Monitoring of Intracellular Components During Fermentation and Some Applications for Control

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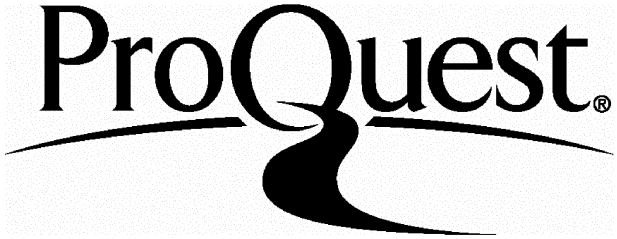

Abstract

At present most of fermentation monitoring is concerned with collecting information obtained from the environment in which the cell is growing; for example pH, dissolved oxygen tension, carbon dioxide evolution rate, oxygen uptake rate, extracellular enzymes, sugars and metabolites.

When the product of interest is located intracellularly, cell breakage forms the major difficulty being laborious and time consuming (Balankenstein and Kula, 1991). This research project focused on rapid and reproducible cell breakage, and the demonstration of some uses for the data generated.

The analysis of cell disruption kinetics within the Micron Lab 40 were found to be as pilot scale homogenisers described by Hetherington *et. al.*, 1971.

Cell disruption within the Micron Lab 40 has been shown to be reproducible. Statistical analysis of the stages from cell breakage, through to intracellular component measurement (assay) yielded an SD of <7%.

The intracellular total soluble protein and G6PDH profiles for *P. putida* ML2, *E. coli*, and *S. cerevisiae* have been obtained with the emphasis on rapid & frequent monitoring. ADH profiles have also been obtained for the yeast fermentations.

A prototype high pressure homogeniser (<10mL working volume) developed within the department was found to produce high levels of cell breakage (>50%) at moderate pressures (300-500 bar) in a single pass.

Acknowledgement

All praise is to God, the Merciful, the Beneficent. May His salutations be to the Holy Prophet and his pure Progeny.

I am indebted to my parents and all the family for their unwavering support not only during this research project but throughout my life. *(I would like to thank my daughter Sakina (aged 2.5 years) for her 'help' in 'typing' parts of this thesis.)*

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"Most surely in the creation of the heavens and the Earth and the alternation of the night and the day are signs for men who understand."

"Those who remember God ... reflect on the creation of the heavens and the Earth: Our Lord! Thou hast not created this in vain! Glory be to Thee; ..."

Holy Qur'an: The family of Imran, 3: 190, 191

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1. Introduction

At present most of fermentation monitoring is concerned with collecting information obtained from the environment in which the cell is growing; for example pH, dissolved oxygen tension, carbon dioxide evolution rate, oxygen uptake rate, extracellular enzymes, sugars and metabolites.

When the product of interest is located intracellularly, then the problem of cell breakage allowing access to the products arises. Some work has been reported in the literature where cell breakage in specific fermentations has been effected by means of sonication, enzymatic lysis and bead milling.

Although numerous cell disruption methods are available (classified into three main categories viz., chemical, biological and physical) the choice of method, has been governed by the following requirements for its inclusion in any on-line fermentation monitoring scheme:

1. Safety: the device must not compromise any safety aspects - containment of any aerosol generated, fail-safe against blockage or processing suspension containing insoluble components;

2. Scale: small sample volumes are needed (not greater than 5mL) as the frequent removal of large amounts of fermentation broth would have serious effects on the working volume especially in batch processes;

3. Effective sell disruption: the technique must be effective for cell breakage. Maximum breakage must be attained and hence maximum product release throughout the entire course of the fermentation regardless of any changes in fermentation phase and condition,

4. Generic: the technique must be effective for most fermentations,

5. Robustness: the technique must be robust and not be subject to wear and tear;

6. Reproducibility: the device must provide reproducible disruption and hence product release results;

7. Rapid operation: the disruption process must be rapid to allow effective on-line fermentation monitoring.

The above criteria narrowed the choice of disruption methods available. The majority of the methods currently in use are either cell-specific (*e.g.* enzymatic lysis, phage infection), possess a potential for product denaturation (*e.g.* chemical methods, sonication, freezing & thawing), have to be optimised for each organism as well as the different stages of fermentation (*e.g.* lysis, sonication, bead milling), produce problems for product separation prior to analysis (*e.g.* chemicals, bead milling), or are slow in their action to effect cell breakage and product release (*e.g.* lysis, sonication, chemicals)

The research work focused primarily on the release of intracellular components using high pressure homogenisation. The analysis of the disruption kinetics was undertaken to compare the Micron Lab 40 with pilot scale homogenisers and to establish criteria under which a small scale (2-5mL working volume) high pressure homogeniser can be designed.

Significance of the work

The research work conducted demonstrates

- the ability for rapid and frequent monitoring of intracellular products during the course of fermentation,
- the level of confidence in the data generated, and
- potential usage of the developed technique in control.

An on-line monitoring schematic which represents the context of the work is shown in Figure 1.1.

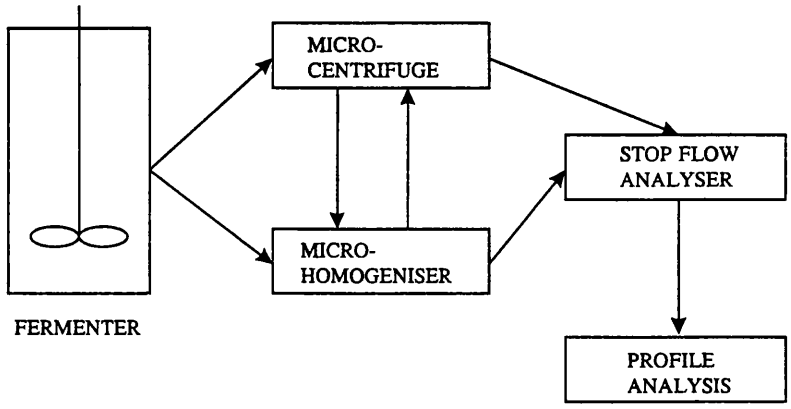


Figure 1.1. Schematic of on-line fermentation monitoring of intracellular and extracellular components.

1.1. Monitoring and Control

1.1.1. On-line Monitoring and Control in Fermentation

Comprehensive bioprocess monitoring is a prerequisite for the understanding of biological phenomena (Locher *et. al.*, 1992) and the improvement of fermentation design / operation. This involves information acquisition and analysis concerned with improving the ability to track the critical variables that affect the fermentation. Whereas the control activity uses the monitored information in order to make decisions that affect the profitability of the process in some way (Royce, 1993). The ability to accurately and reliably monitor a fermentation state is normally greatest hurdle to overcome in automatic control of that state.

In fermentation, a wide range of sensors are used to obtain the necessary information; these can be categorised as those measuring physical and chemical variables. The physical variables comprise general process engineering variables such as temperature, pressure, mass, mass-flow rates, liquid level, and impeller speed. The chemical variables are mainly the concentrations of substrates and metabolites (organic compounds, O_2 and CO_2 in the gas and liquid phase), also including biomass and pH.

Physical Process Variables

Temperature

Temperature is an important parameter in biochemical processes; this is true not only for the reaction itself, but also for auxiliary operations, such as sterilisation and downstream processing. Temperatures are usually measured by standard devices such as resistance thermometers and thermocouples which provide electrical measurement signals transferable to control boards.

The resistance thermometers are accurate and reliable; the use of thermocouples are less frequent.

Pressure

Pressure inside a fermenter vessel is measured by means of conventional pressure gauges. Fouling of the membrane used to transmit the pressure to the manometer

can lead to errors in the measurement. The monitoring of pressure is useful not only from the safety view-point, but also as it influences other parameters such as gas solubility.

Flow Rates (Gas and Liquid)

Gas and liquid flow rates are important in aerobic fermentations, and in continuous and fed-batch fermentations where the rate of nutrient feed is an essential process variable.

Impeller Speed

In stirred tank fermenters the impeller speed is an important process parameter which is often kept constant. It is usually measured by monitoring the number of revolutions of the impeller shaft per unit time; the use of tachometers is made which produce direct current proportional to the number of revolutions or alternating current, the frequency of which is determined by a digital counter;

Liquid Viscosity

Many fermentation broths, e.g. mycelial fermentation broths in antibiotics production or polysaccharide solutions, show non-Newtonian behaviour. Since viscosity is dependent on broth concentration and cell morphology, it can be used as a measure of the state of the culture. For the on-line monitoring of viscosity during fermentation, Langer and Werner, 1975, used essentially a slot-type viscometer through which a sampling stream was pumped. Neuhaus *et. al.*, 1982, tested this system successfully in an antibiotics plant.

The problem of effective degassing of the broth especially in highly viscous solutions does however lead to faulty readings.

Foam

Foaming is a problem in most fermentation broths caused by surface-active metabolites, (e.g. proteins), by components of the medium (e.g. starch, molasses) or by the cells. Foaming must be suppressed in order to prevent contamination of the culture from wetted exit filters, and also loss of culture broth.

Foam destruction can be achieved by means of mechanical methods, usually centrifugal systems, or chemically by anti-foaming agents. The mechanical system

is operated permanently and the chemical antifoam agent is added when mechanical defoaming proves insufficient. In any case, foam control necessitates foam detection which can be afforded by either an electrical conductivity probe, a capacitance probe, or a heat conductivity probe mounted inside the fermenter above the liquid level (Hall *et. al.*, 1973)

Chemical Process Variables

Exhaust Gas Analysis

The determination of gaseous compounds in the off-gas leaving the fermenter can be performed by conventional gas analysis e.g., by using paramagnetism (O_2), infrared absorption (CO_2), or flame ionisation (hydrocarbons). Mass spectrophotometry can be used to analyse all gaseous components of the exhaust stream as well as organic volatiles such as lower alcohols (Reuss *et. al.*, 1975).

pH

The pH value is an important indicator of the state of a biochemical process and its measurement is carried out electrochemically. Sterilisable glass electrodes are used as part of the standard equipment of a fermenter.

Dissolved Gases and Volatiles

Important variables in a fermentation are dissolved oxygen and carbondioxide; usually measured electrochemically. Analysis of oxygen is based on detecting the amounts of oxygen diffusing from the liquid to be analysed through a membrane into an amperometric measuring cell. The analysis of dissolved CO_2 is based on the shift in pH on the diffusion of CO_2 into a bicarbonate solution.

Biomass Determination

Biomass concentration is an important parameter for the state of any fermentation process. The most frequently used methods for determining biomass are the microscopic measurement of the cell number, and the measurement of dry cell weight following aseptic removal of a sample from the fermenter. The disadvantage of both these methods is the time taken to arrive at the measurement, which may be up to several hours. Novel methods such as turbidity and light scattering

measurements can provide a real time measure of biomass, albeit with reduced confidence in the measurement.

Enzymatic Analysis of Substrates and Metabolites

Enzymatic analysis allows very specific determination of many organic compounds. Although enzymatic analysis is usually carried out off-line, on-line methods have been developed.

Many biosensors have been developed providing methods of rapid and continuous measurement of various compounds (Guilbault, 1976), for example, glucose sensor:

The determination of glucose is important for process control. Assimilation of glucose by micro-organisms can be determined by an oxygen electrode because respiration activity increase after assimilation of organic compounds. Therefore, it is possible to construct a microbial electrode sensor for glucose using immobilised whole cells which utilise mainly glucose and an oxygen electrode (Karube, 1985).

On-line Monitoring

Currently, mostly physical and fewer chemical information can be obtained on-line (Locher *et. al.*, 1992). Commonly used industrial on-line measurements are limited to the measurement of physicochemical states by physicochemical means for example measurements of flowrates, temperature, pH, dissolved oxygen, and gas analysis. Beyond this, biologically relevant information is comparably hard to define (e.g. metabolism, viability, morphology) and measures are still the exception rather than the rule. A sound on-line documentation allows for a detailed analysis ranging from balancing over modelling to sensor validation and is fundamental to entirely automated, sophisticated operation. The decision to change a process parameter is for example often based on the occurrence of a special event which is reflected by a certain culture response.

Enzyme-based biosensors have not fulfilled their initial promise (Cook and Livinstone, 1991) for fermentation monitoring because of problems with robustness and long-term stability (Montague *et. al.*, 1988). The enzymes and cells involved in such sensors cannot be steam sterilised and have a limited life (Hatch, 1982). This lack of biosensor reliability and more importantly the financial consequences of sensor failure has served to maintain the prevalence of off-line sample analysis for

the biochemical monitoring of fermentations (Carleysmith, 1987; DiMassimo *et. al.*, 1991).

Much effort has been directed to devising what is basically an automated version of off-line analyses, with automatic sampling of the fermenter. In a production plant with many fermenters the cost and complexity of such a system does not justify the effort (Fox, 1984). Further, many novel techniques do not cope with the need for pressurised steam sterilisation to maintain a sterile barrier for the course of the fermentation (Royce, 1993). Although, Turner *et. al.*, 1993, have demonstrated the use of an aseptic sampling device which is sterilised before and after each sample using live steam.

In industry, the measurement of broth dielectric permeativity at radio frequency to measure biomass concentration in yeast fermentations (Harris, *et. al.*, 1987), and in-situ optical density measurements are used in low-cell-density recombinant *E. coli* fermentations where the low levels of insoluble media components do not interfere with the measurement.

On-line viscometry has been described for mycelial fermentation (Endo, *et. al.*, 1990; Kemblowski, *et. al.*, 1985). Commercially available on-line viscometers are used in some production scale fermentations but, are considered too bulky for use in small fermenters (Tily, 1983). More normally, off-line viscometry is applied as it is often the broth rheology rather than simple viscosity that is of interest (Carr-Brion, 1991).

The potential for in-situ Near-Infrared NIR/FTIR is currently being investigated by a number of companies. It is capable of the rapid (10 spectra per second) multicomponent analysis of aqueous-phase analytes (Schugerl, 1991).

The incentive for further developments in industrial on-line measurement, in the case of secondary metabolite manufacture is to speed process development and scale-up in laboratories and pilot plants, and the increasing desire of companies to specify fermentation control in terms of events (such as the achievement of a certain level of biomass concentration) rather than on a time-basis. Developments are particularly required to facilitate the on-line monitoring of biochemical parameters, rather than strictly physical ones, as it is this information that holds the key to major improvements in feed control strategy, for example.

Off-line measurement

On-line monitoring of all variables would be the desired solution, to follow the events in fermentations, as off-line methods are subject to loss of information density (infrequent), delays, and require a great deal of manpower (Meiners and Rapmundt, 1983). However, the lack of availability of on-line sensors due to absence of suitable sensors and the uneconomically high cost of some well known methods has allowed the domination of off-line methods for the biochemical characterisation of fermentation broth.

Common biochemical analyses in industrial fermentations are for carbohydrates, proteins, phosphate, lipid, enzyme activities, and precursors and intermediates related to the product, and are every 4 to 8 hours, being logged onto computer between 20 to 24 hours after manual sampling. The large number of off-line analyses has served to make laboratory automation a cost effective approach (Carleysmith, 1987) to the extent that robots have been used for sample preparation (Omstead, *et. al.*, 1992).

Chemical analysis of cell components such as DNA, and RNA to indicate biomass concentration requires sophisticated procedures, is labour intensive, and cannot be generated on a real-time basis for control or fault detection (Gbewonyo, *et. al.*, 1989). Faster off-line analyses such as dry weight measurements, cannot be used during the seed stage, where active growth requires an analytical technique taking less than 30 minutes, and sedimentation (for cell mass) or oxygen uptake rate data are used in preference (Cook and Livinstone, 1991). The Flow Injection Analysis (FIA) systems are gaining high acceptance at both research and industrial levels (Bradley, *et. al.*, 1991).

1.1.2. Applications of On-line Intracellular Monitoring as Reported in the Literature

The development of biotechnology has made it possible to use micro-organisms or cells to produce peptides / proteins of medical interest in large quantities (*e.g.* insulin and human growth hormone).

Process control is becoming increasingly important for the rationalisation of biotechnological processes. These processes can be improved by the use of different strategies involving the selection of micro-organism strains with elevated

performance, the study of the configuration of bioreactors, or the control of the physiological conditions in which the cultures grow.

Bioprocesses are generally ill controlled. This is because measurement of the relevant variables is difficult (Locher *et. al.*, 1992).

To successfully carry out, and control a fermentation process it is necessary to measure the development of the product formation and it's concentration in the fermenter. The product is the primary reason for carrying out the fermentation. The optimisation of the fermentation must be based on the product, and not on for instance cell density, which had been the task in the past. The method of analysis has to be fast, accurate, reproducible and be unaffected by the changes in the fermentation conditions.

The characterisation of microbial enzymatic activities is essential to control and direct fermentative processes (*e.g.* bread making) towards products with desirable characteristics and high quality (Torner *et. al.*, 1992).

For optimal process control, values for essential parameters must be reliable and quickly available. For many media components in fermentation, on-line analytical procedures are available, based on wet chemical and electrochemical methods.

During the fermentation the product could be either intracellular or extracellular. In the case where the product is intracellular the cell has to be broken or disrupted to release the product.

On-line monitoring of product formation has traditionally been one of the more difficult tasks in biotechnology, in particular if the product is an intracellular protein. The necessity of cell disintegration forms the major difficulty and the preparation of the cell-free extract for the measurement of enzyme activities is laborious and time consuming (Balankenstein and Kula, 1991).

Methods for continuous monitoring of intracellular enzyme activities are rarely available. Koplov and Cooney (1976) developed a continuous assay for acetate kinase from *E. coli* using sonication for cell disruption. A similar procedure was developed for the on-line determination of the periplasmic enzyme penicillin-G acylase during the cultivation of genetically modified *E. coli* (Ahlmann *et. al.*, 1986). They employed enzymatic chemical treatment for cell breakage.

Flow Injection Analysis (FIA), since its introduction by Ruzicka and Hausen in 1975, has proven to be a promising analytical technique for on-line monitoring of bioprocesses.

The principle of FIA is defined as the injection of a liquid sample into a non-segmented continuous carrier stream of a suitable liquid. After sample injection the peaked shaped sample zone is transported through a reaction coil to the detector, (Figure 1.2.) which continuously records the absorbance, or other physical parameters. The resulting peak, H, is related to the concentration of the species being analysed.

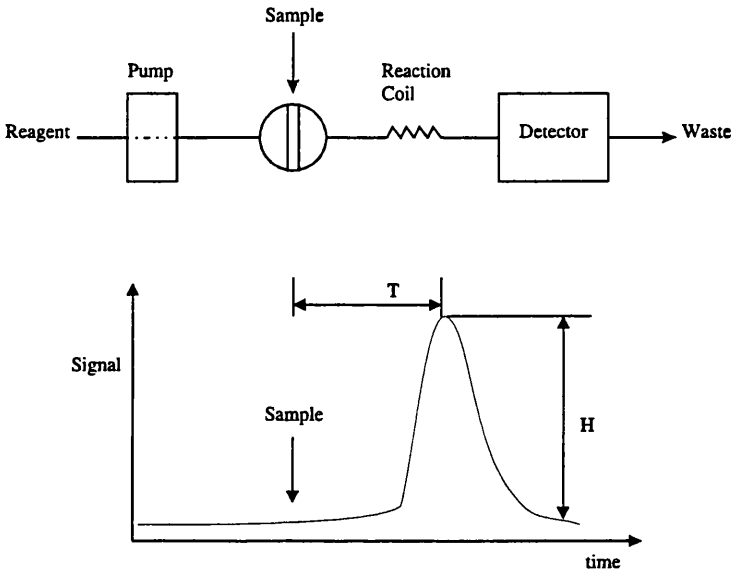


Figure 1.2. General Scheme of FIA technique: T, residence time; H, peak height. (Redrawn from Recktenwald *et. al.*, 1985)

It has been applied to on-line measurement of media components such as glucose (Ludi *et. al.*, 1990), enzyme activities during downstream processing (Stamm and Kula, 1990; Recktenwald *et. al.*, 1985), cellulase activity in precipitated bioreactor preparation, cytoplasmic fumarate dehydrogenase (FDH) activity from *C. boidinii* (Blankenstein and Kula, 1991), and cytoplasmic β-galactosidase activity during recombinant *E. coli* cultivation (Kracke-Helm *et. al.*, 1991).

Blankenstein and Kula (1991) employed permeabilisation with Digitonion and Triton X-100 to disrupt the *C. boidinii* cells to monitor cytoplasmic fumarate dehydrogenase (FDH) activity via FIA techniques combined with membrane separation. They found that to optimise the permeabilisation operating parameters such as permeabilisation agents, detergent concentration, temperature and

incubation time had to be altered. Denaturation of FDH resulted when the incubation time was above 5 min at the optimum conditions. Therefore, for any other organism the permeabilisation conditions must be re-evaluated and re-optimised.

The measurement of FDH activity was semicontinuous with a frequency of six per hour and was found to be sufficient to follow the dynamics of enzyme production process. The activity determined via the FIA system was comparable to those detected manually from the same samples.

Kracke-Helm *et. al.* (1991) found that the β-galactosidase activity determined by on-line FIA assay were on average 9% higher than those determined off-line. The difference was attributed to loss of activity during sample storage and handling.

Recently the rapid on-line monitoring of several dehydrogenases (formate, malate and formaldehyde dehydrogenases (FDH, MDH and FAD)) from a yeast (*Hansenula polymorpha* HD30) has been reported by Stube and Spohn, 1994. Their fully automated system consisted of the on-line fermenter broth sampling device with automated chemical sterilisation, cell disruption chamber and the flow injection analysis set-up. FDH and MDH were sequentially determined for a batch fermentation of *Hansenula anomala.*

Rapid on-line cell disruption was achieved using a customised flow-through bead milling cell. The steel cylinder with a cooling jacket was filled to 90% with glass beads, and the cell debris and beads were excluded from the detector by means of a membrane device (cut off 30,000 D). The authors did not report any fouling or loss of efficiency of the membrane and so its useful life-cycle.

1.2. Cell Disruption Methods

Many useful proteins are intracellular and therefore their monitoring and recovery requires cell disruption. The cell membrane can be made permeable by several mechanical, chemical and enzymatic methods or a combination of these (Becker *et. al.*, 1981).

Over the past few decades several reviews on the methods available for cell disruption have been published. (Wimpenny, 1967; Edebo, 1969; Wiseman, 1969; Hughes *et. al.*, 1971; Edebo and Magnusson, 1973; Coakley *et. al.*, 1977; Engler, 1985; Patel, 1985; Chisti and Moo-Young, 1986; Schutte and Kula, 1990).

A useful classification of these shown in Table 1.1. is given by Kramer and Bomberg, 1988.

Cell Disruption Methods			
Chemical	Biological	Physical	
		Non-Mechanical	Mechanical
Alkali	Enzymatic Lysis	Osmotic Shock	Sonication
Solvents	Phages	Freeze/Thawing	Extrusion
Detergents	Virus	Gas Decompression	Wet Milling
Acid	Inhibition of wall synthesis	Drying	High Pressure Homogenisation

Table 1.1. Cell disruption methods

Ease of disruption of a range of micro-organisms with different methods is given by Edebo, 1983, shown in Table 1.2.

All the methods, classified here into chemical, biological and physical are able to liberate products from the microbial cells with varying degrees of effectiveness (Schutte and Kula, 1990). However, with the restrictions imposed by the requirements for a cell disruption device which will integrate into a system for on-line monitoring and control of bioprocesses, summarised in Table 1.3, many of these processes have obvious limitations for the intended application.

	Liquid pressing	Freeze pressing	Agitation	Sonication
Animal cell	7	7	7	7
Gram -ve bacilli & cocci	6	6	5	6
Gram +ve bacilli	5	4	(4)	5
yeast	4	2.5	3	3.5
Gram +ve cocci	3	2.5	(2)	3.5
Spores	2	1	(1)	2
Mycelium	(1)	5	6	1

Table 1.2. The susceptibility of various kinds of cells in suspension to disintegration by various methods (Edebo, 1983). The numbers indicate the sensitivity of the cell suspension with each group; a high number indicating that the cells are easier to disrupt within the disruption methods so grouped.

Requirements for a cell disruption device for integration into a system for on-line monitoring and control of bioprocesses.
Safety
miniature scale
ability for use with all biological systems (generic application)
maximum product release
reproducibility of results
prevention of product (e.g. protein denaturation)
maximisation of disruption rate for speed of operation
ability to handle a variety of feed concentrations (for example those typically encountered during the course of fermentation).

Table 1.3. Requirements for a cell disruption device for integration into a system for on-line monitoring and control of bioprocesses.

Majority of the methods are either cell-specific (enzymatic lysis, phage infection, virus), possess a potential for product denaturation (chemical methods, sonication,

freezing & thawing), have to be optimised for each organism as well as the different stages of fermentation (lysis, sonication, bead milling), produce problems of product separation prior to analysis (chemicals, bead milling), or are slow in their action to effect cell breakage and product release (lysis, sonication, chemicals).

1.2.1. Chemical Methods of Cell Disruption

Included in the wide range of cell disruption techniques used in the laboratory are chemical methods such as acid and alkali treatment and the use of detergents and solvents. Chemical treatment may be used either to lyse cells or to extract cellular components. However, these chemicals may be expensive, contaminate the product (Chisti and Moo-Young, 1986) and frequently destroy biological activity (Dunnill and Lilly, 1975; Edebo, 1969; Schutte and Kula, 1990).

Treatment with Acid or Alkali

The use of alkali for cell lysis is inexpensive and can easily be applied in almost any scale of operation (Schutte and Kula, 1990). However, an obvious requirement is that the desired product be stable at extreme pH values, ranging for alkali treatment from 10.5 to 12.5, for up to 30 minutes. The therapeutic enzyme L-asparaginase was isolated by exposing the bacteria *Eriwinia carotovora* to 0.5M NaOH (pH 10 to 12.5) for 20 minutes. (Wade, 1971), followed by the addition of 25% acetic acid (pH 4.8). A heavy precipitate was removed by centrifugation, and L-asparaginase was recovered from the supernatant by adsorption on a cation-exchanger.

Sherwood *et. al.*, 1982, reported that a recombinant human growth hormone can be extracted from cells of *E. coli* by raising the pH of the cell suspension to pH11 with NaOH. When a therapeutic protein is being produced from recombinant strains, this technique has the added advantages that no viable cells remain in the process liquor. It has also been suggested that this method may inactivate proteases and reduce pyrogen contamination. However, the concentration of NaOH required will destroy many biological activities and lead to denaturation or degradation of protein. Acids and alkalis are not very selective and tend to damage sensitive proteins and enzymes along with the cell walls (Edebo, 1969; Schutte and Kula, 1990).

Treatment with alkali may solubilise most components except for the cell wall (Edebo, 1969), however, Lee *et. al.*, 1979, reported that alkali did not solubilise protein from *Candida lipolytica* but that 6M HCl was effective in hydrolysing the cells. Acid treatment apparently hydrolysed protein to free amino acids as well.

Solvent Extraction and Detergent Breakdown

Organic solvents have been widely used for the disruption of yeast cells in the isolation of enzymes. Although, in their presence many enzymes are denatured (Edebo, 1969; Schutte and Kula, 1990).

Selective liberation of periplasmic enzymes may be achieved using these methods. Fenton, 1982, achieved the release of β-D-galactosidase from *Kluveromyces fragilis* by treating the yeast cake with 80% Ethanol for 90 minutes at 28°C, thereafter, the cells were filtered to remove the solvent, and the enzyme was extracted with 0.1M phosphate buffer (pH 6.6) at 28°C in a stirred tank. To emphasise the slow nature of this process, good yields of the enzyme were obtained after 17 hours. Fenton, 1982, found that varying solvent treatment times between 5 minutes and 10 hours did not significantly affect β-D-galactosidase yields. On employing several solvents varying degrees of release from 2% for butanol to 90% for methanol, ethanol and i-butanol were achieved.

Detergents often cause lysis or leakage (Engler, 1985) of cell contents by disorganising the cell membrane (Edebo, 1969). The plasma membrane is damaged in such a way that the morphology of the cells remains intact, yet low-molecular-weight molecules can enter and leave the cells (Felix, 1982).

Commonly used detergents such as cholate, sodium dodecyl sulphate, cetyltrimethylammonium bromide (CTAB), Tween and Triton X-100 are able to act on the cytoplasmic and cell membranes. The constituent proteins of the membrane structure are solubilised to a greater or lesser extent, making the cell permeable to the passage of certain proteins (Schutte and Kula, 1990). Buckland *et. al.* reported such a selective extraction of cholesterol oxidase from *Nocardia* by treatment with Triton X-100. Gowda *et. al.*, 1991, permeabilised *S. cerevisiae* with CTAB. Maximum activities of alcohol dehydrogenase, glucose 6 phosphate dehydrogenase and hexokinase from the yeast were observed after treatment with CTAB (2% wet weight of cells) at 24°C for 15 minutes. They also found that *K. fragilis*, a yeast thought to have a much tougher cell wall, was permeabilised under much milder

conditions (CTAB concentration of 1% wet weight of cells, 5 minutes at 4°C). They then clearly showed that the ratio of cell to detergent is important in permeabilising the cells, rather than the detergent concentration.

An advantage of permeabilisation techniques over mechanical disruption is that extensive fragmentation of the cells can be avoided, and hence the nucleic acids are retained within the cell. There is also the obvious advantage of replacing a mechanical disruption device with a simple batch operation in a stirred vessel.

Disadvantages are that detergents are quite expensive and contaminate the product stream which may lead to irreversible loss of product activity and sub-optimum yields (Schutte and Kula, 1990) The need for an incubation period is an added disadvantage. The choice of a special permeabilisation procedure depends on the cell wall and membrane composition of the particular organism, and its optimisation involves parameters such as permeabilisation agents, concentration of detergents, temperature and incubation times (Blankenstein and Kula, 1991).

1.2.2. Biological Methods of Cell Disruption

Enzymatic Lysis

Enzymatic lysis is one of the most widely studied laboratory methods of cell disruption. It represents an alternative of great potential to capital intensive and high running cost mechanical methods for low cost products, such as single cell protein (Asenjo, 1981; Asenjo and Dunnill, 1981).

Numerous enzymes have been identified which attack specific bonds in the cell wall structure of micro-organisms leaving the rest of the cell wall undamaged. Most wall lytic proteases are specific with little or no activity towards intracellular proteins (Schutte and Kula, 1990). Enzymes can be used directly for the release of intracellular or wall associated materials or in conjunction with other techniques, for example mechanical methods, to improve the rate and yield of product extraction (Asenjo *et. al.*, 1985). Kula *et. al.*, 1990, treated a suspension of 20% v/v of gram positive organism *Bacillus cereus* with a low concentration of cellosyl to find the cells more susceptible to mechanical stress in the bead milling as compared to the suspension which had not been treated. Similarly, Baldwin and Robinson, 1990, pre-treated Bakers' yeast with zymolyase for two hours prior to disruption in a newly developed high pressure homogeniser, the Microfluidizer, and found that this enhanced the mechanical disruption from about 35% without pre-treatment to 70% with after 5 passes. (they did not report the homogenising pressure and cell concentration).

The application of enzymatic lysis is restricted by the selection of an appropriate enzyme or enzyme system and determination of specific reaction conditions for efficient lysis (Engler, 1985; Keshavarz-Moore, 1990; Schutte and Kula, 1990), their high costs (Asenjo, 1990) and also their restricted availability (Schutte and Kula, 1990). Enzymatic lysis is improved at lower concentrations, markedly affected by buffer, pH, temperature and to a lesser extent by ionic strength. The growth phase and fermentation conditions affect greatly the sensitivity of micro-organisms towards various lytic enzymes (Schutte and Kula, 1990).

Enzymatic and chemical lysis have not been widely used for large scale intracellular product release and disruption of microbial cells (Asenjo, 1990). Lysozyme is a commercial lytic enzyme which has been used for many years in industry. However,

this enzyme is only active on bacterial cells. Other bacteriolytic and yeast lytic enzymes are only available as laboratory reagents (Asenjo, 1990).

Autolysis

Autolysis is another method of enzymatic lysis in which the lytic enzymes are produced by the organism itself. To allow normal growth processes to occur, most micro-organisms have the capability of producing enzymes which hydrolyse polymeric structures in the cell wall. However, environmental changes may trigger over-production of these enzymes or activate production of other autolytic enzymes (Engler, 1985). Auto lysis is volume independent (Cumming *et. al.*, 1985), however, many factors affect the process such as temperature, pH, incubation time, molarity of buffer, and the metabolic state of the cells which need to be explored to find the optimum conditions for such a process for any given species (Hughes *et. al.*, 1971).

The major disadvantages of autolysis are the long incubation times of 2 to 20 hours, low yields of disrupted cells / protein, and high costs (Engler, 1985; Knorr *et. al.*, 1979) and in addition, considerable protein denaturation may occur (Dunnill and Lilly, 1975; Edebo, 1969).

Inhibition of Cell Wall Synthesis

Inhibition of cell wall synthesis in the late growth phase using antibiotics such as penicillin, which act by blocking synthesis of new cell wall material, may be used to initiate lysis (Engler, 1985). Conditions for lysis will be favourable only if biosynthesis and reproduction continue to take place after the addition of the inhibitor (Hughes *et. al.*, 1971) so that deficient cell walls are present at the time of disruption.

Phage Infection

Infection by a phage consists of its adsorption to the cell wall. The enzyme carried by the phage hydrolyses the host cell wall leading to the penetration and passage of the phage nucleic acid into the cell (Engler, 1985; Hughes *et. al.*, 1971). If the infection is heavy, lysis can be produced before penetration of phage nucleic acid (lysis from without). Alternatively, cell lysis occurs when enzymes produced in the phage reproduction cycle will lyse the host cell (from within). Cellular constituents

may be significantly altered with this process, especially if lysis occurs from within (Engler, 1985).

1.2.3. Physical Methods of Cell Disruption

Physical methods of cell disruption can be further split into mechanical and non mechanical methods.

Mechanical methods include:

- Wet milling - extrusion, bead milling
- High pressure homogenisation
- Counter current two jet impingement
- Microfluidizer
- Sonication

whilst osmotic shock, freezing and thawing, heating and drying, and gas decompression are counted as non mechanical methods.

Extrusion

Another frequently used laboratory method for cell disruption is the use of high pressure to force suspended cells through a needle valve. The sample is bled through the needle valve whilst keeping the pressure constant (Engler, 1985). This principle is applied in the French press. Extrusion of frozen cell suspensions through an orifice is the basis of the Hughes press and also the X-press, which in consequence does not require a pressure controlling valve (Keshavarz-Moore, 1990).

French Press & S-R Fractionator

The French press was developed for the breakage of *Cholera* (Milner *et. al.*, 1950). Later it proved to be very effective in disrupting cells from many species of bacteria (Hughes *et. al.*, 1971), and has been widely used for the preparation of cell extracts in work on enzymes and on membranes.

The French press consists of a hollow cylinder in a stainless steel block having a small orifice and a needle valve at its base (Aiba *et. al.*, 1973; Keshavarz-Moore, 1990). The press is pre-cooled to zero degree centigrade and the piston is pre-set to provide the desired chamber volume. The cylinder is filled with the cell suspension or paste, subjected to high pressures up to 210 MPa (Hughes *et. al.*, 1971) causing the cells to burst as they are extruded through the pre-cooled valve to atmospheric pressure (Milner, Lawrence and French, 1950).

Heating problems encountered have been circumvented in the continuous press based on similar principle as the French press, the Servall-Ribi fractionator (Perrine *et. al.*, 1962); either the needle valve is chilled with nitrogen gas or the extruded cells are ejected directly into chilled liquid.

Duerre and Ribi (1963) reported that with *E. coli* the rupture of all cells occurred at a pressures of 25,000 p.s.i. (ca. 1725 bar), with only a few whole cells remaining at 15,000 p.s.i. (ca. 1034 bar). The loss of enzyme activity was minimal.

Hughes Press & X-Press

The method of disrupting micro-organisms by extrusion of a frozen cell suspension or paste through a narrow slit (1 to 2 mm) at very high pressures (100 to 200 MPa) (White and Marcus, 1988) was developed by Hughes in 1951.

The Hughes press although laborious to use, is very effective in cell breaking and mild with respect to the recovery of liable enzymes. The press consists of a steel body having a cylindrical bore which is connected to a receiving chamber via an annular slit, and a steel plunger. The press is chilled to 25°C to 30°C below zero and the frozen cell suspension or paste (with or without abrasive) (Aiba *et. al.*, 1973; Chisti and Moo-Young, 1986) is put into the chamber, and with the plunger in position high pressures (about 1000 to 1500 bar) are applied with a hydraulic press. Disruption is brought about by the frozen cell suspension or paste being forced through the slit into the recovery chamber.

Edebo (1960) modified the apparatus to facilitate several pressings of the samples through the orifice. This device, the X-Press, consist of two identical cylinders mounted coaxially and separated by a disc with a central orifice. The sample being forced from one chamber to the other. This semicontinuous design is capable of handling 10kg of material every hour (Magnusson and Edebo, 1976a). Unlimited

scale-up of a similar machine has been suggested. However, no industrial size freeze-press is known to be commercially available (Chisti and Moo-Young, 1986).

Freeze pressing is very effective in disrupting cells. In a single pass 90% disruption of undiluted Bakers' yeast was achieved (Magnusson and Edebo, 1976c). Edebo (1983) stated that relative to other processes minimal enzyme inactivation occurs with freeze pressing. The technique is not suitable for use with enzymes sensitive to freezing and thawing (Hughes *et. al.*, 1971). An added advantage is less communition of cell fragments (Edebo, 1969; Hughes *et. al.*, 1971). This is an excellent method for obtaining enzyme protein and bacterial cell wall preparations (Melling and Phillips, 1975). Of all the methods available for cell disruption, freeze pressing appears to offer the most complete rupture of a large variety of cell material from bacteriophage to animal tissue (Hughes *et. al.*, 1971).

The suggested mechanisms of disruption include abrasion by ice crystals (Hughes *et. al.*, 1971), changes in ice crystal states (Edebo, 1960) and plastic flow through the discharge gap (Scully and Wimpenny, 1974). Studies by Magnusson and Edebo (1976), indicate that internal friction (shear) sustained by a high concentration of cells, low temperatures and a high mean pressure, is a promoter of disruption.

Wet Milling

Bead milling

A common laboratory ball mill is the rotary cell homogeniser. In which the cell suspension is filled into a glass or stainless steel container, together with glass beads. This is in turn mounted with a special holder between rubber "cushions". The container is caused to vibrate at a high frequency. Essential cooling is provided to the device by liquid carbon dioxide which is flushed through the system (Engler, 1985; White and Marcus, 1988). Results from the manufacturers show the almost complete breakage of Bakers' yeast cells (5% cell suspension) within only one minute (White and Marcus, 1988). Because of the chamber vibrations, the scale up of this method would be practically impossible (White and Marcus, 1988). A disrupter capable of processing 10mL is commercially available.

As reported in the Monitoring and control section above, a small 1.5mL bead milling was customised to a flow-through cell and successfully used in the on-line disruption of yeast cells (Stube and Spohn, 1994).

Following trial with three methods of cell disruption namely ultrasonic, ultraturrax and mixer mill (all incorporating customised flow-through cells), they concluded that the mixer mill flow-through cell provided the least deactivation of the enzymes (the dehydrogenases FDH, MDH and FAD), and its long term stability (as compared to the other two methods) makes it suitable for its inclusion to on-line fermentation monitoring.

Although Stube and Spohn, 1994, acknowledged the numerous operating variables involved (type of organism, mixing frequency, mixing time, bead size and bead loading as discussed further in the section below), they found that the customised flow-through mixer mill gave the same degree and time of cell disruption as that commercially available, with better reproducibility. This enhanced reproducibility was attributed to precisely defined operating parameters.

They optimised the bead size and bead loading for the yeast *Hansenula polymorpha.* This gave a maximum disruption and protein concentration after nine minutes

Ultraturrax

The evaluation of an ultraturrax flow-through device (as mentioned above) for on-line cell disruption was carried out by Stube and Spohn, 1994. The technique required a constant biomass concentration, absence of significant changes in chemical composition (pH, ionic strength, surfactants) of cell suspension as a necessary prerequisites.

Maximum FDH activity was obtained after 3 minutes at 50,000 r.p.m. They found enzyme deactivation occurred at high rotational speeds. However, the data presented was insufficient to indicate this as the optimum operating procedure.

The degree of cell disruption, and enzyme deactivation depend on the cell concentration (Stube and Spohn, 1994). These combine to make the use of the ultraturrax untenable in fermentation monitoring.

High Speed Bead milling

Many attempts have been made at scaling-up laboratory scale disruption devices but greater success has been achieved in adapting commercially available high speed bead milling and high pressure homogenises (Engler, 1985).

High speed bead milling have been adapted for cell disruption but were originally developed for the communition of pigments for the printing industry, requiring the dispersion of solids into micrometer sized particles with a narrow size range. The development of bead milling equipment and research carried out on their design has been reviewed in recent years (Chisti and Moo-Young, 1986; Dunnill and Lilly, 1975; Engler, 1985; Schutte and Kula, 1990; White and Marcus, 1988).

Cell disintegration in bead milling is one of the most efficient methods for physical cell disruption (Chisti and Moo-Young, 1986; Darbyshire, 1981) and noted for its effectiveness for mycelial disruption (Lambert, 1983; Wang *et. al.*, 1979).

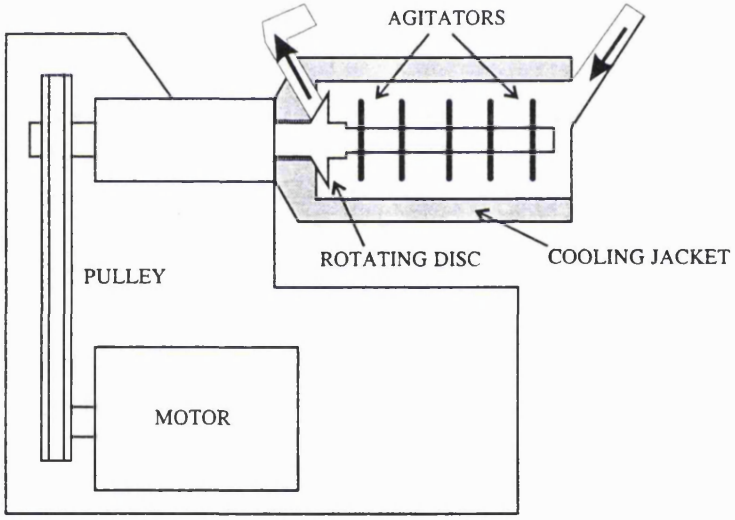


Figure 1.3. The schematic diagram of a horizontal bead milling. The Dyno-mill KD5

In high speed bead milling, cell suspension and glass beads are agitated by discs rotating at high speeds. Various designs of mills consist of either vertical or horizontal cylindrical chamber with a motor driven shaft supporting a collection of discs or other agitating elements. The grinding action is provided by the beads, typically occupying 80 - 85% of the working volume of the chamber. The rotation of the discs causes the grinding beads to move in a circular manner in the chamber. The kinetic energy transferred from the beads creates impact and differential

velocity profiles which generate very high shear forces between individual beads, and between the beads and the microbial cells. This is through to cause cell disruption (Rehacek and Schaefer, 1977).

Relative to the vertical, the horizontal configuration of the mill is known to give a better efficiency (Rehacek *et. al.*, 1969). This is because upward fluid flow tends to fluidise the grinding beads to an extent, and so reducing efficiency (Chisti and Moo-Young, 1986). A further reason for preference is that horizontal mills are fed from above and so there is no need for a screen at the feed end to retain the beads inside the chamber as is the case in the vertical mill. The mills can be operated in batch or continuous mode.

High capacity cooling must be provided for processing temperature sensitive materials. This requirement restricts the size of the mill however, units from 0.1 to 20L are available.

The Kinetics of cell disruption within the bead milling:

The process variables of a high speed bead milling are numerous and given by Schutte and Kula (1990) as: agitator speed and design; bead size; bead density; bead loading; chamber geometry; temperature; and feed flowrate.

Dunnill and Lilly (1975) investigated the kinetics of protein release from Bakers' yeast. The kinetics depend on the construction of the mill. Machines with predominant plug flow gave rise to first order disruption kinetics whereas in machines in which the rotor permits significant back mixing the disruption deviates from first order.

$$\frac{dR}{dt} = k \,.\, (R_m - R)\,; \quad \ln\left(\frac{R_m}{R_m - R}\right) = k \,.\, t \qquad \text{Equation 1}$$

where R is the weight protein per unit weight of packed yeast;

R_m is the maximum protein release;

k is the first order rate constant;

and t is the disruption time.

Limon-Lason *et. al.* (1979) confirmed first order kinetics where the amount of protein release was proportional to the amount of protein remaining, when examining batch and continuous disruption of Bakers' yeast in laboratory and pilot scale mills (0.6 and 5 litre chamber volumes) covering a range of impeller speeds. In the larger mill, at higher impeller tip speeds (above 10 m/s), disruption did not follow first order kinetics.

These first order kinetics observation were confirmed by Kloosterman *et. al.* (1988), Agerkvist and Enfors (1990) both during cell debris studies and by Marffy and Kula (1974), in investigating the release of intracellular enzymes.

The disruption rate constant is known to be a function of several operating parameters such as temperature, impeller rotational speed (Dunnill and Lilly, 1975; Limon-Lason *et. al.*, 1979; Marffy and Kula, 1974; Mogren *et. al.*, 1974; Rehacek and Schaefer, 1977; Schutte *et. al.*, 1983; White and Marcus, 1988), bead loading (Dunnill and Lilly, 1975; Rehacek and Schaefer, 1977; Schutte *et. al.*, 1983), bead size (Dunnill and Lilly, 1975; Marffy and Kula, 1974; Schutte *et. al.*, 1983; White and Marcus, 1988), cell concentration (Dunnill and Lilly, 1975; Schutte *et. al.*, 1983; White and Marcus, 1988), bead material, number and design of discs (Keshavarz-Moore, 1990; Schutte and Kula, 1990) and grinding chamber geometry (Schutte and Kula, 1990).

Agitator speed and design:

In general, a very rapid increase in the rate constant, k, is caused by increasing the agitator speed (Currie *et. al.* 1972; Marffy and Kula, 1974; Mogren *et. al.*, 1974; Rehacek and Schaefer, 1977).

At the high speeds the degree of disruption levelled off (Schutte *et. al.*, 1983). Limon-Lason *et. al.* (1979) found this was the case in a 5L horizontal mill. However, a clear difference in the design of the agitators was observed. The disruption rate constant, k, increased with speed for both the polyurethane open design and the stainless steel closed design agitators at speeds of up to 10m/s. At higher speeds, the polyurethane agitator yielded a constant, k, value while the value increased as expected with the stainless steel agitator. This was attributed to the backflow at high speeds being greater for the stainless steel agitator, so that the increase in the disruption rate constant would increase for both agitators but for the backflow effect. Furthermore, k values from the polyurethane agitator were larger

than those from the stainless steel agitator but, the maximum protein release was greater for the stainless steel agitator. This effect was attributed to the more open design of the polyurethane agitator creating a greater degree of mixing at a distance but lower shear rate close to the agitator. The higher degree of mixing explains the greater disruption rates and the lower shear rates gave lower release. The converse being the case for the stainless steel agitator.

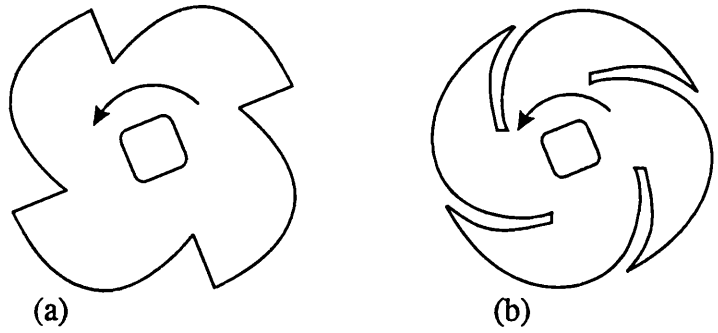


Figure 1.4. Agitator design: (a) Polyurethane 'open' type and (b) stainless steel 'closed' type. (Limon-Lason *et. al.*, 1979)

Schutte and Kula (1988) have extensively analysed the effect of agitator design on cell disruption. A four fold increase in the rate constant for protein release from Bakers' yeast was observed when changing the rotor type from 12 eccentric rings to 6 notched and 2 cone discs (mill Netzsch LME 4).

Bead size:

Currie *et. al.* (1972) using a range of beads (0.5 - 2.8 mm) reported that for yeast concentration of 30% the smaller beads were preferred whilst their advantage was reduced for concentrations above 60%. However, at the low concentrations of 5, 7.1 and 12.5% dry weight of *Enterobacter cloacae*, Woodrow and Quirk (1982) clearly showed that decreasing bead size significantly increases the rate of disruption. However, they do note the practical difficulties in using very small beads for example those noted by Currie *et. al.* (1972) that at high cell concentrations small beads tend to float. Melendres *et. al.* (1991) showed that off 0.375 mm, 0.625 mm and 0.875 mm diameter beads, the smaller beads were effective for faster disruption. This was attributed to the increased frequency of bead collisions. They

went further and postulated that it may be possible to control the rate governing disruption based on the collision frequency. Marffy and Kula (1974) found that beads of 0.25 to 0.5 mm were more effective for Brewers' yeast disruption than either the very small beads (0.1 to 0.25 mm) or larger beads (0.75 to 1.00 mm). Rehacek and Schaefer (1977) suggest that bead size must be selected in relation to cell size and cell concentration.

Bead loading:

Increased bead loading enhances the rate of disruption (Currie, 1972; Dunnill and Lilly, 1975; Rehacek and Schaefer, 1977; Schutte *et. al.*, 1983; White and Marcus, 1988) however, temperature increases and power consumption are strongly influenced (Rehacek and Schaefer, 1977; Schutte *et. al.*, 1983). In a recent study Melendres *et. al.* (1991) found that the disruption rate constant was proportional to the square of the bead loading, for Bakers' yeast (30% w/v) processed in a 150mL Dyno-mill KDL type bead milling operating in batch mode. (They evaluated cell disruption in terms of viable cell count).

Feed flowrate:

It is known that reduction in the disruption rate occurs at low residence times resulting from high flowrates (Currie *et. al.*, 1972; Marffy and Kula, 1974). Schutte *et. al.* (1983) and Limon-Lason *et. al.* (1979) using pulses of dye introduced into the chamber showed that there is a large distribution of residence times for the process material within the chamber.

Temperature:

A general problem associated with agitated mills is the generation of heat due to impacts and friction between the grinding elements. Jacketing the grinding chamber is normally sufficient in small mills to handle the heat load generated. However, for larger mills of capacity in excess of 10 litres the processing of temperature sensitive materials can cause serious problems with regard to cooling. This problem has been overcome in a 20 litre horizontal bead milling in which the agitator shaft and discs are cooled along with jacketing the grinding chamber (Rehacek and Schaefer, 1977).

Denaturation of proteins due to shear and temperature effects is a distinct possibility. In general, no denaturation is noticed at low operational temperatures (Chisti and Moo-Young, 1986) although Marffy and Kula (1974) reported some enzyme inactivation at temperatures as low as 2°C. Limon-Lason *et. al.* (1979) found reduced protein release with increasing temperature. This is apparently due to thermal degradation and/or changes in physical properties such as viscosity causing greater back-mixing and so reducing efficiency. The disruption temperature was reported by Currie *et. al.* (1972) to have little effect on the disruption kinetics within the range 5 - 40°C.

Cell concentration:

The effect of cell concentration on the disruption process are inconsistent. Mogren *et. al.* (1974) reported no effect of cell concentration in the range 40 - 200 kg/m^3, while Limon-Lason (1979) found that the disruption rate constant, k, to be independent of cell concentration greater than 30%w/v for only the stainless steel closed design agitator and decreased with increasing concentration for the open design polyurethane agitator. The effects were attributed to changes in the rheology and agitator type. Release of enzyme from Bakers' yeast yielded an optimum of cell concentration of 40% wet w/v with 30% and 50% yielding lower values. This was more marked at agitator speed of 5.1m/s and much less for 10m/s. Working with a 20L horizontal mill Schutte *et. al.* (1983) showed that the effect of cell concentration was only evident at the lower agitation speeds. The effect of viscosity on the power input in a 0.6L Dyno-mill was the explanation for the increase in the disruption rate as cell concentration increased (Marffy and Kula, 1974). This shows the difficulty in identifying individual factors affecting the disruption process and highlighting the interaction of the different process variables.

Schutte and Kula, 1990, presented a table (Table 1.4.) for the preferred operating conditions for the disruption of yeast and bacteria.

parameter	yeast	bacteria
Agitator tip speed	8-12m/s	10-15m/s
Feed flow	10-15 times chamber vol./hour	2.5-7.5 times chamber vol./hour
Cell concentration	10-15%DCW	10-15%DCW
Size of glass beads	0.4-0.7mm dia.	0.2-0.5mm dia.
Bead loading	80-85% of free chamber vol.	85% of free chamber vol.
Capacity	4-10kg/h/L chamber vol.	2-6kg/h/L chamber vol.

Table 1.4. Preferred operating conditions for the disruption of yeast and bacteria in a bead mill.

High Pressure Homogeniser

High pressure homogenisation is the most widely used method for large scale cell disruption (Scawen *et. al.*, 1980) with probably the Manton Gaulin APV type homogeniser being most commonly employed (Chisti and Moo-Young, 1986). Commercial homogenisers are only differentiated by their capacity, type of homogenising valve, operating pressure range, drive mechanism and number of pistons (Hetherington *et. al.*, 1971; Keshavarz-Moore, 1990).

In principle, a positive displacement piston pump with one or more plungers delivers the cell suspension into a valve assembly. A diagram of the valve assembly is given in Figure 1.5.

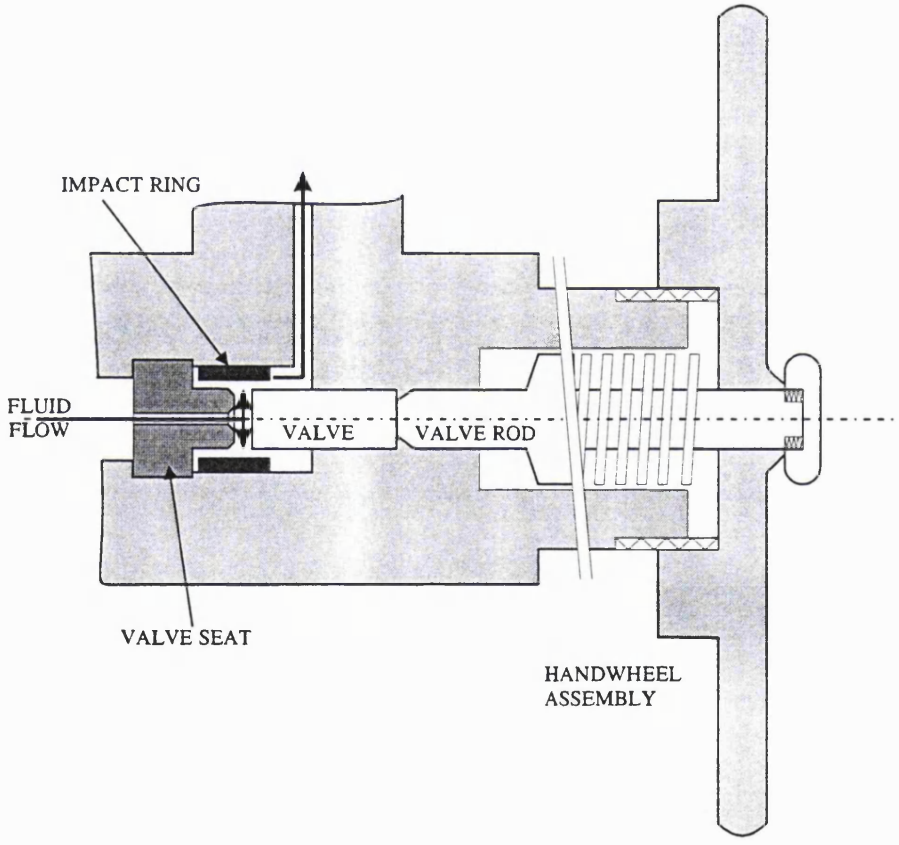


Figure 1.5. Schematic of an APV Manton-Gaulin high pressure homogeniser valve assembly.

During discharge the cell suspension passes between the valve and its seat and impinges on an impact ring. The discharge pressure is controlled manually by a

spring-loaded valve rod or by an automatic hydraulic regulator (Schutte and Kula, 1990).

High pressure homogenises have been used since before the turn of the century in the processing of milk (Keshavarz-Moore, 1990). With time, many modifications have been made with the main emphasis on the homogenising valve design. Knife-edged, flat, tapered, grooved and, tapered and grooved valves have emerged in the quest for efficient and low energy-consuming homogenisation.

Disruption within these devices has generally been found to be a first order process with respect to the number of passes through the valve, with the rate constant being a function of temperature and pressure (Brookman, 1974; Dunnill and Lilly, 1975; Gray *et. al.*, 1972; Hetherington *et. al.*, 1971).

Hetherington *et. al.* (1971) studied the effects of process variables such as operating pressure, cell concentration, number of passes through the homogeniser, and the temperature on the performance of the homogeniser (Manton Gaulin APV high pressure homogeniser), processing Bakers' yeast up to a pressure of 500 bar (50 MPa). They represented the disruption process as a first order kinetics using the equation.

$$\frac{dR}{dN} = k \,.\, (R_m - R)$$

$$\ln\left(\frac{R_m}{R_m - R}\right) = k \,.\, N \qquad \text{Equation 2}$$

where k is the dimensionless disruption rate constant, N is the number of passes through the homogeniser valve, R is the protein release with R_m being the maximum obtainable protein release.

The pressure dependency of the process can be determined by introducing a second disruption rate constant in the following empirical equation.

$$k = K \,.\, P^{\,a}$$

$$\ln\left(\frac{R_m}{R_m - R}\right) = K \,.\, N \,.\, P^{\,a} \qquad \text{Equation 3}$$

K is the dimensioned disruption rate constant which is a function of temperature and possibly of cell concentration. The exponent, a, on the operating pressure term, P, is a function of cell type .

The exponent, a, on the pressure term, was reported to have a value of 2.9 for Bakers' yeast (Hetherington *et. al.*, 1971) and 2.2 for the release of β-galactosidase from *E. coli* mutant (Gray *et. al.*, 1972). Hetherington *et. al.* (1971) based much of their calculations of exponent, a, on continuous flow data. Although it is mathematically possible to convert from recycle times to discrete passes and obtain non integer values for N, in practice this is not strictly valid.

Recalculations based on the data Hetherington *et. al.* (1971) gives values for the pressure exponent to be 2.71 when operating at 5°C and 2.64 when operating at 30°C (both lines having the coefficient of linear regression, r, greater than 0.99).

Hetherington *et. al.* (1971) found that this exponent does not vary between pressures of 100 to 300 bar, for Bakers' yeast, but decreases for pressures above 300 bar and cell concentrations over 210 kg dry weight / m^3 and also that kinetics were no longer first order. Dunnill and Lilly (1975) also reported a decrease in a for Bakers' yeast at operating pressures above 700 bar. This was attributed to the decrease in efficiency of the large scale homogeniser at the higher operating pressures. Recent cell disruption work is presented by Kula *et. al.* (1990) on three different samples of commercial yeast with operating pressures of up to 700 bar. They calculated values for the pressure exponent, a, to be significantly lower than that published earlier. In addition the values for the three samples were different (1.86, 1.88 and 2.08). They tentatively attributed this to possible biological factors. Engler and Robinson (1981a) have suggested that the pressure dependency may not be constant over an extremely wide range of operating pressures.

Engler and Robinson (1981a) did however, confirm the first order relationship for the disruption of *Candida utilis* in a slightly different high pressure flow device operating between pressures of 500 and 1250 bar. The device used was capable of operating at 3000 bar employing a high velocity jet of up to 150mL of cell suspension through an 80μm nozzle to impact onto a stationary surface. They indicated this impingement on a stationary surface is the major cause of cell disruption.

More recent work by Keshavarz *et. al.* (1990b) provided evidence with a detailed study using Bakers' yeast, to confirm this. They investigated the effect of impact

distance and valve geometry (Figure 1.5). Increased disruption was achieved with a smaller impact distance and also with a decreased valve gap width (i.e. the distance between the valve rod and the valve seat).

Of the five geometries studied (Figure 1.6.) they found that at an operating pressure of 460 bar the "knife edge"(KE) valve configuration gave the best disruption results. This was followed by "cell disruption" (CD) and "cell rupture"(CR) valve configurations which gave similar results and then by "cell rupture with flat valve rod" (CRF) configuration and lastly the "flat valve" (FV) valve configuration (Figure 1.6.).

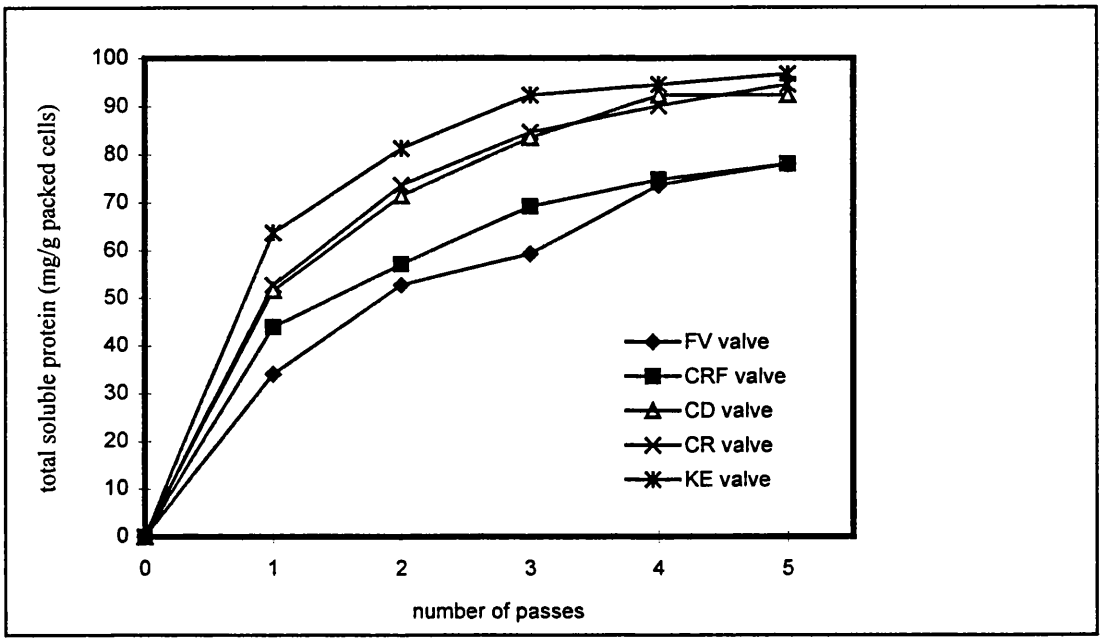


Figure 1.6. Extent of disruption using five valve units at an operating pressure of 460 bar (redrawn from Keshavarz-Moore *et. al.*, 1990b)

These results corresponded to increasing gap width (12.55μm to 21.61μm), decreasing entry and exit velocities, and decreasing mean velocity gradient from the KE to the FV valve configurations.

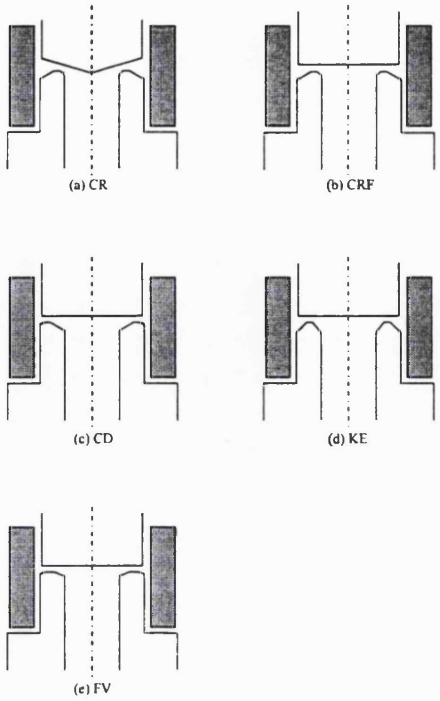


Figure 1.7. Details of the five valves used by Keshavarz-Moore *et. al.*, 1990b

Kula *et. al.* (1990) presented single pass *S. cerevisiae* disruption data for the four valve configurations (CD, CR, CRF and FV configurations) (see Table 1.5.). They employed an APV Gaulin high pressure homogeniser model MC4 TBX operating at 700 bar and a feed flow rate of 150L/h.

valve type	protein release	G6PDH release
CD	89.1%	90.3%
CR	86.4%	80.4%
CRF	79.4%	75.2%
FV	66.2%	63.5%

Table 1.5. The percentage protein and G6PDH activity released using different valve types. Disruption of *S. cerevisiae* in a high pressure homogeniser (single pass, 150L/h, 700 bar). (Kula *et. al.*, 1990).

To analyse the significance of these results, the difference within the valve geometries is noted, the underlying design features are examined, and their impact on the disruption process postulated upon.

The only difference between the CR and CRF valve configuration is the valve rod which is coned in the CR unit and flat in the CRF unit. This has given rise to the above disruption differences. Now, operating with the same feed flow rate (150L/h) the valve gap (*i.e.* the gap between the valve rod and the valve seat) must be smaller in the CR unit to give the same pressure reading as the CRF unit. In addition there will be a reduced shear area (between valve rod and valve seat) in the CR unit. Overall, this should result in greater impact and less shear effects in the CR unit and *visa versa* in the CRF unit causing the cell disruption.

Within the CD valve configuration the valve gap will be smaller than either CR or CRF units to achieve same pressure at the same feed flow rate. Thus the cell suspension velocity must increase through the valve gap. Keshavarz-Moore *et. al.*, 1990b, calculated the gap widths for CD and CRF valve units as 15.15μm and 19.10μm respectively (operating pressure at 460bar). In addition, there is less travel and so less energy loss on exit to the impact ring. They presented a more significant result in the release of G6PDH over a range of operating pressures. They found comparable release results when the feed flow rate was doubled to 300L/h (Figure 1.8.). In this case, the valve gap must be greater at the same pressure. This then clearly shows that impact and impact alone is the major factor determining disruption.

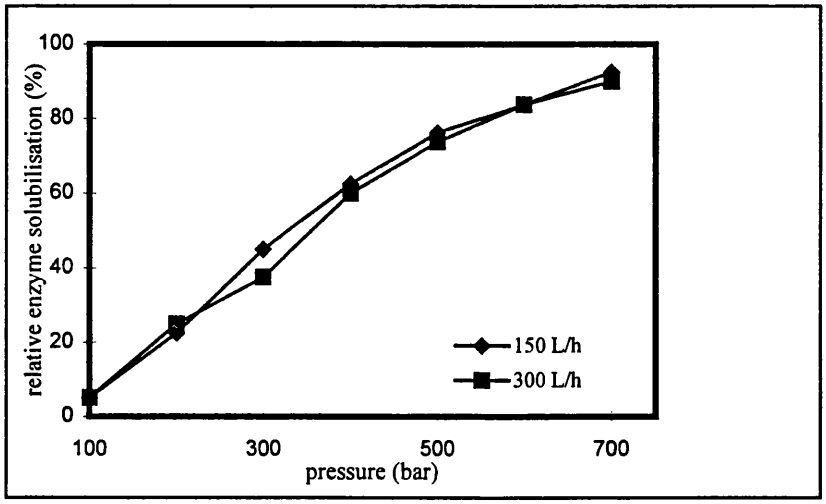


Figure 1.8. Solubilisation of glucose-6-phosphate dehydrogenase as a function of operating pressure and volumetric flowrate through the homogeniser (redrawn from Kula *et. al.*, 1990)

Keshavarz *et. al.* (1990b) demonstrated the influence of the impact ring by operating the CD valve configuration with and without the impact ring. They showed that at an operating pressure of 460 bar the absence of the impact ring resulted in a decrease in protein release of over 50 g/kg packed cell on the first pass (Figure 1.9.).

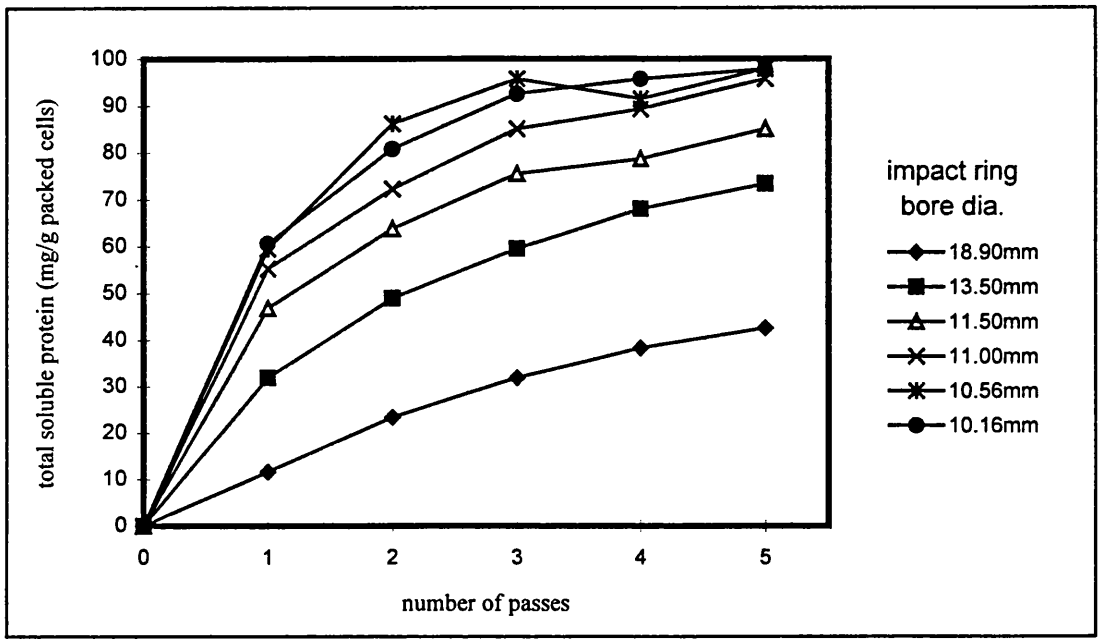


Figure 1.9. Extent of disruption efficiencies using a CD valve unit with different impact rings. Operating pressure 460 bar (redrawn from Keshavarz-Moore *et. al.*, 1990b).

At variance with the simple first order relationship, it has been reported that the maximum amount of protein released R_m during the disruption process increased with increased pressure (Engler, 1985; Whitworth, 1974b). The dependence of R_m values in the study of *C. lipolytica* by Whitworth (1974b) may be attributed to firstly, that at higher pressures micronisation of the cell debris results in the release of intracellular material such as insoluble complex protein and amino acids (Limon-Lason *et. al.*, 1979). Secondly, *C. lipolytica* grown in a hydrocarbon culture medium has a thicker cytoplasmic membrane (Keshavarz, 1990). Thus, complete rupture and protein release are affected at lower pressures giving rise to the apparent changes in R_m.

The rate of release of a number of enzymes from Bakers' yeast were found to be first order similar to protein release (Follows *et. al.*, 1971), but the rate at which a

particular enzymes were released was not the same. When compared with the rate of release of intracellular total soluble protein, enzymes located in the periplasmic space were released more quickly, followed by those in the cell cytoplasm and then by those associated with the mitochondria.

Disruption of filamentous organisms in high pressure homogenisers.

In a comparative study Zetalaki (1969) found that of the methods compared, high pressure homogenisation resulted in the lowest activity yield of glucose oxidase from *A. niger.* Added to this, he found that the valve unit became blocked even at very dilute concentrations. When viewed under the microscope, long and unbroken hyphea were observed. Although high pressure homogenisation was considered unsuitable for the disruption of filamentous micro-organisms, It must however be noted that crucial operating parameters such as pressure and number of passes were not given.

Lilly and Dunnill (1969) compared the disruption of *S. cerevisiae*, *A. niger* and a bacteria in the APV Manton Gaulin high pressure homogeniser operating at 350 bar and 800mL/minute on a continuous basis. They showed that the disruption profile with time of the yeast was similar to that of *A. niger.*

Lew, 1991, used the similarity in the data to assume that the disruption was first order so as to assess the kinetics in more detail in terms of the disruption rate constant, k. He makes the conversion from recycle times to discrete passes based on the curve provided by Hetherington *et. al.* (1971). Lew, 1991, graphically showed that the disruption rate constant for the yeast was greater than that for *A. niger.* This showed that *A. niger* was slightly more difficult to disrupt than the yeast (although he in fact concluded the reverse).

The disruption of *Rhizopus nigricans* was carried out by Thomas, 1988. He found that the release of membrane bound progesterone 11α hydroxylase was slower than the total soluble protein release. This was expected given the work by Follows *et. al.* (1971). First order kinetics were confirmed for the release of total soluble protein with a weak dependency on pressure (a=0.57). Thomas, 1988, also found, Whitworth (1974b) did for *C. lipolytica*, that the maximum protein released (R_m) was a function of pressure that is $R_m = 0.11\ P^{0.14}$. However, the maximum enzyme release was not a function of pressure suggesting a phenomenon first observed by Limon-Lason, 1979, and later confirmed by Keshavarz *et. al.*, 1990a. Their work found similar kinetics (total soluble protein as a weak function of

pressure) and also that R_m varied with pressure. Using ADH as an independent marker of cell disruption they were able to show no variations in the maximum activity levels for pressures between 100 and 500 bar indicating complete release of intracellular material. Thus they attributed the variations in R_m with pressure to micronisation of cell debris releasing insoluble complexes proteins and other compounds giving a positive result on the protein assay.

They also reported blockage of the homogenising valve for cell concentrations beyond 22g/L dry cell weight (DCW) for the filamentous cells and beyond 30g/L for pelletted cells. The filamentous cells formed an interlocked network structure, whereas pelletted cells form a homogeneous suspension much like unicellular organisms.

Counter-current Two Jet impingement

In this system cell disruption is achieved when two jets of cell suspension are coaxially directed to impinge on each other at high relative velocities. The impingement of the streaming jets in the stagnation area creates high turbulence intensity and therefore high turbulent shear forces occur which cause disintegration of the micro-organisms (Kramer and Bomberg, 1990). A need thus exists to achieve maximum conversion of the pressure energy into kinetic energy in order to get maximum cell suspension velocities exiting the nozzles giving a maximum relative velocity for the cell suspension. With this technique the pressure before the nozzle is only 25-30% of that needed in high pressure homogenisers for comparable disruption results (Kramer and Bomberg, 1988a).

Kramer and Bomberg (1988a) achieved sixty percent disruption of *E. coli* cells in a single pass at system pressure of 140 bar corresponding to 180 m/s relative velocity and 95% disruption after four passes.

Studies of product stability using model enzymes showed that after 10 or even 15 passes product deterioration is negligible.

Since the counter-current impingement concept does not involve solid impingement areas as in high pressure homogenisers, Kramer and Brebbermann (1990) expected denaturation of released enzymes and even inclusion bodies due to mechanical and thermal stress to be lower than in established mechanical procedures.

Bomberg *et. al.* (1988) modified Hetherington's relationship for high pressure homogenisers by describing the energy input (pressure) term by a term based on kinetic energy and presented the dramatic velocity dependency, verified up to 195 m/s, of cell disruption with this method.

They extrapolated the information with the *proviso* of dependency remaining constant to give achievable 95% cell disruption in a single pass at about 270 m/s (equivalent to 190 bar) for *E. coli* and 330 m/s (equivalent to 210 bar) for *S. cerevisiae*. These pressures are much lower in comparison with the APV Manton-Gaulin high pressure homogeniser (discussed earlier) where 3 or 4 passes at 500 bar are required to achieve close to 100% disruption of yeast cells.

Microfluidizer

The Microfluidizer is an apparatus suitable for cell disintegration (White and Marcus, 1988). This relatively new development is based on the submerged jet principle in which the process fluid is split into two streams and these then interact at high velocities in precisely defined micro-channels all within the sealed interaction chamber (Keshavarz-Moore, 1990; White and Marcus, 1988).

A preliminary comparison of the extent of disruption of yeast and bacteria in a Microfluidizer and in other mechanical devices show that the former is not suitable for the disruption of yeast. Ninety percent cell disruption was achieved after 30 passes at 600 bar. Whereas with *B. subtilis* breakage as compared with that achieved in APV Manton-Gaulin high pressure homogeniser (discussed earlier) was similar, although levels of protein release were different (Seva *et. al.*, 1986). More recent work shows that similar disruption levels may be obtained at lower pressures and after fewer passes.

Baldwin and Robinson (1990) disrupted yeast cell suspensions ranging in concentration from 0.6 to 15 g/L dry weight in a Microfluidizer (M110T fitted with a heavy duty pump). After 4 passes at 950 bar only 32% disruption occurred. In studies with *E. coli*, recombinant and non-recombinant strains, (employing the same device) a high disruption level of 95% to 98% for the recombinant strain was achieved in two or three passes at 950 bar (Sauer *et. al.*, 1988).

In addition to the high dependency on both pressure and number of passes, which is comparable with findings with high pressure homogenisers, Sauer *et. al.* (1988)

found that the degree of disruption was also dependent on the specific growth rate of the cells during the upstream fermentation stage and on the initial cell concentration of the homogeniser feed.

Sonication

Sonication is one of the most widely used laboratory methods of cell disruption (Engler, 1985; Edebo, 1969; Lambert, 1983).

Ultrasonic disintegraters generally operate at frequencies of 15 to 25 kHz, beyond the range of human hearing, with cell disruption resulting from cavitational effects (Engler, 1985).

As the acoustic power is increased, microbubbles begin to form at various nucleation sites and these then grow to about 10 microns during the rarefaction phase of the sound wave. Then in the compression phase, the bubble content is compressed to a minimum radius where the bubble implodes violently releasing a shock wave of several thousand atmospheres which propagates through the medium. This phenomenon is termed cavitation (Aiba *et. al.*, 1973; Chisti and Moo-Young, 1986; Doulah, 1977; White and Marcus, 1988).

Most of the ultrasonic energy absorbed into the cell suspension ultimately appears as heat, making good temperature control essential (Chisti and Moo-Young, 1986) and so along with the violent shock wave, high localised temperatures occur and free radicals form in the solution (Aiba *et. al.*, 1973; Edebo, 1969). Although chemical effects are known to occur (Palma and Bucalon, 1987), they do not affect the cell disintegration which arises due to mechanical effects of cavitation (Doulah, 1977; Engler, 1985).

Doulah (1977) has suggested cell disintegration is the result of shear stresses developed by viscous dissipative eddies arising from shock waves produced by imploding cavitation bubbles. Doulah (1978) further suggested that ultrasonic disruption follows the statistical theory of reliability, showing that wear-out damage to cell walls is caused by applied stresses. Nappiras and Hughes (1964) had much earlier suggested that rapid oscillations of the bubbles are responsible for cell rupture rather than damage from shock wave or free radicals. Mechanical forces that can cause cell disintegration could arise prior to the collapse of the bubbles. Micro-streaming near the bubble surface generates stresses strong enough to cause cell

disruption (Hughes and Cunningham, 1963; Doulah, 1977). Wase and Patel (1985) reported that the principal determinant of susceptibility to ultrasonic cell disruption was the mean cell volume, irrespective of culture conditions. However, these findings may be the result of changes occurring due to varying fermentation conditions used to obtain the different cell volumes, and not the cell volume itself (Keshavarz-Moore, 1990).

Kracke-Helm *et. al.*, 1991, sonicated samples of *E. coli* discontinuously at 4°C (100W, frequency not given) and found that for sonication times below 3 minutes deactivation in the activity of β-galactosidase was observed.

Hughes (1961, and 1963) and Nappiras and Hughes (1964) applied this technique to bacterial suspensions to disrupt both the cell wall and the cell membrane. They found rods to break more easily than cocci, and gram-negative cells to disintegrate more readily more readily than gram-positive ones. Some bacteria were very resistant to ultrasonic disruption and in addition, membrane-bound enzymes required considerable exposure to achieve solubilisation (Melling and Phillips, 1975). Zetalaki (1969) found ultrasound not as effective for disrupting fungal cell.

Ultrasound is limited to small scale operation due to the difficulties in transmitting sufficient power to larger volumes (Keshavarz-Moore, 1990; Lambert, 1983; Melling and Phillips, 1975) as increasing probe size decreases sonication energy (White and Marcus, 1988) and in dissipating the heat generated (Aiba *et. al.*, 1973; Lilly and Dunnill, 1969).

Denaturation of enzymes occurs (Aiba *et. al.*, 1973; Edebo, 1969; Engler, 1985; Stube and Spohn, 1994) and is compounded by associated problems in downstream processing of the fine cell debris produced (Aiba *et. al.*, 1973; Chisti and Moo-Young, 1986).

The optimal conditions for sonication are different for various organisms and depend on whether full or partial cell breakage is aimed at (White and Marcus, 1988). Dakabu (1976) found conditions under which reproducible cell survival curves were obtained for *E. coli*. He also found a clear relationship between the rate of inactivation and the peak acoustic intensity existed below which no inactivation was observed. With increase in initial cell concentration Dakabu (1976) noted a reduction in inactivation rate. However, it has been reported that the protein release constant, k, for Brewers' yeast (sonicated at powers of up to 200 acoustic Watts at 20 kHz) has been shown to be independent of cell concentration up to 60g/100mL

and proportional to the input power from 60 to 195 acoustic Watts. Maximum throughput is equivalent to 60% protein release from 1.44 kg wet weight yeast/hour (Wang *et. al.*, 1979).

The efficiency of ultrasonic treatment depends upon various parameters including pH, temperature, ionic strength of the suspending medium as well as the time of exposure. The selection of set conditions, an empirical choice will vary with individual organisms (Melling and Phillips, 1975; White and Marcus, 1988). From the prospective of the equipment, repeatability of disruption results depends largely on maintaining constant geometry of vessel and probe (Neppires and Hughes, 1964).

Recently Stube and Spohn, 1994, reported the promising results from an ultrasonic device incorporating a flow-through cell with a 500μL sample volume. They tested the device with a view to its inclusion in an on-line fermentation monitoring configuration. The FDH activity and protein results yielded a plateau after 6 minutes at 93%.

Although, they found a significant level of enzyme deactivation, the most limiting feature of the ultrasonic technique was the stability of the sonitrobes (the probes). They reported an increase in the rate of decrease of power input with decreasing sonitrobe tip diameters and drop in ultrasonic power input from 550W/cm^2 to 50W/cm^2 within an operation time of 40 minutes. With a processing frequency of six every hour and disintegration time of three minutes, they were forced to change the sonitrobe after only six hours.

Osmotic Shock

Physical lysis for micro-organisms has not found wide application on a large scale (Wang *et. al.*, 1979).

Osmotic shock is one of the gentlest methods of cell disruption (Engler, 1985; Keshavarz-Moore, 1990) in which cells are allowed to equilibrate briefly in a medium of high osmotic pressure. When the medium is suddenly diluted, water enters the cell rapidly increasing the hydrostatic pressure which causes disruption. Most micro-organisms however, cannot be disrupted by osmotic shock unless their cell walls are first weakened by for example enzymatic attack or by being grown

under conditions of inhibited cell wall synthesis (Hughes *et. al.*, 1971). Often this method is used in conjunctions with other techniques (Wimpenny, 1967).

Freezing & Thawing, and Heating & Drying

Repeated cycles of freezing and thawing, and heating and drying have been used successfully for the disintegration of microbes (Edebo, 1969; Hughes *et. al.*, 1971) although only low yields are achieved (Dunnill and Lilly, 1975; Engler, 1985) and denaturation of proteins and other components may result (Engler, 1985).

Gas Decompression

Gas decompression or depressurisation is a cell disruption technique in which a batch disrupter containing the cell suspension is pressurised by an inert gas (*e.g.* nitrogen) at high pressure until equilibrium is achieved (Dave and Hong, 1986; Keshavarz-Moore, 1990). Then suddenly, the pressure is released rupturing cell walls and membranes due to the high internal and external cellular pressure difference . The cycle can so be repeated.

The low disruption efficiency (Edebo, 1969) of this gentle technique makes it only suitable for fragile cells and is itself also difficult to scale up (Keshavarz-Moore, 1990).

2. Materials and Methods

2.1. The micro-organisms studied

The main micro-organisms studied were *Pseudomonas putida* ML2, *Escherichia coli* (K12) and *Saccharomyces cerevisiae* (GB4918) (packed yeast was also bought in). *Rhodococcus erythropolis* were briefly studied.

The fermentations carried out were of *P. putida* ML2 and *E coli*. The other organisms and strains were obtained from fermentations carried out by colleagues. Acknowledgement is made to S. Khan, Advance Centre for Biochemical Engineering, University College London, for allowing the use of samples from some of the yeast fermentations

2.1.1. *Pseudomonas putida* ML2

The micro-organism *Pseudomonas putida* ML2 was supplied by Dr Geary, Shell Research limited, Sittingbourne, UK The maintenance of the organism was carried out by Lynch (1995) such that stock cultures were maintained on nutrient agar plates at 4°C and sub cultured weekly.

2.1.2. *Escherichia coli*

The *E. coli* K12 cells were taken from frozen glycerol stocks

A 200mL solution of nutrient broth was added to a 500mL conical flask. Three such flasks were prepared. These along with gilson pipette tips were sterilised in-situ by autoclaving at 121°C for 20 minutes.

The flasks were cooled to room temperature and to allow aeration placed in a New Brunswick (Eddison, New Jersey, USA) incubator for 10 minutes.

A single 5mL universal containing a frozen glycerol stock was defrosted and observing aseptic technique, 1mL was transferred to each shake flask.

The shakes flask were placed in the incubator and shaken at 250 r.p.m. and 37°C for 24 hours.

The samples from the shake flasks were viewed under the microscope (with Dave Pollard, *E. coli* rods were observed with no signs of contamination) and then aseptically transferred to the already prepared nutrient agar plates (10 plates were streaked). These were then placed into a static incubator at 26°C for 24 hours.

The agar plates were then bagged and stored in the refrigerator at 4°C. These plate were then used to inoculate shake flasks for fermentation inoculation. From these fermenter inoculum shake flasks further agar plates were streaked.

2.1.3. *Saccharomyces cerevisiae*

The organism *Saccharomyces cerevisiae* (GB4918) was supplied by the Distillers Co. Ltd., Sutton, Surrey, UK and maintained by S. Khan.

2.1.4. *Rhodococcus erythropolis*

The propagation and maintenance of the micro organism *Rhodococcus erythropolis* was carried out by N. Rosen.

2.2. Growth of micro-organism in shake flask

2.2.1. *Pseudomonas putida* ML2

A 100mL solution of shake flask medium (see Table 2.1) was added to a 500mL conical flask (SF1). This was sterilised in-situ along with a super-seal and gilson pipette tips by autoclaving at 121°C for 20 minutes.

The flask was cooled to room temperature and placed in the incubator for 10 minutes. This allows the aeration of the medium before the super-seals are used.

Observing aseptic technique, the stock culture from an agar plate was transferred into the shake flasks. Then 100μL of benzene was added as the carbon source and the flask sealed immediately with the super-seal. Benzene was used to prevent the growth of other strains of *P. putida* which did not possess the ability to perform biotransformations.

The shake flask was placed in the incubator at 250 r.p.m. and 30°C for 24 hours.

After 24 hours, a 500mL solution of shake flask medium (Table 2.1.) was added to a 2L shake flask (SF2). This was then sterilised *in situ* along with a fructose solution (60% w/v.). SF2 was then cooled to room temperature.

Observing aseptic technique, 20mL of grown culture (SF1) and 20mL of the fructose solution were added to SF2.

The shake flask was placed in the incubator at 250 r.p.m. and 30°C for 24 hours.

Chemical	Formula	weight
Ammonium sulphate	$(NH_4)_2SO_4$	1.0g
Magnesium sulphate	$MgSO_4.7H_2O$	0.2g
Iron chloride	$FeCl_2$	0.016g
Disodium hydrogen orthophosphate	Na_2HPO_4	3.0g
Potassium dihydrogen orthophosphate	KH_2PO_4	3.0g
Calcium chloride	$CaCl_2.2H_2O$	0.015g
Trace elements solution (see Table 2.2.)		2.0mL
RO water to		1.0L

pH pre-inoculation 6.8

Table 2.1. Shake flask media for *P. putida* ML2.

Chemical Name	Formula	weight
Calcium chloride	$CaCl_2.2H_2O$	0.66g
Zinc sulphate	$ZnSO_4.7H_2O$	0.18g
Copper sulphate	$CuSO_4.5H_2O$	0.15g
Manganese sulphate	$MnSO_4.4H_2O$	0.15g
Cobalt chloride	$CoCl_2.6H_2O$	0.18g
Boric acid	$H_3BO_3.H_2O$	0.1g
	$Na_2MoO_4.2H_2O$	0.3g
RO water to	H_2O	1.0L

Table 2.2. Trace elements solution for *P. putida* ML2.

2.2.2. *Escherichia coli*

A 200mL solution of nutrient broth was added to a 500mL conical flask. This was sterilised in-situ by autoclaving at 121°C for 20 minutes.

The flask was cooled to room temperature and placed in the incubator for 10 minutes.

Observing aseptic technique, the stock culture from an agar plate was transferred into the shake flask.

The shake flask was placed in the incubator and shaken at 250 r.p.m. and 37°C for 24 hours.

2.3. Growth of micro-organisms in fermenters

The fermentations of *P. putida* ML2, *S. cerevisiae* and *E. coli* were carried out in 7L and 20L fermenters (LH Eng., Bucks, UK) with working volumes of 5L and 10L respectively.

2.3.1. Fermenter instrumentation and ancillaries

Both fermenters were equipped with Ingold steam sterilisable pH and dissolved oxygen tension (DOT) electrodes (Life Science Labs., Beds., UK). Fermenter exit gases were recorded and analysed using a microprocessor controlled VG MM-80 mass spectrometer (VG Gas Analysis, Cheshire, UK). It was possible to monitor the physical parameters and compute the oxygen uptake rate (OUR), the carbon evolution rate (CER), throughout the fermentation via a link-up to a minicomputer. A photograph of the 20L fermenter is shown in Figure 2.1

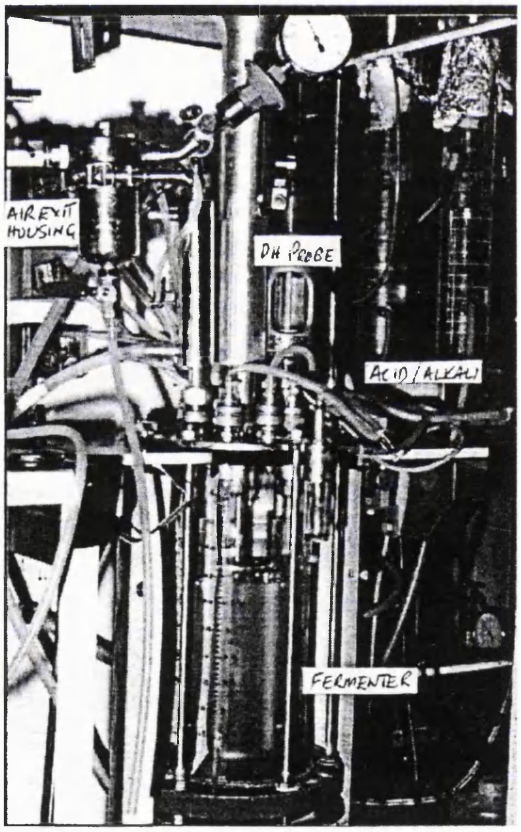


Figure 2.1 A photograph showing the fermenter used during the course of the projects.

2.3.2. Sterilisation

All the media components were sterilised *in situ*, at 121°C at 1bar pressure for 20 minutes, except for the sugars which was dissolved in deionised water, sterilised in an autoclave, and then aseptically transferred into the fermenter. The fermenter trace elements solution was filter-sterilised.

The pH regulating solutions, sulphuric acid solution (2M) was sterilised as the fructose solution above along with the deionised water to which was added the 98% ammonia solution to attain a 5M ammonia solution.

2.3.3. Conditions of Growth

This section will cover the media used for the *P. putida* ML2 and *E. coli* fermentations, the fermenter controlled parameters and the harvesting or sampling from the fermentations

The fermenter medium

P. putida ML2

Details of the fermenter medium used in the *P. putida* ML2 fermentations are given below in Table 2.3., and the fermenter trace elements solution are detailed in Table 2.4. The carbon source was fructose 30g/L media.

Media components	Formula	(/ L)
Ammonium sulphate	$(NH_4)_2SO_4$	1.68g
Dipotassium hydrogen orthophosphate	K_2HPO_4	1.74g
Potassium dihydrogen orthophosphate	KH_2PO_4	1.36g
antifoam (polypropylene glycol)		0.6mL

Table 2.3. The fermenter medium for *P. putida* ML2 growth.

Fermenter trace element components	Formula	
Magnesium sulphate	$MgSO_4.7H_2O$	0.02g
Iron sulphate	$FeSO_4.7H_2O$	0.0016g
Trace elements solution (see Table 2.2)		1.54mL
RO water to	H_2O	2.0mL

Table 2.4. The fermenter trace elements solution

10mL and 20mL of the fermenter trace elements solution was used in the 5L and 10L working volume fermentations respectively.

E. coli

Details of the fermenter medium for the *E. coli* fermentations are detailed below in Table 2.5.

Media components	Formula	(/ L)
Ammonium sulphate	$(NH_4)_2SO_4$	10.0g
Sodium chloride	NaCl	5.0g
Iron sulphate	$FeSO_4.7H_2O$	0.2g
Citric acid		0.2g
Dipotassium hydrogen orthophosphate	K_2HPO_4	0.216g
Potassium dihydrogen orthophosphate	KH_2PO_4	0.64g
antifoam (PPG)		1.0mL

Table 2.5. The fermenter medium for *E. coli* growth.

Magnesium sulphate, 2.0g, was sterilised in 10 mL of RO water and aseptically introduced to the fermenter. 10 mL of trace element solution (see Table 2.2.) was sterile filtered

S. cerevisiae

Details of the fermenter medium, vitamins and salts used in the *S. cerevisiae* fermentations are detailed below in Tables 2.6., 2.7. and 2.8.

Media components	Formula	(/ L)
Ammonium sulphate	$(NH_4)_2SO_4$	17.8g
Sodium chloride	NaCl	0.1
Dipotassium hydrogen orthophosphate	K_2HPO_4	5.7g
antifoam (PPG)		1.0mL

Table 2.6. The fermenter medium for *S. cerevisiae* growth.

Vitamins	(mg/L)
D-biotin	1.0
Calcium pantothenate	20
Nicotinic acid	15
Thiamine HCl	4
Pyridoxine HCl	10

Table 2.7. The fermenter vitamins used for *S. cerevisiae* fermentation.

Glucose and Salts	Formula	(/L)
Iron chloride	$FeCl_3.6H_2O$	100mg
Zinc sulphate	$ZnSO_4.7H_2O$	30mg
Boric acid	H_3BO_4	15mg
Cobalt chloride	$CaCl_2.6H_2O$	5.6mg
	$NaMoO_4.2H_2O$	5mg
Potassium iodide	KI	2mg
Copper sulphate	$CuSO_4.5H_2O$	0.8mg
Manganese sulphate	$MnSO_4.H_2O$	32mg
D+ glucose		30g
Meso-inosotol		0.6g
EDTA Na+2 salt		0.243g
Calcium chloride	$CaCl_2.2H_2O$	0.15

Table 2.8. The glucose and salts used for *S. cerevisiae* fermentation.

R. erythropolis

Details of the fermenter medium for the *R. erythropolis* fermentations are detailed below in Table 2.9.

Media components	Formula	(/ L)
Sodium hydrogen orthophosphate	Na_2HPO_4	2.1g
Dipotassium dihydrogen orthophosphate	KH_2PO_4	1.5g
Sodium hydrogen carbonate	$NaHCO_4$	0.5g
Magnesium sulphate	$MgSO_4.7H_2O$	0.2g
Ammonium sulphate	$(NH_4)_2SO_4$	0.5g
yeast extract		6.0g
glycerol		6.0g
Trace elements solution		5mL

Table 2.9. The fermenter medium for *R. erythropolis* growth.

Controlled parameters

P. putida ML2

The following physical parameters could be controlled on line

i) The pH of the media was controlled at 6.8 using 2M sulphuric acid and 5M ammonium solutions.

ii) The temperature was maintained at 30°C by means of a heating element, and cooling coils running cold tap water).

iii) Dissolved oxygen tension (DOT) was not controlled during the fermentation

iv) Aeration: Oxygen was supplied to the vessel by pumping air through a 0.2μm pore air filter at 3.5L/min in the 5L and 7.0L/min for 10L working volume respectively.

v) Agitation: The fermenter medium was agitated by turbine impellers rotating at 500 r.p.m. and 1000 r.p.m. for the 7L and 20L fermenters respectively.

E. coli

All the *E. coli* fermentations were carried out in the 20L fermenter with a working volume of 10L and 11L. The controlled parameters for these and those of other micro-organism fermentations are given in Table 2.10.

Micro-organism	*P. putida* ML2	*E. coli*	*S. cerevisiae*	*R. erythropolis*
Parameter	Value			
pH	6.8	7.0	4.5	7.0
Temperature	30°C	37°C	28°C	30°C
Dissolved oxygen tension	not controlled			
Aeration (L/min)	3.5 & 7.0	10	7	10
Agitation (r.p.m.)	500 & 1000	1000	700	700

Table 2.10. The values for the controlled parameter in *P. putida* ML2, *E. coli*, *S. cerevisiae* and *R. erythropolis* fermentations

Sampling and harvesting of the micro-organisms

S. cerevisiae (Bakers' yeast)

Packed yeast

Blocks of yeast (1kg / block) were purchased from Distillers Co. Ltd., Sutton, Surrey, UK The blocks were stored at 4°C until required, usually within 7 to 10 days. The yeast was used well before the 'use by date' stamped by the suppliers.

Yeast fermentations

From the yeast fermentations the cell samples were taken from the fermenter via the sampling port directly into 22.5mL sterile universal bottles.

P. putida ML2

P. putida ML2 cell samples were taken from the fermenter via the sampling port directly into 22.5mL sterile universal bottles.

E. coli

E. coli cell samples were taken from the fermenter via the sampling port directly into 22.5mL sterile universal bottles.

R. erythropolis

R. erythropolis cell samples were taken from the fermenter via the sampling port directly into 22.5mL sterile universal bottles.

2.4. Preparation of micro-organism for disruption

In this section, the preparation of the micro-organism cell suspension for the disruption process is detailed. Samples were cooled on ice to below 12°C prior to disruption. In general, the samples taken during the course of fermentations were not treated in any way prior to disruption. Any cooling that took place was before and after homogenisation which formed the disruption stage.

Pre-treatment in the form of centrifugation and subsequent resuspension of the *R. erythropolis* cells was necessary. This was to prevent the denaturation of the product enzyme of interest in the chlorine-ion rich complex medium.

2.4.1. *Saccharomyces cerevisiae* , Packed yeast

Known amounts of packed Bakers' yeast were crumbled and suspended in a 100mM phosphate buffer at 4°C to give a range of concentrations (5-45% w/v packed weight). The suspension was kept on ice prior to disruption.

2.4.2. Alternative procedure for *Pseudomonas putida* ML2

For some of the early trials, the cells were harvested, centrifuged in Europa centrifuge for 30 minutes, stored for 24 hours, resuspended to a known concentration in phosphate buffer and then disrupted.

This procedure was rarely employed as on-line monitoring requirements can not by definition allow such a time delay to occur between harvesting and analysis.

2.4.3. *Rhodococcus erythropolis*

R. erythropolis cells samples were withdrawn from the fermenter via the sampling port. The sample was centrifuged for 20 minutes and then resuspended in glycine buffer prior to homogenisation.

2.5. Disruption of micro-organisms

Although use of a variety of devices was made, most of the cell disruption was carried out in the APV Gaulin Micron Lab 40 high pressure homogeniser.

The main features of the Micron Lab 40 which prompted its detailed investigation were:

1. Similar valve geometry as the pilot and industrial scale high pressure homogenisers

2. The change in operating scale; *i.e.* processing a sample volume of 40mL. The minimum volume processed with this type of device was 2-3L in the APV Lab 60. (The Rannie homogeniser is available with a minimum volume of 150-200mL)

3. The batch disruption process within the Micron Lab 40 is complete within 5 seconds

2.5.1 APV Gaulin Micron Lab 40 high pressure homogeniser.

Equipment

The Micron Lab 40 is commercially available (APV Gaulin GmbH, Lubeck, Germany). It is essentially a bench scale device operating in batch mode only, processing 40mL of cell suspension in a single pass at pressure in the range of 100 to 1600 bar. A photograph of the homogeniser is shown in its working environment in Figure 2.2.

As with some of the larger high pressure homogenisers a positive displacement piston pump with one delivers the cell suspension into a valve assembly. A diagram of the valve assembly is given in Figure 2.3. Photographs of the dismantled valve assembly are shown in Figure 2.3a.

During discharge the cell suspension passes between the valve and its seat and impinges on an impact ring. The discharge pressure is controlled by an automatic hydraulic regulator (the required pressure being dialled up on a digital display).

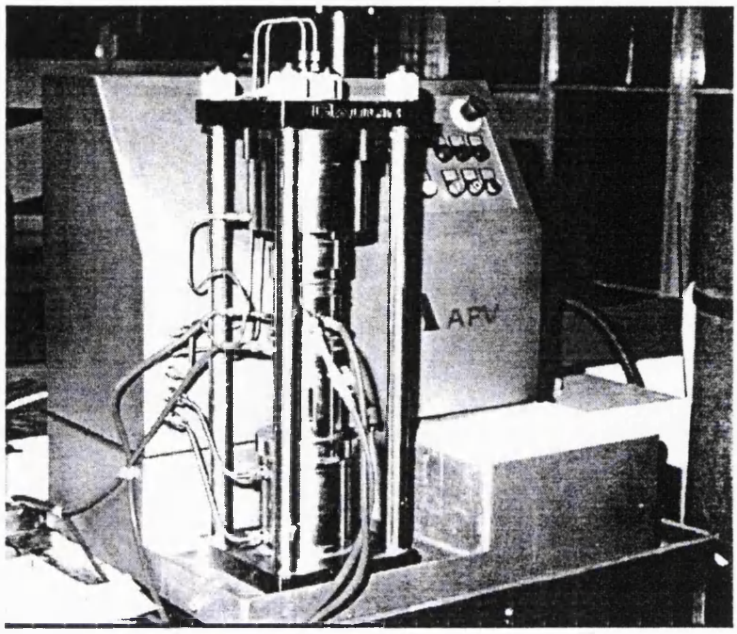


Figure 2.2. A photograph of the Micron Lab 40 high pressure homogeniser shown in its working environment.

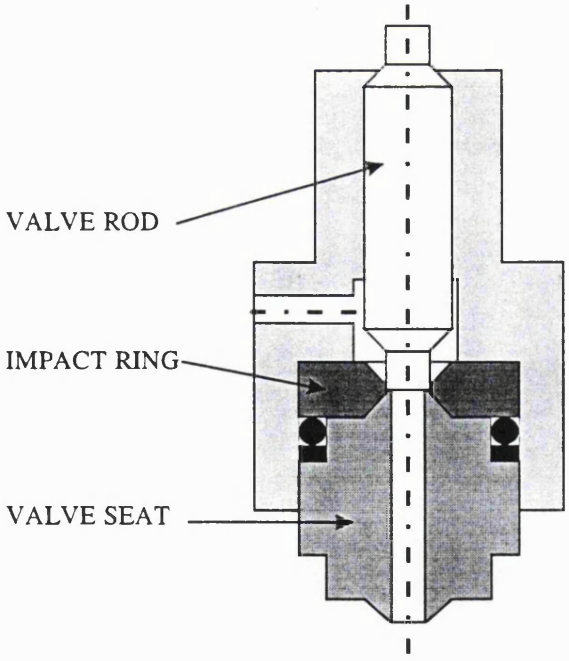


Figure 2.3. A diagram of the Micron Lab 40 valve assembly

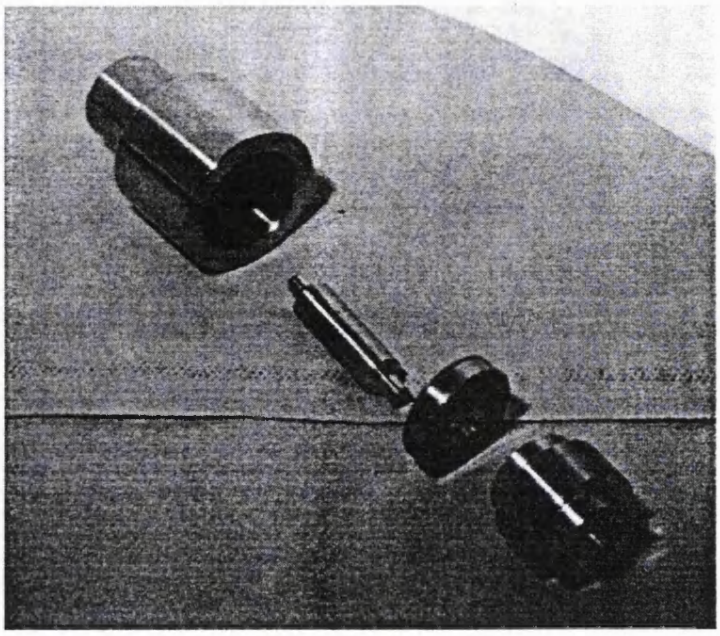


Figure 2.3a. Photograph of the dismantled Micron Lab 40 high pressure homogeniser valve assembly.

To counter the temperature rise during homogenisation (found to be 1°C per 100bar, Figure 3.6. Section 3.2.), cooling coils were installed around the sample chamber and the valve housing, with glycol at -5°C as the coolant. This allowed the process sample temperature to be kept below 12°C and the homogenate, the temperature of which can rise to above 20°C, to be rapidly cooled again to below 12°C.

The impact rings

Three different sized impact rings were employed within the Micron lab 40. All the rings were made of Stellite.

The impact ring internal diameters were 5.5mm (normally used), 7.5mm and 9.5mm. The size of the aperture is restricted to a maximum of 9.5mm because of safety consideration as the impact ring also serves as a spacer to restrict the movement of the valve seat causing the homogenising valve to travel through the valve housing.

During the normal operation of the Micron Lab 40, the impact ring with 5.5mm internal diameter was used. Where the other impact rings were used, this is specified. A photograph of the impact rings and the valve housing for the Micron Lab 40 high pressure homogeniser in shown in Figure 2.4.

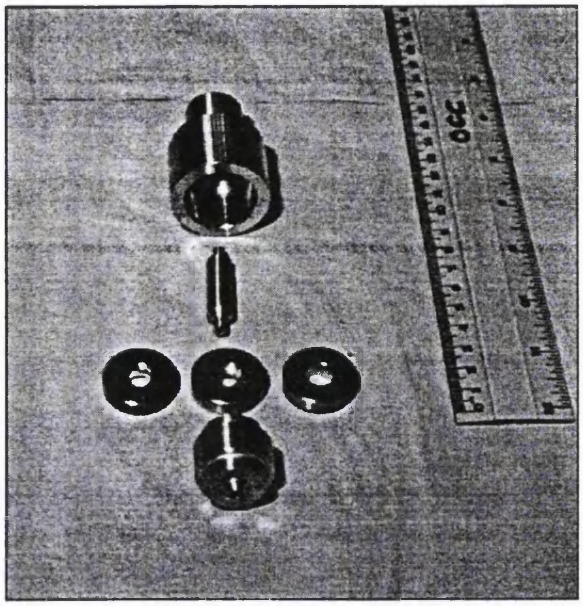


Figure 2.4. A photograph of the impact rings and the valve housing for the Micron Lab 40 high pressure homogeniser.

The removal and replacement of any of the components of the Micron Lab 40 in contact with the process stream is relatively straight forward and need not be mentioned here. However, care has to be taken to ensure that all the parts are centrally aligned.

Disruption procedure

The cooling coil of the Micron Lab 40 was turned on at least 30 minutes before each disruption session, to allow time for the large metal pieces to cool.

The required pressure setting (100 to 1600bar with a 10bar step) was made by means of a dial and read on the digital display. During the disruption process which lasted for only 5 seconds, the pressure reading fluctuated at times to ± 20bar.

The cell suspension was cooled to below 12^{o}C on ice and poured into the product chamber. During fermentation monitoring, the sample was processed directly without this cooling stage; any cooling that took place was just prior to and post homogenisation which formed the disruption stage. The valve housing and cover were placed in position the automatic disruption step was activated by means of a push button. Following the disruption process, the cells collected in the valve housing.

This valve housing was removed and then "swirled" to cool the homogenate prior to any further disruption. The cooling coils on the valve housing were efficient enough to allow rapid cooling in under a minute. On pouring the sample into the sample chamber, the temperature was checked to make sure that it was indeed below 12°C

Samples for analyses were take prior to disruption from the product (piston) cylinder (number of passes, N = 0) and from the valve housing after each pass (for N > 1)

2.5.2. APV Gaulin Lab 60 high pressure homogeniser

The APV Gaulin Lab 60 high pressure homogeniser is described in Section 1.2.3. where a diagram of the valve assembly is given in Figure 1.5.

The cell suspension was processed in a recycle loop at operating pressure of up to 500 bar and for up to 10 passes through the homogenising head.

The homogenised samples were centrifuged for 5 minutes in an MSE Micro Centaur at 13500 r.p.m. The supernatants were then assayed for total soluble protein and alcohol dehydrogenase (ADH).

2.5.3. Microfluidics Microfluidizer ®

Microfluidics Microfluidizer® M110T was on loan from Christisons. The principles of operation have been discussed in 'Cell Disruption' Section 1.2.3.

Limited trials using the Microfluidizer® M110T were conducted. This was primarily due to the short length of the loan. The second important reason was the compressed air requirement to operate the equipment; College air flowrate at the required pressure was unsustainable.

Not withstanding the above mentioned limitations, trials were successfully conducted.

Cooling was applied with extensions of the process lines into coils which could be submerged in glycol. This increases the total hold-up volume and also the volume of sample required. With the heat exchanger, back flush unit, and back pressure unit removed, it was possible not only reduce the minimum sample volume required but obviously with the decrease in the pressure drop across the system, higher operating pressure could be achieved, even with the poor air supply.

Without the heat exchanger in place, sample temperatures would reach over 35°C after processing for four or five passes at high pressures.

The sealed nature of the device allowed it to be submerged in ice with only the sample reservoir and collection points above the surface (Sauer *et. al.*, 1989). The system was cooled so, an hour before any series of trials.

Disruption procedure

A 10% w/v (packed weight) Baker's yeast cell suspension was processed for five passes (N = 0-5) at a range of operating pressure below 1000bar. Prior to the processing, the pump was primed with the test suspension.

2.5.4. MSE Soniprep 150

Cell disruption trials were carried out in a MSE Soniprep 150, (MSE, England). Sonication is a commonly used laboratory method for cell disruption (Engler, 1985; Edebo, 1969, Lambert, 1983).

Disruption procedure

A 300mL, 10% w/v (packed weight) Baker's yeast cell suspension was cooled to 4°C prior to processing. The sample was placed in ice (in a beaker) and sonicated at 20kHz frequency and 18μm amplitude. 1mL samples were withdrawn for total soluble protein and ADH analysis at regular intervals.

2.5.5. B. Braun Mikrodismemberator® II

Although the Mikrodismemberator® II has been designed for a wide variety of low-volume micro-grinding and mixing applications it may well prove useful in cell breakage. In effect, it is very similar to the beadmill system. It is essentially a shaker which very vigorously shakes a sample held within a chamber. For on-line cell disruption the chamber can easily be turned into a flow-through cell.

A range of sample chambers are available from 3 to 20mL in stainless steel or Teflon. To aid the mixing / disruption, the chamber is loaded with glass / steel beads along with the sample. The shaking amplitude is up to 15mm with a frequency of 50Hz.

Disruption procedure

In all the cases described below investigate the disruption of Bakers' yeast cells was carried out using a 10% packed weight suspension which was made up prior to the trials in phosphate buffer.

Preliminary Trials

In these preliminary trials the sample chamber was loaded with the cell suspension and with varying amounts of beads. The device was turned on and the chamber was then shaken at maximum amplitude of 15mm at a frequency of 50Hz for five minutes. After this time, the sample was observed under a microscope to see signs of disruption and once these were indeed observed the sample was centrifuged and assayed for total soluble protein.

- A 2mL suspension was pipetted into the Teflon chamber along with three 3mm diameter tungsten beads. After a processing time of 5 minutes, the sample was examined under the microscope to reveal no signs of breakage.

- A 2mL suspension was pipetted into the Teflon chamber along with 0.5g of 0.4-0.5mm diameter glass beads. After a processing time of one minute, the sample was examined under the microscope to reveal no signs of breakage.

- A 3mL suspension was pipetted into the Teflon chamber along with one gram of 0.4-0.5mm diameter glass beads. After a processing time of two minute, the sample was examined under the microscope to reveal signs of breakage. The sample was centrifuged and assayed for total soluble protein. The protein release was 90mg/g packed weight.

Detailed Trials

From the preliminary trials a sample to bead loading was decided at 2mL of the 10% packed weight Bakers' yeast cell suspension to one gram of 0.4-0.5mm glass beads.

After a processing time of one minute at a maximum amplitude of 15mm at a frequency of 50Hz,, the device was switched off. The sample was centrifuged and the supernatant assayed for ADH and total soluble protein.

A second, fresh, 2mL sample was similarly processed for two minutes, and then a third for three minutes and so on for up to ten minutes. This lengthy procedure was

adopted to avoid sample volume loss and change in the sample to bead ratio to influence the results.

2.5.5. Polytron PT1200 & PT3000

Both the Polytron PT1200 & PT3000 are commercially available grinding devices which were on loan from Philip Harris Scientific, London. The grinding action is brought about by the rotation of an inner cylinder against an outer stationary one. The tip of the inner cylinder is serrated. The cells are trapped between the cylinders and sheared. These grinding tips are easily changeable to three different sizes; the 7mm cell disruption tip was used for all the trials carried out here.

Polytron PT1200

The Polytron PT1200 is a hand-held device. The tip speed was controlled by means of a dial with no indicator.

Disruption procedure

A 5% Bakers' yeast cell suspension was made up in phosphate buffer immediately prior to the trials. The suspension was well mixed and several 1mL samples were taken in eppendorf tubes.

A single eppendorf containing the cell suspension was taken. The grinding tip immersed in the sample and with the setting on maximum the processing was started. The sample was not cooled at all during the processing. The device was locked in a vertical position on it's stand and the eppendorf was held in the hand.

After one minute, the device was switched off. The sample was centrifuged and the supernatant assayed for ADH and total soluble protein.

A second, fresh, 1mL sample was similarly processed for two minutes, and then a third for three minutes and so on for up to six minutes. This lengthy procedure was adopted to avoid sample volume loss to influence the results.

Polytron PT3000

The PT3000 is a larger version of the hand-held PT1200 device described above. The grinding tip attachment is fixed to a motor mounted on a stand. The tip rotation speed was controlled by means of a dial and the speed shown on an analogue display.

Disruption procedure

The same batch of 5% Bakers' yeast cell suspension as in the above trials was used here. Several 10mL samples were taken from the well mixed suspension into plastic 20mL universal tubes.

The sample containing universal was strapped into place and the grinding tip immersed. Some "cling-film" was placed over the top of the universal to prevent any spillage. With the power on, the tip speed control was turned and within several seconds the set tip speed was attained.

The rotation speed was set at 28,000 r.p.m. but varied between 26 and 28,000 r.p.m. through these trials.

After a processing time of one minute, the device was switched off. A 1mL sample was place in an eppendorf and centrifuged The supernatant was assayed for ADH and total soluble protein.

A second, fresh, 10mL sample was similarly processed for two minutes, and then a third for three minutes and so on for up to six minutes. This lengthy procedure was adopted to avoid sample volume loss to influence the results.

Although the samples were cooled on ice prior to the trials, no cooling was applied during processing.

2.6. Analytical Techniques

This section will describe the analytical methods employed to assess the extent of cell disruption with the various cell disruption devices. Total soluble protein and a suitable cytoplasmic marker enzyme were monitored.

2.6.1 Sample preparation for analysis of intracellular content

All the samples were processed in the same manner for the protein and enzyme assays.

The samples from the disruption devices were centrifuged in MSE Micro Centaur for 10 minutes at 13,500 r.p.m. and the supernatant then assayed.

2.6.2 Bio-Rad Protein Assay

This method was used for all the total soluble protein assays.

The Bio-Rad protein assay (Bio-Rad Laboratories Limited, Hemel Hempstead, Herts., UK) is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein (Bradford, 1976). It is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs.

The standard assay method was followed, with only the minor modifications indicated:

1. Each time a set of assays was performed, a protein standard curve was prepared using bovine serum albumin (BSA) (lyophilised powder, Sigma Limited, UK) made up to various known concentrations in deionised water. The procedure described was applied to both the standard BSA solutions and the samples.

2. Fifty micro-litres of standards or appropriately diluted samples (supernatant from the homogenates) were placed in plastic disposable 1.5mL cuvettes. Fifty micro-litres of sample diluent were placed into a cuvette as "blank" (50μL is used in-place of 0.1mL in the standard method).

3. To the cuvettes 1.5mL of 5-times diluted reagent concentrate was added and mixed well (modified from 5mL).

4. After a period of 5 minutes and before half an hour the absorbance at 595nm was measured against the "blank". The colour is stable from 5 minutes to one hour. The assay is performed at room temperature.

5. A plot of absorbance at 595nm versus concentration of standards (i.e. the standard protein curve) was used to read the unknown protein concentrations in the samples.

The absorbances were measured on the following spectrophotometers, with the standard protein curve being determined for each at the time of performing the assays:

Beckman DU-64 Spectrophotometer, Beckman DU-64 Spectrophotometer, Phillips PU8800 UV/VIS Spectrophotometer and LKB Biochrom Ultrospec II.

2.6.3. Enzyme assays

Alcohol dehydrogenase (ADH) was used as a marker enzyme for *S. cerevisiae* (Bakers' yeast) cell disruption and glucose-6-phosphate dehydrogenase (G6PDH) for *P. putida* ML2 cell disruption.

The profiles of these enzymes was monitored during the course of fermentations (Table 2.9.)

Enzyme	Fermentation / organism
ADH	*S. cerevisiae*
G6PDH	*P. putida* ML2 and *E. coli*

Table 2.9. Enzymes monitored during the course of fermentations.

Alcohol dehydrogenase (ADH)

The method for assaying for alcohol dehydrogenase is from Vallen and Hock (1955) in which the formation of acetaldehyde from ethanol is catalysed by ADH. A change in absorbance of the reaction mixture occurs at 340nm due to the formation of NADH.

The following stock reagents are prepared (the concentration in each mL of assay mixture is show in brackets):

1. NAD: 0.1M (1.8mM)
2. Semicarbazide HCl: 1.0M (6.2mM)
3. Glutathione 0.1M (1.0mM)
4. Tris buffer, Tris(hydroxymethyl)aminomethane: 0.05M adjusted to pH 8.8 with HCl (50mM)
5. Ethanol: absolute (0.7M)

In practice the assay mixture was made up and kept at 4°C. The assay mix is stable for several weeks.

The assay method is detailed below:

1. 50µL of appropriately diluted sample was placed into a plastic 3.0mL cuvette and to that was added 3.0mL of assay mixture (25°C) and mix thoroughly.
2. The absorbance change over a period of 1.5 minutes at 340nm in a temperature controlled spectrophotometer (Phillips PU8800 UV/VIS Spectrophotometer) against a "blank" was monitored.
3. The rate of change of absorbance is used to calculate the activity of the enzyme in international units per mL of sample.

One IU = The conversion of 1 micro-mole of NAD in one minute under the specified assay conditions.

This activity (U/mL) is then converted to units per gram dry cell weight (DCW).

Glucose-6-phosphate dehydrogenase (G6PDH)

The method for assaying for glucose-6-phosphate dehydrogenase was taken from Bergmeyer (1983), in which the following stock reagents are prepared (the concentration in each mL of assay mixture is show in brackets):

1. β-Nictotinamide adenine dinucleotide phosphate (NADP), Sigma grade 98-100% sodium salt: 3.8mM (0.38mM).

2. Tris buffer, Tris(hydroxymethyl)aminomethane: 0.5M adjusted to pH 7.5 with HCl (50mM).

3. Magnesium chloride, $MgCl_2.6H_2O$: 0.63M (6.3mM).

4. Glucose-6-phosphate, G-6-P: 33mM (3.3mM).

In practice the assay mixture was made up and aliquoted into 50mL portions and frozen at -20°C, and defrosted when required. Any unused assay mixture was not re-frozen for later use. Note that no maleinimide was used as it did not make any difference to the absorbance readings. Maleinimide is employed to inhibit 6-phospho gluconate dehydrogenase (6-PGDH) which causes further oxidation of the reaction products of G6PDH and hence misleading results.

The method is detailed below:

1. Fifty micro-litres to 100μL of sample were placed into a plastic 1.5mL cuvette and add 1.5mL of assay mixture (25°C) and mix thoroughly.

2. A delay time of 3 minutes was used before reading the absorbance change over a period of 3 minutes at 339nm in a temperature controlled spectrophotometer (Beckman DU-65 spectrophotometer) against a "blank".

3. The rate of change of absorbance was used to calculate the activity of the enzyme in international units per mL of sample.

One IU = The conversion of 1 micro-mole of NADP in one minute under the specified assay conditions.

This activity (U/mL) is then converted to units per gram dry cell weight (DCW).

2.6.4. Optical density measurements

The optical density measurement was obtained reading absorbance values at 670nm against the buffer "blank". As the optical density and absorbance readings have a linear correlation over a limited range, the absorbance reading was kept below 0.3 units by diluting the sample appropriately with buffer; so the resulting reading was corrected accordingly.

The absorbances were measured on the following spectrophotometers: Beckman DU-64 Spectrophotometer, Beckman DU-65 Spectrophotometer, Phillips PU8800 UV/VIS Spectrophotometer and LKB Biochrom Ultrospec II.

2.6.5. Dry weight measurements

Filter paper method

The dry weight measurement was obtained by pipetting 5mL of well mixed sample onto a pre-dried (24 hours in a drying oven at 90°C) and pre-weighed Whatman CF/C, 0.2mm pore size, 47mm diameter membrane filter (Whatman Lab. Products Ltd., Maidstone, England), placed in a Sartorius vacuum filtration apparatus (Sartorius Instruments Ltd., Belmont, England).

After applying a vacuum for several minutes, the liquid component was substantially removed. A further 10mL of deionised water was added to wash the residuals and the vacuum reapplied.

The filter and residual solids were placed in the drying oven, and dried to constant weight (24 hours at 90°C). When dry, the filters were allowed to cool to room temperature in a desiccator and then re-weighed.

The dry weight of the residuals was calculated as the difference between the weight of the filter before and after use. The conversion to concentration was applied with the known initial sample volume.

Centrifuge tube method

Difficulties in using the filter paper method, such as the lengthy filtering times led to the use of centrifuge tubes.

20mL of the sample was pipetted into the pre-dried and weighted tube and centrifuged for 30 minutes at 3,500 r.p.m. in an MSE centrifuge.

Once the supernatant was carefully decanted, the pellet residue was resuspended in deionised water and re-centrifuged for a further 30 minutes.

The supernatant was again decanted and the centrifuge tube and pellet was placed in the drying oven; dried to constant weight (24 hours at 90°C). When dry, the centrifuge tubes were allowed to cool to room temperature in a desiccator and then re-weighed.

The dry weight of the cells was calculated as above.

3. Results and Discussion

The results from a series of trials using commercially available cell disrupters are presented in this section. The detailed work presented here focuses on the Micron Lab 40 high pressure homogeniser, the cell disruption kinetics, the reproducibility of the results, and its use in the rapid monitoring of intracellular components during the fermentation of a number of micro-organisms; the information thus gained being used in fermentation profile analysis.

(The cell disruption results of packed yeast within a prototype high pressure homogeniser capable of operating at pressures of up to 3,000 bar are presented in Section 5).

3.1 Various commercially available cell disruption devices

A number of cell concentrations of *S. cerevisiae* (packed yeast) were disrupted within various commercially available devices which include:

- a high pressure homogeniser, APV Lab 60,
- a Microfluidizer® -M110T,
- a sonicator - MSE Soniprep 150,
- a small scale vibrator, bead mill - Mikrodismemberator
- grinders, Polytron PT1200 & PT3000.

3.1.1. APV Gaulin Lab 60 high pressure homogeniser

The commercially available pilot scale APV Gaulin Lab 60 was tested as an introduction to high pressure homogenisers and cell disruption. The APV Lab 60, with a minimum processing volume of 2.5L to 3.0L, was investigated enroute to a small scale high pressure homogeniser. Even though a significant amount of data

was already available for such devices it proved to be a useful familiarisation exercise.

The results for the disruption of 45% w/v cell suspension of *S. cerevisiae* (packed Bakers' yeast) within the Lab 60 are shown in Figure 3.1.1. The cell suspension was processed for a total of 10 passes through the homogeniser at operating pressure of 500 bar. As illustrated in Figure 3.1.1., there was an increased release of both total soluble protein and ADH levels with increasing number of passes.

3.1.2. Microfluidics Microfluidizer ®

The Microfluidics Microfluidizer® M110T (see Section 2.5.3.) could not be used to its full potential because of the very high compressed air requirement ($0.035cm^3$/min at a pressure of 6.9 bar) which could not be supplied.

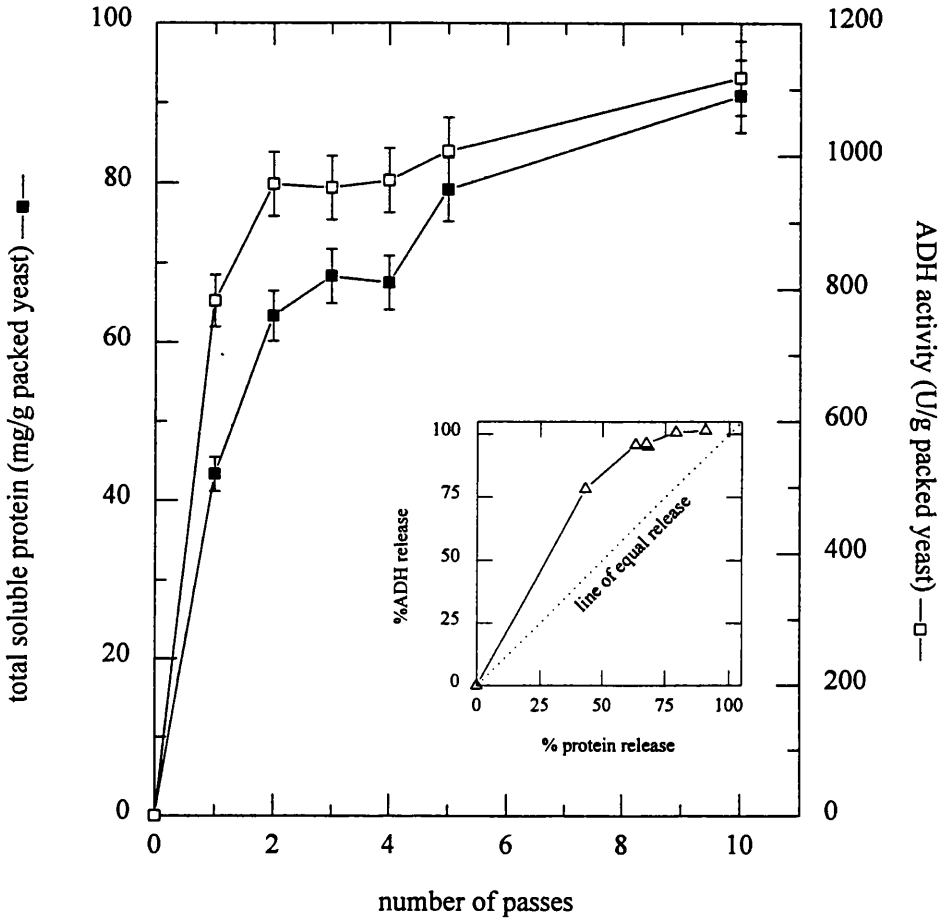
Nonetheless, a series of disruption experiments were carried out with a 10% w/v packed yeast cell suspension. The release of total soluble protein and ADH activity with number of passes over a range of operating pressures is shown in Figure 3.1.2.

The results show a typical gradual release of the intracellular components, with limited cell breakage being evident at the lower operating pressures. Even at the higher pressure of 725 bar, the levels of ADH and total soluble protein were very low after a single pass. Two or more passes were necessary to achieve the levels of release and breakage similar to those attainable after a single pass within the APV Lab 60 at 500 bar operating pressure.

These results do not to agree with those reported by Seva and co-workers (1986) where 90% cell disruption of yeast cells was achieved after 30 passes at 600 bar. Figure 3.1.2. shows that at 725 bar operating pressure almost complete release (96mg/g packed yeast) after 5 passes is achieved.

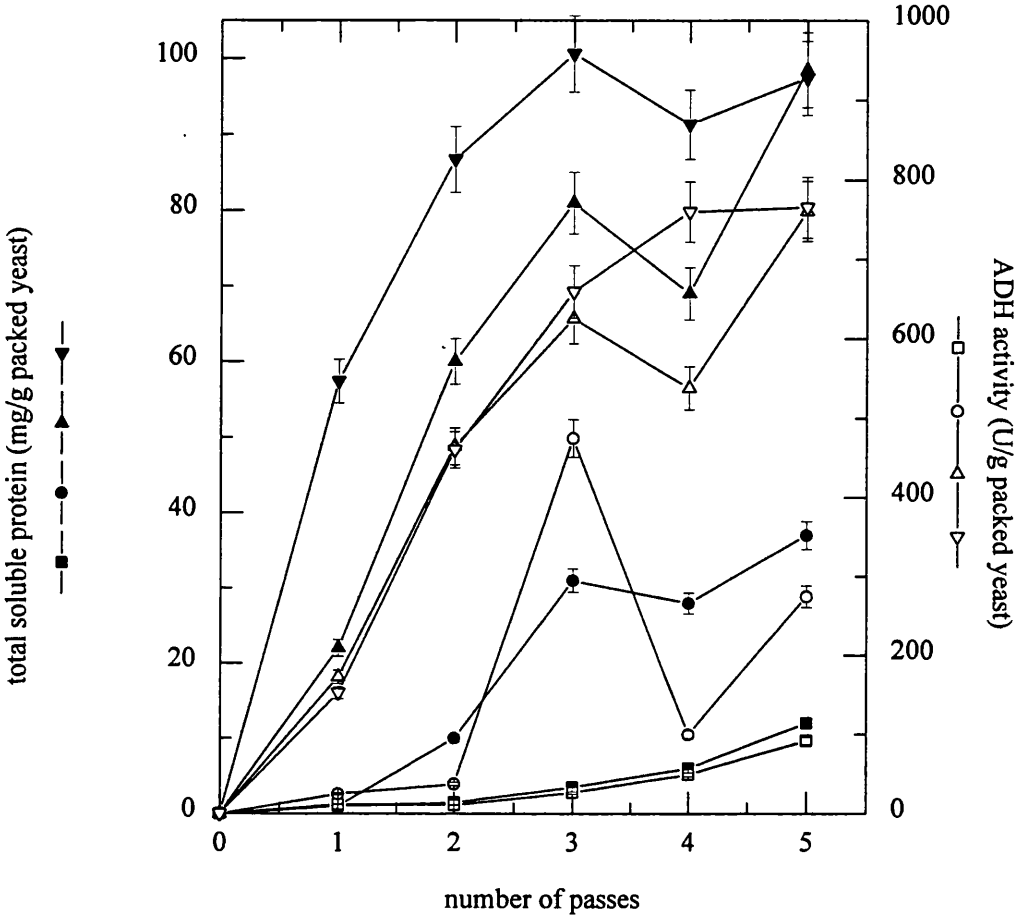
With *B. subtilis*, cell breakage as compared with that achieved in APV Manton-Gaulin high pressure homogeniser was similar, although levels of protein release were different (Seva *et. al.*, 1986).

Baldwin and Robinson (1990) disrupted yeast cell suspensions ranging in concentration from 0.6 to 15 g/L dry weight in a Microfluidizer (M110T fitted with a heavy duty pump). After 4 passes at 950 bar only 32% disruption occurred. In studies with recombinant and non-recombinant strains of *E. coli*, a high disruption



—■— total soluble protein released (mg/g packed cells)
—□— ADH released (U/g packed cells)
error bars represent SD of 5% based on analysis of disruption process
INSET: —△— %ADH vs %protein release
100% based on 1100U/g for ADH and 100mg/g for protein

Figure 3.1.1.
Total soluble protein & ADH release from *S.cerevisiae* (45% w/v packed yeast) in the APV Gaulin Lab 60 at an operating pressure of 500 bar



protein	ADH activity
—■— 276 bar	—□—
—●— 387 bar	—○—
—▲— 724 bar	—△—
—▼— 827 bar	—▽—

error bars represent SD of 5% based on analysis of disruption process

Figure 3.1.2.
Total soluble protein & ADH release from *S.cerevisiae* (10% w/v packed yeast) in the Microfluidizer M110T at a range of operating pressures

level of 95% to 98% for the recombinant strain was achieved in two or three passes at 950 bar (Sauer *et. al.*, 1988).

In addition to the varying levels of release reported in the literature for disruption of various micro-organisms, Sauer *et. al.* (1988) found that the degree of disruption was also dependent on the specific growth rate of the cells during the upstream fermentation stage and on the initial cell concentration of the homogeniser feed.

Figure 3.1.3. correlates the release of ADH with total soluble intracellular protein from a suspension of *S. cerevisiae* (10% w/v packed yeast) disrupted over a range of operating pressures (275 to 827 bar). This shows the correlation to be below the line of equal rate of release; indicating either an unexpected slower release of ADH compared with total soluble protein or simply ADH enzyme deactivation.

3.1.3. MSE Soniprep 150

The results from the sonication of a 300mL *S. cerevisiae* cell suspension 10% w/v (packed yeast) within the MSE Soniprep 150 are presented in Figure 3.1.4. The yeast cell suspension were sonicated for up to 45 minutes at a frequency of 20kHz.

The release of the intracellular content was very gradual; after 45 minutes of processing, the total soluble protein released represented only 46% of the total available (based on a figure of 96mg of protein per gram of packed yeast, Hetherington *et. al.*, 1971).

The inset of Figure 3.1.4., correlating ADH release with total soluble protein for the above results, shows the start of enzyme deactivation as well as emphasising the low levels of intracellular content release.

The poor results from the sonicator were due to:

- the large volumes (300mL) compared to the volume for the sonitrode (the sonicating probe) being only 50mL, and
- the tip of the sonitrode being eroded requiring regrinding and thus not functioning efficiently.

Following these initial trial, the sonicator was serviced and the sonitrode replaced. Other workers within the department have made use of this device for cell breakage.

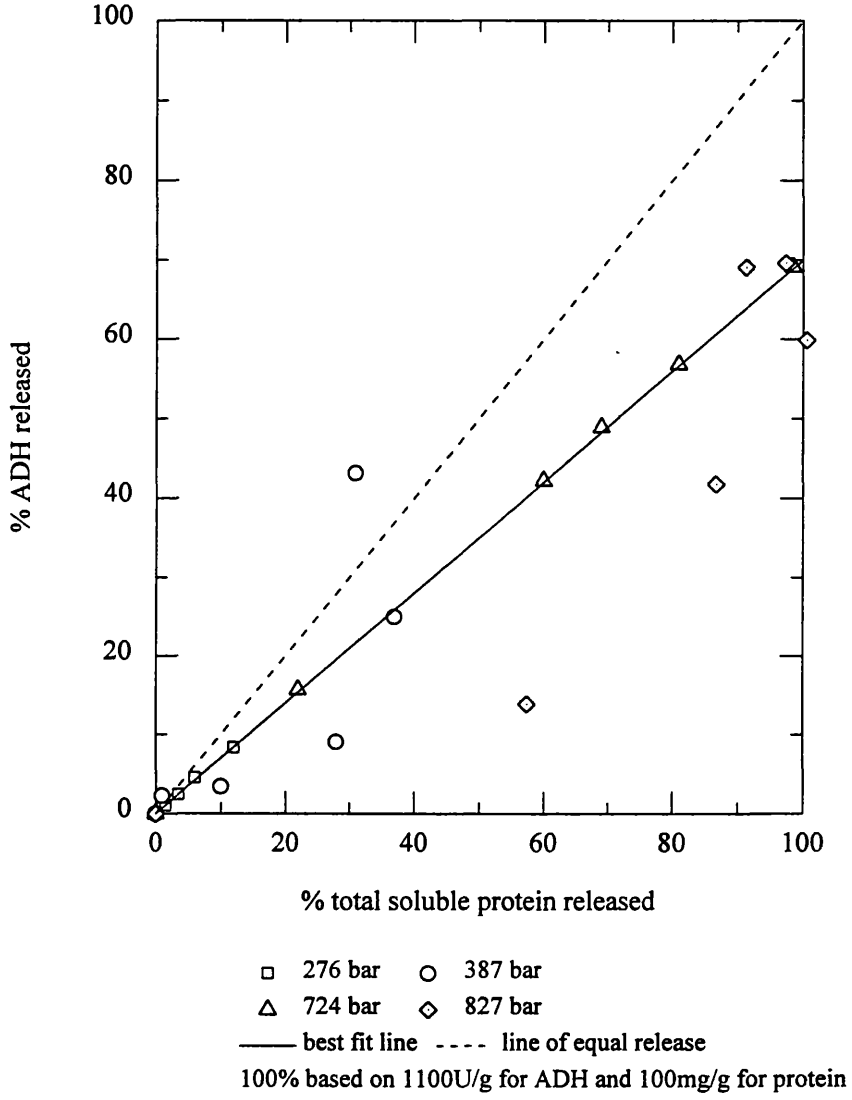
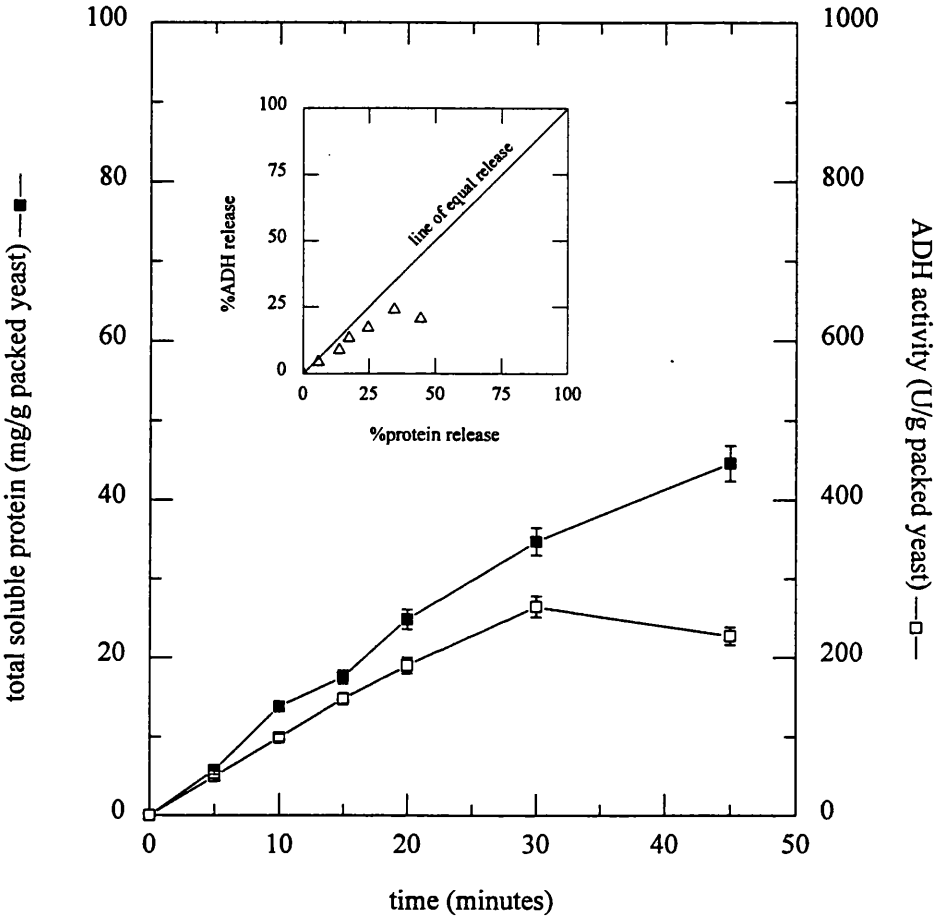


Figure 3.1.3.
Percentage ADH against total soluble protein release from *S.cerevisiae* (10% w/v packed yeast) in the Microfluidizer M110T at a range of operating pressures



—■— total soluble protein released (mg/g packed cells)
—□— ADH released (U/g packed cells)
error bars represent SD of 5% based on analysis of disruption process
INSET: △ %ADH vs %protein release
100% based on 1100U/g for ADH and 100mg/g for protein

Figure 3.1.4.
Total soluble protein & ADH release from *S.cerevisiae* (10% w/v packed yeast) in the MSE Soniprep 150

In conclusion the potential of this method was not adequately investigated during the course of this study. The issues of rapid, complete cell breakage, throughout the course of fermentation, need to be addressed.

As the efficiency of sonication is dependent upon various parameters including pH, temperature, ionic strength of the suspending medium as well as the time of exposure, the selection of set conditions, being an empirical choice, will vary with individual organisms (Melling and Phillips, 1975; White and Marcus, 1988).

As mentioned earlier (Section 1.2.3., in 'Cell Disruption Methods') Stube and Spohn, 1994, reported using sonication for effecting cell disruption during fermentation monitoring. After 6 minutes of processing, they achieved an enzyme activity release plateau at 93%. Although they found a significant level of enzyme deactivation, the most limiting feature of the ultrasonic technique was the stability of the sonitrode. With a processing frequency of six samples every hour and disintegration time of three minutes, they were forced to change the sonitrode after every six hours.

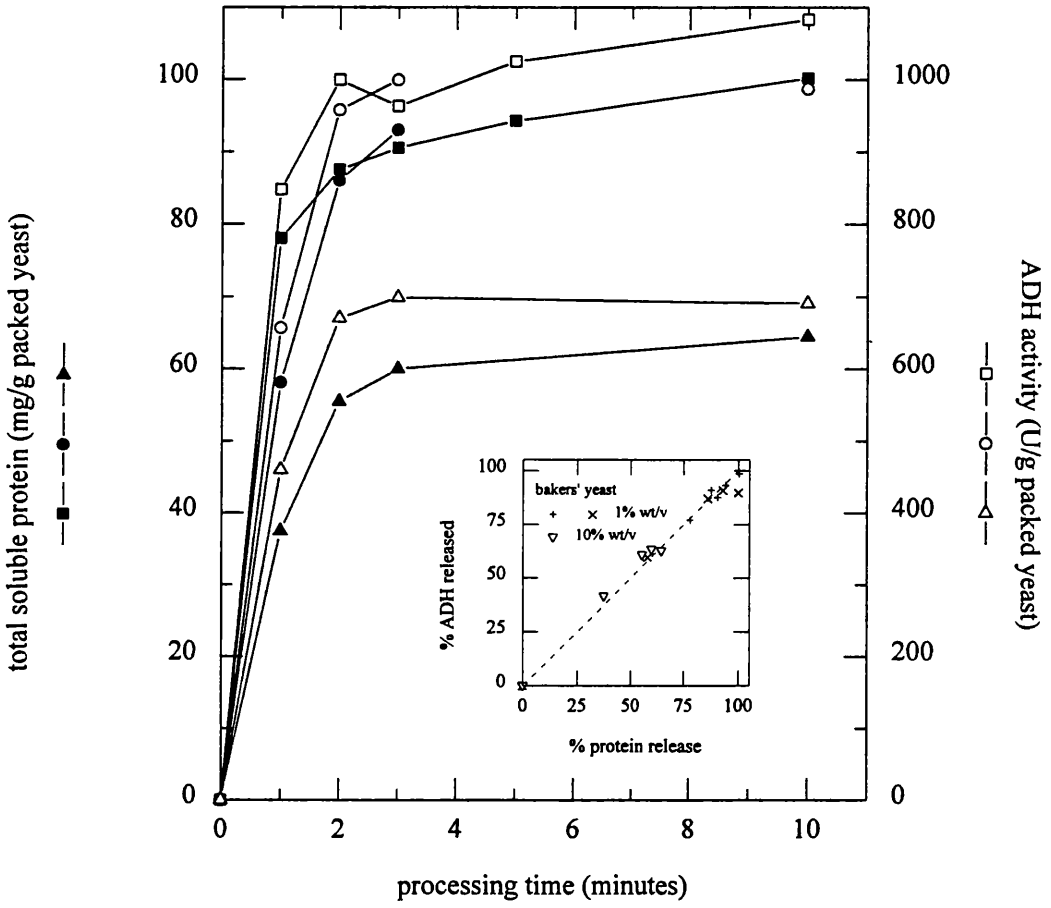
Denaturation of enzymes has also been reported by others (Aiba *et. al.*, 1973; Edebo, 1969; Engler, 1985). This is clearly a serious limitation when the measured component is being denatured.

3.1.4. B. Braun Mikrodismemberator® II

The device relies on the use of intrusive abrasives (glass, Teflon and steel beads) to effect disruption. In principle, it is not dissimilar to the bead milling. As mentioned previously (Section 1.2.3. in 'Cell Disruption Methods'), bead milling has many factors which influence the cell disruption process.

The possible variables requiring assessment were numerous, e.g., processing time, sample to bead ratio, bead size, bead type and cell concentration. After preliminary trials, a sample-to-bead ratio of 2:1 (mL sample/g beads) was adopted for further study and experiments conducted with varying process times.

Figure 3.1.5. shows the results from trials with *S. cerevisiae* (10% w/v packed yeast). The total soluble protein released after 60s of processing was almost 40mg/g packed yeast and reached a plateau of about 60mg/g packed yeast after 3 minutes. A similar trend being observed for the ADH activity.



ADH activity protein release

—□— —○— 1% w/v —■— —●— 1% w/v yeast

—△— 10% w/v —▲— 10% w/v

INSET: + × ▽ %ADH vs %protein released

100% based on 1100U/g for ADH and 100mg/g for protein

Figure 3.1.5.

Total soluble protein and ADH release from 2mL *S. cerevisiae* (1% w/v [two trials] and 10% w/v packed yeast) with processing time in the B Braun Mikro-dismemberator II (1g of 0.45-0.5mm dia. glass beads)

The Figure also shows the results from trials with *S. cerevisiae* (1% w/v packed yeast). In this case, the total soluble protein released after 60s of processing was almost 60mg/g packed yeast and reaching a plateau of about 90mg/g after 3 minutes. A similar trend is observed for the ADH activity released. This is confirmed when ADH release is correlated with the release of total soluble protein (inset of Figure 3.1.5.).

Figure 3.1.5. clearly shows a higher final-level of release both for total soluble protein and ADH in the processing of 1% w/v packed yeast cell suspension when compared to results for the 10% w/v suspension. The disadvantage of a relatively lengthy processing times required for high levels of intracellular release is dwarfed by the inconsistency in the final-levels of release (i.e. different maximum protein release values, R_m). Therefore, cell concentration was found to be a significant parameter in the kinetics of cell disruption within such devices.

3.1.5. Polytron PT1200 & PT3000

Polytron PT1200 & PT3000 are fundamentally grinding devices (see Section 2.5.5.). Both Polytron units were tested to evaluate their potential as on-line disruption devices. They were already commercially available and required minor modification for potential on-line cell disruption. A simple flow-through cell could be designed to house the grinding tips.

Heat generation was a major problem with the hand held device (PT1200), with both the grinding tip and the electric motor becoming hot; the sample temperature also exceeded 25°C and the electric motor itself got heated. Iron fillings from the grinding tip were observed with the cell debris and unbroken cells sediment after centrifugation of the processed samples.

The same temperature problems associated with the PT1200 were not observed with the larger Polytron, PT3000, the sample mounting became hot but there was no heat transfer to the sample itself. And there were no signs of iron fillings in the centrifuged sediment.

Figure 3.1.6. shows the results of processing *S. cerevisiae* cell suspension 5% w/v (packed yeast) for a total of six minutes. The crucial point of the trials was to effect cell breakage. Both devices failed to produce total soluble protein and ADH release yields in excess of 5% of that available by disruption within a high pressure

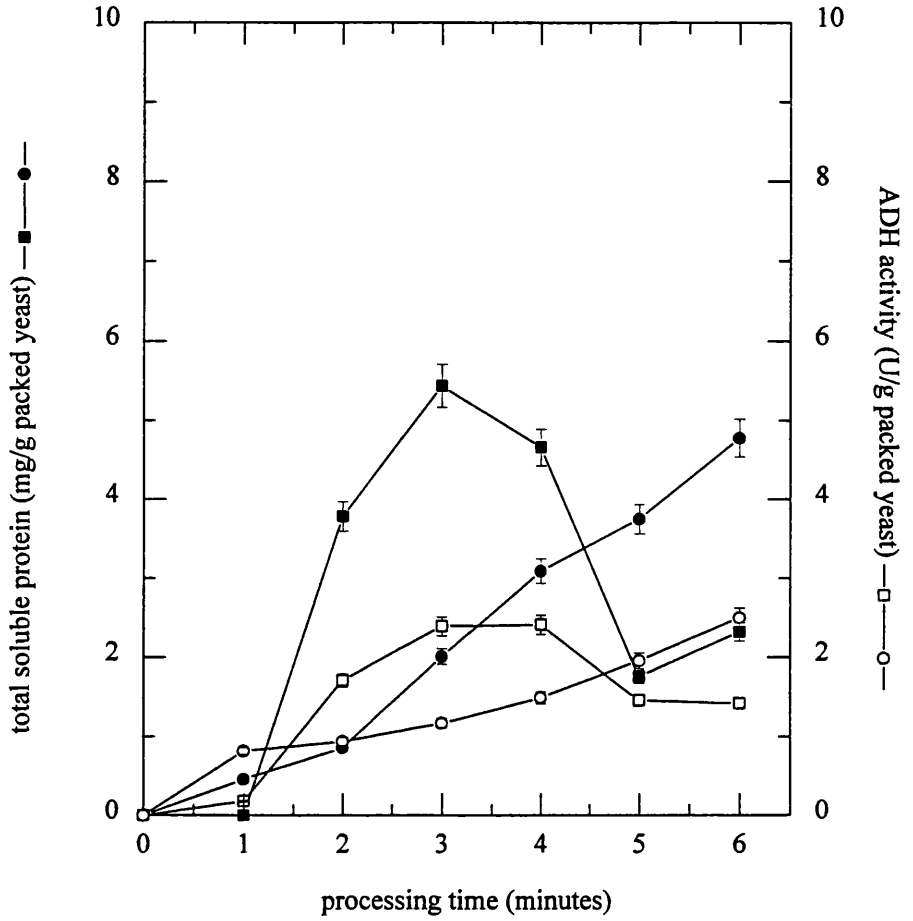


Figure 3.1.6.
Total soluble protein & ADH release from *S.cerevisiae* (5% w/v packed yeast) in the Polytron PT3000 & PT1200 with processing time.
Error bars represent an SD of 5% based on analysis of disruption process

homogeniser. In addition to the poor breakage results, in both cases, there was evidence of ADH denaturation after processing the samples for more than 5 minutes.

From the total soluble protein data, a marked difference in the rates of release between the two devices is observed. The release rate for the larger PT3000 is greater than that for the PT1200.

Neither of these devices was found to be suitable to effect cell breakage and the results were sufficiently poor to discontinue any further evaluation of these systems.

Summary for this section of the results

Several low processing volume commercially available cell disruption devices were assessed. The table below summarises the results observed (Table 3.1.)

Device	Main Findings
Microfluidizer M110T	High levels of cell disruption with pressure and number of passes; ADH release slower than expected or being deactivated.
Soniprep 150	Very gradual release with ADH enzyme deactivation
Mikro-dismemberator	High levels of cell disruption with no evidence of ADH deactivation; however, levels of maximum release significantly affected by cell suspension concentration
Polytron PT1200, PT3000	Neither devices were effective in cell disruption (<5% cell breakage after 6 min.); ADH enzyme deactivation was evident.

Table 3.1. Summary of the main findings for the small scale commercially available cell disruption devices. (Test organism *S. cerevisiae*, packed yeast).

3.2. APV Gaulin Micron Lab 40 high pressure homogeniser

The Micron Lab 40 is "a bench scale" device operating only in batch mode, processing 40mL of cell suspension in a single pass at pressures in the range of 100 to 1600 bar (see section 2.5.1., in 'Materials and Methods').

As with some of the larger high pressure homogenisers a positive displacement single-piston pump delivers the cell suspension into a valve assembly. During discharge the cell suspension passes between the valve and its seat and impinges on an impact ring.

A major concern with the APV Micron Lab 40 was the need to control temperature as the only cooling available was the homogeniser block used as a heat sink. On processing pure water, a temperature rise of 1°C per pass per 100 bar of operating pressure was found (Figure 3.2.1.). This led to the installation of cooling coils around the sample chamber and the valve housing, with glycol as the coolant (see Section 2.5.1., in 'Materials and Methods). This allowed the homogenised samples to be quickly cooled back down to below 12°C.

3.2.1 Disruption Kinetics of *Saccharomyces cerevisiae* within the Micron Lab 40

The release of total soluble protein with number of passes over a range of operating pressures (200bar to 1600bar) is shown in Figure 3.2.2. Similar results to the larger scale APV Lab 60 were observed with the disruption of the 10% w/v packed yeast cell suspension within the Micron Lab 40.

At the lower homogenising pressures of below 600 bar the total soluble protein release is significantly below the maximum available (R_m) even after two passes. At the higher operating pressures, the levels of cell breakage approach maximum as indicated by the total soluble protein releases after the first pass. The total soluble protein release at 1000 bar to 1600 bar operating pressure approach R_m and remain high after further passes through the homogeniser.

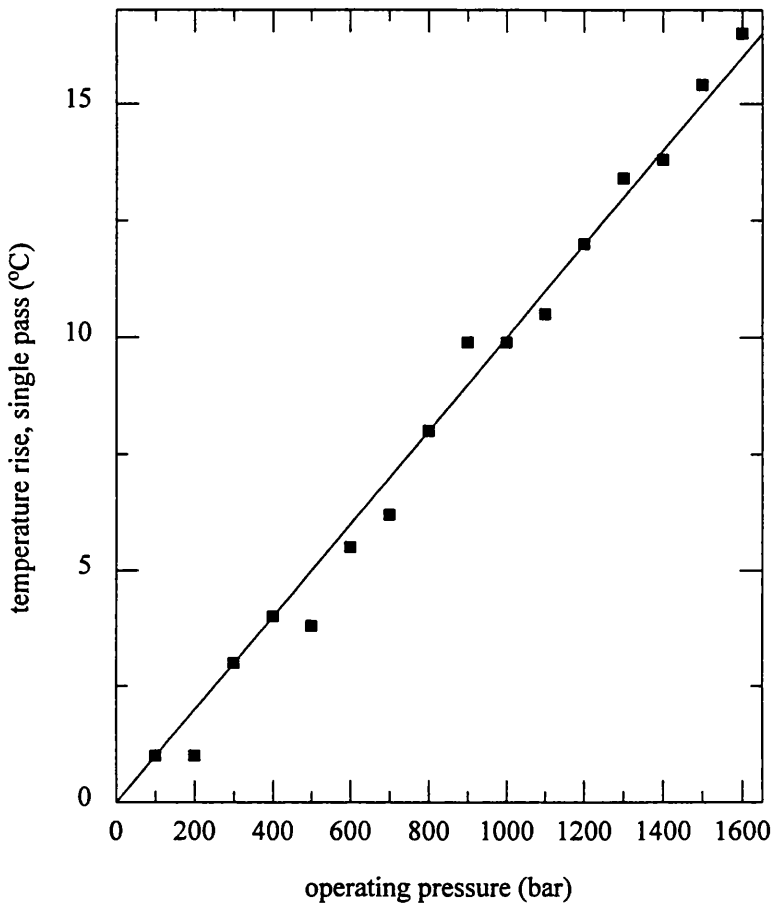
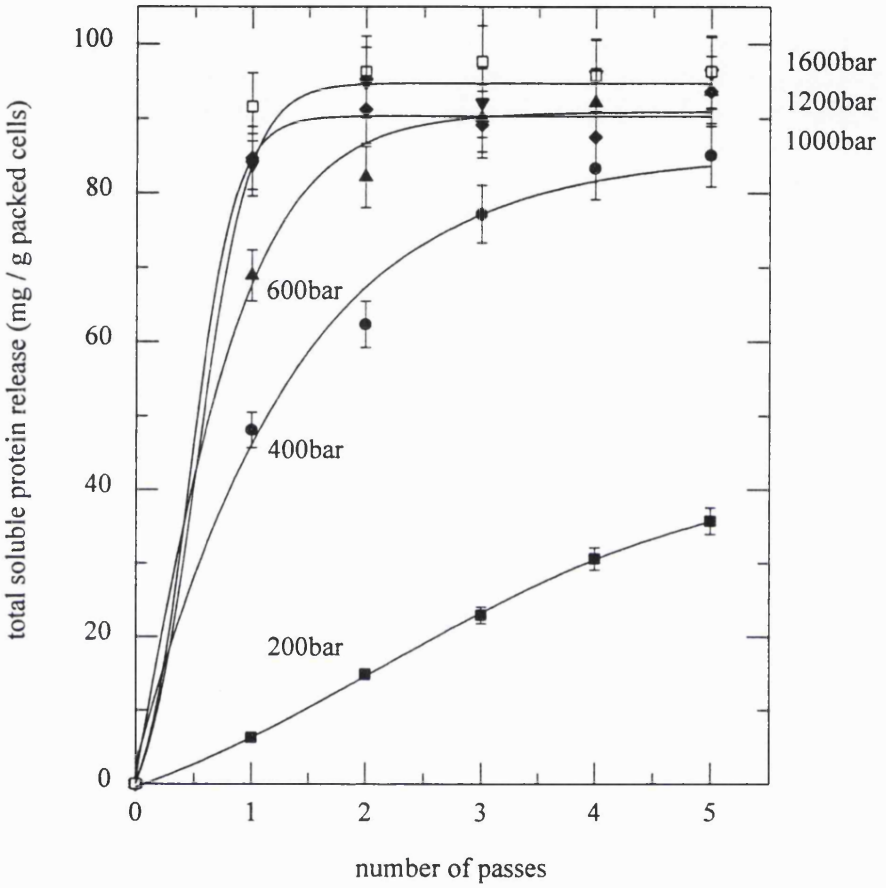


Figure 3.2.1.
The temperature profile within the Micron Lab 40 high pressure homogeniser



Operating pressure

■ 200 bar ● 400 bar ▲ 600 bar

▼ 1000 bar ◆ 1200 bar □ 1600 bar

error bar represent an SD of 5%

based on the analysis of disruption process

Figure 3.2.2.

Total soluble protein release from *S. cerevisiae* (10% w/v packed yeast) in the Micron Lab 40 at various operating pressures

Process Kinetics

Only in the case of lower homogenisation pressures was the release slow enough to allow evaluation of process kinetics.

The rate of release of protein with respect to the number of passes is a first order process with respect to the unreleased protein concentration (i.e. R_m-R) as described by (Hetherington *et. al.*, 1971):

$$\ln\left(\frac{R_m}{R_m - R}\right) = k \,.\, N \qquad \text{Equation 3.1}$$

This is shown (Figure 3.2.2.) to be a suitable representation of the disruption kinetics of *S. cerevisiae* within the Micron Lab 40.

where:

R_m is the maximum possible soluble protein release (mg/packed dry cell weight);

R is the soluble protein release (mg/packed cell weight);

k is the dimensionless disruption rate constant;

N is the number of passes;

The total soluble protein release results for 200 bar to 800 bar operating pressures are plotted.

Of major interest in this study was the evaluation of the Micron Lab 40 to effect the rapid release of intracellular components and provide a representative measure of these components during fermentation. To examine this for high pressures it was necessary to extend the lower pressure data presented in Figure 3.2.3. The first pass total soluble protein release data for the homogenisation of various cell concentrations of *S. cerevisiae* (5% w/v to 60% w/v packed yeast) are plotted for a range of operating pressures (from 100 bar to 1600 bar) in an alternative form

$$\ln\left(\frac{R_m}{R_m - R}\right) = K \,.\, P^{a} \,.\, N \qquad \text{Equation. 3.2..}$$

where:

K is a dimensional rate constant (bar^{-a});

P is the operating pressure;

and 'a' is the exponent on the pressure term.

This yields values for K = 1.06×10^{-5} (bar^{-a}) and 'a' = 1.76 (Figure 3.2.4.) (using all the data points available off the Figure).

The value for 'a' ('a' = 1.76) is comparable to 'a' = 1.72 to 1.79 obtained by Doulah *et. al.*, 1975, and 'a' = 1.86, 1.88 and 2.08 (data for Bakers' yeast from three different sources) obtained by Kula *et. al.* (1990) operating at pressures up to 700 bar. However, 'a' was not comparable to 2.9 obtained by Hetherington *et. al.* (1971) operating at pressures below 500 bar. In both cases, the disruption of Bakers' yeast was carried out in large scale homogenisers. Dunnill and Lilly (1975) reported a decrease in the value of the exponent 'a' for Bakers' yeast at operating pressures above 700 bar. This being attributed to the decrease in efficiency of the large scale homogeniser at the high operating pressures.

The exponent 'a' has a value of 2.53 and K = 1.07×10^{-7} when calculated using results for operating pressures up to 600 bar.

S. cerevisiae (100g/L - 450g/L, packed yeast)	≤600 bar	≤1600 bar
'a'	2.53	1.76
K (bar^{-a})	1.07×10^{-7}	1.06×10^{-5}
SE 'a'	0.228	0.143
SE 'ln K'	0.591	0.946
n	20	50
r^2	0.873	0.758

Table 3.2. The values for the dimensional disruption rate, K and the exponent 'a' on the pressure term in Equation 3.2. for *S. cerevisiae* (100g/L - 450g/L, packed yeast).

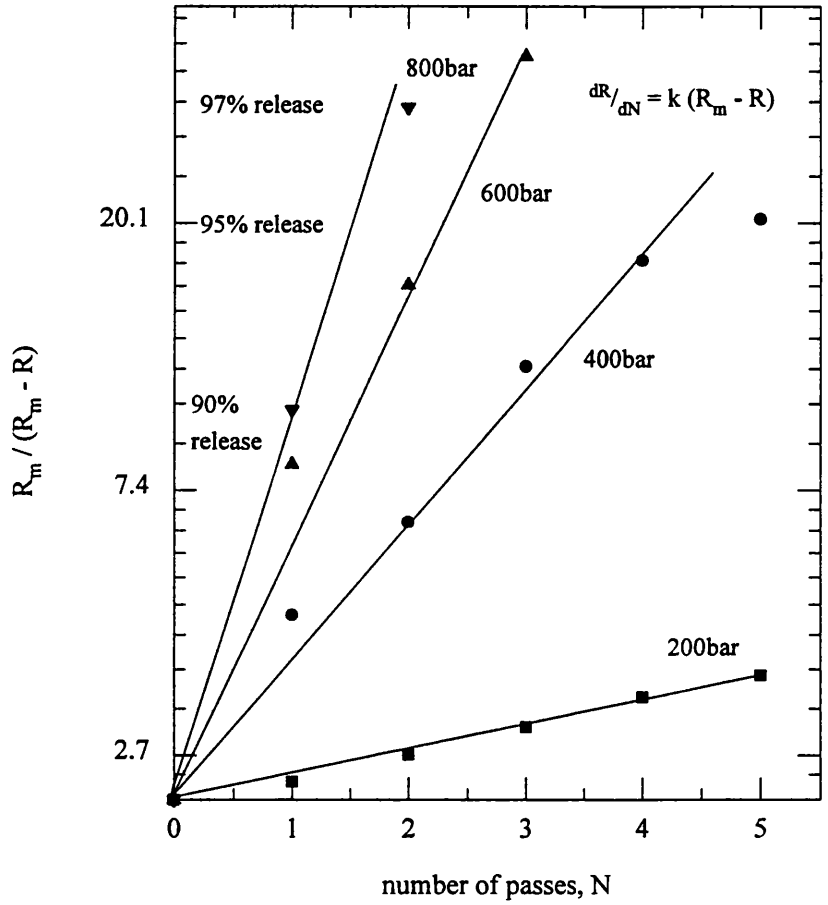


Figure 3.2.3.
First order release of total soluble protein from *S. cerevisiae* (10% w/v packed yeast) in the Micron Lab 40 high pressure homogeniser

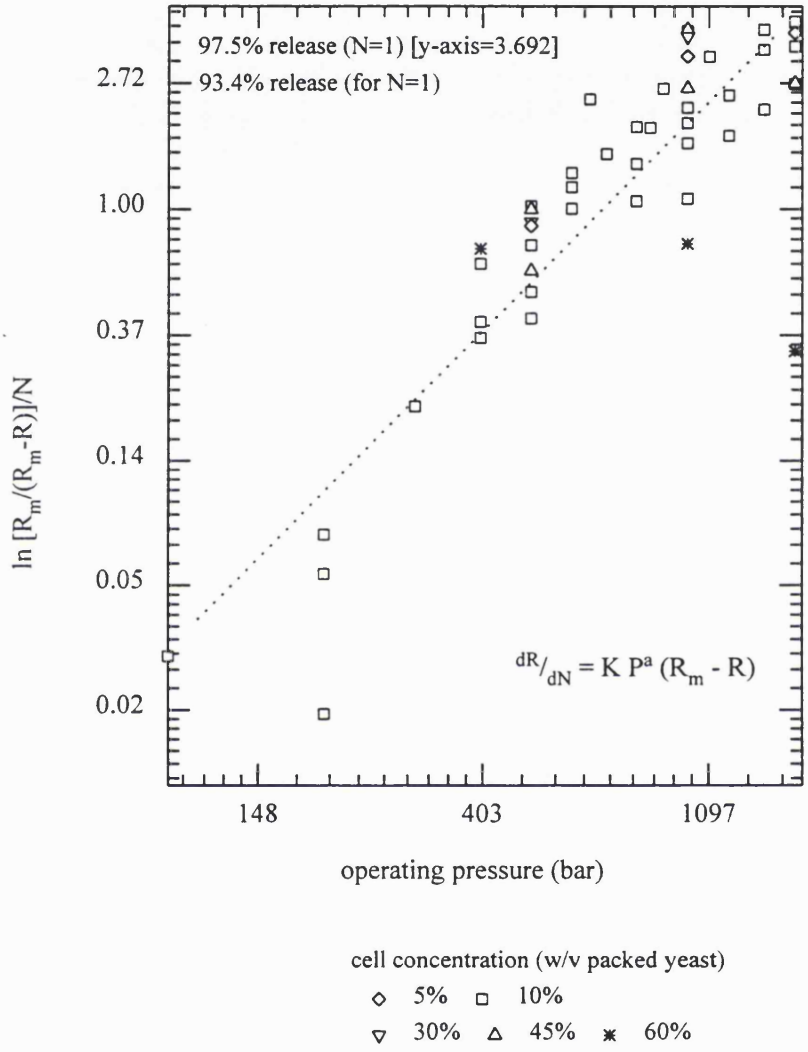


Figure 3.2.4.
The evaluation of pressure dependency of protein release form various concentrations of *S. cerevisiae* in the Micron Lab 40 at range of operating pressures - single pass data.

The average rate of release of ADH activity was shown to correlate to the total soluble protein release (Figure 3.2.5.) as expected for a cytoplasmic enzyme (Follows *et. al.*, 1971).

From the data presented in Figure 3.2.4, the disruption process is seen to be independent of cell concentration within the range of 5% w/v to 45% w/v packed yeast.

3.2.2. Disruption Kinetics of *Pseudomonas putida* ML2 within the Micron Lab 40

Pseudomonas putida ML2 was chosen as the main micro-organism for study within a broader field of study involving other workers, because of its ability to effect biotransformations.

Initial Trials

The initial trials were with *P. putida* ML2 whole cells which had been stored in fermenter broth for 24 hours and then centrifuged for 35 minutes at 4°C, resuspended in phosphate buffer and disrupted in the Micron Lab 40 high pressure homogeniser.

The results are shown in Figure 3.2.6. illustrate that the disruption process was pressure dependent and the exponent on the pressure term, 'a', in Equation 3.2. was 1.45. This when compared to the yeast data indicated that even more extensive disruption conditions would be needed for the rapid release of the intracellular components: (A high value of 'a', indicating a high pressure dependency. Conversely a lower value for 'a' indicating a lower pressure dependency requiring harsher conditions to effect disruption).

Although, these results did merit further investigation, it was clearly not within the scope of on-line monitoring, during which storage of cells would not occur.

The rest of this section on *P. putida* ML2 cells relates only to cells processed immediately after sampling.

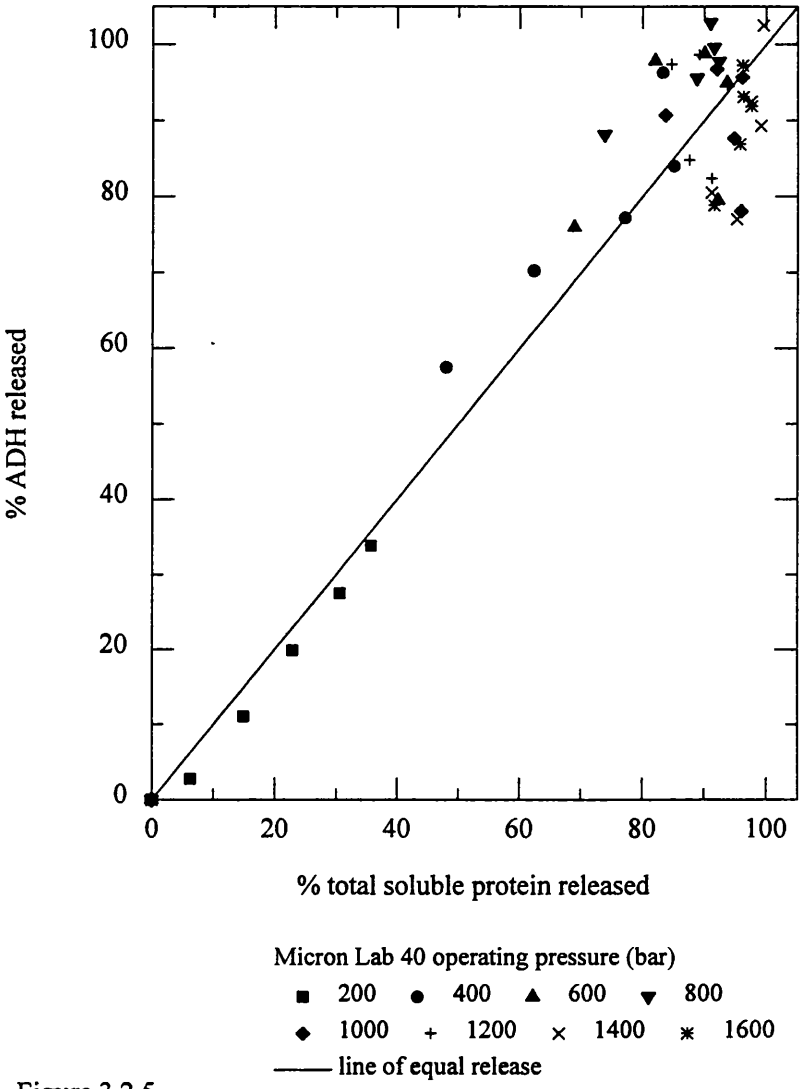
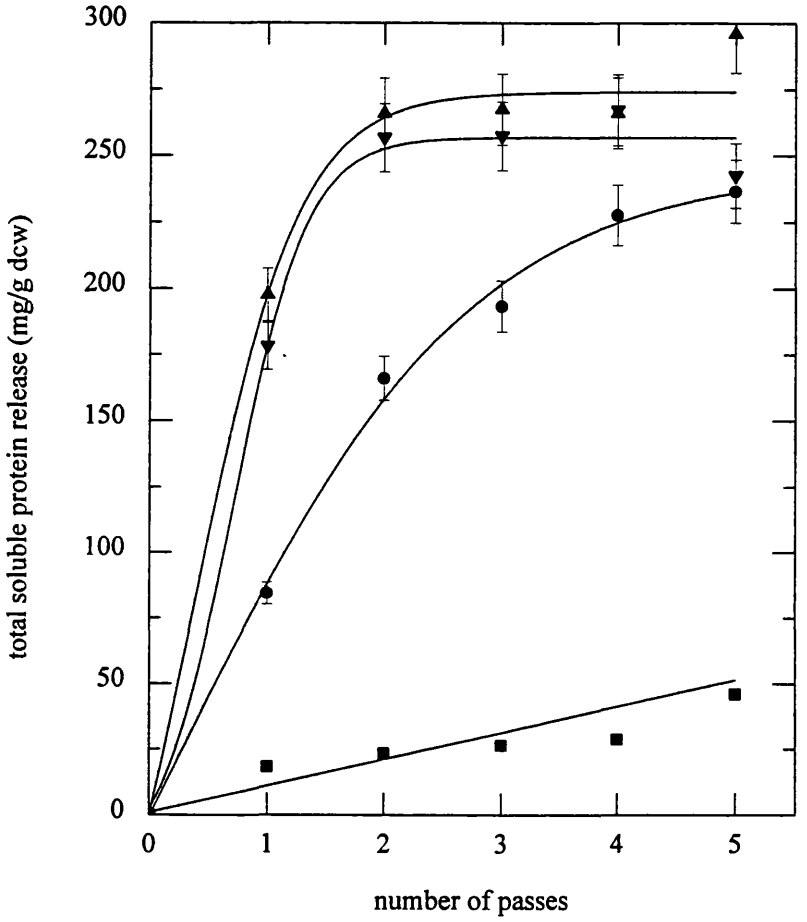


Figure 3.2.5.
Total soluble protein and ADH release from *S.cerevisiae* (10% w/v packed yeast) disrupted within the Micron Lab 40 high pressure homogeniser over a range of operating pressures



operating pressure (bar)

■ 200 ● 600 ▲ 1000 ▼ 1600

error bars represent an SD of 5% based on the analysis of disruption process

Figure 3.2.6.

Total soluble protein release from *P. putida* ML2 (6.3g/L dcw) cells stored in fermenter broth overnight and then resuspended in phosphate buffer prior to disruption in the Micron Lab 40 at various operating pressures.

Effect of pressure and number of passes

The release of total soluble protein with number of passes over a range of operating pressures is shown in Figures 3.2.7. and 3.2.8. for samples from two *P. putida* ML2 fermentations.

The total soluble protein release results are similar to those achieved for *S. cerevisiae* cell breakage.

The glucose 6-phosphate dehydrogenase activity was assayed by detecting the rate of change in absorbance of the sample; the low levels of G6PDH activity within the cell yielding a rate in the region of 0.01U/min, providing a potentially greater source of error in the results. Thus, the results of G6PDH were more erratic than those for the total soluble protein.

The release of intracellular enzyme G6PDH relative to the total soluble protein is shown in Figure 3.2.9., where the results of four different *P. putida* ML2 fermentations are presented in a normalised form. The correlation shows a similar average rate of release for both, as is expected for a cytoplasmic enzyme (Follows *et. al.*, 1971).

Effect of Cell Concentration

The effect of cell concentration on the disruption process was evaluated to show that disruption can be reliably performed across the range of concentrations encountered throughout fermentation.

Figure 3.2.10. presents the combined results of a single sample taken at the end of a *P. putida* ML2 fermentation, diluted to four different concentrations (0.24g/L, 0.48g/L, 4.8g/L & 9.76g/L dcw/v) and disrupted within the Micron Lab 40 for five passes at 1600 bar operating pressure. The disruption process was shown to be independent of cell concentration within the range 0.24g/L to 9.76g/L dry cell weight (dcw), such concentrations being expected during *P. putida* ML2 fermentations.

Although this shows that the process is independent of cell concentration, it cannot be concluded that this will be the case with cells taken from different stages of fermentation. This is because the changes that occur during fermentation (e.g. growth) leading to increases in cell concentration also affect cell wall strength.

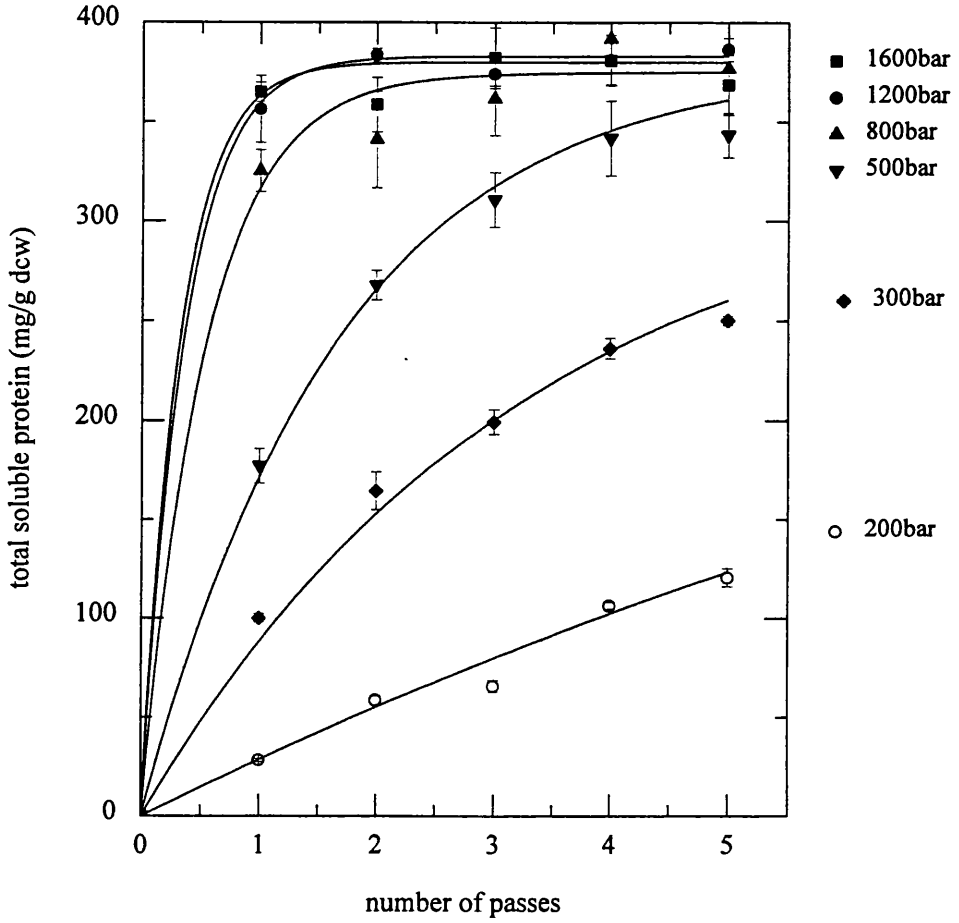


Figure 3.2.7.
Total soluble protein released from *P. putida* ML2 (12.38g/L dcw) with number of passes through the Micron Lab 40 at various operating pressures.
P. putida ML2 cells from fermentation 'B' were processed without any pretreatment.

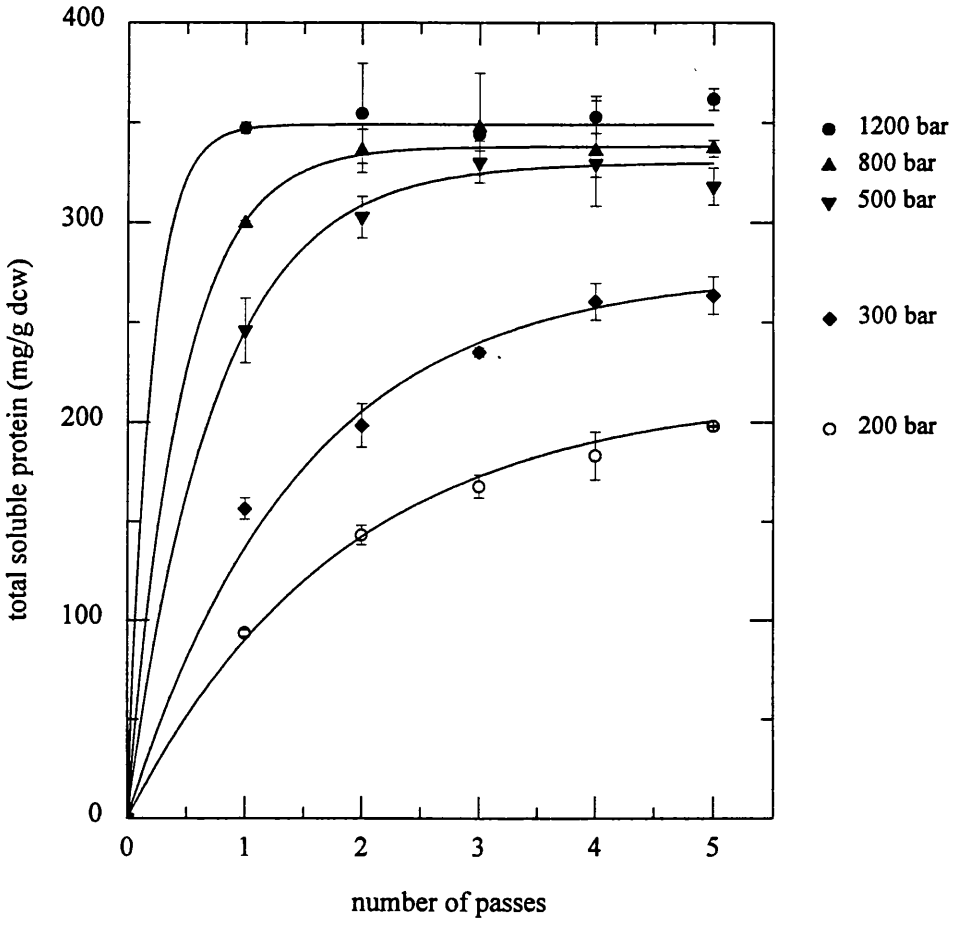


Figure 3.2.8.

Total soluble protein released from *P. putida* ML2 (2.48g/L dcw) with number of passes through the Micron Lab 40 at various operating pressures.

P. putida ML2 cells (9.9g/L dcw) from fermentation 'C' were diluted in fermenter medium to provide a large sample volume, and then processed without further treatment.

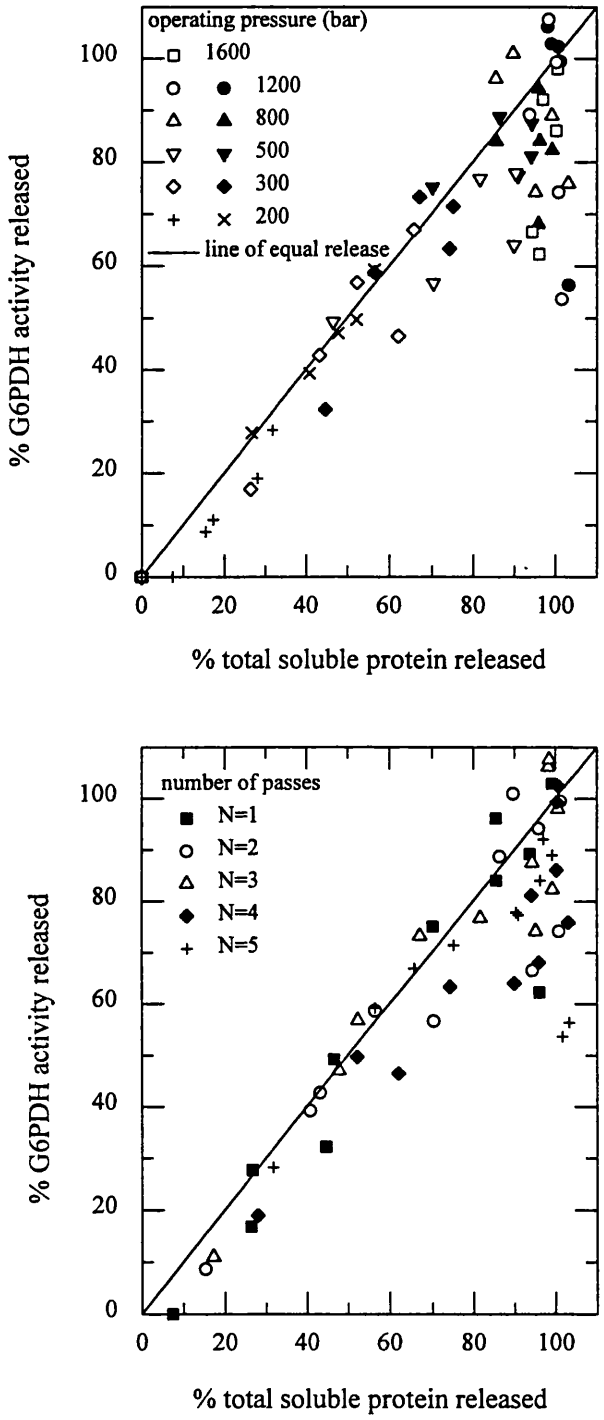
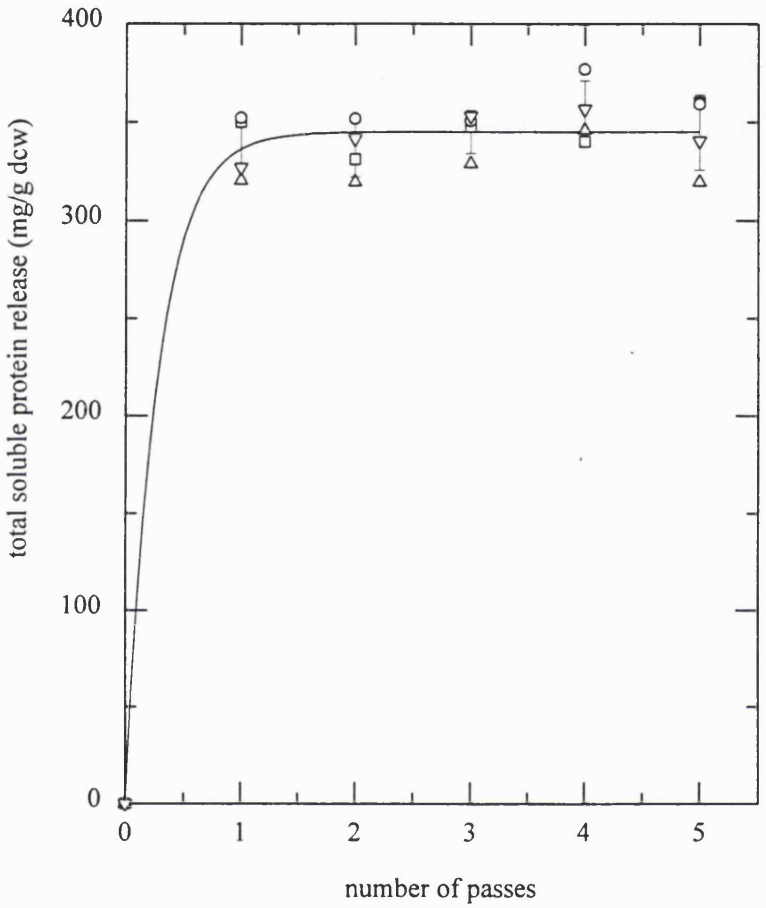


Figure 3.2.9.
Top: Total soluble protein and G6PDH release from *P.Putida* ML2 cells from two fermentations ('B' & 'C') processed in the Micron Lab 40 at various operating pressures;
Bottom: The same data is presented showing the number of passes throught the Micron Lab 40.



cell concentration (g/L dcw)

□ 0.24, ○ 0.48, △ 4.8, ▽ 9.76

error bars represent SD of the protein release for the four concentrations

Figure 3.2.10.

The effect of concentration on the disruption of *P. putida* ML2 in the Micron Lab 40 at 1600 bar operating pressure. (0.24 - 9.76g/L dcw broth concentration)

It is therefore important to obtain representative samples at different stages during fermentation which will necessarily be at different concentrations.

Figure 3.2.11. shows the results from processing *P. putida* ML2 cells for five passes at 1200 bar operating pressure, with the samples covering a range of fermentation conditions from growth to stationary phase. The protein release data are expressed in terms of mg protein per gram dry cell weight. The results confirm the above findings (shown in Figure 3.2.10.) that cell disruption within the Micron Lab 40 is independent of cell concentration and stage of fermentation at these high operating pressures.

The results of the disruption and the resultant protein measurement of *P. putida* ML2 cells covering the range of fermentation conditions, is shown by representing the total soluble protein released after the first pass in terms of percentage release plotted against cell concentration (Figure 3.2.12.). The samples were taken from nine fermentations (also see Section 3.2.3. 'Reproducibility').

Each sample was processed for several passes to ascertain the maximum protein level. This was then used to calculate the percentage release on the first pass. A mean first pass release of 95.6% ± 4.1% was found for the 49 fermentation samples.

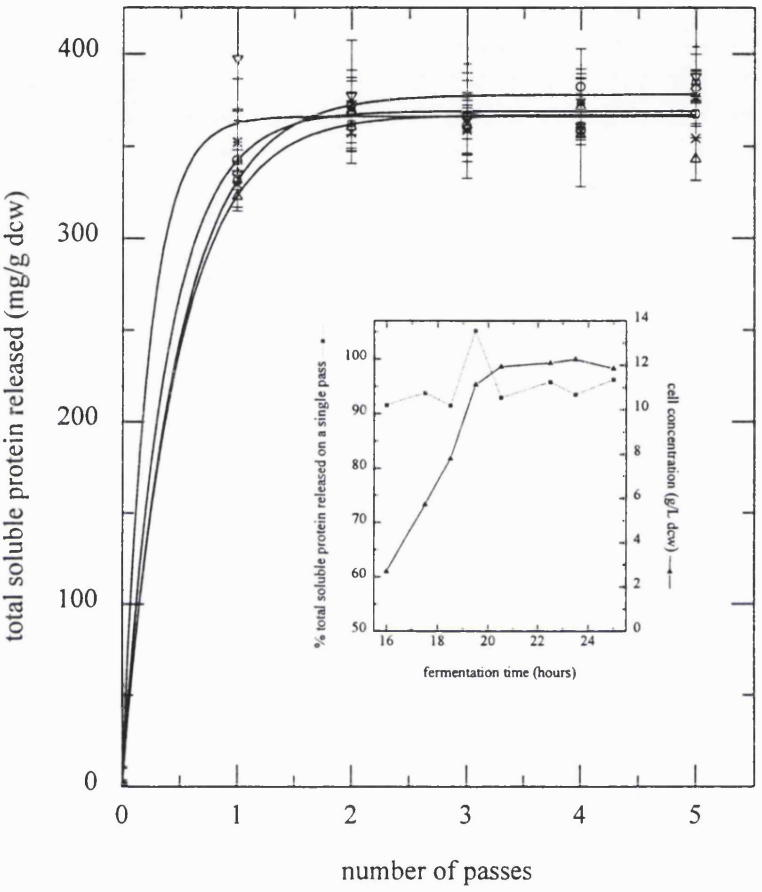
Evaluation of process kinetics

The evaluation of the cell disruption kinetics was undertaken to compare the Micron Lab 40 with pilot scale high pressure homogenisers and to establish criteria under which a small scale high pressure homogeniser with a 2mL to 5mL working volume can be designed. Do we retain the current configuration and simply reduce the working volume or fundamentally alter the design based on the research?

As with *S. cerevisiae*, the evaluation of process kinetics was possible only with the lower homogenisation pressures where there was a slow release rate..

A first order process is shown to be a suitable representation of the disruption kinetics of *P. putida* ML2 within the Micron Lab 40 (Figure 3.2.13.).

Protein release data for cells from *P. putida* ML2 fermentation 'B' for all passes and pressures are plotted in the form of Equation 3.2 (Figure 3.2.14.) to yield values for



samples taken at fermentation times

□ 16 h, ○ 17.5 h, △ 18.5 h, ▽ 19.5 h, ◇ 20.5 h

+ 22.5 h, × 23.5 h, ✱ 25 h, error bars represent SD of the assays; n=3

INSET: Persentage total soluble protein released in a single pass ·· ■ ··

against fermentation time; Cell concentration is also plotted —▲—

Figure 3.2.11.

Total soluble protein released from *P. putida* ML2 fermentation 'A'

with number of passes through the Micron Lab 40 at 1200 bar operating pressure.

Samples taken over nine hours covering growth and stationary phases were processed

without any pretreatment..

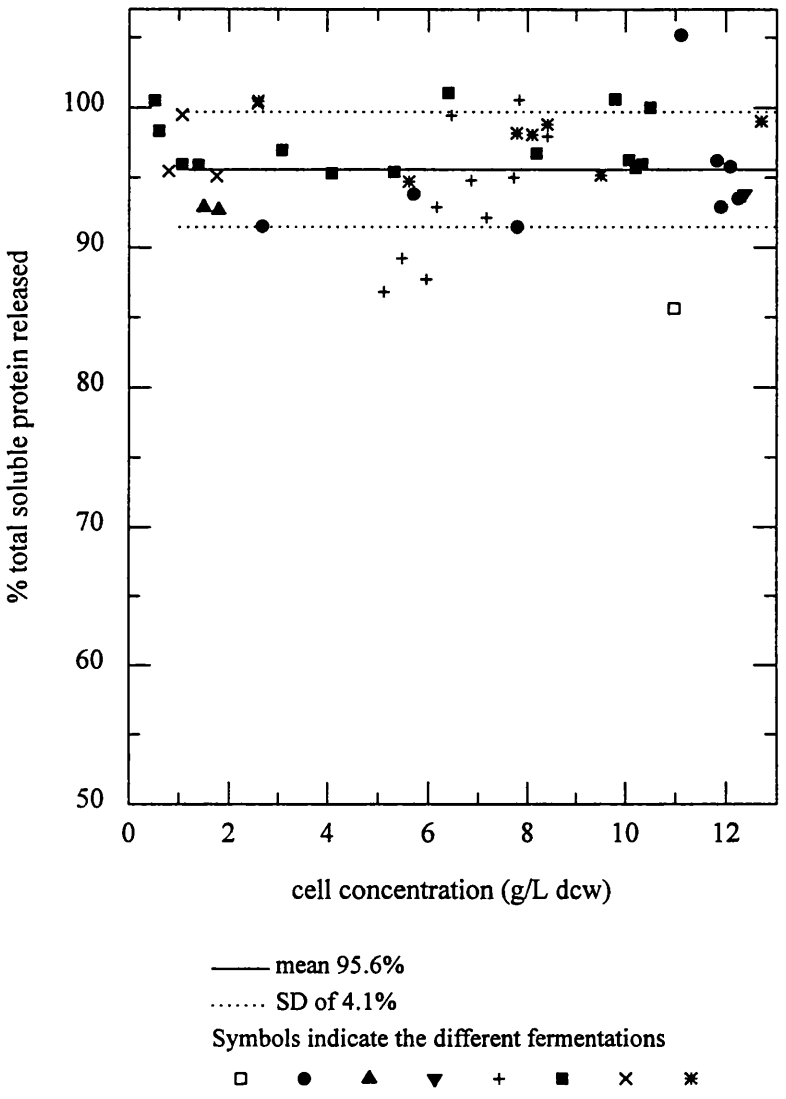


Figure 3.2.12.
Percentage total soluble protein release from *P.putida* ML2
at various stages of nine fermentations plotted against cell concentration
Micron Lab 40 - single pass (1200bar)

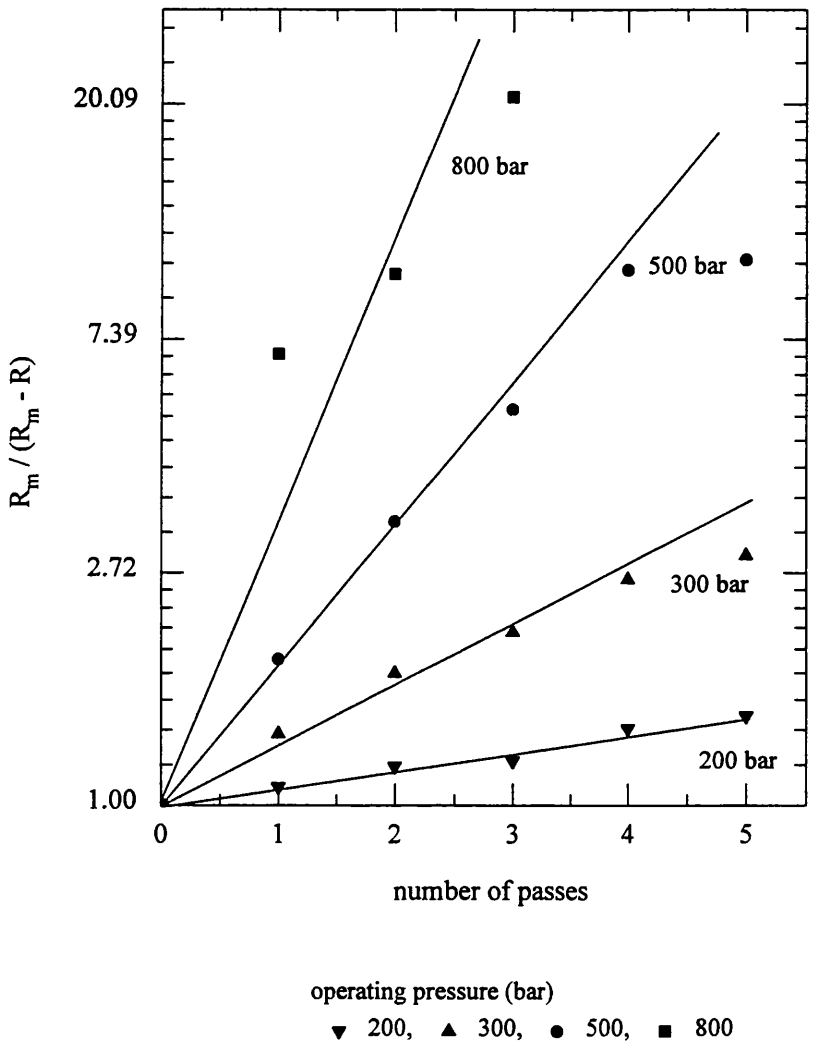


Figure 3.2.13.
First order release of total soluble protein from *P. putida* ML2
(fermentation 'B' - Figure 3.2.7.) in the Micron Lab 40 high pressure homogeniser

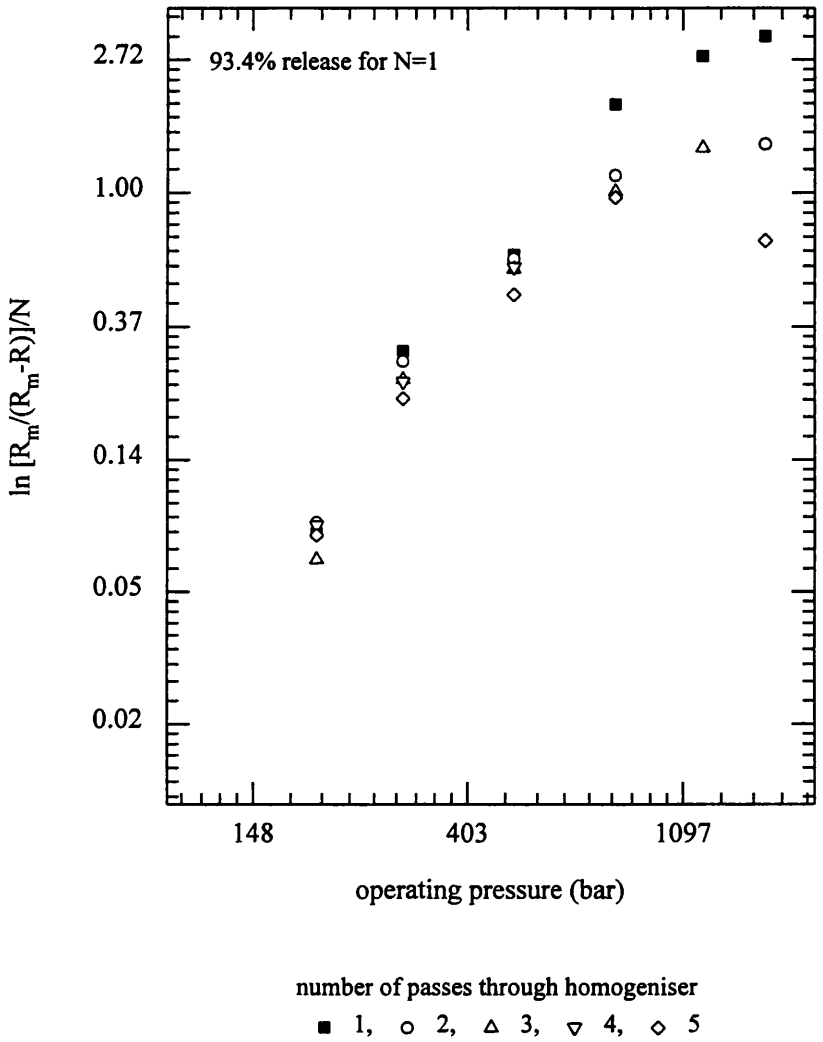


Figure 3.2.14.
Evaluation of pressure dependency for the disruption of *P. putida* ML2 in the Micron Lab 40 - multipass protein release data from Figure 3.2.7.
[operating pressure 100 to 1600 bar]

K = 9.36 x 10^{-6} bar^{-a} and 'a' = 1.77. Similarly, results obtained for the other fermentations are tabulated in Table 3.3.

Fermentation	A #	B *	B #	C	C #	D# ^{exp}	D# ^{stat}
'a'	1.52	1.77	1.52	1.46	1.29	1.12	1.87
K (bar^{-a}) x10^{-4}	0.270	0.094	0.363	1.36	2.4	4.23	0.017
SE 'a'	0.254	0.193	0.130	0.097	0.139	0.209	0.197
SE 'ln K'	1.580	1.243	0.814	0.608	0.849	1.292	1.219
n	23	6	24	5	22	20	21
r^2	0.629	0.955	0.861	0.987	0.812	0.613	0.825

Table 3.3.The values for the dimensional disruption rate, K and the exponent 'a' on the pressure term in Equation 3.2. for *P. putida* ML2 fermentations. [# indicates multipass data; * indicates single pass data for the same samples]. The standard errors (SE's) and other statistical information is also given.

These results are comparable with values obtained for *S. cerevisiae* of 'a' = 1.76 and the dimensioned disruption rate constant K = 1.06 x10^{-5}. (Also see Table 3.2. above).

Using these values for 'a' and K to calculate the theoretical percentage cell breakage (total soluble protein release) emphasises the need to operate the Micron Lab 40 at very high operating pressures (Table 3.4.).

The theoretical values for cell breakage at 500 bar operating pressure in Table 3.4. also point to variations in the cell strength of cells taken from similar *P. putida* ML2 fermentations . In particular, cells taken from fermentation labelled 'D' in exponential phase are substantially weaker than those taken during the stationary phase of the same fermentation.

Fermentation	% Protein released at 500 bar single pass	% Protein released at 1600 bar single pass
A #	29.0	86.5
B *	43.0	98.8
B #	36.8	93.2
C	69.5	99.8
C #	51.7	96.2
D# ^{exp}	36.0	80.6
D# ^{stat}	17.3	81.1
Bakers yeast	44.9	99.0

Table 3.4.The % total soluble protein released on a single pass through the Micron Lab 40 at 500 bar and 1600 bar based on the values for K and 'a' in Table 3.3.

3.2.3. Cell disruption and the effect of the impact ring

In trying to understand the mechanism of cell disruption it was hoped to define the criterion or features for scaling down of the homogeniser; whether to retain the current valve geometry and simply reduce the working volume (e.g. 40mL to 5mL) or given our understanding of cell disruption mechanism, to arrive at a more fundamental design. The Micron Lab 40 was employed as a 'stepping-stone' towards a new design.

To conclude the work with the cell disruption kinetics within the Micron Lab 40 high pressure homogeniser, a series of experiments were conducted to assess the effect of the impact ring design. (Figure 2.3. in Section 2 'Materials and Methods', shows the valve assembly). On the power stroke the cell suspension is forced through the valve seat, then squeezed and accelerated through the valve gap and then impacted against the impact ring at very high velocities.

Work within the Department several years ago gave valuable insight into the mechanism of such a process. In essence, with a similar homogeniser, it was concluded that both the valve gap (dependent on valve geometry) and the impact distance were important in the disruption process (Keshavarz *et. al.*, 1990). The work was conducted at an operating pressure of 460 bar. The Micron Lab 40 with its greater range of operating pressures provided an opportunity to take their work further.

As put forward by Keshavarz and co-workers, 1990, varying the size of the impact ring would have two possible effects:

- variation in the back pressure of the system;
- involvement in the impact phenomenon.

Their investigations led them to conclude that the effects due to the back pressure were insignificant and also assumed that the changes in impact distance, X, had a minor effect on the valve gap. As such investigations employing changes in the impact ring internal diameter would not significantly alter other operating parameters.

In the Micron Lab 40 trials, three sizes of impact ring were acquired from APV (Lubeck, Germany). These included impact ring with an internal diameter of 5.5 mm

(standard size), 7.5mm and 9.5 mm. The largest diameter resulting in the largest impact distance being set at the maximum possible without compromising the safe operation of the homogeniser (the impact ring acts as a spacer which prevents the travel of the valve seat through the valve housing). The impact distances were 0.35 mm, 1.35 mm and 2.35 mm respectively. Because of safety reasons (i.e. severe damage to homogeniser section) it was not possible to acquire an impact ring to provide an even smaller impact distance.

According to the design engineers of the Micron Lab 40 (Jarchau, 1994) the changes in impact ring internal diameter will not significantly alter any other operating parameter within the homogeniser.

S. cerevisiae (packed yeast) was made up to 10% packed weight/volume and poured (well-mixed) into 50 falcon tubes so as to allow 50 disruption cycles to be performed which would include the use of different impact rings. Each aliquot of the cell suspension was processed through the Micron Lab 40 for a single pass at operating pressures covering the entire range from 100 bar to 1600 bar in steps of 100 bar.

Figure 3.2.15. shows the results from these experiments. The error bars represent a standard deviation of 5% based on work reported in Section 3.2.3. Maximum total soluble protein release was achieved in a single pass at pressures exceeding around 1000 bar with the standard impact ring. The protein release using the other two impact rings which had greater impact distances failed to reach the maximum even at an operating pressure of 1600 bar. Thus, the importance of the impact distance (impact ring) has been demonstrated within the homogeniser over the pressure range and the valve configuration studied. Now, if the Micron Lab 40 results can be scaled-up to the Lab 60 then it can be expected to show the importance of the impact ring at an extended range of operating pressures to compliment the work by Keshavarz *et. al.*, 1990 (carried out at 460 bar operating pressure).

A multipass cell disruption within the Micron Lab 40 was carried out on *S. cerevisiae* (10% w/v) at 500 bar and 1200 bar operating pressures using the three different impact rings. Applying the familiar disruption kinetics for the 500 bar results, (Figure 3.2.16.), it was evident that the process remains first order, and that the dimensionless disruption rate constant, k, increased with decreasing impact distance (i.e. with decreasing impact ring internal diameter) as shown in Table 3.2.

impact distance	k (at 500bar)	'a'	K ($x10^{-6}$)	n	r^2
2.35mm	0.06	2.37	0.047	16	0.986
1.35mm	0.11	2.04	0.893	16	0.986
0.35mm	0.32	2.03	2.310	13	0.974

Table 3.4. The first order dimensionless disruption rate constants, k, for the disruption of *S. cerevisiae* within the Micron Lab 40 operating at 500 bar pressure using the three different impact rings. The exponent on the pressure term, 'a', is also given along with the associated dimensioned rate constant. [R_m was taken as 11.00mg/mL]

Figure 3.2.17. shows the pressure dependency of total soluble protein released from *S. cerevisiae* (10% w/v packed yeast) following a single pass through the Micron Lab 40 over a range operating pressure (100-1600bar) for the three different impact rings (i.e. impact distances).

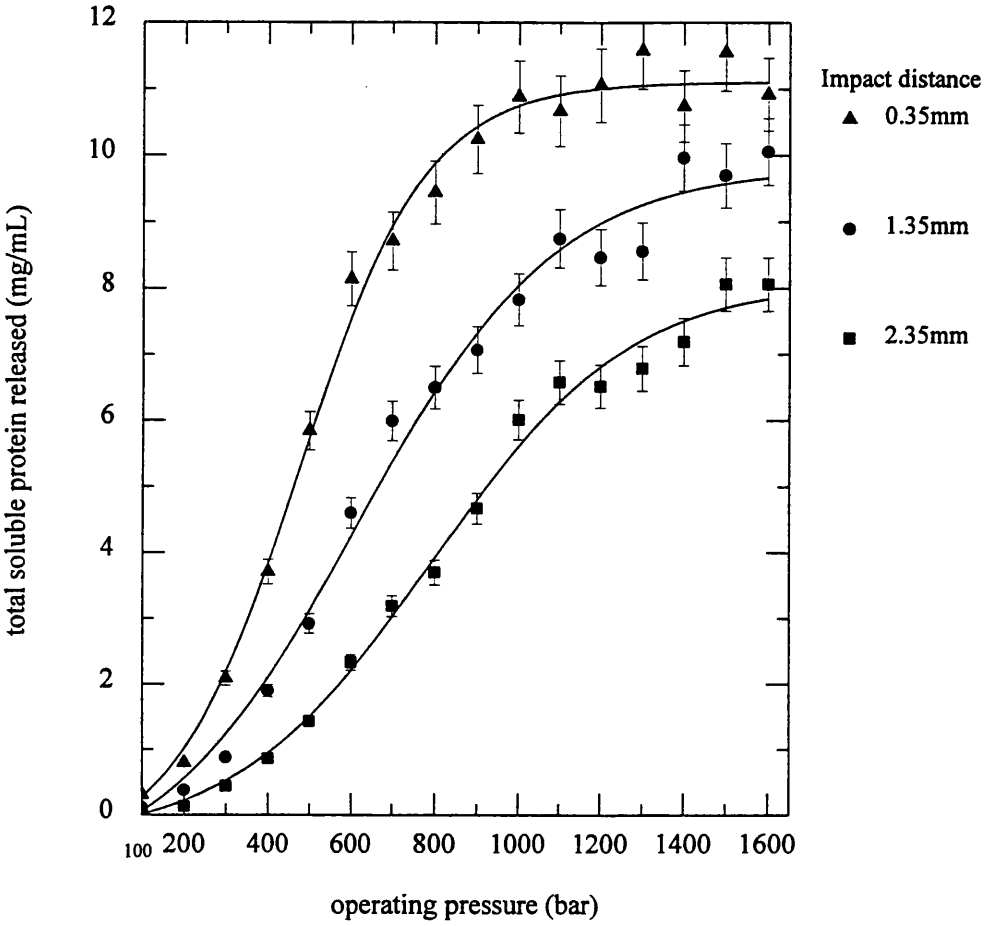


Figure 3.2.15.
Influence of the impact ring, impact distance on cell breakage.
Total soluble protein released from *S. cerevisiae* (10% w/v packed yeast) with a single pass through the Micron Lab 40 employing three different impact rings at various operating pressures.

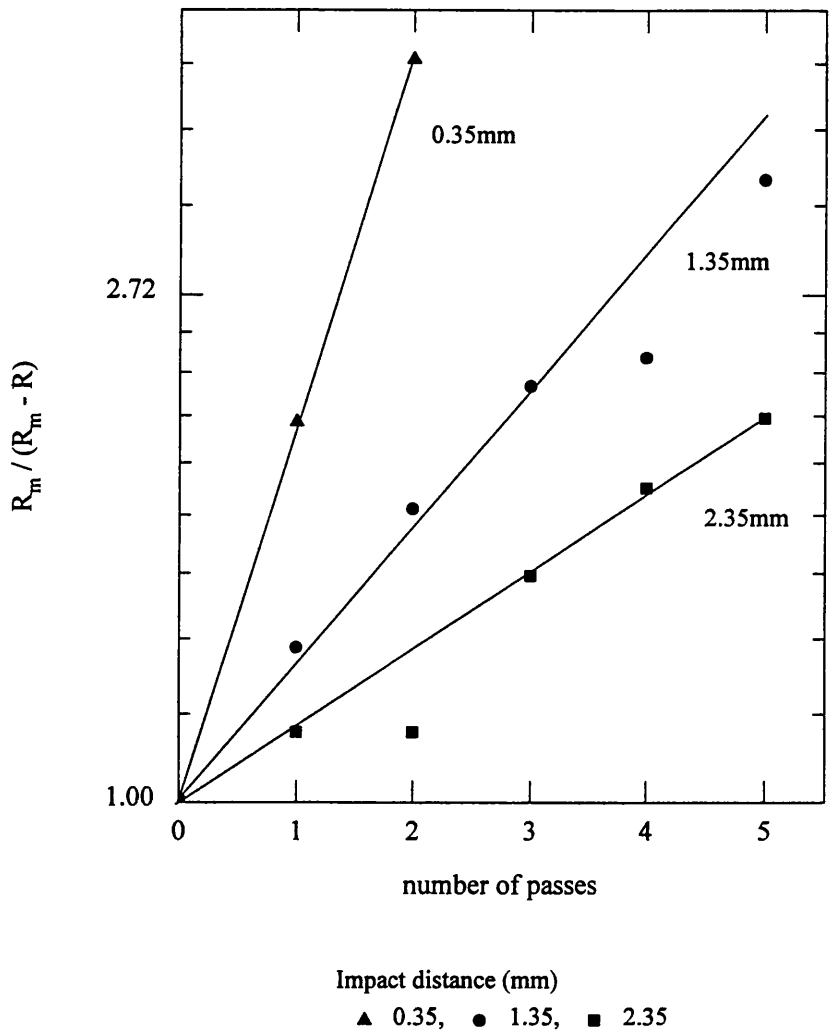


Figure 3.2.16.
Influence of the impact ring, impact distance on cell breakage.
First order release of total soluble protein from *S. cerevisiae* (10% w/v packed yeast) in the Micron Lab 40 employing three different impact rings at 500 bar operating pressure.

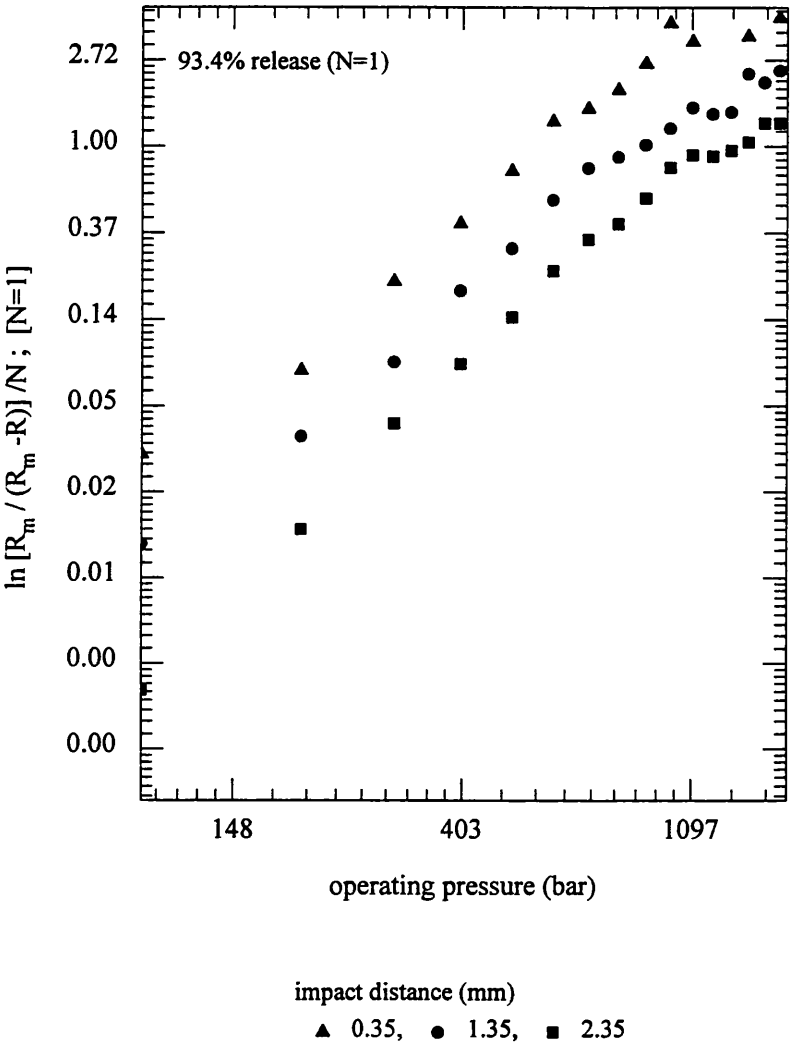


Figure 3.2.17.
Evaluation of the pressure dependency of total soluble protein release from *S. cerevisiae* (10% w/v packed yeast) homogenised over a range of operating pressures in the Micron Lab 40 using three different impact rings - single pass data.

3.3. Reproducibility of cell disruption

In the assessment of the reliability and reproducibility of the cell disruption and intracellular measurement, experiments were repeated numerous times to allow statistical analysis to be carried out. This would evaluate the confidence in the data obtained with cells taken from varying fermentation conditions.

It was decided to use the Micron Lab 40, with single pass at an operating pressure of 1200 bar for the fermentation monitoring work. As discussed elsewhere (in Section 3.2.1., in 'Kinetics of Disruption'), operating at this pressure yielded high levels of cell disruption. It also overcame any of the changes in cell susceptibility to break-up during fermentation discussed later in Section 3.4. (in 'Profile Monitoring'). The use of even higher pressures was possible, but increased pressure did not yield proportional gains in cell breakage within the Micron Lab 40.

Statistical analysis

Several sources of error for any resultant measurement are considered in this section. The statistical analyses were conducted at 5% confidence level. In all cases the final total soluble protein assays were conducted in triplicate and ADH assays in duplicate.

3.3.1. Disruption within the Micron Lab 40 and intracellular component assays.

The disruption will necessarily involve the complete process through to protein or enzyme assay. The stages of analysis under consideration as sources of potential error are:

- the complete process - disruption to measurement,
- sampling from the homogeniser,
- dilution for measurement, and
- the assay itself.

This is illustrated in Figure 3.3.1.

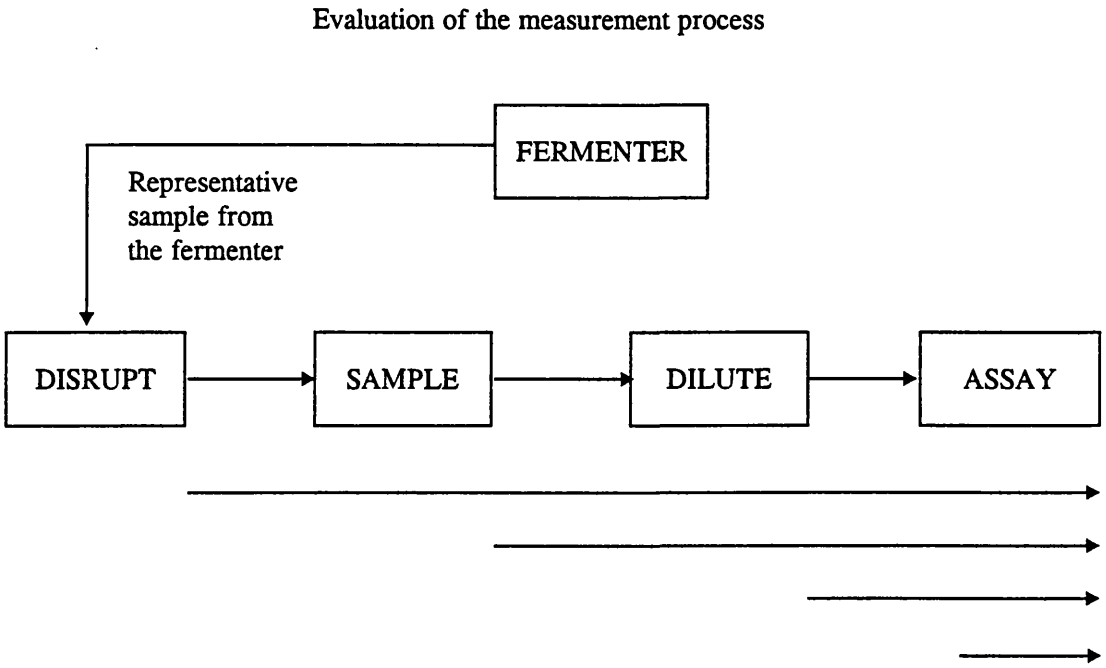


Figure 3.3.1. The evaluation of the measurement process from cell disruption in the Micron Lab 40 to the assay for the intracellular component.

3.3.1.1. The analysis of the complete disruption process using *S. cerevisiae*.

To consider the sources of errors from disruption to measurement, fifty 40mL samples of *S. cerevisiae* 1% and 45% w/v (packed yeast) were processed for a single pass at 1200 bar through the Micron Lab 40.

The release of total soluble protein and alcohol dehydrogenase (ADH) activity was assayed.

The percentage total soluble protein and ADH released on the single pass was calculated based on the maximum protein release from four samples processed for a total of five passes. This gives the R_m value for maximum total soluble protein available for release. This was carried out for both concentrations. Basing the R_m value for the sample population on only four samples, it is possible that these yield lower values for the maximum, which can be the result of product denaturation on multi-pass operations. The mean percentage release of ADH was 105.5 ± 5.7% and 102.1 ± 3.8% and the mean percentage total soluble protein release was 105.5 ± 6.7% and 98.1 ± 5.7% for the two populations respectively (1% and 45% w/v packed yeast), as shown in Figures 3.3.2. & 3.3.3.

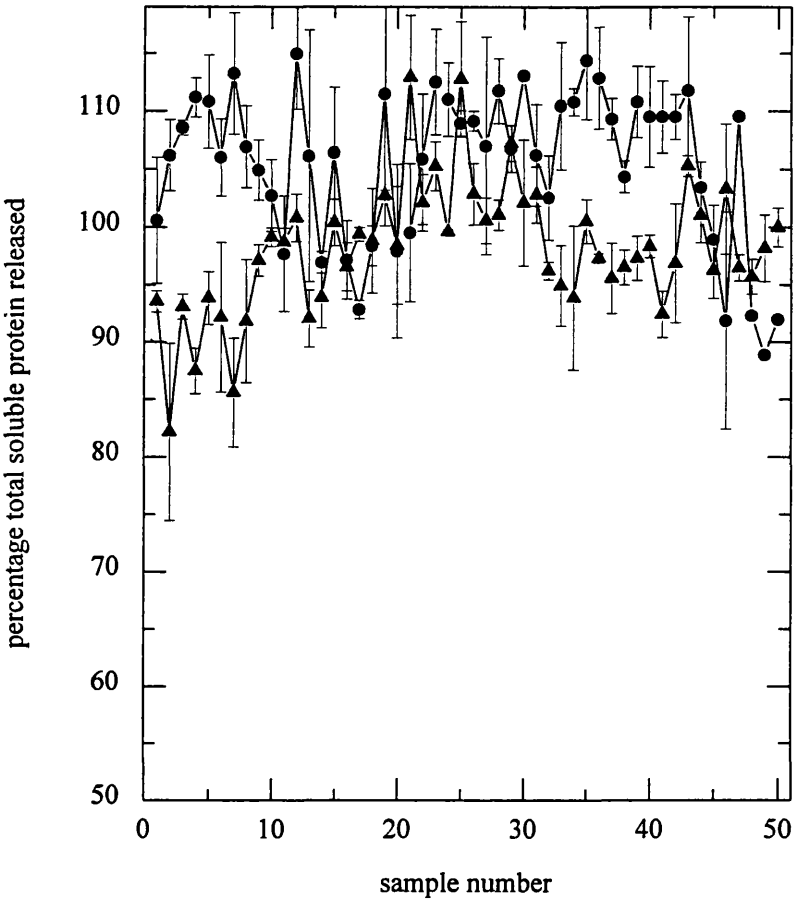


Figure 3.3.2.
Assessment of the disruption process.
Percentage total soluble protein release from *S. cerevisiae* (1% and 45% w/v packed yeast) in Micron Lab 40 at 1200 bar operating pressure - single pass.
Four of these samples were processed for 5 passes to yield the 100% values

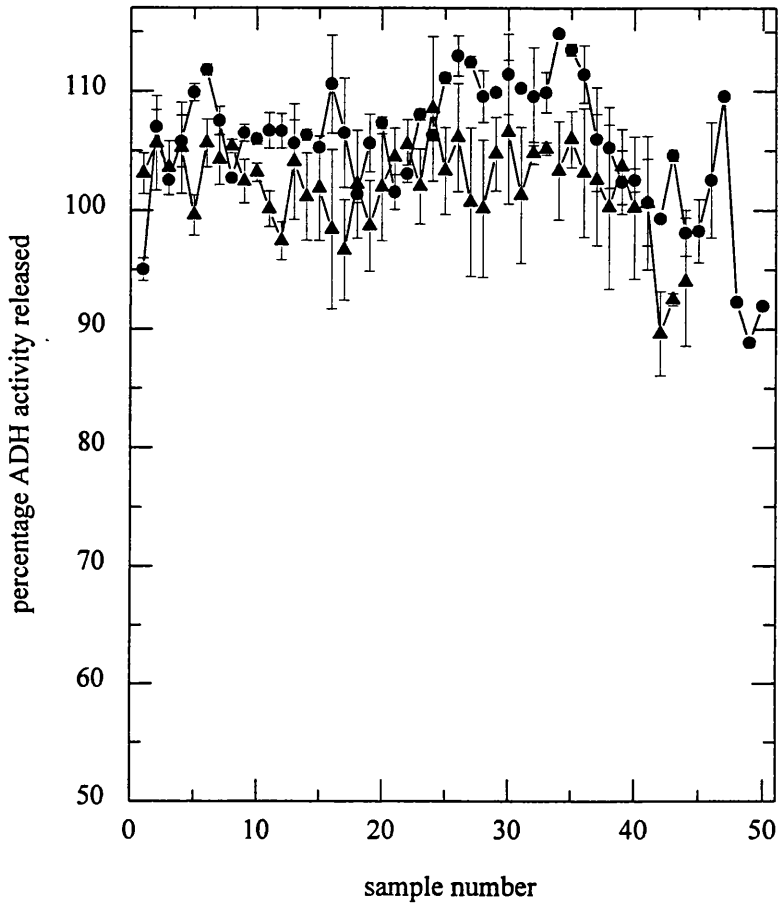


Figure 3.3.3.
Assessment of the disruption process.
percentage ADH activity released from *S. cerevisiae* (1% and 45% w/v packed yeast) in the Micron Lab 40 at 1200 bar operating pressure - single pass.
Four of these samples were processed for 5 passes to yield the 100% values

The analysis of the variances was conducted employing the F-test. The null hypothesis could not be rejected for both total soluble protein and ADH data (i.e., there was no significant difference in the variances).

However, t-tests conducted yielded a rejection of the null hypothesis. Thus indicating that the means of total soluble protein and ADH from 1% w/v and 45% w/v cell concentrations were significantly different. The statistical difference in the mean values is not crucial since this can be attributed to the four samples (for each case), used as the bases for calculating R_m, the maximum protein and ADH release, giving different maximum values for normalisation. These maximum values can also be lower than the means for the population giving rise to normalised population means greater than 100%, as in the above cases.

Figure 3.3.4. shows the percentage ADH released as a fraction of the percentage total soluble protein released against the sample number for the above data (Figures 3.3.2. and 3.3.3.). The yields mean values of 1.00 ± 0.06 and 1.05 ± 0.08 for the 1% w/v and 45% w/v suspensions.

3.3.1.2. The analysis of the complete disruption process using *P. putida* ML2.

Twenty-five 40mL samples of *P. putida* ML2, 4.53g/L dry weight and twenty-one at 9.89g/L dry weight were processed for a single pass at 1200 bar through the Micron Lab 40. Similarly, the mean percentage total soluble protein released on the single pass was calculated based on the disruption results of two samples processed for five passes. The percentage release was 105.1 ± 4.3% and 104.4 ± 3.6% for the two populations respectively (Figure 3.3.5.).

The variances and mean were analysed employing the F-test and t-test respectively. For both cases the null hypothesis could not be rejected, thus showing that there was no significant difference in the variances, and in the means. This is contrary to the findings for *S. cerevisiae* where the means were found to be different, where the difference was put down to different R_m values giving rise to different population means. For the *P. putida* ML2, the two concentrations were not so markedly different as was the case with the yeast.

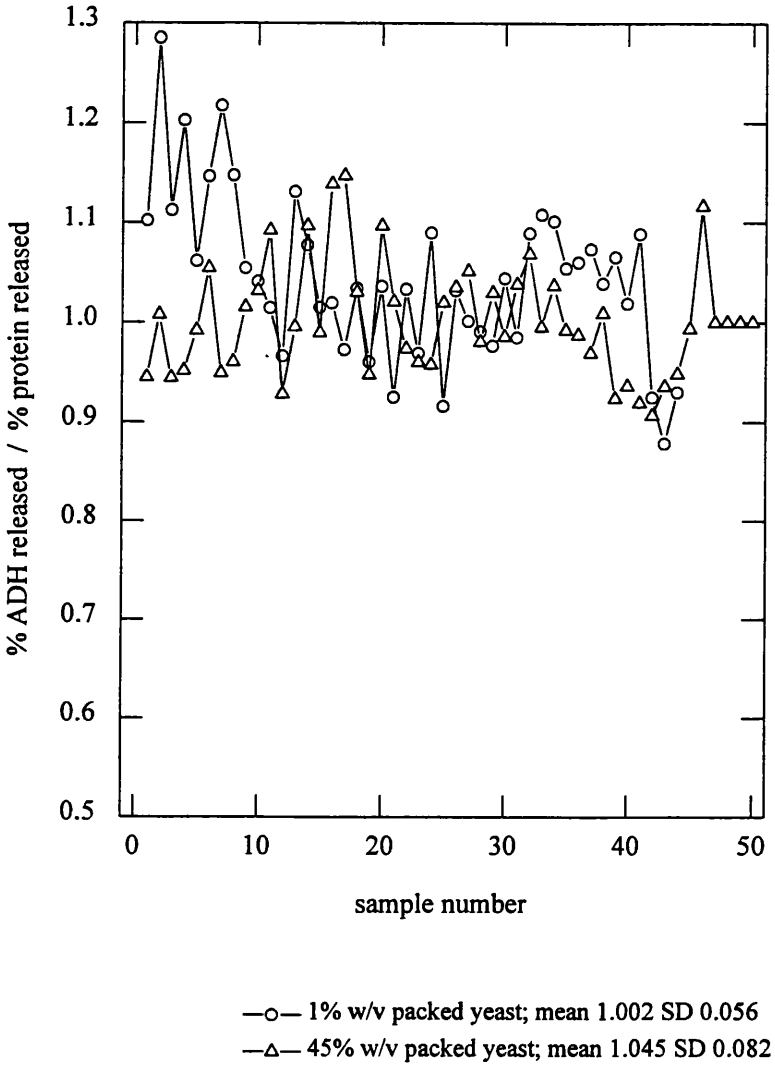


Figure 3.3.4.
Assessment of the disruption process within the Micron Lab 40 - 1200 bar; single pass.
Releative release of ADH to total soluble protein; *S. cerevisiae* (1% and 45% w/v packed yeast)

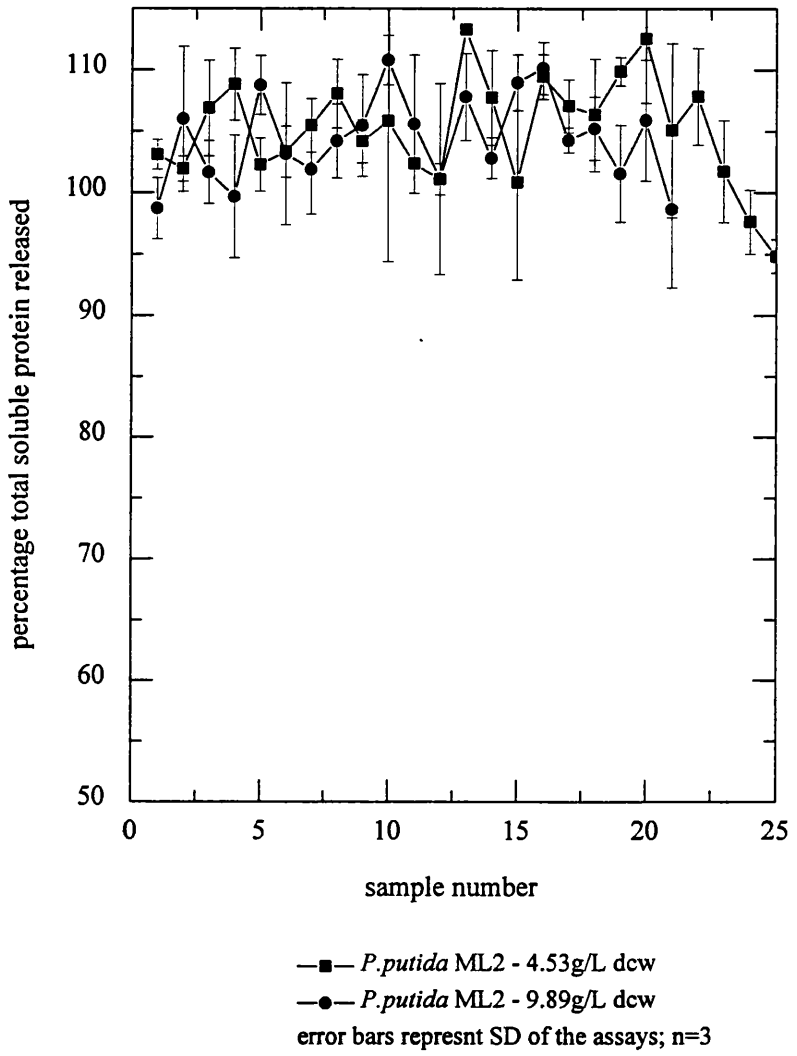


Figure 3.3.5.
Percentage total soluble protein released from *P. putida* ML2 (4.53 and 9.89g/L dcw) disrupted in the Micron Lab 40 at 1200 bar operating pressure - single pass.

3.3.1.3. The analysis of the complete disruption process using *E. coli*

The statistical analysis of the complete disruption process with *E. coli* was carried out to evaluate the confidence in the data obtained with cells taken from varying *E. coli* fermentations.

Twenty 40mL samples of *E. coli* taken from a fermentation at 10.3g/L dcw were processed after a single pass at 1200 bar through the Micron Lab 40. The mean percentage total soluble protein released after a single pass was calculated based on the disruption results of one sample processed for three passes. The mean release was 94.5% ± 5.0% (three values were rejected as outliers being greater than three standard deviations from the mean as shown in Figure 3.3.6.).

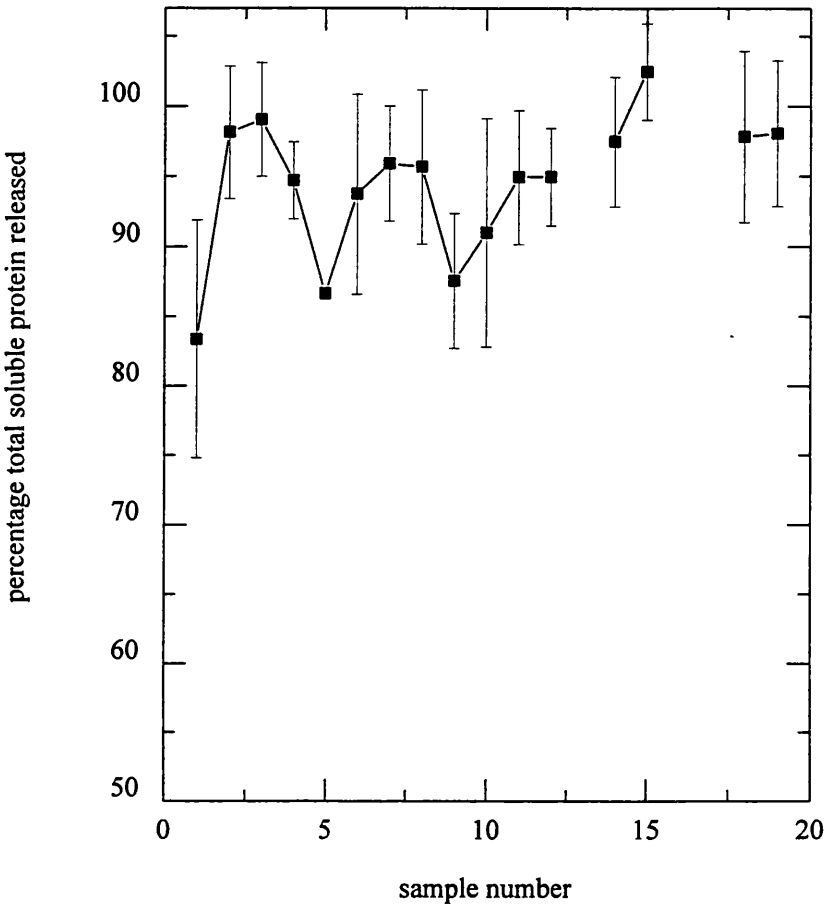
3.3.1.4. Analysis of the sections making up the complete process.

This analysis would identify the errors resulting from each section, and so point out where improvements could be made and where greater operator care was needed.

Taking a well mixed representative sample from the fermenter the measurement process included disruption in the Micron Lab 40, the removal of a sample from the homogenate, the dilution for the protein / enzyme assay, and the assay itself. This concept is illustrated in Figure 3.3.1. (earlier).

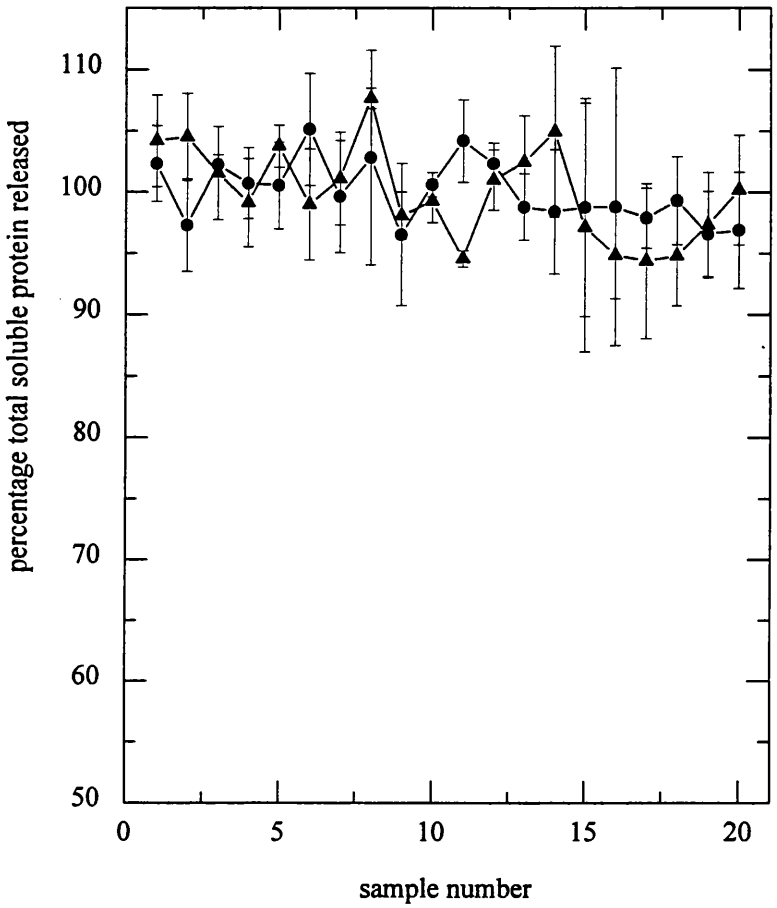
Sampling from the Micron Lab 40 (*i.e.* the homogenate)

Single 40mL samples of *S. cerevisiae* 1% w/v and 45% w/v (packed yeast) were homogenised within the Micron Lab 40 at 1200 bar operating pressure. From each of the resultant homogenates, twenty samples were taken and their supernatants assayed for total soluble protein released. The standard deviations were found to be 2.5% and 3.8% of the means for 1% w/v and 45% w/v cell concentrations respectively. Although these values are low, analysis of the variance resulted in the rejection of the null hypothesis, i.e. the variances of the two populations are significantly different (Figure 3.3.7.).



—■—% total soluble protein; mean = 94.5%, SD = 5.0%
E.coli (10.3g/L dcw)
error bar represent the SD for the repeated assays; n=3.

Figure 3.3.6.
Assessment of the cell disruption process for *E. coli* (10.3g/L dcw) in the Micron Lab 40 - 1200 bar - single pass.



packed yeast
—●— 1% w/v SD of 2.5%
—▲— 45% w/v SD of 3.8%
error bars represent the SD on repeat assays; n=3

Figure 3.3.7.
Assessment of the disruption process - sampling from homogeniser.
Percentage total soluble protein assayed during repeated homogenate sampling from *S. cerevisiae* [1% & 45% w/v packed yeast] disrupted in the Micron Lab 40 - single pass

Dilution for assaying.

A 1mg/mL solution of BSA was diluted to the same level 25 separate times and assayed in triplicate. This yielded a mean value of 99.7 ± 3.4% (Figure 3.3.8.).

BSA was used instead of total soluble protein from yeast, *P. putida* ML2 or *E. coli* simply because it is used in constructing the standard calibration curve for the protein assay.

Assaying for G6PDH

In order to assess the repeatability of the G6PDH assay, 28 separate assays were conducted for a single homogenate supernatant sample. The resultant standard deviation for the assays was 5.1% (Figure 3.3.9.).

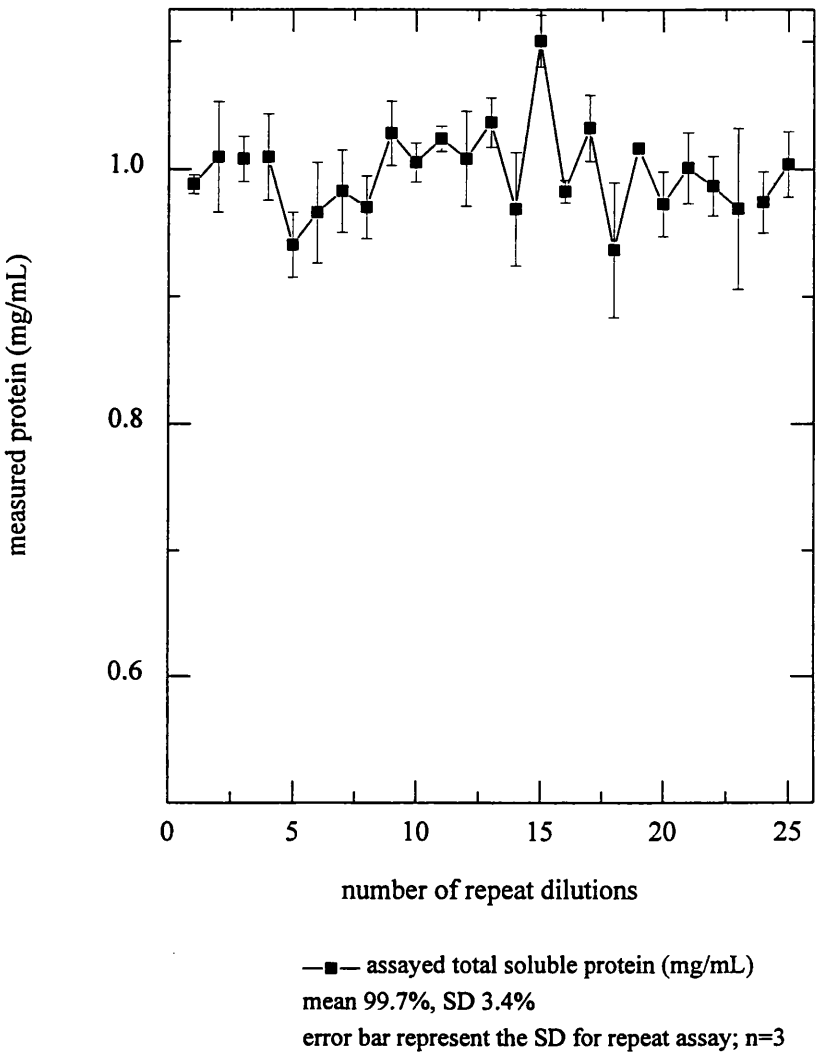


Figure 3.3.8.
Assessment of the disruption process - Dilution.
The results from assays of repeat dilutions of a 1mg/mL BSA solution

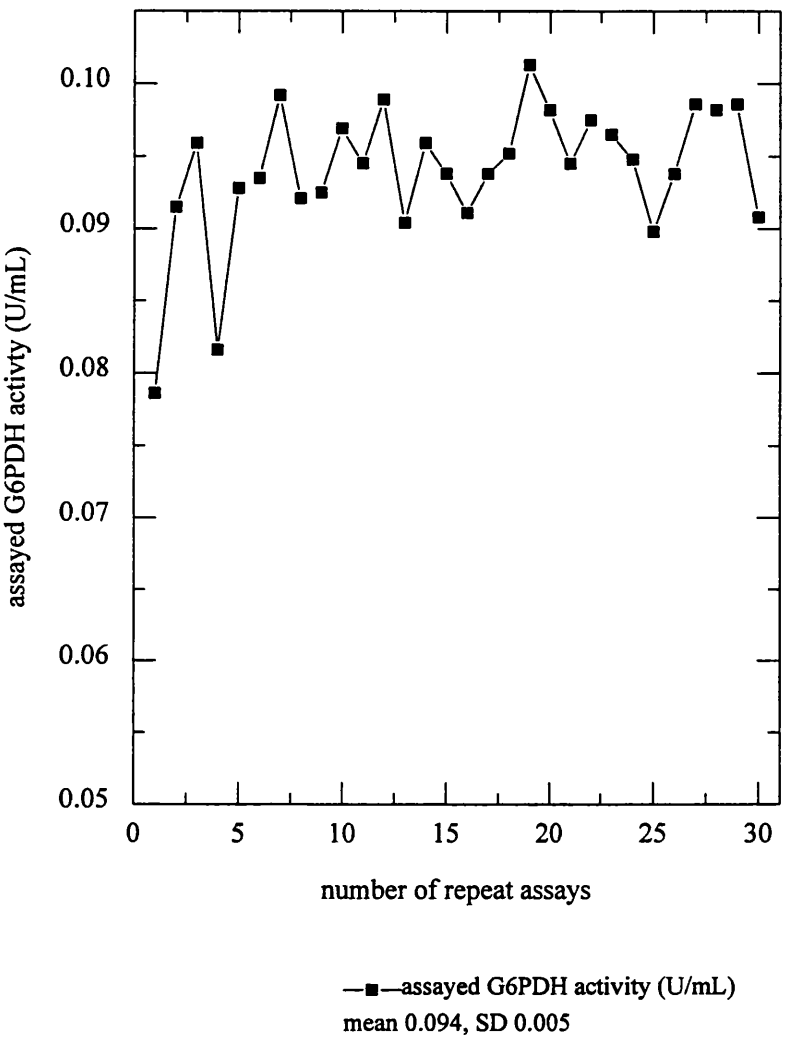


Figure 3.3.9.
Assessment of the disruption process - Assays.
The results from repeated assays for G6PDH activity from a single sample.

The overall results for the statistical analysis are represented in diagram form in Figure 3.3.10.

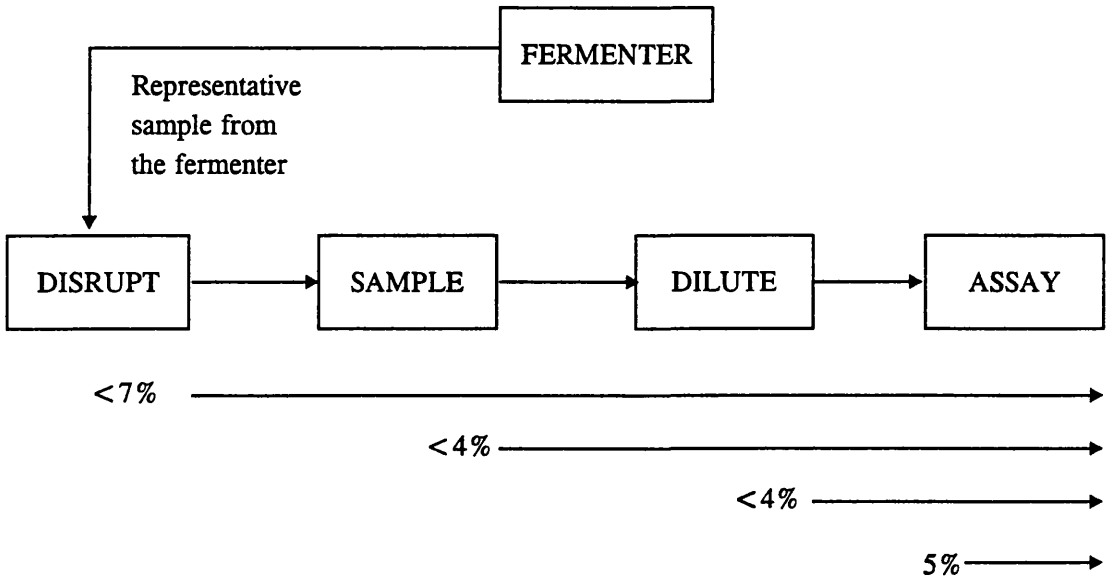


Figure 3.3.10. The evaluation of the measurement process from cell disruption in the Micron Lab 40 to the assay for the intracellular component.

3.3.2. Reproducibility of the disruption across a range of fermentation conditions.

It is clear from the results above that the process has the potential of providing reproducible results. To take this further and show its applicability to fermentation and so inclusion in a monitoring scheme, the disruption process has to be evaluated for with samples taken from fermentations. To continue the assessment of the reliability and reproducibility of the cell disruption technique, experiments were repeated numerous times, with a range of organisms, to allow statistical analyses to be carried out.

Samples from fermentations of *P. putida* ML2, *E. coli*, *S. cerevisiae* and *R. erythropolis* were disrupted at 1200 bar operating pressure, and single pass through the Micron Lab 40. Statistical analysis was carried out on the data. The results are given below:

3.3.2.1. *P. putida* ML2

The reproducibility of the disruption and the resultant protein measurement of *P. putida* ML2 cells covering the range of fermentation conditions from growth to stationary phase, is shown by representing the total soluble protein released after the first pass in terms of percentage release (Figure 3.3.11.). The samples were taken from nine fermentations.

Each sample was disrupted up to five passes to ascertain the maximum protein level (R_m). This R_m value was then used to calculate the percentage release on the first pass. For the 49 samples from fermentation this yielded a mean of 95.6% ± 4.1% release.

This means that the technique used (release on intracellular product using the Micron Lab 40 operating at 1200 bar, single pass) results in over 90% release irrespective of fermentation and the stage of fermentation.

3.3.2.2. *E. coli.*

The reproducibility of the disruption of *E. coli* cells within the Micron Lab 40 operating at 1200 bar pressure, covering the range of fermentation conditions from growth to stationary phase, is shown by representing the total soluble protein

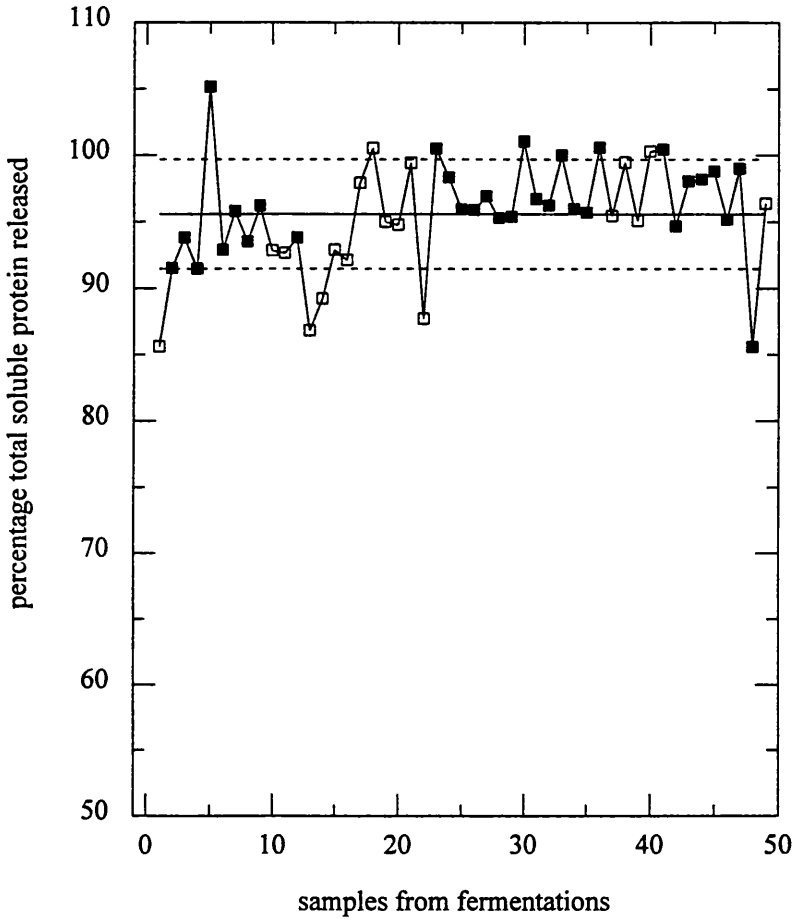


Figure 3.3.11.
Assessment of the disruption process
Percentage total soluble protein release from *P.putida* ML2 samples
taken at various stages of nine fermentations; Micron Lab 40 - 1200 bar - single pass
(each sample was processed for upto 5 passes to obtain the respective maximum values)

released after the first pass in terms of percentage release. A mean of 96.1% ± 6.9% was obtained from 27 samples taken from two fermentations (Figure 3.3.12.).

The analyses of the variances and means employing the F-test and t-test respectively were conducted for the data from two fermentations and the repeated disruption of a single sample. For all cases the null hypothesis could not be rejected, thus showing that there was no significant difference in the variances, and in the means. This indicates that the process of measurement is statistically reliable (as indicated by the test on the variance) and reproducible (as indicated by the test on the means).

3.3.2.3. *S. cerevisiae*

The reproducibility of the disruption of *S. cerevisiae* cells within the Micron Lab 40 operating at 1200 bar pressure, covering the range of fermentation conditions, is shown by representing the total soluble protein released after the first pass in terms of percentage release. The 44 samples taken from three fermentations yield a mean of 96.1% ± 4.4% (Figure 3.3.13.).

3.3.2.4. The analyses of disruption of various micro-organisms

Samples from several fermentations of various micro-organisms were disrupted to assess the level of disruption achieved in a single pass through the Micron Lab 40 (operating at 1200 bar). The total soluble protein releases are shown in Figure 3.3.14. and the mean releases are shown in Table 3.5.

Organism	mean percentage release	standard deviation	n
E. coli (recombinant; samples taken from steady state only)	95.6%	2.9%	9
R. erythropolis (1000L)	84.2%	4.5%	9
R. erythropolis (20L)	84.9%	6.0%	4

Table 3.5. The mean total soluble protein releases and standard deviations for various micro-organisms taken during the course of fermentations.

The analyses of the variances and means were conducted employing the F-test and t-test respectively. The results of the t-tests were not surprising in that the mean total soluble protein releases for *R. erythropolis* and the other organism (the recombinant *E .coli*) were found to be significantly different. The differences in the means for the range of micro-organisms implies that the level of cell disruption on a single pass through the Micron Lab 40 at 1200 bar operating pressure was significantly different, statistically, when comparing all the micro-organisms together.

The t-test results comparing the two fermentation scales for *R. erythropolis* showed them not to be statistically significant. The results from the F-test showed that there was no significant difference in the variance.

Rhodococcus erythropolis is known to be a particularly difficult organism to disrupt and as such provided an interesting challenge to test the Micron Lab 40.

Samples from a single fermentation were disrupted and the maximum protein release value was obtained as described above. The cells were grown on a complex medium, and were resuspended in glycine buffer prior to disruption at 1200 bar operating pressure single pass. Removal of the fermenter broth and resuspension in the glycine buffer was needed to prevent denaturation of one of the enzymes of interest.

The mean total soluble protein release on a single pass was 84.2% with a standard deviation of 4.1%. It could be argued that there was a real decrease in the percentage release as the fermentation continues into stationary phase. Clearly a low value for the standard deviation suggests that the process of cell disruption within the Micron Lab 40 was statistically reproducible. However, what is of significance is the low level of cell breakage as measured by total soluble protein release.

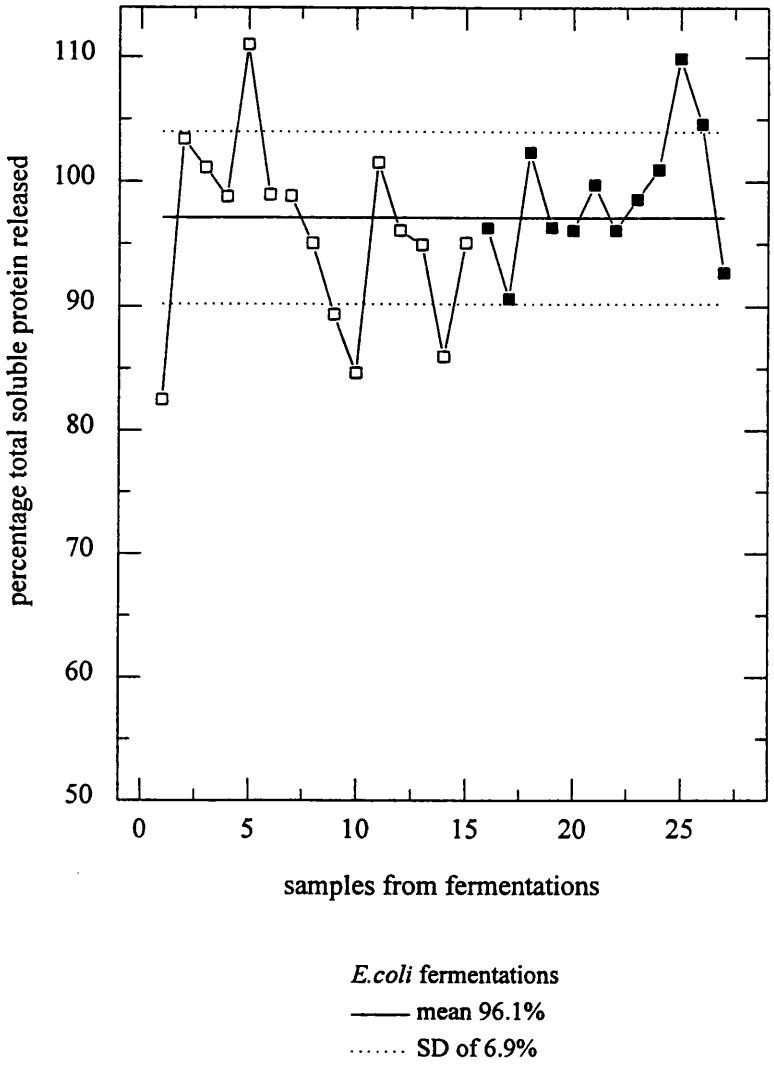


Figure 3.3.12.
Assessment of the disruption process.
Percentage total soluble protein release from *E.coli* samples taken at various stages of two fermentations; Micron Lab 40 - 1200 bar - single pass
(each sample was processed for upto 5 passes to obtain the respective maximum values)

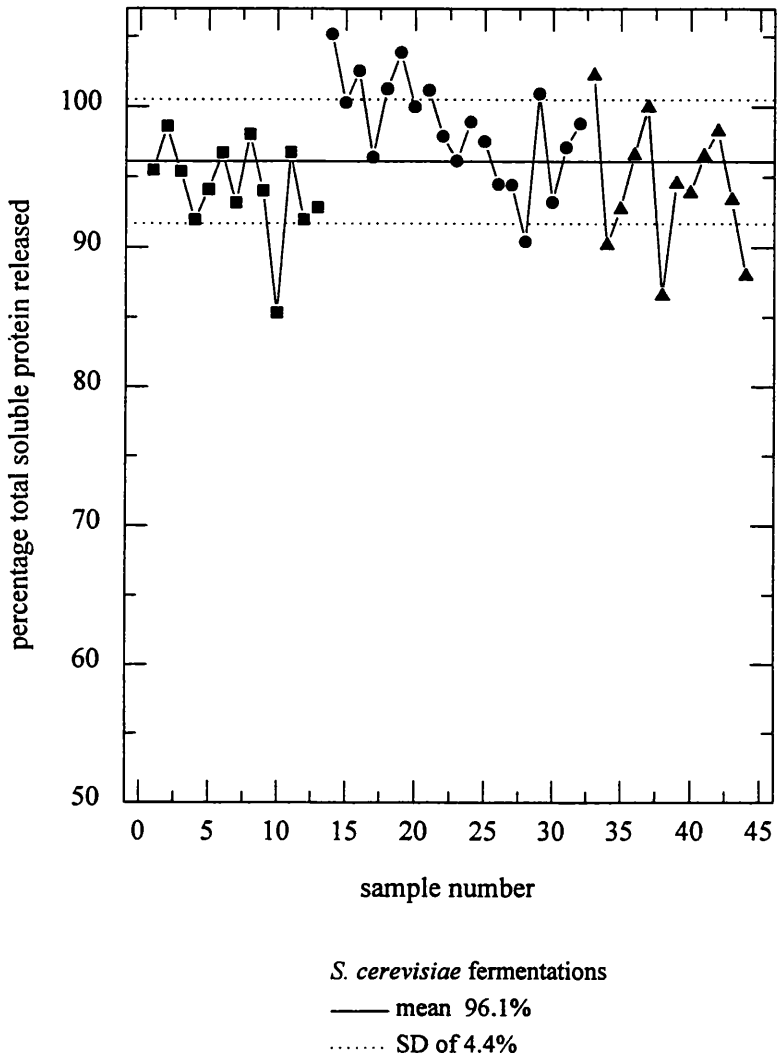
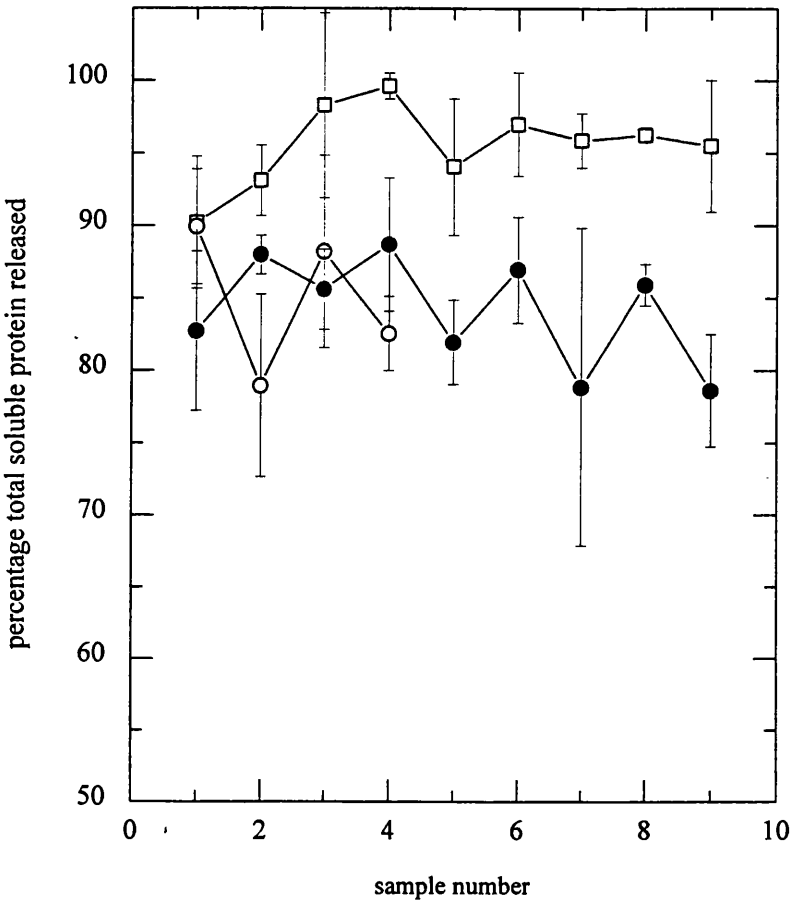


Figure 3.3.13.
Assessment of the disruption process.
Percentage total soluble protein release from *S.cerevisiae* samples taken at various stages of three fermentations; Micron Lab 40 - 1200 bar - single pass
(each sample was processed for upto 5 passes to obtain the respective maximum values)



—□— *E.coli* (recomb.); mean 95.6% +/- 2.9%
—●— *R.erythropolis* (20L); mean 84.2% +/- 4.5%
—○— *R.erythropolis* (1000L); mean 84.9% +/- 6.0%
error bars represent SD on the repeat assays; n=3

Figure 3.3.14.
Assessment of the disruption process.
Percentage total soluble protein release from various micro-organism samples taken from fermentations; Micron Lab 40 - 1200 bar - single pass
(each sample was processed for upto 5 passes to obtain the respective maximum values)

3.4. Profile Monitoring

The confidence in the intracellular data generated using the Micron lab 40 high pressure homogeniser to effect cell disruption showed the suitability of such a device for rapid fermentation monitoring.

For this purpose, fermentations of several micro-organisms were chosen including *P. putida* ML2, *E coli*, *S. cerevisiae*, and *R. erythropolis*. The first three were grown in defined medium fermentations whilst *R. erythropolis* was grown on a complex medium. Samples from the *R. erythropolis* fermentations were kindly made available for monitoring by N. Rosen (University College London).

3.4.1. *P. putida* ML2

Several fermentation profiles, monitoring the levels of intracellular components were constructed. 40mL samples were taken at regular intervals and disrupted directly, without any pre-treatment, within the Micron Lab 40 high pressure homogeniser.

The release of intracellular total soluble protein and the intracellular enzyme glucose-6-phosphate dehydrogenase (G6PDH) were monitored.

The *P. putida* ML2 fermentation shown in Figure 3.4.1. was monitored for 24 hours covering most of the growth phase and several hours of the stationary phase. The intracellular total soluble protein is shown in terms of mg/mL of homogenate supernatant and G6PDH is expressed in terms of specific activity as a fraction of the total soluble protein U/g (single pass data). The carbon dioxide evolution rate (CER) data are also plotted.

The total soluble protein increases as expected during the growth phase (reflecting the increase in biomass) and peaks with the onset of the stationary phase.

Monitoring the fermentations in the early growth phase (CER less than about 20 mmol/Lh) showed that the G6PDH specific activity U/g total soluble protein drops from over 1400 units before stabilising around a much lower value (below 100 U/g total soluble protein). This event has been observed in the fermentation shown between times 25 and 32 hours and is supported by the other fermentations monitored.

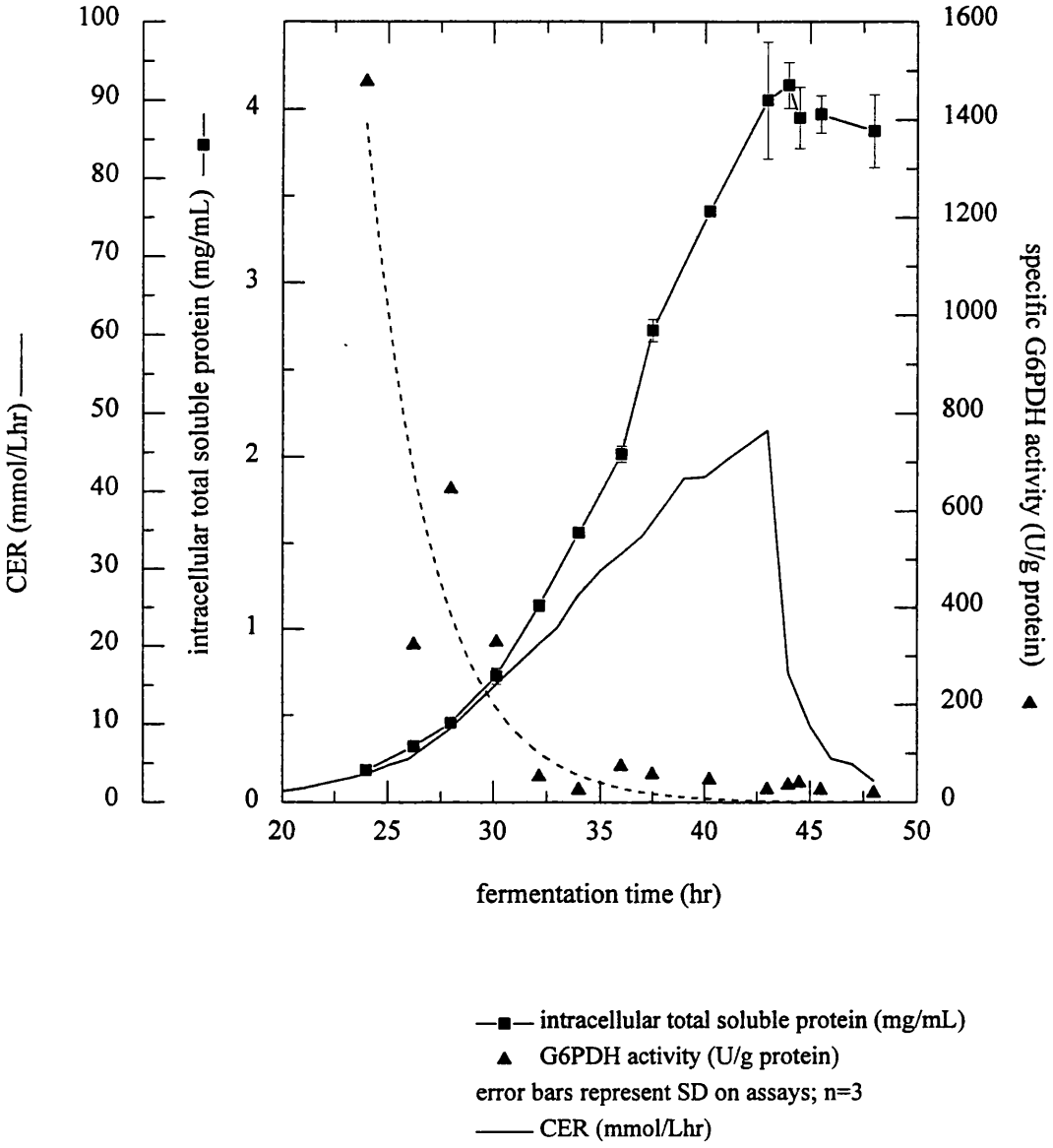


Figure 3.4.1.
Intracellular component monitoring during a *P. putida* ML2 fermentation.
Micron Lab 40 - 1200 bar operating pressure - single pass.

With fructose as the only carbon source for the *P. putida* ML2 fermentations, the fructose would be converted to Fructose 6-phosphate and thus joining the pentose phosphate pathway in the non-oxidative branch. The enzyme G6PDH is required in the oxidative branch of the pentose phosphate pathway where it catalyses the conversion of Glucose 6-phosphate to 6-phosphoglucono-δ-lactone.

3.4.2. *E. coli*

E. coli fermentations were chosen to demonstrate the ability to monitor the intracellular components in extremely dynamic fermentations which peak within six hours. The sampling frequency had to be increased to follow the rapid growth and events within the fermentations. (At best, the sampling frequency of four every hour was achieved).

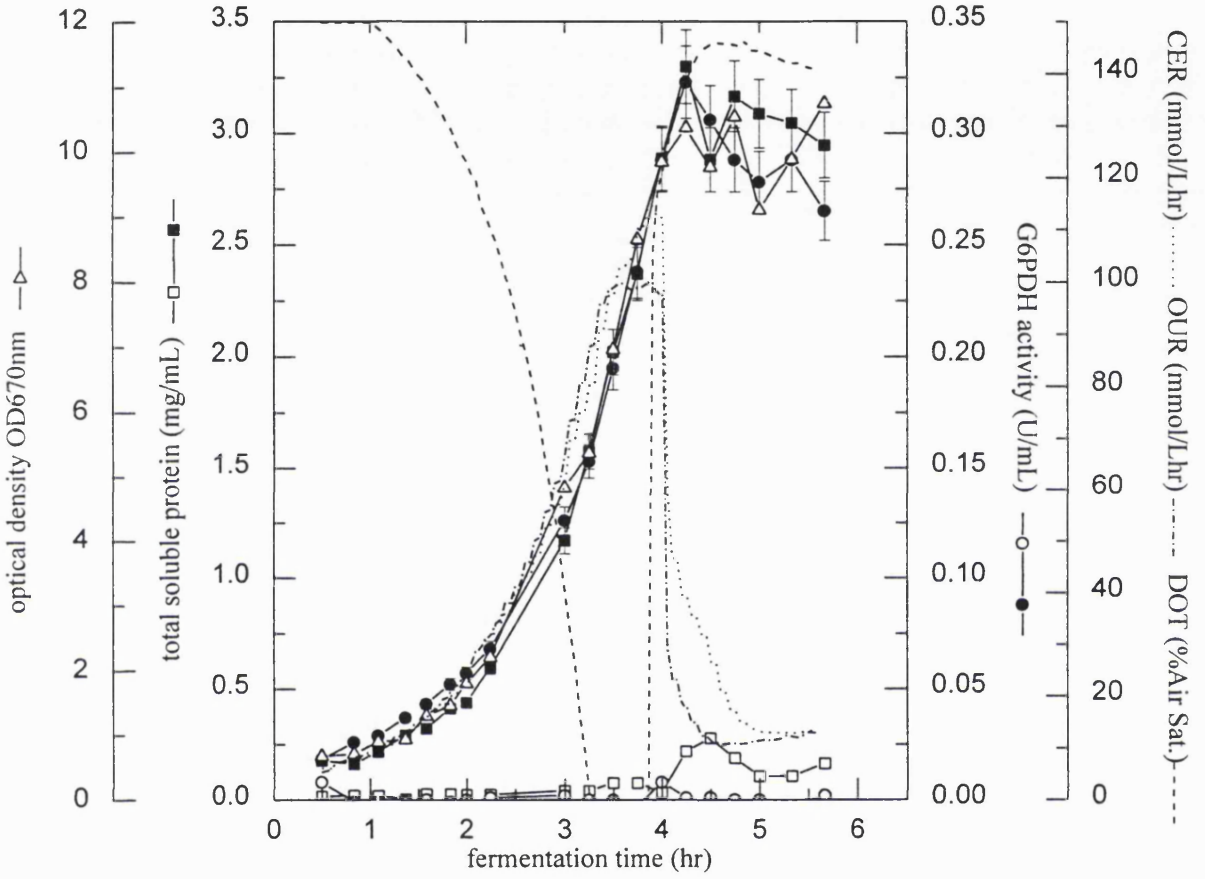
3.4.2.1. Rapid monitoring of *E. coli* Fermentations

Several *E. coli* fermentation profiles monitoring the levels of intracellular components were obtained, in which 15g/L glucose was the single carbon source was used. Once the glucose was consumed the fermentation would move into stationary phase.

To follow the rapid changes during these fermentations the sampling frequency was increased (to a maximum of four per hour) and as such the amount of information taken from each sample was restricted. Intracellular total soluble protein and the enzyme glucose 6-phosphate dehydrogenase were monitored to determine the background level (i.e. in the extracellular environment) and the increase after a single pass through the homogeniser. Optical density (OD) measurements at λ=670nm were also taken as an indicator of growth.

With glucose as the carbon source for the fermentation, the enzyme G6PDH was expected to be constitutive as it would required in the oxidative branch of the pentose phosphate pathway.

40mL samples taken at regular intervals, were disrupted directly, without any pre treatment, within the Micron Lab 40 at 1200 bar operating pressure.



background: —□—protein (mg/mL); —○— G6PDH (U/mL)
intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— OD 670 nm
····· CER (mmol/Lhr), -·-·- OUR (mmol/Lhr), - - - - DOT (% Air Sat.)

Figure 3.4.2.
The intracellular component monitoring during *E.coli* fermentation 'D'.
Micron Lab 40 (1200 bar - single pass)

A typical *E. coli* fermentation profile is shown in Figure 3.4.2. The total soluble protein and G6PDH activity are given in mg/mL and U/mL of fermenter broth respectively. The error bars represent a standard deviation of 5% (this is based on the results for repeated *E. coli* cell disruptions reported in Section 3.3., 'Reproducibility'). The respiratory data CER, OUR and DOT are also shown.

The levels of G6PDH, total soluble protein and the optical density peak approximately three quarters of an hour after the entry into the stationary phase as indicated by the CER data. The levels of the intracellular components had risen by a further third after the drop in CER values. Thus showing direct product monitoring to be a useful tool. Other monitors which are not directly related to the product can potentially provide misleading results.

The profiles of other *E. coli* fermentations are shown in Figures 3.4.3, 3.4.4., 3.4.5, and 3.4.6.

3.4.2.2. *E. coli* grown to induce the enzyme β-galactosidase

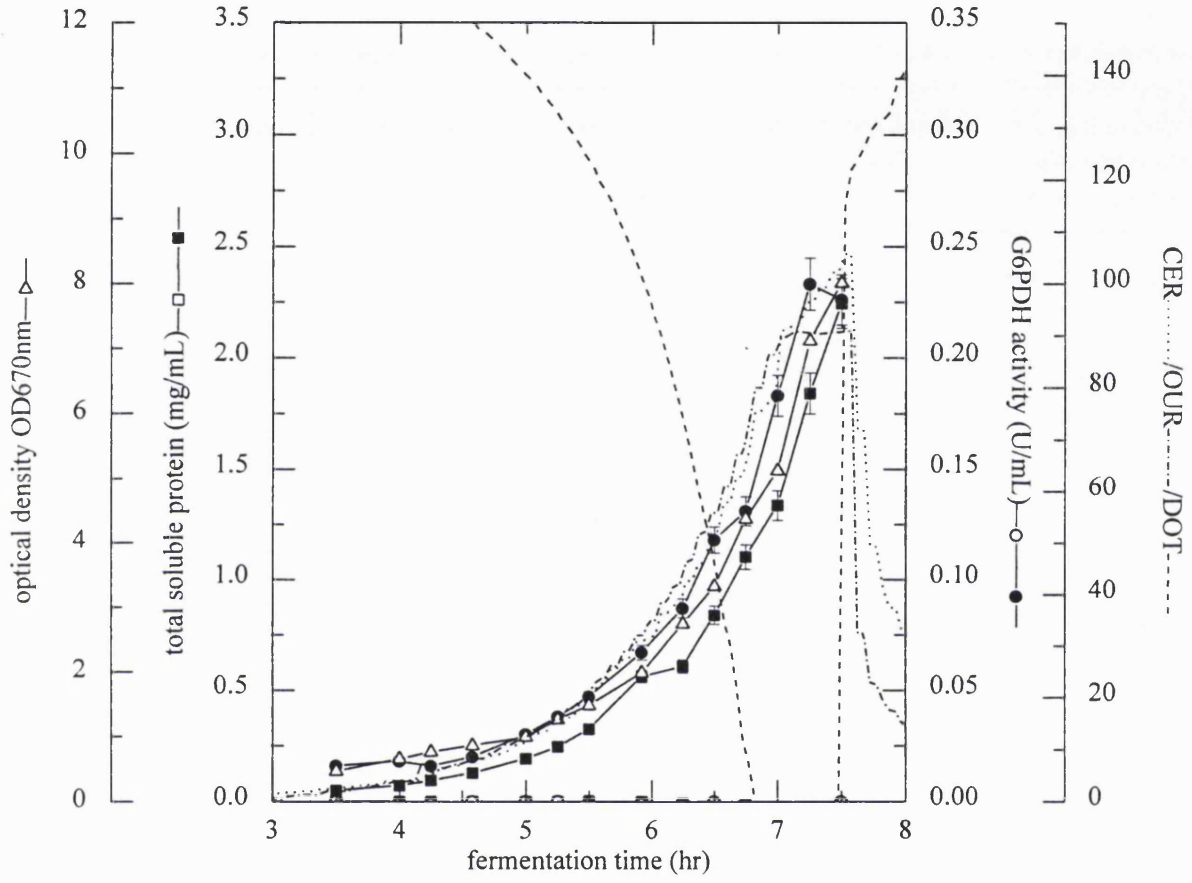
To demonstrate the ability to monitor induced changes in intracellular components two further *E. coli* fermentation profiles were monitored

The carbon sources for these fermentations were glucose (30g/L) and lactose (10g/L). The cells will preferentially utilise glucose, followed by lactose, and grow at a higher growth rate on glucose than on lactose.

The release of total soluble protein and the intracellular enzyme glucose-6-phosphate dehydrogenase (G6PDH) were monitored. Although the intracellular enzyme β-galactosidase was assayed, errors in carrying out the assay procedure invalidated the results.

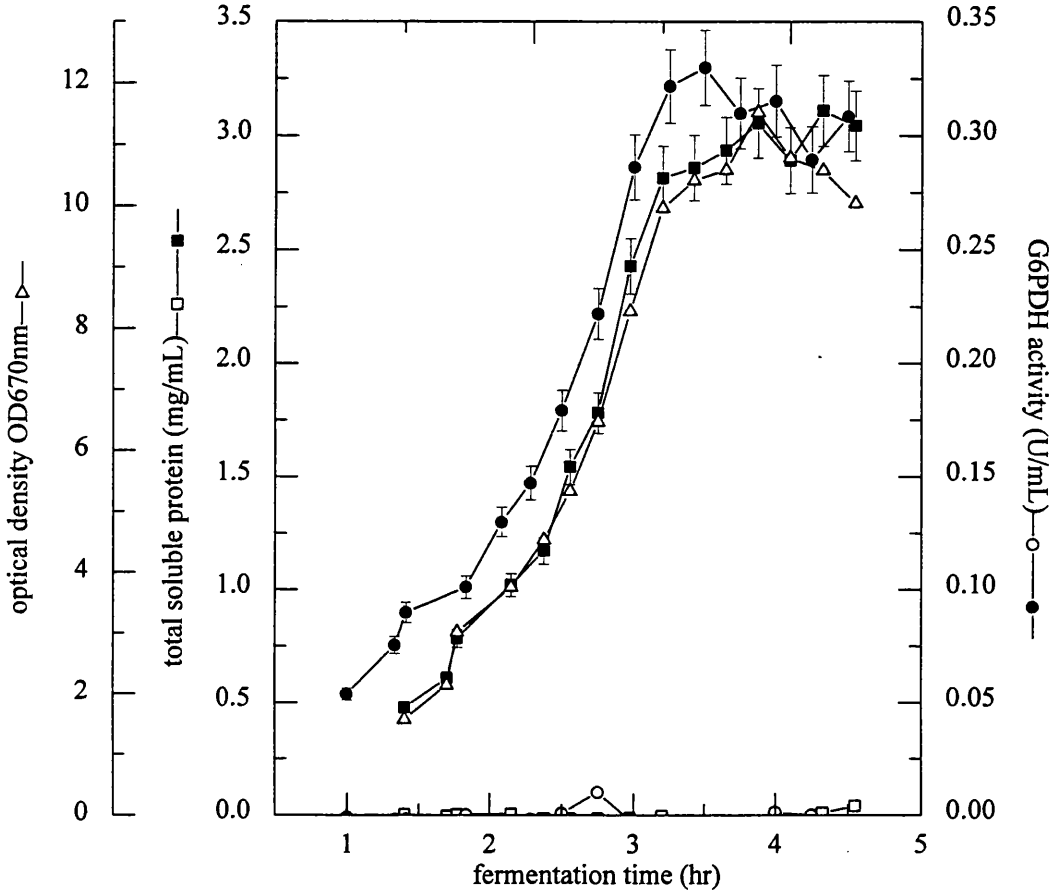
The first fermentation was monitored for 16 hours to represent the growth phase on the two carbon sources. The intracellular total soluble protein and G6PDH activity profiles are shown in terms of mg/mL and U/mL of fermentation broth respectively (single pass data). The CER (mmol/Lh) and dry cell weight data are also plotted in Figure 3.4.7.

The total soluble protein increases as expected during the growth phase reflecting the increase in biomass. Although the cell dry weight data peaks after 8 hours this is not seen in the G6PDH activity data.



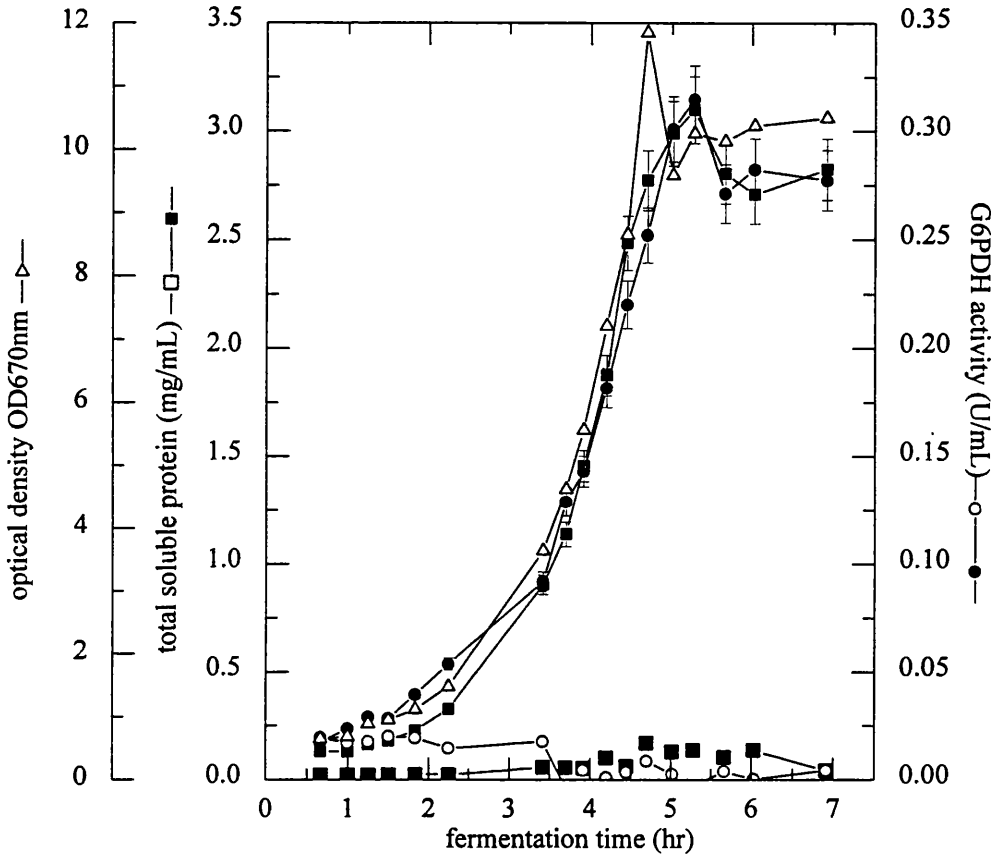
background: —□—protein (mg/mL); —○— G6PDH (U/mL)
intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— OD 670 nm
····· CER (mmol/Lhr), -·-·- OUR (mmol/Lhr), - - - - DOT (% Air Sat.)

Figure 3.4.3.
Intracellular component monitoring during *E.coli* fermentation 'C'.
Micron Lab 40 (1200 bar - single pass)



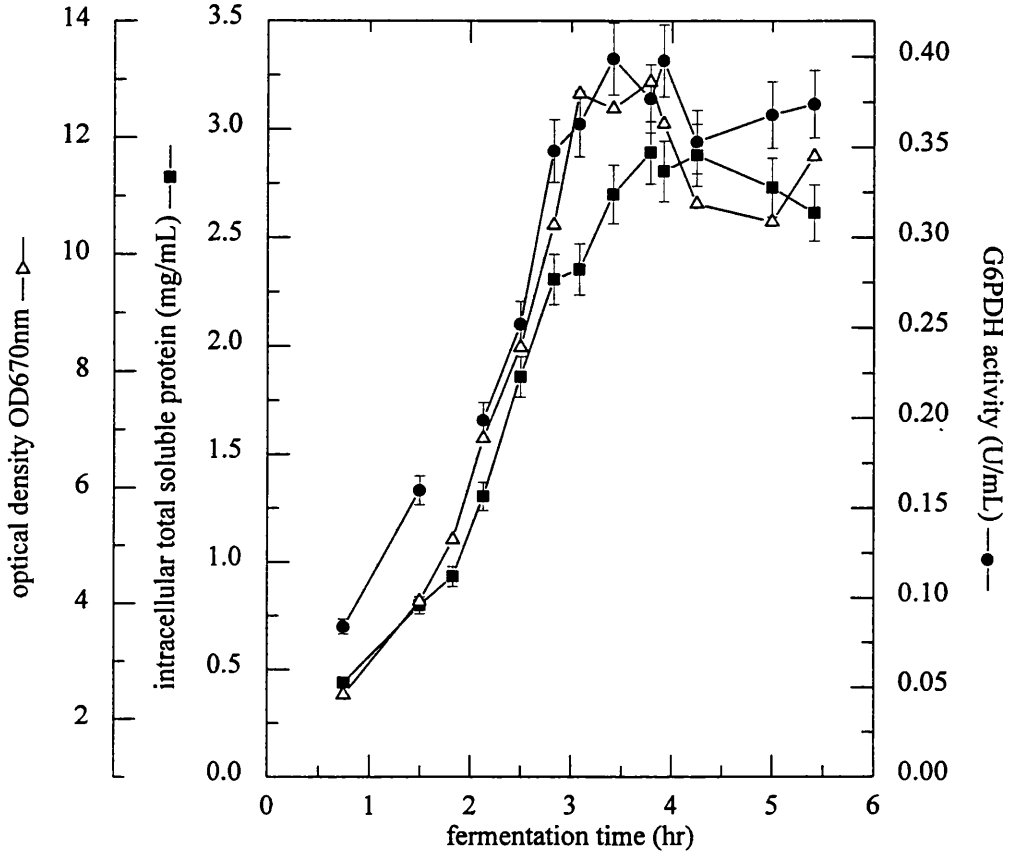
background: —□—protein (mg/mL); —○— G6PDH (U/mL)
intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— OD 670 nm

Figure 3.4.4.
Intracellular component monitoring during *E.coli* fermentation 'E'.
Micron Lab 40 (1200 bar - single pass)



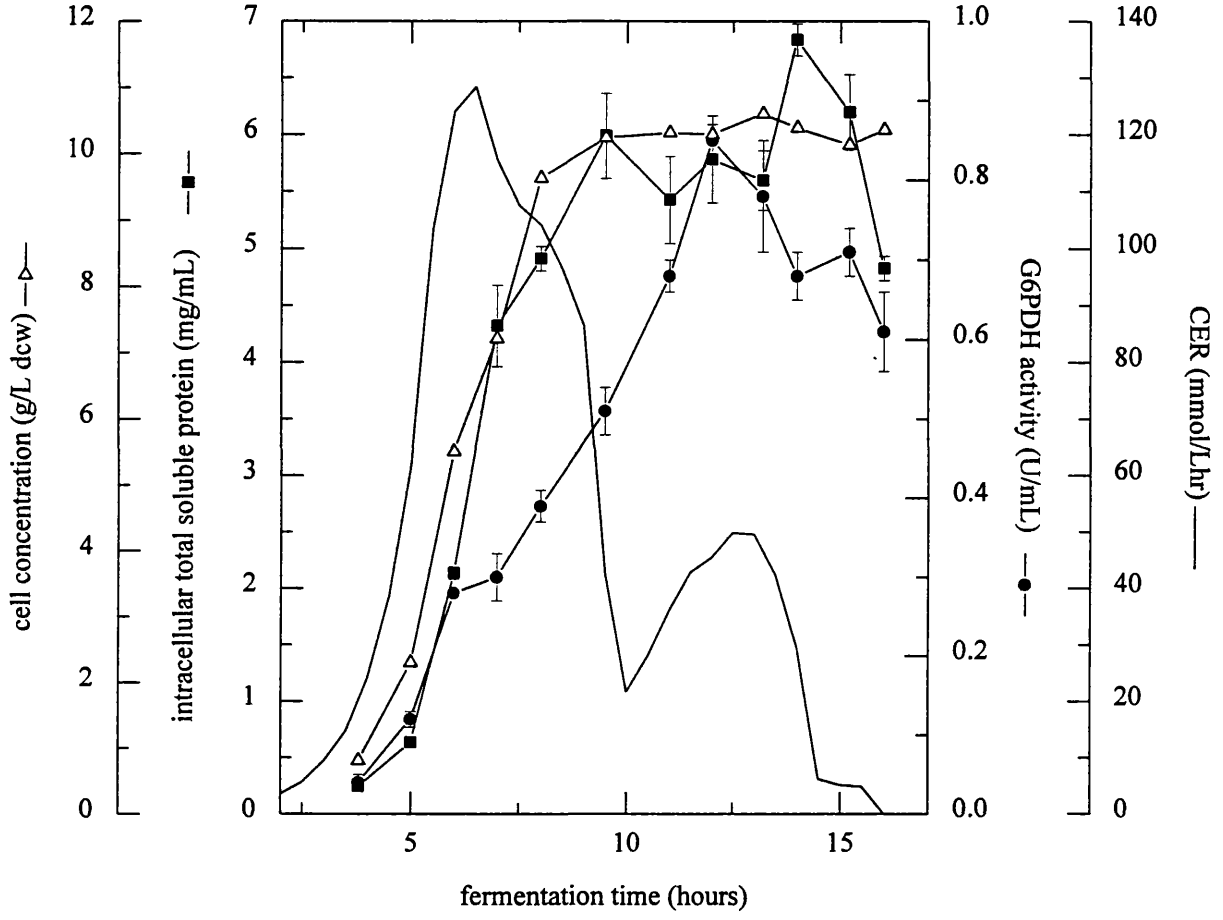
background: —□—protein (mg/mL); —○— G6PDH (U/mL)
intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— OD 670 nm

Figure 3.4.5.
Intracellular component monitoring during *E.coli* fermentation 'F'.
Micron Lab 40 (1200 bar - single pass)



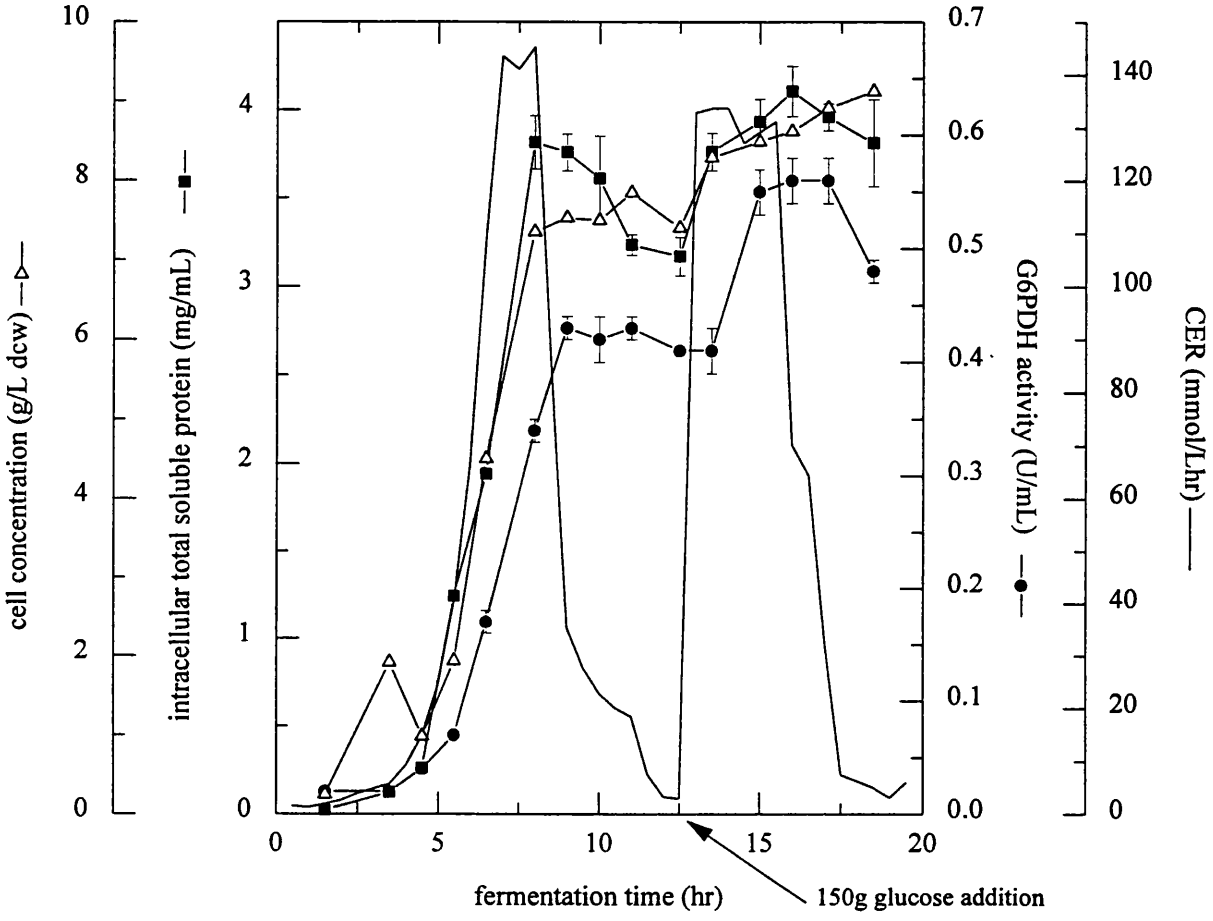
intracellular: —■—protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— OD 670 nm

Figure 3.4.6.
Intracellular component monitoring during *E.coli* fermentation 'G'.
Micron Lab 40 (1200 bar - single pass)



intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— cell concentration (g/L dcw)
—— CER (mmol/Lhr)

Figure 3.4.7.
Intracellular component monitoring during the course of *E.coli* fermentation 'A'.
30g/L glucose and 10g/L lactose were the two carbon sources.
Micron Lab 40 (1200 bar - single pass)



intracellular: —■— protein (mg/mL); —●— G6PDH (U/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— cell concentration (g/L dcw)
—— CER (mmol/Lhr)

Figure 3.4.8.
Intracellular component monitoring during the course of *E.coli* fermentation 'B'. Glucose (150g) added when initial carbon sources (150g glucose & 100g lactose) were deemed exhaused. Micron Lab 40 (1200 bar - single pass)

Whilst the G6PDH activity also follows the growth of the cells it does not reflect the diauxic shift in the experiment. (At present it is not known whether or not the diauxic growth will be reflected in the G6PDH profile).

Significant consumption of alkali (500mL of 5M ammonium solution) to regulate the pH occurred between 5.5 hours and 9 hours, during which time the dissolved oxygen tension (DOT) was zero.

Figure 3.4.8. shows the second *E. coli* fermentation (grown for β-galactosidase induction). In this fermentation the carbon sources were introduced in two stages. At the beginning of the fermentation 15g/L of glucose and 10g/L lactose were present. Then at 12.5 hour into the fermentation, when both the initial carbon sources were deemed exhausted (as indicated by the drop in carbon dioxide evolution rate, CER, to about zero), a further 150g of glucose was added. This regime was adopted to provide an artificial change in conditions to see if the change would be detected.

The G6PDH activity (U/mL) increased with growth and levelled off. Then two hours after the addition of the second batch of glucose, the activity rose very markedly (by 35%). This event was not seen so clearly in the total soluble protein profile which drops slightly (by 10%) and then picked up to a fraction (by 5%) above its first maximum.

Again significant consumption of alkali solution (5M ammonium solution) occurred during the CER peaks. In total, over a litre of alkali was used to regulate the pH. During these periods, it is suspected that the levels of acetate produced during the fermentation became inhibitory to growth and so a drop in DOT was also observed.

3.4.3. *S. cerevisiae*

Several *S. cerevisiae* fermentation profiles were obtained; by monitoring the total soluble protein and the enzyme alcohol dehydrogenase (ADH), to demonstrate the applicability of this methodology to another micro-organism. Optical density measurements and G6PDH were also taken for some of the fermentations.

As before, 40mL samples taken at regular intervals were disrupted directly, without any pre treatment, within the Micron Lab 40 at 1200 bar operating pressure.

The profile shown in Figure 3.4.9. is of fermentation 'yeast D', which covers the period from inoculation and growth on glucose. The total soluble protein and ADH activity are given in mg/mL and U/mL of fermenter broth respectively.

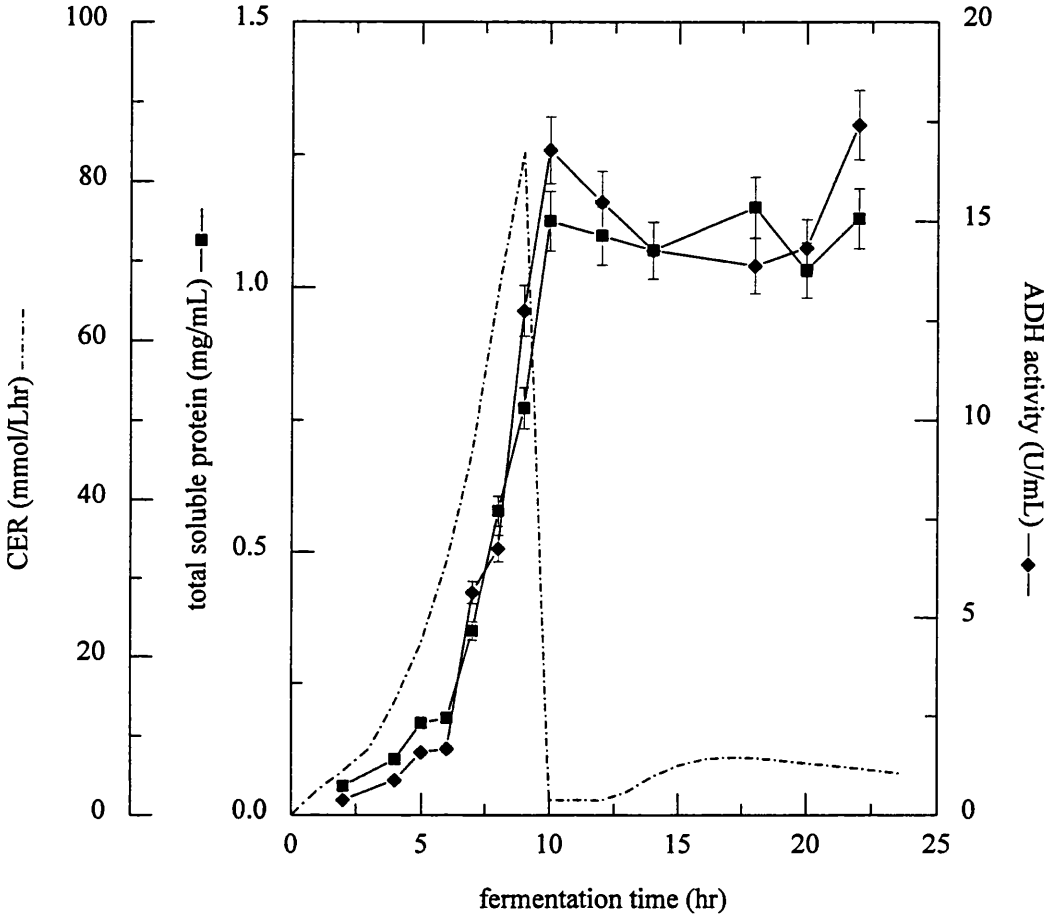


Figure 3.4.9.
Intracellular component monitoring during *S. cerevisiae* fermentation 'D'.
Micron Lab 40 (1200 bar - single pass)

The sampling frequency was limited to hourly and two hourly so as not to loose too much volume during the course of the batch fermentations.

In the yeast fermentations, the enzyme ADH was chosen as it is involved as a catalyst in one of the steps in the conversion of glucose to ethanol. Glucose is converted into pyruvate in the glycolytic pathway. The pyruvate is decarboxylated to acetaldehyde and then in a further step this is reduced to ethanol by NADH. The enzyme Alcohol dehydrogenase, which contains a zinc ion at its active site, catalyses this reaction.

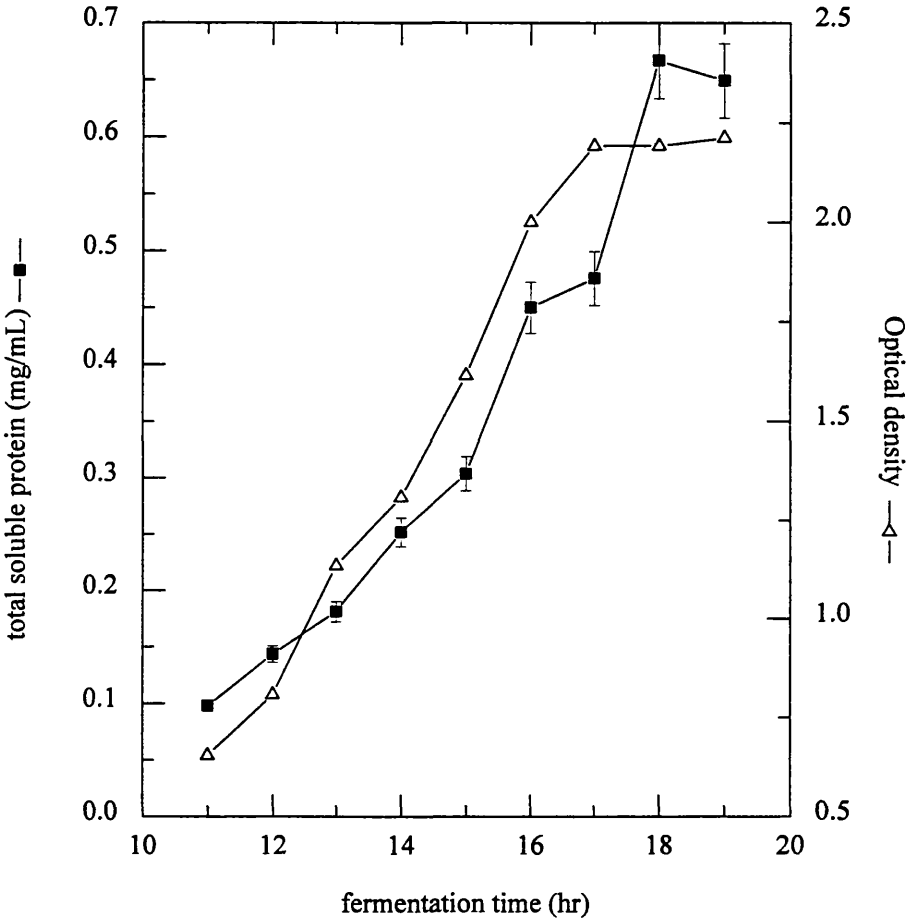
The analysis of the homogenate showed rapid decay in the ADH activity during some parts of the fermentation and so prompt assaying was essential. This decay of ADH activity warrants investigation (within another program).

3.4.4. *R. erythropolis*

To demonstrate the applicability of the monitoring process to a fermentation grown on a complex medium an *R. erythropolis* fermentation was monitored and the total soluble protein profile assayed as shown in Figure 3.4.10. *R. erythropolis* is known to be a particularly difficult organism to disrupt.

Samples from the fermentation required centrifugation and resuspension in a glycine buffer to prevent the intracellular enzyme of interest from degradation. This additional step in the monitoring process introduced an extra 20 minutes to the total monitoring time for each sample. However, the processing time was not optimised, and it is expected that it can be reduced considerably.

The figure includes optical density (OD600nm) measurements as a measure of growth and shows that as expected the total soluble protein (mg/mL) increases with growth.



—■— intracellular total soluble protein (mg/mL)
error bars represent an SD of 5% based on the analysis of disruption process
—△— optical density OD670nm

Figure 3.4.10.
Intracellular total soluble protein monitoring during *R.erythropolis* (20L) fermentation
Micron Lab 40 (1200 bar - single pass)

3.5. Profile Analysis

Process control is important for the rationalisation of biotechnological processes. These processes can be improved by the use of different strategies involving the selection of micro-organism strains with elevated performance, the study of the configuration of bioreactors, or the control of the physiological conditions in which the cultures grow.

Several control options were identified as potential uses for the information generated as a result of the confidence in the technique used to obtain reproducible data for the intracellular products (viz. total soluble protein, ADH and G6PDH). These targets and the techniques developed can also be used to time a sampling policy, to gain more data at times of change and/or predicted change. These included:

- the determination of whether the fermentation is on course; producing the desired product,
- projecting the product profile to predict the time at which the known maximum product level would be attained, and
- to statistically show that the product profile has indeed peaked once a maximum is reached.

3.5.1. Is the fermentation on course i.e. producing the desired product?

The early identification of the problems which may occur during a fermentation is clearly useful to decide for example whether to abort the fermentation, to take corrective measures or to carry on and await further data. This is particularly critical when the desired product is being produced at a reduced level or at worst not at all.

Monitoring the intracellular product directly can identify situations where all other indicators show a normal fermentation but the product of interest is not being produced. An interesting example is in the fermentation involving a recombinant organism where, upon the loss of a plasmid, the recombinant organism reverts to its

natural state. Because of the intracellular location of the product of interest, other monitoring parameters might continue indicating a normal fermentation.

Locus plots have been used to define a region describing a normal fermentation; a locus plot essentially plots such data as will 'fall' around a single centre point on the graph. The specific enzyme activity was plotted against the specific total soluble protein measurements. For the locus to be stationary both the specific enzyme activity and total soluble protein content of the cell to remain constant throughout the fermentation.

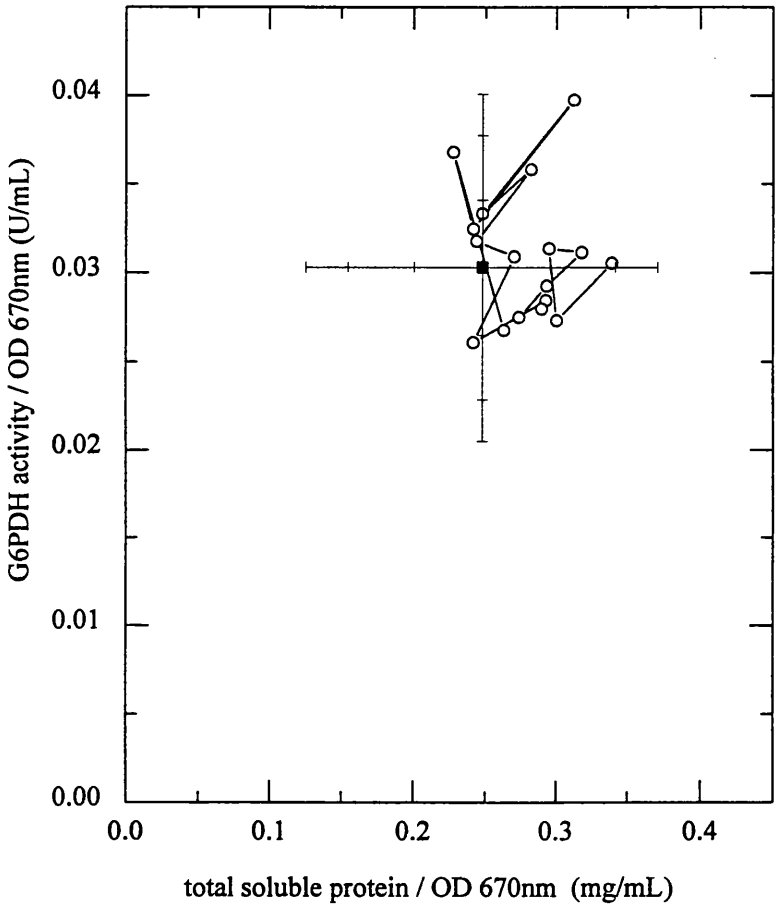
Figure 3.5.1. shows the results from a typical *E. coli* fermentation where specific G6PDH activity is plotted against total soluble protein. Optical density measurements were used to make the values of units activity/mL and mg protein/mL specific. The bounds of the normal operating area is designated by plotting 95% and 99% confidence levels given be 1.96SD and 2.58SD respectively.

Interpreting this type of graph is simple. For example, if the locus moves down the y-axis, vertically away from the expected locus point, it indicates that the desired product (the enzyme G6PDH in this case) is not being produced or expressed to normally expected levels.

Figure 3.5.2. shows a locus plot monitoring a normal *E. coli* fermentation. For this fermentation, the locus starts outside the operating window or template. This then indicates lower levels than expected of total soluble protein, even though the enzyme levels are within normal limits. This information should then give rise to some form of investigation (for example, for contamination of fermentation).

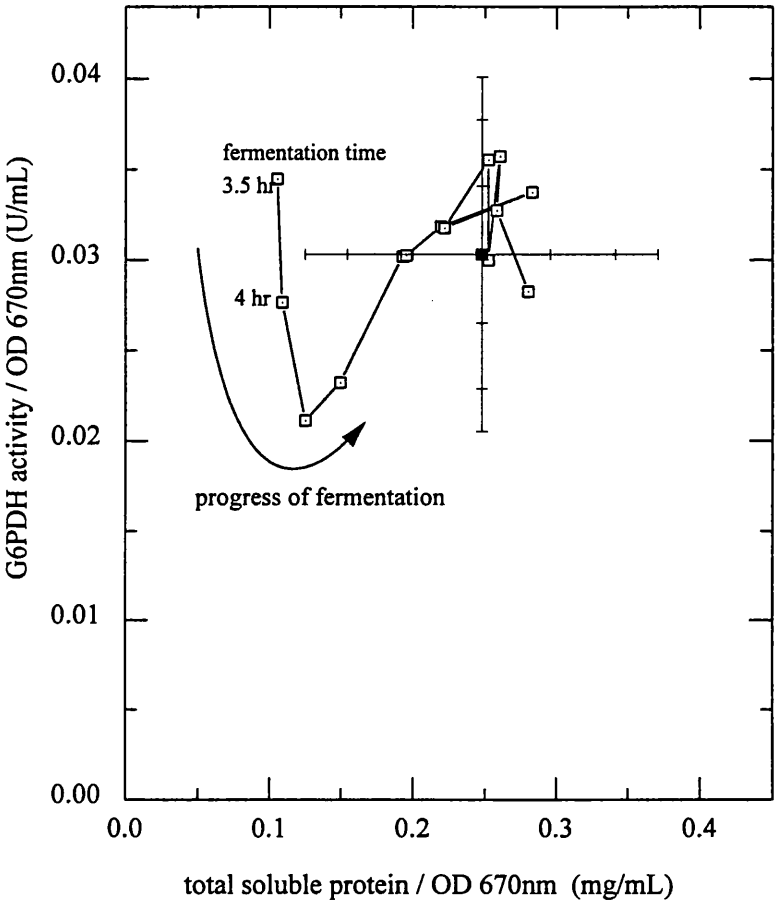
It is observed that within the space of one or two further measurements (within 30 minutes) the locus comes back within the normal specification, and continues to remain within these bounds.

Figure 3.5.3 shows the data used to construct the 'normal' *E. coli* fermentation locus plot for G6PDH and total soluble protein. The plot shows the data points from five fermentations to be concentrated around an area, which will form the criterion upon which further *E coli* fermentations can be assessed for deviations.



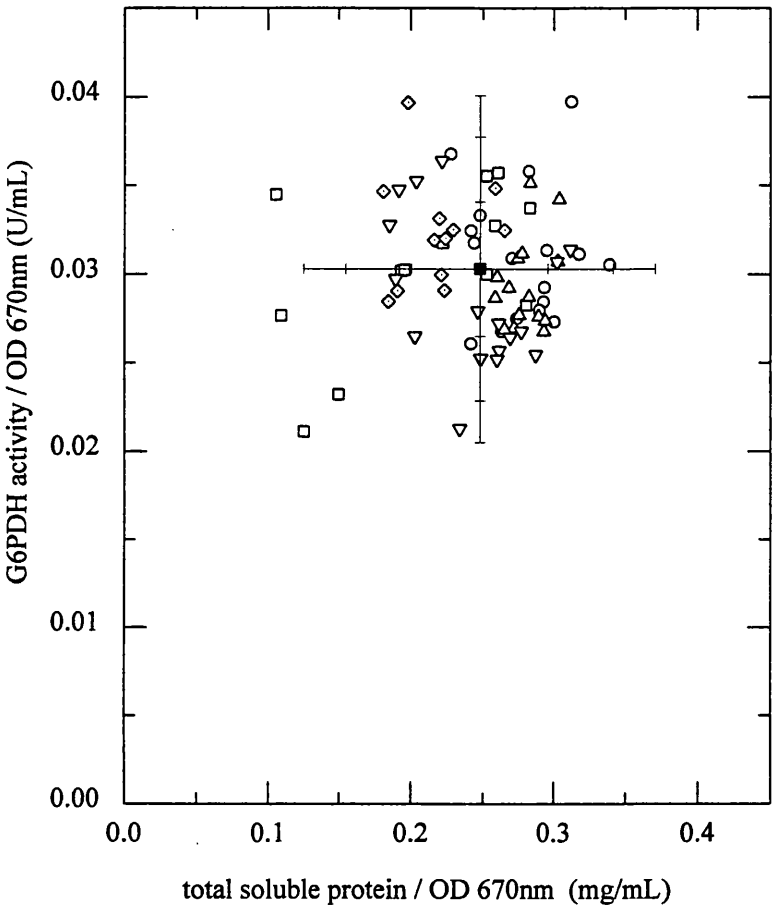
■ mean for 'normal' fermentations
error bars represent
1*SD, 1.96*SD (95%) and 2.58*SD (99% confidence levels)

Figure 3.5.1.
Intracellular total soluble protein and G6PDH activity, normalised by optical density measurements from *E.coli* fermentation 'D'; fermentation within the "norm".



■ mean for 'normal' fermentations
error bars represent
1*SD, 1.96*SD (95%) and 2.58*SD (99.% confidence levels)

Figure 3.5.2.
Intracellular total soluble protein and G6PDH activity, normalised by optical density measurements from *E.coli* fermentation 'C'; detection of departure from "norm".



E. coli fermentations

□ , ○ , △ , ▽ , ◇

■ mean for all five fermentations

error bars represent 1*SD, 1.96*SD (95%) & 2.58*SD (99%)

Figure 3.5.3.

Intracellular total soluble protein and G6PDH activity, normalised by optical density measurements from five *E.coli* fermentations form this locus plot which can be used to detect a fermentations departure from "norm".

3.5.2. Project the product profile to predict the time at which the known maximum product level will be attained

Another useful objective was to identify as early as confidently possible the time at which the harvesting cycle can begin. This will be particularly important in industry where shift working, alerting of the downstream processing staff, etc. are serious considerations. In large scale fermentations, the cooling process prior to harvesting can take hours and so an early indication of this time would be beneficial.

Here, the data were used as though they were being collected in real time. After the first four data points are observed, a best linear or exponential fits can be extrapolated to the maximum product level expected (e.g. 4mg/mL for intracellular total soluble protein during *P. putida* ML2 fermentations) giving the corresponding fermentation time. This procedure is continued as more protein data are obtained, giving revised estimates for the time at which the maximum level is achieved.

A comparison of the projected times based on linear and exponential fits is presented in a series of figures from Figure 3.5.4. to Figure 3.5.18. for *P. putida* ML2, *E. coli* and *S. cerevisiae* fermentations. The (known) maximum product level is plotted along with the developing protein profile; the first projection being made when the fourth sample was taken. The error bars on the data points represent the standard deviation of the fitted line. The comparisons are made for the total soluble protein, G6PDH and optical density profile data for other fermentations.

As might have been expected, the exponential fits provided estimates for the projected time, following only one or two projections, which were seen to be very close to the eventual reality. This also points to the confidence in the data generated.

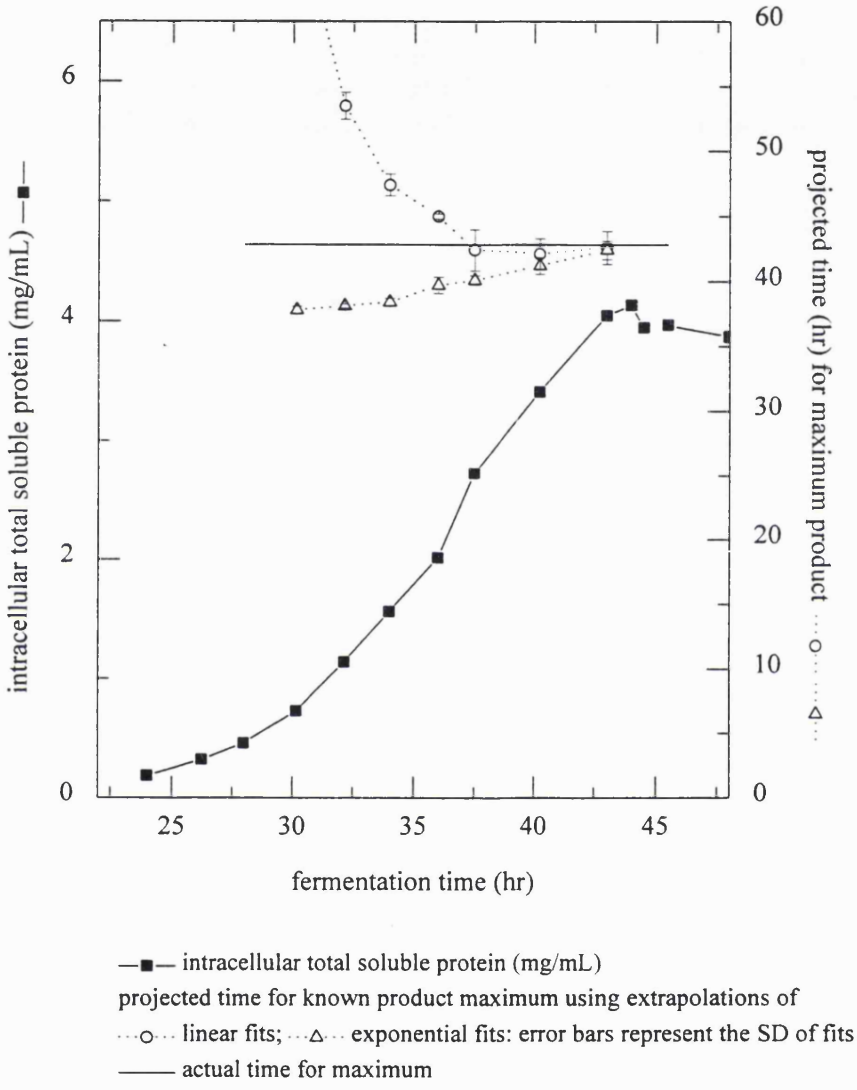
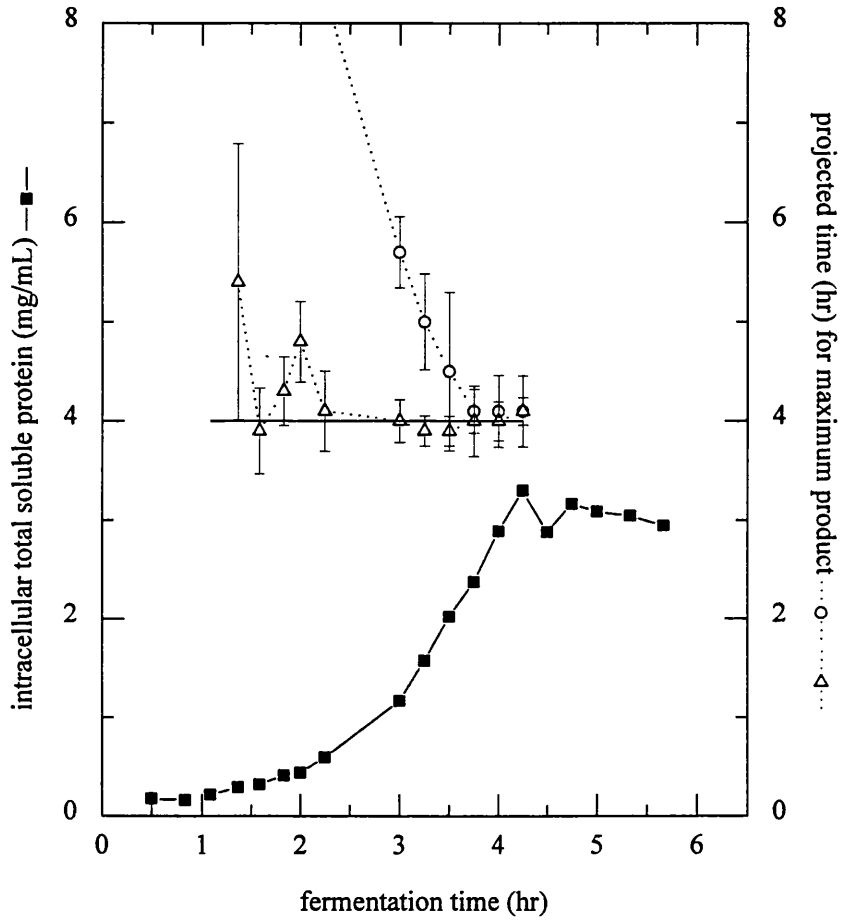
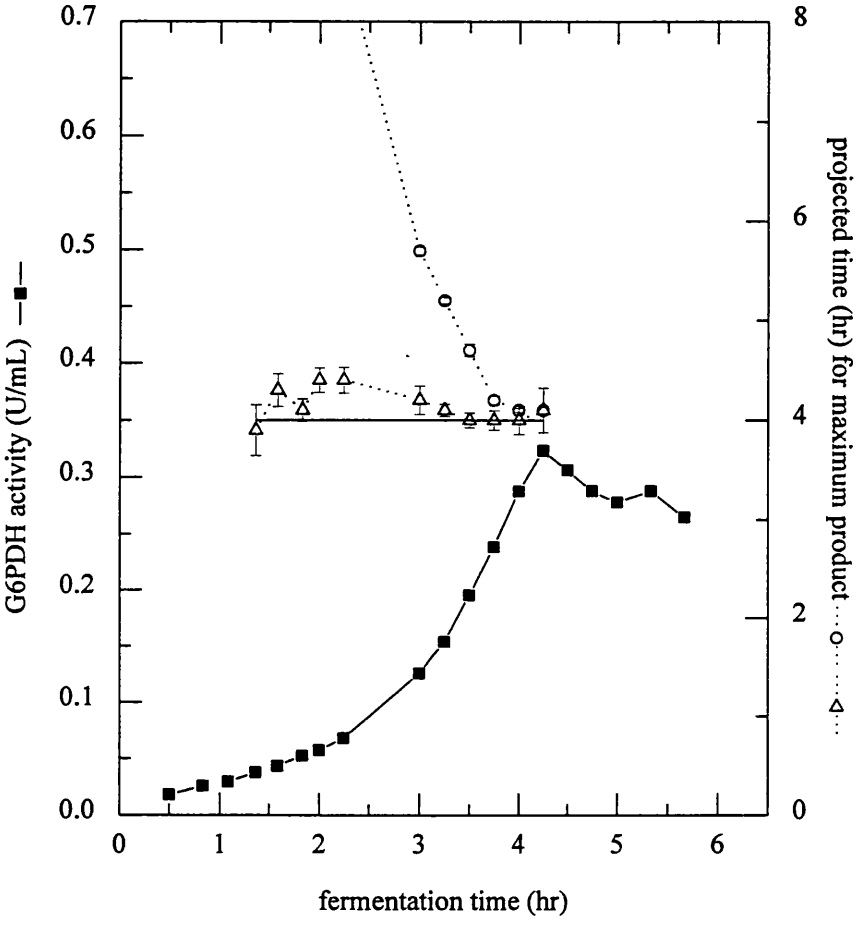


Figure 3.5.4.
PROJECTING TO THE TIME OF MAX PRODUCT; *P.putida* ML2 fermentation 'E'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (4.0mg/mL) would be attained.



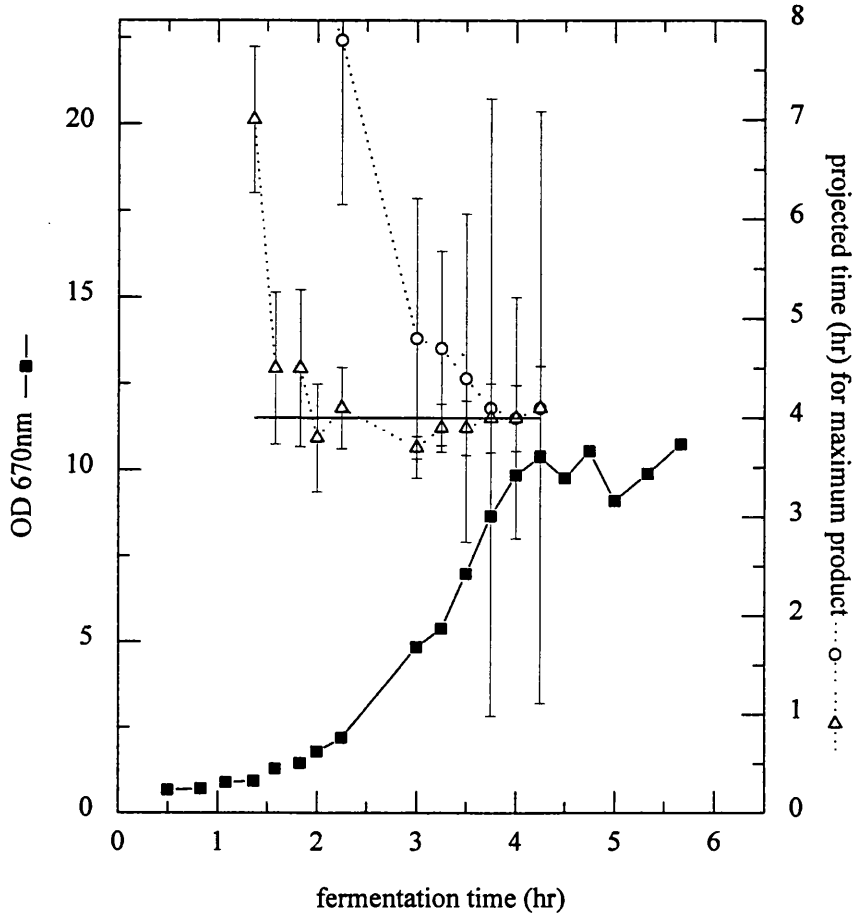
—■— intracellular total soluble protein (mg/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.5.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'D'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (3.0mg/mL) would be attained



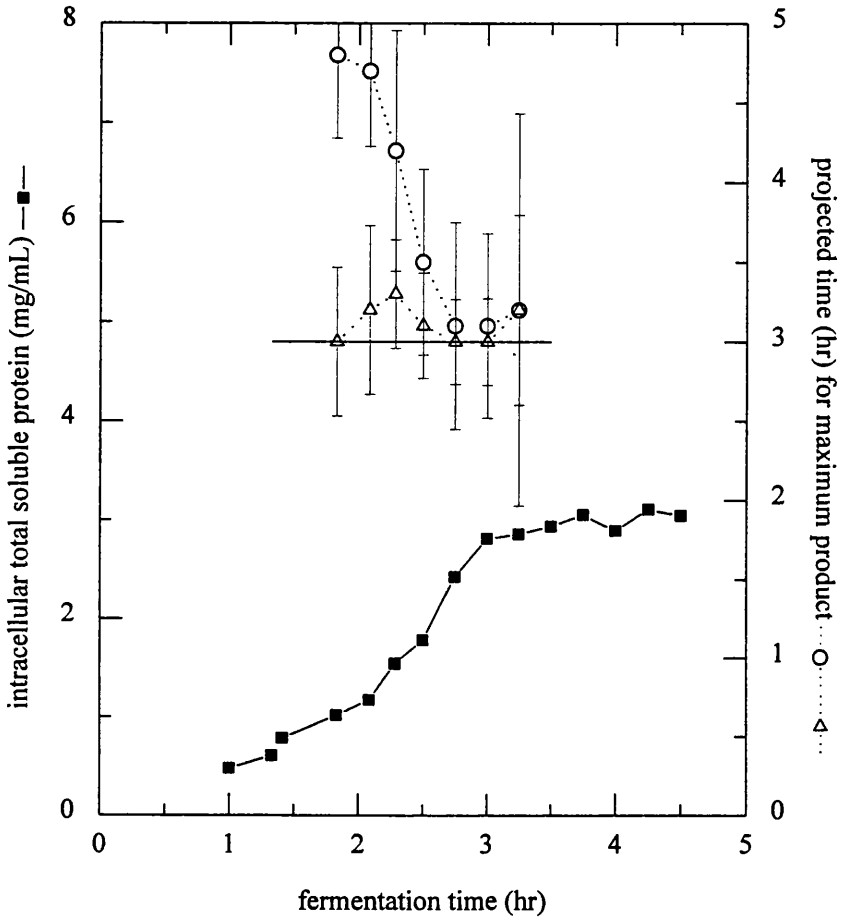
—■— G6PDH activity (U/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.6.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'D'
Comparison between linear and exponential fits for projecting the time when the known maximum G6PDH activity level (0.3 U/mL) would be attained



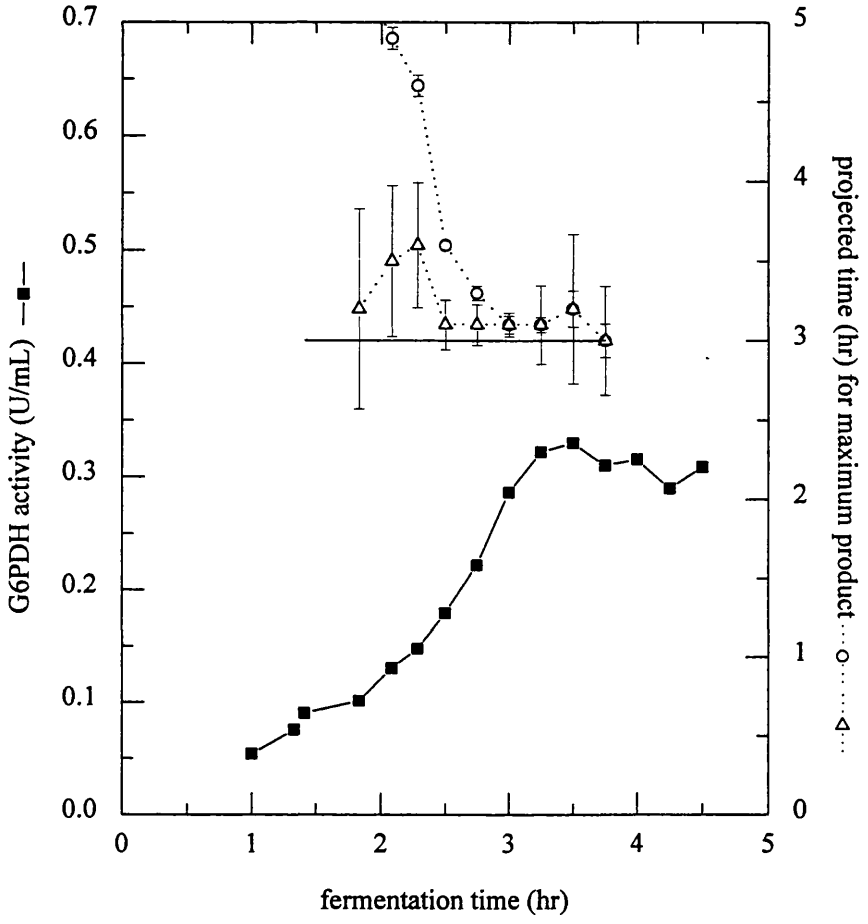
—■— optical density OD670nm (abs. units)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.7.
PROJECTING TO THE TIME OF MAX PRODUCT; *E. coli* fermentation 'D'
Comparison between linear and exponential fits for projecting the time when the known maximum OD 670nm level (10 abs. units) would be attained



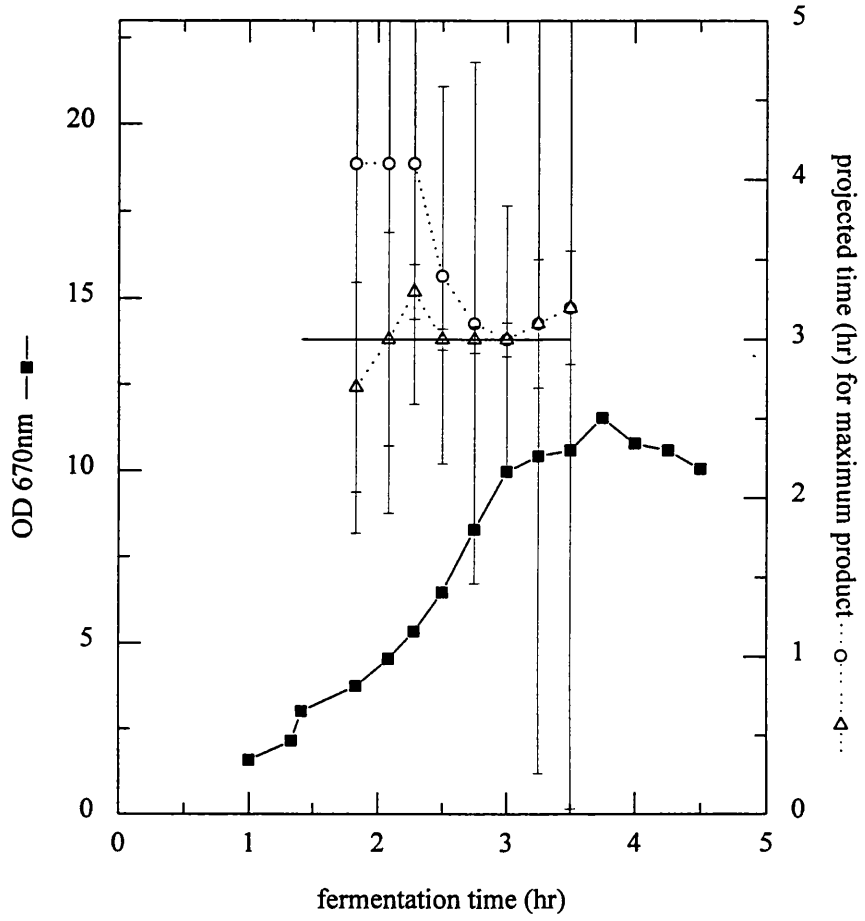
—■— intracellular total soluble protein (mg/mL)
projected time for known product maximum using extrapolations of
·· ○ ·· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.8.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'E'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (3.0mg/mL) would be attained



—■— G6PDH activity (U/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.9.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'E'
Comparison between linear and exponential fits for projecting the time when the known maximum G6PDH activity level (0.3 U/mL) would be attained



—■— optical density OD670nm (abs. units)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.10.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'E'
Comparison between linear and exponential fits for projecting the time when the known maximum OD 670nm level (10 abs. units) would be attained

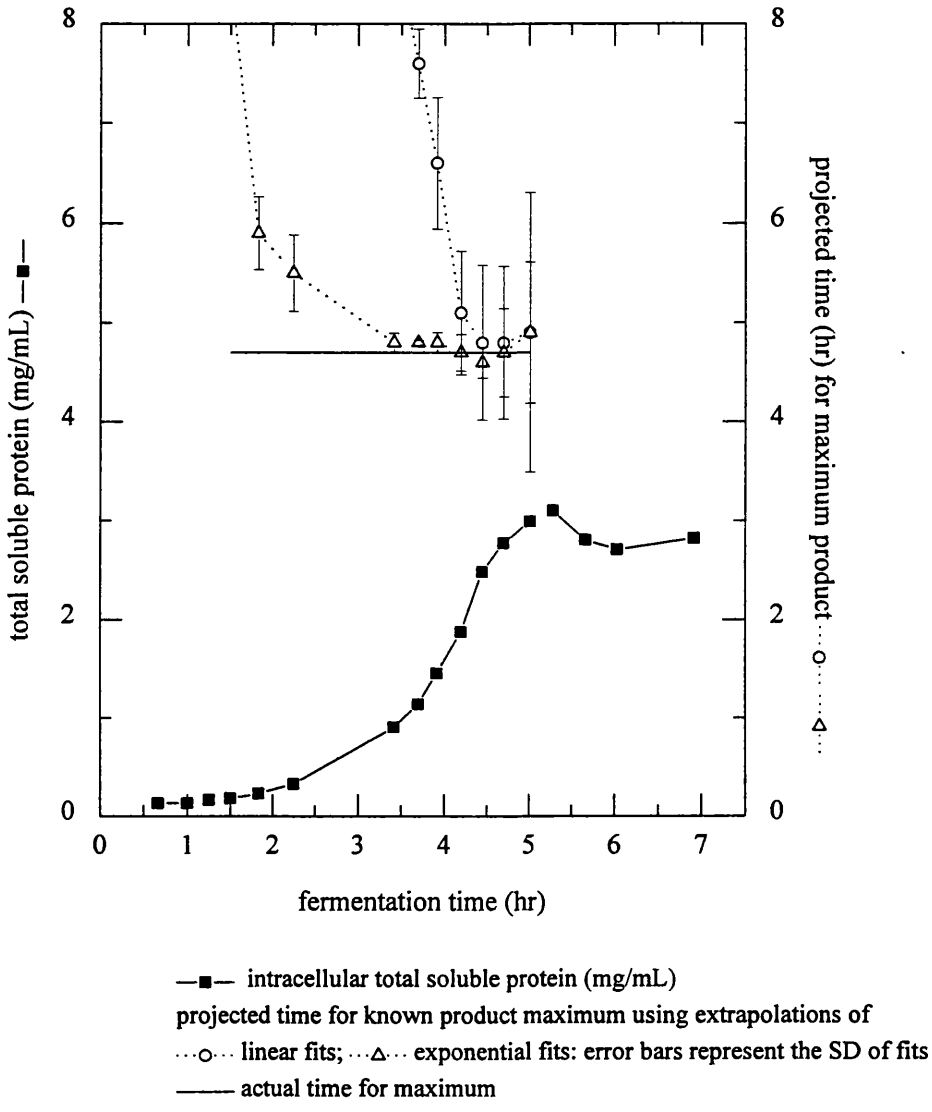
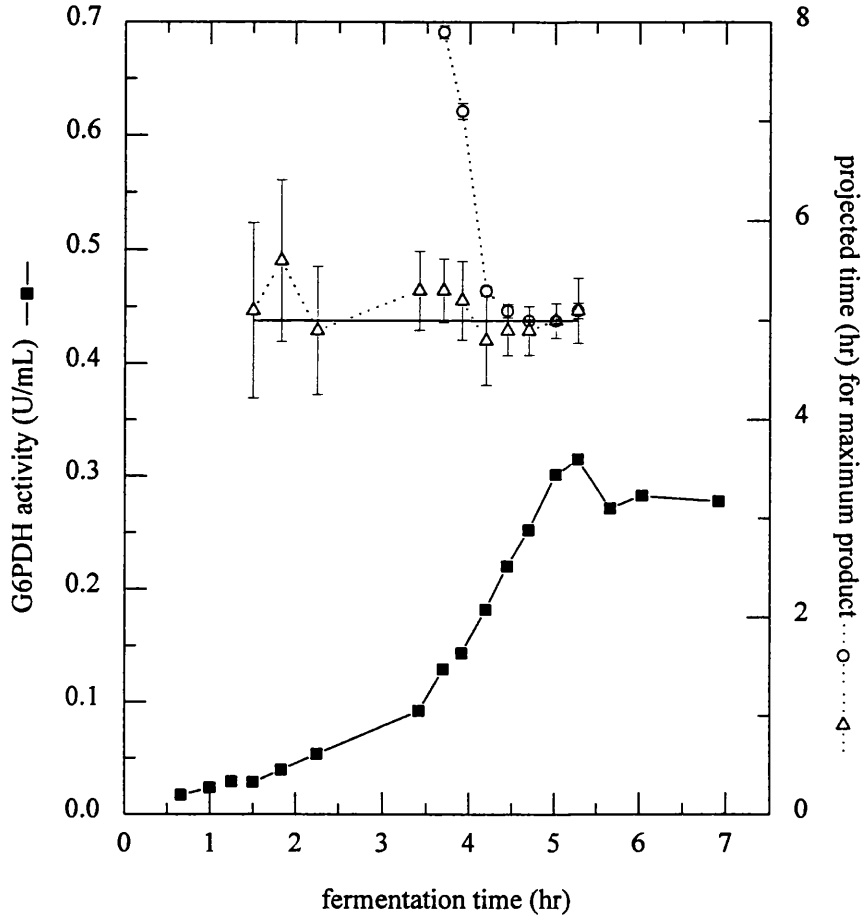
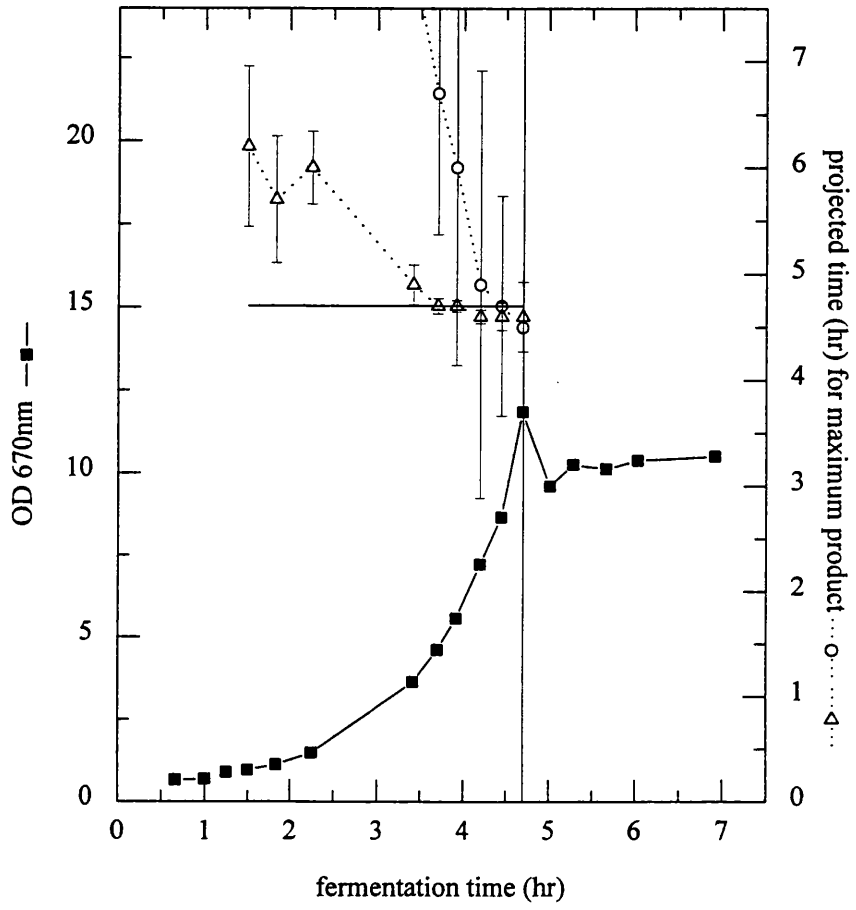


Figure 3.5.11.
PROJECTING TO THE TIME OF MAX PRODUCT; E.coli fermentation 'F'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (3.0mg/mL) would be attained



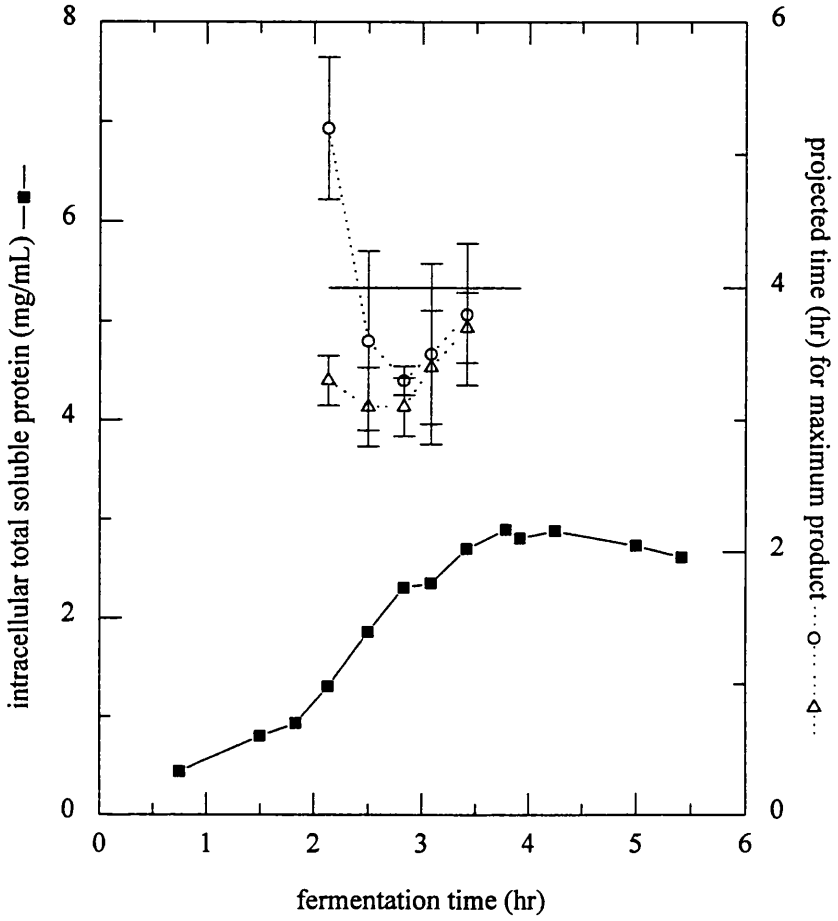
—■— G6PDH activity (U/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.12.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'F'
Comparison between linear and exponential fits for projecting the time when the known maximum G6PDH activity level (0.3 U/mL) would be attained



—■— optical density OD670nm (abs. units)
projected time for known product maximum using extrapolations of
···o··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.13.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'F'
Comparison between linear and exponential fits for projecting the time when the known maximum OD 670nm level (10 abs. units) would be attained



—■— intracellular total soluble protein (mg/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.14.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'G'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (3.0mg/mL) would be attained

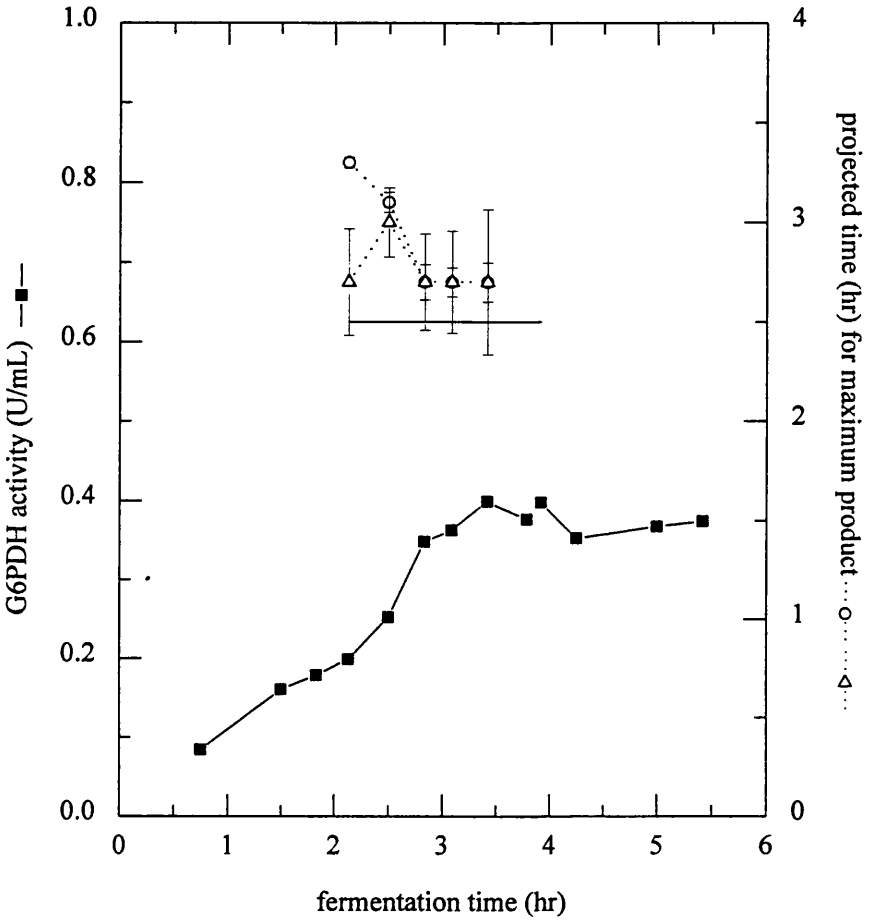
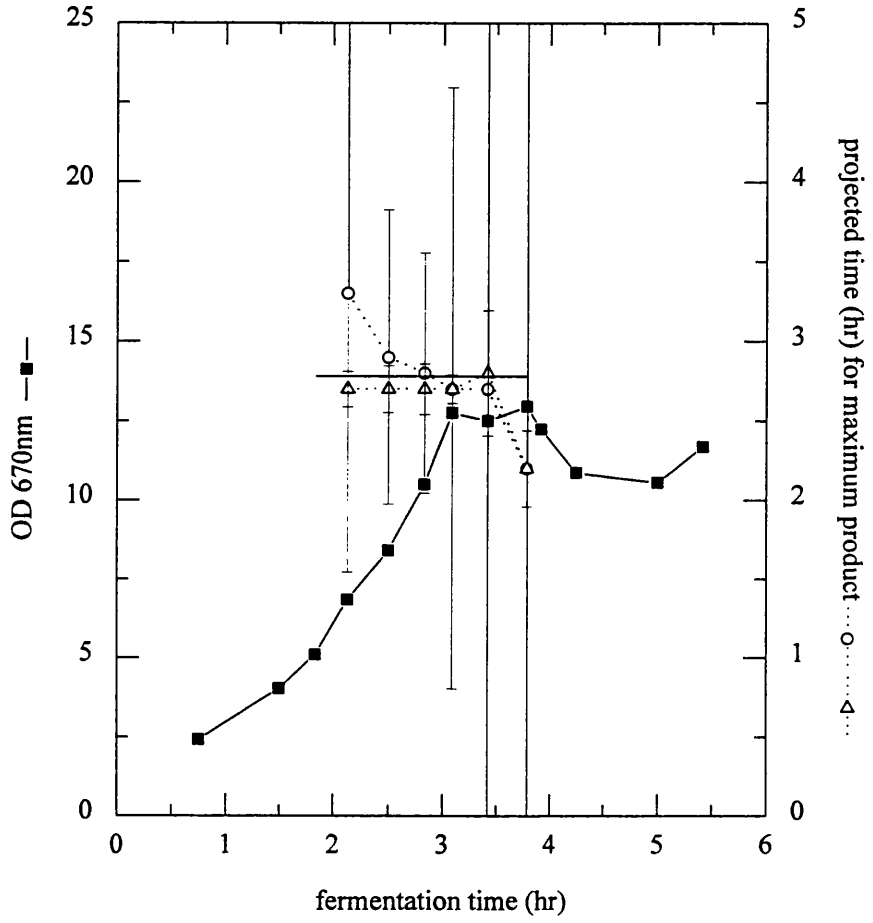
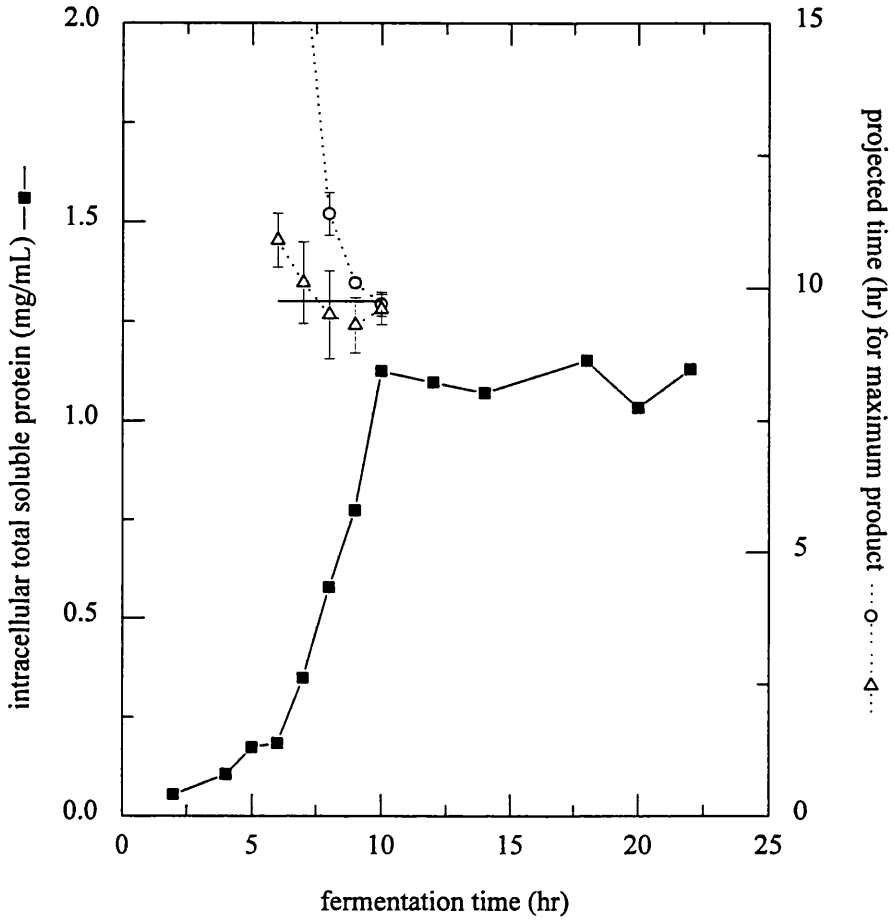


Figure 3.5.15.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'G'
Comparison between linear and exponential fits for projecting the time when the known maximum G6PDH activity level (0.3 U/mL) would be attained



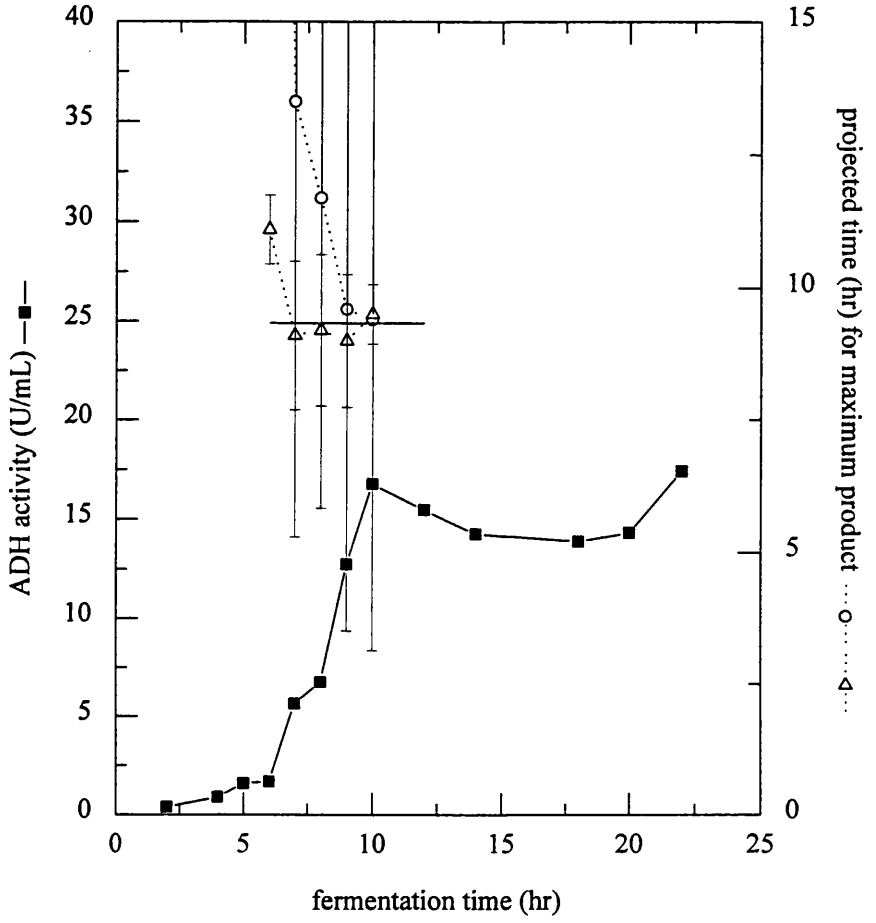
—■— optical density OD670nm (abs. units)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.16.
PROJECTING TO THE TIME OF MAX PRODUCT; *E.coli* fermentation 'G'
Comparison between linear and exponential fits for projecting the time when the known maximum OD 670nm level (10 abs. units) would be attained



—■— intracellular total soluble protein (mg/mL)
projected time for known product maximum using extrapolations of
···○··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.17.
PROJECTING TO THE TIME OF MAX PRODUCT; *S.cerevisiae* fermentation 'D'
Comparison between linear and exponential fits for projecting the time when the known maximum protein level (1.0mg/mL) would be attained



—■— ADH activity (U/mL)
projected time for known product maximum using extrapolations of
···o··· linear fits; ···△··· exponential fits: error bars represent the SD of fits
—— actual time for maximum

Figure 3.5.18.
PROJECTING TO THE TIME OF MAX PRODUCT; *S.cerevisiae* fermentation 'D'
Comparison between linear and exponential fits for projecting the time when the known maximum ADH level (14U/mL) would be attained

3.5.3. Statistically show that the product profile has indeed peaked

A further use of the fermentation data was to identify when the intracellular product profile has in fact started to peak. That is, to statistically show that a series of data points measured were significantly different, or otherwise, from the ones preceding it.

The following statistical criterion was employed to indicate that a real change in the rate has occurred.

$$\frac{\text{Rate}}{\text{CV} * \text{product level}} < -1.64 \quad \text{(i.e. 95\% confidence)}$$

where:

CV or coefficient of variance was taken as 5% throughout the fermentation;

and the rate is given by the gradient of the best linear fit for four points compared to the preceding set.

The results of applying this criterion to several fermentations are shown in Table 3.6. The table incorporates the results for the intracellular total soluble protein, G6PDH or ADH (as applicable) and also optical density profiles for the given fermentations.

	First change in detected at fermentation time Graphically determined change in parenthesis		
fermentation	total soluble protein	enzyme	OD 670nm
E. coli 'D'	4.5 : (4.25)	4.5 : (4.25)	4.25 : (4.25)
E. coli 'E'	3.25 : (3.25)	3.5 : (3.5)	3.25 : (3.5)
E. coli 'F'	5.02 : (5.67)	5.28 : (5.67)	5.02 : (5.02)
E. coli 'G'	3.08 : (3.92)	3.42 : (3.78)	3.42 : (3.42)
P. putida ML2 'A'	20.5^{*+} : (20.5)	22.5^{*+}:(22.5)	
P. putida ML2 'D'	39^{*} : (39)		
P. putida ML2 'E'	44.5^{*} : (44.5)		
S. cerevisiae 'D'	12.0 : (12.0)	12.0 : (12.0)	

Table 3.6. The results showing the times for statistically determined change and its corresponding graphical value (historically) determined for several fermentations. *CV = 2.5% ; $^{+}$ linear fits to three data points.

The fermentation profiles are given in Section 3.4. in 'Profile Monitoring'.

3.6. Rapid automatic analysis of enzymes activities using a "Stop Flow Analyser".

In the drive to automate the assaying and the analysis of enzyme activities (primarily alcohol dehydrogenase), a Stop Flow Analyser, SFA, was developed within the department. Essentially the SFA mimics the actions of manual assaying. A schematic diagram of the SFA is shown in Figure 3.6.1.

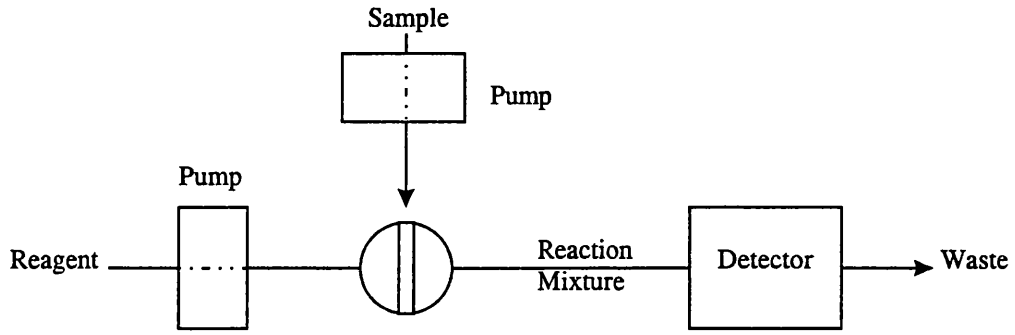


Figure 3.6.1. A schematic diagram of the Stop Flow Analyser.

The SFA was used, during intracellular product monitoring, to assay for alcohol dehydrogenase (ADH) and glucose 6-phosphate dehydrogenase (G6PDH) activities during the *S. cerevisiae* and *E. coli* fermentations respectively. The disrupted samples were split to be assayed both manually and with the SFA (operated by Dr. I. Holwill, of A.C.B.E., University College London). The homogenates for the SFA analysis were kept at room temperature for the duration of the analysis, whilst those samples which required dilution prior to manual assay were diluted in cold buffer immediately after homogenisation. This has raised questions regarding the validity of the direct comparison between manual and automatic SFA analyses for fermentations where product decay occurred mainly because of the homogenate handling conditions.

3.6.1. Monitoring ADH activity during *S. cerevisiae* fermentations.

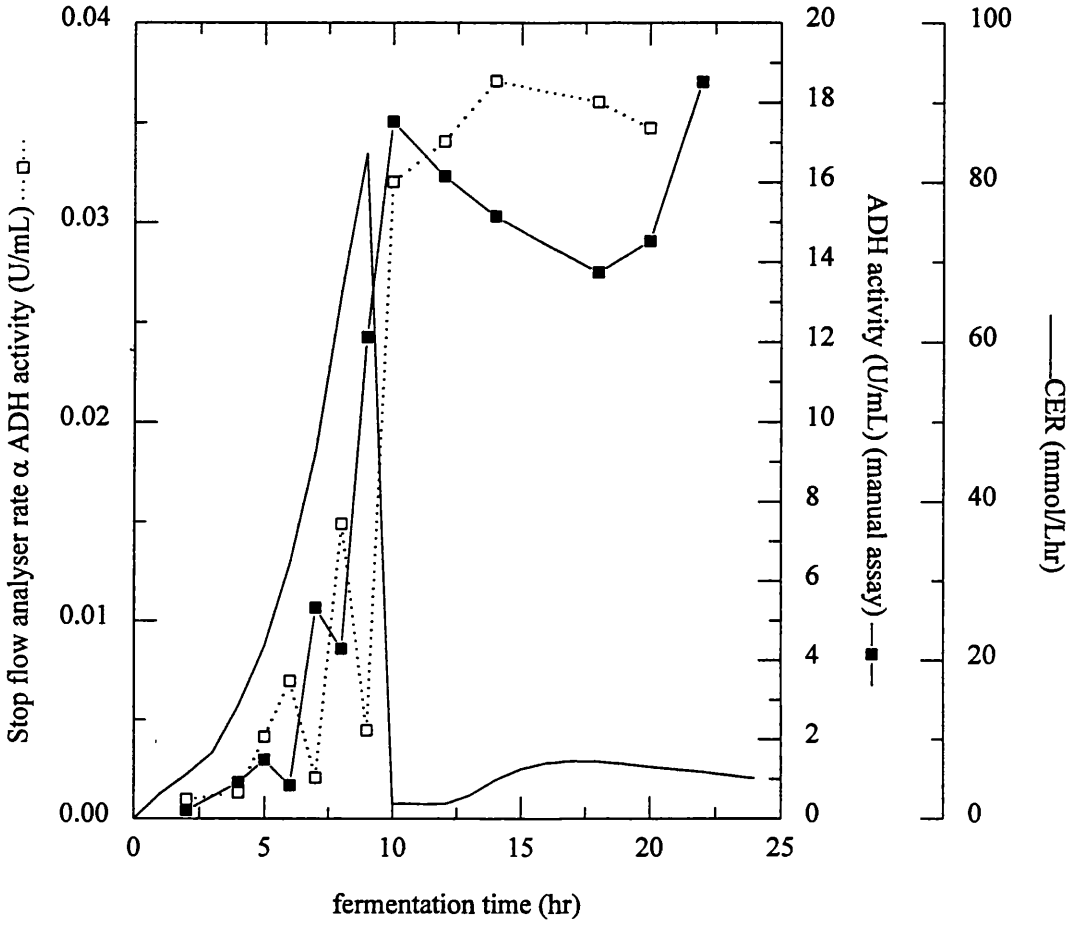
Several of the *S. cerevisiae* fermentations reported in Section 3.4., 'Profile Monitoring', were also monitored using the SFA for the homogenate analysis.

Figure 3.6.2. shows the profiles of ADH activity obtained by both manual and SFA analysis of the disrupted samples from one fermentation (yeast 'D'). During this particular fermentation, the samples from the fermenter were processed for two passes within the Micron Lab 40. The first pass homogenates were assayed once manually, and the second pass homogenates were assayed five times (the mean values are presented). The SFA was used to assay the homogenates on the second pass and as many as ten repeat assays were performed (again, the mean values are presented). As such the comparison is made for the second pass samples. First pass manual results for ADH activity are also shown in the figure given in U/mL.

The SFA results are given in terms of "mean rate per second" which is directly proportional to the ADH activity in U/mL.

The figure shows close agreement between the manual and SFA results over the first 12 hours. However, a marked drop in the manually assayed activities occurs over the following 7 hours which deviates from those registered with the SFA.

Two data points in particular from the SFA (at 7 and 9 hours) are markedly below expected values due to rapid decay in ADH activity over the repeat assay period. The homogenate storage during this period (of 1.5 minutes) has been highlighted as the most probable source for the decay. This effect was also observed with neat homogenates stored on ice as well as those diluted in cold buffer.



···□··· SFA rate (α U/mL)
5 SFA assays in 5 min: neat homogenate stored at room temp.
—■— ADH activity (U/mL)
5 manual assays in 5 min: homogenate diluted in cold buffer and stored on ice.
—— CER (mmol/Lhr)

Figure 3.6.2.
Rapid automatic analysis of ADH production during *S. cerevisiae* fermentation 'D' using Stop flow analyser compared to manual analysis.

3.6.2. Monitoring G6PDH activity during *E. coli* fermentations

A first attempt, made at monitoring the G6PDH activity through the course of an *E. coli* fermentation, proved to be unsuccessful because of

- the large dilution fixed within the SFA for alcohol dehydrogenase assaying work, and
- the fact that the levels of G6PDH are low compared to those of ADH in *S. cerevisiae* fermentations.

The SFA was reconfigured with relatively minor changes to maintain or at least revert very quickly the to ADH monitoring capability. This then enabled a further trial to be conducted to assay G6PDH activity for *E. coli* cells taken at the end of a fermentation. These included changes to the tube bore diameters to allow more of the sample and less of the assay mix for analysis in the photocell. This procedure consumed 30mL of cell homogenate with only four analysis trails.

The changes made proved insufficient to return a measurable rate for the G6PDH activity. Manual assays with a total dilution of 21 gave an adequate response for the activity, which was about at its peak at the end of fermentation.

This, by no means, limits the application of the SFA. The large fixed dilution within the SFA will however, have to be reduced to assay enzymes with lower activities.

In conclusion, the SFA would have to be totally re-configuration to conduct G6PDH assays whilst retaining ADH measuring capability. This would require a second pumping arrangement.

4. Conclusions

Commercial cell disruption devices

This investigation involved the use of several commercially available homogenisers to study their performance in the disruption of *S. cerevisiae* cell suspensions based upon the measurement of intracellular total soluble protein and ADH levels.

Although Table 4.1. shows the summary of the main findings, the devices were not tested in detail nor were their operating parameters optimised. Therefore these main findings must be taken as initial and preliminary.

Device	Main Findings
Microfluidizer M110T	High levels of cell disruption with pressure and number of passes; ADH release slower than expected or being deactivated.
Soniprep 150	Very gradual release with ADH enzyme deactivation
Mikro-dismemberator	High levels of cell disruption with no evidence of ADH deactivation; however, levels of maximum release significantly affected by cell suspension concentration
Polytron PT1200, PT3000	Neither devices were effective in cell disruption (<5% cell breakage after 6 min.); ADH enzyme deactivation was evident.

Table 4.1. Summary of the main findings for the small scale commercially available cell disruption devices. (Test organism *S. cerevisiae*, packed yeast).

Kinetics of cell disruption within the Micron Lab 40

The first order process described by (Hetherington *et. al.*, 1971) was shown to be a suitable representation of the disruption kinetics of *S. cerevisiae* within the Micron Lab 40. (i.e. The rate of release of protein with respect to the number of passes is a first order process with respect to the unreleased protein concentration (i.e. R_m-R)):

This yielded values for K = 1.06 x 10^{-5} (bar^{-a}) and 'a' = 1.76 (Figure 3.2.4. Section 3.2.).

Reproducibility of cell disruption and statistical analysis

The stages of analysis under consideration as sources of potential error were:

- the complete process - disruption to measurement,
- sampling from the homogeniser,
- dilution for measurement,
- the assay itself.

micro-organism and cell concentration		n	protein%	SD%	ADH%	SD%
Disrupt to assay						
S. cerevisiae 1% w/v		50	105.5	5.7	102.1	3.8
S. cerevisiae 45% w/v		50	105.5	6.7	98.1	5.7
P. putida ML2, 4.53g/L dcw		25	105.1	4.3		
P. putida ML2, 9.89g/L dcw		25	104.4	3.6		
E. coli 10.3g/L dcw		20	94.5	5.0		
Sampling from the homogeniser						
S. cerevisiae 1% w/v		20	-	2.5		
S. cerevisiae 45% w/v		20	-	3.8		
Dilution	BSA 1mg/mL	25	99.7	3.4		
Enzyme assay	G6PDH	28	-	5.1		

Table 4.2. The mean total intracellular soluble protein and enzyme activity and standard deviations from various micro-organisms at various stages of the measurement process.

Samples taken during the course of fermentations of *P. putida* ML2, *E. coli*, *S. cerevisiae* and *R. erythropolis* were disrupted at 1200 bar operating pressure, and single pass through the Micron Lab 40. Statistical analysis was carried out on the data. The results are given below:

Micro organism	n	mean protein %	SD %
P. putida ML2	49	95.6	4.1
S. cerevisiae	44	96.1	4.4
E. coli	27	96.1	6.9
R. erythropolis (1000L)	9	84.2	4.5
R. erythropolis (20L)	4	84.9	6.0

Table 4.3. The mean total soluble protein releases and standard deviations for various micro-organisms taken during the course of fermentations.

Profile Monitoring

- The fermentations of several micro-organisms including *P. putida* ML2, *E coli*, and *S. cerevisiae*, were monitored where the intracellular total soluble protein, ADH (for yeast only), G6PDH, and optical density were measured.
- In the *E. coli* fermentations the sampling frequency was one every 20 min, simulating at-line monitoring.

Profile analysis

- "Normal" operating zone has been constructed for *E. coli* fermentations using locus plots of normalised intracellular total soluble protein and G6PDH.
- Exponential fits to four data points, progressively, has allowed rapid time projections to a known maximum product level.

- Statistical ratio has been employed to show a peaking / levelling in the product profile.

Prototype high pressure homogeniser

- The small scale high pressure homogeniser developed with the department yielded in excess of 50% cell breakage at moderate pressures with no enzyme (ADH) deactivation. (Refer to Section 5, below).

5. Prototype Micro-homogeniser

The results from the Micron Lab 40, showing almost complete cell disruption, reproducibly for various micro-organisms, were encouraging. However, the need to develop a disruption device that had the potential for use on-line processing a small sample volume, led to the testing of a new prototype high pressure homogeniser.

5.1. Equipment and Methods

The prototype was developed and constructed by John T. Bailey (Engineering) Ltd., Surrey, UK in conjunction with the Department of Chemical & Biochemical Engineering, University College London, and Jim Malloy of Fisons, Cambridge, UK

The design of this prototype is based around the conventional Micron Lab 40 in that a single piston is driven by a hydraulic force to discharge the contents of the piston chamber through the homogenising head at a pre-set release pressures, the obvious difference between the two homogenisers is the small sample volume required in the prototype (<5mL). Other differences included the cone-shaped piston and a 1mm diameter orifice in the valve seat.

A schematic diagram of its cross section is given in Figures 5.1.a., 5.1.b & 5.1.c.

Figure 5.1.d. shows a photograph of the prototype in its working position.

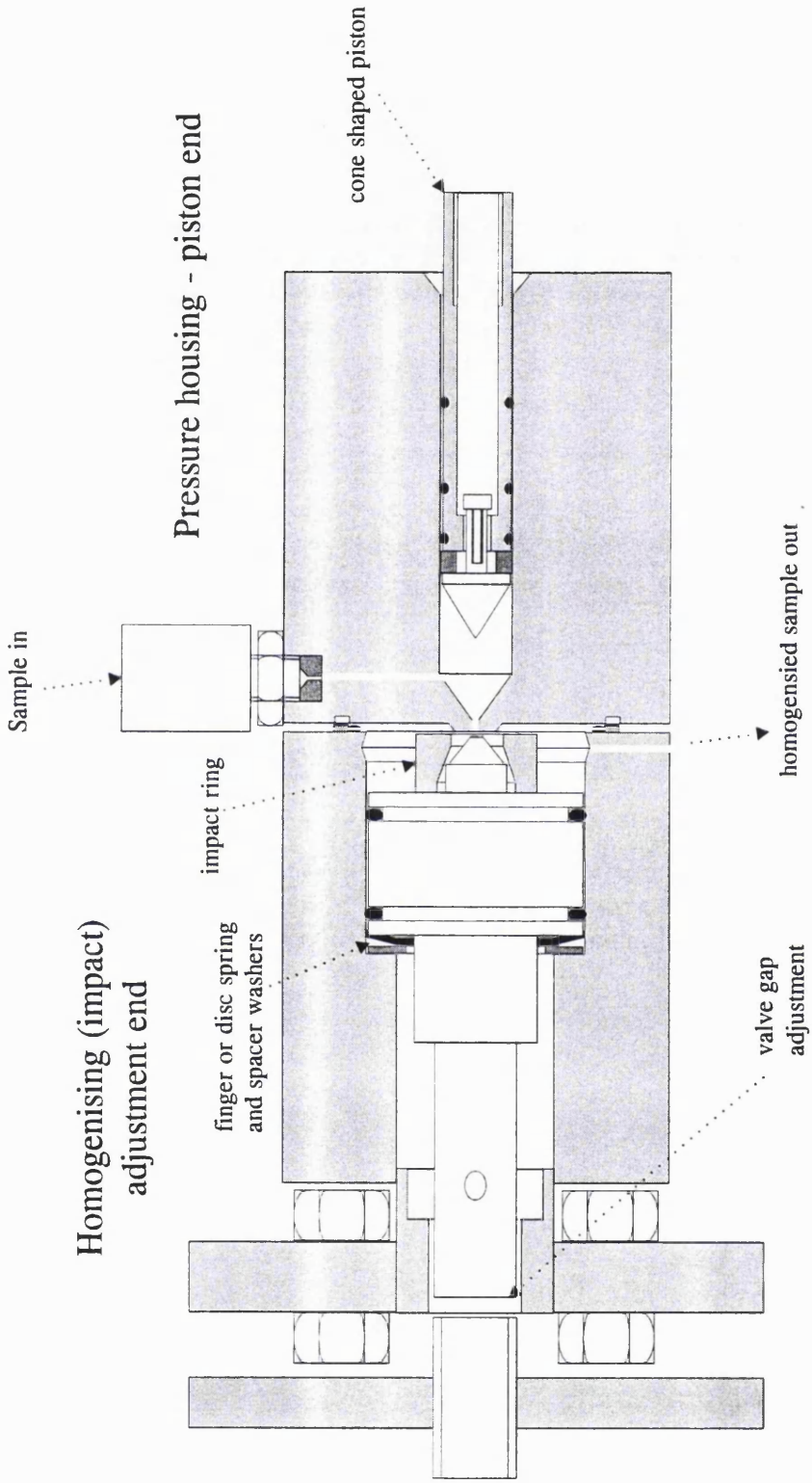


Figure 5.1.a. Schematic diagram of the cross-section of the 'Micro-homogeniser'

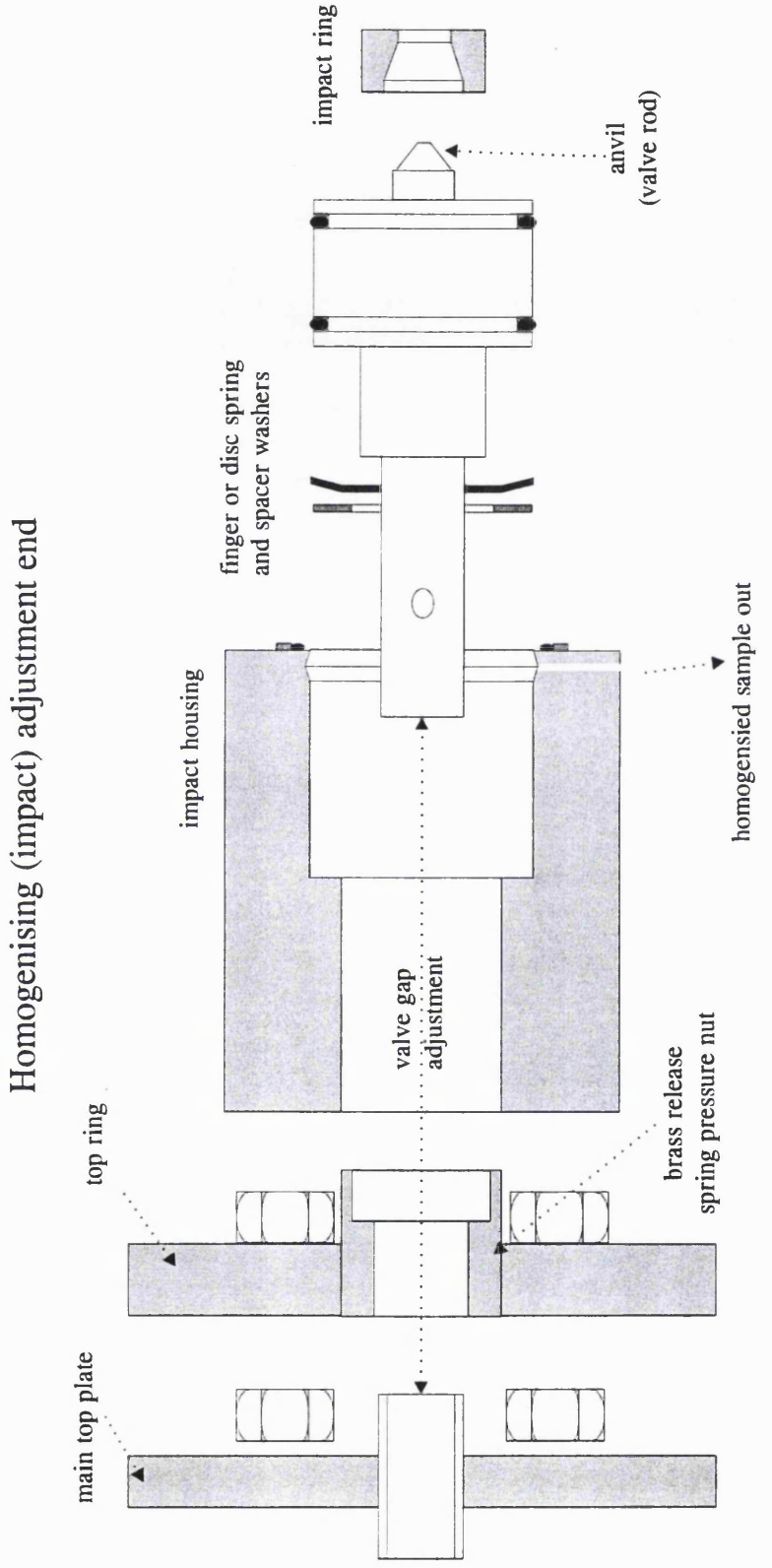
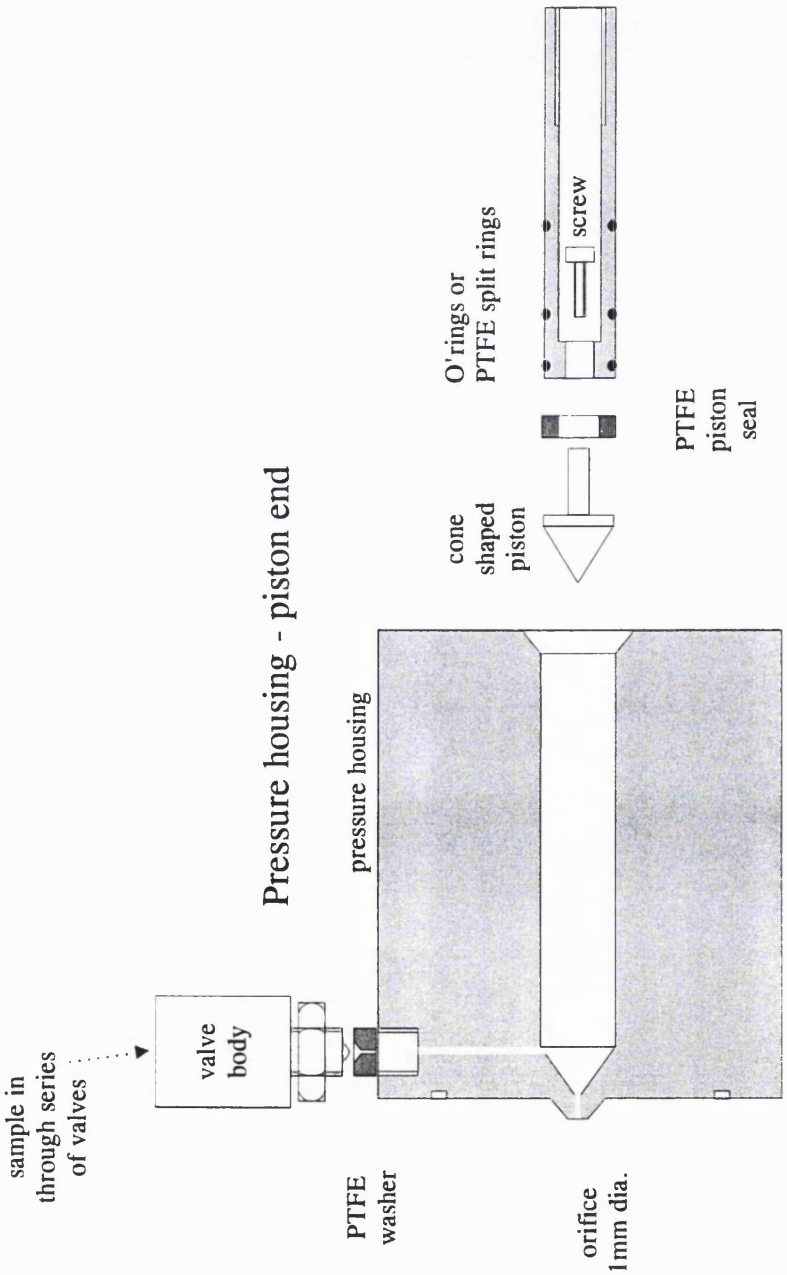


Figure 5.1.b. Schematic diagram of the cross-section of the 'Micro-homogeniser' - homogenising end (exploded view).

Figure 5.1.c. Schematic diagram of the cross-section of the 'Micro-homogeniser' - Pressure housing (exploded view)



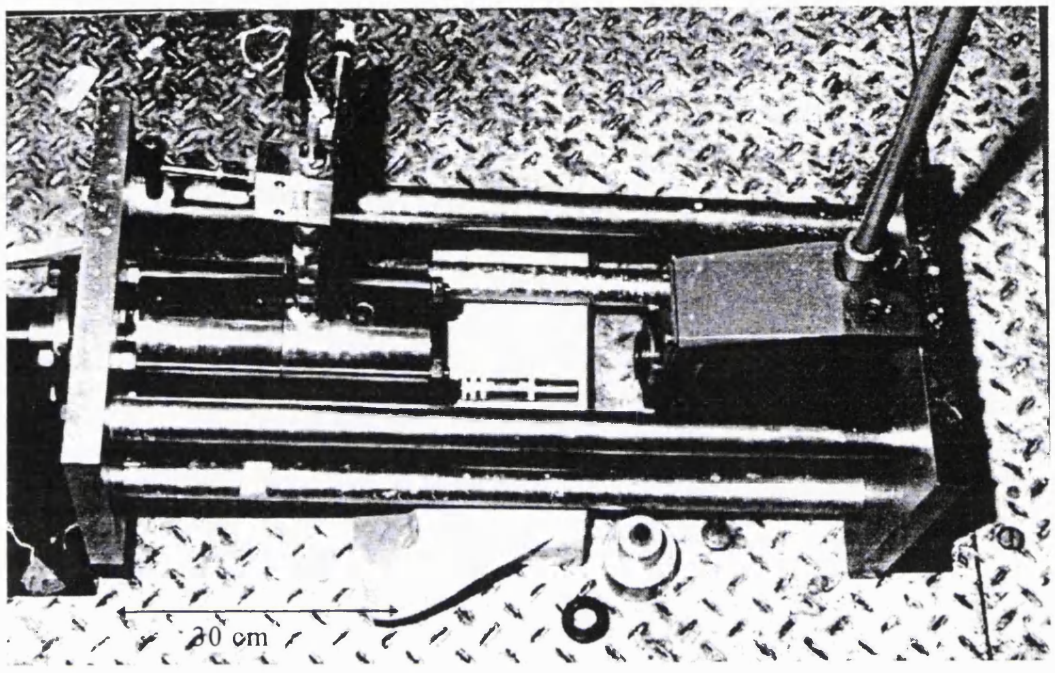


Figure 5.1.d. A photograph showing the "Micro-homogeniser' in its working environment.

5.1.1. Equipment

The crucial features of this prototype are as follows:

- small sample volume (<5mL) which compares very favourably with the 40mL in the smallest commercially available high pressure homogeniser (Micron Lab 40);
- the discharge is governed not by the valve gap between the valve rod and valve seat, but by means of a spring which holds the valve closed until the pressure exceed its compression limit. Thus the discharge pressure can be adjusted by replacing the spring with one of lower or higher strength and / or altering its compression;

- the valve gap is set manually and remains at that setting throughout the disruption cycle; the gap being set by using filer gauges commonly used to set valve clearances in the combustion engine of motor vehicles;
- there was no cooling device on this prototype, so the samples were kept on ice prior to disruption and only processed for a single pass through the homogeniser.
- the impact distance can be altered by replacing the impact rings; and unlike the Micron Lab 40, the device can be operated safely without the impact ring in place.

5.1.2. Disruption procedure

After initial trials the sample loading section was modified to allow use of a pressure gauge mounted in the process stream, measuring the pressure experienced by the cell suspension. This arrangement is shown in a schematic diagram in Figure 5.2.

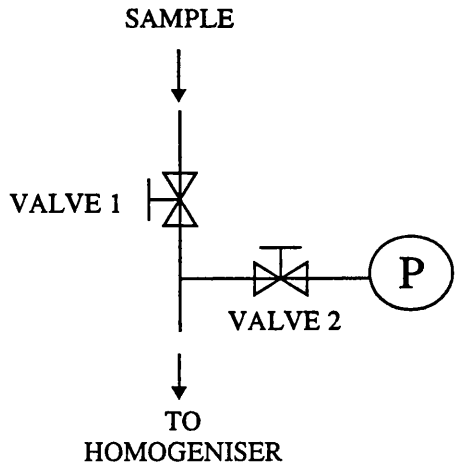


Figure 5.2. Schematic of the sample loading assembly of the prototype homogeniser.

The cell suspension was cooled to below 12°C on ice and drawn into the syringe which was then attached to the valve body leading to the piston chamber. The arm connecting the pressure gauge and the gauge itself required priming prior to the trials. When the piston is physically withdrawn (valve 2 is closed), the combination

of the vacuum generated and the force on the syringe holding the sample, the sample is loaded into the piston chamber.

Now with valve 2 open and valve 1 open, the pressure gauge can be primed by pumping up the piston (no release of cell suspension through the homogenising head occurs). Then valve 2 can be closed and 1 open to allow a further intake of sample to replace the volume used to prime the gauge.

With valve 2 open and valve 1 closed, the homogenising process is started.

Following the disruption process, representative samples of the homogenate were collected in eppendorf tubes. Samples for analyses were take prior to disruption (number of passes, N = 0) and from the discharge stream after homogenisation (for N = 1)

The valve gap was set with the use of filer gauges. The release pressure could only be set after semi-dismantling the equipment.

5.2. Results

The trials conducted involved the use of *S. cerevisiae* (Baker's yeast) cell suspensions to investigate the effects of impact ring, valve gap and the operating pressure.

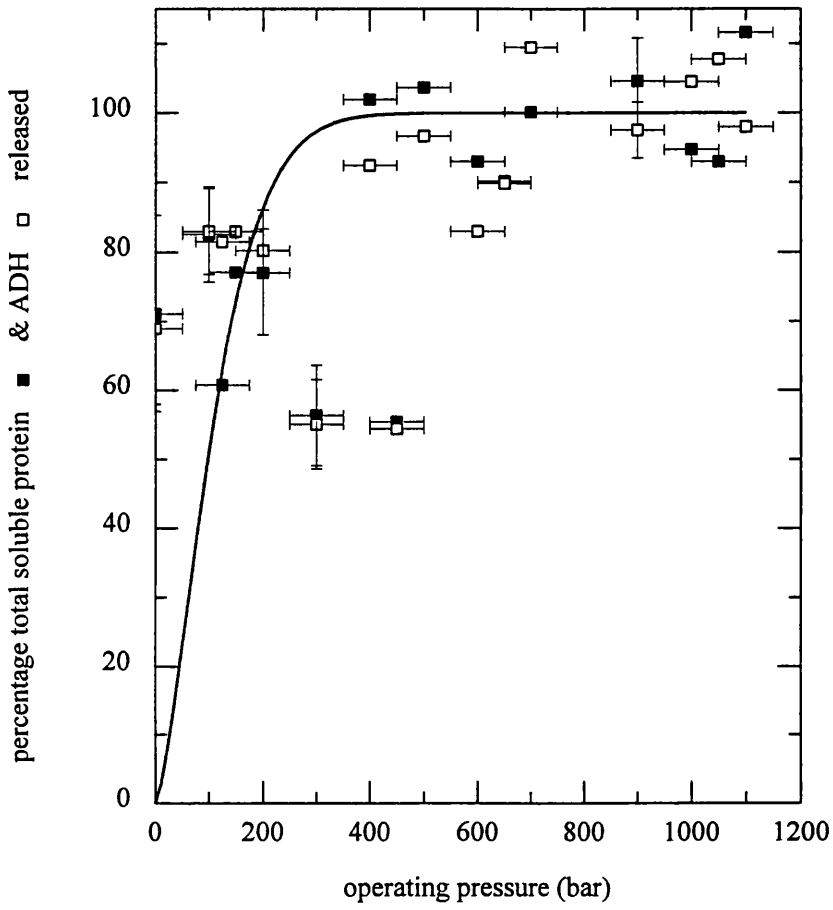
Initial operation of the prototype was without the impact ring. This did not compromise the safe running of the equipment. Changes in release pressure were set from zero to 1100bar. There was pressure variations in the order of 50 to 100 bar during the course of the disruption cycle. These variations are not significant at very high pressures but are significant at operating pressures ~100 to 300 bar.

Figure 5.3. shows the results for the disruption of *S. cerevisiae* (10% w/v packed yeast). Separate experiments were carried out with the valve gap set at 50μm, 150μm, 300μm and 5mm respectively. (The valve gap is the distance to which the valve opens at the release pressure to allow the discharge of the cell suspension). The maximum setting was arbitrarily set to 5mm but this distance exceeded the maximum spring extension (i.e. the disc or finger spring will only extend to their original shapes and no more).

The maximum protein and ADH levels were determined by homogenising a sample of the cell suspension for three passes at 1200 bar operating pressure in the Micron Lab 40 (Figure 5.4.). These results were then used to calculate the percentage protein or ADH released in the prototype homogeniser.

With the high levels of cell breakage found using the prototype it was decided to evaluate the strength of the yeast cells by disruption at low pressures within the Micron Lab 40. Figure 5.4. also includes results for the disruption of a sample of 10% w/v packed yeast for five passes at 400 bar operating pressure in the Micron Lab 40. The gradual release of both protein and ADH with number of passes shows that the yeast was not abnormally weak (the trend in the release is identical to that shown in previous work shown in Figure 3.2.2. in section 3.2.1.)

The results obtained with the prototype homogeniser were important in that the lowest amount of protein release observed during the entire trials was above 50% of the maximum. Even when the release pressure in the prototype was set to zero bar (albeit that zero bar could quite easily be 50-100bar), total soluble protein and ADH activity levels were approximately 70%.



■ intracellular total soluble protein (%)

□ ADH activity (%)

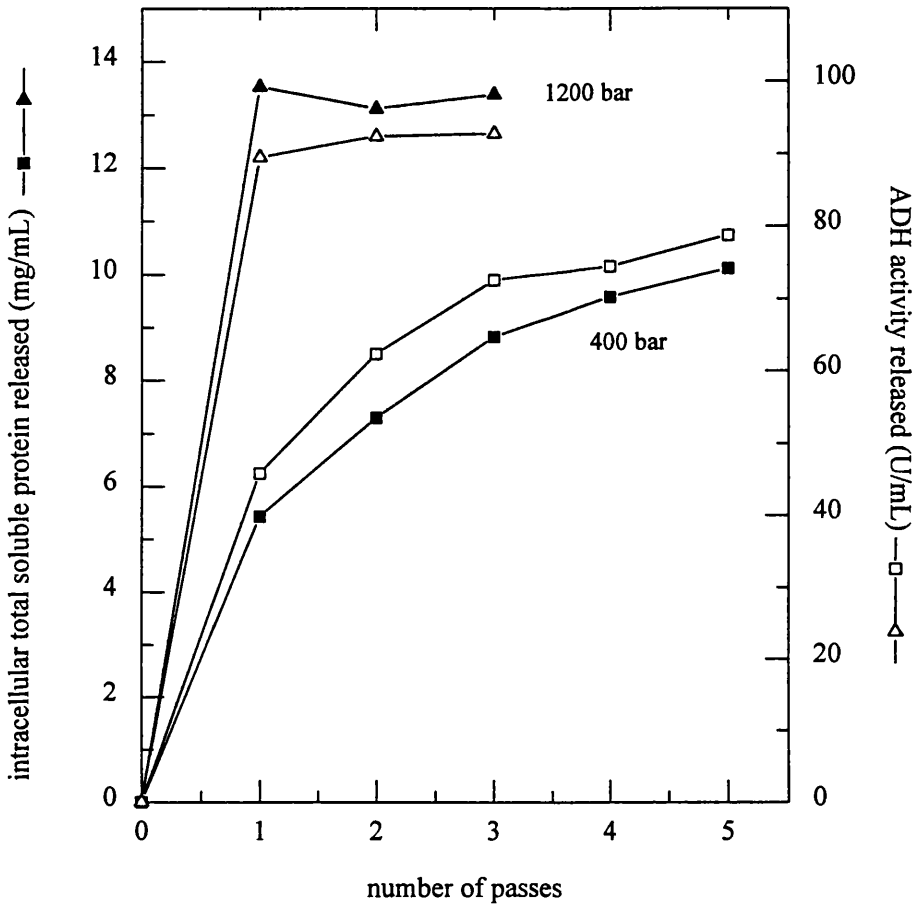
—— fit based on the usual kinetics of high pressure homogenisers

x-error bars indicate the pressure variation of +/- 50bar

y-error bars where shown indicate the SD for several repeats

Figure 5.3.

Intracellular total soluble protein and ADH activity released from *S.cerevisiae* (10% w/v packed yeast) in the prototype high pressure homogeniser (single pass) over a range of operating pressures. Results from Micron Lab 40 (1200 bar, three passes, shown in Figure 5.4.) were used to calculate the percentages.



400, 1200bar operating pressure
—■—,—▲— intracellular total soluble protein
—□—,—△— ADH activity

Figure 5.4.
Intracellular total soluble protein and ADH released from *S. cerevisiae* (10% w/v packed yeast) in the Micron Lab 40. The results at 1200bar operating pressure, three passes were used to calculate the percentages for the results for the prototype homogeniser. The yeast used in the prototype trials is shown not to be abnormally weak.

This result is apparent in Figure 5.5. which shows no data points below 50% on either axis. In Figure 5.6. the percentage release of ADH is plotted against the percentage total soluble protein released and as expected (Follows *et. al.*, 1971), a correlation is shown. This also points to a fact that there is no evidence of ADH damage.

5.2.1. Influence of the impact ring

No significant difference in the cell disruption levels were observed between P = 0 bar ± 50 bar without an impact ring (71% protein, 68% ADH) and P = 100 bar ± 50 bar with impact ring (83% protein and ADH).

The limited number of trials carried out with the impact ring, seem to suggest that in this device the impact ring was not a crucial element in the cell disruption process. Clearly further work need to be carried out over an extended pressure range to support this finding.

5.2.2. Influence of the valve gap

This prototype differs from conventional high pressure homogenisers in a crucial way in that the operating pressure in conventional high pressure homogenisers is regulated by control of the valve gap, whilst this was not the case for the prototype. The operating pressure (or the release pressure) is governed by the tension in the disc-spring; the valve gap being independently set to any chosen distance. The disc-spring keeps the valve closed until the pressure is high enough to overcome the disc-spring-tension. (It is important to mention here that the pressure was measured directly from the liquid-leg).

This difference in the means of regulating the operating pressure allows investigation into the influence of the valve gap on cell disruption within high pressure homogenisers (independent of the operating pressure).

Figure 5.5. shows the release of total soluble protein and ADH with varying valve gap. The valve gap was varied from 100μm to 5mm and the operating pressure ranged form 100 bar to 1100 bar. In general these results (the protein and ADH release together) show the valve gap to have no significant influence on the level of cell disruption at any given operating pressure. This is particularly evident in the

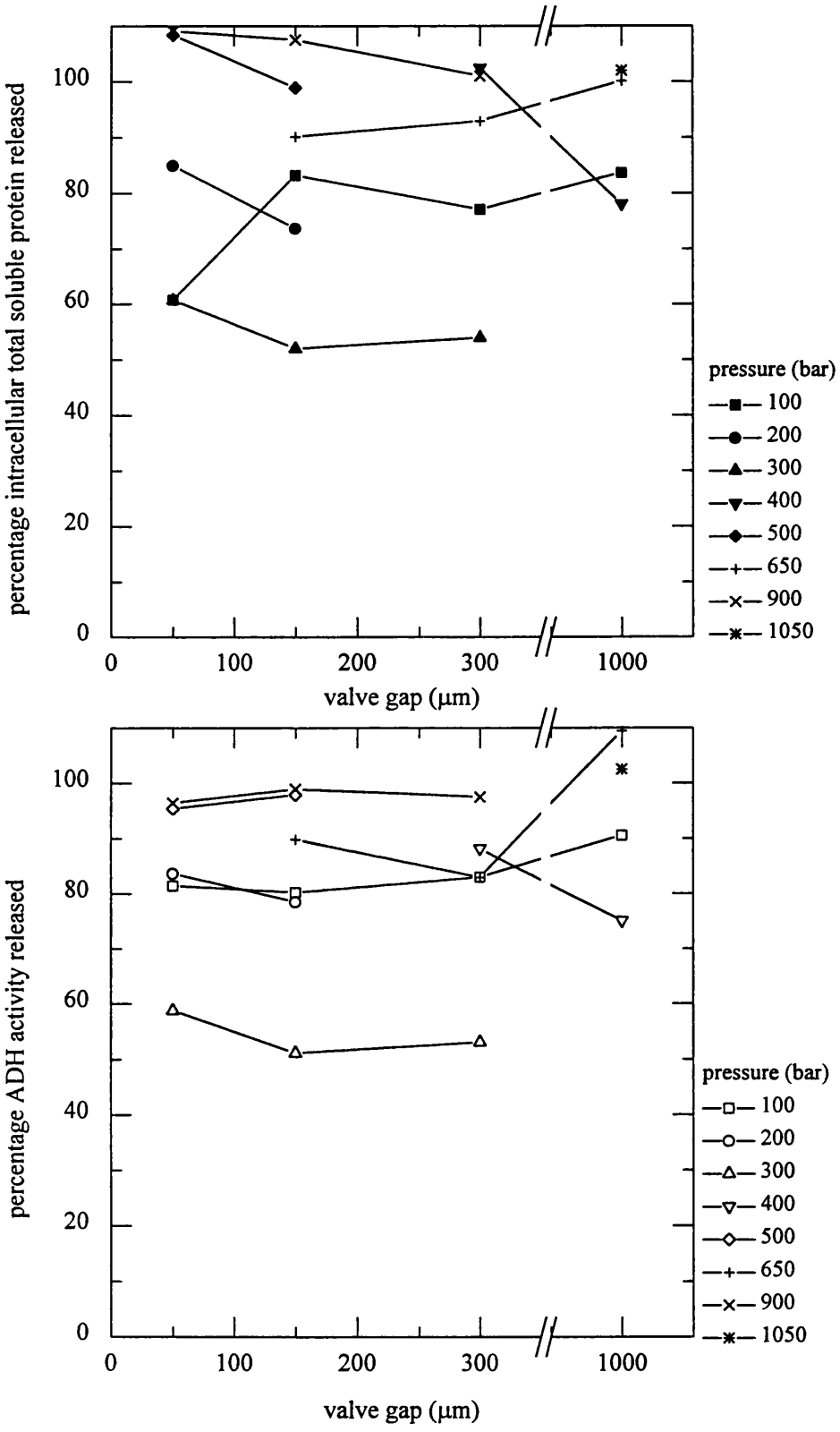


Figure 5.5.
The effect of valve gap on the percentage release of intracellular components from *S. cerevisiae* (10% w/v packed yeast) after a single pass through the prototype high pressure homogeniser; total soluble protein [top], ADH activity [bottom].

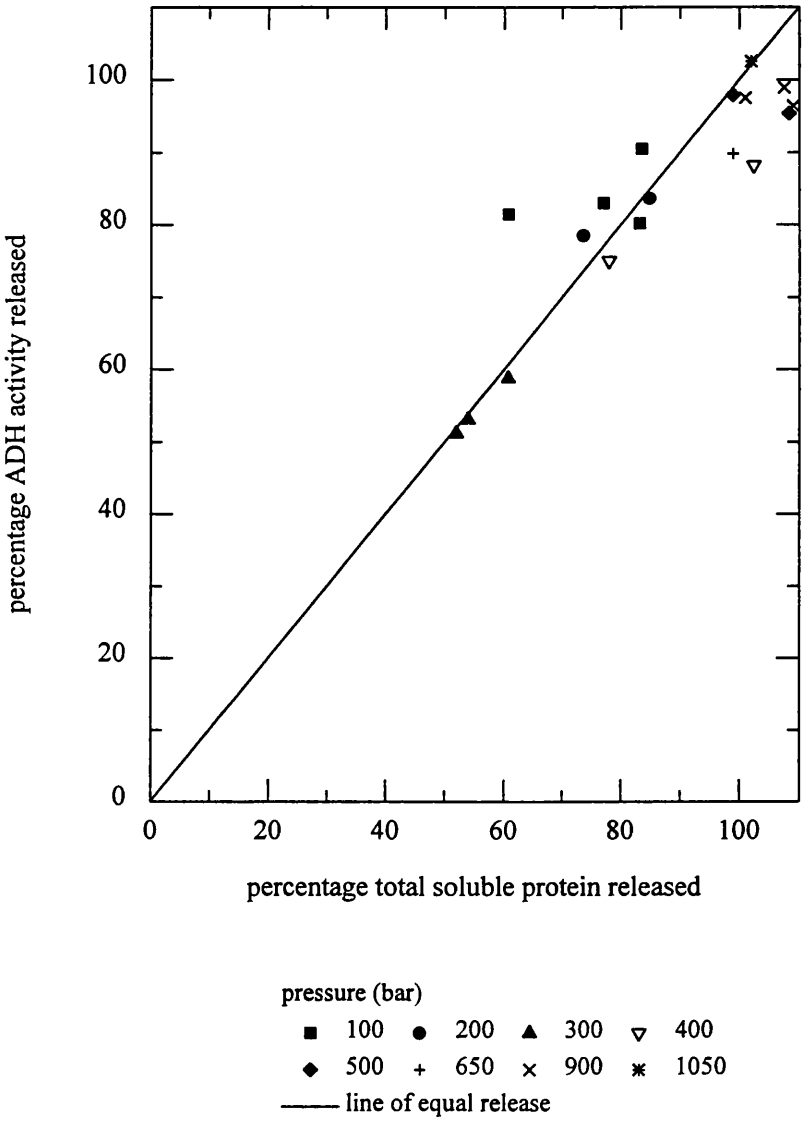


Figure 5.6.
Correlation between intracellular total soluble protein and ADH released from *S.cerevisiae* (10% packed w/v) disrupted in the prototype high pressure homogeniser with varying valve gaps (from results shown in Figure 5.5.)

ADH release data at operating pressures of 100, 200, 300, 500 and 900 bar. The total soluble protein data shows an increase in the percentage released at 100 bar.

With the valve gap within the Micron Lab 40 high pressure homogeniser ranging from 50μm to 25μm for operating pressures of 100 bar to 1600 bar respectively (Section 3.2.2.), it is strictly not valid to compare these findings directly with other homogenisers.

5.2.3. Mechanism of cell disruption

High levels of cell breakage were achieved at relatively low pressures without the use of an impact ring. The valve gap was shown not to influence cell disruption in any discernible way. Another factor effecting cell disruption can be the pressure component.

At very low pressures (0-200 bar) a significant amount of cell breakage was observed, as illustrated by the total soluble protein and ADH released. This suggests that the release pressure although important is not the only (pressure related) factor influencing cell breakage.

The change in pressure, ΔP, or the rate of change of pressure, dP/dt, compression and sudden release of pressure is also an element to be considered.

The cell suspension flow-path is through a 1mm diameter orifice which constitutes the valve seat. (The Micron Lab 40 has a 3mm diameter bore in its valve seat). However, the work reported by Engler and Robinson (1981a), with a high pressure flow device operating at pressures of 500 - 1250 bar employing a high velocity jet of up to 150 mL of cell suspension through an 80μm nozzle to impact onto a stationary surface, indicated this impingement onto a stationary surface was the major cause of cell disruption.

Although, wear and tear to the piston and other seals as well as on the piston & bore prevented further investigations, successful and useful information has been generated.

5.3. Conclusions

- *S. cerevisiae* cell breakage was very significant in that the minimum amount of protein release observed during the entire trial was >50% of the maximum (50% of R_m) without the impact ring *in situ*. Even when the release pressure in the prototype was set to zero bar, total soluble protein and ADH activity levels were approximately 70%.

- No significant difference in the cell disruption levels were observed between P = 0 (±50) bar without an impact ring (71% protein, 68% ADH) and P = 100 bar with impact ring (83% protein and ADH).

- The limited number of trials carried out with the impact ring *in situ*, seem to suggest that the impact ring was not a crucial element of the cell disruption process in this device.

- The results of protein and ADH release showed that the valve gap to have no significant influence on the level of cell disruption at any given operating pressure. This was particularly evident in the ADH release data at operating pressures of 100, 200, 300, 500 and 900 bar. The total soluble protein data showed an increase in the percentage released at 100 bar.

- Although wear and tear to the piston and other seals as well as on the piston & bore prevented further investigations, successful and useful information has been generated.

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6. Recommendations

6.1. Commercially available cell disruption devices

The study of commercially available cell disruption devices was conducted to evaluate the cell breakage efficiency of these devices so as not to limit the focus of this study to high pressure homogenisation.

A further reason for evaluating other cell disruption devices was the need to compare their cell breakage results with those obtained using the Micron Lab 40 high pressure homogeniser.

The study presented in Section 3.1. was however, limited in two significant ways:

- there was only partial confidence in the results because of the limited amount of trials data;
- not every commercially available device could be (nor was) experimentally evaluated.

The most promising device, of those evaluated, was the Mikrodismemberator (Section 3.1.4.) which yielded high levels of cell breakage within a two minute processing time. But, because of initial concerns about the variable levels of breakage the evaluation was prematurely terminated.

The disruption devices, with the exception of the two Polytron devices (Section 3.1.5.), should be evaluated in greater detail to provide sufficient data to ascertain their full potential as useful cell disruption devices.

New devices such as the Nebuliser which produces a large number of gas-liquid surfaces should be evaluated. Although gas-liquid interfaces are thought to cause enzyme deactivation, the extremely large numbers of these surface may in this device, even protect the enzyme from deactivation.

6.2. Automation of cell breakage

This study has successfully demonstrated the ability to monitor intracellular components rapidly and with increased frequency during the course of fermentation using the Micron Lab 40 to effect cell breakage. As automated aseptic sampling and micro-centrifuge already exist and have been successfully used for monitoring extracellular components (Turner et. al., 1994), the next evolutionary step would be to develop a homogeniser so that it lends itself towards the automated sampling and analyses of intracellular products.

The product of interest can either be assayed directly, e.g. in the stop flow analyser, or to eliminate potential problems of cell debris from homogenisation interfering with product analyses, the homogenate can be clarified in the micro-centrifuge prior to analyses.

The development towards automation of the homogeniser will require extensive validation steps which would include reproducibility of cell disruption.

The Micron Lab 40 high pressure homogeniser cannot be automated without significant modification by the manufacturer. These modifications would include the filling of sample into the piston chamber, homogenate removal, system flushing to avoid cross-contamination between two sequential samples, and the necessary electronic adjustments to accommodate this sequencing.

Figure 2.3., in 'Materials and Methods', shows the possibility of homogenate removal directly from the sample assembly; it may be feasible to use this homogenate removal loop to also load the piston chamber.

Some of the commercial devices such as the Microfluidizer and Mikrodismemberator (which is essentially a miniature beadmill) lend themselves rather easier towards automation. In fact Stube and Spohn, 1994, customised a small 1.5 mL beadmill in the form of a flow-through cell and successfully used it in the on-line disruption of yeast cells.

6.2.1. Operator / Environmental Safety

The micro-organisms used in the biotechnology industry and generally accepted as safe (GRAS status). They are not pathogenic in themselves, however, the

compounds they produce or express may present a risk to health, if the micro-organism is able to enter into the body.

In addition, there is the more obvious problem of direct operator exposure to unbroken cell, proteins, cell debris and other products in the form of aerosols, where various toxic or allergic reactions have to be considered after inhalation (Bennet, *et. al.*, 1990). In particular, allergic reactions can be induced in susceptible people following repeated exposure to an allergen, workers may become sensitised and will then react to extremely low challenge concentrations.

The most likely route of occupational exposure of cells, cell debris and intracellular components is via aerosol generated by breaches of containment during cell disruption.

Health hazards to the operators may be reduced by operating the homogenisers in a contained environment, e.g. the homogeniser could have an encapsulating air hood which would drawing any aerosols away from the operator.

6.2.2. Mechanical Safety

Any on-line product monitoring system developed has a potential of being used within the industrial environment employing crude fermentation broths.

The high pressure homogeniser used within this system must be evaluated for potential mechanical risks when processing fermenter broth containing insoluble components or inclusion bodies within the cells, must be addressed.

6.3. Prototype micro-homogeniser

The prototype homogeniser (Section 5.) has performed well even at low operating pressures (See Figure 5.3.). However, contrary to the strong influence of the impact ring and the valve gap in Micron Lab, these seemed not to play a significant role in the prototype homogeniser during the trials conducted. This element requires further evaluation and qualification. The design of the prototype homogeniser, although mimicking the Micron Lab 40, had a valve geometry considerably different to it. It may be that the dimensions of the impact ring are such that it has no significant influence on the operation, and the valve gap, however crudely set, was much larger

(50 μm to 1000 μm) than those expected within conventional homogenisers (<50 μm). Clearly, the design of the valve warrants detailed investigation.

Although further work on the prototype homogeniser has been recommended, the fact that the development of any prototype is invariantly expensive, requiring many improvements and versions, must be considered alongside any potential sales market for the production device. Prototype development is also a very time consuming exercise.

6.4. Determination of the extent of cell breakage

Within this study the use of R_m has been central to the analysis of the extent of cell breakage. Other methods, listed below, can also be used to verify the extent of cell breakage, including:

6.4.1. Viable cell count or plate count

The 'neat' cell suspension is serially diluted to separate and isolate individual cells and then spread over an agar medium. This procedure is based on the theoretical principle that one viable microbial cell can give rise to one colony and that the number of colonies that develop on an agar plate approximates the number of viable cells plated onto the agar.

Although in routine use, viable cell counting is limited in its use to determine the extent of actual cell lysis because:

- some microbes form stable groupings, such as tetrads, cuboidal packets, and chains of cells, which are difficult to break apart. Each group would produce a single colony thus the exact number of cells will remain unknown;
- this is not a direct measure of cell breakage, intact cells which are not viable will not be counted as in plate counts. Hornby, 1995, found that under certain stress levels, cells would remain intact and at the same time be not viable.

6.4.2. Electronic cell counters

Electric cell counters, such as the Coulter counter (Coulter Electronics Inc., Hialeah, Florida, USA), for example, measures the conductivity of an electrolyte

solution as it passes through a small aperture. Cells (viable or non-viable), cell debris and other non-conducting particles suspended in the electrolyte passing through the aperture decreases conductivity of the electrolyte. The electronic counter is able to detect and record these changes, from which both the number and the size of the organisms in suspension can be estimated.

The extent of cell breakage can be estimated by comparing the results of the pre-homogenisation cell suspension with those of post homogenisation.

6.4.3. Particle size distribution

Cell disruption kinetics of high concentrations of *E. coli* cell suspensions (48.4 g/L d.c.w.) in a high pressure homogeniser were effectively followed by Agerkvist and Enfors (1990). They showed a mean size of 1200nm reduced to 500nm in one pass and on subsequent passes the mean size reduced to 200nm.

Given the heights of the cell distribution peaks for pre and post homogenisation cell suspensions, an estimate of the extent of cell breakage can be determined.

6.4.4. Flow cytometry

Flow cytometry, especially when coupled with other analytical techniques (such as culture fluorescence monitoring), is a useful tool in biotechnology because the detailed information on the distribution of cell characteristics provided by this technique contributes to the understanding of the physiology of the organism cultured (Degelau *et. al.*, 1992).

Samples taken directly from the fermenter are analysed without any pre-treatment steps to determine cell size and shape using scattered light and absorbance measurements. Many examples of these measurements have been reported, including cell size studies on *S. cerevisiae* (Ranzi *et. al.*, 1986) and plasmid stability studies in recombinant *E. coli* (Dennis *et. al.*, 1983, *ibid*, 1985; Scheper *et. al.*, 1984, *ibid*, 1987).

The extent of cell breakage could be calculated by flow cytometry by comparison of scattering / absorbance data of pre and post homogenisation samples; i.e., the reduction in overall cell size (or increase in 'smaller' cells) would be indicative of the extent of cell lysis.

6.5. Application to other micro-organisms

The walls of Gram-positive bacteria are relatively thick (15-50nm) and contain 40-90% peptidoglycan with the remainder being primarily polysaccharides and teichoic acids (Ghuysen and Shockman, 1973). In Gram-negative bacteria the wall consists of a much thinner peptidoglycan layer(1.5-2.0nm) and an outer membrane similar in appearance to the cytoplasmic membrane. The major resistance to disruption of bacterial cells appears to be the peptidoglycan network (Engler, 1985). Yeast cell wall thickness is greater than Gram-positive bacteria, as reported by Moor and Muhlethaler (1963), and that this thickness also increases with age. However, it is possible that only a portion of the wall thickness contributes to its rigidity and strength (Engler, 1985).

Fungal wall strength is apparently related to polymer networks, as in both yeast and bacterial cell walls. Additional strength is provided by fibrous structures of either chitin or cellulose in some fungal cell walls.

This study concentrated primarily on the disruption of *S. cerevisiae* (Bakers' yeast), *P. putida* ML2 and *E. coli*. *P. putida* ML2 and *E. coli* are Gram-negative bacteria. It is evident from Table 1.2. (Section 1.) that Gram-positive cocci are harder to disrupt using high pressure homogenisation than yeasts or Gram-negative cocci. (Edebo, 1983).

The cell disruption kinetics of filamentous micro-organisms should be thoroughly investigated as it was not covered in this study.

6.6. Fermentation monitoring

In this study, fermentation integrity and sterile operation was seen to be effective by removing samples under pressure maintaining fermenter sterility (i.e. with the air exit shut off to increase pressure within the fermenter vessel and then the sample removed}, then the samples were viewed under the microscope to observe the shape of the cell and confirm the growth of a monoculture fermentation. With the *E. coli* cell fermentations, as well as observations under the microscope, the large inoculum and the extremely rapid doubling time of the cells (30 minutes) were deemed sufficient to guarantee that *E. coli* cells were being grown and monitored.

Notwithstanding the above comments, confirmation of the growth of a monoculture during the course of fermentation should also be carried out by plating out samples

and observe the subsequent growth. This may not directly lend itself to on-line monitoring but can be used for retrospective confirmation of the cell identity.

Further fermentation work should be carried out to increase the confidence in the fermentation "event" detection methods put forward in this thesis, and test them with deliberately induced "abnormal" fermentations. The use of *E. coli* will provide relatively short and reproducible fermentations which can easily be repeated several times.

6.6.1. Product monitoring

During the current study, alongside intracellular total soluble protein, key intracellular enzymes were targeted as the products of interest, and the monitoring of these was undertaken.

The enzyme Glucose 6-phosphate dehydrogenase was monitored in the *E. coli* and *S. cerevisiae* fermentations. Alcohol dehydrogenase was also monitored in the *S. cerevisiae* fermentations.

With glucose as the carbon source for the fermentations, the enzyme G6PDH was expected to be constitutive for both organisms as it would be required in the oxidative branch of the pentose phosphate pathway where it catalyses the conversion of Glucose 6-phosphate to 6-phosphoglucono-δ-lactone.

In the yeast fermentations, the enzyme ADH was chosen as it is involved as a catalyst in one of the steps in the conversion of glucose to ethanol. Glucose is converted into pyruvate in the glycolytic pathway. The pyruvate is decarboxylated to acetaldehyde and then in a further step this is reduced to ethanol by NADH. The enzyme alcohol dehydrogenase catalyses this reaction. ADH and G6PDH were also chosen because they are located in the cytoplasm and are soluble and stable enzymes.

This study has successfully monitored the levels of these two enzymes in several fermentations. Clearly this has demonstrated the potential of the technique to monitor intracellular products. However, this study needs to be extended to enzymes which are far less stable and located in other parts of the cell.

Application of intracellular product monitoring in recombinant DNA technology can be used to follow progress in inducible cell fermentations; whether the induct was

successful or not, detect leakage due to cell lysis (as the extracellular environment is also monitored) or over expression, and monitor potential enzyme degradation for example.

Potential uses of recombinant DNA techniques industrially are fermentation of biomass to organic solvents and chemicals, processing and production of food, recovery of minerals and oils, and control of pollution.

Recombinant DNA technology could theoretically be used to produce any protein from any cell on a large scale. Already genetic engineering has been used to create bacterial strains that synthesise hormones, such as insulin and human growth hormone, and interferon, an antiviral protein that also shows promise in cancer treatment. Vaccines are also being developed by recombinant DNA to synthesise antigenic protein fractions of a pathogen in a harmless bacterium.

Application of the methodology presented in this study for the monitoring of recombinant DNA fermentation products will have to address the following issues:

- Product location

  The product may be located in the extracellular environment in which case the cell disruption will not be required. If however the product is located within the cytoplasm or in the periplasmic layer the assessment of cell disruption kinetics will be required including evaluation of reproducible selective product release with homogeniser operating pressure, cell age and fermentation stage.

- Plasmid loss

  Plasmid loss into the extracellular environment would be detected using the on-line monitoring scheme presented in Figure 1.1., Section 1., where the fermentation broth can be clarified of suspended solids in the micro-centrifuge and then analysed.

- Soluble or insoluble product

  This study has monitored two cytoplasmic soluble enzyme products but, if the product is insoluble then questions of potential product damage during cell breakage within high pressure homogenisation need to be answered.

- Product stability

  Issues of product stability within the cell during fermentation development work are not directly related to this study. However, the stability of the product within the homogeniser and post homogenisation should be a major source of investigation.

A simple example where intracellular product monitoring can be carried out is the case on a recombinant *E. coli* fermentation, say, where the antibiotic used to suppress the natural strain was omitted, potentially all normal indicators would show that the fermentation was proceeding as planned. Only with direct intracellular monitoring would the omission come to light.

6.6.2. The use of 'Locus' plots

The use of 'Locus' plots (Section 3.5.1.) should be further developed with components which are not growth associated (e.g., secondary metabolites) and thus will have mobile loci. For example, where the intracellular product is to be induced.

Secondary metabolites are not essential for growth or biosynthesis and are usually produced at the end of growth or after growth. The examples of secondary metabolites are penicillin and other antibiotics produced by micro-organisms such as *Penicillium chrysogenum*, *Bacillus subtilis*, *Bacillus brevis*, *Streptomyces griseus*, *Streptomyces fradiae*, *Streptomyces kanamyceticus*, *Streptomyces aureafaciens*, *Streptomyces rimosus*, *Streptomyces noursie*, and *Streptomyces nodosus*.

6.6.3. Predicting the time for the (known) product maximum

The use of exponential fits to four data points to predict the time for the (known) product maximum has proved successful. Increasing the frequency of monitoring can result in an earlier prediction and the use of more data points (say eight or ten) will reduce the size of error. Therefore, giving even more reason for an on-line homogeniser to effect rapid cell breakage and use low volumes (e.g. 1 mL) of fermenter broth so as not to deplete fermenter working volume.

Once a detailed series of fermentations have been successfully tested, a model can be developed to allow predictions to be made following the acquisition of say only one or two points (dependent on the confidence of the model, as well as the required accuracy). The datum point can be mapped onto the model yielding the projected times albeit that subsequent points may be required to confirm this.

6.6.4. Statistical analysis

The statistical testing conducted during this project has focused on the use of very elementary statistical techniques for tests of significance such as the F-test and the t-test.

(The level of confidence used was 95%; i.e. leaving a 5% possibility for a Type I or Type II error to be committed where the null hypothesis is wrongly accepted or wrongly rejected).

Type I and Type II errors

When testing for significance a formal approach is adopted by stating the assumption being made about the population.; this assumption is known as the null hypothesis. For example:

H_0: mean of population one = mean of population two

against the alternative H_1: mean of population one $\neq$ mean of population two

The null hypothesis could of course be completely verified if the entire population is examined. Using statistical methods, however, the hypothesis is tested using the results of one sample taken from the population. Two types of error may result in this approach.

Type I error, is when the null hypothesis is rejected when in fact it is true.

Type II error, is committed when the null hypothesis is accepted when in fact it is false.

Thus using a 5% level of significance, only 5 times in a 100 will a Type I or Type II error be committed.

Rapid on-line intracellular product monitoring data can be used within more complex statistical techniques such as Principal Component Analysis (PCA) which can be made to track the progress of a fermentation. This makes use of existing batch data to develop empirical models for a given fermentation. The approach is based on Statistical Process Control (SPC) under which the behaviour of the fermentation is characterised using data generated in "normal" fermentations that is fermentations which are "in control". Then unusual events in subsequent fermentations are detected by referencing the measured behaviour against the "in control" model.

This leads to the development of new multivariate SPC control charts whose presentation and interpretation are no more difficult than conventional Shewarts charts, and yet are much more powerful in their ability to detect even subtle changes.

The approach is "non-directional" in that it will detect any deviation form the normal behaviour, and as with most "non-directional" SPC procedures no assignment as to the cause of the event is provided. Once a significant event is detected, it will be left to the engineers and operators to use their knowledge to provide a quick diagnosis of the possible causes, and respond to them in the appropriate manner.

6.6.5. Product integrity

In this study intracellular enzyme products were monitored by enzyme activity assays. Verification of the product activity and integrity must be made by other methods, e.g. Gel electrophoresis, rapid HPLC, and biosensors such as immunoassays.

7. Nomenclature / Abbreviations

a	exponent on the pressure term
ADH	the enzyme alcohol dehydrogenase
CER	carbon dioxide evolution rate (mmol/Lh)
CV	coefficient of variance
D.C.W.	dry cell weight (g/L)
d.c.w.	dry cell weight (g/L)
DOT	dissolved oxygen tension (% air saturation)
G6PDH	the enzyme glucose 6-phosphate dehydrogenase
h	the valve gap within high pressure homogenisers (mm, μm)
K	dimensional rate constant (bar^{-a})
k	dimensionless disruption rate constant
N	number of passes
OD	optical density
OUR	oxygen uptake rate (mmol/Lh)
P	operating pressure (bar)
R	soluble protein release (mg/g packed weight or dry cell weight, mg/mL)
R_m	maximum possible soluble protein release (mg/packed weight or dry cell weight, mg/mL)
SD	standard deviation
SFA	stop flow analyser
w/v	unit weight per unit volume (kg/m^3, g/L, mg/mL)
X	the impact distance in high pressure homogenisers (mm, μm)

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9. Appendix

9.1. Fermentations

The fermentation in the text of this thesis has been reported as 'A', 'B', 'C', etc. for the particular organism. The experimental names given to each of the fermentation is tabulated here.

P. putida ML2

A	ALINK6
B	ALINK9
C	RL1409
D	ALINK10
E	ALINK12
F	ALINK13
G	ALINK1
H	ALINK5

E. coli

A	ALINK21
B	ALINK22
C	ALINK23
D	ALINK24
E	ALINK25
F	ALINK26
G	ALINK27
H	ALINK28
I	ALINK29

S. cerevisiae

A	SK114
B	SK215
C	SK216
D	GMMY05

9.2. Statistical Analysis

This section gives details on the statistical analysis carried out on the cell disruption and fermentation data by way of assessing their reliability and reproducibility.

The types of statistical analysis that may be applied to data are the following:

- To test a given hypothesis concerning some observed characteristic;
- To determine a reliable estimate of some factual value;
- To represent a physical situation functionally.

The reason for such an analysis is the fact that all data are to some extent, one way or another, subject to chance error. These chance errors may arise whether the problem involves an estimation - the testing of a hypothesis - or the development of a reliable model. For example, if a value is to be estimated by laboratory analysis, it would first appear that either the "true value" is observed or it is not. However, since no experimentally determined value is absolute, it is frequently necessary to determine by statistical methods the reliability of scientific determination.

Mean

The mean or the arithmetic average can be symbolised by:

$$\text{mean, } \bar{x} = \frac{\sum x}{n}$$

Standard Deviation

Dispersion is the statistical name given for the spread or variability of data, and the most commonly used measure of dispersion is the standard deviation (Francis, 1993), a quantity of fundamental importance (Plews, 1979). The standard deviation or root mean square is the square root of the average squared difference between the individual observations and the average value.

$$\text{standard deviation, } s = \sqrt{\frac{\sum(x-\bar{x})^2}{n}}$$

If the dispersion is small many of the values cluster around the average, whereas if the dispersion is large a considerable proportion of values are markedly different from the average.

The importance of measures of average, such as mean, and measures of dispersion such as standard deviation, can be appreciated when it is known that for all normally distributed variables ~ 68% of values lie within one standard deviation, SD, of the mean, ~95% within two SD's and practically 100 percent within three SD's.

i.e. $\mu \pm \sigma = 0.6827$ or 68.27%

$\mu \pm 2\sigma = 0.9545$ or 95.45%

$\mu \pm 3\sigma = 0.9973$ or 99.73%

these are known as confidence intervals. We are never be 100% certain since Z can have a value from $-\infty$ to $+\infty$.

Variance

Another important quantity in its relation to the standard deviation is the variance or the mean square.

$$\text{variance, } s^2 = \frac{\sum(x-\bar{x})^2}{(n-1)}$$

The quantity (n-1) in this particular case is referred to as degrees of freedom. This quantity allows for the mathematical correction of the data for constraints placed upon this data. In this sense it is stated that, since the given data has been used to calculate the average, one degree of freedom is lost for the calculation of the standard deviation; i.e. a constraint has been placed on the data.

Some more definitions are:

Coefficient of Variance / Variation

$$\text{coefficient of variation, CV} = \frac{\text{standard deviation}}{\text{mean}} \quad 100\%$$

Standard Error

$$\text{standard error of estimate, SE} = \frac{\text{standard deviation}}{\sqrt{n}}$$

Tests of Significance

Tests of significance are directly related to the confidence limits and are based on a Normal Distribution concepts.

To test whether a sample of size n, with a mean $\bar{x}$ and standard deviation s, could be considered as having been drawn from a population with a mean μ, the test statistic

$$Z = \frac{\bar{x} - \mu}{s/\sqrt{n}}$$

must lie in the range -1.96 to +1.96 (95% confidence level). If $-1.96 > Z > 1.96$ then there is evidence of a difference.

Z is also known as the confidence factor (Francis, 1993); 1.96 for 95%; 2.58 for 99%.

The 95% confidence limit means that if many samples are drawn, and the mean of each is found, then it can be expected that 95% of the sample means will be within the stated limits (Owen and Jones, 1990).

Type I and Type II errors

When testing for significance a formal approach is adopted by stating the assumption being made about the population.; this assumption is known as the null hypothesis. For example:

H_0: mean of population one = mean of population two

against the alternative H_1: mean of population one $\neq$ mean of population two

The null hypothesis could of course be completely verified if the entire population is examined. Using statistical methods, however, the hypothesis is tested using the results of one sample taken from the population. Two types of error may result in this approach.

Type I error, is when the null hypothesis is rejected when in fact it is true. The maximum probability of committing a Type I error in a particular test is called the significance level of the test.

Type II error, is committed when the null hypothesis is accepted when in fact it is false.

Thus using a 5% level of significance, only 5 times in a 100 will a Type I or Type II error be committed.

One-tailed significance tests

When merely interested in knowing the probability that the standardised variable Z is not greater than (or less than) some particular value, a "one-tailed" test is performed. If however, the probability that Z lies within a particular range symmetrical around the mean 0, is required then the "two-tailed" test is used.

A flow chart showing the main stages of a test of significance is shown in Figure 9.1.

Students t-test

If the data contained only chance errors, the cumulative estimates $\bar{x}$ and s would gradually approach the limits of μ and σ. In particular, the distribution of yields would also be, within practical limitations, normally distributed with mean μ and standard deviation σ. Suppose then that the true mean μ of the infinite population were known. In this case it would be expected that the averaged means for each group of say 10 yields would also have some symmetrical type of distribution centred around μ. However, it would be expected that the dispersion or spread of this distribution about the mean would depend on the sample size (since larger sample size would result in a better estimate of μ) as well as the standard deviation of the original population. In particular it can be shown that the standard deviation of the distribution of means equals $\sigma / \sqrt{n}$. A British statistician, W.S. Gosset, originally formulated a distribution of this type on the early part of the twentieth century. Writing under the pseudonym "Student" the distribution is referred to as the "Student's distribution" and the corresponding test of significance (a measure of error between μ and $\bar{x}$) as the t test. In general the t test is actually not sensitive to the sampled distribution being normal. In this sense the t test is said to be robust. The distribution of the statistic was determined

$$t = \frac{(\bar{x} - \mu)}{s / \sqrt{n}}$$

When comparing the means from two samplings where there is only minor modifications and the experimental error is assumed not to have changed, the t test can be used to determine whether the observed differences could have been due to chance alone.

The level of significance is selected before applying the statistical test. The hypothesis of this test is that the two population means μ_1 and μ_2 are the same. In applying this test it is assumed that the standard deviations are equal. Under these specified conditions the value of t is calculated as follows:

$$t = \frac{(\bar{x}_2 - \bar{x}_1)}{s / \sqrt{(1/n_1 + 1/n_2)}}$$

where s^2 is the pooled variance estimate of $\sigma^2 = \sigma_1^2 = \sigma_2^2$ given by:

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

Corresponding to the selected probability level (100-α) the theoretical value of t can be found in the distribution table of t. If the calculated t is greater that this value, the hypothesis is refuted, and it is therefore assumed that $\mu_1 \neq \mu_2$

For example, to be *significant* at the 95% probability level, with say 18 degrees of freedom, t would have to be-2.10> t >2.10. So if the calculated t does not satisfy this condition, there is no statistical evidence for disputing the hypothesis of $\mu_1 = \mu_2$.

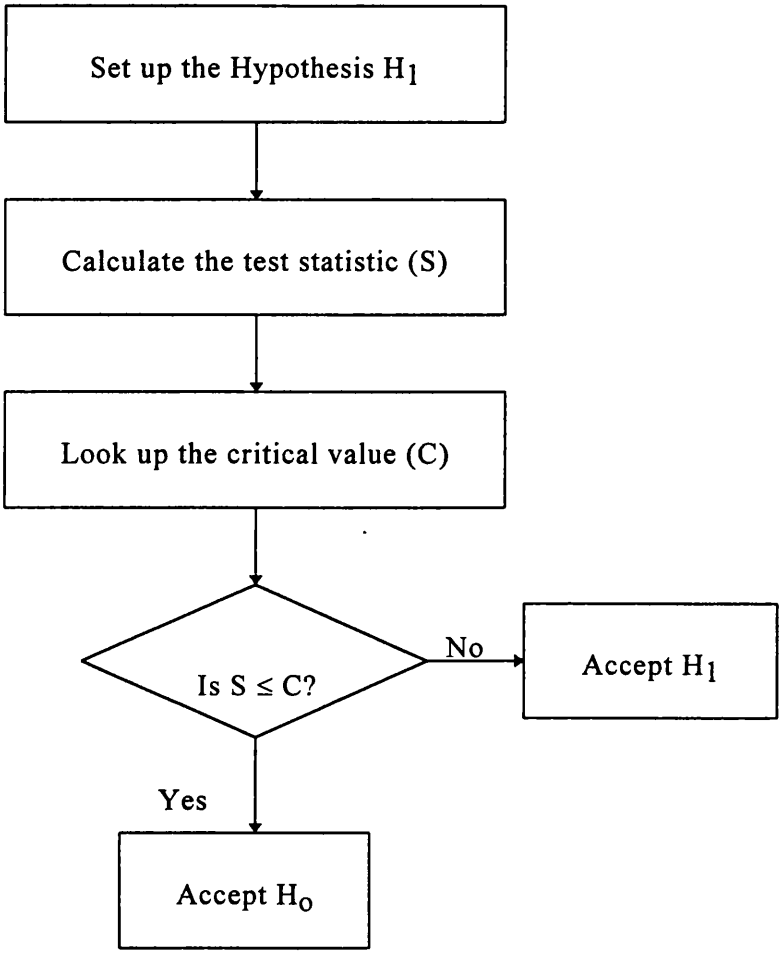

Figure 9.1. Flow chart showing the four main stages of a test of significance (Graham, 1988).