RECOMBINANT PROTEIN PRODUCTION USING *E. COLI* CONTAINING TEMPERATURE-SENSITIVE CONSTRUCTS

by

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Do your best to present yourself to God as one approved, a workman who does not need to be ashamed and who correctly handles the word of truth.

2 Timothy 2, v15.

ACKNOWLEDGEMENT.

I would like to thank my supervisor, Dr Neville Fish, along with Dr Mike Hoare, for their help, guidance, and criticism during this work.

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ABSTRACT.

The principles of large scale process design for the use of *E. coli* strains containing temperature sensitive constructs were developed. Two strains were characterised in a 14L fermenter, one containing a constant copy number plasmid (pCQV2), and the other an amplifiable copy number plasmid (pMG169). Batch fermentations were performed under kinetic limitation to determine key parameters such as optimum conditions for growth and production, oxygen requirements, and the effects of different lengths of time for the implementation of the temperature shift.

These results suggested that on the large scale oxygen limitation was likely to occur. Therefore fermentations were performed with a limiting oxygen transfer rate typical of a $100m^3$ reactor (50-60 $mM.L^{-1}h^{-1}$). Batch productivity under oxygen limitation fell by between 84% and 87%.

The negative effects of oxygen limitation were removed by fermenter operation under glucose limitation, which yielded maximum batch productivity of the three strategies. At all times the strain containing plasmid pMG169 yielded higher batch productivities than the one containing pCQV2.

The heat of fermentation was measured (using a continuous calorimetric method) as a means of partially implementing the temperature shift. The results differed from those obtained from widely used empirical correlations (which should not be used with recombinant strains).

Design calculations were performed that allow batch and annual titres to be predicted under different fermenter configurations, scales, and modes of operation.

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ABBREVIATIONS.

Abbreviation.	Explanation.
Adj OD_{670}	Adjusted OD_{670}.
Ap^R	Ampicillin Resistant.
C	CAT concentration.
CAT	Chloramphenicol Acetyl Transferase.
CER	Carbon Dioxide Evolution Rate.
C_p	Specific Heat Capacity.
D	Dilution Factor.
D'	Accumulated Dilution Factor.
DOT	Dissolved Oxygen Tension.
dcw	Dry Cell Weight.
F	Glucose Feed-Rate.
k	Specific Death Rate.
k	Arrhenius Constant.
m'	Mass Flowrate of Air.
μ	Specific Growth Rate.
N	Number of Cells.
n/a	Not Applicable.
OD_{670}	Optical Density (at 670 nm).
OUR	Oxygen Uptake Rate.
OTR	Oxygen Transfer Rate.
P	Overall Batch Titre.
P_l	Batch Titre Per Litre.
p'	Specific Production Rate.
p''	Volumetric Production Rate.
Q_{ag}	Heat of Agitation.
Q_{cool}	Heat Removed by Cooling Circuit.

Q_{evap}	Heat Loss Due to Evaporation.
Q_f	Overall Heat of Fermentation.
Q_{sens}	Sensible Heat Loss to the Surroundings.
Q_{surr}	Heat Loss to the Surroundings.
q_f	Specific Heat of Fermentation.
RQ	Respiratory Quotient.
r	Linear Regression Coefficient.
Str^R	Streptomycin Resistant.
strain pCQV2	Strain *E. coli* RV308(pCQV2).
strain pMG169	Strain *E. coli* RV308(pMG169).
T	Temperature.
TI	Temperature Indicating.
t	Time.
t_d	Doubling Time.
ts	Temperature Sensitive.
t_1	Time in Glucose Limited Growth phase.
V_1	Working Volume.
w/v	Weight Per Volume.
X	Biomass Concentration.
X'	Biomass Generation Rate. Substrate.
X_{of}	Biomass Concentration at Onset of O_2-limitation.
X_{pf}	Final Biomass Concentration at End of Production Phase.
X_{po}	Initial Biomass Concentration at Beginning of Production Phase.

$Y_{(x/s)}$ Overall Yield of Biomass on Substrate (Glucose).

1 INTRODUCTION

The ability to manipulate the genetic material in microorganisms has had, and will continue to have, far-reaching implications for the biological and fermentation industries. Despite this, Zabriskie DW and Arcuri EJ (1986) found that only 6 of the nearly 250 papers published in 1985 in a leading biochemical engineering journal described research involving recombinant microorganisms. The same journal published nearly 50 papers concerned with cellulose utilisation. This shows there is a deficiency in the amount of published work about process design for the use of recombinant strains, and it is intended that this thesis should go some way towards correcting the imbalance.

Although many different microorganisms are being used as host strains for recombinant DNA studies, the majority employ either *Escherichia coli* or *Saccharomyces cerevisiae* (Klotz LC (1983)). The former is used because of the wealth of information known about its molecular biology and physiology, and also because of the expression systems that exist to control product expression. There are a number of problems associated with using this microorganism, among which are pyrogen production, improper folding of the protein product, product insolubility, and the inability to make certain post-translational modifications (eg glycosylations) (Klotz LC (1983)). Despite this the high growth and production rates (hence short fermentation

times), good expression systems, and comparitive cheapness of the fermentations mean that *E. coli* is likely to be the chosen microorganism for the production of many proteins and small molecules for some time.

S. cerevisiae is also widely used because of the large amount of information about its molecular biology and physiology. In addition it is generally regarded as safe, it has long been used in large fermenters, and it can glycosylate and secrete proteins (Innis MA *et al* (1985)). The disadvantages of this microorganism are the low level of cloned gene product expression, and incorrect protein glycosylations. However as these problems are overcome it is likely that yeasts will become increasingly important vehicles for expressing many different recombinant products.

Bacillus and *Streptomyces* species are also being used for recombinant DNA studies, as well as mammalian cells. It is possible that in the future there will be a variety of different hosts and vectors to choose from and ultimate choice of a suitable system for any product might be made on economical and/or regulatory grounds rather than purely technical ones.

The potential afforded by the ability to engineer microorganisms to produce highly valued proteins of industrial interest is on the way to being realised. This project concerned the process development for *E. coli* strains that contain temperature sensitive (ts) expression

switches for the production of foreign proteins. These constructs also show considerable potential as suitable expression systems for the production of small molecules, like amino acids, on scales similar to conventional antibiotic fermentations.

The techniques required to insert a foreign gene into a microorganism like *E. coli* are now well documented. Problems come in genotypic stability and controlled expression of the cloned gene, and stability of the active, foreign protein. There are also problems associated with the large-scale purification of the product.

Controlled expression of the foreign gene is necessary because constitutive production can interfere with cell growth, resulting in a reduced specific growth rate, or even cell death (Botterman JH *et al* (1985); Uhlin BE *et al* (1981)). In addition, gene expression can also result in plasmid segregational instability (Caulcott CA *et al* (1985)). The net effect of either of these is fermentations of low productivity. A widely used strategy to overcome these problems is to incorporate control systems into the cells that prevent product expression under the normal fermentation conditions, resulting in potentially high biomass concentrations, but which can be switched on and promote a high level of product expression during the production phase. In this way maximum levels of product can be attained.

Several such expression systems are used with varying

degrees of success, and these rely on chemical induction or derepression. However, this study was concerned with physically controlled systems. Expression levels were determined by the temperature of the culture. At the growth temperature the foreign gene was effectively switched off (repressed) and under these conditions high levels of biomass could be obtained. Then at a predetermined cell concentration the temperature was increased, which caused high level expression of the foreign gene. Thus the growth and production phases are conveniently dissociated from one another. For a fuller description of temperature sensitive expression systems see section 1.2.2.

Temperature sensitive (ts) expression systems have been used to very good effect in the shake-flask (eg Wright E *et al* (1986)). However, the process engineering necessary to use them on a large scale (typically 100m^3) has not yet been developed, and that was the aim of this research project.

1.1 EXPRESSION OF EUKARYOTIC DNA IN *E. COLI*.

The methods and problems of expressing eukaryotic DNA in the prokaryote, *E. coli*, are introduced here to allow a perspective on how ts constructs came to be developed.

Since *E. coli* is one of the most studied microorganisms more is known about the control of gene expression in this

bacterium than any other. It has long been known that the genes involved in a metabolic process tend to be congregated in a single transcription unit, known as an operon. Positioned at the beginning of the operon are the major control sites (called the operator and promoter) which govern expression of the structural genes downstream. When the metabolic state of the cell requires it an operon is transcribed into a polycistronic mRNA, which is then translated into the correct proteins at the ribosomes. The type of control exerted on an operon will depend on the function of the proteins expressed from the structural genes, but generally control will be exerted at the transcriptional stage, although translational control is also common (eg attenuation in the tryptophan operon).

The obvious strategy for the production of a recombinant, eukaryotic protein of industrial interest would be to clone the gene for that protein into a highly expressing strain with a high growth rate. Thus protein production would be maximised. However there are many biochemical reasons which greatly complicate this process. Once the techniques of gene cloning had been developed it was relatively easy to obtain high levels of proteins normally expressed in *E. coli* but whose genes had been deleted on the chromosome. It was very different though when the expression of eukaryotic proteins in prokaryotes was desired.

It was found that eukaryotic genomic DNA was not a suitable gene source for the construction of expression vectors.

Firstly it was known that eukaryotic genes contain intervening sequences of DNA (introns) that do not form part of the coding sequence. Eukaryotes possess a series of splicing enzymes that cut out these introns from the mRNA before it leaves the nucleus and is translated. Prokaryotes do not contain these splicing enzymes, so the mRNA would not be modified and a different protein from the one intended would be produced. This problem was overcome by using cDNA. This was constructed by taking the processed eukaryotic mRNA and producing the corresponding DNA sequence using the enzyme reverse transcriptase. The cDNA could then be cloned into a suitable expression vector (Old RW and Primrose SB (1985) or any modern molecular biology textbook).

Secondly transcriptional signals in prokaryotes are different from those in eukaryotes (Corden J *et al* (1980); Breathnach R and Chambon P (1981)), the problem being that *E. coli* does not recognise the eukaryotic signals and would therefore not transcribe the DNA properly, if at all. The solution was to clone the cDNA next to a controllable *E. coli* promoter. A termination signal was also cloned after the gene. Generally the vectors used for cloning were multicopy plasmids so that the gene coding for the product was present in the cell in high dosage (Uhlin BE and Nordstrom K (1979)).

Thirdly eukaryotic mRNA differs from prokaryotic mRNA in that the former has a polyadenylated 3' tail and a cap structure at the 5' end. Breathnach R and Chambon P (1981)

found this affected mRNA stability in eukaryotes, as well as ribosome binding. These difficulties were overcome by fusing the cDNA next to an *E. coli* gene in the vector so that the correct reading frame was maintained and the *E. coli* gene ribosome binding site and Shine-Delgarno sequence was utilised.

Fourthly there was the problem of codon usage. In prokaryotes the codons used in the expression of high level proteins are far from random, as demonstrated by Grantham R *et al* (1981). Certain codons for the same amino acid tend to be preferred to others. Ikemura T (1981) showed that there is a correlation between the popularity of different codons and the abundance of their corresponding tRNA species. This became a problem because codon preference differs between prokaryotes and eukaryotes, implying that popular codons in eukaryotes may not be so popular in prokaryotes. This would affect translational efficiency if there were few tRNA species present which corresponded to a popularly used codon. This problem could be overcome chemically by synthesising genes with codons corresponding to abundant tRNA species.

The problems mentioned so far have all been to do with the differences between the structures of eukaryotic and prokaryotic nucleic acids. There were further problems which were related to the protein products, and the different modifications they undergo after translation, as well as their degradation.

Bacterial proteins undergo a variety of modifications. The formyl group on the NH_2-terminal methionine residue is hydrolysed and one or more NH_2-terminal residues may be removed.

E. coli produces many proteins that are secreted to the periplasmic space, and these usually as large precursors which have additional hydrophobic NH_2-terminal signal sequences. These sequences are cleaved off by a membrane-bound enzyme to yield the active protein in its final form in the periplasmic space. Much use of this has been made in the expression of normally secreted eukaryotic proteins in *E. coli*. This will be dealt with more fully below.

Eukaryotes tend to modify their proteins in very different ways. The most common of these are glycosylation and phosphorylation, which do not occur in *E. coli*, but are thought to be essential to the activity and stability of the functional protein product. This presents problems when it is desired to produce a functional eukaryotic protein in *E. coli* which needs one or more of these modifications for functionality. To overcome this difficulty much work is being done on yeast systems, which are eukaryotic and glycosylate their proteins. However, yeasts do not do so in quite the same way as higher eukaryotes and so there are doubts as to whether they can be used to solve this problem.

There are still further problems concerning the stability

of foreign proteins in *E. coli*. The bacterium contains a group of proteolytic enzymes (also known as proteases) that degrade foreign proteins in the cell. Taniguchi T *et al* (1980) showed human fibroblast pre-interferon to be unstable, while Old RW and Primrose SB (1985) cite experiments which showed that the hormone Somatostatin is degraded by *E. coli* proteases. The main strategy that has been developed to overcome this problem is the fusion of the foreign protein to an *E. coli* protein. This is recognised as being native by the *E. coli* proteases and so gives protection to the foreign protein. Later the fusion protein can be removed *in vitro* to yield the exogenous protein. This was used successfully with the small hormone Somatostatin (14 amino acids), as its degradation was prevented by its fusion to β-galactosidase. The latter was later cleaved off using cyanogen bromide. The main drawback to this method is that while there are other examples of stabilisation occurring in this way, the subsequent cleaving and release of the native protein is not always possible. Also this technique has only been successful with small products. Despite these disadvantages some foreign proteins are formed as fusions.

The ampicillin resistance (Ap^R) gene found on plasmid pBR322 expresses the enzyme β-lactamase which cleaves the β-lactam ring of this type of antibiotic. This enzyme is found in the periplasmic space of cells expressing it and is formed as a preprotein with an additional 23 amino acid leader sequence which directs its transport across the membrane. The leader sequence is removed by a

membrane-bound enzyme yielding native, functional β-lactamase in the periplasmic space. If a foreign gene is cloned into the Ap^R gene a fusion protein should be produced which is secreted to the periplasmic space, away from the host proteases. Experiments of Talmadge K and Gilbert W (1980) showed that it is possible to fuse the gene for a normally secreted eukaryotic protein into the Pst 1 site of the β-lactamase gene giving a hybrid eukaryotic and prokaryotic signal sequence. The result was that the eukaryotic protein was found in the periplasmic space. This work showed that normally secreted eukaryotic proteins could be secreted in *E. coli*. Further investigation of the secretion processes followed (Koshland D *et al* (1982); Tommassen J *et al* (1983); Ito K *et al* (1981)) and it became clear that normally cytoplasmic proteins cannot always be engineered for secretion to the periplasmic space.

For industrial purposes it is necessary for the expression of cloned genes to be maximised. Several factors for this have already been discussed but there are still further points to discuss in the choice of a suitable expression system.

Most industrial expression systems have the foreign gene on a plasmid. Expression of foreign genes causes a reduction in the growth rate of the cell, and often low cellular yields (Botterman JH *et al* (1985)). This means that the level of product from a fermentation would be low. Therefore a good expression system would be one that was

repressed during the growth phase but which could be derepressed to express the foreign protein when there was a high cell concentration. In other words there needs to be a switch between the growth and production phases. This is achieved by cloning the foreign gene downstream of a promoter/operator site on the plasmid. Ideally this control site should completely switch off expression of the foreign gene during the growth phase, so no product is formed, while once derepressed it should promote expression very strongly. There are relatively few promoter/operator systems currently used and the most popular of these are discussed in section 1.2.

As well as having a strong promoter/operator system another way of optimising product formation is to have a high gene dosage (ie high number of copies of the foreign gene being expressed). This is done by using plasmids that are present in the cells in high copy number. However cells containing high copy number plasmids must maintain them at an energy cost to the cell, resulting in lower growth rates. Also some high copy number plasmids are unstable and will be lost. Celltech Ltd have developed a family of plasmids which are present in low copy number during the growth phase but on implementation of a temperature shift plasmid copy number amplification and expression of the foreign gene occur (Yarranton GT *et al* (1984); Wright E *et al* (1986)).

Plasmid stability is important because the expression of high copy number plasmids results in a reduction in the

cell's growth rate. Cells which lose the unstable plasmid would have higher growth rates than plasmid-containing cells, would outcompete them and so dominate the culture. This is obviously undesirable because it would result in low product yields.

1.2 EXPRESSION SYSTEMS.

The more commonly used expression systems are now introduced and discussed.

1.2.1 *Lac* and *Trp* Expression Systems.

Two categories of expression system occur naturally - inducible or repressible, and both types have been used industrially.

Inducible systems tend to come from operons used in the catabolism of substrates and are switched on when required. An example of such a system comes from the *lac* operon (used for the degradation of lactose).

Repressible systems tend to come from operons which control the synthesis of essential cellular components, and are switched off when the level of those components is adequate for the cell's metabolism. An example of a repressible

system comes from the *trp* operon (used for the synthesis of the essential amino acid tryptophan).

Both types of system require the metabolic state of the cell to be controlled to keep expression switched off during the growth phase. However, both switches are "leaky", resulting in some expression of the recombinant gene during this phase. This results in reduced growth rates and ultimately lower product yields. Expensive inducers and derepressors are added to the fermentation in order to switch on expression (IPTG (Isopropylthio-galactoside) in the case of *lac* and IAA (Indole acrylic acid) in the case of *trp*). This not only obviously raises the cost of the fermentation process (Remaut E *et al* (1983)) but also adds inert compounds which could contaminate the product, and increase the downstream processing requirements.

It was found that the *trp* switch prevented product expression during the growth phase more tightly than the *lac* switch, and was a more active promoter of production when derepressed (Harris TJR (1983)). The problem with the *trp* switch was that it was not active enough for industrial production purposes at low copy number but was too "leaky", causing plasmid instability and low product yields at higher copy number (Caulcott CA *et al* (1985)).

For a more extensive discussion of these types of expression systems refer to general biochemistry and molecular biology texts.

1.2.2 Temperature Sensitive Expression Systems.

The alternative to using a chemically activated switch is to use a physically activated one. The temperature switch has been very successfully used in the laboratory and pilot plant, and works on the basis that a repressor binds to an operator site at a temperature that permits growth. The switch is activated by an increase in temperature resulting in the loss of affinity between the repressor and operator such that the former no longer binds to the latter. The operator being derepressed, RNA polymerase binds to the promoter and expression of the gene(s) downstream occurs. The most commonly cited ts system is the phage λcI_{857} repressor coupled to either the early leftward- or rightward-reading promoters, P_L or P_R respectively. At 30°C the repressor binds to the operator, preventing expression from the promoter, while at 42°C complete derepression occurs. These systems tend to be far less "leaky" than the conventional ones already discussed (Vidal-Ingigliardi D and Raibaud O (1985)). This is largely due to the fact that there is enough λ repressor produced from each copy of the cI gene to prevent transcription from the multiple plasmid-borne copies of the λ promoters. This tends not to be true for the other expression systems. Bacteriophage are also a likely source of strong initiation sequences because phage will be in competition with the host cell to redirect its metabolism, especially during the lytic phase of their cycles. McKenney K *et al* (1981) found that the P_L promoter was 8-10 times more efficient than the *lac* promoter. Crowl

R *et al* (1985) found that it was the strongest and most tightly regulated expression system.

To maximise production of a foreign protein it is necessary to have a high copy number vector during the production phase (so that a high number of copies of the foreign gene are present) with a very tight switch so that little foreign protein is produced during the growth phase. High copy number vectors tend to be segregatively unstable unless they contain a marker that is selected for. Therefore plasmids have been constructed that are temperature-sensitive amplifiable copy number vectors (Uhlin BE *et al* (1979,1983); Yasuda S and Takagi T (1983); Larsen JEL *et al* (1984); Yarranton GT *et al* (1984); Wright E *et al* (1986); Remaut E *et al* (1983)). These are maintained at low copy number at the growth temperature, before replicating profusely upon a shift in temperature, along with expression of the foreign gene. Remaut E *et al* (1983) developed a vector to express T4DNA ligase, while cloning the cI repressor gene on a different, compatible plasmid. The cells were grown at 28°C and 3 hours after induction at 42°C, DNA ligase was found to be 20% of the total cellular protein. This represented some 40% of the total protein synthesis during the production phase. They also claimed that these types of vectors are universal in that they can be expressed in any *E. coli* strain. This is not true for vectors using the *lac* operator/promoter, which can only be fully repressed in strains carrying the *lac* I^q repressor gene. Comparison between proteins expressed from the amplifiable copy number vector and a constant copy

number ts plasmid (using the same ts system) showed that the former type produced 2.5 times more protein than the latter. Zabriskie DW and Arcuri EJ (1986) state that high copy number systems are typically more productive than low copy number ones. The copy number will also affect plasmid stability, higher copy number ones often being more stable than lower copy number systems. They then go on to say that the presence of high copy number plasmids can result in a decreased specific growth rate. Thus slight segregational instability can cause a rapid proportional increase in the number of plasmid-free cells.

Two different plasmids containing ts constructs were used in these studies and both were supplied by Celltech Ltd. One was the amplifiable copy number plasmid, pMG169 (Wright E *et al* (1986)), and the other was the constant copy number plasmid, pCQV2 (Queen C (1983)).

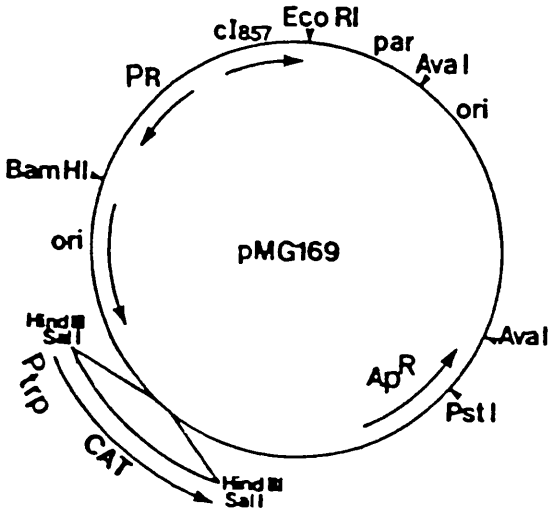


Figure 1.1: Map of Plasmid pMG169, Adapted From Wright E *et al* (1986).

Fig 1.1 shows a map of plasmid pMG169, adapted from Wright E *et al* (1986). The plasmid contains a copy of the cI_{857} repressor gene which is constitutively expressed, and the *par* sequence to ensure the plasmid is partitioned at the point of cell division. It also contains two origins of replication (*ori*), one being a low copy number *ori* (4 copies/cell) and constitutively expressed. The other is a high copy number *ori* whose expression is under the tight control of the λP_R promoter/operator sequence. Expression of the Chloramphenicol Acetyl Transferase (CAT) gene is under the control of the *trp* promoter. Thus at growth temperatures (typically 30°C) the plasmid is present at 4 copies/cell and the *trp* promoter/operator keeps expression of CAT repressed. However, at production temperatures (typically 42°C) the P_R promoter/operator is derepressed, the high copy number *ori* is expressed, and plasmid replication occurs to reach a copy number of around 150 copies/cell. Under these conditions the *trp* switch becomes so leaky that repression is practically nonexistent and CAT accumulates to a high concentration. Wright E *et al* (1986) achieved final CAT concentrations in excess of 20% total cell protein.

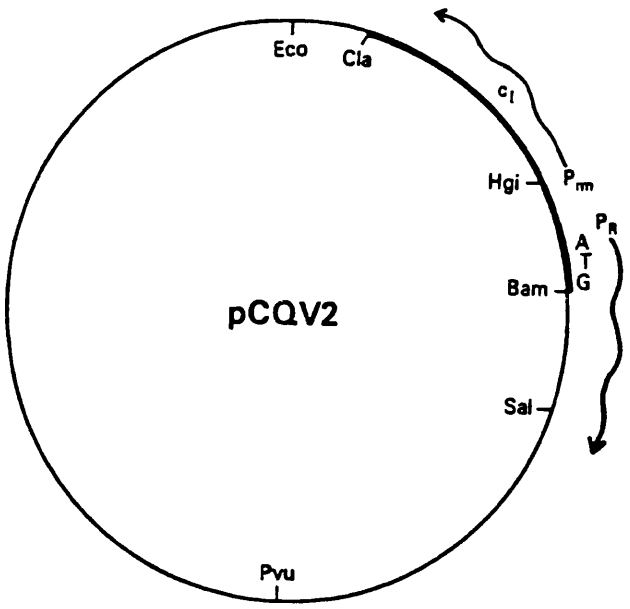


Figure 1.2: Map of Plasmid pCQV2, Adapted From Queen C (1983).

The second plasmid used in this work was pCQV2, developed by Queen C (1983) and shown in fig 1.2. This is a plasmid of constant copy number around 20 copies/cell, and uses the λP_R promoter/operator to control expression of the foreign structural gene, in this case also CAT. The background to the development of this vector was unpublished experiments which showed that the λP_R promoter initiates transcription more frequently than such bacterial promoters as *lac* and *gal*, and that the first gene (*cro*) on the P_R transcript

(mRNA) is translated well in relation to bacterial genes. pCQV2 was therefore constructed with the P_R promoter/ operator and the *cro* ribosomal binding site correctly orientated and the correct distance apart. The ribosomal binding site is just upstream of a BamH1 restriction enzyme site, which can be used for the incorporation of a foreign gene. The cI repressor gene is also present on the plasmid to code for the constitutive production of repressor used in the control of product expression. The vector was used to express β-galactosidase and a temperature shift from 32-42°C caused a 100-fold increase in the level of this protein to 7% total cell protein. No attempt was made to optimise expression from this vector by alterring media, bacterial strain, or fermentation time. The production phase lasted between 1 and 2 hours at 42°C to obtain maximal levels of the expressed protein.

The purpose of using two different types of ts constructs in the same host strain was to see the effects of the different strategies on the fermentation, and the wider effects on production and process economics.

1.3 THE HEAT-SHOCK PHENOMENON IN *E. COLI*.

It has long been observed that eukaryotes express proteins in different amounts when they are subjected to a heat-shock, whereas until recently the phenomenon had not been recognised in prokaryotes. Niedhart FC *et al* (1984)

cite their own work which showed that heat-shock causes dramatic changes (10- to 50-fold increase or decrease) in the levels of individual proteins produced. Proteins that showed enhanced production were termed htp proteins (high temperature production).

It is thought that the heat- shock response allows *E. coli* to adapt to environments at different temperatures (Niedhart FC *et al* (1984)).

There seems to be no threshold temperature which must be achieved before the heat-shock response occurs. A jump from low temperatures to the 35-43°C range causes induction of the htp proteins over and above the accelerated expression of all cell genes at these higher temperatures. Shifts to the temperature range 43-47°C cause reduced growth rates and provoke a more pronounced heat-shock response.

17 htp proteins have been found in *E. coli* (Niedhart FC *et al* (1984)), of which the product of the *lon* gene, protease La, is of primary concern in the use of ts systems for the expression of foreign genes.

Chung CH and Goldberg AL (1981), Charette MF *et al* (1981), and Goldberg AL *et al* (1982) showed the product of the *lon* gene to be an ATP-dependent protease (La) and that changes in the cellular level of La influenced the host's capacity to degrade abnormal and foreign proteins. In mutants lacking the *lon* gene the degradation of abnormal proteins occurs 2 to 4 times more slowly than in wild-type cells.

The level of protease La increases 2- to 3-fold following a temperature jump from 30 to 42°C (Phillips TA *et al* (1984); Goff SA and Goldberg AL (1984))

Furthermore it has been shown (Goff SA and Goldberg AL (1985)) that it is not only heat-shock that causes protease La to be expressed. They found that it causes the degradation of aberrant, incomplete, unfolded, and some foreign proteins that might be expressed. These workers were expressing human tissue plasminogen activator (TPA) in *E. coli*, and found that its expression caused induction of protease La, which then degraded the TPA. From unpublished experiments they found that only certain foreign proteins expressed in *E. coli* stimulated transcription of the *lon* gene. One of the reasons that so much work has been done on protease La is that it is ATP-dependent and this means it is comparitively easy to work with. There are at least 16 other heat-shock proteins, some of which might also show proteolytic activity, and perhaps other proteases still, that cause the degradation of recombinant gene products, but which have not yet been identified and characterised in this role.

Grossman AD *et al* (1984) found that the *htpR* gene product regulated the expression of all the heat-shock genes. This protein was shown to be a sigma factor (facilitates binding of RNA polymerase) required for the initiation of transcription of the heat-shock genes. Mutants that cannot express the active *htpR* protein upon induction cannot produce protease La, and have a reduced capacity for the

degradation of abnormal and foreign proteins. Hence ts strains producing a product that is degraded by protease La must also contain a deficient *htpR* gene to obtain high levels of the foreign protein. However Celltech Ltd (personal communication) have not found strains with a deficient heat-shock response to perform as well as reported.

1.4 EFFECT OF TEMPERATURE ON CELL GROWTH AND DEATH.

This section deals with the implications of different growth and production temperatures on cell growth and death kinetics.

A literature review of this subject revealed that most of the work done is now well-established and little regarding bacterial growth and death kinetics seems to be done at the present time. Unfortunately most of this body of literature (eg Senez JC (1962); Marr AG *et al* (1963)) either ignores or predates Pirt's concept of maintenance energy requirements for cells (Pirt SJ (1965)), which has gained widespread acceptance over the years. Little re-evaluation of the older data has occurred in the light of Pirt's work, though. Fig 1.3 is adapted from Pirt's book (Pirt SJ (1975)) and shows the effect of temperature on the growth of three organisms, the most important of which from the point of this project, is *E. coli*. This shows that the specific growth rate increases with increasing temperature

until it peaks at around 40°C, and then falls sharply as the temperature is further raised. Experiments were carried out to see how the specific growth rate varied with temperature for the ts systems.

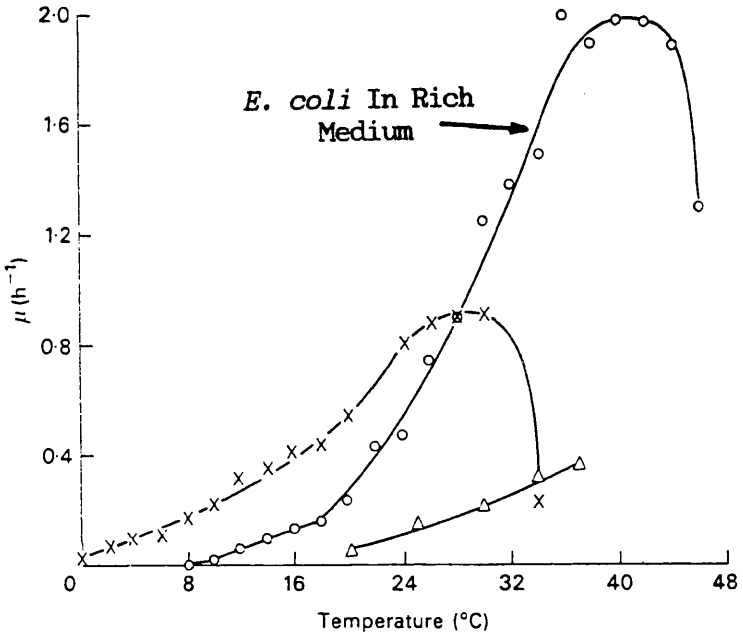


Figure 1.3: The Effect of Temperature on Specific Growth Rate For *E. coli* in Rich Medium, Adapted From Pirt SJ (1975).

If no cell death occurs the growth rate of viable biomass (X) is given by $dX/dt = \mu * X$.

However, if cells die, which of course they do, then the specific growth rate becomes $\mu = \mu(\mu,k)$ where k is the specific death rate. Also from fig 1.3, $\mu = \mu(T)$. As T increases k becomes dominant in its influence on μ and

thermal death occurs. This is caused by the higher activation energy for death compared with growth, meaning that when the temperature is high enough to impart sufficient energy for activation of death, then the rapid fall in specific growth rate seen in fig 1.3 occurs. This effect of temperature on cell death and growth is explained basically in terms of the structural temperature dependence of proteins and lipids for their stability and activity. At higher temperatures proteins denature and lose their activity and structure (Pirt SJ (1975)).

Information about the rate of destruction of microorganisms by heat came about from studies on sterilisation kinetics and can be found well documented in standard biochemical engineering textbooks (Aiba S *et al* (1973); Bailey JE and Ollis DF (1986); Wang DIC *et al* (1979)). The relevant equations are

$$dN/dt = -k * N$$

and $$k = A * e^{-\Delta E / RT}$$

- the slope of the line of ln(k) vs 1/T is $\Delta E/R$ and is a measure of the susceptibility of the organism to heat.

- k shows Arrhenius dependence.

The very obvious link between cell death and enzyme/lipid denaturation is maintained by fig 1.4, adapted from a figure taken from Bailey JE and Ollis DF (1977). This Arrhenius plot for enzyme reactions shows rapid denaturation and loss of activity above 53°C. Protein thermal denaturation, in many cases, begins to occur between 45-50°C, becoming very severe at 55°C, according to

Bailey JE and Ollis DF (1977). They suggest the obvious physical mechanism for this is that with increasing temperature and corresponding molecular energy a point is reached when this energy is sufficient to break the stabilising bonds of the proteins, resulting in denaturation.

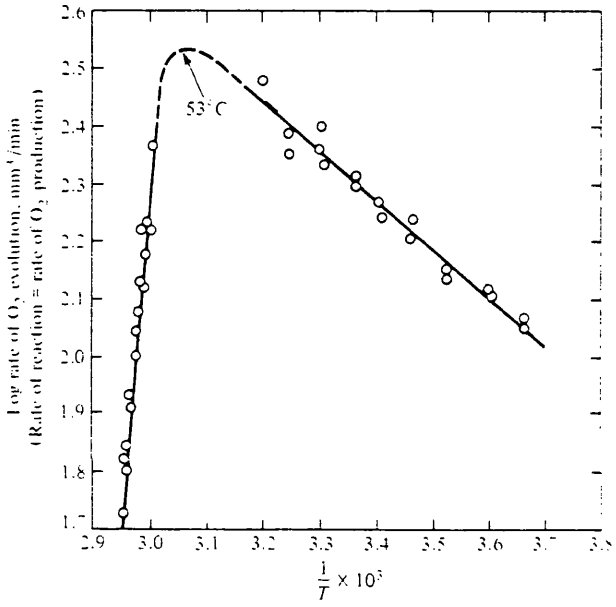


Figure 1.4: Arrhenius Rate Dependence Breaks Down at High Temperatures, Above Which Enzyme Deactivation Predominates (H_2O_2 Decomposition Catalysed by Catalase. Adapted From a Figure Taken From Bailey JE and Ollis DF (1977).

The effects of increasing temperature on the cell's secondary metabolism may be very different to growth. Rowley BI and Pirt SJ (1972) found that *Aspergillus nidulans* grown in glucose limited chemostat culture produced melanin at twice the rate at 37°C compared to 23°C. However, Franks PA *et al* (1980) found that in the

production of an acid used in cheddar cheese manufacture by *Streptococcus cremoris* the specific product formation rate fell by a factor of 5 over a period of around 6 hours subsequent to a shift in temperature from 30-38°C. They state that the reason for this was the progressive damage done to the cell's anabolic and catabolic processes by the higher temperature. However they did not perform a thorough investigation to show this was actually the case.

Pirt SJ (1975) cites Topiwala HH and Sinclair C (1971) who found that for *Klebsiella aerogenes* the observed decrease in growth yield on glucose with increasing temperature was explained by an increase in the maintenance energy requirements of the microbe. Esener AA *et al* (1983) found the same trend for *Klebsiella pneumoniae*. They noted that as the temperature increased the maximum yield of cells on substrate fell and that there was a corresponding increase in a defined normalised maintenance coefficient. In a paper published by Farmer IS and Jones CW (1976) the temperature effects on the energetics of *E. coli* W grown aerobically in glycerol-limited chemostat culture were investigated. The results indicated that over the growth range 20-40°C, the maintenance energy needs increased 12-fold, while the energy requirements for cell production rose only by 1/3. The yields of biomass on glycerol (carbon source) and oxygen fell respectively from 52.3 to 35.3 (g.cells. $mole^{-1}$glycerol) and 46.8 to 33.4 (g.cells.$mole^{-1}O_2$) over the same temperature range.

1.5 METABOLIC HEAT PRODUCTION DURING THE MICROBIAL PROCESS.

This section introduces the subject of metabolic heat production, important for this work because the heat of fermentation was to be investigated as a means, at least partly, of implementing the temperature shift. This can also be used for determining the cooling requirements at different stages of the process.

As microorganisms grow they produce varying amounts of heat. Most of this is released during the catabolism of the organic substrate, which is used as both a carbon and energy source. A proportion of this released energy is conserved by the cell in the form of a high energy sugar-phosphate bond in ATP, and other energy storage compounds, the rest being released as heat. These conserved species provide energy for biosynthesis of cellular components and other microbial processes. Most of the energy conserved in these storage compounds is released as heat when the molecules are used in metabolism.

The quantity of heat released depends on two main factors, related to the type of organism. Firstly, the type of catabolic pathway through which the organic substrate is metabolised will determine the amount of heat evolved, and secondly the way in which cell biosynthesis is coupled to energy-storage compounds like ATP.

Microbial heat production changes reflect the type of metabolic activities of the cell (Luong JHT and Volesky B (1983)). Experiments were carried out to see how the metabolic heat production rate changed during and after the temperature shift, since the metabolic heat will be used, at least in part, to raise the temperature of the fermentation and so implement the desired shift. This obviously has important implications for the fermenter temperature control system.

In section 1.4 it was shown that within the envisaged shift (30-42°C maximum) increases in the growth rate and maintenance requirements of the cells would occur. ATP is the primary fuel for the reactions of both of these processes, so at higher temperatures more of this substance would be expected to be required for growth and biosynthesis. The rate of metabolic heat production would be expected to increase with increasing temperature (within the limits of this shift) because more carbon substrate would be catabolised to form ATP, so releasing energy, and more ATP would be hydrolysed which would release still further energy. Essentially the process of ATP generation and hydrolysis are net exothermic reactions, and since at higher temperatures ATP is expected to be used at a higher rate the metabolic heat production rate would also be expected to increase.

Oxygen is required for the synthesis of ATP in *E. coli*, and so the oxygen uptake rate (OUR) of the cells has been used quite effectively in the calculation of the heat of

fermentation. In the preliminary design the equation

$$Q_f = 0.1439*OUR \qquad (W.L^{-1})$$

has been used to determine Q_f, the heat of fermentation (Cooney CL *et al* (1968)). Estimations of this parameter by other methods were attempted and compared with the measured values determined by the method of Luong JHT and Volesky B (1982).

1.6 REACTOR TYPE SELECTION.

This section outlines the reasons why batch and/or fed-batch operation was chosen instead of continuous fermentation for these studies and design.

It is generally true in the fermentation industries (excluding the waste-water treatment sector) that if the aim is to produce biomass then continuous culture would be used, whereas if a cellular component or secondary metabolite is the desired product, a batch operation would be chosen. Two well-known examples of continuous biomass production are ICI's Pruteen and Rank Hovis McDougall's Mycoprotein processes. Examples of the second category of processes are many, typically antibiotic and enzyme production processes.

There are several reasons for this difference, revolving around the biological systems themselves, the first being due to system instabilities. There are two common types of

instability. The first is structural gene instability, while the second and currently most important type is segregational instability.

Structural gene instability results from this gene being either incorrectly inserted into the vector or from there being a mutation in that structural gene causing production of a mutant, undesired protein. Genetic engineering techniques are now sufficiently advanced for the former case to be avoided. Mutations will occur at low frequency in all biological systems, are very unlikely to affect overall production, and so can be ignored.

Segregational instability is plasmid loss caused by defective partitioning at cell division. Naturally occurring plasmids are stably maintained because they contain a *par* region responsible for ensuring plasmid segregation during cell division. Many high copy number expression vectors do not incorporate a *par*, but they rely on at least a few copies of the plasmid being transferred to the daughter cell during division. Caulcott CA et al (1985) found that high copy number plasmids expressing low levels of the foreign gene product and low copy number plasmids efficiently expressing the foreign gene were sgrgatively stable. Highly expressing high copy number plasmids tend to be segregatively unstable, suggesting that high level translation of the recombinant gene was the cause of the instability. Wouters JTM et al (1980) found that low growth rates or high growth temperatures reduced the stability of *E. coli*(pBR322) in continuous culture.

Segregational instability is both plasmid- and strain-specific, and is very variable. Models for plasmid stability exist (Fish NM and Thornhill N (1986) and the work of Bailey JE and co-workers).

Genetically engineered organisms tend to have lower growth rates than their wild-types. If the plasmid should be lost from some of the cells, these cells would divide at a much faster rate than the production strain. In batch culture this is not a real problem because more often than not considerable growth of the production strain would occur, and so some product would be formed. Instability causes batches with lower productivity. However, if plasmid loss occurred in continuous culture the plasmid-free cells would quickly outgrow the production strain, wash it out of the fermenter and no, or little product, would be obtained. Measures would then have to be taken to remove the plasmid-free cells, and replace them with the production strain. Until strains containing very stable plasmids fulfilling the other constraints for production recombinant organisms have been developed, large-scale continuous culture will not be chosen as the industrial mode of operation. Amplifiable copy number vectors seem to go most of the way towards fulfilling the criteria for continuous culture. Rhodes M (1986) described a system run by Celltech Ltd in which an amplifiable copy number plasmid was found to be very stable at 30°C in continuous culture (less than 1% plasmid-free cells after 100 generations (12 days)). This phase generated the biomass with the expression system repressed. These cells were then transferred to a second

continuous reactor at 42°C which caused rapid plasmid replication, and high level expression of the foreign gene. At this stage the plasmid was unstable, and plasmid-free cells rapidly outgrew the production strain. This might, however, show a way forward towards continuous processes using two reactors in series, one for growth, and the second for production.

Another important reason why continuous culture is not used for recombinant strains is the susceptibility of the fermentation to contamination. However, with careful maintenance and good management this risk can be minimised.

Fed-batch culture offers the possibility of higher productivities than a batch system by supplying additional nutrients at rates that are limiting. Thus higher biomass (and product) concentration can be obtained. The disadvantage of fed-batch culture is that for most of the fermentation the volume of the broth is lower than in a batch case, since space must be left for the additional media pumped in during feeding.

1.7 TEMPERATURE CONTROL DURING FERMENTATION AND THE IMPLEMENTATION OF THE SHIFT.

The efficiency and flexibility of the temperature control system is likely to be very important in the scale-up of temperature sensitive systems. It will be required to

maintain very precise regulation of the temperature during the growth phase, to avoid unacceptably high levels of product expression to occur at this time. Cooling circuits on fermenters tend to be quite flexible in that they are called upon to maintain constant temperature of cultures that are exponentially growing and so producing metabolic heat at an exponentially increasing rate.

Once the temperature switch has been implemented the cells are likely to be producing more heat (see section 1.5) which will need to be removed to maintain the culture at its post-shift temperature. The temperature control system will hence have to be adaptable enough to cope with this unusual task.

The metabolic heat is a very important parameter in the scale-up of ts systems. On a large scale it seems sensible not to ignore it as a means of implementing the temperature shift, and rely on conventional heat exchange equipment. Instead a sensible approach would be to view the metabolic and agitation heat as a means of implementation of the shift, while looking at other heat exchange methods as possible supplements to this. The viability of this approach can only truly be assessed when more information on the metabolic heat production rate is known. Thus this was a key parameter to be investigated. One possible method of supplementing the metabolic heat would be to inject steam into the cooling water stream, so raising its temperature and causing the circuit to work as a heater before switching it back to a cooler subsequent to the

shift. However, the response time of the cooling circuit would have to be very fast to prevent overshoot, which could either be lethal to the cells or detrimental to product formation. The problems of preventing overshoot are made worse by the expectation (section 1.5) that as the temperature increases the metabolic heat production rate will also increase. This means that the nearer the culture becomes to the desired post-shift temperature, the higher will be its metabolic heat production rate, and so the faster it will approach the desired temperature.

It may well be that on a large scale ($100m^3$) the response time of the cooling circuit will be far too low to prevent such overshoot. The extent to which overshoot will be a problem will depend not only on the system response times, but also on the value of the metabolic heat of fermentation and the way it changes during the shift.

It is clear that the heat of fermentation is very important to this study. There are several methods available for determining this parameter, apart from empirical techniques which link it to substrate utilisation rates. The two most applicable to fermentation processes are dynamic and continuous calorimetry.

Cooney CL *et al* (1968) were the first to develop an easy method to measure the rate of metabolic heat production by a growing culture during a fermentation. This was a dynamic calorimetric technique which monitored the broth temperature increase when the controller was turned off.

The heat accumulation measured was then corrected for heat gains and losses to and from the fermenter. The reported accuracy of this method ranges from -4.5% to +1.1%. The great advantage of this technique is that it is very simple, but there are certain disadvantages which do not make it a practical method:

1) Continuous attention is needed throughout the experiment (especially as the approach may not be compatible with automatic process control procedures), which is very inconvenient.

2) This method causes fluctuations in the temperature of the fermentation broth, which may easily disturb the fermentation process.

3) Data collection is slow because it usually takes between 5 and 9 minutes for the rate of temperature rise to become constant.

Luong JHT and Volesky B (1982) have developed a continuous calorimetric method for measuring the heat of fermentation. The culture was overcooled using a constant, known cooling water flowrate. A variable power immersion heater was then placed in the vessel and used to supply the culture with the extra heat required to maintain it at the fermentation temperature. The temperature of the culture was measured by a thermocouple and compared with the set-point of an electrical proportional controller. The difference between these two signals (the error) was used to control the programmable direct current supply to the heater. In the early stages of the fermentation the metabolic heat production rate was low and so the heater was supplying

almost all the heat removed by the cooling circuit. However, as the culture grew the overall heat production rate increased, the temperature of the broth would rise, and so the power to the heater would be reduced. Hence, from the value of the power supplied by the heater a direct calculation of the heat of fermentation could be made. This gives instantaneous values of the heat of fermentation, within the response time limits of the system. The overall accuracy of this method is reported to be 2.04 +/- 1.31%. This method was used to determine the overall and specific heat of fermentation for the scale-up of ts systems.

1.8 THE EFFECT OF TEMPERATURE ON OXYGEN SOLUBILITY.

This section assesses the implications for the fermentation of the reduced O_2 solubility in water and media as the temperature is increased.

Montgomery HAC *et al* (1964) gave information about the solubility of oxygen in water in the range 0-40°C at 1°C intervals. The solubility was measured with the water in equilibrium with air, saturated with water vapour, at a pressure of 760 mm.Hg.

The value of the solubility at 30 and 38°C was 7.57 and 6.67 $mg.O_2.L^{-1}$, respectively. This was a fall of ((7.57 - 6.67)/7.57) * 100% = 11.9% for an 8°C rise.

If it is assumed that the solubility of O_2 in fermentation media changes in the same way as in water, then this suggests a similar 11.9% reduction for an 8°C rise from 30-38°C. This will occur after the temperature switch has been activated and at a time when the OUR will increase to its maximum value. Thus the production phase may be oxygen-limited. This will need to be investigated.

1.9 THE EFFECTS OF SCALE ON TEMPERATURE SENSITIVE SYSTEMS.

Mixing, aeration, and heat transfer are three important areas to be looked at for scale-up during process design.

Ts constructs are mainly used in *E. coli*, which is advantageous for mixing in that the organisms should not be harmed by the high shear regions anticipated in the fermenter. Also, *E. coli* broths are Newtonian in their rheology. The consequences of this are that the fermenter can be operated with a high agitator rate to ensure good mixing and a homogeneous broth.

Heat transfer is a crucial process in this area of investigation, and is also greatly aided by a high agitator rate. This will cause the broth to be of almost uniform temperature throughout, which is important so as not to have regions of the fermenter at temperatures sufficient to increase product expression from the temperature sensitive

construct. The good mixing caused by the agitator will cause scouring of the heat transfer surfaces and aid good transfer.

The metabolic heat production rate is independent of scale (calculated on a per unit volume basis), whereas heat transfer characteristics of a fermentation system are not. As the scale increases the surface area to volume ratio falls. This means that there comes a scale where a cooling jacket alone is inadequate for performing the required cooling duty. Immersed cooling coils need to be added when this point is reached. There could be problems in implementing the temperature shift on a large scale in such a way that would prevent overshoot of the desired post-shift temperature (see section 1.7). Hence the effect of scale on the heat transfer characteristics will become increasingly important with increasingly large scales.

The problem of achieving adequate oxygen transfer on a large scale is a general one for the fermentation industries. It was envisaged that the maximum OUR would occur at the end of the production phase immediately prior to media exhaustion at a time when the cells were growing, and present in their maximum concentration. This is at the point where the temperature would be maximal, which means the O_2 solubility in the fermentation media would be at its minimum value. Hence it may be difficult to achieve adequate oxygen transfer on a large scale, and this parameter may decide the final cell concentration that these systems can be designed for under kinetic limitation.

More important than the air flowrate in achieving good O_2 transfer is the impeller rate. As has already been stated the impeller rate is likely to be high in these systems and this will obviously work in favour of good O_2 transfer to the broth.

1.10 MODEL SYSTEMS.

Celltech Ltd supplied two prototrophic *E. coli* K12 strains containing plasmids pCQV2 (Queen C (1983)) and pMG169 (Wright E *et al* (1986)), both expressing CAT.

The plasmid pCQV2 was supplied to Celltech Ltd by C Queen and was cut with restriction enzyme BamH1. The CAT gene cut from plasmid pACYC184 with Sau3A was inserted at the BamH1 site in the correct orientation. This insert contained not only the CAT structural gene, but also the ribosome binding site and terminator sequence. CAT expression was controlled by the P_R promoter already present on the plasmid. This promoter is in turn controlled by the λcI_{857} temperature-sensitive repressor, whose gene is also carried on the plasmid.

The plasmid pMG169 was supplied in the form described in section 1.2.2 and Wright E *et al* (1986).

These plasmids were transformed into host strain RV308 (ATCC number 31608), prototrophic *E. coli* K12 λ^- F^- str^R

*gal*305. The host was streptomycin-resistant, whereas the plasmids carried a β-lactamase gene, conferring resistance to β-lactam antibiotics (eg Ampicillin, Carbenicillin) and so giving a method for the selection of plasmid-bearing cells.

CAT confers resistance to Chloramphenicol. Hence these organisms were sensitive to Chloramphenicol at 30°C, but resistant at 37°C. The cI repressor confers resistance to phage λ infection at 30°C, but not at 42°C.

CAT was chosen as the product to be expressed for several reasons (Shaw WV (1975)):

1) It is stable to thermal denaturation.
2) It is stable against normal protease attack.
3) It is easily assayed spectrophotometrically.

1.11 AIMS OF THE PROJECT.

The overall aim of this project was to develop process design principles for the large scale use of recombinant *E. coli* containing constant and amplifiable copy number plasmids incorporating temperature sensitive constructs.

A series of fermentations were planned, which were intended to yield information about the optimum conditions of growth and production temperature, length of growth and production phases, the effects of implementing the temperature shift

over different lengths of time, and the oxygen requirements of the cells. This was intended to give a description of the process for optimum reactor productivities under kinetic limitation.

It was expected that oxygen limitation would occur on the large scale (see section 1.9), and so a second aim was to determine the effects of this on the reactor productivity for the two strains.

A third aim was to try to remove the oxygen limitation by the imposition of another nutrient limitation (glucose). The effects of this on the reactor productivities were to be compared with those under both kinetic and oxygen limitation.

A fourth aim was to investigate the metabolic heat output of the two strains, to assess the possibility of using it to raise, at least partially, the temperature of the fermenter, and so initiating the production phase. It was intended that a comparison of these results with those obtained from widely used empirical correlations would be made.

The final aim was to perform some design calculations to predict reactor performance on the large scale. It was intended that this should be done under the three different limitations investigated, and should be extendable beyond the limiting confines of the model system to be more generally applicable.

2 MATERIALS AND METHODS.

2.1 MATERIALS.

2.1.1 Microorganism.

2.1.1.1 Bacterial Strain.

All experiments were performed using Escherichia coli RV308 (ATCC No. 31608), a K-12 strain with the phenotype: λ^-, F^-, str^R, and gal 305.

2.1.1.2 Plasmids.

The investigations using a low, constant copy number plasmid were performed with plasmid pCQV2, which has been described previously (Queen C (1983)). The map of the plasmid is shown in figure 1.2 (section 1.2.2), and it was present in the cells at a copy number of approximately 20 copies per cell.

The CAT1-encoding fragment from pACYC184 (cut with Sau3A) was inserted in the correct orientation in the BamH1 site of pCQV2. The insert contained the CAT structural gene,

ribosome binding site, and terminator sequence, but no promoter. CAT expression was under the control of the λP_R promoter, regulated by the cI_{857} temperature sensitive repressor protein, whose gene was also carried on the plasmid and expressed constitutively. The plasmid also contained a β-lactamase gene conferring resistance to β-lactam antibiotics.

The investigations using an amplifiable copy number plasmid were performed with plasmid pMG169, which has been described previously (Wright E *et al* (1986)). The map of the plasmid is shown in figure 1.1 (section 1.2.2), and it was present in the cells at a copy number of approximately 4 copies per cell at growth temperature, prior to copy number amplification.

Plasmid pMG169, being exactly as described in Wright E *et al* (1986), was transformed into the host strain *E. coli* RV308. Plasmid pCQV2 was not exactly as described in Queen C (1983) because the CAT gene was inserted (as described above) under the control of the λP_R promoter system.

The phenotype of the strains containing the plasmids were:

Streptomycin resistant due to the host coded function,

β-lactam antibiotic resistant due to the plasmid coded β-lactamase,

Chloramphenicol sensitive at 30°C,

Chloramphenicol resistant at 37°C,

Immunity to phage lambda due to cI repressor protein at 30°C but not at 42°C.

2.1.2 Chemicals (Analar grade used).

Substance	Commercial Supplier
Nutrient Agar	Oxoid ltd, Basingstoke, Hants, UK
Yeast Extract	Ditto
Bacto Tryptone (Pancreatic Digest of Casein)	Difco Labs, Detroit, Michigan, USA
Sodium Chloride	BDH Chemicals ltd, Poole, Dorset, UK
Glucose (α-D(+)-glucose)	Sigma Chemical Co ltd, Poole, Dorset, UK
Sodium Dihydrogen Orthophosphate	Fisons PLC,Loughborough Leics, UK
Ammonium Chloride	Ditto
Potassium Chloride	Sigma Chemical Co Ltd, Poole, Dorset, UK
Sodium Sulphate	BDH Chemicals Ltd,Poole, Dorset, UK
Magnesium Chloride	Ditto
Calcium Chloride	Ditto
Manganous Chloride	Ditto
Cupric Chloride	Ditto
Sodium Molybdate	Ditto

Substance	Commercial Supplier
Citric Acid Monohydrate	Sigma Chemical Co Ltd, Poole, Dorset, UK
Zinc Oxide	Ditto
Carbenicillin	Ditto
Ferric Chloride (hydrated)	Fisons PLC,Loughborough Leics, UK
Sodium Lauryl Sulphate	Ditto
Glacial Acetic Acid	Ditto
Cobaltous Chloride	Ditto
Glycine	Ditto
Boric Acid	Ditto
5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB)	Sigma Chemical Co Ltd Poole, Dorset, UK
Chloramphenicol	Ditto
Albumin, Bovine	Ditto
Chloramphenicol Acetyl Transferase	Ditto
Acetyl Coenzyme A (Lithium salt)	Ditto
Sigma 7-9 Biochemical Buffer	Ditto
Tris(hydroxymethyl) Amino Methane	Ditto
2-Mercaptoethanol	Ditto
DL-Dithiothreitol	Ditto
Glu-cinet Reagent	Technicon Chemicals Cy SAB-75 01 Orcq-Tournai (Belgique)
Protein Assay Reagent	Pierce Chemical Co, Rockford, Illinois USA

Substance	Commercial Supplier
Glycerol	BDH Chemicals Ltd, Poole, Dorset, UK
Ethylenediaminetetra acetic acid (EDTA)	Ditto
Ethanol (Absolute)	Ditto
Bromophenol Blue	Ditto
Ethidium Bromide	Ditto
Agarose	Ditto
Sodium Acetate	Ditto
Acrylamide	Ditto
Coomassie Blue (PAGE Blue 83)	Ditto
Acetic Acid	Ditto
Methanol	Ditto
Butanol	Ditto
Ammonium Persulphate	Bio-Rad Laboratories Ltd Watford, Herts, UK
Temed	Ditto
Bisacrylamide	Ditto

2.1.3 Growth Media.

2.1.3.1 Nutrient And Selective Agar Plates.

Nutrient Agar plates were made to a concentration of 28 gL^{-1} Nutrient Agar in deionised water. The agar was

autoclaved for 20 minutes at 121°C.

Selective plates were made the same way as above, but with the addition of carbenicillin to a concentration of 0.2 gL^{-1}.

2.1.3.2 Nutrient Broth.

The composition of the nutrient broth was as follows:

Yeast Extract	5 gL^{-1}
Bacto Tryptone	10 gL^{-1}
NaCl	5 gL^{-1}
Glucose	1 gL^{-1}

in deionised water, adjusted to pH 7.0, and autoclaved at 121°C for 15 minutes.

2.1.3.3 Simple Defined Salts Medium (Evans, CGT, *et al* (1970)).

Stock Solutions

Solution		Concentration (gL^{-1})
1 Phosphorus Source	2M-$NaH_2PO_4.2H_2O$	312
2 Nitrogen Source	4M-NH_4Cl	214
3 Potassium Source	2M-KCl	149
4 Sulphur Source	1M-$Na_2SO_4.10H_2O$	322

Solution		Concentration (gL^{-1})
5 Chelating Agent	1M-Citric Acid	210
6 Magnesium Source	0.25M-$MgCl_2$	23.8
7 Calcium Source	0.02M-$CaCl_2$	2.2
8 Trace Metals (dissolved in 1 L deionised water):		
	Conc HCl	10 ml
	ZnO	0.408 g
	$FeCl_3.6H_2O$	5.4 g
	$MnCl_2.4H_2O$	2.0 g
	$CuCl_2.2H_2O$	0.17 g
	$CoCl_2.6H_2O$	0.476 g
	H_3BO_3	0.062 g
9 Molybdenum Source	0.0017M-Na_2MoO_4	0.4

For runs under kinetic limitation with an initial concentration of glucose of 20 gL^{-1}, the volume of each of the stock solutions needed to make up the media was:

Solution		Concentration ($ml.L^{-1}$)
1	(phosphate)	10
2	(nitrogen)	50
3	(potassium)	10
4	(sulphate)	4.0
5	(chelate)	4.0
6	(magnesium)	10
7	(calcium)	2.0
8	(trace metals)	10
9	(molybdate)	0.2

2.1.4 Disruption Buffers.

The composition of this in deionised water was:

0.01 M-Tris buffer pH 7.8

0.1 mM-DTT (Dithiothreitol)

2.1.5 Batch Culture Equipment.

The stages of inoculum preparation were carried out in a Controlled Environment Incubator Shaker (Model 6-25) (New Brunswick Scientific, Edison, New Jersey, USA).

The fermentations were carried out in a Chemap GF0014 fermenter of 10 L working volume (Chemap, Alfa-Laval Ltd, Brentwood, Middx).

2.2 METHODS.

2.2.1 Microorganism Storage.

Cells were stored at -20°C as a 60% glycerol stock.

2.0 ml of an exponentially growing Nutrient Broth culture containing carbenicillin, were added to a glass bijou bottle containing 3.0 ml sterile glycerol and placed in a freezer at -20°C.

2.2.2 Microorganism Culture.

2.2.2.1 Selection Plates.

A loopful of the glycerol stock was streaked for single colonies onto a fresh carbenicillin plate and grown for 24h at 30°C.

2.2.2.2 Culture In Nutrient Broth.

A single colony from the plate in section 2.2.2.1 was inoculated into 10 ml Nutrient Broth containing 0.2 gL^{-1} carbenicillin in a Universal bottle. This was shaken at 30°C and 200 rpm for 4-5h.

2.2.2.3 Shake-Flask Culture.

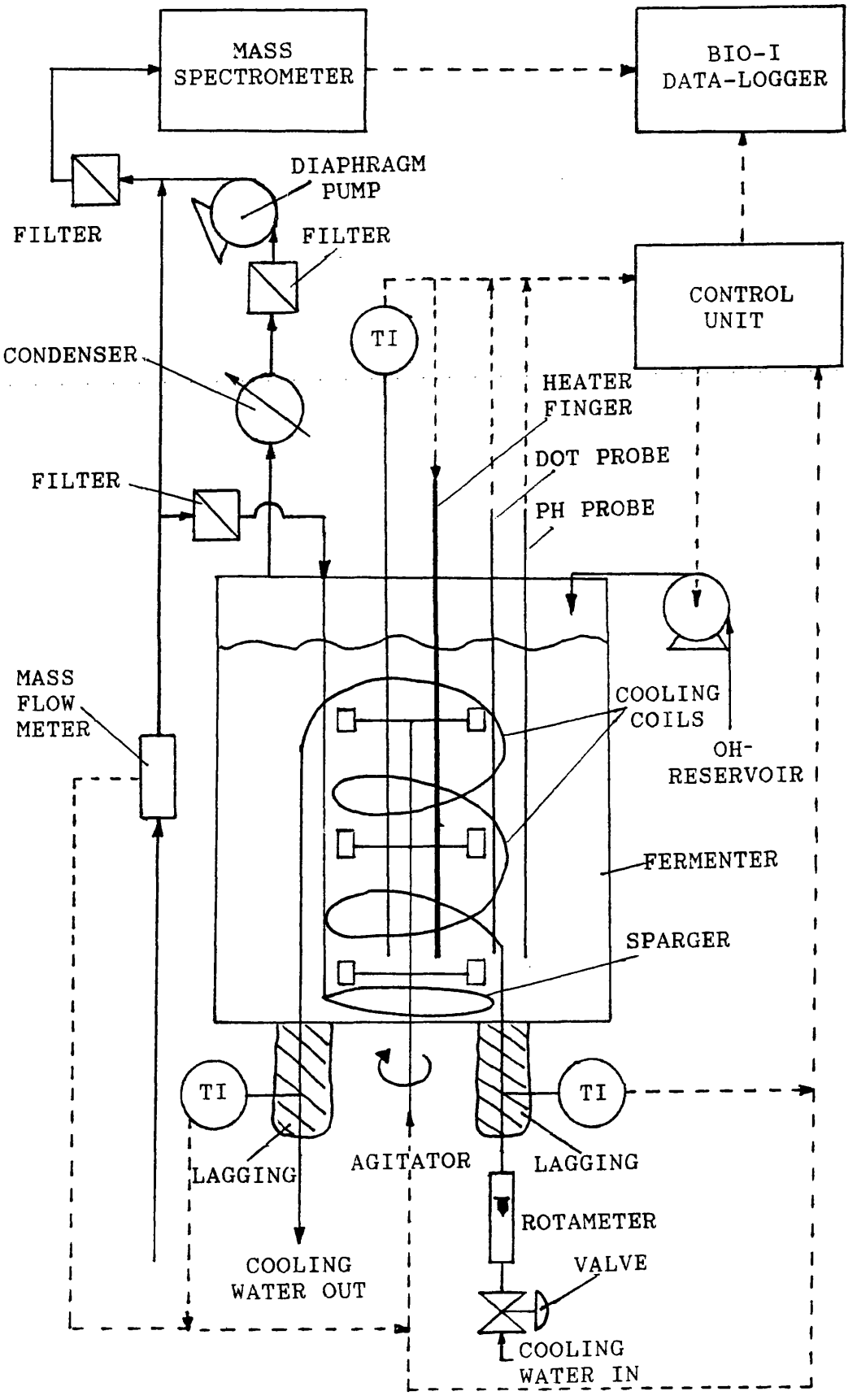
The contents of the Universal bottle in section 2.2.2.2 were inoculated into a 2.5 L baffled, side-arm flask containing 250 ml of the simple defined salts medium containing glucose (autoclaved separately at 121°C for 15 minutes). This was grown overnight at 30°C on the shaker at 200 rpm. The pH was adjusted to 7.0 and buffered using Na_2HPO_4.

2.2.2.4 14 L Batch Fermentation Under Kinetic Limitation.

The fermenter (see figure 2.1) was sterilised in situ containing the salts medium for 20 minutes at 121°C. The ancillaries and glucose were autoclaved separately for 20 minutes at 121°C.

Initially the contents of the shake-flask in section 2.2.2.3 were used to inoculate the fermenter. Later 5 ml from the Universal bottle in section 2.2.2.2 were used. This was done because a low inoculum meant the selective pressure of the carbenicillin was maintained for longer (Pierce J and Gutteridge S (1985)). The fermentation conditions were an agitator rate in the range 1000-1400 rpm, and air flowrate 4.0-5.0 SLPM. The pH was maintained at pH 7.0 using 5M-NaOH. The temperature was maintained at a variety of values between 30°C and 42°C. Carbenicillin was added initially to a concentration of 0.15 gL^{-1}. Polypropylene glycol was added as antifoam when required.

Figure 2.1: Diagram Of Fermentation Apparatus.



2.2.2.5 14 L Batch Fermentation Under Oxygen Limitation.

The fermenter was operated as above except that the agitator rate was used to control the OUR and DOT. Onset of oxygen limitation was initiated when the OUR had reached the desired limiting value by a reduction in agitator rate. Under the fermentation conditions employed prior to this the DOT=100%. The air flowrate was maintained constant throughout to ensure accuracy of the gas analysis system and to prevent the concentration of dissolved CO_2 from becoming inhibitory.

2.2.2.6 14 L Batch Fermentation Under Glucose Limitation.

The fermenter was operated as in section 2.2.2.4 except that glucose was added initially only in sufficient quantity to allow the biomass concentration to rise to a value yielding an OUR of 50-60 moles.$m^{-3}h^{-1}$. At this point glucose was fed at different rates using an Eyela MP-3 peristaltic pump (Eyela, Tokyo).

Adjustment to the OD readings was based on volume at inoculation and included alkali and glucose additions (see section 2.2.4).

2.2.3 Heat of Fermentation.

The apparatus used was the same as in figure 2.1, except that the fermenter was lagged to prevent heat losses to the surroundings.

Heat Balance over the system:

$$Q_{ag} + Q_f = Q_{cool} + Q_{sens} + Q_{evap} + Q_{surr}$$

Energy In = Energy Out

The method used was that of Luong JHT and Volesky B (1982).

The fermenter was set up as described in the above diagram, sterilised, carbenicillin added, and allowed to come to a steady-state at the temperature at which Q_f was to be measured. At this steady-state the cooling water flowrate was constant and continuous, and the heat supplied by the variable power heater was constant and sufficient to maintain the temperature control. At this point the fermenter was inoculated with 5 ml broth from section 2.2.2.2. A low inoculum was used to disturb the steady-state as little as possible. As the cells grew heat was produced and the heater power necessary to maintain temperature control therefore lessened in direct proportion to this. The biomass profile was followed by taking 5-6 Adjusted OD readings towards the end of the batch. Only this number were taken to ensure the system was again disturbed as little as possible.

2.2.4 Gas Analysis.

The fermentation off-gas was analysed using a VG MM-80 fermentation mass spectrometer system (VG, Middlewich, Cheshire). 150 $ml.min^{-1}$ exit gas was pumped to the mass spectrometer and analysed. The inlet air composition was also measured. The OUR, CER, and RQ were calculated by hand initially and subsequently by a BCS BIO-i fermentation data-logging system (BCS, Chiswick, London,), incorporating a DEC PDP-1173 mini-computer.

OUR was determined in the following way. The air flowrate through the fermenter was measured using a thermal mass flowmeter and expressed as Standard Litre Per Minutes (SLPM). Knowing the composition of the gas enterring, this was expressed as molesO_2 m^{-3}broth.h^{-1}. The proportion of this O_2 taken up by the cells was measured by the mass spectrometer, and so the OUR followed.

The same process was followed for CER except the spectrometer measured the amount of CO_2 added to the exit gas.

RQ = CER/OUR

2.2.5 Determination Of Bacterial Growth.

Initially a dry weight versus OD_{670} correlation was carried out. Subsequently the biomass levels were measured using OD_{670} and an occasional dry weight measurement to check the relationship still held.

However, adjustment to the OD_{670} readings was made to account for dilution of the culture due to additions (eg alkali). This meant that adjusted readings were the OD_{670} values based on the initial broth volume. They were determined as follows:

Dilution factor, D, of sample #n =

$$\frac{\text{broth volume at \#n-1}}{\text{broth volume at \#n-1 + sum of additions since \#n-1}}$$

Accumulated dilution factor, D', of #n = product of all dilution factors up to and including #n.

Therefore Adj OD_{670} of #n = (OD_{670} of #n)/D'at #n

The above adjustment was made for dilution of the broth due to additions to its volume. However this did not account for any errors associated with distortion of the growth due to removal of cells for samples. The sample volume was kept to the necessary minimum and never exceeded 10 ml during growth. In order to produce an OD20 pellet the sample volume taken from the fermenter was only large at low biomass concentrations. As growth progressed less sample volume was needed. In any fermentation the total volume removed did not exceed 50 ml (50/10000 = 0.5%), which was

negligible.

The dry weight determination was carried out by filtering 25 ml sample through a dried, weighed 0.2 μm Whatman cellulose nitrate membrane filter (Whatman Laboratory Products Ltd, Maidstone, Kent) in a Sartorius vacuum filtration unit (Sartorius Instruments Ltd, Belmont, Surrey). The filter was then dried overnight, and reweighed to give the dry weight (gL^{-1}).

OD_{670} measurements were carried out using a Pye Unicam PU 8600 UV/VIS Spectrophotometer (Pye Unicam, Cambridge, Cambs). The absorbance was measured at 670 nm, zeroed against water in the range 0-0.4 absorbance units. Dilutions were made using 0.7% (w/v) NaCl solution to maintain the absorbance within this band.

2.2.6 Sample Collection And Preparation.

For the CAT and protein assays samples were prepared as OD20 pellets. These are defined as the quantity of cells which, when resuspended in 1 ml disruption buffer gives an OD_{670} reading of 20 absorbance units. The volume of culture necessary to give such a pellet is given by $20/OD_{670}$ ml broth. Depending on the biomass level the appropriate volume of culture was removed. OD6 samples for PAGE were also taken, being determined in a similar way.

The sample was centrifuged in an MSE Centaur 2 centrifuge (MSE, Crawley, Sussex) at 4200 rpm for 10 minutes. The supernatant was stored at 4°C and later assayed for glucose.

The pellet was stored at -20°C in the centrifuge tube.

2.2.7 Cell Disruption.

The OD20 pellets were thawed and resuspended in 10 ml disruption buffer, and kept on ice. The samples were then sonicated in a MSE Soniprep 150 (MSE, Crawley, Sussex) on full power and amplitude of 20 μm for various lengths of time (to yield the conditions for total disruption), in a cooled double-skinned flask with mains water as the coolant. 1.5 ml of this was spun down at 13,000g in an Eppendorf tube to remove cell debris. The clarified lysate was then used for CAT and protein assays. The protocols for the CAT and protein assays were worked out for disruption of the OD20 pellet to occur in 1 ml buffer. 10 ml had to be used to cover the sonicator probe, which represents a 10-fold dilution of the original sample. Expression of results as $mg.ml^{-1}$ sample have been multiplied by 10 to account for the dilution.

2.2.8 Protein Assay.

The concentration of soluble protein released by the disruption was measured using the method of Bradford M (1976). 60 µl sample was added to 3 ml Pierce Reagent, left for 5 minutes and the absorbance at 595 nm was measured. Protein concentration was determined after comparison to a calibration curve of 0-1.0 $mg.ml^{-1}$ Bovine serum albumin solution.

2.2.9 CAT Assay (Protocol supplied by Celltech Ltd, adapted from Shaw WV (1975)).

20 µl of sample were added to 0.96 ml Reaction Mixture consisting of:

4.0 mg DTNB

1.0 ml 1.0 M-Tris HCl pH7.8

0.2 ml 5 mM-acetyl CoA

Distilled water to 10 ml.

5 mM-acetyl CoA in 0.1 M-Tris HCl pH7.8 was prepared immediately before use .

These two solutions were placed into a 1.0 ml plastic cuvette with 1.0 cm absorbance path, and allowed to equilibrate to 37°C.

Then 20 µl 5 mM-chloramphenicol solution (in 0.1 M-Tris HCl pH7.8) was added, mixed well, and the change in absorbance at 412 nm was followed.

CALCULATION :

The CAT concentration (mg CAT.ml^{-1} sample) was calculated by:

ΔODmin^{-1} 20µl^{-1} * 0.01876 mg CAT.ml^{-1} sample.

Divide CAT concentration by protein concentration to give % protein.

CAT concentration (unit.g^{-1}dcs) was given by:

ΔOD min^{-1} 20µl^{-1} * 4177.8075.

These values were normalised against the mean protein release from cell disruption, since the conditions of this were selected for total disruption (see Appendix 1). Normalised units of CAT are expressed as nu.

2.2.10 Glucose Assay.

The concentration of glucose dissolved in the fermentation broth was measured using the Glu-cinet reagent. 20 µl sample was added to 2.5 ml reagent, left for 30 minutes and the absorbance at 510 nm was measured. Glucose concentration was determined after comparison to a calibration curve of 0-5.0 gL^{-1} glucose solution. The samples were diluted 4-fold to fall within the limits of

the assay.

2.2.11 PAGE (Standard Operating Procedure of Celltech Ltd).

Solution	Components
A	30% w/v Acrylamide 0.8% Bisacrylamide
B	0.75 M-Tris HCl pH8.8 0.2% w/v Sodium Lauryl Sulphate
B'	0.25 M-Tris HCl pH8.8 0.2% w/v Sodium Lauryl Sulphate

Solution	Components
Electrode Buffer	0.217 M-Tris-Glycine pH8.3 0.1% w/v Sodium Lauryl Sulphate

All the above solutions were made up in deionised water.

Stain	0.2% w/v Coomassie Blue 25% w/v Methanol 10% w/v Glacial Acetic Acid

Solution	Components
Destain	10% w/v Glacial Acetic Acid 10% w/v Methanol
Sample Buffer (*2 concentration)	20% w/v Glycerol 10% w/v 2-Mercaptoethanol 6% w/v Sodium Lauryl Sulphate 0.5% w/v Bromophenol Blue dissolved in 0.12 M-Tris HCl pH 6.8

12.5% Gel Preparation: 18 ml Solution A
22.5 ml Solution B
4.5 ml Deionised Water
50 µl Temed
150 µl of 100 mg.ml^{-1} Ammonium Persulphate

The mixture was agitated gently with a spatula so as not to aerate it and poured between the plates. A few drops of a 1:1 Butanol:Water mixture were layered onto the surface to keep the surface edge of the gel free from air bubbles. After the gel has set the Butanol was poured off and the surface rinsed with water before the stacking gel was added.

3% Stacking Gel Preparation:

1 ml Solution A

5 ml Solution B'

4 ml Deionised Water

10 µl Temed

75 µl of 100 mg.ml^{-1} Ammonium Persulphate

After the gel has polymerised the rig is assembled, the buffer added, and the samples loaded.

Sample Preparation: A thawed OD6 pellet was resuspended in 100 µl Water and mixed well. 10 µl this was added to 90 µl Sample Buffer (*1 concentration) and boiled for 1.5-3 minutes. 20 µl this was loaded onto the gel.

The gel was electrophorised at 40 mA for 6h, or until the dye front reached the bottom of the gel.

The gel was carefully removed from between the plates and stained for 1h, before being destained until the protein bands were visible.

3 RESULTS.

Throughout this chapter "strain pCQV2" and "strain pMG169" are used as abbreviations for "strain *E. coli* RV308(pCQV2)" and "strain *E. coli* RV308(pMG169)", respectively.

Also, throughout this chapter reference is made to the growth and production phases. This does not imply that growth was absent during production or that production was absent during growth, and in reality both occurred concurrently. However the growth phase was defined here as the period in the fermentation when biomass was generated with little or no product expression occuring. The temperatures at which this phase was run were 34.5°C and lower, and are referred to as growth temperatures. The production phase was here defined as the period when the temperature was raised to derepress product expression, and occured at temperatures between 37 and 43°C. These temperatures are referred to as production temperatures.

3.1 BATCH FERMENTATION UNDER KINETIC LIMITATION.

The term kinetic limitation refers to the way in which these experimental fermentations were run. They were performed with no nutrient limitation but a medium was used in which the carbon source, glucose, was the first to be exhausted (see section 2.1.3.3). Therefore prior to glucose

exhaustion the cells were under kinetic limitation at each set of different fermentation conditions.

The purpose of these experiments was to characterise the two strains to see how best they should be used in fermenters, and to determine the important parameters for scale-up. A large number of fermentations (around 35) were performed under different conditions of growth and production temperatures, lengths of growth and production phases, and the effect of different lengths of time taken to implement the temperature shift. Certain key parameters like the production kinetics and batch oxygen requirements were followed under these conditions.

It should be noted that production phases run at 37°C were actually raised initially to 42°C for 10 minutes, before being reduced to 37°C for the duration of the fermentation. They are referred to as 37°C for convenience. The reason this was done was to mimic much of the laboratory-scale work done on these types of strains (eg Wright E *et al* (1986)).

3.1.1 Specific Growth Rate and Expression at Different Growth Temperatures.

It was clear that a growth temperature would need to be selected for scale-up, that combined the highest maximum specific growth rate (to minimise the required growth time)

with a minimum acceptable product expression level. Hence the maximum specific growth rate and product expression level were determined at different growth temperatures.

The maximum specific growth rate is defined as $\mu_m = 0.693/t_d$, where t_d was the doubling time (h).

Strain pCQV2

TABLE 3.1

GROWTH AND PRODUCT EXPRESSION CHARACTERISTICS AT DIFFERENT GROWTH TEMPERATURES FOR STRAIN PCQV2.

	GROWTH TEMPERATURE (°C)							
	30	31	31.5	32	33	33.5	34	34.5
Mean Specific Growth Rate (h^{-1})	.486	.561	.582	.536	.609	.576	.681	.644
Sample Size	9	2	1	8	2	1	1	5
Sample Standard Deviation	.051	.021	n/a	.065	.037	n/a	n/a	.053
Specific Growth Rate Range (h^{-1})	.413 to .562	.546 to .575	n/a	.442 to .635	.583 to .635	n/a	n/a	.550 to .671
Mean Doubling Time (minutes)	85	74	71	78	68	72	61	64
Doubling Time Range (minutes)	74 to 100	72 to 76	n/a	65 to 94	65 to 71	n/a	n/a	62 to 76
Mean Expression Level (% cell protein)	.007	.011	.06	.009	.044	.047	.056	.129
Sample Size	4	2	1	8	2	1	1	3
Sample Standard Deviation	.008	.016	n/a	.014	.037	n/a	n/a	.065
Range of Expression Level (% cell protein)	0 to .015	0 to .023	n/a	0 to .038	.018 to .070	n/a	n/a	.063 to .192

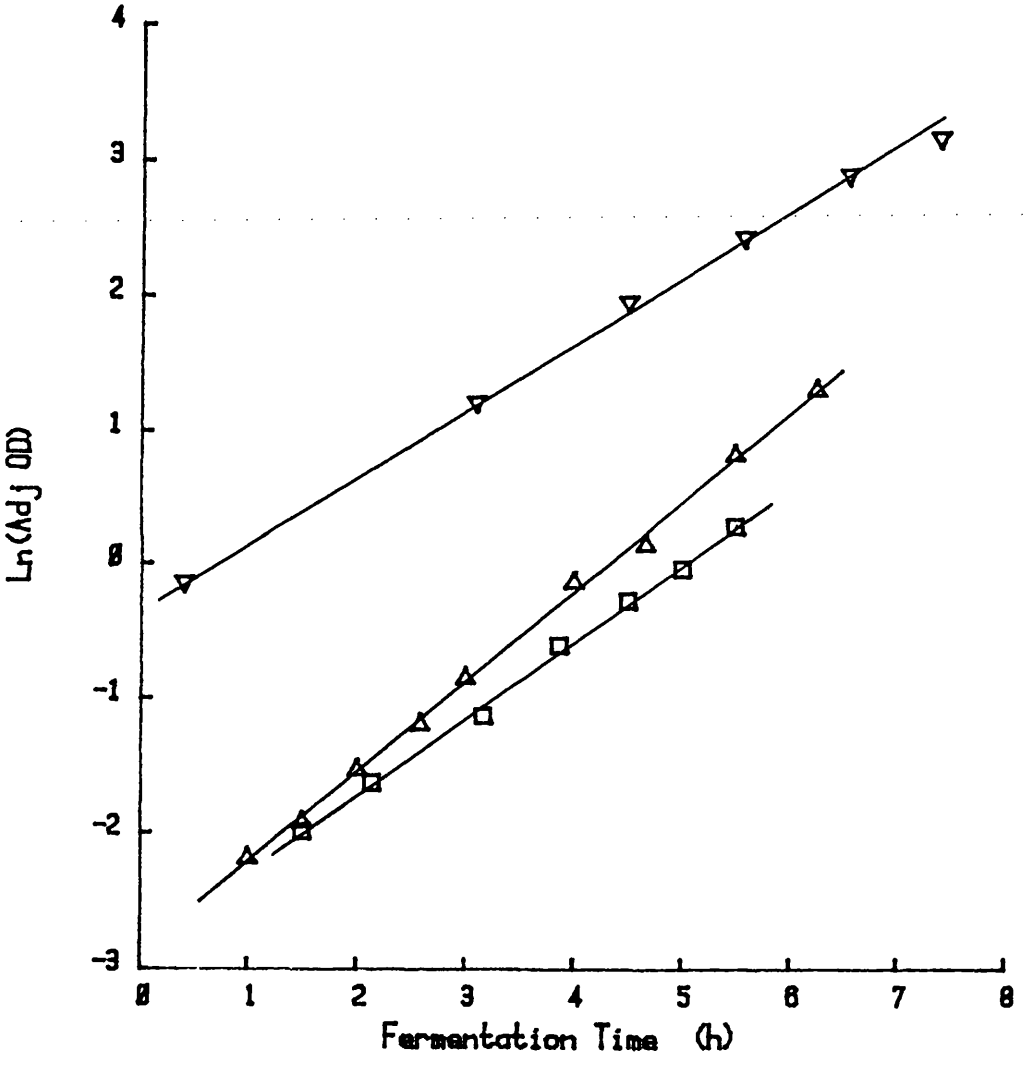


Figure 3.1: Effect Of Growth Temperature On Ln(Adj OD) against Fermentation Time - Strain pCQV2 (▽ ,30°C [r=.9985]; □ ,32°C [r=.9988]; △ ,34.5°C [r=.9992]).

Figure 3.1 is representative of each stated growth temperature. The different sizes of inocula used in the experimental programme (see section 2.2.2.4) resulted in varying lengths of growth phase. The specific growth rate was the slope of the ln(Adj OD) vs time line during exponential growth. Therefore since it was the slope that was being measured some of the fermentation data presented was shifted along the x-axis, so that all the data fitted on a common scale. This was only done to data that came from fermentations that were inoculated in the evening with a small inoculum, and grown overnight. In these cases there was a 16h delay in taking the first OD measurement. Therefore 16h was taken to be time 0h, and the data then appeared as in figure 3.1.

The regression coefficients, r, show the degree to which the points fell on a straight line. (r=1.0000 is a perfect straight line).

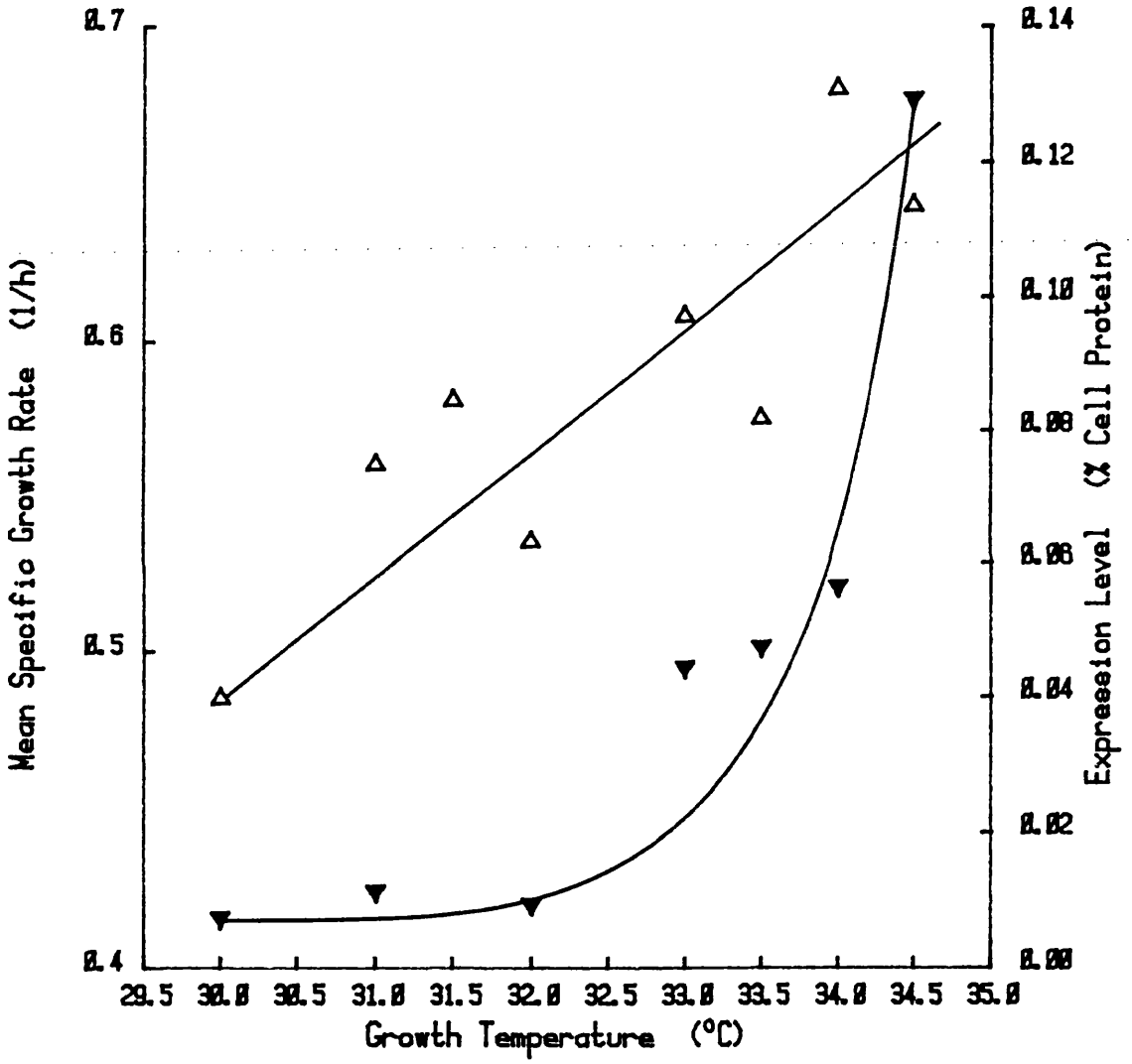


Figure 3.2: Effect Of Growth Temperature On Mean Specific Growth Rate (Δ) and Mean Expression Level (▼) - Strain pCQV2.

Figure 3.2 is a plot of mean maximum specific growth rates and mean expression levels at different growth temperatures. The mean expression level is the mean value of product expression for all the runs at a given growth temperature.

The figure shows that over the range of temperatures investigated there was a rise in mean specific growth rate. There is some scatter but a straight line relationship describes the situation most accurately, even after the variability shown by the sample standard deviation is taken into account. (See table 3.1).

The mean expression levels show that at temperatures up to 32°C there was negligible production during growth. The sample standard deviation shows there was some variability at these temperatures.

At temperatures between 32 and 34.5°C product expression increased. At 34.5°C this became significant (approx 10% final expression level during production). The variability in expression levels, shown by the sample standard deviation, was particularly marked at the temperatures at which expression was significant (33-34.5°C). The expression of certain products is known either to be lethal to cells or to cause reduced growth rates (Botterman JH *et al* (1985); Uhlin BE *et al* (1981)), or to cause plasmid instability (Caulcott CA *et al* (1985)). Whether the expression levels observed here would be sufficient to cause these effects would have to be investigated for other

products.

For individual fermentations there was no correlation between specific growth rate and expression level. For example a run with a lower than average specific growth rate did not necessarily show a higher than average expression level.

Strain pMG169

TABLE 3.2

GROWTH AND PRODUCT EXPRESSION CHARACTERISTICS AT DIFFERENT GROWTH TEMPERATURES FOR STRAIN PMG169.

	GROWTH TEMPERATURE (°C)			
	30	31.5	32	33
Mean Specific Growth Rate (h^{-1})	.467	.476	.555	.508
Sample Size	3	1	2	2
Sample Standard Deviation	.01	n/a	.009	.038
Specific Growth Rate Range (h^{-1})	.462 to .479	n/a	.549 to .561	.481 to .534
Mean Doubling Time (minutes)	89	87	75	82
Doubling Time Range (minutes)	87 to 90	n/a	74 to 76	78 to 86
Mean Expression Level (% cell protein)	.373	.524	.748	.996
Sample Size	2	1	2	1
Sample Standard Deviation	.031	n/a	.107	n/a
Expression Level Range (% cell protein)	.351 to .395	n/a	.672 to .824	n/a

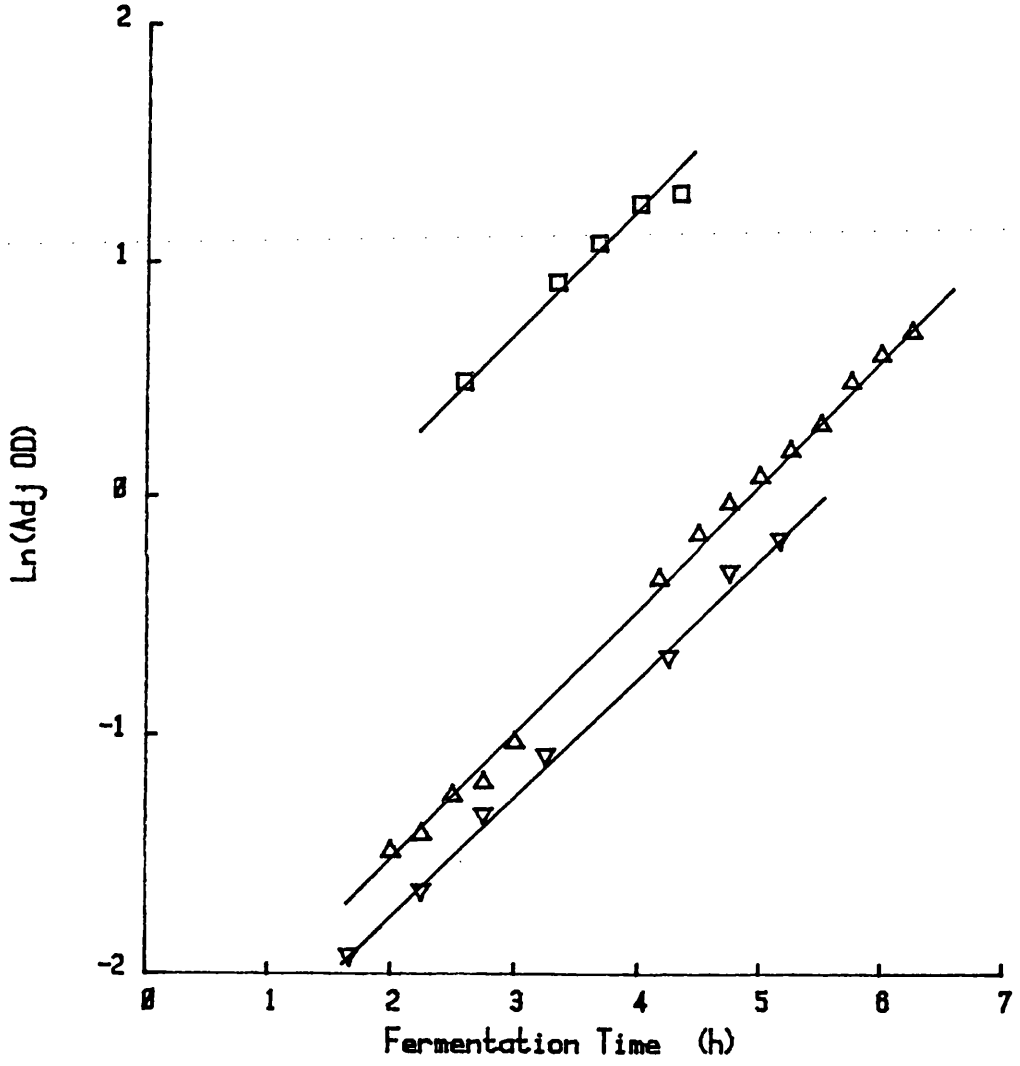


Figure 3.3: Effect Of Growth Temperature On Ln(Adj OD) against Fermentation Time - Strain pMG169 (▽, 30°C [r=.9964]; □, 31.5°C [r=.9874]; △, 33°C [r=.9992]).

Figure 3.3 is representative of each stated growth temperature in that data was selected from fermentations in which the slope was similar to the mean value of specific growth rate shown in table 3.2. It is the same as figure 3.1 except that it shows the results for strain pMG169. The same comments apply in this case regarding the fermentation time as for figure 3.1.

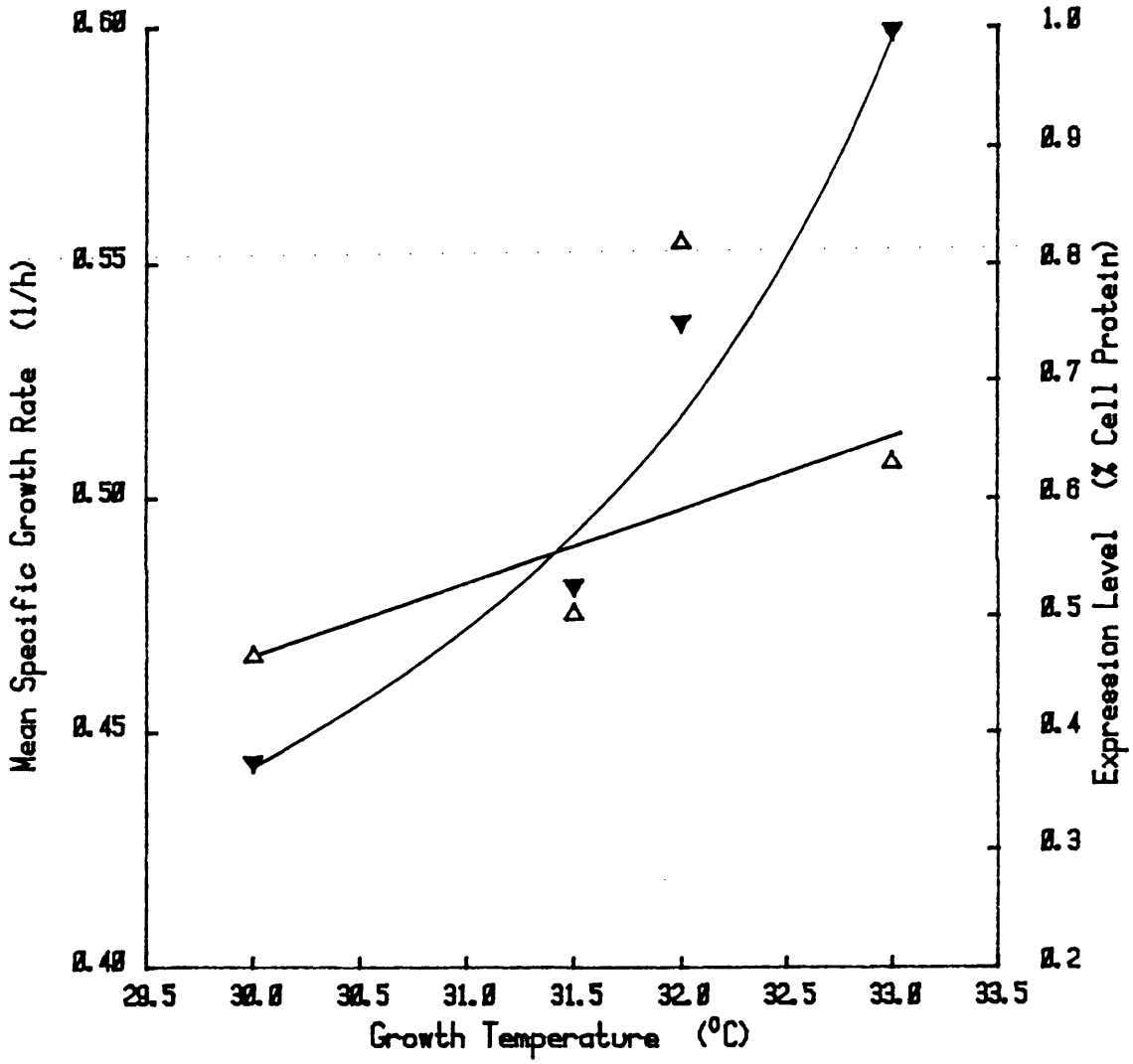


Figure 3.4: Effect Of Growth Temperature On Mean Specific Growth Rate (Δ) and Mean Expression Level (▼) - Strain pMG169.

Figure 3.4 is a plot of mean specific growth rates and mean expression levels at different growth temperatures. This shows that over the range of temperatures investigated there is a slight rise in mean specific growth rate. However the scatter and small size of sample (8 fermentations - see table 3.2) means this result is not conclusive.

The mean expression levels show product to be present at higher levels than for strain pCQV2, even at temperatures as low as 30°C, and as the temperature rose expression levels became even higher. At 33°C the expression level was of the order of 12% final intracellular product level (see figure 3.9), and significantly higher than at any growth temperature for strain pCQV2. Therefore since product expression during growth can have such potentially detrimental effects on fermenter productivity it is even more important to investigate the effects of different products on growth and plasmid stability for this strain.

The sample standard deviation was determined for two of the temperatures, but in each case was calculated from only two different results. However greater variability was apparently shown at 32°C, a temperature yielding significant expression.

3.1.2 Specific Growth Rate at Different Production Temperatures.

The specific growth rate was determined to see how this was affected by production at different temperatures, and for use in design and scale-up calculations.

Strain pCQV2

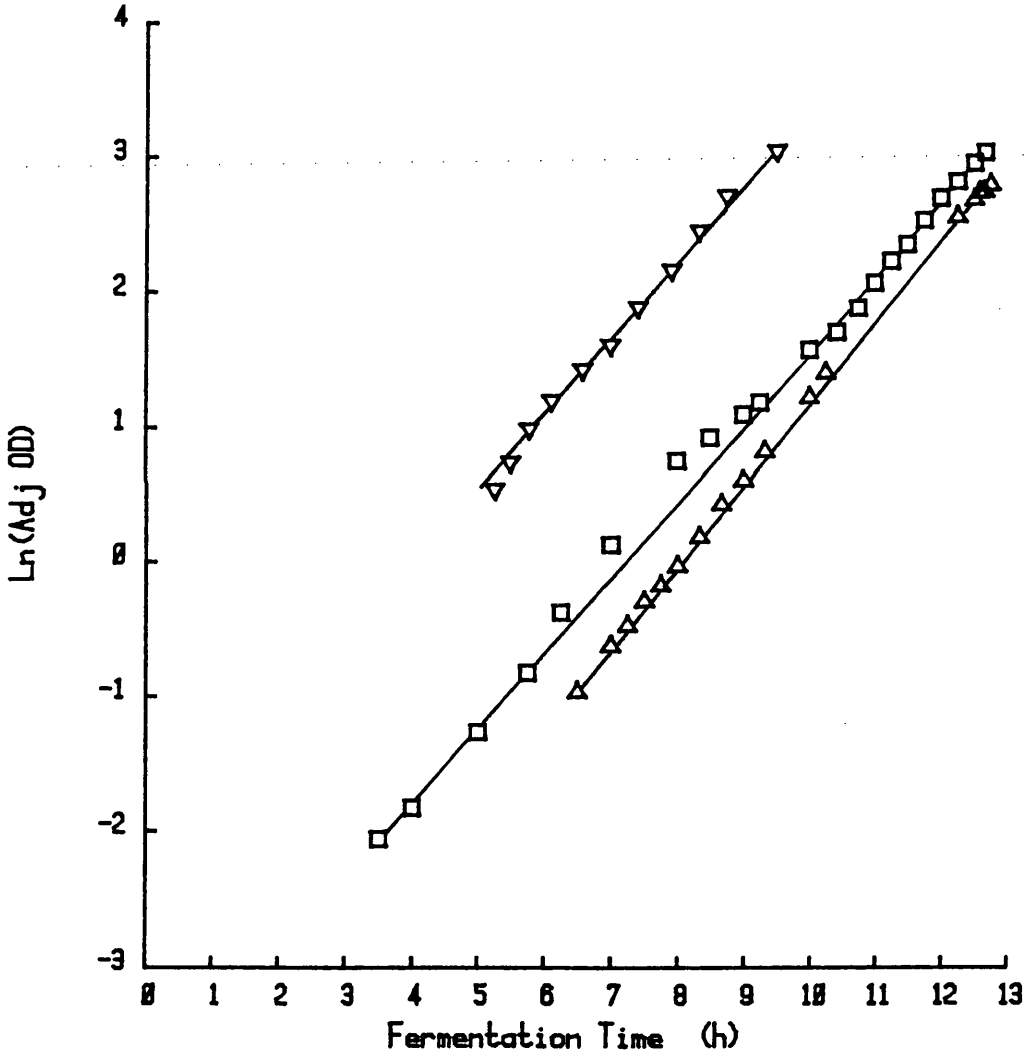


Figure 3.5: Effect Of Production Temperature On Ln(Adj OD) against Fermentation Time - Strain pCQV2 (□ ,37°C [r=.9983]; △ ,40°C [r=.9996]; ▽ ,42°C [r=.9981]).

Figure 3.5 is representative of each stated production temperature. It was clear that exponential growth continued throughout the production phase for strain pCQV2.

TABLE 3.3

GROWTH CHARACTERISTICS AT DIFFERENT PRODUCTION TEMPERATURES FOR STRAIN PCQV2.

	PRODUCTION TEMPERATURE (°C)			
	37	38	40	42
Mean Specific Growth Rate (h^{-1})	.545	.578	.583	.582
Sample Size	3	3	8	13
Sample Standard Deviation	.177	.099	.102	.089
Specific Growth Rate Range (h^{-1})	.367 to .720	.464 to .648	.450 to .713	.512 to .738
Mean Doubling Time (minutes)	76	72	71	71
Doubling Time Range (minutes)	58 to 113	64 to 90	58 to 92	56 to 81

Table 3.3 details the mean maximum specific growth rate, its sample standard deviation and other growth parameters at different production temperatures.

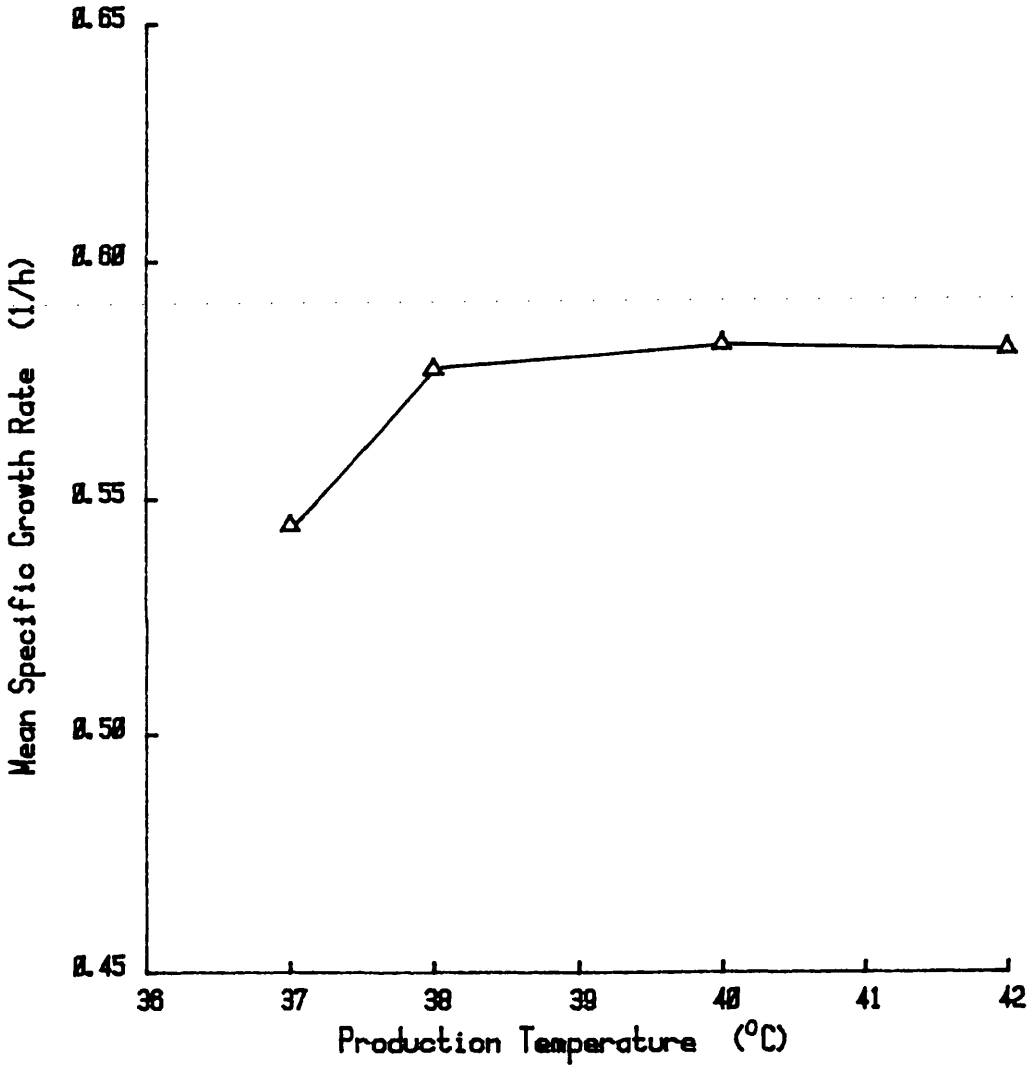


Figure 3.6: Effect Of Production Temperature On Mean Specific Growth Rate - Strain pCQV2.

Figure 3.6 shows that increasing the temperature from 37 to 38°C caused a rise in maximum specific growth rate, but over the range 38-42°C it remained constant. A comparison of figures 3.2 and 3.6 shows the maximum specific growth rate during production to be approximately the same as that over the range of growth temperatures 32-33°C. In the range of growth temperatures 33-34.5°C the maximum specific growth rate was higher than over the range of production temperatures. Section 1.4 in the Introduction contained figure 1.3 which showed that the maximum specific growth rate should be maximal during these higher production temperatures. Clearly the production phase caused a reduction in maximum specific growth rate.

Strain pMG169

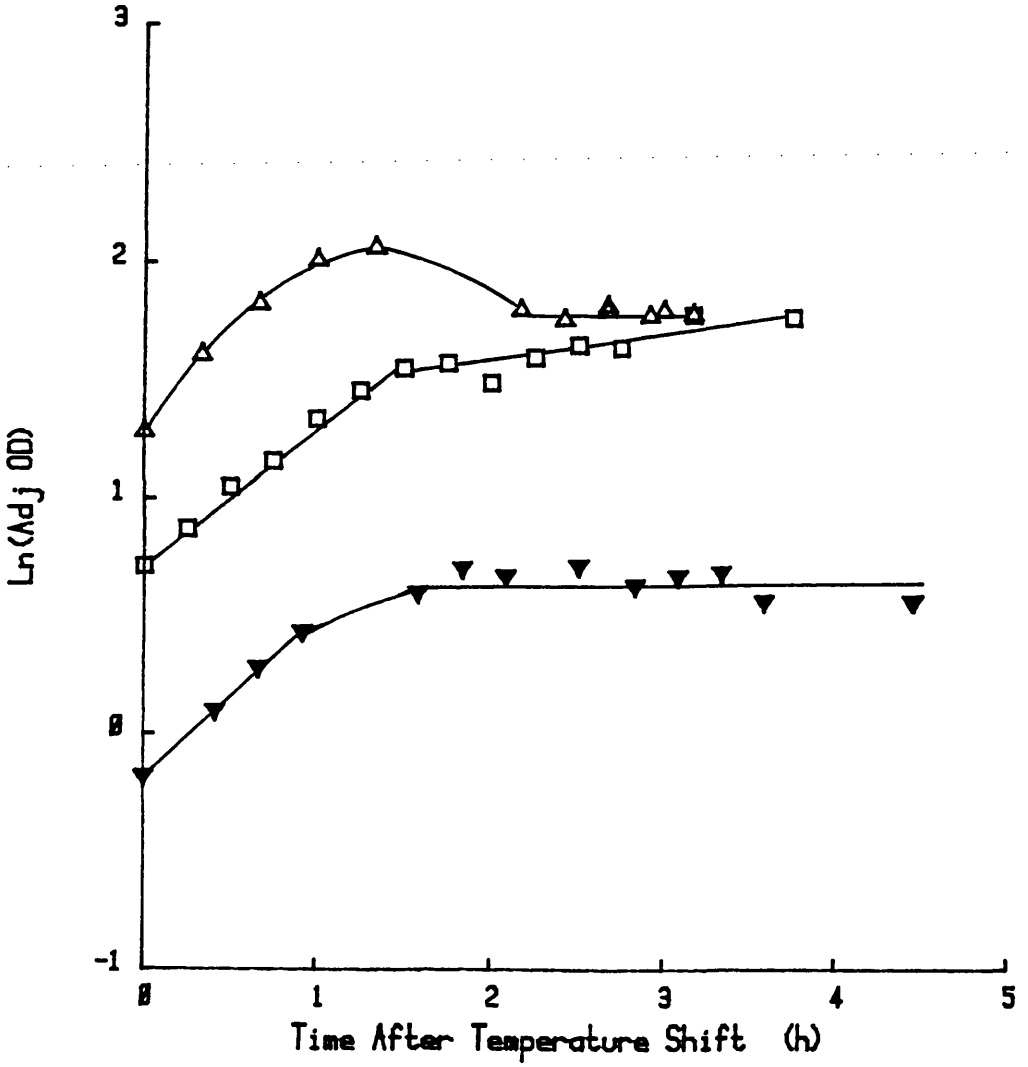


Figure 3.7: Effect Of Temperature On Ln(Adj OD) against Time After Temperature Shift - Strain pMG169 (□,38°C; ▼, 40°C; △, 42°C).

Figure 3.7 shows that after the shift had been implemented the biomass level approximately doubled before growth either ceased or was greatly reduced.

At 38°C the culture grew at a maximum specific growth rate of $0.598h^{-1}$ for 1.25h before it fell to a value of $0.119h^{-1}$ (slopes of the lines on figure 3.7).

At 40°C the culture grew at a specific growth rate of $0.681h^{-1}$ for approximately 1h. Thereafter the biomass level remained constant.

At 42°C the points in figure 3.7 did not lie on a straight line during the initial production phase, when growth was occurring. Instead they laid on a curve such that the slopes of tangents (maximum specific growth rate) fell as time progressed. It took around 1.25h for the biomass concentration to peak. Thereafter it fell slightly, before staying constant for the remainder of the fermentation.

3.1.3 Production Kinetics.

The production kinetics were investigated to determine the effects of different fermentation conditions on the batch productivity, for the two strains.

These were determined by CAT and protein assays, as described in sections 2.2.9 and 2.2.8 respectively, and

verified by polyacrylamide gel electrophoresis (Appendix 1 for result) at different production temperatures.

3.1.3.1 Typical Intracellular Production Profile.

This section deals with the typical shapes of the intacellular CAT concentration profiles during the production phase for the two strains. Intracellular CAT concentration was defined as the amount of CAT measured in disrupted cell samples taken from the fermentations, expressed in terms of the amount of total protein released. This was either expressed as % cell protein (as in figure 3.8) or as normalised unit.g^{-1}dry cell sample (see section 2.2.9). Besides intracellular CAT there was also the possibility of extracellular CAT being produced - that is CAT produced intracellularly and then exported. Appendix 2 shows that there were never any appreciable levels of this. Therefore this was ignored and only intracellular CAT was considered.

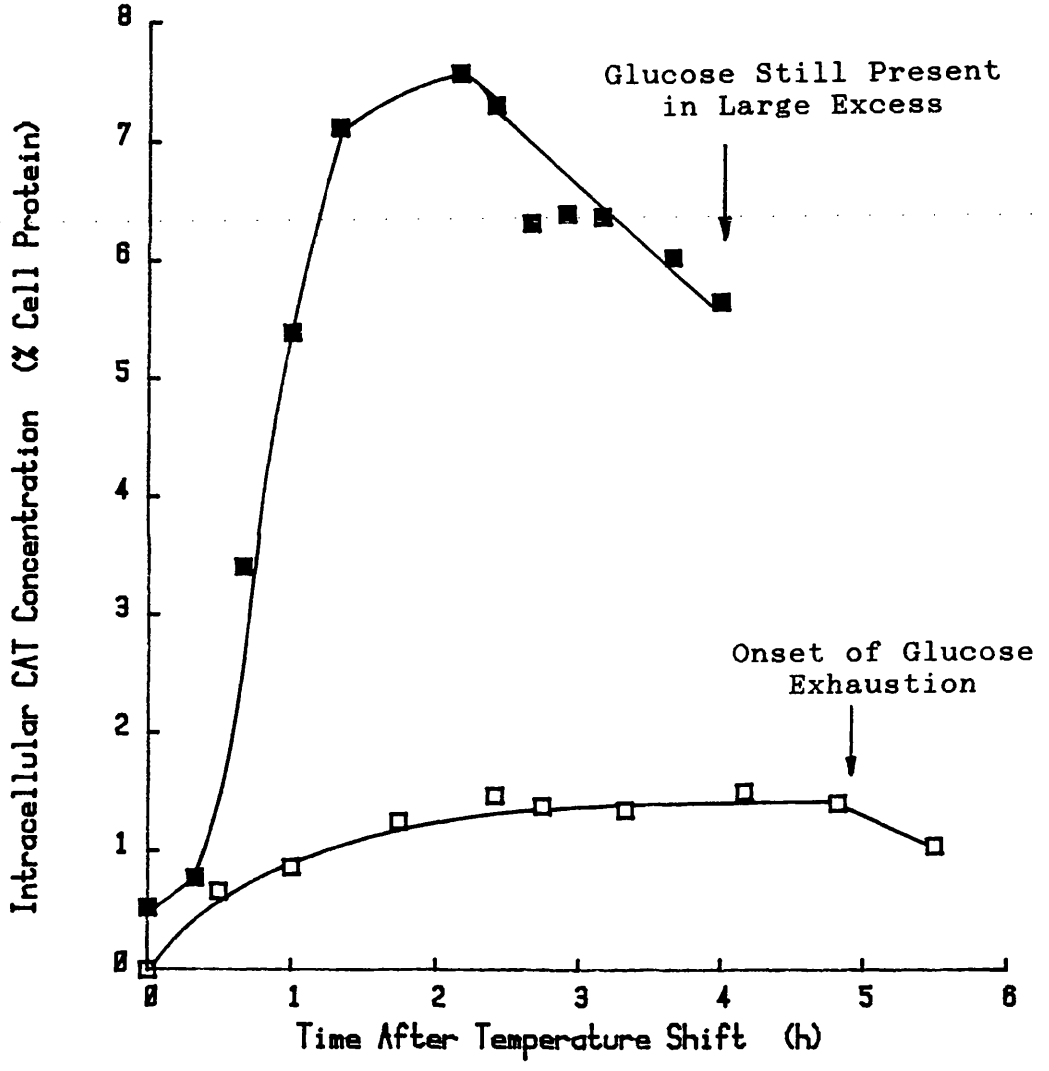


Figure 3.8: Typical Intracellular CAT Concentration Profile against Time After temperature Shift (□,pCQV2; ■,pMG169).

Figure 3.8 shows that for strain pCQV2 CAT accumulated in the cells to peak at its maximum concentration in around 2.5h. The concentration then remained constant for the rest of the fermentation until glucose exhaustion. Thereafter it began to fall. The peak value for the fermentation described here was around 1.45% intracellular protein.

Strain pMG169 showed a different intracellular production profile. The intracellular CAT concentration rose to its peak level in around 1.5 to 2h. It remained at this level for approximately 0.5h before beginning to fall. The peak value shown was around 7.5% intracellular protein and the level then fell to 5.6% intracellular protein in 1.75h. Glucose was still present in the media in large excess, so the fall was not due to degradation caused by nutrient starvation. The turnover may have resulted from heat-shock proteolytic action caused by the rise in fermentation temperature and/or the abnormally high intracellular CAT concentration (see section 1.3).

The peak intracellular CAT concentration for strain pMG169 occurred approximately 0.5h after growth stopped as described in section 3.1.2, meaning that the production phase for this strain under kinetic limitation was only around 2h.

This figure shows strain pMG169 to be a better intracellular CAT producer than strain pCQV2. Peak CAT concentration was around 5-fold higher for the former strain compared to the latter.

3.1.3.2 Peak Intracellular Production at Different Temperatures.

These results describe the mean peak intracellular CAT concentration for the two strains at different temperatures. This is the mean value of the peak intracellular CAT concentrations at each temperature.

TABLE 3.4

PEAK PRODUCT EXPRESSION CHARACTERISTICS AT DIFFERENT PRODUCTION TEMPERATURES FOR STRAIN PCQV2.

	PRODUCTION TEMPERATURE (°C)			
	37	38	40	42
Mean Peak Value (% cell protein)	.5	1.0	1.7	1.3
Sample Size	1	2	4	5
Sample Standard Deviation	n/a	.141	.411	.229
Peak Value Range (% cell protein)	n/a	.9 to 1.1	1.2 to 2.2	1.1 to 1.6

Table 3.4 shows the analysis of this parameter for strain pCQV2.

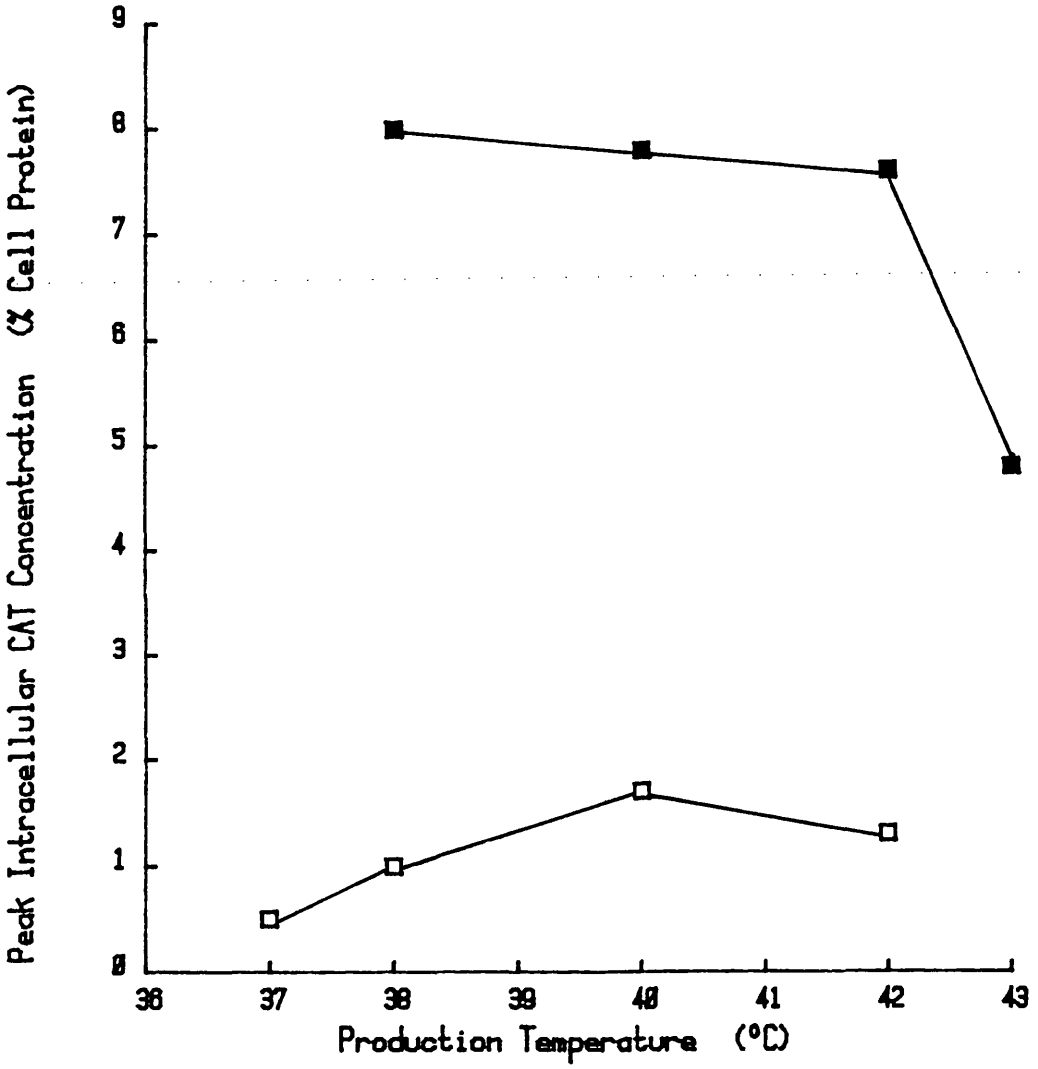


Figure 3.9: Mean Peak Intracellular CAT Concentration against Production Temperature (□ ,pCQV2; ■ ,pMG169).

Figure 3.9 shows that for strain pCQV2 a rise in peak levels over the temperature range 37 to 40°C was followed by a fall from 40 to 42°C. This suggests that 40°C was kinetically the best temperature for the accumulation of CAT within the cell. However the sample standard deviation shows that 40°C displayed the greatest variability in peak values. The poorer production at 42°C might have been due to increased proteolytic action stimulated by the higher temperature.

Strain pMG169 showed a 5% fall in peak level over the temperature range 38 to 42°C from 8 to 7.6% intracellular protein, followed by a fall to 4.8% intracellular protein at 43°C. It is questionable whether the perceived fall in peak values over the range 38 to 42°C was a real effect because as can be seen in figure 3.8 it was difficult to say for certain what the exact shape of the profile was around the peak. Curve fitting was ruled out to obtain the maximum because of the shapes of the profiles, and so individual samples had to be relied upon to be taken at the exact moment of peak intracellular CAT concentration. It is therefore quite likely that a sample was not taken at the exact moment of maximum intracellular CAT concentration, and so the perceived fall in peak values from 38 to 42°C might be erroneous.

These results suggest that 40°C was the best production temperature for strain pCQV2, but that there was little to choose between 38 to 42°C for strain pMG169.

3.1.3.3 Intracellular Production Profile at Different Temperatures.

This section deals with the intracellular CAT concentration profiles against time for the two strains at different production temperatures.

The intracellular CAT concentration, as described in this work might also have been termed specific CAT concentration. However this was not done in order to avoid a possible confusion between specific production profiles and specific production rates (see section 3.1.3.6). In the former the CAT concentrations present within the cell at known times during the fermentation were plotted, whereas in the latter the rates of CAT production within the cell at any time were determined.

The values expressed in this section represent the mean intracellular CAT concentration in the cell sample taken at any time. It is not a measure of the total amount of intracellular CAT produced by each cell in the sample, because in the fermenter the cells were growing and dividing and in so doing donating some of their intracellular CAT to their progeny. Hence there would actually have been a distribution of amounts of CAT produced by each cell according to age, with cells born during the production phase having produced less than those present from its beginning.

Strain pCQV2.

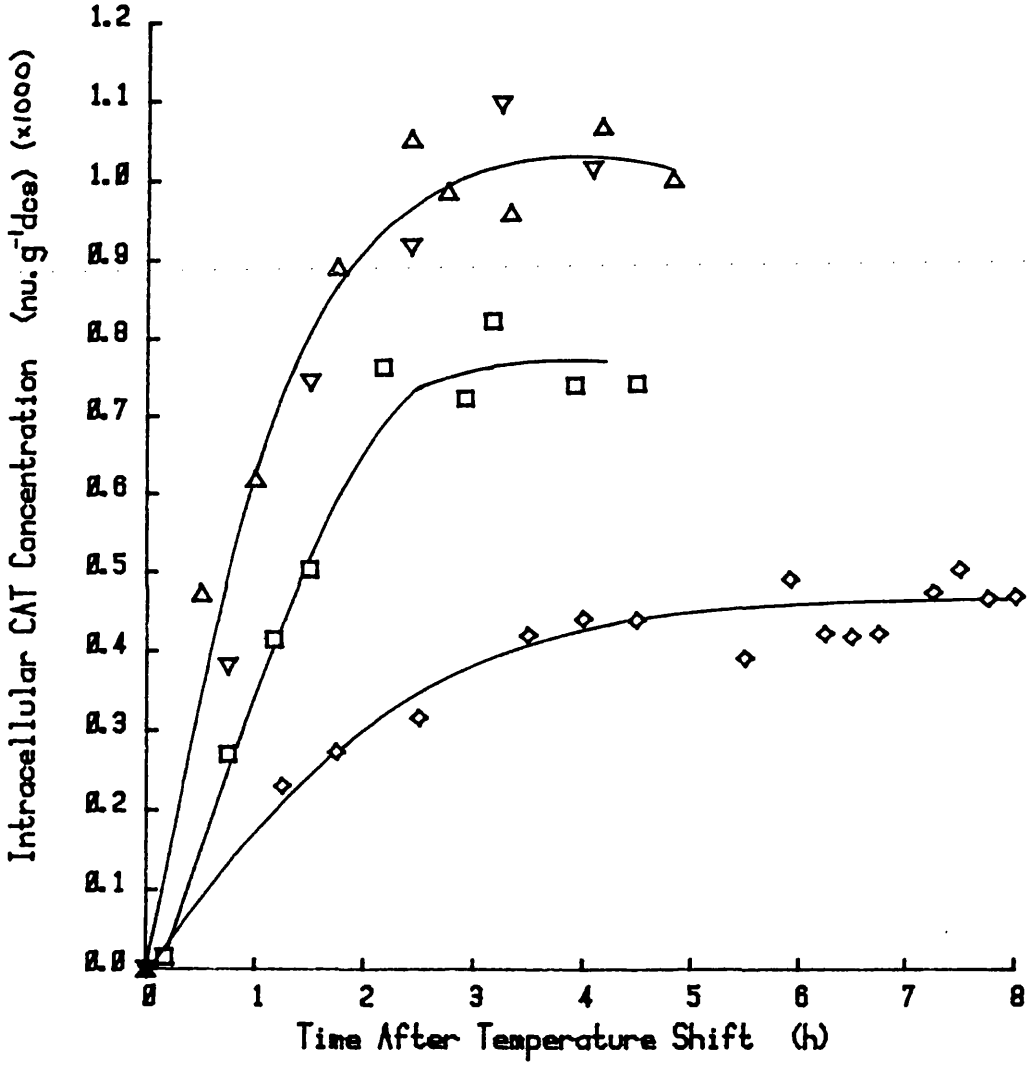


Figure 3.10: Effect Of Temperature On Intracellular CAT Concentration Profile against Time After Temperature Shift - Strain pCQV2 (◇ ,37°C; □ ,38°C; ▽ ,40°C; △ ,42°C).

Figure 3.10 is a plot of intracellular CAT concentration (measured in normalised units of enzyme per gram of dry cell sample) against fermentation time subsequent to implementation of the temperature shift. The figure shows a consistent shape of profile for the four different temperatures. It shows increasing intracellular levels of CAT over the range of temperatures 37 to 40°C. A temperature of 42°C yielded a similar profile to 40°C. The kinetics are such that not only did the higher production temperatures give higher peak values but they were also reached more quickly subsequent to the temperature shift.

Strain pMG169.

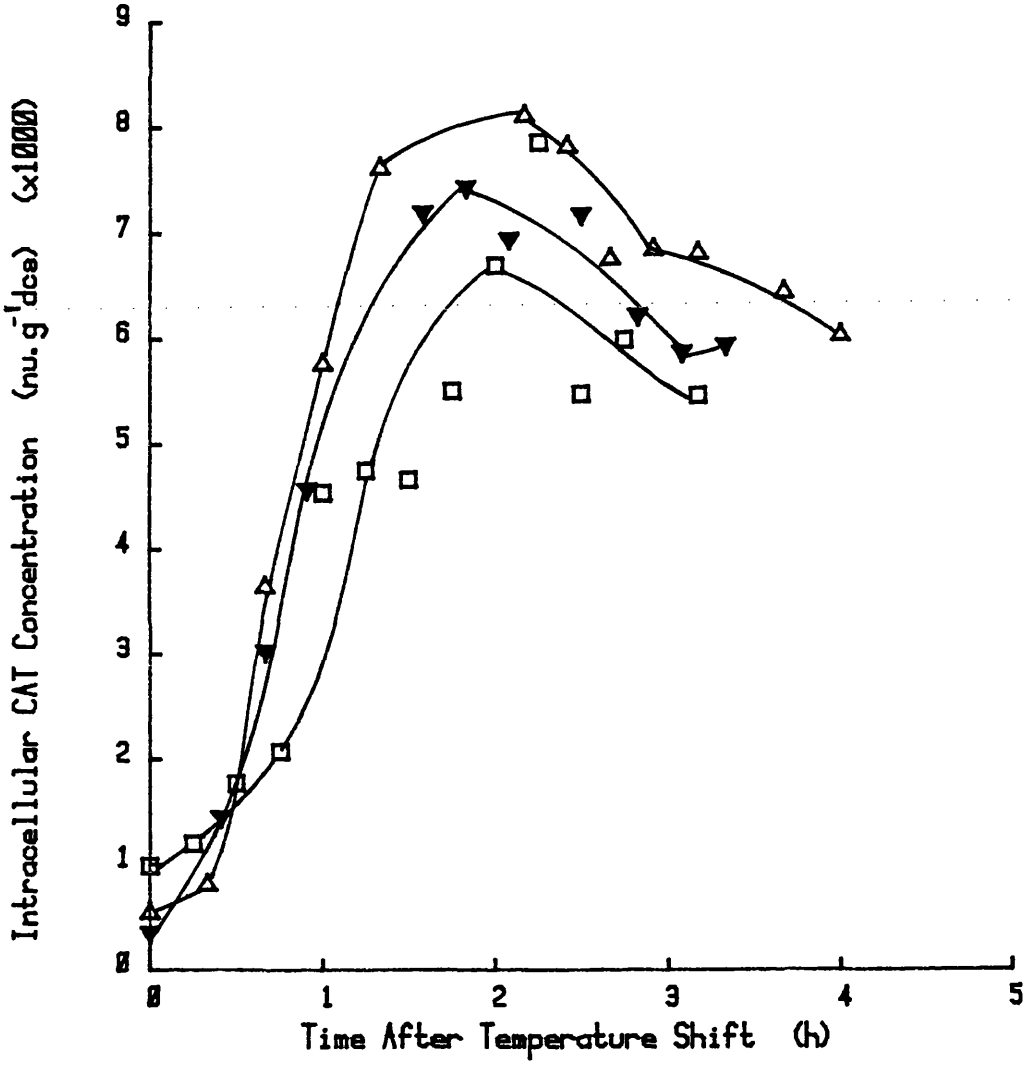


Figure 3.11: Effect Of Temperature On Intracellular CAT Concentration Profile against Time After Temperature Shift - Strain pMG169 (□ , 38°C; ▼ ,40°C; △ ,42°C).

Figure 3.11 is a plot of intracellular CAT concentration (measured in normalised unit of enzyme per gram of dry cell sample) against fermentation time subsequent to implementation of the temperature shift. The figure shows a consistent shape in that the intracellular CAT levels rose to peak, and then fell again. The time to peak varied for the different temperatures. At 38°C it was approximately 2.25h, and at 40 to 42°C it was around 1.75h. This corresponded with a lag of up to 1h subsequent to growth being curtailed, as mentioned in section 3.1.2.

At time 0h (ie the time at which the temperature shift was implemented) the fermentations displayed different intracellular CAT concentrations. The reason for this was that the growth phases were carried out at different growth temperatures, which caused different basal levels of CAT to be expressed prior to commencement of the production phases (see section 3.1.1).

3.1.3.4 Reactor Production Profile at Different Temperatures.

This shows how CAT accumulated in the reactor after the temperature shift had been implemented for the two strains. These values are a combination of the intracellular production profiles seen in section 3.1.3.3 and biomass generation seen in section 3.1.2.

The values of reactor CAT concentration were determined by multiplying the CAT concentration in each sample by the number of samples present in the reactor per litre at that time:

Volume required for OD20 sample = (20/Adj OD) ml

Number of samples per litre = 1000/(20/Adj OD)

= 50*Adj OD

Therefore reactor CAT concentration ($nu.L^{-1}$) =

intracellular CAT concentration ($nu.g^{-1}dcs$) * 50 * Adj OD

($no\ sample.L^{-1}$)

Strain pCQV2.

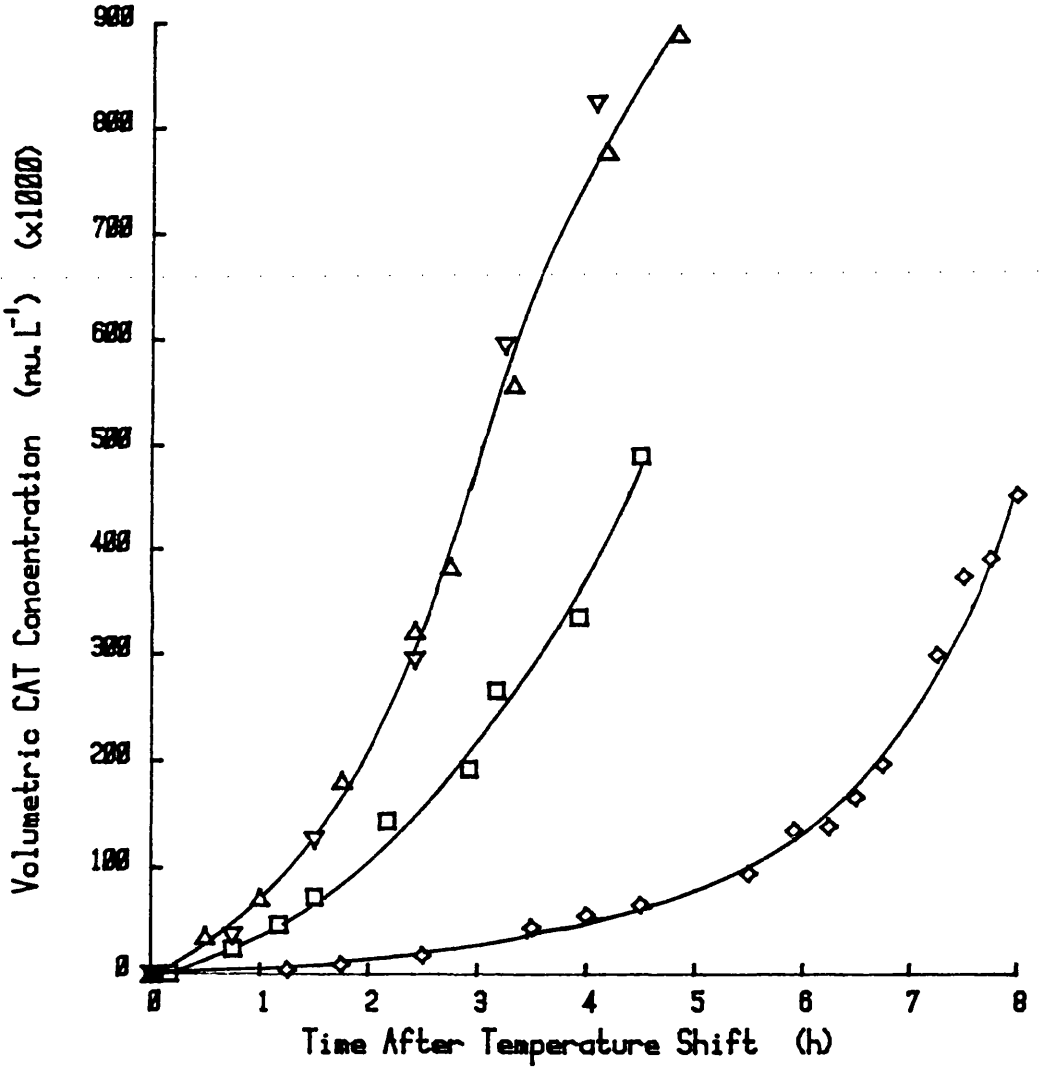


Figure 3.12: Effect Of Temperature On Volumetric CAT Concentration Profile against Time After Temperature Shift - Strain pCQV2 (◇ ,37°C; □ ,38°C; ▽ ,40°C; △ ,42°C).

Figure 3.12 shows that the maximum CAT concentration in the reactor occurred at the end of the fermentation, at the point of glucose exhaustion. This was due to the fact that although the intracellular CAT concentration had peaked and remained constant, the biomass was increasing exponentially, with the result that the reactor CAT concentration continued to rise.

Figure 3.8 shows that intracellular CAT concentration fell subsequent to glucose exhaustion. Obviously growth also stopped at this point, so the implication for reactor CAT concentration is that this too would fall. This means that the fermentation must be deactivated and/or harvested at the point of glucose exhaustion if product degradation due to substrate starvation is to be avoided.

The figure also shows that the higher temperatures of 40 to 42°C caused CAT to appear in the reactor at the fastest rate and to the highest levels.

On a reactor basis, up to 40°C higher production levels were clearly achieved by higher temperatures.

Strain pMG169.

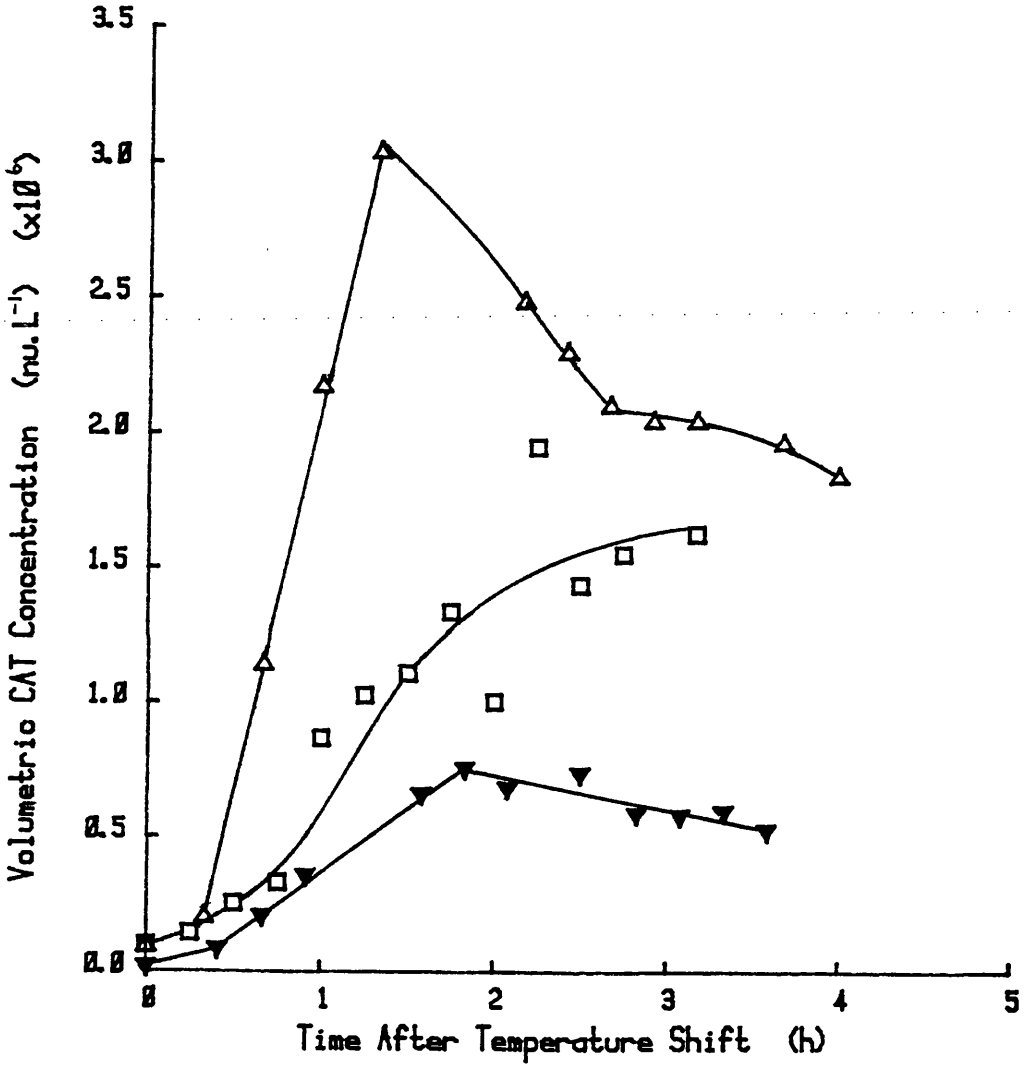


Figure 3.13: Effect Of Temperature On Volumetric CAT Concentration Profile against Time After Temperature Shift - Strain pMG169 (□ ,38°C; ▼ ,40°C; △ ,42°C).

For this strain the point at which the temperature shift was implemented was particularly important in determining the final reactor CAT concentration. This is because growth stopped (or greatly reduced) 1 to 1.25h after the temperature shift (see section 3.1.2). CAT production ceased approximately 1h after this. Thus, unlike strain pCQV2, the final reactor CAT concentration was greatly determined by the biomass present at the time of the shift.

Figure 3.13 shows this clearly. These profiles mimic in shape the intracellular ones shown in figure 3.11. However, the 40°C fermentation had its temperature shift implemented at a much lower biomass level than the 38°C run, and lower still than the 42°C fermentation. Hence the final biomass level for the 42°C run was higher than the 38°C, which was higher than the 40°C run. This explains the differences in reactor CAT concentration profile seen between the three temperatures shown.

In the case of the 38°C fermentation the CAT concentration peaked after 2.25h, fell, and then started to rise again. The fall coincided with the reduction in specific growth rate, and the subsequent rise was due to continued increase in biomass at the lower growth rate (see figure 3.7) .

The lengths of the production phases were 1.75 to 2h at 40°C and 1.3 to 2h at 42°C.

At the time of implementation of the shift (0h) the fermentations displayed different intracellular CAT

concentrations. This was due to the different temperatures that the growth phases were carried out at, which expressed different basal levels of CAT (see section 3.1.1).

3.1.3.5 Volumetric Production Rate Profile at Different Temperatures.

This is defined as the instantaneous rate of CAT formation in the reactor, and was calculated by taking the slopes of the tangents to the curves in figure 3.12. The values are expressed as nu.$L^{-1}h^{-1}$ in figure 3.14.

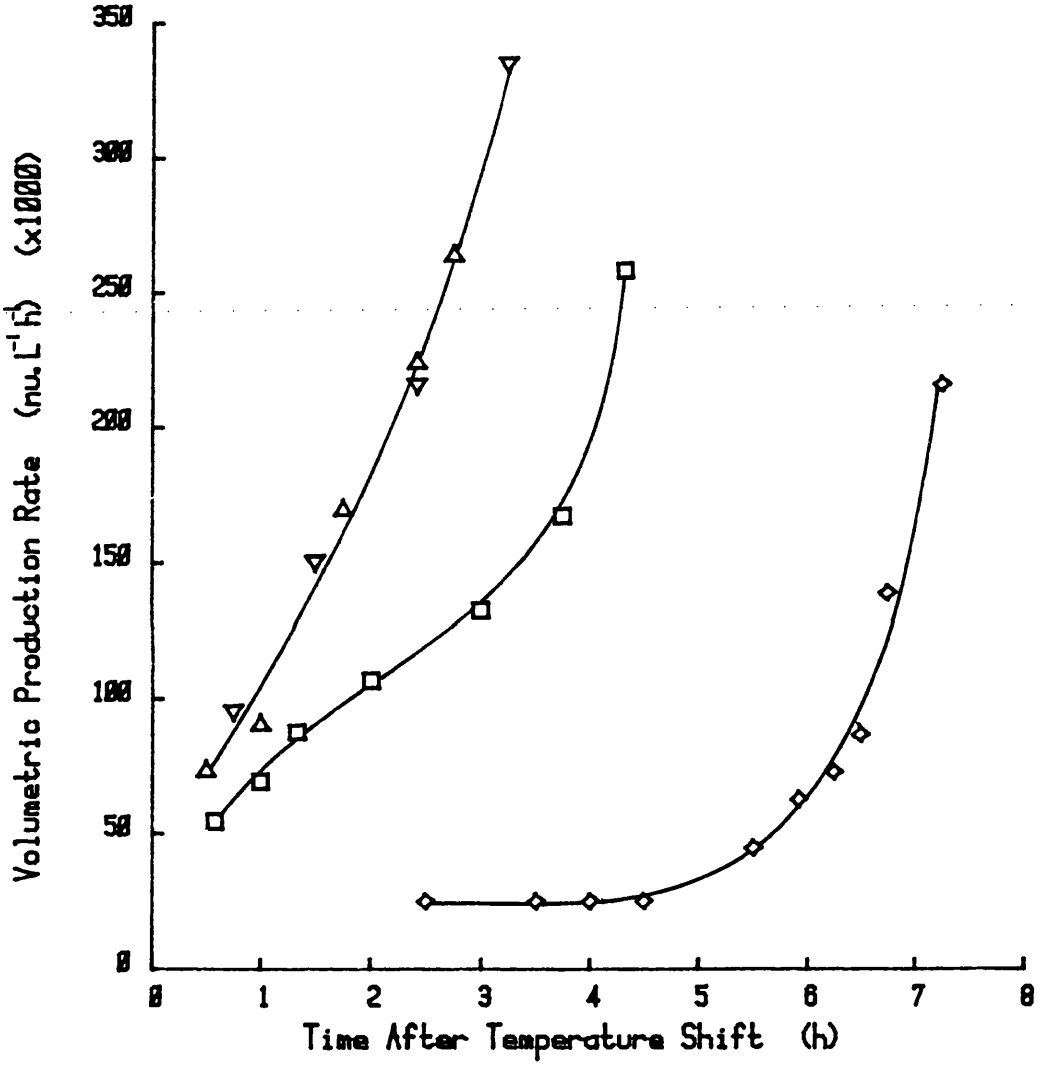


Figure 3.14: Effect Of Temperature On Volumetric Production Rate against Time After Temperature Shift - Strain pCQV2 (◇ ,37°C; □ ,38°C; ▽ ,40°C; △ ,42°C).

Since drawing tangents to curves can be inaccurate, at each point two estimates of the tangent were drawn as extreme as possible while still remaining reasonable. The slopes of the two estimates were measured and the mean value plotted in figure 3.14 as the actual tangent. In no case was the deviation from the mean greater than 5%, and in most cases it was less than 2.5%.

Figure 3.14 shows similarly shaped profiles to those in figure 3.12, with the volumetric production rate maximal in the case of every temperature at the point of glucose exhaustion.

The rates were higher (at any given time) in the range 38 to 42°C than those observed for 37°C because both the intracellular CAT concentrations (figure 3.10), and the specific growth rates (figure 3.6) were higher at these temperatures.

The rates at 40 to 42°C were higher at any given time than those observed for 38°C only because the intracellular CAT concentrations were higher at these temperatures.

The reason that the volumetric production rate was highest prior to glucose exhaustion, even though intracellular CAT concentration was constant, is that this was the time when biomass was being generated at the fastest rate.

Details of volumetric production rates for strain pMG169 have not been presented because as for figure 3.13 the rate

depended largely on the biomass level at which the temperature shift was implemented. However straight lines could be drawn through the data in figure 3.13 during the actual production phase. Therefore the volumetric production rate for strain pMG169 was constant, with the following values (presented for completeness):
38°C - 826,446 nu.$L^{-1}h^{-1}$, 40°C - 478,469 nu.$L^{-1}h^{-1}$, 42°C - 2,999,999 nu.$L^{-1}h^{-1}$.

3.1.3.6 Specific Production Rate Profile at Different Temperatures.

This is defined as the production rate per gram of dry cells, and was calculated by dividing the volumetric production rates by the biomass present at the time to give nu.g^{-1}dcw.h^{-1}. The accuracy was lower than that for the volumetric production rate because inaccuracies associated with estimating the biomass concentration were also incorporated in the specific production rate, even though these were minimalised by good technique.

The specific production rate was important in scaling up the process because it represented a production rate within the cell, and so can be applied to other biomass concentrations to give the volumetric production rate under those conditions. Therefore batch titres at different scales and biomass concentrations can be calculated. The volumetric production rates determined in section 3.1.3.5

were not as general in that the profiles could only be used to calculate batch titres if identical biomass concentrations to those in these experiments were designed for. The problem here was that the OURs achieved in these experiments were higher than are achievable at appreciable scales (see section 3.1.6). Oxygen limitation would result at larger scale, and affect production (see section 3.2.2). Therefore the volumetric production rate profiles would not be accurate for estimating the batch titre at different scales. However a value of the specific production rate could be used, when combined to a particular changing biomass profile, to estimate the batch titre.

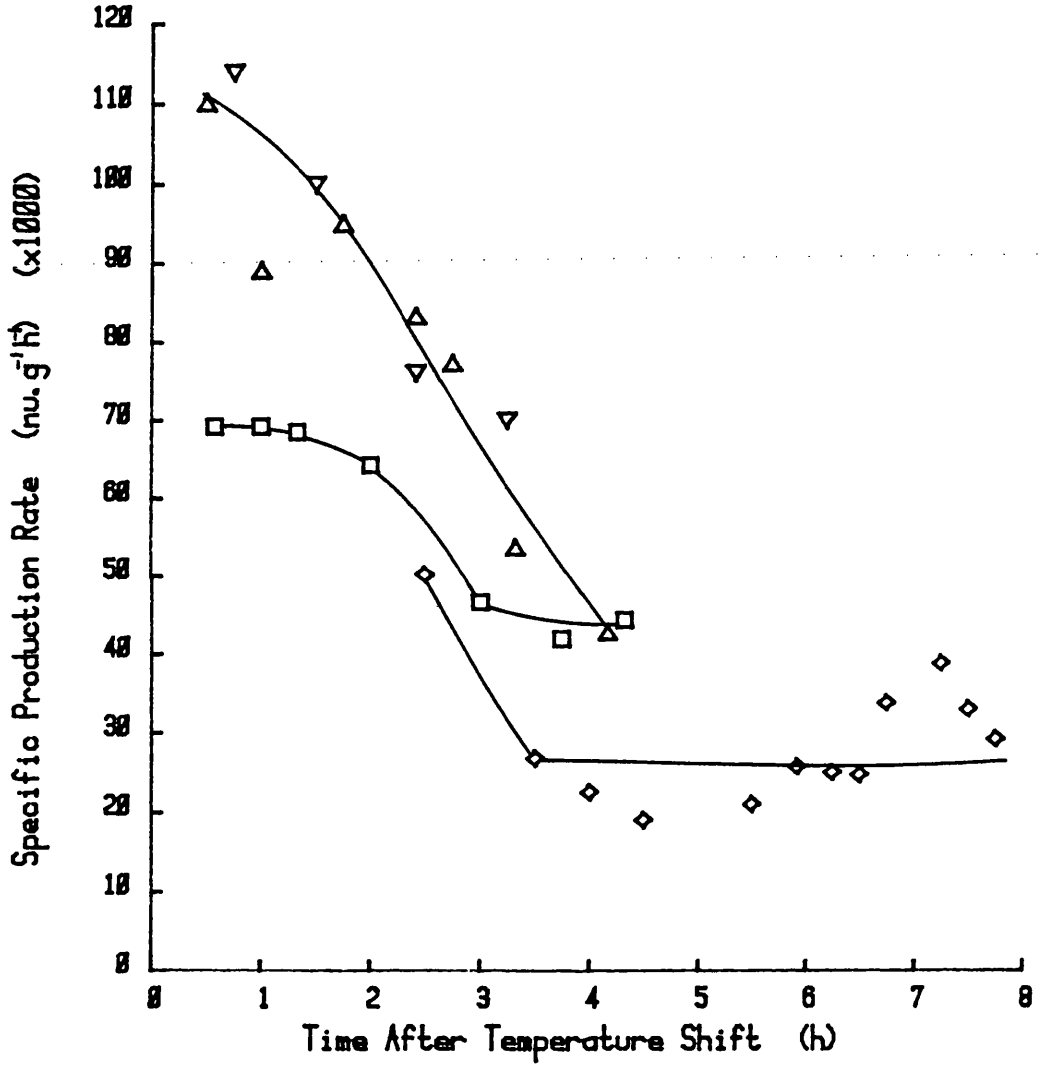


Figure 3.15: Effect Of Temperature On Specific Production Rate against Time After Temperature Shift -Strain pCQV2 (◇ , 37° C; □ , 38° C; ▽ , 40° C; △ , 42° C).

Figure 3.15 shows specific production rate, (1/X)*dC/dt (where X is biomass concentration and C is CAT concentration), against time after the temperature shift, for strain pCQV2.

As expected the specific production rate increased from 37 to 40°C but was approximately the same at 40 and 42°C.

At 37°C it remained approximately constant throughout the production phase.

The temperatures 38 to 42°C caused high initial specific production rates followed by a reduction. This is consistent with the intracellular CAT concentration against time data presented in figure 3.10 which described an initial fast increase in CAT concentration followed by a gradual reduction in the rate of increase until the intracellular CAT concentration peaked and remained on a plateau. At this point it would be expected that the specific production rate would fall to zero.

However figure 3.15 shows this not to be the case. The specific production rates fell from their initially high values but still remained at significant levels. This was because the specific production rate figures contained two components. The first component was the rise in intracellular CAT concentration subsequent to the shift. The second was the CAT produced and given to progeny upon cell division. After production began the two were combined as the CAT produced both increased the intracellular CAT

concentration and was donated to progeny upon division. However once the plateau in intracellular CAT concentration was reached all the CAT produced then went to progeny. Hence initially the specific production rate was highest and then it fell to a constant rate.

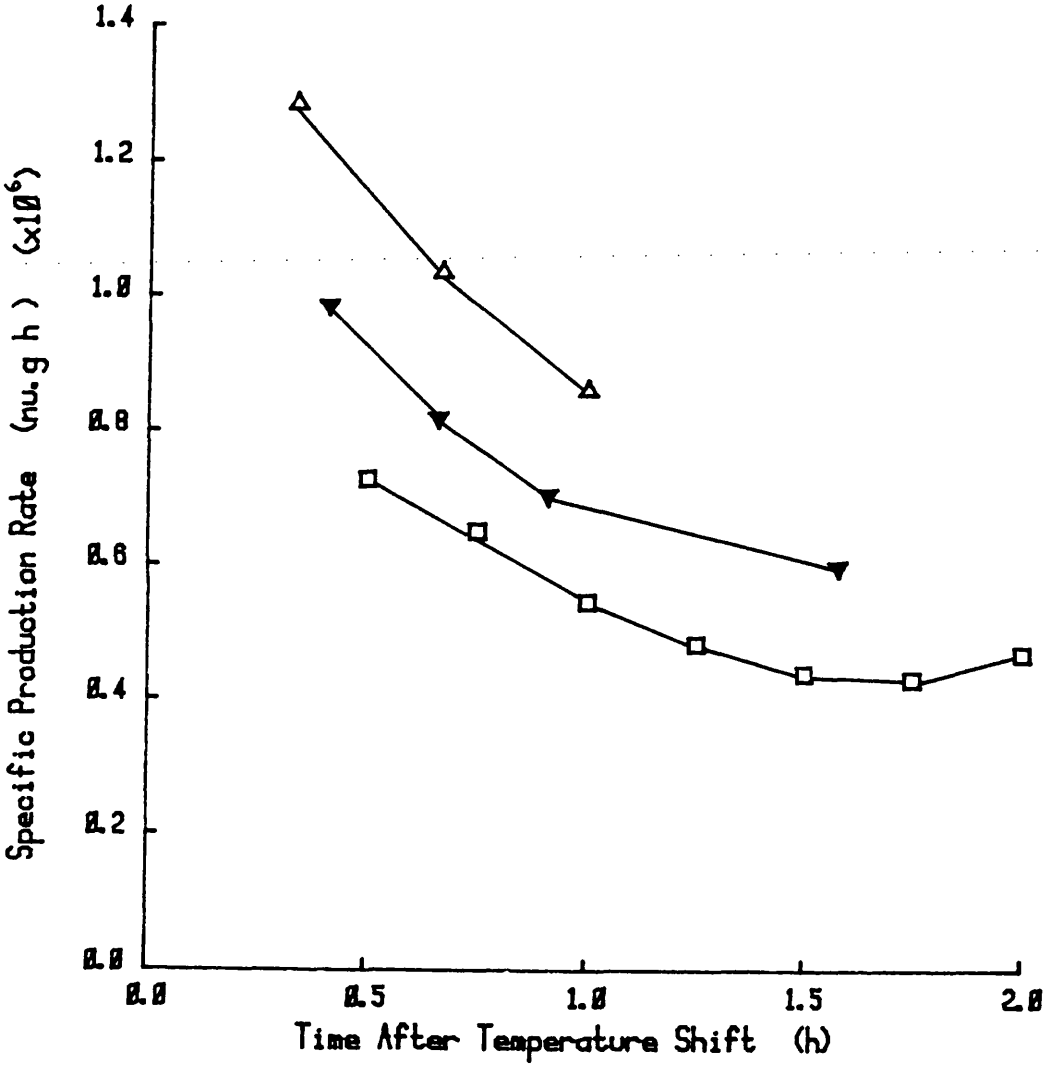


Figure 3.16: Effect Of Temperature On Specific Production Rate against Time After Temperature Shift -Strain pMG169 (□,38°C; ▼,40°C; △,42°C).

Figure 3.16 shows specific production rate, (1/X)*dC/dt, against time after shift for strain pMG169. Since the volumetric production rate was assumed to be constant, then as the biomass concentration increased the specific production rate would clearly fall. Increasing the temperature resulted in increased specific production rates. There are only three points for each of the two highest temperatures because the peaks in volumetric CAT concentration occurred within 1.5 to 2h after the shift, and it was decided that values should only be calculated after production was well under way, and before the peak was achieved. This was decided because the exact shape of the curves at these extremes was not known, and so the tangents would have been speculative.

3.1.4 Overall Yield Coefficient at Different Temperatures.

The overall yield coefficient $Y_{(x/s)}$ is an important parameter because it measures how well the carbon substrate is being used for biomass generation. Conversely, it also shows the level of non growth-associated metabolic activity. (X is the biomass concentration and S is the concentration of substrate, in this case glucose).

The $Y_{(x/s)}$ was defined as gram of biomass produced per gram of glucose removed from the media (g dcw.g^{-1}glucose). Biomass generation was measured from Adjusted OD readings

and from ln(Adj OD) vs time plots like those shown in figures 3.1, 3.3, and 3.5. Glucose removal from the media was measured by assaying the clarified broth for glucose (see section 2.2.10) and plotting the results to give a profile for glucose uptake by the cells. Thus over a significant time-scale an accurate measure of the $Y_{(x/s)}$ was determined.

TABLE 3.5

OVERALL YIELD COEFFICIENT AT DIFFERENT GROWTH AND PRODUCTION TEMPERATURES FOR BOTH STRAINS.

TEMPERATURE (°C)	$Y_{(x/s)}$ ($gdcw.g^{-1}$)	Sample Size	Sample Standard Deviation	$Y_{(x/s)}$ Range ($gdcw.g^{-1}$)
pCQV2				
30	.515	1	n/a	n/a
31.5	.576	1	n/a	n/a
33	.435	1	n/a	n/a
33.5	.547	1	n/a	n/a
34	.622	2	.013	.613 to .631
34.5	.565	3	.076	.500 to .649
37	.521	2	.054	.482 to .559
38	.469	3	.012	.460 to .482
40	.469	7	.058	.395 to .554
42	.471	9	.104	.370 to .695
pMG169				
30	.562	1	n/a	n/a
33	.457	1	n/a	n/a

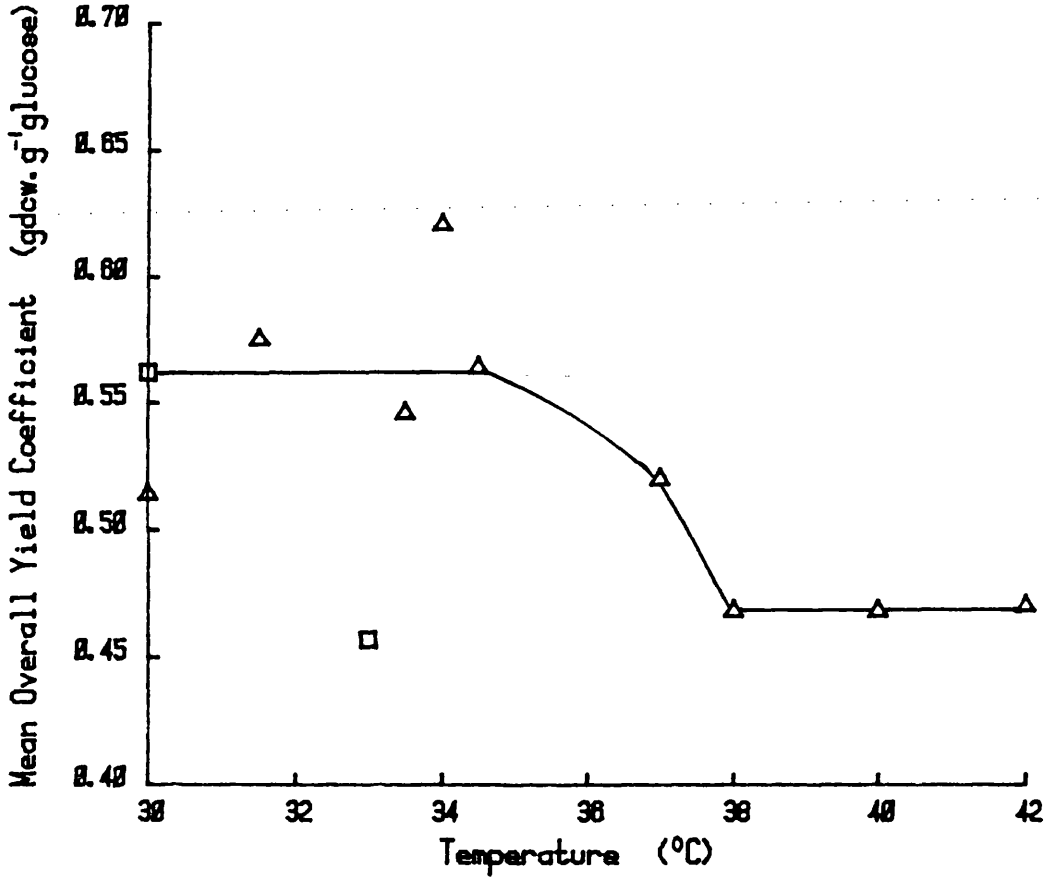


Figure 3.17: Effect Of Temperature On Mean Overall Yield Coefficient (△ ,pCQV2; □ ,pMG169).

Figure 3.17 shows that there was scatter at the growth temperatures. This was largely due to the small sample available (see Table 3.5), caused by the necessity of having to begin the production phases at biomass concentrations around $1.5gL^{-1}$. At this level of biomass it was difficult to accurately measure the change in broth glucose concentration. Hence these results were taken from fermentations with longer growth phases or from the heat of fermentation experiments (see section 3.4)

The production temperatures showed less scatter, although the sample standard deviations showed some variability.

Figure 3.17 shows that the mean $Y_{(x/s)}$ was higher at the growth temperatures and that production caused the mean $Y_{(x/s)}$ to fall. In the range of production temperatures 38 to 42°C, the mean $Y_{(x/s)}$ remained constant.

3.1.5 Effect of Time Taken to Implement the Temperature Shift on Production.

An investigation was carried out to see the effects on production of implementing the temperature shift over different lengths of time. This is important because of the costs and difficulties of raising the temperature quickly at different scales. It would be useful to use the metabolic heat to raise the temperature (see section 3.4), at least partially, but this cannot be done

instantaneously. Work done on ts systems in the laboratory has, for convenience, tended to raise the temperature of the culture quickly (Wright E *et al* (1986)). Therefore it was necessary to find the effects of different lengths of shift time on production.

Strain pCQV2.

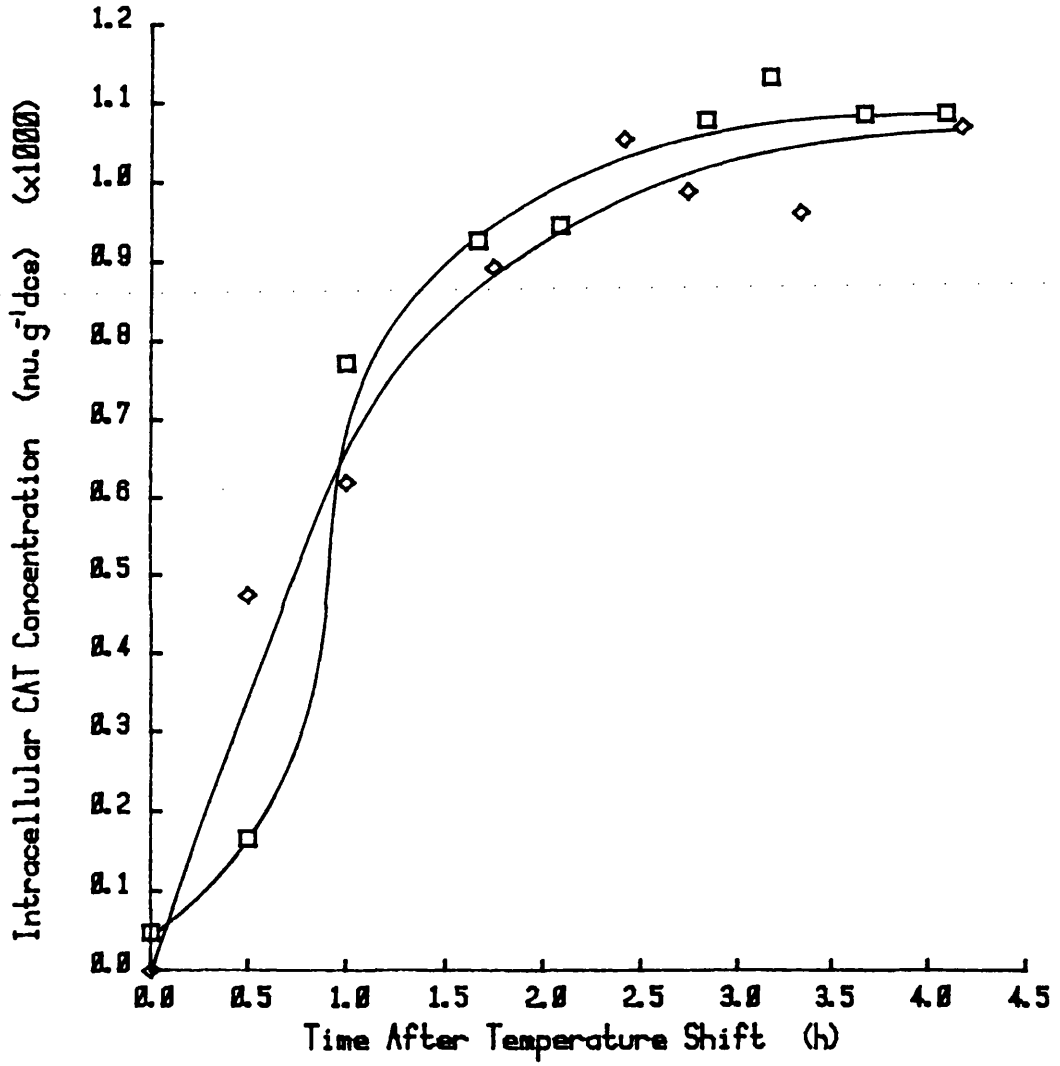


Figure 3.18: Effect Of Speed Of Temperature Shift On Intracellular CAT Production - Strain pCQV2 (□ ,slow shift; ◇ ,fast shift).

Figure 3.18 is a plot of intracellular CAT concentration against time after the beginning of the implementation of the temperature shift. The results shown are for fermentations with production phases at 42°C.

The shift implemented in 3 minutes was achieved using steam passed through the heat transfer coils within the fermenter. The shift implemented in 0.5h was achieved using hot water passed through the same coils. In both cases the rate of temperature rise was linear.

Figure 3.18 shows the profiles to be the same shape except that in the slowly shifted run the profile lags the faster shifted run by around 0.5h. The peak intracellular CAT concentration was almost identical for the two fermentations.

Strain pMG169.

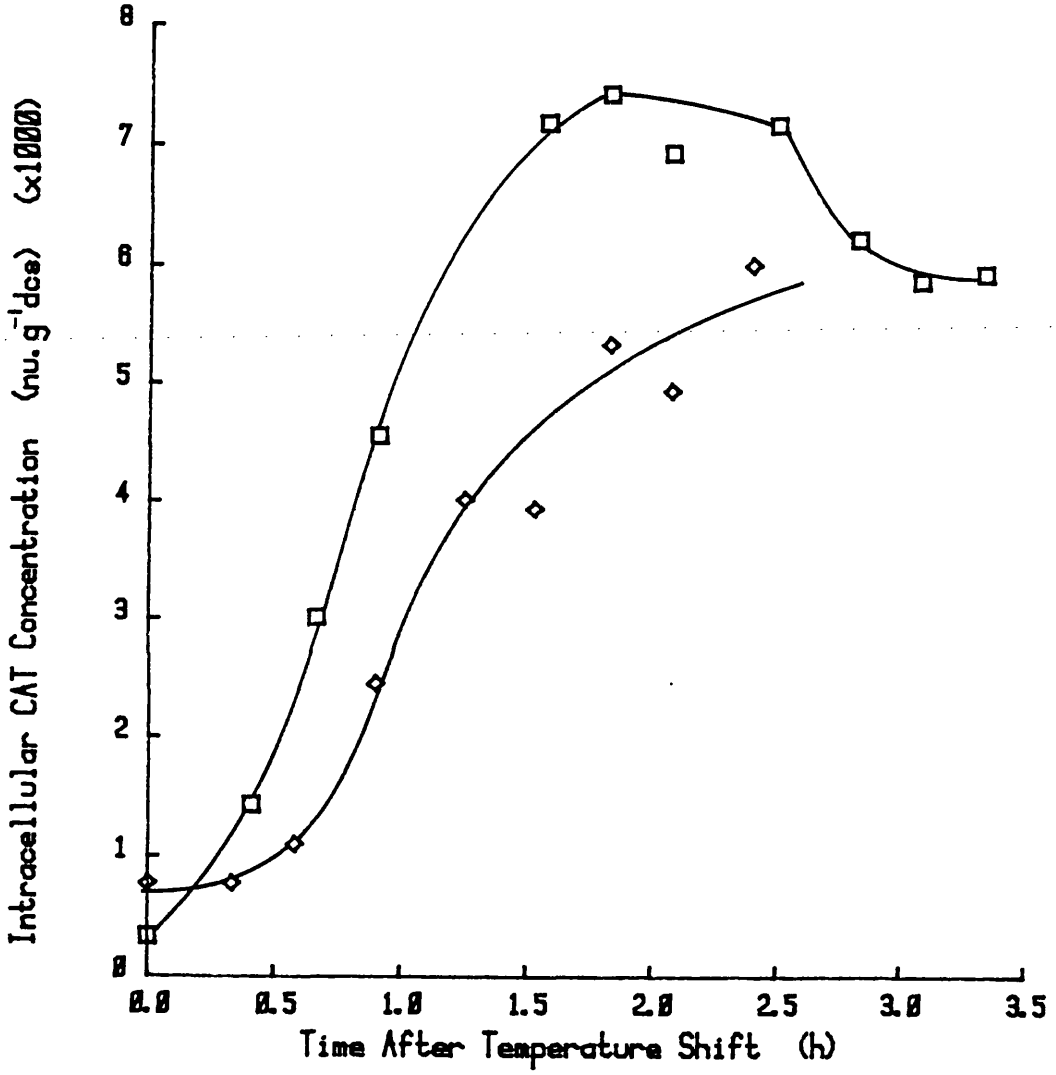


Figure 3.19: Effect Of Speed Of Temperature Shift On Intracellular CAT Production - Strain pMG169 (◇ ,slow shift; □ ,fast shift).

Figure 3.19 is a plot of intracellular CAT concentration against time after the beginning of the implementation of the temperature shift. The results shown are for fermentations with production phases at 40°C.

The shift implemented in 3 minutes was achieved using steam passed through the heat transfer coils within the fermenter. The configuration of the temperature control system was changed to allow the metabolic heat of fermentation to be measured (see section 3.4) and so the slow shift was implemented using a reduced cooling water flowrate and constant power to the immersion heater in the fermenter. The slow shift was implemented in 0.83h. The reason that the slow shift took longer to implement for strain pMG169 compared to strain pCQV2 was that it was difficult to estimate the correct settings of heater power and cooling water flowrate to raise the temperature in exactly the same time for both strains. Since a linear rate of temperature rise was desired the settings were not altered during the shift, and so the slow shifts took differing lengths of time to achieve.

In both the fast and slow shifts the rate of temperature rise was linear.

Figure 3.19 shows in the case of the quickly shifted fermentation a rapid increase in intracellular CAT concentration followed by a peak and then a reduction. The peak was achieved in around 1.75h, similar to the result described in section 3.1.3.3.

In the case of the slowly shifted fermentation it was not until approximately 0.5h after the beginning that a sufficient temperature was reached that caused an increase in intracellular CAT concentration. By the end of the shift production was occurring at its fastest rate. The intracellular CAT concentration increased over a period of 2h and although the rate of rise was slowing it had not peaked when the fermenter was harvested. As usual the growth had stopped but the intracellular CAT concentration had still not peaked. A shift of this length extended the the production phase without an apparent increase in intracellular CAT concentration. It is possible that production would have continued, resulting in higher intracellular CAT concentration, being comparable to the more slowly shifted fermentation. However it would be recommended that a shift taking less time than the 0.83h here should be designed for, of the order of 0.5h, similar to the slow shift for strain pCQV2 (see earlier in this section).

3.1.6 Batch Oxygen Requirements.

This was an important area of investigation because it was expected that in a large-scale batch fermenter, growth and/or production could be oxygen limited. In the 14 L fermenter under the batch conditions stated it was not expected to be a problem.

In the case of strain pCQV2 the shapes of the OUR profiles were exponentially increasing curves mimicing biomass generation. They peaked at glucose exhaustion and thereafter quickly fell away.

In the case of strain pMG169 the OUR profiles showed the same shape except they peaked at the point during production at which growth stopped (see section 3.1.2) and then fell away.

3.1.6.1 Specific OUR Profile at Different Temperatures.

The specific OUR is defined as the rate of oxygen uptake per gram of dry cells. It was calculated by dividing the OUR at any point in time by the biomass concentration present at that time.

Strain pCQV2.

TABLE 3.6

BATCH OXYGEN UPTAKE CHARACTERISTICS AT DIFFERENT TEMPERATURES FOR STRAIN PCQV2.

	TEMPERATURE (°C)				
	30	34	38	40	42
Peak OUR ($mM.L^{-1}h^{-1}$)	88	115	121	105	105
Biomass At Peak OUR ($g.L^{-1}$)	11.97	11.739	11.422	8.114	8.034
Specific OUR At Peak ($mM.g^{-1}h^{-1}$)	7.35	9.80	10.59	12.94	13.07

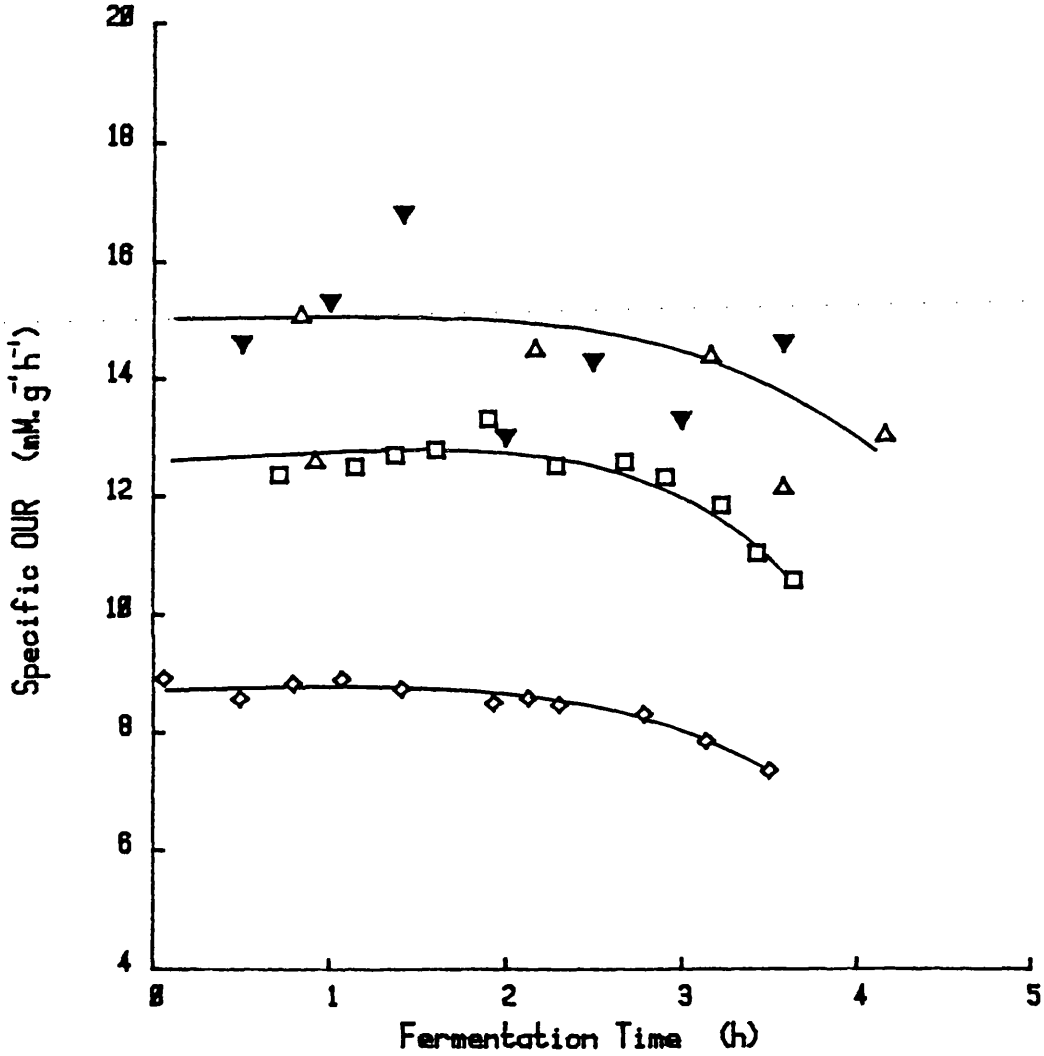


Figure 3.20: Specific OUR against Fermentation Time - Strain pCQV2 (◇ , 30°C; □, 38°C; △ , 40°C; ▼ , 42°C).

Figure 3.20 shows that the trend was for higher specific OURs at higher temperatures. Since the specific OUR is a measure of the oxygen requirements per gram of dry cells the results show that there was a higher degree of non growth-associated metabolic activity at the higher temperatures (given that the specific growth rates (see section 3.1.1 and 3.1.2) were similar at all these temperatures). This was probably partly due to increased requirements for production and partly due to increased maintenance needs.

The figure also shows the values to be constant with a trail-off (of around 15%) towards the end of the fermentations. Since this trailing-off was seen at 30°C it was not an effect of production. Instead it was probably due to errors of measurement. Biomass generation was measured from Adjusted OD readings and from ln(Adj OD) vs time plots like those shown in figures 3.1, 3.3, and 3.5. Towards the end of the fermentations the ln(Adj OD) points still lay on the straight line. Therefore it is unlikely that the biomass concentration was overestimated, even given the errors associated with dilution of the sample for reading, which was kept minimal with careful pipetting techniques. The errors were more likely to originate from the gas analysis system. Part of the exit gas stream was pumped to the mass spectrometer using a diaphragm pump. However this was not a perfect plug-flow system, which meant there would have been some mixing between the gas pumped immediately before and after any given sample. This effect would only cause appreciable errors when the

composition of the gas was rapidly changing, and would give lower OURs than was actually the case, because wall effects would predominate, causing a sample to mix with a previous one. Since the OUR was an exponential function under the fermentation conditions described, it changed at an increasingly rapid rate, so the error became greater as the fermentation progressed.

Strain pMG169.

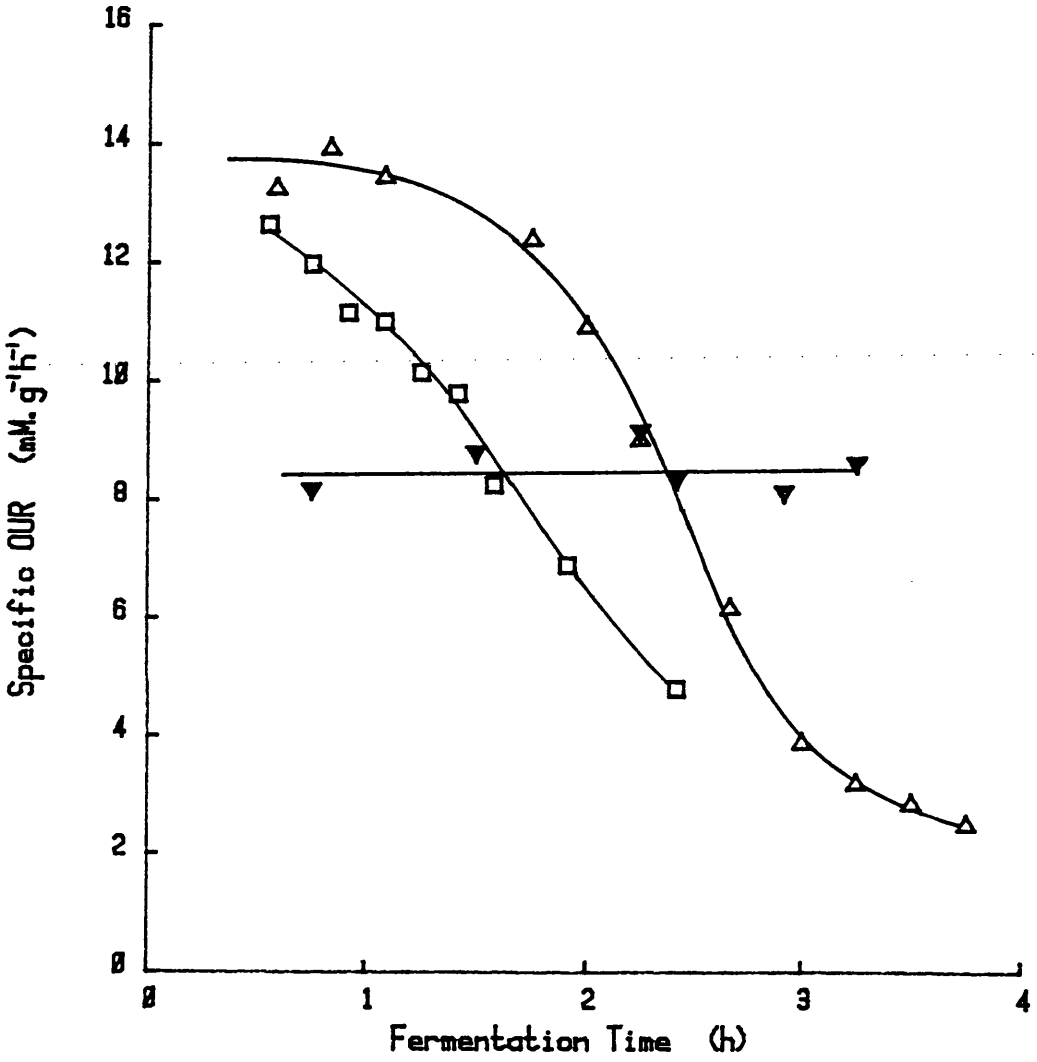


Figure 3.21: Specific OUR against Fermentation Time - Strain pMG169 (▼,30°C; △,40°C; □,43°C).

Figure 3.21 shows the specific OUR at 30°C to be constant over the time-scale plotted, at the same level as strain pCQV2.

However at the production temperatures the specific OUR remained high while growth occurred. Thereafter the biomass concentration remained largely unchanged but the OUR fell, resulting in this reduction of specific OUR.

The value at 40°C initially (t = 0.5 to approximately 1.75h) was around the same as for strain pCQV2.

The error associated with the gas analysis system described in the previous section did not account for artificially high or low values in this case, because the rate of change of gas composition was never as high as when the errors were seen for strain pCQV2.

3.2 BATCH FERMENTATION UNDER OXYGEN LIMITATION.

These experiments were performed because the oxygen requirements under kinetic limitation could not be supplied with air practically on scales up to 100 m^3. Therefore if the processes were run in the way previously described O_2-limitation would result. A worst case situation would be a 100 m^3 fermenter in which the maximum sustainable OUR would be of the order of 50 to 60 $moles.m^{-3}h^{-1}$. In order to achieve the same OUR in the 14 L fermenter the agitator

rate was reduced to 685 rpm during the growth phase for the two strains.

In addition to this, on the large scale it is common to go to biomass concentrations much higher than reported here under limitation of a key nutrient.

Figures 3.22 to 3.25 have consistent time axes.

3.2.1 Effect of Oxygen Limitation on Growth.

It was important to determine the effects of oxygen limitation on growth because this mode of operation offered the possibility of achieving higher biomass concentrations in large fermenters than could be achieved using kinetic limitation. It was possible that this could have led to higher fermenter productivities had the production kinetics been favourable.

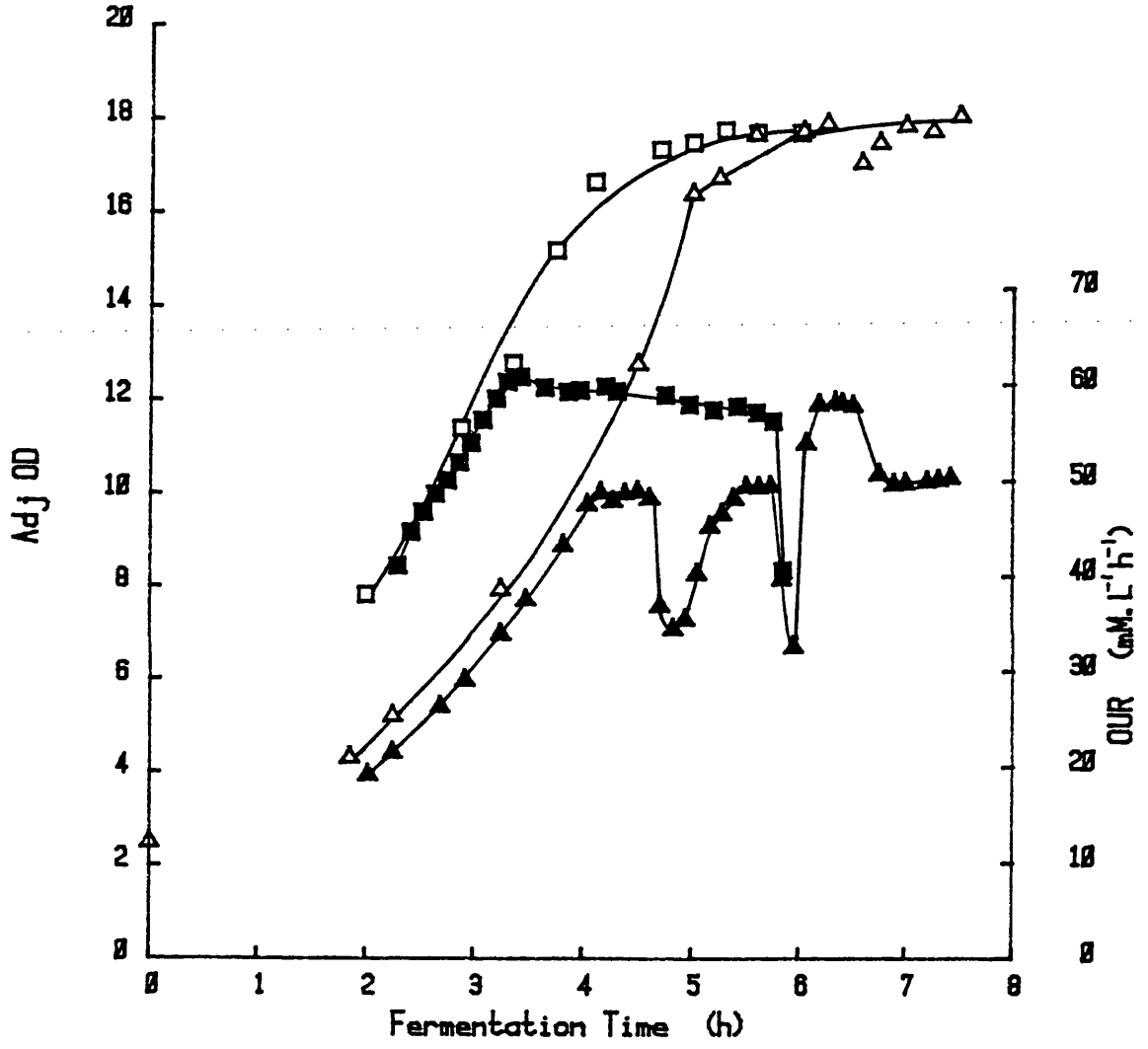


Figure 3.22: Effect Of Oxygen Limitation On Growth - Adjusted OD (△ ,pCQV2; □ ,pMG169) and OUR (▲ , pCQV2; ■ ,pMG169).

Figure 3.22 is a plot of Adjusted OD and OUR against fermentation time for the two strains. It shows the onset of O_2-limitation as the OUR became constant (OUR=OTR [oxygen transfer rate to the broth]) and the corresponding change in growth from exponential to linear. Biomass generation continued throughout the production phase under these conditions, for both strains.

There was a delay between the OTR becoming constant and growth becoming linear (denoting the onset of O_2-limitation). This was due to saturation of the broth with dissolved O_2 (DOT=100%) which occurred under kinetic limitation prior to the onset of O_2-limitation (see section 2.2.2.5). O_2-limitation would not occur until this dissolved O_2 was removed. Therefore the measured OUR was lower than the actual OUR because the latter consisted of removal of dissolved O_2 from the broth as well as that transferred from the gas. It was only after the DOT fell to zero that true O_2-limitation commenced, and this corresponded to the onset of linear growth.

Strain pCQV2 showed two periods of reduced OUR after the onset of constant OTR. The first occurred because the agitator rate was reduced to ensure the DOT fell more quickly, and it was subsequently raised again when DOT = zero. The second occurred because of glucose exhaustion, shown by a rise in the pH and a fall in the fermenter temperature. 1L fresh media containing 100 g glucose as the limiting nutrient was immediately batched in. The fermentation then quickly recovered. 1 L of media of the

same composition was added to the pMG169 fermentation before the first sample was taken to avoid a similar occurrence.

3.2.2 Effect of Oxygen Limitation on Production.

As stated at the beginning of section 3.2.1 it was important to determine how oxygen limitation affected the production kinetics.

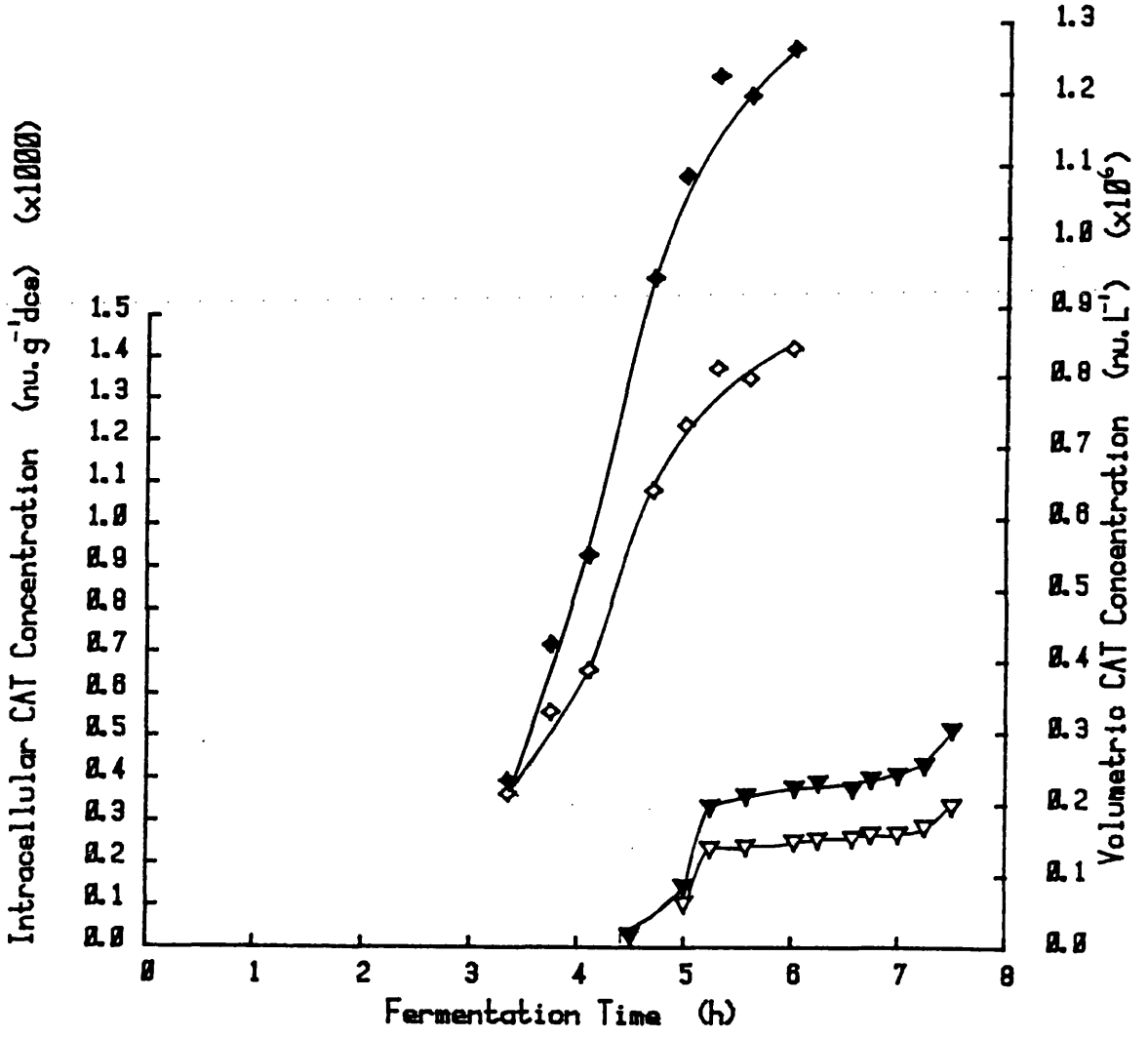


Figure 3.23: Effect Of Oxygen Limitation On Production - Intracellular (▽ ,pCQV2; ◇ ,pMG169) and Volumetric (▼ , pCQV2; ◆ ,pMG169).

Figure 3.23 shows that in the case of strain pCQV2 production initially seemed to occur as under kinetic limitation, for approximately 0.75h, before O_2-limitation caused linear intracellular and volumetric production.

In the case of strain pMG169 production was linear from the start of the production phase. Strain pMG169 was clearly a better producer than strain pCQV2, under oxygen limitation.

The volumetric production rates (slopes of the linear parts of the volumetric profiles of figure 3.23) were different for the two strains.

It was noted that under kinetic limitation the volumetric production rate could not be used to predict batch titres at larger scales (section 3.1.3.5). This was because at the large scale the culture would become oxygen limited because the achievable OTR would be less. In that case the volumetric production profile would change. However under oxygen limitation the volumetric production rate can be used to predict batch titres at larger scales, but only at the limiting OTR supplied in these experiments.

For both strains the volumetric production titres rose at a higher rate than the intracellular titres because the biomass concentration was increasing as well as intracellular CAT concentration.

A comparison of these results with those in sections 3.1.3. show production under O_2-limitation to be worse than under

kinetic limitation in both intracellular and volumetric terms. This suggests that oxygen limitation would not be recommended as the chosen mode of operation for either constant or amplifiable copy number temperature sensitive systems.

For strain pCQV2 the production phase would last until the supplied O_2 was required entirely for maintenance, whereupon growth and production would stop.

For strain pMG169 the production phase lasted approximately 3h.

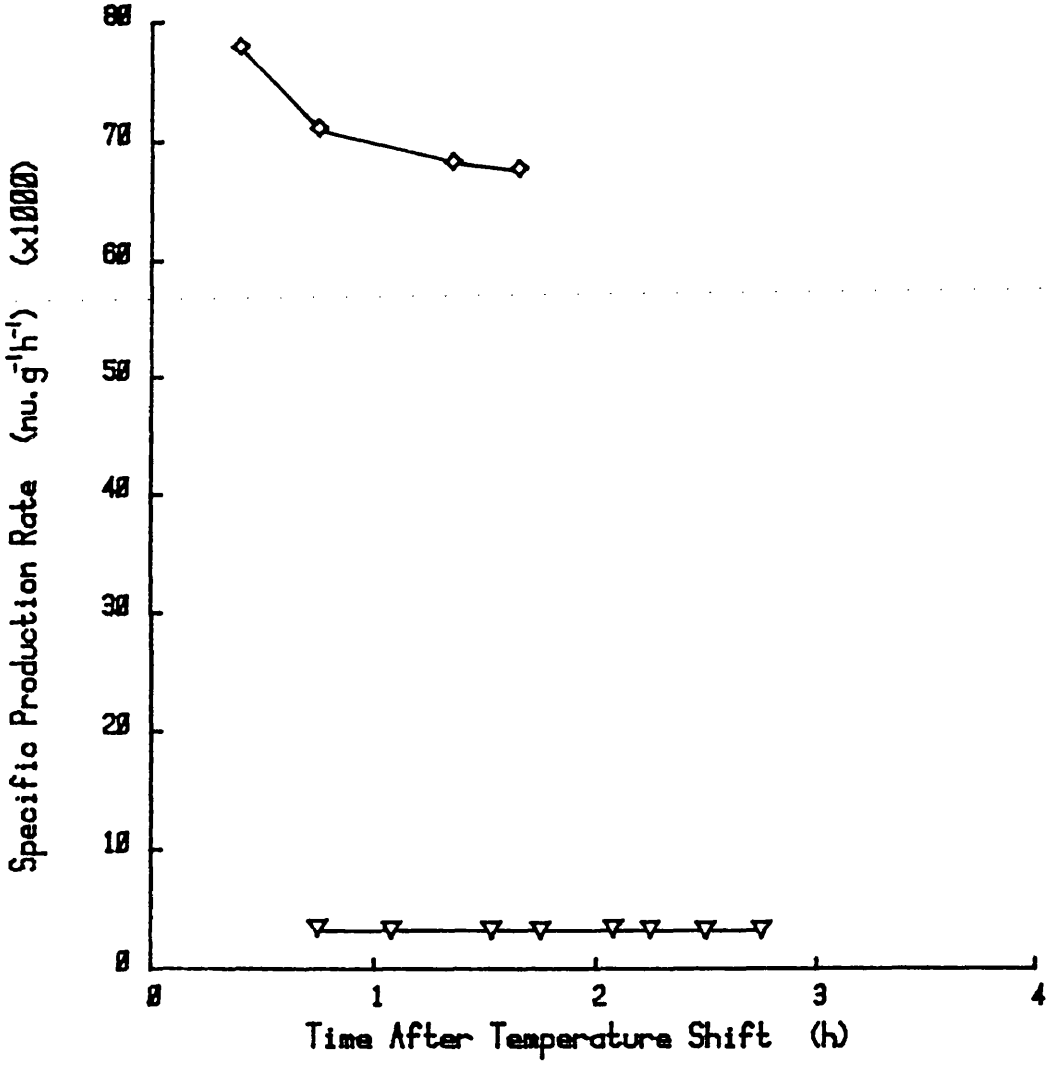


Figure 3.24: Effect Of Oxygen Limitation On Specific Production Rate (▽ ,pCQV2; ◇ ,pMG169).

Figure 3.24 shows a constant, low specific production rate against fermentation time for the two strains (cf section 3.1.3.6).

The specific production rate for strain pMG169 was highest initially. It seems as though plasmid amplification actually caused a reduction in specific production rate. This was possibly due to resources being diverted towards plasmid amplification, therefore depleting the amount available for production. The rate for strain pMG169 was greater than for strain pCQV2, which remained a constant value throughout.

3.2.3 Effect of Oxygen Limitation on Glucose Uptake.

The effect of O_2-limitation on glucose uptake was investigated to assess the efficiency of nutrient utilisation under these conditions.

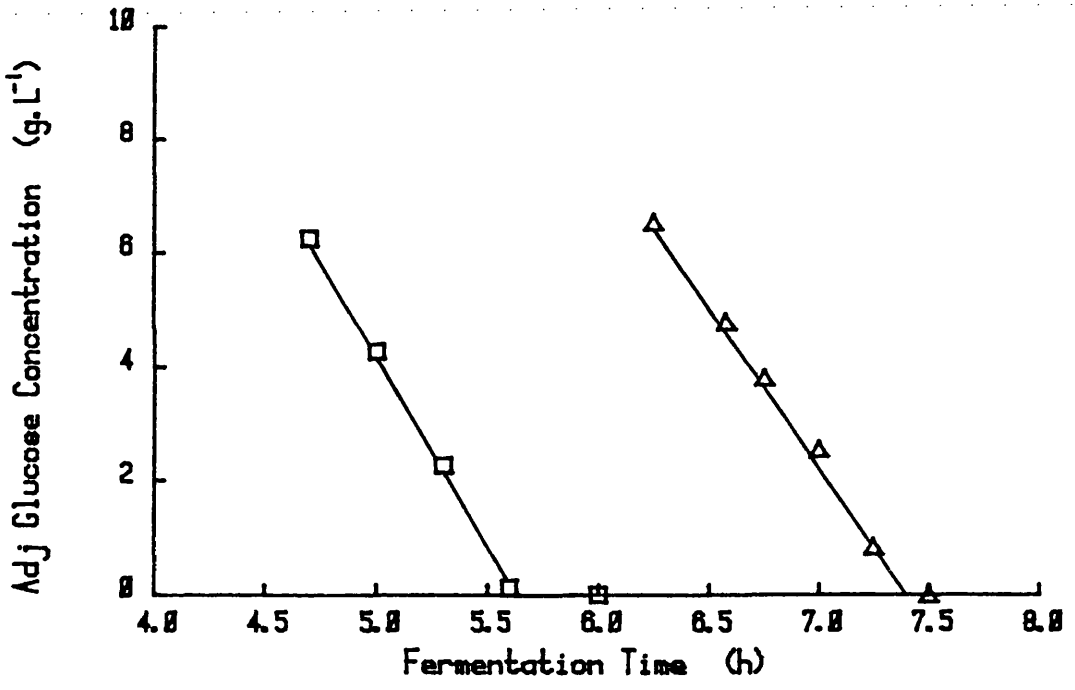


Figure 3.25: Effect Of Oxygen Limitation On Glucose Uptake (△,pCQV2; □,pMG169).

Figure 3.25 is a plot of adjusted glucose concentration against fermentation time for the two strains, and shows, as expected, a linear glucose uptake rate during O_2-limitation.

In these fermentations 300 g glucose was consumed compared to 200 g in the kinetically limited ones, and yielded less biomass. $Y_{(x/s)}$ for the two strains during the O_2-limited phase were:

- pCQV2 0.084 g dcw g^{-1} glucose
- pMG169 0.023 g dcw g^{-1} glucose

These values were considerably lower than the ones reported under kinetic limitation in section 3.1.4.

The value of $Y_{(x/s)}$ for strain pMG169 shown above, was significantly lower than for strain pCQV2 under similar conditions. The reason for this might have been that glucose (and other nutrients) would have been required in greater quantity for plasmid amplification processes and production than in strain pCQV2, resulting in less substrate left for growth, and hence lower yields.

3.2.4 Effect of Oxygen Limitation on pH Control.

It was more difficult to control the pH at 7.0 under these conditions than compared to kinetically limited runs. Approximately three times as much alkali was needed to counteract the effects of greater acidic metabolite

production (mainly| acetic acid).

3.3 BATCH FERMENTATION UNDER GLUCOSE LIMITATION.

Since O_2-limitation had a deleterious effect on production it was intended to remove this limitation by limiting on another nutrient, glucose, and see the effects of this on the fermentation. The glucose feed-rate was used to control the OUR of the culture. As stated in section 3.2 a worst case situation might be a $100m^3$ fermenter in which the maximum sustainable OUR would be of the order of 50 to 60 $moles.m^{-3}h^{-1}$. Therefore feed-rates were investigated that would sustain OURs of that order, as well as lower feed-rates. Fed-batch fermentation of this sort was also investigated as a possible means of achieving higher biomass concentrations than can be obtained in large fermenters under kinetic limitation.

3.3.1 Effect of Different Glucose Feed-Rate on Growth.

As mentioned above it was important to determine the effects of glucose limitation on biomass generation with a view to achieving higher cell concentrations than can be achieved under kinetic limitation in larger fermenters.

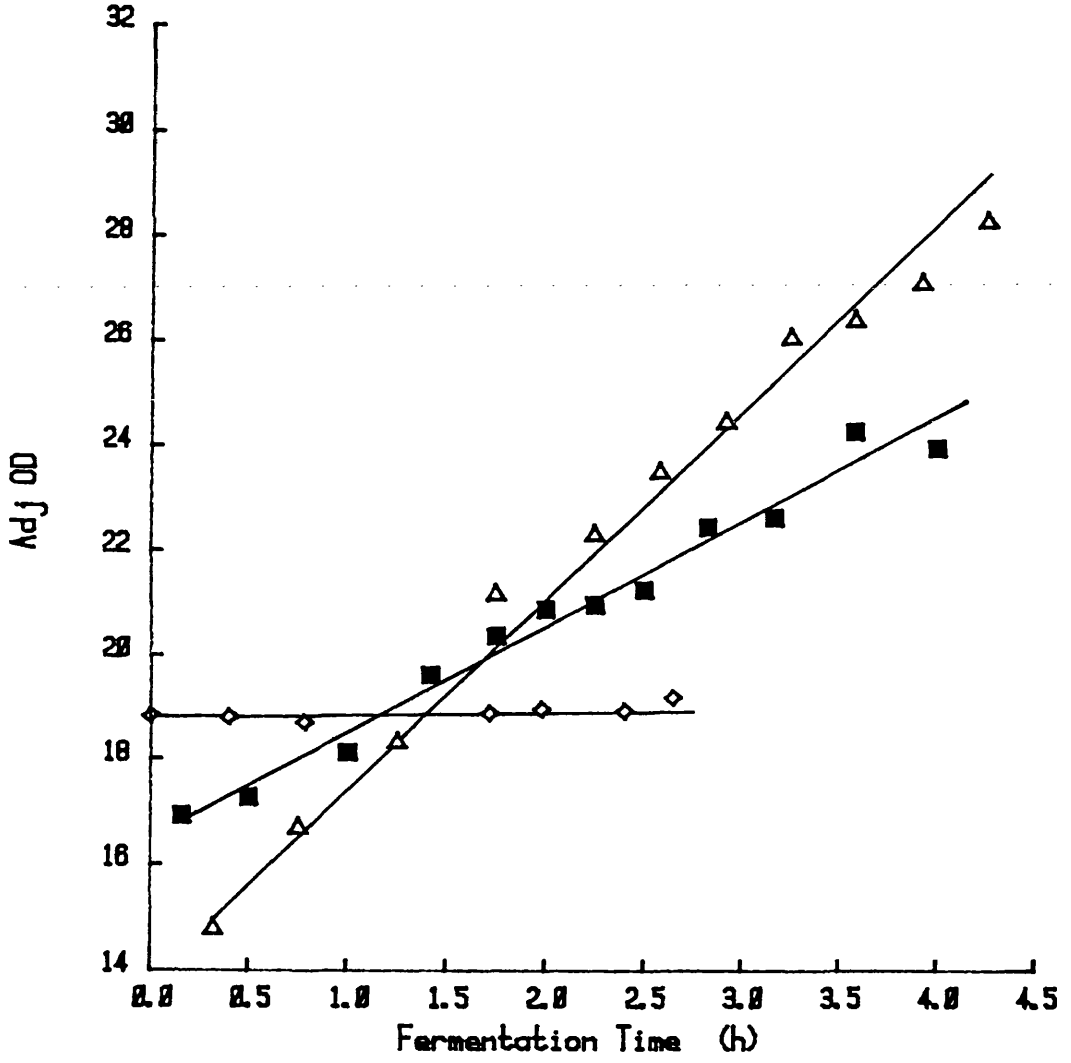


Figure 3.26: Effect Of Glucose Limitation At Different Feed-Rates On Growth - Strain pCQV2 (◇ , 10g.m^{-3}min^{-1} ; ■ , 21g.m^{-3}min^{-1} ; △ , 46g.m^{-3}min^{-1}).

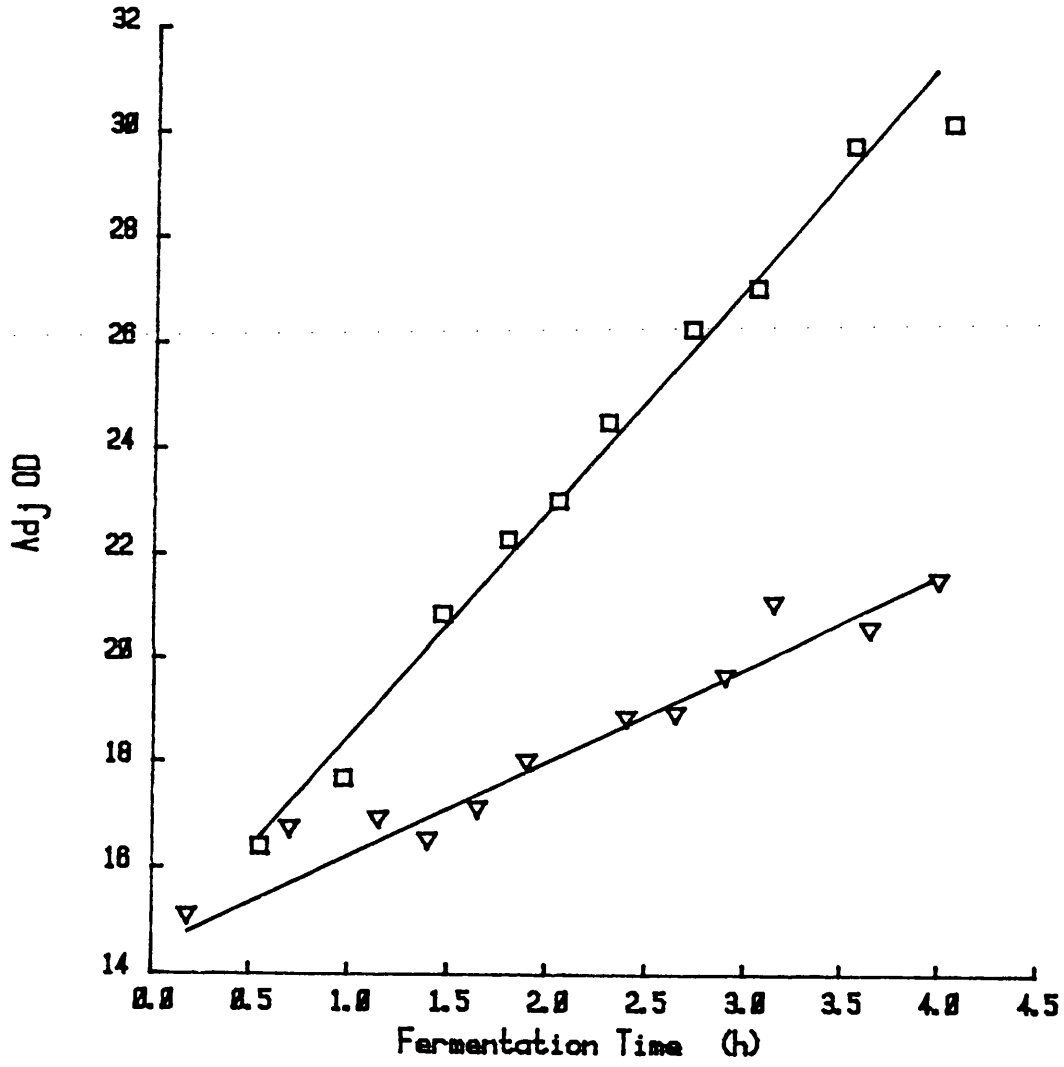


Figure 3.27: Effect Of Glucose Limitation At Different Feed-Rates On Growth - Strain pMG169 (▽ ,21g.m^{-3}min^{-1}; □ , 50g.m^{-3}min^{-1}).

Figs 3.26 and 3.27 are plots of Adjusted OD against fermentation time at three different glucose feed-rates for strain pCQV2 and two different rates for strain pMG169.

Growth was linear, and increased with increasing feed-rate in the manner shown in figure 3.28.

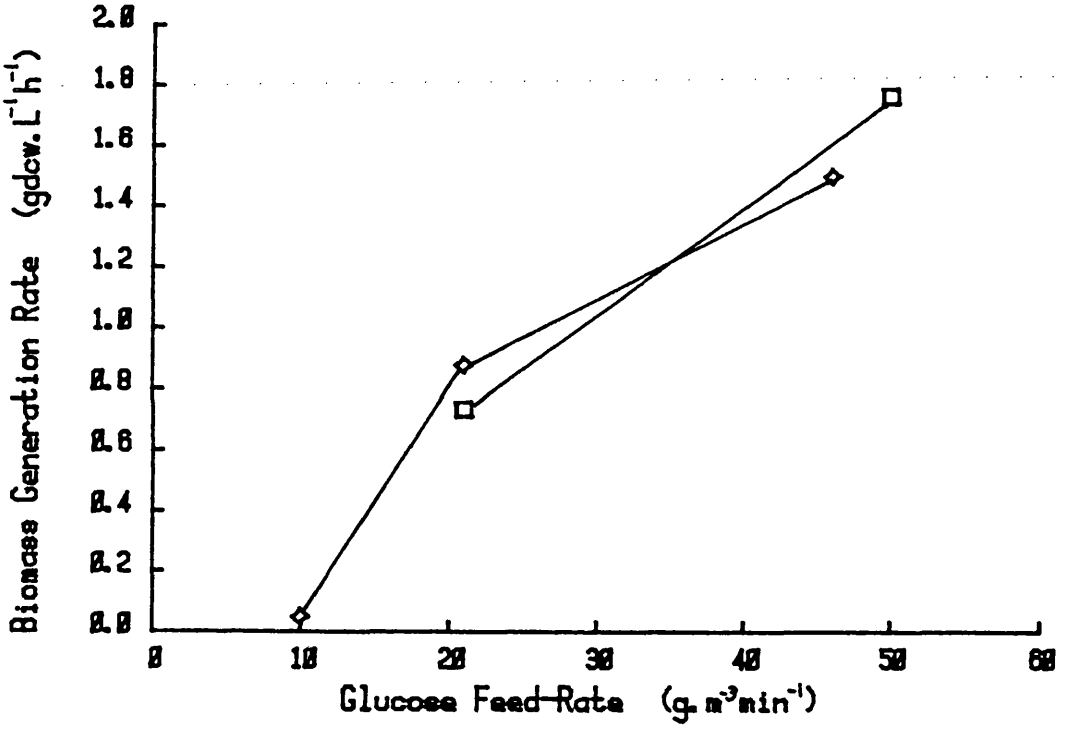


Figure 3.28: Effect of Feed-Rate on Biomass Generation Rate During Glucose Limitation (◇ ,Strain pCQV2; □ ,Strain pMG169).

3.3.2 Effect of Glucose Feed-Rate on OUR.

The glucose feed-rate was constant throughout each of the fermentations and was used to limit the OUR of the culture.

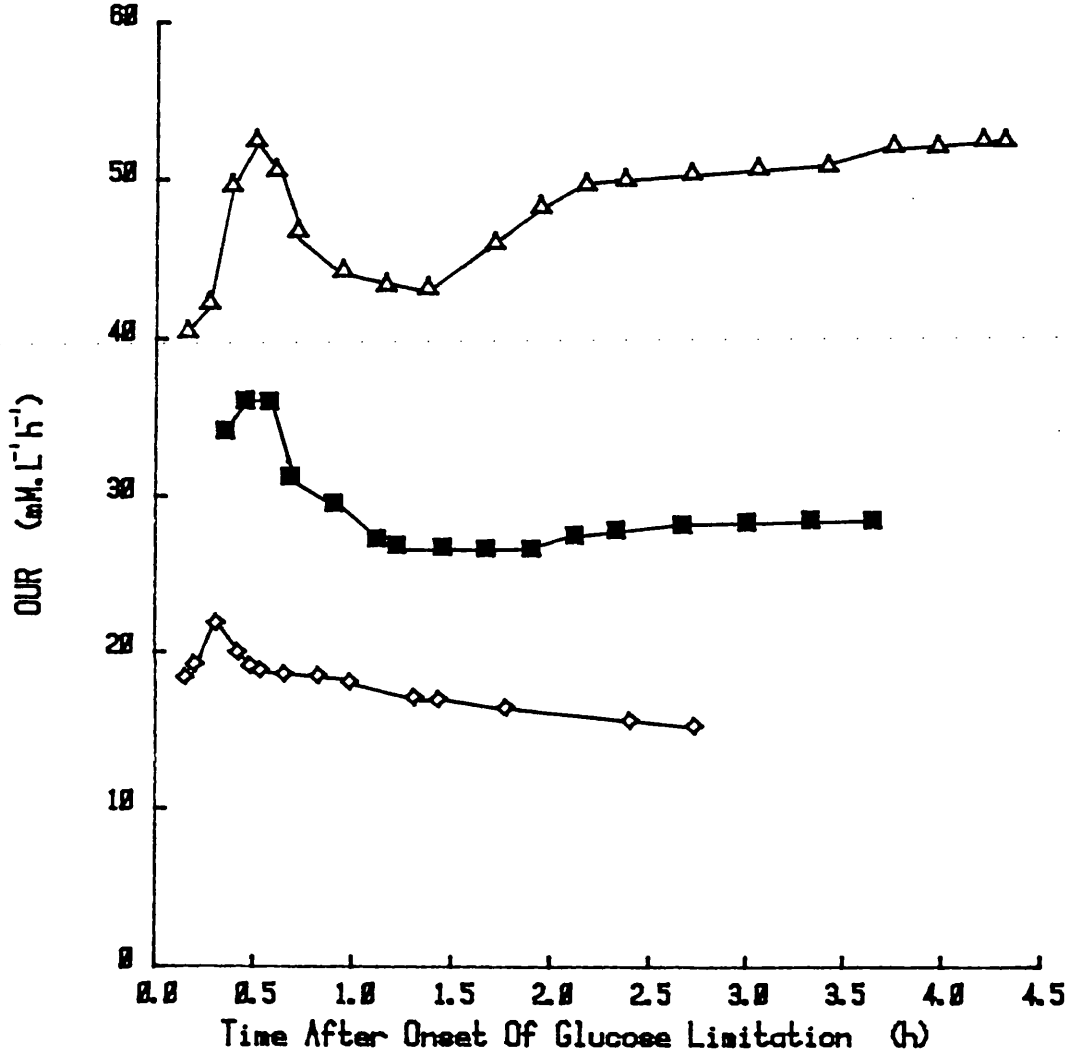


Figure 3.29: Effect Of Glucose Limitation At Different Feed-Rates On OUR - Strain pCQV2 (◇, 10g.m^{-3}min^{-1}; ■, 21g.m^{-3}min^{-1}; △, 46g.m^{-3}min^{-1}).

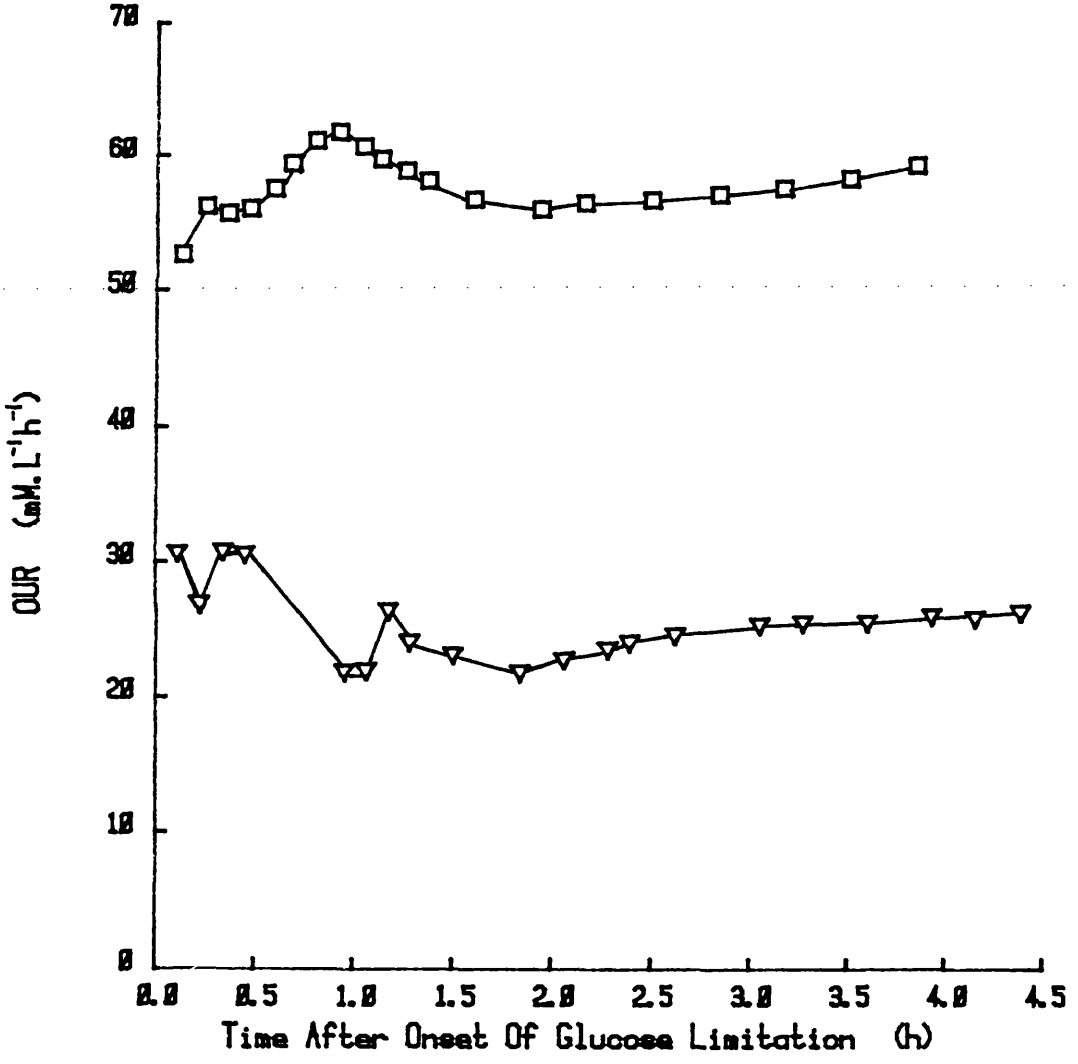


Figure 3.30: Effect Of Glucose Limitation At Different Feed-Rates On OUR - Strain pMG169 (▽ , 21g.m^{-3}min^{-1}; □ , 50g.m^{-3}min^{-1}).

Figures 3.29 and 3.30 are plots of OUR against time at three different feed-rates for strain pCQV2 and two feed-rates for strain pMG169, respectively.

OUR was approximately constant at different feed-rates except for a peak, followed by a fall, at the time of the temperature shift.

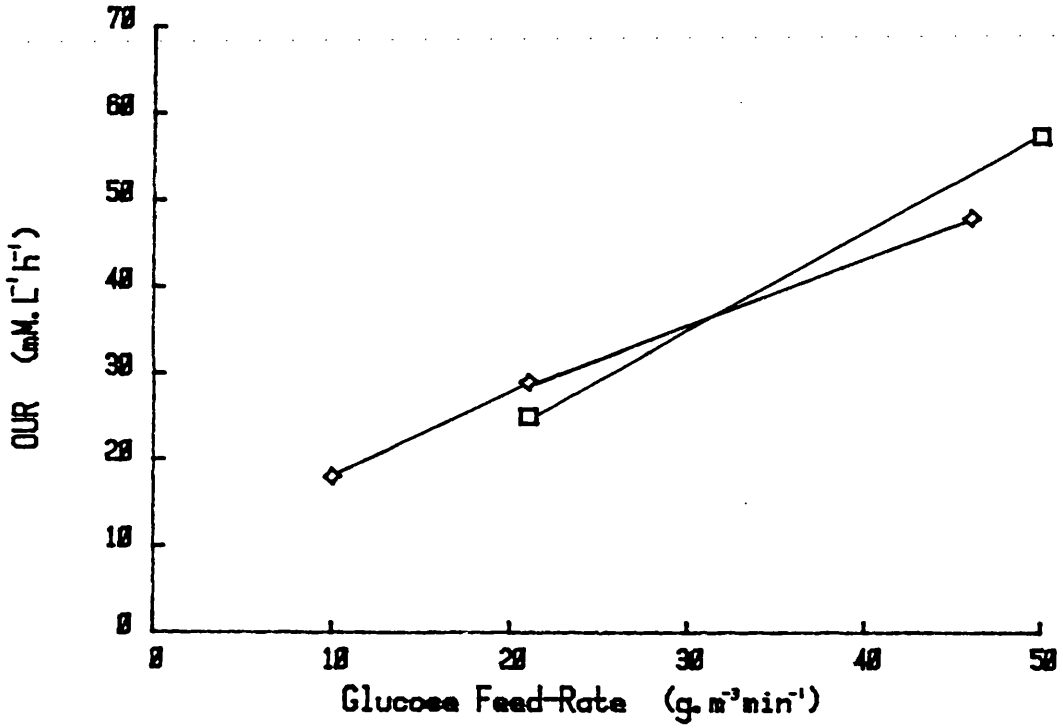


Figure 3.31: Effect of Feed-Rate on OUR During Glucose Limitation (◇ ,Strain pCQV2; □ ,Strain pMG169).

As expected, the glucose feed-rate can be used effectively to maintain the OUR at a constant value, thereby ensuring the avoidance of oxygen limitation.

In order to maintain the OUR within the range 50 to 60 moles.$m^{-3}h^{-1}$ the glucose feed-rate should be set between 46 to 50 g.$m^{-3}min^{-1}$.

3.3.3 Effect of Glucose Feed-Rate on Intracellular Production.

It was important to determine the effects of glucose limitation on the production kinetics since this mode of operation is a possible alternative to kinetic limitation at larger scale.

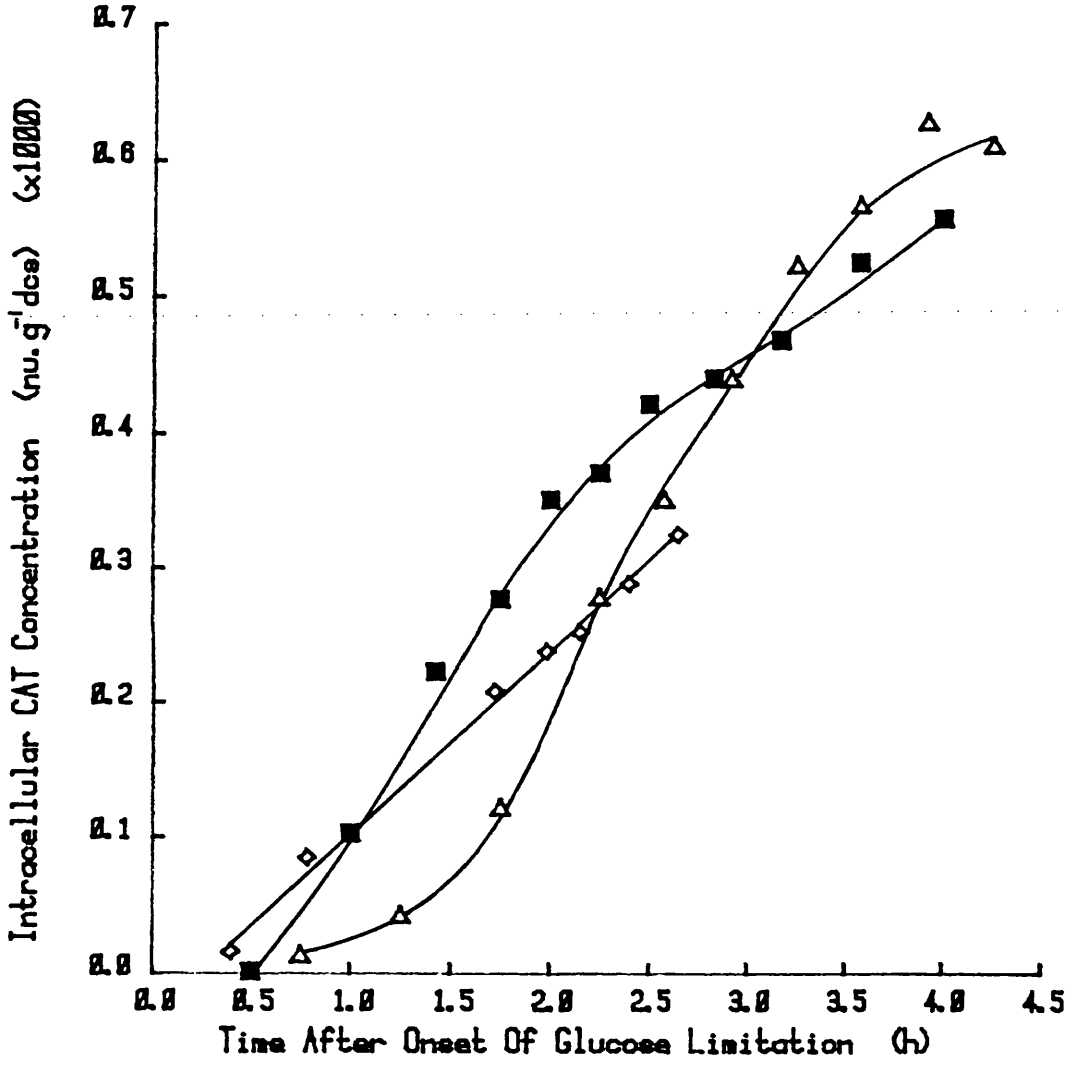


Figure 3.32: Effect Of Glucose Limitation At Different Feed-Rates On Intracellular Production - Strain pCQV2 (◇, 10g.m^{-3}min^{-1}; ■, 21g.m^{-3}min^{-1}; △, 46g.m^{-3}min^{-1}).

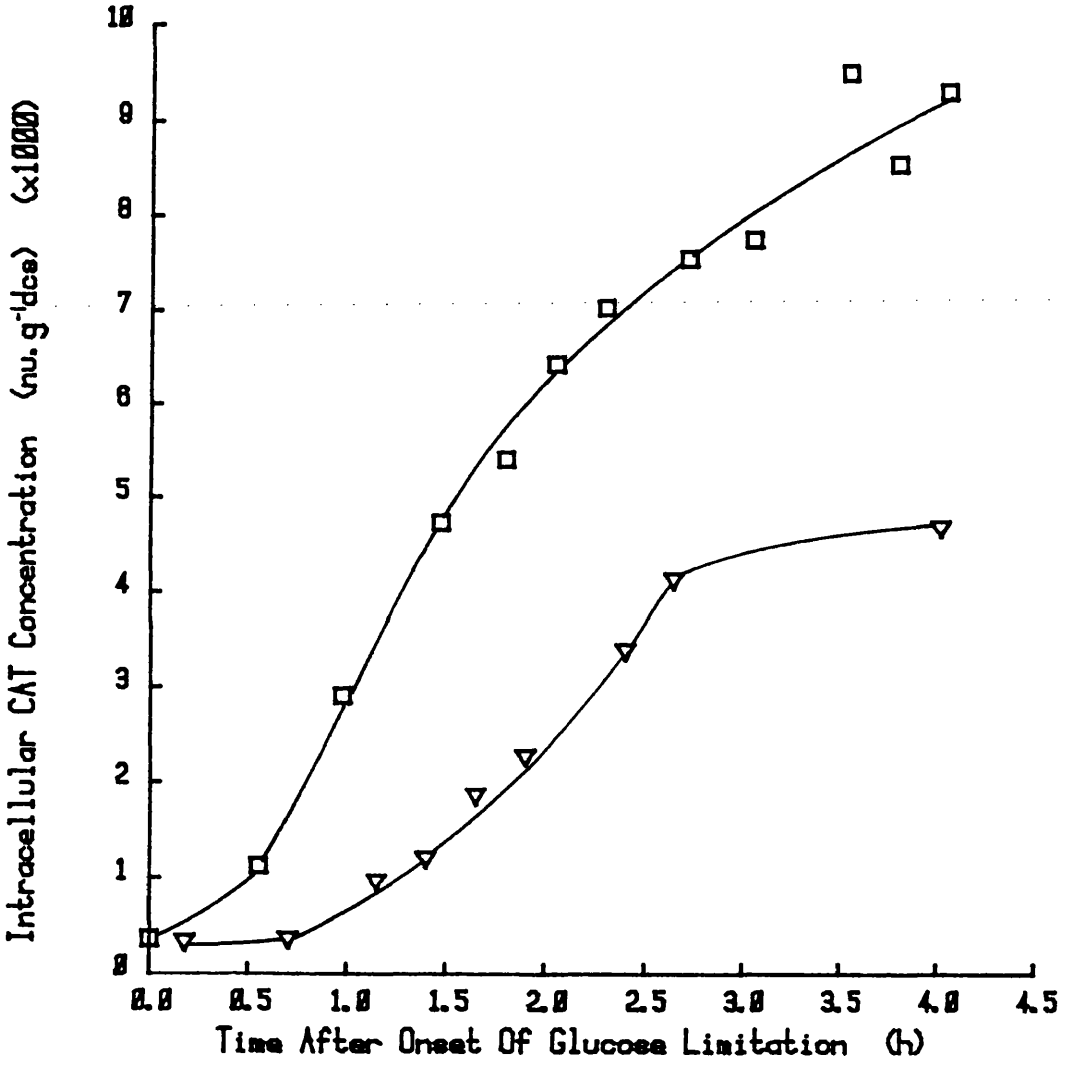


Figure 3.33: Effect Of Glucose Limitation At Different Feed-Rates On Intracellular Production - Strain pMG169 (▽ ,21g.m^{-3}min^{-1} ; □ ,50g.m^{-3}min^{-1}).

Figures 3.32 and 3.33 are plots of intracellular CAT concentration against time at three different feed-rates for strain pCQV2 and two feed-rates for strain pMG169, respectively.

For both strains the higher the feed-rate the higher the peak intracellular CAT concentration.

For strain pCQV2 a comparison between figures 3.10 and 3.32 shows that after 3h production under glucose limitation (feed-rate = 46 $g.m^{-3}min^{-1}$) the intracellular CAT concentration had reached approximately 60% of the level after 3h under kinetic limitation.

For strain pMG169 a comparison between figures 3.13 and 3.33 shows that after 4h production under glucose limitation (feed-rate = 50 $g.m^{-3}min^{-1}$) the intracellular CAT concentration had reached approximately 120% of the level after 2h under kinetic limitation (time of peak level).

The effect on both strains of glucose limitation was to cause the intracellular CAT concentration to increase fastest initially, with the levels trailing off towards a plateau as seen under kinetic limitation. The difference here was that it took longer to achieve that plateau.

3.3.4 Effect of Glucose Feed-Rate on Volumetric Production.

The volumetric production rate for the two strains were determined at a number of different glucose feed-rates. Therefore it could be used to predict the batch titres at different scales. However since the parameter contains a biomass concentration component it can only be used for similar biomass concentrations and fermentations of this sort, ie a kinetically limited growth phase with the glucose becoming exhausted at the point of the desired OUR, followed by a glucose feed at a rate to maintain that OUR, followed by the temperature shift. In section 4.3.3 the volumetric production rates are extrapolated to higher feed-rates.

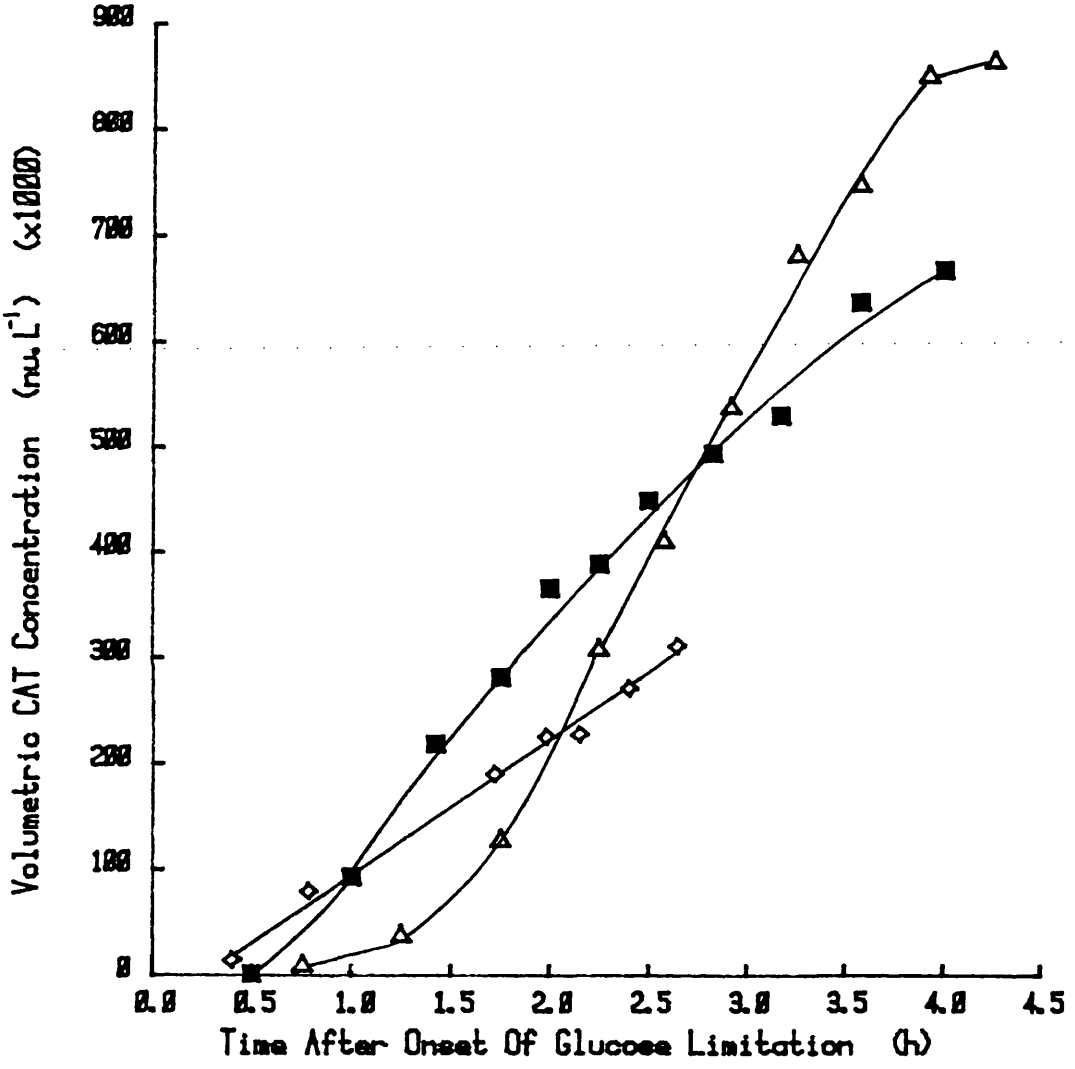


Figure 3.34: Effect Of Glucose Limitation At Different Feed-Rates On Volumetric Production - Strain pCQV2 (◇, 10g.m^{-3}min^{-1}; ■, 21g.m^{-3}min^{-1}; △, 46g.m^{-3}min^{-1}).

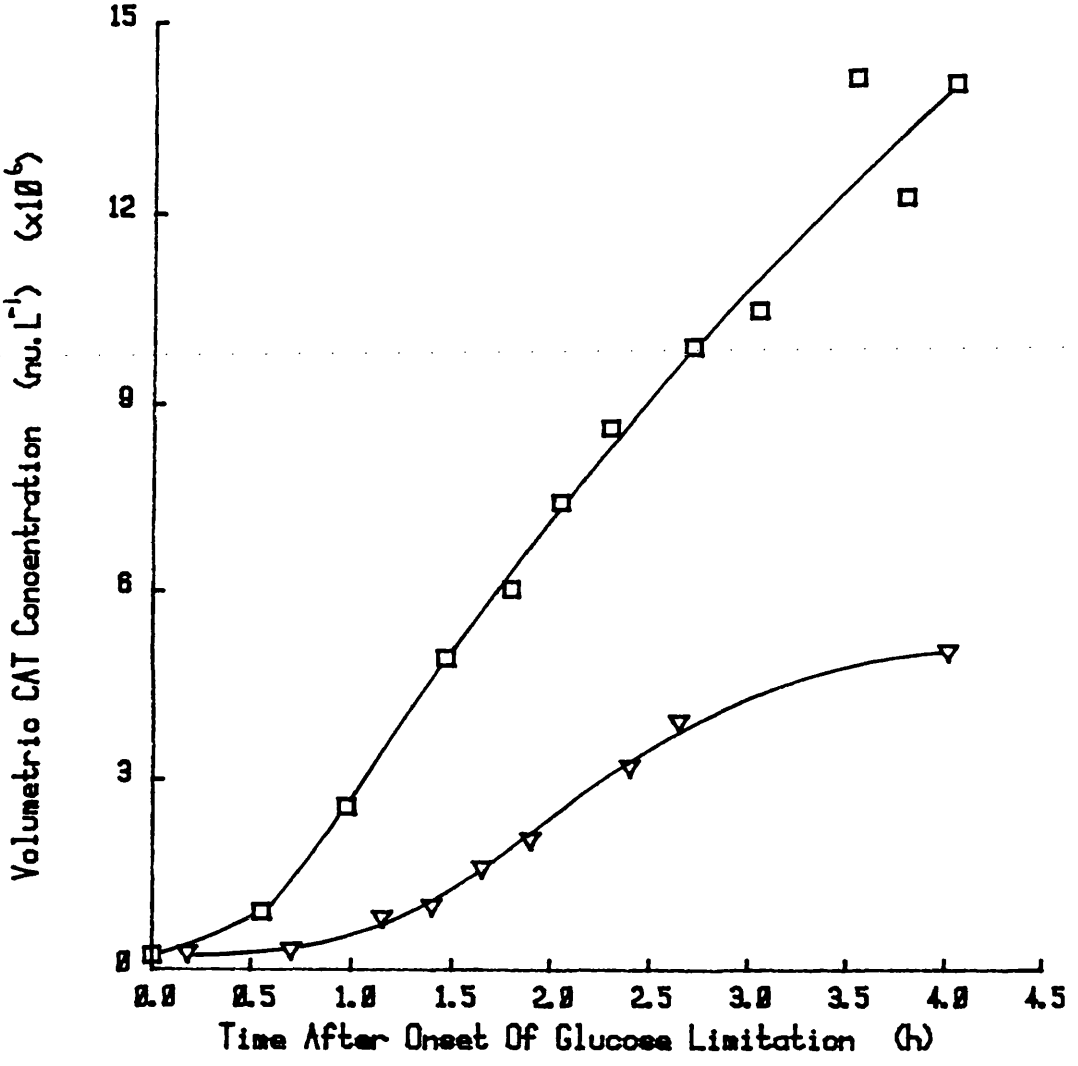


Figure 3.35: Effect Of Glucose Limitation At Different Feed-Rates On Volumetric Production - Strain pMG169 (▽, 21g.m^{-3}min^{-1}; □, 50g.m^{-3}min^{-1}).

The figures are plots of volumetric CAT concentration against time at three different feed-rates for strain pCQV2 and two feed-rates for strain pMG169, respectively.

For both strains the higher the feed-rate the higher the peak volumetric CAT concentration.

Both strains show a constant production rate (slope of line) for the bulk of the production phase, and these rates were greater at higher feed-rates as shown in figure 3.36.

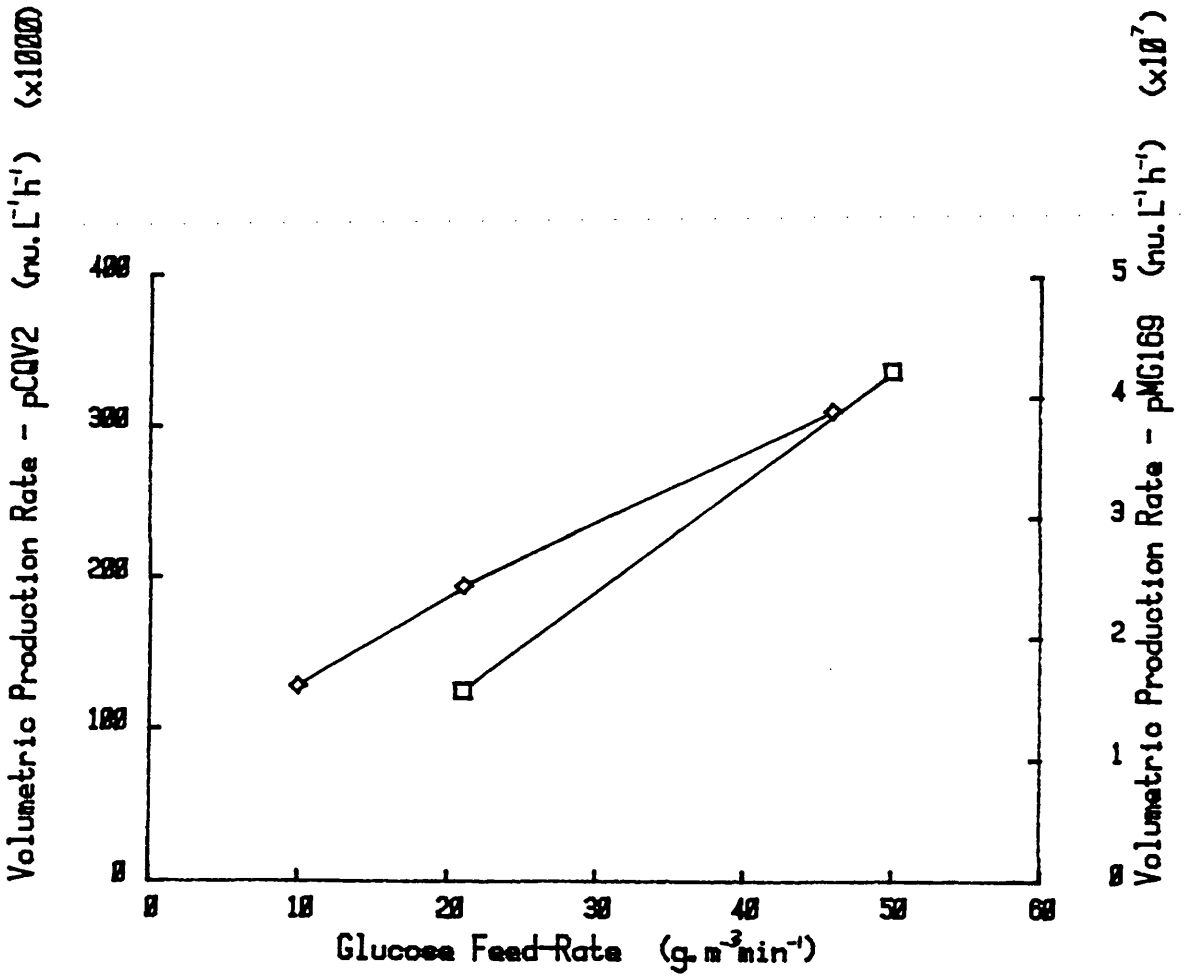


Figure 3.36: Effect of Feed-Rate on Volumetric Production Rate During Glucose Limitation (◇ ,Strain pCQV2; □ ,Strain pMG169).

For strain pCQV2 a comparison between figures 3.12 and 3.34 shows that after 3h production under glucose limitation (feed-rate = 46 $g.m^{-3}min^{-1}$) the volumetric CAT concentration had reached the same level as after approximately 4.5h under kinetic limitation.

For strain pMG169 a comparison between figures 3.13 and 3.35 can be misleading because the biomass concentration at which the shift was implemented was much greater in figure 3.35 than for any of the fermentations shown in figure 3.13. In the 40°C fermentation of figure 3.13 the peak OUR (at the peak volumetric CAT concentration) was 10 $moles.m^{-3}h^{-1}$. Therefore the peak biomass concentration could be 6-fold higher in 100 m^3 without becoming O_2-limited. This suggests the peak volumetric CAT concentration would also be 6-fold higher, at 4.5 million $nu.L^{-1}$. This value compares poorly with the results shown in figure 3.35. After 3.5h production with a glucose feed-rate of 50 $g.m^{-3}h^{-1}$ the volumetric CAT concentration was 14 million $nu.L^{-1}$. This was due to both higher intracellular CAT concentration and biomass concentration achieved under glucose limitation.

3.3.5 Effect of Glucose Feed-Rate on Specific Production Rate.

The specific production rate was calculated at the different glucose feed-rates. This parameter was important

in predicting the batch titres at higher biomass concentrations than were achieved in these glucose limited fermentations. As has already been stated, one of the purposes of nutrient limited fermentation was to obtain higher biomass concentrations and batch titres than can be achieved under kinetic limitation. This could be achieved by incorporating a glucose limited growth phase subsequent to kinetically limited growth, and prior to the production phase. Under such conditions the specific production rate must be combined with the biomass generation profile to predict batch titres (see sections 4.3.3 and 4.4.). Hence the specific production rate was important in design.

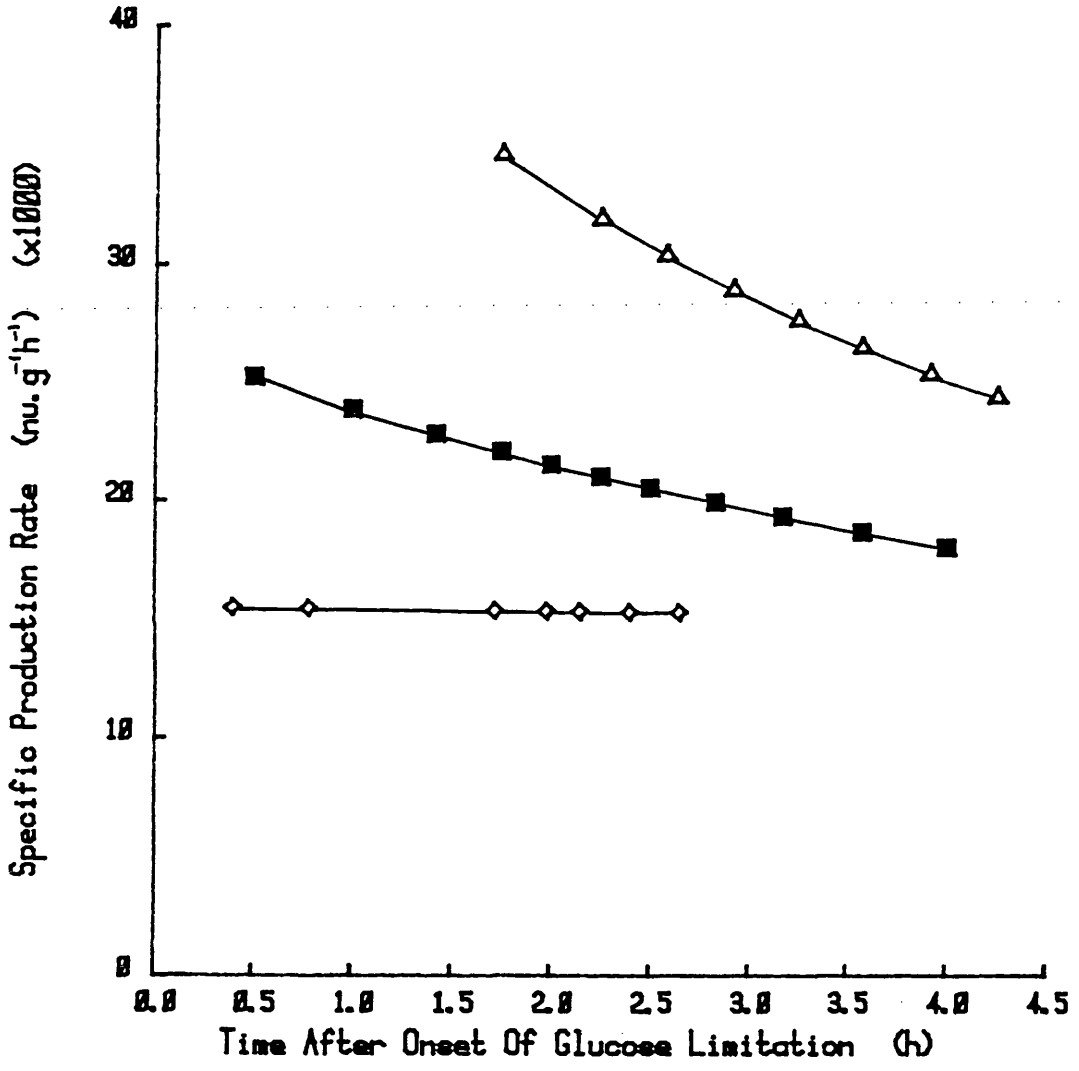


Figure 3.37: Effect Of Glucose Limitation At Different Feed-Rates On Specific Production - Strain pCQV2 (◇, 10g.m^{-3}min^{-1}; ■, 21g.m^{-3}min^{-1}; △, 46g.m^{-3}min^{-1}).

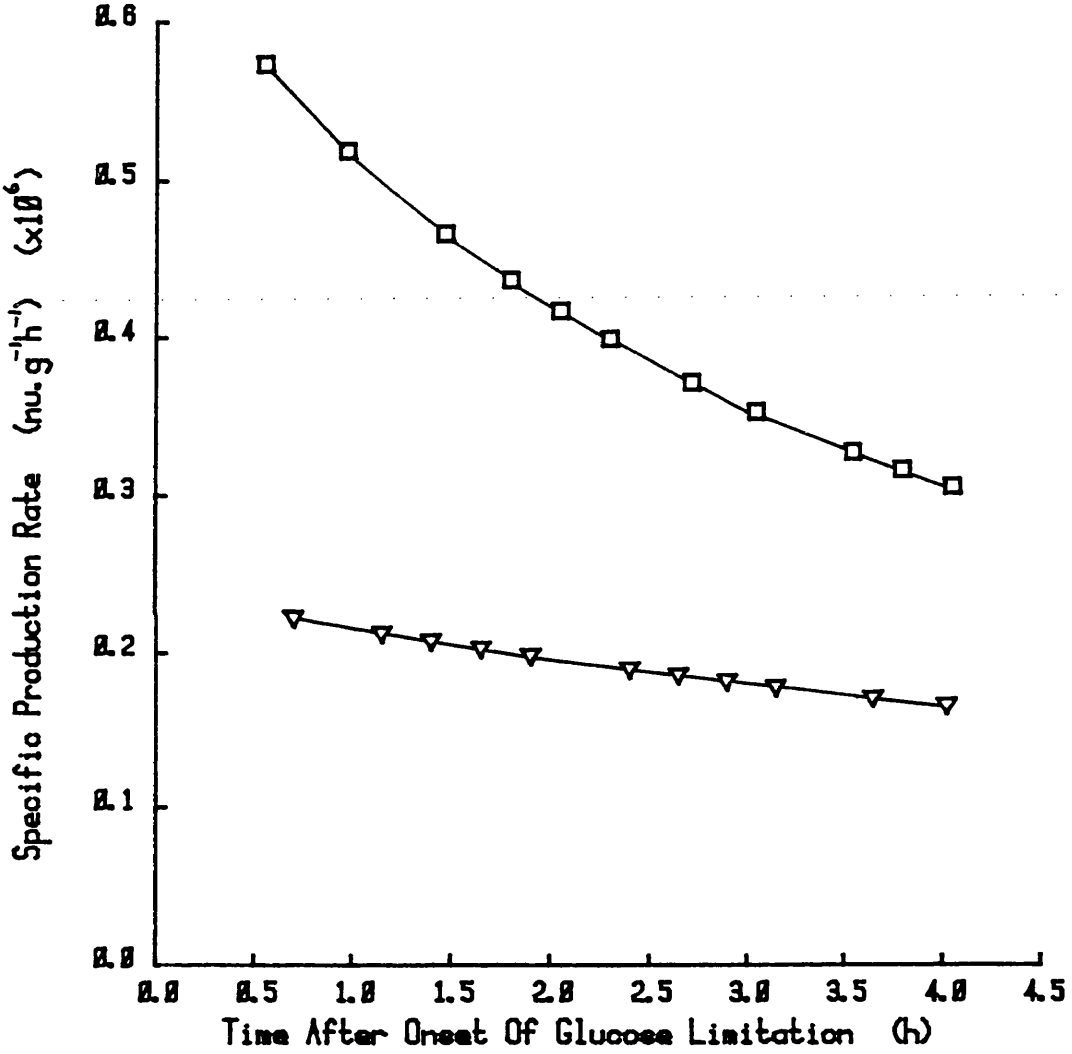


Figure 3.38: Effect Of Glucose Limitation At Different Feed-Rates On Specific Production - Strain pMG169 (▽ , 21g.m^{-3}min^{-1} ; □ , 50g.m^{-3}min^{-1}).

The figures are plots of specific production rate against time at three different feed-rates for strain pCQV2 and two feed-rates for strain pMG169, respectively.

These values were calculated in the way described in section 3.1.3.6.

As mentioned in the previous section the volumetric production rate was constant for most of the production phase. Therefore the way in which growth occurred at the different feed-rates determined the shapes of the specific production rate profiles.

In figure 3.37 at a feed-rate of 10 $g.m^{-3}min^{-1}$ there was so little biomass generation that the specific production rate remained constant throughout. As the feed-rate was increased the specific production rate also increased. At the higher feed-rates the specific production rates were highest initially and fell with time in a similar way as described in figure 3.15. However the fall was not as pronounced in figure 3.37 because it took longer for the intracellular CAT concentration to peak under glucose limitation compared to kinetic limitation.

In figure 3.38 at a feed-rate of 21 $g.m^{-3}min^{-1}$ the specific production rate was constant throughout. At a feed-rate of 50 $g.m^{-3}min^{-1}$ the specific production rate was considerably higher, being greatest initially before falling away towards a constant value.

3.3.6 Effect of Glucose Feed-Rate on Overall Yield Coefficient.

The overall yield coefficient, $Y_{(x/s)}$, is important in assessing the efficiency of nutrient utilisation under glucose limitation. This is vital in assessing the process economics of the various different modes of operation. If one mode of operation produced better titres but with much greater nutrient requirements then it may not be economical to operate in that way, and the more economical though poorer producing method may be chosen instead.

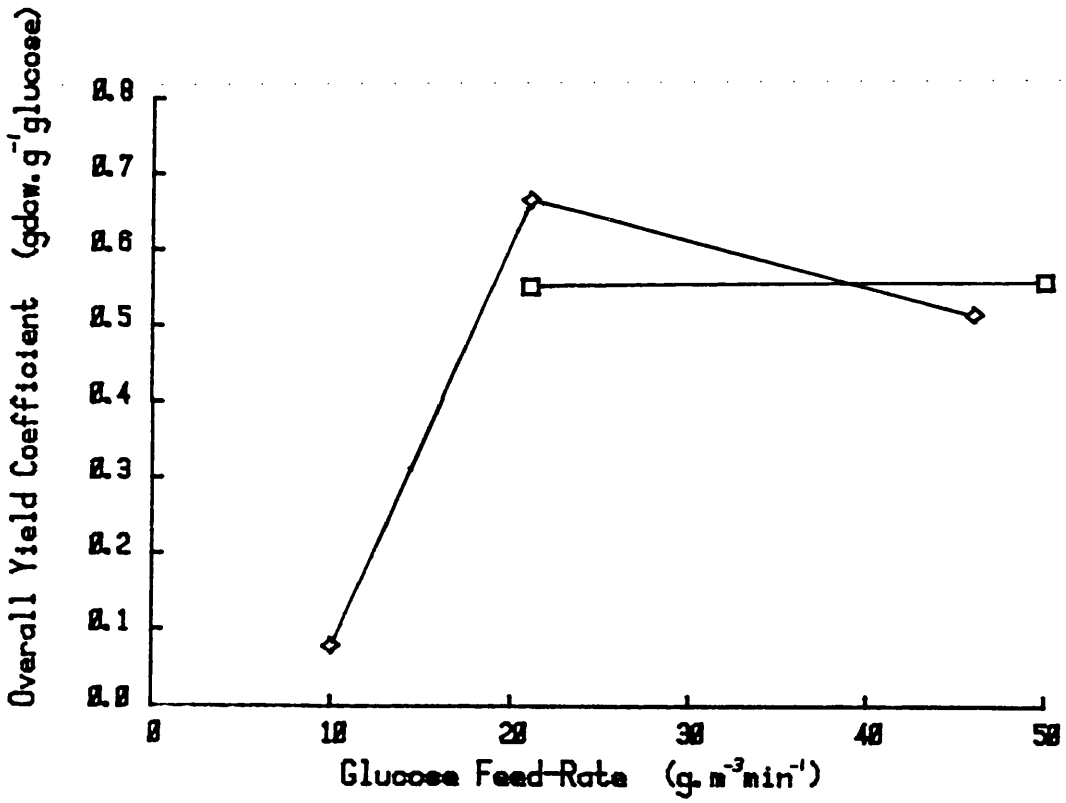


Figure 3.39: Effect Of Glucose Limitation At Different Feed-Rates On Overall Yield Coefficient (◇ ,pCQV2; □ , pMG169).

The figure shows that at the higher feed-rates there was little difference between $Y_{(x/s)}$ at the different rates. The values were very similar to those in figure 3.17 at the growth temperatures, under kinetic limitation. However the glucose limited parts of the fermentations were performed at production temperatures, at which under kinetic limitation the $Y_{(x/s)}$ was lower (see figure 3.17). Therefore glucose limitation resulted in a more efficient conversion of glucose into biomass at production temperatures than kinetic limitation.

Since the $Y_{(x/s)}$ was so low at a feed-rate of 10 $g.m^{-3}min^{-1}$ it is clear this feed-rate was sufficient for maintenance of the culture and production, leaving very little for growth.

3.3.7 Effect of Glucose Feed-Rate on pH Control.

The onset of glucose exhaustion at the end of the fermentations under kinetic limitation was accompanied by a sudden rise in pH as the cells began to metabolise the acidic metabolites produced during earlier metabolism. The onset of glucose limitation caused similar though less pronounced effects in both strains.

At a feed-rate of 10 $g.m^{-3}min^{-1}$ concentrated HNO_3 was added by hand for 45 minutes before the pH was controllable at 7.0 using the OH^- alone, and for 20 minutes at the

feed-rate of 21 $g.m^{-3}min^{-1}$.

At the higher feed-rates the pH did not rise above 7.05 and the continued production of the acidic metabolites meant the pH fell to 7.00 quickly and was controlled as previously, with OH^-.

3.4 HEAT OF FERMENTATION.

3.4.1 Accuracy of Method and Results.

The energy balance over the system was:

$$Q_{ag} + Q_f = Q_{cool} + Q_{sens} + Q_{evap} + Q_{surr}$$

Q_{ag} was constant since the agitator rate remained constant throughout.

Q_{cool} was constant since the temperature and flowrate of the coolant were constant at the times of initial steady-state and final readings.

Q_{evap} was constant since the evaporative properties of the broth remained constant throughout growth. This was checked by increasing the pH to around 10 subsequent to glucose exhaustion, to prevent appreciable metabolic activity, and measuring the new steady-state heater power (see figure 3.40). This was found to be the same as the initial value prior to inoculation.

Q_{surr} was constant since the fermenter was lagged and the process carried out in a temperature controlled room.

Q_{sens} was constant if the temperature of the air entering the fermenter was constant. The following calculation was performed to evaluate the magnitude of any change to this value due to fluctuations in the ambient air temperature:

$$\Delta Q_{sens} = m' * C_p * \Delta T.$$

$$C_p = 1046.7 \text{ J.kg}^{-1}\text{K}^{-1}$$

$$m' = 5.0 \text{ SLPM}$$

$$= 0.2232 \text{ moles air.min}^{-1}$$

$$= 6.473 \text{ g.min}^{-1}$$

$$= 1.079*10^{-4} \text{ kg.s}^{-1}$$

The temperature of the ambient air may have risen by 15°C during the fermentations.

$$\Delta T = 15 \text{ K}$$

$$\Delta Q_{sens} = 1.079*10^{-4} * 1046.7 * 15$$

$$= 1.7\text{W}$$

Table 3.7 below shows the lowest measured Q_f was 118W (in 10 L). Therefore the greatest error due to this effect was approximately 1.5%.

The greatest error in measuring the power was +/- 2W. As above the lowest measured Q_f was 118W, meaning that this error was 1.7%, maximum.

The sample volumes were kept to a minimum so as not to disturb the system greatly and an accurate record of the additions (alkali and antifoam) was kept. Thus an accurate profile of the biomass concentration was obtained. Any further errors were therefore associated with the Adjusted OD against dry cell weight conversion.

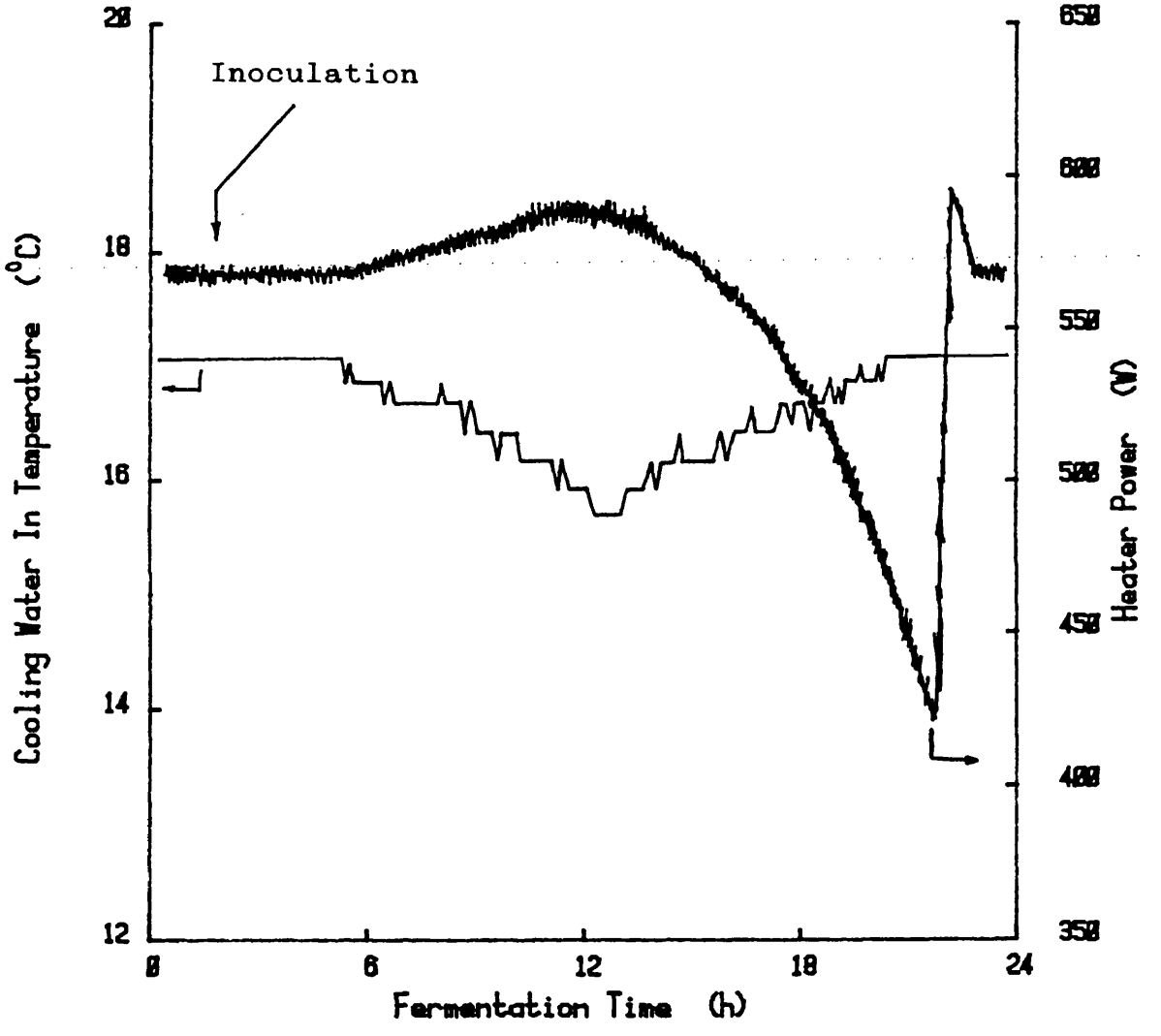


Figure 3.40: Illustration Of Typical Profile Of Temperature Of Cooling Water In And Heater Power - Strain pCQV2.

The figure shows a typical result obtained from the computer logging the fermentation data. The fermenter was inoculated in the late afternoon, and the biomass concentration did not become appreciable until the next morning. During the night, however, the temperature of the cooling water entering the fermenter fell and then rose again the following morning. The magnitude of this change varied with the season, but was generally between 1 to 2°C.

Q_f, the overall heat of fermentation at any time during the run, was measured by subtracting the heater power at that time from the steady-state power reading prior to inoculation. However in order that Q_f was the only variable in the system (see section 3.4.1) the heater power was only read when the temperature of the coolant was the same as at the initial steady-state.

The heater power rose during the night to counteract the effect of falling cooling water temperature in maintaining constant fermenter temperature. At this stage the biomass concentration, while increasing, was not sufficient to produce enough heat to remove the need for this rise in heater power.

The heater power fell as an exponential function mirroring the exponential rise in biomass concentration. Glucose exhaustion was shown to have occurred when the heater power began to rise quickly. This was because heat production by the cells was curtailed, the temperature in the fermenter quickly fell, and the controller increased the heater power

to maintain the temperature at the set-point.

The initial steady-state heater power varied with the temperature of the fermentation and the cooling water flowrate. There was a build-up of a black protein coat on the heater surface when the heater power exceeded around 700W. This caused errors due to the changing heat transfer properties of the heater. To ensure this did not happen the following water flowrates were selected:

Temperature (°C)	Flowrate ($L.min^{-1}$)
30	1.0
34	0.8
38	0.5
42	0.4

3.4.2 Overall Heat of Fermentation - Strain pCQV2.

The measured value of overall heat of fermentation, Q_f, was compared to that determined from the correlations relating it to OUR adapted from (a) Cooney CL *et al* (1968), and (b) and (c) Luong JHT and Volesky B (1980):

(a) $Q_f = 0.518*OUR$ ($kJ.L^{-1}h^{-1}$) (OUR in $moles.m^{-3}h^{-1}$)

$= 0.1439*OUR$ ($W.L^{-1}$)

(b) $Q_f = 0.1292*OUR$ ($W.L^{-1}$) (OUR in $moles.m^{-3}h^{-1}$) using the general result obtained from the different organisms and

(c) $Q_f = 0.1569*OUR$ ($W.L^{-1}$) (OUR in $moles.m^{-3}h^{-1}$) using

the result obtained for *E coli*.

TABLE 3.7

COMPARISON BETWEEN MEASURED AND PREDICTED VALUES OF OVERALL HEAT OF FERMENTATION AT DIFFERENT TEMPERATURES FOR STRAIN PCQV2.

Temperature (°C)	Q_f -measured ($W.L^{-1}$)	OUR (moles. $m^{-3}h^{-1}$)	Q_f -predicted ($W.L^{-1}$)		
			(a)	(b)	(c)
30	11.8	88	12.7	11.4	13.8
34	14.1	103.6	14.9	13.4	16.3
38	15.0	137	19.7	17.7	21.5

3.4.3 Specific Heat of Fermentation.

The specific heat of fermentation, q_f, was defined as the heat of fermentation per gram of dry cells. This was determined by dividing Q_f by the biomass concentration present at the time, and expressed as $W.g^{-1}$dcw. It can also be regarded as the yield coefficient of heat on biomass.

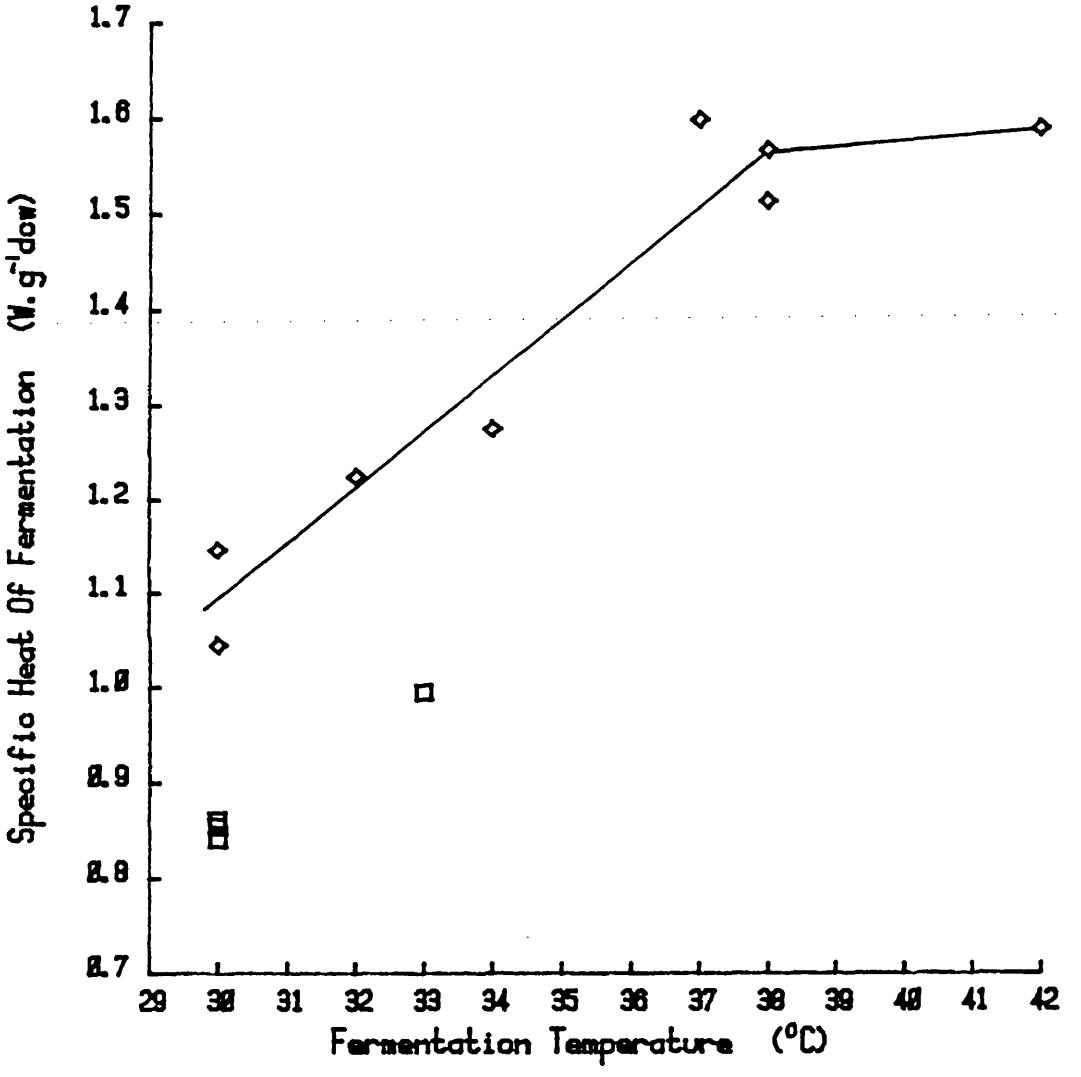


Figure 3.41: Effect Of Fermentation Temperature On Specific Heat Of Fermentation (◇ ,pCQV2; □ ,pMG169).

The figure is a plot of q_f against fermentation temperature for the two strains. However values were only determined for strain pMG169 at growth temperatures because this technique requires exponential growth at the selected temperature. As previous results have shown this was the case for pMG169 at growth temperatures, but not at production temperatures.

A regression analysis on the results for strain pCQV2 in the range 30-38°C yielded the following equation:

$$q_f = -0.6905 + 0.05944*T$$

(T in °C and a regression coefficient of 0.9662)

3.4.4 Rate of Temperature Increase Using Overall Heat of Fermentation.

The time taken to raise the temperature using metabolic and agitation heat is an important parameter in designing the way in which the temperature shift should be implemented on the large scale.

The results described so far have suggested that batch fermentation with glucose feeding would be the best system investigated for process optimisation. Therefore fermentations were performed with the two strains in which the heater and cooling circuit were switched off during glucose limitation (at the highest feed-rate used for each strain) to see the rate at which the temperature rose under

agitation and metabolic heat. The results are shown in figure 3.42.

The actual time taken to rise 10°C was 1.14h for pCQV2, and 1.42h for pMG169, the shifts being implemented at the same biomass concentration.

The rates of temperature rise for the two strains were:

- pCQV2 $8.756°C.h^{-1}$
- pMG169 $7.092°C.h^{-1}$

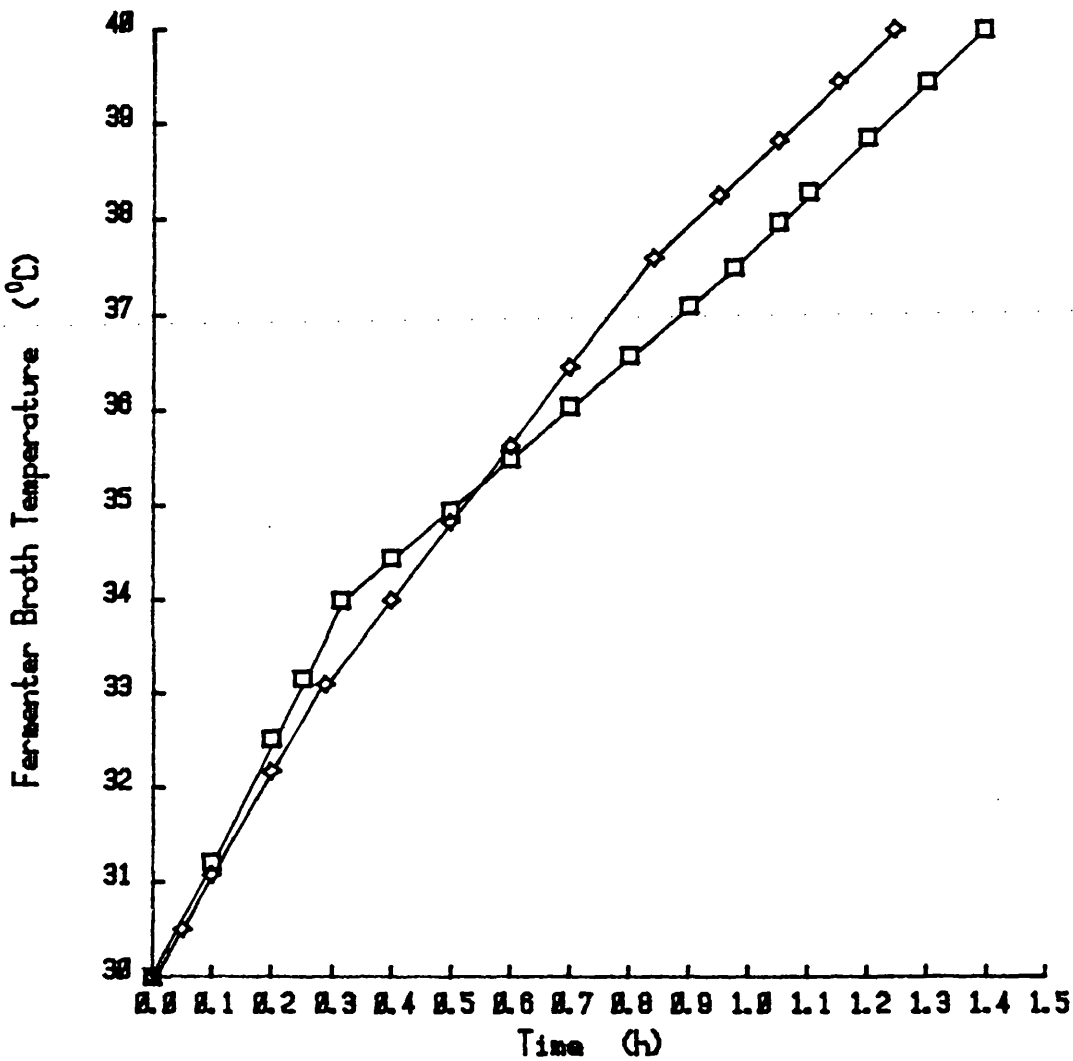


Figure 3.42: Fermenter Temperature Rise against Time Using Metabolic And Agitator Heat Alone (◇ ,pCQV2; □ ,pMG169).

During these experiments the glass jar of the fermenter was unlagged, implying that heat would have been lost to the surroundings. Heat loss on a larger scale would not have been so marked because the surface area to volume ratio is smaller at larger scales, assuming the height to diameter ratio remains within the conventional range. It might be expected that with both the biomass concentration and specific heat of fermentation being at their greatest towards the end of the fermentation, that the greatest amount of heat would be evolved then, and the broth temperature would rise most quickly. However heat losses to the surroundings from a small unlagged fermenter would also be greatest at this point due to the high temperatures of the broth, and might counteract the effect of greater heat generation at the higher temperatures. This is a possible explanation of the result obtained in figure 3.42. It would be expected, therefore, that the temperature shift would be implemented in this way at a faster rate in larger vessels.

4 DESIGN.

Throughout this chapter "strain pCQV2" and "strain pMG169" are used as abbreviations for "strain *E. coli* RV308(pCQV2)" and "strain *E. coli* RV308(pMG169)", respectively.

4.1 INTRODUCTION.

Batch titres were calculated at different OTRs for the two strains at 38 and 40°C under kinetic limitation, 40°C for strain pMG169 and 42°C for strain pCQV2 under glucose limitation. It was also calculated under oxygen limitation at 40°C and OTR=approx 60 $mM.L^{-1}h^{-1}$ for pMG169, and at 42°C and OTR=approx 50 $mM.L^{-1}h^{-1}$ for pCQV2. The titres were calculated against different OTRs since this is determined by choice of scale and design of fermentation process variables (eg agitator rate, air flowrate, head pressure, etc).

These titres and the number of batches per year that could be run were calculated on the scale of $100m^3$. However the results can be extended to other scales because the titres were determined at different OTRs, which can be achieved at other fermenter volumes and conditions. Naveh D (1985) stated that the upper limit of fermenter size is determined by constraints like downstream processing capacity, structural limitations (foundation loadings, space), air

supply (increased height increases static pressure), and heat transfer. Brown DE (1982) found that heat transfer in recombinant *E. coli* fermentations did not become limiting until a scale of 210m³ (4.5m diameter, 14m height). There are no technical reasons why large scale recombinant *E. coli* fermentations cannot be performed. However Pickett A and Haddock B (1983) concluded that for many recombinant DNA products production fermenters will be of the order of 10m³. Although this might be generally true there could well be some such products that have much larger markets than that scale could supply. Fish NM and Lilly MD (1984) thought it likely that recombinant strains will be used for bulk enzyme production.

In the development of process design principles it must be remembered that a process could either be made to work in an existing fermentation plant or in a purpose-built facility. Reference is made in the text and in chapter 5 to the differences associated with these two cases.

The process design below was performed on a conventional stirred tank fermenter. The reasons for this were that considerable fermentation capacity of this type exists in which these processes could be run, and also because of the advantages offerred by this vessel. These are that this type of fermenter is very flexible (many different types of process can be run in it), meaning that the plant would not have to be solely dedicated for the use of these strains. It also has a time-proven record (data and expertise available), is readily available, is comparitively cheap,

and there are a number of manufacturers to choose from (Naveh D (1985)).

4.2 DATA USED FROM RESULTS (CHAPTER 3).

This section describes the results that were used in the calculations to follow. The results are listed and the text or figures they are taken from stated.

Specific Growth Rate:
pCQV2 - taken from figure 3.6 (0.582h^{-1} at 40°C, 0.578h^{-1} at 38°C).
pMG169 - taken from figure 3.7 and calculated as the change in ln(Adj OD) divided by the time taken prior to growth being curtailed as a result of the temperature shift (0.461h^{-1} at 40°C, 0.445h^{-1} at 38°C).

Length of Production Phase Under Kinetic Limitation:
pCQV2 - taken from figure 3.10 (2.5h at 40°C, 2.25h at 38°C)
pMG169 - taken from figures 3.11 and 3.13 (1.8h at 40°C, 2.25h at 38°C)

Length of Production Phase Under Oxygen Limitation:
pCQV2 - taken from figure 3.23 (3h at 42°C)
pMG169 - taken from figure 3.23 (2.25h at 40°C)

Length of Production Phase Under Glucose Limitation:

pCQV2 - taken from figures 3.32 and 3.34 (4h at 42°C)

pMG169 - taken from figures 3.33 and 3.35 (4h at 40°C)

Specific OUR:

pCQV2 - taken from figure 3.20 (14.5 $mML^{-1}h^{-1}$ at 40°C, 12.5 $mML^{-1}h^{-1}$ at 38°C).

pMG169 - taken from figure 3.21 (13.0 $mML^{-1}h^{-1}$ at 40°C, 12.5 $mML^{-1}h^{-1}$ at 38°C [pCQV2 value assumed])

Specific Production Rate Under Kinetic Limitation:

pCQV2 - taken from figure 3.15 (100,000 $nu.g^{-1}h^{-1}$ at 40°C, 60,000 $nu.g^{-1}h^{-1}$)

pMG169 - taken from figure 3.16 (750,000 $nu.g^{-1}h^{-1}$ at 40°C, 500,000 $nu.g^{-1}h^{-1}$ at 38°C)

Specific Production Rate Under Glucose Limitation:

pCQV2 - taken from figure 3.37 (27,500 $nu.g^{-1}h^{-1}$ at 42°C)

pMG169 - taken from figure 3.38 (425,000 $nu.g^{-1}h^{-1}$ at 40°C)

Volumetric Production Rate Under Oxygen Limitation:

pCQV2 - taken from figure 3.23 and section 3.2.2 (25,421 $nu.L^{-1}h^{-1}$ at 42°C)

pMG169 - taken from figure 3.23 and section 3.2.2 (521,918 $nu.L^{-1}h^{-1}$ at 40°C)

Volumetric Production Rate Under Glucose Limitation:

pCQV2 - taken from figure 3.34 and section 3.2.2 (310,733 $nu.L^{-1}h^{-1}$ at 42°C)

pMG169 - taken from figure 3.35 and section 3.2.2 (4,218,649 $nu.L^{-1}h^{-1}$ at 40°C)

4.3 METHOD.

The methods used to calculate batch titres at different OTRs, and the number of batches per year achievable under different fermentation conditions are stated in this section.

4.3.1 Kinetic Limitation.

A scale, working volume, production temperature, and maximum OTR were chosen.

Let X_{of} be the biomass level at onset of O_2-limitation, and therefore represent the final concentration achievable under kinetically limited conditions.
Therefore X_{of} = OTR/specific OUR.
As already stated the OTR is chosen and the specific OUR came from section 3.1.6.1 at any chosen production temperature.

Let X_{pf} and X_{p0} be the final and initial biomass concentrations respectively, during the production phase.
(For kinetic limitation, $X_{pf} = X_{of}$).
Since $X_{pf} = X_{p0} * e^{\mu t}$ $\quad X_{p0} = X_{pf} / e^{\mu t}$

Let P_1 = batch titre per litre, p' = sp prod rate, and t = length of time in the production phase.

$$P_1 = p' * X_{p0} * \int_0^t e^{\mu t}.dt$$

$$= p' * X_{p0} * (e^{\mu t} - 1)/\mu$$

Therefore $P = P_1 * V_1$ where P = batch titre and V_1 = working volume.

Total batch time = length of growth phase + length of temperature shift + length of production phase + turnround time.

Number of batches per year = 365 days * 24 hours * proportion of time plant operating / Total batch time.

The proportion of time the plant was assumed to be operating was 80% (personal communication, M D Lilly, UCL). This takes into consideration loss of productive time through contaminated batches, poor batches caused by biological variability, mechanical failure, staff holidays, and plant maintenance. Good planning and management practices could improve this figure, yielding more batches per year and so increasing annual production.

4.3.2 Oxygen Limitation and Glucose Limitation (Without a Glucose Limited Growth Phase).

These calculations described the actual situations that arose from the fermentations presented in Chapter 3 ie O_2-limitation at OTR = 50 $mML^{-1}h^{-1}$ (pCQV2) and 60 $mML^{-1}h^{-1}$

(pMG169), Glucose limitation at OTR = 50 $mML^{-1}h^{-1}$ (pCQV2) and 57.5 $mML^{-1}h^{-1}$ (pMG169). They differ from those in section 4.3.1 in the following way.

$P_1 = p'' * t$ where p'' = volumetric production rate.

4.3.3 Glucose Limitation Extrapolated for Higher OTR, Glucose Limited Growth Phase, and Longer Production Phase.

Equations for OTR and biomass generation rate at different glucose feed-rates were developed from existing results, and solved simultaneously to give biomass generation rate at different OTRs.

This enabled prediction of batch titres at different OTRs and also allowed a glucose limited growth phase to be incorporated for higher biomass production.

A similar method to that in 4.3.1 was used except that:
$X_{gf} = X_{of} + (t_1 * X')$ where X_{gf} = biomass concentration at the end of the glucose limited growth phase, t_1 = time in glucose limited growth phase, and X' = biomass generation rate.

Since growth would be linear the biomass concentration at the halfway point in the production phase could be used when combined with the specific production rate to describe the volumetric production rate.

Therefore $P_1 = p' * ((X' * t/2) + X_{gf}) * t$.

Batch titres and other results followed the same method as those in section 4.3.1.

A similar extrapolative design was not performed for oxygen limited fermentations due to insufficient information as to how growth and production were changed by different limiting OTRs. The relevant experiments were not performed because of the poor performance of the fermentations already done, when compared to those done under glucose limitation.

In the results shown below (sections 4.4.) OTR = 50 $mML^{-1}h^{-1}$ for pCQV2 and 60 $mML^{-1}h^{-1}$ for pMG169, and the biomass generation rate equations developed in section 4.4.1 were used to predict growth.

4.4 BATCH TITRE AT DIFFERENT OTR.

General Conditions - temperature = 40°C, scale = $100m^3$

Special Conditions - Strain pCQV2

Kinetic limitation:

specific growth rate = $0.522h^{-1}$, specific production rate = 100,000 $nu.g^{-1}h^{-1}$, specific OUR = 14.5 $mM.L^{-1}h^{-1}$, length of production phase = 2.5h.

Oxygen limitation:

max OTR = 50 $mML^{-1}h^{-1}$, volumetric production rate = 25,421 $nu.L^{-1}h^{-1}$, length of production phase = 3h.

Glucose limitation:

volumetric production rate = 310,733 nu.$L^{-1}h^{-1}$, length of production phase = 4h.

TABLE 4.1

BATCH TITRE FOR STRAIN pCQV2 UNDER DIFFERENT LIMITATION AGAINST OTR.

OTR ($mML^{-1}h^{-1}$)	BATCH TITRE ($*10^6$ nu)		
	Kinetic Limitation	Oxygen Limitation	Glucose Limitation
50	47,399	6,101	78,425
55	51,541		86,203
60	56,879		93,981
65	61,619		101,759
70	66,359		109,536
75	71,098		117,314
80	75,838		125,092
85	80,578		132,870
90	85,318		140,647
95	90,058		148,425
100	94,798		156,203

For kinetic limitation the equation of the line of batch titre ($*10^6$ nu) (y) against OTR ($mML^{-1}h^{-1}$) (x) was determined using a least squares regression analysis:

y = 952,321,818.5*x - 380,045,490 (r=0.9999)

For glucose limitation the equation of the line was:

$y = 155,555,002*x + 647,922,545.5$ (r=1.0000)

Therefore, using these equations batch titres can be calculated at OTRs other than those appearing in the table.

Special Conditions - Strain pMG169

Kinetic limitation:

specific growth rate = 0.461h^{-1}, specific production rate = 750,000 $nu.g^{-1}h^{-1}$, specific OUR = 13.0 $mM.L^{-1}h^{-1}$, length of production phase = 1.8h.

Oxygen limitation:

max OTR = 60 $mML^{-1}h^{-1}$, volumetric production rate = 521,918 $nu.L^{-1}h^{-1}$, length of production phase = 2.25h.

Glucose limitation:

volumetric production rate = 4,218,649 $nu.L^{-1}h^{-1}$, length of production phase = 4h.

TABLE 4.2

BATCH TITRE FOR STRAIN pMG169 UNDER DIFFERENT LIMITATION AGAINST OTR.

OTR ($mML^{-1}h^{-1}$)	BATCH TITRE ($*10^9$ nu)		
	Kinetic Limitation	Oxygen Limitation	Glucose Limitation
50	500.584		1,206.4
55	550.642		1,328.7
60	600.701	93.945	1,451.1
65	650.759		1,573.4
70	700.818		1,695.8
75	750.876		1,818.1
80	800.934		1,940.5
85	850.993		2,062.8
90	901.051		2,185.2
95	951.110		2,307.5
100	1,001.168		2,429.8

The same regression analysis was performed for strain pMG169. The equation of the line of batch titre ($*10^9$ nu) (y) against OTR ($mML^{-1}h^{-1}$) (x) was found to be, for kinetic limitation:

$y = 1.001168*10^{10}*x - 272,727.272$ (r=1.0000)

For glucose limitation:

$y = 2.446909*10^{10}*x - 1.706364*10^{10}$ (r=0.9999)

General Conditions - temperature = 38°C, scale = 100m³

Special Conditions - Strain pCQV2

Kinetic limitation:

specific growth rate = 0.578h^{-1}, specific production rate = 60,000 nu.g^{-1}h^{-1}, specific OUR = 12.5 mM.L^{-1}h^{-1}, length of production phase = 2.25h.

Special Conditions - Strain pMG169

Kinetic limitation:

specific growth rate = 0.445h^{-1}, specific production rate = 500,000 nu.g^{-1}h^{-1}, specific OUR = 11.5 mM.L^{-1}h^{-1}, length of production phase = 2.25h.

TABLE 4.3

BATCH TITRE FOR BOTH STRAINS UNDER KINETIC LIMITATION AGAINST OTR, AT 38°C.

OTR ($mML^{-1}h^{-1}$)	pCQV2 Batch Titre ($*10^6$ nu)	pMG169 Batch Titre ($*10^6$ nu)
60	39,862	468,979
65	43,183	508,061
70	46,505	547,142
75	49,827	586,224
80	53,149	625,305
85	56,471	664,387
90	59,792	703,468
95	63,114	742,550
100	66,436	781,632
105	69,758	820,713
110	73,080	859,795

The equation of the line of batch titre ($*10^6$ nu) (y) against OTR ($mML^{-1}h^{-1}$) (x) was determined using a least squares regression analysis.

For strain pCQV2:

$y = 664,363,636.7*x - 272,763.64$ (r=1.0000)

For strain pMG169:

$y = 7,816,314,549*x + 172,362.636$ (r=1.0000).

As mentioned in section 4.1 the reason that batch titre was determined against OTR was that the latter is a fermentation condition that can be chosen on economic, as well as technical grounds. OTR can be increased in one or more of the following ways:

- by sparging pure oxygen into the broth.

- by retro-fitting an existing fermenter with new impeller systems designed to increase mixing and oxygen transfer.

- by increasing the fermenter head pressure. This may require expensive modifications to the fermenter.

- by increasing the gassed power per unit volume. This not only increases power costs but also the cooling costs since much of the power is dissipated as heat. It might also require a new motor and/or gear-box.

- by using a mechanical foam-breaker instead of chemical antifoams, which reduce the DOT, and hence O_2 available to the cells.

- by increasing the air flowrate. However the OTR is not as sensitive to this as the gassed power per unit volume. Also, this would be likely to increase foaming.

- by using novel fermenter designs. The inherent flexibility of the stirred tank fermenter would be lost, and this might make it impossible to run other types of process in the plant.

The increased costs associated with these actions might turn out to be insignificant in terms of overall process costs. However this will vary from process to process.

There will come a point where it is either uneconomical or dangerous, or both to try to increase the OTR further. At this point it would be necessary to build or seek new fermentation capacity.

However since OTR is a crucial variable which can be selected by design, the batch titre for the two different strains was determined against it.

4.4.1 Glucose Limited Growth Phase Incorporated.

The tables above show that a production phase run under glucose limitation would yield a higher batch titre than under kinetic limitation. The reason for this was that under kinetic limitation the biomass concentration at the end of production (the point of glucose exhaustion) would be such that oxygen limitation was just avoided. The biomass concentration ($gdcw.L^{-1}$) would therefore be OTR/specific OUR.

However with glucose-feeding this would be the biomass concentration at the beginning of the production phase. Therefore the final biomass concentration would be higher. This explains the higher batch titres under glucose limitation described above.

The imposition of glucose limitation is also a way of achieving higher biomass concentrations prior to

production. Therefore a possible fermentation strategy for temperature sensitive systems might be a short exponential growth phase followed by glucose feeding at a growth temperature to generate more biomass, followed by a temperature shift and production phase.

From figure 3.28 it was assumed that in the range 21-50 $g.m^{-3}min^{-1}$ increasing the feed-rate (F) resulted in a linear increase in biomass generation rate (X'). It was also assumed that a linear relationship held in figure 3.31 and that both of these linear functions held at higher glucose feed-rates. (In the following equations the units are: OTR, $mML^{-1}h^{-1}$; F, $g.m^{-3}min^{-1}$; X', $gdcw.L^{-1}h^{-1}$). For pCQV2:

$$OTR = 10.600 + 0.8208*F$$

$$X' = 0.357396 + 0.024824*F$$

Therefore $X' = 0.036813 + 0.030244*OTR$

For pMG169:

$$OTR = 1.466 + 1.1207*F$$

$$X' = -0.01627 + 0.03566*F$$

Therefore $X' = -0.0627 + 0.03182*OTR$

Since at any OTR X' can be calculated and assumed to be constant, then a length of glucose limited growth phase can be chosen, followed by a production phase. Hence final biomass concentration and batch titre can be calculated. The results are shown in the tables below.

TABLE 4.4

BATCH TITRE AND FINAL CELL CONCENTRATIONS FOR BOTH STRAINS AGAINST OTR, INCORPORATING A 5h GLUCOSE-LIMITED GROWTH PHASE.

OTR ($mML^{-1}h^{-1}$)	pCQV2		pMG169	
	Final Cell Concentration (gL^{-1})	Batch Titre ($*10^6$ nu)	Final Cell Concentration (gL^{-1})	Batch Titre ($*10^9$ nu)
50	19.7551	146,582	19.5687	2,245.6
55	21.6974	161,013	21.5819	2,476.2
60	23.6398	175,445	23.5952	2,706.7
65	25.5822	189,876	25.6085	2,937.2
70	27.5246	204,308	27.6218	3,167.8
75	29.4669	218,739	29.6351	3,398.3
80	31.4093	233,171	31.6484	3,628.8
85	33.3517	247,602	33.6617	3,859.4
90	35.2941	262,033	35.6750	4,089.9
95	37.2364	276,465	37.6883	4,320.4
100	39.1788	290,896	39.7016	4,551.0

TABLE 4.5

BATCH TITRE AND FINAL CELL CONCENTRATIONS FOR BOTH STRAINS AGAINST OTR, INCORPORATING A 12h GLUCOSE-LIMITED GROWTH PHASE.

OTR ($mML^{-1}h^{-1}$)	pCQV2		pMG169	
	Final Cell Concentration (gL^{-1})	Batch Titre ($*10^6$ nu)	Final Cell Concentration (gL^{-1})	Batch Titre ($*10^9$ nu)
50	30.5982	242,001	30.2668	3,700.6
55	33.5991	265,748	33.3937	4,082.6
60	36.6000	289,494	36.5207	4,464.6
65	39.6009	313,241	39.6477	4,846.6
70	42.6018	336,988	42.7747	5,228.6
75	45.6027	360,734	45.9017	5,610.6
80	48.6037	384,481	49.0287	5,992.6
85	51.6046	408,227	52.1557	6,374.6
90	54.6055	431,974	55.2827	6,756.6
95	57.6064	455,720	58.4097	7,138.6
100	60.6073	479,467	61.5367	7,520.5

4.4.2 Effect of Different Lengths of Production Phase in Glucose Limitation.

Another fermentation strategy would be to extend the production phase beyond 4h.

For these calculations no glucose limited growth was incorporated prior to production. The actual method described in section 4.3.3 was used.

For glucose limitation the equation of the lines of batch titre (nu) (y) against length of production phase (h) (x) was determined using a least squares regression analysis:

- pCQV2

$y = 2.48586*10^{10}*x + 245,381.82$ (r=1.0000)

- pMG169

$y = 3.3749158*10^{12}*x + 1,170,909.1$ (r=1.0000)

However the assumption that production would continue in the same way for longer periods may not be valid. This is largely because in the kinetically limited fermentations intracellular production reached a peak and thereafter remained constant. It is therefore likely that the same thing would happen under glucose limitation, albeit more slowly. Figures 3.32 and 3.33 show signs of this happening after around 3.5-4h production. It would also be expected that at higher feed-rates (assuming O_2-limitation was avoided) the production profiles would increasingly resemble in shape those under kinetic limitation.

4.4.3 Effect of Different Lengths of Glucose Limited Growth Phase on Batch Titre.

The purpose of this was to see the effects on batch titre of extrapolating existing results to include higher biomass generation under glucose limitation prior to the production phase.

Constants: Growth phase = 3.0h, Shift time = 0.5h, Turnround time = 5h, Production phase = 4h.
Effect of length of GLUCOSE LIMITED GROWTH PHASE (h) (x) on Batch titre (nu) (y) - Data from tables A3.7 and A3.8 (Appendix 3).

- pCQV2

$y = 1.3631314*10^{10}*x + 7.84254208*10^{10}$ (r=1.0000)

- pMG169

$y = 2.511239*10^{12} + 1.451088*10^{13}$ (r=1.0000)

4.5 EFFECT OF CHANGING SCALE.

The results calculated at different scales showed that for any OTR the batch titre of broth volume V_1 (m^3) was:

$(V_1/80)$*batch titre from above tables.

4.6 FERMENTER CONFIGURATION.

This section shows how differing lengths of growth phase (kinetic and glucose limited), shift times, turnround times, and glucose limited production phase can affect the number of batches that could be run in a given time. Decisions must be made about the magnitude of these variables on both technical and economic grounds.

For a particular fermenter two possible scenarios can be envisaged which would affect the set-up of the process. These are:

1) A fermenter is solely dedicated to a process using a temperature sensitive system and demand for the product increases so that more batches per year must be operated.

2) A fermenter is used for several different processes at different times throughout the year, and either through increased product demand and/or the need to introduce another process, more runs are to be added to the schedule.

In either of these scenarios different ways of reducing fermentation time must be sought, without adversely affecting the batch titre.

In designing the way in which the fermenter would be run three choices have to be made for kinetically limited processes, which affect the overall fermentation time for each batch:

a) The length of the growth phase. This depends on the size of the final inoculum, and represents unproductive fermenter time. It should therefore be reduced to a minimum.

b) The time taken to implement the temperature shift. This is not as important as a) but nonetheless can have a significant effect on the number of batches that can be run annually.

c) The turnround time between batches. This can have the greatest effect on annual fermenter productivity, and should obviously be reduced to a minimum.

Assuming c) would be kept to a minimum then the number of annual batches using temperature sensitive systems could be maximised by using the final fermenter for production only. This could only be done for strain pCQV2 (where growth continues exponentially) but it would remove a) and b) from the equation. However this assumes there would be no adverse effects of transferring exponentially growing culture into fresh media at a higher temperature.

The reason strain pMG169 would not be suitable for this sort of operation is that during production the biomass concentration only doubles once. This means that the size of the final inoculum would have to be half the peak achievable biomass concentration at the production scale, which is unlikely to be an economical mode of operation.

This mode of operation could also only be used for kinetically or glucose limited runs in which it was

intended that glucose-feeding should be commensurate with production. In chapter 3 the temperature shift was not implemented until the cultures were growing under glucose limitation. Imposing glucose limitation at the point of the temperature shift might have adverse effects on production eg cause plasmid instability.

In addition to choices a) to c) a further choice has to be made of the length of glucose limited growth if one is to be incorporated in the process. Also different products might cause different production profiles, so that a different production phase length (either kinetic or glucose limited) might have to be chosen.

For the purpose of determining the effects of changing each variable on fermenter productivity certain values of the other variables were assumed while the one under investigation was considered. These assumptions were consistent throughout section 4.6 and are discussed in section 4.6.6.

4.6.1 Effect of Length of Growth Phase on Fermenter Productivity.

Constants: Shift time = 0.5h, Turnround time = 5h

Effect of length of GROWTH PHASE (h) (x) on number of batches per year (y) - Data from tables A3.1 and A3.4 (Appendix 3).

- pCQV2 40°C

$y = 813.266 * e^{-0.079215 * x}$ (r = -0.9964)

- pCQV2 38°C

$y = 836.5395 * e^{-0.08085 * x}$ (r = -0.9962)

- pMG169 40°C

$y = 881.872 * e^{-0.08397 * x}$ (r = -0.9959)

- pMG169 38°C

$y = 750.463 * e^{-0.07469 * x}$ (r = -0.9968)

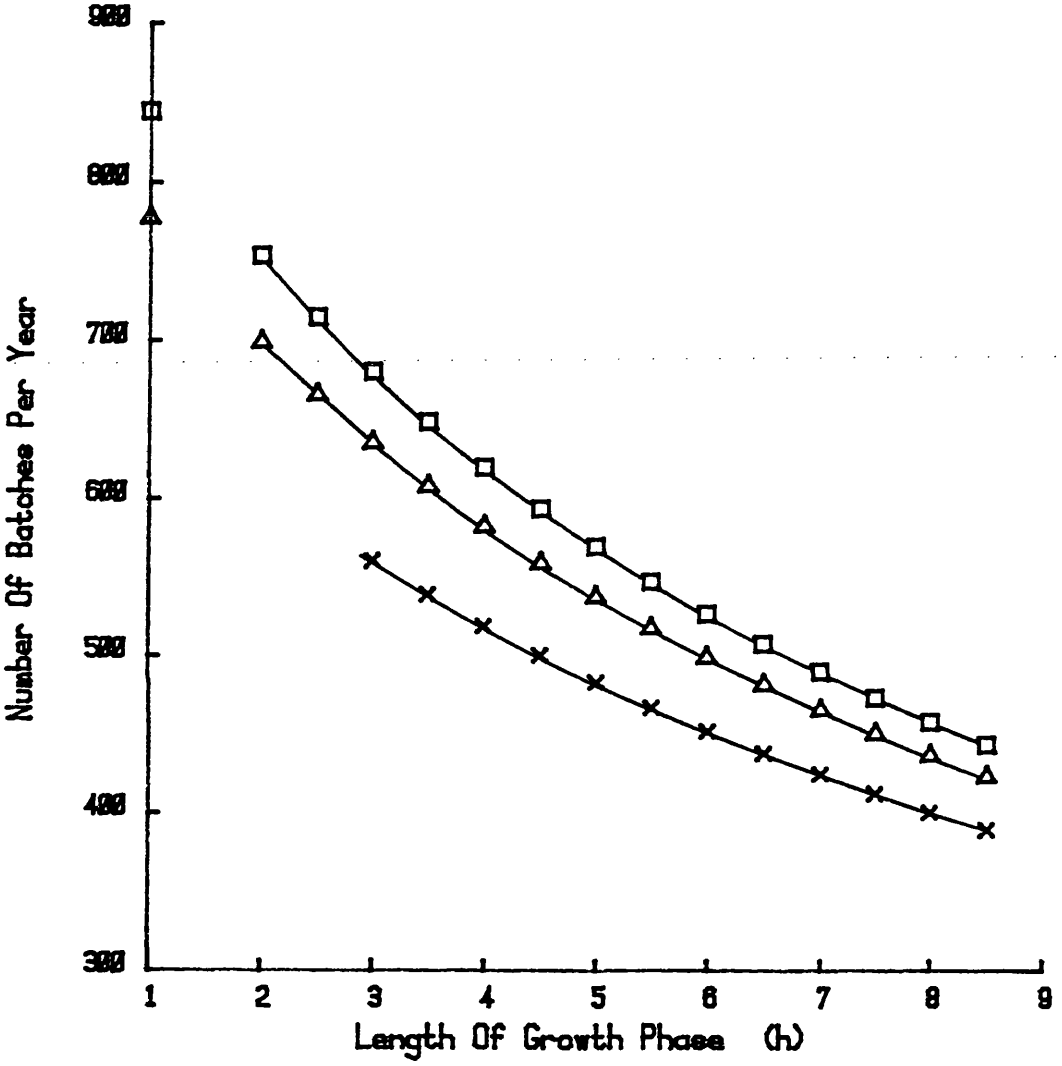


Figure 4.1: Effect of Length of Growth Phase on Number of Batches Per Year (□ ,Strain pMG169 - Kinetic Limitation; Δ , Strain pCQV2 - Kinetic Limitation; x ,Both Strains - Glucose Limitation).

4.6.2 Effect of Length of Shift Time on Fermenter Productivity.

Constants: Growth phase = 3.0h, Turnround time = 5h

Effect of length of SHIFT TIME (h) (x) on number of batches per year (y) - Data from tables A3.2 and A3.5 (Appendix3).

- pCQV2 40°C

  $y = 666.328 * e^{-0.08919 * x}$ (r = -0.9999)

- pCQV2 38°C

  $y = 682.531 * e^{-0.09123 * x}$ (r = -0.9999)

- pMG169 40°C

  $y = 713.757 * e^{-0.09513 * x}$ (r = -0.9999)

- pMG169 38°C

  $y = 622.0327 * e^{-0.08359 * x}$ (r = -0.9999)

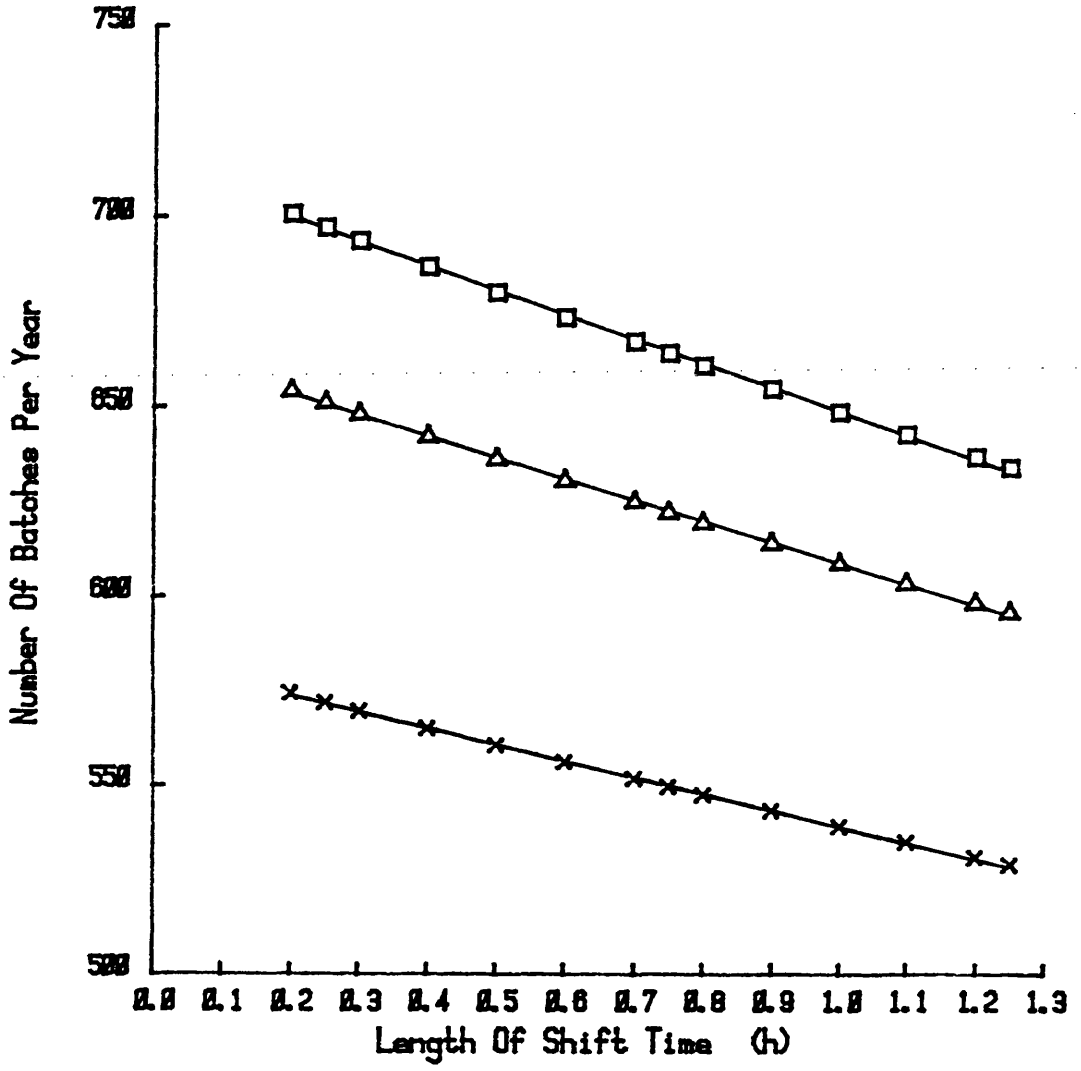


Figure 4.2: Effect of Length of Shift Time on Number of Batches Per Year (□ ,Strain pMG169 - Kinetic Limitation; △ , Strain pCQV2 - Kinetic Limitation; X ,Both Strains - Glucose Limitation).

4.6.3 Effect of Length of Turnround Time on Fermenter Productivity.

Constants: Growth phase = 3.0h, Shift time = 0.5h

Effect of length of TURNROUND TIME (h) (x) on number of batches per year (y) - Data from tables A3.3 and A3.6 (Appendix 3).

- pCQV2 40°C

  $y = 1064.058-269.7716*\ln(x)$ (r = -0.9992)

- pCQV2 38°C

  $y = 1092.187-278.710*\ln(x)$ (r = -0.9990)

- pMG169 40°C

  $y = 1146.2754-295.994*\ln(x)$ (r = -0.9987)

- pMG169 38°C

  $y = 987.005-245.481*\ln(x)$ (r = -0.9995)

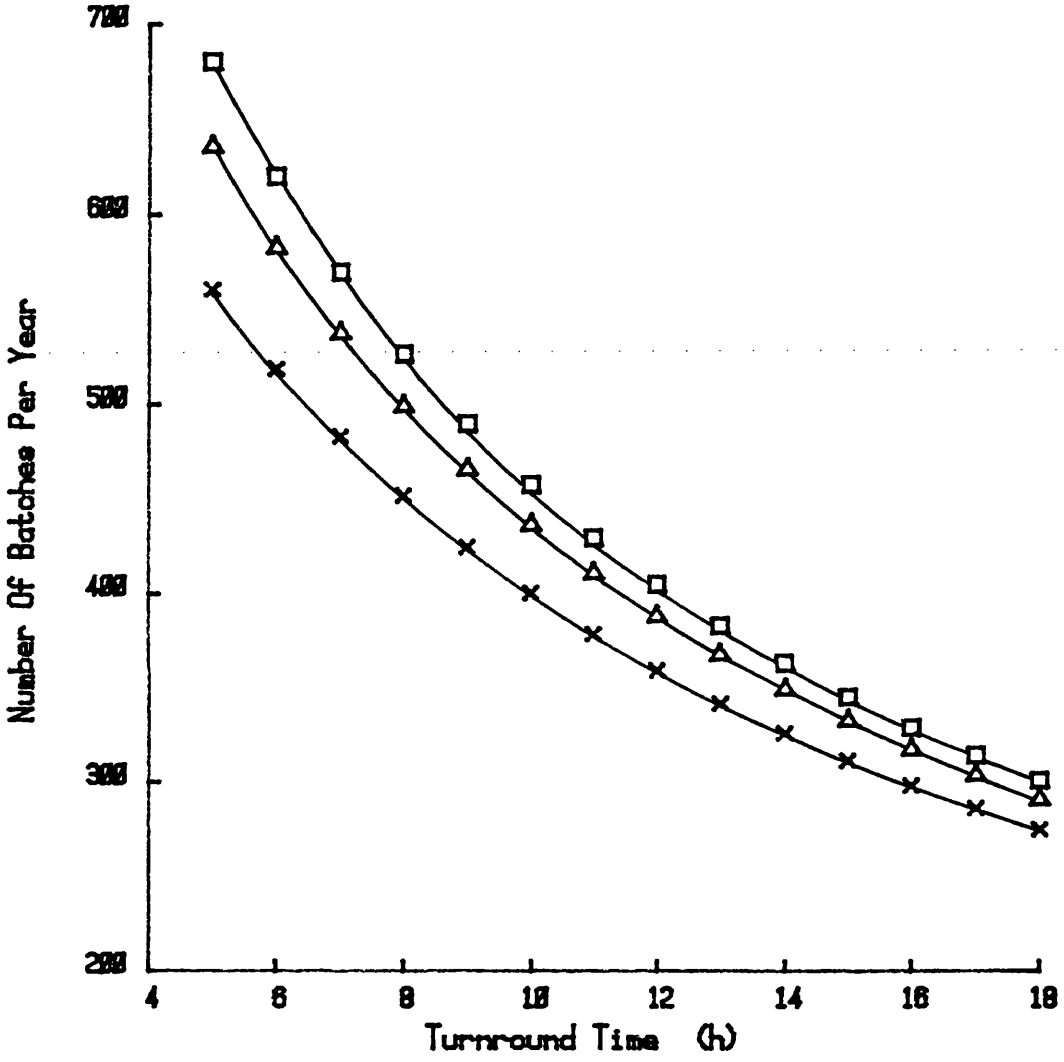


Figure 4.3: Effect of Turnround Time on Number of Batches Per Year (□ ,Strain pMG169 - Kinetic Limitation; △ ,Strain pCQV2 - Kinetic Limitation; × ,Both Strains - Glucose Limitation).

4.6.4 Effect of Length of Production Phase Under Glucose Limitation on Fermenter Productivity.

Constants: Growth phase = 3.0h, Shift time = 0.5h, Turnround time = 5h, Glucose limited growth phase = 0h.

Effect of length of PRODUCTION PHASE (h) (x) on number of batches per year (y) - Data from table A3.9 (Appendix 3).

- pCQV2 and pMG169

$$y = 1/2*[748.505*(e^{-0.071977x})+822.2354-189.8401*\ln(x)]$$

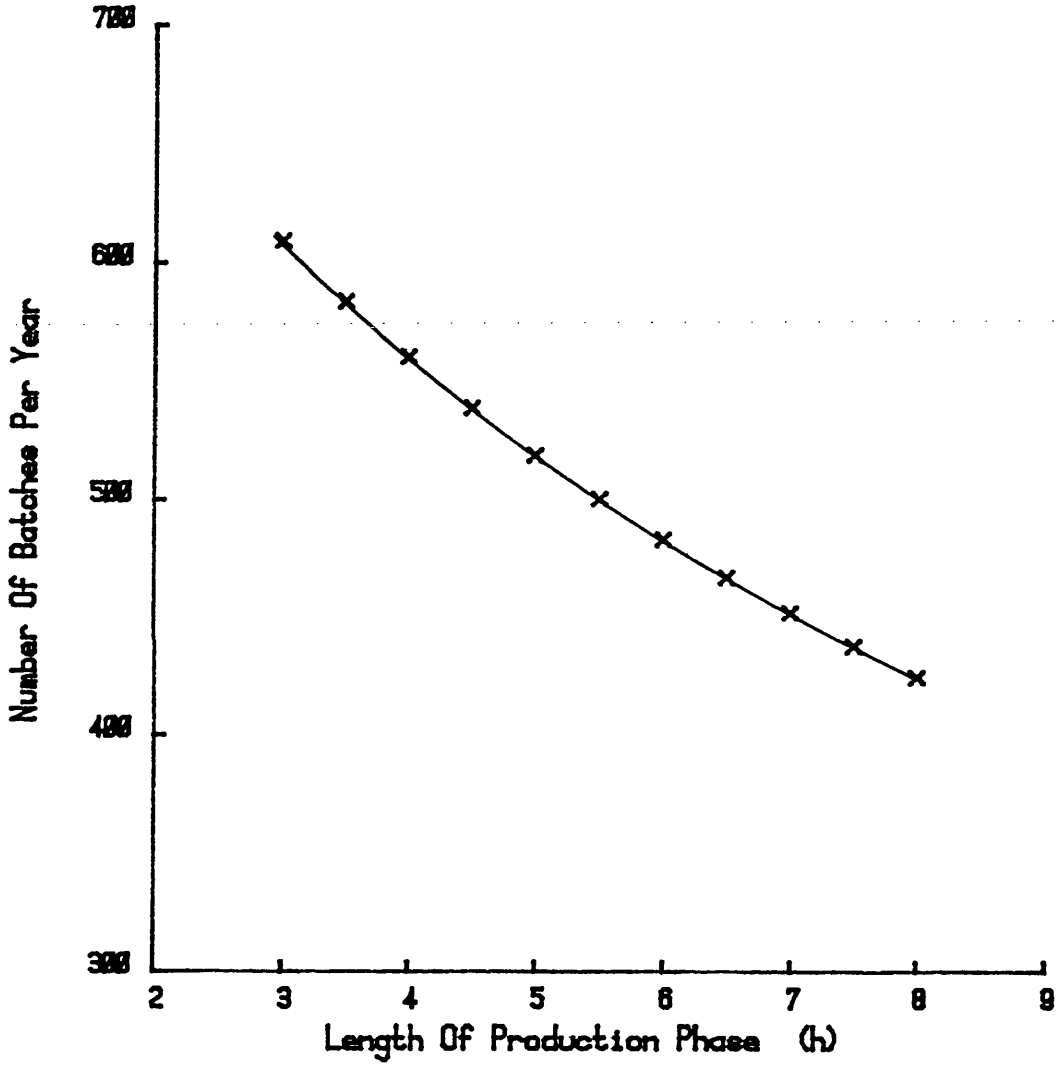


Figure 4.4: Effect of Length of Production Phase Under Glucose Limitation on Number of Batches Per Year - Both Strains.

4.6.5 Effect of Length of Glucose Limited Growth Phase on Fermenter Productivity.

Constants: Growth phase = 3.0h, Shift time = 0.5h, Turnround time = 5h, Production phase = 4h.

Effect of length of GLUCOSE LIMITED GROWTH PHASE (h) (x) on number of batches per year (y) - Data from tables A3.7 and A3.8 (Appendix 3).

- pCQV2 and pMG169

$$y = 613.7195 - 132.2211 * \ln(x) \qquad (r = 0.9996)$$

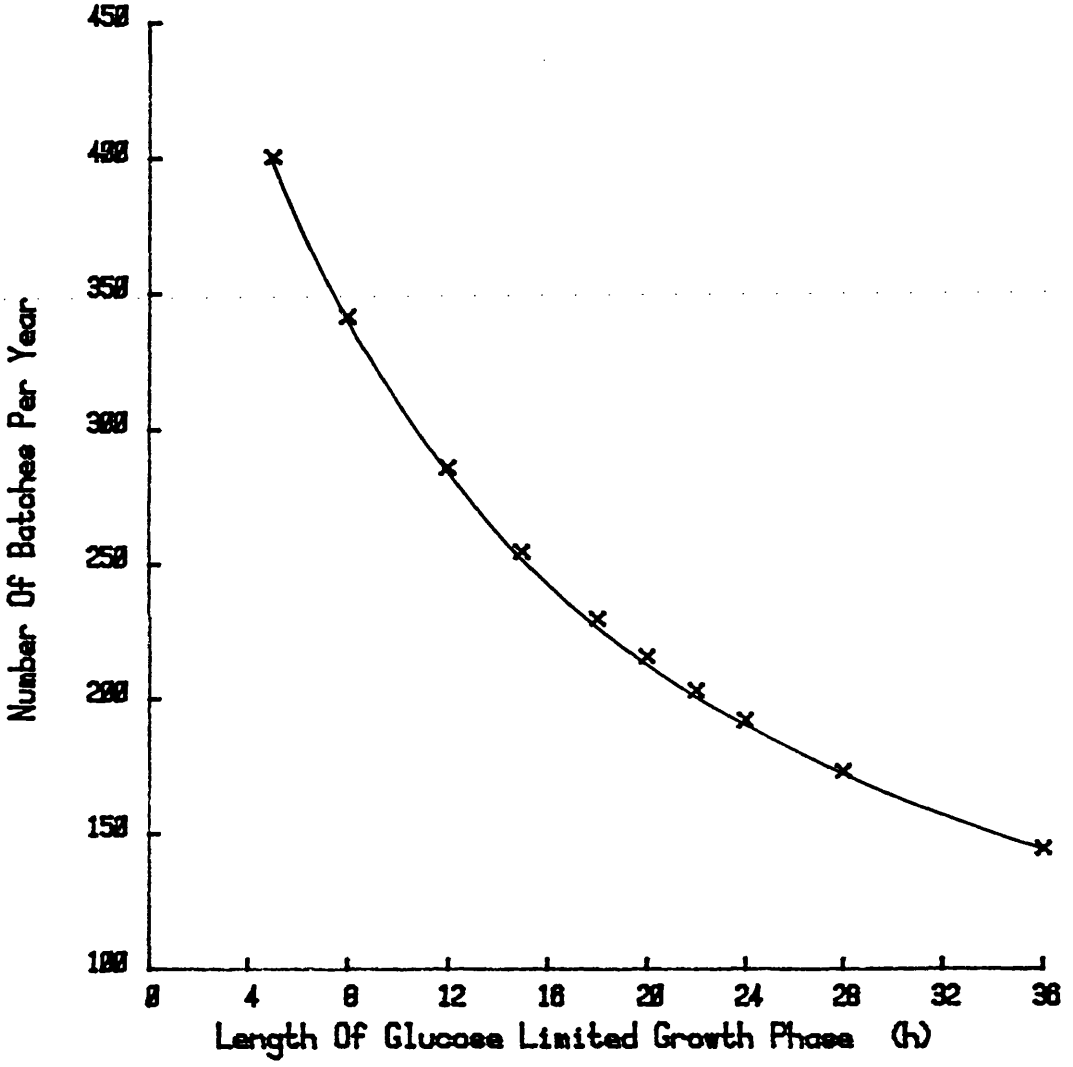


Figure 4.5: Effect of Length of Glucose Limited Growth Phase on Number of Batches Per Year - Both Strains.

4.6.6 Implications of Sections 4.6.1 to 4.6.4

Figs 4.1-4.5 clearly show that the shorter the various time parameters the greater the number of batches per year. This would be important in maximising either annual production or required production in the shortest possible time, releasing the plant for the use of other processes.

In figs 4.1-4.3 the number of theoretical batches is higher for kinetically limited strain pMG169 than kinetically limited strain pCQV2, which in turn is higher than either glucose limited strain. The reason for this is that these fermentations have respectively increasing lengths of production phase, and so increasing batch times. The implications are that although significantly greater batch titres were achieved for glucose limited fermentations the fact that fewer can be done in any given time compared with kinetically limited ones means that overall production figures are closer than the batch titres suggest (see section 4.6.8). However they are not close enough to suggest kinetic limitation as an alternative to glucose limitation. Also, there would be a greater cost associated with running a greater number of batches in the form of increased steam requirements, inoculum preparations, and other running costs.

To maximise the number of batches the most important of these parameters to minimise would be the turnround time (figure 4.3). For example in kinetically limited pMG169

fermentations increasing the turnround time from 5h to 15h would result in an approximate halving of the number of batches per year (or a doubling of the time necessary to produce a desired quantity of product). A value of 5h is an unrealistically low turnround time for a 100m^3 fermenter but it serves to illustrate the importance of minimising this variable.

The second most important time variable to minimise would be the length of the growth phase, because this represents "dead" time from the production point of view. The length of the growth phase is decided by the size and condition of the inoculum into the final fermenter. The inoculum should be growing exponentially (to avoid appreciable lag).

The limit to the reduction in growth phase time would be to run the fermenter at a production temperature from the start. It was calculated that for kinetically limited pCQV2 at 40°C the biomass concentration at the beginning of the production phase would be 0.966 gdcw.L^{-1}. If the 100m^3 fermenter was used simply for production (in the way calculated in 4.3.1) and was operated at 40°C, the inoculum from a 15m^3 seed vessel would need to be at a concentration of 6.44 gdcw.L^{-1}. This should be easily achievable. However this does assume that the transfer of the culture combined with the step change in temperature would not adversely affect the stability of the cells, or induce either a lag in growth or a heat-shock response. This mode of operation would dispense with the need for a temperature shift, which would remove the time involved for this, and the possibly

expensive and sophisticated temperature control system needed to achieve it.

The most productive way to operate in this way would be to have several different sets of seed fermenters (final scale 15m^3) working in staggered fashion with the 100m^3 vessel at a production temperature and turned round as quickly as possible ready to receive the next batch of inoculum.

This method of operation might not be as attractive for glucose limited fermentations with an extended glucose limited growth phase because the transfer of the cells into the production fermenter combined with the onset of the nutrient limitation might cause a lag phase and/or trauma to the cells which might cause plasmid instability or other detrimental effects. This would have to be investigated. In addition the advantage of not needing to implement a temperature shift would be lost.

The third of the time variables to affect productivity is the time taken to implement the temperature shift (figure 4.2). Section 3.1.5 showed that slow shifts would not affect batch production too adversely. However this parameter would affect the batch time, and therefore the number of batches achievable in any given time. Fig 4.2 shows that increasing the shift time from 0.2h to 1.25h would reduce the number of batches per year by 45.52 (glucose limited), 58.52 (kinetically limited pCQV2), and 66.59 (kinetically limited pMG169). Although this effect would not be as pronounced as from the other variables it

would nonetheless be significant.

Fig 4.4 shows how the length of the production phase under glucose limitation affects the number of batches per year. It was assumed that the production kinetics were maintained for the extended production phase. Although batch titre increased with length of production phase the reduction in number of batches per year means the effect on annual production was less pronounced.

Fig 4.5 shows the effect of the length of glucose limited growth phase on number of batches per year. As above the greater batch titre achieved with greater biomass concentration took longer to achieve, and so would not yield such a high annual titre as might be expected.

4.6.7 Prediction of Number of Batches Per Year For Different Conditions.

Two examples are given below to show how the equations described in sections 4.6.1 to 4.6.5 can be used to predict the number of batches achievable per year for a particular fermentation.

Example: kinetically limited pMG169 with 4.5h growth phase, 0.4h shift time, and 18h turnround time.

Table A3.4 shows 593.90 batches per year with this growth

phase.

This assumed a shift time of 0.5h. Table A3.5 shows the difference between this and a shift of 0.4h is 687.06-680.39 = 6.67 batches per year.

The value from table A3.4 also assumed a turnround time of 5h. Table A3.6 shows the effect of an 18h turnround time is to reduce the number of batches per year by 680.39-300.77 = 379.62.

Therefore the total number of batches per year for the example = 593.90 + 6.67 - 379.62 = 220.95 = 220 batches.

Example: glucose limited pCQV2 with 3.5h growth phase, 5h glucose limited growth phase, 0.75h shift time, 4h production phase, and 15h turnround time.

Table A3.7 shows a 5h glucose limited growth phase to yield 400.46 batches per year.

This assumed a growth phase of 3h. Table A3.1 shows a growth phase of 3.5h causes a reduction of 560.64-539.08 = 21.56 batches per year.

Table A3.7 also assumed a shift time of 0.5h. Table A3.2 shows the reduction in number of batches per year caused by a shift time of 0.75h to be 560.64-549.65 = 10.99

Table A3.7 also assumed a turnround time of 5h. The

reduction in the number of batches per year caused by a 15h turnround time is, from table A3.3, 560.64-311.47 = 249.17.

Therefore the total number of batches per year for the example = 400.46 - 21.56 - 10.99 - 249.17 = 118.74 = 118 batches.

In these two examples the tables in Appendix 3 were used for convenience. If the values of the various variables did not fall as exactly those displayed in the tables, then the equations in section 4.6 could be used to calculate the number of batches per year.

4.6.8 Annual Titres.

These values were derived by multiplying the batch titres by the number of batches per year.

Alternatively if a particular production requirement is known then the number of batches needed to achieve that target could be calculated.

General Conditions

Scale = 100m^3, Temperature = 40°C (42°C for pCQV2 glucose limited), OTR = 60 $mM.L^{-1}h^{-1}$, Growth phase = 3h, Shift time = 0.5h.

Special Condition

Turnround time = 5h.

TABLE 4.6

BATCH TITRE, NUMBER OF POSSIBLE BATCHES PER YEAR, AND ANNUAL TITRE FOR BOTH STRAINS UNDER DIFFERENT LIMITATIONS INCORPORATING A 5h TURNROUND TIME.

	Batch Titre ($*10^6$ nu)	Number of Batches/y	Annual Titre ($*10^9$ nu)
pCQV2			
kinetically limited	56,879	637.09	36,237
glucose limited	93,981	560.64	52,689
5h glucose limited	175,445	400.46	70,259
growth phase			
pMG169			
kinetically limited	600,701	680.39	408,711
glucose limited	1,451,090	560.64	813,539
5h glucose limited	2,706,700	400.46	1,083,925
growth phase			

Special Condition

Turnround time = 18h.

TABLE 4.7

BATCH TITRE, NUMBER OF POSSIBLE BATCHES PER YEAR, AND ANNUAL TITRE FOR BOTH STRAINS UNDER DIFFERENT LIMITATIONS INCORPORATING A 18h TURNROUND TIME.

	Batch Titre ($*10^6$ nu)	Number of Batches/y	Annual Titre ($*10^9$ nu)
pCQV2			
kinetically limited	56,879	292.00	16,609
glucose limited	93,981	274.82	25,828
5h glucose limited growth phase	175,445	229.77	40,312
pMG169			
kinetically limited	600,701	300.77	180,673
glucose limited	1,451,090	274.82	398,789
5h glucose limited growth phase	2,706,700	229.77	621,918

It is generally true that batch titres would be greater in the fermentations run under glucose limitation compared to the kinetically limited cases. It would be greater still in those fermentations that included a glucose limited growth phase to generate higher biomass prior to production.

It is also generally true that annual production would be greater in the fermentations run under glucose limitation compared to the kinetically limited cases. It is true it would also be greater in those fermentations that included a glucose limited growth phase to generate higher biomass prior to production.

However the differences between the annual titres for the different types of fermentation were not as great as the differences between the batch titres. For example in the case of strain pMG169 the glucose limited batch titre (without glucose limited growth phase) was approximately 2.42 times the kinetically limited one. The annual titre for the former, though, (given a turnround time of 5h) was only 1.99 times the latter. The improved performance of the kinetically limited fermentation on an annual basis was due entirely to the shorter batch time and hence greater number of batches per annum.

A comparison of the figures from the two tables above shows the importance of minimising the amount of unproductive time, for fermenter productivity. In the case of both the kinetically limited and glucose limited fermentations for both strains the annual production was greater than halved

by the increase in turnround time from 5 to 18h.(The increase in unproductive fermentation time could equally well also include longer growth and shift times). Significant reductions in annual titres were also incurred in the case of the fermentations incorporating the glucose limited growth phase.

For the same growth phase length, shift time, and turnround time the different types of fermentation have different numbers of batches achievable per annum. The processes with more batches per year would incur higher costs due to extra steam for sterilisation, number of inoculum preparations, and other running costs. This makes the fermentations incorporating a glucose limited growth phase even more attractive economically.

When figure 3.39 is compared with figure 3.17 the values of $Y_{(x/s)}$ under glucose limitation were similar to those under kinetic limitation. This suggests that glucose was used as efficiently under both limitations.

When these things are taken together it suggests that the best way to operate both these strains would be with a glucose limited growth phase incorporated prior to production, and with a feed-rate that would sustain an OUR close to the maximum OTR set for the fermenter. However this design assumed certain things, the validity of which are discussed in section 5.8.

It was always the case that strain pMG169 yielded better

production titres than strain pCQV2 and would always be the recommended expression system, except possibly in some circumstances mentioned in section 4.7 below.

4.7 DIFFERENT TYPES OF PRODUCT.

This section deals with some of the implications of expressing different types of product from the model one described in this work.

The production kinetics (in both intracellular and volumetric terms) must be investigated for each individual product. However some general principles can be drawn from the results obtained in these studies. The product expressed in this work was a soluble thermo-stable homologous protein. The other types of potential products can be listed in the following way:

a) soluble heterologous

b) insoluble heterologous (inclusion body)

c) thermo-unstable.

It is quite possible that a product may fall into both categories a) and c).

Each of these types of product could influence the way in which the fermentation was run.

Soluble Heterologous Products.

It has been shown that in the cases of some soluble heterologous products their expression can trigger proteolysis and/or the heat shock response (Goff SA & Goldberg AL (1985)). Their production can also cause plasmid instability (Caulcott CA *et al* (1985)). If either of these effects did occur the fermentation may have to be redesigned for higher biomass concentration at the end of the growth phase followed by a shorter production phase. In that case it might be that kinetic limitation performs better than some of the glucose limitation strategies.

In addition expression of some such products has a detrimental effect on growth, due to toxicity, for example (Botterman JH *et al* (1985)). If this was the case then with strain pCQV2 the volumetric production profile would appear much more similar in shape to that for strain pMG169, peaking and then either remaining constant or falling, rather than exponentially increasing as in these studies. Also, significant product expression occurred at the growth temperatures investigated in the case of strain pMG169 (section 3.1.1). This might cause in some cases greatly reduced productivity, and other types of expression system may have to be considered.

Insoluble Heterologous Products.

The advantage of products being expressed in this form is that they are often protected from proteolysis in the cell

and can be easier to isolate than soluble products (personal communication, SAM Gardiner, Celltech Ltd).

The length of the production phase often depends on plasmid stability and would have to be adjusted to optimise production.

Temperature Sensitive Products.

The action necessary to optimise fermenter productivity would depend on how thermo-stable the product was.

Obviously the production temperatures may have to be reduced from the optimums established in this work. Alternatively other types of expression systems may give better product yields. These studies have dealt with rises in temperature to trigger production, but new temperature sensitve expression systems could perhaps be developed in which product expression was initiated by a reduction in temperature.

A thermo-unstable product might be engineered to be produced as an inclusion body. It would then be in a denatured state, and perhaps immune to further denaturation and proteolysis. The expediency of this approach will depend on process economics and the development of improved large scale processes for inclusion body processing.

Clearly for any potential product the production kinetics would have to be investigated and the optimum temperatures,

length of production phase, and fermentation strategy would be selected.

5 DISCUSSION

Throughout this chapter "strain pCQV2" and "strain pMG169" are used as abbreviations for "strain *E. coli* RV308(pCQV2)" and "strain *E. coli* RV308(pMG169)", respectively.

In this chapter the results and some of their implications were discussed. This was then followed by a discussion of the data (taken from chapter 3) used in the design calculations, and the actual results from the design.

5.1 SPECIFIC GROWTH RATE AND PRODUCT EXPRESSION AT DIFFERENT GROWTH TEMPERATURE.

A comparison of figures 3.2 and 3.4 shows that as the growth temperature was raised the mean specific growth rate increased for both strains. However the rate of rise was lower in the case of strain pMG169 (figure 3.4). Figures 3.2 and 3.4 also show the level of CAT expression at these temperatures, and strain pMG169 consistently produced more. (At 30°C it produced three times as much as strain pCQV2 did at 34.5°C). At 33°C strain pMG169 expressed as much CAT as strain pCQV2 at 38°C (figure 3.9). This higher level of product expression may account for the slower rate of rise of mean specific growth rate with increasing growth temperature for pMG169.

pCQV2 is a constant 20 copies/cell (approximately) plasmid and the temperature switch directly controls product expression (Queen C (1983)). However plasmid pMG169 is present at around 4 copies/cell at 30°C. The temperature switch directly controls plasmid copy number amplification, product expression being under the control of the *trp* promoter (Yarranton GT *et al* (1984)). Obviously plasmid pMG169, present at 4 copies/cell at 30°C, did not exert as tight repression of product expression as plasmid pCQV2, present at 20 copies/cell. This result clearly suggests that the *trp* promoter system is a weaker represser of product expression than the λP_R system, under conditions of repression. If, as might be the case for some products, their expression were to have a detrimental effect on growth and/or plasmid stability, and so ultimately fermenter productivity, then lower growth temperatures and/or constructs of the pCQV2 type with direct temperature control of production would have to be considered.

However, under such circumstances the best possible solution might be a dual-origin plasmid of the pMG169 type with product expression under the control of either another copy of the λP_R or the λP_L promoter, rather than the *trp* system. Therefore both plasmid copy number regulation and product expression would be under temperature control. This would combine the strengths of both vectors investigated here, although it is not certain that this strategy would work well.

5.2 SPECIFIC GROWTH RATE AT DIFFERENT PRODUCTION TEMPERATURE.

Pirt SJ (1975) showed that in the range of temperatures 38-42°C the specific growth rate for *E coli* in rich medium was approximately constant. This was the case for strain pCQV2, in these studies. However he also showed that the specific growth rate was maximal in the 38-42°C range. This was not the case for strain pCQV2 where the higher growth temperatures (33-34.5°C) yielded greater values than the production temperatures (cf figures 3.2 and 3.6). Clearly expression of CAT caused a reduction in specific growth rate such that its usual rise over this temperature range was not seen.

The values of specific growth rate were lower in the results reported here, compared with those found in Pirt SJ (1975). This may be partly due to the different growth media used, but it is also consistent with the work of Imanaka T and Aiba S (1981) who found that plasmid-bearing cells grew more slowly than host cells. However, their result contradicted previous work which found that there was no difference in specific growth rate of *E. coli* cells containing the small, multicopy plasmid, ColE1, and those not carrying it, in batch culture (Adams J *et al* (1978)).

The amplification of plasmid pMG169 copy number resulted in a curtailment of growth. The degree of this curtailment depended on the temperature, ranging from an apparent

reduction in specific growth rate at 38°C to a cessation of growth at 40-42°C (see figure 3.7). This effect is common with amplifiable copy number plasmids (Wright E *et al* (1986), Betenbaugh MJ *et al* (1987)).

Larsen JEL *et al* (1984) found that plasmids that were stable during copy number amplification exhibited the curtailment in growth seen in these results. However they also found that in experiments conducted with unstable plasmids growth was also curtailed in the same way, but then it picked up again as plasmid-free cells grew. The degree of instability could be determined by the rate at which growth picked up again at the higher temperatures. The results obtained in figure 3.7 suggest the plasmid pMG169 was very stable at 40 to 42°C, showing no continuation of growth after the initial cessation. The reduction in specific growth rate seen at 38°C was not consistent with plasmid instability because the growth rate of plasmid-free cells would have been much greater than the value recorded here. Also the fact that growth did not stop at all in these experiments suggests the plasmid would have had to have been very unstable, which is inconsistent with other results.

5.3 PRODUCTION KINETICS.

5.3.1 Intracellular Production.

Queen C (1983) claimed that, using pCQV2, a product could be expressed to a level of 5-10% total intracellular cell protein. Clearly the levels achieved in this work were lower than that (figures 3.8 and 3.9). A possible explanation for this might be that more CAT was actually being produced but that proteolytic activity reduced the measured concentration from both the gels and the activity assay. If this were so then the proteolytic activity was such as to maintain an equilibrium value between CAT production and degradation at the peak values shown for each temperature.

Wright E *et al* (1986) achieved CAT concentrations of 10-13% at 42°C, compared to 7.5% in this work. However that was achieved with complex media and grown in shake-flasks. These studies were performed with minimal media and in a 14L fermenter. This may have affected production. Alternatively it is possible that host strain RV308 might not contain sufficient quantities of the tRNA species required for optimum CAT production. This codon usage problem might explain why for both pCQV2 and pMG169 final product concentration was lower than claimed in the literature. Other explanations might be low rates of transcription and translation, mRNA and product turnover

(Ernst JF (1988)), or copy number control systems may be different in the reported hosts, causing copy number differences. Both strains produced approximately the same proportion less CAT in these experiments compared to the results reported in the literature for these plasmids in other host strains. This suggests that host strain *E. coli* RV308 is a poor expresser of CAT, and possibly of other recombinant proteins as well.

These results clearly suggest that the amplifiable copy number plasmid, pMG169, caused greater accumulation of CAT to occur than the constant copy number plasmid, pCQV2. However it is also clear that the host strain was not the optimum for production for either plasmid. Therefore it is not certain that plasmid pMG169 would still yield the greatest amount of product when both are expressed in their optimum host strains. But the results from these studies do suggest that a low copy number plasmid present during growth, amplified during production, is a better production system than a constant 20 copies/cell plasmid system.

Wright E *et al* (1986) found that the combination of plasmid amplification and CAT expression caused the loss in cell viability observed during the production phase, rather than either of these processes alone. The apparent loss of viability followed by a peak in intracellular CAT accumulation observed in this work occurred more quickly at 40 and 42°C, compared with 38°C. This suggests that the kinetics of plasmid replication and/or CAT production were faster at the higher temperatures. This would explain why

the loss of cell viability also happened earlier at the higher temperatures. It might also explain why an initial phase at 42°C to denature the represser molecules followed by an extended phase at 37°C produced approximately twice the intracellular CAT compared with that achieved by maintaining the higher temperature throughout (Wright E *et al* (1986)). At 37°C the kinetics of plasmid amplification and CAT expression would be slower than at the higher temperatures, which might allow more CAT to be produced prior to the loss of cell viability. If this is a correct explanation then a more slowly replicating plasmid at production temperatures might yield still higher titres.

DiPasquantonio VM *et al* (1987) performed some shake-flask experiments using an amplifiable copy number recombinant plasmid in which they held the cultures at 42°C for differring lengths of time before continuing the production phase at 37°C for the duration. They found the maximum concentration of product was formed at 42°C but that it was maintained for only a short time before it rapidly fell. THe best time at 42°C before the reduction to 37°C was 45 minutes. This did not give as high a product concentration as when the culture was at 42°C for the duration, but it was maintained constant. 15 and 30 minutes at 42°C did not yield as good production. This work was not exhaustive in its range of times at the higher temperature, and there might be a still better time at 42°C before a reduction to 37°C. In addition, if a prediction of the time at which the peak at 42°C occurred (as in this thesis), and the culture could be inactivated quickly enough to avoid product loss, then these

would be the best conditions for optimised productivity. However the quoted study was performed in shake-flasks, using different host and plasmid systems, and at low biomass concentrations, all of which may cause different results from the work presented in this thesis.

The fall in intracellular CAT concentration subsequent to growth curtailment in strain pMG169 suggests some proteolytic degradation of the product. Wright E *et al* (1986) found that CAT accumulation began within the first hour and continued for 4-6h whereupon it stopped. This was clearly not the case in these studies. It is known that one proteolytic mechanism involves the heat-shock response (Goff SA and Goldberg AL (1985)) in which either a heat-shock and/or the production of normal or abnormal proteins in large quantities can stimulate expression of the ATP-dependent lon protease. It is possible that a combination of the higher temperature and the comparitively large quantity of CAT expressed stimulated this proteolytic response. Alternatively other proteolytic mechanisms may also have been responsible.

The difference in the production kinetics reported in this work compared with that in the literature suggests that for a new product several different host strains and plasmid systems should be investigated to see which gives the best production figures. In addition the length of production phase for each different host and plasmid system is likely to be different, and this might affect the process economics and therefore choice of recombinant strain. As an

example the strain used in this work produced CAT to a concentration of 7.5% cell protein in approximately 2h, compared with 10-13% in 6h in a different host strain (Wright E *et al* (1986)). In certain circumstances it might be envisaged that annual production could actually be higher (and more economical) in the former strain than the latter.

The onset of glucose exhaustion caused CAT to be turned over (figure 3.8), which means that the cells would have to be deactivated quickly at the point of harvesting to prevent large quantities of product from being lost (Fish NM and Lilly MD (1984)).

For strain pMG169 43°C was a much poorer production temperature. This may well have been caused by an increased heat-shock response at temperatures above 42°C (Goff SA *et al* (1984)). The kinetics of production at 42°C were such that the peak intracellular CAT concentration occurred sooner than at 40°C. Figure 3.9 showed the peak values to be approximately the same. This means that 42°C would be chosen as the best production temperature, except that figure 3.9 also shows 43°C caused much poorer product expression. Temperature gradients are likely to build up in production scale fermenters, so a production temperature must be chosen which would prevent the temperature rising above 42°C. Therefore 41°C is recommended as the optimum production temperature, based on the intracellular production kinetics.

5.3.2 Volumetric Production.

pCQV2

Figures 3.12 and 3.15 show that 40 and 42°C yield very similar production kinetics except that intracellular production was slightly higher at 40°C. Figure 3.6 shows these two temperatures yield the same specific growth rate, and figure 3.17 the same $Y_{(x/s)}$. Hence the higher intracellular production at 40°C (see figure 3.9) means that this is the optimum temperature for overall production. However the small difference between the two temperatures means that at scales where precise temperature control would be difficult to achieve 41°C might be chosen so that any fluctuations would not adversely affect the production kinetics. This argument assumes that there would be no adverse effects on production of any cyclical exposure to temperature gradients. This assumption should be tested experimentally.

pMG169

A similar outcome follows for this strain because 40 and 42°C both yield almost identical intracellular peak CAT concentration (as far as it is possible to deduce) in almost the same time. Once again 41°C would be suggested as the best temperature for production at large scales, for the same reasons as above.

A production temperature of 42°C for 10 minutes followed by a reduction to 37°C for the duration was not found to

promote greater production in strain pCQV2. However Wright E *et al* (1986) found that product accumulated in approximately twice the concentration (20% cell protein) if the λcI_{857} repressor was denatured at 42°C before the culture was incubated at 37°C. This was not attempted in these studies, and might optimise production in pMG169 in this host. Detailed investigation of the optimum length of time at 42°C is suggested for future work, but may yield a result that is not practically achievable on the large scale and/or in an economic manner.

5.3.3 Specific Production.

An estimation of the specific production rate was used for calculating production titres at different biomass concentration profiles from those actually seen experimentally. This can be done because dividing the volumetric production rate by the biomass concentration present at that time effectively removes the biomass concentration dependency from the production rate. Thus it can be used for other biomass profiles, rather than just the ones in these experimental fermentations.

5.4 OVERALL YIELD COEFFICIENT.

Although figure 3.17 shows some scatter at the growth temperatures it seems that the $Y_{(x/s)}$ was greater at these temperatures than at the higher ones. This is an important result for strain pCQV2 (which gave most of the data) because it suggests that glucose (and possibly nutrients in general) is better utilised at the growth temperatures. Therefore it would be more economical to grow the cells at lower temperatures followed by a short but optimum production phase. This would be true even of products whose presence did not have detrimental effects on cell growth.

The $Y_{(x/s)}$ may have been lower at the higher temperatures because nutrients would have been used for production as well as growth, but also because of generally increased metabolic activity at these higher temperatures. Goff SA and Goldberg AL (1987) found that protein degradation initiated by a heat-shock required metabolic energy, and the ATP-dependence of protease La appeared to account in large part for this requirement. If heat-shock proteolysis also occurred in these studies then this energy requirement might have contributed to the reduced $Y_{(x/s)}$ seen at the production temperatures.

5.5 EFFECT OF DIFFERENT SHIFT TIME ON PRODUCTION.

The result that a slow shift of the order of 0.5h did not adversely affect the peak intracellular CAT concentration was important because had a fast shift been necessary it would have led to significantly increased process costs. This is because an expensive heating system would have had to have been incorporated into the fermenter with a sophisticated controller to prevent overshoot during the shift. This result gives flexibility in implementing the temperature shift at different scales, and does not limit the scale that these systems could be used at by placing impractical constraints on the time over which the shift must be implemented.

5.6 BATCH OXYGEN REQUIREMENTS.

The specific OUR data at different temperatures was important because it enabled the final achievable biomass concentration to be calculated prior to oxygen limitation at any OTR in a fermenter. For both strains kinetically limited fermentations should be designed to reach that biomass concentration at the point of glucose exhaustion, thereby preventing both oxygen limitation and a waste of glucose and other nutrients.

5.7 BATCH FERMENTATION UNDER OXYGEN LIMITATION.

This mode of operation was clearly an inefficient use of resources. Considerable quantities of nutrients were taken up by the cells yielding a poor return of biomass and product (figures 3.22 and 3.23). Most of these nutrients seem to have been used for maintenance under these oxygen starved conditions. A much larger than usual quantity of alkali was added to maintain pH 7.0 suggesting greater than usual production of acidic metabolic by-products, like pyruvate and lactic acid.

When optimising a process it is essential that as far as possible the supply of nutrients is used to produce the greatest yield of product. It seems very unlikely that oxygen limitation would ever perform better than kinetic limitation by achieving higher biomass and therefore eventually a better volumetric titre. Even if such a situation did occur it is questionable whether it could be done economically, given the greater nutrient requirements and length of time that would be necessary.

5.8 BATCH FERMENTATION UNDER GLUCOSE LIMITATION.

According to Pirt's concept of maintenance (Pirt SJ (1975)) nutrients taken up by the cells are partly used to maintain the metabolic functions of the cell, the remainder used for

growth and production (in this case). As the culture grew, more of the available nutrients would be needed for maintenance, leaving less available for growth and production. Then the growth and production rates would start to slow. Therefore to maintain these rates nutrients would have to be supplied at an increasing rate. However this would also increase the OUR of the culture, and if the fermenter was operating at maximum OTR then oxygen limitation would occur.

Another effect comes from the fact that oxygen is also required for maintenance, and no matter what the glucose feed-rate growth would slow for a given OTR as the maintenance oxygen requirements increased. Hence the only way the growth rate could be maintained would be to increase the OTR with the increasing maintenance oxygen requirements. This could be achieved using some of the suggestions in section 4.4. The ability to supply this increasing oxygen requirement would probably determine the final biomass (and product) concentration achieved.

This suggests that some of the design extrapolations to very high biomass concentrations using long glucose-limited growth phases are very unlikely to be achievable on the large scale. However growth under glucose limitation for of the order of 5h followed by a 4h production phase might be achievable under the conditions used in experimentation here. This would have to be more fully investigated.

The fed-batch strategy used in this study was a very simple

one, and served to illustrate the effects of removing oxygen limitation and restricting the growth rate of the cultures. This may not be the optimum fed-batch strategy, since finely tuned systems can be developed to maximise batch productivity. There are feed-forward and feedback systems with constant or exponentially increasing feed-rates (Minihane BJ and Brown DE (1986)). There are also strategies that yield high biomass concentrations using other nutrient feeds, growth temperatures, and pure oxygen (Zabriskie DW and Arcuri EJ (1986)). Therefore future work should determine a fed-batch strategy to optimise batch productivity for constant and amplifiable copy number temperature sensitive constructs.

For strain pMG169 a glucose limited production phase caused growth and product expression to continue well beyond the time at which both stopped under kinetic limitation. If the cause of the curtailment of growth and production seen under kinetic limitation resulted from the rate of plasmid copy number amplification, then the glucose limitation might have caused the kinetics of amplification to be slowed. This would then have allowed both growth and production to continue for longer, allowing greater overall production to result.

Plasmid stability during an extended glucose limited growth phase would also be important in determining the optimum achievable biomass concentration during this period. Both of the plasmids dealt with in this work were very stable under kinetically limited growth conditions (see Appendix

2). At the higher glucose feed-rates onset of limitation seemed to cause little trauma to the cells, shown by the continued high $Y_{(x/s)}$ (figure 3.39) and only a small rise in pH. This suggests that the plasmids would be stable during a glucose limited growth phase, particularly as no signs of plasmid instability are obvious from these results, which were performed at production temperatures, which might be expected to put greater stress on plasmid-bearing cells.

However Caulcott CA *et al* (1985) found that translation of the recombinant gene was the cause of the instability of plasmid pCT70. The construction of plasmid pMG168 (a sister of pMG169) expressing calf prochymosin resulted in stability at low copy number. However the significant levels of product formed in strain pMG169 at the growth temperatures investigated might cause some plasmid instability when producing certain products. This might in turn limit the biomass concentration that could be achieved.

It can be seen from figures 3.26, 3.28 and 3.39 that at the feed-rate of 10 $g.m^{-3}min^{-1}$ very little growth occurred. This suggests that this feed-rate was very close to the maintenance requirement at that biomass concentration.

A comparison of figures 3.17 and 3.39 shows that at feed-rates above 20 $g.m^{-3}min^{-1}$ glucose limitation did not detrimentally affect the $Y_{(x/s)}$. In fact the $Y_{(x/s)}$ was higher at production temperatures under glucose limitation.

This might be explained by the concept of catabolite repression. If an organism is utilising a rapidly metabolisable carbon energy source, such as glucose, the resultant decrease in intracellular cyclic AMP concentration causes enzyme biosynthesis to cease (catabolite repression). Glucose-feeding, by reducing the rate of carbon metabolism and growth rate, derepresses the formation of desired products (Minihane BJ and Brown DE (1986)), and this may result in enhanced growth yield, $Y_{(x/s)}$.

The results shown in figure 3.34 and 3.35 suggest for both strains that the rate of volumetric production was beginning to slow towards the end of the fermentations. If this were the case the extrapolation for longer glucose limited production phase may well not hold for long beyond 4h.

5.9 HEAT OF FERMENTATION.

It can be seen from table 3.7 that the measured value of Q_f at 38°C was low for the observed OUR, when compared to the results at 30 and 34°C. The specific growth rate was lower at 38°C than at 34°C and so the added metabolic activity shown by the increased OUR could not have yielded as much heat as growth did. Part of this added metabolic activity must have come from production, which did not occur in appreciable amounts at the lower temperatures, and might suggest that production was not a very exothermic process,

compared to other cellular activity.

A comparison of the actual and predicted Q_f yields varied results. The most accurate prediction came from the general correlation of Luong JHT and Volesky B (1980) and the worst from the *E. coli* correlation of Luong JHT and Volesky B (1980). The former gave slightly low values until 38°C, when it was higher, although still the lowest of the predictions.

The results used for the correlation of Cooney CL *et al* (1968) came from *E. coli* and *B. subtilis* grown at 37°C, and *A. niger* and *C. intermedia* grown at 30°C. The results used for the correlations of Luong JHT and Volesky B (1980) used the same organisms except that *B. subtilis* was substituted for *C. utilis* and *C. lipolytica*. The only specified temperature was 37°C for *E. coli*. It is unstated but probably safe to assume that the strains studied were wild-type, without the presence of recombinant plasmids, particularly given the dates of the published work. It is therefore clear from the measured results that the presence of even a low expressing recombinant plasmid caused considerable deviation from the predicted Q_f. Although further work needs to be done to measure the extent of the deviation for different plasmid systems, it should not be assumed that these widely used correlations hold for recombinant strains.

Figure 3.41 shows no increase in q_f from 38-42°C. Figure 3.6 shows there is also no increase in specific growth rate

over this range of temperatures. This means that between 38-42°C the overall heat of fermentation would be constant for a given biomass concentration. However, the cooling duty would be less at the higher temperatures because more heat would be lost to the surroundings and sparged air. In addition the specific OUR at 38°C was lower than at the higher temperatures, which means that a higher biomass concentration could be achieved prior to the onset of oxygen limitation at any scale. This higher biomass concentration would therefore generate a greater Q_f , and hence cooling duty.

Any given cooling duty is more difficult to achieve as scale is increased because the surface area to volume ratio of a fermenter falls with increasing volume. In addition poorer mixing and reduced liquid velocities cause a reduction in the heat transfer efficiency of large fermenters, and can allow temperature gradients to build up. It is particularly important for ts systems that this is avoided and good temperature control is maintained. This means that high agitator rates are recommended for minimising the gradients and reducing the resistance to more efficient heat transfer by scouring the film layer at the heat transfer surfaces. An added benefit of higher agitator rates is increased oxygen transfer rates. However it also increases both power and cooling costs since the added agitator power is largely dissipated as heat.

The specific heat of fermentation can be used in a general way to calculate the overall heat of fermentation for the

purpose of cooling duty calculations. The q_f is multiplied by the biomass concentration at any instant to give Q_f at that point. However the difference in the results for the two strains shown in figure 3.41 demonstrates that q_f is a vector- as well as host-dependent parameter.

A possible useful advantage of running fermenters under nutrient limitation is that the Q_f would remain approximately constant. Therefore if the Q_{ag} remained the same, the cooling duty would remain constant throughout the limited parts of the fermentation. Hence glucose feeding is a means of controlling the heat output of the cells. This might become very important under conditions of high OTRs or very large scales (210m^3), when heat transfer could become the limiting condition of operation. In such circumstances controlling the Q_f with glucose feeding may be necessary. Brown DE (1982) found that heat transfer should not become limiting for recombinant *E. coli* fermentations until scales of around 210m^3 (4.5m diameter, 14m height).

5.10 RESULTS USED FOR DESIGN.

In order that batch titres at different scales could be calculated a value of the specific growth rate during the whole of the production phase was needed. This was not a problem for strain pCQV2 because growth continued throughout production. However for strain pMG169 it changed

and eventually fell to zero at the end of the production phase. Therefore it was calculated as the rise in ln(Adj OD) divided by the time during the production phase.

Given that the $Y_{(x/s)}$ for strain pCQV2 was lower at production temperatures it was decided that to minimise nutrient requirements the production phase should be the minimum length of time to maximum intracellular CAT concentration, thereby also maximising volumetric production. In the case of strain pMG169 maximum intracellular (and therefore also volumetric) CAT concentration was achieved 0.5 to 1h after growth was curtailed. In the kinetically limited case the production phase was designed so the final biomass concentration was such that O_2-limitation would just be reached. At this point the glucose would be exhausted, and the fermenter should be harvested. Hence X_{pf} = max OTR/specific OUR.

The specific production rate figures used during the design were chosen as being values representative of the entire production phase. The trend for this parameter was an initially high value falling towards a constant, and an estimate was made of a representative figure for the chosen length of the production phase.

The volumetric production rate was used to scale up the runs done specifically to mimic conditions on the scale of $100m^3$. Both the glucose and O_2 limited runs were set up so that the maximum OTR was in the range 50-60 $mM.L^{-1}h^{-1}$, the sort of value achievable at $100m^3$ (personal commumnication,

M D Lilly, UCL). Hence volumetric production rate could be used to calculate the large-scale titres under these conditions.

5.11 DESIGN RESULTS.

The results from the design are general but quantitative in nature. They show the batch titres achievable at any given OTR (oxygen supply being the primary constraint on final titre) and at any scale for kinetic and glucose limitation. Fermentation under oxygen limitation was unproductive and only one limiting OTR was investigated. In addition to this the effects of certain key parameters (eg turnround time) on the possible number of batches per year enable estimates of annual titres to be made. This approach can be extended to other expression systems.

The experimental and design results clearly show that oxygen limitation must be avoided because of the poor production figures and large, inefficient uptake of nutrients.

A comparison of figures 3.12 and 3.34 (highest feed-rate) shows almost identical maximum titres of approximately 900,000 $nu.L^{-1}$ for strain pCQV2. However in the case of the kinetically limited run (figure 3.12) the final OUR was greater than 100 $mM.L^{-1}h^{-1}$, compared with around 50 $mM.L^{-1}h^{-1}$ for the glucose limited run. Thus at scales and fermentation conditions where the maximum OTR = 50 $mM.L^{-1}h^{-1}$

the kinetically limited run would have become oxygen limited and not yielded anything like as high a titre as the glucose limited case. Therefore although a comparison of figures 3.10 and 3.32 shows better specific production under kinetic limitation, the higher biomass concentration achieved under glucose limitation (at any limiting OTR) means that batch titres were greater under the latter conditions.

In the case of strain pMG169 a comparison of figures 3.11 and 3.33 shows that intracellular production was better under glucose limitation. Since higher biomass concentrations were also achieved under these conditions, glucose limitation yielded even better batch titres compared to kinetic limitation.

It is clear that the optimum fermentation strategy for both these strains is to run the production phase under glucose limitation with a feed-rate that allows an OUR which just avoids oxygen limitation at the chosen scale. The extrapolated results that included a glucose limited growth phase achieved still higher biomass concentrations and batch titres. Further experiments should be performed to determine the extent to which this actually occurs. The actual performance of the two strains under glucose limitation at higher OTR should also be investigated.

There are many factors that must be taken into account when deciding on which type of recombinant strain to use. Clearly the batch titres compared with those of other strains is very important. Strain pCQV2 showed very good repression of

product expression during growth, and strain pMG169 has shown the ability to express product at high levels (Wright E *et al* (1986) and CAT as greater than 20% total cell protein in some host strains (personal communication, Celltech Ltd)). However if the product was thermo-unstable then other expression systems might be better choices. The choice between different expression systems is further complicated by the fact that Rosen C-G (1983) pointed out that for a recombinant DNA product 45% of the equipment costs were associated with product recovery and only 14% with the fermentation. He also reported that the ratio of recovery to fermentation costs for an enzyme is 2.0 and is even higher (appprox 3.0) for pharmacologically pure L-asparaginase.

Furthermore Buckland B (1984) stated that changes in fermentation conditions can have a marked effect on even the final product recovery step. Fish NM and Lilly MD (1984) stated that choices of microorganism and fermentation conditions (eg culture medium, growth rate) will influence the choice of recovery operations and affect the yields achieved in the downstream processing. Hence it will not necessarily be true that a strain yielding the highest batch titre will do so in the most economical way, industrially. Since downstream costs are so important the process economics might determine that a product can be made more cheaply using a strain that does not produce the greatest amount of product.

In addition, this is further complicated by the fact that

the recovery steps can be different at different scales (Fish NM and Lilly MD (1984)). Therefore a strain that turned out to be most economical at one scale might not be so at another.

All of these factors must be considered in the specification of a suitable microbial system and scale for the expression of a desired product, industrially.

An alternative to a $100m^3$ fermenter would be to have a series of smaller ones, say five $20m^3$ vessels, run in a staggered fashion for the same production requirements. This offers several advantages:

- downstream processing equipment can be run continuously and would be smaller.

- improved step yield of harvesting. It can take a long time to harvest large volumes of broth, during which cell lysis and protein release can happen (Fish NM and Lilly MD (1984)). Cell lysis is also more common in defined media of the type used in this study (Gray PP *et al* (1972)), and increased protein turnover is caused by depletion of carbon or nitrogen source. Hence product titres can rapidly decline and it is essential that the broth be deactivated and quickly processed through the first few steps of downstream processing (Fish NM and Lilly MD (1984)). It would be easier to do this with broth from $20m^3$ rather than $100m^3$ fermenters.

- greater flexibility of equipment use. If production requirements fell then one or more fermenters could be used for other processes, and switched back again when demand

necessitated it. Also $20m^3$ fermenters are probably of more general use than $100m^3$ ones for other microbial processes, although this would to some extent depend on the company's business.

- higher OTRs more easily achievable.
- potentially lower cooling costs. Some large scale fermentation processes require expensive chilled cooling water to remove the necessary amount of heat. Since the surface area to volume ratio increases with decreasing fermenter volume then the need for chilled water might be avoided at smaller scales by the improved heat transfer properties of this greater surface area.

However this suggested plant layout has the disadvantage of loss of economies of scale. There would be increased capital costs associated with seed fermenters and support services as well as the added cost of five fermenters. The plant would also require an added degree of automation to run efficiently. There may also be increased labour costs associated with running such a plant, incurred in turnround of fermenters, inoculum preparations, plant operation, etc. Naveh D (1985) stated that capital costs increase at only a power of 2/3 of volume. Therefore a better return on investment might be achieved with one large fermenter compared with five smaller ones. However this might not be so if the product yield from the downstream processing was poorer at $100m^3$ scale compared with $20m^3$, for reasons already discussed.

The conclusion of this argument is that for any product the

economics of the total overall process must be considered before a particular microbial system and fermenter size can be specified.

The equations in section 4.6 can be combined with those in section 4.4 and used as the basis for a model to predict batch and annual titres given different fermentation conditions and fermenter set-ups. Given a required annual production figure it could also be used to predict the number of batches and the time necessary to produce such a figure, for each of the strains and different types of fermentation process. This type of approach could also be used for other types of expression systems, and if production kinetics were known a computer would be able to predict the optimum fermentation process to meet a stated need. It would require a tremendous amount of work but if cost considerations were also included then the initial stages of a fermentation expert system could be developed.

5.12 CONCLUSIONS.

1) The recommended growth temperature for both strains is 30°C, being a compromise between the conflicting interests of high specific growth rates and low product expression during the growth phase. However if a product is to be expressed whose presence has significant detrimental effects on the process then a lower growth temperature than this, particularly for strain pMG169, would be recommended.

2) The λP_R switch was a tighter represser of product expression than the *trp* switch (at 33°C 20 copies/cell pCQV2 expressed CAT as 0.044% cell protein compared to 0.996% from 4 copies/cell pMG169).

3) The recommended production temperature for both strains is 41°C to combine maximum production rates with a +/-1°C degree of flexibilty for the fermenter temperature control system.

4) The amplifiable copy number plasmid, pMG169, yielded higher production titres than the constant copy number plasmid, pCQV2, under all the fermentation conditions tested.

5) *E. coli* RV308 was a poorer expresser of CAT than other reported hosts using the same plasmid systems.

6) The production phases under oxygen limitation resulted in severely limited growth, low product expression and $Y_{(x/s)}$, and high alkali requirement for pH control.

7) The production phase under glucose limitation resulted in increasing linear growth rate with increasing glucose feed-rate, longer production phases, higher production titres at the higher feed-rates tested, and higher $Y_{(x/s)}$ than under kinetic limitation, for both strains.

8) Higher batch titres may be yielded by incorporating a glucose limited growth phase prior to a glucose limited

production phase.

9) In order to optimise productivity fermenters should operate in the production phase for as high a proportion of the time as possible ie minimise turnround time and other non-productive fermenter time.

10) The production kinetics described in this work might not be the same should other types of product be expressed in either of these strains.

11) For a known production need the total overall process economics (including downstream processing) should be considered before a particular microbial system and fermenter size and configuration is specified.

12) A temperature shift lasting 0.5h did not adversely affect production when compared to the much faster shifts performed in the laboratory.

13) The specific heat of fermentation increased linearly with increasing temperature until it became constant in the 38-42°C range.

14) Metabolic heat output was lower from strain pMG169 compared with strain pCQV2. It was vector-dependent.

15) Some widely used empirical correlations to predict the heat of fermentation do not necessarily hold for recombinant strains.

APPENDIX 1

ANALYSIS OF TECHNIQUES

OD_{670} (absorbance units) against dcw (gL^{-1}) data

These results were taken from two fermentations and regularly checked throughout the experimental programme.

FERMENTATION 1		FERMENTATION 2	
OD_{670} (units)	dcw (gL^{-1})	OD_{670} (units)	dcw (gL^{-1})
0.21	0.056	0.307	0.25
0.351	0.18	0.542	0.355
0.49	0.228	0.794	0.42
1.15	0.494	4.42	1.57
4.55	1.97	10.615	5.13
6.466	3.29	18.24	7.98
8.85	3.8		
11.96	5.35		
14.67	6.38		

Regression analysis of these results plotted as dry cell weight (dcw) vs OD_{670} gives the following results:

FERMENTATION 1 - Equation of line: $dcw = 0.4404 * OD_{670} + 0.033$ ($r = 0.9977$)

FERMENTATION 2 - Equation of line: $dcw = 0.4421 * OD_{670} + 0.045$ ($r = 0.9965$)

Fig A1.1 is a plot of these results.

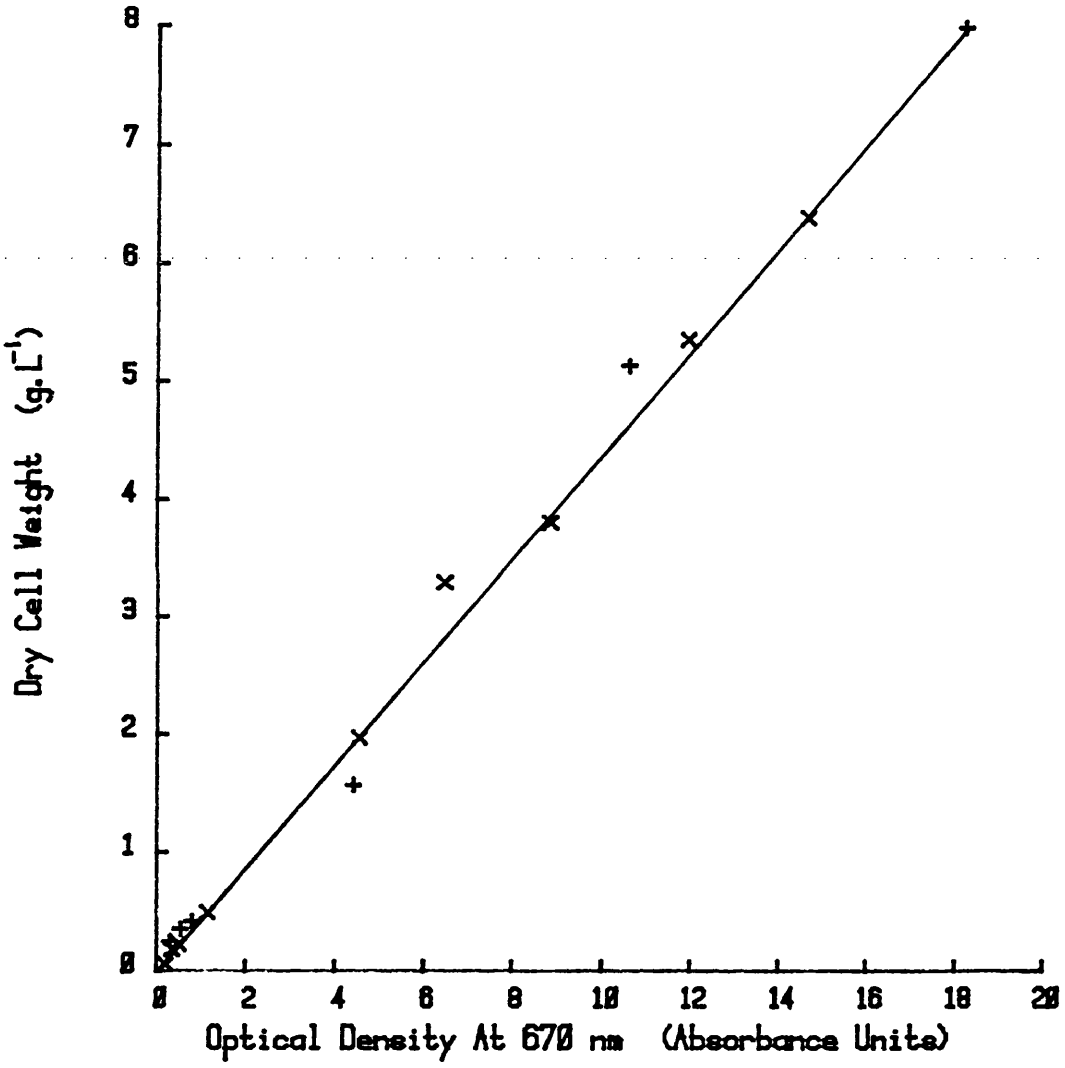


Figure A1.1: Plot of Dry Cell Weight Against Optical Density At 670 nm (× ,Fermentation 1; + ,Fermentation 2).

Therefore in order to obtain the dry cell weight of a sample the OD_{670} was multiplied by 0.44.

Fig A1.1 shows the OD_{670} values go up to approximately 18 units. However towards the end of some fermentations it frequently went higher than this figure. Therefore the relationship shown in the figure was extrapolated, and it was assumed that the dry cell weight was still 0.44 multiplied by OD_{670}. The maximum OD_{670} reading was approximately 30 units, an extrapolation of around 12 units beyond the maximum measured. However this relationship should still hold because meticulous dilutions were made to ensure the OD_{670} readings were taken in the range 0-0.4 units, the absorbance reading then being multiplied by the dilution factor to give the true OD_{670} value. In this way the absorbance reading was still accurate, even at high biomass concentrations, and the extrapolation should be valid.

Characterisation of cell disruption

14 OD20 pellets were prepared from the same sample taken from the end of a fermentation and resuspended as described in section 2.2.7. These were then sonicated for different lengths of time to define optimum disruption conditions. The results are shown in fig A1.2.

From these results 4 minutes was selected as the sonication time necessary for complete disruption and CAT release. This result was consistently reproducible.

The temperature of the disrupted samples was recorded and it was found that the temperature after 4 minutes was approximatey 26°C. This represented a rise of 22°C during sonication.

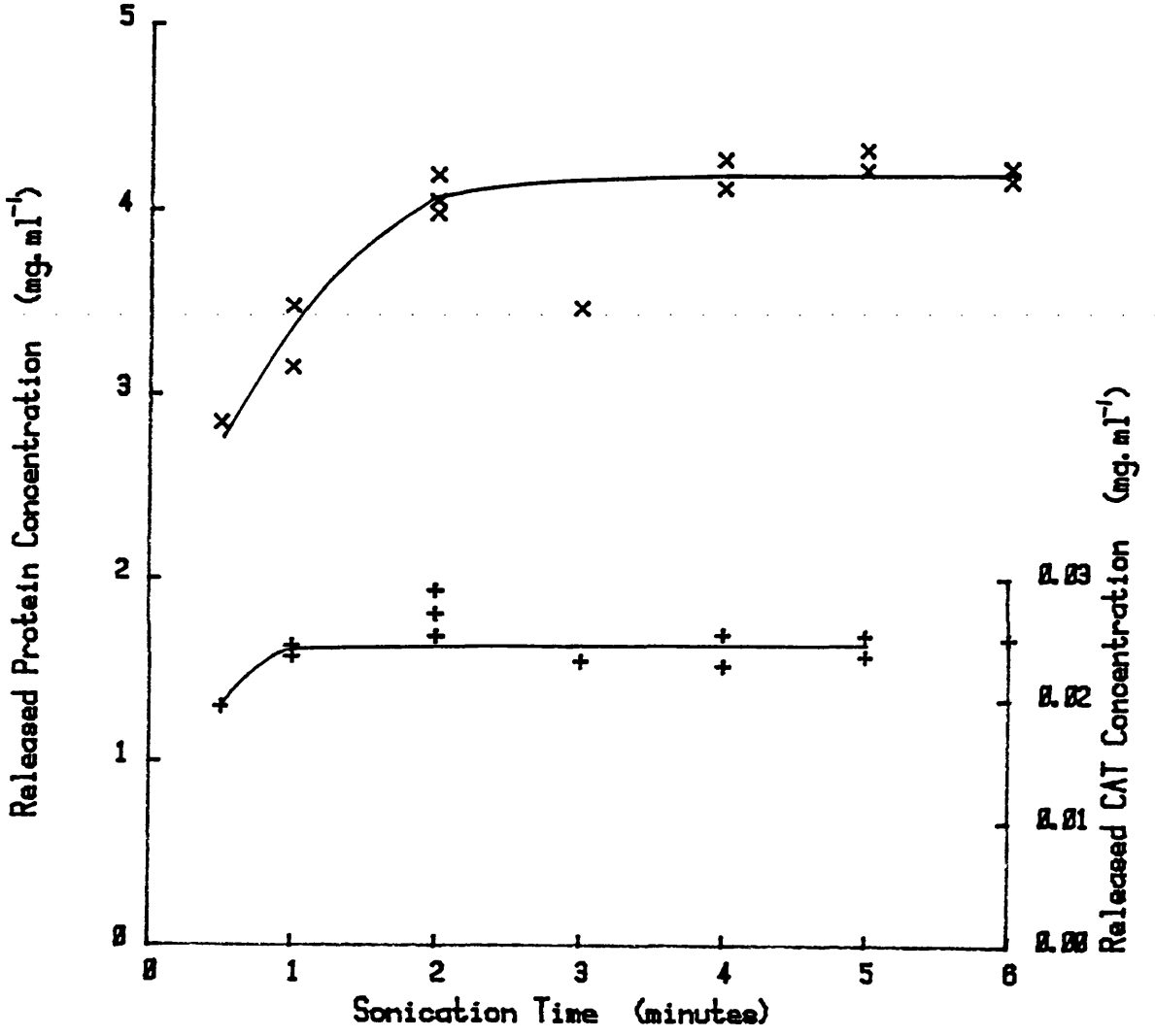


Figure A1.2: Plot OF Released CAT And Protein Concentration Against Sonication Time (+ ,CAT Concentration; × ,Protein Concentration).

CAT assay accuracy studies

The same disrupted sample was assayed 7 times consecutively to assess the reproducibility of results and accuracy of experimental technique.

The results were ($mg.ml^{-1}$): 0.0624, 0.0522, 0.0612, 0.0561, 0.0538, 0.0539, 0.0625.

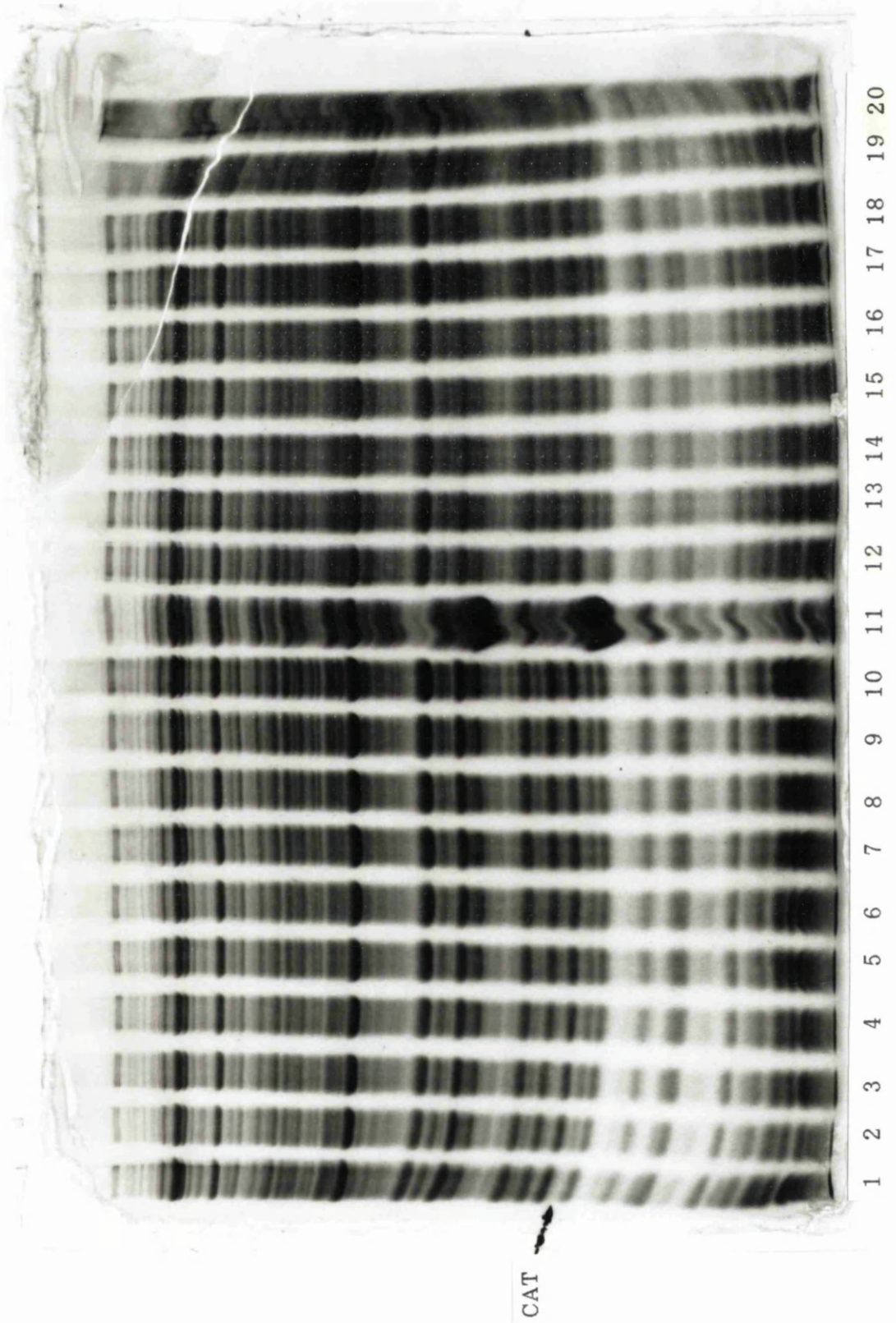
The mean result was 0.0574 $mg.ml^{-1}$ and sample standard deviation 0.00446.

The largest deviation from the mean value was:

(0.0574-0.0522)/0.0574 = 9.1%.

The values of CAT concentration expressed as % cell protein were compared with polyacrylamide gels of the fermentation samples and were seen to be convincingly similar. An example of a photograph of one of the dried gels is shown in figure A1.3.

Figure A1.3: Photograph of a Dried Polyacrylamide Gel.



Tracks 1-10 of figure A1.3 represent samples taken from a kinetically limited fermentation using strain *E. coli* RV308(pCQV2). Track 1 represents a sample taken prior to the temperature shift, tracks 2-10 representing samples taken after increasing lengths of time in the production phase.

Track 11 represents a sample, supplied by Celltech Ltd, taken from a fermentation using plasmid pMG169 in which it was known that CAT was present at a concentration of 10% total cell protein.

Previous gels run with molecular weight markers (as well as samples) had shown the labelled part of the gel to contain the CAT band.

Protein assay accuracy studies

The same disrupted sample was assayed 8 times consecutively to assess the reproducibility of results and accuracy of experimental technique.

The results were ($mg.ml^{-1}$): 4.86, 4.93, 4.86, 5.03, 5.14, 5.21, 5.04, 5.04.

The mean result was 5.01 $mg.ml^{-1}$ and sample standard deviation 0.1256.

The largest deviation from the mean value was:

(5.21-5.01)/5.01 = 4.0%.

These results were within acceptable limits of experimental error.

APPENDIX 2

PLASMID STABILITY AND CAT PRESENCE IN THE BROTH

Plasmid stability.

This was checked for pCQV2 cultures grown in shake-flask and fermenter.

Fermentation samples were diluted, plated for single colonies on nutrient agar plates, and incubated at 30°C. 100 colonies were then plated on selective and nutrient plates and incubated at 30°C. Plasmid stability was measured as the number of colonies that grew on selective plates as a proportion of those that grew on nutrient plates.

In shake-flasks samples were taken after 6h growth at 30°C and 3h growth at 42°C. In both cases the results were 100/100 = 100% stability.

In the fermenter the results at 42°C were 93/100 after 2h and 99/100 after 3.67h growth from the same fermentation. In addition, when the fermentation time was increased by the use of 5ml inoculum (ie many more generations in the fermenter) the production profiles did not change. This suggests that significant numbers of plasmid-free cells were not present.

These combined results suggest that plasmid pCQV2 was stable.

Plasmid pMG169 was known to be stable when supplied. Its stability was also shown by the curtailment of growth during the production phase, following plasmid copy number amplification. Larsen *et al* (1984) found that if this type

of plasmid was unstable then the biomass concentration began to increase exponentially as the plasmid-free cells grew. Since growth was curtailed throughout the production phases with no sign of exponential growth of plasmid-free cells, it can be assumed that this strain was stable during these studies.

CAT presence in the broth.

Samples taken for glucose assay from a pCQV2 fermentation were also assayed for CAT to see whether it appeared in the broth in significant amount. The production phase was operated at 42°C. The results were:

Time after shift (h)	CAT concentration in broth (uL^{-1})
1.17	0.0
1.58	95.5
2.00	141.0
2.50	191.0
3.00	210.5
3.50	248.5
4.00	259.5
5.17	416.5

These results are plotted in fig A2.1 and show an increasing CAT concentration in the broth as the fermentation progressed. However the values represented insignificant levels caused probably by lysis of old cells. CAT did not appear immediately after the shift was implemented, but 1.17-1.58h later.

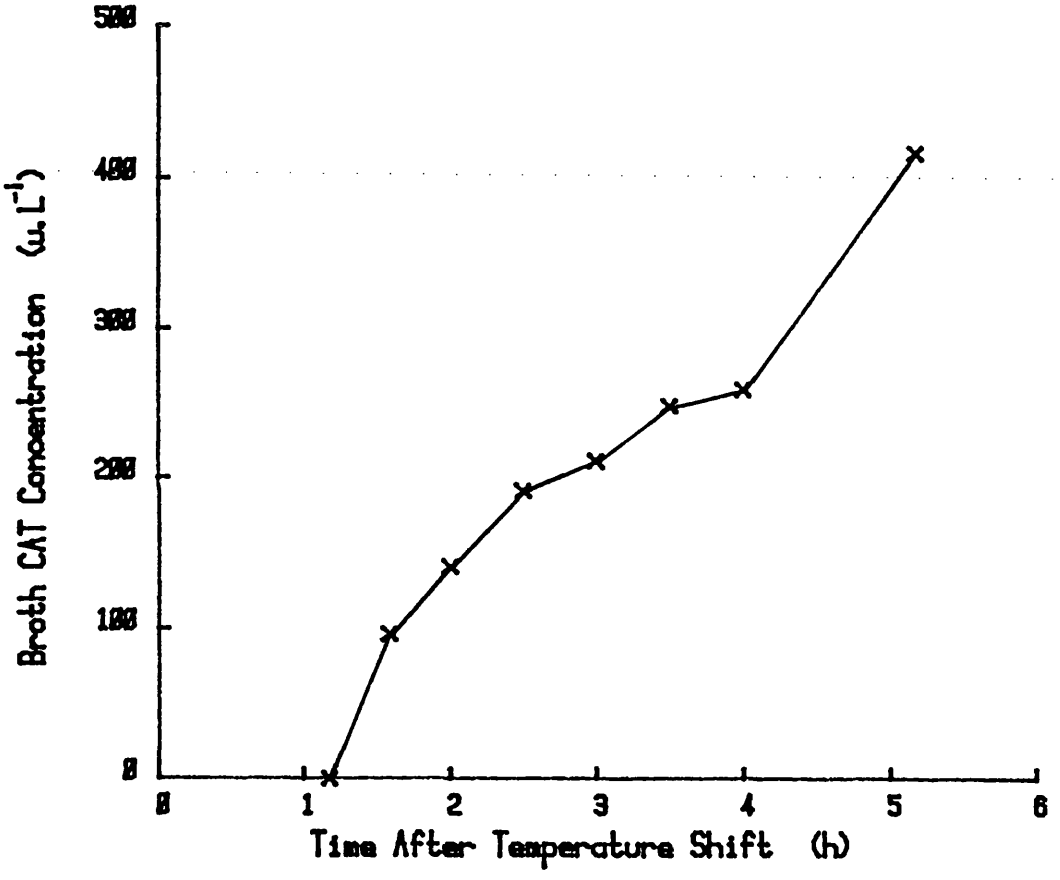


Figure A2.1: Plot of Broth CAT Concentration Against Time After Temperature Shift.

APPENDIX 3

TABLE A3.1

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pCQV2 WITH DIFFERENT LIMITATION AGAINST LENGTH OF GROWTH PHASE.

Growth Phase Length (h)	pCQV2					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y
1	47,399	778.67	6,101		78,425	
2		700.80				
2.5		667.43				
3		637.09		609.39		560.64
3.5		609.39		584.00		539.08
4		584.00		560.64		519.11
4.5		560.64		539.08		500.57
5		539.08		519.11		483.31
5.5		519.11		500.57		467.20
6		500.57		483.31		452.13
6.5		483.31		467.20		438.00
7		467.20		452.13		424.73
7.5		452.13		438.00		412.24
8		438.00		424.73		400.46
8.5		424.73		412.24		389.33

TABLE A3.2

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pCQV2 WITH DIFFERENT LIMITATION AGAINST LENGTH OF SHIFT TIME.

Shift Length (h)	pCQV2					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y
0.2	47,399	654.95	6,101	625.71	78,425	574.43
0.25		651.91		622.93		572.08
0.3		648.89		620.18		569.76
0.4		642.94		614.74		565.16
0.5		637.09		609.39		560.64
0.6		631.35		604.14		556.19
0.7		625.71		598.97		551.81
0.75		622.93		596.43		549.65
0.8		620.18		593.90		547.50
0.9		614.74		588.91		543.26
1.0		609.39		584.00		539.08
1.1		604.14		579.17		534.96
1.2		598.97		574.43		530.91
1.25		596.43		572.08		528.91

TABLE A3.3

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pCQV2 WITH DIFFERENT LIMITATION AGAINST LENGTH OF TURNROUND TIME.

Turn-round Time (h)	pCQV2					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y	Batch Titre (10^6 nu)	Batch/y
5	47,399	637.09	6,101	609.39	78,425	560.64
6		584.00		560.44		519.11
7		539.08		519.11		483.31
8		500.57		483.31		452.13
9		467.20		452.13		424.73
10		438.00		424.73		400.46
11		412.24		400.46		378.81
12		389.33		378.81		359.38
13		368.84		359.38		341.85
14		350.40		341.85		325.95
15		333.71		325.95		311.47
16		318.55		311.47		298.21
17		304.70		298.21		286.04
18		292.00		286.04		274.82

TABLE A3.4

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pMG169 WITH DIFFERENT LIMITATION AGAINST LENGTH OF GROWTH PHASE.

Growth Phase Length (h)	pMG169					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y
1	600.701	844.34	93.945		1,451.1	
2		753.55				
2.5		715.10				
3		680.39		651.91		560.44
3.5		648.89		622.93		539.08
4		620.18		596.43		519.10
4.5		593.90		572.08		500.57
5		569.76		549.65		483.31
5.5		547.50		528.91		467.20
6		526.92		509.67		452.13
6.5		507.83		491.79		438.00
7		490.07		475.12		424.73
7.5		473.51		459.54		412.24
8		458.04		444.95		400.46
8.5		443.54		431.26		389.33

TABLE A3.5

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pMG169 WITH DIFFERENT LIMITATION AGAINST LENGTH OF SHIFT TIME.

Shift Length (h)	pMG169					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y
0.2	600.701	700.80	93.945	670.62	1,451.1	574.43
0.25		697.31		667.43		572.08
0.3		693.86		664.27		569.76
0.4		687.06		658.03		565.16
0.5		680.39		651.91		560.64
0.6		673.85		645.90		556.19
0.7		667.43		640.00		551.81
0.75		664.27		637.09		549.65
0.8		661.13		634.21		547.50
0.9		654.95		625.52		543.26
1.0		648.89		622.93		539.08
1.1		642.94		617.44		534.96
1.2		637.09		612.05		530.91
1.25		634.21		609.39		528.91

TABLE A3.6

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pMG169 WITH DIFFERENT LIMITATION AGAINST LENGTH OF TURNROUND TIME.

Turn-round Time (h)	pMG169					
	Kinetic-Limitation		Oxygen-Limitation		Glucose-Limitation	
	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y	Batch Titre (10^9 nu)	Batch/y
5	600.701	680.39	93.945	651.91	1,451.1	560.64
6		620.18		596.43		519.11
7		569.76		549.65		483.31
8		526.92		509.67		452.13
9		490.07		475.12		424.73
10		458.04		444.95		400.46
11		429.94		418.39		378.81
12		405.09		394.82		359.38
13		382.95		373.76		341.85
14		363.11		354.84		325.95
15		345.22		337.73		311.47
16		329.01		322.21		298.21
17		314.26		308.04		286.04
18		300.77		295.07		274.82

TABLE A3.7

BIOMASS CONCENTRATIONS, BATCH TITRE, AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pCQV2 WITH DIFFERENT LIMITATION AGAINST LENGTH OF GLUCOSE LIMITED GROWTH PHASE.

Glucose-Limited Growth Phase Length (h)	pCQV2			
	[Biomass] at end of growth (gL^{-1})	[Biomass] at end of production (gL^{-1})	Batch Titre (10^6 nu)	Batch/y
5	13.5590	19.7551	146,582.0	400.46
8	18.2061	24.4021	187,475.9	341.85
12	24.4021	30.5892	242,001.2	286.04
15	29.0491	35.2452	282,895.1	254.84
18	33.6962	39.8922	323,789.1	229.77
20	36.7942	42.9903	351,051.7	215.63
22	39.8922	46.0883	378,314.3	203.13
24	42.9903	49.1863	405,577.0	192.00
28	49.1863	55.3824	460,102.2	173.04
36	61.5784	67.7745	569,152.7	144.49

TABLE A3.8

BIOMASS CONCENTRATIONS, BATCH TITRE, AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR STRAIN pMG169 WITH DIFFERENT LIMITATION AGAINST LENGTH OF GLUCOSE LIMITED GROWTH PHASE.

Glucose-Limited Growth Phase Length (h)	pMG169			
	[Biomass] at end of growth (gL^{-1})	[Biomass] at end of production (gL^{-1})	Batch Titre (10^9 nu)	Batch/y
5	16.2092	23.5952	2,706.7	400.46
8	21.7487	29.1347	3,460.1	341.85
12	29.1347	36.5207	4,464.6	286.04
15	34.6742	42.0602	5,218.0	254.84
18	40.2137	47.5997	5,971.3	229.77
20	43.9067	51.2927	6,473.6	215.63
22	47.5997	54.9857	6,975.8	203.13
24	51.2927	58.6787	7,478.1	192.00
28	58.6787	66.0647	8,482.6	173.04
36	73.4507	80.8367	10,491.6	144.49

TABLE A3.9

BATCH TITRE AND NUMBER OF ACHIEVABLE BATCHES PER YEAR FOR BOTH STRAINS AGAINST LENGTH OF GLUCOSE LIMITED PRODUCTION PHASE.

Production Phase Length (h)	pCQV2		pMG169	
	Batch Titre (10^9 nu)	Batch/ y	Batch Titre (10^9 nu)	Batch/ y
3	74.576	609.39	994.1	609.39
3.5	87.005	584.00	1,214.8	584.00
4	99.435	560.64	1,451.1	560.64
4.5	111.864	539.08	1,703.1	539.08
5	124.293	519.11	1,970.8	519.11
5.5	136.723	500.57	2,254.2	500.57
6	149.152	483.31	2,553.3	483.31
6.5	161.581	467.20	2,868.1	467.20
7	174.010	452.13	3,198.6	452.13
7.5	186.440	438.00	3,544.8	438.00
8	198.869	424.73	3,906.7	424.73

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