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Production of a lipase from a Pseudomonad species

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A B S T R A C T

A lipase-producing micro-organism was found to be a strain of *Pseudomonas aeruginosa*. When it was grown batchwise in a stirred 2 dm³ fermenter in a simple defined minimal salts medium containing yeast extract and glucose, it produced lipase at a level of 0.173 LU/cm³ without the need for a lipid substrate. Batch cultures of cells were fed glucose at constant rates. An optimum rate was found to be 0.50 g/dm³/h and this produced a maximum lipase activity of 0.80 LU/cm³.

Medium composition was changed until it was possible to produce 25 g/dm³ dry weight of cells. Evidence of linear growth due to nutrient limitations was obtained. A micro-computer controlled protocol was used successfully to feed glucose solutions at exponentially increasing rates. Problems were encountered with the production of lipase from the interaction with pyocyanin production and from possible trace nutrient limitations.

A C K N O W L E D G E M E N T S

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C O N T E N T S

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ABSTRACT

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N O M E N C L A T U R E

Roman		<u>Units</u>
A	Feed constant	dm^3/h
B	Feed Exponent	h^{-1}
F	Feed Rate	dm^3/h
S	Substrate Concentration	g/dm^3
t	Time	
V	Fermenter Broth Volume	dm^3
X	Cell Mass	g
x	Cell Concentration	g/dm^3
Y_{XS}	Yield Coefficient on Substrate	g/g

Greek

μ	Specific Growth Rate	h^{-1}
μ_{max}	Maximum Specific Growth Rate	h^{-1}

Subscript

F	of the feed
o	at time = 0

Base Units

Time	Hours
Mass	Grams
Volume	dm^3
Temperature	degrees Celcius

CHAPTER ONE

INTRODUCTION

Lipases catalyse the hydrolysis of triglycerides into glycerol, partial glycerides and free fatty acids and are produced by animals and plants as well as micro-organisms. Commercial lipases have been used in dairy and other food processes and lipases produced *in situ* by micro-organisms are important in making fat-containing food both palatable and acceptable. So far their other main application has been in the pharmaceutical industry for inclusion in digestive aids. Other more recent applications are as washing powder and detergent additives and as non-aqueous biocatalysts for inter-esterification reactions. This process, which is used in the oils and fats industry, can modify the composition and hence the physical properties of, triglyceride mixtures in order to increase their utility and value. Lipases have also been used in cosmetics, leather processing, in the production of aliphatic acids and in the treatment of domestic and industrial wastes.

Lipases have traditionally been obtained from animal pancreases and initial interest in microbial lipases was generated because of a shortage of pancreases and the difficulties in collecting available material.

Therefore to be able to provide lipases from micro-organisms in large, easily obtainable amounts, would be of commercial interest.

Much work has been carried out on batch culturing of micro-organisms in complex media to produce lipases. However, lipase production using a fed-batch system is rare and has only been tried with the feeding of olive oil and not glucose.

When a micro-organism is growing in an abundance of glucose, most inducible enzyme systems are repressed. More than 99% of the available genes for enzyme biosynthesis are switched off in this way at any one time. Bacteria and in particular pseudomonads, are known to produce lipases when grown in complex media, the carbon source being presented in the form of, for example, lipids, starch or soybean meal.

Another extracellular enzyme, pullulanase, is commercially produced by growing the organism *Klebsiella planticola* in batch fermentation also using a complex medium. Workers found that glucose fed-batch culture of *K. planticola* was possible and pullulanase productivity was increased four fold.

Therefore the initial aim of the work reported in this thesis was to investigate whether the lipase produced by a lipase-producing pseudomonad was controlled by catabolite

repression or induction. Having established that catabolite repression was the controlling mechanism, a defined minimal salts medium in which the organism could grow was required. From this starting point, the possibility and problems (such as nutrient limitations and pyocyanin biosynthesis) of growing the organism in a stirred fermenter and then feeding glucose at such a rate that the enzyme biosynthesis mechanisms would be de-repressed, was examined.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The overall metabolic activity of a growing micro-organism reflects the simultaneous operation of a large number of interconnected pathways, both energy-yielding and biosynthetic. Each specific pathway comprises a number of individual reactions catalysed by specific enzymes. The product of these many reactions is a new cell.

Successful competition in nature demands that the growth process be both rapid and efficient. The cell must therefore possess appropriate means of balancing the rates of the constituent reactions in each metabolic pathway as well as the overall rates of flow through the various pathways.

Many bacteria can use a wide range of different organic compounds as carbon and energy sources but at any given time, only one of these compounds may be present in the environment. Although the genetic information necessary to synthesise the relevant enzymes for a particular metabolic pathway is always present, its expression may be environmentally determined; that is, a given enzyme may be synthesised in response to a given environmental stimulus.

The cell is able to make qualitative adjustments in its metabolic machinery in response to changing needs imposed by environmental changes. Micro-organisms have evolved a variety of regulatory mechanisms which accomplish this, including the regulation of enzyme synthesis.

The aim of this project is to manipulate one of these regulatory mechanisms so that the micro-organism can produce the enzyme throughout the whole of the growth phase, rather than only at the end. Therefore it is necessary to examine the process of enzyme synthesis and the mechanisms by which it may be regulated.

2.2 Protein Synthesis

The flow of genetic information in normal cells is from DNA to RNA to protein. In the nucleus, a piece of DNA (a gene) is transcribed, forming a mRNA molecule. Translation (protein synthesis) takes place in the cytoplasm and requires the co-ordinated interplay of more than a hundred macromolecules. The mRNA transcript combines with a ribosome to make a mRNA-ribosome complex. Molecules of tRNA present amino acids in the correct order to the complex which then catalyses the production of the polypeptide. Many mRNA molecules in bacteria are polycistronic. That is, they code for two or more polypeptide chains. Within the cell, translation of a molecule of mRNA begins before its synthesis is complete and it is brought about by the simultaneous functioning of a large number of

ribosomes distributed over the length of the mRNA molecule (thus creating a polysome).

2.3 Regulation of Protein Synthesis

The regulation of microbial metabolism includes mechanisms which modulate the enzymatic composition of the bacterial cell and this regulation is effected primarily at the level of transcription rather than translation.

2.3.1 The mRNA-ribosome complex

One of the primary methods of control of protein synthesis is the half-life of the mRNA-ribosome complex. The complex is unstable but the time it takes for the mRNA transcript to be degraded depends largely upon the enzyme for which it codes. The mRNA transcripts that code for many extracellular enzymes (for example proteases and amylases) have half lives measured in hours (but not often more than ten hours (Enatsu and Shinmyo 1978)) whereas transcripts coding for intracellular enzymes are very short-lived, less than two minutes for *beta*-galactosidase (Jacob and Monod 1961). Thus, to continue obtaining a given enzyme, the cell must continue to produce the appropriate mRNA.

2.3.2 Constitutive and inducible enzymes

Whilst studying the enzymes which govern the dissimilation of lactose by *Escherichia coli* Jacob and Monod (1961) found that there were two sorts of enzymes: those which

were produced at a constant rate under all growth conditions, called constitutive enzymes and those which were synthesised only in response to a given environmental chemical stimulus, called inducible enzymes.

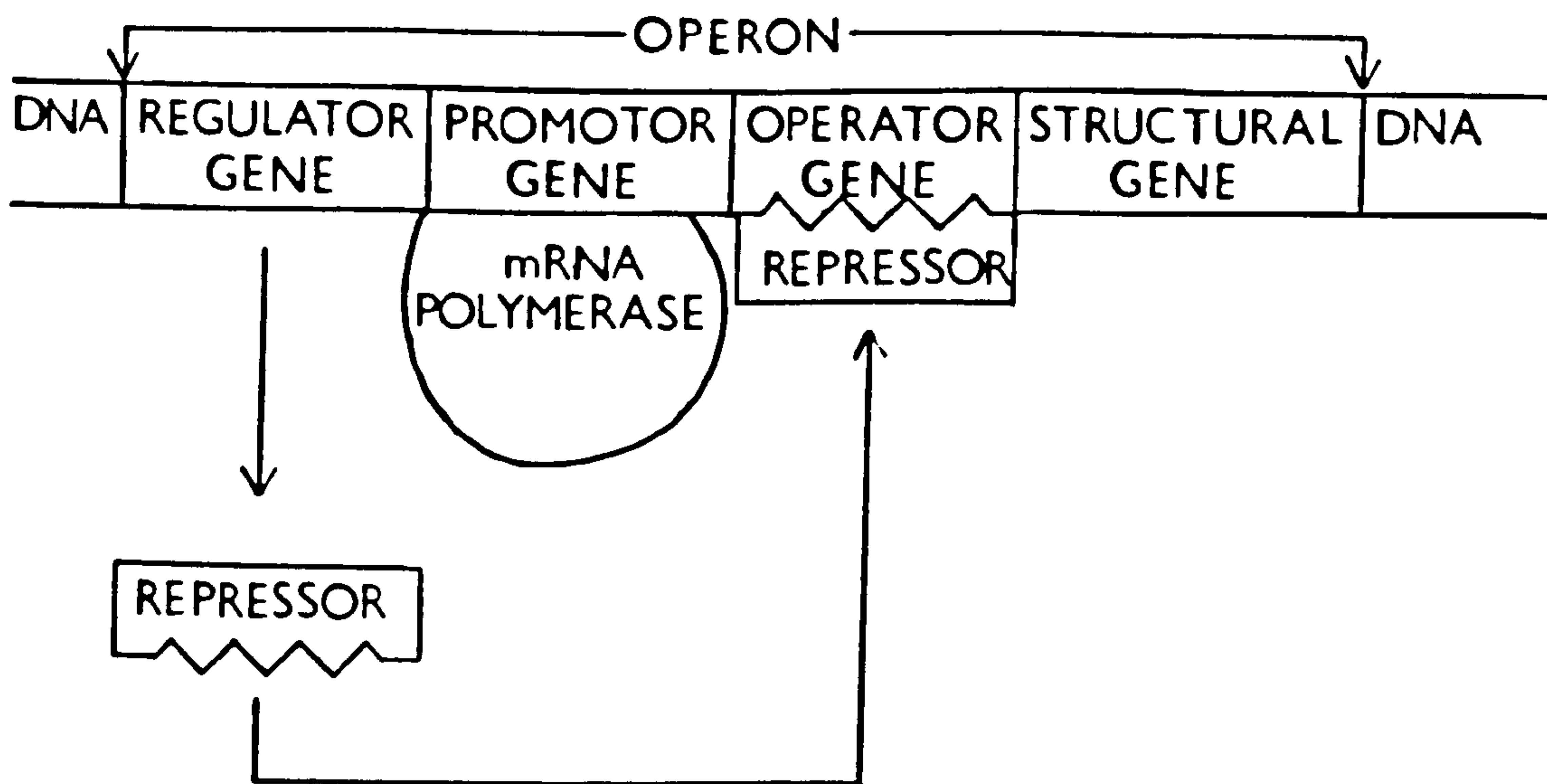
In *E. coli* the enzymes necessary for the metabolism of glucose are constitutive whereas the enzymes for the assimilation of lactose are inducible. Jacob and Monod found that glucose-grown cells contained only barely detectable levels of the enzymes necessary for the initial steps in the metabolism of lactose. However, these enzymes were present at high levels in lactose-grown cells. They therefore concluded that lactose was the inducer for the enzymes involved in the metabolism of lactose.

2.3.3 Biochemical repression

Many genes are organised into operons, which are co-ordinated units of genetic expression. An operon consists of control sites (an operator and a promoter) and a set of structural genes coding for the enzymes. Outside the operon, there is a regulator gene coding for a protein, called a repressor, that can interact with the operator site.

Repressors mediate the induction of enzyme synthesis and they control enzyme synthesis in a negative manner (Figure 2.1). Repressors prevent the transcription of the structural genes by binding to the operator and preventing

REPRESSION



INDUCTION

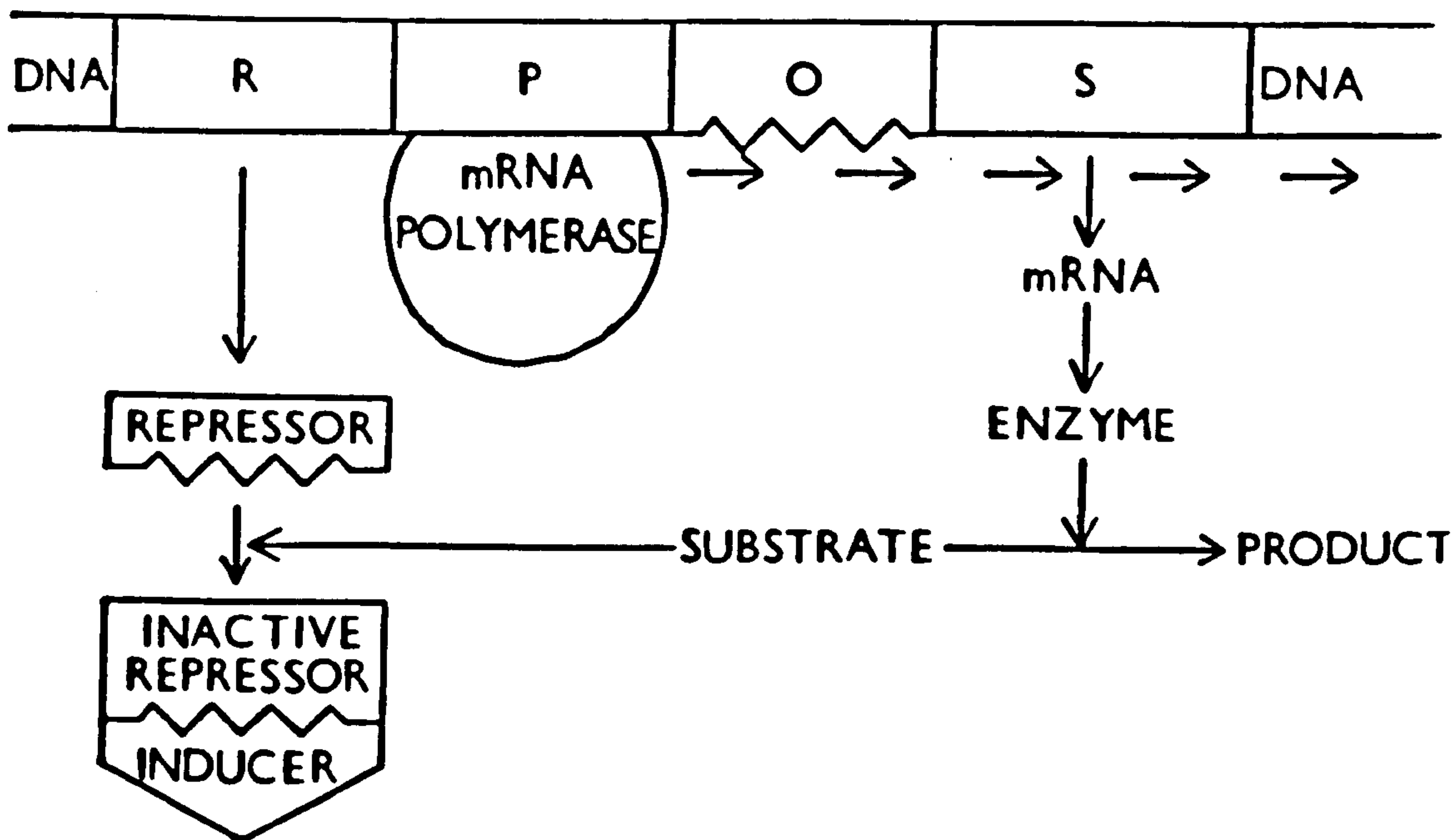


Figure 2.1. Schematic representation of repression and induction (Demain 1972)

the RNA polymerase from binding to the start of the DNA sequence. This system is said to be repressed. Demain (1972) reported that more than 99% of the available genes for enzyme production are switched off in this way at any one time. When a repressor is bound to its specific inducer (that is, the environmental stimulus which triggers the gene's expression), which it does more easily than binding to the operator gene, its ability to bind to the DNA strand is lost and the binding of RNA polymerase is no longer impeded. Therefore, specific enzyme synthesis is initiated.

2.3.4 Catabolite repression

Monod (1942) discovered a phenomenon known as diauxy. When a culture of *E. coli* was grown in a medium containing certain pairs of compounds as carbon sources (for example, glucose and lactose) it underwent two distinct growth cycles, characterised by two exponential phases of growth separated by a distinct lag phase. Glucose was utilised during the first growth cycle and lactose was utilised during the second. The enzymes necessary for the metabolism of lactose are not synthesised (even though the inducer is always present) until the glucose in the medium has been exhausted. While glucose is being metabolised, the induction of the lactose operon is prevented.

Growth curves of similar form are obtained in media containing glucose and various other carbon sources which

are metabolised by inducible enzymes. This kind of repression of enzyme induction was at first thought to be restricted to glucose and was termed the glucose effect. Subsequent work by Pastan and Perlman (1970) has shown that all rapidly metabolisable energy sources repress the formation of enzymes necessary for the dissimilation of energy sources that are more slowly attacked and the phenomenon is now known as catabolite repression. This process obviates the expenditure of energy when a readily utilisable nutrient is available and the production of additional enzymes is not required. However, catabolite repression also regulates the rate at which carbon sources are metabolised. Even when growing on a single carbon source, the levels of enzymes required to generate ATP are regulated through catabolite repression.

The extent of catabolite repression is proportional to the intracellular concentration of ATP. A single allosteric protein, called CAP (catabolite activator protein) is responsible for the regulation of all enzymes under this form of control. Its effector is a cyclic nucleotide, 3',5'-cyclic adenosine monophosphate (cyclic AMP), which is synthesised from ATP. The intracellular concentration of cyclic AMP varies inversely with ATP supply (Figure 2.2). Makman and Sutherland (1965) found that transcription of enzyme systems under the control of catabolite repression could not take place in the presence of high glucose concentrations because the surplus glucose

CATABOLITE REPRESSION

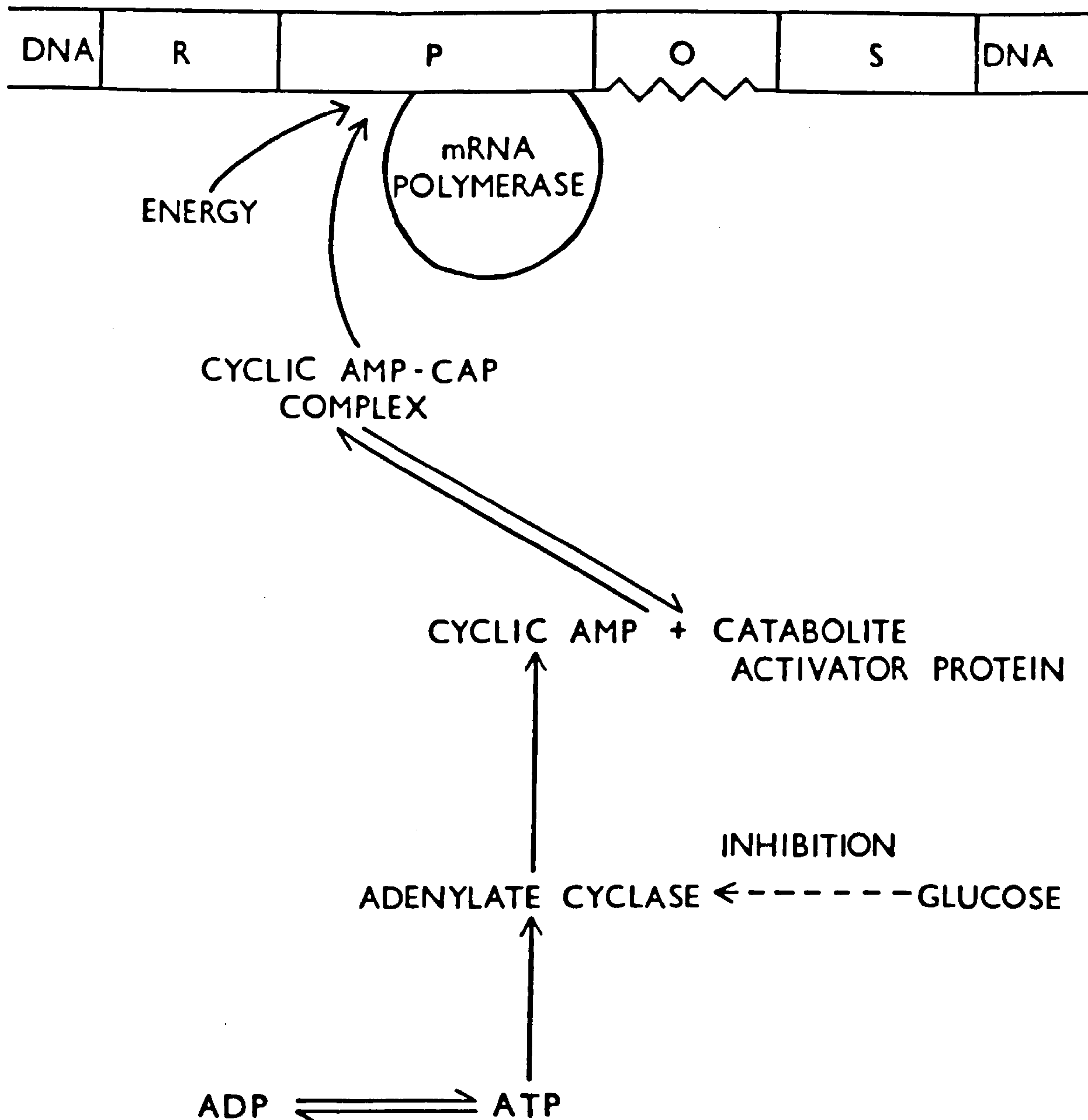


Figure 2.2. Schematic representation of catabolite repression (Makman and Sutherland 1965)

(or its metabolites) inhibits the enzyme adenylate cyclase which produces cyclic AMP from ATP; therefore little or no cyclic AMP is made as all the ATP is being used in the conversion to and from ADP. Hence, when the cell is growing on rapidly metabolisable substrates, intracellular concentrations of cyclic AMP are low; when the cell is growing on slowly metabolisable substrates, the concentrations are high. They also found that the binding of cyclic AMP to CAP caused it to undergo an allosteric transition which allows it to interact with the promotor sites of several inducible operons.

2.3.5 Maximising enzyme production

These cellular control mechanisms have to be overcome in order to get a micro-organism to produce an enzyme continuously. Apart from genetic modification of the organism (for example, stabilising the mRNA-ribosome complex so that it never decayed or replicating the gene for the particular enzyme many times within the bacterial genome), one powerful way to circumvent the depressed formation of enzymes is to limit the growth rate by slow feeding of the carbon source. A low concentration of glucose will create a limitation upon the organism's growth rate, assuming all other nutrients are in excess. Catabolite de-repression can therefore be correlated to a reduction in the specific growth rate of the organism.

Finally, if the enzyme is inducible then the inducer must be present at all times in the medium.

2.4 Fermentation Processes

A fermentation process, in a strict biochemical sense, is one in which energy is generated by the catabolism of organic compounds. The production of alcohol (and carbon dioxide) by the anaerobic catabolism of the sugars present in malt or fruit extracts by yeasts has been carried out on a large scale for very many years and was the first 'industrial' process for the production of a microbial metabolite. Industrial microbiologists have extended the term fermentation to describe any process for the production of a product by the mass culture of a micro-organism. Using the term in a broad microbiological context, brewing, the production of organic solvents and aerobic processes for antibiotic and enzyme production can all be described as fermentation processes.

Fermentations are most commonly carried out in stirred cylindrical vessels made of either glass (laboratory scale) or stainless steel (industrial scale), called fermenters. The main function of a fermenter is to provide a controlled environment for the growth of a micro-organism, or a defined mixture of micro-organisms, to obtain a desired product. It should be capable of being operated aseptically for a number of days, provide adequate aeration and agitation to meet the metabolic

requirements of the micro-organism and there should be control systems for temperature and pH. In the same way, the composition of the medium in which the micro-organism is cultured may be varied, either at the start of each fermentation or by addition of nutrients during the fermentation. The medium may be described as defined (the exact composition is known) or complex (containing components whose composition may vary from run to run). A medium containing both definable and non-definable constituents is termed a semi-defined medium.

2.4.1 Batch processes

Batch culture is an example of a closed system which contains an initial, limited amount of nutrient. The inoculated culture will pass through a number of phases (Figure 2.3). After inoculation there is a period during which no growth appears to take place; this period is referred to as the lag phase and may be considered as a time of adaptation. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum specific rate (designated $\mu_{\max}$) and this period is known as the log, or exponential, phase. During the log phase of growth the products produced are essential to the growth of the cells and include amino acids, nucleotides, proteins (including enzymes), nucleic acids, lipids and carbohydrates. These products are referred to as the primary products of metabolism.

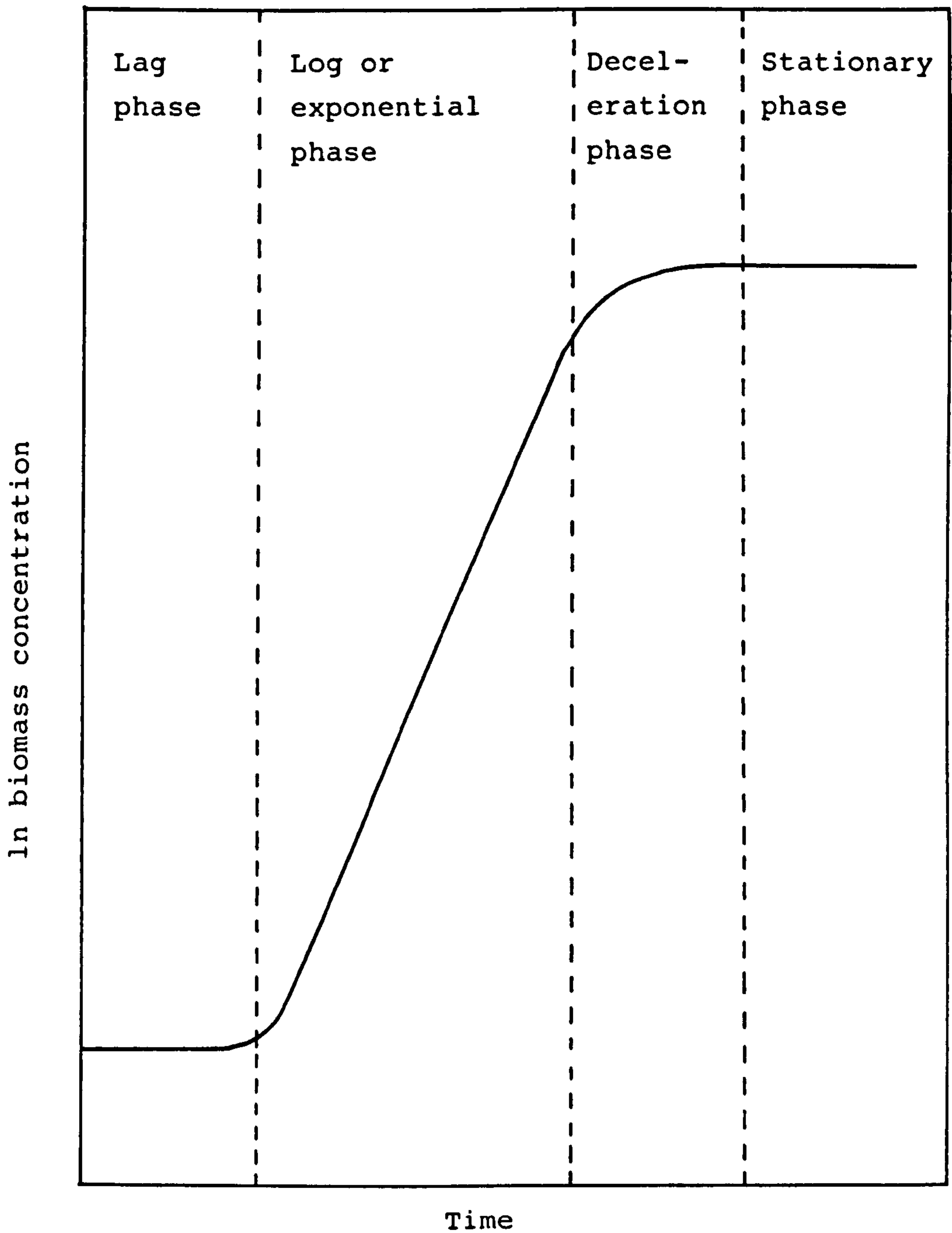


Figure 2.3. Growth of a typical microbial culture under batch conditions (Stanbury and Whitaker 1984)

Theoretically, growth should continue indefinitely. However, growth results in the consumption of nutrients and the excretion of microbial products, events which influence the growth of the organism. After a certain time, the growth rate of the culture decreases until growth ceases. The cessation of growth may be due to the depletion of some essential nutrient in the medium (substrate limitation), the accumulation of some autotoxic product of the organism in the medium (product inhibition) or a combination of the two.

The stationary phase in batch culture is that period in which the growth rate has declined to zero. However, the stationary phase is a misnomer in terms of the physiology of the organism, as the population is still metabolically active during this phase and produces products called secondary metabolites, which are not produced during the exponential phase (Bull 1974). Even though secondary metabolism is exhibited by a very wide range of different micro-organisms, not all micro-organisms undergo secondary metabolism; it is common amongst the filamentous bacteria and fungi and the sporulating bacteria but it is not found, for example, in the *Enterobacteriaceae*. After a further period of time, the stationary phase is followed by the death phase, in which the viable cell number declines. Batch processes may therefore be used to produce biomass, primary metabolites or secondary metabolites.

Secondary metabolites are often confused with the products of catabolite de-repression. As the stationary phase is approached the growth rate declines. If this is due to the carbon source becoming limiting then enzyme production systems will become de-repressed. This can lead to the production of compounds usually associated with secondary metabolism, such as, pigments, antibiotics, biosurfactants and extracellular polymeric substances.

There are several techniques that are used to reduce specific microbial growth rates in order to optimise the production of primary and secondary metabolites under laboratory and industrial conditions.

A. Environmental factors

The metabolic processes can be slowed down by non-optimum temperature and pH. Andersson (1980) when growing *Pseudomonas fluorescens* at different temperatures (4°C to 30°C), found that maximum lipase production was achieved at 8°C whereas maximum growth occurred at 20°C. Brown and Zainudeen (1977) found that all metabolic processes are slowed down at low pH. Entering stationary phase has long been associated with enzyme production (Bu'Lock et al. 1965) and uncontrolled pH in microbial fermentations can result in low pH at the end of the growth phase. Therefore, there may be a connection between low pH and enzyme production.

B. Choice of carbohydrate material

Complex carbon sources such as starch, soyabean meal, barley syrup, corn steep liquor and malt extract are used in many lipase fermentations (Section 2.5.4). Sometimes a mixture of two or more of them is used (LP₂SF, Section 4.1.2). The result is a slow supply of carbon to the organism as the polymers are degraded by hydrolysis.

C. Feeding of glucose

As an alternative to complex carbohydrates as a source of carbon, a glucose solution can be fed at low rates. The specific growth rate of the micro-organism is therefore controlled by the rate at which the glucose solution is fed.

2.4.2 Fed-batch processes

It was recognised as early as 1915 that an excess of malt in the medium for the production of baker's yeast would lead to too much growth resulting in oxygen depletion of the medium, which resulted in the development of anaerobic conditions and the formation of ethanol at the expense of biomass production (Reed and Peppler 1973). The problem was minimised by growing the organism in an initially weak medium such that the growth of the cells was limited by the availability of carbon source rather than oxygen. Additional medium was added at a rate less than that at which the organism could use it. It was not until 1973

that Yoshida et al. introduced the term 'fed-batch culture' to describe batch cultures in which one or more nutrients are fed continuously, or sequentially, to the bioreactor after inoculation without the removal of culture fluid. In modern fed-batch processes for yeast production the feed of molasses is under strict control based on the automatic measurement of traces of ethanol in the exhaust gas of the fermenter.

This concept of batch feed or continuous feed of single components or multiple components has now been used in many fermentation processes resulting in better control of the fermentation process and more efficient utilisation of media components (Table 2.1).

Enzyme production has also been seen to increase by the use of fed-batch processes. For example, when glucose is fed at a limiting rate to the culture of *E. coli*, the differential rate of beta-galactosidase synthesis is 60 times higher than the differential rate of beta-galactosidase synthesis in unrestricted culture containing glucose (Clark and Marr 1964). By feeding a mixture of sucrose and sodium hydroxide (at a predetermined ratio) to maintain the controlled pH, Brown and McAvoy (1990) found it was possible to increase dextransucrase production by *Leuconostoc mesenteroides* from 120 Dextransucrase Units/cm³ (DSU/cm³) to over 300 DSU/cm³.

Table 2.1. Some processes using batch feed or continuous feed or in which they have been tried (Stanbury and Whitaker 1984)

Product	Additions
Yeast	Molasses, nitrogen sources, phosphate and magnesium
Glycerol	Sugar, sodium carbonate
Acetone-butylalcohol	Additions and withdrawals of wort
Riboflavin	Carbohydrate
Penicillin	Glucose and ammonia
Novobiocin	Various carbon and nitrogen sources
Griseofulvin	Carbohydrate
Rifamycin	Glucose, fatty acids
Gibberellins	Glucose
Vitamin B ₁₂	Glucose
Tetracyclines	Glucose
Citric acid	Carbohydrates, ammonia
Single-cell protein	Methanol
Candicidin	Glucose
Streptomycin	Glucose, ammonium sulphate
Cephalosporin	Fresh medium addition

Fed-batch fermentation has also been shown to produce higher concentrations of cellulase.

For example, in cultures of *Pseudomonas fluorescens* with a controlled supply of various carbohydrates, formation of extra-cellular cellulase was enhanced compared with levels obtained in normal batch culture (Yamané, Suzuki and Nishizawa 1970). Also, McLean and Prodruzny (1985) quote productivities up to 160 Units of Filter Paper Activity/L/h (U.FPA/L/h) using intermittent feeding of SolkaFloc whereas when Hendy *et al.* (1982, 1984) used a continuous feed of SolkaFloc, a maximum productivity of 247 U.FPA/L/h was achieved.

When Suzuki *et al.* (1988) were growing *Pseudomonas fluorescens* in batch culture on olive oil, they observed that the cell mass concentration increased in proportion to the amount of olive oil in the medium but the lipase activity did not. The results suggested to them that if the feed-rate of olive oil was varied following cellular growth (by using carbon dioxide evolution rate as a control parameter) then the productivity rate of lipase would increase. They fed olive oil at a rate of 50 g oil/mol.CO₂ until a dry weight of 60 g/dm³ was obtained. Then they reduced the feed rate to 30 g oil/mol.CO₂. Whilst cell production ceased, lipase activity increased from 300 LU/cm³ to 2000 LU/cm³.

Whilst working on the production of *beta*-amylase by *Bacillus megaterium*, Yamané and Tsukano (1977) tried

feeding starch solutions at various constant feed rates and at an exponentially increased feed rate. They found that both feeding methods had comparable effects on the yield of *beta*-amylase from *B. megaterium* but a greatly increased yield of the enzyme (3.6 times that in the batch culture) was obtained by modifying the exponential fed-batch culture.

Another extracellular enzyme, pullulanase, is commercially produced by growing the organism *Klebsiella planticola* in batch fermentation using a complex medium. Research into the production of pullulanase was carried out by Allinson (1990). He replaced the traditional complex medium with a minimal salts medium and tried to imitate the slow supply of carbon to the organism brought about by slow hydrolysis of the hydrocarbon polymers, by feeding glucose. Allinson observed that during rapid growth, when glucose was plentiful, enzyme biosynthesis was repressed, but operated when the rate of metabolism was reduced as the glucose concentration approached zero. In order to confirm that the rate of glucose consumption determined the rate at which the enzyme pullulanase was produced, a number of experiments were carried out in which the glucose was fed to the fermentations at seven different constant rates. The maximum enzyme concentration achieved at each feed rate is plotted in Figure 2.4. The highest overall value of pullulanase activity was 25.2 Pullulanase Units/cm³ (Pu/cm³) at a glucose feed rate of 2.06 g/dm³/h.

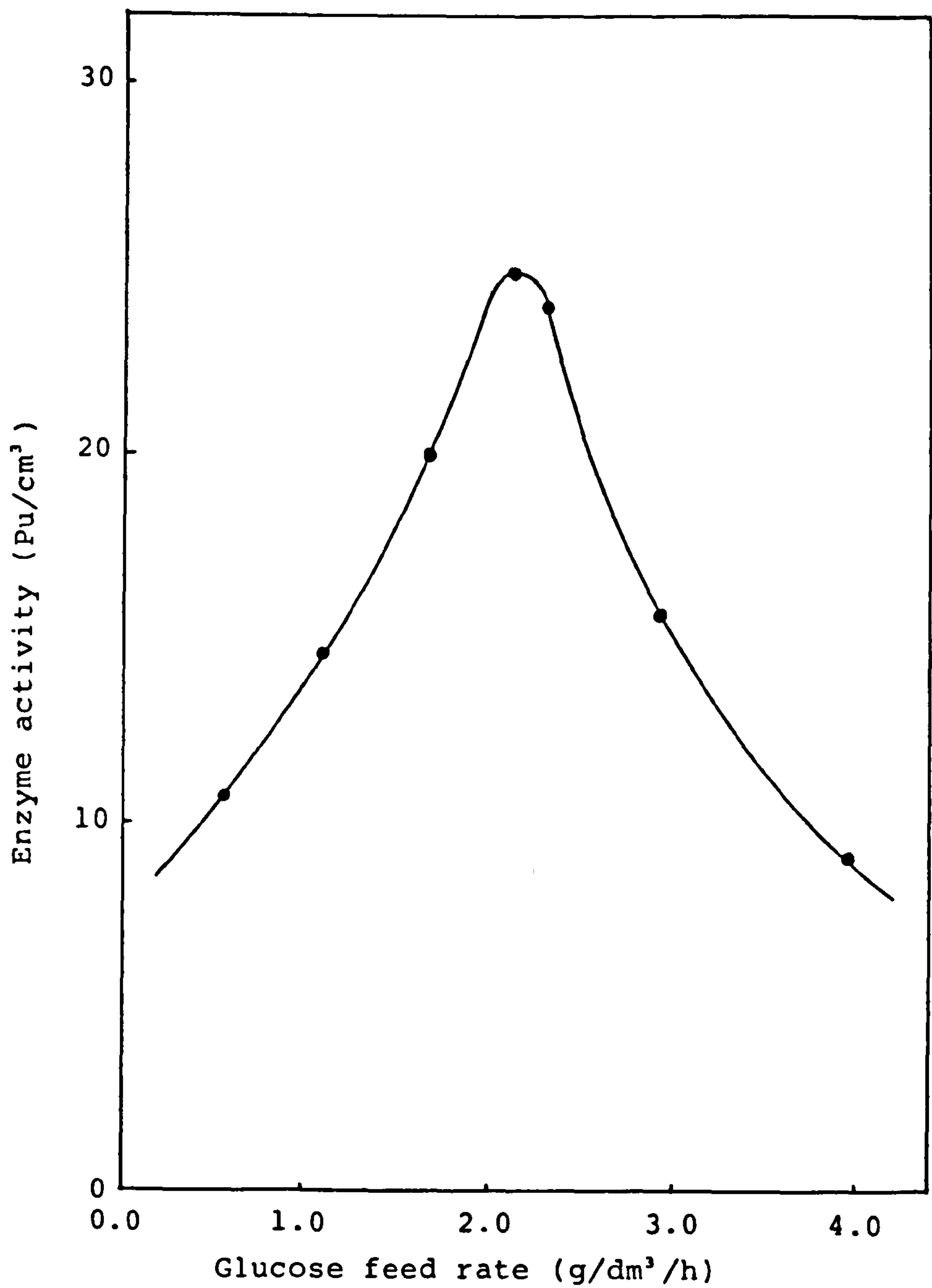


Figure 2.4. Pullulanase activities at different constant glucose feed rates (Brown *et al.* 1988a)

The conclusion drawn from these experiments was that the production of pullulanase may be controlled by both induction and catabolite repression and that the industrial mutant of *K. planticola* was able to produce pullulanase on a simple medium containing only glucose as the sole carbon source.

A further investigation was then carried out by Minihane (1990) into the possibility of feeding glucose at exponential rates in order to give constant specific growth rates. A constant specific feed rate was used to maintain a constant specific growth rate and the feed rate was controlled by an exponential feed equation incorporated into a software package specifically written for the work. *K. planticola* was grown at different constant specific growth rates and an optimum range was found. It was observed that there was a constant specific enzyme production rate for any specific growth rate and this was greater at low specific growth rates than at higher growth rates (Figure 2.5). This was because the concentration of the limiting carbon substrate was low and the enzyme production systems became de-repressed. The enzyme concentration that was produced by this method was twice that of the maximum of a commercial batch fermentation in half the time.

Other fed-batch systems and fed-batch culture technology in general are reviewed in the paper by Minihane and Brown (1986).

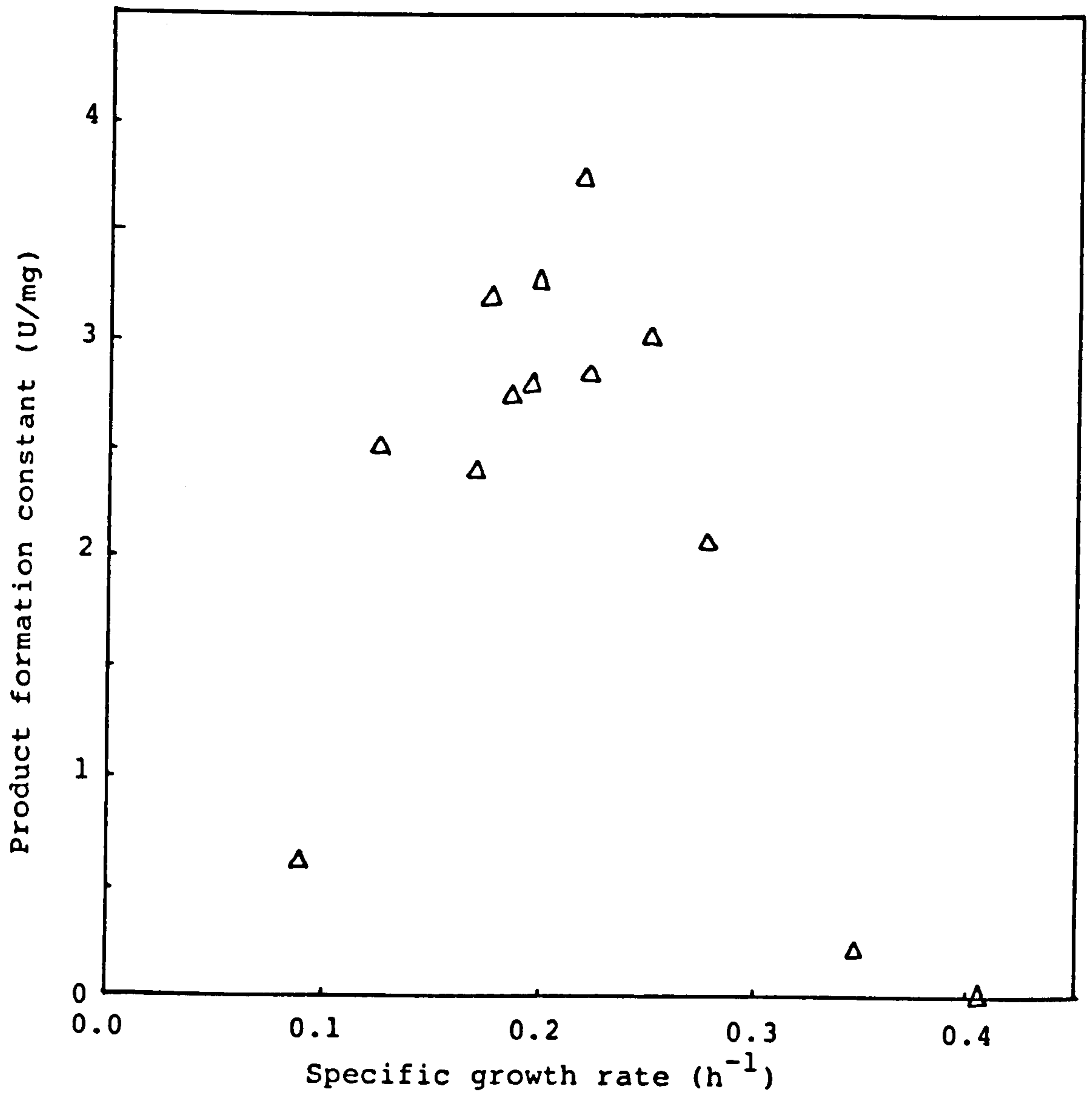


Figure 2.5. Plot of the product formation constant, K_E , against the specific growth rate (Minihane 1990)

The availability of reliable steam sterilisable oxygen and pH electrodes has enabled the direct monitoring of a fermentation and has led to the development of techniques for controlled additions. This means that the concentration of one or more of the nutrients in the medium can be externally manipulated by altering the feed rate during the run according to the feed-back control parameters such as dissolved oxygen (DO), pH, or respiratory quotient (RQ). Alternatively, feed-forward strategies can be employed using pre-programmed feed profiles. The advantages that these techniques give to the operator for improved process technology has resulted in their widespread industrial application.

The penicillin fermentation provides an example of the use of feed systems in the production of a secondary metabolite. The fermentation may be divided into two phases: the 'rapid-growth' phase, during which the culture grows at its maximal rate and the 'slow-growth' or 'production' phase. Glucose feeds may be used to control the metabolism of the organism during both phases. Queener and Swartz (1979) showed that considerable increases in productivity have been achieved by the use of computer controlled feeding of glucose during the rapid growth phase, such that either dissolved oxygen or pH is maintained within certain limits (that is, both control parameters will fall when glucose is in excess, due to an increased respiration rate and the accumulation of organic

acids when the respiration rate exceeds the aeration capacity of the fermenter). During the production phase of the penicillin fermentation the feed rates utilised should limit the growth related oxygen consumption such that a high rate of penicillin synthesis is achieved and sufficient dissolved oxygen is available in the medium.

2.4.3 Linear growth

Brown *et al.* (1988b) reviewed several examples of workers growing various organisms under batch conditions who had found the initial exponential growth rates were followed by linear growth rates. In some cases the linear growth rate was caused by the constant rate of supply of oxygen to the system limiting growth (Sinclair and Kristiansen 1987). Similar effects can also be seen when a batch process is fed with a limiting substrate at a constant rate (Yamané and Hirano 1977 and Esener *et al.* 1981).

When oxygen and carbon were supplied in excess, it was found to be trace nutrients, such as nicotinic acid (Jackson and Copping 1952) or phosphate (Slezak and Sikyta 1957 and Horiuchi 1959) that were the limiting components. The fact that limiting quantities of these trace nutrients caused linear rather than exponential growth suggested that there was another factor to be taken into consideration. It was noticed by the workers growing these organisms that nicotinic acid and phosphate were taken up by the initially small concentrations of cells at rates

out of proportion to their nutritional requirements. This early scavenged uptake of trace nutrients could be followed by their distribution to successive generations thus causing the change from exponential growth rates to linear growth rates. Nyholm (1976) described such components as 'conservative' nutrients.

Gaddum (1988) found that yeast extract contained an unknown trace nutrient which was essential for the growth of *Bacillus subtilis*. This organism was grown under batch conditions at two different initial concentrations of yeast extract. The ratio of the yeast extract concentrations was 0.5, the ratio of the values of the slopes of the linear growth periods was 0.45 and the ratio of the cell concentrations at which exponential growth ceased was 0.54.

Brown et al. (1988b) proposed a simple kinetic model which shows that the mass of the trace material in the initial medium determines both the cell concentration at which exponential growth ends and also the slope of the linear growth phase. The results obtained from the batch growth studies of *B. subtilis* show close agreement with the kinetic analysis.

2.5 Lipases

2.5.1 History of lipases

Enzymes that are capable of hydrolysing triglycerides have been studied for well over one hundred years. In 1846, Claude Bernard demonstrated lipase activity in the pancreas, Marcet described gastric lipase in 1858 and the existence of lipases in plant seeds was noted by Muntz (1871) and Green (1890) (Wills 1965).

Pancreatic lipase has been most extensively studied but lipases in other digestive juices (for example, saliva and gastric juices), animal tissues, plants and micro-organisms have received varying degrees of attention. Relatively little advance in the methods of purification or knowledge of the properties of lipases was made until Willslatter et al. made significant contributions in the early 1920's (Wills 1965). Then, despite the great development of general enzymology during the next thirty years, lipases received only moderate attention and only relatively minor progress was made in this field. The reasons for this were threefold: almost all work on lipases was carried out with crude or partially purified enzyme preparations; kinetic analysis on the enzyme reaction was performed in heterogeneous reaction systems containing insoluble substrates (oils and fats); almost all lipase preparations subjected to these studies were those obtained from animal or plant sources and they were

recognised as unstable. However, during the 1960's mainly as a result of the work of Desnuelle et al., this situation has been largely rectified and more comprehensive information on lipase purification and properties is now available (Wills 1965).

2.5.2 Characteristics of the lipase reaction

Lipases (glycerol ester hydrolase E.C. 3.1.1.3) catalyse the hydrolysis of triglycerides into glycerol, partial glycerides and free fatty acids, as shown in Figure 2.6.

The natural substrates of lipases are triglycerides of long-chain fatty acids. These triglycerides are insoluble in water and lipases are characterised by the ability to catalyse rapidly the hydrolysis of the ester bonds at the interface between the insoluble substrate phase and the aqueous phase in which the enzyme is soluble. However, this reaction is reversible and the enzymes can be shown to catalyse the formation of glycerides from glycerol and free fatty acids (Macrae 1983a).

2.5.3 Bacterial sources of lipases

Comprehensive reviews of bacterial lipases have been carried out by Lawrence (1967) and Sugiura (1984). There has been a large amount of work done on bacterial lipases, a selection of which is presented in Table 2.2. The work most relevant to this thesis is summarised in the next section.

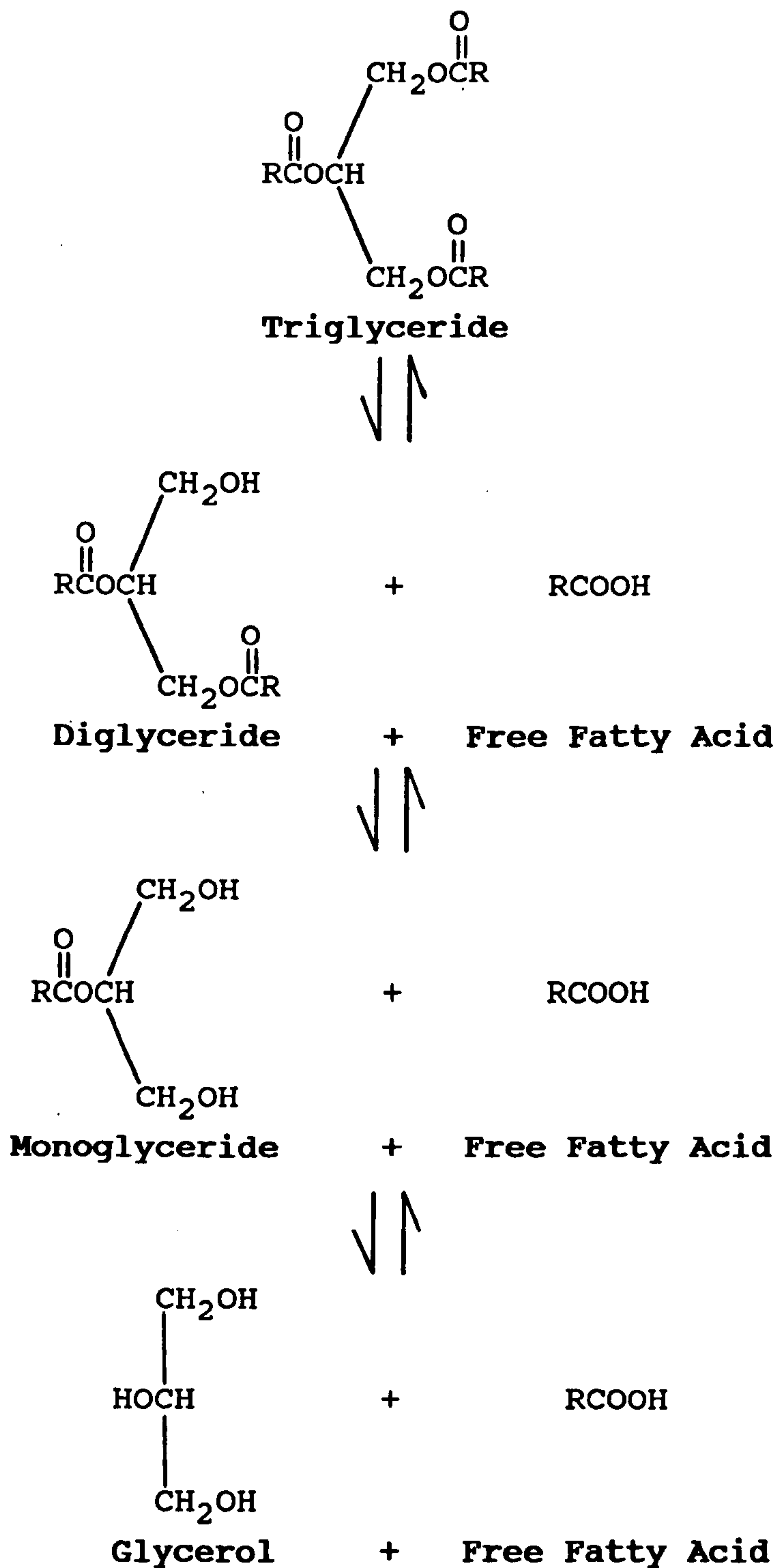


Figure 2.6. The Lipase Reactions
R = hydrocarbon chain

Table 2.2. Bacterial sources of lipases

Organism	Reference
<i>Bacillus</i>	Kennedy and Lennarz 1979
<i>Beauveria</i>	Hegedus and Khachatourians 1988
<i>Serratia</i>	Winkler and Stuckmann 1979
<i>Streptococcus</i>	Chander et al. 1975, 1979a, b
<i>Chromobacterium</i>	Horiuti and Imamura 1977, 1978 Isobe and Sugiura 1977a, 1977b
<i>Staphylococcus</i>	Rollof et al. 1987a, 1987b Vadehra 1974 Vadehra and Harmon 1969
<i>Pseudomonas</i>	Adams and Brawley 1981a, 1981b Kosugi and Kamibayashi 1971 Narasaki et al. 1968
<i>Ps. fluorescens</i>	Andersson 1980 Bloquel and Veillet-Poncet 1983 Bozoglu et al. 1984 Dring and Fox 1983 Fox and Stepaniak 1983 Sugiura and Oikawa 1977 Sugiura et al. 1977 Suzuki et al. 1988 Tan and Gill 1985, 1987
<i>Ps. fragi</i>	Alford and Pierce 1963 Lawrence et al. 1967 Lu and Liska 1969 Mencher and Alford 1967 Nasif and Nelson 1953 Nishio et al. 1987a, 1987b
<i>Ps. aeruginosa</i>	Jager and Winkler 1983 Nadkarni 1971 Schulte et al. 1982
<i>Ps. hydrophila</i>	Hugo and Beveridge 1962

2.5.4 Production of bacterial lipases

Many procedures for the production of lipases in submerged culture have been published in the general and patent literature. The nutrients and culture conditions that have been used vary widely, as do the conclusions that the workers draw with regard to what constitutes the most suitable conditions for the production of lipase.

It is well known that nutritional factors influencing microbial growth and the production of lipase are not necessarily the same. Chander and Klostermeyer (1982, 1983a, 1983b, 1983c) reported the differences in the optimum culture conditions required to get four bacteria to produce lipases and discussed the differences in the characteristics of the lipases produced. One of the major inconsistencies in the production of lipases is whether or not to include a lipid in the medium. In some cases the requirement for an oil or fatty acid does not appear to be absolute but incorporation of such a material increases the level of lipase produced by *Beauveria bassiana* (Hegedus and Khachatourians 1988). For *Rhizopus stolonifer* (Chander et al. 1981) and *Staphylococcus aureus* (Mates and Sudakevitz 1973) the incorporation of synthetic or natural lipids was reported to cause a reduction in lipase production. Chander et al. (1981) suggested that the inhibition may be a physical phenomenon of surface tension, in which certain fatty acids interfere with the

metabolism of the organism. Smith and Alford (1966) showed that the inhibition caused by free fatty acids could be reduced by the presence of protein and/or calcium ions.

Some workers (Macrae 1983b, Watanabe et al. 1977) have found that lipase formation by two pseudomonads is often suppressed by the presence of mono- or di-saccharides or glycerol in the growth medium. In contrast, others have found when growing *Serratia marcescens*, that poly-saccharides act as stimulants, interacting with certain sites on the microbial cell surfaces, thereby detaching cell-bound lipase molecules (Winkler and Stuckmann 1979, Jager and Winkler 1983).

Another interesting controversy concerns whether or not the lipase is constitutive or inducible. Many workers found organisms which produced lipases only in the presence of an inducer. This may be a triglyceride oil (Chander and Klostermeyer 1982), long-chain fatty acid ester (Tsujisaka et al. 1973, Kalle et al. 1972) or a free fatty acid (Iwai et al. 1973). Other workers found a combination, such as olive oil (2% v/v) and kerosene (4% v/v) (Kosaric et al. 1979) or a fatty acid and sterol (Ota et al. 1968) worked well as inducers.

Ibrahim et al. (1987) found that the presence of lipids in the cultivation medium remarkably stimulated the lipase production by *Humicola lanuginosa* but several problems arose because of the high concentration of triglycerides

in the medium. Firstly, the medium formed a heterogeneous mixture which caused some difficulties in enzyme harvesting from the culture broth and secondly, they observed a decrease in enzyme production due to the inhibition by excess fatty acids which were liberated during the hydrolysis of the triglycerides. To overcome these problems they substituted olive oil with sorbitol and corn steep liquor.

Other constituents have been found to be required in the medium in order to enhance lipase production, such as, vitamins (Cutchins et al. 1952), peptides (Tan and Gill 1985), soybean flour (Watanabe et al. 1977) and case peptone (Alford and Pierce 1963).

Extracellular lipase production by bacteria has been examined most extensively in species of *Pseudomonas*, notably *Ps. fluorescens* and *Ps. aeruginosa*. This is because these organisms are the most common psychrotrophs isolated from milk and its derived products, the latter producing what is commonly called 'blue milk'. Therefore enzymes, and in particular lipases, produced by these organisms play a role in the spoilage of fatty foods. Tan and Gill (1985) discovered some inconsistencies between the data obtained by workers growing pseudomonads but they came to the conclusion that the induction of lipases from pseudomonads does not require, and may be suppressed by, the presence of fat in the medium and a rich medium

containing peptides appears to enhance development of lipolytic activity.

Andersson (1980) grew *Pseudomonas fluorescens* in nutrient broth and nutrient broth plus various different oils. He concluded from his results, that because the addition of an oil did not affect the final biomass or the amount of lipase made then the lipase was constitutive. Bloquel and Veillet-Poncet (1983) when growing the same organism also found that the production of lipase did not appear to be induced by lipids in the culture medium but that it was stimulated by the presence of urea. Casitone, peptone and beef extract were found to be necessary requirements for enzyme production.

Work carried out by Winkler and Stuckmann (1979) prompted Schulte et al. (1982) to examine the possibility that glycogen and other polysaccharides could stimulate the formation of the exolipase from *Pseudomonas aeruginosa*. They found that bacteria grown in the presence of the polysaccharides released the enzyme three to four hours earlier than cells of the control experiment and the final exolipase activity was raised four to six times the value of the control even though the bacteria were unable to utilise the polysaccharides tested as sole carbon and energy source. Subsequent work by Jager and Winkler (1983) found hyaluronate to be the most effective polysaccharide for this purpose. They therefore concluded that the

polysaccharide-mediated stimulation of extracellular lipases is a wide-spread phenomena.

Nadkarni (1971) isolated a lipolytic organism from sewage and identified it as *Pseudomonas aeruginosa*. The organism was found to produce a maximal amount of lipase when the medium had an initial pH of 7.0, contained corn steep liquor (2.5%), yeast extract (0.5%) and glucose (0.1%) and the culture flasks were shaken during incubation.

A wide range of workers have studied lipase production, particularly in the last fifteen years, using a number of different organisms. Work carried out in stirred fermenters has been summarised in Table 2.3 which also includes key conditions and medium components.

2.5.5 Assay of lipases

A number of different substrates have been used for the assay of lipases however the preferred methods use emulsions of insoluble triglycerides. Probably the best substrate is triolein but this pure triglyceride is expensive and a cheap, convenient alternative is olive oil which contains over 70% oleate residues.

The progress of the lipase reaction is normally followed by the measurement of the fatty acids released. The reaction is stopped by the addition of solvent (alcohol or acetone) (Mattson et al. 1970) after a set incubation

Table 2.3. A summary of work on the production of lipases from micro-organisms in fermenters

Ref No.	Micro-organism	Fermentation Conditions						Medium			Lipase Activity	
		Time (h)	Temp (°C)	Agitn (rpm)	pH	Aeration (a)	Size (dm ³)	Carbon Source	Nitrogen Source	Lipid	Crude	Pure
1.	Rhizopus arrhizus	40-45	30	✓	7	✓	100	starch	hydolysed caseine	none	350 LU/cm ³	8x10 ⁶ LU/g
2.	Chromobacterium viscosum var. paralipolyticum	96	26	300	6-7	400	400	starch	soybean flour	lard oil	251 LU/g	1,530 LU/g
3.	Humicola lanuginosa	80	45	300	7	1	600	soluble starch	corn steep liquor	soybean oil	55 LU/cm ³	1.2x10 ⁴ LU/g
4.	Mucor meihei	77-114	37	✓	6	✓	n/k	corn starch	soybean meal	none	not stated	
5.	Myriococcum	48	40-43	500	7	0.4-0.6	20	maize meal	soybean meal	linseed oil	220 LU/cm ³	110 LU/mg
6.	Phycomyces	66	24	200	6	100	n/k	wheat bran	poly-peptone	none	160 LU/cm ³	3,750 LU/g
7.	Candida lipolytica	30	30	500	5.8	0.6	10	microbial lipid	yeast extract	n/k	600 LU/cm ³	4.5x10 ⁵ LU/g
8.	Pseudomonas fluorescens	96	26	300	6	7.5	n/k	glucose	poly-peptone	olive oil	10 LU/mg	4,580 LU/mg
9.	Candida lipolytica	cont	25	✓	6	1	1	kerosene and/or olive oil	ammonium sulphate	n/k	3x10 ³ LU (48 h)	n/k
10.	Rhizopus delemar	cont	30	✓	6	✓	3	glucose	soytone	none	460 LU/cm ³	n/k
11.	Pseudomonas fluorescens	40-60	23	1000	6.5	dO ₂ 2-5 ppm	1	olive oil	urea	olive oil	2,000 LU/cm ³	n/k

Key:-
 ✓ = aeration and agitation were used but values were not stated
 cont = continuous culture
 (a) = units of aeration in dm³/dm³/min
 n/k = not known
 LU = Lipase Activity Units - µmoles of free fatty acid released per minute under test conditions

References for Table 2.3:

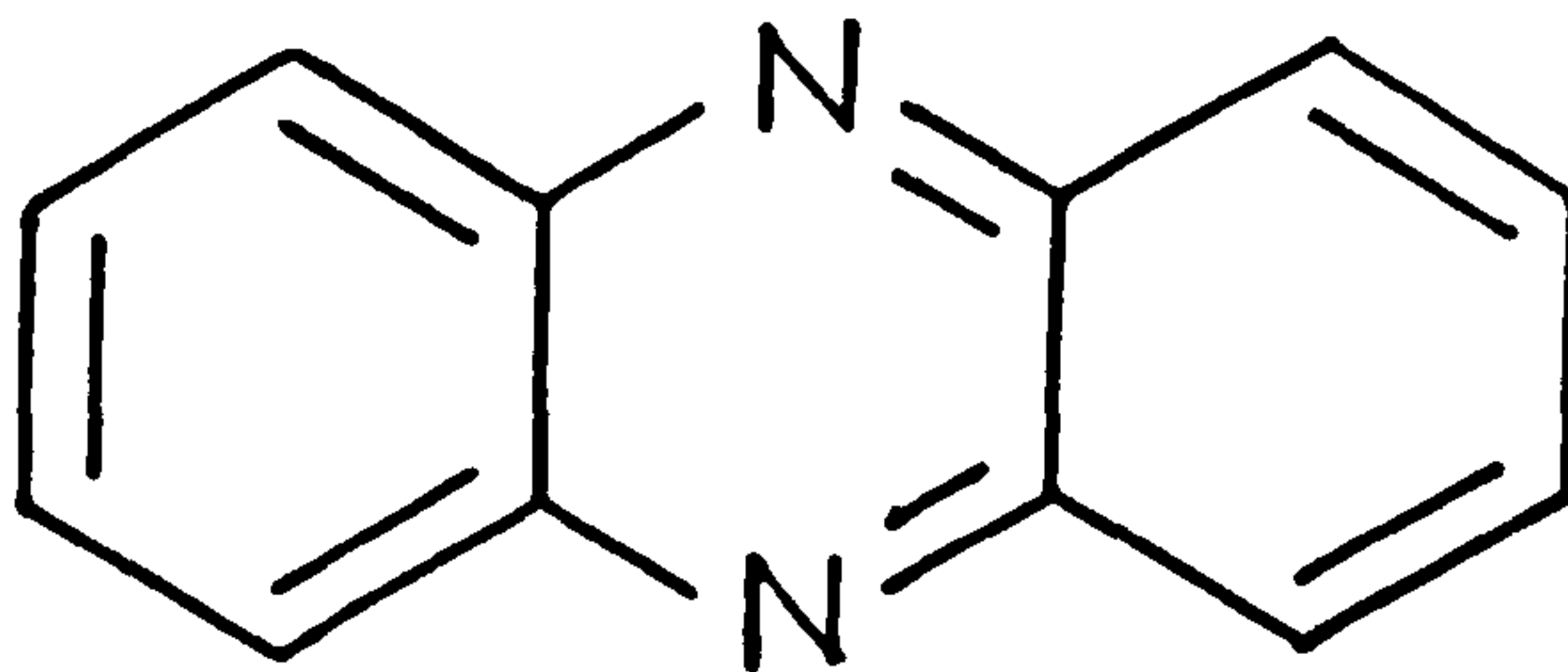
1. Laboureur and Labrousse (1966)
2. Abe et al. (1972)
3. Liu et al. (1972)
4. Moskowitz et al. (1975)
5. Societa' Italiana Resine S.I.R. S.p.A. (1972)
6. Takeda Chemical Industries Ltd. (1976)
7. Pasero et al. (1977)
8. Sugiura et al. (1977)
9. Kosaric et al. (1979)
10. Giuseppin (1984)
11. Suzuki et al. (1988)

period and the released fatty acids are titrated either using an indicator or with a pH-stat.

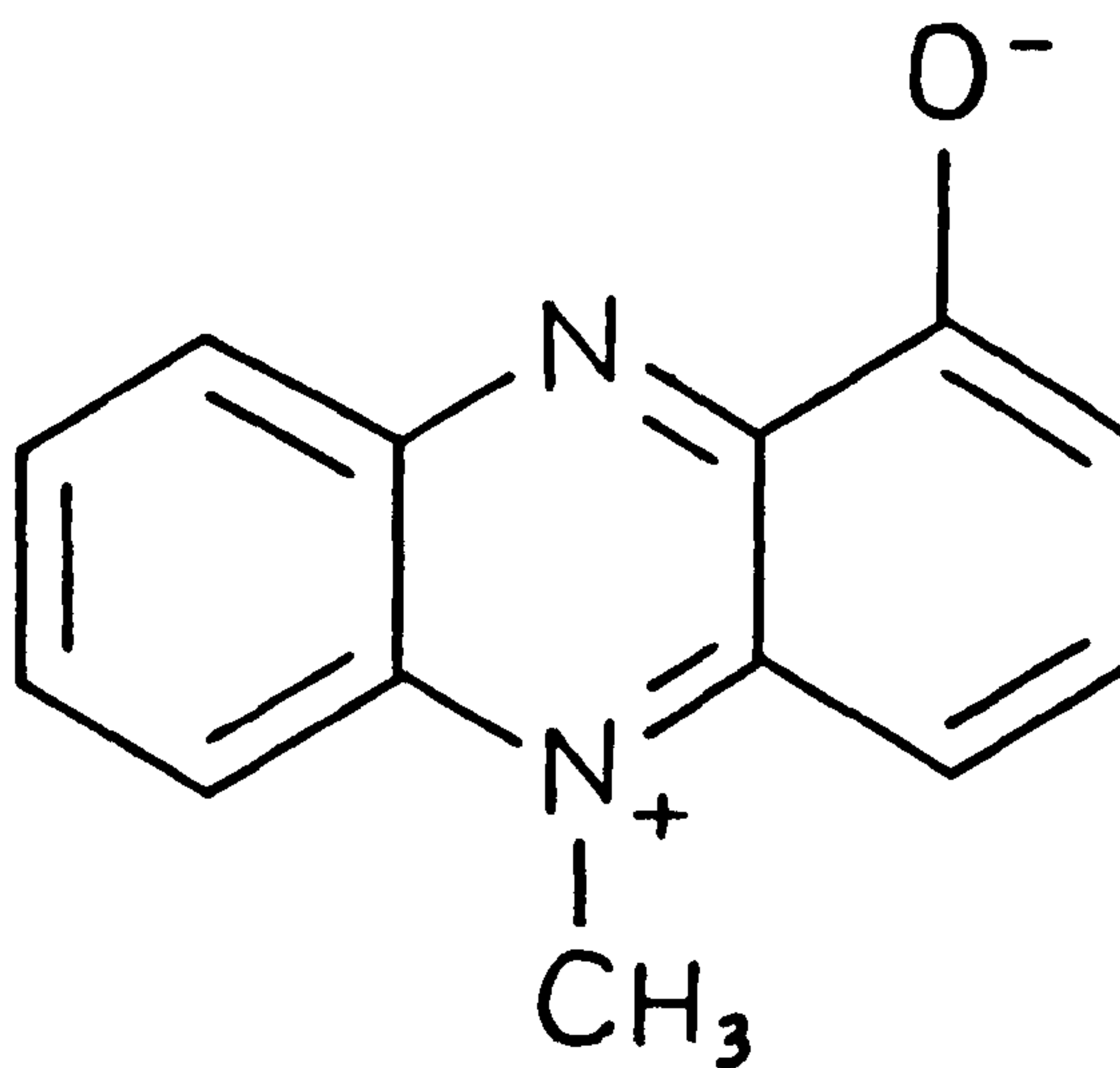
In an alternative method, fatty acid release is continuously monitored throughout the incubation period by titration with a pH-stat coupled to an automatic burette and recorder (Parry *et al.* 1966). This method is invaluable because initial reaction rates can be measured. Other methods include monitoring the disappearance of the triglyceride (Carter and Sykes 1961, Lawrence *et al.* 1967), the glycerol/mono-/di-saccharide production and colourimetric techniques (Dirstine *et al.* 1968, Yang and Biggs 1971).

2.6 Pyocyanin Production

Pseudomonas aeruginosa has been known to produce a blue-green pigment called pyocyanin for over a hundred years. Studies of the pigments of *Ps. aeruginosa* have appeared in the literature with some regularity since Fordos (1860) extracted pyocyanin from surgical dressings. A little later Jordan (1899) described pyocyanin formation during growth on simple chemically defined media for the first time. The original structure of pyocyanin elucidated by Wrede and Strack (1929) was later shown by Hilleman in 1938 (Ingram and Blackwood 1970) to be double the now accepted formula which is illustrated in Figure 2.7 and represents the first reported instance of the occurrence of the phenazine nucleus as a natural product.



Phenazine nucleus



Pyocyanin

Figure 2.7. Structure of the phenazine nucleus and pyocyanin

More than 50 phenazines of bacterial origin are now known, only a few of which have been given trivial names and represent every colour of the visible spectrum. The absorption spectra of phenazines are characteristic, with an intense peak in the range 250 to 290 nm and a weaker peak at 350 to 400 nm (Turner and Messenger 1986) (Figure 5.2). Unlike the various carotenoid and other pigments that colour many bacteria, the phenazine pigments are mostly water soluble and are excreted into the medium. Thus, pyocyanin produced by *Ps. aeruginosa*, diffuses readily into agar-solidified media which become stained blue. The most comprehensive review of the occurrence and biosynthesis of phenazines is that of Ingram and Blackwood (1970).

There are many papers investigating the maximising of the production of pyocyanin but there is no single influential factor upon which they can all agree. One of the first media to be suggested was an ammonium succinate/phosphate/sulphate medium, found to be suitable by Jordan (1899). Burton, Campbell and Eagles investigated the requirements for pyocyanin production by *Ps. aeruginosa*. In one paper (Burton et al. 1947) they looked at the amino acids necessary for pyocyanin production. They found that when glycine, dl-alanine, dl-valine or l-tyrosine were sole sources of nitrogen then pyocyanin was produced. Pyocyanin production was increased markedly when l-leucine was added to a medium already containing glycine or dl-alanine.

But when dl-phenylalanine was added to a synthetic medium pigment production was inhibited. These same workers (Burton et al. 1948) found magnesium, sulphate, potassium, phosphate and iron all to be essential to the formation of the pigment (they were also found to be necessary for growth, that is, no growth, no pigment). They found that in the presence of trace amounts of iron sulphate, the organism is able to grow but not produce pyocyanin.

King et al. (1954) had a different problem. They worked in a hospital laboratory where pyocyanin production is one of the main means of identifying *Ps. aeruginosa*. They observed that not all plates colonised by *Ps. aeruginosa* went blue. They developed a minimal salts medium on which *Ps. aeruginosa* produced pyocyanin every time.

Later work by Frank and Demoss (1959) showed pyocyanin formation commenced when the stationary phase was entered. They observed that the synthesis of pyocyanin, under the conditions investigated, was accompanied by protein synthesis. They concluded that the close association between growth and pigment production effectively obscured any attempt to decide which of the substrates that were tested, might be a specific precursor for pyocyanin.

Gentry et al. (1971) ended their paper on the longevity of *Pseudomonas* cultures with: "The most widely accepted biosynthetic pathway for pyocyanin, in which the aromatic carbon atoms are derived primarily from condensation of

two molecules of shikimic acid (Ingledew and Campbell 1969a), includes at least one phosphatase-dependant reaction. Many synthetic media contain far more inorganic phosphate than is needed for maximal vegetative growth; we suggest that, in those studies involving post-vegetative growth phase cultures, inorganic phosphate available to cells be in carefully controlled amounts and buffer systems other than phosphate be employed." That is, to maximise pyocyanin production, control the amount of phosphate available to the organism. However, a conflicting observation was made by Ingledew and Campbell (1969b) in which pyocyanin formation was reported to occur when the phosphate runs out and growth becomes linear. They concluded that phosphate deficiency may be the trigger for phenazine synthesis in *Pseudomonas aeruginosa*.

Ingram and Blackwood (1970) a year later confirmed Ingledew and Campbell's observations that phosphate deficiency probably triggers pyocyanin production and they also made another observation: they found that increasing the phosphate level beyond a certain point did not increase pyocyanin production and could, in fact, terminate production.

Kanner et al. (1978) isolated a strain of *Ps. aeruginosa* which under different cultural conditions produced three pigments, including pyocyanin. By using the same glucose minimal medium but varying temperature (37°C or 28°C) and

motion (shaking or stationary) they got the organism to produce a different pigment in each different condition.

More recent work was carried out by Messenger and Turner (1983) who ended their discussion with: "From the results, it seems that when resources are limited, i.e. low growth rates, the advantages of phenazine production is outweighed by the need to conserve cell resources and so cell mass is maintained at the expense of phenazine production."

Whooley and McLoughlin (1982) contradicted this finding. They observed that pyocyanin formation occurred (i) only after the exponential phase of growth; (ii) when there was nutrient limitation (carbon or oxygen) and (iii) when growth rates were less than 0.1 h^{-1} . They confirmed that pyocyanin is an energy mediated regulatory mechanism and its production may even be regulated by intracellular ATP levels.

Phenazines, along with most other secondary metabolites, are of uncertain physiological significance. Even so, when pseudomonads are grown in carbon-limiting circumstances and almost half of the substrate ends up in the pigment, either the process or the products of this profligate use of carbon source must be of some importance.

One of the proposed functions for secondary metabolism suggests that the process is more important than the

nature of the product. Secondary metabolism thus prevents excessive accumulation of primary metabolites occurring at the end of the growth phase, by converting them to innocuous end products which are excreted (Turner and Messenger 1986). Gentry et al. (1971) found that in the case of *Ps. aeruginosa*, growth under conditions where pyocyanin production did not occur resulted in a greater loss of viability relative to cultures grown under pyocyanin-producing conditions.

Although many bacterial phenazines were recognised as possessing antibiotic activity towards other micro-organisms (Schoental 1941) and this property motivated further research (Young 1947), there is still little evidence available to identify the value of phenazine formation to the producing micro-organism. Similarly, in spite of all the work, there is no agreement on those physiological factors that regulate the initiation of phenazine biosynthesis and cause its cessation.

CHAPTER THREE

EXPERIMENTAL EQUIPMENT

3.1 Introduction

Cultures of a Pseudomonas species were grown in shake flasks and a stirred fermenter. An overall view of the experimental apparatus used for the fermenter work reported here can be seen in Plate 1. Figure 3.1 shows a schematic diagram of the apparatus.

3.2 Shake flasks

Conical flasks (250 cm^3) containing sterile medium (50 cm^3) were used for the initial growth studies. These were kept sterile with foil-wrapped plugs of non-absorbant cotton wool. The flasks were incubated on an orbital shaker (180 rpm) in a constant temperature room (30°C).

Baffled conical flasks (2 dm^3) containing sterile medium (500 cm^3) were used in later studies on trace element limitation. These larger flasks were treated in the same way as the smaller ones.

3.3 Fermenter Unit: Bioengineering KLF 2000

The fermenter (Plate 1: B) used in this work, was a model KLF 2000 (Bioengineering, Sagenrainstrasse 7, CH-8636, Wald ZH, Switzerland) stirred vessel with a standard

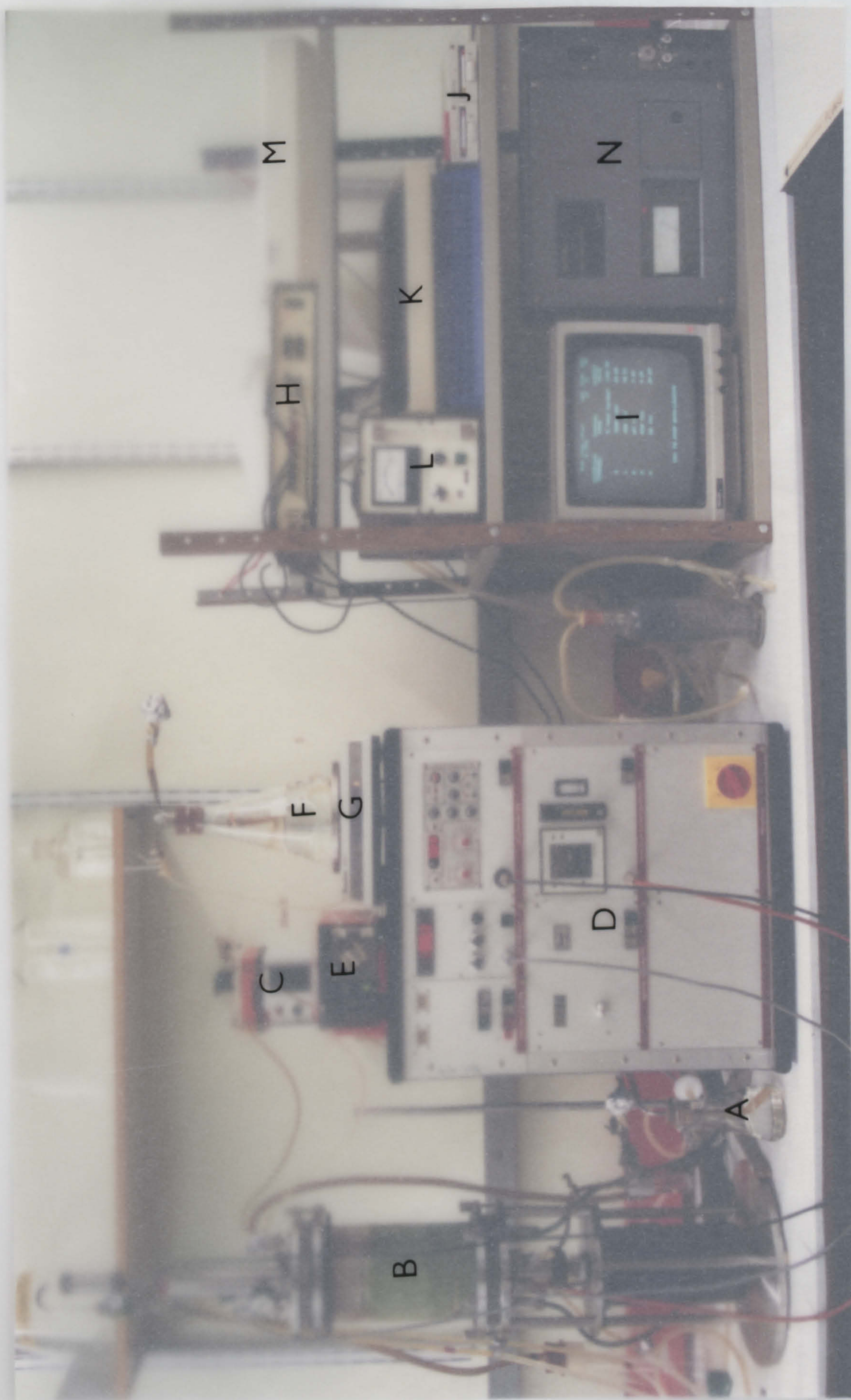


Plate 1. A photograph of the experimental apparatus

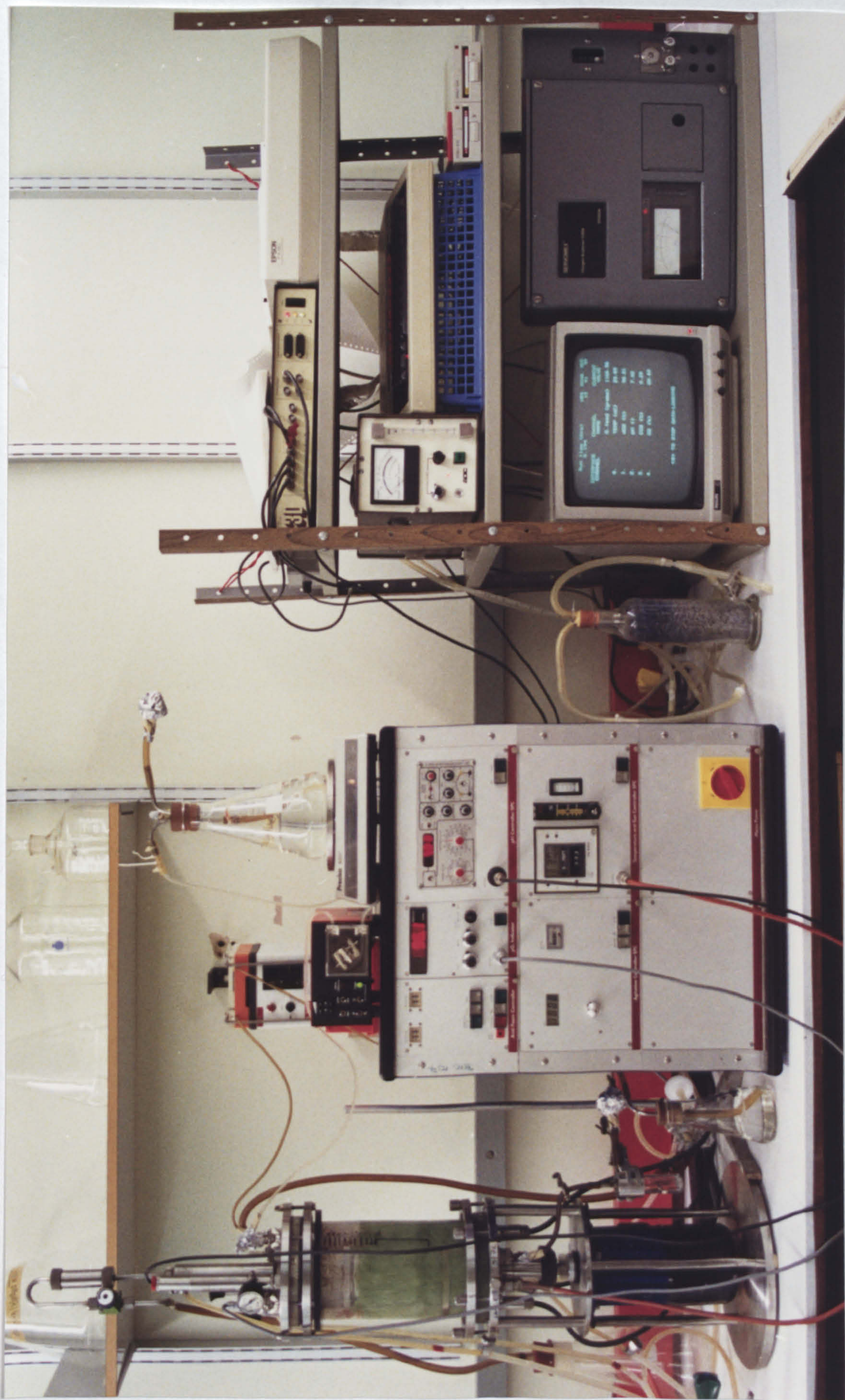


Plate 1. A photograph of the experimental apparatus

Plate 1. Index

- A: Alkali feed bottle
- B: Fermenter
- C: Alkali feed pump
- D: Fermenter control panel
- E: Glucose feed pump
- F: Glucose feed bottle
- G: Top-pan balance
- H: GPIS Interface
- I: VDU
- J: Disc drive
- K: BBC Micro-computer
- L: Infra-red CO₂ gas analyser
- M: Printer
- N: Paramagnetic oxygen analyser

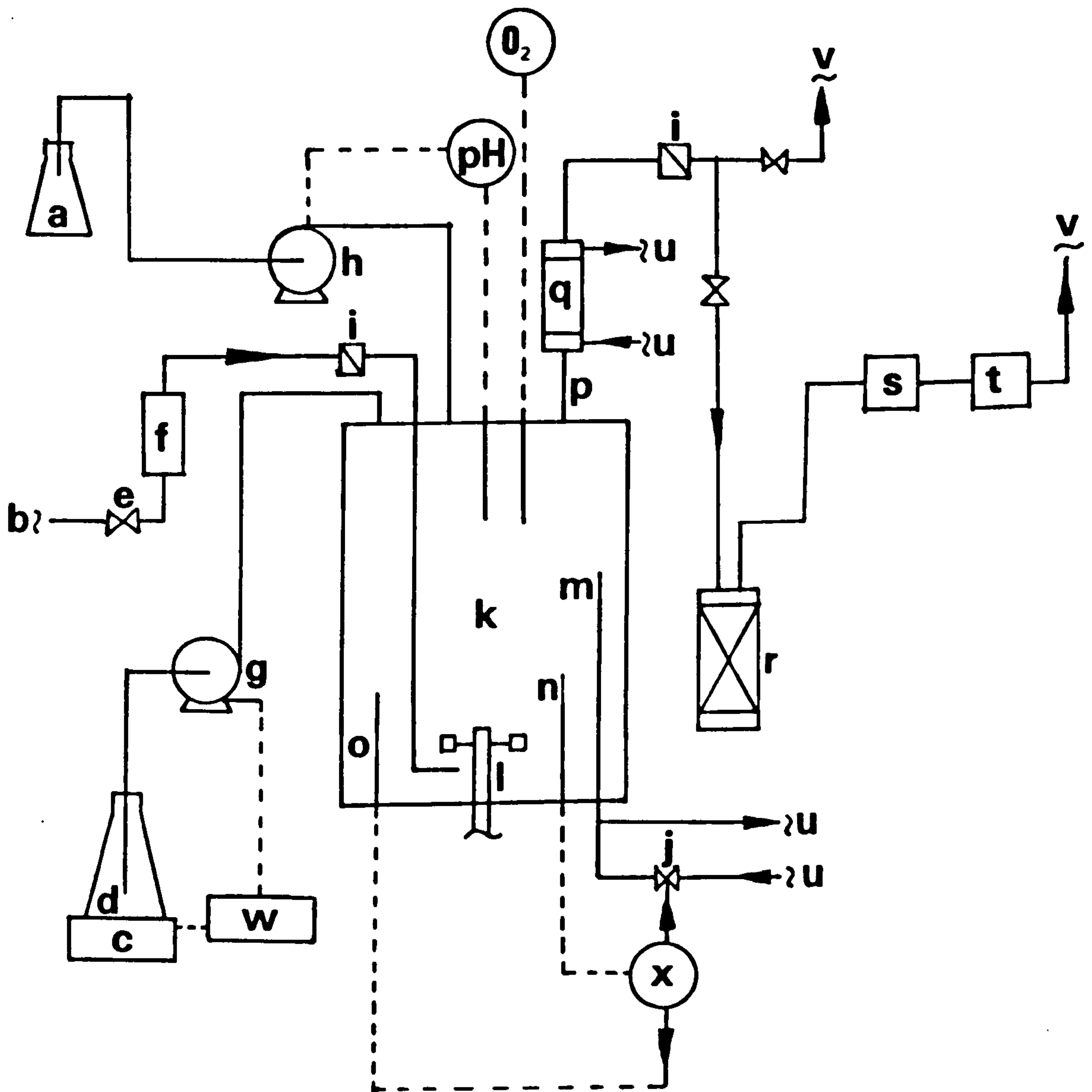


Figure 3.1. Schematic diagram of experimental apparatus used for the fermentations

Figure 3.1. Index

a: Alkali feed bottle
b: Air supply
c: Top pan balance
d: Glucose feed bottle
e: Constant flow regulator
f: Rotameter
g: Glucose feed pump
h: Alkali feed pump
i: Sterile air filter
j: Solenoid valve
k: Bioengineering 2 dm³ fermenter
l: Impeller
m: Cooling water finger
n: Temperature probe
o: Heater
p: Exhaust gas
q: Top condenser
r: Silica gel drying bottle
s: Carbon dioxide meter
t: Oxygen meter
u: Cooling water
v: Exhaust gas vent
w: BBC Micro-computer interface
x: Temperature control box

15 cm diameter cylindrical glass section (3.7 dm^3 , working volume 2.5 dm^3) and stainless steel top and bottom plates. The vessel was stirred by two bottom entry, 6 cm diameter six bladed, disc turbines driven by a variable speed DC motor, had four equally spaced 25 mm baffles and sat upon a base containing the impeller motor drive. The vessel was heated by means of a bottom entry heater rated at 800 Watts. Cooling was achieved by a flow of chilled or tap water through the bottom entry cooling finger assembly and controlled by a solenoid valve.

The top plate contained nine standard openings for fitting accessories such as outlet filter/condenser assembly, safety valve, pH electrode, pO_2 electrode, foam sensor. Other access ports were occupied by diaphragms beneath perforation cups through which needle insertions, such as aeration, sampling, inoculation, dosage, could be made.

The control system consisted of a power unit and five control boards, with a 4 - 20 mA recorder output which provided the signals for the micro-computer data-logging.

3.4 Top-Pan Balance

The glucose feed bottle (Plate 1: F) sat on a top-pan balance (Plate 1: G) which was interfaced directly to the micro-computer (Plate 1: K). The balance (Precisa 2200C, Metragram Instruments, Aspley Guise, Bucks) was accurate

to two decimal places over the range 0 - 1600 g and was fitted with an RS423 interface board.

3.5 Carbon Dioxide Meter

Carbon dioxide was monitored using an infra-red analyser (Plate 1: L) (Analytical Development Company Ltd., Hoddensdon, type SS-200) with two selectable ranges, 0 - 2% and 0 - 10% and a zero calibration channel. The output signal was linearised to 0 - 1 volt. The 0 - 10% channel was used giving an accuracy of 0.2% according to the manufacturer's manual. The instrument was calibrated using an exact gas mixture of CO₂ : N₂ : O₂ (P. K. Morgan Ltd., Rainham; nominal 8% CO₂, 14% O₂, with exact analysis) and the zero calibration channel. Full technical specification is available from the manufacturers' manual (ADC Ltd. 1982).

3.6 Oxygen Meter

Oxygen was monitored using a paramagnetic analyser (Plate 1: N) (Servomex Ltd., Crowborough, type 540A) with five selectable ranges, a zero suppression card and a linearised 0 - 1 volt output. The range 0 - 25% was used, giving an accuracy of 0.2% according to the manufacturer's manual. The analyser was calibrated using the same gas as the carbon dioxide analyser (Section 3.4) and air. Full technical data is available from the manufacturers' manual (Servomex 1984).

3.7 Instrumentation

3.7.1 Temperature

The temperature control range for the fermenter was 0 - 150°C. The temperature of the medium in the fermenter was measured using a resistance thermometer introduced into the vessel via a bottom entry port. The signal went to the temperature control module in the control unit, which had a set point control to 0.1°C maintained by on-off control of the heater or cooling water flow via the solenoid valve.

3.7.2 Agitation

This had set point control from 0 - 2500 rpm.

3.7.3 Dissolved oxygen

The dissolved oxygen tension of the fermentation medium in the fermenter, was measured using an Ingold polarographic amperometric pO_2 sensor fitted with a replaceable gas permeable teflon membrane impermeable to ions. This electrode was connected to an oxygen modular amplifier.

3.7.4 pH

The pH was measured by a pH/rH pressurizable electrode introduced into the fermenter via the top plate. The signal was sent to a programmable pH/mV controller (M 7832 N) which had set point control to 0.1 pH unit. Variation from the set point could be used to switch on

and off an acid or alkali feed pump. For all the fermentations carried out in this work, only an alkali feed pump was used.

3.7.5 Exit gas analysis

The exit gas from the fermenter was passed via a condenser to remove excess moisture and a two litre overflow catch flask, through a sterile air filter (0.2 μm Gelman) and a Dreschel bottle containing silica gel. A small amount (1.5 dm^3/min) of the dry, sterile gas then flowed to the carbon dioxide and oxygen analysers (Sections 3.4 and 3.5) and was finally vented to atmosphere, the rest was also vented to the atmosphere. The voltage signals from the analysers were passed to the GPIS interface (Section 3.7).

3.8 Micro-Computer System

3.8.1 Hardware

The 4 - 20 mA outputs from the fermenter unit were converted to 0 - 1 volts before input to the interface (Plate 1: H) (Digital Design and Development Ltd., London; type GPIS) together with the signals from the analysers. After conversion to a digital signal they were sent to the micro-computer (Plate 1: K) (BBC model 'B', Acorn Instruments, Cambridge) via the 1 MHz bus. All electrical signals were sent via co-axial cable. The GPIS interface provided 12 bit resolution on eight analogue to digital (A/D) inputs and four digital to analogue (D/A) outputs

and was controlled by software run on the micro-computer. Five of the A/D inputs were used (Table 3.1). One D/A output was used to control the glucose feed pump. The inputs were calibrated using the procedure given in the operating manual (Digital Design and Development 1983) to give 12 bit resolution of a 0 - 1 volt signal. The output was similarly calibrated on the 0 - 10 volt output using a short calibration program.

The BBC model 'B' provided 32k of Random Access Memory (RAM), of which 31k was available for the fermentation data-logging. The system was completed by a twin disc drive (Plate 1: J) using 3" floppy discs, a monitor (Plate 1: I) and a dot-matrix printer (Plate 1: M) (Epson, Telford). The micro-computer was fitted with a "Printmaster" EPROM (Computer Concepts, London) which gave enhanced graphical printing ability.

The top-pan balance was interfaced directly to the micro-computer via the RS423 interface. The balance reading was read directly as an ASCII data string. The signal connections are shown in Figure 3.2.

3.8.2 Software

To allow for the control and data-logging of the fermentation a software package was written in BBC - BASIC (Minihane 1990), except for a machine code procedure supplied for the interface (Digital Design and Development 1983).

Table 3.1. A/D Inputs on the GPIS Interface

Channel	Parameter	Recorded Units	Measured Scale
0	Temperature	°C	0 - 100
1	Dissolved O ₂	%	0 - 100
2	pH		0 - 14
3	CO ₂	%	0 - 10
4	O ₂	%	0 - 25

Figure 3.2. Index

- a: Top-pan balance
- b: Glucose feed pump
- c: Alkali feed pump
- d: Dot-matrix printer
- e: Bioengineering 2 dm³ fermenter
- f: Disc-drive
- g: VDU
- h: BBC model 'B' micro-computer
- i: Carbon dioxide analyser
- j: Oxygen analyser
- k: GPIS interface
- l: Voltage box
- m: Fermenter control box

The package consisted of six programs stored on disc. The programs were:

- (a) INFO A guide to the programs
- (b) CHOICE A simple menu program
- (c) DATALOG The major program which controlled the interface calibration, data-logging and glucose feed to the fermentation. The control variables for the feed profile were entered and the operator was then guided through the selection of the data-logging parameters. The program ran with an updating display of the measured values and the feed could be started at any time via the keyboard.
- (d) RECDATA This recalled data from disc and displayed it in tabular form. A hard copy could be obtained.
- (e) GRAPHIC Using the "Printmaster" Eprom, data from a disc file could be displayed graphically and a hard copy obtained, examples of which can be found in Appendix B.
- (f) INDATA Data from off-line measurements could be entered into a disc-file using this program.

A program listing can be obtained elsewhere (Minihane 1990).

3.9 Feed Systems

3.9.1 Glucose feed

The sterile glucose feed solution was contained in a conical flask (1 dm^3) (Plate 1: F) which was placed upon the top-pan balance (Plate 1: G). At the start of the

fermentation the balance reading was tared so that the reading became progressively more negative during the fermentation. The feed line to the vessel was 4 mm sterile silicon tubing. A peristaltic pump (Watson-Marlow Ltd., Falmouth; type 101 U/R) was used. The speed of the pump was controlled by a 0 - 10 volt signal from the interface.

3.9.2 Alkali feed

The alkali solutions (Sections 4.5 and 4.6) were contained in a side-arm flask (250 cm³) (Plate 1: A). The side-arm had a sterile air filter fitted (0.2 µm Gelman). The solution was fed to the fermenter via 5 mm sterile silicon tubing using an EYELA peristaltic pump (Bioengineering UK Ltd., Cambridge; type MP-3). The pump (Plate 1: C) was under on-off control from the pH control board.

3.10 Services

3.10.1 Air supply

The air main to the laboratory was at 15 psig. The flow rate was controlled by a constant flow regulator, measured on a 0 - 300 dm³/h rotameter. The air then passed through a sterile air filter (0.2 µm Gelman) before being sparged into the medium.

3.10.2 Water supply

The cooling water was taken from the laboratory mains or from circulating chilled water (5°C). The feed was split

between the top condenser, which had a constant water supply and the cooling finger assembly, which had an on-off control valve (Section 3.6.1).

3.10.3 Electrical supply

All the mains electrical supply was taken from the wall distribution board. The micro-computer and interface ran from a plug board connected to the mains via an electrical filter (RS Components, Corby) to smooth any mains supply fluctuations.

3.11 Sterilisation Equipment

A Prestige pressure cooker was used to sterilise small amounts of equipment such as Finntips, saline tubes, fermenter utensils, etc. Sterilisation took place at 1 bar for 15 minutes. For larger amounts of equipment, such as shake flasks, an Astell Hearson autoclave was used. Sterilisation took place at 121°C for 20 minutes.

CHAPTER FOUR

EXPERIMENTAL METHODS

4.1 The Organism

4.1.1 Source and classification of organism

The organism was a generous gift from Dr. Helena Sztajer. Dr. Sztajer and her colleagues carried out a screen of micro-organisms found contaminating a tributyrin agar plate left uncovered in a margarine factory in Poland (Sztajer *et al.* 1988). One of the organisms which produced the largest zones of clearance on tributyrin agar plates, they identified to be a strain of *Pseudomonas fluorescens*.

4.1.2 Maintenance of the organism

The organism was grown up "under stress" (that is, without a simple carbon source) in sterile aliquots (50 cm³) of LP₂SF (Table 4.1). Conical flasks (250 cm³) were inoculated with a loop full of organism from an existing maintenance culture and incubated on an orbital shaker (180 rpm for 48 hours at 30°C). Culture aliquots were then serially diluted in 0.9% sterile saline and spread in duplicate on tributyrin agar plates (Table 4.2). After 48 hours at 30°C, the plates with the best spread of colonies were then used as the new maintenance cultures.

Table 4.1. Composition of LP₂SF - Lipase-producing medium containing soybean flour

Chemical	Concentration (g/dm ³)
Soybean Flour	20.0
Peptone	10.0
Soluble Starch	10.0
K ₂ HPO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
CaCO ₃	5.0

Method:

The components were added to 750 cm³ distilled water in the stated order then made up to 1 dm³. The pH was adjusted to 7.0 with NaOH (4N). The medium was sterilised in 50 cm³ aliquots in 250 cm³ conical flasks by autoclaving (121°C for 20 minutes). (Arima et al. 1972).

Table 4.2. Composition of tributyrin agar

Chemical	Concentration (g/dm ³)
Peptone	5.0
Yeast Extract	3.0
Tributyrin	10.0 cm ³
Agar	25.0

Method:

The peptone (Lab M, Bury, Lancs) and yeast extract (Lab M, Bury, Lancs) were dissolved in distilled water (1 dm³). An emulsion of the tributyrin was made in 200 cm³ of the above solution by sonication. Meanwhile, the agar (Lab M, Bury, Lancs) was dissolved in the remaining 800 cm³ of the above solution, by warming. The tributyrin emulsion was then added, with mixing, to the melted agar mixture. This final solution was sterilised by autoclaving (110°C for 20 minutes). Whilst still molten, the tributyrin agar was poured into plates.

The maintenance cultures were stored at 4°C and replaced with freshly prepared cultures after 28 days.

4.2 Inoculum Preparation

4.2.1 Shake flask experiments (250 cm³)

A conical flask (250 cm³) containing a sterile aliquot (50 cm³) of medium to be used in the growth experiment, was inoculated aseptically with a loop full of the organism from a maintenance culture plate and incubated on an orbital shaker (180 rpm at 30°C for 36 hours).

4.2.2 Shake flask experiments (2 dm³)

A conical flask (1 dm³) containing a sterile aliquot (200 cm³) of medium (Pirt's medium (x1) (Table 4.3), 10 g/dm³ glucose, 1 g/dm³ yeast extract) was inoculated aseptically with a loop full of the organism from a maintenance culture plate and incubated on orbital shaker (180 rpm at 30°C for 24 - 36 hours).

4.2.3 Fermenter experiments

A conical flask (1 dm³) containing a sterile aliquot (200 cm³) of medium (Pirt's medium (x1) (Table 4.3), 10 g/dm³ glucose, 1 g/dm³ yeast extract) was inoculated aseptically with a loop full of the organism from a maintenance culture plate and incubated on an orbital shaker (180 rpm at 30°C for 24 - 30 hours).

Table 4.3. Composition of Pirt's medium (x1)

Chemical	Concentration (g/dm ³)
NH ₄ Cl	4.3700
KH ₂ PO ₄	1.1300
MgSO ₄ ·7H ₂ O	0.2320
CaCl ₂ ·2H ₂ O	0.0110
FeSO ₄ ·7H ₂ O	0.0070
MnSO ₄ ·4H ₂ O	0.0020
ZnSO ₄ ·7H ₂ O	0.0020
CuSO ₄ ·5H ₂ O	0.0004
CoCl ₂ ·6H ₂ O	0.0004
EDTA, disodium salt, dihydrate	0.3940

Method:

The components were added to 750 cm³ of distilled water in the stated order. If the medium was to contain glucose and yeast extract, then they were added at this point and the medium made up to 1 dm³. The pH was adjusted to 7.0 with NaOH (4N) (Pirt 1975). Flasks were sterilised by autoclaving (121°C for 20 minutes).

If the medium was for use in the fermenter, the pH was adjusted after sterilisation (Section 4.7.1).

4.3 Medium Preparation

All the chemicals were of the highest possible grade and obtained from the Sigma Chemical Company Ltd., Poole, Dorset unless otherwise stated.

4.3.1 Shake flask experiments (250 cm³)

These were carried out either with glucose and yeast extract media or with Pirt's medium (x1).

The glucose and yeast extract media were made by dissolving the required amount of yeast extract (either 1% or 2%) in the appropriate amount of distilled water and then adding the required amount of glucose (0.3%, 0.5%, 0.75% or 1%). For experiments using either medium, an aliquot (50 cm³) of the medium was added to each of ten conical flasks (250 cm³). The flasks were then sterilised by autoclaving (121°C for 15 minutes). At the start of the experiment the flasks were aseptically inoculated approximately at the same time, with 2.5 cm³ (5%) of the inoculum culture (Section 4.2.1).

4.3.2 Shake flask experiments (2 dm³)

Each medium was set up in triplicate and there were eight media in all and the compositions of which are shown in Table 4.4.

Table 4.4. Composition of shake flask media (2 dm³)

Chemical	Concentration (g/dm ³)	Contained in all media except
EDTA.2Na.2H ₂ O	3.940	
NaCl	5.000	
(NH ₄) ₂ SO ₄	6.000	
KH ₂ PO ₄	22.600	
MgSO ₄ .7H ₂ O	2.320	A
CaCl ₂ .2H ₂ O	0.220	B
FeSO ₄ .7H ₂ O	0.140	C
MnSO ₄ .4H ₂ O	0.040	D
ZnSO ₄ .7H ₂ O	0.040	E
CuSO ₄ .5H ₂ O	0.008	F
CoCl ₂ .6H ₂ O	0.008	G
olive oil	50 cm ³	

Medium A contained everything except magnesium sulphate
Medium B contained everything except calcium chloride
Medium C contained everything except ferrous sulphate
Medium D contained everything except manganese sulphate
Medium E contained everything except zinc sulphate
Medium F contained everything except cupric sulphate
Medium G contained everything except cobalt chloride
Medium H contained everything and was the control

The media were made up and an aliquot (500 cm^3) was added to each of the required number of baffled conical flasks (2 dm^3). The flasks were then sterilised by autoclaving (121°C for 20 minutes). At the start of the experiment the flasks were inoculated with 25 cm^3 (5%) of the inoculum culture (Section 4.2.2).

4.3.3 Fermenter experiments

To the fermenter vessel was added 2 dm^3 of Pirt's medium and yeast extract (except where otherwise stated). The medium, dissolved oxygen and pH probes were sterilised *in situ* by autoclaving (121°C for 15 minutes), with impeller speed set at 500 rpm, all other controls were switched off during sterilisation as was the cooling water flow. The glucose to be used during the batch stage of the fermentation ($1 - 10\text{ g/dm}^3$) was dissolved in 100 cm^3 in a 250 cm^3 side-arm flask and sterilised separately by autoclaving (121°C for 15 minutes).

The medium for Run 39 contained some extra trace salts. These are listed with their concentrations in Table 4.5. These extra salts were found in the defined minimal salts medium FAM II 20/20. The full medium description can be found elsewhere (Talbot 1990).

4.4 Glucose Feed for the Fermenter

For the constant fed fermentations the concentration of the glucose feed was 100 g/dm^3 . For the development of the growth medium experiments the glucose feed concentration was either 100, 200 or 350 g/dm^3 . For the exponential fed fermentations the glucose feed concentration varied from 80 g/dm^3 to as high as 350 g/dm^3 . One litre of the glucose solution to be used was sterilised in a conical flask (1 dm^3) and kept sterile with a foil-wrapped plug of non-absorbant cotton wool. A bung with a sterile air filter ($0.2 \mu\text{m}$ Gelman) and feed tubing were dry sterilised separately by autoclaving (121°C for 20 minutes) and added aseptically to the glucose feed flask just before use.

4.5 Sodium Hydroxide Feed for the Fermenter

A solution of sodium hydroxide (4N) was made up and sterilised in a conical flask by autoclaving (121°C for 15 minutes) kept sterile with a foil-wrapped plug of non-absorbant cotton wool. The bung and tubing was dry sterilised and added aseptically just before use. The flask was then connected to the fermenter via the alkali feed pump.

Table 4.5. Concentrations of extra trace salts added to Run 39

Chemical	Concentration (g/dm ³)
Na ₂ HPO ₄	1.7000
K ₂ SO ₄	0.1720
EDTA, Fe (III)	0.0040
H ₃ BO ₃	0.0030
Na ₂ MoO ₄ ·2H ₂ O	0.0020
NiCl ₂ ·6H ₂ O	0.0002

Method:

The components were added to 750 cm³ of distilled water in the stated order along with the components of Pirt's medium (Table 4.3) x4. The medium was made up to 1 dm³ with distilled water. The complete medium was sterilised by autoclaving (121°C for 20 minutes) in the fermenter. The pH of the medium was adjusted to 7.0 with sterile aliquots of NaOH (4N) after sterilisation.

4.6 Ammonium Hydroxide Feed for the Fermenter

The flask and tubing for the feed were dry sterilised. Aseptically added to this flask were sterile distilled water and '880' ammonium hydroxide in the ratio of 3 : 1. The flask was then connected to the fermenter via the alkali feed pump.

4.7 Start-up and Operation of a Fermenter Run

4.7.1 Fermenter

Before *in situ* sterilisation, the equipment was recalibrated. This was done using the recalibration sub-routine of the "DATALOG" program. The temperature probe was calibrated by cooling (18°C) and warming (40°C) the fermenter medium. The dissolved oxygen probe was calibrated by first passing nitrogen gas (0%) and then compressed air (100%), through the fermenter medium at the flow rate and impeller speed to be used during the fermenter run. The pH probe was calibrated by placing it in pH 4.0 buffer followed by pH 9.0 buffer. The gas analysers were calibrated using calibration gas of exact composition (nominal composition 8% CO_2 : 12% O_2) and compressed air, passed initially through silica gel and soda lime. The calibration constants were then saved on the program disc as a data file.

After *in situ* sterilisation, the temperature controller was set to 30°C and the cooling water flow turned on. The

impeller speed was increased to 1000 rpm to ensure good mixing. Once the fermenter vessel had cooled down, a sample of the sterile medium was removed and labelled 'first medium blank'. The pH and dissolved oxygen controllers were switched on. The pH was probe checked and calibrated by comparison of the indicated pH value with the value of 'first medium blank' measured on a previously calibrated laboratory pH meter. The pH of the medium after sterilising was in the range of 3.0 - 4.0. The pH was corrected to 7.0 by aseptic addition of sodium hydroxide (4N) through one of the spare ports. Once the pH was corrected the alkali line was connected to the fermenter and the line primed with solution. The pH controller set-point was set at 7.0. The sterile glucose was added to the fermenter medium aseptically and another sample was taken and labelled 'second medium blank'.

4.7.2 Computer

The program "DATALOG" was chosen from the software package, the feed profile parameters selected for the fermentation were entered and the set-up procedure worked through.

4.7.3 Manual operation

Except where otherwise stated, for all the fermentations reported here, the temperature, air flowrate and pH were constant, set at 30°C, 200 dm³/h and pH 7.0 respectively.

The agitation was increased manually to maintain a dissolved oxygen level greater than 20% saturation when necessary.

The fermenter was inoculated and a sample was removed and labelled 'zero hours'. For batch fermentations see Section 4.8.3. For constantly and exponentially fed fermentations, an adequate amount of cells (approximately 1.5 g/dm^3) was grown up initially in the batch part of the fermentation. The end of the batch part of the fermentation was signalled by the glucose concentration reaching zero, the dissolved oxygen and pH increasing; this time was noted and feeding was started as soon as possible after this time.

For constantly fed fermentations, feeding involved pumping a glucose solution, using a pre-calibrated pump, at the desired constant rate until the organism had reached stationary phase.

For exponentially fed fermentations, the reading from the balance was tared to zero at the start of feeding. As the glucose solution was fed, the mass became more negative. However, only absolute values were read by the program. Therefore, the total mass of solution transferred at any moment in time is easily obtained. Feeding continued until the glucose feed solution ran out.

4.8 Sampling Procedure

4.8.1 Shake flask experiments (250 cm^3)

One flask was removed every hour. An aliquot (1 cm^3) was used in the turbidimetric method of determining bacterial growth (Section 5.1.2); another aliquot (15 cm^3) was centrifuged ($17,000 \times g$ for 15 minutes). The pelleted cells were used for dry weight estimation (Section 5.1.3) and the cell-free supernatant (containing the lipase) was frozen in liquid nitrogen immediately and stored at -20°C until it could be assayed for pH (when measured), glucose concentration (Section 5.2), lipase activity (Section 5.3) (when measured) and ammonia concentration (Section 5.4) (when measured).

4.8.2 Shake flask experiments (2 dm^3)

Samples of 20 cm^3 were removed aseptically from the flasks at the end of an experiment and treated in the same way as the samples from the 250 cm^3 flasks (Section 4.8.1).

4.8.3 Fermenter experiments

For the batch fermentations, samples were taken every hour until the glucose ran out (this time being noted) and for 3 - 4 hours after this point. For the constant and exponential fed fermentations, the sampling frequency varied according to the length of feeding being at either one, two or three hour intervals. Two aliquots of approximately 20 cm^3 were collected at each sampling time

in sterile universals. The first aliquot was discarded and the second aliquot was treated in the same way as the samples removed from the 250 cm³ conical flasks (Section 4.8.1). The fermentations were constantly monitored for pH and dissolved oxygen (all runs) and the composition of the exhaust gas (Runs 16 to 60 only).

4.9 Shutdown

The end of any fermentation occurred when the dissolved oxygen and glucose concentration in solution started to increase and the bacterial growth, measured turbidimetrically, became stationary. A final sample was taken and the services and analysers were turned off and disconnected. The fermentation vessel was sterilised *in situ* by autoclaving (121°C for 30 minutes), washed out and cleaned.

4.10 Sample Preparation For Analysis

The frozen cell-free supernatants were returned to liquid form in the shortest possible time (to prevent denaturing the enzyme) by placing them in a 30°C water bath. Immediately after liquification, the samples were placed on ice until they were required. The samples were then ready to use in the Sigma Lipase assay (Section 5.3). Some of the samples had to be diluted for the reducing sugar determination (Section 5.2) to allow the glucose concentration to fall within the range of the standards.

All of the samples had to be diluted by 100 with distilled water for the ammonia assay (Section 5.4).

4.11 Dinitrosalicylic Acid (DNS) Solution

Dinitrosalicylic acid (2.5 g) was dissolved in approximately 100 cm³ of distilled water by warming. When the DNS had completely dissolved, 40 cm³ of 10% NaOH was added, followed by 75 g of Rochelle Salts. The solution was made up to 250 cm³ with more distilled water. This solution would keep for 14 days at 4°C.

4.12 Indicating Boric Acid Solution

Pure boric acid, H₃BO₃, (20 g) was dissolved in approximately 1 dm³ of distilled water by warming. When completely dissolved, methyl red (20 cm³ of 0.5 g/dm³) and methylene blue (0.4 cm³ of 15 g/dm³) were added with mixing. The solution was stable for several months.

CHAPTER FIVE

ANALYTICAL METHODS

5.1 Study of Bacterial Growth

Two different methods were used on their own and in conjunction with each other, to observe bacterial growth.

5.1.1 Turbidimetric method

Culture aliquots (0.5 or 1 cm^3) were diluted in sterile saline (0.9% w/v; 9.5 or 9.0 cm^3) and a sample (3 cm^3) of this was transferred to a cuvette with a 1 cm light path. The optical density of the sample was measured by the absorbance of scattered light of wavelength 580 nm using a Beckman DU-8 UV-Vis Computing Spectrophotometer (Analytical Instruments, High Wycombe, Bucks). Sterile saline (0.9% w/v) was used as a blank. Optical density values, where quoted, are for a dilution of 1 in 10 . When a dilution of 1 in 20 was used, the measured optical density value was multiplied by two.

5.1.2 Dry weight method

Culture aliquots (15 cm^3) were centrifuged ($17,000 \times g$ for 15 minutes) using a JA17 rotor in a Beckman J2 - 21 Centrifuge (Analytical Instruments, High Wycombe, Bucks). The supernatant was poured off and frozen immediately in liquid nitrogen. An aliquot (5 cm^3) of sterile saline

(0.9% w/v) was added to the pelleted cells. The cells were re-suspended by gentle shaking. The re-suspended cells were then transferred into pre-dried (70°C for 24 hours), pre-weighed aluminium foil boats. The pellets were then dried to constant weight (70°C for 48 hours). The boats were left to cool down to room temperature for half an hour and then weighed. By subtracting the weight of the boat it was possible to calculate the dry weight of the sample in g/dm³ and a sample calculation is shown in Appendix C. Two saline blanks were prepared for each run.

5.1.3 Correlation between methods

For Runs 17 to 30, the turbidimetric method was the only method used for monitoring bacterial growth. To convert optical density measurements to dry cell weight (g/dm³) a conversion graph was used (Figure 5.1). Data was taken from Runs 31 to 60 of optical density (580 nm) and dry weight (g/dm³). These values were plotted and linear regression calculated. A linear equation relating the optical density measurements to the dry cell weights was produced and can be seen in Figure 5.1.

5.1.4 Interference from pyocyanin

Pyocyanin was extracted from the cell-free supernatants of samples taken during Run 50 using the method described in Section 5.5. The chloroform extracts were scanned from 200 nm to 600 nm using the Beckman DU-8 UV-Vis Computing

Spectrophotometer. Even though the maximum optical density at lower wavelengths was greater than 2.0, at a wavelength of 580 nm, the absorbance of the chloroform extracts never exceeded 0.3 (Figure 5.2).

As samples taken from the fermenter were diluted (at least) by ten, it can therefore be said that the most the pyocyanin contributed to the optical density measurements was 0.03.

5.2 Reducing Sugar Determination

A crude estimation of the glucose content of samples removed from the fermenter was made by using a paper colourimetric indicator assay (Clinistix Reagent Strips, Ames Division, Miles Laboratories Ltd., Stoke Poges, Bucks) which gave a colour change at 10 seconds after wetting.

The concentration of glucose in the samples was determined as a reducing sugar equivalent using the dinitrosalicylic acid reagent method of Sumner and Somers (1944).

5.2.1 Standards

A standard glucose solution of concentration 0.5 mg/cm^3 was prepared. Five standard concentrations, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/cm^3 were prepared using the initial standard solution. A distilled water blank was used.

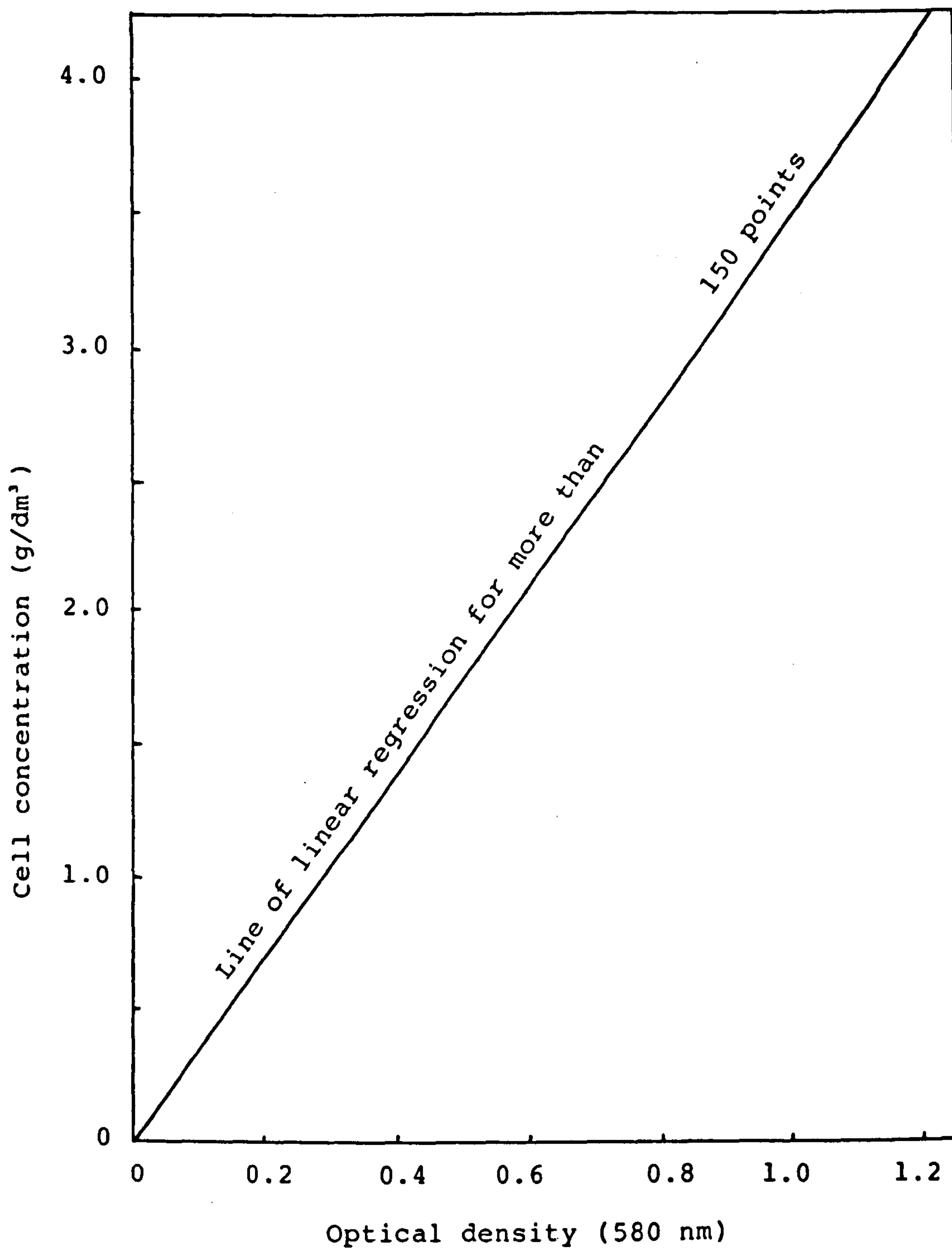


Figure 5.1. Conversion graph of optical density (580 nm) to dry cell weight (g/dm³)

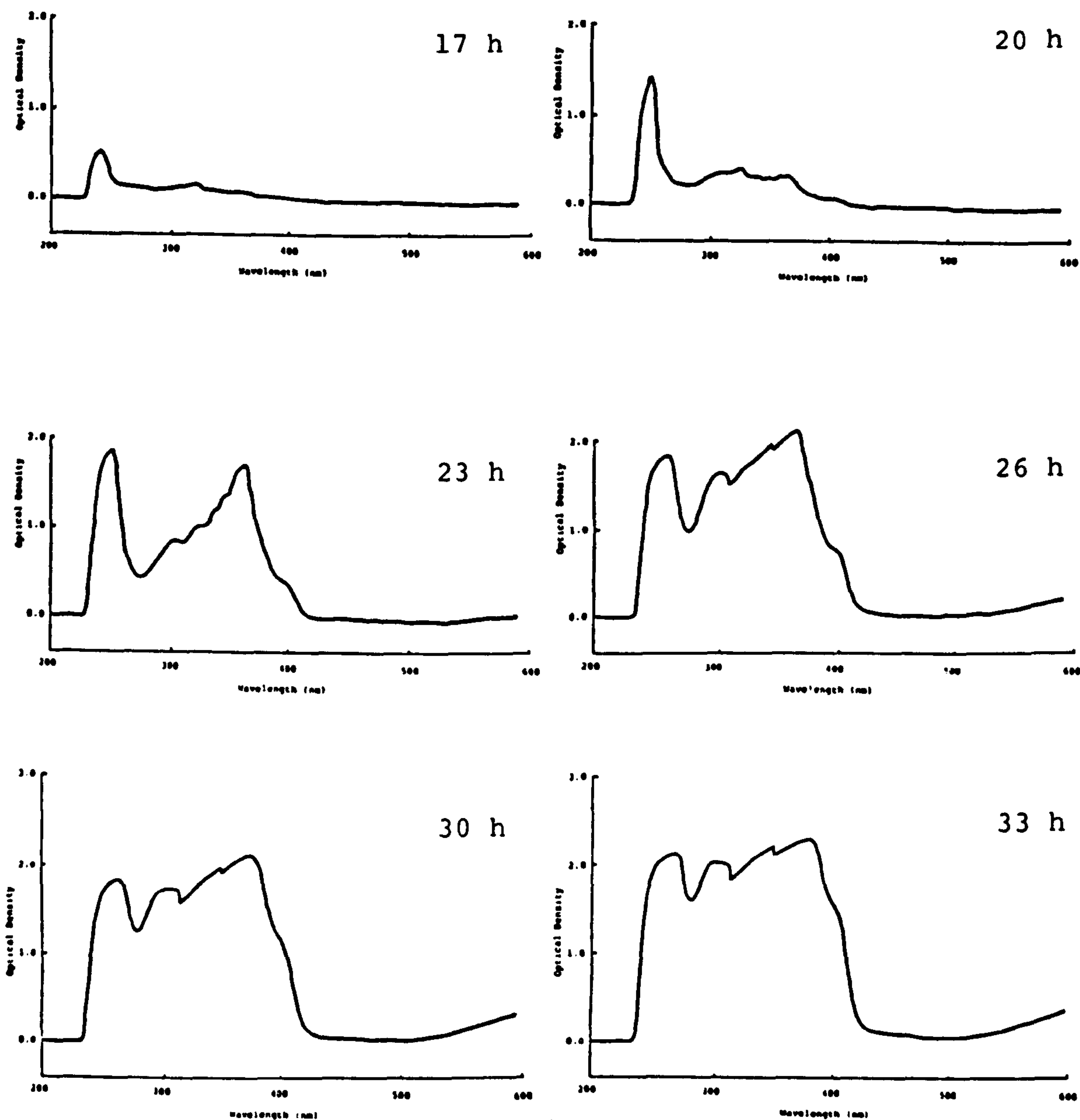


Figure 5.2. Chloroform extracts containing pyocyanin from Run 50. Samples were scanned between 200 and 600 nm (pyocyanin was apparent in the culture medium by 17 hours).

5.2.2 Procedure

To aliquots (1 cm^3) of standards, samples and blank, in test-tubes, DNS solution (1 cm^3) (Section 4.11) was added. To the sample blanks, distilled water (1 cm^3) was added. The contents of all the test-tubes were then thoroughly mixed, boiled for exactly 5 minutes and then cooled rapidly in iced water (to stop the reaction). The contents of all the test-tubes were then diluted with distilled water (10 cm^3) and thoroughly mixed again. The absorbance of all the solutions was read at 540 nm using distilled water as the blank. Values of reducing sugar concentration in the samples, were estimated from the standard curve. A sample calculation is shown in Appendix C.

5.3 Sigma Lipase Assay

There are two Sigma Lipase Assays described here. The first one is the standard procedure specified by Sigma in collaboration with Tietz (Section 5.3.1) and the lipase activity of the samples from the shake flask experiments (250 cm^3) (Section 6.2) and Fermentation Runs 1 to 46 (Sections 6.3, 6.4 and 6.5) were measured using this method. It was decided to use the Sigma Lipase Kit because the Lipase Substrate is stable and requires no preparation. However, this substrate is expensive so that the alternative 3 hour assay (suggested in the Sigma protocol) was considered to be an unnecessary expense

because it uses 10 cm^3 of the Lipase Substrate as opposed to 3 cm^3 used in the Six hour assay.

Work was carried out subsequent to the start of this study on alternative lipase assays but as the substrates made in the laboratory proved unstable, an investigation into reducing the incubation time of the Sigma Lipase Assay was carried out (Talbot 1990). It was found that 90% of the substrate conversion occurred in the first 2 hours of incubation. Thus, for Runs 48 to 60 (Section 6.7) the Two hour assay (Section 5.3.2) was used.

So that the lipase activity that was measured using the two incubation times could be compared with each other and with the results of other workers, the Sigma-Tietz Lipase Units were converted into standard Lipase Activity Units. One Lipase Activity Unit (LU) is defined as the number of μmoles of free fatty acid released per minute under test conditions. Sigma-Tietz Units can be related to the standard Lipase Activity Units by multiplying the number of μmoles in 1 cm^3 of NaOH (0.05M) and dividing by the length of the incubation time in minutes. The precise concentration of the NaOH was checked by titrating against HCl which had been standardised with Na_2CO_3 . It was found consistently that NaOH solution made up as 0.05M was actually 0.048M and the figure of 48 was taken as the number of μmoles in 1 cm^3 .

Therefore, Sigma-Tietz Units (Six hour assay) were converted to Lipase Activity Units (LU) by multiplying by a factor of 0.133 and Sigma-Tietz Units (Two hour assay) were converted to Lipase Activity Units (LU) by multiplying by a factor of 0.40.

5.3.1 Six hour assay

The procedure followed was:

1. To the required number of conical flasks (25 cm^3) (twice the number of samples), Tris - HCl buffer pH 7.0 (0.05M; 4 cm^3) and Sigma Lipase Substrate (olive oil emulsion) (3 cm^3) were added.
2. Half the flasks were covered with parafilm, these were the blanks.
3. To each of the tests, an aliquot (1 cm^3) of the appropriate sample was added. These flasks were also covered with parafilm.
4. All the conical flasks were then placed in a reciprocating water bath (40 strokes per minute and 37°C) for six hours.
5. To stop the reaction, 95% ethanol (BDH Ltd., Poole, Dorset) (3 cm^3) was added to all the conical flasks.
6. To each respective blank an aliquot (1 cm^3) of the appropriate sample was added.
7. Four drops of phenolphthalein indicator (in 1% ethanol) was then added to all the flasks.

8. All the flasks were then titrated against NaOH (0.05M) to the same definite blue end point.
9. To express lipase activity in Sigma-Tietz Units/cm³, the blank values were subtracted from the test values. One Sigma-Tietz Unit is defined as the volume (in cm³) of 0.05M NaOH needed to neutralise the free fatty acids released under test conditions.
10. To express lipase activity in standard Lipase Activity Units/cm³, the Sigma-Tietz Units/cm³ were multiplied by a factor of 0.133.

5.3.2 Two hour assay

The procedure followed was:

1. To the required number of beakers (25 cm³) (twice the number of samples), Tris-HCl buffer pH 7.0 (0.05M; 4 cm³) and Sigma Lipase Substrate (3 cm³) were added.
2. Half the beakers were covered with parafilm, these were the blanks.
3. To each of the tests, an aliquot (1 cm³) of the appropriate sample was added. These beakers were also covered with parafilm.
4. All the beakers were then placed in a reciprocating water bath (40 per minute and 37°C) for 2 hours.
5. To stop the reaction, 95% ethanol (3 cm³) was added to all the beakers.
6. To each respective blank, an aliquot (1 cm³) of the appropriate sample was added.

7. All the beakers were then titrated against NaOH (0.05M) to a pH end point of 10.2.
8. To express lipase activity in Sigma-Tietz Units/cm³, the blank values were subtracted from the test values. One Sigma-Tietz Unit is defined as the volume (in cm³) of 0.05M NaOH needed to neutralise the free fatty acids released under test conditions.
9. To express lipase activity in standard Lipase Activity Units/cm³, the Sigma-Tietz Units/cm³ were multiplied by a factor of 0.40.

5.4 Distillation and Titration Method for Assaying Ammonia

The procedure followed was:

1. Before assembling, the apparatus was thoroughly cleaned with ammonia-free water. The apparatus included a distillation flask, splash head and condenser.
2. A suitable volume of sample (100 cm³) was placed in the distillation flask and diluted with ammonia-free water (total volume 350 cm³).
3. Magnesium oxide (0.25 g) was then added to the flask.
4. The indicating boric acid solution (50 cm³) was then placed in a receiver (250 cm³ conical flask) and the lower end of the delivery tube from the condenser was kept below the surface of the absorbant liquid throughout the distillation.

5. The sample was distilled at a rate of approximately 10 cm^3 per minute.
6. When the level in the receiver had reached 150 cm^3 , the receiver was removed and the distillation stopped.
7. The contents of the receiver were then titrated against sulphamic acid (M/140) until the indicating boric acid solution returned to its original purple colour.
8. The ammonia nitrogen (mg/dm^3) is equal to the volume of M/140 acid used (cm^3), divided by the volume of the sample taken (cm^3), multiplied by 100. As the volume of the samples taken in all cases was 100 cm^3 , then the ammonia nitrogen (mg/dm^3) was equal to the volume of M/140 acid used (cm^3).
9. To convert the concentration of ammonia nitrogen in the samples which had been diluted by 100, to g/dm^3 , then the volume of M/140 acid used was divided by 10.

5.5 Pyocyanin Assays

5.5.1 Observation of pyocyanin

A visual observation of the green colouration of the culture was made for every run (Plate 1). Plate 2 shows the cell free supernatants from Run 24. It was noted that pyocyanin appeared in the fermenter at 17 hours. Samples taken from 18 hours onwards show the green colour.

5.5.2 Extraction of pyocyanin

The procedure followed was:

1. An aliquot (5 cm^3) of the cell-free supernatant of a sample was pipetted into a clean universal bottle.
2. A volume (5 cm^3) of chloroform was added to the bottle and the solvent and aqueous layers vigorously shaken together.
3. The contents of the bottle were then left for 10 minutes to separate.
4. Using a Pasteur pipette, approximately 1 cm^3 of the lower chloroform layer (containing the pyocyanin) was transferred to a quartz cuvette.
5. Using a Beckman DU-8 UV-Vis Computing Spectrophotometer, a scan from 200 nm to 600 nm was made of the sample using chloroform as a blank (also in a quartz cuvette), the change over between the two lamps being made at 315 nm.

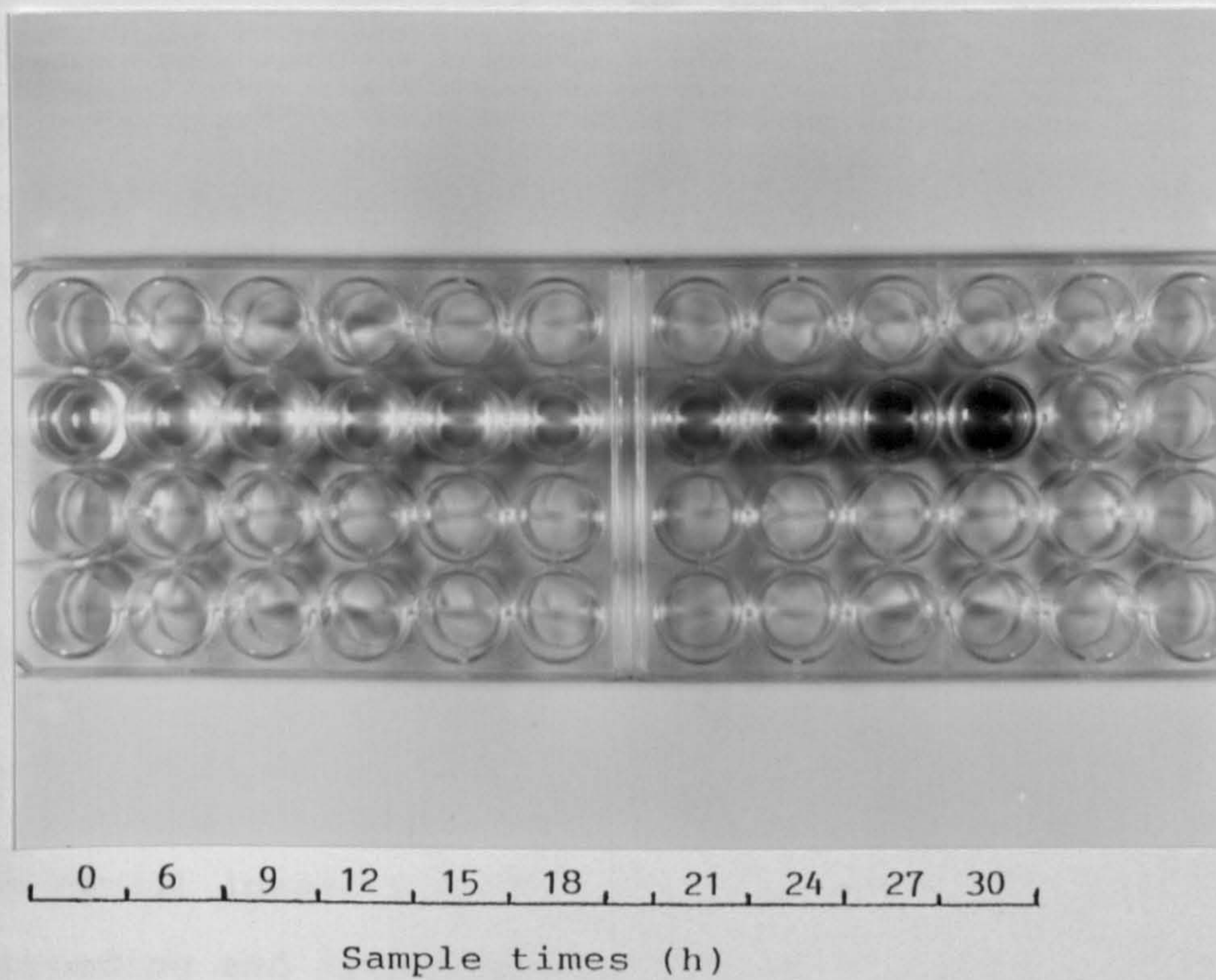


Plate 2. The cell free supernatants taken during Run 24.

CHAPTER SIX

EXPERIMENTAL PROGRAMME AND RESULTS

6.1 Introduction

A large number of experiments were carried out in shake flasks and a 2 dm³ stirred fermenter. During the fermenter work several parameters were continuously recorded.

However, not all the data that was collected is presented in this thesis. The results described here are selected examples to demonstrate particular points of discussion. Subsequent derived results and discussion are presented in Chapter Seven.

6.2 The Organism

6.2.1 Microbiological tests

On visual inspection, the colonies were large, flat-spreading and irregular, greyish green in colour. The blue/green pigment soon diffused into the agar. Older plates were red/brown in colour. The colonies were microscopically inspected and gram stained. The unicellular, curved or straight, motile rods were gram negative.

6.2.2 Biochemical tests

The organism was identified using a commercial biochemical test strip API 20 E which allows for the rapid performance of the biochemical tests described in Bergey's Manual of Systematic Bacteriology. The tests carried out are presented in Table 7.1 together with the results.

6.3 Shake Flask Experiments (250 cm³)

6.3.1 Yeast extract media

The first minimal medium to be tested was a semi-defined yeast extract medium containing glucose. The organism was incubated in media containing glucose (0.3% - 1.0%) and yeast extract (1% and 2%). The media details and results of final biomass, maximum lipase activity and final pH for these time course experiments can be found in Table 7.2. As well as being assayed for cell dry weight (Section 5.1.3), pH and glucose concentration (Section 5.2), samples were also assayed for lipase activity (Section 5.3.1) and small but measurable amounts were found (0.01 to 0.04 LU/cm³) (Table 7.2).

6.3.2 Pirt's medium

The initial experiment in this group used the defined minimal salts medium, Pirt's medium (x1) (Table 4.3) with glucose (22.7 g/dm³) as the carbon source. The results of biomass and pH for this time course experiment (S13) are

shown in Table 7.3. As a comparison, Pirt's medium (x1) with glucose (22.7 g/dm^3) was supplemented with 1 g/dm^3 yeast extract and a time course carried out. The results of this experiment (S14) are also shown in Table 7.3.

6.4 Batch Experiments in the Fermenter

Using Pirt's medium (x1) as the basic medium, the glucose and yeast extract additions were varied in order that a dry weight of approximately 1.5 g/dm^3 of cells could be grown up (prior to the start of the feed) in as short a time as possible.

Fifteen runs were carried out and the initial concentration of glucose (in the range from 22.7 g/dm^3 to 1 g/dm^3), yeast extract (in the range 0 g/dm^3 to 2 g/dm^3) and changes in the pH profile (Figure 7.2) were examined. Data was collected for each run on cell dry weight (Section 5.1.3), glucose concentration (Section 5.2) and lipase activity (Section 5.3.1). Data of particular interest have been extracted from each run and are shown in Table 7.4. At this stage the computer had not been connected to the fermenter so that only the dissolved oxygen and pH of the medium were monitored. Run 12 is shown in Figure 7.1 as an example but the other runs followed very similar patterns.

6.5 Constant Feed Experiments

A series of constant feed fermentations (Runs 14 - 30) was carried out and the glucose feed rate was varied from 0.19 to 4.50 g/dm³/h (the effects of which are shown in Figures 7.5, 7.6 and 7.7). The basic fermenter medium for these runs was Pirt's medium (x1) plus 1.0 g/dm³ each of glucose and yeast extract, except for Runs 25 and 27 when Pirt's medium (x2) was used. Data was collected for each run on optical density (Section 5.1.2), glucose concentration (Section 5.2) and lipase activity (Section 5.3.1). Dissolved oxygen and pH were monitored along with the percentages of carbon dioxide and oxygen in the exhaust gas from the fermenter. The run details and typical results from Runs 14 - 16, 24 - 26 are presented in Table 7.6. Figure 7.4 shows Run 23 as a typical run but the other runs followed very similar patterns.

6.6 Developing the Growth Medium

The aim of the next group of experiments (Runs 31 - 46) was to develop the fermentation medium so that it could support a dry weight of 25 g/dm³ of cells.

Carbon was maintained in excess by increasing the glucose feed pumping rate manually, when necessary, during the progress of each run. Dissolved oxygen was maintained in excess of 20% by increasing the impeller speed when necessary. Samples were assayed turbidimetrically

(Section 5.1.2), for dry cell weight (Section 5.1.3) and glucose concentration (Section 5.2). Dissolved oxygen, pH and the percentages of carbon dioxide and oxygen in the exhaust gas from the fermenter were monitored continuously. The changes to the medium and their effects on the final biomass and linear growth rate are presented in Table 7.7. The profile of Run 31 is shown in Figure 7.10. The biomass data for Runs 33, 38, 42 and 43 are presented in Figure 7.11 and Run 46 in Figure 7.15.

6.7 Exponential Feed Experiments

Once a medium had been derived which would support a dry weight of 25 g/dm^3 of cells, the organisms were fed glucose solutions exponentially via a pump controlled by a micro-computer program. The aim of these experiments (Runs 48 - 60) was to find an optimum specific growth rate for the production of the lipase by growing the organisms at different specific growth rates as dictated by the computer program.

Samples were assayed turbidimetrically (Section 5.1.2), for dry cell weight (Section 5.1.3), glucose concentration (Section 5.2), lipase activity (Section 5.3.2) and ammonia concentration (Section 5.4). Dissolved oxygen, pH and the percentages of carbon dioxide and oxygen in the exhaust gas from the fermenter were monitored continuously. The run details and results of these fermentations are shown

in Table 7.8. A profile of Run 55 is shown in Figure 7.19 as an example of a typical exponential fed experiment.

The accuracy of the glucose feed control is shown in Table 7.9. Figure 7.17 shows the logged balance values of the glucose feed from Run 58 and how the balance readings were converted into quantities of glucose added per hour which were then plotted on a log scale and an exponential function fitted to the data. Figure 7.18 shows Runs 50, 56, 57 and 60 as examples of the biomass and carbon dioxide data plotted on log scales. The specific growth rates during each run were calculated from biomass and carbon dioxide data and these calculated rates for each run are compared to the value of "B" in Table 7.10. The lipase activities of the samples taken from Runs 49 to 60 are presented in Table 7.12.

6.8 Shake Flask Experiments (2 dm³)

The aim of these experiments was to determine whether or not the induction of the production of pyocyanin was caused by a trace salt limitation. The colour of the culture medium was noted and the cell concentrations of the samples were measured using the turbidimetric method (Section 5.1.2). The results of these experiments are shown in Table 7.14.

CHAPTER SEVEN

DISCUSSION

7.1 Introduction

Before the selected organism was grown in a fermenter, biochemical tests were carried out on it and a defined minimal salts medium was identified in which the organism would grow and produce a lipase without the presence of a lipid substrate. The results of the tests and experiments carried out are analysed in Sections 7.2 and 7.3 respectively. Having ascertained that the organism would grow and produce a lipase in the chosen minimal salts medium, the organism was grown in a small laboratory fermenter under batch conditions. The effects of changing the concentration of medium components (such as yeast extract and glucose) and the pH profile, on the biomass and lipase produced, are investigated in Section 7.4. Cells grown up under batch conditions were subsequently fed glucose solutions at different constant feed rates and the correlations between feed rate and linear growth rate and feed rate and lipase production are examined in Section 7.5. Section 7.6 describes how the medium composition was developed in order to increase the dry weight of cells from 5 g/dm^3 to 25 g/dm^3 and the nutrient limitations that were discovered whilst carrying out these

experiments are analysed. In order that lipase production might be maintained throughout the growth period, a computer program controlled the feed of glucose solutions at different exponential rates to cells grown up under batch conditions. The technical and physiological problems encountered are covered in Section 7.7. Finally, in Section 7.8, the problem of pyocyanin is examined and the attempts made to prevent its production are compared with and contrasted to published work.

7.2 The Organism

The organism was found to be a gram negative rod. It liquified gelatin, gave positive oxidase and arginine dihydrolase reactions and produced acid from glucose. It did not form indole or produce an acid from sucrose and the Voges-Proskauer test was negative.

From a comparison between the identification table provided with the commercial biochemical test strip API 20 E and the results listed in Table 7.1, the organism was identified as a species of *Pseudomonas* but no distinction could be made between the species *fluorescens* and *aeruginosa*. Some of the differences between *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, as quoted in Bergey's Manual of Determinative Bacteriology are: *Ps. fluorescens* produces a diffusible fluorescent pigment, pyoverdine, although some strains produce a non-diffusible blue pigment. In contrast *Ps. aeruginosa*

Table 7.1. Results of API 20 E Biochemical Test Strip

Tests	Sustrate	Reaction/Enzymes	Result
ADH	arginine	arginine dihydrolase	+ve
CIT	sodium citrate	citrate utilisation	+ve
URE	urea	urease	+ve
GEL	Kohn's gelatin	gelatinase	+ve
GLU	glucose	oxidation	+ve
OX	on filter paper	cytochrome-oxidase	+ve
IND	tryptophane	indole production	-ve
VP	sodium pyruvate	acetoin production	-ve
ONPG	ortho-nitro-phenyl-galactoside	beta-galactosidase	-ve
LDC	lysine	lysine decarboxylase	-ve
ODC	ornithine	ornithine decarboxylase	-ve
H ₂ S	sodium thio-sulphate	H ₂ S production	-ve
TDA	tryptophane	tryptophane deaminase	-ve
MAN	mannitol	fermentation/oxidation	-ve
INO	inositol	fermentation/oxidation	-ve
SOR	sorbitol	fermentation/oxidation	-ve
RHA	rhamnose	fermentation/oxidation	-ve
SAC	sucrose	fermentation/oxidation	-ve
MEL	melibiose	fermentation/oxidation	-ve
AMY	amygdalin	fermentation/oxidation	-ve
ARA	arabinose	fermentation/oxidation	-ve

produces a diffusible fluorescent pigment and it also makes a soluble phenazine pigment, pyocyanin (blue/green in neutral or alkaline media, red in acid media).

As has already been noted (Section 6.1.1), the tributyrin agar plates on which the organism was maintained soon became blue/green in colour, as did cultures grown in LP₂SF medium. Both of these sources changed to a red/brown colour following a reduction in pH. The plates and cultures did not fluoresce under UV light.

From the above observations, it was concluded that the organism that was used for this project was a strain of *Pseudomonas aeruginosa*. This conclusion conflicted with the one made by Dr. Sztajer (Section 4.1.1). Taxonomic investigations were carried out by Sztajer et al. (1988) mainly according to Bergey's Manual of Systematic Bacteriology and she concluded that this same organism was a strain of *Pseudomonas fluorescens*.

Pseudomonas aeruginosa is known to produce many secondary metabolites. Harvey et al. (1990) demonstrated the production of a glycolipid emulsifier by a strain of *Ps. aeruginosa* that can effectively reduce the surface tension of an oil-water interface and act as a potent dispersant of oil in water. Ramana and Karanth (1989a) found that glycolipid appearance in the fermentation broth of *Ps. aeruginosa* coincided with the exhaustion of nitrogen and the commencement of the stationary phase with

respect to biomass. Ramana and Karanth (1989b) increased the productivity of glycolipids two-fold by transferring cells after the exponential growth phase into a medium devoid of phosphate. Robinson et al. (1984) showed that nitrogen limitation in *Ps. aeruginosa* shifts partitioning of substrate carbon toward exopolysaccharide production, reducing the yield of cellular biomass. Bayer et al. (1989) found that *Ps. aeruginosa* grown at an oxygen tension of 80 mmHg produced more exopolysaccharide than similar cells grown at 40 mmHg.

As well as producing pyocyanin, the colonies of the strain of *Ps. aeruginosa* used in this work also produced a diffusible lipase. When the organism was grown on tributyrin agar plates, the oily emulsion cleared around the colonies demonstrating that the lipase being produced was capable of hydrolysing synthetic lipids. The lipase also hydrolysed natural lipids such as olive oil (as illustrated in the Sigma Lipase Assay (Section 5.3) which uses an olive oil emulsion as the substrate).

7.3 Shake Flask Experiments (250 cm³)

7.3.1 Introduction

To investigate whether the lipase produced by the organism was under the control of induction by a lipid (or its metabolite) or by catabolite repression, the organism was grown up in various minimal media with no lipid substrate present.

It had been demonstrated by Sztajer (1987) that a strain of *Pseudomonas fluorescens* grew very well in the complex medium LP₂SF (Table 4.2) with olive oil as the lipid inducer and produced a lipase in measurable amounts (approximately 0.02 LU/cm³). However LP₂SF medium contains a high percentage of insoluble solids and was therefore considered to be unsuitable for use with controlled nutrient feed systems in a fermenter. A simple medium had to be found that was capable of supporting the growth and lipase production of the strain of *Ps. aeruginosa* described in this work.

7.3.2 Yeast extract media

Starting from a glucose concentration of 1.0% and using the known percentage of nitrogen in yeast extract, the concentration of yeast extract necessary (such that the carbon runs out before the nitrogen, thus preventing the production of other secondary metabolites (Section 7.2)) was calculated to be 1.0% (Appendix A). Typical Enterobacteriaceae, such as *Escherichia coli*, grown in a medium containing 1% glucose, would be expected to have a cell doubling time of one hour and to use all the glucose in about six hours. Because the yield on glucose is 0.5 g/g, a dry mass of approximately 5 g/dm³ of cells would be expected to be produced from such a medium. Experiments were carried out in shake flasks to investigate the effect of yeast extract and glucose concentrations and the results are listed in Table 7.2.

Table 7.2. Results of growing *Ps. aeruginosa* in yeast extract media in shake flasks

Run No.	<u>Medium composition</u>		Glucose left in solution	Final biomass	Final pH	Length of incubation (h)	Maximum lipase activity (LU/cm ³)
	Yeast extract (% (w/v))	Glucose (% (w/v))	(% (w/v))	(g/dm ³)			
S1	1.00	1.00	0.85	2.00	7.39	10.0	0.07
S2	1.00	1.00	0.81	2.20	7.80	9.0	0.08
S3	1.00	1.00	0.79	2.60	7.74	11.0	0.03
S4	1.00	0.75	0.63	1.80	7.63	7.0	0.03
S5	1.00	0.50	0.31	1.80	7.70	6.0	0.03
S6	1.00	0.50	0.32	2.80	7.60	6.0	0.01
S7	1.00	0.30	0.25	1.80	7.71	8.0	0.01
S8	1.00	0.30	0.23	1.60	7.70	10.0	0.01
S9	1.00	0.50	0.30	2.60	7.67	10.0	0.02
S10	2.00	0.50	0.34	3.00	7.81	10.0	0.01
S11	1.00	0.50	0.32	2.60	7.63	9.0	0.03
S12	2.00	0.50	0.38	2.80	7.78	9.0	0.02

The initial pH of the flasks in all the experiments was 7.0.

LU = μ moles of free fatty acid released per minute under the test conditions

When *Pseudomonas aeruginosa* was grown up in a medium containing 1.0% yeast extract and 1.0% glucose, there was little change in the glucose concentration in the culture medium over ten hours of incubation and the pH became alkaline (Table 7.2: Runs S1 - S3).

When yeast extract concentration was kept constant at 1% and the glucose concentration was decreased to 0.75% (Run S4), 0.50% (Runs S5, S6, S9 and S11) and 0.30% (Runs S7 and S8), there was little change in the final pH (it was still alkaline) and there was still more than 50% of the glucose left unused in the culture medium (Table 7.2).

The total biomass that was produced (Table 7.2) could not have been made solely from the glucose used. Therefore it was concluded that the organism was using the yeast extract as a carbon source as well as a nitrogen source. (For example, Run S1: $(1.00\% - 0.85\%) = 0.15\%$ (w/v) glucose used. With an approximate yield of cells on glucose of 0.5 g/g this amount of glucose would produce 0.75 g/dm^3 dry weight of cells, yet 2.0 g/dm^3 dry weight of cells was produced.)

Small but measurable amounts of lipase were produced (approximately 0.03 LU/cm^3 : Table 7.2) from which it was concluded that lipase production in this strain of *Pseudomonas aeruginosa* was not under the control of

induction and was probably controlled by catabolite repression.

These experiments showed that the organism would grow on a yeast extract-glucose mixture. This is in contrast to the media used by many workers studying lipase production (Table 2.3) which tended to be complex and expensive. In an attempt to resolve further the nutritional requirements of this organism, a defined minimal salts medium using glucose as the carbon source was required. Pirt's medium (Pirt 1975) is a typical defined minimal salts medium because it has the basic requirements for growth (carbon, nitrogen and phosphate sources) as well as a range of metal ions.

7.3.3 Pirt's medium

The initial experiment used Pirt's defined salts medium (Table 4.3) with glucose (22.7 g/dm^3) as the carbon source. The result of this experiment (S13) is shown in Table 7.3. The organism grew very slowly and only just produced a dry weight of 1.0 g/dm^3 of cells after ten hours of incubation. However, in the yeast extract-glucose medium a dry weight of at least 1.6 g/dm^3 and as much as 3.0 g/dm^3 of cells had been produced after the same incubation time (Table 7.2: Runs S1, S8 - S10). It was possible that the organism had a need for some component in the yeast extract and it was thus decided to supplement Pirt's minimal salts medium with 1 g/dm^3 yeast extract.

Table 7.3. The Results of growing *Ps. aeruginosa* in Pirt's medium (x1) and Pirt's medium (x1) supplemented with 1 g/dm³ yeast extract

Time (h)	Run Number		
	S13	S14	
	<u>Pirt's medium</u>	<u>Pirt's + 1 g/dm³ Y.E.</u>	
	Biomass (g/dm ³)	Biomass (g/dm ³)	pH
0	0.10	0.10	7.00
1	0.10	0.14	
2	0.14	0.54	6.45
3	0.20	0.86	
4	0.26	1.08	6.42
5	0.32	1.50	
6	0.42	1.78	6.17
7	0.58	2.40	
8	0.72	2.84	5.78
9	0.86	3.14	
10	1.02	3.20	4.84

The results of this experiment (S14) are also shown in Table 7.3. The organism produced a dry weight of 3.2 g/dm^3 of cells and the culture medium pH became acidic after ten hours of incubation.

7.3.4 Conclusion

A semi-defined medium (Pirt's medium plus 1 g/dm^3 yeast extract) had therefore been found which supported the growth of the organism and could be used in a stirred fermenter.

7.4 Batch Experiments in the Fermenter

7.4.1 Introduction

There are many disadvantages in using shake flasks (each flask has a slightly different environment and it is not possible to monitor or control pH changes, aeration rates or foaming) and therefore a stirred fermenter was used to carry out detailed time-course experiments.

The lipase-producing organism was grown up under batch conditions in a laboratory fermenter in the defined minimal salts medium described by Pirt (1975), supplemented with yeast extract. A typical batch run is first described. The effect of varying the glucose concentration on the time it took for the glucose concentration to reach zero and the effect of changing the yeast extract concentration were examined. The factors

affecting lipase production such as the effects of changing the pH profile during seven of the batch experiments are analysed and the occurrence of linear growth during the batch fermentations and its possible causes are investigated in this section.

7.4.2 Profile of a typical run

The results of the various data collected for Run 12 are shown in Figure 7.1 and in Appendix B. The fermentation was run for 13 hours and the maximum lipase activity of 0.173 LU/cm^3 (Table 7.4) was achieved at 12 hours. The lipase was made at two points during the growth phase. The first occurred between 5 and 6 hours when some component other than glucose became limiting and caused the growth and subsequent usage of glucose to slow down dramatically. The biomass was seen to change at this point, from an exponential trend ($\mu = 0.33 \text{ h}^{-1}$: Appendix C) into a linear trend, as did the usage of glucose.

The lipase activity remained approximately constant between 6 and 10 hours, whilst the biomass increased linearly (slope = $0.250 \text{ g/dm}^3/\text{h}$) and the glucose was used linearly. The dissolved oxygen decreased from 100% to 10% saturation between 0 and 10.5 hours. The glucose ran out at 10.5 hours and the second aliquot of lipase was made. Also at 10.5 hours the dissolved oxygen rose rapidly from 10% to 90% saturation and the pH was reduced from its controlled value of 7.0 to 6.0 by manual additions of

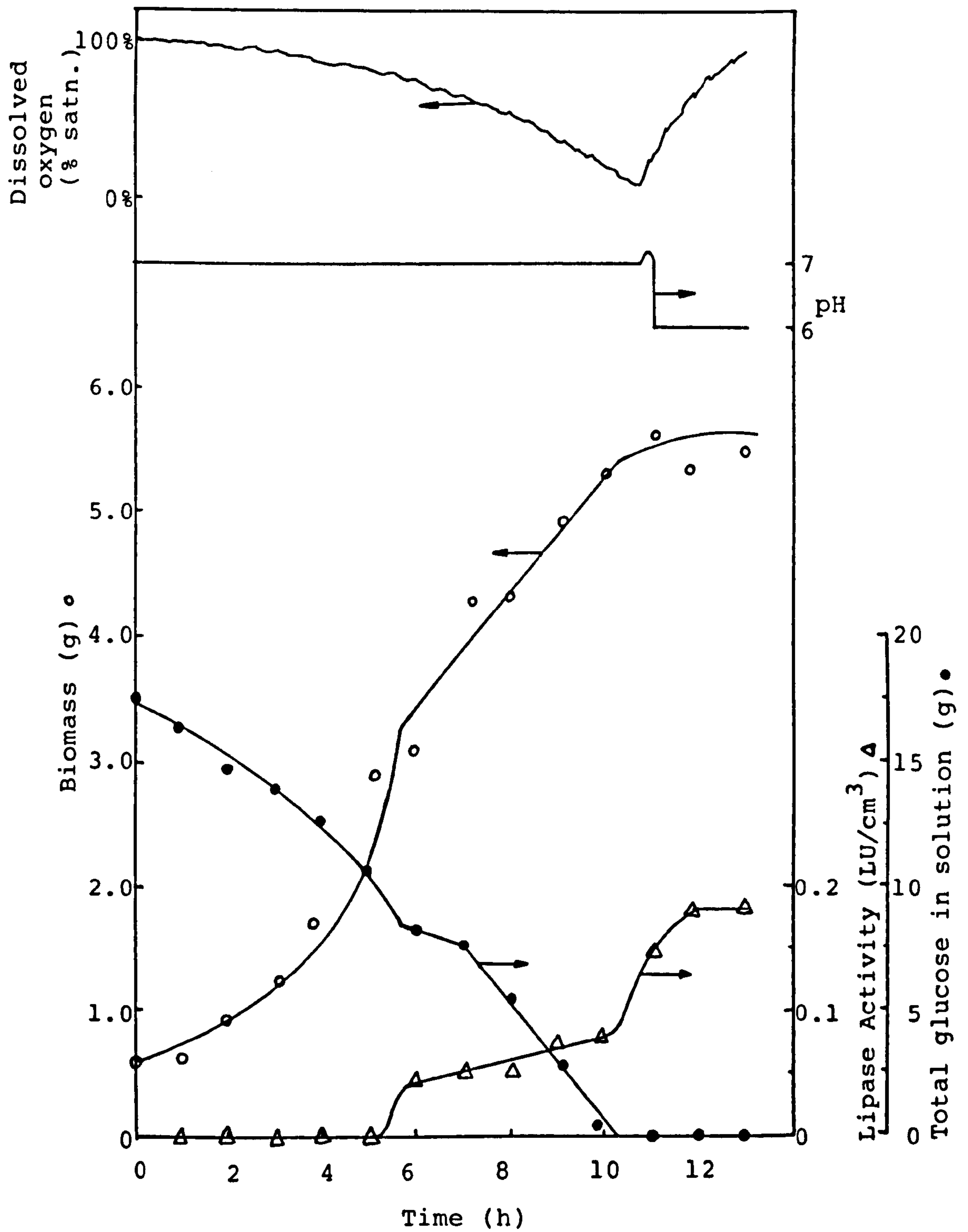


Figure 7.1. Run 12: A typical batch experiment

Table 7.4. Batch Fermentations: Conditions and Results

Run No.	Glucose Concn. (g/dm ³)	Y.E. Concn. (g/dm ³)	Time Glucose Concn. Reached Zero (h)	Linear Growth Rate (g/dm ³ /h)	Biomass at Which Linear Growth Started (g)	Final Lipase Activity (LU/cm ³)
1	22.7	1.0	>48.0	0.298	2.20	0.040
2	11.4	1.0	14.0	0.280	1.50	0.093
3	11.4	1.0	13.0	0.260	2.40	0.080
4	7.5	1.0	9.0	0.352	2.90	0.107
5	5.0	1.0	11.0	0.337	3.10	0.080
6	5.0	2.0	12.0	0.118	2.30	0.147
7	5.0	2.0	9.0	0.293	2.30	0.133
8	5.0	0.5	11.0	0.444	3.60	0.107
9	5.0	0	(a)			
10	5.0	1.0	(b)			
11	5.0	1.0	10.0	0.384	1.70	0.067
12	5.0	1.0	11.0	0.250	3.10	0.173
13	5.0	1.0	12.0	0.323	1.90	0.080
14(c)	2.0	1.0	9.0	(d)	(d)	0.067
15(c)	1.0	1.0	7.0	(d)	(d)	0.080

(a) Did not grow

(b) Fermentation aborted

(c) Subsequently fed as constant feed experiments (Table 7.5)

(d) Not applicable

LU = μ moles of free fatty acid released per minute under the test conditions

acid. The values of the time taken for glucose to reach zero, the slope of the linear growth phase, the biomass at which linear growth starts and the final lipase activity for this and the other batch experiments were abstracted from the run data and are listed in Table 7.4. These results are discussed below.

7.4.3 Effect of medium composition on the length of time of a run

The ultimate aim of this work was to be able to feed glucose solutions to cultures at slow rates, thereby controlling their growth rates. In order to do this, a small amount of cells, sufficient to indicate that the culture was well established, was required to be grown up in as short a time as possible with all the initially added glucose consumed. The concentration of glucose suggested in the original Pirt's medium (22.7 g/dm^3) created a run time of greater than 48 hours (Table 7.4: Run 1) which was far too long for the purposes of the future work. The concentration of the initial glucose addition was eventually reduced to 1.0 g/dm^3 because this produced a dry weight of 1.5 g/dm^3 of cells (considered a sufficient amount) in 7 hours (Table 7.4: Run 15). Following this result, an initial glucose addition of 1.0 g/dm^3 was used in Runs 15 to 60.

Increasing the yeast extract concentration from 1 g/dm^3 to 2 g/dm^3 had little effect on the time it took for the

glucose concentration to reach zero (this can be seen by comparing Runs 6 and 7 with Runs 5, 11, 12, and 13 in Table 7.4) but when yeast extract was omitted altogether the cells failed to grow (Table 7.4: Run 9).

7.4.4 Lipase production

The lipase activity in most of the batch fermentation runs was up to an order of magnitude higher than that measured in the shake flasks (Section 7.3.1) because in these runs the glucose was observed to run out, causing the growth rate to pass through a low enough value for enzyme systems to be de-repressed. The final lipase activities determined in these experiments (Table 7.4) were very similar to Dr. Sztajer's results (Sztajer et al. 1988) that is, approximately 0.133 LU/cm³.

During Runs 2 to 5, it was noted that the lipase activity that was produced (once the glucose had been used up) 'decayed' rapidly (Figure 7.2: Run 4). A possible explanation for this was found in literature concerning commercial lipases (Godtfredson 1988): even though *Pseudomonas aeruginosa* has an optimal growing pH of 7.0, the lipase that it produces could be highly labile at neutral pH. It was decided in this work, to lower the pH of the fermentation medium to 6.0 (by the aseptic manual addition of sterile HCl (1M)) (Figure 7.2: Run 12) once the glucose had run out and the organism had stopped

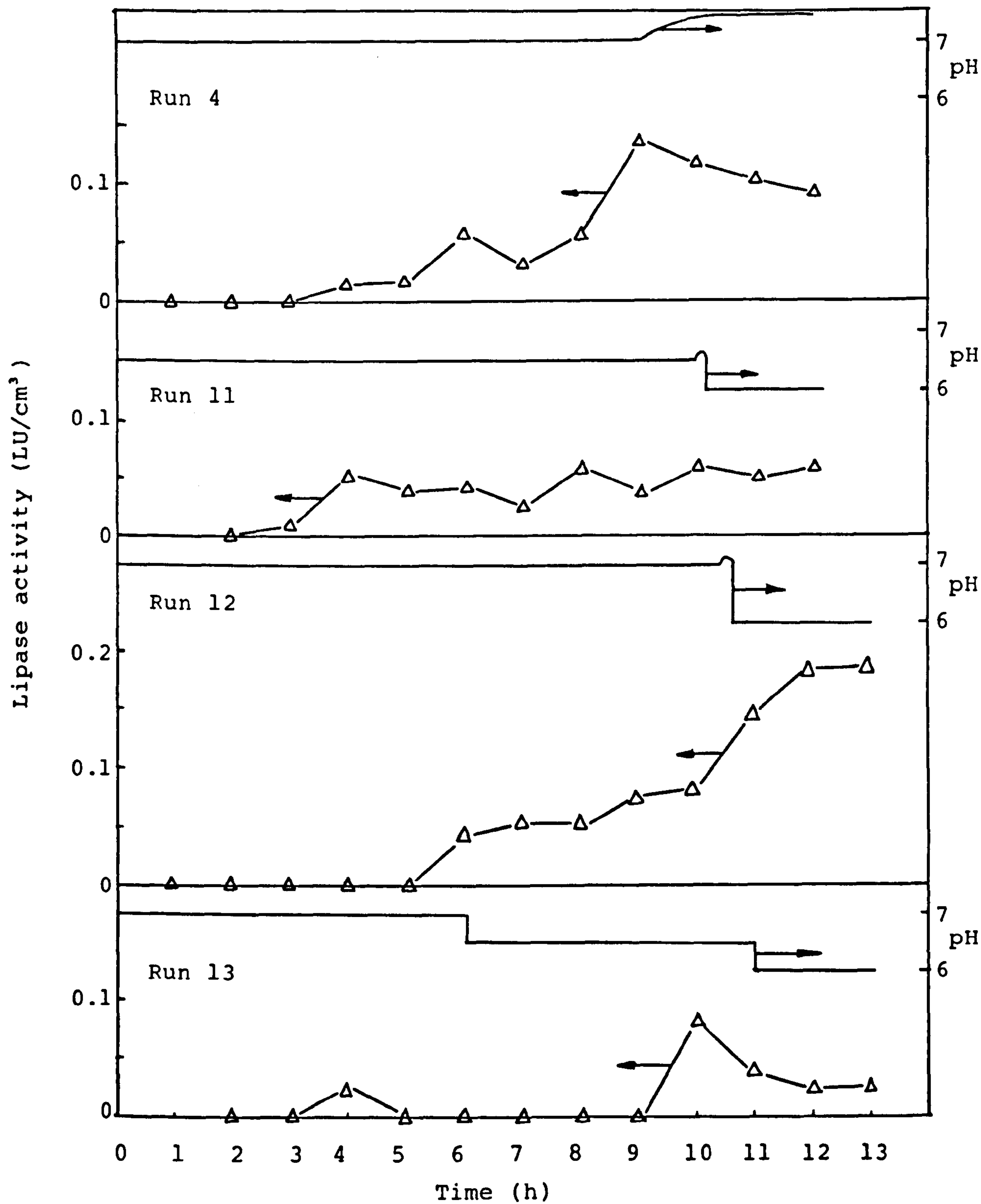


Figure 7.2. The effects of changing the pH profile on lipase production

growing. This, it was hoped, might protect any lipase which had been made. This procedure was followed for Runs 6 to 16.

7.4.5 Effect of changes in the pH profile

In order to investigate the effect of pH on lipase production, Run 11 was carried out at pH 6.5 (Figure 7.2). It was hoped that any lipase produced during the fermentation would remain stable until the pH was reduced to 6.0 when the glucose ran out. The effect of lowering the pH was to reduce the growth rate (which would be expected (Section 2.3.5)) but it also more than halved the amount of lipase produced (a maximum of 0.067 LU/cm^3 as opposed to 0.173 LU/cm^3 (Run 12)) (Figure 7.2). During Run 13 the pH was lowered in a stepwise progression: the pH was maintained at 7.0 for the first six hours, then it was lowered to 6.5 and further reduced to 6.0 at 10 hours when the glucose ran out. The effect was to reduce greatly the overall production of lipase even though there was a sudden burst of production between 9 and 10 hours which rapidly 'decayed' (Figure 7.2). The procedure followed in Run 12 was carried out on Runs 14, 15 and 16 (Section 7.5) but when the lowering of the pH to 6.0 did not prevent the lipase that had been produced from 'decaying' once all the glucose had been consumed, this practice was abandoned.

7.4.6 Linear growth

The expected profile of a batch fermentation is shown in Figure 2.3 and comprises of a series of phases: lag phase, exponential phase, stationary phase and death phase. When the data from the batch fermentations was presented in a graphical form, it could be seen that a fifth phase was passed through. After growing exponentially for a length of time (between 3 and 5 hours) there was a period of linear growth before the stationary and death phases were entered (Figure 7.1).

The implication from the above observation is that the batch fermentations were suffering from a conservative trace nutrient limitation (Section 2.4.3). From the work performed by Gaddum (1988), the conservative trace nutrient may have been found in the yeast extract supplement. Therefore, *Ps. aeruginosa* was grown in Pirt's medium under batch conditions with various initial concentrations of yeast extract (0.5, 1.0, and 2.0 g/dm³) (Figure 7.3). In Gaddum's experiments, when the amount of yeast extract that was added was doubled, the slope of the linear growth was doubled and linear growth commenced at a higher biomass concentration. In the experiments carried out in this work, the effect of increasing the yeast extract concentration has the opposite effect. Relevant data, shown graphically in Figure 7.3 and summarised in Table 7.4, is re-presented for convenience in Table 7.5.

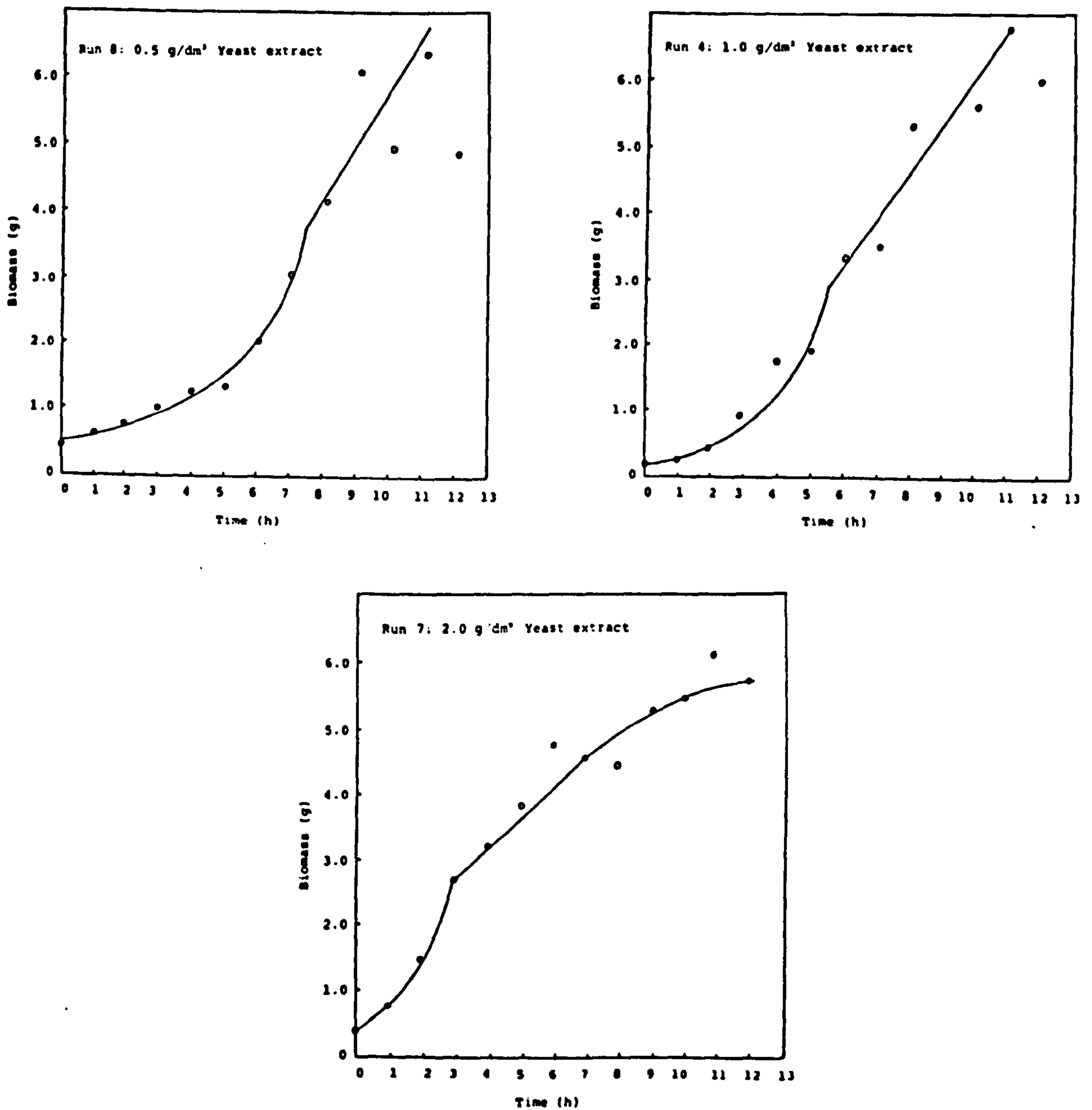


Figure 7.3. Typical examples showing the effect of changing yeast extract concentration on biomass production during the batch experiments

Table 7.5. The effect of changing yeast extract concentration on the commencement and slope of linear growth

Run Number	8	4	7
Concentration of yeast extract (g/dm^3)	0.5	1.0	2.0
Slope of linear growth phase ($\text{g/dm}^3/\text{h}$)	0.44	0.35	0.29
Time of start of linear growth (h)	7.50	5.40	2.40
Biomass at start of linear growth (g)	3.60	2.90	2.30

As the yeast extract concentration is increased the linear growth slopes become less steep and linear growth occurs sooner and at lower cell concentrations.

Assessing the batch fermentations as a whole (Table 7.4), there are differences in the slopes of the linear growth phases and in the cell concentrations at which the linear growth phase starts. Because yeast extract was eliminated as the source of the trace nutrient causing the limitation, another possibility could be that the limiting nutrient was one of the components of Pirt's medium. However, concentrations of the salts were not altered deliberately during any of the batch fermentations carried out here. Therefore, the conservative trace nutrient must have been available in different amounts at the beginning of each run due to reasons other than medium composition. One of these reasons could be the complexing and/or precipitating of salts in the medium during sterilisation. Another reason could be that the yeast extract somehow adsorbs the limiting component and therefore reduces its availability and as the concentration of yeast extract in solution increases so the amount of limiting component decreases.

Unfortunately carbon dioxide concentrations of the exhaust gas were not measured during the batch fermentations, as such data might have confirmed the precise moment when the exponential growth rate changed to a linear growth rate

and the exact value of the slope. But this change can nonetheless be seen clearly in the glucose usage profile of Run 12 (Figure 7.1). Glucose is used at an exponential rate during exponential growth and is used linearly during linear growth and this linear growth stops when the glucose runs out.

7.4.7 Conclusions

It was found that Pirt's recommended glucose concentration of 22.7 g/dm^3 created a glucose consumption time that was too high for the purposes of this work and the glucose concentration was eventually reduced to 1 g/dm^3 . A yeast extract addition of 1 g/dm^3 was found to affect the glucose consumption time the least. It was found that changing the pH profile from a controlled pH of 7.0 resulted in a decrease in lipase production. It was observed that the growth of the organism passed through a linear phase after an exponential phase and it was suggested that the yeast extract could be adsorbing a trace nutrient thus causing its limitation.

Because there was production of lipase around the time that the glucose ran out (that is, when enzyme production systems would become de-repressed) it can be said confidently that lipase production in this strain of *Pseudomonas aeruginosa* is under the control of catabolite repression.

As the change over from exponential growth to linear growth did not seem to prevent lipase production, it was not investigated further at this stage.

7.5 Constant Feed Experiments

7.5.1 Introduction

The glucose and yeast extract concentrations were adjusted such that 1.5 g/dm^3 dry weight of cells could be produced in approximately 6 hours (Section 7.4.3) and from this point the cells can now be fed with more glucose in a controlled manner. Glucose solutions were fed at constant rates from $4.50 \text{ g/dm}^3/\text{h}$ to $0.19 \text{ g/dm}^3/\text{h}$ with the intention of supplying the glucose at various limiting rates. Seven runs are reported here covering the range of glucose feed rates tested.

The profile of a typical constant fed-batch run is first described. The effect of the rate of addition of glucose feed on the growth rate of the organism is examined, as is the correlation between the constant glucose feed rate and linear growth rate and enzyme production.

7.5.2 Profile of a typical run

The results of the various data collected for Run 23 are shown in Figure 7.4. The fermentation was run for 32 hours and a lipase activity of 0.67 LU/cm^3 was achieved at 32 hours. The fermentation took place in two stages.

The first stage was a batch fermentation during which the initial glucose addition was consumed after 5 hours growth. The fact that the glucose concentration had reached zero was signalled by the dissolved oxygen and pH rising. As soon as these signals had been noted, feeding began.

A glucose solution (100 g/dm^3) was fed at a rate of $0.2 \text{ cm}^3/\text{min}$ giving a glucose feeding rate of $0.6 \text{ g/dm}^3/\text{h}$. Initially (5 - 6 hours) the rate of the feeding was a little too high for the concentration of cells present and the glucose was seen to accumulate. The biomass increased linearly from 8 hours to 18 hours with a slope of $0.097 \text{ g/dm}^3/\text{h}$. From 6 hours to 18 hours lipase was made at a rate of $0.043 \text{ LU/cm}^3/\text{h}$. At 18 hours the cells seemed to halt their growth for about 3 hours, there was a sudden loss of lipase activity for 4 hours and pyocyanin was seen in the fermenter culture medium. At 22 hours, lipase production was resumed but at half the previous rate ($0.020 \text{ LU/cm}^3/\text{h}$), glucose was seen to accumulate in the culture medium, a small amount of biomass was made and cell growth gradually decreased to zero. The experiment was terminated at 32 hours. The data for other constant feed experiments carried out at different feed rates, are shown in Figures 7.5 - 7.7. Values of the slope of the linear growth rate, the glucose accumulation and maximum lipase activity were abstracted from these figures and are listed in Table 7.6.

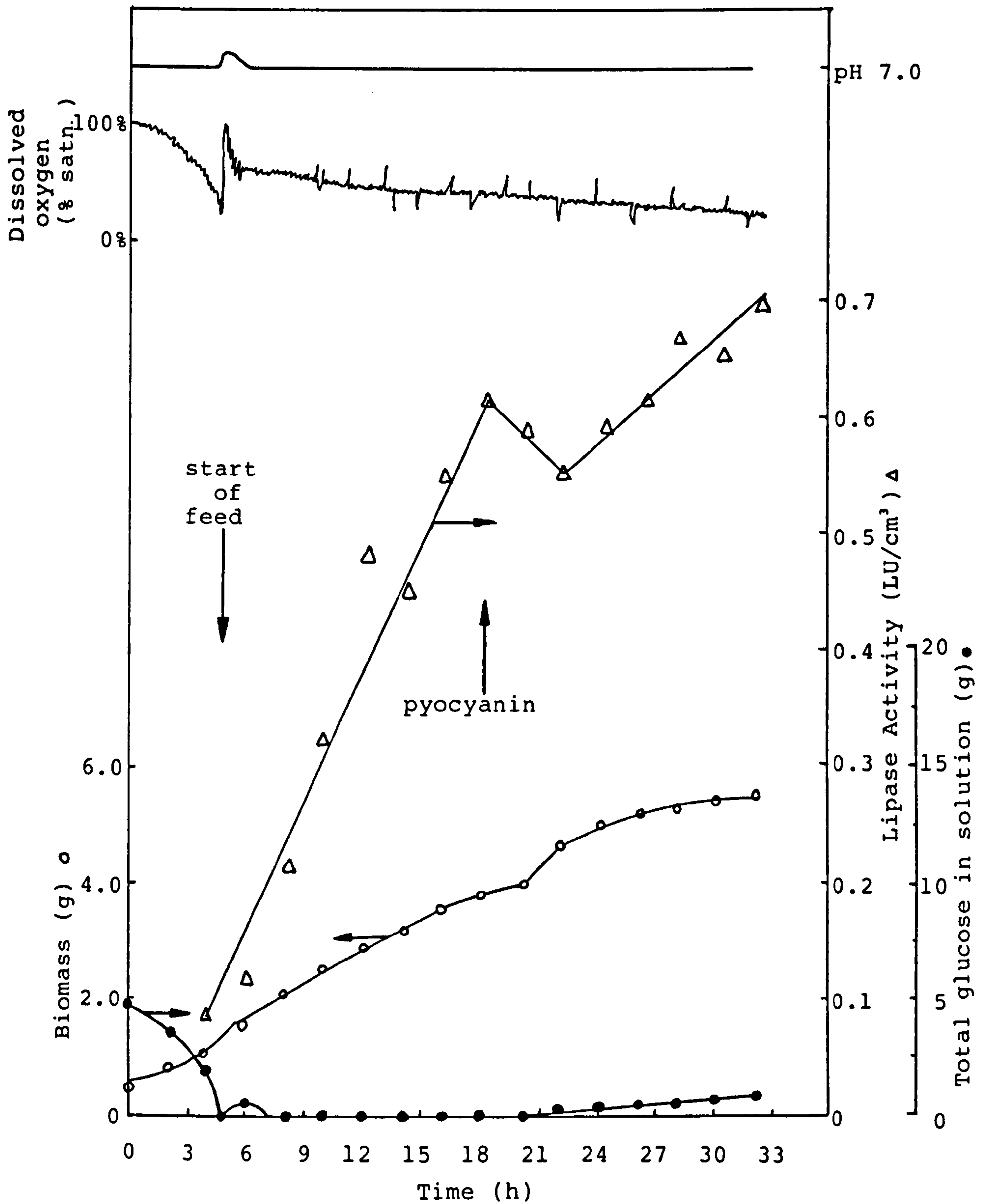


Figure 7.4. Run 23: A typical fed-batch experiment
Glucose feed rate: $0.6 \text{ g/dm}^3/\text{h}$

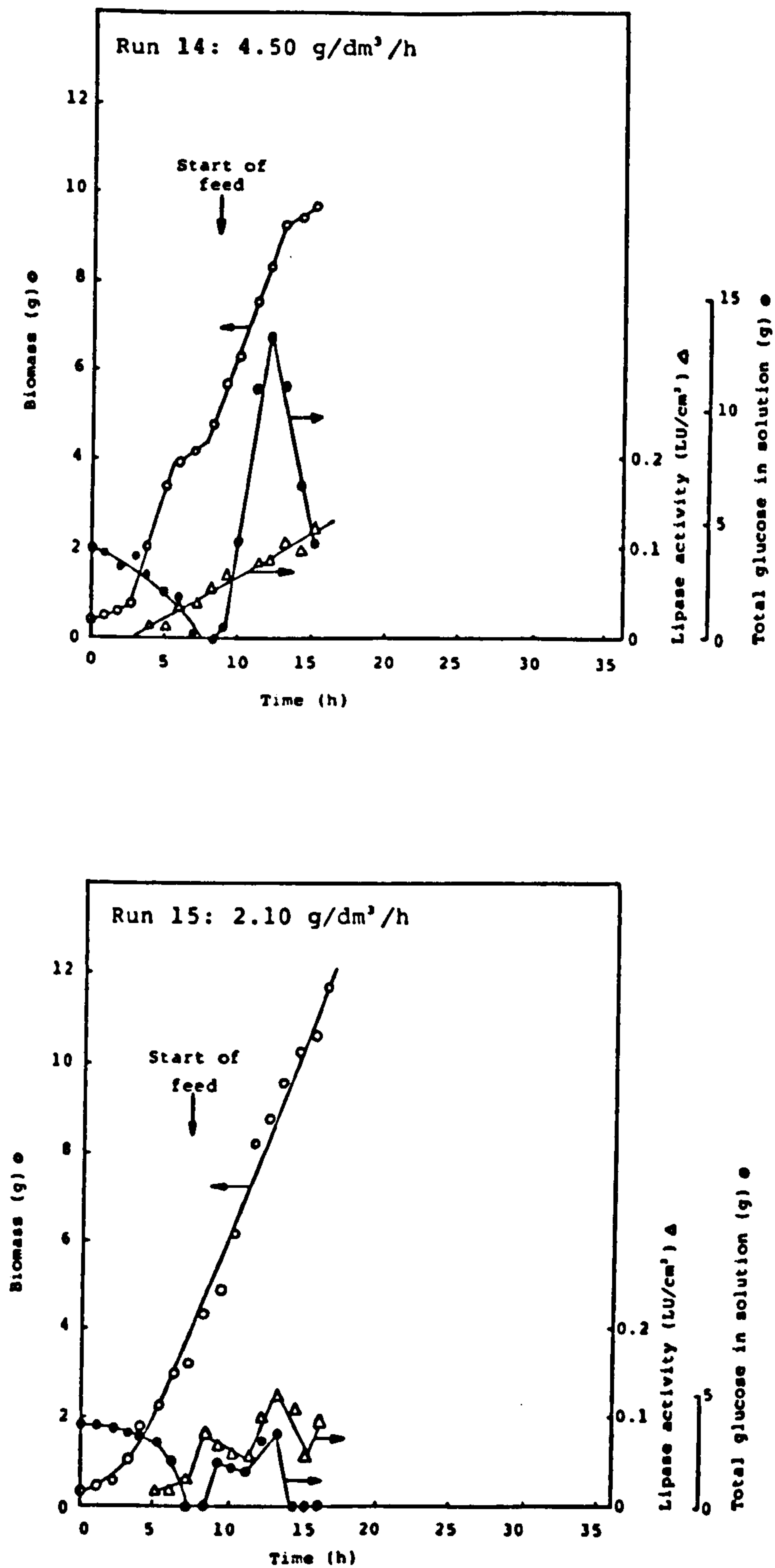


Figure 7.5. Runs 14 and 15: Constant glucose feed rate too high causing maximal growth rate, accumulation of glucose and very little lipase to be made

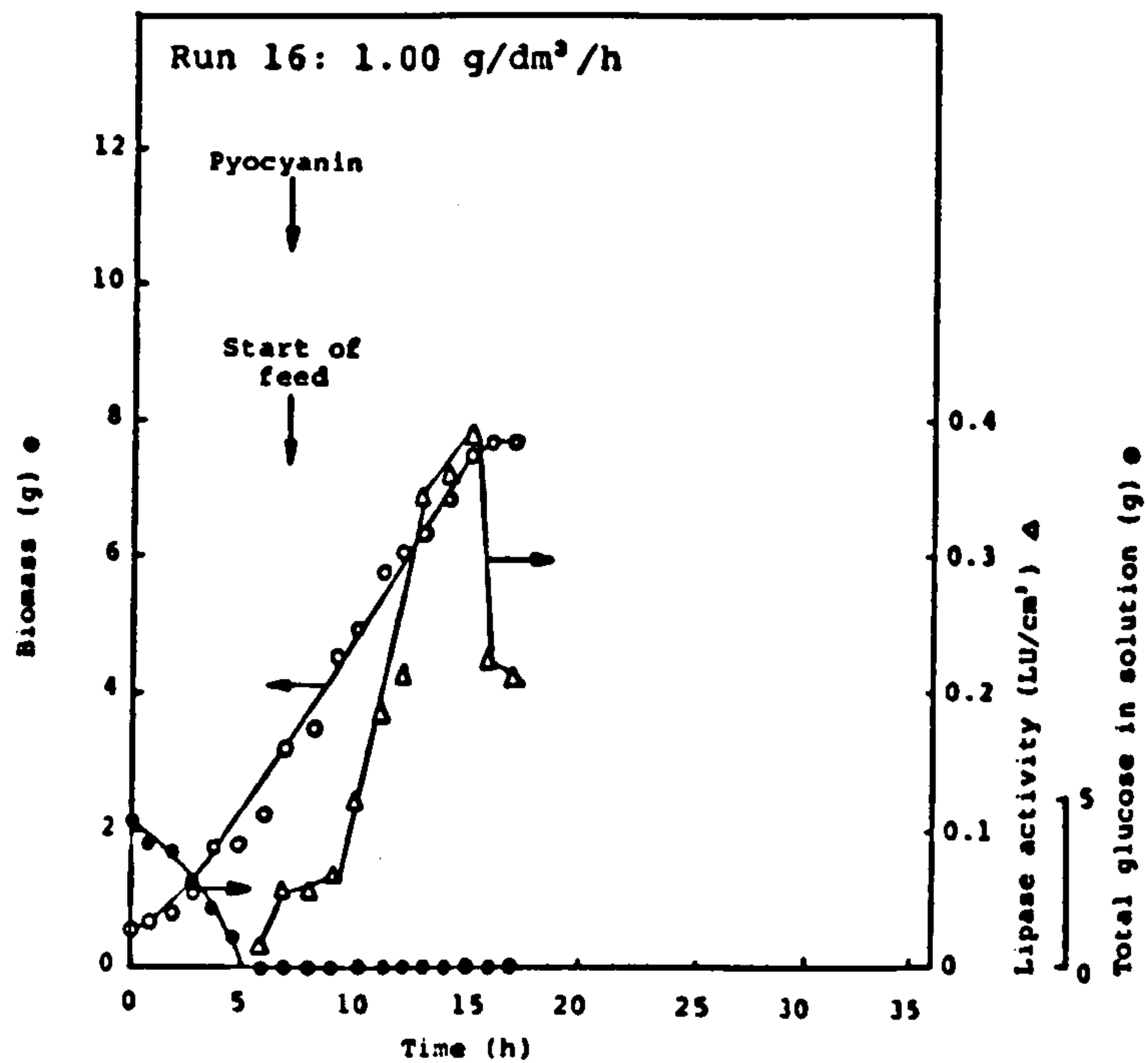
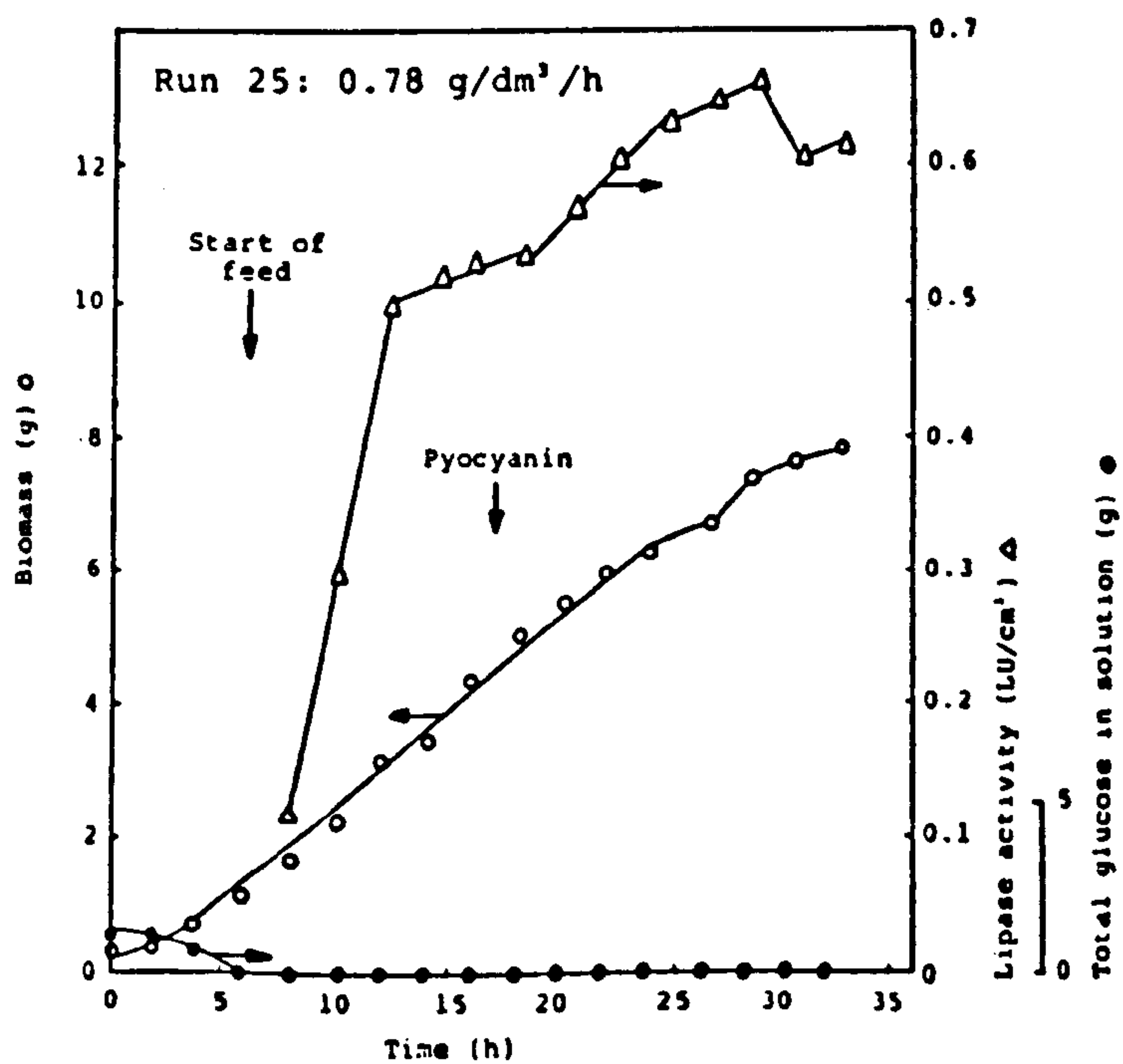


Figure 7.6. Runs 16 and 25: Constant glucose feed rate lowered causing the linear growth rate to decrease, no accumulation of glucose and more lipase to be made

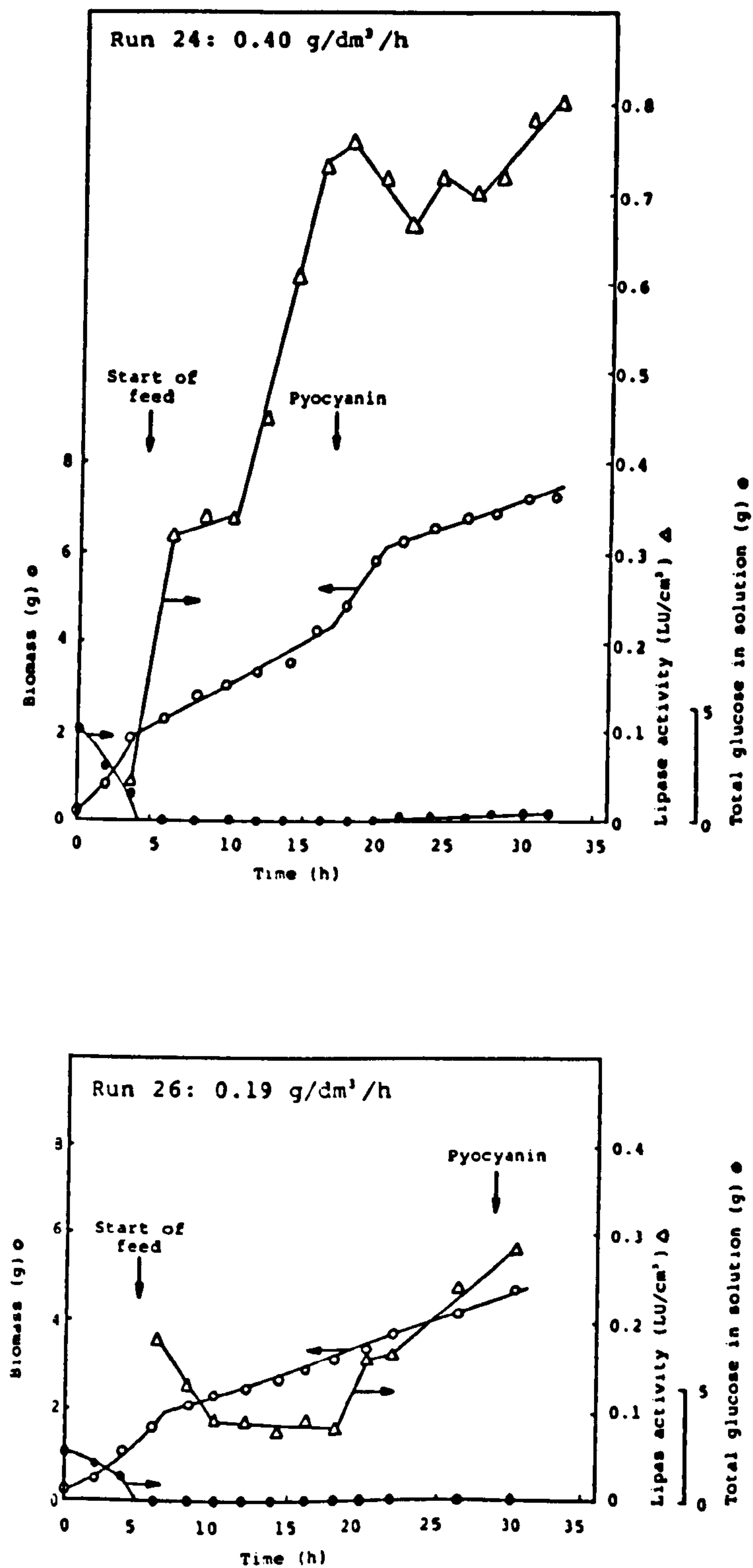


Figure 7.7. Run 24: Optimal constant glucose feed rate
Run 26: Below optimal glucose feed rate

Table 7.6. The effect of varying the rate of addition of the glucose feed on growth rate of the organism, accumulation of glucose in the culture medium and lipase activity

Run No.	Feed Rate (g/dm ³ /h)	Slope of Linear Growth Rate (g/dm ³ /h)	Maximum Amount of Glucose Accumulated in the Medium (g)	Maximum Lipase Activity (LU/cm ³)
14	4.50	0.426	13.5	0.120
15	2.10	0.417	3.1	0.133
16	1.00	0.293	0	0.387
25	0.78	0.130	0	0.667
23	0.60	0.097	0	0.700
24	0.40	0.073	0	0.800
26	0.19	0.046	0	0.280

LU = μ moles of free fatty acid released per minute under test conditions

7.5.3 Linear growth

Linear growth rates were obtained by drawing a line of best fit through the biomass points and calculating the slope of that line. The lines from which the slopes were calculated are shown in Figures 7.5 - 7.7.

The effect of varying the glucose feed rate on the linear growth rate is shown in Figure 7.8. At high glucose feed rates (that is, rates greater than $2.0 \text{ g/dm}^3/\text{h}$) the feed rate is not controlling the growth rate and this is confirmed because glucose was observed to accumulate in the culture medium (Figure 7.5). When the feed rate is below a value of $2.0 \text{ g/dm}^3/\text{h}$ then there is a good linear correlation between the feed rate and the linear growth rate and glucose does not accumulate. It can therefore be stated that at the lower feed rates, the glucose feed rate is controlling the linear growth rate. In Figure 7.8 it is demonstrated that the linear growth rate is approximately the same for feed rates greater than $2.0 \text{ g/dm}^3/\text{h}$.

Therefore, it can also be stated that above feed rates of $2.0 \text{ g/dm}^3/\text{h}$ something other than the glucose feed rate is limiting the linear growth rate.

The constant fed batch runs discussed here contained 1 g/dm^3 yeast extract identical to the batch runs described in Section 7.4. The maximum linear growth rate achieved in the constant fed batch runs was approximately $0.42 \text{ g/dm}^3/\text{h}$ which is very similar to the values obtained

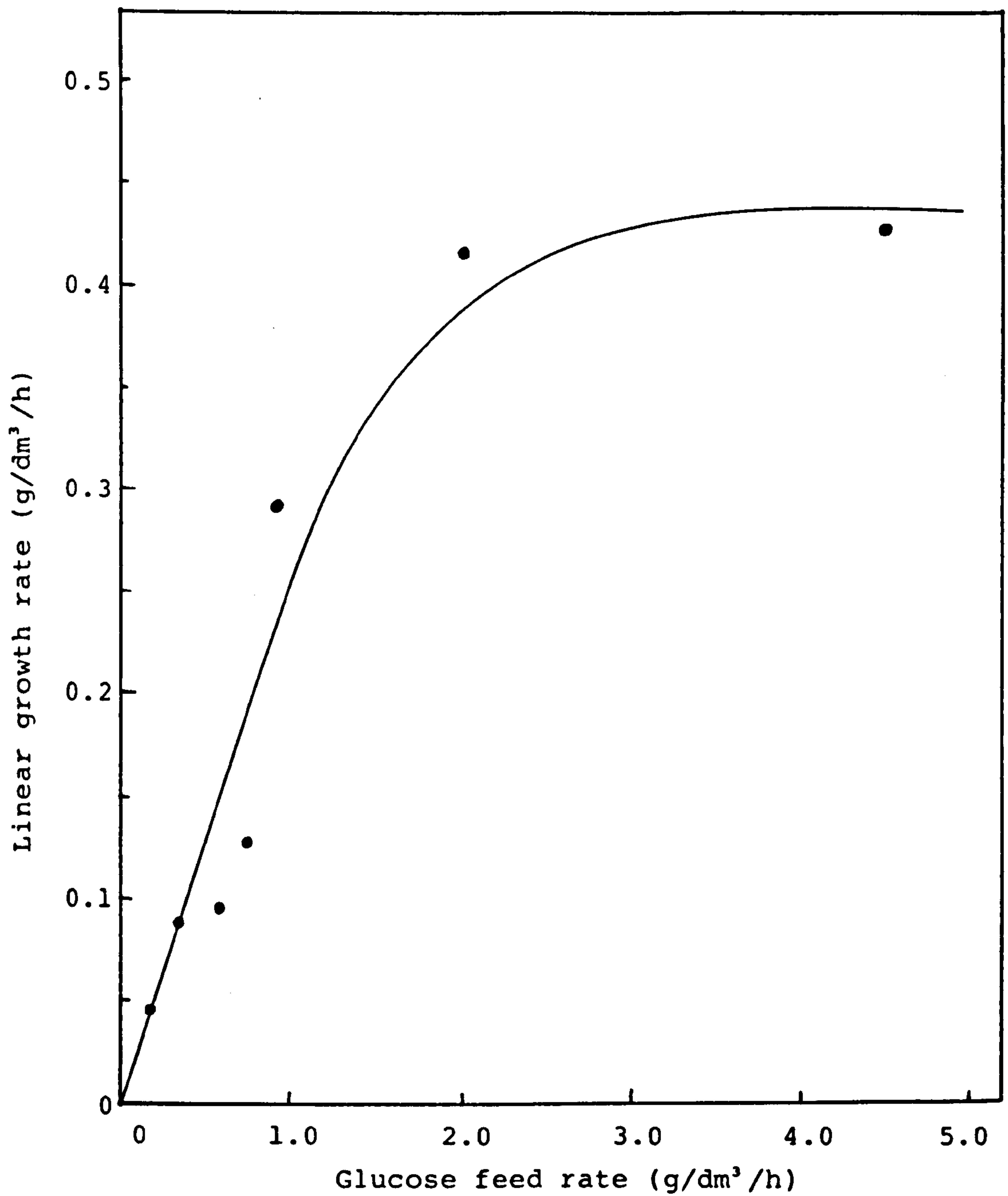


Figure 7.8. The effect of the glucose feed rate on the linear growth rate

for the linear growth phase of the batch runs (Table 7.4) which vary between 0.3 and 0.4 g/dm³/h. Therefore, this maximum value for the constant fed runs is probably controlled by the same limitation that produced linear growth in the batch runs.

7.5.4 Lipase production

When maximum lipase activity of each run is plotted against the constant glucose feed rate (Figure 7.9) the effect of the changing constant feed rate on lipase activity is clearly seen. As the glucose feed rate decreases towards the optimum, there is an increase in the maximum lipase activity that was attained. When the glucose feed rate is too low then the maximum lipase activity rapidly drops.

A similar graph was produced by Brown et al. (1988) for pullulanase production (Figure 2.4). However, there are two differences. The optimum constant glucose feed rate for pullulanase production was found to be 2.0 g/dm³/h (as opposed to the optimum constant glucose feed rate for lipase production in this work which is 0.5 g/dm³/h). The other difference is that the graph produced by Brown et al. was much more 'bell-shaped', that is, the slopes away from the optimum on both sides were gentle. This contrasts starkly with the results produced from this work where the slope away from the optimum glucose feed rate on the low feed rate side is very steep indeed.

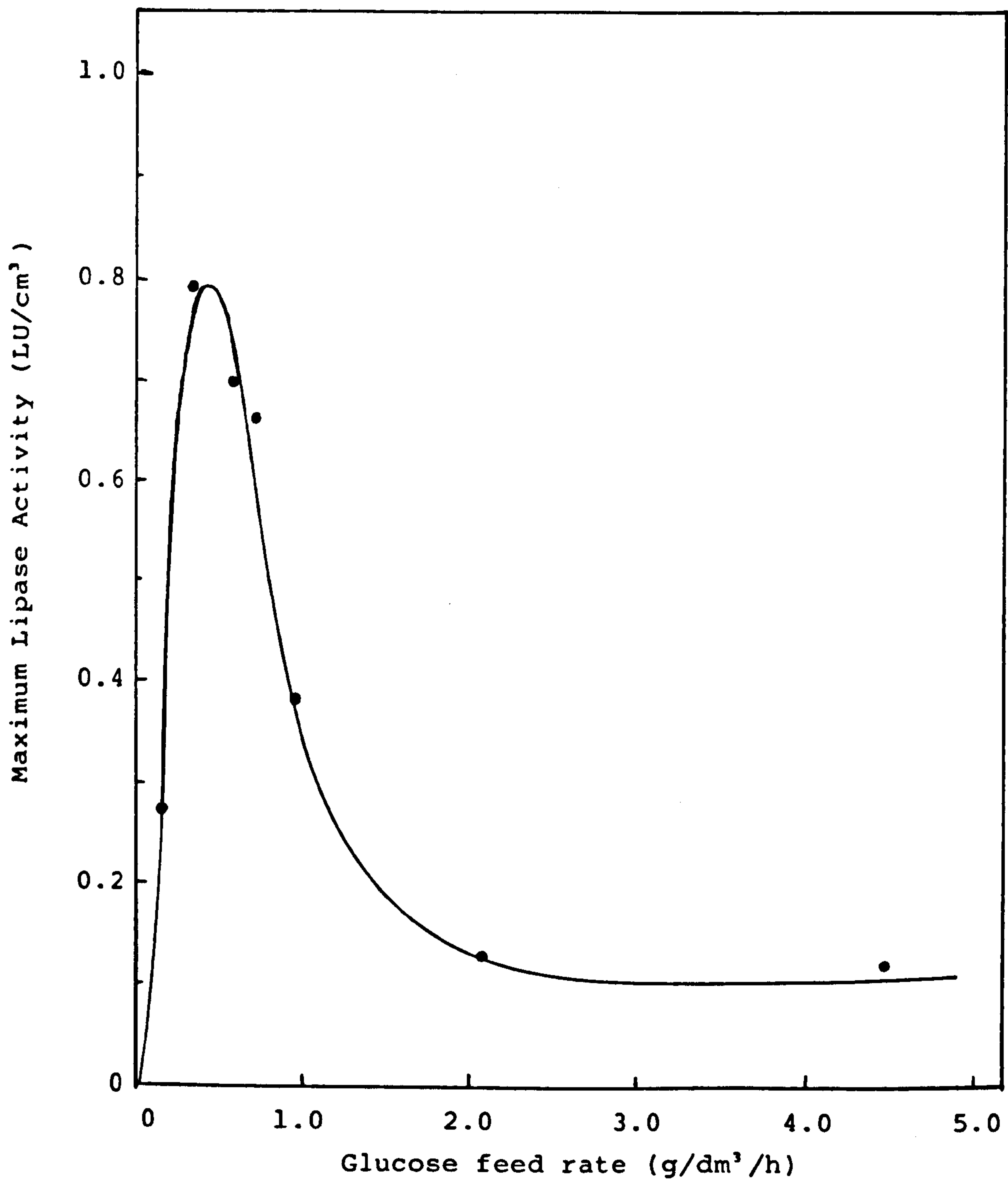


Figure 7.9. The effect of the glucose feed rate on the maximum lipase activity

7.5.5 Extra experiments

For the purposes of the next sets of experiments (Sections 7.6 and 7.7), it was required that the medium be as defined as possible and therefore the yeast extract was omitted from Runs 28 - 30. Runs 28 and 30 were repeats of Run 25 and Run 29 was a repeat of Run 23. Contrary to the results obtained from the shake flask experiments (Section 7.3.2) and Run 9 (Section 7.4.2) the glucose consumption time was only extended to 9 hours. It was concluded that yeast extract was not actually necessary for growth and therefore the yeast extract was omitted from the growth medium for Run 31 onwards.

7.5.6 Conclusions

The growth rate of the organism was shown to be controlled by the rate of addition of glucose feed when the glucose feed was below a rate of $2.0 \text{ g/dm}^3/\text{h}$. When glucose was fed at rates above $2.0 \text{ g/dm}^3/\text{h}$ something else was found to be limiting the growth rate. The glucose feed rate which produced the maximum amount of lipase production was found to be approximately $0.5 \text{ g/dm}^3/\text{h}$. The fact that this effect was seen at all confirms that the production of lipase by this strain of *Pseudomonas aeruginosa* is under the control of catabolite repression. This was encouraging and meant that the organism was ready to be used in the next stage, which was to feed glucose solutions at exponential rates.

However, before this could happen the medium needed to be adapted so that it could support the maximum amount of cells that the fermenter was capable of oxygenating.

7.6 Developing the Growth Medium

7.6.1 Introduction

The maximum dry weight of cells produced during the constant fed-batch experiments was 12 g (Figure 7.5) (approximately equivalent to 5 g/dm^3). It was known that the fermenter could support a dry weight of at least 25 g/dm^3 of cells. Producing this amount of cells would then result in high levels of lipase production. A typical run of excess glucose feed experiments is presented in Figure 7.10. Because of the excess glucose and thus unlimited growth rates, it was expected that lipase production would be very small, of the order of 0.10 LU/cm^3 (Figure 7.9). Lipase activity was thus not measured in this series of experiments. The range of variables that were examined, linear growth rate and final biomass, for these experiments can be found in Table 7.7. The biomass data for Runs 33, 38, 42 and 43 are presented in Figure 7.11 as examples.

7.6.2 Preliminary experiment

Pseudomonas aeruginosa was grown up under batch conditions in Pirt's medium (x1) plus 1.0 g/dm^3 glucose (Run 31). By 6 hours this glucose had been used up and a biomass

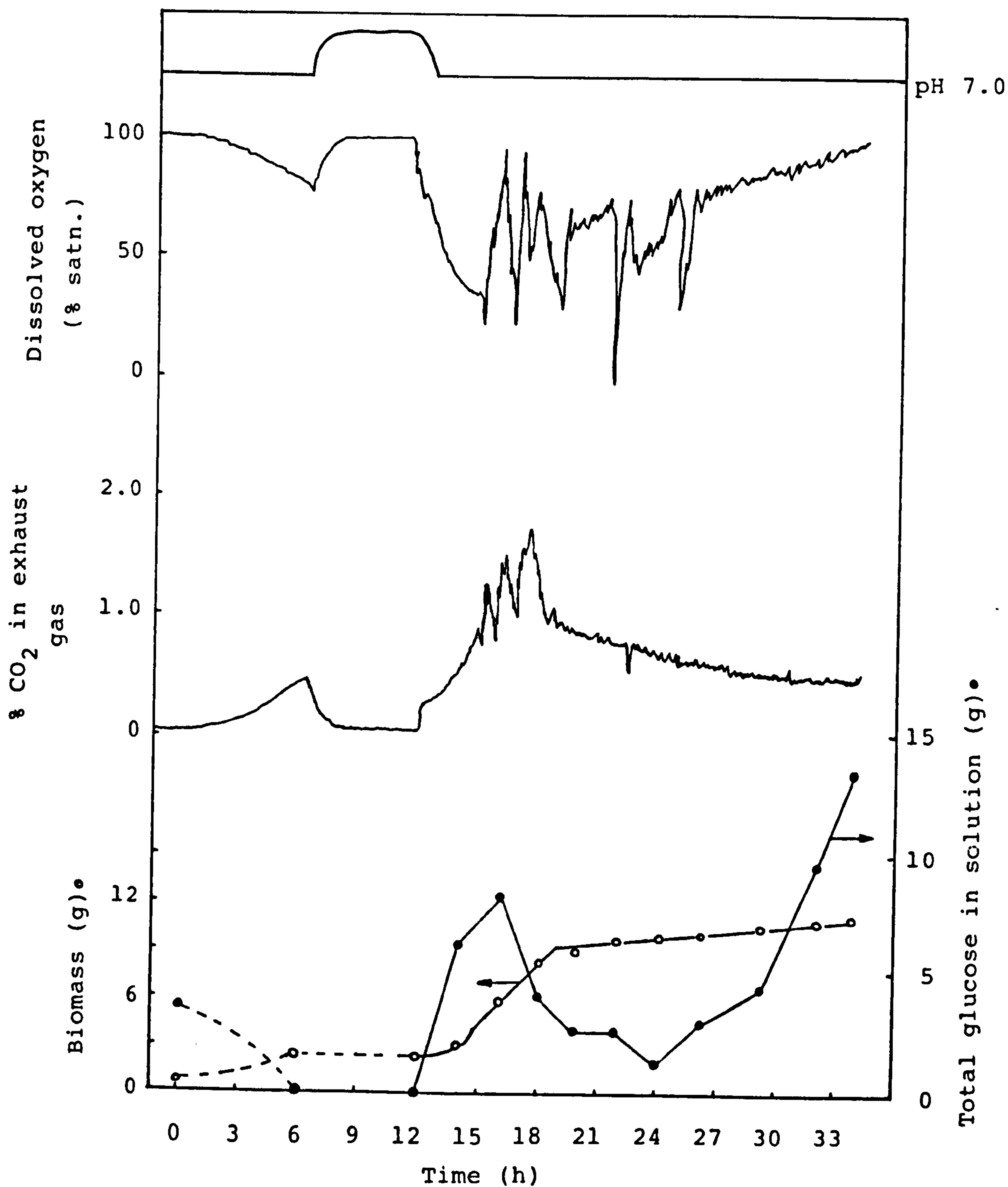


Figure 7.10. Profile of Run 31

Table 7.7. Developing the growth medium

Run No.	Pirt's Trace Salts Incd. By Factor	KH ₂ PO ₄ Conc. (g/dm ³)	Addition of NaCl (5 g/dm ³)	Inoculation Addns. No. of Loops	Yeast Extract	Linear Growth Rate (g/dm ³ /h)	Final Biomass (g)
31(a)	x1	1.13	-	1	-	0.63	8.50
32(a)	x2	1.13	-	1	-	0.65	12.80
33	x2	1.13	-	1	-	0.62	18.61
34	x2	2.26	-	1	-	1.77	27.73
35	x4	4.52	-	1	-	0.73	34.89
36	(b)						
37	x4	4.52	-	1	-	0.85	23.88
38	x4	4.52	+	1	-	1.00	31.75
39(c)	x4	4.52	-	1	-	0.89	25.17
40	x4	4.52	+	2	-	1.25	34.02
41	x14	4.52	+	3	-	1.57	46.34
42	x14	4.52	+	2	-	1.46	46.46
43(d)	x14	4.52	+	1	-	1.69	41.13
44	x14	4.52	+	1	+	1.23	38.28
45	x10	9.04	+	1	+	2.14	58.99
46	x10	9.04	+	1	+	2.66	60.56

(a) Nitrogen source NH₄Cl and pH control with 4N NaOH

(b) Fermentation aborted

(c) Extra trace salts added to medium

(d) Inoculum contained 1 g/dm³ peptone

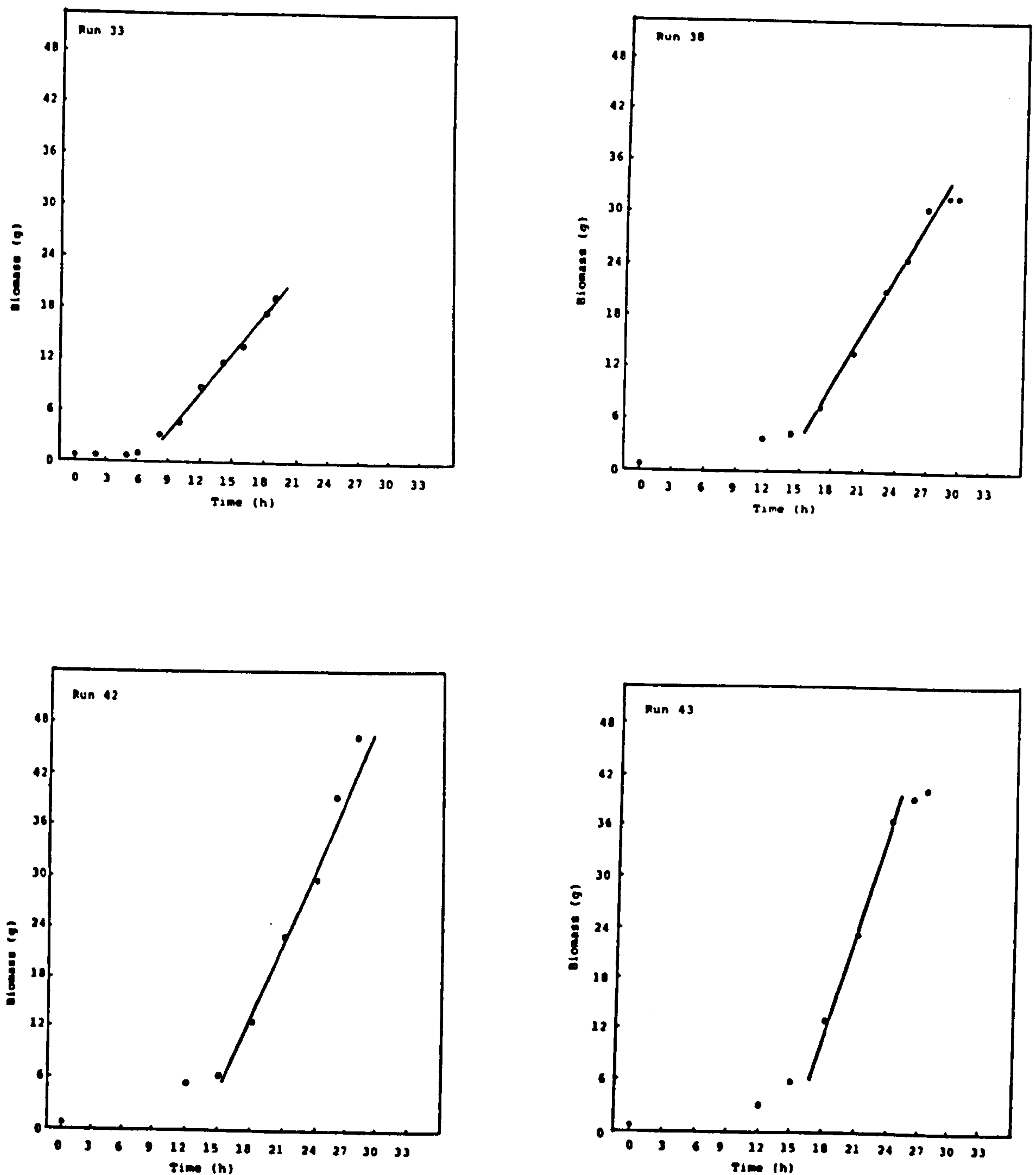


Figure 7.11. Runs 33, 38, 42 and 43 as examples of how changing the medium components increased the linear growth rate and the final biomass achieved (See text for details)

of 2.34 g had been produced. At 12 hours a glucose solution (100 g/dm^3) was fed at increasing rates such that glucose in the culture medium was always in excess (Section 5.2). Between 12 and 15 hours the cells grew exponentially and this exponential growth can be seen in the carbon dioxide and dissolved oxygen data (Figure 7.10). But at 15 hours there was a change to linear growth. The rate of the linear growth was found to be $0.63 \text{ g/dm}^3/\text{h}$ (Table 7.7).

At 19 hours the cells' growth stopped almost completely. This can be seen by the small amount of increase in biomass, the carbon dioxide production rate gradually decreasing to an equivalent of 0.5% and the dissolved oxygen gradually increasing back to 100%. The fermentation was ended at 34 hours because glucose addition (accumulating at a rapid rate in the culture medium) no longer influenced the process. From 15 hours onwards both the dissolved oxygen and the carbon dioxide traces are very noisy. This suggests that the metabolism of the organism could have changed at this point. Also, these traces continue to be noisy until the next limitation occurs at approximately 24 hours.

Because glucose and oxygen were in excess and nitrogen and phosphate were assumed to be in excess, the cause of the linear growth and low biomass yield (approximately 4 g/dm^3 instead of the 10 g/dm^3 Pirt's predicted yield on this

medium) was presumed at this point to be due to a trace salt limitation.

7.6.3 Effect of increasing the trace salts concentration

When trace salt concentrations were doubled from Pirt's x1 to Pirt's x2 (Run 31 compared with Run 32) the cells grew at approximately the same linear rate of $0.65 \text{ g/dm}^3/\text{h}$ but made more biomass before growth stopped. When the trace salt concentrations were increased from Pirt's x4 to Pirt's x14 (Run 40 compared with Run 42) both the growth rate and the final biomass were increased but precipitation of salts was observed. It was found that the maximum beneficial effects of increasing the salts concentration with minimum precipitation was observed with a trace salts concentration of Pirt's x10 (Runs 45 and 46). The problem of a limitation was still present because the cells continued to grow linearly after a short burst of exponential growth, in all the experiments.

It was suggested that the trace salt which was causing the limitation might not be included in the Pirt's medium. Therefore, extra trace salts (potassium sulphate, sodium phosphate, iron (III) EDTA, boric acid, sodium molybdate and nickle chloride) (found in another defined minimal salts medium - FAM II 20/20 - Table 4.5) were added to Pirt's x4 (Run 39 compared with Run 37). However, the cells still grew linearly and a similar final biomass was produced. It was concluded that the trace salt causing the

limitation was not one of those incorporated from the FAM II 20/20 medium.

7.6.4 Effect of pH control changes and NaCl addition

The nitrogen source (ammonium chloride) and pH control (sodium hydroxide solution) of Pirt's medium were replaced with ammonium sulphate and ammonium hydroxide solution respectively (for Runs 33 to 60). This allowed the nitrogen in the medium to be maintained at a constant level even when the organism was growing rapidly. It also allowed for the calculation of nitrogen usage if the weight of the ammonium hydroxide feed bottle was monitored during the fermentation experiments. The benefits of this change can be seen if Runs 32 and 33 are compared. Even though the linear growth rates are approximately the same, 50% more biomass was produced by the end of the run.

When the nitrogen source and pH control were changed, it was realised that there was no longer a presence of both sodium and chloride ions. Therefore, it was decided that Pirt's medium should be supplemented with sodium chloride. Again benefits of this addition can be seen if Run 37 is compared to Run 38. Not only did the growth rate increase but there was a greater final biomass as well.

7.6.5 Effect of increasing the phosphate concentration

The effect of doubling the potassium dihydrogen phosphate concentration from 1.13 g/dm^3 to 2.26 g/dm^3 was very

dramatic indeed (compare Run 33 to Run 34). There was a 50% increase in final biomass and the linear growth rate increased three fold. The potassium dihydrogen phosphate concentration was increased from 2.26 g/dm^3 to 4.52 g/dm^3 (Runs 35 to 44) but as other constituents were changed at the same time the effects of the increased phosphate can not be seen in isolation. For Runs 45 and 46 the potassium dihydrogen phosphate concentration was increased yet again to 9.04 g/dm^3 but at the same time the trace salts concentrations were reduced from Pirt's x14 to Pirt's x10. Thus, the effect of phosphate concentration increase cannot be discussed in isolation from the trace salt concentration effects.

When the final biomass is plotted against the concentration of potassium dihydrogen phosphate in the medium (Figure 7.12) the beneficial effect of phosphate can be seen. There is a definite trend that as the phosphate in the medium increases so the final biomass increases. The three points at 1.24 g/dm^3 potassium dihydrogen phosphate (1.13 g/dm^3 in the medium and 0.11 g/dm^3 from the inoculum) are Runs 31 to 33. Runs 32 and 33 contain twice the concentration of trace salts as Run 31 and this enabled a higher final biomass to be attained. There are eight points at 4.63 g/dm^3 potassium dihydrogen phosphate (4.52 g/dm^3 in the medium and 0.11 g/dm^3 from the inoculum) and these are Runs 35 to 44.

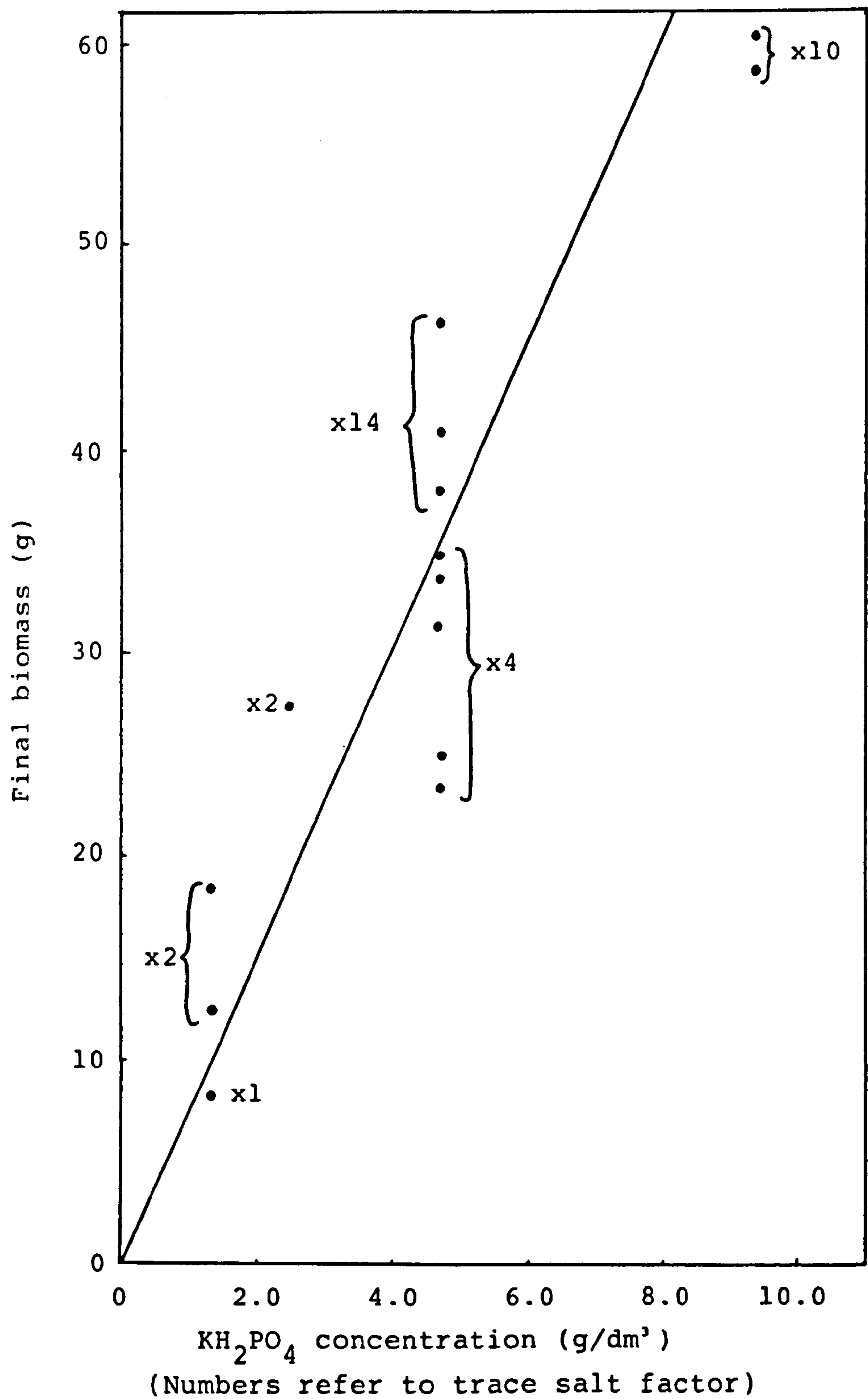


Figure 7.12. The effect of the concentration of potassium dihydrogen phosphate in the medium on the final biomass achieved

Runs 35 to 40 contain a trace salt concentration of Pirt's x4 (the lower five points), whereas Runs 41 to 44 contain a trace salt concentration of Pirt's x14 (the upper three points). However, apart from the trace salt groupings, there are no other observed variations to explain this spread of data.

The graph in Figure 7.12 is a rough plot of biomass yield on phosphate and has a slope of 7.7 g biomass/g potassium dihydrogen phosphate. This value converts to a cell yield for phosphorus of 33.6 g biomass/g phosphorus which compares favourably with the value quoted by Pirt (1975) for the cell yield of *Klebsiella aerogenes* on phosphorus of 39.1 g/g and with the cell yield of *Escherichia coli* quoted by Park and Rogers (1989), of 29.4 g/g.

7.6.6 Effect of size of inoculum

It was postulated that the organism could be storing up some vital component from the agar maintenance plates which was subsequently running out during exponential growth in the fermenter and thus causing it to grow linearly (Section 2.4.3). The effect of inoculating the inoculum flask with 2 loops full of cells was to increase the linear growth rate by 25% and increase the final biomass slightly (compare Run 38 with Run 40).

The other components of tributyrin agar plates apart from tributyrin and agar, are yeast extract and peptone. It was

therefore decided to add peptone and yeast extract in turn to the inoculum flasks. There was little difference in the final biomass achieved (Run 43 compared to Run 44) from these two additions. Thus, to be consistent with previous experiments done in this work, 1 g/dm³ yeast extract was added to the inoculum flasks for Runs 44 to 60.

7.6.7 Linear growth

Nyholm (1976) introduced the term 'conservative substrates'. This was used to define those substrates which are not irreversibly consumed after uptake, but which are accumulated simply as storage materials. Under conditions of starvation, these 'conserved substrates' can be degraded and the limiting substrate re-used for synthesis of new biomass.

Brown et al. (1988) proposed a simple kinetic model which shows that the mass of the limiting trace substrate scavenged from the initial medium determines both the cell concentration at which exponential growth ends, the slope of the linear growth phase and also the final biomass value (Section 2.4.3). Thus the plot of slope of linear growth should be a straight line with the plot of final biomass.

When final biomass is plotted against linear growth rate for Runs 31 to 46 (Figure 7.13) there is a good linear relationship. This confirms that the amount of the

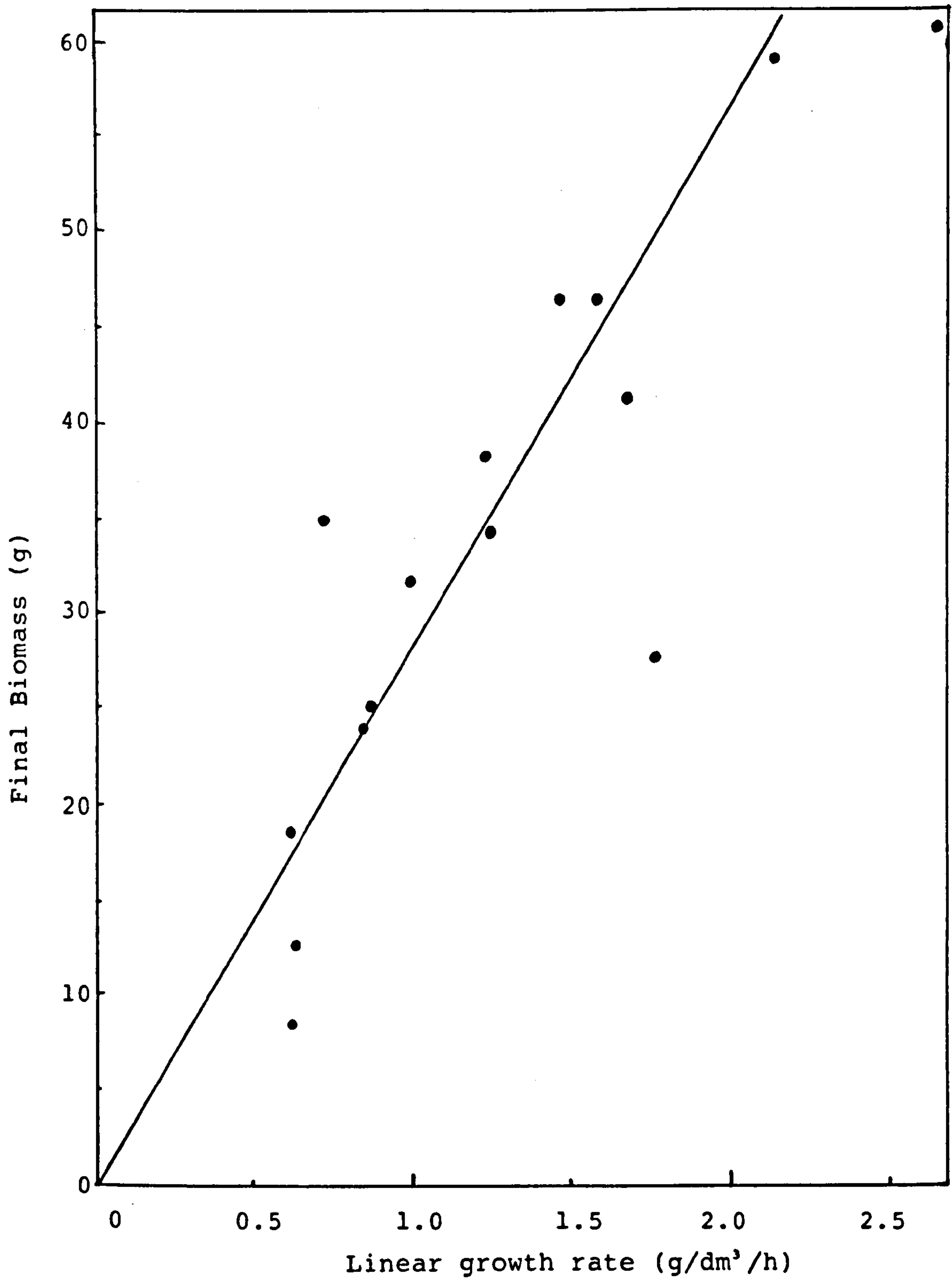


Figure 7.13. The correlation between linear growth rate and final biomass achieved

limiting substrate which was causing the linear growth was being increased in the medium changes and ultimately had an effect on the final biomass.

Because of the correlation between final biomass and potassium dihydrogen phosphate concentration (Figure 7.12), this correlation between the linear growth rate and final biomass would suggest that there is a similar relationship between the linear growth rate and phosphate concentration if phosphate was the controlling limiting component. Figure 7.14 shows that linear growth rate does increase with phosphate concentration approximately linearly but the results are not good enough to say categorically that phosphate is controlling the linear growth rate. The spread of linear growth rates at a potassium dihydrogen phosphate concentration of 4.63 g/dm^3 is again apparent in this graph.

It can be said that the limiting factor affects both linear growth rate and final biomass. However, when it is postulated that the limiting factor is phosphate there is a large amount of scatter when these two variables are plotted against phosphate concentration. Therefore it is quite likely that there is something else causing a limitation as well as phosphate.

A further observation can be made between linear growth and yeast extract concentration. It was noted in Section 7.4.5 (Table 7.5), that increased yeast extract

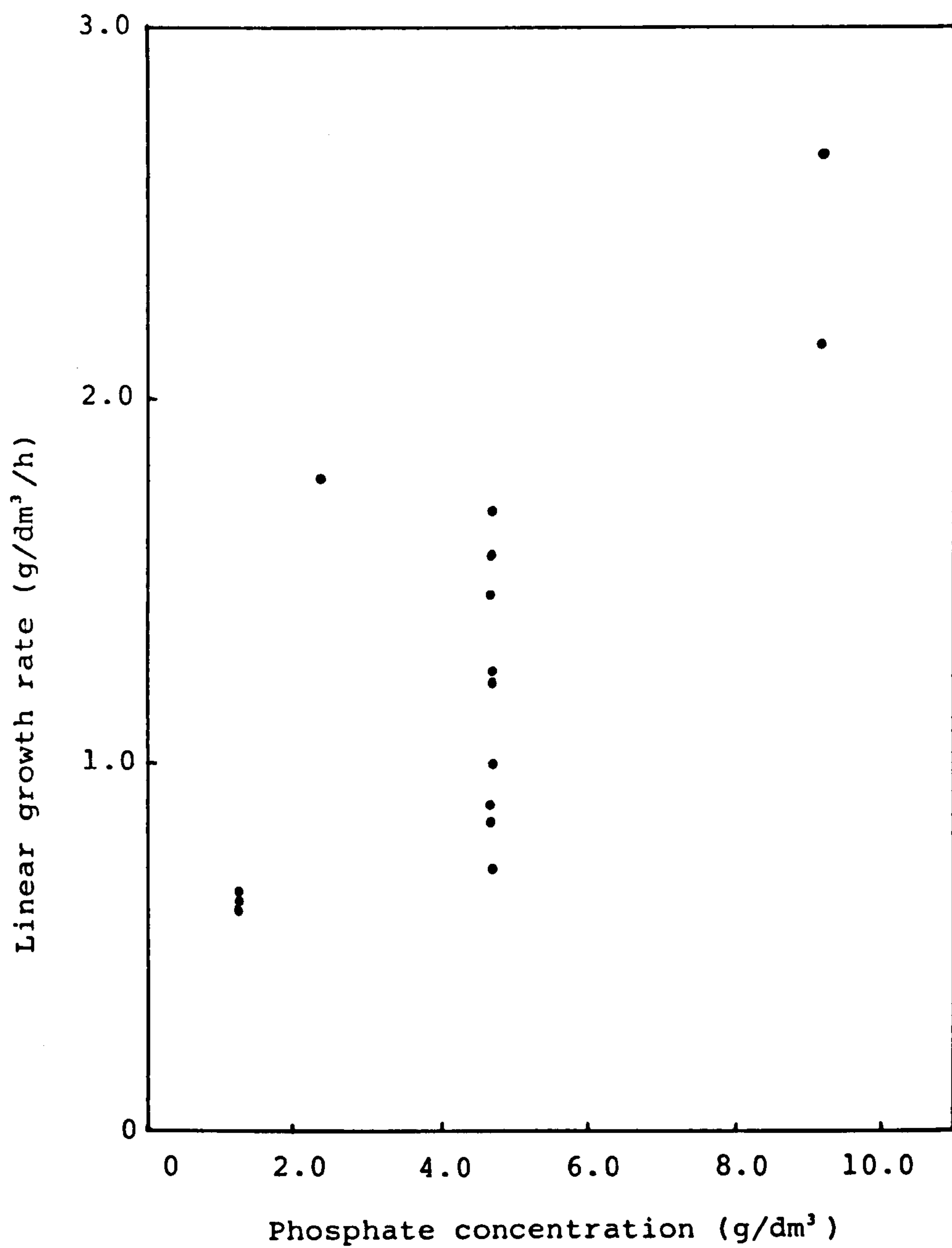


Figure 7.14. The effect of the concentration of potassium dihydrogen phosphate in the medium on the linear growth rate

additions reduced the slope of the linear growth. Those values can be combined with the result from Run 31 (Table 7.7). When the linear growth rates of the batch runs (Table 7.5) together with the linear growth rate of Run 31 (Table 7.7) are plotted against concentration of yeast extract in the medium then an interesting relationship is observed (Figure 7.15). As the amount of yeast extract that was added decreases, the linear growth rate continues to increase, thus supporting further the theory that the yeast extract has some connection with the phenomenon of linear growth as seen in the batch and fed batch fermentations.

7.6.8 Conclusions

When the growth medium contained a potassium dihydrogen phosphate concentration of 9.04 g/dm^3 , a trace salts concentration of Pirt's x10, an addition of 5 g/dm^3 sodium chloride and the inoculum flask had an addition of 1 g/dm^3 yeast extract, the results were quite dramatic (Runs 45 and 46) (Figure 7.16). The linear growth rate increased to over $2 \text{ g/dm}^3/\text{h}$ and a final biomass of 60 g was achieved (that is, a dry weight of approximately 25 g/dm^3 of cells).

There were several observations made about these excess glucose feed experiments. The short exponential phase of growth was followed by a linear growth phase even though glucose was in excess. There seemed to be definite links

Key:- • Runs 4, 7 and 8

* Run 31

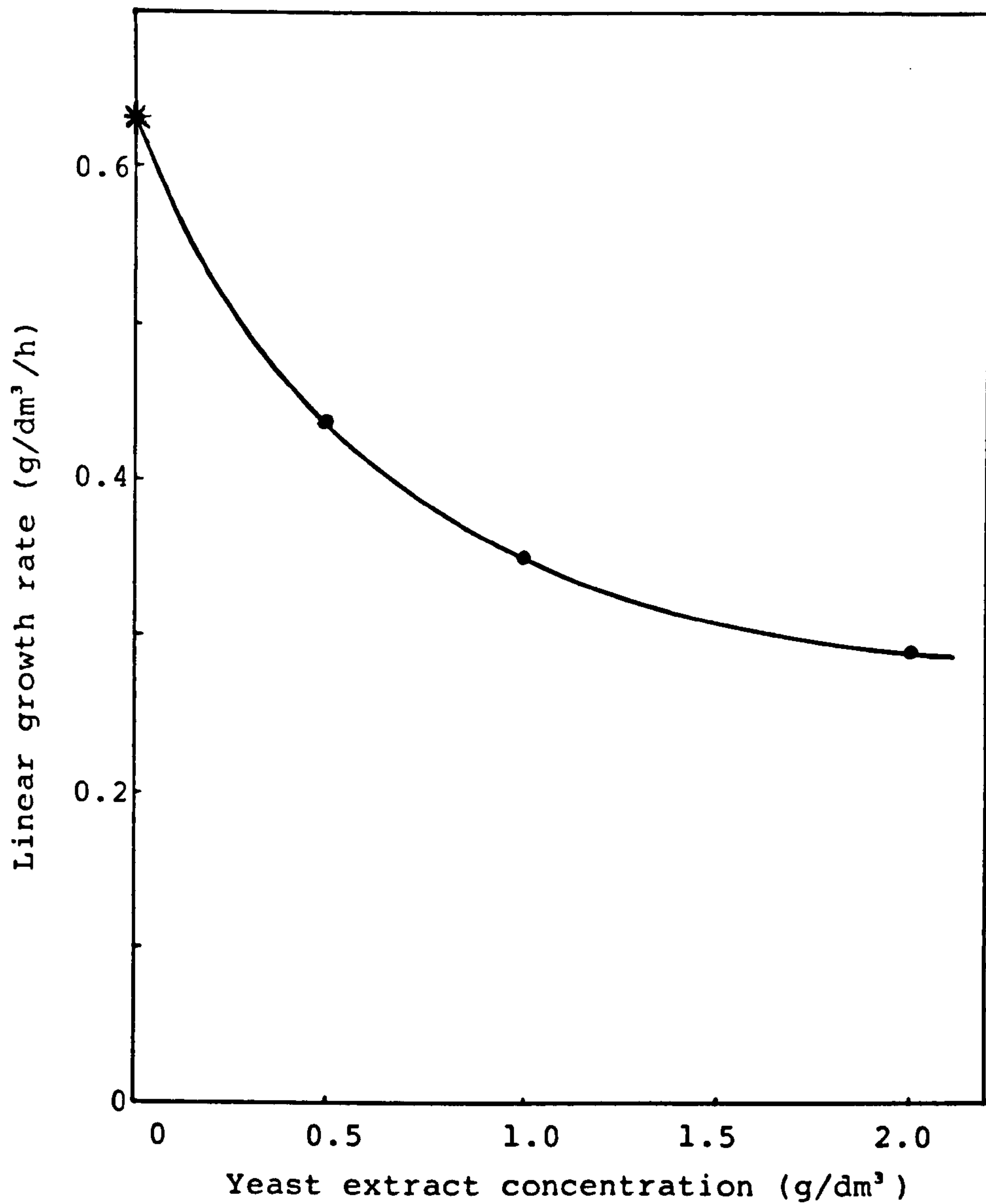


Figure 7.15. The effect of varying the yeast extract concentration on the linear growth rate
 KH_2PO_4 concentration = 1.13 g/dm^3

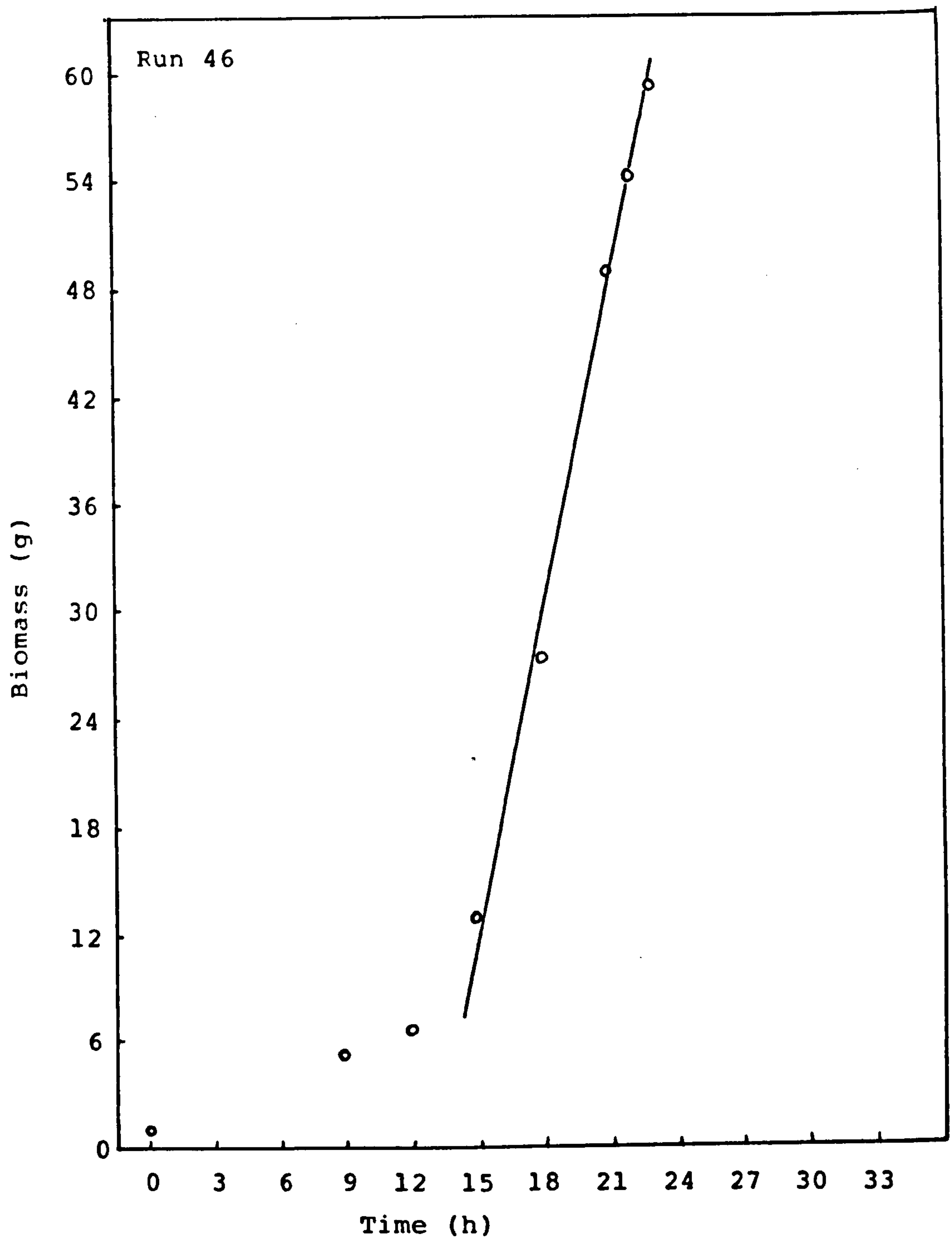


Figure 7.16. Biomass profile of Run 46

between the phosphate concentration, trace salt concentration and the linear growth rate and final biomass achieved. There was also evidence to suggest that yeast extract could be adsorbing a trace nutrient and therefore making it unavailable and subsequently causing a limitation. A change in metabolism was also indicated by the amount of noise on the dissolved oxygen and carbon dioxide traces taken during the progress of Run 31 (Figure 7.10).

7.7 Exponential Feed Fermentations

7.7.1 Introduction

It was confirmed that the selected strain of *Pseudomonas aeruginosa* produced a lipase under the control of catabolite de-repression. A medium had been developed which supported a dry weight of 25 g/dm³ of cells. Up to this point, the results of the experiments were very similar to those obtained by Allinson (1990) and Minihane (1990) and therefore it was considered logical to attempt the next group of experiments which involved feeding glucose solutions at exponential rates.

It had been shown previously (Minihane 1990) that a concentrated solution of glucose could be fed to a laboratory scale fermenter at an accurately known and exponentially increasing flow rate. This was achieved using the feed system described below in which the feed

solution mass was weighed (using a top-pan balance) and the weight was monitored by a micro-computer (and compared with a calculated value) thus creating a feed-back control system. Integral on/off control of the pumping rate through a pre-programmed protocol was shown to produce the desired feed profile with an error less than 0.1%. The glucose solution feed pump was controlled by the micro-computer with a signal via the 3D interface. The voltage output from the interface controlled the roller speed of the pump in direct proportion.

The feed equation for the glucose feed rate F is given by:

$$F = \frac{x_0 \cdot V_0 \cdot \mu}{S_F \cdot Y_{XS}} \cdot e^{\mu t} \quad \dots (1)$$

where:

F = glucose solution feed rate

x_0 = initial cell concentration in fermenter

V_0 = initial culture volume

S_F = concentration of glucose in the feed solution

Y_{XS} = yield coefficient on glucose

μ = specific growth rate

Equation (1) was derived by Minihane (1990) from assumptions made about the physiology of microbial growth which are only approximate. However, this equation is sufficiently accurate to permit the formulation of the actual feed equation as:

$$F = A.e^{Bt} \quad \dots (2)$$

A value of the feed exponent "B" can be selected to be equivalent to the desired specific growth rate, μ , and a working value of "A" can be derived from Equation (1).

Therefore the aim of this next set of experiments was to utilise the previous authors feed technique and demonstrate that the technology would work on various fed-batch systems, as well as maximise the lipase production from this selected strain of *Pseudomonas aeruginosa*.

Thirteen exponential feed experiments were attempted and the experimental details of these runs are shown in Table 7.8. Cells were grown up under batch conditions and as soon as possible after the initial amount of glucose had been consumed, they were fed with glucose solutions at exponential rates.

A relatively wide range of values of "B" would have to be examined to find the exact optimum for lipase production. However, a limited range was covered in this work due in part to the need to develop a working system. The values of "B" that were tested were 0.10, 0.15 and 0.20 h⁻¹ and these values were chosen for a number of reasons. Firstly, the optimum glucose feed rate from the constant feed fermentations (Section 7.5) was 0.5 g/dm³/h, with a yield of cells on glucose of 0.5 g/g, this rate of feeding would

Table 7.8. Run details of the exponentially fed fermentations

Run No.	"B" (h ⁻¹)	Pirt's Trace Salts Incd. By Factor	KH ₂ PO ₄ Concn. (g/dm ³)	Glucose Feed Concn. (g/dm ³)
48	0.10	x10	9.04	300
49	0.10	x10	9.04	80
50	0.10	x10	2.26	250
51	0.10	x10	2.26	250
52	0.20	x10	2.26	280
53	0.20	x10	9.04	280
54	0.20	x20	22.60	350
55	0.20	x20	22.60	350
56	0.20	x40	45.20	350
57	0.10	x30	33.90	350
58	0.10	x40	45.20	200
59	0.15	x40	45.20	200
60	0.15	x40	45.20	200

produce $0.25 \text{ g/dm}^3/\text{h}$, the concentration of cells that produced the maximum amount of lipase activity at this feed rate was 2.6 g/dm^3 (Run 26: Figure 7.7), by dividing the former by the latter, an optimum growth rate of approximately 0.10 h^{-1} is produced. Secondly, the value of "B" which gave optimum pullulanase production by *Klebsiella planticola* was 0.20 h^{-1} (Minihane 1990). Finally, 0.15 h^{-1} was chosen because it was between the two other values.

7.7.2 Glucose feed

For each fermentation the actual profile of the glucose feed that was followed can be determined from the logged balance values. Using this data the balance reading was converted into a quantity of glucose added and a statistical analysis program was used to fit an exponential function to the data. An example of the results of the glucose feed for Run 58 are shown in Figure 7.17.

If perfect control was achieved, the exponent would be equal to the value of "B" used in the feed equation with a regression coefficient of 1.0 and an error of zero. The value of the slope of the data for Run 58 was 0.099 which is recorded, together with the results of the other runs, in Table 7.9. The regressed exponent from the feed was very close to the set-point exponent "B" entered into the computer. In most cases the error was less than

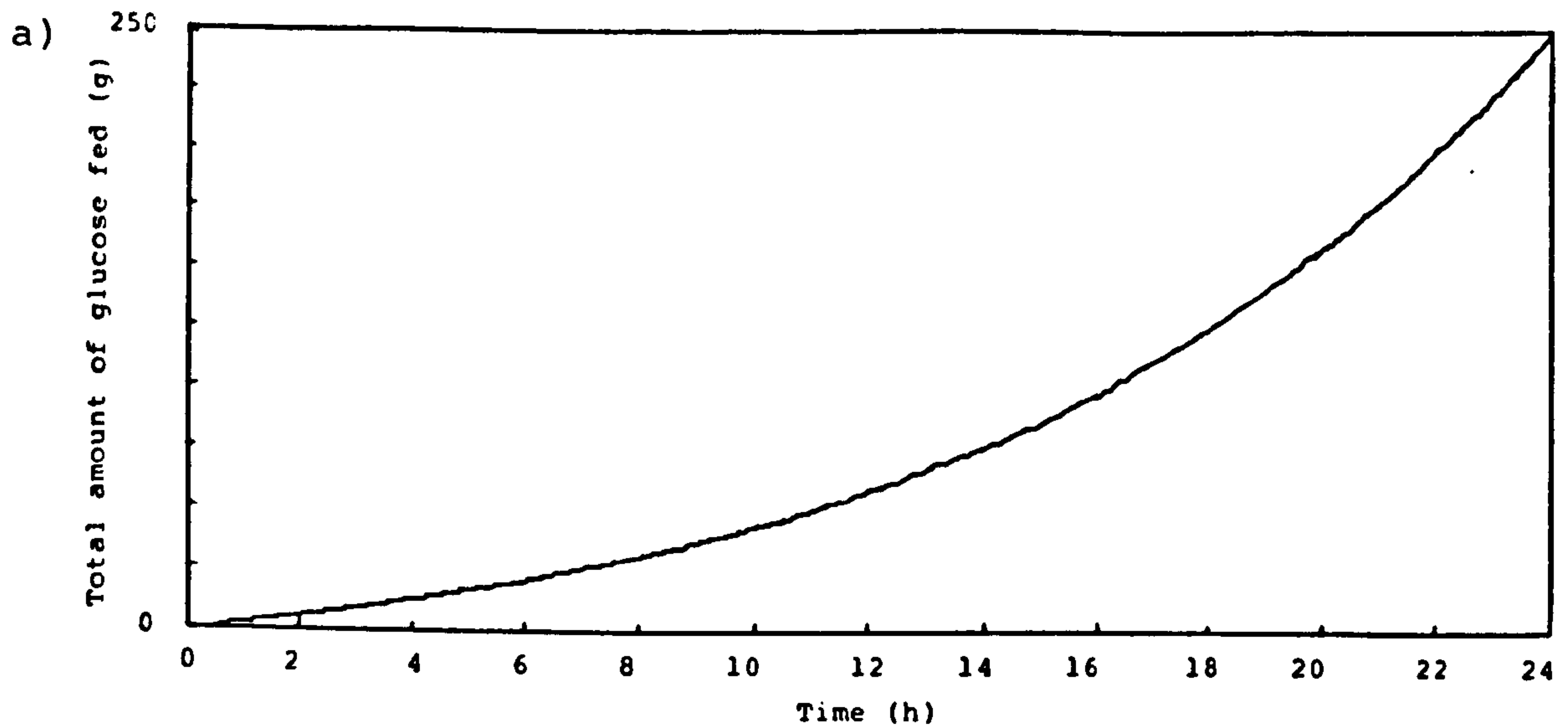
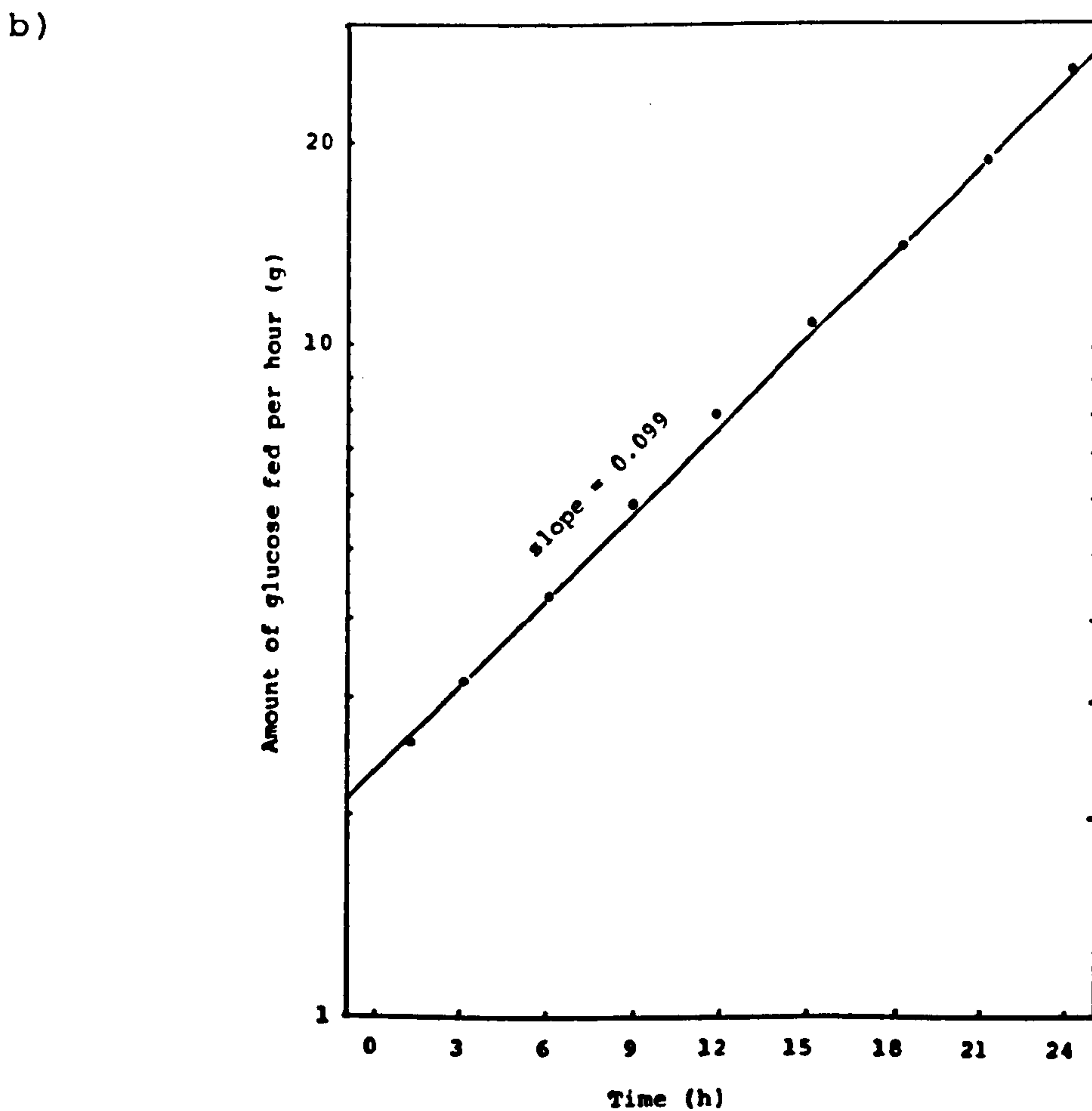


Figure 7.17. (a) The logged balance values of the glucose feed.
(b) The balance reading converted into a quantity of glucose added plotted on a log scale and an exponential function fitted to the data.
Run 58: "B" = 0.1 h^{-1}

Table 7.9. Accuracy of the glucose feed control

Run No.	Set-point exponent "B" (h^{-1})	Regressed exponent (h^{-1})	Error ($\times 10^2$)
48	0.10	0.099	-0.1
49	0.10	0.111	+1.1
50	0.10	0.101	+0.1
51	0.10	0.108	+0.8
52	0.20	0.199	-0.1
53	0.20	0.197	-0.3
54	0.20	0.202	+0.2
55	0.20	0.202	+0.2
56	0.20	0.201	+0.1
57	0.10	0.102	+0.2
58	0.10	0.099	-0.1
59	0.15	0.149	-0.1
60	0.15	0.149	-0.1

$1 \times 10^{-2} \text{ h}^{-1}$. There seems to be no significant pattern to the errors calculated. There is approximately the same number of runs which have a positive error as there is which have a negative error. This is contrary to the results of Minihane (1990), who found that the errors were consistently negative and which he believed was an inevitable result of single sided control action which causes a bias in the error towards underfeeding.

7.7.3 Biomass production

In an ideal situation, that is, one in which the organism has a simple constant stoichiometry and its growth is unlimited by any material other than the glucose feed, then the glucose, biomass and carbon dioxide data of a particular fermentation plotted on a log scale versus time, should all have the same slope value and this value should equal the "B" input into the computer (Minihane and Brown 1990). From Section 7.7.2 above, it was known that the glucose was being added at very accurate exponential rates.

To find out whether or not the biomass was being produced exponentially, the biomass values were drawn on a semi-log plot to check that they were exponential and then the slopes (μ) were calculated and compared to "B".

Figure 7.18 shows four examples of the biomass and carbon dioxide data drawn on semi-log plots. The specific growth rate (the slopes of the lines in Figure 7.18) for each

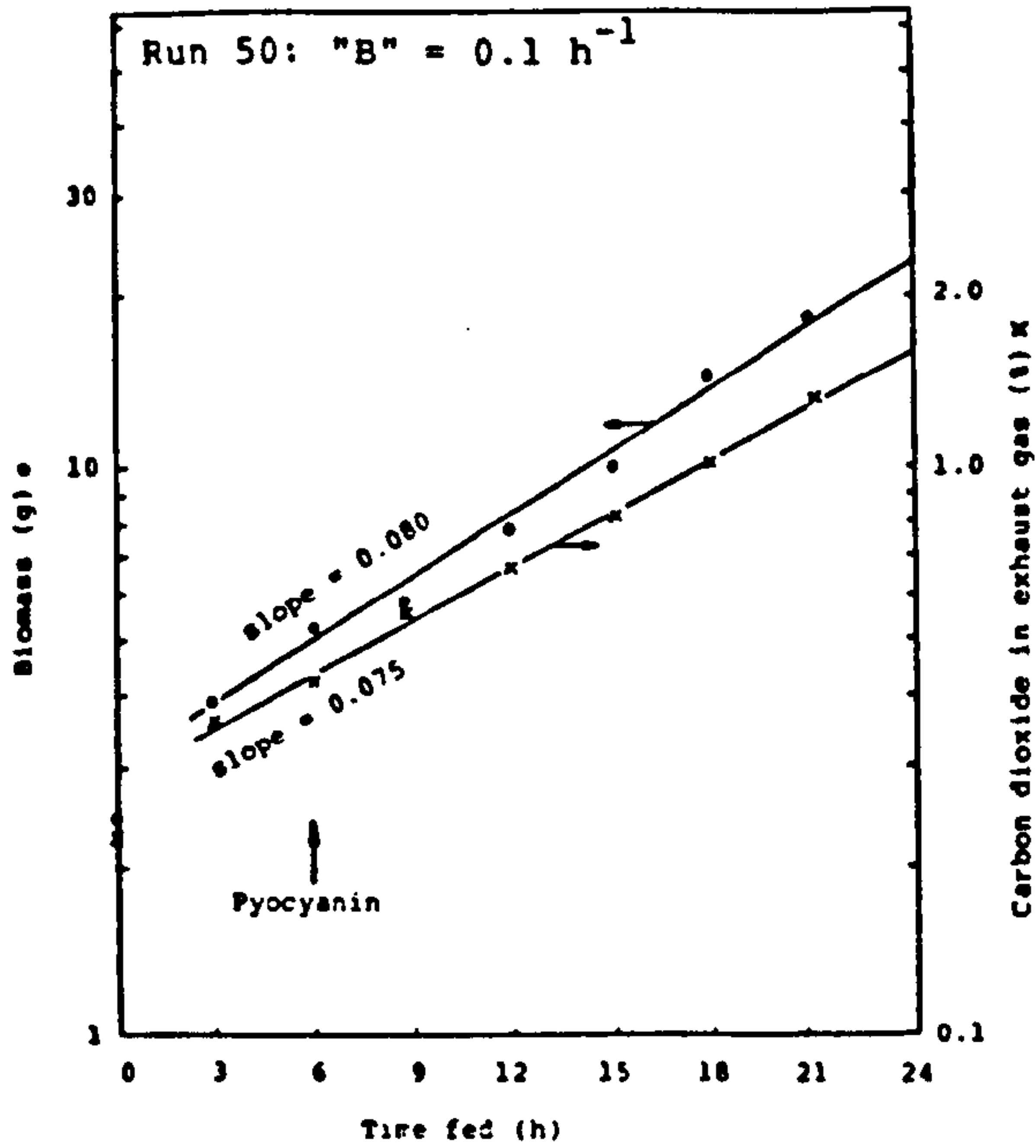
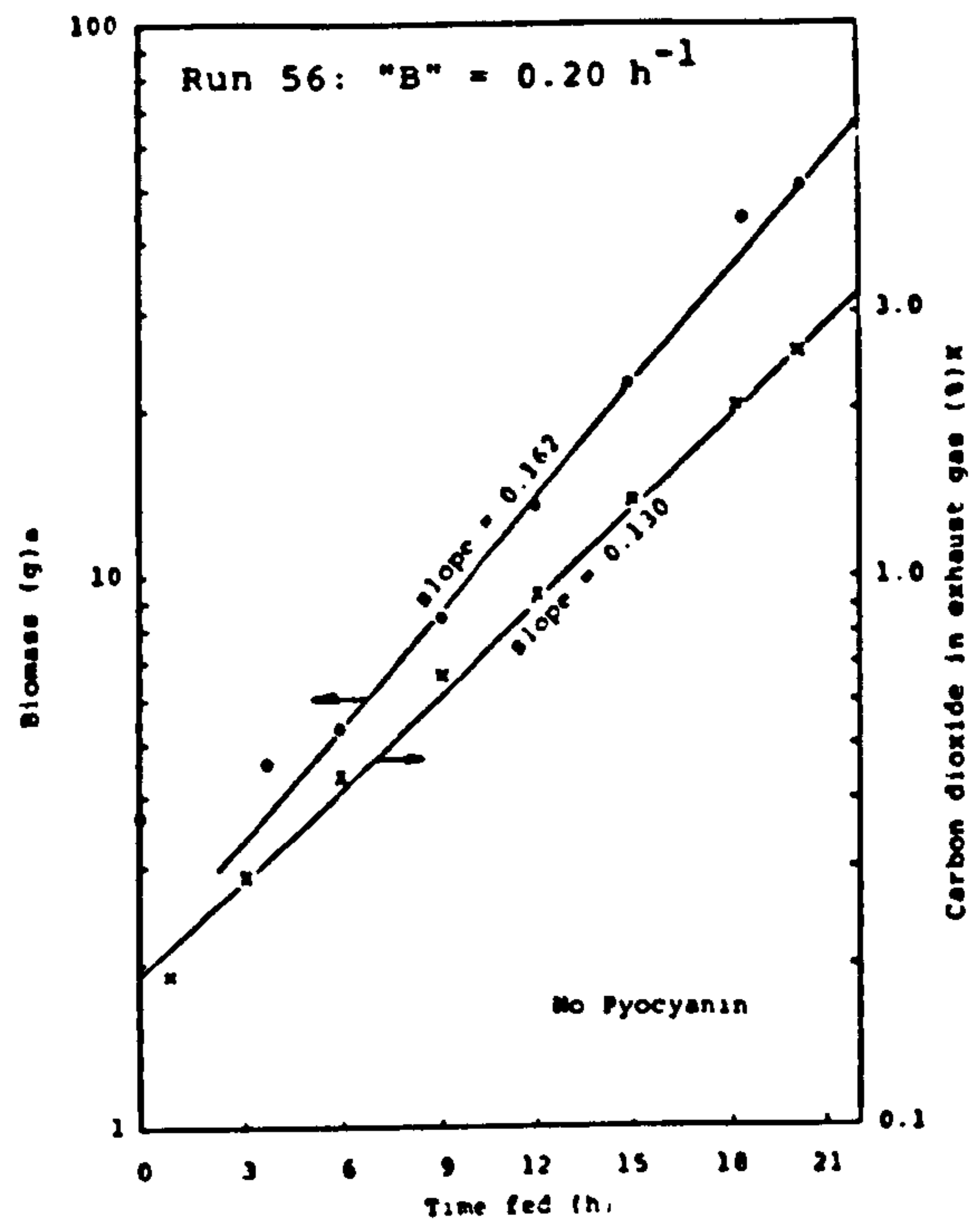
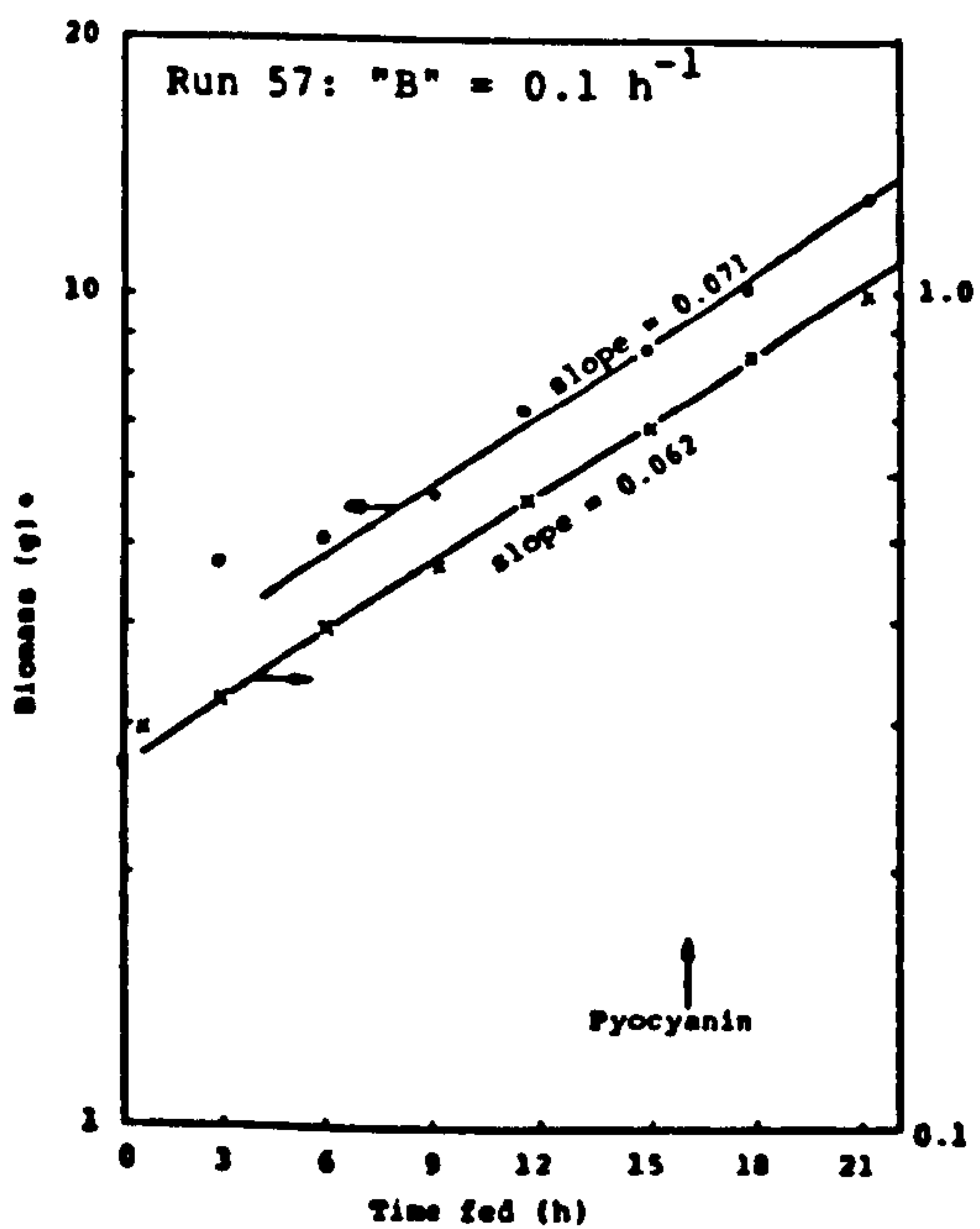
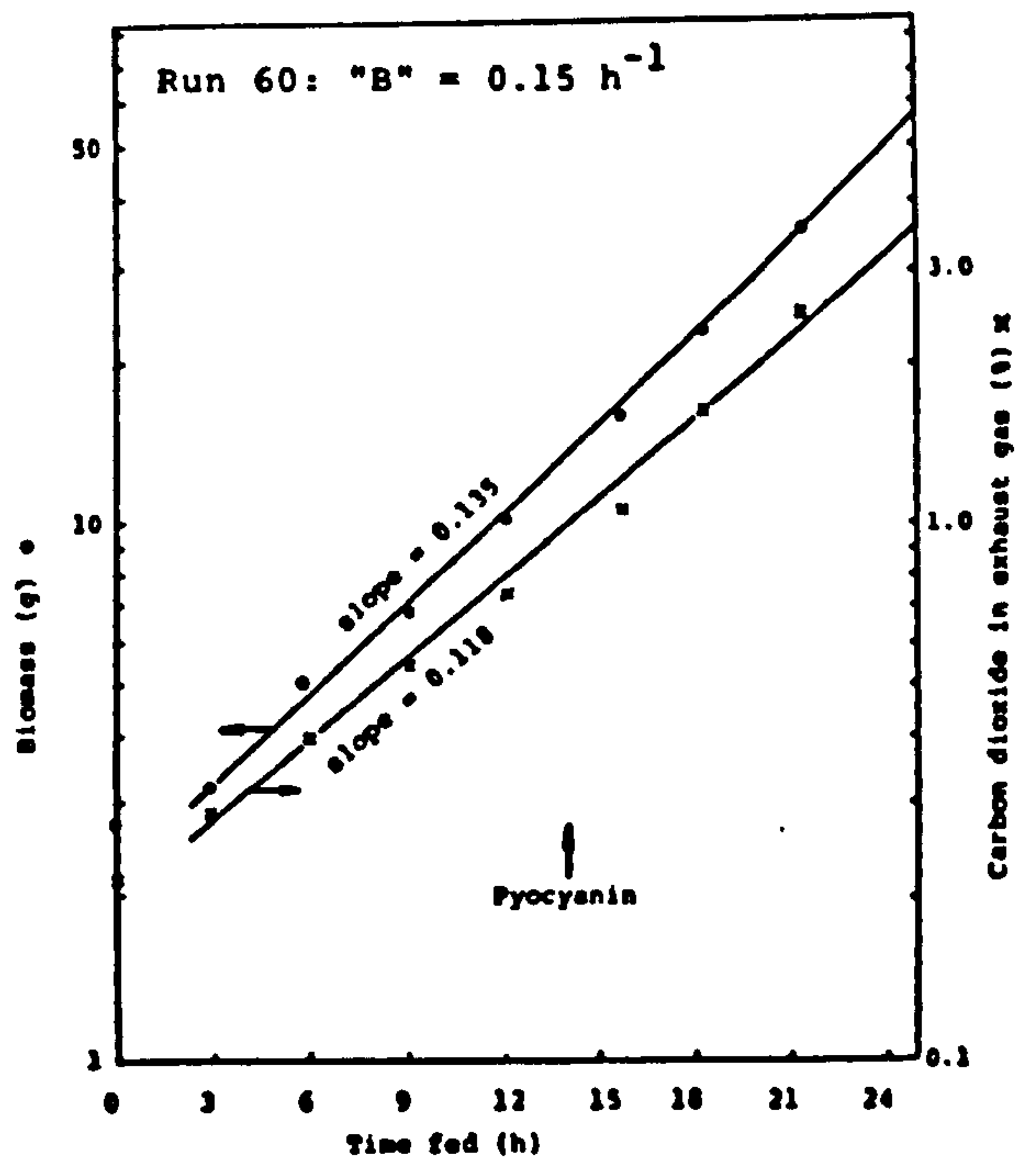


Figure 7.18. Runs 50, 56, 57 and 60
Examples of the biomass and carbon dioxide
data plotted on log scales

fermentation was calculated both directly using the measured biomass and indirectly (assuming a constant stoichiometry) using the carbon dioxide concentration in the exit gas. The results obtained are presented in Table 7.10.

Minihane (1990) observed good correlations between the specific growth rates calculated using the biomass and carbon dioxide data and the value of "B" whereas the specific growth rates calculated from the biomass and carbon dioxide data of this work (Table 7.10) are lower than the value of the set-point exponent "B" and in a few cases they are much lower.

There are several possible reasons for the biomass data from the exponential experiments having a different exponent. This difference can arise in particular from the difficulty in estimating the initial cell concentration that was input into the computer program. Runs 50 and 57 are examples of the cell concentration estimate being higher than the actual cell concentration. This created a situation in which more glucose, than that which was necessary for these cells to grow at the prescribed value of "B", was added. Therefore the growth rate was greater than "B" for the first 3 to 6 hours until the cell concentration caught up. This effect can be seen in Figure 7.18. In Runs 56 and 60 the opposite effect occurred. Here the estimated cell concentrations were

Table 7.10. Specific growth rates calculated from the biomass data and carbon dioxide data

Run No.	Set-point exponent "B" (h^{-1})	μ (biomass) (h^{-1})	μ (CO_2) (h^{-1})
48	0.10	0.087	(a)
49	0.10	0.097	0.090
50	0.10	0.080	0.075
51	0.10	0.095	0.080
52	0.20	0.143	0.138
53	0.20	0.184	0.170
54	0.20	0.195	0.161
55	0.20	0.198	0.188
56	0.20	0.162	0.130
57	0.10	0.071	0.062
58	0.10	0.091	0.083
59	0.15	0.138	0.133
60	0.15	0.135	0.118

(a) Data unavailable

lower than the actual cell concentrations. Therefore the cell concentration increased at a rate less than "B" for the first 3 to 6 hours until the glucose feed rate was sufficient for them to grow at the rate of the set-point exponent "B". This effect can also be seen in Figure 7.18.

There are several factors which indicate that there is a change from glucose controlled exponential to some other limitation of the growth rate. One such factor is the accumulation of glucose in the culture medium. Run 55 is a typical exponential run and data collected from Run 55 is presented in Figure 7.19 and Appendix B. Finite values of glucose concentration in the culture medium after 12 hours of feeding can be seen in Figure 7.19.

Other factors which indicate limitations are occurring are changes in the dissolved oxygen and carbon dioxide traces (Figure 7.19). From 10 hours onwards for the dissolved oxygen trace and from 12 hours onwards for the carbon dioxide trace, there was an increase in noise, which indicates a change in metabolism. Also at 12 hours, pyocyanin was evident in the culture medium. From 17 hours onwards there were dramatic changes in the amount of glucose in solution (accumulating at a rate of 10 g/h), carbon dioxide production (showing a steady decline), dissolved oxygen (rising to a constant amount of approximately 40%) and there was a change in the biomass from an exponential function to a linear function.

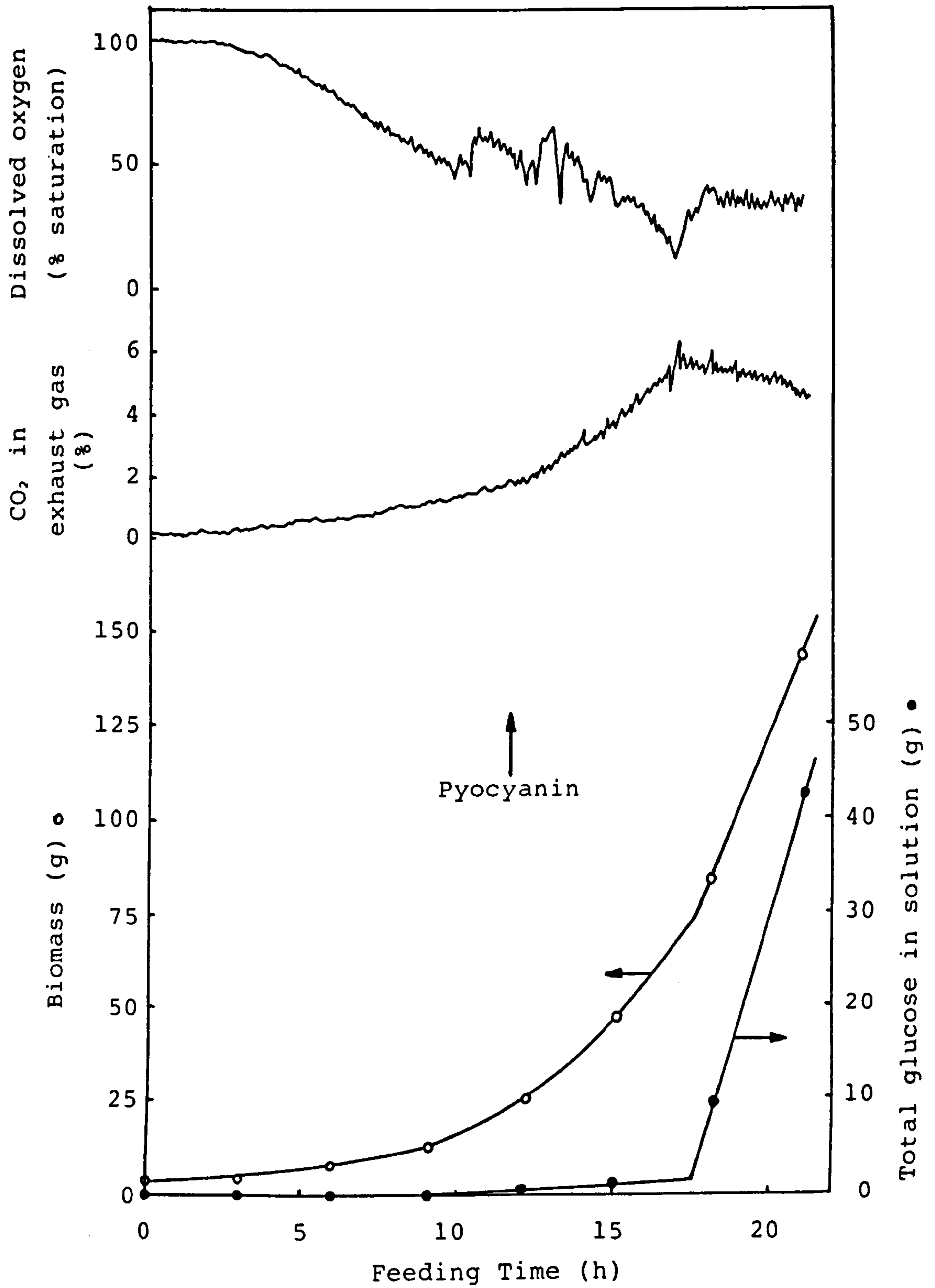


Figure 7.19. Run 55: A typical exponential fed run
"B" = 0.20 h⁻¹

From this evidence there is a suggestion of two limitations; a minor one coming into effect at 10 hours of feeding and a very significant one causing dramatic changes at 17 hours of feeding.

Earlier work discussed in Section 7.6.5 indicated that a limitation occurs during biomass production and there is evidence to suggest that this is possibly caused by phosphate scavenging. So it could be expected that the exponential fed experiments would be prone to this limitation as well.

Whilst the cells are being fed at a specific rate, they will be scavenging the conservative substrate(s). (The rate at which they scavenge these substrate(s) may be governed by the growth rate. Thus, if the growth rate is low the scavenging rate could be low.) When the substrate(s) have all been consumed the cells will have to reconstitute the substrate(s) so that they can use them. This change from using the substrate(s) in the medium to using the scavenged substrate(s) may show its self in the biomass profile. There will be a tendency for the growth rate to change from exponential growth to linear growth due to the cells now using stored substrate(s) and this rate of reconstituting the substrate(s) will control the growth rate.

Therefore, the difference seen between the biomass data and "B" can be explained by the influence of conservative

substrate(s) causing linear growth and thus the growth rate would be controlled by the conservative substrate limitation and not the glucose feed rate.

The timing of the limitation will be dependant on the concentration of the conservative substrate(s) (and in particular, the phosphate concentration) in the medium and the glucose feed rate. With high phosphate and substrate concentrations it will occur later, with low concentrations, sooner. With high feed rates it will become apparent whereas with slower feed rates it may never become apparent. When a limitation occurs the slope of the biomass values will change from "B" and therefore it would be expected that the biomass slope will be less than "B".

Another observation that can be made is that the specific growth rates calculated using the carbon dioxide data are less than those calculated from the biomass data. This could indicate that some of the carbon supplied in the form of glucose was being used by the organism to produce another carbon-containing compound other than carbon dioxide and this therefore leads to the suggestion that the stoichiometry of the organism is not simple.

In Section 7.6.7 it was suggested that yeast extract might be adsorbing some trace nutrient(s) thus taking them out of solution. If this phenomenon was occurring during the

exponential fed fermentations then this too, could have had an effect on the growth rate of the organism.

7.7.4 Cell yield values

When the amount of glucose and nitrogen additions are plotted against the biomass produced during feeding, a yield on glucose and nitrogen can be calculated.

Figure 7.20 is of two example runs (Runs 50 and 60) showing how the amount of glucose and nitrogen fed were plotted against biomass produced (Appendix C has an example which shows how the cell yields on glucose and nitrogen were calculated using the data collected). The cell yields on glucose and nitrogen for Runs 48 to 60 are presented in Table 7.11.

The glucose yield plots show a good linear relationship which is to be expected. Runs 49 to 53 have an average cell yield on glucose of 0.226 g/g and Runs 54 to 60 have an average cell yield of 0.326 g/g (Table 7.11). The most obvious difference between these two groups of runs is the medium composition (Table 7.8). The first group of runs (with the lower cell yield on glucose) have low phosphate and trace salt concentrations whereas the other group of runs (with the higher cell yield) have comparatively high phosphate and trace salt concentrations.

The plots of nitrogen fed against biomass produced on the other hand show non-linear relationships. The cell yields

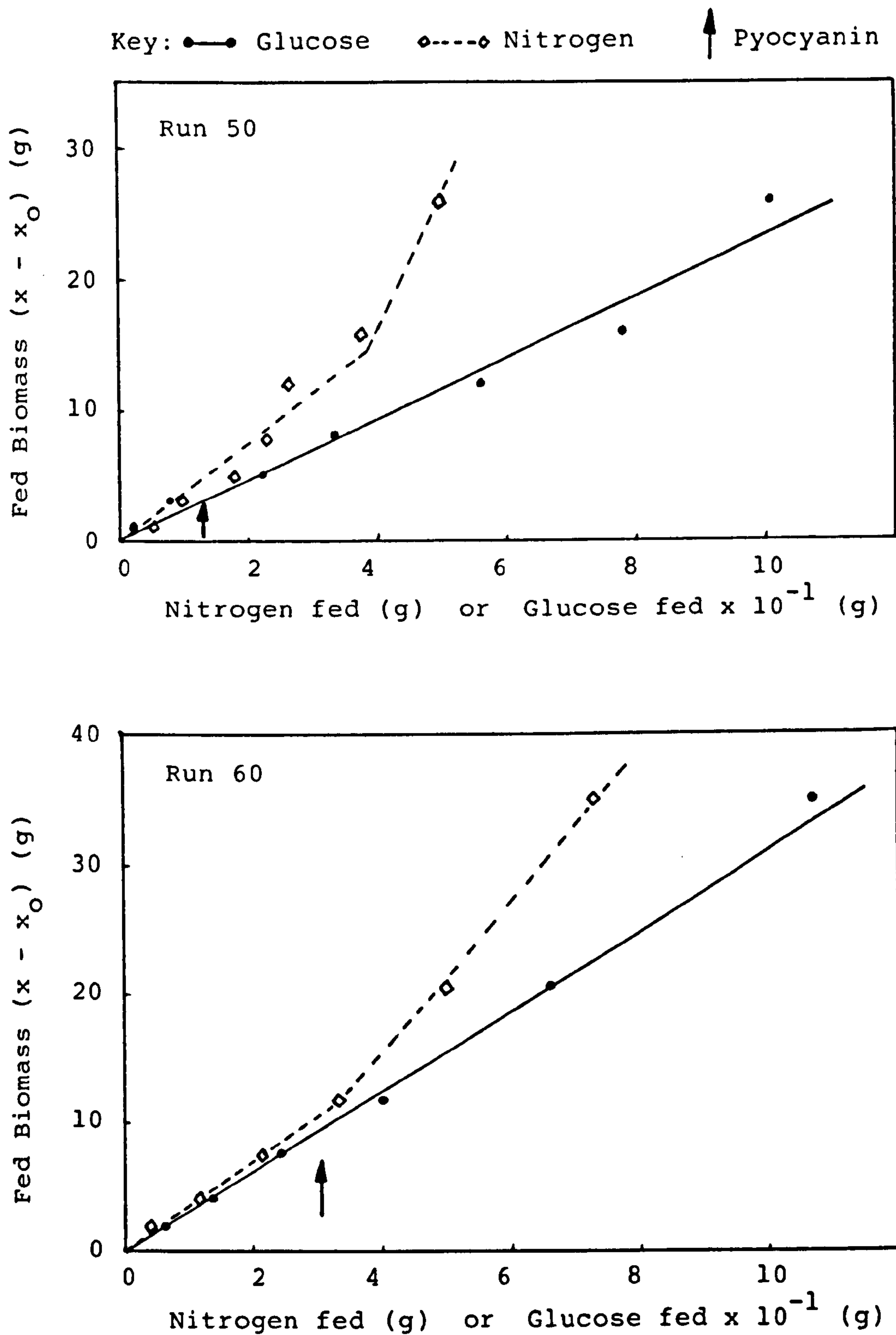


Figure 7.20. Two examples of the cell yield on glucose and nitrogen

Run 50: "B" = 0.10 h^{-1}

Run 60: "B" = 0.15 h^{-1}

Table 7.11. The cell yields on glucose and nitrogen

Run No.	Yield on Glucose (g/g)	Yield on Nitrogen (g/g)		Comments
		Phase 1	Phase 2	
48	(a)			
49	0.23	1.75	3.00	Precipitate
50	0.23	4.50	12.00	
51	0.23	4.00	4.80	
52	0.21	0.65	5.50	Loss of temperature control at 23 hours NH ₃ accumulation in medium
53	0.23	4.50	14.50	
54	0.31	1.60	5.60	
55	0.39	5.60	12.50	
56	0.14	3.75	7.00	No pyocyanin made
57	0.35	3.20	4.80	
58	0.31	2.80	5.40	
59	0.30	3.50	4.40	
60	0.30	3.50	6.25	
Av.	0.30	3.60	6.97	(Not including those with comments)

(a) Data unavailable

start at an average value of 3.60 and at some point during the fermentation the yield increases sharply to an average value of approximately 7.0 (Table 7.11). Pirt (1975) quotes cell yields for *Klebsiella aerogenes* of 0.50 g/g glucose and 8.75 g/g nitrogen. The average cell yields (Table 7.11) of this work for glucose and nitrogen are 0.30 g/g and 3.60 g/g (first phase) respectively. These cell yields are surprisingly low when the cell yield on phosphorus (Section 7.6.5) compared very favourably with other reported cell yields. A reason for the cell yields to be so low could be that the organism is making at least one compound which contains both carbon and nitrogen, for example, pyocyanin.

Because pyocyanin contains a large amount of carbon and nitrogen it would be expected that at the point at which pyocyanin is seen in the culture medium, the yields of biomass on glucose and nitrogen would decrease. But what seems to be happening (Figure 7.20) is that the pyocyanin is being made at relatively low biomass values and the yield on glucose remains unchanged whereas the yield on nitrogen has a tendency to increase at higher biomass values. The point at which the nitrogen yield increases could be an indication that the organism has stopped making this compound (perhaps due to a trace salt limitation) and the nitrogen being added is used only for biomass production from that point onwards. However, this does not explain why the glucose yield remains low. One

possible explanation could be that the organism is making other secondary products, such as exopolysaccharides.

These cell yield results support the view made in the previous section that the stoichiometry of this organism is unusual and far from simple.

7.7.5 Lipase production

The lipase activities of samples taken from Runs 49 to 60 are presented in Table 7.12 (a) and the lipase activities per g of cells of these samples are shown in Table 7.12 (b). In most cases the lipase activities rise during the first few hours of feeding, reaching a maximum of 0.52 LU/cm^3 (Table 7.12 (a): Run 53) and then 'decay' even though the cell concentrations continue to increase. This 'decaying' is more obvious in Table 7.12 (b) where the cell concentrations have been taken into consideration. Apart from this observation, there seems to be no obvious pattern nor does the lipase production seem to be linked to the growth rate of the organism. For many of the runs the 'decay' in lipase activity coincides with the production of pyocyanin, with a few exceptions, Runs 50, 51, 52 and 55. Run 56 is the only run in which pyocyanin was not observed but even during this run the lipase activity 'decayed'. This suggests that there are factors other than pyocyanin production which cause the organism to stop producing the lipase.

Table 7.12 (a). Lipase activity (LU/cm³)

Run No.	49	50	51	52	53	54	55	56	57	58	59	60
$\mu_{\text{(biomass)}}$ h^{-1}	0.097	0.096	0.098	0.150	0.180	0.195	0.198	0.143	0.071	0.091	0.147	0.143
FT (h)												
0	0	0.040	0.080	0	0	0.040	0	0.080	0.080	0.120	0	0
3	0.360	0.120	0.160	0.120	0.080	0	0.200	0.040	0.080	0.040	0	0
6	0.360	0.160	0.240	0.160	0.320	0.120	0.400	0.040	0.080	0.120	0.040	0
9	0.280	0.200	0.080	0.160	0.520	0.320	0.440	0.160	0.040	0.040	0.080	0.040
12	0.200	0.240	0.080	0.280	0.400	0.200	0.200	0.440	0.200	0	0	0.300
15	0.200	0.220	0.160		0.160		0.240	0.440	0.320	0	0.080	0.160
18	0.240						0.080	0.080	0.080	0.040	0	0
21	0.200				0.160		0.120		0.200	0.080		0.040
22		0.280	0.160									
24										0.040		
25		0.760	0.320									
28			0.400									

Key:- — = The point at which pyocyanin appeared in the culture medium
LU = μ moles of free fatty acid released per minute under the test conditions
FT = Length of time that glucose was fed to the culture

Table 7.12 (b). Lipase activity (LU/g cells)

Run No.	49	50	51	52	53	54	55	56	57	58	59	60
μ (biomass) h^{-1}	0.097	0.096	0.098	0.150	0.180	0.195	0.198	0.143	0.071	0.091	0.147	0.143
FT (h)												
0	0	41	100	0	0	25	0	44	65	300	0	0
3	200	75	178	63	47	0	65	18	36	40	0	0
6	171	71	150	67	119	41	105	15	33	80	25	0
9	112	83	44	34	113	56	81	39	15	21	29	13
12	63	73	40	47	51	23	2	71	57	0	0	63
15	54	52	64		12		14	45	76	0	13	22
18	52						3	5	16	9	0	0
21	30				7		3		31	14		3
22		37	30									
24										6		
25		70	44									
28			43									

Key:- — = The point at which pyocyanin appeared in the culture medium
LU = μ moles of free fatty acid released per minute under the test conditions
FT = Length of time that glucose was fed to the culture

For the majority of the exponentially fed fermentations the cells grew exponentially, even if it was not at the rate at which they were being fed (Table 7.10). Therefore, it could have been expected that the lipase would have been produced at an exponential rate. This effect was not observed in any of the fermentations. One reason for this could have been the small number of samples taken. However, there are obviously many interacting factors occurring during these fermentations (as covered in the previous sections) so that it is not surprising that lipase was not made at an exponential rate because the growth of the organism was not under the control of the glucose feed.

There is evidence in the literature that other workers had similar problems with the lipase activity 'decaying'. Some workers thought that this was due to the fermentation conditions being sub-optimal, for example, the lipase may be labile at temperatures greater than 10°C (Nashif and Nelson 1953) and Watanabe et al. (1977) found that many *Pseudomonas* strains produce alkaline lipases, in particular *aeruginosa* and *fluorescens* (that is, they have pH optima around 9.5).

Several workers have found that the production of the lipase is accompanied by the production of a protease. Fox and Stepaniak (1983) found that the secretion of both lipase and protease occurred at the same time during the

fermentation. That is, mainly at the end of the log phase and during early stationary phase.

Whilst growing *Pseudomonas fragi*, Lawrence et al. (1967) noted that after 48 hours of incubation, the number of cells stayed constant but the amount of lipase decreased rapidly. Nahas (1988) found that lipase activity decreased rapidly after the stationary phase was reached on the fourth day of incubation. Neither author suggested theories of why the lipase activity 'decayed' in this way.

7.7.5 Conclusions

The feed technique developed by Minihane (1990) was shown to be very accurate. From the logged balance values, the glucose feed profile that was followed had a regressed exponent very close to the set-point exponent, "B", with an error of approximately 1% in most cases.

The specific growth rates however, when calculated using the biomass and carbon dioxide data, were lower than the value of "B". There were several observations which suggested why this might be so. Previous work showed that the organism was prone to conservative substrate limitation with particular reference to phosphate scavenging. This would mean that the growth of the organism was not controlled by the glucose feed rate.

Another observation that was made was that the cell yields on glucose and nitrogen were nearly half the expected

values. It was suggested that this was due to the organism producing secondary metabolites such as pyocyanin and exopolysaccharide. The overall effects of the growth rate not being under the control of the glucose feed and of other secondary products being made was that the lipase activity was low. The maximum lipase activity that was achieved was 0.52 LU/cm^3 , compared to the maximum lipase activity achieved in the constant fed experiments which was 0.80 LU/cm^3 .

7.8 Pyocyanin production

7.8.1 Introduction

Pyocyanin was observed to be produced by the selected strain of *Pseudomonas aeruginosa* on tributyrin and nutrient agar plates and in shake flasks containing LP₂SF medium.

During the fermentation runs, pyocyanin was assayed using two methods as detailed in Section 5.5. The technique which was used for every run was a visual observation of the appearance of pyocyanin in the culture medium. The information generated using this technique can be found in Table 7.13. A quantitative assay for pyocyanin was not developed because pyocyanin was not the metabolite whose production was being optimised. By using the extraction method for pyocyanin (Section 5.5.2) it would have been possible to quantitatively assess the amount of pyocyanin

Table 7.13. Time of appearance of pyocyanin

	Run No	Time (h)	Length of Feeding (h)	Biomass (g)	Pirt's Trace Salts Incd. By Factor	KH ₂ PO ₄ Concn. (g/dm ³)	Ppt. Formed
	2	11.0	--	4.83	x1	1.13	no
Constant fed	16	7.0	0	3.12	x1	1.13	no
	17	12.0	5.0	7.70	x1	1.13	no
	18	12.0	7.0	6.50	x1	1.13	no
	20	14.0	8.0	n/k	x1	1.13	no
	21	19.0	14.0	11.50	x1	1.13	no
	23	18.0	13.0	3.80	x1	1.13	no
	24	17.0	12.0	4.50	x1	1.13	no
	25	17.0	10.0	4.75	x2	2.26	no
	26	29.0	14.0	4.20	x1	1.13	no
	27	15.0	9.0	6.75	x2	2.26	no
	28	30.0	8.0	6.50	x1	1.13	no
	29	36.0	9.0	5.00	x1	1.13	no
	30	36.0	14.0	7.30	x1	1.13	no
Excess glucose	33	15.0	9.0	12.00	x2	1.13	no
	34	16.0	8.0	18.53	x2	2.26	no
	35(a)	28.5	13.0	17.20	x4	4.52	yes
	37	18.5	8.0	9.00	x4	4.52	no
	38	22.0	11.0	18.50	x4	4.52	no
	39	19.5	8.0	10.00	x4	4.52	no
	40	21.0	9.0	17.50	x4	4.52	no
	41	19.0	7.0	19.50	x14	4.52	no
	42	20.0	8.0	22.50	x14	4.52	no
	43	21.0	9.0	23.50	x14	4.52	no
	44	13.0	7.0	14.50	x14	4.52	no
	45	20.0	8.0	20.00	x10	9.04	yes
Exponential fed	48	21.0	8.0	4.50	x10	9.04	yes
	49	17.0	7.0	4.50	x10	9.04	yes
	50	17.0	6.0	5.40	x10	2.26	no
	51(b)	22.0	11.0	14.20	x10	2.26	no
	52	22.0	9.0	11.00	x10	2.26	no
	53	21.0	9.0	11.20	x10	9.04	no
	54	27.5	10.0	15.00	x20	22.60	no
	55	24.0	12.0	25.00	x20	22.60	no
	56	>35.0	>20.0	>51.30	x40	45.20	yes
	57	32.0	16.0	10.00	x30	33.90	yes
	58	39.0	18.0	9.00	x40	45.20	yes
	59	32.0	17.0	15.00	x40	45.20	yes
	60	34.0	14.0	15.50	x40	45.20	yes

(a) Sterile fermenter left 96 hours before inoculating

(b) From Run 51 onwards CaCl₂ solution added separately

made, but the time at which the pyocyanin production started was considered a more important piece of data than the quantity of pyocyanin that was produced.

During the batch fermentations of this work, the presence of pyocyanin was not usually observed. This could have been for various reasons. For instance, the maximum biomass made during these runs was approximately 5 g and this was low enough for a trace substrate limitation situation not to have occurred. Also, the growth rate of the organism was not being manipulated in any way.

Many workers studying the production of lipase by *Pseudomonas aeruginosa* do not mention production of pigment. This could be for a number of reasons. The majority of workers grew the organism on complex media and produced low cell concentrations (that is, substrate limitation situations did not occur) or, it was expected that *Ps. aeruginosa* produced a pigment and therefore it was not considered necessary to comment on this fact. An exception to this is Nadkarni (1971) who does mention the presence of the pigment and corrected his optical density measurements for its effect. However, it did not seem to affect the amount of lipase produced by *Ps. aeruginosa*.

Some of the factors which could have affected the appearance of pyocyanin are listed for each fermentation run in which pyocyanin was observed and they are presented

in Table 7.13 and considered in the subsequent subsections.

7.8.2 Concentration of trace salts

It was first thought that the trigger for pyocyanin production by the organism was a trace salt limitation. The medium used in the constantly fed-batch fermentations was the same as that used in the batch fermentations and yet pyocyanin was observed in practically every constantly fed run. The cell concentrations produced in the fed-batch runs were much higher than those produced in the batch fermentations due to the large additions of glucose during feeding and this could have caused a limitation of trace salt or salts.

Trace salt concentrations in the growth medium were increased from 1 to 40 times the concentrations recommended by Pirt (1975). During the constant fed fermentations the trace salts were only doubled, but during the process of developing the medium for maximum biomass production (Runs 31 to 46), the trace salts were increased up to fourteen times that suggested by Pirt (1975) and still pyocyanin was formed (Table 7.13). For Runs 54 to 60, the trace salt concentrations were increased by factors greater than 20 fold and it was noticed that precipitations formed and this could have been another cause of possible trace salt limitations.

To establish which trace salt could be causing the limitation, each trace salt was omitted in turn from the basal medium (Section 4.3.2). The results of the large shake flask experiments (Table 7.14) seemed to suggest that the pyocyanin production was induced by an iron limitation. Therefore the amount of iron added to the fermenter medium of this work was increased to 0.28 g/dm^3 (Pirt's x40) (Runs 58, 59 and 60) and pyocyanin was still produced.

This result was in total contradiction to the work done by Burton *et al.* (1948). Their aim was to determine the mineral constituents of a medium which were essential to the formation of pyocyanin and establish the concentrations of salts required for optimum yield of the pigment. By omitting each salt in turn from a basal medium, they were able to establish which ions were essential to pigment formation and to ascertain the optimum levels of concentration. They found that with a ferrous sulphate concentration of 0.00001% or less, maximum growth was obtained but there was no elaboration of pyocyanin. They found that maximum pyocyanin formation occurred between 0.0005% and 0.005% ferrous sulphate and that growth, and therefore pyocyanin formation, was inhibited in the presence of 0.03% ferrous sulphate.

The results of these shake flask experiments (Table 7.14) suggests that pyocyanin production may be triggered also

Table 7.14. Results of shake flask experiments (2 dm³)

Medium	Average Biomass (after 144 h) (g)	Colour (after 24 h)
A (no MgSO ₄ ·7H ₂ O added)	0.70	slightly green
B (no CaCl ₂ ·2H ₂ O added)	1.75	peachy-pink
C (no FeSO ₄ ·7H ₂ O added)	2.25	blue-green
D (no MnSO ₄ ·4H ₂ O added)	1.80	peachy-pink
E (no ZnSO ₄ ·7H ₂ O added)	4.45	peachy-pink
F (no CuSO ₄ ·5H ₂ O added)	1.95	peachy-pink
G (no CoCl ₂ ·6H ₂ O added)	6.90	peachy-pink
H (control)	6.95	peachy-pink

by a magnesium sulphate deficit. Burton et al. (1948) found that when 0.0001% magnesium sulphate was employed only a slight amount of growth occurred and that maximum pyocyanin production was obtained at 2% magnesium sulphate.

7.8.3 Phosphate concentration

Potassium dihydrogen phosphate concentrations from 1.13 g/dm³ to 45.20 g/dm³ were added to various runs in this work. The effect on the feeding time and the biomass at which pyocyanin was observed for the different potassium dihydrogen phosphate concentrations can be seen in Figure 7.21. The fermentation runs have been split into two groups: Runs 14 to 45 (the constant fed and excess glucose runs which have relatively high growth rates) and Runs 48 to 60 (the exponential fed runs which have low growth rates).

The trend of the graph of feeding time against phosphate concentration for Runs 14 to 45 is almost constant and closely resembles the early values of Runs 48 to 60. The trend of the higher phosphate concentrations for Runs 48 to 60 is generally upward. This suggests that when the phosphate concentrations are low, small changes in phosphate concentration and growth rate do not affect the time at which pyocyanin appears. But when the phosphate concentrations are high and the growth rates are low, the pyocyanin takes longer to appear.

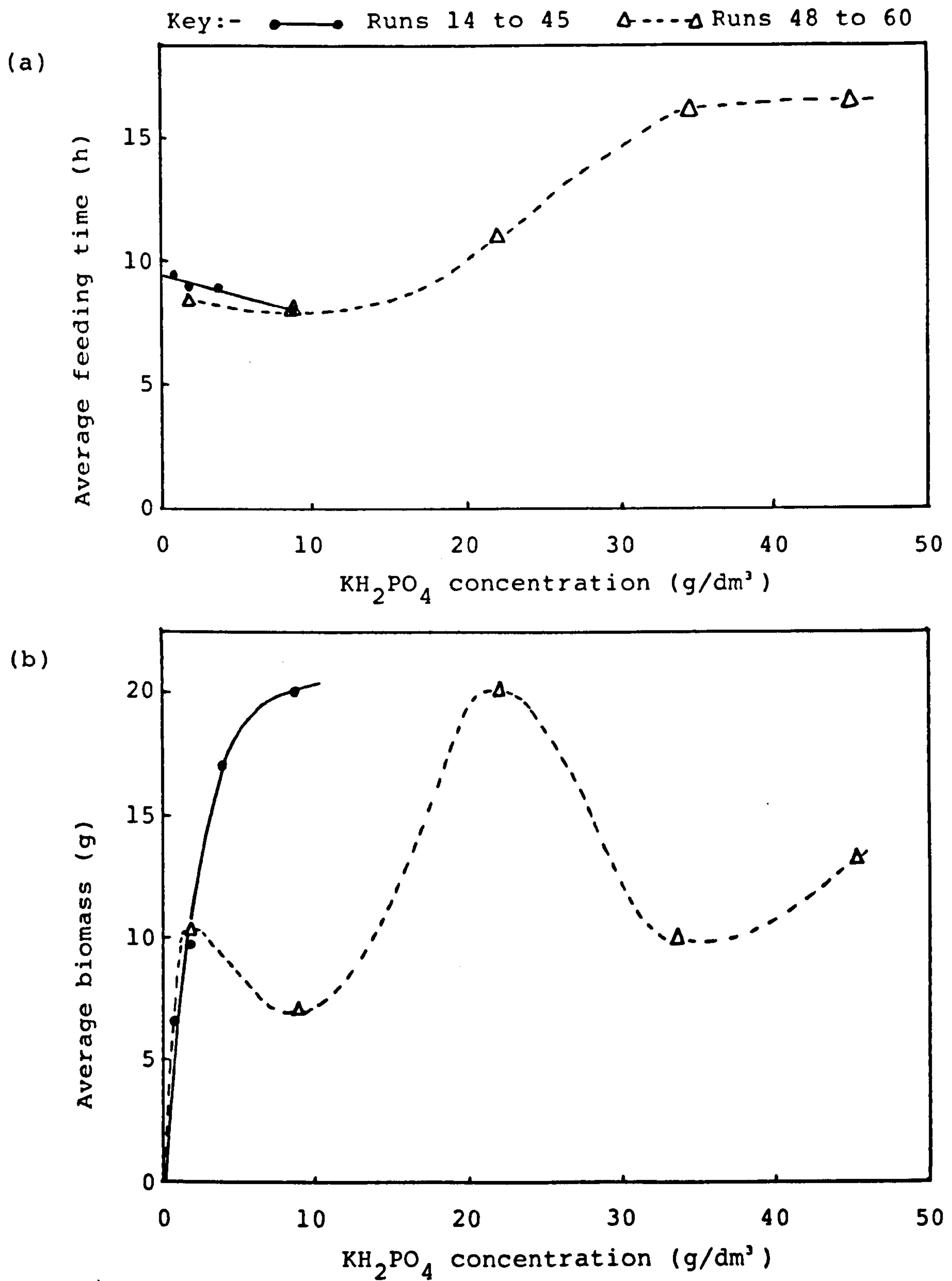


Figure 7.21. Effect of KH_2PO_4 concentration on the appearance of pyocyanin as a function of
(a) feeding time and
(b) biomass

The trend of the graph of biomass against phosphate for Runs 14 to 45 is upward and for Runs 48 to 60 it oscillates but is also generally upward. This suggests that increasing the phosphate concentration in the medium causes the pyocyanin to be formed at higher biomass values. There is evidence from the literature that indicates that the concentration of phosphate in the medium has an effect on the production of pyocyanin.

King et al. (1954) described two semi-synthetic media for demonstration of pyocyanin and fluorescent pigment production by pseudomonads and noted the inhibitory effect of added phosphate on pyocyanin formation. Frank and Demoss (1959) found that omission of phosphate greatly reduced or completely inhibited pyocyanin formation.

Ingledew and Campbell (1969) come to the conclusion that phosphate deficiency may be the trigger for phenazine synthesis in *Pseudomonas aeruginosa* and given this position, virtually every medium described in the literature would be deficient in phosphate during the latter stages of growth. They conclude that phosphate concentration may provide the active control mechanism for initiation and maintenance of synthesis of the enzymes responsible for pigment formation.

An opposing view is put forward by Ingram and Blackwood (1970) who found that there was production of pyocyanin when the phosphate concentration reached a critical

minimum level and this production was terminated as the phosphate concentration in the medium was increased.

7.8.4 Growth rate of the organism

Some workers have suggested that the trigger for pyocyanin production is low growth rates and/or carbon source limitation (Section 2.6). This may have been the case during the constant fed batch fermentations but it does not seem to be the case during the exponential fed fermentations and during the development of the growth medium experiments the glucose was kept in excess throughout the feeding process and the pyocyanin was still produced.

7.8.5 Lipase production

Pyocyanin production seemed to be connected with a reduction in lipase production during constant fed-batch runs. When describing the typical constant fed experiment, Run 23, it was noted that there was a sudden decrease in the lipase activity which coincided with the appearance of pyocyanin in the culture medium (Figure 7.4). The lipase production resumed after four hours but at half the original rate. In the other constant fed fermentations similar effects were observed.

During the exponential fed experiments, there seemed to be a link between the appearance of pyocyanin in the culture medium (marked with a line) and a decrease in the measured

lipase activity (Table 7.12). A possible explanation for these observations is that lipase production and pyocyanin production are mutually exclusive. That is, if the organism is making one product it cannot make the other.

These observations are contrary to those made by Sztajer (1987) who reported a beneficial correlation between pyocyanin and lipase production in LP₂SF medium in shake flasks.

7.8.6 Conclusions

Many workers have tried to establish the factors that trigger *Pseudomonas aeruginosa* to produce pyocyanin but they are unable to agree on what the factor or factors are. From shake flask experiments carried out in this work, it seemed as though the trigger for pyocyanin production was an iron deficiency. However, when the iron was increased in the culture medium with a view to preventing pyocyanin production, the pigment was formed.

In fact, none of the suggestions found in the literature, including high phosphate concentrations (though increasing the phosphate concentration did delay the appearance of pyocyanin) and high growth rates, had any effect on the organism's ability to produce pyocyanin. This was unfortunate as there seemed to be a link between pyocyanin production and lipase non-production. Pyocyanin production is obviously controlled by a very complex mechanism.

7.9 General Discussion

The lipase-producing organism which was used in this work was a gift from Dr. Helena Sztajer. Dr. Sztajer and her colleagues identified the micro-organism to be a strain of *Pseudomonas fluorescens* (Sztajer et al. 1988). They grew the organism in the highly complex medium, LP₂SF, with olive oil as the lipid substrate. She found that when the shake flask cultures turned blue/green, lipase was produced by the organism.

This same lipase-producing organism was microbiologically examined and biochemically tested and found to be a strain of *Pseudomonas aeruginosa*. It is a gram negative rod and it produces a soluble phenazine pigment (pyocyanin), a glycolipid emulsifier, an exopolysaccharide as well as a diffusible lipase.

This organism was inoculated into two semi-defined media (with glucose as the carbon source) and it was found that it was able to grow and produce lipase in small but measurable amounts (0.03 LU/cm³) without the need of a lipid inducer. This suggested that the lipase was not inducible but was constitutive (Jacob and Monod 1961) and under the control of catabolite repression (Pastan and Perlman 1970).

The organism was grown up under batch conditions in a stirred fermenter in a semi-defined medium (Pirt's medium

plus 1 g/dm³ yeast extract) and it was observed that as the glucose in the medium reached zero, an aliquot of lipase was produced (0.173 LU/cm³). This supported the view made by Makman and Sutherland (1965) that transcription of enzyme systems under the control of catabolite repression could not take place in the presence of high glucose concentrations because the surplus glucose (or its metabolites) inhibits the enzyme adenylate cyclase. Hence, when the glucose concentration is low, the concentration of adenylate cyclase and cyclic AMP is high and therefore transcription of the enzyme's genes can take place. Also, when the glucose concentration is low, the growth rate of the organism decreases.

Another way of decreasing the growth rate is if a nutrient (other than glucose) becomes limiting. During the batch fermentations, it was noted there was a second phase of lipase production which occurred before the glucose had run out. This suggests that another nutrient became limiting before the glucose did but the result was the same, a reduction in the growth rate and production of lipase.

When an enzyme's production is under the control of catabolite repression, it is possible to catabolite de-repress the production of the enzyme by using a feed system and get the organism to produce the enzyme throughout its growth phase, not just at the end.

From the work carried out by, for example, Clark and Marr (1964), Yamane and Tsukano (1977), Brown and McAvoy (1990) and Allinson (1990), it is known that if carbon sources are fed at limiting constant rates the amount of enzyme produced dramatically increases.

When this strain of *Pseudomonas aeruginosa* was fed with glucose solutions at various constant rates the lipase production did increase. The lipase activity more than quadrupled, from 0.173 LU/cm³ for the batch fermentations (Run 12) to 0.800 LU/cm³ for the constant fed-batch fermentations (Run 23). Even with this dramatic increase, the lipase activity being produced was still at least 250 times less than that recorded by other workers (Table 2.3). For example, Suzuki et al. (1988) who fed olive oil to *Pseudomonas fluorescens* were able to produce 2,000 LU/cm³.

Several other observations were made about the constant fed fermentations. Firstly, the point at which the pyocyanin appeared in the culture medium, the lipase production rate decreased by as much as half. Secondly, it was found that at constant glucose feed rates below 2.0 g/dm³/h the glucose feed rate controlled the linear growth rate, but at feed rates above 2.0 g/dm³/h the growth rate was under the control of some other limiting substrate, a substrate which was limiting so much that it did not allow the linear growth rate to exceed

0.45 g/dm³/h. Thirdly, the constant glucose feed rate which produced the maximum lipase activity was found to be 0.5 g/dm³/h which was a quarter of the value which gave maximum pullulanase production by *Klebsiella planticola* (2.0 g/dm³/h) (Brown et al. 1988). This much lower optimum constant feed rate meant that the slope away from the optimum on the low feed rate side was very steep.

Developing the medium such that it would support a dry weight of 25 g/dm³ of cells produced a lot of information about the physiology of the organism. It was found that there was a dominating nutrient limitation which consistently caused linear growth. From the literature, this is known to occur and the most obvious nutrients are glucose (Yamané and Hirano 1977 and Esener et al. 1981) and oxygen (Sinclair and Kristiansen 1987). But when oxygen and glucose are supplied in excess, it has been found to be trace nutrients, such as nicotinic acid (Jackson and Copping 1952) or phosphate (Slezak and Sikyta 1957 and Horiuchi 1959) that are the limiting components.

The fact that limiting quantities of these trace nutrients caused linear rather than exponential growth suggested that there was another factor to be taken into consideration. It was noticed by the workers growing these organisms that nicotinic acid and phosphate were taken up by the initially small concentrations of cells at rates out of proportion to their nutritional requirements.

This early scavenged uptake of trace nutrients could be followed by their distribution to successive generations thus causing the change from exponential growth rates to linear growth rates. Nyholm (1976) described such components as 'conservative' nutrients.

In this work, there did seem to be some connection between the final biomass achieved, the linear growth rate and the phosphate concentration but not enough to categorically say phosphate scavenging was causing the limitation. However, the cell yield of the organism on phosphorus compared favourably to published data.

From the work of Gaddum (1988), there was a suggestion that yeast extract was the limiting nutrient which was causing the linear growth in the batch fermentations. When Gaddum (1988) increased the amount of yeast extract in his growth medium, the linear growth rate (and other indicating factors) increased accordingly. However, when the yeast extract concentration was increased in this work, the linear growth rate decreased. It was suggested that the yeast extract was absorbing trace nutrient(s) and thus causing a limitation.

Even though the indications were that this organism was going to follow the pattern of *Klebsiella planticola* for control of enzyme production from results up to and including the constant fed-batch fermentations, it was clear from the nutrient limitations described above, that

there would be some problems associated with feeding glucose solutions exponentially.

The exponential feed technology developed by Minihane (1990) was shown to be very accurate. Minihane (1990) suggested that the specific growth rate of the organism would be the same as the value of "B" input into the pre-programmed protocol. However, when the specific growth rates were calculated using the biomass and carbon dioxide data they were lower than the value of set-point exponent, "B". It was presumed that the reason for the specific growth rate of the organism not being controlled by the glucose feed rate was the nutrient limitations causing changes in the stoichiometry of the organism.

The possibility of secondary metabolite production, such as pyocyanin and exopolysaccharide, is made more likely by the fact that the cell yields on glucose and nitrogen were much below published values.

The overall effect of the growth rate not being controlled by the glucose feed, was that the lipase was made in very small amounts, the maximum lipase activity achieved being 0.52 LU/cm^3 compared to the maximum lipase activity achieved in the constant fed experiments which was 0.80 LU/cm^3 .

Finally, work was carried out to try and identify the factor which triggers pyocyanin production. None of the attempts at preventing pyocyanin production succeeded.

The intention of this work was to produce an exponential fed-batch lipase production system. The overall concept was good. The preliminary studies gave excellent results, the technology could not be faulted but the organism had a far more complex stoichiometry than was expected. For this technique to succeed, the metabolism of the organism would have to be studied in much more detail. Therefore, it can be concluded that the basic concept was sound, but that the maximisation of lipase production by *Pseudomonas aeruginosa* was not possible with the present state of knowledge.

CHAPTER EIGHT

CONCLUSIONS

1. The lipase-producing micro-organism was identified as a strain of *Pseudomonas aeruginosa*..
2. When the organism was grown on nutrient and tributyrin agar plates and in the complex medium LP₂SF, it turned the cultures and plates blue/green due to the production of pyocyanin.
3. The organism was able to grow in shake flasks on a simple yeast extract and glucose medium with no lipid substrate present.
4. The organism produced a lipase activity of 0.03 LU/cm³ under the conditions described in Conclusion No. 3.
5. Lipase production in this strain of *Ps. aeruginosa* is under the control of catabolite repression.
6. The organism was able to grow in a simple defined minimal salts medium (Pirt 1975) supplemented with 1 g/dm³ yeast extract.
7. In order to produce 1.5 g/dm³ dry weight of cells in approximately six hours an addition of 1 g/dm³ of glucose had to be made to the semi-defined medium.

8. The maximum amount of lipase activity produced under batch conditions was 0.173 LU/cm^3 .
9. The lipase was produced in two aliquots, one aliquot coinciding with the glucose running out.
10. The batch fermentations were seen to be suffering from a conservative trace nutrient limitation.
11. When this limitation occurred the growth rate and glucose consumption rate changed from exponential functions to linear functions and the second aliquot of lipase was made.
12. Increasing the yeast extract concentration from 0.5 g/dm^3 to 2.0 g/dm^3 decreased the slope of the linear growth phase, time of start of linear growth and the biomass at which linear growth started.
13. It was postulated that yeast extract was somehow adsorbing a trace nutrient and thus reducing its availability.
14. The optimum constant feed rate of glucose for maximum lipase production was $0.5 \text{ g/dm}^3/\text{h}$.
15. The maximum amount of lipase activity produced under constant glucose fed batch conditions was 0.80 LU/cm^3 .

16. Constant glucose feed rates less than $2.0 \text{ g/dm}^3/\text{h}$ controlled the growth rate of the organism.
17. When the organism was fed glucose at constant rates greater than $2.0 \text{ g/dm}^3/\text{h}$, the nutrient limitations controlled the growth rate.
18. When the organism was grown in Pirt's medium x1 (Table 4.3), the linear growth rate reached a maximum of $0.45 \text{ g/dm}^3/\text{h}$.
19. The medium composition was modified by making the changes itemised below:
 - (a) trace salt concentrations were increased ten fold;
 - (b) the potassium dihydrogen phosphate concentration was increased eight fold;
 - (c) an addition of $5 \text{ g/dm}^3 \text{ NaCl}$ was made;
 - (d) the pH control was changed to ammonium sulphate and ammonium hydroxide solution;
 - (e) an addition of 1 g/dm^3 yeast extract was made to the inoculum flask.
20. A fed-batch fermentation carried out with the medium composition as outlined in Conclusion No. 19 was able to support 25 g/dm^3 of cells with no oxygen limitation.
21. When the organism was grown in the medium outlined in Conclusion No. 19, the linear growth rate reached was in excess of $2.00 \text{ g/dm}^3/\text{h}$.

22. The linear growth rate slope changed with the changes in the medium composition confirming that some nutrient was limiting.
23. There was a increasing linear relationship between the final biomass and the potassium dihydrogen phosphate concentration.
24. There was also an increasing linear relationship between the linear growth rate and the potassium dihydrogen phosphate concentration.
25. The relationships described in Conclusions No. 23 and 24 were not strong enough to categorically say that phosphate was the conservative trace nutrient causing the limitation but there was obviously some connection between the limitation and the phosphate concentration.
26. Some other trace nutrient was limiting as well as phosphate.
27. A change in the metabolism of the organism was indicated by an increase in noise on the dissolved oxygen and carbon dioxide traces during the progress of the runs.
28. The cell yield on phosphorus was 33.6 g/g which compared favourably to the values quoted by Pirt

(1975) of 39.1 g/g and by Park and Rogers (1989) of 29.4 g/g.

29. The feed technique developed by Minihane (1990) was shown to be very accurate.
30. There was very good agreement between the glucose rate fed into the fermenter and the intended feeding rate.
31. The specific growth rates calculated using the biomass and carbon dioxide data did not agree with the set-point exponent in contradiction to the work done by Minihane (1990).
32. These deviations from the expected growth rate were thought to be due to trace nutrient limitations and the complex stoichiometry of the organism.
33. The average cell yield for glucose was 0.30 g/g, nearly half the expected value of 0.50 g/g (Pirt 1975).
34. The cell yields on nitrogen had two slopes. The average yield for the first slopes was 3.60 g/g and the average yield for the second slopes was 6.97 g/g both well below the expected value of 8.75 g/g (Pirt 1975).
35. The cell yields were thought to be low because the organism was making secondary metabolites such as pyocyanin and exopolysaccharide.

36. The change in the nitrogen yields was thought to coincide with the cessation of pyocyanin production.
37. Lipase production was low during the exponential fed fermentations because the growth rate was not controlled by the glucose feed rate but by some other limitation.
38. The maximum lipase activity produced during the exponential fed fermentations was 0.520 LU/cm^3 .
39. A drop in lipase production seemed to be correlated with the commencement of pyocyanin production.
40. Various changes to the medium composition and culture conditions proved ineffective against prevention of pyocyanin production.
41. Phosphate was shown to delay the onset of pyocyanin production.

CHAPTER NINE

SUGGESTIONS FOR FURTHER WORK

1. A pyocyanin assay should be developed so that the concentrations of pyocyanin can be calculated accurately and the times when pyocyanin production commences and finishes can be measured.
2. A greater understanding of the physiology of the organism is necessary and the stoichiometry of the organism needs to be explored, so that the secondary metabolites and the factors affecting their production can be identified.
3. Further experiments into the nutrient limitations should be carried out so that a medium can be developed which supports a large concentration of cells without these limitations occurring.
4. A wider range of set-point exponents, "B" values, needs to be examined and the optimum value of "B" which gives maximum lipase production identified.

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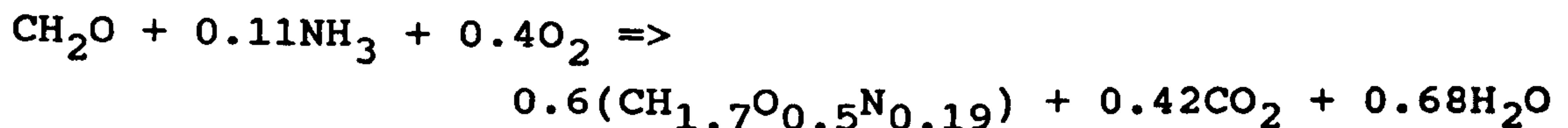
Pigment production and antibiotic activity in cultures of *Pseudomonas aeruginosa*

Journal of Bacteriology 54 109-117

A P P E N D I X A

Concentration of yeast extract necessary to ensure that the carbon is used up before the nitrogen in a 1% glucose solution

A stoichiometric equation for the growth of yeast on hydrolysed starch (MacLennan 1976) gives:



Thus:- 12 g carbon combines with 0.11×14 g nitrogen

$$\text{Ratio C/N} = \frac{12}{0.11 \times 14} = 7.79$$

If $\text{C/N} > 8$ then nitrogen will run out first and storage compounds will be made from surplus carbon

If $\text{C/N} < 8$ then carbon will run out first and growth process stops

To make 5 g/dm^3 of cells:

$$Y \text{ g glucose} = 0.5 \text{ g cells/g glucose}$$

$$\text{will need } \frac{5}{0.5} = 10 \text{ g glucose/dm}^3 \text{ in medium}$$

$$= 10 \times \frac{72}{180} = 4.0 \text{ g carbon/dm}^3$$

For carbon to run out first $\text{C/N} < 8$, say 6

Therefore, when $\text{C/N} = 6$, the amount of nitrogen should be

$$\text{N} = 4/6 = 0.67 \text{ g nitrogen/dm}^3$$

Nitrogen content of yeast extract = 0.85%

$$\text{Thus, } \frac{8.5}{100\%} = \frac{0.67}{x} \Rightarrow x = \frac{100 \times 0.67}{8.5} = 7.88 \text{ g/dm}^3$$

Thus, a concentration of yeast extract of 10 g/dm^3 should result in the carbon running out before the nitrogen.

A P P E N D I X B

S A M P L E D A T A

Sixty fermentation runs were carried out under various conditions and a large amount of data was collected. The raw data is stored in laboratory notebooks but two example runs, Run 12 and Run 55, are shown here and the calculation methods are developed in Appendix C.

Runs 1 to 46 had the same data collected and Run 12 is shown as an example. Runs 48 to 60 had slightly different and more detailed data collected and Run 55 is shown as an example. Dissolved oxygen and pH were monitored continuously during all the fermentation runs. The concentration of oxygen and carbon dioxide in the exhaust gas was monitored continuously during Runs 16 to 60.

1. Example of data collected from the batch fermentations: Run 12

Initial volume: 2.3 dm³

a) Cell concentration (optical density)

Time (h)	Sample No.	O.D. (580 nm)
0	1	0.057
1	2	0.085
2	3	0.128
3	4	0.160
4	5	0.206
5	6	0.253
6	7	0.330
7	8	0.430
8	9	0.488
9	10	0.596
10	11	0.751
11	12	0.811
12	13	0.786
13	14	0.747

b) Cell concentration (dry weight)

Sample number	Empty weight (g)	Dry weight (g)	Cell dry weight (g)
Blank	0.4095	0.4542	0.0447
Blank	0.4420	0.4835	0.0415
1	0.4290	0.4755	0.0465
2	0.3262	0.3734	0.0472
3	0.3787	0.4295	0.0508
4	0.3963	0.4476	0.0513
5	0.3263	0.3813	0.0550
6	0.3234	0.3858	0.0624
7	0.3951	0.4602	0.0651
8	0.3814	0.4564	0.0750
9	0.3240	0.3989	0.0749
10	0.3296	0.4097	0.0801
11	0.3226	0.4064	0.0838
12	0.3471	0.4334	0.0863
13	0.3307	0.4159	0.0852
14	0.3505	0.4448	0.0943

c) Glucose concentration

(i) Absorbance of the Standards

Glucose (g/dm ³)	Absorbance (A) (540 nm)	A - A ₀
0	0.0093 (A ₀)	0
0.1	0.0373	0.028
0.2	0.0749	0.066
0.3	0.0983	0.089
0.4	0.1524	0.143
0.5	0.1786	0.169

(ii) Absorbance of the Samples

Sample number	Absorbance (A) (540 nm)	$A - A_0$
1st med. blank	0.0093	0
2nd med. blank	0.0230	0.014
1	0.0994	0.090
2	0.1089	0.100
3	0.0961	0.087
4	0.1087	0.099
5	0.0869	0.078
6	0.0733	0.064
7	0.0623	0.054
8	0.0587	0.049
9	0.0453	0.036
10	0.0295	0.020
11	0.0119	0.003
12	0.0106	0.001
13	0.0093	0
14	0.0078	0

Glucose concentrations were calculated from the adjusted absorbance values by the method described in Appendix C.

d) Lipase activity

For Run 12 the lipase activity was assayed using the Six hour assay (Section 5.3.1)

Sample number	NaOH added (cm ³)		Lipase activity (Sigma-Tietz Units)
	Blank	Test	
1	6.3	6.3	0
3	6.3	6.3	0
5	6.3	6.3	0
6	6.2	6.2	0
7	6.0	6.3	0.3
8	6.1	6.5	0.4
9	6.0	6.4	0.4
10	5.9	6.3	0.4
11	5.9	6.4	0.5
12	5.7	6.8	1.1
13	5.7	7.0	1.3
14	5.7	7.0	1.3

Section 5.3 describes how Sigma-Tietz Units of lipase activity can be converted into standard Lipase Activity Units (LU).

2. An example of the exponential fed fermentations: Run 55

a) Basic data

Target exponent "B" = 0.2 h⁻¹
 Initial conditions: x_0 = 1.4 g/dm³
 V_0 = 2.46 dm³
 S_F = 350 g/dm³
 density of glucose solution = 1135 g/dm³

Time (h)	Sample No.	O.D. (580 nm)	Ammonia added (g)	Balance Reading (g)	Comments
0	1	0.015	0	0	1000 rpm
12	2	0.154	5	0	Start feed
13			6	3.24	
14			7	7.53	
15	3	0.332	10	12.92	
16			12	19.32	
17			14	27.09	
18	4	0.649	16	36.71	
19			19	48.55	
20			23	62.82	
21	5	1.270	29	80.62	
22			35	101.83	1200 rpm
23			41	127.99	
24	6	2.200	48	159.88	Green
25			58	198.95	1400 rpm
26			70	246.89	1600 rpm
27	7	4.314	86	304.98	
28			100	376.55	1800 rpm
29			125	463.36	
30	8	7.023	142	569.87	2000 rpm
31			161	699.86	
32		8.590	179	858.94	
33	9	9.180	197	1053.23	
34		9.200	213		

b) Cell concentration (dry weight)

Sample number	Empty weight (g)	Dry weight (g)	Cell dry weight (g)
Blank	0.2847	0.3342	0.0495
Blank	0.2929	0.3424	0.0495
1st med. blank	0.2858	0.3519	0.0661
2nd med. blank	0.2837	0.3400	0.0563
1	0.2971	0.3694	0.0723
2	0.3016	0.3788	0.0772
3	0.2471	0.3504	0.1033
4	0.2625	0.3750	0.1125
5	0.3117	0.4489	0.1372
6	0.3142	0.5203	0.2061
7	0.2808	0.5894	0.3086
8	0.2650	0.7509	0.4859
9	0.2869	0.9909	0.7040

c) Glucose concentration

(i) Absorbance of the Standards

Glucose (g/dm ³)	Absorbance (A) (540 nm)	A - A ₀
0	0.0108 (A ₀)	0
0.1	0.2908	0.02
0.2	0.0533	0.04
0.3	0.1017	0.09
0.4	0.1440	0.13
0.5	0.1662	0.16

(ii) Absorbance of the Samples

Sample number	Absorbance (A) (540 nm)	A - A ₀
1st medium blank	0.0108	0
2nd medium blank	0.2324	0.22
1	0.4058	0.40
2	0.0121	0
3	0.0295	0.02
4	0.0124	0
5	0.0305	0.01
6	0.0527	0.03
7	0.1148	0.09
8	1.0805	1.04
9	2.5010	2.44

d) Lipase activity

For Run 55 the lipase activity of the samples was assayed using the Two hour assay (Section 5.3.2)

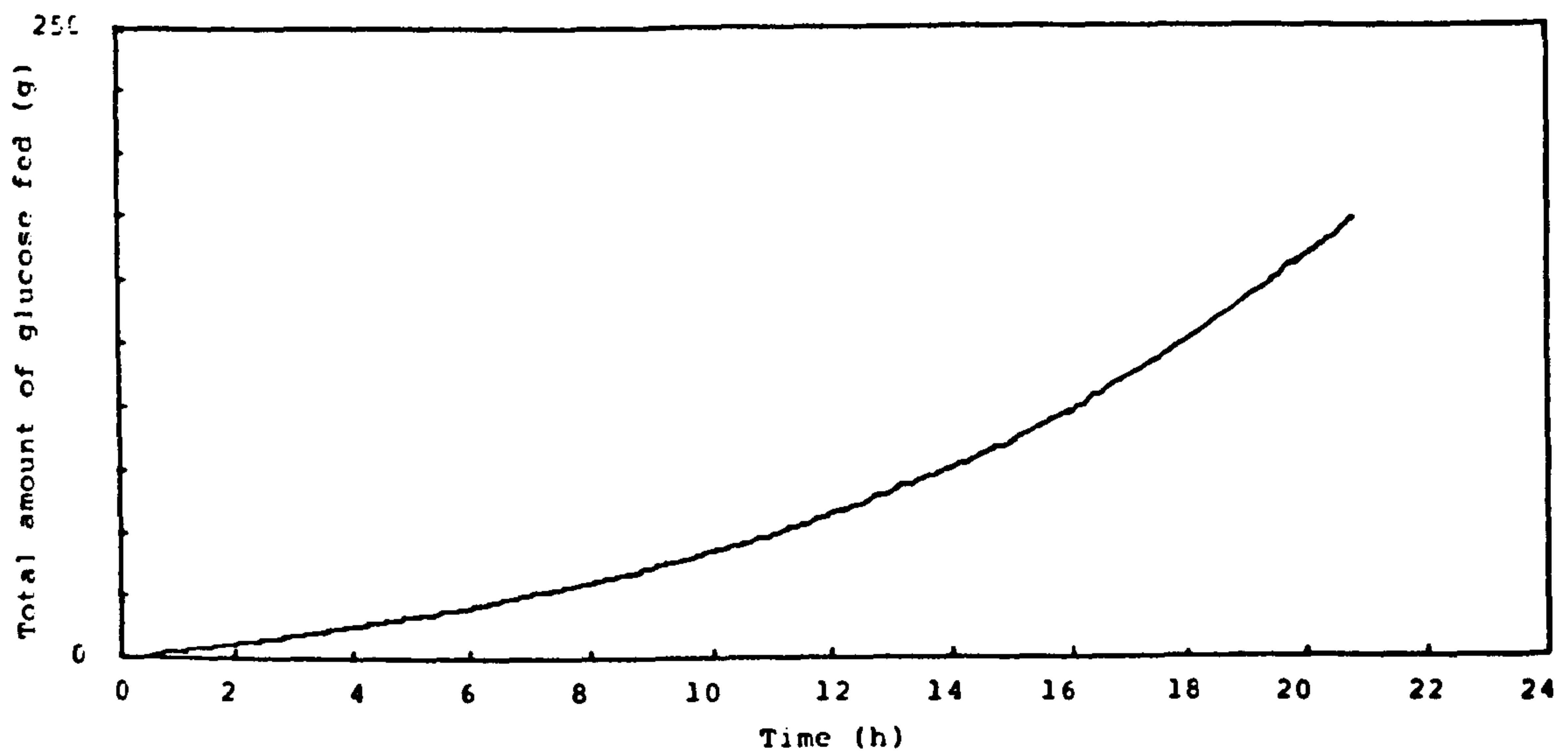
Sample number	NaOH added (cm ³)		Lipase activity (Sigma-Tietz Units)
	Blank	Test	
1	7.8	7.9	0.1
2	7.9	7.9	0
3	7.6	8.1	0.5
4	7.8	8.8	1.0
5	7.7	8.8	1.1
6	7.6	8.1	0.5
7	7.4	8.0	0.6
8	7.8	8.0	0.2
9	7.7	8.0	0.3

e) Ammonia concentration

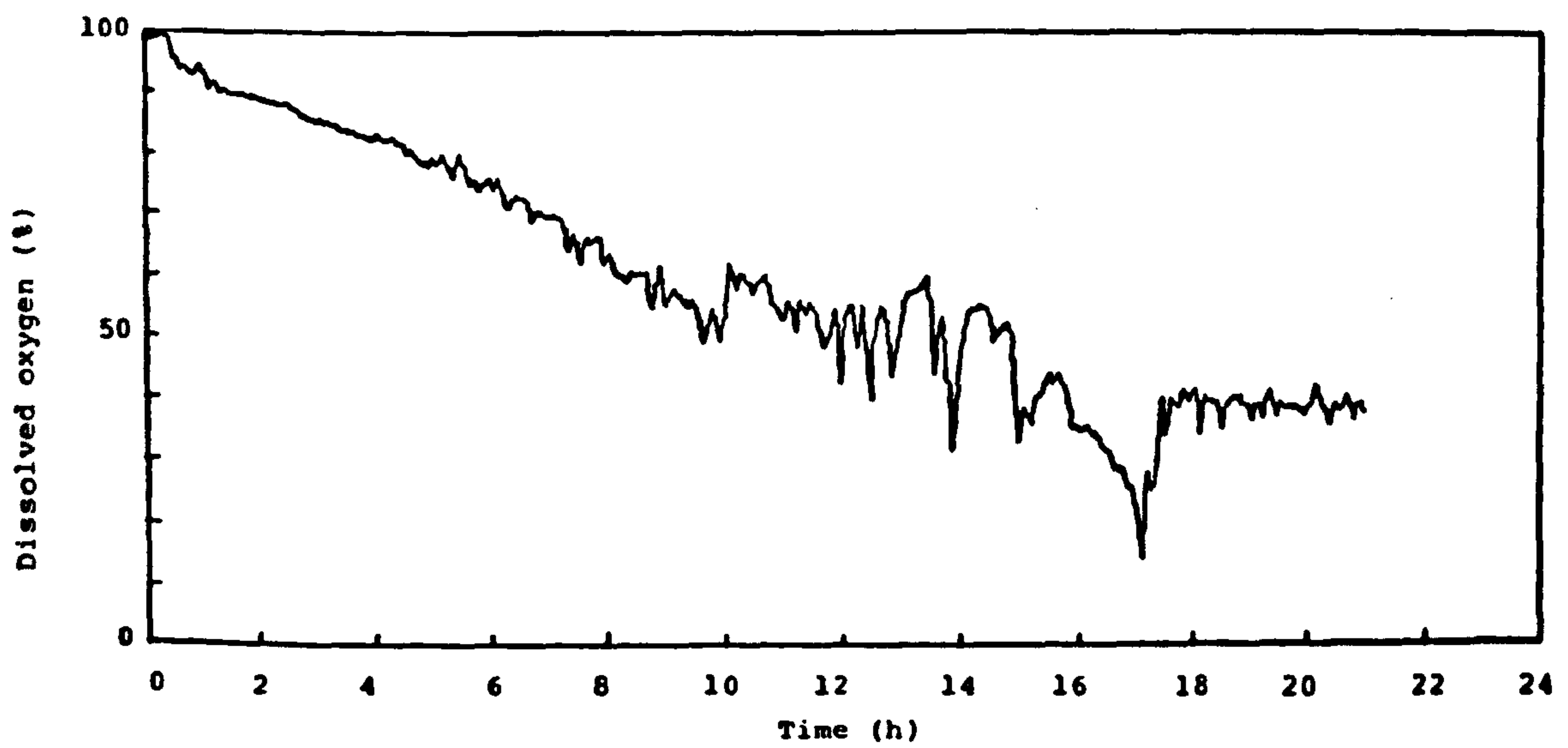
Sample number	Boric acid (cm ³)	Ammonia (g/dm ³)
1st med. blank	13.6	1.36
2nd med. blank	11.4	1.14
1	11.8	1.18
2	11.3	1.13
3	11.4	1.14
4	12.9	1.29
5	11.3	1.13
6	10.9	1.09
7	9.2	0.92
8	7.9	0.79
9	5.5	0.55

Section 5.4 describes how to convert boric acid (cm³) into ammonia (g/dm³).

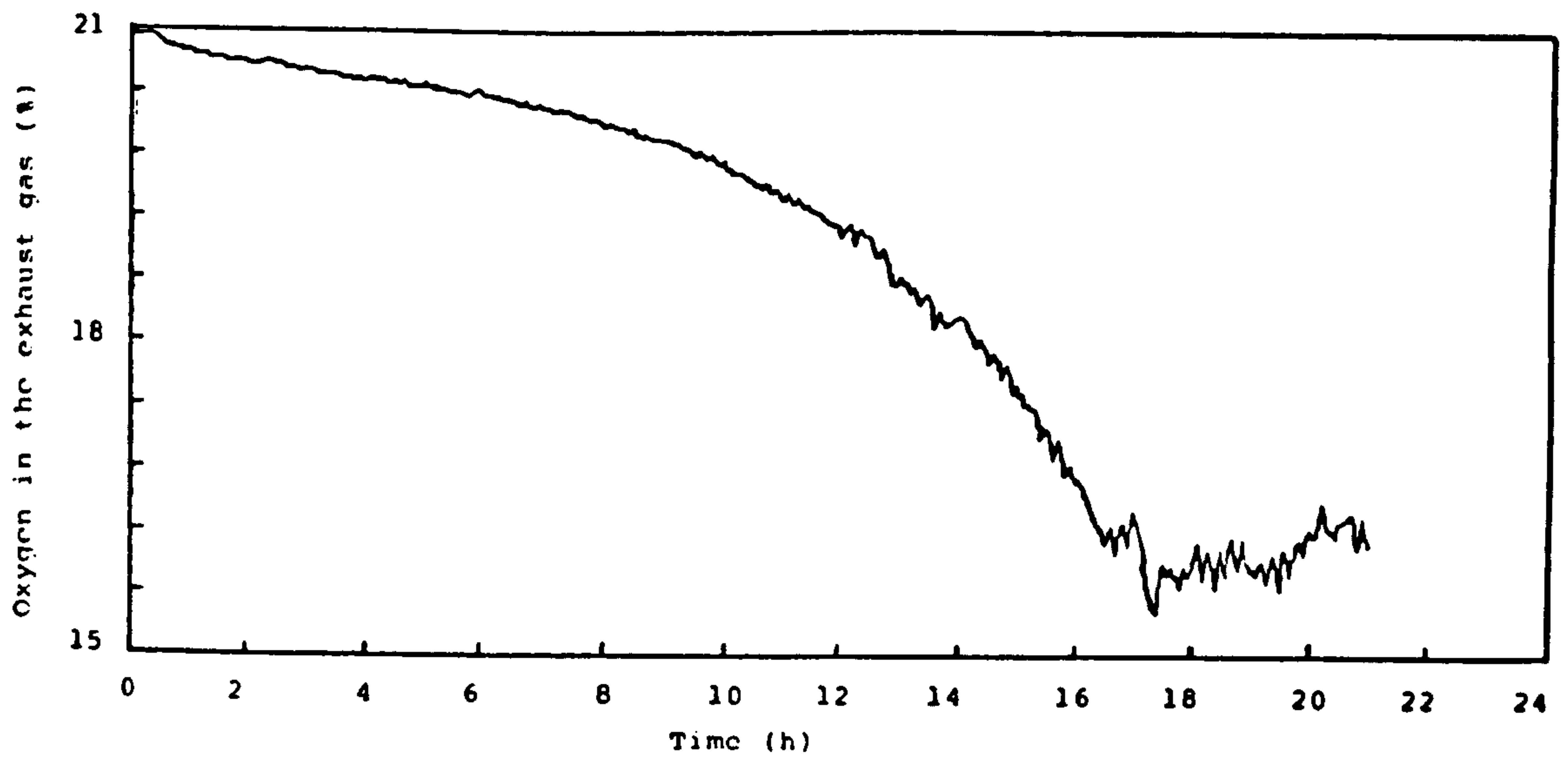
f) Glucose balance reading (g)



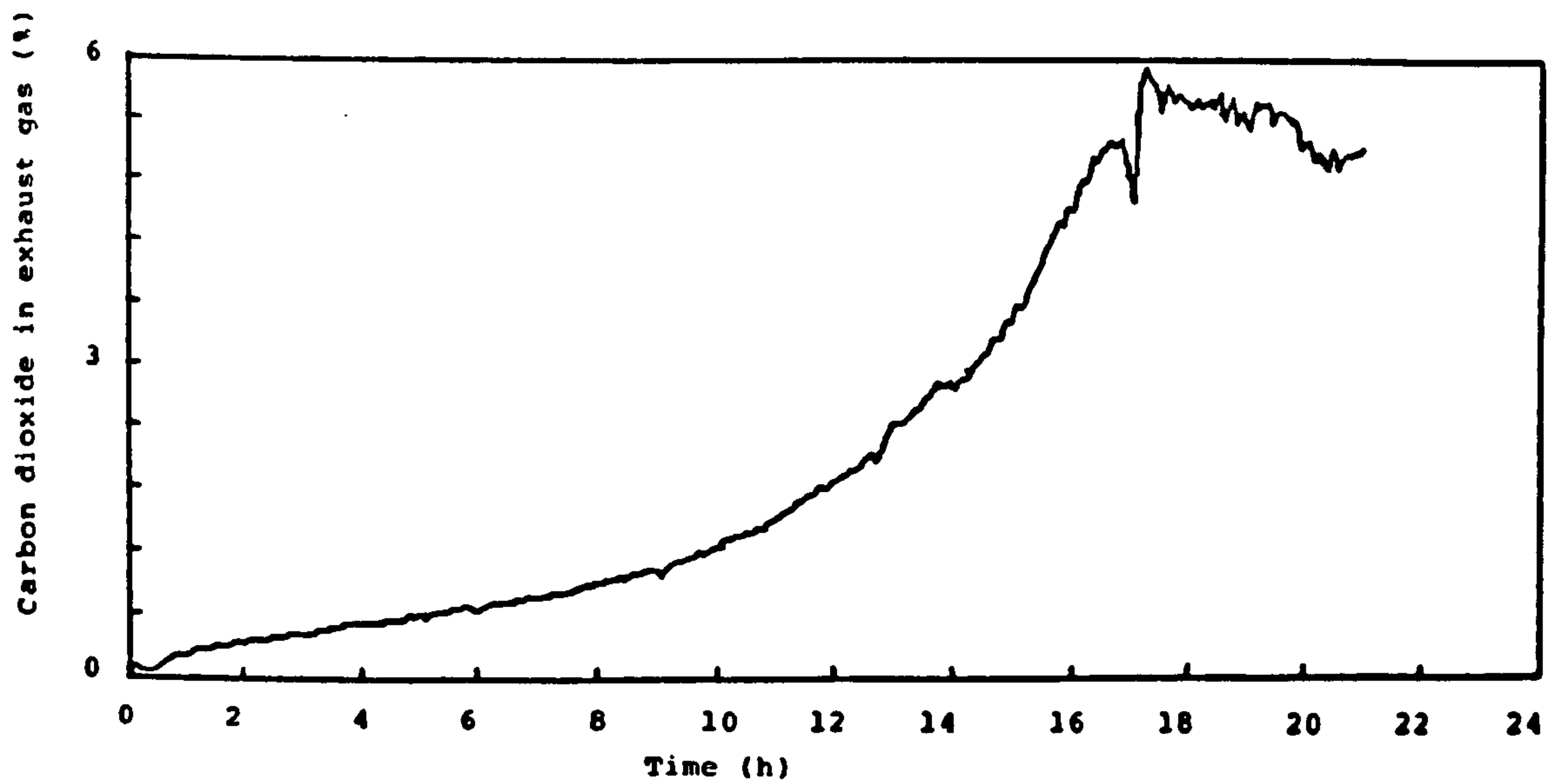
g) Dissolved oxygen reading (%)



h) Exhaust gas, oxygen concentration (%)



i) Exhaust gas, carbon dioxide concentration (%)



A P P E N D I X C

S A M P L E C A L C U L A T I O N S

The sample calculations are shown using the recorded data for fermentation Runs 12 and 55 shown in Appendix B.

1. Cell concentration

The cell concentration was calculated from the dry weights of the 15 cm³ samples as shown:

$$\text{Cell conc (g/dm}^3\text{)} = \frac{1000}{15} \times ((\text{dry wt.} - \text{empty wt.}) - \text{blank})$$

Example (i): Run 12, sample 10

dry wt. of broth + foil boat = 0.4097 g

dry wt. of empty foil boat = 0.3296 g

average dry wt. of blanks = 0.0431 g

(The blank is calculated by averaging the two saline blanks.)

$$\text{Cell concentration} = \frac{1000}{15} \times ((0.4097 - 0.3296) - 0.0431)$$

$$x = 2.47 \text{ g/dm}^3$$

Example (ii): Run 55, sample 5

dry wt. of broth + foil boat = 0.4489 g

dry wt. of empty foil boat = 0.3117 g

average dry wt. of blanks = 0.0554 g

(The blank is calculated by averaging the two saline blanks and the medium blanks.)

$$\text{Cell concentration} = \frac{1000}{15} \times ((0.4489 - 0.3117) - 0.0554)$$

$$x = 5.45 \text{ g/dm}^3$$

2. Broth volume

The broth volume at any time was calculated from the volumes fed and sample volumes removed by:

$$\text{Volume} = \text{Initial Volume} + \text{Volume Fed} - \text{sample volume}$$

It was assumed that each sample volume was 40 cm^3

The density of the ammonium hydroxide was 944 g/dm^3

The density of the glucose solution was 1135 g/dm^3

Example (i): Run 12, sample 10, Time = 9 h

$$\text{Initial Volume} = 2.3 \text{ dm}^3$$

$$* \text{ Wt. of NH}_3\text{OH fed (approx)} = (9 \times 5) \text{ g}$$

$$\text{Volume of ammonium hydroxide} = \frac{45}{944}$$

$$= 0.05 \text{ dm}^3$$

$$\text{Volume of broth} = 2.3 + 0.05 - (9 \times 0.04)$$

$$V = 1.99 \text{ dm}^3$$

* Ammonia fed was not monitored, so a value of 5 cm^3 per hour was used to calculate the broth volume for Runs 1 to 46.

Example (ii): Run 55, sample 5, Time = 21 h

$$\text{Initial Volume} = 2.4 \text{ dm}^3$$

$$\text{Balance reading} = 80.62 \text{ g}$$

$$\begin{array}{rcl} \text{Volume of glucose fed} & = & 80.62 \\ & & \hline & & 1135 \end{array}$$

$$\text{Volume of glucose fed} = 0.071 \text{ dm}^3$$

$$\text{Weight of ammonium hydroxide fed} = 29 \text{ g}$$

$$\begin{array}{rcl} \text{Volume of ammonium hydroxide} & = & 29 \\ & & \hline & & 944 \end{array}$$

$$= 0.031 \text{ dm}^3$$

$$\text{Volume of broth} = 2.4 + 0.071 + 0.031 - (4 \times 0.04)$$

$$V = 2.34 \text{ dm}^3$$

3. Biomass

The total cell weight was calculated from the cell concentration and the broth volume at that time

$$\text{Total cell mass} = xV$$

Example (i): Run 12, sample 10

$$\text{Total cell mass} = 2.47 \times 1.99$$

$$X = 4.91 \text{ g}$$

The biomass values for Run 12 have been plotted on semi-log graph paper (Figure C1) and the slope of the exponential portion of the line has been calculated. This is equivalent to the specific growth rate, μ .

From Figure C1, $\mu = 0.33 \text{ h}^{-1}$

Example (ii): Run 55, sample 5

$$\text{Total cell mass} = 5.45 \times 2.42$$

$$X = 13.19 \text{ g}$$

Four examples of biomass values against time semi-log plots for the exponential runs can be found in Figure 7.18

4. Glucose concentration

(a) The standard curve was plotted (Figure C2).

(b) The absorbances (at 540 nm) of the samples were adjusted to take into account the blanks. This involved subtracting the standard blank (0 g/dm³ glucose) from the sample absorbances.

Example: Run 12,

Absorbance (540 nm) sample 5 = 0.0869

Absorbance (540 nm) blank = 0.0093

Adjusted absorbance, sample 5 = 0.0776

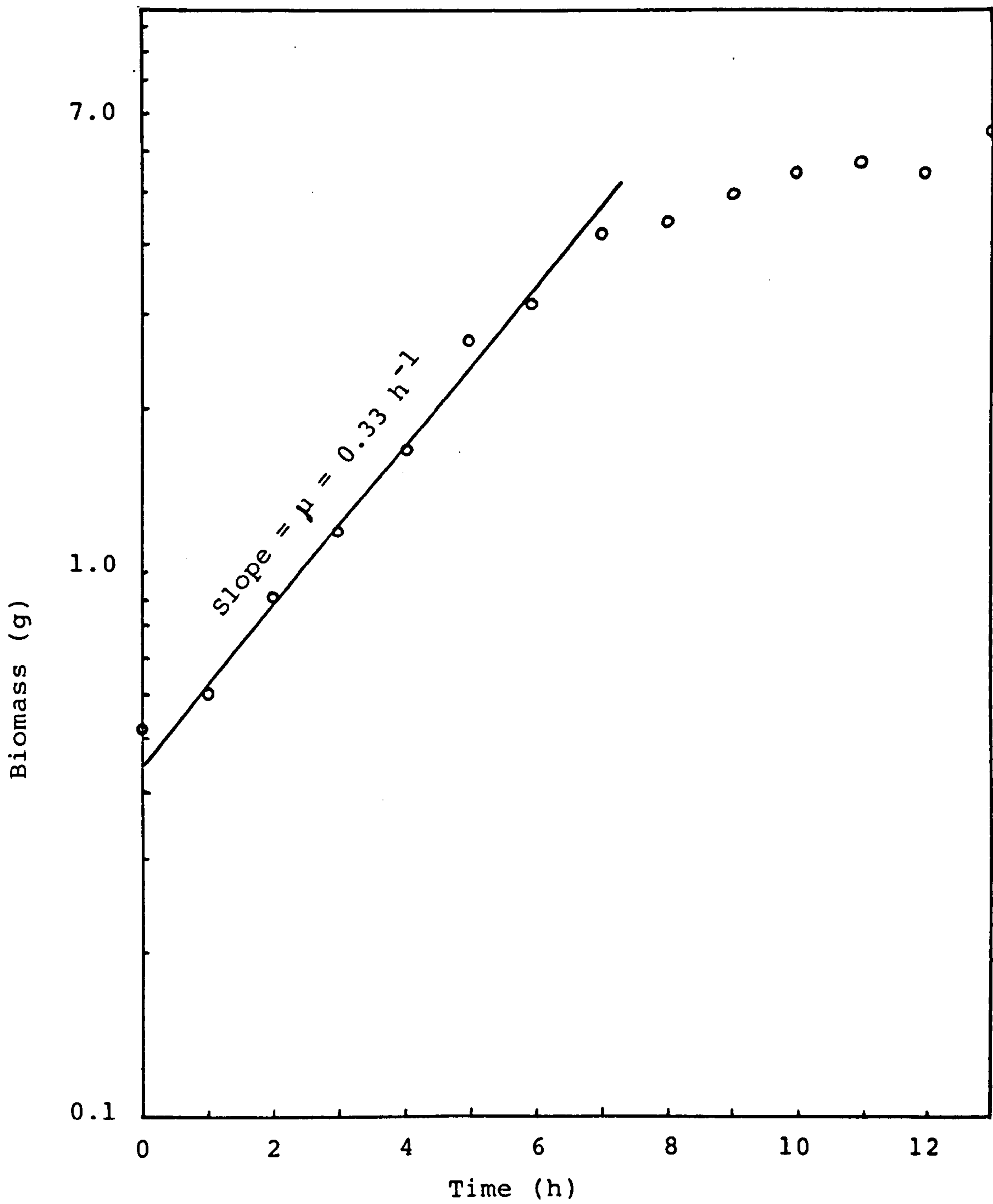


Figure C1. Run 12: A semi-log plot of the biomass values against time
 $\mu = 0.33 \text{ h}^{-1}$

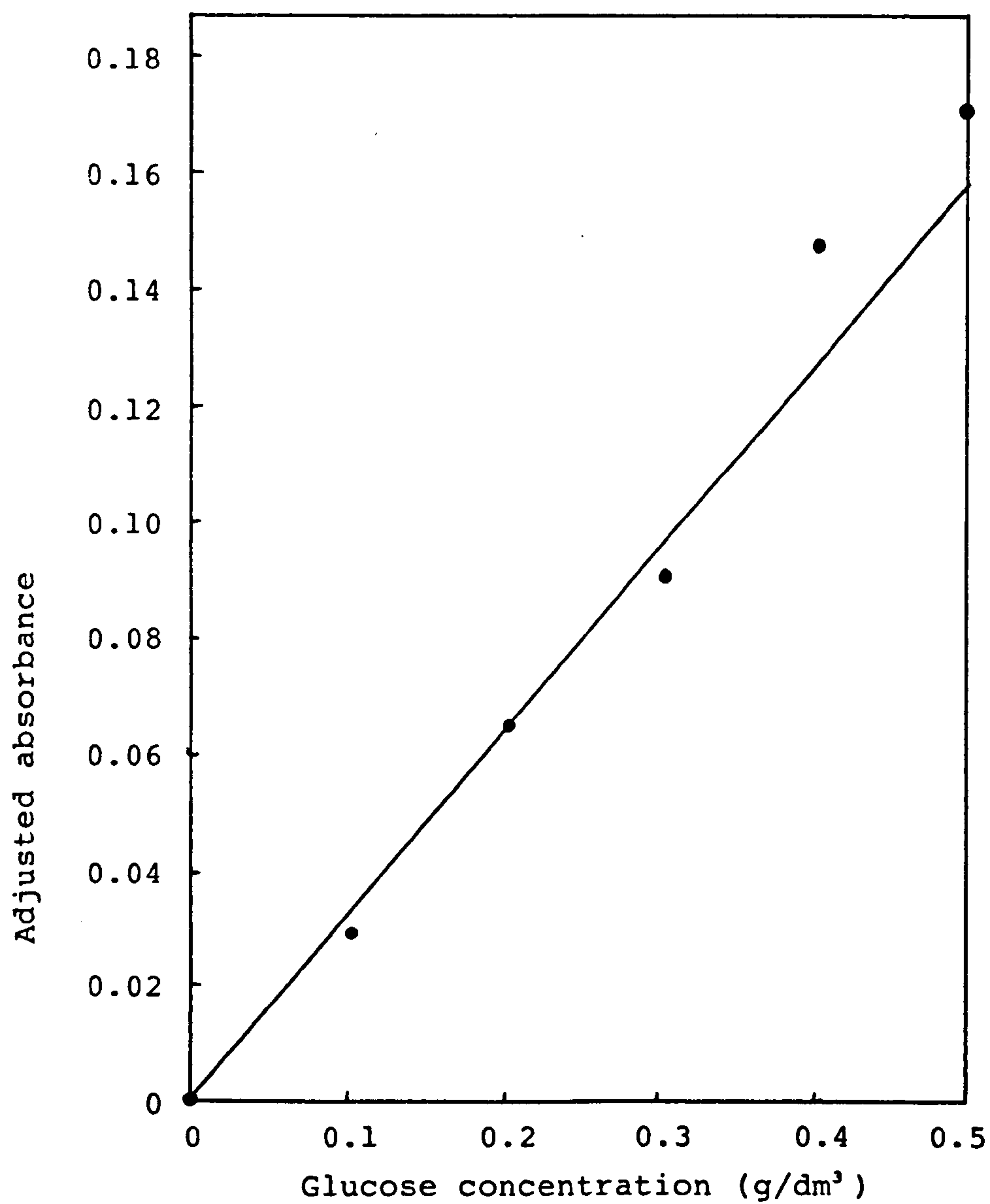


Figure C2. Run 12: The glucose standard curve

(c) The standard curve was then used to convert this absorbance into glucose concentration (g/dm^3)

Example: Run 12, sample 5

Adjusted absorbance = 0.0776

Glucose concentration = 0.25 g/dm^3

5. Cell yield on glucose

For each of the exponential fed fermentations (Runs 48 to 60) a cell yield on glucose was calculated from the regression line of a plot between the biomass produced during the fed-batch phase ($X - X_0$) and the glucose fed.

Example: Run 55

Time (h)	$X - X_0$ (g)	Glucose fed (g)
12	0	0
15	4.12	4.52
18	5.79	12.85
21	9.74	28.22
24	21.53	55.96
27	40.79	106.70
30	79.40	199.50
33	141.40	368.60

The yield is then obtained from the slope of the above plot

From the cell yield plot: $Y_{XS} = 0.39 \text{ g/g}$

Two examples of glucose yield plots can be found in Figure 7.20

6. Cell yield on nitrogen

For each of the exponential fed fermentations (Runs 48 to 60) a cell yield on nitrogen was calculated from the regression line of a plot between the biomass produced during the fed-batch phase ($X - X_0$) and the nitrogen fed.

The amount of nitrogen fed was calculated from the amount of ammonium hydroxide fed as shown:

$$\text{Nitrogen fed (g)} = \frac{\text{NH}_3\text{OH fed (g)} \times \text{molarity} \times 14}{\text{NH}_3\text{OH density}}$$

The molarity of the ammonium hydroxide solution was 7.5 M

Example: Run 55

Time (h)	$X - X_0$ (g)	NH ₃ OH fed (g)	Nitrogen fed (g)
12	0	0	0
15	4.12	5	0.44
18	5.79	11	0.96
21	9.74	24	2.10
24	21.53	45	3.94
27	40.79	83	7.26
30	79.40	140	12.21
33	141.40	195	17.06

The yield is then obtained from the slopes of the above plot

From the cell yield plot: $Y_{XS} = 5.60 \text{ g/g}$ (Phase 1)
and $Y_{XS} = 12.50 \text{ g/g}$ (Phase 2)

Two examples of nitrogen yield plots can be found in Figure 7.20