THE APPLICATION OF A NOVEL SAMPLING DEVICE TO THE ON-LINE ANALYSIS OF FERMENTATION BROTH

A thesis submitted to the University of London

for the degree of DOCTOR OF PHILOSOPHY

by

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September 1993

ACKNOWLEDGEMENTS

I would like to thank Mrs Nina Thornhill, Professor Mike Turner and Dr Neville Fish for their help and support during this project. I would also like to express my thanks and appreciation to Dr Malcolm Gregory, who developed the LabView process control system used in this project, and who collaborated with me on some of the fermentations described in the thesis.

Special thanks are most certainly due to Martyn Vale for his unending patience and for providing me with expert assistance throughout the project. I would also like to thank Mark Spurgeon, John Bailey, Billy Doyle, Alison Clayton and everyone in the Chemical Engineering Workshop for their help.

The financial support of the Science and Engineering Research Council through the Interdisciplinary Centre for Biochemical Engineering at UCL is also acknowledged.

Finally, I would like to thank Mark for all his support.

for Florence Adeline Slatter

ABSTRACT

This thesis describes the development of an automated, aseptic, sampling, centrifugation and analysis system for fermentation broth, and its link to a process control system for the control of sugars and organic acids in fermentation processes. Experiments were done to determine the separation efficiency of the microcentrifuge on suspensions of *E. coli* and *S. cerevisiae* cells. Reproducibility tests showed the system to be suited to on-line use, and on-line HPLC results compared well with off-line enzymatic assays of glucose and acetate for samples taken during an *E. coli* K12 fermentation.

The on-line monitoring system was linked to a control system developed by another researcher to enable on-line HPLC results to be used to control a fermentation process. The thesis presents two examples of the use of the combined system in controlling acetate build-up in a fed-batch fermentation.

Operating fed-batch fermentations provided suitable conditions for the testing of the on-line monitoring system in controlling the build-up of galactose and acetate in the fermenter. Experiments looked at the effects of various fermentation conditions on the expression of α-amylase produced in a recombinant *E. coli*, JM107 + pQR126, and secreted into the periplasm. Fed-batch fermentations were performed at different growth rates and feed profiles. Further physiological studies were then performed using continuous culture techniques to look at the effect of acetate concentration on the maximum specific growth rate and α-amylase production; and the effect of dilution rate on the overflow of substrate to acetate and production of α-amylase. Results show that high and low growth rates inhibit α-amylase production, as do high acetate and galactose concentrations. High acetate concentrations also reduce the maximum specific growth rate.

These experiments highlight the need for monitoring and controlling galactose and acetate concentrations in fermentation processes.

AIM OF THESIS

The aim of this thesis is to describe the design and development of an on-line monitoring system for the automated, aseptic, sampling, centrifugation and analysis of fermentation broth. The thesis demonstrates the suitability of each of the components in the system for its purpose, and each component is tested to ensure that the whole system functions under the best operating conditions.

The system has been developed to automatically measure concentrations of sugars and organic acids in fermentation broth; on-line data can then optimise the process by improving the control over the fermentation. The system was integrated with a commercial process control system, LabView (programmed by Dr. M. Gregory at UCL). This enables the on-line HPLC data to be incorporated into an algorithm written to control the feeding of substrate (galactose) in a fed-batch fermentation. The thesis describes how this closed-loop control is achieved, and gives two examples of control strategies used in demonstrating the system.

The reasons for wanting to control sugar and organic acid concentrations are explained by describing some experiments performed to elucidate the roles of high concentrations of sugar (galactose) and organic acid (acetate) in a fermentation process. The role of specific growth rate on the production of recombinant protein is also looked at. Apart from using the system to determine these roles, these experiments demonstrate the use and need for the on-line monitoring system in certain fermentation processes.

LAYOUT OF THESIS

This thesis is divided into four sections, plus two appendices. The first section is concerned with the development of the on-line sampling, centrifugation and analysis of fermentation broth, and its link to a process control system. It begins with a literature review of research in the field of on-line sampling and analysis from fermentation processes. An overview of the monitoring system is then given, followed by sub-sections dedicated to each of the components of the on-line monitoring system in turn. The first use of the system in directly monitoring a fermentation is presented in section 1.7., and results from the on-line HPLC are compared to analyses of off-line samples using enzyme based assay kits. Section 1.8. describes the linkage of the monitoring system to the process control system, LabView, and demonstrates the use of the combined system in closed loop control of acetate/galactose concentrations in fed-batch culture.

Section 2. deals with the effects of high acetate and galactose concentrations on a fermentation process, as well as the effects of different specific growth rates on the production of recombinant protein. Firstly, there is a brief literature review of the subject, and then a materials and methods section. Next, comes a description of a "standard" batch fermentation to provide basic information about the organism and for later comparison. Subsequent sections deal with the effects of different conditions on both the growth of the cell and its production of recombinant protein. The experiments were performed in fed-batch culture, using the on-line monitoring and/or control systems; continuous culture was also used in this study. The section ends with a summary of the main results obtained from the experiments described in this section, and a discussion of their importance.

Section 3. is a discussion of the development and use of the on-line monitoring system, describing the various problems encountered during its use. Main conclusions are drawn about the project.

The main body of the thesis ends with a short section (section 4.) on the future.

Both future work to be done on the project in the form of modifications required to improve the speed and efficiency of on-line monitoring, and future potential of the system are discussed. There is a brief description of how the system could be modified to monitor intracellular proteins or enzymes on-line.

There are two appendices. Appendix A provides details of the relay ladder logic program written for the programmable logic controller to sequence events necessary for sampling and analysing fermentation broth. Included in this appendix is the HPLC method file used in the PE Nelson chromatography software, and the short QuickBasic program used to send on-line HPLC data to the LabView computer.

Appendix B describes some additional work done to develop an HPLC assay for prochymosin, an important fermentation product. This work was done at the same time as the main project, but it is not directly relevant to the on-line monitoring of fermentations, and hence appears in an appendix. It is hoped, however, that future work on the HPLC assay will eventually enable prochymosin to be monitored on-line during a fermentation, and for this reason, it is included in this thesis.

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1. THE DEVELOPMENT OF AN ON-LINE MONITORING SYSTEM FOR THE AUTOMATED SAMPLING, CENTRIFUGATION AND ANALYSIS OF FERMENTATION BROTH AND ITS USE IN CLOSED-LOOP CONTROL OF FERMENTATION

1.0. INTRODUCTION

The aim of this project was to develop an on-line monitoring system for the automated measurement of sugars and organic acids, and to demonstrate its effectiveness in controlling a fermentation process. It has also been used to identify fermentation conditions that can affect the growth and recombinant protein production of the organism.

Section 1 describes the main bulk of the work in the project *ie* the development of the on-line monitoring system and its linkage to a control system (developed by another researcher). The section starts with a literature review (section 1.1.) which describes variables that need to be measured during a fermentation, and why such measurements are necessary. It then looks at different sampling methods, and how samples are prepared for analysis. A very few researchers have been able to measure intracellular protein product, and this review describes how that was achieved. It then examines different analytical methods available for the measurement of a wide range of variables from glucose to biomass concentration. The review ends with a very brief mention of control techniques used in fermentation. This part of the review is deliberately brief, because the programming of the high level LabView controller was done by another researcher (although low-level programmable logic controller programs were written by the author).

After the literature review, there is an overview of the on-line monitoring system (section 1.2.), to provide the reader with a brief summary of the components of the system. The next three sections are mostly descriptive, providing details

about each of the components of the system; how they have been developed and put together, and any problems that arose from their use. Section 1.3. describes the sampling device; section 1.4. looks at the microcentrifuge;, and section 1.5. examines the chromatography system used. Section 1.6. provides details on how the whole system is put together and programmed to take samples from the fermenter and analyse sugar and organic acid concentrations. Appendix A provides full details of the programs used.

The first use of the on-line monitoring system in sampling and analysing fermentation broth automatically is shown in section 1.7., where results from on-line HPLC are compared to off-line analyses done using enzyme assay kits. The final part, section 1.8. describes how the on-line monitoring system was linked to a control computer (running LabView process control software), and how the combined system was able to demonstrate effective closed-loop (feedback) control.

1.1. LITERATURE REVIEW

This literature review provides a background into research that has been done in the field of sampling and analysis of fermentation broth. The scope of the material surveyed is broader than the subject of the thesis, which focuses on a particular method for monitoring specific substances (sugars and organic acids) in fermentation broth. Many of the developments in the review are reports from the published academic literature, although commercially available devices / systems have been mentioned when they are particularly useful or novel. The conclusions drawn at the end of the review take the line that biosensors are generally inferior to techniques such as HPLC, and this provides the background for much of the work reported in the thesis.

The review first looks at a number of variables that are (or need to be monitored) in a fermentation. These include physical, chemical and biological variables. The physical variables, plus pH, DOT and DCO_2 are not examined in any detail, and are only covered in this part of the literature review. This first section only deals with the importance of the other variables; analytical methods come later.

The next part deals with sampling methods, and the way fermentation broth is prepared for analysis *ie* how solid and liquid components are separated. The need for and means of achieving asepsis during sampling procedures is shown. This part then describes how a very few researchers have managed to monitor the concentrations of intracellular proteins/enzymes, and briefly discusses ways of disrupting cells that may be suitable for small sample volumes.

Then follows a review of methods of analysing important fermentation variables, with emphasis on on-line techniques. Analytical techniques are described for the measurement of the following: glucose, acetic acid, ethanol, ammonia and phosphate, penicillin and cephalosporin C, amino acids, protein, biomass and fermenter exhaust gas. General comments about on-line analysis are made, and there is a conclusion about the most suitable technique for the on-line

measurement of a number of variables.

The final part of the literature review is a very brief look at some control techniques employed in fermentation.

1.1.1. Fermentation

Fermentation is used in the production of various materials, such as enzymes, antibiotics and food products, as well as in processes involved in waste water treatment. Recently, however, substances which were traditionally manufactured by chemical processes are now fermentation products, or products of other biological processes because of the high specificity, and often the lower cost of production that may be associated with these processes.

Fermentation broth is typically a system of at least three phases consisting of cells, organic and inorganic media components dissolved in the liquid phase, and gases. In order for a fermentation to be successfully monitored and controlled, complex analytical methods often need to be employed to assess the state of the fermentation. Currently, very few of these methods are on-line, and this means that the time taken between manual sampling and the result of an analysis can take too long to prevent problems, for example in nutrient deficiency or product formation. Effective fermentation control needs the development of rapid and frequent on-line monitoring, and additionally, automatic sampling and analysis means that fewer personnel are required to maintain the fermentation. Less broth is usually removed from the fermenter with automated sampling, and the chance of causing fermenter contamination is reduced.

Fermentations are typically much more difficult to monitor and control than chemical processes largely because of the great complexity of the microorganism compared to single chemical reactions.

<u>Monitoring of fermentation variables</u>

There is a very wide range of variables that can be monitored during fermentation

or other biological processes. This introduction will concentrate on the most widely measured fermentation variables.

Such variables may be categorised into three groups, namely:
1) Physical variables *ie.* those related to the physical state of the fermentation.
2) Chemical variables, relating to the chemical environment of the cells, *eg.* pH.
3) Biochemical and biological variables *eg.* biomass concentration.

1.1.1.1. Physical variables

This subsection provides a very brief overview of methods used for measuring physical variables. Most techniques have been around for many years, but are included here because of the importance of monitoring and controlling the physical environment of the cell.

It is crucial to accurately monitor and control temperature in many fermentation processes because different microorganisms have different temperature optima for growth. In addition, product formation and expression of a plasmid may be under temperature control *eg* the temperature inducible t_R promoter/cI857 repressor expression system (Rinas *et al*, 1989; Strandberg and Enfors, 1991). An example of this is in the growth of mouse-mouse hybridoma cells in culture. Growing cells at 3-9 °C below the optimum of 37°C causes an increase in viability but a decrease in the production of monoclonal antibodies (Reuveny *et al.*, 1986). Temperature sensors used in fermentation include the platinum resistance thermometer and the thermistor, a semiconductor device operating on the principle that changing resistance is a function of temperature as well as thermocouples and thermometer bulbs of Hg in stainless steel. Obviously, they should be steam sterilisable *in situ.*

Monitoring reactor head pressure is especially important during sterilisation and subsequent operation of the fermenter. Sterile conditions are easier to maintain if there is a positive head pressure, and it is also important with respect to the partial pressures of oxygen and CO_2, and consequently oxygen transfer. Pressure measurement devices used are pressure transducers based on strain gauges, and

piezoelectric manometers.

Other important physical variables include flow rate (air and liquid), liquid level, power input and viscosity. Inlet airflow rate is particularly important in aerobic processes (and is required for the calculation of carbon dioxide evolution rate, for example), and the simplest measurement technique is the use of a variable air meter, or rotameter, on the air supply. Flow rates when determined by mass flow meters, orifice plates or vortex shedding meters, for example, are easily obtained as an electrical output.

Due to the problems of making measurements on a gas containing a significant level of water vapour, exit gas flow rates are normally determined indirectly. The most common method involves a mass balance, or an inert gas in the inlet and exit gas, *eg.* argon or nitrogen. Coppella and Wang, (1990) describe a method of spiking the off-gas flow with an accurately determined flow of argon to measure exit gas flow rate.

Liquid flow rates of nutrients or pH controlling solutions can be measured using load cells or a well calibrated pump, although such measurements can be difficult if two or more phases or solids are present in the liquid.

The measurement of liquid level may be determined by conductivity sensors, capacitance probes, ultrasound or two pressure transducers - the pressure difference being equivalent to the hydrostatic head. This is only possible if the fermenter headspace is large enough. The first two are also used in detecting foam build up in a fermentation process.

Power input determination is important for economic considerations, and also in looking at oxygen transfer between gas and liquid, possible mechanical damage to cells, and how it influences microbial morphology. Agitator power can be measured by a Hall effect transducer. Shaft power input is sometimes measured by a torsion dynamometer. Often, however, the external power drain by the motor is the only relevant measurement that is available.

On-line viscosity measurements are not yet done routinely, although in some fermentations, they would be useful, for example where there is mycelial growth. A device known as a vibrating rod viscometer has been used, but requires specific calibration for each fermentation process.

1.1.1.2. Chemical and biological variables

This section concentrates on the monitoring of chemical variables, some of which (such as pH) are measured routinely, and are not discussed in any detail. More relevant to the work described in the thesis is the analysis of non-routinely measured variables, and these are looked at in much more detail; both in this section (where the reasons for measurement are examined) and in section 1.1.3. where current analytical methods are discussed.

pH

pH is a critical fermentation variable as most cells have narrow pH optima, and some cells systems cannot survive more than a moderate change in pH. For example, it has been known for several years that the growth of normal, virus transformed and cancerous mammalian cells is markedly affected by relatively small variations in pH in the range 6.8 to 8.2 (Eagle, 1971). Many other researchers report that modest fluctuations in animal cell culture pH may have a great effect on growth rate, viability and final cell yield (Harbour *et al.*, 1988; Birch and Edwards, 1979 and Miller *et al.*, 1988). Expression of a recombinant β-galactosidase in *E. coli* can be induced by a pH change when controlled by the regulatory region of the *cad*A gene (Tolentino *et al.*, 1992).

pH probes used in a fermenter should be robust and steam sterilisable. Calibration should last for the duration of the fermentation, although this often is not the case (Clarke *et al.*, 1984). Probes are also easily fouled by protein in the broth. An alternative to the glass probe is that proposed by Clarke *et al.* (1984), where a glass pH probe is replaced by disposable solid state electrodes and semiconductor devices.

Dissolved oxygen

Measurement of dissolved oxygen can provide useful information on the growth rate and metabolic state of the fermentation. Conditions of low DOT can have a major effect on a fermentation process *eg.* yeast cells may produce excess ethanol if conditions become more anaerobic, and similarly, *E. coli* produce more acetic acid. Two types of probe are in frequent use; galvanic and polarographic. As with pH probes, fouling of the probe can easily occur, and short calibration longevity is a problem. Kok and Hogan (1987/88) developed a probe calibrator to allow for *in situ* cleaning and calibration of a probe during a fermentation.

Dissolved CO_2

Steam sterilisable electrodes are now also available for the measurement of dissolved CO_2, but they require careful maintenance (Lorenz *et al.* 1987). The probe functions by measuring the pH of a standard bicarbonate and NaCl solution separated from the medium by a gas permeable membrane. Calibration of the pH electrode during a fermentation is difficult.

Nothing further will be written about the above variables. The variables following this are mentioned immediately below with respect to the reasons for their measurement. Section 1.1.3. then examines analytical techniques for determining them.

Glucose

Glucose is one of the most widely used carbon substrates in fermentation; substrate depletion can lead to a number of undesirable effects, yet an excess could have an inhibitory effect on cell growth rate (Strandberg and Enfors, 1991) and leads to the bacterial Crabtree effect where the formation of TCA cycle enzymes may be repressed and the formation of plasmid encoded product inhibited, either directly or by the accumulation of excreted acids (Rinas *et al.*, 1989; Gerson *et al.*, 1988; Kleman *et al*, 1991; Bech Jensen and Carlsen, 1990). In baker's yeast cultivation, the Crabtree effect means excess ethanol accumulates, but if the sugar concentration is too low, however, the biomass productivity is

again limited (Mizutani *et al.* 1987). It is often essential, therefore, to rapidly and accurately be able to estimate, or measure, glucose concentration inside the reactor. Further details of these effects are discussed in section 2.1.

Another reason for measuring glucose concentration in a fermentation is described by Garn *et al.* (1989). They used glucose measurements for on-line estimation of fermenter biomass concentration, by using glucose concentration values directly in a simple carbon balance equation:

$$\text{glucose} \rightarrow \text{biomass} + CO_2 + \text{acetate}$$

They obtained reliable estimates of biomass in the batch phase of the fermentation of a recombinant strain of *E.coli* using this method. Valero *et al.* (1990 a), used a similar method in fermentations of *Candida rugosa*.

In these examples, rapid results are of utmost importance. On-line analysis of glucose concentration would considerably aid in obtaining good fermentation results. The on-line measurement of glucose and other sugars, and reasons for doing so, form a major part of this thesis.

Acetic acid

Acetic acid is the main by-product excreted by *E. coli* (the organism used in the project) when the concentration of sugar substrate in the medium is too high, and the oxidative capacity of the cells is exceeded; or when the growth rate of the culture is too high. Its presence in the fermentation broth is detrimental to the cells at high concentrations, and needs to be carefully controlled. A more detailed discussion of this is given in section 2.1. Acetate measurement (like glucose and other sugars) also forms a large part of this thesis.

Nitrogen, ammonia and phosphate

Nitrogen is a major component of microbial biomass, and phosphate is an important component in many substances involved in metabolism, and is a component of nucleic acid. It can also be used to control the expression of

certain recombinant proteins, whose genes are linked to the alkaline phosphatase promoter in *E. coli* (Forman *et al.*, 1991). Nitrogen or phosphate limited growth is often used as an important strategy in the formation of fermentation products. Ammonium ions also appear as a product of metabolic pathways *eg.* in the decay and metabolism of glutamine in cell culture media (Doblhoff-Dier *et al.* 1989). In *E. coli* fermentations, growth is inhibited if ammonia concentration exceeds 170 mM (Thompson *et al.*, 1985), so it needs to be fed in high cell density culture.

Ethanol

There are a number of reasons for the interest in the analysis of ethanol: it is one of the earliest and most important fermentation products. In the early 1980s, the world market for ethanol produced by biotechnology was *ca.* \$5 x 10^8, (Scheller and Kirstein, 1987) and current figures are likely to be much higher; ethanol is also important medically, hence the interest in methods of measurement. Although it is often the product in yeast fermentations, ethanol may be a byproduct in fermentations to produce yeast biomass, and its concentration needs to be closely monitored.

Protein

The term protein is one given to a macromolecular substance composed of a polymer of covalently linked amino acids. This includes structural proteins, enzymes, antibodies *etc.* Proteins and peptides make up a large slice of the biotechnology market, and fermentation accounts for products such as monoclonal antibodies and many different enzymes. Microorganisms are now also able to produce proteins or peptides with enzymic or hormonal activity, and antigens which are of use in the production of vaccines (Gustafsson *et al.* 1986).

Antibiotics

Scheller and Kirstein, (1987), estimate the world market for antibiotics in the early 1980s at approximately \$8 x 10^9 annually; the current annual tonnage of penicillin produced by fermentation is approximately 25 000 tonnes. Antibiotics have been produced by fermentation for many years, and because of the importance of antibiotics to the biotechnology industry, there has been much

work on the determination of the concentrations of penicillin and other antibiotics during the fermentation.

Amino acids

Amino acids are also important biotechnological products; their world turnover in 1981/82 estimated at DM 1.63 x 10^9 (Finn and Präve, 1988). Annual tonnages of some amino acids produced in 1987 are: glutamate, 350 000; lysine, 87 000; and phenylalanine, 3 000. Fermentation is sometimes a preferable way of producing amino acids for many reasons, including cost. An important advantage is that only 1 optical isomer of the amino acid is formed during fermentation (usually the L-form, except glycine which is achiral), as opposed to racemic mixtures produced in chemical reactions. Very often, optically pure compounds are required (although racemates are used in feed-stocks). Only the L-form is found in protein.

Many amino acids, such as glutamic acid, lysine, phenylalanine, aspartic acid, and tryptophan are used in the food industry, *eg.* phenylalanine and aspartic acid are major components of aspartame. Many others are required as substrates in fermentations for the manufacture of other products.

Exhaust gas

CO_2 and O_2 are routinely measured on-line from a fermenter and with the measurement of a reference air stream, allows the calculation of such parameters as carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and respiratory quotient (RQ), which provide a measure of the relative respiratory or fermentative activity of the organism in the fermenter. This may be important for monitoring and control *eg.* in Baker's yeast production, where, for example, RQ correlates well with ethanol production rate; and OUR and CER are calculated to indirectly assess cell growth (Wang *et al.*, 1979).

The measurement of exhaust gases is also crucial in mass balancing, and has been used in estimating biomass concentration (Wu *et al.* 1989).

Biomass

Biomass is monitored routinely in fermentation processes. It is important to monitor biomass concentration as close to real time as possible - either it must be maximised if cells are required *eg.* for single cell protein production, or it must be tightly controlled so that there is enough biomass to produce the required product, but not so much that the available nutrients are wasted on producing more cells at the expense of product formation. Monitoring biomass is important in many processes *eg.* in the production of some protein products in recombinant *E. coli* fermentations to limit the growth rate to provide maximum protein production. An accurate estimate of biomass is required for good control over cell growth in fed-batch fermentations.

1.1.2. Sampling and sample preparation for analysis

This subsection deals with ways of getting a sample out of the fermenter and preparing it for analysis. Topics examined here include sampling techniques, methods for the separation of solid and liquid components of whole broth samples, and the maintenance of sterility, subjects which are closely related to much of the work described in the thesis. There is also a short description of cell disruption techniques, and their relevance to the on-line analysis of intracellular components.

1.1.2.1. Manual sampling

Most commercially available sampling devices are manually operated. They are generally either bottom or side sampling valves, or hooded samplers (Seifert and Matteau, 1988). The latter may either be sterilised separately, or with the fermenter, and are operated according to the vacuum principle. The bottom or side sampling valves are usually found on larger vessels, and use steam sterilisable or piston valves.

There are, however, a number of drawbacks to manual sampling: 1) it is expensive, requiring operator time, especially overnight 2) culture contamination may occur 3) it is not always easy to regulate sample volume, especially with

bottom sampling valves 4) hooded samplers are ineffective when used with airlift fermenters, because of the high airflow rates (Seifert and Matteau, 1988) 5) off-line analysis with manual sampling takes longer, and delay could lead to the characteristics of the sample altering 6) good laboratory safety practice - microbiologicals material should not come into contact with personnel. The development and use of automated on-line sampling would solve most of these problems.

1.1.2.2. Automated sampling

Sampling can generally divided into two aspects - removal and transportation of the sample; and the separation of the solid and liquid components, although both operations are often present in the same device. Samples can either pumped to the analyser continuously or discontinuously depending on the process and what is being measured (Bradley *et al.*, 1991).

Removal of a sample and transportation from the fermenter has been accomplished in a number of ways. Coppella (1990) described a sampling system consisting of a recycle loop and a debubbler to provide a bubble-free sample stream which was analysed spectrophotometrically to measure cell concentration. The sampling system had a number of advantages: low dead volume; representative samples from continuous flow; and operation under pressure, reducing the likelihood of fermenter contamination. Oakley (1990) has patented a system for the sampling of animal cell cultures aseptically. Burns (1987) has used air bubbles in a fluidic transit system that moves samples at a 0.3 ms^{-1} from a fermenter to a remote analysis station aseptically. The bubbles preserve sample integrity and maintain system cleanliness. An advantage of this system is the potential to link up a number of fermenters to the same analysis system, as can that described by Dinwoodie and Mehnert (1985). Samples are removed and filtered by a membrane of porosity 0.2μm, and cells continuously recycled to the fermenter. The clean filtered liquid sample stream passes through a flow-through vial, and is then returned to the fermenter. During this latter stage, an HPLC system is programmed to withdraw and analyze samples at any desired time interval. Another systems capable of multi-fermenter sampling is that described

by Reda *et al.* (1991), in which an automated, aseptic sampling method for whole broth samples from up to six fermenters is based on a piston-type sampling valve, a local sample loop, and the ability to send the sample with sterile air through the sample line into a remote, chilled tube for later analysis.

Marshall *et al.* (1990) developed a commercial sampling system consisting of a peristaltic pump and recycle loop which passes through a 12 vial sampling device. At programmable intervals, a sample can be diverted to a vial in the sampling device; the vials being cooled for later analysis off-line. The commercial sampler has the advantages that it is comparatively inexpensive, asepsis is maintained, there is no dead volume, samples are representative, and it can be fitted to any fermenter. A similar device was described by Strudsholm *et al.* (1992) where a needle connected to the fermenter by a tube breaks the seal on a sterile sample vial in a refrigerated fraction collector; the vacuum in the vial causes a sample to be taken. Other automated sampling devices involve various combinations of pumps and valves. Appelqvist *et al.* (1989), used two pneumatically operated three way diaphragm valves, and peristaltic pumps, all under computer control to provide samples from a fermenter for FIA. Other examples in the literature include the recycle loop of 3 way valves and a pump (Ghoul *et al.*, 1986); and the systems of valves and pumps developed by Kroner and Papamichael (1988); Seifert and Matteau, (1988) and Beitle and Ataai (1991).

Not all analyses require the separation of solid and liquid components. Nielsen *et al.* (1989a), described the removal of a sample from a fermenter and pumping cells into a small stirred chamber. There, cells were deactivated by cooling and the addition of 0.02M chloramine solution. The subsequent mixture was then pumped to the analytical system which measures OD (optical density) and the concentrations of glucose, lactic acid and protein. Another sampling device that does not remove solids before analysis is that described by Håkanson *et al.* (1991). This device consisted of two concentrically arranged catheters, the inner one slightly shorter than the outer. A sample was sucked through the inner lumen, and an inhibitor added to terminate metabolic processes before analysis and is shown in figure 1.1.a. This is not usually done however; solid and liquid

components are generally separated before analysis.

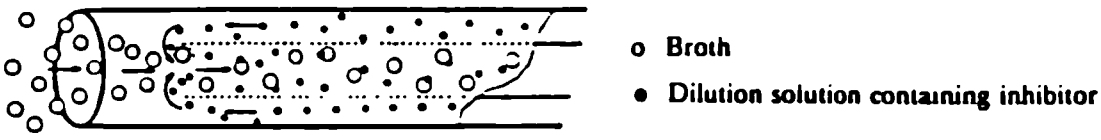


Fig. 1.1.a. Sampling device of concentrically arranged catheters (Håkanson *et al.*, 1991)

1.1.2.3. Sample preparation by solid-liquid separation

The previous subsection described the removal of a sample from the fermenter. This subsection describes techniques available for separating sold and liquid components of the samples, either inside or outside the sterile fermenter barrier.

Extracellular components of cell broth include substrates, inhibitors, certain products and various metabolites. It may be necessary to analyze one or all of these during a fermentation, and in order to do so, the biomass and other solids nearly always need to be removed first. Separation devices may be placed either inside the fermenter (which is often preferred for viscous broths of filamentous organisms) which filter the broth; or outside the fermenter. Internal devices are not replaceable during a fermentation, but do not require repeated sterilisation. External devices have the advantage that they can be replaced during the fermentation; but pumps, tubing and other materials that come into contact with broth outside the fermenter, generally require sterilisation (Lorenz *et al.*, 1987).

Microfiltration

Microfiltration is filtration defined according to the pore size of the filtering material, which can be nylon, acrylnitryl copolymer, propylene, cellulose acetate, cellulose nitrate etc. Many pore sizes are available, however the most commonly used is 0,2 μm which guarantees the separation of medium from biomass and most other suspended material. Cells may be filtered directly from a fermenter *eg* in the device described by Garn *et al.* (1989) which is shown in figure 1.1.b., although membrane fouling and protein build-up can readily occur. To reduce

fouling, liquid flow can be made tangential to the membrane (cross-flow microfiltration). Millipore has developed a steam sterilizable filtration device using a cross-flow filtration module to filter fermentation broth outside the fermenter, which is analysed by HPLC; and unfiltered material is returned to the fermenter (Kalynaput, 1989). Other examples of the use of cross-flow filtration are given by Bayer *et al.*, (1986), Schmidt *et al.*, (1985), Forman *et al.*; (1991) and Freitag *et al.*, (1991). Kroner and Papamichael (1988), describe a sampling device (BIOPEM) which is an autoclavable, sterilizable, magnetically stirred filtration cell for the continuous aseptic separation of samples of fermentation media by dynamic filtration. BIOPEM has been used by a number of researchers (Koliander *et al.*, 1990; Lundström *et al.*, 1990) and is shown in figure 1.1.c. In spite of the wide usage of BIOPEM, it has still been shown to start to block during fermentation, especially where filamentous fungi are sampled (Christensen *et al.*, 1991).

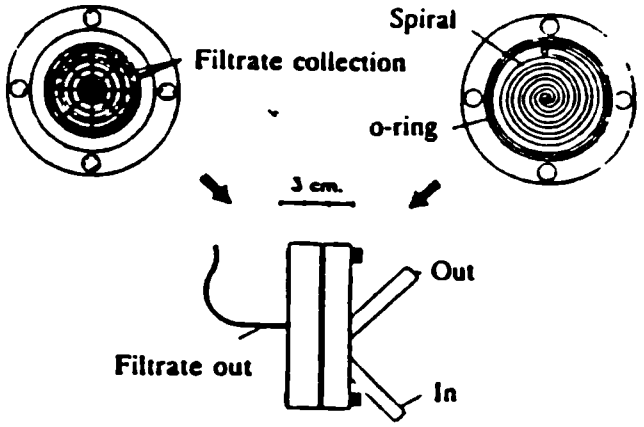


Fig. 1.1.b. *In situ* sampling device described by Garn *et al.* (1989); reproduced from Bradley *et al.*, 1991)

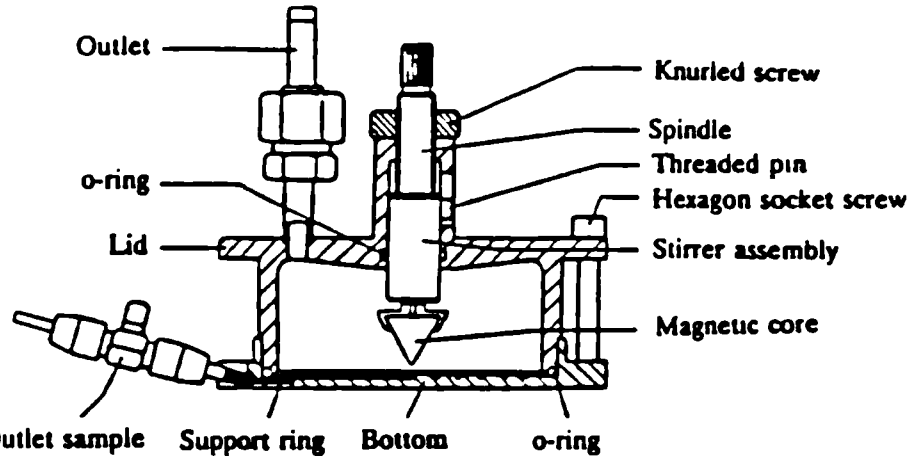


Fig. 1.1.c. Commercially available sampling device, BIOPEM, developed by Kroner and Papamichael (1988); reproduced from Bradley *et al.* (1991).

The problem of fouling has been tackled in other ways. Sulzer AG, Switzerland, has developed a filter unit that decreases the effects of fouling for use in separating samples from a fermenter for analysis by FIA. It consisted of two coaxial cylinders, the outer one fixed and the inner one rotating; a membrane being fixed to the outer wall of the inner one. Filtration by centrifugal forces results, with the membrane surface being continually rinsed, so there is no clogging or fouling (Ogbomo *et al.*, 1990). Kroner *et al.* (1987), described a similar device with a rotating shear filter which improves mass transfer at the membrane surface and may be used at the sampling point of the fermenter. This device seems to be rather complicated for its use in separating solid and liquid components in samples.

Some filtration devices are actually placed inside the fermenter, the solid-free samples being pumped out for analysis. Möller *et al.* (1986) sampled through a module containing a replaceable filter placed at the bottom of a tower loop reactor, and *in situ* filtration "probes" have been used in an animal cell bioreactor (Graf *et al.*, 1991) and Cephalosporin C and *E. coli* fermentations (Holzhauer-Rieger *et al.*, 1990). Filters have also been placed near the mechanical stirrer to reduce fouling (Schmidt *et al.*, 1984; Hustedt *et al.*, 1985). Picque and Corrieu

(1992) compared two *in situ* liquid sampling systems consisting of an inorganic membrane filter that is either rotating or stationary, and found the rotating device to give a better filtration performance. The failure of these systems during fermentations, however, may require the termination of the fermentation.

Ultrafiltration

Ultrafiltration separates large macromolecules and suspended solids from solution by forcing the solution through the membrane using a pressure gradient that dominates over osmotic differences across the membrane. This is particularly useful for colloidal or proteinaceous liquid streams as well as larger particulate matter. The materials used in ultrafiltration are graded according to cut-off point of molecular weight, usually between 10^4 and 10^6 daltons, that is unable to pass through the membrane (Kroner *et al.*, 1987).

Many examples of the use of ultrafiltration for the production of solids-free samples exist in the literature. Ogbomo *et al.* (1990), gave a number of applications for using ultrafiltration of samples *eg.* when analysing ethanol produced by *Saccharomyces cerevisiae* or in the analysis of glucose. Other examples include the use of an agitated ultrafiltration cell fed sequentially to provide a solids-free sample stream for process liquid chromatography (Gressin, 1988); other ultrafiltration based sampling systems for on-line HPLC (Dinwoodie and Mehnert, 1985 and Favre *et al.*, 1990), and with prefiltration to reduce clogging (Wang, 1984). Wang in this paper also suggested that because ultrafiltration rate is inversely proportional to the concentration of (yeast) cells in the fermentation, measurement of flux may be used to indirectly monitor the concentration of microbial biomass in the fermenter.

Dialysis

Another "filtration" alternative is dialysis. Whereas microfiltration membranes are graded according to pore size, dialysis as well as ultrafiltration membranes are graded in terms of molecular weight cut off points; dialysis being used to separate even smaller particles. In addition, dialysis membranes are diffusion driven as opposed to the majority of filtration devices which are pressure driven. With

dialysis, a semi-permeable membrane is used which generally retains material larger than about 15 000 daltons. The driving force for dialysis is a difference in concentration of a component on either side of the membrane. Some membranes are made from cellulosic material, but applications are limited due to hydrolysis by many organisms.

A number of sampling devices make use of dialysis, either on its own, or in conjunction with another technique. Bayer *et al.* (1986) has used microfiltration to remove cells from a sample stream and then dialysis to remove proteins, and the device is shown in figure 1.1.d. Similarly, Niehoff *et al.* (1986) used dialysis to dilute and remove proteins from a sample already prepared by ultrafiltration.

It is probably better to precede dialysis with initial filtration of the sample as dialysis membranes are very easily fouled. Dialysis has the advantage of being able to dilute a sample for later analysis if careful selection of dialysis membrane type, area and dialysis fluid flow rate is made. Lorenz *et al.*(1987) suggested that sampling devices using the dialysis principle should only be used in small vessels, where the membranes remain stable and any decrease in liquid volume can be avoided.

Dialysis sampling systems have been constructed for use either inside or outside the fermenter. Mandenius *et al.* (1984) mounted a steam sterilisable dialysis probe inside a fermenter, protected from fouling by a strong tangential flow of the sample solution (caused by placing a magnetic stirrer bar close to the membrane surface). The probe provided continuous samples for HPLC. Zabriskie and Humphrey (1978a) modified a stainless steel baffle in a fermenter vessel to construct a dialyser that could generate solids-free samples of fermentation broth. A dialysis probe of perspex and nylon has also been constructed, with a membrane stretched over the end; the whole thing being screwed into the headplate of an Anglicon fermenter. It was not steam sterilisable however, and had to be fitted in a laminar flow cabinet after vessel sterilisation (Gibson and Woodward, 1988), something that greatly limits its use. On-line dialysers may also be placed outside the fermenter *eg.* between an analysis device such as FIA, and a sterile barrier to the fermenter (Appelqvist *et al.*, 1989). Dialysis membranes are also used in conjunction with various biosensors (Cleland and Enfors, 1984).

1.1.2.4. Problems with sample separation techniques using filtration

A serious problem has emerged with all the above different types of sample filtration (microfiltration, ultra filtration and dialysis). It has frequently been mentioned that clogging or fouling of the different membrane types causes difficulties with the acquisition of representative samples for analysis, and very few of the techniques described to overcome it are practical, especially in production applications, or where the fermentations are long. Clogging or fouling of membranes is not only caused by cells and solids in the fermentation media, but also by components such as antifoam (Hoffman *et al.*, 1987). Antifoam effects are cumulative and cause a decrease in trans-membrane flux (Cabral *et al.*, 1985) *eg.* polyethylene glycol, a typical antifoam, lowers the permeability of many membranes commonly used for this purpose (Kroner and Kula, 1984). The problems can in many cases be eased by backflushing, or by the use of rotating filter systems (Kroner *et al.*, 1987; Rushton and Zhang, 1990), although the latter is not very practical for the typically small sample volumes. Even so, filters often need to be cleaned or even replaced during or between fermentations. Another

problem with filtration for sample preparation is that it only provides analysis of the filtrate, and hence only extracellular broth components; cells and solids are either returned to the fermenter or discarded. Clearly, a completely new approach is needed to overcome this problem.

1.1.2.5. Maintenance of sterility

A very important consideration in sampling from fermenters is the prevention of contamination. Many of the filters or probes described earlier are constructed either so they may be steam-sterilised *in situ*; or fitted outside the fermenter so that valves *etc.* may be steam sterilised outside the fermenter. An example of this is a sampling system consisting of a recirculation loop, a pump and four three-way valves that are steam sterilisable (Ghoul *et al.*, 1986). Some researchers, however, have not found satisfactory methods for maintaining sterility; for example, a dialysis probe constructed by Gibson and Woodward (1988) was not steam sterilisable and had to be attached to the fermenter in a laminar flow cabinet after vessel sterilisation. This obviously limits its use to small portable fermenters.

Other workers have concentrated on this problem. Methods of maintaining asepsis use discontinuous operating valve combinations flushed with steam, sterile water, sterile air, detergents and disinfectants (Hustedt *et al.*,1985). Appelqvist *et al.* (1989) developed a sampling system consisting of two three-way valves and stainless steel tubing through which a sample may be drawn and subsequently analysed. After the sample had been taken, the valves were switched to pump through an external system of 5% formaldehyde, which had the effect of sterilising the sampling system, and maintaining a sterile barrier to the fermenter. Another way achieving asepsis is to construct a system where the components are easily isolated and removed. An example of this is the dynamic filtration sampling device connected to a steam sterilisable by-pass system so that filters may be removed and replaced without contamination (Kroner and Kula, 1984). In *E. coli* fermentations, preventing contamination of the fermenter is not difficult, and in some cases researchers have made no attempt to render a sampling device aseptic, merely pumping a sample out for on-line analysis (Valero

et al., 1992). This is an interesting approach, and it raises the question of how much emphasis should be placed on aseptic sampling when rapid growing organisms are used, especially when grown on a defined medium containing an antibiotic.

1.1.2.6. Analysis of intracellular components

The methods discussed so far are only useful for the analysis of extracellular components, because the cells are either retained by the fermenter, or discarded. Apart from non-invasive methods, very little work has been done on the analysis of intracellular components on-line, primarily because of the difficulties in automated sampling and on-line cell disruption. In spite of these difficulties, Ahlmann *et al.* (1986), were able to monitor intracellular enzyme activity of recombinant *Escherichia coli*, by on-line automated sampling and disintegration of cells, followed by transport to an analyzer system which monitored coloured products of the enzyme spectrophotometrically. Different methods of cell disruption were examined for this application: lysozyme plus EDTA; $CHCl_3$ and toluene plus EDTA; and ultrasonic disintegration. The last of these gave the best results although exact details of the method were not given. Although some success was obtained, particularly early on in the fermentation, later on-line analyses showed substantial deviation from off-line values. Greater success was obtained by Kracke-Helm *et al.* (1991) and Valero *et al.* (1992) who monitored intracellular β-galactosidase on-line during in *E. coli* fermentations by using automatic ultrasonic cell disruption and FIA analysis. In spite of these successes, it is still very difficult to do on-line analysis of intracellular components, and performing routine analyses of this type is probably a long way off.

Most work in the field of cell disruption has been done on large scale cell disruption and intracellular product recovery, but many methods may be adapted for small scale use. Methods for cell disruption include:

- Chemical disruption using alkali, detergents and solvents, although such harsh techniques may result in damage to or destruction of biological compounds. This method may be more suitable where small, more stable molecules need to be

analysed.

- Enzymatic methods, *eg.* the use of lysozyme in cell disruption. This is more effective on gram positive organisms, although some success can be obtained with gram negative cells if EDTA is also used.
- Osmotic shock, although this is generally only effective on fragile organisms.
- Ultrasonication, which is useful for small scale cell disruption only.
- Agitation of microorganism suspensions with small glass beads. This is effective at small scale, although a lot of heat may be produced, therefore a cooling jacket may be required (Kula and Schütte, 1987).
- High pressure homogenisation, used for large scale cell disruption, although it may be adapted for disrupting fermenter sample size volumes of cell suspensions.
- Freeze-thawing using a freeze press (Magnusson and Edebo, 1976) or freeze-blasting (Omori *et al.*, 1989), which are both small scale techniques.

Applicability of current cell-disruption techniques to the analysis of samples of fermentation broth

Nearly all the literature on cell-disruption deals specifically with that required to release products from cells during downstream processing. Because cell disruption for this purpose has completely different requirements from those for on-line analysis of intracellular substances in a sample from a fermenter, a very different approach ought to be taken. Where possible, the simplest techniques ought to be used (for example, the addition of alkali to the sample) to cause cell lysis and the release of the analyte. Unfortunately, the simplest techniques tend to be the least generally applicable to all cell types, so if a tool suitable for any cell system is to be developed, it would probably either be necessary to combine two or more techniques, or use a much more complicated device. Possibilities for such a tool may include addition of a chemical followed by ultrasonication of a sample; or the use of a miniature homogeniser. These still have their problems: no generally applicable techniques currently exist for routinely monitoring intracellular substances on-line from samples of fermentation media. Clearly, there is a lot of research to be done in this area.

1.1.3. Analytical methods

This section deals with analytical techniques available for some of the more important fermentation variables that are not generally currently routinely measured on-line. The reasons for wanting to measure these variables have already been discussed in section 1.1.1.

1.1.3.1. Glucose

As mentioned previously, glucose is one of the most widely used substrates in fermentation and because of its clinical importance, a tremendous amount of work has been done on analysis methods, but in this review, only those that have been used in fermentation will be examined. There are a wide range of analysis techniques available, from the use of immobilised enzyme biosensors, to on-line HPLC.

Possibly the most widely used glucose analysis methods are enzymatic, either by using an enzyme electrode, or enzymic reaction in an FIA system or a glucose analyser. The most studied and developed method of enzymatically monitoring glucose is the electrochemical monitoring of H_2O_2 at a Pt anode which is produced in the reaction:

$$\text{glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2$$

The reaction is catalysed by the enzyme glucose oxidase (GOD), a stable flavoprotein, the most widely used enzyme in the field of biosensors (Wilson and Turner, 1992). A problem with the GOD reaction with glucose is the dependence on oxygen concentration in the medium. One way of overcoming this problem is described by Rishpon *et al.* (1990). The electrode uses immobilised GOD "sandwiched" between two membranes - the inner one to separate the fermentation medium from the enzyme compartment and to reduce the glucose content reaching the enzyme by diffusional limitations; and the outer one being exposed to the air, allowing unlimited oxygen to reach the enzyme whilst preventing liquid leakage. Although it is not dependent on DOT in the

fermenter, a modified reactor is required for its use to enable contact between the outer surface of the probe and the air. This probe is capable of monitoring high levels of glucose (up to 0.8M), useful for fermentation monitoring where high levels of glucose frequently occur.

Another way of overcoming the problem of variation in oxygen concentration within the fermenter causing inaccuracies in GOD based glucose measurements is by using a non-oxygen dependent glucose electrode, *eg.* a ferrocene mediated sensor. Although GOD is highly specific for glucose as opposed to other carbohydrates, oxygen as co-substrate and electron acceptor may be replaced by a number of other compounds. Ferrocene has been found to be excellent for this purpose, being a very good electron acceptor, and is suitable for use in a glucose sensor. Developments in this field have greatly improved the use of glucose probes in fermentation. Gründig and Krabisch (1989) developed an amperometric enzyme electrode for fermentation medium incorporating ferrocene or tetratriafulvene in a graphite epoxy matrix subsequently coated with immobilised GOD. Probes with steam sterilisable housings for *in situ* monitoring of glucose in fermentation broth using ferrocene mediated electrodes have been described by Brooks *et al.* (1987/88); Filippini *et al.* (1991) and Bradley and Schmid (1991). Cleland and Enfors (1984) developed a sterilisable housing for a GOD based glucose probe with oxygen content of the enzyme preparation kept constant, instead of using ferrocene.

FIA or glucose analyser systems (where glucose is analysed outside the fermenter hence oxygen concentration isn't a problem) based on the GOD reaction include the on-line glucose analyser for control of *E. coli* fermentations (Luli *et al.*, 1987), the FIA system used in on-line monitoring of glucose in animal cell cultures where peroxide is measured amperometrically (Rennenberg *et al.*, 1991;) or by chemiluminescent detection of peroxide after its reaction with luminol in the presence of a suitable catalyst (Huang *et al.*, 1991;). Galactose can be measured in the same way (Nielsen *et al.*, 1990). Another FIA system using GOD makes use of the peroxide produced in the following reaction:

2 H_2O_2 + 4-aminophenazone + phenol → monoimino-p-benzoquinone-4-phenazone + 4 H_2O (catalysed by peroxidase)

giving a coloured product (Valero *et al.*, 1990 b). A similar FIA system makes use of colorimetric determination of a product after the sample has been passed over immobilised GOD and then mixed with ABTS (2,2-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid) (Filippini *et al.*,1991).

Glucose dehydrogenase (GDH) is used less frequently in enzyme electrodes probably because it is less stable, less tolerant to a wide pH range, has a lower current density and is less available commercially. It does have some advantages, however, namely its lower oxygen sensitivity and faster response time (Bradley *et al.*, 1989).
The reaction catalysed by the enzyme is:

glucose + NAD^+ → d-gluconolactone + NADH + H^+

The resulting NADH may be determined either spectrophotometrically or amperometrically. Appelqvist *et al.* (1989) monitored glucose on-line in fermentation broth by using an FIA system and GDH based biosensor. NADH is produced and re-oxidised to NAD^+ by the electrode, the oxidation current being measured to give glucose concentration. A similar system was developed by Kittsteiner-Eberle *et al.* (1989) where NADH was measured either by means of a modified graphite electrode, or spectrophotometrically.

Glucose in fermentation broths may also be determined colorimetrically, without the use of enzymes. Methods used include the reaction of glucose with p-hydroxy-benzoic acid hydrazide (pHBAH) in slightly alkaline solution, the colour change being monitored at 410 nm (Schmidt *et al.*, 1985; Kuhlmann et al., 1984). pHBAH is selective for reducing sugars, and has a sensitivity similar to that of enzymatic methods, but is cheaper and the assay is easier. Another method is monitoring the reaction of glucose with neocuproin (Ghoul *et al.*, 1986). In concentrations greater than 2 $g.L^{-1}$, glucose can be determined by measuring the

polarisation angle α at 385nm using a flow through polarimeter cuvette (Kuhlmann *et al.*, 1984).

A relatively new technique in the analysis of glucose is use of the affinity sensor. Glucose diffuses into a chamber where the lectin concanavalin A is attached to the wall. Con A is reversibly bound to dextran labelled with a fluorescent dye. Glucose displaces the dextran, and the fluorescence of the free dextran may be measured (Wang, 1984; Merten and Palfi, 1987; Vadgama 1989).

Glucose and other sugars may be determined by HPLC relatively easily. Paalme *et al.* (1990); Motte *et al.* (1984); Koliander *et al.* (1990) and Favre *et al.* (1990) all describe similar methods for the isocratic elution of glucose and various acids using a Biorad Aminex HPX-87H cation exchange column with dilute sulphuric acid as the mobile phase; detection based on change in refractive index. Plaga *et al.* (1989) used the similar aminex HPX 87C column for analysis of several different carbohydrate species. Dinwoodie and Mehnert (1985) used on-line HPLC to detect glucose in fermentation broth. The sample was filtered before injection, and the column used was an amino alcohol analysis column. The mobile phase was a dilute solution of HNO_3, and a refractive index detector was used. There was good correlation between on and off-line samples.

1.1.3.2. Acetic acid

Acetate can be measured by HPLC using the same method and column described above for measurement of glucose, namely the Biorad Aminex HPX-87H column (Marsili *et al.*, 1981). It is also often measured by gas chromatography, *eg.* using a glass column packed with Poropac QS in a GC equipped with a flame ionization detector (Fieschko and Ritch, 1986; Konstantinov *et al.*, 1990 a).

1.1.3.3. Ethanol

Like glucose, there are a number of different techniques for the analysis of ethanol. Because ethanol is the oldest fermentation product, there is an old, traditional method of measuring ethanol (indirectly). Brewers measured the specific gravity (density) of the wort before fermenting it; the higher the specific

gravity, the greater the starch content, and therefore the greater the ethanol concentration at the end of the fermentation. More recently, ethanol has been measured using other methods.

Alcohol oxidase (AOD) has been used extensively in probes for placement either inside the fermenter, or in an external FIA system. The reaction catalysed by AOD is similar to that of GOD, with the production of H_2O_2 and acetaldehyde. Künnecke and Schmid (1990) used an FIA based system with enzyme coils, monitoring H_2O_2 spectrophotometrically using the ABTS reaction. Using this system, the lifetime of the enzyme was considerably longer than with conventional autoanalyser systems because of the short contact time between enzyme and sample stream. Gibson and Woodward (1988) used a similar analytical system on dialysed fermenter samples. They found that the dialysis membrane prolonged the life of the enzyme coils, but reduced the sensitivity. Peroxide produced in the AOD reaction has also been measured amperometrically *eg.* in the enzyme electrodes for fermentation process control described by Belghith *et al.* (1987) and Scheller and Kirstein (1987). As with the similar GOD electrodes, a drawback with *in situ* probes is the dependence on oxygen concentration in the vessel. Alcohol dehydrogenase (ADH) could be used instead with the NADH formed monitored fluorimetrically to give ethanol concentration (Kittsteiner-Eberle *et al.*, 1989). An amperometric ADH sensor has been developed that has an ADH layer, an NAD^+ layer, and a BSA layer, where ethanol may be measured without the addition of further NAD^+ (Miyamoto *et al.*, 1991).

Ethanol may also be measured chromatographically. Gas chromatography (GC) has been used most often, although the procedure is difficult to do on-line. Samples must be removed from the fermenter, butanol added, and then mixed and centrifuged and allow to settle into the aqueous and butanol layers. The latter is drawn off and injected into the gas chromatograph (Varma *et al.*, 1984). GC has also been used for headspace analysis of ethanol and other volatile components (Motte *et al.*, 1984), and some success has been achieved with a gas membrane connected to a GC (Groboillet *et al.*, 1990). In contrast, ethanol is easily assayed on-line with HPLC, which can measure other components such as

glucose, glycerol and acetate, on-line simultaneously (Dinwoodie and Mehnert, 1985; Picque and Corrieu, 1992).

Mass spectrometry is used to measure gases and volatiles including ethanol in fermenter exhaust gases on-line (Kuhlmann *et al.*, 1984). It is straightforward, automated, rapid and results are easily stored in a computer. Large errors have been reported, however, because of the necessity of heating the fermenter exhaust lines (Coppella and Dhurjati, 1987; Heinzle, 1987). Response time and reproducibility can be improved by using PTFE tubing to transport gas samples from the fermenter to the mass spectrometer (Camelbeeck *et al.*, 1991).

Reuss (1988) describes a successful semi-conductor gas sensor manufactured by Figaro (Japan) consisting of sintered SnO_2 and working on the principle that the adsorption of flammable or reducing gases or organic solvent vapours lowers the electrical resistance. A similar sensor has been used to monitor ethanol concentration during yeast fermentations (Paul and Maerz, 1991; and Mandenius, 1988).

A novel, non-invasive method for monitoring ethanol in a fermenter is described by Cavinato *et al.* (1990) using short wave infrared spectroscopy. A photodiode array spectrometer is connected to a fibre optic probe that is attached to the glass wall of the fermenter, and using this technique, good correlation was found between the intensity of backscattered light at 905 nm and the actual ethanol concentration. Yu and Phillips (1992) have also used near infra-red to measure and predict biomass, and mid-infra red in monitoring ethanol, glucose and glycerol in fermentations with some success, although they believed a lot of work was still needed to perfect the technique.

1.1.3.4. Ammonia and phosphate

Ammonia may be measured with an ion sensitive electrode (ISE) off-line after diluting the filtered sample with water, adding NaOH and EDTA and pumping the mixture through a channel to the ISE (Kuhlmann *et al.*, 1984; Schmidt *et al*, 1984). Another method for the analysis of ammonia in fermentation broth is the

use of an ammonia gas electrode in conjunction with a pH controller (to ensure pH high enough to convert ammonium ions to ammonia gas) on a sample stream from a fermenter (Thompson *et al.*, 1985; Gostomski *et al.*, 1992).

Many phosphate analysis methods in the literature rely on the reaction of phosphate with a molybdenum reagent to produce a coloured molybdate-phosphoric acid compound that can be monitored spectrophotometrically (Schmidt *et al.*, 1984; Garn *et al.*, 1989; Niehoff *et al.*, 1986, Williams *et al.*, 1993). This can be done on-line from a fermenter using an FIA system (Forman *et al.*, 1991).

1.1.3.5. Penicillin and cephalosporin C

As one of the most important and cheapest antibiotics that has been made by fermentation for a number of years, penicillin has attracted much interest in the development of measurement techniques. As early as 1979, Enfors and Nilsson report the construction of an autoclavable enzyme electrode for the measurement of penicillin concentration in fermentation broths. It is based on a pH electrode on which β-lactamase is immobilised. The principle is that penicillin diffuses onto the enzyme layer which causes a split in the β lactam ring, and the resulting decrease in pH may be measured (potentiometric method). The probe is steam sterilised *in situ*, after which the enzyme is introduced. This also enables recharging of the electrode during operation if required. This work has later been emulated by Scheller and Kirstein, (1987) and Vadgama, (1989). The hydrolysis of penicillin causing a pH change has also been used as the basis for enzyme optrodes (optical fibre probe) where the pH change affects the fluorescence of bound fluorescein isothiocynanate (Scheper *et al.*, 1991; Höbel *et al.*, 1992). The pH change principle has been used in an FIA system, where penicillinase is immobilised on a glass pH electrode, and fermentation samples flow through detection cells (Meier and Tran-Minh, 1992).

Other FIA systems have been described (Carlsen *et al.*, 1993) comparing different detection methods (spectrophotometric, iodimetric and potentiometric) of the products of hydrolysis of penicillin V using β-lactamase, and here, the iodimetric

method was found to be the best for measurement in fermentation media; the potentiometric method was found to be unsuitable for fermentation media because of the changing buffering capacity of the fermentation medium.

Penicillin and its precursors may also be analysed by HPLC on-line relatively easily (Reuss *et al.*, 1987; Schügerl *et al.*, 1989; Möller *et al.*, 1986; Holzhauer-Rieger *et al.*, 1990), and this is probably still the easiest method for penicillin analysis. Other techniques less commonly used include the on-line affinity sensor using an enzyme immunoassay (Wang, 1984) and an enzyme thermistor (Schügerl *et al.*, 1989).

Cephalosporin C (shown in figure 1.1.3.5.a.), like penicillin is a β-lactam antibiotic, but with a relatively weak bacteriostatic effect. It may be measured by the absorbance at 260nm of the cepham chromophore, O=C-N-C=C, which appears in cephalosporins but not penicillins. However, because other media components may influence this reading, after the measurement, a β lactamase such as cephalosporinase should be used to destroy the cephalosporin, and then the residual absorbance can be measured. The difference will be due to the cephalosporin. (Bayer *et al.*, 1986; Herold *et al.*, 1988).

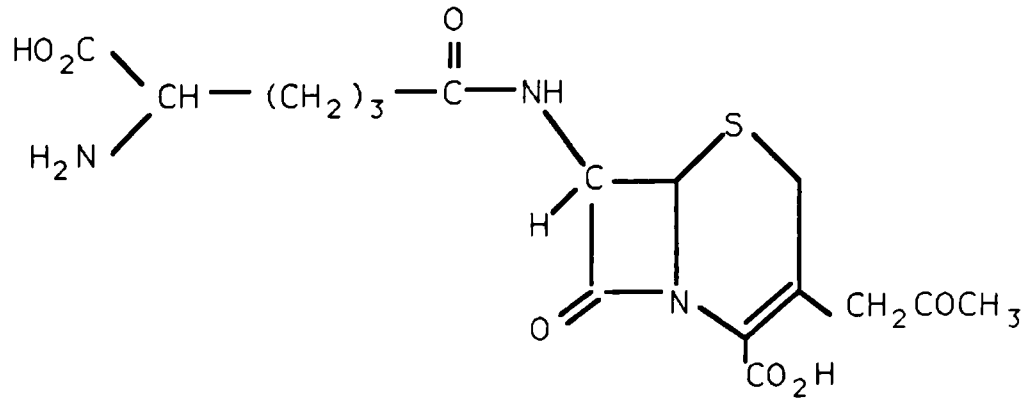


Fig. 1.1.3.5.a. The structure of cephalosporin C

1.1.3.6. Amino acids

There are many different analytical methods for amino acids, reflecting their different chemical natures. Only a very few methods will be looked at here.

Methionine may be determined spectrophotometrically by being mixed with sodium nitroprusside and NaOH reagent and then acidified. The red dye generated can be measured at 505nm (Bayer *et al.*, 1986).

Glutamate may be measured by means of an enzyme electrode containing L-glutamate oxidase; the reaction produces H_2O_2 which can be measured amperometrically. The electrode can be used in an FIA system and could therefore be adapted to measure glutamate in fermentation broth (Yao *et al.*, 1990). Similarly, L-lysine has been determined by immobilising lysine oxidase and catalase in a support attached to an oxygen sensor (Vrbová *et al.*, 1992). Glutamate and glutamine have been measured using FIA with peroxide produced from the reactions of immobilised glutamate oxidase and glutamine, coupled with peroxidase detected by luminol chemiluminescence (Blankenstein *et a*l., 1993).

Amino acids may be measured with or without derivatisation by HPLC without difficulty (Joseph and Marsden, 1987). The aromatic amino acids - phenylalanine, tyrosine and tryptophan are easily measured by reverse-phase HPLC (Joseph and Marsden, 1987). A similar method for tryptophan is given by Patrick and Lagu (1991).

1.1.3.7. Protein

The term protein is a general one and refers to a wide range of biological macromolecules composed of polypeptide chains arranged in different structures and with diverse functions.

Protein

A number of assays are available for the detection of total free protein content in a sample of fermentation broth using FIA, and these are based on the Biuret, (Nielsen *et al.*, 1989b) Lowry, and Bradford assays (Recktenwald *et al.*, 1985). In

the latter case on-line protein assays were used to detect the end of the fermentation, when cell lysis starts to occur. The BCA (bicinchoninic acid) protein assay has been used in the on-line measurement of extracellular cellulases in fermentation broth (Stamm *et al.*, 1989).

Fast protein liquid chromatography (FPLC) is an alternative to the above assays as a means of determining intracellular protein concentration. Gustafsson *et al.* (1986) used FPLC for the monitoring of protein formation during fermentation using a strong anion exchange column, although they were unable to measure it on-line because of the necessity of cell disruption. Similar work was done by Low (1986). Affinity chromatography has also been used in monitoring fermentation products, sometimes on-line; *eg*, the excreted fusion protein, IGF-1 has been monitored on-line during an *E. coli* fermentation process using this technique (Lundström *et al.*, 1990). Ultrafast protein analysis using microbore chromatography columns is a promising technique for rapid on-line analysis (Nugent and Olsen, 1990). Rapid protein separations are also achieved using the comparatively new technique of capillary electrophoresis; and routine separations should soon be possible within a few minutes (Paliwal *et al.*, 1993). Bioactive peptides have been determined by this technique, coupled with mass spectrometry (Moseley *et al.*, 1991).

Immunoassays in the form of ELISA or the electro-chemiluminescent immuno assay (CIA) are used for monitoring product formation in animal cell cultures (Lindell *et al.*, 1990). ELISA has been fully automated and rapid analyses (6-10 minutes) are now possible (Nilsson *et al.*, 1991). Other immunotechniques used include the assay based on the interaction between the analyte protein and an antibody raised to that protein; aggregates are formed which increases the turbidity, which can then be measured (Freitag *et al.*, 1991).

Enzymes

A method for the on-line analysis of β-galactosidase, a cytoplasmic enzyme frequently monitored in *E. coli* in fermentation broth, has been described (Valero *et al.*, 1992; Kracke-Helm *et al.*, 1991;), making use of FIA; the substrate, ONPG,

being hydrolysed by the enzyme to produce *o*-nitrophenol, a yellow compound. The activity of intracellular penicillin-G-acylase has also been measured (Ahlmann *et al.*, 1986). The methods of cell disruption were discussed previously; the assay of the enzyme using 6-nitro-3-phenylacetamidobenzoic acid (NPAB) as substrate. Few other intracellular enzymes have been measured on-line. Extracellular lipase produced by *Candida rugosa* has been measured on-line (Valero *et al.*, 1991) using a device that measures the turbidity change of a triolein emulsion after an lipase sample has been added. Kroner and Kula (1984) developed a method for the on-line determination of extracellular hydrolases, an assay based on the reaction of the enzyme with its natural high molecular weight substrate.

1.1.3.8. Biomass

Biomass is routinely measured in most fermentations, often as a dry weight determination. Although considered fairly accurate, dry weight determinations are tedious, and results are not immediately available. Optical density, or turbidity measurements are also frequently used, and can provide a reasonable indication of biomass, although many factors can influence the turbidity of the broth. Many researchers have used on-line turbidity measurements in fermentations because of the ease, rapidity and non-invasiveness of the technique (Ortmanis *et al.*, 1991; Yamane, 1993; Yamane *et al.*, 1992; Konstantinov *et al.*, 1992). A related technique is that described by Lima Filho and Ledingham (1987), and Cox *et al.* (1989) where a light emitting diode is coupled with a photodiode detector to measure interference by biomass in the fermentation broth.

NADH and culture fluorescence

The use of culture fluorescence due to NADH (which is related to viable cell count) in determining biomass concentration has been around for some time, and as early as 1981 (Beyeler *et al.*, 1981), a sterilisable probe for continuous measurement of culture fluorescence in fermentation was developed. The technique is sensitive, instantaneous and non-invasive, but, the practical application of culture fluorescence techniques to complex, typically industrial,

fermentation media is that many of the media components fluoresce. Their utilisation during the course of the fermentation may interfere with any correlation between culture fluorescence and biomass concentration.

Microcalorimetry

Microcalorimetry is also used to determine biomass concentration in fermentations, having been used for some time to determine enthalpy changes in some organisms which can in some cases be used to provide an estimate of biomass activity (Clarke *et al.*, 1986). Samson *et al.* (1987) used a flow calorimeter to measure heat output in batch cultures of *Pseudomonas putida* and *Saccharomyces cerevisiae*. Heat output could be quantitatively related to phases of the growth cycle.

Dielectric permittivity

The dielectric properties of ionic solutions are affected by the presence of cellular material; the dielectric permittivity or capacitance of cell suspensions being a linear function of the biomass present and is independent of the presence of non-cellular material. This principle has been used by Fehrenbach *et al.* (1992); Mishima *et al.* (1991) and Harris *et al.* (1987) to measure biomass on-line. Markx *et al.* (1990) have developed a commercial on-line sterilisable sensor, the Bugmeter, using the above principle which is selective, rapid, simple and robust. Although this is a promising technique, the signal can be affected by ionic strength of the medium, and problems arise in fermentations where large ranges in biomass concentration occur. This technique is also sensitive to the morphology of the organism, and is difficult to accurately apply it to any organisms other than yeast.

Other techniques

Kilburn *et al.* (1989) monitored cell mass in mammalian cell cultures using acoustic densitometry, which depends on the relationship between density and resonant frequency of a sample enclosed in a test chamber electromagnetically excited to vibrate at its natural frequency. Biomass is determined by measuring the density of the culture and subtracting the density of cell-free culture filtrate.

Viscosity differences have also been used in determining biomass at high cell densities (Groot *et al.*, 1991), and in dextran fermentations (Endo *et al.*, 1990). The analysis of cellular ATP is used in measuring viable biomass in hybridoma culture (Sonderhoff *et al.*, 1992).

1.1.3.9. Fermenter exhaust gas

The measurement of fermenter exhaust gas component concentration is a good way of obtaining useful information on the state of the fermentation. For example, Wu *et al.* (1989) used the measurement of CO_2 and O_2 to estimate cell mass after a linear relationship between cell concentration and CER was found, although this is not the case in all fermentations. There is also a correlation between oxygen uptake rate (OUR) and dry cell weight during the early growth phase of a fermentation. Specific growth rate during growth phase may also be calculated from the OUR (Buckland *et al.*, 1985).

Methods used to determine the concentration of exhaust gas components include mass spectrometry, the use of a paramagnetic analyser for O_2, an infrared analyser for CO_2 and gas chromatography.

Mass spectrometry has a number of advantages: it offers rapid response times (<1min); it has high sensitivity (10^{-5} M detection limit); it has the ability to analyse several different components practically simultaneously; it has a linear response over a broad concentration range; it suffers from minimal calibration drift; and it is possible to connect several fermenters to one instrument. In addition to the analysis of oxygen, nitrogen, carbon-dioxide and argon, various volatile compounds such as ethanol, methanol, acetone, volatile fatty acids and ammonia may be monitored (Reuss, 1988; Heinzle, 1987; Camelbeeck *et al.*; 1988).

Carbon-dioxide may be measured with an infrared spectrophotometer. Oxygen partial pressure in the gas stream can be measured with a paramagnetic analyser, although samples require drying to minimise error. Signals must be compensated for variations in atmospheric pressure. Both types of analyser suffer from severe

calibration drift, difficulty of maintenance and slow response time. Some workers consider neither method to be particularly reliable (Humphrey, 1987), and they do not have the flexibility of mass spectrometry but these techniques have been used extensively, presumably because of their reduced cost in comparison to a mass spectrometer (Schmidt *et al.*, 1984; Schügerl *et al.*, 1989).

Gas chromatography has also been applied to analyse many components in an exhaust gas stream including hydrogen and methane. For example, Griot *et al.* (1988) used GC to monitor the concentrations of volatile materials in fermentation broths.

1.1.4. General comments on on-line analysis

The on-line monitoring of fermentations is generally quite difficult. Apart from the routine on-line measurement of DOT, pH, temperature and exit gas analysis, other variables have proved very difficult to measure on-line. A variable can either be measured inside the fermenter, or a sample removed and the analysis performed externally. For the analysis of typical fermentation substrates and products, the former approach will often need the use of a biosensor, and the latter would generally require solid-liquid separation of the sample. Both approaches have their associated problems. Biosensors have been developed to monitor chemicals such as glucose, ethanol, penicillin *etc.*, but a lot of development is still required before these sensors can be relied on for use in industrial fermentation. Problems associated with the development of biosensors and the reasons for the small number of probes include: 1) steam or chemical sterilisation is necessary if the sensor is to be used in the fermenter, and will denature the biological component (usually an enzyme); 2) the temperatures during fermentation will decrease the activity; 3) enzyme techniques may run into difficulty in providing a linear signal in the high concentrations normally present in biological processes, and it is difficult to dilute *in situ*; 4) fermenter broths readily clog, foul or poison biological sensors 5) probe calibration often will not last for the duration of the fermentation and it is difficult to calibrate *in situ* 6) other components of the broth can interfere, leading to a spurious result 7) in

aerobic fermentations, the DOT in the broth may change rapidly in a very short time, which would cause problems for the majority of enzyme electrodes 8) a biosensor can often only analyze one component; 9) generally only extracellular components may be monitored (the non-invasive measurement of NAD(P)H dependent culture fluorescence being an exception); 10) probes cannot usually be removed from a fermenter during a fermentation if a problem arises.

Although of many of these problems would be solved if a biosensor is incorporated into an external analytical device *eg.* an FIA system, a general disadvantage with all enzyme based systems remains: enzymes are potentially very easily inactivated by other substances in the fermentation broth, although filtration or dialysis of the sample prior to analysis by enzyme based methods does help. It would, therefore, generally be better to use non-enzyme based analytical methods, *eg.* a non-enzymic chemical reaction with a colour change that can be measured spectrophotometrically, incorporated into an FIA or other autoanalyser. A disadvantage of this however, is the reduced specificity in comparison with an enzyme based technique. In addition to using non-enzyme based analytical methods, techniques which allow sample removal from the fermenter are generally preferable to enable multiple analyses to be performed on the same sample.

The obvious choices of analytical techniques (especially where more than one substance needs to be measured) are the use of FIA or HPLC systems. FIA and HPLC can analyse many components simultaneously, and are generally simple, universal, rapid, accurate and selective with the minimum of sample pretreatment. They can be made continuous, are easily automated, and can be made remote, providing some safety for analytical equipment and personnel during the analysis of harmful substances. HPLC particularly requires little adaptation for use on different fermentation systems analysing completely different components; a change of column may be all that is required. All these reasons provide strong justification for using HPLC as the analytical technique in an on-line sampling and analysis system that can monitor certain components in fermentation broth on-line.

1.1.5. Fermentation control

Fermentation control is a vast subject. The key features relevant to the work in this thesis are the concepts of 1) a hierarchical control structure, comprising a supervisory system interacting with lower level dedicated controllers and 2) the idea of closed loop (or feed-back) physiological control of a culture. Section 1.1.5.1. discusses the reasons for high level control, while section 1.1.5.2. reviews examples from the literature of controlling the growth of a culture. These examples will be relevant to section 1.8. of the thesis.

Armiger and Moran (1979) have stated that there are three different levels of process control that may be incorporated into a process. The first, most basic level deals with sequencing operations such as the opening or closing of valves, or the starting or stopping of pumps. A device such as a programmable logic controller (plc) is very suitable for such tasks.

The second control level involves the connection into the system of individual control loops, that function to maintain the environmental parameters at a particular value, or set-point. Such parameters include temperature, pH and dissolved oxygen. Digital Set-point Control (DSC) is a frequently used technique here; the computer scans the output from a control loop (obtained from the sensor) and compares it to a previously defined set-point. If they differ, control functions may be effected by the local controller, *eg.* proportional, integral and derivative control (PID). If the computer fails, control may be returned to each control loop. Another method is Direct Digital Control (DDC) where the sensors are connected directly to the computer. Better control may be obtained, but problems arise if computer failure occurs.

The highest control level is concerned with the optimisation of the process, which is a comparatively young, but rapidly growing field. It is concerned with using more complex measurements *eg.* those that have a direct bearing on the growth and productivity of microorganisms such as the concentrations of biomass, substrate and metabolites in the medium, and using these measurements to

control variables such as growth rate. It is this highest level of control that will be discussed here briefly.

1.1.5.1. Reasons for control

Requirements for supervisory control and data logging vary from site to site. At UCL, for example, the research pilot plant has requirements for flexibility and user written routines. Such flexibility would probably not be required in a production plant.

High level control is usually implemented during fed-batch or continuous fermentations and is obtained by controlling the feed-rate of one or more nutrients. The reasons for wanting to do this are firstly: controlling the feed rate will control the growth rate of the organism, and it is widely known that specific growth rate has an effect on the production of recombinant protein. Secondly, controlling the feed-rate (particularly with closed loop control) will prevent overfeeding that can lead to the build-up of substrate in the fermenter causing excretion by the organism of unwanted metabolites. An example of this is in yeast fermentations; ethanol can be produced even under aerobic conditions if glucose concentration is too high. This is known as the Crabtree effect; the equivalent in *E. coli* is the production of acetic acid. Apart from wasting nutrients, these excreted metabolites can have a detrimental effect on the fermentation process. Further discussion of this appears in section 2.1.

1.1.5.2. Methods of controlling feed rate

Open loop control

The simplest way of controlling a feed rate is by feeding nutrients at a particular rate, without any closed loop, or feed-back control. A constant, linear or exponential rate can be used; if a constant specific growth rate is required, the nutrients need to be added exponentially. The following basic equations will then be required in the operation of the control algorithm:

$$F = \frac{\mu}{Y_x} x(t).V(t) \qquad (1)$$

$$x(t)=x(t-\Delta t).e^{\mu \Delta t} \quad (2)$$

F = substrate feed rate ($g.h^{-1}$)

μ = specific growth rate required (h^{-1})

x = biomass concentration ($g.L^{-1}$)

V = culture volume (L)

Y_x = growth yield on substrate (g biomass.g $substrate^{-1}$)

Open loop control in which nutrients are fed to allow the organism to grow at a particular specific growth rate can still allow substrate to build up, particularly if the original estimate of biomass is incorrect, or if little is known about the process. For this reason, closed loop control is used if possible.

Closed loop (feedback) control

The same equations may be required for exponential growth in closed loop control, however, modifications are made to the algorithm according to the feedback control mechanism in use. Again, an accurate on-line estimate of biomass is important. In some cases, feedback control merely controls the concentration of a component to a setpoint, and does not need to calculate exponential feed rate. With closed loop control, the feeding profile is altered according to specific measurements made. Some measurements used are given below.

Closed loop control based on dissolved oxygen tension (DOT) measurements are common because of the ease of measurement. The basis for the control is the action on abrupt DOT increases due to substrate depletion; when these increases occur, a pulse of feed can be added to the fermenter. The DOT will then drop until the nutrients are used up, when it again abruptly increases. This should prevent overfeeding. The use of this technique has been described in the control of acetic acid excretion in *E. coli* (Konstantinov *et al.*, 1990 b); and in the controlled feeding of the toxic substrate, methanol, in fermentations of the yeast *Hansenula polymorpha* (Hopkins, 1981).

In yeast fermentations, feeding can be controlled by measuring exhaust gas and

deriving the value of the metabolic indicator, respiratory quotient (RQ). Respiratory quotient is a measure of how carbon dioxide evolution rate (CER) compares with the oxygen uptake rate (OUR). If it exceeds unity, it implies that carbon metabolism is producing ethanol instead oxidising glucose fully. Hence feed rate can be reduced when RQ increases above 1. This was done originally and successfully by Aiba *et al.* (1976) and Wang *et al.* (1979). An advantage of this method is that, like DOT, exhaust gas analyses are routinely performed.

Direct measurement of substrate, which is the focus for this project, is more desirable, because less needs to be known about the fermentation process for adequate control, and fine control can be achieved much more easily. As has been discussed earlier, such measurements are more difficult, however a number of researchers have achieved control by monitoring substrate concentration. Simple control using on-line glucose measurements to keep a constant glucose concentration in the reactor, without controlling growth rate has been performed by Kobayashi *et al.* (1987); Mizutani *et al* (1987) and Luli *et al.* (1987). Kleman *et al.* (1991) described the development of a more complex two component system that predicts the future glucose demand in *E. coli* fermentations on the basis of recent glucose consumption rates (feed-forward component) and then corrects for minor offsets after rapid measurement of glucose concentrations (feedback component). The controller performed well, but no advanced control profile was attempted.

Other measurements can be used in closed-loop control. These include analysis of the concentrations of ethanol, acetate and other excreted metabolites. An ideal controller would be able to act on many different measurements to achieve an optimisation of the process. A good understanding of the cell physiology would be required for this; so far, this has been largely absent in control strategies, treating the cell as a "black box".

Recent developments in on-line monitoring of intracellular protein products (described earlier in section 1.1.3.7.) mean that real time protein analyses can now be used in control. The actual development of a control strategy based on

such measurements has not really been examined to date, probably because no direct application has yet been found for this, beyond identifying the optimal harvest time. Better process optimisation is currently achieved by controlling factors affecting protein production rather than protein production itself.

1.2 AN OVERVIEW

The rest of section 1 describes a novel system that has been developed for the on-line, automated, aseptic removal, separation and analysis of samples of fermentation broth, and the subsequent linkage of such a device to a process control system. Previous systems, as discussed in the introduction (Section 1.1) take a different approach to automated sampling in that they generally filter (microfiltration and ultrafiltration) or dialyse the sample to separate its solid and liquid components. The drawbacks of those approaches are firstly that intra-cellular components are usually unavailable for analysis; and secondly that filtration systems are readily clogged leading to poor performance and often compromising fermenter asepsis. The novelty of the system developed here rests on its alternative method for separating solid and liquid components of fermentation broth, using a purpose built, small volume, high speed, pneumatically driven microcentrifuge. A schematic representation of the on-line monitoring system is shown in figure 1.2.a., and a photograph is shown in figure 1.2.b. Table 1.2.(i). summarises the main components of the on-line monitoring and control system.

Using this new system, a sample may be pumped from the fermenter, through a sampling device (designed to provide a sterile barrier between the fermenter and exterior) and into the microcentrifuge. The microcentrifuge then spins the sample at high speed for a short time to separate solid and liquid components of the broth, and a pump on the centrifuge pumps the supernatant into an HPLC injection loop. A pneumatic valve injects the sample onto an HPLC column for analysis of various components in the fermentation broth supernatant. The cells and any other solid components of the broth remain on the side walls of the centrifuge, and may be dislodged by a high pressure jet of liquid. The solids are then vacuumed to waste.

A programmable logic controller (PLC) sequentially operates all components of the system, and is programmed by software installed on an IBM PS/2 personal computer (PC). The PC also analyses raw data generated by the HPLC and

calculates the concentrations of previously identified and calibrated components by using commercially available software.

The on-line monitoring device was linked to a process control system, LabView, so that the results from the on-line HPLC can be used to control the concentrations of sugars and organic acids in fermentation broth.

Detailed descriptions of all components of this system appear in subsequent sections: the sampling device in section 1.3; the microcentrifuge in section 1.4; the chromatography equipment in section 1.5; and the PLC and sequencing of events in section 1.6. Section 1.7. describes the use of the on-line sampling system in monitoring glucose and acetate concentrations in a fermentation; section 1.8. provides details of the link of the monitoring system to LabView.

COMPONENT	FUNCTION
sampling device	provides aseptic barrier between fermenter and external environment
microcentrifuge	separates solid and liquid components in sample of fermentation broth
HPLC	measures concentrations of sugars and organic acids in broth
PLC	sequences the operation of individual valves and pumps *etc.* in above 3 devices
LabView	can be programmed to alter feeding strategy according to HPLC results

Table 1.2.(i). Summary of the chief function and main components of the on-line monitoring and control system described in sections 1.3. to 1.8. of this thesis.

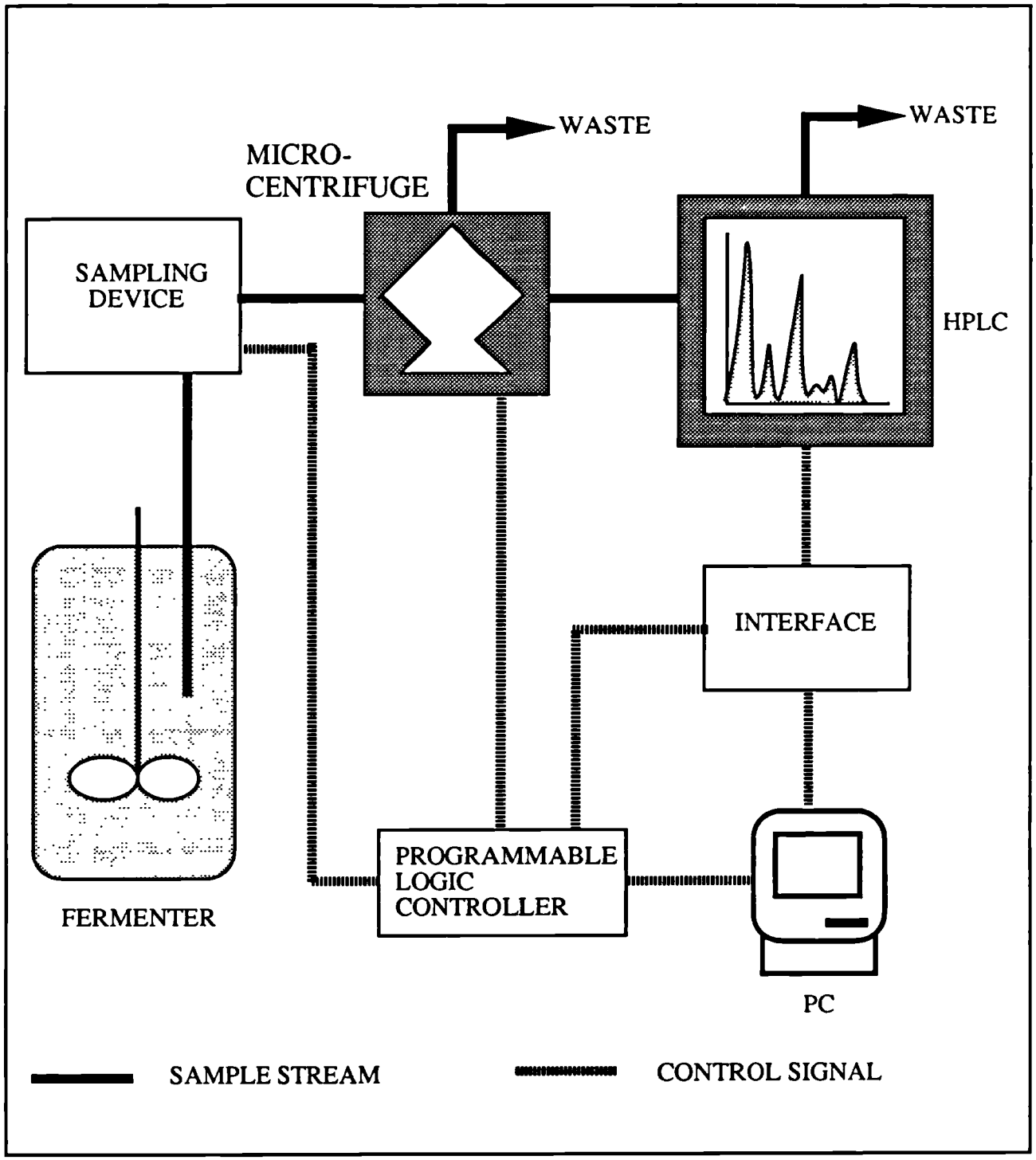


Fig. 1.2.a. Overview of main components of sampling and analysis system for fermentation broth. System is composed of a sampling device, allowing aseptic removal of sample from fermenter, microcentrifuge for the separation of solid and liquid components, and HPLC for analysis of supernatant. The system's sequencing is controlled by a programmable logic controller.

Fig. 1.2.b. Photograph of on-line monitoring equipment for fermentation broth. The fermenter is clearly visible on the right of the picture. On the left is the trolley showing the top two shelves. On the top shelf is the sampling device (large stainless steel box connected via tubing to the fermenter). To the left of the sampling device is a smaller unit: the microcentrifuge. The HPLC equipment rests on the shelf underneath (to the left is the autosampler used for calibration, and to the right is the water bath for the HPLC column). The programmable logic controller rests on the bottom shelf of the trolley (not shown).

1.3. THE SAMPLING DEVICE

The sampling device was designed by a number of people at UCL (including the author). The chief functions of the sampling device are firstly to provide an aseptic barrier between the fermenter and its surroundings; and secondly to interface the fermenter to the microcentrifuge allowing the passage of a sample.

The sampling device consists of five pneumatically actuated valves, a steam trap and a docking device; all connected by stainless steel tubing of internal diameter 1/16 inch. The layout is shown in figure 1.3.a. Apart from the docking device, the whole system is enclosed in a stainless steel box (500 mm long x 435 mm wide x 165 mm high) which has inlets for steam and air supply; and outlets for steam via the steam trap, and of sample to the microcentrifuge. The sample enters the sampling device via the docking device mounted on the top surface of the box. A photograph of the sampling device unit is shown in figure 1.3.b.

1.3.1. Valves

The valves used are air actuated Nupro 2P4T plug valves (from Swagelok), constructed primarily from 316 stainless steel. The pneumatically actuated valves require a minimum air pressure of 40 psi. AC (240 volt) solenoid actuated control valves electrically operate the air actuators, and the connection and operation of these is described more fully in Section 1.6. (PLC). Compressed air for the valves is provided by a BAMBI air compressor, model 225/1000, which also provides air for the microcentrifuge. As the centrifuge uses up to 90 psi air, an additional air regulator is needed to reduce the air pressure to the sampling device. The valves enable passage of steam and sample at different times through the relevant parts of the sampling device.

1.3.2. Steam Trap

The steam trap is a Spirax Sarco TD42A thermodynamic steam trap, attached to valve 5 by a length of stainless steel tubing, and to a lagged stainless steel steam

outlet pipe on the other, leading out of the sampling device, and down to the drain.

1.3.3. The Docking Device

The docking device was specifically designed and constructed for this application, by Jim Molloy of Fisons Instruments. It consists of a cylinder of diameter 50 mm and height 60 mm, mounted on top of the sampling device. Through the cylinder is a small hole, leading down into the sampling device, to connect the docking device to the internal sections of tubing, and valves 2 and 3. Through the hole, a rigid piece of plastic tubing of internal diameter 0.8 mm (Pharmacia) is inserted. The tubing is attached to a length of silicon tubing, which in turn is attached to a fermentation inoculation needle. All the tubing is previously sterilised in the autoclave prior to feeding the piece through the hole on top of the docking device. Once threaded through, it may be secured by tightening screws down onto the cylinder on either side of the hole, each with a washer and rubber seal. A closeup photograph of the docking device is shown in figure 1.3.3.a.

When the tubing is in place, valves 1, 2, 3 and 5 (see figure 1.3.a) are opened to allow steam to sterilize the outside of the tubing threaded through, the interior having already been sterilised in the autoclave. Once sterilised, a lever mounted on top of the cylinder is turned through 45°, cutting a hole through the inserted tubing (this is analagous to the action of opening and closing the tap on a glass burette), opening up a passage between the fermenter needle (inserted into the sampling port of the fermenter), through the sampling device, and out into the microcentrifuge, blocked off by valves when necessary.

When performing a fermentation, the tubing is "loaded" by threading it into the docking device, and it is subsequently sterilised just prior to the inoculation of the pre-sterilised fermenter.

1.3.4. Volume of sampling device and system

The volume of the sampling device is approximately 2 mL, and the time taken to pump a sample through it, using the inlet pump on the microcentrifuge, is approximately 20 seconds.

The total volume of the system, including sample tube in the fermenter, sampling device, and all tubing as far as the microcentrifuge bowl will obviously vary depending on length and internal diameter of tubing used. However, easily the largest volume is that of the sampling tube in the fermenter. To reduce this, a new sample tube was made from stainless steel of much smaller internal diameter. In addition the sample tube was shorter, and the end bent into a loop, so sample was removed from the middle of the fermenter, as opposed to just above the sparger, where the sample would be full of air. With the new sample tube, the total volume of the system may be reduced to less than 5 mL, which means that each representative sample removed from the fermenter may have a volume of less than 6 mL. This is an acceptable volume considering the large sample volumes generally taken when sampling manually.

A potential problem seen with the sampling device is if the distance between the fermenter and microcentrifuge is too great; the inlet pump on the microcentrifuge responsible for pumping the sample has problems removing the sample from the fermenter. This problem is exacerbated if the agitation speed and air flow into the fermenter are both high (as is often the case with *E. coli* fermentations). Hence the tubing between each piece of equipment should be as short as possible, also reducing sample size. This has been made possible in this case as a stainless steel three tier trolley has been manufactured for the equipment to stand on, and this enables the sampling device to stand within a few inches of the sample port the Chemap 14L fermenter used in this study, and the other equipment to be as close as possible to that. Figure 1.2.b. shows the relative position of fermenter, sampling device and trolley.

1.3.5. Operation of the Sampling Device

After connection to the fermenter and sterilisation of the docking device and tubing threaded through it, the sampling device is ready to be operated. A description of operation and programming is described in Section 1.6.; the operation routines for docking, sterilisation and sampling *etc.* are shown in figure 1.3.5.a., as well as the passage of steam or sample with each operation.

1.3.6. Problems with the sampling device

Because the sampling device described here is a prototype, there have been two problems during operation that could be addressed if another device is constructed:

Firstly, although the steam line is filtered, the filtration is not adequate, and the steam trap blocks up very quickly. Hence it was removed during the course of this project, and sterilisation occurred by free steaming. This did not cause any sterility problems during *E. coli* fermentations, however it could cause some difficulty with a more slow-growing organism. Hence mounting the steam trap outside the sampling device box is recommended as a solution; the trap could readily be removed and unblocked if required.

The second operating problem that has been encountered is the difficulty in ensuring an air tight seal when the fermenter is connected to the sampling device via the docking device. Tubing is threaded into the docking device, and then screwed in. The internal area is conically shaped, and it is believed that a seal would be easier to achieve if it was flat.

1.3.7. Sterility

The sampling device did not infect any of the *E. coli* fermentations, however such organisms are not readily contaminated because of their rapid growth. Fermentations of other organisms have not been performed with the device.

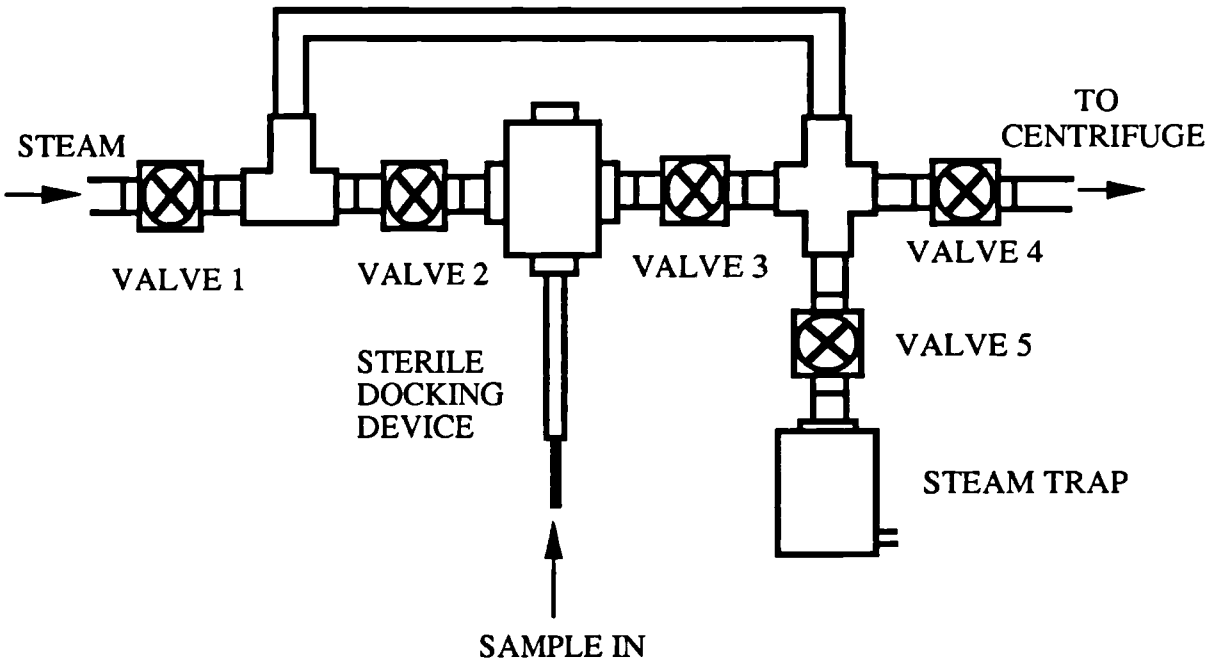


Fig. 1.3.a. Layout of valves, steam trap and docking device in the aseptic sampling device (valve solenoids not shown). An explanation of how it works is given in figure 1.3.5.a.

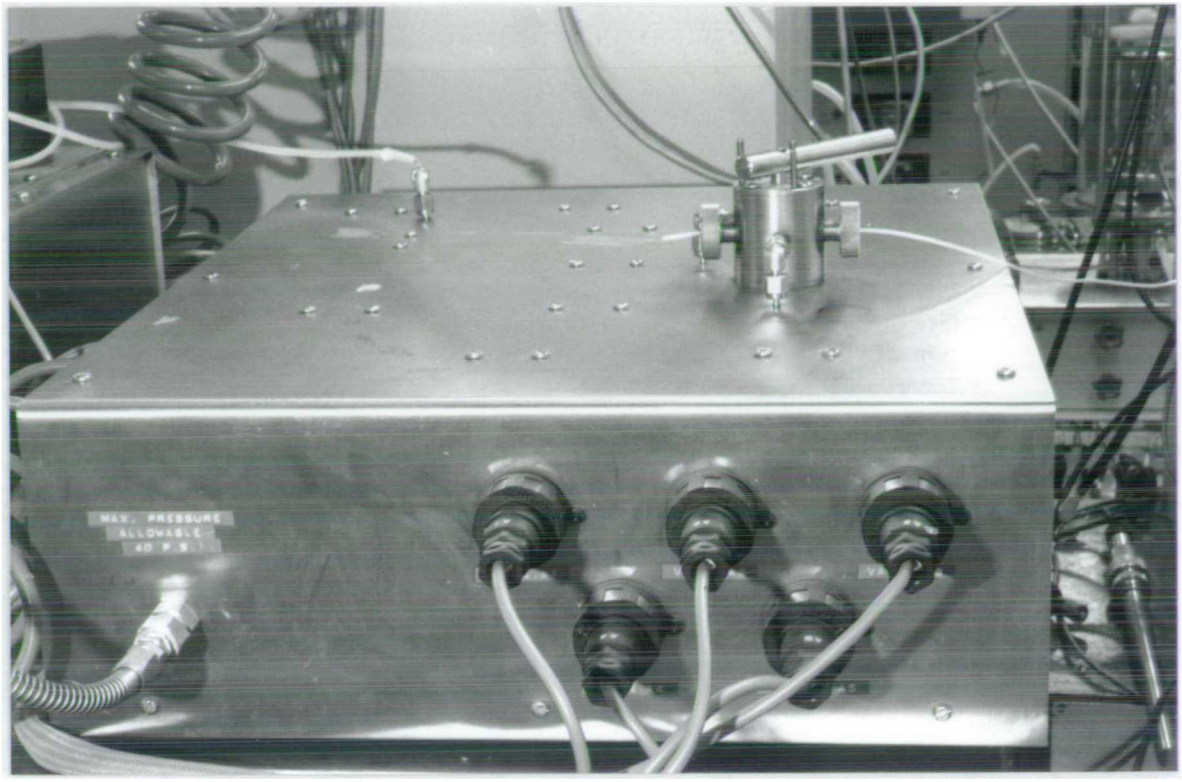


Fig. 1.3.b. Photograph of sampling device. On the vertical side of the unit, facing the camera, the five black connectors on the right link the PLC to the solenoids for each of the five valves; on the left, the air inlet can be seen. On the top of the box on the right, the docking device can be seen; on the left towards the rear is the sample outlet pipe. The steam enters and leaves the device through connections at the back (not shown).

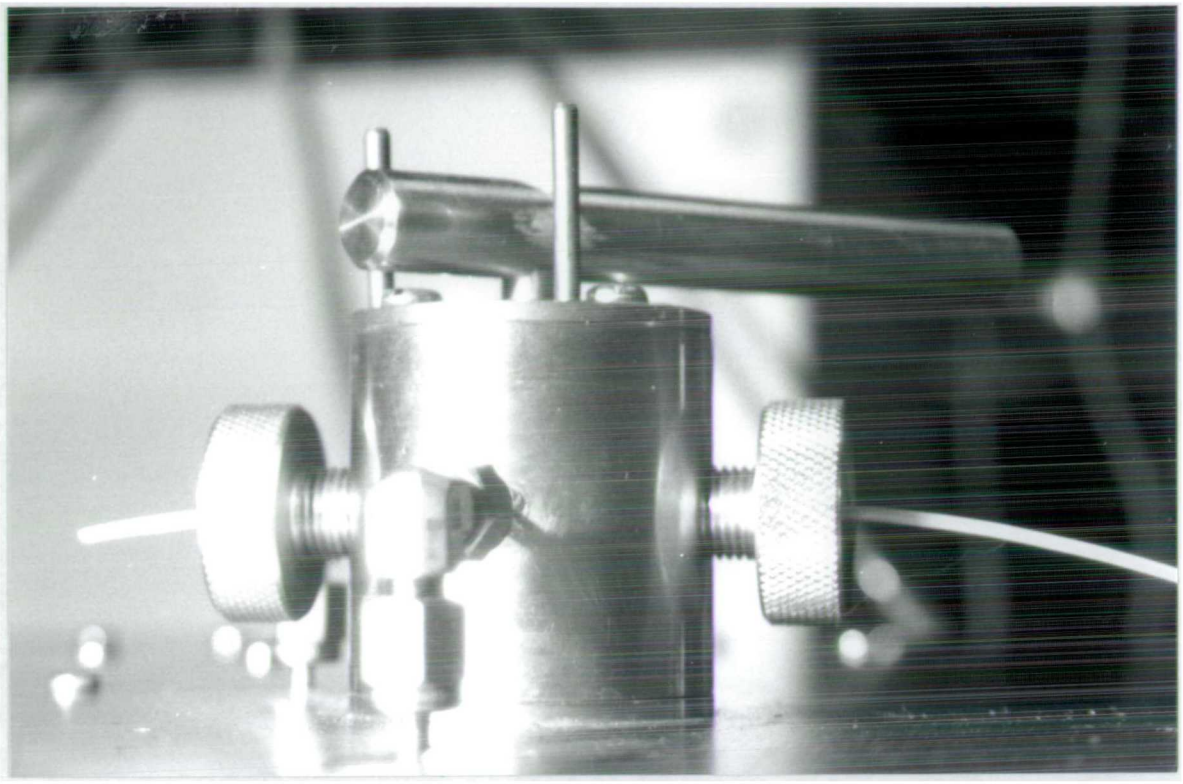


Fig. 1.3.3.a. Photograph of docking device (mounted on sampling device unit), for connection of sampling device to fermenter. On either side of the stainless steel cylinder, two screws are fixed. In the centre of the screws is a hole leading into the device, and through which the connecting tubing to the fermenter is threaded (the tubing is seen on the right). Once the tubing is threaded through one screw out the other screw, and the screws tightened, valves are opened for sterilising the outside of the tube. The lever on the top is then turned through 45° to cut the tube, and make a through passage from fermenter to sampling device.

OPERATION PERFORMED	V1	V2	V3	V4	V5	PASSAGE OF SAMPLE/STEAM
pre-docking tube sterilisation	O	O	O	C	O	STEAM→V1→V2→DD→V3→ ↓ V5→ST
docking of tube	C	C	C	C	C	no passage
pre-sampling sterilisation	O	C	C	C	O	→→→ ↑ ↓ STEAM→V1→ ↓ V5→ST
sampling from fermenter	C	C	O	O	C	DD→V3→V4→MC ↑ SAMPLE
post sampling sterilisation	O	C	C	C	O	as for pre-sampling sterilisation
all other times	C	C	C	C	C	no passage

Fig. 1.3.5.a. Operation routine for sampling device at different stages of use. The valves numbers shown correspond to those given in fig 1.3.a. Passage of sample/steam indicates valves and other components steam and sample pass through during use of sampling device. ST refers to steam trap; DD refers to docking device; MC refers to microcentrifuge. O indicates the valve is open and C indicates the valve is closed for that particular operation.

1.4. THE MICROCENTRIFUGE

The microcentrifuge is a purpose built piece of equipment for the separation of small volumes of samples of biological material. It was designed by Jim Molloy of Fisons UK specially for UCL for use in both this project for the separation of whole fermentation broth samples, and a project involving on-line fractional protein precipitation. This section describes the main features of the microcentrifuge, and how it is operated. The dependence of rotation speed on air pressure is given, and then follows a description of a series of experiments performed to determine how well the centrifuge separates suspensions of different cell types and concentrations. In addition, it describes some problems encountered during the preliminary experiments, and explains how they were solved. A brief summary and discussion of results concludes the section.

1.4.1. Description

1.4.1.1. External features

The air driven microcentrifuge is housed in a stainless steel box of dimensions 340 mm x 290 mm x 190 mm, and a photograph of the unit is shown in figure 1.4.1.a. Projecting from this box are a number of external features to provide inputs for air, power, computer control and speed control. In addition, a vacuum valve is mounting externally, as are 3 peristaltic pumps and the centrifuge bowl.

The air inlet (not shown in fig. 1.4.1.a.) is connected to an air compressor (Bambi model 225/1000) by a flexible hose. The unit is powered by standard domestic supply; there is an external 15 pin (7 x 8) male socket for parallel connection to a computer (or other logical device) for overall control of the microcentrifuge.

The microcentrifuge has been designed to provide fine speed control by rotating a metal valve clockwise to reduce the air supply, and thus speed; or counterclockwise to increase speed. However, this has been shown to be ineffective; speed control can only really be achieved by varying air pressure going into the device.

Not everything that enters the centrifuge bowl is removed as a sample. Before a sample comes from the fermenter, liquid in the lines needs to be removed, as does unwanted material adhering to the walls after centrifugation. It is therefore necessary to be able to remove unwanted waste material by means other than the sample out pump, and for this purpose, a vacuum valve has been designed; this is mounted externally on the top of the casing, and can be seen in figure 1.4.1.a. It consists of a metal cylinder containing a mobile plastic part fitted inside. There are two horizontal slits in the side of the plastic, and fitted through the top one is a piece of silicon tubing connected to a vacuum device. When the plastic component is in the "down" position (moved by means of a solenoid), the tube is crimped, and the vacuum is effectively "switched off". Raising the plastic part restores the vacuum.

The peristaltic pumps used are pumps are Watson Marlow 303D pumps, the pump heads being mounted externally. There are three pumps: one for pumping the sample into the centrifuge bowl, one to remove the supernatant after centrifugation, and the third to pump in wash solution. The pump adjacent to the vacuum valve runs at 50 rpm and is used for sample removal in this study; the other two run at 100 rpm.

The bowl of the centrifuge is shown in figure 1.4.1.b. It is mounted on a platform composed of DELRIN (a polyacetal resin), itself mounted on a rubber seal. In the front side of the mounting is a small hole containing the friction brake adjustment screw (not shown in photograph). The bowl is mounted in the centre of the platform, and immediately coming away from the bowl is a tapered slit cut into the DELRIN platform to allow excess liquid to drain away, should the bowl be overfilled. The freely rotating bowl is composed of stainless steel of external diameter 25 mm, and sits on a stainless steel base. The stainless steel bowl mounting has 3 lugs mounted equilaterally around it for attachment of the bowl head.

The bowl head has a roughly octagonally shaped DELRIN exterior, shown in figure 1.4.1.c.; and a metallic interior (shown in figure 1.4.1.d.); the two being

separated on the underside by an O-ring. The inlet, outlet and vacuum pump lines are attached to the top via plastic hexagonal nuts with screwed in fittings. On the interior of the bowl head, the distance the metal tubes extend is crucial. The vacuum and sample outlet pipes must be adjusted so that when the head is attached to the bowl, the metal tubing is as close to the bottom of the bowl as possible, without actually touching it, enabling maximum volume to be removed. The wash pipes and sample inlet pipe should be positioned so that they are not touching liquid when the bowl is filled. This is to reduce the amount of unspun sample adhering to the pipes which will mix with the clear supernatant after centrifugation, causing turbidity (this problem will be discussed later in greater detail). A diagrammatic representation of the bowl showing inlet and outlet pipes and other connections is shown in Fig. 1.4.1.e.

On the inside of the bowl head three slits are cut into the metal area for attachment of the cover onto the corresponding lugs on the centrifuge bowl base. The roof on the inside of the cover tapers, and is not circular, but is cut away so that the wash pump tubing is able to enter on the side. The tubes enter from the non-metallic part at the very top of the bowl head cover.

1.4.1.2. Internal features

Air enters the microcentrifuge at the external air inlet connector, and once inside the box, passes through a T-piece to separate the stream into two. Each stream passes through a solenoid valve, but in addition, one stream passes through the adjustable speed control device. Two metal plates secure the air tubes and fittings mounted onto the inside of the metal casing. The air streams then join up and pass through a length of flexible air hosing to reach the air turbine, which drives the centrifuge bowl. The air turbine is a commercially available turbine grinder, model 7980-A, manufactured by ARO (Redditch, Worcestershire). The only modification made to the grinder is the placement of the microcentrifuge where the chuck for the grinder had ben positioned. It is rated for a maximum operating speed of 85 000 rpm (although in practice this was never attained), with a maximum air pressure of 90 psi. The turbine is mounted internally on a DELRIN block of dimensions 50 mm x 60 mm x 30 mm.

A friction brake is mounted onto the microcentrifuge shaft, and is controlled by a solenoid operating at 12 V DC. A switch is released which pushes out a metal strip with a piece of TUFNOL attached at one end to act as the friction brake. This makes contact with the base of the bowl, slowing it down. The brake solenoid has been set up so that when it is off, the brake is in an "on" position. Hence when the microcentrifuge is in operation, the solenoid should be switched on to move the brake away from the base of the bowl.

1.4.1.3. Electrical components

The right hand third of the box contains the electrical components. A TTL digital interface receives a 0-5 Volt signal from the computer and sends it to an RS 632-102 board which, with a domestic power input, converts it to 12V DC signals for the centrifuge break solenoid and vacuum pump to operate, and 240 V AC signals for the pumps and air valve solenoids to operate. The step down to the 12V DC signal requires the signal to be stepped down with a transformer, and then through to another board to be converted to DC.

1.4.2 Method for operation of microcentrifuge

1.4.2.1. General operation

The inlet pump mounted on the top of the centrifuge pumps a sample into the centrifuge bowl. In order to obtain a representative sample, the first few mLs of sample (equivalent to the volume of line between fermenter and centrifuge bowl) is removed by the vacuum device (attached to a mains water tap) through the pipe in the bowl head, to waste. The vacuum is then switched off, and the bowl is filled. The maximum amount of sample that can be spun in the centrifuge without losing sample out the top is 0.54 mL. The valves in the centrifuge attached to the air supply are then opened, and the bowl is spun round at high speed. When the valves are closed, the bowl slows down, and comes to a stop in a time period dependent on whether the brake is used or not; the sample supernatant may then be pumped out. The solid material may then be washed off the walls of the bowl using a high pressure jet of liquid pumped through the

small internal diameter wash tubes using the wash pump. The best results are obtained by rotating for a fraction of a second when pumping the wash solution in. The cellular material may then be removed to waste using the vacuum valve.

Another problem that has not been addressed in this piece of work is the issue of containment. The microcentrifuge generates aerosols, which is obviously highly undesirable especially if recombinant organisms are used. For the duration of this project, this was dealt with by using cotton wool and industrial methylated spirits. What really is required is for the centrifuge to be contained within a sealed box in which all air inlets and outlets are protected by microbial filters.

1.4.2.2. Rotation speed of microcentrifuge

It is possible to measure the rotation speed of the microcentrifuge. Two holes have been drilled in the top of the centrifuge turbine that "line up" twice per revolution. This enables speed of rotation to be measured optically using an LED, photodiode and oscilloscope.

Rotation speed of the microcentrifuge at different air pressures has been measured by Holwill and Chard (1992), and the results are shown in Figure 1.4.2.a.

Maximum speed at 90 psi has been measured as 68 630 rpm. Using the equation,
centrifugal field, $g = 1118 \times 10^{-8} R N^2$,
where R = radius of bowl in cm (1.05 cm)
and N = revolutions per minute,
g force pulled by centrifuge at maximum speed is: 55 300

1.4.3. Measurement of separation efficiency of microcentrifuge

The function of the microcentrifuge is to separate solid and liquid components of fermentation broth. In order for it to be of any use in the application of on-line HPLC analysis, it is necessary for as many solids to be removed as possible, or rapid fouling of the HPLC column will result. Hence experiments were

performed to discover the best possible separation conditions for both *Escherichia coli* and *Saccharomyces cerevisiae*.

1.4.3.1. Materials and Methods

It was impractical to perform these experiments on real fermentations. Instead, fresh cell paste was used and resuspended in phosphate buffered saline (0.1 M Na_2HPO_4, 0.9% NaCl, pH 6.9) to a number of different optical densities, measured at 600 nm on a PYE UNICAM SP6-250 visible spectrophotometer.

S. cerevisiae was obtained as packed yeast from DCL. Cell samples of *E. coli* strain K12 used in the centrifuge characterisation experiments were obtained by fermentation in a Chemap 14 L fermenter using the following medium ($g.L^{-1}$): lactose, 30; $MgSO_4.7H_2O$, 0.2; yeast extract, 1; $(NH_4)_2SO_4$, 10; NaCl, 5; Na_2HPO_4, 2.16; KH_2PO_4, 0.64; $FeSO_4.7H_2O$, 0.2; citric acid (anhydrous), 0.2; $CaCl_2.2H_2O$, 0.01; H_3BO_3, 0.004; $MnCl_4.4H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.0004; $CoCl_2.6H_2O$, 0.0004 and $NaMoO_4.2H_2O$, 0.0002. All chemicals were obtained from SIGMA, except yeast extract, which was obtained from DIFCO. $MgSO_4.7H_2O$ and lactose were autoclaved separately and added just after inoculation of the fermenter.

The cells were harvested when the lactose was exhausted and concentrated in the Sharples tubular bowl 1P centrifuge to produce a thick cell paste.

The initial experiments were performed by removing all pipes from the centrifuge bowl head, by manually injecting 0.5 mL of cell sample at a number of different optical densities (A_{600}) and hence cell concentrations into the bowl and centrifuging it under different conditions. The supernatant was then manually removed by syringe, and the absorbances were read at 600 nm. Similar experiments were performed for cell samples loaded into the microcentrifuge bowl automatically, with all pipes screwed into the bowl head.

1.4.3.2. Results

Figs 1.4.3.a.1-4 show the separation of *E.coli* at conditions of varying turbine air

pressure (and hence spin speed) and duration of pressure applied for samples loaded and removed manually. Figs 1.4.3.b.1-4 show the separation of *S. cerevisiae* under similar conditions. Separations achieved for *E.coli* were fairly good, however, results were much poorer for *S. cerevisiae*, particularly at intermediate cell concentrations.

Figs. 1.4.3.c.1-3 (*E. coli*) and 1.4.3.d.1-3 (*S. cerevisiae*) where samples were added and supernatants removed automatically show that the supernatant is much more turbid than when samples were loaded and removed manually. A number of experiments led to the cause of this problem. The sample out pipe, and vacuum to waste pipe need to be as close to the bottom of the bowl as possible, and will be submerged in the bowl when the sample is pumped in. As a result, droplets of fermentation broth were adhering to the pipes when the sample is flung against the side of the bowl during centrifugation. When the sample had been spun, and the bowl had come to rest, the droplets of broth that had adhered to the pipes mixed with the clear supernatant to form a turbid solution. If the volume of supernatant is 0.5 mL, then a series of drops of total volume 50 µl would cause the final sample to have an OD of at least 10% of the original sample. This is clearly unacceptable.

Attempts were made to solve this problem by coating the pipes with substances to prevent the adherence of broth. Firstly, the pipes were dipped in a silicone compound in heptane from Sigma called Sigmacote. Three coats were applied, drying in between each. This had some effect for a short time, but the coating became waterlogged very quickly. Subsequently, the outside surfaces of the pipes were highly polished to reduce surface features, and then coated several times over a period of 3 days (drying between each coat) with a 5% solution of dipalmitoyl phoshatidyl choline (from SIGMA) in chloroform (from BDH). Again, this had some effect although the supernatants were still unacceptably turbid.

A further reduction in turbidity of the supernatant was obtained by switching on the vacuum valve while the bowl was spinning, to remove broth from inside and

at the end of the vacuum pipe, the largest in the bowl head. However, this still did not provide a suitably clear supernatant, so it was decided to spin the bowl twice, allowing it to come to rest between the spins so the broth on the pipes could be resuspended. The second spin would then remove the majority of the solids. Figs 1.4.3.e.1-3 and 1.4.3.f.1-3 for *E. coli* and *S. cerevisiae* show that this provided a good solution; post spin supernatants were much better than those obtained with a single spin for both *S. cerevisiae* and *E. coli*. Dual spin results were similar to those obtained by manually adding and removing samples, as would be expected.

1.4.3.3. The use of the brake

The microcentrifuge friction brake can control the rate at which the bowl deccelerates, and has been used in a number of experiments with both *E. coli* and *S. cerevisiae* cell suspensions, to determine whether its use has any effect on the final supernatant OD.

Fig. 1.4.3.g.1. presents data demonstrating the effect of a 15 second brake on *E. coli cell* samples centrifuged under different conditions, while fig. 1.4.3.g.2. presents data showing the effect of time for the bowl to come to rest using the brake, on the post spin OD. Figs. 1.4.3.h.1. and 1.4.3.h.2. present similar data for *S. cerevisiae*. As can be observed, the separation achieved using the brake is very poor, probably because of vibrations caused by the friction brake acting to resuspend the separated broth.

A problem encountered with the brake was the difficulty in setting it such that the degree of braking power set remains consistent. The reason for this is the difficulty in keeping the brake adjustment screw in a fixed position. When the bowl is spinning and the brake is acting, the screw works loose from its set position, altering the braking power. Much of the data presented was obtained by locking the adjustment screw with a screwdriver while the bowl is spinning. Hence, if the brake is to be used, some modification will be required.

1.4.3.4. Comparison of microcentrifuge with Denley microcentrifuge

The separation efficiency of the microcentrifuge should be compared with the separation efficiency of a commercially available small scale laboratory microcentrifuge. The eparation of 1.5 mL samples of *E. coli* and *S. cerevisiae* in a Denley microcentrifuge, model BM 402, spun for 8 minutes at 10 000 rpm (equivalent to approximately 7500 g) is shown in Fig. 1.4.3.j. This is compared to the best separations obtained with a dual spin in the microcentrifuge (Figs. 1.4.3.e.1. and 1.4.3.f.1.). The comparison clearly shows that the Denley provides clearer supernatants. This could be because of the longer spin time, however, with the high g force of the microcentrifuge, that is not thought to be the reason. Instead, it is believed to be as a result of the contact area between solid and supernatant. In the microcentrifuge, the area to volume ratio is large because the sample is spread in a thin layer around the walls of the bowl. In contrast, the Denley concentrates solids to a small volume, with a very low area to volume ratio, at the bottom of an Eppendorf tube. This provides less area for resuspension.

1.4.3.5. Comparison of microcentrifuge with filtration

The microcentrifuge has been compared to filtration. Samples of fresh *S. cerevisiae* suspended in phosphate buffered saline (PBS) to provide samples of different cell concentrations were filtered through 47 mm Millipore GVWP 0.2 μm pore size filters, and the A_{600} of each filtrate was measured. Fig. 1.4.3.k. shows results of this, compared to the best dual spin results for the microcentrifuge with *S. cerevisiae* samples.

1.4.4. Summary and discussion of microcentrifuge separation efficiency

Results have been presented showing the ability of the microcentrifuge to separate samples of *E. coli* and *S. cerevisiae* suspended in PBS. The cell concentrations chosen were designed to emulate typical concentrations that would be obtained throughout the course of a fermentation. The A_{600} optical densities used were related to dry weight (measured by filtering a known volume of sample through a pre-weighed 47 mm diameter Millipore GVWP 0.2 μm pore size filter,

heating for 100° C oven for twenty four hours and re-weighing), and the relationship between OD and dry weight is shown in Fig. 1.4.4.a.

The choice of the two cell types was based on the fact that these are two frequently used organisms in fermentation, and they are of different cell shape; *E. coli* cells being rod shaped, while *S. cerevisiae* cells are spherical. Preliminary studies were performed on filamentous *Saccharopolyspora erythraea* (reclassified from *Streptomyces erythraeus*) cell samples, but results (not presented here) indicate that separation of the fermentation broth of this organism is more difficult, and will probably require a much longer spin time.

As may be inferred from the results presented, supernatants of *E. coli* cell samples were clearer than equivalent samples of *S. cerevisiae*. The reason for this has not been established with certainty, but it is likely that the rod-shaped *E. coli* cells are better able to pack against the centrifuge bowl wall than are the *S. cerevisiae* cells, so are less likely to be resuspended when the centrifuge is slowing down. In general, supernatants of *S. cerevisiae* samples of the higher initial cell concentrations are less turbid than those of intermediate cell concentrations, and proportionately more solids are removed with large initial cell concentrations. It is therefore possible that they pack better against each other than against the cell wall, and this improves the clarity of the supernatant.

For *E. coli* cell samples, short spin times and a low air turbine pressure of 45 psi corresponding to a spin speed of about 52 000 rpm (lower pressures than this could not be used because of the requirement of the sampling device for a minimum pressure of 45 psi to open the valves) are the optimal conditions for separation. Separation occurs very quickly, and spinning for a longer time at a faster speed appears to resuspend some cells. Results for *S. cerevisiae* samples follow that tendency, but are not quite so clear cut.

In comparison with filtration and a commercial off-line microcentrifuge, the Denley, the separated solutions contain a higher percentage of solids. While the clearer the supernatant the better, later results (see section 1.5) show that

supernatants of *E. coli* samples are acceptably clear for HPLC analysis. Yeast samples may cause a problem; on-line HPLC has not been performed on *S. cerevisiae* fermentations during this project.

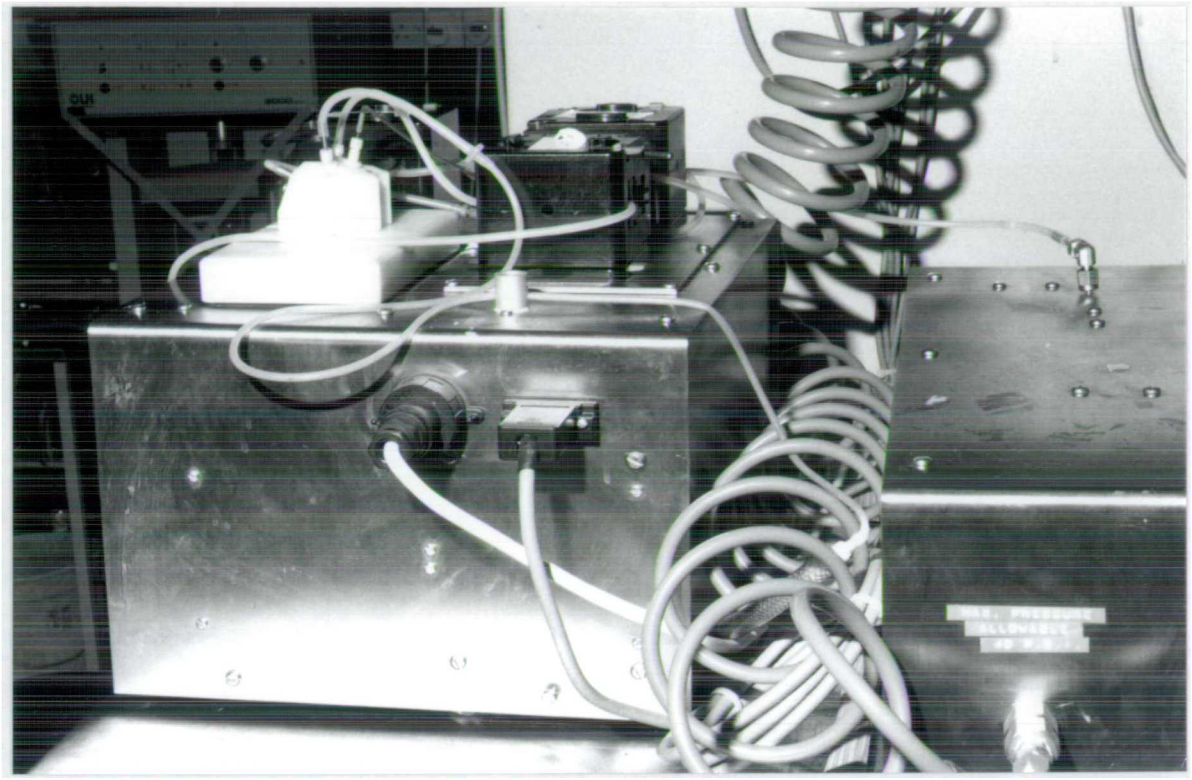


Fig. 1.4.1.a. Photograph of microcentrifuge seen from the side. On the right of the picture, the connection to the sampling device can be seen. At the left, the bowl cover is visble, on top of the Delrin mounting. On the side panel facing the photograph is the power supply connection on the left, and the link to the PLC on the right. The three black units on the top of the centrifuge are the pump heads; the vacuum valve is the small cylindical device in front of the nearest pump.

Fig. 1.4.1.b. Photograph of centrifuge bowl mounted on Delrin platform. Two of the metal lugs on the bowl base for attachment of bowl head are clearly visible. A two pence piece is included in the picture for scale.

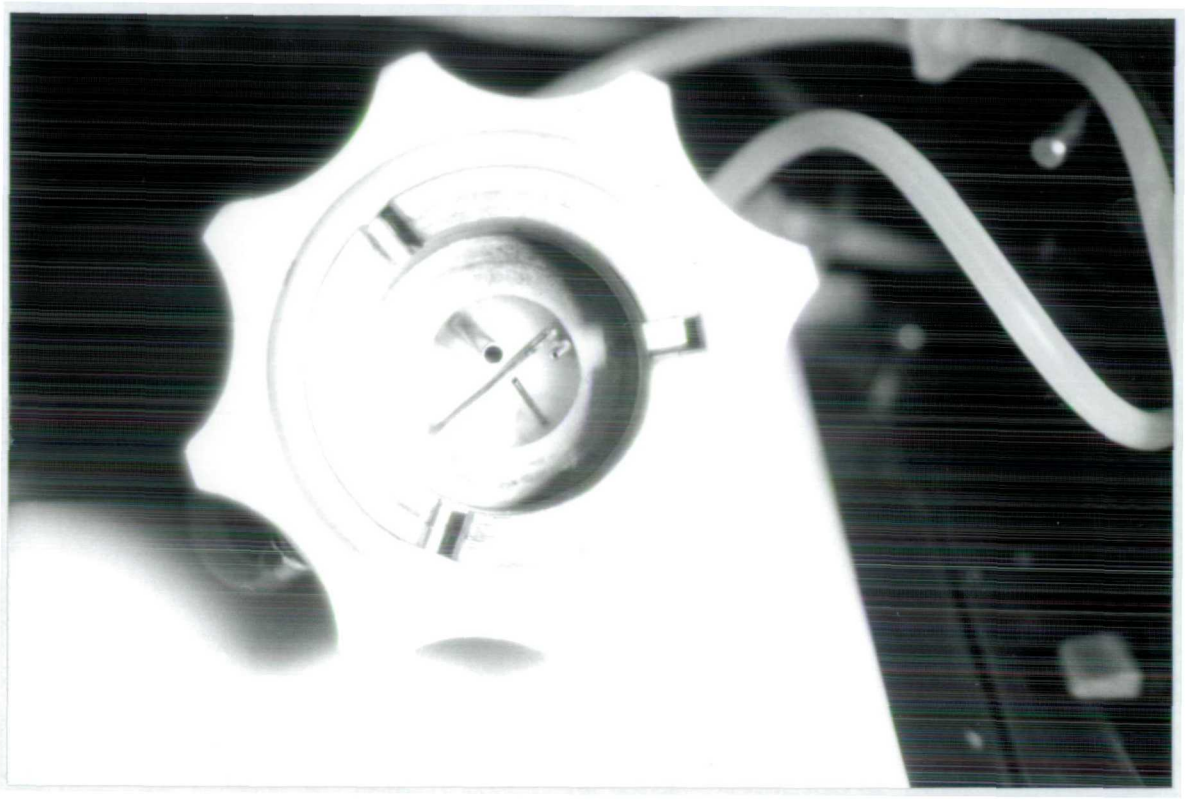


Fig. 1.4.1.c. Photograph of interior of microcentrifuge bowl head showing (clockwise from right) inlet, outlet, and vacuum pipes. Wash pipes are visible going across bowl head, from left to right.

Fig. 1.4.1.d. Photgraph of exterior of microcentrifuge bowl head, showing (clockwise from left) wash pipe, outlet pipe, inlet pipe and vacuum pipe connections.

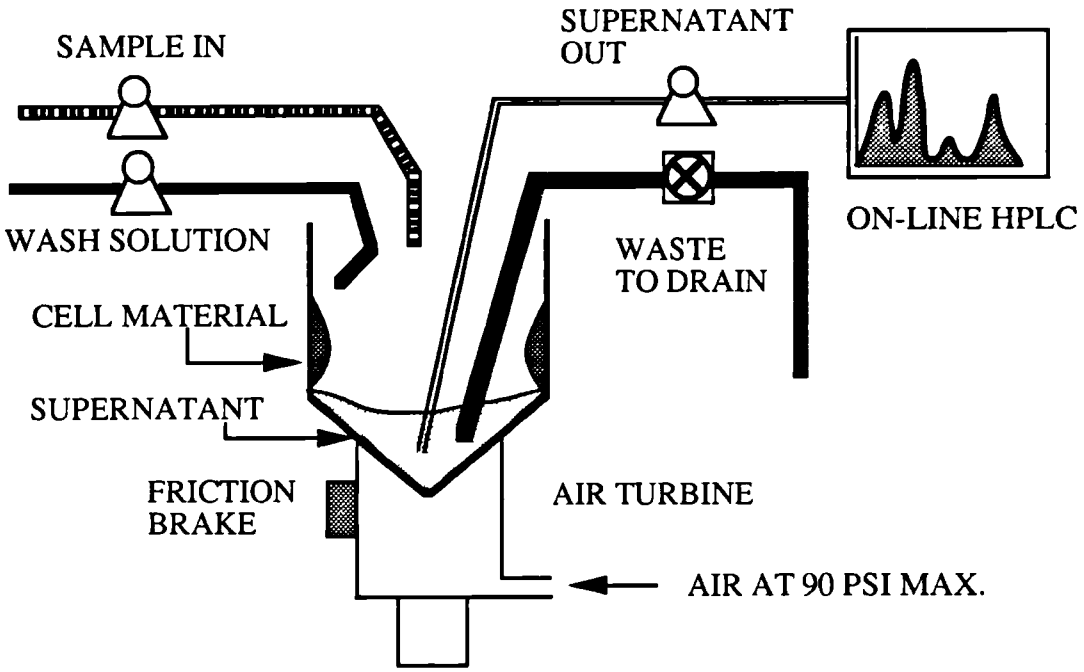


Fig. 1.4.1.e. Diagrammatic representation of microcentrifuge bowl showing inlet and outlet pipes. The sample is pumped in, the centrifuge spun, and the supernatant removed for analysis. The cells and any other solid material collect on the side of the bowl. The high pressure wash jet dislodges the solids which can then be vacuumed to waste.
As can be seen , the sample outlet pipe and vacuum pipes are below the liquid level in order to remove sample supernatant and waste.

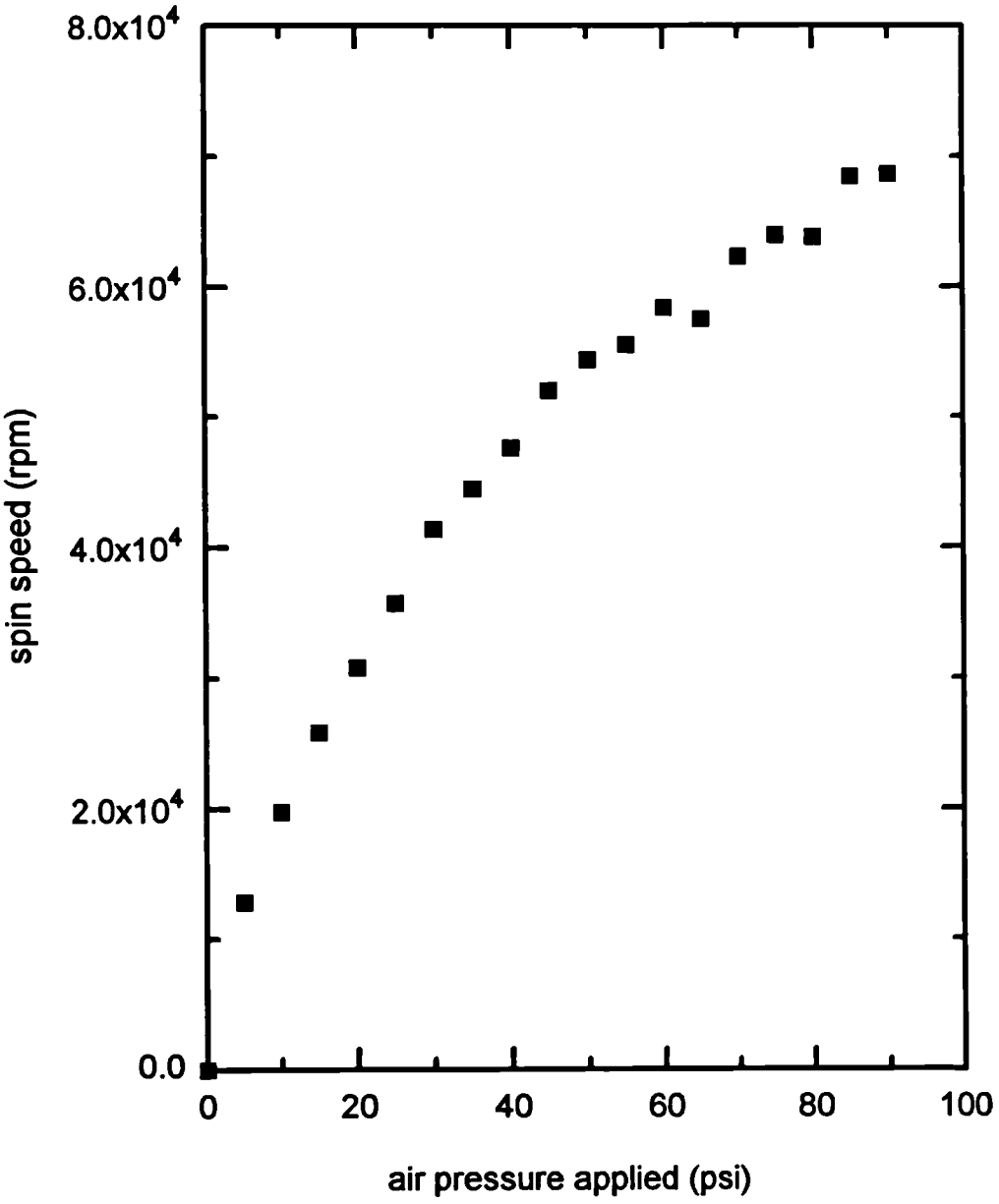


Fig. 1.4.2.a. Relationship between air pressure applied to microcentrifuge air turbine, and speed of rotation of bowl.

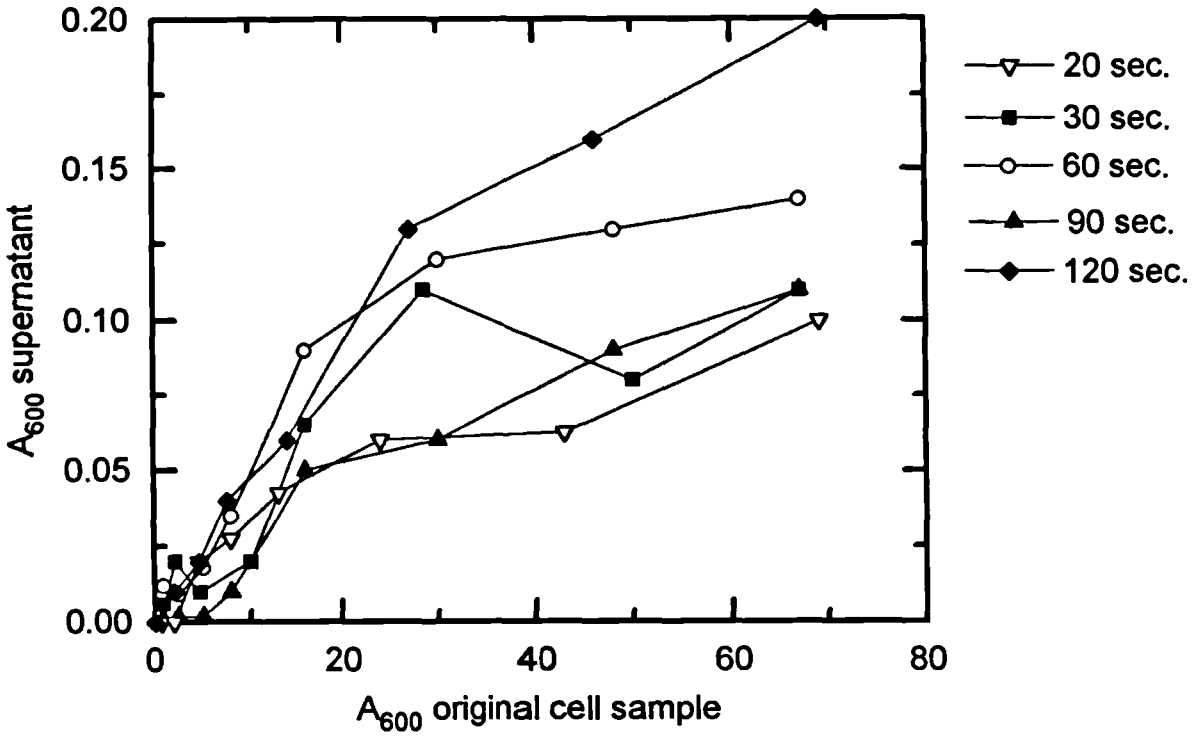


Fig. 1.4.3.a.1. Separation efficiency of microcentrifuge at 45 psi, different spin times with different concentrations of *E. coli*. Samples were loaded and removed manually.

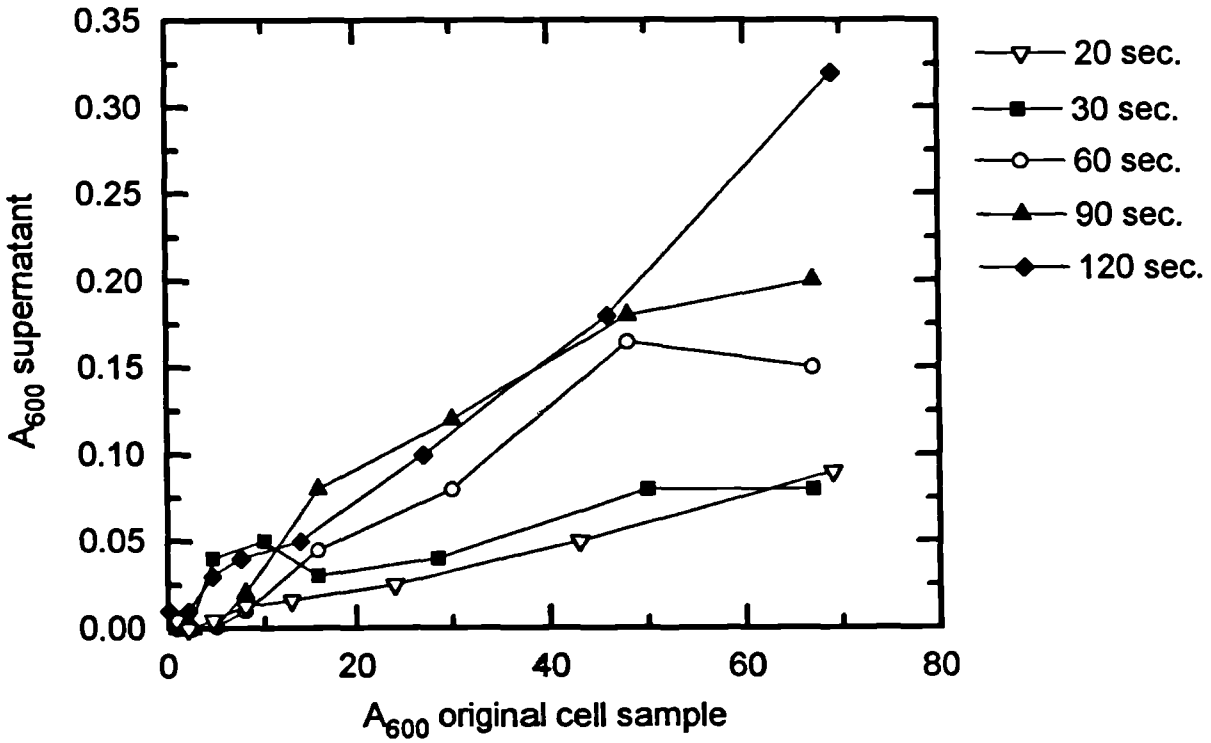


Fig. 1.4.3 a.2. Separation efficiency of microcentrifuge at 70 psi, different spin times with different concentrations of *E. coli*. Samples were loaded and removed manually.

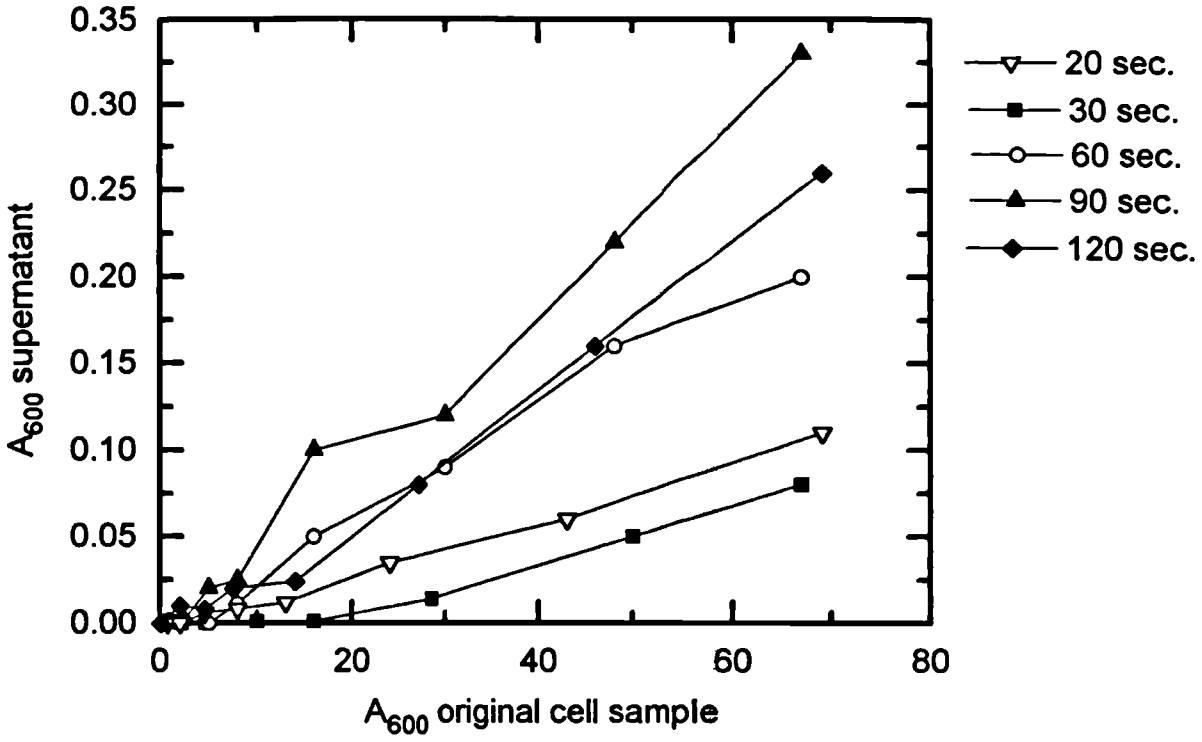


Fig. 1.4.3.a.3. Separation efficiency of microcentrifuge at 90 psi, different spin times with different concentrations of *E. coli*. Samples were loaded and removed manually.

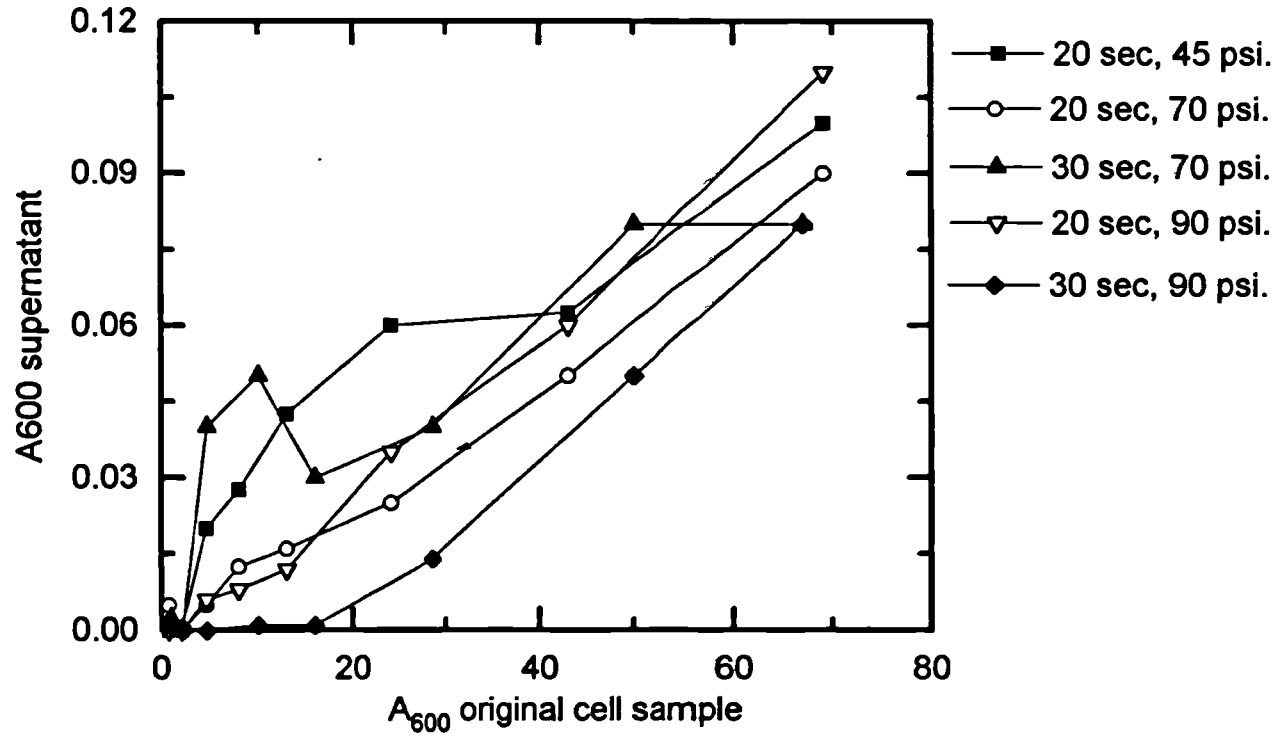


Fig. 1.4.3.a.4. Separation efficiency of microcentrifuge at 45, 70 and 90 psi; best spin times with different concentrations of *E. coli*. Results show that short spin times are best, with low air pressures. Samples were loaded and removed manually.

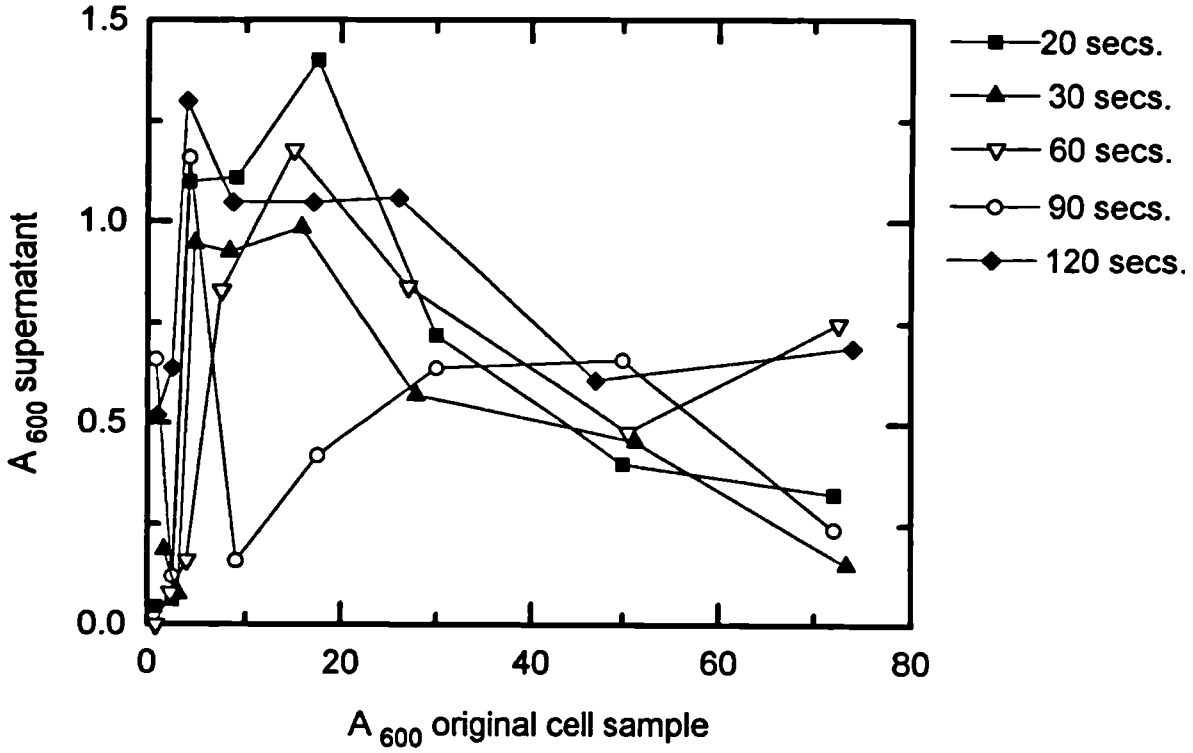


Fig. 1.4.3.b.1. Separation efficiency of microcentrifuge at 45 psi, different spin times with different concentrations of *S. cerevisiae*. Samples were loaded and removed manually.

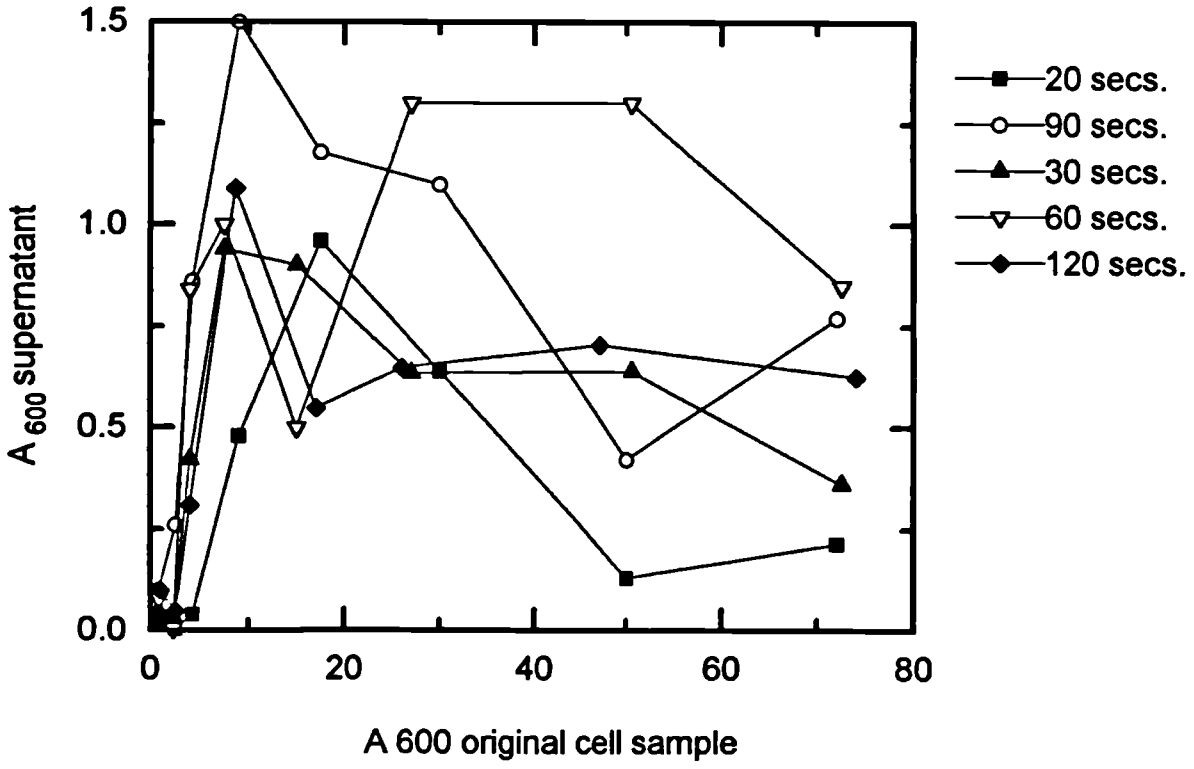


Fig. 1.4.3.b.2. Separation efficiency of microcentrifuge at 70 psi, different spin times with different concentrations of *S. cerevisiae*. Samples were loaded and removed manually.

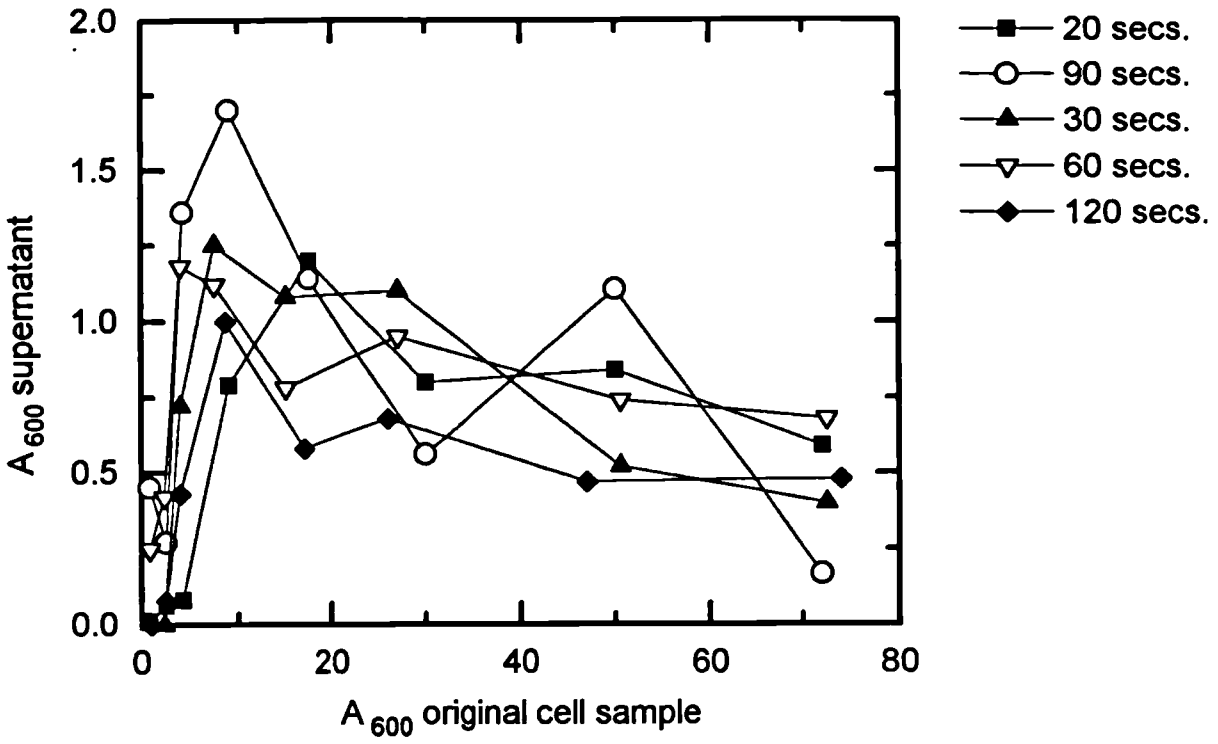


Fig. 1.4.3.b.3. Separation efficiency of microcentrifuge at 90 psi, different spin times with different concentrations of *S. cerevisiae*. Samples were loaded and removed manually.

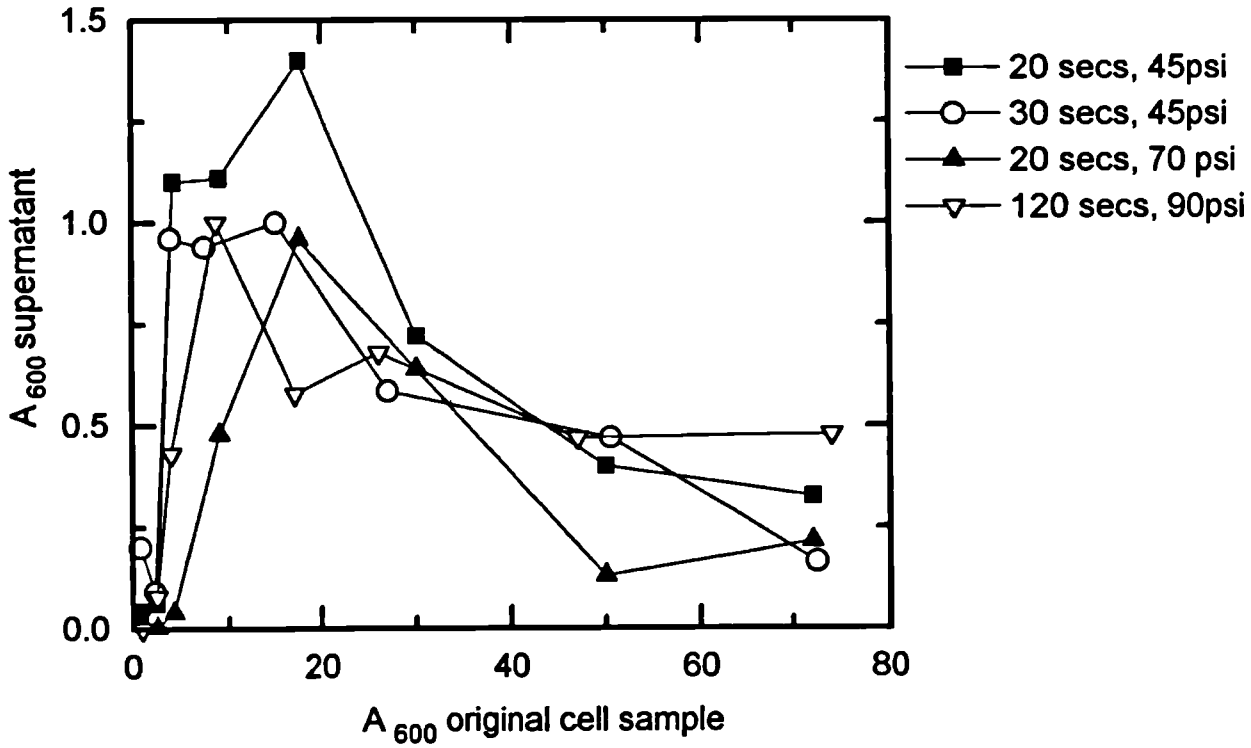


Fig. 1.4.3.b.4. Separation efficiency of microcentrifuge at 45, 70 and 90 psi; best spin times with different concentrations of *S. cerevisiae*. Results show that short spin times and low air pressures are best. Samples were loaded and removed manually.

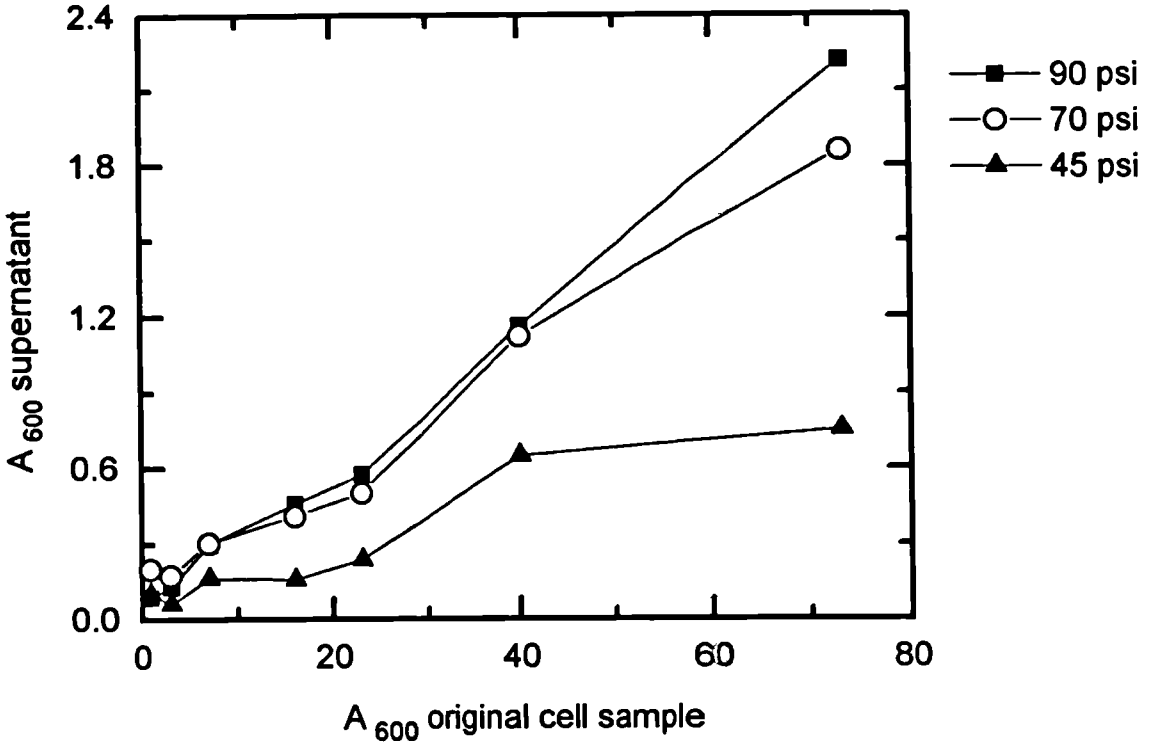


Fig. 1.4.3.c.1. Separation efficiency of microcentrifuge with 20 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of *E. coli*.

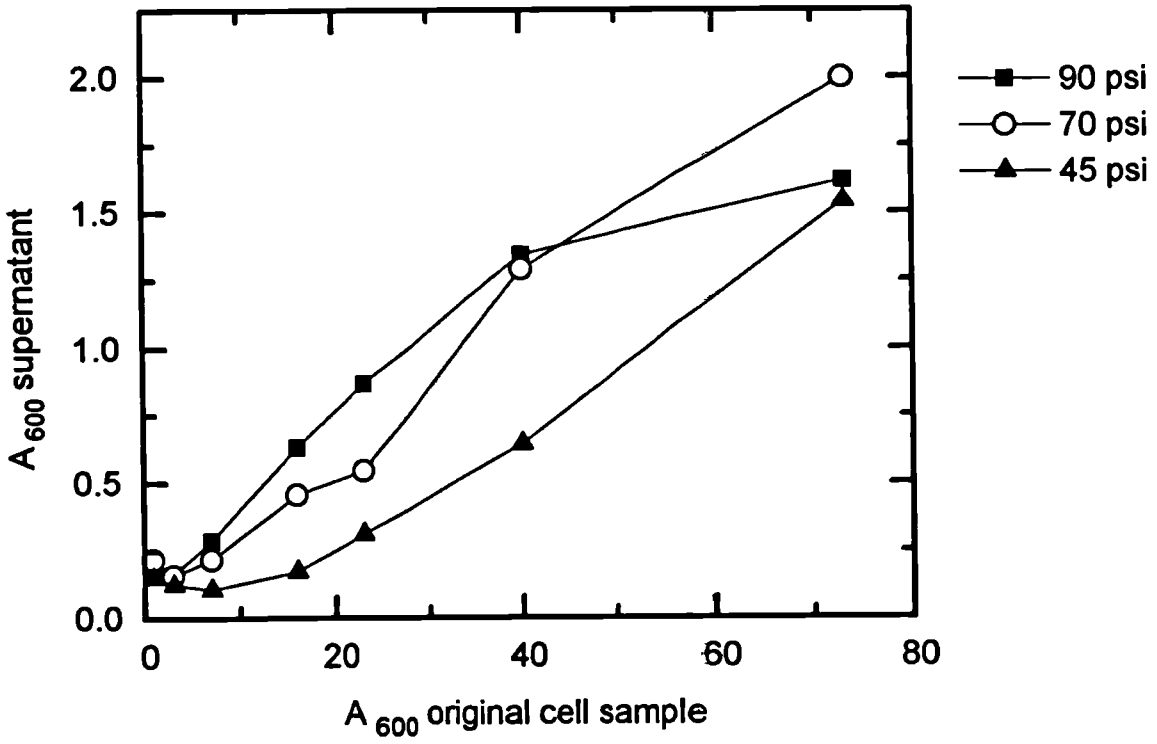


Fig. 1.4.3.c.2. Separation efficiency of microcentrifuge with 60 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of *E. coli*.

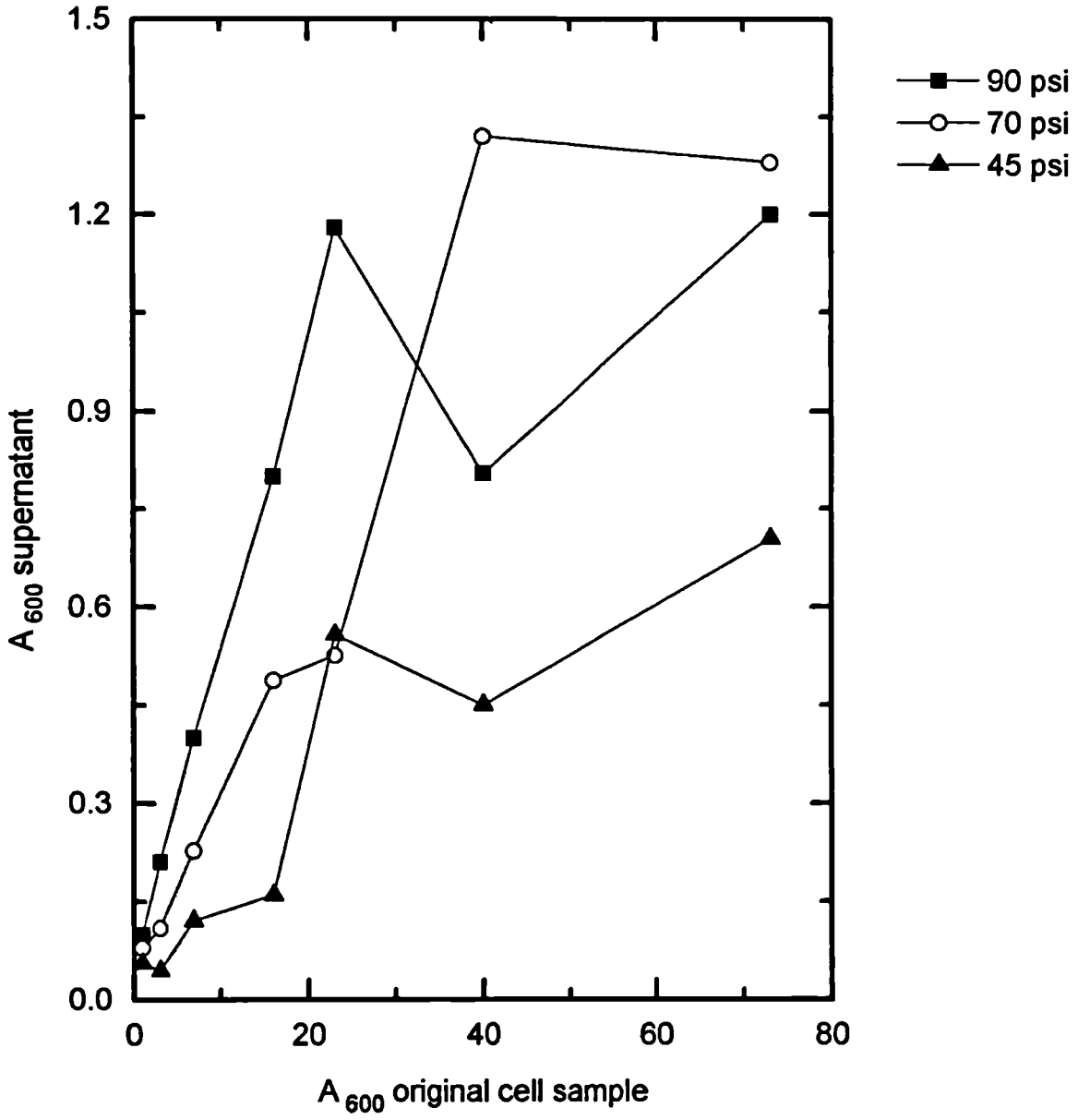


Fig. 1.4.3.c.3. Separation efficiency of microcentrifuge with 90 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of *E. coli*.

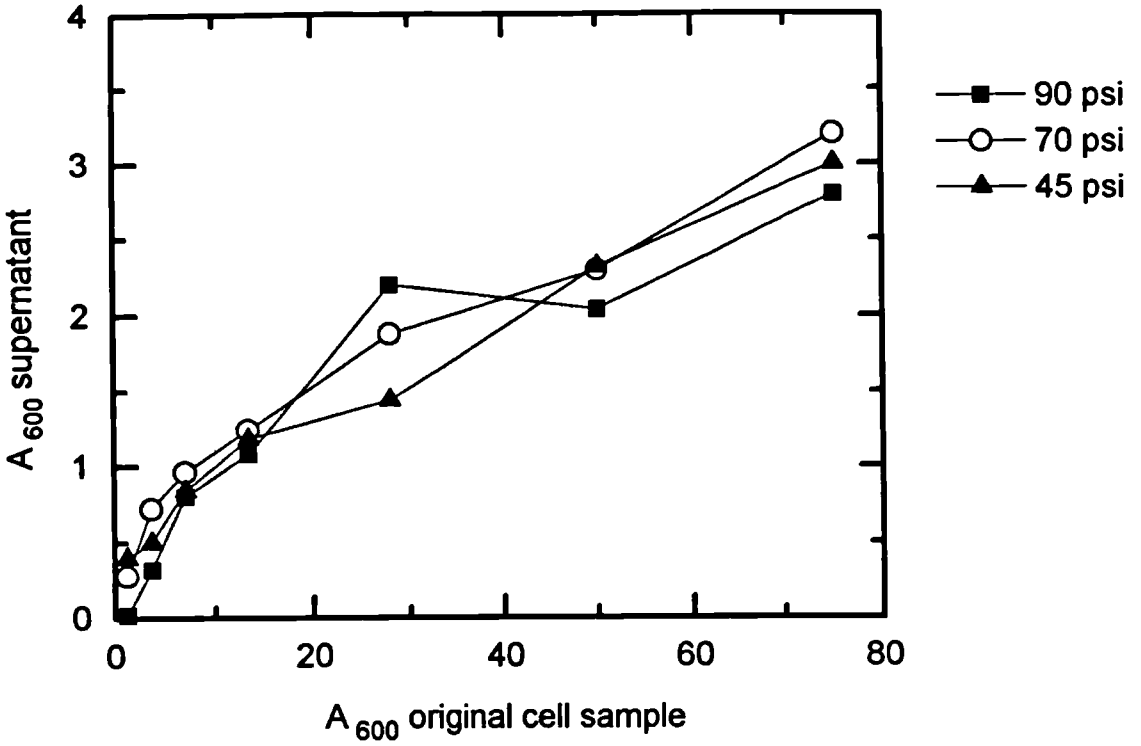


Fig. 1.4.3.d.1. Separation efficiency of microcentrifuge with 20 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of *S. cerevisiae*.

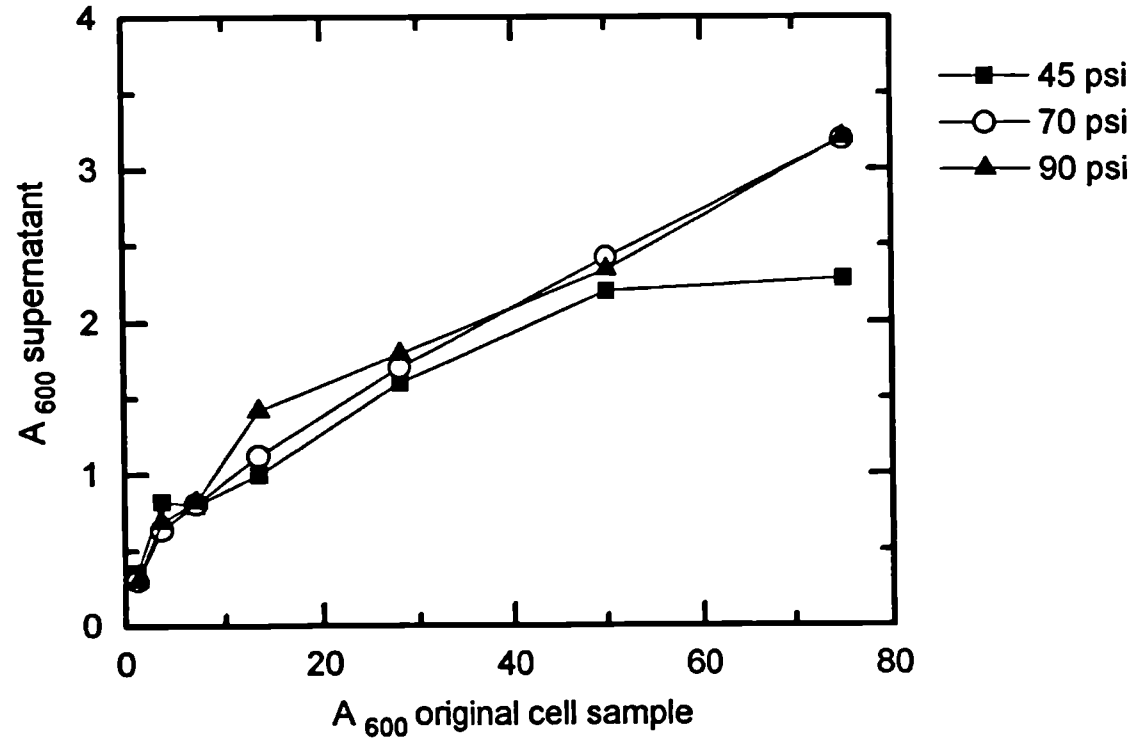


Fig. 1.4.3.d.2. Separation efficiency of microcentrifuge with 60 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of. *S. cerevisiae*.

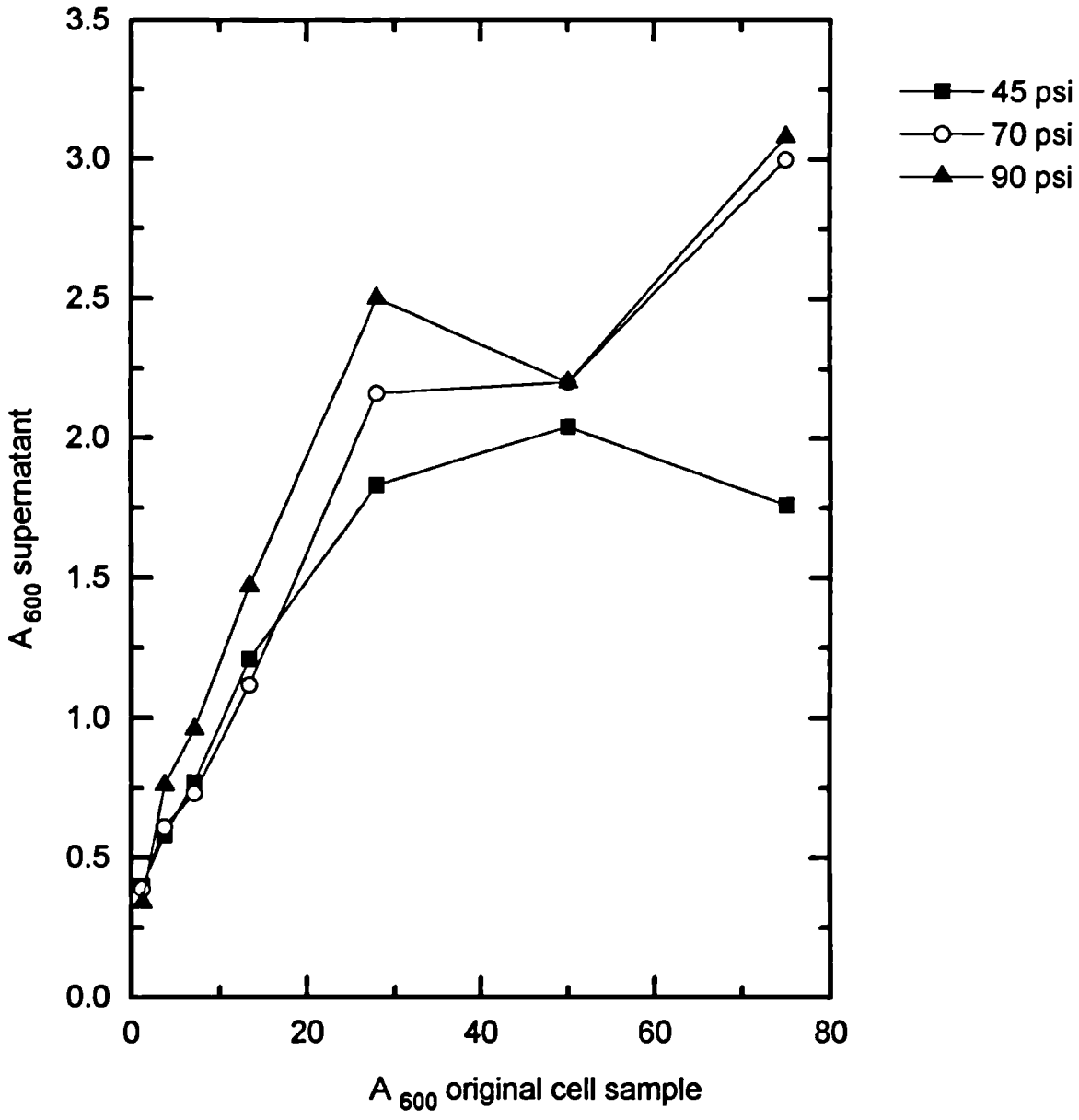


Fig. 1.4.3.d.3. Separation efficiency of the microcentrifuge with 90 sec. spin at different air turbine pressures with automatic sample loading of different concentrations of *S. cerevisiae*.

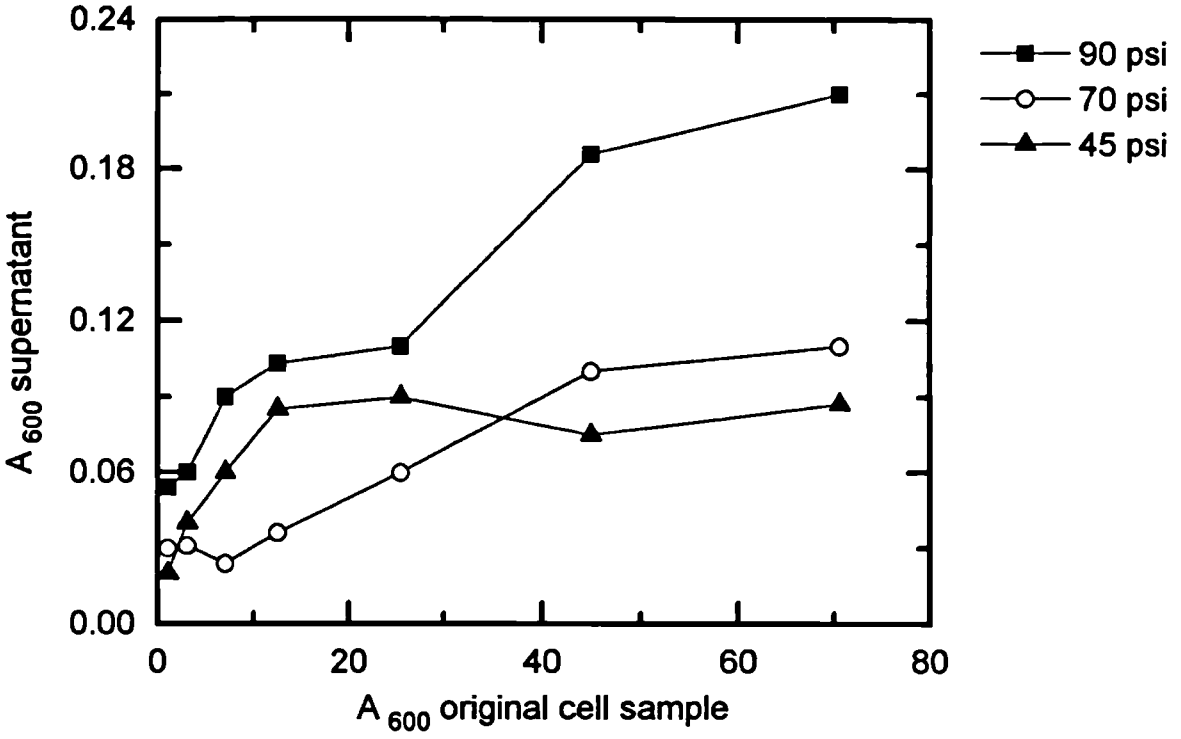


Fig. 1.4.3.e.1. Separation efficiency of microcentrifuge with two 20 sec. spins at different air turbine pressures with automatic sample loading of different concentrations of *E. coli*.

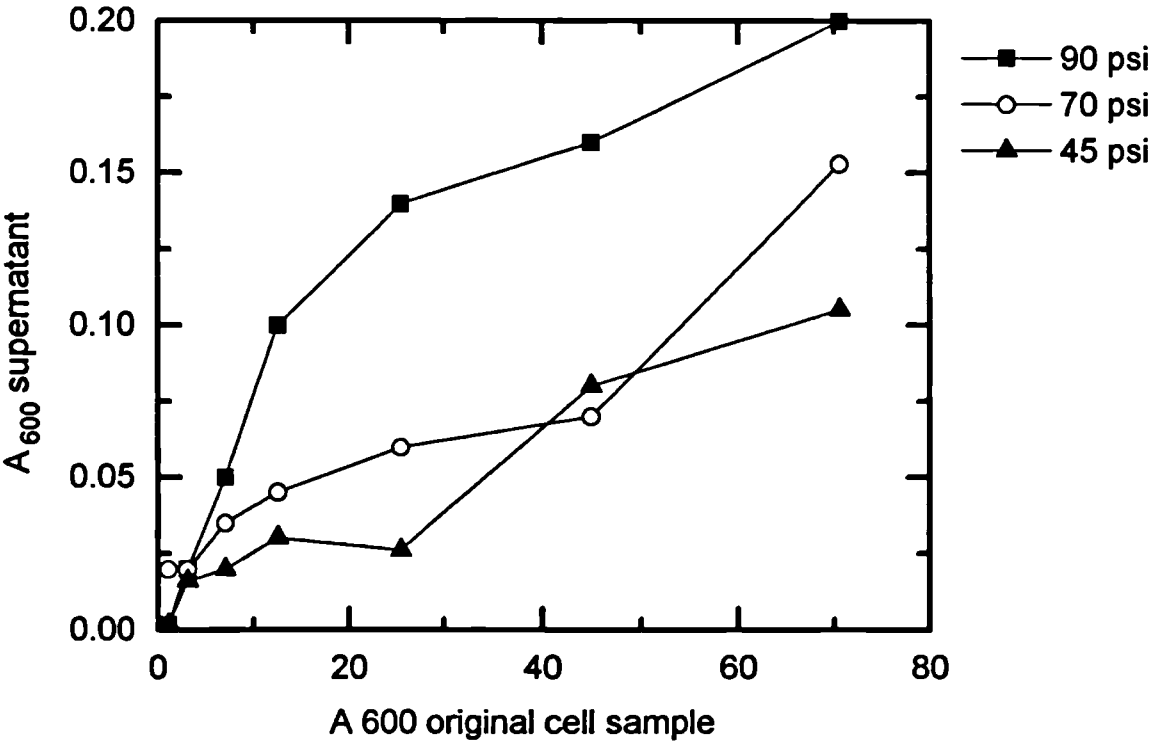


Fig. 1.4.3.e.2. Separation efficiency of microcentrifuge with two 60 sec. spins at different air turbine pressures with automatic sample loading of different concentrations of *E. coli*.

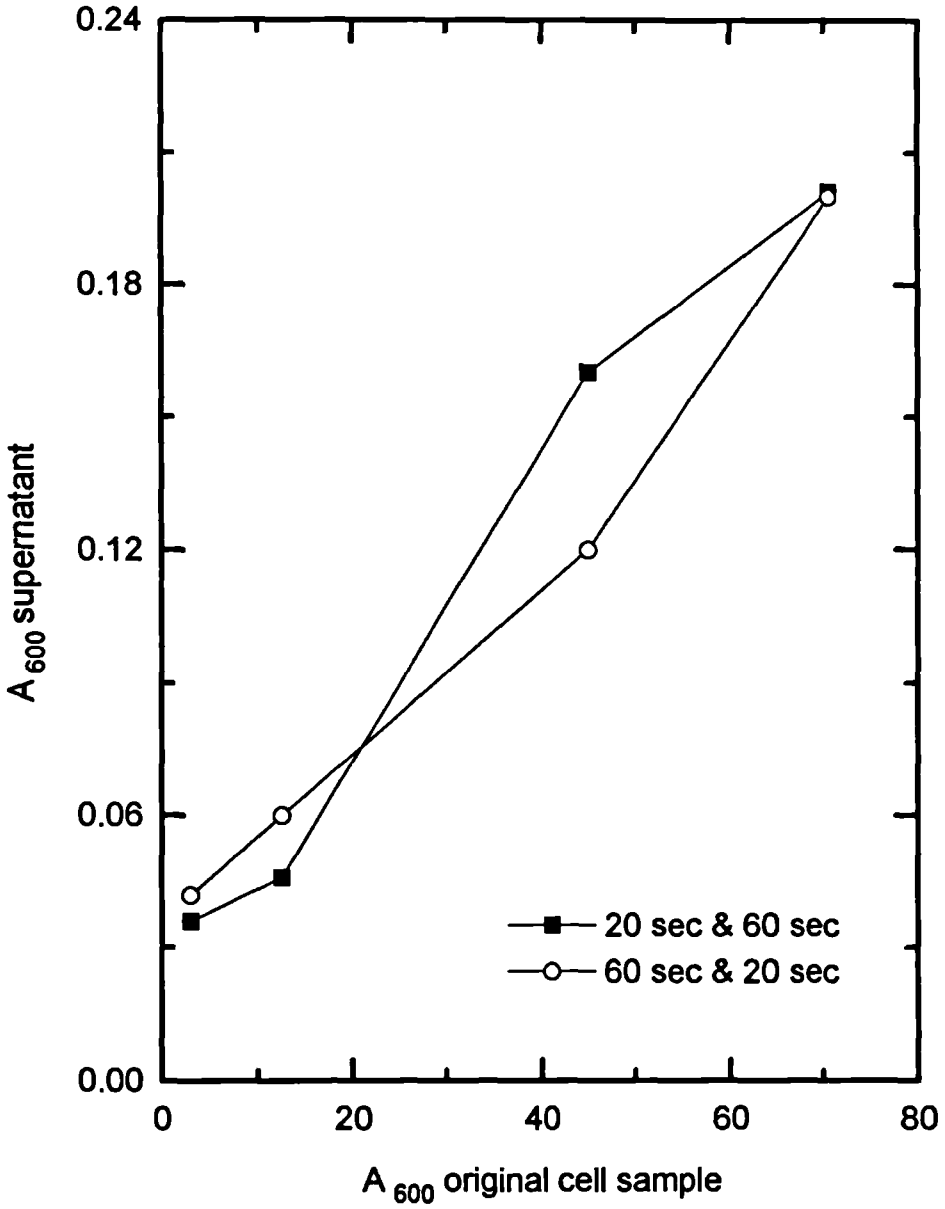


Fig. 1.4.3.e.3. Separation efficiency of microcentrifuge with two spins of different duration at 45 psi with automatic sample loading of different cell concentrations of *E. coli*.

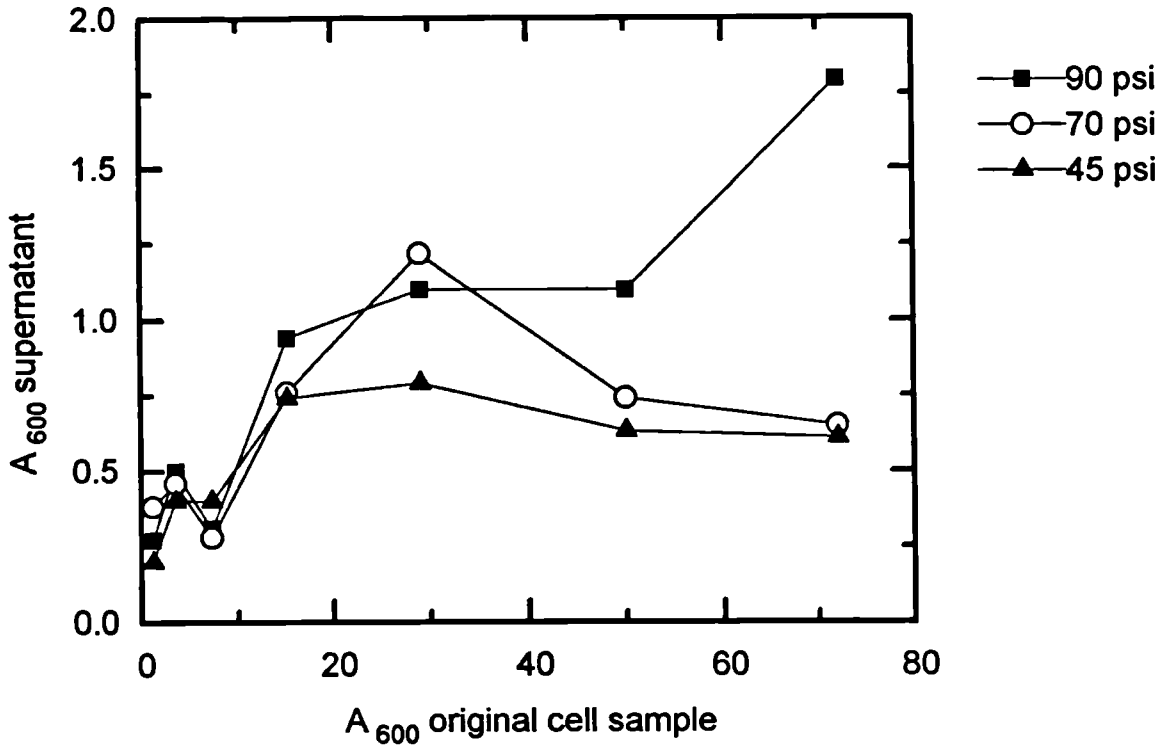


Fig. 1.4.3.f.1. Separation efficiency of microcentrifuge with two 20 sec. spins at different air turbine pressures with automatic sample loading of different concentrations of *S. cerevisiae*.

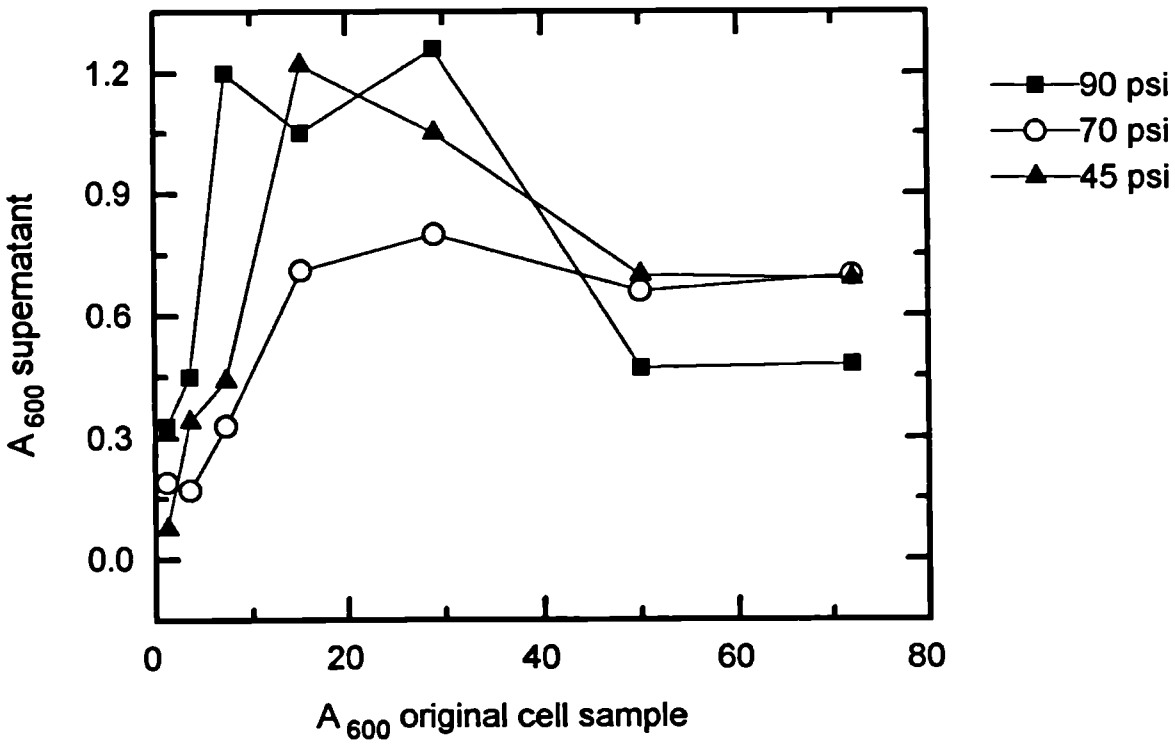


Fig. 1.4.3.f.2. Separation efficiency of microcentrifuge with two 60 sec. spins at different air turbine pressures with automatic sample loading of different concentrations of *S. cerevisiae*.

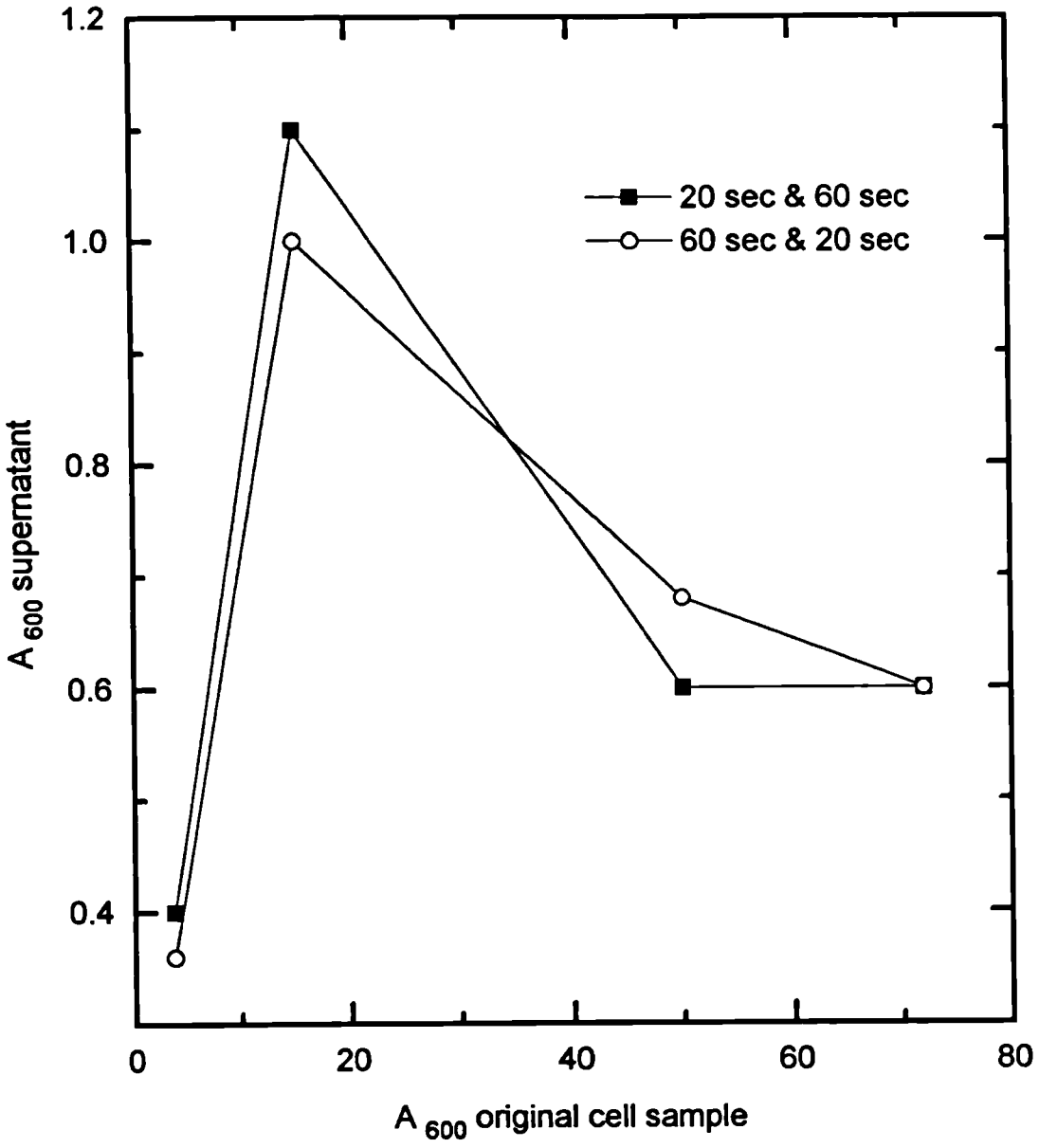


Fig. 1.4.3.f.3. Separation efficiency of microcentrifuge with two spins of different duration at 45 psi with automatic sample loading of different cell concentrations of *S. cerevisiae*.

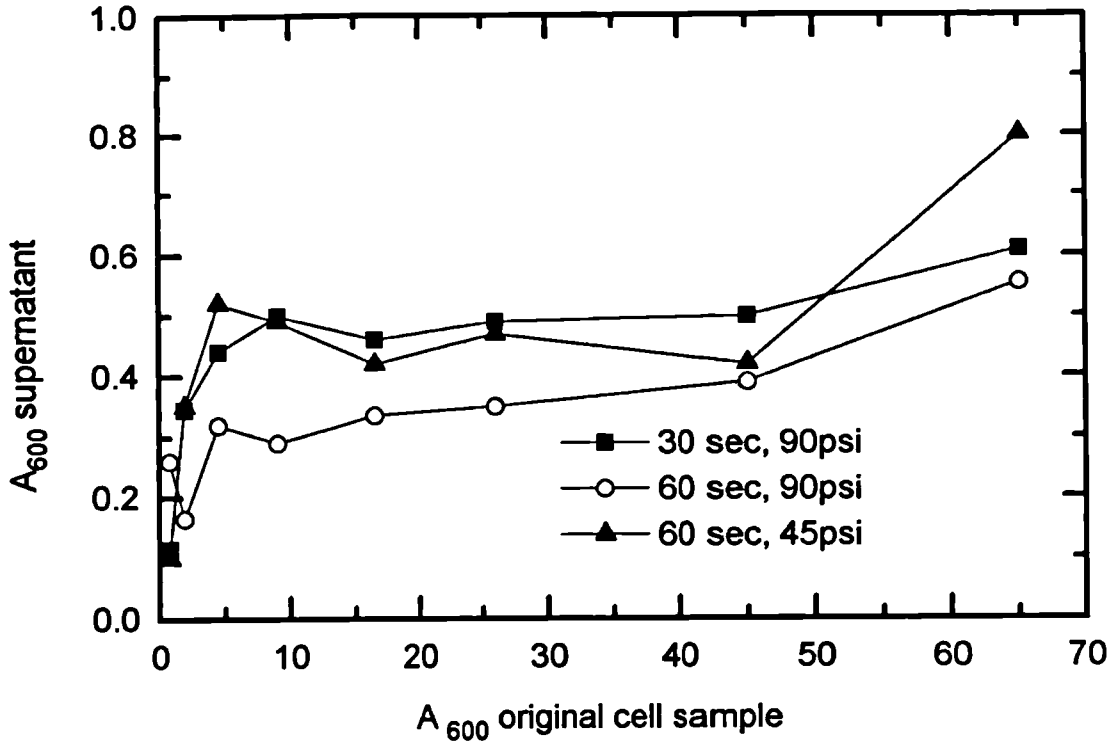


Fig. 1.4.3.g.1. Separation efficiency of microcentrifuge with brake on to stop bowl in 15 secs. Plot of pre and post spin A_{600} values for different concentration cell samples of *E. coli*.

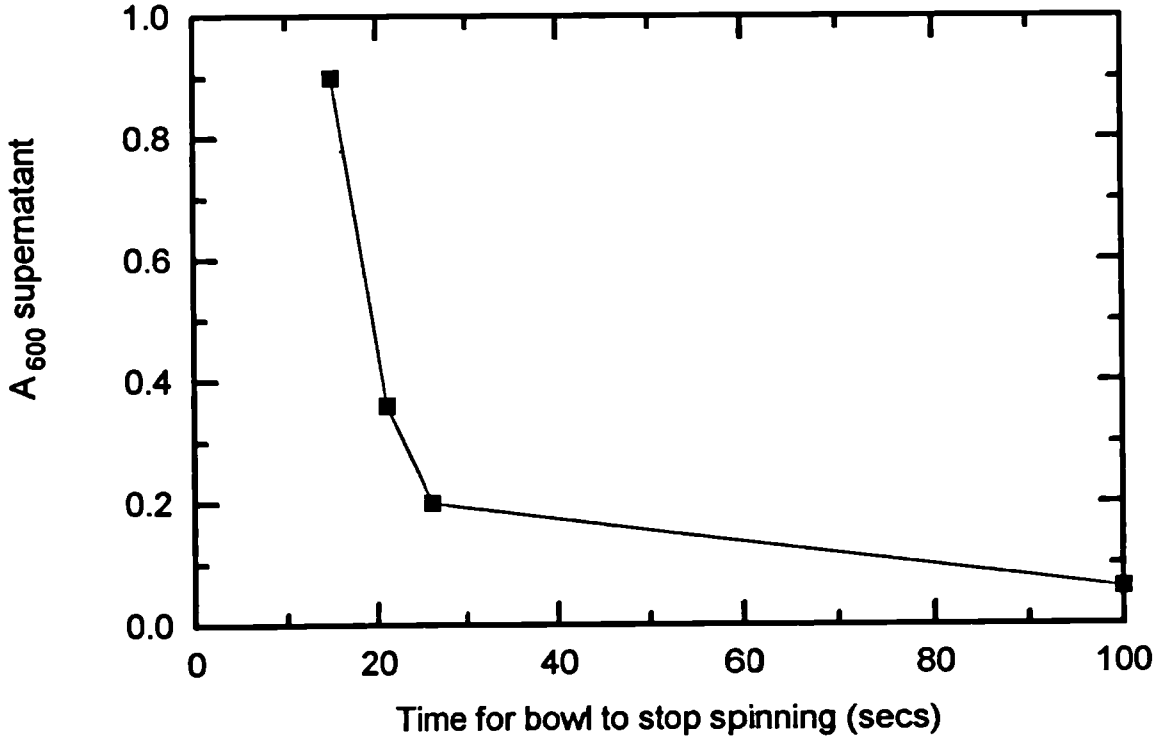


Fig. 1.4.3.g.2. A_{600} of supernatant of *E. coli* cell sample of original A_{600} of 21 after brake applied to centrifuge bowl spinning for 20 secs. at 45 psi, to stop in different times.

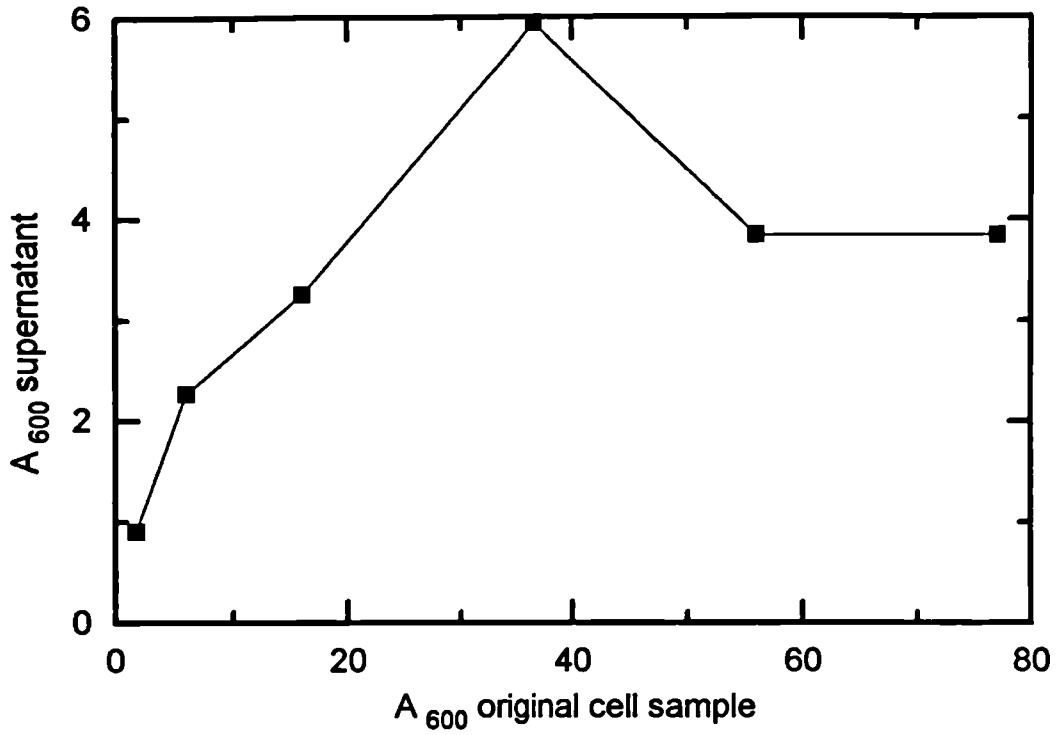


Fig. 1.4.3.h.1. Separation efficiency of microcentrifuge with brake on to stop bowl in 19 secs, spinning for 30 secs at 90 psi. Plot of post spin A_{600} vs different concentrations of *S. cerevisiae*.

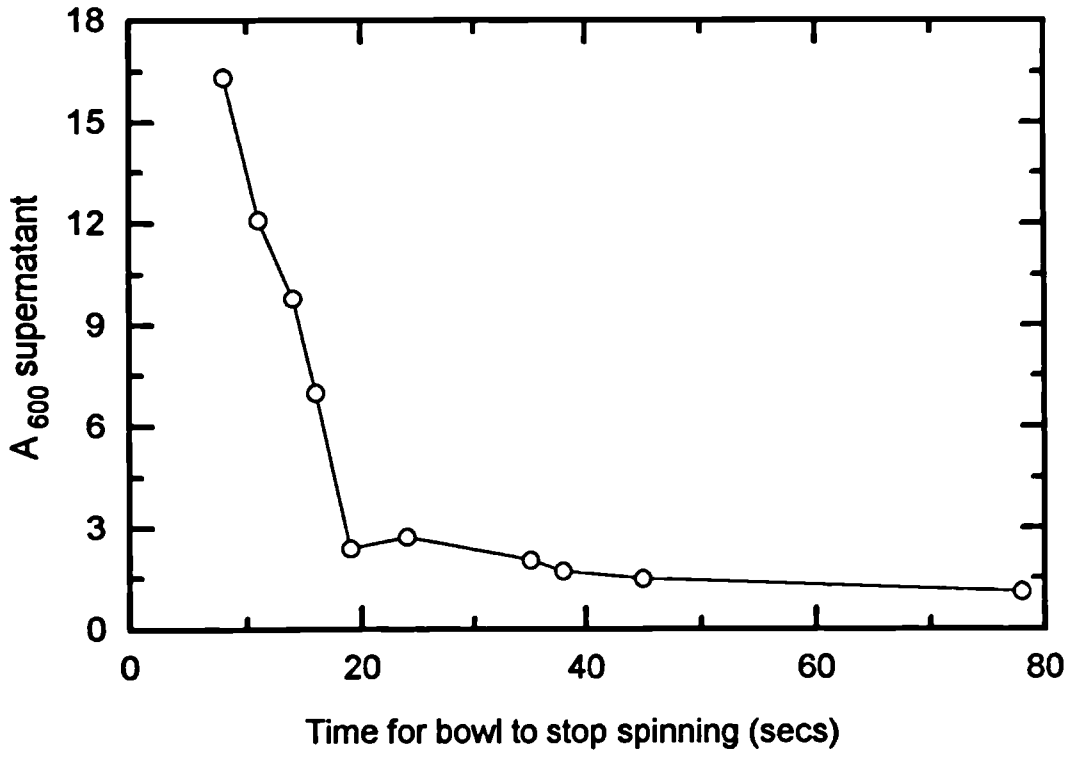


Fig. 1.4.3.h.2. A_{600} of supernatant of *S. cerevisiae* cell sample of original A_{600} of 36.5 after brake applied to centrifuge bowl, spinning for 30 secs. at 90 psi to stop in different times.

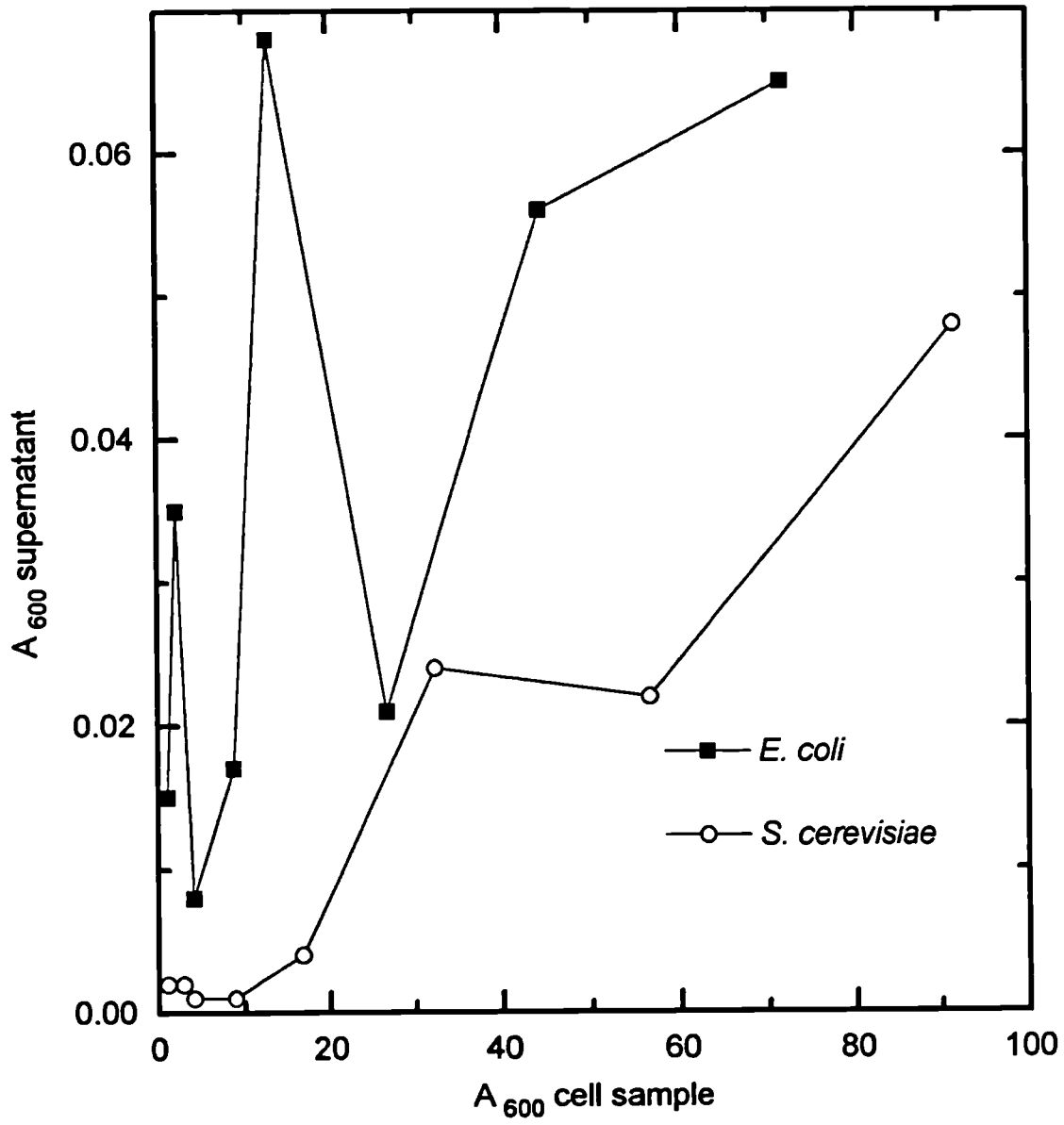


Fig. 1.4.3.j. Separation efficiency of Denley microcentrifuge at 10 000 rpm for 10 minutes.

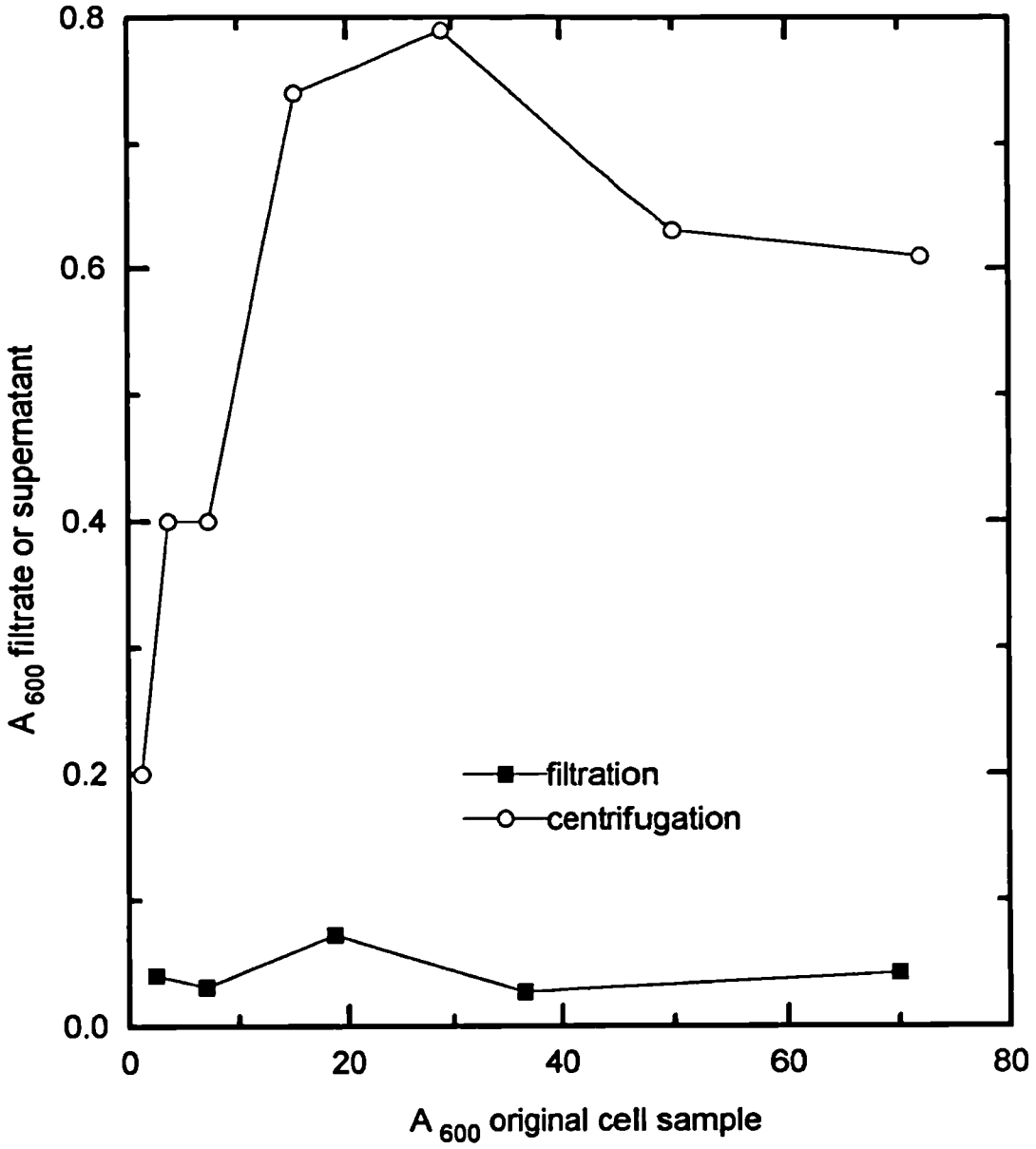


Fig. 1.4.3.k. Comparison of centrifugation of *S. cerevisiae* sample (dual spins for 20 secs. at 45 psi) with filtration through a 0.2 μm filter. This graph shows that filtration removes a much higher proportion of solids than centrifugation.

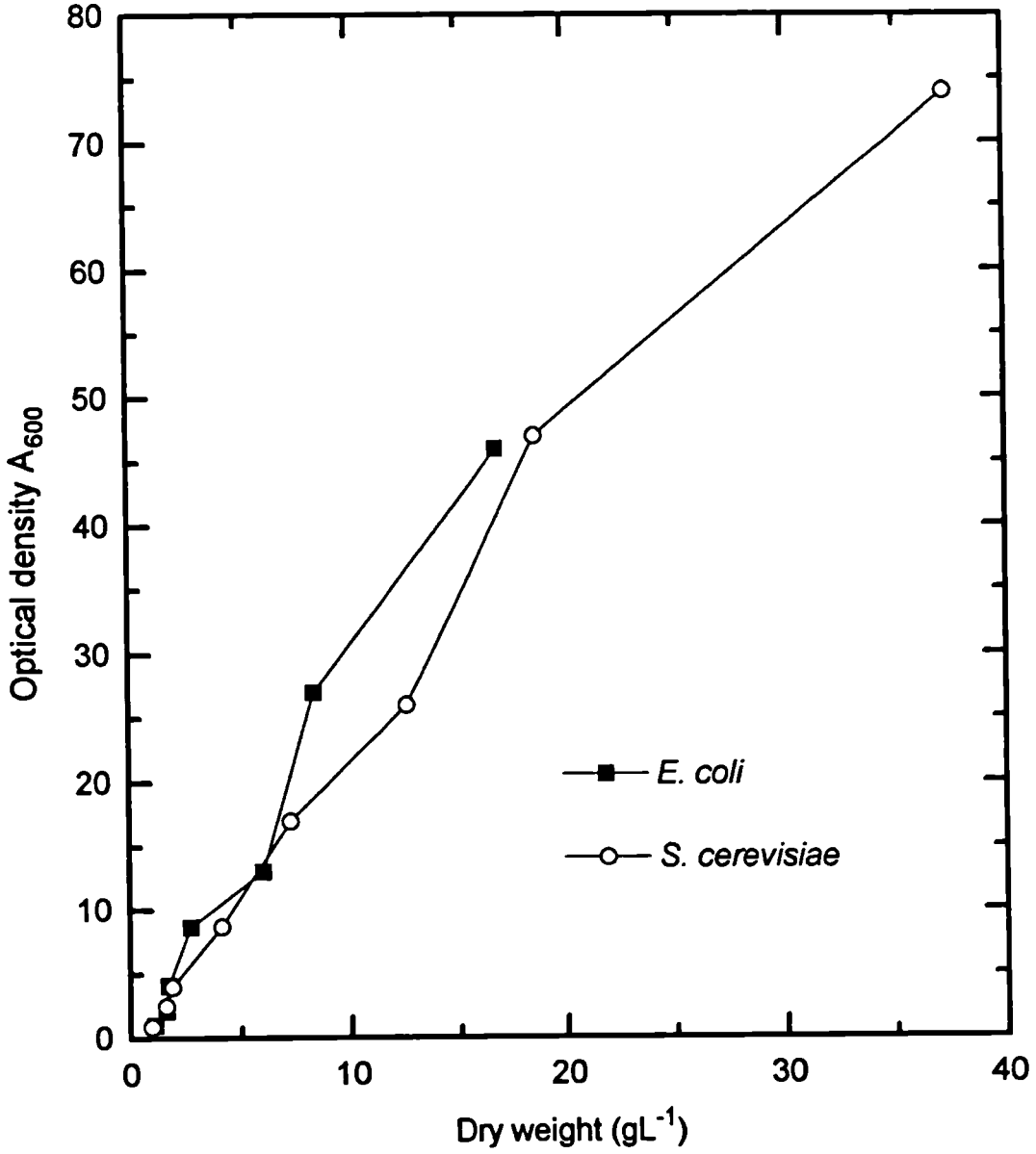


Fig. 1.4.4.a. Relationship between optical density (600nm) and dry weight ($g.L^{-1}$) for *E. coli* and *S. cerevisiae*.
In this instance, 1 OD unit for *E. coli* represents 0.35 $g.L^{-1}$ cells and 1 OD unit for *S. cerevisiae* represents 0.49 $g.L^{-1}$ cells.

1.5 CHROMATOGRAPHY SYSTEM

1.5.1. Equipment used

The equipment used in the on-line HPLC system is as follows:

- LDC Milton Roy isocratic HPLC pump, model Constametric III
- LDC Milton Roy ultra-violet detector, model spectromonitor III
- LDC Milton Roy refractive index detector, model RefractoMonitor IV
- LDC Milton Roy autosampler, model LC 241
- LDC Milton Roy automatic rheodyne valve, model AutoInjector A1000
- Perkin Elmer Nelson 2100 interface box model 950
- Grant water bath
- Jun-Air air compressor to operate rheodyne valve and autosampler
- IBM Model 55 SX personal computer, running Perkin Elmer Nelson 2100 HPLC analysis software.

The HPLC plumbing is conventional; the solvent is pumped first through the autosampler (for injection of calibration standards or off line samples), then through the automatic rheodyne valve unit (for injection of on-line samples from the microcentrifuge), before passing through the column, incubated at a particular temperature in the water bath. The solvent then passes first through the UV detector, and then the RI detector, before being collected in a waste reservoir. The UV and RI detectors are wired to the interface box "Channel A and Channel B" connections. Outputs from the PLC and autosampler are connected via a switch (which allows for the selection of only one device) to the "Start" connection on the interface box. This switch allows either device to start the box acquiring data from the detectors according to a method file programmed in.

In addition, the interface box has eight relays which can be connected to pieces of equipment. The first and second relays are connected to the solenoid valves in the automatic rheodyne valve that switch the valve from the "load" to "inject" position, and back again. The third relay is connected to the input card of the programmable logic controller (PLC), to start or stop a PLC program running

(see section 1.6). The other relays are unused. Opening or closing of the relays may be effected by programming timed events into the method.

1.5.2. Chromatography method used

The chromatography system in this project is used to measure the concentrations of sugars such as glucose and galactose, and small organic acids, such as acetate and pyruvate. The method practised here uses an isocratic separation of those components. The columns used during this project were obtained from Biorad. The first is an Aminex HPX-87H column, a 30 cm column which provides good resolution between a number of components, however the time required for analysis is relatively long (at the usual flow rate used *ie* 0.65 $mL.min^{-1}$ galactose elutes at about 10 minutes, and acetate at about 15.5 minutes). The other column used is Biorad's fermentation monitoring column, which is 15 cm long, and provides analyses in about half the time (about 5 minutes for galactose and 8 minutes for acetate), but at the expense of some resolution. A Biorad cation H^+ guard column is attached to the front of the HPLC column to protect it from solids and proteins in the fermentation broth.

Both columns used the same operating conditions. The mobile phase used is 0.004 M H_2SO_4 (HPLC grade H_2SO_4 obtained from Fisons). The flow rate was 0.65 $mL.min^{-1}$, and the column was kept in the water bath at 50°C; UV detection was at 210 nm. UV detection is more sensitive than RI detection, however, UV is only able to detect sugars at relatively high concentration. RI detection gives much more stable baselines, and provides more accurate results for the main substances measured during this project, namely galactose and acetate. For these reasons, RI results were used to calculate the concentration of components of the broth; the UV data was used as a backup.

A 20 μL injection loop was used in both the autosampler and automatic rheodyne valve. The linear range of the calibration of galactose and acetate is high; galactose calibrations range from 0.1 $g.L^{-1}$ to 35 $g.L^{-1}$ and the relationship between peak area and concentration is linear over a wide concentration range. Acetate

calibrations have been done up to 10 $g.L^{-1}$, also with linear calibration curves. Typical calibration curves for these two compounds are provided in figure 1.5.2.a.; and figure 1.5.2.b. and c illustrate typical chromatograms for a galactose and acetate standard solution; and a fermentation sample. The detection limit of both compounds is somewhat below 0.05 $g.L^{1}$, however it is dependent on the sample quality and the presence of interfering compounds.

1.5.3. Conditions of operation

The on-line HPLC system receives samples that have been clarified in the microcentrifuge and directly injects them onto the column without further sample treatment. The solid components present in the sample were low enough to allow a single HPLC column to be used throughout the course of this project (except the replacement of the HPX-87H column with the fermentation monitoring column for more rapid analysis). However, after approximately 200 injections, the column resolution starts to deteriorate. This is corrected by replacing the guard column. If the column has not been used for some time, the column may be cleaned by reversing the flow and replacing the mobile phase with a cleaning solvent of 0.004M H_2SO_4 containing 30% acetonitrile and operating under conditions of low flow rate and high temperature. Subsequently, the column can be regenerated with a solution of 0.025M H_2SO_4.

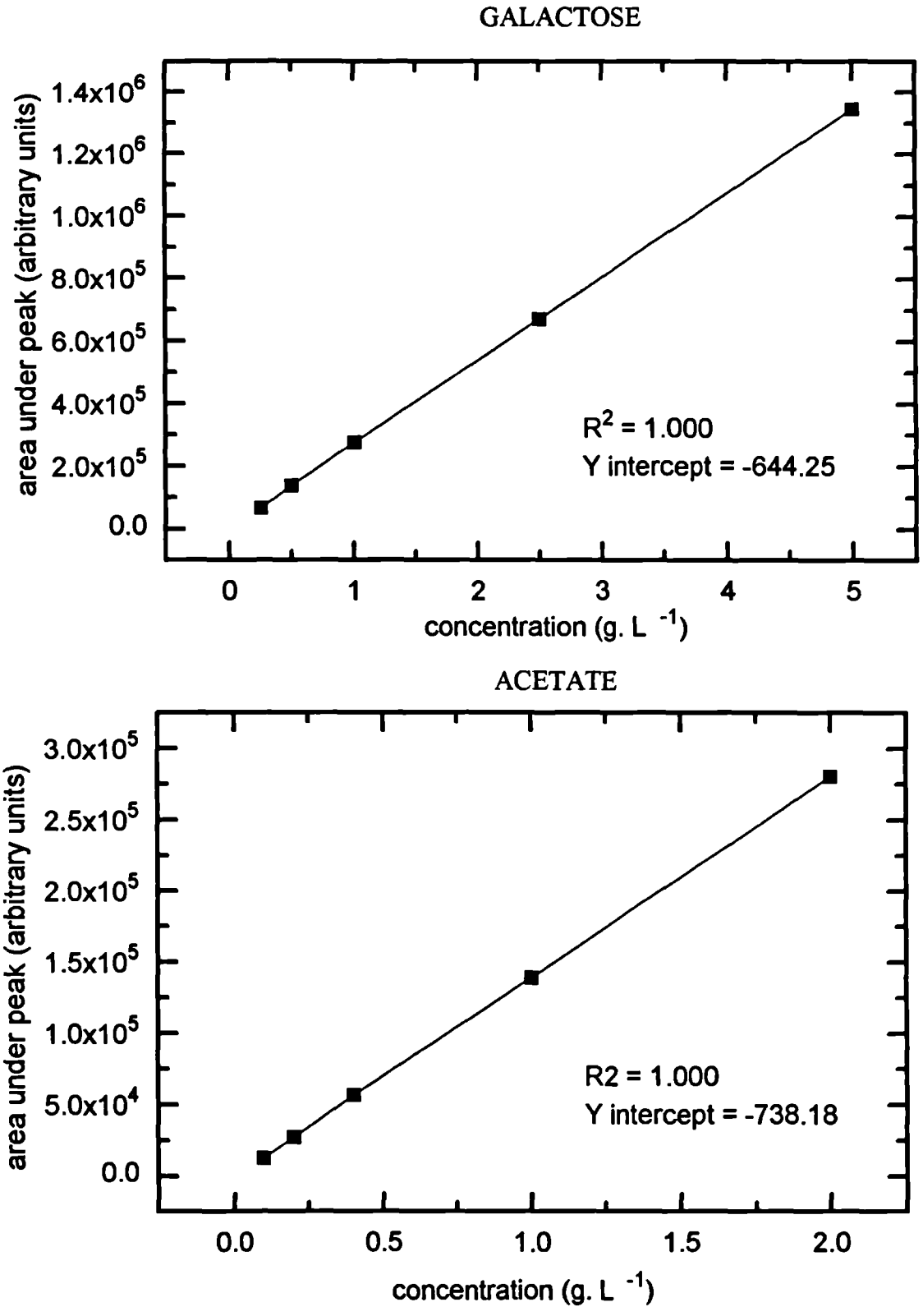


Fig. 1.5.2.a. Typical calibration curves for galactose (upper graph) and acetate (lower graph) low concentration standards run on HPLC system, using fermentation monitoring column. Lower concentration standards were used more frequently because of low concentrations of both components in fed-batch fermentations. R^2 values and Y intercept from linear regressions are given on the graphs.

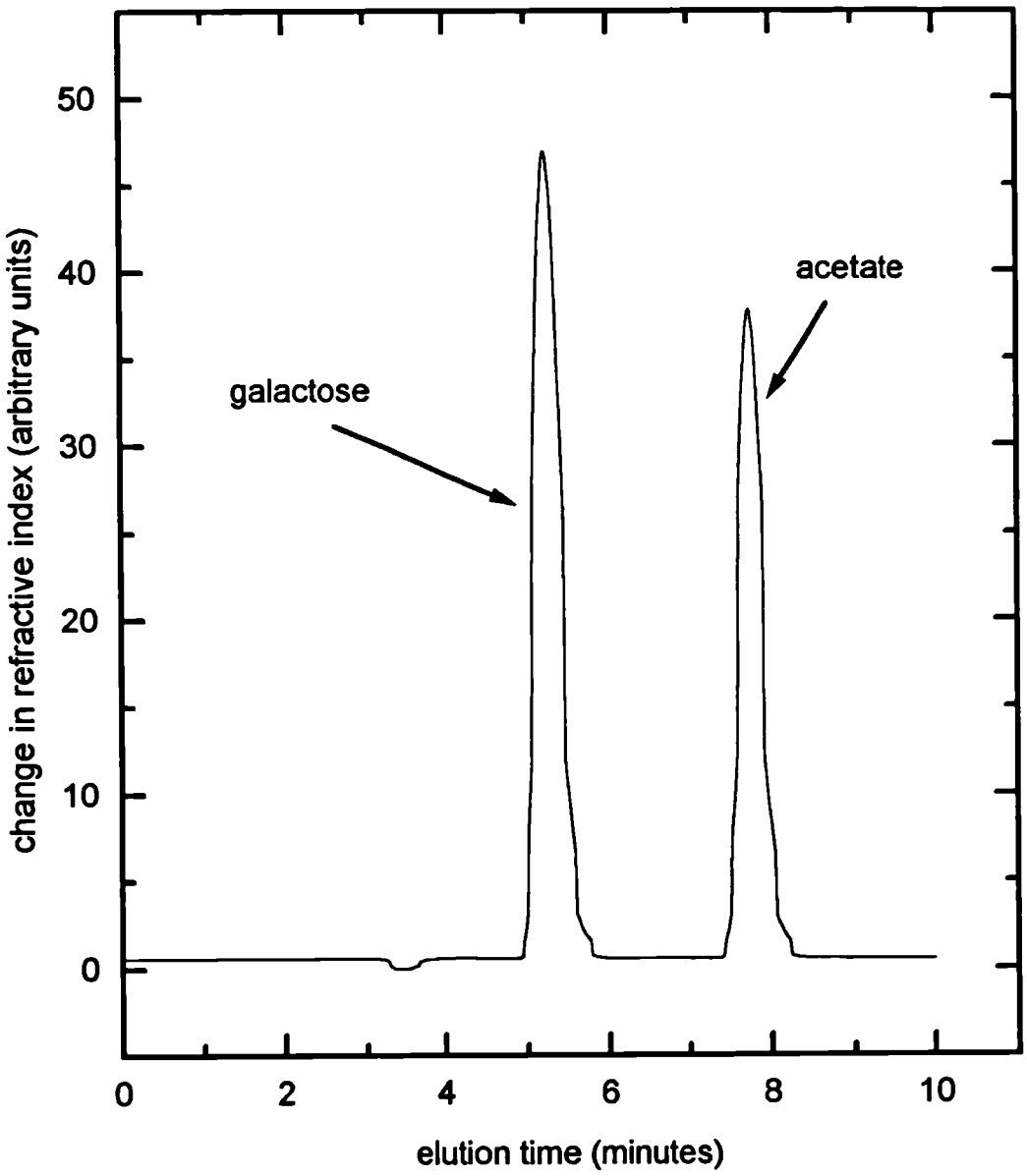


Fig. 1.5.2 b. Typical chromatogram of a standard solution of galactose and acetate used for calibrating the HPLC prior analysis of samples of fermentation broth. The fermentation monitoring column was used. Acetate (8 $g.L^{-1}$) and galactose (5 $g.L^{-1}$) peaks are marked.

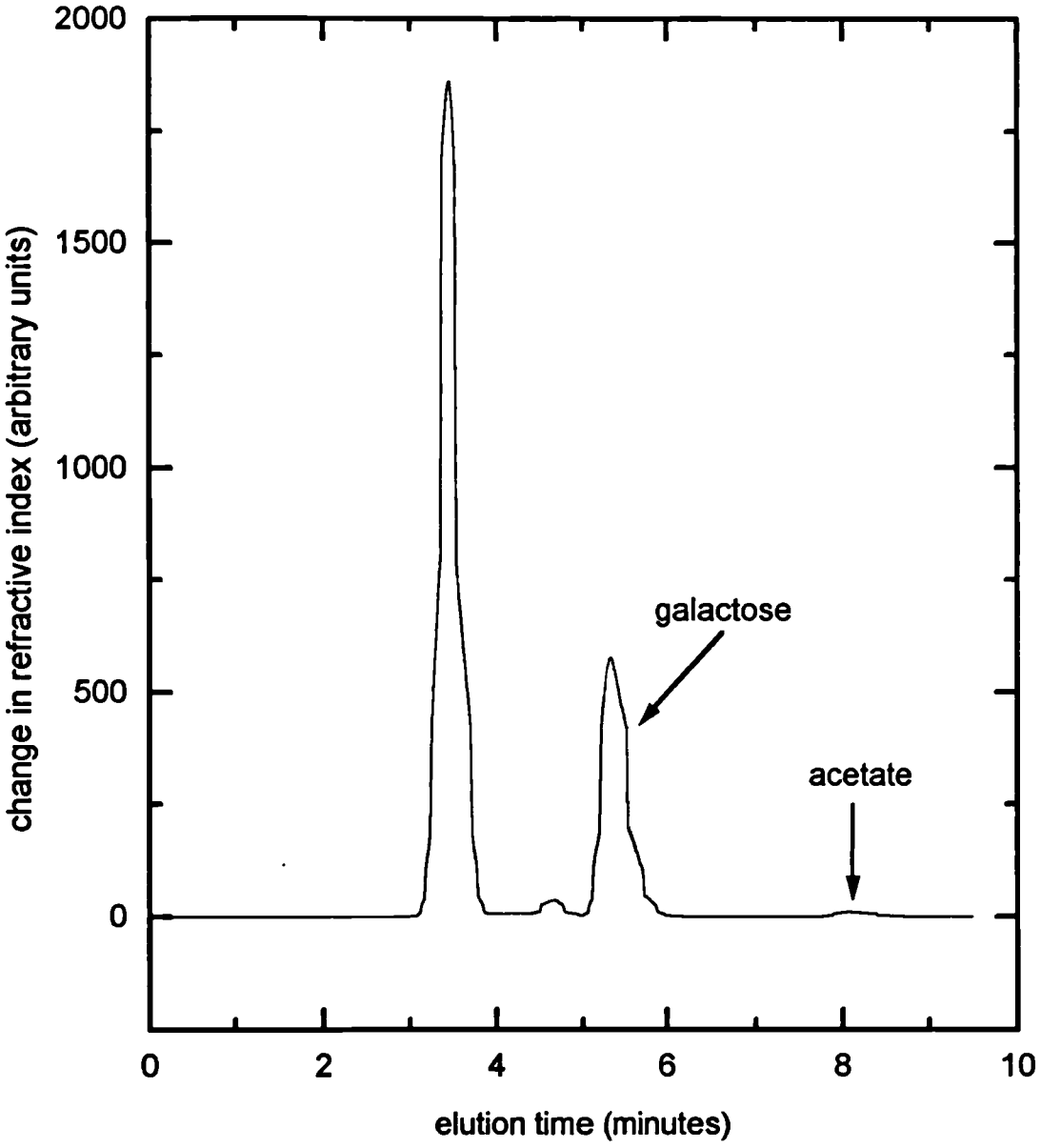


Fig. 1.5.2.c. Typical chromatogram of supernatant of sample of fermentation broth prepared using the on-line monitoring system. HPLC column used is the fermentation monitoring column. Peaks corresponding to galactose and acetate are marked on the graph.

1.6. COMPONENT INTEGRATION AND SEQUENCE CONTROL

1.6.1. Physical and control connections

The physical connections between the components of the system were made with flexible silicon tubing of different sizes; *ie.* between sampling device and microcentrifuge, and from microcentrifuge to the 20 μL injection loop on the rheodyne valve. Samples are pumped directly into the loop, and injected onto the HPLC column on a control signal programmed into the HPLC method program.

The control links were made by connecting all individual components of each device to a programmable logic controller. The sampling device has five pneumatic valves operated by 240V AC solenoids, and because the PLC control signal is 24 V DC, relays have been installed in the PLC to enable the correct signal to be supplied. The components the other devices consist of the two air inlet valves for the microcentrifuge air turbine, the three pumps, the vacuum valve and brake solenoid in the microcentrifuge, and the input and output connections to the HPLC interface box (the output from the PLC being connected to the "START" relay, and the input to the PLC connected to relay 3 on the interface box). The connections of each device and component to the programmable logic controller is shown in figure 1.6.1.a. The HPLC interface box was connected via serial link to an IBM Model 55 SX PC, for transfer of HPLC data and subsequent analysis in addition to its connection to the PLC.

1.6.2. The Programmable Logic Controller (PLC)

As has been previously mentioned, the sequence control for the operation of the system is provided by a programmable logic controller. The reason for the choice of a PLC is its reliability (the device has not failed throughout the project), its robustness, making it preferable to a computer for use in a harsh environment such as a pilot plant, and finally because a personal computer under most circumstances is unable to perform multi tasking operations. Hence the computer will be free to analyse HPLC data while equipment components are being

operated by the PLC.

The PLC used was a Texas Instruments model TI330 (obtained from Realm Control Systems, Crawley, Sussex), with 3.7 K RAM, one digital input card, and three digital output cards (one used for connections to each of sampling device, microcentrifuge and HPLC interface box), and these function with 24 V DC signals. In addition, analogue input and output cards were fitted, but these were not used in this project. The PLC is programmed off-line on Texas Instruments TISOFT software loaded onto the IBM PC and then downloaded to the data communications unit of the PLC by serial link. Once the program has been downloaded, the computer isn't required again until the program needs to be altered. The programming language is relay ladder logic, which is based on Boolean algebra. It functions by receiving inputs, and operating devices attached to outputs at programmed times. The PLC programs used during this project were written by the author of this thesis, with a lot of assistance from Martyn Vale of the department's electronics workshop.

1.6.3. Operation of System

The system is operated by the interaction of the PLC program with the HPLC method file downloaded to the interface box. This method file programs the interface box to operate relays and acquire data at specified times. Details of both the PLC program and HPLC method file used during the main part of this project are given in Appendix A.

The operator starts the system from the interface box (having previously downloaded a method file to the box, and a program to the PLC). The interface box then starts acquiring data from the HPLC detectors and operates relays in the box according to "timed events" programmed into the HPLC method. The first timed event occurs immediately at the start of the "run", and that is the injection of sample (that has been pumped into the injection loop by the microcentrifuge pump) onto the column, although there will obviously be no sample at the start of the very first "run". The valve is then pneumatically

actuated back into the load position for the next "run". Then some minutes later, another timed event closes relay 3 on the interface box, which will send a signal to the PLC which will start its program off to remove a sample from the fermenter, separate it in the microcentrifuge, and then pump the supernatant into the HPLC injection loop. By this time, the previous HPLC "run" will have ended, and the PLC sends an output signal to the interface box to start the next run. The timed event will then load the sample onto the columns, and the whole process is repeated continuously until the "stop" button is pressed at a particular time in the sequence at the end of the fermentation. At the end of each HPLC run, the output from the UV and RI detectors is downloaded to the PC for analysis. Figure 1.6.3.a. illustrates the operation of the equipment using the PLC and HPLC interface box.

1.6.4. Reproducibility of system

1.6.4.1. Method

In order to determine the reproducibility of results obtained by the system, a solution of approximately 2 $g.L^{-1}$ of galactose in water was made up and placed in a flask. The sampling device was connected to the flask, and the system was set up to remove a sample from the flask, centrifuge it and then pump it into the HPLC injection loop, for loading onto the column for analysis (*ie* it was run in the same way as a fermentation). The column used in this experiment was the longer Aminex HPX-87H column. A similar solution was pipetted into several HPLC vials for comparison of results with those obtained from a commercially available autosampler (LDC Milton Roy).

1.6.4.2. Results

Results of repeated analysis of the galactose sample in the on-line monitoring system show that fairly good reproducibility was obtained for 68 consecutive samples from the flask (at 14 minutes per sample, this means an operation time of approximately 16 hours). After the 68th sample, the reproducibility deteriorated, but it improved if the sample out pump on the microcentrifuge was left on for a longer period.

By comparison, the autosampler (three consecutive injections per vial) was far less reliable. Although the majority of injections was very reproducible, a number of "rogue" vials (where the sample was apparently not injected properly) was responsible in a reduction of the overall reproducibility. This is clearly unacceptable. Fig. 1.6.4.a. shows a comparison of results for the two systems. Table 1.6.4.(i). presents the mean concentrations of galactose solution and associated standard deviations for the all analyses using the on-line monitoring system, the first 68 analyses with the on-line monitoring system, the autosampler for all analyses, and the autosampler excluding the "rogue" vial analyses.

On the basis of the results presented, the on-line monitoring system outperforms the commercially available sampling device with respect to analysis reproducibility.

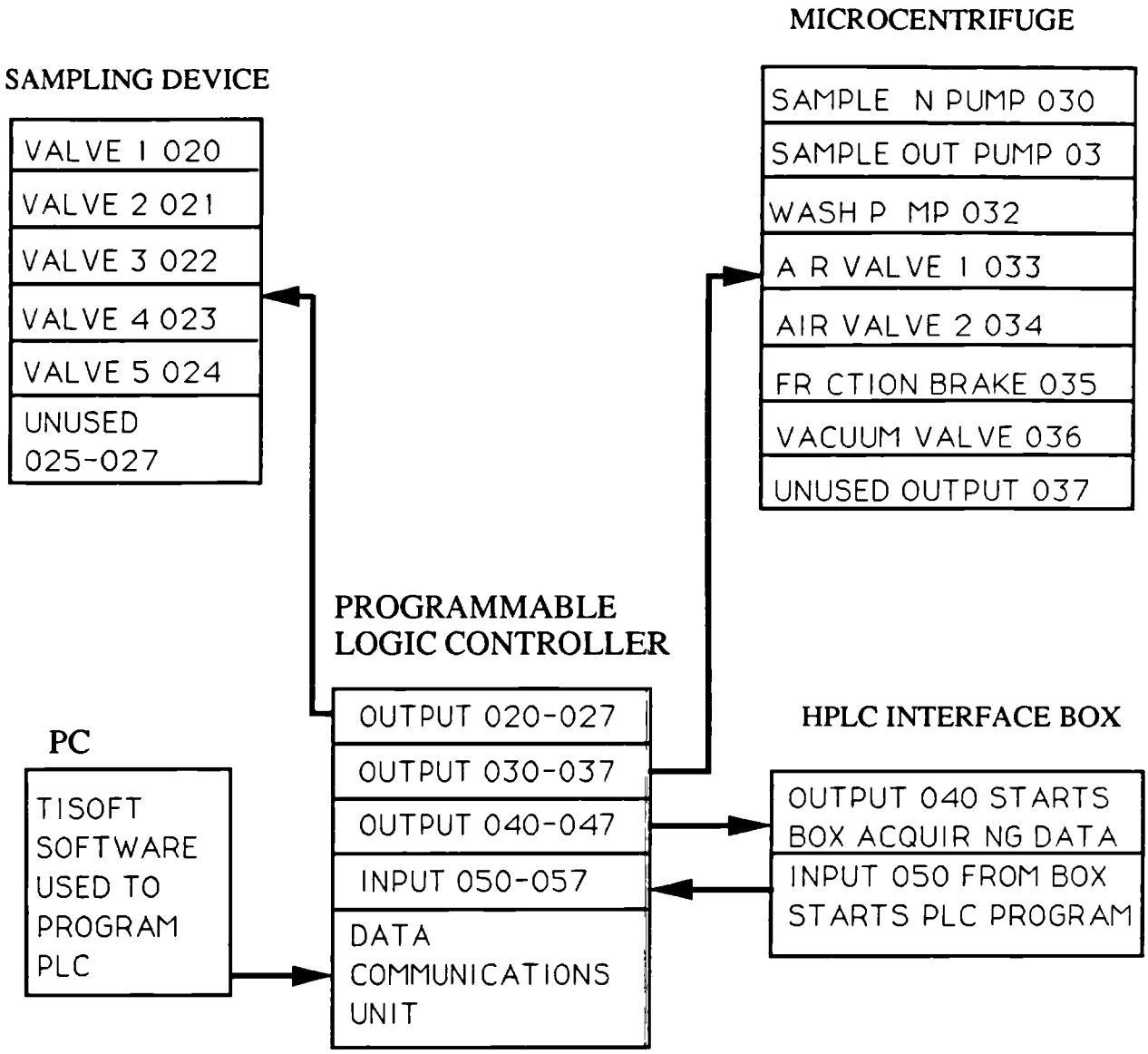


Fig. 1.6.1.a. Layout of control signals for the operation of on-line monitoring equipment. The PLC is connected to 4 different devices: the computer (through the data communications device); the HPLC interface box (through both input and output cards); and the sampling device and microcentrifuge through output cards. Each card has a total 8 inputs or outputs and the addresses of the individual components of each device are given, eg. Valve 4 in the sampling device has address 023.

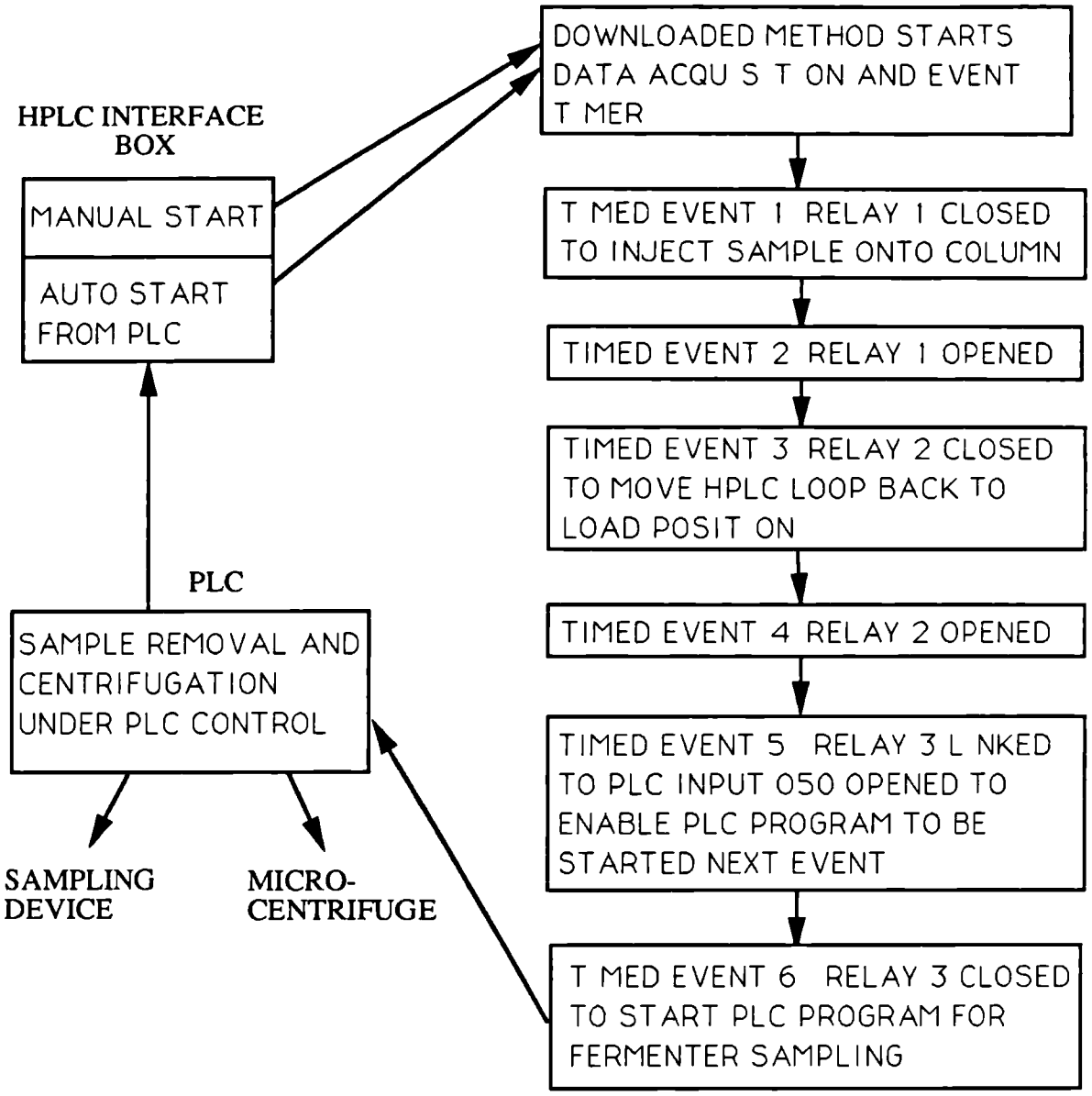


Fig. 1.6.3.a. Operation scheme of on-line monitoring equipment. Sampling is initiated by manually starting the interface box acquiring data and implementing operations programmed into timed events. The timed events operate devices connected to relays in the interface box at times programmed into the HPLC method file. The first relay operates a solenoid that injects a sample in the rheodyne valve injection loop onto the HPLC column. The second relay turns the injection valve back to the load position ready for the next injection. The third relay is connected to the input card on the PLC so that the PLC sampling program may be started. The HPLC method program runs for a time interval, stops, and then downloads the data. When a sample is ready for loading, the PLC signals to the interface box to start acquiring data again, and the program is repeated until stopped by manual intervention.

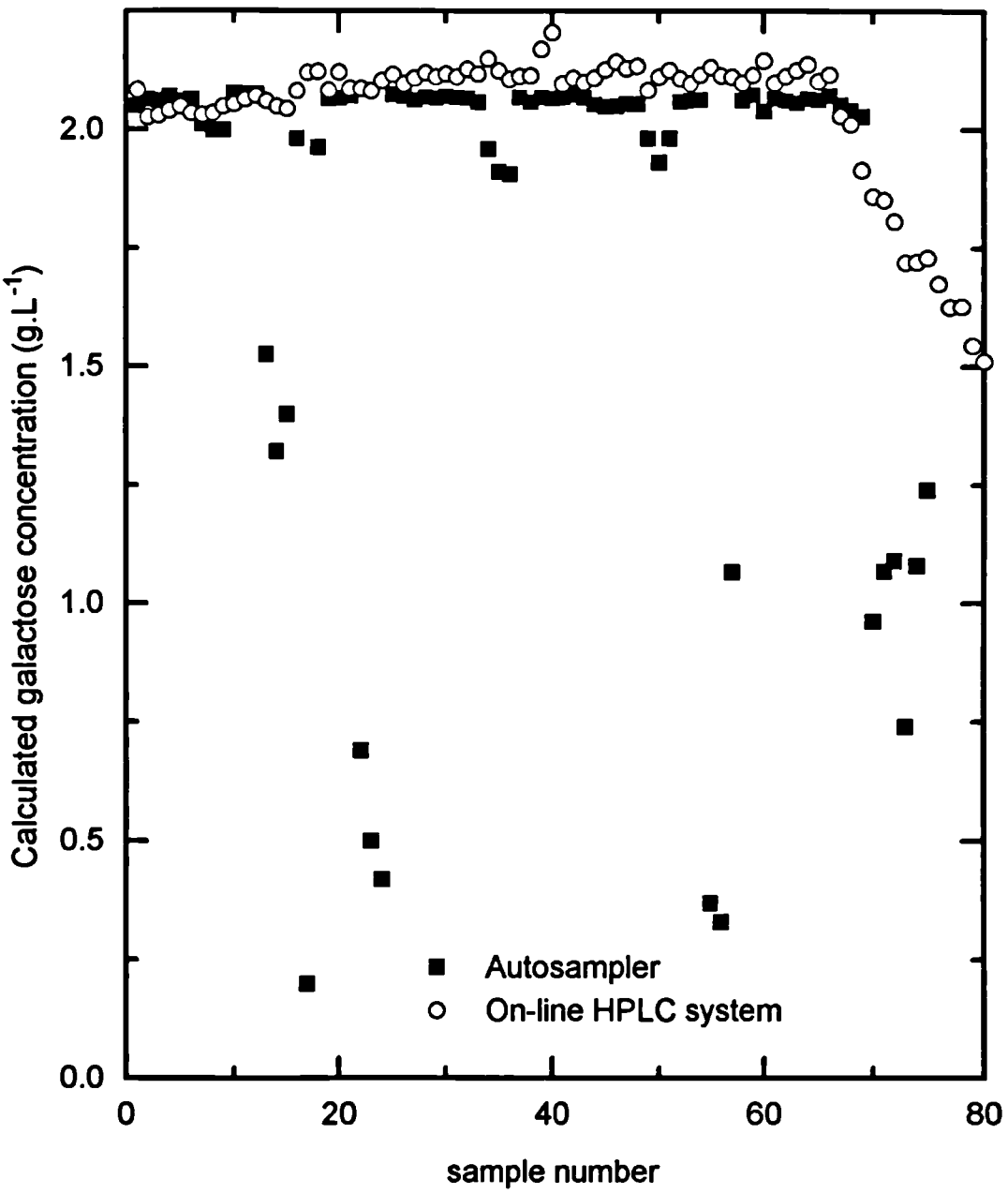


Fig. 1.6.4.a. Comparison of reproducibility of analysis of a solution of galactose in water using a commercial autosampler and on-line HPLC system. The "rogue" data points for the autosampler arose where the sample was not injected properly. The reproducibility of the on-line system deteriorated after 68 samples.

SYSTEM USED	MEAN CONCENTRATION	STANDARD DEVIATION
All samples analysed with on-line monitoring system	2.0407	0.149
First 68 samples analysed with on-line monitoring system	2.098	0.037
All injections made with commercial autosampler	1.7922	0.522
Injections excluding those from "rogue" vials using commercial autosampler	2.050	0.032

Table 1.6.4.(i). Mean concentrations and standard deviations calculated from HPLC data analyses from the on-line monitoring system, and the commercially available autosampler.

1.7. USE OF THE SYSTEM DURING A FERMENTATION

After testing the reproducibility of the system on a synthetic sample in a flask, the it was necessary to determine how well the system performed during a real fermentation, and to compare the concentrations of glucose and acetate obtained from data using the on-line HPLC analysis equipment with samples taken manually; analysis performed by off-line enzymatic type assays. This section describes how this was done, and presents the results that were obtained.

1.7.1. Materials and Methods

The organism used in this test fermentation was *E. coli* K12, grown for 12 hours in nutrient broth as a seed solution with which to inoculate the fermenter. The fermenter used was a 14L Chemap fermenter, and the fermentation medium was as described in Section 1.4.3., under Materials and Methods, with the exception that the carbon source was glucose (15.5 $g.L^{-1}$) instead of lactose. Glucose and $MgSO_4.7H_2O$ were sterilised separately and added immediately after inoculation of the fermenter.

Standard fermentation variables (*ie* pH, DOT, temperature and off-gas composition) were monitored, and data was collected by a DEC PDP 11-73 computer running the fermentation monitoring software, Bio-i, from BCS (Biotechnology Computer Systems).

The on-line HPLC system in this experiment was fitted with the longer Biorad Aminex HPX-87H column, and on-line samples were taken at twenty minute intervals; off-line samples were taken 1 minute after each on-line sample. Glucose and acetic acid concentrations were monitored on and off-line throughout the fermentation.

The glucose and acetic acid were assayed off line using assay kits for comparison with on-line HPLC results. The glucose assay kit used was SIGMA kit catalogue number 115A, and analysis of glucose is based on the following enzyme reactions

coupled with the reduction of iodonitrotetrazolium (INT) to form a coloured INT formazan:

$$Glucose + ATP \rightarrow Glucose\text{-}6\text{-}phosphate + ADP \quad (1)$$

$$Glucose\text{-}6\text{-}phosphate + NADP^{+} \rightarrow 6\text{-}phosphogluconate + NADPH \quad (2)$$

$$NADPH + PMS \rightarrow NADP^{+} + PMSH + INT \rightarrow PMS + INT\text{-}formazan$$

The enzyme for reaction (1) is hexokinase and for (2) is glucose-6-phosphate dehydrogenase.

The acetic acid assay kit was obtained from Boehringer Mannheim, catalogue number 148 261, and analysis of acetate is based on the following reactions:

$$Acetate + ATP + Co\text{-}A \rightarrow acetyl\ Co\text{-}A + AMP + pyrophosphate \quad (1)$$

$$Acetyl\ Co\text{-}A + oxaloacetate + H_2O \rightarrow citrate + Co\text{-}A \quad (2)$$

The oxaloacetate for the above reaction is obtained from:

$$Malate + NAD^{+} \rightleftarrows oxaloacetate + NADH + H^{+} \quad (3)$$

The enzyme for reaction (1) is acetyl Co-A synthetase; for (2) is citrate synthase, and for (3) is malate dehydrogenase. The determination is based on the formation of NADH measured by an increase in absorbance at 340 nm.

1.7.2. Results

The comparison between on and off line analyses of glucose and acetic acid throughout the fermentation are shown in Fig. 1.7.2.a. for glucose, and Fig. 1.7.2.b. for acetic acid.

Results for acetic acid show close agreement between on-line and off-line analyses; however there is a slight discrepancy between the two sets of data for glucose, particularly early on in the fermentation. It is believed, however, that the on-line HPLC result is more likely to be closer to the true value because of the known amount of glucose put in the fermenter (although it is accepted that evaporation of liquid during sterilisation of fermenter could lead to a slight concentration of components). In addition, the published coefficient of variation of the glucose assay is between 2% and 4% (possibly as a result of the multi-stage nature of the assay), which could account for the discrepancy between the results.

The correlations between glucose and acetate determined by on-line HPLC, and by off-line enzymatic assays are given in figure 1.7.2.c. For the purposes of monitoring and controlling an *E. coli* fermentation, the results were considered acceptable, and no further action was taken to improve the correlation.

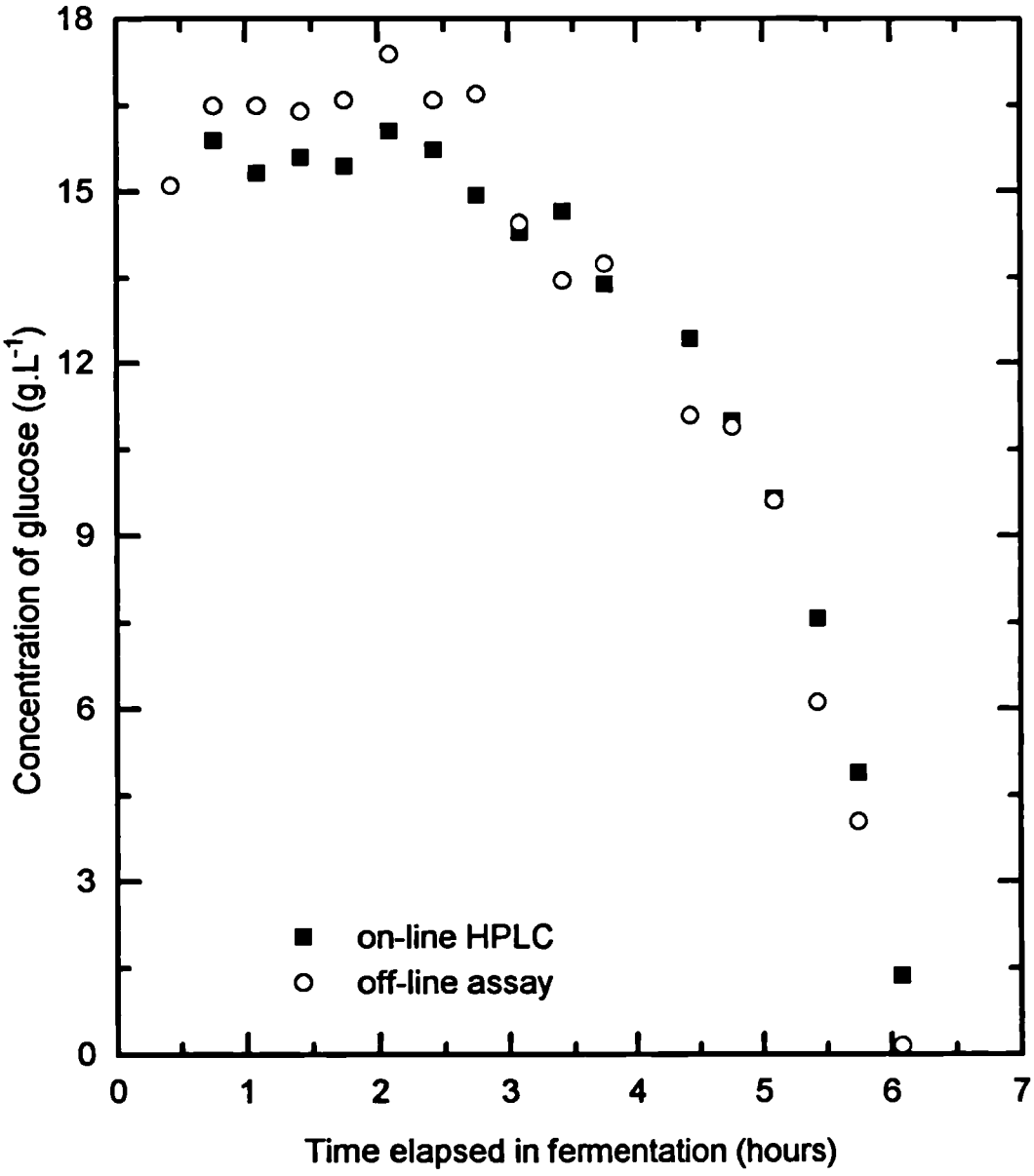


Fig. 1.7.2.a. Comparison of on-line and off line analysis of glucose during an *E. coli* fermentation. Off-line assay results at the start appear to give higher concentrations than the known fermenter contents. The correlation between on and off-line data was considered adquate for the purposes of on-line monitoring.

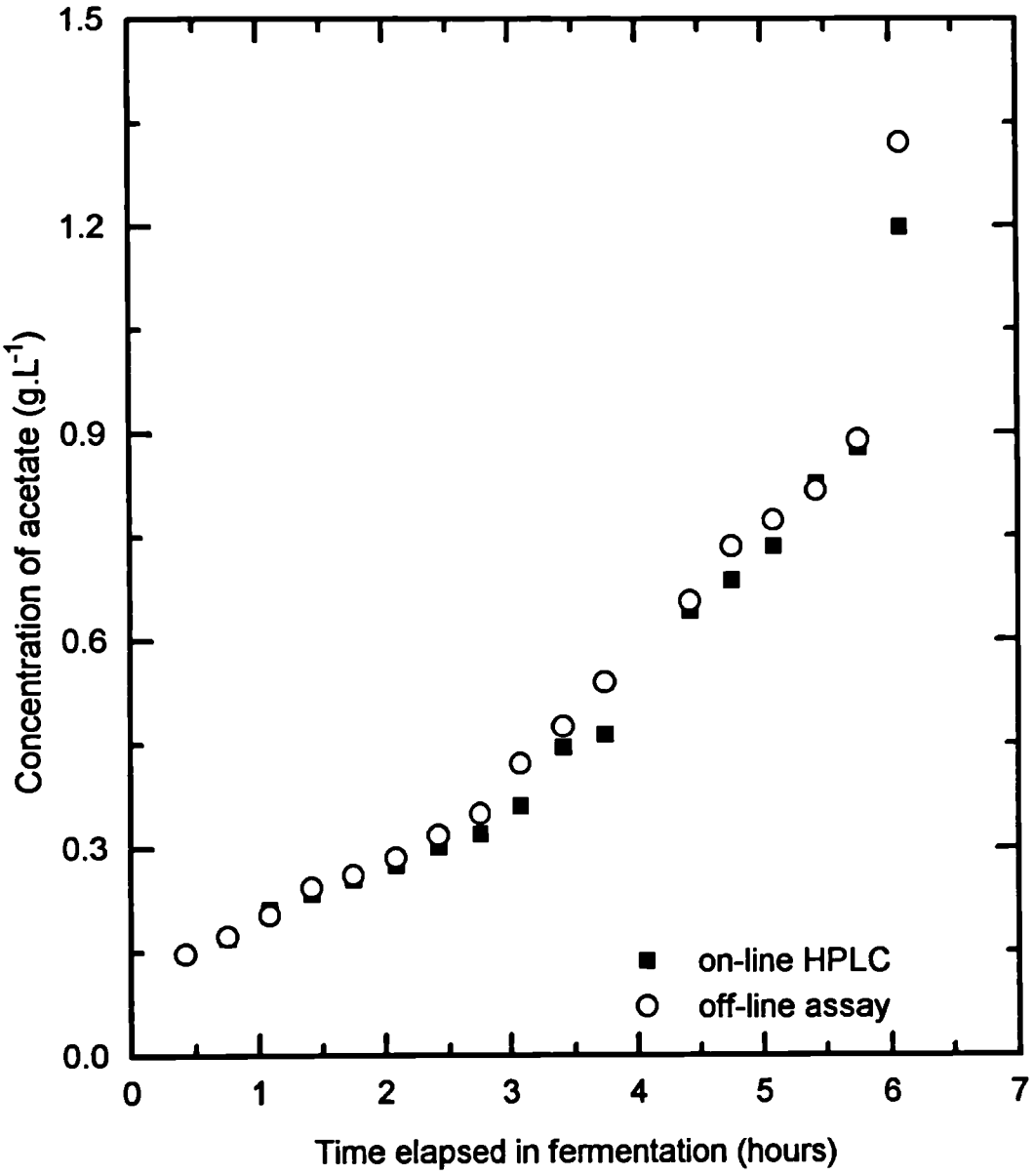


Fig. 1.7.2.b. Comparison of on-line and off-line analysis of acetate during an *E. coli* fermentation. Correlation is good throughout the fermentation.

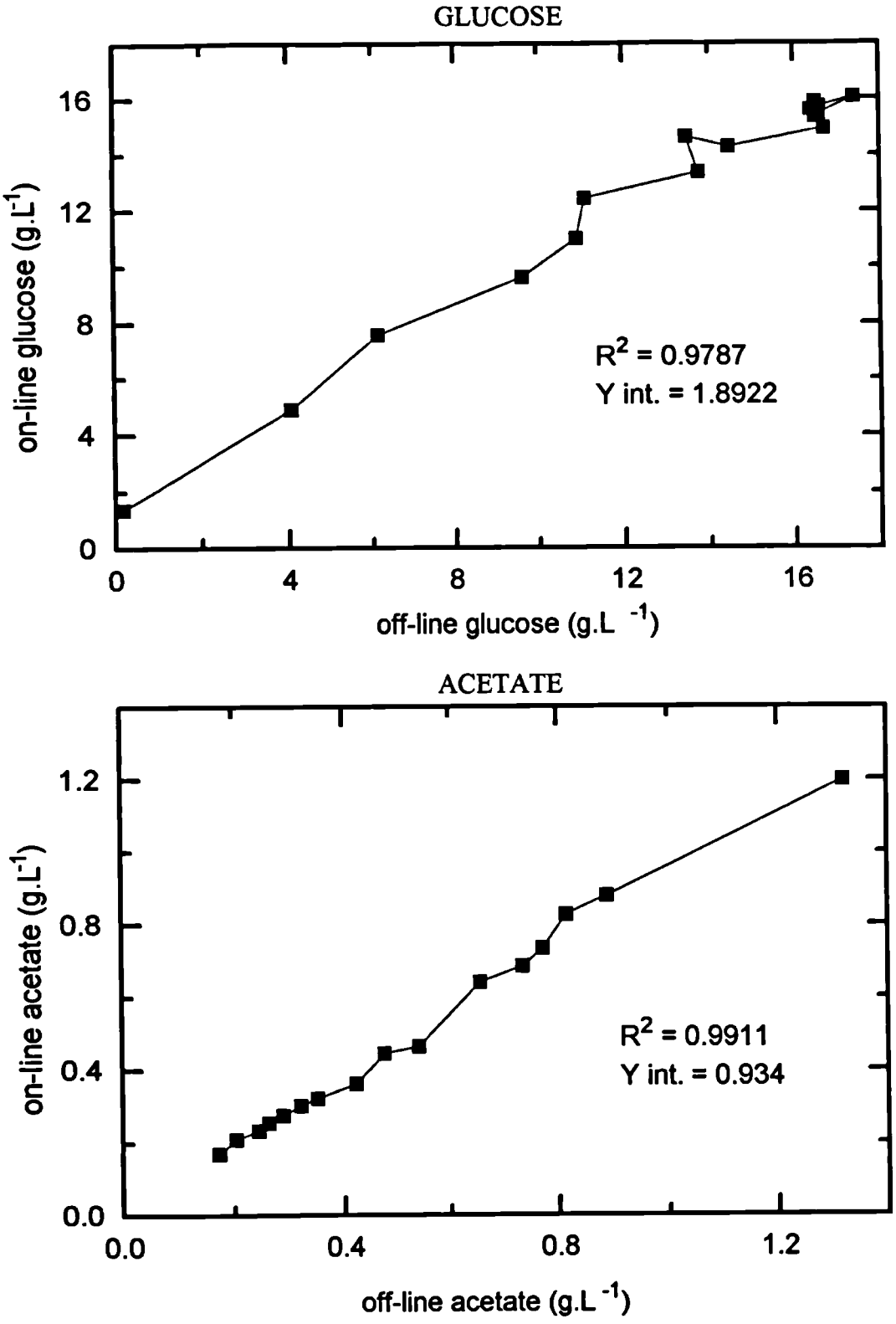


Fig. 1.7.2.c. Graphs showing the correlation between on and off-line glucose data (top) and on and off-line acetate data (below). The correlation is better for the acetate measurements.
R2 and Y intercept values from linear regressions are shown on the graphs.

1.8. LINKAGE OF ON-LINE MONITORING SYSTEM TO A CONTROL SYSTEM

A system capable of automatically monitoring sugars and excreted metabolites (such as acetic acid) on-line is useful, but its usefulness can be greatly extended by using the HPLC results in a feedback control loop to enable the monitoring *and* control of growth rate, sugar substrate or excreted metabolite concentrations. The reasons for wanting to do this are discussed in section 2; here, a description is provided of how this may be achieved.

1.8.1. The Control System

The control system used has been programmed by Malcolm Gregory at UCL, and is based on the LabView graphical programming software installed on an Apple MacIntosh IIi personal computer, with 5 MByte RAM, and using a 4 port RS232 serial card for data acquisition. Originally, a series of control algorithms were written for open loop control of growth rate and closed loop control based on respiratory quotient (RQ) data in *S. cerevisiae* fermentations. The programs were constructed from hierarchically linked sub-routines, either written by Dr. Gregory, or from those available in standard libraries. Further details of the control system appear in Gregory *et al.* (1993, manuscript in preparation), and it will not be discussed in great detail here because it is not the work of the author of this thesis. Basically, however, the algorithm calculates the exponential feed rate required for a particular growth protocol. Inputs required are:

- growth protocol required
- initial biomass concentration
- culture volume (hence total biomass)
- feed concentration
- pump calibration data
- growth yield on substrate
- correction for volume changes caused by feeding, base addition and sampling

Feeding substrate to provide a constant specific growth rate is an example of feed-forward trajectory control. The substrate feed rate required is calculated as follows:

$$F=\frac{\mu}{Y_x}.x(t).V(t).R \qquad (1)$$

$$x(t)=x(t-\Delta t).e^{\mu \Delta t} \qquad (2)$$

$$V(t)=V(t-\Delta t)+\frac{F}{S_f}.\Delta t \qquad (3)$$

F = substrate feed rate ($g.h^{-1}$)

μ = specific growth rate (h^{-1})

x(t) = dry biomass concentration ($g.L^{-1}$) at time t

V = culture volume (here, only addition of substrate used to modify calculated volume)

S_f= concentration of fed substrate ($g.L^{-1}$)

Y_x= growth yield on substrate fed ($g.g^{-1}$)

R = scaling factor (used to close the loop *eg.* to adjust feed rate to achieve desired RQ)

The substrate solution can then be fed under the control of LabView algorithms using two peristaltic pumps (Watson Marlow), one a 2 rpm pump, the other has a maximum speed of 32 rpm, to cover all the flow rates required. The flow rate required is set by sending new values at preset time intervals for the pumps as ASCII commands from the LabView computer to the pump controller (an 8 loop TCS 6258 controller) which varies the pump speed by analogue voltage signal. In order to use the on-line monitoring system for control, it was decided to link Dr. Gregory's control system to the on-line HPLC system described earlier.

1.8.2. Linkage of on-line monitoring system to control system

The PE Nelson HPLC software used to calculate concentrations of analytes from raw data from the detectors has the capability to "hook" on a user program. The method file allows for a link to a user program when the component concentrations have been calculated. Hence a user program supplied with the PE Nelson software (written in Microsoft QuickBasic) was modified to send data in the form of an ASCII string containing the analyte's name and concentration down the parallel port of the IBM PC (both serial ports of the computer are already used: firstly for a connection to the HPLC interface box, and secondly for downloading programs to the PLC). The modified program is given in Appendix A. The ASCII text was converted to RS232 serial format, and is then picked up by the serial card inside the Apple MacIntosh LabView computer. The card is scanned at intervals (which can be programmed into LabView) for the data, which is then extracted and cut into a format that can be used by the control algorithms. Hence the HPLC data may be directly incorporated into the LabView programming environment, and may then be acted upon. The control algorithm was modified to allow alteration of the feed rate depending on the analytical data from the on-line HPLC. Hence closed loop control is achieved. A diagram illustrating the entire system is shown in fig. 1.8.2.a.

1.8.3. The use of on-line HPLC with closed loop control in an E. coli fermentation

The use of this closed-loop controller in elucidating some aspects of an *E. coli* fermentation is demonstrated in Section 2. Before looking at the uses for such a controller, it is necessary to first describe how it may be used. To illustrate this, there are two examples demonstrating the use of the controller under different conditions: firstly, coarse control; and secondly, a much more complicated growth profile where much finer control was achieved. The basic fermentation protocol is similar for both, and is described below:

1.8.3.1. Organism used

E. coli strain JM107 with plasmid pQR126 is the organism used. The cloning of the organism was performed by Bahri and Ward (1990). The strain was maintained on slopes and plates of nutrient agar containing kanamycin A ($1\mu g.mL^{-1}$) and potato starch (Sigma, 1%). The organism has a gene for α-amylase production on a high copy number plasmid. For plasmid selection, a gene for kanamycin resistance also resides on the plasmid. Induction of the plasmid is under the control of the *lac* promoter, which is repressed in the presence of glucose.

1.8.3.2. Fermentation

Fermentations were performed in a Chemap 14 L fermenter, with standard fermentation variables monitored and controlled as described previously in Section 1.7.1. The agitation and aeration rates were kept sufficiently high throughout the fermentations to maintain aerobic conditions (DOT > 20% saturation). The start volume of the fermentations is 8 litres, 500 mL of which comes from the inoculum.

1.8.3.3. Growth medium

The growth medium initially in the fermenter is the same in both fermentations described in this section. It comprised ($g.L^{-1}$):

$(NH_4)_2SO_4$, 10; NaCl, 5; Na_2HPO_4, 2.16; KH_2PO_4, 0.64; $FeSO_4.7H_2O$, 0.2; citric acid, 0.2; $MgSO_4.7H_2O$. 0.2; thiamine, 0.1; kanamycin, 0.01; $CaCl_2$, 0.01; H_3BO_3, 0.004; $MnCl_4.4H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.0004; $CoCl_2.6H_2O$, 0.0004; $NaMoO_4.2H_2O$, 0.0002.

The reagents apart from $MgSO_4.7H_2O$, thiamine and kanamycin were steam sterilised and cooled. The remaining reagents were then added to the fermenter through a sterile filter.

The inoculum flask contained 500 mL of the above medium, in addition to 2 $g.L^{-1}$ galactose, which was the substrate used in the fermentations. Galactose was fed into the fermentation broth according to the feeding strategy in the controller.

1.8.3.4. Aim of the fermentations

The aim of both fermentations described below is to illustrate the on-line HPLC and fed-batch controller by trying to limit the amount of acetic acid that builds up in the fermentation medium by controlling the amount of substrate fed.

1.8.4. Example of use of on-line HPLC with coarse closed-loop control

When the on-line HPLC had been linked to the controller, the first step was to see whether it actually worked. In order to do this unambiguously, it was decided to use a strategy in which the control would be very coarse, so that any effects of changes in the feed rate on the excretion of acetate would be easily seen. In this experiment, no attempt was made to tune the controller as deliberately large alterations in feed rate were chosen to ensure as large an effect as possible. The control action chosen was to reduce the feed pump setpoint by 50% when a threshold concentration of acetate detected by the HPLC was exceeded.

In order to allow sufficient cultivation time for demonstration of the control, the fermentation was extended to produce about 30 $g.L^{-1}$ dry cell weight. This meant that the feed medium needed to contain nutrients other than galactose to prevent growth limitation on anything other than the carbon source. The feed comprised ($g.L^{-1}$):

Galactose, 375; $(NH_4)_2SO_4$, 264; Na_2HPO_4, 42; KH_2PO_4, 13; $FeSO_4.7H_2O$, 2.5; Citric acid, 2.5; $MgSO_4.7H_2O$, 5; Thiamine, 2.5.

1.8.4.1. The fermentation and algorithm conditions

The fermenter was inoculated to give an approximate biomass concentration at the start of 0.2 $g.L^{-1}$. The feed pump was set to a constant speed for the first 12 hours to add 3.8 g galactose to the culture to provide sufficient biomass for the controller to feed accurately to the culture when engaged. After 12 hours, the OD was 4.7, and the controller was started with the following initial values:

$x(o) = 2.5\ g.L^{-1}$

$\mu = 0.5\ h^{-1}$

$Y = 0.5\ g.g^{-1}$

pump rate update every 15 seconds

scan serial card for HPLC data every 15 seconds

critical analyte = acetate

threshold critical analyte concentration = $0.5\ g.L^{-1}$

control action: reduce pump rate by 50% (*ie.* scaling factor R = 0.5) when threshold exceeded, and revert to the current calculated feed rate if the concentration goes back below the threshold level.

1.8.4.2. Results of fermentation

Results of the fermentation are best illustrated graphically. Fig. 1.8.4.a. plots the concentrations of acetate and galactose as determined by the HPLC for the duration of the controlled part of the fermentation. The control actions can clearly seen in the oscillating concentrations of galactose and acetate. The reason for the increase in acetate to over 1 $g.L^{-1}$ at certain stages of the fermentation when the threshold is set at 0.5 $g.L^{-1}$ is because of the 12.5 minute delay time between the sample being removed from the fermenter, and action on the feed pump. No attempt was made to address this during this fermentation. In spite of this, however, the concentration of acetate did not exceed 1.2 $g.L^{-1}$ throughout the fermentation, and at the end of the fermentation, it had been reduced to zero in spite of the biomass concentration having reached 32 $g.L^{-1}$ by the time the fermentation was terminated. A plot of biomass and optical density of samples taken during the fermentation is shown in fig. 1.8.4.b. Linear regressions of natural logarithms of OD and dry weight data have slopes of 0.267 h^{-1} for OD data and 0.276 h^{-1} for dry weight data; the slopes give the overall specific growth rate obtained. A plot of specific growth rate obtained from dry weight and OD data from hourly samples taken during the fermentation is shown in fig. 1.8.4.c. The specific growth rate calculated from estimated biomass data obtained from LabView over this time is shown on the same graph. It does not take into account the reductions in μ due to the acetate threshold being exceeded and the pump speed being reduced; major changes observed are due to the algorithm being stopped and restarted with a new estimate of biomass obtained from the OD data (see below). The algorithm requires modification to enable it to

recalculate biomass estimates after the pump has been reduced, and is beyond the scope of this work.

Fig. 1.8.4.d. plots the feed pump controller set point converted to $g.h^{-1}$ galactose being fed. The control action on the pump when the acetate threshold has been exceeded, or reverts back to below the threshold concentration, can clearly be seen. In addition, some changes in feed rate are due to the algorithm being restarted with a new estimate of biomass.

1.8.4.3. Stopping and restarting the algorithm

At certain times during the fermentation, it became necessary to stop and restart the algorithm with a new estimate of biomass, because the biomass concentration estimated by the controller (using yield, feed rate, tank volume and growth rate data, as described earlier) started to deviate from the biomass concentration estimated from OD measurements. The reason for the deviation of the LabView biomass estimate from the true value is due to inaccuracies in the pump (particularly at low dilution rates), the difficulty of obtaining a reliable biomass estimate from OD data at low cell concentrations, any change in growth yield (*eg.* when acetate is produced) and if the cells do not grow at the growth rate set, and substrate starts to build up in the medium. An under-estimate of biomass is not likely to cause problems, and will only deviate by a certain amount. An over-estimate, however can lead to overfeeding and build up of substrate in the medium, which is also a problem of open-loop feed control. Hence at certain times during this fermentation, the algorithm was stopped and restarted with a new estimate of biomass based on the OD. The times at which the algorithm was restarted are shown in fig. 1.8.4.d. (marked with the numbers 2, 3, 4, 7, 9, 11 and 13).

1.8.5. Example of the use of on-line HPLC with fine closed-loop control

After it had been demonstrated that the closed-loop controller functioned as it had been intended to, a more complex experiment was attempted. Observations made after the coarse control experiment allowed modifications to be made to

the algorithm. These are:

1. Acetate only appeared in the medium when galactose started to accumulate. Hence galactose ought to be the critical analyte for control of the feed pump.
2. Because the biomass estimate calculated in LabView was found to deviate from the real biomass concentration, it was found to be necessary to restart the algorithm at various intervals with a new estimate of biomass. Therefore, the algorithm needs to be able to reduce the rate of increase of the LabView biomass estimate at a certain signal *eg.* when galactose and/or acetate build up in the medium.
3. Although a 50 % decrease in the feed rate eventually stopped the accumulation of acetate and galactose in the coarse control experiment, finer control would be required to reduce the size of oscillations in the concentrations of acetate and galactose. This finer control can be achieved by acting earlier and reducing the feed rate when a lower concentration of galactose is detected in the fermentation broth, and by having an additional reduction in feed rate at a slightly higher threshold concentration.

In addition to this, a more complex feeding strategy was required to see how an *E. coli* culture would cope with a ramped increase in specific growth rate (see Part 2). This would also serve to illustrate that LabView is capable of feeding to a much more complex profile.

1.8.5.1. The algorithm

The central feed algorithm assumed exponential growth (as described earlier). In addition, the LabView algorithm was modified to provide a linear increase in the specific growth rate setpoint from 0.2 h^{-1} to 0.45 h^{-1} over a period of 10 hours (*ie.* 0.025h^{-2}). Initially, galactose was selected as the critical analyte *ie.* feed rate changes were based on the concentration of galactose in the fermentation broth. When a concentration of 0.25 $g.L^{-1}$ was detected, the specific growth rate setpoint ramp was disabled. If the galactose concentration then rose above 0.4 $g.L^{-1}$, a second control decision was made: the biomass estimate calculated in LabView was decreased by 5 % every 12 minutes (corresponding to the HPLC analysis

time) until the galactose concentration dropped below 0.4 $g.L^{-1}$ again. (In practice, the off-line measurement of optical density could be used to correct the algorithm's estimate of biomass; this was done crudely in the previous experiment where the algorithm was stopped and started with a new estimate of biomass. This experiment is an attempt to correct biomass automatically by seeing galactose build-up). This acts to prevent the biomass estimate from deviating from the true value by too much, which reduces overfeeding. When the galactose concentration drops below 0.25 $g.L^{-1}$ again, the μ setpoint ramp is re-enabled.

1.8.5.2. Fermentation conditions

As in the previous fermentation, 7.5 litres of fermentation medium was inoculated with a 500 mL seed solution, to give an approximate biomass concentration at the start of 0.1 $g.L^{-1}$. The feed solution was 250 $g.L^{-1}$ galactose and the feed pump was set at a constant speed overnight to add 8 g galactose to the culture to provide sufficient biomass for the controller to feed accurately to the culture when engaged. After 12 hours, the OD was 1.5 and the controller was started with the following initial values:

$x(o) = 0.8\ g.L^{-1}$
$\mu = 0.2\ h^{-1}$
μ ramp 0.2 to 0.45 h^{-1} over 10 hours ($= 0.025h^{-2}$)
$Y = 0.5\ g.g^{-1}$
pump rate update every 15 seconds
scan for HPLC data every 15 seconds
critical analyte = galactose
critical analyte threshold concentration 1. : if exceeded, disable μ ramp
critical analyte threshold concentration 2. : if exceeded, decrease estimated biomass by 5 % every 12 minutes.

1.8.5.3. Results

The performance of the fine closed loop control algorithm is illustrated by plotting the concentrations of acetate and galactose measured by on-line HPLC during the controlled part of the fermentation, and this can be seen in fig. 1.8.5.a.

As can be seen on this graph, for the first 22 hours, the galactose concentration barely exceeded the second threshold level (reaching a maximum of 0.54 $g.L^{-1}$), and for this period, the maximum acetate concentration was 0.23 $g.L^{-1}$. Hence the control action proved efficient for this part of the fermentation. After 21.9 hours, however, the critical analyte (*ie* the analyte LabView uses to change the feeding strategy) was changed to acetate to see what effect this would have. As can be seen on the graph, this change allowed overfeeding to occur; the galactose concentration reached 3.66 $g.L^{-1}$ before acetate reached the first threshold value (0.25 $g.L^{-1}$). Acetate did not appear until 2.1 hours after the increase of galactose above 0.25 $g.L^{-1}$ (possible reasons for this lag are discussed in Part 2). In this case, therefore, better control is achieved by making galactose the critical analyte.

The feed rate in $g.h^{-1}$ galactose is shown in fig. 1.8.5.b.; the times where the control action occurred is marked on the plot. The changes in feed rate are much more subtle in this experiment, and can barely be seen. Fig 1.8.5.c. plots dry weight and OD data from samples taken throughout the fermentation. Linear regressions performed on the natural logarithms of the data give a slope (and hence overall specific growth rate) of 0.249 h^{-1} for dry weight data, and 0.215 h^{-1} for OD data. Fig. 1.8.5.d. plots the specific growth rate calculated from biomass estimate data in LabView and the growth rates calculated from dry weight and OD data obtained from samples taken at various times throughout the fermentation. The LabView estimates reflect the specific growth rate the pumps are feeding to, set by the algorithm (not the actual specific growth rate). Visible on the graph is where the biomass estimate was reduced yielding a negative growth rate according to LabView, although of course it will not really be negative because of the excess galactose in the fermenter. Changes in the linear specific growth rate ramp due to the threshold concentration 1 or 2 being exceeded (or the measured concentration dropping below the threshold value) are clearly seen on the plot of μ obtained from LabView biomass estimates.

1.8.6. Discussion

The coarse closed loop control experiment was reasonably successful in

controlling acetate concentration throughout the fermentation. Quite large fluctuations of galactose and acetate occurred, but the overall acetate concentration did not exceed 1.2 $g.L^{-1}$. It was observed that acetate was only excreted after galactose built up in the medium, hence it was thought that the critical analyte (on which LabView acts) would lead to finer control if it was galactose instead of acetate, so that low galactose levels would not allow acetate to overflow. This was done in the fine control experiments, and was as much, or more responsible for the very successful control of acetate concentration as the dual level control system. This was confirmed by changing the critical analyte to acetate after 22 hours, and seeing a large build-up of galactose before acetate was excreted, and then a rapid acetate accumulation.

The dual level control system was primarily useful in correcting the algorithm's estimate of biomass, but this particular system was designed for the complex action of increasing the specific growth rate linearly throughout the fermentation. the dual level action could be modified for use with constant specific growth rate feeds by using the first level to reduce the feed rate by a certain amount, and the second to reduce the algorithm's estimate of biomass.

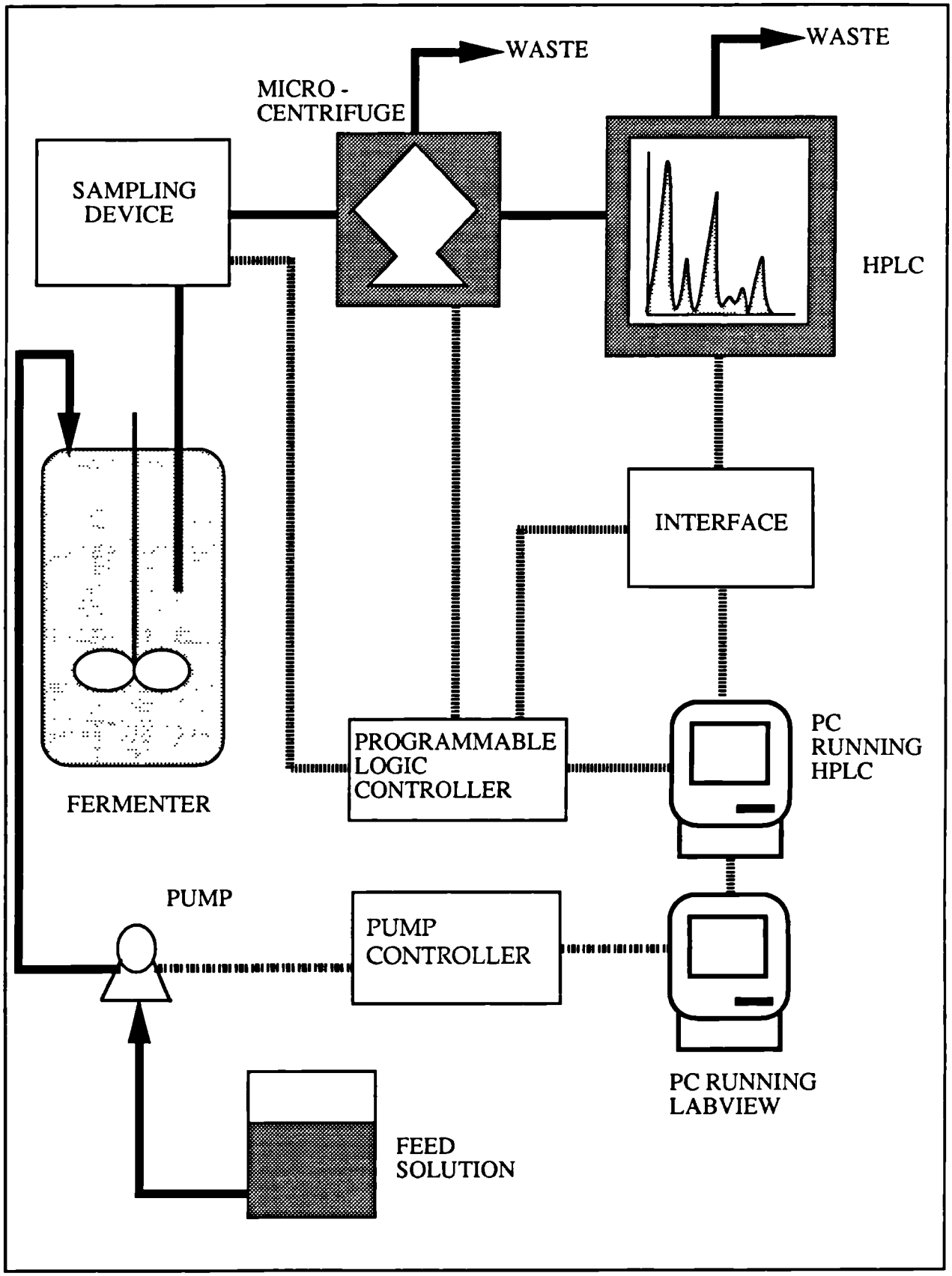


Fig. 1.8.2.a. Overview of main components of on-line monitoring system linked to fed-batch closed loop feed-pump controller

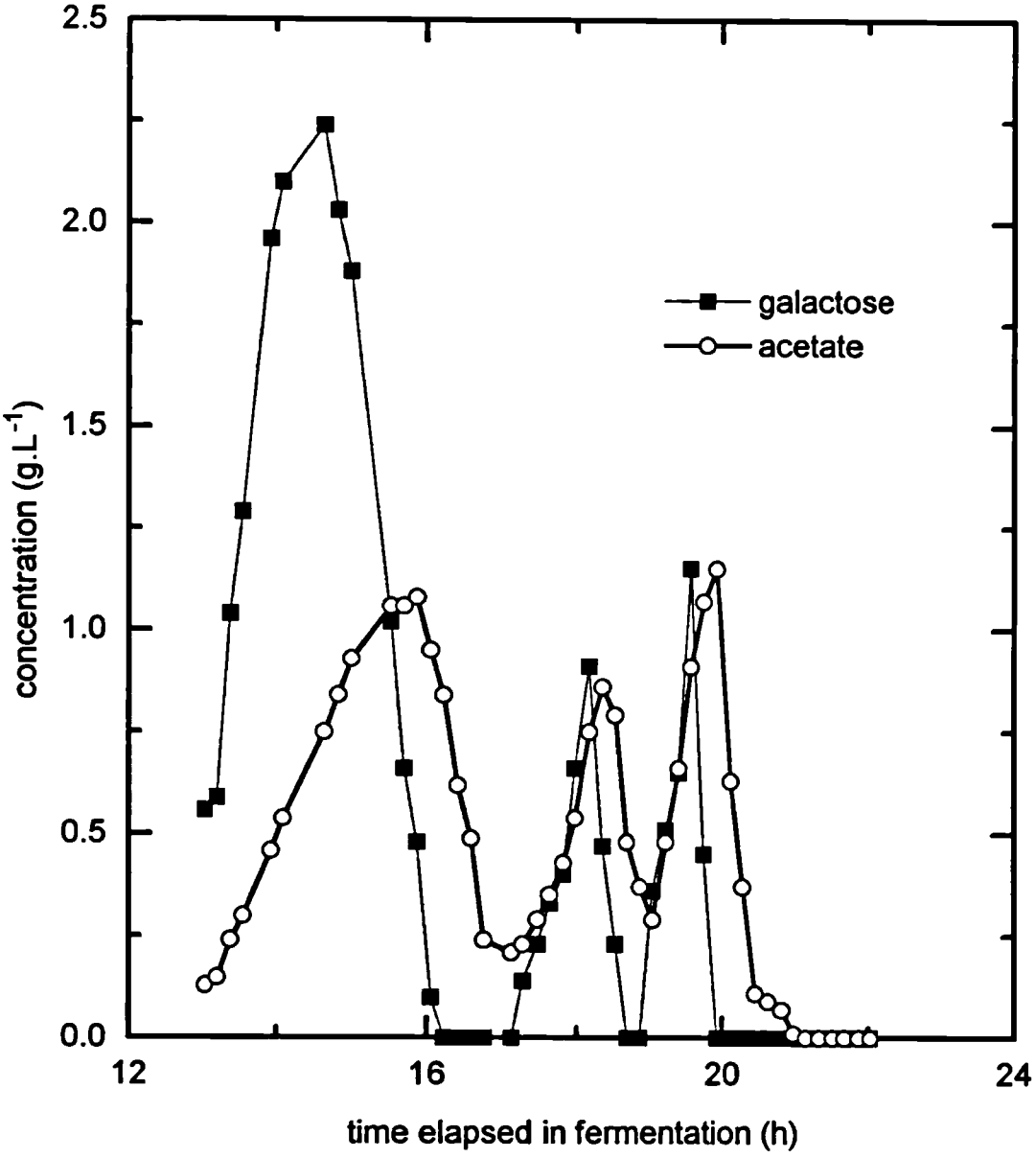


Fig. 1.8.4.a. Plot of galactose and acetate concentrations obtained from on-line HPLC analysis during the fermentation run to demonstrate the coarse control obtained by the control algorithm. The results show that the control algorithm worked - the large oscillations in concentrations of galactose and acetate result from the pump being cut by 50 % when the threshold acetate concentration was exceeded, and then reverting to normal.

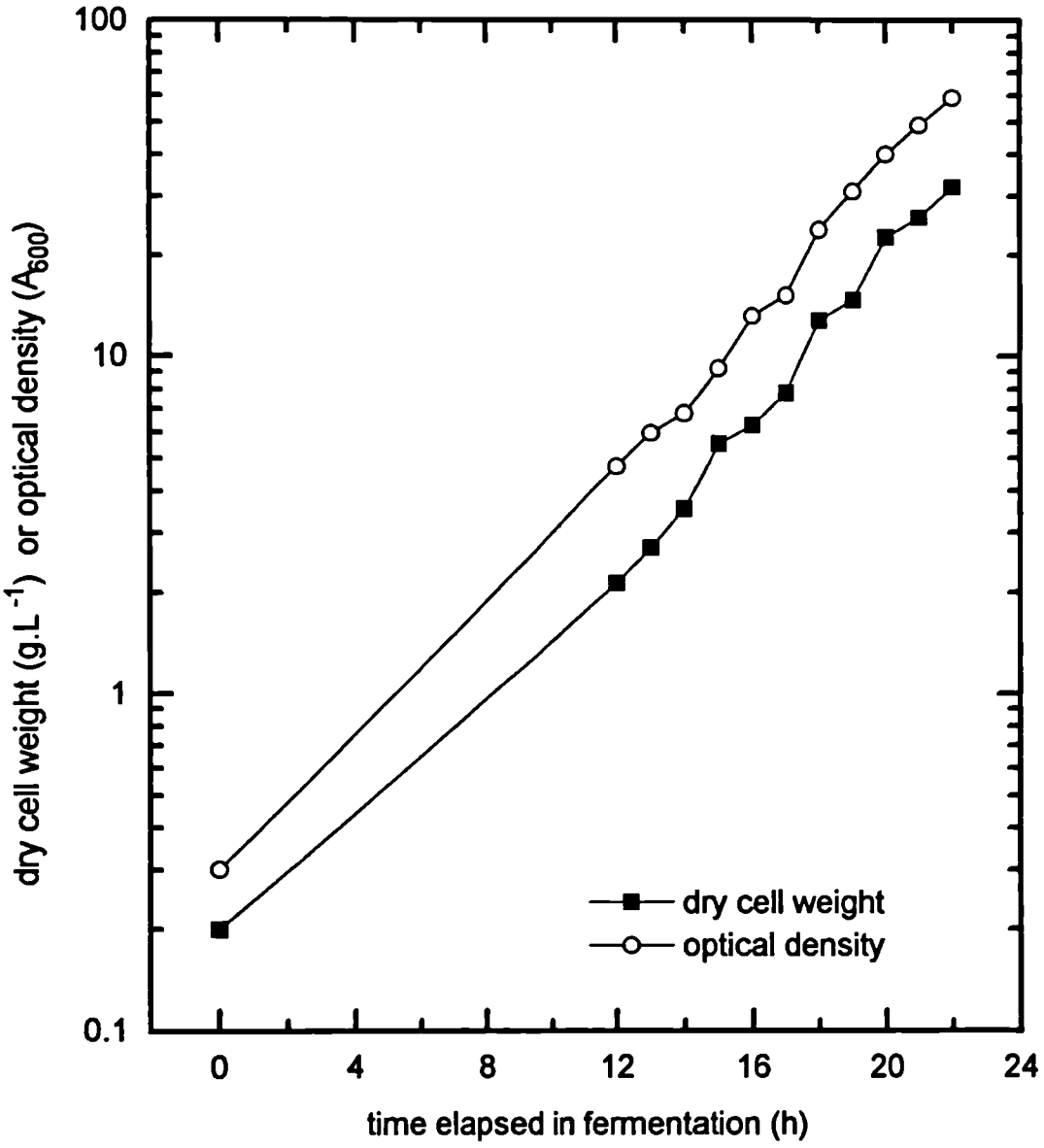


Fig. 1.8.4.b. Plot of dry cell weight and optical density of samples taken at intervals throughout fermentation run to demonstrate coarse control obtained by control algorithm.

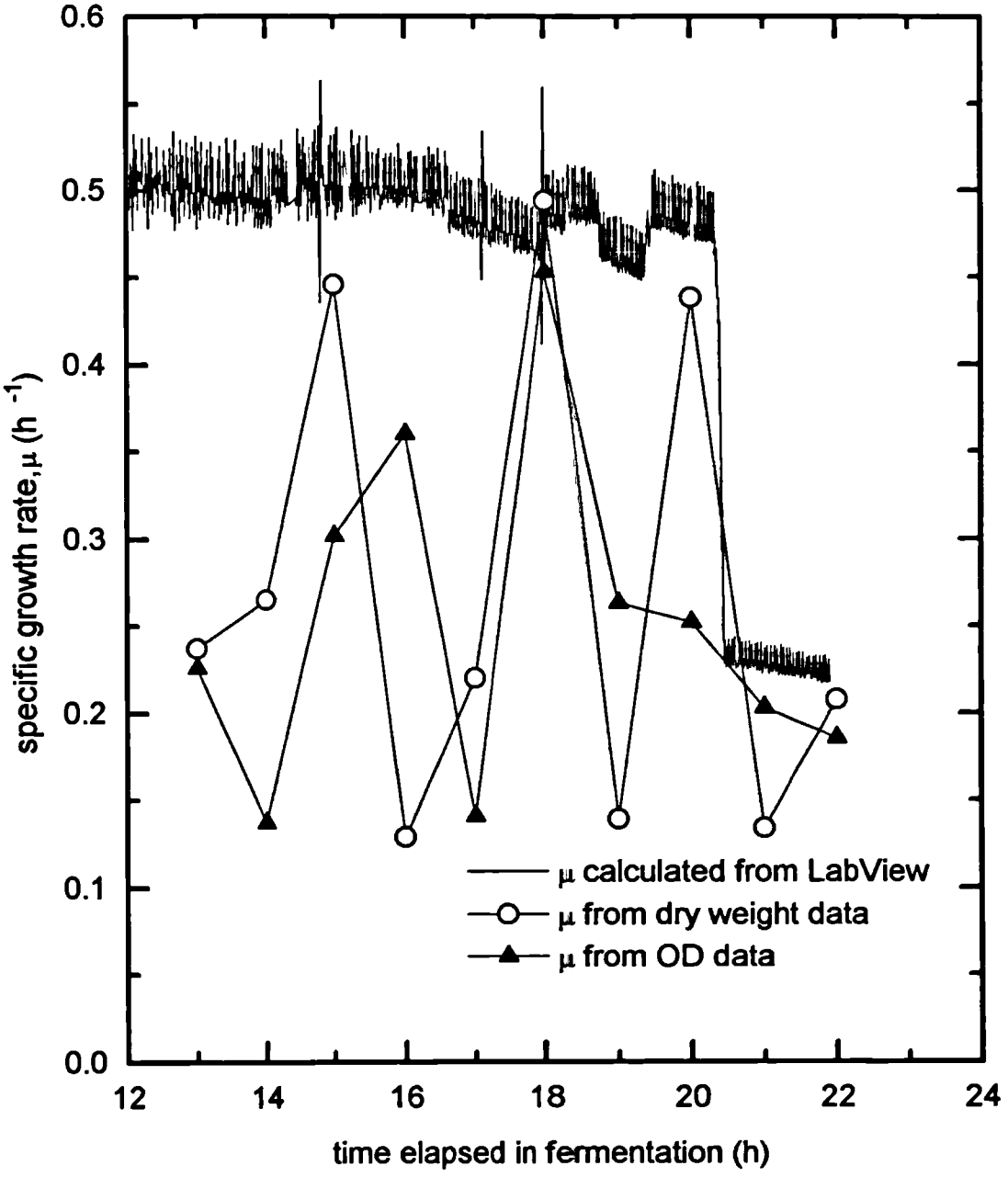


Fig. 1.8.4.c. Plot of μ calculated from LabView biomass estimate data compared to μ observed from dry weight and optical density data in fermentation run to demonstrate coarse control using control algorithm. The estimate differs from the real value because the LabView algorithm does not recalculate the specific growth rate it is feeding to when the pump is cut by 50 %. The changes on the graph are due to the program being stopped and restarted with a new estimate of biomass.

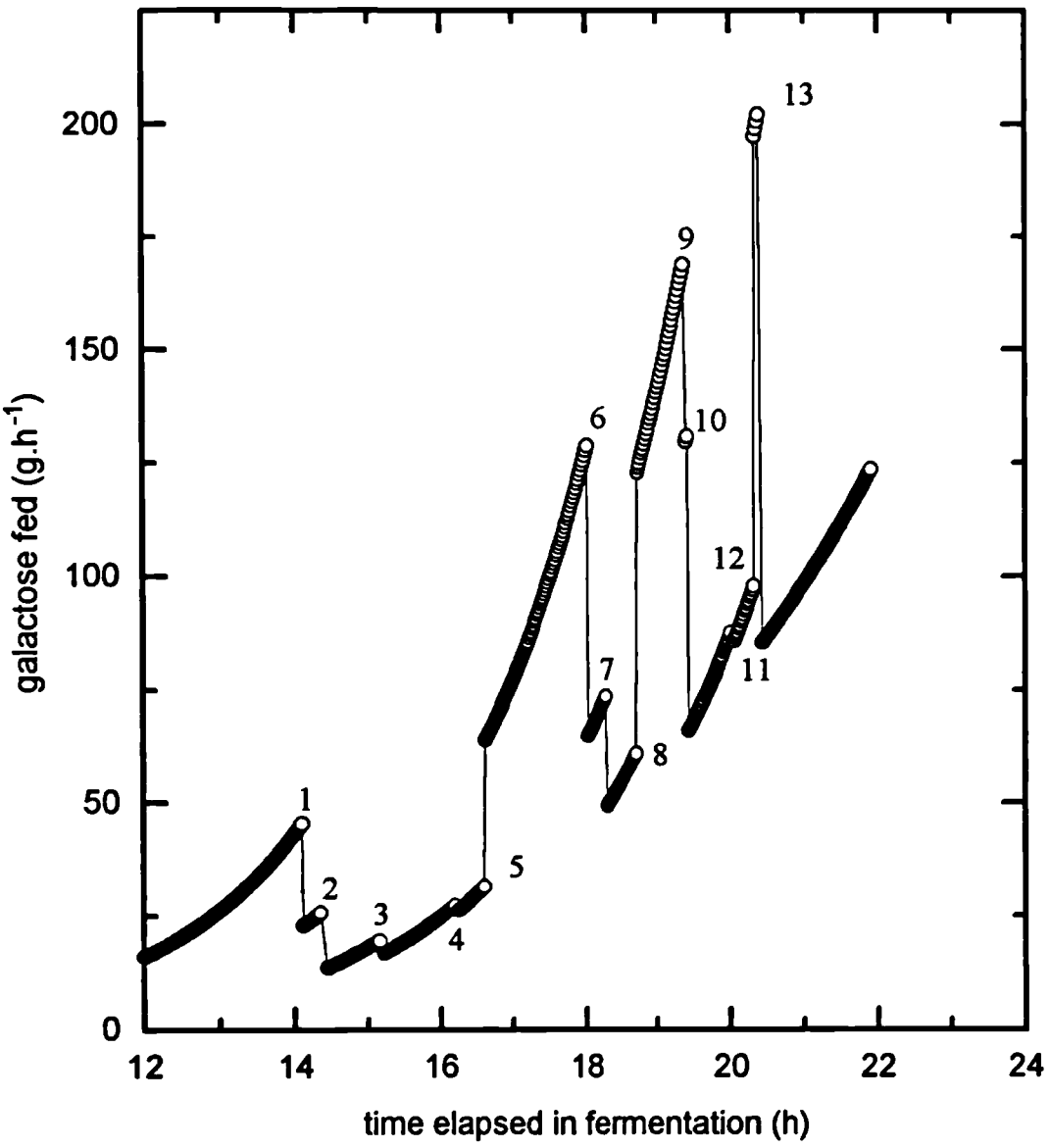


Fig. 1.8.4.d. Profile of setpoint feed rate in g.h $^{-1}$ galactose pumped to demonstrate coarse control obtained by algorithm. Numbered step changes in the exponential feed trajectory are due to either the threshold acetate conc. being exceeded or acetate conc. going below threshold (TH); or the algorithm being restarted with a new estimate of biomass (RE): 1=TH; 2=RE; 3=RE; 4=RE; 5=TH; 6=TH; 7=RE; 8=TH; 9=RE; 10=TH; 11=RE; 12=TH; 13=RE.

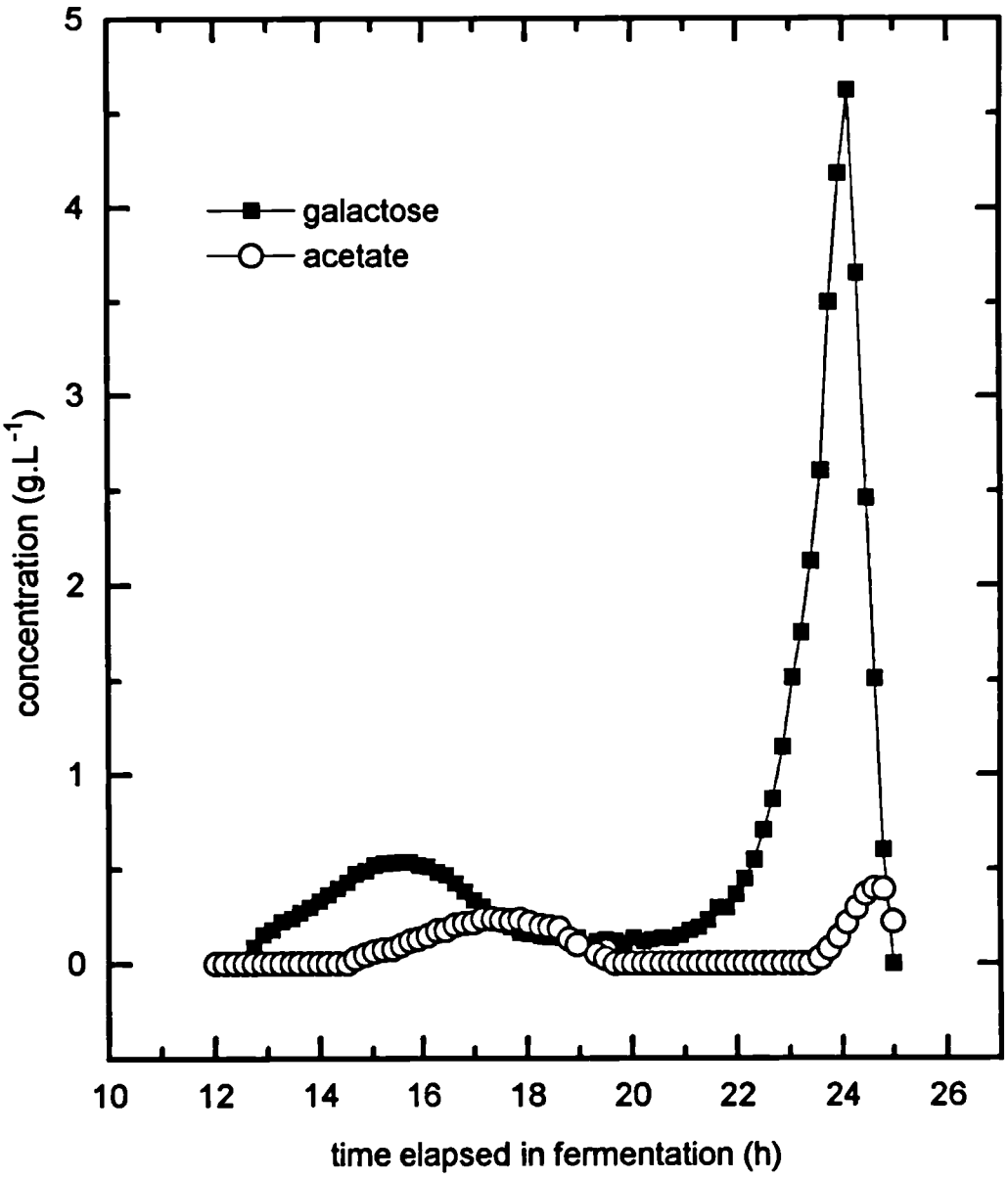


Fig. 1.8.5.a. Plot of galactose and acetate concentrations obtained from on-line HPLC analysis during the fermentation run to demonstrate the fine control obtained by the control algorithm. The acetate concentration was well controlled and did not exceed 0.23 $g.L^{-1}$ for the period when galactose was the critical analyte. It did increase above this level when the critical analyte was changed to acetate.

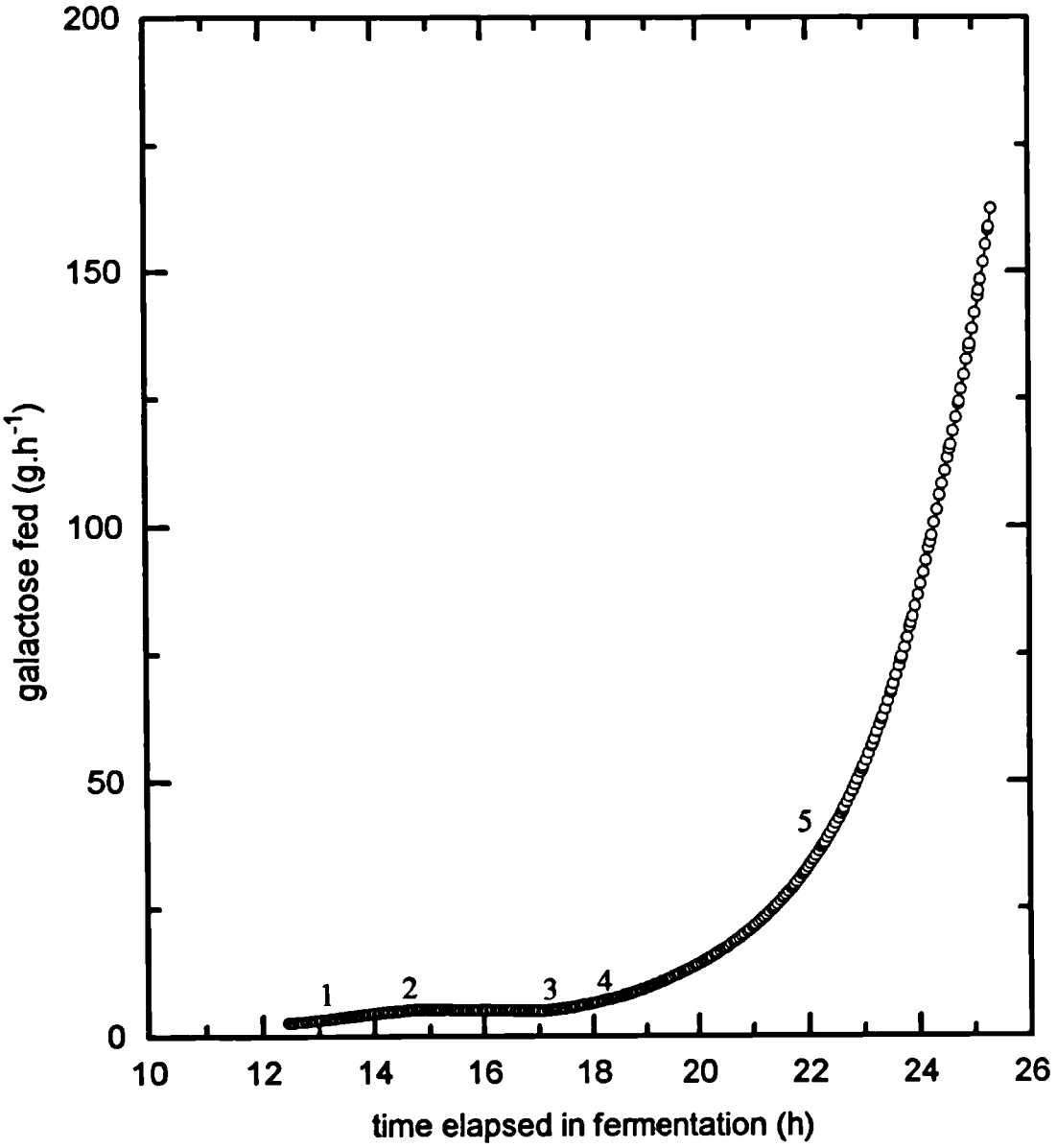


Fig. 1.8.5.b. Profile of setpoint feed rate in g.h $^{-1}$ galactose pumped during fermentation run to demonstrate fine control obtained by control algorithm. Numbers represent where:

1: threshold 1 exceeded and μ ramp disabled

2: threshold 2 exceeded and biomass estimate reduced

3: galactose concentration below threshold 2; biomass estimate no longer reduced

4:galactose concentration below threshold 1; μ ramp re-enabled

5: critical analyte changed to acetate

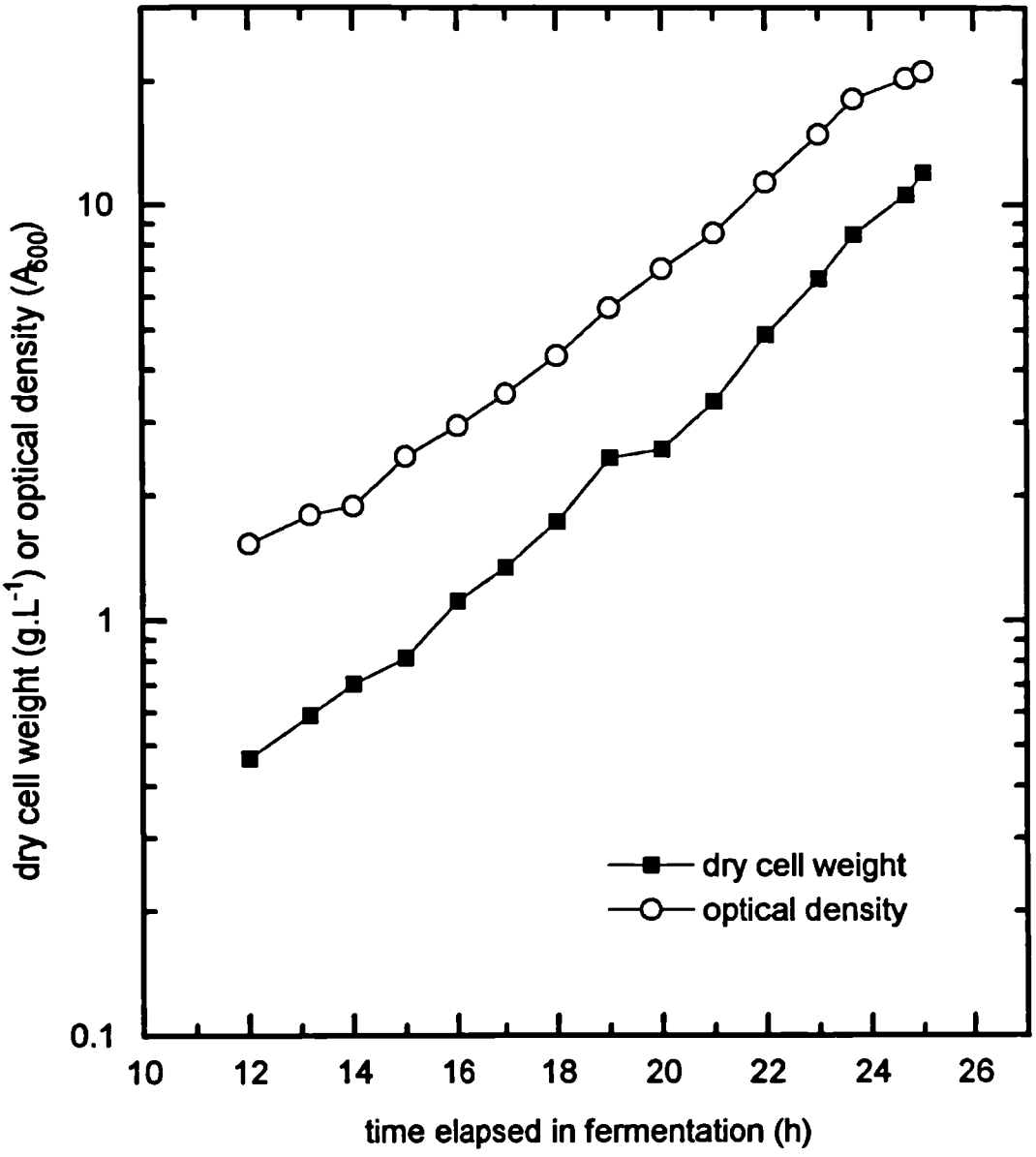


Fig. 1.8.5.c. Plot of dry cell weight and optical density of samples taken throughout fermentation run to demonstrate fine control obtained by control algorithm.

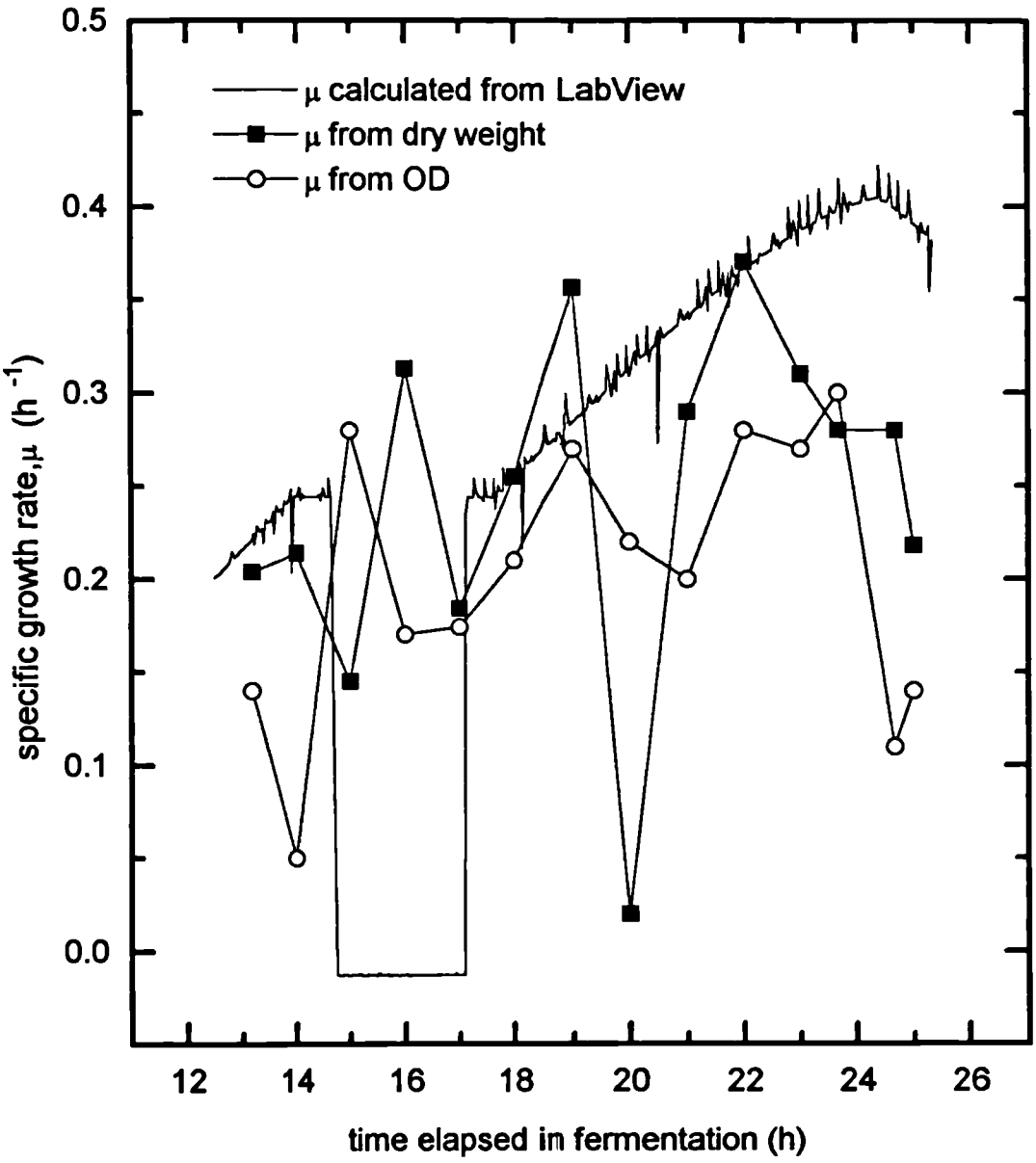


Fig. 1.8.5.d. Plot of μ calculated from LabView biomass estimate data compared to μ obtained from dry weight and optical density data during fermentation run to demonstrate fine control using control algorithm. The sudden drop in the LabView μ estimate data results from galactose exceeding the second threshold, and LabView reducing its estimate of biomass.

2. A STUDY OF THE EFFECT OF SOME GROWTH CONDITIONS ON A RECOMBINANT FERMENTATION PROCESS USING ON-LINE HPLC

2.0. INTRODUCTION

The on-line monitoring system described in section 1 of this thesis has been used to monitor (and control, with the LabView process control system) a number of recombinant *E. coli* fermentations. These experiments, plus some additional experiments in which the on-line monitoring system was not used (batch and continuous fermentations) have provided some information about the production of α-amylase, a recombinant protein coded for by a plasmid gene in the *E. coli* strain JM107, and the factors affecting production and its secretion from the periplasm into the surrounding medium. These factors include specific growth rate and the concentration of acetate present in the fermentation medium. Further information has been obtained about the fermentation process itself, such as the relationship between acetate and specific growth rate; and the effects of substrate concentration.

This section (section 2) of the thesis provides a background literature review relevant to the above issues, and goes on to describe the experiments performed to determine the effects the different growth conditions have on the state of the micro-organism, and the production of recombinant protein. A brief discussion and summary outlines the main results, and discusses how they may best be used in the optimisation of the fermentation process.

2.1. LITERATURE REVIEW

This literature review describes work done to look at the growth of *Escherichia coli* on glucose (and other sugars), and the effects observed with different growth conditions. Conditions discussed include substrate concentration, acetate production and specific growth rate (dilution rate in continuous culture). Plasmid stability in recombinant *E. coli* is also discussed because of its importance in the synthesis of non-native proteins in *E. coli*. The production of recombinant periplasmic and secreted proteins is also examined, and the final section of the literature review deals with α-amylase formation in *E. coli*, the enzyme produced by recombinant *E. coli* in this study.

2.1.1. Growth of *E. coli* on glucose

When *E. coli* are grown on certain carbon substrates (such as glucose) in fully aerobic conditions, acetic acid may be produced as a by-product. This occurs under two different, but closely related conditions (Doelle *et al.*, 1982): firstly, where the concentration of glucose in the medium exceeds a characteristic threshold concentration (Luli and Strohl, 1990); and secondly, where the growth rate of the organism is too high. The first condition is known as the bacterial Crabtree effect, which was first identified in yeast, where excess glucose under aerobic conditions leads to overproduction of ethanol. Because the production of acetic acid is undesirable (see below), many researchers have examined this occurrence to determine the cause, and several possible reasons for acetate production have been reported. Firstly, it may be caused as a result of the limited capacity of oxidative metabolism (and perhaps the capacity of the TCA cycle) being exceeded and the limited capacity of oxygen uptake rate (Han *et al.*, 1992; Reiling *et al.*, 1985; Konstantinov *et al.*, 1990 b; Andersen and von Meyenburg, 1980; Majewski and Domach, 1990; Landwall and Holme, 1977 b). Secondly, the formation of TCA cycle enzymes are repressed (Rinas *et al.*, 1989) resulting in a high intracellular NADH + H^+ concentration (Doelle *et al*, 1982). One of the key TCA cycle enzymes repressed in the Crabtree effect is α-ketoglutarate dehydrogenase (KDH) (Doelle et al., 1982; Majewski and Domach,

1990), and repression of isocitrate dehydrogenase synthesis has also been implicated. The above result in acetyl-CoA produced from the decarboxylation of pyruvate in the glycolytic pathway being diverted from the TCA cycle to acetyl phosphate, and then acetate with the production of ATP. Although the pathways leading to the production of acetate are energetically unfavourable compared to full oxidation of the glucose, formation of acetate leads to the second largest amount of ATP (and NADH + H^+) production and faster growth (Han *et al.*, 1992; Andersen and von Meyenburg, 1980). *E. coli* may even have evolved to produce acetate to enable faster growth - a teleological viewpoint (Holms, 1986).

Acetate production is also affected by the type of medium used. Han *et al.* (1992) added yeast extract to cultures and found that acetate production was diminished by reducing the specific glucose uptake rate (through lowering the cell's anabolic requirements). Addition of methionine also reduced acetate formation, although this was by enhancing the capacity of oxidative metabolism. Other researchers found that complex media resulted in acetate production at lower dilution rates (or specific growth rates) than for defined media (Riesenberg *et al.*, 1991; Meyer et al., 1984; Rieling *et al.*, 1985). In addition, it has also been found that the maximum specific acetate production rate is about twice as high on complex medium as on defined medium (Meyer *et al.*, 1984)

Other by-products are excreted by certain strains of *E. coli*, but in lower concentrations, such that on their own, they do not usually have a detrimental effect on the organism. These compounds include ethanol, propionic acid, isobutyric acid, lactic acid (Pan *et al.*, 1987) and pyruvate and succinate (Robbins and Taylor, 1989; Landwall and Holme, 1977 a).

2.1.2. Effect of glucose concentration on recombinant protein production

Apart from the effect of glucose concentration on acetate production, there are other effects on the *E. coli* culture. Firstly, a high concentration of glucose in the culture medium (*eg.* in batch fermentations) reduces the maximum specific growth rate (substrate inhibition). Growth limiting the culture on glucose (or any

nutrient) will control the specific growth rate. Possibly more important is the effect of glucose concentration on the production of recombinant protein. One issue here is that of catabolite repression. For example, if *E. coli* are grown in medium containing both glucose and lactose, the glucose will be catabolised first; lactose under these conditions will not induce β-galactosidase production, and formation of cyclic AMP (cAMP) is inhibited, blocking induction of the *lac* operon. Induction will only occur when the glucose is exhausted. Because the *lac* promoter is widely used in recombinant DNA technology, it follows that recombinant proteins induced by the *lac* promoter are influenced by catabolite repression (Warnes *et al.*, 1991). The Crabtree effect has also been found to be responsible for repression of a plasmid encoded product even when synthesis of the recombinant protein is under the control of the temperature inducible lambda P_R promoter/cI857-repressor expression system (Rinas *et al.*, 1989). Yang (1992) has also found that growth and recombinant protein production in an E. coli culture were inhibited by a high glucose concentration. Bech Jensen and Carlsen (1990) found that the specific yield of recombinant protein was dependent on constant glucose feed rate, and maximal yield occurred at an intermediate feed rate, implying that both glucose starvation and glucose excess were detrimental to the cells' performance.

2.1.3. Acetate production in E. coli and its affect on the cell

2.1.3.1. Reasons for wanting to keep acetate excretion to a minimum

The production of acetic acid in a recombinant *E. coli* fermentation is undesirable for four reasons. Firstly, the overflow of carbon source to acetic acid means that the process is inefficient, and nutrients that could be used for producing more biomass or product are being wasted in the production of acetate. In many strains, this production of acetic acid is not particularly severe, however, some researchers have reported a massive overproduction of acetic acid representing a very large wastage and reduced yield *eg.* Konstantinov *et al.* (1990 b) found that a strain they used produced a large amount of acetate resulting in a concentration of up to 35 $g.L^{-1}$ at the end of a fermentation.

The second reason for wanting to minimise the production of acetic acid is that above a certain concentration, usually between 1.2 and 14 $g.L^{-1}$ dependent on strain, acetate inhibits cell growth (Landwall and Holme, 1977 a and b; Konstantinov *et al.*, 1990 b; Luli and Strohl, 1990; Yang, 1992; Pan *et al.*, 1987). Koh *et al.* (1992) found that the inhibitory effect of acetate on batch growth could be modelled by the following equation:

$$\frac{\mu}{\mu_{max}} = \frac{1}{1 + k[Ac]}$$

where k is the growth inhibitory constant

It has long been known that growth inhibition of *E. coli* occurs at a low pH (where there is a higher proportion of protonated acid than the dissociated form), even at low concentrations (60 μmoles) of acetate and other volatile fatty acids (Wolin, 1969). However, acetic acid at neutral pH in high enough concentrations is toxic to the cell. Researchers believe that undissociated acetic acid (present at neutral pH along with the dissociated form; pKa of acetic acid is 4.73) can cross the cell membrane because of its weak lipophilic nature, and can then function as an uncoupler of the proton motive force. When the protonated form passes into the interior of the cell, it dissociates at the higher internal pH, decreasing the intracellular pH. The passage of protonated acetic acid across the membrane causes dissociated acid in the medium to be protonated by the equilibrium causing a net electroneutral hydrogen ion influx. The external pH is unlikely to change much because of the medium buffering, but the decrease in intracellular pH causes an uncoupling effect. This may act to reduce the capacity of an *E. coli* culture to sustain balanced growth (George *et al.*, 1992; Luli and Strohl, 1990). Acetate inhibition of cell growth has also been found to be more marked with recombinant cells than for host cells (Koh *et al.*, 1992).

The third reason for reducing acetate production, is because it is well documented that the presence of a high concentration of acetic acid in

recombinant *E. coli* fermentations can have a major effect on the production of recombinant protein. This is probably caused by the uncoupling effect on the cell's metabolism described above, which would extend to all cellular activities including the production of recombinant protein. Examples of this effect in the literature include the inhibition of recombinant: β-galactosidase production in *E coli* HB101 with plasmid pTREZ1 (Shimizu *et al.*, 1988); human growth hormone production in *E. coli* MC1061 from a plasmid derived from pAT153 (Bech Jensen and Carlsen, 1990); phenylalanine production in *E. coli* AT2471 with plasmid pSY130-14 (Konstantinov *et al.*, 1990 b).

The fourth reason is that production of excess acetic acid requires the addition of alkali to maintain the culture pH. Ammonium hydroxide is frequently used in this role, however ammonia is known to be inhibitory to cell growth above a concentration of 170 mM (Thompson *et al.*, 1985). That is one of the reasons for its use as a pH regulator, because it cannot all be added at the start of the fermentation; however if excess acetate is produced, excess ammonia added to control pH may cause the same problem. Excess salt concentrations are also undesirable because they can reduce the expression rate of a recombinant protein (Bech Jensen and Carlsen, 1990), so even the addition of quantities of sodium or potassium hydroxide to maintain culture pH after production of acetate can have detrimental effects on the culture.

2.1.3.2. The assimilation of acetic acid

Once produced, acetate can be re-assimilated in many *E. coli* strains when glucose is absent (Luli and Strohl, 1990; Shimizu *et al.*, 1988; Andersen and von Meyenburg, 1980), although one research group (Lee and Chang, 1990) observed that continual substrate feeding is required for acetic acid reuse, while Nancib *et al.* (1991) found that acetate was not re-assimilated in a yeast extract-free medium. Interestingly, Yang (1992) found that when *E. coli* switched carbon source from glucose to acetate, the total carbon yield for biomass was reduced but the carbon yield for recombinant protein expression was maintained, suggesting that acetate is used preferentially in producing β-galactosidase, the

recombinant protein. If the concentration of acetate in the medium is too high, the cellular metabolic activities are inhibited, including those for using acetate, and so acetate assimilation will be very slow (Lee and Chang, 1990).

Mechanism of acetate reuse

Acetate is actively transported into the cell, and is converted to acetyl-CoA by acetyl-CoA synthetase with the concomitant hydrolysis of ATP in the following reaction:

$$\text{acetate} + \text{HS-CoA} + \text{ATP} \rightarrow \text{acetyl-SCoA} + \text{AMP} + \text{PPi}$$

The acetyl-CoA can then be fed into TCA cycle yielding NADH. The problem with this, however, is regenerating the intermediates of the cycle that also serve as materials for biosynthesis. Oxaloacetate cannot be produced in the anaplerotic sequence from phosphoenolpyruvate (PEP), and PEP cannot directly be synthesised from acetate. Instead, another anaplerotic sequence is invoked: the glyoxylate bypass. Two enzymes are produced for this bypass: isocitrate lyase, which cleaves isocitrate to succinate and glyoxylate; and malate synthase, which catalyses the condensation of acetyl-CoA with glyoxylate to yield L-malate. The TCA cycle and glyoxylate bypass are shown in figure 2.1.3.2.a. To illustrate the function of the glyoxylate bypass: two oxaloacetates react with two acetyl-CoAs to yield two citrate molecules, which are then converted to two isocitrates. One of these is oxidised through the TCA cycle, regenerating one oxaloacetate. The second is cleaved to succinate and glyoxylate; the oxidation of succinate providing a second oxaloacetate molecule. The glyoxylate condenses with more acetyl-CoA, forming L-malate, from which a third oxaloacetate is produced. This means that one oxaloacetate is available for biosynthesis (Gottschalk, 1986). The activation of the glyoxylate bypass is effected by phosphorylating isocitrate dehydrogenase, inactivating it (Walsh and Koshland, 1985).

2.1.4. The effect of specific growth rate on recombinant protein production

It has been found that by controlling the specific growth rate (in a fed-batch

fermentation) or dilution rate (in a continuous fermentation) it is possible to affect the production of recombinant protein quite considerably (Fu et al., 1993; Nancib and Boudrant, 1992; Brown *et al.*, 1985; Park and Ryu, 1990), and yields are generally higher at lower growth rates/dilution rates. Conversely, specific growth rate decreases linearly with concentrations of intracellular plasmid content and protein product *ie* if a high plasmid copy number results (through growth conditions, for example), the maximum specific growth rate of the cell will be reduced, (Betenbaugh and Dhurati, 1990; Summers, 1991), probably as a result of the high levels of protein being made. High production of recombinant protein product can be very detrimental to the cell, which can suffer severe stress or even die (Kim and Ryu, 1991; Togna *et al.*, 1993). The plasmid concentration in turn often has a direct bearing on the production of recombinant protein (Zabriskie and Arcuri, 1986) although regulatory mechanisms may impose an upper limit (Friehs and Reardon, 1993), and promoter strength can have a greater influence on production than plasmid content (Kim and Ryu, 1991; Togna *et al.*, 1993). Apart from using control of growth rate to limit overflow to acetate, and feeding nutrients to a slow growth rate in a high cell density culture to maintain dissolved oxygen concentration above a certain amount (both of which would improve protein yields) specific growth rate has a direct bearing on the plasmid copy number in the cell. Many researchers have controlled specific growth rate in *E. coli* fermentations in order to prevent acetate excretion, and found improved recombinant protein yields which they attributed to the control of acetate. Although acetate effects are important, an improvement in productivity could have been mostly, or in part, due to a reduced specific growth rate.

The relationship between specific growth rate and recombinant protein production is quite complex. Another important issue is that of how specific growth rate affects plasmid stability. Plasmid stability has been observed to decrease with increasing growth rate (Nancib and Boudrant, 1992) because there is a growth advantage of plasmid free cells over those containing plasmid; the rate of plasmid loss increases with dilution rate (Friehs and Reardon, 1993; Nancib and Boudrant, 1992; Nam *et al.*, 1987). The reasons for the observed instability will be discussed in the following section.

2.1.5. Plasmid stability

An important issue affecting the productivity of a recombinant *E. coli* fermentation is that of plasmid stability, which is a function of the genetics of the plasmid and is strongly influenced by the genetics and physiology of the host (Zabriskie and Arcuri, 1986). Productivity of a recombinant plasmid has been found to decrease with age of the culture (Nam *et al.*, 1987). Two types of plasmid instability have been observed. The first is called structural instability, which is due to the insertion, deletion or rearrangement of DNA. This can be difficult to detect in a culture because the growth rate and marker phenotype are the same as in the original cells (Friehs and Reardon, 1993). It has been found that structural stability can be affected by the growth environment of the cell *eg.* it has been found in glucose and phosphate limited chemostats (Friehs and Reardon, 1993; Zabriskie and Arcuri, 1986).

The second type of structural instability is segregational instability, which is more frequently reported and examined by fermentation scientists because it is a major problem in recombinant fermentations, and results from defective partitioning during cell division. A number of factors affect segregational stability, including specific growth rate or dilution rate. Nancib and Boudrant (1992); Mosrati *et al.* (1993) and Warnes *et al.* (1991) found that stability improved with decreasing dilution rate; in contrast, Park and Ryu (1990) found that plasmid stability improved with *increasing* dilution rate. Wild type, low copy number plasmids show high stability (Friehs and Reardon, 1993), however, most recombinant plasmids are lost to some extent during cell culture. Segregational instability is undesirable, because a lower average copy number will usually result, causing reduced yields of recombinant protein; and also because the plasmid free cells that ultimately result from this instability will have a higher specific growth rate leading to a higher concentration of plasmid free cells.

There are a number of ways of improving or overcoming segregational instability. The best known way is by including a gene for antibiotic resistance on the plasmid, and by growing the cells in the presence of the antibiotic thereby

selecting for cells containing the plasmid. Problems with this approach are that antibiotics are expensive, they need to be separated from the desired product, and they can be inactivated during a process. It is also generally undesirable to use antibiotics because of the biological action of the substance. Another selective approach is by cloning a gene essential for the host cell onto the plasmid and this has been very successful (Friehs and Reardon, 1993). Selection can also be achieved by killing plasmid free cells *eg.* by including the bacteriophage λ repressor on the plasmid, and infecting the host cells with the phage. Although these techniques prevent plasmid loss, they do not maintain copy number. A method called runaway plasmid replication allows good plasmid stability and high productivity. A promising, non-selective, genetic approach for enhanced plasmid stability is the incorporation of the *cer* locus of the plasmid ColE1, a natural multicopy plasmid of *E. coli* which is maintained stably under most growth conditions. The accumulation of plasmid multimers gives rise to the unstable inheritance of high copy number plasmids, which reduces the number of plasmids that segregate independently at cell division. The *cis* acting plasmid site, *cer*, and the *trans* acting product of the chromosomal *xer* gene participate in the conversion of dimers and higher oligomeric forms to monomers, resulting in plasmid stability (Summers and Sherratt, 1988).

2.1.6. The growth of *E. coli* on galactose

The carbon source used in the recombinant *E. coli* fermentations in this project was galactose, therefore, some mention should be made of the growth of *E. coli* on galactose. Andersen and von Meyenburg (1980) did some studies on the growth of *E. coli* on a number of different carbon sources, including glucose and galactose, and compared several different growth parameters. The main differences are that firstly, the cell needs to expend energy for the transport of galactose into the cell, whereas the transport system that actively moves glucose into the cell is coupled to the phosphorylation of glucose to glucose-6-phosphate, phosphoenolpyruvate being the phosphate donor. Secondly, the specific growth rate on galactose was less than that on glucose, possibly as a result of the different transport mechanism. Thirdly, Andersen and von Meyenburg (1980)

found that growth on galactose produced marginally more by-product excretion (acetate) than on glucose, causing a slightly lesser biomass yield on galactose as carbon source than glucose. The reason for this was not speculated on.

2.1.7. Secretion of proteins in *E. coli*

E. coli remains one of the most used hosts for the commercial production of recombinant products because of the knowledge of the organism. The majority of systems express the protein in the cytoplasm, however, a consequence of overproduction of cytoplasmic proteins is that inclusion bodies, or insoluble aggregates of protein are formed; and the protein is in a denatured form, and needs careful treatment after extraction from the cell to ensure reasonable efficiency in refolding. Because of this problem, there has been a move towards using systems which secrete the native, biologically active protein into the periplasmic space. There are problems with the secretion of recombinant proteins, *eg.* how high is the proportion of mature protein with the signal peptide removed.

The secretion of proteins across the cytoplasmic membrane uses a number of different proteins working together (Zhu *et al.*, 1993), *eg.* the chaperones that mediate the correct folding of proteins by specifically and non-covalently binding to the surface of the protein that is exposed transiently during certain cellular processes such as transport across the membrane. The proteins of the membrane translocase complex are also required and they transport proteins across the cytoplasmic membrane into the periplasmic space, possibly by forming secretion channels to facilitate protein transport (Zhu *et al.*, 1993).

Aside from the avoidance of inclusion bodies, there is another reasons why it is favourable to have recombinant proteins secreted into the periplasmic space, and that is the relative ease with which the protein can be removed from the organism for processing. With periplasmic proteins, the cells can be separated from the fermentation medium by filtration or centrifugation, and then it is fairly simple to release the comparatively few periplasmic proteins from which the protein of

interest is to be separated, leaving the cytoplasmic membrane intact. This protects the product from contamination by cytoplasmic proteins and nucleic acids, simplifying the downstream processing.

2.1.8. α-Amylase and its production in *E. coli*

Thermostable α-amylase, the recombinant protein produced in recombinant *E. coli* fermentations in this project, is an endoamylase which hydrolyses α-(1→4)-glycosidic linkages of amylose and amylopectin molecules of starch. It is widely used in the paper, textile, starch, distillation and brewing industries for the liquefaction of starch (Mukhopadhyay, 1992).

The gene for the α-amylase was cloned from *Streptomyces thermoviolaceus* strain CUB74 into *E. coli* JM107 using the high copy plasmid pUC8 as the cloning vector. *E. coli* with the plasmid pQR300 was found to express the gene, and exported the enzyme into the periplasmic space, some of which leaked into the culture medium. It was found to have the same molecular mass as that expressed by the parent strain, suggesting a similar processing (Bahri and Ward, 1990). Additional work by French and Ward (1991, unpublished) resulted in the use of *E. coli* JM107 containing the new plasmid pQR126 with the gene described above, for the production of α-amylase.

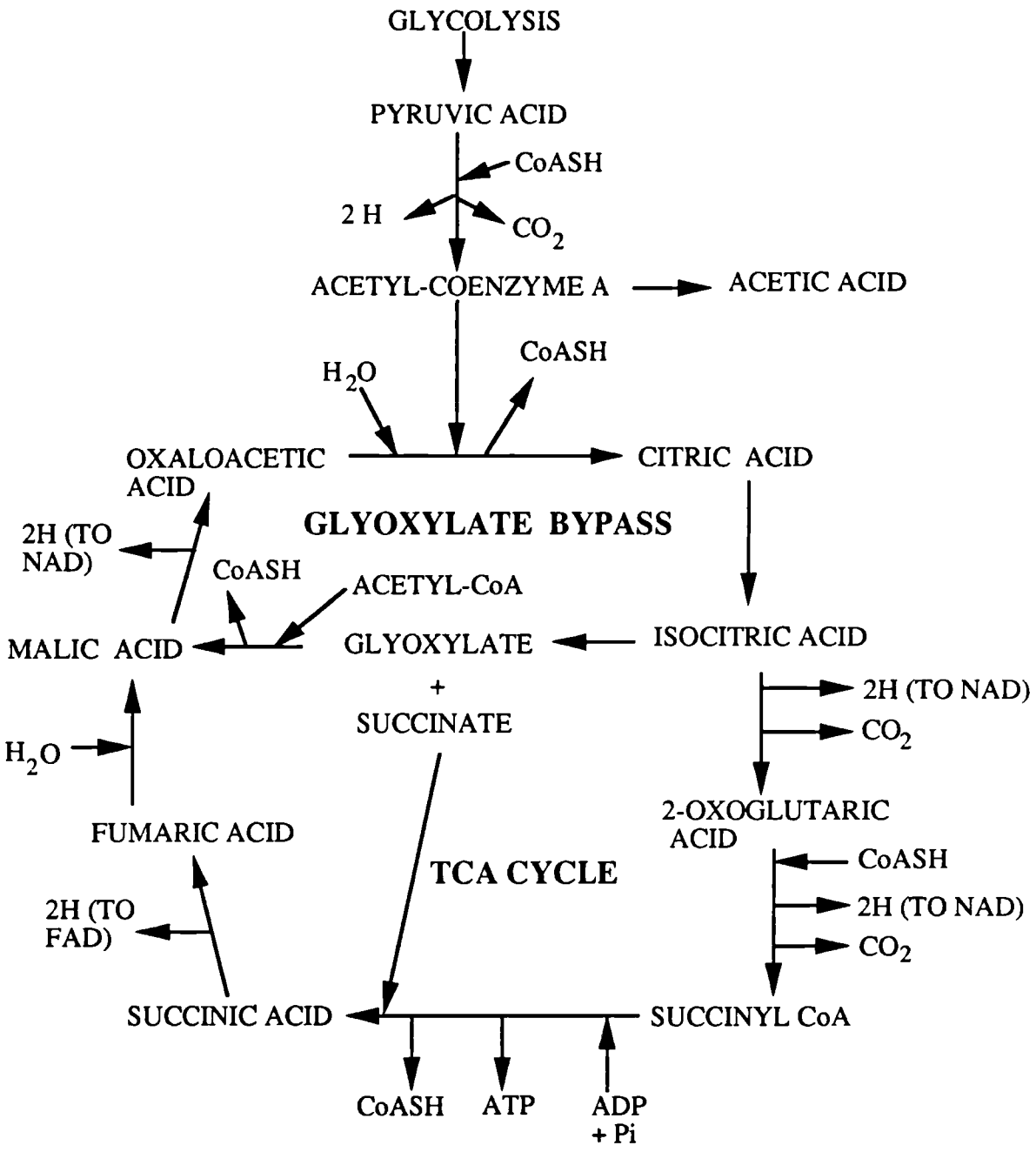


Figure 2.1.3.a. TCA cycle and glyoxylate bypass. The TCA cycle is used by cells to oxidise glucose, provide reducing power in the form of NADH, and for the production of precursors of biosynthetic intermediates. It is shown as the series of reactions around the perimeter of the diagram; from acetyl Co-A to citrate, then converted to isocitrate, 2-oxoglutarate, succinyl CoA, succinate, fumarate, malate, oxaloacetate and back to citrate. The glyoxylate bypass enables *E. coli* to grow on acetic acid, and produce the necessary biosynthetic intermediates. It is shown in the centre of the diagram, converting isocitrate to glyoxylate and succinate. The glyoxylate then condenses with acetyl-CoA to produce malate.

2.2. MATERIALS AND METHODS

The materials and methods in this section relate to the batch, fed-batch and continuous fermentations used to examine fermentation conditions affecting growth and recombinant protein production in *E. coli.*

2.2.1. Organism used

The organism used in this study was *E. coli* JM107 + pQR126. Cloning was done in the Biochemistry Department at UCL, and is described in Bahri and Ward (1990). The high copy number plasmid contains a gene for α-amylase, a starch degrading enzyme and a gene for kanamycin resistance for plasmid selection. The α-amylase gene is controlled by the *lac* promoter, and is therefore subject to catabolite repression by glucose. Catabolite repression of the *lac* operon decreases with carbon source as follows:
gluconate > glucose > fructose > maltose > lactose > galactose > glycerol > succinate (Dawes and Sutherland, 1992).
For this reason, the organism was grown on galactose during this project.

2.2.2. Growth media

The medium used in the fermenter and seed vessel (for batch, fed-batch and continuous culture) is as follows ($g.L^{-1}$):
$(NH_4)_2SO_4$, 10; Na_2HPO_4, 2.16; KH_2PO_4, 0.64; NaCl, 5; citric acid, 0.2; $FeSO_4.7H_2O$, 0.2; $MgSO_4.7H_2O$, 0.2; kanamycin, 0.01; thiamine, 0.1; trace element solution, 1 $mL.L^{-1}$.
The trace element solution is a 1000 x stock to provide final concentrations of the following ($g.L^{-1}$):
$CaCl_2$, 0.01; H_3BO_3, 0.004; $MnCl_4.4H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.0004; $CoCl_2.6H_2O$, 0.0004; $NaMoO_4.2H_2O$, 0.0002.
Galactose was the carbon substrate used, and was added in different concentrations and in different methods according to the protocol used. Galactose and $MgSO_4.7H_2O$ were sterilised separately in the autoclave;

kanamycin and thiamine were filter sterilised (Gelman Acrodisc, 0.2 μm pore pre-sterilised filters) into the medium prior to inoculation. All chemicals were obtained from Sigma.

2.2.3. Seed solution

The volume of seed solution used in batch and fed-batch culture was 500 mL, which gives a 6.25 % inoculum (total fermenter volume 8 litres). The seed solutions contained 4 $g.L^{-1}$ galactose, and were inoculated from agar plates (nutrient agar, kanamycin (10 $\mu g.mL^{-1}$) and starch (1 %)), and incubated at 37°C for about 12 hours.

2.2.4. Fermentation

2.2.4.1. Batch and fed-batch culture

The fermenter used was a Chemap 14 litre fermenter; the volume after inoculation being 8 litres. Standard fermentation variables were monitored and controlled; pH was set to 7, controlled with 4 M NaOH; temperature was controlled at 37°C. Dissolved oxygen (DOT) was monitored and kept above 20% by manually adjusting aeration and agitation rates. Exhaust gas was analysed by a VG MM 8-80 mass spectrometer, and results were used to obtain the derived parameters carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and respiratory quotient (RQ). A DEC PDP 11-73 operating the Bio-i software (Biotechnology Computer Systems) was used to monitor all these variables. For fed-batch culture, two Watson Marlow 101 UR pumps were used to add feed solution to the fermenter; one a 2 rpm (max) pump, and the other a 32 rpm (max) pump, to cover the whole range of feed rates required.

2.2.4.2. Continuous culture

Continuous culture experiments were performed in an LH series 2000 2 litre fermenter, at a running volume of 1.5 litres. Dilution rates were set by altering flow rates of two Watson Marlow 101 UR pumps (for inflow and outflow). Only exhaust gas composition was monitored by computer; other variables were logged

manually. As before, temperature was controlled at 37°C, pH at 7 with 3 M NaOH. DOT was kept high (> 20 % as before). The medium contained 5 $g.L^{-1}$ galactose in addition to the other medium components. At each steady state, at least three successive samples were taken for amylase assays (in duplicate), and dry weight and optical density readings. Samples in each steady state were usually taken at intervals of one pot volume (although this was not always possible; sometimes samples were taken at shorter intervals than this). OD readings and CER profiles were used to determine whether the culture was in steady state; in addition, the steady state was only considered achieved when amylase activities of the samples were very close.

Continuous culture was also used to determine μ_{max}, the maximum specific growth rate of the organism. This is done by increasing the dilution rate to approximately twice the expected μ_{max}, and then taking samples every fifteen minutes and measuring the optical density. The natural logarithm of optical density is plotted against time, and the slope obtained from a linear regression. μ_{max} is then obtained from:

$$\mu_{max} = d + m$$

where d = dilution rate and m = slope of ln OD versus time.

Acetate studies in continuous culture were done by adding sodium acetate (Sigma) to the fermenter, or to the medium in sterile aspirators.

2.2.4.3. Control of pH in fermentation

The pH in batch and fed-batch fermentations was controlled at 7 using 4 M NaOH. In continuous culture, 3 M NaOH controlled the pH.

2.2.5. Assays

2.2.5.1. Dry weight, optical density and specific growth rate

Dry weight determinations were made by filtering a known volume of broth through a pre-weighed 0.2 μm filter (Millipore Type GVWP), drying at 105°C for 24 hours, and reweighing. Optical density measurements were made by

measuring the absorbance at 600 nm, diluting with distilled water when necessary. Specific growth rates were calculated from the equation:

$$\mu = \frac{\ln x(t2) - \ln x(t1)}{\Delta t}$$

μ = specific growth rate

x(t) = biomass concentration at time t

Δt = time difference between t2 and t1 (hours)

2.2.5.2. Amylase assay

The starch degrading enzyme, α-amylase, the gene for which is cloned into the organism used in this study, is secreted into the periplasm of *E. coli*, but some leaks into the surrounding medium. Both periplasmic and extracellular α-amylase are measured.

Release of periplasmic (and some cytoplasmic) amylase:

Method: A 1 mL sample of fermentation broth is spun in the Eppendorf microcentrifuge for 5 minutes. The supernatant is decanted and kept for α-amylase analysis; this is the "extracellular fraction". To the remaining cell pellet, 200 μL fractionation buffer (see below) is added and the pellet resuspended. After 10 minutes, 200 μL deionised water is added, mixed in and allowed to stand for a further 10 minutes. The suspension is then centrifuged for 10 minutes; the resulting supernatant is termed the "periplasmic fraction", although this method of fractionation (where tris is included in the fractionation buffer) does cause some cell lysis, and may release any cytoplasmic amylase.

Fractionation buffer: Sucrose (20 %); EDTA (1 mM); tris (200 mM); lysozyme (500 $\mu g.mL^{-1}$).

Assay: The assay is based on the colour change of a starch-iodine complex in solution, due to the degradation of starch by α-amylase.

Materials: (All chemicals obtained from Sigma)
Starch solution: 0.5 % soluble starch in 15 mM sodium phosphate buffer at pH 5.8; solution heated in the microwave for 90 seconds and filtered (Whatman No.1) when hot.
Phosphate buffer: 15 mM Na_2HPO_4, pH 5.8 (titrated with 1 M HCl).
Solution A: 2.2 % I_2 and 4.4 % KI in deionised water.
Solution B: 2 % KI in deionised water.
Solution C: 200 μL solution A into 100 mL solution B.

Method: A volume of sample is added to phosphate buffer to bring the final volume to 0.5 mL, and incubated at 50°C. Starch solution is also incubated at 50°C. 0.5 mL starch solution is added to the sample, mixed, and a timer is started. At time intervals over 15 minutes, a 50 μL aliquot is removed and added to 1 mL solution C in a cuvette. The absorbance of each cuvette is read at 620 nm; a plot of absorbance versus time is linear, the negative slope giving the α-amylase activity in units.min^{-1}.mL^{-1}.

2.2.5.3. Galactose and acetate

Galactose and acetate are measured either on-line or off-line by HPLC according to the method described in section 1.5.

2.2.5.4. Plasmid stability

Plasmid stability is measured by doing serial dilutions, and pipetting an equal volume of diluted sample onto agar plates, containing nutrient agar, starch (1 %) and kanamycin (designated kan+ plates); or just nutrient agar and starch (designated kan- plates). The difference between the number of colonies growing on kan- plates and kan+ plates over a 24 hour period gives an indication of the percentage of organisms that have lost the plasmid.

2.2.6. Control

Some fermentations have made use of the LabView control system described in

section 1.8., either using feed-forward control, without corrective feedback action; or using feed-forward control with automatic feedback from the on-line monitoring system. In one fermentation (designated **FB2**), the on-line monitoring system was used to ensure the concentration of substrate was in the correct limits, and by using these results to manually operate a feed-pump.

2.2.7. Fermentation nomenclature

This section describes the profiles of the fermentations performed; and gives each fermentation (an) initial(s) to describe the type of fermentation (**B** = batch; **FB** = fed-batch; **C** = continuous) and a number to identify a specific fermentation. This nomenclature is used throughout section 2 when presenting results for a fermentation.

B1 refers to a typical batch fermentation (in which all medium components including 35 $g.L^{-1}$ galactose were added to the fermentation at the start) which was performed to provide a "standard" for comparison of later fed-batch and continuous fermentations.

FB1a refers to a fermentation in which the medium and inoculum were prepared as described earlier (2.2.2 and 2.2.3). After fermenter inoculation, LabView (described in 1.8) set the feed pump to feed the substrate (galactose, 500 $g.L^{-1}$) to allow the cells to grow at a μ of 0.2 h^{-1} overnight (for 11.5 hours) without the use of the on-line monitoring system. The following morning, the on-line HPLC was started, and when the galactose concentration began to rise due to a deviation between actual and estimated biomass concentration, the algorithm was stopped and restarted with a new estimate of biomass. When the optical density of the broth reached 20, the fermenter was drained until about 250 mL remained in the tank, and fresh, sterile medium added to bring the volume to 8 litres. This formed the starting point for the fermentation **FB1b**.

FB1b was prepared as described above. At the start of the fermentation, LabView set the feed pump to feed to a μ of 0.4 h^{-1}, and on-line HPLC was used

to determine when the galactose was building up, indicating the algorithm needed to be restarted with a new estimate of biomass. As in **FB1a**, no direct, automated feedback control was used. The fermentation was terminated at an optical density of 19.

FB2 was a fed-batch fermentation in which the concentration of galactose was maintained above 15 $g.L^{-1}$ throughout the experiment, by feeding in a solution containing ($g.L^{-1}$):
galactose, 400; $MgSO_4.7H_2O$, 4; Na_2HPO_4, 33.3; KH_2PO_4, 10.3; $(NH_4)_2SO_4$, 200; trace element 1000 x stock (for composition, see 2.2.2.), 9.3 $mL.L^{-1}$; $FeSO_4.7H_2O$, 2; citric acid, 2.
Initially, the fermenter was operated in a batch mode in which 35 $g.L^{-1}$ galactose was added at inoculation. The on-line monitoring system was used to determine when the feed pump needed to be switched on (manually) to maintain the galactose concentration above 15 $g.L^{-1}$. The fermentation was run until an optical density of 60 has been reached.

FB3 was a fed-batch fermentation in which the LabView controller was set to feed in a galactose solution (250 $g.L^{-1}$) to provide a μ of 0.1 h^{-1} from inoculation. The kanamycin and thiamine were added to the fermenter 9½ hours after inoculation, resulting in the death of about 33% of the cells (based on the reduction of CER). For this reason, the fermentation was considered to have been started at this point. The on-line monitoring system was not in use in this fermentation as it was assumed that no galactose or acetate would be detected at such a low feed rate. The fermentation was run until an optical density of 18 was reached.

FB4 fed-batch fermentation was previously described in section 1.8. It was run to demonstrate coarse closed loop control in which the LabView controller was set to feed to a μ of 0.5 h^{-1}. The on-line HPLC was operated to provide data for feedback control - when the acetate concentration exceeded a pre-set threshold of 0.5 $g.L^{-1}$, the feed pump was cut by 50%. When it dropped below 0.5 $g.L^{-1}$, it was increased again. Details of the fermentation are given in section 1.8.

FB5 was a fermentation in which μ was initially set to 0.2 h^{-1} in LabView, by feeding a solution of galactose (250 $g.L^{-1}$), and then manually increased until, using the on-line HPLC, galactose and acetate were detected in the medium. The fermentation continued until μ had reached 0.38 h^{-1}, and the optical density had reached 11.

FB6 has also previously been described in section 1.8.; it being the fermentation used to demonstrate dual-level, fine closed-loop control. In this experiment, the feed pump was set to feed to a particular μ, and then automatically increase μ at a linear rate over 10 hours. The on-line monitoring system provided galactose and acetate data; when the concentration of galactose exceeded the first threshold (0.25 $g.L^{-1}$), the μ ramp was disabled until the level was again below the threshold. If the galactose concentration rose above a second threshold set at (0.4 $g.L^{-1}$), the algorithms estimate was reduced by 5 % until the concentration dropped below that level. For the first 21.9 hours of the fermentation, galactose was the critical analyte; then the critical analyte was changed to acetate. for further details of the fermentation, refer to section 1.8.

C1 was an extended continuous culture in which medium components were kept constant, and the dilution rate varied. Details of equipment and media for this culture are given in sections 2.2.4.2 and 2.2.2.

2.3. BATCH FERMENTATION

Batch fermentations (in which all medium components were added at the start of the fermentation, including the carbon source, 35 $g.L^{-1}$ galactose) were performed to provide a comparison for fed-batch and continuous cultures.

The α-amylase profile for a typical batch fermentation is given in figure 2.3.a, which presents total amylase activity, total specific amylase activity, and the periplasmic and extracellular fractions versus time after fermenter inoculation. The percentage of amylase leaked from the periplasm into the extracellular medium is shown on the same graph.

Figure 2.3.b. presents the galactose and acetate profiles for the same batch fermentation. Specific rates of galactose consumption (q_{gal}) and acetate (q_{ac}) production (g_{gal} or $g_{ac}.gDCW^{-1}.h^{-1}$) versus time are shown in figure 2.3.c. These results are calculated by taking the difference in galactose concentration between two sample points, and dividing this by the average dry weight of those sample points; this method will only give an approximate rate. Using this method, the average specific galactose consumption rate (after 11.3 hours) is 0.56, and the average specific acetate production rate over the same time period is 0.049. These rates can also be calculated from the equations:

$$q_{gal} = \frac{\mu}{Y_{gal}} \qquad (1)$$

$$q_{ac} = \frac{\mu}{Y_{ac}} \qquad (2)$$

μ is the overall specific growth rate; Y_{gal} is the yield of cells on galactose; and Y_{ac} is the yield by cells of acetate ie how many grams of cells produce one gram of acetate. μ is calculated from the slope obtained from a linear regression of natural logarithm (ln) dry weight versus time; Y_{gal} from galactose concentration

versus dry weight; and Y_{ac} from acetate versus dry weight. These calculations give a q_{gal} of 0.577 $g.g^{-1}.h^{-1}$, and a q^{ac} of 0.047 $g.g^{-1}.h^{-1}$, which are close to the approximate values calculated.

Figure 2.3.d. shows the optical densities and dry weight data from samples taken throughout the experiment. From these data, specific growth rates have been calculated, and are shown in figure 2.3.e.

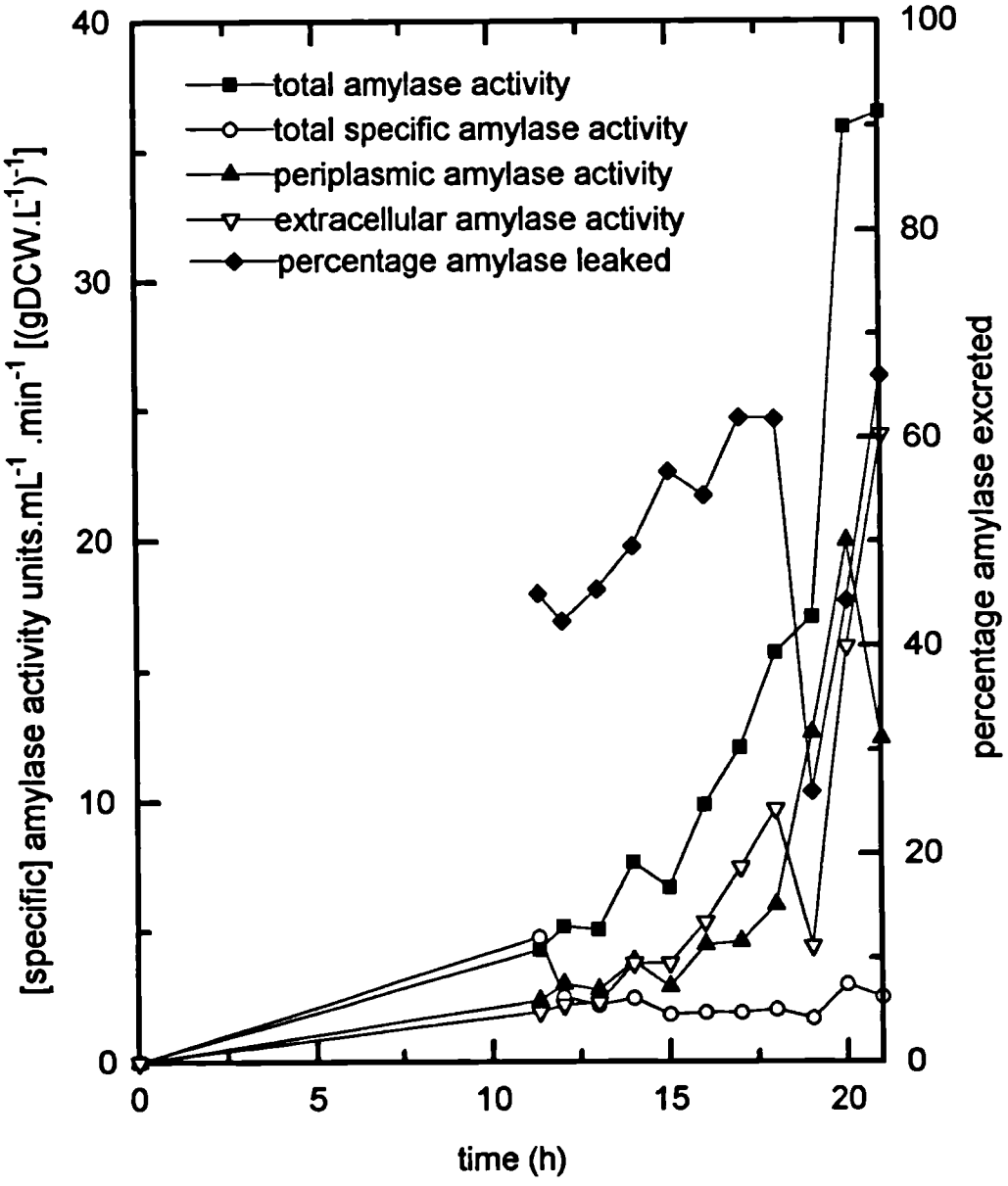


Fig. 2.3.a. Amylase profile for batch fermentation. Profiles of the total amylase activity, the total specific amylase activity, the periplasmic and extracellular amylase activities, and the percentage of amylase leaked into the extracellular medium in a typical batch fermentation of *E. coli* JM107 + pQR126.

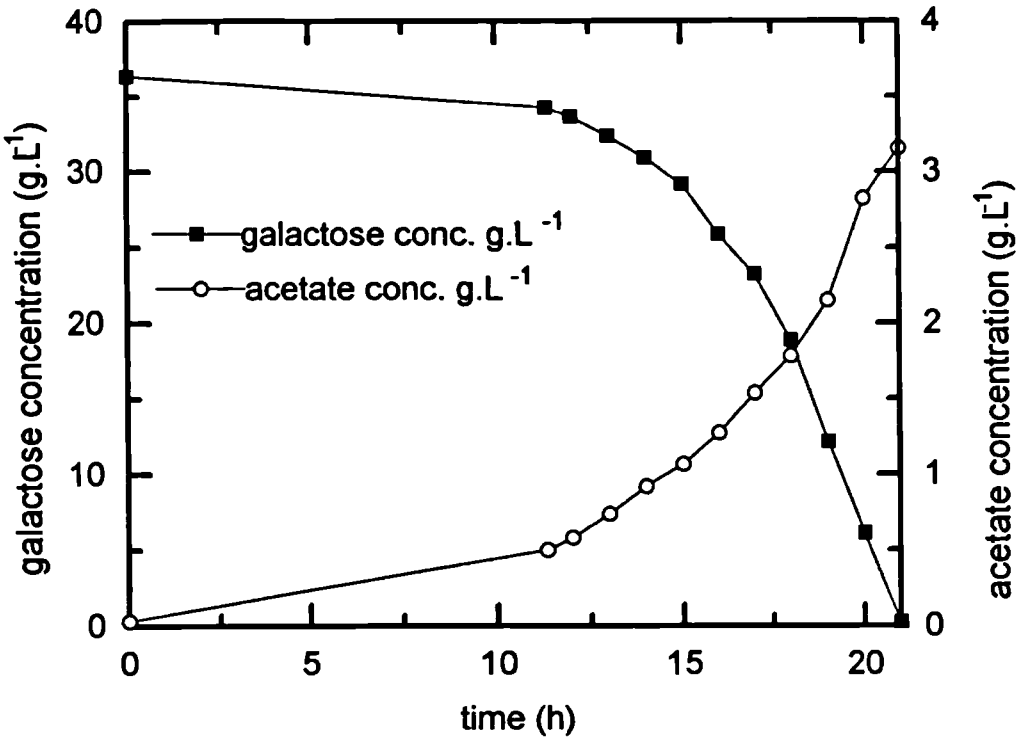


Fig. 2.3.b. Galactose and acetate profiles for a typical batch fermentation of *E. coli* JM107 + pQR126.

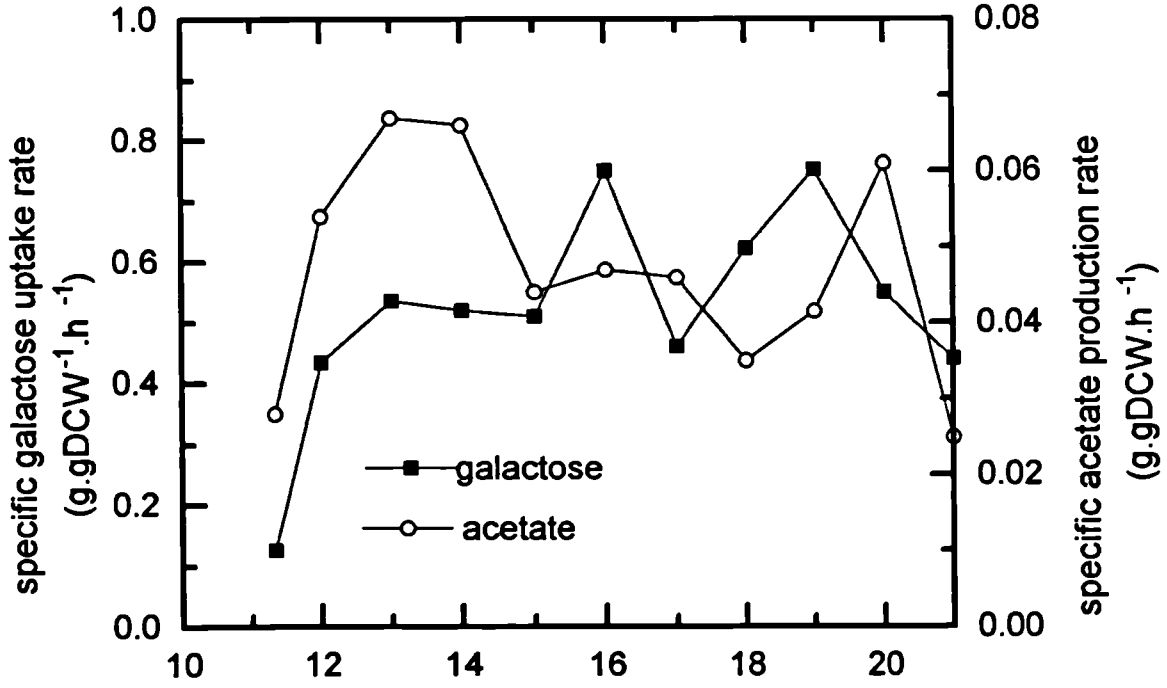


Fig. 2.3.c. Specific rates of galactose uptake and acetate production for a typical batch fermentation of *E. coli* JM107 + pQR126. Average rate for galactose from 11.3 hours is 0.56, and for acetate, is 0.049 g.gDCW $^{-1}$.h $^{-1}$.

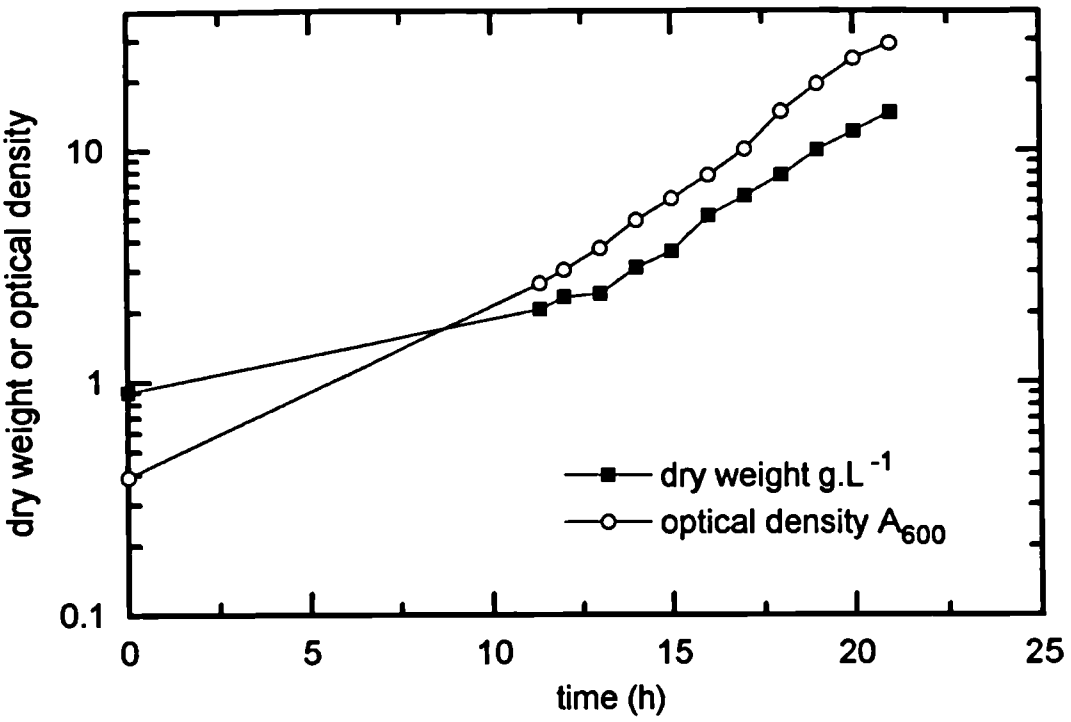


Fig. 2.3.d. Dry weight (g.L $^{-1}$) and optical density (A_{600}) profile for a typical batch fermentation of *E. coli* JM107 + pQR126.

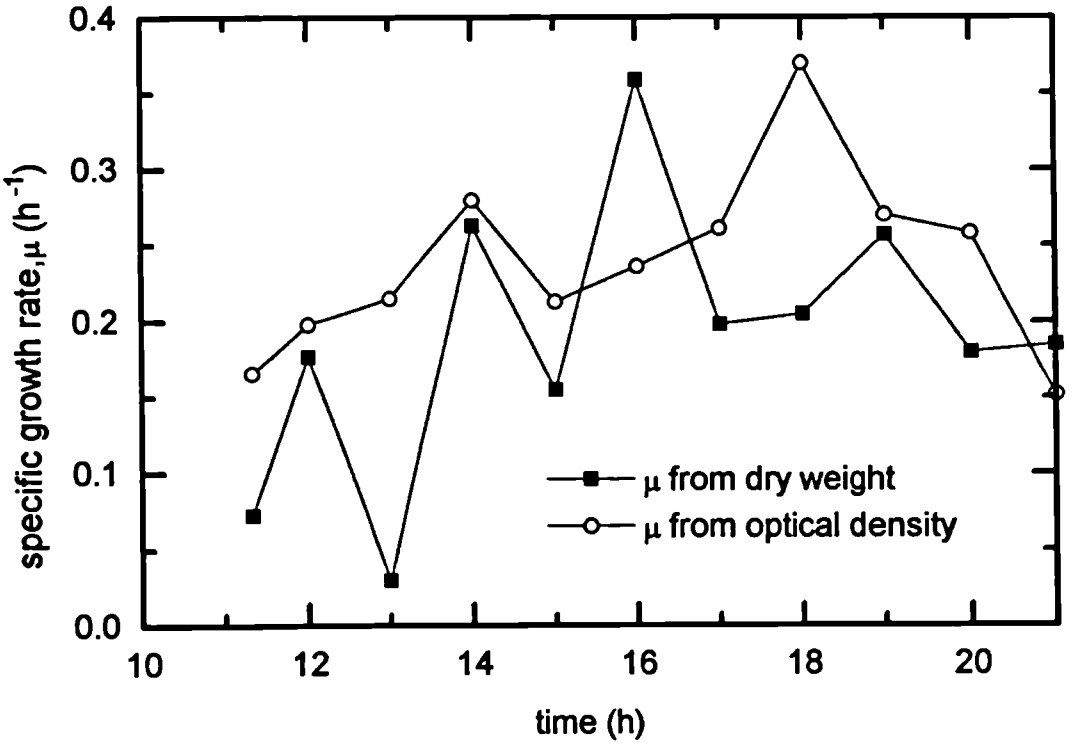


Fig. 2.3.e. Specific growth rates calculated from dry weight and optical density data for a typical batch fermentation of *E. coli* JM107 + pQR126.

2.4. THE EFFECT OF SPECIFIC GROWTH RATE ON AMYLASE PRODUCTION

2.4.1. Introduction

Many researchers have found that recombinant protein production is greatly affected by dilution rate (equivalent to specific growth rate, μ) in continuous culture, and most have reported that productivity is higher at lower dilution rates (see section 2.1.4.). Less work has been done on growing cells at a constant specific growth rate in fed-batch cultures, because of the greater complexity of the control required, and the increased equipment burden. As a result, little has been published on the effects of varying specific growth rate on recombinant protein production in fed-batch culture. With the development of the equipment described in section 1 of this thesis *ie* the on-line monitoring system and the LabView controller, it has been possible to grow the recombinant, α-amylase producing *E. coli* in fed-batch culture at different specific growth rates, and compare the differences in amylase production. These results were then compared to results obtained from a continuous culture in which dilution rates were varied and amylase activity measured at different steady states. Fermentation nomenclature is given in section 2.2.7.

2.4.2. Results

2.4.2.1. Fed-batch cultures

Results of three fed-batch fermentations are presented in this section - **FB1a** in which the cells were grown to a specific growth rate of 0.2 h^{-1}, **FB1b** where the cells are grown at a μ of 0.4 h^{-1} and **FB3** in which the cells are grown at a μ of 0.1 h^{-1}; control of μ in all cases being provided by LabView.

Figures 2.4.2.1.a,b and c plot the amylase profiles for each growth rate versus time. Extracellular, periplasmic and total amylase activities are shown, as well as the specific total amylase activity for each sample throughout the fermentation.

Total amylase concentrations are plotted against dry weight for each fermentation in figure 2.4.2.1.d., and the percentage of total amylase (activity) leaked into the extracellular medium for each fermentation plotted against dry weight is shown in figure 2.4.2.1.e. This data shows that in all fermentations, most of the amylase had leaked from the periplasm by the end of the fermentation. In the fermentations of lower μ (*ie* **FB1a**; 0.2 h^{-1} and **FB3**; 0.1 h^{-1}), the amylase was retained in the periplasm for the early part of the fermentation, and more and more leaked out towards the end. However, in **FB1b** (μ = 0.4 h^{-1}), amylase was leaked from the periplasm very early on. This may be due to the fact that this fermentation used **FB1a** as an inoculum, but does not account for the greater percentage retention at the end of the fermentation than the beginning.

Figures 2.4.2.1.f and g plot the actual specific growth rates obtained in from optical density and dry weight data respectively for each of the fermentations to demonstrate how well the μ controller functioned. The average μ obtained from dry weight and optical density data for each fermentation is shown in table 2.4.2.1.(i).

FERMENTATION	AVE. μ from DRY WEIGHT DATA (h^{-1})	AVE μ from OD DATA (h^{-1})
FB1a (μ = 0.2 h^{-1})	0.197	0.183
FB1b (μ = 0.4 h^{-1})	0.333	0.406
FB3 (μ = 0.1 h^{-1})	0.106	0.090

Table 2.4.2.1.(i). Average specific growth rates obtained from dry weight and optical density data for each of the three fermentations in which specific growth rate was controlled by LabView.

The final graphs presented in this section (figures 2.4.1.h and i) show the galactose and acetate concentrations measured by on-line HPLC for fermentations **FB1a** and **FB1b**. Although the on-line HPLC was not used in closed loop control in these fermentations, it was very useful in pinpointing when

LabView's estimate of biomass had deviated from the true value, indicating that the algorithm needed to be restarted with a new estimate of biomass.

The results from these three fermentations indicate that the greatest amylase productivity occurred when the cells were grown at a specific growth rate of 0.2 h^{-1}. This is a surprising result. The expectation was that the higher growth rates would yield lower amylase activities because the copy number is likely to be lower. However, the converse of that is that at very low growth rates, the highest amylase activities would be expected. This was not observed; the amylase activities at a μ of 0.1 h^{-1} were significantly lower than those at 0.2 h^{-1}.

2.4.2.2. Continuous culture

Steady states in continuous culture were obtained states at the following dilution rates (h^{-1}): 0.09; 0.15; 0.22; 0.29; 0.31; 0.44 and 0.5. Figure 2.4.2.2.a plots the total specific amylase activity versus dilution rate. Figures 2.4.2.2.b plots the periplasmic, extracellular and total amylase activities (respectively) versus dilution rate, and figure 2.4.2.2.c. plots the percentage of total amylase activity secreted into the extracellular medium versus the dilution rate.

The continuous culture looked at more dilution rates than the growth rates in fed-batch culture, however, a similar trend emerged. The greatest amylase productivity (based on its activity) occurred when an intermediate dilution rate was used (0.29 h^{-1}). Again, as expected, amylase activities at high dilution rates were low. In addition, it was found that low dilution rates resulted in relatively low amylase activities; a similar result to that obtained from fed-batch culture.

The percentage of α-amylase leaked from the periplasm is lower in continuous culture for all dilution rates compared to fed-batch culture. The reason for this is unclear, but the cell population is constantly changing, therefore is generally younger in continuous culture. Further evidence for this is the fact that in fed-batch culture, the 0.4 h^{-1} fed-batch fermentation has lower percentage secretion at the end of the batch, which occurs after a much shorter time than in the other

fed-batch fermentations.

2.4.3. Summary

The data presented here provides information about a growth condition (specific growth rate / dilution rate) which has a profound effect on the production of recombinant protein. Although a lot has been published on the effect of dilution rate on recombinant protein production, little has been done on the effects of specific growth rate in fed-batch culture. Results show that the amylase activity varies considerably with dilution rate / specific growth rate, and although the absolute values differ, results from the continuous fermentation show similar trends to the fed-batch fermentation results. At low and high dilution rates / μs (specific growth rates), amylase activity is relatively low, while intermediate dilution / μs show produce greater activities.

The results also show that the LabView controller was quite well able to control growth at a particular specific growth rate, when used in collaboration with the on-line monitoring device. The μ values obtained from dry weight and optical density data were (after averaging because of noise) close to those set by the controller, and the concentrations of acetate and galactose did not build up significantly in either fermentation monitored. Clearly, the development of a system that can control the growth rate of cells in fed-batch culture while maintaining low concentrations of substrate and excreted metabolites could prove very useful.

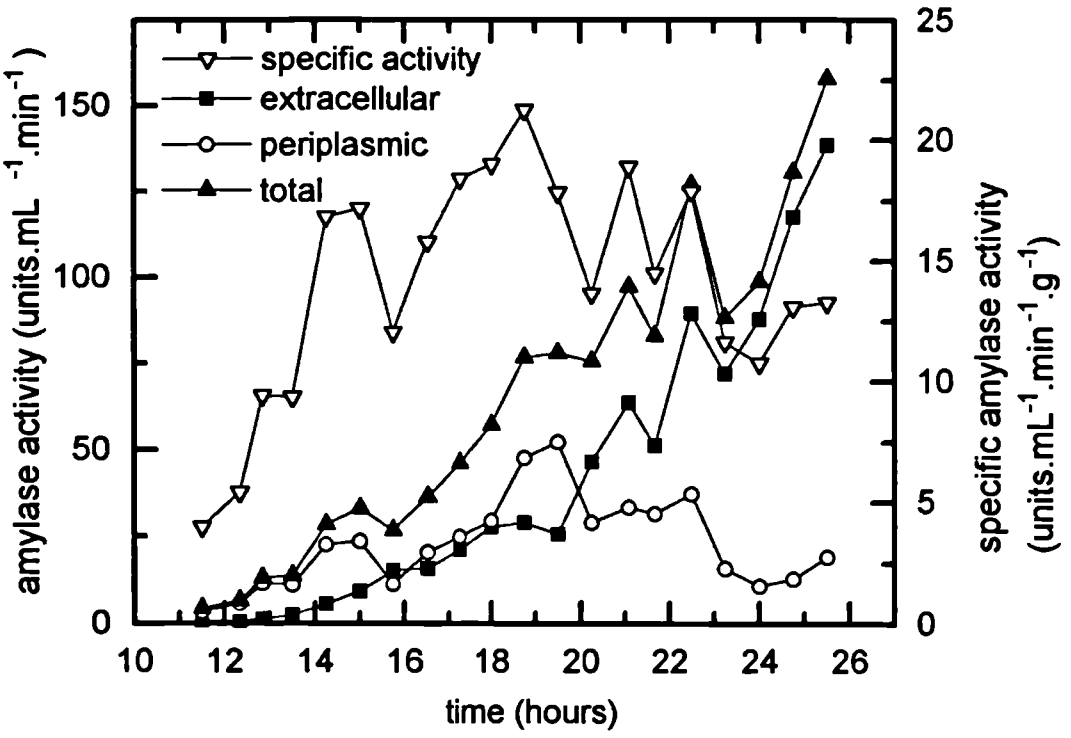


Fig. 2.4.2.1.a. Amylase profile for fermentation **FB1a** in which cells were grown at a controlled specific growth rate of 0.2 h^{-1}.

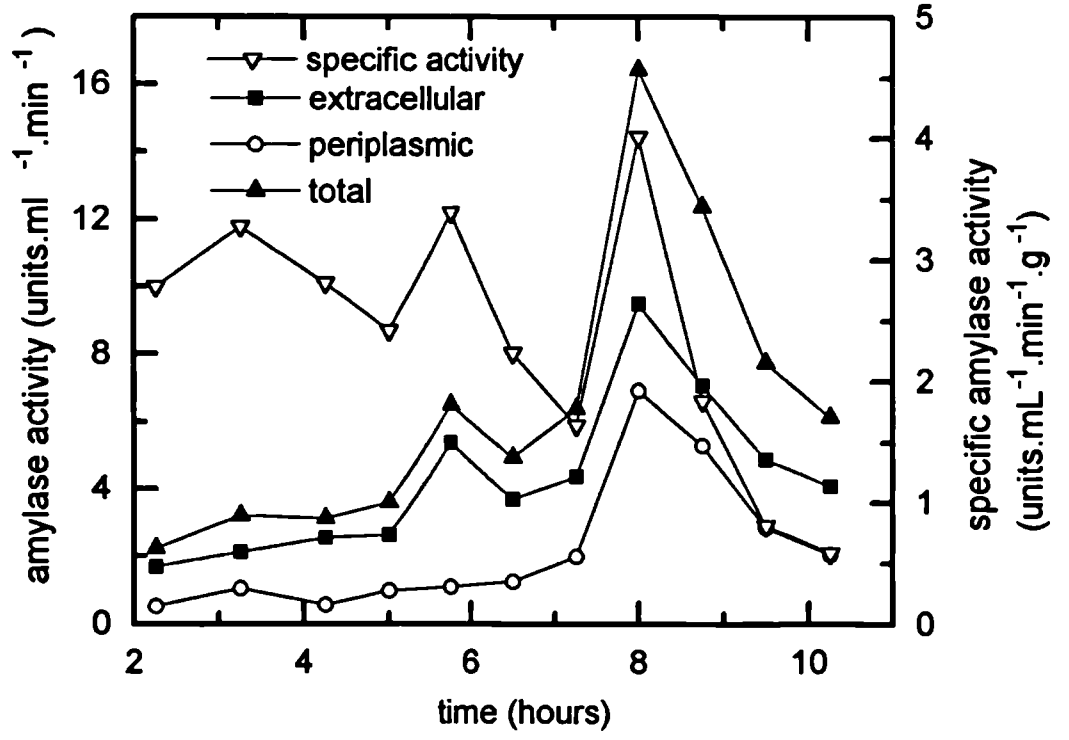


Fig. 2.4.2.1.b. Amylase profile for fermentation **FB1b** in which cells were grown at a controlled specific growth rate of 0.4 h^{-1}.

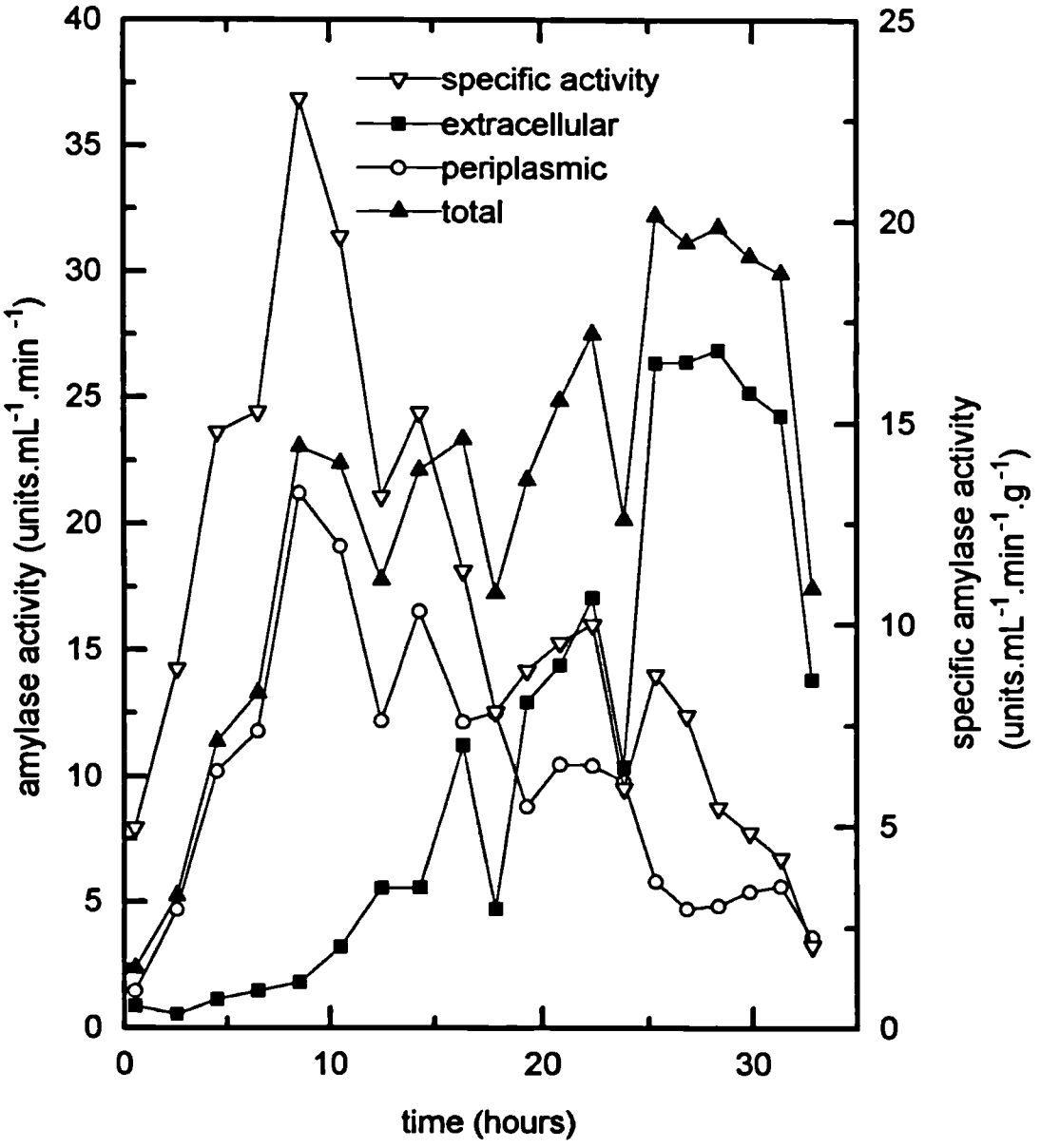


Fig. 2.4.2.1.c. Amylase profile for fermentation **FB3** in which cells were grown at a controlled specific growth rate of 0.1 h^{-1}.

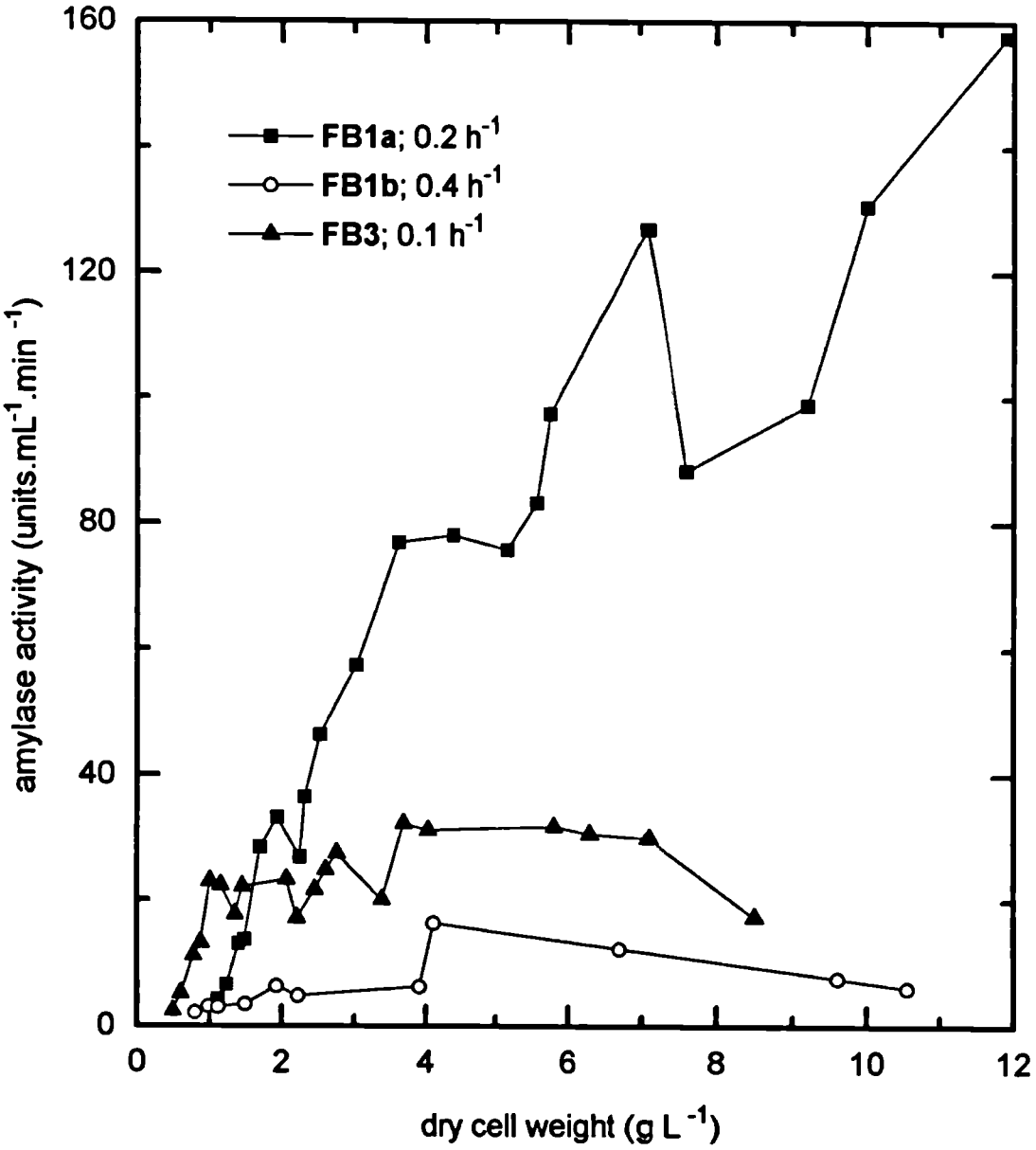


Fig. 2.4.2.1.d. Total amylase concentrations plotted against dry cell weight for each of the three fermentations controlled at different specific growth rates using LabView.

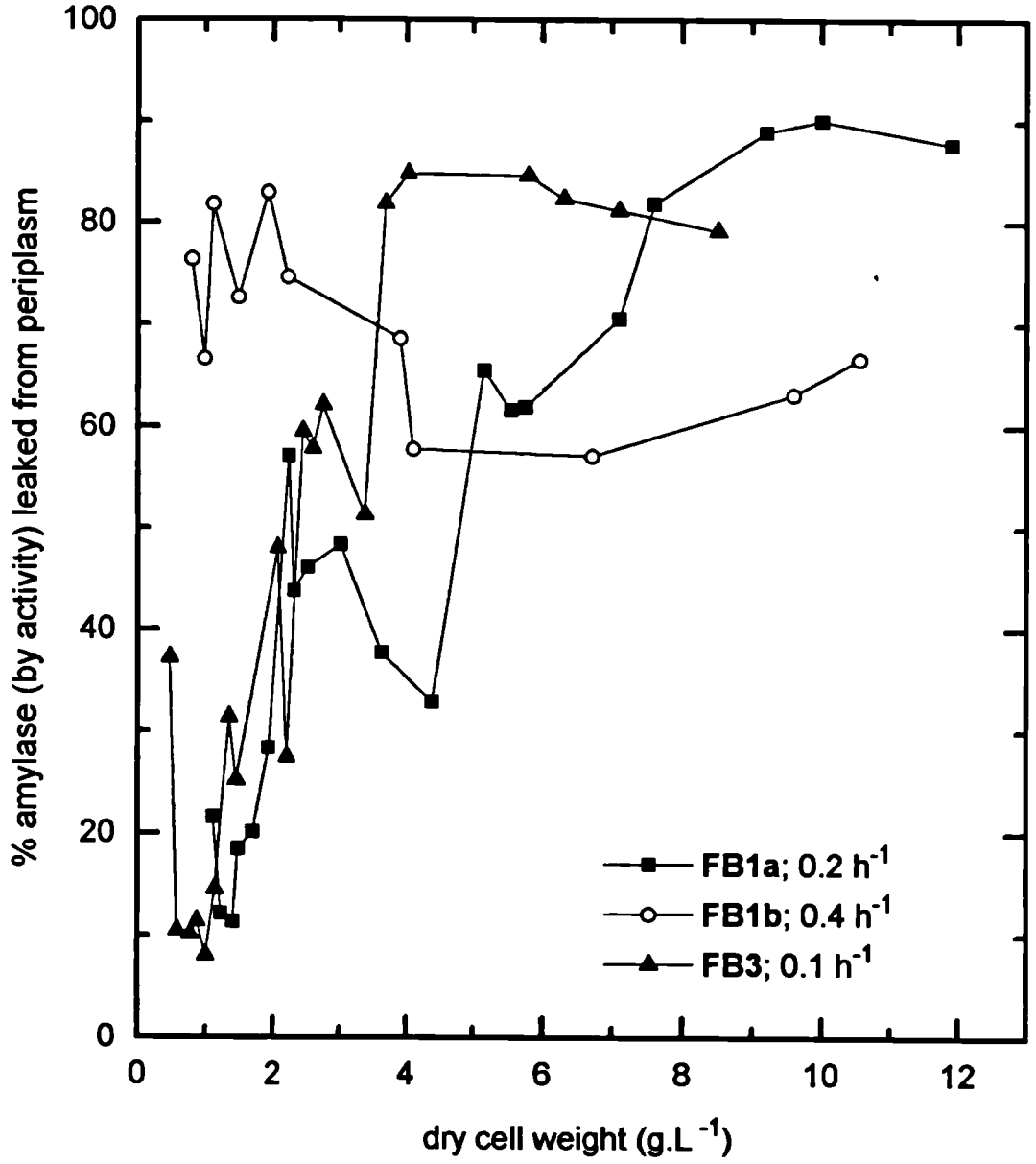


Fig. 2.4.2.1.e. Profile of percentage amylase leaked out of the periplasmic space versus dry cell weight for the course of each of the three fermentations controlled at different specific growth rates.

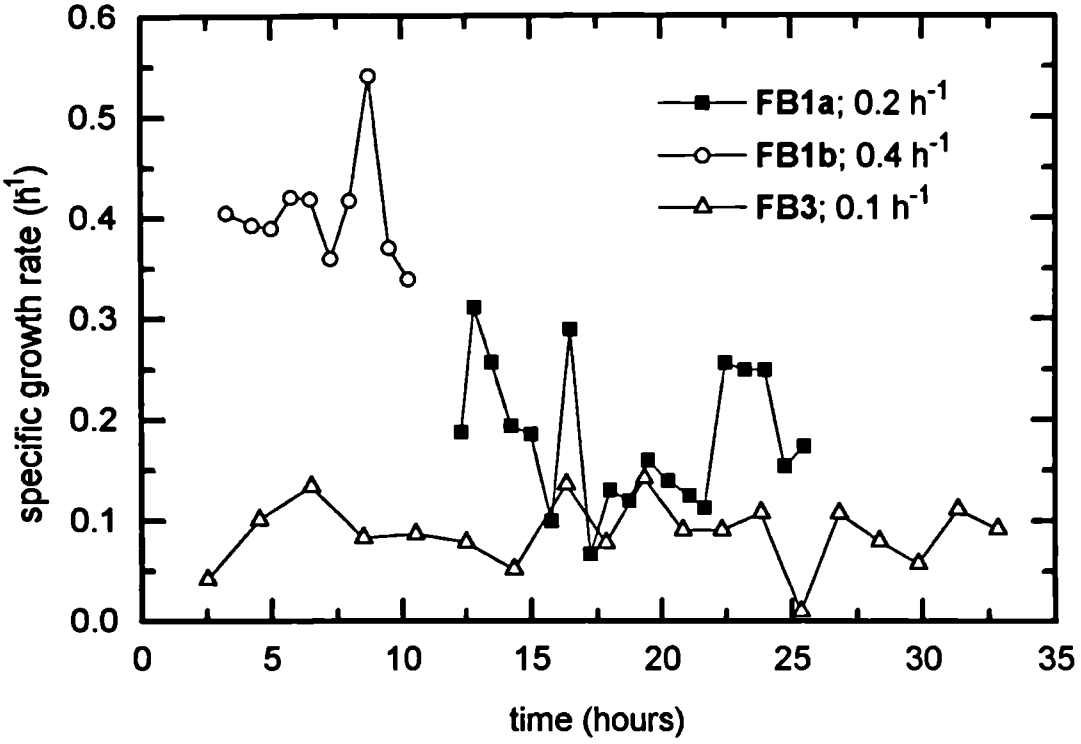


Fig. 2.4.2.1.f. Actual specific growth rates obtained from optical density data for each of the three fermentations controlled at different specific growth rates.

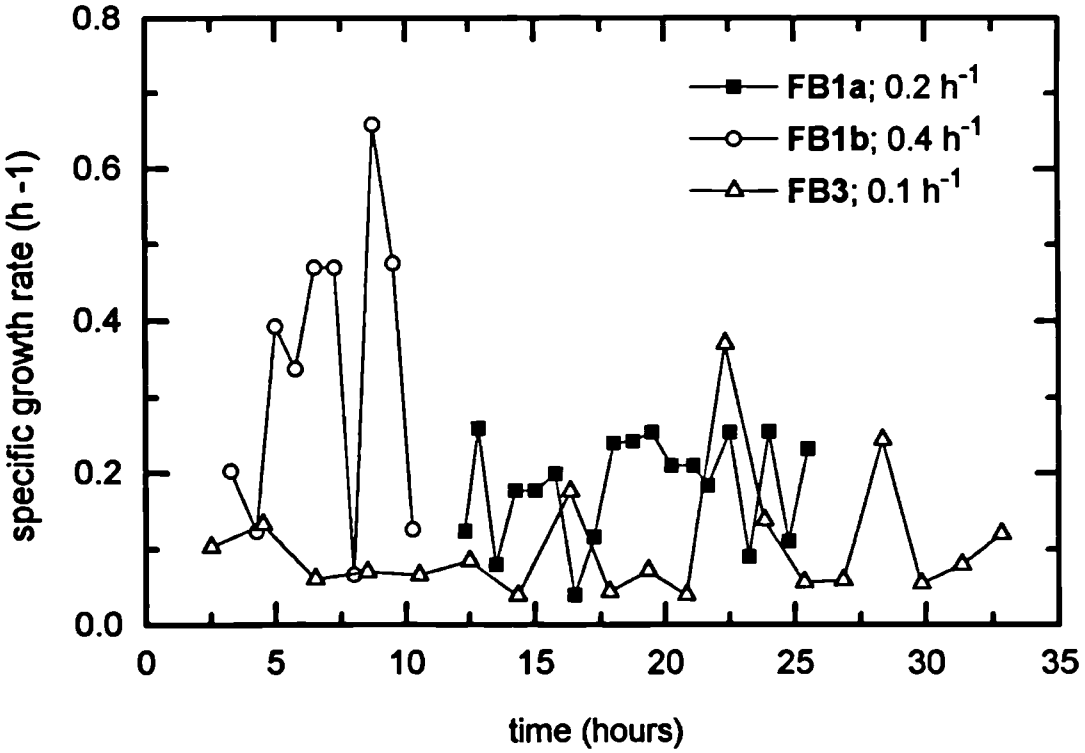


Fig. 2.4.2.1.g. Actual specific growth rates obtained from dry cell weight data for each of the three fermentations controlled at different specific growth rates.

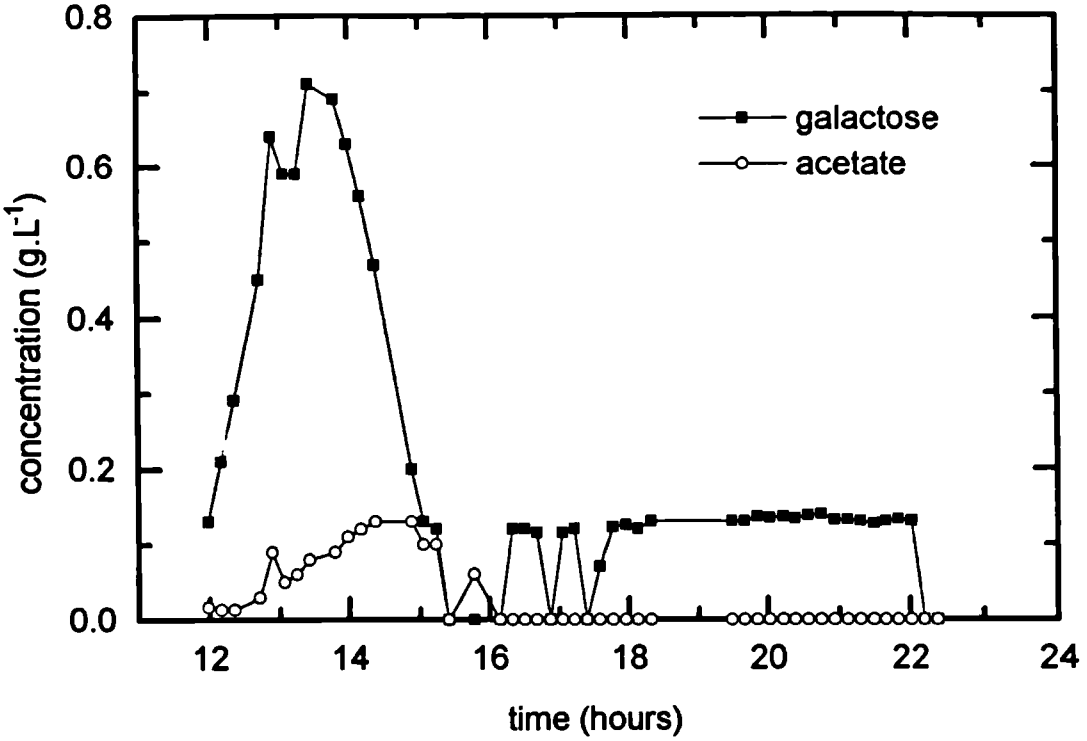


Fig. 2.4.2.1.h. Galactose and acetate profiles for fermentation **FB1a** in which the fermentation was controlled at a specific growth rate of 0.2 h^{-1}.

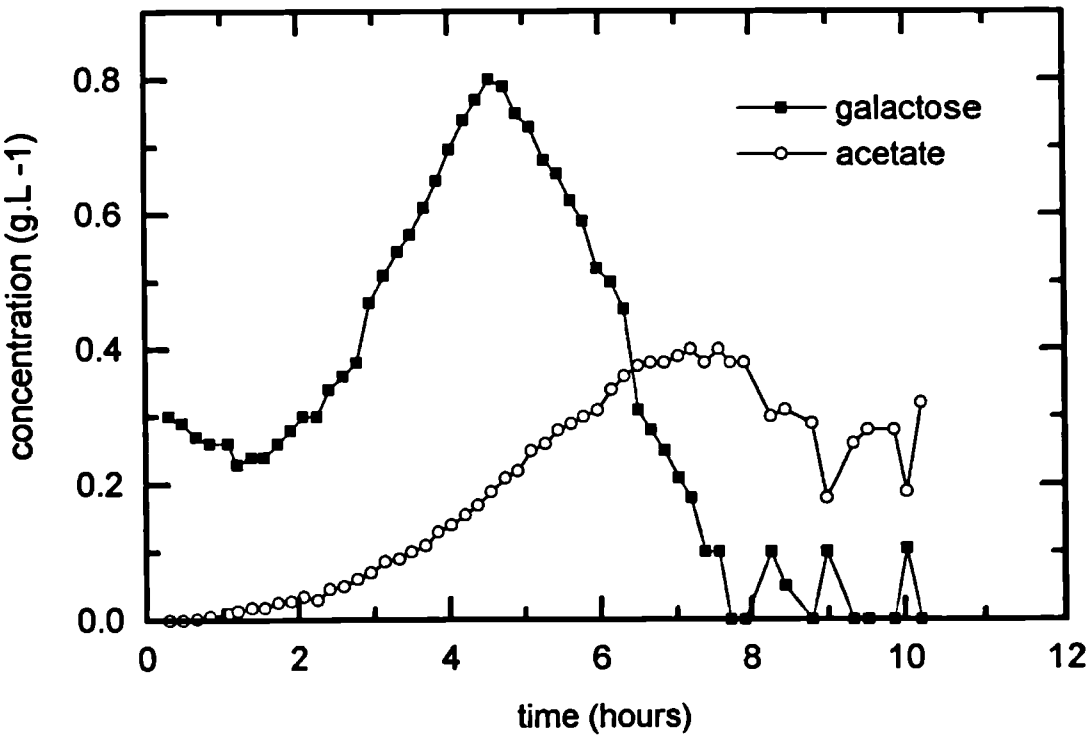


Fig. 2.4.2.1.i. Galactose and acetate profiles for fermentation **FB1b** in which the fermentation was controlled to a specific growth rate of 0.4 h^{-1}.

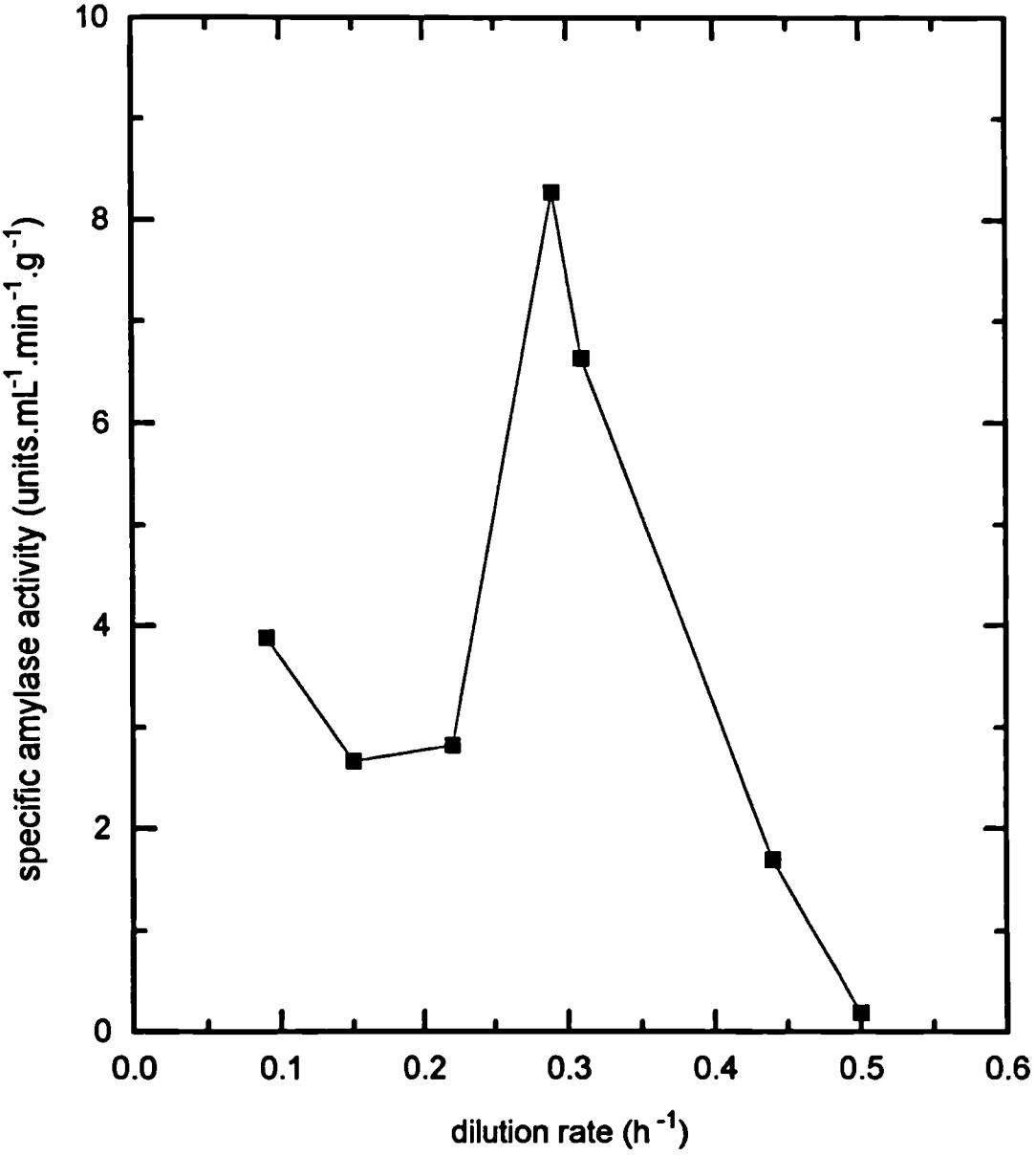


Fig. 2.4.2.2.a. Plot of dilution rate versus total specific amylase activity obtained from samples taken at steady states during continuous culture, C1.

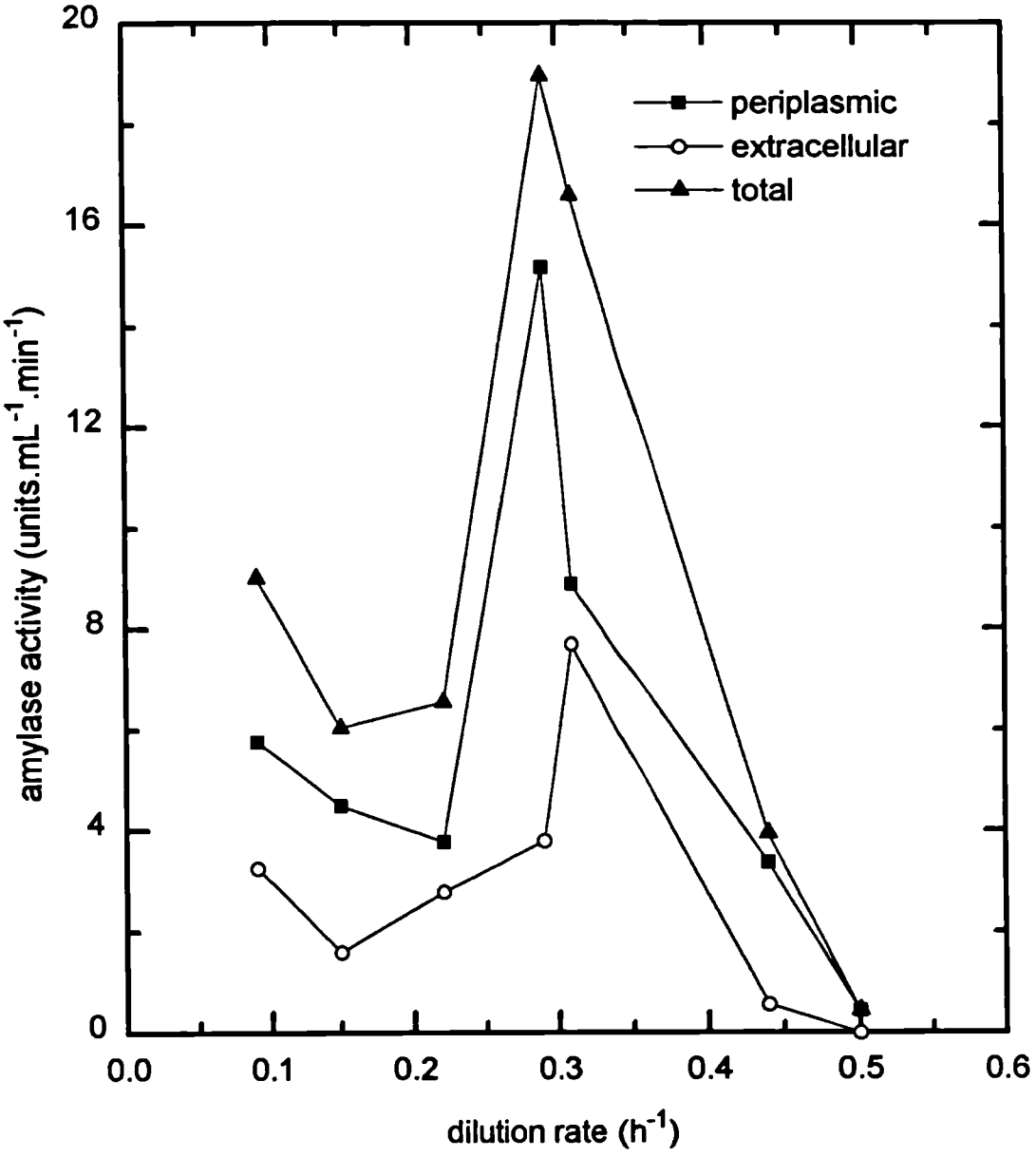


Fig. 2.4.2.2.b. Plot of amylase activities (periplasmic, extracellular and total) versus dilution rate obtained from samples taken at steady states during continuous culture, **C1**.

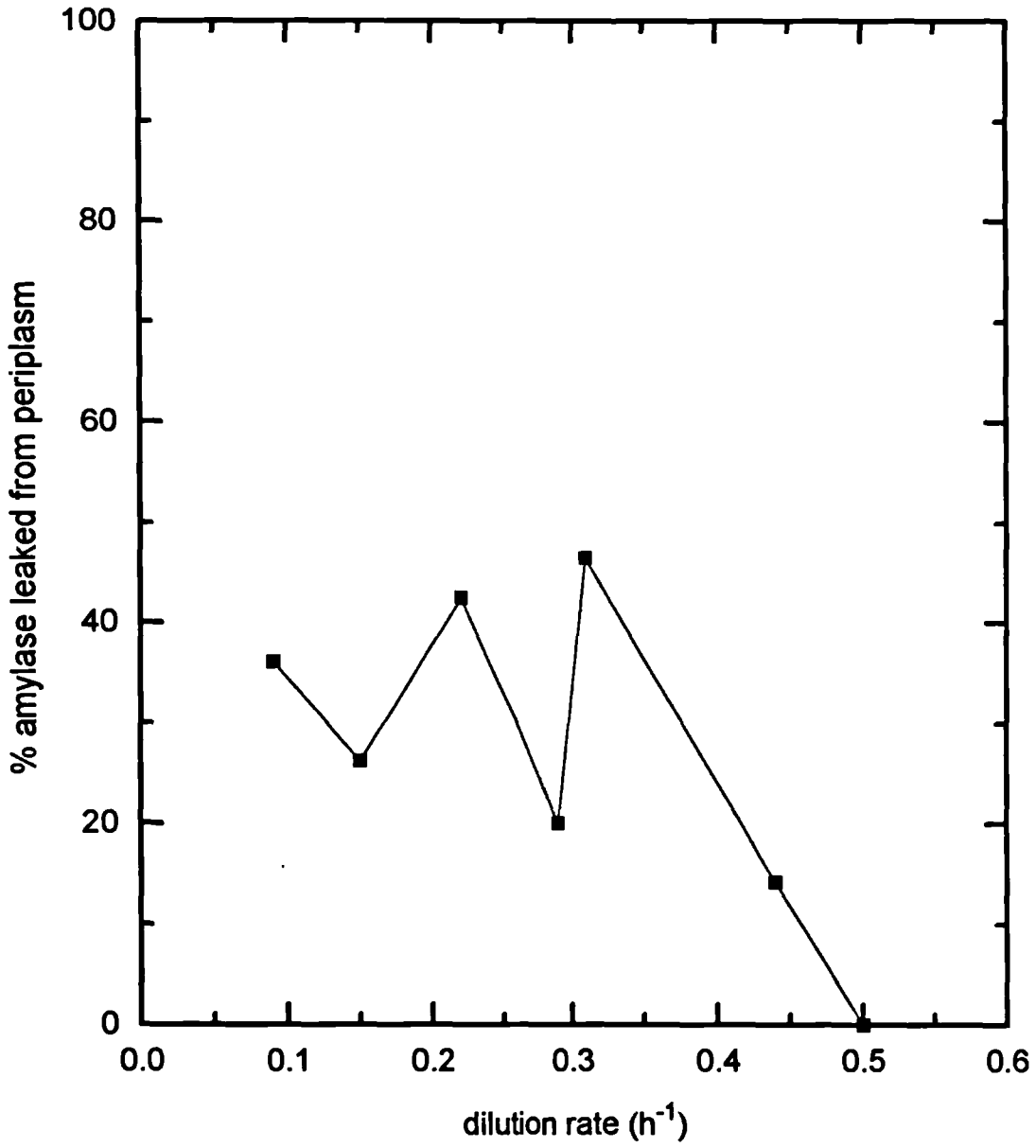


Fig. 2.4.2.2.c. Plot of percentage of total amylase leaked from periplasm into extracellular medium versus dilution rate. Results were obtained from samples taken at different steady states during continuous culture, **C1**.

2.5. THE EFFECT OF HIGH SUBSTRATE CONCENTRATION ON SPECIFIC GROWTH RATE

2.5.1. Introduction

In batch fermentations, (or fed-batch fermentations where substrate concentration is not carefully controlled), a high substrate concentration will be present for at least part of the fermentation. As this may have some effect on μ (and acetate production), specific growth rates in a batch fermentation where the starting substrate (galactose) concentration was 35 $g.L^{-1}$ were compared to 1) the maximum specific growth rate, μ_{max}, obtained from carbon limited continuous culture, 2) μ in a controlled fed-batch culture (where μ was set to 0.4 h^{-1}, with little galactose build-up) and 3), μ obtained from a fed-batch culture in which the concentration of galactose was maintained above 15 $g.L^{-1}$.

2.5.2. Determination of μ_{max}

μ_{max} was determined in continuous culture as described in section 2.2.4.2. The μ_{max} was found to vary depending on the conditions preceding the μ_{max} experiment. When μ_{max} was determined after the cells had been grown at low dilution rates, and then d increased to 0.44 h^{-1}, it was found to be 0.53 h^{-1}. However, when the cells had been grown at higher dilution rates for some time prior to the washout experiment, μ_{max} was found to be in the range of 0.63 to 0.67 h^{-1}. This could indicate segregational instability of the plasmid causing variable copy number. Lower copy numbers would enable the cells to grow faster (as well as growth rate affecting copy number, copy number also affects growth rate). Later evidence for this emerged when it was discovered that, after a series of washout experiments, going back to a dilution rate previously operarted, lower levels of amylase, and hence plasmid, were detected. Although plasmid stability tests using serial dilutions and plate counts indicated no plasmid loss occurred, plasmid copy number was not directly measured.

2.5.3. Results

Batch fermentation, **B1**, is compared to **FB1b** (where the specific growth rate is controlled at 0.4 h^{-1} with very little galactose build-up) and **FB2** where the fermentation had a batch phase, followed by feeding to control the galactose concentration above 15 $g.L^{-1}$. The specific growth rates obtained from dry weight data for batch and fed-batch fermentations are shown in figure 2.5.3.a., while figure 2.5.3.b. presents μ obtained from optical density data for the same fermentations. Table 2.5.3.(i) gives the average specific growth rates from both dry weight and optical density data from the three fermentations. The galactose concentration profiles of **B1**, **FB1b** and **FB2** are given in figure 2.5.3.c.

FERMENTATION	AVE. μ from DRY WEIGHT DATA (h^{-1})	AVE. μ from OD DATA (h^{-1})
B1	0.239	0.238
FB1b	0.33	0.406
FB2	0.266	0.279

Table 2.5.3.(i). Average specific growth rates obtained from dry weight and optical density data for the three fermentations run to determine the effect of high galactose concentration on specific growth rate.

The results show that the μ_{max} is somewhere in the region of 0.53 h^{-1} to 0.66 h^{-1}; in addition, the cells were easily able to grow in fed-batch culture at a controlled μ of 0.4 h^{-1} with very little galactose build-up. Conversely, the cells in batch culture grew more slowly, the difference being the high concentration of galactose in the fermenter. To discount the possibility of a lag for other reasons (*eg.* cells in stationary phase being present in the inoculum), **FB2** was designed to prolong a fermentation (to a biomass concentration in the region of 35 $g.L^{-1}$) with a high galactose concentration throughout. Growth rates for this fermentation, **FB2**, are lower than for **FB1b** for nearly the duration of the fermentation. Although the end of the fermentation saw a build-up in acetate concentration (which could

cause some reduction in growth; see section 2.7), the majority of the fermentation had little acetate build-up. These results, therefore, strongly suggest an inhibitory effect on growth by a high concentration of galactose.

2.5.4. The effects of high galactose concentration on α-amylase production

It would seem that this growth inhibition should lead to improved yields of recombinant protein, however specific amylase activities were slightly lower in fermentation **B1** than **FB1b**, shown in figure 2.5.4.a., therefore it seems that high substrate concentrations may also detrimentally affect recombinant protein production. This has been reported in the literature (see section 2.1.2.).

2.5.5. Summary

This section (2.5) describes the effect of high galactose concentration on specific growth rate: it reduces it. In doing so, the production of recombinant protein is not increased; if anything, a slight decrease is found. Therefore the presence of high substrate concentrations has an undesirable effect on the organism under study here, and its production of α-amylase. This provides strong support in favour of performing well-controlled fed-batch fermentations in which substrate concentration is carefully controlled.

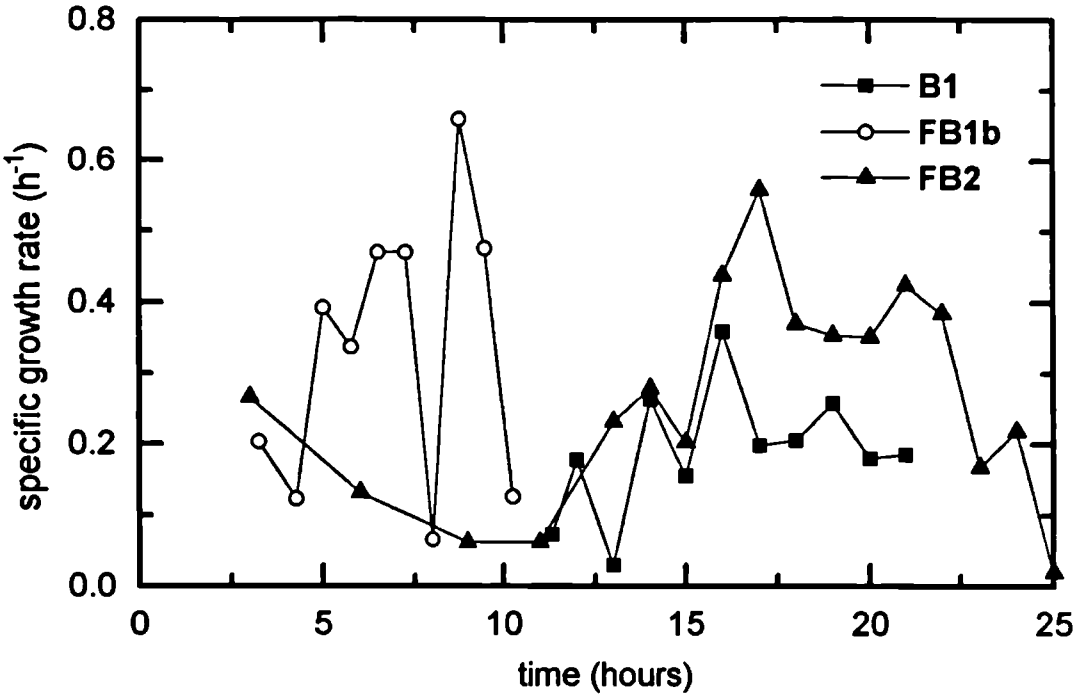


Fig. 2.5.3.a. Plot of specific growth rate (calculated from dry weight data) versus time for three fermentations: **B1**, a batch, **FB1b**, a fed-batch with low galactose concentrations throughout, and **FB2**, a fed-batch with high galactose concentrations throughout.

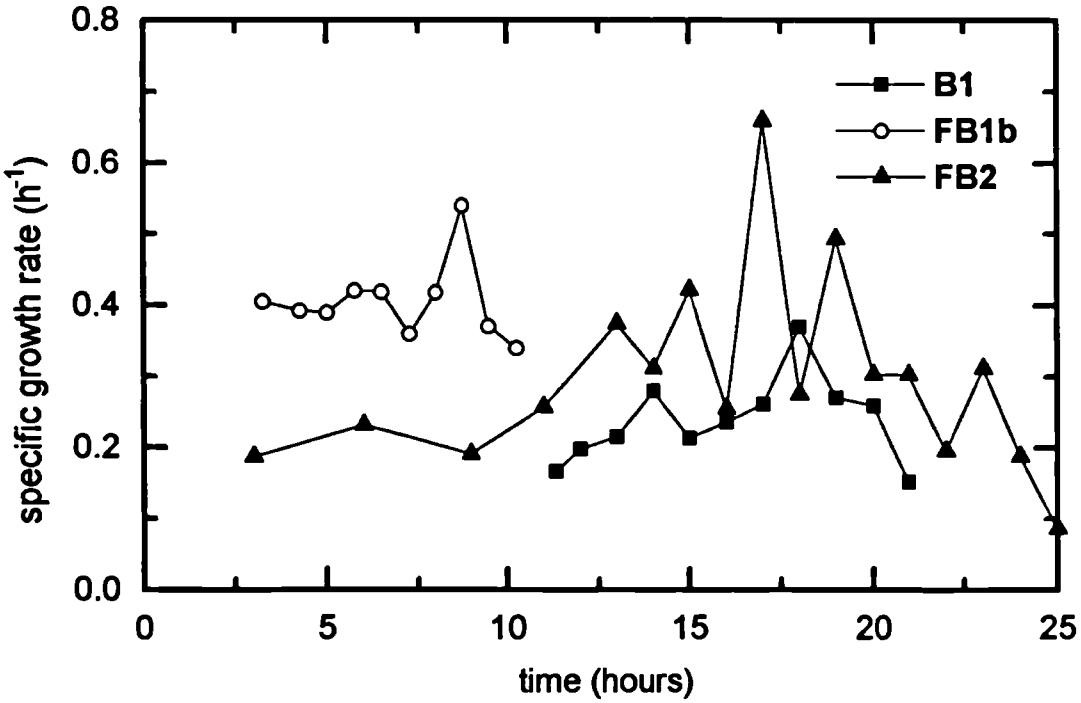


Fig. 2.5.3.b. Plot of specific growth rate (calculated from optical density data) versus time for three fermentations: **B1**, a batch; **FB1b**, a fed-batch with low galactose concentrations throughout; and **FB2**, a fed-batch with high galactose concentrations throughout.

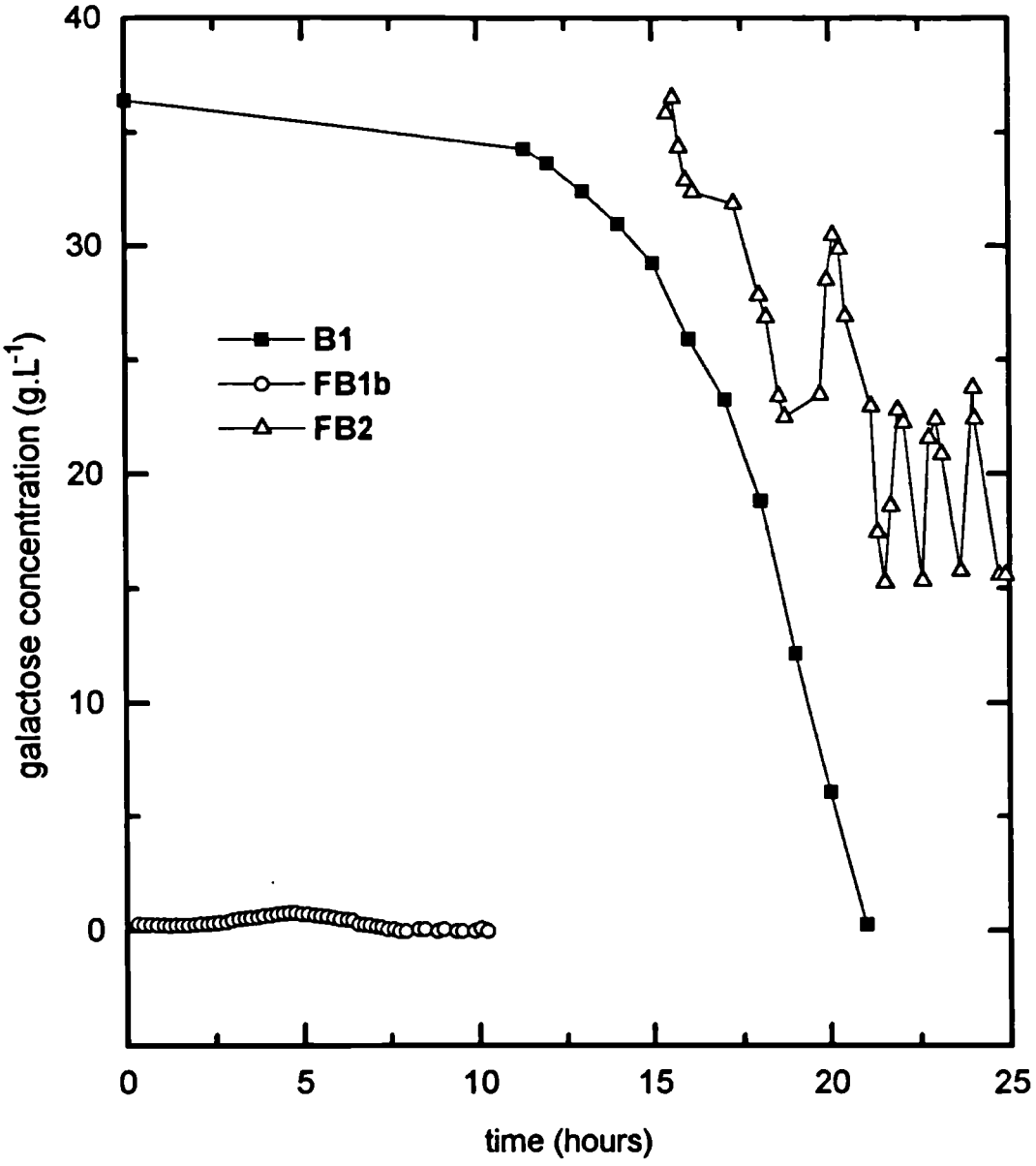


Fig. 2.5.3.c. Galactose concentration profiles for each of three fermentations: **B1**, a batch; **FB1b**, a fed-batch where galactose concentration was controlled using LabView; and **FB2**, a fed-batch where manual feed-control was used to keep the galactose concentration high. On-line HPLC was used in both fed-batches.

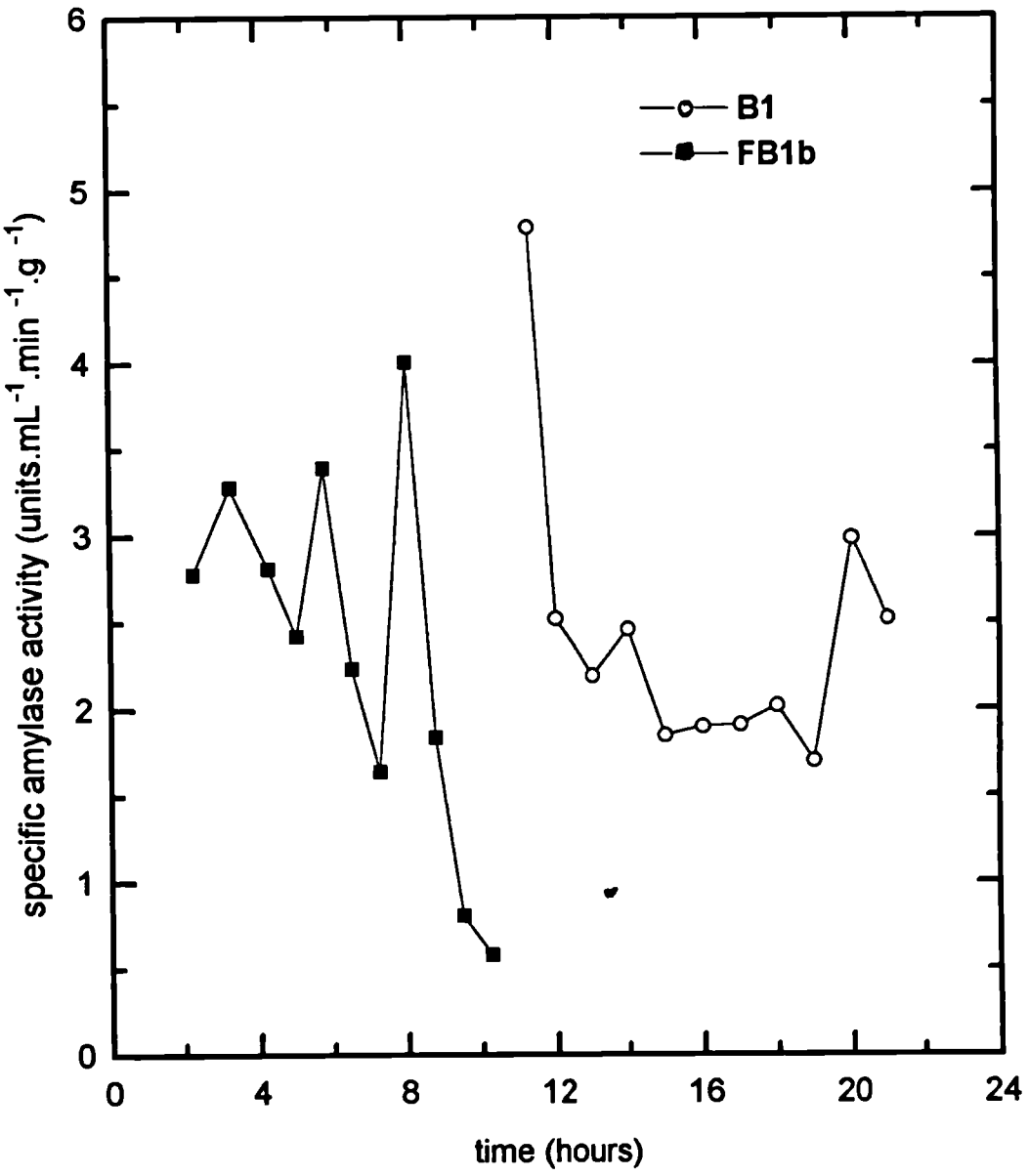


Fig. 2.5.4.a. Plot of specific amylase activities versus time for **B1**, the batch fermentation; and **FB1b**, the fed-batch fermentation controlled at a specific growth rate of 0.4 h^{-1}.

2.6. THE EFFECT OF SPECIFIC GROWTH RATE ON ACETATE PRODUCTION

2.6.1. Introduction

As discussed in section 2.1.1., if *E. coli* grow at a rate faster than a threshold specific growth rate, acetate is excreted by the cells. Because the production of acetate is undesirable, it is important to discover that threshold specific growth rate in order to devise a suitable feeding strategy to prevent overflow.

This threshold growth rate (dilution rate) was obtained from continuous culture, **C1**. In addition, an attempt was made to determine the threshold μ in fed-batch culture, however, it was found that the overflow of acetate is more complex in fed-batch culture.

2.6.2. Continuous culture results

By gradually increasing the dilution rate and waiting for a steady state, it was possible to find the dilution rate at which acetate was produced by the cells without any build-up or detection of galactose in the medium. This dilution rate was 0.49 - 0.5 h^{-1}. This experiment was done before the cells had been subjected to a series of washout studies, hence this threshold rate may be even higher if the cells were to undergo repeated washout experiments (where they had grown at μ_{max}) before this threshold rate was found. Segregational instability seems to cause a variation in plasmid copy number resulting in a change in μ_{max}, as seen in section 2.4. (where μ_{max} varied depending on how the cells had been grown prior to the washout experiment). The average concentration of acetate found in the samples taken during the steady state was 0.1 $g.L^{-1}$; no galactose was detected during this steady state. This indicated that high growth rate above the threshold value is responsible for acetate overflow, and not excess substrate.

2.6.3. Fed-batch culture results

Fed-batch fermentations were also performed to try and discover the threshold rate at which acetate is excreted by the cells without galactose build-up. Fed-batch experiments **FB5** and **FB6** used in this study were controlled by LabView, with on-line HPLC monitoring of galactose and acetate concentrations. In both experiments, the μ was gradually increased in LabView throughout the experiment, (**FB5** manually) to try and reach the μ at which acetate is first excreted without galactose build-up. **FB6** had automatic feed-back (closed-loop) control described more fully in section 1.8. The galactose and acetate profiles for both fermentations are shown in figure 2.6.3.a. (**FB5**) and figure 2.6.3.b. (**FB6**). Figure 2.6.3.c. plots μ calculated from optical density data versus time, while figure 2.6.3.d. gives μ from dry weight data for both fermentations (although the dry weight data for **FB5** is unreliable because of equipment problems). The μ values set by LabView, entered either manually (**FB5**) or automatically (**FB6**) are given in figures 2.6.3.e. and f. Figures 2.6.3.g. and h. presents some data for fermentation **FB4**, the original fermentation run to demonstrate simple closed-loop control (details given in section 1.8.). Figure 2.6.3.g. shows galactose and acetate concentrations while figure 2.6.3.h. gives specific growth rates from dry weight and optical density data. In this experiment, μ was set at 0.5, and the pump was cut when acetate built up.

These graphs show a number of things. Firstly, fig. 2.6.3.a shows that the cells in **FB5** were overfed during the night, and when the feed-pump was switched off, the cells were able to increase their growth rate **rapidly** to consume the excess galactose (and acetate), and μ (from OD data) increased to greater than 0.4 h^{-1}. Hence, it is known that the cells are able to rapidly increase their specific growth rate without building up galactose, and even consume acetate. However, both **FB5** and **FB6** show that the cells are wholly unable to adjust to a slow increase in specific growth rate, and galactose and acetate build up (unless control actions are taken such as in **FB6**). This in turn means that it is going to be very difficult to determine the threshold μ at which acetate overflows without galactose build up in fed-batch culture with a slow μ ramp. The cells also found it very difficult

to grow at a specific growth rate set at 0.5 h^{-1} set in **FB4** and the build-up of galactose and acetate meant the feed-pump was cut, so a μ of 0.5 h^{-1} was never achieved.

2.6.4. Summary

The μ at which acetate starts to overflow without detection of galactose in the medium was found for continuous culture. Attempts to find the equivalent μ in fed-batch culture by slowly increasing the specific growth rate proved fruitless, as the cells were unable to cope with a slow increase in feed, and could not assimilate all substrate. These results indicate that continuous culture is a much better method for obtaining these results. Fed-batch culture could not work, because several pseudo steady states cannot be achieved in the relatively small number of cell generations that can be included in this type of fermentation.

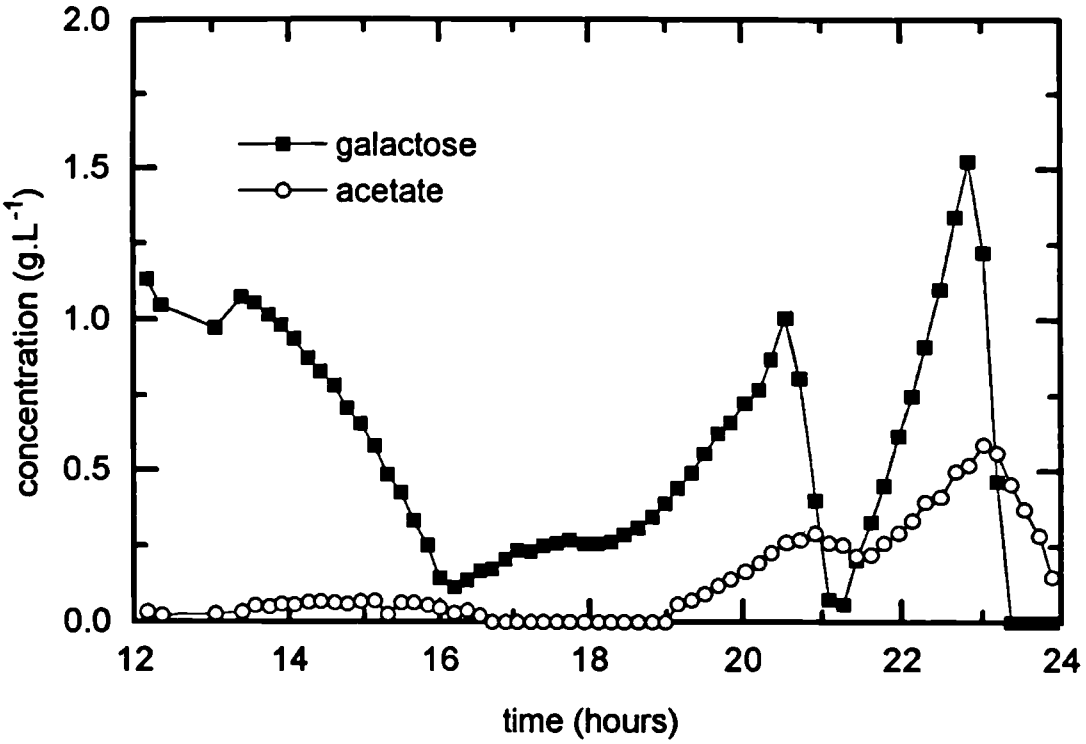


Fig. 2.6.3.a. Galactose and acetate profiles for fed-batch fermentation **FB5** in which the specific growth rate was slowly ramped up manually in LabView.

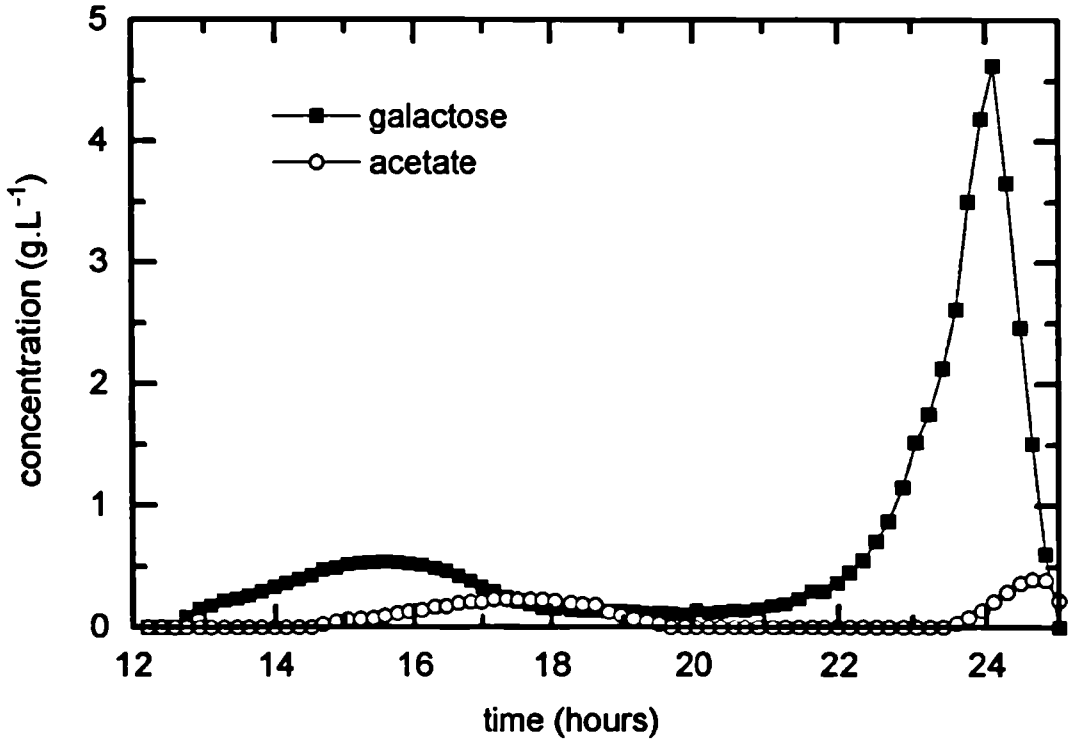


Fig. 2.6.3.b. Galactose and acetate profile for fed-batch fermentation **FB6** in which the specific growth rate was slowly ramped up automatically in LabView.

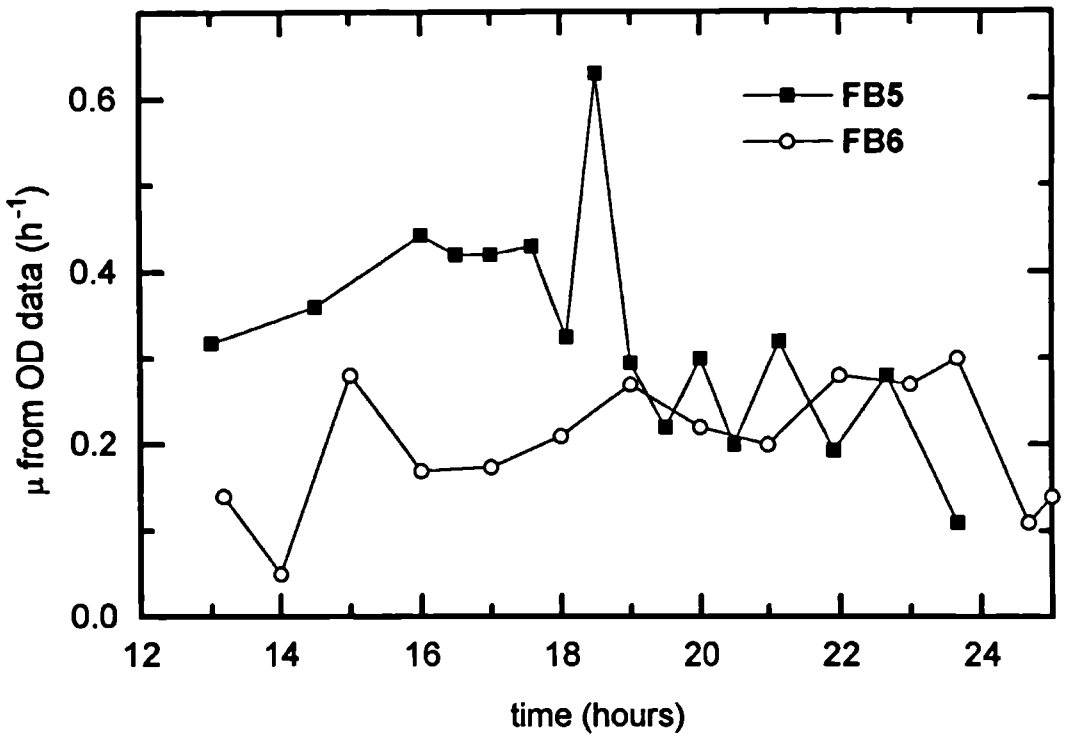


Fig. 2.6.3.c. Specific growth rates obtained from optical density data for fed-batch fermentations **FB5** and **FB6**.

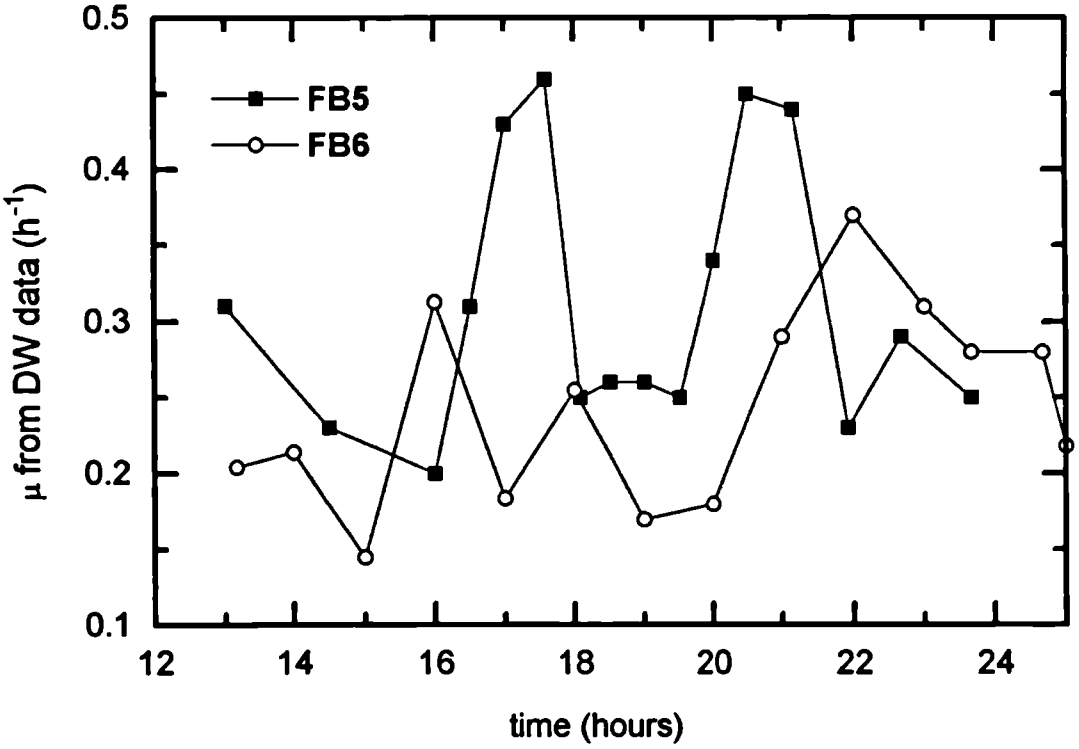


Fig. 2.6.3.d. Specific growth rates obtained from dry weight data for fed-batch fermentations **FB5** and **FB6**.

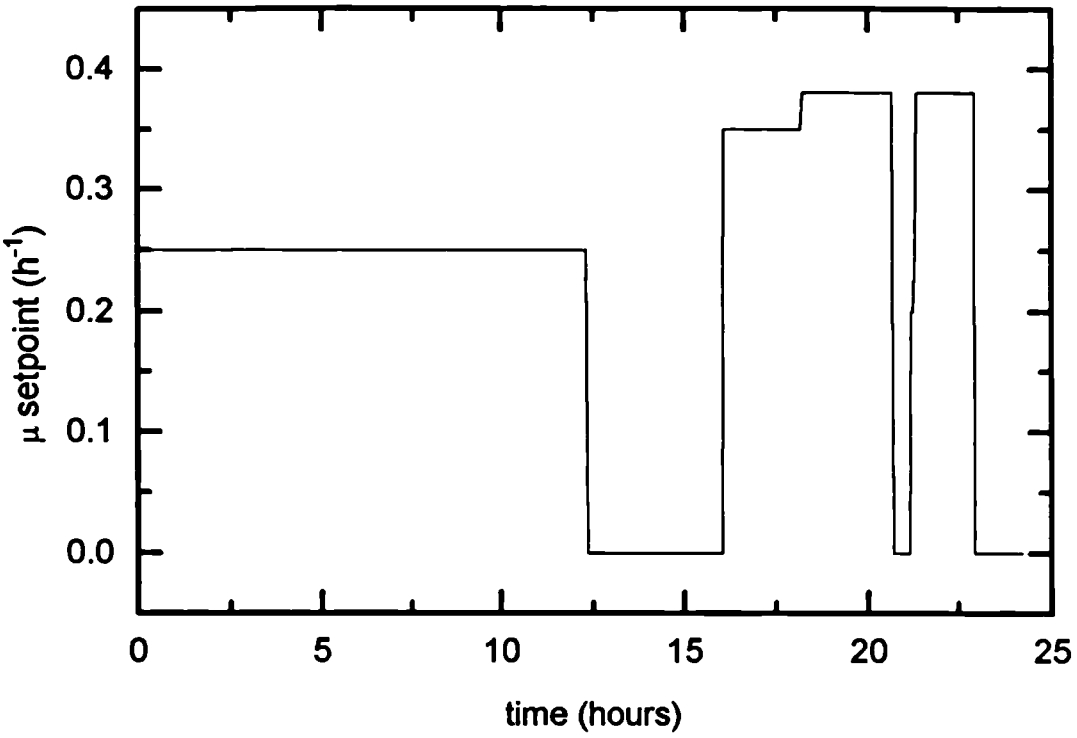


Fig. 2.6.3.e. Plot of μ setpoint entered manually into LabView for fermentation **FB5**. The stages on the graph where μ = 0 indicate where the pump feed was set to zero to allow excess galactose in the medium to be assimilated.

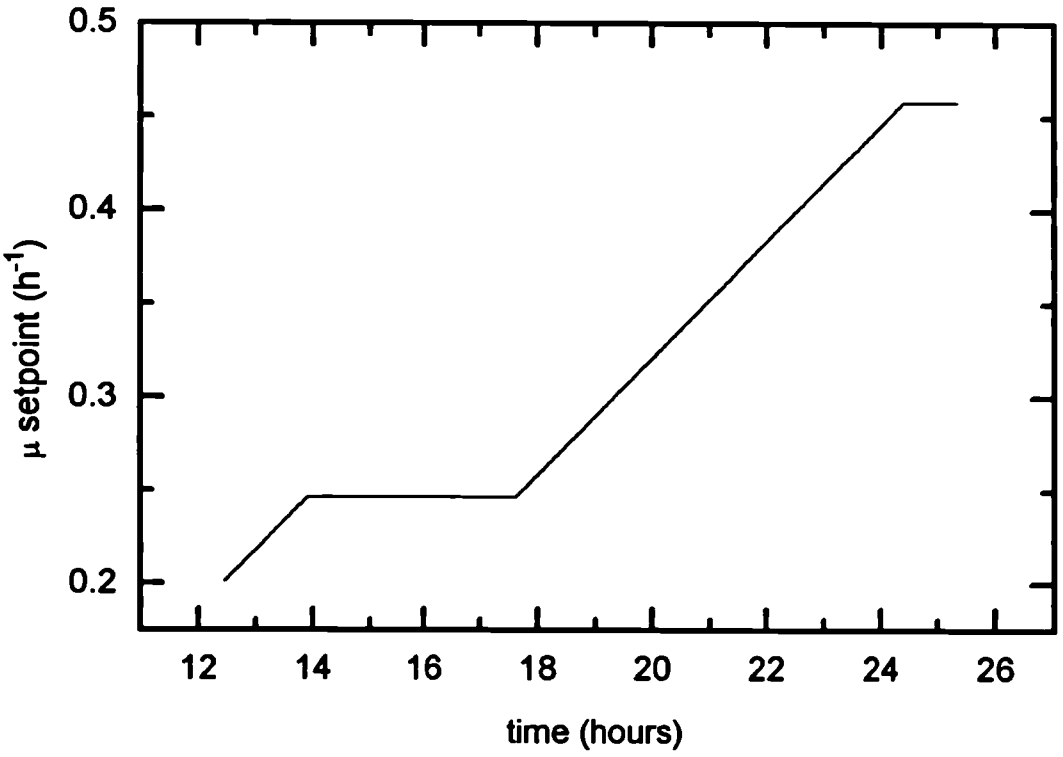


Fig.2.6.3.f. Plot of μ setpoint ramped up automatically by LabView for fermentation **FB6**. The "plateau" areas on the graph indicate where the critical analyte threshold 1 was exceeded, and the μ ramp was disabled.

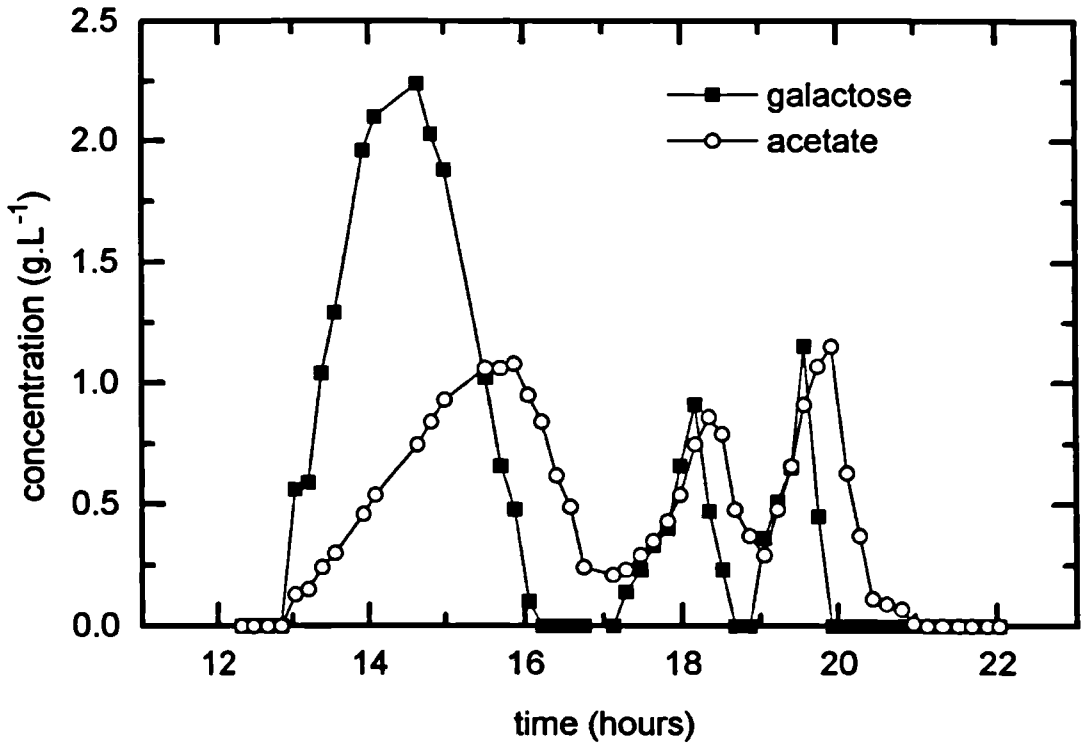


Fig. 2.6.3.g. Galactose and acetate profile of fed-batch fermentation **FB4** in which cells were set to grow at a μ of 0.5 h^{-1}, however μ was reduced as a result of feed-back control.

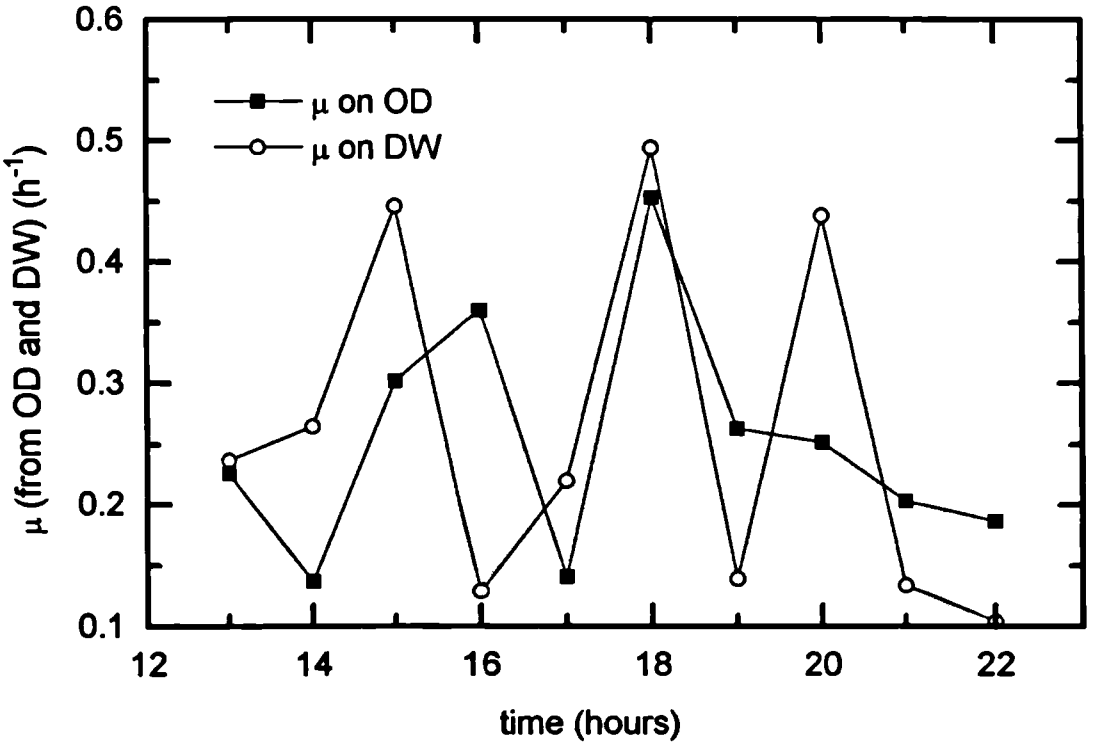


Fig. 2.6.3.h. Specific growth rates calculated from optical density and dry weight data for fed-batch fermentation **FB4** with no μ ramp.

2.7. THE EFFECTS OF ACETATE ON SPECIFIC GROWTH RATE

2.7.1. Introduction

The previous section (2.6.) looked at the effects of specific growth rate on acetate production. Equally, or perhaps more important is the effect of acetic acid on specific growth rate. A section in the literature review (section 2.1.3.1.) described work by a number of researchers that has shown that high levels of acetic acid in fermentation medium inhibits the growth rate of the microorganism, and in some cases, growth ceases altogether. This section describes the measurement of the effects of acetate on μ_{max} in continuous culture, **C1**, and presents some evidence linking high acetate concentration to a reduction in specific growth rate in fed-batch culture, **FB2**.

2.7.2. Continuous culture: method of measuring reduction in μ_{max}

The continuous culture, **C1**, provided the most suitable way of examining the effects of acetate concentration on the maximum specific growth rate. This was achieved as follows: firstly, the cells were subjected to a washout experiment on normal medium; taking samples for optical density measurements at frequent intervals. The washout was then stopped and the cells allowed to grow back to almost maximum OD *ie.* until just before the culture became galactose limited (the cells still growing at μ_{max}). The medium was then supplemented with a particular concentration of acetate (as sodium acetate) and the washout experiment repeated, again with frequent sampling to obtain optical density measurements. This procedure was performed for three different concentrations of acetate - 2; 5 and 8 $g.L^{-1}$, and also for a control experiment in which the equivalent sodium in 8 $g.L^{-1}$ sodium acetate (as sodium chloride) was added to the medium. Plotting the natural logarithm of optical density (of samples taken at intervals throughout each washout, both with and without acetate or sodium addition) versus time, and doing a linear regression, gives a slope, m. Using this value, and the dilution rate used, the maximum specific growth rate can be derived as described in section 2.2.4.2. From this data, the difference between

μ_{max} with and without additions of acetate and sodium can be seen; and hence the effects of acetate (at different concentrations) and sodium on the maximum specific growth rate of the cells.

2.7.3. Results of continuous culture

Plots of natural logarithm of optical density versus time are given for the washout experiments prior to acetate addition, and after it for each of the acetate concentrations, in figures 2.7.3.a, b, and c. The results obtained from linear regressions of these graphs, plus the known dilution rate of the culture, are summarised in table 2.7.3.(i).

EXPERIMENT	μ_{max} (h^{-1}), NORMAL MEDIUM	μ_{max} (h^{-1}), MEDIUM PLUS ACETATE/NaCl	% REDUCTION IN μ_{max}
2 $g.L^{-1}$ acetate	0.67	0.67	0
5 $g.L^{-1}$ acetate	0.656	0.58	11.6
8 $g.L^{-1}$ acetate	0.639	0.303	52.6
8 $g.L^{-1}$ sodium equivalent	0.649	0.652	-0.56

Table 2.7.3.(i). Results from washout experiment in which μ_{max} was measured in normal medium which was then supplemented with different concentrations of acetate (as sodium acetate) and then the subsequent μ_{max} measured. This was repeated for a sodium control.

These results show that no reduction in μ_{max} occurred with the presence of 2 $g.L^{-1}$ acetate, however, when this was increased to 5 $g.L^{-1}$, there is an 11.6 % reduction in μ_{max}. This increases to at least a massive 52.6 % with the presence of 8 $g.L^{-1}$ acetate. This latter figure is an approximation: it could be substantially higher than this. The μ_{max} was decreasing during the course of the experiment: 0.303 h^{-1} was the final figure recorded; after this point the optical density was too low to

provide meaningful data.

A control experiment was performed with sodium chloride to ensure that the effects observed were due solely to the acetate, and not to sodium. The same concentration of sodium added in the 8 $g.L^{-1}$ sodium acetate was added as sodium chloride. The results shown in figure 2.7.3.d. and table 2.7.3.(i). indicate that no reduction in μ_{max} occurred, confirming that any reduction in μ_{max} when sodium acetate had been added was solely due to the acetate.

2.7.4. Results from fed-batch fermentation FB2

FB2 (the fermentation in which the galactose concentration was maintained above 15 $g.L^{-1}$ throughout) had a large build-up of acetic acid at the end of the fermentation, and this corresponded to a decrease in specific growth rate. Figure 2.7.4.a. plots the acetate profile of the fermentation, and μ obtained from both dry weight and optical density data. The reduction in μ toward the end of the fermentation is evident, as is the high acetate concentration. In the light of the results for the continuous culture, it does not seem surprising that the specific growth rate drops considerably towards the end of the fermentation.

2.7.5. Summary

This section presents results indicating that acetate can cause a reduction in growth rate in concentrations as low as 5 $g.L^{-1}$, and this reduction becomes much larger at 8 $g.L^{-1}$. Both fed-batch and continuous cultures provide data to show the effects of acetate on specific growth rate. These results again suggest that in order to obtain optimal results from a fermentation, acetate concentration should be minimised by on-line monitoring and controlled feeding.

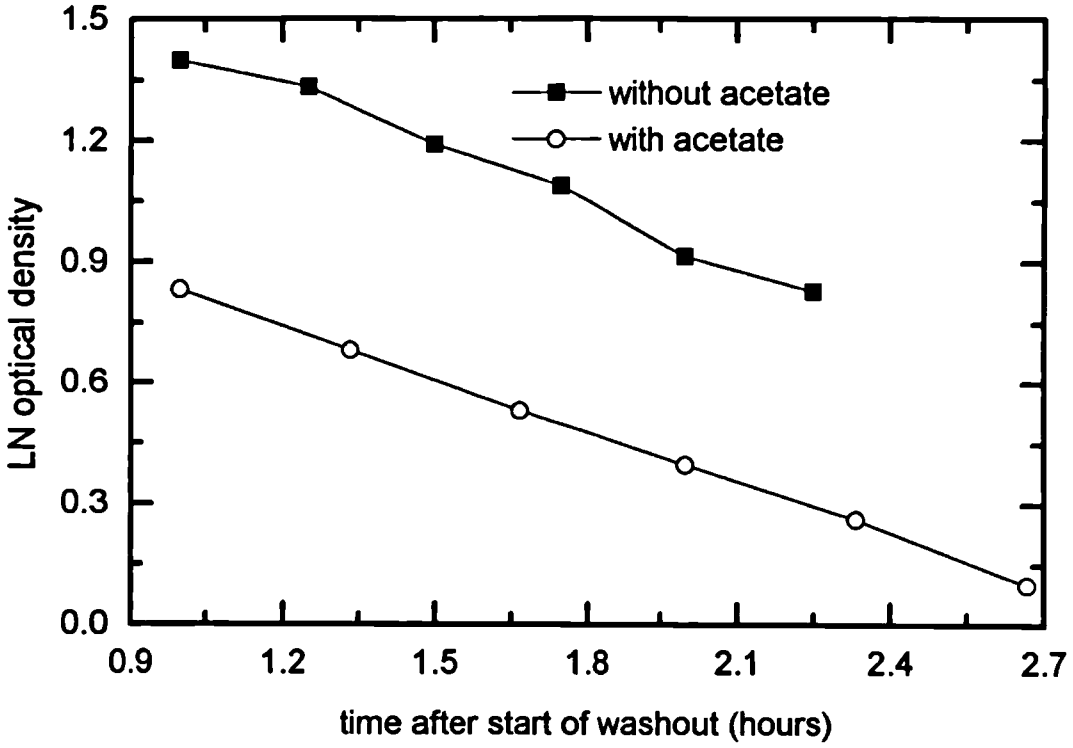


Fig. 2.7.3.a. Plot of natural logarithm of optical density versus time for washout experiments with and without 2 $g.L^{-1}$ acetate.

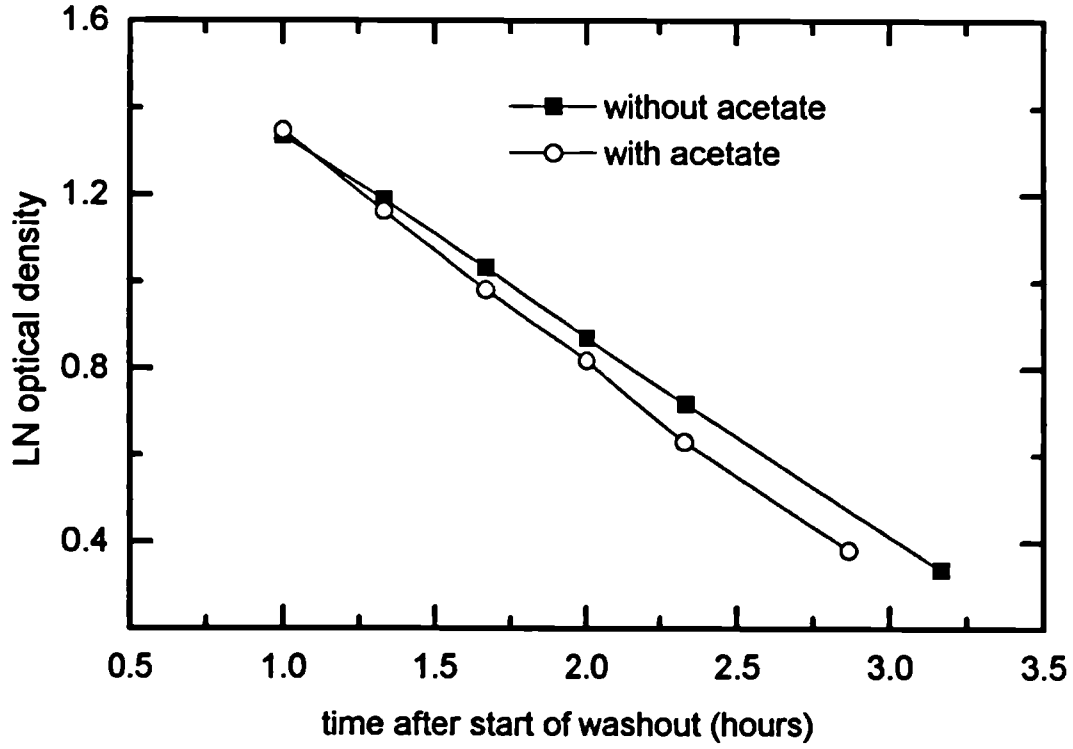


Fig. 2.7.3.b. Plot of natural logarithm of optical density versus time for washout experiments with and without 5 $g.L^{-1}$ acetate.

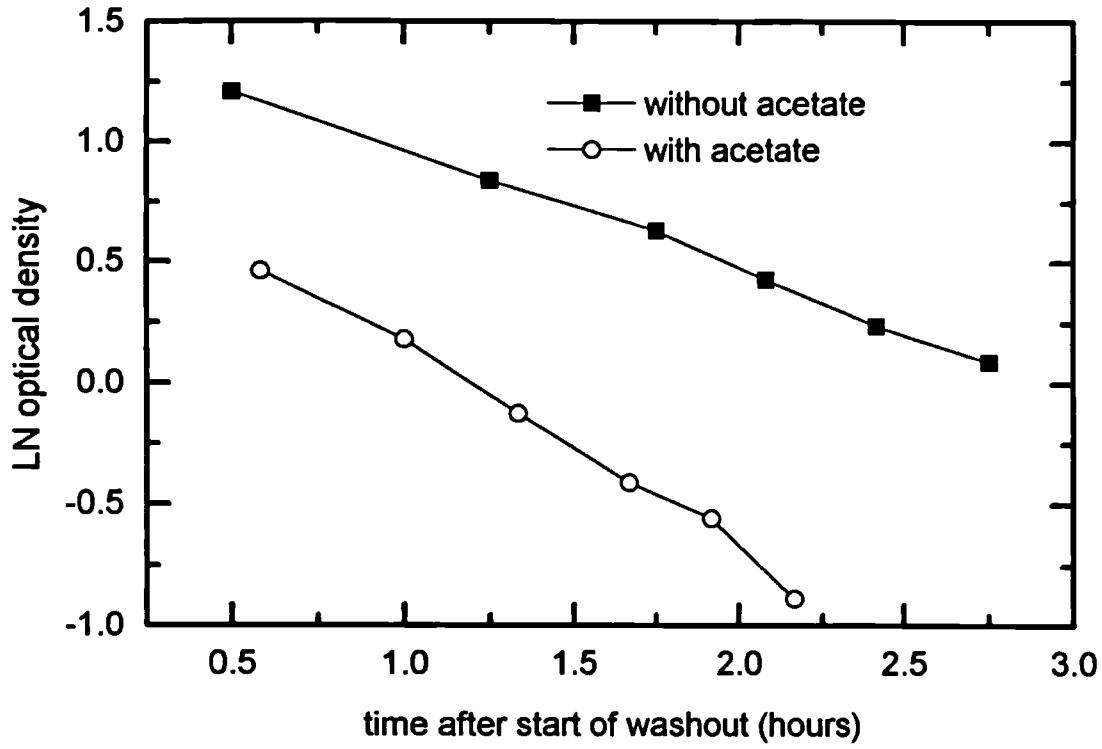


Fig. 2.7.3.c. Plot of natural logarithm of optical density versus time for washout experiments with and without 8 $g.L^{-1}$ acetate.

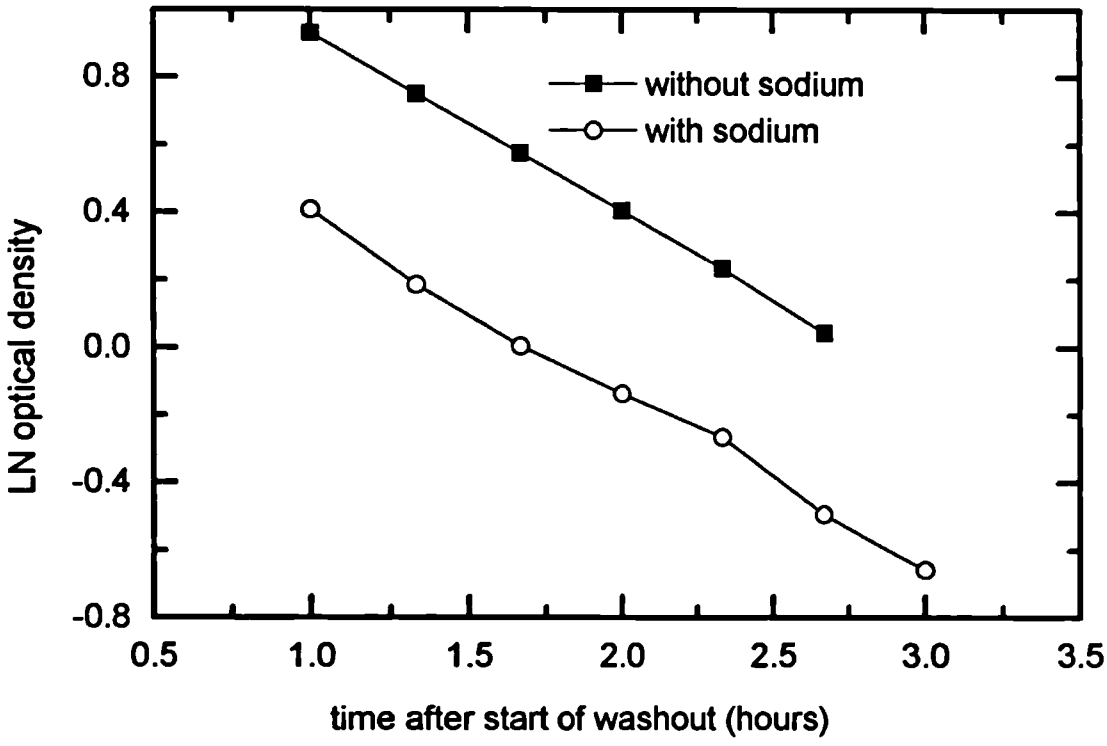


Fig. 2.7.3.d. Plot of natural logarithm of optical density versus time for washout experiments with and without 8 $g.L^{-1}$ sodium equivalent.

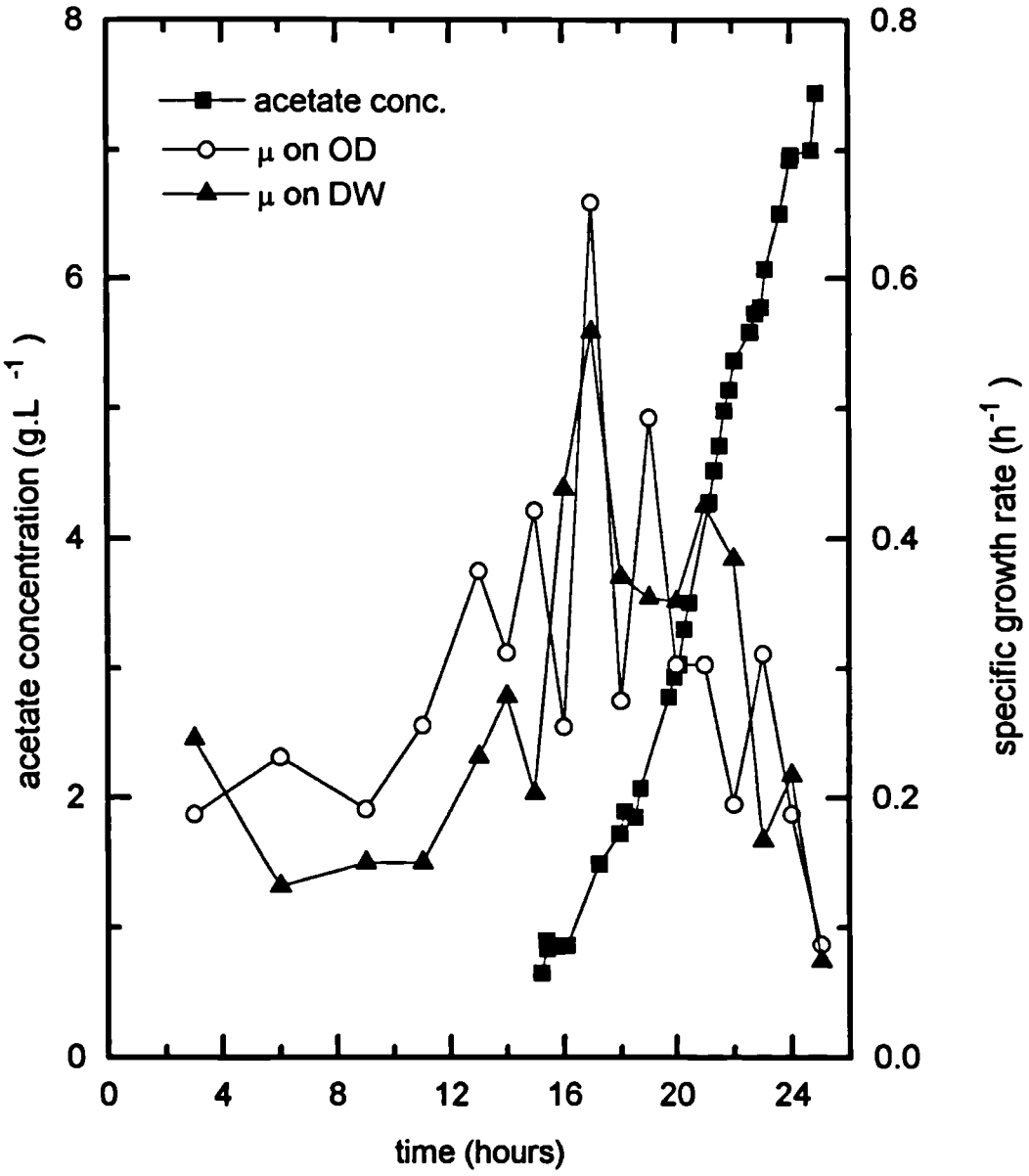


Fig. 2.7.4.a. Plot of acetate concentration and specific growth rates (obtained from optical density and dry weight data) for fed-batch fermentation, **FB2**.

2.8. THE EFFECT OF ACETATE ON AMYLASE PRODUCTION

2.8.1. Introduction

As has been demonstrated in section 2.7., the presence of acetate in fermentation broth in concentrations of 5 $g.L^{-1}$ or higher can have a profound effect on the maximum specific growth rate of the organism used in this study. Another effect of acetate on fermentations reported in the literature is its role in reducing the production of recombinant protein. This role was examined for one concentration of acetate on the production of α-amylase in *E. coli* JM107 in continuous culture.

2.8.2. Method

Cells were grown in continuous culture, as previously described, at a dilution rate of 0.3 h^{-1} on the usual medium, samples being taken to ensure a steady state had been reached. At a certain time (time zero) the experiment started with the addition of a single dose of acetate (7.4 $g.L^{-1}$, as sodium acetate). Samples taken over the twenty hours following the acetate addition provided optical density, amylase and acetate profiles for the experiment.

2.8.3. Results

Figure 2.8.3.a. plots the total amylase activity versus time throughout the experiment; a theoretical line appears on this graph, representing what the amylase activity measurement would be, due to dilution effects, if no α-amylase was produced after acetate addition. Figure 2.8.3.b. plots the percentage of original total amylase (prior to acetate addition) present in each sample. Figure 2.8.3.c. plots the optical density for the experiment, and the total specific (per optical density unit) amylase activity. The acetate concentrations are given in figure 2.8.3.d.

These results show the great effect that the addition of acetate has on α-amylase

production. Figure 2.8.3.b. indicates that the amylase activity drops to just 60 % of its original value. This does not mean that amylase production has halved; figure 2.8.3.a. plots the amylase activities that would be found if no amylase was produced after addition of acetate *ie* due to dilution effects as well as those actually measured. These two profiles are not too different for the time period that acetate is still present in the culture, indicating that α-amylase production had almost ceased, particularly after 1½ - 2 hours after acetate addition.

Unfortunately, the results are not entirely clear cut. Addition of acetate caused an initial reduction in optical density due to dilution of the culture, and then a further decline in OD (see figure 2.8.3.c.). During this time, galactose was detected in the broth indicating that the cells were unable to maintain the dilution rate set. This was not unexpected in the light of results presented in section 2.7. However, after 1½ - 2 hours, the cells were able to use (consume) acetate as a carbon source to produce biomass, and the increase in optical density can be seen. This caused an increase in actual specific growth rate (ie it became faster than the 0.3 h^{-1} set by the dilution rate); this can be seen in figure 2.8.3.e. The increase in μ can lead to a reduction in amylase production, as previously discussed, so the loss of amylase production could partly be due to an increase in μ. However, it is not believed to be the main cause of reduction in amylase activity.

The final result from this experiment is the effect of acetate addition on the leakage of the cells from the periplasm. Figure 2.8.3.f. plots the percentage of total amylase activity leaked into the extracellular medium plotted on the same graph as total amylase activity. As can be seen from this data, a greater percentage of amylase is leaked from the periplasm with a reduction in total amylase activity, implying that cells subjected to stress (shown by loss of amylase activity) have greater leakage of amylase from the periplasm.

2.8.4. Summary

These results show that acetate (at a concentration of 7.4 $g.L^{-1}$) has an effect on

amylase activity. This effect is large, but cannot be fully measured because of the change in actual specific growth rate, when the cells were able to utilise the acetate as a carbon source. The cell culture was fully able to recover from the acetate addition, and amylase activity was back to the original amount when the acetate had been diluted out and used up by the cells.

The experiment also provides evidence that the presence of acetate reduces the amount of amylase that is retained in the periplasm; this could also be partly due to a change in specific growth rate of the culture.

A final point is worth noting. The original starting amylase activity at this dilution rate was significantly less than when the culture was operated at a similar dilution rate some time before this experiment had been performed. In between these experiment, many washout experiments had been performed, and although the plasmid had not been lost (from plasmid stability studies), it is likely that segregational instability had caused a variable copy number, enhanced by repeatedly growing the cells at μ_{max}. This would account for the differing amylase activities.

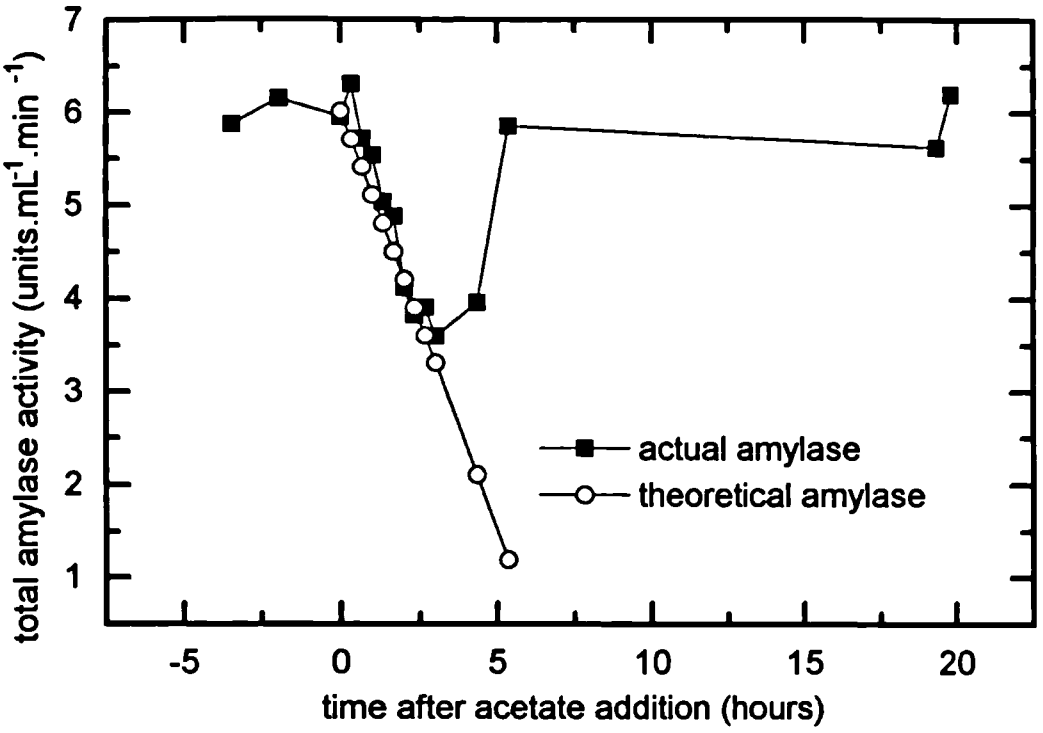


Fig. 2.8.3.a. Plot of actual amylase activity versus time after acetate addition. Also shown on the graph is the theoretical profile that would result from dilution effects if no amylase was produced after acetate addition.

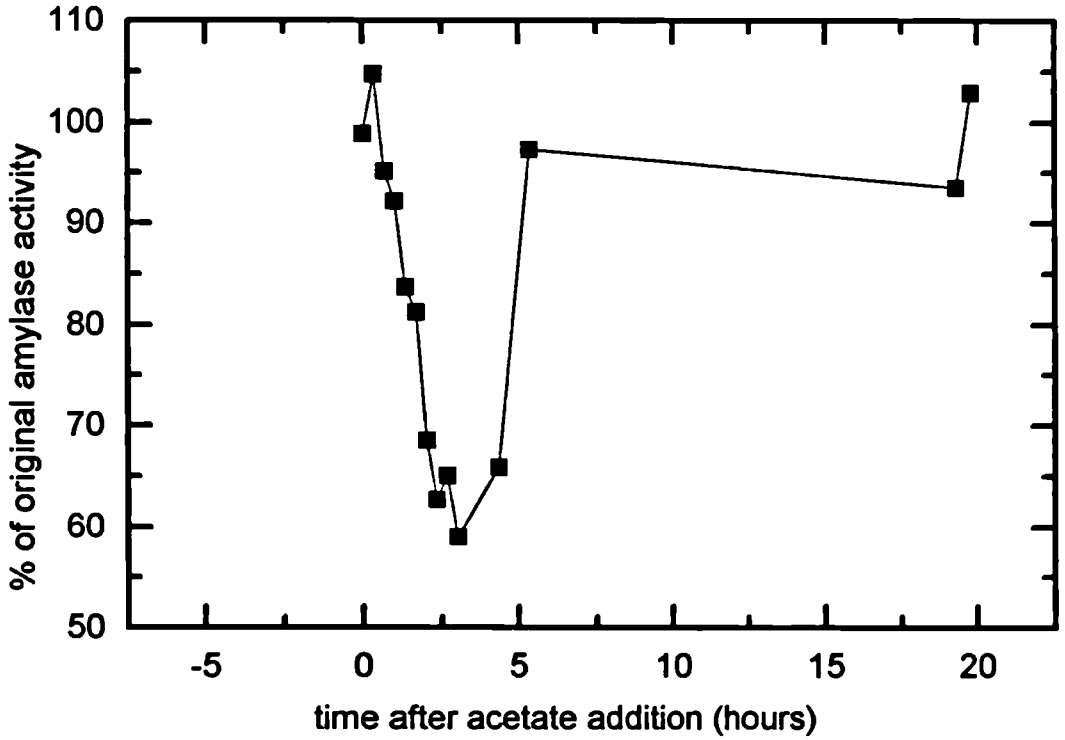


Fig. 2.8.3.b. Plot of % of original amylase activity found in sample after addition of 7.4 g L^{-1} acetate to culture.

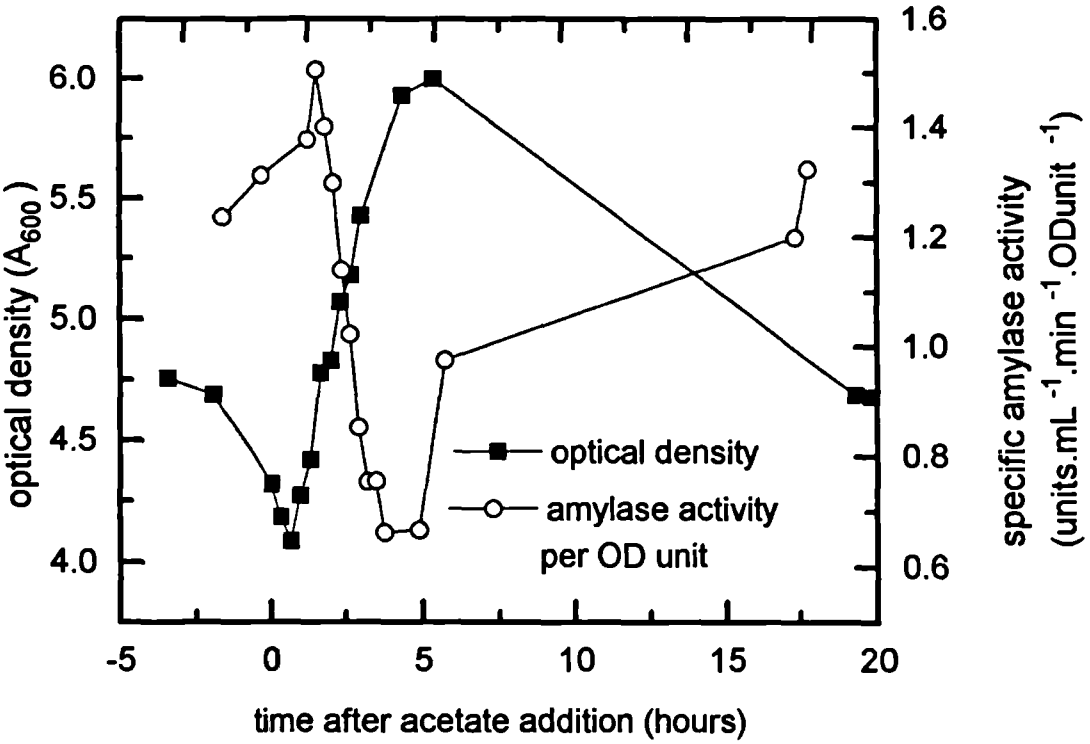


Fig. 2.8.3.c. Plot of optical density and specific amylase activity (based on optical density) versus time after addition of 7.4 g.L^{-1} acetate.

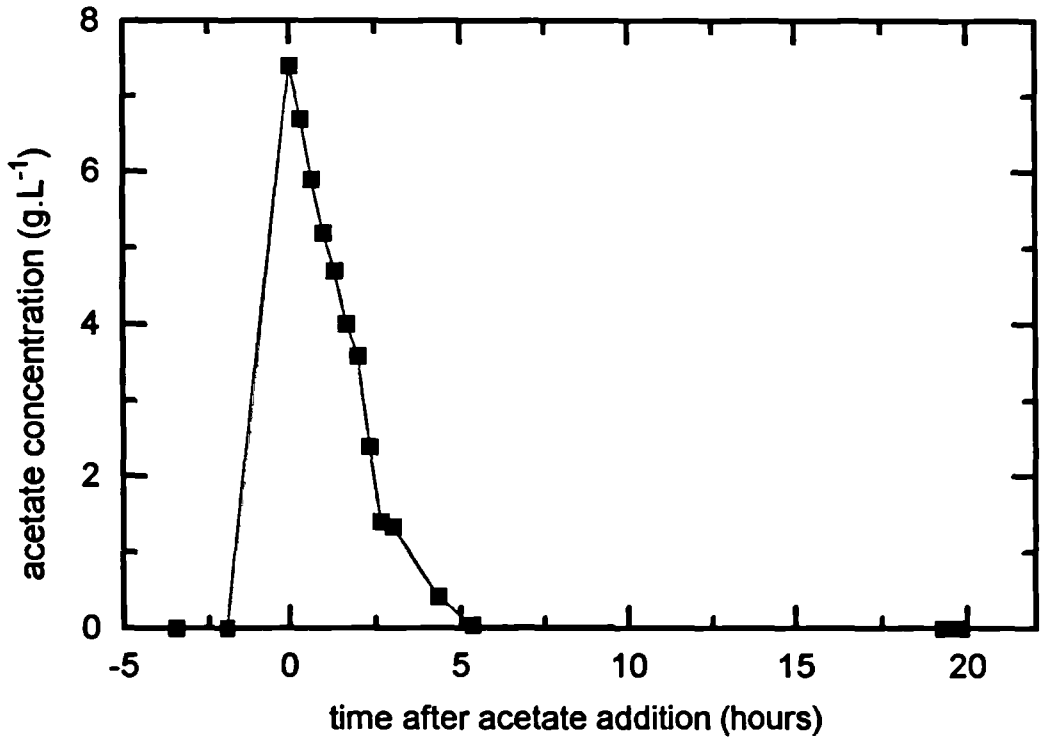


Fig. 2.8.3.d. Acetate profile for experiment in which a dose of 7.4 g.L^{-1} acetate was added to fermenter. Some acetate is washed out, while some is used by the *E. coli* as carbon source.

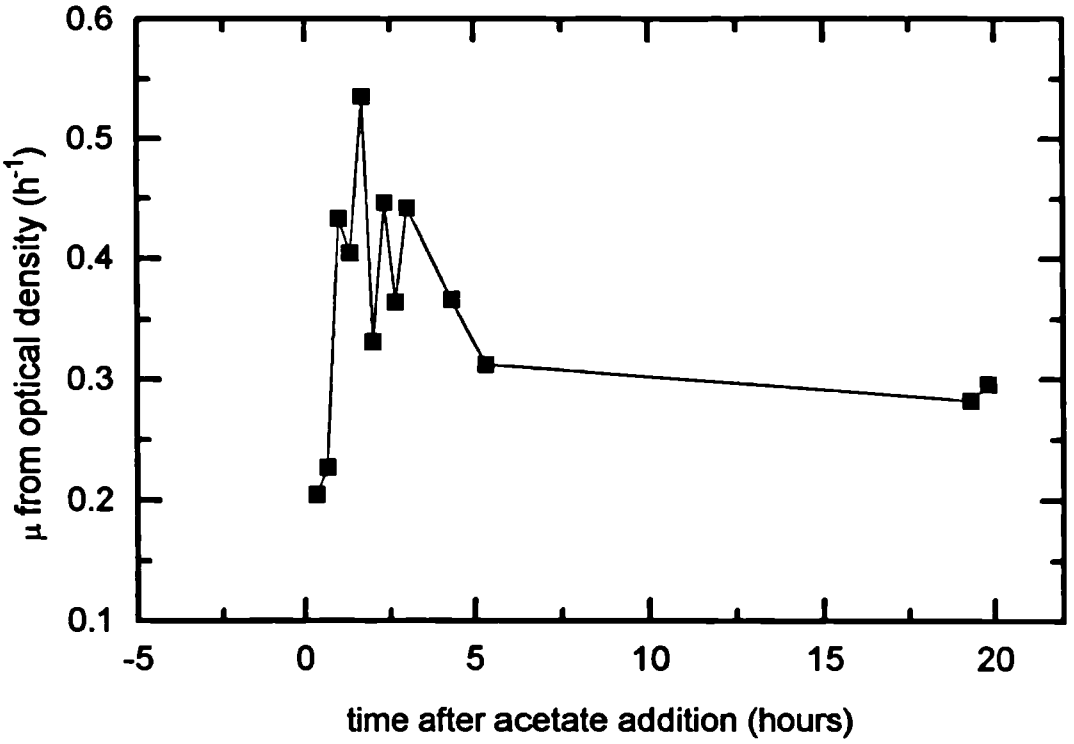


Fig. 2.8.3.e. Plot of specifife growth rate (from optical density data) after addition of 7.4 $g.L^{-1}$ acetate added to fermenter. Change from dilution rate set at 0.3 h^{-1} is due to cells using acetate as an extra carbon source.

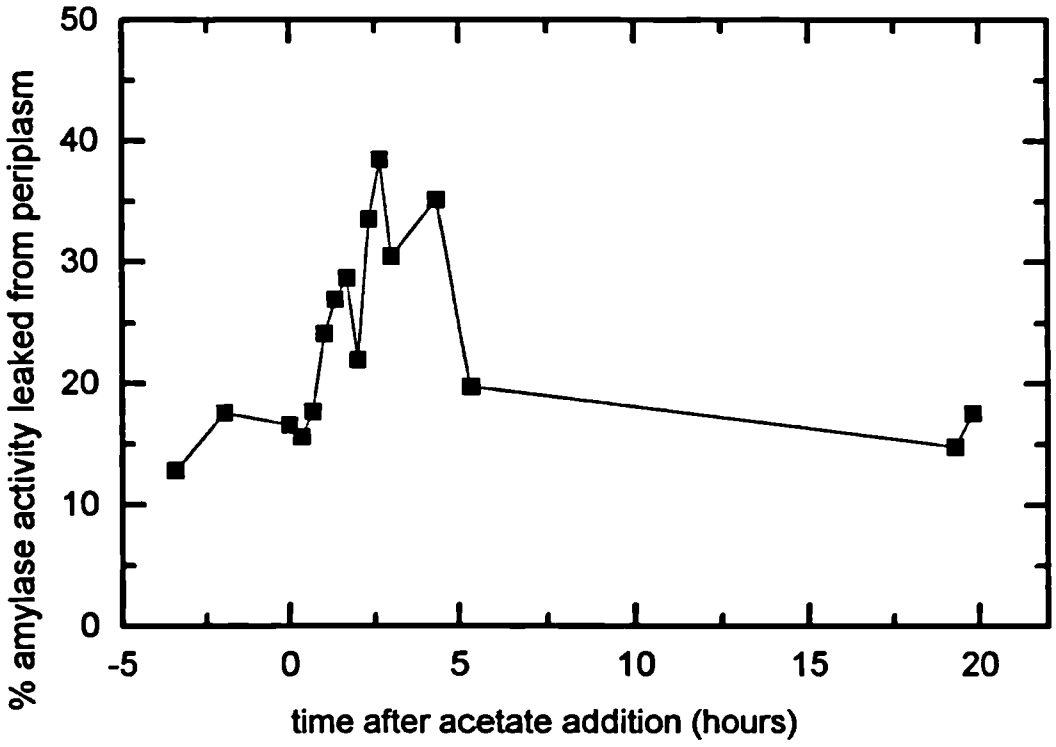


Fig. 2.8.3.f. Plot of percentage amylase activity leaked from periplasm versus time after 7.4 $g.L^{-1}$ acetate added to fermenter.

2.9. SUMMARY AND DISCUSSION

This section has examined the effects of certain fermentation conditions on the growth and production of recombinant α-amylase in *E. coli* JM107 + pQR126. Many of these results were arrived at by using the on-line monitoring system described in section 1, with or without the use of the LabView process control system. The results from section 2 are summarised below, and then implications of the results are discussed.

2.9.1. Summary of main results

- The production of α-amylase is strongly dependent on specific growth rate / dilution rate; being fairly low at low dilution rates / μs, increasing with dilution rate / μ to a maximum, and then decreasing again at high dilution rates / μs.

- High carbon substrate (galactose) concentration reduces the specific growth rate of the culture, and also reduces the production of α-amylase.

- Acetate was found to be excreted by *E. coli* JM107 at a dilution rate of 0.49 - 0.5 h^{-1} in continuous culture, without galactose being present. The experiment could not be repeated in fed-batch culture because of the cells' inability to rapidly change the rate at which they grow. Fed-batch is an inappropriate medium for obtaining pseudo steady states because of the number of generation times needed for each one.

- Acetate at a concentration of 5 $g.L^{-1}$ or greater reduces the maximum specific growth rate of the cell.

- High acetate concentrations reduce the production of recombinant α-amylase.

2.9.2. Discussion

The results obtained from this series of fermentations provide the basis for

developing a feeding control strategy to maximise recombinant protein production. The main points to emerge are the need to control the specific growth rate of the organism, while maintaining a low substrate concentration in the broth, and preventing acetic acid from being excreted. If the cells are grown at a suitable specific growth rate, galactose and acetate concentrations should not build up in the fermenter, because as has been seen, the cells are easily able to grow at the growth rate optimal for amylase production. However, if the estimate of biomass deviates from the true value, or if the cells are not "pre-conditioned" to grow at the required growth rate, both galactose and acetate can build up in the medium. The on-line measurement of galactose and acetate provides a suitable method of determining when the controller is deviating from its setpoints, especially as devices for the on-line measurement of biomass are still not robust enough for ubiquitous, commercial use. The on-line monitoring system has also shown itself to be very useful in performing fermentations to research aspects of the physiology of the organism.

Recombinant protein production's dependence on specific growth rate in fed-batch culture is an important result. As previously mentioned, organisms are seldom grown in fed-batch culture at a particular specific growth rate, and as a result, some results reported in the literature have been incompletely examined and the wrong conclusions drawn. For example, researchers write that by measuring and controlling glucose concentration in a culture, they have reduced the cells' excretion of acetate, resulting in much higher yields of recombinant protein. Although those results are certainly true, it is very likely that a lot of the improved yield is due to the reduced specific growth rates arising from the revised feeding strategy. This probability is seldom considered - a clear omission.

3. DISCUSSION

3.0. INTRODUCTION

This thesis has described the development of an on-line monitoring system which automatically aseptically removes and centrifuges small samples of fermentation broth. The supernatants can then go forward for analysis of sugars and organic acids by HPLC. It has then gone on to describe the the use of the system in the monitoring and control of galactose and acetate concentrations in fed-batch recombinant *Escherichia coli* fermentations by linking the system to the process control package, LabView. The combined monitoring and control package has then been used in examining the effects of certain fermentation conditions on the growth and recombinant α-amylase prodcution of the recombinant *E. coli* in fed-batch culture. Batch and continuous cultures without the use of the on-line monitoring and control system were also performed to provide additional information about the effect of fermentation conditions on the organism.

This discussion looks at the advantages and disadvantages of the system as a whole and then discusses each of the components of the system in turn. The two main operating problems are described, and alternatives suggested. A description of the usefulness of the system in monitoring fermentation follows, as well as how applications of the system can be extended by linking it to a control system. The discussion culminates in a brief description of the main conclusions drawn from the work done.

3.1. THE ON-LINE MONITORING SYSTEM

3.1.1. General comments

An on-line monitoring system based on microcentrifugation as opposed to the more traditional filtration techniques has two main advantages. Firstly, there is no filter to clog, so the free passage of a sample is assured. This is a particular problem when a filtration device is required to maintain a sterile barrier as well as to provide a means of separating solid and liquid components of the broth - if the filter gets clogged, its replacement would mean compromising fermenter asepsis. The second advantage is that removing a whole broth sample from the fermenter and centrifuging it provides an easily accessible sample of cellular material separated from a clear supernatant. This means that there is potential for future development to modify the system to analyse some component of the cellular material, and this possibility will be discussed in section 5. The drawback of using microcentrifugation is that its separation efficiency is inferior to filtration; some solids are still present in the supernatant; this is discussed in section 4.1.3.

Using HPLC to analyse components of the broth allowed the simultaneous measurement of both galactose and acetic acid within twelve minutes. Had *E. coli* JM107 been a strain that excreted high concentrations of other TCA (and associated) compounds, the HPLC column would have been able to analyse those at the same time. Another advantage of HPLC is its ability to analyse almost any compound by changing the column and separation and elution method, which means it can be used for the on-line monitoring of a range of substances from sugars to organic acids to proteins to antibiotics. HPLC does have the limitation of taking several minutes per sample, which isn't a problem in most fermentation systems, but can be a limitation in very fast growing systems and short-lived fermentations. Recent advances in HPLC technology (the development of microbore columns, for example) means that this drawback is rapidly disappearing. There are also control techniques available for dealing with delayed measurements, although some knowledge of the fermentation is required before these have much use. The twelve minute delay time between sampling and

control action was not a major problem in the fermentation system described in this thesis, although a fairly complex dual level control strategy was required for fine control.

3.1.2. The sampling device

The purpose of the sampling device was to prevent the contamination of a fermentation occurring during sampling. Initially, the sampling device was operated with a steam trap (to maintain steam pressure in the lines); however, large particles in the steam supply frequently blocked the steam trap. For this reason, the steam trap was removed, and sterilisation was the achieved by "free-steaming". This did not cause the contamination of any recombinant *E. coli* fermentations, and this fact raises the question of how robust the sterilisation system needs to be for such a fast growing organism, especially when grown on defined medium containing an antibiotic. The sampling device was not tested with any other type of organism, so its efficiency in maintaining asepsis cannot be reliably assessed.

The stainless steel tubing in the sampling device was easily able to allow free passage of *E. coli* and *S. cerevisiae* samples *ie* the device was not blocked. If the device was to be used to sample filamentous fermentations, replacement tubing of wider internal diameter would need to be used, however, this could easily be achieved.

3.1.3. The microcentrifuge

The microcentrifuge was tested extensively with *S. cerevisiae* and *E.coli* at different cell concentrations and under different operating conditions to determine how well it performed with each of the two cell types. The microcentrifuge was also compared to filtration and a commercially available off line microcentrifuge (the Denley). The main drawback of the microcentrifuge is that the separation obtained from its use is inferior to both filtration and the Denley. It is understandably inferior to filtration, but the *g* forces obtained in the

Denley are much lower than in the microcentrifuge. A possible explanation of this separation difference is the smaller area of contact between solid and liquid in the Denley than the microcentrifuge, allowing less area across which resuspension of solids can occur, probably due to vibrations after separation of the sample when the centrifuge is slowing down.

It was also found that the separation of *E. coli* was significantly better than the separation of *S. cerevisiae*. This is probably due to morphological differences in the cells - the rod shaped E. coli probably pack together much tighter than the spherical *S. cerevisiae* can, allowing the *E. coli* to either better pack against the wall of the centrifuge bowl in the first place, or being less easily dislodged and resuspending when the bowl is slowing down.

The other issue concerning separation efficiency is the unexpected result that separation is better at lower spin speeds and shorter spin durations. Separation is believed to occur very quickly, implying that short spins would be as efficient as longer spins, therefore the declining separation efficiency is not readily explained. Higher spin speeds possibly have worse efficiencies than lower spin speeds, because the bowl has to pass through more "nodes of vibration" which could cause resuspension of solids.

The centrifuge was only used in fermentation with *E. coli* cells; the clarity of the supernatant being sufficiently good for direct injection onto an HPLC column without prior treatment (although a guard column was in use). The clarity of the supernatant from yeast separations was much poorer, and it is doubtful whether supernatants could be directly used; the column would rapidly be fouled. This problem needs to be addressed before the system can be used to monitor yeast fermentations.

The overall performance of the centrifuge was good. There are a number of design problems which, if solved, could improve the lifespan of the air-turbine and reduce the amount of time spent preparing the sample for HPLC analysis. These will be discussed in section 4.

3.1.4. The HPLC system

The HPLC system and column also performed very well during the on-line analysis of *E. coli* fermentations. Each guard column operated for approximately 200 injections before the column resolution deteriorated and the guard column needed replacing. The need to clean and regenerate the column was even more infrequent.

A comparison of on-line HPLC analysis of glucose and acetate with off-line enzymatic assays of samples of fermentation broth showed excellent agreement for acetate analyses. There was poorer agreement for the glucose results, although the differences were at about the edge of the quoted coefficient of variation for the off-line enzymatic assay kit at high glucose concentrations; it is not clear which assay method is more accurate. At lower concentrations, the agreement was much closer, and it is at the lower concentrations that accurate measurements of substrate are more important, both in terms of knowing when your substrate is going to run out, and also because it is at these levels of substrate that control of feed supply in fed-batch fermentations is operated.

The data obtained from the refractive index detector was more reliable, with less noise than data from the ultra violet detector; although the latter is more sensitive. The sensitivity limitation of the RI detector was more than compensated for by its stability, and detection was possible at levels as low as 0.1 $g.L^{-1}$ galactose, and even less for acetate.

3.1.5. The programmable logic controller

There is little that needs to be said about the programmable logic controller. It has show itself to be very well suited to its task of low level control of sequencing the operation of each of the components of the on-line monitoring system. It was relatively easy to program, and did not fail throughout the duration of this project. Its robustness and reliability make it ideally suited for pilot plant function.

3.2. PROBLEMS ARISING DURING THE USE OF THE ON-LINE MONITORING SYSTEM

As the on-line monitoring system was a prototype, problems during its operation were inevitable. There were two main failures which resulted in most of the problems encountered during the monitoring of acetate and galactose concentrations during fermentations. Firstly, the air compressor used to supply air pressure for operation of the microcentrifuge and valves in the sampling device easily overheated; when this occurred, it became necessary to discontinue sampling. Fortunately, this was not a serious problem when low spin speeds (and therefore air pressures) were used with a short spin duration. In addition, the current drawn by the compressor when it switched on was very large, and the 13 Amp fuse frequently blew. When this occurred and was not immediately detected, samples could not be pumped out of the fermenter, because the air pressure became too low to open the valves in the sampling device. This was usually detected by the lack of a "sample" peak on the chromatography trace. This problem can best be solved by changing the air supply. The pilot plant air supply was considered as an alternative, but the pressure varies greatly, and is sometimes not high enough to open the sampling device valves (40 psi). In addition, pilot plant air contains high levels of water, which is known to rust the turbine bearings, so adequate measures need be taken to remove water from the air supply. This problem clearly needs some attention.

The other problem responsible for operation failure is the docking device in the sampling device. The tubing to connect fermenter to sampling device is threaded through some "screws" in the docking device, and tightened down (shown in figure 1.3.3.a.). The screws are tightened down onto a spherically shaped surface, separated by a rubber seal. This spherical shape means the airtight seal is difficult, and if it is not perfect, the samples cannot be removed from the fermenter. In order to improve the airtight seal, it is recommended that in the next version of the sampling device, the screws can be tightened down onto a flat surface.

3.3. APPLICATIONS OF THE USE OF THE ON-LINE MONITORING SYSTEM AND ITS LINKAGE TO A CONTROL SYSTEM

The ability to measure sugars and organic acids rapidly on-line provides useful information about the fermentation process. Section 2 has shown how the concentrations of galactose and acetic acid affect the fermentation process, and the on-line monitoring system was invaluable in determining these effects in a fed-batch process. A major advantage of an on-line monitoring system is if it can provide rapid analyses for linkage to a control system so that rapid, on-line control decisions can be made. These control decisions can be used either in refining and optimising a process, in finding out about aspects of the growth of the organism in a fermenter, or in running a process under predetermined conditions.

The control system that the on-line monitoring device has been linked to is LabView. The link was made by using a simple QuickBasic program which sent down the parallel port the analyte's name and its concentration. A parallel to serial converter changed the format to serial form, and LabView was programmed to pick up the strings and convert them to names and values. This was fairly easily achieved, and once done, the values could be used to alter the feeding strategy. The level of control achieved was quite complex, having moved beyond the simple function of feeding to maintain a specific concentration of analyte in the fermenter (as in PID control). Complex control was achieved in LabView by programming it to alter a feed-pump to feed along an exponential trajectory (as required to feed to a particular specific growth rate) and including a feedback loop in the algorithm to keep the concentration of one or more analytes low. This control was found to be most effective, particularly when a refined dual level control was employed. This has been done only infrequently in the literature; most researchers only attempt growing organisms at a controlled specific growth rate in continuous culture. Industry is not always set up to run processes continuously, and the development of a system that can mimic continuous culture in a fed-batch fermentation may prove very useful.

The experiments described in section 2 have fulfilled two purposes. Firstly, they have demonstrated the use of the on-line sampling, microcentrifugation and analysis system in monitoring the concentrations of sugars and organic acids in fed-batch *E. coli* fermentations. The second purpose of those experiments was to illustrate the importance of monitoring and controlling the concentrations of sugars and organic acids in a fermentation process, because of the direct effects of high concentrations of sugar substrate (galactose) and acetate on the growth of the organism and production of recombinant protein. Monitoring galactose and acetate concentrations has also been useful in fermentations where specific growth rate is controlled by LabView. When either substance builds up in the medium, it is an indication that either the cells are unable to grow at that particular specific growth rate, or that the algorithm's estimate of biomass has deviated from the true value. Under either condition, feed-back control using on-line HPLC can correct the problem and keep the concentrations of sugar and organic acid present in the medium low.

3.4. MAIN CONCLUSIONS

This thesis has demonstrated the development and use of an on-line sampling, microcentrifugation and analysis (HPLC) system in the automated monitoring of the concentrations of sugars and organic acids in *E. coli* fermentations. The microcentrifuge has been tested under a wide range of conditions with two organisms: *Saccharomyces cerevisiae* and *Escherichia coli* and the best conditions determined for operation of the microcentrifuge *ie* a dual spin, of short, 20 second spin time each, at an air pressure of 45 psi. *E. coli* are separated from the liquid fermentation phase better than *S. cerevisiae*. The HPLC system is shown to be reproducible by running repeated assays of the same sample with less deviation than that obtained from using a commercially available HPLC autosampler. The entire system was demonstrated by using it to monitor the concentrations of glucose and acetate in a batch fermentation of *E. coli* K12, and comparing the results from the on-line HPLC to those obtained from similar samples taken manually and assayed by off-line enzymatic assays. The comparisons were very close for acetate and acceptably close for glucose.

Once established as a functional system, the on-line monitoring system was linked to a process control system, LabView (programmed by Dr. M. Gregory at UCL) for use in closed-loop control of a fed-batch fermentation. Closed-loop control using the on-line HPLC was demonstrated in two fermentations: one in which simple, single level control was employed, and the second in which a more complex control strategy was used with dual level feedback control. In both fermentations, the combined system was successfully able to control the concentrations of galactose and acetate in the fermenter.

The combined on-line monitoring and control system was then used to examine how the concentrations of galactose and acetate affect growth and recombinant protein production in fed-batch *E. coli* fermentations, and the role of specific growth rate in recombinant protein production was also looked at. The results were compared with equivalent and additional experiments performed in continuous and batch cultures. The main results from these experiments are that

high galactose and acetate concentrations reduce the maximum specific growth rate seen in the fermentation, and the production of recombinant α-amylase is also detrimentally affected. It has also been found that there is an optimal specific growth rate at which maximal recombinant α-amylase is produced, and this is an intermediate growth rate, not a low growth rate as expected.

These results provide evidence for the need for monitoring and controlling galactose and acetate concentrations in fed-batch *E. coli* fermentations; a role fulfilled by the on-line monitoring system developed during the course of this project.

4. THE FUTURE

4.1. FUTURE MODIFICATIONS REQUIRED TO IMPROVE THE SYSTEM

Although the on-line sampling and analysis system has performed well in monitoring sugar and organic acid concentrations in fermentations, there are a number of changes that could be made to the system that would improve sample times and prolong the lifetime of the system in use. These suggested changes are described below.

4.1.1. The sampling device

The main change suggested for the sampling device has been mentioned in section 3.2. *ie* the change in design of the docking device to allow the "screws" that attach the tubing from the fermenter to the docking device to be tightened down onto a flat surface rather than the current spherical surface, so that a better seal can be achieved.

The other suggested modification would need to be made if the sampling device is to be used with slower growing microorganisms than *E. coli* (or possibly if complex medium is used). This change is to re-attach the steam trap to the sampling device, but mounting it outside the stainless steel sampling device box to enable it to be easily be dismantled if it becomes necessary to unblock it.

4.1.2. The microcentrifuge

There are four suggested modifications to the microcentrifuge: two relating to the improvement (reduction) in the time taken to prepare the sample for analysis, one to increase the lifespan of the air-turbine, and one to improve the clarity of the supernatant. An additional possible modification is discussed, relating to containment of microorganisms within the microcentrifuge.

Most of the time in preparing the fermentation sample for analysis is taken up

by the need for a dual spin of the centrifuge bowl (because of the droplets of broth sticking onto the pipes, which then mix with the clear supernatant after the first spin, reducing the clarity); and because of the amount of time needed to allow the microcentrifuge bowl to come to rest after it has been spun (at least 60 seconds). If the vacuum and sample out pipes in the centrifuge bowl head were made retractable (the sample inlet and wash pipes have already been positioned to rest above the liquid level), the vacuum pipe need be the only pipe immersed when the broth is pumped into the bowl. When sampling has stopped, the vacuum pipe should be retracted. Then when the bowl comes to a stop, the sample out pipe could be lowered to remove the clear sample supernatant from the bowl. This could be achieved by attaching each of the two pipes to a robot arm that could be operated in on / off mode (which could be programmed by the programmable logic controller). This way, the second bowl spin would be avoided.

The second modification suggested to reduce sample separation times is to redesign the brake. The brake is a very crude structure, and operates by touching only one side of the centrifuge bowl when engaged. Friction then slows the bowl, but because of the uneven contact of the brake on the bowl, vibrations develop in the bowl when it is slowing down with the result that the solids are resuspended and the clarity of the supernatant is poor. For this reason, the brake was not used during the fermentation monitoring experiments. If the brake is redesigned to come into contact with as much of the circumference of the bowl base as possible, it should reduce the bowl vibrations so that the cellular material does not resuspend. These two modifications could save up to two minutes of the sample preparation time.

The third suggested modification involves extending the lifespan of the air turbine in the microcentrifuge. During the course of this project, the air turbine in the microcentrifuge was replaced twice due to the bearings inside having rusted and seized. Water enters the turbine bearings from two sources: firstly, if the bowl is overfilled, liquid spills over and drops down into the turbine bearings; and secondly from its presence in the air supply. The high humidity of the fermenter

room and pilot plant areas mean that water condenses in the air storage tanks of the compressor, and is therefore present in the air that drives the turbines. Both of these factors may be responsible for the bearings rusted. Apart from reducing the water content of the air supply (*eg.* by better filtration), the mild steel bearings in the air turbine should be replaced with stainless steel bearings of the same speed rating. So far, however, a supplier of these alternative bearings has not been found.

The fourth modification may increase the clarity of the supernatant. As has been shown, the Denley microcentrifuge provides a much clearer supernatant than the microcentrifuge used in this project. The probable reason for this is the small area of contact between cells and liquid in the Denley, therefore allowing less area for cells to resuspend. Redesigning the microcentrifuge bowl shape such that cells collect in a small, spherical groove rather than the large surface area at the sides of the bowl, should, therefore, reduce supernatant turbidity.

The other change that needs to be made arises from the need for containment of the organisms spun in the microcentrifuge. The high speed the centrifuge runs at generates aerosols, which are undesirable, particularly where recombinant microorganisms are used. The centrifuge, therefore, should be placed inside an enclosed box, where all air inlets and outlets are adequately filtered.

4.1.3. The HPLC system

The replacement of the current HPLC system and column with a microbore system would be an expensive option, but could reduce the analysis time to less than five minutes. This means that the total time for sampling and analysis would be less than seven minutes. As previously mentioned, this would be useful in the monitoring of rapid fermentations but is not strictly necessary in fermentations of slower growing organisms. In this case it is more important to reduce the time taken to prepare the sample for analysis than the analysis time itself, because of the presence of cells and adequate conditions for the composition of the sample to be altered (by the cells' metabolic activities).

4.2. FUTURE POTENTIAL

The use and importance of the on-line sampling and analysis system in monitoring the concentrations of sugars and organic acids in fermentations has been discussed. Also mentioned in section 3. is that one of the advantages of a fermentation sampling system employing microcentrifugation instead of filtration is that cells are available for disruption and analysis of an intracellular component. Although this possibility has not been attempted during this project, a very brief explanation of how it may be achieved is given here.

One way of doing on-line disruption is by first separating cells from the liquid component of the broth (as before) and pumping out the clear supernatant. The cells could then be resuspended by pumping in a buffer while spinning the bowl using the wash tubes, so that cells are dislodged from the walls of the bowl. The cell suspension could then be pumped out of the bowl and into a so-called micro-homogeniser for disruption. The homogenate can then be again pumped into the bowl, spun, and the supernatant removed for analysis of an intracellular component such as a protein product or enzyme marker of interest. The extra equipment needed would be one additional pump head on each of the existing pumps (inlet, outlet and wash pumps) and extra tubing connected via two valves to select the sample stream from the fermenter or homogeniser *etc.* and T or Y connectors. The valves could easily be connected to and operated by the programmable logic controller, and the homogeniser should be able to operate from an on / off signal too. Ideally, the means of analysis of the protein would be chromatographic because of its ability to analyse a wide range of compounds.

The main reason for wanting to measure protein product on-line at the moment is to determine the optimal harvest time of the fermentation. It will also relieve personnel from doing possibly lengthy and tedious assays. However, because this technology is so new, few applications have so far been found. This needn't hinder research, however. Once the ability to measure protein product on-line becomes routine, more applications are likely to be found, and it is possible that the results from the determination of product concentrations could at some time

in the future be used in control. The system may, however, be more useful in monitoring the activity of a marker enzyme on-line. This marker enzyme could be anything known to have an influence on or be influenced by the process, and this could provide as much information about the process as, say, the concentrations of sugars or organic acids. Such analyses could, too, be used in optimisation and control of that process.

Another future possibility is to couple the system to alternative analytical instrumentation. A candidate for this is the use of capillary zone electrophoresis, instead of HPLC. It can be used to measure almost anything that HPLC can measure, but in a faster time and with lower running costs, both important considerations.

The final potential improvement discussed here is the option of modifying the on-line monitoring system to enable it to monitor more than one fermentation. A sampling device would be needed for each fermenter to maintain asepsis, but the sampling device outlet pipes could be coupled to the same microcentrifuge and HPLC system. Extra valves would be required, and the timing would have to carefully controlled; the HPLC system would have to be modified to provide more rapid results, or replaced with capillary zone electrophoresis. However, if these changes were implemented, it could again reduce running costs and improve control over a larger number of fermentations.

APPENDIX A - PROGRAMS FOR ON-LINE MONITORING AND CONTROL

This appendix details some of the programs and methods used in the operation of the on-line HPLC equipment, and the connection to LabView, the process control software.

A.1. PROGRAMMABLE LOGIC CONTROLLER PROGRAM

As described in section 1.6., the PLC is connected to the individual components of each device in the on-line monitoring system: the sampling device (5 valves); the microcentrifuge (2 air valves, 3 peristaltic pumps, a vacuum valve and a brake solenoid); and the HPLC interface box (relay 3 on the rear of the box, and the "start" connection). The program written in relay ladder logic (based on Boolean algebra) used by PLCs to control the sequence of operation of the individual components uses the following symbols:

020 - 024 : Valves 1 to 5 in sampling device (see figure 1.3.a. for numbering of valves)
030 - 037 : Microcentrifuge. 030 = inlet pump; 031 = wash pump; 032 = sample outlet pump; 033 and 034 = air turbine valves; 035 = brake solenoid; 036 = vacuum valve
040 : signal to HPLC interface box to start acquiring data and going through method
050: signal from HPLC interface box to commence operation of PLC program

Symbols T600 to T620 refer to timers used in ladder logic for timed sequencing of operations. The number after "K" on the right below the "T" number (*eg.* K0300 below T600 on rung 5 of the ladder) refers to the length of time the timer is operating for in tenths of a second.

Numbers 200 through to 211 refer to internal relays used to start timers *etc.*

Explanation of the program

As mentioned before, the function of the PLC program is to co-ordinate the removal, centrifugation and injection into the HPLC system of a sample of fermentation broth and sterilisation of the sampling device. In this short section, there is a brief explanation of the function of each rung on the PLC program ladder; and on the three pages following the explanation, the program is given.

Rung 0 : Input from 050 (HPLC interface box) opens valves 1 and 5 of the sampling device for 30 seconds, for pre-sampling sterilisation.
Rung 5 : Defines the timer for operation of rung 1.
Rung 8 : Defines the timers for two further operations.
Rung 13 : Opens valves 3 and 4 of sampling device, and switches on inlet pump of microcentrifuge for 65 seconds, to remove sample from fermenter.
Rung 18 : Switches on vacuum valve for 58 seconds for removal of broth from bowl while it is being pumped in (to allow "dead volume" broth to be removed). Time difference of 7 seconds allows bowl to fill.
Rung 24 : Defines the timers for two more operations.
Rung 29 : Opens air turbine valves and switches off brake in microcentrifuge to spin bowl for 20 seconds.
Rung 36 : Starts a timer, which runs for 8 seconds.
Rung 39 : Defines a timer the next operation.
Rung 42 : Switches on vacuum valve for 10 seconds after centrifuge has been spinning for 8 seconds to remove as much excess broth on pipes as possible.
Rung 45 : Defines the next timer.
Rung 48 : After air valves have been switched off, allows centrifuge to come to rest for 65 seconds to allow any droplets on pipes to mix with supernatant.
Rung 51 : Defines two more timers.
Rung 56 : Starts centrifuge spinning again for 20 seconds; brake off.
Rung 59 : Starts a timer, which runs for 8 seconds.
Rung 62 : Defines the next timer.
Rung 65 : Again, switches on vacuum valve for 10 seconds after bowl has been spinning for 8 seconds.

Rung 68 : Defines the next timer.

Rung 71 : After air valves switched off, allows 65 seconds for bowl to come to rest.

Rung 74 : Defines the next timer.

Rung 77 : Switches on the sample out pump for 28 seconds to pump sample into HPLC injection loop.

Rung 80 : Defines the next timer.

Rung 83 : Sends a signal to the HPLC for it to start the method program which injects the sample onto the column, and starts acquiring data.

Rung 86 : Defines the next three timers.

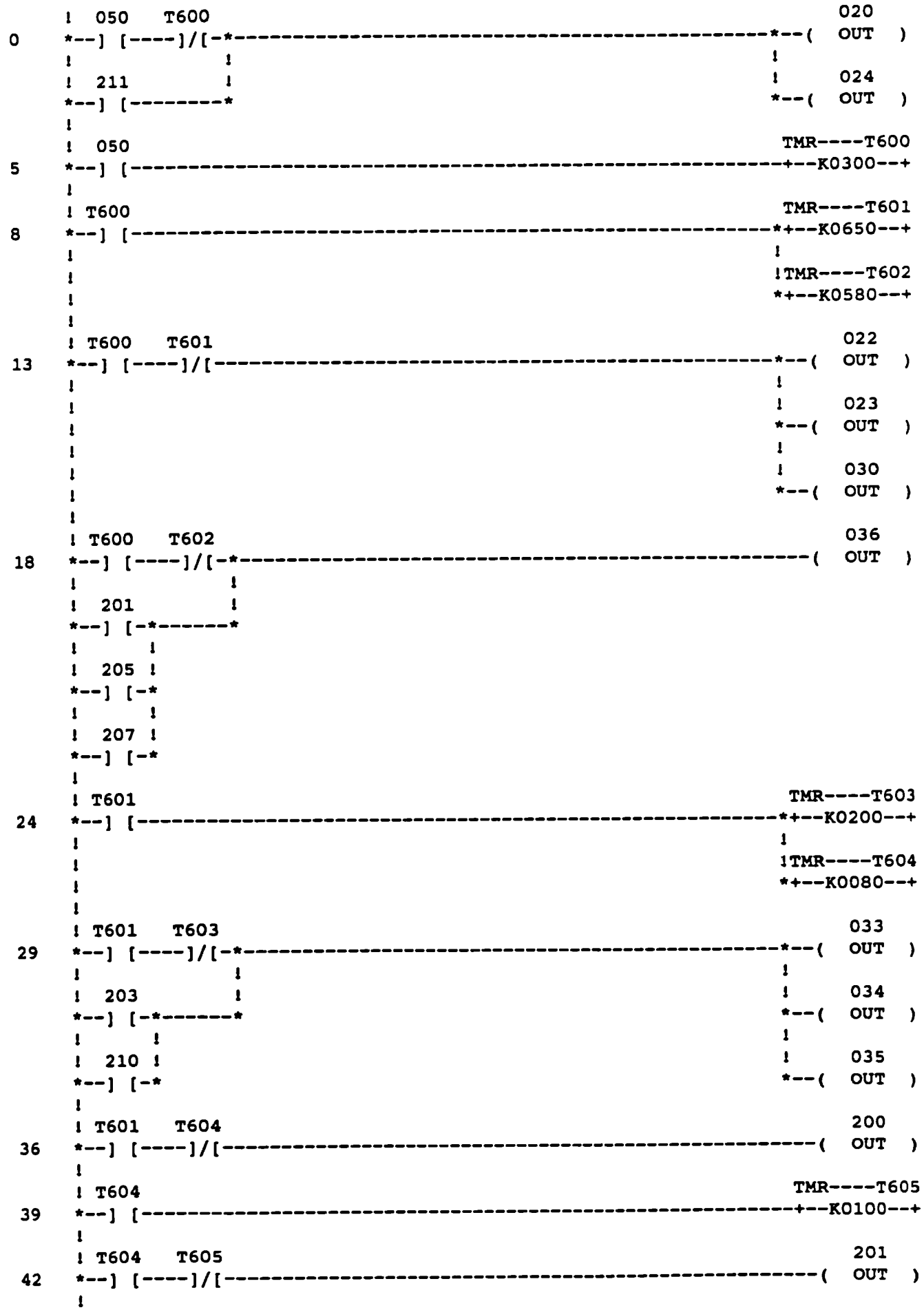
Rung 93 : Switches on the wash pump, to pump wash solution into the bowl.

Rung 96 : Switches on vacuum valve to remove cells suspended in wash solution.

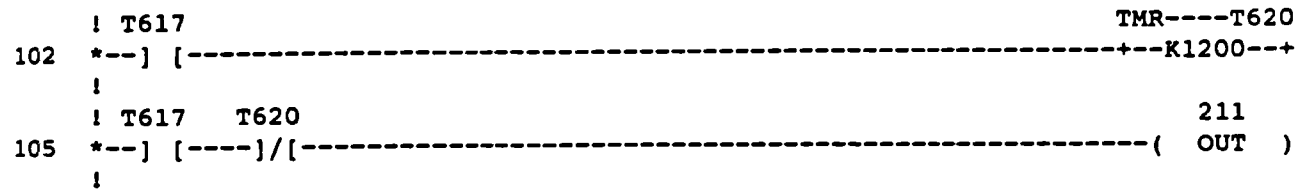
Rung 99 : Opens air valves for 0.1 second to spin bowl while wash solution is being added.

Rung 102 : Defines the next timer.

Rung 105 : Opens valves 1 and 5 in the sampling device to allow sterilisation before the next sample is taken.


050 T600
020 OUT
211
024 OUT
050
TMR----T600 K0300
T600
TMR----T601 K0650
TMR----T602 K0580
T600 T601
022 OUT
023 OUT
030 OUT
T600 T602
036 OUT
201
205
207
T601
TMR----T603 K0200
TMR----T604 K0080
T601 T603
033 OUT
203
034 OUT
210
035 OUT
T601 T604
200 OUT
T604
TMR----T605 K0100
T604 T605
201 OUT

```
    ! T603                                                          TMR----T606
45  *--] [-----------------------------------------------------------+--K0650--+
    !
    ! T603   T606                                                        202
48  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T606                                                           TMR----T607
51  *--] [-----------------------------------------------------------*+--K0200--+
    !                                                               !
    !                                                               !TMR----T610
    !                                                               *+--K0080--+
    !
    ! T606   T607                                                        203
56  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T606   T610                                                        204
59  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T610                                                           TMR----T611
62  *--] [-----------------------------------------------------------+--K0100--+
    !
    ! T610   T611                                                        205
65  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T607                                                           TMR----T612
68  *--] [-----------------------------------------------------------+--K0650--+
    !
    ! T607   T612                                                        206
71  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T612                                                           TMR----T613
74  *--] [-----------------------------------------------------------+--K0280--+
    !
    ! T612   T613                                                        032
77  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T613                                                           TMR----T614
80  *--] [-----------------------------------------------------------+--K0002--+
    !
    ! T613   T614                                                        040
83  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T614                                                           TMR----T615
86  *--] [-----------------------------------------------------------*+--K0150--+
    !                                                               !
    !                                                               !TMR----T616
    !                                                               *+--K0200--+
    !                                                               !
    !                                                               !TMR----T617
    !                                                               *+--K0001--+
    !
    ! T614   T615                                                        031
93  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T614   T616                                                        207
96  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
    ! T614   T617                                                        210
99  *--] [----]/[-----------------------------------------------------(  OUT  )
    !
```


! T617
TMR----T620
102
*--] [----------+--K1200--+
!
! T617 T620
211
105
*--] [----]/[----------(OUT)
!

A.2. HPLC METHOD FILE

Apart from using the HPLC method file in the PE Nelson 2100 chromatography software to acquire data, it is also crucial to the timing of the automated sampling and analysis from the fermenter. The next page lists all the operating parameters relevant to the acquisition of data by the interface box, and how that data is calculated; the method is printed out directly from the PE Nelson 2100 software.

The page following that shows typical calibration data used in an HPLC method file to calculate amounts of various components in the samples. Underneath the calibration data is a description of the "timed events" programmed into a method file than can be used to perform a number of tasks while the method is running. All the timed events in the methods used in this project operate relays in the interface box. Relay 1 is connected to the "inject" solenoid in the rheodyne valve that injects a sample onto the column. Relay 2 is connected to the "load" solenoid, to return the valve back the original position, ready for another injection. Relay 3 is connected to input 050 in the PLC. The operation of the relays is as follows:

Time zero: relay 1 closed; rheodyne solenoid loads sample onto column.
Time 0.1 minutes : relay 1 opened for closure on next operation of method.
Time 0.2 minutes : relay 2 closed to bring rheodyne valve back to load position.
Time 0.3 minutes : relay 2 opened for closure on next operation of method.
Time 3 minutes : relay 3 opened which "switches off" PLC input 050, so it can be "switched on" and restarted when needed.
Time 5.7 minutes : relay 3 closed to "switch on" PLC input 050 to start PLC program.

The timings of the relays have been designed to minimise the total amount of time between sampling and receiving the result of the analysis.

Method file name: F:BFERM
Default Sample Name: ONLINEFERMENTATION
Operator: CT

ACQUISITION PARAMETERS

SINGLE OR DUAL CHANNEL (1 OR 2)	1.00
RUN TIME (minutes)	9.50
END TIME FOR PLOTS (default=RUN TIME)	9.50
SOLVENT DELAY TIME (minutes)	0.00
PEAK DETECTION THRESHOLD (microv/sec)	1.00
Area Threshold	100.00
MINIMUM PEAK WIDTH (seconds)	10.00
TIME FOR ONE SAMPLE (seconds)	1.00
NUMBER OF REAL TIME CRT PAGES TO PLOT (0 TO 99)	1.00
REAL TIME PLOT FULL SCALE FOR CH.0 (millivolts)	50.00
REAL TIME FULL SCALE FOR CH.1 (millivolts)	200.00
HARD COPY REAL TIME PLOT	NO
AUTO ZERO REAL TIME PLOT	YES
Pre Version 4 method	YES
RECORD AREA TABLES ON DISK	YES
RECORD RAW DATA	YES
NUMBER OF CRT PAGES FOR REPLOT (1 TO 99)	1.00
VERTICAL SCALE FACTOR FOR REPLOT (units of largest peak)	1.00
OFFSET FOR THE REPLOT (millivolts)	0.00
PUT NAMES ON REPLOT?	NO
PRINT AREA PERCENT REPORT	YES
PRINT EXTERNAL STANDARD REPORT	YES
PRINT INTERNAL STANDARD REPORT	NO
FINAL REPORT AREA REJECT (microvolt-sec)	0.00
LINK TO USER PROGRAM	YES
FORCE DROP LINE INTEGRATION	NO
FORCE COMMON BASE LINE	NO
FULL SCALE RANGE FOR A.D.C. (3=1VOLT, 1=2VOLT, 0=10VOLT)	3.00
AREA REJECT FOR REFERENCE PEAKS?	1000.00
% RET TIME WINDOW FOR REFERENCE PEAKS	0.00
RET TIME WINDOW IN SECONDS FOR REF. PEAKS	0.00
AREA OR PEAK HEIGHT QUANTITATION (0 OR 1)	0.00
PRINT GROUP REPORT	NO
NUMBER OF CALIBRATION LEVELS (1 TO 6)	5.00
LIST COMPONENTS NOT FOUND IN SAMPLE?	YES
INCLUDE UNKNOWN PEAKS IN REPORTS?	YES
UPDATE RESPONSE FACTORS WITH REPLACEMENT (0) OR AVERAGE (1)	0.00
DEFAULT DILUTION FACTOR	1.00
DEFAULT SAMPLE WEIGHT	1.00
DEFAULT AMOUNT INJECTED	1.00
DEFAULT AMOUNT OF INTERNAL STANDARD	1.00
PRINT GPC MW DISTRIBUTION	NO
PRINT SIMULATED DISTILLATION REPORT	NO

LINK TO PROGRAM MLINK
SAVE RESULTS IN: U6N.ATB
SAVE RAW DATA IN: U6N.PTS

```
---------------------------------------------------------------------------
 1 GALACTOSE          Ret. Time =    5.40 min.  Fit.type =  1
   Ref. peak: GALACTOSE          Int Std: GALACTOSE          Window size:  6.0%
   AREA  =  2.8469D+05 * AMOUNT + -1.6178D+04
   Correlation (R squared) =  0.9978
                  LEVEL    AREA       AMOUNT          RATIO (amount/area)
                    1      67225       0.25000           0.00000372
                    2     122662       0.50000           0.00000408
                    3     267534       1.00000           0.00000374
                    4     530303       2.00000           0.00000377
                    5     853063       3.00000           0.00000352

 2 ACETATE            Ret. Time =    8.00 min.  Fit.type =  1
   Ref. peak: GALACTOSE          Int Std: GALACTOSE          Window size:  6.0%
   AREA  =  1.1530D+05 * AMOUNT + -2.2390D+03
   Correlation (R squared) =  1.0000
                  LEVEL    AREA       AMOUNT          RATIO (amount/area)
                    1      12136       0.12500           0.00001030
                    2      26187       0.25000           0.00000955
                    3      56118       0.50000           0.00000891
                    4     112750       1.00000           0.00000887
                    5     170754       1.50000           0.00000878

Response factor for unknowns=  1.0000E-04
Component Units = G/L
---------------------------------------------------------------------------
```

Ev#	Time	Event	Description
1	0.00	1C	relay (Close/Open)
2	0.10	1O	relay (Close/Open)
3	0.20	2C	relay (Close/Open)
4	0.30	2O	relay (Close/Open)
5	3.00	3O	relay (Close/Open)
6	5.70	3C	relay (Close/Open)

A.3. LINK TO LABVIEW

The link to the LabView computer for closed loop control of the fermentation process based on on-line HPLC analysis was achieved by modifying a user program supplied with the PE Nelson software, and linking it to the method file. The method allows for a link to a user program, and looks for the program if the user enters the name of the program in the method. This can be seen in the method printed out on A.2. At the bottom of the page, the method prompts "LINK TO PROGRAM" and the program "MLINK" has been entered.

The user program "MLINK" is given on the following page. The modifications made to the supplied program (called "USER.BAS") are merely to reduce the amount of information sent down LPT1 port of the computer. The lines responsible for supplying information such as "sample name", "method used" *etc.* have been commented out *ie* the computer does not execute them. The modified program only supplies the name of the analyte, and its concentration.

The information is sent down the parallel port, LPT1, and then converted to serial by means of a parallel to serial converter. The data in serial form can then be acquired by the serial card in the Apple MacIntosh computer running LabView.

```
'*   This Program is an example of a COMPILED BASIC program that will
'* produce a report showing the names and amounts detected in a run.

' compile with these commands:
'  qb cuser /l nairun.exe;
'  link cuser;

'*** Include all common variables required for automatic runs. ****
     ' $INCLUDE: 'DIMCOM.inc'

10   'LPRINT "THIS IS AN EXAMPLE OF A LINKED USER PROGRAM"
     'LPRINT "data was stored in file "; cur.file$
' Note: An external standard or internal standard table must have been
'       printed in order for vector% to be set.
     'LPRINT "Sample Name: "; NAM$
     'LPRINT "Method Used: "; method$
     'LPRINT "Total number of peaks detected = "; npeaks
     'LPRINT
     'LPRINT "Compound Name          Amount          Area      Ret Time"
     FOR Q% = 1 TO NUM.COMPOUNDS
         comp% = vector%(Q%)
         IF comp% = 0 THEN
            LPRINT NAMES$(Q%); "   NOT FOUND"
         ELSE
            LPRINT NAMES$(Q%);
                  LPRINT " "; AMOUNT(comp%)
         END IF
     NEXT Q%
     LINKFLG = 1              '* Link flag set to indicate user program was run.
     CHAIN "TRAFFIC"          '* Chain back to TRAFFIC for further processing.
```

APPENDIX B. THE DEVELOPMENT OF AN HPLC ASSAY FOR PROCHYMOSIN

This thesis describes the on-line monitoring of fermentation broth and some applications of it. As discussed in the "future potential" section, the next major developmental step in the use of the on-line monitoring (and control) system is its extension to the monitoring of a protein product on-line, using an assay technique such as HPLC. Because of this, some time was spent developing an HPLC assay for the determination of an important recombinant, intracellular protein produced in an *E. coli* fermentation.

The protein is prochymosin, a precursor of the economically important enzyme, chymosin, used in cheese manufacture. Chymosin is an aspartic proteinase, and clots milk. Traditionally obtained from calf stomachs, it is now produced in fermentation, the gene for chymosin and prochymosin having been cloned into *Escherichia coli*, because of the serious shortage of the naturally produced enzyme. When prochymosin is produced inside the cell, in common with most proteins, it is not excreted. Instead, it forms inclusion bodies, in which the proteins co-precipitate in a denatured state with other cytoplasmic proteins, ribosomes and nucleic acids. As a result, analysis (and recovery) of prochymosin is a daunting task. One of the reasons for choosing to develop an HPLC assay for prochymosin (apart from wanting to develop an on-line assay) is because of the lack of a suitable assay for this protein. Up until now, the semi-quantitative method of SDS polyacrylamide gel electrophoresis has been used; another assay in use is the milk clotting assay, which can only measure active enzymic protein in the form of chymosin. Clearly, a better assay is highly desirable.

Although the development of the assay is still in an early stage and some way from being used in on-line monitoring, there is progress in the off-line analytical technique. This appendix presents the developmental work done on the assay, as well as details of the fermentation procedure used to make the prochymosin and its extraction from the cells.

B.1. MATERIALS AND METHODS

B.1.1. Fermentation and production of prochymosin

Organism

The organism used to produce prochymosin is *E. coli* HB101 containing the plasmid pMG168, which was kindly donated by Dr. G.T. Yarranton (Celltech Ltd). The plasmid contains a gene for ampicillin resistance for plasmid selection. Induction of the plasmid is by a temperature shift from 30°C to 42°C, at which temperature the culture is kept for 20 minutes to inactivate the repressor protein. Subsequently, the temperature is reduced to 37°C for the remainder of the fermentation. This will start the production of prochymosin.

Agar, starter, seed and fermentation culture media

The starter culture medium is LB broth and contains ($g.L^{-1}$): bacto tryptone (Difco) 10; bacto yeast extract (Difco) 5; NaCl (Sigma) 10. The medium is supplemented with ampicillin (sodium salt, Sigma, 0.1 $g.L^{-1}$). Cultures were made on agar plated containing 1.2 % Technical agar number 3 (Oxoid) and LB medium supplemented with ampicillin, as above.

The seed and fermentation media contain the following components ($g.L^{-1}$): KH_2PO_4, 3; $Na_2HPO_4.10H_2O$, 12.06; NaCl, 0.5; $CaCl_2.6H_2O$, 0.022; yeast extract, 1; glucose, 30; casamino acids, 30; $MgSO_4.7H_2O$, 0.25; proline, 0.5; thiamine, 0.01; ampicillin (sodium salt) 0.1.
Casamino acids and yeast extract were obtained from Difco; the other compounds were obtained from Sigma.
Glucose and MgSO4.7H2O were autoclaved separately, and proline, thiamine and ampicillin were filter sterilised (0.2 μm Gelman Acrodisc) into the fermenter prior to inoculating. All other components were steam sterilised in the fermenter.

Fermentation

A starter culture (10 mL) was grown for 16 hours at 30°C in an orbital shaker after inoculating from a colony on an agar plate. The starter culture was used to inoculate a 200 mL seed culture, grown under similar conditions, which was then used to inoculate the fermenter, to give a final working volume of 8 litres. The fermenter used was a Chemap 14 litre fermenter, with monitoring and control of standard fermentation variables. The culture temperature was 30°C until it reached an optical density of 6 at 600 nm, and was then raised to 42°C to induce the plasmid. After 20 minutes, the temperature was dropped to 37°C for the remainder of the fermentation. During this stage of the fermentation, the culture produced prochymosin in the form of inclusion bodies. When the glucose had been exhausted, the culture was harvested by centrifuging the broth in a Sharples 1P tubular bowl centrifuge, and the cell paste was stored at -20°C.

B.1.2. Extraction of prochymosin inclusion bodies from the cells

Solutions used

Sonication buffer: 100 mM tris HCl, 5 mM EDTA, pH 7.5
Washing buffer: 0.5 % Triton X-100, 50 mM tris HCl, 10 mM EDTA, pH 8.0
Solubilisation buffer: 8 M urea, 50 mM KH_2PO_4, pH 10.7
Tris, EDTA and Triton X-100 were obtained from BDH; KH_2PO_4 was from Sigma and HPLC grade urea was obtained from Camlab.

Method

Frozen *E. coli* cells (3 g) were suspended in sonication buffer (100 mL), and the resulting suspension was sonicated on ice for 15 minutes, and then centrifuged at 4000 rpm using an MSE Centaur 2 centrifuge for 12 minutes. The supernatant was then discarded, and the pellet resuspended in washing buffer (100 mL), sonicated for 10 minutes, and the supernatant discarded. This was then repeated twice. The final supernatant was discarded, and the pellets were resuspended in

deionised water (100 mL). They were then recentrifuged for 12 minutes at 4000 rpm, and the supernatant again discarded. The pellet was then resuspended in solubilisation buffer (50 mL), and left stirring for 1 hour at room temperature. The solution was filtered, and stored at -20°C. Protein assay of the sample gave a protein concentration of 3.8 $mg.mL^{-1}$.

B.1.3. Standards and antibodies

The prochymosin standard was kindly supplied by Celltech Ltd. Crystalline chymosin, and prochymosin antibodies raised in rabbits for Western blots were obtained from Chr. Hansen's Laboratorium.

B.1.4. SDS-PAGE assay of prochymosin

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect, and estimate prochymosin concentration. If necessary, samples were first prepared for this technique by subjecting them to TCA (trichloroacetic acid) precipitation, which is performed as follows:
TCA (BDH, 0.333 mL, 100 %) was added to protein solution (1 mL) in an Eppendorf tube, mixed, and left for 1 hour at 4°C. The resulting suspension was spun in a Denley microcentrifuge for 7 minutes at 10 00 rpm. The supernatant is discarded, and acetone/HCl (1 mL acetone containing 5 mM HCl, Sigma) added, and mixed. The tubes were spun again at 10 000 rpm for 7 minutes, and the supernatant again discarded. Acetone (1 mL) is added, mixed and the tube is again centrifuged. The supernatant is discarded, and the solid protein dried by vacuum.

<u>SDS-PAGE solutions</u>

Sample buffer: 2 % SDS, 5 % dithiothreitol, 10 % sucrose, 0.002 % bromophenol blue, 0.0625 M tris HCl, pH 6.8.
Stacking gel buffer stock: 0.5 M tris HCl, pH 6.8.
Resolving gel buffer stock: 3 M tris HCl, pH 8.8.

Reservoir buffer: 0.025 M tris, 0,192 M glycine, 0.1 % SDS.
Acrylamide stock: 30 % acrylamide, 0.8 % bisacrylamide in deionised water.
Stacking gel: 2.5 mL acrylamide stock, 5 mL stacking gel buffer stock, 0.2 mL 10 % SDS, 1 mL 1.5 % ammonium persulphate, 11.3 mL deionised water, 0.015 mL TEMED.
Resolving gel (15 % gel): 15 mL acrylamide stock, 3.75 mL resolving gel buffer stock, 0.3 mL 10 % SDS, 1.5 mL 1.5 % ammonium persulphate, 9.45 mL deionised water, 0.015 mL TEMED.
Stain solution: 0.1 % Coomassie blue in water, methanol and acetic acid (5:5:2 by volume) filtered through Whatman no. 1 filter paper.
Destain solution: 30 % methanol, 10 % acetic acid, 60 % deionised water.
Silver stain: method according to Wray *et al.* (1982).

SDS, dithiothreitol, acrylamide, bis acrylamide, TEMED and Coomassie blue were obtained from Biorad. Sucrose, tris, glycine, methanol and acetic acid were obtained from BDH. Bromophenol blue and ammonium persulphate were obtained from Sigma.

SDS-PAGE method

The samples are prepared by adding sufficient sample buffer to the sample to give an approximate final concentration of 1 $mg.mL^{-1}$ protein, and then heating the sample in a water bath at 70°C for twenty minutes. Prior to the sample being loaded into wells, it is spun in the Denley centrifuge for 1 minute. Gel solutions are made, degassed, and ammonium persulphate and TEMED added just prior to pouring. The resolving gel is allowed to set for a minimum of 1 hour, and the stacking gel for half an hour. Samples are loaded after running buffer is poured into the reservoir. For mini gels (used in these experiments), each gel is run at a current of 15-20 mA. When the bromophenol blue marker dye reaches the bottom of the gels, they are removed from the apparatus, stained for 1 hour, and destained until a clear background is visible, changing the destain solution as often as possible.

B.1.5. Western blotting

Blotting was done to determine which bands visible on an SDS-PAGE gel were prochymosin, or prochymosin breakdown products. SDS-PAGE gels that had not been stained were used in the blotting procedure. Concentrations of protein on the gels used for blotting were much lower - of the order of 50 ng protein.

Solutions used in blotting procedure

Transfer buffer: 5.82 $g.L^{-1}$ tris, 2.93 $g.L^{-1}$ glycine, 0.0375 $g.L^{-1}$ SDS, 200 $mL.L^{-1}$ methanol
Blocking solution: 1 % casein, 0.01 M Na_2HPO_4, 0.13 M NaCl, 0.5 % v/v TWEEN 20, pH 7.5
PBS-TWEEN stock solution: 160 g. L^{-1} NaCl, 23 g. L^{-1} Na_2HPO_4, 4 g. L^{-1} KCl, 4 g. L^{-1} KH_2PO_4, 100 g. L^{-1} $CoCl_2$, 12 mL TWEEN 20
Developing buffer: 2.5 mL PBS-TWEEN stock solution, 47.5 mL deionised water, 0.8 mL 2 % diaminobenzidine, 0.1 mL H_2O_2

Casein, $CoCl_2$ and diaminobenzidine were obtained from Sigma. TWEEN 20 was obtained from BDH.

Western blot method

After electrophoresis, gels are placed in transfer buffer, and shaken gently for 1 hour. Transfer of the proteins onto nitrocellulose membrane is then performed, ensuring gel-nitrocellulose membrane is covered with Whatman 3MM blotting paper. Transfer is done at 4°C for 1 hour at 20 V, 0.8 A. It is then necessary to block the remaining sites on the nitrocellulose by covering the membrane with blocking solution for 1 hour at room temperature. After blocking, the membrane is placed between plastic sheets and sealed as a bag containing blocking buffer (8 mL) containing anti-prochymosin antibody (8 μL). The bag is shaken gently for 1 hour, after which membrane is washed three times for three minutes each by blocking buffer (200 mL). The membrane is then resealed in another bag with

blocking buffer (8 mL) containing donkey-anti rabbit horseradish peroxidase-linked antibody (30 μL). After shaking for an hour, the membrane is again washed three times in blocking buffer. The membrane colour is then developed by adding developing buffer. Bands corresponding to prochymosin show up on the blot as a dark band.

B.1.6. Reverse phase HPLC using a polymeric support

Reverse phase HPLC was attempted using the polymeric support PLRP-S 300 (Polymer Laboratories) 15-25 μm particle size. This support was chosen because previous work (Salt and Turner, 1990) suggested that this support was the most promising for analysing denatured prochymosin.

PLRP-S 300 was packed into a column of dimensions 25 cm x 4.6 mm using a Shandon HPLC packing pump at 2000 psi pressure using a sonicated solution of acetonitrile and water in a ratio of 4:1 as packing solvent. Samples were loaded onto the column which was heated in a water bath to 60°C, and eluted with a two solvent gradient under different conditions. Solvent A was acetonitrile, with or without 50 % 2 M urea in water and solvent B was 2 M urea, 50 mM Na_2HPO_4, pH 10.7, or pH 11.7. Acetonitrile was obtained from Fisons, urea from Camlab, and Na_2HPO_4 from Sigma. Samples loaded onto the column were dissolved in 8M urea, 50 mM sodium phosphate at pH 10.7, and filtered through 0,2 μm filters.

B.1.7. Affinity dye chromatography

All the affinity dye chromatography was performed on a PIKSI kit (Affinity Chromatography Limited), a low pressure chromatography test kit containing ten mini columns packed with 6 % cross-linked agarose coupled to ten different MIMETIC, or triazine dye ligands. The 10 gels contained the following Mimetic ligands: red 2; red 3; orange 1; orange 2; orange 3; yellow 1; yellow 2; green 1; blue 1; blue 2. These ligands are proprietary ligands whose structures have not been released by the manufacturers.

Pure, native prochymosin standard was loaded onto the column after first dialysing against 10 mM NaH_2PO_4 buffer, pH 6.0. The same buffer (5 mL) was pipetted onto each dye column prior to sample loading for equilibration. The samples, which had been filtered through 0.2 μm filters, were loaded onto the column, and fractions collected. Then, eluting buffer (10 mM NaH_2PO_4, 0.2 M NaCl, pH 6) was added, and further fractions collected. Finally, gel cleaning, caustic rinse solution was added (1 M NaOH), and fractions were collected from this. A similar procedure applied for denatured prochymosin, with the exception that all solutions contained 8 M urea. Chemicals were obtained from Sigma, except urea (from Camlab).

Absorbances at 280 nm were taken of some fractions to obtain a rough estimate of protein. Fractions from the PIKSI kit were analysed on SDS-PAGE gels, and on Western blots.

B.2. RESULTS

B.2.1. Reverse phase (RP) HPLC

Throughout the RP-HPLC experiments, a number of different elution conditions were used to try and optimise the analysis of prochymosin. The pH of the mobile phase was either 10.7 or 11.7; the urea concentration in the mobile phase was varied, as was the gradient - from an isocratic separation, through to a change from 0 to 50 % acetonitrile. Although the chromatographic separation of prochymosin was obtained under all these conditions, the results indicate a number of problems with this procedure. Repeated injections of the same volume of identical samples yielded different retention times, very variable peak areas and peak heights, and the subsequent presence of ghost peaks in blank injections run after the sample.

Prochymosin was generally eluted as a dual peak (in both the prochymosin standard, and inclusion body sample), at 20.9 and 22 minutes (with 0-100% A for 20 minutes followed by 100% A for 10 minutes, A = 50 % acetonitrile, 50 % 2

M urea; B = 2 M urea, 50 mM sodium phosphate, pH 11.7). This can be seen in figures B.2.1.a and b. The time difference between the two peaks increases when 3 M urea is used in the mobile phase.
Because of the inconclusive results obtained from the HPLC traces, it was necessary to take fractions of eluant for subsequent SDS-PAGE analysis. Figures B.2.1.a. shows the trace in which fractions were taken of eluant from a sample of prochymosin standard injected onto the column. Fraction 1 was taken of the 0.72 minute peak, fraction 2 of the 16.69 minute peak, and fraction 3 over 20.6 to 23.5 minutes. Figure B.2.1.b. gives a similar trace for an inclusion body sample; fraction 1 taken of 0.73 minute peak; fraction 2 of 16.87 minute peak, and fraction 3 over 20.3 to 23.5 minutes.

Results of the SDS-PAGE gel (silver stained because of the low amounts of protein present) indicated that each fraction taken of both standard and sample had the same overall pattern of bands whose proportion did not change from one fraction to another. In addition, a band corresponding to prochymosin was present in all fractions. This strongly suggests that the column was not separating the components to any great degree.

B.2.2. Affinity dye chromatography

Affinity dye chromatography was used to try and obtain a better chromatographic separation and elution of the prochymosin inclusion body sample under denatured conditions, and the prochymosin standard under native and denatured conditions than that obtained with reverse phase chromatography.

Denatured conditions

Initially, the separation and elution of samples was attempted by loading inclusion body samples onto the PIKSI kit gels in loading buffer containing 1 M urea. The elution buffer contained 1 M urea with 0.2 M NaCl, and the rinsing buffer was 1 m NaOH. On all gels except for yellow 2, all the protein was retained on the column until the caustic rinse, indicating that 1 M urea may be too low a

concentration, and protein may be precipitating out on the column. The prochymosin inclusion bodies were then loaded on the column in 8 M urea; all other solutions also contained 8 M urea. Fractions were collected from each solution loaded onto the gel columns (*ie* fraction 1 from loading buffer; fraction 2 from eluting buffer, and fraction 3 from caustic rinse). A_{280} readings were taken of all fractions, and results are presented in table B.2.2.(i).

GEL COLUMN	FRACTION 1 A_{280}	FRACTION 2 A_{280}	FRACTION 3 A_{280}
RED 2	0.474	0.145	0.059
RED 3	0.451	0.172	0.051
ORANGE 1	0.499	0.140	
ORANGE 2	0.457	0.151	0.062
ORANGE 3	0.463	0.155	0.062
YELLOW 1	0.488	0.144	0.07
YELLOW 2	0.443	0.162	0.065
GREEN 1	0.079	0.495	0.185
BLUE 1	0.494	0.138	0.086

Table B.2.2.(i) Absorbances at 280 nm of fractions taken from eluted solutions loaded onto PIKSI gel columns. Fraction 1 was taken from loading buffer that had passed through column; fraction 2 from elution buffer; and fraction 3 from caustic wash.

These results show that the only column which retained, and then eluted prochymosin when a salt solution was applied, was green 1. The experiment was repeated on green 1, and similar results were obtained. From these results, green 1 therefore appears to be the most promising candidate for developing an HPLC assay. The fractions were loaded onto SDS-PAGE gels for analysis. Results of the gels indicate that fraction 1 from green 1 mostly contains protein other than

prochymosin, although some prochymosin was visible. The main prochymosin band was in fraction 2; the remaining protein was washed off the column in fraction 3, the caustic rinse.

As an additional test, Western immunoblots were performed on the fractions. Previous tests on the prochymosin antibody indicated that it bound to both chymosin and prochymosin, and was found not to bind to a series of marker proteins, indicating its specificity. Results of the Western blot show that firstly, many of the bands previously seen on SDS-PAGE gels are prochymosin, or its breakdown products; and secondly, the prochymosin standard contains many breakdown products. From this blot, it was not possible to determine the relative quantities of prochymosin in each of the fractions, except to say that the highest concentration appears in fraction 2. Additional blots were run to determine the relative concentrations, and results indicate that the concentration of prochymosin in fraction 2 was approximately 3 or 4 times greater than that in fraction 1. This either indicates non-selective absorption of prochymosin, or an excess load on the column. A further experiment, where the same sample size as before was divided in two, and added in two separate loadings on the column, and fractions were taken. The first fraction had an A_{280} of 0.032, and the second, 0.185. This strongly suggests that the elution of protein in loading buffer was due to an overloading of protein.

Undenatured conditions

Native prochymosin, dialysed against 10 mM sodium phosphate, pH 6, was loaded onto gel columns yellow 1, yellow 2 and green 1. Elution was obtained using the loading buffer containing 0.2 M NaCl, and a caustic wash was also used. As before, fractions were taken at each stage, and subjected to TCA precipitation. The relative amounts of protein in each fraction are given in table B.2.2.(ii).

The fractions were run on SDS-PAGE gels, and results show that there is one main band corresponding to prochymosin in fraction 2 from both yellow 1 and yellow 2 gel columns. Smaller amounts of prochymosin were visible in fraction

1 and 3 from yellow 1, and fraction 3 from yellow 2. These results indicate that yellow 2 is the most promising ligand for the analysis of undenatured prochymosin, although yellow 1 may also have some potential. Green 1 does not seem suited for undenatured prochymosin.

GEL COLUMN	FRACTION 1 (A_{280})	FRACTION 2 (A_{280})	FRACTION 3 (A_{280})
YELLOW 1	little	lot	little
YELLOW 2	none	lot	very little
GREEN 1	little (0.071)	little (0.108)	lot (0.400)

Table B.2.2.(ii). Relative amount of protein as seen by TCA precipitation on fractions taken from loading samples onto PIKSI gel columns yellow 1, yellow 2 and green 1. Fractions were taken from loading (fraction 1), eluting (fraction 2) and caustic rinsing (fraction 3) steps.

B.3. DISCUSSION

The development of the HPLC assay for prochymosin inclusion bodies has been difficult. Salt and Turner (1990) tried a number of different supports and methods to separate proteins in prochymosin inclusion bodies (Mono Q, C_4 and C_8 silica, diol silica, sax silica, PLRP-S polymer) and the most success was obtained with the ion exchange Mono Q column, and PLRP-S polymer reverse phase support. Repeating the work with the PLRP-S support indicated that there was still a long way to go in developing the assay, if indeed all the problems are ever solved. Ghost peaks in subsequent runs, variable peak heights, areas and retention times need to be addressed. SDS-PAGE gels of peak fractions from inclusion body and pure prochymosin injections indicated that separation was, at best, incomplete. There is the additional problem of column-life: after fewer than 20 injections, column resolution markedly deteriorated, indicating short column lifespan.

For these reasons, a completely different chromatographic method was examined: affinity dye chromatography. Affinity dye chromatography is a promising technique for the separation of proteins because dye ligands are relatively inexpensive, stable, can be made very selective, and can be used with a wide selection of proteins. Mimetic ligands suffer minimal leakage from the support, they are stable over a wide pH range, and can be operated with 1 M NaOH; 8 M urea; organic solvents; detergents; thiols; and can be autoclaved. Because of these advantages, there was a reasonable chance that at least one of the dyes under certain conditions would be able to retain and separate denatured prochymosin from other proteins present in inclusion bodies; and results show that this is the case. Mimetic green 1 appears to be the best ligand for the retention and subsequent elution of denatured prochymosin in inclusion bodies. The loss of prochymosin from the column straight after loading was shown to be at least in part due to the overloading of protein onto the column. Yellow 1 and yellow 2 ligands are potentially suitable for the analysis of native prochymosin.

The identification of three possible ligands for the separation of prochymosin is an encouraging sign. The next step in the development of this assay is to chemically couple the relevant Mimetic dyes to some suitable HPLC support (eg. PLRP-S), and then modify the conditions used in the soft-gel columns to make them suitable for separation and elution under HPLC conditions.

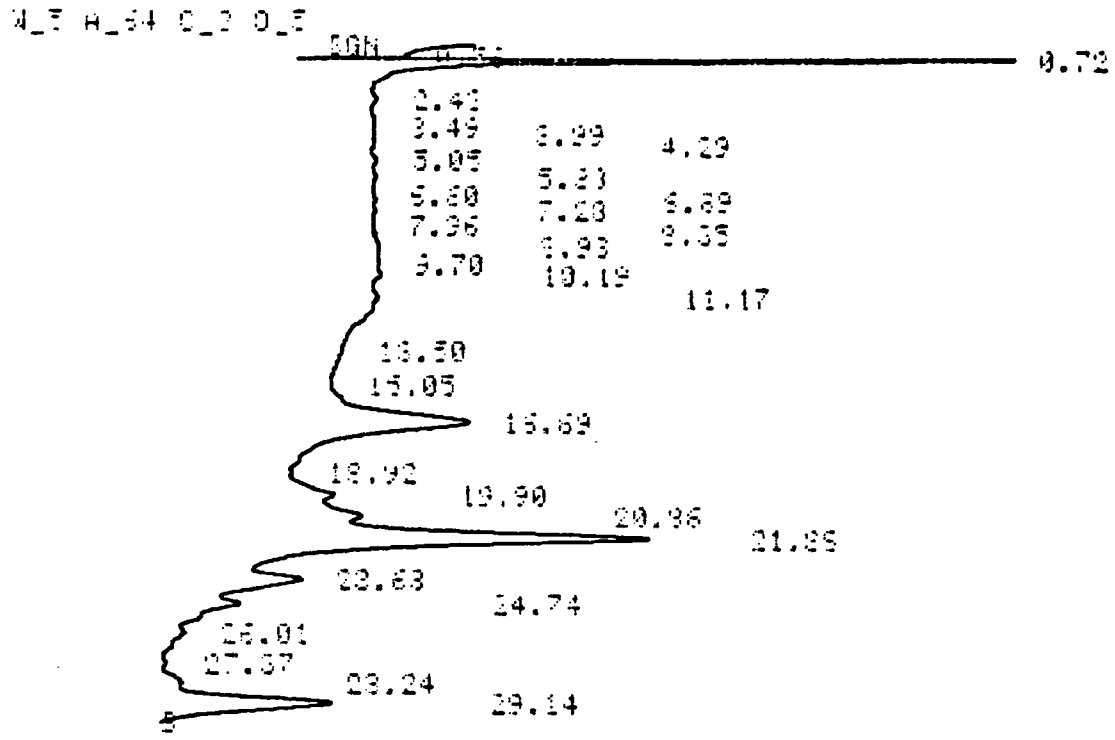


Fig. B.2.1.a. HPLC trace from a 30 μL sample of prochymosin standard injected onto a PLRP-S column. Fractions for SDS-PAGE analysis were taken of the 0.72 minute peak (fraction 1), the 16.69 minute peak (fraction 2), and from 20.6 to 23.5 minutes (fraction 3).

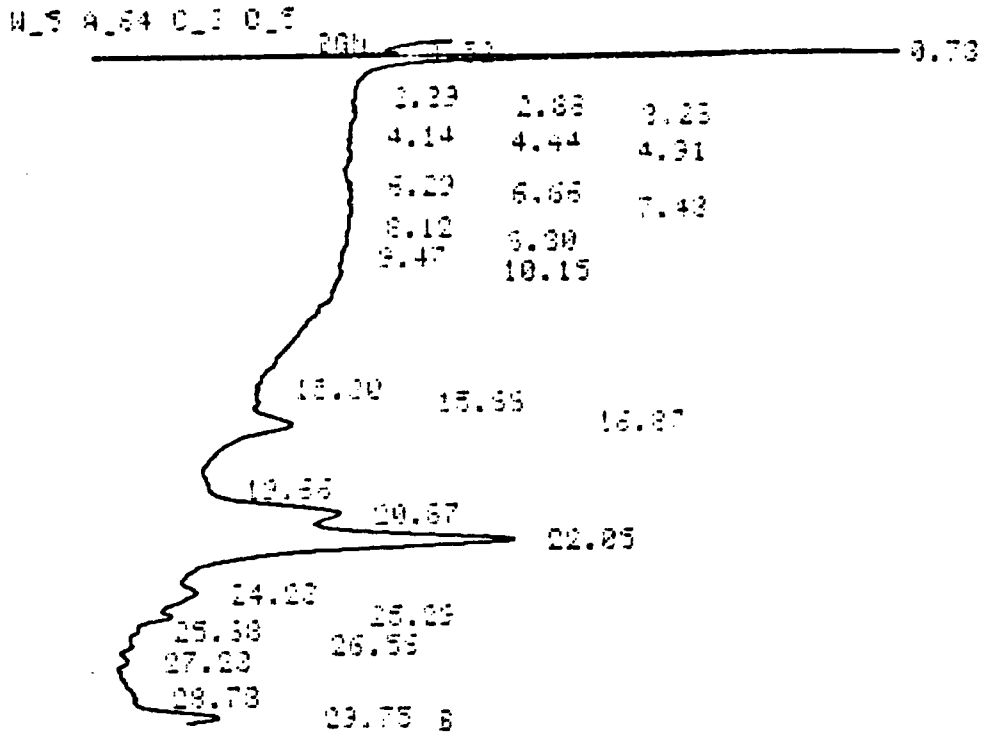


Fig. B.2.1.b. HPLC trace from a 30 µL sample of prochymosin inclusion bodies injected onto a PLRP-S column. Fractions for SDS-PAGE analysis were taken of the 0.73 minute peak (fraction 1), the 16.87 minute peak (fraction 2), and from 20.3 to 23.5 minutes (fraction 3).

NOMENCLATURE

A_{600}	absorbance at 600 nm
ABTS	2,2-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid
AC	alternating current
ADH	alcohol dehydrogenase
AOD	alcohol oxidase
ATP	adenosine tri-phosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine mono-phosphate
CER	carbon dioxide evolution rate
ConA	concanavalin A
d	dilution rate
DC	direct current
DCO_2	dissolved carbon dioxide
DDC	direct digital control
DOT	dissolved oxygen tension
DSC	digital setpoint control
DW	dry weight
EDTA	ethylene diamine tetra acetic acid
FPLC	fast protein liquid chromatography
FIA	flow injection analysis
g	gravity constant
GOD	glucose oxidase
GC	gas chromatography
GDH	glucose dehydrogenase
HPLC	high performance liquid chromatography
ISE	ion selective electrode
LED	light emitting diode
μ	specific growth rate
μ_{max}	maximum specific growth rate
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NPAB	6-nitro-3-phenylacetamidobenzoic acid

OD	optical density
OUR	oxygen uptake rate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pHBAH	p-hydroxy-benzoic acid hydrazide
PEP	phospho enol pyruvate
PID	proportional, differential, integral (control)
PLC	programmable logic controller
PPi	inorganic phosphate
psi	pounds per square inch
q_x	specific rate of production or consumption of x
RAM	random access memory
RI	refractive index
rpm	revolutions per minute
RQ	respiratory quotient
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TEMED	N,N,N',N'-tetramethylethylenediamine
tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
V	volts
V	volume
x(t)	biomass at time t
Y_x	growth yield on x

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