Downstream Processing Effects On Microbial Viability

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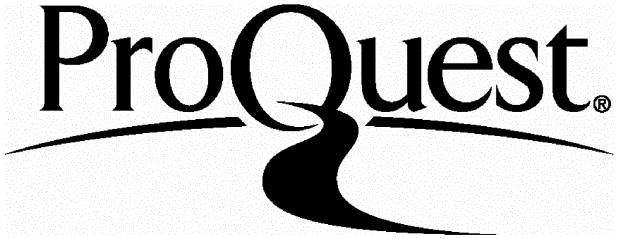

Abstract

An increasing number of biotechnology production processes require a whole cell recovery step during downstream processing. Viability loss at this stage is generally undesirable as it can lead to reduced yields and separation difficulties further downstream. Comparisons between the effects of different types of equipment on a single strain of cells is rare. Such information can be invaluable when designing new processes or equipment. This work was carried out in the following stages:

- Production of identical batches of cells for testing
- Use cells to develop a reproducible assay test system
- Use the assay system to assess the damage of various concentration techniques on the viable cells

A reproducible batch fermentation for the production of *Pseudomonas putida* ML2 cells was developed. Both the inoculum and fermentation stages were carried out in fully defined media. The fermentation used a minimum salts media and fructose as a carbon source.

The bioluminescent ATP assay was tested as a novel method for measuring cell damage. The action of separation was simulated at laboratory scale by the use of a high pressure homogeniser. The ATP assay was tested on growth and stationary phase cells and results were compared to standard assay methods such as plate counts and protein release. Cells in stationary phase appeared to undergo a metabolic activation and so ATP analysis proved to be unreliable. Protein assay and plate count techniques were developed to increase reproducibility and accuracy by the use of multiple repeats and statistical analysis.

Batch centrifugation was examined, multichamber bowl centrifugation proved to be less damaging to cells than tubular bowl centrifugation. Continuous centrifugation was carried out using a disk stack centrifuge with intermittent solids discharge. Damage was observed between 0-20% loss of viability. Cells were sampled directly from the separation bowl as well as from the discharged solids. Little difference was observed, suggesting that damage occurs in either the separation or feed zones prior to discharge.

Microfiltration was carried out using a specially devised laboratory scale rig, designed at UCL. Runs were carried out under a number of different operating conditions. Increased cell damage was observed at higher cell concentrations and transmembrane pressures. Unlike centrifugation, results the protein release levels are less than the measured loss of viability suggesting a different mode of inactivation.

Hydraulic shear stresses were estimated across the range of operations tested, as well as in the fermenter. Examination of the microfiltration results suggested that the action of the system was similar to that of a high speed fermenter.

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1 Introduction

1.1 Biotechnology

Biotechnology is the provision of goods and services that rely on the use of biological materials. There are many important industrial applications of biotechnology:

- Food — altering appearance, taste
- Drink — brewing
- Health — drug production
- Agriculture — pest resistance, crop yields
- Sewage treatment — reduction in chemical usage

Production processes generally involve the culturing of cells and the subsequent extraction and purification of a product. The end product is usually one of the following:

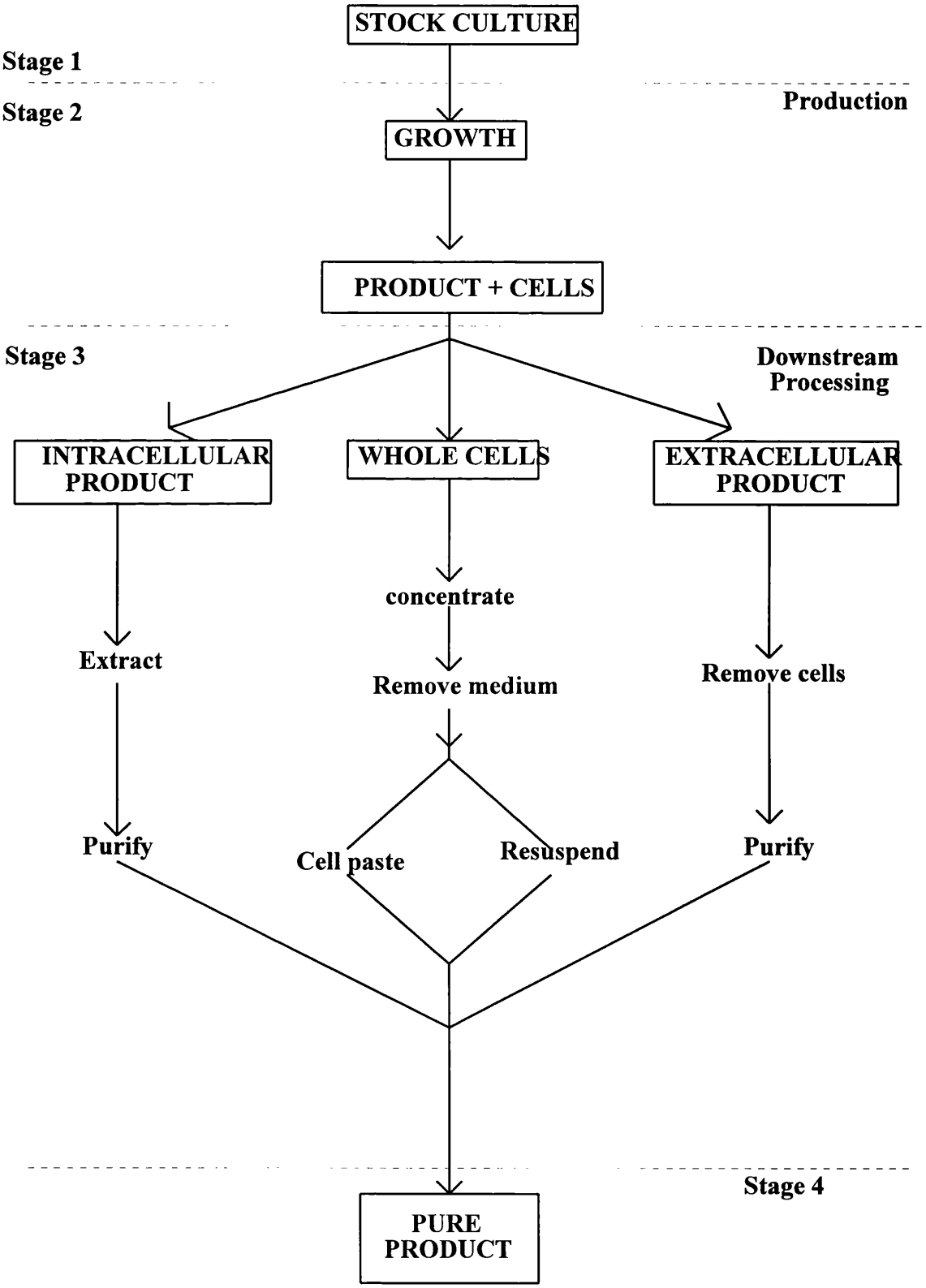
- Intracellular product
- Extracellular product
- Whole cell

The first stage of production is growth of the cells. Microbial cells are often used to produce biotechnology products, as they are relatively straightforward to grow and process compared to mammalian or plant cells. The resistance to shear stresses of microbial cells allows large scale liquid culture.

Unfortunately many products, such as vaccines and drugs are mammalian in origin. Advances in recombinant DNA technology have helped solve many problems. The expression of mammalian genes in microbial hosts is widespread. Once foreign genes are incorporated into the host it can then be rapidly grown by fermentation culture. The mass production of many complex biological substances is now possible. Products often occur in very low concentrations in the culture and the ability to produce cells in large quantities compensates for low yields.

Downstream processing is the series of events, following the cell production, that involves the extraction and purification of the end product from other cellular materials. Figure 1.1 outlines the series of events that lead from a stock culture to the production of a pure product.

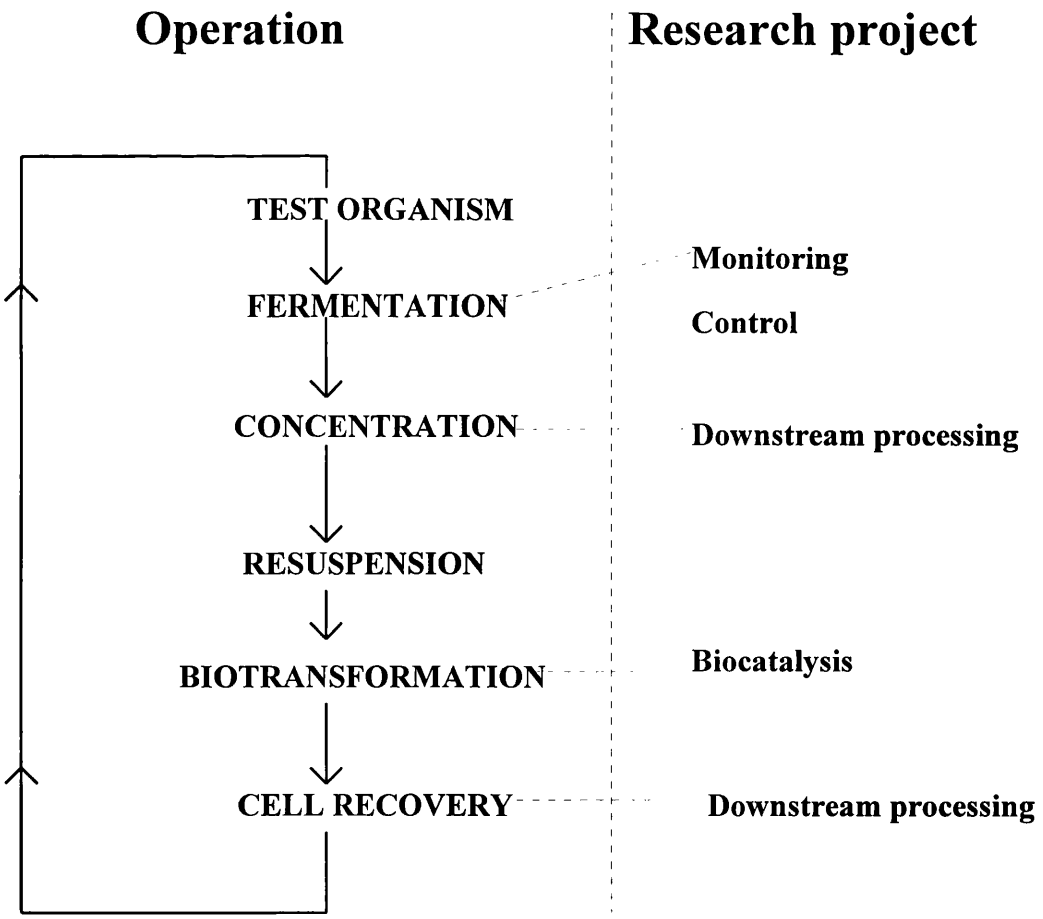
Fig 1.1 Biotechnology production processes



1.2 Context of this research

This work was carried out as part of a LINK project. Five separate projects investigated particular aspects of a model production process, which consisted of a microbial fermentation followed by a biotransformation. Figure 1.2 outlines the model.

Fig 1.2 LINK model production system



This work will examine one downstream processing step - the concentration of cell suspensions. In particular the damage effects of microbial cell recovery from a fermentation broth. There are a number of ways of separating cells in a suspension

from the aqueous phase, centrifugation and microfiltration are the most frequently used.

Successful isolation of biological particles has proved to be a challenge for the biochemical engineer. The target substances are often difficult to isolate and separate from other cellular components. This is often due to small differences in size and density as well as problems caused by temperature and mechanical sensitivity.

Damage caused to cells by centrifugation and microfiltration often cause problems in subsequent processing steps. It is important to be able to concentrate suspensions without damaging the cells, for a variety of production processes.

Whole cells

Cells are often the desired product. Whole cell recovery from fermentation broth is an important stage of many production processes. Concentration of the cells allows separation from their liquid medium. This is carried out when :

- Nutrients are depleted, to allow resuspension in a fresh medium
- Product production is a two stage process, that requires a medium change. For example, secondary metabolite production. Cells are grown to the desired concentration and a different medium is used to induce formation of the product
- Accumulation of autotoxic metabolites excreted into the extracellular environment causes problems by limiting growth or production of product. Cells can simply be resuspended in fresh medium
- Cells are to be used as whole cell catalysts in biotransformations. The cells are grown to a desired concentration, separated from their growth medium and then resuspended in the reaction medium for production of the product

There are a number of advantages of being able to successfully produce cells in a concentrated form, without breakage or damage:

- No loss of product, eg yeast production
- Ease of handling a concentrated suspension
- Ease of transporting a concentrated suspension
- Ease of processing, eg freeze-drying. Concentration is a relatively rapid process. A concentration step followed by freeze-drying a concentrated cell suspension is likely to be quicker than freeze-drying a dilute suspension

Intracellular product

Production of intracellular material requires a number of processing steps, usually extraction followed by purification. Product is inevitably lost at each stage. The product is more concentrated when situated within cells than it is outside them. If cells can be concentrated before the product is extracted then loss is reduced.

Extracellular product

One source of contamination when purifying an extracellular product is intracellular components that have been released from lysed cells. Removal of cells without breakage removes potential contaminants before they come into contact with the target material.

1.3 Mechanism of cell damage

The action of microfiltration and centrifugation often damage a microbial population. Comparisons between the effects of different types of equipment on a particular strain of cells is rare. An understanding of the causes of this damage would assist in the optimisation of any production process or design of equipment. Defining and quantifying cell damage is not a straightforward task. The most obvious method of expressing damage is in terms of loss of viability. The term viability is not rigidly defined and is generally regarded as an organism's ability to:

- Reproduce
- Metabolise

The ability of a cell to reproduce itself depends on a fully functioning metabolic system enclosed by an intact cell membrane. However the ability to metabolise does not depend on the entire metabolic system being in perfect functioning order. Roszak and Colwell (1987) stated that any natural population of bacteria contains a majority of underdeveloped, undernourished cells that are incapable of division when transferred onto culture media. This section of the microbial population is however still classified as viable when measured by indirectly by respiration or photosynthetic methods.

It is possible to study effects of a process on a cell population. This can be carried out by quantification of the number of reproducing and hence useful cells. Any method adopted would ideally be:

- Accurate
- Consistent and reproducible over a long period of time
- Rapid
- Easy to use
- Inexpensive

The most widely used method of quantifying viable microbial cells is the Standard Plate Count (SPC), also known as the Viable Cell Count (VCC). There are a number of inherent problems with this test which will be discussed later. An alternative approach to viability quantification in this situation would be to assume that any viability loss is caused by the lysis of cells. The assay for released substances could be correlated to the viability loss of a fraction of the cell population. The assay of total soluble protein has been shown to be simple and accurate. Many industrial processes do not require cells to possess the ability to reproduce, only metabolise. One such application is a biotransformation carried out by *Pseudomonas putida* ML2. This requires the presence of an intact cell membrane and respiration system but not the ability to reproduce.

The effects of mechanical stress on microbial cells can reduce their ability to metabolise (Thomas, 1993). When viability is measured by colony formation on solid media or as an inoculum in liquid media these effects can manifest themselves as an extended lag phase even before signs of growth are detected. It is important to incubate cells for a fixed period of time when carrying out these growth-based viability tests as the growth delay can be longer than the incubation period. The length of this delay can vary considerably, depending on the levels of stress the cells have been subjected to. Non-lethally stressed cells can show no signs of growth on solid media for hours or even days. Viability assays based on growth characteristics can provide sufficient information on the state of the cell population. However due to large scattering of measurements they seldom do. To compensate for this most approaches combine an assay for reproducibility with the one for an intracellular product to test for mechanical cell damage. The degree of cell lysis is quantified and any changes in the reproduction capabilities of surviving intact cells are revealed. This has particular application when the number of intact cells varies from the number of reproducing, growing cells.

1.4 Summary of work

The test organism used throughout this work was *Pseudomonas putida* ML2 which is a gram negative bacteria. The organism is capable of carrying out a industrially useful biotransformation. This is the whole cell biocatalytic conversion of fluorobenzene to fluorocatechol. This is mediated by the enzyme benzene dioxygenase which is constitutively expressed by the *Tol* plasmid.

This work was carried out in a number of stages:

1. Develop method of testing cell damage
   - reproducibly produce cells
   - identify a test procedure
   - test assays and develop
2. Investigate effects of pilot-scale operations
   - centrifugation
   - microfiltration
3. Data analysis over range of operations

Initially a method was developed to reproducibly produce the test organism by fermentation culture. A literature survey highlighted the potential of a recently developed assay approach for the quantification of microbial viability. Cells were subjected to stresses at laboratory scale similar to those encountered during downstream processing. Results of analysis using the novel technique were compared to a number of established techniques, and the results were compared. A test system was then proposed.

Using the test system the effects of processing *P. putida* ML2 cells by microfiltration and centrifugation were examined. A variety of different centrifuge configurations were used and then a specially designed laboratory scale microfiltration rig was tested. Estimates were made of the stresses generated within certain components of the equipment in an attempt to understand the mechanisms of cell damage.

2 Fermentation Development

2.1 Introduction

This chapter describes the development of a protocol for the reproducible production of *Pseudomonas putida* ML2 cells. For the consistent production of cell batches the following parameters need to be well defined:

- Quantity of cells
- Physiological state of the cells
- Purity of the culture

Cells can be produced by a number of different methods. Solid culture uses agar plates, bottles and tubes. Liquid culture involves shake flasks and fermentation techniques. Fermentation can be carried out as either batch, fed-batch or continuous culture.

The quantity of cells required will determine the method of production. Solid and shake flask culture can only provide enough cells for small scale laboratory work. In order to test biochemical engineering equipment cells need to be produced in liquid culture in quantities exceeding tens of litres. The most controllable method is batch fermentation, which has a number of advantages over continuous culture or fed batch:

- Simplicity of operation
- Homogeneity of harvested culture

Simplicity of preparation and operation of batch fermentations reduces the risk of contamination because of shortened operating times and decreased contact with potential sources of infection, eg during media addition and washout. Equipment and running costs are also reduced.

Factors that lead to variability in the cell production process are:

- Revival of stock culture
- Inoculum seed culture regime and transfer to vessel
- Consistent medium components for inoculum and fermentation culture
- Sterilisation procedures
- Vessel operating parameters

Cells are required weekly, over a period of a number of years. The culturing of stocks must ensure that no change in physiological characteristics takes place. In the case of *P. putida* ML2 this could be loss of *Tol* plasmid activity.

The inoculum seed culture regime must provide uncontaminated cell suspensions in sufficient quantities to be used as fermenter inocula. It is important to use the inoculum at the same point in the growth cycle of each fermentation. Cells in growth phase are preferable because they grow on introduction to the fermenter. The quantity and stage of growth of the cells is dependent on incubation temperature and time.

Components of the fermentation media must be in sufficient quantity to allow maximum cell yield without limiting growth or wastage. Growth time should be as short as possible to reduce the risk of vessel contamination. Harvesting the cells should occur at precisely the same time for each of the fermentations.

The vessel operating parameters ideally should be fixed. If an organism has a high oxygen requirement the agitation or aeration is often increased during the fermentation to prevent the Dissolved Oxygen Tension (DOT) falling below critical levels, which can effect the physiological characteristics of the cells. The increase must take place at a set time during the fermentation. To remove this variable it is preferable to start with high levels of agitation and aeration throughout the run.

A protocol for the standardised production of batches of *P. putida* ML2 cells has been developed. The development is described in two parts. Firstly the development of a fermenter inoculum production regime. Changes were made to media composition, revival of stock cultures and the scale of operation. Fermentation development involved modifications to the media composition, preparation of the equipment and the inoculum regime used. A description of the fermentation development is outlined in the following pages.

2.2 *Pseudomonas putida* ML2 fermentations

2.2.1 Organism

The organism used throughout this project was *Pseudomonas putida* ML2, provided by Shell Research Ltd (Sittingbourne, UK). The stock cultures were provided and stored as freeze-dried samples.

When required, the culture was revived by resuspension in 1.3% (w/v) Nutrient Broth (Oxoid, Unipath Ltd., Basingstoke, UK) and used as an inoculum for shake flask culture. To screen for plasmid retention the culture was streak plated onto Indole agar plates. The composition of which is listed in Appendix 2.1. Cells containing the plasmid formed indigo colonies. These were subcultured onto 2.8% (w/v) nutrient agar plates (Oxoid, Unipath Ltd., Basingstoke, UK). These were stored at 4°C and used as a working stock culture for upto one month.

2.2.2 Inoculum preparation

The preparation of inoculum for fermentations was carried out in two stages. Cells were grown in a two stage liquid culture system using shake flasks. The first shake flask stage was used to revive the stock culture and screen for *P. putida* ML2 *Tol* plasmid activity. The second stage involved growing screened cells to a concentration suitable for fermenter inoculation.

Shake flask 1 (SF1)

The first stage consisted of culturing cells in a liquid minimal salt media containing an aromatic compound as a carbon source. This provided a screening mechanism for pure *P. putida* ML2 cells as the aromatic's toxicity inactivated contaminating cells or any that had lost plasmid activity. Cells containing a fully functioning plasmid were able to utilise the aromatic as a carbon and energy source and grow.

Shake flask 2 (SF2)

Due to the toxicity of the aromatic carbon sources in high concentrations only small amounts could be used for the initial stock culture revival step. This gave rise to low

cell concentrations in the first set of shake flasks. A secondary shake flask culture (SF2) was then used to grow cells to higher concentrations.

The base medium of both sets of flasks was identical and is listed in Appendix 2.2. The quantity and composition of the shake flasks and the incubation time was developed during the course of this work. Six different regimes were used and designated the titles PPInoc1-PPInoc6. Shake Flask 1 media composition and incubation time was varied until a satisfactory cell yield was obtained. The composition of the Shake Flask 2 base medium remained unchanged however the media and inoculum volume was varied. Full details of the preparation of the flasks and media is outlined in Appendix 2.2.

Table 2.1 Variations In Shake Flask 1 (SF1) media composition and incubation times

	Carbon Source	[carbon vol] $\mu L\ L^{-1}$	Peptone added	incubation time / h
PPInoc1	benz	500	✓	24
PPInoc2	fluoro	500	✓	24
PPInoc3	benz	2000	×	24
PPInoc4	benz	1000	×	72
PPInoc5	benz	1000	×	72
PPInoc6	benz	1000	×	72

500 mL sealed conical flasks containing 100 mL base media were used. Peptone when added 50 μL of 0.07% (w/v) solution per flask. The flasks were inoculated with approximately five loopfulls of cells, sealed and incubated in a shaker cabinet at 30°C and 200 rpm.

Table 2.2 Variations In Shake Flask 2 Media Composition

	Flask volume / mL	Base media mL	Inoculum mL	Carbon source	Number of flasks
PPInoc1	500	100	5	Fructose	2
PPInoc2	500	100	5	Fructose	2
PPInoc3	500	100	5	Fructose	1
PPInoc4	500	100	10	Benzene	1
PPInoc5	500	100	25	Fructose	2
PPInoc6	2000	400	100	Fructose	1

Shake flask cultures were grown to a chosen cell concentration as measured by optical density measurements at 670 nm. They were then used as a fermenter inoculum.

2.2.3 Fermentation protocol

Pseudomonas putida ML2 cells were produced by batch fermentation. Fermentations were carried out over a range of scales using LH fermenters (Reading, UK). At 5 litre scale in a LH07L fermenter, 10 litre in a LH20L fermenter and 25 litre in a LH42L fermenter.

Table 2.3 Fermentation scale-up conditions

Fermenter	Total volume L	Working volume L	Agitation rpm	Airflow L min^{-1}
LH7L02	7	5	1000	5
LH20LO2	20	10	1000	10
LH42L01 / 2	42	25	800	20

Fermentations were carried out in the three different fermenters at a pH of 6.8 and a temperature of 30°C. Aeration of the culture was kept in excess to avoid changing

any of the settings during the course of the run. The media composition was optimised until a reproducible fermentation was developed. Changes were made to the quantities of nitrogen and iron source used. Some small changes were made to the sterilisation regime of certain components. Each regime was designated the name PPF(number). Six different media compositions were used, PPF1-PPF6.

Table 2.4 Compositional changes in fermenter media

Fermentation Regime	Nitrogen source $(NH_4)_2SO_4$ g L^{-1}	Iron (II) source $FeSO_4$ g L^{-1}
PPF1	8.4	0.032
PPF2	1	0.032
PPF3	8.4	0.032
PPF4	5	0.032
PPF5	8.4	0.08
PPF6	8.4	0.08

Appendices 2.3 gives full details of media composition, equipment preparation and variations in inoculum regime used.

2.2.4 Assay And Monitoring Techniques

Samples were regularly monitored for dry weight, optical density and viable cell concentration. Details of these techniques can be found in Section 3.2.

The exit gas composition was monitored using a VG mass spectrometer (Northwich, Cheshire, UK). The data from this as well as temperature, pH, DOT, air flow and agitation were logged by a PDP 11-73 (DEC, Maynard, Mass., USA) computer running the Bio-i fermentation monitoring software, (Biotechnology Computer Systems, High Wycome, UK). Bio-i was later replaced by software from Real Time Data Acquisition Systems (RTDAS, Talton/Louley Engineering, Fleet, Surrey, UK).

2.3 Results

The production of inoculum and the fermenter operating conditions were varied until the fermentations produced consistent cell batches.

2.3.1 Inoculum Development

The inoculum regime was modified during this work because a significant number of fermentations failed due to lack of growth. To standardise fermentations the inoculum seed culture must be in the same stage of growth and contain the same quantity of cells on each occasion.

Seed cultures were produced in two stages each consisting of growth in liquid shake flask culture. The first shake flask culture was designated Shake Flask 1 (or SF1) and was used to revive stock cultures and simultaneously screen for *P. putida* ML2 by selection for *Tol* plasmid activity. These first shake flasks consisted of 100 mL of base medium in 500 mL conical flasks inoculated with loops of stock culture. The carbon source was either benzene or fluorobenzene, the concentrations of which were varied.

Culture from Shake Flask 1 was then used to inoculate a second series of shake flasks. These were incubated until the cell concentration had reached a certain value, before being used to inoculate the fermenter. This was designated Shake Flask 2 (SF2). These flasks were either 100 mL of base media in a 500 mL conical flasks or 400 mL of base media in a 2000 mL conical flask, both containing fructose as sole carbon source at 30 g L^{-1}. One inoculum regime, PPInoc4, substituted benzene for fructose.

Table 2.5 shows how media composition and incubation times affected the final cell yield for each of the inoculum regimes, PPInoc1 to PPInoc 6.

Table 2.5 Cell yields obtained during inoculum development

Regime, PPInoc:	SHAKE Carbon, Amount µL L^{-1}	FLASK Incubation time / hours	1 Yield / g dry wt L^{-1}	SHAKE SF1 vol as inoc m L	FLASK Final volume m L	2 Yield / g dry wt L-1	Final Inoculum g dry wt
1	Benzene 500	24	0.2	5	220	0.75	0.165
2	Fluoro-benzene 500	24	0	5	220	0	0
3	Benzene 2000	24	0.01	5	220	varied	0.001-0.01
4	Benzene 1000	72	0.4	10	20	0.4	0.008
5	Benzene 1000	72	0.4	25	250	1.75	0.455
6	Benzene 1000	72	0.4	100	510	1.75	0.89

In all cases the basal minimal media remained the same. The type and amount of carbon source was varied along with the volumes used for inocula.

PPInoc1

The initial formulation suggested by Shell resulted in a low cell yield in the first shake flask, and a correspondingly low yield in the second.

PPInoc2

The substitution of fluorobenzene for benzene in the SF1 was suggested. No cell growth was detected using this carbon source. The ML2 strain was selected because of its inability to metabolise fluorocatechol, hence leading to its accumulation. The sequence of enzyme reactions required for metabolising fluorobenzene is broken due to defective enzyme production, at the fluorcatechol stage (Lynch, 1994).

PPInoc3

Benzene was reused and the quantity was quadrupled and peptone was removed from the SF1 media. It appeared that this benzene concentration was slightly toxic to the cells as it yielded a very small amount of growth.

PPInoc4

The concentration of benzene in the first shake flask was therefore halved. The incubation time was increased from 24-72 hours. This allowed the growth of cells to a concentration of 0.4 g dry wt L^{-1}. The volume of Shake Flask 1 culture used as inoculum for Shake Flask 2 was increased from 5-10 mL. Concerns over plasmid stability during fructose growth lead to benzene being tested as the carbon source in the second shake flask. The storage of identical 20 mL inoculum samples at -70°C in glycerol was carried out in an attempt to make fermentation times more uniform. When a fermenter needed to be inoculated a sample could be revived and used directly. However the use of a 0.4 g dry wt L^{-1} inoculum with a final volume of 20 mL proved to be inadequate and resulted in variable fermentation times.

PPInoc5

Fructose was used as the carbon source in the second shake flasks, SF2, from this point. The volume of benzene-grown cell suspension used to inoculate these flasks was increased from 10-25 mL. These SF2 flasks were incubated to a final cell yield of 1.75 g dry wt L^{-1}. Two inoculum culture flasks per 5 L of fermenter medium were used for inoculation.

PPInoc6

The final volume of cell suspension was increased. 2000 mL shake flasks containing 400 mL of media were used as SF2's instead of 100 mL of media in 500 mL flasks. The entire SF1 culture was used as the inoculum. The flasks were incubated for 24 hours until the cell concentration reached 1.75 g dry wt L^{-1}.

This proved to be the optimum regime for growing inocula in terms of reproducibility and convenience. All fermentations were subsequently inoculated with cells grown by this final method (PPInoc6). For exact media composition and equipment details see Appendix 2.2.

2.3.2 Fermentation development

The development of a standard fermentation for *P. putida* ML2 proved to be problematic. Fermentations showed a high degree of variability in terms of time taken and final yields. A standard reproducible fermentation was required if identical batches of cells were to be produced for testing processing equipment over a number of years, i.e. in a similar physiological state and with similar disruption characteristics. Changes were made in the media composition and sterilisation regime until a reproducible fermentation was developed. The main problems encountered were due to:

- Inoculum
- Nitrogen source
- Iron (II) source

Inoculum

The inoculum regime has already been discussed. The main problem with the inoculum during the initial stages of fermentation development was variable concentrations which lead to variation in fermentation time and insufficient cell concentration. This caused pre-growth phases lasting for days before any detectable change in cell concentration took place. This was deemed undesirable due to the increased possibility of vessel contamination.

Nitrogen source

Ammonium sulphate was used as the main nitrogen source. The concentration was in excess (8.4 g L^{-1}) as listed in the original Shell medium (PPF1). Ammonium ions were also introduced into the fermenter by addition of alkali in the form of ammonia solution. This lead to problems when collaborative work, with another LINK team member was carried out. This work involved the monitoring of fermentations using a Biomass Probe (Aber Instruments, Aberystwyth, UK).

The Biomass Probe is a novel device that can be used to determine the concentration of a cell suspension. The presence of a non-conducting, intact cell membranes interferes with an electrical signal passing between two electrodes protruding from the end of the probe. The degree of disturbance is dependent on a number of factors, one of which is the number of cells in the electrical path between the two electrodes, and hence the cell concentration. This is an on-line device for the measurement of

cell concentration changes during a fermentation and is a potentially more useful indicator of growth than exit gas measurements.

Unfortunately the high concentration of ammonium sulphate increased the electrical conductivity of the medium which prevented the device from functioning. The concentration was lowered and this appeared to cause reduced cell growth. Ammonium levels were varied in an attempt to find a compromise.

Iron (II) source

A number of difficulties arose from the transfer of sterile iron (II) sulphate into the fermenter. Iron (II) sulphate precipitates from aqueous solution when heated therefore it could not be sterilised in the vessel with the media or separately by autoclaving. Sterile filtration was the most suitable method. Unfortunately the iron sulphate precipitated rapidly when dissolved in water and so it had to be filtered within a matter of minutes. Precipitate inevitably formed and was trapped in the filter. This reduced the amount of iron transferred into the fermenter. The amount of trapped precipitate varied and so therefore did the concentration of Fe^{2+} ions in the vessel.

A number of fermentations showed linear growth during mid exponential growth phase. This appeared to be a nutrient limitation effect. The addition of extra iron (II) sulphate on the detection of this linear growth lead to the resumption of exponential growth. To prevent further iron limitation effects two steps were taken.

Initially magnesium sulphate and the iron sulphate were mixed together before sterile filtering into the vessel, before inoculation. The two components were separated. Magnesium sulphate was subsequently sterilised by autoclaving instead of sterile filtering. Whereas the iron (II) sulphate was dissolved in water and filtered alone.

The concentration of iron sulphate was increased so that it was in excess. The concentration was finally doubled to 0.08 g dry wt L^{-1}. These actions prevented further slowing down of exponential growth.

The fermentation regimes were designated the title "*P. putida* fermentation Media Number" which was abbreviated to PPF1-PPF6. Table 2.6 summarises the fermentation media, the inoculum regime, the time taken to reach stationary phase and the final cell yields.

Table 2.6 Summary of fermentation yields and inoculum regimes used

Fermentation Protocol	Figure number	Inoculum g dry wt	Max OUR mmol L^{-1} h^{-1}	Time hours	Yield g dry wt L^{-1}
PPF1	2.1	0.17	55	26	11
PPF2	2.2	0.17	60	26	10.5
PPF3	2.3	0.01	60	50	10.5
PPF4	2.4	0.46	51	40	10.9
PPF5	2.5	0.46	65	25	11.5
PPF6	2.6	0.9	100	24	13.5

Time time taken post-inoculation to reach stationary phase, hours
Yield final cell concentration, g dry wt L^{-1}
OUR maximum Oxygen Uptake Rate reached at peak of stationary phase, mmol $L^{-1}h^{-1}$

The media formulation and preparation of equipment for all six regimes is outlined in Appendix 2.3. The figures, 2.1-2.2 are presented after the following description of the fermentation development.

PPF1 Figure 2.1

The original media suggested by Shell (PPF1) was a minimal salts media containing 30 g dry wt L^{-1} of fructose as a carbon and energy source. Fructose was chosen because, unlike glucose it does not affect the ability of the organism to carry out the biotransformation. This was not relevant in the case of this work except for collaboration with other projects within the LINK program. The fermenter was inoculated with 0.17 g dry wt of cells grown by PPInoc1 regime. This fermentation regime proved to be adequate for the provision of cells for testing and resulted in a final cell yield in the region of 11 g dry wt L^{-1}.

PPF2 Figure 2.2

The electrical conductivity of the PPF1 medium was too high to allow use of the Biomass Probe. The medium was changed and renamed PPF2. The ammonium sulphate concentration was decreased from 8.4 to 1 g dry wt L^{-1}.

Excessive foaming, when the culture reached stationary phase, lead to frequent blockage of the fermenter air exit filter. Antifoam concentration was increased from 0.6 to 2 mL L^{-1}.

PPF3 Figure 2.3

Fermentations were prone to failure due to lack of growth and variable inocula. Ammonium sulphate concentration was returned to the original 8.4 g dry wt L^{-1}. The inoculum regime was changed so that stock culture could be stored and inoculated directly into the fermenter without the need for growth in shake flasks. 20 mL aliquots of benzene-grown cells at a concentration of 0.4 g dry wt L^{-1} were stored with glycerol at -70°C for use as inocula.

The resultant fermentations were approximately 50 hours long. The growth profiles were similar to previous runs but with increased pre-growth phases. It was not clear whether these pre-growth phases were genuine lag phases or periods when growth was occurring but was undetectable. It was not possible to detect the presence of cells at such a low concentration after the vessel inoculation. The fermentation run time being extended was probably due to a combination of the small amount of cells used as an inoculum and the possibility of the cells being damaged as a result of the cold storage.

The increased period of time before growth was detected was considered to be undesirable because it increased the chances of the vessel becoming contaminated. It would appear likely that some sort of lag phase was taking place after refrigeration of the inocula at such low temperatures.

PPF4 Figure 2.4

The lack of consistency of the inoculum culture lead to a number of unreliable fermentations. Stock cultures were once again revived in a benzene-containing shake flasks before being grown in fructose medium. This lead to the largest amount of inoculum culture (0.455 g dry wt L^{-1}) being used to inoculate the vessel. (PPInoc5)

The Biomass Probe appeared to function at an ammonium sulphate concentration of 4 g dry wt L^{-1} and so the fermentation medium was changed to allow use of this device.

PPF5 Figure 2.5

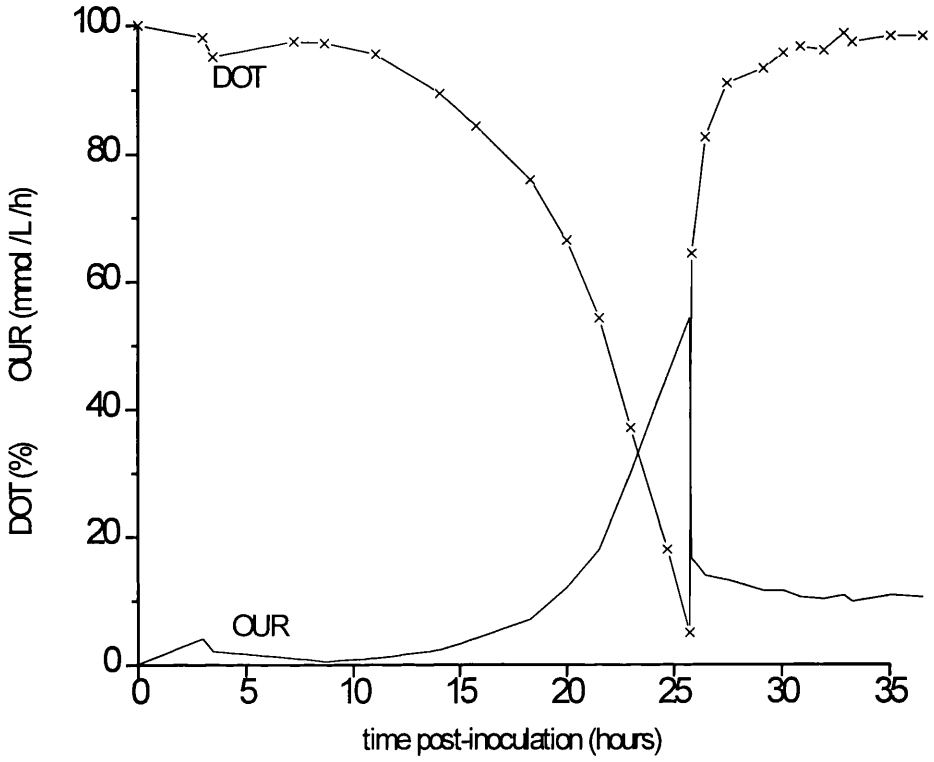
The Biomass Probe was still in development at this time and appeared to be unable to detect bacterial cells of this size at such low concentrations (0-15 g dry wt L^{-1}). No more fermentations were monitored using the instrument. This allowed the ammonium sulphate concentration to be increased to the original 8.4 g L^{-1}. Problems with iron (II) sulphate limitation effects during the fermentation lead to the an increase from 0.0032-0.08 g L^{-1}.

PPF6 Figure 2.6

Fermentation times were still varying by upto three hours. This was due to changing pre-growth phase times. In order to prevent this happening and to ensure that the culture moved directly to growth phase, as soon as possible after inoculation, the seed culture regime was changed. A larger volume of inoculum culture was introduced into the vessel. This corresponded to a change from inoculum regime PPInoc5 to PPInoc6 (see Section 2.3.1). The media formulation and preparation of equipment for regime PPF6 is identical to that for PPF5 and is outlined in Appendix 2.3. This proved to be the most reliable and reproducible method and was used for production of all cell batches for processing. Final cell yield was 13.5 g dry wt L^{-1}.

The following figures (Fig 2.1-2.6) are fermentation profiles obtained using each of the six different protocols. Summary information was given in Table 2.6.

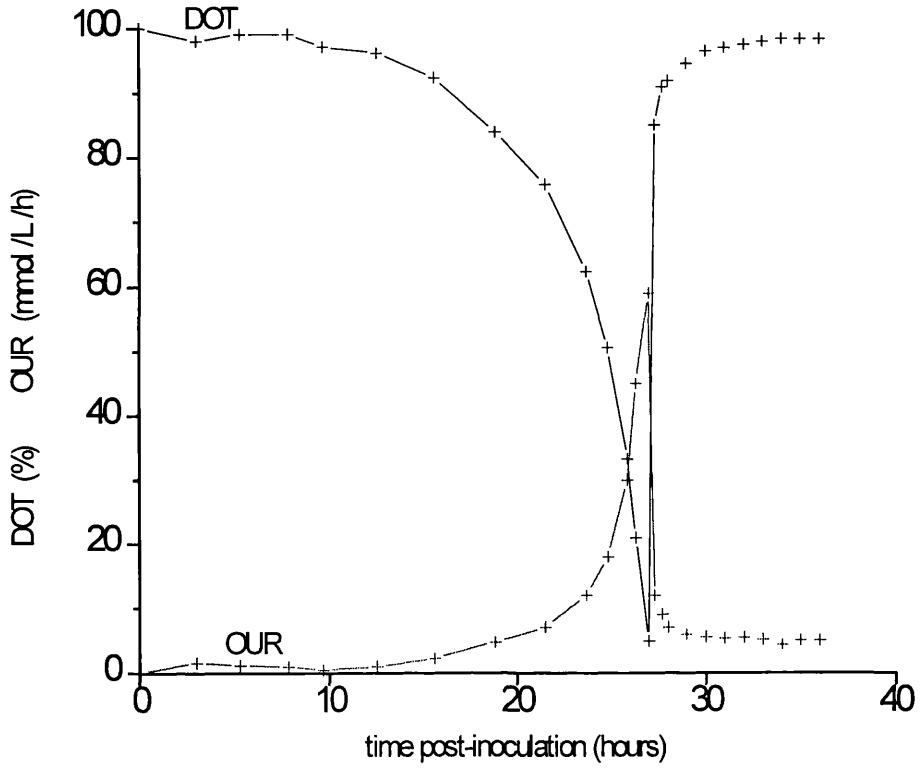
Fig 2.1 ***P. putida*** **ML2 fermentation profile**
Fermentation regime PPF1



Fermentation LINK4
Maximum OUR 55 mmol $L^{-1}h^{-1}$ Cell yield 11 g dry wt L^{-1}

DOT Dissolved Oxygen Tension (Percentage saturation)
OUR Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

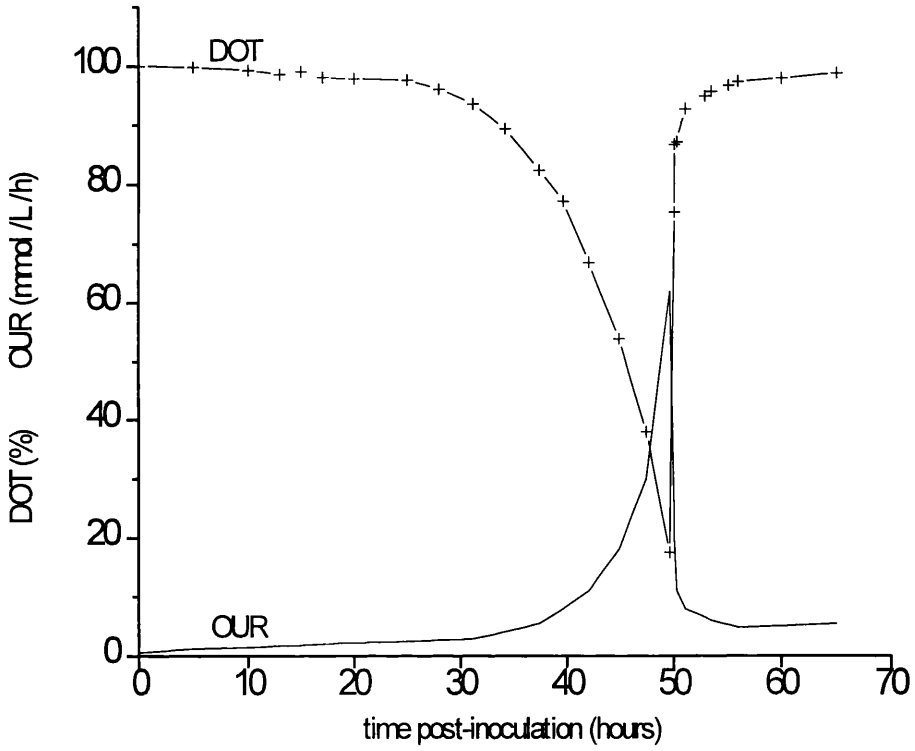
Fig 2.2 ***P. putida*** **ML2 fermentation profile**
Fermentation regime PPF2



Fermentation LINK17
Maximum OUR 60 mmol $L^{-1}h^{-1}$ Cell yield 10.5 g dry wt L^{-1}

DOT	Dissolved Oxygen Tension (Percentage saturation)
OUR	Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

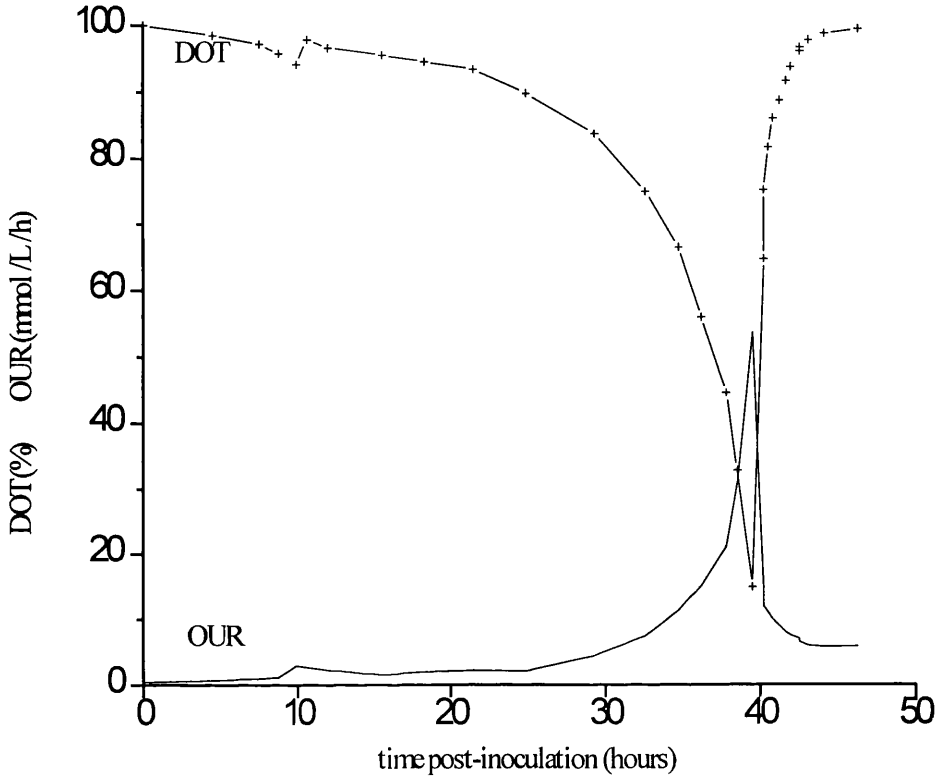
Fig 2.3 ***P. putida*** **ML2 fermentation profile**
Fermentation regime PPF3



Fermentation LINK44
Maximum OUR 60 mmol $L^{-1}h^{-1}$ Cell yield 10.5 g dry wt L^{-1}

DOT	Dissolved Oxygen Tension (Percentage saturation)
OUR	Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

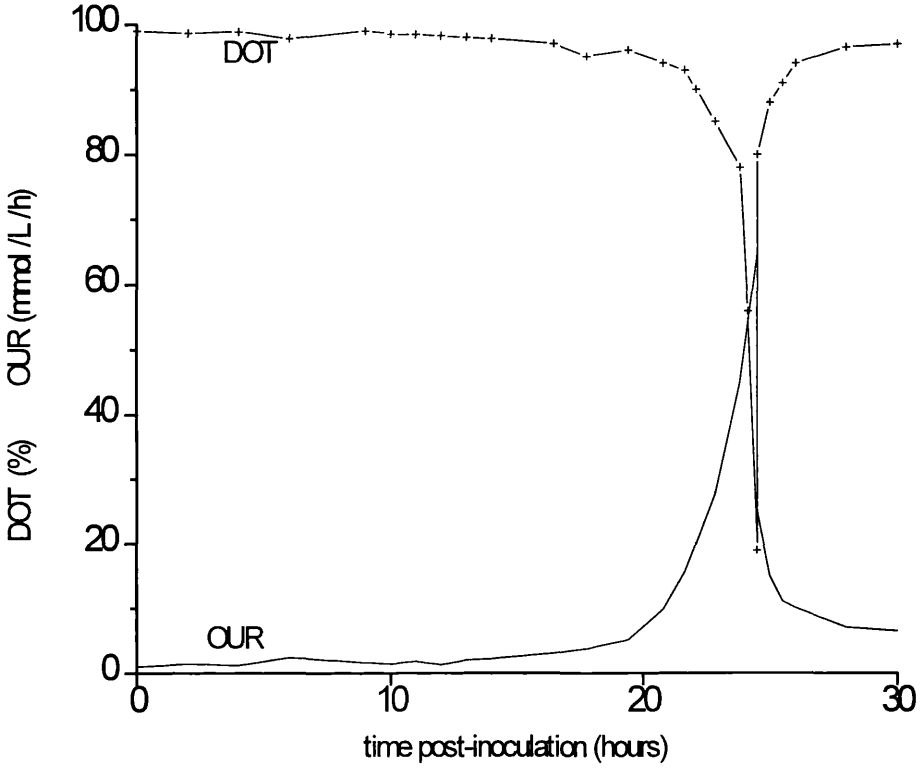
Fig 2.4 ***P. putida* ML2 fermentation profile**
Fermentation regime PPF4



Fermentation HOMOG3
Maximum OUR 51 mmol $L^{-1}h^{-1}$ Cell yield 10.9 g dry wt L^{-1}

DOT	Dissolved Oxygen Tension (Percentage saturation)
OUR	Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

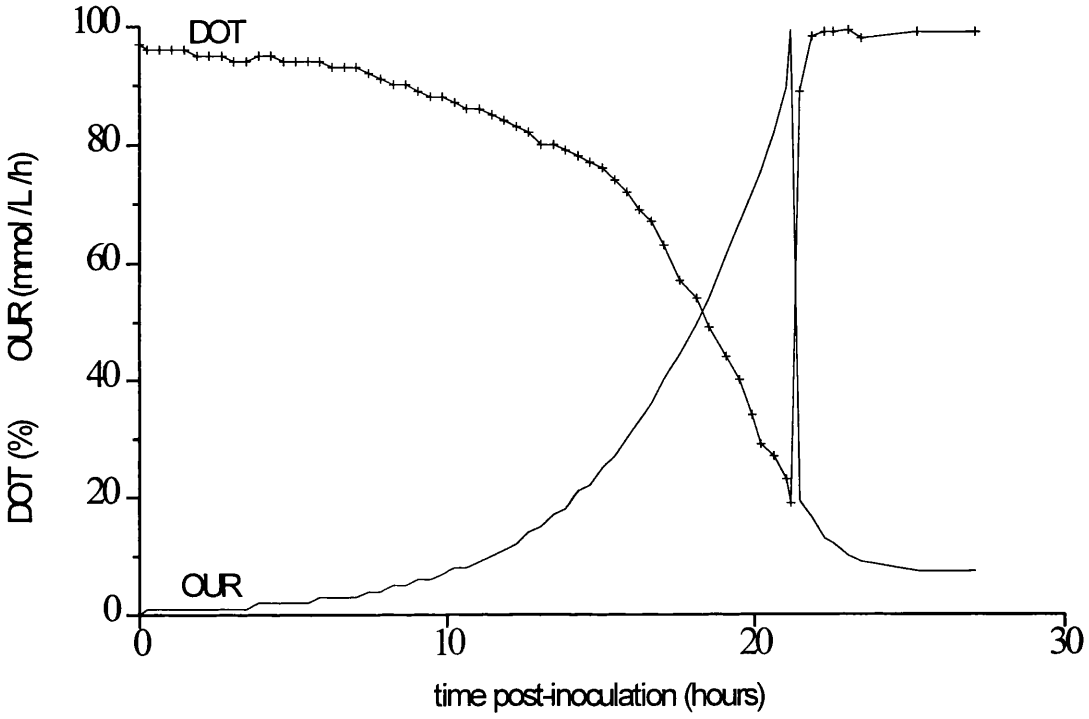
Fig 2.5 ***P. putida*** **ML2 fermentation profile**
Fermentation regime PPF5



Fermentation 92OCT2
Maximum OUR 65 mmol $L^{-1}h^{-1}$ Cell yield 11.5 g dry wt L^{-1}

DOT	Dissolved Oxygen Tension (Percentage saturation)
OUR	Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

Fig 2.6 ***P. putida*** **ML2 fermentation profile**
Fermentation regime PPF6



Fermentation SHPP30
Maximum OUR 100 mmol $L^{-1}h^{-1}$ Cell yield 13.5 g dry wt L^{-1}

DOT Dissolved Oxygen Tension (Percentage saturation)
OUR Oxygen Uptake Rate (mmol $L^{-1}h^{-1}$)

In order to demonstrate the reproducibility of fermentation regime PPF6 a number of parameters are compared in the following graph:

Table 2.7 Fermentation data

	DCW $g\ L^{-1}$	OUR $mmol\ L^{-1}h^{-1}$	DOT %	Time hours	Vessel L
SHPP30	13.5	99	19	21.5	20
SHPP31	13.8	102	22	21.5	20
SHPP34	13.4	101	30	22	42
SHPP35	13.3	105	10	22.5	42
SHPP49	13.77	99	18	22	42
SHPP51	13.72	102	21	22.5	42
SHPP52	13.7	104	23	22.5	42
SHPP53	13.55	100	22	22.5	42

DCW	final dry cell weight yield on harvest
OUR	maximum growth phase OUR
DOT	minimum growth phase DOT
Time	time taken to reach stationary phase
Vessel	fermenter vessel volume

Nb Table 2.6

2.4 Discussion

A reproducible batch fermentation for the production of *P. putida* ML2 has been developed. The factors that lead to variation in the production process were:

- Revival of stock culture
- Inoculum seed culture regime and transfer to vessel
- Consistent medium components for inoculum and fermentation culture
- Fermenter operating parameters

Stock culture

Stock cultures were stored on nutrient agar plates at 4°C for one month. After one month colonies of *P. putida* ML2 cells were selected by testing for *Tol* plasmid activity by plating on indole agar streak plates. These colonies were then plated onto nutrient agar plates and stored at 4°C. LINK researchers studying the biotransformation ability of *P. putida* ML2 reported no loss of plasmid activity when stored in this way.

An attempt was made to avoid the shake flask inoculum culturing stage by inoculating stock culture directly into the vessel. Cells were grown in 1000 μ L^{-1} benzene to 0.4 g dry wt L^{-1}. 20 mL samples were stored under glycerol at -70°C and used to inoculate fermenter vessels. This regime was designated PPInoc3. The resulting fermentation times were increased by approximately 24 hours. A long pre-growth phase was observed. This was probably not a true lag phase because cells may have been repairing any damage caused by cold storage (Takano, 1982). In addition the inoculum was too small for detection by exit gas analysis or OD readings. No increase in cell concentration could be measured and a long period of apparent inactivity took place before growth phase.

This proved to be an unsuccessful method of running reproducible fermentations. Although the growth profiles were identical, the length of the pre-growth phase varied. When a vessel containing growth media is running for long periods of time there is a danger of it becoming contaminated, this is increased when there is only a small number of cells in it. The overall run time was also increased by the time taken to switch from metabolising benzene to fructose.

Inoculum regime

Growth of *P. putida* ML2 in benzene culture had been slow and the final cell yield low. It was believed that *P. putida* ML2 was capable of utilising fluorobenzene as a carbon and energy source. The use of benzene in the first set of shake flasks was substituted for fluorobenzene. However fluorobenzene gave rise to no detectable cell growth. Later correspondence (Lynch, 1994) revealed that *P. putida* ML2's industrial importance is an enzyme defect that prevents it metabolising fluorocatechol, the product fluobenzene oxidation, hence leading to its accumulation.

In order to grow cells on entirely defined media the peptone was excluded from the first set of shake flasks. When benzene was used as a sole carbon source it was found that concentrations in the region of 2000 $\mu L\ L^{-1}$ inhibited growth and did not increase the cell yield. Sampling meant that the vaporised benzene was lost when the Subaseal was removed and so it was not possible to construct growth curves in this first shake flask stage.

The use of fructose-grown cells was resumed because inoculum cultures could be grown to greater densities. It was easier to grow larger volumes of cell suspension with fructose than benzene because of faster growth and equipment limitations of the flask culture system. Due to the volatility of benzene the shake flasks had to be sealed. Scaling up the stoppered benzene flasks was difficult, as two litre flasks could not be stoppered effectively. The volume of SF1 used to inoculate the SF2's was increased and this shortened the overall incubation time. The only other variation in the inoculum regime was the use of a larger volume of SF2 flask. (IPP6) Finally the inoculum was used at high enough densities at mid growth phase to allow very rapid growth.

Cells could only be grown to 0.4 g dry wt L^{-1} in benzene shake flasks. The inhibitory nature of high benzene concentrations and oxygen starvation in the stoppered flasks limited growth. This was not the case with the fructose-grown cells which could be cultured to concentrations upto 400% higher. The use of two phase system of flask culture proved to be effective. The first allowed cells to be screened for *Tol* plasmid activity (only cells with the plasmid can utilise benzene) and the second increased the cell concentration. This reproducible approach provided stable populations of cells in mid growth phase for use as an inoculum.

Fermenter medium composition

The initial media formulation and method suggested by Shell (PPF1) adequately produced cells. In order to allow collaborative work within the department it was decided to work with a researcher investigating on-line monitoring of biomass during fermentations. The method of testing for biomass changes was the measurement of capacitance using a device, known as a Biomass Probe. The presence of an excess of ammonium sulphate increased the electrical conductivity of the fermenter medium and interfered with the operation of the Biomass Monitor.

The majority of problems with fermentations appear to have arisen from variable inocula. A lack of standardisation in the inoculum regime can lead to low cell concentrations and cells possibly not being in the chosen growth phase. The fermenter media composition was not significantly changed during this work but the preparation of the components was altered. The main changes were in the nitrogen source. Ammonium sulphate at a concentration of 1 g L^{-1} appears to be adequate provided an strong inoculum is introduced into the vessel and extra nitrogen is fed in the form of ammonia solution for pH control. To prevent any slowing of growth due to nitrogen limitation an excess was eventually used. The initial concentration of ammonium sulphate of 8.4 g L^{-1} proved satisfactory.

Fermenter operating parameters

It was considered important to standardise the fermenter operating conditions as well as the inoculum regime. *P. putida* ML2 is an aerobic organism and has a large oxygen requirement. In order to avoid oxygen limitation effects it was decided to prevent the DOT dropping below 20% and fix the operating parameters from the start of the run. Aeration rates were in excess, in the region of 1 vessel volume $minute^{-1}$ were used with agitation speeds of upto 1000 rpm. This gave rise to two problems. One of the reasons for the slow growth of benzene grown cells was believed to be because of the high stirrer speeds damaging the solvent weakened cell membranes. During scale up to 25 L fermentations the use of an aeration rate of 25 L min^{-1} lead to foaming problems when the culture reached stationary phase. It was reduced and compensated for by increased stirrer speed.

Another advantage of the final regime was the convenience of being able to divide the work into weekly units:

- Friday revive stock cultures in the benzene shake flasks (SF1)
- Monday inoculate fructose shake flasks (SF2)
  prepare the fermenter and allow the probes to settle over night
- Tuesday inoculate the vessel
- Wednesday harvest cells
  downstream processing or assay testing
- Thursday cell damage testing
- Friday results analysis
  revive stock cultures in the benzene shake flasks

The fermentation protocol PPF6 successfully produced cells for assay and downstream processing testing. There was no reported loss of biotransformation activity and the method proved to be inexpensive in terms of media costs and preparation time.

3 Analytical development

3.1 Introduction

3.1.1 Methods for quantifying cell viability and damage

As was mentioned in Chapter 1, quantifying the damage suffered by a cell population is not a straightforward task. Simply measuring loss of viability can provide insufficient information about the damage process. The term viability is not rigidly defined and has a number of different meanings.

The following chapter sets out to show how a reproducible assay test system was developed to quantify cell damage. The introductory section provides a review of current 'viability' and 'cell damage' tests and is divided into the following sections:

- Measurement of cellular viability
- Measurement of cellular disruption
- Measurement of the ATP content of cells
- Methods of cell disruption at laboratory scale

3.1.1.1 Growth analysis

It is often necessary to report on the size of the bacterial population in a sample. A Viable Cell Count assumes that a visible colony will develop from each organism. Bacteria are however, rarely separated from each other and are often clumped together in large numbers particularly if they are actively reproducing (Collins *et al.*, 1989). A single colony may therefore develop from one organism or from hundreds or even thousands of organisms. Each colony develops from one viable unit, known as a Colony Forming Unit (CFU). As any agitation, eg in the preparation of dilutions, can break up or induce the formation of clumps, it is obviously difficult to obtain reproducible results. Bacteria are seldomly evenly distributed throughout the sample and as only small samples are usually examined very large errors can be introduced. Many of the bacteria present in a sample may not grow on the medium used at the pH or incubation temperatures employed or in the time allowed.

In Viable Cell Count methods, it is recognised that large errors are inevitable, even if numbers of replicate plates are used. Some of these errors are, as indicated above

inherent in the material, others in the technique. Errors of +/- 90% in counts of the order of 10^4-10^5 mL^{-1} are not unusual even with the best possible technique (Collines *et al.*, 1989). It is therefore, necessary to combine the maximum of care in technique with a statistical interpretation of results. They can be interpreted only if the product is regularly tested and the normal range is known.

The basis for these techniques is; material containing the bacteria is serially diluted and a portion of each dilution is placed in or on suitable culture media. Each colony developing is assumed to have grown from one viable unit, which as indicated earlier, may be one organism or a group of many.

Standard Plate Count

This is the industry standard method for the enumeration of viable cells. It has been shown to be an effective tool for isolating organisms, obtaining them in pure cultures, classifying and identifying them (Hattori, 1988). The Standard Plate Count (SPC) is often known as the Viable Cell Count (VCC) or simply as plate counting.

The plate count technique is relatively straightforward. A cell sample is serially diluted until single colonies can be detected on solid culture medium. Sterile, sealed universal bottles or test tubes containing 9 mL of sterile diluent as well as a number of agar culture plates and sterile pipettes are pre-prepared. 1 mL of the cell sample is aseptically pipetted into 9 mL of sterile diluent. 1 mL of this first dilution is then transferred to the next sample of diluent until the initial sample has been diluted 10 times. The number of dilutions obviously depends on the concentration of the cell sample which can be estimated by spectrophotometry. Usually the last six diluted samples are tested. 0.1 mL of a dilution is evenly spread over the entire surface of the plate. This is accomplished by the used of a spreader that has been sterilised by immersing in methanol and flaming. The process is repeated for the other dilutions. In practice usually the last few dilutions need be plated out.

Plates are incubated for a set period of time (depending on the growth rate of the organism). The number of colonies on each plate is identified by dividing the underside of the dish with gridlines and marking colonies in a particular grid with an indelible pen and tallying with a hand-held counter. To be statistically accurate a plate needs to contain between 30 and 300 visible colonies (Cappuccino and Sherman, 1987). The cell concentration of the original sample is calculated by

multiplying the dilution factor by the number of colonies on any plate. Colonies are counted.

For large work loads automated systems are essential. Semi-automatic systems replace the pen that marks the glass above the colonies with an electronic counter which displays the numbers counted on a small screen. Fully automated systems use a television camera or laser beam to scan the plate and results are displayed on a screen.

Plate counts are the most commonly used method for determining viability. Advantages of this method include:

- Only viable / reproducing cells are counted
- Allows isolation of discrete colonies
- Easy to master, only requires knowledge of basic aseptic technique
- Useful test for contamination of samples
- Indole plates provide a rapid test for loss of enzyme activity. In the case of biotransformation ability analysis of *P. putida* ML2

There are however a number of disadvantages of plate counting:

- Accuracy
- Labour intensive
- Suitability of organism
- Incubation time
- State of stress of organism
- Culture conditions

Accuracy, as has been mentioned, can be very low. This is often due to the inherent problems with the test itself. Aggregation of cells gives rise to single colonies. Bacteria are seldomly evenly distributed throughout a sample. Operator errors are also responsible for dilution inaccuracies and the inability to discriminate between colonies too small for analysis.

The equipment preparation, serial dilution and the subsequent plating of samples are all extremely labour intensive.

Suitability of the organism for the test. This method cannot be applied to cells forming inseparable chains or cells which attach themselves to certain solid material. When testing natural cell populations, many of the bacteria present in a sample may not grow on the medium used, at the pH or incubation temperatures employed or within the time allowed (Folt *et al.*, 1988). This is not a problem for known bacteria. Dehority and Grubb (1980) showed how aggregation of cells can lead to problems. Cold storage of anaerobic bacteria from bovine rumens for short periods of time gave rise to increased plate count values when compared to unstored samples. This is claimed to be due to the fact that the cells form aggregates, which equate to one colony forming unit, but the action of cold storage somehow separates the aggregates, increasing the number of CFU's.

Long incubation times are not only inconvenient but can lead to inaccuracies. The state of stress of the organism can affect the time taken for visible colonies to appear. This is due to the phenomenon of growth delay, which will be discussed in more detail later. This is the time taken before the organism's lag phase in which it repairs any stress-induced damage. In the case of severely stressed cells this growth delay could be as long as the incubation time allotted for the SPC analysis. In which case a reduced number or even no visible colonies would have appeared by the counting time, although a lengthened incubation could change this. Although this factor has an adverse effect on results, growth delay analysis can be used as a quantitative measure of the stress placed on a cell population (Takano and Tsuchido, 1982).

Culture conditions, the recovery of stressed cells depends on the ambient temperature and the composition of the diluent as well as the culture conditions. Certain diluents may cause problems eg saline or distilled water as they may be lethal for some organisms. Diluents, when used direct from the fridge can cause cold shock which may prevent organisms from reproducing.

Other methods of determining viable bacterial numbers based on a serial dilution followed by culture on suitable medium include:

- Pour Plate Method
- Roll-Tube Method
- Drop Count Method
- Droplette Method
- Surface Count Method
- Plate Loop Count

- Membrane filter counts
- Most Probable Number Estimates
- Rapid Automated Methods

Many of these techniques are only of use for organisms that are thermally robust enough to retain viability after immersion in molten agar. *P. putida* ML2 is unlikely to survive this sort of treatment, however other organisms, such as *E. coli,* is unaffected by temperatures upto 45°C. All of the techniques retain the same advantages and disadvantages of the basic plate count technique. More information has been included as Appendix 3.1 to provide reference for future work using different organisms.

Biophotometry

A semiautomatic method for quantifying viable bacteria is described by Vosbeck *et al.* (1984). The bacterial effect of the antibiotic rifampicin was tested on the organism *Staphylococcus aureus.* Viable cell numbers were quantified by the analysis of growth curve data. Growth curves were constructed using absorbance measurements made by a multichannel biophotometer. Use of this particular instrument allowed the possible simultaneous monitoring of upto 176 samples. Vosbeck claims a very close correlation with SPC results.

As with the SPC the biophotometric method is based on the organism's growth kinetics. It therefore shares many of the advantages and disadvantages associated with plate counts. These potentially include long incubation periods and dilution errors at high concentrations.

A more specific disadvantage would be the suitability to *P. putida* ML2. After agitated and aerated growth the change to static culture in a cuvette could lead to metabolic changes which would be reflected in altered growth kinetics.

One problem that this technique does not share with plate counts is cost. The purchase and maintenance cost of a multichannel biophotometer is prohibitively high for use in this study. However there are a number of advantages of this technique:

- Semi-automation of the process allowing the analysis of upto 176 small samples simultaneously.
- Growth kinetics give an indication of the levels of cell stress.

Growth Delay Analysis

Takano and Tsuchido (1982) developed the concept of Growth Delay Analysis for estimation of the total injury of a bacterial cell population, which had been subjected to some form of bactericidal stress. In order to avoid some of the problems associated with plate counts automated equipment was used to plot growth curves. The length of the observed delay in growth before the exponential growth phase seems to be a function of the levels of stress that the surviving cells have been subjected to.

Standard Plate Count (SPC) is the method widely used for enumeration of surviving cells that have been exposed to various physical and chemical stresses. These include heat, ultraviolet and ionising irradiations, freeze-thawing detergents and other bactericidal chemical agents. Growth delay analysis attempts to avoid some of the inherent problems of colony counting, such as the inability to test organisms that form inseparable chains and the overall lack of accuracy. In particular it addresses the problem of lengthened incubation times, which can delay the appearance of colonies during the allotted incubation time.

All types of cells from bacterial to human possess a set of intracellular proteins which are expressed in response to certain types of environmental stress - these are known as the Stress Response Proteins (Passini and Goochee, 1988). When a cell population is subjected to stress and then placed in an appropriate liquid assay medium, initiation of growth is delayed compared with that of an unstressed cell population. This stress-induced delay reflects not only a decrease in the number of reproducible cells but also the increased lag time which is required by non-lethally injured cells for the repair processes before the first division.

The delay time for growth can be estimated quantitatively and expressed as an integrated viability, υ:

$$\upsilon = \sum_{i=1}^{k} n_i^{-\mu\lambda_i} \qquad (3.1)$$

Where:

λ_i increment of the lag time for the stressed cells in the i-th fraction, n_i

μ specific growth rate

The growth delay was $-G_{10} \log_{10} \upsilon$ (G_{10} is delay time corresponding to a one tenth reduction in the size of the inoculum). Takano and Tsuchido claim a close correlation with plate count techniques.

The delay time was measured turbidimetrically from growth curves of both stressed and untreated cell populations. Other techniques can be used for detecting microbial growth such as microcalorimetry, radiometry, change in electrical impedance in the medium or electrochemical detection.

An automated system has been developed (Tsuchido *et al.*, 1989) that links an incubator to an automated OD analyser and a computer-assisted data analysis system. This allows evaluation of not only lethal but also growth inhibition by drugs or chemical agents.

The viability of stressed cell populations, as enumerated by the standard plate count method, often coincides with data estimated from growth curves. Hirano *et al.* (1958) determined the viability of a thermophilic bacterium at sub-optimal growth temperatures using the growth delay model. The results obtained were identical to those of the colony count method. However the agreement of data between these two methods is not always the case for other types of stress or test organisms. (Tsuchido *et al.*, 1989)

The theoretical approach to Growth delay analysis is outlined in Appendix 3.2.

Growth delay analysis still retains one of the main disadvantages of the viable cell count. Results are still dependent on the growth kinetics of the particular organism under investigation. This means that results take hours or even days to be collected. However there are two factors that are of specific interest in the context of this project:

- It is one of the few published works that proposes a method of evaluating the stress put on a bacterial culture as opposed to simply quantifying loss of viability.
- The sample size. When using plate count techniques it is only possible to count less than 500 colonies on a single plate. This is too small to estimate a population of 10^7cells per mL. The data has to include a substantial error to take into account inaccuracies mainly produced during the dilution process. Growth delay analysis can be conducted with an inoculum containing 10^6 or

more cells per mL, indicating a much higher reliability for detecting total damage of a bacterial population.

3.1.1.2 Physical Analysis

Coulter Counter

A Coulter counter, or electric sensing zone, is an instrument capable of rapidly counting the number of cells suspended in a conducting fluid, usually a saline solution, that passes through a minute orifice through which an electric current is flowing (Cappaccino & Sherman, 1987). The orifice is an aperture measuring between 30-100 μm in diameter (Kubitshek, 1989). Increased resistance is proportional to cell volume allowing these devices to count both the number and the range of sizes of cells in any given volume of fluid. A Coulter counter can count between 3-5 $x10^8$ cells per second. The device cannot distinguish between metabolising and non metabolising cells.

Seydell & Wempe (1980) claim to have devised a method to differentiate between total and viable cell populations by the use of a Coulter counter. The paper describes the testing of the bactericidal effects of antibiotics on *E. coli* cells that avoids the use of SPC. The concentration of cells per fixed sample volume is measured over time to give growth curves. In a similar way to those obtained by optical density measurements.

Disadvantages of using this method include:

- Dependent on growth kinetics and is therefore subject to the same advantages and disadvantages
- Preparation and sample processing
- Machine difficult to operate and familiarisation time lengthy
- Price of equipment
- Bacteria are close to minimum detection size
- Only unicellular organisms can be counted
- Clumping of cells can give rise to false readings and blocked aperture
- Cannot differentiate between viable and non viable biomass, cell debris, non biomass solids and gas bubbles

Advantages of this technique:

- Avoids plate counts
- Avoids serial dilutions
- Rapid - 3-5000 cells second^{-1}
- Operational over full range of biomass concentrations

There appear to be very few advantages in using this method over conventional turbidity testing for evaluating simple growth kinetics of an sample of microorganisms.

Biomass Probe

The Biomass Probe, also known as the Dielectric Conductivity Probe or Biomass Monitor is a novel device used for detection of biomass in suspension. It functions by detecting changes in capacitance between two electrodes which are caused by the presence of intact cells (Mathew, 1995). Intact cells have a non conducting membrane which changes the capacitance signal. These capacitance measurements can be correlated with viable cells.

At the time of writing the probe was in its developmental stage and unfortunately results only seemed reliable after the probe had been left to settle for a number of hours. It was therefore not a suitable method of measurement for a number of different samples.

3.1.1.3 Respiration Measurements

The respiration rate of a viable cell population is indicative of the number of active cells. There are a number of techniques that can be used to quantify respiratory activity (Zimmerman *et al.*, 1978, Matsunaga, 1981).

The most common form of test for respiratory activity is the uptake of labelled nutrients. Labelling can be fluorescence or radioactively based. One paper reports a combination of the two techniques for the determination of aquatic bacteria (Tabor and Neihof, 1982). However these tests are still based on the organism's growth kinetics.

Another example is when microorganisms metabolise they produce heat, known as the heat of enthalpy. This heat production can be related via enthalpic and elemental balances to give an estimation of biomass. These methods require extremely sensitive and specialised equipment and appear to have very few advantages over conventional cell enumeration techniques.

Possibly the most straightforward respiratory measurement is the measurement of the oxygen utilisation of an aerobic cell culture, which can be easily carried out using an oxygen electrode. There are a number of disadvantages of using an oxygen electrode. Measurement of respiratory activity is dependent on the stage of growth of the cell population. This coupled with the problems associated with oxygen electrode can make the accurate estimation of biomass extremely difficult.

The most suitable methods for detection of respiratory activity are testing for metabolic activity. There are a great number of different methods of doing this. A few of which will be discussed in the following pages.

3.1.1.4 Active Dye Staining

Bacterial metabolism can also be assessed by measuring the activity of the electron transport system, which is based on the reduction of tetrazolium salts. Electron transport system activity has been shown to be directly related to respiration. Active dye staining is a technique that allows the differentiation of metabolically active and inactive cells by their ability to combine with certain unnatural substances. The substance forms a complex with part of the cell which can be detected by microscopy. The complex forms with either the active or inactive cell population, not both.

Epifluorescence Microscopy

Epifluorescence microscopy is a form of active dye staining which uses fluorescent dyes to differentiate between metabolising and non-metabolising cells (Hobbie *et al.*, 1977). The direct count method using a fluorescent or epifluorescent dye has been widely reported. Bacteria are stained with a fluorochrome, retained on a membrane filter and counted under epifluorescence illumination (Fry, 1988). The criteria for a successful direct-counting technique on a filter are simple:

- All the bacteria must be retained by the filter
- All the bacteria must be visible at the filter surface
- Staining and optical conditions must produce high contrast between the bacteria and the background

There are a variety of stains currently in use the intensity of fluorescence varies between the stains and has been ranked as DAPI > mithramycin > ethidium bromide > acridine orange. Although most of these stains are supposedly specific to DNA, other living and non-living material is commonly visible as well. DAPI and acridine orange are the most widely used because they stain bacteria and other particulate debris differentially (Fry, 1990).

Acridine orange (3,6-bis(dimethylamino)acridinium chloride) is the most commonly used stain. Bacteria grown at high growth rates in batch or continuous culture will fluoresce red-orange due to the predominance of RNA. The random coil of the RNA allows so many acridine orange molecules to attach and interact that the acridine orange fluoresces as a dimer. In contrast inactive bacteria have mostly DNA and fluoresce green, the rigid structure of the double helix allows fewer acridine orange molecules to attach, they do not interact and the acridine orange fluoresces as a monomer. In living bacteria the DNA fluorescence is always present but is always masked by the great amounts of RNA. The fixation by aldehyde does not change the nucleic acids, so the green-red distinction will continue after cell death

A fluorescence-based viability assay for the determination of mammalian cells using the carboxyfluorescein derivative BCECF (2'-7'-biscarboxyethyl-5(6)-carboxyfluorescein) by Leeder *et al.* (1988).

Other Methods Of Active Dye Staining

Due to the fact that respiration is closely bound to active metabolism the cytochemical detection of dehydrogenase activity was combined with a technique for direct counting of aquatic bacteria by Zimmerman *et al.* (1978). Zimmerman has pioneered an active dye staining technique for the determination of numbers of viable bacteria occurring in marine ecosystems. The active electron transport system of respiring organisms reduces 2(p-iodophenyl)-3-(-p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. INT acts as a hydrogen acceptor and respiring (active) bacteria will accumulate water-insoluble INT-formazan crystals intracellulary as dark red spots. Corresponding to electron transport activity these

deposits attain a size and a degree of optical density which allows them to be examined by light microscopy. If polycarbonate filters and epifluorescence microscopy are applied to analyse an INT-treated sample, it is possible to differentiate between respiring and non-respiring bacteria. This differentiation, which permits determinations of the total number of bacteria and the proportion involved in respiration, is seen directly in one microscopic image.

Using similar techniques, a method was developed by Bitton and Koopman (1982) to assess the activity of filamentous bacteria in activated sludge. It was previously necessary to identify the bacteria in the sample by epifluorescence microscopy and then switch to bright field microscopy to observe the formazan crystals. Bitton and Koopman set out to develop a technique that would allow observation, in the same field, active (with red formazan crystals) and inactive filamentous bacteria. Malachite green was found to be the most suitable counter stain that would dispense with epifluorescence dying. The activated sludge was incubated with INT followed by staining with malachite green. Both cells and INT-formazan crystals can then be observed by using bright field microscopy.

The dye NPN (1-*N*-phenylnaphthylamine) was used to investigate the effects of heat stress on *E. coli* by Tsuchido *et al.* (1989). NPN is an uncharged lipophilic dye which fluoresces weakly in aqueous environments and strongly in hydrophobic and non polar ones, eg cell membranes. Membranes are among the critical targets of heat stress in bacterial cells, as shown by the leak of intracellular substances and loss of membrane components. Following heat stress the outer membrane's permeability to the dye increased and was reduced following repair. This appears to provide information about the state of damage to the cell following this form of damage.

Active dye staining is a technique which is potentially useful for this type of work however the disadvantages have to be taken into account. These include the potential for operator error and the safety problems with the toxicity of the reagents. The main advantage of this method is the fact that there is no reliance on growth kinetics and is therefore more rapid than plate counts.

3.1.1.5 Summary

Many technological advances have been made for enumeration of viable cells in recent years. In spite of these, the Standard Plate Count is still the most widely used method for the following reasons:

- As the standard technique used throughout the world for many years all novel techniques have to be calibrated against it. When a new method is tested SPC's still have to be carried out as a confirmation for publication purposes
- No complex equipment is required and it is therefore inexpensive. Training time is short and inexperienced operators can rapidly produce acceptable results
- Information concerning sterility of the culture and changes in colony morphology are rapidly apparent
- The methods and materials pose no hazard to the health of the operator. Unlike the use of active dye staining and radiolabels

All of the techniques that have been discussed in previous chapters have fundamental drawbacks. The plate count, although far from an ideal choice, appears to be the most acceptable form of enumerating viable cells. A possible approach would be to supplement the information provided by plate counts with another test.

As stated at the beginning of this review, cell viability is generally regarded as the ability of an organism to reproduce itself and metabolise. It has been shown that organisms are capable of metabolising without possessing the ability to reproduce themselves. The reverse does not hold true. Cells that are no longer intact are capable of neither. The damage caused to cells during downstream processing appear to be mainly caused by the action of mechanical stresses which lead to cell lysis. There is therefore a strong possibility that remaining, intact cells will retain viability. If this is the case then there is a chance that the processing techniques do not have to be assessed by viability tests and can be examined by testing for structural damage. The following section examines methods of testing for cell lysis.

3.1.2 Testing for intact cells

Concentrating cell suspensions frequently results in the lysis of the cells. One approach is to quantify the number of intact cells before and after processing. This assumes that any loss of viability is due to physical destruction of cells. This is not necessarily the case but testing for loss of intact cells from a population is generally quicker than testing for loss of viability by measuring growth kinetics and provides a fairly reasonable indication of the levels of damage. If it is assumed that the intracellular composition of the organisms remains roughly constant during the processing then assaying for intracellular components released into the extracellular environment will give an accurate indication of the number of intact cells remaining. A target metabolite is chosen that can be easily and reliably assayed after release into the extracellular environment.

A typical approach would be carried out in the following way. A cell sample would be taken from the culture before the processing step is carried out. The sample is centrifuged and the background levels of the target metabolite are assayed. The presence of background levels are due to cells that have lysed after normal cell death. Another sample is taken and all the cells are completely disrupted, by multiple passes at very high pressures (eg 3 passes at 1600 bar). The levels of the target metabolite in the disrupted sample are measured. By subtracting the background levels from the totally disrupted levels the total amount of target metabolite within the intact cell population can be obtained. After processing the levels of target metabolite in the extracellular environment are measured. The proportion of cells remaining intact can be calculated by comparison with the control (unprocessed) sample.

The following section provides an overview of the major types of target metabolites that can be used in this way. A survey of major types of cellular disruption techniques can be found in Section 3.1.5.

3.1.2.1 Protein Assay

3.1.2.1.1 Total Soluble Protein

The majority of the dry weight of a cell is made up of protein. Structural and metabolic components contain protein. A non-specific assay for the total protein of the cells is an obvious choice of target metabolite. There are a number of methods available for the measurement of total protein.

Table 3.1 Properties of protein assays (Robyt & White, 1978)

Test	Chemical Interference	Protein/Protein Interference	Speed	Complexity	Detection Limit mg/mL
Spectroscopic	great	great	rapid	simple	varies
Biuret	moderate	low	moderate	simple	1-6
Lowry	great	signif	moderate	complex	1-10
BCA	moderate	moderate	moderate	complex	0.1-1.2
Kjeldahl	moderate	low	slow	complex	0.2-0.1
Bradford	slight	signif	rapid	simple	0.2-1.4

Spectroscopic measurements

Measurement At 280 nm Most proteins absorb at 280 nm due to the phenolic group of tyrosine and the indolic group of tryptophan. Amino acid composition determines the extinction coefficient-usually 0.4-1.5. However nucleic acids absorb at 260 nm and cause interference. Therefore this method is limited for relatively pure protein solutions.

Far UV Peptide bonds absorb at 191-194 nm, but oxygen, carboxylic acids, buffer ions and alcohols interfere and therefore measurements have to be at 205 nm.

2 wavelength absorption Concentration of proteins in solutions or cell free extracts contaminated by rRNA's or tRNA's can be simply, rapidly and accurately determined by measuring absorption at 229 and 235 nm, two wavelengths where absorptions are identical and therefore interference can be eliminated. (Ehresman *et al,*, 1973) The method is claimed to give results comparable to the Lowry method.

Biuret Test

Under alkali conditions Cu(ll) SO_4 and peptide nitrogen absorb at 540-560 nm to give a purple colour. The colour is caused by the formation of a complex of a Cu(ll) ion with four nitrogen atoms, two from each of the two peptide chains. This reaction is similar to the reaction of four ammonia ions with Cu(ll) to give the deep blue complex ion cupric tetramine. Therefore ammonia and ammonium ions will interfere with the formation of a blue complex with the Cu(ll) of the Biuret reagent. There is no interference from amino acids and it is independent of amino acid composition as reaction is of the peptide chain not the side groups. Low sensitivity can be increased by separation of Cu-Protein complex by gel filtration followed by colorimetry. As well as absorbance measurements in near UV at 310 nm. Disadvantages are the low sensitivity of the test requires relatively large amounts of protein, interference by buffers and interference by ammonia, which is in excess in the fermenter medium.

Lowry

Biuret reactions of proteins with Cu(ll) under alkali conditions and its subsequent production of heteropolymolybdenum blue by the copper catalysed oxidation of the aromatic amino acids (Lowry *et al*, 1951) However there are disadvantages for example the variation in batches of Lowry reagents leads to a variation in responses to different amino acids. Also interference from buffers, detergents drugs, lipids. sugars, salts. Most of the problems with the Lowry procedure are due to the Folin-Ciocalteau detection agent therefore it is possible to substitute bicinchoninic acid

Kjeldahl Method

Protein samples are digested in concentrated sulphuric acid by heating for several hours. During the digestion protein nitrogen is converted to ammonium sulphate. The solution is made alkali which causes the ammonium sulphate to be converted into ammonia. The ammonia is steam distilled into HCl which is then estimated. As most proteins contain about 16% nitrogen, 100 mg of protein contains 16 mg of nitrogen, and so 1 mg of nitrogen =100/16 or 6.25 mg of protein. The mass of nitrogen in the sample is multiplied by 6.25 to give the mass of protein. Unfortunately this method requires large samples.

Bradford Assay

The method uses a dye, Coomassie Brilliant Blue G-250 which has a negative charge and binds to the positive charges on the protein. The dye exists in a red form (A_{max} 465 nm) and a blue form (A_{max} 595). The red form is the predominant form in solution and when its negative charges bind to the positive charges on the protein it is converted to the blue form. Many proteins have nearly identical response curves so the technique can be applied widely using a single set of standards. Furthermore it is much less susceptible to interfering substances. The reaction is highly reproducible and is complete in 2 minutes with colour stability for over one hour.

3.1.3.1.2 Marker Enzyme

An alternative approach to the assay of total soluble protein is to target a specific intracellular enzyme for analysis. Criteria for this selection are:

- Reasonably high intracellular levels
- Cytoplasmic
- Ease of assay
- Cost of assay

The fermentation of *P. putida* ML2 uses fructose as a carbon source and therefore a possible target enzyme would be a catabolic enzyme involved in the metabolic pathway eg glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase both are cytoplasmic and occur at reasonably high levels to allow utilisation of fructose. *P. putida* ML2 possesses a plasmid-bound enzyme system

which allows the organism to metabolise aromatic substances, which is of potential industrial importance. One of the enzymes of this group is benzene dioxygenase. At the time of writing no assay for benzene dioxygenase activity is available. Although work is in progress within the Chemical and Biochemical Engineering Department at University College London.

3.1.2.2 Nucleic Acid Analysis

A problem with protein assays is that very small or surface proteins could be released during a separation and an organism still retain its metabolic activity. Nucleic acids are larger molecules than proteins and their release into the medium would almost certainly be accompanied by cell death.

Nucleic acids contain three distinct chemical components: bases (purines and pyrimidines), sugars (2-ribose or 2 deoxyribose) and phosphorous. Therefore methods for determining nucleic acids are based on UV absorbance of the bases, specific reactions of the pentoses or determination of the phosphorous. Nucleic acids can also be quantified by HPLC analysis, however other intracellular components could lead to inaccuracies.

Distinction between the ribose and deoxyribose is by specific colour reactions. RNA is determined by the orcinol reaction with ribose in which a blue-green colour is produced. DNA is determined by the diphenylamine reaction in which a blue-green colour is produced. (Robyt & White, 1987)

3.1.2.2.1 Deoxyribonucleic Acid (DNA) Assay

There are three commonly used assays for the determination of deoxyribonucleic acid, ethidium bromide fluorescence, diphenylamine analysis and UV absorbance.

Ethidium Bromide Fluorescence Of DNA

A rapid semi-quantitative method for estimating the amount of DNA in samples containing contaminating substances is to utilise the UV induced fluorescence emitted by ethidium bromide molecules that have intercalculated into the DNA.

Comparison of the intensity of the samples with that of a set of known standards determines the quantity. This technique is accurate within the range 0.5-20 $\mu g\ mL^{-1}$.

A simplified rapid procedure involves the spotting equal volumes of standards onto a sheet of transparent wrap stretched over an ultraviolet light source. An equal volume of buffer containing ethidium bromide is added to each standard and sample. The concentration of the unknown sample is estimated by comparison to the intensity of fluorescence of the standards when irradiated with 254 nm UV light.

Diphenylamine Analysis Of DNA

Treatment of DNA with strong, hot acid causes the depurination of DNA, cleavage of the sugar-phosphate backbone, and dehydration of the deoxyribose to hydroxylevulinyl aldehyde. The aldehyde condenses with diphenylamine to yield products with an absorption maximum of 595 nm. A method is described by Leyva and Kelley (1974) that allows complete separation of the DNA from the soluble cell extract which can then be used for other biochemical determinations.

Absorbance Of Ultraviolet Radiation At 260 and 280 nm

Pure samples of nucleic acids and nucleotides absorb light in the UV because of the purine and pyrimidine bases. The concentration of the sample can be determined if the extinction coefficient is known. Double stranded DNA has an extinction coefficient of 200 at 260 nm. Single stranded DNA and RNA have a coefficient at 260 nm of 250. Pure preparations of DNA and RNA have A260/280 values of 1.8 and 2.0 respectively; If protein of phenol is present, the absorbance at 280 nm of either substance will interfere with the accurate quantification of the nucleic acid present. Concentrations of pure nucleic acids as low as 5 $\mu g\ mL^{-1}$ can be accurately measured with this method.

3.1.2.2.2 Ribonucleic Acid (RNA) Assay

Ribonucleic acid is assayed by orcinol analysis. The colour complex formed with orcinol and RNA has a maximum absorbance at 665 nm and reaches a maximum colour after 15 minutes.

3.1.2.3 Metabolite Analysis

Assays for intracellular metabolites other than proteins and nucleic acids are useful. These can include commonly found carbohydrates such as Glucose-6-Phosphate or phospholipids.

There has been a considerable interest recently in the use of the bioluminescent assay of ATP. All cells use ATP as the energy source for metabolic reactions. A small number of publications suggest the use of ATP as a target metabolite for testing for cellular integrity. A thorough investigation into the potential for ATP analysis has been carried out and forms Section 3.1.3.

3.1.2.4 Whole Cell Tests

There are a number of methods of testing for intact cells. These include the use of Coulter Counters and Biomass Probes which have been discussed in Section 3.1.1.3

3.1.2.5 Most Suitable Test

Assaying for total soluble protein is a simple, accurate and reproducible task, and its suitability for this type of work is widely reported (Harrison *et al.,* 1990, Keshavarz-Moore *et al.*, 1987, 1990). For examining cell breakage it appears to be preferable to assay for protein rather than other intracellular components such as nucleic acids due to time, accuracy and cost. The most acceptable method of assaying is the Bradford Test.

The use of ATP as a target metabolite for assessing cell damage appears to have a great deal of potential for this work. A few publications, at the time of writing have highlighted this potential application. The bioluminescent ATP assay is claimed to be superior to the Bradford test in terms of speed and accuracy and in addition provides information about the metabolic rate of the surviving, intact cells. The following section is devoted to work that has been published on this subject.

3.1.3 ATP Analysis

3.1.3.1 Introduction

A considerable amount of interest is currently being shown in the use of the bioluminescent ATP assay. This technique appears to have some potential in the context of this project to supplement or possibly replace plate counts and total soluble protein assay. All living cells contain ATP which may be extracted and then assayed. The amount of ATP that can be extracted from a biological sample is directly related to the number of microbial cells.

The intracellular concentration of ATP regulates the activity of many enzymes. It is therefore fairly constant in all living cells (Knowles, 1977). The amount of ATP per cell is essentially determined by the volume of the intracellular space. ATP concentrations have been shown to vary between individual bacterial cells (McEntee *et al.*, 1989) and this is most likely due to changes in intracellular volumes or metabolic effects. Cell death leads to the rapid degradation of ATP by intracellular enzymes. In naturally occurring biological samples the extracellular ATP is very low due to intracellular ATP-degrading enzymes being released, eg from dead cells. Differences between ATP content of individual cells are small enough to allow the total intracellular ATP of a population to be used as an accurate indicator of cell numbers. A theoretical basis for using ATP as a measure of the living biomass is strong. (Lundin, 1989)

The concept of using ATP as an index of biomass was first suggested by NASA groups working on methods of detecting life on other planets (Lundin, 1989) as well as for testing for contamination of water in space. The main advantage of this technique is its speed and sensitivity. Most other microbiological techniques generally require the growth of microbes to produce the amplification needed to bring the cell numbers within the scale of sensitivity of the test. Most ATP assays require no amplification due to the intrinsic sensitivity of the firefly luciferase enzyme which may be used to detect and measure ATP to below 1 pg. This is equivalent to 1000 'average' microbial cells. (Stanley, 1989a)

3.1.3.2 Applications

To date no paper has been published on the use of the ATP bioluminescent assay for measuring microbial damage after exposure to mechanical stress. Lundin (1989) has listed the following applications:

- Index of biomass
- Index of cell membrane leak
- Index of energy status

Index of biomass

This was first proposed by NASA, who developed the assay and has subsequently been reported in the following contexts:

- Activated sludge monitoring
- Soil microbiology
- Water contamination detection
- Fermentation monitoring.
- Clinical research

Activated sludge monitoring.

Nelson and Lawrence (1980) investigated the effects on a steady state kinetic model of slurry type biological waste water treatment processes. ATP concentration was used as an indicator of microbial viability. It was found that microbial viability of activated sludge Mixed Liquor Volatile Suspended Solids (MLVSS) determined by ATP analysis exhibits a close relationship with standard viability determinations.

Soil microbiology

The use of ATP analysis in soil microbiology has been reported by Brookes and Ocio (1989). The soil microbial biomass comprises between 1-3% of the total soil organic matter. It is an important store of plant nutrients, especially nitrogen, phosphorous and sulphur. Methods are needed to measure soil microbial biomass and the use of ATP has provided a suitable indicator. In soil, extracellular ATP has a very short half-life and therefore occurs only in living cells. The ATP extracted from soil comes therefore entirely from living organisms.

Water contamination detection

A rapid Microbiological Quality Assurance (MQA) test for the microbial quality of process water is vital in the manufacture of personal and liquid detergent products (Wooldrige, 1989). Water is used in large quantities by most manufacturing industries and is a particularly important source of gram negative bacteria, with *Pseudomonads* being particularly responsible for the biodeterioration of food, petroleum products, pharmaceuticals and cosmetics. The detection of these and other microorganisms at the earliest opportunity is therefore important so that good quality process water can be maintained for product manufacture. In order to avoid long incubation times associated with plate counting Wooldridge developed a rapid method for the MQA of process water based on the detection of ATP, similar to that used widely in the food industry. As process water is generally chemically simple the luciferase enzyme is not frequently subjected to inhibition which is a problem with many product analyses. Similar advantages are applicable for the use of ATP monitoring in marine microbiology.

Fermentation monitoring

The monitoring of a seed culture of *E. coli* and a subsequent fermentation has been reported by McEntee *et al.* (1989). The levels of intracellular ATP in shake flask seed cultures was shown to vary during the growth cycle. Unfortunately this is a poorly presented paper with simple reporting of results and little discussion.

Shortly after inoculation of a shake flask from an agar plate the ATP concentration per cell rises and reaches a maximum after 90 minutes of incubation. This peak corresponds with the onset of the exponential growth phase when correlated with absorbance readings taken at 670 nm. Once the cells have reached the exponential growth phase the intracellular ATP levels are rapidly diminished. A ten thousand fold difference between levels at the start of growth phase and the onset of stationary phase was observed. On the addition of more glucose to the culture a 10 fold increase in intracellular ATP was observed after 8 hours. *Candida utilis* was cultivated in a glucose and mineral salts medium and it was found that specific growth rates were similar when determined by absorbance dry weight or ATP measurements. ATP concentration per unit dry weight of cells was found to be constant during early exponential phase but declined in late exponential and stationary phase. (Prior *et al.*, 1988). In a similar fashion Ng *et al.* (1985) report on the estimation of *Campylobacter* species in broth culture by bioluminescent ATP analysis and Gikas and Livingstone estimated activated sludge.

There appears to be a certain amount of confusion in most other published work on this most essential point. The majority of papers on the subject have been written by Stanley and Lundin who state at the beginning of almost every paper that intracellular ATP concentration remains constant in cells which is what makes it a good index of biomass. These two authors frequently list factors that effect ATP levels. This seems indicative of the levels of knowledge of this subject. More details are included later.

Clinical research

There are a number of clinical applications for the ATP assay approach. Details are given in the section on index of cell leakage.

Index of cell membrane leakage

A number of papers have been published outlining the use of ATP assays as an index of cell membrane leakage. These are of particular interest in the context of this project. An assay of antimycotic effects has been described by Ansehn and Nilsson (1984). Adding tioconazole to a cell suspension caused a loss of intracellular contents to the extracellular environment. This loss could be monitored by testing for ATP inside and outside the cell. This loss corresponded directly to loss of viability as determined by plate counts. Other published works that assess loss of microbial viability after exposure to a harmful substance include:

- The effects of antibiotics on *E. coli* is evaluated in a similar way by Hanberger *et al.* (1990)
- The effect of warfarin on ATP content and viability was studied in suspensions of rat hepatocytes by Gjerde and Helegland (1984)
- Sorbic acid sensitive cultures of *Penicillium roqueforti* were monitored for loss of viability and reduction in intracellular ATP content (Liewin and Marth, 1985)

These papers are particularly interesting in the context of this work as they highlight two points. Firstly, the measurement of a reduction in intracellular ATP levels can be obtained rapidly and then correlated with loss of viability as measured by plate counts. Secondly, the leakage of ATP from cells exposed to membrane-damaging substances can be correlated with loss of integrity, as measured by intracellular protein release. This loss of intracellular contents is potentially similar to the effect

of mechanical stress leading to structural damage of the cell membrane leading to increased porosity or even cell lysis. Therefore the ATP assay could possibly be used to assess microbial viability loss after cells had been exposed to mechanical stress.

Apart from Lundin's three generalised applications there have also been reports of a method of harnessing the luciferase system in vivo. Stewart (1990) reviews a number of papers claiming to have utilised a reporter of microbial recovery during and following stress exposure. There are two ways in which in vivo bioluminescence can report on the components of microbial stress.

The first requires the genetic coupling of the bacterial luciferase gene tandem to a transcriptional promoter whose activity is controlled either directly or indirectly in response to environmental stress. Luciferase expression acts as a reporter of stress. For example, luciferase genes from *Vibrio fisheri* were coupled to the *E. coli* pro U osmoregulated promoter. Transformation of the *E. coli* with the recombinant chimera provided a bioluminescent phenotype responsive to the levels of media osmolites such as NaCl.

The second application in this context probes the capacity of cells to supply high energy biochemical intermediates during or after some cellular insult. In vivo luminescence depends not only on the production of the luciferase enzyme but also the on the availability of an energy source to drive production of the excited state that, on return to the ground state, results in a photon emission. Robinson (1981) states that lux genes from *V. harvei* and *V. fisheri* have been expressed in a wide range of bacteria including *Pseudomonas*.

Index of energy status

The Adenylate Energy Charge has been proposed as the best index of the energy status of bacterial cells (Lundin, 1984). A firefly luciferase assay of ATP, ADP and AMP in the same cuvette has been described that allows very accurate determinations of the energy charge values. This method could find applications whenever physiological conditions affect intracellular levels of adenine nucleotides, eg in environmental studies or in fermentation monitoring.

As previously described the energy status of the cell is dependent on the total adenylate charge which is partially dependent on the ATP content of the cell in

relation to the total adenylate population. Brookes and Jenkinson (1989) reported that eukaryotic and prokaryotic microorganisms, growing exponentially in vitro, have a mean ATP concentration of about 12 μmol ATP g^{-1} biomass carbon. The Adenylate Energy Charge (AEC) provides an approximate measure of the metabolic energy stored in the adenylate nucleotide pool, being made up of ATP, ADP and AMP and represented by the equation:

$$\text{Adenylate Energy Charge} = \frac{\text{ATP} + (0.5\ \text{ADP})}{\text{ADP} + \text{AMP}} \qquad (3.2)$$

AEC's range from about 0.8 - 0.95 in metabolically active organisms in vitro to about 0.4 when cell death occurs in most organisms

3.1.3.3 ATP Content Of Cells

There is a general consensus of opinion that ATP levels within cells remain constant, hence its use as an index of biomass (Stanley, 1989c). This appears to be an over-simplification. ATP plays a central role not only in the energy status of the cell but also as a regulator of enzyme activity. It is not surprising therefore that the overall internal cellular level of ATP remains fairly constant for a given set of environmental conditions and it is this factor that makes ATP a potentially useful index for microbial biomass. Studies have shown that ATP levels change during cell division and during the growth cycle of a culture. The former situation is not encountered in routine work because samples consist of a large number of microbial cells dividing in an asynchronous fashion and an "average" is always assayed. Changes in nutritional status can also effect ATP levels and a culture deprived of oxygen (eg during centrifugation or filtration), will suffer a rather rapid depletion of cellular ATP. This may be rapidly relieved (minutes) by resuplying oxygen or in some circumstances glucose. (Stanley, 1989b)

It therefore appears that ATP assaying is only an effective measure of biomass as long as the content remains unchanged. Factors that effect the ATP levels per unit cell: (Stanley, 1986)

- Age of cells or stage of growth
- Stage of cell division
- Concentration of cells
- Phage, virus and microbial infection
- Action of agents that could change the cell type eg tumour promoting agents
- Antibiotics
- Disinfectants, pesticides and herbicides
- Heavy metals
- Metabolic inhibitors, congeners of metabolites and toxins
- Radiation eg UV, microwave, X and gamma rays

Stanley (1986) also lists a number of environmental changes that are known to affect the levels of intracellular ATP per cell:

- Change of growth rate
- Change of nutrient(s) or their concentration.
- Change of gaseous environment eg DOT
- Change of temperature
- Change of pH
- Change of pressure
- Change of light flux (photosynthetic organisms)

In most situations it is not necessary to convert ATP back to colony forming units (CFU's). The value reported can be in ATP units or instrument readings. In such situations there is nothing special about having results expressed as CFU's. An average ATP content for a bacterial cell is 10^{-15} g ATP $cell^{-1}$, yeast and mammalian cells have an average content of 10^{-12}. (Stanley, 1989a)

Fluctuations In Cellular ATP Content

Work carried out by Jirku (1989) examined the changes in adenylate pool size, energy charge values, and the whole sum of nucleotide concentrations of free and covalently immobilised *Saccharomyces cerevisiae*. Free and immobilised cells were

starved by shaking in citrate-phosphate buffer. During the 6 days of starvation the ATP, ADP, and AMP levels of free cells remained constant. More prolonged starvation produced a gradual decrease of ATP and an increase of ADP and AMP levels. In the immobilised cells the ATP, ADP and AMP levels remained constant for 11 days after which the levels abruptly changed. The EC values showed that the biosynthetic capacity and viability of the immobilised cells remained. The ATP/ADP ratio decreased, showing an increase in catabolic activity. The results indicated that immobilisation leads to a prolonged stabilisation of cell energy status of starving yeast's. The more efficient maintenance of cell energetics is probably due to a specific physiological state of cells immobilised by this procedure.

Analysis of the ATP content of the fungus *Trichoderma reesei* was carried out on batch-grown cultures by Gaunt *et al.*. (1985). The ratio of ATP/ unit weight was found to remain constant during exponential growth.

It is apparent that some variation in intracellular ATP occurs. In work described by van Schie *et al.* (1991), cells of *A. calcoaceticus* were grown in acetate-limited culture and then starved for 2-5 hours in order to lower the steady state concentration of intracellular ATP from 16 to 2 nmol ATP mg^{-1} dry wt. Addition of glucose resulted in a rapid increase of intracellular ATP to the original maximal level. This approach to ATP analysis could be of use in quantifying cells that have varying intracellular ATP concentrations by raising the ATP in all the cells in the sample to a maximum level. To determine viability of a cell sample after processing, raise to maximum ATP levels both control and processed cell samples. The difference between the two peak values is the proportional loss of viability.

3.1.3.4 Assay Techniques

There are a number of different methods of testing for ATP:

- Radiolabelling
- Fluorescence labelling
- Chemiluminescence
- Bioluminescence

Radiolabelling

Classic methods for the determination of metabolites have usually depended on the use of radioisotopes to label target analytes and their subsequent detection by microautoradiography. Such methods have been shown to be rapid and have great sensitivity. A method of ATP determination is described by Gonzalez and Garcia-Sancho (1980), using a radioenzymic assay based on the phosphorylation of radioactively labelled adenine sugar in the presence of the appropriate kinase. The labelled sugar-phosphate is then separated from the unreacted sugar using an ion exchange resin. This method has been shown to be extremely sensitive with detection in the range 10^{-12} to 10^{-14} mol ATP. The authors claim that accuracy could be increased using a radioactive sugar of higher specific activity.

Fluorescence labelling

A number of other methods are currently available for ATP determination. There are fluorimetric methods based on the coupling to the reduction of the pyridine nucleotides. Photoluminescence measuring procedures in the presence of the firefly and bacterial luciferin-luciferase system have also been well reported.

Chemi- and Bioluminescence

The trend away from isotopic labelling of proteins and other molecules, particularly for kits, is increasing and considerable interest is currently being shown in bioluminescence and fluorescence labelling. In addition to the health risks associated with the day to day use of radioisotopes there are the staff training and radioactive waste-disposal problems as well as the isotope half-lives which limit the useful shelf life of diagnostic kits. (Robinson, 1991) Chemiluminescence (CL) is the emission of radiation, usually visible or near infra-red, caused by the decay of a chemical reaction product from an exited state to ground state. Bioluminescence (BL) is the CL produced by a wide range of organisms. Although known for several hundred

years, only in the past two decades has there been analytical interest in luminescent reactions. Chemiluminescence coupled in assays with enzymatic or immunological methods allow the reactions to be adapted to detect and quantify a variety of analytes with great precision.

The most successful application of bioluminescence has been its use for ATP assay using the firefly luciferin-luciferase system. Under optimal conditions light intensity is proportional to ATP concentrations in the region of $5x10^{-14}$ to 10^{-6} molar. However contrary to the situation encountered in spectrophotometry and other photometric methods, the light emitted by bio- and chemiluminescent reactions depends not on the concentration of the substrates but also on their reaction rate. This further implies a more stringent control of the assay conditions, a situation further complicated by the fact that bioluminescence enzymes usually show a limited stability and a high sensitivity to various sample components, and that the emitted luminescence may more or less rapidly in the course of time. (Schram & van Witzenberg, 1989)
Using this reaction, two types of luminescence curve can be obtained. With high concentrations of the luminescent reactants, all the ATP is utilised within a few seconds and a high rapid peak occurs, so called "flash kinetics". With low concentrations of luciferin and luciferase, a continuous signal is produced which eventually diminishes as the ATP is utilised. The flash method offers greater sensitivity over the continuous output system. However, the continuous signal is more convenient and facilitates internal standardisation, and its introduction by Lundin in 1976 was considered to be somewhat of a milestone in ATP methodology (Jago, 1989).

Details of the reaction system are given in Appendix 3.3.

3.1.3.5 Comparison Between Firefly ATP Assay And Conventional Culture Techniques

There are a number of factors to consider when deciding to use a novel assay technique such as bioluminescent ATP assay:

- Sensitivity
- Reliability
- Basis of test
- Suitability
- Presence of toxins

Sensitivity

The detection limit for culture techniques is a single colony forming unit in a sample volume that may be several millilitres of more. The average ATP content of a bacterial cell is in the region of 10^{-15} g $cell^{-1}$. With the presently available commercial firefly reagents the detection limit is approximately 10 bacterial cells. (Lundin, 1989) When making comparison with conventional culture techniques it should be realised that a colony forming unit may in some samples derive from clusters of tens or hundreds of cells in the original sample. Furthermore new production procedures may result in considerably more active firefly reagents at reasonable prices. Improvements in luminometer sensitivity are also envisaged but at higher costs. Thus in routine microbiology detection limits of the order 100 to 1000 colony forming units per mL are certainly possible from future luminescence units.

Reliability

A comparison between the reliability of the firefly assay for microbial ATP and conventional culture techniques is rather difficult. Firefly luciferase is almost entirely specific for ATP and light emission from other naturally occurring nucleotides can be neglected. As with any other enzymatic reaction, both the rate of the reaction and the intensity of the light produced can be affected by various substances. Turbidity or light absorbance in samples may also reduce the measured light intensity. However these problems are easily overcome by the addition of a known amount of ATP standard in each assay. Thus the firefly assay in itself is highly accurate. However the firefly assay of microbial ATP also involves the pre treatment to remove non-microbial ATP, followed by an extraction of the microbial

ATP. These two steps are critical with respect to the overall accuracy of the technique and will be considered in detail below.

Basis of test

The microbial ATP content ideally reflects the total intracellular volume of the cells, while culture techniques count the number of colony forming units. Both techniques are affected by substances in the sample eg antibiotics. Neither techniques count the number of cells in the sample. Microbial cells adhere to each other, to other cells and to other particles, particularly in complex clinical or environmental samples. Thus conventional culture may give a considerable underestimation of the number of cells in samples. One of the benefits of colony counting is the fact that micro organisms can often be identified directly on the agar plate the culture technique has definite advantages. The ATP assay on the other hand would be a method of choice in analytical situations where appropriate culture conditions for unknown organisms are not understood or cannot easily be obtained.

Suitability

Bioluminescent ATP assays provide a rapid and highly reproducible means of quantifying the viability of a cell population. In the case of this particular project, which will look into the effects of dewatering systems on bacterial cells, it appears particularly suitable.

For example the effects of oxygen starvation will be studied. The effects of oxygen starvation, both temporary and permanent can be quantified. ATP levels show decline in a matter of minutes when aerobic bacteria are starved of oxygen this is initially a temporary state of cell stress and subsequently a cause of cell death.

Presence of toxins

ATP assays have been used to quantify the effects of varying dosages of antibiotics that cause cell membrane damage, by measuring the sudden leakage of intracellular ATP into the culture medium. This is analogous to measuring the effects of impact damage, eg in a centrifugation system. The usual method of quantifying the effects of a disruption system eg an industrial homogeniser is by the assay of intracellular metabolites such as marker compounds (eg glucose-6-phosphate) or total soluble protein. These methods will be discussed in detail later on. The bioluminescent ATP

assay is particularly useful in this application as it has the ability to distinguish between intra- and extra cellular ATP and can thus not only give indications on the levels of cellular disruption but also the levels of stress on any remaining intact cells.

There is another useful aspect of this test. It would be useful to determine the effects of oxygen starvation on a population of aerobic bacteria. Cells would be harvested from the fermenter and their ATP levels tested before they were processed (Sample 1). Another sample of cells would be taken at this time held unagitated and unaerated at an identical temperature to the test culture (Sample 2). When the cells had been processed they would be retested (Sample 3). Any differences between Samples 1 and 2 can be considered to be due to the effects of oxygen starvation. Protein assay measurements would be unable to measure any change as no mechanical stress has lead to cell breakage and viability tests would take a number of days.

In summary the bioluminescent assay of ATP appears to have many advantages over conventional assay techniques, the main ones being improved accuracy and reduced assay time. Assays are available which can distinguish between intra- and extracellular ATP which will allow measurement of cellular disruption. The determination of non-lysed cells is generally carried out by plate counting, which takes days to complete. ATP testing gives a rapid indication of the metabolic rate of the viable cells in a population.

3.1.4 Cell Disruption Techniques For Analytical Development

Cell disruption is believed to be one of the major causes of loss of cell viability when cells are subjected to downstream processing stresses. The previous sections have reviewed analytical techniques that could be used to test for loss of cell viability and damage to a population of cells after downstream processing.

When developing analytical techniques it is wasteful in terms of time and resources to carry out testing using pilot scale equipment. It would therefore be preferable to use a laboratory based device that simulates the type of stresses that cells would encounter during processing. The rapid pumping action of a high pressure homogeniser can be considered to be similar to the sediment discharging stage of disc stack centrifugation, which is the step believed to cause the majority of cell damage.

The resistance to mechanical stress of different cell types shows a high degree of variation. The cell wall is responsible for strength, rigidity and shape and is the major barrier to release of intracellular proteins. Compositional variation in the cell wall are responsible. Bacterial cells are surrounded by a cell wall, which owes its strength and rigidity to peptidoglycan. In gram-positive bacteria the major component of the cell wall is peptidoglycan (40-90%) together with teichoic acids, teichuronic acids as well as other carbohydrates. This layer is 20-50 nm thick depending on the species and growth conditions. Gram-negative bacteria have a peptidoglycan layer 2-3 nm thick which is surrounded by a lipopolysaccharide layer containing proteins and lipids.

The following table shows the mechanical strength of different cells when processed by three different types of disruption device.

Table 3.2 Resistance of cell types to mechanical stress

(Salusbury, 1989)

Cell type	Sonication	Agitation	liquid extrusion
Animal cells	7	7	7
Plant cells	7	7	7
Gram-negative bacteria	6	5	6
Gram-positive bacilli	5	4	5
Yeast	3	3	4
Gram-positive cocci	3	2	3
Spores	2	1	2
Mycelia	1	6	1

7 very susceptible, 1 very resistant

3.1.4.1 Homogenisation

The advent of recombinant DNA technology and the overproduction of proteins as inclusion bodies has lead to increased interest in efficient and cost effective cell rupture. The disruption of yeast by high pressure homogeniser is well documented (Hetherington *et al.*, 1971 and Keshavarz-Moore *et al.*, 1987 & 1990). Less information is to be found concerning the disruption of bacteria Homogenisation is considered to be one of the fastest and most effective methods of cell rupture.

Homogenisers consist of reciprocating positive - displacement piston type pump which incorporate an adjustable restricted orifice discharge valve. The cell suspension is forced through the valve at high pressure and low velocity, it collides with the valve face and is directed away at right angles, with a resultant rapid increase in flow velocity and shear.

Factors Affecting Homogenisation

Harrison *et al.* (1990) report on the homogenisation of a gram negative bacteria *Alcaligenes eutrpohus.* Parameters investigated were:

Energy input soluble protein was detected after homogenisation at 100 bar, DNA at 300 bar and disruption by phase contrast microscopy at 200 bar. It was shown that that the energy input required for cell rupture by homogenisation was a function of pressure, the number of passes and the input broth temperature.

Pressure Cell rupture was detected in the pressure range 50-600 bar.

Number of passes Maximum protein release was achieved after two passes at 620 bar and maximum DNA release was achieved after three passes. Hetherington *et al.* (1971)devised the following expression:

$$\ln\left(\frac{R_{max}}{R_{max}-R}\right) = kNP^{a} \qquad (3.3)$$

R_{max} maximum total soluble protein release (mg g^{-1})
R soluble protein release (mg g^{-1})
k rate constant
N number of passes
P operating pressure
a pressure exponent

Temperature The temperature of the culture broth affected its disruption characteristics. Cell rupture increased at higher temperatures, by one and a half times when the temperature was increased from 13.5 to 26°C. During homogenisation broth temperature increases as a linear function of operating pressure, and is independent of initial broth temperature.

Cell concentration Cell suspensions within the biomass concentration of 9-260 g dry wt L^{-1} showed no difference in rupture characteristics

when homogenised, although the viscosity of the ruptured broths varies.

Cell pretreatment The strength of the cell wall is a very important factor in the efficiency of mechanical rupture. Incubation with detergents lead to increased disruption.

Elevated temperature incubation at 45°C for one hour results in protein release of 17% of R_{max}. After homogenisation at 620 bar incubated samples showed a 1.8 fold increase over control cultures.

Alkaline conditions. Within 30 seconds of incubation at 8°C and pH 10 30% disruption occurs. 1% sodium dodecyl sulphate (SDS) incubation for 20 minutes resulted in total cell disruption after a single pass at 350 bar

Sodium chloride. The addition of 8% sodium chloride to the cell broth showed a disruption of 20% after an incubation period of one hour.

Fermentation conditions E. coli grown on a defined medium was found to be easier to disrupt than cultures grown on a complex medium. Cultures grown at high specific growth rates were easier to disrupt than those grown at low rates and log phase cells were easier to disrupt than stationary phase cells (Keshavarz-Moore *et al.*, 1987)

The main disruption mechanism is impingement and the rate of cell breakage relates to the stagnation pressure or maximum wall stress of the fluid jet. (Keshavarz-Moore *et al.*, 1990) Decreased valve gap width, as estimated from the flow conditions in the valve, and decreased impact distance, both increase the cell disruption rate. Knife-edge geometries yield better disruption than flat-edge configurations. Disruption valves can be constructed from ceramic composite material which give greater resistance to wear and extend the lifetime for industrial use.

3.1.4.2 Other Methods Of Cell Disruption

Other methods of cell disruption are:

- Bead Mills
- Chemical permeabilisation
- Osmotic shock
- Double cone
- Capillary flow
- BioNebulizer
- Ultrasound
- Electrical pulsing

Bead Mills

These devices consist of either a vertical or horizontal grinding chamber containing rotating discs or impellers mounted concentrically or off-centered on a motor-driven shift. The grinding action is provided by beads typically occupying 80-85% of the free working volume of the chamber

Chemical Permeabilisation

SDS this is a commonly used technique in molecular biology. Disruption is achieved by the dissociation of the outer membrane, protein denaturation and removal of the lipopolysaccharide. (Hettwer and Wang, 1985, 1986)

Lysozyme breaks down peptidoglycan. If used in an isotonic medium will remove outer cell wall and release the contents of the periplasmic space (between the cell wall and the plasma membrane). Gram-negative bacteria are less susceptible to lysozyme due to the lipopolysaccharide layer, which can be destabilised by the presence of calcium-chelating agents eg EDTA.

Osmotic Shock

Sodium chloride is believed to cause disruption by altering the size of the cell.

Double Cone (Shimizu *et al.*, 1992)
This apparatus consists of two cones an inner rotating cone separated from a stationary depressed outer cone by the test liquid.

Three types of cells were tested using this apparatus, *S. cerevisiae, E. coli and B. subtilis*. A threshold value of breakage was recorded for each type of cell. This corresponded to the shear stress at which release of intracellular contents could be detected.

The paper concluded that excessive shear stress broke the bacterial cells and induced the release of sugar, protein and ATP into the culture medium. Bacterial viability was shown to remain unchanged for 30 hours when cells were subjected to shear stresses below the threshold values.

Capillary Flow
The viability of *Pseudomonas putida* cells after exposure to low shear stresses was investigated by forcing them through 0.5 mm x 1.1 m capillary tubes at 10 to 30 bar (Svetlichnyi *et al.*, 1991). It is claimed that cells in the population are heterologous in their sensitivity to shear stresses and they can be divided into two fractions. This work is important to the study of cross flow membrane filtration as low shear rates are studied such as those measured in a cross flow membrane rig. (10^{-3}-$10^{-5}$$sec^{-1}$). At these stresses it has been shown that bacterial inactivation can be measured without explicit damage to the cell wall, which occur at rates of $10^{8}sec^{-1}$) Shear velocities such as these are virtually unobtainable in membrane devices. Cell inactivation was found to be due to accumulation of structural damage to the cell membrane.

The BioNebulizer
This is a newly reported apparatus for breaking cells and large molecules from a variety of sources. (Togasaki *et al.*, 1991) Shear forces created by laminar flow in microcapillary channels are responsible for cell and molecule breakage. Such channels can be created by nebulization or microdroplet formation of a cell suspension, or other molecular solution, under gas pressure. The channel size can be regulated by adjusting the gas pressure. Nebulization is rapid (180 mL min^{-1}) and h the apparatus does not generate heat. 90% of *Chlamydomonas reinhardtii* cells were

broken in the test. The BioNebulizer is adjustable for many cell lysis conditions, and was shown to break cells of *Escherichia coli, Saccharomyces cerevisiae, C. reinhardtii, Chlorella pyrenoidosa, Asparagus officinalis,* and soybean tissue culture.

Ultrasound

A method for disrupting cells involves subjecting the cells to ultrasonic energy in the presence of beads is reported in a patent from Gen-Probe (1986). The beads can be formed from glass, plastic, latex, crystals, metals, metal oxides, sand or silicates and have diameters of 0.05-1 mm. The ultrasonic energy is delivered to the cells from an ultrasound cleaning batch with a power density of less than 0.2 W mL^{-1}. Prior to delivery of the ultrasonic energy, the cells or microorganisms can be suspended or placed in solution. The cells release their constituents, including RNA and DNA, into solution within min. The nucleic acids can be readily detected by hybridisation techniques and are not destroyed. Cell breakage occurs easily with all microorganisms including mycobacteria.

Electrical pulsing

A system for the selective lysis of cells in solution is described in a patent by Akira-Mizuno (1987). The application of short duration, high voltage pulses to the cell suspension, instantaneously creates a high voltage electric field. The unit consists of a high voltage and an earth electrode within an insulating case. By altering the wave amplitude and pulse time the selective lysis of cells can be carried out. (Salusbury, 1989)

3.1.4.3 Most Suitable Disruption System

A system for the disruption of cells is required. Disrupted and partially disrupted cell samples are to be used for the development of a test system for assessing the damage to viable cells after cell suspension concentration processing. The use of the laboratory-scale high pressure homogeniser appears to be the best solution to this problem. The Biochemical Engineering Department at University College London and APV-Gaulin have developed such a device, called the Micron Lab 40, which appears suitable for the following reasons:

- Sample volume
- Operating range
- Ease of operation
- Reproducibility
- Background

Sample volume
Laboratory scale and small, 20-40 mL. Small scale cell production required.

Operating range
The device operates at a wide range of pressures from 100-1600 bar. This will allow different proportions of broken and intact cell samples to be obtained from the same batch.

Ease of operation
The homogeniser is extremely simple to use and operator familiarisation time is short.

Reproducibility
Has been demonstrated by Sayed (1995) in *P. putida* ML2 disruption trials.

Background
The operation of homogenisers is well defined and documented. (Hetherington *et al.*, 1971, Harrison *et al.*, 1990, Keshavarz-Moore *et al.*, 1987, 1990)

3.1.5 Summary Of Analytical Development

A reliable production system of cells has been developed. Before testing of downstream processing can begin a methodology for the testing of damage has to be devised.

Cells will be produced and then subjected to stresses at laboratory scale. These stresses will be similar to those that they would encounter during industrial centrifugation and microfiltration processing. The APV-Gaulin Micron Lab 40 homogeniser appears to be the most suitable mimic of downstream processing stresses at laboratory scale. This approach will reduce costs of large scale cell production and the operation of pilot scale equipment as well as being more reproducible.

The standard plate count and Bradford total soluble protein assay will be used to assess levels of cell damage. This will be carried out over a range of operating pressures, which will allow different cell samples to contain varying proportions of intact to broken cells. A novel viability test, the bioluminescent assay of ATP, will also be investigated alongside these established techniques with a view to replacing one or both of them.

3.2 Materials And Methods

3.2.1 Homogenisation

The homogeniser used during the course of this work was the Micron Lab 40 (APV-Gaulin GmbH., Lubeck, Germany). The device consists of a high pressure reciprocating positive displacement pump with a restricted orifice valve. Cell suspension is drawn throuh a check valve into the pump cylinder and on the return of the piston it is forced through the discharge valve. The discharge pressure is controlled using a spring loaded valve rod which positions the valve in relation to the valve seat. During discharge the suspension passes between the valve and its seat and impinges on an impact ring. The unit is fitted with a knife edge cell disruption configuration. (Keshavarz-Moore *et al.*, 1989)

Sample volume is 20-40 mL. The homogeniser operates in batch mode over a range of pressures ranging from 100-1600 bar and is adjustable in units of 10 bar. A glycol cooling jacket has been added around the disruption chamber and product bowl. This allows cooling of the samples. A temperature increase of 1°C 100 bar^{-1} $pass^{-1}$ was demonstrated by Sayed (1995).

3.2.2 Analytical Assay Techniques

3.2.2.1 Optical Density

Cell samples were diluted with phosphate buffer made to the same concentration as the fermenter medium. Optical density readings were made against phosphate buffer blank at 670 nm, using a UV2 spectrophotometer (ATI Unicam, Cambridge, UK). Samples were diluted so that readings were within the range 0.3-0.5 in accordance to the Beer-Lambert law. At least three repeats were carried out per sample.

3.2.2.2 Dry Weights

The dry weights of samples were calculated by spinning down cells, removing the supernatant and drying the solids. Labelled 1.5 mL micro-centrifuge tubes were dried overnight in a Townson & Mercer (Croydon, UK) vacuum oven at 50°C and 30 mmHg and then weighed to one thousandth of a milligram using a Mettler AE166

digital balance (Mettler-Toledo, Zurich, Switzerland). 1 mL cell samples were pipetted into the tubes which were then spun in an Sigma 113 microcentrifuge (Sigma GmbH., Funkenstorgrad, Germany) at 10000g for fifteen minutes. The supernatant was removed and the tubes were dried in the oven for for at least 24 hours before reweighing. Six repeats were carried out for each sample.

Dry weight measurements were used to normalise readings from other assays to allow comparisons of samples at different cell concentrations.

3.2.2.3 ATP Assay

ATP assaying was carried out using a commercially available test kit supplied by Bio Orbit (Labsystems, Basingstoke, UK). In order to assay a sample of cells for intracellular ATP a total and an extracellar assay are required.

The assay is based on the luciferin-luciferase firefly extract method. The detection is based on the chemical release of ATP from the cell sample and its detection by a light producing reaction catalysed by firefly luciferase. Full details of the assay technique are given in Appendix 3.4.

3.2.2.4 Viable Cell Counts

The viable cell count of cell samples was determined by the plate count method. 1 mL of cell suspension was serially diluted in 9 mL of phoshate buffer at the same concentration as the fermenter medium. 0.1mL dilutions were plated onto petri dishes containing 25 mL of 2.8% w/v nutrient agar (Oxoid, Unipath Ltd., Basingstoke, UK). In order to increase the sensitivity of the test certain optimisation tests were made throughout the period of this work. A large number of duplicates were made. Three sets of dilutions were carried out from each sample. From each of these dilution series three plates were made at each of the estimated target dilutions. Hence nine plates for any dilution were made.

Plates were incubated at 30°C for thirty six hours and then colonies were counted on plates containing between 30-300 colonies.

Statistical analysis was applied in order to validate the measured data. Of particular concern was the large spread of results for a single sample.

Personal correspondence within the department suggested that application of the f-test would be the most suitable statistical regime to apply to the data. The *f-test* was applied in the following way:

- Standard deviations of the plate count values were calculated.
- A multiplication factor was obtained from *f-test* tables. This allowed presentation of data to a confidence limit of 95%, which is acceptable for this type of work. The value of the multiplication factor is obtained from the 95% confidence column for a value of n-1, where n is the number of samples.
- An acceptable range of colonies was calculated for each sample. This was obtained by both addition and subtraction of the *f-test* multiplication factor to the mean value of colonies for the sample.
- Any values falling outside this range were considered to be unacceptable. These were removed from the data.
- The analysis would be applied to the remaining data and another range would be calculated. Unacceptable data points would be removed.
- The test would be reapplied *ad infinitum* until all the remaining data points fell within the calculated range.

Viable cell counts of processed cell samples were expressed as a percentage of the unprocessed value.

3.2.2.5 Protein Assay

The Bradford Protein Assay (Bio-Rad Ltd., Hemel Hempstead, UK) was used to test for extracellular total soluble protein. Cell samples were spun down in micro-centrifuge tubes for ten minutes. 50 μL of supernatant was added to 1.5 mL of diluted assay reagent. The absorbance of the mixture at 595 nm was measured using an UV2 spectrophotometer (ATI Unicam, Cambridge, UK) after five minutes of incubation.

Protein release data was expressed as percentage of the original (unprocessed) total intracellular protein value. This control value was obtained by completely disrupting an unprocessed cell sample by a single 1600 bar pass through the homogeniser,

spinning down and testing the supernatant for protein. This gave a total protein value. Further 1600 bar homogenisation passes resulted in no more protein release and therefore maximum disruption was assumed. The background value was calculated by testing the supernatant from an identical unprocessed sample. The intracellular protein value of this control sample was calculated by subtracting the total (completely disrupted sample) from the background (unprocessed sample).

Samples were taken from the viable cell count dilution bottles, four measurements for each of the three dilutions giving a total of 12 readings per sample. Statistical analysis was then applied to the raw data in an identical way to the viable cell count data.

3.2.2.6 Viscosities

Viscosity measurements were made using a couette viscometer (Contraves Rheomat 115, Contraves AG, Zurich, Switzerland), operated according to the manufacturers instructions.

3.2.2.7 Biotransformation Ability (BTA)

The capacity of the *P. putida* ML2 cells to oxidise flurobenzene to fluorocatechol was known as biotransformation ability. This was monitored during one of the microfiltration runs (SHPP52). The capacity of a unit mass of cells to carry out the conversion was measured by the method of Lynch (1994). This involved the reaction taking place in shake flasks and measurement of substrate and product by gas chromatography. The mass of fluorocatechol produced per unit dry weight of cells was expressed as a percentage of the original unprocessed value.

3.2.3 Analytical Development

3.2.3.1 Fermentation Monitoring

Cell suspension was aseptically sampled at timed intervals during fermentations and measured for intracellular ATP. Results were compared to optical density, viable cell counts, dry weights and on-line gas analysis data.

3.2.3.2 Homogenisation

Growth Phase Cells

Cells were sampled from the fermenter during exponential growth phase and diluted with phosphate buffer. 40 mL of diluted cell suspension was transferred to the feed bowl of the Micron Lab 40 homogeniser.

Operating pressure was varied between 100 and 1600 bar. The cell suspension was passed through the homogeniser and 2 mL sample was withdrawn from the collecting bowl and tested for total and extracellular ATP The remains of the sample were stored on ice for later assaying for protein release and viable cell count. The homogenised cell suspension was then returned to the feed bowl and passed through the homogeniser, 2 mL was removed for sampling. The homogenised cells were then once again returned to the feed bowl for subsequent passes, upto five passes maximum.

Stationary Phase Cells

Analysis of homogenised stationary phase cells was carried out in two ways. The first was by direct sampling of multipass homogenisations and the second was by monitoring ATP levels in processed and unprocessed cells after inoculation into a shake flask.

Direct sampling

Cells were harvested from the fermentation during stationary phase and processed by the multipass operation of the Micron Lab 40 homogeniser at different operating pressures. Sampling and operation of the homogeniser was carried out in an identical fashion to that described for growth phase cells.

Activation of cells

Cells were processed by a single pass through the Micron Lab 40 homogeniser at a number of different operating pressures. 5 mL of cells were used to inoculate a 500 mL shake flask containing 100 mL of fermentation inoculum shake flask medium (SF2) containing 30 g L^{-1} of glucose instead of fructose. The shake flasks were agitated in a rotary shaker-incubator at 30°C and 200 rpm and then sampled at regular intervals and assayed for ATP. The rate of increase and the final ATP levels of unprocessed cell samples were compared to an unprocessed control culture. Cell samples were also tested for viable cell count and protein release as described above.

3.3 Results

This results section is split into a number of sub-sections. In order to avoid expressing cell damage in terms of intracellular content release and viable cell counts the bioluminescent ATP assay was tested as a method for quantifying viable cells. Results are presented in chronological order and demonstrate how an assay test system was developed for the quantification of cell damage.

ATP standard curve Section 3.3.1 Figure 3.1

The sensitivity of the ATP assay equipment was tested. A standard curve of luminometer response to a series of serially diluted ATP standards tested using a commercially available ATP test kit.

Fermentation monitoring Section 3.3.2 Figures 3.2-3.5

In order to test the ATP assay approach as a suitable method for quantifying viable cell concentration a number of fermentations were monitored. ATP concentration was compared to cell concentration, as measured by dry weights and optical density. Initial monitoring of fermentations in their growth phases showed the test to be reliable. Fermentation monitoring was extended from pre-growth phase to late stationary phase. ATP concentration was compared to carbon dioxide evolution rate and viable cell counts.

Homogenisation Section 3.3.3

Multiple pass homogenisation Figures 3.6-3.13

Homogenisation was chosen as a method of providing damaged cell populations in a controllable fashion. Cells were harvested from fermentations at various stages of growth and subjected to multiple pass homogenisation at a number of different pressures. Varying the operating pressure provided a reproducible method of altering the proportions of the intact and disrupted sections of cell population.control

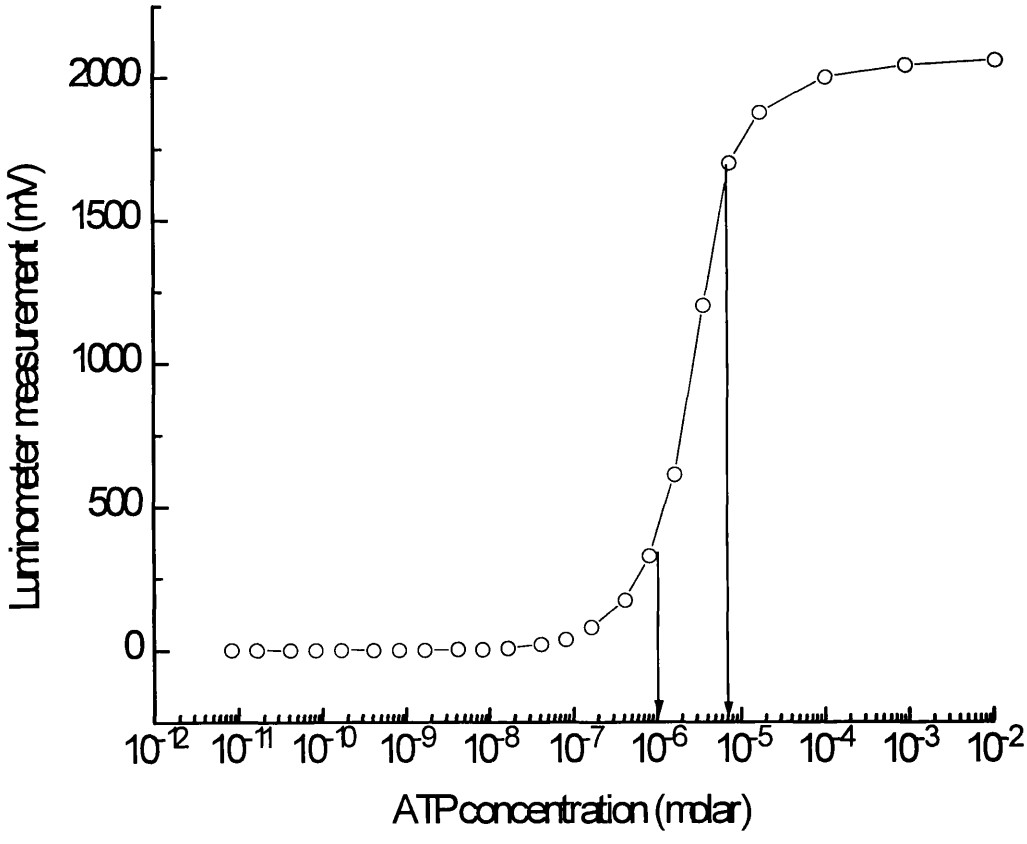
Single pass homogenisation Figures 3.14-3.16

ATP assaying proved to be unreliable in assessing cell damage caused by multiple pass homogenisation. As an alternative to directly assaying cells after homogenisation stationary phase cells were revived by use as inoculum in shake flasks. ATP levels were monitored until they rose to a maximum. This maximum level was compared to that from an unprocessed sample.

3.3.1 ATP Standard Curve

In order to establish the sensitivity and working range of the ATP test system a standard curve of emitted light (luminometer response) was plotted against ATP concentration. Serial dilutions of ATP standard were made up in the range of 10^{-12} to 10^{-2} M. The dilutions were assayed using a Bio-Orbit test kit as described in Appendix 3.4. The response of the luminometer for each dilution was recorded and plotted against concentration.

Fig 3.1 **ATP standard curve**
Luminometer readings (mV) vs ATP concentration (molar)



3.3.2 Fermentation Monitoring

While a reproducible batch fermentation was being developed for *P. putida* ML2 fermentations were monitored for a number of different parameters. Physical parameters such as optical density and dry cell weight were compared to on-line gas analysis, viable cell counts and total intracellular ATP concentration.

Two types of *P. putida* ML2 fermentations were monitored, production batch fermentations and extended batch fermentations. Production batch fermentations were runs that were carried out in order to provide cells for the purpose of analtytical development, fermentation monitoring and fermentation development. Harvesting was carried out one hour after stationary phase had started.

Extended fermentations were similar to the production runs. The runs were carried out specifically for the purpose of monitoring and were generally carried out in collaboration with another researcher from within the department. In addition to the parameters mentioned above the on-line capacitance of the broth was measured using a Biomass Probe (Aber Instruments,Aberystwyth, UK). Unfortunately the probe was still in a developmental stage and no results could be obtained during this stage of the work.

Production batch fementations

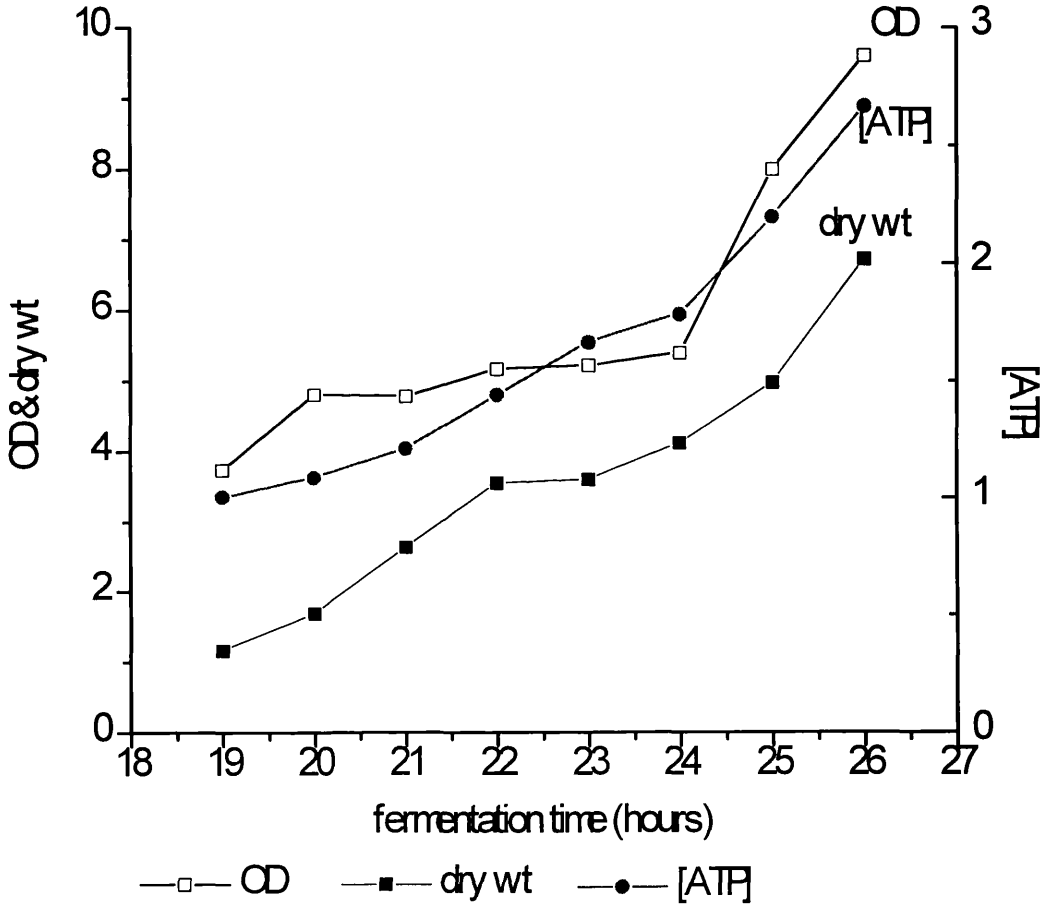
In order to test the ATP assay over a range of cell concentrations and different stages of growth a number of fermentations were monitored. Initially ATP concentration was only monitored during exponential growth phase.

The following two figures show how intracellular ATP concentration of the broth changes over time and how this variation relates to changes in cell concentration as measured by optical density and dry weight:

Figure 3.2 Early growth phase monitoring.
OD, dry weight and ATP variation with fermentation time
Fermentation LINK27

Figure 3.3 Mid growth phase monitoring.
OD, dry weight and ATP variation with fermentation time
Fermentation LINK17

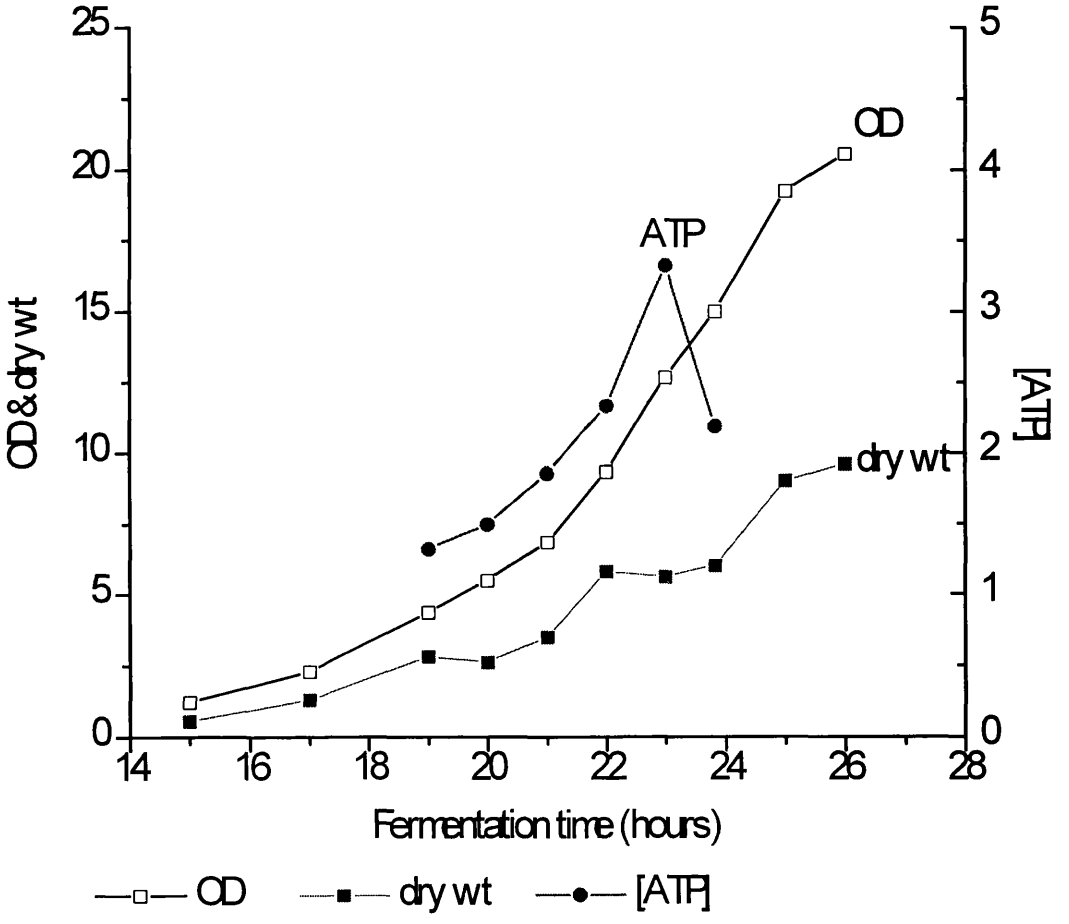
Fig 3.2 **Production batch fermentation monitoring**
Intracellular ATP, OD and dry cell weight variation with time
Early exponential growth phase monitoring



***P. putida* ML2 fermentation, number LINK27**

time	time post-inoculation, hours
OD	optical density measurements at 670 nm
dry wt	cell concentration (dry weight measurements, g dry wt L^{-1})
[ATP]	ATP concentration per unit volume of broth (μM)

Fig 3.3 Production batch fermentation monitoring
Intracellular ATP, OD and dry cell weight variation with time
Mid exponential growth phase monitoring



***P. putida* ML2 fermentation, number LINK 17**

time	time post-inoculation, hours
OD	optical density measurements at 670 nm
dry wt	cell concentration (dry weight measurements, g dry wt L^{-1})
[ATP]	ATP concentration per unit volume of broth (μM)

These figures (3.2 and 3.3) show how cell concentration, as measured by dry weight and optical density, increased exponentially during the growth phase of the fermentations. Intracellular ATP measurements per unit broth volume were also shown to increase exponentially in phase with increased cell concentration. Intracellular ATP measurements appear to be a useful measure of cell concentration during exponential growth. The decreased ATP value of the last sample of Fig 3.3 is assumed to be due to a sampling error.

Extended runs

Fermentations were run for 50-60 hours. This allowed pre-growth, growth and approximately twenty hours of stationary phase monitoring to be carried out. Cell concentration measurements were made by optical density readings. Viable cell concentration (VCC) was estimated by plate counts. In addition on-line gas analysis was carried out by a mass spectrometer to give Carbon dioxide Evolution Rate (CER) values. The following figures show the monitoring results for two identical fementations which were run for 60 hours:

Fig 3.4 Monitoring of extended fermentation
OD, CER, VCC and ATP variation with fermentation time
Fermentation SHHPP07

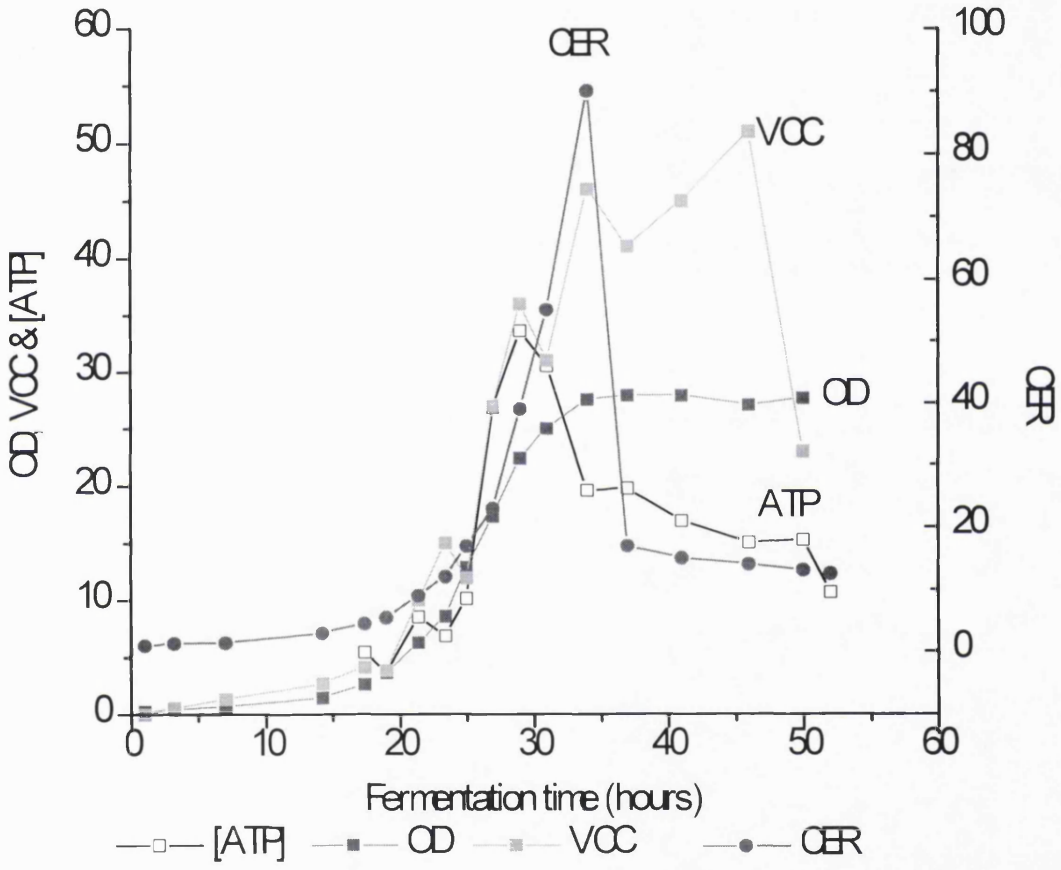
Fig 3.5 Monitoring of extended fermentation
OD, CER, VCC and ATP variation with fermentation time
Fermentation SHHPP08

As shown in the previous two figures a relationship between cell concentration, as measured by optical density, and ATP was seen during pre-growth and growth phases. In addition to these two measurements, viable cell concentration and CER levels were shown to increase in line with OD and ATP.

During stationary phase OD remained constant at its peak value, however CER was shown to drop to approximately 10% of its maximum level, at the peak of growth phase. ATP was shown to follow the CER trend. VCC trends appears unclear during stationary phase.

This suggests that ATP measurements are a reliable estimation of viable cell concentration for growth phase cells. It is not clear if ATP is a good measurement for viable cell concentration during stationary phase.

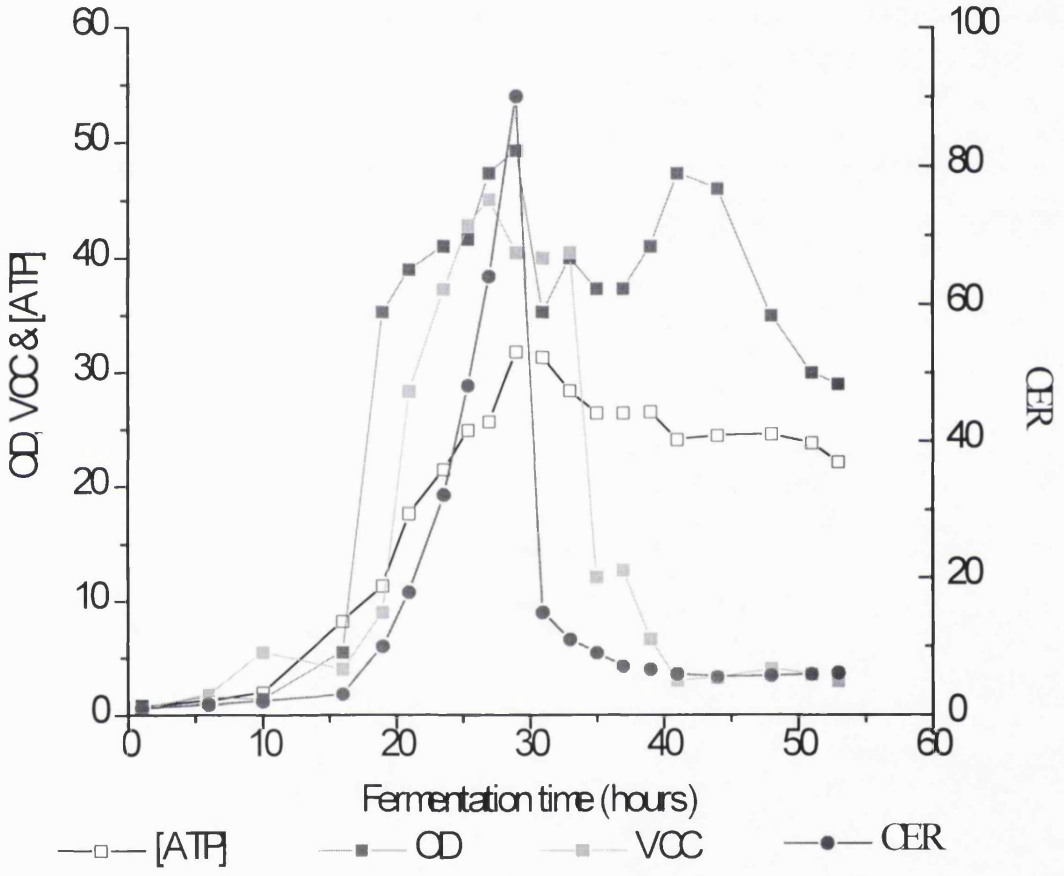
Fig 3.4 **Extended fermentation monitoring**
Intracellular ATP, optical density, viable cell count and carbon dioxide evolution rate variation with time



***P. putida* ML2 fermentation SHPP07**

OD	Optical density measurements at 670 nm
VCC	Viable Cell Counts measured by plate count method (Colonies x 10^9)
[ATP]	ATP per unit broth volume (μM)
CER	Carbon Dioxide Evolution Rate (mmol L^{-1} h^{-1})

Fig 3.5 **Extended fermentation monitoring**
Intracellular ATP, optical density, viable cell count and carbon dioxide evolution rate variation with time



***P. putida* ML2 fermentation SHPP08**

OD	Optical density measurements at 670 nm
VCC	Viable Cell Counts measured by plate count method (Colonies x 10^9)
[ATP]	ATP per unit broth volume (μM)
CER	Carbon Dioxide Evolution Rate (mmol L^{-1} h^{-1})

3.3.3 Homogenisation

A lab scale high pressure homogeniser was used to disrupt cells. Varying the operating pressure of the device allowed the proportion of intact to disrupted cells to be changed.

Intracellular ATP release was measured and compared to total soluble protein release. The following test conditions were varied:

- Growth phase — stationary or exponential growth phase cells were tested
- Mode of operation — multiple pass or single pass
- Sampling method — directly from homogeniser or following revival of intact cells

3.3.3.1 Growth Phase Cells

Cells were harvested from the fermentation in growth phase and processed by multiple pass homogenisation using the Lab 40 homogensier. Comparisons were made between extracellular and intracellular ATP as well as released protein. All values are expressed as percentages. Extra- and Intracellular ATP are expressed as a percentage of the unprocessed ATP value. Protein is the percentage of the original intracellular and is calculated:

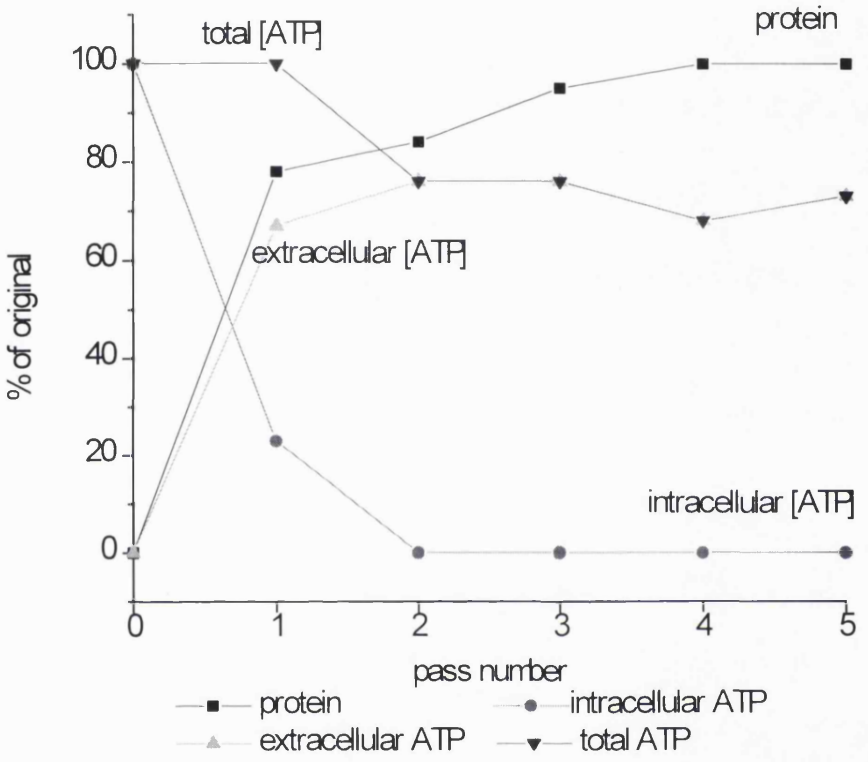
$$\text{Protein} = \frac{(\text{resuspended} - \text{background}) / \text{unit dry weight}}{(\text{maximum} - \text{background}) / \text{unit dry weight}} \times 100$$

Fig 3.7 shows the relationship between number of passes and intracellular content release (protein and ATP).

Figs 3.8 - 3.10 show how the levels of a particular component (protein, intracellular and extracellular ATP) change with different operating pressures.

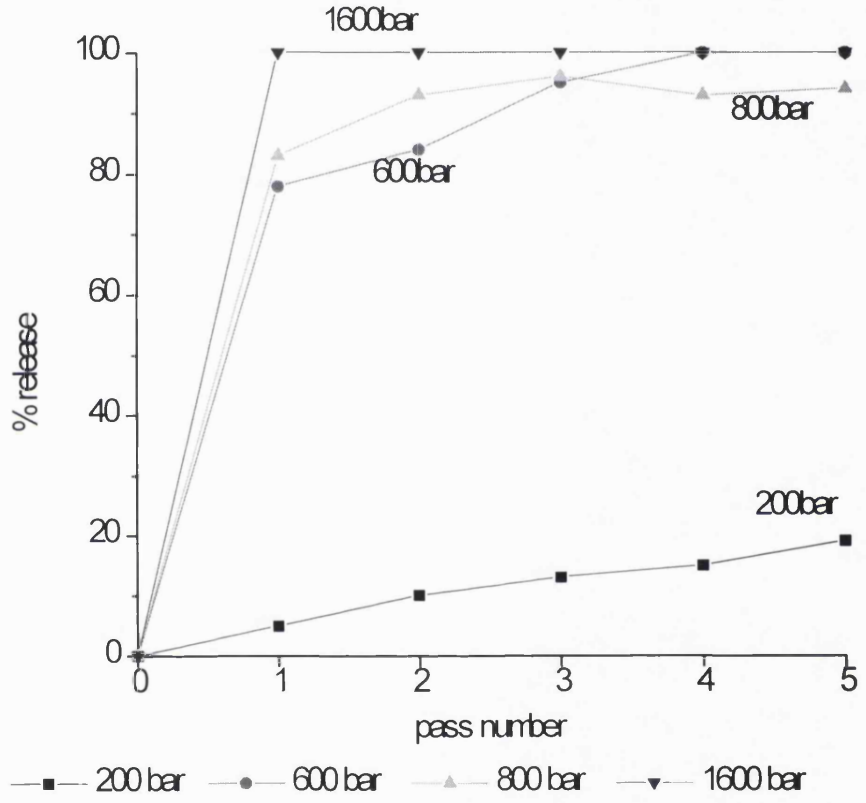
Measured intracellular ATP and protein release were shown to have an inverse relationship.

Fig 3.6 **600 bar multiple pass homogenisation of growth phase cells**
Intracellular content release
Percentage release vs number of passes



protein	protein release as percentage of original intracellular
intracellular ATP	intracellular ATP as percentage of original intracellular
extracellular ATP	extracellular ATP as percentage of original intracellular

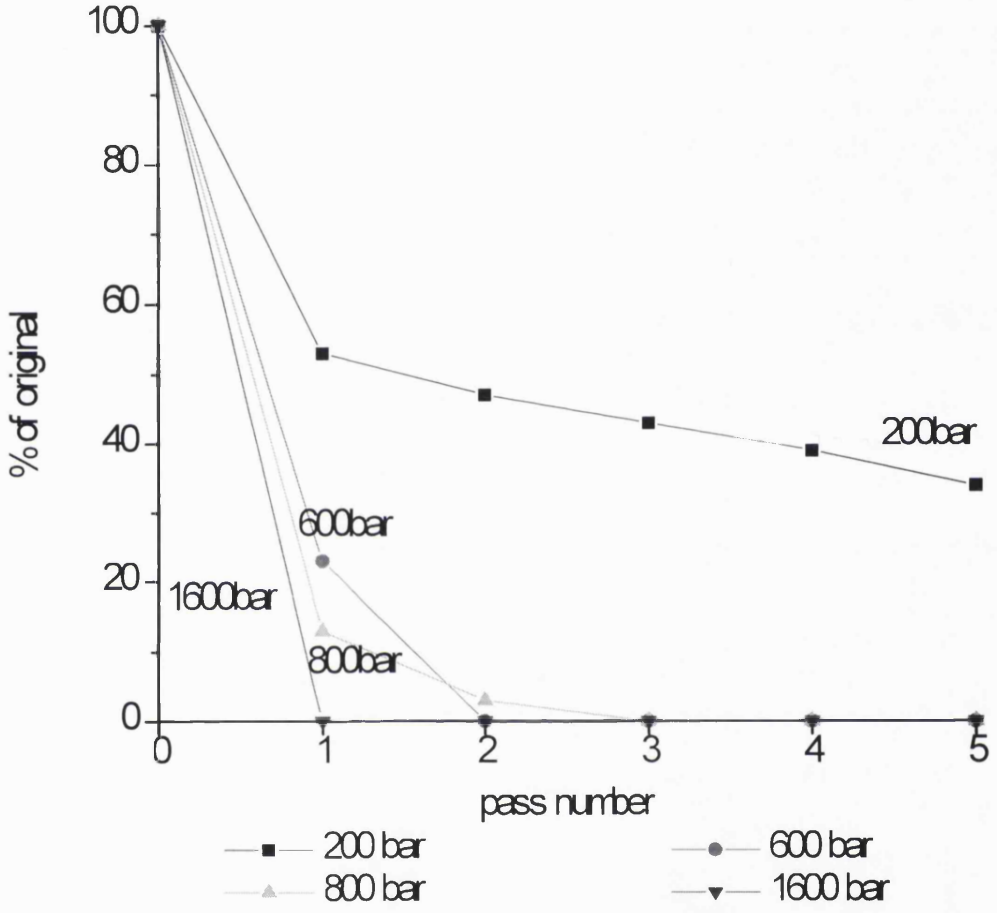
Fig 3.7 **Multiple pass homogenisation**
Percentage protein release vs number of passes
Growth phase cells



Protein release percentage of original intracellular.

Cell disruption was shown to increase with increased operating pressure.

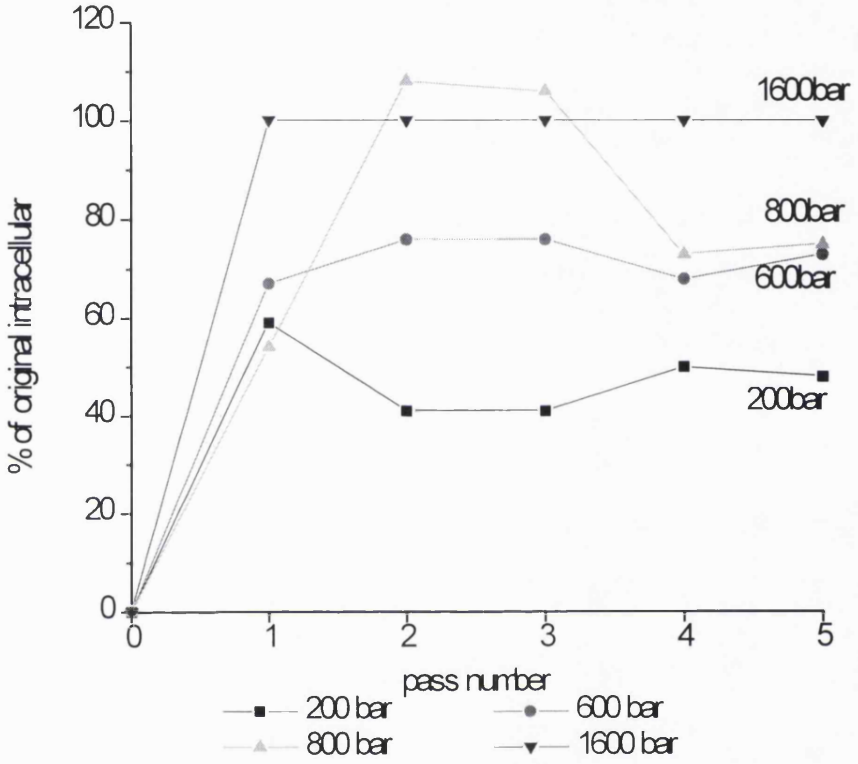
Fig 3.8 **Multiple pass homogenisation**
Intracellular ATP vs number of passes
Growth phase cells



Intracellular ATP as percentage of original

Intracellular ATP falls with increased number of passes with protein release

Fig 3.9 **Multiple pass homogenisation**
Extracellular ATP vs number of passes
Growth phase cells



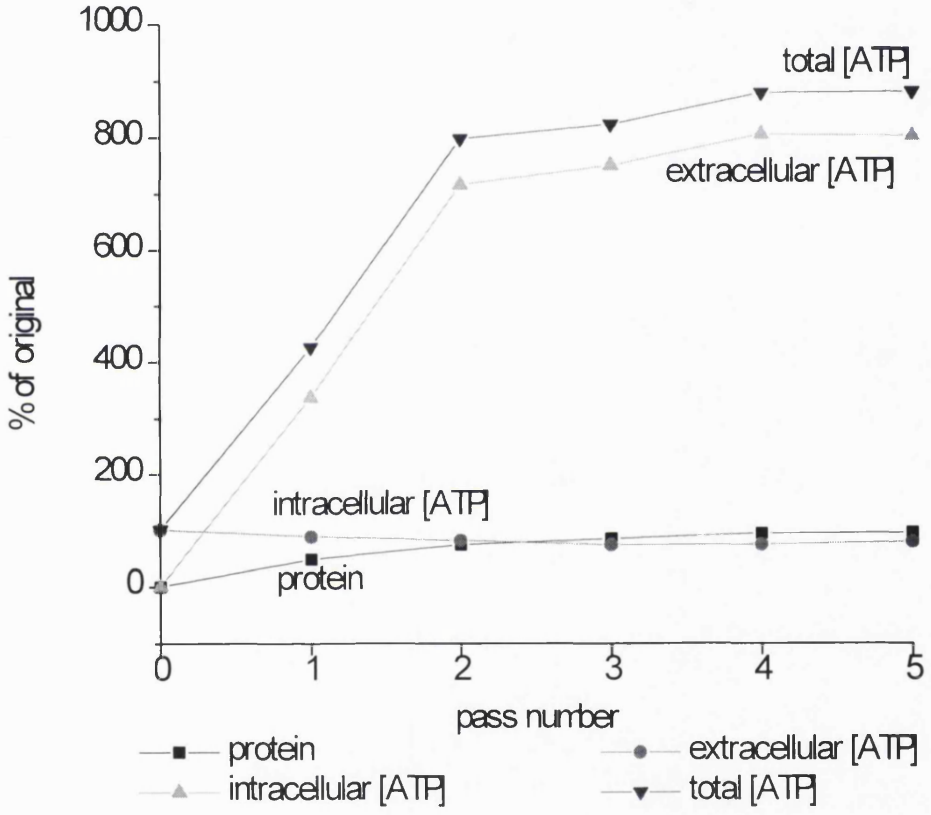
Extracellular ATP as percentage of original intracellular ATP

3.3.3.2 Stationary Phase Cells

3.3.3.2.1 Direct Sampling

Stationary phase cells were processed by multiple pass homogenisation using the Lab 40.

Fig 3.10 500 bar multiple pass homogenisation of stationary phase cells
Intracellular content release
Percentage release vs number of passes



There is no apparent correlation with protein and ATP loss from within the cells. Intact cells after 1 pass show raised levels of intracellular ATP. Final extracellular ATP after five passes was 750% of original intracellular.

Levels of intracellular ATP after one pass remain at 100% of the original value. However 50% of the cells have been broken (according to protein release data) therefore there is an approximate doubling of intracellular ATP in the remaining intact cells.

Fig 3.11 **Multiple pass homogenisation**
Percentage protein release vs number of passes
Stationary phase cells

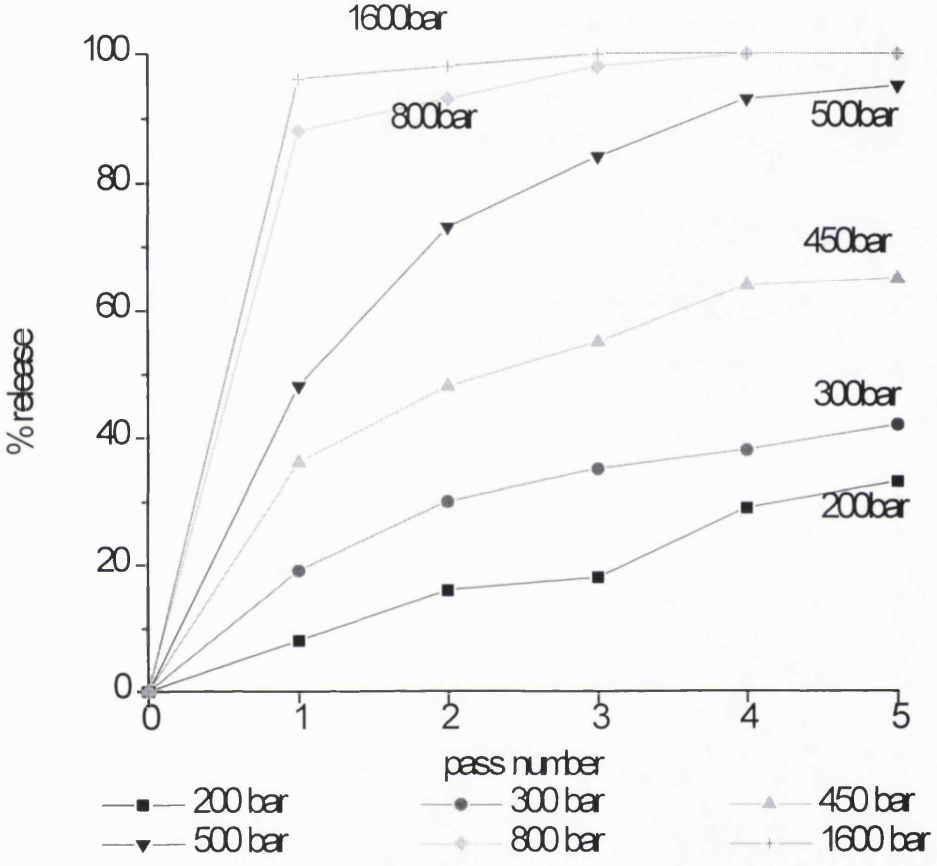
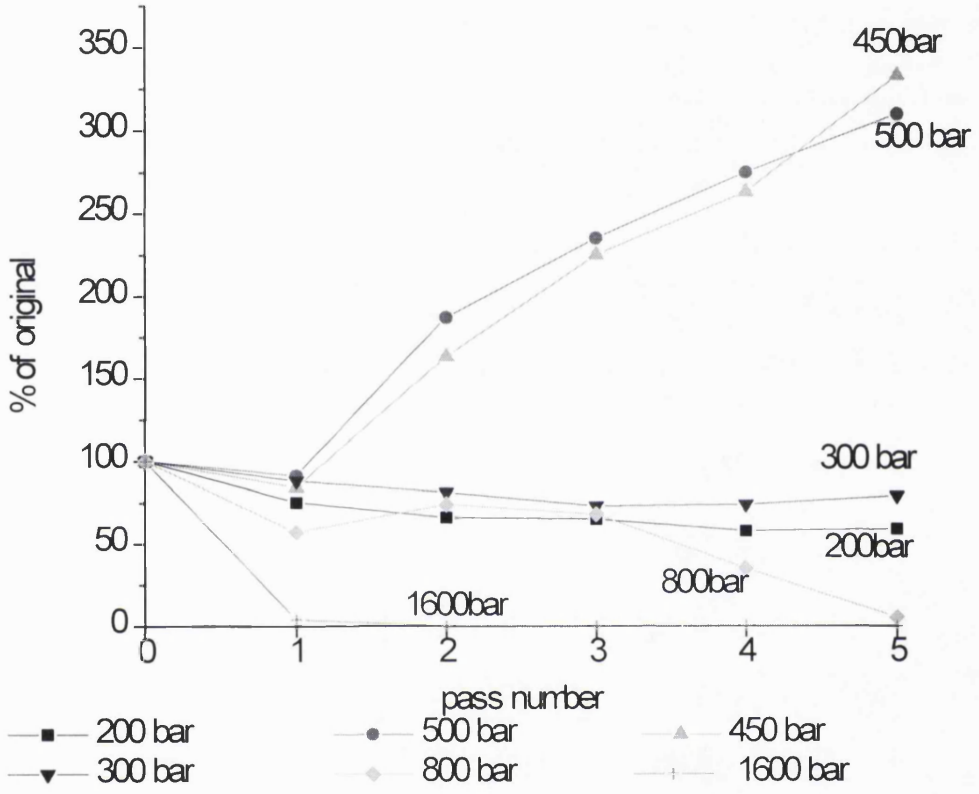
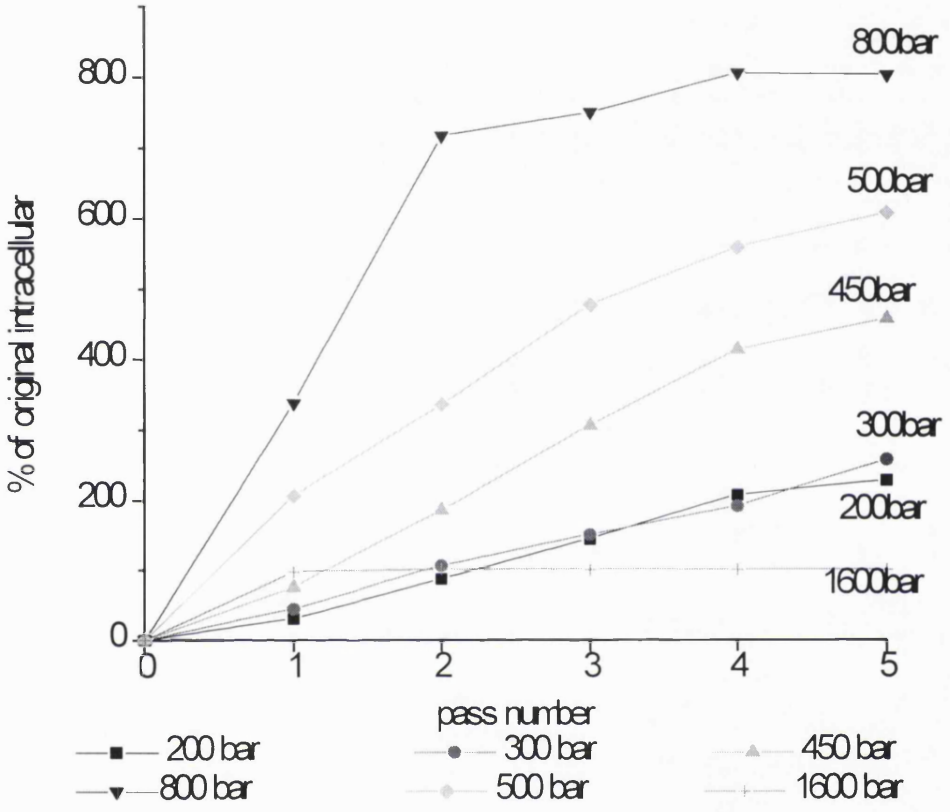


Fig 3.12 Multiple pass homogenisation
Intracellular ATP vs number of passes
Stationary phase cells



It appears that activation of cells leading to increased intracellular ATP levels is only occurring for mid-range pressures (450 & 500 bar), this is not the case. Vlues are total ATP for the remaining intact cells after each pass. Almost 90% of the cells are disrupted after one pass at 800 bar. A value of 50% of the original ATP is therefore from only 10% of the original cells. Refer to Fig 3.22 for ATP/intact cells after each pass to see true activation levels.

Fig 3.13 **Multiple pass homogenisation**
Extracellular ATP vs number of passes
Stationary phase cells



At medium pressures a huge increase in ATP levels in intact cells was observed. If not completely disrupted by initial passes the largest intracellular ATP increases were seen at high pressures.

3.3.3.2.2 Activation

Stationary phase cells were processed by a single homogeniser pass. The cells were then used to inoculate a minimal media shake flask containing glucose as a carbon source. The rate of increase and final levels of intracellular ATP were compared to that of an unprocessed cell sample.

Viable cell counts were carried out and released intracellular protein was assessed.

Table 3.3 Summary of homogenisation trials using cells from fermentations SHPP17, SHPP21 and SHPP22

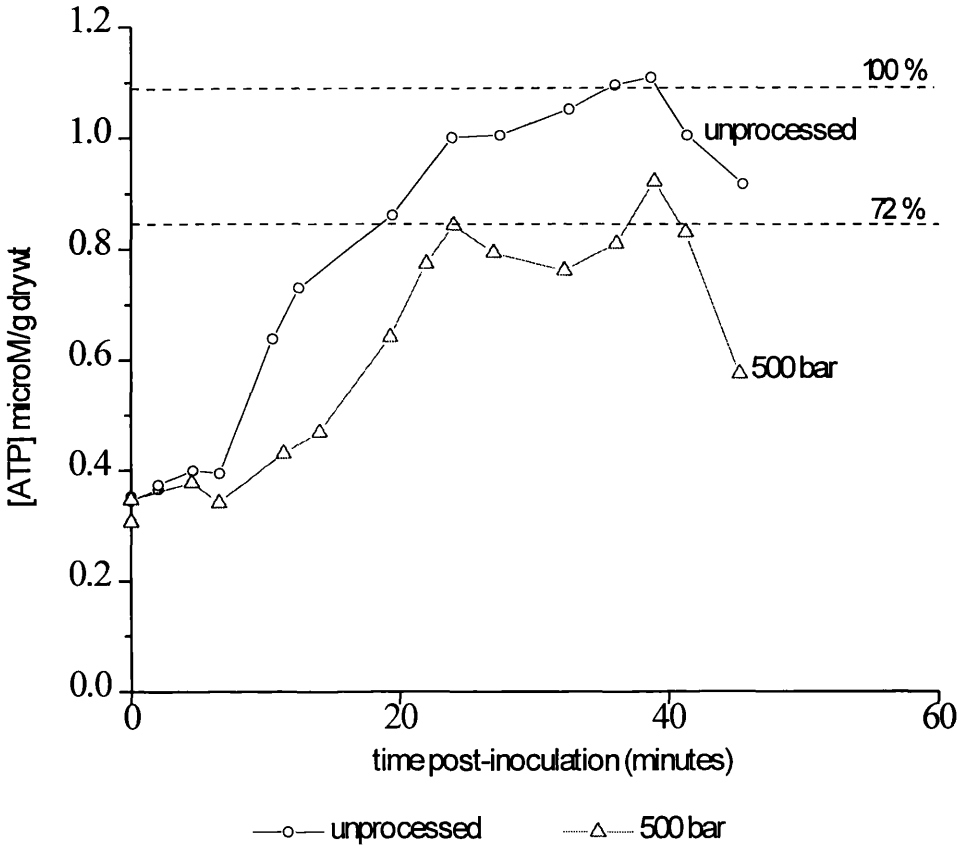
FERMENTATION	HOMOGENISATION PRESSURE	% VIABILITY (CFU'S)	% INTEGRITY (PROTEIN)	% VIABILITY (ATP}
SHPP17	500	41 (16)	54 (4)	{80}
SHPP21	300	80 (14)	83 (2)	{71}
	500	59 (22)	54 (2)	{42}
SHPP22	300	49 (6)	44 (6)	{25}
	500	22 (41)	26 (5)	{13}

( ) standard deviation as %

{} Indicates a lack of confidence in the data

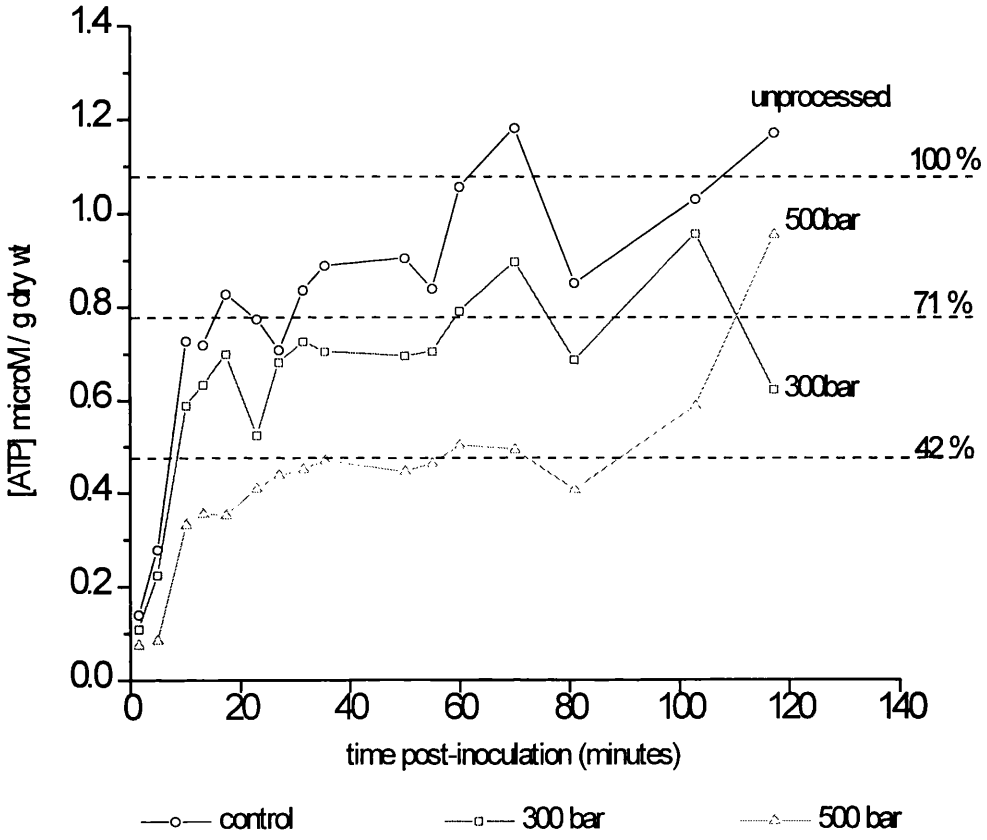
A close relationship can be seen between percentage protein release and percentage viability for each of the operations. There does not appear to be any correlation with these two tests and the ATP measurements. Figures 3.16 - 3.18 show the activation curves obtained in the three runs. The scatter on the results means that ATP maxima are impossible to estimate with any degree of confidence.

Fig 3.14 Use of ATP activation profiles for the study of damage of stationary phase cells - single pass homogenisation
Intracellular ATP vs time
Fermentation SHPP17



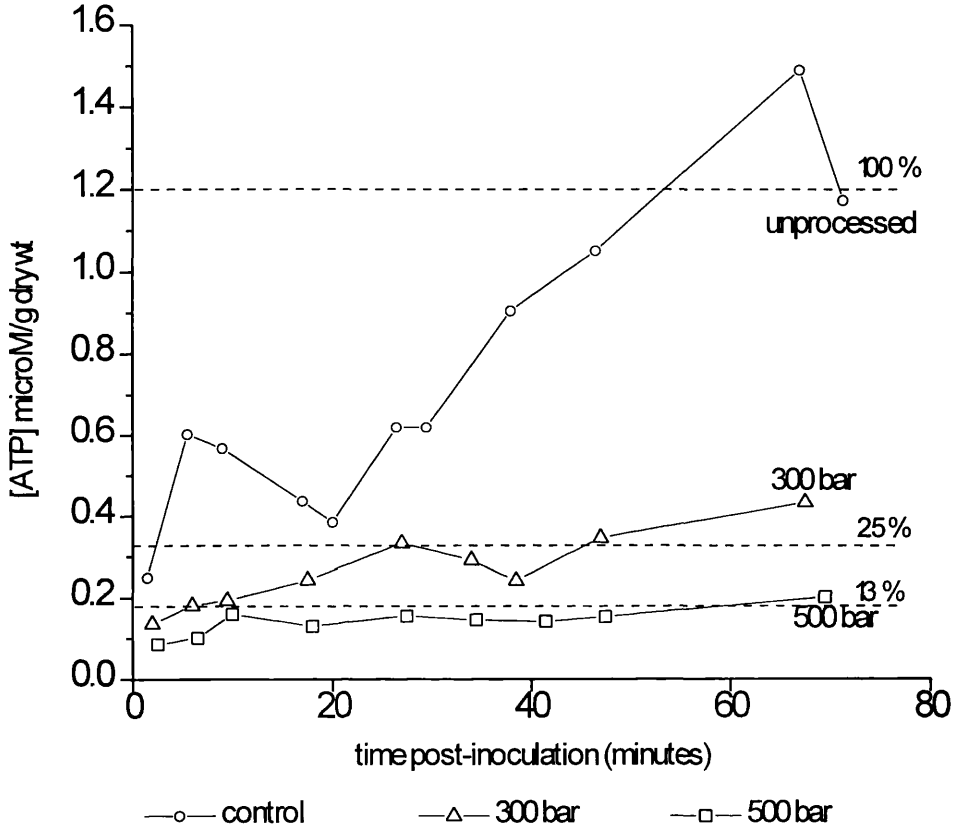
Dotted lines are estimates of the maximum ATP levels reached by the cell samples, corresponding to their growth phase maximum. This allows the intracellular ATP levels to be used for determination of viable biomass.

Fig 3.15 Use of ATP activation profiles for the study of damage of stationary phase cells - single pass homogenisation
Intracellular ATP vs time
Fermentation SHPP21



Dotted lines are estimates of the maximum ATP levels reached by the cell samples, corresponding to their growth phase maximum. This allows the intracellular ATP levels to be used for determination of viable biomass.

Fig 3.16 Use of ATP activation profiles for the study of damage of stationary phase cells - single pass homogenisation
Intracellular ATP vs time
Fermentation SHPP22



Dotted lines are estimates of the maximum ATP levels reached by the cell samples, corresponding to their growth phase maximum. This allows the intracellular ATP levels to be used for determination of viable biomass.

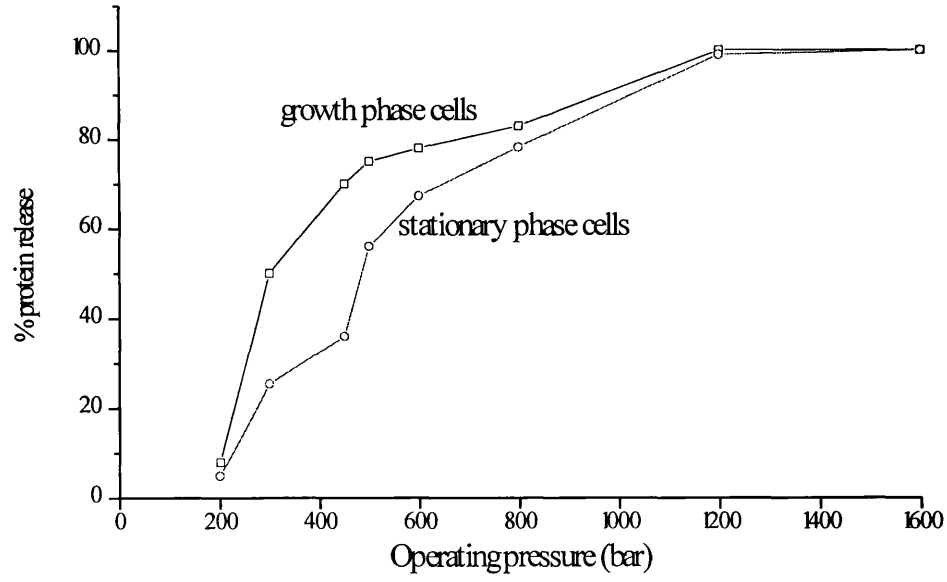
3.3.3.3 *P. putida* ML2 disruption characteristics

The disruption characteristics of stationary and growth phase cells are different. Fig 3.18 shows *P. putida* ML2 disruption characteristics after a single homogeniser pass over a range of pressures. Cells were disrupted during two stages of the growth cycle, stationary and exponential growth.

The disrupted cell population is expressed as a percentage of the maximum protein release. The operating pressure of the Lab 40 homogeniser is expressed in bar.

Stationary phase cells appear to be more resistant to mechanical rupture at all operating pressures.

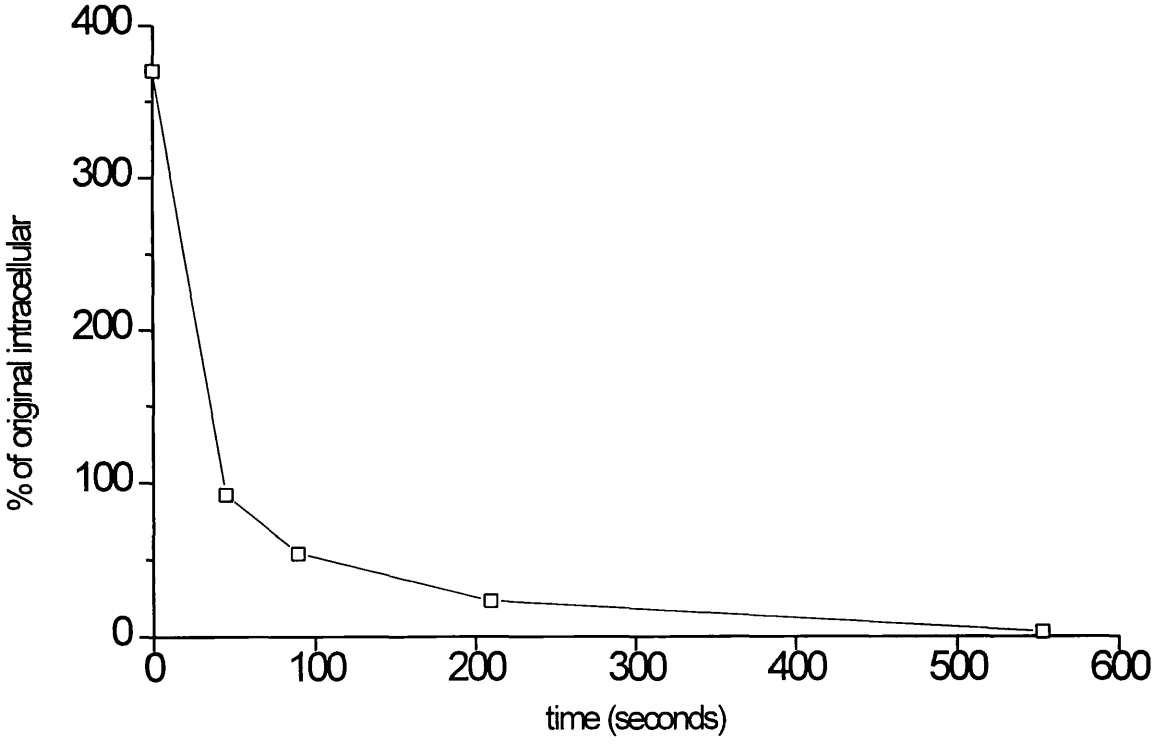
Fig 3.17 ***P. putida* ML2 resistance to mechanical stress**
Single pass homogenisation of growth and stationary phase cells
% protein release vs operating pressure



3.3.3.4 The fate of extracellular ATP

ATP has a short half life within metabolising cells and is reported to be almost non existent outside the cellular environment due to the presence of ATPases. It has been shown that ATP can be measured outside the cell. It was considered important to examine the rate of decay of intracellular ATP from disrupted cells. 500 bar single pass homogenisations were carried out and the rate of decay of the released ATP was monitored against time.

Fig 3.18 Stability of ATP released from disrupted cells
Single pass homogenisation of stationary phase cells at 500 bar
Extracellular ATP concentration vs time



3.4 Discussion

3.4.1 Fermentation Monitoring

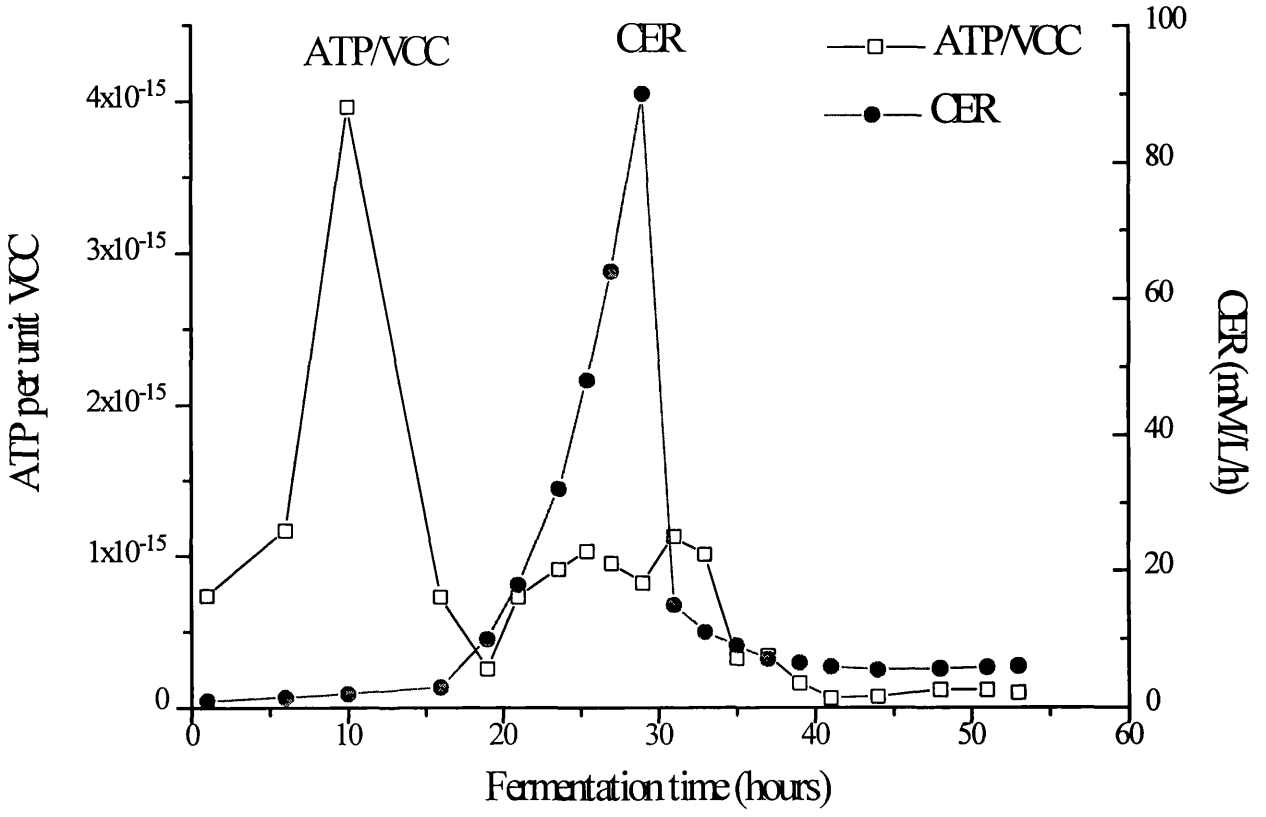
The assay of ATP was investigated with the intention of providing a method of quantifying cell damage and potentially replacing viable cell counts. ATP is a dynamic substance that only occurs in viable, metabolically active cells. Convenience and accuracy were key factors in this choice of technique, the assay time is minutes rather than days.

The relationship between total intracellular ATP and VCC, OD and CER throughout growth phase suggests that cells contain a fixed amount of ATP and therefore its assay can be used as an index of biomass. However during stationary phase ATP levels drop and follow CER only. It appears that levels are not fixed throughout all stages of growth and that ATP is actually a measure of metabolic activity.

McEntee *et al.* (1989) studied the variation in cellular ATP content of *E. coli* when grown in shake flask culture. Shortly after inoculation, ATP / viable cell begins to rise until it reaches a maximum. This peak corresponds to onset of exponential growth phase. ATP $cell^{-1}$ rapidly drops in growth phase.

This variation was identical to that measured during *P. putida* ML2 fermentation as shown in Figs 3.4 & 5. Measured ATP per viable cell was calculated for *P. putida* ML2 and plotted against fermentation time. A maximum value was observed at the onset of growth phase. The lowest value was observed during stationary phase, where ATP drops because metabolic activity is limited due to lack of nutrients.

Fig 3.19 Fermentation monitoring SHPP08
ATP per unit VCC and CER vs time



ATP/VCC ATP concentration per viable cell (μM)
CER Carbon dioxide Evolution Rate (mmol $L^{-1}h^{-1}$)

The apparent decrease in ATP/viable cell during growth phase seems to suggest that cells are functioning at a lower metabolic rate. This is unlikely, it is more likely to be due to the fact that a cell culture at this stage of its growth does not contain a homogenous population. The overall population is in growth phase but individual cells are going through a constant cycle of lag, growth and division. Intracellular ATP must remain reasonably constant to allow regulation of metabolic activity, so the ATP content of a viable cell is dependent on the cellular volume. Cells that have recently divided have a smaller volume than the parent cell. When cells are fully

developed they immediately divide and hence each of the new cells has a smaller amount of ATP than the parent cell. This is the most likely explanation for the variations between lag and growth phase cells.

During a fermentation the total ATP of growth phase cells are at a maximum level of 2.7 μM g^{-1} dry wt drops to 1.15 μM g^{-1} dry wt in stationary phase. This data was obtained from a number of monitored fermentations and does not match that of Knowles (1977) who stated that for prokaryotic and eukaryotic cells growing exponentially in vitro a figure of 12 μM g^{-1} dry wt was obtained. Many papers state that cells do not vary their ATP content much and the only variation is between species. A possible reason for the lowered ATP content with *P. putida* ML2 and other bacteria such as *E. coli* might be due to growth conditions. A large variaion in doubling times has been demonstrated by *P. putida* ML2 when grown on different carbon sources. This would affect the measured values. *E. coli* generally has a shorter doubling time than *P. putida* and this might be responsible for the differences.

3.4.2 ATP Assay Development

Growth phase cells were harvested and homogenised while at their maximum total ATP levels. Measured intracellular ATP and protein release show an inverse relationship, with the amount of remaining intracellular ATP being proportional to the fraction of unbroken cells as measured by protein release. This suggests that ATP could be used as a measure of cell damage for cells harvested at this stage of growth.

Stationary phase cells were processed by homogenisation. At medium pressures (400-800 bar) they appeared to undergo a metabolic activation. The largest activation observed was at 800 bar when the intracellular ATP levels of intact cells rose by 3500% (actual measured values 0.08-2.69 $\mu M\ g^{-1}$ cells). This final activation was equivalent to the levels observed at the growth phase maximum.

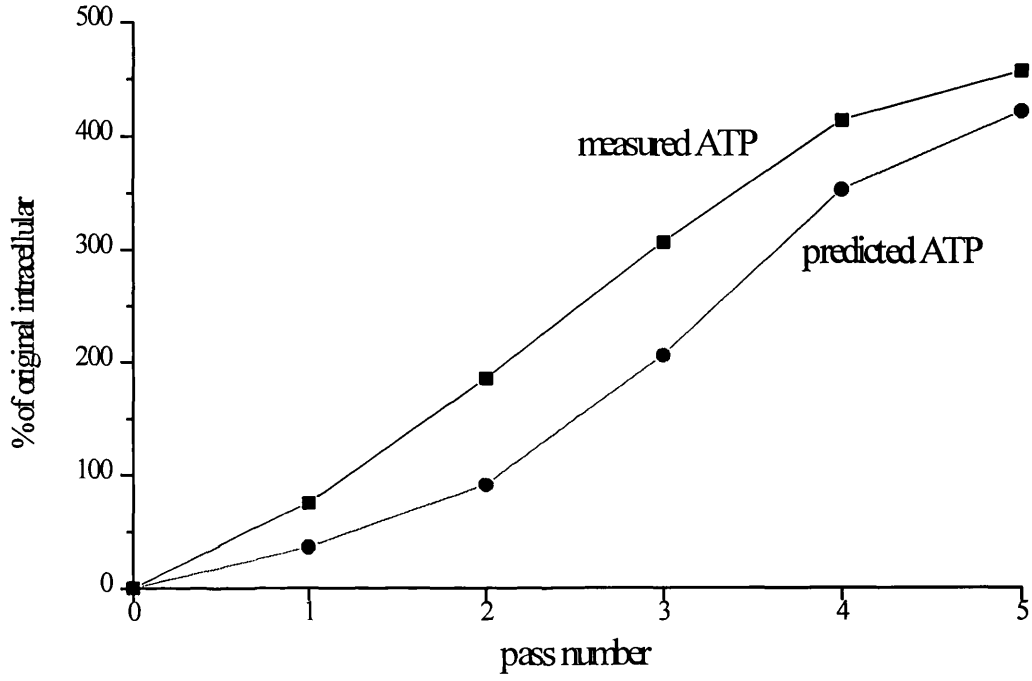
This observed activation of cells means that the measurement of released ATP can not be used as a tool for damage assessment. A fundamental requirement of any test system would be that the intracellular levels of the target metabolite remain the same before and after processing, unless they are released due to cell lysis.

An understanding of the mechanism of this physiological effect would possibly allow the prediction of ATP release. The modeling of such a system could make sense of the measurements. Step to step mass balancing of ATP and protein was used as follows:

- The percentage protein release was used to identify the percentage of cells broken.
- The percentage release of extracellular ATP was calculated on the basis of percentage cells broken and intracellular ATP measured. The extracellular release was calculated as cumulative released ATP.
- The predicted intracellular ATP was not calculated as it was evident that ATP concentration was rising - the results were used to estimate the ATP concentration of remaining intact cells.
- Step to step calculations are detailed in Appendix 3.5

The following graph (Fig 3.20) compares predicted and measured intracellular ATP levels over multiple passes for stationary phase *P. putida* ML2 cells.

Fig 3.20 Multiple pass homogenisation at 450 bar operating pressure Predicted and measured intracellular ATP values



There are several features worth noting:

- There is a constant underprediction by approximately (50%) of ATP release at each pass.
- The high measured values of extracellular ATP release are probably due to the increased intracellular ATP content of the remaining intact cells. Figure 3.22 indicates ATP / remaining whole cells with 6-10 fold increase. This is evidently due to the cells coming from a stationary phase and it appears that interstage activation is causing ATP generation.

This approach to analysis of the results does not allow the test system to be used to assess cellular damage.

The cellular activation is most likely to be caused by a combination of oxygenation, sudden supply of fresh nutrients and a stress response. *P. putida* ML2 has a high oxygen requirement, the optimised conditions of the fermenter provide aeration and agitation. Cells have access to available oxygen and are stopped from aggregating. During storage before and during processing on ice this does not occur. Cells are in stationary phase because their growth is limited by lack of carbon source. Intracellular metabolites from lysed cells become available for absorption. Finally, stress stress damage is known to increase metabolic activity and hence ATP levels (Takano ,1982).

The majority of problems with this test system arise from the fact that the turnover of ATP within metabolising cells is so high. In the case of exponentially growing *E. coli* its half life is less than one second (Lehninger, 1979). This bestows on the cells an ability to react quickly to a changed environment. Problems with this test system occur in two parts; the operation of the system and the design of the assay itself.

Problems with the test system are basically the inability to standardise the procedure. To ensure that each cell batch undergoes exactly the same procedure would require processing to within a fraction of a second each time. Areas of variation in the sampling procedure occur before and after the homogenisation:

- Before homogenisation pass has taken place.
- The time taken between harvesting the cells and storing them on ice.
- The loss of aeration and agitation during storage.
- Time stored in ice between tests can lead to variability of starting temperature

After operation. The process of homogenisation is known to heat the samples. An value of 1°C $100bar^{-1}$ $pass^{-1}$ (Sayed, 1995) has been observed during Lab40 use. The addition of glycol cooling coils surrounding the collector bowl is unlikely to be able to instantly cool the homogenate before sampling. Ideally the taking of samples as soon as possible after processing would reduce the degradation of released extracellular ATP by ATPases. The action of the homogeniser pass in aerating the cell suspension is likely but the extent is unknown. The susceptibility of the cell sample to breakage will ultimately effect the quantity of released nutrients.

A problem with the assay procedure is the variation in the time between passes. To reproduce the assaying technique over large numbers of operations to within seconds each time is impossible. The nature of such a rapidly changing adenylate pool means

that readings almost need to be instantaneous. The testing of samples as soon as possible after a pass, during multipass homogenisation, makes it difficult to carry out multiple repeats. This has lead to a high degree of scatter in results but however is possible with more "off-line" techniques such as plate counts and protein assays.

The kinetics of the enzyme reaction may not be as simple as the luminometer and test kit manufacturer suggest. The idea that by manipulation of reagent quantities it is possible to produce an almost instantaneous peak of light emission that does not decay for minutes has been refuted in many publications. Krones *et al.* (1990) suggest that a better analytical approach is automatic data acquisition from the luminometer and the fitting of an exponential decay equation. They claim that an integral of the curve was found to be a more reliable indicator of the amount of ATP in a sample than testing for peak light emission. This held true regardless of the overall shape peak height of the curve or the rate of initial rise. It also seems possible that the extractant may not be as effective on unprocessed samples and fermentation samples. This could be due to damage to the membrane leading to increased permeabilisation after processing.

Expressing the results as a percentage to allow comparison between samples containing different cell concentrations may also be a problem. This is perfectly acceptable for metabolites who's concentration is unlikely to change during the time of the sampling eg protein. It was assumed that cells are harvested in their stationary phase and contain a minimal level of ATP and were then processed and activated. However at the start of processing the ATP levels within the cells could and did drop below the stationary phase level that they were harvested at. When expressed as a percentage far larger increase appears to be taking place after processing. Comparison between batches is not valid due to unavoidable variations in the operating procedures, even though cells were harvested from the fermentation at identical times in the same physiological state.

The rapid turnover of ATP within the cell means that to standardise processing all operations need to be carried out within fractions of a second. Publications have highlighted this nutrient mediated activation of microbial cells. To activate a nutrient starved *A. calcoaceticus* culture with glucose takes less than a minute and with acetate 6 minutes (van Schie *et al.*, 1991). The glucose activation was so fast that it could not be measured accurately. It would appear that the homogenisation activation could be happening within a similar time scale.

The huge ATP variability within the cells that occurs during the processing means that it cannot be used as an index of viable biomass or as a measure of cell damage. In order to use it as such an analytical tool cellular levels must be constant. The only time that you can be sure that they are a t a constant level is when they are at their maximum. It was decided to process cells and then activate them to their maximal growth phase levels by the addition of fresh nutrients and then compare their maximal levels to those of an unprocessed sample. All start at about 0.4 $\mu M\ g^{-1}$ and activate to 1-2 $\mu M\ g^{-1}$ and this is less than stationary phase (2.7 $\mu M\ g^{-1}$). This is probably due to oxygen limitation. When fermentations were run during the development stage and aeration became a limiting factor growth rate was slowed down. It seems unlikely that shake flask culture would produce cells metabolising at their maximum metabolic rate as fermenters produce optimal growth conditions.

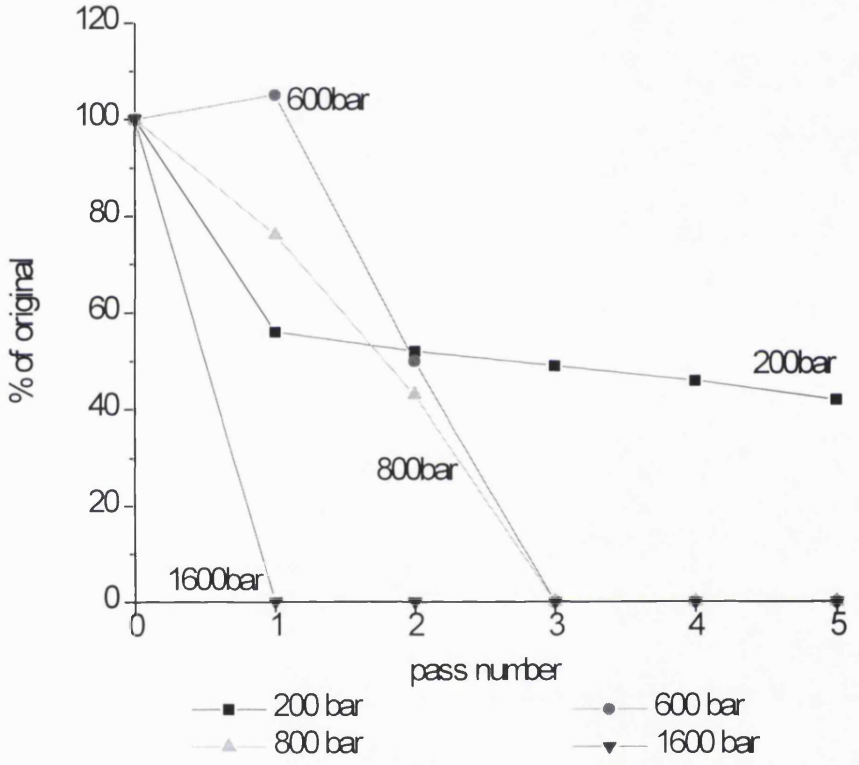
The spread on results for the shake flask activation experiments proved to be too big for estimation of final values. Brookes and Jenkinson (1989) suggested that ATP variability in natural populations of bacteria was too high for it to be used as an index of biomass and suggested the use of total adenylate content instead. This however is far more lengthy assay and does not have any of the inherent advantages that made ATP analysis so appealing at the start of this work.

It should be noted that extracellular ATP levels were not considered to be a reliable means of measuring loss of cellular integrity of stationary phase cells. This was due to the presence of ATP-degrading enzymes, which reduced extracellular ATP levels in a cell sample processed by a single homogenisation pass (Fig 3.18). However extracellular levels remained high, and did not appear to decrease, as expected, during multiple pass homogenisations (Figs 3.9 & 3.13). The reasons for these anomalies was not considered to be within the scope of this project and therefore extracellular ATP analysis wa not investigated further.

The protein release and viable cell count data was shown to correlate well. See Table 3.1. This was due to the use of large numbers of repeat samples and statistical analysis. This optimisation of the viable count technique suggested that errors could be reduced to acceptable levels and the system could be workable. ATP analysis was therefore considered unsuitable for this work and subsequent downstream processing studies used large numbers of repeats of total soluble protein release and viable cell counts.

The following graphs shows the variation in ATP per intact cell for the multiple pass homogenisation of growth phase cells (Fig 3.21) and stationary phase cells (Fig 3.22).

Fig 3.21 Multiple pass homogenisation of growth phase cells ATP per intact cell vs pass number

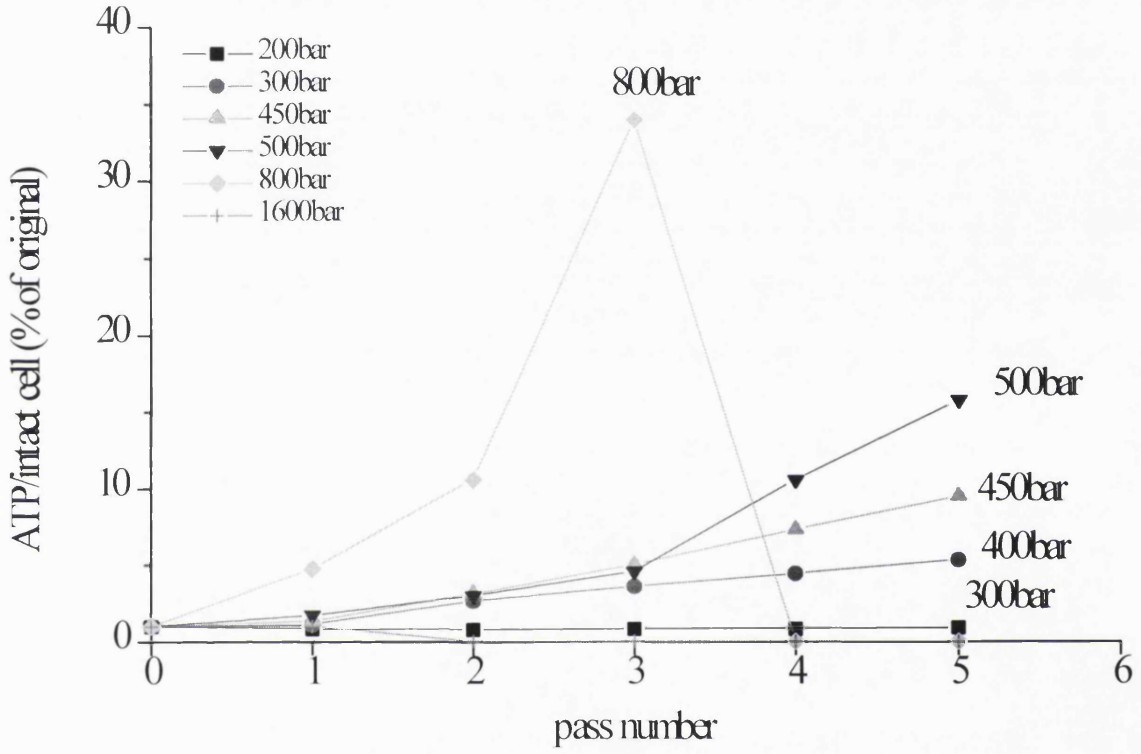


At the lowest pressure, 200 bar, intracellular ATP levels showed a 50% drop after the first pass and remained reasonably constant over the subsequent four passes. Although there did appear to be a small decline.

At 600 bar levels remained steady after pass one and then dropped to zero at pass three which corresponded to complete disruption of all the cells.

At 800 bar levels dropped by 20% per pass until complete disruption at pass 3.

Fig 3.22 Multiple pass homogenisation of stationary phase cells ATP per intact cell vs pass number



At medium pressures (400-800 bar) there levels of ATP per intact cell were progressively higher at each pass. The rate of increase was dependent on the operating pressure. 800 bar homogenisation showed the highest levels of activation, however the cells were completely disrupted after 3 passes At highest pressures complete disruption at pass one so no effect on intracellular environment so levels stay at 0%. Extra cellular even more exaggerated

3.5 Conclusion

This section dealt with the development of an analytical test system for the determination of damage inflicted on a population of *P. putida* ML2 cells after exposure to mechanical stresses. Measurement of cellular damage is not a straightforward task, and it was suggested that loss of viability alone was not a sufficient means of expression. Standard methods of determining cellular damage are to measure loss of ability to reproduce by plate counting and loss of integrity by measuring levels of released intracellular components, such as total soluble protein. There are inherent problems with these tests and the bioluminescent ATP assay was examined with a view to their replacement.

Total intracellular ATP levels were found to closely correspond with optical density, dry weight, viable cell count, oxygen uptake and carbon dioxide evolution rate during pre-growth and exponential growth phases of fermentation. ATP levels were found to only follow exit gas profiles during early stationary phase.

Downstream processing stresses were simulated at laboratory scale using a high pressure homogeniser, which was considered to be an approximation to the sediment discharge of a disc stack centrifuge. For multiple pass processing of growth phase cells the release of intracellular ATP was found to correlate well with total soluble protein. However when stationary phase cells were processed in a similar fashion activation of the cells lead to an increase in their ATP content, therefore the test was of no use.

Stationary phase cells were processed by a single homogenisation pass and used to inoculate shake flasks containing growth medium. Intracellular ATP levels were measured over time and the peak values were compared to those of an unprocessed sample. Scatter of data made interpretation of the results very difficult. Further development of the ATP assay was stopped at this point due to time constraints.

Viable cell counts and protein release data had been found to closely match during homogenisation trials and it was decided to use both of these techniques to express cellular damage.

4 Centrifugation

4.1 Introduction

4.1.1 The Use Of Centrifuges In The Biotechnology Industry

Centrifuges have had an important role in industrial separations since 1896, when they were first used to separate yeast from fermentation broths (Knight, 1988). Since that time mechanical separation has proved extremely reliable for a number of biotechnology applications. The most commonly used machine for the separation of biological particles is the disc stack centrifuge, which can be used for the continuous recovery or removal of a wide range of biological particles. Common applications include:

- Separation of cells from fermentation broths (Higgins *et al.*, 1978)
- Removal of debris after a disruption step (Datar and Rosen, 1987)
- Separation of precipitate particles (Bell and Dunnill, 1982)

The basis for centrifugal sedimentation is the density difference between solids and liquids. Centrifugal forces subjected on particles make them move radially through the liquid either outwards or inwards, depending on whether they are heavier or lighter than the liquid. Centrifugation may be considered to be an extension of gravity sedimentation. There are a number of basic machine types, each of which has different operational characteristics (Bell *et al.*, 1983). All suspensions have distinctive processing properties and efficient separation is dependent on a suitable choice of machine. Recent centrifuge development has given rise to a number of different centrifuge configurations which can operate in a variety of different modes.

- Batch mode
  - Tubular bowl (manual discharge)
  - Multichamber bowl
  - Disc stack - solids retaining
- Semi continuous mode
  - Imperforate basket (intermittent discharge)
  - Disc stack — solids ejecting (intermittent discharge)
- Continuous mode
  - Scroll
  - Disc stack — Nozzle type - (continuous discharge)

There are a number of problems associated with the centrifugal separation of biological particles. This is mainly due to their small size, the small density difference between the solid and liquid phase, high suspension viscosity and the sensitivity of many biological particles to shear (Mannweiler *et al.*, 1989). However there are many advantages of centrifuge use for industrial separations:

- Continuous processing-reduced down time for cleaning and harvesting
- Short retention times-lack of stability of most biological products
- No filter aid used
- Adjustable separation efficiency
- Small space requirement
- Closed system-containment (Erikson, 1984)

Choice of centrifuge type depends on a number of different factors. Lavanchy *et al.*, (1964) devised a graphical guide which gives limiting flow rates and settling capabilities of various types of equipment.

4.1.2 Mechanisms Of Cell Damage Due To Centrifugation

There are a number of factors that are likely to lead to loss of cellular viability during centrifugation processing. There are two such factors that are of interest for this study:

- Oxygen starvation
- Mechanical stress

Oxygen starvation is a problem that arises when processing aerobic organisms. This occurs due to lack of gas transfer when cells are held as a sedimented cell paste before harvesting, when pasted in a centrifuge bowl . Oxygen requirements of aerobic micro organisms can be considerable. For example, the test organism used in this study is *P. putida* ML2. Cells are grown in fermenter in a defined medium containing oxygen as sole carbon source. When the fructose is fully utilised the cells reach stationary phase this occurs at a cell concentration of approximately 10 g dry wt L^{-1}. An oxygen requirement of 10 mmol $L^{-1}h^{-1}$ is measured by on-line gas analysis. When the cells are sedimented to 100 g dry wt L^{-1} in the bowl of a disc stack centrifuge the oxygen requirement of the paste rises to 100 mmol $L^{-1}h^{-1}$. Gas

transfer is almost negligible throughout this period which can last for upto 20 minutes before discharge. This is a potential cause of damage to a population of strictly aerobic bacteria.

Another cause of loss of cellular viability during centrifugation is mechanical forces acting on the cells during processing. The mechanical stresses are likely to manifest themselves as:

- Simple shear
- Extensional shear
- Compaction

Unlike microfiltration the shear effects of centrifugation have been widely documented. The majority of work appears to concentrate on the shear break up of aggregated flocs and not on whole cell recovery. The first stage of recovery of many proteins is a flocculation step followed by centrifugation. Spielman (1978) reviewed the behaviour of aggregates in shear fields and stated that break up is by a combination of mechanisms, which are also likely to act on whole cells:

- deformation and compaction leading to rupture by fluctuating dynamic pressures, such as those caused by sudden rapid, acceleration on entry to the centrifuge bowl
- impaction forces as cells collide with each other and solid surfaces
- hydraulic shear from rapid liquid movement

Cell damage results from the combination of these effects acting on the cells. Continuous centrifugation can be seen as a process that takes place in three stages, feed, separation and discharge. No particular force is acting solely at any one stage, and all act to a greater or lesser extent throughout the separation process.

- Feed rapid radial acceleration upon entry
- Separation hydraulic shear stresses due to liquid flow
- Discharge hydraulic shear due to pumping of viscous cell paste

This also holds true for batch centrifugation with the omission of the discharge step. Bell and Dunnill (1982) found that the maximum conditions of shear in the centrifuge occur in the feed zone. It has been shown that the amount of cell damage is dependant on the time of exposure to the shear-field. Although the suspension

residence time is short, large stresses are generated. The suspension is subjected to an extremely rapid and violent increase in its tangential and radial velocity components. In the case of a fast spinning tubular bowl centrifuge a solid particle may be undergo a radial acceleration from zero to 50000 rpm in as little as 10^{-3} seconds. Many centrifuges designed for biotechnology use are equipped with "soft feed zones" which generate lower shear conditions than standard machines. (See section 4.1.5)

The feed zone is not the only area in a centrifuge where cells are subjected to shear stress. In continuous processing, such as disc stack centrifugation shear is encountered during the separation stage due to fluid flow through the disc stack. Bell (1982) reviewed the roles of simple and extensional shear in mixing and dispersion systems and showed that the influence of extensional shear is greater than that of simple shear in dilute suspensions. As the suspension becomes more concentrated the role of simple shear can increase. This is of particular concern as high cell concentration sediment is pumped through discharge ports or nozzles (Knight, 1988). High shear forces are also associated with centripetal pumping (Bell and Brunner, 1983) such as that required to feed large industrial centrifuges.

Compaction of the cells can lead to structural deformation and even rupture. This has been shown to occur in the feed zone of many centrifuges due to inertia as the cells are accelerated to high speeds. In batch centrifuges cells are retained within the spinning bowl and are subjected to such forces.

4.1.3 Centrifuge Theory

The rate of particle sedimentation is defined by the balance between the centrifugal force, the Brownian diffusional force and the kinetic drag of the fluid (Bell, 1982). The most useful way of describing the sedimentation process that occurs within a centrifuge is by Stokes Law, which assumes that the particles being processed are small, inert spheres, flowing in the laminar flow regime, and that the degree of hindered settling is negligible. Stokes law gives the sedimentation velocity, v_g, for a solid particle settling under the influence of gravity as:

$$v_g = \frac{(\rho_a - \rho_f)}{18\mu} d^2 g_a \qquad (4.1)$$

Where :

ρ_a density of particles

ρ_f density of suspending fluid

μ viscosity of the suspension

d particle diameter

g_a acceleration due to gravity

This equation only holds true for laminar flow. For turbulent flow alternative equations have been derived by Sokolov (1971) and Hsu (1981). Particle movement in centrifuges is generally considered to be in the laminar regime (Ambler, 1952)

For sedimentation in a centrifugal field the sedimentation velocity, v_z, is given by:

$$v_z = \frac{(\rho_a - \rho_f)}{18\mu} d^2 \omega^2 r \qquad (4.2)$$

Where:

ω angular velocity

r radial position of the particle

Unfortunately this does not hold true for practical trials, so the sigma concept was developed.

The Sigma Concept

This was developed by Ambler (1952) to account for lack of accuracy of Stoke's Law when used to test industrial centrifuges. The basic assumptions of lack of particle-particle interference does not hold true for real systems. It defines the relationship between centrifuge performance, total volumetric flow rate and the size of the centrifuge, and allows prediction of performance from small scale testing. This model has been widely used for scale-up purposes by centrifuge manufacturing companies (Clarkson, 1994).

The Grade Efficiency Concept

The separation ability of a particular centrifuge is highly dependent on the particle size of the feed material. The separation efficiency of a particular machine will be

governed by the particle size. It is therefore not possible to give a single separation efficiency value for an individual machine so the Grade Efficiency Concept is used instead. When the mass efficiency is found for each particle size present in the feed then the gravimetric grade efficiency function, T_d, can be obtained. This value is independent of the solids size distribution and density and is constant for a given set of operating conditions. This has also been used in the scale up of industrial centrifuges.

A full description of sedimentation and centrifugation is not relevant to this work and can be found in many engineering text books.

4.1.4 Types Of Centrifuge

The following section will provide a brief overview of centrifuge types frequently use by the biotechnology industry:

- Tubular bowl
- Imperforate basket
- Multichamber bowl
- Scroll type
- Disc Stack

Table 4.1 Properties of centrifuges

	Solids content (%)	Speed (rpm)	applications
Tubular bowl	1	50000	very fine solids
Imperforate basket	3-5	450-3500	dewatering of sludge recovery of solids from a waste stream
Multichamber	4-5	4500-8500	clarification of beer, wine, fruit juice
Scroll type	2-50	1600-6000	dewatering of both municipal and industrial sludge
Disc Stack	1	8000-12000	whole cell recovery flocculated cell debris

(Svarovsky, 1977)

4.1.4.1 Tubular Bowl Centrifuge

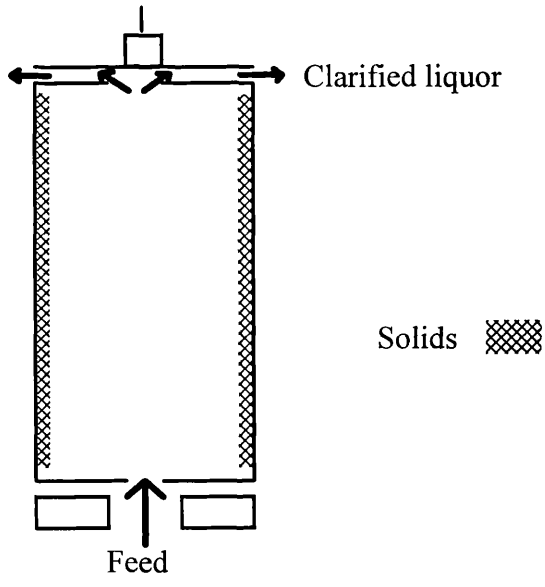
The tubular bowl centrifuge is the simplest batch centrifuge design, consisting of a vertical tube with a large length to diameter ratio which is suspended from a shaft and motor or air turbine. The cylinder rotates at high speed about its vertical axis. The rotor is set in a drag bearing usually made of brass, which by design allows the rotor to find its own rotational axis. This bearing design allows the rotor to find its own rotational axis and allows rotor recentering due to any temporary imbalance.

The liquid is introduced through a nozzle at the bottom of the cylinder and the solids are sedimented to the walls as the liquid rises. Clarified liquid is forcibly ejected ate the top of the centrifuge. The use of plastic liners allows solids to be removed easily once the unit has been dismantled.

The high levels of turbulence in the feed zone of such machines can disrupt delicate particles such as precipitates or shear-sensitive cells and leads to reduced separation efficiency. The use of soft feed designs provide a more gentle acceleration of the feed to the bowl speed.

One of the assumptions is that the amount of sedimented solids in the bowl is negligible throughout the operating cycle. In practice however these devices have to be stopped and cleaned (usually manually) when the solids in the bowl reaches a certain level. During the operation period a cake (or heel) of solids is gradually building up in the bowl, thus reducing the area available to flow. This in turn reduces the residence time of the liquid in the bowl and the efficiency of separation gradually drops. Reduction in efficiency results in increased solids content in the overflow which can be monitored, for example by turbidity measurements.

Fig 4.1 Simple Tubular Bowl Centrifuge

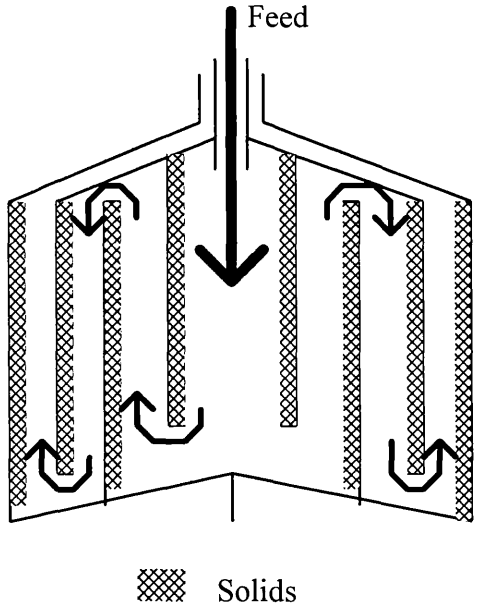


4.1.4.2 Multichamber Centrifuges

The multichamber bowl centrifuge is a development of the tubular bowl device and uses a closed bowl which is sub-divided into a number of concentric vertical cylindrical compartments through which the suspension flows in series. The complexity of the machine limits its rotational speed.

The feed suspension is fed in through a central pipe. Solids are deposited on the inside of the bowls and each outer bowl has a progressively higher acceleration and therefore a greater sedimenting force. This results in a classification effect with the coarsest fraction being deposited in the inner chamber and the finest fraction in the outermost chamber.

Fig 4.2 Multichamber Centrifuge



An advantage of this configuration is the large solids holding capacity. In a large machine this may be as much as 751 m^3. Westfalia Separator AG make models with either a six chamber bowl (5 cylindrical inserts) or a 2 chamber bowl (1 cylindrical insert) which also incorporates a centripetal pump on the discharge side of the bowl (this converts the kinetic energy of the liquid on discharge into pressure. Another advantage is that due to the fact that they operate in flooded condition there is no air-liquid interface as in a tubular bowl. This reduces turbulence and lowers shear. The cleaning of multichamber centrifuges is more difficult and takes longer than tubular ones. The manual discharge generally limits the use of this type of machine.

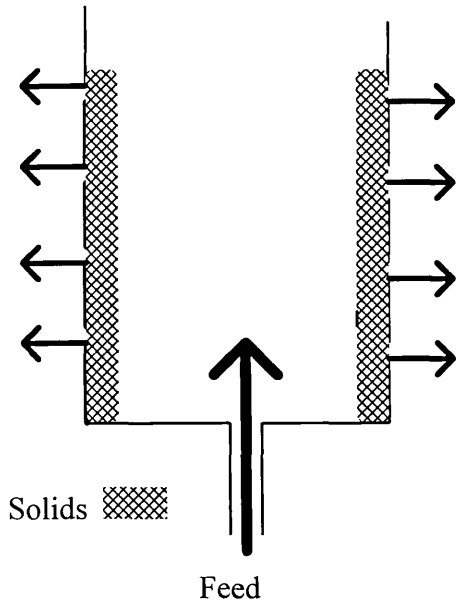
Multichamber centrifuges have high efficiencies because of the long residence time of the liquor in the unit. A mathematical model similar to that used for the tubular centrifuge can be applied to the multichamber centrifuge provided that satisfactory assumptions and modifications are made.

4.1.4.3 Imperforate Basket Centrifuge

This is an adaption of the standard basket centrifuge used for centrifugal filtration made by replacing the bowl with an imperforate one. The resulting arrangement is very similar to a tubular bowl centrifuge but the length to diameter ratio is far smaller. Usually 0.6, compared to 4-8 for a tubular centrifuge. Separation efficiency is generally lower because of the shorter clarification length of the bowl compared with the zone disturbed by feeding and it varies widely with different methods of feeding.

Basket centrifuges are usually operated with a vertical axis of rotation. The feed is introduced near the bottom of the bowl the solids separate at the bowl wall and the clarified liquid overflows the lip of the ring dam at the top and is discharged continuously.

Fig 4.3 Imperforate Basket Centrifuge

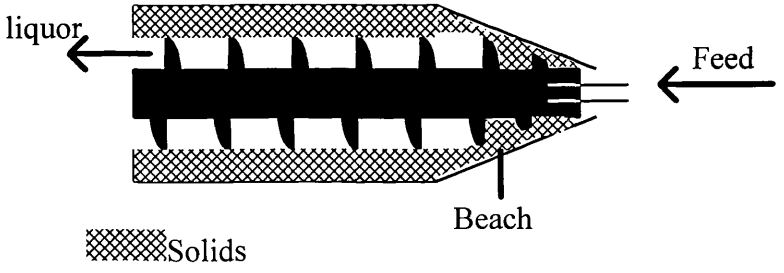


The mode of solids discharge depends on the type of solids handled soft and plastic solids are skimmed at full speed and coarse and fibrous are removed at slower speeds by a ploughing knife the cake drops though the open bottom of the bowl.

4.1.4.4 Scroll Type Centrifuges

Often called decanter-type centrifuges and characterised by their ability to continuously discharge liquids and solids. The centrifuge consists of an archimedian screw within a rotating bowl, which rotates at a slightly different speed to the screw (a differential speed of 5-100 rpm). Solids are scrolled along the bowl and clarified liquor is washed out of the other end. Generally the bowls have a conical end section so that solids are scrolled up a "beach", above the liquid layer for good dewatering, before being discharged. Some machines consist entirely of this conical end section.

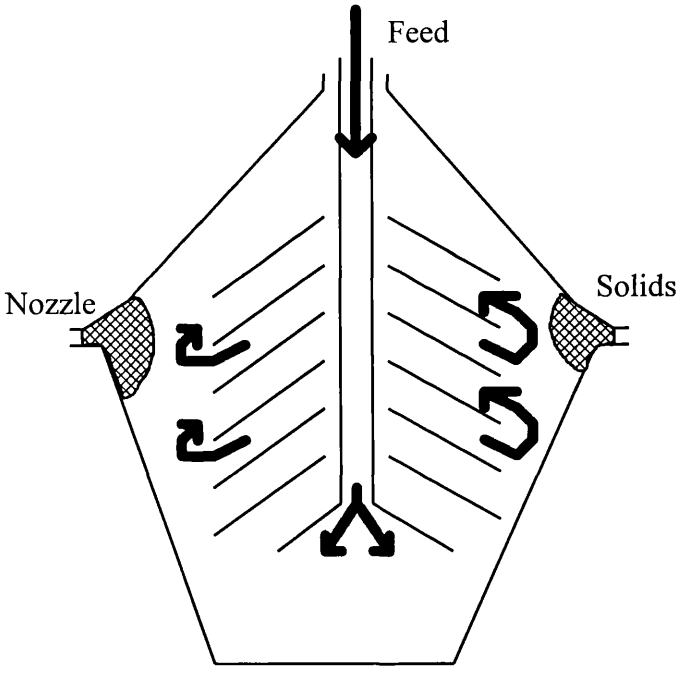
Fig 4.4 Scroll Type Centrifuge



4.1.4.5 Disc Stack Centrifuges

Disc stack centrifuges consist of a set of conical discs, with spacing strips between each disc, so that a cone is formed. Suspension enters the machine through a central pipe, which passes through the centre of the cone. The material leaving the pipe then moves upwards passing inbetween the discs. The close spacing of the discs helps the solids sediment rapidly, which then pass to the edge of the bowl as well as increasing the sedimentation area .

Figure 4.5 Disc stack centrifuge



There are three main designs of disc centrifuge:

- Solids retaining type
- Nozzle type
- Solids ejecting type

Solids retaining type

This is the simplest type and operates in batch mode. The device has a non perforate bowl parallel parallel to the axis of rotation. In order to avoid frequent manual

cleaning this type is used with low solids concentrations, <1% by volume. Cleaning is sometimes facilitated by using disposable paper liners typical dirt holding capacity is 5-20 L. The most frequent application is the separation of cream from milk.

Nozzle type

Continuous discharge of the solids as a slurry is possible with the nozzle type disc centrifuge. The shape of the bowl is modified so that the sludge space has a conical section. The walls of the bowl slope towards a peripheral zone containing a number of evenly spaced orifices, known as nozzles. The number and size of the nozzles is optimised in order to avoid build up of the cake or blockage and to obtain a reasonably concentrated sludge. The number of nozzles ranges from 12 to 24, the size from 0.75 to 2 mm.

Solids ejecting type

Intermittent solids ejection is achieved by the inclusion of a number of peripheral ports which are closed with valves. These are controlled either by a timer or by a device that senses the cake depth. The solids ejecting type is used where there is a medium concentration of solids, such as 2-6% so that neither continuous discharge nor batch operation would be optimum. It is also useful for solids which break down or deaggregate under the shear forces of nozzle discharge. Depending on the length to time the rotor remains open either total discharge (of the entire bowl contents) or partial discharge (solids only) may be obtained. Applications of this device include the concentration of cell suspensions, clarification of various juices and food extracts as well as the purification of marine fuels.

4.1.5 Modifications To Centrifuges

Standard industrial centrifuges have been modified for optimum use for biotechnological applications. There are a number of problems, the main one being prevention of mechanical disruption of particles when they are subjected to shear stresses. This has been achieved in the following ways:

- Redesign of high shear areas
- Enclosure
- Temperature control

Redesign of high shear areas

The area that generates the highest shear levels in most centrifuges is the feed zone. The redesign of devices to include "soft" feed zones prevents shear damage and prevents air entrapment which could cause oxidisation and denaturation at gas/ liquid interfaces of the biomaterials (Erikson, 1984). For example, in the case of the scroll decanter centrifuge redesign of the feel zone has lead to a conical shaped inlet device which gently accelerates feed to bowl speed.

Also the hydro-hermetic disc centrifuge, which combines the advantages of the fully-hermetic and semi-hermetically sealed centrifuges (Mannweiler, 1990). As for semi-hermetic feed zone design the feed suspension is fed into the rotating bowl via a stationary feed pipe. Discharge of the clarified feed is performed by a centrifugal pump. Unlike a semi-hermetic design the bowl is filled to the centre with feed suspension, as a full-hermetic device, but without the latter's need for mechanical seals. Since the feed suspension is injected into a liquid filled distributor the conditions experienced by the entering suspension are thought to be far less violent compared with the semi-hermetic feed zone design. The formation of air-liquid interfaces is also reduced to a small area created by the free surface of the liquid circle around the rip-body in the upper neck section of the distributor.

Differences between hydro-hermetic and semi-hermetic centrifuge are

- Rib-body design
- Hydro-hermetic disc
- Neck section wall

All are located in the neck section of the distributor. The rib-body enables the feed section to fill the fill the space underneath the hydro-hermetic disc, to the centre of the bowl, and to immerse the stationary feed pipe into the feed suspension. It also compensates the pressure generated by the radial ribs located in the conical foot-section of the distributor. (Or feed would overflow from the distributor) The ribs of the rib-body cause fluid to rotated with the same angular velocity as the bowl generating a sealing liquid circle above the hydro-hermetic disc and the stationary feed pipe.

The walls of the neck section are smooth and not axially ribbed like semi- and fully-hermetically sealed designs.

Enclosure

Many centrifuges are now hermetically sealed. This has two main advantages. Hermetic sealing eliminates the central vortex and reduces the impact of the suspension on the rotating bowl as well as reducing turbulence (Placek and Teague, 1988). Manweiler (1990) studied the effects of shear breakage on soya protein precipitates in three different feed zones. Results showed that most breakage occurred in non-hermetic feed zones and the least in fully-hermetic feed zones. Hermetically sealed systems prevent the formation of aerosols. This protects workers from pathogenic substances and also prevents airborne contamination of a sterile product.

Temperature control

Bowl cooling is available on many devices. The majority of biological products are thermally unstable and the action of centrifugation leads to heating of the suspension. Liquid temperature rises by 3-6°C and solids 15-20°C during disc stack centrifugation (Erikson, 1984). Cooling is facilitated by the inclusion of cooling jackets surrounding the centrifuge bowl through which cooled water or glycol is pumped.

Other improvements

- Improved metallurgy to achieve higher acceleration forces
- Capability of steam sterilisation
- In-place-cleaning

4.1.6 Summary

A considerable amount of work has been carried out on understanding the mechanism of operation of centrifuges. Centrifugation is a major downstream processing step in many biotechnological processes. Disc stack centrifuges are ideally suited to the recovery of whole cells.

This work will investigate the effects of centrifuge recovery on viability of whole cell *P. putida* ML2 cells after harvesting from a fermentation. The following centrifuge types will be used:

Batch	Tubular bowl
	Multichamber bowl
Continuous	Disc stack with intermittent solids discharge

An attempt will be made to calculate some of the shear stresses generated in these devices and to examine the possibility of correlation with cell viability and disruption data.

4.2 Materials And Methods

4.2.1 Analytical Assay Techniques

The following assay techniques were used foranalysis of the centrifugation work:

- Optical density
- Dry weight measurements
- Viable cell counts
- Protein assay

Methodology details are given in section 3.2.2

4.2.2 Bench Top Centrifuge

MSE Hi Spin 21

P. putida ML2 cells were harvested from the fermentation in early stationary phase. Viable cell count and protein assay measurements were made, as described in the methods and materials section of Section 2. 250 mL of cells were transferred into six centrifuge bottles and spun down at 12000 rpm for 0.3 hrs using the MSE Hi Spin (Fisons, Loughborough, England). The centrifuge was fitted with an angled bowl with a diameter of 145 mm, this gave a maximum RCF of 3150g. The cells were resuspended using a Whirlimixer (BDH Ltd., Poole, Dorset, England).

Loss of viability was measured by calculating the difference in VCC before and after centrifugation. Loss of cell integrity was measured by subtracting the background protein value (before centrifugation) from the final value (after centrifugation and resuspension) and comparing the amount released with that of a fully disrupted sample as determined by multiplle pass homogenisation at 1600 bar. This procedure was used for all the centrifugation experiments.

Microcentrifuge

P. putida ML2 cells were harvested from the fermentation in early stationary phase. 1 mL of *P. putida* ML2 cells were transferred into six eppendorf tubes and spun down at 4000 rpm for 0.3 hrs using a Sigma 113 (Funkenstorgrad, Germany). The cells were resuspended using a Whirlimixer (BDH Ltd., Poole, Dorset, England). Asays for protein release and viable cell count were made before and after centrifugation.

4.2.3 Multichamber Bowl Centrifuge

The effects of processing *P. putida* ML2 cells using a multichamber bowl centrifuge was studied. The model used was a KDD 605 (Westfalia Separator Ltd, Milton Keynes, Buckinghamshire, England) which is a hermetically sealed centrifuge with a low shear feed zone. The machine can be used with either two or four chamber bowl configurations. The bowl is mounted on a drive spindle from a gearbox below, this is driven by an end mounted three speed motor. Operational speed is 10000 rpm and this equates to a Relative Centrifugal Force of 9500g. This is a small scale centrifuge with a bowl volume of 1.5 L.

Cells were harvested from the fermentation in early stationary phase and transferred to a feed tank. Viable cell count and protein assay measurements were made, as described in the methods and materials section of Section 2. A peristaltic pump (Watson-Marlow 502S, BDH, Poole, Dorset, England) was used to transfer cells into the bowl until it was filled. The centrifuge motor was started and when it reached operational speed the cell suspension was pumped through the centrifuge at a flow rate of 500 mL min^{-1} (8.33×10^{-6} m^3 s^{-1}). Supernatant was sampled from the outlet at timed intervals. The separation bowl was deemed to be full of cells when the supernatant appeared to be cloudy when judged by visual inspection. At this point the motor was stopped and the head stripped and dismantled. Cell solids were removed and resupended in the original volume of supernatant by agitation with a 300 W Silverson mixer (Model AXR, Chesham, Buckinghamshire, England) operating at 200 rpm.

The resuspended cell suspension was then sampled and tested for viability and protein release, as described earlier. Dry weight and optical density measurements were made to allow comparison of viability results before and after processing.

4.2.4 Tubular Bowl Centrifuge

The effects of processing *P. putida* ML2 cells using a tubular bowl centrifuge was studied. The model used was a 1P (Sharples-Pennwalt Ltd., Camberley Surrey England). This is a lab scale, non-hermetically sealed high shear feed zone centrifuge, with a bowl volume of 0.3 L. Operational speed is 50000 rpm and this equates to a Relative Centrifugal Force of 62500g.
As for the multichamber bowl investigation cells were harvested in stationary phase, assayed for viability and protein release, processed, resuspended and reassayed as described above.

4.2.5 Continuous Disk Stack Centrifuge

The effects of processing *P. putida* ML2 cells using a multichamber bowl centrifuge was studied. The model used was a CSA1 (Westfalia Separator Ltd, Milton Keynes, Buckinghamshire, England) which is a non hermetically sealed continuous operation centrifuge capable of intermittent solids discharge. Operational speed is 10000 and this equates to a Relative Centrifugal Force of 9500g.

As for the two previous runs cells were harvested from the fermenter in early stationary phase and transferred to a feed tank. Viable cell count and protein assay samples were taken as described previously. As this is a continuous operation centrifuge the sampling regime was different to that described for the two batch devices tested (the multichamber and tubular bowl centrifuges).

Cell suspension was pumped into the centrifuge with a Watson-Marlow 502S peristaltic pump at a flow rate of 500 mL min^{-1} (8.33×10^{-6} m^3 s^{-1}). When the separation bowl was full of cell suspension the rotor was started. One cycle of operation was completed. That is the centrifuge was run until the bowl full of cell solids. This was judged to occur when the viewing port in the supernatant output pipe appeared to be cloudy. The solids were then discharged from the bowl. This was a partial discharge and the feed was not stopped at this time. Another cycle was completed and solids were again discharged. The cell paste was then resupended in its original volume of supernatant using the Silverson mixer Assays were carried out for dry weight, OD, protein release and viable cell count.

The centrifuge was allowed to complete another separation cycle, however instead of discharging the cell paste, the rotor was stopped. The head was stripped and the cells were recovered from within the separation bowl and resuspended and assayed as before.

The use of these two sampling regimes allowed testing of the damaged caused to the concentrated cells by the discharge stage alone.

4.3 Results

Bench top - MSE Hi Spin 21

The percentage viability and intactness of the cell population is given in the table below. % viability is the percentage of the original unprocessed cells. % intact corresponds to the percentage of the maximum protein release as measured by complete disruption of the cell sample in a high pressure homogeniser. The standard deviation of the measured values is depicted by σ .

Table 4.2 Mse Hi Spin 21 Bench Top Centrifugation Results

	% VCC	σ	% INTACT	σ
FEED	100	21	100	3
RESUSPEND	103	33	100	7

Bench top - Sigma 113

A similar technique to that described above was used for separtion of cells using a Sigma 113 centrifuge.

Table 4.3 Sigma 113 Bench Top Centrifugation Results

	% VCC	σ	% INTACT	σ
FEED	100	21	100	3
RESUSPEND	112	39	100	5

Multichamber bowl - Westfalia KDD605

The mean of the results of three centrifugation runs of *P. putida* ML2 using the Westfalia KDD605 multichamber bowl centrifuge is given below.

Table 4.4 Westfalia KDD605 Multichamber Bowl Centrifugation Results

	% VCC	σ	% INTACT	σ
FEED	100	29	100	3
RESUSPEND	91	20	97	9

Measured values are given in Appendix 4.1

Tubular bowl centrifuge - Sharples-Pennwalt 1P

The mean of the results of three centrifugation runs of *P.putida* ML2 using a Sharples-Pennwalt 1P tubular bowl centrifuge is given below.

Table 4.5 Sharples-Pennwalt 1P Tubular Bowl Centrifugation Results

	% VCC	σ	% INTACT	σ
FEED	100	19	100	4
RESUPEND	87	40	93	10

Measured values are given in Appendix 4.1

Continuous, intermittent discharge disk stack centrifuge - Westfalia CSA1

The mean of the results of three centrifugation runs of *P. putida* ML2 using a Westfalia CSA1 disc stack centrifuge capable of intermittent solids discharge is given below.

Table 4.6 Westfalia CSA1 Disc Stack Centrifugation Results

	% VCC	σ	% INTACT	σ
HARVEST	100	27	100	7
NO DISCHARGE	79	16	98	11
DISCHARGE	78	12	95	5

Measured values are given in Appendix 4.1

Of all the centrifugation operations tested the least cell damage was measured after cells had been processed and resuspended by the multichamber bowl centrifuge. The most measured damage was caused by processing with the tubular bowl centrifuge. When processing with the disc stack centrifuge there was a surprisingly small, almost negligable, difference between damage measurements from the discharged cells and those sampled directly from the separation bowl.

4.4 Discussion

No cell damage was detected after *P. putida* ML2 cells had been centrifuged in either of the two bench top devices. This appears to indicate the effects of possible oxygen starvation are minimal over the separation period. A degree of cell damage was measured after separation of cells from their fermentation broth using a multichamber bowl, tubular bowl and disc stack centrifuge. The smallest amount of cell damage was measured after processing with the multichamber bowl centrifuge. The maximum was by the tubular bowl. Both of these devices operate in batch mode, that is neither discharge solids. Cell paste accumulates within the bowl while the supernantant is ejected. The maximum relative centrifugal force within the multichamber centrifuge is 9500 g whereas in the tubular bowl it is 62000 g, at 50000 rpm.

Cellular damage was also measured after processing with the CSA1 intermittent discharge disc stack centrifuge which operated in continuous mode. The measured loss of cell viability was approximately 15% with approximately 5% disruption. The majority of papers reporting on damage caused to cells by centrifugation with disc stack centrifuges state that the maximum shear levels are generated in the feed zone (Bell, 1982). However the discharge of viscous cell paste from such a machine might also be expected to contribute to the overall cellular damage. The concentration of cell paste recovered after discharge was at approximately 100 g dry wt L^{-1}. Datar and Rosen (1987) measured the release of β-galactosidase from *E. coli* cells after processing with similar machine to the CSA1. They reported an overall loss of intracellular enzyme in the region of 15% and attributed 10% of the loss to the discharge stage.

Total cell damage was measured by resuspension of the solids after a complete separation-discharge cycle. The damage caused by the feed and separation stages alone was measured by sampling the solids directly from the bowl without discharging. This involved allowing the bowl to fill with cell paste, stopping the motor before discharge, dismantling the bowl, recovering the cells, resuspending and assaying.

It is perhaps surprising that the rapid pumping of a viscous, high concentration, cell paste from the centrifuge bowl did not result in any significant damage to the cells. The magnitude of the shear forces generated were obviously below that of the critical stress value (see Chapter 6).

The total damage to the cells was seen to be in the region between that caused by the multichamber and tubular bowl centrifuges. The expected cell damage caused by the rapid discharge of the sedimented cells was not observed. Cell damage appears to occur during separation prior to discharge.

The reasonably large standard errors given alongside the viable cell count results highlight the difficulty involved with sampling and analysis. The maximum loss of viability is approximately 20% and the errors are all in this region.

Chapter 6 contains a more detailed analysis of these centrifugation results. An attempt is made to estimate the magnitude of the shear forces generated by operation of the centrifuges.

5 Microfiltration

5.1 Introduction

5.1.1 Use Of Membrane Separation In Biotechnology

Particle separation steps often play an important part of the downstream processing stage of a biotechnology production process. The advent of recombinant DNA technology has highlighted the importance of being able to separate shear-sensitive cells from their surroundings. Particular interest is currently being shown in membrane technology for replacing more traditional particle separation methods such as centrifugation and flocculation.

Membrane technology is currently being used at each stage of a fermentation process:

Pre-fermentation	sterile filtration of nutrient solutions
	separations during medium preparation.
Fermentation	biomass recycle
	on-line analysis
	removal of inhibitory products
Post-fermentation	recovery of biomass
	recovery of products (Bashir and Reuss, 1992)

Particular interest is being shown in cross-flow, pressure-driven membrane filtration for the following reasons:

- No phase or temperature change is involved, eg freeze drying, spray drying.
- The separation process is direct and there is no requirement for additives (eg coagulants and flocculation), foam fractionation and filter aid filtration.
- The property difference exploited is molecular weight or size. This can be very large when comparing whole cells with products or waste molecules. Continuous centrifugation relies on a relatively small density difference between the species. Energy required to create the high gravitational forces to overcome the low density difference of the culture broth system can constitute upto one third of the operating costs. (Haarstrick *et al.*, 1991)

- The use of closed modules greatly reduces the formation of aerosols such as those formed by centrifugation or foam fractionation.

This work sets out to investigate the effects of concentration processing on microbial viability. The damage effects of microfiltration are not well understood unlike those of centrifugation. The following pages are an overview of current membrane filtration technology with details of operation and problems to be addressed.

5.1.2 Types Of Membrane Processing

A membrane is best considered as a thin film of material interposed between two fluid phases, which allows the selective permeation of particles. The process of separation is governed by the interaction of a number of factors (Stitt, 1980):

- Particle or molecular size
- Chemical or physical affinity to the membrane material
- Mobility of the permeating species within the membrane.

As with any separation process work has to be done on the system and this energy requirement is met chemically, mechanically or electrically. The method of supplying energy provides a driving force for the mass transfer of a concentration, potential or pressure gradient. It is possible to crudely classify the separation process by the nature of this driving force.

Table 5.1 Separation Processes That Have An Energy Requirement

	Driving force	Operation
Pressure driven	Pressure gradient	Microfiltration Ultrafiltration Hyperfiltration
Electrically driven	Voltage gradient	Electrodialysis
Chemically driven	Concentration gradient	Dialysis Liquid membranes Pervaporation

There are four membrane separation techniques that are frequently used throughout the biotechnology industry. These are hyperfiltration, ultrafiltration, microfiltration and conventional or "dead end" filtration. The differences are outlined in the following table:

Table 5.2
Biotechnology Industry Applications Of Membrane Separation Processes

Operation	Particle size / μm	Application
Conventional filtration	$<10^5$	also known as "dead end" filtration
Microfiltration	0.1-10	filtering of fine or colloidal suspensions. Especially cell suspensions
Ultrafiltration	0.005-1	fractionation of dissolved molecules according to size
Hyperfiltration	<0.1	separation of solvent (usually water) from dissolved solutes.

The use of a particular operation is generally dependent on particle size. Boundaries between different applications are not distinct due to the interaction of other factors such as affinity to the membrane material and mobility of the permeating species.

Conventional filtration

Due to low flux rates and scale-up difficulties conventional filtration is only used when shear-sensitive separations are to be carried out.

Microfiltration

Has the largest number of applications in biotechnological downstream processing. The technique is limited to the concentration of suspensions or slurries, not complete solid-liquid separations. It is widely used for the concentration of cell suspensions and in particular the concentration of bacterial cell suspensions.

Ultrafiltration

Often used as an alternative to centrifugation for the recovery of protein precipitates. (Devereux *et al.*, 1986a) The choice is usually dependent on the performance of the membrane at high precipitate concentrations.

Ultrafiltration membranes are generally characterised by the size of the smallest molecule that they will retain. This is known as molecular weight cut-off (MWCO) and is a crude way of describing the pore diameter as molecular weight and size of macromolecules is roughly similar. There is a relatively wide distribution of pore diameters in most membranes and so it is difficult to separate components of similar size. No standard has been set for the determination of MWCO. Molecular shape and rigidity have an important influence on a membranes steric rejection The retention of linear, flexible molecules is normally an order of magnitude less than that for a globular or cyclic molecule of the same molecular weight. This generally holds true for microfiltration as well.

Hyperfiltration

Also known as reverse osmosis, uses in the biotechnology industry include:

Purification of water	for research and production processes.
Wine and beer treatment	tartar removal
	modification of alcohol content
Dairy	dewatering cheese whey
Concentration	fruit juices and sugar solutions

5.1.3 Problems With Conventional Filtration Techniques

The basis of "dead end" conventional filtration is the flow of solution across the membrane surface. Solvent passes across the membrane and solute is trapped. Inevitably the membrane becomes fouled by the trapped solute particles, which form a "cake" over the surface of the membrane. Any force applied to increase fluid flow across the membrane will cause compression of the cake of trapped particles making it even more impenetrable to the solvent. This greatly reduces efficiency and therefore increases the time taken for the separation.

This unavoidable problem with conventional filtration has become the driving force for the advances made in membrane technology of recent years. To prevent fouling membrane operations now generally employ cross-flow rather than 'dead end' flow. The solution flows tangentially, rather than perpendicularly to the membrane. Shear forces generated by fluid motion across the membrane surface inhibit fouling of the membrane by preventing the deposition of the suspended particles onto the membrane surface. Permeation rates are increased and so is separation efficiency The shear forces that remove the fouling layer can unfortunately have deleterious effects on the cells leading to disruption. The cross-flow velocity and the applied pressure are determined by the nature of the separation. (Nagata *et al.*, 1989)

As has been mentioned before in any biotechnological process there are three possible products:

- intracellular product
- extracellular product
- whole cell product

In the context of this project the whole cell is the product of interest. Not only do we want whole, intact cells but also fully functioning viable ones. Laminar shear stresses are the major cause of disruption of the cells. This can limit flow rate and hence flux rate. (Maiorella *et al.*, 1990) When the cells are disrupted they release their intracellular contents into the media. One of the major constituents of the intracellular contents is protein. Protein release can cause problems by possible interaction with the membrane surface and blocking of pores. If protein is retained by the membrane its concentration could increase until it forms an impenetrable gel layer close to the surface of the membrane, decreasing flux rates.

The majority of membrane research is concerned with flux rate production and fouling characteristics of membrane fouling. A great deal of work has been published concerning ultrafiltration, the mechanisms of which are widely understood. (Bentham *et al.*, 1987, Devereux *et al.*, 1986a & b) Microfiltration modelling is less certain but there are a number of papers that describe certain behaviours. (Nagata *et al.*, 1989, Asaadi & White (1992), Jaffrin *et al.*, (1992)) Very little work has been published which describes the effects of microfiltration on bacterial viability alone and almost all publications to date have concentrated on flux prediction and fouling mechanisms. The purpose of this section of the work is to investigate effects of process conditions on viability. The following pages describe the construction of microfiltration units, effects of processing conditions and problems encountered.

5.1.4 Microfiltration For Whole Cell Separation

To efficiently concentrate a bacterial cell suspension using a membrane process microfiltration is generally carried out. Experimental work will focus on a comparison of microfiltration with established centrifuge techniques.

Widespread industrial applications of cross-flow microfiltration are:

Concentration of slurries and suspensions	Harvesting of bacterial cells Sterile filtration
Removal of suspended matter from effluent waters	ZnS in television screen production Recovery of metallic colloids
Clarification of process streams	Vinegar Fruit juices

Cross-flow microfiltration has a number of advantages over centrifugation:

- The use of 0.2μm pores ensures complete elimination of bacteria (provided there are no faults in the membrane). This is not possible with other separation techniques eg centrifugation, deep bed filtration etc Therefore the use of chemicals for flocculation, coagulation etc is minimal. As the product of interest is viable cells no contact with toxic treatments will take place during downstream processing.
- The physical and chemical tolerance of ceramic membranes allows easy cleaning, maintenance and sterilisation.

The main problem associated with microfiltration is the lack of efficiency at high cell concentrations. This due to reduced flux rates because of a number of fouling effects which cannot be relieved by increasing operating parameters such as cross flow velocity or transmembrane pressure.

5.1.5 Construction Of Membrane Rigs

5.1.5.4 Membrane Unit

Microfiltration membranes are generally constructed of polypropylene and PTFE. Membranes are usually manufactured by a casting technique. Other methods of production:

- Track etching
- Stretching a sheet of polymer to yield a thin microporous film.
- Liquid phase separation

Final thickness of the membrane is 100-250 μm. To improve mechanical properties this is then bonded to a porous support. Flow resistance of the support is very small compared to that of the membrane itself.

Inorganic membranes, usually referred to as ceramic or mineral membranes, were developed for the French nuclear industry and are now widely available. Advantages of ceramic over organic membranes is their temperature stability (especially for heat-based sterilisation techniques) and their resistance to chemical attack.

Disadvantages include sensitivity to mechanical shock and high purchase price.

Unit Configuration

The membrane modules can be made in a variety of different forms, depending on their intended use.

- Flat sheet
- Plate and frame
- Tubular unit
- Hollow fibres
- Cartridge Membrane

Figure 5.3 (overleaf) shows how the characteristics of different membrane configurations compare to each other.

Table 5.3 Characteristics Of Different Membrane Configurations

	area / volume m^2 / m^3	Cost to buy	Cost to run	flow control	ease of clean
Flat sheet	-	low	low	poor	good
Plate and frame	400-600	high	low	good	good
Tubular unit	25-100	high	high	good	good
Hollow fibre	600-1200	low	low	good	fair
Spiral wound	800-1000	low	low	poor	poor
Cartridge membrane	300-600	high	fair	good	good

area / volume surface area of membrane per unit volume of module

Membrane filtration plants are generally built up from a number of different modules. Scale up simply involves the addition of further modules to increase the membrane surface area. There are two types of process plant configuration:

Closed loop feed liquor is withdrawn from a feed tank, passed through a membrane unit, recycled to the inlet of the membrane unit. This reduces the damage caused to shear sensitive cells by the repeated passage through the filtration loop.

Open loop As above but retentate recycled to the feed tank.

A common requirement for ultrafiltration and microfiltration processes is that the concentrated, retained product should have a high level of purity so frequently a volume of water (or solvent) is gradually added to the feed tank and permeable impurities are flushed through. This is known as dilution mode or diafiltration and is used in batch and continuous processing and is analogous to cake washing in conventional filtration.

Single stage continuous processes are inefficient due to the high average retentate concentration in the modules. To obtain higher average fluxes multi-stage systems are often used, with each stage having its own closed loop recycle. Each stage

operates at successively higher retentate concentrations, with only the final stage operating at a retentate concentration close to that of the product. Continuous operation is less efficient than batch processing due to the higher average concentrations and the reduction in time-averaged fluxes. The use of multistaging allows greater efficiency, a three stage plant will typically attain 80% of the efficiency of a batch plant in terms of membrane area utilisation.

5.1.5.2 Pump

Correct choice of pump is vital for the sucess of a microfiltration operation. Factors affecting choice of pumping equipment for a microfiltration rig used for cell recovery are:

- Quantity of liquid to be handled
- Head to which liquid is to be pumped
- Nature of liquid
- Nature of power supply
- Corrosion possibilities if used intermittently
- Cost of purchase and maintenance
- Shear stresses generated during operation

The following types of pump are widely used in industrial applications:

- Piston pump
- Diaphragm pump
- Positive displacement pumps:
  - Gear pumps
  - Peristaltic pumps
  - Mono pumps
  - Screw pumps
  - Centrifugal pumps
  - Rotary vane pumps

Piston pump

Consists of a cylinder with a reciprocating piston connected to a rod. Liquid enters from a suction line through a suction valve and is discharged through a delivery valve. Flow rate 0.02 m^3 s^{-1} at a pressure of 100 MN m^{-2}.

Diaphragm pump

Two section construction separated by a diaphragm. One section a plunger or piston operates in a cylinder in which a non corrosive fluid is displaced. The movement of the flied is transmitted by means of the flexible diaphragm to the liquid to be pumped. The only moving parts in contact with the liquid are the valves. Diaphragm moved by direct mechanical action or by air driven.

Positive displacement pumps

Gear pumps

These consist of two gear wheels operate inside a casing, one of the gear wheels is driven and the other operates in mesh with it. The liquid is carried around the space between consecutive teeth and the casing and is then ejected as the teeth come into mesh. The pump has no valves and depends for its seal on the small clearance between the gear wheels and the case. Delivery is almost independent of pressure and priming is unnecessary. Suitable for high viscosity liquids, however cannot be used for suspensions because spaces between the gears are small.

Peristaltic pump

A length of elastic tubing (usually silicon tubing) is compressed in stages by means of a rotor. Tubing is fitted into a curved track mounted concentrically with ac rotor carrying three rollers. As the rollers move they flatten the tube against the track at the points of contact. Theses flats move the fluid by positive displacement, flow can be precisely controlled by the speed of the motor. Not generally suitable for high pressure and flow rate operations.

Mono pump

A helical metal rotor revolves eccentrically within a double helix resilient rubber stator, of twice the pitch length of the metal rotor. A continuous forming cavity is

created as the rotor turns, the cavity progressing towards the discharge, advancing in front of a continuously forming seal line and thus carrying the material with it.

Screw pump

Fluid is sheared in a channel formed between the screw and the wall of the barrel.

Centrifugal pump

Most widely used design. Fluid is fed to the centre of a rotating impeller and is thrown out by centrifugal action.. The liquid acquires high kinetic energy from the high speed of rotation and the pressure difference between the suction and delivery sides arises from the conversion of kinetic into pressure energy.

The impeller consists of a series of curved vanes, to allow as smooth a flow as possible. The more vanes the less energy is lost due to turbulence and circulation between the vanes. Efficiency depends on the vane angle, the throughput and the speed of rotation.

Advantages:

- Simple construction
- Absence of valves
- High speeds
- Steady delivery
- Low maintenance costs
- No damaged if blocked
- Small size
- Suitable for processing suspensions

Disadvantages:

- Not suitable for operation at high pressures
- Highly specific conditions for high efficiency operations
- Not usually self priming
- Needs a non return valve to stop liquid sucking back when motor tuned off
- Not very good for viscous liquids

Rotary vane

Similar construction to centrifugal pumps. The impeller is mounted centrally in the casing. Vanes can slide in and out of the central housing and therefore touch side of casing when thrown out by centrifugal forces during operation. This seals the unit, hence there is no need for valves to prevent suck back when motor is off.

This design has many of the advantages of centrifugal pump but with far lower shearing characteristics during operation. This is because the majority of shear forces generated within a centrifugal pump are believed to reside between the impeller tip and the pump casing, and theoretically there is no such gap in a rotary vane pump.

Tests on cellular inactivation after pumping suspensions with different types of pump were carried out by Shimizu *et al.* (1992). It was found that least damage was inflicted on *P. putida* cells when using a rotary vane pump, followed by a centrifugal, followed by a screw type pump.

The rotary vane pump is the most suitable choice for pilot and laboratory scale microfiltration rigs with the main disadvantage being the higher purchase cost than for equivalent centrifugal pumps.

5.1.6 Effects Of Membrane Process Conditions

Membrane theory has not been successfully investigated for many aspects of microfiltration. As has been mentioned earlier a sucessful membrane separation procedure requires a careful balance of operating conditions. Simply running the operation at high transmembrane pressures can lead to formation of an inpenetrable layer of solids fouling the membrane. In some cases this can be removed by increasing the cross-flow velocity of the feed liquid. The resultant high shear forces exerted on whole cells at high velocites often leads to cellular disruption, which in turn causes increased fouling by intracellular components. The study of fouling rates and prediction of permeate flux rates is not within the scope of this work. The effects of cellular content release on operating parameters will be noted. The following section is a very brief overview of the effects of operating parameters on the separation procedure.

Transmembrane pressure Δp_{tm}

This is the pressure difference across the membrane. It is the mean of the inlet and outlet pressures taking into account any back pressure on the permeate side of the membrane.

$$\Delta p_{tm} = \left(\frac{p_{inlet} + p_{outlet}}{2} \right) - p_{permeate} \qquad (5.1)$$

p_{inlet} Feed (inlet) pressure

p_{outlet} exit (outlet) pressure

$p_{permeate}$ permeate pressure

(Gaddis, 1992)

Higher pressures increase the rate of fouling. This is probably due to the compaction of the fouling layer at higher pressures leading to greater hydraulic resistance and lower flux. High pressures result in a high initial flux which rapidly drops to a very low one. An example is the microfiltration of polycarbonate beads by Wakeman and Tarleton (1991):

at 8.6 kPa it took 238 min to concentrate from 10-70 g L^{-1}
at 103 kPa it took 477 min to concentrate from 10-70 g L^{-1}

Increasing the filtration pressure results in an increased permeate flux, but the increase can be small and not in proportion to the increase in filtration pressures. This is particularly so for smaller particles At lower pressures an equilibrium flux is established more rapidly. Fouling takes place much more rapidly at higher flux rates achieved by increased transmembrane pressures.

Transcartridge Pressure

The pressure drop along the module is known as the transcartridge pressure, Δp_c.

$$\Delta p_c = p_{inlet} - p_{outlet} \qquad (5.2)$$

Flow Rate.

Higher flow rates reduce the rate of flux decline. This is due to the removal of the fouling layers by higher shear stresses.

Cross-Flow Velocity

Cross-flow velocity is the speed of liquid flow through the membrane unit and is a function of the trans cartridge pressure, Δp_c

Increasing the cross-flow velocity can either increase or decrease the filtrate flux. The effect observed is attributable to the size and size distribution of the dispersed phase and the behaviour of that phase at the membrane surface. There is evidence that a critical particle size will exist for any set of operating conditions, such a that cross-flow velocity will have no effect on the permeate flux decline curve.

Feed Composition

Permeate fluxes are generally lower with feeds containing smaller particle sizes, although equilibrium fluxes are established more rapidly. Flux levels are also apparently lower with feeds containing smaller particle sizes, but if the size distribution of the coarser particles includes any smaller particles the equilibrium flux reached may be small as that obtained with a feed containing a smaller mean size. However, the rate of approach to equilibrium is more rapid when no coarse particles exist.

Taddei *et al.* (1990) claimed that in the case of processing cider containing brewers yeast, the proportion of nonviable cells had an effect on the resultant flux. The higher the proportion of non-viable cells the higher the flux. In the same paper they investigated the effects of aeration and agitation. The yeast fermentation was sparged with air for 48 hours before filtration was carried out. This had no effect on the yeast content or the viability. A decrease in air flow rate during this "aerobic" phase improved the flux by 200%. This is due to the composition of the cell membrane. Synthesis of unsaturated fatty acids and sterols in yeasts only occurs in the presence of oxygen. The reason why this increased flux rates was not mentioned.

Feed Concentration

For all membrane filtration systems increasing feed concentration may lead to a reduction in both permeate production rate and purity. In ultrafiltration and microfiltration the situation may be made worse by changes in feed stream rheology. Viscosity of macromolecular solutions and particle suspensions can be very sensitive

to concentration. Increases in viscosity leads to decreased Reynold's number and mass transfer coefficient and therefore more concentration polarisation.

Higher cell concentration leads to greater fouling rates but there is an interaction between cell concentration, transmembrane pressure and flow rate. This suggests there may be a critical pressure-velocity combination above which the rate of fouling may increase at higher velocities. A similar critical pressure-velocity combination was found to exist for spiral-wound ultrafiltration of cottage cheese whey (Kuo and Cheryan 1983)

Reismeier and Kroner (1987) showed that with increasing particle concentration the initial flux decline becomes more marked. The differences in initial flux decline will be reduced with increasing particle concentration. They postulated that in filtration of a suspension with a low particle concentration a single particle has several possible locations at which to make contact with the membrane surface. The competition for an optimal position is not very high and it takes longer to fully develop a fouling sub layer. This will change with increasing particle concentration until a point is reached where the difference between the cell concentrations is negligible.

5.1.7 Membrane Processing Problems And Their Solutions

Problems

There are a number of factors that lead to a loss in flux, and therefore decreased process efficiency and increased running costs. Two limiting phenomena are generally considered:

- concentration polarisation
- membrane fouling

The accumulation of material on the membrane surface accounts for a gradual flux decline while processing under steady-state conditions. It is generally regarded as an additional resistance R_f to the membrane resistance R_m. Flux stability is an important factor in plant running costs. The rate of flux decline and the consequent frequency of shutdown for cleaning or membrane replacement are directly attributable to the nature and severity of the membrane fouling. Huge differences in performance of claimed similar membranes occur as well as marked differences in

supposedly similar membranes, this is due to susceptibility to fouling and the process liquor.

Membrane Fouling

Membrane fouling is distinguishable from concentration polarisation by the fact that it is an irreversible and time dependent process. This is the first part of the flux decline process and is due to small species in the feed stream being captured and embedded in the membrane pores. In particular adsorption by intracellular contents, organisms and colloids are the major causes of fouling (Mackley and Sherman, 1992).

Concentration Polarisation

This is the second stage of fouling. The performance of a membrane system is dependent on the solute concentration at the membrane-solution interface, C_W. Concentration polarisation is a phenomenon which results in wall concentrations being substantially higher than those in the bulk phase (McDonogh *et al.*, 1992). This is due to the build up of layers of rejected solids on the membrane surface due to the (transmembrane) pressure gradient. These layers can be either mobile or stagnant and the fouling is reversible. (Wakeman and Tarleton, 1991) Flux is lowered due to The hydrodynamic resistance of the boundary layer is increased by the addition of this polarised layer. (Patel, 1987)

Solutions

When designing any membrane separaration process the efficiency of the separation must be balanced against possible damage to the product by extreme process conditons. Prevention of membrane fouling is the most obvious method of increasing efficiency. Concentration polarisation seems unavoidable in any pressure driven membrane process. However the initial, irreversible membrane fouling due to adsorption of small particles can be controlled. These small particles inevitably are intracellular components such as proteins. These are released when cells are lysed. There are a variety of different ways of combating the problems of lowered flux rates due to membrane fouling. The most common involve the pre treatment or the process fluid and fluid management.

There is no need for pre-treatment if the foulants can be readily removed by periodic flushing or cleaning of the unit. Two methods of cleaning exist back-flushing and recycling, depending on the nature of the foulant and the nature of the membrane and its resistance to chemical cleaning elements.

Back-flushing

Back-flushing involves forcing permeate back through the membrane in order to lift off or loosen any cake or gel layer that has formed on the surface of the membrane. This has been shown to be an extremely effective technique. Examples of the advantages are widely reported (Matsumoto *et al.*, 1988, Boothanon *et al.*, 1991, Finnegan and Howell, 1989) For example for the microfiltration of apple juice by Gupta *et al.*, 1992. Pulsing by a pneumatically operated piston in a sinusoidal motion at 1Hz increased the permeate flux by fifty percent. The stroke volume was twice the internal volume of membrane.

Recycling

Involves switching off the permeate outlets and allowing the feed stream to recirculate. Permeate is continuously produced, resulting in a build up of pressure on the permeate side of the membrane. Eventually both sides of the membrane will be at equal pressures and the cross flow may effectively clean the membrane. This process can be made more effective by the use of suitable cleaning agents. Ceramic membranes are particularly advantageous in this situation as they can withstand severe cleaning methods such as steam or moderately concentrated acid or alkaline solutions.

5.2 Materials and Methods

5.2.1 Analytical Assay Techniques

The following analytical assay techniques were used to monitor cell damage during microfiltration trials:

- Optical density
- Dry weight
- Viable cell count
- Protein assay
- Viscosity
- Biotransformation ability (BTA)

Details of methodology are given in Section 3.2.2.

5.2.2 Membrane Rig

The membrane rig was designed at UCL and manufactured by Biodesign Ltd. It was upgraded at UCL during the course of this work. It consisted of a simple loop with a membrane unit, pump, feed tank and control apparatus. Components of the rig were constantly updated throughout the time of this work and eventually the configuration was changed to two allow the use of another membrane unit with a surface area approximately ten times the size of the original. This allowed cell concentration work to be carried out.

The configuration of the rig components are shown in the following two figures:

Fig 5.1 Original membrane filtration loop with 0.005 m^2 membrane unit
Fig 5.2 Modified rig with additional 0.05m^2 membrane unit

Details of individual components and their operation follows.

Fig 5.1 Biodesign Cross-Flow Microfiltration Rig Configuration

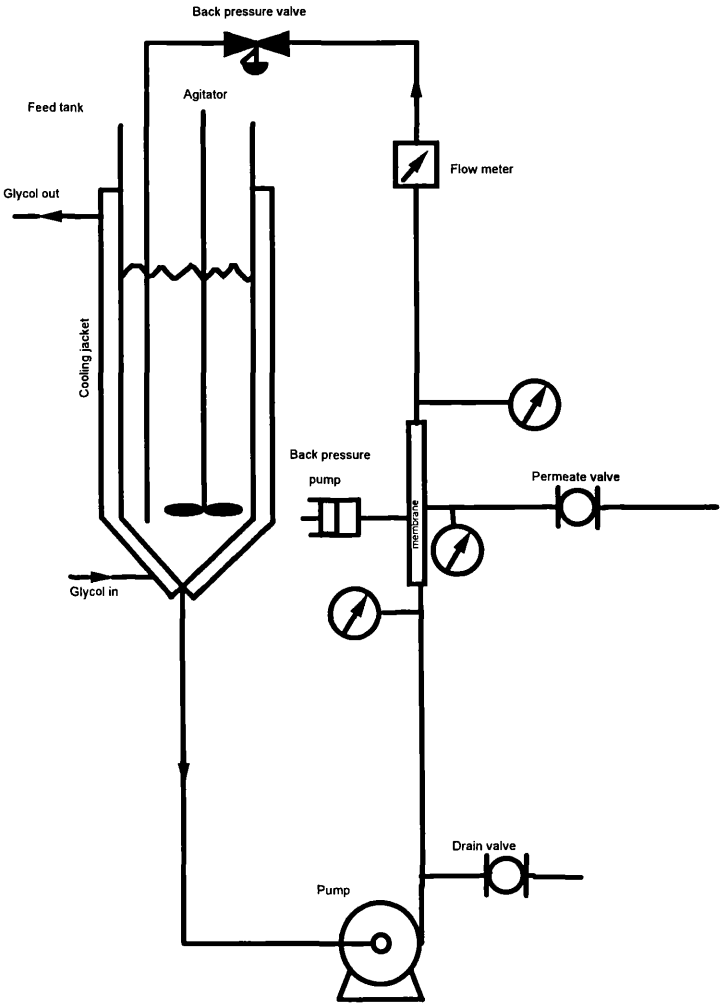
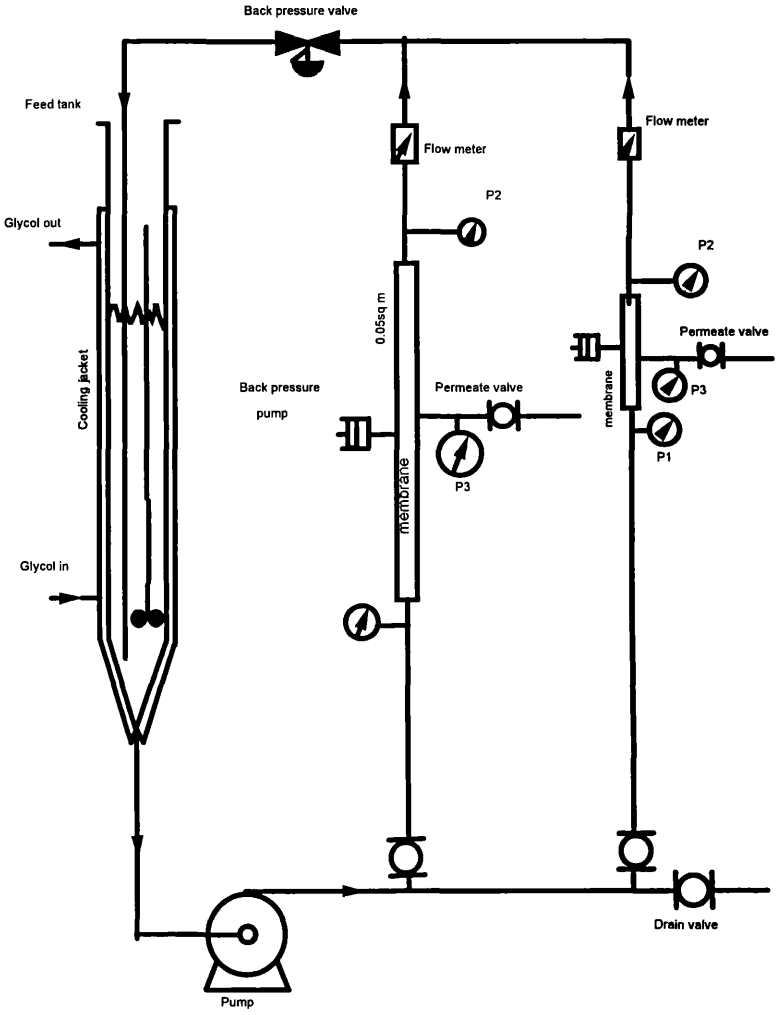


Fig 5.2 Membrane Rig After Addition Of Extra Membrane Module



Details of the components

Membrane Cartridges

Two membrane cartridges were fitted to the rig. Liquid flow could be directed through either cartridge by the operation of valves V1 and V2 (Fig 5.2).

Table 5.4 Properties Of Membrane Modules

	small	**large**
manufacturer	Fairey Tecramics, Stoke, UK	SCT, Bazet, France
material	ceramic (α-alumina)	ceramic (α-alumina)
X-section shape	circular	star
pore size	0.2 μm	0.2 μm
length	0.25 m	0.6 m
diameter	0.006m	0.006m
surface area	0.0047m^2	ID 0.002m, OD 0.006m 0.06 m^2
flowmeter	Autolux	7 star channels Turbo Senser MG711-F

Feed Tank

The 9 L stainless steel feed tank held liquid that was not being pumped through the pipework. The tank was a vertical cylinder with a conical bottom. Liquid left the tank and entered the pipework at the bottom and was returned by a pipe which ejected retentate towards the bottom of the tank. Temperature of the liquid was regulated by a thermostat which controlled flow of cooled glycol through a series of stainless steel pipes coiled around the tank. The whole tank was insulated with cotton wadding which was held on with plastic sheeting for water resistance. Heat was provided by the addition of a "Red-Rod" BD series electothermal immersion heater (BDH Ltd., Poole, Dorset). A 300 W Silverson agitator was fitted to the top to constantly mix the feed suspension. Rotor speed was approximately 200 rpm.

Pump

The pump was a P01011 (Becktech, Chichester, UK) which consisted of a 300 watt electrical motor and a positive displacement rotary vane fitted with carbon impellers. Controlled by a VS31-YAA Mspeed controller (SKF Automation Systems, UK).

Pipes

Stainless steel construction, 0.006 m internal diameter. This is the same as the diameter of the smaller membrane unit.

Balance

Permeate production was automatically measured against time using a AE166 (Mettler-Toledo, Zurich, Switzerland) digital balance linked to an IBM 386 personal computer running a specially formulated Labview (Austin, Texas, USA) datalogging program.

Pressure Measurements

Pressure measurements were made using Wika 316SS gauges (Crawlsden, UK). Labelling is identical in both the diagrams, Figs 5.1 and 5.2. Feed inlet pressure is measured by pressure gauge situated in the flow directly before the liquid enters the membrane unit, labelled P_{in}. Retentate outlet pressure is measured by pressure gauge situated in the flow directly after the liquid exits the membrane unit, labelled P_{out}. Permeate pressure was measured on a gauge $P_{permeate}$, placed in the permeate flow between the permeate valve and the membrane unit itself.

Transmembrane pressure could be varied between 0-5 bar and was measured thus:

$$\Delta p_{tm} = \left(\frac{p_{inlet} + p_{outlet}}{2} \right) - p_{permeate} \qquad (5.1)$$

Flow Meter

The flow rate of the liquid flow was measured in L s^{-1} using a flow meter situated directly in the retentate exit flow from the membrane cartridge. The large membrane loop had a Turbo-Senser MG711-F (Tekflow, Weymouth, UK) unit fitted and the small one had an Autlolux (Khrone, Germany). Flow rates were changed by varying the pump speed.

Back Pulsing

Back pulsing of permeate across the membrane surface into the retentate stream was possible, using an air driven diaphragm pump. The duration and frequency of the strokes was controlled using a 6100BG-D pressure transducer (Trans Instruments, Basingstoke, UK). Maximum operating frequency was 5 Hz.

Temperature

Temperature of the feed liquid was manually regulated at first and then the rig was modified to allow automatic temperature control. Initially a manual temperature measurements were made using a digital thermometer placed in the cell suspension. The action of pumping the cells though the membrane unit and the pipework resulted in a temperature rise. The temperature was lowered passing cooled glycol throught the coils of the cooling jacket. Heat was supplied to the system though electric heating coils placed in the liquid. After modification a thermostatic controller (MC810 Mk2, BDH Ltd., Poole, Dorset, UK) was configured to operate a valve that controlled flow of glycol though the cooling jacket.

Mode

The membrane rig could be run in total recycle or cell concentration mode. Total recycle of permeate and retentate was achieved by returning the permeate to the feed tank after flux measurements had been made. Concentration runs could only be achieved by the use of the larger membrane.

Cell Concentration

Feed cell concentration was varied Cells suspension was harvested from the fermenter in early stationary phase at a dry weight of 14 g L^{-1}. Dilution with phosphate buffer to 5 and 7 g dry wt L^{-1} allowed low cell concentrations to be tested. For higher cell concentrations cells were harvested from the fermenter and pre concentrated in a Westfalia KDD605 multichamber bowl centrifuge. This was chosen as the pre-concentration method because results from chapter 4 suggested that this was the fastest and least damaging form of cell concentration. Solids were collected and resuspended in a smaller volume of supernatant upto 113 g dry wt L^{-1}.

5.3 Results

5.3.1 Overview

A number of microfiltration trials were carried out using *P. putida* ML2 cells harvested from a fermentation in stationary phase. A number of operating parameters were varied:

- cell concentration
- transmembrane pressure
- cross flow velocity
- membrane cartridge
- mode of operation - total retentate and permeate recycle
  cell concentration

Cell concentration

Cells were harvested from the standardised fructose batch fermentation one hour after reaching stationary phase. The cell concentration on harvesting was 13 g dry wt L^{-1}. For low cell concentration runs this was diluted with phosphate buffer. For high cell concentration runs the suspension was concentrated by centrifuge using a Westfalia KDD605 multichamber centrifuge. Solids were retrieved and resuspended in a reduced volume of supernatant. Total recycle microfiltration runs were carried out in the concentration range 5-113 g dry wt L^{-1}.

Transmembrane pressure

The transmembrane pressure could be varied by adjustment of the back pressure valve situated after the membrane cartridge. Trials were carried out at 1 and 4 bar transmembrane pressure.

$$\Delta p_{tm} = \left(\frac{p_{in} - p_{out}}{2} \right) - p_{permeate} \qquad (5.1)$$

Cross flow velocity

The cross flow velocity of the system was dependent on the pump speed. Components of the membrane rig were updated during the time of this work. A number of fixed speed pumps were used and finally a variable speed pump was fitted. Attempts were made to keep the cross flow velocity constant at 3 m s^{-1}.

However this was not possible and the velocities varied between 2.6 and 4.9 m s^{-1} depending on which pump was used.

Mode of operation

The membrane rig initially was fitted with a ceramic membrane module which had a identical diameter to the pipes. This allowed defined flow characateristics throughout the system. The membrane had a relatively small surface area which produced small volumes of permeate, making cell work concentration impractical. Runs that used this module were operated in total recycle mode ie. the permeate was returned to the feed tank for mixing with the retentate. No change in cell concentration over the period of the run took place.

An additional module with ten times the surface area of the original was added to the membrane rig. This did not replace the original module but was configured to allow flow through either cartridge by adjustment of valves. This larger unit produced enough permeate to allow cell concentration runs to be carried out. Two cell concentration runs were carried out.

Other operating variables

Temperature was maintained at 20°C throughout the runs. The action of pumping the cell suspension through the pipework raised the temperature of the liquid. Initially manual temperature measurements were using a digital thermometer and the fluid was cooled by allowing the glycol to flow through the cooling pipes surrounding the feed tank. The rig was finally modified to allow automatic temperature control via a thermostatically operated glycol valve.

The operating time was also varied. Initial work involved runs lasting for one hour but it became necessary to operate for longer periods and eventually runs were extended to three hours. In order to test for any effects of processing time a control sample was taken at zero time and incubated at 20°C during the run. After three hours no considerable difference was observed between the viability of the control and the feed sample at time zero.

The small membrane unit was fitted with a backpulsing device. This forces permeate in pulses, across the membrane, to the retentate side. The duration, frequency and pressure of pulsing was controllable.

5.3.2 Total Recycle Of Retentate And Permeate

5.3.2.1 1 bar Transmembrane Pressure

Microfiltration runs were carried out with a transmembrane pressure drop of 1 bar in total recycle mode. The following runs were carried out:

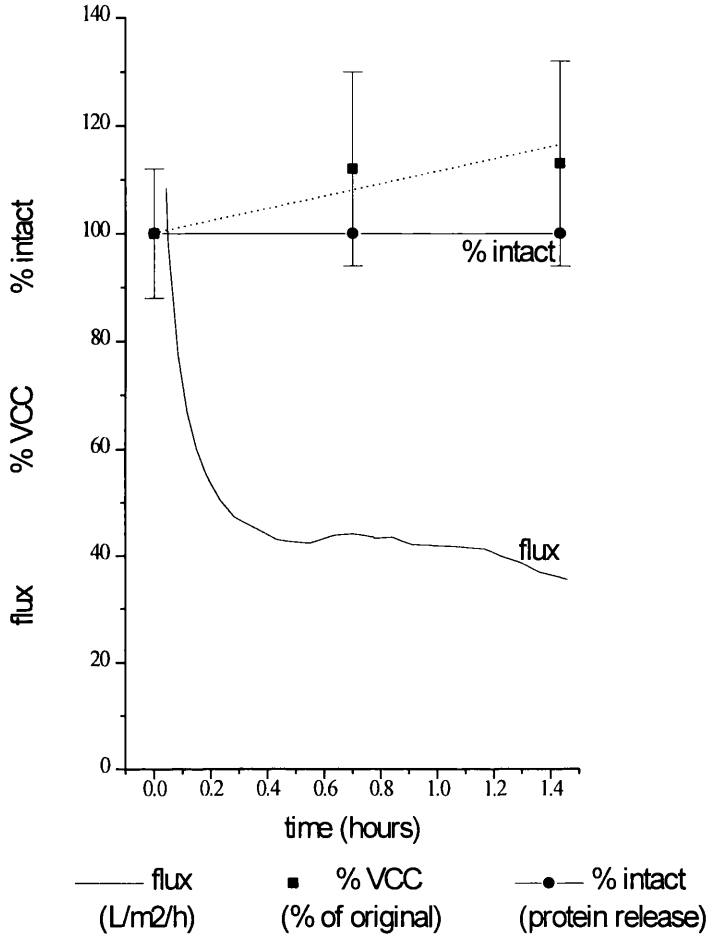
Table 5.5 1 bar Total Recycle Microfiltration Trials

Fermentation number	Cell concentration g dry wt L^{-1}	p_{in} bar	p_{out} bar	Cross flow velocity m s^{-1}
SHPP22	5	1.2	0.8	3.05
SHPP25	7	1.32	0.71	3.05
SHPP35	13	1.2	0.8	3.89
SHPP50	113	1.4	0.6	4.92

The following graphs show the variation in viable cell count, intact cell damage and flux rate against time for the microfiltration runs. In all cases percentage viable cell count is depicted by solid circles and the errror bars correspond to one standard deviation either side of the measured value. Percentage intact is depicted by solid squares and flux rate by a solid line with no point markers.

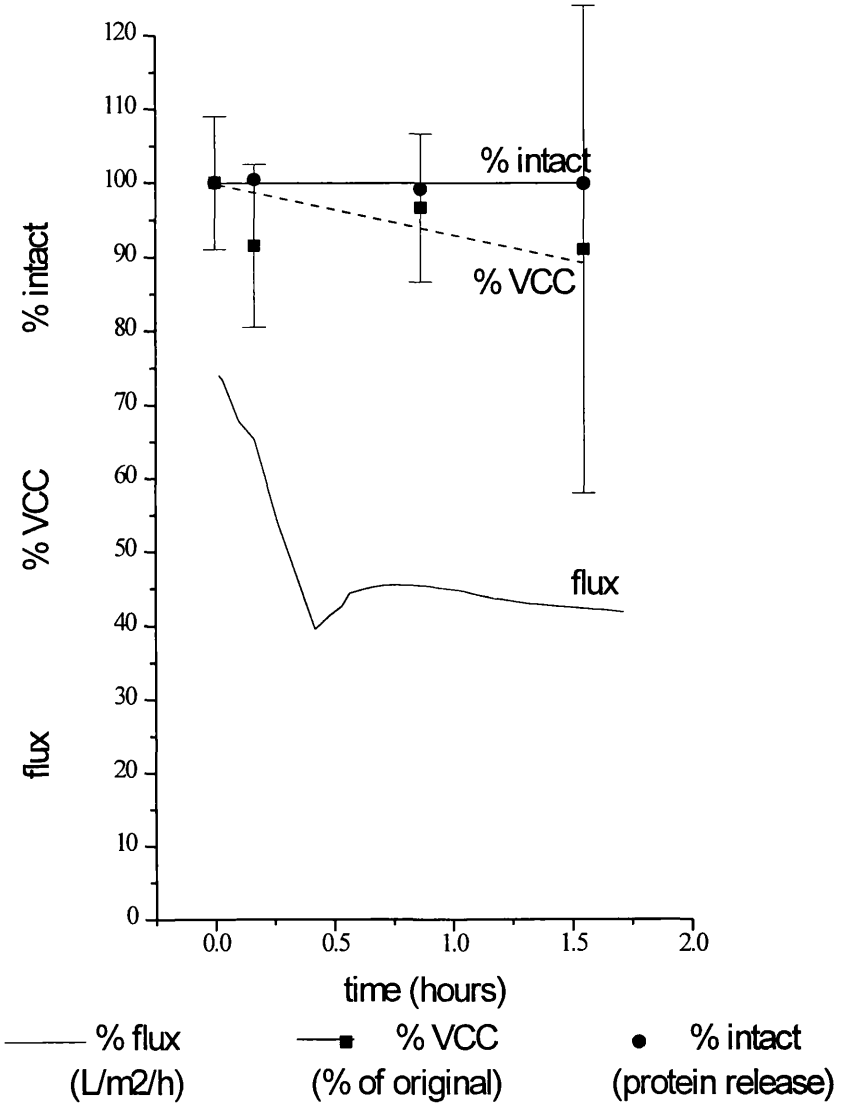
In the following figures VCC data is shown as a linear regressed line for convenience. In the discussion section of this chapter loss of viability is expressed as a rate constant.

Fig 5.3 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (5 g dry wt L^{-1}) Fermentation SHPP22**



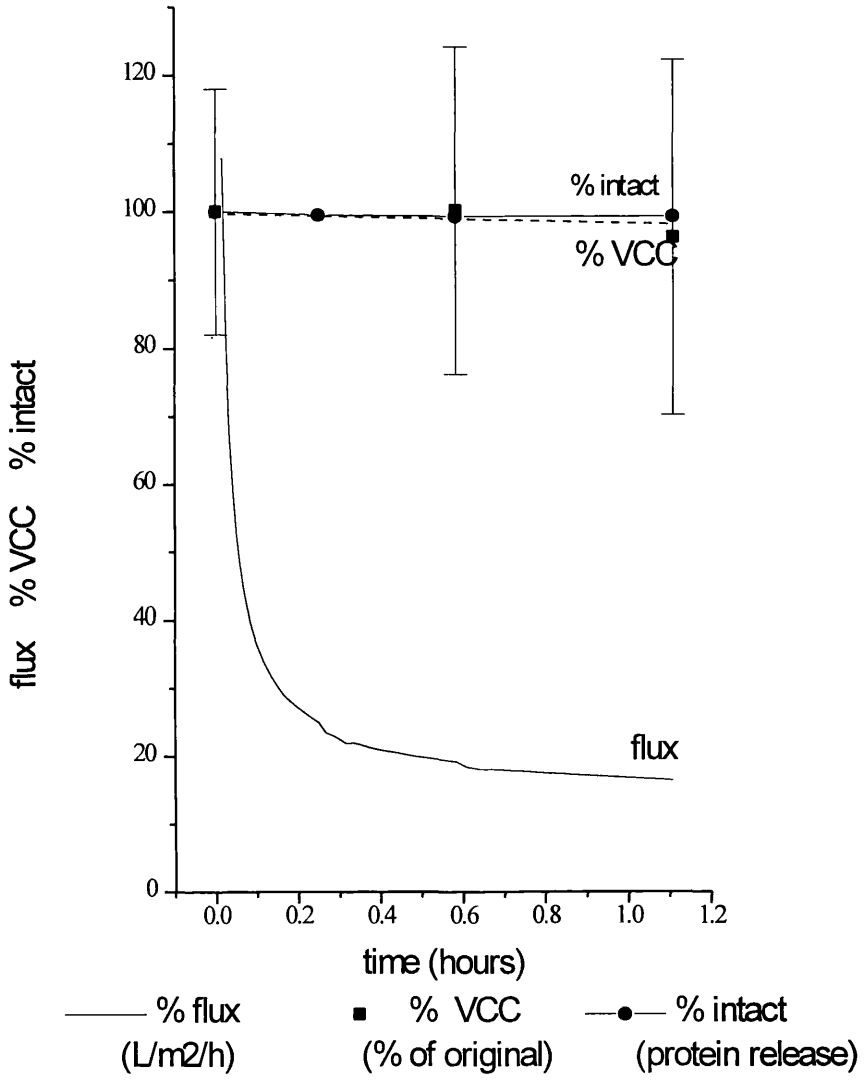
P_{in} = 1.2 bar P_{out} = 0.8 bar Cross flow velocity 3.05 m s^{-1}
Total recycle of retentate and permeate
% VCC percentage viability of original (t=0) sample.
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.4 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (7 g dry wt L^{-1}) Fermentation SHPP25**



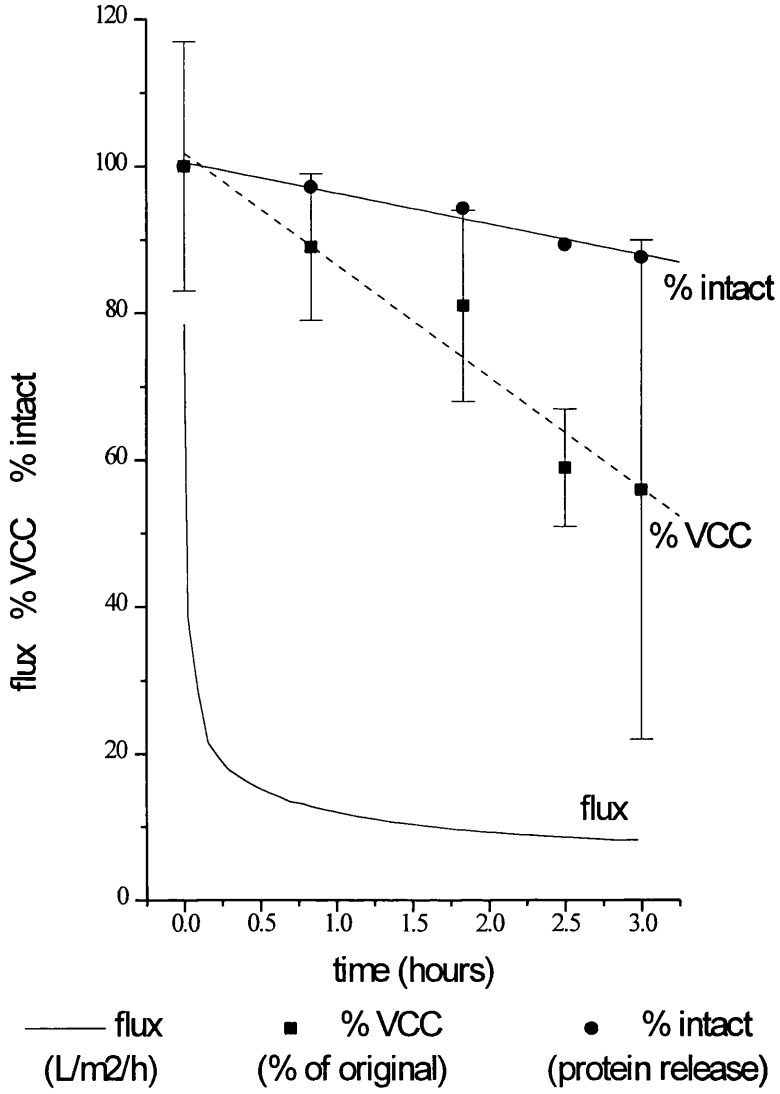
p_{in} = 1.32 bar p_{out} = 0.71 bar Cross flow velocity 3.05 m s^{-1}
Total recycle of retentate and permeate
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.5 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (13 g dry wt L^{-1}) Fermentation SHPP35**



p_{in} = 1.2 bar p_{out} = 0.8 bar Cross flow velocity 3.89 m s^{-1}
Total recycle of retentate and permeate
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.6 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (113 g dry wt L^{-1}) Fermentation SHPP50**



p_{in} = 1.4 bar p_{out} = 0.6 bar Cross flow velocity 4.92 m s^{-1}
Total recycle of retentate and permeate
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Summary of 1 bar total recycle microfiltration trials

Fig 5.3 SHPP22 5 g dry wt L^{-1}

Run time- one hour. No loss of intact cells. Percentage VCC appears to increase by 20%. This is most likely to be due to poor asssay technique.

Fig 5.4 SHPP25 7g dry wt L^{-1}

Run time 1.5 hours. The spread of measured VCC data coupled with large standard errors made it impossible to report a trend with any confidence. No loss of intact cells. Flux shows rapid decline in early stages of runa and inexplicably rises before reaching equilibrium. Membrane cartridge replaced.

Fig 5.5 SHPP35 14g dry wt L^{-1}

Run lasted one hour. No apparent loss of intact cells or VCC.

Fig 5.6 SHPP50 113 g dry wt L^{-1}

Run time was extended to three hours. 10% loss intact cells and 50% loss of VCC. Reduced permeate flux.

5.3.2.2 4 bar Transmembrane Pressure

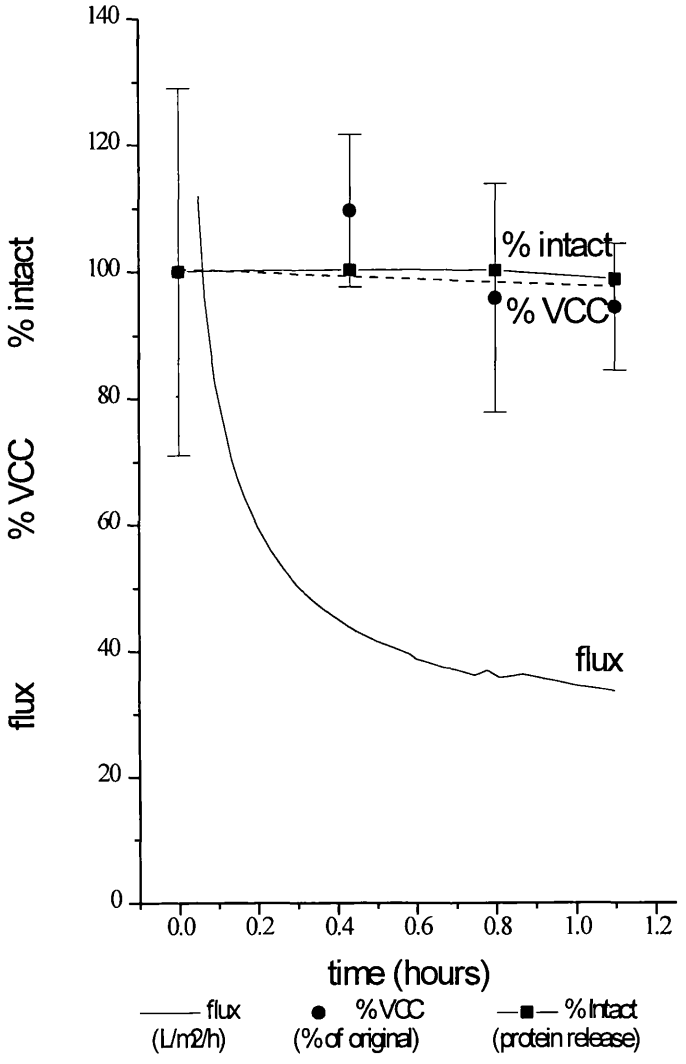
A number of microfiltration runs were carried out on cell suspensions at different concentrations. The transmembrane pressure was 4 bar for all the runs. Permeate was returned to the feed tank and remixed with the retentate.

Table 5.6 4 bar Total Recycle Microfiltration Trials

Fermentation number	Cell concentration g dry wt L^{-1}	p_{in} bar	p_{out} bar	Cross flow velocity m s^{-1}
SHPP26	7	4.27	3.73	2.64
SHPP34	61	4.15	3.85	3.71
SHPP35	14	4.2	3.8	3.82
SHPP42	81	4.19	3.82	3.71
SHPP51	113	4.25	3.8	4.61

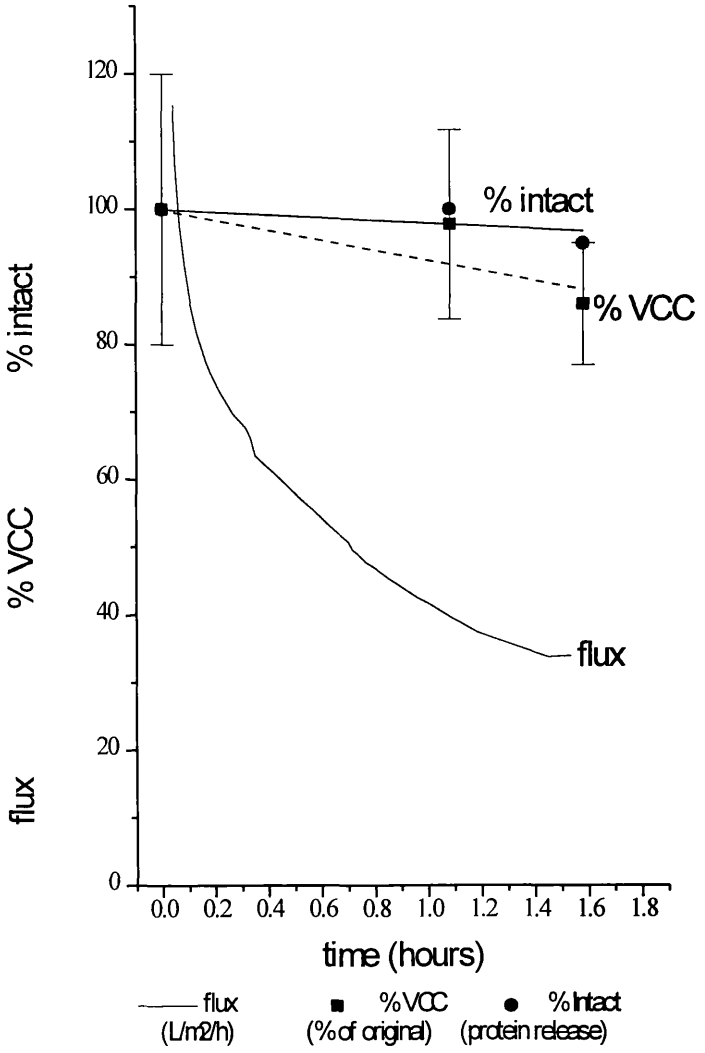
Figures 3.5-3.8 show flux rates, percentage of the original viability and percentage intact cells varying during the operation time.

Fig 5.7 Cross flow microfiltration trial
4 bar transmembrane pressure
***P. putida* ML2 (7 g dry wt L^{-1}) Fermentation SHPP26**



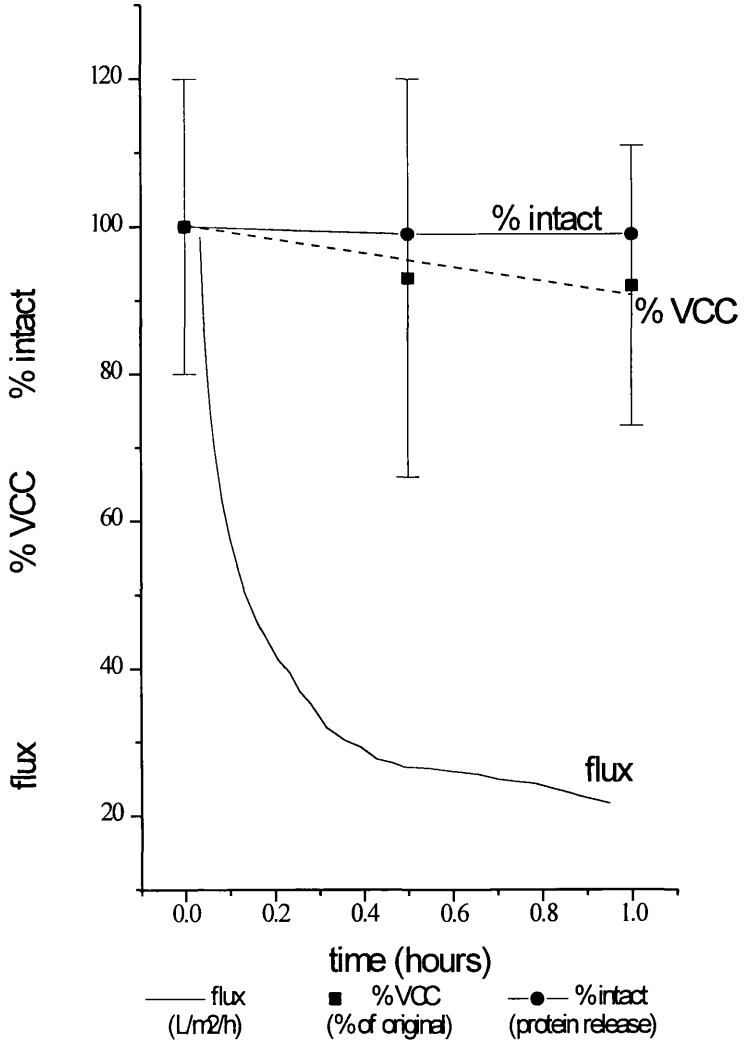
p_{in} = 4.27 bar p_{out} = 3.73 bar Cross flow velocity 2.64 m s^{-1}
Total recycle of retentate and permeate
Flux permeate flux, L m^{-2} h^{-1}
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Linear regressed slope. Error bars correspond to one standard deviation either side of the mean.

Fig 5.8 **Cross flow microfiltration trial**
4 bar transmembrane pressure
***P. putida* ML2 (61 g dry wt L^{-1}) Fermentation SHPP34**



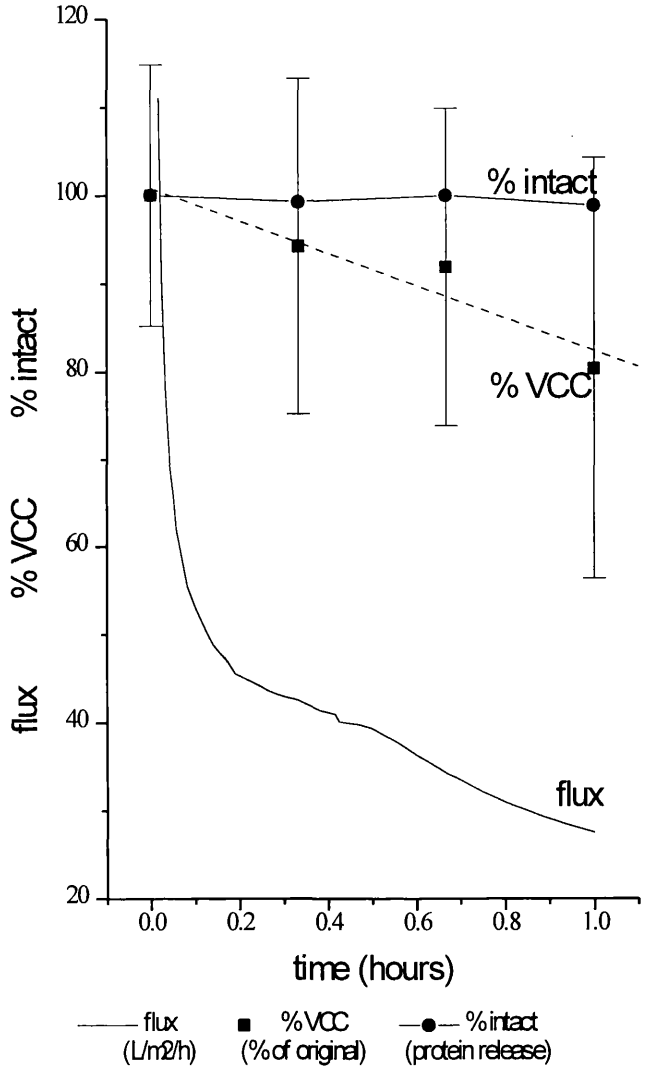
p_{in} = 4.15 bar p_{out} = 3.85 bar **Cross flow velocity 3.71 m s^{-1}**
Total recycle of retentate and permeate
% VCC **percentage viability of original (t=0) sample**
% intact **percentage of intact cells compared to original (t=0) sample**
flux **permeate flux (L m^{-2} h^{-1})**
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.9 **Cross flow microfiltration trial**
4 bar transmembrane pressure
***P. putida* ML2 (14 g dry wt L^{-1}) Fermentation SHPP35**



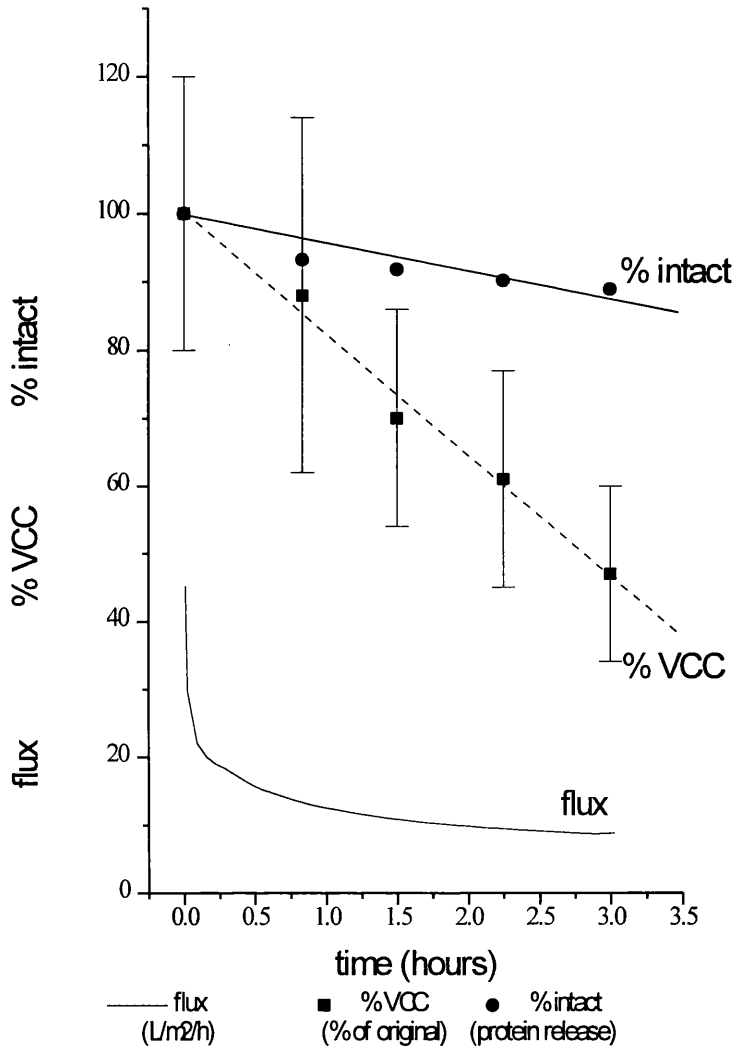
P_{in} = 4.2 bar P_{out} = 3.8 bar Cross flow velocity 3.82 m s^{-1}
Total recycle of retentate and permeate
% VCC **percentage viability of original (t=0) sample**
% intact **percentage of intact cells compared to original (t=0) sample**
flux **permeate flux (L m^{-2} h^{-1})**
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.10 **Cross flow microfiltration trial**
4 bar transmembrane pressure
***P. putida* ML2 (81 g dry wt / L) Fermentation SHPP42**



P_{in} = 4.19 bar P_{out} = 3.82 bar **Cross flow velocity 3.71 m s^{-1}**
Total recycle of retentate and permeate
% VCC **percentage viability of original (t=0) sample**
% intact **percentage of intact cells compared to original (t=0) sample**
flux **permeate flux (L m^{-2} h^{-1})**
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.11 **Cross flow microfiltration trial**
4 bar transmembrane pressure
***P. putida* ML2 (113 g dry wt L^{-1}) Fermentation SHPP51**



P_{in} = 4.25 bar P_{out} = 3.8 bar **Cross flow velocity 4.61 m s^{-1}**
Total recycle of retentate and permeate
% VCC **percentage viability of original (t=0) sample**
% intact **percentage of intact cells compared to original (t=0) sample**
flux **permeate flux (L m^{-2} h^{-1})**
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Summary of 4 bar transmembrane pressure total recycle microfiltration runs

Fig 5.7 SHPP26 7 g dry wt L^{-1}

No loss of viability or intact cells.

Fig 5.8 SHPP34 61 g dry wt L^{-1}

No apparent loss of intact cells but 10% loss of viability.

Fig 5.9 SHPP35 14 g dry wt L^{-1}

No loss of viability or intact cells.

Fig 5.10 SHPP42 81 g dry wt L^{-1}

Apparent 20% loss of viability over one hour. The flux decrease is due to short run time, which would probably have settled after 1.5 hours.

Fig 5.11 SHPP51 113 g dry wt L^{-1}

Run time was extended to three hours. 10% loss of intact cells and 50% loss of viability.

5.3.3 Cell concentration mode

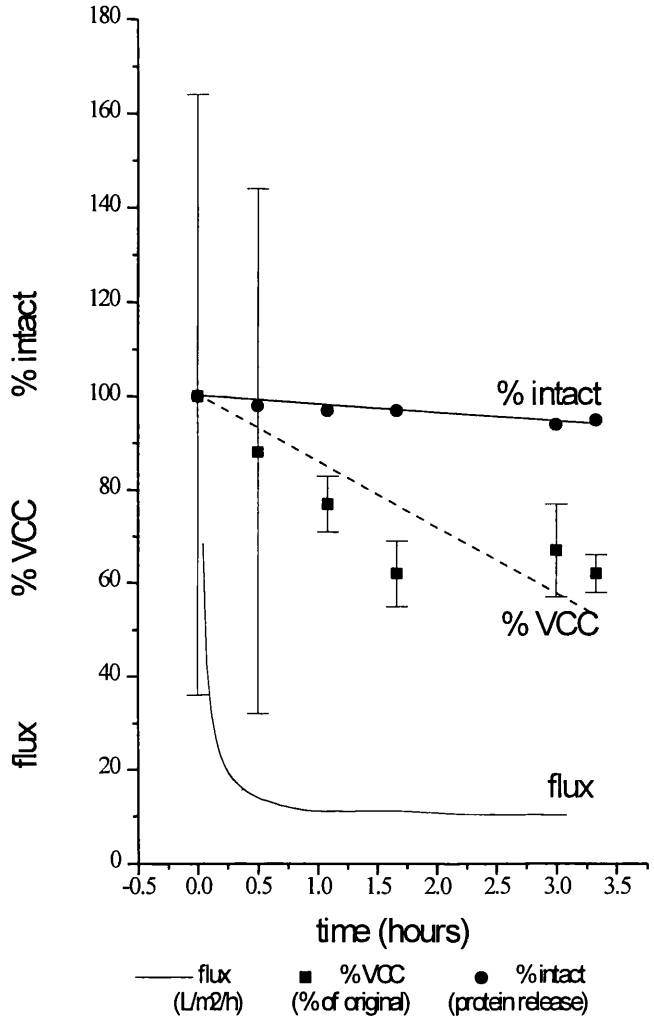
The following two microfiltration runs used a membrane module with approximately ten times the surface area of the one used for the total recycle runs.

Table 5.7 Microfiltration trials carried out in cell concentration mode

Fermentation number	Cell concentration g dry wt L^{-1}	p_{in} bar	p_{out} bar	Cross flow velocity m s^{-1}
SHPP49	32-40	1.1	0.9	2.1
SHPP52	87-325	varied	varied	3.8

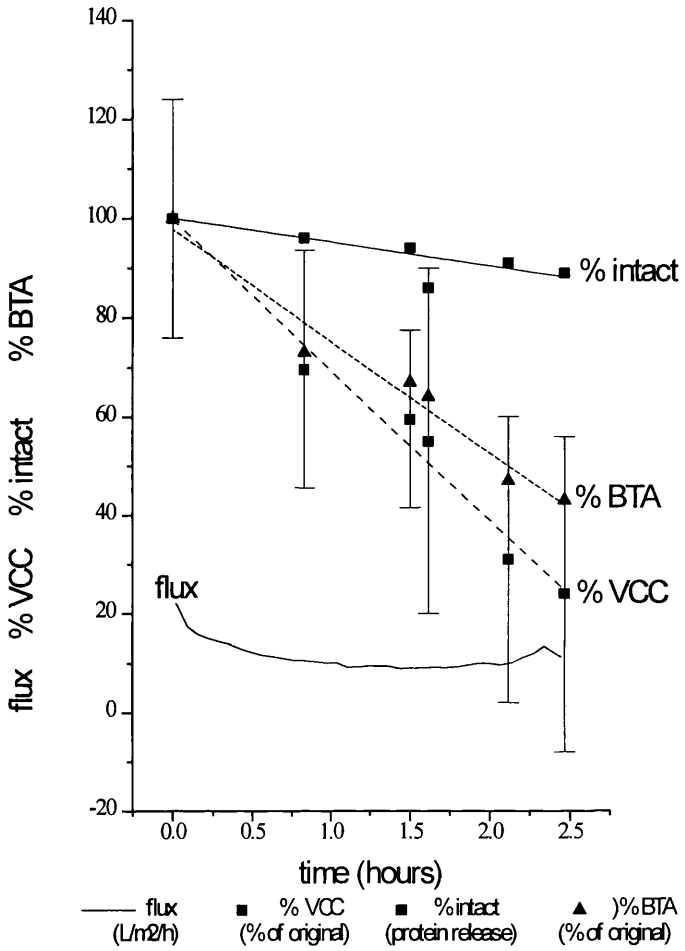
In both cases cells were pre-concentrated using a multichamber bowl centrifuge and then the cells were resuspended in a reduced volume of supernatant.

Fig 5.12 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (32-40 g dry wt L^{-1}) Fermentation SHPP49**



p_{in} = 1.1 bar p_{out} = 0.9 bar Cross flow velocity 2.1 m s^{-1}
Cell concentration run
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Fig 5.13 Cross flow microfiltration trial
4 bar transmembrane pressure
***P. putida* ML2 (87-325 g dry wt L^{-1}) Fermentation SHPP52**



P_{in} and P_{out} varied throughout the run and were constantly adjusted to allow a ΔP_{tm} of 4 bar

Cell concentration run **Cross flow velocity 3.8 m s^{-1}**

% BTA **percentage of the original (t=0) biotransformation ability of the sample**

% VCC **percentage viability of original (t=0) sample**

% intact **percentage of intact cells compared to original (t=0) sample**

flux **permeate flux (L m^{-2} h^{-1})**

VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.

Summary of the results from the cell concentration microfiltration trials

SHPP49 **32 - 40 g dry wt L^{-1}**

Pre-concentrated cell suspension was used at a concentration of 32 g dry wt L^{-1}. The rig was operated in concentration mode which increased the cell concnetration to 40 g dry wt L^{-1} at the end of the run.

It was not known at the time of carrying out this run that the back pressure valve had been changed to a needle valve. Other workers in the department noted highly increased cell damage (in the region of 20%) when processing E. coli cells. Slightly higher levels of cellular damage were noted for *P. putida* ML2 processing.

SHPP52 **87 - 325 g dry wt L^{-1}**

The cell suspension was preconcentrated to 87 g dry wt L^{-1} and increased until the solution became too viscous to allow operation of the pump at approximately 325 g dry wt L^{-1}. The transcartridge pressrure varied thoughout the run increasing with increased viscosity. The flux rated showed an increase towards the end of the run. The perecentage of intact cells dropped by approximately 10% over the cource of the run. The viability decreased by almost 80%. The biotransformation ability of the cells was also tested and this was found to decrease over time the rate of which appeared to relate to the viability loss.

5.3.4 Microfiltration Using Back Pulsing

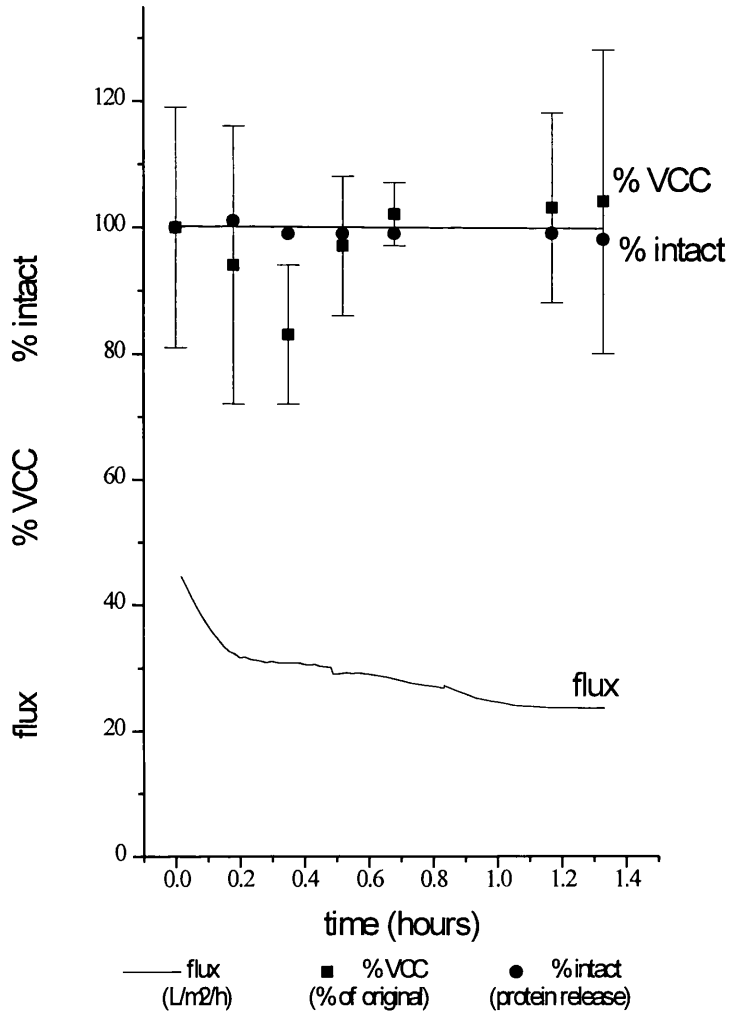
Table 5.8 Microfiltration Using Back Pulsing

Fermentation number	Cell concentration g dry wt L^{-1}	P_{in} bar	P_{out} bar	Cross flow velocity m s^{-1}
SHPP27	7	1.3	0.7	3.03

One run was carried out using the back pulsing equipment attatched to the small membrane unit. Cells were grown and diluted to a concentration of 7 g dry wt L^{-1}. Back pulsing is used extensively in membrane processing operations to prevent and remove fouled solids on the retentate side of the membrane.

The permeate valve was opened for 40 seconds per minute. A 0.93 bar pulse of air was sent across the membrane from the permeate side for 0.1 second while the valve was shut. Flux rates were similar to those observed for other low cell density runs. No loss of intact cells or viability was observed.

Fig 5.14 Cross flow microfiltration trial
1 bar transmembrane pressure
***P. putida* ML2 (7 g dry wt L^{-1}) Fermentation SHPP27**

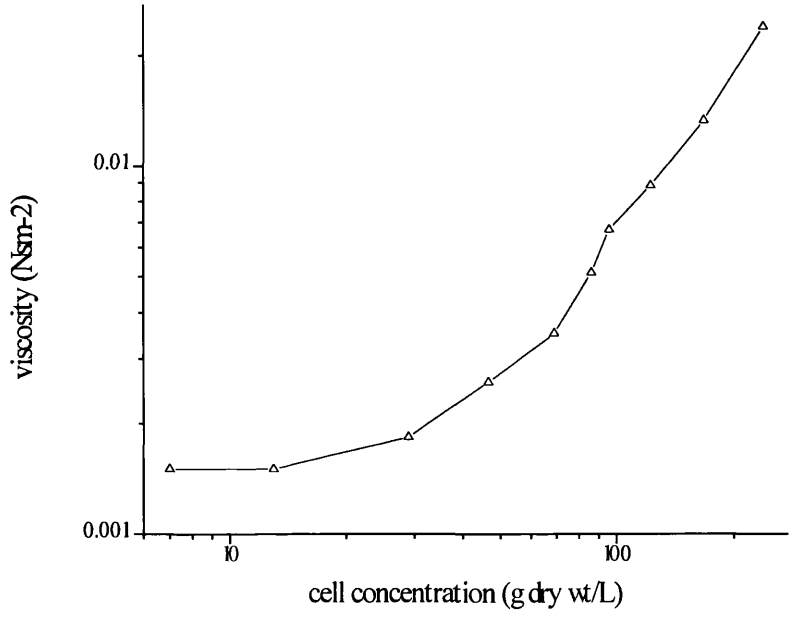


P_{in} = 1.23 bar P_{out} = 0.7 bar Cross flow velocity 3.03 m s^{-1}
Total recycle of retentate and permeate
% VCC percentage viability of original (t=0) sample
% intact percentage of intact cells compared to original (t=0) sample
flux permeate flux (L m^{-2} h^{-1})
VCC, mean of nine dilutions. Error bars correspond to one standard deviation either side of the mean.
This run was carried out under similar conditions (cell concentration, cross flow velocity and transmembrane pressure) as SHPP25 (Fig 3.2).

5.3.5 Viscosity Measurements

Viscosity measurements of cell concentrations at different concentrations were made using a Contraves Rheomat 115 rheometer. Viscosity was shown to increase with increased cell concentration.

Fig 5.15 **Viscosity increase with increased cell concentration**
Log viscosity vs log cell concentration



5.4 Discussion

5.4.1 Measured Results

There appears to be an increased loss of viability with increased cell concentration. In all of the runs there was a smaller associated loss of protein. The fact that loss of viability and protein release data do not equal each other, but do seem to correlate, suggests that the mechanism of cell inactivation is different from the one that occured in the homogenisation and centrifugation trials. The measured loss of viability and intact cells was approximately equal in these cases.

It was also noted that the standard deviations of the viability samples from the runs that used preconcentrated cell suspensions decreased over time. This was most likely due to the fact that aggregation of cells occurred when the cell suspension was centrifuged. These aggregated cell clumps were not completely separated during the resuspension step. The action of the pump and the shear stresses encountered during flow though the pipes lead to further breakage of the aggregates. The presence of aggreagated cell clumps in the plate count samples gives rise to a wide spread of plate counts as the clumps are inevitably broken during the serial dilution. Clumps of cells in some repeat samples will increase the spread of the results.

5.4.2 Mechanism Of Cell Damage

Maiorella *et al.* (1990) proposed four potential causes of cell damage during cross flow filtration:

- laminar shear within the filter unit
- deformation of cells into filter pores by transmembrane pressure
- turbulent flow in the pipes
- passage through pump

Laminar shear within the filter unit

In the case of the small membrane unit the type of flow would be similar to that seen in the pipework. This is because the membrane rig was designed with a membrane unit with the same internal diameter as the pipework.

Deformation of cells into filter pores by the transmembrane pressure.

The degree of deformation is a function of the applied strain and residence time over filter pore. The effects of this would seem to be negligible. In all the runs there seemed to be almost no relationship between protein release and loss of viability. This contrasted with the centrifugation results, where the two measured values were approximately the same. It is likely that a different form of damage is occurring.

During centrifugation cells are subjected to a combination of shear and compaction stresses, during microfiltration it mainly shear. Deformation of the cells due to compaction forces is generated by pressure within the equipment. The pressures generated in the feed zone of an industrial centrifuge are many orders of magnitude higher than that generated by the transmembrane pressure within a microfiltration rig.

Shear damage inflicted, during centrifugation, causes the formation of small ruptures in the cell membrane. The compaction stresses would lead to deformation of the cells. A combination of the ruptures caused by the shear and the deformation caused by the compaction of the cells would lead to intracellular contents being forced out. As measured by protein release. The lack of measured protein release, during microfiltration, suggests that the effects of compaction are minimal.

The effects of turbulent flow in the piping

Shear stresses generated by pumping viscous solutions through piping can be estimated by a number of different methods. The shear stress generated by flow of cell suspension through the microfiltration rig pipework woud be expected to increase with increasing viscosity of the suspension (ie. when the cell concentration was increased). This would appear to be a likely cause of cell damage.

The action of the pump

This is a likely source of damage. Shimizu *et al.* (1992) found that a rotary vane pump similar to the one used in the Biodesign rig resulted in no inactivation of *E.coli* cells at low concentrations. The same was found for *P. putida* ML2 cells when tested in similar a concentration range. At higher concentrations cell damage was detected.

Effect of pre-concentrating the cells

The multichamber bowl centrifuge was used because it caused least damage to cells during processing of all the centrifuge types tested (Chapter 4). However there is a possibility that this stage could have caused the cells to be slightly weakened compared to their fermentation harvest state. Also the long term effects of this pre-concentration step were not investigated.

One of the aims of this work was to report the damage effects inflicted on a cell population in suspension that had been processed by a variety of apparently unrelated dewatering processes. If the cell damage effects for an individual process could be expressed in engineering terms then they could be correlated with the measured damage. Little work has been published that quantifies the hydraulic forces generated within a microfiltration rig or an industrial centrifuge. A detailed analysis of the results is presented in Chapter 6, which will:

- Express the damage inflicted on the cells, during microfiltration, in terms of viability loss as a first order rate constant of cellular inactivation.
- Estimate the stresses generated within the microfiltration rig in a number of different ways and then compare these values with the stresses induced by the action of the fermenter.
- Correlate viability loss and input stress and determine whether there is a relationship between process conditions and cell damage.

6 Analysis Of Results

6.1 Introduction

As was mentioned in Chapter 1 mechanical stresses are the major cause of cellular damage during microbial production and recovery. Exposure to shear stresses generally leads to disruption of cells and release of intracellular components. This can cause loss of product and increased difficulty in purification downstream.

The objective of this section is to explore the relationship between cell inactivation and exposure to shear stress over three types of operation, homogenisation, centrifugation and microfiltration.

In all cases the inactivation of cells was measured and was believed to be caused by exposure to shear stress within the processing equipment. The nature of the liquid flow is turbulent and ill defined. It is proposed to examine one common way of characterising turbulent flow and explore the inter relationship between the operations.

6.2 Cell Damage

Cells have been produced and processed by three different types of device, homogenisers, centrifuges and microfiltration units.

Damage has been measured by loss of viability and release of intracellular protein. Measured inactivation levels are similar to those of protein release for homogenisation. There appears to be a discrepancy between loss of viability and released protein for microfiltration.

A number of homogenisation trials were carried out using *P. putida* ML2. The disruption observed followed first order kinetics with respect to the number of passes and can be described by the general derivation (Hetherington *et al.*, 1971):

$$\frac{dR}{dN} = k\,(R_m - R) \qquad (6.1)$$

$$\int_{R_m}^{R} \frac{dR}{R_m - R} = k \int_{0}^{N} dN \qquad (6.2)$$

$$\left[\ln R\right]_{R_m}^{R} = \mathrm{kN} \qquad (6.3)$$

$$\ln \frac{R_m}{R_m - R} = \mathrm{k\,N} \qquad (6.4)$$

R	amount released
R_m	maximum release
R_m-R	amount available for release
N	number of passes
k	disruption rate constant

If loss of viability was measured instead of protein release then a rate constant of inactivation could be derived in a similar way to give the following expression:

$$\mathrm{kN} = \ln \frac{x}{100} \qquad (6.5)$$

x	% of viable cells compared to unprocessed
100	% viable cells in unprocessed sample

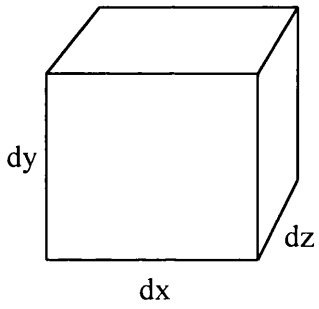
This allows an evaluation of k to be made, even for single pass homogenisations.

6.3 Shear Stress

6.3.1 Characterisation of fluid stresses

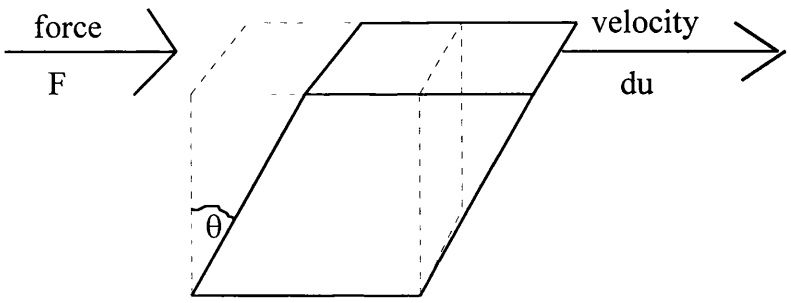
Shear is the action of a force on an object parallel to a surface. Imagine a small element of fluid:

Fig 6.1 Small element of fluid



A force F acts on the top surface of the element, of surface area dxdz, causing rotation by an angle of θ over a time dt.

Fig 6.2 Force acting on small element of fluid



Torque applied to element = Force x distance = F dy (6.6)

Shear stress, τ = F/A = $\frac{F}{dx\,dz}$ (6.7)

Torque applied = τ dx dz dy (6.8)

Power input = Torque x rate of rotation (6.9)

= Torque x $\frac{d\theta}{dt}$ (6.10)

$\approx$ Torque x $\frac{du}{dz}$ (6.11)

$$= \quad \tau \, dx \, dz \, dy \, \frac{du}{dz} \qquad (6.12)$$

$$\therefore \quad \tau \, \frac{du}{dz} = \frac{P}{dxdzdy} = \frac{P}{V} \qquad (6.13)$$

P = power

V = volume

$$\frac{du}{dz} = G = \text{velocity gradient} \qquad (6.14)$$

$$\tau = \mu G \qquad (6.15)$$

μ=viscosity

$$\mu G^2 = \frac{P}{V} \qquad (6.16)$$

$$\therefore \quad G = \left(\frac{P}{V\mu}\right)^{1/2} \qquad (6.17)$$

$$\text{Stress in a fluid, } \tau = \mu G \qquad (6.18)$$

$$\tau = \left(\frac{P}{V\mu}\right)^{1/2} \mu \qquad (6.19)$$

$$\text{Therefore} \quad \tau = \left(\frac{P\mu}{V}\right)^{1/2} \qquad (6.20)$$

NB Shear rate, G is a common basis for characterisation of fluids in a turbulent flow for flocculation i.e. Gt=Camp number which is the basis of design of flocculation reactors (Bailey and Ollis, 1986).

The key for analysis across the operations is to characterise fluids in terms of P/V i.e. power dissipation per unit volume. In all operations hydrodynamic forces (most probably shear) are generated by the movement of fluid through the device. The rate of fluid displacement is a function of the power input on the system. This gives ready characterisation of the shear stress. The real challenge when looking at the systems tested is to define the magnitude of the power and estimate over what volume the power is dissipated.

The following pages outline methods for the estimation of the shear stresses generated by the following devices:

- Pump
- Fermenter
- Homogeniser
- Centrifuge
- Microfiltration unit

Full details of calculations can be found in the appropriate appendices.

6.3.2 Pump

The power input for a pump, P, can be estimated in the following way:

$$P = \Delta pQ \qquad (6.21)$$

P = power
Δp = pressure developed across pump head
Q = flow rate

The shear stress,τ, can therefore be estimated:

$$\tau = \left(\frac{\Delta pQ\mu}{V}\right)^{1/2} \qquad (6.22)$$

The volume, V, over which the power is dissipated is likely to equal the whole volume of the pump head in the case of a centrifugal pump. It therefore acts like a wide diameter impeller in a stirred vessel. For other configurations eg a gear pump this is less simple as it is likely that nearly all the power is dissipated into the gaps near the edge of the impellers.

Ultimately the pump would introduce energy as hydrostatic stress rather than shear stress but ΔpQ does reflect the overall fluid stress in the system arising from dissipation of the energy introduced by the pumping.

6.3.3 Fermenter Appendix 6.1

For a fully turbulent fluid (Reynolds number $>10^4$) in a single impeller system the power is given by the following expression:

$$P = kN_p \rho N^3 d^5 \quad (6.23)$$

N_p power number (for a turbine impeller, Np=6 (Aiba *et al.*, 1973))
ρ density of liquid
N impeller speed (revs per unit time)
d diameter of impeller
k correction for number of impellers
(for the three turbine impeller used in this work, k=2 (Aiba *et al.*, 1973))

It is generally accepted that 90% of the energy is dissipated in 10% of the fluid volume, therefore:

$$\tau_{max} = \left(\frac{9P\mu}{V}\right)^{1/2} \quad (6.24)$$

6.3.4 Homogeniser Appendix 6.2

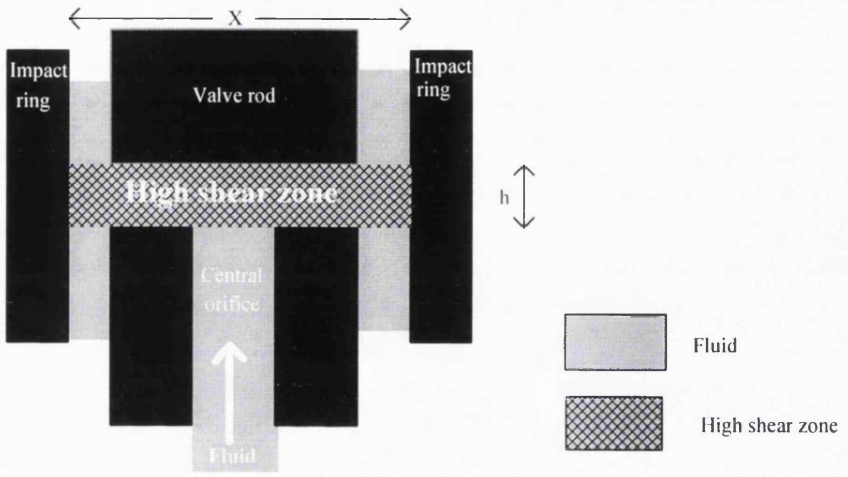
The action of the homogeniser can be considered to be similar to a pump. The power dissipation model can be used to estimate generated shear stresses.

$$P = \Delta p\, Q \quad (6.21)$$

Δp operating pressure
Q fixed flow rate

The volume over which the shear forces act is considered to be that over which power is dissipated. This is not easily defined and has to be estimated. It is depicted in the following diagram by the shaded area. The gap widths were estimated by Keshavarz *et al.* (1990). Much of the breakage occurs by impact suggesting that power dissipation is in a small volume across the valve seat and jet upto the impact ring.

Fig 6.3 Lab 40 homogeniser high shear area



The volume, V of the high shear zone:

$$V = \pi \frac{X^2}{4} h \qquad (6.25)$$

When V has been calculated the shear stress is estimated by the power dissipation model:

$$\tau = \left(\frac{\Delta p Q \mu}{V} \right)^{1/2} = \left(\frac{4 \Delta p Q \mu}{\pi X^2 h} \right)^{1/2} \qquad (6.26)$$

There are a number of problems encountered in the estimation of the volume of the disruption chamber. It is not possible to directly measure the impact distance, h, which is varied as the operating pressure is changed.

However it can be assumed:

$$\Delta p \propto \frac{1}{h^2} \quad \text{(Keshavarz } et\ al.\text{, 1990)} \qquad (6.27)$$

or

$$h \propto \frac{1}{\Delta p^{1/2}} \qquad (6.28)$$

$$h = \frac{k'}{\Delta p^{1/2}} \qquad (6.29)$$

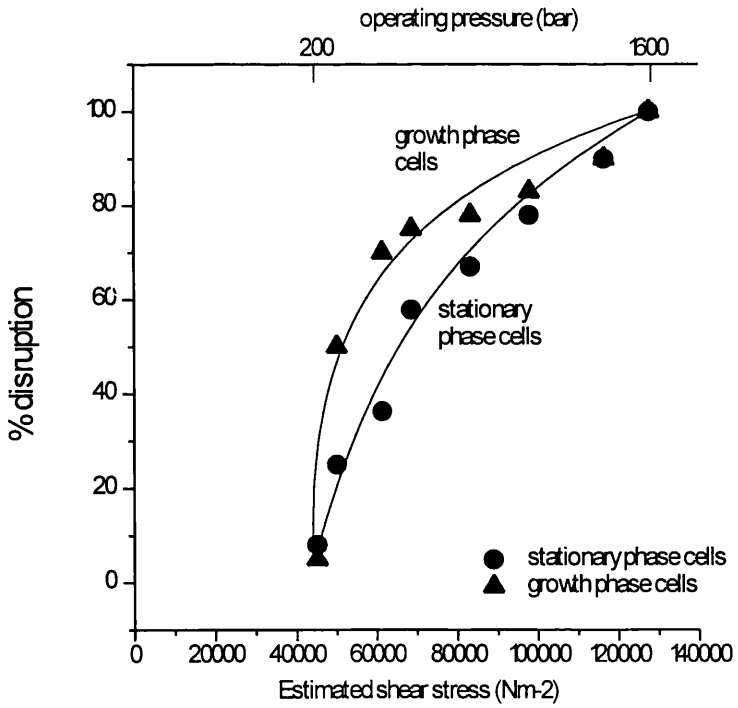
Hence the shear expression can be modified to:

$$\tau = \left(\frac{4\Delta p^{3/2} Q\mu}{\pi X^2 k'} \right)^{1/2} \qquad (6.30)$$

One set of values of h and Δp may be used to estimate k'. For the configuration used here when Δp is 560 bar h is 15μm.

The shear stresses for the range of operating pressures has been estimated (see appendix 6.2) and plotted against percentage disruption for *P. putida* ML2.

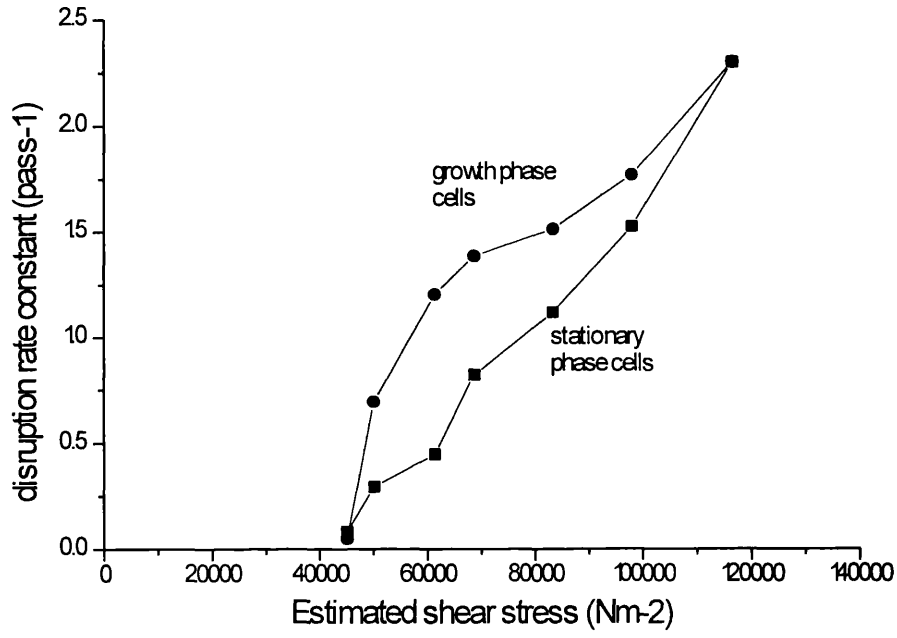
Fig 6.4 Percentage cellular disruption after single homogeniser pass vs estimated shear stress and operating pressure



The shape of the two curves suggests that there is a stress value above which cellular disruption can be measured. This is known as a critical stress value and will be discussed later in this section.

Alternatively the disruption rate constant can be plotted against the estimated shear stress:

Fig 6.5 Disruption rate constant vs estimated shear stress

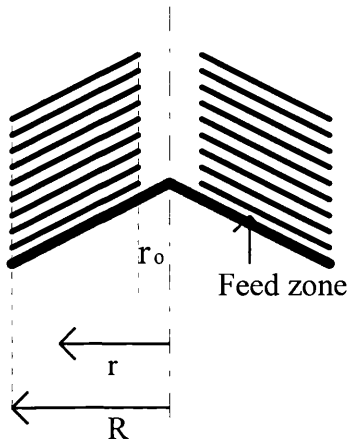


6.3.5 Centrifuge Appendix 6.3

A number of models have been proposed for the estimation of shear stresses generated within a centrifuge. If the action of the centrifuge is considered to be similar to that of a pump it should be possible to estimate shear by the pump model, mentioned earlier. This can be carried out in two stages:

- Calculation of change in pressure
- Use pump calculation to estimate shear rate

Fig 6.6 Westfalia CSA 1 disc stack centrifuge



Pressure drop across distributor to edge of disc is given by:

$$\Delta p = \rho \omega^2 R (R-r_o) \qquad (6.31)$$

Where: (see Appendix 6.3)

ρ	density
g	$\omega^2 r$
ω	rotational speed (rads per unit time)
R	radius of outer disc (i.e. the point to where fluid is pumped)
r_o	distance from centre to start of disc
$(R-r_o)$	head of fluid

Estimation of the volume of the feed zone is not straightforward. It can be considered to be the volume of the six feed pipes situated underneath the feed discs and not the actual feed zone. The shear is then calculated as before:

$$\tau = \left(\frac{\Delta p Q \mu}{V}\right)^{1/2} = \left(\frac{\rho \omega^2 R(R-r_o) Q \mu}{V}\right)^{1/2} \qquad (6.32)$$

$$= 400 \text{ Nm}^{-2}$$

The Spinning Disc Model is an alternative method for estimating generated shear stresses within the disc stack centrifuge and was proposed by Bell (1982).

$$\tau_{max} = 2\,\mu^{1/2}\,\rho^{1/2}\,r\,\omega^{3/2} \qquad (6.33)$$

$$= 840\ Nm^{-2}$$

The two models give similar results but the pump analogy method is probably more suitable because it takes into account the effects of flow rate.

Stresses due to solids discharge from the centrifuge bowl have not been calculated because of the negligable amount of damage caused to the cells.

6.3.6 Microfiltration Rig Appendix 6.4

The mode of action of the microfiltration unit is more complex than the other two devices. The rig is made up of a number of different components which are likely to contribute to the overall stress encountered by the cells. Stress is believed to be generated by the following:

- Turbulent flow through the pipework
- Turbulent flow through the back pressure valve
- Action of the pump

Estimations were made of stress due to the pipework and the pump. It is not possible to distinguish between stress inactivation relationships in the pump and in the back pressure valve, because for the experiments reported here the two values should be directly proportional. There is a possibility that the damage occurs exclusively within the valve.

Pipe Wall Shear Stress Calculation

Using a model derived from the Blasius equation for turbulent flow shear stress at the pipe walls was estimated.

$$\tau_{wall} = 0.0396\,\rho\,v^2\,Re^{-0.25} \qquad (6.34)$$

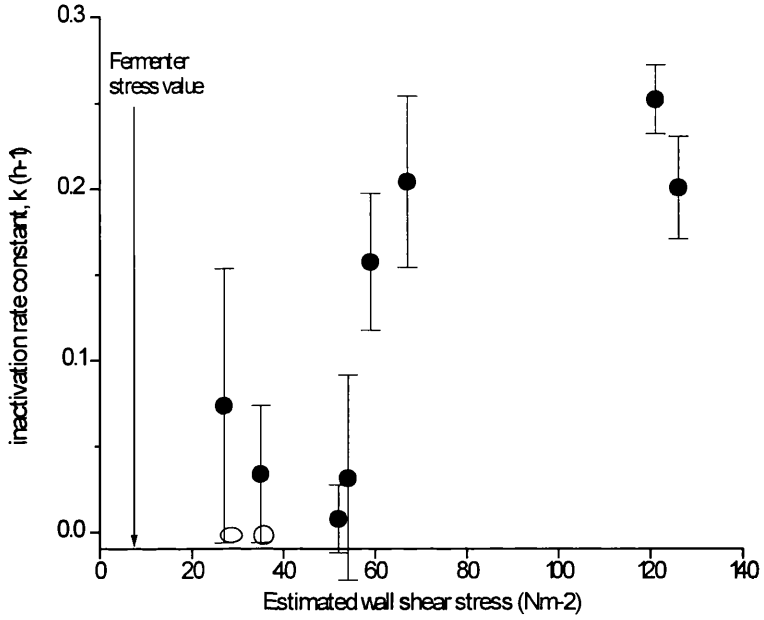
ρ density
v velocity
Re Reynolds Number

$$= \frac{\rho\,v\,d}{\mu} \qquad (6.35)$$

d pipe diameter

Viability loss for the fixed cell concentration microfiltration runs shown in Figures 5.3-5.11 was expressed as an inactivation rate constant k, $\left(\ln\frac{x}{100}\right)$ which was plotted against the estimated wall shear stress to give the following graph:

Fig 6.7 Inactivation rate constant vs estimated wall shear stresses Data from fixed cell concentration microfiltration runs



The error bars correspond to one standard deviation either side of point. Two of the calculated inactivation rate constants had a negative value. This suggests cell growth during the experiment, this seems unlikely as the cells are in stationary phase. The control sample showed no overall increase in cell number during the trial. These two trials have been given a value of zero and are depicted in the graph as unshaded dots.

Variation in the back pressure in the rig appears to keep the stress in the pipes at a consistent level. This is not the case for the pump and valve. Different rates of inactivation were observed for two high cell concentration runs the only difference in operating conditions was the transmembrane pressure. It appears that this shear stress estimation method is not appropriate.

Calculation of shear stress within the pump

Two different engineering models for the calculation of pump shear stress can be applied to this system.

- Tip speed calculation
- Power dissipation model

The tip speed method of estimating the shear stresses was considered:

$$\tau = \frac{v\,\mu}{l} \qquad (6.36)$$

v tip speed
l distance between the pump wall and the tip of the impeller
μ viscosity of liquid

However, as with the wall stress model, the effects of the process conditions are not considered. The calculated stresses for the 113 g dry wt L^{-1} runs at 1 and 4 bar transmembrane pressure are similar. This does not help to explain the differences in measured loss of viability.

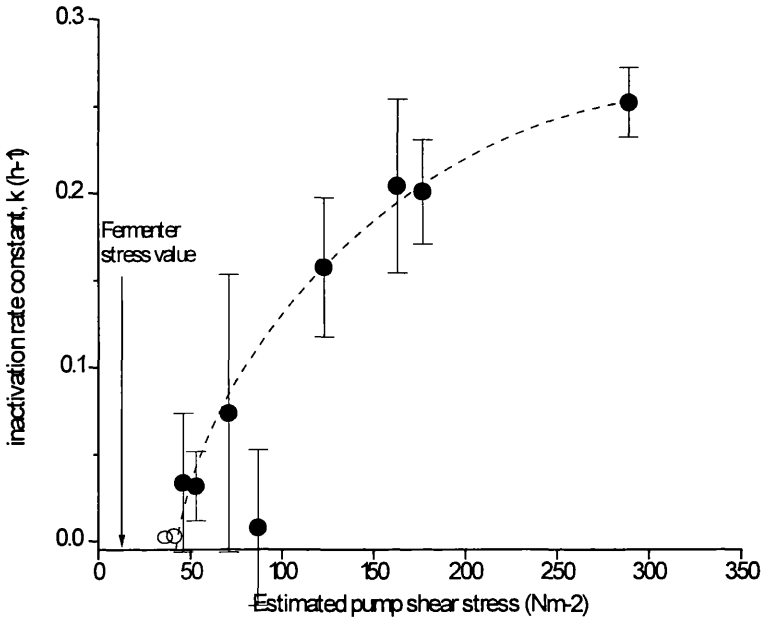
Estimation of pump shear by power dissipation model

Power dissipated, $P = \Delta p\, Q$ (6.21)

Shear stress: $\tau = \left(\frac{\Delta pQ\mu}{V}\right)^{1/2}$ (6.22)

The stress values appear to be of the same order of magnitude as those for the pipework. The inactivation rate constants for the fixed cell concentration microfiltration runs (Figs 5.3-5.11) were plotted against the estimated pump shear stresses and are shown in Fig 6.7. This model appears to be sensitive to changes in the process conditions. The inactivation rate constants were plotted against the estimated pump shear stresses.

Fig 6.8 Inactivation rate constant vs estimated pump shear stress
Data from fixed cell concentration microfiltration runs



The error bars correspond to one standard deviation either side of point. Two of the calculated inactivation rate constants had a negative value. This suggests cell growth during the experiment, this seems unlikely as the cells are in stationary phase. The control sample showed no overall increase in cell number during the trial. These two trials have been given a value of zero and are depicted in the graph as unshaded dots.

The graph appears to suggest that above a certain shear stress value (50 Nm^{-2}) the inactivation of cells can be measured. This implies that there is a critical shear stress value for cell inactivation. This is a widely reported concept. In a paper by Shimizu *et al.* (1992) the effects of shear stress on a number of microbes were investigated. The authors proposed that there was a value of stress above which the release of intracellular components can be measured. However there was no effect on bacterial viability when cells were subjected to sub-critical stress for prolonged periods, upto thirty hours.

Merchuk (1991) also recognised the critical shear value and noted that subjecting cells to sub-critical stress levels can affect a number of cellular characteristics such as growth rate, volume, metabolite production rate and membrane permeability. This critical stress value is characteristic for individual organisms. There is also likely to be some variation depending on the growth phase of the organisms as has been

demonstrated by the difference in disruption characteristics of growth and stationary phase *P. putida* ML2 cells.

It has been shown that it is possible to measure the damage inflicted on a *P. putida* ML2 cell population by the following operations, homogenisation, centrifugation and cross flow microfiltration. Shear stress analysis does not help identify the mechanism of cellular inactivation.

Svetlichnyi *et al.* (1991) concluded that cellular inactivation during microfiltration was caused by shear stresses. They carried out *P. putida* cell disruption trials using capillary flow disrupters operating at typical shear rates generated during microfiltration. It is suggested that inactivation occurs due to the accumulation of cell wall structural damage, in the form of ruptures. The number of ruptures suffered per cell was estimated by electron microscopy. Histogram plots were used to show that the cell population was heterogeneous and could be divided into a number of fractions based on their susceptibility to this form of damage. To test this hypothesis cell suspension was passed though capillary tubes a number of times. After twenty passes the rate of inactivation was shown to decrease. It was suggested that this was due to the least mechanically robust cells in the population being inactivated by initial passes. Svetlichnyi *et al.* suggested that each fraction of the cell population has its own critical shear stress value of inactivation.

There appears to be a close relationship between the observed inactivation and the estimated shear stress in the pump. The shear effects due to the back pressure valve have been mentioned earlier as a possible cause of cell damage. The power input of the system is given by the following expression:

$$P = \Delta p\, Q \qquad (6.21)$$

Δp will be similar for the pump and the valve as the pressure drop along the pipe is fairly small compared to pressure drop across pump head or valve

Q will be the same for the pump and the valve

It is difficult to estimate the volume over which the power is dissipated within the valve. Therefore it has to be assumed that:

$$\tau_{valve} = K\,\tau_{pump\ shear\ stress} \qquad (6.37)$$

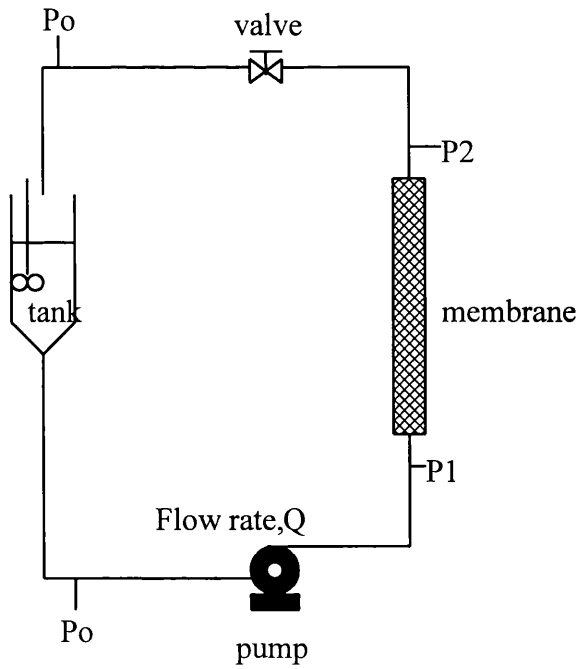
and

$$K = \left(\frac{\text{volume of pump head}}{\text{volume of valve seat}}\right)^{1/2} \qquad (6.38)$$

Figures 6.6 and 6.7 show the estimated average value of the fermenter shear stress (7 Nm^{-2}) in relation to the shear stresses generated within the pump and the pipework. It is interesting to note that the maximum shear stress within the fermenter could be upto 100 times this value. This would still be in the region of the critical stress value.

Pressure changes within the membrane rig are shown in the following diagram:

Fig 6.9 Pressure changes within the microfiltration rig



Stress @ pump $\propto (P_1-P_o) \cdot Q$

Stress @ wall $\propto (P_1-P_2) \cdot Q$

Stress @ valve $\propto (P_2-P_o) \cdot Q$

(P_1-P_2) is constant for constant Q (unless μ changes)

If the valve is closed off and the flow rate kept constant then the stress for pump and valve goes up (i.e. increased P_1 and P_2) but (P_1-P_2) remains the same, therefore the stress at the wall does not change. It is likely that:

$$(P_2-P_o) \quad \propto \quad (P_1-P_2)$$

Po is small compared with P_1 or P_2 and as $(P_1 -P_2)$ is approximately the same it is likely that

$$P_2 \quad \propto \quad P_1$$

When the flow rate increases for the same P_1 then the value of (P_1-P_2) increases and stress within pump increases more rapidly than the stress in valve. The same effect will be seen for an increase in viscosity.

6.3.7 Summary of shear stresses across range of operations

The shear stresses generated within the equipment tested were estimated for a range of operating conditions and are included in the following table.

Table 6.1 Estimated shear stresses across range of operations

Operation and conditions $Q=m^3s^{-1}$ $\Delta p=Nm^{-2}$	Total power input /W	Estimated volume of power dissipation /mL	Viscosity μ Nsm^{-2}	Shear rate G s^{-1}	Shear stress τ_{max} $N\ m^{-2}$
42 L Fermenter	94	27500	0.0015	$4.55x10^{-3}$	6.8
Centrifugation					
CSA1 $Q=1.38x10^{-5}$	23	0.21	0.0015	$2.6x10^{5}$	4000
KDD, $Q=1.38x10^{-5}$	17	310	0.0015	$6.0x10^{3}$	9
1P, $Q=1.38x10^{-5}$	$8.8x10^{6}$	0.71	0.0015	$2.9x10^{6}$	4310
Homogeniser					
$\Delta p=2x10^{7}$ (200 bar)	1600	use k' value	0.0015	$2.6x10^{7}$	45100
$\Delta p=1.6x10^{8}$(1600bar)	12800	use k' value	0.0015	$9x10^{7}$	128000
Microfiltration (Pump calculation)					
$Q=8.55x10^{-5}$ $\Delta p=1.7x10^{5}$	9.4	10	0.0015	35160	53
$Q=8.55x10^{-5}$ $\Delta p=4.8x10^{5}$	41	10	0.0015	58425	88
$Q=1.04x10^{-4}$ $\Delta p=4.7x10^{5}$	48.9	10	0.0031	39747	120
$Q=1.04x10^{-4}$ $\Delta p=4.82x10^{5}$	44.5	10	0.0053	30691	160
$Q=1.38x10^{-4}$ $\Delta p=2.57x10^{5}$	35.5	10	0.0088	20074	180
$Q=1.29x10^{-4}$ $\Delta p=5.38x10^{5}$	69.4	10	0.0015	24053	290

key overleaf...

G	Shear rate	$= \left(\frac{P}{V\mu}\right)^{1/2}$	(6.17)
	Indicative of the amount of exposure		
τ	Shear stress	$= \mu G$	(6.18)
	Indicative of the harshness of the exposure		
Q	Flow rate / m^3 sec^{-1}		
Δp	Pressure / Nm^{-2}		
CSA1	Disc stack centrifuge		
KDD	Multichamber bowl centrifuge		
1P	Tubular bowl centrifuge		

Microfiltration shear values are calculated by power dissipation through pump

6.4 The Effects Of Exposure To High Shear Zones On Cells

It appears to be possible to estimate the shear stresses generated within the processing equipment tested by assuming that the stresses are a function of the power dissipation per unit volume within the equipment. Cell damage is assumed to occur in the following ways:

Homogenisation

The action of pumping cells through the disruption chamber exposes them to shear and compaction stresses (on impact with the valve rod). This leads to inactivation and disruption. The operation can be repeated by carrying out further passes. Measured loss of viability and release of intracellular protein appears to be identical following homogenisation.

Centrifugation

The majority of cellular damage occurs in the feed zone of a centrifuge. Pumping cell suspension through this region has a similar effect to the action of the homogeniser. Centrifugation can be considered to be equivalent to a single homogenisation pass.

Microfiltration

In contrast the action of microfiltration is more complex. The site of damage is unclear. Cells are pumped through the pipework loop and are exposed to damaging shear stresses. This could occur at a number of points in the circuit. The estimated shear values within the pump and the pipework are considerably lower than those from the homogeniser or disc stack centrifuge. If the action of pumping the cells through the loop and exposing them to shear is considered to be similar to that of a single homogeniser pass then the action of continually pumping cells through the pipework loop during processing can be envisaged as a multiple pass homogenisation operation.

When the measured inactivation rates were plotted against the estimated wall shear stresses encountered within the microfiltration rig there appeared to be no relationship between the two. The shear stress values estimated by power dissipation within the pump appear to indicate that there is a relationship between increased shear stress in the device and cellular inactivation. However it is unclear as to whether the pump or the valve is the point at which the damaging stress occurs.

As mentioned earlier, Svetlichnyi *et al.* (1991) concluded that the cell population is heterogeneous in its resistance to mechanical stress. The population consists of a number of fractions of cells with each fraction possessing its own individual critical shear stress level.

In addition to assuming that the cells show variation in their mechanical strength characteristics, the magnitude of the shear stresses generated within processing equipment could show variation, with some areas generating significantly higher levels than others.

Homogenisation processing involves exposing the cells a high stress level for a very short period of time, and it is likely that the majority of the cells are exposed. During centrifugation, the time of the exposure is still short (not as short as the homogeniser) but the stress levels are lower. It is likely that the majority of cells in this case are exposed to damaging stress levels.

Microfiltration processing involves much longer exposure times but at lower stress levels, which appear to be many orders of magnitude less than those generated by a homogeniser or centrifuge. It is probable that the a broad range of stresses are generated within the microfiltration rig. Stresses are believed to be generated by

flow though the pipework and within the pump and back pressure valve. If the fluid flow through the pipework is considered, maximum stress values are generated close to the surface of the wall. At any given time during operation the majority of the cells reside in the middle of the pipe and are therefore exposed to considerably smaller stresses.

In the pump the highest stresses are likely to be generated in the space between the impeller tips and the walls of the pump casing. Consider this volume to be in the region of 0.1 mL whereas the total volume is approximately 10 mL. If the stresses occurring in this region are greater than the critical stress value then all cells residing in this region will be damaged. However the majority of the cells reside in the impeller blades and are not subjected to these high stress levels and survive undamaged. Damage caused by the pump is very small however it is the continuous exposure of many passages through the pump over a number of hours that leads to measurable damage.

Shear stress related damage to a cell population appears to be a function of the following parameters:

- Magnitude of the stress generated within the high shear area compared to the populations critical stress value
- Probability of exposure to that stress (number of passes)

If a cell passes through a region of high stress, i.e. above its critical stress, it will be damaged. In the microfiltration pipework loop these high shear regions are very small when compared to the total volume. It is the continual process of pumping the cells around the loop that increases any cell's chance of coming into contact with a high shear area.

The following table demonstrates the interaction of the shear stress and number of passes on cellular damage.

Table 6.2 Cell damage effects across range of operations

	High shear stress region	Shear stress, τ	Number of exposures, N	Inactivation rate constant, k
Homogenisation	disruption chamber	high	low	high
Centrifugation	feed zone	medium	low	medium
	discharge	low	low	low
Microfiltration	pump wall, pipe wall, valve	low	very high	low

6.5 Observed And Actual Inactivation Rate Constants

During microfiltration it assumed that the action of the pump has an intrinsic inactivation constant, K, such that:

$$\frac{dy}{dN} = -Ky \qquad (6.39)$$

Where:

N number of passes through the pump

The observed inactivation constant, k, is modified by the dilution effect of the feed tank and is a function of the tank volume V, the flow rate, Q and the intrinsic or actual rate constant, K. Assuming perfect mixing within the tank:

$$k = -\frac{Q}{V}\left(1-e^{-K}\right) \qquad (6.40)$$

therefore

$$K = -\ln\left(1-\frac{V}{Q}k\right) \qquad (6.41)$$

(See Appendix 6.5)

Assume that

$$K = b(\tau-\tau_{crit}) \qquad (6.42)$$

Where

τ_{crit} the critical shear stress for inactivation

b inactivation / breakage constant

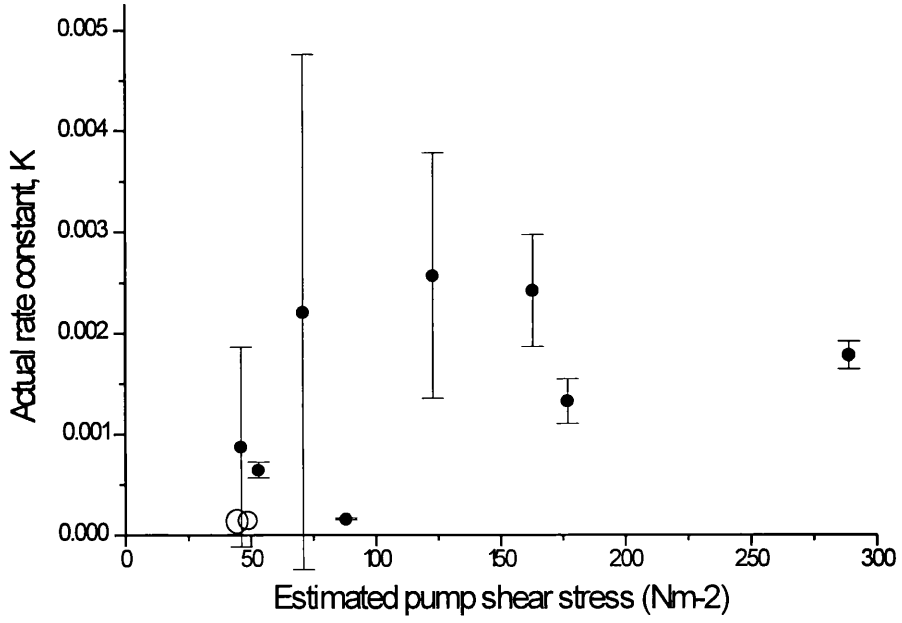
Plotting of the observed rate constant, k, against estimated shear stresses within the pump (Fig 6.6) suggested that significant inactivation occurred at shear stresses in excess of 50 Nm^{-2}. Therefore:

$$\tau_{crit} = 50\ Nm^{-2}$$

Table 6.3 Observed And Actual Rate Constants

Dry weight g dry wt L^{-1}	Observed rate constant k, s^{-1}	Actual rate constant K	Inactivation constant b m^2N^{-1}	Pump shear stress, τ Nm^{-2}	Pump shear rate G s^{-1}
5	$-2.37x10^{-5}$	-0.00222	0.00037	44	29300
7	9.3x10-6	0.00874	-0.00022	46	30700
7	2.03x10-5	0.0022	0.000105	71	47300
7	-2.03x10-5	-0.003	0.0000757	46	30700
14	8.7x10-6	0.000644	0.000215	53	35300
14	2.09x10-6	0.000156	$4.12x10^{-6}$	88	58400
61	$4.4x10^{-5}$	0.00258	$3.52x10^{-5}$	123	39700
84	$5.7x10^{-5}$	0.00252	$2.14x10^{-5}$	163	30700
113	$5.6x10^{-5}$	0.001327	$1.05x10^{-5}$	177	20100
113	$7x10^{-5}$	0.001784	7.46x10-6	289	24100

Fig 6.10 Actual rate constant vs estimated shear stress



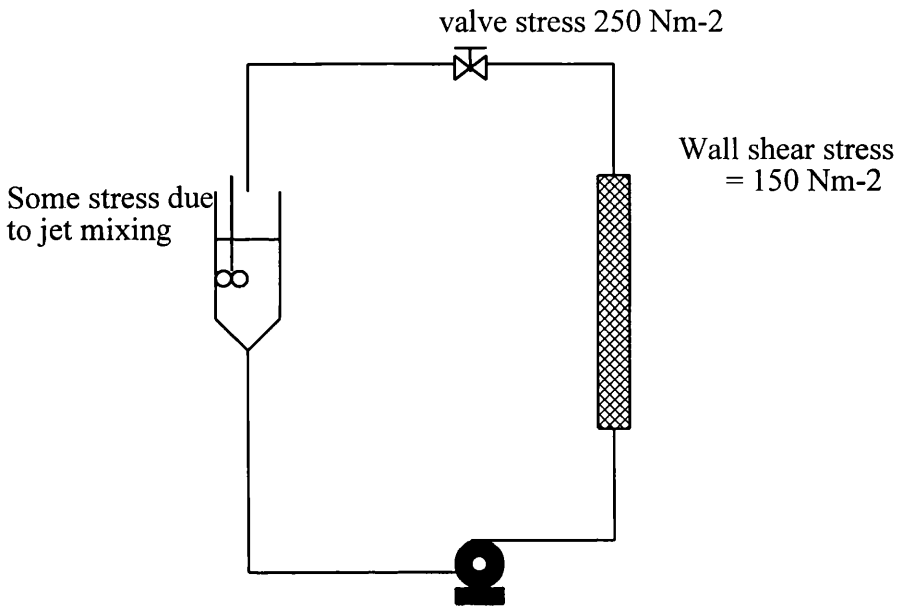
As with Figures 6.6 and 6.7 negative rate constants are depicted as unshaded dots adjacent to their respective shear stress values at K=0. Error bars correspond to one standard deviation.

As $K = b(\tau-\tau_{crit})$ then $K = b\tau-b\tau_{crit}$

This should plot a straight line graph with a slope b and an intercept of $-b\tau_{crit}$

It is difficult to take this interpretation further and a more convincing analysis is gained by considering Figure 6.7 further (observed inactivation rate constant, k, vs estimated pump shear stress). It seems likely that stresses within the rig as a whole are likely to be equal throughout.

Fig 6.11 Microfiltration rig stresses



It seems that the best correlation can be made when the observed rate constant, k is plotted against pump shear stress. It would appear that the system is acting in a similar fashion to a fermenter but with the stress levels considerably raised hence leading to cellular inactivation.

7. Discussion

This work examined the effects of concentrating a cell suspension. The separation processes tested were microfiltration and centrifugation. A high pressure homogeniser was used to mimic the action of microfiltration and centrifugation in order to develop an assay test system. Damage to *P. putida* ML2 cells was observed when the three processes were tested.

The measurement of cell damage is not a straightforward task. It is generally regarded as the loss of viability, which can be defined as an organism's ability to reproduce or remain metabolically active. It is therefore possible for cells to suffer different degrees of damage. These can result in a loss of:

- Ability to reproduce
- Metabolic activity
- Physical integrity

These three factors are inter-related. Loss of ability to metabolise prevents the organism reproducing but the reverse does not necessarily hold true.

It is important to be clear which properties are required from the cell population after an initial separation step. Whether the damage inflicted on the cells is a problem depends on the application of the organism. It is necessary to determine whether the degree of damage inflicted on the cell population is justifiable.

For example, if the cells are to be used as an inoculum and their ability to reproduce is lost, then the processing technique or operating conditions are unsuitable. Many processes do not require cells to have the ability to reproduce only to metabolise. Whole cell biocatalysis is one such example, in the case of this work, the oxidation of fluorobenzene by *P. putida* ML2.

Whole cells are frequently used for the production of a product. When damage takes the form of disruption or breakage of the cells a number of problems can arise. If the product is intracellular then disruption causes dilution of product and can add to recovery problems. Conversely the release of intracellular components can interfere with the recovery of extracellular products.

A convenient and reproducible test was required for the quantification of cellular damage after exposure to mechanical stress. The use of viable cell counts and assaying for released intracellular protein is well documented. In addition to investigating these techniques the potential for the use of the bioluminescent assay of ATP was examined.

A number of *P. putida* ML2 fermentations were monitored. This determined the values of intracellular ATP and correlated ATP with viable count, optical density, dry weights and exit gas analysis. As expected total intracellular ATP levels rose exponentially during growth phase (along with VCC, OD, CER and dry weights). During stationary phase ATP levels fell in line with CER and OUR. The viable counts remain almost unchanged during this period of sampling. The amount of ATP / viable cell was shown to vary by one thousand percent from growth to stationary phase. ATP levels appear to be a measure of metabolic activity of the cell sample rather than an index of biomass, due to the variations seen during growth phase changes.

A high pressure homogeniser operating at low pressures was used as an approximation to sediment discharge from a high speed disc stack centrifuge. Multiple pass operation was used with a view to examining whether cell breakage or loss of cell viability could be analysed using plate counts, ATP and protein measurement for cells in stationary phase.

- Cell disruption was determined by release of total soluble protein as measured by the Bradford assay.
- The ability of cells to reproduce was measured by the ability of cells to form colonies on solid complex growth medium, following serial dilutions. (Plate counts).
- Metabolic integrity was examined using the bioluminescent ATP assay. Metabolic activation of stationary phase cells after low pressure homogenisation meant that intracellular ATP levels could not be used as a quantitative method of determining cell viability or disruption. In order to avoid the problem a similar method of analysis to that reported by van Schie *et al.* (1991) was attempted. In order to activate these stationary phase cells to their maximum metabolic rate, a control and a homogenised cell sample were used to inoculate a shake flask containing fermentation medium with glucose as the sole carbon source. Levels of intracellular ATP were monitored at timed intervals after inoculation and subsequent incubation in

agitated incubators. The degree of activation of the cells was considered to be proportional to the amount of metabolically active cells in the original inoculum.

Plate counts correlate well with protein release figures although they are an extremely labour intensive method. For increased accuracy the plate count regime was changed. At least three sets of dilutions were carried out for each sample and statistical analysis was applied to the results. The following conclusions were reached for the analytical development stage of this work:

- Extracellular ATP has proved to be an unreliable measurement for loss of cell viability due to the presence of ATP degrading enzymes, and unstable levels.
- Intracellular ATP measurement is an unreliable measure of cell viability for cells processed in stationary phase due to sharp ATP increase on activation of the cells. Believed to be caused by the rapid supply of nutrient (in the form of lysed cell contents), oxygenation and a stress response.
- Intracellular ATP measurements appear to be a reliable measurement of cell viability for cells in the active growth phase.
- Decline in cell viability as measured by plate counts has been found to correlate well with protein release data, when using single-pass, low pressure homogenisation
- The shake flask activation of stationary phase cells and the measurement of maximum ATP levels shows some potential as a method of determining viability. However the development of a reproducible method is not the objective of this project.

Determination of cell damage therefore involved measuring loss of cellular integrity (by total soluble protein release) and loss of cellular viability (by plate counts).

Shear stresses are believed to be the main cause of the cellular damage during processing. However it was not possible to account for the exact mechanism of damage over the range of operations. It has been noted that the levels of cell breakage and loss of viability are identical for homogenisation. Levels of cell breakage are considerably less than inactivation during microfiltration and somewhere between the two for centrifugation. It was been suggested previously that a possible explanation is that during microfiltration shear stress damage inactivates cells without causing disruption, but lack of impaction and compression (as in homogenisation and centrifugation) prevents release of intracellular protein.

However the assumption that the homogeniser is a good mimic of processing effects could be inappropriate and the mechanism of damage in the homogeniser could be entirely due to impaction of the cells on the valve rod and impact ring.

It is possible to characterise the shear stress within fluids, τ, in terms of the power dissipation per unit volume using the following equation:

$$\tau = \left(\frac{P\mu}{V}\right)^{1/2} \qquad (6.20)$$

P power
μ viscosity
V volume

The most common method of mechanically transferring fluids is by the use of a pump. The shear stresses generated across the pump head can be estimated using the above expression. The power input for a pump is the product of the pressure developed across the pump head and the flow rate:

$$P = \Delta p\, Q \qquad (6.21)$$

The main difficulty encountered is estimation of the volume over which the power is dissipated within the system. The fermenter was considered first, as cells are exposed to shear stresses for long periods of time due to agitation of the impeller. It is unlikely that the stresses generated by agitation will be of sufficient magnitude to result in damage to the cell population, otherwise cell growth would not occur. It is possible that some cells will be damaged in the very highest shear zones of the vessel. These areas occupy a very small fraction of the total volume, approximately ½ % (van't Reit and Smith, 1975) and therefore only a small proportion of the cell population would reside within this region at any given time. Power is dissipated within a known volume of fluid in a fermenter and can be easily estimated.

A homogeniser was used to mimic the effects of centrifugation and microfiltration. The action of this device was considered to be similar to that of a pump, i.e. fluid undergoes rapid acceleration and is directed through a high pressure zone. This acceleration takes place across the disruption chamber. Power is dissipated within the high pressure zone and shear stresses are generated leading to cellular damage.

Estimation of the volume of the high shear zone (over which power is dissipated) is difficult in the case of the homogeniser and is assumed to be the volume of the disruption chamber. This volume changes with operating pressure and so the pump expression was modified to allow the substitution of a constant in the shear expression. Generated shear stresses were estimated for the range of operating pressures tested.

The majority of cell damage has been shown to occur within the feed zone of the disc stack centrifuge. Before separation occurs cells are pumped through the feed zone where they undergo a rapid radial acceleration and are exposed to shear forces. It is possible to estimate the change in pressure across the feed zone and once its volume has been estimated then the shear stress value can be calculated.

The action of the microfiltration rig is more complex than that of a homogeniser or a centrifuge. A proportion of the cell suspension is pumped though the pipework and returned to the tank. Stresses are believed to be generated in three areas, across the pump head, at the pipe walls and in the valve seat. It is difficult to estimate the volume of the valve over which power is dissipated and it was assumed that stresses generated were similar to those across the pump head. A number of fixed cell concentration microfiltration trials were carried out under different operating conditions (flow rate, transmembrane pressure and cell concentration) and the cell damage was measured over time by assaying for protein release and plate counting.

Shear stresses were estimated at the pipe walls and were plotted against measured cellular inactivation rates. A high degree of scatter was observed and there was no apparent trend. However the method of wall stress estimation failed to take into account one of the operating variables, transmembrane pressure. All of the operating variables were used to calculate stresses across the pump head and when these were plotted against measured inactivation rates a trend could be inferred. It was possible to measure cellular damage beyond a critical level of stress (approximately 50 Nm^{-2}) and inactivation rate appeared to increase with rising stress levels.

The shear stress values calculated were estimates of order of magnitude and a high degree of variation was observed across the range of operations.

Table 7.1 Relative stress levels generated by process equipment

Process equipment	Relative stress level
Fermenter	1
Microfilter	10
Centrifuge	100
Homogeniser	1000

The mechanism of cell damage has been considered across the range of operations. In all cases cells are exposed to stress and the amount of damage is measured. The extent of the damage is dependent on the number of exposures and the magnitude of the stress levels.

The homogeniser functions by pumping cells suspension through the disruption chamber where the cells are exposed to high shear and impact stresses leading to damage. The amount of damage is proportional to the number of passes through the disruption chamber and the processing conditions (operating pressure). The action of a centrifuge can be considered to be similar as cells are pumped though a high shear area, in this case the feed zone, exposed to stress, leading to cellular inactivation. This can be considered to be analogous to the action of a single homogeniser pass.

When the stresses within the microfiltration rig were estimated they were found to be orders of magnitude less than those of the homogeniser or centrifuges. It was not possible to predict with any certainty the exact stage at which cell damage occurs. Cell suspension is taken from the feed tank and pumped through the pipework exposing it to stress effects from the pump, tubing and back pressure valve. Even though the stress levels are considerably lower than those of the homogeniser or centrifuge, the number of times that the cells are exposed to these stresses is very much higher, as cells are pumped though the pipework many times during processing.

The dilution effects of the feed tank were examined and the concepts of an actual and observed rate constant were proposed. It appeared that the majority of the stress exposure was due to the pump or valve. The difference in viability between cells before and after passage though the pump head would not be truly represented by the observed viability levels of cells sampled directly from the tank as the small proportion of damaged cells returning from the pipework would be instantly diluted by the majority of cells residing in the tank. An expression for the actual inactivation

rate taking place was derived and plotted against estimated pump shear stresses. Less of a trend was apparent than was seen when the observed rate constant was plotted against pump stress.

The conclusion that can be drawn is that, due to the fact that the shear stresses within the microfiltration rig are reasonably similar to each other that the system is acting in a similar fashion to a fermenter i.e. the levels of stress are low but the number of exposures is very high. This contrasts with homogenisation and centrifugation where the stress levels are very high and the number of exposures is very low.

There are a number of consequences of this work. It has been shown that the levels of cell damage are related to the type of process and the operating conditions. Cell damage has been shown to occur in different forms. It is possible to estimate shear stress levels generated over whole operations, and also within individual components of process equipment. This can also be performed over a range of operating conditions. Correlation of the damage caused to a cell population with process conditions can aid the design of equipment and choice of a process operation.

8. Conclusion

P. putida ML2 was used as a test organism throughout this study identical batches of cell were produced by batch fermentation in fully defined media. A laboratory scale high pressure homogeniser was used as a mimic for downstream processing stresses. An assay test system was developed for measuring cellular inactivation. The test system was used to test the effects of a number of pilot scale operations, including microfiltration and centrifugation in bench top, multichamber bowl, tubular bowl and disc stack centrifuges. Stresses generated within the processing equipment were estimated and correlated with measured inactivation rate constants.

8.1 Main Findings

1. It is possible to reliably produce *Pseudomonas putida* ML2 cells by fermentation in defined media.

2. Use of laboratory scale high pressure homogeniser to mimic the effects of downstream processing is reliable and convenient.

3. ATP appears to be an indicator of metabolic activity and follows CER and OUR levels during a fermentation.

4. The bioluminescent assay of ATP appears to be a reliable indicator of the viability of *P. putida* ML2 cells in their active growth phase, following disruption.

5. Extracellular ATP has proved to be an unreliable measurement for loss of cell viability due to the presence of ATP degrading enzymes.

6. Intracellular ATP measurement is an unreliable measure of cell viability for cells processed in stationary phase due to sharp ATP increase on activation of the cells.

7. Shake flask activation of stationary phase cells and the measurement of maximum ATP levels shows some potential as a method of determining viability.

8. Decline in cell viability as measured by plate counts has been found to correlate well with protein release data, when using single-pass, low pressure homogenisation.

9. The plotting of cellular disruption against estimated homogeniser input shear stress allows the evaluation of a critical stress value, above which disruption can be observed.

10. Stationary phase *P. putida* ML2 cells show a greater resistance to rupture than growth phase cells.

11. Damage was detected when *P. putida* ML2 cells were processed using a multichamber bowl, tubular bowl and a disc stack centrifuge.

12. The action of discharging cell paste from a disc stack centrifuge appears to result in no detectable damage to the cells. Cellular damage appears to occur in the feed zone.

13. The action of the centrifuge on cells can be considered to be analogous to the action of a single homogeniser pass.

14. Microfiltration trials revealed that damage to cells occurred at high cell concentrations, when fluid viscosity increased, and loss of viability was higher than protein release in all cases.

15. Loss of biotransformation ability of *P. putida* ML2 cells appeared to follow the trends set by protein release and loss of viability during high cell concentration microfiltration trials.

16. The mechanism of cellular damage appears to differ between homogenisation, centrifugation and microfiltration.

17. Shear stress can be quantified across the range of operations tested (homogenisation, centrifugation and microfiltration) by estimation of the power dissipation per unit volume of fluid. The estimated values differed by many orders of magnitude.

18. Estimations of shear stress were made for individual components of the microfiltration rig. When inactivation rate constants were plotted against the shear stresses caused by the action of the pump it became apparent that a critical shear stress could be estimated.

19. Modelling of the cellular inactivation within the microfiltration system revealed a complex process. The action of microfiltration appears to be analogous to that of a fermenter, with repeated exposures to low levels of stress.

8.2 Importance Of Work

Design Of Process Equipment

Downstream processing equipment is increasingly designed with concessions to shear damage, the use of 'soft' feed zones in industrial centrifuges is commonplace. Using the principles outlined in this thesis estimation of shear effects within specific areas or individual components of a microfiltration rig is possible. Design of these areas can now be carried out to minimise the stresses that will be inflicted on the cells being processed. In particular components such as pump units, membrane cartridges can be evaluated alongside considerations such as dimensions of pipework.

Choice Of Processing Technique

The application of engineering principles to try to explain the relationship of processing conditions and cell inactivation rates assists in the planning of a recovery stage. For example whether to recover cells from a fermentation broth by centrifugation or microfiltration the additional factor of loss of cellular activity or disruption can be taken into account. Information about critical stress values for cell lines and stresses generated by particular operations can be invaluable in this situation.

8.3 Future Work

1. Development of bioluminescent ATP assay as a measure of cell damage. In particular the use of cells activated to their maximal metabolic rates and comparison between processed and unprocessed peak values.

2. Development of a model for estimating actual inactivation rate constants within microfiltration rigs.

3. Assessing damage effects of other process operations.

4. Use of other test organisms.

5. Establish mechanism of cell damage for process operations.

Appendix 2.1

Indole Agar Composition

To make one litre of indole agar:

Potassium dihydrogenorthophosphate	KH_2PO_4	1.33 g
Dipotassium hydrogenorthophosphate	K_2HPO_4	2.65 g
Yeast extract		3.0 g
Magnesium sulphate	$MgSO_4$	0.4 g
Peptone		0.04 g
Indole		0.1 g
Agar		15 g
RO Water		1.0 L

Autoclave for 20 minutes at 121°C and 1 bar. Cool to 50°C and pour approximately 25 mL into each sterile disposable petri dish. (BDH Ltd., Poole, Dorset, UK).

Peptone and agar were supplied by Oxoid (Unipath Ltd., Basingstoke, UK). All other chemicals supplied by Sigma Chemical Co. (Poole, Dorset, UK).

Trace Element Solution

Trace element solution used in fermentations and shake flask media

To make one litre

Calcium chloride	$CaCl_2.2H_2O$	0.66 g
Zinc sulphate	$ZnSO_4.7H_2O$	0.18 g
Copper sulphate	$CuSO_4.5H_2O$	0.15 g
Manganese sulphate	$MnSO_4.4H_2O$	0.15 g
Cobalt chloride	$CoCl_2.6H_2O$	0.18 g
Boric acid	$H_3BO_3.H_2O$	0.1 g
Sodium molybdinate	$Na_2MoO_4.2H_2O$	0.3 g
RO water	H_2O	1 L

All chemicals supplied by Sigma Chemical Co., Poole, UK.

Sterilise RO water and magnetic strirrer flea in Schott Duran Bottle (BDH Ltd., Poole, Dorset, UK) at 121°C and 1 bar for 20 minutes, allow to cool to room temperature. Add chemicals. Store at room temperature for upto one month or until precipitate is visible.

Appendix 2.2

Inoculum Shake Flask Preparation

All of the six shake flask culture media types were composed of the same base medium. Each regime consisted of the growth of two seperate cultures before transfer to the fermenter vessel. The only variations were in carbon source and the medium volume.

Shake Flask Base Medium Composition

All chemicals supplied by Sigma Chemical Co., Poole, UK.

Ammonium sulphate	$(NH_4)_2SO_4$	1 g
Magnesium sulphate	$MgSO_4\ 7H_2O$	0.2 g
Iron chloride	$FeCl_2$	0.016 g
Disodium hydrogen orthophosphate	Na_2HPO_4	3.0g
Potassium dihydrogen orthophosphate	KH_2PO_4	3.0g
Calcium chloride	$CaCl_2\ 2H_2O$	0.015 g
Trace element solution		2.0 mL
RO water	to	1.0 L

pH pre inoculation 6.8

Appendix 2.2

Flask Preparation

Shake flask 1

100 mL of base media was added to a 500 mL Quickfit flask (BDH Ltd., Poole, Dorset, UK)and autoclaved at 121°C and 1 bar. Each flask was inoculated with colonies from the nutrient agar stock culture. Benzene and peptone mixture were added in an aseptic manner.

The flask was sealed with a sterile Suba Seal (BDH Ltd., Poole, Dorset, UK). Two flasks were made up and incubated for 48 hours in a rotary shaker cabinet at 30°C and 200 rpm.

Shake flask 2

For 500 mL flasks, 100 mL of base media was added to a 500 mL Quickfit flask and autoclaved at 121°C and 1 bar. Sterile fructose solution was added so that the final mixture contained a concentration of 30 g L^{-1}. Each flask was inoculated with Shake Flask 1 culture broth. For 2000 mL flasks 400 mL of base media was used. In the case of PPInoc4 a benzene flask of identical composition to SF1 was used and inoculated with SF1 broth.

Appendix 2.2

Variation in media composition for the six different inocula regimes PPInoc1-6

		PPInoc					
		1	**2**	**3**	**4**	**5**	**6**
	flask vol	500	500	500	500	500	500
SF1	**base media**	100	100	100	100	100	100
	carbon	benz	fluoro	benz	benz	benz	benz
	[carbon]	500	500	2000	1000	1000	1000
	peptone	50	50	0	0	0	0
	volume	100	100	100	100	100	100
	incubation	24	24	24	72	72	72
	flask vol	500	500	500	500	500	2000
SF2	**base media**	100	100	100	100	100	400
	inoculum	5	5	5	10	25	100
	carbon	F	F	F	benz	F	F
	flasks	2	2	2	1	2	1
	volume	220	220	220	20	260	510

PPInoc...	*P. putida* ML2 Inoculum regime...
SF1	Shake Flask 1
SF2	Shake Flask 2
flask vol	volume of shake flask / mL
base media	volume of base media / mL
carbon	benzene (benz) or fluorobenzene (fluoro)
[carbon]	concentration of carbon source $\mu L\ L^{-1}$
peptone	volume of 0.07 % (w/v) peptone mix / μL
inoculum	SF1 culture used to inoculate SF2 / mL
carbon	fructose (F) at 30 g L^{-1} or benzene (benz) at 1000 $\mu L\ L^{-1}$
flasks	number of flasks used per 5 L of fermenter media
volume	final volume of inoculum / mL

Peptone supplied by Oxoid, Unipath Ltd., Basingstoke, UK.

Appendix 2.3 Fermenter Media

Fermenter Media Composition PPF1

Fermentations were carried out at 5, 10 and 25 L scale. The following components are required to make up 5 L of fermentation media:

All chemicals supplied by Sigma Chemical Co. (Poole, UK) unless otherwise stated.

Solution A - Base media

Ammonium sulphate	$(NH_4)_2SO_4$	42 g
Dipotassium hydrogen orthophosphate	K_2HPO_4	8.7 g
Potassium dihydrogen ortho phosphate	KH_2PO_4	6.8 g
Antifoam	polypropylene glycol	3 mL
RO water	to	4 L

Solution B

Magnesium sulphate	$MgSO_4.7H_2O$	4.6 g
Iron sulphate	$FeSO_4.7H_20$	0.016g
RO water	to	20 mL

Solution C - trace element solution

Trace element solution (See Appendix 1.2)	7.7 mL
RO water	3.3 mL

Solution D - carbon and energy source

Fructose	150 g
RO water	1 L

Appendix 2.3

pH solutions

Alkali

Ammonium solution	$(NH_4)OH$	600 mL
RO water		400 mL

Acid

Sulphuric acid	H_2SO_4, 2.5M	250 mL

Preparation

Sterilise the base media (Solution A) in the fermenter at 121°C and 1 bar for 20 minutes. Cool to 30°C. Overnight, hold temperature, agitate and sparge air through vessel. This prevents paper air filters blocking with condensation and helps to prevent contamination.

The following day, recalibrate pH and DOT probes. Sterilise ancilliaries (inoculum vessel, sample port, antifoam cylinder and acid and alkali cylinders). Autoclave fructose solution (Solution D), aseptically transfer to fermenter via inoculum addition vessel.

Sterile filter trace element solution (Solution B) and magnesium/iron sulphate mixture (Solution C) with a 0.2 μm filter (Gelman Accrodisc) into the inoculum addition vessel and introduce into the fermenter.

Adjust the pH of the media to 6.8 by addition of acid/alkali. Set stirrer speed and aeration rate. Log onto mass spectrometer and RTDAS.

Inoculate vessel by aseptically transferring the inoculum culture into the inoculum vessel.

Appendix 2.3

Variations on PPF1

The media composition and preparation was changed until a reproducible fermentation protocol was established. The original protocol was designated "PPF1" and five variations on this regime followed "PPF3-PPF6". The main variations in media composition and inoculum used are summarised in the following table.

	PPF1	PPF2	PPF3	PPF4	PPF5	PPF6
$(NH_4)_2SO_4$ g L^{-1}	8.4	1	8.4	4	8.4	8.4
$FeSO_4$ g L^{-1}	0.0032	0.0032	0.0032	0.0032	0.08	0.08
Trace elements mL L^{-1}	1.5	2	2	2	2	2
Antifoam mL L^{-1}	0.6	2	2	2	2	2
Inoculum regime	1	1	4	5	5	6
Inoculum g dry wt	0.17	0.17	0.008	0.46	0.46	0.89

To prevent Fe (II) limitation effects the sterilisation regime was changed for PPF5. $MgSO_4$ was sterilised separately by autoclaving in aqueous solution. $FeSO_4$ concentration was increased from 0.0032 to 0.08 g L^{-1}. Sterilisation was by sterile filtering in aqueous solution.

Appendix 3.1

Variations on the Standard Plate Count

The following techniques are viability tests which are based on the standard plate count:

- Pour Plate Method
- Roll-Tube Method
- Drop Count Method
- Droplette Method
- Surface Count Method
- Plate Loop Count
- Membrane filter counts
- Most Probable Number Estimates
- Rapid Automated Methods

Pour Plate Method

This is a variation on the standard plate count where 1 mL of diluted cell samples are added to 10 mL samples of molten agar culture medium which is then poured into petri dishes, allowed to set and then incubated and analysed as before. This method has all the advantages and disadvantages of the standard plate count but has one extra disadvantage apart from the obvious requirement for extra equipment. Organisms that are adversely effected or are unable to grow at temperatures much above their optima. Agar is kept molten at temperatures in the region of 45°C. A temporary mixing with media at this temperature would be acceptable for organisms such as *E. coli* which would be subsequently incubated in the region of 37°C. However less thermally robust organisms may be temporarily or lethally stressed by this change of environment and which could result in further inaccuracies creeping in. (Postgate, 1969) This is particularly relevant in the case of the test organism *P. putida* ML2 which does not grow at 37°C.

Roll-Tube Method

Instead of using plates, tubes or bottles containing media are inoculated with diluted material and rotated horizontally until the medium sets. After incubation the colonies are counted. This method is popular in the dairy industry.

Drop Count Method

This is also known as the Miles-Misra method. Small drops of material are placed on agar plates and colonies are counted in the inoculated areas after incubation. At least five drops from each dilution of sample are dropped from not more than 2 cm (to avoid splashing) onto each plate using an accurate digital pipette. When the drops are dry the plates are incubated. The colonies in each drop are counted with the aid of a magnifying glass and the data is analysed as before.

Droplette Method

Serial replicate dilutions of the sample are made mechanically in agar medium in 0.1 mL amounts and 0.1 mL drops are placed in petri dishes automatically. The apparatus offers savings in time and labour but not expense. Image analysis can be used to enumerate the number of visible colonies.

Surface Count Method

This is not a particularly accurate method and has found application for on-line sampling in food processing plants. 0.1 mL or another suitable amount of the sample measured with a standard loop or a micropipette in the centre of a well dried plate of suitable medium and spread with a loop or spreader all over the surface and then incubated. When counting colonies, difficulties arise with spreading organisms and large smears of small colonies caused when a large viable unit is broken up but not dispersed during manipulation.

Plate Loop Count

The plate loop count is a smear technique in which a wire loop is used to spread a calibrated volume of culture onto an agar plate surface as a means of measuring viable bacterial numbers. Brodsky and Ciebin (1980) report a survey conducted using thirteen commercial labs and showed a good correlation between this technique and standard plate count.

Membrane Filter Counts

Liquid containing the bacteria is passed through a filter which will retain the organisms. The filter is then allowed to absorb the culture medium and incubated, when the colonies which develop can be counted. The filter carrying apparatus is made of metal, glass or plastic and consists of a lower funnel which carries a fitted

glass platform surrounded by a silicone rubber ring. The filter disc rests on the platform and is clamped by its periphery between the rubber ring and the flange of the upper funnel. The upper and lower funnels are held together by a clamp.

The filters are thin porous cellulose ester discs varying in diameter and about 120 μ m thick. The pores in the upper layers are 0.5-1 μm diameter enlarging to 3-5 μm in diameter at the bottom. Bacteria are thus held back on top, but culture medium can easily rise to them by capillary action. Filters are stored interleaved between absorbent pads in metal containers. A grid to facilitate counting is drawn on the upper surface of each filter.

This is an effective technique for analysis of cultures that contain chemicals toxic to the cells. Craven *et al.* (1981) report an application for the quantification of viable *Staphylococcus aureus* after subjection to antibiotics.

The use of filters in this way has found particular application in aquatic microbiology where the number of organisms in any particular sample is to small for analysis and requires concentration. Hobbie (1977) and others have combined filter techniques with active dye staining and particularly epifluorescence microscopy. This is described later in section 3.1.1.5.

Commercial filter kits are available and the use of one of these is described by Ginn *et al.* (1984). The filter's base is coated with dried nutrients, overlaid with a top film coated with a water soluble gelling agent and a tetrazolium indicator dye to facilitate counting. This is only really a substitute for the SPC that reduces the amount of preparation required.

Most Probable Number Estimates

Are based on the assumption that bacteria are normally distributed in liquid media ie repeated samples of the same size from one source are expected to contain the same number of organisms on average. Some samples will obviously contain more and some will contain less. The average is the Most Probable Number. If the number is very large the difference between samples will be small and all the individual results will be close to the average. If the number is smaller then the differences between samples will be relatively larger.

If a liquid contains 100 organisms / 100 mL, then 10 mL samples will contain on average ten organisms each. Some will contain more, perhaps one or two samples will contain as many as twenty; some will contain less, but a sample containing none is most unlikely. If a number of such samples is inoculated into suitable media every sample would be expected to show growth .

Similarly, 1 mL samples will contain on average, one organism each. Some may two or three and others may contain none. A number of tubes of culture media inoculated with 1 mL samples would therefore yield a proportion showing no growth. Samples of 0.1 mL however could be expected to contain only one organism per ten samples and most tubes inoculated would be negative. It is possible to calculate the most probable number of organisms / mL for any combination of results from such sample series. Tables are available in most microbiology handbooks for these calculations.

Rapid Automated Methods

Conventional viable counts are time consuming and slow. Rapid automated methods use one or other of the following electronic particle counting, bioluminescence, changes in optical properties, changes in pH and iH by bacterial growth, detection of radiolabels, microcalorimetry and changes in conductivity.

Statistical analysis of results

Large numbers of repeats are required for most of these techniques and results are generally examined using statistical analysis. A guide to choosing the statistical method for analysing results is available (Schmehl *et al.*, 1989)

Appendix 3.2

Growth delay analysis

The theory of Growth Delay Analysis is; if a bacterial population is inoculated into an appropriate culture medium under favourable conditions it starts to grow after an apparent lag period. This can be detected as an increase in absorbance. When the untreated culture grows exponentially from the inoculated level (X_o or N_o) to an arbitrary level (X or N)in time t the relationship may be written as follows:

$$N = N_0^{[\mu(t-l]} \qquad \text{(A32.1)}$$

We assumed here that an untreated culture is a homogenous population in which every cell has a uniform lag time, l(t>l), before the first division and grows with a uniform specific growth rate μ.

If time t is replaced with t_c when N is a fixed viable cell number N_a

$$N_a = N_0^{[\mu(t_c-l)]} \qquad \text{(A32.2)}$$

The value of t_c thus correlates inversely with the logarithm of the inoculum size at a fixed level of N_a

$$\ln N_o = -\mu (t_c - l) + \ln N_a \qquad \text{(A32.3)}$$

When the inoculum size is reduced from N_o to N_o', t_c may increase by τ_c. Thus the delay time for growth is

$$\ln (N_o'/N_o) + -\mu (t_c'-t_c) = -\mu\tau_c \qquad \text{(A32.4)}$$

When $t_c' = t_c = \tau_c$ c and τ_c c.0. Here we defined the growth delay time corresponding to the reduction of the inoculum size by one tenth as G_{10} similarly to the increase in lag time L_{10} defined by Shida et al Or when $N_o' = N_o/10$, τ_c G_{10} thus

$$\mu G_{10} = 2.303 \qquad \text{(A32.5)}$$

When a cell population is exposed to stress its subsequent growth estimated by absorbance can be plotted. Indicating that the time required to reach X_a may be prolonged compared with an untreated culture. On the other hand the increase in actual viable cell number corresponding to that in absorbance during the growth may be indicating the growth delay time of τ resulting from not only an increase in the dead cell number but also the repair of sublethally injured cells.

Takano and Tsuchido then perform a further analysis assuming that instead of there being a homogenous cell population of equally stressed cells the population is heterogenous since individual cells are exposed to variations in the stress factor.

Key

G_{10}	the growth delay time corresponding to the reduction of inoculum size or viable cell fraction by one-tenth, h
l	lag time before the first cell division for untreated cells
N	cell concentration at time t, mL-1
N_a	a fixed level of bacterial cell concentration at which tc, t' and tor are measured during the exponential growth phase, mL-1
N_o,N_o'	a fixed level of bacterial cell concentration at which tc, t' and tor are measured during the exponential growth phase
t	time, h
t_c	he time required for growth of an untreated culture from N_o to N_a or from X_o to X_a, h
t'	the time required for growth of a stressed culture from N_o to N_a or from X_o to X_a, h
X	the optical density of a culture at time t
X_a	the optical density corresponding to N_a
X_o	initial OD of an untreated culture
μ	specific growth rate, h^{-1}
τ	growth delay time of a stressed culture referred to an untreated culture, t_c-t', h
τ_c	growth delay time of an untreated culture by a decrease in inoculum size, t_c'-t_c', h

For the application of Growth Delay Analysis for evaluation of total injury the following criteria should be satisfied:

- Cultivation of untreated and stressed cell populations is started with an identical inoculum size including dead and injured cells, and is conducted under identical assay conditions.
- The untreated cell population starts to grow unifor mLy after a relatively short lag time (l). In contrast, every cell in the stressed cell population has a differently prolonged lag time (l =λi) depending on the degree of injury.
- The sublethally injured cells may repair their lesions during the prolonged lag time before the first cell division and then van grow at the same rate as that of the untreated cell population.
- The growth of the untreated or repaired cells is not influenced by the cell concentration or the viable cell number.
- The OD value at a given cell concentration during the exponential growth phase reflects substantially the viable cell concentration.

Appendix 3.3

Firefly Luciferase Reaction

ATP is degraded in the presence of luciferase from the firefly *Photinus pyralis* (Photinus luciferin : oxygen 4-oxidoreductase (decarboxylating, ATP hydrolysing), EC 1.13.12.7). The overall reaction for the firefly luciferin-luciferase ATP assay is represented in the following way:

$$ATP + Luciferin + O_2 \xrightarrow{luciferase} Oxyluciferase + AMP + PPi + CO_2 + LIGHT \quad \text{(A33.1)}$$

LIGHT = 0.9 Photons of 562nm

(Stanley, 1986)

The reaction can be broken down into three distinct steps, in the presence of luciferin ATP reacts to give pyrophosphate and adenyl luciferin. Adenyl luciferin is then oxidised by molecular oxygen in the presence of enzyme to give CO_2, AMP and oxyluciferin in the electronically excited state. Upon relaxation, 0.9 photons of wavelength are emitted per molecule of ATP. The time span from the initial coming together of the reactants to final relaxation is 300 ms.

$$ATP + Luciferin \xrightarrow{luciferase} Pyrophsophate + Adenyl - luciferin \quad \text{(A33.2)}$$

$$Adenyl\text{-}luciferin + O_2 \xrightarrow{luciferase} CO_2 + Oxyluciferin^* \quad \text{(A33.3)}$$

$$Oxyluciferin^* \rightarrow Oxyluciferin + Light \quad \text{(A33.4)}$$

Oxyluciferin = Oxyluciferin in the electronically excited state*

(Stanley, 1986)

<u>Luciferase Enzyme</u>

Luciferase from the firefly Photinus pyralis. (Photinus luciferin : oxygen 4-oxidoreductase (decarboxylating, ATP hydrolysing) EC 1.13.127) is reported to have a half life of 35 days at 20°C and 140 days at -18°C. (Stanley, 1989c) The use of the enzyme for sensitive ATP assays is highly dependent on the buffer in which the assay is carried out. The use of arsenate originated in the 1950's in order to inhibit the enzyme so that measurements could be made with the instruments available at the time. The use of arsenate buffers is no longer required with the current light

detectors and its presence now lowers sensitivity. Webster *et al.* (1980) cites a paper reporting a 90% quenching of the luminescence when arsenate buffer was used instead of glyglycine buffer. Phosphate buffer at an equivalent concentration produced a 70% inhibition. Hence the composition of the reaction mixture has a marked effect on the light output. In this paper it was shown that the light production by firefly luciferase from a given quantity of ATP is greater in Tris-methyl glycine (Trycine) buffer than in other buffers, without significant changes in the kinetics of light emission or in the wavelength of light emitted. The enzyme has an anion binding site that influences its conformation. Evidence has been obtained that luciferase has a slightly different conformation in Tricine buffer and this would explain its stimulation of light production.

It appears that the inhibitors of firefly luciferase shift the emission spectrum peak towards the red, which usually decreases the light yield because of the characteristics of the phototubes used in the measurement. (Webster *et al.* 1980).

Extraction Of Intracellular ATP

The extraction of ATP from microbial cells generally requires a substantially more vigorous extractant than that required for eukaryotic ones, because of the more robust nature of the microbial cell walls and membranes. Many different papers have been written on the specific nature of many extraction agents. Stanley (1986) reports on the properties of the ideal extractant for removing intracellular ATP from a viable bacterial cell:

- It should penetrate the cell wall and membrane more or less instantaneously.
- It should extract ATP more or less instantaneously.
- It should extract the target intracellular ATP pool completely.
- It should instantaneously and irreversibly inactivate all enzymes that use ATP as a substrate or produce ATP from other sources.
- It should not cause breakdown of ATP (eg hydrolysis) either in short term (at the extraction time) or long term (during storage).
- It should not have an inhibitory (quenching effect) on firefly luciferase during ATP assay.

- It should not have any effect on the kinetics of firefly reaction. Such an effect will cause problems of signal distortion and consequently internal standardisation.
- It should not extract undue quantities of extraneous materials which in themselves affect the firefly assay (by quenching or inhibition) and / or the result, eg coloured agents, turbidity.

Nb properties 1-4 are associated with the extraction process alone whereas 5-8 have an effect on the ATP assay. Microbial ATP extractants can generally be divided into four classes:

1. Organic compounds
2. Acids such as trichloroacetic, sulphuric, nitric etc
3. Boiling buffers and steam
4. Cationic detergents and mixtures of them (Stanley, 1989a)

Organic compounds are reasonably effective for the majority of microorganisms. Substances such as ethanol are used for alga, acetone for yeast, chloroform for mycobacteria and dimethylsulphoxide and butanol for bacteria. They have the disadvantage, however that they are usually potent inhibitors of luciferase. It is therefore necessary to substantially dilute or remove entirely these compounds before the addition of luciferase. Removal may be labour intensive and tedious and could comprise the sensitivity of the assay.

The ability of dilute acids to remove small molecules from cells while simultaneously inactivating any enzymes that can act on the target molecule has been known to biochemists for many years. Of the six ATP extractants that were investigated by Lundin only trichloroacetic acid (TCA) was universally effective (Lundin 1984). However it must be noted that TCA is known to be a potent inhibitor of luciferase and hence must be diluted or removed. In addition the pH of the assay medium must be returned to neutrality where luciferase has optimal activity.

Both boiling buffer and steam are effective extractants (Lundin 1989). The most commonly reported boiling buffer extractant is Tris-Cl with EDTA. (Nelson & Lawrence, 1980, Ansehn & Nilsson, 1984, Gjerde & Helgeland, 1984) When treating a cell sample with boiling buffer it is essential that the sample is brought upto 100°C within a few seconds in order to denature cellular enzymes that may deplete the ATP pool at elevated temperatures eg 40-50°C. Samples are pipetted into a 10 fold excess of boiling buffer in a water bath. Only a few minutes at boiling

temperatures are required to extract ATP after which samples can be immediately assayed or frozen for later analysis. Buffer extractants, unlike the previous agents do not inhibit luciferase but the sample is still subject to a dilution effect which can lead to a certain loss of accuracy.

The use of cationic detergents is widely reported (Stanley, 1986). Bacterial cells often need contact for a few seconds but thicker walled microbes such as yeast and spores may need a minute or even longer to effect a certain amount of extraction. However the majority of commercial ATP test kits include a cationic based releasing agent for extraction of intracellular ATP. Many detergents are powerful luciferase inhibitors and effect the light output from constant output reagents.

No standard method for extraction of ATP from microbial cells has been established and most of the listed methods are effective. Extracts made using organic solvents, acids and boiling buffer all require extra processing and this inevitably leads to a loss of accuracy. Detergents appear to be a particularly promising solution as they are easy to use and avoid large dilutions. This convenience must be balanced against occasional lack of accuracy and possible deleterious effects on luciferase kinetics.

Reaction Sensitivity

Under optimal conditions light intensity is proportional to ATP concentrations in the region of $5x10^{-14}$ to 10^{-6}molar. The number of bacteria detectable will depend on the sensitivity of the reagent (number of photons/ ATP concentration x time) and usually be limited either by the blank value of the reagent (residual or contaminating ATP) or by the background of the instrument and by the latter's efficiency (number of photons necessary to obtain one count). The sensitivity of reagents can be increased by using higher enzyme concentrations but this will result in a faster consumption of ATP and a steeper decay of the luminescence. The sensitivity attainable can easily be estimated starting from the most favourable conditions encountered in practice:

- Luminescence decay: 1% min^{-1} (i.e. 1% of ATP consumed per minute)
- Instrumental background: 2000 counts min^{-1}
- Instrumental efficiency: 1 count /1000 photons
- Reagent blank approximately equal to instrumental background
- Sample volume 100 μmol

If the detection limit is considered to be equal to the sum of the instrumental background and the reagent blank we may conclude that the lowest number of ATP molecules detectable is 2×10^8 for a sample volume of 100 $\mu M\ L^{-1}$ this amounts to concentration of $2 \times 10^8 \times 10^4 / 6 \times 10^{23} = 3 \times 10^{-12}$ mol L^{-1}, or 2×10^3 bacteria mL^{-1}. A more realistic figure would lie around 10^{-11} mol L^{-1}, ie between 5 and 10000 bacteria mL^{-1} which fits the goal usually pursued. Higher sensitivities can only be obtained at the cost of increased enzyme concentration, with the associated drawbacks. Another difficulty arises from the fact that for the measurement of concentrations around 10^{-12} mol L^{-1} and lower, extreme precautions must be observed : water and reagents must be devoid of contaminating ATP . The absolute efficiency of luminometers can be estimated in a rough way by integrating the number of counts registered for a given amount of ATP consumed, considering the fact that, in a first approximation, the luminescence decay corresponds to ATP consumption, and that the quantum efficiency for firefly luciferase approaches unity.

Sources Of Error

- Absorption by quenching-if substrate is present who's absorption spectrum overlaps that of luciferase reaction
- Luciferase inhibition-of ATP/luciferase binding sites
  - -by neutral salts-ionic concentration
  - -specific effect of anion in accordance with cheotropic series

All of the above can be accounted for by the use of an internal standard.

Appendix 3.4

Instructions for carrying out the ATP assay

Equipment and reagents

All measurements were made using a commercially available test kit supplied by Bio-Orbit ATP monitoring kit (1243-110) and a LKB Wallac 1250 luminometer. Both available from Labsystems Ltd.(Basingstoke, UK). Assays were developed from methodology outlined by Bio-Orbit (1992).

The ATP test kit consisted of the following reagents:

- Monitoring reagent
- Releasing agent
- ATP standard
- Tris buffer

Samples

Cell samples were diluted with phosphate buffer at the same concentration as the fermenter medium. This was to bring the target ATP withing the sample within the linear range of the equipment and also to prevent quenching of the emitted light by cellular components. This resulted in cell samples that were in the range of 0-1 g dry wt L^{-1}. At the end of the fermentation growth phase (13.5 g dry wt L^{-1}) this was a 10-20 fold dilution.

Monitoring Reagent (Bio-Orbit 1243-200)

This is a lyophilized mixture composed of the following ingredients:

- Firefly luciferase
- D-Luciferin
- Bovine Serum Albumin 50 mg
- Magnesium Acetate 0.5 mmol
- Inorganic pyrophosphate 0.1 μmol

Stored at -18°C and reconstituted by the addition of 10 mL of tris buffer. Ten 1 mL aliquots of reagent are pipetted into sterile, new disposable universals before freezing

at -18°C. Storage is possible for upto 2 months. Each sample is reconstituted by the addition of 4 mL of Tris buffer and stored on ice during use.

Releasing Agent (Bio-Orbit 1243-208)

Undisclosed ionic surfactant for permeabilising microbial membranes for the release of ATP, mixed with ATPase inhibitors. Stored at 4°C, no preparation or reconstitution required, used directly from bottle.

Tris buffer (Bio-Orbit 1243-227)

100 mM tris-actetate buffer, 2 mM EDTA, pH 7.75 at 25°C

ATP Standard (Bio-Orbit 1243-201)

Supplied in bottles containing:

0.1 μmol ATP
Magnesium sulphate 2 μmol

Stored at -18°C. Reconstituted by the addition of 10 mL of Tris buffer to give a 10^{-5}M ATP solution. 1 mL aliquots were transferred into sterile, unused 30 mL universal bottles which were stored at -18°C. Reconstitution was with 9 mL of Tris buffer to give a 10^{-6}M solution which was stored on ice during use.

Luminometer

Switched on 15 minutes before taking a reading. Cuvette containing 500 μL of measuring reagent inserted into measuring chamber, cover closed, press "Background" button to zero instrument.

Pipette Tips

ATP is heat stable and contamination of equipment should be avoided wearing examination gloves while inserting disposable pipette tips into racks before sterilising by autoclaving at 121°C and 1bar for 20 minutes.

Assay Procedure

Extracellular [ATP]

1. 100 μL of buffer into cuvette.
2. 100 μL of sample into cuvette.
3. Add 500 μL of Monitoring reagent. Agitate.
4. Place cuvette into measuring chamber of luminometer. Press "Start" button. Read measurement.
5. Make duplicate samples

Total [ATP]

1. 100 μL of sample into cuvette.
2. Add 100 μL of Releasing Agent. Agitate for 30 seconds.
3. Add 500 μL of Monitoring reagent. Agitate.
4. Place cuvette into measuring chamber of luminometer. Press "Start" button. Read measurement.
5. Make duplicate samples

ATP Standard

1. 100 μL of buffer into cuvette.
2. 100 μL of ATP standard into cuvette.
3. Add 500 μL of Monitoring reagent. Agitate.
4. Place cuvette into measuring chamber of luminometer. Press "Start" button. Read off measurement.

Intracellular [ATP] calculation

1. Subtract extracellular reading from total to give intracellular [ATP]
2. Multiply by Standard reading/10^{-6} to give [ATP] in mol x10^{-6}

Appendix 3.5

ATP release prediction for 450 Bar Homogenisation

Step to step mass balancing was used to predict the release of ATP

Pass 1

	Protein %	I/C [ATP]	E/C [ATP]
Before	0	0.51	0.255
After	54.7	0.53	0.728

If it is assumed that protein release is equivalent to number of broken cells and 54.7% are broken, then ATP released

$$= \text{broken cells x initial intracellular [ATP]}$$

$$= \frac{54.7}{100} \times 0.51$$

$$= 0.278$$

$$+ \text{E/C [ATP] } (0.2555) = 0.534$$

$$\text{measured} = 0.728$$

Pass 2

	Protein %	I/C[ATP]	E/C[ATP]
Before	54.7	0.53	0.728
After	73.6	1.028	1.42

Difference before and after pass = $\frac{(100-54.7)-(100-73.6)}{(100-54.7)}$ x 100

= 41.7 %

ATP released = Broken cells x Initial I/C [ATP]

= $\frac{41}{100}$ x 0.53

= 0.221

+ initial E/C [ATP] (0.728) = 0.949

measured = 1.42

Pass 3

	Protein %	I/C[ATP]	E/C[ATP]
Before	73.6	1.028	1.42
After	83.38	1.42	2.18

% Broken = $\frac{(100-73.6)-(100-83.38)}{(100-73.6)}$ x 100

= 37.04 %

ATP release = $\frac{37.04}{100}$ x 1.028

= 0.38

+E/C [ATP] (1.42) = 1.8

measured = 2.18

Pass 4

	Protein %	I/C[ATP]	E/C[ATP]
Before	83.38	1.42	2.18
After	98.28	1.66	2.86

$$\% \text{ broken} = \frac{(100-83.38)-(100-98.28)}{(100-83.38)} \times 100$$

$$= 89.65\ \%$$

$$\text{ATP release} = \frac{89.65}{100} \times 1.42$$

$$= 1.27$$

$$+ \text{E/C [ATP] (2.18)} = 3.45$$

$$\text{measured} = 2.86$$

Pass 5

	Protein %	I/C[ATP]	E/C[ATP]
Before	98.28	1.6	2.86
After	100	0	3.316

$$\% \text{ Broken} = \frac{(100-98.28)-(100-100)}{(100-100)} \times 100$$

$$= 100\ \%$$

$$\text{ATP release} = \frac{100}{100} \times 1.6$$

$$= 1.6$$

$$+\text{E/C [ATP] (2.86)} = 4.46$$

$$\text{measured} = 3.136)$$

Appendix 3.5

Summary - predicted and measured extracellular ATP

Pass	Predicted	Measured
1	0.534	0.728
2	0.949	1.42
3	1.8	2.18
4	3.45	2.86
5	4.46	3.136

Appendix 4.1 Centrifugation Results

Multichamber Bowl Centrifugation

Centrifugation Of *P. putida* ML2 Using a Westfalia KDD605 Multichamber Bowl Centrifuge.

SHPP32	dry wt	% VCC	σ	% INTACT	σ
HARVEST	13.2	100	23	100	3
RESUSPEND	13.1	90	20	97	11

SHPP34	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	13.8	100	34	100	2
RESUSPEND	13.4	87	18	99	6

SHPP42	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	13.3	100	29	100	5
RESUSPEND	12.9	85	23	97	9

Dry wt — Cell concentration as dry weight (g dry wt L^{-1})

* — non-resuspended solids

σ — standard deviation (percent)

% VCC — % viable cell count (of original harvest value)

$$= 100 \times \frac{\text{cell counts / unit dry weight (sediment)}}{\text{cell counts / unit dry weight (feed)}}$$

Intact — % of intact cells (% protein release of maximum)

$$= 100 \times \frac{\text{(resuspended - background) / unit dry weight}}{\text{(maximum - background) / unit dry weight}}$$

Tubular Bowl Centrifugation

Centrifugation Of *P.putida* ML2 in The Sharples-Pennwalt 1P Tubular Bowl Centrifuge.

SHPP31	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	13.6	100	25	100	6
RESUSPEND	12.6	90	14	94	6

SHPP37	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	12.4	100	16	100	3
RESUSPEND	12.2	87	22	97	3

SHPP46	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	13.5	100	17.3	100	2
RESUSPEND	11.3	83.5	66.3	89	21

Dry wt — Cell concentration as dry weight (g dry wt L^{-1})

* — non-resuspended solids

σ — standard deviation (percent)

% VCC — % viable cell count (of original harvest value)

$$= 100 \times \frac{\text{cell counts / unit dry weight (sediment)}}{\text{cell counts / unit dry weight (feed)}}$$

Intact — % of intact cells (% protein release of maximum)

$$= 100 \times \frac{\text{(resuspended - background) / unit dry weight}}{\text{(maximum - background) / unit dry weight}}$$

Appendix 4.1

Disk Stack Centrifugation

Centrifugation of *P. putida* ML2 using a Westfalia CSA1 disc stack centrifuge.
For all measurements three samples were taken and analysed three or four times

SHPP43	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	11.3	100	15	100	16
DISCHARGE	10.13	74	18	95	3

SHPP45	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	14.4	100	24.8	100	2
NO DISCHARGE	78.67*	68	7	97	5
DISCHARGE	97.33*	72	10	95	5

SHPP46	DRY WT	% VCC	σ	% INTACT	σ
HARVEST	12.75	100	41	100	3
NO DISCHARGE	8.925	90	24	99	17
DISCHARGE	11.625	89	7	96	6

Dry wt — Cell concentration as dry weight (g dry wt L^{-1})

* — non-resuspended solids

σ — standard deviation (percent)

% VCC — % viable cell count (of original harvest value)

$$= 100 \times \frac{\text{cell counts / unit dry weight (sediment)}}{\text{cell counts / unit dry weight (feed)}}$$

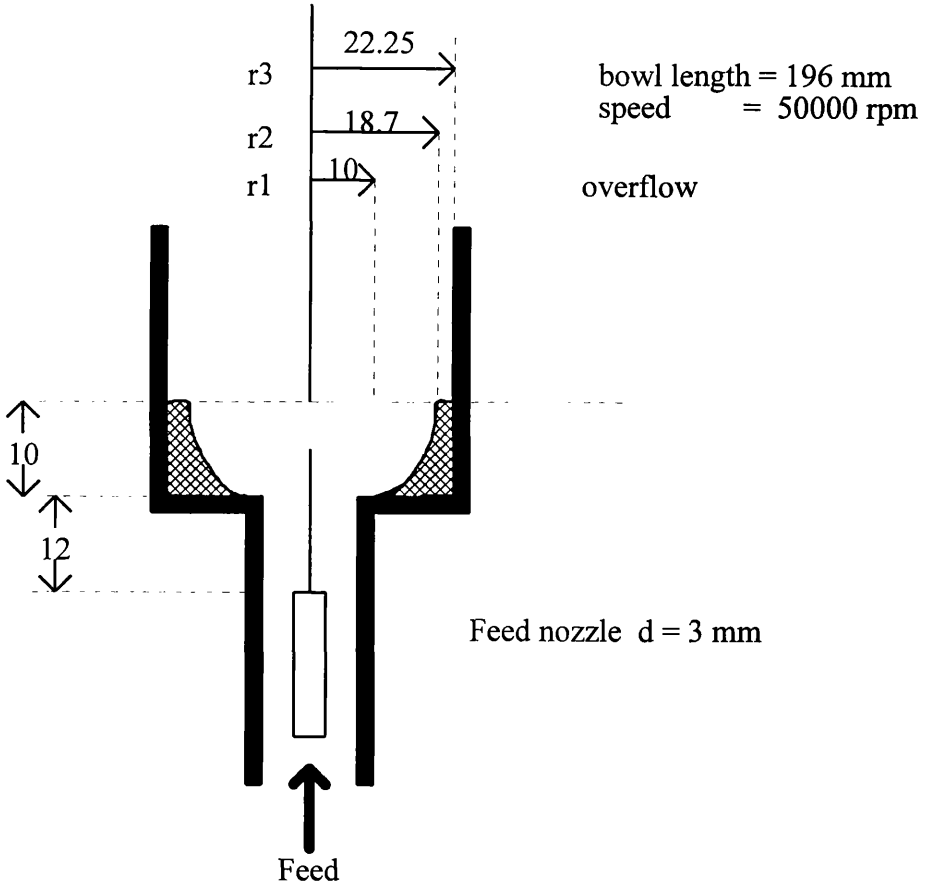
Intact — % of intact cells (% protein release of maximum)

$$= 100 \times \frac{\text{(resuspended - background) / unit dry weight}}{\text{(maximum - background) / unit dry weight}}$$

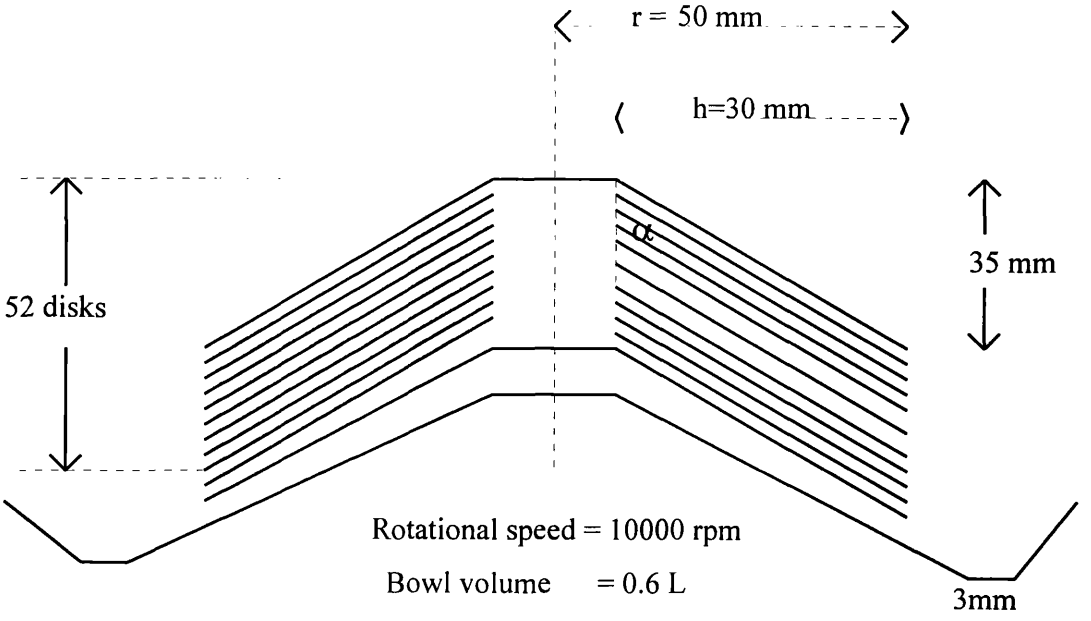
Appendix 4.2

Centrifuge Dimensions

1P tubular bowl centrifuge dimensions



CSA1 disk stack centrifuge



6 Feed tubes 20 mm long and 3mm diameter

Westfalia KDD605 Multichamber bowl centrifuge

Radii:

Chamber	1	0.035
	2	0.05
	3	0.062
	4	0.075

Appendix 6.1 Estimation of fermenter shear stress

The shear stresses generated in the fermenter during cell growth have to be below any critical value for inactivation. The value for shear stress in the LH42L fermenter was estimated for the conditions of *P. putida* ML2 growth in the following way:

1. Determination of nature of fluid flow
2. Calculation of power number
3. Assume 90% of power is dissipated within 10% of the volume
4. Calculate shear stress

1. Determination of nature of fluid flow

In order to calculate the shear stresses generated within a fermenter it is necessary to determine the nature of the fluid flow. This is carried out by calculation of the Reynolds Number.

Fermenter dimensions:

d impeller diameter, 0.08 m

N impeller speed, 13.33 rps

ρ density of liquid, 1000 kg m^{-3}

μ viscosity of 0-14 g dry wt L^{-1} cell suspension, 1.5 $x10^{-3}$ Nsm^{-2}

$$\text{Reynolds number} = \frac{d^2 N \rho}{\mu} \qquad (6.35)$$

$$= \frac{0.08^2 \ 13.33 \ 1000}{1.5 \times 10^{-3}}$$

$$= 5.69 \times 10^4$$

The Reynolds number is a function of the type of fluid flow within the vessel. For a fully baffled vessel:

Reynolds number	<	10	system is laminar
Reynolds number	=	10 - 10^4	system is transitional
Reynolds number	>	10^4	system is fully turbulent

The Reynolds number was calculated to be 5.69 x10^4. Therefore the system is fully turbulent.

2. Calculation of power number

The power number is a dimensionless constant which is the ratio of the external to inertial forces of an agitated liquid (Aiba *et al.*, 1973). The power and Reynolds numbers for a variety of different impeller geometries has been correlated. In turbulent flows each type of impeller has a characteristic power number which is independent of Reynolds number.

The power number, N_p, is calculated:

$$N_p = \frac{P}{d^5 N^3 \rho} \qquad \text{(A61.1)}$$

For a flat blade impeller in a fully turbulent system (i.e. when Reynolds number is greater than 10^4)

Power number, N_p = 6

For single impeller Power = $N_p\, d^5 N^3 \rho$ (A61.2)

(Aiba *et al.*, 1973) = 6 x 0.08^5 x 13.33^3 x 1000

= 47 W

For 3 impellers multiply power by 2 = 94 W
(Aiba *et al.*, 1973)

3. Assume 90% of power is dissipated within 10% of the volume

In order to calculate the maximum shear rates within a fermenter vessel it is assumed that 90% of the power is dissipated in 10% of the volume of the liquid. Shear rates are highest near the tips of the impellers (van't Reit and Smith, 1975, Tomi and Bagster, 1978).

Near impellers $\frac{P}{V} = \frac{P \times 0.9}{V \times 0.1}$ (A61.3)

$$= \frac{94 \times 0.9}{27.5 \times 10^{-3} \times 0.1}$$

$$= 3.1 \times 10^{4} \text{ W m}^{-3}$$

The maximum power per unit liquor volume in fermenter (27.5 L or)

$$= 31 \text{ W L}^{-1}$$

4. Calculation of shear stress

Shear stress within a fermenter, G, is given by the following equation

$$\tau = \left(\frac{P\mu}{V}\right)^{1/2} \quad (6.20)$$

P power

μ viscosity

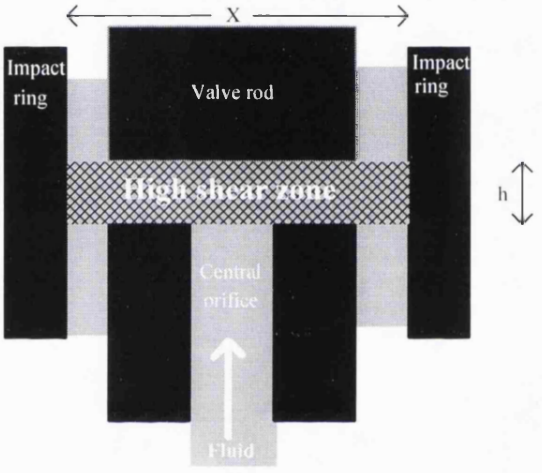
$$= \left(3.1 \times 10^{4} \quad 1.5 \times 10^{-3}\right)^{1/2}$$

$$= \mathbf{6.82 \text{ Nm}^{-2}}$$

Appendix 6.2 Estimation of homogeniser shear stress

The volume over which the shear forces act is considered to be that over which power is dissipated. This is not easily defined and has to be estimated. It is depicted in the following diagram by the shaded area.

Lab 40 homogeniser high shear area



To calculate volume over which power is dissipated:

$$V = \pi \frac{X^2}{4} h \qquad \text{(A62.1)}$$

When V has been calculated the shear stress is estimated by the power dissipation model:

$$\tau = \left(\frac{\Delta p Q \mu}{V} \right)^{1/2} = \left(\frac{4 \Delta p Q \mu}{\pi X^2 h} \right)^{1/2} \qquad \text{(A62.2)}$$

However there is a problem with estimating the volume of the disruption chamber because the distance, h, is too small to measure and is varied over the range of operating pressures.

However it can be assumed that the operating pressure:

$$\text{i.e. } \Delta p \propto \frac{1}{h^2} \qquad \text{(A62.3)}$$

(Keshavarz *et al.*, 1990)

or

$$h \propto \frac{1}{\Delta p^{1/2}} \qquad \text{(A62.4)}$$

$$h = \frac{k'}{\Delta p^{1/2}} \qquad \text{(A62.5)}$$

k' can be calculated, Keshavarz Moore *et al.* (1990). For example at an operating pressure of 300 bar (30×10^6 Nm^{-2}):

h 15 μm (15×10^{-6} m)

$$k' = h \times \Delta p^{1/2} \qquad \text{(A62.6)}$$

$$= 15 \times 10^{-6} \times (30 \times 10^6)^{1/2}$$

$$= 0.0822\ N^{1/2}$$

Therefore the shear expression can be modified:

$$\tau = \left(\frac{4 \Delta p^{3/2} Q \mu}{\pi X^2 k'} \right)^{1/2} \qquad \text{(A62.7)}$$

Using power dissipation model to calculate the generated shear.
For example at 300 bar:

τ shear stress, Nm^{-2}

μ viscosity of cell suspension, 0.0015 Nsm^{-2}

Δp Pressure change ≡ Operating pressure (1 bar = 10^5 Nm^{-2})

Q Flow rate (Volume displaced / homogenisation time)

eg 80 mL sec^{-1} ≡ 8×10^{-5} m^3 s^{-1}

X diameter of impact ring (1 cm) (Keshavarz-Moore *et al.*, 1990)

$$\tau = \left(\frac{4\Delta p^{3/2} Q \mu}{\pi X^2 k'} \right)^{1/2} \qquad \text{(A62.8)}$$

$$= \left(\frac{4 \quad 300\text{x}10^5 \quad 8\text{x}10^{-5} \quad 0.0015}{\pi \quad 0.01^2 \quad 0.0822} \right)^{1/2}$$

$$= 55300 \ Nm^{-2}$$

This calculation can be performed over the whole range of operations.

Appendix 6.3 Estimation Of Centrifuge Shear Stress

A number of models have been proposed for the estimation of shear stresses generated within a centrifuge. Two will be outlined in this Appendix:

- Spinning Disc Model
- Pump Model

Spinning Disc Model

The Spinning Disc Model was proposed by Bell (1982) for estimation of shear stresses within the CSA 1 disc stack centrifuge. Dimensions of all the centrifuges used can be found in Appendix 4.2.

$$G_{max} = 2\,\mu^{-1/2}\,\rho^{1/2}\,r\,\omega^{3/2} \qquad \text{(A63.1)}$$

$$= 2\,(1.5\times10^{-3})^{-1/2}\;1000^{1/2}\;0.05\;1050^{3/2}$$
$$= 51.63 \times 31.6 \times 0.05 \times 33878$$
$$= 2.8 \times 10^{6}\ s^{-1}$$

$$G_{average} = 0.2\,G_{max} \qquad \text{(A63.2)}$$

$$= 0.2 \times 2.8 \times 10^{6}$$
$$= 5.6 \times 10^{5}$$

$$\text{Mean shear stress, } \tau = \mu G \qquad (6.18)$$
$$= 840\ N\ m^{-2}$$

This model does not take into account the effects of flow rate.

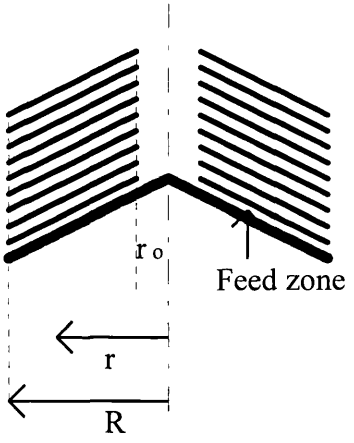
Pump Model

If the action of the centrifuge is considered to be similar to that of a pump it should be possible to estimate shear by the pump model, mentioned earlier. This can be carried out in two stages

- Calculation of change in pressure
- Use pump calculation to estimate shear rate

Calculation of change in pressure

Westfalia CSA 1 Disc Stack Centrifuge



To calculate the pressure generated in a disc stack

Where

R radius of disc stack

r_o distance from centre of disc stack to start of discs

r point within the fluid flow along the disc

g $\omega^2 r$

For an element of fluid, width dr, and radius r (where $r<R$, $r>r_o$)

$$dp_r = \rho g (r + dr - r_o) - \rho g (r - r_o) \quad \text{(A63.3)}$$

$$= \rho \omega^2 (r + dr)(r + dr - r_o) - (\rho \omega^2 r(r - r_o) \quad \text{(A63.4)}$$

$$\frac{dp_r}{\rho\omega^2} = r^2 + dr^2 + 2rdr - r_o r - r^2 + r r_o - dr r_o \quad \text{(A63.5)}$$

$$dp_r = \rho \omega^2 (2r - r_o)dr \quad \text{(A63.6)}$$

$$\int_{p_0}^{p_r} dp_r = \rho\,\omega^2 \int_{r_o}^{r} (2r - r_o)dr \qquad \text{(A63.7)}$$

$$p_R\text{-}p_O = \rho\,\omega^2 \left[r^2 - r\,r_o\right]_{r_o}^{R} \qquad \text{(A63.8)}$$

$$= \rho\,\omega^2\,(R^2\text{-}Rr_o\text{-}r_o^2 + r_o^2) \qquad \text{(A63.9)}$$

$$= \rho\,\omega^2\,R\,(R\text{-}r_o) \qquad \text{(A63.10)}$$

$R\text{-}r_o$ head of fluid

$p_R\text{-}p_O$ pressure generated in feed zone of centrifuge

Where:

ρ = 1000 kg m^{-3}

g = ω^2 r

speed = 10000 rpm

ω = angular velocity (1047 rad s^{-1})

R = 0.05 m

r_o = 0.02 m

$$= 1000 \left(\frac{2\,\pi\ 10000}{60}\right)^2 0.05\ (0.05 - 0.02)$$

$$\Delta p = 1.6 \times 10^6\ \text{N m}^{-2}$$

Use pump calculation to estimate shear stress

$$\text{Shear stress, } \tau = \left(\frac{\Delta p\, Q\mu}{V}\right)^{1/2} = \left(\frac{\rho\omega^2 R(R - r_o)Q\mu}{V}\right)^{1/2} \qquad \text{(A63.11)}$$

Δp = 1.63 x 10^6 N m^{-2}

Q = 50 L h^{-1}

= 1.38 x 10^{-5} m^3 s^{-1}

μ = 1.5 x 10^{-3} Nsm^{-2}

V = 2.1 x10^{-7} m^3

$$= \left(\frac{1.63 \times 10^6\ \ 1.38 \times 10^{-5}\ \ 1.5 \times 10^{-3}}{2.1 \times 10^{-7}}\right)^{1/2}$$

$$= \mathbf{400\ N\ m^{-2}}$$

The two results are of the same order of magnitude but the pump analogy model is probably more suitable because it takes into account the effects of flow rate.

Similar analyses were carried out for the multichamber bowl and the tubular bowl centrifuges:

Westfalia KDD605 multichamber bowl centrifuge

Δp	=	1.2×10^{6} N m^{-2}
Q	=	50 L h^{-1}
	=	1.38×10^{-5} m^3 s^{-1}
μ	=	1.5×10^{-3} Nsm^{-2}
V	=	3.1×10^{-4} m^3
τ	=	**9 Nm^{-2}**

The feed zone size for the multichamber bowl centrifuge is the volume of the first chamber.

Sharples-Pennwalt 1P Tubular Bowl centrifuge

Δp	=	6.73×10^{8} N m^{-2}
Q	=	50 L h^{-1}
	=	1.38×10^{-5} m^3 s^{-1}
μ	=	1.5×10^{-3} Nsm^{-2}
V	=	7.1×10^{-7} m^3
τ	=	**4310 Nm^{-2}**

The feed zone size for the tubular bowl centrifuge is given by Bell (1982).

Appendix 6.4 Estimation Of Shear Stresses Within Microfiltration Rig

The following shear estimations were made:

- Pipe wall shear stress
- Pump shear stress -by tip speed
  - by power dissipation

Pipe Wall Shear Stress Calculation

Blasius equation for turbulent flow:

$$\phi = 0.0396\ Re^{-0.25} \qquad \text{(A64.1)}$$

(Coulson et al., 1990)

$$\phi = \frac{\tau_0}{\rho v^2} \qquad \text{(A64.2)}$$

(Stanton and Pannel, 1914)

Shear stress

$$\tau = 0.0396\ \rho\ v^2\ Re^{-0.25} \qquad \text{(A64.3)}$$

Re Reynolds number

$$= v\ d\ \rho / \mu \qquad \text{(A64.4)}$$

ϕ friction factor

ρ density 1000 kg m^3

v velocity m s^{-1}

τ shear stress N m^{-2}

μ viscosity

Estimated wall shear stresses for the microfiltration runs

Cell concentration g dry wt / L	Δp_{tm} bar	cross flow velocity, m s^{-1}	Shear stress, τ, N m^{-2}	Rate constant, k
5	1	3.05	35	-0.08527
7	1	3.05	35	0.03361
7	4	2.64	27	0.07337
7	1	3.03	32	-0.1157
14	1	3.89	54	0.031555
14	4	3.82	52	0.00753
61	4	3.71	59	0.1575
84	4	3.72	67	0.2042
113	1	4.92	126	0.20089
113	4	4.61	121	0.25232

Calculation of shear stress within the pump

Two different engineering models for the calculation of pump shear stress can be applied to this system. The first based on the shear encountered between the tips of the pump vanes and the second based on energy dissipation per unit volume as for a stirred tank reactor.

Tip speed calculation

Pump speed, n 920 rpm, 15.33 rps
diameter 50 mm, 0.05 m
no of vanes 4
gap, l 0.1 mm, 0.1×10^{-3} m

$$\omega = 2 \pi n \qquad \text{(A64.5)}$$

$$\text{Tip speed, } v = r \omega \qquad \text{(A64.6)}$$
$$= 2.5 \times 10^{-3} \times 2 \pi \times 15.33$$
$$= 2.4 \text{ m s}^{-1}$$

Shear rate, G $= \frac{v}{l}$ (A64.7)

$= \frac{2.4}{0.1 \times 10^{-3}}$

$= 24 \times 10^{-3}\ s^{-1}$

Shear stress = viscosity x shear rate

$= \mu G$ (6.18)

Estimated pump shear stress using tip speed calculation

Cell concentration g dry wt L^{-1}	Viscosity, μ Nsm^{-2}	Shear stress, τ $N\ m^{-2}$
7, 14	1.5×10^{-3}	36
61	3×10^{-3}	74
84	5.3×10^{-3}	130
113	8.8×10^{-3}	210

The obvious problem with this method of estimating the shear stresses is the fact that it does not take into account the operating pressures. This is possibly the reason why it fails to explain the different measured viability losses when cell suspensions of 113 g dry wt L^{-1} were processed at 1 and 4 bar.

Estimation of pump shear by power dissipation

The change in pressure across the pump head can be considered to be simply the membrane inlet pressure. This is assuming that any effects of the weight of the fluid in the tank above the pump are ignored. The membrane inlet pressure is measured by a gauge 1.4 m from the pump exit. A drop in pressure would be expected from the pump to the gauge. To perform any stress calculations the outlet pressure at the pump needs to be estimated by equating the forces in the pipework.

Length, l 1.4 m
pipe, d 0.006 m
wall stress τ_W

Total frictional force at the walls is the product of τ_W and surface area ($\tau_W \pi dl$). This results in Δp. (Coulson *et al.*, 1990)

$$\tau_W \pi dl = \frac{\Delta p \pi d^2}{4} \quad \text{(A64.8)}$$

$$\Delta p = \frac{4 l \tau_w}{d} \quad \text{(A64.9)}$$

$$= \frac{4 \times 1.4 \times \tau_w}{0.06}$$

$$= 933 \tau_W \ Nm^{-2}$$

$$= 0.0933 \tau_W \ bar$$

$$p_{pump} = p_{inlet} + (0.00933 \tau_W) \quad \text{(A64.10)}$$

Therefore the shear stress, τ, for the pump can be expressed as:

$$\tau = \left(\frac{\Delta p_{pump\ outlet} Q \mu}{V} \right)^{1/2} \quad \text{(A64.11)}$$

V volume
τ pump shear stress
p pressure
Q volumetric flow rate
μ viscosity

Appendix 6.4

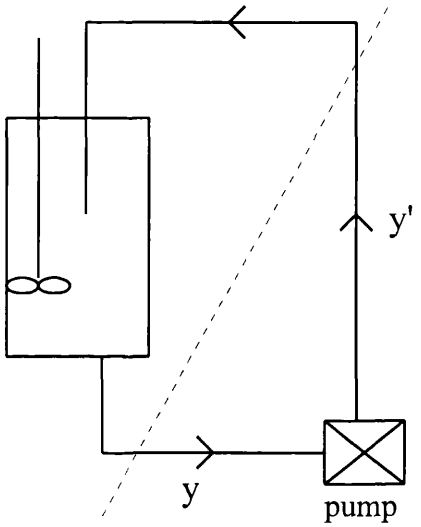
Estimated pump shear stresses by analogy to stirred tank reactor

Cell concentration g dry wt L^{-1}	Δp_{tm} bar	p_{pump} bar	cross-flow velocity, m s^{-1}	τ N m^{-2}	k h^{-1}
5	1	1.53	3.05	44	-0.08527
7	1	1.65	3.05	46	0.03361
7	4	4.52	2.64	71	0.07337
7	1	1.62	3.03	46	-0.1157
14	1	1.70	3.89	53	0.1575
14	4	4.79	3.82	88	0.03155
61	4	4.70	3.71	123	0.00753
84	4	4.82	3.72	163	0.2042
113	1	2.58	4.92	177	0.20089
113	4	5.38	4.61	289	0.25232

Appendix 6.5 Modelling the system

The mechanism of cell damage in the homogeniser and the centrifuges appears to be fairly straigtforward. The majority of cells in the processed samples are subjected to similar amounts of shear and damage is fairly uniform throughout the population. However the microfiltration rig is a more complex system. If the action of the pump alone is considered:

Action Of The Microfiltration Pump



Viable cells, of concentration y, leave the tank and flow through the pump. The action of the pump results in damage to the cell population and the concentration of viable cells leaving the pump is y'. If N is the number of times the cells are passed through the pump and K is the inactivation rate constant:

$$\Delta y = -K\, y\, \Delta N \qquad \text{(A65.1)}$$

$$\frac{dy}{dN} = -K\, y \qquad \text{(A65.2)}$$

$$\int_{y}^{y'} \frac{dy}{y} = -K \int_{N}^{N+1} N \qquad \text{(A65.3)}$$

$$[\ln y]_{y}^{y'} = -[KN]_{N}^{N+1} \qquad \text{(A65.4)}$$

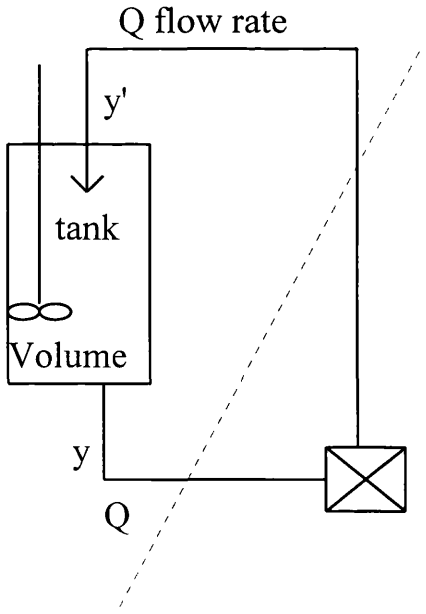
$$\ln\frac{y'}{y} = -K(N+1-N) = -k \qquad \text{(A65.5)}$$

$$-k = \ln\left(\frac{y'}{y}\right) \qquad \text{(A65.6)}$$

$$\text{or} \quad y' = y\,e^{-k} \qquad \text{(A65.7)}$$

The presence of the tank prevents the system acting as a closed loop. As y' viable cells leave the pump they flow through the pipework and are returned to the tank. Assuming perfect mixing within the tank they are instantly diluted with the other cells. The majority of the cells reside within the tank. Approximately 4m of pipework corresponds to approximately 100 mL of fluid compared to 8 L within the tank. The fluid leaving the tank therefore contains some proportion of the population that has just returned from the pipework. The observed inactivation rate constant is measured from cell samples from the tank and reflects this dilution effect. The rate constant, K, is the actual rate constant which reflects the levels of damage occurring within the pump.

Fig 6.7 The action of the microfiltration tank



Where:

V volume of tank

Q flow rate

y viable cells leaving tank

y' viable cells returning to tank

$$V\frac{dy}{dt} = Qy'-Qy \qquad \text{(A65.8)}$$

$$Q(y_{'}-y) = V\frac{dy}{dt} \qquad \text{(A65.9)}$$

$$(y'=y\,e^{-K})$$

$$Q(y\,e^{-K}-y) = V\frac{dy}{dt} \qquad \text{(A65.10)}$$

$$\frac{Q}{V}y(e^{-K}-1) = \frac{dy}{dt} \qquad \text{(A65.11)}$$

$$\int_{y=100}^{y=y}\frac{dy}{\left(ye^{-K}-1\right)} = \frac{Q}{V}\int_{t=0}^{t=t}dt \qquad \text{(A65.12)}$$

$$\int_{y=100}^{y=y}\frac{dy}{y} = \frac{Q}{V}(e^{-K}-1)\int_{t=0}^{t=t}dt \qquad \text{(A65.13)}$$

$$\left[\ln y\right]_{y=100}^{y=y} = \frac{Q}{V}\left(e^{-K}-1\right)t \qquad \text{(A62.14)}$$

$$\ln\frac{y}{100} = \frac{Q}{V}\left(e^{-K}-1\right)t \qquad \text{(A65.15)}$$

The observed rate constant, k, that has been calculated:

$$\ln\frac{y}{100} = -k\,t \qquad \text{(A65.16)}$$

K= actual rate constant

k= measured rate constant

$$k = -\frac{Q}{V}\left(1 - e^{-K}\right) \quad \text{(A65.17)}$$

$$e^{-K} = 1 - \frac{V}{Q}k \quad \text{(A65.18)}$$

$$K = -\ln\left(1 - \frac{V}{Q}k\right) \quad \text{(A65.19)}$$

$$= \ln\left(\frac{1}{1 - \frac{V}{Q}k}\right) \quad \text{(A65.20)}$$

Assume $K = b(\tau - \tau_{crit})$ (A65.21)

with $\tau_{crit} = 50 Nm^{-2}$

Microfiltration data

Dry weight g dry wt L^{-1}	**Observed rate constant k,** s^{-1}	**Actual rate constant K**	**Inactivation constant b** m^2N^{-1}	**Pump shear stress, τ** Nm^{-2}
5	-2.37×10^{-5}	-0.00222	0.00037	44
7	9.3x10-6	0.00874	-0.00022	46
7	2.03x10-5	-0.0022	0.000105	71
7	-2.03x10-5	0.003	0.0000757	46
14	8.7x10-6	0.000644	0.000215	53
14	2.09x10-6	0.000156	4.12×10^{-6}	88
61	4.4×10^{-5}	0.00258	3.52×10^{-5}	123
84	5.7×10^{-5}	0.00252	2.14×10^{-5}	163
113	5.6×10^{-5}	0.001327	1.05×10^{-5}	177
113	7×10^{-5}	0.001784	7.46x10-6	289

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Abbreviations

AEC	Adenylate Energy Charge
ADP	Adenosine Di Phosphate
ATP	Adenosine Tri Phosphate
CER	Carbon dioxide Evolution Rate
CFU	Colony Forming Unit
DOT	Dissolved Oxygen Tension
E/C	Extra Cellular
I/C	Intra Cellular
OD	Optical Density
OUR	Oxygen Uptake Rate
PPF	*P. putida* ML2 Fermentation regime
PPInoc	*P. putida* ML2 Inoculation regime
rpm	Revolutions Per Minute
SF1	Shake Flask 1
SF2	Shake Flask 2
SPC	Standard Plate Count
VCC	Viable Cell Count
v/v	Volume / Volume
wt	Weight
w/v	Weight / Volume
X-flow	Cross flow

Units

bar	bar (0.1 MPa)
C	centigrade
g	grammes
h	hours
m	metres
min	minutes
mol	moles
M	molar
N	newtons
Pa	pascals
s	seconds
W	watts

Prefixes

p	pico	$(1x10^{-12})$
m	milli	$(1x10^{-3})$
μ	micro	$(1x10^{-6})$
c	centi	$(1x10^{-2})$
n	nano	$(1x10^{-9})$
k	kilo	$(1x10^{3})$
m	mega	$(1x10^{6})$

Symbols

Δ	delta, difference
Δp_{tm}	transmembrane pressure
π	pi
ω	omega, angular velocity
ρ	rho, density
μ	mu, viscosity
τ	tau, shear stress
σ	standard deviation
°	degrees
G	shear rate
k	rate constant
log	logarithm to base 10
ln	natural logarithm