The Physiology of clavulanic acid production by *Streptomyces clavuligerus*

Thesis submitted in fulfilment of the requirements for the degree of PhD.

July 2000
To My Family
Acknowledgements

I would like to thank Mike for his guidance and support throughout this research and to SmithKline Beecham for their sponsorship.

A big thank you to everyone in the lab for making it a very enjoyable 3 years especially to Di, Noel and Claudio not only for their help but also great friendship and amusing conversation.

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Summary

Clavulanic acid is a potent inhibitor of many clinically important β-lactamases produced by both Gram positive and Gram negative pathogenic bacteria. Augmentin™ and Timentin™, commercial preparations of amoxycillin and ticarcillin with clavulanic acid are currently marketed by SmithKline Beecham. These antibiotics will shortly attain generic status and are, therefore, of widespread interest.

As a result of its commercial importance, there is a restricted amount of information in the literature surrounding the biosynthesis of the clavulanic acid molecule. Influencing biosynthetic pathway fluxes using bioreactor feeding strategies in order to enhance clavulanic acid titre has therefore only occurred on a hit or miss basis.

Work carried out by Ives and Bushell (1997), used cluster analysis for the identification of intermediary metabolites involved in clavulanic acid production. The results were used to construct feed which resulted in improved clavulanic acid titres being produced. Despite this, it was still difficult to predict the actual effect of the feed on the biochemical pathway and the way in which it was influencing clavulanic acid production.

The aim of this study was to investigate the effect of various amino and organic acids on the production of clavulanic acid. A model was developed which enabled the effect of a range of feeds on clavulanic acid production to be determined, in addition to the effect on the organism's biochemical pathway (Chapters 6 & 7).
This research identified 5 amino/organic acids which were shown to have a stimulatory
effect upon clavulanic acid production. These were isoleucine, threonine, asparagine,
oxaloacetate and ornithine. These feeds were all identified as having the ability to
increase the flux of carbon into either the C3 or C5 precursors of clavulanic acid.

The physiology of clavulanic acid production was examined starting initially with the
investigation of nutrient limitation (Chapter 3). Phosphate was found to yield the highest
titre of clavulanic acid produced per gram of biomass in comparison to the other 3
limitations examined. This was expanded by examination of the effect of temperature on
clavulanic acid formation and breakdown (Chapter 4). It was discovered that whilst an
incubation temperature of 30 °C resulted in a greater biomass concentration being
produced, it facilitated the degradation of the clavulanic acid molecule. A two stage
temperature profile was suggested to optimise clavulanic acid formation whilst prevent
its rapid degradation.

Novel bioreactor techniques were also examined for their effect on clavulanic acid
production. Chapter 5 examines the performance of S. clavuligerus in Cyclic Fed Batch
Culture. Due to the nature of CFBC with its changing volume, the initial results obtained
using this technique were disappointing. After optimisation of this system, CFBC
resulted in titres 172 % of batch value being produced.
1.0 INTRODUCTION

Antibiotics are products of secondary metabolism and are produced by a wide variety of organisms including higher plants and animals. Their distribution within taxonomic groups is shown in Figure 1.

Fig 1. The number of known antibiotics and the relative importance of the various taxa that produce them (Berdy, 1985).

<table>
<thead>
<tr>
<th>Type of producing organism</th>
<th>Approximate number</th>
<th>Percentage</th>
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<tr>
<td>Bacteria</td>
<td>950</td>
<td>9</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>4600</td>
<td>43</td>
</tr>
<tr>
<td>Fungi</td>
<td>1600</td>
<td>15</td>
</tr>
<tr>
<td><strong>All microorganisms</strong></td>
<td><strong>7150</strong></td>
<td><strong>67</strong></td>
</tr>
<tr>
<td>Lichens</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Algae</td>
<td>250</td>
<td>2</td>
</tr>
<tr>
<td>Higher plants</td>
<td>2500</td>
<td>23</td>
</tr>
<tr>
<td>Animal organisms</td>
<td>700</td>
<td>7</td>
</tr>
<tr>
<td><strong>All “higher organisms”</strong></td>
<td><strong>3550</strong></td>
<td><strong>33</strong></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>10700</strong></td>
<td><strong>100</strong></td>
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The greatest variety in structure and number of antibiotics is found within the Actinomycetales, especially within the genus Streptomyces. Streptomyces are thought to account for approximately 93% of reported secondary metabolite producing cultures (Bushell, 1992), making Streptomyces a genus of great commercial importance.

Despite the economic value of antibiotics, it is still unknown why these secondary metabolites are produced. The term secondary metabolite was first introduced by Bu'Lock (1961), and is loosely applied to microbial products which are non-essential to the growth of the producer organism. These products have since provoked much debate in the literature concerning not only the biosynthetic pathways involved, but also the reasons for their existence (Zahner, 1979, Campbell, 1984, Vining, 1990 and Demain, 1995).
The most obvious role of antibiotics is as weapons against competitors in the environment (Katz and Demain, 1977), but matters have been complicated by suggestions that some antibiotics have other functions in the producing organism. Examples of this are the peptide antibiotics which appear to be involved in the sporulation process (Katz and Demain, 1977), and the antibiotic methylenomycin, which has been implicated in the formation of aerial mycelium (Wright and Hopwood, 1976). These studies suggest that the primary function of these secondary metabolites is as regulators of differentiation, and antibiotic production is purely serendipitous.

Zahner (1979), has suggested that secondary metabolism functions as a playground for evolution. In this scheme, enzymes of low specificity form new compounds from available metabolites. Some of these new compounds may eventually find a use, either inside the cell or in the external environment. Zahner therefore predicts that many secondary metabolites will have no function at all, while others may have more than one.

Several authors have suggested that the process of secondary metabolism may be far more important to the producing organism than the final products. Dhar and Khan (1971) proposed that secondary metabolites are formed in order to reduce the accumulation of a more toxic intermediate (the `detoxification hypothesis'). Bu'Lock (1961) proposed that secondary metabolism maintains a reasonable balance of primary metabolites when conditions cannot support balanced growth (the `unbalanced growth hypothesis'). The unbalanced growth hypothesis suggests that metabolic intermediates accumulate when a nutrient becomes growth - limiting through failure of control mechanisms which adjust flow through pathways. Instead of being excreted directly, accumulated metabolites are acted upon by enzymes of low substrate specificity. A succession of reactions leads to the formation of a complex metabolite termed a 'shunt' product which is excreted. All propositions that the process of secondary metabolism is of importance do not preclude the possibility that secondary metabolites have subsequently acquired a functional role.
Until now, the study of secondary metabolism has been mainly descriptive. Compared with primary metabolism, the enzymology and regulation of pathways is poorly understood (Hunter, 1992). Recent advances in molecular biology may increase our knowledge of how gene expression is controlled which in turn may illuminate the role of secondary metabolites for the producing organism. Indeed, the demonstration that antibiotic biosynthesis, regulation and resistance genes are clustered (Hopwood, 1988) has implied an antagonistic function in nature (Stone and Williams, 1992).
1.0.1 DIVERSITY OF SECONDARY METABOLISM

To date there has been a vast diversity of secondary metabolites identified. These range from those with unusual structures, different chemical features and bioactivities. A classification of antibiotics according to their chemical structure, and examples of each are shown in Figure 2.

Figure 2. Classification of antibiotics according to chemical structure.

<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
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<tr>
<td>Carbohydrate Containing:</td>
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<td>Aminoglycosides</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>C-Glycosides</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>Macrocyclic lactones:</td>
<td></td>
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<tr>
<td>Macrolide</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Polyene</td>
<td>Candidcidin</td>
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<tr>
<td>Quinones:</td>
<td></td>
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<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Naphthoquinones</td>
<td>Actinorhodin</td>
</tr>
<tr>
<td>Amino Acid &amp; Peptide:</td>
<td></td>
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<tr>
<td>Amino acid derivatives</td>
<td>Cycloserine</td>
</tr>
<tr>
<td>ß-lactam</td>
<td>Penicillin</td>
</tr>
<tr>
<td>Heterocyclic containing Nitrogen</td>
<td></td>
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<tr>
<td>Nucleoside</td>
<td>Polyoxins</td>
</tr>
<tr>
<td>Heterocyclic containing Oxygen</td>
<td></td>
</tr>
<tr>
<td>Polyether</td>
<td>Monensin</td>
</tr>
<tr>
<td>Alicyclic Derivatives</td>
<td></td>
</tr>
<tr>
<td>Cycloalkane derivatives</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Aromatic</td>
<td></td>
</tr>
<tr>
<td>Benzene derivatives</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>Fosfomycins</td>
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It is evident from the foregoing discussion that the vast range of secondary metabolites produced by different micro-organisms are likely to serve a number of different roles in these organisms. Varied roles of secondary metabolites could imply that there will also be considerable variation in the mechanisms controlling their biosynthesis.

1.1 CONTROL OF SECONDARY METABOLISM

At present there is no universally accepted theory for the control of antibiotic biosynthesis. One theory by Chater and Bibb (1996) suggests that there is a global regulatory influence on antibiotic synthesis due to growth rate with pathway specific regulation influencing the production of individual antibiotics. These pathway specific regulators may be influenced by the nature of the growth limiting substrate or catabolite regulation.

1.1.1 CATABOLITE REGULATION

Catabolite regulation is a general regulatory mechanism in which a key enzyme involved in a catabolic pathway is repressed, inhibited or inactivated when a commonly used substrate is added. Substrates that have been found to elicit catabolite repression include carbon, nitrogen and phosphate.

1.1.2 CARBON REGULATION

In many cases, the presence of a rapidly utilisable carbon source such as glucose can result in the repression of antibiotic synthesis. However glucose is not the only carbon source to elicit this effect. Novobiocin production in *Streptomyces niveus* is suppressed by citrate, which is more readily assimilated than glucose (Kominek, 1972) and glycerol and starch were reported to repress the production of cephamycin C in *Streptomyces clavuligerus* (Lebrihi et al 1988., Ahoronwitz and Demain, 1978).
The sugar utilisation pattern of *Streptomyces clavuligerus* is unusual as it is unable to utilise glucose for growth or antibiotic production. A study carried out by Pruess and Kellet (1983), found that whilst antibiotic production occurred when glycerol, maltose and dextrin were used as carbon sources, there was no antibiotic production detected when glucose, myoinositol, mannitol, sucrose or L-sorbose were used. It is thought that the inability of the organism to utilise glucose lies in its inability to complete glucose transport (Garcia-Dominguez *et al*., 1989).

Direct carbon catabolite repression has also been demonstrated with known enzymes of an antibiotic biosynthetic pathway. Examples include the actinomycin producer *Streptomyces antibioticus* where glucose has been shown to repress phenoxazinone synthase which is involved in the formation of the phenoxazinone ring of the antibiotic (Gallo and Katz, 1972). It has also been suggested that in streptomycin biosynthesis, glucose regulates the enzyme mannosidastreptomycinase which converts inactive mannostreptomycin into active streptomycin (Demain and Inamie, 1970).

Production media for such secondary metabolites sensitive to carbon catabolite repression generally combine rapidly used and slowly used carbon sources in the same medium (Demain, 1985). The rapidly used carbon source is usually used first in a rapid growth phase where secondary metabolism does not occur. After the favoured carbon source is depleted, growth proceeds at a slower rate on the second carbon source and secondary metabolite production is stimulated. These production media are then referred to as being "carbon limited" as it is the carbon source which is the nutrient that is controlling the organisms' growth rate.

The effect of carbon limitation upon the formation of clavulanic acid has been explored in chapter 3.
1.1.3 NITROGEN REGULATION

Nitrogen regulation is the term used when secondary metabolism is suppressed by the presence of high concentrations of ammonium and certain amino acids. Interfering nitrogen sources usually act by repressing synthesis and inhibiting activity of synthases of secondary metabolism (Demain, 1985). Antibiotics that are sensitive to this form of nitrogen regulation include cephamycin C (Aharonowitz and Demain, 1978), actinomycin (Katz et al., 1984), tetracycline (Behal et al., 1983) and tylosin (Omura et al., 1984).

It has been found that secondary metabolism can be suppressed by the presence of high concentrations of ammonium in the medium. It is not known exactly how this occurs, but it is generally accepted that in most instances, it is repression rather than inhibition of antibiotic synthesising enzymes that is implicated (McDermott et al., 1993), (Wilson and Bushell, 1995). This effect has been seen in the production of many antibiotics e.g. cephamycin C (Castro et al., 1985), erythromycin (Flores and Sanchez, 1985), leucomycin & tylosin (Tanaka et al., 1981).

Evidence for ammonium repression is apparent in Streptomyces clavuligerus which has two enzyme systems for nitrogen assimilation. These are glutamine synthetase glutamate aminotransferase (GS - GOGAT) and glutamate dehydrogenase (GDH). These enzymes operate under ammonium limited and ammonium rich conditions respectively. Optimal cephalosporin production occurs when the GS - GOGAT system is highly active however in ammonium rich conditions optimal for GDH action, cephalosporin production ceases (Aharonowitz, 1980). Other studies of these enzymes have given contradictory results of their importance in secondary metabolite production (Flores et al., 1985) and to date, no clear mechanisms have been identified.

The effect of nitrogen limitation upon clavulanic acid formation has been explored in chapter 3.
1.1.4 PHOSPHATE REGULATION

Phosphate regulation is a term used to describe the suppression of secondary metabolism by high concentrations of phosphate. Phosphate appears to be an important growth-limiting nutrient in many antibiotic cultures (Martin and Demain, 1980). It was noted that concentrations that ranged from 0.3 – 300mM of phosphate would support extensive cell growth, but at concentrations of 10mM and above, the synthesis of many antibiotics was inhibited. There are many mechanisms that have been proposed to explain this phenomenon. However there appear to be two major theories proposed to account for this effect (Martin, 1977).

The first is based upon the idea that phosphate has an inhibitory or a repressive effect on the phosphatases that are involved in secondary metabolism. This may be expected wherever phosphorylated intermediates are a component of the biosynthetic pathway for example in streptomycin biosynthesis where phosphate inhibits the phosphatase responsible for converting streptomycin phosphate to streptomycin (Walker and Walker, 1971).

The second theory involves the role of adenine triphosphate (ATP) as an effector in phosphate regulation. Through the measurement of intracellular ATP levels, it has been discovered that after the addition of 10mM phosphate to a culture of *Streptomyces griseus*, a rapid doubling of the intracellular ATP levels prior to the inhibition of antibiotic synthesis was detected. The increase in energy charge (0.80 to 0.85) noted was small which lead the authors to propose a role for ATP itself rather than for the adenylate energy charge in the cell (Martin and Demain, 1976).

With the wealth of data that is available on the role of organic and inorganic phosphorus in secondary metabolism, the number of mechanisms proposed to explain this phenomenon is large (reviewed by Demain, 1986). At present we can only assume the role of phosphate in secondary metabolism until further research is carried out. The effect of phosphate limitation upon clavulanic acid formation has been explored in chapter 3.
1.1.5 GROWTH RATE

Since many secondary metabolites are produced at low growth rates, it is possible that nutrient limitation merely acts by lowering growth rate to a level at which secondary metabolite production is stimulated. A study on *Streptomyces cattleya* (Lilley et al., 1981) has indicated that some secondary metabolite processes are stimulated by low growth rates irrespective of the type of nutrient that is limiting growth, whereas others require a specific type of nutrient limitation. Often mutants which over produce secondary metabolites display lower growth rates which may suggest that growth rate could be the key factor in antibiotic production (Kralovkova and Vanek, 1979).

1.1.6 THE STRINGENT RESPONSE

Secondary metabolite production, morphological differentiation and changes in nucleotide pools often coincide and as a result, have sparked an extensive research programme. One of the findings of this research is the 'stringent response'. The stringent response can be described as the microbial mechanism for surviving difficult conditions whereby an organism protects its resources by undergoing only the minimum of metabolic activity until conditions improve (Lamond & Travers, 1985). When an organism is transferred from a casamino acid containing medium to a non-casamino containing medium, a nutritional downshift occurs and a higher proportion of uncharged transfer-RNA (t-RNA) to charged t-RNA (aminoacyl-t-RNA or aa-t-RNA) results due to the reduced availability of free amino acids. The uncharged t-RNA reacts with the 'A' site of the 70S ribosomal sub-unit in what is termed to be the 'idling reaction' which initiates the production of ppGpp by ppGpp synthetase. Subsequently ppGpp was found to be diminished during growth phase, and reached peak concentrations after the exponential phase due to nutrient limitation, at which point secondary metabolism was also induced (Ochi, 1987). Unfortunately, further investigations also revealed that the biosynthesis of cephalosporin by *Streptomyces clavuligerus* occurs with no accompanying increase in ppGpp levels (Bascaran et al., 1991).
The fact that changes in the level of ppGpp have an effect on some *Streptomyces* species but not others would indicate that ppGpp is not the messenger molecule in secondary metabolism that it was initially thought to be. It has also been suggested that the effects observed may be due to ppGpp affecting the expression of a gene that is developmentally regulated rather than a regulatory gene (Hodgson, 1992). It may be the case that the transient increase in ppGpp at the end of exponential phase and its occurrence in stationary phase could indicate that it might be a requirement for the expression of genes involved in secondary metabolism (Strauch *et al.*, 1991).

Studies into t-RNA levels in *Escherichia coli* (Rojiani *et al.*, 1989) have shown that it is not the concentration of uncharged t-RNA that is important in initiating ppGpp production, but it is the ratio of uncharged to charged t-RNA that is critical. A study carried out by Wilson & Bushell (1995) which investigated the effect of decreasing growth rate via nutrient limitation upon antibiotic production, protein synthesis rate and the ratio of uncharged to charged t-RNA in *Streptomyces hygroscopicus* and *Saccharopolyspora erythraea*, found that the onset of antibiotic production coincides with minimal protein synthesis rate. It was also found in *S. erythraea* that this period of minimal protein synthesis rate corresponded to the minimum ratio of charged to uncharged t-RNA i.e. when uncharged t-RNA accumulated. Uncharged t-RNA may therefore in itself exert some effect upon secondary metabolite production.

### 1.1.7 AUTOREGULATORS

Many *Streptomyces* species produce endogenous molecules called autoregulators, that are implicated in the onset of secondary metabolite production. The best studied of these is A-Factor which was first isolated in *S.griseus* strains by Khoklov (1982). Hara and Beppu (1982), showed that in mutants of *S.griseus* that were incapable of sporulating or streptomycin production, there was no A-Factor detectable in the cells. Such mutants strains deficient in the ability to synthesise A-Factor (afs mutants) had both phenotypes restored by the addition of exogenous A-Factor. Although it appears that the presence of A-Factor is needed for both phenomena to occur, A-Factor alone will not initiate secondary metabolism or morphological differentiation.
Ochi (1987) stressed the significance of the stringent response in morphological
differentiation and secondary metabolite production and suggested a possible co-
ordinated role of this response with the action of A-Factor. Ochi (1987) concluded
that morphological differentiation of *S. griseus* resulted from a decrease in the pool of
GTP in the cell as a consequence of the stringent response, whereas physiological
differentiation and secondary metabolite production resulted from a more direct
function of ppGpp as mentioned earlier in *S. griseus* which do not exhibit the stringent
response. It was therefore suggested that A-Factor may render the cell sensitive to
receive and respond to these specified signal molecules i.e. ppGpp and GTP.

From the initial work by Miyake *et al.*, (1989), it appears that A-Factor is not essential
in the induction of physiological and morphological differentiation, but that it may be
involved as an additional ‘check’ in the process. It is also possible that it may act as a
unifying communicator to channel remote signals, such as stringent response, into the
effecting of sporulation or secondary metabolite production.

1.1.8 GLOBAL CONTROL ASPECTS OF SECONDARY METABOLISM

Although there is no universally accepted model for the physiological control of
antibiotic biosynthesis, several models have been proposed that attempt to take into
account both pathway specific and global control of antibiotic synthesis. Chater and
Bibb (1995) proposed a model (Figure 3) that takes into account both pathway
specific and global regulation. Their model suggested that the effect of growth rate
on certain pleiotropic genes initiates a cascade of events that leads to the initiation of
antibiotic biosynthesis.
An example of a pleiotropic gene can be seen in the action of the *bld* genes. Mutation of *bldA* in *S.coelicolor* resulted in the loss of both morphological and antibiotic synthesis. This indicated that the *bldA* gene is involved in the control pathways of both processes. In *S.coelicolor* and *S.lividans* the *bldA* gene encodes for the only tRNA that can translate the rare leucine codon UUA efficiently (Lawlor *et al.*, 1987, Leskiw *et al.*, 1991a). It has been shown that the TTA codon is also found in several genes that are expressed late in the growth of the organism (Lawlor *et al.*, 1987), hence it has been suggested that *bldA* regulates antibiotic production by allowing the translation of mRNA containing the UUA codon only under certain conditions (Leskiw *et al.*, 1991b).
The bldA gene can then be seen to be acting as a global regulator for the pathway specific genes that contain the UUA codon. The production of actinorhodin and undecyiprodigiosin by S. coelicolor requires the expression of the pathway specific genes actII-ORF and redD respectively (Takana et al., 1992, Gramajo et al., 1993). Both of these genes contain the UUA codon and require the expression of the bldA gene to ensure their translation.

The expression of such global regulators such as bldA however, still requires some form of physiological trigger. The models proposed by Chater and Bibb (1995) and Wilson and Bushell (1995), (Figure 4) are both complementary in identifying the reduction in growth rate of the organism as this potential trigger. Wilson and Bushell (1995), proposed that the protein synthesis rate of the organism acts as a global initiator of secondary metabolism, with this presumably having an effect on the expression of global regulatory genes, such as those as identified by Chater and Bibb (1995).

Fig 4. Proposed regulatory cascade (Adapted from Wilson and Bushell, 1995)
Wilson and Bushell (1995), proposed that differences in the substrate affinities for the growth rate limiting substrate may also be responsible for the differences in production kinetics observed. They observed non-growth associated kinetics under carbon limitation and growth associated kinetics under nitrogen limitation. It was suggested that the differing production kinetics were possibly as a result of differing relative substrate affinities for carbon and nitrogen sources. A lower affinity for the nitrogen source would lead to growth associated production under nitrogen limitation and non-associated production under carbon limitation.

A possible hypothesis for the control of secondary metabolite production is that growth rate down regulation acts as a global trigger for the induction of secondary metabolism, with other mechanisms such as catabolite, phosphate and nitrogen repression, acting on individual steps in biosynthetic pathways once they have been induced (Wilson & Bushell, 1995).
1.2 BIOREACTOR TECHNIQUES

McDermott et al., (1993) confirmed that the kinetics of erythromycin production were dependant on the identity of the growth rate limiting nutrient during batch culture of *Saccharopolyspora erythraea*. Wilson and Bushell (1995), provided an explanation to support this theory by examining changes in protein synthesis rate and its relation to antibiotic synthesis rate in *Saccharopolyspora erythraea* and *Streptomyces hygroscopicus*. They concluded that for antibiotic production to be initiated, a downshift in growth rate and a decrease in the cells protein synthesis rate is required.

The effect of down regulation of growth and protein synthesis rate as effectors for antibiotic production has been further investigated by Lynch and Bushell (1995). In this study, a Cyclic Fed Batch Culture system was employed to provide conditions which would result in continual and varied changes in growth rate. This system enhanced antibiotic production compared to batch culture, and also indicated that the magnitude of the shift in growth rate was of great significance to the level of antibiotic production obtained.

1.2.1 BATCH CULTURE

A batch culture can be considered to be a “closed system”. Organisms are inoculated into growth medium and incubation is allowed to proceed under optimal physiological conditions. Monod, (1949), reported that a typical batch fermentation is composed of four stages. These stages collectively form what is known as the growth cycle.
The first stage in the growth cycle is known as the lag phase. This is where cells undergo a period of biochemical readjustment before resuming growth. During this period the growth rate is zero. This is then followed by a phase of rapid growth known as the exponential phase. During this phase, the culture biomass concentration doubles at a constant rate as medium components are metabolised. This can be described mathematically:

\[ \mu = \frac{d x}{d t} \cdot \frac{1}{x} \]

Where \( \mu \) is the specific growth rate constant, \( t \) is time, \( x \) is the initial concentration of the organism.

The specific growth rate is significantly effected by the concentration of essential nutrients in the medium and so a decrease in one nutrient also results in a decrease in specific growth rate. Monod showed that the specific growth rate of an organism could be related to the concentration of a single limiting nutrient using an equation equivalent to that used to describe enzyme kinetics, known as the Michaelis-Menten equation:

\[ \mu = \frac{\mu_{\text{max}} S}{K_s + S} \]

Where \( \mu_{\text{max}} \) is the maximum value of \( \mu \) when the substrate concentration \( S \) is no longer growth limiting, and \( K_s \) is the saturation constant or substrate affinity constant, this is numerically equal to the growth limiting substrate concentration at \( 0.5\mu_{\text{max}} \). Therefore in the exponential phase of batch culture, there must be sufficient nutrients present to enable the organism to grow at the growth rate equal to \( \mu_{\text{max}} \).
The two final stages of the cycle are the stationary and death phases. Here growth can no longer be maintained due to depletion of nutrients or due to the formation of inhibitory conditions. Growth of the culture no longer proceeds at a maximum rate and so remains static since it is balanced by autolysis. During the death phase, the cells maintenance energy requirements are not met and autolysis dominates. Since antibiotic synthesis appears to be regulated by a down-shift in growth rate (Lynch and Bushell, 1995, Ives and Bushell, 1997), a culture technique that can continually induce a down-shift in growth rate of an organism is required. Although batch culture will eventually result in a decrease of the growth rate of an organism, the down regulation only occurs for a short period during the overall incubation time before cell lysis occurs.

1.2.2 CONTINUOUS CULTURE - THE CHEMOSTAT

The chemostat is a method for the continuous culture of micro-organisms. It involves a continual supply of substrate to a culture with the continuous removal of spent medium and culture at the same rate. During a chemostat culture, a steady state is established in which biomass and substrate concentration remain constant. Growth is balanced by wash out and specific growth rate is limited by a single nutrient component. With a chemostat in the steady state where the volume $V$ is constant, it can be said that:

$$\frac{dx}{dt} = (\mu - D)x$$

Where $\mu$ is the specific growth rate, $D$ the dilution rate. In the steady state, where $dx/dt$ is equal to zero then:

$$\mu = D$$

And since $D$ is equal to flow rate ($F$) divided by volume ($V$) specific growth rate $\mu$ also equals dilution rate.
Chemostat culture has, in the past been used as a tool in which physiological studies have been carried out and have also been used to determine growth parameters such as specific growth rate and yield coefficients. The earlier studies on the production of tetracycline antibiotics in chemostat culture (Sikyta et al., 1961), suggested the importance of this technique as a means of controlling antibiotic production by controlling the organisms growth rate during exponential phase.

The chemostat principal has also been used in other bioreactor feeding strategies in order to avoid oxygen limitation during yeast fermentations (Reed and Peppler, 1973). Because the relationship between specific growth rate and oxygen uptake rate is stoichiometric (Bushell and Fryday, 1983), the oxygen requirements of the culture can be matched to the aeration capacity of the bioreactor.

The principal of the chemostat has been further developed into the 2-stage chemostat. This involves a chain of 2 chemostats in series and enables a range of growth rates and conditions to be generated in each stage. The majority of the literature on multi-stage chemostats has been concentrated upon bacterial cell cultivation (Park et al., 1990., Hortacsu and Ryu, 1991., Togna et al., 1995), and unfortunately there has been little work carried out on Streptomyces.

A recent study designed to examine the effect of growth rate down-regulation of *Streptomyces clavuligerus* in 2-stage chemostat resulted in a significant increase in clavulanic acid production (Ives and Bushell, 1997). In this investigation, the 2-stage chemostat was used to provide a non-inhibitory culture system for complete ammonia utilisation. With an ammonium chloride feed concentration of 3.8g/L^-1 it was found that the specific ammonium uptake rate decreased in stage 2, (2.88g/L^-1 utilised in stage 1 and 0.79g/L^-1 in stage 2). Despite this, clavulanic acid production rate increased by 161% from the first stage to the second. A possible explanation for this increase in production is that the clavulanic acid biosynthetic pathway is subject to ammonium repression and that this is relieved by the lower uptake rate in stage 2. However there is at present no literature to support this theory.
The 2 stage chemostat was thus proven to be successful in utilising residual ammonia, and provided a disproportionate increase in clavulanic acid production rate possibly arising as a consequence of growth rate down regulation from 0.05h\(^{-1}\) to 0.022h\(^{-1}\) between the 1\(^{st}\) and 2\(^{nd}\) stages.

1.2.3 CONTINUOUS CULTURE - CYCLIC FED BATCH CULTURE

Cyclic Fed Batch Culture (CFBC) is a bioreactor method used for varying the growth rate of an organism to a defined profile. Pirt (1974) first introduced CFBC as a continuation of the fed batch process where a volume of culture is removed at intervals to prevent bioreactor capacity limitations. By using constant feed rates, cyclic variations in culture volume, dilution rate and specific growth rate can occur during repeated fed batch culture. This culture technique provided a means of maintaining the fed batch system by removal of a portion of culture from the vessel on reaching maximum capacity. The remaining volume of culture in the vessel provided an inoculum for the proceeding cycle, which eliminates any lag phase associated with this inoculum. This semi-continuous culture method provided nutritional perturbations in the vessel since the flow remained constant whilst the volume and therefore total biomass increased resulting in a constant down regulation of dilution rate and growth rate. Pirt (1974), also stated that biomass concentration remained constant throughout CFBC and a “quasi steady state” was demonstrated to exist, with the dilution rate and growth rate equivalent and constantly decreasing. Pirt (1979) suggested that the constant decrease in growth rate throughout each cycle might provide a continual trigger for secondary metabolite formation.
1.2.3.1 DILUTION RATE

The dilution rate in CFCB can be calculated as follows:

\[ D_t = \frac{F}{V_t} \]

Where \( F \) = flow rate and volume \( V_t \) at time \( t \).

Since \( V_t = (V_{\text{INIT}} + F \cdot t) \) where \( V_{\text{INIT}} \) is the initial volume then:

\[ D_t = \frac{F}{V_{\text{INIT}} + F \cdot t} \]

The growth rate can also be calculated by including volume changes with time. The volume of culture in the vessel controls growth rate in CFBC, as the growth limiting substrate concentration and therefore biomass concentration remain constant.

The total biomass concentration increases as the volume increases since \( X = xV \) and so the equation for CFBC can be written as:

\[ \mu = \frac{d}{dt} \frac{x}{x} \cdot \frac{1}{x} \]

Despite being used in industry for the production of penicillin (Pirt 1975), CFBC has had little published recognition. A recent study on CFBC has however produced exciting results by increasing the yield of erythromycin from *Saccharopolyspora erythraea* compared to batch culture and chemostat culture (Lynch and Bushell, 1995). With the relationship between specific growth rate profile and initiation of antibiotic production already established (McDermott *et al.*, 1993), this study certainly proved consistent with these theories.
If down-regulation of growth rate is the trigger for initiation of 2° metabolites, high antibiotic production rates at the end of batch culture and throughout CFBC cycles would be expected. Chemostat with its steady state conditions would not be expected to support high antibiotic production as there would be no down regulation of growth rate and therefore no trigger for antibiotic production.

In CFBC, erythromycin concentration was found to be 180% of that achieved in batch culture, when a low dilution rate range (0.025-0.01h⁻¹) was used (Lynch and Bushell, 1995). When the dilution rate was increased (0.06-0.027h⁻¹, 0.1-.04h⁻¹, the erythromycin concentration fell to 113% and 88% respectively of batch value. There have been very few studies using CFBC for the production of 2° metabolites but the evidence from this study and from Gray & Vu-Tong (1987) who enhanced tylosin yields in Streptomyces fradiae cultures suggests that CFBC could be a useful tool for the over production of many other antibiotics.

Cyclic Fed Batch Culture has been investigated for the production of clavulanic acid from S.clavuligerus in chapter 5.
1.3 *S. clavuligerus* - CHARACTERISTICS

Isolated from a South American soil sample, *S. clavuligerus* (ATCC 27064, NRRL 35851), produces a network of branched aerial mycelium. The name given to the organism described the spore bearing structures (L. fem. n. *clavula* little club: L. suffix -igerus, bearing) which appeared as 1-4 spores borne on short club shaped hyphal branches (Higgins and Kastner, 1971).

This species of *Streptomyces* is unusual in its ability to produce as many as 21 2° metabolites (Hopwood, 1981), and at least 6 different antibiotics of the β-lactam class. These are cephamycin C, penicillin N, clavulanic acid, deacetoxycephalosporin C, cephalosporin C and deacetyl-3-carbamoyl-cephalosporin C. The most commercially significant of these is the β-lactamase inhibitor clavulanic acid. This compound possesses 3R, 5R stereochemistry (Brown, 1984), and was the first reported naturally occurring fused β-lactam containing oxygen instead of sulphur, and lacking in the acyl amino-side chain present in both the penicillins and cephalosporins.

Clavulanic acid displays only a low order of antibacterial activity, but it is a potent inhibitor of many clinically important β-lactamases produced by Gram +ve and Gram –ve bacteria. In combination with low concentrations of clavulanic acid, the activity of β-lactamase labile penicillins and cephalosporins is greatly improved. Commercial products such as Augmentintm and Timentintm, combinations of clavulanic acid together with amoxycillin or ticarcillin, respectively, have made clavulanic acid a product valued in excess of a billion dollars/annum. These products are prescribed world-wide where antibiotic resistant infections are suspected.
1.3.1 STRUCTURE AND ACTIVITY

The clavulanic acid molecule Z- (2R,5R)- 3 -(β-hydroxyethylidene) -7- o xo -4- oxa
-1- azabicyclo- {3, 2, 0} heptane -2- carboxylic acid) consists of a fused bicyclic
nucleus comprising of a β-lactam moiety and an oxazolidine ring.

Figure 5. A molecule of clavulanic acid.

Clavulanic acid has a high affinity for the active site of the β-lactamase enzyme,
binding to the catalytic centre and functioning initially as a competitive inhibitor.
A rapid reaction with a serine-hydroxyl group of the enzyme results in acylation of the
enzyme and opening of the β-lactam ring via the carbonyl bond. This is followed by
the opening of the oxazolidine ring, which exposes the reactive groups that can form
stable covalent bonds with the active site.

Based on the frequent occurrence of clavulanic acid or other clavams in species
which also produce conventional β-lactam antibiotics and given the structural
similarity between the two groups of compounds, there was some initial speculation
that clavulanic acid and β-lactam antibiotics may arise from a shared biosynthetic
pathway.
This hypothesis was eventually disproved in part by the demonstration that purified enzymes known to be responsible for penicillin/cephamycin biosynthesis showed substrate specificity that was incompatible with the precursors of clavulanic acid. At the same time feeding studies implicated an amino acid such as ornithine or arginine together with a 3-C glycolytic pathway intermediate such as glycerate or pyruvate as the precursors of clavulanic acid. Recent studies now point to arginine and pyruvate as the direct precursors of clavulanic acid (Valentine et al., 1993, Pitlik and Townsend, 1997, Thirkettle et al., 1997).

Aside from feeding studies, progress on elucidating the biosynthetic pathway to clavulanic acid was hindered for many years by the lack of recognisable pathway intermediates analogous to δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in penicillin/cephamycin biosynthesis, which would support in vitro biosynthesis of clavulanic acid. Early intermediates in clavulanic acid biosynthesis were first discovered in a study where metabolites containing guanidino groups were purified from the culture filtrate of a S. clavuligerus mutant blocked in clavulanic acid production (Elson et al., 1993). The metabolites were recognised by their ability to participate in the Sakaguchi colour reaction specific for compounds bearing guanidino groups. Based on the structures of the compounds isolated, labelled forms of three putative intermediates were synthesised and shown to be incorporated directly into clavulanic acid thereby confirming their involvement in the biosynthetic pathway (Elson et al., 1993). The structure of the earliest of these intermediates (N₂-(2-carboxyethyl)-arginine) suggests that the condensation of the pyruvate and arginine precursors must occur by a mechanism very different from the amide bond formation catalysed by a non-ribosomal peptide synthetase, such as that in involved in the early steps of penicillin biosynthesis. This is consistent with genetic studies which have found no evidence for the existence of a gene encoding a peptide synthetase within the clavulanic acid gene cluster.
The second of these guanidino intermediates, deoxyguanidinoproclavaminic acid, is a monocyclic \( \beta \)-lactam compound which arises from carboxyethylarginine via closure of the \( \beta \)-lactam ring. The enzyme responsible for the formation of the \( \beta \)-lactam ring, called \( \beta \)-lactam synthetase (BLS), has recently been described (Bachmann et al., 1998), and shows some similarity to asparagine synthetases in both primary amino acid sequence and in reaction catalysed. As such, the reaction is very different from that involved in penicillin/cephamycin production since ring closure requires the formation of an amide bond. Greater parallels may be apparent with the formation of the \( \beta \)-lactam ring as it occurs in the carbapenam pathway. Like clavulanic acid, understanding of the carbapenam biosynthetic pathway is at an early stage of development when compared to the penicillin/cephamycin pathway. However, the gene cluster which encodes the carbapenam biosynthetic enzymes has been located and sequenced and the putative gene products bear little resemblance to the enzymes of the penicillin/cephamycin cluster, but do show significant homology to proteins encoded by the clavulanic acid gene cluster. McGowan et al., (1996) suggest that these observations are consistent with the existence of a second major pathway to the biosynthesis of \( \beta \)-lactam containing compounds, including the carbapenams and clavulanic acid.

Deoxyguanidinoproclavaminic acid is then hydroxylated in the first of three reactions catalysed by the enzyme clavaminate synthase (CAS; Baldwin et al., 1993). CAS is an unusual iron-containing, 2-ketoglutarate-dependent molecular dioxygenase, reminiscent of the ring expansion and hydroxylation enzymes involved in cephalosporin biosynthesis. The product, guanidinoproclavaminate (the third of the guanidino intermediates) contains a hydroxyl group in which the oxygen atom is derived from molecular oxygen, analogous to the hydroxylation of desacetoxycephalosporin C to desacetylcephalosporin C. Despite this similarity in the reactions catalysed, the two enzymes show very little sequence similarity.
Guanidinoproclavaminic acid is then converted to proclavaminic acid by the action of an amidinohydrolase which removes the guanidino group from the arginine-derived end of the molecule. The enzyme involved has been named proclavaminic acid amidinohydrolase (PAH). The existence of this enzyme was first deduced by sequence analysis of the corresponding gene (Elson et al., 1993, Aidoo et al., 1994). In monomeric form, the enzyme has a molecular weight of 33.3 kDa as deduced from the gene sequence, but the active enzyme must be multimeric since it was isolated by Aidoo et al., (1994) as a 270 kDa protein which would correspond to an octameric structure. When a gene encoding an arginase or amidinohydrolyase type enzyme was first detected in the clavulanic acid gene cluster, ornithine was believed to be the immediate precursor of clavulanic acid rather than arginine. Therefore PAH was investigated as an arginase capable of converting arginine to ornithine. However, arginase activity is very low in cell extracts of S. clavuligerus unless cells are given arginine as a nitrogen source (Romero et al., 1986), and correspondingly, PAH has no detectable arginase activity. The interpretation of these observations became clear once it was understood that arginine, not ornithine is the immediate precursor of clavulanic acid. The actual function for PAH in catalysing the conversion of guanidinoproclavaminic acid to proclavaminic acid was demonstrated using PAH purified from S. clavuligerus (Elson et al., 1993).

Proclavaminic acid was one of the first intermediates in the clavulanic acid biosynthetic pathway to be identified following isolation from mycelial extracts of S. clavuligerus together with clavaminic acid (Elson et al., 1978). The structural similarity between proclavaminic acid and clavaminic acid led to a search for an enzyme able to interconvert them and thus to the discovery of CAS. Proclavaminic acid is converted into clavaminic acid in a two step reaction involving the transient intermediate, dihydroclavaminic acid (Baldwin et al., 1993, Salowe et al., 1991). Both steps are catalysed by CAS and therefore require iron, molecular oxygen and 2-ketoglutarate as co-substrate, generating succinate as a side product. It was only realised later that CAS is also responsible for a third activity, the hydroxylation of deoxyguanidinoproclavaminic acid, which occurs earlier in the pathway.
Full characterisation of CAS did not become possible until a chemical synthesis for proclavamine was available to provide a reliable assay for the enzyme (Baggaley et al., 1990). The purification of CAS was first achieved by Salowe et al., (1991) and revealed the surprising fact that there are two forms of the enzyme which differ only slightly in kinetic properties and molecular weight. Although this remarkable enzyme catalyses three distinct steps in the pathway, there was no indication that the two forms of the enzyme differed in their ability to catalyse any of the three steps. Possible reasons for having two forms of this enzyme include a gene dosage effect to provide additional amounts of a rate limiting enzyme, or a gene duplication with one form of CAS arising as part of the clavulanic acid biosynthetic pathway while a second form is associated with production of non-clavulanic acid clavams.

Clavaminic acid has the fused bicyclic β-lactam/oxazolidine ring system but differs from clavulanic acid in the stereochemistry of the ring system. In this property it resembles all of the other clavam metabolites accumulated by S.clavuligerus and other Streptomyces spp., and it also shares with them its ineffectiveness as a β-lactamase inhibitor. The similar nuclear structure and stereochemistry of clavaminic acid and the other clavam metabolites has led to investigation of the possibility that all clavam metabolites share a common biosynthetic pathway. Janc et al., (1993) has shown that clavulanic acid and the clavams share a common pathway at least to the stage of proclavaminic acid because labelled proclavamine is incorporated directly into clavams as well as into clavulanic acid. Evidence that clavaminic acid is a shared intermediate in the biosynthesis of both clavulanic acid and the other clavams has been proved by Egan et al., (1997) based in part on studies in S.antibioticus, where a single isozyme of CAS, called CAS3 has been isolated (Janc et al., 1995). This strain of S.antibioticus produced hydroxyethylclavam and valclavam, but not clavulanic acid and so the presence of CAS activity implies that clavaminic acid is an intermediate in the biosynthesis of one or both of the clavam metabolites as well as its known involvement in clavulanic acid biosynthesis.
The conversion of clavaminic acid into clavulanic acid requires the inversion of stereochemistry of the ring system as well as conversions of the side chain substituent from an amino to a hydroxyl group, but as with the earliest steps in the pathway, the reactions involved are unclear. The existence of an aldehyde intermediate with the same stereochemistry as clavulanic acid has been demonstrated, and the presence of a NADPH dependent dehydrogenase called clavulanic acid dehydrogenase (CAD) which reduces the clavaldehyde to clavulanic acid has been documented (Nicholoson et al., 1994). However, it remains unclear how the aldehyde is formed from the amine substituent of clavaminate, and how and when the inversion of stereochemistry is accomplished. Interestingly, clavaldehyde is a highly unstable but β-lactamase inhibitory compound, lending support to the suggestion that β-lactamase inhibitory activity is associated with the 3R,5R stereochemistry of the ring system. A schematic diagram of the biosynthetic pathway to clavulanic acid and the other clavams is shown in figure 6.
Fig 6. The biosynthetic pathway to clavulanic acid and the other clavams.

1.3.2. OPTIMISATION STRATEGIES

There have been many different approaches aimed at the optimisation of antibiotic production. These strategies range from media development (Chapter 8), nutritional feeds (Chapter 6) and the analysis of metabolic fluxes (Chapter 7). One of the most successful strategies used to increase the yields of clavulanic acid obtained from *S. clavuligerus* involved the feeding of certain amino acids to influence the clavulanic acid biosynthetic pathway. (Ives and Bushell, 1997). This study resulted in significant increases in clavulanic acid titre to be obtained, and by analysing the intracellular pools of particular amino acids, enabled speculation into the roles of certain areas of the metabolic pathway in clavulanic acid production.

1.3.2.1 NUTRITIONAL FEEDING - amino acids

It is known that particular amino acids can exert a stimulatory or inhibitory effect upon the production of clavulanic acid. A study carried out by Ives and Bushell (1997) examined the relationship between intracellular amino acid production rate and clavulanic production. Amino acid feeds (arginine, isoleucine, serine, valine) were used in chemostat culture (\(D=0.03\)h\(^{-1}\)) to give a final concentration of 10mM.

It was discovered that valine, leucine and isoleucine feeds resulted in an increase in clavulanic acid production compared to that of the control where no amino acid was added.

Ives and Bushell (1997) also put forward a postulated biosynthetic scheme for clavulanic acid production, which indicates the fluxes which they believed to compete with clavulanic acid production (Figure 7).
Using this scheme it was postulated that the supply of C3 precursor (glycerol) is rate limiting for clavulanic acid production and by feeding leucine or isoleucine to *S. clavuligerus*, the anaplerotic carbon flux of pyruvate will decrease towards the synthesis of these amino acids, and thus allowing for more C3 precursor for the synthesis of clavulanic acid. Increased leucine and isoleucine pool sizes were observed in the fed cultures, and the synthesis of both amino acids is repressed in many microbial species under these conditions (Umbarger 1978).

The arginine feed provided the evidence to support that the C3 carbon supply and not the C5 supply is rate limiting to clavulanic acid production. Whilst the arginine feed increased the arginine pool size (which is the direct precursor to clavulanic acid synthesis), no increase in clavulanic acid production was noted.

The effect of amino feeding upon the intracellular amino acid pools and carbon fluxes in *S. clavuligerus* has been explored in chapters 6 and 7. As parts of the clavulanic acid biosynthetic pathway are still unknown, we can only speculate the effects of particular feeds on the formation of clavulanic acid until further conclusive research is carried out.
2.0 MATERIALS AND METHODS

This study was based on *Streptomyces clavuligerus* (SmithKline Beecham) strain ATCC 27064, NRRL 35851.

2.1 CULTURE MAINTENANCE

*Streptomyces clavuligerus* was grown on M5D\textsuperscript{-} agar, which comprised of (g/l) Dextrin 10g, K\textsubscript{2}HPO\textsubscript{4} 1g, MgSO\textsubscript{4}.6H\textsubscript{2}O 1g, NaCl 1g, CaCO\textsubscript{3} 4g, (NH\textsubscript{4}) SO\textsubscript{4} 1g, Oxoid agar No. 3 20g, and distilled water to 1L. pH was adjusted to 7.4 before autoclaving at 121°C for 15 minutes. 1ml of trace salts solution was added post-sterilisation consisting of (g/l) FeSO\textsubscript{4}.7H\textsubscript{2}O 1g, MnCl\textsubscript{2}.4H\textsubscript{2}O 1g, ZnSO\textsubscript{4} 1g. A single colony isolate was streaked onto M5D\textsuperscript{-} agar and allowed to grow at 30°C for up to 10 days. The plates were then transferred to 26°C for 7 days to enable sporulation. Plugs of the sporulating *Streptomyces clavuligerus* were stored in brain heart infusion broth (Oxoid) containing 10% glycerol and frozen at -20°C. Sufficient stock was prepared for all experiments described.
2.2 EXPERIMENTAL MEDIA FORMULATION

Defined synthetic nutrient limited media used in this study for the culture of *Streptomyces clavuligerus* are summarised in Figure 8.

Fig 8. Experimental Media formulations.

<table>
<thead>
<tr>
<th>Component</th>
<th>Carbon Ltd</th>
<th>Nitrogen Ltd</th>
<th>Phosphate Ltd</th>
<th>Nutrient Rich/Oxyden Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>15.0</td>
<td>45.0</td>
<td>60.0</td>
<td>50.0</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>7.0</td>
<td>1.5</td>
<td>7.0</td>
<td>10.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0</td>
<td>3.0</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>7.0</td>
<td>7.0</td>
<td>-</td>
<td>7.0</td>
</tr>
<tr>
<td>MOPS*</td>
<td>-</td>
<td>-</td>
<td>21.0</td>
<td>-</td>
</tr>
<tr>
<td>Trace Solution (ml)**</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*3-(N-morpholino) propanesulphonic acid (Sigma Chemical Co.)*

** Trace element solution was made up as a x 100 concentrate and added to give the following final concentrations (g/l) : MgSO₄·7H₂O 0.25g, FeSO₄·7H₂O 0.25g, CoCl₂ 0.00055g, CuCl₂ 0.0053, CaCl₂·2H₂O 0.0138g, ZnCl₂ 0.0104g, MnCl₂ 0.0062g, Na₂MoO₄ 0.0003g.
2.3 INOCULUM PREPARATION

To eliminate the effects of nutrient carry-over into nutrient limited media, a two-stage inoculum procedure was used. The contents of one vial of *Streptomyces clavuligerus* was defrosted at room temperature for 2 minutes and inoculated into a baffled flask containing 25 ml of SV2 broth which consisted of (g/l) glycerol 15g, glucose 15g, soya peptone 15g, calcium carbonate 1g and distilled water to 1L. pH was adjusted to 7.0 before autoclaving at 121° C for 15 minutes. The flask was incubated at 30° C on an orbital shaker at 250 r.p.m for 2 days. 2ml of the pre-culture was then inoculated into 25ml of experimental medium and incubated as above for a further day. Bioreactors were inoculated at 5.0% volume.

2.4 FLASK CULTURE

Flask culture experiments were carried out using 250ml Pyrex flasks with 4 baffles. The maximum working volume of each flask was 25ml. Bungs for shake flasks were made of non absorbent cotton wool for optimal oxygen transfer. Flasks were placed on an orbital shaker platform at 250 r.p.m at 30° C unless otherwise stated.
2.5 BIOREACTOR SET-UP

The bioreactor used throughout this study was a two litre (1.5L working volume) Braun Biolab (B. Braun Biotech) bioreactor (Figure 9). Agitation of the culture, was maintained at 750 r.p.m, via a centrally mounted stirrer shaft fitted with two pitched blade impellers. The distance between impellers was 65mm, and the pitched blades were at 45° angles to each other.

Sterile air was supplied to the culture using a sparger directly below the impellers using a Capex 2D-C air pump. Airflow was maintained at 1.0 v/v mm⁻¹ and the dissolved oxygen was monitored with an Ingold polarographic electrode. The calibration of the oxygen probe was achieved by sparging with oxygen free nitrogen following the polarising period for the probe. Exit gasses were monitored using CO₂ (ADC Carbon dioxide analyser) and O₂ (Sybron/Taylor Servomex Oxygen analyser) detection meters. Culture temperature was maintained at 25°C +/- 0.2°C. Culture pH was monitored using a liquid filled Ingold pH probe. To control foaming, Breox FMT30 antifoam (International Speciality Chemicals) was added to the medium at 0.001% v/v.
2.5.1 BATCH CULTURE

The working volume used for batch culture was 1.5L. At each sample point, 20ml of culture was removed for biomass determination and supernatant was stored at -20° C for further analysis. Batch cultures were allowed to proceed to between 150-200 hours until death and lysis of the culture was observed.
2.5.2 CHEMOSTAT CULTURE

Chemostat culture was carried out with the above vessel using an overflow to maintain a constant volume. Media flowing into the vessel contained antifoam at the appropriate concentration and was continually stirred via a magnetic stirrer (LH Fermentations) to prevent precipitation of medium components. Medium feeds were added to the vessel via peristaltic pumps (Gilson Minipuls 2), which were fitted with Masterflex Norprene tubing.

Between dilution rate changes, 300ml of culture was removed and the vessel was allowed to fill up at the new dilution rate. This allowed a period of unlimited growth between dilution rates with a view to minimising mutant selection. Steady state conditions were achieved when the growth limiting substrate could no longer be detected and biomass and exit gas production remained constant. As the working volume of 1.5L and the flow rate of medium to the vessel were constant, the dilution rate/growth rate of the culture could be calculated using the equation:

\[
\text{Dilution rate } \frac{D}{\text{Growth rate } \mu} = \frac{\text{Flowrate (L/hr)}}{\text{Working volume } V (L)}
\]

Adjusting the flow of fresh medium to the vessel could therefore alter the growth rate of the culture. Chemostat cultures can be maintained indefinitely in theory as the exponentially growing culture is supplied with fresh medium.
2.5.3 CYCLIC FED BATCH CULTURE (CFBC)

CFBC is a method of controlling the growth rate of a culture at varying rates to a profile between two specific values. This system is based on the concept that a down regulation of culture growth rate stimulates secondary metabolite production.

CFBC culture was also carried out in the Braun vessel but with some adjustments. The medium feed was pumped into the vessel at a constant rate using a Gilson Minipuls 2 peristaltic pump fitted with Masterflex Norprene tubing.

The culture volume increased with time from the initial value $V_{\text{min}}$ (0.42L), where assuming the flow rate remains constant, the dilution rate will be at $D_{\text{max}}$, to the final volume $V_{\text{max}}$ (1.5L), where the dilution rate will have reached $D_{\text{min}}$. The time taken to complete this volume change constituted a single CFBC cycle. The medium reservoir was continually stirred via a magnetic stirrer to prevent precipitation of trace metals from the solution.

At the end of a cycle ($V_{\text{max}}$), the bioreactor was emptied from $V_{\text{max}}$ to $V_{\text{min}}$ using a pump (Watson Marlow) connected to the waste reservoir. This pump was controlled by a socket timer (Superswitch) to ensure removal of culture at precise times. An over flow pipe connected to the waste reservoir was also included in case of pump failure.

A freshly inoculated CFBC was allowed to proceed without feeding until the first increase in biomass was detected. It was at this point that the feed was switched on. This helped prevent the problem of the culture washing out.
The timing of the initiation of feeding was proven to be critical for a successful CFBC (Lynch and Bushell 1995). When a dilution series was changed, one cycle was allowed to proceed before samples were taken to ensure that a reproducible cycle was being monitored.

The dilution rate range is determined by the minimum and maximum culture volumes used. As flow rate remains constant throughout a cycle, the dilution rate changes with changing volume.

**Minimum volume:**

- Dilution rate = 0.025 h\(^{-1}\)
- Working volume = 0.42 L
- Flow rate = 0.0105 L/hr

**Maximum volume:**

- Dilution rate = 0.007 h\(^{-1}\)
- Working volume = 1.5 L
- Flow rate = 0.0105 L/hr

**Cycle Time = 103 hrs.**

By measuring the culture volume, the dilution rate/growth rate of the culture can be determined at any point.
The parameter changes in CFBC can be observed in Figure 10.

Fig 10. Parameter Changes in CFBC Over Time.

2.6 CULTURE ANALYSIS

2.6.1 CULTURE BIOMASS CONCENTRATION

To determine the concentration of biomass, membrane filtration was used. Membrane filters of 0.4\(\mu\)m porosity (Gelman Sciences) were dried in a microwave oven (Toshiba, 850W) at power level 6 for 6 minutes and desiccated until required. Each filter was then weighed before it was used and washed with 10ml of Reverse Osmosis water (RO). 5 ml of sample was filtered and washed with a further 3 x 10ml of RO water. Wet filters were then subjected to two drying cycles as above, and allowed to cool in a desiccator before re-weighing. Dry weight analysis was carried out in triplicate.
2.6.2 COLLECTION OF CULTURE SUPERNATANT

Samples for analysis were taken from the bioreactor and centrifuged at $10,000 \times g$ for 10 minutes. 1ml aliquots of the resulting supernatant were then dispensed into sterile 1.5ml Eppendorf tubes and either analysed immediately or frozen at -20°C. As clavulanic acid has been noted to degrade upon storage (Ives, 1995), it was analysed immediately.

2.6.3 CLAVULANIC ACID ANALYSIS

The high performance liquid chromatography (HPLC) assay for the measurement of clavulanic acid has developed from a method originating from Foulstone and Reading (1982). This method utilises a single step derivatisation with imidazole Figure 11.

Fig 11. Derivatisation of clavulanic acid.

Potassium Clavulanate

+ 

Imidazole

\[ \downarrow \]

1-(8-hydroxy-6-oxo-4-azaoct-2-enoyl)-imidazole + CO$_2$
The HPLC system used included a Waters 600 pump and injections were made with a Waters Wisp 710B autoinjector. Column eluent was monitored using a Waters 486 tunable absorbance detector with the wavelength set at 313nm and 0.1 AUFS. The flow rate was maintained at 2mls per minute throughout the total run time of 6 minutes. Assays were carried out at room temperature and derivatised clavulanic acid ran at approximately 3.5 minutes.

**2.6.3.1 HPLC METHOD**

For this method, a Waters 5µm C18 bondapak column of 250mm in length with an internal diameter of 4.6mm was used.

The mobile phase consisted of one buffer which was run isocratically containing, 100mM NaH$_2$ orthophosphate (BDH Analar grade) and 10% methanol. The pH was adjusted to 6.8 with acetic acid, and the buffer was degassed with oxygen free helium (Foulstone and Reading, 1982).
2.6.3.2 DERIVATISATION PROCEDURE

The working solution of imidazole was prepared using 5g of 99% purity imidazole (Sigma) which had been dissolved in 6ml of Milli-Q water. The pH of the reagent was adjusted to 6.8 using concentrated HCl, and Milli-Q water was added to bring the final volume to 10ml. 160µl of sample was added to 40µl of imidazole reagent, and after 10 minutes at room temperature, 20µl of the sample was ready to be analysed by HPLC.

To calculate the clavulanic acid concentration in experimental samples, an external standard of a known concentration of clavulanic acid was injected and programmed into the Waters 740 data module. The clavulanic acid concentration in the samples was then automatically calculated. This was regularly checked by injecting “spiked” samples of known concentrations of clavulanic acid. This assay enabled clavulanic acid concentrations as low as 0.1 µg/ml to be detected.

2.6.4 AMINO ACID ANALYSIS

In order to measure the intracellular pool of amino acids in *Streptomyces clavuligerus*, the cells had to be broken open in order to release their contents. Fermentation samples for analysis were taken and immediately centrifuged at 10,000 x g using a Labofuge centrifuge. The supernatant was removed and the pellet was washed in ice cold methanol and centrifuged again at 10,000 x g.
This cycle was repeated twice. The washed biomass was then immediately freeze-dried and remained so until analysis.

The freeze dried hyphae were then weighed and resuspended in Milli-Q water. 3ml volumes were then exposed to 2 minutes sonication at intensity 3 with a sonicating probe fitted with a micro tip (Heat systems). Cells were maintained in ice throughout sonication and were exposed for 30-second periods. To remove debris, samples were then subjected to centrifugation at $12,000 \times g$ for 15 minutes at $4^\circ C$. The supernatant was removed and freeze dried before analysis. Amino acid concentration was then determined using Waters PICO.TAG amino acid analysis system. A solution was prepared containing a 2:2:1 mixture by volume of ethanol, Milli-Q water and triethylamine. 10µl of this was added to the freeze-dried sample and vortex mixed. The sample was then dried under vacuum using the PICO.TAG workstation. The derivatisation reagent was prepared containing 7:1:1:1 by volume of ethanol, triethylamine, Milli-Q water and phenylisothiocyanate (PITC).

20µl of the derivatisation reagent was added to each redried sample and mixed by vortexing. The samples were allowed to stand for 20 minutes at room temperature. The sample was dried under vacuum until all traces of PITC had been removed. Sample diluent was prepared by weighing 710mg of disodium hydrogen phosphate in 1 litre of Milli-Q water. pH was adjusted to 7.4 with 10% phosphoric acid. The resulting solution was mixed with acetonitrile so that acetonitrile equalled 5% by volume.
Eluent A was prepared by adding 19g of sodium acetate trihydrate and 0.5ml of triethylamine to 1L of Milli-Q water. The pH was titrated to 6.4 with glacial acetic acid, and the resulting solution was filtered to remove particulates. 940mls of the solution was then added to 60mls of acetonitrile to give the working solution.

Eluent B was prepared by adding 710mg of disodium hydrogen phosphate to 1L of Milli-Q water. The pH was titrated to 7.4 with 10% phosphoric acid. The resulting solution was mixed with acetonitrile so that acetonitrile equalled 5% by volume.

The derivatised sample was then reconstituted in 200µl of sample diluent.

The reaction of amino acids with the PITC derivatising agent is shown in Figure 12.

Fig 12. Production of PITC amino acids.
The HPLC system used included a Waters 600 pump, and injections were made using a Waters 710B autoinjector. Column eluent was monitored with a Waters 486 tunable absorbance detector set at 254 nm and 0.1 AUFS.

To enable the separation of all the amino acids, a gradient elution consisting of the 2 mobile phases was required. This was achieved using a Waters 600E controller. The gradient programme can be observed in Figure 13.

Fig 13. Pico.Tag Amino Acid analysis HPLC gradient table.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Flow</th>
<th>% A</th>
<th>% B</th>
<th>% C</th>
<th>Curve (profile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>*</td>
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<tr>
<td>10.0</td>
<td>1.0</td>
<td>54</td>
<td>46</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
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<td>1.0</td>
<td>0</td>
<td>100</td>
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<td>6</td>
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<td>100</td>
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<td>1.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

4 µl of the sample was then injected onto the Waters Pico Tag column, which was controlled at 38°C. An amino acid standard solution (Sigma) was treated as above and used to calculate amino acid concentrations in experimental samples.

This assay enabled amino acid concentrations as low as pico mole quantities to be detected.
2.6.5 AMINO ACID ANALYSIS 2

During this study it became necessary to determine the level of amino acids that remained in the culture medium after amino acid feeding experiments had been completed. Unfortunately, due to the high concentrations of glycerol and salts which were present in the culture supernatant, HPLC analysis could not be used to determine amino acid concentration in supernatant. Instead a colourimetric estimation of amino acids was used using ninhydrin which allowed quantification spectrophotometrically (Jayaraman, 1981). Although this method is non specific for any particular amino acid, as only one amino acid was fed per run, it was assumed that only one amino acid would be present in sufficient quantity to be detected by this assay.

A solution of ninhydrin was prepared by dissolving 2g in 25ml of acetone. To this solution, 25ml of 0.2M acetate buffer, pH 5.5 was added. 1ml of the ninhydrin reagent was then added to 4ml of sample, mixed and incubated in a boiling water bath for 15 minutes. The tubes were then cooled and 1ml of 50% ethanol was added to each tube. The pink colour developed was measured at 550nm spectrophotometrically. A standard curve was constructed for each amino acid to be quantified.
2.7 MORPHOLOGICAL ANALYSIS

Simple stains were prepared using methylene blue for all culture samples taken. Slides were then kept for later examination of hyphal micro-morphology.

Viability was determined using baclight, a fluorescent viability stain which utilises a mixture of SYTO 9 fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population including those with intact membranes and those with damaged membranes. In contrast, propidium iodide stains bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus with an appropriate mixture of stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480nm/500nm for SYTO 9 and 490nm/635nm for propidium iodide. 5µl of stain consisting of 50:50 of SYTO 9 and propidium iodide were added to 50µl of sample and after gentle mixing were allowed to stand for 10 minutes at room temperature in darkness. Samples were then placed on a microscope slide and were viewed immediately using a fluorescence microscope fitted with the appropriate filter sets.
2.7.1 DNA ASSAY

The concentration of DNA that was released into the culture supernatant was used as an indication of the level of fragmentation that was occurring to hyphae during a bioreactor run. If the level of fragmentation was high, then it would be expected that an increase in concentration of DNA in the supernatant would be detected.

2.5 mls of cell free supernatant was collected and 278µl of 3M sodium acetate was added. This was mixed briefly by vortexing. 2.5 mls of cold propan-2-ol was added and vortex mixed for 20 seconds. The sample was then centrifuged at 13000 r.p.m for 10 minutes in a microfuge to precipitate the DNA. The propan-2-ol/water mix was then carefully removed from the DNA pellet. The pellet could then be rehydrated in sterile Milli Q water, and the concentration was determined at 360nm in a spectrophotometer (Pharmacia biotech). This assay may also have caused precipitation of protein from the supernatant, but as it was used for all samples, it gave an approximate indication of fragmentation.

2.7.2 PHOSPHATE ANALYSIS (Merk No 16978)

The phosphate concentration in experimental samples was measured using the Rqflex reflectometer (Merck). This analysis is based upon the principal that in a solution acidified with sulphuric acid, orthophosphate ions (PO$_4^{3-}$) and molybdate ions form molybdophosphoric acid. This is reduced to phosphomolybdenum blue, the concentration of which is determined reflectometrically.
Firstly the sample to be analysed was checked to be within the measuring range of the method (containing less than 120mg/L PO$_4^{3-}$). Samples containing more than this were diluted with distilled water. 10 drops of reagent P0$_4^{3-}$ were added to 5ml of test sample and shaken for a few seconds. A test strip was immersed in the treated sample for 2 seconds, and the strip was shaken to remove excess liquid. After 90 seconds the strip was inserted into the Rqflex reflectometer and the result recorded. A phosphate standard solution was diluted to give phosphate concentrations ranging from 50 to 120 mg/L$^{-1}$. These were treated as above and the results used to produce a standard curve. Phosphate concentration could then be determined.

2.7.3 AMMONIUM ANALYSIS (Merk No 16977)

To measure the concentration of ammonium ions in a culture the Rqflex reflectometer was used once again. In this method NH$_4^+$ ions react with Nessler's reagent to form a yellow brown compound, the concentration of which is determined reflectometrically. 10 drops of Nessler's reagent were added to 5ml of sample supernatant. The solutions were mixed by gently swirling. Once analytical test strip was immersed into the solution for 2 seconds. The strip was shaken to remove excess liquid and after 15 seconds, was placed into the Rqflex reflectometer.

An ammonium standard solution was diluted to give a range of ammonium concentrations from 20 to 180 mg/L$^{-1}$. This was treated as above and analysed in the Rqflex reflectometer. A standard curve could then be constructed, and ammonium concentration could then be calculated.
2.7.4 GLUCOSE ANALYSIS (Merk 1.16720.0001)

Under the catalytic effect of glucose oxidase, glucose is converted to gluconic acid lactone. The resultant hydrogen peroxide reacts with an organic redox indicator in the presence of peroxidase to form a blue-green dye, the concentration of which is determined reflectometrically (Merk).

A glucose test strip was immersed in the sample solution for 15 seconds so that both reaction zones became wetted. The strip was then inserted into the Rqflex reflectometer and the result was noted. A standard curve was constructed between the measuring range of 1 - 100 mg/l glucose.

2.7.5 GLYCEROL ANALYSIS (Sigma No. 148270)

Glycerol concentration was determined enzymatically and was quantified spectrophotometrically. Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase (GK).

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L-glycerol-3-phosphate} + \text{ADP}
\]

The adenosine-5'-diphosphate (ADP) formed in the above reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) into ATP with the formation of pyruvate.
ADP + PEP $\xrightarrow{PK}^{P}$ ATP + pyruvate

In the presence of the enzyme L-lactate dehydrogenase (L-LDH), pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{L-LDH} \text{L-lactate} + \text{NAD}^+$$

The amount of NADH oxidised in the above reaction is stoichiometric to the amount of glycerol. NADH is determined by means of its light absorption at 340nm.

2.7.6 UREA NITROGEN ANALYSIS (Sigma No. 640)

This analysis is based primarily on methods described by Fawcett and Scott 1960, and based upon the reaction that urea is converted to ammonia and carbon dioxide in the presence of water by the enzyme urease.

$$\text{Urea} + \text{H}_2\text{O} \xrightarrow{Urease} 2\text{NH}_3 + \text{CO}_2$$

Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside [Na$_2$Fe(CN)$_5$NO 2H$_2$O], to form indophenol. The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrophotometrically at 570nm.
2.8 ORGANIC ACID ANALYSIS

Krebs intermediates were quantified using enzymatic methods. These methods rely on the spectrophotometric measurement of NADH and NAD, the former having a high $A_{340}$ and the latter a low $A_{340}$. All measurements were made on a Pharmacia biotech Ultrospec 2000 spectrophotometer.

2.8.1 PYRUVATE (Sigma No. 726)

This procedure utilises the enzyme, lactate dehydrogenase, which catalyses the following reaction:

$$\text{Pyruvate} + \text{NADH} \overset{\text{LD}}{\longrightarrow} \text{Lactate} + \text{NAD}$$

In the presence of excess NADH, substantially all pyruvate is converted to lactate. The reduction of absorbance at 340nm due to oxidation of NADH to NAD becomes a measure of the amount of pyruvate originally present.

2.8.2 MALATE (Boehringer Mannheim No 139 068)

L-Malate is oxidised by NAD to oxaloacetate in the presence of L-malate dehydrogenase (L-MDH).

$$\text{L-Malate} + \text{NAD} \overset{\text{L-MDH}}{\longrightarrow} \text{oxaloacetate} + \text{NAD} + \text{H}$$
The amount of NADH formed is stoichiometric to the amount of L-malate. The increase is measured by means of its light absorbance at 340nm.

2.8.3 SUCCINATE (Boehringer Mannheim No 176 281)

Succinate is converted by the enzyme succinyl-CoA synthetase to succinyl-CoA with a simultaneous formation of inosine-5'-diphosphate and inorganic phosphate:

\[
\text{Succinate} + \text{ITP} + \text{CoA} \xrightarrow{SCS} \text{IDP} + \text{succinyl-CoA} + \text{Pi}
\]

Inosine-5'-diphosphate reacts with phosphoenolpyruvate in the presence of pyruvate kinase to pyruvate and ITP:

\[
\text{IDP} + \text{PEP} \xrightarrow{PK} \text{ITP} + \text{pyruvate}
\]

Pyruvate is reduced by NADH in the presence of lactate dehydrogenase:

\[
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{LDH} \text{L-lactate} + \text{NAD}^+
\]

The amount of NADH oxidised in the above reaction is stoichiometric to the amount of succinic acid. NADH is measured by its light absorbance at 340nm.
2.8.4 ISOCITRATE (Sigma No 735)

D-Isocitric acid is oxidatively decarboxylated by nicotinamide-adenine dinucleotide phosphate (NADP) in the presence of the enzyme isocitrate dehydrogenase (ICDH).

\[
\text{D-Isocitrate} + \text{NADP}^+ \xrightarrow{\text{ICDH}} \text{2-oxoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+
\]

The amount of NADPH formed in the reaction is stoichiometric to the amount of D-isocitrate. NADPH is determined by means of its light absorbance at 340nm.

2.8.5 LACTATE (Sigma No 735)

Lactic acid is converted to pyruvate and hydrogen peroxide (H₂O₂) by lactate oxidase. In the presence of the H₂O₂ formed, peroxidase catalyzes the oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum at 540nm. The increase in absorbance at 540nm is directly proportional to lactate concentration in the sample.
2.8.6 CITRATE (Boehringer Mannheim No 139 076)

Citric acid is converted to oxaloacetate and acetate in the reaction catalysed by the enzyme citrate lyase (CL).

\[
\text{Citrate} \xrightleftharpoons{\text{CL}} \text{oxaloacetate} + \text{acetate}
\]

In the presence of the enzymes L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate respectively, by reduced nicotinamide-adenine dinucleotide (NADH).

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-MDH}} \text{L-malate} + \text{NAD}^+
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+
\]

The amount of NADH oxidised in the above reactions is stoichiometric to the amount of citrate. NADH is determined by means of its light absorbance at 340nm.
2.8.7 FUMARATE

(The following three assays were taken from Methods in Enzymology XIII, Assays of intermediates of the citric acid cycle and related compounds by fluorometric methods, Williamson J.R, Corkey B.E ). The principal of this assay relies on Fumarase catalysing the reversible hydration of fumarate to form malate:

\[
\text{Fumarate} + \text{H}_2\text{O} \xrightarrow{\text{Fumarase}} \text{L-malate}
\]

Malate dehydrogenase (MDH) then catalyses the oxidation of malate by NAD\(^+\) to oxaloacetate and NADH:

\[
\text{L-Malate} + \text{NAD}^+ \xrightarrow{\text{MDH}} \text{oxaloacetate} + \text{NADH} + \text{H}^+
\]

The equilibrium of this reaction lies far to the left so that quantitative oxidation of malate is possible only if oxaloacetate is removed from the reaction medium. An alkaline reaction medium is used to decrease the H\(^+\) concentration and oxaloacetate is removed by converting it to the hydrazone derivative with hydrazine hydrate. Quantitative measurement of fumarate is then possible according to the following:

\[
\text{Fumarate} + \text{H}_2\text{O} + \text{NAD}^+ + \text{hydrazine} \xrightarrow{\text{Fumarase} \text{ MDH}} \text{oxaloacetate-hydrazone} + \text{NADH} + \text{H}_2\text{O}^+
\]

The accompanying increase in fluorescence of NADH can be observed spectrophotometrically by following the increase in optical density at 340 nm.
**2.8.8 α-KETOGLUTARATE**

This reaction depends upon the conversion of α-ketoglutarate and NADH by the enzyme glutamate dehydrogenase (GD) to succinyl CoA and NAD:

\[
\alpha\text{-ketoglutarate} + \text{NADH} \xrightarrow{GD} \text{Succinyl CoA} + \text{NAD}
\]

The change in optical density at 340nm can be observed and used to determine concentration of α-ketoglutarate in the sample.

**2.8.9 OXALOACETATE**

In this reaction malate dehydrogenase catalyses the reduction of oxaloacetate to L-malate in the presence of NADH:

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xleftarrow{L\text{-Malate}} \xrightarrow{L\text{-Malate}} + \text{NAD}^+
\]

The equilibrium of this reaction lies far to the right and quantitative conversion of oxaloacetate to malate is achieved in the presence of a slight excess of NADH. The optical density change at 340nm is used to follow this reaction.
2.9 REPRODUCIBILITY OF EXPERIMENTS AND ASSAYS

Individual assays were replicated in triplicate. Where error bars (which represent standard error) are not shown, they were too small to be visible on the figures presented.
3.0 RESULTS

3.1 THE EFFECT OF NUTRIENT LIMITATION UPON *S. clavuligerus*

3.1.1 INTRODUCTION

Synthetic, defined nutrient-limited media were evaluated in this study to determine the effect of specific limitations on the growth of *S. clavuligerus* and the production of clavulanic acid. Growth and production profiles were established under carbon, nitrogen, oxygen and phosphate limitation from experiments carried out in shake flask and bioreactor culture.

Shake flasks contained 25 ml of nutrient limited media (Chapter 2), which were limiting in either carbon, oxygen, nitrogen or phosphate. Flasks were inoculated with 2 ml of *S. clavuligerus* cultivated in SV2 broth as described in Chapter 2. Flasks were incubated at 30°C on an orbital shaker at 250 r.p.m. Samples for analysis were taken every 24 hours for a maximum of 5 days. The flask results are displayed in Figures 14, 15, 16.

Bioreactors were set up according to Chapter 2. Temperature was maintained at 30°C and the pH was controlled at 6.5. pH 6.5 was shown in a previous study (Ives 1995) to minimise the breakdown of the clavulanic acid molecule when compared to cultures maintained at pH 7.0. Bioreactor results can be observed in Figures 13-22.
3.1.2 SHAKE FLASK STUDY

Fig 14. The Effect of Nutrient Limitation upon Biomass Concentration

Fig 15. The Effect of Nutrient Limitation Upon Clavulanic Acid Production
Of the four limitations examined in shake flask culture, only phosphate and nitrogen limitation were capable of supporting clavulanic acid production (Fig 15). Biomass under all four limitations reached its maximum concentration between 72 and 96 hours of incubation, and in all cases this declined until the end of the 120 hour incubation period (Fig 14). Similarly, clavulanic acid production under both phosphate and nitrogen limitation also reached its maximum concentration between 72 and 96 hours of incubation. Maximum biomass and clavulanic acid production therefore occurred simultaneously under these conditions.

Nitrogen limitation supported the greatest maximum clavulanic acid titre of 0.013 g/l compared to 0.007 g/l obtained under phosphate limitation. However, when biomass concentration was taken into consideration, phosphate limitation yielded a maximum of 0.008 g·g biomass⁻¹ of clavulanic acid compared to 0.005 g·g biomass⁻¹ under nitrogen limitation which was 60% less.
3.1.3 BIOREACTOR STUDY

Fig 17. The Effect of Oxygen Limitation Upon *S. clavuligerus*

In bioreactor culture, oxygen limited conditions supported the greatest concentration of biomass (6.5 g/l), which was observed after 90 hours of incubation.

In order to maintain conditions of oxygen limitation, a rich medium was used (Chapter 2) which was designed to provide all nutrients in excess quantities to promote a high biomass yield which would have a high oxygen demand. In addition, dissolved oxygen in the bioreactor was maintained at a concentration below 25% saturation.

Clavulanic acid was not detected by HPLC under oxygen limiting conditions in bioreactor culture.
Under conditions of carbon limitation, clavulanic acid production was not detected by HPLC. Maximum biomass concentration of 3.6 g/l was reached after 100 hours of incubation, and the residual glycerol was exhausted by 120 hours. This corresponded with the decrease in biomass concentration and the start of culture lysis. Nitrogen and phosphate sources were demonstrated to be in excess throughout the 160 hour incubation period (not shown), confirming that carbon was the growth limiting substrate.
Fig 19. The Effect of Nitrogen Limitation Upon *S. clavuligerus*

Clavulanic acid verses Ammonium chloride concentration.

![Graph showing the effect of nitrogen limitation on *S. clavuligerus*.](image1)

Fig 20. The Effect of Nitrogen Limitation Upon *S. clavuligerus*

Biomass verses specific clavulanic acid concentration.

![Graph showing the effect of nitrogen limitation on *S. clavuligerus* on biomass and specific clavulanic acid concentration.](image2)
Under nitrogen limiting conditions, clavulanic acid production was observed to increase from the point just prior to nitrogen exhaustion (Fig 19). Maximum biomass concentration of 3.2 g/l was reached some 20 hours after nitrogen exhaustion and coincided with the maximum clavulanic acid concentration of 0.04 g/l (Fig 20). It appears unusual for biomass concentration to continue to increase after NH₄Cl in the media was no longer detected. Culture biomass is not a good indicator of true growth in a culture, and variations in biomass concentration may give an impression that the culture is continuing to grow.

It is thought by some, that a more accurate way of determining growth is by measuring the DNA content (Martin and McDaniel, 1975). This would reduce fluctuations that are commonly observed when measuring biomass concentration.

Both biomass and clavulanic acid declined in concentration from 100 hours until the end of the 160 hour incubation. The maximum specific productivity obtained under nitrogen limiting conditions was 0.013 g·g biomass⁻¹ of clavulanic acid. Carbon and phosphate sources were demonstrated to be in excess throughout the 160 hour incubation (not shown), confirming that nitrogen was the limiting substrate. The fall in clavulanic acid concentration throughout the latter part of the incubation indicates that the molecule may be degraded or perhaps re-metabolised by the organism. During the later stages of incubation the exhaustion of nitrogen could result in the abolition of amino acid synthesis. As arginine has been shown to be a C5 precursor of clavulanic acid (Elson.1993), without sufficient quantities of this, clavulanic acid synthesis may be restricted.
Fig 21. The Effect Of Phosphate Limitation Upon *S. clavuligerus*

Specific clavulanic acid verses Biomass concentration

![Graph showing the effect of phosphate limitation on biomass and specific clavulanic acid concentration.](image)

Fig 22. The Effect Of Phosphate Limitation Upon *S. clavuligerus*

Clavulanic acid verses phosphate concentration

![Graph showing the effect of phosphate limitation on clavulanic acid and phosphate concentration.](image)
The phosphate limited batch culture yielded a maximum clavulanic acid titre of 0.03 g/l, which was obtained after 59 hours when phosphate was no longer detected in the culture medium (Fig 22). This was approximately 9 hours after the peak in biomass concentration was detected (Fig 21). Again both biomass and clavulanic acid concentration declined steadily after 59 hours where culture lysis and reduction in clavulanic acid concentration occurred.

In comparison to the other nutrient limitations, phosphate reduced the length of the batch culture and as a result, the maximum biomass and clavulanic acid concentration were obtained earlier on in the batch than had previously been observed. This may possibly be due to the very low initial phosphate concentration of 0.1 g/l, which was exhausted very quickly. As a result, growth rate would have been down regulated earlier on in the culture and so antibiotic production may have been initiated earlier.

Both carbon and nitrogen sources were proven to be in excess throughout the culture and so phosphate was assumed to be the limiting substrate.
Of the 4 limitations examined, carbon and oxygen limitation failed to support clavulanic acid production in both shake flask and bioreactor culture. The exclusive exhaustion of the appropriate nutrients confirmed the identity of the respective growth limiting substrates.

Despite a greater biomass and hence clavulanic acid concentration being present in bioreactor culture compared to shake flask culture, trends obtained from the data of the two systems remained comparable.

Fig 23. Bioreactor Study: A Comparison Of Maximum Clavulanic Acid Titres Obtained Under Nutrient Limitation
Results confirmed that of the nutrient limitations that were examined, phosphate limitation promoted the highest specific clavulanic acid titre in both bioreactor and shake flask culture (Fig 16 & 24). Phosphate limited bioreactor culture produced a maximum specific clavulanic acid titre of 0.02 g: g biomass\(^{-1}\) which was 0.007 g: g biomass\(^{-1}\) greater than that obtained under nitrogen limitation. This reflected the trends that were observed in shake flask culture.
These results are consistent with those in the literature reflecting the sensitivity of clavulanic acid production to medium phosphate concentration (Lebrihi et al., 1988). In this study, Lebrihi et al., demonstrated that the production of cephamycin and clavulanic acid by *S. clavuligerus* can be controlled by phosphate concentration. In the presence of 2 mM phosphate, the specific activity of cephamycin synthase, expandase and clavulanic acid synthetase were higher than in the presence of 78 mM phosphate. This would indicate that there could be a degree of phosphate catabolite repression occurring.

Phosphate appears to be a crucial growth limiting nutrient in many antibiotic producing systems (Martin and Demain 1980). Concentrations ranging from 0.3-300 mM generally support extensive cell growth, but concentrations of 10 mM or more, usually provide conditions in which the synthesis of many antibiotics does not take place (Martin, 1977).

The negative effects of phosphate on antibiotic production has been covered in many studies in the past which have included cephamycin production by *S. clavuligerus* (Aharonwitz and Demain, 1978), streptomycin by *S. griseus* (Inoue et al., 1982), thienamycin by *S. cattleya* (Lilley et al., 1981) and vancomycin by *S. orientalis* (Mertz and Doolin, 1972).
It has also been suggested that glycolysis may be restricted under phosphate limiting conditions (Ives and Bushell, 1995). If this were true, then under these phosphate limiting conditions, the flux of glycerol would be restricted into glycolysis and the TCA cycle, which would enable the flux of glycerol into clavulanic acid to increase. Under nitrogen limiting conditions, it is assumed that these restrictions would not apply and so clavulanic acid production would have to compete with glycolysis for glycerol. This may explain the higher clavulanic acid titres obtained under phosphate limitation when compared to nitrogen limitation. The effect of nutrient limitation upon intracellular carbon fluxes has been explored in chapter 7.

Ammonium ions have also been reported to repress the synthesis of specific biosynthetic enzymes for secondary metabolite production. Examples of this include the production of cephalosporins by *S. clavuligerus* (Aharonwitz and Demain, 1978 and Brana et al., 1985) and the production of erythromycin by *Saccaropolyspora erythraea* (Flores and Sanchez, 1985). Production media for secondary metabolites that are sensitive to nitrogen regulation classically contain soybean meal. As soybean meal is broken down slowly, this prevents an accumulation of ammonium or amino acids (Demain, 1986). Alternatively, nitrogen regulation is sometimes overcome by adding ammonium-trapping agents to culture media. Examples of these include tribasic magnesium phosphate and natural zeolites. Media designed to overcome nitrogen regulation are often described as being ‘nitrogen limited’ because the nitrogen source is the nutrient limiting growth.
Clavulanic acid synthesis has been reported to be affected by high nitrogen concentration, but it is also likely that its production will be affected by a shortage of available nitrogen. The immediate precursor molecules of clavulanic acid are considered to be glycerol, which provides the β-lactam carbons, and the five carbon amino acid arginine, which provides the oxazolidine ring. If nitrogen supply was limited, it is possible that the flux of nitrogen into the synthesis of amino acids would be reduced. With less nitrogen available for the synthesis of arginine, there would be a reduction in the supply of the C5 precursor. This may then have a detrimental effect on the quantity of clavulanic acid produced.

A nitrogen source should not therefore be available in large quantities that would cause the repression of antibiotics synthesising enzymes, but must provide sufficient nitrogen to enable the synthesis of precursor molecules. This clearly indicates the complexities of secondary metabolism and explains why these compounds are only produced under a very particular set of conditions.

Clavulanic acid synthesis was not detected under oxygen limiting conditions. Dissolved oxygen concentration has been observed to effect both growth and antibiotic production in *Streptomyces* species. In cultures of *S. clavuligerus*, a 50% dissolved oxygen concentration which was maintained throughout the incubation, increased the rate of cephalosporin production two fold, whilst a constant 100% dissolved oxygen increased production three fold when compared to the uncontrolled system (Rollin *et al.*, 1988).
The reason behind these increases was found in 1988 by Rollin et al., who discovered that the activities of the enzymes involved in the synthesis of the antibiotic, increased with increasing percentage of dissolved oxygen.

In the case of clavulanic acid production, the enzyme clavaminate synthase is required to catalyse the conversion of proclavaminic acid to clavaminic acid. This enzyme has been shown to have a requirement for ferrous iron and molecular oxygen for its activity (Elson et al., 1978, Salowe et al., 1991). Although there is no indication of the concentrations of molecular oxygen required, it is likely that the formation of clavulanic acid may be dependent on the concentration of available oxygen. In the case of oxygen limitation, the activity of clavaminate synthase would be inhibited and hence the conversion of precursors into clavulanic acid would be restricted.

Under carbon limiting conditions there was also no clavulanic acid production detected. Under such condition, many metabolic pathways including clavulanic acid synthesis, gluconeogenesis and glycolysis would presumably be competing for a limited resource. If this is the case then all of these pathways would suffer from a lack of available carbon. However, as secondary metabolite production is not immediately essential for the survival of the organism, clavulanic acid production may be sacrificed in order for the organism to maintain its survival. This may possibly explain the abolition of clavulanic acid production observed under these conditions.
3.2 SUMMARY

Production of clavulanic acid by *S. clavuligerus* in batch culture occurred under conditions of nitrogen and phosphate limitation but not under carbon or oxygen limitation.

When cultured under phosphate limited conditions, *S. clavuligerus* produced significantly less biomass than under equivalent nitrogen limiting conditions. As a result, there was more clavulanic acid detected under nitrogen limitation than phosphate limitation (0.04 g/l and 0.03 g/l respectively). However, when the biomass concentration was taken into consideration, there was a greater specific productivity occurring under phosphate limitation (0.02 g:g biomass$^{-1}$), than under nitrogen limitation (0.013 g:g biomass$^{-1}$).

Many theories have been suggested in this study to account for the results obtained under conditions of nutrient limitation. A more detailed investigation has been carried out later in this study to determine the actual metabolic consequences of these limitations and the production of clavulanic acid (Chapter 7).
4.0 THE EFFECT OF TEMPERATURE UPON THE GROWTH OF

*S. clavuligerus* AND THE PRODUCTION OF CLAVULANIC ACID.

4.1 INTRODUCTION

Microorganisms have particular temperature ranges under which optimal growth will occur. Invariably, the growth rate of an organism increases with temperature up to some well-defined optimum value, beyond which it decreases sharply to zero. Thus the difference between the optimum temperature for growth and the maximum that can be tolerated is small when compared with the difference between the optimum and minimum temperatures for growth. The lower limit of temperature at which growth will occur is not easily defined, but generally speaking the temperature range within which observable growth of *Streptomyces* will occur seldom exceeds 45 °C.

The optimum growth temperature varies widely between different microbial species. The terms thermophile, mesophile and psychrophile are used to refer, respectively, to species which grow optimally at high, intermediate and low temperatures. *Streptomyces* fermentations are usually operated at the mesophile range (temperature optimum 20 - 45 °C). The appropriate temperature must be chosen to achieve maximum growth on one hand and optimal product formation on the other.

Commercial batch fermentation processes are ordinarily operated under essentially constant temperature conditions, while pH and the concentrations of nutrients, cell mass and products are allowed to change with little or no control as the fermentation progresses. Apart from tradition, there is no inherent reason why batch fermentations should be run at constant temperature (Constantinides *et al.*, 1970).
In many modern processes, higher temperatures are used to obtain increased growth of the culture and then the temperature is decreased at the onset of the idiophase. A temperature profile was designed for the production of penicillin by Constantinides, Spencer and Gaden (1970). In this study, the culture was started at a high temperature (30 °C) which favoured faster growth, and then was reduced to a lower temperature (25 °C), which favoured a high level of cell concentration and a low rate of penicillin destruction. An increase in penicillin yield of 15% was obtained when this temperature profile was followed.

The importance of temperature is apparent when observing the penicillin acylase fermentation. An increase of 1 °C above the optimum temperature results in a 20% lower yield in the penicillin acylase formation (Crueger and Crueger, 1989). Thus it is crucial that the temperature profile is considered when an increase in product formation is required.

For this investigation, a temperature block apparatus was used. This consisted of a metal block which contained a series of incubation ports within which a test tube could be inserted. The temperature within the incubation ports increased with increasing port number. This enabled the effect of a range of temperatures upon *S. clavuligerus* to be examined simultaneously. Incubation temperatures of 12 °C to 52 °C were examined in this study.
Before using the temperature block, 25 ml phosphate limited shake flask cultures were inoculated as described in Chapter 2. Phosphate limitation was chosen for this study as it yielded the highest specific concentration of clavulanic acid (Chapter 3). The cultures were then incubated at 30 °C on an orbital shaker at 250 r.p.m for 24 hours. The flask cultures were then pooled together to produce a single uniform culture. 10 ml volumes of this culture was then transferred into sterile test tubes and placed into the temperature block. The cultures were left for a further 62 hours incubation before analysis.

Cultures were analysed for biomass concentration and clavulanic acid formation.
4.2 RESULTS

4.2.1 THE EFFECT OF TEMPERATURE ON THE GROWTH OF *S. clavuligerus* AND PRODUCTION OF CLAVULANIC ACID.

Fig 25. Biomass production over a range of temperatures.

(Initial biomass concentration before incubation in the temperature block = 0.3 g/l)

Fig 26. Clavulanic acid concentration over a range of temperatures.
Fig 27. Specific production of clavulanic acid over a range of temperatures.

It can be observed from these results (Fig 25, 26, 27), that temperature has a significant effect upon the growth of *S. clavuligerus* and the production of clavulanic acid. Upon examination of these findings, it immediately became obvious that there was significantly less biomass and clavulanic acid produced in the temperature block than in bioreactor batch culture, (0.5 g/l biomass compared to 1.5 g/l biomass from bioreactor culture). As the cultures in the temperature block were contained in static test tubes, they received no mixing. This would have prevented the efficient transfer of nutrients and oxygen to all of the culture. It is therefore not surprising that *S. clavuligerus* did not perform as well in the temperature block as has been previously observed in the highly controlled environment of the bioreactor.
Although the cultures had not performed as well as those cultivated within a bioreactor, they did display some obvious trends. The greatest concentration of biomass (0.57 g/l), was obtained when the culture was incubated at 30 °C (Fig 25). This was an increase in biomass of 0.27 g/l compared to the initial biomass concentration of 0.03 g/l. The lowest biomass concentration that was observed in this study was obtained when the cultures were incubated at the high temperature ranges of 43, 46, 48 and 52 °C. These temperatures resulted in only 0.1 g/l of biomass being produced. As this concentration was lower than the initial biomass concentration, it is possible that very high incubation temperatures can not only prevent growth but can also cause cell lysis.

Clavulanic acid production was only observed in this investigation between the temperature ranges of 23 to 35 °C (Fig 26). Clavulanic acid was not produced at temperatures below 23 °C or above 35 °C. The maximum clavulanic acid titre (0.0051 g/l) was observed from the culture incubated at 30 °C (Fig 26). However, as a large biomass concentration was also observed with an incubation temperature of 30 °C, it did not result in the greatest specific concentration of clavulanic acid (0.009 g·g biomass⁻¹, Fig 27). The greatest specific clavulanic acid production was obtained at an incubation temperature of 26 °C (0.0106 g·g biomass⁻¹, Fig 27).
The reason why specific clavulanic acid production was lower when the culture had been incubated at a slightly elevated temperature is unclear from this experiment. As 30 °C and 26 °C appeared from these results to be two successful incubation temperatures for the growth of S. clavuligerus and the production of clavulanic acid, these were investigated further in bioreactor batch culture.
4.3 BIOREACTOR BATCH CULTURE

Bioreactor cultures were set up according to the procedure outlined in chapter 2.

Fig 28. The growth of *S. clavuligerus* and production of clavulanic acid at 30 °C.

When incubated at 30 °C under phosphate limited conditions, a maximum biomass concentration of 1.6 g/l was obtained after 50 hours (Fig 28). Clavulanic acid peaked at 59 hours and reached a concentration of 0.03 g/l. As a result the maximum specific clavulanic acid titre was 0.02 g:g biomass⁻¹. After the maximum antibiotic production had reached its peak at 59 hours, the concentration of clavulanic acid detected within the culture supernatant fell to 0.026 g/l by 65 hours. The concentration of clavulanic acid fell rapidly for the remainder of the batch culture until only 0.0077 g/l of clavulanic acid was detected at 130 hours.
At an incubation temperature of 25 °C, maximum clavulanic acid production (0.026 g/l) and maximum biomass production (1.2 g/l), were both detected at 70 hours (Fig 29). Specific clavulanic acid production reached a maximum concentration of 0.022 g·g\text{biomass}^{-1} which was also obtained at 70 hours of incubation.

The concentration of clavulanic acid detected within the culture supernatant did decrease after the maximum peak, although this occurred very gradually. Even after 98 hours of incubation, 0.02 g/l of clavulanic acid was still detected. At the end of the batch culture at 140 hours, there was still 0.015 g/l of clavulanic acid present within the culture supernatant.
It can be clearly observed from figures 28 & 29, that two different fermentation profiles were obtained with bioreactor batch culture incubated at 25 °C and 30 °C. When incubated at 30 °C, growth of S. clavuligerus occurred rapidly and reached its maximum peak 20 hours before it was reached at 26 °C. In addition, 0.4 g/l more biomass was obtained at the higher incubation temperature.

As a result of the faster growth rate of the organism at a higher incubation temperature, the maximum clavulanic acid production was also obtained earlier at 30 °C than was achieved at 26 °C (59 hours and 70 hours respectively). The culture incubated at 30 °C also produced 0.004 g/l more clavulanic acid than was produced under the lower incubation temperature. However, because there was less biomass produced at 26 °C, there was very little difference in the production of clavulanic acid per gram of biomass produced between the two incubation temperatures (0.02 g:g biomass⁻¹ at 30 °C compared to 0.022 g:g biomass⁻¹).

The most noticeable difference between the two production profiles was the rate at which clavulanic acid concentration declined after the production phase. At 30 °C, the concentration of clavulanic acid fell rapidly after the initial peak had been reached, and by the end of the fermentation, only 0.0077 g/l of clavulanic acid remained.
In the batch culture incubated at 25 °C, clavulanic acid concentration also fell after the maximum clavulanic acid peak had been reached, but this was gradual when compared to that previously observed at 30 °C (Fig 28). In addition, 0.015 g/l of clavulanic acid remained at the end of the fermentation.

It appears from this investigation that if a single temperature is to be chosen to carry out an *S. clavuligerus* cultivation, then there has to be a compromise between biomass production and minimisation of clavulanic acid degradation.

An investigation into the simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations was carried out by Mayer et al., (1996). They concluded that the decomposition of clavulanic acid could be attributed to re-consumption of the molecule by the organism to form other secondary metabolites, or the modification of its structure to detoxify the medium.

Mayer et al., examined the rate of clavulanic acid decomposition in two complex media formulations. It was discovered that the rate of decomposition was not fixed under all conditions but could be manipulated depending on the formulation of the media. Those formulations containing high concentrations of particulates, were found to minimise the rate of clavulanic degradation. The presence of these particles may have enabled adsorption of clavulanic acid, which is thus protected from microbial attack. It was thus concluded that production levels of clavulanic acid could be enhanced by selecting medium compositions and operating conditions which could minimise the degradation effect.
It was apparent from this study that at the end of the fermentation operated at 30 ° C, only 25% of the maximum clavulanic acid yield remained. In comparison, at the end of the batch culture operated at 26 ° C, 57% of the maximum clavulanic acid titre remained. The rate of clavulanic acid degradation appeared partly dependent upon temperature.

To investigate the effect of temperature further, a shake flask study was carried out to monitor the effect of five temperatures on clavulanic acid decomposition.
4.4 THE EFFECT OF TEMPERATURE UPON THE DEGRADATION OF CLAVULANIC ACID.

A shake flask study was carried to determine the effect of temperature upon the degradation of clavulanic acid.

25 ml of phosphate limited media was spiked with 0.1 g of clavulanic acid. This was sterilised by membrane filtration. Duplicate flasks were incubated at one of five temperatures. These were, 20 °C, 25 °C, 30 °C, 37 °C and 45 °C. Flasks were incubated for a total of 96 hours before being analysed for clavulanic acid concentration.

Fig 30. The effect of temperature upon the degradation of clavulanic acid.
It can be clearly observed from fig 30, that after 5 days incubation, there was a greater concentration of clavulanic acid present within flasks incubated at the lower temperatures. At 45 °C, only 0.3% of the initial clavulanic acid concentration was remaining after 96 hours of incubation. In comparison, at 20 °C, 25% of the initial titre remained after the 5 day incubation period.

It is apparent from these results that by lowering the incubation temperature, it is possible to minimise the breakdown of the clavulanic acid molecule. However, as previously discussed, a lower incubation temperature (25 °C) slows the growth of the organism and does not encourage the level of biomass production that occurs at the higher temperatures (30 °C). There must therefore be a compromise between biomass production and the reduction of clavulanic acid degradation.

If 30 °C encourages greater biomass production, and a lower temperature reduces the breakdown of clavulanic acid, then by using both temperatures in phases throughout the fermentation, it may be possible to combine the advantages of both temperatures. This system has been used in several studies. Park, Rye et al., (1990), utilised a two stage continuous culture system in a recombinant E.coli fermentation. The first stage was operated at temperatures lower than 37 °C for cell growth without cloned gene expression, while the second stage is maintained at temperatures higher than 38 °C to induce the biosynthesis and accumulation of the product. This system was further optimised by Hortacsu and Rye et al., (1991), who successfully carried out the two stage system producing high biomass yields and product formation.
Although these studies have been carried out in two stage continuous culture, the principles can still be applied to batch culture. Constantinides, Jordan et al., (1970), optimised a two stage temperature profile for the production of penicillin. In this case, the optimum temperature remained at 30 °C for the first 5 hours of the fermentation and then dropped to 25 °C, where it remained for 35 hours before shifting to 20 °C. This temperature profile encouraged a high cell formation at the start of the fermentation and then optimal product formation in the second phase.

As the two stage temperature profile was proven to be a success with other organisms, it was decided to investigate the effect of a two stage temperature profile on the performance of *S. clavuligerus*. 
4.5 THE INVESTIGATION OF A TWO STAGE TEMPERATURE PROFILE
UPON THE GROWTH OF *S. clavuligerus* AND THE PRODUCTION OF
CLAVULANIC ACID

In order to investigate the two stage temperature profile, a phosphate limited
bioreactor batch culture was carried out. This was set up according to chapter 2 and
allowed to initially proceed at 30 °C. Samples were taken regularly and analysed for
biomass and clavulanic acid concentration. The fermentation was allowed to proceed
for 55 hours until the peak in biomass was detected. At this point, the temperature
was reduced to 25 °C for the remainder of the incubation.

Fig 31. Two stage temperature profile bioreactor batch culture.
Fig 31, displays the fermentation profile for the phosphate limited bioreactor culture carried out using a two stage temperature profile. It can be observed from this profile that by switching temperatures during a fermentation, optimal biomass production can be obtained, while reducing the rate at which the clavulanic acid molecule is broken down.

A maximum biomass concentration of 1.66 g/l was obtained in this fermentation which was comparable with yields obtained with other batch cultures incubated at 30 °C. In addition, clavulanic acid production was also of a comparable titre. However, unlike the fermentation carried out at 30 °C only, there was over twice the concentration of clavulanic acid left remaining at the end of the bioreactor run as had been previously observed (0.0077 g/l at 30 °C compared to 0.016 g/l at 25 °C).

Although there is not an improvement in clavulanic acid titre obtained when using a dual temperature profile, if clavulanic acid is harvested at the end of a bioreactor run then this profile would have obvious benefits.

It can therefore be confirmed that an individual temperature profile would be ideal for maximising biomass production and minimising product degradation within batch cultures. If this were to be employed on an industrial level, then there would be a need for an automated feed-back system to enable the switch in incubation temperatures to occur at the correct stage in the culture life cycle based, for example, on changes in the alkali usage rate for pH control.
5.0 CYCLIC FED BATCH CULTURE (CFBC)

5.1 INTRODUCTION

Cyclic Fed Batch Culture is a continuous culture system which can be exploited for the production of growth dissociated secondary metabolites. It is a system which allows the growth rate of the culture to be controlled at varying rates between two specific values (Pirt 1974, Gray et al., 1987). It has been previously reported that Cyclic Fed Batch Culture can significantly enhance erythromycin production by *Saccharopolyspora erythraea* through the down regulation of growth and protein synthesis rate (Lynch and Bushell 1995). As down regulation of growth rate is the trigger for antibiotic production in CFBC, there is no need for nutrient exhaustion as in batch cultures. This means that the culture remains viable and many cycles can be performed. The CFBC has been employed to investigate the performance of *S.clavuligerus* under these conditions with the aim of increasing clavulanic acid titre.

This study was used to determine the effect of a number of parameters on *S.clavuligerus* in CFBC. These included, the effect of impeller design on morphology and productivity, the effect of dilution rate and the variations in titre observed with changes in growth limiting substrate concentration. Cyclic fed batch cultures were examined for clavulanic acid titre, biomass, growth limiting substrate concentration and morphology. The levels of DNA in the culture supernatant were also measured as an indication of the level of hyphal breakage that was occurring throughout a CFBC cycle.
As the phosphate limited medium was previously shown to give the highest specific clavulanic acid titre (g·g⁻¹ biomass⁻¹) in chapter 3, this was chosen for investigation in CFBC culture. Phosphate analysis was carried out throughout the CFBC cycles in order to confirm that phosphate limitation was maintained.

5.2 RESULTS
EFFECT OF IMPELLER DESIGN

Many publications since the 1950’s have been devoted to the study of morphophology of filamentous organisms. Factors thought to influence morphology include strain type, engineering variables and nutritional effects. Amongst the engineering variables thought to affect morphology, mixing and shear stresses are probably the two most reported.

Optimal impeller design is vital to ensure that sufficient mixing occurs within a bioreactor and that the transfer of nutrients and oxygen is maximised. However, it is also crucial that this is achieved with the minimum of damage to fragile cells. There are many different impeller designs which have very different performance capabilities. Some have excellent mixing characteristics but create high shear stresses within the bioreactor. This may make certain impellers suitable for particular processes but unsuitable for others (Crueger and Crueger 1989). Impellers that have superior mixing characteristics but cause filamentous microbial cells to fragment are not suitable as they can seriously reduce the culture viability.
This study compared the performance of two impeller designs used in CFBC.

5.3 RUSHTON IMPELLERS

Figure 32. A Photograph of Rushton Impellers

The following table displays results taken from representative CFBC cycles at a range of dilution rates. The fresh medium feed was turned on after 20 hours as this was the point at which the first increase in biomass was noted. Two cycles were allowed to proceed before samples were taken for analysis as this was when biomass concentration remained constant, and the growth limiting substrate (phosphate) was no longer detected. It was assumed that quasi steady state conditions were then achieved.
Fig 33. Results of CFBC with Rushton impellers - Results of 1 cycle at each dilution rate.

Minimum volume (Vmin) = 0.42 l Maximum volume (Vmax) = 1.5 l

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Flow rate (L/hr)</th>
<th>Cycle Time (hr)</th>
<th>Max clav conc (g/l)</th>
<th>Max specific clav conc (g gbiomass⁻¹)</th>
<th>Clav concⁿ expressed as % of Batch control</th>
<th>Specific clav concⁿ expressed as % of Batch control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pltd Batch Control</td>
<td>-</td>
<td>-</td>
<td>0.03</td>
<td>0.016</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pltd CFBC 0.025-0.007</td>
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<td>0.02</td>
<td>0.012</td>
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<td>75</td>
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<tr>
<td>Pltd CFBC 0.055-0.0154</td>
<td>0.0231</td>
<td>46.75</td>
<td>0.0142</td>
<td>0.007</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td>Pltd CFBC 0.09-0.0252</td>
<td>0.0378</td>
<td>28.57</td>
<td>0.01</td>
<td>0.006</td>
<td>33</td>
<td>38</td>
</tr>
</tbody>
</table>

5.3.1. DNA RELEASE (HYPHAL BREAKAGE) RESULTS

A CFBC with a dilution rate of 0.025-0.007 h⁻¹ was examined to determine the concentration of DNA present in the supernatant. This was found to be upon average 2.4 µg/ml and despite the changing volume throughout the CFBC cycle, this concentration did not fluctuate more than 0.2 µg/ml at any given sample point indicating that the rate of fragmentation remained constant throughout a CFBC cycle.
Three dilution rates were examined in this study which represented low (0.025-0.007 h\(^{-1}\)), medium (0.055-0.0154 h\(^{-1}\)) and high (0.09-0.0252 h\(^{-1}\)) dilution rate ranges. Unfortunately, these dilution rate ranges were not capable of producing clavulanic acid titres that were equal to, or an improvement on the titres obtained from the batch control (Fig 33). Despite this, it was evident that the lowest dilution rate range of 0.025-0.007 h\(^{-1}\) was capable of supporting the greatest maximum clavulanic acid titre when compared to the other dilution rates that were examined. However, at 0.02 g/l, this was only 67% of the titre obtained with batch culture. It was also evident that by increasing the dilution rate, clavulanic acid titres decreased. At the highest dilution rate range of 0.09-0.0252 h\(^{-1}\), only 33% of clavulanic acid was obtained in comparison to the yield obtained from the batch control, and only 50% of the titre obtained at the lowest dilution rate range.

Lynch and Bushell (1995), found that by using *S. erythraea*, erythromycin titres of 180% of batch value could be achieved using CFBC with a low dilution rate, and titres of 113% of batch value when a medium dilution rate was used. When they examined performance of CFBC with a high dilution rate, only 88% of batch titre could be produced.
Evidence has been obtained that antibiotic production is associated with a down-regulation of growth and hence intracellular protein synthesis rate (Wilson and Bushell 1995). Induction of antibiotic production occurred if a minimum ratio of charged to uncharged tRNA was obtained (when uncharged tRNA accumulated). In CFBC, which allows the growth rate of the organism to be manipulated in the absence of substrate exhaustion, the protein synthesis rate increased at the start of each cycle, then decreased throughout the remainder of the cycle (Bushell, Smith and Lynch 1997).

As in batch culture, the antibiotic synthesis rate had an inverse relationship with growth rate, increasing during the phase of decreasing growth rate and decreasing only when the growth rate increased. Although in this study the dilution rate and hence growth and protein synthesis rate would have decreased throughout the CFBC cycle at a high dilution rate, the dilution rate still appeared to be too high for significant secondary metabolite formation. This indicates that maximum clavulanic acid concentrations can be obtained when low to medium dilution rate ranges are used.

When using S. clavuligerus in CFBC with Rushton impellers many factors were observed that may account for the organism's apparent poor performance in comparison to that of batch culture. When the bioreactor was emptied to its minimum volume (Vmin) of 0.42 l, severe hyphal fragmentation and loss of culture viability were detected. In order to investigate this further, biomass samples were taken at intervals throughout the CFBC cycles and washed in sterile saline to remove excess medium.
Samples of the biomass were then photographed using bright field microscopy, whilst other samples were stained with the BacLight viability kit, a novel two-colour fluorescence assay which would enable the general viability of the culture to be determined. This method has been successfully used to examine physiological states of many organisms. These have included, *Salmonella typhimurium* cells under starvation-survival conditions in artificial seawater (Joux et al., 1997), *Heliophrya chapmani* (Fox et al., 1987), *E.coli* (Lebaroni et al., 1998, Roth et al., 1997) and has also been used to investigate glutamate-induced neuronal death (Ankarcrona 1995). The results of the morphological analysis confirmed what had been previously suspected and were as follows (35 - 40).
Figure 35. *S. clavuligerus* at the start of a cycle.

**Bright Field**

Figure 36. Baclight.
Figure 37. *S. clavuligerus* at the end of a cycle.

**Bright field**

Fig 38.

**Baclight**
Fig 39. *S. clavuligerus* after several CFBC cycles.

**Bright Field**

Fig 40.

**Baclight**
It is apparent from the morphological analysis that as the CFBC cycle progressed, the normal viable hyphae were broken into very small fragments. These fragmented hyphae tended to clump together to form pellets after a few cycles had taken place. Pellets have been previously reported in the literature to have devastating effects on antibiotic production as the mass transfer of oxygen and nutrients are restricted to the cells present at the centre of the dense pellet (Moore and Bushell 1997). As a result of the pellet formation, clavulanic acid production was poor, and the constant fragmentation seriously reduced the culture viability to a point where it could only survive a maximum of two cycles at any dilution rate. Factors contributing to hyphal damage in bioreactors have been extensively reviewed by Bushell (1988) and Prosser and Tough (1991). Most of the available information has been obtained during studies on fungal cultures, however, the leakage of intracellular metabolites as a result of bioreactor agitation has been described in *Streptomyces* liquid cultures (Tanaka *et al.*, 1975).

Furthermore, it has been reported that antibiotic production in liquid culture is correlated with mycelial fragment diameter in actinomycete cultures (Martin and Bushell 1996, Bushell, Dunstan and Wilson 1997). These studies implied that although mycelial fragments with a diameter of less than 80-90 µm are capable of growing at the same rate as larger particles, they are incapable of significant antibiotic production. If this is the case, then *S.clavuligerus* hyphae that have been reduced to a diameter of less than 80-90 µm in CFBC, may not be capable of clavulanic acid production. This may account for the poor antibiotic titres obtained in CFBC with Rushton impellers.
In an attempt to quantify the level of fragmentation that was occurring during CFBC, the concentration of DNA that was present in the culture medium was quantified. It was assumed that as the cells were subjected to fragmentation, they would break open and their DNA would be released into the medium. When the Rushton impeller blades were used, there was on average 2.4 µg/ml of DNA released into the medium. This concentration could be compared to those obtained with other impeller configurations to determine the extent of the shearing effect.

In an attempt to overcome the fragmentation, several changes to the CFBC setup were made. When the bioreactor was emptied to its minimum volume (Vmin), the top Rushton blade would be partially above the surface of the culture and severe shearing and splashing at the Vmin culture surface was noted. To reduce this, the distance between the top and bottom impellers was reduced from a distance of 65 mm to 35 mm. Unfortunately, by reducing the distance between the two impellers, when the bioreactor filled to its maximum volume of 1.5 litres, there was then insufficient agitation at the top half of the culture, which created “dead spaces”. This was not desirable as it prevented sufficient oxygen and nutrient transfer to all of the culture and so again, antibiotic production was poor.

The minimum volume of the CFBC was then raised from 0.42 l to 1 l so that the Vmin did not fall below the level of the top Rushton impeller blade. This would stop the fragmentation that occurs when the culture volume increases and reaches the level of the top Rushton impeller.
However, by implementing this, the working volume of the bioreactor had been reduced to only 500 mls. This volume did not allow for sufficient changes in dilution rate and for optimal down regulation of growth/protein synthesis rate and therefore clavulanic acid production to occur. Although there was a small improvement made regarding fragmentation with this arrangement, there was no improvement noted in antibiotic production. Due to fragmentation, *S. clavuligerus* in CFBC was only able to survive for 2 cycles (Fig 34). After this period, the viability of the culture was reduced to a level where it was impossible for the hyphae to recover and they were washed out of the system. This effect has been investigated in previous studies which have also found that mechanical shear can seriously reduce culture viability of *Idriella bolleyi* (Jadubansa 1994). The viability of cells in this study were also reduced to a point where they were no longer able to recover and proliferate after being exposed to great shear forces in batch culture.

Belmar-Beiny and Thomas (1991), observed the effect of stirrer speed on morphology of *S. clavuligerus* in batch culture. They concluded that despite observing changes in morphology with increases in shear stresses, there was no effect on clavulanic acid production. These results have since been disputed by Martin (1994), who concluded from the results of Belmar-Beiny and Thomas that a difference in clavulanic acid production was clearly evident and that clavulanic acid production can be effected by morphological changes induced by shear stresses.

From these results it appeared that Cyclic Fed Batch Culture with Rushton impellers was unsuccessful for the growth of *S. clavuligerus* and for the production of clavulanic acid.
In an attempt to overcome fragmentation, Rushton impellers were substituted with Pitched blade impellers. It was claimed that when the blades were positioned at a 45° angle to each other, optimal culture agitation and oxygen transfer could be achieved with minimal damage to the shear sensitive cells.

Figure 42 shows one complete CFBC cycle at a low dilution rate range of 0.025-0.007 h⁻¹. The fresh medium feed was turned on after 20 hours of growth in batch culture, as this was the point in which the first increase in biomass was noted. Two CFBC cycles were allowed to proceed before samples were taken for analysis to allow for the establishment of steady state conditions necessary for CFBC. This was also necessary for the culture to become phosphate limited and to remain so for the proceeding cycles.
PHOSPHATE LIMITED CFBC CULTURE OF *S. clavuligerus*
(DILUTION RATE RANGE 0.025-0.007 h⁻¹)

PITCHED BLADE IMPELLERS

<table>
<thead>
<tr>
<th>CYCLE TIME = 102.86 h</th>
<th>MINIMUM VOLUME</th>
<th>MAXIMUM VOLUME</th>
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</thead>
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<td>0.007 h⁻¹</td>
</tr>
<tr>
<td>WORKING VOLUME</td>
<td>0.42 l</td>
<td>1.5 l</td>
</tr>
<tr>
<td>FLOW RATE</td>
<td>0.0105 l/h</td>
<td>0.0105 l/h</td>
</tr>
</tbody>
</table>

(Fig 42)

PHOSPHATE LIMITED CFBC CULTURE OF *S. CLAVULIGERUS*
(DILUTION RATE RANGE 0.025-0.007 h⁻¹)

![Graph showing biomass and clavulanic acid production over run hours]

Clav acid g/l vs Run Hours
Biomass g/l vs Run Hours

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Throughout the CFBC cycle, biomass values remained constant with changing dilution rates indicating that steady state conditions were obtained (Fig 42). Clavulanic acid production increased towards the end of the cycle where it peaked at 0.058 g/l. This was almost double the maximum clavulanic acid titre obtained with batch culture. It was also noted that there was a great improvement in specific clavulanic acid production in comparison to that obtained from batch culture. The maximum specific clavulanic acid concentration obtained in a Pltd batch reached 0.016 g·g \text{biomass}^{-1}. In comparison, CFBC produced a maximum specific clavulanic acid concentration of 0.041 g·g \text{biomass}^{-1} (Fig 43). This was an increase in specific clavulanic acid of 156%.
Clavulanic acid titre was observed to increase towards the end of a cycle where the dilution rate was at its lowest, as clavulanic acid production appears to increase linearly when the dilution rate reaches 0.0085 h\(^{-1}\) (71 hours) to 0.007 h\(^{-1}\). These results possibly indicate that clavulanic acid production is being stimulated by a down regulation in both protein synthesis and growth rate which would confirm that clavulanic acid is a typical secondary metabolite being produced under growth dissociated conditions.

Clavulanic acid production has also been proven to be stimulated via a down regulation in growth rate from 0.05 to 0.022 h\(^{-1}\) in a 2-stage chemostat (Ives and Bushell 1997). This system was successful in utilising the residual ammonia from substrate limitation and provided a disproportionate increase in the rate of clavulanic acid production. This non-stoichiometric increase in antibiotic production is also consistent with findings in other studies where growth rate is down regulated. These include Bushell et al., (1997) and Lynch and Bushell (1995) with studies on S. erythraea, the production of Tylosin in CFBC (Gray and Vu Trong 1986) and the optimisation of a two stage recombinant E.coli fermentation process (Hortacsu et al., 1991).

Although clavulanic acid production can be stimulated by low growth rates, if dilution rate is reduced too far then extreme nutrient exhaustion will occur. At this point, the organism’s maintenance energy requirements will not be met and the biomass would be washed from the system.
PLTD CFBC CULTURE OF *S. clavuligerus*
(DILUTION RATE 0.055-0.0154 h⁻¹)

PITCHED BLADE IMPELLERS

<table>
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<tr>
<th>CYCLE TIME = 46.75</th>
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<tr>
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<tr>
<td>WORKING VOLUME</td>
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<tr>
<td>FLOW RATE</td>
<td>0.0231</td>
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</tr>
</tbody>
</table>

(Fig 44)

PLTD CFBC CULTURE OF *S. clavuligerus*
(DILUTION RATE 0.055-0.0154 h⁻¹)

![Graph showing biomass and clav production over run hours.](image-url)
The performance of *S. clavuligerus* was investigated at medium dilution rates ranging from 0.055-0.0154 h\(^{-1}\). Fig 44 shows a representative cycle carried out at this range.

Biomass concentration remained relatively constant throughout the changing dilution rates ranges, and only fluctuated between 1.38 g/l and 1.4 g/l (Fig 44). Clavulanic acid production slightly increased with the fall in dilution rate, the maximum clavulanic acid yield of 0.045 g/l being detected at the end of the CFBC cycle. Although the maximum clavulanic acid titre obtained was greater than that obtained from batch culture (0.045 g/l compared to 0.03 g/l from batch), the titre obtained was lower than had been obtained previously with the low dilution rate range of 0.025-0.007 h\(^{-1}\).
PLTD CFBC CULTURE OF *S. clavuligerus*
(DILUTION RATE 0.09-0.0252 h\(^{-1}\))

PITCHED BLADE IMPELLERS

<table>
<thead>
<tr>
<th>CYCLE TIME = 28.57</th>
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<th>MAXIMUM VOLUME</th>
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<tr>
<td>DILUTION RATE</td>
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<td>0.0252</td>
</tr>
<tr>
<td>WORKING VOLUME</td>
<td>0.42</td>
<td>1.5</td>
</tr>
<tr>
<td>FLOW RATE</td>
<td>0.0378</td>
<td>0.0378</td>
</tr>
</tbody>
</table>

PLTD CFBC CULTURE OF *S. clavuligerus*
(DILUTION RATE 0.09-0.0252h\(^{-1}\))

(Fig 46)
Figure 46 shows a representative CFBC cycle at a high dilution rate range of 0.09-0.0252 h\(^{-1}\). It can be seen that the biomass concentration remained constant throughout the cycle indicating that steady state conditions were obtained. Clavulanic acid production was low when compared to the lower dilution rate ranges and only produced a maximum clavulanic acid titre of 0.03 g/l, which was similar to that obtained from batch culture.

The specific clavulanic acid concentration increased with decreasing dilution rate (Fig47), a trend which has been observed in CFBC at all dilution rate ranges examined, indicating that antibiotic production is most certainly triggered by a down regulation of the organism’s growth and presumably protein synthesis rate. Maximum clavulanic acid titre obtained at the high dilution rate ranges (0.03 g/l) was almost 50% less than the maximum antibiotic titre that had been obtained at the low dilution rate ranges (0.058 g/l).
The concentration of DNA released into the supernatant was examined from one cycle of a CFBC at a dilution rate range of 0.025-0.007 h$^{-1}$. When using the Pitched blade impellers, the DNA concentration in the culture supernatant was on average 0.3 µg/ml, and was only 12% of the levels that were observed with the Rushton impellers (2.5 µg/ml). This would indicate that the level of shearing was significantly reduced with a change in impeller design and would add confidence to the use of pitch blades in hyphal fermentations especially where fragile cells are being used. It was also noted that the level of DNA released throughout a cycle showed slight fluctuations. The concentration of DNA detected in the culture supernatant peaked at 30 hours where 0.6 µg/ml DNA was present (Fig 48). It is possible that this ‘peak’ in DNA released coincides with culture volume reaching the level of the top impeller blade. This causes temporary splashing at the culture surface until the culture volume increases above the blade. This phenomenon may not have been so apparent during cycles carried out using Rushton impellers as the concentration of DNA being released was so high that it masked any slight fluctuations. Despite this effect which would be expected in any CFBC regardless of impeller design, Pitch blade impellers drastically improved culture morphology and viability which enabled excellent performance of *S. clavuligerus* in CFBC.
Morphology and viability of *S. clavuligerus* throughout a CFBC cycle using Pitch blade impellers are shown below (49 - 52).

Figure 49. *S. clavuligerus* at the start of a cycle

**Bright Field**

![Bright Field Image](image)

Figure 50. *S. clavuligerus* at the start of a cycle

**Baclight**

![Baclight Image](image)
Figure 51. *S. clavuligerus* at the end of a cycle

**Bright Field**

![Bright Field Image]

Figure 52. **Baclight**

![Baclight Image]
It can be seen that hyphal morphology remained filamentous throughout CFBC and that the cells maintained their viability even after several cycles (Figures 49 - 52), indicating that Pitched blade impellers had drastically reduced fragmentation in CFBC. The following shows a comparison of clavulanic acid titres that were obtained in CFBC with Pitched Blade impellers over a range of dilution rates (Figure 53).

**Clavulanic acid concentration in a single CFBC cycle with different growth rate ranges in \textit{S. clavuligerus}.**

(Fig 53)

It can be observed in figure 53 that clavulanic acid concentration varied with growth rate range for the three CFBC cultures of \textit{S.clavuligerus} that were examined. The lowest dilution rate produced the highest concentration of clavulanic acid, whilst the highest dilution rate produced the lowest clavulanic acid titre. Product concentration did not only vary with dilution rate but also varied throughout an individual CFBC cycle. At the early stages of a cycle there was little or no increase in product concentration, whilst concentration increased during the latter stages.
This could probably be due to the shift up in nutrient availability that occurred as a result of an increase in dilution rate and a slight metabolic disturbance within the cells at the start of the cycle. On adjustment to the nutrient environment and a subsequent decrease in dilution rate, the clavulanic acid concentration increased for the remainder of the cycle and hence greater clavulanic acid production was observed towards the end of the cycle.

As the dilution rate decreases in CFBC, the protein synthesis rate of the organism has been proven to fall (Bushell, Smith and Lynch 1997). The pattern of protein synthesis rate observed during a CFBC cycle provides evidence that down regulation of protein synthesis rate results in the initiation of antibiotic production, a concept first developed by Wilson and Bushell (1995). The study found that a down regulation of protein synthesis rate would only effect initiation of secondary metabolism if it results in a significant increase in uncharged tRNA. This observation supports the assertion of Ochi (1987) that ppGpp probably plays a crucial role in initiation of antibiotic synthesis, since accumulation of uncharged tRNA initiates production of ppGpp (leading to the stringent response, Lamond and Travers 1985).

Varying erythromycin concentration within CFBC cycles was also observed in CFBC studies carried out by Lynch (1995). Erythromycin concentration peaked at the end of a CFBC cycle where the dilution rate was at its lowest, and then fell at the start of a new cycle where dilution and growth rate was up regulated.
The following table is a summary of the clavulanic acid productivity data obtained with both Rushton and Pitched blade impellers (Figure 54).

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>Maximum clavulanic acid concentration (mg l(^{-1}))</th>
<th>Clavulanic acid overall specific productivity (mg g(^{-1}) h(^{-1})) (^*)</th>
<th>Clavulanic acid gravimetric productivity in 1.5 l (mg h(^{-1})) (^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch (Pitched Blade)</td>
<td>0.030</td>
<td>0.580</td>
<td>0.630</td>
</tr>
<tr>
<td>CFBC (Rushton)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025-0.007 h(^{-1})</td>
<td>0.020</td>
<td>0.300</td>
<td>0.220</td>
</tr>
<tr>
<td>0.055-0.0154 h(^{-1})</td>
<td>0.142</td>
<td>0.470</td>
<td>0.330</td>
</tr>
<tr>
<td>0.09-0.0252 h(^{-1})</td>
<td>0.010</td>
<td>0.580</td>
<td>0.400</td>
</tr>
<tr>
<td>CFBC (Pitched Blade)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025-0.007 h(^{-1})</td>
<td>0.058</td>
<td>0.490</td>
<td>0.680</td>
</tr>
<tr>
<td>0.055-0.0154 h(^{-1})</td>
<td>0.045</td>
<td>0.770</td>
<td>1.080</td>
</tr>
<tr>
<td>0.09-0.0252 h(^{-1})</td>
<td>0.030</td>
<td>1.000</td>
<td>1.210</td>
</tr>
</tbody>
</table>

\(^*\) \{[titre (g l\(^{-1}\)) at V_{max} \times V_{max}] - [titre (g l\(^{-1}\)) at V_{min} \times V_{min}]\}/\{biomass conc x cycle time\}.

\(^\dagger\) \{[titre (g l\(^{-1}\)) at V_{max} \times V_{max}] - [titre (g l\(^{-1}\)) at V_{min} \times V_{min}]\}/\{cycle time\}.

It can be observed in figure 54 that the greatest maximum clavulanic acid concentration was obtained in CFBC with Pitch blade impellers at low and medium dilution rate ranges. In comparison to a phosphate limited batch control, CFBC using Rushton impellers resulted in a reduction in the maximum clavulanic acid concentration that was obtained at all the dilution rates examined.
Overall specific productivity and gravimetric productivity both increased with increasing dilution rates. Specific productivity was respectively 52%, 81% and 100% of the batch culture value at the low, medium and high dilution rate ranges with Rushton impellers, and 84%, 132% and 172% of batch value with Pitched blade impellers.

However, the parameter with the most commercial significance is gravimetric productivity, which indicates the concentration of clavulanic acid produced per hour. CFBC gravimetric productivity values were respectively 35%, 52% and 63% of batch culture value at low, medium and high dilution rate ranges using Rushton impellers, whilst with Pitched blade impellers, 108%, 171% and 192% of batch culture value was obtained. This represents 1.21 mg antibiotic h⁻¹ produced in CFBC compared to 0.63 mg antibiotic h⁻¹ produced in batch culture.

Despite the observation that maximum clavulanic acid concentration decreased with CFBC cycle time, overall specific and gravimetric productivity increased (Fig 54). This indicates the overriding economic importance of process rate compared to the significant yield increases obtainable in this system.
5.5 STABILITY OF *S. clavuligerus* IN CFBC

5.5.1. Production of 'mutants'

In order to determine the stability of *S. clavuligerus* in CFBC, a culture was set up at the dilution rate range 0.025-0.007 h\(^{-1}\) and allowed to proceed for several cycles (Figure 55).

![Graph](image_url)

Fig 55. PHOSPHATE LIMITED CFBC CULTURE OF *S. clavuligerus*  
(DILUTION RATE 0.025-0.007 h\(^{-1}\))

The results indicate that there were slight fluctuations in biomass concentration over the 6 cycles analysed. The biomass concentration tended to drop toward the end of each cycle which was probably due to the fall in dilution rate. The severe lack of nutrients at the end of each cycle resulted in the cell maintenance energy requirements not being entirely met. This in turn resulted in a reduction in cell number. However when a new cycle began, the dilution rate was increased again, and this enabled the biomass concentration to recover. However in general, overall biomass concentration remained relatively constant over several cycles and was not significantly reduced with each proceeding cycle as with the Rushton impellers.
Quasi steady state conditions (Pirt, 1974) were observed after the 2nd cycle where biomass concentration remained relatively constant, and the growth limiting substrate (phosphate) was no longer detected.

Clavulanic acid production displayed an inverse relationship with biomass concentration in CFBC. Unlike biomass concentration which tended to decrease with a decrease in dilution rate, clavulanic acid concentration increased towards the end of a cycle where dilution rate was at its lowest. This can be attributed to the down regulation in growth and protein synthesis rate that occurs at the end of a cycle. Clavulanic acid concentration fell slightly at the start of a new cycle where dilution rate was increased once again.

It is also apparent that there is an increase in product concentration with each cycle number (approx 0.01 g/l per cycle) (Fig 55). This can be partially accounted for by the carryover effect of 0.42 l of culture from the previous cycle (containing clavulanic acid) being present at the start of each new cycle. However even when taking this into consideration, an increase in product formation is still observed. This phenomenon was also observed in studies carried out by Lynch and Bushell (1995), who predicted that an increasing proportion of the culture could adapt to CFBC with each cycle. This may result in an enhanced ability of the culture to respond to the constantly changing conditions by more rapid initiation of antibiotic production. If this is the case, then prolonged exposure to CFBC may result in the selection of a ‘mutant’ strain of the organism which may be perfectly adapted to conditions in CFBC and produce greater quantities of antibiotic when compared to the wild type.
5.5.1. PRODUCTION OF 'MUTANTS'

It has been suggested that throughout many CFBC cycles, an increasing quantity of the culture may become well adapted to CFBC conditions and may be capable of greater antibiotic production. Unlike chemostat culture where the organism is subjected to exponential growth conditions for long periods of time, CFBC provides a continual culture system with a continual down-shift in growth rate. As a result of this, unlike chemostat culture, it would be unlikely for a CFBC system to select for non-producing 'mutants'. This effect was examined over several cycles in a Plt CFBC at 0.025-0.007 h⁻¹.

RESULTS

At various points throughout the CFBC, samples were taken and hyphal morphology was examined (Figure 56 - 60)

Fig 56. Morphology at the start of CFBC
Fig 57. Morphology at cycle 3

Fig 58. Morphology at cycle 4
Fig 59. Morphology at cycle 5

Fig 60. Morphology at cycle 6
Upon examination of the culture it became apparent that there were morphological changes occurring as the CFBC cycles progressed. The majority of the initial culture consisted of long unbranched hyphae that represented a usual *S. clavuligerus* culture. During subsequent cycles, the majority of the hyphae became much shorter in length, and more highly branched (Fig 57).

These changes in morphology could be explained by the selection of cells that are more suitably adapted to survival in the CFBC system. It has been shown earlier in this chapter that despite switching to pitched blade impellers, some hyphal fragmentation will always take place in CFBC because of the nature of the system with its continual changing volume. It is possible that the short hyphal particles which are present in small quantities at the start of a CFBC, are less prone to breakage from the impeller blades, and so do not suffer as much damage from the impellers. If these particles have an advantage in terms of survival then they would survive to seed the following cycles and eventually, these particles would form the majority of the culture. Hyphae that were better suited to CFBC conditions would then have been selected.

A sample of the CFBC culture after six cycles was taken and stocks were made from a single colony isolate. The new isolate (SPM), was compared in Pltd CFBC to the wild type. Shown below are representative CFBC cycles at a dilution rate of 0.025-0.007 h⁻¹. These cycles represent the first cycles where quasi steady state conditions were reached.
Fig 61. The performance of wild type *S. clavuligerus* in CFBC.

<table>
<thead>
<tr>
<th>Run Hours</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>102</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific clav</td>
<td>0.033</td>
<td>0.036</td>
<td>0.042</td>
<td>0.039</td>
<td>0.044</td>
<td>0.044</td>
</tr>
</tbody>
</table>
It can be noted that SPM performed well in CFBC and produced more biomass and clavulanic acid in comparison to the wild type (Fig 61 & 62). Both organisms displayed the same trends that had been observed in previous CFBC, where biomass slightly decreased towards the end of a cycle, and was inversely related to clavulanic acid production which slightly increased.
Biomass concentration from the SPM CFBC reached a maximum of 1.3 g/l during the early part of the cycle (Fig 62). The maximum biomass obtained from the wild type CFBC was 1.2 g/l although this fell to 1 g/l throughout the majority of the cycle (Fig 61). Because of the slightly greater concentration of biomass in the SPM CFBC, there was a greater quantity of clavulanic acid produced. When specific production of the two CFBC were compared, the concentrations of clavulanic acid produced per gram of biomass were almost identical. This indicates that it is simply the cell density in the SPM culture that is responsible for the increase in yield of clavulanic acid. As SPM was already present in its shortened highly branched form at the very start of the CFBC, it is likely that its cells were already suited to CFBC conditions and so more of the biomass survived to produce clavulanic acid. The wild type, was perhaps only beginning to select for suitable hyphae, and so a proportion of this culture was still undergoing fatal damage and would have been washed out the system or broken into fragments incapable of antibiotic production. As a result, the wild type CFBC would have a lower biomass than would have been expected from the SPM mutant.

The cell density of *S. clavuligerus* has been shown to influence antibiotic biosynthesis in a study carried out by Sanchez and Brana (1996). They postulated that a rapid activation of secondary metabolite formation was favoured by a high inoculum density. This was thought to be due to the production of a conditioning factor that is able to accelerate the acquisition of antibiotic biosynthetic capacity. As each CFBC cycle is inoculated with culture from the previous cycle, it may be possible that if there are more cells carried over to seed the next cycle, the initiation of clavulanic acid production may occur earlier on in the cycle and so allow more time for the accumulation of clavulanic acid.
Although the biochemical differences between the wild type and SPM were not investigated in this study, a previous study carried out by Pickup and Bushell (1995), found that non-fragmenting variants of *Streptomyces* hyphae have enhanced activity of an enzyme phospho-N-acetylmuramyl pentapeptide translocase. This enzyme catalyses the first membrane bound reaction of cell wall synthesis. By using a hyphal enrichment process, a strain was selected that possessed increased translocase activity and whose cell wall had the mechanical strength necessary to avoid fragmentation. It could be possible that the SPM non-fragmenting mutant may also show an increase in activity of this enzyme.

It can be concluded that CFBC can be successfully used to select for organisms that are most suitably adapted to a particular set of CFBC conditions. Because of the changing dilution rate, unlike chemostat, it is unlikely that non-producing mutants will be favoured.
5.6. ENHANCED CFBC

The concentration of the growth limiting substrate was investigated to determine its effect on growth and production of wild type *S. clavuligerus* in CFBC. The growth limiting substrate (phosphate) was increased from 0.1 g/l to 0.5 g/l, and its effects were examined in CFBC at 0.025-0.007 h⁻¹.

Fig 63. Graph to show clavulanic acid titres obtained at 0.1 g/l and 0.5 g/l phosphate.
Fig 64. Graph to show a comparison in biomass obtained at 0.1 g/l and 0.5 g/l phosphate.

![Graph showing biomass comparison](image1)

Fig 65. Graph to show a comparison of specific clavulanic acid production 0.1 g/l and 0.5 g/l phosphate.

![Graph showing specific clavulanic acid production](image2)
Increasing the growth limiting substrate concentration to 0.5 g/l in CFBC resulted in an increase in both clavulanic acid and biomass concentration (Fig 63 & 64). Upon average, the biomass and clavulanic acid concentration from the CFBC containing 0.5 g/l of phosphate was approximately double the concentration that was obtained at a phosphate concentration of 0.1 g/l. These results highlight one of the significant potentials of the CFBC technique for secondary metabolite production.

In batch culture, the growth limiting substrate is exhausted before antibiotic production is initiated. In order to increase biomass and hence antibiotic production, the growth limiting substrate concentration must be increased. However the time taken for assimilation of growth limiting substrate levels to growth limiting concentrations will increase with increasing growth limiting substrate and so will require longer process times for antibiotic initiation.

As the growth rate decay profile during a CFBC cycle is the same once a quasi steady state has been achieved irrespective of growth limiting substrate concentration, biomass concentration and therefore antibiotic titre may be increased simultaneously by increasing the growth limiting substrate concentration.

When the specific clavulanic acid production is examined, it is clear that g·g biomass⁻¹, both cyclic fed batch cultures are producing equally and yielding approximately 0.04 g·g biomass⁻¹. The two fold increase in titre observed by increasing the phosphate concentration was then most definitely due to the increase in biomass concentration that was obtained with the increase in growth limiting substrate.
Similar effects have also been noted in *S. erythraea* by Lynch and Bushell (1995). Here erythromycin titres were significantly increased with an increase in the carbon source, which in this case was the growth limiting substrate. The increase in antibiotic titre was also due to the increase in biomass that was achieved by increasing the growth limiting substrate concentration from 6 g l\(^{-1}\) to 15 g l\(^{-1}\) glucose.

### 5.7 SUMMARY

CFBC can be successfully used to increase antibiotic production of *S. clavuligerus*. It enables the growth rate profile of an organism to be accurately controlled and optimised to gain maximum antibiotic yield, as well as enabling an increase in growth limiting substrate to increase biomass concentration and hence antibiotic production. CFBC can also be used to select for adapted mutants. This can be used to isolate organisms that are better suited to a set of conditions in comparison to a wild type organism.

The production of antibiotic as a response to growth rate down regulation is compatible with the 'biowars' theory for secondary metabolites proposed by Bushell (1989b). The arrival of a competitor organism in a micro-habitat results in a sufficient down regulation of growth rate of the original inhabitant due to competition for nutrients. This would elicit the production of antibiotics as a defensive measure. If this theory is correct, then the growth rate down regulation profile observed in CFBC is simulating the arrival of a competitor, resulting in initiation of antibiotic production.
6.0. METABOLIC PATHWAY ANALYSIS

The performance of *Streptomyces clavuligerus* in phosphate limited chemostat culture was investigated over the growth rate range of 0.03 to 0.09 h\(^{-1}\). Steady state conditions were achieved after four volume changes, and were monitored over at least one additional volume change. The production of clavulanic acid across the growth rate range is shown in Figure 66.

Fig 66. The growth of *S.clavuligerus* and the production of clavulanic acid in phosphate limited chemostat culture. Culture pH was maintained at pH 6.5 and the vessel working volume was 1 litre.

![Graph showing the relationship between growth rate and clavulanic acid production](image)

The highest specific production of clavulanic acid occurred when the growth rate was 0.03 h\(^{-1}\) and the production of the antibiotic decreased almost linearly as the growth rate increased to 0.09h\(^{-1}\) indicating that growth rate has an effect on clavulanic acid production.
At low growth rates where medium flow into the vessel is very low, the mixing characteristics of the vessel could have a significant effect on the distribution of fresh medium throughout the culture and hence affect biomass concentration. As biomass remained relatively constant across the dilution rate ranges and fluctuated between 0.9 g/l and 1.1 g/l at its highest and lowest points, this effect would not appear to be a significant factor.

6.1 RESIDUAL PHOSPHATE CONCENTRATION

Phosphate concentration was analysed over the dilution rate ranges and was shown to be undetectable at all the dilution rates examined. This indicates that even at a high dilution rate, the culture remained phosphate limited. Phosphate could therefore be confirmed as the growth limiting substrate.
6.1.1 RESIDUAL AMMONIUM CONCENTRATION

Fig 67. Clavulanic acid production and residual ammonium concentration in chemostat culture.

![Graph showing Clavulanic acid production and residual ammonium concentration](image)

The concentration of residual ammonium detected in the supernatant increased with increasing growth rate and decreasing clavulanic acid production (Fig 67). It was always shown to be in excess of the culture's requirements at all of the dilution rates examined, and so indicates that nitrogen limitation did not occur.

It has been found that secondary metabolism can be suppressed by the presence of a high concentration of ammonium in the medium. It is not known exactly how this happens, but it is generally accepted that in most instances, it is repression rather than inhibition of antibiotic synthesising enzymes that is implicated (McDermott et al., 1993, Wilson and Bushell 1995).
This effect has been noted for the production of many antibiotics for example, cephamycin C (Castro et al., 1985), erythromycin (Flores and Sanchez 1985), leucomycin (Tanaka et al., 1981) and tylosin (Tanaka et al., 1986).

Ives and Bushell (1997), correlated the sensitivity of *S. clavuligerus* to ammonium ions with the urea cycle, and proposed that the evolution of a pathway for synthesising an antibiotic using arginine as a precursor was consistent with the ecology of the species. The presence of a urea cycle in prokaryotes is unusual and is required for the removal of ammonia from cells in which toxic intracellular concentrations are likely to occur.

The toxicity of large concentrations of intracellular ammonia probably arises from its ability to raise intracellular pH which can result in inhibition of some metabolic enzymes (Mathews et al., 1997).
6.1.2. RESIDUAL GLYCEROL CONCENTRATION

Fig 68. Clavulanic acid production and residual glycerol concentration in chemostat culture.

The carbon source (glycerol), was provided in excess at all growth rates examined to ensure that carbon limitation did not occur. Although there were fluctuations noted in concentration over the growth rates examined, in general the concentration increased with growth rate and was inversely related to clavulanic acid production.

In many cases the presence of a rapidly utilisable carbon source can result in the repression of antibiotic synthesis. Lebrihi et al., (1988) and Ahoronwitz and Demain (1978), have reported that cephamycin C production in S.clavuligerus can be repressed in the presence of high concentrations of glycerol and starch. In addition, it has also been discovered in one study, that despite glycerol being a direct precursor of clavulanic acid, it will have a negative effect on production when its concentration exceeds 100mM (Romero et al., 1984).
6.2. INTRACELLULAR ANALYSIS - FREE AMINO AND ORGANIC ACID POOLS

Using cell free extracts prepared from freeze dried portions of culture biomass, a profile of the intracellular amino acid and organic acid pools across the growth rate ranges was obtained.

Figure 69. Composition of the intracellular pools of amino acids of *S. clavuligerus* grown in phosphate limited chemostat at different growth rates. (Amino acids expressed as mg of amino acid per mg of culture biomass.)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>0.03</th>
<th>0.04</th>
<th>0.05</th>
<th>0.06</th>
<th>0.07</th>
<th>0.08</th>
<th>0.09</th>
</tr>
</thead>
<tbody>
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<td>ASP</td>
<td>0.000048</td>
<td>0.000025</td>
<td>0.00002</td>
<td>0.000046</td>
<td>0.00005</td>
<td>0.00025</td>
<td>2.3E-06</td>
</tr>
<tr>
<td>GLU</td>
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<td>0.00031</td>
<td>0.00023</td>
<td>0.00019</td>
<td>0.00025</td>
<td>0.00023</td>
<td>0.00015</td>
</tr>
<tr>
<td>SER</td>
<td>0.00002</td>
<td>0.00024</td>
<td>0.00016</td>
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<td>0.00007</td>
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</tr>
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<td>CIT</td>
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<td>0.00013</td>
<td>0.0001</td>
<td>0.00007</td>
<td>0.00007</td>
<td>0.00011</td>
<td>0.0001</td>
</tr>
<tr>
<td>GLY</td>
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<td>5E-07</td>
<td>0.000001</td>
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<td>2.5E-06</td>
<td>1.2E-06</td>
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<td>TYR</td>
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<td>0</td>
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<td>0.00025</td>
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<td>0.00002</td>
<td>0.00005</td>
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<td>0.00001</td>
<td>5E-07</td>
<td>9E-07</td>
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<tr>
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<td>0.00001</td>
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<td>0.00001</td>
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<td>0</td>
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<tr>
<td>Sum</td>
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<td>0.00168</td>
<td>0.00165</td>
<td>0.00154</td>
<td>0.00193</td>
<td>0.00197</td>
</tr>
</tbody>
</table>

140
The results obtained indicated that glutamate and cysteine contributed significantly to the intracellular pools of amino acids (Fig 69). Glutamate constituted 15.6% of the intracellular pool at a growth rate 0.03 h\(^{-1}\) where clavulanic acid production was at its greatest, but was only 7.6% of the intracellular pool at 0.09 h\(^{-1}\) where production was at its lowest. Cysteine concentrations did fluctuate over the dilution rates, although there was no obvious trend.

Fig 70. The percentage of glutamate in the free amino acid pool of *S.clavuligerus* grown in phosphate limited chemostat culture.

<table>
<thead>
<tr>
<th>Growth rate (h(^{-1}))</th>
<th>Total amino acid pool (mg/mg biomass)</th>
<th>Glutamate (mg/mg biomass)</th>
<th>% composition of glutamate in pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.00154</td>
<td>0.00024</td>
<td>15.6</td>
</tr>
<tr>
<td>0.04</td>
<td>0.00183</td>
<td>0.00031</td>
<td>16.9</td>
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<td>0.05</td>
<td>0.00168</td>
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<td>13.7</td>
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<td>0.00165</td>
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<td>0.07</td>
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<tr>
<td>0.08</td>
<td>0.00193</td>
<td>0.00023</td>
<td>11.9</td>
</tr>
<tr>
<td>0.09</td>
<td>0.00197</td>
<td>0.00015</td>
<td>7.6</td>
</tr>
</tbody>
</table>
Glutamate has been identified as a predominant amino acid in the microbial amino acid pool in a number of bacteria. Tempest et al., (1970), proposed that glutamate accounted for between 52% and 89% of the total amino acid content of a wide range of gram positive and gram negative bacteria, whilst Brana et al., (1985), reported that glutamate contributed a major part of the free amino acid pool in exponential phase shake flask cultures of S. clavuligerus grown on 80mM ammonium chloride as the sole nitrogen source. Although the values present here are lower than has been previously reported, different medium compositions will obviously contain different concentrations of nutrients and so have varying effects on the intracellular metabolite pools.

Fig 71. The percentage of cysteine in the free amino acid pool of S. clavuligerus grown in phosphate limited chemostat culture.

<table>
<thead>
<tr>
<th>Growth rate (h⁻¹)</th>
<th>Total amino acid pool (mg/mg biomass)</th>
<th>Cysteine (mg/mg biomass)</th>
<th>% composition of cysteine in pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.00154</td>
<td>0.00035</td>
<td>22.8</td>
</tr>
<tr>
<td>0.04</td>
<td>0.00183</td>
<td>0.00047</td>
<td>25.6</td>
</tr>
<tr>
<td>0.05</td>
<td>0.00168</td>
<td>0.00014</td>
<td>8.3</td>
</tr>
<tr>
<td>0.06</td>
<td>0.00165</td>
<td>0.00020</td>
<td>12.2</td>
</tr>
<tr>
<td>0.07</td>
<td>0.00154</td>
<td>0.00019</td>
<td>12.3</td>
</tr>
<tr>
<td>0.08</td>
<td>0.00193</td>
<td>0.00030</td>
<td>15.5</td>
</tr>
<tr>
<td>0.09</td>
<td>0.00197</td>
<td>0.00050</td>
<td>25.4</td>
</tr>
</tbody>
</table>
Cysteine accounted for a greater percentage on average of the total intracellular pool of metabolites than any other amino acid examined. The concentrations of cysteine did initially appear to fall with an increase in dilution rate, however concentrations increased at the highest dilution rates examined (0.08 & 0.09 \text{ h}^{-1}).

The concentration of glutamate and cysteine were examined in comparison to specific clavulanic acid production.

Fig 72. The effect of growth rate on the specific production of clavulanic acid and the concentration of intracellular glutamate and cysteine in \textit{S.clavuligerus} cultivated in phosphate limited chemostat culture.

It can be noted that in general, glutamate concentration declined with a decrease in specific clavulanic acid production.
As glutamate is a precursor of arginine, which has been shown to directly proceed into the C5 portion of clavulanic acid (Valentine et al., 1993), an increase in availability of this precursor could potentially increase the supply of available arginine for clavulanic acid biosynthesis. In addition, as glutamate is also a precursor of ornithine, the additional flow of metabolites throughout the urea cycle derivatives, could also have a stimulatory effect on clavulanic acid biosynthesis. The effect may be a direct promotion of clavulanic acid biosynthesis, or indirectly via the ability of glutamate to compensate for the drain of other metabolites. This drainage could be into either clavulanic acid biosynthesis, or other cellular biosynthetic pathways. The growth rate mediated control of clavulanic acid biosynthesis could therefore be viewed as a growth rate at which the flow of metabolites favours clavulanic acid formation.

The cysteine pool was also found to fluctuate over the dilution rates examined, but there was not any obvious correlation to specific clavulanic acid production observed. It is possible that the intracellular concentration of cysteine is a reflection of the metabolic activities of the organism at that growth rate, rather than an indicator of clavulanic acid biosynthesis.
Fig 73. Organic Acids expressed as µmole per gram of biomass.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Growth rate (h⁻¹)</th>
<th>0.03</th>
<th>0.04</th>
<th>0.05</th>
<th>0.06</th>
<th>0.07</th>
<th>0.08</th>
<th>0.09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td></td>
<td>1.25</td>
<td>0.98</td>
<td>3.6</td>
<td>1.5</td>
<td>5.2</td>
<td>1.04</td>
<td>0.47</td>
</tr>
<tr>
<td>Malate</td>
<td></td>
<td>4.4</td>
<td>7.4</td>
<td>22</td>
<td>6.7</td>
<td>2.2</td>
<td>4.4</td>
<td>5.9</td>
</tr>
<tr>
<td>Isocitrate</td>
<td></td>
<td>0.4</td>
<td>0.6</td>
<td>0.4</td>
<td>0.8</td>
<td>1</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>0.5</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Pyruvate</td>
<td></td>
<td>2.1</td>
<td>1.85</td>
<td>1.93</td>
<td>1.91</td>
<td>1.83</td>
<td>1.89</td>
<td>1.88</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td></td>
<td>1.3</td>
<td>1.7</td>
<td>1.5</td>
<td>1.9</td>
<td>1.9</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
<td>3.9</td>
<td>3.2</td>
<td>3.2</td>
<td>4</td>
<td>4.4</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>13.45</td>
<td>16.43</td>
<td>32.73</td>
<td>18.61</td>
<td>19.53</td>
<td>19.53</td>
<td>16.65</td>
</tr>
</tbody>
</table>

Fig 74. The total organic acid and clavulanic acid concentration at a range of dilution rates.
It can be observed that despite the dilution rate 0.05 h⁻¹, the total concentration of TCA cycle organic acids remained relatively constant (Fig 74). It may possibly be expected that as the organism's growth rate increases, the concentration of organic acids in the TCA cycle may also increase due to the increase of available carbon. However, metabolism is very complex and strictly regulated so an increase in the availability of carbon may not necessarily mean that there will be a greater flux through the TCA cycle.

6.3 DATA ANALYSIS

With such a large quantity of data, trends and patterns become almost impossible to detect. To facilitate the analysis of this data, cluster analysis was used.

6.3.1 CLUSTER ANALYSIS (Hair et al., 1995)

Cluster analysis is a multivariate technique used to group objects based on a number of characteristics. The degree of similarity between all of the characteristics of each case to those of every other case is calculated. This similarity coefficient is then used to calculate a hierarchical linkage map (a dendrogram), in which the relationship of all cases to each other can be visualised. The resulting cluster of objects should then exhibit high internal (within clusters) homogeneity, and high external (between clusters) heterogeneity. If plotted geometrically, the objects within clusters will be close together and different clusters will be far apart.
One aspect of the application of cluster analysis to experimental data is the sensitivity of distance measures to differing scales or magnitude among the variables. Since there were significant variations in the original data from parameter to parameter, the results were transformed by standardisation. This is achieved by subtracting the mean and dividing by the standard deviation for each variable. The mean and standard deviation of each data set was calculated from each row in the table. The standardised results are shown in Fig 75.

Fig 75. Standardised data.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>0.03</th>
<th>0.04</th>
<th>0.05</th>
<th>0.06</th>
<th>0.07</th>
<th>0.08</th>
<th>0.09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>-0.248366</td>
<td>-0.667004</td>
<td>-0.458397</td>
<td>-0.468544</td>
<td>1.230435</td>
<td>1.483760</td>
<td>0.104547</td>
</tr>
<tr>
<td>Glu</td>
<td>-1.686727</td>
<td>-2.05072</td>
<td>-0.468544</td>
<td>1.230435</td>
<td>1.483760</td>
<td>0.104547</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>-0.778502</td>
<td>0.417733</td>
<td>-0.113927</td>
<td>1.015851</td>
<td>1.647197</td>
<td>-0.911418</td>
<td>-1.276934</td>
</tr>
<tr>
<td>Cit</td>
<td>-1.703434</td>
<td>-0.108245</td>
<td>-0.188004</td>
<td>-0.507042</td>
<td>-0.227884</td>
<td>1.327425</td>
<td>1.407185</td>
</tr>
<tr>
<td>Gly</td>
<td>-0.841969</td>
<td>0.366000</td>
<td>-1.372775</td>
<td>-0.732147</td>
<td>0.604021</td>
<td>1.830367</td>
<td>0.145450</td>
</tr>
<tr>
<td>His</td>
<td>-1.381511</td>
<td>-0.477845</td>
<td>0.736035</td>
<td>1.734114</td>
<td>-0.275531</td>
<td>0.601159</td>
<td>-0.836421</td>
</tr>
<tr>
<td>Arg</td>
<td>0.646799</td>
<td>1.610116</td>
<td>-0.357804</td>
<td>-0.839462</td>
<td>-0.013762</td>
<td>0.619275</td>
<td>-1.665163</td>
</tr>
<tr>
<td>Thr</td>
<td>-0.116204</td>
<td>1.781795</td>
<td>1.103938</td>
<td>0.104547</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>-1.365901</td>
<td>-0.938686</td>
<td>0.717974</td>
<td>-0.814287</td>
<td>-0.078802</td>
<td>1.269587</td>
<td>1.208297</td>
</tr>
<tr>
<td>Pro</td>
<td>0.247156</td>
<td>-1.867399</td>
<td>-0.714006</td>
<td>-0.329541</td>
<td>1.400549</td>
<td>0.439388</td>
<td>0.823653</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.296156</td>
<td>0.357130</td>
<td>1.271731</td>
<td>0.113236</td>
<td>-2.081806</td>
<td>-0.618444</td>
<td>0.661997</td>
</tr>
<tr>
<td>Val</td>
<td>0.001190</td>
<td>0.459476</td>
<td>-0.207121</td>
<td>1.875996</td>
<td>-1.756961</td>
<td>-0.373771</td>
<td>0.001190</td>
</tr>
<tr>
<td>Met</td>
<td>0.362301</td>
<td>0.925880</td>
<td>-0.466492</td>
<td>0.859577</td>
<td>-2.008047</td>
<td>-0.532796</td>
<td>0.859577</td>
</tr>
<tr>
<td>Cys</td>
<td>-0.683174</td>
<td>0.011965</td>
<td>-0.976304</td>
<td>-0.557546</td>
<td>-0.448669</td>
<td>0.447473</td>
<td>2.206256</td>
</tr>
<tr>
<td>Ile</td>
<td>0.699015</td>
<td>1.655857</td>
<td>0.611120</td>
<td>0.171646</td>
<td>-1.091402</td>
<td>-1.146776</td>
<td>-0.909460</td>
</tr>
<tr>
<td>Orn</td>
<td>-1.059967</td>
<td>-0.899540</td>
<td>-0.538578</td>
<td>-0.097402</td>
<td>-0.482570</td>
<td>1.667300</td>
<td>1.346445</td>
</tr>
<tr>
<td>Leu</td>
<td>-1.281915</td>
<td>-1.281915</td>
<td>0.411180</td>
<td>-0.469229</td>
<td>1.088418</td>
<td>0.072561</td>
<td>1.460899</td>
</tr>
<tr>
<td>Glu</td>
<td>-1.668727</td>
<td>-0.205072</td>
<td>-0.458397</td>
<td>-0.486544</td>
<td>1.230435</td>
<td>1.483760</td>
<td>0.104547</td>
</tr>
<tr>
<td>Trp</td>
<td>1.294444</td>
<td>-1.238980</td>
<td>-0.340933</td>
<td>0.075343</td>
<td>0.149800</td>
<td>1.064892</td>
<td>1.584643</td>
</tr>
<tr>
<td>Citrate</td>
<td>-0.744800</td>
<td>-0.729351</td>
<td>0.550194</td>
<td>-0.287697</td>
<td>2.222325</td>
<td>-0.329493</td>
<td>-0.701179</td>
</tr>
<tr>
<td>Malate</td>
<td>-0.958776</td>
<td>-0.420022</td>
<td>2.221188</td>
<td>-0.071803</td>
<td>-0.886504</td>
<td>-0.236057</td>
<td>0.351974</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>-1.323306</td>
<td>-0.833624</td>
<td>-0.996852</td>
<td>0.145739</td>
<td>1.043488</td>
<td>0.472193</td>
<td>1.492363</td>
</tr>
<tr>
<td>Succinate</td>
<td>-0.764443</td>
<td>-0.677903</td>
<td>-0.764443</td>
<td>-0.548091</td>
<td>0.750020</td>
<td>2.120248</td>
<td>-0.115388</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>-1.401297</td>
<td>-1.104640</td>
<td>-0.480830</td>
<td>0.020992</td>
<td>0.395278</td>
<td>1.035724</td>
<td>1.534773</td>
</tr>
<tr>
<td>Urea</td>
<td>-0.857114</td>
<td>0.109605</td>
<td>-1.501594</td>
<td>1.030291</td>
<td>-0.688322</td>
<td>1.521323</td>
<td>0.385811</td>
</tr>
</tbody>
</table>
The standardised data was then plotted for each amino and organic acid at each dilution rate examined (Figure 76).

Fig 76. Plots of amino and organic acids at the chemostat dilution rates examined.

This plotted standardised data enabled trends to be more easily identified and correlations between clavulanic acid and several of the amino and organic acids were more easily observed. Following standardisation, the data was then used to calculate intracellular production rates of the amino and organic acids. These were compared to the clavulanic acid production rates. It was then possible to construct a high resolution dendrogram that provided a graphical representation of the results of the clustering. This dendrogram is shown in Figure 77.
6.3.2. Key to dendrogram terms

1. **Euclidean measure**

   The Euclidean distance is the most common measure of similarity between two objects and is essentially a measure of the length of a straight line drawn between two objects.

2. **Average linkage**

   A single procedure is based on the minimum distance between two individuals and places them in the first cluster. The next shortest distance is then found and so on. The average linkage is a measure of the average distance from all individuals in one cluster to all individuals in another. This method is not affected by extreme values since partitioning is based on all members of the cluster.
Thus those amino/organic acids that were most closely correlated to the clavulanic acid production rate, are those in which the cluster distance (y-axis) is the lowest around the clavulanic acid cluster. These amino/organic acids are threonine, oxaloacetate, asparagine and isoleucine, with threonine being the most strongly correlated with antibiotic production.

6.3.3 EVALUATION OF DENDROGRAM LINKAGE MODEL

The dendrogram identified amino and organic acids that were significantly linked to clavulanic acid production rate. A series of chemostat cultures were undertaken to test the correlation's that had been identified from the dendrogram. Chemostats were operated as previously described at a growth rate of 0.03 h^{-1}. This was the growth rate at which the highest specific rate of clavulanic acid production was observed (Fig 66). The following amino and organic acids were added individually to phosphate limited media at a concentration of 10mM.

- Aspartate
- Isoleucine
- Arginine
- Threonine
- Asparagine
- Ornithine
- Oxaloacetate
The effect of these amino and organic acids feeds on culture biomass concentration and clavulanic acid titre is shown in figure 78.

Fig 78. The effect of amino/organic acid feeds on the production of clavulanic acid in a phosphate limited chemostat operated at a growth rate of 0.03 h⁻¹.

Of the amino and organic acids fed to the culture, aspartate, arginine, ornithine and glutamate were shown to be the most poorly related to clavulanic acid. Experimental results confirm this as these amino acids all resulted in a reduction in clavulanic acid titre when compared to a non-fed control.
Fig 79. The effect of amino acid feeds on the specific productivity of clavulanic acid in a phosphate limited chemostat operated at a dilution rate of 0.03 h\(^{-1}\).

From fig 79, it can be observed that threonine feeding resulted in a 0.024 g/l increase in clavulanic acid titre over the control culture (Phosphate limited with no feeds). This was the greatest increase in titre seen with any of the feeds, and also confirmed the dendrogram correlation as threonine was the most highly correlated to clavulanic acid formation (Fig 77).

All amino acids within the clavulanic acid dendrogram cluster (Thr, Asn, Ileu & Oaa) (Fig 77), resulted in an increase in clavulanic acid titre being produced. Similarly, those which were found outside of the clavulanic acid cluster (Asp, Arg & Glu), were shown to have a detrimental effect of clavulanic acid formation.
The exception to this was ornithine. Despite this amino acid being present in a different cluster from clavulanic acid and therefore not being highly correlated to clavulanic acid formation, feeding ornithine resulted in an increase in clavulanic acid production. However, when the biosynthetic pathway for clavulanic acid is examined (Fig 80), it can be seen that arginine, the C3 precursor to clavulanic acid is also a precursor to ornithine biosynthesis. It is possible that ornithine itself does not feed clavulanic acid, but prevents the flow of arginine into its own biosynthesis, hence leaving more arginine for the formation of clavulanic acid. This indirect effect may account for the increase in clavulanic acid titre obtained in this study.

When evaluating the effects of different feeds upon a culture, it is also important to consider the ease in which a particular feed is taken up into the cell and metabolised. The residual concentration of amino acid feed in the culture medium was analysed to determine if it was being effectively transported into the cell. When the residual concentrations of the amino acids were compared, it was apparent that there was very little difference between the amino acid feeds. It can therefore be assumed that all of the feeds were as efficiently taken up into the cell. This however does not mean that all of the amino acids were as easily metabolised once they were inside the cell.

On the basis of the relative performance of the amino acid feeds in the previous section, a simplified biochemical model was mapped for *S. clavuligerus*. This model, based on existing microbial pathways was used to identify fluxes that are important to clavulanic acid biosynthesis. This model is shown in figure 80.
Fig 80. A simplified pathway model showing the biosynthetic steps that are involved in clavulanic acid biosynthesis. Figures given beside amino acid compartments represent the specific productivity of clavulanic acid (grams per gram of biomass) produced when that amino acid was fed to chemostat culture. The value given for glycerol represents the control productivity achieved in the absence of additional amino acid feeds.
6.3.4 ASPARTATE FEED

Fig 81, displays the effect of a 10 mM aspartate feed on the intracellular profile of free amino acids. All plots show the relative amino acid pool size (µ g / 10 mg biomass) with respect to a non fed control that was considered to be the base line.

The intracellular profile revealed that there was a significant increase in aspartate pool size in cultures fed with aspartate in comparison to phosphate limitation without an amino acid feed. In addition, an increase in the pool sizes of threonine, methionine and to a lesser extent isoleucine could also be observed under these conditions.
Aspartate, asparagine, methionine, threonine and partly isoleucine constitute what is known as the aspartate family of amino acids. Their synthesis is formed from a highly branched pathway, which consists of numerous branch points that are potential sites for regulation of carbon flow. Several patterns of control over this branching pathway have evolved in various organisms.

Aspartate biosynthesis occurs via a glutamate aspartate transaminase which catalyses the conversion of oxaloacetate to aspartate. This reaction is freely reversible. In addition, aspartate synthesis has also been demonstrated to be repressed by aspartate in the medium which represses bulk glutamate-aspartate transaminase activity. Therefore under conditions of excess asparate, carbon flow will be restricted from oxaloacetate in the TCA cycle for the formation of this family of amino acids.

The increase in availability of aspartate in the medium has resulted in an increase in the pool size of aspartate and subsequently methionine, threonine and isoleucine of which aspartate is a precursor. There did not however, appear to be an increase in the pool size of asparagine when compared to the control culture.

On the basis of the pathway shown in fig 80, it may appear that by feeding aspartate the drainage of pyruvate will be alleviated as carbon will not be required for the synthesis of aspartate and subsequent amino acids. As the oxaloacetate-aspartate reaction is freely reversible, the presence of excess aspartate may actually increase carbon flow into the TCA cycle enabling even more carbon to be available for clavulanic acid formation.
Unexpectedly, there was no increase observed in the intracellular pool size of arginine or ornithine under these conditions of excess aspartate. This indicates that the increase in carbon availability in the aspartate family does not pass directly through arginosuccinate and into the urea cycle as may be predicted from the pathway.

Aspartate feeding resulted in a reduction in the titre of clavulanic acid produced (0.009 \text{ g:g biomass}^{-1} \text{ compared to } 0.03 \text{ g:g biomass}^{-1} \text{ obtained from the control culture, fig78}). The possible increase in carbon availability that may have occurred as a result of aspartate feeding may have increased the availability of the C3 precursor by reducing carbon drain from the TCA cycle, but as there was no increase in the availability of the C5 precursor, it is likely that the formation of clavulanic acid was restricted by this.
6.3.5 ISOLEUCINE FEED

Fig 82. The relative amino acid pool size (μ g / 10 mg biomass) of an isoleucine fed chemostat culture.

When isoleucine was fed to chemostat culture, it increased the titre of clavulanic acid in comparison to the non fed control (0.045 g/l compared to 0.03 g/l control) (Fig 78), and as was expected, the intracellular pool of isoleucine was also significantly increased.

Isoleucine is considered to be a member of the aspartate family of amino acids although it obtains 2 of its 6 carbons from pyruvate. The control of carbon flow into the isoleucine pathway is achieved by inhibition of threonine deaminase by isoleucine itself, forming part of a negative feedback response.
When the intracellular amino acid pools were examined, it was observed that there was an increase in the pool size of threonine in comparison to the control. If there was an excess of isoleucine within the cell, it is possible that there would be less drain on pyruvate and threonine to provide carbon for its synthesis. It is therefore reasonable to expect an increase in the threonine pool size as a result of this. As less carbon is required for the synthesis of this amino acid from pyruvate or the aspartate family of amino acids, the surplus carbon resulting from this could be available for the synthesis of clavulanic acid.

An unexpected finding from this intracellular profile, was the decrease in the pool sizes of aspartate and methionine observed under these conditions. It may be possible that because there is an increase in the pool size of isoleucine and threonine, there is less carbon required for the synthesis of the aspartate family of amino acids. There may therefore be a reduction in the quantity of carbon drawn from oxaloacetic acid in the TCA cycle, which may in turn result in a decrease in the pool size of aspartate. Presumably, methionine formation would also suffer under these conditions as aspartate is a precursor to this amino acid.

The most likely explanation for the increase in clavulanic formation under these conditions is therefore the increase in pyruvate, the C3 precursor to clavulanic acid.
6.3.6 ARGININE FEED

Fig 83. The relative amino acid pool size (µ g / 10 mg biomass) of an arginine fed chemostat culture.

An arginine feed increased the intracellular arginine pool, although this was less dramatic than had been observed with other amino acid feeds (Fig 83). The increase in arginine pool size with arginine feeding had also been demonstrated by Bascaran et al., 1989.

In addition to this, it was also observed that there was only a small increase in the ornithine pool size of which arginine is a precursor. Arginine, ornithine and citrulline constitute what is known as the urea cycle. In the urea cycle, ornithine is synthesised from glutamate which serves as a carrier upon which are assembled the carbon and nitrogen atoms that will eventually constitute urea.
Carbamoyl phosphate reacts with ornithine via ornithine carbamoyltransferase to yield citrulline. Arginosuccinate synthetase then reacts within citrulline to form arginosuccinate. Arginosuccinase cleaves arginosuccinate in a non-hydrolytic, non-oxidative reaction to give arginine and fumarate. Arginine is cleaved hydrolytically by arginase to regenerate ornithine and urea.

Excess arginine has been proven to inhibit the biosynthesis of ornithine from glutamate via the inhibition of glutamate acetyltransferase (Mandelstam et al., 1986). This is consistent with the results obtained from this experiment where an excess of arginine has resulted in a large increase in the glutamate pool, presumably due to the regulation of the urea cycle by arginine.

Despite this, the increase in arginine availability did cause a significant increase in the pool sizes of many amino acids. All of the amino acids analysed except citrulline, alanine and asparagine had their intracellular pool size increased by an arginine feed when compared to the non fed culture. The metabolic pathways of amino acids are complex and interrelated. An increase in the pool size of the 5 carbon branched side chain amino acid valine for example caused by the increase in arginine availability may also result in an increase in the pool size of the 6 carbon branched chain isoleucine, as their synthesis occurs via a parallel set of reactions and that require an almost identical set of enzymes. The knock on effect of this then can become clear, with threonine conversion into isoleucine inhibited by isoleucine itself resulting in an increase in the threonine pool.
Although an arginine feed resulted in a stimulatory effect on the pool sizes of many amino acids, it did not have a stimulatory effect on the formation of clavulanic acid despite being found in the clavulanic acid cluster in the dendrogram (Fig 77).

Clavulanic acid obtained in an arginine fed chemostat reached a maximum concentration of 0.024 g·g\textsuperscript{-1} biomass\textsuperscript{-1} which was 0.006 g·g\textsuperscript{-1} biomass\textsuperscript{-1} less than that observed in a non fed control. It then appears from these results that under phosphate limitation, increasing the availability of the C5 precursor, does not result in an increase in clavulanic acid formation. This possibly indicates that in these circumstances, it is the C3 precursor which is rate limiting to clavulanic acid formation.
6.3.7 THREONINE FEED

Fig 84. The relative amino acid pool size (μ g / 10 mg biomass) of a threonine fed chemostat culture.

A threonine feed resulted in the greatest increase in clavulanic acid formation than was observed with any of the other amino acid feeds (0.054 compared to 0.03 g/l control) (Fig 78) and also resulted in a significant increase in the intracellular pool size. The synthesis of threonine occurs via oxaloacetate and through 6 enzymatic steps from asparate, aspartyl-P aspartic semialdehyde, homoserine, o-phosphohomoserine and threonine. The final control of carbon flow into threonine is achieved by inhibition of homoserine kinase by threonine. Threonine can then be converted into isoleucine via threonine deaminase.
The increase in threonine availability dramatically increase the threonine pool and to a lesser extent the isoleucine pool. This finding was not unexpected given the biosynthetic pathways of these amino acids. Threonine feeding did not result in an increase in the pool sizes of many amino acids. It resulted in a decrease in the pool size of aspartate possibly as a result of decreasing the carbon flow from the TCA cycle into the formation of the aspartic acid family of amino acids. This coupled with the reduction in carbon drawn directly from pyruvate which is no longer required for the synthesis of isoleucine, may presumably allow for greater carbon availability to be used in clavulanic acid formation.

From this analysis it is also apparent that the pool sizes of alanine, tyrosine and proline were found to be reduced when threonine was fed to a phosphate limited culture. From the metabolic pathway (Fig 80), it is apparent that the carbon required for the synthesis of these amino acids is usually drawn off of gluconeogenesis. As a threonine feed reduces the formation of these amino acids, it may be possible that under these conditions, gluconeogenesis is much less active when compared to the non fed control conditions.

Threonine feeding decreased the pool size of arginine the C5 precursor of clavulanic acid when compared to the control culture. It can be assumed that as a greater concentration of clavulanic acid was obtained under these conditions possibly due to the increase in the C3 precursor, it may be possible that as a result, the C5 precursor then becomes rate limiting to clavulanic acid formation under these conditions.
Fig 85. The relative amino acid pool size (µg / 10 mg biomass) of an asparagine fed chemostat culture.

When asparagine was fed to a *S. clavuligerus* culture, clavulanic acid formation was stimulated (0.048 compared to 0.03 g/l control). Although the asparagine intracellular pool was increased, the pool sizes of most of the other amino acids were reduced.

If asparagine feeding reduces the drain of carbon from aspartate and as a result oxaloacetate, this may increase the availability of pyruvate as less carbon would be required to replenish the TCA cycle. There may therefore be an increase in the quantity of pyruvate available for clavulanic acid synthesis.
As the pool sizes of many amino acids are reduced under these conditions, this may mean that there is a reduction in the flux of carbon entering the TCA cycle. If this is the case then this would result in a lack of carbon skeletons available for amino acid synthesis. The result of this may possibly be an increase in carbon availability for the synthesis of the C3 precursor and clavulanic acid.
The effect of feeding 10 mM oxaloacetate on the intracellular free amino acid pool of *S. clavuligerus* is shown in fig 86. As would be expected, the intracellular pool sizes of amino acids that have oxaloacetate as their precursor (Asp, Thr, Ileu, Meth) were increased with oxaloacetate feeding. This could have two effects upon the metabolic pathway of clavulanic acid.

Firstly as there is an abundant supply of oxaloacetate, the anaplerotic reaction (the ATP dependent carboxylation of pyruvate to oxaloacetate) would not be required. This would conserve the pool of pyruvate. Secondly, as the culture is fed with a TCA cycle intermediate, this may reduce the carbon flux required for the synthesis of other TCA cycle intermediates and so again conserve the pyruvate pool.
An oxaloacetate feed would then presumably increase the carbon flow into the formation of the C3 precursor.

Although there was a probable increase in C3 availability, clavulanic acid formation was not significantly increased (0.032 g g\text{biomass}^{-1} vs 0.03 g g\text{biomass}^{-1}). As the arginine availability was reduced, the C5 precursor had become rate limiting to clavulanic acid formation under these conditions.
6.4.0 ORNITHINE FEED

Fig 87. The relative amino acid pool size (μg / 10 mg biomass) of an ornithine fed chemostat culture.

A 10 mM ornithine feed was observed to have little effect upon the formation of clavulanic acid (0.036 g·g biomass⁻¹ compared to 0.03 g·g biomass⁻¹ control). It may be assumed that as ornithine is part of the urea cycle, it may cause an increase in clavulanic acid formation by increasing the pool of arginine, the C5 precursor. However, when the results are analysed, it appears that this is not the case as the arginine pool is actually decreased when compared to that of the control (Fig 87).

An ornithine feed was however found in this study to have a stimulatory effect on the production of citrulline. This is not an unusual finding as it can be observed from the biochemical pathway that ornithine is a precursor to citrulline synthesis.
An increase in the glutamate pool was detected under these conditions. As ornithine in synthesised from glutamate, the presence of increased ornithine may have inhibited its own synthesis by negative feedback and therefore increased the pool size of glutamate. The reduction in the arginine pool observed in comparison to the control, probably resulted in the C5 precursor becoming the rate limiting step in clavulanic acid formation and therefore would explain the little increase observed in clavulanic acid production under these conditions.
Cluster analysis of the intracellular profile of free amino acids in *S. clavuligerus* identified a number of amino acids that could be directly correlated to the production of clavulanic acid. The subsequent feeding of these amino acids to a culture resulted in an increase or decrease in clavulanic acid formation as predicted by the cluster analysis.

The poor performance of particular amino acids such as arginine and ornithine that may have been expected to increase clavulanic acid formation can be attributed to the fact that under phosphate limiting conditions, the C5 supply may not be rate limiting to clavulanic acid formation.

The amino acids that can increase the carbon availability for the synthesis of the C3 precursor, often also exert a stimulatory effect upon clavulanic acid formation. This indicates that the C3 precursor is possibly rate limiting to clavulanic acid formation under these conditions.

Feeding of the amino acids to the phosphate limited culture resulted in the accumulation of increased intracellular pools of that particular amino acid (relative to the control). This may indicate that all the amino acids tested were transported into the cell.
This study provided an indication of the roles of various amino acids in clavulanic acid biosynthesis and enabled several theories to be predicted. To investigate these theories further and analyse the distribution of carbon within the metabolic pathway under these conditions, metabolic flux analysis was employed (Chapter 7).
7.0 METABOLIC FLUX ANALYSIS

7.1 INTRODUCTION

Metabolic flux analysis is a sophisticated mathematical methodology that provides a method of determining steady state fluxes in a metabolic network by measuring some external key fluxes (Vallino and Stephanopoulos 1990). These fluxes can be steady state uptake rates of substrates for example glycerol and oxygen and formation rates of various products for example carbon dioxide and clavulanic acid.

Mutation / selection and genetic engineering techniques employed to increase product yield in microbial processes often only alleviate metabolic regulation that occurs at, or near the end of a biosynthetic chain that leads to a particular product. However, microorganisms have evolved biosynthetic pathways to produce energy and metabolites necessary for growth and replication, not for overproduction of specific biocompounds. Consequently, not only does the regulation of the product of interest need to be removed, but also the main fuelling reactions of the cell must be re-routed in such a way as to channel the main carbon flux into the biosynthetic pathway required for product synthesis.
In previous chapters the effect of certain conditions on the intracellular amino acid pool sizes has been investigated (Chapter 6). These changes in pool sizes have enabled predictions to be made regarding the flow of metabolites to various parts of the metabolic pathway under these conditions and the resulting effect on clavulanic acid production. These predictions were purely derived from the results obtained and did not take into consideration products other than clavulanic acid being exported from the cell. Metabolic flux analysis was used to provide further evidence of the carbon distribution occurring within the cell under particular sets of conditions. The aim of this was to provide a more complete picture of metabolism and clavulanic acid production under the conditions examined.

This study aims to investigate carbon fluxes through the biosynthetic pathways of *S. clavuligerus* under a range of conditions. These conditions included, carbon, nitrogen and phosphate limitation, in addition to phosphate limitation supplemented individually with 10 mM amino acids which included, threonine, aspartate, arginine, as well as 10 mM oxaloacetic acid. These feeds were chosen for analysis because of their correlation to clavulanic acid production obtained from cluster analysis carried out in chapter 6.

In order to carry out metabolic flux analysis, firstly a simplified metabolic network for *S. clavuligerus* was constructed which consisted of the main fuelling and metabolite generating bioreactions i.e. Gluconeogenesis, Glycolysis, Tricarboxylic acid cycle (TCA) and the Pentose phosphate pathway (PPP).
The chosen stoichiometry used in this study consisted of 43 metabolites and 36 separate reactions. A list of all the reactions can be found in Appendix 1. As information regarding certain parts of the *S. clavuligerus* metabolic pathway were not available, the majority of the metabolic reactions included in the model were assumed to be of a similar nature to other bacteria such as *E. coli*. This is common practice in MFA where there is no data available for a particular organism (Ingraham et al., 1983, Bailey and Ollis 1986). However this has to be taken into consideration when interpreting results.

The assumptions made regarding the *S. clavuligerus* metabolic pathway were as follows. The existence of a complete TCA cycle in both *S. lividans* and *Streptomyces C5* was proved in a study by Dekleva and Strohl (1988 a) where they found that citrate synthase, the gateway enzyme to the TCA cycle had an especially high activity. The TCA cycle was therefore assumed to be present in *S. clavuligerus* (reaction 8).

The presence of a full Pentose phosphate pathway (PPP) was reported by Salas et al., (1984) for *S. coelicolor, S. antibioticus, S. scabies* and *S. reticuli*. However Holmes (1996), found that when glycerol is used as a carbon source, it yields glucose 6 phosphate by gluconeogenesis, but can feed the Pentose phosphate pathway from Triose phosphate without oxidative decarboxylation. This was also taken into consideration in the model (reaction 13).
Amino acid synthesis was assumed to be similar to reactions occurring in other organisms, and so equations were taken from standard biochemistry text (Mandelstam and McQuillen 1986) (reaction 18). In addition, polysaccharide production which was observed in this study was also assumed to be as other organisms Mandelstam and McQuillen 1986) (reaction 34).

Reaction 31 represents the conversion of monomers into biomass. It accounts for everything from amino acid integration into protein, to cell wall synthesis. The energy required is also taken into account. The reaction stoichiometry was obtained using the *E.coli* biomass composition and assembly requirements given by Ingraham *et al.*, (1983).

Reactions 28 and 29 represent the oxidative phosphorylation reactions of the cell. A P/O ratio of 2 was assumed, and has been assumed in other models for *Streptomyces* (Bailey and Ollis 1986, Daae and Ison 1999). This assumes that the number of ATP molecules produced per molecule of NADH is 2. It was further assumed that compared to NADH, FADH produced 50% of the amount of ATP. These assumptions are fairly standard relationships in a number of bacteria (Bailey and Ollis1986).

The enzyme phosphoenolpyruvate carboxylase (PEPC) (reaction 27), has been identified as the main anaplerotic enzyme in a number of *Streptomyces* species (Dekleva and Strohl 1988 a, 1988 b, Bramwell *et al.*,1993, Coggins *et al.*, 1995). This was therefore assumed to be present in *S.clavuligerus* as well.
Reactions for clavulanic acid synthesis (reactions 32 and 33) were obtained from a study carried out by Jensen et al., (1999), on the biosynthesis and molecular genetics of clavulanic acid.

Metabolic Flux Analysis (MFA) was carried out using FluxMap (CAD environment) software.
The stoichiometric model is most conveniently formulated in matrix notation (Nielsen and Villadsen 1994). In a stoichiometric model comprising J cellular reactions between N substrates (s), M extracellular products (p) and L intracellular metabolites (x), the matrix notation reads:

\[(s)\]
\[
\begin{pmatrix}
T & 0
\end{pmatrix}
\begin{pmatrix}
(s) \\
p \\
x
\end{pmatrix}
= 0
\]

The stoichiometric coefficients are listed in the total stoichiometric matrix T with each reaction and metabolite constituting respectively rows and columns. The coefficients are either positive, negative or zero depending on whether the metabolite is formed, consumed or does not participate in the given reaction. When all the biochemical fluxes through the J reactions in the model are collected in the vector r, the net specific production rate \(r_i\) (mol/g DW/h) of the i’th extracellular component i.e. either a substrate or a product can be calculated as:

\[r_i = T_i^t \cdot r ; \quad i = 1, ..., N + M\]
whereas the net specific production rate of the $i$'th intracellular metabolite $X_i$ is given by:

\[
T_i' \cdot \mathbf{r} - \mu \cdot X_i \quad ; \quad i = N + M + 1, ..., N + M + L
\]

with $T_i$ as the $i$'th column vector of $T$, as the specific growth rate (h$^{-1}$) and $X_i$ as the intracellular concentration of the $i$'th metabolite (mol/g DW). Since the turnover of most of the intracellular pools is rapid (Nielsen and Villadsen 1994), the last term in fig 90 accounting for dilution of the intracellular metabolite due to growth is often negligible. This assumption is however, not valid for macromolecular components and for other metabolites present in high intracellular concentrations. Another assumption often applied and is also applied here, is that the intracellular metabolites are in a pseudo steady state (PSS), i.e. $r_i = 0$.

By assuming PSS a considerable simplification of the metabolic network can be done since all metabolites which are not at a branch point of the metabolic pathways can be eliminated from the model by lumping of reactions without loss of information concerning the flux distribution. If the assumption of PSS applies for $C$ intracellular metabolites it imposes $C$ constraints on the $J$ biochemical fluxes and the degrees of freedom is thus $F = J - C$. The degrees of freedom represents the minimum number of measurements which are required in order to calculate the entire set of metabolic fluxes.
It is seldom necessary to measure the net specific formation rates of all the substrates and products considered in the metabolic network in order to quantify the intracellular flux distribution, i.e. to make the system observable. The best set of measurements for the MFA which makes the system observable, can be chosen on the basis of knowledge on the effort involved in attaining the different sets of data, or on a sensitivity analysis (Vallino and Stephanopoulos 1990), whereby the set of measurements from which the metabolic flux distribution is the least sensitive towards measurement errors can be found.

The set of measurements chosen for MFA in this study included carbon uptake as well as the basic outputs from the system i.e. biomass, clavulanic acid and CO₂. These were chosen because they were relatively simple to quantify, and provided accurate data which we could have confidence in.

A prerequisite for MFA is that the elemental balances close. If a large carbon flux is not considered, the calculated flux distributions may be artefacts. Experimental analysis of the medium revealed large quantities of an extracellular polysaccharide. This has not been previously considered. This was included in the model and enabled the carbon balances to be closed to within 10%. All experimental data was converted to carbon mole to avoid confusion during MFA. Using the stoichiometric model for the metabolism of *S. clavuligerus*, calculated fluxes could be obtained from experimental data (Fig 91).
Fig 91. Carbon balance of *S. clavuligerus* under various conditions

**RESULTS**

**CARBON BALANCES**

( Cmole l⁻¹ h⁻¹)

<table>
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<th>Conditions</th>
<th>Pld</th>
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<th>Cltd</th>
<th>Pld + Thr</th>
<th>Pld + Asp</th>
<th>Pld + Arg</th>
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<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
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<td>0.019</td>
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<td>2.9</td>
<td>1.29</td>
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<td>1.4</td>
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<td>1.3</td>
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<td>1</td>
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<td>0</td>
<td>14.5</td>
<td>26.5</td>
<td>18.09</td>
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Unfortunately, there were no true polysaccharide measurements available from the amino acid fed chemostat cultures. However polysaccharide accounted for a very substantial portion of the carbon utilised within the system and without reasonable estimates of polysaccharide concentrations under these conditions, the carbon balance would not close i.e. the proportion of carbon entering the cell did not match the quantity of carbon used in product formation. In order for MFA estimations to be made, the carbon mole used in product formation were compared to the carbon mole uptake in the amino acid fed chemostats. The difference in these values was then considered to be carbon used in polysaccharide production for the purpose of this study. The values obtained from this were considered to be very reasonable when compared to actual data obtained from previous chemostats.

Due to the number of assumptions made when carrying out MFA, the results obtained are purely theoretical and intended only to give an indication of what may possibly happen in the intracellular environment. Until confirmation is obtained on the actual metabolic reactions that occur specifically within *S. clavuligerus*, we can only speculate using data obtained from other organisms.

Using the above data (Fig 91), MFA could be attempted. The calculated fluxes obtained for each equation in appendix 1, are listed as follows (Fig 92).
METABOLIC FLUX DATA

(Fig 92) Calculated carbon fluxes for *S. clavuligerus* biosynthetic pathway.

<table>
<thead>
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<th>React1</th>
<th>C-lim</th>
<th>P-lim</th>
<th>N-lim</th>
<th>P-lim THR fluxes in C-mole</th>
<th>P-limASP</th>
<th>P-limARG</th>
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A list of reactions can be found in appendix 1.
From the calculated flux data for metabolic equations, a summary was produced for areas of particular interest in the *S. clavuligerus* metabolic pathway. Areas considered to be of interest were, gluconeogenesis, glycolysis, clavulanic acid production, polysaccharide production and the carbon fluxes into aspartic acid, arginine, threonine and oxaloacetate synthesis. A summary table containing estimated carbon fluxes into these particular areas of the pathway was constructed and is displayed in Figure 93.
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(Fig 93) A summary of *S. clavuligerus* metabolic flux analysis

Reactions are listed in appendix 1.
7.3 RESULTS

7.3.1 CARBON LIMITATION

(Red arrows indicate major carbon fluxes, Blue arrows: minor carbon fluxes, White arrows indicate no carbon flux, Black arrows indicate no flux data available.)

Fig 94. Carbon flux distribution under carbon limitation.
Under carbon limitation it can be observed from fig 94, that the model predicts that the flux of carbon through gluconeogenesis is considerably lower than that observed under any of the other nutrient limitations. The ratio of gluconeogenesis to glycolysis (reaction 2 / reaction 4) obtained indicates that almost all available carbon assimilated by the cell is directed through glycolysis and into the TCA cycle at the expense of gluconeogenesis. As the TCA cycle constitutes the most important single mechanism for the generation of ATP in aerobic organisms, it is not surprising that this should take precedence over other areas of the pathway under this particular limitation to maintain the cells basic energy requirements. This observation can also be substantiated by the predicted concentration of polysaccharide produced under carbon limitation compared to other nutrient limiting conditions. Only 3.1 E-04 Carbon mole/l/h of polysaccharide was obtained under carbon limitation in comparison to 2.6 E-02 Carbon mole/l/h produced under phosphate limitation. This certainly indicated that gluconeogenesis is much less active under carbon limiting conditions.

As there are insufficient quantities of carbon to supply all areas of the metabolic pathway in excess, it is not surprising that the production of clavulanic acid, a secondary metabolite, is affected. The model predicts that only 4.78% of clavulanic acid would be produced under carbon limiting conditions compared to that produced under phosphate limitation. Despite this, the flux of carbon through the C3 precursor is greater than the flux of carbon into clavulanic acid indicating that there is possibly an intermediate stage between the formation of the C3 precursor and clavulanic acid formation.
Despite this, the flux of carbon through the C3 precursor is lower than that predicted under any other nutrient limiting conditions studied because of the total shortage of carbon available to the cell. It can therefore be assumed that there is not enough carbon available in general to support portions of the pathway that are not essential to the immediate survival of the organism, and so clavulanic acid production is practically abolished.

The flux of carbon through the anaplerotic reaction was shown to be greater under carbon limitation than under either phosphate or nitrogen limitation. Intermediates of the TCA cycle are continuously drawn off and used as precursors of amino acids etc. If the TCA cycle is to go on functioning, then these intermediates must be replenished. This usually happens by carboxylation of pyruvate or PEP to yield oxaloacetate. Reactions having this function are often referred to as anaplerotic (from the Greek for filling up) since they replenish intermediates drained off for biosynthesis. Under carbon limitation, the anaplerotic carboxylation of PEP is positive. TCA cycle intermediates are drawn off for the synthesis of essential amino acids and this in turn initiates the production of phosphoenolpyruvate, the main anaplerotic enzyme, and subsequently begins the reaction in an attempt to replenish oxaloacetic acid. This could explain why the flux of carbon through the anaplerotic reaction is high when compared to the other conditions examined. Despite the fact that the cell was under such severe carbon limitation, there were still carbon fluxes predicted for the synthesis of aspartate, threonine, arginine. It would appear that these amino acids are required for the survival of the cell and so they are produced even when the organism is under such severe metabolic strain.
7.3.2 PHOSPHATE LIMITATION

Fig 95. Carbon flux distribution under phosphate limitation.
Under phosphate limitation (Fig 95), the ratio of gluconeogenesis to glycolysis ($r_2/r_4$) was predicted to be 1.42, which indicated that there was a much greater carbon flux through gluconeogenesis compared to glycolysis. This finding is consistent with the high polysaccharide production that was predicted by the model and observed from experimental data under these conditions.

It was also predicted that the flux of carbon through the TCA cycle was lower than the flux of carbon through glycolysis under these conditions. This is commonly observed in organisms capable of producing acids as carbon used for its synthesis is drawn off of glycolysis (Mathews et al., 1999).

It has been found in previous studies (Goel et al., 1993), that the flux of carbon through the TCA cycle in continuous culture is much in excess of cell energetic and biosynthetic demands for precursors. The carbon flux into the TCA cycle must be strictly regulated to prevent the TCA cycle from turning too quickly and accumulating ATP. Control of the TCA cycle is exerted principally at the citrate synthase stage, i.e. on the first enzyme of the cycle. It has been discovered that citrate synthases of certain bacteria are inhibited by NADH, whereas in other species, the enzyme is inhibited by ATP. Thus under conditions which result in an accumulation of reducing power and ATP, e.g. when biosynthesis is curtailed, the overall operation of the TCA cycle will be inhibited in susceptible bacteria, thus conserving energy and carbon (Mandelstam et al., 1986)
Although the oxidation of pyruvate to acetyl CoA is not part of the TCA cycle, it does furnish one of the substrates for citrate synthase, and is itself also subject to regulation. Acetyl CoA, the product of the oxidation, inhibits pyruvate dehydrogenase, the first enzyme of the complex by negative feedback inhibition. This enzyme is activated by AMP and inhibited when the energy charge is high.

It is possible that under phosphate limiting conditions where there is an abundance of available carbon, the TCA cycle is subject to regulation. Under these conditions, it is possible that the carbon not required for energetic purposes is used to form a by-product such as polysaccharide.

Exopolysaccharide production has been observed in many microbial species (Jansson et al., 1975, Sturgeon 1983, Lindberg 1990). In a study on Aureobasidium pullulans (Reeslev et al., 1996), the concentration of polysaccharide produced was examined under conditions of nitrogen and carbon limitation. It was observed that under nitrogen limitation, polysaccharide production was constant and about 36 mg g\(^{-1}\) l\(^{-1}\), but under carbon limitation, no polysaccharide was detected, a phenomenon which has been observed in this study. An extracellular polysaccharide has also been isolated from culture broth of a Streptomyces species (Inoue et al., 1992). It was proven to consist of many sugars including D-mannose, D-galactose, D-galacturonic acid, D-xylose and D-glucose.
Although polysaccharides produced by *Streptomyces* have not been fully studied, they have been detected in some *Streptomyces* species. One species of *Streptomyces* was shown to produce a mannoglucan, whilst Harris and Gray (1977), extracted acetylated methylmannose polysaccharide from *Streptomyces griseus*. Although a hypothesis has not been given on the reasons why *Streptomyces* should produce these compounds, it could be possible that they are produced to regulate metabolism in some way.

Phosphate limiting conditions were the most supportive of clavulanic acid production (Chapter 3). The flux of carbon predicted for the formation of clavulanic acid totalled $1.80^{-0.4}$ C mole l/h, and when this is compared to the flux of carbon directed into the C3 precursor ($1.85^{-0.4}$ mole l/h), it can be observed that these fluxes are evenly matched. This indicates that there is little loss of carbon between the C3 precursor and clavulanic acid synthesis predicted, and could indicate that under these conditions, the C3 precursor may be rate limiting to clavulanic acid production as all carbon utilised for the formation of the C3 precursor is used in clavulanic acid formation. Despite this, there was a greater carbon flux predicted for the formation of clavulanic acid than observed under either carbon or nitrogen limitation.

It has been postulated in an earlier chapter (chapter 3), that glycolysis may be restricted under phosphate limitation because of the shortage of available phosphate. Although this has not been proven, it is the case that before entering the TCA cycle, pyruvate is converted to acetyl coenzyme A.
This oxidative decarboxylation is brought about by a multi-enzyme complex which comprises three enzymes and five different cofactors. One of these cofactors is thiamine pyrophosphate which is used in the first stage of the oxidative decarboxylation of pyruvate which is catalysed by pyruvate dehydrogenase. If there was a shortage of phosphate within the cell, then it may be possible that the synthesis of thiamine pyrophosphate is restricted. Without sufficient quantities of cofactor available, the conversion of pyruvate may also be restricted and hence the flux of carbon into the TCA cycle may also be affected.

It appears from this study that the shortage of phosphate has not restricted the entry of carbon into the TCA cycle as the flux appears to be similar to that observed carbon and nitrogen limitation.

Phosphate limitation also resulted in a large carbon flux entering aspartate synthesis from oxaloacetate when compared to the other nutrient limitations. As the flux through the anaplerotic reaction is negative, this indicates that it was unnecessary to replenish oxaloacetate that was being utilised for amino acid synthesis.

It is also apparent from these predictions, that this large quantity of carbon used for aspartate synthesis was not being fed from aspartate into threonine synthesis as might be expected, but from aspartate through arginosuccinate and into arginine synthesis. There is a greater carbon flux used for the synthesis of arginine under these conditions than under any of the other nutrient limitations studied.
It is this abundance of arginine (C5 precursor) and greater flux of carbon used for clavulanic acid formation which results in high clavulanic acid titres obtained when compared to other nutrient limitations studied.
7.3.3 NITROGEN LIMITATION

Fig 96. Carbon flux distribution under nitrogen limitation.
As was predicted with phosphate limitation, nitrogen limitation also encouraged a large carbon flux through gluconeogenesis compared to the flux through glycolysis. Again, the huge polysaccharide concentration obtained in experimental results and predicted by the model would confirm this finding. As with phosphate limitation, it could be assumed that there is an abundance of carbon within the cell that is restricted from entering the TCA cycle. As a result, this carbon is forced up through gluconeogenesis and into polysaccharide production which enables the cell to export unwanted carbon.

As there is a significant quantity of carbon within the cell, there is also good clavulanic acid production (1.81 E-04 Cmole/l/h). Carbon fluxes predicted by MFA do follow experimental data but do not match exactly. This is due to the very low percentage of carbon going to the clavulanic acid product when compared to the percentage of carbon going to other portions of the pathway such as polysaccharide production for example. This prediction is intended to give an indication of the proportions of carbon used in the pathway and not an exact prediction of the concentration of product formed.

The carbon flux directed into the formation of the C3 precursor is also substantial under nitrogen limitation and when this is compared to the flux estimated into clavulanic acid formation, it is obvious that the C3 precursor is greatly in excess of carbon directed into clavulanic acid formation. This possibly indicates that under nitrogen limitation, the C3 precursor is not the rate limiting step in clavulanic acid formation.
It can be observed from fig 96, that there is a large carbon flux predicted through the TCA cycle. This could result in the anaplerotic reaction being abolished under nitrogen limiting conditions and is also confirmed by the negative flux value obtained for this reaction. In addition to this, there were no carbon fluxes predicted into aspartate or threonine synthesis. As aspartate is a precursor to threonine formation, the fact that threonine is not being formed is not unexpected. For the synthesis of amino acids, carbon and nitrogen supplied by NH₄ are required. As there is a shortage of intracellular nitrogen, it is possible that this is the cause of the abolition of the synthesis of these two amino acids. Despite this, there was still a very small carbon flux directed towards arginine synthesis. Because of this, it was possible that clavulanic acid synthesis could still occur.

It has been hypothesised in an earlier chapter (chapter 3) that clavulanic acid synthesis is effected by low intracellular nitrogen concentration. As arginine is the C5 precursor to clavulanic acid, with less nitrogen available for its synthesis, it is possible that the C5 precursor may become the rate limiting step in clavulanic acid formation under these conditions.

Upon examination of the flux data, it appears that this hypothesis is also predicted by the MFA model. It has already been mentioned that it is unlikely that the C3 precursor is rate limiting under nitrogen limitation due to large carbon flux directed to its synthesis, but when the carbon flux into arginine synthesis is examined, it is apparent that it is lower under nitrogen limitation than under either of the other two limitations studied.
It would then appear from these predictions that it is actually the C5 precursor, arginine, which is rate limiting under nitrogen limiting conditions.

From this metabolic flux analysis, it also appears that the anapleurotic reaction is not required for oxaloacetic acid synthesis. If carbon from oxaloacetate is not being used as a precursor for amino acid synthesis because of the lack of available nitrogen, then it would not be necessary for extra carbon to be directed into its synthesis as there would not be a requirement to ‘top up’ the TCA cycle.
7.3.4 THREONINE

Fig 97. Carbon flux distribution under phosphate limitation with threonine feed.
When threonine was fed to a phosphate limited chemostat culture, the ratio of gluconeogenesis to glycolysis fell from 1.42 in phosphate limitation to 0.91 indicating that the flow of carbon had changed from being primarily directed into gluconeogenesis, to being directed into glycolysis. In addition, the quantity of carbon entering the TCA cycle had also fallen when compared to the non amino acid fed cultures. This indicates that there appears to be a loss of carbon between glycolysis and the TCA cycle. Phosphate limitation with a threanine feed provided the conditions under which a very large quantity of clavulanic acid was produced (0.07 g/l). There was a large flux of carbon which was directed into the synthesis of the C3 precursor. It is likely that a substantial portion of carbon from glycolysis was drawn off here in order for substantial clavulanic acid production to take place.

As the flow of carbon into gluconeogenesis was low, it is not surprising that under these conditions there was very little polysaccharide produced and predicted. When the intracellular pool size of threonine was examined, it was found that a threonine feed elevated the intracellular pool size. This increase in pool size may have inhibited the further synthesis of threonine by feed back inhibition. There was no carbon flux predicted into the synthesis of threonine, and as aspartate is a precursor to threonine, it is not surprising that there is no carbon flux predicted towards the synthesis of this amino acid either.
The fact that there is such a large carbon flux directed towards the C3 precursor, when this is compared to the flux entering clavulanic acid formation, it is apparent that it is unlikely to be the rate limiting step. This is also substantiated by the decrease in carbon flux directed into arginine synthesis when compared to the non fed control, indicating that the C5 precursor may be the controlling factor in clavulanic acid synthesis. It was postulated in the previous chapter (6), that a threonine feed may cause an increase in clavulanic acid formation by preventing carbon from being directed into threonine synthesis and allowing more carbon flux to be fed into the formation of the C3 precursor. The model predicted that this feed did result in an increase in carbon flux directed into the synthesis of the C3 precursor when compared to the phosphate limited control.

Threonine feeding resulted in a decrease in arginine synthesis compared to the control and the examination of the intracellular pool size. It is therefore possible that the C5 precursor is rate limiting to clavulanic acid formation under these conditions.
7.3.5 ASPARTATE

Fig 98. Carbon flux distribution under phosphate limitation with aspartate feed

- Polysacc → Glu6P → PPP
- Glycerol
- Fru6P
- G3P
- PEP
- PYR
- AccoA
- Citrate
- Isocit
- AlphaKG
- TCA
- Oxaloacetate
- Malate
- Fumarate
- Succinate
- Citrulline
- Ornithine
- Arginine
- Arginosuccinate
- Urea
- Clavulanic Acid
- C3 Prec
- Aspartate
- Threonine

Flux values:
- Polysacc → Glu6P: 1.35E-04
- Polysacc → Clavulanic Acid: 1.35E-04
- Glycerol → Fru6P: 2.08E-02
- Fru6P → G3P: Ratio : 0.70
- PEP → PYR: -1.59E-03
- Arginosuccinate → Oxaloacetate: 2.55E-03
- Citrate
- Isocit
- AlphaKG
- TCA
- Oxaloacetate
- Malate
- Fumarate
- Succinate
- Citrulline
- Ornithine
- Arginine
- Arginosuccinate
- Urea
- Clavulanic Acid
- C3 Prec
- Aspartate
- Threonine

Biomass: 202
When aspartate was fed to a phosphate limited chemostat it was found to have a detrimental effect upon clavulanic acid synthesis. Aspartate is indirectly linked to clavulanic acid formation as it is involved in the biosynthetic pathway of arginine synthesis, which is the C5 precursor. This was an unusual finding as threonine, an amino acid which also has aspartate as its precursor was found to have a positive effect upon clavulanic acid biosynthesis.

Again, as would be expected, when aspartate is fed to the culture, the flow of carbon into the synthesis of aspartate and threonine is abolished. Despite this, there is still a flow of carbon directed into arginine synthesis which is quite low when compared to the flux under other limitations and feeds examined, but is actually substantial when compared to the fluxes under these particular conditions.

The very low ratio of carbon directed into gluconeogenesis accounts for the very low concentration of polysaccharide predicted with this particular amino acid feed. As there is very little carbon available for the synthesis of the C3 precursor, and there is no loss of carbon from the C3 precursor into clavulanic acid formation, it is probable that in this case, it is this which is the rate limiting step in clavulanic formation. Under phosphate limiting conditions, the C3 precursor was also found to be the rate limiting step in clavulanic acid formation. It appears that an aspartate feed does not alleviate this shortage.
7.3.6 ARGININE

Fig 99. Carbon flux distribution under phosphate limitation with arginine feed
A 10mM arginine feed in a phosphate limited chemostat culture was observed in the previous chapter (6) to have little effect upon the formation of clavulanic acid in comparison to a non fed culture (0.03 g/l compared to 0.033 g/l without feed).

Again, as was observed with the other amino acid fed cultures, the majority of the carbon flow was not directed through gluconeogenesis, but directed down into glycolysis which was apparent from the low ratio obtained and the low production of polysaccharide. The majority of this carbon flow was then directed through the TCA cycle.

There was slightly less carbon flux observed which was involved in the synthesis of the C3 precursor in comparison to the non fed control, and as arginine was being supplied to the culture and the arginine pool size was elevated (Chapter 6), it is likely that the C3 precursor must have been rate limiting to clavulanic acid formation under these conditions. The C3 precursor was also found to be rate limiting under phosphate limiting conditions without an amino acid feed.

Despite the large availability of arginine, a small flux entering arginine synthesis and the urea cycle was still detected. As the urea cycle is required to eliminate potentially toxic quantities of ammonia from building up within the cell, it is possible that despite the increase in arginine availability, this function is still required.
A significant flow of carbon was predicted to enter aspartate synthesis, and as there was no flux into the formation of threonine, it is possible that the remaining flow is required for the formation of other amino acids for example methionine. This large flux to aspartate synthesis was also observed under phosphate limitation without an amino acid feed and so likely to be due to the phosphate limitation, rather than as an effect of the arginine feed.
7.3.7 OXALOACETIC ACID

Fig 100. Carbon flux distribution under phosphate limitation with oxaloacetate feed

Polysacc → Glu6P → ppp

Glycerol

G3P

Clavulanic Acid → C3 Prec

PEP

Threonine

Aspartate

Arginosuccinate

Arginine

Urea

Citrulline

Ornithine

Oxaloacetate

Malate

Fumarate

Succinate

Citrulline Succinate

Biomass

Clavulanic Acid -O$m C3 Prec

7.00E-04

2.08 E-02

Ratio :1.54

2.11E-04

1.68 E-04

5.30 E-05

1.50E-04

6.52E-03

4.72E-04

1.77E-03

5.67E-03
Oxaloacetate was found in the previous chapter (6) to have a positive effect on clavulanic acid biosynthesis. The ratio of gluconeogenesis to glycolysis indicates that unlike the other feeds, oxaloacetate feeding causes a substantial carbon flux into gluconeogenesis. Although this is the case, it does not stimulate the production of the polysaccharide when compared to the non fed control.

A very large proportion of carbon is also directed into the formation of the C3 precursor. This flux is greater with this feed than observed under any other limitations and feeds. As a result, the C3 precursor is substantially in excess of the flux into clavulanic acid formation. As there is an abundant supply of oxaloacetate available to the cell, it is available to be drawn from the TCA cycle, and utilised as a precursor for the synthesis of amino acids. There is a very large flow of carbon directed into the formation of aspartate, and as a result, substantial fluxes into arginine and threonine synthesis. Despite this, the flux entering arginine synthesis is still not as great as that entering the formation of the C3 precursor.

From this analysis it is also apparent that although oxaloacetate is available in substantial quantities, it is still being replenished by the anaplerotic reaction. The reason why this is predicted is unclear. One explanation may be that the conversion of large quantities of oxaloacetate actually initiates the anaplerotic reaction despite there being large quantities of oxaloacetate available in the culture medium.
7.4 SUMMARY

Metabolic flux analysis has enabled the major carbon fluxes of *S. clavuligerus* to be examined under several nutrient limitations and amino acid feeds. Several trends can be observed from this analysis.

Firstly, the ratio of gluconeogenesis to glycolysis is consistent with high polysaccharide production both predicted and experimentally detected. Conditions which encourage a large carbon flux into gluconeogenesis often result in large quantities of polysaccharide being produced. This was observed under phosphate and nitrogen limitation. Surprisingly, by feeding amino acids to a phosphate limited culture, the ratio of gluconeogenesis to glycolysis fell. This resulted in a change of direction for the major carbon flow, and a drastic reduction in the quantity of polysaccharide produced.

Secondly, the carbon fluxes through the TCA cycle appeared to be consistent under carbon, nitrogen and phosphate limitation with little difference observed between all three. It was discussed earlier (Chapter 6), that entry into the TCA cycle is strictly regulated, and so even though there may be more carbon available under certain conditions, this does not necessarily mean that a greater carbon flux will be allowed to enter the TCA cycle.
When amino acids were fed to a phosphate limited culture, the flux through the TCA cycle was significantly reduced. Intermediates of the TCA cycle are continuously drawn off of the cycle to provide carbon skeletons for the synthesis of amino acids and other molecules. As a result, this carbon lost from the cycle must be replenished in order for it to fulfil its role as the major generator of ATP. If amino acids are already provided to a culture, then this reduces the need for carbon to be drawn off of the TCA cycle, as it is these which can then be utilised as precursors for other molecules. As a result of this, a smaller carbon flux is required to replenish the cycle. This may account for the smaller TCA cycle flux observed under these conditions.

It also became apparent from this analysis that under all conditions examined, the flux through the TCA cycle was lower than that observed through glycolysis. This is commonly observed in acid producing strains where carbon is drawn off of glycolysis for its production. It is possible that *S. clavuligerus* produces acids other than clavulanic acid as part of its metabolism. This can be observed by the significant fall in pH during the bioreactor run which cannot be attributed to clavulanic acid itself as it is present in only very small quantities.

It also appears from this analysis that the rate limiting step in clavulanic acid formation is not fixed but changes under different conditions. It was proven that under carbon and nitrogen limitation, the carbon flux directed into the C3 precursor greatly exceeded that directed into clavulanic acid formation. It can only be concluded that under these 2 conditions, it must be the C5 precursor which controls the rate of clavulanic acid formation.
Under phosphate limitation however, the carbon flux directed into the formation of the C3 precursor is equal to that involved in the formation of clavulanic acid. It is possible that under these conditions it is the C3 precursor which is the rate limiting step in clavulanic acid production.

When amino acids are fed to a phosphate limiting culture, it appears that in all cases, the C3 precursor remains the rate limiting step in clavulanic acid formation. Both aspartic acid and arginine caused a reduction in the flux of carbon into the C3 precursor and in the case of aspartate, caused a reduction in the titre of clavulanic acid produced when compared to the non fed phosphate limiting conditions. Arginine caused little effect upon the titre of clavulanic acid obtained. Of the three amino acids examined, threonine was the only amino acid to increase the flux of carbon into the formation of the C3 precursor. As a result of this, clavulanic acid production was also greatly improved. Although there was an increase in the formation of the C3 precursor, a large carbon flux was also directed into the formation of arginine. As a result of this, the C3 precursor was still rate limiting to clavulanic acid formation.

After an oxaloacetate feed into a phosphate limited culture, the rate limiting step switched from being the C3 precursor to the C5 precursor. The oxaloacetate feed caused a greater increase in the carbon flux directed into the formation of the C3 precursor than any of the other limitations and amino acid feeds. This feed did give an increase in clavulanic acid production, but as there was not a large flux directed into arginine synthesis, clavulanic acid formation was still restricted by the C5 precursor.
Synthesis Of Amino Acids

The carbon flux directed into the formation of amino acids was also taken into consideration in this analysis. Aspartate is indirectly linked to clavulanic acid formation through arginine production. It was apparent from this study that there was no aspartate synthesis detected under nitrogen limited conditions. This was almost certainly due to the lack of intracellular nitrogen which could be used for amino acid synthesis. In addition, there was no aspartate synthesis detected under phosphate limitation with both threonine and aspartate feeds. This is not an unusual finding, as it would be unnecessary to produce aspartate if it is readily available within the culture media. As aspartate is a precursor for threonine synthesis, it would also be wasteful for carbon to be utilised for the production of this amino acid either.

Threonine production was found in this analysis to be inversely linked to clavulanic acid synthesis by means of aspartate. The synthesis of threonine was found to occur only under carbon limitation and oxaloacetate fed chemostats. Carbon limitation caused a reduction in clavulanic acid synthesis when compared to non fed phosphate limited conditions, whereas an oxaloacetate fed chemostat did give an increase in antibiotic formation. When threonine was fed to an *S. clavuligerus* culture, it resulted in a significant increase in clavulanic acid formation. This would indicate that if carbon is required for the synthesis of threonine, then clavulanic acid formation would be reduced. If this is entirely as a result of competition for carbon or due to a more complex reason is difficult to tell from these findings.
The formation of arginine was found to be positive under all conditions examined. In addition to this, the rate of arginine synthesis was directly related to the rate of clavulanic acid formation. This is in agreement with the biochemical pathway for clavulanic acid biosynthesis. As this is the case, it appears from this finding that the C5 precursor is rate limiting to clavulanic acid production under all conditions examined. However, where there is significant arginine formation, in some cases there is an insufficient flux of carbon entering the synthesis of the C3 precursor. Under these conditions it is the C3 precursor which is then still rate limiting.

The anaplerotic carboxylation of PEP was found to occur only under carbon limiting conditions and during the oxaloacetate fed culture. Under carbon limitation the anaplerotic reaction would be required to top up carbon within the TCA cycle. Why this reaction is required under oxaloacetate fed conditions is unclear, but as mentioned earlier, could be triggered by the conversion of large concentrations of oxaloacetate into amino acids.

Surprisingly, the anaplerotic reaction only occurred under conditions which supported the synthesis of threonine. This reaction could then not only appear to be competing for carbon with the C3 precursor, as conditions that encouraged threonine synthesis had a detrimental effect on clavulanic acid formation, but also directing the carbon produced from this reaction into the synthesis of threonine. It is unusual however, that this carbon should stimulate threonine synthesis and not aspartate synthesis which is its precursor.
This application of metabolic flux analysis has given an insight into the distribution of carbon within the metabolic pathway of \textit{S.\textit{clavuligerus}} under several nutrient limiting conditions and feeds. Although it has not provided clear explanations as to why a particular feed has given rise to an increase or decrease in clavulanic acid production, it has enabled the rate limiting steps to be identified under these different conditions. By doing this, it may then be possible to devise culture methods that can alleviate these shortages and increase the rate of clavulanic acid formation.

Fig 101. Summary of Rate Limiting Steps In Clavulanic Acid Synthesis.

<table>
<thead>
<tr>
<th>Limitation / Feed</th>
<th>Clavulanic Acid Rate Limiting Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>C3 &amp; C5</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>C5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>C3</td>
</tr>
<tr>
<td>P + Threonine</td>
<td>C5</td>
</tr>
<tr>
<td>P + Aspartate</td>
<td>C3</td>
</tr>
<tr>
<td>P + Arginine</td>
<td>C3</td>
</tr>
<tr>
<td>P + Oxaloacetic Acid</td>
<td>C5</td>
</tr>
</tbody>
</table>
The culture medium determines the chemical or nutritional environment and is thus vital to research into and the manufacturing of microbial metabolites. It has always been the critical component of an industrial or commercial fermentation process, directly affecting not only productivity but also process economics.

Depending on the nature of the materials used, the fermentation medium can be chemically defined (synthetic, defined), or undefined (natural complex). The media employed to support high productivity’s in commercial fermentations are predominantly formulated with inexpensive complex carbon and nitrogen sources (Miller and Churchill, 1986). These enable high biomass concentrations to be obtained, and therefore high concentrations of product formation from relatively inexpensive substrates.

The aim of this study was to investigate the effect of a range of complex carbon and nitrogen sources upon the growth of *S. clavuligerus* and the production of clavulanic acid. Using the Plackett-Burman fraction factorial technique, the optimisation of a complex media formulation was carried out.
The majority of research and investigative studies into a particular response (e.g. product yield or growth rate), require the optimisation of the system being used (Deming, 1985). In the "classical" approach, the problem would be resolved by holding the majority of the variables at a constant value and observing the effect of altering one of the independent factors, i.e. a single dimensional study. This arrangement is both costly and time consuming and may not prove to be fully satisfactory due to changes in the background conditions that may occur during the lengthy experimental period (Davies, 1971).

An alternative to this approach is the concept of factorial designs where the different variables are varied independently in order to determine their effect. This can lead to an extremely high number of trials. Evaluating seven nutrients at two concentrations for example, would require $2^7$ trials. In this case an experimental design is needed so that a large number of variables can be examined. One trend has been the development of more efficient screening designs, that is the number of experiments is only slightly greater than the number of variables studied. Box (1960), has used the term "saturated" to describe a design in which all of the available degrees of freedom are used to determine main effects. Chemical research workers now have available a wide variety of screening designs differing in experimental layout and degree of saturation for any particular problem.
Williams (1963), compared three designs, a fractional factorial, a Plackett-Burman and a random balance. Of the three designs studied, the most information was obtained from the Plackett-Burman design. The random design was ineffective because the lack of orthogonality confounded main effects and led to the determination of only a few significant variables. The fractional factorial design was no better because main effects and two-factor interactions were completely confounded. Therefore, an experimental situation where there are many variables and high variance, is best suited to the Plackett-Burman design.
The Plackett-Burman design is a highly fractionated form of a complete two factorial design and allows the investigation of up to N-1 variables in N experiments. Designs for 8, 16, 20, 24 and up to 100 experiments (at increments of four) may be selected, depending on the number of independent variables of interest. A number of variables can be tested at two levels allowing the interactions of all the main components at the appropriate level. Each design consists of a grid with N (the number of trials) rows and columns. Each row represents a trial and each column represents an independent or dummy variable. Each grid is generated by cyclically moving the first row one position at a time. Finally a row of minus signs are added to complete the grid. The first row of the smaller Plackett-Burman designs are listed below:

\[
\begin{align*}
N=8 & \quad +++-+-- \\
N=12 & \quad ++-+++-+--+-- \\
N=16 & \quad +++++-+-++-++---
\end{align*}
\]

At least three dummy variables should be used to estimate the experimental error. The elements + and - represent two different levels of the independent variable under investigation.
This design requires the frequency of each level in a given column to be equal and that each row having the same level of one variable must have an equal frequency of each level of other independent variables. Although the difference between the levels of each variable should be large enough to ensure that the effect of the variable can be identified, caution must be exercised when setting the level different for sensitive variables, since a differential too large could mask the results of the other variables. Once the independent variables and corresponding levels have been selected, the trials are performed in random order to eliminate any bias in experimentation. This response(s) of interest, such as improved productivity, is measured for each trial (Greasham and Inamine, 1986).

**EXPERIMENTAL DESIGN**

The Plackett-Burman technique was used for the investigation of 8 variables in 12 experiments (N=12). Flasks were prepared using the component concentrations in accordance with the Plackett-Burman grid (Fig 103). These were incubated for 96 hours at 30°C. Each row of the grid represents an experimental trial (flask) and each column represents an independent variable (component). Three dummy variables were included to estimate the experimental error. The dummy variables were placed randomly across the grid and were used for calculation purposes only. The elements + and - represent the two different levels of the independent variables under investigation (Fig 103).
Fig 102. The concentration of independent variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Concentration + (i.e. high concentration)</th>
<th>Concentration - (i.e. low concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Glycerol</td>
<td>20 g</td>
<td>5 g</td>
</tr>
<tr>
<td>2 FeSO₄</td>
<td>0.28 g</td>
<td>0.07 g</td>
</tr>
<tr>
<td>3 CaCO₃</td>
<td>4 g</td>
<td>2 g</td>
</tr>
<tr>
<td>4 Modified Starch</td>
<td>10 g</td>
<td>5 g</td>
</tr>
<tr>
<td>5 Soy Meal</td>
<td>15 g</td>
<td>3.75 g</td>
</tr>
<tr>
<td>6 Casein Hydrolysate</td>
<td>4 g</td>
<td>2 g</td>
</tr>
<tr>
<td>7 KH₂PO₄</td>
<td>0.1 g</td>
<td>0.025 g</td>
</tr>
<tr>
<td>8 Threonine</td>
<td>10 mM</td>
<td>2.5 mM</td>
</tr>
</tbody>
</table>

Fig 103. Plackett-Burman design for 12 trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Glycerol</th>
<th>Mod Stch</th>
<th>Dummy</th>
<th>Casein</th>
<th>FeSo₄</th>
<th>Dummy</th>
<th>CaCO₃</th>
<th>Threon</th>
<th>KH₂PO₄</th>
<th>Soy Meal</th>
<th>Dummy</th>
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<tbody>
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</table>
As shown in Fig 102, eight variables were selected for this study leaving three dummy variables from which the error was estimated. Inspection of variable 1 (glycerol), shows that during the twelve experiments, it appears at its high concentration six times and at its low concentration six times. This is also true for all of the other variables. The effect of a variable on the response is simply the difference between the average value of the response for the runs at the high level and the average value of the response for the six runs at the low level. This is represented by Fig 104:

Fig 104.

$$E_1 = \frac{R.at.(+)}{6} - \frac{R.at.(-)}{6}$$

Where $E_1$ = The effect of $x_1$ and $R$ = response or result.

The question of ‘how can one variable be distinguished when all the other variables are changing at the same time?’ can be answered upon examination of the matrix (Fig 103). When variable 1 (glycerol) is at its high level, variable 2 (FeSO\textsubscript{4}) is at its high level three times and at its low level three times. Likewise, when glycerol is at its low level, FeSO\textsubscript{4} is at its high level three times and at its low level three times. Thus the net effect of changing the concentration of FeSO\textsubscript{4} cancels out in calculating the effect of glycerol. The remaining variables balance out in the same way so that the net difference is the effect of glycerol only (Stowe and Mayer, 1966).
In the matrix, three dummy variables are included. The effects of the dummy variables are calculated in the same way as the effects of the real variables. If there are no interactions and all levels are reproduced perfectly with no error in measuring response, the effect shown by the dummy variable will be zero. If the effect is not equal to zero, it is assumed to be a measure of the lack of experimental precision plus any analytical error in measuring the response. Usually, the three estimates of experimental error, that is the three dummy variables will provide adequate confidence.

Fig 105 indicates how the dummy effects are combined to estimate the variance of an effect:

Fig 105.

\[ V_{\text{eff.}} = \sum (Ed)^2 / n = Ed_1^2 + Ed_2^2 + Ed_3^2 \]

where \( V_{\text{eff.}} \) = variance of an effect, \( Ed = \) effect shown by a dummy and \( n = \) number of dummy variables (Greasham and Inamine, 1986). The relationship between the variance of an effect and the standard error of an effect is shown in Fig 106:

Fig 106.

\[ \text{S. Eff.} = \sqrt{V_{\text{eff.}}} \]

Thus from the responses of the twelve runs, it is possible to calculate the effect of each of the eight real variables and to find the standard error of the effects of the dummies. The significance of each effect can be determined by using the familiar t-test (Greasham and Inamine, 1986), as shown in Fig 107.
In this case, the three dummy variables provide three degrees of freedom for entering the tabulated values of $t$. The t-test identifies whether there is a significant difference between the mean of the high (+) levels and the mean of the low (-) levels. It is a measure of the likelihood of the observed results occurring by chance. For example, a 95% significance means that 95% of the time, the observed results are likely to happen and are only likely to occur purely by chance 5% of the time.

According to Stowe and Mayer (1966), confidence levels should be 70% or greater and therefore any significance above 70% is accepted. If the probability of the event occurring by chance is sufficiently small, then the idea that the effect is caused by a change in the levels is accepted. Any positive T-tests indicate that the high level has had an effect compared to the low level, and any negative T-test values indicate that the low level has had an effect compared to the high level. A high negative T-test implies that the low concentration is more beneficial and the high concentration may be inhibitory. Low significance either positive or negative T-test reveals that there is no difference between the low and the high concentrations of the variables.
8.2 RESULTS

Fig 108. Results of the Plackett-Burman complex medium development.

<table>
<thead>
<tr>
<th>Flask Number</th>
<th>Biomass g/l</th>
<th>Clavulanic acid g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.20</td>
<td>0.095</td>
</tr>
<tr>
<td>2</td>
<td>5.00</td>
<td>0.000</td>
</tr>
<tr>
<td>3</td>
<td>4.90</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>4.70</td>
<td>0.035</td>
</tr>
<tr>
<td>5</td>
<td>6.85</td>
<td>0.060</td>
</tr>
<tr>
<td>6</td>
<td>5.53</td>
<td>0.030</td>
</tr>
<tr>
<td>7</td>
<td>5.42</td>
<td>0.045</td>
</tr>
<tr>
<td>8</td>
<td>7.92</td>
<td>0.070</td>
</tr>
<tr>
<td>9</td>
<td>6.60</td>
<td>0.065</td>
</tr>
<tr>
<td>10</td>
<td>6.85</td>
<td>0.010</td>
</tr>
<tr>
<td>11</td>
<td>4.60</td>
<td>0.080</td>
</tr>
<tr>
<td>12</td>
<td>4.76</td>
<td>0.000</td>
</tr>
</tbody>
</table>

It is clear from Fig 108, that the different combinations of medium components affected biomass concentration and clavulanic acid formation. The highest clavulanic acid titre obtained was 0.095 g/l from flask 1, whereas the lowest obtained was 0 g/l. Similarly, the highest biomass concentration reached 7.92 g/l from flask 8, whilst the lowest only produced 4.6 g/l. In order to determine the effects of the media components upon the culture, this data was further analysed using the Plackett-Burman technique.
Fig 109. The effect of medium components upon biomass formation and clavulanic acid production.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect Upon Biomass</th>
<th>Effect Upon Clavulanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>0.448</td>
<td>0.0375</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>0.992</td>
<td>0.0092</td>
</tr>
<tr>
<td>Dummy</td>
<td>-0.078</td>
<td>-0.0098</td>
</tr>
<tr>
<td>Casein Hydrolysate</td>
<td>-0.0508</td>
<td>0.002</td>
</tr>
<tr>
<td>FeSO4</td>
<td>-0.75</td>
<td>0.0235</td>
</tr>
<tr>
<td>Dummy</td>
<td>0.46</td>
<td>0.0035</td>
</tr>
<tr>
<td>CaCO3</td>
<td>-0.71</td>
<td>-0.0235</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.53</td>
<td>-0.0099</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.39</td>
<td>0.031</td>
</tr>
<tr>
<td>Soy Meal</td>
<td>0.81</td>
<td>0.02</td>
</tr>
<tr>
<td>Dummy</td>
<td>0.345</td>
<td>0.0025</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.33</td>
<td>0.0062</td>
</tr>
</tbody>
</table>

* The standard error of the effect on biomass and clavulanic acid was calculated according to Fig 106.

Again, a considerable difference was observed in terms of the effect that the different media components were having upon the *S. clavuligerus* culture. Some components such as modified starch had a high positive effect, whilst others such as CaCO3 displayed a negative effect on both biomass and clavulanic acid production. It was necessary to determine the significance of these findings and so a t-test was carried out (Fig 110).
Fig 110. T-test and the significance of the effect of the variables upon biomass and clavulanic acid production.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Biomass t-test</th>
<th>Biomass Significance</th>
<th>Clavulanic acid t-test</th>
<th>Clavulanic acid Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1.36</td>
<td>86%</td>
<td>6.05</td>
<td>99.54%</td>
</tr>
<tr>
<td>Mod. Starch</td>
<td>3.00</td>
<td>97%</td>
<td>1.48</td>
<td>88.47%</td>
</tr>
<tr>
<td>Dummy</td>
<td>-0.236</td>
<td>57.29%</td>
<td>-0.0036</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Casein Hydrol.</td>
<td>-1.54</td>
<td>88%</td>
<td>0.322</td>
<td>60.81%</td>
</tr>
<tr>
<td>FeSO4</td>
<td>-2.3</td>
<td>94%</td>
<td>3.8</td>
<td>98.4%</td>
</tr>
<tr>
<td>Dummy</td>
<td>1.4</td>
<td>87.2%</td>
<td>0.56</td>
<td>70%</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>-2.15</td>
<td>93.95%</td>
<td>-3.8</td>
<td>98.4%</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.6</td>
<td>89.60%</td>
<td>-1.45</td>
<td>88%</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.18</td>
<td>84%</td>
<td>5</td>
<td>99.23%</td>
</tr>
<tr>
<td>Soymeal</td>
<td>2.45</td>
<td>95%</td>
<td>3.2</td>
<td>97.53%</td>
</tr>
<tr>
<td>Dummy</td>
<td>1.045</td>
<td>80%</td>
<td>0.4</td>
<td>64.2%</td>
</tr>
</tbody>
</table>

Fig 110. displays the t-test results and the percentage significance of each variable upon biomass production and formation of clavulanic acid. It can be observed again that a significant variation was obtained over the various medium components, and that although a particular component may have a high significance for biomass production, it does not necessarily result in a high significance for clavulanic acid production. In order to simplify this data further, the components were tabulated in order of their significance for both biomass production and clavulanic acid formation (Fig 111 & 112).
### Fig 111. Ranking of variables: Clavulanic acid

<table>
<thead>
<tr>
<th>Variable</th>
<th>t-test value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>6.05</td>
<td>99.54%</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>5.00</td>
<td>99.23%</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>3.80</td>
<td>98.4%</td>
</tr>
<tr>
<td>Soy Meal</td>
<td>3.20</td>
<td>97.53%</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>1.48</td>
<td>88.47%</td>
</tr>
<tr>
<td>Casein Hydrolysate</td>
<td>0.32</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Threonine</td>
<td>-1.45</td>
<td>88%</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>-3.8</td>
<td>98.4%</td>
</tr>
</tbody>
</table>

### Fig 112. Ranking of variables: Biomass

<table>
<thead>
<tr>
<th>Variable</th>
<th>t-test value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Starch</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>Soy Meal</td>
<td>2.45</td>
<td>95</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.6</td>
<td>89.60</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.36</td>
<td>86</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.18</td>
<td>84</td>
</tr>
<tr>
<td>Casein Hydrolysate</td>
<td>-1.54</td>
<td>88</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>-2.15</td>
<td>93.95</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>-2.3</td>
<td>94</td>
</tr>
</tbody>
</table>
The significance of the effect of medium components on biomass formation and clavulanic acid production are displayed in Fig 111 & 112. In order to evaluate the actual effect of the medium components, it was reasonable to categorise the significance of the effect as low, high or none. Confidence levels below 70% were not accepted and were considered to be insignificant, levels between 70-90% were considered to be low significance and above 90% is considered to be high significance as the probability of occurrence by chance is negligible.

It can be observed from Fig 112 that in terms of biomass, all the variables tested were shown to be significant. Those which were less significant for the formation of biomass included, glycerol, threonine, KH$_2$PO$_4$ and casein hydrolysate. As there was sufficient carbon supplied to the culture as modified starch and protein and phosphate present in soy meal, it is possible that it would be slightly less significant to the culture if glycerol, KH$_2$PO$_4$ and casein hydrolysate were not present when compared to the other components. In addition, it is likely that threonine would be supplied to the culture via soy meal and so extra threonine would be unnecessary.

Modified starch was proven to have the highest significance for the formation of biomass (97%) and its high positive t-test score (3) indicates that it is most beneficial to the culture at its highest concentration of 10g/l. This must act as the preferable carbon source for cell formation.
When the variables involved in clavulanic acid formation were examined (Fig 111), the significance of many components changed. Casein hydrolysate which was shown to be of a low significance for biomass formation was found not to be of any significance for the production of clavulanic acid. Threonine was also found to be less significant than other medium components for the production of clavulanic acid. This is an unexpected finding as results indicate in a previous investigation (chapter 6), that threonine had a very positive effect upon the yields of clavulanic acid produced. It is very likely that as mentioned in biomass formation, there were significant concentrations of threonine present within the soy meal, and so the extra threonine did not have any significant effect on clavulanic acid formation.

Of all of the medium components examined, glycerol was proven to be of 99.54% significance and had a positive t-test value of 6.05. This indicates that glycerol had its most beneficial effect upon clavulanic acid production when it was present at its high value (20 g/l). KH₂PO₄, FeSO₄, and soy meal also displayed similar results (Fig 111). Glycerol has been shown to be an essential component for clavulanic acid production in chapter 7. It was demonstrated that under carbon limitation, the formation of clavulanic acid is abolished as the cell attempts to conserve the loss of an essential resource. It has also been shown in chapter 7, that under certain conditions, it is the C3 precursor (glycerol), which is rate limiting to clavulanic acid formation. The main effect of glycerol in the culture medium however, is to prevent the culture from becoming carbon limited.
Phosphate is also an essential component for living organisms. It appears from this study that it is also essential for the production of clavulanic acid. In an earlier chapter (Chapter 7, Metabolic Flux Analysis), it had been discussed that phosphate was required for the conversion of pyruvate to phosphoenolpyruvate for entry into the TCA cycle. It would be necessary for substantial fluxes of carbon to flow through the TCA as not only does it provide the ATP required for the cell, but also the precursor molecules required for the synthesis of arginine, the C5 precursor of clavulanic acid. Phosphate is therefore essential for the formation and supply of precursor molecules involved in clavulanic acid formation.

Although there is this requirement for phosphate, it is also inhibitory to have an excessive concentration of phosphate present within a production medium. Under conditions of excessive phosphate, catabolite repression occurs. This results in the abolition of clavulanic acid production (Demain, 1985).

Iron is known to be a key material for secondary metabolite formation in bacteria including Actinomycetes. In *Streptomyces* species, iron is required for the production of the antibiotics actinomycin, neomycin, streptomycin, chloramphenicol and mitomycin (Weinberg 1970). Ferrous iron has also been proven to be required for the production of cephamycin C from *S. clavuligerus* in a study carried out by Rollins et al., (1988).
It has also been proven that iron plays an essential role in the formation of the bicyclic nucleus of clavulanic acid (Elson et al., 1987). Pro-clavaminic acid, a monocyclic β-lactam has been shown to be the precursor molecule to clavaminic acid, the direct precursor molecule to clavulanic acid. The conversion of pro-clavaminic acid into clavaminic acid has been shown to be catalysed by a ferrous and α-ketoglutarate dependant oxygenase clavaminate acid synthase (CAS). Iron is therefore an essential required for the successful conversion of precursors into clavulanic acid, and it is therefore not surprising that it has found to be of high significance to clavulanic acid production in this study.

CaCO₃, was also shown to be of 98.4% significance regarding the formation of clavulanic acid. However, it also obtained a negative t-test value of -3.8 (Fig 110). This indicates that the lower level of CaCO₃ is beneficial for clavulanic acid production, whilst the high concentration may have a negative effect upon its production. As CaCO₃ also obtained a high negative t-test value for biomass formation, it would be advisable to use CaCO₃ at its low concentration in the new medium formulation.
Although the benefits of CaCO₃ are not obvious, it has been found to be a beneficial component of clavulanic acid production media since the first fermentations using *S. clavuligerus* were carried out. As discussed in chapter 6, *S. clavuligerus* has evolved with a full urea cycle presumable to enable the organism to deal with excessive toxic intracellular ammonia. If this is the case, and the organism is often exposed to high concentrations of ammonia, then the internal environment would experience a significant rise in pH. If CaCO₃ were to be taken up into the cell, it could certainly be beneficial in the serious task of re-stabilising the organisms internal pH. It may be possible that CaCO₃ may prove to be beneficial to the internal environment of the cell by having a buffering effect and helping to maintain intracellular pH.

Soy meal is a very important component of many commercial production media. It is the residue from soy beans after the extraction of soybean oil, and is a very complex substrate. Analysis shows that soy meal frequently contains a protein content of 50%, a carbohydrate content of 30%, a residual fat content of 1% and 1.8% lecithin. It is frequently used in antibiotic producing fermentations because of the slow metabolism of the mixture. This prevents catabolite repression from occurring (Crueger and Crueger 1989). Because it is rich in many essential nutrients, soy meal was shown to be highly beneficial not only for biomass production, but also for the formation of clavulanic acid in this study (Fig 111 & 112).
From this data, it has become possible to identify a basic complex media formulation which encourages substantial growth of *S. clavuligerus* and good production of clavulanic acid. Plackett-Burman analysis has indicated that several of the medium components would have their most beneficial effect by being present in their high concentrations. These are glycerol, KH$_2$PO$_4$, FeSO$_4$, Soy meal and modified starch.

Although glycerol was shown to be of low significance for biomass production, it was essential for clavulanic acid formation and so it was decided to keep it within the medium. KH$_2$PO$_4$ was also shown to be essential for clavulanic acid formation, although it was only shown to be of 84% significance to biomass production. Despite this, it did obtain a high t-test value for biomass production, and so it was also included in the new formulation. Soy meal and FeSO$_4$ were shown to be of great significance to both biomass and clavulanic acid formation and so they were included at their high concentrations. Although modified starch was not shown to be significantly involved in the formation of clavulanic acid, it was the primary source of carbon for biomass production and so it was also included.

CaCO$_3$ was shown to be of high significance for biomass and antibiotic production in this investigation. However, in both cases it obtained a high negative t-test value (Fig 111 & 112). This indicated that it would be most beneficial to all aspects of the culture at its low concentration. This was taken into consideration.
Threonine was shown to be of a relatively low significance for both clavulanic acid and biomass production, and as amino acids would be supplied to the culture via soy meal, it was decided to remove this from the complex medium. In addition, as individual amino acids are expensive, it is unlikely that threonine would be considered to be a realistic component in a commercial fermentation.

Casein hydrolysate was another component which failed to be of substantial significance in this study. It was decided to omit this from future complex media designs.
8.3 OPTIMISED COMPLEX MEDIUM FORMULATION

After the Plackett-Burman analysis, the new complex medium formulation was obtained (Fig 113).

Fig 113.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>20 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.1 g</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>0.28 g</td>
</tr>
<tr>
<td>Soy Meal</td>
<td>15 g</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>10 g</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>2 g</td>
</tr>
</tbody>
</table>

This formulation was tested in shake flask culture according to Chapter 2. The results of the new medium formulation were compared with the Plackett-Burman flask results (Fig 114).
Fig 114. Comparison of optimised complex formulation against Plackett-Burman flask results.

<table>
<thead>
<tr>
<th>Flask Number</th>
<th>Biomass g/l</th>
<th>Clavulanic acid g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.20</td>
<td>0.095</td>
</tr>
<tr>
<td>2</td>
<td>5.00</td>
<td>0.000</td>
</tr>
<tr>
<td>3</td>
<td>4.90</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>4.70</td>
<td>0.035</td>
</tr>
<tr>
<td>5</td>
<td>6.85</td>
<td>0.060</td>
</tr>
<tr>
<td>6</td>
<td>5.53</td>
<td>0.030</td>
</tr>
<tr>
<td>7</td>
<td>5.42</td>
<td>0.045</td>
</tr>
<tr>
<td>8</td>
<td>7.92</td>
<td>0.070</td>
</tr>
<tr>
<td>9</td>
<td>6.60</td>
<td>0.065</td>
</tr>
<tr>
<td>10</td>
<td>6.85</td>
<td>0.010</td>
</tr>
<tr>
<td>11</td>
<td>4.60</td>
<td>0.080</td>
</tr>
<tr>
<td>12</td>
<td>4.76</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Optimised Formulation</strong></td>
<td><strong>7.5</strong></td>
<td><strong>0.12</strong></td>
</tr>
</tbody>
</table>

As it can be observed from (Fig 114), that the new complex formulation performed very well in comparison to the Plackett-Burman flask experiment.
Although the biomass concentration was not as high as had been observed from flask number 8 (7.5 g/l compared to 7.9 g/l), clavulanic acid production was 0.025 g/l greater than obtained from the best Plackett-Burman flask.

Plackett-Burman analysis therefore provided a successful tool for the improvement of complex media and as a result, clavulanic acid yields.

8.4 FURTHER MEDIA DEVELOPMENT

When using the Plackett-Burman statistical approach it is important not to include very similar components that may interact with one another and produce inaccurate results. It would therefore be inadvisable to examine a range of complex nitrogen sources for example.

In this investigation traditional methods were used to examine the suitability of a range of different complex nitrogen sources. Their performance was compared to that of soy meal to determine if further improvement of the complex media formulation could be made.
In addition to soy meal, 3 complex nitrogen sources were examined. These were, Edamin, an enzymatic digest of lactalbumin, Primatone, an enzymatic digest of selected animal tissue, and Hy-Yest, a spray-dried extract from primary grown yeast. The nitrogen sources were kindly donated by Quest International, Sheffield.

The complex nitrogen sources all were slightly different in terms of chemical composition. Quantities of these were adjust so that each nitrogen source provided the same concentration of total nitrogen, phosphate and potassium to the culture. This was carried out so that the only difference between the nitrogen sources were the distributions of the amino acids. The distributions of amino acids are displayed in figures 115 - 118.
Soy meal consisted of a range of amino acids in different quantities. The amino acids that were present in the greatest quantities were, glutamate, aspartate and arginine. In total, soy meal consisted of 597.8 mg/g of total amino acids.
Primatone consisted of 801.2 mg/g total amino acids. Many of the amino acids were present in high quantities, but glutamate, glycine, serine, isoleucine and aspartate were predominant.
Glutamate, aspartate and lysine were the most predominant amino acids contained within Hy-Yest. There is 621 mg/g of total amino acid present within this nitrogen source.
Edamin consists of 863.2 mg/g total amino acids. The most predominant of these are glutamate, leucine and aspartate.

The complex nitrogen sources were then tested in shake flask culture using the new complex medium formulation but substituting soy meal for each of the alternative nitrogen sources. Shake flasks were incubated for 4 days before analysing for culture biomass and clavulanic acid production. The results are displayed in Fig 119.
8.5 RESULTS

Fig 119. The effect of nitrogen source upon biomass and clavulanic acid formation

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Biomass</th>
<th>Clavulanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy Meal</td>
<td>7.5 g/l</td>
<td>0.15 g/l</td>
</tr>
<tr>
<td>Primatone</td>
<td>6.8 g/l</td>
<td>0.08 g/l</td>
</tr>
<tr>
<td>Hy-Yest</td>
<td>6.6 g/l</td>
<td>0.1 g/l</td>
</tr>
<tr>
<td>Edamin</td>
<td>7.2 g/l</td>
<td>0.23 g/l</td>
</tr>
</tbody>
</table>

It can be observed from fig 119, that all of the nitrogen sources examined in this study were capable of supporting a similar concentration of biomass.

Of all the products tested however, Edamin resulted in the greatest yield of clavulanic acid being produced. When the amino acid profile of Edamin is examined, the reason behind its success becomes clear. Compared to the other nitrogen sources examined, Edamin contained the highest concentration of threonine (45.3 mg/g) and isoleucine (51 mg/l) (Fig 118). These two amino acids have been previously shown to stimulate clavulanic acid formation possibly by allowing a greater flux of carbon to be directed into clavulanic formation (Chapter 7). Edamin also contains the highest concentration of total amino acids in comparison to the other nitrogen sources examined. As this product provides a substantial quantity of amino acids to the culture, it is possible that carbon and nitrogen can be diverted from the synthesis of these amino acids within the cell, into the formation of other compounds.

This could result in greater resources being available for clavulanic acid production.
Primatone was the product which did not support substantial clavulanic acid formation when compared to the other nitrogen sources tested (0.08 g/l compared to 0.15 g/l) (Fig 119). It did contain a high concentration of total amino acids, however when the distribution of these were examined, it was found to particularly low in both threonine and isoleucine (Fig 116). The amino acids which were found to be present in substantial quantities within this product were glutamate, glycine and proline.

Upon examination of the dendrogram produced in chapter 6 which correlates amino acids by their significance upon clavulanic acid formation, these three amino acids appear to be very poorly correlated to clavulanic acid production (Fig 77). It is possible that this is the reason behind this product's poor performance.

Hy-Yest supported 0.1 g/l of clavulanic acid. This was not as high as titres obtained with either soy meal or Edamin, but was greater than that observed with Primatone (Fig 119). Although in terms of its amino acid distribution it was highest in glutamic acid, which is not highly correlated to clavulanic acid formation, it did not exert an inhibitory effect upon clavulanic acid synthesis.
CONCLUSION

By using Plackett-Burman analysis, it had become possible to optimise a complex medium suitable for the production of clavulanic acid.

By using data obtained from the metabolic pathway analysis (Chapter 6), it has become possible to identify the best nitrogen source for clavulanic acid production based upon its amino acid distribution. This has enabled a specialised medium formulation to have been developed.

The final medium formulation is displayed in Fig 120.

Fig 120. Final complex medium formulation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>20 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.1 g</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.28 g</td>
</tr>
<tr>
<td>Edamin</td>
<td>15 g</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>10 g</td>
</tr>
</tbody>
</table>
9.0 FURTHER INVESTIGATION

To fully optimise the metabolic flux model there are a number of areas which could be further investigated which fell outside of the scope of this project.

It was discovered that under certain conditions *S. clavuligerus* produces significant quantities of extracellular polysaccharide presumably as a method of balancing cell metabolism. This was not experimentally quantified under all conditions examined and so for particular models this had to be predicted by carbon balancing. This meant that any carbon that could not be accounted for in other reactions was considered to be used in polysaccharide production. This was acceptable under these circumstances but to improve the accuracy of the model, polysaccharide measurements should be made under all experimental conditions.

The stochiometric model itself was based upon current literature from a number of organisms and not necessarily only *S. clavuligerus*. This is because at present, a complete set of data on the biosynthetic pathway of clavulanic acid is not readily available in the literature. This model has therefore been constructed with the best data available. As further research is carried out upon the biosynthesis of clavulanic acid, more information regarding the biochemical pathway will become available. This will enable a more accurate model to be constructed, and an even more precise picture of *S. clavuligerus* metabolism to be identified.
10.0 REFERENCES


Box, G. Behnken, D. (1960) Some new three level designs for the study of quantitative variables. Technometrics 2, 455 - 475.


Ives, P. R., Bushell, M. E. (1997) Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*. Microbiology, 143, 3573-3579.


Jones, C., Thompson, A., England, R. (1996) Guanosine 5'-diphosphate 3'-diphosphate (ppGpp), guanosine 5'-diphosphate 3'-monophosphate (ppGp) and antibiotic production in *Streptomyces clavuligerus*. Microbiology, 142, 1789-1795.


This is the model for *S. clavuligerus* incorporating glycerol as the carbon source.

**Glycerol in.**

1. \[ \text{GLYC} + 0.333 \text{ ATP} + 0.333 \text{ FAD} \rightarrow \text{GAP} + 0.333 \text{ ADP} + 0.333 \text{ FADH} \]
2. \[ 2 \text{ GAP} + 0.167 \text{ ADP} \leftrightarrow \text{FRU6P} + 0.167 \text{ ATP} \]
3. \[ \text{FRU6P} \leftrightarrow \text{GLUC6P} \]
4. \[ \text{GAP} + 0.333 \text{ ADP} + 0.333 \text{ NAD} \rightarrow \text{G3P} + 0.333 \text{ ATP} + 0.333 \text{ NADH} \]
5. \[ \text{G3P} \leftrightarrow \text{PEP} \]
6. \[ \text{PEP} + 0.333 \text{ ADP} \rightarrow \text{PYR} + 0.333 \text{ ATP} \]
7. \[ \text{PYR} + 0.333 \text{ NAD} \rightarrow 0.667 \text{ ACCOA} + 0.333 \text{ CO2} + 0.333 \text{ NADH} \]
8. \[ \text{ACCOA} + 2 \text{ OAA} \rightarrow 3 \text{ ISOCIT} \]
9. \[ \text{ISOCIT} + 0.167 \text{ NADP} \rightarrow 0.833 \text{ ALFAKG} + 0.167 \text{ NADPH} + 0.167 \text{ CO2} \]
10. \[ \text{ALFAKG} + 0.2 \text{ NAD} + 0.2 \text{ ADP} \rightarrow 0.8 \text{ SUC} + 0.2 \text{ CO2} + 0.2 \text{ NADH} + 0.2 \text{ ATP} \]
11. \[ \text{SUC} + 0.25 \text{ FAD} \rightarrow \text{MAL} + 0.25 \text{ FADH} \]
12. \[ \text{MAL} + 0.25 \text{ NAD} \rightarrow \text{OAA} + 0.25 \text{ NADH} \]

**Pentose phosphate pathway**

13. \[ \text{RIB5P} \leftrightarrow \text{RU5P} \]
14. \[ \text{XYL5P} \leftrightarrow \text{RU5P} \]
15. \[ 4 \text{ SED7P} + 0.6 \text{ GAP} \leftrightarrow \text{XYL5P} + \text{RIB5P} \]
16. \[ 0.857 \text{ FRU6P} + 0.571 \text{ E4P} \leftrightarrow \text{SED7P} + 0.428 \text{ GAP} \]
17. \[ 1.2 \text{ FRU6P} + 0.6 \text{ GAP} \leftrightarrow \text{XYL5P} + 0.8 \text{ E4P} \]
Amino acid synthesis

18: \( \text{PYR} + 0.1667 \text{NADPH} + 0.833 \text{GLU} + 0.333 \text{ACCOA} \rightarrow \text{LEU} + 0.833 \text{ALFAKG} + 0.333 \text{CO}_2 + 0.1667 \text{NADP} \)

19: \( \text{OAA} + 1.25 \text{GLU} \Leftrightarrow \text{ASP} + 1.25 \text{ALFAKG} \)

20: \( \text{ASP} + 0.5 \text{NADPH} + 0.25 \text{ATP} + 1.25 \text{GLU} + 0.75 \text{PYR} \rightarrow 1.5 \text{LYS} + 1.25 \text{ALFAKG} + 0.25 \text{CO}_2 + 0.25 \text{ADP} + 0.5 \text{NADP} \)

21: \( \text{ASP} + 0.5 \text{ATP} + 0.5 \text{NADPH} \rightarrow \text{THR} + 0.5 \text{ADP} + 0.5 \text{NADPH} \)

22: \( \text{G3P} + \text{GLU} + \text{ACCOA} + \text{NAD} \rightarrow \text{ALFAKG} + \text{NADH} + \text{CYS} \)

23: \( \text{ASP} + 0.25 \text{ATP} + 0.5 \text{NADPH} + 0.75 \text{CYS} \rightarrow \text{MET} + 0.75 \text{PYR} + 0.25 \text{NH}_4 \)

24: \( \text{ALFAKG} + 0.2 \text{NH}_4 + 0.2 \text{NADPH} \Leftrightarrow \text{GLU} + 0.2 \text{NADP} \)

25: \( \text{GLU} + 0.2 \text{ATP} + 0.2 \text{NH}_4 \rightarrow \text{GLN} + 0.2 \text{ADP} \)

26: \( \text{GLU} + \text{GLN} + 0.2 \text{CO}_2 + 0.8 \text{ASP} + 0.8 \text{ATP} + 0.2 \text{NADPH} \rightarrow 1.2 \text{ARG} + 0.8 \text{ADP} + 0.2 \text{NADP} + 0.8 \text{MAL} + \text{ALFAKG} \)

Anaplerotic reaction

27: \( \text{PEP} + 0.333 \text{CO}_2 \rightarrow 1.333 \text{OAA} \)

Oxydative phosphorylation

28: \( 2 \text{NADH} + \text{O}_2 + 4 \text{ADP} \rightarrow 4 \text{ATP} + 2 \text{NAD} \)

29: \( 2 \text{FADH} + \text{O}_2 + 2 \text{ADP} \rightarrow 2 \text{ATP} + 2 \text{FAD} \)

ATP dissipation

30: \( \text{ATP} \rightarrow \text{ADP} \)
Biomass production

31: 0.03 GLUC6P + 0.011 FRU6P + 0.113 RIB5P + 0.036 E4P + 0.01 GAP + 0.113 G3P + 0.039 PEP + 0.094 PYR + 0.165 ACCOA + 0.075 ALFAKG + 0.11 OAA + 0.05 LYS + 0.2 NH4 + 0.031 GLU + 0.031 GLN + 0.973 ATP + 0.343 NADPH + 0.078 NAD + 0.065 LEU + 0.019 MET + 0.024 THR => BIOM + 0.973 ADP + 0.343 NADP + 0.078 NADH + 0.036 CO2

Clavulanic acid production

32: PYR => C3 PREC

33: C3 PREC + 2 ARG + 0.667 NADPH + 0.333 FAD => 3 CLAY + 0.667 NADP + 0.333 FADH

34: GLUC6P + 0.167 ATP => POLYSACE + 0.167 ADP

35: NADPH ⇔ NADH

Amino acid feed

36: AMAE => AMA (Where AMAE is the amino acid being fed)
KEY

ACCOA - Acetyl CoA
ATP - Adenosine triphosphate
ADP - Adenosine diphosphate
ALFAKG - Alphaketoglutarate
Arg - Arginine
Asp - Aspartate
Biom - Biomass
C3 prec - C3 precursor
Clav - Clavulanic acid
CO2 - Carbon Dioxide
CYS - Cysteine
E4P - Erythrose-4-phosphate
FAD - Flavin adenine dinucleotide
FADH - Flavin adenine dinucleotide reduced
Fru6p - Fructose-6-phosphate
G3P - Phosphoglycerate
GAP - Glyceraldehyde-3-phosphate
GLU - Glutamate
GLN - Glutamine
Gluc6p - Glucose-6-phosphate
Glyc - Glycerol
<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Chemical Name</th>
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<tbody>
<tr>
<td>Isocit</td>
<td>Isocitrate</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LYS</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAL</td>
<td>Malate</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
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</tr>
<tr>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>Ammonia</td>
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<td>O2</td>
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<td>Sedheptulose-7-phosphate</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>XYL5P</td>
<td>Xylulose-5-phosphate</td>
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