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The scale-up of microbial batch and fed-batch fermentation processes.

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I Introduction.

Micro-organisms are important for both human health and to industry so the fed-batch cultivation of microbial strains, often over expressing recombinant or natural proteins, to high cell density has become an increasingly important technique throughout the field of biotechnology, from basic research programmes to large-scale pharmaceutical production processes (Hewitt *et al.*, 1999). The scale-up of such a process is usually the final step in any research and development programme leading to the large-scale industrial manufacture of such products by fermentation (Einsele, 1978). It is important to understand that the process of scaling-up a fermentation system is frequently governed by a number of important engineering considerations and not simply a matter of increasing culture and vessel volume. Therefore, it is perhaps surprising when the large-scale does not perform as well as the small-scale laboratory process. It is often observed that the biomass yield and any growth associated products are often decreased on the scale-up of an aerobic process (Enfors *et al.*, 2001). For *Saccharomyces cerevisiae*, the biomass yield on molasses increased by 7% when the process was scaled-down from 120 m^3 to 10 l even when a seemingly identical strain, medium and process were employed (George *et al.*, 1993). In an *E. coli* fed-batch recombinant protein process, the maximum cell density reached was found to be 20% lower when scaling-up from 3l to 9 m^3 and the pattern of acetic acid formation had changed. (Bylund *et al.*, 1998). During another study (Enfors *et al.*, 2001), the performance of a recombinant strain of *E. coli* during fed-batch culture was found to vary on scale-up from the lab-scale to 10-30 m^3 industrial bioreactors. This included lower biomass yields, recombinant protein accumulation and surprisingly perhaps a higher cell viability. These findings are typical of those found when scaling-

up most fermentation processes yet only a few mechanisms have been presented that can satisfactorily explain these phenomena.

In this Chapter, we will briefly discuss the main engineering considerations involved in fermentation scale-up and then critically review those mechanisms thought to be responsible for any detrimental change in bioprocessing at the larger-scale. Though it addresses mainly *E. coli* fed-batch fermentations, much of the discussion also applies to batch and other single celled aerobic microbial fermentations too.

II Engineering Considerations Involved in Scale-up
A. Agitator Tasks in the Bioreactor.

The agitation system in the bioreactor provides the liquid motion that enables many different tasks to be fulfilled. An example of a typical stirred bioreactor is shown in diagrammatic form in Figure 1. It is important to understand the interaction between the fluid motion, the agitator speed and the power input into the bioreactor and these tasks. It is also necessary to know how a change of scale affects these relationships. Many of these aspects can be studied without carrying out a specific bioprocess and these physical aspects most relevant to bacterial fermentations are listed in Table 1. Table 2 sets out those aspects that are specific to the organism being grown and will usually be different for each case. The more important of these aspects with respect to scale-up are discussed later.

The physical aspects in Table 1 have been discussed extensively for conditions relevant to a wide range of organisms elsewhere (Nienow, 1996; Nienow, 1998;

Nienow and Bujalski, 2004). Here, their relevance to microbial fermentations for which the viscosity essentially does not go much higher than that of water is discussed, e.g. bacteria and yeast. Thus viscous polysaccharide and filamentous systems are excluded from consideration in this Chapter. With such low viscosities, the flow in the fermenter is turbulent from a 5 L bench bioreactor to the largest scale, i.e. Reynolds number, $Re = \rho_L ND^2/\mu > \sim 10^4$ where ρ_L is the growth medium density (kg/m^3), μ is its viscosity (Pa s), D is the impeller diameter (m) and N, its speed (rev/s). For scale-up purposes, as long as the flow is turbulent, the actual value of the Reynolds number does not matter and turbulent flow theories can be used to analyse the fluid mechanics in the bioreactors across the scales. The topics listed in Table 1 will be considered first for such flows.

1. Mass Transfer of Oxygen into the Broth and Carbon Dioxide out.

The transfer of oxygen into a fermentation broth has been studied since the 1940s when 'submerged fermentations' were first established. The topic was recently reviewed by Nienow (2003). The overall oxygen demand of the cells throughout the batch or fed-batch fermentation must be met by the oxygen transfer rate and the demand increases as long as the number of cells is increasing. Roughly, for every mole of O_2 utilised, 1 mole of CO_2 is produced, i.e the respiratory quotient, $RQ \approx 1$ (Nienow, 2006). Thus, a maximum oxygen transfer rate must be achievable and this rate depends on the mass transfer coefficient, $k_L a$ (1/s), and the driving force for mass transfer, ΔC, since

$$OUR = k_L a. \Delta C \qquad\qquad Eq. 1$$

The value of $k_L a$ is similar for both O_2 transfer from air to the broth and CO_2 from it. For oxygen transfer, the driving force conceptually is the difference between the oxygen concentration in the air bubbles and that in the broth, which must always be held above the critical dO_2 value throughout the fermenter for the duration of the process. In a similar way, the dCO_2, must be kept below that which will lead to a reduction in fermentation rate or productivity.

It has been shown many times (Nienow, 2003) that in low viscosity systems, $k_L a$ is only dependent on two parameters. These are, firstly, the total mean specific energy dissipation rate imposed on the system, $(\bar{\varepsilon}_T)_g$ (W/kg) and secondly v_s (m/s), the superficial air velocity (= (vvm/60)(volume of broth)/(X-sectional area of the bioreactor). $(\bar{\varepsilon}_T)_g$ and v_s must together be sufficient to produce the necessary $k_L a$ where,

$$k_L a = A(\bar{\varepsilon}_T)_g^{\alpha}(v_s)^{\beta} \qquad \text{Eq. 2}$$

This equation applies independently of the impeller type and scale and α and β are usually about 0.5 ± 0.1 whatever the liquid. On the other hand, A is extremely sensitive to growth medium composition (Nienow, 2003) and the addition of antifoam which lowers $k_L a$ or salts which increase it, may lead to a 20 fold difference in $k_L a$ for the same values of $(\bar{\varepsilon}_T)_g$ and v_s. Typical values of $(\bar{\varepsilon}_T)_g$ are up to ~ 5 W/kg and for the air flow rate about 1 volume of air per volume of growth medium (vvm). Since the value of $k_L a$ is similar for both O_2 and CO_2 transfer, provided scale-up is undertaken at constant vvm (or close to it), the driving force for transfer in of O_2 and of CO_2 out will remain essentially the same across the scales. In this case, since vvm scales with

fermenter volume and v_s scales with its cross-sectional area, v_s increases. There is some debate as to whether $(\bar{\varepsilon}_T)_g$ should include a contribution from the sparged air ($\approx$ $v_s g$ where g is the acceleration due to gravity (9.81 m^2/s)), which only becomes significant on scale-up at constant vvm. This approach should also eliminate problems with high dCO_2 on scale-up (Nienow, 2006).

2. Heat Transfer

The oxygen uptake rate (OUR in (mol O_2 /m^3/s)) largely determines the metabolic heat release Q (W/m^3) (RQ $\approx$ 1) which is proportional to it (Van't Riet and Tramper 1991), i.e.,

$$Q \approx 4.6 \times 10^5 \text{ OUR} \qquad \text{Eq. 3}$$

This cooling load has to be removed by heat transfer at an equivalent rate given by,

$$Q = U A \Delta\theta \qquad \text{Eq. 4}$$

where U is the overall heat transfer coefficient (which is hardly affected by the agitation conditions), $\Delta\theta$ is the difference between the temperature of the cooling water and the broth temperature (it being critical to control the latter) and A is the heat transfer area available. At the commercial scale, heat transfer is often a problem as Q scales with the volume of the reactor, i.e., for geometrically-similar systems with T^3 (bioreactor diameter, T m) whilst cooling surface area scales with T^2. Hence, on the large-scale for such systems, cooling coils are often required and sometimes cooling baffles. The inability to meet the cooling requirements at the large-scale (especially for example, in

high cell density (>50g/L dry cell weight) fed-batch fermentations) is a very serious problem because it is extremely expensive to resolve.

B. Unaerated Power Draw P (or Mean Specific Energy Dissipation Rate, $\bar{\varepsilon}_T$ W/kg).

These parameters are both dependent on the impeller power number, Po (dimensionless). Po depends on the agitator type, and in the turbulent regime for any one type, it is essentially constant, regardless of the diameter, D (m), relative to the bioreactor diameter, T or the speed, N and of the bioreactor size (scale) provided geometric similarity is maintained across the scales (Nienow, 1998). The power input, P (W), into the bioreactor imparted by the impeller is given by,

$$P = Po\rho_L N^3 D^5 \qquad \text{Eq. 5}$$

The mean specific energy dissipation rate, $(\bar{\varepsilon}_T)$ (W/kg) from the impeller is the given by,

$$(\bar{\varepsilon}_T) = P / \rho_L V \qquad \text{Eq. 6}$$

where V is the volume of growth medium in the reactor (m^3). The maximum local specific energy dissipation rate, $(\varepsilon_T)_{\max}$, is close to the impeller, is very high relative to the mean and depends on the agitator type. Also, the Kolmogoroff or micro-scale of turbulence, λ_K, which is often considered as an indicator of the potential for mechanical damage to cells (see below for more details) is given by,

$$\lambda_K = ((\varepsilon_T)/\nu)^{-1/4} \qquad\qquad \text{Eq. 7}$$

where (ε_T) is the local specific energy dissipation rate and ν is the kinematic viscosity, $\approx 10^{-6}$ m^2/s when the cell/medium suspension is water-like.

The traditional impeller for fermentation processes has been the Rushton turbine (Figure1) and it has a relatively high power number (~ 5). Recently, it has been clearly shown to have many weaknesses (Nienow, 1996) and it is being superseded by the impellers shown in Figure 2, each of which can be considered an example of a generic type (Nienow, 1996; Nienow and Bujalski, 2004). All these impellers have lower Po values and so can easily replace a Rushton turbine running at the same speed, torque and power by one of a larger diameter which gives certain processing advantages as set out below.

C. Aerated Power Draw P_g (or Aerated $(\bar{\varepsilon}_T)_g$ W/kg)

Particularly with the Rushton turbine, upon aeration at around 1 vvm, the power P_g, (and therefore $Po_g (= P_g/\rho_L N^3 D^5)$ and $(\bar{\varepsilon}_T)_g (= P_g/\rho_L V)$) falls significantly, typically by 50%. The relationship with D/T, scale and impeller speed is very complex and difficult to predict (the commonly-used Michel and Miller correlation is dangerously inappropriate (Nienow, 1998; Middleton and Smith, 2004), especially at large-scale and with multiple impellers (Nienow, 1998)). Thus, it is difficult to obtain $(\bar{\varepsilon}_T)_g$, which is a critical requirement for calculating mass transfer on scale-up. Also, it has safety implications since, if air flow is lost, the power drawn by the impeller doubles. Therefore, often a much more powerful motor is installed in

order to cope with this possibility. The advantage of the up-pumping, high solidity

ratio (SR = plan area of impeller/ area of circle swept out by its blade tips) hydrofoil

(Figure 2b) and the hollow blade impeller (Figure 2c) is that impellers of both these

types lose very little if any power on aeration (Nienow, 1996; Middleton and Smith,

2004; Nienow and Bujalski, 2004). Thus, they finesse the problem of the loss of

power found with the Rushton impeller. Finally, it is worth noting that during the

earlier stages of a fermentation, lower $(\bar{\varepsilon}_T)_g$ values will suffice for achieving the

required O_2 transfer, so a variable speed drive motor for the impeller gives additional

flexibility and a reduction in running costs.

D. Flow Close to the Agitator-single Phase and Air-liquid

The turbulent flow field close to the agitator depends on its shape and

determines its power number and the mechanism by which the air is dispersed and

hence the aerated power draw too. This process is described in detail elsewhere

(Nienow, 1998; Middleton and Smith, 2004) and is beyond the scope of this Chapter.

E. Variation in Local Specific Energy Dissipation Rates, ε_T W/kg

The region of $(\varepsilon_T)_{\max}$ where mechanical damage due to agitation is most

likely to occur is also close to the agitator and $(\varepsilon_T)_{\max}/(\bar{\varepsilon}_T) = \Phi$ is similarly

dependent on the agitator type. It is difficult to determine and values of the order of

about 20 to 70 have been reported for Rushton turbines with similar values for other

impellers (Nienow, 1998; Kresta & Brodkey, 2003). The significance of $(\varepsilon_T)_{\max}$ for

damage to micro-organisms is discussed below. Well away from the agitator, Φ is

much less than 1. These differences in Φ have important implications for the feed location of nutrients, pH control chemicals, etc., as discussed below.

F. Air Dispersion Capability

The flow close to the agitator also determines whether the agitator speed is sufficient to disperse the air. A variety of air dispersion conditions can be usefully identified (Figure 3). If the air flow rate is too high, the air flow dominates the bulk flow pattern and the air is poorly dispersed (Figure 3a). This condition is known as flooding and is to be avoided. For Rushton turbines, the correlation (Nienow, 1998),

$$(Fl_G)_F = 30(D/T)^{3.5}(Fr)_F \qquad \text{Eq. 8}$$

enables the minimum agitator speed required to prevent flooding, N_F to be calculated. In this equation, Fr is the dimensionless Froude number, N^2D/g, proportional to the ratio of the inertial to buoyancy forces; and Fl_G is the dimensionless gas flow number, Q_G/ND^3, proportional to the ratio of the air flow rate, Q_G (m^3/s), from the sparger to the pumping capacity of the agitator. This correlation also works quite well for hollow blade agitators too but because of their lower Po, to draw the same power at the same speed, a larger diameter must be used so that they can handle much more air before flooding (Nienow, 1996, 1998). It can also be shown that on scale-up at constant $\bar{\varepsilon}_T$ and vvm, impellers are much more likely to be flooded. A similar equation, which leads to similar conclusions (Nienow, 1998), applies to the complete dispersion condition, N_{CD} (Figure 3c).

For axial flow hydrofoil impellers, a similar correlation has not been established. However, down pumping high solidity hydrofoils (Figure 2a), made of a larger diameter as described above, are somewhat similar to the Rushton turbine; whilst the large diameter up-pumping configurations (Figure 2b) perform similarly to the hollow blade impellers and are significantly better than Rushton turbines, especially as $N_{CD} \approx N_F$ (Nienow and Bujalski, 2004).

G. Bulk Fluid- and Air-phase Mixing

The mixing of the air (gas) phase is important for mass transfer (Nienow, 2003) but is beyond the scope of this chapter. On the other hand, the ability of the agitator to mix the contents of very large (up to 400 m^3) fermenters with multiple impellers compared to the bench-scale is the most challenging and important of all the scale-up issues. A measure of this difference is the parameter, the mixing time θ_m (s), which indicates after the addition of a tracer how long it takes to be evenly dispersed throughout the fermenter. For a fermenter containing broth to a height H (m) =T,

$$\theta_m = 5.9 T^{2/3} \left(\bar{\varepsilon}_T\right)^{-1/3} \left(D/T\right)^{-1/3} \qquad \text{Eq. 9}$$

Equation 9 also holds for aerated conditions if $\left(\bar{\varepsilon}_T\right)_g$ is used. It implies for fermenters with broth up to an aspect ratio, AR = 1, at constant $\left(\bar{\varepsilon}_T\right)_g$, all impellers of the same D/T ratio give the same mixing time which larger D/T ratios can reduce. Most importantly, θ_m increases with (linear scale)$^{2/3}$. For fermenters with AR > 1, with multiple impellers (Nienow, 1998),

$$\theta_m \propto (H/D)^{2.43} \qquad\qquad \text{Eq. 10}$$

Equation. 10 indicates the great sensitivity of mixing time to fill height and this increase in θ_m would be even greater with fed batch fermentations when towards its end, a large portion of the broth often does not experience any direct agitation (Nienow, 2005). The use of multiple high solidity ratio axial flow hydrofoils reduces the mixing time by about a factor of 2 compared to radial flow impellers and this has led to their use (Nienow, 2005). Unfortunately, in the down-pumping mode (Figure 2a), they lose power (though not by as much as the Rushton impeller) and more importantly, they are prone to two-phase flow instabilities which leads to a large variable loading of the impeller drive motor. These problems are eliminated by the use of up-pumping configurations (Nienow and Bujalski, 2004), which also helps reduce foam formation (Boon *et al.*, 2002). The implications for this loss of homogeneity on scale-up and small-scale experiments to mimic it are discussed below. It is also important to consider where additions are made. Though 'final' mixing time does not depend on where addition is made, addition near the impeller in the regions of $\Phi \gg 1$ dramatically reduces the maximum concentration of the additive as it mixes whilst addition onto the top surface where $\Phi \ll 1$ and which is much easier and therefore preferred industrially, results in very high local concentrations of additives for some considerable time before they are dissipated (Nienow, 2006). The latter feed position magnifies the lack of homogeneity at the large-scale and significantly increases the chances of a different biological performance compared to the small.

H Main Differences Across the Scales

If these considerations are assessed for the changes that occur across the scale, the following points emerge. Firstly, mass transfer requirements can be met at similar or even lower specific power inputs or $(\bar{\varepsilon}_T)_g$. Thus, $(\bar{\varepsilon}_T)_{g,\max}$ will be the same or less and since even at the bench scale, the cells are very small compared to the size of the bioreactor, if 'shear damage' is not an issue at the small scale, then it should not be on scale-up. This aspect is discussed in more detail below. Heat transfer is not an issue provided sufficient area for cooling is provided. However, the mixing time is always very significantly longer and therefore the spatial and temporal homogeneity is generally much worse on scale –up. Again, this aspect is discussed in detail below.

III Process Engineering Considerations for Scale-up

A. Fluid Mechanical Stress or so Called 'Shear Damage'.

Anecdotal reference to the damaging effects on cells of fluid mechanical stress or so called 'shear damage' are frequently made to explain poor process performance when mechanical agitation and aeration are introduced into a bioreactor as compared to the non-agitated and non-sparged conditions in a shake flask or microtitre plate (Thomas, 1990). Thomas (1990) suggested that cells might be considered to be unaffected by fluid dynamic stresses if they were of a size smaller than the Kolmogoroff microscale of turbulence, λ_K. The microscale of turbulence is related to the local specific energy dissipation rate ε_T by Eq. 7. Therefore, if ε_T is 1 W/kg in a water like medium, $\lambda_K \simeq 30$ μm. However, even though bacterial cells, of size ~1 -

2μm, are well below the Kolmogoroff microscale of turbulence, it has been reported that the mean cell volume of two strains of *E. coli* and of two other species of bacteria, increased linearly with impeller speed during continuous cultivation with a concomitant increase in intracellular potassium and sodium ion concentration (Wase and Patel, 1985; Wase and Rattwatte, 1985). Toma *et al.* (1991) also studied the effect of mechanical agitation on two species of bacteria, *Brevibactarium flavium* and *Tricherderma reesii*. In each case, they found that under conditions of high agitation intensity during batch culture, both growth and metabolism, were inhibited. They even coined the term 'turbohypobiosis' to describe this phenomenon and suggested that excessive turbulence may cause this inhibition by damaging the membranes of the cell. However, in these cases, the results are difficult to interpret because any changes in agitation and aeration rate will also effect levels of dissolved oxygen (dO_2) *via* Eqs. 1 and 2 and depending on the critical dO_2 value, this parameter may also affect biological performance. Thus, any experimental protocol for investigating the impact of fluid dynamic stress on cell response should be undertaken under steady state (continuous culture) conditions, including the control of dO_2, if the cause of the change is to be determined conclusively. Therefore in the cases discussed above the results were probably based on poor experimental design and their controversial findings may have been due to the lack of controlled dO_2 (Wase and Patel, 1985; Wase and Rattwatte, 1985) or the use of the constantly changing conditions experienced during batch culture (Toma *et al.*, 1991).

Recently, studies concerning the impact of agitation and aeration (because animal cells are potentially more easily damaged by bursting bubbles rather than rotating impellers (Nienow, 2006)) on microbial fermentations have been carried out.

in a stirred tank bioreactor. The bioreactor was operated as a chemostat, with blending of sparged air and nitrogen to control the driving force. Thus, again *via* Eqs. 1 and 2, the dO$_s$ was controlled to a constant value. Firstly, the impact of high levels of agitation and aeration intensity (fluid mechanical stress) on *E. coli* fermentation performance were addressed as measured by standard microbiological techniques and the physiologically sensitive technique of multi-parameter flow cytometry (Hewitt and Nebe-von-Caron 2001, 2004; Hewitt *et al*., 1998). The initial work in glucose limited continuous culture at the 5L scale showed that agitation intensities, expressed as mean specific energy dissipation rates, $\bar{\varepsilon}_T$, up to 30 W/kg and aeration rates up to 3 vvm, served only to strip away the outer polysaccharide layer (endotoxin) of the cells but did not lead to any significant change in the physiological response of individual cells which could lead to a detrimental change in bioprocessing. Estimates of the Kolmogoroff microscale of turbulence based on $\bar{\varepsilon}_T$, at 30 W/kg gives $\lambda_K = 13.5$ μm, well above the size of the cell ($\sim$ 1-2 μm). Even if the maximum local specific energy dissipation rate is used ($\sim$ 30 $\bar{\varepsilon}_T$,), to estimate λ_K, a value of $\sim$ 6 μm is obtained, still greater than the cell size. This agitation intensity is an order of magnitude or more greater than those typically found on the industrial scale and the range of aeration rates tested was much higher than those normally used, thus eliminating the possibility that damage due to fluid mechanical stresses may occur under the normal range of operating conditions.

Further studies were also undertaken during continuous cultivation with the Gram +ve bacterium *Corynebacterium glutamicum* (Chamsartra *et al*., 2005) with essentially similar results. In this case, it was shown that variations in agitation, aeration rate, or dO$_2$ concentrations down to $\sim$1% of saturation do not cause a

significant change in physiological response of *C. glutamicum* even though the mean cell size was slightly reduced (Figure 4 and 5).

Similar work with the larger (~7 μm) *Saccharomyces cerevisiae* showed that under steady state conditions, specific power inputs in the range 0.04 to 5kW/m³ (λ_K = 16 μm) were found to have little effect on either cellular morphology or physiology even though at the upper end of the agitation range there was a small, but transient measurable effect on cell division (Boswell *et al* 2002). This was probably because budding cells may be more susceptible to hydrodynamic stress or that as a cell increases in size during division (~10-12 μm) it approaches the scale at which the Kolmogoroff microscale of turbulence may have an effect. Since the microscale of turbulence decreases with increasing power input and impeller speed, it is expected that such an effect is more likely at high impeller speeds. With this system, at the highest impeller speed used, the microscale is less than 20 μm i.e. within the range that might interact with budding yeast cells. Therefore this work indicates that the potentially deleterious effects of high agitation rates can again be discounted provided $\bar{\varepsilon}_T < \sim 5.0$ kW/m³) for propagation cultures.

All three of these studies concluded that any change in the biological behaviour of non-filamentous microbial cells within the $\bar{\varepsilon}_T$ range representing the normal operating window for mechanical agitation found in bioreactors as compared to the relatively-gentle behaviour found in shake flasks (Buchs *et al.*, 2001) is not due to fluid dynamic stresses, whether arising from agitator generated turbulence or bursting bubbles. In all cases, any changes in biological performance were only found under the most extreme of agitation intensities at values far above the normal

operating range required to satisfy the mass transfer requirements. A recent review of the issues involved in large scale, free suspension animal cell culture in stirred bioreactors reached essentially the same conclusions (Nienow, 2006).

It is also worth noting that the work reported above was carried out with Rushton turbines, so-called high shear impellers. Even so damage was not found. It is also now understood that many of the so-called low shear impellers have higher values of $(\varepsilon_T)_{max}/(\bar{\varepsilon}_T) = \Phi$ than Rushton turbines. The concept of 'low shear impellers', a description which is intended to imply that they cause less damage to cells than other impellers, is essentially a manufacturers sales pitch (Simmons *et al*, 2007). Overall, since all non-filamentous cell types have been demonstrated experimentally not to be 'damaged' by the fluid mechanical stresses found in bioreactors, an alternative explanation for any detrimental change in bioprocessing performance at the large-scale must be found.

B. Operational Constraints at the large-scale

The fed-batch, high cell density cultivation of microbial strains is the preferred industrial method for increasing the volumetric productivity of such bacterial products as nucleic acids (Elsworth *et al.*, 1968), amino acids (Forberg and Haggstrom, 1987) and heterologous recombinant proteins (Riesenberg and Schulz, 1991). The salient feature of this type of process is the continuous feed of a concentrated growth limiting substrate, usually the carbon source, characterised by an ever increasing level of energy limitation and an ever decreasing specific growth rate. This type of feeding regime avoids problems associated with catabolic regulation, oxygen limitation and

heat generation that can occur during unlimited batch processes (Minihane and Brown, 1986). Importantly, the build up of toxic concentrations of metabolic bi-products *via* so-called 'overflow' metabolic routes can also be avoided. Overflow metabolism has been reported for *Saccharomyces cerevisiae*. (George *et al*., 1993) as well as for *Escherichia coli* and occurs at glucose concentrations above ~30 mg/L. For *E. coli*, an accumulation of an inhibitory concentration of acetic acid occurs *via* the redirection of acetyl CoA from the Krebbs cycle, during fast aerobic growth when a rapidly metabolisable carbon source, such as glucose, is available in excess (Andersson, 1996). For *S. cerevisiae*, overflow metabolism is known as the 'Crabtree Effect' and the inhibitory bi-product is ethanol but produced in a similar way to acetate in *E. coli*. In batch fermentation, overflow metabolism can be avoided by the use of a slowly metabolisable carbon source such as glycerol (Elsworth *et al*., 1968), but the preferred method is the use of a fed-batch process where growth can easily be controlled by substrate feed rate (see Lee, 1996, for a comprehensive review).

Although the optimal position for the addition of any feed in order to ensure its subsequent rapid dispersal is in the region near to the impeller of $(\varepsilon_T)_{max}$ which leads to the rapid reduction of the high concentrations in the feed towards the desired mean value, (Nienow, 1998, 2006), most large-scale industrial processes still use surface additions (because of concern for contamination, pipe blockage, mechanical stability and so on (Nienow, 1998)). Further, bioreactor configurations have traditionally been designed to satisfy oxygen mass transfer using radial flow Rushton turbines, with the inherent assumption that they were well mixed or if not, it was not important. Indeed, such a view is easy to understand as on-line measurements and

control actions appear to show near to steady state conditions or slow progressive changes in those parameters being measured.

Around the mid-1980s, Kossen and co-workers using the concept of 'regime analysis', where the rate of oxygen uptake is compared with that of oxygen transfer and the level of dO_2, suggested that, at the large scale, differences in dO_2 would be found in batch fermentations (see Oosterhuis *et al.*, (1985) for example). Sometime later in the early 1990s, Enfors and co-workers came to similar conclusions for fed-batch fermentations with respect to the nutrient feed (see for example, George *et al.* (1993)). At around this time, it was shown that replacing Rushton turbines by high solidity ratio hydrofoil impellers which enhanced bulk mixing (spatial homogeneity) improved fermentation performance (Buckland *et al.*, 1988),

Only fairly recently, however has it been established experimentally that spatial and temporal chemical gradients exist in large-scale fed batch bio-reactors (Xu *et al.*, 1999) where additions of a concentrated, often viscous, carbon source at a single point onto the top surface of the growth medium means that mixing times are high (>~50s even at the 20m³ scale (Vrabel *et al.*, 2000)). Studies using computational fluid dynamics (CFD) based on Large Eddy Simulation (LES) also showed that considerable glucose gradients could be expected even when a standard 500 g/L glucose solution was fed to the liquid surface in a 22 m³ bioreactor fitted with four Rushton turbines (Enfors *et al.*, 2001). Such studies also showed that the region (compartment) around the top impeller would have a much higher glucose concentration when compared with the bulk (remainder) of the vessel (Figure 6). The use of LES also shows the temporal as well as the spatial concentration fluctuations of

the glucose concentration in the vicinity of the feed point. Indeed, this was confirmed experimentally showing that cells were frequently exposed to peak glucose concentrations several times higher than the mean in the addition zone (Xu *et al.*, 1999) and that spatially dependent concentration gradients exist in large-scale fed-batch fermentation processes with a declining glucose concentration found with increasing distance from the feed point (Bylund *et al.*, 1998). In laboratory scale bioreactors on the other hand, where much development work is done, mixing times are low (<~5s) and essentially significant temporal or spatial variations in concentration do not exist (Nienow, 1998).

Additionally, at the large-scale, any pH controlling action is often based on the point measurement of local pH by a single probe situated adjacent to an impeller and hence in a well mixed, high (ε_T) region. By contrast, the controlling agent, like the feed components, is usually added at the poorly mixed surface of the liquid. Additionally, the amount of controlling agent added is not continuous but added as a pulse, the volume of which is largely dependent on biomass concentration and its relative metabolic activity and hence will vary throughout the duration of the process, whether batch or fed-batch. The inherent inertia in such a system can lead to over feeding of the pH controlling agent and therefore zones of high and low pH, again with temporal fluctuations superimposed on the spatial ones. Indeed, such regions of fluctuating high and low pH have now been measured in 8 m^3 bioreactors for animal cell culture (Langheinrich and Nienow, 1999).

It is our contention that the composition of a cell's micro-environment is a product both of the fluid dynamics and a cell's physiological response to it, so cells

circulating around a large-scale bioreactor will experience rapidly changing micro-environments. Therefore knowledge of if/how a cell reacts to such changes is essential if we are to understand the problems associated with bioprocessing on scale-up.

C. The Physiological Response of Cells to the Large-scale Environment.

Complex networks of regulatory systems often known physiologically as the so called 'stress responses' are phenomena that have evolved to help micro-organisms withstand conditions when their immediate environment becomes sub-optimal for growth (Wick and Egli, 2004). However, descriptions and discussions regarding such responses are usually confined to the mainstream microbiological literature. It is only now that it is being understood that the chemical and physical heterogeneities found within a poorly-mixed large-scale bioreactor can cause microbial cells to alter their physiology as a response to these environmental stimuli (Enfors, 2004) and that this can have a detrimental effect on bioprocessing. Until recently any physiological response of microbial cells to changes in environmental conditions within a bioreactor was mostly measured indirectly by measurement of external variables outside of the cell. However, recent developments in the so called "omics" analytical technologies have allowed the direct measurement of internal variables within the cell. So it has now been shown that *E. coli* cells respond very quickly to changes in local glucose concentration known to exist within a large-scale bioreactor by the fast transcriptional induction of an alternative set of genes (Schweder *et al.*, 1999). mRNA molecules associated with the expression of stress proteins, sensitive to oxygen limitation, are synthesised rapidly, when a cell passes through a local zone of high glucose

concentration (Figure 7). At the 20m^3 scale, the overall biomass yield was reduced by approximately 24% as compared to an otherwise identical laboratory scale process and formate accumulated, to 50 mg/L. In this case, it was concluded that a high local glucose concentration induced local oxygen limitation so that acetate synthesis was not due to overflow metabolism but to mixed acid synthesis through fermentative metabolic pathways. Fermentative metabolism in *E. coli* differs in comparison to overflow metabolism in that formate, D-lactate, succinate and ethanol are produced in addition to acetate under anaerobic conditions. However, both acetate and D-lactate are re-assimilated much more quickly than formate when *E. coli* cells re-enter an oxygen sufficient zone, leading to an accumulation of formate in the culture medium. It was concluded that the repeated synthesis and consumption of these mixed acids in response to a cells exposure to oxygen sufficient/oxygen deficient zones was responsible for the lower biomass yields experienced at the large-scale (Xu, 1999).

Proteomic and metabolomic techniques can also be used which can reveal post-transcriptional or post-translational events in cells, which cannot be revealed by transcriptomics alone. Using such sensitive molecular biological techniques investigations have shown that under such poorly mixed conditions, *E. coli* cells may induce one of a number of interlinked regulatory stress response pathways characterised by rapid increases in the concentration of certain intracellular signalling molecules, such as ppGpp and cAMP, as well as the induction of alternative sigma factors such as RpoS (Hoffman and Rinas, 2004; Schweder *et al*., 2004). These changes often result in the transcription and expression of a number of stress proteins, the consequences of which can include the inhibition of DNA replication initiation, a reduction in rRNA synthesis and protein production. In addition, glycolytic activity,

DNA metabolism and the synthesis of structural components may be greatly reduced allowing cells to survive conditions sub-optimal for continued growth leading inevitably to low biomass and product yields at the large-scale.

D. Small-scale Experimental Simulation Models of the Large-scale

It has long been the goal of biochemical engineers to be able to quantitatively model and predict large-scale process performance from data obtained from small-scale laboratory experiments. However, in general, mathematical models that are currently used to predict biomass production and protein synthesis during the scale-up of laboratory scale fermentation processes (Anderson *et al.*, 1994) make two basic assumptions. Firstly, that throughout the course of a fermentation, a bacterial population is homogenous with respect to its physiological state and its ability to divide. Secondly, that the physiological state of a bacterial population is independent of the scale of cultivation. Such assumptions have now largely been shown to be invalid (Hewitt and Nebe-von-Caron 2001, 2004). The latter would still hold true if the large and the small-scale had identical process conditions but for the reasons discussed earlier this is very rarely the case. Techniques such as multi-parameter flow cytometry that make measurements on individual cells have now shown unequivocally that different physiological sub-populations exist and evolve throughout the course of many microbial fermentation processes (Hewitt *et al.*,1999, 2000; Enfors *et al.*, 2001; Onyeaka *et al.*, 2003; Sundstrom *et al.*, 2004; Lopes da Silva *et al.*, 2005; Reis *et al.*, 2005). For example, it was shown that during a 40 hr 5l laboratory-scale fed-batch process to grow *E. coli* W3110 to high cell density (>50g/L dry cell weight) that there was a progressive change in cell physiological state with respect to cytoplasmic

membrane potential and permeability (Figure 8). With ~16% of the population characterised as being dead at the end of the process and ~5% being in a fluctuating dormant state throughout (Hewitt *et al.*, 1999). Other work has demonstrated a catastrophic drop (to ~ zero) in the number of cfu/ml in the middle of a fed-batch recombinant fermentation whilst all other measurements showed that the majority of the cells were viable and metabolising as normal (Sundstrom *et al.*, 2003). Therefore this type of study casts doubt on the use of mathematical models with the above assumptions for the reliable prediction of biomass production and product yield on scale-up. So the only definitive way of finding out how an industrial process is going to perform remains to carry out actual large-scale trials of the final process which are often difficult and expensive to carry out. Additionally, when the results differ from the bench-scale, they are often difficult to interpret. Therefore, equipment and techniques that allow large-scale studies to be simulated at small-scale have become important research tools.

For simulating the phenomenon of poor spatial and temporal homogeneity at the large-scale, a technique used is to divide the large-scale reactor into two compartments, firstly by Kossens and co-workers (Oosterhuis *et al.*, 1985) and later by Enfors and co-workers (George *et al.*, 1993). The conditions established in each compartment depend on the type of poor mixing situation on the large-scale that is to be simulated. For fed-batch fermentations and for pH control heterogeneities associated with feed streams, these two compartments can represent an addition zone where the most severe pH and nutrient concentration gradients exist and the bulk region where the system can be considered to be essentially well mixed, so therefore at a much lower concentration with respect to the nutrient feed and pH. The relative size

of the addition zone in the simulation should be of the order of size of the region in which higher concentrations exist at the large-scale. The size of this region may be estimated by intuition, by flow visualisation (again on the small-scale) or by computational fluid dynamics (Enfors *et al*., 2001). Typically, in the small-scale simulation, the addition zone is represented by a relatively small plug-flow reactor (PFR), and the bulk region by a stirred tank reactor (STR) (Amanullah *et al*., 2003). The volumetric ratio between these two reactors is equal to the estimated ratio of the addition zone to the bulk region in the large-scale reactor with the rate of circulation between them related to the circulation time of cells in the broth at the large scale due to agitation.

A similar approach is to use two stirred vessels side by side (STR-STR). This method has been particularly used to simulate dO_2 inhomogeneities associated with the slow rate of mixing compared to oxygen utilisation. In this case, the volume of the well oxygenated region is typically made of the order of 25% of the poorly oxygenated region. The STR-STR and STR-PFR have been compared for batch fermentations of *Bacillus subtilis* with respect to pH and dO_2 fluctuations (Amanullah *et al*., 2003). In both cases, significant differences were found compared to the well mixed case, which depended on the relative size of the 2 zones and the recirculation rate between them. However, it is not possible to say which is the best technique and both are only rather crude approximations of the real variations actually seen by the cells at the large-scale.

E. Results from Small-scale Experimental Trials of

Large-scale *E. coli* Fed-batch Processes.

1. Experimental Set-up

Recently, we have carried out a number of small-scale simulation studies of large-scale fed-batch fermentations with *E. coli*. For this work a two-compartment reactor system (Hewitt *et al*., 2000; Onyeaka *et al*., 2003) was used (Figure 9). This system consisted of a stirred tank reactor (STR, the 5L fermenter) linked in series to a plug flow reactor (PFR). The STR consisted of a 5L cylindrical glass bioreactor (162 mm diameter x 300 mm total height), with an initial working volume of 2.5L rising to 4L at the end of the fermentation. The vessel was fitted with two 82 mm, six bladed radial flow paddle type impellers which were 80 mm apart, with the lower impeller situated 80 mm above the bottom of the vessel. The vessel was also fitted with three equally spaced baffles, width 15 mm. The PFR was made up of a glass cylinder consisting of five equally sized sections each containing a set of removable stainless steel static mixer modules, each with 24 individual mixer elements to give a total of 120 mixing elements and a liquid volume of 544 ml (~14 − 22% of the total working volume). These elements were included in order to reduce radial concentration gradients, enhance oxygen transfer (where appropriate) and to encourage plug flow. Provision was made so that either the pH controlling agent, or the substrate or air, or all three, could be introduced at the inlet of the PFR as well as into the STR. Medium was pumped through the PFR (the residence time in the PFR could be varied between 60 − 110s) *via* a short length of silicone tubing. The PFR was thermally insulated along its length to avoid temperature gradients. All additions were made at 90° to the PFR flow,

again in order to minimise disturbances, *via* energy input, to the plug-flow characteristics of the reactor (George *et al.*, 1993). Large-scale fermentations were carried out in a 30m³ cylindrical stainless steel bioreactor (2090 mm diameter x 9590 mm total height), with an initial working volume of 20m³ rising to 22m³ at the end of the fermentation. The vessel was fitted with four Rushton turbines (diameter 690 mm) which were 1460 mm apart with the lower impeller 1110 mm above the bottom of the vessel. The vessel was also fitted with four baffles 90° apart, width 170 mm. Laboratory scale fermentations were started as batch cultures and an exponential feeding profile was calculated in order to maintain the growth rate below 0.3 /h from the following equation,

$$F = \left(\frac{1}{s}\right) \times \left(\frac{\mu}{Y_{xs}} + m\right) \times X_o \times e^{\mu t}$$

Eq.11

where F is the feed rate (L/h), s is the substrate concentration in the feed solution (g/L), μ is the required specific growth rate (/h), Y_{xs} is the maximum biomass yield on the limiting substrate (g/g), X_o is the total amount of biomass (g) at the start of feeding, m is the maintenance coefficient (g/g/h) and t is the time after feeding commences (h). Exponential feeding was started when the initial glucose had been exhausted. When the DOT had fallen to the 20% saturation level in the STR in all the small-scale cases, the feed rate was held constant for the remainder of the experiment. Large-scale fermentations were started as batch cultures and an exponential feed profile calculated as above was started 1 h after inoculation and continued for 8.5 hrs. This corresponded to a final feed rate of 180 L/h which was continued for a further 2.5 hrs. This was then reduced to 170 L/h for the remainder of the fermentation. For all

fermentations, a synthetic medium (Hewitt *et al.*, 1999; 2000) was used and they all ran for ~ 40 hrs.

2. Experimental Results

Firstly (Table 3), we compared the results obtained from a small-scale, well-mixed, 5L *E. coli* W3110 fed-batch fermentation with those found from carrying out a similar fermentation at the 20 m^3 scale where the pH and dO$_2$ were controlled at the same values (Hewitt *et al.*, 2000). However, at the 20 m^3 scale, at similar levels of agitation intensity (expressed as W/kg), mixing times are much longer compared to the small-scale (see Eqs. 9 and 10 and for results in this 20 m^3 bioreactor, Vrabel *et al.*, 2000). Thus, the spatial and temporal heterogeneity of the environment in the vessel increases and locally higher glucose concentrations are found near the addition point with concomitant lower dissolved oxygen concentrations (Enfors *et al.*, 2001). Differences in pH were also observed between the region close to the addition point of the pH controlling agent and the bulk environment where pH is often measured. At the 20m^3 scale, counter-intuitively, relatively very few dead cells (as measured by flow cytometry) were found (< 0.5%) but the level of biomass was significantly less compared to the 5L scale (32 g/L compared to 53 g/L respectively). These differences in cell biomass and viability were ascribed to the physiological effect on the cells of the combination of the three main heterogeneities, viz., dO$_2$, glucose and pH that occur simultaneously at the large-scale but not at all at the small-scale.

In support of this explanation, the poor mixing was mimicked on the small-scale by using the STR-PFR scale-down experimental model (Table 4), the PFR representing the poorly mixed addition zone and the STR, the well mixed zone in the region of the

impeller. The ratio of the size of the PFR to the STR and the circulation time between them was based on the results from large-scale physical mixing time trials (Vrabel *et al.*, 2000). This scale-down configuration gave very similar results to those found at the large-scale when all three major heterogeneities, dO_2, glucose (Hewitt *et al.*, 2000) and pH (Onyeaka *et al.*, 2003) were introduced into the PFR simultaneously (Table 3). From all of these studies, it was concluded that the STR-PFR scale-down model enabled the impact of the inherently poorer mixing found in large-scale fermenters to be satisfactorily mimicked at the bench-scale.

However, these studies were carried out using the untransformed wild-type bacterial strain, *E. coli* W3110. So the work did not take into account the additional physiological stress imposed on a cell by having to synthesize a foreign protein when studying process performance on scale-up. It is known that the synthesis of foreign proteins at high concentrations exerts a severe metabolic stress on the host cell (Borth *et al.*, 1998; Lewis *et al.*, 2004). Therefore, it was decided to carry out a similar study with a recombinant *E. coli* BL21 (MSD3735) which contains a plasmid coding for an isopropylthiogalactopyranoside (IPTG) inducible, model mammalian protein, AP50. This recombinant system is further complicated because under normal growth conditions as used here, the protein is misfolded, accumulating in the form of insoluble, biologically inactive inclusion bodies in the cytosol of the cell. Relatively low values of agitation intensity, $\bar{\varepsilon}_T$ (~ 1 W/kg) and aeration rate (~1 vvm) were used so that it could be realistically assumed that none of any of the observed effects on biomass or viability could be ascribed to fluid mechanical stresses in the system. In this way, the effect of any physiological or physical stress imposed by the synthesis of

AP50 in addition to that imposed by scale of operation could be investigated (Hewitt *et al*., 2007).

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It is clear that the expression of AP50 as insoluble inclusion bodies exerts a profound physiological stress on the host cell during high cell density fed-batch cultures; and that the extent of these effects are dependent on which combinations of the three major spatial heterogeneities associated with large-scale bio-processing (pH, glucose and dissolved oxygen concentration) are imposed (Table 5). However, the detrimental effect of AP50 production on viability and physiological response can be reduced by the introduction of a spatial or temporal chemical heterogeneity, the extent of which is again dependent on the number and type of heterogeneities imposed. This result is again probably related to the induction of the one of the interlinked regulatory so called 'stress responses' by a proportion of the cells as they pass through the chemically heterogeneous zone of the PFR, such that the resultant dormant cells have a reduced capability for AP50 production and are hence protected from the associated physical or physiological stresses (Hewitt *et al*., 2007).

In the earlier work, simulating at the small-scale the impact of such heterogeneities on the large-scale performance of a fed-batch fermentation, data from an equivalent 20 m^3 commercial fermenter were available for comparison. For this work, such commercial scale data are not available. Therefore, it is not possible to say which of the three scale-down configurations best mimics performance at the large-scale.

IV Conclusions and Future Perspective

The scale-up of single celled aerobic microbial fermentation processes is complicated and unpredictable process performance can result. However, this is not due to the introduction of fluid dynamic generated stresses (or so called 'shear damage'), whether arising from agitator generated turbulence or bursting bubbles, rather it is because the large-scale fed-batch bioreactor provides a very dynamic environment with large spatial and temporal heterogeneities. Such environmental heterogeneities can induce multiple physiological responses in cells. These responses consume energy and resources such that biomass concentration as well as product yields can be reduced. These phenomena are not observed in well-mixed homogeneous laboratory-scale reactors where much process development is done and their effects are difficult to model mathematically. Actual large-scale trials are expensive to carry-out and often not available to the small business or university. Therefore the ability to obtain data on how a recombinant laboratory process may perform at the large-scale, dependent on feeding regime employed or controlling action taken, is invaluable for any detailed and informed development programme. From the work discussed here, it is clear that the scale-down two-compartment model can be used to study the impact of any range or combination of potential heterogeneities known to exist at the large-scale at relatively low cost. Unfortunately, such experiments cannot give precise predictions because the spatial and temporal heterogeneities are only a rather crude approximation of the real ones found at the large scale. It is probable that with increasingly sophisticated CFD becoming available, knowledge of the detailed concentration fields on the large-scale will become available (Schütze and Hengstler, 2006). However, such information will be of limited use until there is either a much

increased knowledge of how cells will respond to such an environment or such conditions can be mimicked on the small-scale. The authors believe that for the foreseeable future, the experimental modelling approach set out here or a variant on it offers the best way forward.

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Figure Legends.

Figure 1. Schematic representation of multiple Rushton turbine impellers in a fermenter.

Figure 2. Newer impellers: a) 'Down-pumping, high solidity ratio hydrofoil' Lightnin' A315 (Po = 0.85); b) 'Up-pumping, high solidity ratio hydrofoil' Haywood Tyler B2 (Po = 0.85); c) 'Hollow-blade' Scaba 6SRGT (Po = 1.5).

Figure 3. The flooding-loading-complete dispersion transitions for a Rushton turbine. a) -flooded; b) -loaded; c) -completely dispersed (Nienow, 1998).

Figure 4. Operating parameters (agitator speed (rpm), aeration rate (vvm), %dO_2,) during continuous cultivation of *C. glutamicum* MCNB 10025 and the resulting OD_{600nm}, CDW g/L and c.f.u./ml (Chamsartra *et al*., 2005).

Figure 5. Scanning electron micrographs of samples taken from different conditions of 'fluid mechanical stress' during continuous cultivation: (a) cells grown under 'standard' operating conditions after 15 h (stirred at 410 rpm ($\bar{\varepsilon}_T = 1$ W/kg), 1 vvm, 40% dO_2); (b) cells from a sample taken during growth under high intensity agitation at 33 h (stirred at 1,200 rpm ($\bar{\varepsilon}_T = 20$ W/kg), 1 vvm, 40% dO_2) and (c) cells from a sample taken during growth under high aeration rates at 51 h (stirred at 410 rpm ($\bar{\varepsilon}_T = 1$ W/kg), 3 vvm, 40% dO_2) (Chamsartra *et al*., 2005).

Figure 6. Large Eddy Simulation of instantaneous glucose concentration in a 22 m^3 bioreactor fed with a 500 g/L solution at a rate of 180 L/h typically used in large-scale fed-batch processes. Four Rushton turbines and the location of the feed point are indicated. The simulation did not include the microbial consumption of the glucose and can therefore only be used to illustrate mixing efficiency (Enfors, *et al*., 2001).

Figure 7. Analysis of mRNA concentrations of four stress sensitive genes at three levels of the 22 m^3 fed-batch culture of *E. coli*. The concentrations averaged from quadruplicate samples were normalized to 100% at the bottom port (Enfors, *et al*., 2001).

Figure 8. Density plots of cell samples taken at times 5hrs (a), 16hrs (b) and 36hrs (c) during a high cell density fed-batch fermentation with *E. coli* W3110 stained with propidium iodide (635nm) and bis-oxanol (575 nm) and analysed using multi-parameter flow cytometry. Three main sub-populations of cells can be distinguished, corresponding to healthy cells (**A**), no staining, cells with no cytoplasmic membrane potential (**B**), stained with bis-oxanol; and cells with permeablised cytoplasmic membranes (**C**), stained with both propidium iodide and bis-oxanol (Hewitt *et al.*, 1999).

Figure 9. Experimental set-up for scale-down simulation studies. a) Large scale STR. Va is the addition zone where the most extreme concentration gradients are known to exist and Vb is the bulk region which can be considered to be well-mixed. b) Scale-down simulation equipment. Here Va is represented by a 0.544L PFR and Vb is represented by a 4L STR (Hewitt *et al*., 2000).

Table 1. Physical aspects of the agitation/agitator requiring consideration (Nienow 1998).

Table 2. Biological aspects that are system specific (Nienow 1998).

Table 3. Experimental protocol and summary of the results for the effect of dO_2, glucose and pH fluctuations on fed-batch fermentations with *E. coli* W3110 (data from Onyeaka *et al.*, 2003).

Table 4. Summary of the scale-down conditions for all of the PFR simulation studies. (Onyeaka *et al.*, 2003).

Table 5. Experimental protocol and summary of the results for the effect of dO_2, glucose and pH fluctuations on fed-batch fermentations of the recombinant *E. coli* strain BL21 (MSD3735) (Hewitt *et al.*, 2006).

Table 1.

1) Mass transfer performance

2) Heat transfer

3) Unaerated power draw (or mean specific energy dissipation rate, $\bar{\varepsilon}_T$ W/kg)

4) Aerated power draw (or aerated $(\bar{\varepsilon}_T)_g$ W/kg)

5) Flow close to the agitator-single phase and air-liquid

6) Variation in local specific energy dissipation rates, ε_T W/kg

7) Air dispersion capability

8) Bulk fluid- and air-phase mixing

Table 2.

1) Growth and productivity
2) Nutrient and other additive requirements including oxygen.
3) CO_2 evolution and RQ
4) Sensitivity to O_2 and CO_2 concentration.
5) pH range and sensitivity.
6) Operating temperature range.
7) 'Shear sensitivity'.

Table 3.

Experiment	Glucose Feed Point	NH₄ Feed Point	Final % Viability**	Final Dry Cell Weight g/L	Air Feed to PFR at 1 vvm*	Residence time in PFR
Well Mixed 5L	STR	STR	84%	55	-	-
PFR1	STR	STR	95%	38	Yes	50s
PFR2	PFR	STR	97%	52	Yes	50s
PFR3	PFR	STR	89%	50	Yes	25s
PFR4	PFR	STR	>99%	37	No	50s
PFR5	PFR	PFR	99%	32	No	~50s
PFR6	PFR	PFR	94%	16	No	~110s
PFR7	PFR	PFR/STR	97%	24	No	~110s
Large Scale	STR	STR	>99%	35	-	-

In all cases the STR was also sparged at 1 vvm and agitated to keep dO_2 in it >20%, a dO_2 level which was also maintained everywhere in the well-mixed 5L and in the large-scale fermentation at the dO_2 electrode.

*1 vvm with respect to the PFR
**With respect to cytoplasmic membrane permeability

Table 4.

PFR1   -   a region of low glucose: pH~7.

PFR2   -   a region of high glucose/ dO_2: pH~7.

PFR3   -   a region of high glucose/ dO_2 but experienced for a shorter time: pH~7.

PFR4   -   a region of high glucose/ low dO_2: pH~7.

PFR5   -   a region of high glucose/ low dO_2: pH>7.

PFR6   -   as PFR5 but for a longer time.

PFR7   -   as PFR6 initially and after 28hrs reverting to PFR4.

PFR8   -   a region of low glucose: pH~7.

PFR9   -   a region of high glucose/ low dO_2: pH~7.

PFR10   -   a region of high glucose/ low dO_2: pH>7.

Table 5.

Experiment	Glucose Feed Point	NH$_4$ Feed Point	Final % Viability[*]	Final Dry Cell Weight g/L
Well Mixed 5L not induced	STR	STR	95.1	48
Well Mixed 5L induced OD$_{550nm}$ ~15	STR	STR	75	18
PFR8 not induced	STR	STR	100	36
PFR8 induced OD$_{550nm}$ ~15	STR	STR	100	15
PFR9 not induced	PFR	STR	99.9	41
PFR9 induced OD$_{550nm}$ ~15	PFR	STR	82.9	14
PFR10 not induced	PFR	PFR	100	34
PFR10 induced OD$_{550nm}$ ~15	PFR	PFR	82.5	14

In all cases the PFR was unaerated whilst the STR was sparged at 1 vvm and agitated to keep dO$_2$ in it >20%. The residence time in the PFR was 60s
[*]With respect to cytoplasmic membrane permeability.

Figure 1.

a b c
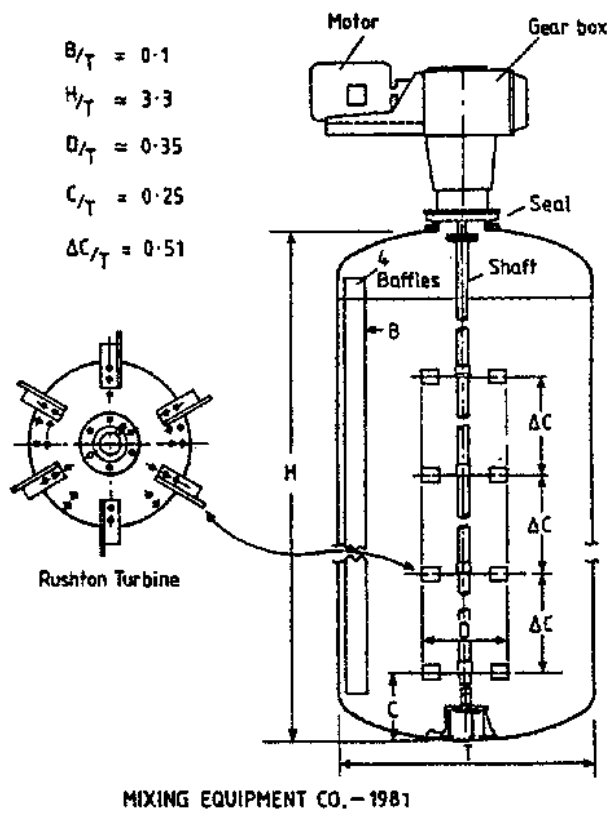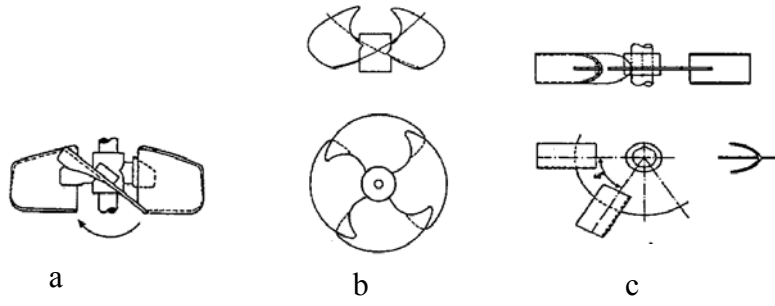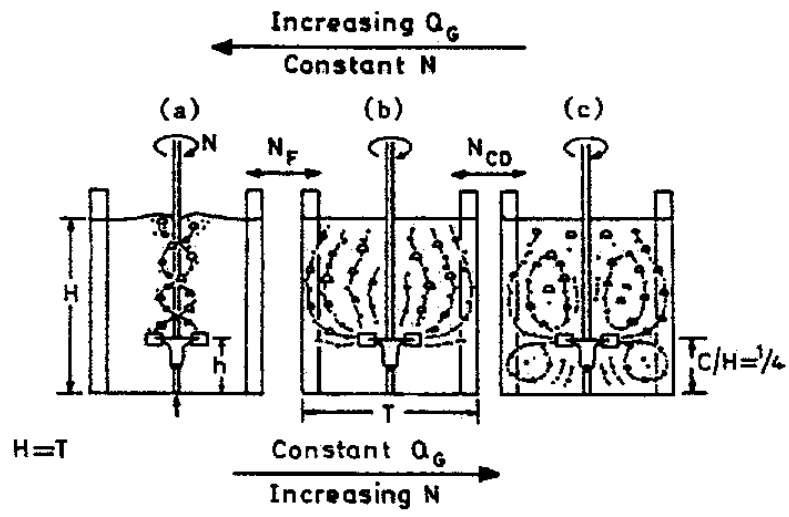
Figure 2

Figure 3

Figure 4.

Figure 5.

Feed
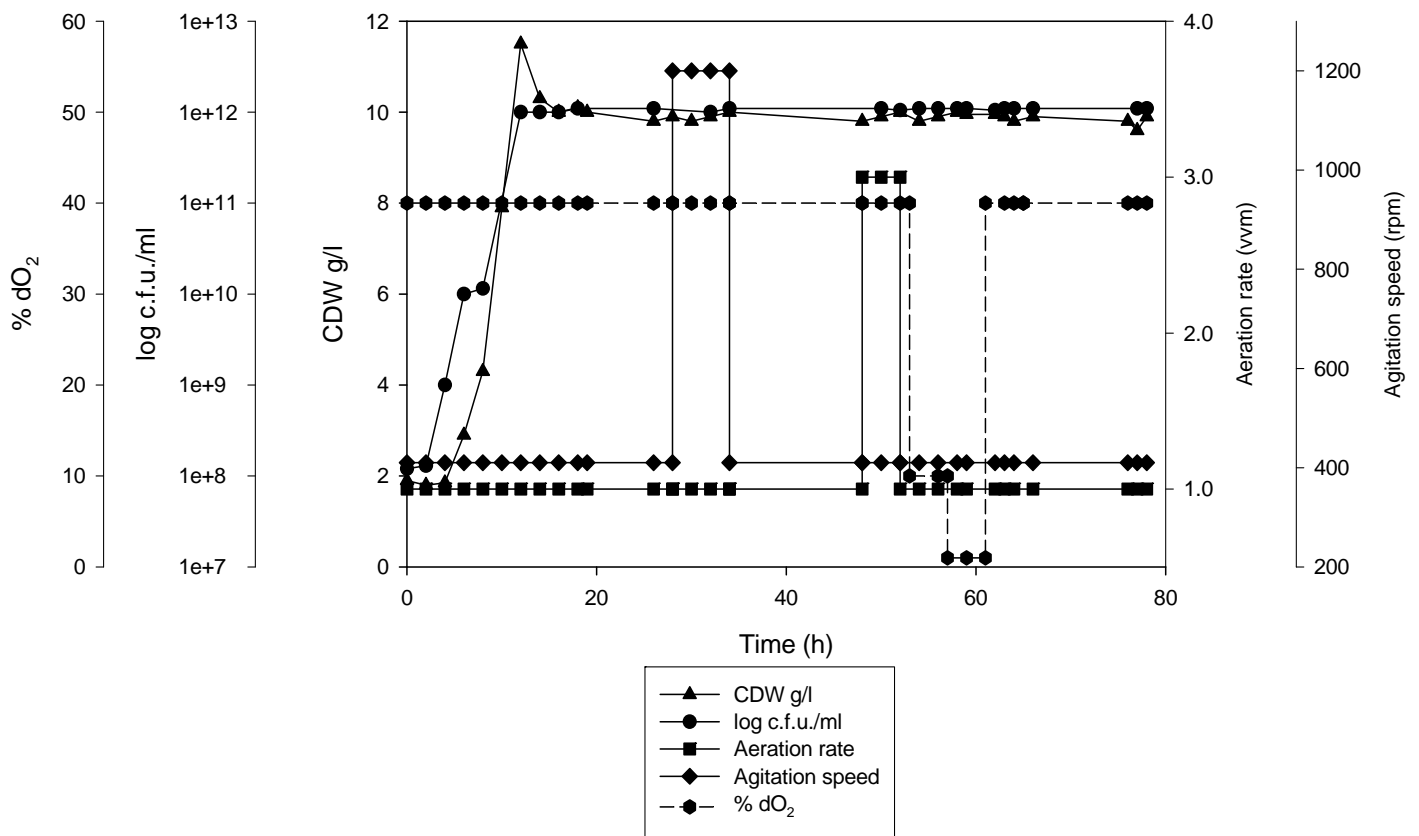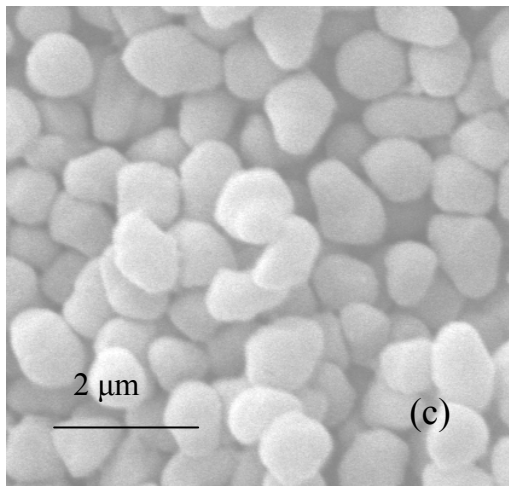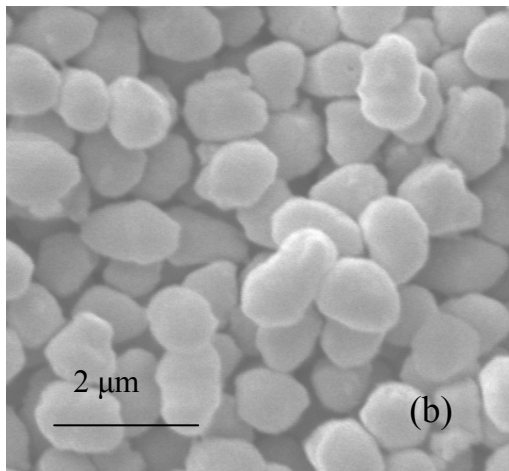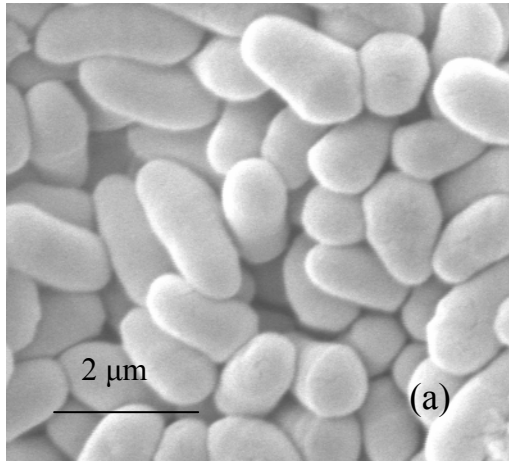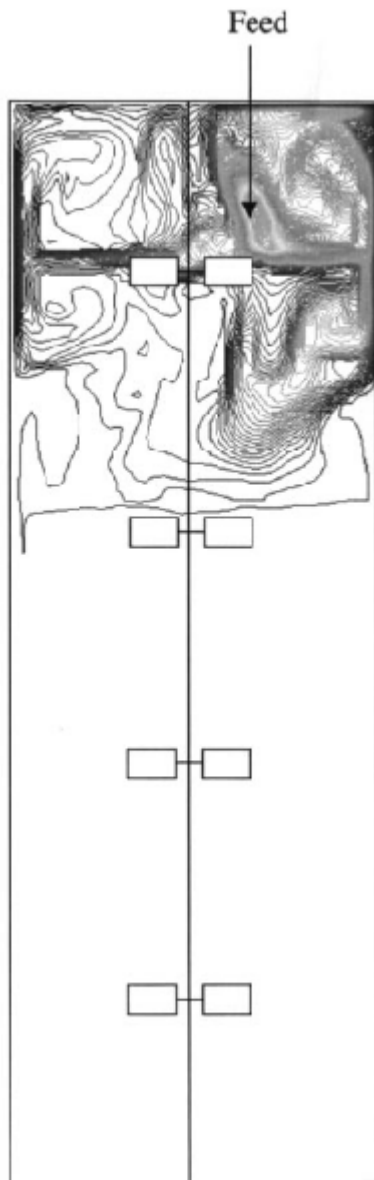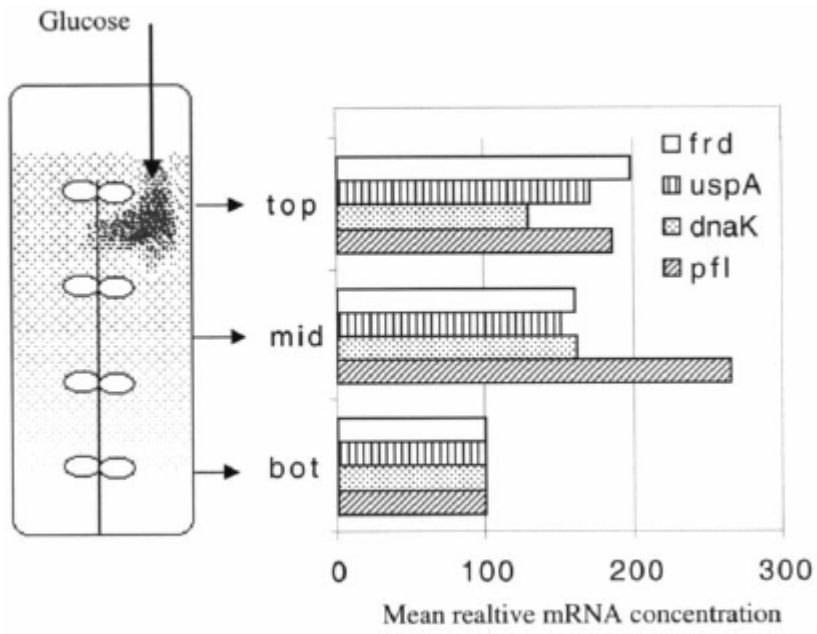
Figure 6.

Figure 7.

Figure 8.

Figure 9.