#### THE INFLUENCE OF SHEAR ON THE FERMENTATION OF

#### Penicillium chrysogenum

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by

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To my husband, Irwan and my families

#### ABSTRACT

Intensive mechanical agitation in the stirred tank fermenters is known to improve mass and heat transfers. However, it can also detrimentally influence productivity and morphology of shear sensitive microorganisms. The influence of shear, meaning the mechanical forces resulting from the rotation of (multiple) turbine impellers, was investigated in batch fermentations of *Penicillium chrysogenum* Panlab P-1 using semidefined media. Experiments were carried out in three different scales of fermenter, 7 L, 150 L and 1500 L total volume, with the impeller tip speed ranging from 2.5 to 6.3 m/s. Throughout all fermentations, the dissolved oxygen concentration never fell below the critical values necessary for growth and penicillin production.

Morphological measurements using image analysis showed that the main hyphal length, total hyphal length and hyphal growth unit increased during the fast growth period and then decreased to a relatively constant value dependent on the agitation intensity. The specific rate of penicillin production  $(q_{pen})$  and the average main hyphal length during the linear penicillin production phase decreased with increase in agitation speed. A higher degree of agitation promoted more rapid mycelial fragmentation and a higher branching frequency. The percentage of clumps was relatively high in all fermentations and was independent of the sample dilution.

Comparison of the results from the three scales of fermenter shows that scale up cannot be based adequately on the impeller tip speed. A model based on the power input per unit mass ( $\varepsilon$ ) was derived using experimental data from the three fermenter scales. It was found that for  $\varepsilon < 10$  W/kg, q<sub>pen</sub> and the mean main hyphal length was practically independent of power input per unit mass, and for  $\varepsilon \ge 10$  W/kg, q<sub>pen</sub> and the mean main hyphal length were proportional to  $\varepsilon^{-0.3}$ . Experimental data from previously published articles were analysed in the light of the model and were found to follow the same general trends as found in this work. However, the transition point from no dependency on  $\varepsilon$  to relatively weak dependency was strain dependent. The data obtained from this work were also found to be reasonably well correlated to the model based on the mycelial circulation through the zone of high energy dissipation (P/D<sup>3</sup>t<sub>c</sub>).

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#### **1** INTRODUCTION

## 1.1 Foreword

Filamentous fungi and Streptomycetes are the most common microorganisms used for antibiotic production. Due to their complex morphologies in submerged cultures during industrial processes, control of fungal fermentations requires great attention. Filamentous microorganisms exhibit two distinct types of morphology in submerged cultures, pellet and filamentous forms. There is no clear boundary between the two types. The filamentous form is commonly referred to as free mycelia. These mycelia often form entangled clumps that can cause the fermentation broth to be highly viscous or non-Newtonian, resulting in poor mass and heat transfer in the fermentation. The pellet type of growth consists of compact discrete masses of hyphae which lead to Newtonian behaviour of the broth. However, as the size of the pellets increases, the diffusion of nutrients into the centre of pellets becomes limited resulting in lower productivity.

The morphology of filamentous microorganisms in submerged culture has been shown to play a critical role in many industrial fermentations. In commercial production of some metabolites it is critical to grow the fungus in a desired morphological form. For example; penicillin requires the filamentous form of *Penicillium chrysogenum* (Dion *et al.*, 1954; Smith & Calam, 1980) while the pellet form of *Aspergillus niger* is required for an optimum production of citric acid (Clark *et al.*, 1966; Al Obaidi & Berry, 1980). The work carried out by Smith (1985) on the effect of agitation on the morphology and productivity of the penicillin fermentation at 10 L and 100 L scale showed that an increase of agitation reduced the penicillin production and the effective hyphal length. Belmar Campero & Thomas (1991) found that an increase in agitation in a 7 L fermentation of *Streptomyces clavuligerus* caused earlier fragmentation, but did not affect clavulanic acid production.

The aim of this project was to study the influence of shear on the morphology and penicillin production of *Penicillium chrysogenum* in 7 L, 150 L, and 1500 L batch fermentations. Semi-defined media were used in order to determine whether, and if so to what extent, agitation actually affects the specific production of secondary metabolites.

# **1.2** Penicillin biosynthesis and process kinetics

# 1.2.1 Biochemical regulation of penicillin synthesis

The industrial development of semi-synthetic and other new penicillin derived antibiotics means that the demand for penicillin is still very high. The demand for penicillin also reflects its lack of toxicity and the susceptibility of the molecule to chemical modification, e.g to semi-synthetic penicillin, which upgrades its utility. Since its discovery in the 1930s, much research work has been carried out to improve the titre or concentration of penicillin as well as to elucidate its biosynthesis. Studies on the biosynthesis have not made a major contribution to the development of the penicillin fermentation. Researchers have been concerned about improvement of the conversion yield of penicillin from glucose as the substrate (Cooney & Acevedo, 1977).

In the mid 1940s, the structure of penicillin was discovered to be a bicyclic structure containing a  $\beta$ -lactam ring which exhibits antibacterial properties (Figure 1.1). In this section the biochemistry and reaction kinetics of penicillin production will be reviewed briefly in order to gain a better understanding of the penicillin fermentation. Studies on penicillin biosynthesis were begun by adding potential precursors to the culture and noting the effect on penicillin production. Early studies were aimed at providing an insight into the mechanism of penicillin formation. Successful results were then obtained when tracer techniques were applied by using amino acid labelling, such as valine and cysteine at various position (Roberts, 1984). It was later concluded that the formation of penicillin is initiated with the incorporation of L- $\alpha$ -aminoadipic acid, L-cysteine, and valine into the tripeptide L- $\alpha$ -aminoadipoyl-L-cysteinyl-D-valine or LLD-ACV.  $\alpha$ -Aminoadipic acid is obtained by diverting an intermediate component from lysine biosynthesis. Higher producing strains of P. chrysogenum were reported to contain higher intracellular  $\alpha$ -aminoadipate pool concentrations during penicillin fermentation, and the level of this pool was found to be very important in determining the flux to penicillin (Hönlinger et al., 1988). The tripeptide is subsequently cyclised and the aminoadipic side chain is exchanged for a suitable precursor, such as phenoxyacetic acid, but the mechanism involved in the formation of the cyclic nucleus remains unknown.



R is  $CH_2OC_6H_5$  for Penicillin-V by addition of phenoxyacetic acid as precursor R is  $CH_2C_6H_5$  for Penicillin-G by addition of phenylacetic acid

Figure 1.1 Basic structure of penicillin (Reproduced from Crueger & Crueger, 1984)

A summary of the biosynthetic route to penicillin is described in Figure 1.2. Since the main precursors of penicillin (valine, cyteine, lysine) are results of primary metabolism, the biochemical regulation of primary pathways also affects penicillin biosynthesis (Vining *et al.*, 1990). Thus, penicillin production is not completely growth dissociated. Demain *et al.* (1983) pointed out that failure to obtain a fully biphasic growth and production pattern may arise from the difficulty of obtaining a true assessment of growth in cultures of filamentous microorganisms. Major biochemical controls that have been observed are feedback inhibition by lysine (Demain, 1957) or by penicillin itself (Martin *et al.*, 1979). At the level of gene expression, lysine biosynthesis in *P. chrysogenum* is regulated mainly by general amino acid control, i.e. by depression of certain enzymes upon starvation of at least one of several amino acids, e.g. histidine or arginine (Jaklitsch *et al.*, 1987).

One of the main regulatory controls of penicillin biosynthesis that has been known for a long time is its dependency on the carbon source used (Jarvis & Johnson, 1947; Soltero & Johnson, 1953). When glucose is depleted or its concentration falls below a repressing threshold, a second carbon source might be used and penicillin production occur. In a medium containing glucose plus a second carbon source, glucose is generally used first, thereby suppressing penicillin biosynthesis. Revilla *et al.* (1984) emphasized that fructose, galactose and sucrose exerted the regulatory effect to the same extent as glucose (~ 70 %), while lactose did not exert suppression of the penicillin biosynthesis.

L-cysteine or L-cystine



Figure 1.2 Biosynthetic route of penicillin

Penicillin-synthesizing activity in control cultures with lactose reached a peak at 24 h of incubation and decreased slowly thereafter as studied with resting cell cultures. Other regulation that has been found is the inhibition by the two main precursors, cysteine and valine (Roberts, 1984), but the side chain precursors, e.g: phenoxyacetic acid or phenylacetic acid tend to simulate the synthesis of the central ring system. In their review, Vining *et al.* (1990) summarized that the control mechanism of precursor supply and enzymes involved in the secondary metabolite production is strain dependent.

# 1.2.2 Process kinetics of penicillin production

The kinetics of product formation and substrate utilisation of filamentous microorganisms in general are very complex. In batch and fed-batch fermentations, a dynamic change in the ability to synthesize and to excrete the secondary metabolites is commonly observed. A typical figure of penicillin fermentation which represents three different regimes of substrate uptake is given in Figure 1.3. During the first 20 h, rapid sugar utilisation is accompanied by an active growth of the culture. As the growth passes into the stationary phase, substrate utilisation slows down while the product formation rate is at its highest peak. Subsequently, the sugar is again consumed rapidly until it is exhausted. No general rule is applicable for all filamentous fermentations, although several exhibit similar patterns.



<u>Figure 1.3</u> The typical kinetic of penicillin fermentation (Reproduced from Bailey & Ollis, 1986)

Bailey & Ollis (1986) summarised some of the peculiar features of filamentous fermentations based on their experimental findings. Firstly, there is an optimum initial substrate concentration for a maximum product yield. Excess substrate results in the inhibition of product formation, but too little substrate will result in inadequate biomass which is essential for product formation. It seems that the substrate is primarily used for biomass production. Secondly, there needs to be an optimal inoculum since product formation can be maximized by minimizing branching in the actively growing hyphae in the inoculum, but the smaller the degree of branching, the longer the lag phase and hence the longer the required batch time.

An early study on penicillin production kinetics was published by Righelato *et al.* (1968) who used the following mass balance equation for the main uses of energy in the antibiotic fermentation,

$$\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)\left(\frac{1}{y}\right) + \mathrm{m}x + \mathrm{p} = \frac{\mathrm{d}O_2}{\mathrm{d}t} \tag{1.1}$$

where x is biomass concentration, y is the yield of cells produced per g nutrient, m is the maintenance coefficient, and p is an allowance for antibiotic production. This equation assumed that the main uses of energy were for cell production, cell maintenance and antibiotic production or other secondary metabolite. Later, it was found that 1 g of penicillin required 2.2 L of oxygen (Ryu & Hospodka, 1980). In the case of penicillin fermentation the value of Respiratory Quotient (RQ) is usually high and steady. The value of unity for RQ which represents the ratio of carbon dioxide produced to oxygen consumed, is reasonable for penicillin fermentation.

The production of penicillin by *Penicillium spp.*, like the synthesis of other secondary metabolites, was considered to be dissociated from the growth of the organism (Pirt & Righelato, 1967). Antibiotic was produced at its maximal rate in batch culture after an initial period of rapid growth of fungi. In a medium where glucose was used as a substrate for growth, the biomass concentration increased in proportion to the glucose supplied. Eventually, the growth rate fell off as a greater proportion of glucose was diverted to the maintenance functions. Wright & Calam (1968) in agreement with results found by Pirt & Righelato (1967) stated that the specific production rate in batch culture was usually higher than that observed in a chemostat. Unlike batch cultures, penicillin formation in continuous cultures appeared to be growth associated. There was a relationship between production and growth rate in which the productivity was constant above a critical value. Later, this was used to optimise the length of the fermentation.

Current industrial practice for penicillin fermentation is to conduct fed batch operations. The use of this system prolongs the penicillin fermentation provided that the extent by which growth rate changes is controlled within certain limits (Court & Pirt, 1976). However, commercial penicillin fermentations normally run for about 7 days because the penicillin-forming enzyme is subject to instability as the fermentation goes on. Humphrey & Jefferis (1973) found that penicillin batches showed genetic run-down by 250 h.

Bajpai & Reuss (1980, 1981) introduced substrate inhibition kinetics for penicillin growth. Their work was based on assumptions that diffusional limitations to the cell occur at high cell densities, and that these effects can be accounted for by elevating the apparent value of the Monod saturation constant. A mechanistic model based on substrate inhibition kinetics found by Bajpai & Reuss (1980, 1981) was used to predict the time course of fed-batch penicillin fermentations. The specific rate of penicillin formation was estimated using the following equation,

$$\frac{dP}{dt} = \frac{q}{K_{p+}} \frac{S}{S(1+S/K_{i})} - KP - \frac{P}{V} \frac{dV}{dt}$$
(1.2)

where P is penicillin concentration  $(kg/m^3)$ , q is maximum specific penicillin synthesis (kg/kg/h), S is substrate concentration  $(kg/m^3)$ , X is biomass concentration  $(kg/m^3)$ , K is first order penicillin decay rate constant (/h), K<sub>p</sub> is saturation constant for penicillin  $(kg/m^3)$ , K<sub>i</sub> is substrate inhibition constant for penicillin synthesis  $(kg/m^3)$ , and V is culture volume  $(m^3)$ .

By considering changes of hyphal ultrastructure, Nestaas & Wang (1983) developed a structural model for penicillin fermentation. The model combines process kinetics of three different cell types, i.e. hyphal tips, penicillin-producing cells, and degenerated, metabolically inactive cells. Changes in the hyphal density measured using a 'filtration probe' monitor the proportion of healthy cells (cells filled by cytoplasm have a relatively high density, ~0.35 g/cm<sup>3</sup>) to degenerated cells (cells with no cytoplasm left and with low density, ~0.18 g/cm<sup>3</sup>). During rapid growth, cell degeneration and penicillin synthesis are assumed to be negligible. It was observed that most of the cell material lost due to hyphal degeneration was protein, while the concentration of cell wall material was relatively constant. This phenomenon might be related to the leakage of intracellular material in the form of nucleotides in filamentous cultures as observed by Tanaka et al. (1975). Nestaas & Wang (1981) suggested that the loss of cytoplasm meant the loss of active components as the hyphal density correlated linearly with the carbon dioxide evolution rate (CER). In addition, the rate of penicillin synthesis (mg/cm<sup>3</sup>/h) was also observed to have a linear relationship with the hyphal density. Pirt & Righelato (1967) considered mycelial vacuolization as a common phenomenon in the penicillin fermentation, and not an indication of a complete lysis of the cells.

# 1.3 Effects of fermentation conditions on the growth, production, and morphology

A wide range of fermentation conditions such as medium composition, pH, temperature, agitation, dissolved oxygen, rheology and foaming can influence the growth, production of secondary metabolites and morphology of filamentous organisms. The following will review the effects of fermentation conditions, other than agitation, especially with regard to *Penicillium chrysogenum*.

## 1.3.1 Carbon source

Studies on media development indicated that di, oligo and polysaccharides were better than glucose for penicillin fermentation (Soltero & Johnson, 1953). Stefaniak *et al.*(1946) compared the use of glucose and lactose as carbon sources. They concluded that the superiority of lactose over glucose as a carbohydrate source lies in its slow availability to the culture. Pentoses, hexoses, dissacharides, polyols, dextrins, starch hydrolysates, and molasses can be metabolized comparably or less efficiently than glucose (Soltero & Johnson, 1953; Perlman, 1970; Queener & Swartz, 1979; Matelová, 1976).

Acetone and ethanol have also been employed as carbon feed sources (Martin *et al.*, 1953; Matelová, 1976). Cell growth and penicillin production was found to be significantly increasing with the addition of n-hexadecane into fermentation (Ho *et al.*, 1990). Solubility of oxygen in the n-hexadecane is approximately eight times higher than that in pure water, therefore the addition of this hydrocarbon improves the oxygen solubility, thus enhancing oxygen transfer rates.

Acetic acid is quickly metabolized by *P. chrysogenum* (Jarvis & Johnson, 1947; Hockenhull *et al.*, 1954) and is therefore quickly removed from the fermentation in the initial stages before glucose feeding is started. Jensen *et al.* (1981) also used acetic acid to improve fed batch fermentations of a low production strain. They found that when 20% of the carbon source was acetic acid, yields of penicillin-V were 25 % higher than in fermentations in which glucose was the only carbon source in the feed. This was explained by an increased availability of precursors of cysteine and valine for penicillin biosynthesis. The pathways for valine and cysteine are known to be related by branching at pyruvate which may be transformed into acetyl Co-A (Hockenhull, 1963). Further work by Norregaard *et al.* (1984) suggested that the acetate effect might be seen with other acids which participate in the material balances around the citric acid cycle. Results of their work gave 50 % increase in penicillin-V yields when propionate and formate were used together with glucose. The yield with succinate was improved to the level observed with acetate (25 %).

Complex media have been reported to be satisfactory for growth and production of secondary metabolites. However, in the interest of metabolic and biochemical studies, the use of complex media is unsuitable. This is due to their unspecified composition. Synthetic media tend to show only some transient penicillin-V production compared to, for example corn steep liquor and the peanut meal based medium (Calam & Ismail, 1980). Through a mass balance on carbon dioxide they also found that cultures in synthetic media required high maintenance energy. Stefaniak *et al.* (1946) and Koffler *et al.* (1945) claimed that the addition of corn steep liquor (CSL) to the lactose media increased the penicillin titre. However, Hosler & Johnson (1953) showed that a fed batch fermentation using a synthetic media medium could give equally good penicillin titres.

#### 1.3.2 Nitrogen and Phosphate

Some amino or ammonium nitrogen was found to be essential for penicillin production, but high concentrations can reduce the yield of penicillin. It has been reported that the ammonium ion is more available to *P. chrysogenum* than nitrate as a nitrogen source (Koffler *et al.*, 1945). Ammonium sulphate provides both nitrogen and sulphur in the proportions necessary for penicillin synthesis (Lurie *et al.*, 1976). Corn steep liquor can act both as carbon and nitrogen source and at the same time provide buffering when no pH control is applied.

Other nitrogen sources that have been reported are cotton seed meal (Foster *et al.*, 1946), Pharmamedia (Queener & Swartz, 1979) and different kinds of brans with a high content of phytic acid and phosphorus (El-Saied *et al.*, 1977). Ammonia has proven to be usable both for pH control and as a nitrogen source in fed batch cultures (Wright & Calam, 1968). In agreement with Lurie *et al.* (1976), Court & Pirt (1981) observed that it was essential to maintain the residual concentration of ammonia at about 0.3 - 0.4 kg/m<sup>3</sup> in order to obtain a high specific penicillin production rate and a continued synthesis of penicillin and to prevent nitrogen/carbon limitation.

Phosphate plays *en* important role in secondary metabolite production by affecting carbohydrate metabolism, inhibition or repression of phosphatases, stimulation of primary metabolism, and inhibition of antibiotic precursor formation (Weinberg, 1974; Martin, 1977). The recommended range of phosphate concentration for antibiotic production varies from process to process. In continuous culture when phosphate was a limiting nutrient, the respiration rate was unaffected by oxygen concentration, when sucrose availability in the medium was between 0.1 and 0.3 M (Mason & Righelato, 1976). However, in fed batch culture, ammonium and sucrose colimitation resulted in a significant reduction of the specific penicillin production rate (Court & Pirt, 1977).

According to Weinberg (1974), the highest amount of inorganic phosphate that favours secondary metabolism is 1.0 mM. An extensive review on the control of antibiotic synthesis by phosphate (Martin, 1977) reveals that inorganic phosphate in the range of 0.3 to 500 mM promotes excellent growth of both procaryotic or eucaryotic microorganism. In addition, 10 mM phosphate will often suppress antibiotic synthesis.

#### 1.3.3 pH

An early study of the penicillin fermentation reported the optimum pH for penicillin production to be in the range from 7.2 to 7.5 (Hosler & Johnson, 1953). Penicillin fermentations nowadays are generally run at a lower value of pH. According to Pan *et al.* (1972), the lower value of pH can probably be attributed to introduction of improved mutant strains, better quality of fermentation nutrients and improved engineering technology.

Moreover, Pan *et al.* (1972) used the pH change phenomenon to control the glucose feed to the fermentation. However, using pH as an indicator of the process during penicillin production is insufficient to guarantee a stable fermentation (Hersbach *et al.*, 1984) since pH is not a very sensitive indicator. If the process becomes unbalanced, it is not always possible to restore the productivity by changing the carbon feed.

Pirt & Callow (1959) observed a change in morphology when growing *P. chrysogenum* in chemostat culture at a dilution rate of 0.05 /h. The density of branching and the diameter of hyphae increased with pH over the range 6.0 to 7.4. Extensive formation of swollen cells occurred at pH values above 7.0 and pellet formation occurred above pH of 6.7. However, these pH changes were not considered as the only factor affecting morphology as nutrition and the strain were also found to be important. According to Miles & Trinci (1983), the effect of pH change is more profound on hyphal diameter than other morphological parameters, and they saw no significant effects on either mean hyphal extension rate or hyphal length.

# 1.3.4 Temperature

It is apparent that the culture utilizes the available nutrients more rapidly at the higher temperatures. Stefaniak *et al.* (1946) demonstrated that the rate of sugar utilization and the rate of carbon dioxide production increased when the temperatures of penicillin fermentations were raised in the range of 20 - 32 °C, but penicillin yields were not appreciably affected at temperatures below 30 °C. Owen & Johnson (1955) found that by starting at a higher temperature (30 °C), followed by operating at constant, low, temperature (20 to 25 °C), the penicillin yield was higher compared to controlled fermentation at 25 °C. Mc Cann & Calam (1972) observed a sharp fall of penicillin yield above 30 °C and recommended an optimum temperature of 25 - 27 °C.

In relation to morphology, Miles & Trinci (1983) showed that in glucose-limited chemostat cultures, the hyphal growth unit (total hyphal length of mycelium divided by the number of branches) and the wall thickness of *P. chrysogenum* increased with an increase of temperature over the range 15 to 30 °C.

## 1.3.5 Carbon dioxide

Carbon dioxide is an essential intermediate in the metabolism of all organisms and consequently it must be present in all culture media. In microbial metabolism, carbon dioxide in general is involved as a biosynthetic substrate in carboxylation reactions or as a metabolic product from decarboxylation reactions e.g. the TCA cycle. It has been widely reported that excessive utilisation of carbon dioxide from the medium has led to low levels of intracellular carbon dioxide, resulting in depressed growth and morphological alteration.

Jones & Greenfield (1982) summarize in their review that inhibitory effects of carbon dioxide include inhibition of cell growth and cellular metabolic processes, and alteration of membrane properties. The TCA cycle of fungi is known to be very sensitive to  $CO_2$  inhibition, with succinate dehydrogenase activity decreasing markedly (Sietsma *et al.*, 1977 and Kritzman *et al.*, 1977). King & Nagel (1967) found that inhibition by carbon dioxide in the *Pseudomonas aeroginosa* cultures was due to a disturbed equilibrium reaction with decarboxylating enzymes. Meanwhile, Mitz (1979) postulates that carbon dioxide reacts with the primary amino groups of membrane proteins by forming carbamine acid anions (RNHCOO<sup>-</sup>), thereby affecting active transport of ions through the cell membranes.

In submerged cultures, the concentration of carbon dioxide will depend on the balance between its production and removal by gas transfer from the liquid to the gaseous phase or air as follows, (per unit volume):

or,

$$R = (q_{CO_2}) X - K_L a (c - c_s)$$
(1.4)

where,  $q_{CO_2}$  is the rate of carbon dioxide production, X is biomass concentration, K<sub>L</sub> is a constant, a is gas liquid interfacial area per unit volume, c is actual concentration of dissolved carbon dioxide, and c<sub>s</sub> is concentration of dissolved carbon dioxide in equilibrium with gas phase. In the liquid phase carbon dioxide forms carbonic acid which dissociates to give bicarbonate and carbonate ions as follows,

$$CO_2 + H_2O \iff H_2CO_3$$
 (1.5)

$$H_2CO_3 \qquad \Leftrightarrow \quad HCO_3^- + \quad H^+ \qquad (1.6)$$

$$HCO_3^- \Leftrightarrow H^+ + CO_3^{-2}$$
 (1.7)

The total concentration of carbon dioxide in the solution is taken as the sum of the concentration of carbon dioxide in aqueous solution plus the concentration of carbonic acid. The total concentration can be related to the external partial pressure of carbon dioxide by the following equation,

$$S = [CO_2] + [H_2CO_3] = HP_{CO_2}$$
 (1.8)

 $P_{\text{CO}_2}$  indicates the partial pressure of dissolved carbon dioxide and H is the solubility of carbon dioxide with a value of approximately thirty times that for oxygen (at 30 °C, the solubility is 0.03 mol carbon dioxide/atm).

During fermentations of *Penicillium chrysogenum* and also in most other fungal fermentations, cultures are exposed to elevated levels of carbon dioxide mostly as a byproduct of their own metabolism. Accumulation of carbon dioxide in the penicillin fermentation in particular has been widely reported. An early study by Koffler *et al.* (1945) found that inlet air containing 0.25 % of carbon dioxide provided the optimum condition for penicillin production. Lower or higher levels could decrease the yield. Stefaniak *et al.* (1946) observed, using a laboratory scale fermenter, that the effect of carbon dioxide was less significant than in an industrial scale unit. This occurred because at low aeration rates (at laboratory scale) the efficiency of air utilization was higher.

According to Lengyel & Nyiri (1965), the presence of 4 - 5 % carbon dioxide measured in the air above the liquid culture medium adversely influenced the respiration, and the rates of sugar uptake and penicillin biosynthesis of *Penicillium chrysogenum* even though the dissolved oxygen in the culture liquid was well above its critical value of 0.02 mM.

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In a later study, Lengyel & Nyiri (1966) observed that the carbon dioxide effect was also dependent on the strategy of antifoam addition. The carbon dioxide concentration changed throughout the fermentation. It accumulated during the evolution of foam bubbles and was liberated when the foam was destroyed, giving an increase in the carbon dioxide concentration of the effluent gas. It was concluded that in order to avoid the damaging effect of accumulated carbon dioxide in the culture liquid, the degree of foaming should be assessed to maintain a good ventilating effect of the aeration.

Using a chemostat culture at fixed values of pH and dissolved oxygen, Pirt & Mancini (1975) pointed out that penicillin synthesis rate (Units penicillin/mg dry mycelium/h) was decreased by 50 % at a carbon dioxide partial pressure of 0.08 atm in the effluent gas compared to the control case of 0.07 atm. It was claimed that the effects of carbon dioxide on the penicillin synthesis was reversible.

Extensive studies on the effect of carbon dioxide on the penicillin fermentation were done by Ho & Smith (1986a, 1986b). The influent gas composition was varied to contain of 3, 5, 12.6, and 20 % carbon dioxide. The total inlet pressure was approximately atmospheric. They found that the carbon dioxide concentrations of 3 % and 5 % in the influent gas gave no effect on penicillin production rate, but 12.6 % concentration inhibited penicillin production by 40 %. The 20 % concentration of carbon dioxide in the influent gas caused an increase in the lag time for cell growth and therefore decreased the maximum cell mass

A high concentration of carbon dioxide in the liquid has been reported to decrease cell size and low cell density and substrate utilisation (Onken & Jostmann, 1984). Ho & Smith (1986b) found that at 0.03 % and 8 % carbon dioxide concentration in the influent air, the predominant morphological form was filamentous, whereas at 15 % concentration swollen and stunted hyphae predominated (Ho & Smith, 1986b). Highly branched mycelia and pellets were also observed in cultures exposed to a high carbon dioxide concentration. They hypothesized that increase in branching frequency was results of induced hyphal growth due to a higher quantity of protons (H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>) released by carbonic acid. Another hypothesis was proposed based on findings that some varieties of fungi have been found to have the ability to attract protons entering the apical zone of growing hyphal tips (Jaffe & Nuccitelli, 1974). The incorporation of protons from the environment has a role in initializing the formation of hyphal tips (Jaffe & Nuccitelli, 1974).

## 1.3.6 Oxygen

#### 1.3.6.1 Mass transfer of oxygen in the fermenter

Oxygen is often an important limiting factor in aerobic fermentations. In order to achieve good productivity, oxygen must be supplied at a rate that meets the demand of the culture system. To ensure the dissolution of oxygen from a gas bubble and its transfer to an individual cell, several independent mass transfer resistances must be overcome (Figure 1.4): 1) resistance from the bulk gas to the gas-liquid interface; 2) penetration of the gas-liquid interface; 3) transfer from the gas-liquid interface to the liquid (fermentation broth); 4) movement within the liquid; 5) movement through the liquid-solid (liquid-cells) interface; 6) diffusive transport into cell aggregates or mycelia; 7) resistance of cell wall; and 8) movement to the intracellular reaction site. The high viscosity of most filamentous fermentations makes the resistance of gas-liquid interface (on the liquid side) more pronounced.



<u>Figure 1.4</u> Mechanism of oxygen transfer from the air bubble to the microbial cell (Reproduced from Bailey & Ollis, 1986)

Based on Fick's Law, mass transfer of oxygen into the liquid can be characterized as follows:

OTR = 
$$k_L a (C^* - C_L)$$
 (1.9)

where OTR is the oxygen transfer rate (mM oxygen/ h),  $k_L$  is oxygen transfer coefficient, a is the gas-liquid interfacial area per unit volume dispersion, C<sup>\*</sup> is the saturation value of the dissolved gas in the gas-liquid interface, and C<sub>L</sub> is the concentration of the dissolved gas (mM).  $k_La$ , which is the volumetric oxygen transfer coefficient (/h), is an important parameter in the design of bioreactors. A number of studies have been done to correlate  $k_La$  with other parameters (Calderbank & Moo-Young, 1961; Fukuda *et al.*, 1968; Robinson & Wilke, 1973; Smith *et al.*, 1977). The mass transfer coefficient has been reported to be dependent on the diameter of the impeller, capacity of the vessel, power input, aeration system, and aeration rate as well as on the density, viscosity, the composition of the nutrient solution, the structure of the microorganism, the antifoam agent used, and the temperature.

Cooper *et al.* (1944) investigated the effect of air flow rate on the  $k_La$  value using a sulphite oxidation method which was not directly relevant to fermentation. The value of  $k_La$  was found to be strongly affected by the degree of agitation, as well as by viscosity. Furthermore, Cooper *et al* (1944) observed that at constant gas flow rate and constant power input per unit volume, the value of  $k_La$  increased as the ratio of liquid height and vessel diameter increased up to a limit: there was no further increase in the  $k_La$  beyond the ratio of liquid height to vessel diameter of two. Maxon & Johnson (1953) found that at constant pressure, the value of  $k_La$  decreased rapidly with increasing volume. This decrease was more pronounced at lower air flow rate.

Air flow rate is frequently kept constant on a volume-volume basis on increasing the scale of fermentation. The range of air flow rate employed rarely falls outside the range 0.5 to 1.5 volumes of air per volume of medium per minute. However, too high an air flow rate can lead to flooding of the impeller by gas, at which point the impeller passes its maximum dispersing capacity. This will reduce the ability of the impeller in assisting the transfer of gas into solution. The flooding point exists in the transition and turbulent flow regions, and is dependent on the D/T ratio (Hempel, 1988). Poor mixing, as found in many mycelial fermentations (due to high viscosity) makes flooding of impeller more pronounced.

Foam formation due to excessive aeration and agitation especially in the fermentation using complex substrates such as corn-steep liquor is very undesirable. A badly foamed system can result in the loss of media and contamination since the foam may overflow from the fermenter via the air outlet or sample lines. Another disadvantage of foam formation is its adverse effect on the oxygen mass transfer as the residence time of bubbles is increased because they become entrapped in the continuously recirculating foam. This will gradually deplete the bubbles of oxygen. In industrial practice the foam is broken down using an antifoam or mechanical foam breakers.

All antifoams have characteristics of surfactants or surface-active materials, which by their nature can also decrease the oxygen transfer rate. Therefore, their level in the culture must always be kept very low. Antifoam may reduce oxygen transfer by interfacial blockage, which results in an increase in mass transfer resistance, or by hydrodynamic changes of the system, thus suppressing the mobility of the surface (Kawase & Moo-Young, 1990). There have been no specific reports on the mechanism of inhibition of mass transfer in penicillin fermentations due to the addition of antifoam. Goldschmidt & Koffler (1950) observed that the use of surface active agents or antifoam in the form of lard oil or oleic acid markedly increased growth and penicillin production of *P. chrysogenum*. However, it was not clearly explained whether these stimulatory effects were due to improved mass transfer or as a result of the ability of the cultures to use those antifoam inaterials as substrates.

## 1.3.6.2 Effect of dissolved oxygen

Dissolved oxygen tension (DOT) is an important parameter in industrial production of various secondary metabolites by filamentous fungi and streptomycetes. Most of these metabolites are produced by differentiated cells, thus it is evident that the critical DOT for growth and the critical DOT for product formation are two distinct parameters and in general the latter is significantly higher. Dissolved oxygen in the fermentation of *Streptomyces clavuligerus* was reported to have a greater effect on the enzyme catalysing the conversion of final intermediate to cephamycin C than on those involved in the earlier steps of the pathway (Rollins *et al.*, 1989). The specific role of dissolved oxygen in penicillin synthesis is not known, however many works have reported the critical values of dissolved oxygen in the penicillin fermentation.

A value of 5 - 10 % air saturation was considered as critical for penicillin production (Squires, 1972), while Bernard & Cooney (1981) stated that 10 % air saturation might be the point of irreversible damage, and König *et al.* (1981) gave the value of 8-10 %. Vardar & Lilly (1982) showed that the DOT value below 10 % air saturation gave irreversible decrease of penicillin production. They also found that 30 % air saturation of DOT was a critical value below which the specific penicillin production rate decreased significantly in a 7 L batch fermentation.

van Suijdam & Metz (1981b) studied the effect of DOT in a continuous culture of *Penicillium chrysogenum* and found that the dissolved oxygen tension in the range of 0.2 to 40 % air saturation had no significant influence on the morphology. Ryu & Hospodka (1980) observed that in a penicillin fermentation operating at a high oxygen concentration, the mycelial growth was very high, but the penicillin production was low. They hypothesized that the mycelial growth was too fast to allow adequate conversion of substrate to product. They also pointed out that beyond a certain concentration of mycelium, the overall rate of penicillin synthesis will decrease because there will be insufficient oxygen to support the increase of specific production rate. Giona *et al.* (1976) found that the specific penicillin production rate increased with increase in dissolved oxygen concentration and was irreversibly damaged below a dissolved oxygen concentration of  $0.22 \times 10^{-4}$  mM.

Dissolved oxygen concentration is also one of the key parameters in scale up strategy. The growth and metabolite production of microorganisms are frequently controlled by the availability of orygen supplied from bubbling air into the broth. Scale up is observed to be more successful based upon maintaining a constant dissolved oxygen concentration in the broth no inatter what the volume of the system is. However, scale up of fermentation systems is not always a matter of maintaining one or two parameters since the phenomena happening in the large scale are very complex. Large scale fermentations are subject to fluctuations of environmental conditions around the vessel. Vardar & Lilly (1982) simulated the conditions which occur in the large scale by sinusoidally cycling the head pressure of a 7 L fermenter to obtain fluctuating DOT around the critical value of 30 % air saturation. Eventhough the mean DOT was still 30 % air saturation, there was a reduction in penicillin production due to cycling. While Ryu & Humphrey (1972) found that 70 % of oxygen uptake rate (OUR) was used for maintenance and observed that this condition should be satisfied in order for biomass production and penicillin synthesis to occur, Vardar & Lilly (1982) proposed that the OUR has to be above 95 % of its maximum value in order to obtain reasonable titres, since 85 % of the OUR being used is required for other purposes in the cell.

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## 1.4 Growth and morphology of filamentous organisms

In submerged cultures, filamentous microorganisms may grow in the form of pellets or filamentous forms. Although the boundary between the two forms is not clear, it is common to define a pellet as a compact discrete mass of hyphae, while filamentous forms exhibit disperse growing mycelia. There are many factors affecting morphology of filamentous microorganism in submerged culture. Camici *et al.* (1952) found that the type of medium and spore concentration affected the morphology of *Penicillium chrysogenum*. In a corn-steep dextrin medium with a spore concentration of more than  $10^3$  /mL, growth will be in the filamentous form, whilst for spore concentrations below  $10^3$  /mL growth is more likely to be in the pelleted form. Meanwhile, for Czapek-dox medium the minimum spore concentration to obtain filamentous growth was 3 x  $10^2$  /mL and for glucose-lactose-ammonium lactate medium it was 2 x  $10^3$  /mL.

In agreement with Camici *et al.* (1952), Smith & Calam (1980) also reported that inoculum size was an important factor in determining the morphology of *Penicillium chrysogenum* and *Penicillium patulum* cultures. They observed different enzyme patterns for Glucose 6-phosphate dehydrogenase (G6PDH), Iso-citrate dehydrogenase (ICDH) and Aldolase in the two strains with the change of spore concentration. The amount of enzymes required and the penicillin production increased with an increase in spore concentration of *P. chrysogenum*. A spore concentration of  $5 \times 10^3$  /ml was claimed to be critical for good growth and productivity.

Fungal morphology also appears to respond to changes in the environment. Caldwell and Trinci (1973) observed that *Geotrichum candidum* exhibited different growth patterns in surface and submerged cultures. The growth in surface cultures is filamentous. In submerged culture, hyphal fragmentation spontaneously occurs and the morphology can alter from the filamentous form to an abnormal shape of hyphae which resembles chains of budding cells. This phenomenon was observed in fermentations with different carbon sources such as galactose, mannose, sorbitol acetate, lactate and pyruvate. As previously discussed, morphological alteration also takes place when an excessive concentration of soluble carbon dioxide is present in the fermentation broth. Oxygen starvation and carbon limitation have been reported to cause degradation of ribosomes, but the cell membrane was found to be particularly resistant to break up (Trinci & Righelato, 1970).

## 1.4.1 Hyphal growth and duplication cycle

The hypha is the basic unit of the filamentous fungal structure. It emerges from a germinated spore at a specific location which becomes the hyphal tip. The spores germinate by increasing the wall thickness with the incorporation of some new wall material. Hyphae can be septate or non septate. *Penicillium chrysogenum* as a member of Deuteromycotina is septate. The hyphae of fungi usually have diameters of 3 to 10  $\mu$ m but some can reach a diameter of 100  $\mu$ m. Oliver & Trinci (1985) concluded that based on the mode of growth, a hyphae could have an extension zone and peripheral growth zone (Figure 1.5). An extension zone which consists of the apical compartment may vary from 2 to 35  $\mu$ m. The extension of hyphae is concentrated at the tips of apical zones, whereas material synthesized in distant regions of the hyphae is transported to the tips and incorporated into the tip walls. During growth, the cell wall does not vary significantly and the growth of hyphae exhibits a linear profile. When hyphal growth is non-linear, Oliver & Trinci (1985) conclude that there must be wall rigidification occurring along a hypha at the same rate as hyphal extension during growth. Intercalary growth mostly occurs in the aerial structures, e.g., conidiophores and sporangiophores.



<u>Figure 1.5</u> Extension zone of a leading hypha *Neurospora crassa* spco 9 from the margin of a mature colony (Reproduced from Oliver & Trinci, 1985)

The peripheral growth zone or the non-apical part of hyphae contains membrane-bound vesicles with diameter between 100 and 400 nm and microvesicles between 30 to 100 nm in diameter. These in turn contain or consist of glycoproteins and enzymes required for wall synthesis. Vesicles contents are released on fusion with the tip wall, while the vesicle membrane contributes to the expansion of the plasma membrane. The incorporation of large number of vesicles containing material required for hyphal elongation into the tip allows fungi to attain extension rates of up to 100  $\mu$ m/min.

Like fungi, actinomycetes also perform apical growth. Incorporation of cell wall material (tritiated N-acetyl glucosamine or GLcNAc) towards the apical region of the parent and branch hyphae of *Streptomyces antibioticus* (Hardisson *et al.*, 1984) and *Streptomyces coelicolor* (Gray *et al.*, 1990) have been reported. The migration of cell wall material in fungi and actinomycetes is reported to be due to hydrostatic pressure acting on an extension zone (Prosser & Tough, 1991). Regrowth of hyphae of actinomycetes after treatment with  $\beta$ -lactam antibiotic caused apical swelling of hyphae and, in some cases, lysis. It was found that  $\beta$ -lactam prevented cell wall rigidification, therefore internal hydrostatic pressure generated by the migration of vesicles caused hyphal swelling and when the internal pressure became greater than cell wall rigidity, cell lysis occurred.

It is believed that fungal hyphae exhibit a similar pattern of integration between growth and cell division that is found in yeast and bacteria. The term duplication cycle is used to describe these events (Trinci, 1978). Prosser & Tough (1991) extensively reviewed the growth mechanisms and kinetics of filamentous microorganisms. The duplication cycle is slightly different among monokaryotic, dikaryotic and coenocytic species of fungi. In monokaryotic and dikaryotic species of fungi, the nucleus is seen to remain in a constant position relative to the tip and consequently must migrate at a rate equivalent to the rate of tip growth. When the apical compartment reaches a critical state, nuclear division occurs followed by septation. The timing of these events is regular indicating a cyclic nature. The situation in coenocytic fungi, e.g., Aspergillus nidulans, where there are many nuclei present (in some instances up to 90) is identical but with slight modification. Nuclear division is not completely synchronous and where a large number of nuclei are involved the division event may last for up to 20 min, until each nucleus has divided. The formation of several septa in the coenocytes divides the compartment into an apical segment and a number of other segments each of variable size and nuclear number. In all monokaryotic, dikaryotic and coenocytic cases, the apical compartment is reduced to half its length at septation thus maintaining constancy in size.

# 1.4.2 Hyphal branching, growth unit and fragmentation

Branching of hyphae can be considered as a modified form of tip growth in which similar types of cytoplasmic vesicles still fuse with the plasma membrane along the sides of the hypha. Hypha branches arise either adjacent to the septa or on the lateral walls of apical or sub-apical hyphal compartments. In the species where branches form behind the septa, vesicles migrating towards the apex may become trapped behind septa, and their accumulation may trigger wall lysis, swelling and subsequent branch formation (Trinci, 1978). Branches usually form only in the sub-apical zone. In the older parts of the hypha, there is often a zone of vacuolization containing small amounts of cytoplasm and vacuoles that become larger and more numerous with increasing distance from the hyphal apex. This zone has been reported to be important in the production of secondary metabolites. Hyphal branching of *Geotrichum candidum* is closely linked to the septation with the evidence that a single branch is formed per septum, usually behind the septum, and the time between septation and branch formation is relatively constant (Fiddy & Trinci, 1976). Aspergillus nidulans strains is an example of fungi where branch formation is independent from septation (Trinci & Morris, 1979). Vesicles containing cell wall material can continue to migrate towards the hyphal tip in the presence of septa, and the period between septation and branch formation varies considerably.

Katz et al. (1972) and Trinci (1974) observed that if the hyphal growth unit exceeds a critical length, a new branch is initiated. For an exponentially growing mycelium the rate of extension of a hyphal tip reaches a maximum characteristic of the strain (Katz et al., 1972). In batch cultures, it is difficult to observe exponential growth of microorganisms especially for *Penicillium chrysogenum*. Based on observations on the growth of Aspergillus nidulans, Katz et al. (1972) stated that the specific growth rate ( $\mu$ ) can be considered as the ratio of the mean tip extension rate, E (mm/h) and the hyphal growth unit, G ( $\mu$ m). They found that the number of branches per unit of hyphal length increased with growth rate. Meanwhile, Metz (1976) found that the total hyphal length of *Penicillium chrysogenum* in a chemostat culture increased with growth rate, but the hyphal growth unit did not vary. In other words, the hyphal growth unit was independent of specific growth rate. Caldwell & Trinci (1973) defined the length of the hyphal growth unit as the ratio between the total hyphal length and number of hyphal tips. Furthermore, it was observed that there were two possible relationships between the hyphal growth unit and the specific growth rate in the mycelial cultures. First, the hyphal growth unit is maintained at constant value while other parameters such as the

specific growth rate, the mean extension rate of the apical cells and the number of lateral branches increase. This phenomenon was observed in the cultures of Geotrichum candidum in which fragmentation occurred naturally. Second, the hyphal growth unit decreases although the specific growth rate increases. In this case the mean extension rate of apical cells remains constant and the number of branches per unit hyphal length becomes larger. It was concluded that data observed by Katz et al. (1972) represented the second phenomenon. The results found by Fiddy & Trinci (1975) in glucose limited chemostat cultures followed this second theory. In contrast, Robinson & Smith (1976) did the same experiment as Fiddy & Trinci (1975) but the result did not fit either the first or the second phenomenon. A repeat experiment later confirmed their previous results and they concluded that by increasing specific growth rate, more branching, thicker and shorter mycelia were observed but the mean extension rate of the apical cells increased (Robinson & Smith, 1979). In deriving a model to describe the influence of growth rate and shear on the morphology, van Suijdam & Metz (1981b) considered that a combination of the two phenomena described above could occur, and found that branching frequency was independent of specific growth rate of P. chrysogenum grown in a glucose limited chemostat cultures.

The mechanism of fragmentation in filamentous systems is not as clear as in fungal pellets. Taguchi (1971) described that fragmentation of pellets involved two shear-dependent processes : chipping off pieces of hyphae from outside the pellet and total rupture of the pellet. Rupture of pellets follows a first order decrease in pellet numbers. The factors that cause total rupture to take place can be a high circulation frequency of the pellet passing the zone of high shear rates in the fermenter, or a considerable increase in the pellet size which results in the decrease of tensile strength or oxygen limitation in the centre of the pellet. Fragmentation. This mechanism is known as dimorphism and has been found in the cultures of *Geotrichum candidum* (Caldwell & Trinci, 1973) and *Cephalosporium acremonium* Corda (Drew *et al.*, 1976) due to the presence of inhibitors.

Shear in fermenters has been studied as one of the factors causing fragmentation, but the progress so far is not sufficient to conclude that high shear rate is responsible for mycelial break-up. Similar size of mycelial fragments could be found in shake flasks and stirred tank fermenters, although the shear rates in the shake flask are likely to be smaller. It is also possible that fragmentation only takes place on a particular region along the hyphae where the degree of vacuolization is significant. This may happen as a

result of autolysis of some hyphal compartments and degradation of the wall of the lysed compartment. However, vacuolization is not necessarily a phenomenon of hyphal breakup or 'total' autolysis of a hyphae. It has been observed that occasional compartments showing advanced signs of autolysis can be produced in the middle of an intact and growing hyphae of *P. chrysogenum* (Righelato *et al.*, 1968; Trinci & Righelato, 1970). Controlled fragmentation in the fermentation would be of considerable value in terms of reduced viscosity and good oxygen transfer, but this might require an enormous increase in energy input. Therefore, van Suijdam & Metz (1981b) stated that increasing shear stress in order to generate fragmented mycelia and hence reduced viscosity of the broth would not be practical.

# **1.4.3** Factors affecting cell wall structure of filamentous microorganisms

Mechanical agitation is not the only factor affecting microbial structure. Environmental factors such as the presence of antibiotic or toxic compounds and osmotic shock have been reported to affect cell wall strength. The effect of agitation on the microbial structure will be discussed separately in section 1.5.1. The results of the effects of cell environment or physical forces exerted on the cell wall can be either a decrease in the wall strength which may lead to complete break up or an increase in one component of the cell wall composition such as chitin in order to protect the cell content. Moss & Badii (1980) examined the effect of Rubratoxin B on the cell wall of Aspergillus niger. Rubratoxin B is a toxic metabolite produced by some strains of Penicillium rubrum which grow as parasites in cultures of A. niger and A. flavus. The results were an increase in the neutral carbohydrate component and a decrease in the protein content. They found that in the presence of Rubratoxin B, the hyphal tips of A. niger became swollen and some vacuolization occurred along the mycelium. Such swelling diminished the strength of the cell wall and therefore the cell wall became very sensitive to the turgor pressure of the cytoplasm. Swollen cells were also found in cultures of P. chrysogenum when the pH was increased above the optimum value (Pirt & Callow, 1959). They postulated that this was because the cell wall became non-rigid and the decrease of hyphal length was considered as a sign of weakening of the cell wall. Under microscopic examination, the hyphae of *Penicillium chrysogenum* grown at low specific growth rates exhibit more vacuolization than those grown at high specific growth rates (Righelato et al., 1968). Cell wall composition was found to be unaffected by osmotic

shock as shown by the continuity of protein synthesis in the cell (Katz & Rosenberger, 1971). The decrease in cell wall rigidity of *Streptomyces coelicolor* due to the addition of a  $\beta$ -lactam antibiotic in the medium was reported to be accompanied by hyphal swelling (Gray *et al.*, 1990). Edwards & Ho (1988) observed a marked increase in the chitin synthesis along the apical and sub apical zone of *P. chrysogenum* due to carbon dioxide exposure. The swollen and highly branched mycelia were thought to be the result of stimulatory effects of high concentration of chitin in plasticizing the cell wall. Tanaka (1976) observed the phenomenon of a reduced cell wall rigidity resulting in leakage of nucleotides from mycelia which were exposed to a range of mechanical agitation. However, there has been no report on the cell wall characteristics of filamentous microorganisms under high shear exposure.

# 1.4.4 Morphological measurements of filamentous cultures

The influence of process conditions on the morphology of microorganisms were examined only qualitatively until Metz *et al.* (1981) established quantitative morphological parameters for this purpose (Figure 1.6). They selected morphological parameters such as: length of the main hypha or effective length, the total hypha length which is the main hypha length plus length of all the branches, mean length of the segments, the number of branches and the hyphal growth unit which is the ratio of the total hypha length and number of branches. The method used was based on an electronic digitizer where photographs of the microorganisms were taken and projected onto a digitizing table so that the image could be traced with a cursor (Figure 1.6). The system was applied to *Penicillium chrysogenum* batch and continuous fermentations. It was found that using this digitizing method the reproducibility of data for continuous fermentations was satisfactory, but for batch culture was poor.

An image analysis method has been developed by Packer & Thomas (1990) to characterize mycelial morphology in terms of parameters established by Metz *et al.* (1981) and the proportion of biomass in clumps which might be related to the permanent aggregates in a fermenter. This method has many advantages in terms of time and accuracy, compared to the digitizing table. The image analysis system consists of a television camera mounted on the microscope and a computer which is able to digitize an image from the microscope both in space and tone. The output of this enhanced image is then segmented to separate the regions of interest from the background.



Figure 1.6 Layout of digitizing table with a projected mycelial particle (Reproduced from Metz *et al.*, 1981)

A binary image results which defines the objects of interest in the original grey image. The system is then able to find the lines in the middle of opposing edges of the objects in the binary image (skeletonization), and the results are stored, measured and analysed statistically. The image analysis has been proved successful in studying the effect of stirrer speed on the morphology of *Streptomyces clavuligerus* (Belmar Campero & Thomas, 1991). The application of image analysis has also been tested on a batch culture of *Penicillium chrysogenum* P1 and a fed-batch culture of *Penicillium chrysogenum* P2 containing solid media (Packer & Thomas, 1990). It was shown that the presence of solid media ingredients did not interfere with the measurement. Both *Penicillium chrysogenum* P1 and P2 gave very high proportions of clumps (above 70%). It was shown that those clumps were permanent aggregates since an increase in dilution had no effect on the percentage of clumps found.

Using an image processing system connected to a growth chamber, the early growth and branching of *Streptomyces tendae* was studied (Reichl *et al.*, 1989). The use of a growth chamber (size of 40 x 95 mm) was aimed at simulating the conditions of submerged fermentation. It was claimed that most of the parameters could be measured on line with an accuracy comparable to those observed by Adam & Thomas (1988), but

the method was much slower than that of Packer & Thomas (1990). Compared to previously published results of solid growth, Reichl *et al.* (1989) concluded that mechanisms for growth of streptomycetes were the same for both solid and liquid media. However, this is not necessarily true; although the growth chamber used was equipped with temperature control and half filled to ensure oxygen supply, the absent of mixing could lead to the aerial growth of the cultures. Thus, it did not represent a truly submerged growth.

## **1.5** Effect of agitation on mycelial fermentations

In submerged fermentations, agitation is important to provide good mixing, mass transfer and heat transfer. For an aerobic system, mixing is required to ensure sufficient oxygen transfer throughout the vessel. In the case of a stirred tank fermenter, there are two important regions. The area around the impeller represents a region of very good mixing hence good mass and heat transfer. On the other hand, the area close to the vessel wall may suffer from an inadequate supply of oxygen, because mixing is relatively poor in that part of the vessel. In large scale fermenters, the gradient of mass transfer across the vessel becomes significant and this can affect mycelial growth and product formation. Therefore, the application of multiple impellers is needed to ensure good overall mixing.

Secondary metabolites are mostly produced by filamentous microorganisms which, due to their morphological nature, tend to form entanglement. The entanglement of mycelia creates shear thinning (pseudoplastic) behaviour. As the cell concentration in the broth increases, the viscosity increases resulting in a decrease in oxygen transfer coefficient. Increasing stirrer speed can reduce broth viscosity up to a point, as found by Gbewonyo *et al.* (1986) in Avermectin fermentation. They suggested that the use of a hydrofoil impeller, instead of the standard Rushton turbine, could improve the oxygen transfer efficiency in mycelial cultures probably because of better bulk mixing. The use of small pellets can be advantageous from mass transfer point of view since it can reduce broth viscosity. However, the formation of large pellets during fermentation can result in oxygen starvation in the interior of the pellets, thus leading to lower productivity.

Agitation applied during fermentation creates shear forces which can affect microorganisms in several ways, e.g. damage to cell structures, morphological changes and variation in growth and product formation. Märkl & Bronnenmeier (1985) observed that damage to microorganisms in a fermenter agitated with a turbine agitated impeller could be the result of two mechanisms. Firstly, the change of pressure which occurs rapidly over the blade, from the back of the blade (low pressure region) to the impeller tips (high pressure region created by a strongly accelerating stream). Secondly, the specific shape of turbine tips, for example the Rushton turbine, tends to create shear stresses. Furthermore, they considered several possibilities for the way in which the microorganisms would respond to these stresses. Firstly, microorganisms exposed to the high energy dissipation zone may be damaged but may manage to recover. The ability to recover will depend on the degree of damage and the recovery rate. Secondly, exposure to a high shear zone will not cause instantaneous damage: damage will occur gradually due to hydrodynamic stresses. This means that the microorganism has the ability to adapt to a certain level of mechanical stress. Finally, these effects may depend on the age of the cell. Some filamentous microorganisms can respond to agitation by increasing chitin synthesis of the cell wall, as was observed by Pitt & Bull (1982) in an ammonia limited chemostat cultures of Trichoderma aureovide. Increasing stirrer speed from 800 RPM to 1000 RPM in a 1.5 L fermenter with a 0.5 cm impeller diameter resulted in the formation of non growing tips. At 1200 RPM an increase in hyphal diameter became significant and partial autolysis was observed at 1300 RPM. When the agitator speed was finally increased to 1500 RPM, the formation of growing tips resumed suggesting an ability to counteract the shear applied onto the system.

# 1.5.1 Agitation and release of intracellular material

Mechanical agitation has been reported to cause the release of intracellular substances consisting of RNA-related nucleotides, mostly mononucleotides with a maximum absorption at 260 nm or intracellular low molecular nucleotides (Tanaka, 1976). Tanaka *et al.* (1975) introduced an index refereed to as R to represent mycelial strength in maintaining the physiological activity. R is defined as the ratio of the leakage rate of low molecular nucleotides under fixed agitation conditions (measured as extinction at 260 nm or E260) and the total amount of nucleic acid-related substances contained in the mycelia. By examining 18 strains of filamentous microorganisms, they claimed that leakage of nucleotides due to agitation was a common phenomenon. The value of R was claimed to
be independent of hyphal diameter but was influenced by the viscosity of the culture liquid. In the case of *Mucor javanicus*, leakage increased with an increase in apparent viscosity of culture liquid in the range of 0.6 to 600 cp. They considered *P*. *chrysogenum* as the weakest strain in comparison to *M. javanicus*, *M. alternans* and *Aspergillus oryzae* at the same nominal mycelial length. Using *Mucor sp.* and *Rhizopus sp.*, Tanaka (1976) later proved that as the agitator speed in a 5 L (jar) fermenter was increased in the range of 0 to 800 RPM, the release of nucleic-acid related substances also increased. The release of nucleotides was considered to be dependent on the strain, scale, and the aeration/agitation system. Continuous agitation at 600 RPM for 120 h resulted in a considerable release of low molecular nucleic acid related substances from the cells. A small amount of high molecular nucleic acid related substances was also found in the supernatant. When zinc ions were added into the system, the excretion of intracellular material stopped. Further increase in tip speed appeared to increase the excretion.

In contrast, Musílková *et al.* (1981) found that a high agitation condition led to a morphologically compact and strong mycelium and therefore the cell wall was able to provide a greater resistance to the action of hydrolysis. Ujcová *et al.* (1980) also did not find evidence to suggest that an increase in agitation speed is responsible for the release of intracellular materials. One strain of *Aspergillus niger* S59 used in their experiments showed a minimum leakage of intracellular material after being exposed at the highest speed in a shake flask fermentation. They also observed that the ability to release intracellular material was strain dependent and not directly correlated to production. Several different *Aspergillus niger* strains were examined. In each case, maximum production of citric acid was achieved at a different range of stirrer speeds. In addition, young hyphae was unlikely to be affected although their morphology reflected the effect of mechanical forces.

Reuss (1988) investigated the influence of mechanical stress on the growth of *Rhizopus* nigricans in 15 L, 75 L and 300 L fermenters. The impeller to tank diameter ratio was a variable. The results showed that increasing stirrer speed resulted in the reduction of maximum specific growth rate ( $\mu_{max}$ ). However, it was suggested that the molecular nucleotides measured in the supernatant, as proposed by Tanaka *et al.* (1975), could not be considered as an absolute parameter of the effect of mechanical stress on microorganisms. The presence of nucleotide in the supernatant should be regarded as a result of growth activity as well as excretion. Consequently, mycelial growth rate will depend on the balance of nucleotide excretion due to mechanical stress and growth associated nucleotides.

# 1.5.2 Effect of agitation on the growth, production and morphology

Unlike for fungal pellets, the superimposed effects of agitation in filamentous fermentation are difficult to quantify. For example, in many works better mycelial growth due to improved oxygen transfer is often claimed as a positive effect of agitation. In contrast, Tanaka (1976) et al. found that mycelial growth of different species of fungi decreased with increase in agitation. König et al. (1981) investigated the influence of agitation and viscosity on the penicillin fermentation. Different type of impellers, fermenters and strains were used in order to generalize the results. High and low production strains gave similar responses to agitation, except that higher production strains showed a dissociated growth pattern more clearly. High agitation speed at 1250 and 1500 RPM resulted in lower penicillin production and higher cell growth in comparison to those at 900 and 1000 RPM. At 700 RPM, oxygen limitation was observed and penicillin production was as low as that found at 1500 RPM. In conclusion, the results were not sufficient to claim that high shear rate due to intense agitation caused low productivity. High impeller speed was found to promote mycelial growth and possibly to stimulate the occurrence of unclear pathways which ended in low productivity of penicillin. For equivalent impeller speeds, disc turbine impellers gave higher value of maximum penicillin concentration compared to propellers. The authors assumed that this was due to a much higher specific power input and hence oxygen transfer rate for turbines although the two batches showed very similar profiles in carbon consumption rate and biomass concentration.

The effect of impeller geometry on a filamentous fermentation was reported as early as 1962 by Steel & Maxon. In a fermentation of *Streptomyces niveus* producing novobiocin at 20 L scale, the use of a larger impeller diameter at constant agitator speed resulted in a greater degree of reduction in oxygen availability rate (OAR). However, there was no direct correlation between novobiocin yields and impeller diameter at a constant value of OAR. OAR was used to represent the rate of oxygen uptake measured under conditions in which the culture was limited in its respiration rate. This seems to suggest that *Streptomyces niveus* is more sensitive to oxygen supply than to any shear effects possibly due to their relatively small size. In a 7 L batch fermentation of *S. clavuligerus*, Belmar Campero & Thomas (1991) did not find a direct effect of agitation on clavulanic acid production, but an increase in stirrer speed in the range of 490 and 1300 RPM showed significant changes in morphological parameters measured using an image analyser.

When *P. chrysogenum* was grown in a complex medium in a glucose fed batch fermentation, a decrease in the penicillin production rate and a drop in the effective hyphal length due to agitation did occur (Smith *et al.*, 1990). Morphological measurements in a 150 L scale (100 L working volume) fermenter showed a slower decrease in the effective hyphal length in comparison to those from a 14 L scale (10 L working volume) vessel. Earlier, Dion *et al.* (1954) investigated changes in morphology and penicillin production of *P. chrysogenum* over a wide range of fermentation scales (10, 3000, and 12,000 L), although most observations were carried out in 10 L scale. It was found that biomass concentration did not vary with agitation speed. Penicillin production was best produced by short hyphae with sufficient aeration and optimum agitation. Under intense agitation, the cultures consisted of predominantly short, thick and highly branched mycelia which were assumed to be relatively young hyphae.

In order to obtain a reasonably uniform shear rate throughout the culture liquid, Mitard & Riba (1988) carried out an *A. niger* fermentations in an annular bioreactor constructed from two coaxial cylinders. The culture broth was placed between the two cylinders and subjected to a range of shear rates by rotating the outer cylinder up to 1500 RPM. Increasing rotational speed in the range of 100 to 745 RPM resulted in the earlier peak of specific growth rate ( $\mu$ ). There were two peaks of specific growth rate observed during every fermentation: the first one was claimed to be due to pellet growth, and the second peak was due to filamentous growth. In between the two peaks, there was a stagnation period when the pellet was releasing filaments which then grew filamentously to reach the second peak. As the speed was increased, the first peak became more and more invisible and at 1500 RPM they observed only one peak. With all these results, they concluded that higher speeds prevented spore aggregation and promoted earlier filamentous growth. However, at the end of every fermentation the microorganisms would adapt themselves to the high shear rates by producing more branches leading to pellets formation to improve mechanical resistance.

In addition, Mitard & Riba (1988) agreed with Tanaka *et al.* (1975) that the release of nucleotides does take place but only as a response to excessive shear rates both from filamentous and pellets or in the case when a filament must adapt to a high shear rate.

## 1.6 Scale up and modelling of fungal break up

### 1.6.1 Scale up of bioreactors

The aim of scale up is to reproduce successfully on production scale results obtained in a laboratory size or pilot plant equipment. Usually, laboratory fermentation is used to screen strains and optimise media and fermentation conditions, while pilot plant fermentation is carried out to verify whether the process conditions are optimum and to produce sufficient data for evaluation. There are three main factors involved in the scale up process, namely biological, chemical and physical factors. Scale up is usually based on the physical factors of the process although not all physical factors can be used as scale up parameters. For example, geometric similarity is likely to change as the scale gets bigger, the ratio of liquid height to tank diameter (H/T) goes up and the ratio of the impeller diameter to tank diameter (D/T) goes down. Some conditions required for growth such as temperature, pressure, and nutrient concentration that have been proved successful in a laboratory scale are often maintained during a scale up process. Scale up may be based on maintaining constant any one, or combination, of the following groups:

- (1) Power input per unit volume (P/V)
- (2) Impeller tip speed,  $V_{tip} = \pi ND$
- (3) Mixing times, t<sub>m</sub>
- (4) Reynolds number, Re =  $\rho ND^2/\eta$
- (5) Volumetric oxygen transfer coefficient ( $k_La$ )
- (6) Oxygen transfer rate (OTR)
- (7) Critical dissolved oxygen concentration (DOT)
- (8) Carbon dioxide concentration or partial pressure

The two most widely reported methods are based on the constant power consumption per unit volume and constant oxygen transfer rate. Mixing time is reported not suitable as a scale up parameter since its value increases with reactor size (Einsele, 1978). Reynolds number and tip speed are also unsuitable, because in large scale units, shear damage may be significant: scale up based on a constant tip speed means that the level of shear experienced by the mycelia increases with scale up. Typical values of tip speed in many production fermenters were in the range of 5 - 7 m/s. Several studies have reported the successful scale up based on power input per unit volume. Maxon (1959) found that at a power input per unit volume below 1.1 kW/m<sup>3</sup>, novobiocin production by *Streptomyces niveus* was dependent on the impeller diameter (three fermentations were

carried out with different impellers diameter: 0.3T, 0.4T, and 0.5T). At a power input per unit volume of 1.1 - 2.1 kW/m<sup>3</sup>, the three fermentations gave approximately the same value of maximum yield of novobiocin. Later, Steel & Maxon (1962, 1966) reported that the limiting factor in the novobiocin fermentation was the mass transfer from the liquid to the cell agglomerate of *Streptomyces niveus*, and proposed a scale up formulation based on the oxygen uptake rate and impeller tip speed. The results were further analysed by Wang & Fewkes (1977) who observed that the limiting oxygen transfer to the pellet surface of *Streptomyces niveus* was dependent on the turbulent shear stress ( $\tau$ ) in the fermenter and pumping capacity (Q<sub>p</sub>). The ratio of  $\tau$ /Q<sub>p</sub> was reported proportional to the ratio of impeller speed and impeller diameter (N/D).

Successful scale up of flavomycin fermentation has also been reported from 4 to 40 m<sup>3</sup> scale, based on constant P/V (Sukatsch *et al.*, 1976). In the case of the penicillin fermentation, Gaden (1961) and Humphrey (1964a & b) reported that P/V could be a good scale up parameter. Gaden (1962) proposed an optimum P/V value between 1.5 and 3 kW/m<sup>3</sup>, while a value between 1 and 2.5 kW/m<sup>3</sup> was established by Humphrey (1964b). However, a comparative study of many production plants with operating volumes in the range of 0.5 to 300 m<sup>3</sup> showed that no constant P/V ratio was found (Einsele, 1978), and the following relationship was observed:

$$\left(\frac{\mathrm{P}}{\mathrm{V}}\right) \propto \mathrm{V}^{-0.37}$$
 (1.10)

Successful scale up of the penicillin fermentation based on constant oxygen transfer rate (OTR) and constant volumetric oxygen transfer coefficient ( $k_La$ ) has been reported by several groups (Karow *et al.*, 1953; Wegrich & Shurter, 1953 and Bylinkina *et al.*, 1976). Karow *et al.* (1953) found that at a OTR value of more than 0.5 mol/L/h, the penicillin yield was maximal and nearly constant for the fermentation scales in the range of 5 x 10<sup>-3</sup> m<sup>3</sup> to 57 m<sup>3</sup>. Wegrich & Shurter (1953) kept the superficial gas velocity and P/V constant, which is essentially equivalent to constant  $k_La$ . Bylinkina *et al.* (1976) also maintained a constant  $k_La$  for scale up of penicillin and streptomycin fermentations. Takei *et al.* (1975) found that an increase of OTR above 0.13 mmol/L/s markedly decreased the yield of protease by *Streptomyces sp*, and the effect was more profound in the larger scale.

In some cases, at the same  $k_La$  value, the oxygen transfer rate in large scale fermenters is often higher than that at the laboratory scale, because the higher hydrostatic pressure in a larger scale fermenter results in a higher driving force. However, higher mixing time in large scale fermenters should also be taken into account, because it can prolong the residence time of air bubbles, which may lead to insufficient mass transfer.

Lilly (1983) in his review summarised the problems in scale up by interrelating mixing, liquid shear and gas transfer. As mentioned earlier, mixing time increases with scale. This can result in poor mixing during which significant temperature and nutrient concentration gradients, fluctuating pH and insufficient supply of oxygen take place. This, in turn, may lead to reduced product yields. Mixing time can be defined as the time required to minimize concentration differences throughout the entire liquid volume and the value depends on the agitation system and broth rheological properties. The decrease of power input per unit volume as the scale increases results in longer mixing time because the pumping capacity per unit volume also decreases. Einsele (1978) observed this phenomenon when the vessel volume increased from 0.01 to 1 and then to 100 m<sup>3</sup>, the mixing time (t<sub>c</sub>) increased from 5 to 20 and to 100 s respectively. The correlation between mixing time (t<sub>c</sub>) and fermenter volume (V) can be expressed as

 $t_c \propto V^{0.3} \tag{1.11}$ 

Agitation has to provide adequate mixing as well as good heat and mass transfer. However, excessive agitation can develop liquid shear forces which according to some authors can have destructive effects on the morphology and hence can reduce product yields. In large scale vessels, the average impeller-zone shear rates are relatively smaller than those in laboratory scales, but the gradient of shear rates throughout the vessel becomes greater. A scale up study based on the shearing forces represented as impeller tip speed was reported by Midler & Finn (1966). The fractional survival of protozoa cells of *Tetrahymena pyriformis* decreased with increase in impeller tip speed in the range of 0 to 5 m/s. Three stirrer of different size (50.8 mm, 101.6 mm and 127.0 mm) were used. In the case of mycelial fermentations, impeller tip speed has been reported as an unsuitable scale up parameter. One of the reasons is the important role of circulation frequency in determining the damage to mycelia. Although impeller tip speed applied in the production scale is usually higher in comparison to that of laboratory scale, the damage due to shear is often less, because of the longer mixing time and lower pumping capacity in large scale fermenters which results in lower frequency of circulation of mycelia through the high shear region.

In large scale operations, it is often difficult to provide adequate oxygen transfer throughout the vessel. As the scale of operation increases, it is not possible to maintain the ratio of liquid height and tank diameter (H/T) constant. The implications of this are the presence of dissolved oxygen gradient along the vertical direction and the possibility of carbon dioxide inhibition resulting from hydrostatic pressure. The relatively high hydrostatic pressure in large scale units is generated from liquid depth and from increased head pressures which are commonly applied in large scale operations in order to avoid contamination and to have a better oxygen transfer. The variation in dissolved oxygen can be minimized by improving the vertical bulk mixing. However, in the case of mycelial fermentation the presence of dissolved oxygen gradients is often inevitable. Fox (1978) proposed a scale up strategy as described schematically in Figure 1.7. The combination of aeration and agitation should ideally lie inside the boundary which was set up by considering the effects of shear, carbon dioxide, oxygen, bulk mixing, foam, and cost. However, these boundaries depend on the scale, and for highly viscous fermentations there might be no region inside the boundaries.



## Figure 1.7 Interrelationship of fermentation conditions in scale up (Reproduced from Fox, 1978)

#### 1.6.2 Fluid mixing in the impeller region

Fluid mixing in the fermenter can be divided into two parts, namely microscale and macroscale mixing (Oldshue, 1989). Macroscale mixing involves particles with the size of 1000  $\mu$ m and above, while microscale mixing involves particles with the size of around 100  $\mu$ m and below. Each scale of mixing has its own variables which have to be considered during scale up. Macroscale mixing is affected by power input, impeller speed, impeller diameter, impeller blade shape, impeller blade width or height, thickness of the material used to make the impeller, number of blades, impeller location, baffle location and number of impellers. Microscale mixing is influenced by power input per unit volume, the root-mean square (rms) velocity fluctuation, energy spectra, dissipation length, and the smallest microscale eddy size.

Viscosity plays an important role in the performance of impellers both in terms of fundamental fluid mechanics and also by affecting blending time, flow pattern and heat transfer. Highly viscous systems can generate viscous shear effects in turbulent flow. Viscous shear on the microscale of mixing allows the conversion of power applied by an agitator to a fluid into heat. According to Oldshue (1989) the conversion is approximately 1.0 W of heat for 1.0 W of power input. The region around the impeller which could be about 5 % of the total vessel volume is subjected to shear rates 100 times higher than the average value for the rest of the vessel. Current industrial practice of secondary metabolites production by filamentous microorganisms considers both macroscale and microscale mixing. The impeller design becomes important as shown by the introduction of axial hydrofoil impellers. The advantages of using these hydrofoil impellers with high solidity ratio (the ratio of impeller surface to impeller swept-out area), are reported to give better performance in enhancing gas-liquid mass transfer than the conventional disc turbine impellers.

The area around the impeller has the highest shear rate and many works have been focussed on studies of flow around the impeller region. Although the maximum impeller-zone shear rates will be higher in a large tank, the average shear rate will be lower. Variation in shear rates across large tanks is higher than in small tanks. In the case of the standard Rushton turbine, as the fluid is discharged radially outward towards the tank walls, a pair of counter-rotating vortices are formed, one along the top edge and



Figure 1.8 Fluid flow around Rushton turbine impeller (Reproduced from van't Riet & Smith, 1975)

one along the bottom edge of the blade (Figure 1.8). The vortices then will move outward and reach the region of high turbulence where they will break down to small vortices. This highly turbulent area is also subjected to the highest energy dissipation. The results are high fluid deformation and strong hydrodynamic forces which create shear. As reported by van't Riet & Smith (1975), the area behind the impeller blades produces a strong vortex through which a considerable fraction of the flow from the impeller passes.

If air is introduced to the system, air bubbles occupy the cavities behind the impeller blades, and as a result the vortices disappear. Thus, dispersion in an aerated vessel takes place in the flow stream coming from the impeller. In mycelial cultures, dispersion will be limited by the rheological characteristics of the mycelial cultures (Metz *et al.*, 1976; Taguchi, 1971). Mycelial cultures often exhibit pseudoplastic or Bingham plastic flow behaviour. Metzner & Taylor (1960) reported that in a pseudoplastic culture, the fluid velocity gradient around the impeller markedly increased causing pressure fluctuations in that particular region.

## 1.6.3 Concepts of turbulence dispersion in bioreactors

Mechanical forces acting on microorganisms in agitated vessels might originate from collisions of microorganisms with agitator, vessel wall or with other solid objects in the fermenter. Gas bubbles generated during aeration may also cause surface tension forces and their motion throughout the fermenter may result in fluid mechanical forces. It has been shown that break up of mycelia or pellets could be explained in terms of the theory of turbulence (Taguchi et al., 1968; van Suijdam & Metz, 1981a &b, and Smith et al., 1990). In a typical stirred tank fermenter, especially with high impeller tip speed, turbulent, or at least locally turbulent fluid flow can be assumed in the impeller region. Turbulence in the area around the impeller might be considered as isotropic (there is no preference of flow direction). In this condition, the large eddies that are initially released from behind impeller blades are gradually broken up into smaller eddies until they reach a stage where the small eddies lose their energy due to viscous dissipation at a certain distance from the impeller. The range of these small eddies is completely independent of the external conditions and is called the Universal Equilibrium Range (Shinar & Church, 1960). Taylor's energy spectrum showing the size of eddies, presented as wave number as a function of energy is given in Figure 1.9.



Figure 1.9 Taylor's turbulent energy spectrum (Reproduced from Metz, 1976)

The universal equilibrium range consists of the inertial convection subrange and the viscous dissipation subrange. The size of eddies in the viscous dissipation subrange is defined by the Kolmogoroff microscale ( $\eta$ ). The Kolmogoroff microscale ( $\eta$ ) is the eddy size at which the ratio of inertial forces and viscous forces is one (Re<sub>eddy</sub> = 1). It can be calculated by the following equation,

$$\eta = \left(\frac{\upsilon^3}{\varepsilon}\right)^{0.25}$$
(1.12)

where  $\eta$  is Kolmogoroff microscale (m),  $\upsilon$  is kinematic viscosity (m<sup>2</sup>/s) and  $\varepsilon$  is local energy input per unit mass (W/kg). The root mean square of the velocity of eddies (u) in the inertial convection and viscous dissipation subranges are given by the Equations 1.13 and 1.14 respectively.

inertial convection subrange	:	u	×	$(\epsilon d_e)^{0.33}$	(1.13)
viscous dissipation subrange	:	u	∝	$(\epsilon/\upsilon)^{0.5} d_e$	(1.14)

where  $d_e$  is the eddy size and is equivalent to 1/k (k = wave number, as described in Figure 1.9). According to van Suijdam & Metz (1981b), the size of eddies which are responsible for mycelial break up should be about the same as the size of mycelia themselves: much smaller eddies will not have enough energy for break up and the much larger ones will not create fluctuations in the dynamic pressures which are required for break up.

Break up of mycelia will take place when the turbulent shear force acting on the mycelia exceeds the strength of the hyphal cell wall ( $\sigma_w$ ). The shear stress acting on the particle (mycelia) is given by,

$$\tau_{Le} \propto \rho u^2$$
 (1.15)

The conditions of break up can be summarized by the following equations,

$$\left(\frac{\tau_{Le}}{\sigma_w/Le}\right) = \text{constant} = C$$
 (1.16)

$$d_e \approx Le \tag{1.17}$$

The shear stress ( $\tau_{Le}$ ) in inertial convection subrange can be derived by substituting Equation 1.13 into 1.15, while for eddies in the viscous dissipation subrange,  $\tau_{Le}$  can be derived by substituting Equation 1.14 into 1.15. The results are as follows,

inertial convection subrange :	$\tau_{Le}$	∝	ρ (ε Le) <sup>0.66</sup>	(1.18)
viscous dissipation subrange :	$\tau_{Le}$	×	ρ (ε/υ) Le <sup>2</sup>	(1.19)

Particle size or in this case the main hyphal length (Le) after break up can be predicted by substituting Equation 1.18 into 1.16 for the inertial convection subrange, or by substituting Equation 1.19 into 1.16 in the case of viscous dissipation range. The main or effective hyphal length after break up are given by Equations 1.20 & 1.21.

- inertial convection subrange : Le  $\propto (\sigma/\rho)^{0.6} \epsilon^{-0.4} \propto C_1 \epsilon^{-0.4}$  (1.20)
- viscous dissipation subrange : Le  $\propto (\sigma/\rho)^{0.33} (\epsilon/\upsilon)^{-0.33} \propto C_2 \epsilon^{-0.33}$  (1.21)

where  $C_1 \& C_2$  are constants. Power per unit mass ( $\epsilon$ ) which represents local energy dissipation can be expressed as

$$\frac{P}{\rho V} = \epsilon \propto \left(\frac{P_o N^3 D^5}{D^3}\right) \propto C_3 N^3 D^2 \qquad (1.22)$$

Subsequently, the size of particle or length of hyphae after break up can also be related to impeller speed and impeller diameter by substituting Equation 1.22 into Equations 1.20 and 1.21 for inertial convection and viscous dissipation subranges respectively. The results are given by Equations 1.23 and 1.24.

inertial convection subrange : Le  $\propto$  C<sub>4</sub> N<sup>-1.2</sup> D<sup>-0.8</sup> (1.23)

viscous dissipation subrange : Le 
$$\propto$$
 C<sub>5</sub> N<sup>-1</sup> D<sup>-0.6</sup> (1.24)

Typically, the size of Kolmogoroff microscale of eddies in stirred vessels, including this work, is about 400 - 800  $\mu$ m. This is larger than the typical size of filamentous microorganisms used in the present investigation. This suggests that eddies in the viscous dissipation subrange are likely to be responsible for damage to the microorganisms.

#### 1.6.4 Models of fungal break up due to shear in the fermenter

The models of pellet break up are more widely reported compared with filamentous type. A dispersion model for the break up of pellets of *Aspergillus niger* was developed by Elmayergi (1975). The rate of spores agglomeration was found to be proportional to  $(P/V)^{-0.5}$ . The result was examined in terms of dispersion phenomena of Taylor's turbulent energy spectrum as described in the Section 1.6.2. However, the addition of Carbopol into the system reduced the exponent of P/V from 0.5 to 0.2 and prevented a definite conclusion to be drawn about the validity of the model. Carbopol was postulated to enhance spore dispersion through an impartial electrostatic repulsion between its ionized carboxyl groups (COO<sup>-</sup>) and the spores of *A. niger* which carried a negative charge. Taguchi (1971) described that intense agitation applied on fungal pellets with diameter  $D_p$  could result in two separate damage phenomena, i.e. abrasion from the pellet surface and rupture of the whole pellets. Empirical models based on the impeller speed (N) and impeller diameter (D) were developed to describe the rate of abrasion noted as  $d(D_p)/dt$  and the rate of of pellets rupture noted as dn/dt.

abrasion of pellets surface : 
$$d(D_p)/dt = -C_9 (N D)^{5.5} Dp^{5.7}$$
 (1.25)  
rupture of pellets :  $dn/dt = -C_{10} (D_p^{3.2} N^{6.65} D^{8.75})$  (1.26)

Earlier data from Taguchi *et al.* (1968) were examined by van Suijdam & Metz (1981a) and it was found that the mean pellet diameter was proportional to  $\varepsilon^{-0.15}$ . van Suijdam & Metz (1981a) using their own data found that the mean pellets diameter of *P*. *chrysogenum* subjected to a range of agitation speed in a 1 L and a 10 L fermenter decreased with increase in power per unit mass ( $\varepsilon$ ). The mean pellet diameter was reported to be a function of  $\varepsilon^{-0.4}$ . This model was in good agreement with a turbulent break up mechanism with eddy size in the inertial convection subrange, Equations 1.20 and 1.23.

van Suijdam & Metz (1981b) proposed models describing the effects of growth rate and shear stress on the morphology of filamentous microorganisms. Their concept is based on the dynamic equilibrium between growth and break up of the hyphae. They assume that break up of mycelial hyphae occurs when the local shearing forces in the vessel are higher than the forces necessary to break the hyphae cell wall given by Equation 1.16 and 1.17. The local shearing force is dependent on the local energy dissipation and the size of particle.

Two models were proposed by van Suijdam & Metz (1981b). In the first model, it is assumed that there is a steady state period of morphology during which the effective length of mycelia is stable, while the second model implies that no steady state period exists (this generally occurs in the break up of mycelial pellets). In this later case, the break up continues even at low shear stresses, but at a slower rate. However, the variation of tensile strength of the hyphae with age and culturing conditions prevented them from getting conclusive agreement with the model. The models proposed by van Suijdam & Metz are given by Equations 1.27 & 1.28.

without fatigue phenomena	a :	Le	∝	ε <sup>-0.25</sup>	∝	C <sub>11</sub> N <sup>-0.75</sup> D <sup>-0.5</sup>	(1.27)
with fatigue phenomena	:	Le	∝	ε-0.6	×	$C_{12} N^{-1.8} D^{-2.6}$	(1.28)

In deriving models describing the effects of specific growth rate ( $\mu$ ) on the morphology, three possibilities are considered (van Suijdam & Metz, 1981b). First, hyphal growth unit decreases with increase in  $\mu$ , while the rate of hyphal linear extension (dLe/dt) remains constant. Second, hyphal growth unit is constant or independent of  $\mu$ , while dLe/dt increases with increase in  $\mu$ . Third, both phenomena occur in the system. Their data from continuous culture experiments at 1000 RPM qualitatively confirmed the second phenomenon.

By assuming that nearly all the energy is dissipated in the bulk of the fluid rather than in the dispersion zone around the impeller as proposed by van Suijdam & Metz (1981b), Reuss (1988) found that the maximum specific growth rate of *Rhizopus nigricans* ( $\mu_{max}$ ) diminished from its upper limit ( $\mu_{max}^*$ ) linearly as energy input per unit volume expressed as P $\theta_c$ /V increased (Equation 1.29). The value of  $\theta_c$  represents circulation time which was determined by taking into account geometrical and operating parameters of the fermenters as described in the Equation 1.30.

$$(\mu_{max}^* - \mu_{max}) = C_{13} (P\theta_c/V)$$
 (1.29)

$$N\theta_c = 0.76 \ (H/T)^{0.6} \ (T/D)^{2.7}$$
 (1.30)

In contrast, Smith *et al.* (1990) suggested that the break up of mycelia should be inversely proportional to circulation time, because the damage occurring in the microorganism is not only determined by the energy dissipated in the impeller region but also by the frequency with which microorganisms circulate around the high shear region.

Their data for volumetric penicillin production rate (Units/ml/h) from 10 L and 100 L working volume of fermenters showed good qualitative relationship with the proposed model which is a combination of power input per unit volume and circulation frequency as given in Equation 1.31.

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Penicillin production rate 
$$\propto \frac{P}{(D^3 t_c)}$$
 (1.31)

where P is power dissipation rate for one impeller,  $t_c$  is circulation time calculated from pumping capacity of the fermenter as described by Nagata (1975). The profile of relationship given in Equation 1.31 is shown in Figure 1.10.



<u>Figure 1.10</u> Penicillin production rate as function of  $P/D^3t_c$ (Reproduced from Smith *et al.*, 1990)

#### 2 MATERIALS AND METHODS

#### 2.1 Materials

#### 2.1.1 Organism

*Penicillium chrysogenum* P1 (Microbiological Department, Polytechnic of Central London) was used throughout the experiments. The culture was originally developed by Panlabs Inc. The strain is capable of producing Penicillin-V with phenoxyacetic acid (P.O.A) as a precursor.

#### 2.1.2 Chemicals

The list of major chemicals and the suppliers is given in Table 2.1. The other chemicals were obtained from BDH Chemicals, Poole, Dorset. Analar grades of (Fe,Na, Zn, Mn, Cu, Mg) sulphates and calcium chloride were used.

#### 2.2 Equipment

## 2.2.1 Fermentation Equipment

#### 2.2.1.1 Fermenter Vessels

The fermenters used were manufactured by Chemap and LH Engineering. The total working volume for the production fermenters ranged from 7 L to 1500 L. Shake flasks were used to grow inocula for the 7 L production fermenter. Fermenters of 20 L and 150 L working volume were used to grow inocula for 150 L and 1500 L fermentations respectively. All production fermenters had four baffles and three impellers of six bladed disc turbine design. Vessel and impeller geometries are shown in Figures 2.1 and 2.2. The geometry and dimension are listed in Table 2.3.

Chemical	Supplier
Sucrose	BDH Chemicals, Poole, Dorset
Lactose monohydrate	PSV Ltd, Milton Keynes
Mycological peptone	Oxoid Ltd, London
Technical grade agar No.3	Oxoid Ltd, London
Phenoxyacetic acid (P.O.A)	Fluka Company, London
Ammonium sulphate	Sigma Chemical Company
Penicillin V	Sigma Chemical Company
Ethylenediaminetetraacetic acid (EDTA)	Fisons Scientific Apparatus, UK
Potassium dihydrogensulphate	Fisons Scientific Apparatus, UK
Sodium sulphate	Fisons Scientific Apparatus
T.M.S Antifoam	Supplied courtesy of Beecham
	Pharmaceuticals

Table 2.1 Major chemicals and the suppliers

Description	Manufacturer	Working volume (L)
Shake flask	Fisons Ltd	0.25
7 L	LH Engineering	5
20 L	LH Engineering	14
150 L	LH Engineering	100
1500 L	Chemap AG	1000

Table 2.2 List of fermenters

Methods



Figure 2.1 Vessel geometry



Figure 2.2 Impeller Geometry

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Plate 2.1 The 7 L fermenter

## 2.2.1.2 Fermenter Instrumentation

Each fermenter was equipped with Ingold steam sterilizable polarographic dissolved oxygen and pH probes. The fermenters were connected to the on line data logging system Bio-I capable of monitoring the profiles of dissolved oxygen tension (DOT), temperature, air flow rate, pH and agitator speed continuously throughout fermentation.

#### 2.2.2 Gas Analysis System

A VG Gas Analysis Ltd MMG-80 mass spectrometer was connected to all fermenters to measure the composition of outlet gas periodically. The output of the mass spectrometer was recorded continuously by the Bio-I system described in Section 2.2.1.2 and the calculations of Carbon dioxide Evolution Rate (CER), Oxygen Uptake Rate (OUR), and Respiratory Quotient (RQ) were done. There were times during the experiments when the Bio-I system was unable to read data from the mass spectrometer. In these cases, the values of CER, OUR and RQ were calculated off-line. However, the CER, OUR and RQ values given in Results were mostly calculated on line by Bio-I approximately every 15 min.

## 2.2.3 High Performance Liquid Chromatography (HPLC)

A HPLC unit with automatic sampling was used to determine the Penicillin-V titre. The pump was a Perkin Elmer liquid chromatographic pump model series 10 (Perkin Elmer Corporation, Connecticut, USA) connected to an automatic sampling system type ISS-100. The unit was also equipped with a Spectro Monitor III variable wavelength detector (LDC/Milton Roy). A Perkin Elmer LCI-100 laboratory integrator was incorporated into the unit to quantify the detector output. A Spherisorb S5C8 column from Hichrom was used. The column was 20 cm in length and 4.6 mm inside diameter. The preparation and condition of analysis using HPLC are given in Section 2.3.3.



Plate 2.2 The 150 L fermenter

Code	Т	HL	D	Hi	Hb	Li	Wi	Wb
7 L	150	280	68	90	30	16.5	13	25
20 L	225	390	68	100	65	16.5	13	22
150 L	410	780	182	230	100	46	36	43
1500 L	888	1615	300	560	300	76	60	90

Table 2.3 Fermenter Geometry (All dimensions in mm)



Plate 2.3 The 1500 L fermenter

### 2.2.4 Image Analyser

A Magiscan 2A Image Analyser (Joyce Loebl Ltd, Gateshead, UK) attached to a Nikon Optiphot microscope (Nikon UK Ltd, UK) was used. The system was run automatically with the set up procedure given in Section 2.3.4.

## 2.2.5 Viscometer

Rheological measurements on the broth were carried out using a modified Rheometer. It comprised a Motomatic motor generator E850MG (Electrocraft Corporation, USA) on which a disc turbine impeller of 0.063 m diameter was mounted and a Masterservodyne Controller Model 4445/30 (Cole Palmer Instrument Corporation, USA) with a speed range of up to 3000 RPM. The unit was connected to the voltmeter to refine the reading of the Masterservodyne Controller.

#### 2.3 Methods

#### 2.3.1 Fermentation

#### 2.3.1.1 Spore production and storage

The medium for spore production comprised

	<u>g/L</u>
glycerol	7.5
molasses	2.5
yeast extract	1.0
MgSO <sub>4</sub> . 7H <sub>2</sub> O	0.05
KH <sub>2</sub> PO <sub>4</sub>	0.06
bacteriological peptone	5
NaCl	10
FeSO <sub>4</sub> . 7H <sub>2</sub> O	0.003
CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.001
agar	20

The medium ingredients except agar were dissolved in deionised water and the pH was adjusted to 6.70 with 2 M NaOH. Every 100 mL of liquid medium was added to a 500 mL mediflat bottle together with a 2 g of agar. The mixture was shaken vigorously and sterilised in the autoclave at 121 °C for 25 minutes. Each mediflat bottle was inoculated with 0.5 mL of spore suspension from the stock cultures. The incubation was carried out at 26 °C for 10 days after which the agar surface was covered with green spores.

The spores were harvested with sterile glass beads and mixture of solution which was made of the following :

sucrose	20	%	(w/v)
Tween-80	0.1	%	(v/v)

dissolved into phosphate buffer saline solution at pH 7.0. The buffer solution was prepared by dissolving one tablet of phosphate buffer saline into 100 mL of deionised water. The spore suspension was pipetted and dispensed into universal bottles

containing 4 glassbeads of 3 mm in diameter. The spore suspension was then stored at -20 °C. When required the spore suspension was thawed and shaken vigorously for few minutes using a vortex mixer. Spore count using haemocytometer was carried out on every batch of spores and the concentration was found to be fairly reproducible around  $(2 \pm 0.4) \times 10^8$  spores/mL. Observations under a microscope revealed negligible amounts of lysed hyphae present. All fermentations carried were inoculated with the same generation of spores. The master stock of spores were stored in several 7 mL bijou bottles at -70 °C.

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## 2.3.1.2 Preparation of seed cultures for the producing fermentation

The seed culture was grown in the medium adapted from Hosler and Johnson (1953) with some modifications. The composition of the seed medium (g/L) was as follows :

sucrose	10
lactose	10
KH <sub>2</sub> PO <sub>4</sub>	3
Na <sub>2</sub> SO <sub>4</sub>	0.5
FeSO <sub>4</sub> . 7H <sub>2</sub> O	0.1
ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.02
MnSO <sub>4</sub> . 7H <sub>2</sub> O	0.02
CuSO <sub>4</sub> . 7H <sub>2</sub> O	0.005
MgSO <sub>4</sub> . 7H <sub>2</sub> O	0.25
CaCl <sub>2</sub>	0.05
EDTA	0.55
mycological peptone	5
$(NH_4)_2SO_4$	15
antifoam	1 mL/L

Sucrose solution was prepared in 10 % of the total working volume and sterilised separately for 20 minutes and so was the mixture solution of peptone and ammonium sulphate. The pH was adjusted at 6.70 before sterilisation. The volume of seed culture was 10 % of total working volume of the producing fermentation as listed in Table 2.4.

The seed culture medium in the shake flask was sterilised at 121 °C for 20 min. The holding times during sterilisation at 121 °C and 1 bar for seed culture medium in the 20 L and 150 L fermenters were 20 min and 30 min respectively. Spores were inoculated at  $0.8 \times 10^{6}$ /mL and the fermentation was carried out at 26 °C.

Seed cultures grown in shake flasks for inoculating the 7 L fermenters were put in an orbital shaker incubator at 200 RPM for 39 h. Preparation of seed cultures in the 20 L (for 150 L scale producing fermentations) and 150 L (for 1500 L scale producing fermentations) fermenters were carried out at 700 RPM and 400 RPM respectively.

Fermentations	Scale (L)	Working volume (L)	Speed (RPM)	Comments
A700	7	5	700	
A700a	7	5	700	reproducibility of system
A850	7	5	850	
A850a	7	5	850	reproducibility of system
A850b	7	5	850	media sterilisation effect
A1000	7	5	1000	
A1150	7	5	1150	
A1300	7	5	1300	
A1300a	7	5	1300	reproducibility of system
B265	150	100	265	
B265a	150	100	265	effect of overpressure
B400	150	100	400	
B400a	150	100	400	effect of overpressure
B530	150	100	530	
B530a	150	100	530	media sterilisation effect
C300	1500	1000	300	
C100	1500	1000	400	
C400	1300	1000	400	

Table 2.4 Fermentations carried out in this study

#### 2.3.1.3 Penicillin Production Fermentation

The production medium comprised of the following in g/L :

sucrose	10
lactose	100
KH <sub>2</sub> PO <sub>4</sub>	7.5
Na <sub>2</sub> SO <sub>4</sub>	1
FeSO <sub>4</sub> . 7H <sub>2</sub> O	0.18
ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.05
MnSO <sub>4</sub> . 4H <sub>2</sub> O	0.05
CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.008
MgSO <sub>4</sub> . 7H <sub>2</sub> O	0.7
CaCl <sub>2</sub>	0.05
EDTA	0.55
mycological peptone	1
$(NH_4)_2SO_4$	13
phenoxyacetic acid	4.8
antifoam	1 mL/L

As for the seed culture medium, sucrose and the mixture of mycological peptone and ammonium sulphate at pH 6.70 were sterilised separately. Phenoxyacetic acid was converted to potassium phenoxyacetate prior to mixing with the remainder of the medium components. Phenoxyacetic acid was dissolved in one third of the working volume and excess potassium hydroxide pellets were added until the pH of the solution was around 10. The pH was then brought back to neutral by titration with concentrated solution of hydrochloric acid. The potassium phenoxyacetate solution was mixed with the remainder of medium components and the pH was adjusted to 6.70 using 4 M NaOH.

The pH probe was calibrated at pH 4.0, 7.0 and 11.0 prior to every sterilisation. The DOT probe was calibrated prior to sterilisation in the 150 L and 1500 L fermenters, and in the 7 L scale the calibration was carried out before and after sterilisation. During sterilisation the medium was held at 121 °C and 1 bar for 18 min, 25 min and 60 min for the 7 L, 150 L and 1500 L scales respectively.

During fermentation the pH was maintained at  $6.70 \pm 0.05$  using 5 M NH<sub>4</sub>OH and 2 M H<sub>2</sub>SO<sub>4</sub>. Samples taken during the fermentations were quickly placed in an ice bath. Dry cell weight determination and sample preparation for Penicillin-V analysis were carried out immediately.

#### 2.3.2 Determination of Dry Cell Weight

The dry cell weight was determined by filtering 10 mL of broth through 0.45  $\mu$ m glass microfibre filters (Whatman, grade GF/A 4.7 cm diameter) using a Sartorius SM vacuum filter holder. The filtered mycelia were washed with 3 x 10 mL of distilled water before oven drying at 105 °C for 24 hours. The filter and dry biomass were placed in the desiccator until the weight remained constant. The determination was duplicated for each sample.

#### 2.3.3 Determination of Penicillin-V

#### 2.3.3.1 Sample Preparation and Storage

To provide samples for Penicillin-V determination, the filtrate from dry cell weight measurement was refiltered through 0.2  $\mu$ m cellulose nitrate membrane filter using a Sartorius SM vacuum filter holder placed in an ice bath. The filtrate was stored in 7 mL bijou bottle at -20 °C until required.

#### 2.3.3.2 HPLC Assay

The buffer solution for the HPLC assay was prepared by dissolving 20.4 g KH<sub>2</sub>PO<sub>4</sub> (HPLC grade) to 500 mL using deionized water. The pH was adjusted to 5.0 with 1 M potassium hydroxide. Another 300 mL of deionized water was added into the mixture and finally 200 mL acetonitrile (HPLC grade) was added while the mixture was stirred. The buffer solution was then filtered through a 0.45  $\mu$ m HV type Milipore filter (solvent resistant filter). Sonication for 30 minutes was carried out to remove air bubbles. Samples stored at - 20 °C were quickly thawed and diluted 4 : 1 with buffer solution before being injected into the column. The conditions of analysis were as follows :

Flow rate	1.5 mL/min
Temperature	5 °C
Volume of injection	10 µL
Wave length of absorbance	220 nm
Analysis time	10 min

The standards were prepared by adding a solution of potassium salt of penicillin V to the buffer solution. A fresh standard solution was prepared for each batch of analysis, although the variation among them was very little. A typical standard curve for the Penicillin-V assay using HPLC is given in Figure 2.3. The concentration of Penicillin-V was regarded as being proportional to the area under the peak appearing between 7 - 8 min on the integrator.

## 2.3.4 Determination of morphology

#### 2.3.4.1 Sample preparation and storage

Samples from fermentations were immediately mixed with an equal volume of fixative solution before being stored at 4 °C until required for analysis. The fixative solution comprised of

40 % formaldehyde	13 mL
glacial acetic acid	5 mL
50 % (w/v) ethanol	200 mL

The fixed samples were then diluted to 20-fold with fixative solution and stained with Tryphan Blue which was made by dissolving of 0.25 g Tryphan Blue into 100 mL solution with lactophenol. Wet-mount slides for image analysis were prepared by spreading one drop of fixed and stained sample from a Pasteur pipette onto a slide and covered with a cover slip.

Methods



Figure 2.3 Standard curve of Penicillin-V assay using HPLC

## 2.3.4.2 Morphological measurements

The measurements were carried out using a Magiscan 2A Image analyser (Joyce Loebl Ltd., Gateshead, U.K.) with a fully automatic method developed by Packer and Thomas (1990). A slide of a wet sample was placed under a microscope and an attached television camera sent a video signal of the field of view to a computer capable of image processing and analysis. The microscope magnifications used were 60 and 100 times. Manual adjustment of the contrast was necessary.

The main steps involved in the measurements of morphological characteristics using image analysis are segmentation, skeletonization, selection of clumps and measurement within a predefined active measuring frame. In the segmentation phase, the image is separated from undesirable objects such as dust and media particles based on a preset level of greyness. Skeletonization of the image clarifies the edges of the objects. The processed binary image from skeletonization phase is then divided into measurable microorganisms and clumps.

For each sample at least 125 microorganisms were characterized. The morphological parameters measured were main hyphal length, total hyphal length, hyphal growth unit, number of tips and percentage of clumps.

#### 2.3.5 Rheological measurement

The rheological measurements of fermentation broths were determined using a method developed by Kemblowski and Kristiansen (1986). In this technique, a sample is placed in the 2 L glass beaker and stirred at various speed in the range of 0 - 500 RPM. The torque reading at a number of shear rates ( $\gamma$ ) is converted into shear stress ( $\tau$ ). The flow curve is constructed by plotting shear rate against shear stress on log-log graph paper and the values of consistency index (k) and flow index (n) are calculated from the intercept and slope of the curve. A sample calculation of fermentation broth viscosity is given in Appendix 1.

#### 3 RESULTS

#### **3.1** Effects of agitation at 7 L scale

#### **3.1.1** Inoculum development in shake flask cultures

In order to inoculate a 7 L fermenter (5 L working volume) with 10 % v/v inoculum, a seed stage fermentation was carried out in two 2 L shake flasks each with only 250 mL broth to ensure oxygen sufficiency. The seed culture was incubated at 26 °C in a 200 RPM orbital shaker incubator. In a preliminary experiment, growth of the shake flask culture was monitored for up to 100 h to determine the point of transfer. Sucrose and lactose were used as carbon sources and therefore the fermentation showed diauxic type of growth.

Twenty hours after inoculation with  $0.8 \times 10^6$  /mL spores, germination followed and fast growth took place until sucrose in the media became limited, and then lactose started being consumed at a slower rate than sucrose. Growth of shake flask cultures was quite reproducible as shown in Figure 3.1. During the fast growth period, the dry cell weight, main hyphal length, number of tips and hyphal growth unit increased (Figures 3.1 & 3.2). Fragmentation occurred when fast growth ended and for the remainder of fermentation the main hyphal length and hyphal growth unit were reasonably constant. The number of tips decreased after the fast growth period due to fragmentation, and remained constant for a short period of time before hyphal branches started being produced again during the late period of fermentation. A high degree of entanglement was observed in the shake flask cultures as shown by the high percentage of clumps (Figure 3.3).

Seed cultures were transferred to the 7 L fermenters after 39 h of fermentation when they were still in the fast growth period. This point of transfer was chosen to avoid the second lag phase occurring (the second lag phase took place as soon as the fast growth ended, and during this stage a slight decrease in the dry cell weight was observed). This early transfer was also used to minimize predetermined morphology affecting hyphal characteristics in the production stage.

The following symbols have been used for all fermentation figures unless otherwise stated:

- Dry cell weight (g/L)
- Yield of Penicillin-V (mg/g biomass or mg/g dry cell weight)
- $\blacktriangle \qquad C. E. R (mmol/L/h)$
- $\Delta$  O. U. R (mmol/L/h)
- + D. O. T (% Air saturation)
- O Main hyphal length (μm)
- \* Number of tips
- Hyphal growth unit ( $\mu$ m)
- ▼ Clumps (%)
- ◆ Exit gas CO<sub>2</sub> (%)
  - R.Q


<u>Figure 3.1</u> Dry cell weight and main hyphal length of shake flask cultures



Figure 3.2 Number of tips and hyphal growth unit of shake flask culture



Figure 3.3 Percentage of clumps of shake flask culture

The typical hyphal morphology of seed cultures at the point of transfer were as follows:

Main hyphal length (µm)	110	±	12.7
Hyphal growth unit (µm)	43	±	4.8
Number of tips	3.2	±	0.4

As seen in Figures 3.10 to 3.15 in Section 3.1.2 and Figures 3.18 and 3.19 in Section 3.1.4, the mean values of main hyphal length and hyphal growth unit of seed cultures (for '0 h of fermentation') varied within 95 % confidence interval of the values listed above.

# 3.1.2 Reproducibility of the system: fermentations at 700, 850 and 1300 RPM

To ensure the reproducibility of the system, repeated runs in the 7 L fermenter (5 L working volumes) were carried out at 700 (A700 & A700a), 850 (A850 & A850a), and 1300 (A1300 & A1300a) RPM. Tip speeds of these fermentations were 2.5 m/s, 3.0 m/s, and 4.6 m/s for 700, 850, and 1300 RPM respectively. The aeration rate was kept constant throughout the fermentations at 2.5 L/min.

#### Growth and penicillin production:

The fermentation at 1300 RPM showed the best reproducibility for dry cell weight and Penicillin-V production (Figure 3.8) although all fermentations showed good reproducibility. The yield was calculated as Penicillin-V produced (mg) per dry cell weight or biomass (g). Degrees of agitation and aeration chosen for these fermentations did not appear to have caused oxygen limitation to the system and subsequently the differences in culture behaviour may be considered to be mainly due to agitation changes.

All of the DOT profiles in this 'Results' section i.e. Figures 3.5, 3.7, & 3.9 were constructed of data points monitored continuously by BiO-I system and the symbols do not indicate measured points. The DOT in the fermentation at 700 RPM dropped to 38 % air saturation (Figure 3.5) which was well above the critical value of 30 % air saturation proposed by Vardar & Lilly (1982).

Fast growth took place during approximately the first 25 h for all three impeller speeds used. During this period the dry cell weight increased rapidly to ~6 g/L. When the fast growth period ended, the DOT at 700 RPM started increasing up to 80 % air saturation and the dry cell weight slightly decreased. This was followed by the second growth stage during which the DOT gradually decreased down to about 45 % air saturation. At the same time the average dry cell weight increased from 5.2 g/L to 7.7 g/L. The maximum value of CER at 700 RPM was 16 mmol/L/h and it decreased rapidly for a period of 15 h before increasing gradually again. The average value of the RQ during the second lag phase (between 25 to 50 h) dropped down to 0.5 - 0.6 and increased steadily for the remainder of fermentation.



Figure 3.4Reproducibility of dry cell weight and Penicillin-V yield of<br/>fermentations operating at 700 RPM in the 7 L fermenter



Figure 3.5 Gas analysis and DOT profile of the fermentation operating at 700 RPM in the 7 L fermenter (A700)



Figure 3.6Reproducibility of dry cell weight and Penicillin-V yield of<br/>fermentations operating at 850 RPM in the 7 L fermenter



Figure 3.7 Gas analysis and DOT profile of the fermentation operating at 850 RPM in the 7 L fermenter (A850)



Figure 3.8Reproducibility of dry cell weight and Penicillin-V yield of<br/>fermentations operating at 1300 RPM in the 7 L fermenter



<u>Figure 3.9</u> Gas analysis and DOT profile of the fermentation operating at 1300 RPM in the 7 L fermenter (A1300)

A similar trend of growth was observed at 850 and 1300 RPM. Average values of RQ around 1.0 were found during the period of Penicillin-V synthesis at 850 RPM and at 1300 RPM the value was constant around 0.7 - 0.8 (Figures 3.7 & 3.9). The DOT during the fast growth period (first stage of growth) dropped down to 70 % air saturation at 850 RPM and to 83 % air saturation at 1300 RPM. At both 850 RPM and 1300 RPM the DOT remained above 80 % air saturation during the second stage of growth.

Fermentations at 850 RPM seemed to recover from the second lag phase faster than those at 700 RPM and the second stage of growth at 850 RPM appeared to be at a higher rate than either 700 RPM or 1300 RPM cases. Specific growth rates ( $\mu$ ) were 0.007 /h, 0.008/h and 0.004 /h for 700, 850 and 1300 RPM respectively. All values of  $\mu$  were calculated with respect to both the second linear growth and the linear penicillin production.

For all three fermentations speeds (700, 850 and 1300 RPM), it can be seen that Penicillin-V was synthesized at high rate of production up to 75 h of fermentation. This was subsequently followed by a slower rate of production. At 700 RPM (Figure 3.4) and 850 RPM (Figure 3.6), the onset of Penicillin-V production occurred after 25 h of fermentation which coincided with the end of the fast growth period. The highest rate of production was observed in the 850 RPM fermentation although the production started to level off after 100 h. The maximum specific Penicillin-V produced at 850 RPM was 185 mg/g compared with 172 mg/g at 700 RPM. In the 1300 RPM fermentation (Figure 3.8), a 20 h delay in the penicillin production was observed. The rate of production at 1300 RPM fermentation was the slowest compared to both the 700 RPM and 850 RPM, and a maximum specific production obtained was only 151 mg/g.

#### Hyphal morphology :

The profile of total hyphal length in every fermentation resembled that of main hyphal length, therefore the presentation of total hyphal length in this 'Results' section is unnecessary. For all the three fermentations, the main hyphal length and hyphal growth unit increased during the first stage of growth (Figures 3.10 to 3.15). In the fermentation at 700 RPM, reproducibility of morphological measurement was poor, particularly for the samples taken during the fast growth period. The mean main hyphal length of the three fermentations at different speeds increased to reach values in the reasonably same range,  $(158 \pm 17.8) \mu m$  in 700 RPM,  $(153 \pm 15.2) \mu m$  in 850 RPM, and  $(140 \pm 19.5) \mu m$  in 1300 RPM.



<u>Figure 3.10</u> Reproducibility of main hyphal length and number of tips of fermentations operating at 700 RPM in the 7 L fermenter



Figure 3.11Reproducibility of hyphal growth unit and percentage of clumps of<br/>fermentations operating at 700 RPM in the 7 L fermenter



Figure 3.12Reproducibility of main hyphal length and number of tips<br/>of fermentations operating at 850 RPM in the 7 L fermenter



Figure 3.13 Reproducibility of hyphal growth unit and percentage of clumps of fermentations operating at 850 RPM in the 7 L fermenter



<u>Figure 3.14</u> Reproducibility of main hyphal length and number of tips of fermentations operating at 1300 RPM in the 7 L fermenter



Figure 3.15Reproducibility of hyphal growth unit and percentage of clumps<br/>of fermentations operating at 1300 RPM in the 7 L fermenter

Comparisons of results on the main hyphal length indicated that fragmentation appeared to occur earliest at 1300 RPM. In the case of 700 RPM, the mean value of main hyphal length did not decrease below 100  $\mu$ m until after 75 h of fermentation while at 1300 RPM this phenomenon occurred after 33 h of fermentation and the mean main hyphal length dropped to ~80  $\mu$ m. At 850 RPM, the mean value of main hyphal length decreased to below 100  $\mu$ m after 60 h of fermentation, and at 700 RPM this occurred after 72 h of fermentation. For approximately the last 25 h of fermentations at 700,850 and 1300 RPM, the main hyphal lengths were found to be fairly constant. The mean main hyphal length at the end of fermentation of 1300 RPM was (65 ± 4.4)  $\mu$ m. The final mean main hyphal length at 850 RPM and 700 RPM were (81.5± 7.5)  $\mu$ m and (82.8 ± 8.4)  $\mu$ m respectively.

The hyphal growth unit during fermentation followed a similar pattern observed for the main hyphal length. There seemed to be no significant differences in the hyphal growth unit at different speeds of fermentations during the first stage of growth. At 1300 RPM, the mean hyphal growth unit increased to  $(49.3 \pm 3.4) \mu m$ . At 700 RPM and 850 RPM similar values were also obtained with lengths of  $(48.7 \pm 2.4) \mu m$  and  $(50.4 \pm 4.2) \mu m$  respectively. During the period of penicillin production, the hyphal growth unit for the 1300 RPM dropped down more rapidly compared to 700 and 850 RPM. At the end of the fermentation, the mean hyphal growth unit at 1300 RPM was  $(26.5 \pm 1.8) \mu m$ . Higher values of mean hyphal growth unit were observed at 700 RPM with  $(33.2 \pm 2.2) \mu m$  and at 850 RPM with  $(32.1 \pm 2.6) \mu m$ .

Reproducibility of the number of tips in these fermentations was poor. The mean values of the number of tips ranged between 2.7 to 3.7. Therefore the average number of branches in the culture morphology varied between 0.7 to 1.7. During the period of penicillin production this phenomenon was likely to happen since hyphal fragmentation resulted in the occurrence of diffused hyphae with only one or two branches.

In the fermentation operated at 1300 RPM, the number of tips increased during the fast growth period. Fragmentation at that speed seemed to reduce the number of hyphal branches as shown by a smaller number of tips observed between the period of 48 to 75 h as compared to the fast growth period. After 75 h of fermentation at 1300 RPM more branches were produced.

In fermentations operated at 700 and 850 RPM, the reproducibility was very poor and therefore it was difficult to see the trend of branch formation during fermentation. One of the fermentations at 850 RPM (Figure 3.12 with '\*' symbol) showed an increase of

mean number of tips during the first 50 h of fermentation and a decrease between 50 to 75 h during which the main hyphal length was diminishing. After 75 h, the number of tips increased again for the remainder of the fermentation period.

The percentage of clumps ranged between 60 to 95 % throughout all fermentations regardless of whether a high or low biomass concentration was present. In addition further dilutions did not reduce the percentage of clumps significantly. The morphological measurements were carried out by means of an automatic method which required absolutely free and diffused mycelium. Even a simple entanglement of two mycelia would be taken into account as a clump (Plate 3.2). Despite all of this, the automatic method improved the efficiency of measurement. More than 125 unclumped organisms were measured for each sample.

# 3.1.3 Growth and penicillin production at 7 L scale

In order to have data at a reasonable number of agitator speeds, two additional fermentations at 7 L scale were performed at 1000 RPM (3.6 m/s tip speed) and 1150 RPM (4.1 m/s tip speed) at the same aeration rate and temperature as the previous runs described in Section 3.1.2 (700, 850 and 1300 RPM). Similar trends of growth were observed as shown on figures 3.16 and 3.17. Specific growth rates of the second stage of growth were 0.005 /h for 1000 RPM and 0.006 /h for 1150 RPM. The overall penicillin production at 1000 RPM was higher than at 1150 RPM. The maximum specific Penicillin-V production achieved in the 1000 RPM fermentation was 184 mg/g which was higher than that at 1150 RPM, 157 mg/g. As with previous fermentations the period of rapid penicillin synthesis was followed by a slower one.

The first stage of growth of the fermentations was not found to be affected by agitation. For all fermentations, the dry cell weight increased to  $\sim 6g/L$  during the first 25 h of fermentations. A lag phase was briefly observed, followed by a second stage of growth during which penicillin was produced. The specific growth rate during the second stages of growth appeared to decrease with increase in agitation speed in the range of 850 to 1300 RPM although the differences were not significant. The specific growth rate of the second stage of growth was higher at 850 RPM (0.008 /h) compared to 700 RPM (0.007/h).



Figure 3.16 Growth and penicillin production of the culture in the 7 L fermenter operating at 1000 RPM



Figure 3.17 Growth and penicillin production of the culture in the 7 L fermenter operating at 1150 RPM (A1150)

Penicillin-V production in this system was fairly reproducible as described in Section 3.1.2. To compare the specific penicillin production rate  $(q_{pen})$  at 7 L scale the best fitted line was drawn within a linear period of production in every fermentation. The values of  $q_{pen}$  are summarized in Table 3.1. The values of  $q_{pen}$  decreased with increase in agitation speed in the range of 850 to 1300 RPM. The value of  $q_{pen}$  at 700 RPM was lower than that at 850 RPM but higher than those of other fermentations at this scale.

Fermentation	Tip speed (m/s)	Time interval (h)	q <sub>pen</sub> (u/mg/h)	μ (/h)
A700	2.5	22 - 119	3.70	0.007
A850	3.0	25 - 100	3.73	0.008
A1000	3.6	32 - 124	3.49	0.006
A1150	4.1	26 - 107	2.99	0.005
A1300	4.6	40 - 121	2.92	0.004



# 3.1.4 Hyphal morphology at 7 L scale

The morphology of fermentations operated at 1000 RPM and 1150 RPM are shown in Figures 3.19 and 3.20. The characteristics of hyphal morphology at these speeds followed similar trends to those of previous fermentations (700, 850 and 1300 RPM) described in Section 3.1.2. At the end of the fast growth period, the main hyphal length at 1150 RPM decreased more rapidly than at 1000 RPM. The mean main hyphal length dropped down to below 100  $\mu$ m after 36 h at 1150 RPM and after 56 h at 1000 RPM. Increase in the mean number of tips during the fast growth period at 1000 RPM corresponded to a relatively constant hyphal growth unit and an increase of main hyphal length.



Figure 3.18 Hyphal morphology of the culture in the 7 L fermenter operating at 1000 RPM

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Figure 3.19 Hyphal morphology of the culture in the 7 L fermenter operating at 1150 RPM

In both fermentations when fragmentation took place, the number of tips diminished and then increased again until the end of fermentation. The main hyphal length was fairly constant. More branches were observed at 1150 RPM after approximately 64 h of fermentation compared to 1000 RPM.

Reproducibility of hyphal morphology was best in the high agitation fermentation as shown by the results of fermentations at 1300 RPM in Figures 3.14 and 3.15. Poor reproducibility of the morphology at the early stage of fermentations at 700 RPM was observed, during this time the entanglement of mycelia was very high. However, during the second stage of growth main hyphal length and hyphal growth unit were reproducible.

Fragmentation occurred in all fermentations as indicated by the considerable decrease in the mean main hyphal length. Earlier fragmentation and more branches were observed in fermentations operated at higher speed. In the second lag phase after the fast growth period ended, mycelia began to show some vacuolization and the proportion of vacuolized region seemed to increase with fermentation time. In the late period of fermentations, considerable amount of empty hyphae were observed in fermentations at 1150 RPM and 1300 RPM.

At the end of the fast growth period, the main hyphal length and hyphal growth unit reached similar values regardless of the agitation speed, indicating that agitation had little effect on the hyphal morphology during the fast growth period. However, during the second stage of growth where penicillin production took place, the average values of main hyphal length and hyphal growth unit were higher in the fermentations operating at 700 RPM and 850 RPM compared to those in the other fermentations. The percentage of clumps in every fermentation varied between 60 to 95 %. In the fermentations operating at 700 RPM and 850 RPM, the average percentages of clumps during the period of penicillin production were lower than those during the fast growth period. However, the increase in agitation speed did not significantly affect the percentage of clumps.

### 3.2 Effects of agitation at 150 L scale

#### 3.2.1 Inoculum development in 20 L fermenter

A series of 20 L fermentations at 14 L working volume were carried out to predetermine the best fermentation conditions for transfer to 150 L scale. Three different sets of fermentations (500, 700 and 900 RPM) each inoculated with  $0.8 \times 10^6$  spores/mL, were performed for 72 h. The aeration rate was kept constant at 0.5 vvm throughout the three fermentations. Results from the three fermentations with respect to growth and morphology are given in Figures 3.20 to 3.25.

Germination of spores began approximately 15 h after inoculation. Fermentation at 700 RPM gave the highest peak of CER (~16 mmol/L/h) at the end of the fast growth period which corresponded to 6g/L of dry cell weight (Figure 3.21). At this point the DOT fell to 39 % air saturation. The DOT then increased indicating that the system had switched to the second stage of growth and this was followed by a marked decrease as the second growth continued. At the end of fermentation, the growth ceased due to nutrient limitation, which was confirmed by the decrease and increase of CER and DOT respectively.

The trends of the growth of the seed cultures at both 700 and 900 RPM were similar. The DOT values at 900 RPM remained above 80 % air saturation throughout fermentation (Figure 3.22). The maximum value of the CER in the fermentation operated at 900 RPM was close to that at 700 RPM. The RQ value increased exponentially after spore germination at both 700 RPM and 900 RPM until 25 h of fermentation and then remained fairly constant at around 1.0 (Figures 3.21 & 3.22).

Values of dry cell weight during the fermentation at 500 RPM were lower than those at 700 RPM or 900 RPM probably due to oxygen limitation. The DOT dropped to  $\sim 10 \%$  air saturation at the end of the fast growth period and the maximum CER at this point was 12.5 mmol/L/h which corresponded to  $\sim 4.6$  g/L dry cell weight (Figure 3.20). The second lag phase in this fermentation during which the DOT increased to  $\sim 50 \%$  air saturation was longer than the other two fermentations.





Figure 3.20 Growth of the seed culture in the 20 L fermenter operating at 500 RPM



Figure 3.21 Growth of the seed culture in the 20 L fermenter operating at 700 RPM



Figure 3.22 Growth of the seed culture in the 20 L fermenter operating at 900 RPM

When the second growth took place the DOT fell rapidly to 10 % air saturation. This phenomena occurred for a period of 20 h. The death phase in this fermentation was found to be earlier than in the other two fermentations. After 60 h of fermentation, the DOT increased while the dry cell weight, CER and OUR decreased.

The hyphal morphology of the seed cultures in the 20 L fermenter are given in Figures 3.23 to 3.25. Main hyphal length in the three fermentations increased after spore germination. Fragmentation took place during the fast growth period when the hyphal length became shorter and then remained fairly constant until the end of fermentation. The average main hyphal length after fragmentation at 500, 700 and 900 RPM were 110  $\mu$ m, 125  $\mu$ m and 95  $\mu$ m respectively (Figures 3.23 to 3.25). The increase in agitation speed from 700 to 900 RPM decreased the average main hyphal length after fragmentation. In contrast, increase in agitation speed from 500 to 700 RPM increased the average main hyphal length.

The short, thick and highly branched mycelia observed in the culture at 500 RPM were probably due to lack of oxygen (DOT fell to  $\sim 10$  % air saturation). The number of tips at 500 RPM increased during first stage of growth and then decreased when fragmentation occurred. Subsequent to that event, the number of tips increased again continuously for the remainder of the fermentation (Figure 3.23). The number of tips during the fermentations at 700 RPM and 900 RPM exhibited similar patterns, but their average values were lower than that at 500 RPM (Figures 3.24 & 3.25). It was observed that the mycelia at 900 RPM were thicker and more branched than those at 700 RPM.

The hyphal growth unit increased during the first stage of growth. After fragmentation in the fermentation operated at 700 RPM, the hyphal growth unit remained fairly constant for the remainder of fermentation (Figure 3.24). Due to the higher branching frequency at 500 RPM and 900 RPM, their hyphal growth units after fragmentation diminished towards the end of the fermentation (Figures 3.23 & 3.25).

It was evident that at 500 RPM, the culture experienced oxygen limitation. Meanwhile the agitation at 900 RPM, due to its high shear forces, caused a rapid mycelial fragmentation. Such situation was avoided at 700 RPM. Therefore, the latter agitation speed was selected for growing the seed inoculum before transferring it into a 150 L fermenter. After CER had reached its maximum peak at the end of the fast growth period (approximately 37 h after spore inoculation), 10 L of broth was transferred aseptically into 90 L medium in a 150 L vessel giving a total working volume of 100 L.



Figure 3.23 Hyphal morphology of the seed culture in the 20 L fermenter operating at 500 RPM



Figure 3.24 Hyphal morphology of the seed culture in the 20 L fermenter operating at 700 RPM



Figure 3.25 Hyphal morphology of the seed culture in the 20 L fermenter operating at 900 RPM

# 3.2.2 Growth and penicillin production at 150 L scale

By applying the same air flow rate per unit volume of fermenter (0.5 vvm), pH and temperature as in 7 L scale experiments, three fermentations at 265, 400 and 530 RPM were carried out in the 150 L fermenter of 100 L working volume. The tip speeds of these fermentations were 2.5, 3.8 and 5.0 m/s respectively. The seed inoculum in a volume of 10 L was transferred aseptically from the 20 L fermenter. This vegetative inoculum was obtained by inoculating the seed medium using  $0.8 \times 10^6$  spores/mL as applied in the inoculum preparation for 7 L scale.

Similar patterns of growth and penicillin production to 7 L scale were observed. The time course of the DOT in the fermentation operating at 265 RPM (B265) was not available due to probe failure after the fermenter had been sterilised. During the first 20 h of fermentation the biomass increased up to 6.6 g/L which corresponded to the CER value of 20 mmol/L/h. As soon as this fast growth ended, penicillin production began (Figure 3.26).

A linear profile was found at the beginning of penicillin production in B265, but after 92 h of fermentation it began to flatten out. A maximum penicillin production of 171 mg/g was achieved in fermentation B265. Increasing agitation speed at this scale was also found to reduce the rates of growth and production as also observed in the 7 L scale experiments. The specific penicillin production rate ( $q_{pen}$ ) at this scale was determined using the same procedure as applied for the 7 L scale and the results are given in Table 3.2.

Fermentation	Tip Speed (m/s)	Time interval (h)	q <sub>pen</sub> (u/mg/h)	μ (/h)
B265	2.5	21 - 92	3.80	0.007
B400	3.8	40 - 100	3.75	0.006
B530	5.0	41 - 112	3.51	0.005

<u>Table 3.2</u> Specific penicillin production rate  $(q_{pen})$  at 150 L scale



operating at 265 RPM



operating at 400 RPM


Figure 3.28 Growth and penicillin production in the 150 L fermenter operating at 530 RPM

In the fermentations operating at 400 RPM (B400) and 530 RPM (B530), the DOT dropped to nearly 70 % and 80 % air saturation respectively at the end of the fast growth period and then increased when the culture switched to the second carbon source which was also indicated by the declining profile of CER and OUR. As the second stage of growth proceeded, the CER and OUR recovered and the DOT began to decrease again but maintained at the values above 70 % air saturation and 85 % air saturation in B400 and B530 respectively (Figures 3.27 and 3.28).

The aeration and agitation conditions in this scale had no marked effect on the respiration of the culture. The RQ during the three fermentations (B265, B400, and B530) was relatively constant at around 1.0. However, it was noticed that the fermentation operating at 530 RPM showed a lag phase of production for approximately 20 h and reached a maximum penicillin production of 156 mg/g (Figure 3.28). In fermentation B400, a value of maximum penicillin produced close to the one in B265 was noted but it was achieved later, and therefore the  $q_{pen}$  value of B400 (3.75 u/mg/h) was smaller than the one of B265 (3.80 u/mg/h).

## 3.2.3 Hyphal Morphology at 150 L scale

The time courses of the main hyphal length, number of tips and hyphal growth unit at 265 RPM, 400 RPM and 530 RPM are given in Figures 3.29 to 3.31. While the longest mycelium observed during the fast growth period at 7 L scale was around 175  $\mu$ m, lengths of up to 350  $\mu$ m were found in the 150 L scale experiments. The main hyphal length and hyphal growth unit increased more rapidly during the first stage of growth in the 150 L scale experiments than those in the 7 L.

The mean main hyphal length of samples taken at 16 h from fermentations, where cultures were still in the fast growth period (as shown by increasing CER), were longer than those at 20 h fermentation. The mean main hyphal lengths at 16 h at 265 RPM, 400 RPM and 530 RPM were ( $352 \pm 26.5$ )  $\mu$ m, ( $292 \pm 20.8$ )  $\mu$ m and ( $279 \pm 18.4$ )  $\mu$ m respectively. On the other hand the values of mean main hyphal lengths at 20 h were ( $270.1 \pm 24.2$ )  $\mu$ m, ( $260.8 \pm 18.1$ )  $\mu$ m and ( $228.8 \pm 16.6$ )  $\mu$ m respectively.



Figure 3.29 Hyphal morphology of the culture in the 150 L fermenter operating at 265 RPM



Figure 3.30 Hyphal morphology of the culture in the 150 L fermenter operating at 400 RPM



Figure 3.31 Hyphal morphology of the culture in the 150 L fermenter operating at 530 RPM

In the three fermentations at 150 L scale, the main hyphal length decreased rapidly from 16 to 40 h. It was possible that this rapid fragmentation was associated with the culture stage adaptating to the second carbon source. Between 40 and 75 h of fermentation, the main hyphal lengths of B265, B400 and B530 were fairly constant, during which the penicillin production increased rapidly. At this period of time the average mean values of main hyphal lengths were 150  $\mu$ m, 120  $\mu$ m and 105  $\mu$ m for B265, B400 and B530 respectively.

After 75 h of fermentation there was a tendency for the main hyphal length to continue to drop due to fragmentation, only this time at a much slower rate in comparison to that between 16 to 40 h. After 100 h of fermentation high degrees of vacuolization were observed in the short and swollen mycelial cultures. This severe condition of the cultures was possibly linked with the slowing rate of penicillin yield at the same period.

The average number of tips was higher in the fast growth period than during penicillin production. When the mean main hyphal length decreased sharply between 16 and 40 h due to fragmentation, the mean number of tips also decreased and then remained fairly constant for the rest of fermentation. More branches were observed in fermentation B530 in comparison to those in B265 and B400.

The hyphal growth unit during fermentation had a similar profile to the main hyphal length. The average mean values of hyphal growth unit increased with a decrease in agitation. Fermentation B265 showed hyphal growth unit ~122  $\mu$ m at 16 h (Figure 3.29), while at this time of fermentation the values were ~93  $\mu$ m and ~86  $\mu$ m for B400 and B530 respectively (Figure 3.30 and 3.31). The cultures grown in the 150 L fermenter were observed to have a significantly higher hyphal growth unit during the fast growth period compared to 7 L cultures.

The percentage of clumps obtained from morphological measurements showed that the higher the agitation speed the lower the average amount of clumps. The values fluctuated throughout the formentations. The ranges of percentage of clumps from the samples of morphological neasurement were 74 - 91 %, 69 - 88 % and 56 - 88 % for B265, B400 and B530 respectively.

# 3.3 Effects of agitation at 1500 L scale

# 3.3.1 Growth of seed inoculum in 150 L fermenter

In order to provide 10 % v/v vegetative inoculum for the 1500 L scale fermentations,  $0.8 \times 10^6$  spores/ml were inoculated into 100 L of seed medium in the 150 L fermenter. An agitation speed of 400 RPM was chosen to accelerate the growth and to minimize predetermination of morphology. Temperature and pH were kept constant at 26 °C and  $6.70 \pm 0.5$  throughout the fermentation. An air flow rate of 0.5 vvm was introduced at atmospheric pressure. The exit gas CO<sub>2</sub> and DOT profiles of the seed inoculum are given in Figure 3.32.



Figure 3.32 DOT profile and exit gas CO<sub>2</sub> of seed cultures in the 150 L fermenter operating at 400 RPM

The spores began to germinate approximately 12 h after inoculation and ~6 hours of lag phase followed. After that a phase of rapid growth took place and reached its peak after 30 h of fermentation at which point this vegetative inoculum was inoculated into the 1500 L fermenter. The cultures at this point of transfer had approximately the same growth condition in terms of carbon dioxide produced as the inoculum grown in the 20 L fermenter for 150 L experiments. The exit gas  $CO_2$  at this time was ~1.1 % which corresponded to CER value of ~16 mmol/L/h. The DOT at this point was approximately 74 % air saturation (Figure 3.32).

## 3.3.2 Effects of agitation at 300 RPM

Two fermentations (C300 and C400) were carried out at 1500 L scale at agitation speeds of 300 RPM and 400 RPM. The tip speeds of these fermentations were 4.7 m/s and 6.3 m/s respectively. The same air flow rate per unit volume (0.5 vvm), pH and temperature as in previous experiments were applied.

During the fast growth period or the first 20 h of fermentation at 300 RPM the biomass increased to ~6.2 g/L (Figure 3.33) whilst DOT fell to ~55 % air saturation. The mean value of the main hyphal length at this point was ~320  $\mu$ m. The 'Chemap' 1500 L fermenter used in this fermentation was not equipped with a condenser or outlet-air heater to prevent the membrane filter from becoming wet. As a result, by the time the fast growth ended the head pressure reading showed 0.2 bar g. Venting the filter was attempted but did not improve the situation permanently. Since the upper scale reading of the air flow rate in this fermenter was only 500 L/min, it was not possible to increase the air flow rate any further in order to have a similar carbon dioxide partial pressure as was obtained in the 150 L experiments in Section 3.2.2. When the head pressure reading showed ~0.6 bar g, after 100 h of inoculation, the fermentation was terminated.

Fragmentation took place as in previous runs and after 45 h of fermentation the main hyphal length was fairly constant at ~150  $\mu$ m. There was no indication of cell lysis as the dry cell weight still increased and an observation under microscope showed intact mycelia. The degree of vacuolization was very high and a large amount of swollen parts along mycelia was observed (Plate 3.5).



Figure 3.33 Growth, production and morphology of cultures in the 1500 L fermenter operating at 300 RPM

There were no data available from a 'standard' fermentation (fermentation with unblocked filter) at 300 RPM in this scale. However, the pressure build up due to the blocked filter did not have a large effect on morphology.

The yield of Penicillin-V was very low in comparison to that in the previous fermentation. It was possible that the overpressure which began to take place after 20 h of fermentation did not give marked effects on the culture performance until after ~50 h of fermentation. Possibly, only when the head pressure approached the value of ~0.35 bar g and kept increasing did the high CO<sub>2</sub> concentration dissolved in the broth become significant, and result in the slump of penicillin production.

# 3.3.3 Effects of agitation at 400 RPM

The agitation at 400 RPM (6.3 m/s tip speed) was the highest that could be applied in this 1500 L 'Chemap' fermenter. To avoid the problem of overpressure that occurred in fermentation C300, a presterilised line was opened between the head of the fermenter and a presterilised 150 L vessel. The connecting line was adjusted manually to ensure that there was enough air coming through the outlet filter to the gas analyzer system. In order to prevent contamination, the fermenter was kept just above atmospheric pressure but below 0.1 bar g throughout the fermentation.

#### Growth and penicillin production :

The profiles of growth and penicillin production are given in Figure 3.34. Dry cell weight increased rapidly to 5.01 g/L during the first 22 h which corresponded to a CER value of  $\sim$ 12 mmol/L/h. The DOT fell to  $\sim$ 72 % air saturation before increasing and stabilising to 105 % air saturation at 0.1 bar g during the second growth period.

Penicillin-V production began early in the second stage of growth during which the dry cell weight increased at a very slow rate. A linear production of Penicillin-V was observed up to 110 h of fermentation reaching a maximum value of 173.5 mg/g biomass. During the last 15 h of the fermentation, it was found that Penicillin-V production fell to 140 mg/g biomass.



Figure 3.34 Growth and penicillin production at 400 RPM in the 1500 L fermenter

The RQ was fairly steady at around 1.0 during penicillin production and CER and OUR showed a constant value of around 4.0 mmol/L/h. By using the same procedure as before, the  $q_{pen}$  value calculated between 37 to 110 h in this fermentation was 3.73 u/mg/h.

#### Hyphal morphology :

The time course of the main hyphal length, number of tips and hyphal growth unit of fermentation C400 are given in Figure 3.35. During the first 22 h of fermentation, the mycelia elongated reaching a mean value of ~230  $\mu$ m in main hyphal length. This value was smaller in comparison to the one obtained in C300 at the same time of fermentation which was ~320  $\mu$ m. More branches were observed during the fast growth period and the number of tips decreased after fragmentation occurred.

During linear penicillin production of 37 to 110 h the average mean of the main hyphal length was ~146  $\mu$ m. The decrease in the main hyphal length, number of tips and hyphal growth unit after 110 h of fermentation are possibly linked with the diminishing profile of penicillin production at the same period of time in Figure 3.34. The average mean number of tips during the second stage of growth was ~4.0. As before, the time course of the hyphal growth unit showed a similar profile to that of the main hyphal length. After fragmentation took place the mean value of the hyphal growth unit fell from 84.1  $\mu$ m to the average value of ~50  $\mu$ m.

The rate of decrease in the main hyphal length and the hyphal growth unit was slower in comparison to those observed in 7 L and 150 L scale experiments. The percentage of clumps varied throughout fermentation between 62 to 88 %. Observation under microscope showed highly entangled mycelia from the samples taken during the fast growth period. However, for the same amount of objects the measurements using automatic image analysis on these samples were faster than those on 7 L and 150 L samples. As the cultures reached 40 h of fermentation, microscopic observation revealed predominantly free mycelia with only simple entanglement or cross over between them. The average percentage of clumps during the period of penicillin production was ~65 %.



Results

Figure 3.35 Morphology of cultures in the 1500 L fermenter operating at 400 RPM



<u>Plate 3.1</u> Morphology of vegetative inoculum grown in the 20 L fermenter at 700 RPM (Magnification : 100 X)



<u>Plate 3.2</u> Simple entanglement of two mycelia recognized as clumps by the automatic image analysis system (Magnification : 100 X)



<u>Plate 3.3</u> Morphology of non-producing penicillin mycelia at 16 h in the 150 L fermenter at 265 RPM (Magnification : 100 X)



<u>Plate 3.4</u> Morphology of penicillin producing mycelia at 72 h in the 150 L fermenter at 265 RPM (Magnification : 100 X)

Results



Plate 3.5 Morphology of mycelia at 100 h in the 1500 L fermenter at 300 RPM (Magnification : 400 X)



Plate 3.6 High percentage of clumps of cultures from the 1500 L fermenter operating at 400 RPM (Magnification : 100 X)

# 3.4 Effects of media sterilisation and overpressure condition on growth, production and morphology

## 3.4.1 Effects of media sterilisation

One of the impacts of using different scales of fermenters is the alteration of culture performance due to variations in the degree of media degradation during sterilisation. The 7 L laboratory fermenter used was made of glass while the 150 L and 1500 L were of stainless steel, therefore the heat transfer and time-temperature profiles during sterilisation would be different.

Batch steam sterilisation was applied to all scales. The fermenters were subjected to 1.0 bar pressure exposure at 118 - 121 °C. Since the rate of temperature increase from room temperature to 121 °C at 1 bar varied with the scale, the holding time to obtain the same degree of sterilisation had to be different. The holding time at this condition for every fermenter was chosen to meet both the sterility demand and as little nutrient degradation as possible.

The medium composition was also designed to be adequate for fermentations of longer than 125 h (Section 2.3.1.3), but at the same time not to exceed critical inhibition concentrations for growth or penicillin production if any. This way, lack of nutrients due to heat degradation was avoided.

Comparisons were made between 7 L and 150 L fermenters (Fermentations A850 & A850b), and between 150 L and 1500 L fermenters (Fermentations B530 and B530a). Effects of media sterilisation in 7 L and 150 L fermenters were compared, by running a fermentation at 850 RPM in the 7 L fermenter using a production medium which had been sterilised in the 150 L fermenter. Transfer of the medium from the 150 L to the 7 L fermenter was carried out aseptically. The results of this fermentation at 1500 L scale. This time, the medium sterilised in the 1500 L fermenter was aseptically transferred into the 150 L fermenter. A 'standard' fermentation at 530 RPM was then carried out in this fermenter. The results were compared to those from B530 in which in situ media sterilisation was used.

#### Growth and penicillin production:

Figure 3.36 shows that there is no significant difference in growth profile between the two fermentations in the 7 L at 850 RPM whether the medium was sterilised in situ or sterilised in the 150 L fermenter. Similarly, Figure 3.37 shows that there is no significant difference in growth between the two fermentations in the 150 L fermenter at 530 RPM whether the medium was sterilised in situ or in the 1500 L fermenter. The profiles of penicillin production of the two fermentations at 150 L fermenter from different sterilisation vessels were fairly similar (Figure 3.39). There was no marked effects of sterilisation in the 1500 L fermenter on the penicillin production. The onset of penicillin production of the two batches in Figure 3.39 were very close.

The more scattered values of dry cell weight in Figure 3.36 resulted in greater deviations between the profiles of penicillin production of Figure 3.38 and was particularly significant towards the end of fermentation. However, this was unlikely to cause significant errors in  $q_{pen}$  values, since the  $q_{pen}$  values were always taken within the linear period of production.

Figures 3.36 to 3.39 also indicated that the growth and penicillin production at 850 RPM in the 7 L fermenter are better than those at 530 RPM in the 150 L fermentation. The growth rate during penicillin production as well as the maximum Penicillin-V achieved were higher at 850 RPM in 7 L fermenter.

#### Hyphal morphology :

The effects of media sterilisation on the hyphal characteristics are given in Figures 3.40 to 3.47. Based on the main hyphal length profile of 7 L fermentations at 850 RPM in Figure 3.40, there seemed to be some effects of sterilisation on the morphology. In situ sterilisation in the 7 L fermenter probably caused more nutrient degradation in comparison to the sterilisation in the 150 L fermenter. This was particularly significant during the first 30 h of fermentation in Figure 3.40. After that, considering 90 % confidence intervals, the values of the main hyphal length of the two fermentations were fairly similar for the rest of the fermentation. In section 3.1.2 concerning reproducibility in the 7 L fermenter, it was found that during the fast growth period at 700 RPM and 850 RPM, poor reproducibility of morphological measurement in this particular period of time was also observed. Therefore, the differences in the mean main hyphal lengths of the two fermentations during the first 30 h in Figure 3.40 were probably due to poor reproducibility of measurement.



Figure 3.36 Effect of media sterilisation on the growth at 850 RPM in the 7 L fermenter (Both symbols represent dry cell weight)



Figure 3.37 Effect of media sterilisation on the growth at 530 RPM in the 150 L fermenter (Both symbols represent dry cell weight)



Figure 3.38 Effect of media sterilisation on penicillin production at 850 RPM in the 7 L fermenter (Both symbols represent yield of Penicillin-V)



Figure 3.39 Effect of media sterilisation on the penicillin production at 530 RPM in the 150 L fermenter (Both symbols represent yield of Penicillin-V)



Figure 3.40 Effect of media sterilisation on the main hyphal length at 850 RPM in the 7 L fermenter (Both symbols represent main hyphal length)



<u>Figure 3.41</u> Effect of media sterilisation on the main hyphal length at 530 RPM in the 150 L fermenter (Both symbols represent main hyphal length)

Figure 3.41 shows that there is no effect of media sterilisation on the main hyphal length of cultures of two fermentations at 530 RPM in the 150 L fermenter. It also indicates good reproducibility of measurement on the main hyphal length at 530 RPM in 150 L . scale. Microscopic observation revealed diffused cultures with predominantly free or simply crossed-over mycelia. Both main hyphal length profiles in Figure 3.41 showed two stages of fragmentation, the first one with a rapid decrease occurring between 20 and 50 h and the other with a slower rate between 100 and 125 h.

The differences in number of tips between the two fermentations in Figure 3.42 may be due to the media sterilisation or to poor reproducibility of morphological measurement of cultures grown in the 7 L fermenter, as mentioned previously. Figure 3.43 indicated that there was no significant effect of sterilisation in the 1500 L fermenter on the number of tips. Comparison between Figures 3.42 and 3.43 revealed that the average number of tips of the fermentations at 530 RPM in 150 L fermenter was higher than that at 850 RPM in the 7 L fermenter.

Hyphal growth unit profiles in Figures 3.44 and 3.45 indicated that media sterilisation had no effects on the main hyphal length and hyphal growth unit. In general, the similarity of morphological parameters in the large scale fermentations (150 and 1500 L) were fairly good.

Figures 3.46 and 3.47 show the effects of media sterilisation on the percentage of clumps. The percentages of clumps in the 7 L fermenter appeared to fluctuate more (Figure 3.46). In Figure 3.47 there is a clear decrease in percentage of clumps with fermentation time. The lowest percentage of clumps obtained was ~50%. Seed cultures consisted of predominantly very entangled mycelia (Plate 3.1), and when they were transferred into a production fermenter, the entangled mycelia were still present and became more open. However, when the cultures began to produce penicillin and the fragmentation took place, the entanglement of mycelia diminished and the measurement for the same number of organism became faster.



Figure 3.42Effect of media sterilisation on the number of tips at 850 RPMin the 7 L fermenter (Both symbols represent number of tips)



Figure 3.43 Effect of media sterilisation on the number of tips at 530 RPM in the 150 L fementer (Both symbols represent number of tips)



Figure 3.44 Effect of media sterilisation on the hyphal growth unit at 850 RPM in the 7 L fermenter (Both symbols represent hyphal growth unit)



Figure 3.45 Effect of media sterilisation on the hyphal growth unit at 530 RPM in the 150 L fermenter (Both symbols represent hyphal growth unit)



Figure 3.46 Effect of media sterilisation on the percentage of clumps at 850 RPM in the 7 L fermenter (Both symbols represent percentage of clumps)



Figure 3.47 Effect of media sterilisation on the percentage of clumps at 530 RPM in the 150 L fermenter (Both symbols represent percentage of clumps)

# 3.4.2 Effects of overpressure

It is a common practice to pressurize the vessel in a large scale aerobic submerged fermentation in order to ensure high oxygen solubility, hence improving oxygen transfer in the system. However, an increase in the hydrostatic pressure can lead to a high solubility of carbon dioxide which has been reported to give negative effects on the growth and production. Increasing air flow rate is often necessary to minimize build up of carbon dioxide in the liquid. However, the 'Chemap' 1500 L fermenter can only be operated to a maximum air flow rate of 500 L/min or 0.5 vvm for 1000 L working volume.

To investigate whether running at overpressure in the 150 L fermenter was beneficial compared to the 'standard' fermentation at an atmospheric pressure, two fermentations were carried out at 265 RPM and 400 RPM at 0.4 bar g overpressure. The nominal air flow rate of 0.7 vvm was applied to achieve the same partial pressures of carbon dioxide as in the 'standard' fermentations, i.e. atmospheric pressure with 0.5 vvm air flow rate. The results were compared to the two previous 'standard' fermentations in the 150 L fermenter at 265 RPM (B265) and 400 RPM (B400).

#### Growth and penicillin production :

Figures 3.48 to 3.51 show the comparisons between the 'standard' fermentations (atmospheric pressure ; 0.5 vvm) and overpressure run (0.4 bar g; 0.7 vvm) in terms of dry cell weight and penicillin production. At 265 RPM, the dry cell weight profiles of the two fermentations were similar (Figure 3.48). At 400 RPM, the profiles were also similar except between 60 - 100 h of fermentation where there was approximately 10 - 15 % differences in dry cell weight (Figure 3.49).

The onset of penicillin production of the two fermentations with different conditions were the same (Figures 3.50 and 3.51). The yield of Penicillin-V of standard fermentations (B265 and B400) was always higher throughout fermentations in comparison to those of overpressure runs. However, the differences did not appear to be significant. All the fermentations in Figures 3.50 and 3.51 show diminishing profiles of penicillin yield after 100 h of fermentation.



<u>Figure 3.48</u> Effect of overpressure on the growth at 265 RPM in the 150 L fermenter (Both symbols represent dry cell weight)



Figure 3.49 Effect of overpressure on the growth at 400 RPM in the 150 L fermenter (Both symbols represent dry cell weight)



Figure 3.50Effect of overpressure on the penicillin production at 265 RPM in the150 L fermenter (Both symbols represent yield of Penicillin-V)



Figure 3.51 Effect of overpressure on the penicillin production at 400 RPM in the 150 L fermenter (Both symbols represent yield of Penicillin-V)

### Hyphal morphology :

Figures 3.52 to 59 show the effects of vessel overpressure on the morphological parameters. Figures 3.52 and 3.53 show that the hyphal elongation is not influenced by overpressure condition. The data also indicate a good reproducibility of morphological measurements on the samples from the 150 L fermenter. The mean main hyphal length increased to  $(360.3 \pm 35.4) \mu m$  during the first 16 h of fermentation at 265 RPM (B265), and to  $(370.1 \pm 39.0) \mu m$  at 400 RPM (B400). These phenomena indicate that there is little effect of agitation during the first stage of growth.

The average number of tips in fermentations at 0.4 bar overpressure were higher than those in 'standard' fermentations (Figure 3.54 and 3.55). When fragmentation took place between 16 and 40 h of fermentation, the mean number of tips in all fermentation decreased and then remained fairly constant for the remainder of the fermentation.

Figures 3.56 and 3.57 show good reproducibility of the hyphal growth unit. The deviation of number of tips in Figure 3.54 does not greatly influence the profile of hyphal growth unit in Figure 3.56. At 265 RPM, the mean hyphal growth unit decreased between 16 - 40 h, remained constant for about 35 h, decreased again during the 75 - 92 h and finally remained fairly constant until the end of fermentation (Figure 3.56). At 400 RPM, the hyphal growth unit decreased between 16 - 40 h, remained constant for the next 57 h and continued to decrease for the rest of the fermentation. The possible link of the above behaviour with penicillin production will be discussed later in the Discussion.

Figures 3.58 and 3.59 show the profiles of percentages of clumps for the 'standard' and the overpressure fermentations at 265 RPM and 400 RPM respectively. Similar percentage of clumps for samples taken at the same time of fermentation were observed. There was a clear the decrease in percentages of clumps with fermentation time shown in both Figures 3.58 and 3.59. The average percentage of clumps at 400 RPM (70 %) was lower than that of 265 RPM (80 %).

resuits



Figure 3.52Effect of overpressure on the main hyphal length at 265 RPM in the 150 Lfermenter (Both symbols represent main hyphal length)



Figure 3.53 Effect of overpressure on the main hyphal length at 400 RPM in the 150 L fermenter (Both symbols represent main hyphal length)



Figure 3.54Effect of overpressure on the number of tips at 265 RPM in the 150 Lfermenter (Both symbols represent number of tips)



Figure 3.55 Effect of overpressure on the number of tips at 400 RPM in the 150 L fermenter (Both symbols represent number of tips)

Results



Figure 3.56Effect of overpressure on the hyphal growth unit at 265 RPM in the 150 Lfermenter (Both symbols represent hyphal growth unit)



Figure 3.57 Effect of overpressure on the hyphal growth unit at 400 RPM in the 150 L fermenter (Both symbols represent hyphal growth unit)



Figure 3.58 Effect of overpressure on the percentage of clumps at 265 RPM in the 150 L fermenter (Both symbols represent percentage of clumps)



Figure 3.59 Effect of overpressure on the percentage of clumps at 400 RPM in the 150 L fermenter (Both symbols represent percentage of clumps)

#### 4 **DISCUSSION**

In this work, a soluble semi-defined medium was chosen rather than a complex medium as used by Smith *et al.* (1990). This was done to allow dry cell weight (biomass) measurements to be made. Although results indicated low titres for Penicillin-V, glucose feeding was not considered necessary as biomass and Penicillin-V measurements were reproducible.

In investigating the effects of shear, it is necessary to isolate factors, other than agitation, which might contribute to changes in the behaviour of culture during fermentation. These factors include inoculum preparation, media sterilisation and pressure. In the section that follows a brief discussion is presented on each of these factors. This is then followed by a discussion on the effects of shear on growth and morphology.

#### Effects of inoculum preparation

All fermentations in this work were inoculated with a 10 % (v/v) seed inoculum. Seed cultures were prepared by inoculating the seed medium with 0.8 x 10<sup>6</sup> spores/mL. Variation in spore viability was minimized by using the same generation of spores for all experiments. Reproducibility was checked and was found to be satisfactory using the 7 L scale fermenter. Calam & Smith (1981) found that the spore concentration and the percentage of inoculum were critical to penicillin production and the morphology of P. chrysogenum. Studies in shake flask cultures showed that 10<sup>4</sup> and 10<sup>5</sup> spores/mL gave approximately equal results in terms of main hyphal length, number of tips and hyphal growth unit. In comparison, when the spore concentration was reduced to  $10^3$  /mL, the main hyphal length was shorter and more branching was observed in the early period of growth. In addition, mycelial cultures became pelleted after 22 h of fermentation. The rate of viability loss as a percentage of the original number of spores was faster for a spore concentration of  $10^3$  /mL than for  $10^5$ /mL. Therefore, a spore concentration of 10<sup>4</sup>/mL was considered as critical for the filamentous type of growth. Inoculum concentration has also been found to be significant in determination of culture production and morphology of P. chrysogenum P1 (Vardar & Lilly, 1982). Specific penicillin production rate (q<sub>pen</sub>), dry cell weight and percentage of free mycelia increased with inoculum concentration in the range of 2.5 and 10 % (v/v).

By using  $0.8 \times 10^6$  spores/mL inoculation in this work, the seed inoculum always exhibited filamentous growth. Although the microorganisms formed highly entangled mycelia, the pellet type of growth was never found. The use of 10 % (v/v) inoculum concentration was to ensure filamentous growth during penicillin production.

Another important aspect of inoculum preparation is the age of the inoculum transferred into the production stage. The age of cells has been reported to be a significant factor in the secondary metabolite fermentations. Brown & Vass (1973) introduced a model of maturity and product formation. It is said that only matured cells play a role in product formation, and the degree of maturity depends on the type of product. The time required to reach this stage is often reflected in the lag phase of secondary metabolite formation.

In this work, the vegetative inocula were transferred from the seed culture vessel to the production scale (150 L and 1500 L) when sucrose consumption ceased. This point was indicated by a peak in the carbon dioxide evolution rate (CER) during the course of seed culture fermentation: CER was monitored continuously using the data logging system. The results were confirmed also by monitoring the RQ value which was almost unity at the point of transfer.

Inoculum for the 7 L fermentations was prepared in shake flasks and no data on CER were available. However, preliminary experiments were carried out to evaluate the growth profiles in the shake flask cultures. In this way, profiles of dry cell weight were developed. The seed culture was transferred at the end of the fast growth period which was ~39 h after inoculation. In the seed fermentation at 20 L scale (inoculum for 150 L fermentations), the peak of CER occurred after ~37 h of inoculation, and at the 150 L scale (inoculum for 1500 L fermentation) it occurred after ~30 h of fermentation. From the three different scales of inoculum preparation, the dry cell weight at the point of transfer was found to be approximately in the range between 6.0 to 6.5 g/L of biomass.

Morphological observations using the microscope between 14 to 24 h after inoculation indicated that the time required to reach germination decreased as the scale of operation increased. This might have been due to more vigorous mixing during spore inoculation in stirred tank fermenters, which gave more effective spore dispersion and germination compared to shake flasks. However, this phenomenon did not appear to affect the growth in the production stage, neither did it influence the onset of penicillin production. It can therefore be assumed that in all cases, at the point of transfer, the inoculum cultures had reached the same level of cell maturity.

#### Effects of media sterilisation

The denaturation of nutrient during media sterilisation can result in changes in culture behaviour. In this work the fermentation medium was batch sterilized in situ at 121 °C and 1 bar pressure with a minimum holding time to prevent degradation of nutrient. The time required to reach 121 °C increased with scale of operation. Fermentations were conducted to investigate the effects of sterilisation conditions in different scales. Comparison of sterilisation effects between 7 L and 150 L was carried out by running a fermentation in the 7 L scale using media that had been sterilised in the 150 L fermenter. The same principle was applied in comparing sterilisation effects between 150 L and 1500 L fermenters.

In most industries batch sterilisation is no longer used for large scale fermentations. Instead continuous sterilisation is often applied. It is also common practice to sterilise certain media components separately in order to prevent unfavourable interactions which can result in the loss of nutrient quality. Carbonyl groups (reducing sugars) in the medium can react with the amino groups from amino acids and protein during sterilisation: this is known as Maillard browning reaction. The converted form of the reducing sugar from this reaction is not a suitable substrate for microorganisms and can result in considerably reduced yields in the fermentation. In this work, the nitrogen sources in the forms of ammonium sulphate and mycological peptone were sterilised separately from the remainder of the medium components. Sucrose was also sterilised separately to avoid losses due to caramelization. This was particularly important because the biomass concentration of cultures for penicillin production was very dependent on the sucrose consumption during the fast growth period. By ensuring the same concentration and quality of carbon source, uniformity in cultures behaviour during penicillin production can be improved.

In this work, once sucrose consumption ceased, the mycelial cultures were able to recover and began to consume lactose as the second carbon source. The nature of slow growth in lactose prevents the culture from runaway metabolism. Media sterilisation did not appear to affect biomass concentration during the course of fermentation (Figures 3.36 & 3.37). There was no significant difference in the penicillin production between fermentation with the media sterilised in the 150 L fermenter and the one sterilised in the 1500 L fermenter (Figure 3.39). Comparison of sterilisation in the 7 L and the 150 L showed that, near the end of fermentation with the media sterilised in 150 L fermenter, there was no sign of diminishing profile of penicillin production (Figure 3.38).
However, the onset of penicillin production and the titre between 25 and 100 h for the two fermentations from different sterilisation vessels were similar. The reason for a nondiminishing profile of penicillin production in the fermentation using media sterilised in the 150 L vessel was not clear since nutrient degradation was likely to occur in the larger scale fermenters due to extended heating and cooling periods. The concentration of media components in this work was designed to be sufficient for more than 125 h so that the loss of nutrient due to sterilisation would not result in cell lysis.

The decline in penicillin production at a particular time during the course of fermentation has been reported by many authors. Pirt & Righelato (1967) found that to maintain penicillin synthesis, a minimum growth rate was required. In chemostat cultures, the growth rates should not fall bellow 0.015 /h otherwise penicillin production would decrease sharply. The rate of decrease in penicillin synthesis was reported to be inversely proportional to the growth rate prior to the decline in the penicillin synthesis. Furthermore, Righelato *et. al.* (1968) proposed that growth rate also influenced the morphology of *Penicillium chrysogenum*. At a growth rate of 0.014 /h and below, conidiation, which represents a severe restriction on the vegetative growth, would occur. There was no conidiation observed in the cultures with growth rates between 0.023 to 0.075 /h, however the proportion of pellets and swollen mycelia increased with increasing growth rate.

In relation to this study, there was no significant difference in the growth rates between the two fermentations with different sterilisation conditions (Figures 3.36 & 3.37). Therefore it was unlikely that the decline in the penicillin production towards the end of fermentation, seen in Figure 3.38, was due to the difference in growth rate. Hyphal morphology did not appear to be affected by the sterilisation conditions although there was a difference in the main hyphal length at the early growth stage. This was mainly due to poor reproducibility of morphological measurement at this particular stage in the 7 L fermentations.

In contrast to the results found by Pirt & Righelato (1967), Mou (1979) showed that the decrease in penicillin production after a certain time was independent of growth rate. Smith (1985) conducted fermentations with increased amount of Phenoxyacetic acid (P.O.A.), nitrogen and sulphur in order to investigate the causes for the decline in penicillin synthesis. The results proved that an excess amount of the main nutrients required for penicillin synthesis did not prevent the decline in penicillin production in fed batch cultures.

#### Effects of pressure

In order to minimize the risk of contamination, large scale operation at an overpressure is a common practice. However, with large scale fermenters there is normally a relatively high hydrostatic pressure and this has to be taken into account in order to control the oxygen and carbon dioxide solubility in the nutrient solutions. There have been many studies on the effect of pressure on the growth and metabolism of microorganisms: the main concern is usually the impact of high concentrations of carbon dioxide. An overpressure combined with high air flow rate is often applied to achieve better oxygen transfer with a controlled carbon dioxide solubility.

In a series of experiments in this study, the effect of pressure became particularly important at 150 L and 1500 L scale. In order to completely isolate the effect of agitation, it is preferable to apply the same fermentation conditions such as temperature, pH and air flow rate during experiments. Two experiments in the 150 L fermenter (265 RPM and 400 RPM) at 0.5 vvm and atmospheric pressure were compared to experiments carried out at 0.7 vvm and 0.4 bar g overpressure: all other fermentation conditions including partial pressure of carbon dioxide remained unchanged. The results (Figures 3.48 to 3.59) show that operating at 0.5 vvm air flow rate and atmospheric pressure in 150 L fermenter did not create an adverse condition for growth and production, and did not affect the morphology of *Penicillium chrysogenum*.

Pirt & Mancini (1975) reported a 50 % decrease in the specific penicillin production rate  $(q_{pen})$  at a carbon dioxide partial pressure of 0.08 atm in a chemostat culture with automatic control of pH and dissolved oxygen tension. The effect was found to be reversible. However, in the large scale fermenters the effects of carbon dioxide are difficult to evaluate because its partial pressure varies with liquid depth. In a 10 m high fermenter operated at atmospheric pressure the partial pressure of carbon dioxide at the bottom is twice that at the top. Increasing hydrostatic pressure in the range of 1.15 bar to 8.0 bar absolute in batch cultures of *Pseudomonas fluorescens* in an airlift reactor resulted in an extended lag phase, decreased dry cell weight at stationary phase and reduced cell size (Onken & Jostmann, 1984). The effects of increasing pressures caused a decrease in cell density and substrate yield. It was postulated that unfavourable conditions due to increasing pressure were attributed to high partial pressure of carbon dioxide and oxygen.

In the 1500 L (1000 L working volume) fermenter used in this work, it was not possible to carry out fermentations with air flow rates above 500 L/min (0.5 vvm). Even though the capacity of the fermenter allows operation up to 1 vvm, the flow meter controller was designed for a maximum value of 500 L/min. In addition, an agitation speed of 400 RPM (6.3 m/s tip speed) is the highest possible value which can be applied in the 1500 fermenter. A fermentation in the 1500 L fermenter at 400 RPM (C400) was operated just under 0.1 bar overpressure. The profile of penicillin production under these conditions (Figure 3.34) was better than those obtained from the 7 L fermentation (Figures 3.4, 3.6, 3.8, 3.16 and 3.17). It is possible that in this range of pressure, the concentration of carbon dioxide does not severely affect penicillin synthesis. The decline in penicillin production towards the end of fermentation was also observed (Figure 3.34).

A fermentation at 300 RPM in the 1500 L fermenter demonstrated the severe effect of carbon dioxide on penicillin production (Section 3.3.2). The pressure increased to 0.6 bar due to a filter blockage during fermentation. This resulted in an increase of carbon dioxide solubility which might have been expected to affect mycelial characteristics. Ho & Smith (1986a) found that compared with cell growth, penicillin production was affected more severely by an excess amount of carbon dioxide. A high degree of vacuolization in the bulbous mycelial network was observed in the fermentation at 300 RPM. However, vacuolization is a common phenomenon observed in this work during penicillin production at all levels of operation. The profile of main hyphal length at 300 RPM was also similar to that at 400 RPM (Figure 3.35). Based on these observations, it is therefore not possible to conclude that overpressure adversely affected the morphology.

# 4.1 Effects of shear on the *P. chrysogenum* fermentation at different scales of fermenters

All penicillin producing fermentations carried out in this study were operated under sufficient oxygen supply. The critical DOT of 30 % air saturation proposed by Vardar & Lilly (1982) was always exceeded in the production fermentations. The following discussions concern the effects of shear generated by mechanical agitation upon the growth, production and morphology of *P. chrysogenum*. The good degree of reproducibility at the 7 L scale, discussed in Section 3.1.2, and the investigation on the effect of media sterilisation (Section 3.4.1) support the idea that variations in growth, production and culture morphology during the fermentations were due to shear in the fermenter.

### 4.1.1 Growth and penicillin production

Fermentations at 7 L scale (laboratory scale):

A series of fermentation experiments were carried out at 700, 850, 1000, 1150 and 1300 RPM. The results of these experiments shown in Figures 3.4 to 3.19 indicate that  $q_{pen}$  decreases with increasing agitation speed. A decrease in the specific growth rate (calculated with respect to both the second linear growth and the linear penicillin production) was also observed: 0.008 /h at 850 RPM and 0.004 /h at 1300 RPM.

The lower growth rate at 700 RPM in comparison to that at 850 RPM was probably due to less growing points being produced during fragmentation. Studies by some authors have indicated that an increase in agitation speed results in low productivity but does not necessarily affect growth (König *et al.*, 1981). The matter is often complicated by problems associated with oxygen limitation. The increase in biomass concentration with increasing agitation in the fermentations of filamentous fungi as reported by many authors (König *et al.*, 1981, Belmar Campero & Thomas, 1991) is often due to an improved oxygen transfer in the system. The superimposed effects of biomass concentration, apparent viscosity, agitation speed and possible formation of small mycelial pellets complicate the relationships between mechanical agitation and growth and between agitation and product yield. The use of complex media by most researchers also causes difficulties in assessing the agitation effect on cell growth.

In this work, using a soluble medium, a decrease in the specific growth rate with increase in agitation speed was observed. In agreement with König *et al.* (1981), the maximum penicillin production at the 7 L scale decreased with an increase in agitation.

#### Fermentations at 150 L and 1500 L scales (pilot plant scales)

Figure 4.1 shows that the specific growth rate ( $\mu$ ) generally decreases with increasing tip speed, with the exception of the data for the 7 L fermenter operating at 700 RPM. The possible reason for this apparent anomaly is that during this specific run, the DOT dropped to 38 % air saturation at the end of the fast growth period (Figure 3.5). This inevitably causes a decrease in the solubility of oxygen in the medium, and consequently a drop in the rate of mass transfer between the mycelia and the dissolved oxygen.

The growth rate during penicillin production in the 150 L fermentations was not strongly affected by agitation. Fermentations at 265 RPM, 400 RPM and 530 RPM in the 150 L fermenter gave second stage growth rates of 0.007 /h, 0.006 /h and 0.005 /h respectively. In contrast, Reuss (1988) observed a significant decrease of specific growth rate in a 137 L (working volume) fermentation of Rhizopus nigricans. The value of  $\mu_{max}$  decreased from 0.339 /h to 0.245 /h with increase in agitation speed from 217 RPM to 373 RPM. However, when the volume of liquid in the fermenter was reduced from 95 L to 63 L at a constant agitation speed of 800 RPM, the culture also showed a decrease in the specific growth rate from 0.263 /h to 0.224 /h. This result indicates that agitation speed was not the only factor affecting the specific growth rate. In the fermentation at 400 RPM (6.3 m/s tip speed) in the 1500 L fermenter used in this work, the growth rate during penicillin production, 0.0029 /h, was low in comparison to the fermentations at 7 L and 150 L scales. It is however interesting to note that the value of  $q_{pen}$  for the 1500 L fermentation (3.73 u/mg/h) was similar to that at 850 RPM in 7 L for which the specific growth rate was 0.008 /h. It seems that the effects of agitation on the specific growth rate and on  $q_{pen}$  decrease with an increase in the scale of fermentation. The possible causes for this trend will be discussed in Section 4.1.3.

Figure 4.2 shows that  $q_{pen}$  also decreases with an increase in tip speed. In comparison to those at 7 L scale, the effects of agitation on  $q_{pen}$  at 150 L scale were mild (Figures 3.26 to 3.28). Doubling the tip speed from 2.5 m/s (265 RPM) to 5.0 m/s (530 RPM) caused a decrease of only 7.7 % in  $q_{pen}$ , whilst at 7 L scale increasing tip speed from 2.5 m/s (700 RPM) to 4.6 m/s (1300 RPM) caused a decrease of 21 % in  $q_{pen}$  (Figure 4.2).







Figure 4.2 Specific penicillin production rate as a function of tip speed (Agitation speed is indicated in RPM for each point)

Smith *et al.* (1990) found that the reduction in  $q_{pen}$  value due to agitation was less pronounced in a 150 L than in a 14 L fermenter. By increasing the tip speed from 3.35 m/s (800 RPM) to 5.03 m/s (1200 RPM) in a 14 L fermenter,  $q_{pen}$  reduced by 52.7 %, whilst in a 150 L fermenter, increasing tip speed from 3.34 m/s (350 RPM) to 5.24 m/s (550 RPM) only reduced  $q_{pen}$  by 16 %. The strain of *P. chrysogenum* used by Smith *et al.* (1990) was the same as in this work. The differences in the degree of dependency of  $q_{pen}$  on the tip speed in this work and in Smith's were possibly due to the difference in the mode of operation (batch vs fed-batch).

#### 4.1.2 Morphology

#### Fermentations at 7 L scales (laboratory scales)

Fermentation experiments revealed that the higher the intensity of agitation in the fermenter, the sooner will be the point of initiation of fragmentation (Figures 3.10, 3.12, 3.14, 3.18 & 3.19). This observation is in agreement with the results of Belmar Campero & Thomas (1991) for *Streptomyces clavuligerus* fermentation in a 7 L fermenter. The decrease in the effective or main hyphal length and the increase in branching frequency with increase in agitation intensity were observed by these workers. These phenomena were also found in the work carried out by Smith *et al.* (1990). These workers observed that the rate of decrease in the main hyphal length was highest at 1200 RPM (10 L working volume vessel) in comparison to data obtained at 800 RPM and 1000 RPM. The limited number of observations during the fermentations however caused difficulties in quantitative assessment of the results.

In the present investigation it proved rather difficult to observe a trend in data relating to the rate of decrease in the main hyphal length. First, the maximum hyphal length achieved in each fermentation run was different and the point in time at which it occurred was also difficult to determine. Second, during the elongation period (prior to fragmentation) the microorganisms exhibited highly entangled mycelial networks. The mean value of the main hyphal length for this period had a higher standard deviation compared with data measured during the penicillin production phase. Therefore, it is difficult to be precise about the exact location of the peak in the time course of the main hyphal length. However, the observations in this work do support the general view that the main hyphal length decreases with increasing impeller speed. Morphological characteristics of *Penicillium chrysogenum* appear to be dependent on the strain and fermentation conditions. For similar hydrodynamic conditions, the main hyphal length and the total hyphal length observed by Smith (1985) were approximately 30 % longer than those found by van Suijdam & Metz (1981b). In the present investigation, the strain used was the same as that employed by Smith (1985), but batch culture was applied instead of fed-batch. As a result the profiles of morphological characteristics during the course of fermentation are different in the two systems. In a fed-batch fermentation using complex media, Smith (1985) found that the main hyphal length and hyphal growth unit continued to decrease during the period of penicillin production, between 30 to 120 h of fermentation. In the experiments carried out by Smith (1985) in a 14 L fermenter (10 L working volume), the main hyphal length decreased from ~220  $\mu$ m to ~95  $\mu$ m, and the hyphal growth unit was in the range of ~90  $\mu m$  to ~40  $\mu m$ . The average main hyphal length and hyphal growth unit in the 7 L fermentations in this work were respectively 20 % and 30 % shorter than those observed by Smith (1985). These differences are probably due to the use of different media and the benefit of glucose feeding over lactose in batch fermentation.

The main hyphal length and hyphal growth unit during the linear penicillin production stage were fairly constant: a slight decrease in these parameters were observed towards the end of some runs (approximately after 100 h of fermentation), for example as shown in Figure 3.31. In agreement with this work, van Suijdam & Metz (1981b) using batch culture experiments also identified a period of constant morphology. However, the poor reproducibility of measurements observed means that the values of the morphological parameters during that period could not be used to represent the morphological condition of a particular fermentation (van Suijdam & Metz, 1981b). The morphological measurements during period of constant morphology in this work were found to be reproducible (Section 3.1.2). In this present investigations, the average value of the main hyphal length and hyphal growth unit (during the linear period of penicillin production) decreased with increase in agitation. The value of the main hyphal length is used to represent the effect of agitation on the morphology in this work.

#### Fermentations at 150 L and 1500 L scales (pilot plant scales)

Measurements of the main hyphal length and hyphal growth unit during the fast growth period in the pilot plant scale fermentation were approximately twice the values observed in the 7 L scale. The high percentages of permanent mycelial aggregates in the fast growth period resulted in poor reproducibility of measurements at the 7 L scale.

In the 150 L and 1500 L scales, these aggregates could be disentangled to a certain extent by increasing the dilution factor. It is possible that the permanent entanglement in the 7 L scale is a response to the higher degree of mechanical stresses in this unit, expressed in terms of energy input per unit mass. Dion *et al.* (1954) also observed *P. chrysogenum* cultures of predominantly longer mycelia during the early growth period in a 30,000 L fermenter in comparison to those in a 10 L scale unit.

The fast growth period in the 150 L scale fermentations lasted for approximately 20-25 h (Figures 3.29 to 3.31). The mean main hyphal length of samples measured after 16 h of fermentations, while cultures were still in the fast growth period, were longer than those after 20 h fermentation (Section 3.2.3). This phenomenon indicated that the initiation of fragmentation was not triggered by limitation of the first carbon source. Since there were no samples taken between 0 and 16 h, it is not possible to distinguish between the two possible effects of agitation in this stage of growth. Firstly, agitation affects the rate of hyphal elongation and the values of the main hyphal length at 265, 400 and 530 RPM showed that lower agitation was beneficial for hyphal growth. Secondly, in each case, at some time between 0 and 16 h, the main hyphal length, for each fermentation, reached its maximum value, and the point in time at which it occurred could not be determined.

From microscopic observations, the vacuolized region along the mycelia began to show significantly when the cultures started producing penicillin. Moreover, the proportion of the vacuolized region seemed to increase during penicillin production. As the biomass concentration also increased during this period, the vacuolization was likely to be a specific cytoplasmic event rather than a sign of autolysis. The average values of the main hyphal length and the hyphal growth unit during penicillin production decreased with increasing agitation speed in the 150 L fermentations. At 400 RPM in the 1500 L fermentation, the rate of decrease in the main hyphal length and the hyphal growth unit (Figure 3.35) was slower in comparison to those at 150 L scale (Figures 3.29 to 3.31). The average value of the main hyphal length during penicillin production in this run was similar to that found in the 150 L scale operating at 265 RPM.

Figure 4.3 shows the average main hyphal length during the linear period of penicillin production as a function of tip speed for the three scales of fermentation studied in this work. The main hyphal length appears to decrease with increasing tip speed in the 7 L and the 150 L scale, while in the 1500 L fermenter the limited data indicate that an increase in tip speed causes little change in the apparent main hyphal length. The 7 L and 150 L fermenters had the same ratio of impeller to tank diameter (D/T) of 0.45, while in the 1500 L fermenter the D/T ratio was 0.33.



Figure 4.3 Main hyphal length as a function of tip speed

The effect of agitation on morphology also depends on the strain and the mode of operation during fermentation. Batch fermentations using lactose as the second carbon source in this work allow a period of constant morphology, while a glucose fed-batch system prevents such a phenomenon. Figure 4.3 also shows that the main hyphal length is scale dependent since at constant tip speed there is no apparent relationship between the average value of main hyphal length and the scale of operation. This phenomenon emphasizes that tip speed is not a good scale up parameter.

# 4.1.3 Relationships between growth, morphology and the rate of penicillin production

#### Growth rate and specific penicillin production

Figure 4.4 shows a plot of  $q_{pen}$  against  $\mu$  with impeller speed as a variable. The data for this plot are obtained from Figures 4.1 & 4.2, and refer to the penicillin production period. Evidently, an increase in the specific growth rate causes an increase in  $q_{pen}$  up to a limit ( $\mu \sim 0.007$  /h), beyond which  $q_{pen}$  becomes nearly independent of  $\mu$ .

These observations support the view suggested previously by Vi ning *et al.* (1990). The three primary amino acid precursors for penicillin production i.e: valine, cysteine and lysine are generated from the primary metabolic pathway, each with its regulatory control mechanism. Although there have been many reports that mechanical stress can lead to lower secondary metabolite yield of filamentous fungi, there has been few reports which verify the direct influence of mechanical stress on growth or productivity with the exception of works by Tanaka *et al.* (1975) and Ujcová *et al.* (1980).

From the discussion presented so far,  $q_{pen}$  may be affected by agitation either directly or indirectly through the decrease in  $\mu$  (resulting from an increase in agitation speed). It is not yet possible to distinguish between the two causes.

### Growth rate and mycelial morphology

The average value of the main hyphal length during the linear penicillin production stage generally increases with an increase in the specific growth rate ( $\mu$ ) (Figure 4.5). The interaction between  $\mu$  and the main hyphal length appears to be scale dependent.

The effect of the specific growth rate on the morphology has been reported to be dependent on strain and fermentation condition and the mode of operation of the fermentation. Observation in a continuous culture at a constant agitator speed of 1000 RPM in a 5 L fermenter showed that the main hyphal length increased with an increase in dilution rate ((Metz, 1976). Results shown in Figure 4.5 are in agreement with this observation, but the increase in the specific growth rate in the present work was a result of less intense agitation.



Figure 4.4Relationship between specific growth rate ( $\mu$ ) and specific penicillinproduction rate (Agitation speed is indicated in RPM for each point)



<u>Figure 4.5</u> Relationship between specific growth rate ( $\mu$ ) and main hyphal length (Agitation speed is indicated in RPM for each point)

#### Mycelial morphology and penicillin production

Figure 4.6 shows the relationship between  $q_{pen}$  and the main hyphal length. As the main hyphal length increases, initially there is an increase in  $q_{pen}$ . For the main hyphal length above ~ 100  $\mu$ m,  $q_{pen}$  becomes relatively independent of the main hyphal length.

In addition, it should be emphasized that it is likely that the increase in  $q_{pen}$  with increasing main hyphal length only applies for a limited range of the main hyphal length, because as agitation speed decreases sooner or later oxygen limitation will result. This, as reported by many authors, causes a decrease in  $q_{pen}$  and creates predominantly short, thick and highly branched mycelia.



<u>Figure 4.6</u> Relationship between main hyphal length and specific penicillin production rate (Agitation speed is indicated in RPM for each point)

# 4.2 Models of shear influence on penicillin production and morphology of *P. chrysogenum*

Break up of mycelia is believed to be caused by mechanical damage to the cells in the region around the impeller. Agitation and aeration are required to provide sufficient oxygen transfer during fermentation, however excessive agitation can have detrimental effects on the microorganisms. Thus, increase in agitation speed above an optimum value should in theory decrease the size of the microorganisms, i.e. main hyphal length.

Furthermore, agitation has been reported to be directly correlated to the ability of the cultures to produce penicillin, although the exact mechanism is yet unknown. Agitation of the broth in the fermenter is commonly achieved by the rotary action of a mechanical impeller and from the air expanding and rising through the vessel, and often the power input per unit mass of fermenter broth ( $\varepsilon$ ) is used to characterize the degree of agitation. The concept of a trailing vortex behind an impeller blade described by van't Riet & Smith (1975) as shown in Figure 1.8 (Section 1.6.2) has been used by many authors to generate models for the discharge flow from turbine impellers. The area around impeller is regarded as a highly turbulent zone. The energy generated in this zone is proportional to the power input per unit mass,  $\varepsilon$ , delivered by the impeller to the fluid.

Figure 4.7 presents data for  $q_{pen}$  and the main hyphal length (Le) plotted against power input per unit mass ( $\varepsilon$ ) on log-log paper. The power input used is the total power drawn by the three impellers: for this purpose the bottom impeller is assumed to be under aeration and the top two impellers are considered to be ungassed. The calculation of the power input per unit mass is given in Appendix 1. The data of the main hyphal length (Le) refer to periods during which there was no change in morphology of the culture, and were obtained during the linear period of penicillin production for the three scales of operation used in the present investigation.

Data in Figure 4.7 indicate that the main hyphal length is less likely to be affected by mechanical agitation as scale of fermenter increases and power inputs are less than 10 W/kg. Evidently, there is a critical value of  $\varepsilon$  beyond which  $q_{pen}$  and the main hyphal length fall as  $\varepsilon$  increases, and below which  $q_{pen}$  and the main hyphal length are only weakly dependent on  $\varepsilon$ . Figure 4.7 suggests that the magnitude of the critical  $\varepsilon$  in this experiments is about 10 W/kg.



Figure 4.7Main hyphal length and  $q_{pen}$  as function of power per unit mass ( $\epsilon$ ) $q_{pen}$  : ( $\bullet$ ) 7 L ; ( $\blacksquare$ ) 150 L ; ( $\blacktriangle$ ) 1500 LMain hyphal length : ( $\circ$ ) 7 L ; ( $\Box$ ) 150 L ; ( $\triangle$ ) 1500 L



Figure 4.8Comparison of  $q_{pen}$  values from this work and Smith *et al.* (1990)This work: (•) 7 L ; (•) 150 L ; (•) 1500 LSmith *et al.* (1990) : (0) 14 L ; (□) 150 L

Therefore, the following relationships are proposed based on these findings:

$$q_{pen} = K_{pen} \ \epsilon^{Apen} \quad ; K_{pen} = 8.6 \text{ and } A_{pen} = -0.3 \qquad \text{for } \epsilon \ge 10 \text{ W/kg, and}$$

$$K_{pen} = K_{pen max} = 3.8 \text{ and } A_{pen} = 0 \qquad \text{for } \epsilon < 10 \text{ W/kg} \qquad (4.1)$$

$$Le = K_{Le} \ \epsilon^{ALe} \qquad ; K_{Le} = 300 \text{ and } A_{Le} = -0.3 \qquad \text{for } \epsilon \ge 10 \text{ W/kg, and}$$

$$K_{Le} = K_{Le max} = 140 \text{ and } A_{Le} = 0 \qquad \text{for } \epsilon < 10 \text{ W/kg} \qquad (4.2)$$

 $K_{pen}$  and  $K_{pen max}$  are in u/mg/h,  $A_{pen}$  is in ukg/(Wmgh).  $K_{Le}$  and  $K_{Le max}$  are in  $\mu m$  and  $A_{Le}$  is in  $\mu mkg/W$ . The values of  $K_{pen}$ ,  $A_{pen}$ ,  $K_{Le}$  and  $A_{Le}$  are likely to be strain dependent, but it is encouraging to see that the simple concept of power input per unit mass has brought together data from different scales.

Substituting into Equations 4.1 & 4.2 for  $\varepsilon$  using Equation 1.22, gives the following relationships,

 $q_{pen} \propto 8.6 [P_0 N^3 D^2]^{-0.3}$  or  $q_{pen} \propto N^{-0.9} D^{-0.6}$  for  $\varepsilon \ge 10 W/kg$  (4.3)

Similarly, Le  $\propto 140 [P_0 N^3 D^2]^{-0.3}$  or Le  $\propto N^{-0.9} D^{-0.6}$  for  $\epsilon \ge 10 W/kg$  (4.4)

Equations 4.1 to 4.4 should be considered in the light of the relatively few experimental data points used in their derivation. This is especially true for the region  $\varepsilon \ge 10$  W/kg.

The plots in Figure 4.8 include data from a previous publication (Smith *et al.*, 1990), using the same strain, but a different mode of operation (fed-batch vs. batch) and, in some cases, a different ratio of impeller to tank diameter. The fermenters used by Smith *et al.* (1990) had different ratios of impeller to tank diameter: D/T was 0.35 for 14 L scale unit and was 0.45 for 150 L scale unit. In addition, one of the fermentation at 150 L scale was carried out with D/T of 0.35 (Smith *et al.*, 1990). The 7 L and 150 L fermenters used in this present investigation had the same D/T ratio (0.45), and the 1500 L fermenter had 0.33 D/T. It is encouraging to see that the trends in data are rather similar in both studies. The exponent of  $\varepsilon$  (-0.3) obtained from this work implies that

hyphal break up is likely to be due to turbulent-induced forces with eddies in the viscous dissipation subrange of the Taylor's energy spectrum (Equation 1.21, Section 1.6.3). The exponent of  $\varepsilon$  in Smith *et al.*'s data is close to -0.5 which implies that hyphal break up is due to turbulent eddies in the inertial subrange. It is however important to emphasize that the difference between exponents of  $\varepsilon$  (-0.3 vs -0.5) is based on a limited observations. Clearly more data in this range of  $\varepsilon$  are required to confirm these observations.

Figure 4.9 shows experimental data on the main hyphal length plotted against  $\varepsilon$  for various microorganisms, including data from the present investigation. Unfortunately, previous researchers whose data are presented in Figure 4.9 did not provide measurements of  $\varepsilon$ . In the absence of this information, and for the purpose of Figure 4.9, values of  $\varepsilon$  were estimated using the procedure given in Appendix 1. The geometrical and operating parameters of the works in Figure 4.9 are given in Table 4.2. Profiles in Figure 4.9 are similar to those given in Figure 4.7, and indicate that critical  $\varepsilon$  value at which the dependency of main hyphal length (Le) on  $\varepsilon$  changes is strain dependent. It is however remarkable to see that within the range of experimental data covered, the form of the relationship between  $\varepsilon$  and Le is strain independent. In other words, Equation 4.2 is applicable to all these cases, the only parameter that changes is the coefficient K<sub>Le</sub>. Table 4.1 below gives the values for K<sub>Le</sub> obtained from data in Figure 4.9.

Strain	E <sub>critical</sub> (W/kg)	K <sub>Le</sub> (μm)	K <sub>Lemax</sub> (μm)
P. chrysogenum P1 (this work)	10	300	140
P. chrysogenum Q176 (sparger)	not reached	300	not reached
P. chrysogenum Q176 (surface)	not reached	350	not reached
A. glaucus	0.5	500	350
A. fumigatus	0.5	1000	400
A. niger	0.5	500	350
P. varioti	0.5	500	350
Pellets of L. edodes	0.004	6000	3500

<u>Table 4.1</u> The values of  $\mathcal{E}_{critical}$  and  $K_{Le}$  for various strains



References	Dion <i>et al.</i> (1954)	Dion <i>et al.</i> (1954)	Dion & Kaushal (1959)	Dion & Kaushal (1959)	Dion & Kaushal (1959)	Dion & Kaushal (1959)	Taguchi <i>et al.</i> (1968)	
د (W/kg)	0.33 1.66 5.05	0.23 0.23 1.43 1.37 4.61	2.31 3.28 0.45 0.15	2.31 3.28 0.45 0.15	2.31 3.28 0.45 0.15	2.31 3.28 0.45 0.15	0.004 0.033 0.117 0.886 0.540	
Le or dp (µm)	250 170	127.9 93 110 80	130 130 300 370	156 158 400 400	160 160 225 315	160 130 270 305	4380 4000 2500 2100	:
dN	بوه	)	0.5 0.5 0.9	0.5 0.5 0.9	0.5 0.5 0.9	0.5 0.5 0.9	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	
Baffles	Yes 	2:::	Yes . No	Yes No	Yes No	Yes No	Yes	
Air flow rate (L/min)	2.5 "	2:5	5 . 5 5	5 . 5 5	2.5	2.5 2.5	1 1 1 1 1 1	<u> Table 4.2</u>
Aeration system	sparger "	surface	surface " sparger	surface " sparger	surface " sparger	surface " sparger		
N (RPM)	360 600 800	600 500 750	750 1450 750 360	750 1450 750 360	750 1450 750 360	750 1450 750 360	200 300 500 500 500 500 500 500 500 500 5	
D (mm)	75 "	65 " 90	90 65 75	90 65 75	90 65 65 75	90 65 65 75	60 90 90 60	
impeller	disc turbine (1)	disc turbine (2) "	propeller (2)	propeller (2)	propeller (2)	propeller (2)	disc turbine (1)	oeriments is 5 L
Strain*	P. chrysogenum Q176	P. chrysogenum Q176 "	Aspergillus niger "	Aspergillus fumigatus "	Aspergillus glaucus "	Paecilomyces varioti "	Lentinus edodes (pellets) " "	*The working volume for all cx1

Discussion

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The data plotted in Figures 4.7 & 4.9 provide a means for testing the various models of break up presented in Introduction, Section 1.6.3. For data on Le obtained in the present investigation, Equation 1.21 appears to describe experimental observations quite well in the region of  $\varepsilon \ge 10$  W/kg. For some strains shown in Figure 4.9, Equation 1.21 is still applicable, but for  $\varepsilon \ge 0.5$  W/kg. This implies that hyphal break up is likely to be due to turbulent-induced forces with eddies in the viscous dissipation subrange of the Taylor's energy spectrum. It is emphasized that this conclusion is based on the limited data presented in Figure 4.7 and 4.9. It is recommended that further experiments be carried out with the aim of extending the range of  $\varepsilon$  in order to confirm this finding.

An alternative model of hyphal break up  $(P/D^3t_c)$  has recently been proposed by Smith *et al.* (1990). The theory behind the model suggests that hyphal break up depends on the frequency of mycelial circulation, through the zone of high energy dissipation around the impeller. The frequency of circulation  $(1/t_c)$  was obtained from the pumping capacity of the impeller, while the energy dissipation per unit mass was based on the swept volume of the bottom aerated impeller. In terms of N and D, the model proposed by Smith *et al.* (1990) can be derived as follows:

(4.6)

$$\frac{1}{t_c} \propto \frac{Q_p}{V} \propto \frac{N D^3}{D^3} \propto N$$
 (4.5)

and,

$$\propto P_{o} N^{3} D^{2}$$

 $\frac{P}{D^3}$ 

Thus,

$$\frac{P}{D^3 t_c} \propto P_0 N^3 D^2 N \propto P_0 N^4 D^2 \qquad (4.7)$$

Figures 4.10 & 4.11 show data obtained in the present investigation, and data on  $q_{pen}$  of Smith *et al.* (1990) plotted in terms P/D<sup>3</sup> t<sub>c</sub>. The total power dissipation is used instead of the gas power of the bottom impeller (Appendix 3). It is interesting to see that the trends in data are similar to those of Figure 4.7. There are some minor differences between Figures 4.10 & 4.11, on the one hand, and Figure 4.7, on the other. For example in Figure 4.7, the dependency of  $q_{pen}$  and main hyphal length (Le) on  $\varepsilon$  changes at almost the same point ( $\varepsilon \approx 10$  W/kg). Figures 4.10 & 4.11 however suggest that the critical value is different in the two cases ( $\varepsilon \approx 80$  kW/m<sup>3</sup>s for  $q_{pen}$  and  $\varepsilon \approx 40$  kW/m<sup>3</sup>s for main hyphal length). These phenomena are similar to the results observed by Smith *et al.* (1990) which imply that the decrease in the main hyphal length and in  $q_{pen}$  are not







Figure 4.11 Correlation between main hyphal length and energy dissipation  $(P/D^3t_c)$ 

simultaneous:  $q_{pen}$  begins to decrease after the main hyphal length breaks down to a certain value (Figure 4.10 & 4.11). The plot in Figure 4.11 suggests that above the critical value (  $P/D^3t_c \ge 40 \text{ kW/m}^3$ s),

Le 
$$\propto \left(\frac{P}{D^3 t_c}\right)^{-0.3}$$
 (4.12)  
Le  $\propto N^{-1.2} D^{-0.6}$  (4.13)

i.e.

which may be compared with the exponents in Equation 4.4. Apparently, data from different scales may be correlated either by Equation 4.4 or Equation 4.13. In other words, given the fact that Le has only a weak dependency on the energy dissipation and that the only difference between the two models is in the exponents of N (-0.9 vs -1.2), the data from the present work are not sensitive enough to distinguish between the two models. The same conclusion applies to data on  $q_{pen}$ .

Reuss (1988) argues that mechanical damage to microorganisms results from energy dissipated in the bulk of the vessel with a rate proportional to the ratio of the liquid volume (V) and the average circulation time ( $\theta_c$ ). Reuss (1988) used geometrical and operating parameters of fermenter to estimate  $\theta_c$  (Equation 1.32, Section 1.6.4). His theory of mechanical damage would suggest the following relationships,

Le 
$$\propto \frac{P \theta_c}{V}$$
 (4.10)

and,

$$q_{\text{pen}} \propto \frac{P \theta_c}{V}$$
 (4.11)

Figures 4.12 & 4.13 show that the model proposed by Reuss (1988) can not be used to generalize the phenomenon of shear influence in this present work.

There are several other models of mycelial break up that have been proposed in the past (Appendix 3). For example the model of van Suijdam & Metz (see Equation 1.29, Section 1.6.4) suggests that:

Le 
$$\propto$$
 N<sup>-0.75</sup> D<sup>-0.5</sup>

which may be compared with Equation 4.4.



<u>Figure 4.12</u> Correlation between  $q_{pen}$  and power dissipation (P $\theta_o$ /V)



<u>Figure 4.13</u> Correlation between main hyphal length and energy dissipation (P $\theta$ /V)

Once again the differences between the exponents of N and D in the two equations are relatively small, and it is therefore rather difficult to experimentally distinguish between these models given some of uncertainties associated with some of measurements. What is important is the fact that models that based on the turbulence dissipation appear to be generally more successful in describing experimental observations.

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Conclusions

### CONCLUSIONS

In this work, the influence of shear on the growth, morphology and penicillin production was investigated by means of fermentations using *Penicillium chrysogenum* P1 in three different scales, i.e. 7 L, 150 L and 1500 L. The agitation speed was varied within the range which ensured sufficient dissolved oxygen. Good reproducibility of the fermentations was achieved by the use of semi-defined media and consistent generation of spores. Sterilisations in the three different scale of fermenters did not significantly affect the specific penicillin production rate  $(q_{pen})$  and morphological characteristics of the cultures. The experiments carried out at atmospheric pressure and 0.4 bar g overpressure at the same agitation speed and carbon dioxide partial pressure showed no significant differences in productivity and morphology.

Increase in agitation resulted in a decrease in the maximum penicillin production and in the specific penicillin production rate. The decrease was more profound in the 7 L scale than in the 150 L. The main hyphal length, total hyphal length and hyphal growth unit increased during the fast growth period of fermentation and then decreased to a relatively constant value dependent on the agitation speed. Hyphal fragmentation and vacuolization took place in all fermentations with the onset occurring earlier in the fermentations carried out at higher agitation speeds.

Generalization of the results from the three different scales of fermentations showed that tip speed was not a good scale up parameter. A simple model based on the mean energy dissipation rate calculated as power input per unit mass ( $\varepsilon$ ) was derived. Two regions were identified showing different behaviour of specific penicillin production rate (q<sub>pen</sub>) and mean main hyphal length (Le) with change of energy dissipation. In the region of  $\varepsilon$ < 10 W/kg, specific penicillin production rate (q<sub>pen</sub>) and mean main hyphal length were found to be relatively independent or only weakly influenced by energy dissipation rate ( $\varepsilon$ ). In the region of  $\varepsilon \ge 10$  W/kg, q<sub>pen</sub> and mean main hyphal length were proportional to  $\varepsilon^{-0.3}$ . Good agreement was also found between results from the present study and those reported previously in the open literature. The results of this work were reasonably well correlated by the model based on the frequency of mycelial circulation, through the zone of high energy dissipation around the impeller.

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#### **Recommendations:**

The data obtained in this work suggest that both main hyphal length (Le) and specific penicillin production rate  $(q_{pen})$  are at best only weak functions of energy input per unit mass (Le  $\propto \epsilon^{-0.3}$ ;  $q_{pen} \propto \epsilon^{-0.3}$ ). Therefore very accurate measurements of  $\epsilon$  are needed in order to be confident about the precise level of dependency. Power input is an important parameter in the proposed model. However, the fermenters used in this project were not equipped with instruments for direct measurements of power input. Instead power input was calculated using previously published methods. Future experiments should include direct measurements of the torque on shaft to improve the validity of the model.

The period of constant morphology during penicillin production in this work was found to be important in providing a representative morphological parameter (main hyphal length). This parameter was used to represent the influence of shear during fermentation. However, it is desirable to investigate the influence of shear on the rate of fragmentation of filamentous microorganisms. On line measurements on morphological parameters are probably required to assess the initiation point of fragmentation.

Secondary metabolites are mostly produced by filamentous microorganisms, either fungi or Streptomycetes. It will be interesting to examine whether the proposed model of this work is applicable to fermentations of secondary metabolites by different strains of fungi or Streptomycetes. A controlled fed batch fermentation is also recommended to prolong the linear period of secondary metabolite production, therefore ensuring the influence of shear on the production rate of secondary metabolite.

## APPENDIX 1: Calculation of Agitator Power Dissipation in Fermenters

The power input used is the total power drawn by the three impellers: for this purpose the bottom impeller is assumed to be under aeration and the top two impellers are considered to be ungassed. The gas power was calculated based on the Michel & Miller correlation (Michel & Miller, 1962).

#### A.1.1 Fermentations at 7 L Scale

The characteristics of 7 L vessel are as follows

Impeller:	number	= 3
	diameter	= 6.8  cm
	number of bla	ades = 6
Aeration:	5 L/min	

The Reynolds number (Re) is defined as

$$\operatorname{Re} = \frac{\rho \operatorname{N} \operatorname{D}^2}{\mu_a}$$

A correlation of rheological behaviour for pseudoplastic fluid shows that the rate of shear increases more than in proportion to the shearing stress or in mathematical terms can be described as

$$\tau = K \gamma^n$$

where,

 $\gamma_a = k_s N$  and  $\tau = \mu_a \gamma_a$ 

Therefore  $\mu_a$  can be calculated as,

$$\mu_a = \frac{\tau}{\gamma_a} = K \frac{\gamma^n}{\gamma} = K (k_s N)^{n-1}$$

where n and K are the flow behaviour and consistency indexes of power law fluid respectively. Figure A.1.1 shows the behaviour of fermentation broth examined over a range of shear conditions.



Figure A.1.1 Typical flow curve for penicillium culture broth

The values of n and K were obtained from the slope and intercept of the curve respectively,

$$n = 0.36$$
;  $K = 3.30$ ;  $k_s$  (for turbine impeller) = 11

Therefore, at 700 RPM,

 $\mu_a = 0.148 \text{ Pa s}$  and Re = 384

From Reynolds Number Correlation for flat six-blade turbine (Rushton *et al.*, 1950) the value of power number  $(N_p)$  can be obtained. Power number or newton number  $(N_p)$  can be defined as,

$$N_p = \frac{P_o}{\rho N^3 D^5}$$

Therefore, the ungassed power (Po) for one impeller at 700 RPM is,

$$P_{o} = 8.1 W$$

Michel Miller correlation for gassed power can be described as follows,

$$P_{g} = 0.706 \left[ \frac{P_{o}^{2} N D^{3}}{Q^{0.56}} \right]^{0.45}$$

The gassed power for one impeller at 700 RPM is,

$$P_g = 4.7 W$$

The total power dissipation can be calculated as,

$$P = P_g + P_o + P_o$$

and the total power at 700 RPM is,

$$P = 21 W$$

The calculation to determine the total power dissipation at the other agitation speed at 7 L scale are summarised in Table A.1.1.

N (RPM)	μ <sub>a</sub> (Pa s)	ρ (kg/m <sup>3</sup> )	Re	Np	P (W)	q <sub>pen</sub> (u/mg/h)	Le (µm)
700	0.148	1050	383	3.35	21	3.70	114
850	0.130	1055	530	3.53	40	3.73	96
1000	0.118	1042	683	3.95	72	3.49	78
1150	0.107	1040	858	4.15	115	2.99	66
1300	0.099	1030	1039	4.2	168	2.92	58

<u>Table A.1.1</u> Power dissipation at 7 L scale

### A.1.2 Fermentations at 150 L scale

The characteristics of 150 L vessel are as follows

Impeller:	number	= 3
	diameter	= 18 cm
	number of bl	ades = 6
Aeration:	50 L/min	

The power dissipation was determined as before and the results are summarised in Table A.1.2.

N (RPM)	μ <sub>a</sub> (Pa s)	ρ (kg/m <sup>3</sup> )	Re	Np	P (W)	q <sub>pen</sub> (u/mg/h)	Le (µm)
265	0.275	1065	567	3.80	177	3.80	150
400	0.211	1030	1077	4.25	665	3.75	120
530	0.176	1020	1692	4.61	1676	3.51	100

Table A.1.2 Power dissipation at 150 L scale

#### A.1.3 Fermentations at 1500 L scale

The characteristics of 1500 L vessel are as follows

Impeller:	number	= 3
	diameter	= 30  cm
	number of bla	ades = 6
Aeration:	500 L/min	

The calculation to determine the power dissipation at 300 and 400 RPM are summarised in Table A.1.3.

N (RPM)	µ <sub>a</sub> (Pa s)	ρ (kg/m <sup>3</sup> )	Re	Np	P (W)	q <sub>pen</sub> (u/mg/h)	Le (µm)
300	0.254	1045	1816	4.96	3871	-	150
400	0.211	1045	2969	5.03	9380	3.73	145

Table A.1.3 Power dissipation at 1500 L scale

# A.1.4 Recalculation of data from Smith *et. al* (1990) on power dissipation

#### A.1.4.1 Fermentations at 14 L scale (10 L working volume)

Impeller:	number	= 3		
	diameter	= 8  cm		
	number of bl	ades = 4		
Aeration:	5 L/min			

The results of recalculation on 14 L data are summarized in Table A.1.4.

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N	$\mu_a$	ρ	Re	Np	Р	q <sub>pen</sub>
(RPM)	(Pa s)	$(kg/m^3)$			(W)	(u/mg/h)
800	0.271	1020	321	3.51	432	6.88
1000	0.237	1020	459	3.66	883	4.44
1200	0.213	1020	613	3.82	1602	3.25

## Table A.1.4

## A.1.4.2 Fermentations at 150 L scale (100 L working volume)

Impeller:	number	= 3		
	diameter	= 18  cm		
	number of blades $= 6$			
Aeration:	70 L/min			

N (RPM)	μ <sub>a</sub> (Pa s)	ρ (kg/m <sup>3</sup> )	Re	Np	P (W)	q <sub>pen</sub> (u/mg/h)
350	0.440	1000	448	3.66	323	8.63
450	0.380	1020	667	4.00	782	7.63
550	0.337	1020	919	4.61	1657	7.25
565*	0.332	1020	543	3.70	328	8.44

\* impeller diameter = 13.7 cm

Table A.1.5

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## APPENDIX 2: Comparison of calculation of agitator power dissipation in fermenters to other method

The results of gassed power calculation based on the Michel & Miller's correlation was compared to those using a procedure adapted by Smith (1985) from Schugerl (1981) and the comparison is given in Figure A.2.1.



Figure A.2.1 Comparison between gassed power dissipation for one impeller (W) calculated using Michel & Miller correlation and Schugerl's.

## APPENDIX 3: Correlations between data of this work to the previously reported models

#### A.3.1 Calculation of circulation frequency

Circulation frequency  $(1/t_c)$  was determined from pumping capacity of a impeller  $(Q_p)$  which can be described by the equation

$$Q_p = K N D^3$$

The impeller discharge coefficient (K) for a 6 bladed turbine with the ratio of impeller to tank diameter (D/T) between 0.2 to 0.5 is  $0.75 \pm 0.15$  (Revill, 1982). The mean circulation frequency was calculated as the ratio of pumping capacity and the volume affected by one impeller, which was assumed as one third of the working volume of fermenter.

Circulation frequency calculated from physical parameters of fermenter ( $\theta_c$ ) as described by Reuss (1988) gave approximately twice the values of those from pumping capacity. The equation proposed by Reuss is as the following

$$N\theta_c = 0.76 \ (H_L/T)^{0.6} \ (T/D)^{2.7}$$

The comparison between  $1/t_c$  and  $1/\theta_c$  are summarised in Table A.3.1. The correlations between  $q_{pen}$  and main hyphal length to  $1/t_c$  and  $1/\theta_c$  are given in Figures A.3.1 & A.3.2.

Scale	Working	D	N	1/t <sub>c</sub>	$1/\theta_{c}$
(L)	volume (L)	(cm)	(RPM)	(1/s)	(1/s)
<u> </u>					
7	5	6.8	700	0.55	1.21
7	5	6.8	850	0.67	1.45
7	5	6.8	1000	0.78	1.72
7	5	6.8	1150	0.90	1.96
7	5	6.8	1300	1.01	2.22
150	100	18	265	0.20	0.42
150	100	18	400	0.30	0.64
150	100	18	530	0.40	0.85
1500	1000	30	300	0.10	0.23
1500	1000	30	400	0.14	0.31

Table A.3.1



<u>Figure A.3.1</u> Correlation of q<sub>pen</sub> and main hyphal length to circulation frequency calculated from pumping capacity (1/t<sub>c</sub>)



Figure A.3.2Correlation of  $q_{pen}$  and main hyphal length to circulation frequency<br/>calculated from fermenter physical parameters  $(1/\theta_c)$ 

A.3.2	Correlation	to	derived	physical	parameters
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N	P/D <sup>3</sup> t <sub>c</sub>	Pθ <sub>c</sub> /V	٤	P/V	N <sup>0.75</sup> D <sup>0.5</sup>
(RPM)	(kW/m <sup>3</sup> s)	((kWs/m <sup>3</sup> )	(W/kg)	(W/m <sup>3</sup> )	(s <sup>-0.75</sup> m <sup>0.5</sup> )
7 L scale					
700	36.5	3.5	4.0	4.2	1.65
850	84.8	5.5	7.6	8.0	1.90
1000	178.0	8.4	13.8	14.4	2.15
1150	331.1	11.8	22.2	23.1	2.40
1300	539.8	15.1	32.6	33.6	2.62
150 L scale					
265	5.85	4.2	1.7	1.8	1.30
400	33.1	10.4	6.5	6.6	1.77
530	111.6	19.8	16.4	16.8	2.19
1500 L scale					
300	14.5	16.5	3.7	9.4	1.83
400	46.9	30.3	9.0	3.9	2.27

Та	hl	e	Α	3	2
<u>1</u> a	$\mathbf{U}_{\mathbf{I}}$	U.	1 1	• •	• 4
## A.3.3 Recalculation on data of Smith et al. (1990)

N	1/t <sub>c</sub>	P/D <sup>3</sup> t <sub>c</sub>	3
(RPM)	(/s)	(kW/m <sup>3</sup> s)	(W/kg)
14 L scale			
800	0.57	479.3	42.4
1000	0.71	1231.6	86.8
1200	0.85	2675.4	157.2
150 L scale			
350	0.29	15.7	3.3
450	0.38	48.9	7.7
550	0.46	126.7	16.3
565*	0.20	25.7	3.2

\* impeller diameter = 13.7 cm

Table A.3.3

References:

- 1.  $P/D^3t_c$ : Smith *et al.* (1990)
- 2.  $P\theta_c/V$  : Reuss (1988)
- 3. N<sup>0.75</sup> D<sup>0.5</sup> : van Suijdam & Metz (1981b)



<u>Figure A.3.3</u> Correlation between  $q_{pen}$  and  $P/D^3t_c$ 



<u>Figure A.3.4</u> Correlation between main hyphal length and  $P/D^3t_c$ 

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<u>Figure A.3.5</u> Correlation between  $q_{pen}$  and  $N^{0.75}D^{0.5}$ 



<u>Figure A.3.5</u> Correlation between main hyphal length and  $N^{0.75}D^{0.5}$ 

## NOMENCLATURE

a	interfacial area per unit volume for mass transfer
A <sub>pen</sub>	constant (u kg/W mg h)
A <sub>Le</sub>	constant (μm kg/W)
$C_1, C_2,, C_{12}$	proportional constants
CER	carbon dioxide evolution rate (mmol/L/h)
D	impeller diameter (mm)
d <sub>e</sub>	eddy scale (m)
DOT	dissolved oxygen tension (% air saturation)
E(k,t)	Taylor's energy spectrum function
H <sub>b</sub>	height of impeller from bottom of the tank (mm)
H <sub>i</sub>	inter-impeller distance (mm)
HL	liquid height (mm)
k	wave number
k <sub>L</sub> a	volumetric mass transfer coefficient
K <sub>pen</sub>	constant (u/mg/h)
K <sub>Le</sub>	constant (µm)
L <sub>i</sub>	length of impeller (mm)
Le	effective length of hypha or main hyphal length ( $\mu$ m)
mМ	millimolar
М	molar
Ν	impeller speed (RPM)
OUR	oxygen uptake rate (mmol/L/h)
Р	total power dissipation (W)
Pg	gassed power (W)
Po	ungassed power (W)
POA	phenoxyacetic acid
Q	air flow rate $(m^3/s)$
Q <sub>p</sub>	pumping capacity of impeller (m <sup>3</sup> /s)
q <sub>pen</sub>	specific penicillin production rate (u/mg/h)
Re	Reynolds number
RQ	respiratory quotient
Т	tark diameter (mm)
1/t <sub>c</sub>	circulation frequency based on pumping capacity (s)
u	root mean square velocity of particle (m/s)
V	liquid volume (L)

,

vvm	volume of air per volume of medium per minute
W <sub>b</sub>	width of baffle (mm)
Wi	width of impeller (mm)
Х	biomass concentration (g/L)
u/mg/h	International units of penicillin-V per mg biomass per hour
	(1 mg ~ 1600 units)

## **Greek** Letters

3	power per unit mass (W/kg)
γ	shear rate (/s)
η	Kolmogoroff's microscale (m)
μ	specific growth rate (/h)
$\mu_a$	apparent viscosity (Pa s)
$\theta_{c}$	circulation time calculated from vessel physical parameters (s)
ρ	density (kg/m <sup>3</sup> )
$\sigma_w$	the strength of hyphal cell wall $(N/m^2)$
τ	shear stress (N/m <sup>2</sup> )
υ	kinematic viscosity (m <sup>2</sup> /s)

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