjected to centrifugal acceleration and impact onto the agar strip at a high velocity.

The major advantage of this sampler is that it is small, portable, and easy to use. Stewart *et al.*(1988) observed higher recoveries of aerosolised *B. subtilis* var *niger* spores when sampling with the RCS compared to the AMS. A disadvantage is that it does not give information on the size distribution of the microorganisms. Clark

et al. (1981) assessed its performance with environmental samples and aerosols of known size to look at the effective volumetric sampling rate and particle size range over which it is maintained. Air flow measurements show a higher air flow rate drawn into the sampler than that claimed by the manufacturers. The effective sampling rate for larger particles is greater than that claimed by the manufacturers, although for particles <0.5 μm it is lower. So although the RCS is convenient to use, any results obtained should be interpreted with caution (Decosemo, 1992).

Houwink (1988) used the Biotest RCS for daily air monitoring in laboratories and biotech plants, finding it to be most practical. This sampler was updated in 1992 and underwent trials carried out by Bennett (1992). When the RCS plus was compared with the Casella slit sampler for aerobiological monitoring, the results showed that the two samplers have similar efficiencies particles over 4 μm. For particles less than 4 μm the sampling efficiency falls off gradually, the efficiency of sampling 1 μm particles being 50 %.

Settle Plates

The use of settle plates to sample airborne microorganisms has long been criticised as being coarse (Ambler and Vernon, 1951; du Buy, 1945). The ability of settle plates to collect airborne microorganisms is governed by the gravitational force on the particle, which decreases with velocity. Since particle velocity is mass-dependent, settle plates are biased towards collecting larger particles and are also sensitive to wind movement. The method also relies on the growth of the microorganisms for detection. Other disadvantages are that settle plates will become overcrowded very quickly, making them difficult to enumerate accurately. The agar surface of the plates also dries out after a short time.

Marple Personal Cascade Impactor

This sampler (Sierra Anderson Div., Anderson Samplers Inc., Atlanta.) works on the same principles as the AMS, but it is designed to be worn on the body within the breathing zone. Each stage (4, 6, or 8 are available) has 6 nozzles arranged radially, staggered from stage to stage. The area between the nozzles form the impaction surface for the preceding stage. Stainless steel and Mylar collection media are available. Air is drawn into the sampler at 2 $L.min^{-1}$. Macher and First

(1984) used membrane and gelatin filters with this sampler to collect *B. subtilis* and *E. coli* from aerosols.

May Ultimate Impactor

Designed by May (1975), this is a static sampler which separates airborne particles into 7 size stages and deposits them on microscope slides. It works using the same principle as cascade impactors, air is drawn through stages and collected in series. Air velocity and particle inertia increase at each stage through the jet. The largest particles impact on the first slide and the smallest on the last. The particles are separated into the following size bands: 32, 16, 8, 4, 2, 1, 0.5 μm. Lacey and Lacey (1987) used this impactor to collect airborne microorganisms in cotton mills.

IOM Personal Inspirable Dust Spectrometer

Aerosols are separated into 8 size fractions (Gibson *et al.* 1987) onto aluminium plates. Within each plate there are circular orifices which act as impactor jets. Each stage acts as a collection plate for the preceding stage. The application of a sticky surface minimises particle bounce on the stages. The sampler operates at 2 $L.min^{-1}$ and is worn in the breathing zone. It has been designed to collect the inhalable fraction of aerosols consistent with ISO recommendations (ISO, 1983), and to provide a size distribution. The inlet is designed to collect the inspirable fraction and minimise wall losses between entry and the first stage of the impactor. It has been characterised using polystyrene latex beads (Vincent, 1989) and was used to show worker exposure to coal dust, but has not yet been used for airborne microorganisms.

3.1.3 Samplers Collecting into Liquids

Cyclones

These are a type of air centrifuge (Leaver *et al.* 1988) which impose a circular path onto the airstream entering the sampler. The effective mass of any particles or droplets within the airstream is increased compared to that under gravity. Air is drawn in tangentially to the body of the sampler, striking the inside surface of the sampler wall. A tangential velocity component is then achieved, causing the airstream to move in a circular path within the sampler. Larger particles impact on

the wall due to their inertia. A spray of liquid is injected across the air inlet of the cyclone to remove particles deposited on the internal surfaces.

Errington and Powell (1969) first described the use of cyclones to collect airborne microorganisms. They used two made of either stainless steel or perspex, which operate at 45 and 350 $L.min^{-1}$ with collection liquid injected at a flow rate of 0.5 - 2.0 $ml.min^{-1}$. Stewart and Salusbury (1989) have adapted the original design to operate at 750 $L.min^{-1}$ and to recirculate collection liquid so that an increase in concentration of collected microorganisms occurs.

Fannin (1980) found cyclone centrifugal samplers useful in monitoring airborne virus but the recovery of culturable bacteria was variable. Stetzenbach *et al.* (1992) points out that fluid loss due to evaporation and foaming can be a problem with this sampler. The XM2 prototype sampler, used by Brenner *et al.* (1988) is a newly developed sampler which combines scrubbing and impingement into a collection fluid. This sampler processes large volumes of air without ozone generation but is difficult to sterilise. Brenner *et al.* (1988) used the sampler to collect aerosolised bacteria and coliphages from a waste water spray irrigation site. Airborne particles were removed from the airflow and concentrated (in a ratio of 8:1) particles in the range 2-12 μm. The sampler processed 1050 $L.min^{-1}$ of air and sampling liquid was collected at a rate of approx. 2 $mL.min^{-1}$.

The Porton All Glass Impinger (AGI)

The AGI-30 (May and Harper, 1957) is widely accepted as a "standard impinger". Brachman *et al.* (1964) recommend its use as a reference sampler, against which other samplers can be compared. Air is drawn through an inlet tube into collection liquid via a 1 mm orifice. When the pressure difference across the orifice reaches 0.5 bar the air flow reaches sonic velocity, which then becomes limiting. An air flow rate of 12.5 $L.min^{-1}$ is usually sufficient. The inlet tube to the impinger is curved to simulate the nasal passage, (Cox, 1987), large particles deposit here and can be recovered by washing. One disadvantage to using impingers is that they are fragile.

The Multistage Liquid Impinger

Designed by May (1966), this consists of 3 stages intended to correspond to the 3 principle deposition sites for airborne particles in the human respiratory system. These are: the upper respiratory tract, the bronchioles and the alveoli. Air is drawn into the sampler through the inlet tube over a sintered glass impaction disc. This stage is continuously washed by the collection liquid as it is agitated by the sampling process. The larger particles are collected on this first stage. The second collection stage has a narrower inlet tube, which increases the air velocity. Larger particles are therefore collected. The particles remaining in the air are deposited in the final stage which has a gently tapered jet tangentially set to the surface of the collection liquid.

Compared to the AGI, both air velocity and splashing are reduced, making the collection process more gentle on the cells (Cox, 1987). One disadvantage of these impingers is that they are hand-made from glass, and hence difficult to compare to each other.

3.1.4 Samplers Collecting onto Filters

A major disadvantage of collecting onto filters is that cells become desiccated if large volumes of air pass over them. Microorganisms are either killed or rendered unculturable. Cells are usually recovered from the filter by washing, although some may remain trapped by the filter. Gelatin filters can be dissolved into a liquid so total cell numbers can be measured. Rotter *et al.* (1973) report a greater number of viable cells sampled using nucleopore filters compared to gelatin filters.

Aerosol Monitors

Aerosol analysis monitors are disposable polypropylene filter casings manufactured by Millipore Ltd to collect asbestos, nuisance dusts, and other contaminants (Millipore, 1990). The airflow of 1 - 2.3 $L.min^{-1}$ is evenly distributed over the filter by a thin cellulose pad. The filter is worn in the breathing zone and faces vertically downwards. Blomquist *et al.* (1984a), Palmgren *et al.* (1986a, 1986b) and Strom (1986) have used nucleopore filters to collect airborne fungal spores in highly contaminated environments.

HSE 7-Hole Total Dust Sampler

Manufactured by Casella London Ltd, this has been recommended by the HSE (1986) for sampling total inhalable dust. It has been tested in a wind tunnel (Mark and Vincent, 1986; Chung *et al.* 1987) and shows good agreement with the ACGIH inspirability curve at low wind speeds. Variation in the results obtained may occur from wall losses (Mark and Vincent, 1986).

3.2 Detecting Microorganisms in Aerosols

3.2.1 Choosing a Method

Once the microorganism has been collected from the air detection methods are used to find the number of cells present. The detection method must be:

1. Specific and sensitive enough to detect low concentrations of microorganisms.
2. Able to give fast results and cheap to carry out.

If it is a GMO that is to be detected, the method must be:

1. Capable of differentiating specific GMOs from other organisms in the environment.
2. Able to discriminate the GMO from other cells of the same species.

Table 3.2 shows a summary of the detection methods described in this section.

3.2.2 Microscopy

By simply examining microorganisms under a microscope, their cell shape, size, Gram reaction, and motility can be discovered. The use of different staining techniques will aid the identification if more than one species, or non-cellular material is present. Counting chambers, e.g. a haemocytometer or Thoma are used, with a microscope, to enumerate microorganisms in liquid culture. The chamber consists of a glass slide in which one area is ground to a known depth. This area contains a ruled grid of known area for counting surrounded by a deeper moat for excess fluid to drain into. When a cover slip is positioned correctly, the depth and volume of the chamber are known so the concentration of cells in the chamber can be calculated (Koch, 1981). Errors may occur if the coverslip is not positioned correctly, i.e. when 'Newton's Rings' appear, the correct depth in the chamber is achieved.

Lange *et al.* (1993) compared four methods of estimating cell viability. These were: a plate count, methylene blue staining, epifluorescent staining and flow cytometry. The experimental error associated with each technique was estimated, and found to be 8% for both of the staining methods and 13% for the plate count. The methods give different errors because they depend upon different criteria. Plate counts are based on cell replication whereas methylene blue staining is based on the cell's ability to exclude dye from the plasma membrane.

Table 3.2: Detection Methods for Aerosols

Method	Assay time	Detection Limit	Specificity	Application
Counting Chambers	<30 minutes	1.6×10^5 cells.mL^{-1}	Used for pure cultures only.	Universal.
Agar Plates	Upto 2 days	300 cells.mL^{-1}	Could detect GMOs if selective plating techniques used.	Universal.
Most Probable Number	Upto 2 days	<1 cell.mL^{-1}	If combined with selective plating techniques, or DEFT, could be specific for GMOs.	Used by Darbyshire *et al.* (1974) to estimate coliforms and faecal streptococci concentrations in water and food samples.
Bioluminescence	<1 hour	2×10^{-14} g ATP.mL^{-1} (enzymatix)	Not species specific.	Used by Salusbury *et al.* (1989) to detect *Bacillus subtilis* var *niger* spores from air samples.
Chemiluminescence	<1 hour	10^3 cells.mL^{-1}	could be used to detect specific species, or GMOs, if combined with antibody labelling technique.	Andersson *et al.* (1972) and Ewertz and Lundin (1972) to enumerate air samples collected with a cyclone.
Immunoassays		10^3 cells.mL^{-1} (Morgan *et al.* 1989).	ELISA or Fluorescent monoclonal antibodies are used to detect GMOs in the presence of mixed populations. The cells are enumerated by fluorescent microscopy.	Morgan *et al.*(1989) used ELISA to detect recombinant *Pseudomonas putida* in a mixed population.
Polymerase Chain Reaction	Few hours	0.3pg target DNA (Steffan and Atlas, 1990).	Target DNA can be detected in a large population of mixed organisms.	Steffan and Atlas (1990).

Bright field stains are the simplest, e.g. methylene blue, victoria pure blue or 1% erythrosine in 5% phenol. In this method the cell suspension is filtered and the filter stained. The disadvantage of bright field staining is that it does not allow differentiation between cells and debris. When staining with an epifluorescent stain, however cells can be differentiated from debris. The cell suspension is filtered onto a polycarbonate filter stained with irgalan black. Cellulose filters are not used because their surface is too rough for microscopic examination. The filter is then stained with a fluorochrome such as acridine orange, which is specific for nucleic acid, and examined using a fluorescent microscope.

3.2.3 Growth-Reliant Methods

Agar plates were originally used by microbiologists to grow and isolate microorganisms, but they are now used almost universally to quantify colony forming units (CFUs). This method assumes that each colony is formed from an individual cell. Thus the number of colonies is related to the number of cells or particles containing cells in the original sample. The method is easy to use and does not require any specialised equipment. More recently it has been recognised that the method can greatly underestimate the number of cells present in the sample. Errors involved in the use of this method include the following:

1. Parkes and Taylor (1985) have estimated that culturable counts account for between only 0.0001 - 10 % of the total population within environmental samples.
2. Inoculated plates can take upto 48 hours to grow.
3. Particles containing more than one cell collected directly onto an agar surface will form one colony only and will therefore be counted as one CFU. (Collection of cells into liquid before plating on agar generally avoids this problem as cell clumps are broken up).
4. Colony counting will only estimate the number of viable cells in a sample. Stressed cells will not grow on agar. Colwell *et al.* (1985) have found *V. chloerae* and related pathogens to enter a viable, but non-culturable state.
5. Growth will depend on the agar medium and conditions used.

Buck (1979) discusses the problems associated with the use of plate counts to estimate cell concentration. One difficulty with spread plates is the relatively small volume of sample examined (<1.0 mL). Therefore for areas of low bacterial density counts are not accurate.

The AMS, CSS, SAS and Biotest RCS all collect airborne microorganisms onto an agar surface. The collection fluid from samplers that work by impingement can be diluted and spread onto agar for enumeration by the same method. Some problems particular to the use of colony counting to enumerate aerosolised microorganisms include: Microorganisms that have been aerosolised are stressed and so their growth on agar will be slow. Samples of aerosolised process organisms are also likely to contain other background microorganisms. When these are cultured the fastest growing microorganisms present will out compete the others for nutrients. Selective nutrients could be used to culture a specific organism although growth will still be slow if the cell has already undergone the stress of aerosolisation.

The Most Probable Number (MPN) Index is another enumeration method which relies on the growth of microorganisms. It estimates the concentration of viable cells from a sample capable of growth in a given liquid medium (Koch, 1981). A growth medium is inoculated with replica dilutions of a sample and probability theory is used to determine the number of viable cells present. The calculation is based on the number of tubes containing bacterial growth and the degree of dilution used. The MPN method has a low order of precision if few tubes and dilutions are employed. The turbidity of the liquid sample is then determined to indicate growth, so it is important that organisms grow visibly or cause a colour change to the medium.

Herbert (1990) found the MPN method particularly useful for determining low cell concentrations in water samples when plate counts were unpracticable. Less than one cell.mL^{-1} of water can be detected by this method. The major disadvantage is the large sampling error, although this is reduced by increasing the number of replicates or the dilution factor. The method can be made selective by monitoring a particular metabolite or using selective media. The MPN method has been used to estimate coliform and faecal streptococci concentrations in water and food samples (Darbyshire *et al.,* 1974).

The method may be useful for the detection and enumeration of GMOs released into the environment if a unique, selectable physiological trait is present. Therefore different physiological groups, species and recombinant organisms can be enumerated. By combining MPN with subsequent plating on selective media the potential exists for estimating select groups of microorganisms or GMOs (Jain *et*

al., 1988). If the specificity of DEFT could be combined with MPN, even more attractive and useful methods for detecting and enumerating GMOs in the environment could be developed.

All of the methods described in sections 3.2.1 and 3.2.3, although well established, are limited by their non-selectivity. Agar methods may only culture less than 10 % of the total bacteria present in environmental samples (Jones, 1977). These methods are also of no use for detecting GMOs, either qualitatively or quantitatively. The next four methods described are more specific/selective.

3.2.4 Luminescence

Several chemical reactions exist in which light is produced quantitatively in response to a certain factor, which may be used as an indicator of biomass. These include:

1. The firefly luciferase ATP assay.
2. the luminol chemiluminescent bacterial iron prophyrin assay.
3. Chemiluminescent immuno-enzymatic assays.

Bioluminescence

ATP plays a central role not only in the energy status of the cell but as a regulator of enzyme activity. Therefore, the overall internal cellular level of ATP remains fairly constant for a given set of environmental conditions, making ATP quantitation a useful index for microbial numbers. Although studies have shown that cellular ATP levels change during cell division, during the growth cycle, and as changes in nutrient levels occur (Stanley, 1989), an average bacterium contains 10^{-15} g ATP/cell.

The enzyme luciferase is specific for ATP and catalyses its hydrolysis, producing yellow-green light. The reaction requires the presence of a co-factor, D-luciferin. ATP is an energy rich molecule due to its two phosphanhydride bonds. A large amount of energy is liberated when ATP is hydrolysed to adenosine diphosphate (ADP) or adenosine monophosphate (AMP) (Allen, 1972):

$$ATP + luciferin + O_2 = oxyluciferin + AMP + PP_i + CO_2 + light$$

Errors in the estimated biomass may arise from:

1. Incomplete ATP extraction.
2. Quenching by extraction chemicals and buffers.
3. The use of impure luciferase (Picciolo *et al.,* 1978).
4. Stressing cells e.g. aerosolisation.
5. The activity of ATPases and other kinases.
6. Variation of cellular ATP content with physiological conditions.
7. The presence of free ATP of non-microbial origin.
8. Degradation of ATP by extraction reagents.

Many types of sample contain ATP other than microorganisms, e.g. human cells. This must be removed before the assay is carried out. This is effected by adding ATP-ase to hydrolyse the free ATP. Lundin and co-workers (Lundin and Thore 1975, Lundin 1984) compared a wide range of ATP extractants for many bacterial species and have concluded that trichloroacetic acid is preferable. It has the advantage of instantly denaturing apyrase, preventing further ATP breakdown.

Another method which uses firefly luciferase has been developed (Karl and Holm-Hansen, 1978) to overcome the problem of variation in cellular ATP content. Although ATP content varies with physiological conditions, it has been observed that the total concentration of adenine nucleotides (AMP, ADP, ATP) remains constant (Atkinson and Walton, 1967; Chapman *et al.,* 1971), and so may be used as an indicator of metabolic activity and the potential for growth. The concentrations of AMP and ADP are determined by separation, enzymatic conversion to ATP and subsequent assay with luciferase (Karl and Holm-Hansen, 1978). The adenylate energy charge is then calculated from:

$$\text{Adenylate energy charge} = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

Chapman *et al.,* (1971) measured the Adenylate Energy Charge (AEC) of *E. coli* during growth and starvation. During growth the AEC was 0.8. During stationary phase, when growth stops or during starvation in carbon-limited cultures, the AEC slowly declined to 0.5 and then fell more rapidly. During the slow decline all the cells are capable of forming colonies, while the cells lose viability during the rapid drop in AEC. Results suggest growth can only occur at AEC values greater than

0.8 and that from 0.8 to 0.5 viability is maintained, with cells dying at AEC values of 0.5.

Detection of microorganisms using ATP measurement can be applied to airborne microorganisms if they are collected into a liquid. Salusbury *et al.,* (1989) used an impinger and cyclone to sample airborne *Bacillus subtilis* var *niger* spores, and compared ATP measurements with colony counts. Generally, the number of microorganisms recovered is less than the actual airborne concentration. This is due to a loss of viability in the aerosolised state, 'slippage' through the sampler, wall losses, and microorganisms being killed or made non-culturable in the sampler. The ATP assay carried out took 2 minutes to complete, compared to 18 hours for traditional culturing techniques.

Both techniques showed a decrease in the percentage recovery with increased retention time in the cyclone. Other results have shown that physical collection efficiency (using NaCl) remained constant with increased sampling time. Thus Salusbury *et al.,* (1989) conclude that long retention times (up to 60 minutes) decrease sample viability despite the apparent protection afforded by the endospore. Recovery rates obtained by bioluminescence and culture techniques decreased, and indicate that viability not just ability to grow were being lost. Some of these cells may be viable but not culturable, since recovery rates obtained by bioluminescent assay were higher than those from culture techniques.

Chemiluminescence

The chemiluminescent luminol method measures light emitted by the haem-protein-catalysed oxidation of luminol in the presence of hydrogen peroxide. Haem protein is attached to cytochromes. The cytochrome system is the main path by which atmospheric O_2 enters the metabolism of the cell. The intensity of the luminescent output is proportional to the reaction rate. An automated luminol chemiluminescent method for measuring the microbial content of water samples was developed by Oleniacz *et al.,* (1968). Two systems were proposed, both capable of detecting 10^3 - 10^5 cells.mL^{-1}. The light emitted from the reaction of alkaline luminol in the presence of sodium perborate or sodium pyrophosphate peroxide was measured. Both methods are not specific to a particular organism. The method showed a linear response to varying cell number.

Andersson *et al.* (1972), and Ewertz and Lundin (1972) used chemiluminescence to enumerate air samples collected using an Errington-Powell cyclone. The results showed that light emissions from air samples did not correlate with numbers of viable cells, but were stronger. This suggested the presence of dead cells and other iron containing material which may add to the light emission. The effects of inorganic compounds were minimised by utilising the fact that they gave a rapid flash of light whereas that from biological material is a prolonged and stable emission. Ewertz and Lundin felt that the presence of dead cells amplified the sensitivity of the method in comparison to colony counting.

Immuno-enzymic assays for the detection of small numbers of specific bacteria present an attractive solution to the rapid enumeration of coliforms (Cundell, 1981). Halmann *et al.,* (1977) describe a chemiluminescent method for the measurement of peroxidase labelled antibody bound to *Serratia marcesens* that enabled as few as 100 cells to be detected in an hour. The peroxide activity was measured by determining the number of photons emitted during the oxidation of pyrogallol with hydrogen peroxide.

3.2.5 Nucleic Acid Probes

GMOs containing genetic markers can be tracked in the environment. This is done either phenotypically, relying on the expression of the marker gene, or genotypically, where the GMO is identified by the expression of that gene by biochemical methods (Jain *et al.,* 1988). In many systems the genetically engineered trait itself is the characteristic on which the detection system is based. Pickup and Saunders (1990) describe the different types of genetic marker:

1. Functional markers provide a selective characteristic (e.g. antibiotic resistance, or the ability to metabolise a different substrate), or a non-selective characteristic in the form of a unique biomarker. This type of detection method relies on the growth of the organism.
2. Chromogenic markers use genes encoding enzymes that produce a colour change in a substrate, providing a colorimetric change in the appearance of bacterial colonies. Like 1 above, this method also relies on the growth of the organism.
3. Unique oligonucleotides can be detected by nucleic acid hybridisation probes.

Pickup and Saunders (1990) recommend insertion of the DNA into the genomic DNA rather than the plasmid. This is because there is increasing evidence that some plasmid marker systems are inherently unstable due to the segregation of the plasmid at cell division once the organism is released into the environment (Morgan *et al.,* 1989; Pickup and Saunders, 1990). It is also possible that the introduction of new genetic material will reduce the viability of the GMO in the environment, for two reasons: firstly, maintenance of the marker system may impart a bigger metabolic burden on the cell; and secondly, the continual expression of the marker gene may reduce the ability of the cell to survive. These problems may be reduced by locating the marker on the genome, so the maintenance budget may be reduced and expression more easily controlled.

Many detection methods assume that metabolically active or viable microorganisms can be cultured and that they are able to transcribe marker genes. Often selective and non-selective media are used. Selective enrichment may also be used before plating to increase the number of target organisms above the detection limit. However, a large number of dormant, or non-culturable cells maintain their viability in the environment (Colwell *et al.,* 1985). Obviously any detection method relying on the growth of these organisms would underestimate the number of viable cells present. Detection methods not requiring growth include immunological and hybridisation techniques. In these techniques the cell or total DNA are extracted from a sample.

DNA hybridisation technology is based on the re-annealing of two complementary, denatured DNA strands. The target DNA is either transferred to a filter directly after extraction and purification, or following restriction analysis and electrophoretic separation. Both methods are labour-intensive, limiting the number of bacterial isolates that can be screened. Colony hybridisation, where cells are cultured and lysed directly onto the filter, can screen up to several thousand colonies at a time (Hanahan and Meselson, 1980). Probe DNA is labelled and in its single stranded form applied to target DNA. Excess probe DNA is washed from the filter leaving the complementary sequences attached. The presence of the probe is visualised using either x-ray film or by chemical colour production.

If suitable probes are available it is possible to use hybridisation to detect the presence of specific nucleic acid sequences, from oligonucleotides to functional recombinant genes. The requirement for culturable cells in the colony hybridisation

method makes it possible to assess quantitatively the extent to which the trait has been transferred within the indigenous population. In contrast, total DNA extraction and subsequent probing for a particular trait can only be used to monitor the characteristic on a presence/absence basis. The detection sensitivity can be increased in a number of ways, one of which is the Polymerase Chain Reaction (PCR). PCR is used to enrich target sequences in order to increase the active concentration of target DNA in the sample (Mullis and Faloona, 1987).

The target nucleic acid sequences extracted with the total DNA from the sample are hybridised by complimentary oligonucleotide dimers. The target sequence is then multiplied in a series of cycles in which a primer-directed enzymatic polymerising step is alternated with one which separates the complementary strands.

3.2.6 The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was invented by Mullis, while working at the Cetus Corp. in California and was first reported in *Science* in 1985 (Saiki, 1985). In simple terms, when a double stranded piece of target DNA is heated, the two stands separate (denature). Two short primer sequences of DNA, added to the reaction mixture are extended by the enzyme DNA polymerase in the presence of deoxy Nucleoside TriPhosphates (dNTPs). The primers anneal to the DNA either side of the target sequence. Two new strands are synthesised to complement the template or target DNA. In this way more template is generated to which more primers can anneal, permitting the next round of polymerisation. The resulting chain reaction allows the amplification of desired nucleic acids from undetected levels to detectable quantities of a target sequence. The presence of the target organism is inferred from this target sequence, taking just a few hours (Pickup and Saunders, 1990).

Some key factors that affect the level of reproducibility, precision and accuracy of the PCR are outlined below:

1. Optimisation of the amplification step can be achieved by changes in nucleic acid preparation, primer design, buffer, and temperature cycling parameters.

2. Undesirable hybridisation events often occur in the first cycle of the reaction and can carry potentially devastating effects. One method of eliminating these is "hot-start" PCR, in which the reaction is started at a higher temperature once the denaturation step is complete. Mullis *et al.* (1986) have achieved a 1000-fold improvement in sensitivity by using hot-start PCR. An alternative to hot-start PCR involves boiling the cell lysate for around 2 hours before the amplification step, instead of the usual 5-10 minutes. A 10-fold increase in sensitivity can be achieved due to cleavage of the DNA molecule and a reduction in the overall background generated. This means that very few molecules besides the target would possess enough sequence homology for both primers to work.

3. The level of accuracy is affected by the exponential nature of the PCR. The amplification reaction is described by the equation $Y_n = (1 + R)^n$, where Y_n is the amplification factor after n cycles and R is the efficiency of amplification at each cycle. If the amplification proceeds with an efficiency of 100% (i.e. R = 1), the amount of PCR product doubles at each cycle. However, this is rarely the case, efficiencies are typically 70-80% from the 15th to the 30th cycle, depending upon the starting material present.

The increase is exponential for a limited number of cycles only, then a plateau is reached. Factors contributing to the onset of this plateau include substrate saturation of enzyme, product strand re annealing and incomplete product strand separation. In this exponential phase the amount of amplified product is no longer proportional to the amount of target molecules present at the start of the reaction. It is therefore important to identify the linear range of the reaction in which the amount of amplified target is directly proportional to the initial amount of target.

The PCR reaction results in μL volumes of the DNA target sequence. This can be detected by a number of different methods, some of which are quantitative. In this study PCR products were examined by gel electrophoresis, which indicates the presence or absence of DNA molecules. The size of these molecules can be compared to a mixture of DNA molecules of known size, which also loaded on the gel. The quantity of each molecule present can only be estimated by comparing the intensity of each band on the gel. Steffan and Atlas (1990) detected 0.3 pg of target DNA, equivalent to 100 target organisms in 100 g soil against a background of 10^{11} non-target organisms.

3.2.6.1 Quantitative PCR

The aim of this study was to quantify the number of microorganisms released from bioprocessing equipment. This has not yet been achieved using the PCR method. However, there are reports in the literature of PCR methods in which the DNA is quantified. Some of these are outlined in this section.

Ferre (1992) discusses various methods now used to carry out PCR and quantify the amount of target DNA produced. Ferre points out that the literature is full of statements like " PCR is not really a quantitative method", and argues that this is not the case. One contributory factor to this attitude is an inherent part of the PCR; that is it's ability to amplify small amounts of nucleic acids exponentially. Anything capable of interfering with the exponential amplification might ruin the intrinsic quantitative ability of PCR. The PCR has been compared to classical nucleic acid quantification methods like Northern blots and *in situ* hybridisation and has been shown to be quantitative. It is also possible to find the absolute amount of DNA present, not just comparative values (Wang *et al.*, 1989).

The possibility of quantifying a PCR product is dependent upon the reproducibility, precision and accuracy of the PCR. These can be improved by methods outlined in section 5.5, and include the optimisation of the amplification step, use of hot start and similar techniques and the linear range of amplification of the reaction.

It is possible to measure the amount of PCR product in the linear phase of the reaction. Before this can be carried out the PCR must be characterised and the length of the linear phase determined. The length of the linear phase will be different for different samples and reaction conditions. It is possible to use DNA standards to overcome this, however, because the products are measured early on in the PCR, there may not be sufficient product for detection by gel electrophoresis or ethidium bromide fluorescence. Expensive detection methods may therefore be necessary.

Romanowski *et al.* (1993) used laser densitometry to quantify PCR produced in the linear phase. This was carried out for a product from a genetically engineered *E. coli* released into the soil. PCR results were correlated with electroporation measurements of the plasmid molecules. Up to two rounds of PCR were carried

out, with a hot-start, the amplification product of the first round being the DNA substrate for the second. Target sequences in the range 1.9 x 10^3 - 10^5, corresponding to 1 fg - 1 pg of the plasmid, could be detected. Romanowski *et al.* found that they could detect as few as 10 target sequences with two rounds of PCR, however this did not fall into the proportionality range of the reaction.

Another method used to quantify PCR products is the Most Probable Number (MPN) technique. This is based on the traditional MPN technique used to enumerate cells by growth in a liquid medium (see section 3.3.3). A dilution series of known cell concentration is prepared and a PCR carried out. The PCR result for the test sample is then compared to this dilution series. This method relies on two assumptions: Firstly that the target DNA sequences are distributed randomly throughout the solution and secondly that the presence of at least one target DNA sequence is enough to generate an amplification product which can be visualised on an agarose gel.

Picard *et al.* (1992) used this MPN PCR technique to detect and enumerate *Agrobacterium tumefaciens* and *Frankia* spp. in soil samples. They observed a strong correlation between inoculum size in the dilution series and PCR result. They initially encountered problems obtaining a positive PCR result with less than 10^3 cells. However, this was improved by using biphasic PCR, in which they reduced primer-dimer synthesis and other spurious products which consume primer stock and monopolise the enzyme. In biphasic PCR the primers are more dilute in the first 10 cycles and then increased in concentration for the remainder. Picard *et al.* (1992) also found that addition of 1% formamide improved the yield of PCR products by lowering the DNA melting temperature. With these improvements, plasmid DNA corresponding to 1 bacterial cell could be detected.

Recorbet *et al.* (1993) used plate counts, immunofluorescence and a MPN PCR technique to quantify bacterial survival and DNA persistence of a genetically manipulated *E. coli* (EL 1003) in soil. Plate counts of *E. coli* dropped below $10^2.g^{-1}$ soil (the limit of detection) after 15 days, but 10^3 extracellular DNA and 5 x 10^5 total DNA target sequences could still be detected after 40 days. The cell numbers dropped below the limit of detection by immunofluorescence by the 30th day.

The MPN PCR technique used by Recorbet *et al.* (1993) was that of Picard *et al.* (1992). It showed a PCR product visible on an electrophoretic gel generated from 1 fg of purified genomic *E. coli* DNA, equivalent to 1 target sequence in each reaction volume. However, when DNA from soil bacteria was used, the limit of detection was found to be 10^3 target sequences for total DNA and 10^2 target sequences for extracellular DNA per 100 mg of dry soil. If an average number of target copies of 10 per cell is assumed, then the number of DNA sequences estimated by MPN PCR does correspond to the number of inoculated cells in the dilution series.

The implications of this work are very interesting. Recorbet *et al.* (1993) suggest three possible explanations for the difference between number of CFU's and number of target DNA molecules:

1. The *E. coli* population may have become viable but non-culturable, the physiological state described by Colwell *et al.* (1985).
2. Genetic transfer may have occurred between the *E. coli* and wild type bacteria present in the soil, (however this seems unlikely since transfer of antibiotic resistance could not be detected).
3. The DNA may be able to persist outside the cell.

Number three above is probably the most likely explanation. PCR is unable to distinguish between live or dead cells. Work at the Institute of Food Research at Reading (IFR News, 1994) has shown that if *E. coli* or *Listeria monocytogenes* is treated with acid or hydrogen peroxide, the PCR signal is lost soon after the organisms can be detected. However, strong signals are retained for long periods post-death if the cells are starved, desiccated or heat-killed. Workers at Reading have found that levels of mRNA, which has a half life of one to two minutes, provides a much more reliable indication that a cell is alive.

Alvarez *et al.* (1994) compared the traditional method of plate counts with solid phase (SP) PCR for the detection of a recombinant *E. coli* in samples collected from the air using an All Glass Impinger. They recommend the use of SP PCR for samples of this type because the cells are often non-culturable due to stress from the aerosolisation and collection processes. The cells are collected into a liquid and then filtered. The PCR is carried out directly on the filter surface after 2 hours incubation in bovine serum albumin to block non-specific DNA binding. The method is purely qualitative, with the presence/absence of a PCR product on an

agarose gel compared with colony growth on agar plates. PCR products were obtained when it was not possible to culture cells on agar.

It is generally accepted that the most reliable QPCR method involves the use of an internal competitor DNA molecule as a quantifying standard. The competitor molecule is amplified in the same tube as the target DNA and should have a similar DNA sequence and use the same primer set as the target. The competitor DNA is designed to generate a PCR product of a different size to the target DNA. It is the ratio between the two DNA products that is important. Any non-specific binding of the primers to the non-target sequence will not affect the result.

Competitor DNA can be obtained by site-directed mutagenesis of the target DNA, so that a restriction site is either added or deleted or by the generation of a genomic PCR product containing a short insert. Alternatively a non-homologous DNA fragment of the desired size can be engineered to contain the primer templates or can be obtained by PCR amplification using target primers and cross-species genomic DNA under reduced annealing stringency. Siebert and Larrick (1993) describe a method for generating non-homologous competitive PCR fragments. Synthetic DNA fragments complementary to previously established PCR primers are ligated together with the primers to both ends of a generic DNA fragment. The length of this generic fragment differs from the natural target gene PCR product.

Mahon and Lax (1993) use competitor DNA consisting of the *spvR* target gene with a 94 base pair deletion situated between the primer recognition sites cloned into a plasmid vector. Amplification of the two sequences in the same PCR gave products of 500 and 406 base pairs. Quantification was achieved by a visual assessment of the ratio of the two products on ethidium bromide stained agarose gels. Mahon and Lax (1993) found quantification to be accurate in estimating the number of DNA molecules present in a sample when the gene was incorporated into a plasmid vector, but less so when whole cells were used. They also point out that QPCR allows detection of false negatives caused by technical failure or error, as the competitive DNA acts as an internal control. The absence of a product from the competitive DNA in a reaction confirms inhibition, or failure of the PCR.

In the example described in the previous paragraph the PCR products are assessed purely on a visual basis. There are however more quantitative alternatives available. The most common tag used for the detection of PCR products is ^{32}P

labelled nucleotides. The ^{32}P is quantified by either cutting the bands from a gel and counting cpm or, by the use of radio imaging. Chemiluminescence can also be used to quantify PCR product. This method relies on the emission of photons which can be counted. Arnold *et al.* (1989) labelled oligonucleotides with a highly sensitive non-radioactive acridinium ester (AE) reporter group. This AE group is sensitive to alkaline hydrolysis when single stranded only. Once hydrolysed, the AE group is no longer chemiluminescent.

3.2.7 Immunoassays

The use of poly or monoclonal antibodies offers a potentially sensitive and specific means of identifying microorganisms. Antibodies of either type are used to identify specific marker gene products or intact organisms expressing the appropriate antigen. There is now increasing interest in raising poly and monoclonal antisera against ecologically important microorganisms, particularly pathogens (Brayton *et al.,* 1987; Lin *et al.,* 1986) and subsequently GMOs (Morgan *et al.,* 1989).

Enzyme linked immunosorbant assays (ELISA) are used for the detection of specific strains e.g. *Rhizobium* (Martensson *et al.,* 1984) and recombinant bacteria e.g. *Pseudomonas putida* (Morgan *et al.,* 1989) in the presence of mixed populations. The detection limit was 10^3 cell.mL^{-1} in lake water samples, using epifluorescence microscopy to enumerate the cells present. Specificity was achieved using antibodies coupled chemically to a fluorochrome. This method however has the following problems (Pickup and Saunders, 1990):

1. The detection of marked strains depends on the presence of an antigen specifically introduced into the GMO. It is likely to be difficult to ensure that the antigen will be expressed as a cell surface component which could therefore be detected. The identifying antigen must be a unique cell surface protein and therefore host specific.
2. The unique cell surface antigen must be stable in the GMO if it is going to be monitored over time.
3. A reduction in the specificity of the antibody may occur due to interference from particulate matter, natural auto fluorescence of co-existing material, cross-reactivity with other agents and failure to reach the target organism due to the presence of extracellular material.

Monoclonal antibodies raised against Flavobacterium P25 (from soil) have been shown to be active even when the organism encountered starvation conditions and cell densities as low as 20 bacteria.g^{-1} (Mason and Burns, 1990). The cross reactivity of the monoclonal antibody was tested against other Gram negative and positive bacteria, but none found. Fluorescent monoclonal antibodies specific for the 01 antigen of *Vibrio cholerae* have been used in conjunction with fluorescence microscopy. This is a more sensitive method of assessing water quality than standard culture methods (Brayton *et al.,* 1987).

An alternative immunological method for detecting and enumerating airborne microorganisms is that involving the measurement of endotoxin (Speight, 1992). Endotoxin forms part of the cell wall of Gram negative microorganisms. It has caused respiratory illnesses in plant operators working on processes involving Gram negative organisms. Airborne endotoxin can be measured using an enzyme-linked immunosorbant assay (ELISA). Some of the assays described by Speight (1992) are sensitive to ng of endotoxin.m^{-3}, and are rapid (2 hours) and reproducible.

3.2.8 Biosensors

Behizad *et al.* (1989) think that one reason for the lack of safety data and exposure limits for the emission of biochemicals may be the absence of sensitive monitoring devices for these biochemicals. Biosensors could be used to monitor containment and to set safe exposure limits in the work environment. Biosensor technology has made considerable advances to produce increasingly sensitive devices in the last decade (Behizad *et al.,* 1989).

Since emissions from processing equipment are likely to be in the form of aerosols, the selection of a sampler to collect the biochemical is important. It is thought that impingers are unsuitable for collecting biochemicals because they may be damaged by high shear rates at the gas/liquid interface. Behizad *et al.,* (1989) have devised a bead bubbler consisting of a Dreschel bottle packed with 350 mm ballotini. This was used effectively to collect a protease, mainly due to the large surface area available for reaction. The main disadvantage being that it was difficult to clean and rejuvenate with fresh medium.

Cyclones would be ideal for collecting biochemicals if a biosensor could be located in the recirculation loop. Devices that collect onto a solid surface, like the slit impactor and the sieve impactor, could be used for biochemicals. This is possible using a solid phase immobilised sensing mechanism (Guilbault and Kauffman, 1987). Samplers that work by electrostatic precipitation could also be used. Air is passed through a sampling chamber and particles are collected by attraction to two electrostatically charged plates. This an effective method even at low flow rates.

Some biosensors now used for detecting biochemicals (Behizad *et al.,* 1989) include the following:

1. Ion selective electrodes, e.g. ammonium selective electrodes for urea monitoring.
2. Conductimetric biosensors, e.g. for glucose in blood and urine.
3. Amperometric biosensors, e.g. to monitor urea, glucose and lipids.
4. Colorimetric test strips, e.g. to monitor glucose, total protein and uric acid in urine.
5. Fluorimetric biosensors, e.g. to monitor serum thyroxine concentrations.
6. Luminometric biosensors, e.g. for glucose monitoring.
7. Fibre optic sensors, e.g. to monitor blood gases and acetone in diabetics.
8. Thermometric biosensors, e.g. for exothermic enzymatic reactions.

An on-line device to detect airborne hazardous biochemicals should preferably incorporate a single step reaction to facilitate continuous monitoring. The detection device should be very sensitive since it is likely that the biochemical is at a low concentration in environmental air. Alternatively it must sample a large volume of air to deposit sufficient biochemical for the biosensor to detect. The sensor should be capable of distinguishing between short term high doses and the background dosage which may exist in working environments.

The biochemical signal generation system can be either antibody, enzyme or receptor based (Behizad *et al.,* 1989). It is important that the signal generator has a rapid turnover of binding to the analyte or a large active capacity so it can monitor for long periods of time before regeneration is necessary.

1. Antibody based: Antibodies can be produced for any molecule with a weight greater than 250 Da. They can be highly specific and are robust. They can therefore be incorporated into a biosensor system using the Enzyme Linked Immunosorbent

Assay (ELISA), which combines antibody specificity with biological amplification via the enzyme catalytic cycle. The antibody to a specific biochemical is labelled with a fluorescent probe, the reaction between the biochemical and its antibody causes a change in the fluorescent signal proportional to the quantity of biochemical present. A disadvantage of this method is that the reaction is not readily reversible and therefore regeneration of the system is not practicable.

2. Enzyme based: An immobilised enzyme system is used to convert a competitive substrate to product with a colour end-point that can be detected spectrophotometrically. Thus the signal is inversely proportional to product concentration. This method has the advantage that it is highly specific, although one problem is that the enzyme may not be stable if exposed to the air for long periods of time.

3. Receptor based: These are normally water soluble, e.g. Con-A is used to detect glucose. The glucose in the test solution is used to displace dextran, which in the absence of glucose is bound to an immobilised receptor, generating a signal. Membrane receptors have the advantage of increased sensitivity. The signal is generated by conformational changes in the receptor which is monitored by changes in fluorescence by a fluorescent probe. One problem with this type of method is that pure active receptors are difficult to obtain, although rDNA techniques could be used. They are useful because they have a catalytic cycle of activity suitable for continuous monitoring.

If these problems associated with the use of biosensors to monitor aerosolised biochemicals could be resolved, biosensors could be extremely useful detection methods. This is because they do not measure whole, or live cells, but a cellular constituent, or a cell product. If they were used in conjunction with cell counts, a more detailed picture of a microbial release could be described.

3.3 Cell Detection in This Study

Throughout this study cell numbers were enumerated by total counting in a haemocytometer. Compared to the techniques described in the previous section (3.2) this is a simple and non specific detection method. However in a clean environment such as a contained cabinet a more specific method is not necessary to detect released cells.

Another advantage of the haemocytometer method is that it gives the total number of cells present irrespective of their viability (ability to grow on agar) or the stability of a particular cell component. Total cell numbers can easily be related to the number of cells released from the atomiser into the cabinet and collected in the cyclone. In this study it is these numbers that are crucial for improving systems of containment.

It is only when cells are released into a dirty environment, perhaps in low numbers, that it is desirable to use a specific method to detect them. In this situation a method such as PCR becomes beneficial because it can quantify a specific GMO, which may be present in low numbers in a mixed microbial environment. A qualitative PCR method is used in sections 4.2 and 4.3 to detect released cells.

3.4 Mass Balancing Experimentation

3.4.1 Mass Balancing in Bassaire Cabinet

3.4.1.1 Aerosol Release Using the Collison Nebuliser

Table 3.3: Collection of *S. cerevisiae* in the Errington-Powell Cyclone

The Collison nebuliser (figure 2.4) was used to spray a suspension of *S. cerevisiae* cells into the Bassaire cabinet (figure 2.1), from which the aerosol was collected with the Errington-Powell cyclone (section 2.2.5 and figure 2.6). All four cell suspensions came from the same stock solution, but those in experiments c. and d. were diluted further to give a less concentrated cell suspension. Total counts were carried out on the collected sample using the haemocytometer (figure 2.9) and were repeated four time for each sample. The figures in the table are the means of these four counts. The Recovery % was calculated based on the assumption that the volume loss that occurred in the nebuliser during spraying was due to loss of cells as well as the suspending liquid. The low recoveries obtained (<10%) suggest that the nebuliser did not release many cells.

Expt	a	b	c	d
Cell concentration in Nebuliser before spraying (cell.mL^{-1})	1.2×10^9	1.35×10^9	1.1×10^8	1.0×10^8
Cell concentration in Nebuliser after spraying (cells.mL^{-1})	1.4×10^9	1.65×10^9	1.2×10^8	1.1×10^8
Volume of liquid aerosolised (mL)	4.9	4.9	6.4	6.9
Total cells in Nebuliser before spraying	5.2×10^{10}	5.0×10^{10}	5.6×10^9	5.8×10^9
Total cells in Nebuliser after spraying	5.5×10^{10}	5.3×10^{10}	5.4×10^9	5.1×10^9
Total cells collected in cyclone	4×10^8	5×10^8	5×10^7	3×10^7
Total cell recovery in cyclone (%)	7	7	8	4

Table 3.4: Release of *S. cerevisiae* from the Collison Nebuliser

The aim of this experiment was to examine the number of cells present in the reservoir of the Nebuliser before and after the spraying period. The nebuliser was used to spray 14 mL of a *S. cerevisiae* suspension into the Bassaire cabinet for 1 hour. Total counts were carried out before and after the spraying period. Ten grids of each sample were counted in the haemocytometer and the mean values for these are shown in the table below.

Nebuliser	Before spraying	After spraying
Total number of cells	$4.9 \times 10^9 \pm 6 \times 10^8$	$5.1 \times 10^9 \pm 4 \times 10^8$
Concentration of cells (cells.mL^{-1})	$1.5 \times 10^8 \pm 1.8 \times 10^7$	$2.6 \times 10^8 \pm 2.1 \times 10^7$

The table above shows little change in the number of cells present in the Nebuliser even after 14 mL of liquid had been lost by aerosolisation. The number of cells after the aerosolisation period is slightly larger than the number of cells present at the start. This is probably reflects inaccuracies in the total counting method. Table 3.3 indicates that a low level of *S. cerevisiae* cells are released from the Nebuliser, because they were detected in the cyclone. It is thought that since the Nebuliser will not atomise particles greater than 3μm diameter (Errington, 1992), it is unlikely to release aerosols of *S. cerevisiae* cells (approximate average diameter = 5μm).

The size of particles present in an aerosol is dependant upon the contents of the liquid from which the aerosol is formed. This includes both microbial cells and suspended solids (e.g. those present in fermentation broth).

Table 3.5: Collection of NaCl in the Cyclone

This experiment was carried out to examine the effect of air pressure on release of NaCl from the Collison Nebuliser and hence it's recovery in the cyclone.

A 1M NaCl solution (6 mL on average) was sprayed directly into the cyclone (operated at 750 $L.min^{-1}$) using 20 psi of compressed air through the Collison Nebuliser. In b. 10 psi of compressed air only was used. In d. the NaCl was released into the Bassaire cabinet and then sampled with the cyclone. The mass of NaCl collected in the cyclone was estimated from the conductivity of the circulating fluid. The calibration curve of conductivity versus molarity for NaCl is shown in Appendix D.

The calculation below was used to estimate the mass of NaCl recovered in the cyclone. Molarity was obtained from the calibration curve.

$$\text{NaCl recovered (g)} = \frac{\text{molarity} \times 58.44 \times \text{volume collected in cyclone}}{1000}$$

Expt	Mass of NaCl sprayed (g)	Mass of NaCl collected (g)	Recovery in cyclone (%)
a	1.17	0.12	10.7
b	0.38	0.42	11.1
c	0.53	0.13	13.2
d	0.58	0.05	25.0

The recoveries are not greatly affected by a change in air pressure on the nebuliser. Spraying into the cabinet and then sampling with the cyclone does seem to improve the recovery. However, the conductivity probe used in these experiments is thought to be accurate to no more than +/-10% when used to measure conductivities >50$mS.cm^{-1}$. A slight change in conductivity was observed over a

few hours as the RO water used to prepare the NaCl solutions was thought to have absorbed CO_2 from the air.

It is not obvious why the recoveries in table 3.5 are so low. It was thought that the Nebuliser was only inefficient in releasing particles with a diameter greater than 3 μm. It was expected that these recoveries of NaCl would be higher because it was thought that the particle size of the NaCl aerosol would be smaller than 3 μm. However, these NaCl recoveries are similar in size (less than 25%) to those for *E. coli* (see table 3.6).

Table 3.6: Collection of *E. coli* in the Cyclone

E. coli cells suspended in Luria broth (and 0.5 mL of a 1% polyethylene glycol solution to prevent foaming) were sprayed into the Bassaire cabinet using the Collison nebuliser (6.9 mL in experiment a. and 7.7 mL of cell suspension in experiment b.). The cyclone (operated at 750 $L.min^{-1}$) was operated for 30 minutes to collect the cells. Ten Ringers' plates were used to estimate cell numbers on the floor of the cabinet and samples from each of these was counted in the haemocytometer. The number for recovery of cells from the floor is based on the mean of these ten counts.

To check that the nebuliser did release cells, total counts were carried out on the cell suspension in the nebuliser both before and after spraying. It can be seen in the table that there in no increase in cell concentration during the spraying period, as there was in table 3.3. This confirmed that the Collison Nebuliser did release the *E. coli* cells.

The cell suspension that was sprayed into the cabinet contained approximately 65% viable cells. Of the cells that were collected from the air in the cabinet using the cyclone, approximately 10% were viable. This drop in viability reflects how stressed the *E. coli* cells become during the aerosolisation and collection process.

Enumeration method	**Total Cells**		**Viable Cells (CFUs)**	
Expt	**a**	**b**	**a**	**b**
Cell concentration in Nebuliser at start (cells.mL^{-1})	1.8 x 10^9	2 x 10^9	1.3 x 10^9	1.5 x 10^9
Cell concentration in Nebuliser at end (cells.mL^{-1})	1.8 x 10^9	1.8 x 10^9	1.1 x 10^9	1.2 x 10^9
Cell number sprayed into cabinet	1.2 x 10^{10}	1.5 x 10^{10}	9.3 x 10^9	9.2 x 10^9
Cell number collected in cyclone	2.1 x 10^9	1.2 x 10^9	2.8 x 10^8	1.1 x 10^8
Cell number collected from floor	1.3 x 10^9	7.7 x 10^8	2.4 x 10^7	1.7 x 10^7
Recovery in cyclone (%)	17	8.1	3	1
Recovery from floor (%)	10	5	0.3	0.2

3.4.1.2 Aerosol Release Using the Glass Atomiser

Table 3.7: Collection of NaCl in the Cyclone

This experiment was carried out because it was thought that loss of aerosolised particles to the air pump inlet may occur.

A 1M NaCl solution (on average 6 mL) was sprayed into the cabinet using the atomiser (figure 2.5) and collected in the cyclone (operated at 750 $L.min^{-1}$) for a 30 minute period. Two cyclones were operated in series to collect the aerosolised NaCl. The outlet of the first cyclone was connected to the inlet of the second. The first number represents the recovery in the first cyclone and the following number the recovery in the second cyclone.

Expt	Mass of NaCl sprayed (g)	Mass of NaCl collected in 1st cyclone (g)	Mass of NaCl collected in 2nd cyclone (g)	Recovery in cyclone (%) (1st + 2nd cyclone)
a	0.41	0.14	0.0034	33.7 + 0.8
b	0.29	0.081	0.0027	27.7 + 0.9

The recovery of NaCl in the second cyclone was less than 1% and so loss of aerosolised particles to the air pump was negligible. This experiment should perhaps be used intermittently to assess the efficiency of the cyclone in collecting aerosols. The result could then be used along with the liquid volume loss during a sampling period to decide if there is cause for concern. This approach was not however used in this investigation.

Table 3.8: Collection of *S. cerevisiae* in the Cyclone

A 10 mL suspension of *S. cerevisiae* was sprayed into the cabinet using the atomiser and collected in the cyclone (operated at 750 $L.min^{-1}$). The number of cells that fell to the floor of the cabinet, escaping capture in the cyclone were estimated using Petri dishes containing Ringers' solution. A total count was estimated for each of the 12 Ringers' plates placed on the floor of the cabinet during these experiments. The mean of these is shown in the table above.

In the table below, in expt. b. the mixing fan in the ceiling of the cabinet was not operated. In c. small areas on the back wall of the cabinet were swabbed to estimate cell number. In d. the cyclone was operated for 30 minutes extra after spraying had finished.

Total cells	**Expt a**	**Expt b**	**Expt c**	**Expt d**
Cells sprayed into cabinet	1.5×10^9	1.4×10^{10}	2.1×10^{10}	1.2×10^{10}
Recovery in cyclone (%)	33	49	25	50
Recovery from floor (%) (walls in Expt. c)	8	8	1	8

In these experiments the recovery from the floor is less than 10% in all cases. The recovery in the cyclone varies from 25 to 50%. The extended sampling time in d. did not increase the number of microorganisms recovered from the cabinet. This was expected since, during a sampling period of 30 minutes the cabinet undergoes 30 air changes.

Assessment of cells on cabinet walls:

Observation showed that a proportion of the cells released from the atomiser during these experiments was to be found on the back wall of the cabinet. A circular pattern of droplets was easily visible on this wall, radiating out from a central point opposite the atomiser. However, coverage of the walls was not uniform, making estimation of the total number of cells on this wall difficult. An attempt was made to estimate the number of cells on the cabinet wall by swabbing small areas and then multiplying to get an approximate number for the whole wall.

This proved to be very difficult to achieve and could be improved, possibly using plastic strips stuck on to the cabinet wall.

Assessment of cells on the cabinet floor:

Ringers' plates collect cells into a liquid, so that a total count as well as a viable count can be determined. The viable counts from Ringers' plates are more accurate than settle plates because settle plates quickly become overcrowded when a high concentration of aerosolised microorganisms is present. If a viable count is required, the liquid from Ringers' plates can be diluted before plating out on growth media to avoid overcrowded plates. In the experiments carried out in this section (3.4.1) the number of cells falling into each floor plate was counted separately and a mean displayed in the tables.

Effect of mixing fan on destination of cells:

A higher recovery in the cyclone is obtained when the mixing fan in the ceiling of the cabinet is not switched on. It is thought that this fan may create air flows in the cabinet causing more of the cells to impinge on the walls and ceiling of the cabinet. If the cell recovery in the cyclone is compared for expt. a and b. above, it can be seen that the recovery in a. is much lower than b. However, the number of cells detected on the cabinet floor seems unaffected. The mixing fan may cause more cells to impact on the cabinet walls.

Table 3.9: Collection of *E. coli* in the Cyclone

A 20 mL suspension of *E. coli* cells in Luria broth was sprayed into the cabinet using the atomiser and collected in the cyclone (operated at 750 $L.min^{-1}$). Sampling with the cyclone continued until the HIAC particle size analyser showed that there were no airborne particles left in the cabinet. The mixing fan on the cabinet ceiling was not operated.

		Expt a	**Expt b**	**Expt c**
Sprayed cells	Total cells	2.1×10^{10}	2.5×10^{10}	1.7×10^{10}
	Viable cells (CFUs)	2.6×10^{10}	n.d.	1.4×10^{10}
Recovery in cyclone (%)	Total cells	68	63	31
	Viable cells (CFUs)	0.4	n.d.	12
Recovery from floor (%)	Total cells	9.1	8.3	5.8
	Viable cells (CFUs)	2.2	n.d.	1.6

The recoveries of 30-60% in the cyclone and 5-10% on the floor are comparable with those obtained for yeast cell suspensions in table 3.8. The results above can be compared with those in table 3.6 for *E. coli* cells released from the Collison Nebuliser. The recoveries for cells released with the atomiser are significantly greater than the recoveries of cells released from the Nebuliser. This is probably due to the inefficiency of the Nebuliser in releasing cells rather than an inefficiency in the cyclone collection method.

In table 3.9 above, experiment a has a viable cell count which is greater than the total cell count, whereas in experiment c. the reverse is true. This difference is probably due to inaccuracies in the plating method used to count viable cells.

3.4.2 Mass Balancing in Soft Film Cabinet

The mass balancing experiments described in the previous section were repeated using the larger soft film cabinet (volume 8.3 m^3). This cabinet was used because it was large enough to contain a piece of processing equipment such as the APV 30CD high pressure homogeniser. When the mass balancing/calibration experiments were completed it would then be possible to examine release from the homogeniser in a known environment.

Table 3.10: Collection of *S. cerevisiae* Sprayed with the Atomiser

A 20 mL suspension of packed yeast cells (71g.L^{-1} wet weight) in Ringers' solution were sprayed into the soft film cabinet (figure 2.2) using the atomiser and collected in the cyclone. The cyclone was operated for the spraying period plus a further 10 minutes. In expt. b the cabinet was not under a positive pressure.

Enumeration method	**Total cells**		**Viable cells (CFUs)**	
Expt	**a**	**b**	**a**	**b**
Sprayed cells	2.1×10^{10}	1.7×10^{10}	1.4×10^{10}	1.0×10^{10}
Recovery in cyclone (%)	17	20	3	3
Recovery from floor (%)	25	20	10	7

The recoveries obtained are approximately half of those from the smaller Bassaire cabinet. The soft film cabinet is 16 time larger in volume than the Bassaire cabinet. In a 30 minute sampling period 1.8 air volume changes occur in the soft film cabinet. The floor recoveries are similar in both cabinets. The reduction in cell viability that occurs during aerosolisation is the same for the Bassaire cabinet, i.e. from around 80% in the spray suspension to 10% in the cyclone sample.

Table 3.11: Persistance of *S. cerevisiae* as an Aerosol

An 18 mL suspension of packed yeast cells (71g.L^{-1} wet weight) in Ringers' solution (5g/70mL) was released into the cabinet at time = 0. Cyclone samples taken at time = 0, 10 minutes and 20 hours.

Enumeration method	Total cells	Viable cells (CFUs)
Cells sprayed	**3.3 x 10^{10}**	**2.5 x 10^{10}**
Recovery in cyclone (%) at t=0	11	1
Recovery from floor (%) at t=0	25	10
Recovery in cyclone (%) at t=10 minutes	0.5	0
Recovery in cyclone (%) at t=20 hours	0.01	tftc

It is interesting to note that although 20 hours after releasing cells into the cabinet, the recovery was reduced to 0.01%, this was equivalent to 3.6 x 10^6 cells. This number of cells is however on the limit of detection using total counts. Further work would be required to accurately predict the length of time that cells will remain suspended in the air.

3.4.3 Mass Balancing in the ACBE

The mass balancing experiments were then scaled up to the Downstream Processing room of the ACBE. This room has a volume of 280m^3 and has been designed to be used for work carried out at Large Scale Containment Category B3. The room is unlikely to be sterile, so that any released microorganisms need to be differentiated from naturally occurring microorganisms present.

Table 3.12: Air Sampling Using the Cyclone

The cyclone was used to sample the air in the Downstream Processing Room of the ACBE for 30 minutes. This experiment was carried out to examine the microbial

content of the air in the processing area. Very little processing work had been undertaken in this room at this stage. The position of the cyclone is shown in figure 3.1. Both total and viable counts were carried out on the collected sample. The HEPA filters in the room were not working during these experiments.

In experiment c. cells were collected into 0.88M sucrose. The sucrose was approximately the same density as any microorganisms that may be present in the air, so that when centrifuged they would sediment together. The sample was then centrifuged at 8000 rpm for 15 minutes before counting in a haemocytometer.

Expt	Total cells ($cells.m^{-3}$)	Viable cells ($CFU.m^{-3}$)
a	8×10^5	4×10^2
b	3×10^5	9×10^3
c	1×10^6	2×10^4

This experiment showed that any yeast cells released into the Downstream Processing Room would be easy to detect because there were no naturally occurring yeast present. The cells counted were all bacterial, with some other particles, probably dirt, of around the same size. This experiment indicated that it would be easy to differentiate released yeast cells from the background population in the recovery room.

Table 3.13: Collection of *S. cerevisiae* from the Downstream Processing Room of the ACBE

A 20 mL suspension of packed yeast cells ($71g.L^{-1}$ wet weight) in Ringers' solution was sprayed into the recovery room using the atomiser and collected in the cyclone. This cell suspension was followed by 5mL of Ringers' solution to clean cells from the atomiser and tubing. The cyclone sampled the room air during spraying and for a further 10 minutes. Both total and viable counts were carried out for the spray suspension and the cyclone sample. The position of the cyclone is shown in figure 3.1.

In the experiments neither supply or extract fans were operated. The recoveries in the cyclone appear to be independent of released cell concentration.

Aerosol	**Expt a**		**Expt b**	
	Total cells	Viable cells (CFUs)	Total cells	Viable cells (CFUs)
	1.7×10^{10}	1.1×10^{10}	1.6×10^{8}	1.6×10^{6}
Recovery in cyclone (%)	5.0	2.4	3.9	1.6

Table 3.14: Effect of Air Circulation on Collection of *S. cerevisiae* from the Downstream Processing Room

These experiments were intended to examine how operating the extract and supply filters in the ceiling of the Downstream Processing Room affected cell recovery in the cyclone.

18 mL of a stock suspension of *S. cerevisiae* in Ringers' solution was released into the Downstream Processing room. The atomiser was used to generate an aerosol of *S. cerevisiae*, and the cyclone used to sample the air. The mass balance was carried out in three different positions, these were: under an air supply filter, under an extract filter and away from either type of filter. Experiments a and b are repeats. Figure 3.2 shows a floor plan of the room.

Cyclone Location	**No Filter**		**Supply Filter**		**Extract Filter**	
Expt	**a**	**b**	**a**	**b**	**a**	**b**
Recovery % (total cells)	1.8	5.0	2.4	2.4	6.1	6.1
Recovery % (viable cells)	1.4	n.d.	1.1	n.d.	2.6	6.9

The results in table 3.14 show that the highest recovery in the cyclone was obtained when the mass balance was carried out underneath the extract filter. This recovery was more than twice that obtained when the mass balance was carried out either under a supply filter, or in the absence of any type of filter.

The extract filter may cause air to flow upwards drawing air containing released cells into the cyclone, producing the highest cell recovery shown in table 3.14. The supply filter may cause air to be dispersed away from the area under the filter. This would cause a decreased recovery of released cells in the cyclone, seen in the table above. However, there is little difference in cell recovery in the cyclone when the mass balance is carried out under a supply filter or without any air movement. The detection and enumeration of cells released into a process environment will depend greatly both on the position of the release and the position of the sampler in relation to the release point and the airflow patterns in the room.

These experiments were not continued due to the health problems that they posed. The deliberate release of even a harmless, non-pathogenic microorganism such as *S. cerevisiae* can cause serious health hazards.

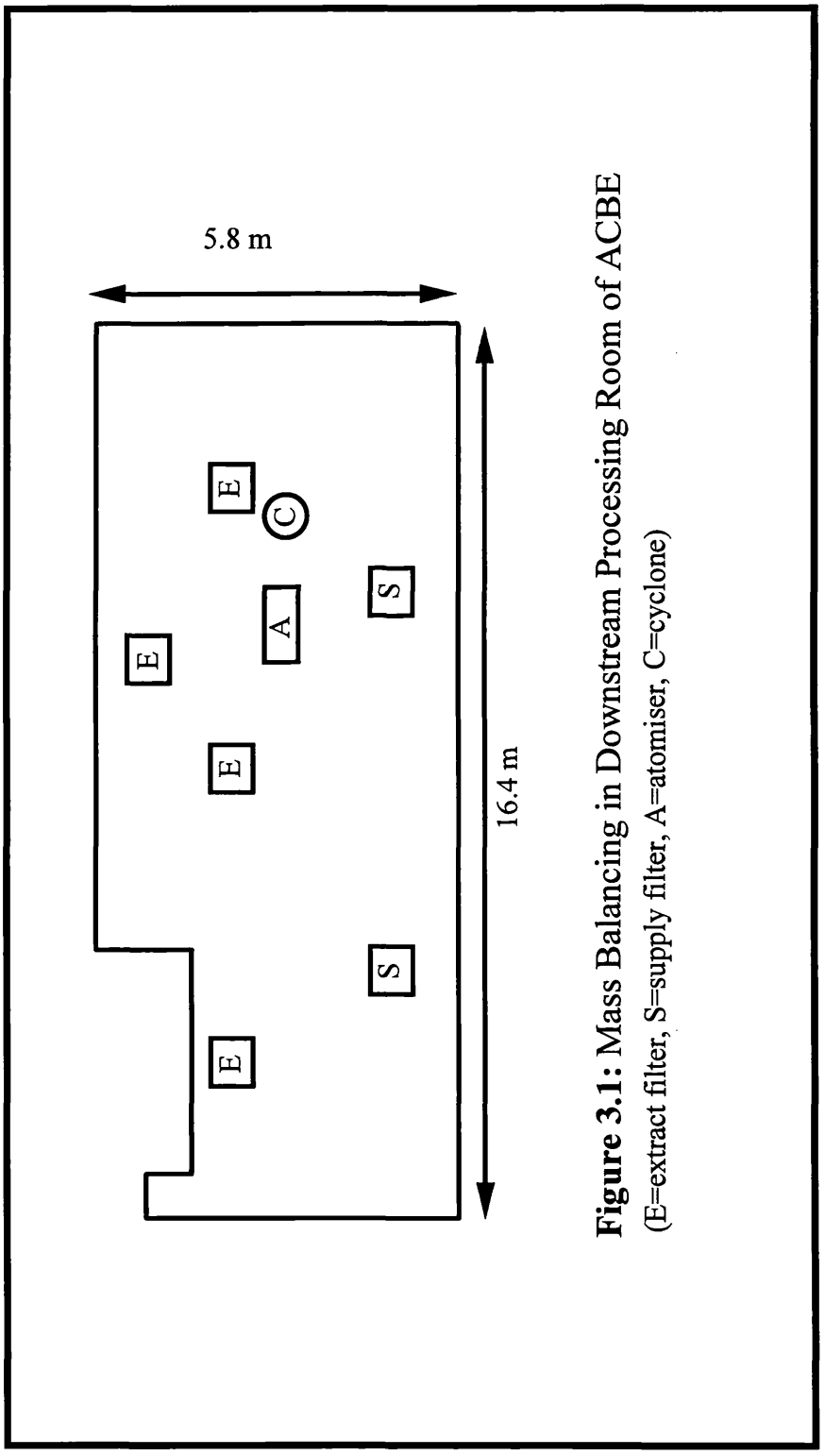


Figure 3.1: Mass Balancing in Downstream Processing Room of ACBE
(E=extract filter, S=supply filter, A=atomiser, C=cyclone)

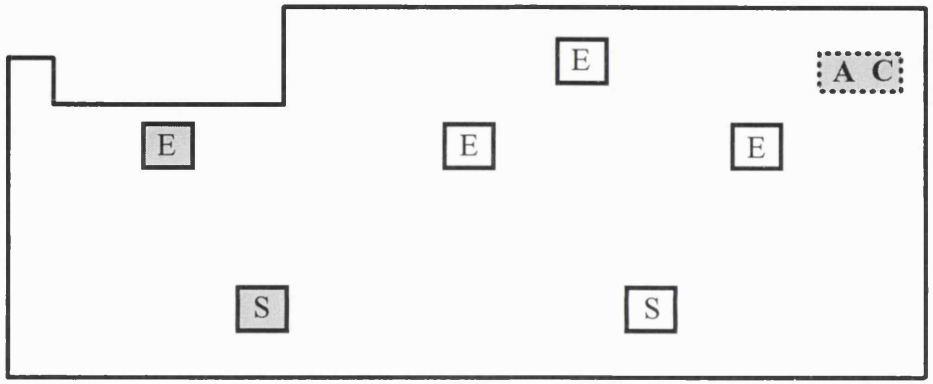


Figure 3.2: Mass Balancing Locations in Downstream Processing Room of ACBE

(E=extract filter, S=supply filter, A=atomiser, C=cyclone)
Mass balances were carried out underneath the two shaded filters and in the right hand corner of the room.

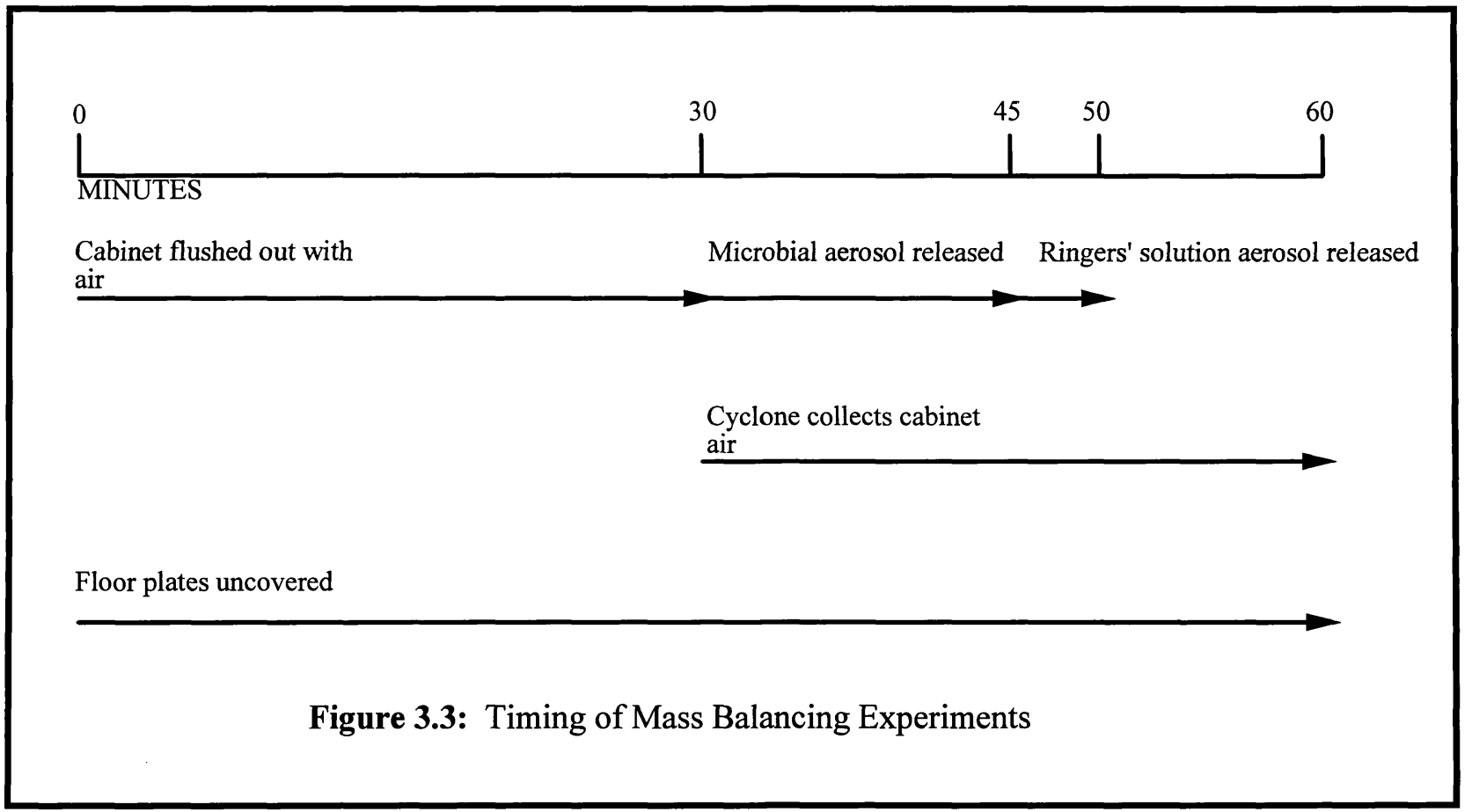


Figure 3.3: Timing of Mass Balancing Experiments

3.5 Factors Affecting Recovery of Microbial Aerosols

3.5.1 Bassaire Cabinet

3.5.1.1 Enumeration by Total Counts

Three different concentrations (approximately 10^7, 10^8, 10^9 cells.mL^{-1}) of *S. cerevisiae* were prepared by diluting a stock cell suspension. After centrifugation, cell suspensions were re suspended in either Ringers' solution or the fermenter broth that they were grown in. These were then sprayed into the Bassaire cabinet using the atomiser (in 18 mL aliquots) and collected with the cyclone. Five Ringers' plates were used to estimate the number of cells that fell to the floor of the cabinet. The floor plates were placed in the same position in each experiment. At the end of the experiment the liquid from the plates was added together. Total counts were enumerated for the cyclone sample and the bulked floor sample. The recoveries shown in the table are the means of 5 experiments. The same procedure was carried out for *E. coli*. Figure 3.3 shows the timings for the experiment.

Table 3.15: Recovery of *S. cerevisiae* and *E. coli* Aerosolised in Ringers' Solution

The cells in these experiments were released into the Bassaire cabinet in a suspension of Ringers' solution.

Aerosol	***S. cerevisiae***			***E. coli***
Total cells released	1.1×10^8	1.1×10^9	1.1×10^{10}	5.5×10^{10}
Mean recovery in cyclone (%)	64 ± 6	43 ± 5	38 ± 4	40 ± 3
Mean recovery from floor (%)	tftc (<20% of the release)	tftc (<2% of the release)	14 ± 1	11 ± 3

These means were compared statistically using a Students' t-test. The recovery for 10^8 (total cells released) was found to be significantly different from both that for 10^9 and 10^{10} at the 0.1% confidence level. There is therefore a significant reduction in recovery with an increase in cell concentration. It is likely that larger

diameter aerosol particles are produced from suspensions with higher cell concentrations. These large particles will be heavier and therefore more likely to fall to the floor of the cabinet. This is seen as an increase from an undetectable number of cells (<1.6 x 10^5 cells.mL^{-1}) on the floor of the cabinet for 10^8 and 10^9 (total cells released), to a recovery of 14% for 10^{10}. This argument is backed up by particle size distribution data for different yeast cell concentrations (section 3.6.2). The mean cyclone recovery for *E. coli* of 40.4% in these experiments compares well with that of 38.2% for a release of 10^{10} yeast cells.

Table 3.16: Recovery of *S. cerevisiae* Aerosolised in Broth

In these experiments the *S. cerevisiae* cells were released in the fermenter broth (malt extract broth) that they were grown in. The recoveries shown in the table are the means of 4 experiments.

Aerosol	***S. cerevisiae***		
Total cells released	1.8 x 10^8	1.8 x 10^9	1.8 x 10^{10}
Mean recovery in cyclone (%)	41 ± 8	33 ± 3	29 ± 3
Mean recovery from floor (%)	tftc (<20% of the release)	~ 20	21 ± 6

Table 3.17: Recovery of *E. coli* Aerosolised in Ringers' Solution

The *E. coli* cells in these experiments were released from Ringers' solution. The recoveries shown in the table are the means of 3 experiments.

Aerosol	***E. coli***		
Total cells released	3.6×10^9	3.6×10^{10}	3.6×10^{11}
Mean recovery in cyclone (%)	23 ± 11	30 ± 7	35 ± 9
Mean recovery from floor (%)	tftc (<20% of the release)	~ 20	24 ± 4

Table 3.18: Recovery of *E. coli* Aerosolised in Broth

The *E. coli* cells used in these experiments were released in the fermentation broth that they were grown in. The recoveries shown are the means of 4 experiments.

Aerosol	***E.coli***		
Total cells released	4.9×10^9	4.9×10^{10}	4.9×10^{11}
Mean recovery in cyclone (%)	39 ± 9	28 ± 5	27 ± 8
Mean recovery from floor (%)	tftc (<20% of the release)	~ 20	14 ± 7

Several points arise from the results in tables 3.15 - 3.18:

1. The recovery of *S. cerevisiae* and *E. coli* aerosols in the cyclone is dependent upon the concentration of the released cells.
2. The recovery of these aerosols is also dependent upon the composition of the medium that the cells are suspended in when aerosolised.

Beginning with the effect of released cell concentration on recovery in the cyclone: Table 3.15 shows clearly that as the released concentration of *S. cerevisiae* increases from 10^6 to 10^8 cells.mL^{-1} (equivalent to 10^8 and 10^{10} total cells respectively), there is a significant decrease in recovery in the cyclone. This is accompanied by an increase in the recovery of cells from the floor of the cabinet. Table 3.16 also shows a decrease in recovery of *S. cerevisiae* cells as the released cell concentration increases. In these experiments the cells were suspended in fermenter broth.

The results for *E. coli* are less clear. When cells are released in Ringers' solution there is an increase in recovery in the cyclone as released cell concentration increases. However when cells are released in Fermenter broth, the reverse is true, i.e. the recovery decreases as released cell concentration increases.

Recoveries from the floor of the cabinet increased to around 20% for *E. coli* in Ringers' solution and *S. cerevisiae* in fermenter broth, whilst those for the remaining suspensions were unchanged at around 15%.

These results show that the recovery of cells in the cyclone is highly dependent upon released cell concentration and the contents of the liquid in which the cells are suspended. The data suggest that these two factors affect the destination of the released cells rather than the efficiency of the cyclone.

3.5.1.2 Enumeration by Viable Count

The experimental procedure described in 3.5.1.1 was followed. However in these experiments cell numbers were estimated by plating the samples on growth media. Colonies were counted after the plates were incubated.

Table 3.19: Recovery of *S. cerevisiae* Aerosolised in Ringers' Solution

Aerosol	**1.09 x 10^8**	**1.09 x 10^9**	**1.09 x 10^{10}**
Mean recovery in cyclone (%)	1.3 ± 1.9	0	2.3 ± 3.7
Mean Recovery from the floor (%)	120 ± 212	12 ± 7	14 ± 5

Table 3.20: Recovery of *S. cerevisiae* Aerosolised in Broth

Aerosol	**1.23 x 10^8**	**1.23 x 10^9**	**1.23 x 10^{10}**
Mean recovery in cyclone (%)	0	0.7 ± 0.8	5.2 ± 0.7
Mean Recovery from the floor (%)	15 ± 4	18 ± 6	25 ± 20

Table 3.21: Recovery of *E. coli* Aerosolised in Ringers' Solution

Aerosol	**9.36 x 10^8**	**9.36 x 10^8**	**9.36 x 10^9**
Mean recovery in cyclone (%)	34 ± 28	1 ± 1	4 ± 1
Mean Recovery from the floor (%)	42 ± 40	52 ± 63	13 ± 3

Table 3.22: Recovery of *E. coli* Aerosolised in Broth

Aerosol	**2.6 x 10^8**	**2.6 x 10^9**	**2.6 x 10^{10}**
Mean recovery in cyclone (%)	10 ± 4	1 ± 1	4 ± 1
Mean Recovery from the floor (%)	4 ± 3	3 ± 3	5 ± 2

When cell recoveries are calculated based on viable counts rather than total cell counts, the effect of the cyclone on cell viability can be seen. In tables 3.19-3.22 the recoveries from the floor are greater than those from the cyclone. This is because cells recovered from the floor are more likely to retain their viability than those recovered in the cyclone. It was seen in table 3.9 that the viability of *E. coli* cells is reduced far more when the cells are collected in the cyclone compared to floor plates. The particles falling to the floor are probably the large ones and therefore cells are coated with a layer of salts which may protect the cell.

The recovery of 1.1 x 10^8 *S. cerevisiae* cells in Ringers' solution from the floor of the cabinet is greater than 100%. This may be due to a particularly large particle containing many cells not having been broken up before it was spread on the agar. This effect could also explain the overall variability in these viable count data. Although viability is used frequently to count cells, plate counts are difficult to repeat accurately and viability itself is a very difficult state to define.

The standard deviations shown in the tables are very large, as large as the means in some cases. This implies that these data are not very consistent. This lack of consistency probably reflects on the cell enumeration method used rather than the efficiency of the cyclone in sampling the cabinet air. The cyclone samples the total cabinet volume in each minute that it is operated.

3.5.2 Soft Film Cabinet

Table 3.23: Recovery of *S. cerevisiae* and *E. coli* Aerosolised in Ringers' solution

The experimental procedure described in section 3.5.1.1 was followed for these experiments, except that the aerosols were released into the soft film cabinet. The numbers in the table are the mean recoveries, based on total counts, for 5 repeats.

Aerosol	***S. cerevisiae***			***E. coli***
Total cells released	1.4 x 10^8	1.4 x 10^9	1.4 x 10^{10}	8.9 x 10^{10}
Mean recovery in cyclone (%)	31 ± 6	19 ± 5	15 ± 3	17 ± 2
Mean recovery from floor (%)	tftc (<20% of the release)	tftc (<2% of the release)	~ 30	14 ± 5

A Student' t-test was carried out to test if the difference between the mean recoveries was significant. The recovery for 10^8 total cells released into the cabinet was found to be significantly different from that for 10^9 cells at the 1.0% confidence level and significantly different from that for 10^{10} cells at the 0.1% confidence level. Again, this indicates that there is a decrease in recovery in the cyclone as the concentration of the released cells increases. The mean cyclone recovery for *E. coli* of 17.1% compares well with that of 15.4% for a release of

10^{10} yeast cells. Although the mean recovery from the floor is much lower for *E. coli* compared with yeast cells at the same concentration.

When the cell recoveries for aerosols released into the soft film cabinet (table 3.23 above) are compared with those in table 3.15 for aerosols released into the Bassaire cabinet, it can be seen that the recoveries are approximately halved. This is interesting because the soft film cabinet has a volume 23 x that of the Bassaire cabinet. When the cyclone samples from the Bassaire cabinet, the whole volume of air is sampled each minute. This is not the case when sampling from the soft film cabinet and for this reason collection data from the soft film cabinet are expected to be less consistent. It is likely that more cells will be recovered from the floor of the soft film cabinet because less than 2 air changes occur inside the cabinet, during the whole sampling period.

The experiment described above was repeated with the APV 30CD homogeniser inside the soft film cabinet. The results are shown in table 3.24.

Table 3.24: Recovery of *S. cerevisiae* from Soft Film Cabinet Containing the APV 30CD Homogeniser

Cells Released (*S. cerevisiae* in Ringers' solution)	**1.3 x 10^{10} total**	**1.1 x 10^{10} viable**
Mean recovery in cyclone (%)	21 ± 2	6 ± 2
Mean recovery from floor (%)	21 ± 5	16 ± 3

These results show that a significant proportion of a microbial aerosol can be recovered from the soft film cabinet and that the results are consistent when the mass balance is repeated. This then suggests that if an aerosol of significant proportions was released from a piece of bioprocessing equipment, it would be possible to measure that release using the mass balancing technique developed in this chapter. This is investigated in section 4.3, using the APV 30CD high pressure homogeniser.

3.6 Particle Size Analysis of Microbial Aerosols

3.6.1 Aerosol Release and Sampling in Bioaerosol Test Chamber

S. cerevisiae and *E. coli* cells were grown in shake flasks and prepared as described in section 2.3.1. Each suspension of cells in fermenter broth was split into two parts. One half of each cell type was centrifuged and the cells re-suspended in Ringers' solution. The remaining half of each was kept in fermenter broth. The cell concentration for each of the four suspensions was estimated in a counting chamber. The suspensions were then diluted to give the following approximate cell concentrations for each: 10^7 (*S. cerevisiae* only), 10^8, 10^9 and 10^{10} (*E. coli* only) cells.mL^{-1}.

The glass atomiser was used to release the cell suspensions into the Bioaerosol Test Chamber (figure 2.3). For these experiments the atomiser was positioned vertically, with the outlet pointing downwards. Cells were released at the top of the cabinet and air was sampled from the bottom section by the Aerodynamic Particle Sizer (APS) described in section 2.2.9. The suspensions were aerosolised for at least 15 minutes to ensure that a representative sample reached the APS. The APS sampled cabinet air for 10 minute periods at the same time that cells were released. The settling time for particles released at the top of the cabinet is approximately 30 seconds. During these experiments the temperature inside the cabinet was maintained at 20^{o}C and the humidity maintained at 50%.

This procedure was carried out for all four cell suspensions and for Fermenter broth and Ringers' solution blanks. The particle size distributions are shown in section 3.6.2.

3.6.2 Particle Size Distributions

For each cell type and the blanks, mass concentration (mg.m^{-3}) divided by channel width (μm) was plotted against particle midsize (μm). For *S. cerevisiae*, the particle size distribution starts at a diameter of 2μm, and for *E. coli* the lower limit is 1μm. Mass concentration rather than number concentration were chosen for these data because it is easier to relate changes in the solids contents of the broth to mass concentration rather than number concentration. A density of 2.3 kg.m^{-3} was

assumed for all cell suspensions in converting numbers of particles to a mass. The data are shown in Appendix B.

Figures 3.4 and 3.5 show particle size distributions for *S. cerevisiae* cells aerosolised in Ringers' solution and fermenter broth. The curves for the controls (either Ringers' solution of fermenter broth) and for the two lower cell concentrations (10^7 and 10^8 cells.mL^{-1}) are very similar. It is only when the cell concentration reaches 10^9 cells.mL^{-1} that the cells make an impact on the size distribution of particles in the aerosol.

In both figures 3.4 and 3.5 there is a large peak at 5μm for the highest cell concentration, corresponding to the approximate diameter of a *S. cerevisiae* cell. Apart from this peak at 5μm, there are no other distinct peaks on the curves. This indicates either that there is little clumping of cells in the aerosol particles or that the larger particles settle out very rapidly and are not captured.

The mass concentration/channel width for *S. cerevisiae* cells in broth are approximately four times those for the same concentration of *S. cerevisiae* cells in Ringers' solution. This must be due to the larger mass of solids present in fermenter broth compared to Ringers' solution (see table 3.26). An equivalent volume of cells suspended in broth contains a greater mass of solids compared to cells suspended in Ringers' solution. It is the solids alone that give rise to the particles because any water in the droplets will have evaporated probably as soon as the aerosol is produced, or at least before it is sampled.

Figures 3.6 and 3.7 show the particle size distributions for *E. coli* cells aerosolised in Ringers' solution and fermenter broth. Like those for *S. cerevisiae*, the curves for the lower two cell concentrations (10^8 and 10^9 cells.mL^{-1}) are very similar to the curves for Ringers' solution and fermenter broth only. The curve for the higher cell concentration (10^{10} cells.mL^{-1}) does break away from those for the lower cell concentration and forms many peaks. Unlike that for *S. cerevisiae*, there is no one distinctive peak but a series of smaller ones. This suggests that the *E. coli* cells clump together in the aerosol particles much more than the *S. cerevisiae* cells.

This effect described, of only the highest cell concentrations producing distinctive particle size distributions could either be due to a larger sample volume being presented to the APS, or to a larger number of cells present in the same volume.

Since the same sample size was used in each case (i.e. of a 10 minute time interval), the effect must be due to the higher cell concentration.

Two factors suggest that the proportion of cells released from the atomiser that reach the APS detector may be small:

1. The APS takes in air from the cabinet at a flow rate of only $5L.min^{-1}$.
2. The aerosol is released at the top of the Bioaerosol Test Chamber and is not collected until it reaches the bottom. There are several surfaces inside the cabinet which particles could attach to.

The following calculation supports this argument.

Assuming that all particles have a diameter of 5 μm (similar to *S. cerevisiae* cells), the volume of a particle = $4/3 \times \pi \times r^3 = 65.45\ \mu m^3$.

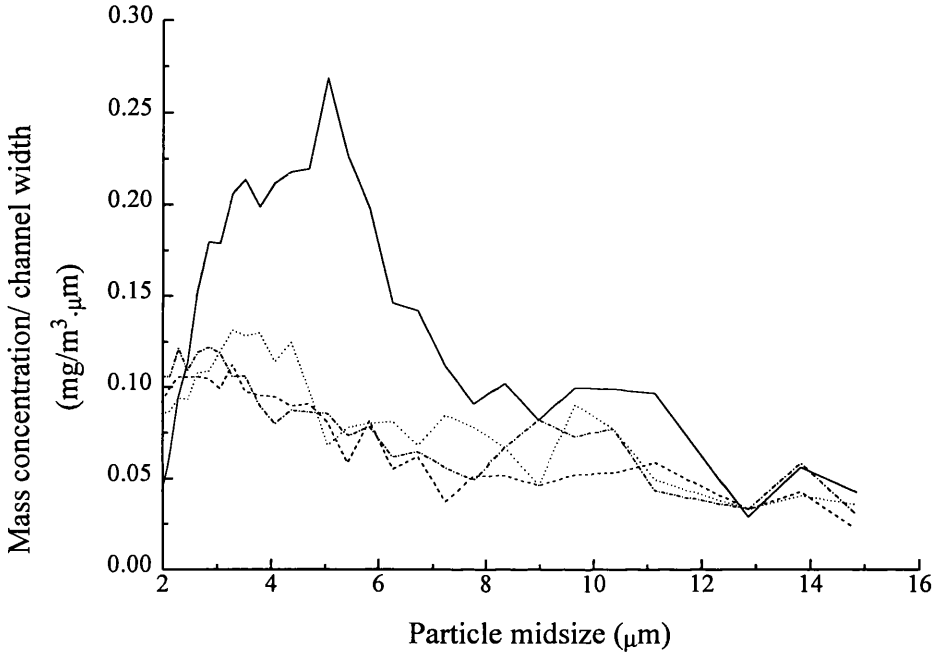
The number of particles contained in a 1mL volume,
$1mL = 1cm^3 = 10^{12}\ \mu m^3$
therefore there are $10^{12}/65.45 = 1.53 \times 10^{10}$ particles of 5 μm in 1mL.

A 50mL volume of each cell suspension was released into the Bioaerosol Test Chamber,
therefore, in 50mL there would be $50 \times 1.53 \times 10^{10} = 7.63 \times 10^{11}$ particles.

The Bioaerosol Test Chamber has a volume of $3m^3$. If 7.63×10^{11} particles are dispersed evenly in this volume, the concentration is $(7.63 \times 10^{11})/(3 \times 10^6)$
i.e. 2.54×10^5 particles.mL^{-1}.

The APS did not detect more than 20 particles.mL^{-1} for any of the cell suspensions, therefore the APS has a sampling efficiency of approximately 0.008%.

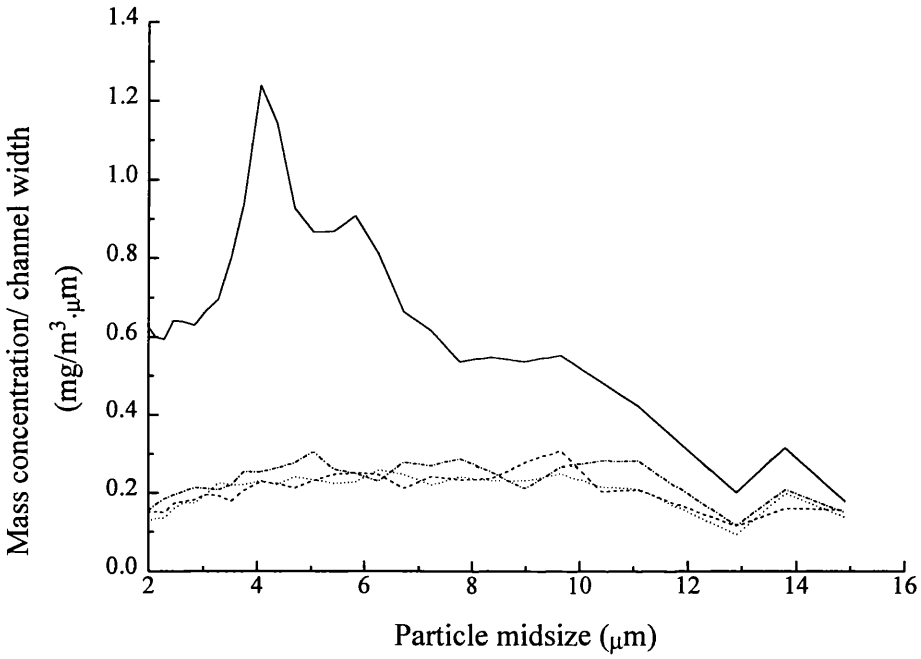
Figure 3.4: Particle Size Distribution of *Saccharomyces cerevisiae* Aerosolised from Ringers' Solution



Approximately 50 mL of cells in liquid suspension were released into the top of the Bioaerosol Test Chamber using the atomiser. The Aerodynamic Particle Sizer sampled air from the bottom of the chamber.

---- Ringers' solution only, ... 1.6×10^7 cells.mL^{-1}, ... 1.6×10^8 cells.mL^{-1}, —— 1.6×10^9 cells.mL^{-1}

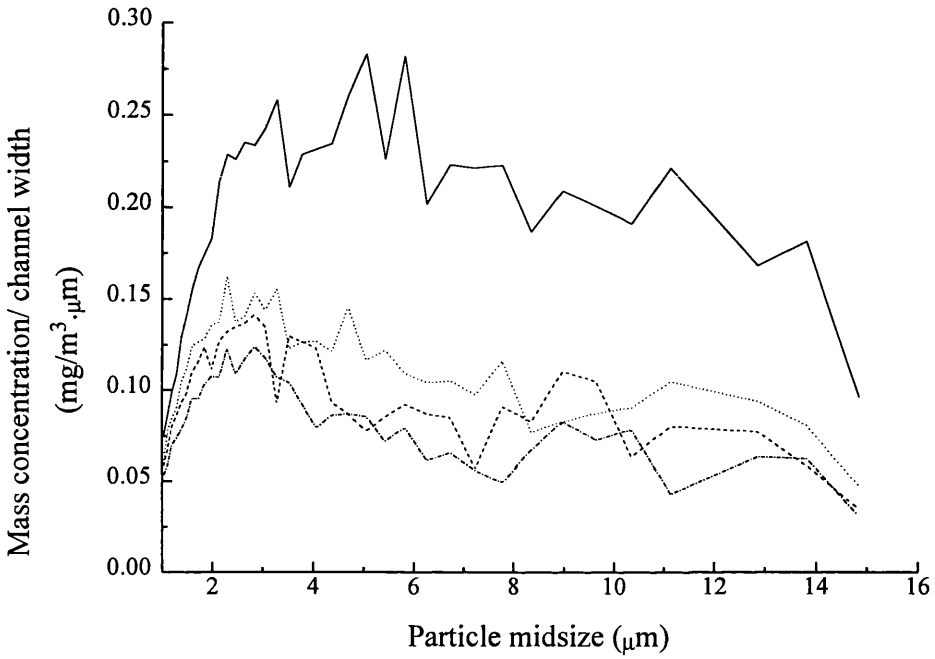
Figure 3.5: Particle Size Distribution of *Saccharomyces cerevisiae* Aerosolised from Broth



Approximately 50 mL of cells in liquid suspension were released into the top of the Bioaerosol Test Chamber using the atomiser. The Aerodynamic Particle Sizer sampled air from the bottom of the chamber.

——— Malt extract broth only, ····· 2.4×10^7 cells.mL^{-1}, ····· 2.4×10^8 cells.mL^{-1}, —— 2.1×10^9 cells.mL^{-1}

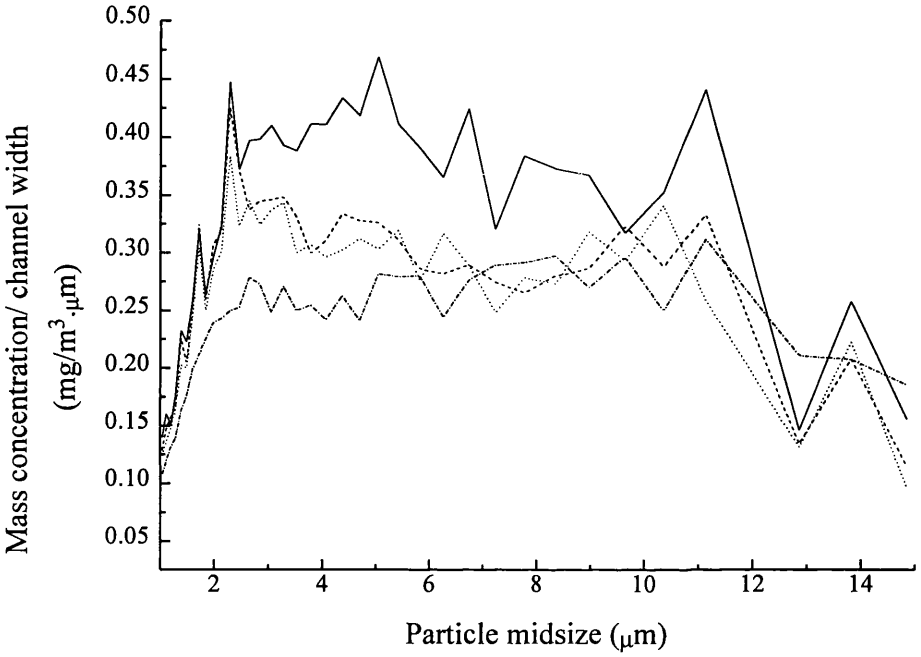
Figure 3.6: Particle Size Distribution of *Escherichia coli* Aerosolised from Ringers' Solution



Approximately 50 mL of cells in liquid suspension were released into the top of the Bioaerosol Test Chamber using the atomiser. The Aerodynamic Particle Sizer sampled air from the bottom of the chamber.

--------- Ringers' solution only, ... 2.9×10^8 cells.mL^{-1}, ... 2.9×10^9 cells.mL^{-1}, —— 2.9×10^{10} cells.mL^{-1}

Figure 3.7: Particle Size Distribution of *Escherichia coli* Aerosolised from Broth



Approximately 50 mL of cells in liquid suspension were released into the top of the Bioaerosol Test Chamber using the atomiser. The Aerodynamic Particle Sizer sampled air from the bottom of the chamber.

-------- Luria broth only, ... 3.8×10^8 cells.mL^{-1}, ... 3.8×10^9 cells.mL^{-1}, —— 3.8×10^{10} cells.mL^{-1}

3.6.2.1 Aerosol Masses

Bennett and Norris (1989) showed that the deposition and size distribution of aerosol particles depend upon the concentration of dissolved solids in the original suspension. The possiblity of a microbial cell occurring in each airborne particle will depend upon the concentration of microorganisms in the aqueous suspension.

Hambleton *et al.* (1992) discuss the particle size distribution of aerosols produced from liquid suspensions. The ultimate particle size of the droplet will depend not only on the initial droplet size, but also on the mass concentration of the original suspension. This includes the solution that the cells are dispersed in, as well as the cells themselves.

Tables 3.25 and 3.26, below, show aerosol masses calculated for each concentration for both cell species. This mass includes both the mass of the cells and of the suspending solution.

$$\text{Cell volume} = \frac{\pi d^3}{6}$$

Where d = 5μm = 5×10^{-6} m for *S. cerevisiae* (1×10^{-6} m for *E. coli*)

$$\text{Volume} = \frac{\pi \times (5 \times 10^{-6})^3}{6} = 6.545 \times 10^{-17}\ m^3$$

Density = 1400 $kg.m^{-3}$

Therefore mass of one yeast cell = $6.545 \times 10^{-17} \times 1400 = 9.163 \times 10^{-14}$ kg

If there are 2.4×10^7 $cells.mL^{-1}$, mass of 1mL = $9.163 \times 10^{-14} \times 2.4 \times 10^7$
= **2.2 $mg.mL^{-1}$**

Malt extract or Luria broth is added at a concentration of $20g.L^{-1}$, therefore $20mg.mL^{-1}$.

Tables 3.25 and 3.26 below show that the broth present in the suspensions makes up the majority of the total mass at the lower cell concentrations. It is only when there is more than 10^9 $cells.mL^{-1}$ present that the cell mass becomes the larger factor.

Table 3.25: Mass Concentration of *S. cerevisiae* Suspensions

***S. cerevisiae* concentration (cells.mL^{-1})**	**Mass of cells only (mg.mL^{-1})**	**Mass of cells and broth (mg.mL^{-1})**
2.4×10^7	2.3	22
2.4×10^8	23	43
2.1×10^9	192	212

Table 3.26: Mass Concentration of *E. coli* Suspensions

***E. coli* concentration (cells.mL^{-1})**	**Mass of cells only (mg.mL^{-1})**	**Mass of cells and broth (mg.mL^{-1})**
3.8×10^8	0.28	20.3
3.8×10^9	2.8	22.8
3.8×10^{10}	28	47.8

3.6.2.2 Particle Size Distribution and Cell Recovery in the Cyclone

This particle size distribution study was carried out to discover if there was a difference in the particle size of aerosols produced from cell suspensions of different concentration. It was thought that this may be the reason for a decrease in cell recovery in the cyclone as released cell concentration increased. Larger diameter particles would fall to the floor of the cabinet more easily and evade capture in the cyclone. If more of these larger particles were produced from the higher cell concentrations this would explain the reduced cyclone recovery.

Figures 3.4 and 3.5 show more smaller particles (3-8 μm) present at the higher concentration of both *S. cerevisiae* samples at the highest cell concentration only. At the larger particle diameters there is a less marked difference in the mass of particles present at the higher *S. cerevisiae* concentrations. This does not correlate with the cyclone recoveries for *S. cerevisiae* cells in both broth and Ringers'

solution (tables 3.15 and 3.16), which decrease as released cell concentration increases.

The particle size distributions of the highest cell concentration for *E. coli* aerosols (figures 3.6 and 3.7) show a larger number of all particles sizes. This is more marked for *E. coli* suspended in Ringers' solution rather than fermenter broth. Again, these particle size distributions do not correlate well with the cyclone recovery data in tables 3.17 and 3.18. Table 3.17 shows that recovery of *E. coli* suspended in Ringers' solution in the cyclone increase as released cell concentration increases. Table 3.18, shows that recovery in the cyclone of *E. coli* suspended in fermenter broth decreases as released cell concentration increases. This data are discussed further in section 5.2.

3.7 The Polymerase Chain Reaction

3.7.1 Detection of Transketolase *E. coli* Cells Using Kanamycin Primers

These experiments were carried out to investigate:
1. Whether Transketolase (TK) *E. coli* cells could be detected in the presence of unmodified (UM) *E. coli* cells.
2. The limit of detection of the Polymerase Chain Reaction (PCR) to TK *E. coli* cells.

Both TK and UM *E. coli* cells were grown up in shake flasks containing Luria broth and Nutrient broth (with Kanamycin) respectively. At harvest, the TK *E. coli* had reached a concentration of 2.9 x 10^9 CFU.mL^{-1} and the UM had reached a concentration of 3.6 x 10^9 cells.mL^{-1}. The two fermentation broths were then diluted from 10^9 CFU.mL^{-1} to 10^3 CFU.mL^{-1} in sterile RO water. Each of the 6 TK *E. coli* dilutions was prepared using one of the following UM *E. coli* concentrations as a diluant: 10^3, 10^5, 10^7 and 10^9 CFU.mL^{-1}.

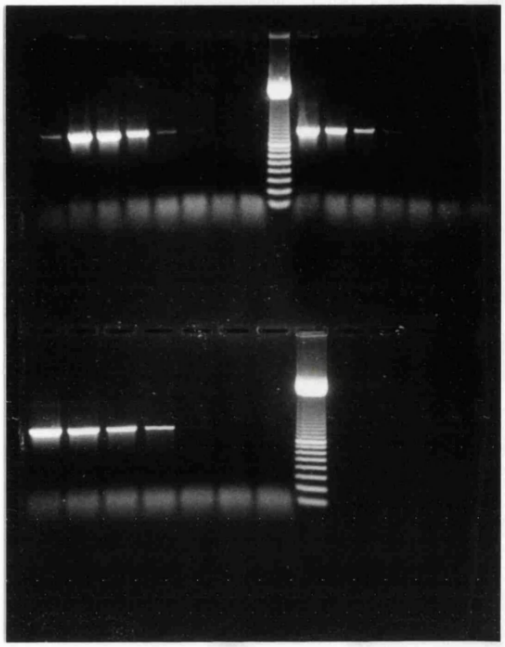
PCR was carried out on these 24 combinations using the KM1 and KM2 primers (see section 2.3.4.3 for method). The PCR products were loaded on electrophoretic gels shown in figures 3.8(i) and (ii).

This experiment demonstrates that, using PCR, it is possible to detect 2.9 x 10^3 TK *E. coli* cells, in a background of 3.2 x 10^7 UM *E. coli* cells. This is equivalent to 1 TK *E. coli* cell in 10^3 UM *E. coli* cells.

The pUC plasmid, which contains the DNA fragment recognised by KM1 and 2, has a copy number of 2-300 per cell. It is therefore not unrealistic to expect to detect less than 10^3 target cells. One way of improving the sensitivity of the PCR reaction may be to lyse the target cells before carrying out the reaction.

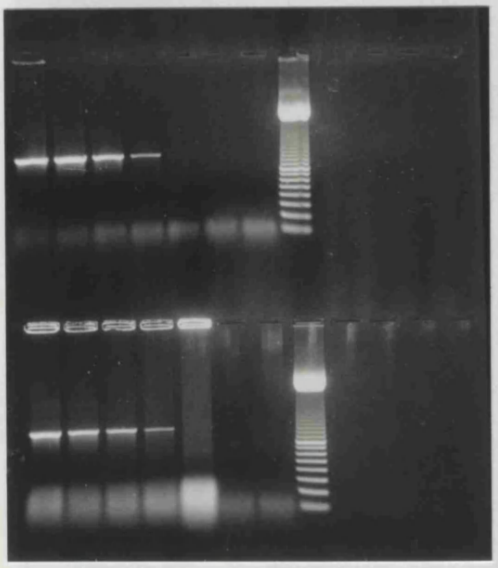
Figure 3.9 shows the electrophoretic gel for the PCR products produced when the cell suspensions are heated to 99°C for 30 minutes before carrying out the PCR. It can be seen that 2.9 x 10^2 TK *E. coli* cells can be detected in a background of 3.2 x 10^7 UM *E. coli* cells. Thus, a lysis step before carrying out the PCR improved the sensitivity 10-fold. However, there was no improvement in the sensitivity of the PCR for TK *E. coli* cells in the absence of UM *E. coli* cells.

Figure 3.8 (i): Detection of Kanamycin Resistance Gene from Whole Transketolase *E. coli* Cells



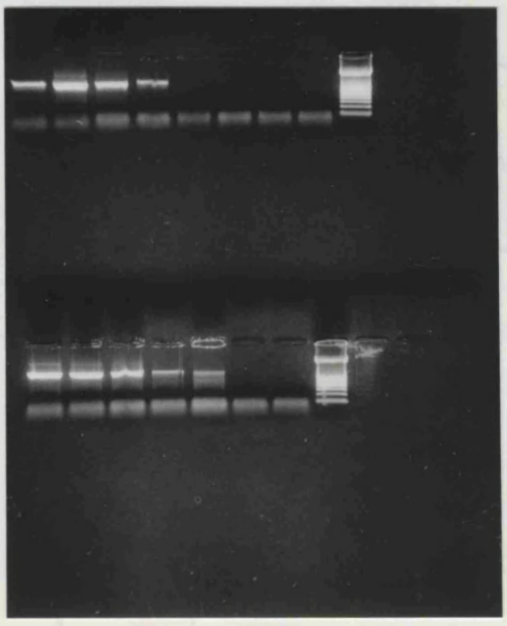
Well	Sample	UM *E. coli* cells	Well	Sample	UM *E. coli* cells
1 (top left)	*Pseudomonas aureofaciens* (positive control)	-	14 (bottom left)	2.9 x 10^2 TK *E. coli* cells	3.24 x 10^1
2	2.9 x 10^6 TK *E. coli* cells	-	15	2.9 x 10^1 TK *E. coli* cells	3.24 x 10^1
3	2.9 x 10^5 TK *E. coli* cells	-	16	Blank	-
4	2.9 x 10^4 TK *E. coli* cells	-	17	2.9 x 10^6 TK *E. coli* cells	3.24 x 10^3
5	2.9 x 10^3 TK *E. coli* cells	-	18	2.9 x 10^5 TK *E. coli* cells	3.24 x 10^3
6	2.9 x 10^2 TK *E. coli* cells	-	19	2.9 x 10^4 TK *E. coli* cells	3.24 x 10^3
7	2.9 x 10^1 TK *E. coli* cells	-	20	2.9 x 10^3 TK *E. coli* cells	3.24 x 10^3
8	Blank	-	21	2.9 x 10^2 TK *E. coli* cells	3.24 x 10^3
9	100 base pair ladder	-	22	2.9 x 10^1 TK *E. coli* cells	3.24 x 10^3
10	2.9 x 10^6 TK *E. coli* cells	3.24 x 10^1	23	Blank	-
11	2.9 x 10^5 TK *E. coli* cells	3.24 x 10^1	24	100 base pair ladder	-
12	2.9 x 10^4 TK *E. coli* cells	3.24 x 10^1			
13	2.9 x 10^3 TK *E. coli* cells	3.24 x 10^1			

Figure 3.8 (ii): Detection of Kanamycin Resistance Gene from Whole Transketolase *E. coli* Cells



Well	Sample	UM *E. coli* cells
1 (top left)	2.9 x 10^6 TK *E. coli* cells	3.24 x 10^5
2	2.9 x 10^5 TK *E. coli* cells	3.24 x 10^5
3	2.9 x 10^4 TK *E. coli* cells	3.24 x 10^5
4	2.9 x 10^3 TK *E. coli* cells	3.24 x 10^5
5	2.9 x 10^2 TK *E. coli* cells	3.24 x 10^5
6	2.9 x 10^1 TK *E. coli* cells	3.24 x 10^5
7	Blank	-
8	100 base pair ladder	-
9 (bottom left)	2.9 x 10^6 TK *E. coli* cells	3.24 x 10^7
10	2.9 x 10^5 TK *E. coli* cells	3.24 x 10^7
11	2.9 x 10^4 TK *E. coli* cells	3.24 x 10^7
12	2.9 x 10^3 TK *E. coli* cells	3.24 x 10^7
13	2.9 x 10^2 TK *E. coli* cells	3.24 x 10^7
14	2.9 x 10^1 TK *E. coli* cells	3.24 x 10^7
15	Blank	-
16	100 base pair ladder	-

Figure 3.9: Detection of Kanamycin Resistance Gene from Lysed Transketolase *E. coli* Cells



Well	Sample	UM *E. coli* cells
1 (top left)	*Pseudomonas aureofaciens* (positive control)	-
2	2.9 x 10^6 TK *E. coli* cells	-
3	2.9 x 10^5 TK *E. coli* cells	-
4	2.9 x 10^4 TK *E. coli* cells	-
5	2.9 x 10^3 TK *E. coli* cells	-
6	2.9 x 10^2 TK *E. coli* cells	-
7	2.9 x 10^1 TK *E. coli* cells	-
8	Blank	-
9	100 base pair ladder	-
10 (bottom left)	2.9 x 10^6 TK *E. coli* cells	3.24 x 10^7
11	2.9 x 10^5 TK *E. coli* cells	3.24 x 10^7
12	2.9 x 10^4 TK *E. coli* cells	3.24 x 10^7
13	2.9 x 10^3 TK *E. coli* cells	3.24 x 10^7
14	2.9 x 10^2 TK *E. coli* cells	3.24 x 10^7
15	2.9 x 10^1 TK *E. coli* cells	3.24 x 10^7
16	Blank	-
17	100 base pair ladder	-

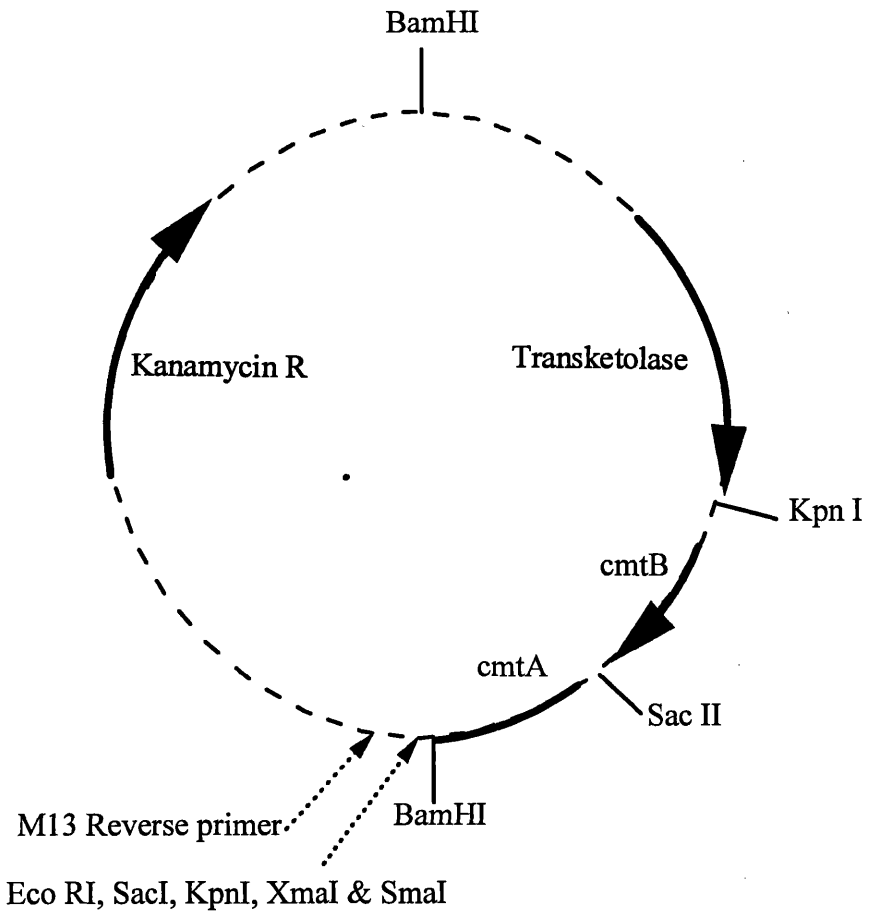


Figure 3.10: Plasmid pQR 701

3.7.2 Detection of TK *E. coli* Using the Primers LF1, M13F and M13R

The PCR was carried out using the new primer, LF1, with both M13F and M13R. The sequences of these primers are shown in section 2.3.2 and the structure of the pQR 701 plasmid carrying the transketolase gene is shown in figure 3.10.

Combinations of LF1 with M13F and M13R were prepared with both purified plasmid pQR700 and with whole TK *E. coli* cells. The plasmid pQR700 has the transketolase gene in opposite orientation compared to the plasmid pQR701 present in the TK *E. coli* cells. The efficiency of the PCR is not however affected by this difference.

When the PCR products were loaded onto a gel, no bands were obtained for the transketolase fragment, from either the pQR700 plasmid or the pQR701 TK *E. coli* cells. It was thought that the PCR may be affected by a change in annealing temperature and extension time. The annealing temperature was reduced from the standard 50°C to 45°C and the extension time reduced from 3 minutes to 30 and 60 seconds. The PCR was unaffected by these changes. During gel electrophoresis a band the for the transketolase fragment did not appear.

The possibility of a PCR reaction working is dependent upon the concentration of magnesium present in the reaction. The concentration of Mg present may change due to:

1. Mg binding to dNTPs.
2. Tris-EDTA buffer (used for diluting reactants) chelating Mg^{2+}.

For these reasons the concentration of Mg present may need optimising. The standard [Mg] of 1.5mM was increased to: 2, 4, 6, and 8mM. The PCR was, however, unaffected by these changes in [Mg].

The primer LF1 did not detect the pQR 701 plasmid because the corresponding DNA sequence was not cloned into the plasmid. The whole cmtA gene sequence was not included when the transketolase and cmtB genes were cloned into pQR 701.

CHAPTER FOUR: RELEASE OF MICROORGANISMS FROM BIOPROCESSES

4.1 Process Design for Containment

Containment can be defined as the act of preventing the spread, beyond certain limits, of a biological process stream regarded as hazardous to either workers, or the external environment. The purpose of containment is to reduce exposure of workers and other people, to prevent release of potentially hazardous agents into the outside environment and to protect the product. It can be achieved either by biological or physical containment. Physical containment reduces the chance that GMOs will escape from the laboratory or plant. Biological containment reduces the probability of survival of GMOs in the environment, if they were to escape.

4.1.1 Biological Disablement

Natural barriers exist which limit an organisms ability to survive or transfer genetic information in specific environments. These highly specific barriers can be employed to aid in containing the organism, and include characteristics such as auxotrophy and UV sensitivity. When such barriers exist naturally or are introduced specifically into the organism, it is said to possess biological containment. The degree of biological containment possessed by an organism can be influenced by manipulation of the organism or the vector. The most commonly employed biological containment modifications limit either the survival and multiplication of the organism in the environment or the transmission of the genetic information to other organisms.

The term "biological containment" can be easily misunderstood, because the organism to which it applies is released into the environment. A more appropriate term would be biological disablement. The principle of biological containment therefore relies on our ability to introduce into certain environments, microorganisms that would survive less well than the wild type of the species. One way of decreasing long term survival could be to introduce mutations that prevent cyclic AMP (cAMP) and cAMP Receptor Protein (CRP) synthesis (Curtiss, 1988). Strains of enteric bacteria with *cya* and *crp* mutations have a generation time 50% longer than the wild type (Curtiss and Kelly, 1987). Microorganisms with the cAMP-CRP method for positive gene regulation use it to regulate the expression of

a diversity of genes and operons for amino acid and carbohydrate transport and utilisation (Perlman and Pastan, 1969; Alper and Ames, 1970; Pastan and Perlman, 1970; Yokota and Gots, 1970; Rickenberg, 1974; Gemmil *et al.,* 1984), and cell surface structures such as fimbriae (Saier *et al.,* 1978), flagella (Yokota and Gots, 1970; Komeda *et al.,* 1975) and outer membrane proteins (Movva *et al.,* 1981; Bremer *et al.,* 1988).

A much better strategy is to include a conditionally lethal phenotype which kills released cells at a desired time. These phenotypes are made by placing lethal genes under the control of promoters activated under certain environmental conditions and would trigger cell death when those conditions are reached. Many bacterial genes are potentially lethal if they are expressed at altered levels or times in the bacterial life cycle, or without the expression of concomitant 'protecting' genes. Some examples suggested by Cuskey (1992) are the genes encoding bacteriocins, restriction endonucleases, and many lethal genes found on large low copy number plasmids (Figurski *et al.* (1982), Ogura and Hiraga (1983), Winans and Walker (1985) and Gerdes *et al.* (1986)). The function of these plasmids is not known, they cannot be cloned unless a cell contains a concomitant protecting gene which may be associated with plasmid replication and maintenance.

One example is the *hok* (host killing) gene located on plasmid R1 which encodes a 52 amino acid protein. This disrupts the cell membrane's electrical potential and results in cell death. Plasmid R1 also contains the protective *sok* (suppression of killing) gene, which produces antisense RNA that binds to *hok* messenger RNA, preventing protein synthesis (Gerdes, *et al.* 1986). Due to the greater stability of the *hok* RNA, daughter cells not receiving a copy of R1 at cell division will die as a result of *hok* expression (Gerdes *et al.* 1988). Molin *et al.* (1987) have shown the *hok* gene to be lethal in a wide variety of bacterial cells, making it an attractive method of biological containment.

It has often been suggested that engineering a microorganism to produce something not originally produced by the wild-type, would serve to debilitate the microorganism and lessen the probability of survival. Curtiss (1988) states that not a single, specific example of a possible harmful consequence, in the use of any GMO designed to achieve a beneficial purpose, has yet come to light. There is therefore no need unless valid perceived risks are apparent, to introduce into

GMOs, attributes not necessary to achieve the beneficial purpose of the construct, but rather to provide enhanced biological containment and safety.

4.1.2 Physical Containment: Design Aspects

After genetic modification, microorganisms are very often crippled with only a small chance of surviving outside the process. These organisms fall into the lowest containment level corresponding to Good (Industrial) Large Scale Practice (G(I)LSP), providing that they are not pathogenic. The containment levels actually used are often very similar to OECD containment levels 1 and 2, to minimise and prevent release. The principal of good safety and occupational hygiene form a starting point for G(I)LSP, those outlined by OECD (OECD, 1986) include keeping workplace and environmental exposure to any physical, chemical, or biological agent to the lowest practicable level, using engineering control levels at source, and ensuring that the measures and equipment are adequately tested and maintained.

The term physical containment includes three elements: equipment design and assembly, operating practices and facility design. It refers to the protection of personnel and the immediate vicinity of exposure to the process organism and its products. Secondary containment refers to the protection of the environment external to the facility and is provided by a combination of facility design and operating practices. Secondary containment is often provided by enclosing a piece of equipment in a cabinet. It is generally used when a piece of equipment is known to leak.

4.1.3 Equipment Design

The fermentation and processing equipment used for an industrial production utilising GMOs serves as the principal means for achieving physical containment. The design varies with the process involved and the scale of operation. The effectiveness of the primary containment provided by equipment is maintained by the complementary use of practices and procedures. Leaver and Hambleton (1992) discuss the mechanical design of fermenters with respect to containment. They point out that one problem with the current regulations is that terms such as "prevent release" and "minimise release" are used, which are not directly translatable into mechanical engineering design.

4.1.3.1 Fermenters

Leaver and Hambleton (1992) describe features of bioreactors designed for containment. Agitator shafts can be top or bottom mounted. If there is a failure in the shaft seal of a bottom driven agitator a major release will occur. However, bottom driven agitators provide easier access to the fermenter top plate, leaving more space for installation of probes, inlet lines and mechanical foam breakers. Top driven bioreactors are favoured by Van Deelen and Logtenberg (1989), Liberman *et al.*,(1983), and Elliot *et al.*, (1990).

Most modern bioreactor drives use mechanical seals rather than packed glands, because packed glands are not easy to contain. The double mechanical seal is an example where requirements of sterility and containment are achieved together. A rotating seal connected to the shaft via a bellows assembly, interfaces with a stationary seal, connected to the body of the bioreactor. The lower seal assembly provides a back-up to the upper. The chamber between the two seal assemblies can be filled with a lubricant, e.g. food grade condensate or pressurised steam. Leaver and Hambleton (1992) suggest that monitoring the flow of lubricant is a means of checking for the failure of seals.

For bioreactor top-plate connection, Leaver and Hambleton (1992) suggest the use of double O-ring seals with steam or sterile flush. For probe and entry ports, double O-ring seals with steam tracing have been described (Allner, 1983), but are not considered practicable by Pennman (1989). Hambleton *et al.*, (1991) report the use of triple seals on high containment facilities and also suggest the use of back-up probes rather than replacement in the event of failure.

Magnetic drives eliminate the need for penetration of the vessel wall, although the required torque may not be achievable with a bottom driven impeller and also suspended solids may be trapped between the stirrer and support. Top mounted magnetic drives are available but not widely used. Walker *et al.* (1987) describe a magnetic coupling design which reduces decoupling problems. A cylindrical stainless steel sleeve is inserted into the fermenter wall and a probe attached to a stirrer motor inserted from the outside. The probe is made from mild steel and is prefabricated with a series of magnets inserted at predetermined locations. An identical stainless steel sleeve with probe and magnets is placed over the external cylindrical sleeve inside the tank, with agitators. The system overcomes torque

problems previously experienced, allowing higher rotational speeds. The agitator rotates in its own magnetic flux, so less problems of friction and wear occur. To date Walker *et al.,* (1987) have demonstrated speeds of 350 rpm in a 2.5 m^3 vessel.

For G(I)LSP operations, the exit air supply is vented straight to the external environment, unless it needs scrubbing for smells. For higher containment categories it is vented to a kill tank. Most traditional fermentation processes do not treat exhaust gas before venting to the external environment. Winkler (1988) found 10^6 bacteria.m^{-3} present in a fermenter headspace. Impaction of organisms on the walls and kinks in the pipelines will reduce the number of aerosolised microorganisms, especially larger ones. Winkler (1988) found a siphon device to reduce the number of aerosolised microorganisms 1000 fold to less than 100 bacteria.m^{-3}, although he does not say what the fermenter conditions were. Filtration is normally the practical option, pretreatment of exhaust gas by condensers, gas-liquid separators and reheaters are considered necessary for optimum filter performance (Rollinson, 1989). Winkler (1988) carried out an environmental risk assessment of GMOs released from the exhaust gas line at a rate of 100 cells.$second^{-1}$. Using a plume dispersal model, he predicted that 100 m from the source, the airborne concentration was less than 1 cell.m^{-3}, with dry deposition of approximately 0.4 cells.$m^{-2}h^{-1}$. Leaver and Hambleton (1992) suggest the use of one filter to minimise release and two to prevent release, but point out that the filters will need to be tested in situ.

Fermenters used for the production of cytotoxic agents (Flickinger and Sansone, 1984) are isolated in high efficiency, particulate air (HEPA) filtered-exhausted barriers. The fermenter is the primary biological barrier, with the controlled access, HEPA-exhausted building containing the fermenters being the secondary biological barrier. Modifications such as parallel exhaust filters, overpressure sensor, and automatic sparge shut-off systems should be made. The recovery equipment used for production of anti-tumour drugs must be further isolated within a secondary containment barrier to protect it from containment hazards and to prevent the release of spills and the generation of toxic aerosols.

4.1.3.2 Centrifuges

Centrifuges tend to operate at high pressures and high liquid flow rates. At these conditions aerosols are easily formed, and containment breached if a leak forms. The use of disc-stack centrifuges predominates in bioprocessing. There are three types: solid bowl, nozzle-discharge and solids ejecting. The solid bowl type cannot be considered for contained operation because the solids must be removed manually from the bowl. The nozzle-discharge and solids ejecting types are more widely used, having containment features such as double mechanical seals, Clean In Place (CIP) facilities and are steam sterilisable.

Solids ejecting disc-stack centrifuges are common in large and pilot-scale bioprocessing plants. They are the most widely researched in terms of contained operation. The difference in design between solids ejecting and solid-bowl or nozzle discharge centrifuges is that peripheral ports in the solids collection area are held closed by water or air pressure, to retain the sediment during separation. Desludging occurs at a predetermined time, the feed stream stops and the ports open to allow the solids to eject. It is possible to get intermittent discharge devices with seals to ensure contained operation, however, this is only one way an aerosol may be released from a centrifuge.

Dunnill (1982) reported that although the centripetal pump is immersed in liquid and is thus unlikely to lead to the release of aerosols, the fitting of a hermetically-sealed head, and a cyclone with vent filter to the solids ejection port dramatically improved containment. Most centrifuges have cyclone receivers to contain the sediment discharged. A considerable shock wave is generated by the centrifuge, and air that is displaced from the cyclone may contain aerosols of cells or debris unless suitable vent filters are fitted. Lawrence and Barry (1982) found shock waves greater than 1.4×10^5 $N.m^{-2}$ during discharge from an Alpha Laval AX 213 separator. It is thought that a shock wave of this size is large enough to allow aerosols to escape from the cartridge housing air vents. This problem of aerosol production was solved by connecting the cartridge housing and cyclone vents to a collection vessel.

Walker *et al.* (1987) describe modifications made to a Westfalia CSA 19-47-476 centrifuge. These were made because the vent filter was blocking due to aerosol formation during desludging. The modifications included removing the vent filter

and attaching it to the main drain, this increased the distance between the solids receiver and the filter. Van Hemert (1982) reduced aerosol production by using a purpose built receiving vessel with a Westfalia SA 47-476. Damping was achieved by a rubber membrane ring mounted concentrically around the connecting pipe from the centrifuge solids line. Wetting of the vent filter is reported to be almost zero due to aerosol deflection plates mounted below the filter. Recently a cyclone design for the catcher chamber has been incorporated to help absorb the energy of discharge (Tinnes and Hoare, 1992).

Krook (1988) discusses the features of Alpha Laval centrifuges which make them contained. The main features are outlined below:

1. The normal labyrinth spindle sealing is replaced by a mechanical sealing. For lower containment categories, a single mechanical seal would be used, although a double mechanical seal with a barrier fluid is used. During sterilisation the barrier fluid is replaced by steam, so that the seal on the product side is sterilised from both sides at the same time.
2. The bowl cover and solids receiving cyclone are designed as pressure vessels, so that the separator can be steam sterilised at 121^{o}C.
3. An air recirculation tube running from the cyclone to the bowl cover has been added. This reduces the shock wave arising when solids are discharged from the bowl into a closed environment.

Many studies have examined the issues of containment of centrifuges. Significant releases of viable organisms are identified during sediment discharge, dismantling and cleaning of the bowl (Dunnill, 1982, Bennett *et al.*, 1990). TNO (1989), in the Netherlands, used helium gas to identify the tightness of welds, joints, and seals, but not during normal centrifuge operation. The problem areas highlighted using helium were then tested with three different microorganisms (*S. cerevisiae, Pseudomonas diminuta,* and *B. subtilis* var *niger*), under normal operating conditions. Tinnes and Hoare (1992) have monitored the release of a modified *E. coli* strain from a high speed disc-stack centrifuge, during normal operating conditions. These issues of release are discussed further in section 1.2.2.

4.1.3.3 Homogenisers

Homogenisers consist of a reciprocating positive displacement piston-type pump. The cell suspension is forced through an adjustable valve at high pressure and low velocity. The suspension hits the valve face and is directed away at right angles, with a rapid increase in velocity and shear. There is a near cubic relationship between rate of disruption and pressure, therefore high pressures are often used (500-1000 bar). These high pressures make aerosol production likely.

The Manton-Gaulin 15M has a ram pump which forces cells through a one-way valve into a homogenising valve at a pressure of 533 bar. As the cell suspension passes between the homogenising valve and its seat there is an increase in velocity and decrease in pressure which result in cavitation. This causes the cells to impact on a ring and disrupt. The gap between the valve and valve seat is adjusted by a spring loaded hand wheel. Pandolfe (1989) found 3 problems with the design of the 15M:

1. If the plunger packing fails, cells can pass from the pump chamber along the plunger and leak out, possibly as an aerosol.
2. Flat gaskets, which are prone to leaking, are used on the cylinder caps.
3. The intersection of 2 bores in a cylinder block is a high stress area. As mentioned earlier, pressures of up to 1000 bar are required for cell disruption. The intersecting bores cannot withstand stresses of continuous operation at these high pressures.

The APV-Gaulin 30CD (figure 2.8) was designed with these problems in mind. Operation is similar to the 15M but the 30CD has a triple action ram pump (see figure 4.4 of piston) capable of providing operating pressures of 1000 bar. The valve has been redesigned for high efficiency of disruption. It has double, self-energising elastomeric O-rings to prevent aerosol formation if leaks occur. This design means that leakage would not spray into the air but would drip from the seal opening (Pandolfe, 1989) due to a change in the direction of the liquid from one O-ring to its backup, i.e. they are counter bored. Flat gaskets and O-rings without counter bores will leak aerosols. The 30CD also has a leak detection system. It consists of a pair of O-ring with a channel between them. Any leakage past the first O-ring is directed to the channel.

Stewart and Deans (1990) used an AMS and a Laser Particle Counter to detect yeast emitted from the 15M and 30CD. They found an increase in particle emission with operating time and pressure. As the primary packing wore in emissions decreased. Often an increase in released cells was followed by a decrease, as the leak hole blocked with cell debris. The primary packing was found to fail after 10-25 hours of operating, contaminating the cooling water. The 30CD was found to release the same number of cell as the 15M, although problems with the seals were not observed for the 30CD. A failure mode and effects analysis on the 30CD showed that the primary packing in the ram pump of the stuffing box was the most likely cause of leaks. The plunger seal is not thought to prevent aerosol release. Stewart and Deans recommend redesigning the ram pump to improve containment.

4.1.3.4 Seals and Valves

Seals are critical in preventing the process coming into contact with operators and the general public (Sinclair, 1992). Chapman (1989) matched the OECD containment categories 1, 2 and 3 to different types of seal (table 4.1). A basic single seal provides a barrier between the pipe or reactor contents and the work environment. Failure of this seal causes an outward flow to the environment, due to the difference in pressures. The process fluid remains uncontaminated, but a biohazard occurs. To prevent this biohazard, Chapman suggests a second back-up seal, or for higher containment, a steam flush, or barrier fluid could be used. Chapman's suggestions are shown in table 4.1:

Table 4.1: Chapman's Seal Suggestions

OECD Containment Category	Static Seals	Dynamic Seals
1. Minimise Release	Single 'O' ring seal	Double mechanical seal.
2. Prevent Release	Double 'O' ring seal	Double mechanical seal with steam flush, or double mechanical seal in ventilated housing.
3. Prevent Release	Double 'O' ring seal with steam tracing	Double mechanical seal with steam flush in ventilated housing.

One issue currently being argued, (Leaver and Hambleton, 1992) is whether a back-up seal without steam tracing offers any containment advantage over the single seal system. There is no means of checking the integrity of the product side or back-up seal *in situ*. If the product-side seal fails, containment is not breached, but the space between the two seals is contaminated and must be sterilised before further use. It is not known whether the back-up seal is likely to fail at the same rate as the primary seal, due to repeated sterilisation cycles. Leaver and Hambleton (1992) suggest that because steam tracing on static-sealed components is not a practicable option, double, or triple seals would provide more protection from inadvertent release than single seals. Preventative maintenance should be adopted to compensate for a lack of direct integrity testing.

Sinclair (1992) states that the selection of equipment to operate at Large Scale Containment Category 2 (LSCC2) is based on: a robust design, a proven track record, and ability to be cleaned and decontaminated. Sinclair (1992) and Titchener-Hooker *et al.*, (1993) are opposed to Chapman's model. The model is based on Chapman's own interpretation of the approach used in the United States. This is that double seals are used in BL2-LS facilities (equivalent to LSCC2 in the UK). In fact (Sinclair, 1992) single seals are used universally in these facilities.

Chapman's analysis is that two seals (without seal flush) offer more protection than one. He assumes that each component is equal to another in terms of potential failure rate. A complex rotating mechanical seal is a stressed component and therefore more likely to fail than a static seal (Sinclair (1992) and Titchener-Hooker *et al.,* (1993)). A single mechanical seal always leaks material in the direction of the pressure differential, whereas a correctly specified and installed single static seal does not leak. Sinclair's argument is backed up by the fact that in mainland Europe and the US, single seals are used on production facilities handling GMOs.

Bursting discs and spring loaded safety relief valves are currently used for pressure relief in fermenters. Bursting discs are more hygienically designed and the relief pressure setting cannot be altered manually. Spring loaded safety relief valves are prone to sticking open, due to material on the valve seat. Chapman (1989) noted that valves are often the weakest point in bioreactor design for containment. Diaphragm valves are preferred by Bamsley (1990) since unlike ball valves they are crevice free. Sinclair (1992) promotes the use of diaphragm valves, and adds that some of the vendors of these have test data and are able to give guarantees on performance of their product.

Piping connections provide a primary containment barrier, so when designing for contained operation, it is advisable to minimise the number of joints used (Sinclair, 1992). Leaver and Hambleton (1992) suggest butt welding connections with an orbital welder would limit the number of potential failure points, and has in fact been used on a high containment facility (Hambleton *et al.,* (1991)). Problems occur when new connections are required and the pipes have to be cut open. Jefferis and Schlager (1986) suggest using International Dairy Federation (IDF), In-Line Cleaning (ILC), and TriClamp fitting for GILSP. Sinclair (1992) lists the advantages of the TriClamp:

1. They are full-face gasket, giving a large sealing area compared to O-rings.
2. The gasket contains a circular key which locks into both faces of the flange.
3. It is of hygienic design, approved by the US Good Manufacturing Practice for the manufacture of parenteral products.
4. The TriClamp joint has a proven track record, it still seals after many sterilisation cycles.
5. The mechanical design pressure of 17 bar g is in excess of system operating pressures of most bioprocessing plants (<2 bar g).

4.1.4 Facility Design

The degree of sophistication of design should match the production activities and should contribute to the functioning of a secondary barrier. The following points should be considered (Hill and Beatrice, 1990):

1. Identification of work areas where physical containment of manufacturing processes is required.
2. Location of areas where containment is not required.
3. Flow and direction of air in controlled and non-controlled areas.
4. Flow of materials, equipment, products and waste.
5. Flow of personnel.

An obvious problem encountered when designing a bioprocessing plant is whether the plant is going to be used for one product only or for many. Ideally the organism used in each process should be propagated in premises where no other microorganisms are handled. Recovery and purification should occur in a self contained area in which controlled temperatures of say 2 to 10^{o}C maybe also be required (Hill and Beatrice, 1990).

In the containment areas the ceiling, walls, and floors should be made of non-shedding material which is non-porous, smooth and easily cleaned. All joints should be sealed and all corners coved, to aid cleaning. Only necessary equipment should be brought into the contained areas. A collection and treatment area should be located below large scale fermenters in case of spills or leaks, and should be capable of immediate start-up to handle twice the production capacity.

4.1.4.1 Air Supply

Air flow and pressure boundaries are critical to the performance of an effective containment barrier. Areas with the highest potential to release the organism or product should be maintained at a lower pressure. In the past aseptic filling areas and contained areas have been separated. This is however no longer thought to be necessary. The flow components, equipment, and product should be unidirectional, leaving only via autoclaves.

Veerman (1991) describes the design and construction of the EuroCetus pilot plant which is designed for large scale operation with GMOs. The contained area is

under negative pressure relative to the outside and a flow of air towards the fermentation area is always maintained. More than twenty air changes per hour are achieved. The air is HEPA filtered at the inlet as well as the outlet.

Hill and Beatrice (1990) also discuss the airflow in contained areas, emphasising that to control airborne viable and non-viable particles, the particle content of the air must be minimised, the size and number of particles present must be reduced. The air supply should be HEPA filtered and the velocity sufficient to sweep particles away. If the organism is hazardous, 50 air changes.hour^{-1} are recommended. Rooms for inoculum preparation, fermentation, harvesting and purification must have separate air handling systems.

4.1.4.2 Water Supply, Effluent Disposal and Sterilisation

Veerman (1991) recommends that all liquid utilities (mains water, deionised water, chilled water) should be membrane filtered at either exit or entry. The status of the membrane filters should be monitored continually using differential pressure sensors, and the filters sanitised with steam before the cartridges are replaced. The contained area must have a separate collection system: waste water is connected in a small pit and pumped to one of two sterilisation vessels or kill tanks (Walker *et al.,* 1987 and Dick and Hanel, 1970). After filling, the vessel and contents are sterilised and the other vessel is filled. The liquid waste is then drained to the normal sewer line, the system is controlled automatically.

Equipment and supplies must be sterilised using autoclaves or dry heat ovens on entering contained areas (Hill and Beatrice, 1991). The cool down phase for sterilised materials should also be carried out in a class 100 laminar air flow. Provision should be made for the disposal and sterilisation of all contaminated liquid and solid waste and the sterilisation of equipment and materials used or generated in the process. The treatment reservoir must be large enough to accommodate all possible loads. The cleaning and decontamination of work areas between operations should be carried out according to written Standard Operating Practices (Hill and Beatrice, 1991).

Flickinger and Sansone (1984) describe the containment measures required during the large scale production of cytotoxic and oncogenic cells. Cell culture incubators and fermenters are located in a HEPA-filtered-exhausted secondary barrier

equipped with air locks, changing room, showers, and double door autoclaves and connected to a sterilising shower or kill tank system (NIH, 1979; Dick and Hanel, 1970; Runkel and Phillips, 1969). Potentially hazardous biological materials are transferred between contained process areas using seamless 100 L stainless steel drums or welded double-pipe transfer lines that are steam sterilised before and after use. If operators enter this area they must wear protection suits, respirators, shoe and head covers or complete inflatable suits (Flickinger and Sansone, 1984).

4.1.4.3 Validation of Containment

The FDA describe validation as: "The provision of documentary evidence that provides a high degree of assurance that a process will consistently manufacture a product of a certain predetermined quality". As applied to containment this means ensuring that the process operates to the necessary containment level.

Qualification or validation should be an integral part of preliminary plant concepts and specifications. When carried out properly, it helps reduce maintenance costs, down time and utility costs. According to Hill and Beatrice (1990) there are 3 parts to validation:

1. Installation Qualification: this relates to the proper installation of equipment and systems. It includes checking to see they meet appropriate design specifications after installation. Preventative maintenance and equipment calibration programs should be drawn up. Complete reliance on outside contractors for validation of all systems and equipment removes an important internal quality control check. If they are used, Hill and Beatrice (1990) recommend that the manufacturers quality control unit receive and evaluate raw data. Rather than rely on a certificate of acceptance, written validation procedures utilised by the outside contractors should be part of the manufacturers SOP manual.
2. Operational Qualification: this relates to the testing of equipment and systems to determine the operational limits. Are the equipment and systems fully capable of repeatedly performing the production functions for which they are intended?
3. Process Validation: this should not begin until all the equipment and systems have been satisfactorily tested and determined to meet the established specifications. It is carried out to verify that the equipment and systems can perform the intended functions within established specifications during the actual production conditions and operations. Test runs should be performed enough times

to assure that equipment, systems, and operations will consistently produce product within established limits during all employee shifts.

All 3 stages must include documentation of the test runs and their results, including written protocols describing the intended objectives, instructions for performing the tests, established equipment specifications and acceptance criteria. Once validated there should be no significant changes in operating procedures without written approval from authorised persons responsible for plant operations.

Levchuk and Lord (1984) discuss validation of sterilisation processes involved in drug production, it must include the initial sterilisation operation and the filling and closing operation under aseptic conditions. Companies typically use microbial growth medium instead of a drug product during validation, a procedure called "media fill". Any microbial growth in the medium will indicate contamination.

Validation activities carried out on the EuroCetus pilot plant before its use were as follows (Veeman, 1991): SOPs and maintenance protocols were written for all pieces of equipment and experimental work carried out. Validation of all sterilising equipment was carried out by means of thermal mapping. The integrity of the fermenter during sterilisation was checked using a rich medium. Environmental monitoring was used to validate the primary containment and sanitation/disinfection procedures. Although they demonstrated that complete primary containment could be achieved, a number of problems had to be solved:

1. The plunger seal of the homogeniser was found to emit aerosols at lower temperatures.
2. Pump seals were found to emit aerosols after prolonged use.
3. Sampling devices currently available were not found to give satisfactory containment.

Strict adherence to SOPs and techniques is an important element of containment (OECD, 1986). Those working with potentially infectious agents, allergenic or toxic materials should be aware of potential hazards and should be trained and proficient in the practices and techniques for the safe handling of such material.

Some physical methods of containment validation are summarised by Leaver (1991). The pressure hold test is widely used in the biotechnology industry, although the criteria for passing one is dependent on the operators experience and

the method used. The vessel to be tested is pressurised to a certain pressure, e.g. with air and any changes in pressure over a given time period noted. LH Fermentation provides details of a pressure test. For a 140 L fermenter they suggest a test is carried out before each fermentation, and recommend a pressure of 20 psig is maintained for at least 3 hours. If the pressure loss is greater than 3.5 x 10^3 $N.m^{-2}$, the manufacturers recommend checking for bubbles using a Savlon solution of 0.5 %.

A lack of information on occupational and environmental exposure limits in the literature and regulations does not help the development of quantitative monitoring methods. At OECD containment category 1 equipment should be designed to minimise release, but there is no mention of the number of cells that could be released. Equipment can be tested, both before its operation, and during operation to detect the release of process material. Leaver (1991) explains how, by calculating the pressure loss through an orifice as a function of time, an equivalent orifice diameter can be obtained for all process equipment. This provides an overall leak tightness index. The size of the diameter could be used as a criterion for passing a pressure test.

Tracing techniques can be used to locate leaks. Helium detection is a highly sensitive method if used in conjunction with a helium pressure test. Sulphur hexafluoride leak detectors are less expensive, and are routinely used on high containment fermenters at PHLS-CAMR Porton Down (Hambleton *et al.,* 1991). Another test that could be used in conjunction with the pressure hold test is the ultrasonic detector. The ultrasonic sound produced by discharging gas can be detected. This method is useful because it can be used during the operation of the equipment being tested.

Kastelein and Logtenberg (TNO, 1989) describe experiments carried out to validate an Alpha Laval BTPX 205 centrifuge. These included a Failure Mode and Effects Analysis (FMEA), leakage tests, checks on the completeness of sterilisation and an analysis and quantification of human error. The centrifuge has a gas tight housing, a double-axial seal and uses water as the coolant and sealant liquid.

The FMEA examines the effect of equipment design on possible undesired events and the consequences of technical or human failure. The system is divided into functional blocks to try to find the weak spots which would lead to a breach of

containment, during a specific stage of the process. It is evident from the long list of tests and checks to be carried out on starting up, that accidents due to human error may occur. The analysis also shows that pipe joints and couplings, the off-gas filter and the double mechanical seal of the centrifuge are also problem areas.

The sealing liquid is sterile water, which at a pressure of 3-7 bar keeps the seals closed. It was found that small amounts of water could leak through the bearing, into a small space, where material from the product side is also present. The contaminated sealing water could then be discharged to the waste tank. If insufficient pressure led to product leaking from the bowl to the sealing liquid, re pressurising the sealing liquid would cause the product to be discharged to the drain. Rather than a drain, this liquid should go to a kill tank. Other possible faults include severe damage to the double mechanical seal caused by an absence of sealing water, or the centrifuge bowl imbalancing. This could be caused if the sediment discharge line blocked. Alpha Laval claim that an imbalance would happen if sludge accumulated unevenly, if the sludge did not slide off the bowl wall or if there was a mechanical fault in the discharge line. Vibrations caused by an imbalance may cause a small increase in sealing water leakage to the product side, although viable cells would not be expected. An automatic stop at a certain vibration level is recommended by Kastelein and Logtenberg to rectify this problem.

The investigation of sterilisation revealed that the condensate may contain viable cells. To prevent release to the external environment, this condensate should be discharged to the kill tank. The report also highlights the need for temperature indicators at the bottom of the sediment collection chamber, at the discharge point of the operating chamber and in the leakage water discharge line of the axial seal. The authors also make the following general comments. Care must be taken with regard to: the flexibility of the system re. vibrations, axial damage from falling objects, the correctness of welding and static seals, discharge of all condensate and drain material to the kill tank, the use of membrane filters, induction flow meters and membrane pressure indicators, valves that discharge material to the environment should also have a connection to the kill tank. They conclude that due to problems with the double mechanical seal, a containment cabinet for the whole system should be used if hazardous material is handled.

Kastelein and Logtenberg (TNO, 1989) report on the leakage tests carried out using helium. The areas that were found to leak were then examined further with microorganisms. Microorganisms were not detected using either an AMS or a Laser Particle Counter.

The human failure analysis carried out consists of a task analysis and quantification of the probability of human error. In a task analysis, each step of a procedure is analysed for possible errors and the consequences of that error. This analysis was carried out using written procedures from Alpha Laval. The analysis showed that the different failure modes will not lead to a breach of containment. If the operator performs an action not in the procedure, a breach of containment could occur. The probability of failure was calculated to be 0.04 - 0.18. It was found that the probability of carrying out sterilisation and cleaning wrongly, was high. This contributes to the high probability of failure.

In concluding the study, the following recommendations are made: The manufacturer should offered design support, a maintenance contract and a training program. Leakage tests and checks on the completeness of sterilisation should be carried out. Ideally the authors would like to see a complete system delivered and guaranteed by the manufacturer, for contained operation.

4.2 Measurement of Release from Fermenter Head-spaces

4.2.1 Fermenter Operation

Growth media, either YEP for *S. cerevisiae*, or Luria broth for *E. coli* was sterilised inside the fermenter pot. The pH and oxygen electrodes were calibrated before fermenter sterilisation. All outlets from the fermenter were closed except the exit gas line which was used to vent steam during sterilisation. A liquid volume (growth media and inoculum) of either 1.5 or 5 L was used in the fermentations.

An inoculum, of 1% of *S. cerevisiae*, 6% of unmodified *E. coli,* or 10% by volume of modified TK *E. coli,* was added to the fermenter when the broth had cooled to the appropriate growth temperature. At this stage pre-sterilised glucose was also added to the *S. cerevisiae* fermentations to give a final concentration of 20 $g.L^{-1}$. Filter sterilised kanamycin was added to the modified TK *E. coli* fermentations to give a final concentration of $10mg.L^{-1}$. Antifoam addition was kept to a minimum and only added if foaming became excessive. Broth samples of a few mLs were taken during the fermentation to estimate the number of cells present. This was carried out by counting in a haemocytometer. The fermentation profiles are shown in Appendix A.

4.2.2 Detection of Released *S. cerevisiae* by Total Counting

4.2.2.1 Sampling Strategy

Table 4.2: Fermenter Conditions Tested for Release
The fermentations were carried out at the conditions described in the table below.

Organism	Growth Conditions	Growth Period (hours)	Stirrer Speed (rpm)	Antifoam (mL)	Airflow Rate (vvm)	Cells.mL^{-1}
S. cerevisiae	pH 7 28°C	16-45	500-1500	0-1.5	0.3-1.0	4.9×10^{7}- 1.1×10^{8}
UM *E. coli*	pH 7 37°C	3-22	750-1500	0-1.0	0.3	5.2×10^{8}- 4.6×10^{9}

The pH in the *S. cerevisiae* fermentations was controlled by addition of 3M NaOH, and in *E. coli* fermentations by addition of 2.5M H_2SO_4.

The cells (either *S. cerevisiae* or *E. coli*) were allowed to grow for approximately 20 hours before aerosol samples were taken from the headspace of the fermenter. A 30 minute control sample of the fermenter room air was taken, using the cyclone, before sampling from the headspace. The cyclone was then connected to the exit gas line from the fermenter (with the air filter removed) and the headspace air sampled for 30 minute periods. Figure 2.7 shows the apparatus used to connect the exit gas line to the cyclone. The effects of antifoam addition, condenser operation, cell number, stirrer speed and fermenter size on aerosol release were examined.

The effect of cell number on aerosol release from the fermenter was examined by operating the fermenter in the usual way, but containing a suspension of packed *S. cerevisiae* in sterile broth.

Total Counts

1 mL of each sample was centrifuged at 10000 rpm for 2 minutes. 0.9 mL of the supernatant was discarded and the remaining 0.1 mL counted as previously in either a haemocytometer (*S. cerevisiae*) or a Helber bacteria counting chamber (*E. coli*). This method was validated using *S. cerevisiae* suspensions of a known concentration. The lower 0.1 mL 'pellet' was found to contain approximately 10 times as many cells as the uncentrifuged cell suspension.

4.2.2.2 Results

S. cerevisiae

In the majority of the cyclone samples taken from the head-space above the *S. cerevisiae* fermentations, it was not possible to detect released *S. cerevisiae* cells. Detection was limited by the sensitivity of the quantification method used. The detection limit for the haemocytometer was set at 15 *S. cerevisiae* cells on the grid, which approximates to a release of less than 1.5×10^6 cells.h^{-1} released from the fermenter.

It is likely that in the single case in which cells were detected in the cyclone sample, these were due to ineffective cleaning of the cyclone at the start of the experiment. This problem of cleaning the cyclone is inherent in the cyclone design

and becomes more important as the number of cells to be measured approaches the detection limit of the counting method.

The air in the fermenter room was also examined for *S. cerevisiae* cells. The cyclone operated at an air flow rate of 360 $L.min^{-1}$. This air supply was made up of both air from the fermenter and air from the room that the fermenter is situated in. Again in the majority of samples there were less than 10^6 $cells.mL^{-1}$, equivalent to 1.4×10^5 $cells.L^{-1}$ air.

It was thought that the release of microorganisms from the surface of the liquid in a fermenter would be affected by addition of antifoam, stirrer speed, airflow rate, and also the concentration of cells present. All these factors were examined, and in all cases, detection was limited by the sensitivity of the total counting method. In one experiment, the condenser on the exit gas line from the fermenter was not used. It was thought that the condensing water vapour in the exit gas line may also trap cells, preventing them from reaching the cyclone.

UM *E. coli*

Table 4.3: Measurement of Release of Unmodified *E. coli* Cells from the Head-space of a Fermenter

Expt	Time elapsed from inoculation (hours)	Sample type	Stirrer speed (rpm)	Total cells released from fermenter.h^{-1}
A	17	Fermenter room	-	1.8×10^7
	17.5	Head-space	750	4×10^7
	18	Head-space	1000	1.8×10^7
	18.5	Head-space	1500	2.4×10^7
	20	Head-space	750 (no condenser)	1×10^7
	20.5	Head-space	1000 (no condenser)	9.6×10^6
	21.5	Head-space	1500 (no condenser)	$< 9.5 \times 10^6$
B	3	Fermenter room	-	4.8×10^7
	3.5	Head-space	750	9.6×10^6
	4	Head-space	1000	$< 9.2 \times 10^6$
	4.5	Head-space	1500	$< 9.7 \times 10^6$

Table 4.3 shows that it is possible to detect cells in the cyclone samples taken from the head-space of UM *E. coli* fermentations. The number of cells detected fell just above the limit of detection of the total counting method, i.e. approximately 10^7 $cells.h^{-1}$. The total counting detection method is not species-specific, so cells detected may have been part of the natural microflora present in the Fermenter

room. Total counts of the cyclone samples from the fermenter room were as high if not higher, than the fermenter head-space samples.

It was initially thought that *E. coli* cells would be aerosolised from a liquid more readily than *S. cerevisiae*. This was because *E. coli* cells were released from the Collison Nebuliser in mass balancing experiments (section 3.4.1.1, tables 3.3 and 3.6) more readily than *S. cerevisiae*. This is thought to be due to their different dimensions. This, however has been very difficult to determine due to the problems described in measuring *S. cerevisiae* cell numbers. It would have been advantageous to carry out the same experiment with a recombinant *S. cerevisiae* fermentation, where a more sensitive, PCR test could be applied. Unfortunately this has not been possible, but a recombinant *E. coli* was available, and was used in the experiments described below.

4.2.3 Detection of Released TK *E. coli* by the Polymerase Chain Reaction

4.2.3.1 Sampling Strategy

Table 4.4: Fermenter Conditions Tested for Release

Fermentations were carried out as described in section 4.2.1. Cyclone samples were taken in exactly the same way as those from the unmodified *E. coli* and *S. cerevisiae* fermentations. In addition, an air sample from the fermentation room, Downstream Processing room, or inocula laboratory was taken before a sample from the fermenter.

Organism	Growth conditions	Growth period (hours)	Stirrer speed (rpm)	Antifoam (mL)	Airflow rate (vvm)	Cells.mL^{-1}
TK *E. coli*	pH 6.8-7.0 37^{o}C	17-26	750-1500	0.2-0.5	0.3	1.6 x 10^{9}- 3.9 x 10^{9}

Due to the highly sensitive nature of the PCR reaction, it was necessary to ensure that all equipment was DNA free before each cyclone sample was taken. This was achieved by soaking the cyclone and fermenter sampling device in 10% chloros (sodium hypochlorite) for 30 minutes before their use. The same chloros solution was pumped through the cyclone tubing for 30 minutes. Residual chloros was then

rinsed off with tap water. The cyclone samples were collected in chloros-treated, sterile bottles, containing sterile Ringers' solution. The use of a 10% chloros solution for the elimination of DNA templates for PCR experiments is recommended by Prince and Andrus (1992).

TK *E. coli* cells were detected both by total counts and by PCR. the total counts were carried out as described in section 4.2.2.1. The PCR method is described in section 2.3.4.3.

4.2.3.2 Results

Table 4.5: Measurement of Release of Transketolase *E. coli* Cells from the Head-space of a Fermenter *(_=no product, +=faint product band, ++=medium size product band, +++=heavy product band)*

Expt	Time elapsed from inoculation (hours)	Sample type	Stirrer speed (rpm)	Total cells released from fermenter.h^{-1}	PCR product
A	17.5	DSP room	-	9.1×10^7	_
	18	Fermenter room	-	1.6×10^7	_
	19.5	Head-space	750	$< 9.3 \times 10^6$	+
	21	DSP room	-	nd	_
	22.5	Head-space	1000	$< 8.4 \times 10^6$	+
	24	DSP room	-	nd	_
	24.5	Head-space	1500	$< 7.1 \times 10^6$	+
	25.5	DSP room	-	nd	_
	26	Head-space	750	$< 8.4 \times 10^6$	+
B	18	DSP room	-	nd	+
	18.5	Fermenter room	-	1.1×10^7	+
	19.5	Head-space	750	$< 9.3 \times 10^6$	++
	22	Inocula lab	-	nd	+
	22.5	Head-space	1000	1.5×10^7	++
	24	Inocula lab	-	$< 1 \times 10^7$	+
	24.5	Head-space	1500	1.2×10^7	++
	25.5	Fermenter room	-	nd	+

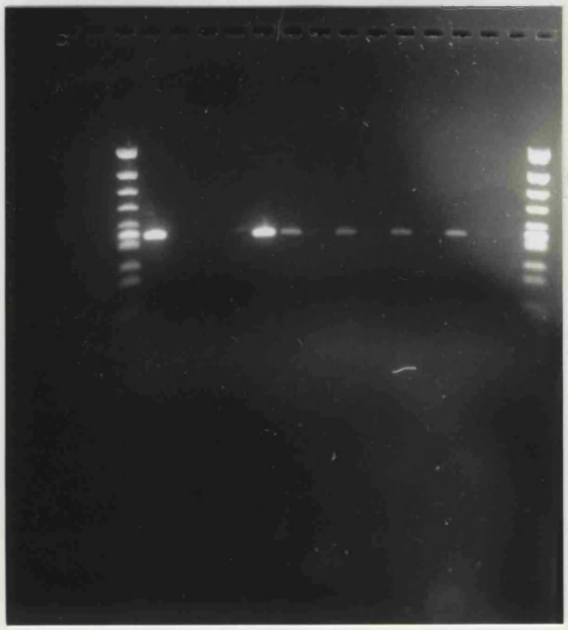
The electrophoretic gels for the experiments described in table 4.5 are shown in figures 4.1 and 4.2. Table 4.5 shows that it is possible to use PCR to detect recombinant *E. coli* from a fermenter head-space. Release occurred at stirrer speeds of 750 to 1500 rpm, both with and without the condenser on the exit gas line. The PCR was able to detect released TK *E. coli* cells in all samples in which they were undetectable by total counting. When enumerated using the counting chamber, approximately 10^7 cells.hr^{-1} are collected in the cyclone. This figure of

10^7 does however include a significant number of cells from the room air. These numbers of TK *E. coli* cells released into the fermenter head space are similar those of UM *E. coli* cells shown in table 4.3.

As in previous experiments, it was necessary to sample from either the Fermenter room, the Inocula laboratory or the Downstream Processing room. This was to show that if TK *E. coli* cells were detected in the cyclone sample, that these were from the fermenter head-space, not the air that the cyclone took from the room. A sample from the fermenter room was least preferable because TK *E. coli* cells detected here may have originated from the fermenter itself, or previous experiments. TK *E. coli* cells detected in the Downstream Processing room or inocula laboratory sample were likely to be due to insufficient cleaning of the cyclone. The PCR was also able to detect TK *E. coli* cells in the fermenter room samples, which could not always be correlated with total counts of cells collected in the cyclone.

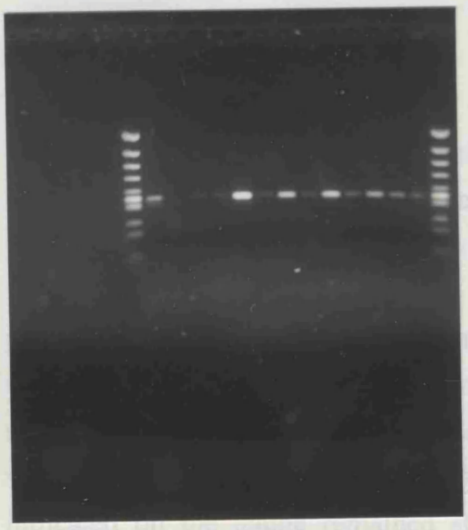
Much difficulty was experienced in cleaning the cyclone and tubing sufficiently. In some samples, e.g. experiment B, a faint signal was detected for the PCR product, in both the Downstream Processing room and fermentation room samples. This is likely to be due to residual DNA or *E. coli* cells in the cyclone, rather than *E. coli* cells present in the air itself.

Figure 4.1: Detection of Transketolase *E. coli* from a Fermenter Head-Space (Experiment A)



Well	Sample
1 (right)	Molecular weight marker
2	DSP room
3	Fermenter room
4	Fermenter head-space, 750 rpm
5	DSP room
6	Fermenter head-space, 1000 rpm
7	DSP room
8	Fermenter room, 1500 rpm
9	DSP room
10	Fermenter head-space, 750 rpm
11	10^{-4} TK *E. coli* cell dilution into DSP room sample
12	Ringers' solution
13	Ringers' solution
14	Sterile RO water
15	10^{-4} *E. coli* cell dilution in Ringers' solution
16 (left)	Molecular weight marker

Figure 4.2: Detection of Transketolase *E. coli* from a Fermenter Head-Space (Experiment B)



Well	Sample
1 (right)	Molecular weight marker
2	DSP room
3	Fermenter room
4	Fermenter head-space, 750 rpm
5	Inocula lab
6	Fermenter head-space, 1000 rpm
7	Inocula lab
8	Fermenter head-space, 1500 rpm
9	Fermenter room
10	10^{-4} cell dilution in inocula lab sample
11	Ringers' solution
12	Ringers' solution
13	Sterile RO water
14	10^{-4} cell dilution in broth
15 (left)	Molecular weight marker

4.3 Measurement of Microbial Release from 30CD APV Homogeniser

4.3.1 Detection of Released *S. cerevisiae* by Total Counting

4.3.1.1 Homogeniser Operation

The homogeniser was serviced by the manufacturers, APV, before use in these experiments. Before the machine was started the flow of lubrication/cooling fluid was adjusted to a slow trickle. This lubrication/cooling liquid consisted of sterile water and was circulated from a dedicated source using a Watson-Marlow peristaltic pump. The Cell Disruption (CD) valve was opened by rotating the hand wheel anti-clockwise until a little resistance was met. The pump motor was then started. When all the air was purged from the pipework, the pressure in the CD valve was increased by rotating the hand wheel clockwise, until the pressure indicated on the gauge remained steady. The pressure was always decreased before the product flow stopped.

The *S. cerevisiae* suspension to be homogenised was held in tank n^{o} 1. Homogenised product was returned to the same tank. The product was cooled by passing through a heat exchanger on exiting from the CD valve and via a jacket on the holding tank. Chilled water at 6^{o}C was used to cool a water supply dedicated to the homogeniser. This cooled water was then passed through the heat exchanger and to the tank jacket to chill the homogenate. The temperature of the yeast suspension was maintained below 20^{o}C whilst the homogeniser was running.

The homogeniser and associated pipework was cleaned using a 1% (w/v) NaOH solution. This was pumped through the CD valve at a flow rate of 60L.h^{-1}for approximately 20 minutes at zero pressure. This was then flushed out with water until the liquid leaving the homogeniser was clear.

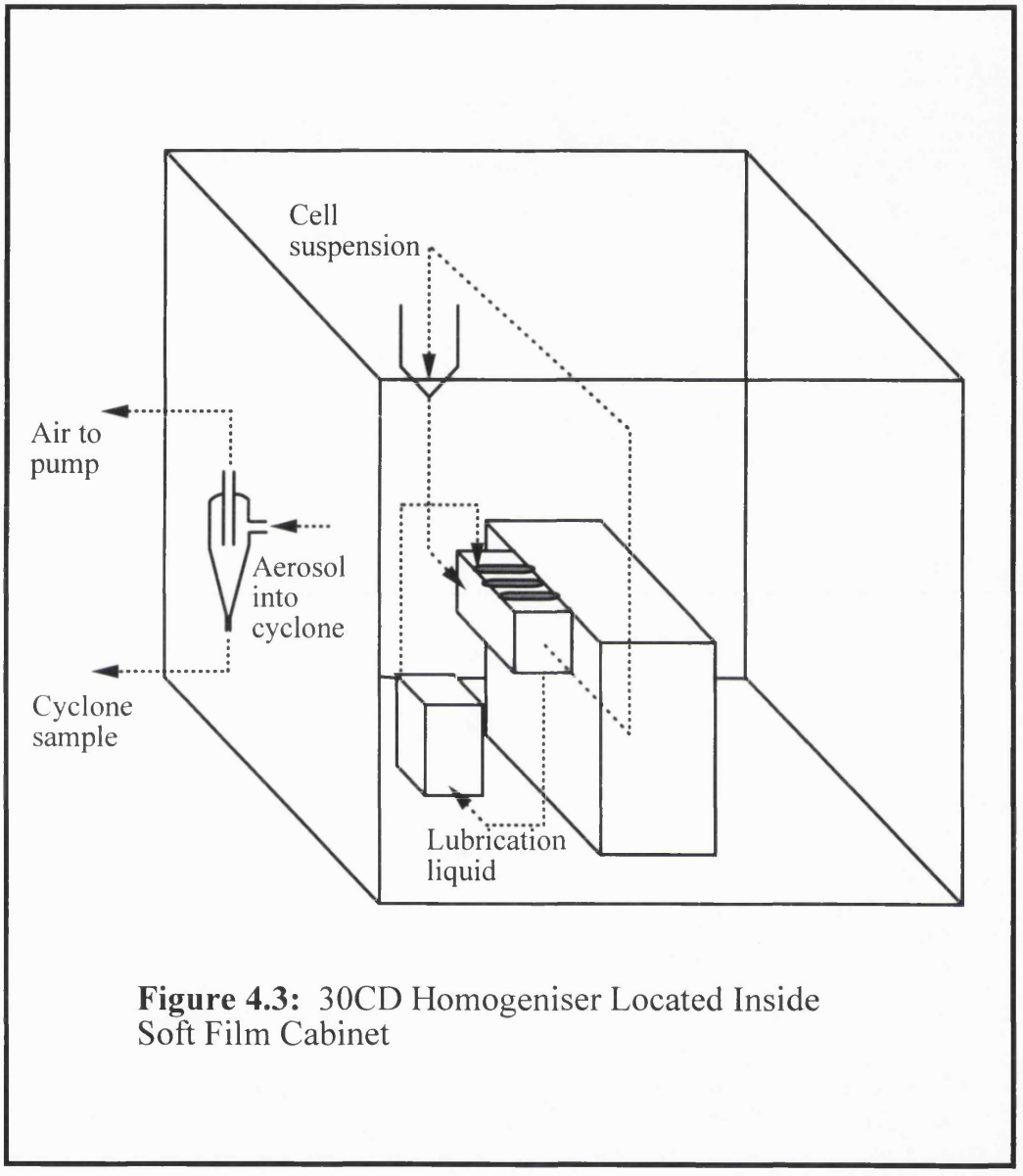


Figure 4.3: 30CD Homogeniser Located Inside Soft Film Cabinet

4.3.1.2 Sampling Strategy

The homogeniser was operated inside the soft film cabinet (see figure 4.3). Air entered the cabinet via the inlet pump at 40 $L.min^{-1}$ and exited through the outlet filter. During a 30 minute period before operation of the homogeniser, the cabinet was allowed to fill with air. During processing air entered through the inlet pump and left through the cyclone only. The effect of this was to keep a positive pressure inside the cabinet during processing.

This cabinet provided a contained environment which was then sampled using the cyclone and 5 Ringers' plates. Processing lasted for 1 hour and during this time two 30 minute cyclone samples were taken. The homogeniser was operated at a pressure of 48-55MPa and a flow rate of $113L.h^{-1}$ to disrupt a suspension of $450g.L^{-1}$ of packed yeast in RO water (equivalent to 10^{12} $cells.mL^{-1}$). The cyclone samples and the Ringers' plates were examined for yeast cells by both counting cells in a haemocytometer and by plating out on malt extract agar plates. The lubrication/cooling liquid for the pistons was also examined for released yeast cells by these two methods.

4.3.1.3 Results

Twenty-four runs were carried out without changing the piston packing rings. Microbial release was not detected from the homogeniser during any of these runs. Both the piston packing and the secondary piston seal must fail before microbial aerosols will be released. If the packing seal only fails, liquid release into the piston lubrication/cooling fluid will occur. After the 24 hour-long runs one of the packing rings was then removed from the homogeniser and replaced with one that had been autoclaved for 20 minutes at 121°C. The homogeniser was then operated in the same way as before, for a further six 1 hour runs. A significant number of cells ($>10^6$ $cells.mL^{-1}$) was still not detectable in the lubrication fluid, the cyclone sample or the floor samples.

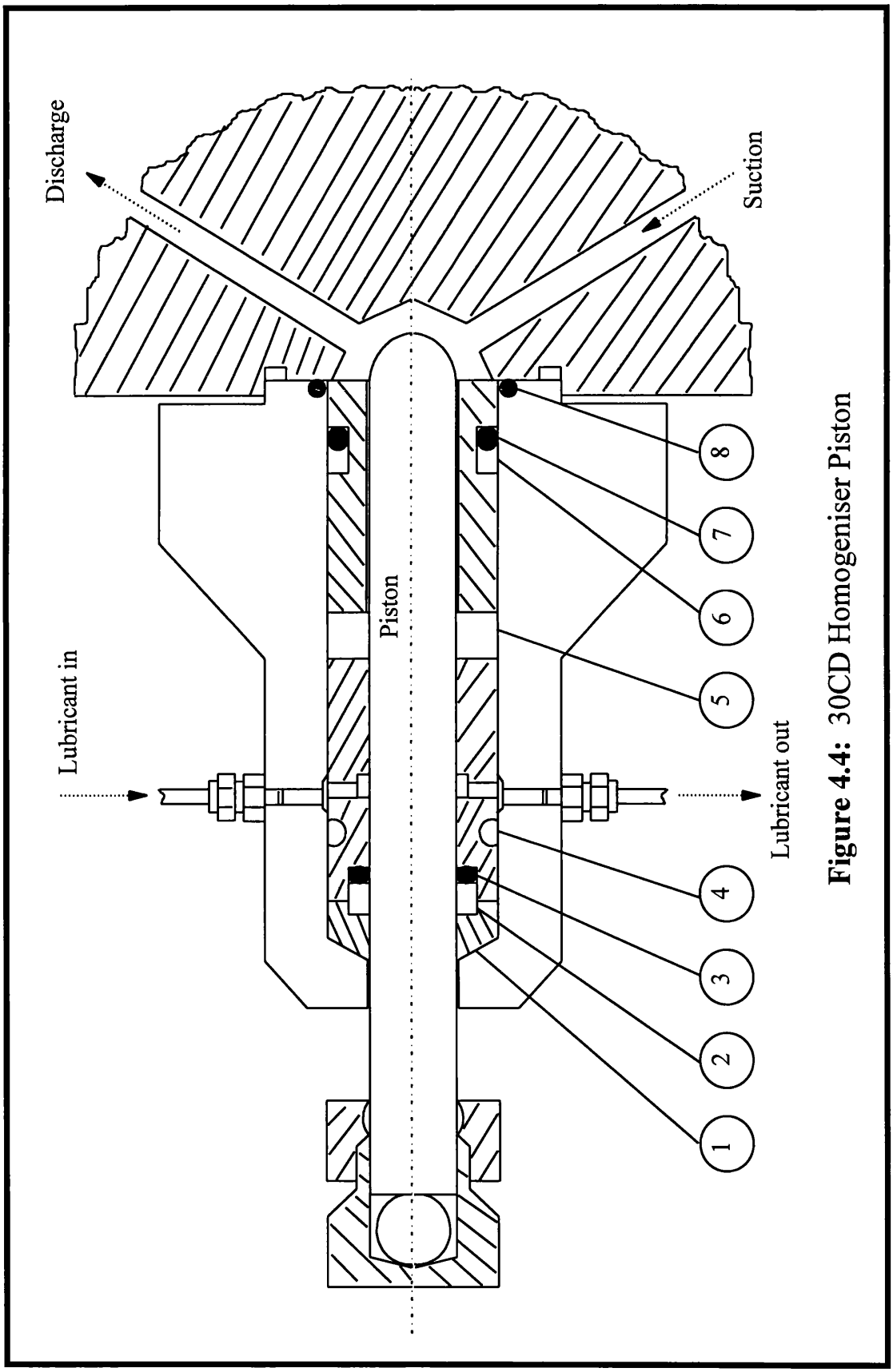


Figure 4.4: 30CD Homogeniser Piston

Figure 4.4: 30CD Homogeniser Piston

1	Stuffing box seat
2	O-ring support ring
3	Secondary seal O-ring
4	Packing support O-ring
5	Piston primary packing
6	Top plug O-ring
7	Top plug backup ring
8	Stuffing box primary O-ring

As in the fermenter experiments described in section 4.2, the detection of released *S. cerevisiae* cells was limited by the detection method used. This method of counting cells in a haemocytometer had a detection limit of 2×10^6 cells.mL^{-1}. If 2×10^6 cells were released in one hour, and the lubrication liquid was assumed to be lost at a flow rate of approximately 25 mL.h^{-1}, the minimum level of release detectable in one day of operation is 6×10^8 cells. This is equivalent to 1 mL of a fairly dilute fermentation broth.

The findings of this section, i.e. that it is very difficult to detect cells released from the homogeniser, lead logically into the next experimental section, in which PCR is used to detect a recombinant *E. coli*.

4.3.2 Detection of Released TK *E. coli* by the Polymerase Chain Reaction

4.3.2.1 Fermentation

E. coli cells were grown in nutrient broth in a 5 L fermenter. 5 mL of antifoam was added and 0.5 mL.L^{-1} sterile filtered kanamycin. The stirrer speed was set at 500 rpm and the air flow rate at 5 L.min^{-1} throughout the fermentation. The pH was maintained at 6.8 and the temperature at 37^{o}C. The cells were harvested after approximately 24 hours and stored at 4^{o}C. At this point cell numbers had reached approximately 10^9 cells.mL^{-1}

4.3.2.2 Homogeniser Operation and Sampling Strategy

The homogeniser was operated in the same way as for disrupting *S. cerevisiae* (section 4.3.1.1). A positive pressure was created inside the cabinet using the air inlet fan. The piston lubrication/cooling water was changed for each homogeniser run, however it was not possible to clean the pipelines. This lubrication liquid was examined for *E. coli* cells both before and after processing by both total counts and PCR. However, Ringer's plates were not used to sample cells that fell to the floor of the cabinet, as in the previous experiments.

Total counts were carried out using the Helber bacteria counting chamber as described in section 4.2.2.1. All samples, except that of the *E. coli* suspension to be processed, were centrifuged at 6630 g for 2 minutes before counting. The PCR was carried out as described in section 2.3.4.3.

The cyclone was used initially to sample the air in the Downstream Processing room, where the homogeniser is situated. The cyclone and associated tubing were cleaned in chloros (10%) for 30 minutes after each sample. The chloros was washed off thoroughly with water. For the first 30 minutes the homogeniser was operated at maximum flow rate of 113L.min^{-1}, without any pressure applied to the disruption valve. During this time the cyclone was used to sample the air around the homogeniser. The homogeniser was then operated at 48 MPa for 30 minutes and a cyclone sample was taken. The homogeniser was then switched off while the cyclone and tubing were cleaned in chloros. Finally the homogeniser was operated for a further 30 minutes while a cyclone sample was taken.

4.3.2.3 Results

Table 4.6: Measurement of Release of Transketolase *E. coli* Cells from 30CD Homogeniser (Run A)*(_=no product, +=faint product band, ++=medium size product band, +++=heavy product band)*

Sample	Total count (cell.mL^{-1})	PCR product
E. coli cells before homogenisation	3.9 x 10^9	+++
E. coli cells after 60 mins of homogenisation	nd	+++
Inocula lab*	nd	+
DSP room*	< 7.8 x 10^4	+
Homogeniser control*	nd	+
Lubrication liquid before homogenisation	1.44 x 10^5	+
Lubrication liquid after homogenisation	2.2 x 10^6	+++
Homogenisation without pressure	< 7.8 x 10^4	_
Homogenisation for 30 minutes at 48 MPa	< 7.8 x 10^4	_
homogenisation for 60 minutes at 48 MPa	< 7.8 x 10^4	+

The samples in table 4.6 marked with an asterix were carried out in a separate experiment. The cyclone and tubing were initially cleaned in chloros. The Inocula lab sample was used to demonstrate the effectiveness of the cleaning process. The

Downstream Processing room sample was used to see if there was a background of TK *E. coli* cells present in the room in which the homogeniser is situated. The homogeniser was then operated with water only, at 48 MPa for 30 minutes and a further cyclone sample taken. At this point the homogeniser had not previously been used to process TK *E. coli* cells. The PCR results for these samples indicate that it is very difficult to get the cyclone and tubing completely *E. coli* or DNA-free. Figures 4.5 and 4.6 show the electrophoretic gels for the two homogenisation runs.

Table 4.7: Measurement of Release of TK *E. coli* Cells from 30CD Homogeniser (Run B)*(_=no product, +=faint product band, ++=medium size product band, +++=heavy product band)*

Sample	Total count (cells.mL^{-1})	PCR product
E. coli cells before homogenisation	5.8×10^9	++
E. coli cells after 30 minutes homogenisation	nd	++
E. coli cells after 60 minutes homogenisation	nd	++
DSP room	$< 7.8 \times 10^4$	+
Lubrication liquid before homogenisation	2.7×10^5	+
Lubrication liquid after homogenisation	9.2×10^5	++
Homogenisation without pressure	$< 7.8 \times 10^4$	+
Homogenisation for 30 minutes at 48 MPa	$< 7.8 \times 10^4$	+
Homogenisation for 60 minutes at 48 MPa	$< 7.8 \times 10^4$	++

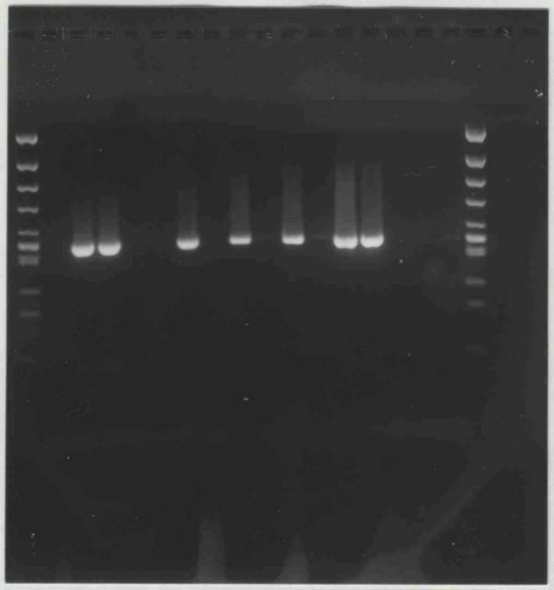
Both homogeniser runs A and B show that it is possible to detect the release of TK *E. coli* cells from the APV 30CD homogeniser using PCR. This release occurs both as an aerosol and as a liquid. Liquid release, from the process stream, into the lubrication liquid for the homogeniser pistons is indicated by the increase in strength of the PCR product band on the electrophoretic gel, for the lubrication liquid sample, after homogenisation has taken place. The faint band present on the gel, for the lubrication liquid sample, before homogenisation had occurred, was due to TK *E. coli* cells present in the piston lubrication pipework. There is no arrangement for cleaning these pipelines, so that cells are carried over from one

experiment to the next. This liquid release consisted of cells numbers high enough to detect using the counting chamber. The occurrence of this liquid release can be explained by the failure of one of the piston packing rings. This had previously been autoclaved and was in a deteriorated state. Liquid was seen leaking from all pistons during the course of the two experiments.

The design of the 30CD homogeniser piston shown in figure 4.4 has now been improved (Pandolfe, 1995). The most recent design uses an adjustable nut to tighten the components in the stuffing box to ensure a good seal on the piston. Apparently, in older designs it was possible to have the piston components less than rigid when assembled. This was due to discrepancies in tolerances of the parts making up the piston. The components of the pistons in the homogeniser used in this study may not have been as rigid as they should and this may hence explain the tendency of the homogeniser pistons to leak lubrication liquid.

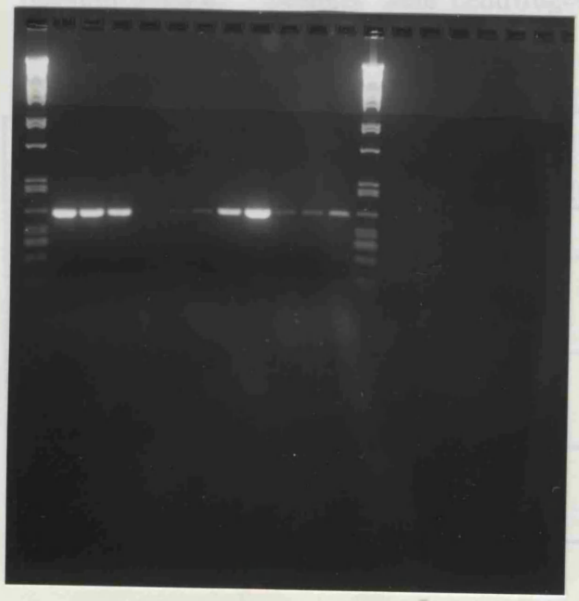
In runs A and B there was an increase in the strength of the PCR product band, for the cyclone samples, during the course of the homogenisation. In run A there was no band until the homogeniser had been operating for 60 minutes. In run B there was a faint band for the cyclone samples taken while the homogeniser was running with no pressure, and also for the first 30 minutes running at 48 MPa. The band appears stronger after 60 minutes of homogeniser operation. However, in both runs A and B, this aerosol release from the homogeniser is not large enough in cell number to be counted with the counting chamber. It is likely that the release of microbial aerosols from the homogeniser is dependent both on operating time and homogenisation pressure.

Figure 4.5: Detection of TK *E. coli* Release from APV 30CD Homogeniser (Run A)



Well	Sample
1 (left)	Molecular weight marker
2	Sterile RO water
3	Cells before homogenisation
4	Cells after homogenisation
5	Ringers' solution
6	Inocula lab
7	10^{-3} *E. coli* cell dilution in Inocula lab sample
8	DSP room
9	10^{-3} *E. coli* cell dilution in DSP room sample
10	Homogeniser control
11	10^{-3} *E. coli* cell dilution in homogeniser control sample
12	Lubrication liquid before homogenisation
13	10^{-3} *E. coli* cell dilution into 12 above
14	Lubrication liquid after homogenisation
15	Homogenisation without pressure
16	Homogenisation for 30 minutes at 48 MPa
17	Homogenisation for 60 minutes at 48 MPa
18 (right)	Molecular weight marker

Figure 4.6: Detection of TK *E. coli* Released from APV 30CD Homogeniser (Run B)



Well	Sample
1 (left)	Molecular weight marker
2	Cells before homogenisation
3	Cells after 30 minutes homogenisation
4	Cells after 60 minutes homogenisation
5	Ringers' solution
6	Sterile RO water
7	DSP room
8	Lubrication liquid before homogenisation
9	Lubrication liquid after homogenisation
10	Homogenisation without pressure
11	Homogenisation for 30 minutes at 48 MPa
12	Homogenisation for 60 minutes at 48 MPa
13 (right)	Molecular weight marker

Table 4.8: Protein Release from TK *E. coli* During Homogenisation

Supernatant protein concentration was measured using the Bio-Rad protein assay (section 2.3.4.4). Samples were centrifuged at 6630 g for 10 minutes and the supernatant assayed.

Sample	Protein released from cells to supernatant ($mg.mL^{-1}$)	
	Run A	Run B
E. coli cells before homogenisation	0.054	0.22
E. coli cells after 30 minutes homogenisation	n.d.	0.51
E. coli cells after 60 minutes homogenisation	0.30	0.50

Table 4.8 shows that during the homogenisation of the TK *E. coli* cells, release of protein from the cells into the broth increases. The concentration of protein in the supernatant doubles, indicating that the cells are being disrupted. It would be interesting to know at what point DNA is destroyed by homogenisation, since this would affect detection using PCR. One of the advantages of using PCR, for the detection of microbial release from a homogeniser, is that it will work as well with cell fragments as with whole cells. It is however, unlikely that a homogeniser would be operated for longer than 1 hour with the same process stream, if the cells had been disrupted in say, the first 30 minutes. It is more likely that the homogeniser would be run for prolonged periods when processing a large volume of fermentation broth. In this case then, PCR could still be used as an effective detection method.

It is evident from these experiments of detecting TK *E. coli* cells released from bioprocesses that the cyclone is not an ideal collection method if the cells are to be detected using PCR. It is almost impossible to ensure that the cyclone and it's tubing are DNA or *E. coli*-free. The cyclone is a hand-blown piece of glassware, and as such consists of many internal surfaces which are very difficult to clean. Some recommendations for improvements to the cyclone design are discussed in section 5.1.1.

CHAPTER FIVE: DISCUSSION

5.1 The Mass Balancing Method

It was necessary to develop a method for tracing a whole release from a piece of bioprocessing equipment, so that it's destination, and most importantly, its magnitude could be determined. The majority of studies reported in the literature (Dunnill, 1982; Lawrence and Barry, 1982; Cameron *et al.*, 1987 and Tinnes and Hoare, 1992) have not used quantitative methods. With one exception (Ashcroft and Pomeroy, 1983), the number of cells detected are not related back to the number that were initially released from the process. At best, concentrations of microorganisms only (per unit volume of air or liquid) are quoted.

This study initially demonstrated that:
1. It was possible to relate the number of cells collected in the cyclone back to the number originally released by the atomiser.
2. Over 50% of the cells released by the atomiser into a contained environment could be collected in the cyclone.
3. This recovery of over 50% was repeatable if the conditions of release were kept constant.

This was achieved using a mass balancing system that consisted of 4 components:
1. A method for producing a microbial aerosol, i.e. the atomiser or the Collison Nebuliser.
2. A method for collecting the microbial aerosol, i.e. the cyclone and Ringers' plates.
3. A contained environment in which to carry this out, i.e. the Bassaire or soft film cabinet.
4. A method for counting cells., i.e. a haemocytometer.

5.1.1 Aerojet-General Cyclone

The Aerojet-General cyclone was chosen to collect aerosols because it operates at a high air flow rate, (flow rates upto 750 $L.min^{-1}$ are possible) and concentrates the particles collected into a relatively small volume of liquid (approx. 50 mL in this study).

The fact that the cyclone collects cells into a liquid rather than onto a solid surface is advantageous. Many other commonly used air samplers collect the contents of the air onto an agar plate or a filter surface. This means that the total number of cells present cannot be estimated, only the number that form colonies on the agar surface. Cells that have undergone aerosolisation are also highly stressed and unlikely to form colonies on agar. They often dry out if left exposed to air currents for long periods of time.

Cameron *et al.,* (1987) suggest that the cyclone has a sampling efficiency of 65% for *Bacillus subtilis* var *niger* spores, although they do not say how this was calculated. However, this figure is similar to the recoveries of approximately 50% consistently achieved in this study.

Errington and Powell (1969) discuss the design of cyclones for the efficient collection of airborne particles. They recommend that a cyclone be small in size and work at a high gas velocity. A means for removing deposited particles from the internal surfaces, such as recirculating a scrubbing liquid is also recommended. Errington and Powell (1969) describe a cyclone which differs from that used in this study in the following way: Their cyclone has an overflow tube which widens gradually into a cylindrical enlargement in which the outlet tube is set tangentially. This design minimises the pressure drop across the cyclone and encourages entrained liquid to creep along the walls rather than break up and become airborne. It would have been of benefit to have this overflow tube included in the design of the cyclone used in this study. During cyclone operation a small amount of liquid was often lost via the air outlet. This flow of liquid appeared to be dependent upon the position of the needle injecting the liquid into the airstream. When examined for the presence of cells, it was not found to contain a significant number (see table 3.7).

It would be advantageous to make the cyclone more easily sterilised and cleaned free from DNA (i.e. using chloros). Glass tends to be prone to chipping and is likely to weaken when autoclaved. However, if the cyclone is transparent it is possible to see immediately if the flow of liquid does not remove material stuck on the cyclone walls. An ideal material of construction would be transparent, autoclavable and chloros resistant.

5.1.2 Counting Chambers

Counting chambers have the advantage of being fairly quick to use compared with growing cells on agar plates and counting colonies. They are also one of only a few methods by which whole cells can be enumerated.

Throughout this study total counts of whole cells are quoted. This does not give an indication of the physiological state of the cells. However, in section 3.4.1.2 viable counts are shown which suggest that only a small percentage of cells (*S. cerevisiae* and *E. coli*) collected in the cyclone (in most cases <10%) are able to form colonies on agar. If the inability to form colonies on agar is an indication of stress, it can be seen that the cells collected in the cyclone were stressed far more than those collected from the floor of the cabinet. Colwell *et al.*, (1985) observed a similar discrepancy between cell counts carried out by microscope and those carried out on agar plates. They suggest that cells collected in environmental samples often enter a resting state called "viable but non-culturable".

All of the total counts presented in this study have been enumerated in at least duplicate for each sample and were found to be repeatable. However, total counts of *E. coli* cells were found to be more difficult than *S. cerevisiae* due to the small size (1μm) of the cells. Cells moved in and out of focus, especially if the cover slip was not properly attached to the counting chamber. The use of phase contrast microscopy did improve the situation, but these counts were more time consuming than those for *S. cerevisiae*.

5.1.3 Bassaire Cabinet

After carrying out the experiment shown in table 3.8 it became obvious that it would not be possible to account for all the cells that were known to have been released into the cabinet. These cells were not on the floor (these were estimated with Ringers' plates) and were not found to be present in the air at the end of the sampling period (table 3.8, expt d). They were also not present in the exit line from the cyclone (table 3.7). It was thought that they were on the walls of the cabinet.

One expt (table 3.8 expt c) was carried out to estimate the number of cells that stuck to the cabinet walls. However, it was difficult to estimate the total number of

cells on the walls with any level of accuracy. Approximately 1% of the cells released into the cabinet were recovered from the back wall. Areas of this wall were swabbed, but it could be seen from the pattern of droplets on the wall that it was not covered uniformly. The aerosol left the atomiser at high velocity with some cells hitting the wall in a circular pattern which radiated out from the centre of the wall, directly opposite the atomiser. The side walls of the cabinet were made of HEPA filter and therefore could not be swabbed. These filters may contain a proportion of the cells released into the cabinet. It was decided that since the mass balance consistently recovered over 50% of the release, it was not necessary to spend an excessive amount of time tracing the remainder.

5.2 Factors Affecting Recovery in the Cyclone

The cyclone design and operating conditions probably have contributed to the inconsistencies in the data described in section 3.4. The cyclone has been used to collect aerosols of different microbial species at different concentrations, suspended in different solutions and sprayed into different size environments. The results show convincingly that viability is not an accurate method of cell detection, even though numerous workers still use this method. The plate counts data expressed in section 3.4.1.2 are too inconsistent to show trends in the way that the total count data does.

Cell recovery in the cyclone is thought to be affected by the following factors:

i. Cell size

There is no evidence in section 3.4 (for example, table 3.15) that cell recovery in the cyclone is different for the two microbial species examined. Even though *S. cerevisiae* and *E. coli* have different diameters (5 μm compared with 1 μm, respectively).

ii. Solids content of the suspending solution

For *S. cerevisiae* suspensions, the cyclone recoveries for suspensions in broth are reduced compared to those in Ringers' solution (tables 3.15 and 3.16). This reduction is 20% for the lowest cell concentration of 10^7 cells.mL^{-1} and 10% for the highest cell concentration of 10^9 cells.mL^{-1}. The cell recovery from the floor for the *S. cerevisiae* cells suspended in broth was 5% greater for 10^9 cells.mL^{-1}.

If the particle size distributions (figures 3.4 and 3.5) for *S. cerevisiae* cells suspended in broth and Ringers' solutions are compared, it can be seen that for cells suspended in broth there are more particles in the size range 8 - 14 μm at the higher concentration (10^9 cells.mL^{-1}) compared to the two lower ones and the broth blank. There is little difference in the particle size distributions for different concentrations of *S. cerevisiae* cells suspended in Ringers' solution. This may account for the reduced recoveries in the cyclone for *S. cerevisiae* cells suspended in broth compared to Ringers' solution as the heavier particles in the broth fall more rapidly to the floor. These heavier particles will also carry more cells.

The *E. coli* data are much more difficult to interpret. The particle size distribution shows more of all particle sizes present for the highest concentration of *E. coli* suspended in Ringers' solution (figure 3.6). For suspensions of *E. coli* suspended in broth (figure 3.7), the particle size distribution again shows more of all particles sizes at the highest cell concentration, with peaks at 2-4 μm and 10-12 μm. Neither of these particle size distributions correlate with cell recovery in the cyclone (tables 3.17 and 3.18).

The errors in the *E. coli* experiments are much larger than those in the *S. cerevisiae* experiments. These errors are probably due to inaccuracies in the total counting method. A more accurate detection method for *E. coli* would allow differences in cell recovery in the cyclone to show up. It may then be possible to correlate the cyclone recovery results with the particle size analysis.

iii. Cell concentration

The evidence for released cell concentration affecting recovery in the cyclone is two-fold. Firstly, the results in tables 3.15 and 3.24 show significant differences in cell recovery depending on the concentration of the released cells. Recovery in the cyclone of cells released into the Bassaire cabinet is reduced by approx. 15% when the *S. cerevisiae* concentration is increased from 10^7 to 10^9 cells.mL^{-1}. It was thought that this difference was due to increased clumping of cells, forming larger aerosol particles in the more concentrated suspensions. These larger particles would fall to the floor of the cabinet more easily and evade capture in the cyclone. The APS was used to examine this particle size distribution in the microbial aerosols.

The particle size distributions show a very similar content for the lower cell concentrations, almost exactly the same as that for buffer only. It is only at the highest cell concentrations that the cells affect on the particle size distributions. For example, that of *S. cerevisiae* suspended in Ringers' solution shows a large peak at 5 μm, however, the distribution for the 8 to 14 μm range is very similar for the lower concentrations.

It not possible to make further conclusions using the particle size analysis data. It does not correlate well with the cyclone recovery data, indicating that there may be a number of errors present. The calculation in section 3.5.2 shows that the APS samples less than 0.1% of the aerosol particles present in the Bioaerosol Test Chamber. The sample analysed by the APS therefore may not be a representative one.

5.3 Releases from Bioprocessing Equipment

5.3.1 APV 30CD Homogeniser

Homogenisers are used to disrupt cells thereby releasing intracellular proteins. They operate at high pressures (the 30CD can be operated up to 120 MPa for short periods) making aerosol production possible if leaks occur. In the processing stages between fermentation and precipitation large volumes of material containing cell wall debris and potentially bioactive substances are handled. The formation of aerosols containing these materials is even less desirable than the production of aerosols containing whole cells.

Homogenisers are commonly believed to release microbial aerosols. Stewart and Deans (1990) show strong evidence for liquid release of *S. cerevisiae* from the 30CD homogeniser. Walker *et al.* (1987) discuss the containment of microorganisms during fermentation and downstream processing. They recommend that when disrupting a genetically modified organism in a homogeniser (a Manton-Gaulin), the homogeniser must be contained within an isolator. This is because at that time (1987) it had not been found possible to contain organisms passing through the homogeniser. Vranch (1992) points out that care should be taken to ensure that the piston of the homogeniser pump is not exposed and that any lubrication fluids are collected and disinfected, although he

continues to say that modern homogenisers can be sterilised *in situ* and are contained.

The above discussion justifies a quantitative investigation into microbial release from a high pressure homogeniser. In this study when a homogeniser was used to process *S. cerevisiae,* release was not detected using the cyclone and counting chamber. The homogeniser was operated for 24 hour-long runs. During this time the seals inside the piston stuffing boxes were not changed. The manufacturers recommend that the packing seals are changed after 8 hours of operation. Release was still undetectable after autoclaving one of the piston packing rings to accelerate failure. At this point lubrication liquid was leaking from all of the pistons at a steady flowrate.

When TK *E. coli* was homogenised it was possible to detect release into the air surrounding the homogeniser (using the cyclone to sample the air) and in the lubrication liquid, using PCR as a detection method. This liquid and aerosol release was found to be dependent upon operating time and pressure. The aerosol release into the soft film cabinet only became detectable with PCR after the homogeniser was operated for 60 minutes (table 4.6 and figure 4.5) at 48 MPa. Liquid release into the lubrication fluid increased with operating time.

The three pistons which form the ram pump of the 30CD homogeniser are the most likely place for aerosols to be generated. Figure 4.4 is a cross section through one of these pistons and shows the seals involved in preventing contamination of the piston lubrication fluid by process liquid. As discussed above, release occurred both in the form of liquid into the piston lubrication fluid, and in the form of an aerosol detected in the air surrounding the homogeniser. For the lubrication fluid to become contaminated with the process stream, the piston packing plus the O-ring and PTFE backup ring must fail. If the piston secondary seal and bush also fail, then the process liquid would travel to the end of the piston and become aerosolised by the movement of the piston in the stuffing box.

The liquid leaking from the three pistons in the ram pump of the homogeniser should be directed to the contained drain provided. However as the pistons move backwards and forwards into the stuffing box, liquid travels along the piston and from there flows down the front casing of the homogeniser. The contaminated liquid collects on the floor of the soft film cabinet from which there is no provision

for contained disposal. If the 30CD homogeniser is operated at containment levels above B1, secondary containment should also be used. However, it should be noted that the number of cells in this liquid release from the pistons was found to be very low.

It is still unclear whether *S. cerevisiae* cells are released but not detected, or simply not released. To answer this question it would be necessary to operate the homogeniser with a recombinant *S. cerevisiae* strain which could be detected using PCR. It is also not known if cell debris is released from the homogeniser. The PCR investigation tells us that fragments of DNA are released which contain the 350 base pair sequence used in the PCR. It would be interesting to know how long the homogeniser could be operated before the 350 base pair fragment is destroyed because this will affect the suitability of PCR as a detection method for microbial release from a homogeniser. This last point is only valid when a cell suspension is recycled through the homogeniser for long periods of time, as in the experiments carried out in this study.

This leads into the argument of whether it is of consequence that cell fragments are released from bioprocessing equipment. This study has, in the main, only attempted to trace whole cells. However the release of aerosols containing cell debris and bioactive components could pose a serious health risk. Indeed, both ACGM Note 6 (1989) and the GMO (Contained Use) Regulations (HSE, 1992) state that when a risk assessment is made, the toxic or allergenic effects of non-viable organisms and/or their metabolic products must be considered.

5.3.2 Fermenters

The reason for carrying out this study of microbial release from fermenters is two-fold: Firstly, there is much published in the literature on the subject of fermenter design for containment (see section 4.1.3.1), however, few, if any of these studies use a quantitative approach; and secondly, the experiments carried out in section 3.3.1.1 of this study suggested that it was unlikely that *S. cerevisiae* cells would be aerosolised from a body of liquid such as that in a fermenter. This was thought to be due to the size of the *S. cerevisiae* cells since *E. coli* cells did aerosolise from a liquid surface under similar conditions.

The release of a microbial aerosol into the head-space of a fermenter is dependent upon:

1. The liquid surface area available for aerosolisation (i.e. the scale of fermenter).
2. The flow rate at which air is sparged through the broth.
3. The size of the organism or particles if cells aggregate together.
4. The number of cells available for aerosolisation, (i.e. the concentration of cells present in fermenter).
5. The surface tension of the liquid (which is affected by antifoam addition).
6. The stirrer speed.

Pilacinski *et al.* (1990) describe the mechanism of droplet formation that causes an aerosol to form above a liquid. Air bubbles produced in a liquid rise to the surface and form a hemispherical film cap. The bubble is kept at the surface by the two forces of buoyancy and surface tension. Gravity and suction from the negative curvature of the film surface cause liquid to drain from the bubble film and it bursts. Fragments of the film spread out forming a large number of small particles. As the bubble bursts, there is a sudden pressure change and the liquid depression is filled by the surrounding liquid with a jet at the centre. Disintegration of this jet creates a few large droplets. This description is however less appropriate for industrial processes where there may be a layer of foam on the liquid surface. Addition of antifoam will also affect this.

Pilacinski *et al.* (1990) looked at changes in air flow rate, agitation rate and liquid properties on the release of aerosol particles from aerated broth. Using an APS they found a higher number concentration of particles present in the head-space for agitated broths compared with non-agitated broths. For dilute broths, the particle concentration in the outlet air was lower (<10 particles.cm^{-3}) compared to undiluted broths (~100 particles.cm^{-3}). For a typical broth, mass mean diameter increased with agitation rate (75-130 rpm) and air velocity (0.15-0.25 cm.s^{-1}), so that 30-40% of the measured particles were greater than 2 μm diameter.

Szewszyk *et al.* (1992) investigated changes in concentration and size distributions of aerosols generated in a 150 L fermenter. The head-space air was taken from a point 15 cm above the liquid surface, the air dried and sampled by an APS. Particle size distributions for head-space air from an uninoculated broth, and then 4 hours and 10 hours after inoculation with *E. coli* K12. A significant decrease was seen in aerosol generation after the growth phase.

Szewszyk *et al.* (1992) found that for particles <2 μm diameter, the particle distribution was unaffected by agitation rate. However in the 5 μm size range the particles concentrations at 375 and 450 rpm were double those at <300 rpm. This shows that agitation of the broth with a stirrer does cause aerosol generation from the liquid surface.

Szewszyk *et al.* (1992) also examined the effect of air flow rate on particle concentration in the head-space air. For particles <2 μm diameter aerosol concentration was independent of air flow rate. However, for particles >2 μm diameter, a five-fold decrease in air flow rate caused increases in aerosol concentration of up to 100%. The authors explain that as air flow rate decreases, generated particles are distributed in a smaller volume of air and therefore become more concentrated.

In the work of both Pilacinski and Szewczyk the particle sizes only were measured. It is not known whether the particles measured contained cells from the fermentation.

In the experiments described in this thesis, a cyclone was used to collect aerosolised organisms from the head-spaces of fermenters. At the conditions used, recovery was found to be unaffected by all of the factors mentioned above except the species of organism grown in the fermenter.

It was not possible to detect *S. cerevisiae* cells in the fermenter head-space using the cyclone and the total counting method. Unmodified *E. coli* may have been released into the fermenter head-space but unfortunately the cells were difficult to distinguish from particles of dirt and background microflora also present in the cyclone sample. This is an inherent problem due to the way that the cyclone is used to sample from fermenters. This is because the air intake must be supplemented with room air to provide the cyclone with 360 $L.min^{-1}$. This problem would be eliminated if the air intake for the cyclone was filtered. Filtration of air at such a high air flow rate is however difficult to achieve.

When a recombinant TK *E. coli* was grown in the fermenter, release was detected in the head-space. This was made possible by the use of the technique of PCR, which detects a specific piece of DNA which only the TK *E. coli* contains. The

following calculation suggests that it is unlikely that *S. cerevisiae* cells will be released from a fermenter head-space at the conditions used in the *E. coli* experiments described in section 4.2.3.

Stokes Law can be used to calculate the settling velocity of a single *S. cerevisiae* or *E. coli* cell. This settling velocity can be compared to the velocity of the gas leaving the broth surface. If the settling velocity of the cell is greater than the rising velocity, the cell will not become aerosolised and will not leave the fermenter in the exit gas line. If the settling velocity is less than the rising velocity the cell will become aerosolised and will be released from the fermenter.

Stoke's Law, $u = \frac{\Delta\rho\, d^2\, g}{18\,\mu}$

Settling Velocity for *E. coli* Cells

Density, $\rho = 1400\ kg.m^{-3}$

Diameter, $d = 1 \times 10^{-6}$ m (*E. coli* cells assumed to have a diameter of 1μm and a spherical shape)

Viscosity, $\mu = 189 \times 10^{-7}\ Ns.m^{-2}$ (or $kg.ms^{-1}$) (at 37°C)

Velocity, $u = \frac{9.81 \times 1400 \times (1 \times 10^{-6})^2}{18 \times 189 \times 10^{-7}} \sim$ **$4 \times 10^{-5}\ m.s^{-1}$**

Settling Velocity for *S. cerevisiae* Cells

$\rho = 1400\ kg.m^{-3}$

$d = 5 \times 10^{-6}$ m (*S. cerevisiae* cells assumed to have a diameter of 5μm)

$\mu = 185 \times 10^{-7}\ Ns.m^{-2}$ (at 28°C)

$u = \frac{9.81 \times 1400 \times (5 \times 10^{-6})^2}{18 \times 185 \times 10^{-7}} \sim$ **$1 \times 10^{-3}\ m.s^{-1}$**

Velocity of Gas Leaving Broth Surface

Velocity = $\frac{\text{Volumetric flow rate}}{\text{Cross-sectional area}}$

Where,
Volumetric flow rate = 8.33×10^{-6} $m^3.s^{-1}$
(0.3 vvm, or 0.5 $L.min^{-1}$ of air was sparged into the fermenter)
Cross-sectional area = $\pi \times 0.1^2$ m^2

So, velocity = **2.65×10^{-4} $m.s^{-1}$**

Comparing the settling velocity for each cell type with the exit gas velocity, it can easily be seen that *S. cerevisiae* cells will not escape from the liquid surface because their settling velocity is greater than the exit gas velocity. However, *E. coli* cells will be released from the liquid surface because their settling velocity is less than the exit gas velocity.

The calculation above makes the assumption that cells aerosolise from a liquid surface as single cells only. However, the particle size distributions shown in figures 3.5 and 3.7 show that the particle distribution produced from the atomiser is of a more heterogeneous nature. Particles larger than 5μm and 1μm are found for *S. cerevisiae* and *E. coli* respectively. These are probably due to cells clumping together, aided by the solids present in the fermentation broth. Clumps of cells are unlikely to escape from the liquid surface due to their larger size and therefore higher settling velocity.

Evans *et al.* (1981) measured 10^9 viable particles.m^{-3} exhaust gas from a small scale fermenter and estimated that from a 1000 m^3 working volume fermenter, 1000 m^3 of effluent gas may emerge in one minute, carrying 10^{12} particles with it. Evans *et al.* (1981) also refer to a personal communication from a J. Shennan who measured 10^5 viable yeast cells.m^{-3} escaping from a 150 m^3 fermenter. An aerojet cyclone was used to reduce the number of organisms escaping, although it was also found to increase the risks of culture contamination.

It is difficult to compare the work of Evans *et al.* (1981) with the release rates found in this study because the authors do not say what organism they were fermenting or how they calculated their estimate of release from the 1000 m^3 fermenter. In this study, less than 1.5×10^6 total *S. cerevisiae* cells were released in a 30 minute period with an air sparge rate of 0.5 $L.min^{-1}$. This is a release of less than 100 cells.m^{-3} of air exhausted from the fermenter. For both modified and

unmodified *E. coli* approximately 10^7 cells.hr^{-1} were released from the fermenter. This is approximately 333 cells.m^{-3} of air exhausted from the fermenter.

Winkler (1988) found 10^6 bacteria.m^{-3} present in the head-space of a fermenter. However there is no further information given on either the fermenter conditions, process organism, or how this figure was arrived at. This makes it impossible to compare Winkler's value for the content of fermenter head-space air with that measured in this study. Winkler (1988) does add that many aerosol particles fall back, or impact on the wall of the fermenter or in bends in pipelines. This is more likely to happen to larger organisms such as yeasts.

These experiments should be repeated using a recombinant *S. cerevisiae* that could be detected using PCR. This would confirm whether or not an organisms the size of *S. cerevisiae* is released into the head-space of a fermenter. If it was not possible to detect cell released into the fermenter head-space, the theory based on particle settling velocity would be confirmed.

5.3.3 Detection Limits

Table 5.1 shows the % efficiency of the cyclone in collecting aerosolised cells from the soft film cabinet and from a fermenter head-space. The efficiency for the cyclone in collecting *S. cerevisiae* from the soft film cabinet was determined with the homogeniser inside (see table 3.25). The cyclone is assumed to be 100% efficient when sampling from a fermenter head-space, which is perhaps unrealistic since losses will occur as the aerosol travels between the fermenter and the cyclone.

Table 5.1: Cyclone Efficiencies

Aerosol Location	*S. cerevisiae* from Soft Film Cabinet	*E. coli* from Soft Film Cabinet	Fermenter Head-space
% Recovery in the cyclone	20	10	100

The limit of detection of the total counting technique is shown in table 5.2. The limit is different depending on cell species and whether the cell sample has been

centrifuged before counting in the haemocytometer (*S. cerevisiae*) or Helber bacteria counting chamber (*E. coli*). It should be noted that these numbers are approximate only.

Table 5.2: Limit of Detection of Total Counting Method

Location	Soft Film Cabinet/Homogeniser		Fermenter Head-space	
Aerosol	*S. cerevisiae*	TK *E. coli*	*S. cerevisiae*	TK & UM *E. coli*
Lowest number of cells that can be counted (cells.mL^{-1})	2 x 10^6	7.8 x 10^4	1.5 x 10^4	7.8 x 10^4
Equivalent release in cells.hr^{-1} (taking into account cyclone efficiency)	1 x 10^9	7.8 x 10^7	1.5 x 10^6	7.8 x 10^6

i. Loss from Homogeniser

i.i Aerosol

Using total counts to enumerate cells, there were less than 2 x 10^6 *S. cerevisiae* cells.mL^{-1} collection liquid in the cyclone sample from the air around the homogeniser. If it is assumed that the cyclone collects 20% of the cells released into the soft film cabinet (see tables 5.1 and 5.2), this is equivalent to a release of less than 1 x 10^9 cells.h^{-1} or 1.0 µL process liquid.h^{-1} (the process liquid contained 10^{12} cells.mL^{-1}).

Less than 7.8 x 10^4 cells.mL^{-1} were detected in the cyclone sample from the air around the homogeniser when it was used to process TK *E. coli*. This is equivalent to the release of less than 7.8 x 10^7 process organisms.h^{-1}, or approx. 16 µL process liquid.h^{-1}.

i.ii Lubrication Fluid

At the end of a processing period of 1 hour the lubrication liquid for the homogeniser pistons contained approx. 10^6 TK *E. coli* cells.mL^{-1}. If it is assumed that there is approx. 5L of lubrication liquid present in the reservoir at the start of the processing period (this is very approximate because liquid is constantly being

lost during homogeniser operation), this represents a total release of 5 x 10^9 cells.h^{-1}. This is equivalent to the loss of around 1 mL of processing liquid.

ii. Loss from Fermenter

Released organisms detected from TK *E. coli* fermentations corresponded to the escape of approx. 0.01 mL of fermenter broth (broth contains approx. 10^9 cells.mL^{-1}) from the fermenter in one hour. The level of release from UM *E. coli* fermentations is probably of a similar level but was difficult to detect due to other debris present in the samples.

The PCR method used has a detection limit of 500 cells.mL^{-1}, which approximates to 5 x 10^5 cells.h^{-1} when the cyclone is used to sample aerosolised *E. coli* from the soft film cabinet, or 5 x 10^4 cells.h^{-1} for cells in a fermenter head space (when sampling from the fermenter a 100% sampling efficiency is assumed for the cyclone). This means that the level of release of TK *E. coli* from the homogeniser falls between 5 x 10^5 - 7.8 x 10^7 cells.h^{-1}, and for the fermenter between 5 x 10^4 - 7.8 x 10^6 cells.h^{-1} released.

5.4 PCR and Microbial Population Balancing

The major disadvantage of carrying out PCR on a sample collected in the Aerojet-General cyclone was that the cyclone, sample container and the cyclone tubing must be made DNA-free. Autoclaving kills cells but does not destroy DNA and also tends to weaken the cyclone glass. Prince and Andrus (1992) found that a 10% v/v sodium hypochlorite (chloros) solution eliminated all ethidium bromide stainable DNA after 10 minutes contact. The chloros compared favourably to concentrated HCl. Prince and Andrus (1992) recommend the use of a 10% solution of chloros for a contact time of 30 minutes. It was then necessary to rinse the cyclone and tubing free from chloros because chloride ions inhibit the PCR.

The mechanism of action of sodium hypochlorite in the destruction of DNA is poorly understood but thought by Prince and Andrus (1992) to be multifactorial. It has been shown that bases and free nucleotides are destroyed and in some cases chlorinated derivatives are formed, such as 5-chlorocytidine. The end result is the destabilisation and breakdown of the DNA helix.

The PCR method could not be used with cyclone samples taken from the Bassaire cabinet because the cabinet could not be completely cleaned free of DNA. It was also not possible to clean out the 30CD homogeniser lubrication system with chloros because this would cause corrosion. The results expressed in section 4.3.2 show an increase in the number of TK *E. coli* cells in the lubrication system both during the sampling period and from one experiment to the next due to ineffective cleaning of the lubrication system.

Another factor to bear in mind when PCR is used to detect cells is that the PCR does not detect whole cells, but the target DNA sequence. This means that a positive results in the PCR may represent dead cells or even cell fragments as well as live cells. This is especially relevant to the measurement of release from the 30CD homogeniser, which, by it's nature disrupts cells. The DNA detected in the PCR carried out on the sample from the air surrounding homogeniser may be entirely free from cell fragments.

5.5 Implications for Legislators and Industry

The findings of this study have important implications concerning the Contained Use Legislation. The current UK legislation for the contained use of GMOs (HSE, 1992) describes control measures to minimise or prevent the incidental release of GMOs from bioprocesses, and to contain the release once it has occurred, for example by placing the equipment in a containment cabinet (secondary containment).

The study presented here has demonstrated that the release of (G)MOs during normal operating conditions (i.e. incidental release) is actually very low. This has been shown to be the case for both a high pressure homogeniser and for small scale fermenters. It could be argued that when one of the piston packing rings in the homogeniser was autoclaved to accelerate its failure, these were not 'normal' operating conditions. However, this packing wear could also occur through repeated use of the homogeniser. The head-space air from the fermenter was sampled unfiltered because when operating under GILSP, the exhaust gas from a fermenter does not require filtering before it is vented into the external environment.

The lowest containment category described by ACGM Note 6 (1987), GILSP, requires no extra containment measures above those required by the process itself to maintain sterility. Organisms used at GILSP can be GMOs and should have limited survival outside the process environment. By using this criterion, ACGM Note 6 therefore recognises that microbial release into the environment does occur. It can be said that GILSP is not a containment category at all and use of a (G)MO at GILSP constitutes release.

5.5.1 Incidental Versus Accidental Release

The work presented in this study suggests that accidental release is the more likely form of release. Accidental release has the potential to release a much larger number of organisms, far in excess of those released incidentally.

However, the legislation includes little instruction on preventing accidental releases. Some simple exercises often considered to be good engineering practice could be recommended, such as: regular maintenance, the use of Standard Operating Procedures, and a HAZOP-style approach to plant and process design.

A more complex design of processing equipment for contained use can lead to processes which are difficult to operate and maintain and therefore more prone to accidents. An often-quoted example is the seal categories suggested by Chapman (1989). Chapman proposed that the three OECD containment categories required increasingly complex static sealing arrangements. For LSCC1 (minimise release), a single seal was thought to be acceptable. For LSCC2 (prevent release), a double seal and for LSCC3 (prevent release), a double seal with steam flush were suggested. However, the double seal without a steam flush would give a false sense of security because the two seals, if installed at the same time, are likely to fail at the same time. However, if one seal did fail before the other, a build up of hazardous material between the seals would remain undetected and untreated without a steam flush.

The findings of the study presented here are reflected in the literature referred to in section 1.1.1. All of these studies report the release of microorganisms from bioprocessing equipment only when operated under less than ideal conditions. Tinnes and Hoare (1992) could not detect, even qualitatively, the release of a labelled *E. coli* from a disc stack centrifuge when operated normally. Cameron *et*

al., (1987) found that when a fermenter sampler was operated under normal conditions, release could not be detected. Similar results were obtained by Cameron *et al.,* (1987) when a novel valve type was tested in the same way. Even after 6700 operational cycles of opening and closing, release could not be detected and the valve showed no signs of wear-induced failure.

5.5.2 Why We Need More Sensitive Detection Methods

In spite of the previous discussion we still need more sensitive methods to detect microbial release from bioprocesses. This is because currently there is no quantitative data available on cell numbers released at different containment levels. When this data is available the OECD minimise/prevent table which is used almost universally (table 1.2) could be replaced with release limits for each containment category. ACGM Note 6 is currently under revision but is likely to retain this OECD minimise/prevent table because there is to be no alternative at present.

The MPN-PCR method described in section 3.6.1.2 and used in chapter 4 to measure microbial release from a high pressure homogeniser and the headspace of small scale fermenters seems to be a promising method to achieve the desired level of sensitivity.

Quantitative release data would show the difference in magnitude between cells released accidentally and incidentally. This would show clearly that an accident will release cell numbers far in excess of those are released incidentally to process operation.

5.6 Conclusions

1. Microbial Population Balancing

- The mass balancing technique used in this study can account for 50% of the cells released into a contained environment

- The recovery of this release in the cyclone is dependant upon the released cell concentration and the presence of suspended solids in the liquid.

- These two factors, cell concentration and suspended solid content, affect the particle size distribution of aerosols produced from the liquid cell suspensions.

- The particle size distributions for *S. cerevisiae* show a definite peak at 5μm for a 10^9 cells.mL^{-1} in both Ringers' solution and fermenter broth. The distributions show a very heterogeneous mixture of particle size for the lower cells concentrations.

- The particle size distributions for *E. coli* in both Ringers' solution or fermenter broth show a heterogeneous mixture of particle sizes for all cell concentrations.

2. Release from Bioprocessing Equipment

2.1 30CD Homogeniser

- The level of aerosol release of *S. cerevisiae* from the 30CD homogeniser during normal operating conditions is less than 1 x 10^9 cells.h^{-1} (taking cyclone collection efficiency into account).

- The level of liquid release of *S. cerevisiae* into the piston lubrication fluid is less than 2 x 10^{10} cells.h^{-1}.

- The level of aerosol release of TK *E. coli* from the 30CD homogeniser during normal operating conditions is between 5 x 10^5 (PCR limit of detection) and 7.8 x 10^7 cells.h^{-1} (taking into account cyclone collection efficiency).

- The level of liquid release of TK *E. coli* into the piston lubrication fluid is between 5 x 10^6 (PCR limit of detection) and 5 x 10^9 cells.h^{-1} (assuming lubrication liquid volume = 5L).

- When TK *E. coli* cells are released into the soft film cabinet, DNA equivalent to about 5 x 10^5 cells.h^{-1} is released, but this may be associated with cell fragments rather than with whole cells.

2.2 Fermenters

- The level of aerosol release into the head-space of a *S. cerevisiae* fermentation is less than 1.5 x 10^6 cells.h^{-1}.

- The level of aerosol release into the head-space of an unmodified *E. coli* fermentation is less than 7.8 x 10^6 cells.h^{-1}.

- Release into the head-space of a recombinant *E. coli* fermentation is between 5 x 10^4 (PCR limit of detection) and 7.8 x 10^6 cells.h^{-1}.

3. Detection Methods

- PCR is a more effective technique for cell detection (via DNA detection) than counting in a haemocytometer when cell concentrations are below 1.5 x 10^4 cells.mL^{-1} for *S. cerevisiae* and 7.8 x 10^4 cells.mL^{-1} for *E. coli*.

- Viable counts are not an accurate method to quantitatively detect either *S. cerevisiae* or *E. coli* cells.

5.7 Future Work

1. Cyclone Improvements

The operation of the cyclone could be made more efficient with the following design changes:

i. The needle position could be fixed at 45^{o} to the vertical. This would prevent variation in cyclone efficiency between samples.

ii. It would be beneficial to construct the cyclone from a material which is less prone to breakage than glass. It should be more easily cleaned, both in detergent and by autoclaving, especially if it is used with PCR.

iii. Addition of either an overflow tube (see section 2.4.3.3) similar to that of Errington and Powell (1969) or a lip around the air exit might also reduce efficiency differences between samples and reduce losses to the air exit line.

2. Aerosol Particle Size Distributions

The graphs of mass concentration/channel width against particle midsize shown in figures 3.4-3.7 would be more complete if they included a plot for cell concentrations between approx. 2×10^{8} and 2×10^{9} for *S. cerevisiae* and 3×10^{9} and 3×10^{10} for *E. coli*. As the graphs stand there is a sudden difference in the particle size distributions between the two highest cell concentrations.

3. Release of Microorganisms into Fermenter Head-spaces

The cyclone, and if possible QPCR, should be used to measure cells released into a head-space during a recombinant *S. cerevisiae* fermentation. This would confirm the relationship between release of microorganisms into fermenter head-spaces and cell size (or species).

4. Release of Microorganisms from 30CD Homogeniser

The cyclone, and if possible QPCR should be used to measure release of a recombinant *S. cerevisiae* from the 30CD homogeniser. The outcome of this experiment could be compared with the results in this study for the release of TK *E. coli* from the homogeniser. This could then be used to investigate the relationship between cell size and aerosolisation.

It would be interesting to examine the relationship between processing time in the homogeniser and debris size produced. This would indicate whether release measured by PCR represents the release of whole cells or cell fragments. It would

also show at what point the DNA is broken up into fragments of less than 350 base pairs. In this study, cell suspension was recycled through the homogeniser for a period of one hour (i.e. many passes through the CD valve). However, in a production process a homogeniser may be operated for one or two passes only.

The results presented in this study indicate that there is a relationship between operating pressure and microbial release from the homogeniser. This could be examined further.

5. Quantitative PCR

QPCR is a sensitive enough technique to be used to actually match the number of cells released from bioprocesses to different containment categories. It would then be possible to evaluate different physical containment measures against microbial release levels. The effectiveness of the current engineering containment practices could be determined.

6. Microbial Population Balancing in the Processing Environment

The cyclone should be used to measure release of microorganisms in a processing environment. The problems likely to be encountered if this is attempted are that the cells will be released at a very low concentration (in normal operating conditions) and are likely to mixed with the natural microflora of the room. This microflora will include debris (from cells and just plain dirt) and other cell species. QPCR would be very useful for solving both of these problems. It is both highly sensitive and can be made specific for the process organism.

The technique of Computational Fluid Dynamics (CFD) could be used together with the suggestion above to build up a picture describing the destination of cells once they are released from a piece of processing equipment. The destination of aerosolised organisms will be affected by such things as air flow patterns from HEPA filters and differential room pressures. The efficiency of air samplers, such as the cyclone, in collecting released microorganisms will be dependent upon factors such as their position in relation to the processing equipment and air filters in the processing room.

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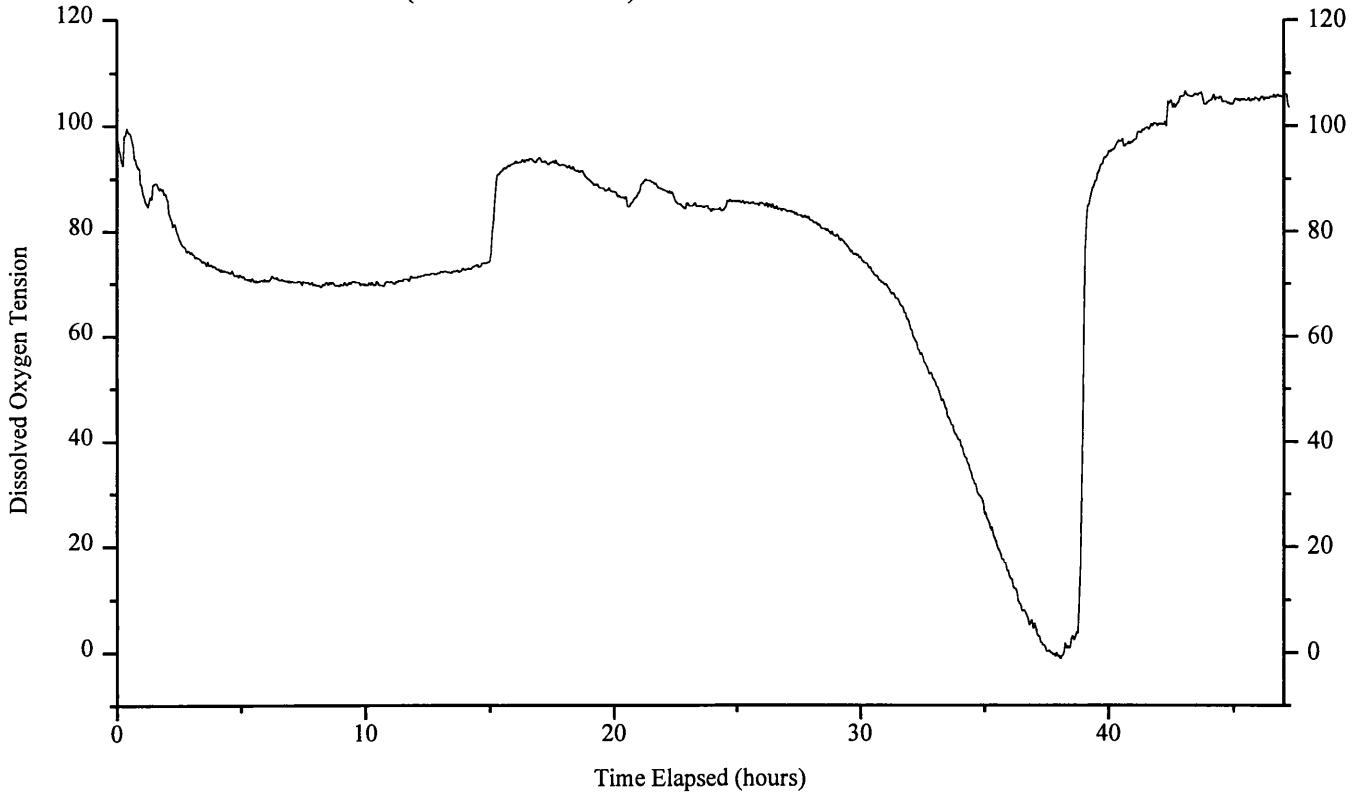
Appendix A
Fermentation Profiles

The fermentations are described in Chapter 4, section 4.2.

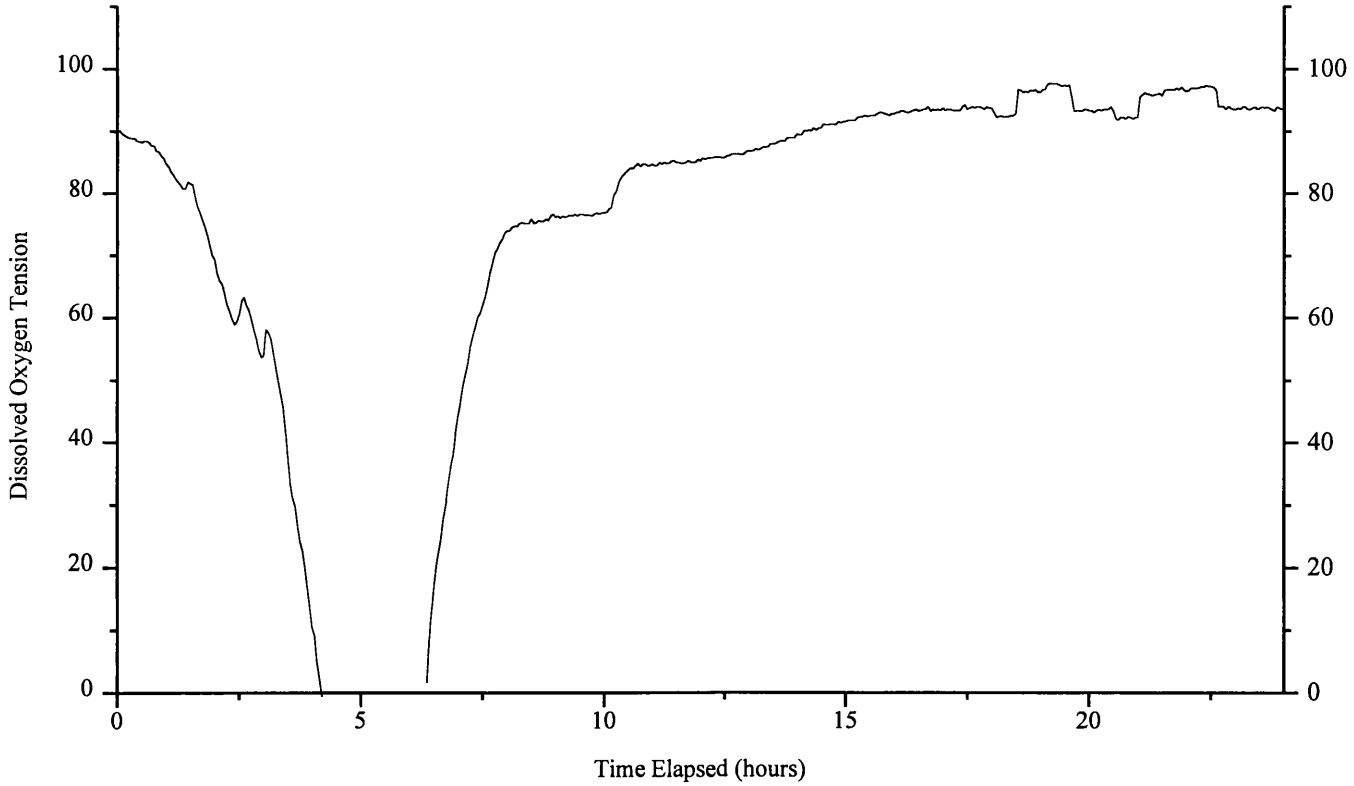
DOT (Dissolved Oxygen Tension) is plotted against time elapsed from inoculation of the fermenter.

Figure A1: *S. cerevisiae* Fermentation
(see section 4.2.2.2)



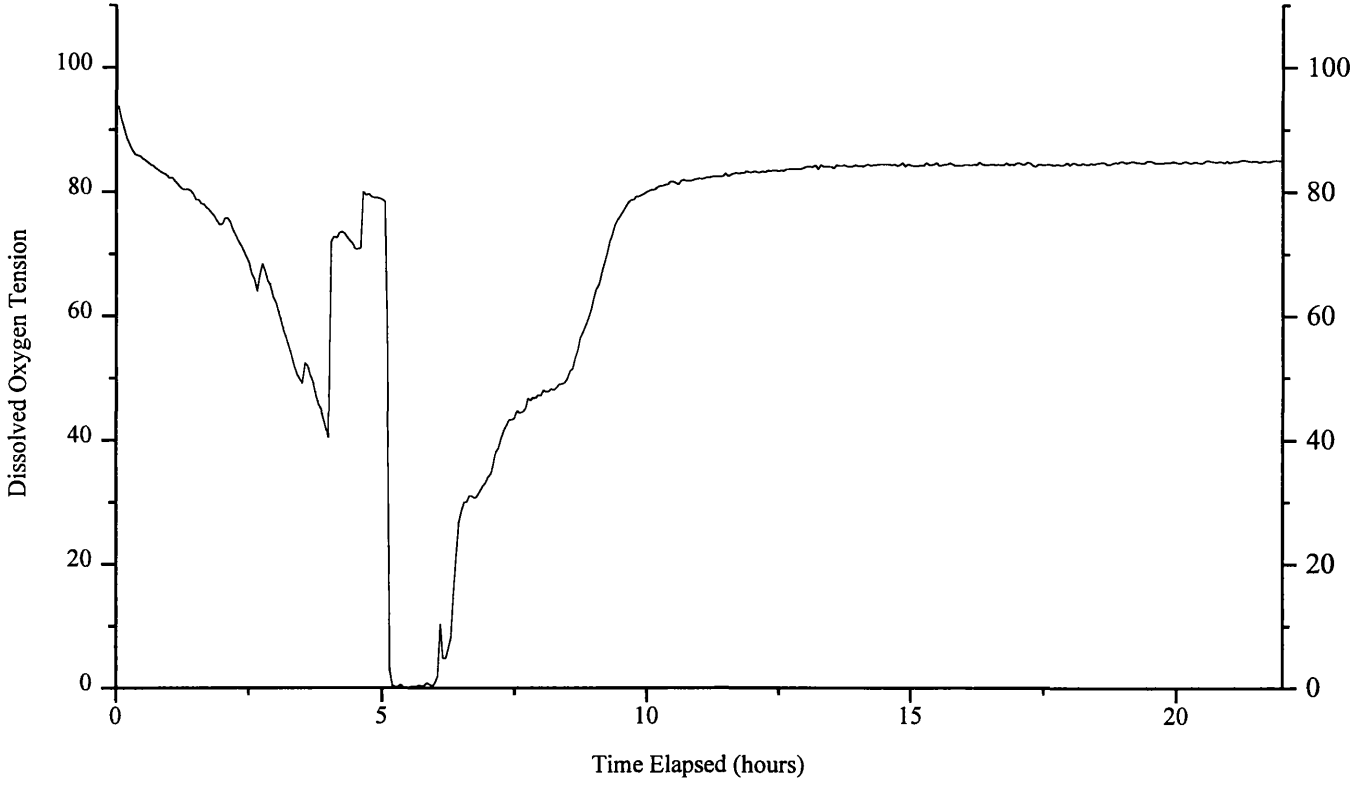
(1000 RPM, no antifoam added, 0.5 $L.min^{-1}$ air sparged, 1.5 L working volume, pH 7, 28°C)

Figure A2: *E. coli* Fermentation A
(see section 4.2.2.2 and table 4.3)



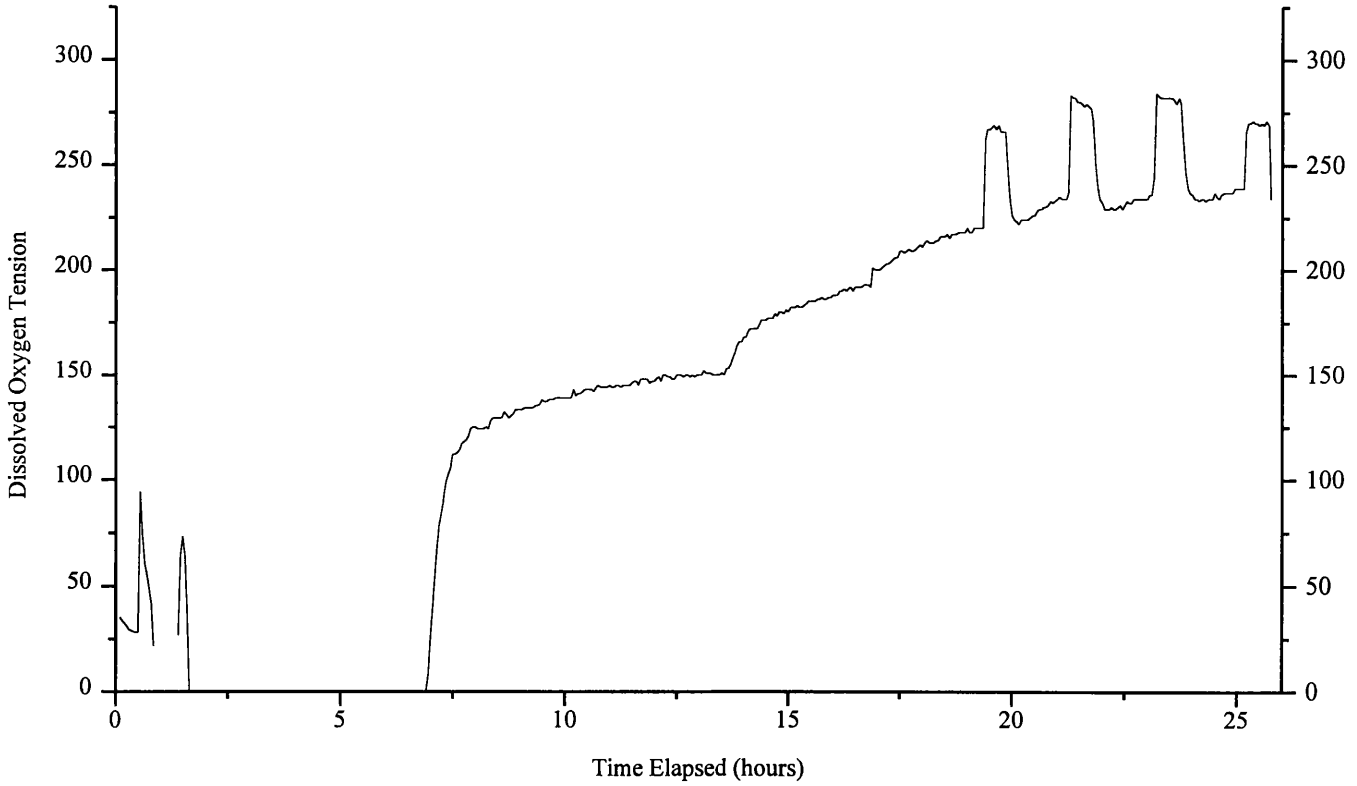
(750 RPM except when sampling, 1mL antifoam added, 0.5 $L.min^{-1}$ air sparged, 1.5L working volume, pH 7, 37°C)

Figure A3: *E. coli* Fermentation B
(see section 4.2.2.2 and table 4.3)



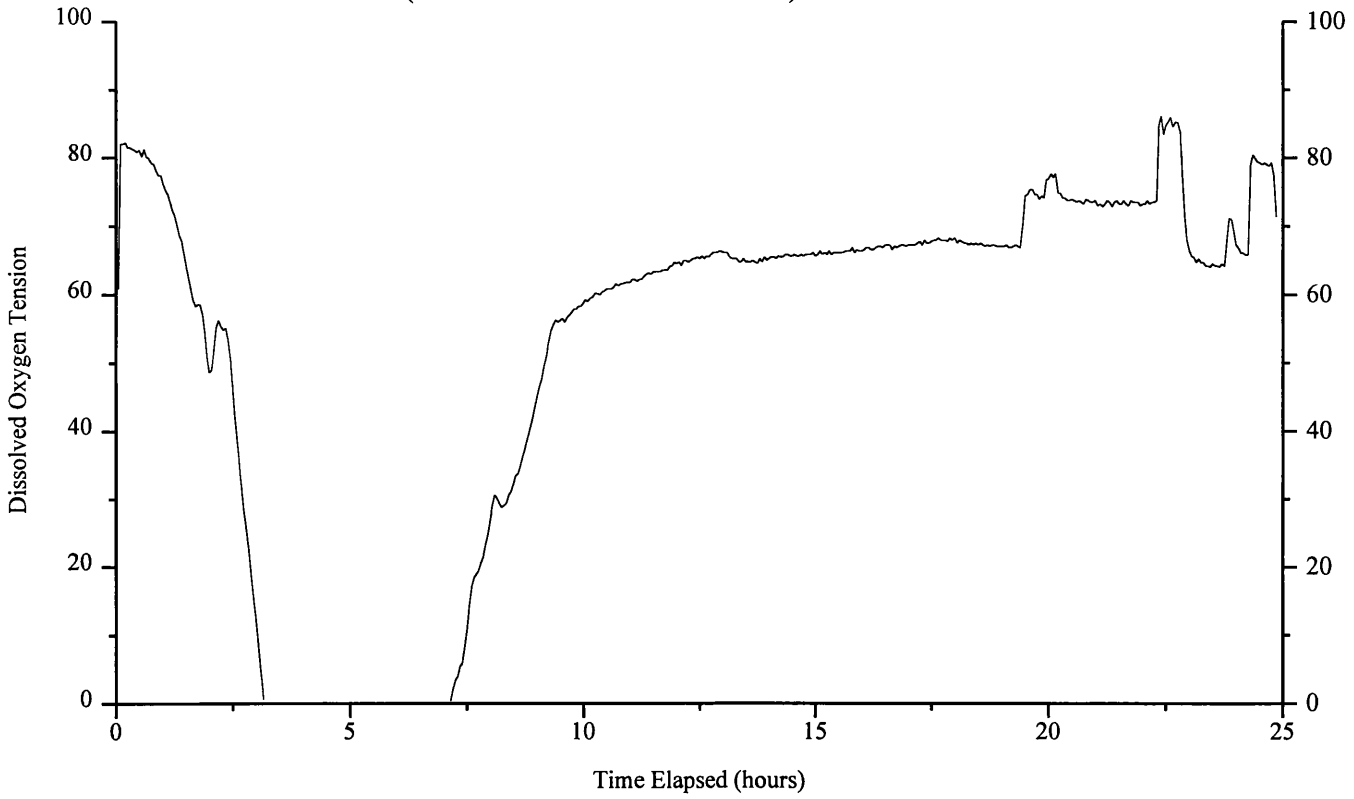
(750 RPM except when sampling, no antifoam added, 0.5 $L.min^{-1}$ air sparged, 1.5L working volume, pH 7, 37°C)

Figure A4: Transketolase *E. coli* Fermentation A
(see section 4.2.3.2 and table 4.5)



(500 RPM except when sampling, 2 drops antifoam added, 0.5 $L.min^{-1}$ air sparged, 1.5L working volume, pH 6.8, 37°C)

Figure A5: Transketolase *E. coli* Fermentation B
(see section 4.2.3.2 and table 4.5)



(500 RPM except when sampling, few drops antifoam added, 0.5 $L.min^{-1}$ air sparged, 1.5L working volume, pH 6.8, 37°C)

Appendix B
Particle Size Analysis Data

The method used to obtain these data is described in Chapter 3, section 3.6.

The tables show the data used to plot figures 3.4, 3.5, 3.6 and 3.7.

Table B1

PARTICLE SIZE ANALYSIS: *S. cerevisiae* IN RINGERS SOLUTION									
midsize	mass conc (mg.m-3)				channel	mass conc x channel width (mg/m3.microns)			
(microns)	ringers	cells in ringers solution			width	ringers	cells in ringers		
		1.6 x 10-7	1.6 x 10-8	1.7 x 10-9	(microns)		1.6 x 10-7	1.6 x 10-8	1.6 x 10-9
1.98	0.0148	0.0128	0.0119	0.006	0.14	0.10571	0.09143	0.085	0.04286
2.13	0.0158	0.0149	0.013	0.0093	0.15	0.10533	0.09933	0.08667	0.062
2.29	0.0194	0.0169	0.015	0.0151	0.16	0.12125	0.10563	0.09375	0.09438
2.46	0.0185	0.0179	0.0158	0.0193	0.17	0.10882	0.10529	0.09294	0.11353
2.64	0.0214	0.019	0.0194	0.0274	0.18	0.11889	0.10556	0.10778	0.15222
2.84	0.0244	0.0209	0.0217	0.0359	0.2	0.122	0.1045	0.1085	0.1795
3.05	0.0249	0.0208	0.0251	0.0375	0.21	0.11857	0.09905	0.11952	0.17857
3.28	0.0243	0.0259	0.0302	0.0473	0.23	0.10565	0.11261	0.1313	0.20565
3.52	0.0255	0.0234	0.0308	0.0512	0.24	0.10625	0.0975	0.12833	0.21333
3.79	0.0242	0.0257	0.035	0.0536	0.27	0.08963	0.09519	0.12963	0.19852
4.07	0.0223	0.0265	0.0319	0.0592	0.28	0.07964	0.09464	0.11393	0.21143
4.37	0.0262	0.0268	0.0374	0.0653	0.3	0.08733	0.08933	0.12467	0.21767
4.7	0.0284	0.0299	0.0325	0.0723	0.33	0.08606	0.09061	0.09848	0.21909
5.05	0.0299	0.0281	0.0238	0.094	0.35	0.08543	0.08029	0.068	0.26857
5.42	0.027	0.0216	0.0288	0.0837	0.37	0.07297	0.05838	0.07784	0.22622
5.83	0.0321	0.0334	0.0328	0.081	0.41	0.07829	0.08146	0.08	0.19756
6.26	0.0265	0.0237	0.0348	0.0628	0.43	0.06163	0.05512	0.08093	0.14605
6.73	0.0304	0.0291	0.0319	0.0666	0.47	0.06468	0.06191	0.06787	0.1417
7.23	0.0279	0.0185	0.0421	0.0556	0.5	0.0558	0.037	0.0842	0.1112
7.77	0.0264	0.0277	0.0418	0.0488	0.54	0.04889	0.0513	0.07741	0.09037
8.35	0.0387	0.0298	0.0387	0.0589	0.58	0.06672	0.05138	0.06672	0.10155
8.98	0.0513	0.0291	0.0291	0.0513	0.63	0.08143	0.04619	0.04619	0.08143
9.65	0.0484	0.0349	0.0601	0.0664	0.67	0.07224	0.05209	0.0897	0.0991
10.37	0.056	0.0387	0.056	0.0717	0.73	0.07671	0.05301	0.07671	0.09822
11.14	0.0331	0.045	0.0379	0.0739	0.77	0.04299	0.05844	0.04922	0.09597
12.86	0.0566	0.0566	0.0566	0.0492	1.72	0.03291	0.03291	0.03291	0.0286
13.82	0.0559	0.0409	0.0387	0.0538	0.96	0.05823	0.0426	0.04031	0.05604
14.86	0.0311	0.0226	0.0368	0.0439	1.04	0.0299	0.02173	0.03538	0.04221

Table B2

PARTICLE SIZE ANALYSIS: *S. cerevisiae* IN BROTH									
midsize	mass conc (mg.m-3)				channel	mass conc x channel width (mg/m3.microns)			
(microns)	broth	cells in broth			width	broth	cells in broth		
		2.4 x 10-7	2.4 x 10-8	2.1 x 10-9	(microns)		2.4 x 10-7	2.4 x 10-8	2.1 x 10-9
1.98	0.0215	0.0209	0.0179	0.088	0.14	0.15357	0.14929	0.12786	0.62857
2.13	0.0245	0.0227	0.02	0.09	0.15	0.16933	0.15133	0.13333	0.6
2.29	0.0277	0.0237	0.0216	0.095	0.16	0.18438	0.14813	0.135	0.59375
2.46	0.0306	0.0292	0.0268	0.109	0.17	0.19353	0.17176	0.15765	0.64118
2.64	0.0323	0.032	0.0314	0.115	0.18	0.20333	0.17778	0.17444	0.63889
2.84	0.0362	0.0362	0.0346	0.126	0.2	0.213	0.181	0.173	0.63
3.05	0.0417	0.0411	0.041	0.14	0.21	0.21143	0.19571	0.19524	0.66667
3.28	0.0472	0.0443	0.052	0.16	0.23	0.20783	0.19261	0.22609	0.69565
3.52	0.0475	0.0426	0.0527	0.192	0.24	0.21958	0.1775	0.21958	0.8
3.75	0.0577	0.0554	0.0597	0.253	0.27	0.25407	0.20519	0.22111	0.93704
4.07	0.058	0.0641	0.0646	0.347	0.28	0.25321	0.22893	0.23071	1.23929
4.37	0.0765	0.0671	0.0662	0.343	0.3	0.264	0.22367	0.22067	1.14333
4.7	0.083	0.0698	0.0794	0.306	0.33	0.27667	0.21152	0.24061	0.92727
5.05	0.0757	0.0809	0.0814	0.303	0.35	0.30571	0.23114	0.23257	0.86571
5.42	0.0863	0.0913	0.0831	0.321	0.37	0.26054	0.24676	0.22459	0.86757
5.83	0.0931	0.103	0.0932	0.372	0.41	0.24878	0.25122	0.22732	0.90732
6.26	0.116	0.106	0.111	0.349	0.43	0.22977	0.24651	0.25814	0.81163
6.73	0.101	0.0988	0.116	0.312	0.47	0.27872	0.21021	0.24681	0.66383
7.23	0.12	0.121	0.11	0.307	0.5	0.268	0.242	0.22	0.614
7.77	0.128	0.126	0.13	0.289	0.54	0.28519	0.23333	0.24074	0.53519
8.35	0.139	0.137	0.134	0.317	0.58	0.25345	0.23621	0.23103	0.54655
8.98	0.149	0.173	0.145	0.337	0.63	0.20952	0.2746	0.23016	0.53492
9.65	0.172	0.205	0.167	0.369	0.67	0.26567	0.30597	0.24925	0.55075
10.4	0.177	0.148	0.157	0.352	0.73	0.27945	0.20274	0.21507	0.48219
11.1	0.171	0.159	0.161	0.322	0.77	0.27922	0.20649	0.20909	0.41818
12.9	0.19	0.195	0.158	0.343	1.72	0.11453	0.11337	0.09186	0.19942
13.8	0.185	0.153	0.19	0.303	0.96	0.20729	0.15938	0.19792	0.31563
14.9	0.172	0.161	0.142	0.184	1.04	0.14904	0.15481	0.13654	0.17692

Table B3

PARTICLE SIZE ANALYSIS: *E. coli* IN RINGERS' SOLUTION									
midsize	mass conc (mg.m-3)				channel	mass conc x channel width (mg/m3.microns)			
(microns)	ringers	cells in ringers solution			width	ringers	cells in ringers solution		
		2.9 x 10-8	2.9 x 10-9	2.9 x 10-10	(microns)		2.9 x 10-8	2.9 x 10-9	2.9 x 10-10
1.0368	0.0038	0.00416	0.00436	0.00538	0.071	0.05352	0.05859	0.06141	0.07577
1.1143	0.0045	0.00516	0.00598	0.00659	0.0761	0.05913	0.06781	0.07858	0.0866
1.1972	0.0058	0.00664	0.007	0.00816	0.0829	0.06996	0.0801	0.08444	0.09843
1.2867	0.0066	0.00772	0.00813	0.00973	0.0895	0.07374	0.08626	0.09084	0.10872
1.3826	0.0075	0.0089	0.01	0.0123	0.0959	0.07821	0.09281	0.10428	0.12826
1.4855	0.0087	0.0101	0.0115	0.0145	0.1029	0.08455	0.09815	0.11176	0.14091
1.5965	0.0106	0.0122	0.0138	0.0172	0.111	0.0955	0.10991	0.12432	0.15495
1.7154	0.0113	0.0137	0.015	0.0198	0.1189	0.09504	0.11522	0.12616	0.16653
1.8433	0.0132	0.0158	0.0164	0.0223	0.1279	0.10321	0.12353	0.12823	0.17435
1.9812	0.0148	0.0153	0.0187	0.0252	0.1379	0.10732	0.11095	0.13561	0.18274
2.1291	0.0158	0.0187	0.0203	0.0316	0.1479	0.10683	0.12644	0.13725	0.21366
2.2875	0.0194	0.0209	0.0257	0.0362	0.1584	0.12247	0.13194	0.16225	0.22854
2.4579	0.0185	0.0229	0.0234	0.0385	0.1704	0.10857	0.13439	0.13732	0.22594
2.6413	0.0214	0.025	0.0257	0.0431	0.1834	0.11668	0.13631	0.14013	0.23501
2.8387	0.0244	0.0279	0.0302	0.0461	0.1974	0.12361	0.14134	0.15299	0.23354
3.0505	0.0249	0.0285	0.0305	0.0514	0.2118	0.11756	0.13456	0.144	0.24268
3.2779	0.0243	0.0211	0.0353	0.0587	0.2274	0.10686	0.09279	0.15523	0.25814
3.5227	0.0255	0.0317	0.03	0.0516	0.2448	0.10417	0.12949	0.12255	0.21078
3.7856	0.0242	0.0332	0.0332	0.0601	0.2629	0.09205	0.12628	0.12628	0.2286
4.0679	0.0223	0.0347	0.0357	0.0653	0.2823	0.07899	0.12292	0.12646	0.23131
4.3717	0.0262	0.0283	0.0368	0.0712	0.3038	0.08624	0.09315	0.12113	0.23436
4.698	0.0284	0.0284	0.0476	0.0849	0.3263	0.08704	0.08704	0.14588	0.26019
5.0482	0.0299	0.0271	0.0407	0.0991	0.3502	0.08538	0.07738	0.11622	0.28298
5.4245	0.027	0.032	0.0457	0.085	0.3763	0.07175	0.08504	0.12145	0.22588
5.8292	0.0321	0.0372	0.044	0.114	0.4047	0.07932	0.09192	0.10872	0.28169
6.2644	0.0265	0.0376	0.0451	0.0876	0.4352	0.06089	0.0864	0.10363	0.20129
6.7317	0.0304	0.0396	0.0489	0.104	0.4673	0.06505	0.08474	0.10464	0.22256
7.2338	0.0279	0.0284	0.0487	0.111	0.5021	0.05557	0.05656	0.09699	0.22107
7.7735	0.0264	0.0488	0.0624	0.12	0.5397	0.04892	0.09042	0.11562	0.22235
8.3536	0.0387	0.0477	0.0444	0.108	0.5801	0.06671	0.08223	0.07654	0.18617
8.9772	0.0513	0.0684	0.0513	0.13	0.6236	0.08226	0.10969	0.08226	0.20847
9.6468	0.0484	0.0697	0.0581	0.134	0.6696	0.07228	0.10409	0.08677	0.20012
10.366	0.056	0.0452	0.0646	0.137	0.7192	0.07786	0.06285	0.08982	0.19049
11.14	0.0331	0.0616	0.0805	0.171	0.774	0.04276	0.07959	0.10401	0.22093
12.864	0.0566	0.0689	0.0837	0.15	0.893	0.06338	0.07716	0.09373	0.16797
13.824	0.0599	0.0559	0.0774	0.174	0.96	0.0624	0.05823	0.08063	0.18125
14.855	0.0311	0.0354	0.0481	0.099	1.031	0.03016	0.03434	0.04665	0.09602

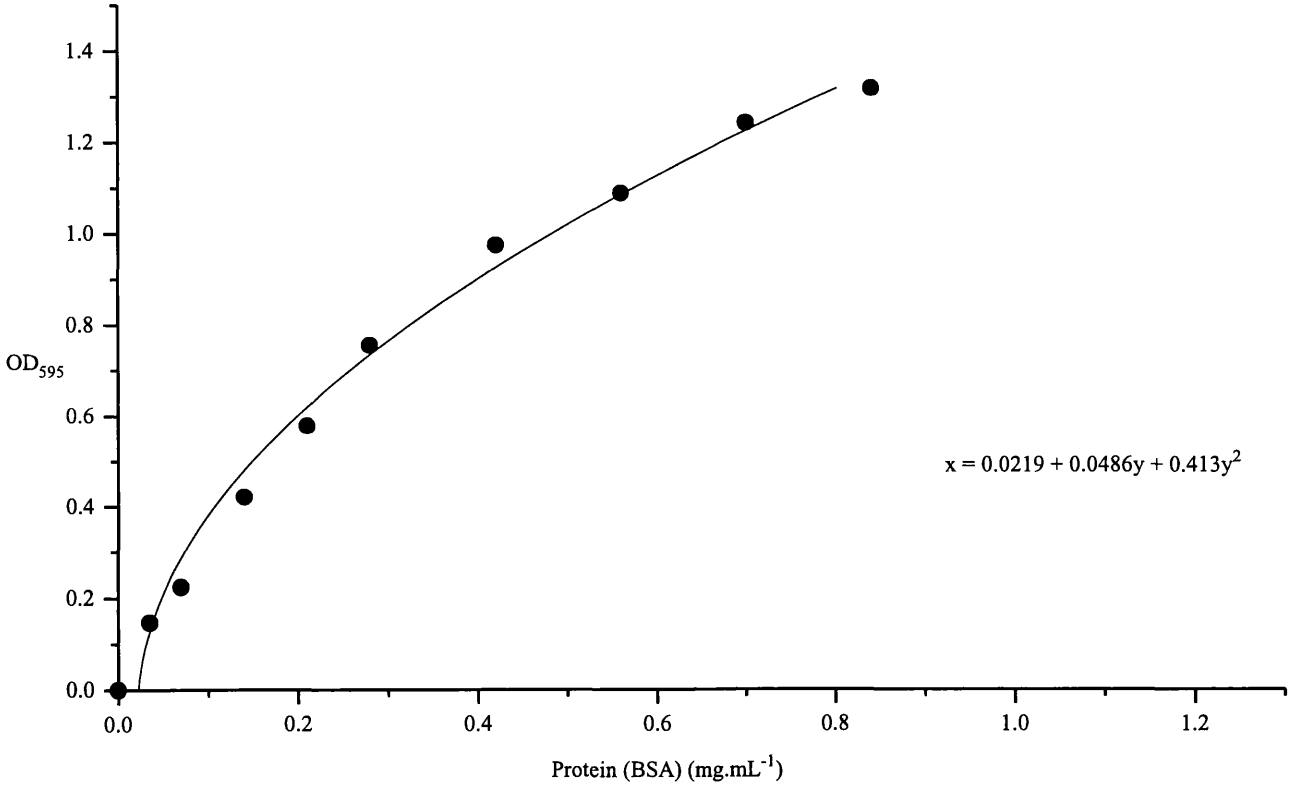
Table B4

PARTICLE SIZE ANALYSIS: *E. coli* IN BROTH									
midsize	mass conc (mg.m-3)				channel	mass conc x channel width (mg/m3.microns)			
(microns)	broth	cells in broth			width	broth	cells in broth		
		3.8 x 10-8	3.8 x 10-9	3.8 x 10-10	(microns)		3.8 x 10-8	3.8 x 10-9	3.8 x 10-10
1.037	0.008	0.009	0.009	0.01	0.071	0.1083	0.1285	0.1239	0.1401
1.114	0.009	0.011	0.01	0.0122	0.0761	0.1192	0.1472	0.1367	0.1603
1.197	0.011	0.014	0.013	0.0136	0.0829	0.1315	0.1578	0.1478	0.1511
1.287	0.013	0.015	0.015	0.0159	0.0895	0.1397	0.1667	0.1644	0.1767
1.383	0.016	0.02	0.018	0.0209	0.0959	0.1627	0.2256	0.2022	0.2322
1.486	0.018	0.023	0.022	0.0245	0.1029	0.1759	0.2046	0.2	0.2227
1.597	0.022	0.028	0.027	0.0285	0.111	0.1991	0.2536	0.2464	0.2591
1.715	0.025	0.033	0.03	0.032	0.1189	0.2111	0.325	0.304	0.32
1.843	0.029	0.036	0.035	0.037	0.1279	0.2252	0.2593	0.25	0.2643
1.981	0.033	0.043	0.04	0.0414	0.1379	0.2393	0.3064	0.285	0.2957
2.129	0.036	0.048	0.045	0.0483	0.1479	0.2427	0.318	0.3007	0.322
2.288	0.04	0.055	0.05	0.0581	0.1584	0.2494	0.4246	0.3839	0.4469
2.458	0.043	0.063	0.055	0.0633	0.1704	0.2524	0.3718	0.3229	0.3724
2.641	0.051	0.061	0.062	0.0714	0.1834	0.2786	0.3367	0.345	0.3967
2.839	0.054	0.069	0.065	0.0796	0.1974	0.2731	0.3445	0.324	0.398
3.051	0.053	0.073	0.071	0.086	0.2118	0.2484	0.3457	0.3367	0.4095
3.278	0.062	0.08	0.079	0.0903	0.2274	0.2709	0.3483	0.3435	0.3926
3.523	0.061	0.079	0.072	0.0931	0.2448	0.2496	0.3308	0.3	0.3879
3.786	0.067	0.081	0.083	0.111	0.2629	0.2545	0.2989	0.3067	0.4111
4.068	0.068	0.087	0.083	0.115	0.2823	0.2419	0.3104	0.2961	0.4107
4.372	0.08	0.1	0.091	0.13	0.3038	0.2627	0.3333	0.3023	0.4333
4.698	0.079	0.108	0.103	0.138	0.3263	0.2409	0.3273	0.3121	0.4182
5.048	0.099	0.114	0.106	0.164	0.3502	0.2816	0.3257	0.3029	0.4686
5.425	0.105	0.115	0.118	0.152	0.3763	0.279	0.3108	0.3189	0.4108
5.829	0.113	0.117	0.113	0.16	0.4047	0.2792	0.2854	0.2756	0.3902
6.264	0.106	0.121	0.136	0.157	0.4352	0.2436	0.2814	0.3163	0.3651
6.732	0.129	0.133	0.133	0.195	0.4673	0.2761	0.2891	0.2891	0.4239
7.234	0.145	0.137	0.124	0.16	0.5021	0.2888	0.274	0.248	0.32
7.774	0.157	0.143	0.15	0.207	0.5397	0.2909	0.2648	0.2778	0.3833
8.354	0.172	0.162	0.158	0.216	0.5801	0.2965	0.2793	0.2724	0.3724
8.977	0.168	0.18	0.2	0.231	0.6236	0.2694	0.2857	0.3175	0.3667
9.647	0.198	0.216	0.196	0.212	0.6696	0.2957	0.3224	0.2925	0.3164
10.37	0.179	0.207	0.245	0.253	0.7192	0.2489	0.2875	0.3403	0.3514
11.14	0.241	0.256	0.199	0.339	0.774	0.3114	0.3325	0.2584	0.4403
12.86	0.188	0.236	0.231	0.257	0.893	0.2105	0.1341	0.1313	0.146
13.82	0.199	0.187	0.2	0.232	0.96	0.2073	0.2078	0.2222	0.2578
14.86	0.191	0.126	0.105	0.171	1.031	0.1853	0.1146	0.0955	0.1555

Appendix C
Protein (BSA) Calibration Curve

See section 2.3.4.4 for method and section 4.3.2.3, table 4.8 for results.

Figure C1: Protein Calibration Curve



Appendix D
NaCl Conductivity Calibration Curve

See section 3.4.1.1, table 3.5 and section 3.4.1.2, table 3.7 for results.

Calculation:

$$\text{NaCl recovered (g)} = \frac{\text{molarity} \times 58.44 \times \text{volume collected}}{1000}$$

Figure D1: NaCl Conductivity Calibration Curve