

Practical Fermentation Technology

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Strathclyde Fermentation Centre, Strathclyde University, UK



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For David & Louise

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Preface

Fermentation is a very ancient practice indeed, dating back several millennia. More recently, fermentation processes have been developed for the manufacture of a vast range of materials from chemically simple feedstocks, such as ethanol, right up to highly complex protein structures. The advent of this latter range of products and processes has revolutionised the practice of clinical medicine and many areas of fundamental research, and has also significantly increased the need for skilled individuals in the fermentation area.

The key question is ‘How can we deliver these skills to those who need them?’ In essence, this question hints at the potential difficulties: fermentation itself is an applied science, an underpinning technology. Many of the new entrants have no track record in the area, and being an applied science, in this context at least, publicly supported training in this area has been subject to the usual neglect in funding terms in many countries.

Typically, the acquisition of a set of practical skills might involve the skills being passed on or down from an experienced practitioner to a relative newcomer via demonstration, explanation and repetition. However, given the expansion in the use of fermentation techniques, this bespoke one to one approach is not always possible, especially for the many new scientists, engineers and technicians entering the fermentation area, in labs which have no previous experience of this area to draw upon.

This book is aimed at helping these relative newcomers to fermentation. It is not intended as a substitute for the type of training described above, but it may help the newcomer avoid some of the more obvious mistakes and pitfalls we have all made, and especially, to prevent them from ‘re-inventing the wheel’.

The contributors to this book are academic and industrial scientists and engineers with many years practical experience of actually carrying out fermentation processes of different types, at a range of scales from bench-top, right up to the largest industrial production scales. This book is intended to help the beginner or less experienced fermentation scientist, by bringing together and setting down our practical experiences in fermentation technology. It is not intended to cover fundamental or theoretical aspects underpinning fermentation, which are, in any respect, already well covered by a range of accessible books and reviews. Instead, we have focused on the practical skills and associated problems, cross referencing to appropriate reading material dealing with the underpinning science or engineering where relevant.

This book proceeds from a brief background on the development of fermentation, through the criteria for selection of lab based fermenters, with a chapter on the more specialised needs and challenges of equipping a lab for membrane protein expression (a very common *raison d’être* for the setting up of small fermentation labs or suites

nowadays) before moving on to practical aspects of cultivation modes, medium preparation and sterilisation, and culture preservation and inoculum work up techniques. The modelling of fermentation processes is then discussed, followed by a discussion of practical aspects of scaling up or scaling down fermentations. The typical sensors used to monitor fermentations are then described and associated practical challenges discussed. Finally, rather more specialised, and less frequently discussed areas relating to fermentation are described, including the selection and use of Supervisory Control and Data Acquisition systems (SCADA). The penultimate chapter focuses on a brief discussion of the variability inherent in fermentation processes, its consequences and how this can be described and quantified. The final chapter deals with a description of the various continuous culture systems (e.g. chemostats), and of how powerful these technologies can be in helping us understand better the physiology of microbes, or cultured cells (animal or plant) via definition and control of their environment in the fermenter.

This book essentially moves from the pre-fermentation stages (equipment selection, lab set up, culture techniques, medium and inoculum preparation) to discussion of how we describe, scale up, and monitor fermentations. Finally ending with some rather neglected areas post fermentation relating to what we do with data arising from the fermentation, how we assess acceptable degrees of variability in our process, and finally how we can use fermentations as powerful tools in microbiological research.

This work is not intended to be a handbook, but we hope that by reading how we deal with some of the challenging aspects of the practice of fermentation, the learning curve of the newcomer might be accelerated, and their path to competence smoothed a little.

Brian McNeil

1

Fermentation: An Art from the Past, a Skill for the Future

Brian McNeil and Linda M. Harvey

The origins of fermentation are lost in ancient history, perhaps even in prehistory. We know that the ancient Egyptians and Sumerians both had knowledge of the techniques used to convert starchy grains into alcohol. For most of history these processes, or similar ones based on fruit juice conversion, have represented the most commonly accepted interpretation of the word ‘fermentation’.

However, ‘fermentation’ has many different and distinct meanings for differing groups of individuals. In the present context we intend it to mean the use of submerged liquid culture of selected strains of microorganisms, plant or animal cells, for the manufacture of some useful product or products, or to gain insights into the physiology of these cell types. This is a relatively narrow definition, but would include the ‘traditional’ fermentations described above. By contrast, the modern fermentation industry, which is largely a product of the Twentieth century, is dominated by aerobic cultivations intended to make a range of higher value products than simple ethanol.

In recent years there has been a tremendous expansion in the use of fermentation technology by individuals with less training in the subject than previous exponents of these techniques. This book is aimed at scientists and engineers relatively new to the subject of fermentation technology, and is intended to be the text equivalent of the briefings and chats that mentors in this area would have with newcomers. It is meant to be a means of passing on the experiences we have had in many years of fermentation to relative newcomers to the subject, so that perhaps, you will be able to avoid some of the more obvious pitfalls we fell into. It is specifically not intended as a reference text to the principles underlying fermentation science and engineering, as such volumes already exist. Each

chapter in this book is accompanied by a short ‘further reading section’ or supporting reference section, which generally contain a list of a few book chapters, or relevant reviews supporting the material in the chapter.

The fermentation industry today is very much in a state of flux, with rapid changes in location, product spectrum, and scale of processes occurring. To a large extent this has been brought about by macroeconomic forces compelling the relocation of large scale bioprocesses outside high labour cost regions, but also by the successful deciphering of the human genome with its myriad of new therapeutic targets, and the significant advances in the construction of advanced fermentation expression systems for making novel proteins and antibodies.

Thus, fermentation skills and knowledge are now essential to driving forward systematic research into drug/receptor interactions, function of membrane proteins in health and disease, and are powering an unparalleled expansion in our capability to combat serious diseases in the human population, including cancers, degenerative illnesses such as Alzheimer’s, and increasingly common complaints of developed societies such as asthma. The new fermentation-derived medicines, including biopharmaceuticals, hold out the prospect of improved specificity of treatment, and decreased side effects. It is truly a revolutionary period in clinical medicine as these new agents manufactured by fermentation routes enter the market. The ‘new’ fermentation products, therapeutic proteins, antibodies (simple and conjugated) are more complex and costly than previous products, but, in essence, the need to focus upon the fermentation step is now clearer than ever. Basically, the ‘quality’ of these products (the potency, efficacy, stability and immunogenicity) is determined by the upstream or fermentation stage, so the need for a clear understanding of what happens in that stage, how it can best be monitored, controlled, and carried out in a reproducible fashion, is greater than ever. It is in exactly these areas that the many often highly capable individuals entering fermentation are unwittingly deficient in background.

The long heralded era of personalized medicine may well be imminent due to recent advances in cultivation and replication of stem cells. This will make the need for scientists and engineers who understand culture techniques even greater in coming years. Thus, the demand for fermentation skills is likely to increase in the immediate future. Fermentation has contributed much to the well-being and wealth of human populations over millennia; it will continue to do so to an even greater extent in the future. We hope this book will help those coming recently to this field to contribute more effectively to that process.

2

Fermentation Equipment Selection: Laboratory Scale Bioreactor Design Considerations

Guy Matthews

2.1 Introduction

This chapter will cover the design considerations of small-scale bioreactors, and illustrate the kind of logic involved in selecting fermentation equipment for a small scale 'general purpose' laboratory where the fermentation equipment will typically be usable for several fermentation processes. Equipping a research scale laboratory for, specialist purposes, for example, for expression of recombinant proteins, particularly, membrane proteins, is covered in detail in Chapter 3.

Bioreactors in some form or another have been around for thousands of years, although up until the 1900s their use was limited to the production of potable alcohol. It was really from the 1940s onwards that fermentation as it is known today began to appear with the need to produce antibiotics during World War Two. At this point the need for process development to improve yields drove research. As it would not be practical to carry out this research on production scale equipment, small scale bioreactors became common place. Small scale for the purposes of this chapter is defined as having a total volume of between 1 litre and 20 litres.

Bioreactors at this volume can be used for a number of purposes, scale up/down studies, clone selection, medium development, process development and, in some cases, production.

The bioreactor should be capable of the following as a minimum:

- aseptic production for extended periods of time;
- meeting the local containment regulations;
- monitoring and controlling the following parameters:
 pH by either acid/base addition or CO₂/base addition;
 mixing such that the culture remains in suspension and DO₂ is maintained; all this
 should be achieved without damage to the organisms;
 temperature regulation;
 sterile sampling capability.

Thought should also be given to the future in terms of spare head plate ports, so that the configuration of the system is not fixed.

Scale up and scale down should also be considered such that the aspect ratio of the vessels being used is consistent with any larger systems, which the small scale system is designed to mimic. This is discussed in much greater detail in Chapter 8.

Other issues that should be considered are the available utilities, the turn around time of the vessel from finishing one run to starting another, and the manual handling issues especially at the 10-L plus scale where the weight of the system may require two people for safe manual handling.

2.2 Types of Bioreactor

By far the most common type of bioreactor in use today, and the focus of this chapter, is the STR (stirred tank reactor). This essentially consists of a vessel with an aspect ratio of around 3 : 1, and a mixing system typically driven through the headplate, although with some steam in-place systems the mixing will be driven through the base. The head plate will have ports that allow for the addition of probes, reagents and gas as well as the removal of samples.

Numerous other types of bioreactor are in existence, the main alternatives to the STR are discussed below.

Tower fermenters, as the name suggests, are vessels characterised by a high height-to-diameter ratio, anywhere from 6 : 1 to 15 : 1. They are aerated by gas sparging via a simple sparger usually located near the fermenter base. These systems can be operated continuously by the creation of settling zones by using baffles, which allow the product to be taken off and the cells returned to the main body of the vessel.

In *airlift fermenters* the mixing system (Motor, driveshaft and impellers) is replaced by a constant flow of gas introduced into a riser tube. Within the vessel flows develop that, as the air rises and then the medium containing cells falls, ensure thorough mixing. The airlift vessel may be baffled to improve mixing. These vessels provide very gentle mixing, and so are particularly suited to cells that are too shear sensitive to be mixed by an impeller.

Hollow fibre chambers are used to grow anchorage-dependent cells. The system consists of a bundle of fibres and the cells grow within the extra capillary spaces (ECS) with in a cartridge. Medium and gas perfuse through the capillary lumen to the ECS, where they are available to the cells. The size of the lumen can be selected such that any product is retained in the ECS or passes through the lumen such that the system acts as a *perfu-*

sion bioreactor. An alternative to hollow fibres would be to use microcarriers in a stirred tank reactor. Microcarriers are typically chromatographic grade DEAE sephadex beads. The beads are positively charged, and so are attracted to negatively charged animal cells and provide a surface on which the cells can grow.

Small scale stirred tank bioreactors may be divided in to three basic groups.

2.2.1 Autoclavable Systems

These represent perhaps the most commonly utilised systems, perhaps due to their relative simplicity, cost and basic utility requirements. Full functionality of such glass systems may be via an actuation panel, which houses all the devices for the control of liquid or gas flow, namely pumps, solenoid valves, and rotameters or mass flow controllers. All of this is controlled by a local controller potentially connected to a SCADA (Supervisory Control and Data Acquisition package), a specific software package that will log all data generated by the system and control the system. The use of a SCADA allows a greater level of functionality than that delivered by just the local controller, and is discussed in more detail in Chapter 10.

Figure 2.1(a) represents a typical small-scale autoclavable system. The vessel is made from glass, with a stainless steel head plate, and is serviced by an actuation panel with a local controller that is connected to a SCADA (Supervisory Control and Data Acquisition package).

2.2.2 Stainless Steel Steam in Place Systems

Figure 2.1(b) shows a vessel of approximately the same volume as that in Figure 2.1(a), but in stainless steel. The key difference between this and the glass system is that the steel vessel has a steam supply piped to it so sterilisation will occur *in-situ*, thus removing the need for a large on site autoclave. Its main advantage over the glass system is the ability to apply over pressure. The application of over pressure (running the system at a higher than atmospheric pressure) is used to enhance the oxygen transfer rate. For obvious safety reasons it would not be acceptable to apply an overpressure to a glass vessel. The removal of manual handling considerations, as the stainless steel system is sterilised *in-situ* rather than in an autoclave, would be another reason to move to a stainless steel system at these small volumes. Some operators prefer an autoclavable system to SIP bioreactors as they feel that the former suffer less contamination than the latter. The evidence for this largely anecdotal and subjective.

The steam supplied to such a system needs to be particle and oil free as either of these can affect the product being made by leaving a residue in the vessel or damage the system by blocking valves. The steam also needs to be dry, as water will condense which could potentially lead to a cold spot that does not achieve the sterilisation temperature, usually 121 degrees centigrade.

2.2.3 Single Use Bioreactors

Figure 2.1(c) shows a single use system where the vessel has been replaced by a bag. This bag is multi-layered and meets all the criteria for a product in contact with a biological process, namely that it should be nonshedding, leaching, or chelating. The bag construction is discussed in the next section covering materials of construction. The bag is

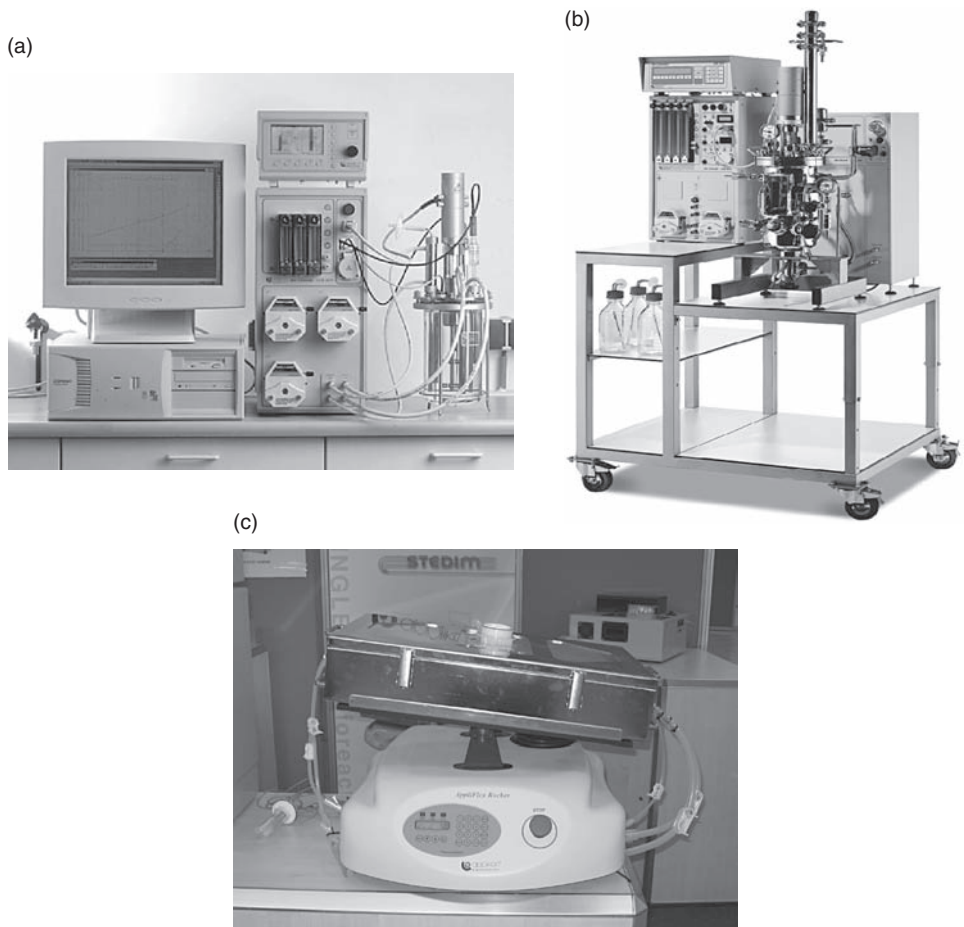


Figure 2.1 (a) Typical small scale bench mounted glass autoclavable bioreactor. (b) Small scale steam in place bioreactor. (c) Bench top single use bioreactor

supplied presterilised, so does not require an autoclave or steam supply. The benefit being that the turn around time (time between runs) is minimised. At the moment these systems tend to be limited to mammalian and insect cell culture.

Before any of the above systems can be considered there are a number of fundamental points to decide upon and these are discussed below.

2.3 Construction Aspects

2.3.1 Materials of Construction

The materials from which a bioreactor is constructed have been widely discussed, for example, by Cowan and Thomas (1988). The material of construction is selected on the

basis that it displays the following physical properties: it must be chemically inert such that it does not leach elements into the medium or chelate elements (principally metal ions) from the medium. So long as the materials of construction meet these requirements then the choice of material for the bioreactor is dictated by the scale at which the process is to be operated, the process itself and economic considerations. This requirement for the material of construction to be chemically inert, as well as being common sense, is also a regulatory requirement, as laid down by the FDA (Food and Drug Administration) and MHRA (Medicine and Healthcare Products Regulatory Agency) where the equipment is being used for the manufacture of a medicinal or diagnostic product.

Larger scale bioreactors, 20 litres and above, have their material of construction dictated primarily by the pressure codes and manual handling considerations. Bioreactors at the 20-L plus scale will almost exclusively be made from stainless steel and primarily 316L grade stainless steel or equivalent. Exceptions to this are systems used in corrosive environments, when such materials such as Hasteloy will be used, or in the single use technologies where a plastic will be the material of construction.

At the smaller scale the systems will be made from borosilicate glass, with a head plate made from 316-L stainless steel and O-rings from PTFE. However systems can be and are made of 316-L stainless steel at this scale. Generally the only reasons for choosing stainless steel at this scale are the lack of a large enough autoclave, the need to apply an overpressure or a culture that is prone to adhere strongly to borosilicate glass. The production of 316L stainless steel is very closely regulated and can be defined as a chromium-containing steel alloy. 316L contains other elements all of which contribute to its properties (Table 2.1).

The resulting metal is then electropolished to give an ultrasmooth finish. These finishes are described as having an *ra* (roughness average) value. This means that when something is quoted as have a 0.4*ra* finish, on average no point will be higher than 0.4 μm above the surface of the metal. It is possible by using mechanical polishing instead of electropolishing (which dissolves peaks on the metal surface) to achieve a higher *ra* finish. However mechanical polishing will potentially lead to areas where contaminants can gather as the surface of the metal is folded over rather than dissolved away. The electropolishing process leads to an open structure on the surface, which can be easily cleaned.

The benefits of this are improved cleanability as there are no surfaces on which debris can settle. It also enhances corrosion resistance.

Borosilicate glass is chemically inert, easy to clean and robust. The glass is made up of SiO_2 81%, B_2O_3 13%, $\text{Na}_2\text{O} + \text{K}_2\text{O}$ 4% and Al_2O_3 2%. The glass itself will have to meet criteria for the thickness of the glass, the number of air bubbles within the glass,

Table 2.1 *The make up and function of the components of 316L stainless steel*

Component	Content (%)	Function
Steel	65	
Chromium	16–18	Corrosion resistance
Nickel	12–14	Improves formability and ductility
Molybdenum	2–3	Improves chlorine resistance
Carbon	0.03 maximum	Reduces sensitisation, which is carbon precipitation during welding.

and how many of these bubbles are on the surface. The glass thickness and number of air bubbles determine the strength of the glass, while a bubble that has formed and burst on the surface of a vessel will leave an area that is difficult to clean and be a potential source of contamination.

Until recently the two main materials of construction were glass and steel. Approximately 4 years ago a single use technology was introduced by Stedim (www.stedim.com) that added a third material, plastic, in the form of bags. To describe these as plastic bags would be to misinterpret them to the same degree as describing a stainless steel bioreactor as being made of a steel drum. The single use fermenter is a 300- μm thick six-layered gas impermeable bag that meets all the validation criteria for materials compatibility as discussed above. This has made major inroads into the traditional STR (stirred tank reactor) market, because (a) the turn around time on single use systems is minimal B) the utility requirements are greatly reduced C) the validation route is very simple for such systems thus, they are used in validated production environments. The only drawback with these systems is the lack of an impellor and baffles to achieve higher OTR's, and, as such, there use has so far been limited to mammalian and insect cell systems with relatively modest oxygen demands.

Any type of system, be it glass, steel or plastic, should be validatable, that is, any manufacturer's claims in relation to pH, temperature control, mixing and O_2 transfer capability must be supported by a documented validation protocol. In such a protocol, all the claimed performance criteria are assessed against independent measuring devices, which themselves are calibrated against recognised international standards. All this work is documented and the records stored. Only when a system is fully validated can it be used in the production of any product that will be used either as a medicine or as part of a clinical trial.

2.3.2 Location Considerations

The system should be placed in an environment that is suited to its use. By this it is meant that the room chosen as the fermenter's location should have suitable containment facilities such that the operation of the system poses no threat to the health of those working with it or those working in the same building.

2.3.3 Utilities

The location should have suitably specified utilities, typically electrical power, chilled water, air, oxygen, nitrogen or carbon dioxide, and possibly, in the case of small scale steam in place (SIP) systems, steam at the correct volume, pressure and temperature.

The power requirements of a low volume system are typically based around 13-amp power, although in some cases 3-phase power may be required. The gas requirements are detailed later on, but typically air is supplied via a compressor with the other gases supplied from cylinders. All gases should be supplied as particulate, oil and moisture free. They are routinely required to be supplied at 1–3 bar as process gases. If pneumatic actuation is used on valves within the system then the air supply will need to be at 6 bar, but the use of pneumatic valves at this volume is unusual.

The water supplied to the fermenter, although never in product contact, should be of a quality that meets the requirements of the process in terms of temperature, particulates, hardness, pH and organic carbon content. If an integrated heating element is used the

water source should be checked for hardness (dissolved mineral content) as hard water (defined as greater than 120mg/L) will cause the system to fur up over time.

2.3.4 Autoclave Size

An important consideration and one that can be easily overlooked when specifying a system, is the size of the available autoclave. Consideration should also be given to the type of autoclave, and the manual handling implications of a front- or top-loading system, especially when dealing with the top end of the volumes under discussion here. A way round this is the use of a pulley or lift system to load the vessel into the autoclave. The restrictions imposed by the need to use an autoclave are one reason to use a steam in place system. However, typically on a cost basis the investment required for a 20-L glass system and a suitably sized autoclave, will be less than that required for 20-L steam in place system.

2.3.5 Risk and Containment

Advice should be sought on the local containment requirements given the classification of the organism with which you are working. East *et al.* (1984) give useful information on this.

The European Federation of Biotechnology classifies microorganisms into five groups based on the perception of risk to the human population.

European Federation of Biotechnology – Risk Classes of Microorganisms

Harmless microorganisms (EFB Class 1). Microorganisms that have never been identified as causative agents of disease in man, and that offer no threat to the environment.

Low-risk microorganisms (EFB Class 2). Microorganisms that may cause disease in man and might, therefore, offer a hazard to laboratory workers. They are unlikely to spread in the environment. Prophylactics are available and treatment is effective.

Medium-risk microorganisms (EFB Class 3). Microorganisms that offer a severe threat to the health of laboratory workers but a comparatively small risk to the population at large. Prophylactics are available and treatment is effective.

High-risk microorganisms (EFB Class 4). Microorganisms that cause severe illness in man, and offer a serious hazard to laboratory workers and people at large. In general effective prophylactics are not available and no effective treatment is known.

Environmental-risk microorganisms. Microorganisms that offer a more severe threat to the environment than to man. They may be responsible for heavy economic losses. This group includes several classes, Ep 1, Ep 2, Ep 3, to accommodate plant pathogens.

In assessing the risk involved in cultivation of a chosen strain in a fermenter there may be a requirement to justify the volume at which you intend working if the organism is in a sufficiently high category. It is usually held that the cultivation of a given microorganism at large volumes (this can be as low as one litre) raises the risk level. The reasons for this are fairly obvious, fermenters are vigorously aerated and stirred thus there is a potential, if leakage occurs, to cause serious environmental contamination via aerosols.

Depending upon the type of organism you are working with the room in which the bioreactor will be located will require certain features to ensure containment. Along with this all personnel should be familiar with good laboratory practices (Table 2.2).

2.4 Vessel Design

2.4.1 Basic Design

Nearly all vessels at the small scale regardless of whether they are glass or steel, for mammalian or bacterial use, share a basic vessel design (Figure 2.2). They will consist of a head plate that is flat, straight vessel walls and a hemispherical base. This gives good mixing characteristics without creating excessive shear forces. Where the stainless steel and glass systems start to differ is in construction of the walls of the vessel. Two options exist for the glass systems, nonjacketed and jacketed, and both relate to how temperature is controlled. The simplest and cheapest system, nonjacketed, relies on a wrap-around heater tape or equivalent to supply heat into the system; heat can be removed by having a heat exchanger sited within the vessel. The vessel wall is constructed of one single layer of glass.

The second option when using glass vessels is to have a jacketed vessel (Figure 2.3) whereby the vessel has a water-filled outer jacket. The water performs the temperature regulation by both heat input and removal. The water temperature is regulated by an external thermal circulator.

The jacketed vessel was considered the industry standard as the temperature control was regarded as more accurate and stable when compared to the nonjacketed system. The drawback of the jacketed system was the higher investment cost and the fragility of the water connections. As control technology has developed, the stability and the accuracy of the nonjacketed system has reached a level where it now matches the jacketed system. The industry norm is now the nonjacketed system; the only reason a jacket system would be required is for elevated temperatures ($+70^{\circ}\text{C}$) that cannot be achieved by direct heating, or if the process requires a rapid temperature shift, which can only be achieved by a water-jacketed vessel due to its large heat transfer area.

Flat bottomed vessels may also be bought, but characterisation of mixing is most clearly understood in hemispherical-based vessels, and they represent more closely what will be done in the larger scale, so it is the hemispherical-based system that is most widely used.

A small scale stainless steel vessel (Figure 2.4) in terms of its heat input/removal works in much the same way as the jacketed glass system, with an externally regulated water supply to control heat input/removal. The reason why wrap-around jackets have not been developed for these systems is that they are sterilised *in situ* and for sterilisation steam is supplied to the jacket.

2.4.2 Aspect Ratio

Convention states that the aspect ratio of a vessel (the ratio between its height and its diameter) should be 1 to 1 at the working volume for cell culture, and 2.2 to 1 at the working volume for microbial systems. The reasoning behind this is simply that the aspect

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