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High Cell Density Growth of Micro-organisms

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What is high cell density growth? The scope of this chapter

It is difficult to define exactly what is meant by high cell density growth because it is a relative term. Thus when working with protozoa a cell density of 2.2×10^7 and 54 g dry weight l^{-1} has been considered as growth at high cell density, whereas with bacteria and fungi this would be only a moderate growth density (Chang and Furusaki, 1991; Kiy and Tiedtke, 1992). The highest biomass levels ever recorded in a bioreactor were those described by Chung, Chang and Kim (1987). In these experiments a *Nocardia* strain was grown in a dual hollow-fibre bioreactor, reaching densities of 550 g dry weight l^{-1}. No record of the cell number was given, but 10^{12} *Escherichia coli* cells ml^{-1} of accessible reactor space has been reported, again for a hollow-fibre based system (Inloes *et al.*, 1983). The common factor in most of these reports is the comparison of a system with batch culture in a shake flask, or a stirred tank reactor. Therefore I will use this approach when discussing the alternatives available for increasing the culture cell density of micro-organisms. As far as possible, all types of micro-organisms will be covered, although inevitably the information available relates mainly to a relatively narrow range of bacteria and fungi.

It is rare that figures are given for both the dry weight and cell numbers present in a particular reactor, yet the two are not always proportional. Therefore the culture conditions are sometimes given in more detail than may seem necessary to allow readers to get as good an impression as possible about the physiological state of the microbes in the systems described. The demand for high cell density growth is mainly to improve the volumetric productivity of a process, although there is growing evidence that it can also be a strategy for stabilizing recombinant organisms, slowing down the loss of plasmid-encoded genes (Huang, Perelti and Bryers, 1993). Interest in high cell density culture is also growing because of a need to obtain increased biomass levels of microbes that are reputed to be difficult to cultivate, for biochemical

Abbreviations: ATP, adenosine triphosphate; GRAS, generally regarded as safe.

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as well as commercial studies. A good example of this is the protozoan *Tetrahymena thermophila* (Kiy and Tiedtke, 1992). It is therefore likely that interest in developing such systems will continue for some time.

Factors that limit culture cell density

Microbial cells are complex entities. *Table 1* shows the elemental composition of a dried mass of *Escherichia coli*, which has to be supplied to the cell from the culture medium. This sort of balance is seen in most types of microbial cell. Neidhardt, Ingraham and Schaechter (1990) illustrate nicely the task required of a micro-organism during growth where, again using *Escherichia coli* as an example and referring only to macromolecules, over 25 million molecules representing more than 1550 different types need to be replicated in order to construct a new cell. Even under conditions allowing for slow growth there is a significant need for cell structure repair and turnover of molecules.

Table 1. The approximate elemental composition of dried *Escherichia coli* biomass

Carbon	50–70%
Nitrogen	15–20%
Oxygen	10–20%
Hydrogen	6–8%
Phosphorus	2–4%
Sulphur	1–3%
Potassium	1–2%
Iron	0.1–0.3%
Calcium	0.01–0.05%
Magnesium	0.01–0.05%
Chlorine	0.02–0.03%
Trace elements	0.2–0.5%

Growth conditions, both nutritional and physical, will affect the chemical composition of the cells and the metabolic needs of the bacterium. Similar elemental compositions are found in other micro-organisms.

The driving force for molecular synthesis has to be provided from the components present in the growth media. When concentrating on adenosine triphosphate (ATP) synthesis, one of the most important molecules used by living cells in this context, it is apparent that different micro-organisms have developed alternative strategies for providing enough synthetic capacity. The most efficient way of making ATP is via oxidative phosphorylation, using oxygen as the terminal electron acceptor. If electron transport based synthesis is not possible, recourse is made to substrate-level phosphorylation. It is no accident that many biotechnological processes have developed around microbial strains whose biochemistry matches the efficient synthesis of the desired product. Thus *Clostridium* has been used for the synthesis of acetone and butanol, yeast for ethanol production and *Acetobacter* for vinegar manufacture, a strictly aerobic process. Problems usually arise when the physiological characteristics of a microbe are in conflict with the intensity of the process needed for a commercially viable operation.

One of the biggest difficulties is found with the supply of oxygen to cells. Tempest, Neijssel and Zewenboon (1983) recorded the fact that at 30°C the concentration of oxygen in water/air equilibria is only 0.23 mM. This figure quickly decreases as the

temperature is increased and/or the salt concentration rises. *Alcaligenes faecalis* consumes 140–170 nmol O_2 min^{-1} (mg protein)$^{-1}$ in an aerobic denitrification process (van Niel *et al.*, 1992) and *Saccharomyces cerevisiae* during aerobic growth needs 2.5 mmol (g dry weight)$^{-1}$ h^{-1} (Verdwyn *et al.*, 1992), which are fairly typical values. It can be seen therefore that oxygen can quickly become the main growth rate or cell maintenance limiting nutrient. Transfer of oxygen into microbial growth media is a continuing problem in the development of biotechnological processes that demand a constant supply. A pragmatic solution to this problem is to select micro-organisms that can grow well under oxygen-limited conditions. *Escherichia coli*, a facultative anaerobe, has proven an excellent choice where high-density growth is required but under conditions where oxygen supply will be limited. The lactic acid bacteria, whose biochemistry allows them to grow almost equally well with or without oxygen, are another group of microbes used under these conditions (Linton *et al.*, 1987). However, in both cases significant changes can occur in the metabolic profile of these bacteria grown under oxygen sufficient or deficient conditions (Harrison and Loveliss, 1971; Tseng and Montville, 1992). Nevertheless many clever solutions have been found to supply oxygen to high cell density cultures with some success. These will be reviewed later.

The supply of other nutrients to high cell density cultures can also be a problem and requires good mixing regimes in order to avoid the development of cultures with heterogeneity of physiological states. Again, many of the systems described later have found solutions to this difficulty, but in some bioreactors it cannot be avoided. It is often difficult to ascertain from reports on high cell density systems which nutrient is limiting the growth and/or the biotransformation potential of the microbial cells. It is, however, important to know the answer to this facet of bioreactor operation because the quality of the cells performing the desired function will, in most cases, be affected radically by the growth-limiting nutrient.

Earlier studies on the effects of culture conditions on the composition and size of microbial cells showed that merely recording the biomass levels in terms of dry weight does not always give an accurate indication of the useful biomass present. Herbert (1961) showed that growth rate dramatically influenced the chemical composition of *Aerobacter aerogenes*. At a growth rate of 0.8 h^{-1} this bacterium, under the conditions used, contained nearly three times more RNA than when grown at 0.1 h^{-1}. Under nitrogen-limited conditions Herbert showed that *Escherichia coli* had nearly five times as much cellular glycogen at low growth rates, than when grown at 0.5 h^{-1}. The mean cell volume can be affected by both the growth environment and the growth rate. The review by Tempest *et al.* (1983) recorded many examples of how microbial cells perform in laboratory culture. For example, van Liere (1979) showed that the cyanobacterium *Oscillatoria agardlia* changes size from 10μm^3 at a growth rate of 0.005 h^{-1} with 5 pg dry weight/cell, to 35 μm^3 at a growth rate of 0.025 h^{-1} with 21 pg dry weight/cell. More recently, Cooper (1991) has reported that bacterial cell density, within the bounds of experimental error, stays constant under a wide range of growth conditions. Finally, the viability of cells is also influenced by the growth-limiting nutrient. Postgate and Hunter (1962) showed that the death rate of *Aerobacter aerogenes* was unaffected by the oxygen in the atmosphere over the range of 2–100%. Populations at cell densities greater than 20 g dry weight l^{-1} survived longer than lower-density cultures, and in cultures where the growth-limiting nutrient was

carbon, phosphorus or sulphur the viability declined linearly, whereas with nitrogen limitation the decline was sigmoidal.

These observations show how variable a high cell density community can be in comparison to the same cells grown under less extreme conditions. It is also likely that the gross changes in cell composition indicate significant changes in the biochemistry of the cells and so reveal the complex task of understanding the rationale behind productivity changes in high- and low-density cultures of a given micro-organism. In many systems the classical techniques of microbiology employed to study changes in the biochemistry of microbial cells under different growth conditions cannot be used. This is usually because the recovery of cells from the bioreactor imposes stresses that change the cell. Physical techniques, such as nuclear magnetic resonance and microcalorimetry, have been used on a limited basis to follow microbial metabolism in such bioreactors. However, changes in the product profile in cultures of well-characterized microbes probably still gives the best indication of the metabolic status of cultures. Examples of such studies will be given in the following sections.

Bioreactors for high cell density growth

Many cultivation methods have been employed in the quest for increased cell density. They fall into two groups: those that are a modification of the stirred tank reactor and those that are developed around a very different concept. The former type of reactor is attractive to most investigators because it employs equipment that, for the main part, is already available in a standard microbial cultivation facility and therefore this type will be dealt with first.

FED BATCH BIOREACTORS

This type of bioreactor has proved to be one of the most successful for increasing the volumetric productivity of a standard batch fermentation. In the basic system the micro-organism is added to the culture medium, usually in a stirred tank reactor when volumes greater than 500 ml are involved, and allowed to grow until one or more of the original nutrients becomes growth limiting. This is an excellent way of producing growth-associated products, but does have limitations when attempting the production of products which are partially or non-growth associated. In the fed-batch process the first stage is that which would occur in a standard batch process. This part of the fed-batch procedure is used to develop the majority of the biocatalytic potential used in the second part of the process. When the culture has reached a point where nutrient limitation begins, the growth-limiting nutrient (and often other metabolites) is fed to the culture to maximize the biomass development and/or product formation (see *Figure 1*). Penicillin synthesis is a good example of one of the most thoroughly designed fed-batch systems and shows how this type of bioreactor can be used to increase catalytic density in addition to directing metabolism to product formation (Swartz, 1985). It has also found extensive use in the processes, rapidly coming on-line, that are the result of recombinant gene technology. Most of these systems are still based on plasmid-encoded genes and fed-batch technology has proved to be reasonably useful in maintaining the population in a productive state, overcoming some of the problems related to segregational and structural instability problems associated

with many plasmids (Chang *et al.*, 1991). Many examples illustrate the use of this type of bioreactor for enhancing productivity, with enhanced cell density being a major factor, but the examples cited here have been selected to show the range of applications rather than to provide an exhaustive review.

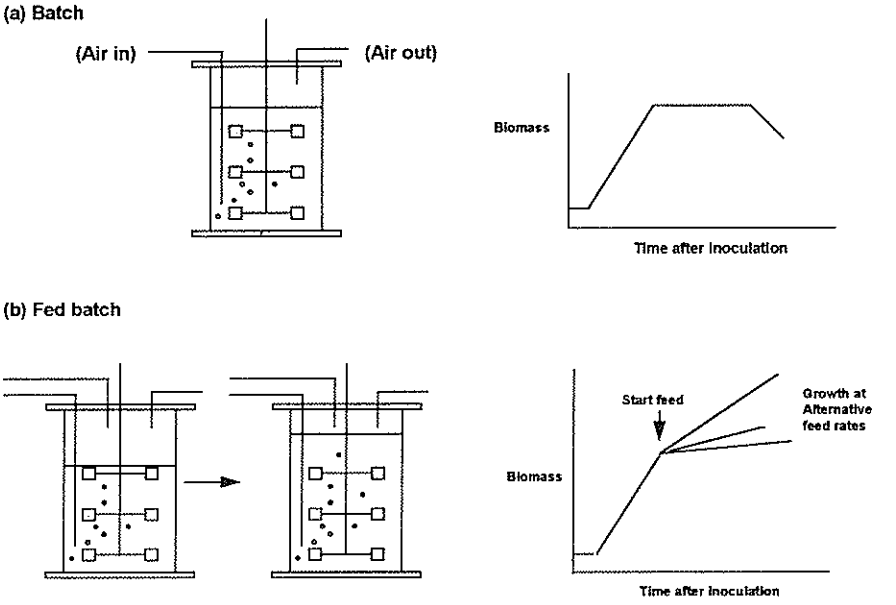

Figure 1. Reactor conformation and biomass development in batch and fed-batch culture. A stirred tank reactor is used to illustrate the stylized representation of batch and fed-batch culture. The design and biomass production profiles are usually more complex than the illustrations indicate. Air supply is an option depending on the micro-organism being grown, inert gases can sometimes be used to remove toxic gaseous end products.

The most straightforward application for fed-batch reactors is the synthesis of growth-associated natural products of microbial metabolism. Lactic acid is a major end product of the metabolism of many bacteria. As well as being produced during the manufacture of dairy products, it is an important chemical in its own right (Vickroy, 1985). Frequently the exploitation of this microbial metabolite is limited by the availability of a commercially viable manufacturing procedure. One of the biggest problems is that focused on by Ohara, Hiyama and Yoshida (1992) and involves inhibition of growth and substrate uptake by the end product, lactic acid. By using the information gained from this study the authors were able to predict suitable operating conditions for maximizing productivity. Substrate inhibition causing limited bioreactor operation is also a problem that can be partially overcome by using fed-batch systems. This can be illustrated by reference to the catabolism of the toxic molecule salicylic acid by *Pseudomonas cepacia* (Tocaj *et al.*, 1993). When grown in batch culture with salicylic acid as the sole source of carbon and energy, concentrations greater than 2 g l^{-1} are inhibitory (Kamin, White-Stevens and Presswood, 1978). At concentrations greater than 10 g l^{-1} no growth occurs. By carefully controlling the feed rate to the reactor, using a monitor that measured the salicylate concentration in the reactor, a much higher conversion rate to biomass was recorded. However, it is interesting to note that modifications had to be made to the culture medium due to the nature of fed-

batch fermentation. Previous studies on the system in batch and cell recycle fermentation used potassium nitrate as the nitrogen source for growth (Kamin, White-Stevens and Presswood, 1978; Berg, Holst and Mattiasson, 1989). In the fed-batch reactor, where the elevated biomass conentrations led to oxygen limitation, the nitrate was converted to nitrite (due to its use as an electron acceptor), which caused toxicity problems. Therefore ammonium sulphate was used as the sole source of nitrogen for growth. The use of biomass recycle can also lead to oxygen-limited growth (see later) but by enabling constant product removal it can overcome this type of problem.

Conversion of cellulosic substrates to acetic acid and ethanol by the fungus *Fusarium oxysporum* is relatively inefficient at high substrate concentrations in batch culture. Using fed batch, Kumar, Singh and Schugerl (1991) increased the levels of end products and useful hydrolytic enzymes at high substate levels. Once again, a complex feed regime was used to avoid nutrient limitations during the production phase which presumably helps to support higher biomass levels and biocatalytic activity in a more efficient state.

Nikkomycin is a nucleoside-peptide antibiotic active against various fungi and insects. It is classified as being a secondary metabolite of *Stretptomyces tendae* and, in common with many useful microbial products of this type, is difficult to produce in large quantities in batch culture. Using an optimized feeding regime, fed-batch fermentation could be used to increase the productivity of the culture from 3 to 6.7 g l^{-1}. Subsequent studies in continuous culture showed that at the optimal growth rate the higher productivity could be maintained for nearly twice as long (Schüz, Fiedler and Zahner, 1993).

An interesting variant of batch/fed-batch fermentation is the self-cycling fermenter systems described by van Walsum and Cooper (1993). In this process the metabolic activity of the culture is monitored (in this case oxygen consumption) and the cycle time matched to the doubling time of the organism. *Bacillus subtilis* and *Acinetobacter calcoaceticus* were used in this study. When a cycle has reached the end point, half of the reactor volume is removed and the volume in the reactor immediately restored with fresh growth medium. Using a holding tank for the removed medium, the culture is allowed a further period of incubation before harvesting. The products investigated were surfactants, classified as being secondary products of the organisms concerned. The additional incubation time was shown to markedly improve the yield from the fermentation.

In these first examples of the use of batch culture the enhancement of cell density in the reactors is often not stated. They were selected to illustrate that although there is often some implication of increased cell densities, increased productivity is a combination of providing the right physiological environment and increasing the biomass level. The fact that these two facets of fed-batch operation are important is often understated and yet both are crucial for maximizing the yield of recombinant products as well as the types of natural product illustrated above.

Recently work relating to recombinant DNA technology was published in the *Annals of the New York Academy of Sciences* (Prokop and Bajpai, 1991). The majority of papers were concerned with the construction of new producer strains. A much smaller number of papers was concerned with the actual production of the recombinant products. Fed-batch fermentation methods were highlighted as a means of improving productivity. *Escherichia coli* was the recombinant host in two of the papers con-

cerned. Concentrating on the high cell density that can be achieved, Knorre *et al.* (1991) stated that this has the advantages of reducing reactor volume, improving space–time yield, and media costs and simplifying downstream processing. They showed how optimizing the growth rate in the fed stage and designing the process to avoid oxygen limitation can improve the productivity of fermentations where the cell density reaches 110 g dry weight l^{-1}. Chang *et al.* (1991) alluded to the complexity of bacterial cells mentioned earlier. They showed how the nature of the selection pressure for the retention of plasmid-containing cells can dramatically influence the proportion of the population capable of synthesizing the recombinant protein, porcine somatotropin, in the production phase. Recombinant protein synthesis was induced by heat shock, the efficiency of which was affected by the cell density at the time of induction. Their studies showed how media design can reduce the catalytic development phase of the fed-batch culture. An interesting observation was why the location of the temperature-sensitive repressor on a plasmid, rather than the chromosome, determined the factors that affect expression of the recombinant protein. The growth rate at which the culture was switched to production is also important in optimizing expression. At the higher growth rates tested expression was greatest, but the final growth yield was lowest. A compromise was needed to maximize productivity. The authors showed that computer control to maintain the optimum nutrient balance in the fermentation could improve productivity further.

Yeasts have proved to be an almost equally popular host for recombinant protein synthesis as *Escherichia coli*, where their GRAS status (generally regarded as safe) is an attractive feature. Increasing numbers of species are employed in this context but reference to *Saccharomyces cerevisiae* illustrates the results obtained in fed-batch cultivation. Once again, good productivity has been obtained in cultures with cell densities in excess of 100 g dry weight l^{-1} (Alberghina *et al.*, 1991). Nurmianto, Greenfield and Lee (1994) showed that, as with the bacteria, there is often an optimum growth rate, supported by optimum nutrient feed rate, for maximal stable recombinant protein synthesis. A recurring observation that is made when reviewing the literature in this area of biotechnology, is the variety of variable factors that can determine the productivity of high cell density cultures. For example, Ibba *et al.* (1993) have shown that the culture medium can affect the plasmid content and stability in recombinant *Saccharomyces cerevisiae*, but cells with the highest copy number are not always most productive. There is clearly a need in many systems to gain a better understanding of the interaction of plasmid copy number, gene expression and protein synthesis. It was also reported that in a similar study repeated fed batch was a better way of producing recombinant protein than continuous culture (see the case of nikkomycin production above). Blondeau *et al.* (1994), from their studies on the yeast *Kluyveromyces lactis*, also remind us that it is possible to increase cell density over that seen in batch culture by using either fed batch or continuous culture, while the physiological conditions need to be optimized for optimal expression of heterologous proteins.

Fed-batch bioreactors need fairly minor modifications to conventional batch reactors. The modifications needed to allow continuous operation of such reactors are also often straightforward. For commercial production of high-value products, continuous culture has never been employed extensively. Reasons for this relate both to the working practices of industry and to the biological properties of micro-

organisms, which can often show unstable productivity in such systems. Nevertheless continuous culture, in its various operation modes, remains a useful tool for studying the behaviour of cells growing at high cell densities and may become more attractive as a production method in the future.

CONTINUOUS CULTURE SYSTEMS FOR ATTAINING HIGH CELL DENSITIES

It has already been shown that continuous culture systems of the sort shown in *Figure 2* can be designed to attain moderately high cell densities. In chemostat culture, growth is usually limited by a single nutrient. Ethanol production by *Saccharomyces cerevisiae* has been compared in batch and continuous systems (Gupta and Chand, 1994). It was shown that some of the best productivities were obtained as the system was perturbed under conditions of glucose limitation. In addition, changes in cell density that resulted from 'shift-up' or 'shift-down' did not immediately affect the productivity of the system. The rates of synthesis in the continuous processes were competitive with the best batch process reported by these authors. It is therefore not surprising that optimization of continuous culture has allowed big improvements in the yields of some microbes that grow comparatively poorly in batch culture. An extreme example of this is the growth of the hyperthermophilic *Pyrococcus furiosus*. Raven *et al.* (1992) showed that growth of the archaeum was limited by the build-up of fermentation products, especially hydrogen. Choosing the best gas to remove this metabolic end product continuously, cell concentrations in the bioreactor could be increased ten-fold to 3×10^9 cells ml^{-1}. In a modified type of continuous culture, using a perfusion device, *Tetrahymena thermophila* could be grown to cell densities 30–40 times that attainable in standard batch reactors. Total cell retention was used in this system and the study revealed that culture growth was apparently limited by both the glucose and protein content of the medium, although the production of excreted protein by the cells when grown at high cell densities gave problems in determining the exact limiting factors (Kiy and Tiedtke, 1992a,b). Many further modifications can be performed on the basic chemostat reactor. One interesting variation briefly reported by Cubarsi and Villaverde (1993) uses a two-stage system for growing and then inducing cells for product formation. However, the most interesting evolutions of the basic continuous culture design are those that incorporate artificial membranes

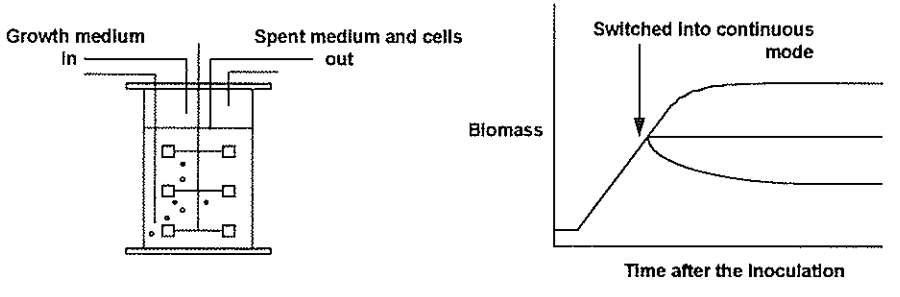

Figure 2. Continuous culture fermentation. The basic design is again illustrated by using a stirred tank reactor. In reality the steady state obtained after switching the reactor into continuous operation can often last for a very short time before mutants appear that affect both the biomass levels and/or product synthesis.

for the continuous separation of products from the microbial catalyst. At this point it is worth reviewing briefly the technology that is available for such separations.

Many techniques can be used to separate cells from culture media. Immobilization of micro-organisms has been used widely where cell retention or product removal on a continuous basis is required. There is, however, often a problem in using such a technique. Few immobilization methods can maintain long-term retention of microbial cells. The systems frequently underutilize the potential biocatalyst present (e.g. due to mass-transfer problems) and it is often difficult to monitor the physiological state of the immobilized cells. In the latter case it causes difficulties in rational process development. Flocculation of microbial cells has been used successfully, but is restricted to microbial species which either readily form natural workable aggregates, or aggregate with the limited range of chemical flocculants available. Alternative physical methods, such as centrifugation, can be too expensive for the application envisaged. From an operational point of view artificial membranes have offered great potential. The difficulty usually arises when long-term continuous operation at high cell densities is envisaged. Frequently the materials available are not robust enough, too expensive, or in a device that quickly fouls during operation. Chang and Furusaki (1991) have comprehensively reviewed the types of reactors that have been constructed incorporating artificial membranes. Heath and Belfort (1992) give details of many of the membranes available and the materials from which they can be constructed. Cross-flow filtration is the most widely applicable separation mode used in bioreactor design and I will limit this discussion to developments in this area of filtration, given their potential for improvement of high cell density growth in continuous culture.

Broadly speaking, the materials available for the construction of filtration units fall into two categories, plastic or inorganic based. The ever increasing range of plastics allows the construction of membranes in a wide variety of conformations, ranging from flat sheet structures to hollow fibres. In addition, the surface properties vary with the polymer used, and many polymer surfaces can be chemically modified to increase the range of surface properties. This facility can be extremely important in separation processes. The inorganic supports available are in some respects less varied, but are still available in a number of conformations, usually in the form of tubular structures. Ceramic membranes have been the main types used in the applications relevant to the topic of this chapter, but newer materials such as zirconium–carbon structures are also available; both types can often have their surfaces modified. The main advantage of the inorganic membranes is that they are usually much more robust than the plastic-based membranes, allowing them to be used under harsher conditions and making their sterilization more straightforward. *Figure 3* shows the basic details of a cross-flow filter unit. There are guidelines for the design of such units, which are essential for the attainment of optimal filtration fluxes. This is particularly important for systems employing tubular structures where increased flow rates through a separation device of high cell density cultures will not result in efficient media removal with small bore filters (Warren, Macdonald and Hill, 1991). It is fair to say that the complete mechanism of cross-flow filtration has not been fully worked out (Patel, Mehaia and Cheryan, 1987; Riesmier, Kroner and Kula, 1989, 1990). Tanaka *et al.* (1993) have investigated the filtration of *Saccharomyces cerevisiae* in cross-flow systems employing a flat-sheet membrane. Careful selection of flow rates at low cell

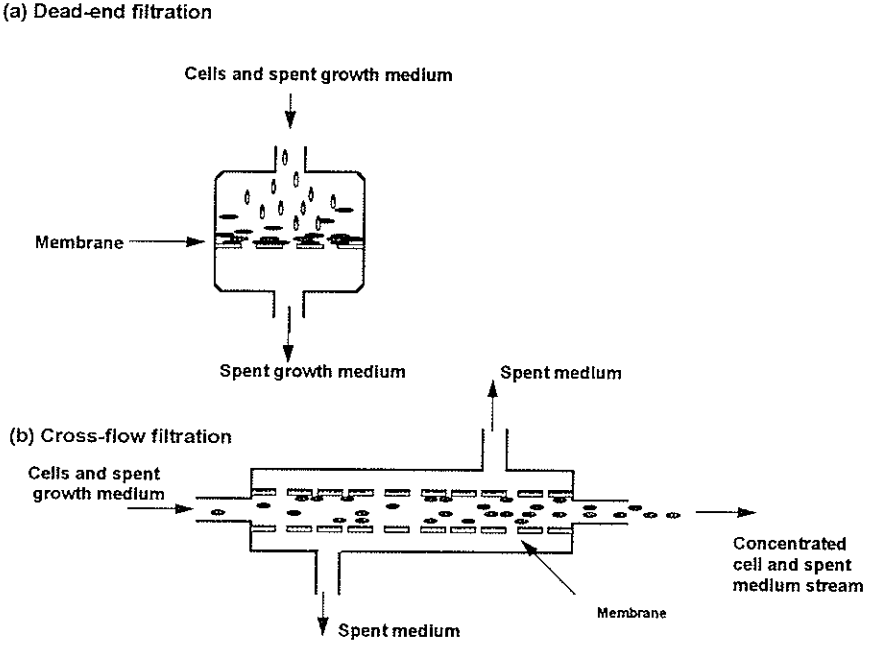

Figure 3. A comparison between dead-end and cross-flow filtration. Cross-flow filtration systems can be constructed from a wide variety of membrane types in both flat-sheet and tubular configurations.

densities allows efficient operation of the separator. As the cell density increases to that often encountered in high cell density culture systems fluxes dramatically decrease. Backflushing of such systems is possible but usually becomes unsuitable when continued long-term filtration is needed in a bioreactor system. There have been several attempts to increase the flow instability in cross-flow systems with the objective of reducing membrane fouling (Heath and Belfort, 1992). Some systems allow the use of ceramics. The rotating ceramic membrane unit described by Nakano, Matsumara and Kataoka (1993) has been shown to allow high cell densities of *Corynebacterium glutamicum* (120 g dry weight l^{-1}) and *Propionobacterium freudenreichii* (53 g dry weight l^{-1}) to be obtained in perfusion culture lasting several hundred hours. By using flat-sheet dimpled membranes and an oscillating flow, Millward *et al.* (1994) have described a cross-flow filtration device that is extremely efficient. Current studies employing this device have shown that it is capable of operation for over 100 h while operating in a cell-recycle continuous culture device with either yeast or lactic acid bacteria (Ruanglek *et al.*, unpublished observations). The membrane arrangement and surface detail are shown in *Figure 4*. Further details, including gas transfer characteristics, have also been described, illustrating the potential operational flexibility of the device (Bellhouse *et al.*, 1973; Beeton *et al.*, 1991, 1994). Thus advances in membrane technology are likely to increase the attractiveness of continuous systems using filters to achieve high cell densities. Of these systems, continuous culture with cell recycle probably offers the greatest potential when fitted with an efficient separations device.

In his classic text, Pirt (1975) outlined the mathematical properties predicted for

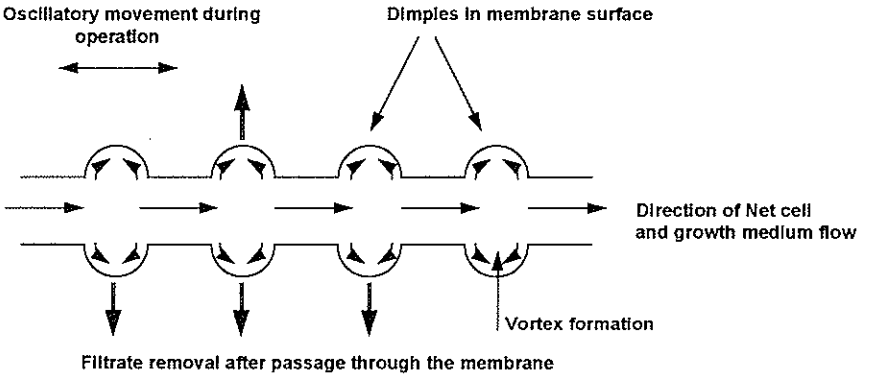

Figure 4. Detail of the twin membrane arrangement in cross-flow filtration with vortex wave formation. The polysulphone membranes are moulded to produce a regular dimpled structure over the surfaces of the two membranes used in this filtration device. During operation the bi-membrane unit oscillates in the direction shown, causing the formation of vortexes, which delay the formation of polarization layers and enhance the filtration rate. Full details can be found in Millward *et al.* (1994).

continuous culture with cell recycle and some of the advantages of using such a system. An example of the basic nature of this type of reactor is given in *Figure 5*. Although the illustration shows an external separation device, internal designs have also been used. It is not appropriate in a review of this sort to expound the potential of these systems in detail. They have great applicability to the fundamental study of microbial physiology as well as to the manufacture of microbial products. The cell-recycle ratio (R) is the proportion of biomass loss from a continuous culture that is recycled back into the main reactor. From chemostat theory it is possible to define the specific growth rate (μ) of a microbe in such a device as:

$$\mu = (1-R)D$$

where D is the dilution rate of the culture. Thus the specific growth rate can be altered by either changing the dilution rate of the system or the recycle ratio. With full recycle one has zero growth rate at steady state, a sort of perfusion culture. It was very difficult in early types of cross-flow filtration to achieve 90–100% cell recycle while attaining a steady state. Thus, as we will see, most of the recent reports in the literature refer to cultures that are not at steady state under conditions of 100% cell retention. These processes are analogous to those used for the attainment of high cell density animal cell cultures using the perfusion technique (Zhang, Handa-Corrigan and Spier, 1993). Some of the predicted properties of the system are, in any case, clearly unattainable. For example, this theory would predict that the steady-state biomass concentration at 100% cell recycle will be infinite. But other properties of the system merit continued study. These systems, by allowing high biomass densities and delaying washout at a given dilution rate, offer attractive prospects for dealing with poorly water-soluble growth substrates, toxic products and the enhancement of microbial products, negatively correlated with, or independent of, the growth rate of the microbe. A few selected examples will be used to illustrate the current status of this technology in microbial cell cultivation, as Chang and Furasaki (1991) have reviewed many earlier cell-recycle systems.

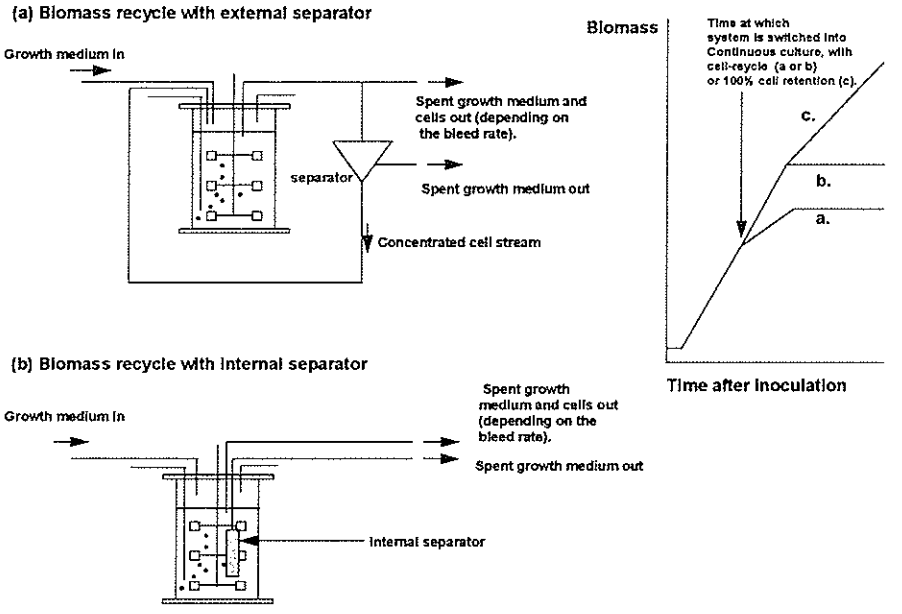

Figure 5. Continuous culture with cell recycle using either external or internal separators. A wide range of devices are available for separating cells from spent growth media. External devices can include centrifugation as well as filtration, and with both internal and external set-ups it is possible to use cell flocculation followed by sedimentation to retain or separate biomass produced during the fermentation. The biomass profiles shown indicate that where 100% cell retention is used no steady state is obtained. With partial cell-recycle the attainment of a steady state can take some time (graph a).

Tin and Mawson (1993) investigated the production of ethanol from whey by *Kluveromyces marxianus* growing in continuous culture with cell recycle, in an attempt to overcome the problems relating to the poor conversion rates observed in batch and continuous culture systems without cell recycle. From an application standpoint it was important to avoid, as far as possible, high sugar levels in the product stream, which in commercial processes would lead to problems of waste disposal. Both centrifugation and flocculation have been used in similar processes to achieve cell recycle. (Howell, 1981; Barry, 1982; Mawson, 1987). Tin and Mawson (1993) used tubular ceramic membranes in the filtration device used to separate the yeast cells from ethanol and unused substrates. Setting the recycle ratio to either 0 or 100%, the effects of increased lactose concentration and increased dilution rate on productivity were examined. The cell densities employed in this study were fairly low, explaining why filter blockage was apparently not a problem. Productivity was increased under cell-recycle conditions and predictably the residual substrate concentrations at a given dilution rate were lower. Others have shown that increasing the biomass density in recycle systems allows higher dilution rates to be used while keeping sugar levels low in the effluent from the reactor (Cheryan and Mehaia, 1983; Mehaia and Cheryan, 1990).

As mentioned previously, lactic acid is a commercially important microbial product. The conversion of starch to lactic acid in batch fermenters is often limited by the low concentration of microbes in the system. Immobilization methods have been used but have often failed to achieve the productivities expected, due to problems

with substrate access to cells and loss of cell activity. Zhang and Cheryan (1994) have investigated the use of biomass recycle continuous culture for improving the efficiency of this process. In this example selection of the membrane properties was predicted as being as important as the filtration unit design. This is because many of the enzymes used in the biotransformation are extracellular and would be continually lost from the reactor if the membrane pore size is too large. Using a hollow-fibre cartridge, operating at relatively high transmembrane pressures, a workable laboratory reactor system was developed. Redesign of this system would be necessary for commercial use. Continuous culture studies identified the range of dilution rates that resulted in low residual starch levels in the outflow from the reactor. Full retention of cells in the reactor was maintained and, more surprisingly, so was the biotransformation potential of the system even when membranes with molecular weight cut-offs much larger than the hydrolytic enzymes likely to be involved were used. The productivity under optimal conditions was nearly twice that observed in comparable batch reactors. A cell density of 38.8 g dry weight l^{-1} was calculated towards the end of the fermentation.

An interesting application of cell-retention continuous culture is that described by Kang, Lee and Chang (1993). *Bacillus thuringiensis* has been used for many years as a source of spores for use as insecticides. It is a fermentation that needs optimal productivity to allow the small profit margins to be maximized. Two systems were compared. The first involved spore production in the cell-retention system operating on its own, the second coupled this reactor to a second-stage continuous culture process with no cell-recycle. Earlier experiments in glucose-limited fed-batch culture obtained a maximum of 5.5×10^9 spores ml^{-1} (Kang, Lee and Chang, 1992). This process was limited by the volume of the reactor. In the total cell retention system the spore count rose to over 1×10^9 spores ml^{-1} but with three times the number of colony-forming units present at the same time. Converting the remaining cells to spores could not be achieved by switching the reactor into batch mode at the time of maximum spore counts. High levels of glucose in the feed to cells in the total cell retention system prevented spore formation. In this case when the reactor was subsequently switched into batch mode spore formation was quickly observed and reached 7.2×10^9 spores ml^{-1}, with over 70% of cells converted to spores. To further decrease the stress on cells in the culture and improve their subsequent conversion to spores, higher levels of glucose in the total cell retention system were used. The best productivity was observed at a glucose feed concentration of 50 g l^{-1}. The reactors that used a continuous culture rather than batch culture in the second stage were an attempt to obtain continuous spore production. A bleed ratio of 0.33 (67% cell retention) from the first reactor was used to feed the continuous culture spore-production phase. Continuous production of spores could be achieved, but at lower spore concentrations in the continuous culture reactor, than were in the experiments mentioned earlier. Problems arose due to the almost inevitable generation of asporogenous mutants. Increasing the bleed ratio led to a gradual decline in productivity. At lower bleed ratios the internal ceramic filter device caused problems, presumably due to the membrane fouling. A maximum cell mass of 82.2 g l^{-1} was recorded in this device and the authors reported that the spore yields under optimum operating conditions were the highest recorded so far for this bacterium.

Using a similar reactor design, fitted with an internal stainless-steel membrane

device, Chang, Lee and Kim (1993) investigated the ability of *Saccharomyces cerevisiae* to produce ethanol. The membrane pore sizes were much larger than usually used and were either 2 or 10 μm. Filter fouling could be reduced by increasing the agitation speed used to mix the components present in the reactor, although there are both operational and cost limits (for commercial production) to using this approach. Cell densities in these reactors reached a maximum of 157 g dry weight l^{-1}. For best productivities, slightly lower cell densities were preferable, which could be controlled by increasing the cell bleed rate (reducing cell retention). The use of co-culture of the yeast *Saccharomyces diastaticus* and *Pichia stipitis* in a cell retention bioreactor, using flocculation to effect cell/media separation, yielded less impressive cell densities but still an efficient process for converting glucose and xylose to ethanol (Laplace *et al.*, 1993). Uribelarrea *et al.* (1993) have investigated the carbon and energy balances in cultures of *Schizosaccharomyces pombe* growing in cell-recycle fermenters at different bleed and glucose feed rates. In previous studies where total cell retention was employed, it was shown that growth, under conditions of glucose excess, was limited by the inhibitory effects of ethanol (De Queiroz, Uribelarrea and Pareilleux, 1991). With partial cell recycle at different growth rates under glucose limitation, little ethanol was produced and the maximum cell density recorded was 32.7 g dry weight l^{-1}. Over a similar range of growth rates with glucose excess, over 40 g l^{-1} ethanol and 165 g dry weight l^{-1} biomass were recorded, the highest values at the lowest growth rates. It was shown that a variety of maintenance requirements were greatest when glucose was in excess. The use of recycle fermentation to examine effects on the specific growth rate independent of dilution rate was highlighted as being of great value in assessing the influence of environmental parameters.

To complete this section three processes have been selected to illustrate the range of variations in fermentation design that can be employed. Babu and Panda (1991) have illustrated the use of fermentation broth recycling in overcoming waste generation problems for the production of penicillin amidase by *Escherichia coli*. In this system the carbon and energy source (phenylacetic acid) has a complex influence on enzyme synthesis and cell growth rate. Centrifugation was used to separate cells from product and the medium nutrient composition analysed and restored to the starting concentrations after cells were removed. It was shown that the process water could be successfully re-used, but some of the products that accumulate influence penicillin amidase synthesis.

Itaconic acid production by *Aspergillus terreus* has been studied in many bioreactor systems. The use of cell immobilization has resulted in disappointing productivities, identified as being caused by oxygen limitation. Using an air-lift reactor in three modes of operation Park *et al.* (1994) showed that repeated batch culture, without cell recycling, gave the most reproducible activities. When cell recycling was attempted, at the end of each batch run, using either centrifugation or filtration to separate the cells from the culture medium, problems arose due to the increased mycelium concentration.

The synthesis of glutamic acid by *Brevibacterium flavum* is an example of a biotechnological process that has been optimized from a variety of approaches ranging from genetic manipulation to growth medium optimization (Beppu, 1986; Minoda, 1986). There is still room, however, for further improvements in productivity by redesign of the bioreactors employed. Glutamate causes end-product inhibition

of its synthesis. The continued removal of this amino acid should therefore help improve the productivity of the fed-batch process routinely used for its manufacture. Glutamic acid can be separated from culture media by electrodialysis. This requires prior separation of cells from the media for maximal efficiency. Ishazaki, Takasaki and Furuka (1993) have designed a total cell recycle system using filtration, to both perform cell separation and maintain an adequate oxygen supply while the cells are in the recycle loop. Their results showed that a separation device incorporating vortex flow (by filtration unit rotation) maintained good productivity, even when the unit was not used to supply air to the cells in the filtration loop.

Many variations of reactor design have been developed to improve the continuous production of a wide range of microbial products. Some of the improvements have been achieved solely by increasing the biomass density in the reactors. However, in most cases it has been appreciated that this strategy is most successful when the undesirable problems associated with extreme biomass densities are avoided, thus avoiding the generation of biomass in a form that is not able to make the desired product because of its location or physiological state. The final type of reactors covered has been shown to have impressive abilities in generating extremely high cell densities, but has much less potential for modulating the accessibility of substrate to cells.

HIGH-DENSITY CULTURE IN IMMOBILIZED CELL BIOREACTORS

Some of the earliest attempts to solve the problem of toxic product removal on a continuous basis involved the use of dialysis culture (Chang and Furusaki, 1991). Technically this sort of bioreactor generates high cell densities with only a minority of the cells attached to the reactor structure, the rest remaining in suspension. However, in many immobilization systems a similar type of culture develops and therefore some reference to this type of cultivation is relevant. Because of the materials available to construct dialysis culture reactors, systems have tended to be restricted to the laboratory scale. Using a two-chamber device, with the growth chamber constructed from a cylindrical dialysis membrane, Märkl *et al.* (1993) grew *Escherichia coli* to densities in excess of 170 g dry weight l^{-1}. They calculated, making generalizations on bacterial cell composition, that the theoretical maximum cell density was between 160 and 200 g dry weight l^{-1}. Growth of this bacterium was obtained by using glycerol as the carbon and energy source. It was shown that for maximal biomass development both low and high concentrations of glycerol should be avoided. The authors attributed the capacity to feed a larger proportion of the culture evenly and without large fluctuations in the nutritional environment, plus continued product removal, to the attainment of such high cell densities when compared to techniques such as fed-batch culture. They also highlighted the fact that high substrate concentrations in the outer part of the reactor (which have to be maintained to allow adequate supply to the bacterial cells) is a problem with the system. Ogbonna and Märkl (1993) showed that adding glycerol directly to the dialysis chamber of a reactor, in which *Escherichia coli* was growing, could reduce nutrient loss while maintaining the high biomass concentrations mentioned above. Fitting an external dialysis chamber to the reactor resulted in much lower biomass densities being recorded. It was proposed that the changes in parameters, including

oxygen tension, in the external dialysis loop were responsible for the lower biomass levels.

Dialysis culture of micro-organisms has many similarities to cultures of microbes growing immobilized in hollow-fibre bioreactors, the mode of cultivation credited with the record for the highest cell density ever reported. A schematic view of this type of bioreactor is shown in *Figure 6*. Past applications of this technique have been reviewed previously (Bunch, 1988; Chang and Furusaki, 1991). Although impressive biomass densities have been reported in these reactors, the individual cell productivities are usually low. This is mainly because mass-transfer limitations prevent all the cells in such reactors from participating in the process involved. It is possible to improve the supply of nutrients to the immobilized cells by changes in the way the bioreactor is constructed or operated (see below). One of the biggest difficulties with these reactors is knowing what factors are limiting productivitity. the first problem is how to determine the biomass levels during operation. This has to be performed by indirect means. Linton *et al.* (1989) used a pressure sensor to follow biomass development. It measured the back pressure as the increasing number of cells restricted the flow of media through the reactor. A calibration graph could be constructed over a fairly wide range of operating cell densities to predict the biomass levels at any stage of the incubation. The results confirmed the fact that in many of these systems the cells grew in a way similar to that observed in dialysis culture. A second problem relates to the physiological state of cells growing in hollow-fibre bioreactors at high cell densities. Linton *et al.* (1987) used a species of lactic acid bacteria, *Streptococcus faecalis*, as a biological sensor. Other bacteria in which the relationship between excreted end-product synthesis and environmental stress has been investigated could be used. Lloyd and Bunch (unpublished observations) using *Escherichia coli* in this way have shown that it is possible to optimize reactor design using this technique for the production of non-growth-associated products, including secondary metabolites and recombinant proteins. In these and other cases it has been shown that, as expected, the supply of oxygen to cells in hollow-fibre bioreactors is most often the major problem. By operating hollow-fibre bioreactors in transverse rather than direct mode (see *Figure 7*), it is possible to improve the delivery of nutrients to cells immobilized on or around the fibres. However, in many cases this can cause fibre rupture to occur (Inloes *et al.*, 1983). Roberson and Kim (1983) designed a dual hollow-fibre system which used one type of fibre for oxygen supply and carbon dioxide removal, the other acting as the immobilization support. Chang and Furusaki (1991) have shown that antibiotics and citric acid can be produced successfully in these reactors and that the productivity can be over 30 times that seen in batch reactors. Other membranes can also be used to replace hollow fibres. Higton *et al.* (1992) compared several types of membrane for the growth of *Streptococcus faecalis*, showing that ceramic membranes, but not glass or metal membranes, gave good results and had the advantage of increased robustness.

A variant of the artificial membrane immobilization method has been reported by Tiltscher and Storr (1993) for high-density growth of the slime mould *Dictyostelium discoideum*, when expressing recombinant human antithrombin III. In this study cells were loaded into inorganic porous aggregates. Up to 15 times the biomass density could be achieved using this method as compared to reactors employing suspended cells. It was shown that although cells growing in these porous structures had a longer

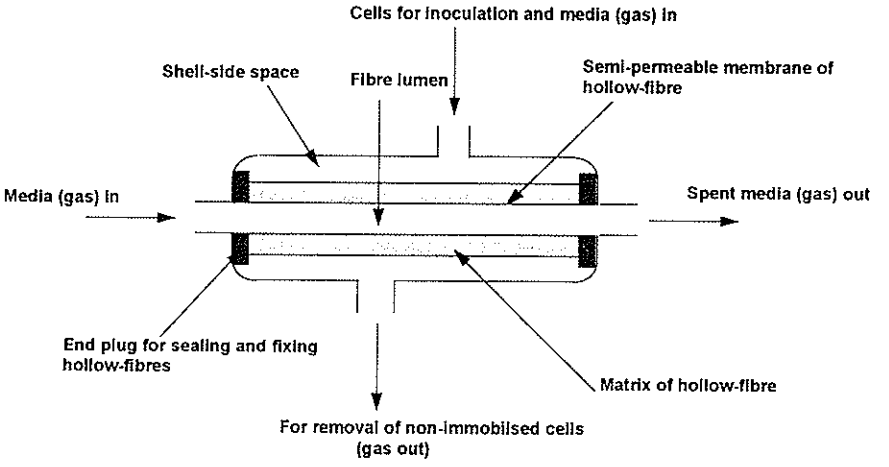

Figure 6. A schematic representation of a hollow-fibre bioreactor for the immobilization of microbial cells. For convenience a single-fibre reactor is illustrated. Although there are reports of such reactors being used for microbial and enzyme immobilization, most reactors will have between 8 and 20 fibres to provide a reasonable immobilization surface plus even media flow at laboratory scale. If anisotropic hollow fibres are used, cells may penetrate into the matrix structure. In most systems cell growth is associated with the surface of the fibre and its surroundings. The semipermeable membrane can be selected to retain all molecules greater than 1 kDa or only retain particles several microns in diameter. Examples of fibre structures and the range of membranes available can be found in Bunch (1988). In dual hollow-fibre reactors a mixture of fibres is employed to allow enhanced gas supply (Chung, Chang and Kim, 1987).

lag phase than suspension batch cultures or cultures with cells absorbed on to the porous aggregates, the cells could be regenerated after storage for use in subsequent experiments. Repeated batch production of the recombinant protein could be achieved using this method. Using a packed bed reactor, operating in an qnalogous way to 100% biomass retention systems, it was possible to obtain continuous production of antithrombin III at levels nearly 20 times higher than that obtained in suspension culture.

A large range of immobilization techniques has been used to enhance biomass densities in reactors. The use of a popular method, gel entrapment, usually leads to a restricted proportion of the biomass being active in a given process. Once again, oxygen limitation is a recurring problem. Although techniques such as co-immobilization of different cell types or the use of agents that can liberate oxygen at the necessary sites have been attempted (Omar, 1993), these systems are likely to have restricted uses in biotechnology. This is particularly true because in microbial cells making natural or recombinant products any loss of competitive advantage between producing and non-producing mutants (or strains that have lost a plasmid containing a recombinant gene) will contribute to the reduction of the cultures' production stability. Using engineered clones of *Escherichia coli*, Varma and Palsson (1994) have shown that careful maintenance of the nutritional environment, not only with respect to the oxygen supply, can stabilize productivity during screening and fermentation procedures. This paper is fascinating as it emphasizes the way that metabolism must be integrated with environment to achieve optimum results. It is an encouraging fact that more and more emphasis is being placed on gaining a better understanding of how microbial metabolism functions under different growth condi-

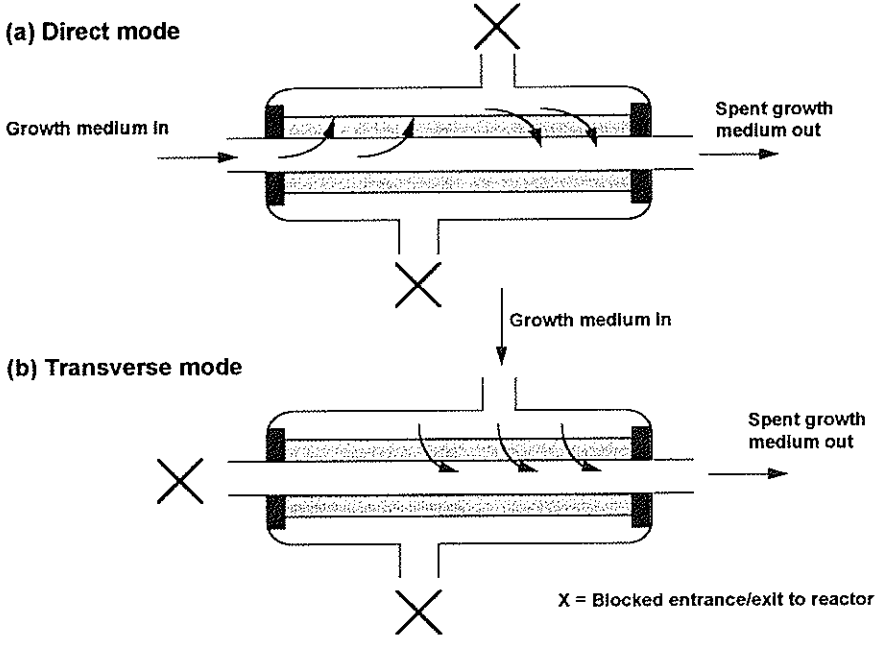

Figure 7. Operational modes for hollow-fibre bioreactor operation. In both operation modes cells are inoculated into the shell side space where they grow on or around the fibres. When using the direct mode, growth medium is supplied via the fibre lumen, where it diffuses to the cells. Products diffuse out into the fibre lumen where they are removed in the outflow from the reactor. Several reports show that this mode of operation can lead to relatively fast fibre breakdown in comparison to the transverse mode systems (Bunch, 1988). Gasses can be supplied or removed via the shell side space if required. In transverse mode the growth medium is passed directly to the cells. The spent medium and products are collected after filtration through the semipermeable part of the hollow-fibre structure. Gasses can be supplied via either the fibre lumen or with the growth medium. To avoid operational problems other solutions to gas supply have been found (see text).

tions, coupled with defining the limits within which it is possible to engineer rationally new metabolic functions (Majewski and Domach, 1990; Bailey, 1991; Stephanopoulos and Vallino, 1991; Varma and Palsson, 1994). In the meantime one of the most interesting ways of holding microbial cells in a form which reduces mass-transfer problems, biofilms, is worthy of some mention as a method of avoiding loss of biocatalytic potential in bioreactors. Tyagi, Gupta and Chand (1992) compared the ethanol productivity of cells immobilized in alginate beads and on the surface of raw and activated bagasse (the latter positively charged with an amino hexyl group in an attempt to gain better establishment of cells). Oxygen and carbon dioxide uptake rates were highest in the 'bagasse'-based reactors where high cell density biofilms were generated. More cells could be loaded on to the activated bagasse support than was the case with the raw material. This compared well with other types of support, such as ion-exchange resins and ceramics. Cell retention of 0.410 g cells/g carrier was reported for the activated bagasse. Loss of cells from the biofilms present in raw bagasse was higher than in the activated bagasse systems. In both cases when operating in a packed bed reactor the flow rates used influenced the rate of cell loss from the system. Although the ethanol productivity of these reactors was comparable

to those reported by other workers, its operational stability was, in almost all cases, superior. Biofilms form quickly on virtually any surface and therefore their study is applicable to a wider range of interests than their application to biotechnological processing. The rate of biocatalysis in biofilms is, not surprisingly, related to the access of substrates to the cells and the growth rate of the microbial population. There have been several attempts to describe the nature of these microbial populations (Characklis and Marshall, 1990; Rittmann and Manem, 1992). Microscopic examination of biofilms reveals a complex arrangement of cells (Lawrence *et al.*, 1991) and it was proposed that the development of such systems is directed towards providing optimum access of nutrients. With the development of techniques such as confocal scanning laser microscopy, tools are now available to study these systems in much more detail, relating structure to operational function. It is interesting to compare the information gained from confocal microscopy with that achieved by scanning electron microscopy methods (Zellner *et al.*, 1994). The outcome of this type of investigation is nicely illustrated by the work of de Beer *et al.* (1994). Interestingly they used a continuous culture system with a recycle loop to deliver a mixed culture of cells to a glass coverslip on which the biofilm development was observed by using a confocal microscope. Microelectrodes were employed to study the oxygen tension in the system. Mature biofilms were developed over a period of several days and fluorescence exclusion with fluorescein was used to enhance the visualization of the internal structures. Penetration of the biofilm was investigated by using fluorescent microspheres of 0.87 μm diameter. Cell clusters 80 μm thick and 100–300 μm in diameter were a key feature in these biofilms, separated from each other by open spaces 50–100 μm wide. The clusters covered approximately 50% of the slide surface. Subsequent development of the biofilm increased the thickness of the cell clusters but not the percentage of the surface covered. Penetrability studies showed that the larger clusters were not closely attached to the substratum. At the point when observation using confocal microscopy becomes severely limited (600 μm), it was shown that an interconnecting channel system had developed in the biofilm. In an elegant study these authors showed that in the channels running from the surface to the substratum the oxygen concentration was always much higher than in the cell clumps and was maintained at the level seen in the supply medium until close to the coverslip surface. Thus it shows that there is potential for 'self-design' of high cell density biofilms to limit mass-transfer problems. The use of this type of microbial culture therefore has the potential to overcome many of the disadvantages of standard immobilization procedures while allowing high biomass densities to be employed.

Weuster-Botz (1993) described the application of biofilm technology to the continuous production of ethanol using *Zymomonas mobilis* growing on porous glass beads in a fluidized bed reactor. The productivity of this system is amongst the highest so far reported, and with the addition of a second reactor stage high productivity with almost total substrate conversion is possible. Demirci, Pometto and Johnson (1993a) have used a biofilm-based system for another product employed as a chemical feedstock, with the same objective of reducing production costs to allow greater industrial use; a mixed culture of *Streptomyces viridosporus* and *Lactobacillus casei* was employed.

Polypropylene chips were used as the support and were blended with a range of agricultural materials, employed as carbon and nitrogen sources on the support

surfaces. *Streptomyces* was used to generate an extensive biofilm in which *Lactobacillus* was immobilized. This process gave a three- to five-fold increase in production rates when compared to suspension culture, and using a mixed culture system improved production over reactors operating with pure culture. As with all biofilm systems, some microbial cells were found in the product stream. In a subsequent paper by Demirci, Pometto and Johnson (1993b) alternative mixed cultures were constructed using a variety of biofilm generators and lactic acid producers. Once again the best production was observed with the mixed cultures when compared to pure cultures of lactic acid producers.

Product removal in biofilms is another feature of these systems that needs careful investigation. It may be envisaged that in biotechnology, in addition to the treatment of environmental wastes, removal of products that inhibit biofilm activity will be of great importance. There have been several attempts to model the consequences of toxic substrates on biofilm activity (van Ede, Bollen and Beenackers, 1993). Continued effort towards toxic product removal is critical for full exploitation of these systems. An extreme example of the sort of possible application of biofilm technology is the biotransformation of waste sulphuric acid by sulphate-reducing bacteria (Stucki, Hanselmann and Hurzeler, 1993). These authors illustrate how such a process can be integrated with physical and chemical systems for the bioremediation of wastes, a rapidly growing concept in environmental technology. The isolation of sulphate-reducing bacteria capable of growth on cheap and abundant waste products is relatively recent (Widdel and Pfennig, 1977). Processes using fixed biofilms show that the key parameter to monitor and control is the production of hydrogen sulphide, which is inhibitory to even those bacteria that synthesize it as a natural product. Using acetate as a co-substrate the process can be summarized as follows:

$$SO_4^{-2} + CH_3COO^- + 3H^+ \rightarrow H_2S + 2CO_2 + 2H_2O$$

Thus the products, as gases, should be easily removed, achieved in this case by incorporating a gas stripping column in the recycle loop. This example illustrates how a knowledge of biofilm development and operation allows clear guidance for the overal design of the bioreactor.

Some reference should be made to solid state fermentations. In some respects these can be considered as the ultimate type of biofilm reactor, employing localized high cell densities. It is a specialized, but important, area of biotechnology. A relatively narrow range of substrates can be used for this type of microbial cultivation. The substrates become heterologous during the fermentation, and excessive heat build-up can cause problems and, once again, monitoring what is happening is extremely difficult (Sargantanis *et al.*, 1993). The latter authors showed how very high biomass densities can occur in these systems, requiring careful process design to avoid premature loss of reactor operation. Scale-up strategies have been discussed by Lonsane *et al.* (1992). Using sugar-cane bagasse impregnated with growth nutrients, Soccol *et al.* (1994) have shown that this type of reactor can be used as a 'low-tech' method of producing lactic acid. Their process used the fungus *Rhizopos oryzae*. An alternative system using bacteria has been described by Xavier and Lonsane (1994), while Sarangbin, Kirimura and Usami (1993) described a process for the manufacture of citric acid using the fungus *Aspergillus niger*. Both accounts again highlight the fact that although the technology is

simple, the design of an efficient process needs careful consideration.

The future of high cell density cultivation of microbial cells

In many examples, investigators have been driven by curiosity about how much biomass can be developed in a given type of reactor. There have been some surprises. Generation of over 500 g dry weight biomass l–1 in dual hollow-fibre reactors (Chung, Chang and Kim, 1987) is much higher than would be predicted using the available data on microbial cell composition. What is more surprising is that these cultures were still productive. With more information about the way microbial cultures establish in these systems, some of the productivities observed in high cell density culture become more understandable. A study of biofilms using confocal laser microscopy is a good illustration of how mass-transfer problems, for example, can be reduced by the way colonies develop.

The operation of few microbial processes has been compared in a wide range of different bioreactors. Chang and Furusaki (1991) have performed such an exercise for citric acid production by micro-organisms; Bunch (1988) for lactic acid synthesis. Using our examples, a limited comparison is possible for the production of ethanol. However, even for this small range of microbial products it is not surprising to learn that the generation of high biomass densities is not the only way of further improving a fermentation process. Some of the most efficient ways of synthesizing lactic acid use low cell density continuous culture, although the highest reactor volumetric productivity is obtained in a hollow-fibre system. Whether the benefits associated with high-density cultivation are worthwhile can only be assessed in the light of factors such as substrate costs, substrate conversion rates, fermentation run-time, reactor stability and process integration with working practices.

Given the fact that enhancing biomass density will still remain part of the approach to process intensification in the future, it is encouraging to see continued development of materials for use as reactors or as separation devices in reactors. As such systems become more robust their application to cultivation problems is likely to grow rapidly. For example, cell recycle/cell retention systems are likely to find extensive use for growing micro-organisms which generate only low biomass densities in batch and chemostat culture. Several examples have been cited which show how reactor selection can lead to useful cell yields of micro-organisms, such as protozoa and extremophiles. New techniques for improving cell separation continue to appear. An ingenious example is that used by Trampler *et al.* (1994) to increase the cell density of hybridoma cultures. Using an acoustic flow-through device they caused aggregation of cells to take place, allowing separation by sedimentation to occur. The result was a 70-fold increase in productivity with 99% cell retention. It is possible therefore to predict that the development of new types of filtration devices will enable further improvements to high cell density systems.

The diversity of reactor types shows that there is great potential for applying high cell density technology. The use of solid-state fermentations shows that relatively low-tech processes can be attractive for the generation of microbial products. Ohno, Ano and Shoda (1993) have shown that such systems can be used with aerobes as well as anaerobes or facultative anaerobes. They show that the productivity of *Bacillus subtilis* cultures making the antifungal peptide antibiotic, iturin, growing on soybean

curd, can be enhanced 6–8 times over that achieved in cell suspension reactors. Nevertheless, it is inevitable that the best increases in productivity will use high cell density culture in combination with other approaches for improving process efficiency. It is not possible to review all these other methods comprehensively here. Nevertheless, two will be mentioned because they reflect the way that process optimization is being approached.

At the beginning of this chapter the complexity of microbial cells was highlighted. We are still a long way from being able to comprehend fully the intricate way that the metabolic processes of micro-organisms are integrated, especially in the vast range of physiological environments provided in bioreactors. One approach is that taken by Varma, Boesch and Palsson (1993). They remind us that only in the case of the red blood cell is it possible to characterize the dynamic capabilities of a cell. Using *Escherichia coli* they performed a flux balance analysis to calculate the production capability of this bacterium in respect of selected amino acids and related products. Their paper reveals how much further this approach has to go before it can be generally applied to microbial fermentations, but gives an insight as to how increasing information on microbial metabolism can be used to determine rationally the capabilities of a given reactor system. This will allow guidelines to be developed to evaluate the potential of metabolic engineering to overcome productivity limitations relating to a single enzyme activity (or lack of activity). It is likely in the near future that larger changes in the natural metabolic capability of a microbial cell could be made.

The use of artificial neural networks is the second approach to process design. In a lucid account of this technique Zhang *et al.* (1994) examined its application to *Bacillus thuringiensis* fermentations. These authors identified three major problems in the development of reliable automated control systems for microbial fermentations: lack of reliable mathematical models to describe cell growth and product formation, a general lack of appropriate on-line sensors and the complicated regulatory systems found within microbial cells. They stated that the use of neural network analysis is well matched to processes where there is much data but poor system knowledge. The best features of this approach are that: it allows deductions without a precise mathematical model of the process, works with incomplete information and it can handle large amounts of information quickly through parallel processing. A neural network solves problems by adapting ('training' in the terminology of this subject) to the input data; in this example, five parameters that affect culture optical density. They showed by selecting one of a variety of different networks that they could model accurately optical density changes in the culture during its development. This technique takes advantage of computer power that is now available, and has been applied to many problems.

All these developments in the drive towards more productive and efficient microbial product synthesis make it possible to predict that limitations of existing cultivation methods will be overcome, to the mutual benefit of those who are seeking to understand more about how micro-organisms function and those who desire to design a commercially viable process.

References

ALBERGHINA, L., PORRO, D., MARTEGANI, E. AND RANZI, B.M. (1991). Efficient production of recombinant DNA proteins in *Saccharomyces cerevisiae* by controlled high-cell density fermentation. *Biotechnology and Applied Biochemistry* **14**, 81–92.

BABU, P.S.R. AND PANDA, T. (1991). Effects of recycling fermentation broth for the production of penicillin amidase. *Process Biochemistry* **26**, 7–14.

BAILEY, J.E. (1991). Toward a science of metabolic engineering. *Science 252*, 1668–1675.

BARRY, J.A. (1982). Alcohol production from cheese whey. *Dairy Industry International* **47**, 19–22.

BEETON,S., MILLWARD, H.R., BELLHOUSE, B.J., NICHOLSON, A.M., JENKINS, N.J. AND KNOWLES, C.J. (1991). Gas transfer characteristics of a novel membrane bioreactor. *Biotechnology and Bioengineering* **38**, 1233–1238.

BEETON, S., BELLHOUSE, B.J., KNOWLES, C.J., MILLWARD, H.R., NICHOLSON, A.M. AND WYATT, J.R. (1994). A novel membrane bioreactor for microbial growth. *Applied Microbiology and Biotechnology* **40**, 812–817.

BELLHOUSE, B.J., BELLHOUSE, F.H., CURL, C.M., MACMILLAN, T.I., GUNNING, A.J., SPRATT, E.H., MACMURRAY, S.B. AND NELEMS, J.M. (1973). A high efficiency membrane oxygenator and pulsatile pumping system and its application to animal trials. *Transactions of the American Society of ARtificial Internal Organs* **34**, 747–754.

BEPPU, T. (1986). Breeding of amino acid-producing strains of coryneform bacteria by recombinant DNA technology and protoplast fusion. In *Biotechnology of Amino Acid Production* (K. Aida, I. Chibata, K. Nakayama, K. Takinami and H. Yamada, Eds), pp. 29–33. Kodansha-Elsevier, Tokyo.

BERG, A.-C., HOLST, O. AND MATTIASSON, B. (1989). Continuous culture with complete cell-recycle cultivation of *Pseudomonas cepacia* ATCC 29351 on salicylate for production of salicylate hydroxylase. *Applied Microbiology and Biotechnology* **30**, 1–4.

BLONDEAU, K., BOUTUR, O., BOZE, H., JUNG, G., MOULIN, G. AND GALZY, P. (1994). Development of high-cell-density fermentation for heterologous interleukin 1β production in *Kluyveromyces lactis* controlled by the PH05 promoter. *Applied Microbiology and Biotechnology* **41**, 324–329.

BUNCH, A.W. (1988). The uses and future potential of microbial hollow-fibre bioreactors. *Journal of Microbiological Methods* **8**, 103–119.

CHANG, H.N. AND FURUSAKI, S. (1991). Membrane bioreactors: present and prospects. *Advances in Biochemical Engineering/Biotechnology* **44**, 28–64.

CHANG, H.W., LEE, W.G. AND KIM, B.S. (1993). Cell retention culture with an internal filter module. Continuous ethanol fermentation. *Biotechnology and Bioengineering* **41**, 677–681.

CHANG, L.-L., HWANG, L.Y., HWANG, C.-F. AND MOU, D.-G. (1991). Study of high density *Escherichia coli* fermentation for production of porcine somatotropin protein. *Annals of the New York Academy of Sciences* **646**, 259–272.

CHARACKLIS, W.G. AND MARSHALL, K.C. (1990). Biofilms: a basis for an interdisciplinary approach. In *Biofilm* (W.G. Characklis and K.C. Marshall, Eds), pp. 3–15. Wiley, New York.

CHERYAN, M. AND MEHAIA, M.A. (1983). A high-performance membrane bioreactor for continuous fermentation of lactose to ethanol. *Biotechnology Letters* **8**, 519–524.

CHUNG, B.H., CHANG, H.N. AND KIM, I.H. (1987). Rifamycin production by *Nocardia mediterrenei* in a dual hollow-fibre bioreactor. *Enzyme and Microbial Technology* **9**, 345–349.

COOPER, S. (1991). *Bacterial Growth and Division: Biochemistry and Regulation of Prokaryotic and Eukaryotic Division Cycles*. Academic Press, New York.

CUBARSI, R. AND VILLAVERDE, A. (1993). Conditions for a continuous production of transient microbial products in a two-stage fermentation system. *Biotechnology Letters* **15**, 827–832.

DE BEER, D., STOODLEY, P., ROE, F. AND LEWANCHOWSKI, Z. (1994). Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and Bioengineering* **43**, 1131–1138.

DEMIRCI, A., POMETTO, A.L. AND JOHNSON, K.E. (1993a). Evaluation of biofilm reactor solid support for mixed-culture lactic acid production. *Applied Microbiology and Biotechnology* **38**, 728–733.

DEMIRCI, A., POMETTO, A.L. AND JOHNSON, K.E. (1993b). Lactic acid production in a mixed culture biofilm reactor. *Applied and Environmental Microbiology* **59**, 203–207.

DE QUEIROZ, J.H., URIBELARREA, J.L. AND PAREILLEUX, A. (1991). High cell density cultures of *Schizosaccharomyces pombe* in a cell-recycle reactor. Growth kinetics and metabolic status. *Applied Biochemistry and Biotechnology* **30**, 283–295.

GUPTA, S.K. AND CHAND, S. (1994). Bioconversion of sugars to ethanol in a chemostat employing *S. cerevisiae* – dynamic response to perturbations in process parameters. *Process Biochemistry* **29**, 343–354.

HARRISON, D.E.F. AND LOVELISS, J.E. (1971). Effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. *Journal of General Microbiology* **68**, 35–43.

HEATH, C.A. AND BELFORT, G. (1992). Synthetic membranes in biotechnology: realities and possibilities. *Advances in Biochemical Engineering* **47**, 46–85.

HERBERT, D. (1961). The chemical composition of microorganisms as a function of their environment. *Society of General Microbiology Symposia* **10**, 391–416.

HIGTON, G., BALL, J., BUNCH, A.W. AND KNOWLES, C.J. (1992). A method for growth and immobilisation of *Streptococcus faecalis* var. *zymogenes* in bioreactors containing hollow-fibre, ceramic, metallic or glass-fibre membranes. *Journal of Microbiological Methods* **16**, 1–11.

HOWELL, M.J. (1981). High-grade ethanol from whey. *Chemical Industry (N2)* **15**, 4–6.

HUANG, C.-T., PERELTI, S.W. AND BRYERS, J.D. (1993). Plasmid retention and gene expression in suspended and biofilm cultures of recombinant *Escherichia coli* DH5α(pMJR1750). *Biotechnology and Bioengineering* **41**, 211–220.

IBBA, M., BONARIUS, D., KUHLA, J., SMITH, A. AND KUENZI, M. (1993). Mode of cultivation is critical for the optimal expression of recombinant hirudin by *Saccharomyces cerevisiae*. *Biotechnology Letters* **15**, 667–672.

INLOES, D.S., SMITH, W.J., TAYLOR, D.P., COHEN, S.N., MICHAELS, A.S. AND ROBERTSON, C.R. (1983). Hollow-fibre membrane bioreactors using immobilised *Escherichia coli* for protein synthesis. *Biotechnology and Bioengineering* **25**, 2653–2681.

ISHIZAKI, A., TAKASAKI, S. AND FURUKA, Y. (1993). Cell-recycled fermentation of glutamate using a novel cross-flow filtration system with constant air supply. *Journal of Fermentation and Bioengineering* **76**, 316–320.

KAMIN, H., WHITE-STEVENS, R.H. AND PRESSWOOD, R.P. (1978). Salicylate hydroxylase. *Methods in Enzymology* **53**, 527–543.

KANG, B.C., LEE, S.Y. AND CHANG, H.N. (1992). Enhanced production of spores of *Bacillus thuringiensis* by fed-batch culture. *Biotechnology Letters* **14**, 721–726.

KANG, B.C., LEE, S.Y. AND CHANG, H.N. (1993). Production of *Bacillus thuringiensis* spores in total cell retention culture and two-stage continuous culture using an internal ceramic filter system. *Biotechnology and Bioengineering* **42**, 1107–1112.

KIY, T. AND TIEDTKE, A. (1992a). Continuous high cell density fermentation of the ciliated protozoon *Tetrahymena*: I. A perfused bioreactor. *Applied Microbiology and Biotechnology* **38**, 141–146.

KIY, T. AND TIEDTKE, A. (1992b). Mass cultivation of *Tetrahymena thermophila* yielding high cell densities and short generation times. *Applied Microbiology and Biotechnology* **37**, 576–579.

KNORRE, W.A., DECKWER, W.-D., KORZ, D., POHL, H.-D., RIESENBERG, D., ROSS, A., SANDERS, E. AND SCHULZ, V. (1991). High cell density fermentation of recombinant *Escherichia coli* with computer-controlled optimal growth rate. *Annals of the New York Academy of Sciences* **646**, 301–321.

KUMAR, P.K.R., SINGH, A. AND SCHUGERL, K. (1991). Fed-batch culture for the direct conversion of cellulosic substrates to acetic acid/ethanol by *Fusarium oxysporum*. *Process Biochemistry* **26**, 209–216.

LAPLACE, J.M., DELGENES, J.P., MOLELTA, R. AND NAVARRO, J.M. (1993). Ethanol production

from glucose and xylose by separated and co-culture processes using high-cell density systems. *Process Biochemistry* **28**, 519–525.

LAWRENCE, J.R., KORBER, D.-R., HOYLE, B.D., COSTERTON, J.W. AND CALDWELL, D.E. (1991). Optical sectioning of microbial biofilms. *Journal of Bacteriology* **273**, 6558–6567.

LINTON, E.A., KNOWLES, C.J., BUNCH, A.W. AND HIGTON, G. (1987). The biotechnological potential of microbial hollow-fibre bioreactors. In *Bioreactors and Biotransformations* (G.W. Moody and P.B. Baker, Eds), pp. 299–309. Elsevier Applied Science, Amsterdam.

LINTON, E.A., HIGTON, G., KNOWLES, C.J. AND BUNCH, A.W. (1989). Monitoring microbial growth in a hollow-fibre reactor using an electronic pressure sensor. *Enzyme and Microbial Technology* **11**, 257–320.

LONSANE, B.K., SAUCEDO-CASTANEDA, G., RAIMBAULT, M., ROUSSOS, S., VINIEGRA-GONZALEZ, G., GHILDYAL, N.P., RAMAKRISHNA, M. AND KRISHNAIAH, M.M. (1992). Scale-up strategies for solid state fermentation systems. *Process Biochemistry* **27**, 259–273.

MAJEWSKI,. R.A. AND DOMACH, M.M. (1990). Simple constrained-optimization view of acetate overflow in *Escherichia coli*. *Biotechnology and Bioengineering* **35**, 732–738.

MÄRKL, H., ZENNECK, C., DUBACH, C.H. AND OGBONNA, J.C. (1993). Cultivation of *Escherichia coli* to high cell densities in a dialysis reactor. *Applied Microbiology and Biotechnology* **39**, 48–52.

MAWSON, A.J. (1987). Ethanol production from whey in New Zealand. *Australian Journal of Biotechnology* **1**, 64–66.

MEHAIA, M.A. AND CHERYAN, M. (1990). Ethanol from hydrolysed whey permeate using *Saccharomyces cerevisiae* in a membrane recycle bioreactor. *Bioprocess Engineering* **5**, 57–61.

MILLWARD, H.R., BELLHOUSE, B.J., NICHOLSON, A.M., BEETON, S., JENKINS, N. AND KNOWLES, C.J. (1994). Mammalian cell damage in a novel membrane bioreactor. *Biotechnology and Bioengineering* **43**, 899–906.

MINODA, Y. (1986). Raw materials for amino acid fermentation. In *Biotechnology of Amino Acid Production* (K. Aida, I. Chibata, K. Nakayama, K. Takinami and H. Yamada, Eds), pp. 51–66. Kodansha-Elsevier, Tokyo.

NAKANO, K., MATSUMARA, M. AND KATAOKA, H. (1993). Application of a rotating ceramic membrane to dense cell culture. *Journal of Fermentation and Bioengineering* **76**, 49–54.

NEIDHARDT, F.C., INGRAHAM, J.L. AND SCHAECHTER, M. (1990). *Physiology of the Bacterial Cell: A Molecular Approach.* Sinawer Associates, Massachusetts.

NURMIANTO, L., GREENFIELD, P.F. AND LEE, P.L. (1994). Optimal feeding policy for recombinant protein production via the yeast *Saccharomyces cerevisiae* in fed-batch culture. *Process Biochemistry* **29**, 55–68.

OGBONNA, J.C. AND MÄRKL, H. (1993). Nutrient-split feeding strategy for dialysis cultivation of *Escherichia coli*. *Biotechnology and Bioengineering* **41**, 1092–1100.

OHARA, H., HIYAMA, K. AND YOSHIDA, T. (1992). Kinetics of growth and lactic acid production in continuous culture and batch culture. *Applied Microbiology and Biotechnology* **37**, 544–548.

OHNO, A., ANO, T. AND SHODA, M. (1993). Production of the antifungal peptide antibiotic iturin by *Bacillus subtilis* NB22 in solid state fermentation. *Journal of Fermentation and Bioengineering* **75**, 23–27.

OMAR, S.H. (1993). Oxygen diffusion through gels employed for immobilization. *Applied Microbiology and Biotechnology* **40**, 173–181.

PARK, Y.S., ITIDA, M., OHTA, N. AND OKABE, M. (1994). Itaconic acid production using an air-lift bioreactor in repeated batch culture of *Aspergillus terreus*. *Journal of Fermentation and Bioengineering* **77**, 329–331.

PATEL, P.N., MEHAIA, M.A. AND CHERYAN, M. (1987). Cross-flow membrane filtration of yeast suspensions. *Journal of Biotechnology* **5**, 1–16.

PIRT, S.J. (1975). *Principles of Microbe and Cell Cultivation*, pp. 42–57. Blackwell Scientific Publications, Oxford.

POSTGATE, J.R. AND HUNTER, J.R. (1962). Survival of starved bacteria. *Journal of General Microbiology* **29**, 233–263.

PROKOP, A. AND BAJPAI, R.K. (1991). Recombinan DNA Technology I. *Annals of the New York Academy of Sciences* **646**, whole volume.

RAVEN, N., LADWA, N., COSSAR, D. AND SHARP, R. (1992). Continuous culture of the hyperthermophilic archaeum *Pyrococcus furiosus*. *Applied Microbiology and Biotechnology* **38**, 263–267.

RIESMEIR, B., KRONER, K.H. AND KULA, M.-R. (1989). Tangential filtration of microbial suspensions: filtration resistances and model development. *Journal of Biotechnology* **12**, 153–171.

RIESMEIR, B., KRONER, K.H. AND KULA, M.-R. (1990). Harvest of microbial suspensions by microfiltration. *Desatination* **77**, 219–233.

RITTMAN, B.E. AND MANEM, J.A. (1992). Development and experimental evaluation of a steady-state multispecies biofilm model. *Biotechnology and Bioengineering* **39**, 914–922.

ROBERSON, C.R. AND KIM, I.H. (1983). Dual aerobic hollow-fibre bioreactor for cultivation of *Streptomyces aureofaciens*. *Biotechnology and Bioengineering* **37**, 1012–1020.

SARANGBIN, S., KIRIMURA, K. AND USAMI, S. (1993). Citric acid production from cellobiose by 2-deoxyglucose-resistant mutant strains of *Aspergillus niger* in semi-solid culture. *Applied Microbiology and Biotechnology* **40**, 206–210.

SARGANTANIS, J., KARIM, M.N., MURPHY, V.G. AND RYOO, D. (1993). Effect of operating conditions on solid substrate fermentation. *Biotechnology and Bioengineering* **42**, 149–158.

SCHÜZ, T.C., FIEDLER, H.-P. AND ZAHNER, H. (1993). Optimised nikkomycin production by fed-batch and continuous fermentation. *Applied Microbiology and Biotechnology* **39**, 433–437.

SOCCOL, C.R., MARIN, B., RAIMBAULT, M. AND LEBEAULT, J.-M. (1994). Potential of solid state fermentation for production of L-(+)-lactic acid by *Rhizopus oryzae*. *Applied Microbiology and Biotechnology* **41**, 286–290.

STEPHANOPOULOS, G. AND VALLINO, J.J. (1991). Network rigidity and metabolic engineering in metabolite over production. *Science* **252**, 1675–1681.

STUCKI, G., HANSELMANN, K.W. AND HURZELER, R.A. (1993). Biological sulphuric acid transformation: Reactor design and process optimization. *Biotechnology and Bioengineering* **41**, 303–315.

SWARTZ, R.W. (1985). Penicillins. In *Comprehensive Biotechnology* (M. Moo-Young, Ed.), volume 3, pp. 8–43. Pergamon Press, Oxford.

TANAKA, T., KAMIMURA, R., ITOH, K. AND NAKANISHI, K. (1993). Factors affecting the performance of cross flow filtration of yeast cell suspension. *Biotechnology and Bioengineering* **41**, 617–624.

TEMPEST, D.W., NEIJSSEL, O.M. AND ZEWENBOON, W. (1983). Properties and performance of microorganisms in labaratory culture: their relevance to growth in natural ecosystems. In *Microbes in their Natural Environment* Society for General Microbiology Symposium. Cambridge University Press **34**, 119–153.

TILTSCHER, H. AND STORR, M. (1993). Immobilisation of the slime mould *Dictoyostelium discoideum* for the continuous production of recombinant human antithrombin III. *Applied Microbiology and Biotechnology* **40**, 246–250.

TIN, C.S.F. AND MAWSON, A.J. (1993). Ethanol production from whey in a membrane recycle bioreactor. *Process Biochemistry* **28**, 217–221.

TOCAIJ, A., HOF, A., HAGANDER, P. AND HOLST, O. (1993). Fed batch cultivation of *Pseudomonas cepacia* with on-line control of the toxic substrate salicylate. *Applied Microbiology and Biotechnology* **38**, 463–466.

TRAMPLER, F., SONDERHOFF, S.A., PUI, P.W.S., KILBURN, D.G. AND PINET, J.M. (1994). Acoustic cell filter for high density perfusion culture of hybridoma cells. *Biotechnology* **12**, 281–284.

TSENG, C.-P. AND MONTVILLE, T.J. (1992). Enzymatic regulation of glucose catabolism by *Lactobacillus plantarum* in an aerobic chemostat. *Biotechnological Progress* **8**, 126–131.

TYAGI, R.D., GUPTA, S.K. AND CHAND, S. (1992). Process engineering studies on continuous ethanol production by immobilised *S. cerevisiae*. *Process Biochemistry* **27**, 23–32.

URIBELARREA, J.L., DE QUEIROZ, H., GOMA, G. AND PAREILLEUX, A. (1993). Carbon and energy balances in cell-recycle cultures of *Schizosaccharomyces pombe*. *Biotechnology and Bioengineering* **42**, 729–736.

VAN EDE, C.J., BOLLEN, A.M. AND BEENACKERS, A.A.C.M. (1993). Analytical effectiveness

calculations concerning the degradation of an inhibitive substrate by a steady state biofilm. *Biotechnology and Bioengineering* **42**, 267–278.

VAN LIERE, L. (1979). On *Oscillatoria agardhii* gomont; experimental ecology and physiology of a nuisance bloom-forming cyanobacterium. Ph.D. thesis, University of Amsterdam, The Netherlands.

VAN NIEL, E.W., BRABER, K.J., ROBERTSON, L.A. AND KWENEN, J.G. (1992). Heterotrophic nitrification and aerobic denitrification in *Alcaligenes faecalis* strain Two. *Antonie van Leeuwenhoek* **62**, 231–237.

VAN WALSUM, G.P. AND COOPER, P.G. (1993). Self-cycling fermentation in a stirred tank reactor. *Biotechnology and Bioengineering* **42**, 1175–1180.

VARMA, A. AND PALSSON, B.O. (1994). Predictions for oxygen supply control to enhance population stability of engineered production strains. *Biotechnology and Bioengineering* **43**, 275–285.

VARMA, A. BOESCH, B.W. AND PALSSON, B.O. (1993). Biochemical production capabilities of *Escherichia coli*. *Biotechnology and Bioengineering* **42**, 59–73.

VERDWYN, C., POSTMA, E., SCHEFFERS, W.A. AND VAN DIYKEN, J.R. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501–517.

VICKROY, T.B. (1985). Lactic acid. In *Comprehensive Biotechnology* (M. Moo-Young, Ed.), volume 3, pp. 8–43. Pergamon Press, Oxford.

WARREN, R.K., MACDONALD, D.G. AND HILL, G.A. (1991). Cross-flow microfiltration of *Saccharomyces cerevisiae*. *Process Biochemistry* **26**, 337–342.

WEUSTER-BOTZ, D. (1993). Continuous ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part I. Kinetic studies of immobilisation in macroporous glass beads. *Applied Microbiology and Biotechnology* **39**, 679–684.

WIDDEL, F. AND PFENNIG, W. (1977). A new anaerobic, sporing, acetate-oxidising, sulphate-reducing bacterium, *Desulphotomaculum (emend) acetoxidans*. *Archives of Microbiology* **112**, 119–122.

XAVIER, S. AND LONSANE, B.K. (1994). Sugar-cane press mulch as a novel and inexpensive substrate for production of lactic acid in a solid-state fermentation system. *Applied Microbiology and Biotechnology* **41**, 291–295.

ZELLNER, G., DIEKMANN, H., AUSTERMANN-HAUN, U. AND SEYFRIED, C.F. (1994). Scanning electron microscopy of biofilm development in anaerobic fixed-bed reactors: influence of the inoculum. *Biotechnology Letters* **16**, 315–320.

ZHANG, D.X. AND CHERYAN, M. (1994). Starch lactic acid in a continuous membrane bioreactor. *Process Biochemistry* **29**, 145–150.

ZHANG, Q., REID, J.F., LITCHFIELD, J.B., REN, J. AND CHANG, S.-W. (1994). A prototype neural network supervised control system for *Bacillus thuringiensis* fermentations. *Biotechnology and Bioengineering* **43**, 483–489.

ZHANG, S., HANDA-CORRIGAN, A. AND SPIER, R.E. (1993). A comparison of oxygenation methods for high-density perfusion cultures of animal cells. *Biotechnology and Bioengineering* **41**, 685–692.