The Effect of Pretreatment Used in Combination with Mechanical Methods on the Extent of Cell Disruption and Subsequent Release of Intracellular Protein

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Thesis submitted in fulfilment of the requirements for the Degree of Masters of Science in Chemical Engineering - Bioprocess Engineering Division

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Submission Date: October 2004

UNIVERSITY OF CAPE TOWN

Abstract

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October 2004

The disruption of microbial cells to obtain intracellular products is well documented and widely used to extend the microbial by-product range. Mechanical disruption processes result in complete cell breakage and all intracellular products are released, resulting in difficult separation and purification of the desired product. The process is also energy intensive and reduces the particle size considerably, further increases the challenge of solid-liquid separation. The combination of non-mechanical methods to permeabilise or weaken the cell envelope with mechanical disruption methods can decrease the energy required, increase the intracellular release and decrease the time required for breakage, to attain maximum intracellular release. The pretreatments were selected and the optimum conditions determined through a screening process for each microorganism, Baker's yeast and *Escherichia coli*. The pretreatments were used in combination with high pressure homogenisation and hydrodynamic cavitation.

The chemical pretreatments selected for permeabilisation of Baker's yeast did not result in an increase in the extent of disruption or rate of release, due to significant enzyme deactivation and interference by the chemicals. The EDTA and CTAB permeabilisation method resulted in strengthening of the cell wall and little or no breakage occurred at pressures of 13.8 MPa and 34.5 MPa following treatment with the chemicals. This was confirmed by light microscopy.

The pretreatments used for permeabilisation of *Escherichia coli* were successful with the EDTA pretreatment achieving maximum release at 13.8 MPa, while untreated bacteria achieved maximum release at 34.5 MPa. Significant energy reductions were observed with the use of this combination method. The G-HCl and Triton X-100 also resulted in increased intracellular release and decreased energy usage when compared with untreated bacteria at the same pressure. Therefore, effective disruption can be achieved with the use of combined non-mechanical methods with mechanical disruption processes with clear advantages in terms of increased intracellular release and decreased energy consumption.

Acknowledgements

Firstly, I would like to thank Professor S.T.L. Harrison for being my supervisor and providing me with the necessary resources for this thesis.

I would also like to thank my family for their continuous support and encouragement. To my mom and dad, thank you for providing the opportunity to be educated and for always making it a priority. Samir and Kushma, thanks for enduring my aggravation at times and for being so understanding. Sabine, thanks for all the help with proof-reading.

Aneet, thank you for always being so optimistic and helping me see the light when I was stressed and frustrated. I am extremely grateful for all your patience, help and advice. Thank you for constantly encouraging and believing in me.

I would especially like to thank Balasundaram for his assistance throughout my research, his invaluable advice and guidance. Many thanks to Chantal Reynard and Sue Jobson for their help in and out of the laboratory, my work would have been much harder if it were not for you. I gratefully acknowledge the assistance of Mark Pickering and Anchor Yeast for the supply of Baker's yeast.

Lastly, I would like to recognise the University of Cape Town for providing me with funding assistance during my studies.

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Nomenclature

a	pressure exponent of soluble protein release	-
C_v	cavitation number	-
E	energy per unit volume	J/m^3
k	rate constant	$1/MPa^a$
k'	effective disruption rate constant	1/pass
N	no. of passes	-
P	operating pressure	Pa
P_3	downstream pressure	Pa
P_v	vapour pressure	Pa
R	soluble protein release	kg/kg
R_B	soluble protein release by mechanical rupture	kg/kg
R_C	soluble protein remaining in cell	kg/kg
R_D	soluble protein denatured	kg/kg
R_H	soluble protein released by combined disruption process	kg/kg
R_i	maximum soluble protein release under specific conditions	kg/kg
R_{max}	maximum soluble protein release	kg/kg
R_o	soluble protein release during pretreatment	kg/kg
T	temperature	°C
t	time	min
v	velocity	m/s
ρ	density of suspension	kg/m^3

Abbreviations

CTAB	cetyltrimethylammonium bromide
EDTA	ethylenediaminetetraacetic acid
G6PDH	glucose-6-phsophate dehydrogenase
G-HCl	guanidium hydrochloride
HPH	high pressure homogenisation
KI	potassium iodide
U	unit of enzyme activity
v/v	volume by volume
w/v	weight by volume

Chapter 1

INTRODUCTION

1.1 Introduction

Microorganisms provide a wide range of biological products that can be exploited commercially in the industrial, food, pharmaceutical and chemical industries. The recovery and purification of these biological products from their microbial source can be achieved through downstream processing. For the exploitation of intracellular microbial products, cell disruption is required as an early step in the downstream process. This thesis describes a new approach to the method by which intracellular proteins are released from microorganisms, through the combination of two different disruption techniques, specifically a chemical pretreatment to destabilise the cell envelope followed by mechanical disruption. The chemical pretreatment of the cell is expected to permeabilise or weaken the cell wall, making the cell more susceptible to disruption. The subsequent mechanical disruption should then result in increased intracellular release under less severe operating conditions.

1.2 The Need for Microbial Cell Disruption

Some microbial by-products can be found extracellularly due to the natural transport of products from the cell to the bulk environment. However, microorganisms do not secrete the majority of their products into the extracellular medium. In these instances, the proteins of interest, including products of recombinant DNA, intracellular enzymes and other intracellular products, can only be obtained by breaking of the cell structure. The intracellular products are separated from the external medium by a cell wall and membrane. Cell disruption is the breakdown of these structures for intracellular release. An efficient and cost effective method for cell disruption can therefore provide an extended microbial product range for commercial exploitation. Cell disruption is thus an important step in the downstream processing of intracellular proteins and enzymes for the recovery and purification of these products.

1.3 Current Cell Disruption Techniques

There are two main categories of cell disruption: mechanical methods and non-mechanical methods. These are categorised in Figure 1.1. Mechanical methods are most widely used on an industrial scale, specifically high pressure homogenisation (HPH) and bead milling. These methods are preferred as they are easy to operate, give good levels of disruption, with high product recovery, and can be implemented on a large scale. However, they are energy intensive making the process costly. Further, they provide no selectivity in intracellular products released. Due to these high energy requirements, a new less energy intensive method is being explored. Experiments using hydrodynamic cavitation have shown promising results with energy consumption reduced by up to two orders of magnitude, compared to other mechanical methods. The scale-up potential, ease of operation due to the simplicity of the equipment and cost effectiveness make hydrodynamic cavitation a potential large-scale cell disruption method.

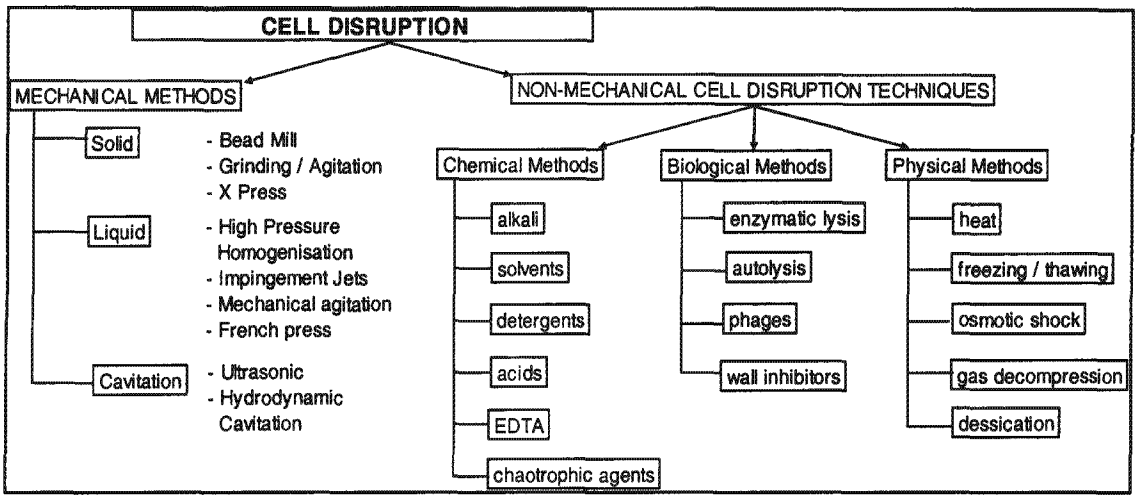


Figure 1.1 Classification of microbial cell disruption techniques

The use of mechanical methods for microbial cell disruption has several disadvantages. When a cell is disrupted mechanically, its intracellular contents are released. Due to the complete breakage of the cell, separation and purification of the desired product becomes increasingly challenging. As the cells are repeatedly passed through the disruption equipment to increase the extent of the disruption, the particle size of the debris decreases considerably, increasing the challenge in solid-liquid separation unit operations.

Non-mechanical cell disruption can be separated into three categories: chemical, physical and biological methods. These methods are considered gentler and some can be used for selective intracellular release over mechanical methods but they are generally limited to application on a small scale, often restricted by process economics or their efficiency. Consequently, they have found limited commercial application to date.

Gentler and more selective methods of cell disruption are being sought. The combination of mechanical and non-mechanical methods could synergistically produce potential improvement over the currently used methods. It is proposed that the use of the non-mechanical method should permeabilise or weaken the cell, allowing the mechanical method to disrupt the cell more easily, with a lower energy requirement for complete disruption. In microbial cell disruption, it is important to take into account the effect the disruption methods will have on subsequent unit operation in downstream processing.

A thorough assessment of this impact and of process economics will aid selection of the most appropriate method.

1.4 Problem statement

Mechanical cell disruption techniques have high energy requirements. Typically a reduction of energy expended in cell disruption compromises the release of intracellular products from the cell.

Researchers have suggested the combination of a mechanical method with a non-mechanical pretreatment has potential for improved protein and enzyme release in combination with a reduction in the energy requirement (Asenjo, 1990; Baldwin and Robinson, 1990; Harrison, 1990a). It can therefore be proposed that the use of specific pretreatment methods can weaken or permeabilise the cell walls of various microorganisms, resulting in easier subsequent mechanical disruption. Advantages are sought in terms of improved energy efficiency and reduced micronisation of cell debris.

1.5 Scope of research

In this study, the combined cell disruption approach was investigated using two different microorganisms: *Saccharomyces cerevisiae* and *Escherichia coli*. Areas of greatest resistance to disruption in their cell envelopes were identified such that chemical methods could be chosen based on their ability to attack the specific resistant areas. These pretreatment methods were implemented for the permeabilisation or weakening of the cell wall and membrane. The permeabilised cells were further exposed to either high pressure homogenisation or hydrodynamic cavitation to enable cell disruption. The effectiveness of cell disruption was determined by both the extent and rate of release of total soluble protein and of specific enzymes from defined locations within the microorganism.

The main objectives of this research were to:

- Increase the amount of protein and enzyme released by using the combination of methods,
- Decrease the amount of energy used for the process while maximising the release,
- Reduce the cost and treatment time,
- Avoid extensive cell fragmentation,
- Minimise product damage and
- Selectively release specific enzymes from different locations within the cell.

In addressing these objectives the following key questions are posed and investigated through a thorough examination of the literature and ensuing experimentation:

- Which pretreatment will weaken each type of cell?
- Does the pretreatment combined with HPH or hydrodynamic cavitation result in an increase in disruption?
- Is energy dissipation or usage kept to a minimum?

The following hypothesis is developed from the literature and challenges associated with microbial cell disruption and the desired outputs of continuing research in this area. Yeast, bacterial and fungal cells are weakened by specific pre-treatment methods making them more susceptible to disruption.

1.6 Structure of Thesis

The thesis begins by introducing the topic of cell disruption. It literature on cell disruption techniques is then reviewed in detail. This is followed by the methods used to carry out the investigation, followed by the presentation and an in-depth discussion of the results. The thesis is then concluded and referenced. All relevant appendices are presented at the end of the thesis.

Chapter 2

LITERATURE REVIEW OF CELL DISRUPTION

2.1 Introduction

Microbial cell disruption is an important process for the exploitation of microbial by-products produced and found within the cell. This process involves the physical breakage of the cell envelope, thereby releasing the intracellular contents into the surrounding medium.

Improvements in the efficiency and financial viability of methods to extract these products would expand the microbial product range for commercial exploitation. The efficiency of the method is often evaluated in terms of the activities of the enzymes released into the disrupted suspension, the amount of energy used in the process and the degree of disruption. The subsequent purification and recovery steps will also be affected by the nature of the disruption process.

A detailed description of the cell wall compositions of the microorganisms (*Saccharomyces cerevisiae and Escherichia coli*) used, as well as a review of the various methods and techniques of cell disruption follows.

2.2 Structure of Microorganisms

The knowledge of the composition and structure of a cell envelope is essential in selecting the appropriate disruption method. The cell wall of an organism is a rigid structure providing the main resistance to disruption, hence an extensive understanding of the cell wall composition and structure is required. Further knowledge of external envelope structures such as the outer cell membrane, where present, is required. The microorganisms used for this research span different classes, hence have significant differences in their cell wall structures.

2.2.1 The Structure of Yeast (*Saccharomyces cerevisiae*)

The Cell Wall

The cell wall of yeast cells is generally quite thick, approximately 100 to 200 nm, comprising of 15 to 25% of the total dry mass of the cell (Walker, 1998). It has been reported that cell walls of the Baker's yeast strain are 70 nm (Moor and Muhlethaler, 1963 cited by Engler, 1985). The main structural components are polysaccharides, which are predominantly glucans and mannans with a small amount of chitin. The polysaccharides account for roughly 80 to 90% of the cell wall. The glucan component is arranged in a microfibrillar network and provides strength to the cell wall. There are two types of glucan links which are identified by their solubility in acid and alkali, namely β-1,6 and β-1,3 linkages. The mannans are present as an α-1,6 linked inner core with α-1,2 and α-1,3 sides chains (Walker, 1998). Phosphodiester links also occur in the mannan and most of the proteins in yeast cell wall are complexed to the mannan (Engler, 1985; Walker, 1998). The small quantities of chitin, which is a polymer of N-acetylglucosamine, are present in bud scars. Other components such as protein, lipids and inorganic phosphate are present in variable quantities depending on the strain of yeast.

The protein–mannan outer layer and the inner layer of glucan, shown in Figure 2.1, are the main resistance to disruption. Therefore, the resistance of yeast cell walls to

disruption appears to be a function of how tightly cross-linked and how thick the structural portion is (Walker, 1998).

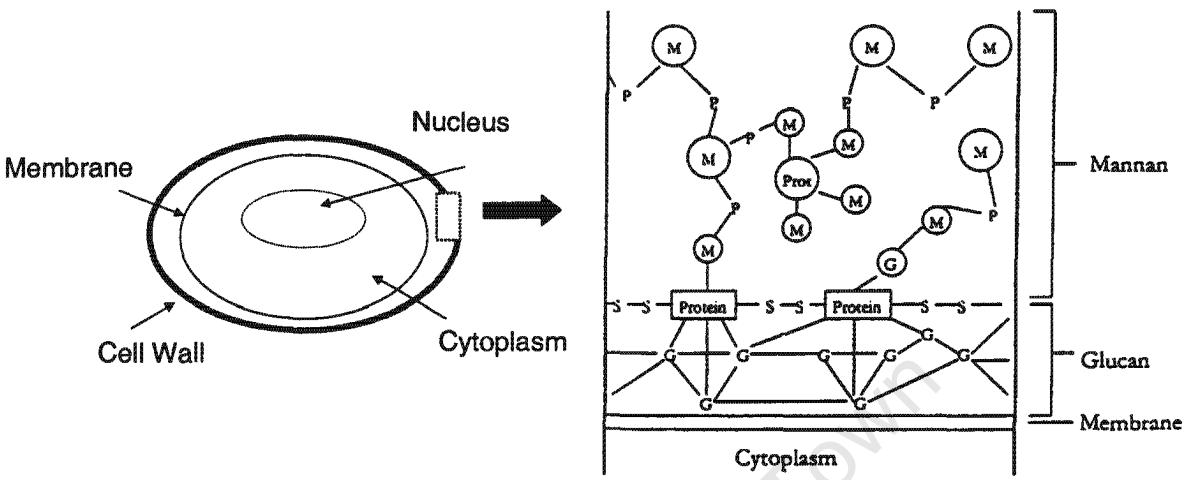


Figure 2.1 Structure of yeast cell, showing detail of the protein-mannan complex and the glucan component (Lampen, 1968)

There are various models of the molecular arrangement of the *S. cerevisiae* cell wall. All describe the wall as a layered structure consisting of an outer section of cross linked mannoproteins, linked together by hydrophobic interaction and disulphide bonds. This determines the surface properties of the cell wall, such as porosity and is covalently bonded to the strong glucan network, which is complexed with chitin. Chitin is mostly found in bud scars but it is also found in small amounts in the cell wall. It has a number of functions, as a killer toxin receptor and in maintaining the osmotic and morphological integrity of the cell (Walker, 1998).

The physiological functions of the yeast cell wall include physical protection of the protoplast and maintenance of cell shape. It provides osmotic stability, preventing protoplast lysis. It is selectively permeable, with solutes larger than 600 Da unable to permeate through the wall, and controls the passage of water into the cell. The cell wall supports enzymes such as glucanases and hydrolases (invertase) which are immobilised in the wall matrix. Several cations are also retained by the cell wall, including heavy metals (Walker, 1998).

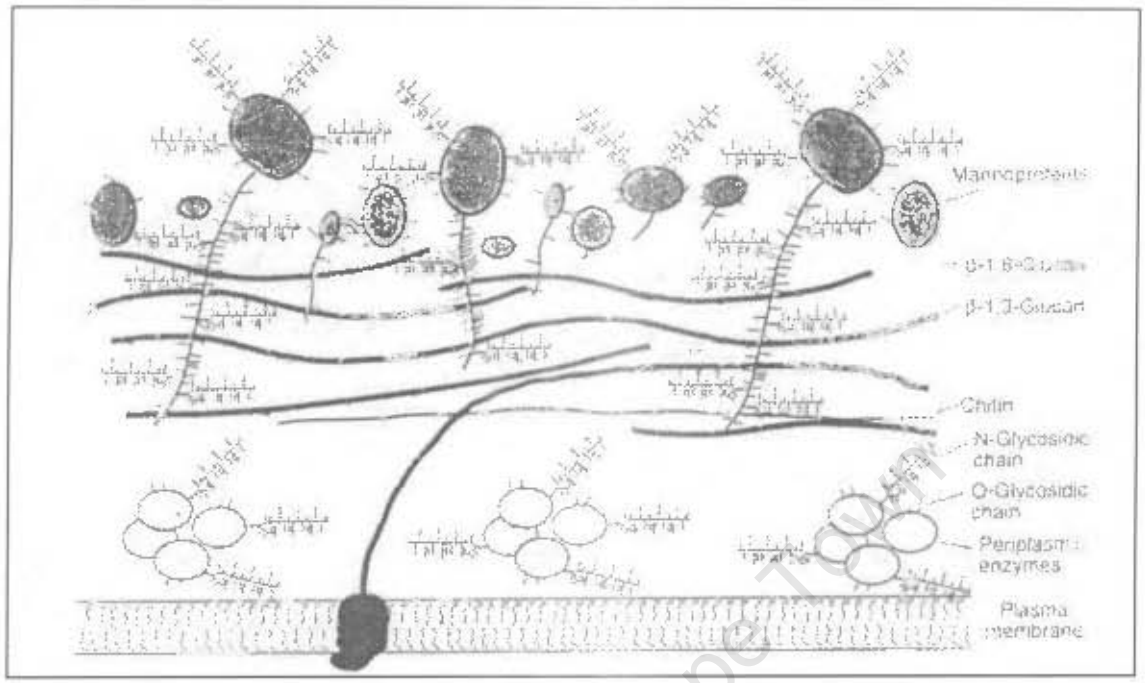


Figure 2.2 **Detailed cell wall structure of *Saccharomyces cerevisiae* (Walker, 1998)**

The Periplasm

The periplasm is a thin region (35 to 45A) between the cell membrane and the cell wall, (Figure 2.2). It is compromised of proteins, such as mannoproteins which cannot permeate the cell wall.

The Plasma Membrane

This is a barrier for the passage of hydrophilic molecules into the cell. It also prevents the cytoplasmic contents combining with the aqueous environment. The membrane is approximately 7.5 nm thick and it is described as a phospholipid bilayer scattered with globular proteins to form a fluid mosaic. The protein components are involved in solute transport, cell wall biosynthesis, transmembrane signal transduction and cytoskeletal anchoring. *S. cerevisiae* also has ATP transporting proteins.

The plasma membrane changes structurally and functionally depending on the conditions of growth. For example, membrane lipid compositions with changing growth rates, temperature and oxygen availability. The main function of the membrane is to control the entrance and exit of substances to and from the membrane through selective permeability. The uptake of sugars, nitrogenous sources, ions and solutes is controlled by the membrane. The membrane has a physiological function in signal transduction of external stimuli to mediate a number of internal biochemical reactions. Other transport functions of the membrane include exocytosis and endocytosis.

2.2.2 The Structure of Gram-negative Bacteria (*Escherichia coli*)

The basis of bacterial cell walls is a rigid matrix of peptidoglycan, a network of glycan (amino sugars) chains cross-linked by short peptides. The degree of cross-linking varies considerably among different organisms. These chains form a continuous network and provide shape and strength to the cell wall. The glycan chains are made up almost exclusively of alternating residues of N-acetylglucosamine and N-acetylmuramic acid in β-1,4 linkages (Engler, 1985).

Adjacent glycan chains are cross linked by peptide bridges comprised of tetrapeptide units linked to the glycan strands at the lactyl groups of the N-acetylmuramic acid residues. The degree of cross linking varies among the organism. In *Escherichia coli*, 50% of the tetrapeptide units are not crossed and the rest are linked as dimers (Ghuysen, 1968 and Weudel and Pelzer, 1964 cited by Engler, 1985).

Bacteria are classified as Gram-positive and Gram-negative, based on their cell envelope structure, as shown in Figure 2.3. Gram-positive bacterial cell walls are generally thick, at 15 to 50 nm. They consist of 40 to 90% peptidoglycan while the remaining portion of the wall is made up of primarily polysaccharides and teichoic acids. They also have no outer membrane and the peptidoglycan represents the outer barrier. The peptidoglycan layer provides greater structural resistance to cell disruption. This dominant peptidoglycan layer in Gram-positive bacteria can be clearly seen in Figure 2.4.

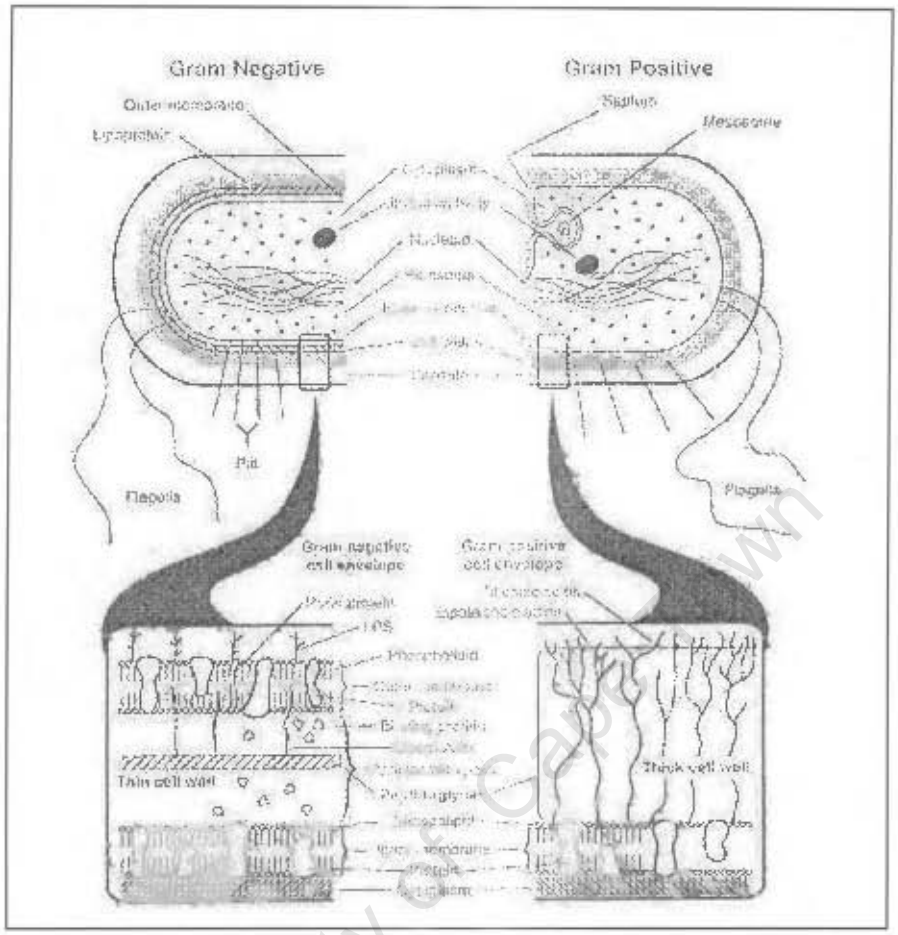


Figure 2.3 Basic structural differences between Gram-positive and Gram-negative bacterial cells (Salton and Kim)

Gram-negative bacteria have a much thinner peptidoglycan layer, between 1.5 to 2.0 nm. They also possess a thin outer membrane. The peptidoglycan layer has lipoprotein covalently attached to it. The presence of the outer membrane, shown in Figure 2.3 and 2.5, on the Gram-negative bacteria does protect the inner layers from direct chemical attack. The major structural resistance to disruption of bacterial cells appears to be the peptidoglycan layer, making the Gram-positive bacteria more difficult to disrupt, even though it does not have protection from the external environment by an outer membrane. The tightness and strength of peptidoglycan network depends both on the frequency with which peptide units occur on the glycan chains and also the frequency with which the units are cross-linked (Engler, 1985).

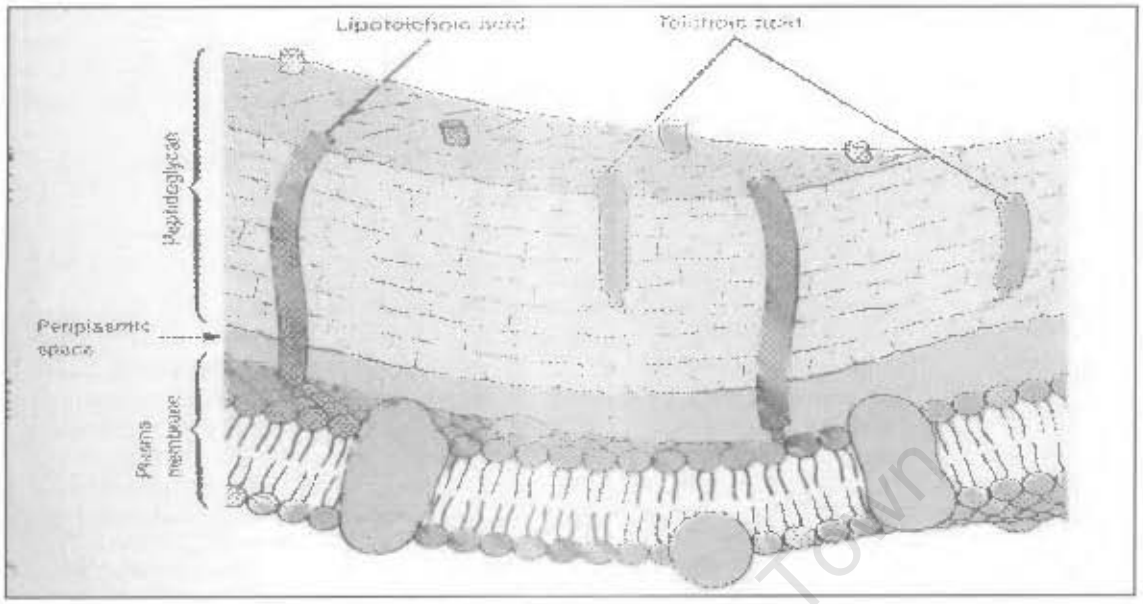


Figure 2.4 **The peptidoglycan structure of Gram-positive envelope (Taken from Prescott *et al.*, 1990).**

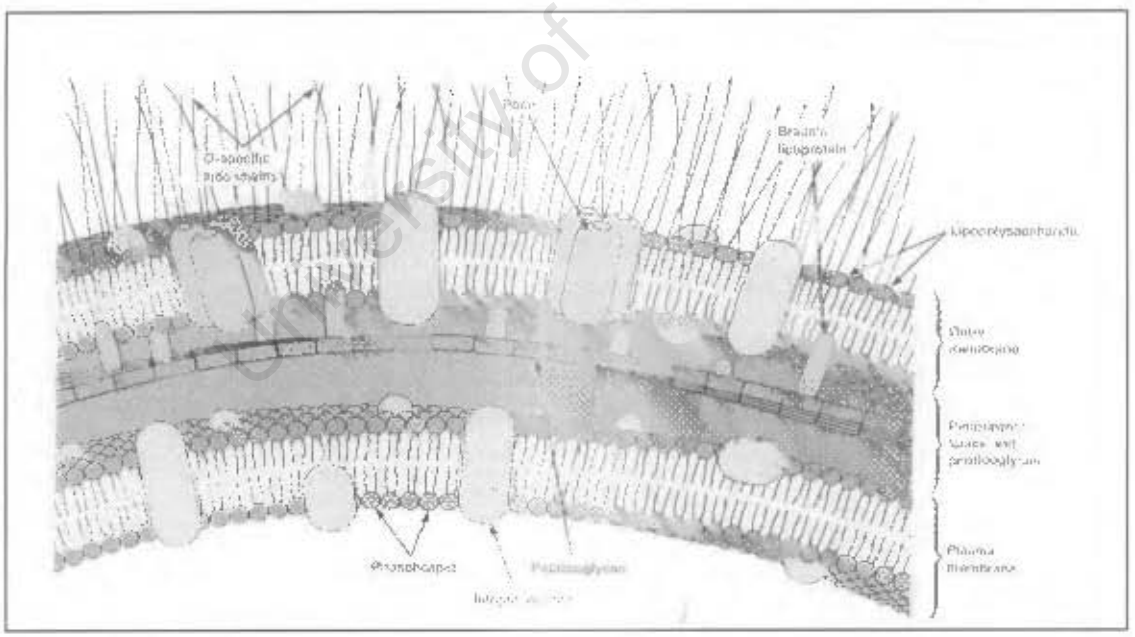


Figure 2.5 **The peptidoglycan structure of a Gram-negative envelope (Taken from Prescott *et al.*, 1990).**

2.2.3 Disruption and Wall Structure

The main resistance to cell disruption is provided by the cell envelope, comprised of the cell wall and one or more plasma membranes. The main resistance to mechanical disruption in the organisms resides within the nature of structural polymers of the wall and the degree to which they are cross linked. The covalent bonding of the structural network is the main barrier to overcome when disrupting a cell. This structure is dependent on genetic information and the growth environment.

To disrupt cells more easily, the susceptibility to the cell disruption must be increased. This can be manipulated using an understanding of the cell wall structure in combination with chemical methods and enzymatic pretreatments (Engler, 1985). Based on structural information, careful selection of a chemical method can be made specifically to 'attack' a component of the wall to weaken it and therefore increase susceptibility to mechanical disruption.

The rate and extent of disruption varies for different cell types due to cell characteristics, such as the cell wall components that present the resistance to disruption and the size and shape of the cell. The disruption characteristics can be adjusted for an organism by altering the growth conditions. Cells that grow rapidly do not form adequate strengthening components for the cell structure and therefore are easier to disrupt (Engler and Robinson, 1981). It has been observed that Gram-negative cells are easier to disrupt than Gram-positive cells and fungi, which in turn are easier to disrupt than yeast cells.

2.3 Mechanical Methods for Microbial Cell Disruption

Cell disruption includes many different methods which can be classified as either mechanical or non-mechanical. The mechanical methods include high pressure homogenisation, hydrodynamic cavitation, bead milling and ultrasonics. Figure 2.6 shows a detailed representation of some of the most commonly employed methods of

mechanical disruption. Microbial cells may be efficiently disrupted on an industrial scale by high pressure homogenisation and bead milling (Hetherington *et al.*, 1971; Limon-Lason *et al.*, 1979).

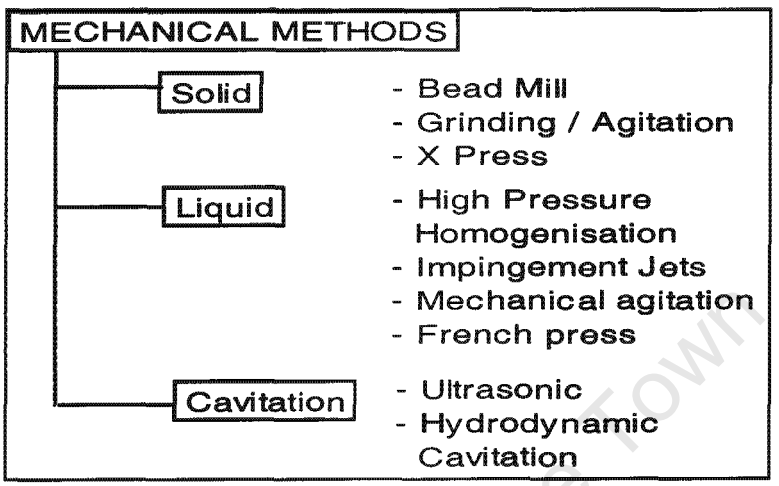


Figure 2.6 Classification of microbial mechanical cell disruption techniques

2.3.1 High Pressure Homogenisation (HPH)

High pressure homogenisation (HPH) is a widely known and employed large-scale method of disrupting cells. The microbial disruption of *Saccharomyces cerevisiae*, Baker's yeast, is extensively reported and well documented (Hetherington *et al.*, 1971; Follows *et al.*, 1971; Brookman, 1974; Doulah and Hammond, 1975; Limon-Lason *et al.*, 1979; Engler and Robinson, 1981; Keshavarz-Moore *et al.*, 1990a). Homogenisation has been studied since the 1950's, where Loo *et al.* (1950) investigated the theory of cavitation during the homogenisation of milk in the dairy industry. It has been widely used in the food and pharmaceutical industries (Shutte and Kula, 1987).

Homogeniser Equipment

The homogeniser is made up of two important parts: a high pressure positive displacement piston pump and a homogeniser valve. The cell suspension is forced through an adjustable orifice discharge valve under high pressure. The suspension is delivered to the valve by the positive displacement piston pump. The pressurised liquid

enters the valve and a change in velocity occurs at the preset pressure. The fluid flows radially across the valve and strikes an impact ring. The suspension exits the valve assembly and is discharged (Middelberg, 1995). A number of different valve geometries can be used: the most common being the cell disruption (CD) and knife edge configurations (CR), presented in Figure 2.7.

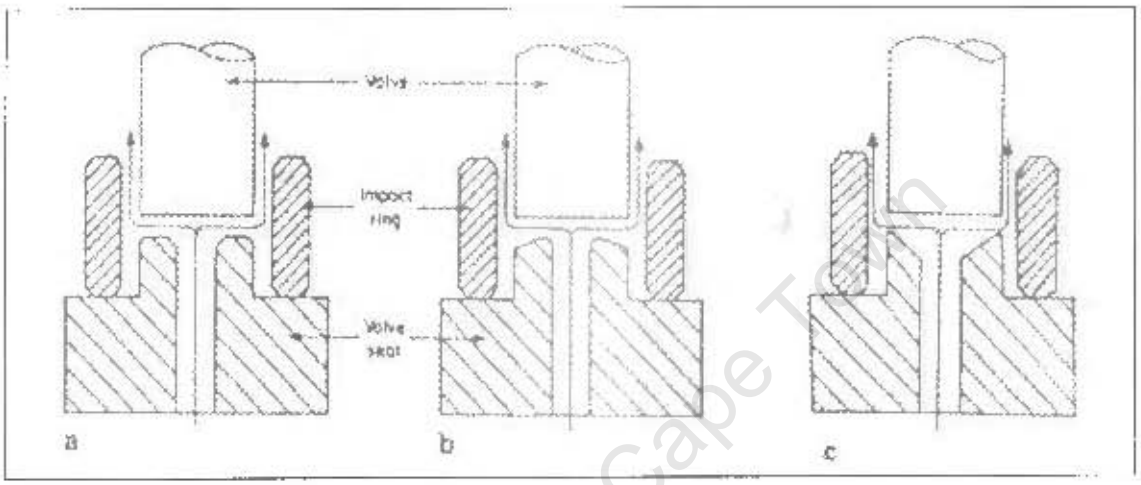


Figure 2.7 **Details of homogenizing valve units (a) flat-edge 'Standard' unit; (b) knife-edge 'Cell Rupture' unit; (c) knife-edge 'Cell Disruption' unit (Keshavarz-Moore *et al.*, 1987)**

The most commonly used HPH is the Manton-Gaulin range manufactured by APV (Middelberg, 1995). The temperature rise due to adiabatic compression in the Gaulin homogeniser is about 2°C per 10 MPa of pressure (Chisti and Moo-Young, 1986). Temperature increases of 0.230°C m^3/MJ for the CD configuration and 0.177°C m^3/MJ with CR are reported in temperature range of 5 to 37°C by Harrison (1990a). Similar values of 0.210°C m^3/MJ are reported by Brookman (1974) and 0.150° m^3/MJ by Keleman and Sharpe (1979). With increasing pressure, a lower number of passes through the homogeniser is required for disruption, but the rise in temperature increases, therefore cooling is needed to prevent the enzymes being denatured. This limits the increase in pressure (Chisti and Moo-Young, 1986).

Another common HPH system is the Microfluidizer which consists of two streams of cell suspension which impact at high velocity against a stationary surface. A schematic representation of the Microfluidizer is presented in Figure 2.8. The operating pressure, achieved by using a high pressure pump, is a function of flow rate, disruption unit and backpressure unit when used. It requires a very short residence time and efficient cooling is easy to achieve. The fraction of disrupted cells increases with an increase in pressure and the number of passes. The efficiency is dependent on the initial cell concentration and specific growth rate of cells (Geciova *et al.*, 2002). The cell suspension impinges at high velocity against a stationary surface and the energy input is dissipated almost instantaneously at the point of impact leading to disruption of cells. This leads to the larger particle sizes of cell debris after disruption than the Manton Gaulin homogeniser resulting in more effective separation during centrifugation (Engler and Robinson, 1981).

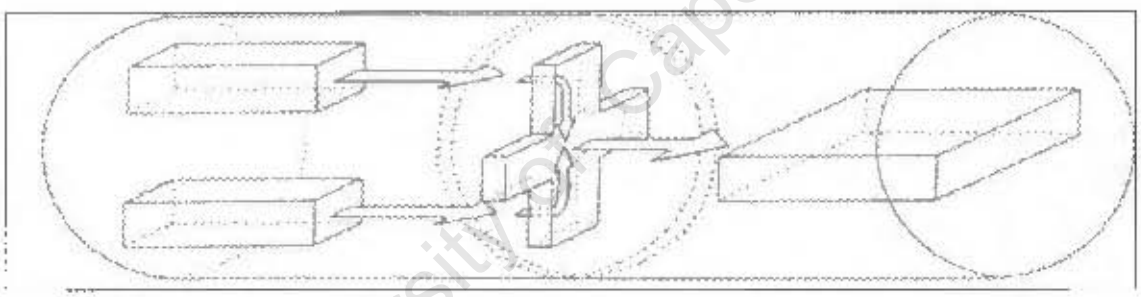


Figure 2.8 Schematic view of the disruption chamber of a high pressure homogeniser – Microfluidizer (Sauer *et al.*, 1989)

Operating Variables and Disruption Mechanism

In the HPH, the cells are subjected to shear, cavitation, impingement, turbulence and a rapid pressure drop (Doulah and Hammond, 1975; Asenjo, 1990; Engler, 1990; Harrison, 1990a; Shutte and Kula, 1990; Middelberg *et al.*, 1999). The principal mechanism of disruption has not yet been ascertained but an important contribution results through the magnitude of pressure drop (Brookman, 1974), impingement (Engler and Robinson, 1981; Keshavarz-Moore *et al.*, 1990a), cavitation (Harrison, 1991a; Middelberg, 1995) and turbulence (Doulah and Hammond, 1975). Evidence of cavitation as the mechanism of disruption in a HPH valve has been confirmed through the liberation of iodine by decomposition of KI (Shirgaonkar *et al.*, 1998).

Operating variables reported to influence the rate and extent of cell disruption include the operating pressure and number of passes (Engler, 1985; Keshavarz-Moore *et al.*, 1987; Harrison *et al.*, 1991c), temperature of suspension (Hetherington *et al.*, 1971; Harrison, 1990a), homogeniser valve geometry (Keleman and Sharpe, 1979; Keshavarz–Moore, 1990a; Harrison, 1990a). An increase in the operating pressure increases cell disruption. The cell wall composition affects the pressure required to disrupt a cell (Keleman and Sharpe, 1979). A lower pressure requires more passes to achieve the same level of disruption as with a higher pressure. At a pressure of 69 MPa, a single pass was required for 75% release of total protein available on disruption of *Ralstonia eutrophus*. Lower pressures of 27.6 MPa required almost three passes to achieve the same level of disruption (Harrison *et al.*, 1991c). Different valve units are available, but the knife-edged unit has proved to be the design that releases the most protein due to the more rapid pressure drop (Chisti and Moo-Young, 1986; Shutte and Kula, 1987). The pressure drop is determined by the narrowness of the orifice or the valve opening. At a constant flow rate, the increase in pressure results in a decrease in the valve opening and therefore an increase in the velocity of the suspension through the valve. This confirms the increased cell disruption trends observed with increased operating pressure (Harrison, 1990a).

The amount of protein released was found to be dependent on pressure, temperature, the number of passes and cell concentration. The results found that an increase in pressure, number of passes and slight increase in temperature released a larger amount of protein. If the number of passes is increased too much, the micronisation of cell debris results in complex downstream processing (Harrison, 1991a). Cells harvested in the stationary phase cells were more resistant to mechanical disruption (Sauer *et al.*, 1989; Harrison *et al.*, 1991c; Bailey *et al.*, 1995). Maximum protein release was found to be independent of pressure for fungal cells grown in shake flasks, but dependent on pressure for fungal cells grown in fermentation. Cells in a fermenter are subjected to increased shear leading to increased wall toughness (Keshavarz-Moore *et al.*, 1990b).

It has also been found that disruption is independent of cell concentration over a wide range (Engler, 1985). Typical concentration ranges are 300 to 600g/l (equating to 75 to 150g/l dry weight) of packed yeast (Hetherington *et al.*, 1971) and 96 to 257 g/l dry weight for the disruption of *Ralstonia eutrophus* (Harrison *et al.*, 1991c). One pass through the homogeniser typically ruptures the cell at a distinct point, while further passes results in micronisation of the cell debris which can be difficult for downstream processing separation (Baldwin and Robinson, 1990; Harrison, 1990a). Micronisation has been noted to complicate solid liquid recovery (Agerkvist and Enfors, 1990).

Cell Disruption Kinetics

The kinetics analysis of microbial cell disruption in the high pressure homogeniser was first studied using Baker's yeast in 1971 by Hetherington *et al.* and shown to be a first order process with respect to the number of passes.

$$\frac{dR}{dN} = k' R \qquad \textbf{Equation 2.1}$$

Further experimental evidence illustrated that protein release is best described as a function of pressure raised to an exponent. Rearrangement and integration, gives Equation 2.3.

$$\frac{dR}{R} = kdN \qquad \textbf{Equation 2.2}$$

$$\ln \frac{R_m}{(R_m - R)} = kNP^a = k' N \qquad \textbf{Equation 2.3}$$

where R_m is the maximum amount of protein that can be released on complete mechanical disruption, R is the protein released following N passes, k is the rate constant which is a function of temperature, with units of Pa^{-1}, k' is the effective disruption constant with units of $pass^{-1}$, considered dimensionless unless the passes are converted to time, P is the operating pressure and a the pressure exponent. The exponent a has been found to vary with microorganism and growth conditions. It is reported to be in the range 1.9 to 2.9 for Baker's yeast (Hetherington *et al.*, 1971; Engler and Robinson, 1981) and in the range 1.6 to 2.2 for Gram-negative bacteria (Gray *et al.*, 1972; Sauer *et al.*, 1989).

Table 2.1 Summary of the values found of the pressure exponent with different microorganisms found in literature

Microorganism	Exponent	Reference
Saccharomyces cerevisiae	2.9	Hetherington *et al.*, 1971
Saccharomyces cerevisiae	1.87	Engler and Robinson, 1981
Ralstonia eutrophus		Harrison *et al.*, 1991c
- exponential growth	3.08	
- stationary phase	1.6 to 1.7	
Escherichia coli	2.2	Gray *et al.*, 1972
Escherichia coli	1.43	Sauer *et al.*, 1989
Recombinant Escherichia coli	1.41	Sauer *et al.*, 1989

The ease of disruption is related to the composition of the cell wall, cell size and shape (Engler, 1985). Gram-negative bacteria are easier to disrupt in the HPH than Gram-positive bacteria and fungal cells, which in turn were easier to disrupt than yeast cells (Engler, 1985). The disruption of Gram-negative bacteria occurs in two stages: during the first pass the fracture of the peptidoglycan layer dominates with the release of soluble cytoplasmic material. During further passes, the disintegration of the cell structure and liberation of the particulate intracellular contents occurs (Harrison *et al.*, 1991c). Follows *et al.* (1971) found that the disruption process did not cause loss of activity of the enzymes released from Baker's yeast. The release rates of the enzymes correlated with the location of the enzymes in the yeast cell. All release rates were measured relative to total soluble protein. The enzymes in the cell wall were released more rapidly than the total soluble protein while those in the cytoplasm were released at a similar rate (Follows *et al.*, 1971). It has also been shown that cells at a higher specific growth rate can be disrupted rapidly (Engler and Robinson, 1981; Sauer *et al.*, 1989; Harrison *et al.*, 1990a). Disruption characteristics for a given organism can be altered significantly by changing growth conditions. For yeasts, it was found that a high growth rate produced cells with weaker cell walls. It is expected that the same trend would be followed by fast growing bacterial cells, as the cells would not have enough time to produce material for reinforcing the cell wall structure (Engler and Robinson, 1981). The disruption of fungi in a homogeniser can cause problems with mycelia blocking the disruption valve (Chisti and Moo-Young, 1986).

2.3.2 Hydrodynamic Cavitation

Studies using HPH have shown cavitation to be one of the major mechanisms causing disruption of a microbial cell in the high pressure homogeniser. The application of a purpose-designed hydrodynamic cavitation system for microbial cell disruption is fairly recent, first investigated by Harrison and Pandit (Harrison, 1990a; Harrison and Pandit, 1992). It has shown positive results (Harrison and Pandit, 1992; Save *et al.*, 1994; Kumar *et al.*, 2000; Balasundaram and Pandit, 2001).

Cavitation occurs with the formation, growth and collapse of micro bubbles. This leads to the generation of high pressures and temperatures which can cause cell damage (Jyoti and Pandit, 2000). Cavitation occurs frequently, causing damage in pumps and pipe work (Harrison and Pandit, 1992).

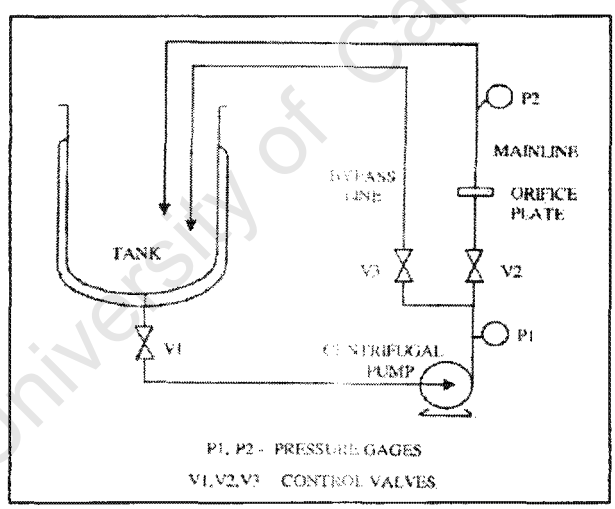


Figure 2.9 The hydrodynamic cavitation set-up using orifice plates (Balasundaram and Pandit, 2001)

Hydrodynamic cavitation occurs when liquid is passed through a throttled valve or constriction in a flow loop system. Figure 2.9 presents a typical hydrodynamic cavitation set-up. Consistent with the Bernoulli equation, the increased liquid velocity is associated with a decreased pressure. If the pressure drops below the vapour pressure of the liquid,

the formation of very small bubbles or vapour cavities results. This is the inception of cavitation. The cavities oscillate through collapse and rebound cycles until they are eventually destroyed by the recovered pressure. Noise and vibration accompany cavitation. An increase in liquid velocity and an increase in the pressure drop across the orifice result in a further reduction in pressure and more intense cavitation (Gogate and Pandit, 2001).

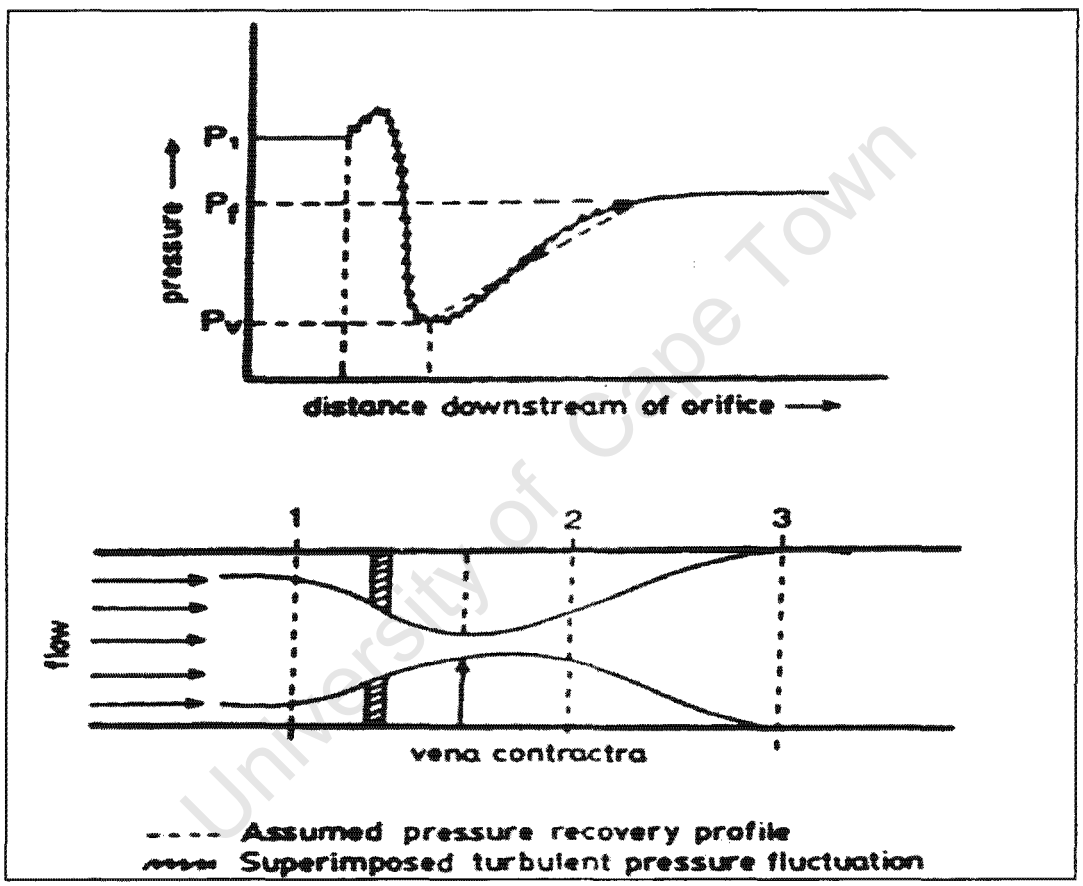


Figure 2.10 Typical axial profile downstream of the orifice during hydrodynamic cavitation (Gogate and Pandit, 2001)

The cavitation system comprising orifice plates used by Balasundaram and Pandit (2001) is shown in Figure 2.9. It is similar to the system used in the studies of Harrison and Pandit (1992) and consists of a closed loop circuit made up of a tank that can hold a large volume of suspension, a centrifugal pump, control valves and flanges to house the orifice

plate. The suction side of the pump is connected to the bottom of the tank. The pump discharge can be separated into two flow streams: one serves as a bypass line for flow control and the other is the main line in which cavitation occurs with the flange and orifice plate. Both of these streams return into the reservoir tank, below the liquid level to avoid air induction into the system. The bypass line can be used for discrete pass study. Cooling coils are placed within the tank to maintain the temperature of the circulating fluid (Balasundaram and Pandit, 2001).

The presence of cavitation can be predicted by use of the dimensionless number, the cavitation number. This relates the flow conditions to the intensity of cavitation through the ratio of the collapsing forces and initiating forces. The cavitation number, C_v, at the inception of cavitation is given by:

$$C_V = \frac{(P_3 - P_V)}{\frac{1}{2}\rho v^2} \qquad \textbf{Equation 2.4}$$

Where P_3 is the recovered pressure downstream of the constriction at position 3 in Figure 2.10., P_v is the vapour pressure of the liquid, v is the orifice velocity and ρ is the suspension density.

Cavitation inception values are in the range 1 to 2.5. At a C_v below 1, significant cavitation effects can be detected. A low cavitation number is achieved when the operating pressure is increased, causing the velocity of the suspension through the orifice to increase. Cell breakage is increased with an increase in the pressure drop across the orifice, an increase in the number of passes as well as an increase in suspension temperature (Gogate and Pandit, 2001). The precise value of cavitation number required is dependent on the scale of the apparatus and the geometry of the constriction (Harrison and Pandit, 1992). The cavitation inception number decreases with a decrease in the orifice diameter or opening size and increases with increased sharpness of orifice entrance (Jyoti and Pandit, 2000).

Hydrodynamic cavitation has been found to be more energy efficient than high-pressure homogenisation and ultrasonication by up to two orders of magnitude less, making it more cost effective (Harrison and Pandit, 1992). It is a simple and cheap cell disruption method, with reduced energy input required for equivalent breakage and a decrease in energy dissipated (Save *et al.*, 1994). It has also been found to be selective in the release of invertase from *S. cerevisiae* (Balasundaram and Pandit, 2001).

A first order relationship has been found for the release of proteins during mechanical disruption processes (Hetherington *et al.*, 1971; Limon-Lason *et al.*, 1979) and is given by:

$$\ln\left(\frac{R_m}{R_m - R}\right) = kt \qquad \textbf{Equation 2.5}$$

Where R_m is the maximum protein available for release, R is the protein released at a specific time t and k is the release rate constant. Hydrodynamic cavitation does not completely break a cell and therefore the maximum soluble protein that is released is the amount released under the specific conditions, R_i (Pearce, 1993). The amount released under the specific conditions can be represented as a fraction of the total maximum available in a cell, R_m as R_i/R_m.

2.3.3 Bead Milling

Bead milling is a very efficient cell disruption method. The mill consists of either a vertical or horizontal cylindrical chamber with a central shaft driven by a motor. The shaft supports a collection of off-centred discs or other agitating elements. The chamber is filled with beads of the desired material such as glass, plastic or steel (Chisti and Moo-Young, 1986). The collisions, caused by an agitator, between shear force layers and rolling and grinding elements cause the cells to disrupt. Cell disruption is affected by bead diameter, loading, cell concentration in feed, residence time, agitator speed, configuration and temperature (Shutte and Kula, 1983). The degree of disruption increases with bead loading due to increased bead-bead interaction. An increase in heating and power consumption also occurs as a result. A bead loading of 80 to 85% is

considered optimal since higher values cause excessive heating and power usage (Shutte and Kula, 1987).

The horizontal configuration is more efficient since the upward flow in the vertical machine fluidises the beads to some degree, thereby reducing grinding efficiency. The residence time, cell envelope structure and organism type affects the process efficiency. Heat produced during milling must be removed by a cooling system such as a cooling jacket. If there is insufficient cooling, thermal denaturation can occur. Shear denaturation of heat labile products can also occur (Schutte and Kula, 1983, 1990). To increase the milling time it is more effective to use a number of mills in series than to decrease the flow rate to avoid back mixing with reduced flow rate (Shutte and Kula, 1983, 1990).

It has been found that the optimum bead size for the release cytoplasmic enzymes is related to the size of the microbial cell: 0.1 to 0.15 mm in diameter for bacteria and 0.25 to 0.75 mm for yeast. This equates to a ratio of bead size to cell size in the range 0.05 to 0.15 mm/μm. To release enzymes from the periplasm or those bound to the cytoplasmic membrane, larger glass beads can be used. Increasing agitator speed increases disruption and agitator design affects efficiency (Geciova *et al.*, 2002). An increase in bead size has resulted in a decrease in the release rate constant for Baker's yeast in the range 0.5 mm to 3 mm. It was also found that increasing the bead loading up to 70% (v/v) increased the extent of disruption (Currie *et al.*, 1972). Moderate to high cell concentrations are optimal for maximum disruption efficiency (Middelberg, 1995). The bead mill is more efficient for the disruption of yeast in comparison to bacteria. The small dimensions of bacterial cells impede their own disruption (Geciova *et al.*, 2002). The disruption of fungi is preferred in a bead mill due to potential blockage of the homogeniser valve.

Disruption in a bead mill is generally first order with respect to time (Currie *et al.*, 1972), and is modelled by Equation 2.6. The release rate constant is dependent on the organism and the design and speed of the impeller, bead loading, bead size, cell concentration and temperature (Shutte and Kula, 1987).

$$\ln\left(\frac{R_m}{R_m - R}\right) = kt \qquad \textbf{Equation 2.6}$$

The first order equation for continuous disruption can be extended according to Equation 2.7, using the concept of CSTR's in series:

$$\ln\left(\frac{R_m}{R_m - R}\right) = \ln D = kt = 1 + \left(\frac{k\tau}{j}\right)^j \qquad \textbf{Equation 2.7}$$

Where τ is the mean residence time (volume of mill/total throughput, q), k is the disruption rate constant and j is the number of CSTR's in series.

2.3.4 Ultrasonication

Ultrasound, the sound of frequency higher than 15 to 20 kHz, causes inactivation and disruption of microbial cells in suspension. The mechanism of ultrasound cell disruption is associated with cavitation. The formation of cavities by ultrasound differs from hydrodynamic cavitation in cavity symmetry and free radicals formation. The cavities form, grow and are then compressed and eventually collapse releasing a shock wave. This is caused by pressure fluctuations. When the bubbles collapse, the sonic energy released is converted to mechanical energy, and eventually disrupts the cell (Chisti and Moo-Young, 1986). Small eddies create disruptive shear forces. Increasing the power shifts the size distribution towards the smaller eddies, which increases the disruption (Doulah, 1977). An increase in the sample volume decreased the protein release since there was a reduction in power dispersed per unit volume. This results in the formation of a smaller number of larger eddies, thereby decreasing the disruption efficiency. The rate constant decreased with increasing volume and increased with increasing power input (Geciova *et al.*, 2002).

The use of ultrasonication has been found to follow first order release kinetics. A release rate constant of 0.45 min^{-1} has been found at an ultrasound frequency of 200 W and an *E. coli* cell concentration of 26 g/l dry weight. At 200 acoustic watts and 20 kHz ultrasound, protein released is independent of the brewer's yeast concentration up to

600 kg/m^3 wet weight (Chisti and Moo-Young, 1986). Ultrasonic cavitation used for cell disintegration occurs by shear stresses developed by viscous dissipative eddies arising from shock waves (Doulah, 1977). Ultrasonication is also known to give rise to chemical effects and form free radicals.

There are a number of factors that affect the disruption of microbial cells using ultrasonication. These include power input per volume, the cell concentration and the temperature of the suspension. Much of the ultrasonic energy is converted to heat and therefore good temperature control is required to avoid denaturation of proteins. Micronisation of cell debris can result. It is difficult to transmit sufficient power to a large volume of cell material (Harrison, 1991a). Sonication is inefficient and largely ineffective for large or pilot scale use but is still the most commonly used laboratory technique.

2.4 Non-Mechanical Methods

The non-mechanical methods are a broader division and can be categorised into three main sections. These include chemical, physical and biological methods and are detailed in Figure 2.11.

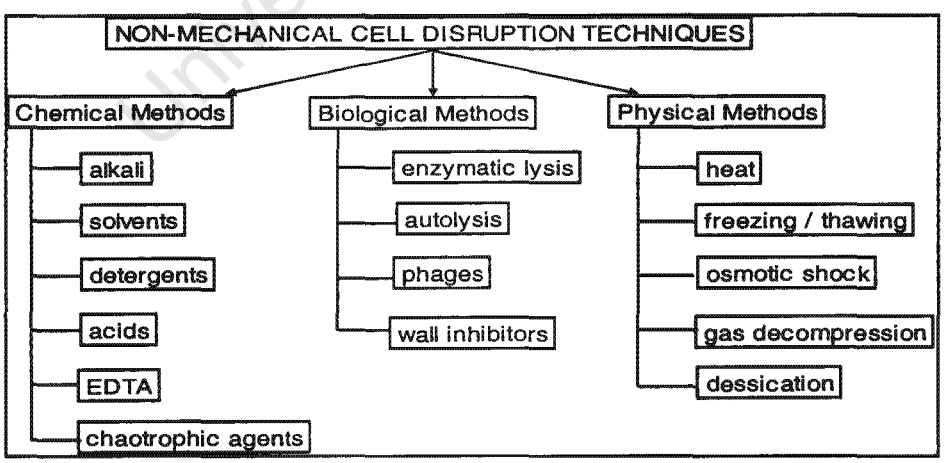


Figure 2.11 Classification of microbial non-mechanical cell disruption techniques

2.4.1 Chemical Methods

In general, chemical treatment of microbial cells does not disrupt the cell. The cell either becomes permeabilised and potentially lyses, or it is weakened and destabilised. Permeabilisation occurs through the outer wall developing perforations, allowing the diffusion of molecules smaller than the pores, such as metallic ions and cofactors, in and out of the cells, while retaining those molecules larger than the pores. Therefore, substrate can diffuse into a cell and react and the product formed can diffuse out. Enzymes retained by the cellular wall may be easily accessed by substrates. The weakening or destabilisation of the cell wall can occur with little or no intracellular leakage. This approach can be used with much less harsh chemicals than the former, and there is also no loss of proteins by chemical deactivation.

There are physical, chemical and biological methods of carrying out this permeabilisation or destabilisation. The treatment conditions required are very specific to an organism. One of the main disadvantages of chemical treatment is the need to remove these chemicals during the purification of the final product. Furthermore, the chemicals can cause the irreversible loss of activity of the product.

2.4.1.1 pH Treatment

The use of alkali for cell lysis is an inexpensive method that can be applied easily in almost any scale of operation. However, the product must be stable in high pH ranges such as 10.5 to 12.5 for at least 30 minutes. L-asparaginase can be isolated from *Erwinia carotovora* by treating with 0.5M NaOH for 20 minutes, followed by the addition of 25% acetic acid. After centrifugation, L-asparaginase can be recovered from the supernatant (Schutte and Kula, 1990).

A recombinant growth hormone has been extracted from *E. coli* pH 11 using NaOH. This method of lysis can inactivate proteases and the concentration of the NaOH can destroy

biological activities and lead to denaturation or degradation of the protein (Shutte and Kula, 1990).

Acidic treatment with 6N HCl hydrolyses *Candida lipolytica*, but the process is slow requiring 6 to 12 hours and proteins are hydrolysed to amino acids (Engler, 1985). Acidic pH should not be used if separation of the soluble and insoluble components is desired, as acidic pH precipitates macromolecules (Harrison, 1991a).

2.4.1.2 Detergents (Surfactants)

Detergents cause cell lysis or leakage through the solubilisation of proteins and perturbation of protein-lipid interaction. However, biological activity may be destroyed (Engler, 1985). Common detergents such as SDS (sodium dodecyl sulfate), CTAB (Cetyl trimethyl ammonium bromide), Triton X-100 and Tween act on the plasma membrane and find application in laboratory methods. Detergents self associate and bind to hydrophobic surfaces. They are comprised of a polar hydrophilic head group and a non-polar hydrophobic tail and are categorised by the nature of the head group. In ionic (cationic and anionic) detergents, the hydrophilic property is conferred by the ionised part of the molecule. In non-ionic detergents, hydrophilicity is based on the presence of multiple hydroxyl groups or other hydrophilic groups.

Anionic Detergents

Anionic detergents act by disorganising the phospholipids on the plasma membrane and causing release of intracellular compounds. Once the surfactant reaches the inner phospholipid layer, the permeability of the membrane is increased by disorganisation of the phospholipids, causing protein release (Bansal-Mutalik and Gaikar, 2003). They do not cause complete breakage of the outer membrane as they are responsible for the disorientation of structures causing large passages to allow the passage of molecules such as proteins and nucleic acids. SDS dissolves the plasma membrane of *E. coli* cells in an alkaline environment with the rapid release of intracellular contents. The release time of approximately 30 to 40 seconds is dependent on cell type (Woldringh and van Iterson,

1972; Ciccolini *et al.*, 1998). While SDS solubilises the plasma membrane, it does not disrupt the peptidoglycan layer of bacterial cells (Harrison, 1991a). Woldringh (1970) also reported SDS to cause the specific disruption of the inner cell membrane, in the presence of Mg^{2+} ions. A contradictory finding showed that anionic detergents, like SDS, in the presence of Mg^{+} ions, augment the membrane structure by stabilising the lipopolysaccharide (LPS) monolayer (Nixdorff *et al.*, 1978). The phospholipids in the inner portion of the outer membrane are the sites where the disruption is most likely to take place, once the detergent passes through the LPS monolayer (Nixdorff *et al.*, 1978).

Cationic Detergents

Cetyltrimethylammonium bromide (CTAB) is a cationic surfactant and it has been used to permeabilise Baker's yeast (*S. cerevisiae*). CTAB is known to cause permeabilisation, however, the exact site of attack and mechanism of the surfactant is debated. Cationic surfactants are proposed to act on the LPS in the outer membrane, thereby changing the conformation of membrane proteins and causing cell disruption (Felix, 1982). The positively charged surfactant is expected to bind strongly to the negatively charged lipids and phospholipids in the cell envelope, disrupting the membrane structure (Bansal-Mutalik and Gaikar, 2003).

Literature on the permeabilisation of cells using CTAB is summarised in Table 2.2. In general, the findings have shown that CTAB has been used for the permeabilisation of both yeast and bacteria. The optimum concentration range falls within 0.1 and 0.4%, with a debate as to whether release is dependent on concentration of CTAB or dependent on the ratio of cells to detergent. As concentration and treatment time increase, it has been found that deactivation also increases. The optimum temperature and pH ranges found are 24 to 37°C and pH 4 to 10 respectively, with typical treatment times of 15 minutes.

The treatment of *S. cerevisiae* by 0.02% to 0.4% CTAB for 15 minutes at 26°C has shown increasing catalase activity, with a maximum at 0.4%. At higher concentrations, the catalase and ADH activities decreased. This was attributed to the inactivation by CTAB and where maximum activity observed may represent the conditions at which the

plasma membrane might have been permeabilised (Sekhar *et al.*, 1999). It was also found that permeabilisation is dependent on the ratio of cells to detergent (1:0.02 w/w) rather than on the concentration of detergent. Therefore, the detergent has a very high affinity for binding to cells (Gowda *et al.*, 1991). However, another study showed that the permeabilising ability of the surfactant was dependent on its concentration, reaching a peak at 0.1% (Alamae and Jarviste, 1995).

Table 2.2 Summary of CTAB Literature Findings

Microorganism	Conc (%)	Conditions	Effects	Reference
Baker's yeast (*Saccharomyces cerevisiae*)	0.2	24°C, 15 minutes	Permeabilisation with increased activities in hexokinase, G6PDH and decreased ADH activity with increased CTAB concentration, due to deactivation.	Gowda *et al.*, 1991
Pichia pinus	0.1	15 minutes	Maximum alcohol oxidase and formaldehyde dehydrogenase activity. Reduced cell viability.	Alamae and Jarviste, 1995
Baker's yeast (*Saccharomyces cerevisiae*)	0.4	26 to 37°C, 15 minutes pH : 4 to 10	Maximum intracellular catalase activity, but decreased ADH activity.	Sekhar *et al.*, 1999
E. coli	10 mmol/dm^3	30°C, 2 hours	100% Protein release, 18% penicillin acylase release – possible deactivation by CTAB.	Bansal-Mutalik and Gaikar, 2003
Azotobacter vivelandii		Reverse micelles	Release of cytoplasmic enzymes.	Giovenco and Verheggen, 1987

Azotobacter vivelandii was treated with CTAB to extract enzymes selectively using reverse micelles. Three dehydrogenases tested showed varying activities, due to unknown reasons. However, it has been inferred that the differences in the activities can be attributed to the hydrophobic interactions among the enzyme, reactants and surfactant. These interactions are distance dependent and therefore, the kinetic behaviour of smaller enzymes are less affected than larger ones (Giovenco and Verheggen, 1987).

Contradiction is found in the literature in terms of enzyme activity at specific CTAB concentrations. Gowda *et al.* (1991) found an increase in activity of the ADH, a

cytoplasmic enzyme, at 0.1 to 0.2% CTAB, while higher concentrations caused decreases in activity or maximum activity. Decreased ADH activity was observed with increases in CTAB concentration beyond, 0.2%, this decrease was attributed to inactivation of the enzyme by the surfactant (Sekhar *et al.*, 1999). Enzyme inactivation is evident in the some instances, specifically when the CTAB concentration is greater than 0.4%. Bansal-Mutalik and Gaikar (2003) found enzyme activity to be 18% but protein release was 100%, indicating that enzyme deactivation by CTAB had occurred.

Non-ionic Detergents

Non-ionic detergents act on the cytoplasmic and cell membranes, and incorporate into the bilayer, and cause changes in physical properties (Helenius and Simons, 1975). The constituent proteins of the membrane structure are solubilised to an extent, making the cell permeable to the passage of certain proteins (Schutte and Kula, 1990).

Triton X-100 acts on the cytoplasmic membrane of bacterial and yeast cells and results in protein release. General observations have shown detergents only affect the inner membrane of Gram-negative bacteria, the lipopolysaccharides of the outer membrane providing resistance to the detergent (Naglak and Wang, 1990). The treatment of cells with Triton X-100 alone results in fairly low protein release. Often, the detergent requires prior removal of the cell wall and therefore the detergent is used in combination with other chemicals to produce a synergistic effect. Membrane removal was found to be independent of detergent concentration in the range 0.5 to 2% (Harrison, 1991a). Common chemicals with which it is used in combination include guanidium hydrochloride and EDTA. Table 2.3 reviews specific examples of use of Triton X-100.

Treatment of *Nocardia rhodocrous* with Triton X-100 released 2% total protein and 70% cholesterol oxidase, an enzyme associated with the surface of *Nocardia* cells (Buckland *et al.*, 1976). Chemical treatment of *E. coli* using Triton X-100 at 23°C resulted in the solubilisation of 15 to 25% of the protein. Prior removal of the cell wall and direct treatment of the cytoplasmic membrane resulted in 60 to 80% of the protein released on treatment with Triton X-100. Electron micrographs have shown that the

detergent does not affect the characteristic morphology of the cell wall but removes fragments of the cell membrane (Schnaitman, 1971b). Effective permeabilisation of *S. cerevisiae* using 0.3 to 1.0% Triton X-100 for 30 minutes at 30°C allowed β-galactosidase activity to be maximised (Chow and Palecek, 2004).

Table 2.3 Summary of Triton X-100 Studies Conducted in Literature

Micro-organism	Conc	Other Chemicals	Conditions	Enzyme	Enzyme Release	Protein	Reference
Nocardia rhodocrous				cholestrol oxidase	70%	2%	Buckland *et al.*, 1976
E. coli	2% (w/v)	12.5% K_2HPO_4 (w/v)	19 hours, 15°C	L-asparaginase	70%	70%	Zhao and Yu, 2001
E. coli	0.50%		2 hours/ 43 hours, 4°C	β-Lactamase		10%	Naglak and Wang, 1990
E. coli	0.50%	0.2M G-HCl	2 hours/ 43 hours, 4°C	β-Lactamase	80%	50%	Naglak and Wang, 1990
E. coli	0 to 4%		Prior removal of OM 10 minutes, 23°C			60 to 80% cytoplasmic protein	Schnaitman, 1971b
E. coli	2%	5mM EDTA	10 minutes, 23°C			50% cell wall protein	Schnaitman, 1971a
S. cerevisiae	0.3 to 1.0%		30 minutes, 30°C	β-Galactosidase	maximum activity		Chow and Palecek, 2004
Pichia pastoris	0.80%		150 minutes, 21°C			-	Shepard *et al.*, 2002
S. cerevisiae	0.2% (w/w)		30 minutes, 20°C	ADH	effective		Laouar *et al.*, 1996
S. cerevisiae	0.02 to 0.5%			Fluorescence dye			King *et al.*, 1991
Yarrowia lipolytica	0.1 to 0.2%		20 minutes, r.t	Phosphatase	100%	80%	Galabova *et al.*, 1996
E. coli	0.5 to 2%	0.1M G-HCl	1 hour, r.t			50 to 60%	Schutte and Kula, 1990
E. coli	2%	2M G-HCl	2 hours, 4°C			30%	Hettwer and Wang, 1989

The use of Triton X-100 for the permeabilisation of *Yarrowia lipolytica* allowed maximum release of acid and alkaline phosphatases. Maximal permeabilisation was achieved using 0.1 to 0.2% Triton X-100 and 30 minutes incubation at room temperature.

The maximum activity was dependent on the detergent concentration. The morphological changes of the treated cell were observed using electron microscopy. The treated cells had altered cell shapes and folded cell walls. Cell surface membranes became thin and translucent compared to control cells. Triton X-100 causes membrane permeability to the substrate rather than release of enzymes into the supernatant. The mild, rapid permeabilisation procedure claims to yield 100% acid and 82% alkaline phosphatase present (Galabova *et al.*, 1996).

Since Triton X-100 acts on the inner membrane only, it is often used in combination with another chemical to enhance release. It was found that combined treatment of *E. coli* Triton X-100 and EDTA solubilises about half of the cell wall protein. Therefore, EDTA permeabilises the outer layer and thus allows passage for the Triton X-100 to the cell membrane, resulting in solubilisation of about half of the protein and all of the lipopolysaccharide and phospholipids (Schnaitman, 1971a).

The chemical permeabilisation of *E. coli* cells to release L-asparaginase can be carried out using Triton X-100 and dipotassium hydrogen phosphate, K_2HPO_4. A 12.5% (w/v) concentration of K_2HPO_4 and 2% (w/v) Triton X-100 at a pH of 8.6 in a cell suspension containing 3 x 10^8 cell/ml released approximately 70% of the enzyme. The amount of enzyme released with the combined use is much greater than the individual effects. Electron micrographs have shown that *E. coli* cells were not structurally fractured, but the surface structure was altered (Zhao and Yu, 2001).

Triton X-100 has a high binding affinity for hydrophobic species and is very effective in binding to and solubilising the phospholipids of *E. coli* inner membrane and outer wall fragments. The combination of Triton X-100 with the chaotrophic agent, G-HCl, which is capable of solubilising protein from *E. coli* membrane fragments, has proved to be a very useful permeabilisation method. No loss of enzyme activity is reported and cell debris fragmentation is avoided (Harrison, 1991a). Electron microscopy showed that guanidine disrupts the outer membrane and exposes the inner membrane to Triton X-100. Extensive disruption of the outer wall or peptidoglycan layer was not observed and therefore the

disruption occurs on a molecular level. Varying concentrations were used, up to 4M for G-HCl and up to 2% for Triton X-100. At G-HCl concentrations above 2M, significant protein release was observed and the Triton X-100 merely acts as an enhancement of this release. At lower concentration of G-HCl of 0.1M and 0.5 to 2% Triton X-100, high protein release yields are observed but at slow rates. There is a synergistic effect between the two chemicals as they are both individually capable of permeabilisation (Hettwer and Wang, 1989). Similar results were also found by Naglak and Wang (1990) under the same range of conditions.

A non-ionic surfactant Pluronic F-68 has been found to be effective in permeabilising yeast cells (Laouar *et al.*, 1996). Its effect has been compared to Triton X-100 and both have been shown to have similar effects as cell permeabilising agents. The growth of yeast in the presence of either detergent increased the permeability of the cell and this was confirmed by the activity of alcohol dehydrogenase. The effect of these surfactants on cell growth was studied at a 0.2% (w/v) concentration with incubation for 30 minutes at 20°C. The results showed that Triton X-100 delayed the logarithmic growth phase while Pluronic F-68 had no effect on growth.

2.4.1.3 Chaotrophic Agents

Chaotrophic agents are described as cell lysis mediators (Hettwer and Wang, 1989), capable of solubilising some hydrophobic compounds, possibly due to interaction with the hydrogen bonding of water (Naglak and Wang, 1990). These agents, specifically guanidine hydrochloride (G-HCl), are known to solubilise protein from membrane fragments of *E. coli* and alter hydrophobic interactions. The potency degree of cell lysis is as follows: trichoroacetate> perchlorate-thiocyanate> nitrate > urea. Chaotrophic salts weaken hydrophobic associations to promote cell lysis and inhibit the cross-linking of the peptidoglycan and wall assembly (Ingram, 1981).

A combination of agents is more effective than individual treatments. The chemical extraction of soluble and insoluble protein from *E. coli* cells was carried out using

7M G-HCl and 7M urea. The mechanism of lysis is believed to be the weakening of hydrophobic interactions resulting in the inhibition of cross-linking and thus inducing lysis. Therefore, the membrane-associated activities are affected (Middelberg *et al.*, 1999).

G-HCl concentrations greater than 2M have shown alterations in the cell structure of *E. coli* on a molecular level, resulting in the release of a substantial amount of protein. Concomitant decrease in activity of some enzymes is attributed to the well known ability of G-HCl to denature proteins. Lower concentrations of G-HCl such as 0.1M are associated with some protein release (Hettwer and Wang, 1989).

A G-HCl concentration of 10mM extracted 90% of the total extractable protein from *E. coli* (Novella *et al.*, 1994). Further increases in the G-HCl concentration increased the protein release. A concentration of 0.4M caused the enzyme recovery values to correlate inversely with the concentration, possibly due to aggregation of protein or denaturation of the enzyme. This agent is often used in combination with EDTA for permeabilisation using a 10mM concentration for each over a 24 hour time period and releasing almost 100% of proteins and penicillin acylase.

2.4.1.4 Solvents

Solvents including many alcohols such as ethanol, methanol, isopropanol and butanol cause disruption of the cell membrane structure by extracting the lipid component of the cell, allowing leakage of intracellular components (Fenton, 1982). Solvents have been used widely for cell disruption to isolate enzymes. The use of solvents can be hazardous as these substances are flammable. Further, enzyme denaturation may result. Table 2.4 summarises the literature findings for chemical treatment of cells using solvents. A discussion of these findings follows.

Table 2.4 Summary of Solvent Studies Conducted in Literature

Microorganism	Solvent	Conc (%)	Conditions	Enzyme Release	Enzyme (%)	Reference
Kluveromyces bulgaricus	n-butanol	10%	3 minutes, 25°C	Maximum β-galactosidase activity	good perm.	Decleire *et al.*, 1987
	propanol	20%			good perm.	
	isopropanol	30%			good perm.	
	tert-butanol	30%			good perm.	
	ethanol	40%			good perm.	
	acetone	40%			good perm.	
	Dimethyl-sulphoxide	70%			good perm.	
Kluveromyces fragilis	Ethanol	80%	17 hours, 28°C	Slow enzyme release (β-galactosidase), but maximum release achieved after 17 hours	90%	Fenton, 1982
	Methanol				90%	
	Isopropanol				85%	
	Butanol				2%	
	tert-butanol				90%	
	acetone				20%	
	Methyl-ethyl ketone				20%	
	Toluene				15%	
Kluveromyces lactis	Ethanol	40%	15 minutes, 5, 30°C	β-galactosidase	effective	Flores *et al.*, 1994
	Toluene	2%	15 minutes, 30 and 37°C		max enzyme activity	
	Chloroform	2%	15 minutes, 5, 30 and 37°C		max enzyme activity	
	Chloroform + Ethanol	2%, 10%	15 minutes, 5, 30 and 37°C		max enzyme activity	
	Toluene + Ethanol	2%, 10%	15 minutes, 30 and 37°C		max enzyme activity	
Escherichia coli	Toluene/ Ethanol	15:85 (v/v)	5 minutes, r.t	penicillin acylase	good perm.	Novella *et al.*, 1994
Saccharomyces cerevisiae	isopropanol	40%	10 minutes, 4°C		good perm.	Kondo *et al.*, 2000
	Ethanol	40%			good perm.	
Saccharomyces cerevisiae	Ethanol	70%, 100%	30 minutes, 25°	β-galactosidase	ineffective	Chow and Palecek, 2004

Microorganism	Solvent	Conc (%)	Conditions	% Enzyme release	Enzyme (%)	Reference
Saccharomyces cerevisiae	Toluene + Ethanol		5 minutes, r.t	ADH	effective perm	Murakami *et al.*, 1980
	Toluene + Ethanol		5 minutes, 37°C		effective perm	
	Toluene		5 minutes, 40-45°C		v. good perm	
Escherichia coli	Toluene	1%	1 hour, 0°C	Protein	4.20%	De Smet *et al.*, 1978
	Toluene + EDTA		1 hour, 0°C		27.9%	

The general, the findings have shown successful permeabilisation of yeast and bacterial cells with solvents. The most common solvents used were ethanol and toluene. In some instances, two solvents are used in combination to achieve increased release. The alcohol concentrations used for effective treatment range from 10 to 80%, with temperatures of 25 to 30°C and treatment times of 5 to 15 minutes. Toluene concentrations of 1 or 2% at 30 to 37°C, with treatment times of 15 minutes to 1 hour caused permeabilisation. Combination treatments showed maximum enzyme activity. However, there is much contradiction of concentrations and treatment times required for permeabilisation.

A high yield of β-galactosidase solubilisation was found, with up to 90% released on treatment of yeast cells with varying concentrations of isopropanol, ethanol and methanol. The release of enzyme was slow at 10 to 20 hours to achieve maximum enzyme release, which occurred at a solvent concentration of 80 to 90%. Concentrations in excess of this value require removal of the solvent to prevent enzyme denaturation (Fenton, 1982). The slow release was confirmed by Chow and Palecek (2004) where little or no release of β-galactosidase from *S. cerevisiae* with 70% ethanol concentration for 30 minutes at 25°C. High β-galactosidase activity was detected with 100% ethanol and 0.3% Triton X-100, by solubilisation of the inner membrane (Chow and Palecek, 2004).

The treatment of *S. cerevisiae* using isopropanol and ethanol has been studied and showed a 317 fold increase in the production rates of S-lactoylgultathione, compared with untreated cells. The high activity was attributed to the reduction in the permeability barrier of the cell envelope. The type of alcohol and its concentration greatly affects the

treatment. It was found that a concentration of 40% in both alcohols produced the maximum results. Concentrations above 40% resulted in most cells being dead. The length of time of treatment showed little effect (Kondo *et al.*, 2000), which contradicts the findings of Fenton (1982), where 17 hours was required for good permeabilisation.

Improved permeabilisation of yeast in the presence of toluene and heat was observed with temperature in the range 25 to 45°C. This allowed substrate molecules to diffuse into the cell and no intracellular protein was released from the cell. The electron microscope showed damage to the cell membrane but the outer membrane remained intact (Murakami *et al.*, 1980). Similar results were found with toluene and heat permeabilisation within 5 minutes and with a mixture of 2% toluene and 10% ethanol (Flores *et al.*, 1994). Maximum β-galactosidase activity was found, equal to that of a disrupted suspension, but no extracellular enzyme was detected. Therefore, the enzyme was contained within the boundaries of the cell and the membrane barrier was damaged (Flores *et al.*, 1994). The treatment of *E. coli* with toluene removed little protein, phospholipid or lipopolysaccharide in the presence of Mg^{2+} ions. Cells treated with toluene and EDTA altered the outer membrane permeability. Electron microscopy showed the same results observed by Murakami *et al.* (1980). It is possible that toluene disorganises the bilayer through phospholipid removal from the cell membrane (De Smet *et al.*, 1978).

Combinations for enhanced release have been reported. A mixture of 1:4 (v/v) toluene: ethanol at 26°C for 5 minutes was used for the permeabilisation of *S. cerevisiae*. The treatment showed the release of large amounts of catalase in comparison to the untreated cells (Sekhar *et al.*, 1999). The same mixture was used to permeabilise *Kluveromyces bulgaricus* and a small amount of β-galactosidase was detected after 3 minutes. When a smaller amount of toluene was mixed with a large amount of ethanol at a ratio of 4:96, high enzyme activity was found (Decleire *et al.*, 1987). The combination treatment has been used to extract protein and was tested for penicillin acylase in *E. coli*. A small amount of enzyme was released but the release was not significant when compared with other techniques used (Novella *et al.*, 1994).

2.4.1.5 Chelating Agent - EDTA (Ethylenediaminetetraacetic Acid)

EDTA causes the chelation of Mg^{2+} and other divalent ions. Through this, it increases the permeability of Gram-negative bacteria and causes lysis in yeast and fungal cells by acting on the cell wall and inner membrane. In coliform bacteria, this results in destabilisation and removal of the outer membrane with a 33 to 50% loss in lipopolysaccharide content. The changes in the outer membrane can affect the cytoplasmic membrane (Felix, 1982).

EDTA has been used in combination with detergents and chaotrophic agents. When used with G-HCl for the permeabilisation of *E. coli*, the periplasmic enzyme, penicillin acylase was successfully extracted showing high activity (Novella *et al.*, 1994). It has also been used with Triton X-100 to efficiently release periplasmic protein with high yields (Bansal-Mutalik and Gaikar, 2003). It has been used with toluene to increase the release of intracellular malate dehydrogenase (De Smet *et al.*, 1978).

2.4.1.6 Antibiotics

Antibiotics may assist in permeabilisation of microbial cells in two ways: through inhibition of cell wall synthesis and through disorganisation or distortion of the cell membrane. Antibiotics such as penicillin block the synthesis of new cell wall material. To initiate cell lysis, these antibiotics must be added during the growth phase (Engler, 1985). Polyene antibiotics such as nytasin permeabilise fungal cells through forming complexes with the membrane and steroids to distort the cell membrane. Polypeptide antibiotics, such as polymyxin, gramicidin and tyrocidine result in disorganization of the cell membrane due to the ability to bind to the cytoplasmic membrane of bacteria. Polymyxin binds to the negatively charged lipid layer. The specificity of the antibiotic against Gram-negative bacteria can be traced to the presence of a lipid at the cell surface of the microorganism (Felix, 1982).

2.4.2 Physical Methods

2.4.2.1 Osmotic Shock

This is a gentle cell lysis method. The cells are allowed to equilibrate briefly in a medium of high osmotic pressure such as 1M glycerol or sucrose. The medium is diluted suddenly and water enters the cells rapidly, increasing the hydrostatic pressure and thereby causing the disruption of the cells. This method is generally used on fragile organisms, when the cell wall has been weakened enzymically, or when cell wall synthesis inhibition has occurred. Osmotic shock has a small effect on microbial cells with a peptidoglycan or glucan wall present. Its effect is further reduced in cells in the stationary phase owing to the stronger cell wall (Felix, 1982). Product contamination may result due to high salt concentrations (Engler, 1985).

2.4.2.2 Freezing and Thawing

This method involves the formation and subsequent melting of ice crystals. Larger crystals form with gradual freezing resulting in more extensive cell damage. Typically a number of freeze-thaw cycles are used. This method is implemented on a small scale. It gives low yields, with a possible loss in enzyme activity (Harrison, 1991a). Protein denaturation may result (Engler, 1985).

2.4.2.3 Dessication

Freeze-drying results in minimal damage to bacterial cells and is commonly used to preserve active cells. Slow drying in air, drum drying, drying with a dessicant or treatment with a dehydrating solvent is more effective being driven by the evaporation process. The subsequent extraction of the microbial product results in a low yield recovery (Engler, 1985). Permeabilisation results with cells remaining morphologically intact (Felix, 1982).

2.4.2.4 Temperature

The thermolysis of *E. coli* cells at 90°C has been reported to release a significant amount of protein in 20 minutes. Longer treatments times of approximately 1 hour at a lower temperature, between 30°C and 70°C resulted in larger protein release. Protein solubility changes at higher temperatures and denaturation results in loss of activity of many proteins and interference with protein measurement (Harrison, 1990a). *E. coli* cells exposed to heat stress of 42°C to 47°C for 10 minutes have shown the translocation of β-galactosidase from the cytoplasm to the periplasm. The movement is attributed to changes in the surface hydrophobicity of the enzyme and inner cytoplasmic membrane of the cells. The hydrophobicity of the enzyme and inner membrane are increased during the treatment. The enhanced hydrophobicity aids the passage of the enzyme across the membrane (Umakoshi *et al.*, 1998).

A commercial process plant for the production of PHB used a heat shock technique for the disruption of *Ralstonia eutrophus*. Steam at 150°C and 0.48 MPa was injected into a fermentation culture for 60 seconds and thereafter discharged. This process is difficult to control, expensive in energy and entraps impurities which can affect polymer properties. Further experimentation using this method revealed that at pH 9.0, a temperature of 140°C and a holding time of 30 seconds enhanced cell disruption and the extraction process, with a 10% increase in protein release. Molecular weight degradation was restricted to 18% (Harrison, 1990a).

2.4.3 Biological Methods

2.4.3.1 Enzymatic Lysis

Enzymatic cell lysis is advantageous because it is a controlled method of disruption, is biologically specific, requires mild operating conditions, has low energy requirements and low capital investment. Further, it avoids harsh physical conditions such as high shear stress that occurs in mechanical disruption processes (Engler, 1985). Its application requires selection of an appropriate enzyme or enzyme system and the determination of

specific reaction conditions for efficient lysis. It is often limited to releasing periplasmic or surface enzymes (Asenjo, 1990). Enzymatic hydrolysis of cell walls is attractive in terms of its mild operating conditions, specificity for only the wall structure and limited deactivation. It has potential as an alternative to mechanical breakage. However, currently extensive enzymatic lysis is a slow and costly process (Baldwin and Robinson, 1990).

Enzymatic Lysis of Bacteria

There are three groups of bacteriolytic enzymes available:

- Glycosidases - hydrolyse polysaccharide chains of the peptidoglycan backbone,
- Acetylmuramyl-L-alanine amidases - cleave polysaccharide polypeptide junction and
- Endopeptidases that split polypeptide chains with peptidoglycan

The most important bacteriolytic enzyme is lysozyme, produced from hen egg white and available from other natural sources. Other bacteriolytic enzymes include those from *Cytophaga sp.*, *Staphylococcus sp.*, and *Streptomyces sp.*, which can lyse Gram-positive bacteria. A lytic protease from *Micronospora sp.* can lyse lyophilised cells of Gram-negative bacteria, and a lytic protease produced by *Bacillus subtilis* is reported to lyse cells of the bacterium *E. coli* without the need for pretreatment (Asenjo, 1990). Bacteriolytic enzymes have a pH optima around 6 or 7 and optimum temperature range of 35 to 60°C (Asenjo, 1990). Most bacteriolytic enzymes are not active on viable cells and therefore, the biomass requires sensitisation by heat inactivation, chemical pretreatment, freezing or lyophilisation (Andrews and Asenjo, 1987). Lytic protease from *Micromonospora sp.* lyses both viable and non-viable Gram- positive and Gram-negative bacteria as well as yeast and fungal cells (Andrews and Asenjo, 1987).

The enzyme lysozyme is the only commercially available bacteriolytic enzyme for large scale application. It hydrolyses β-1-4 glucosidic linkages of polysaccharide chains of peptidoglycan. Gram-positive cells may be attacked directly by lysozyme as contain peptidoglycan forms the outermost structure. Gram-negative cells have an outer

membrane and are therefore more resistant to attack. Their lysis with lysozyme requires prior removal or destabilisation of the outer membrane to expose peptidoglycan (Shutte and Kula, 1990). This can be achieved by the use of a chelating agent EDTA or a non-ionic detergent, Triton X-100. Electron microscopy has been used to confirm the morphological changes to the cell wall (Andrews and Asenjo, 1987).

A lytic enzyme system from *Cytophaga sp.* has been used for the lysis of Gram-positive bacteria, *Bacillus* and *Corynebacterium.* The optimum pH and temperature for the lytic reaction were 9.2 and 50°C respectively (LeCorre *et al.*, 1985). Complete lysis of the Gram-negative bacteria *Ralstonia eutrophus* was achieved using the enzymes from *Cytophaga* without prior removal of the outer membrane or removal of the divalent cations. The destabilisation using EDTA resulted in no significant improvements in protein release (Harrison *et al.*, 1991b). Combinations of lytic enzymes are more effective (Engler, 1985). The *Cytophaga* lysing enzyme preparation contains a combination of enzymes able to attack different targets.

When *Pseudomonas putida* was resuspended in 50mM phosphate buffer (pH 8.0) to a concentration of 25% and lysed at 30°C with 30 μg/ml of lysozyme, the addition of EDTA could be avoided (Shutte and Kula, 1990). Actively growing microorganisms produce enzymes to hydrolyse polymeric structures of their own cell wall for normal growth. Changes in the organism's environment can result in an overproduction of these enzymes or inactivation of the production of other autolytic enzymes and autolysis can occur. The parameters that affect autolysis are pH, temperature, molarity of the buffer and the metabolic state of cells (Engler, 1985).

The Gram-positive bacteria, *Bacillus cereus* were incubated for 90 minutes with a low concentration of cellosyl and the lytic enzyme preparation from *Streptomyces* strain. This resulted in hydrolysis of the glycosidic linkage in murein sacculus forming the cell wall. After random nicking of the murein, the cells became more susceptible to mechanical stress (Schutte and Kula, 1990).

Enzymatic Lysis of Yeast

The ability of enzymes to lyse yeast cells has been studied in batch and continuous culture with *Cytophaga sp.* and *Oerskovia xanthineolytic* lysing enzymes. Lytic enzymes from *Cytophaga* were shown to rupture yeast cells (Asenjo and Dunhill, 1981).

The rate of protein release in the presence of the enzyme was a linear function of enzyme concentration. Yeast cell walls consist of two main layers: an outer protein-mannan complex and an inner β-glucan layer. The enzyme system for yeast lysis is usually a mixture of different enzymes including β-1-3-glucanase, protease, β-1-6-glucanase, mannanase and chitinase. These enzymes act synergistically to lyse the cell wall. Essentially, only two enzymes are required for yeast cell breakage, a wall lytic protease to degrade the outer protein-mannan complex and a lytic β-1-3 glucanase to degrade the inner layer (Shutte and Kula, 1990).

The enzyme Zymolase partially hydrolyses the glucan and mannan components of the carbohydrate-protein-mannan complex of yeast cell walls. The process requires 2 hours and gentle shaking at 25°C to be effective (Baldwin and Robinson, 1990). The combined use of Zymolase and lysozyme released 40% more carbohydrates than Zymolase alone. The lysozyme hydrolyses the glucosidic bonds of mannoprotein and glucan complexes of the cell wall after the partial disorganization of the cell wall by Zymolase. Enzyme treatment of Brewer's yeast using Zymolase and lysozyme for 2 hours at 37°C, followed by extraction at pH 9 resulted in nitrogen release comparable to those obtained by conventional mechanical disruption methods. The method results in negligible protein denaturation and has potential to be implemented on a larger scale (Knorr *et al.*, 1979).

The drawbacks of enzymic lysis are cost of the lytic enzymes and their limited commercial availability. Gram-negative bacteria generally require prior removal of the outer membrane. If the enzyme can be reused through immobilization or is readily produced by organisms, this technique would become more economically viable (Engler, 1985).

2.5 Summary of Effective Non-Mechanical Methods

Table 2.5 summarises the target point of action for a range of non-mechanical cell disruption techniques showing promise with bacterial, yeast and fungal cells. The effect of the pretreatments as non-mechanical methods of cell disruption is indicated.

Table 2.5 Summary of pretreatment effects on the structural components of bacteria, yeast and fungi

Pretreatment		Structural Component (Resistance)	
		Peptidoglycan	Glucans, Mannans, Chitin
		Bacteria	Yeast and Fungi
Method	**Chemicals**	**Effects**	
Detergent + Chaotrophic agent	**Triton X-100 + G-HCl**	Molecular alteration of outer membrane	
		Solubilisation of cytoplasmic membrane	
Detergent + EDTA	**Triton X-100 + EDTA**	Permeabilisation of outer membrane	Permeabilisation of outer membrane
		Solubilisation of cytoplasmic membrane	Solubilisation of cytoplasmic membrane
	CTAB	Permeabilisation of cell wall	Permeabilisation of cell wall
Solvents	**Ethanol**	Inhibition of cell wall synthesis	Permeabilisation of cell wall
	Toluene		Solubilisation of cytoplasmic membrane
Enzymes	**Lysozyme or Zymolase used separately**	Attack of β1,4 linkages	Hydrolysis of β1,3 linkages
	Zymolase combined with Lysozyme		Hydrolysis of bonds and complexes
Autolysis	**EDTA**	Solubilisation	Permeabilisation of cell wall

2.6 Combined Methods for Mechanical Disruption

Limited research is presented in the literature on the combination of non-mechanical and mechanical methods of cell disruption. The aim of this research centres on improving intracellular product release, reducing energy requirements and enhancing selectivity of disruption. Advantages of chemical permeabilisation over mechanical disruption are evident in the protein release and reduced cell fragmentation using a simple batch process like permeabilisation. Some of the disadvantages include removal of chemicals from the final product, irreversible loss of product activity caused by the chemicals and suboptimum yields (Hettwer and Wang, 1989). In this approach, non-mechanical methods have been used to treat cell to weaken their cell walls and make them more susceptible to subsequent mechanical disruption, thereby increasing the amount of intracellular protein released. Mechanical disruption requires large amounts of energy, efficient cooling and results in the micronisation of cell debris. By combining non-mechanical and mechanical methods of disruption, complete disruption is sought on a single pass through a HPH or mill, thus saving energy, reducing enzyme costs and treatment time (Shutte and Kula, 1990).

The pretreatment of *E. coli* using 1.5M G-HCl and 1.5% Triton X-100 followed by high-pressure homogenisation was found to decrease the number of passes required for disruption and operating pressure. The operating pressure used was 41 MPa and a protein release of 82% was found after one pass through the homogeniser, compared to two passes required for untreated cells at the same pressure (Bailey *et al.*, 1995). High G-HCl concentrations of 4.0M produced cell debris that was much smaller than the debris from untreated cells due to premature solubilisation of the inclusion bodies. It was also mentioned that the smaller particle size may be due to differences in measurement techniques and possible shrinkage of debris during sample preparation for electron microscopy. One pass through the homogeniser at a low operating pressure allows for shorter process times, lower energy consumption and less reduction in the size of the cell debris as compared to that of multiple passes at higher pressure (Bailey *et al.*, 1995).

Enzymatic treatment of the Gram-negative bacterium, *Ralstonia eutrophus*, was carried out using the lytic enzyme system of *Cytophaga*. The results showed that complete cell lysis occurred within 60 minutes. The mild operating conditions prevented deleterious effects on the product. Protein release exceeded that obtained on mechanical disruption (330g protein/kg non- PHB biomass) after more than two passes in the HPH at 60 to 70 MPa. Protein release with enzymatic treatment was found to be approximately 360g protein/kg non-PHB biomass). The increased release is attributed to solubilisation of additional membrane bound protein by the enzyme preparation (Harrison *et al.*, 1991b).

Enzymatic pretreatment of Baker's yeast (*S. cerevisiae*) was used prior to high pressure homogenisation. Mechanical disruption without pretreatment gave a protein yield of 40% after 5 passes at 95 MPa, whereas the pretreatment with gave a yield of 100% after 4 passes under the same operating conditions (Baldwin and Robinson, 1990). A combination of two methods, using a pretreatment in the form of partial enzymatic lysis of the cell wall by Zymolase followed by mechanical disruption using a Microfluidizer high pressure homogeniser gave 95% total disruption of *Candida utilis* compared to 65% with mechanical disruption only for the same number of passes. A model was developed to predict the fraction disrupted by the combination and experimental results agreed quite favourably with the predictions over a wide range of operating conditions (Baldwin and Robinson, 1993). The modification was necessary to account for protein release during the pretreatment alone.

A range of operating pressures was considered with the effect of pretreatment with cations and an average increase of 30% in soluble protein was obtained in the presence of 0.137M salt (NaCl or KCl) compared to its absence in the suspension. Cell weakening was observed when EDTA or lysozyme and EDTA were used and an increase in the extent of protein release was observed with one pass through homogeniser at a pressure lower than 70 MPa (Harrison *et al.*, 1991d).

Chemical treatments alter the permeability of microorganisms, allowing the release of soluble products. Cell wall strength is one of the major factors that influence mechanical

disruption and the chemical or enzymatic pre-treatment of the cell wall to increase its susceptibility to mechanical stress has been considered. Alkaline treatment of *Ralstonia eutrophus* at pH 11.5 and 23°C for 15 minutes resulted in protein release corresponding to 26% of R_{max}. An average of 37.5% increase in soluble protein release was found with homogenisation, at a lower pressure with fewer passes, subsequent to alkaline treatment on *Ralstonia eutrophus* compared with homogenisation in the absence of pretreatment.

SDS treatments of 0.24 and 1% (m/v) at 70°C for 20 minutes gave a soluble protein release of 4 and 11% of the R_{max} respectively. Subsequent homogenisation at 62 MPa for a single pass enhanced the release by 15 and 38% over the control. Protein release (Table 2.6) on a single pass at 62.8 MPa following treatment with 1% SDS approached that found on two passes with untreated cells (Harrison *et al.*, 1991d).

Table 2.6 Homogenisation conditions required for maximum cell disruption in terms of soluble protein release after pretreatment with SDS (Redrawn from Harrison *et al.*, 1991d)

SDS Conc (%)	Pretreatment		HPH conditions required for max. cell disruption	
	Temp (°C)	Time (min)	Pressure (MPa)	Number of passes
0.0	20	-	69	3
0.0	70	20	69	>2
0.1	70	20	62	1
1.0	70	20	34.5	1

Therefore, the use of pretreatment to enhance mechanical disruption has been shown to have great potential for cell disruption. Advantages in the use of pretreatments combined with mechanical disruption relate to the lower pressure and the number of passes required. This results in a reduction in energy usage, cost and treatment time. It also ensures minimum product damage and extensive cell fragmentation is avoided. Energy reduction calculations were performed using literature data. Using Equation 3.1, the energy requirement for the mechanical process and combined process and therefore a percentage reduction was calculated. These data are presented in Table 2.7.

Table 2.7 Summary of Combined Methods of Microbial Cell Disruption in Comparison to Mechanical Cell Disruption

Micro-organism	Non-Mechanical	Release by Pretreatment alone	Mechanical	Release by Mechanical	Combined Release	Reference	Energy Reduction (%)
E.coli	2M G-HCl and 2% Triton X-100	35% protein release (2 hours)	Bead Mill	70% release, 4 minutes	Not performed, release by two methods compared	Hettwer and Wang, 1988	-
E.coli	1.5M G-HCl and 1.5% Triton X-100	62% protein release	HPH	41 MPa, 2 passes	82% protein release, 41 MPa, 1 pass	Bailey *et al.*, 1995	50
Ralstonia eutrophus	Enzymes from Cytophaga	Complete cell lysis in 60 minutes	HPH	Max. release, 60 to 70 MPa, < 2 passes	Not performed, release by two methods compared	Harrison *et al.*, 1991b	-
S. cerevisiae	Zymolase	6% fraction disrupted	Microfluidizer	32%, 95 MPa, 4 passes	100%, 4 passes, 95 MPa	Baldwin and Robinson, 1990	75
Candida utilis	Zymolase	3.5% fraction disrupted	Microfluidizer	65%, 95 MPa, 4 passes	95%, 4 passes, 95 MPa	Baldwin and Robinson, 1993	33.3
Ralstonia eutrophus	Alkaline treatment	26% of R_{max} of soluble protein	HPH	100% (330g protein/kg non-PHB biomass) 2 passes, 62 MPa	increase by 37.5% in soluble protein	Harrison *et al.*, 1991d	40
	SDS	0.24% and 1% released 4 and 11% of R_{max} of soluble protein			38% increase, 1 pass, 62 MPa		33.3
	Monovalent cations	20% soluble protein (60°C, 60 min)			30% increase in soluble protein		33.3
	EDTA or EDTA and lysozyme	No significant DNA release			1 pass, 70 MPa		53.3

2.7 Consideration of Upstream and Downstream Processes

Other factors that must be considered in selection of the cell disruption protocol are the upstream and downstream processes. The upstream processes involve all factors that must be considered prior to disruption as well as the processes preceding disruption. It has already been noted that the cell wall of the organism affects the ease of rupture and therefore the organism type greatly influences disruption. Other considerations include the size of the organism, the culture medium, the growth conditions and the growth phase. There is reduced resistance to disruption in the logarithmic phase of both bacterial and yeast cultures through the increase in the disruption rate constant of actively growing cultures relative to stationary phase cultures (Engler and Robinson, 1981; Sauer *et al.*, 1988; Harrison *et al.*, 1991a). As the cell wall is a major influencing factor of disruption, growth phase impacts resistance to breakage because the growth phase determines the status of the cell wall. The cell disruption technique must be carefully selected in order to maximise the release of the desired intracellular product but minimise cost of downstream processing.

The selection of a cell disruption method involves a number of considerations, including the tolerance of the desired product to conditions used, the extent of product release and the process economics. The processes chosen have an effect on the subsequent downstream process. The downstream process involves the separation and purification of the product taking into consideration the solubility and stability of the desired product and debris as well as the micronisation of insoluble cell debris to obtain a high product yield. The disruption of cells can also alter the product and result in an undesirable high viscosity suspension due to nucleic acid release. Extensive purification schemes may be required to isolate the product from the complex soluble cellular protein milieu released. Therefore, techniques for monitoring the protein purification as well as design considerations for a protein purification scheme must be planned.

2.8 Conclusions

A review of the literature has shown copious information on mechanical breakage. Mechanical methods are currently preferred on a large scale but these are energy intensive, non-selective and cause micronisation of cell debris. There are obvious benefits in researching alternatives to allow for decreased energy consumption and micronisation as well as increased selectivity. The analysis of cell wall compositions and structures allow the main locations of resistance to cell disruption to be identified. This knowledge provides the basis for the choice of pretreatment method due to their specificity to maximise the weakening of the cell wall during permeabilisation.

The pretreatment methods have been used for intracellular release by a variety of methods. The release of intracellular contents may be specific and the process of permeabilisation does not result in fine debris. The results of effective pretreatments found in literature are presented in Table 2.5. The mechanical methods have shown high product release with the different methods. The reduction in the energy usage of these methods is desirable, but not at the cost of reduced product release. The combination of the methods to develop a new technique can be applied for increased release and decreased energy consumption.

The results and findings of the literature give further motivation for continuing research in cell disruption, and the primary objectives of this research are explicitly defined:

- Increase the amount of protein and enzyme released by combining the methods,
- Decrease the amount of energy used for the process while maximising the release,
- Reduce the cost and treatment time of the process,
- Avoid extensive cell fragmentation,
- Selectively release specific enzymes from different locations within the cell.

These objectives can be used to develop the following hypothesis:
Yeast, bacterial and fungal cells are weakened by specific pretreatment methods making them more susceptible to mechanical disruption.

Chapter 3

METHODOLOGY

3.1 Introduction

This thesis centres on interrogating the hypothesis that, following the chemical or enzymatic pretreatment of microbial cells to weaken their cell envelope, improved mechanical disruption will result requiring a reduced energy input and cause less micronisation of cell debris. Through a review of the literature, chemical and enzymatic pretreatments were chosen according to the mechanism of attack, taking into account the structure of the cell used. The cell wall provides most resistant to cell disruption while additional elements of the cell envelope complement its function. Pretreatments were primarily selected from the data and are summarised in Table 3.3. These non-mechanical disruption methods were combined with one of two mechanical methods of cell disruption, high pressure homogenisation (HPH) and hydrodynamic cavitation, to investigate the extent of disruption and subsequent release of intracellular protein.

HPH is most widely used for microbial cell disruption on a commercial scale. It requires a large amount of energy, and subsequent heat removal to avoid denaturation of the product. Hydrodynamic cavitation is a novel cell disruption method providing a promising alternative with energy consumption up to two orders magnitude less than other disruption methods.

The extent of disruption was determined in terms of total soluble protein release, release of enzymes from particular locations and microscopic observation. Cell wall associated, periplasmic and cytoplasmic enzymes were assayed to verify the degree of disruption. The enzymes tested were specific to the organism used. Release rates of the enzymes and protein were calculated.

This chapter details the methodology used. The organisms used and their culture conditions are described in Section 3.2. The mechanical cell disruption equipment used is described in Section 3.3. The chemical methods used for each organism are described in Section 3.4, as well as the experimental approach to combined cell disruption, methods used and analysis of release kinetics are given. In Section 3.5, all analytical procedures, including enzyme assays and their calibration curves, and microscopic methods are detailed. Mixing studies (Section 3.6) on the high pressure homogeniser and cavitation systems are also given to ensure that good mixing and an even distribution of disrupted cells was achieved.

3.2 Microorganisms Studied

3.2.1 Yeast

Baker's yeast (*Saccharomyces cerevisiae*) was obtained from Anchor Yeast (Epping, Cape Town, South Africa). The yeast suspension was obtained as a 'sales' cream, with a concentration of approximately 200g/l dry mass. The yeast was separated from the suspending medium using a Beckman cooling centrifuge (Avanti–J25) with a JA-20 rotor at 7000 rpm for 10 minutes at 20°C. It was washed twice with a 0.025 M sodium phosphate buffer (NaH_2PO_4), pH 7, to remove impurities. The yeast was stored for a maximum of one week at 4°C. All yeast experiments were performed using a 1% cell concentration (wet weight, w/v).

3.2.2 Bacteria

Escherichia coli (E.coli CSH 36) were obtained from the Molecular and Cellular Biology Department of the University of Cape Town (UCT). It was sub-cultured from agar slants using sterile Tryptone Yeast Extract (TYE) growth media containing: Tryptone at 10 g/l, Yeast Extract at 5 g/l and sodium chloride at 5 g/l.

A pre-inoculum was grown on 50 ml of the media for 12 hours at 120 rpm at 37°C. This was used to inoculate 200 ml in 2 litre shake flasks, and shaken for 12 hours at 120 rpm and 37°C. The yield from the shake flask culture prepared under these conditions was approximately 3g from a total of 250ml of media, or 12g/l biomass.

3.3 Cell Disruption Equipment

3.3.1 High Pressure Homogeniser

A Rannie high pressure laboratory homogeniser (Model MINI-LAB, type 8.30 H), manufactured by APV, was used (Figure 3.1 and 3.3). The working pressure range of the apparatus is 0 to 1000 bar (0 to 100 MPa). The capacity of the apparatus is 400 ml and its maximum flow rate is 10 l/h. A minimum test sample size of 120 ml can be used. The homogeniser consists of a two piston set up with a single stage homogenising system including the ceramic Rannie homogenising valve. A schematic diagram of the homogeniser is presented in Figure 3.1. The valve housing contains Stellite ball valves with a flat edge valve unit with an orifice diameter of 3 mm, (Figure 3.2). The cooling water is available at 5l/h.

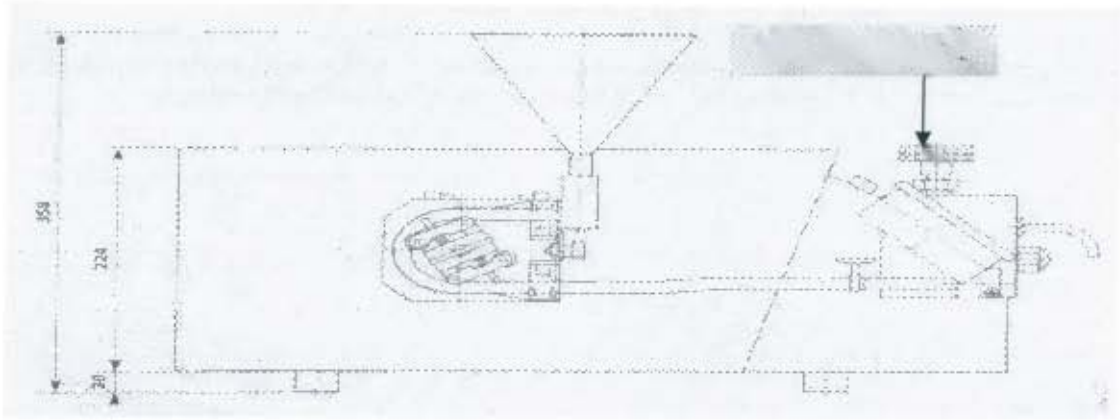


Figure 3.1 Schematic of Rannie HPH used (Model MINI-LAB, type 8.30H)

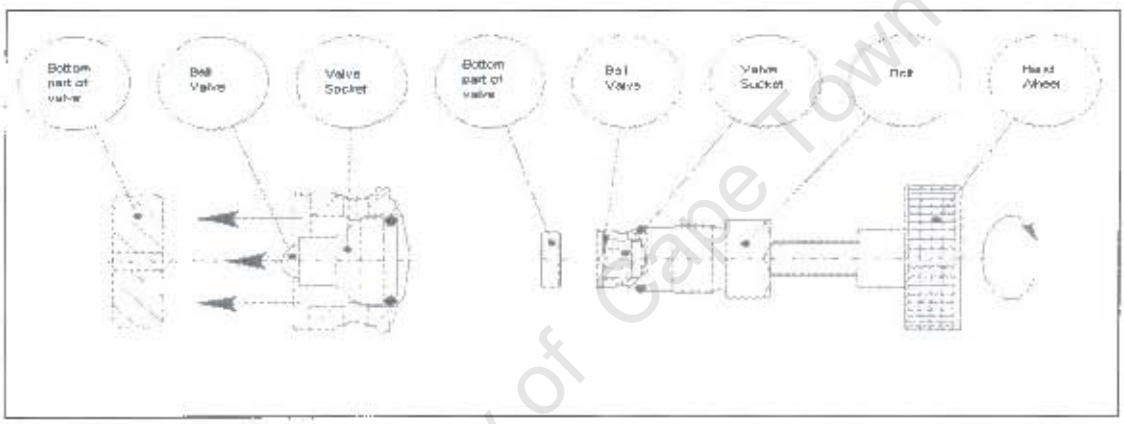


Figure 3.2 The ball valve and valve housing in the HPH used

The energy consumption in a HPH can be calculated using the following equation:

$$E = P.N \qquad \textbf{Equation 3.1}$$

Where E is the amount of energy consumed (MJ/m^3), P is the discharge pressure (MPa) and N is the number of passes used.

Using sample sizes of 300 ml for all experiments, cells were passed through the homogeniser using a continuous flow with recycle for the time equating to the required number of passes, N, that ensured complete cell breakage. Temperature control was necessary in the form of cooling water through the system and ice packs in the holding funnel to maintain the temperature below 37°C to prevent protein denaturation. While temperature is known to enhance cell disruption, it also increases protein denaturation and therefore high temperatures should be avoided. At operating

temperatures higher than 40°C, protein denaturation during disruption is reported (Hetherington *et al.*, 1971).

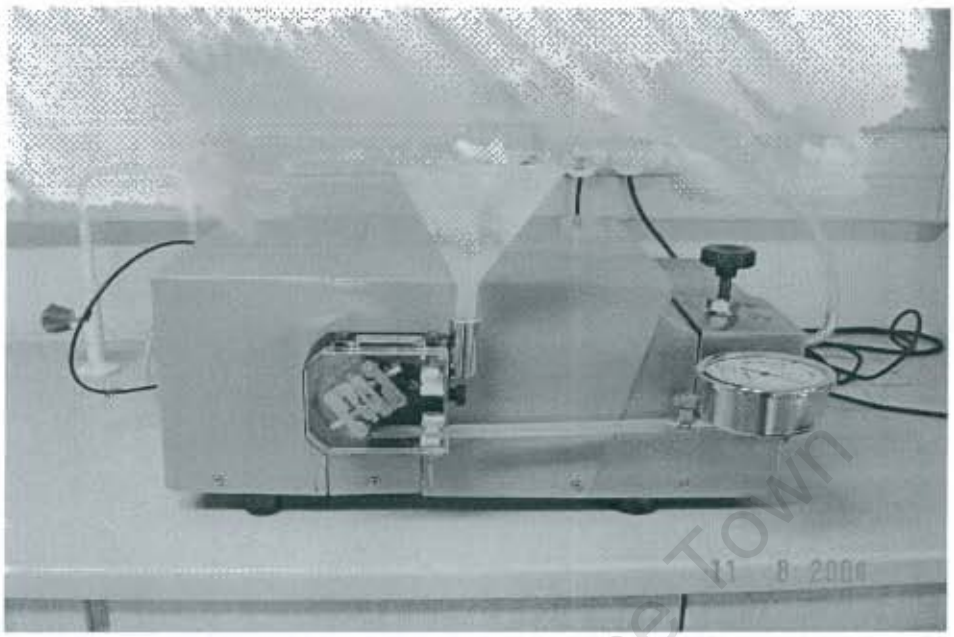


Figure 3.3 Set up of high pressure homogeniser apparatus in the laboratory

Experiments were performed with *Saccharomyces cerevisiae* (Baker's yeast) and *Escherichia coli* using the high pressure homogeniser using pressure ranges of 13.8 to 69.0 and 13.8 to 34.5 MPa respectively. The results of these pressure tests allowed selection of pressure to use in combination with the pretreated cells. Table 3.1 summarises the experiments performed on each microorganism and the pressure which was selected for combination with the pretreatment. All experiments were performed using a 1% cell concentration (wet weight, w/v).

Table 3.1 Operating conditions for the homogeniser for each microorganism

Microorganism	Pressure MPa	Cell concentration (%)	
Yeast	13.8	1	pretreatment
	34.5	1	pretreatment
	69.0	1	
Bacteria	13.8	1	pretreatment
	34.5	1	

3.3.2 Hydrodynamic Cavitation

Hydrodynamic cavitation was only used with yeast, owing to the large quantities of suspension required for the cavitation rig and the ready availability of the yeast. The hydrodynamic cavitation apparatus was made up of a centrifugal multistage high pressure pump (Model 14x7), obtained from Southern Pumps S.A (South Africa). The pump is attached to a 15KW pole Ip55 380/3/50 TFC electric motor. The net positive suction head (NSPH) required was calculated from the pump curve to be 2m and the available NSPH was 5.4m. The available NPSH was greater than that required to prevent the formation of cavities in the pump. Flanges were used to house the orifice plates. A bypass line was available. Pressure gauges were located in the bypass line, before and after the orifice plate. The pressure gauges were placed at these positions to measure the pressures upstream and downstream of the orifice plate to determine the increase in pressure over the plate.

Two orifice plates were used, these being chosen from a range of 10 orifice plates available according to the results reported by Balasundaram (2004) working in the same laboratory. These are detailed in Table 3.2. A 1% cell concentration (wet weight) was used for all experiments (Balasundaram, 2004). The working volume used with the 20 litre holding tank was 18 litres. Cooling coils were immersed in the liquid in the holding tank to maintain the temperature at approximately 32°C by recirculation of chilled water. Maintenance of a lower temperature in the hydrodynamic cavitation rig than in the high pressure homogeniser was achieved. Through recycling of the suspension through the hydrodynamic cavitation set up, the suspension was exposed to 1000 passes, and sampled every 100 passes for analysis.

The extent of cavitation can be measured by the dimensionless cavitation number, calculated according to Equation 3.2:

$$C_V = \frac{(P_3 - P)}{\frac{1}{2}\rho v^2} \qquad \textbf{Equation 3.2}$$

Where P_3 is the recovered downstream pressure (Pa), P is the vapour pressure (Pa), ρ is the density of fluid (kg/m^3) and v is the velocity at throat constriction (m/s). Cavitation inception values are in the range 1 to 2.5. At a C_v below 1, significant

cavitation effects can be detected. The calculated cavitation numbers for the orifice plates used are presented in Table 3.2.

Table 3.2 Orifice plates design and operating conditions

Orifice Plate	Description	Flow Rate (l/s)	Cavitation Number
1	3 mm, 4 orifice	1.32	0.09
2	2 mm, 25 orifice	2.98	0.13

Each orifice plate corresponds to a different flow rate, recovered downstream pressure and cavitation number. The orifice plate geometry affects the intensity of cavitation and therefore, by using two different geometries, two different levels of cavitation intensity were achieved. The lower the cavitation number, the greater the number of cavities formed. In the design of the apparatus, the ratio of the size of the orifice to the inside pipe diameter and flow rate through the pipe must be considered.

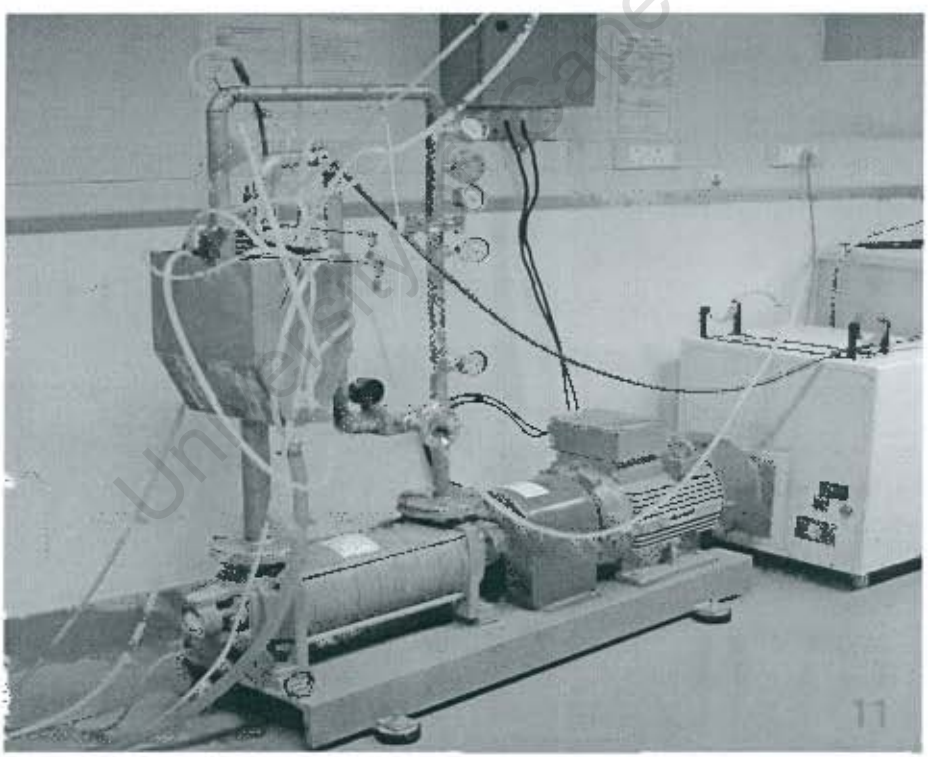


Figure 3.4 Laboratory hydrodynamic cavitation apparatus

3.4 Experimental Methods

The chemical and enzymatic pretreatment methods used were chosen due to their ability to attack specific resistant areas of the cell envelope of the microorganism. The pretreatments were used individually or in combinations. In the latter case, they were chosen such that one permeabilised or weakened the cell wall and another attacked the membrane. All pretreatment methods were first screened to determine optimum concentrations and incubation times through analysis of the release of enzymes and total soluble protein. Selected pretreatments were used in combination with either the HPH or hydrodynamic cavitation. The experimental conditions used to carry out these procedures are detailed in Table 3.3 and 3.4 which show the combination of pretreatments used, the microorganisms attacked and the concentrations of the chemicals used.

Table 3.3 The pretreatments and the range of concentrations tested on each microorganism

Micro-organism	Pretreatments				
		Outer	Concentrations tested	Inner	Concentrations tested
Yeast	1	Ethanol	20, 40, 60, 80%	Toluene	1, 5%
	2	EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	Triton X-100	0.10%
	3	EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	CTAB	0.1, 0.2%
	4	Lyticase	0.01, 0.1, 1 mg/g yeast (wet wt)	-	-
Bacteria	1	EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	-	-
	2	EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	Triton X-100	2%
	3	G-HCl	0.1, 0.5, 1.0, 1.5, 2.5M	Triton X-100	2%

The pretreatments detailed in Table 3.4 were combined with mechanical cell disruption by: HPH (for yeast and bacteria) and hydrodynamic cavitation (for yeast only). The effect of pretreatment, number of passes, operating pressure (HPH) and orifice plate design (cavitation) on the protein and enzyme release was investigated. Combined treatment conditions are summarised in Table 3.5.

Table 3.4 The pretreatments, conditions of experiments and the concentrations selected for combined cell disruption approach for each microorganism

Micro-organism		Outer	Concentration Used	Inner	Concentration Used	Time (minutes)	Temp (°C)
Yeast	1	Ethanol	60%	Toluene	1%	15	30
	2	EDTA	0.020M	Triton X-100	0.10%	60	30
	3	EDTA	0.020M	CTAB	0.10%	15	30
	4	Lyticase	0.1 mg/g yeast (wet wt)	-		120	37
Bacteria	1	EDTA	0.040M	-	-	10	37
	2	EDTA	0.040M	Triton X-100	2%	60	4
	3	G-HCl	0.10M	Triton X-100	2%	60	4

Table 3.5 Combined experiments performed, with operating conditions

Micro-organism	Combination Method					
	Pretreatment	Mechanical Method				
		HPH			HC (C_v = 0.09 and 0.13)	
		Pressure (MPa)	No. of Passes	Temperature (°C)	No. of passes	Temperature (°C)
Yeast	1	13.8	24	15 - 28		
	2	13.8, 34.5	24	19 - 29	1000	
	3	13.8, 34.5	24	20 - 31	1000	18 - 32
	4	13.8, 34.5	24	19 - 26		20 - 31
Bacteria	1	13.8	20	23 - 28		
	2	13.8	20	23 - 28		
	3	13.8	20	22 - 29		

Table 3.6 shows the enzyme assays used for each organism and the location of the enzyme in the cell. The amount of enzyme released from a specific location indicates the extent of disruption of the cell. This can be an indication of the success of the method and provides an insight into its potential for selective release of intracellular compounds.

Table 3.6 Enzymes tested and their location in the cell

Microorganism	Enzymes		
	Cell wall	Periplasmic	Cytoplasmic
Yeast	Invertase	α-glucosidase	Glucose-6-Phosphate Dehydrogenase
Bacteria		Acid Phosphatase	β-galactosidase

3.4.1 High Pressure Homogenisation of Yeast Protocol

A 1% cell suspension (wet weight, w/v) of untreated yeast cells or permeabilised cells was prepared in sodium phosphate buffer solution (0.025M NaH_2PO_4, pH 7.0). This suspension was homogenised at a pressure of 13.8 MPa (2000 psi) for 24 passes and in some instances at 34.5 MPa (5000 psi) as indicated in Table 3.5. Aliquots of 1 ml were taken every 4 passes for analysis. The total soluble protein and the enzymes invertase, α-glucosidase and glucose-6-phosphate dehydrogenase were tested and their releases were measured.

3.4.2 Pretreatment Permeabilisation of Yeast Prior to HPH

A 2% cell suspension (wet weight, w/v) was used for permeabilisation. The final concentrations of chemicals used, time and temperature of incubation are given in Table 3.4. Following the treatment, the suspension was centrifuged at 10000 rpm using the JA-10 Beckman rotor for 10 minutes at 20°C to remove the chemicals. A sample of the supernatant was analysed to determine the amount of enzyme release during the permeabilisation procedure. The total soluble protein was also quantified. The cells were subsequently washed twice with a sodium phosphate buffer (0.025M NaH_2PO_4, pH 7.0) to remove any remaining chemicals prior to further breakage by homogenisation.

3.4.3 Hydrodynamic Cavitation of Yeast Protocol

A 1% suspension of the untreated and permeabilised cells was made up (180g, wet weight, 18 l) in sodium phosphate buffer (0.025M NaH_2PO_4, pH 7.0). This suspension was passed through the cavitation unit, using each orifice plate for 1000 passes. Aliquots of 5 ml were taken every 100 passes for analysis. The release of total soluble protein and the enzymes invertase, α-glucosidase and glucose-6-phosphate dehydrogenase were assayed quantitatively.

3.4.4 Pretreatment Permeabilisation of Yeast Prior to Hydrodynamic Cavitation

A 15% cell suspension (wet weight, w/v) was used for the permeabilisation process. The yeast (300g, wet weight) was suspended with 2 litres of the permeabilising solution. The amounts of chemicals added were calculated to make up the final concentrations specified in Table 3.4 when diluted to an 18 litre sample size. These amounts were added to the 2 litre cell suspension. These cell suspensions were incubated according to the temperatures and times specified in Table 3.4. The suspension was then centrifuged at 10000 rpm using the JA-10 Beckman centrifuge rotor for 10 minutes at 20°C to remove the enzyme. A sample of the supernatant analysed to determine the amount of enzyme release during the permeabilisation procedure. Total soluble protein was also quantified. The cells were subsequently washed five times in a phosphate buffer (0.025M NaH_2PO_4, pH 7.0) and centrifuged to remove any remaining chemicals prior to further breakage by hydrodynamic cavitation. A 1% permeabilised cell suspension was made up (180g, wet weight) and the cells were resuspended in 18 litres of phosphate buffer (0.025M NaH_2PO_4, pH 7.0) and passed through the hydrodynamic cavitation unit.

The reduced sample size used for incubation was selected as it would be too difficult to incubate, agitate, centrifuge and wash 18 litres of suspension. The washing procedure would require 90 litres in total of washing buffer and subsequent separation by centrifugation.

3.4.5 High Pressure Homogenisation of Bacteria Protocol

A 1% cell suspension (wet weight, w/v) of the untreated or permeabilised *E. coli* cells was prepared in sodium phosphate buffer (0.025M NaH_2PO_4, pH 7.0). This suspension was homogenised at a pressure of 13.8 MPa (2000 psi) for 20 passes as indicated in Table 3.5. Aliquots of 1ml were taken every 4 passes for analysis. The

release of total soluble protein and the enzymes acid phosphatase and β-galactosidase were determined.

3.4.6 Pretreatment Permeabilisation of Bacteria Prior to HPH

A 2% *E. coli* cell suspension (wet weight, w/v) was used for permeabilisation. The final concentration of chemicals used in each pretreatment is detailed in Table 3.4. The cell suspension was incubated according to temperatures and times detailed in Table 3.4. The suspension was then centrifuged at 7000 rpm using the JA-10 Beckman centrifuge rotor for 10 minutes at 20°C to remove the chemicals. A sample of supernatant was analysed to determine the amount of enzyme release during the permeabilisation procedure. Total soluble protein was also quantified. The cells were subsequently washed twice in a phosphate buffer (0.025M NaH_2PO_4, pH 7.0) and centrifuged to remove any remaining chemicals prior to further breakage by homogenisation.

3.4.7 Interference of Chemicals

The interference of all the chemicals used was tested. Protein and enzyme containing solutions were prepared by homogenising samples of yeast, bacteria and fungi at the same conditions under which the experiments were performed. The chemical concentrations used in the permeabilisation process were added to the homogenate and these incubated at the temperatures, agitation rates and times used for each particular method. Samples were centrifuged to remove the cell debris and the supernatant was analysed for the enzymes specific to that organism. These results were compared with protein and enzyme concentrations determined in an untreated homogenised sample to determine the fraction of enzymes deactivated and the amount of interference caused by the chemicals. However, direct denaturation and interference was measured on the supernatant only and the results may be compounded by extra material solubilised.

3.5 Analytical Methods

The effect of the pretreatment and the extent of disruption were quantified in terms of the total soluble protein and enzymes released. In order to calculate both the rate and extent of release to quantify disruption, the total protein and specified enzymes present in the supernatant were assayed.

3.5.1 Total Soluble Protein

Total soluble protein was analysed by the Bradford method using Coomassie Brilliant Blue G-250. This exists in the form of two colours. The red form is converted to blue when the dye binds to the protein molecule. The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein. The process is quick, requiring only 2 minutes and the protein-dye complex will remain intact for approximately 1 hour (Bradford, 1976). The Bradford Coomassie Brilliant blue protein-binding dye exists in three forms: cationic, neutral and anionic. Although the anion is not freely present at the dye reagent pH, it is this form that complexes with protein. Dye binding requires a macromolecular form with certain reactive functional groups. The binding behaviour is attributed to Van der Waals forces and hydrophobic interactions (Compton and Jones, 1985).

The assay requires 0.1 ml of the sample and 1 ml of the Coomassie solution to be added together and left to stand for 2 minutes. A bluish colour results if protein is present. The absorbance is read at 595 nm in 1 ml cuvettes. Standard calibration curves were prepared using the same technique and Bovine Serum Albumin (BSA). Dilutions of the samples are required for the absorbance readings to be within the range of the calibration curve. The coefficient of variance for triplicate samples was calculated to be 4.0 %. The method of analysis and calibration curve are presented in Appendix A.

3.5.2 Invertase

Invertase catalyses the hydrolysis of sucrose to glucose and fructose. The activity of invertase is measured by using the DNS (dinitrosalicylic acid) reducing sugar assay to measure the amount of glucose and fructose released during the hydrolysis reaction. The DNS assay is based on the reduction of 3,5-dinitrosalicylic acid by the sugars to 3-amino-5-nitrosalicylic acid which has a strong absorbance at 540 nm. The assay is colorimetric showing a bright orange colour when 3, 5-dinitrosalicylic acid is reduced.

The assay is conducted in:
Sodium acetate buffer (0.1M, pH 5.0) using sucrose as a substrate. The enzyme reaction took place at 55°C for 10 minutes. Following termination, the glucose released was quantified using the DNSA assay and quantified spectrophotometrically at 540nm. One mole of sucrose forms one mole of glucose and one mole of fructose. Therefore, one mole of sucrose utilises two moles 3,5-dinitrosalicyclic acid. The amount of enzyme present is calculated using the standard calibration curve equation, using standard glucose concentrations. The detailed assay protocol and calibration curve are presented in Appendix A. The unit of enzyme activity (U) is defined as 1 micromole of sucrose hydrolysed per minute under the reaction conditions. The coefficient of variance for triplicate samples was calculated to be 6.5 %.

3.5.3 α-glucosidase

The α-glucosidase assay uses p-nitrophenyl α-D-glucosidase as substrate. The enzyme catalyses the hydrolysis reaction of terminal non-reducing 1,4-α-D-glucosides to form α-D-glucose according to the following reaction:

$$p-Nitrophenyl-\alpha-D-Glucosidase \xrightarrow{\alpha-Glucosidase} \alpha-D-Glucose + p-Nitrophenol$$

Equation 3.3

In the assay, 0.1ml sample and 2 ml 5mM p-nitrophenyl α-D-glucoside, made up in NaH_2PO_4 buffer (0.05M, pH 6.8) are mixed. This is incubated at 30°C for 10 minutes.

The reaction is terminated by the addition of 2 ml of 0.1M Na_2CO_3 and the absorbance is read at 410 nm. A calibration curve was prepared using a range of concentrations (0 to 10 moles) of p-nitrophenol solutions. The calibration curve is presented in Appendix A. The unit of enzyme activity is defined as the amount of enzyme that produces 1 mole of p-nitrophenol in one minute. The coefficient of variance for triplicate samples was calculated to be 7.6 %.

3.5.4 Glucose-6-Phosphate Dehydrogenase

The principle behind this enzymatic assay is the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase, with concomitant reduction of the cofactor β-NADP to β-NADPH. The reaction is monitored through the absorbance of β-NADPH at 340 nm. The reaction proceeds as follows:

$$Glu\ \cos\ e-6-Phosphate\ +\ NADP^{+} \xrightarrow{gluose-6-dehydrogen\ ase} Gluconate-6-Phosphate\ +\ H^{+}\ +\ NADPH \qquad \textbf{Equation 3.4}$$

The reaction is carried out adding 1ml of 0.25M Tris-HCl buffer, pH 7.6 to 0.3 ml of 10mM glucose-6-phosphate, 0.2 ml of 0.1M $MgCl_2$, and 0.12 ml of 10mM β–NADP. A 1.38 ml aliquot of the sample or enzyme standard is added to the cuvette and mixed to start the reaction. The absorbance is read at 340 nm every 15 seconds for 2 minutes or more if necessary. One unit of G6PDH catalyses the absorbance by 0.1 units in 1 minute under reaction conditions. The enzyme activity of G6PDH is defined as the conversion of the number of micromoles of substrate, glucose-6-phosphate to D-gluconate-6-phosphate in the presence of NADP, per minute under the assay conditions. The simultaneous reduction of β-NADP to β-NADPH is monitored by measuring the increase in absorbance at 340 nm (Shutte *et al.*, 1983). The coefficient of variance for triplicate samples was calculated to be 4.4 %.

3.5.5 Acid Phosphatase

This enzyme assay catalyses a basic reaction to produce inorganic phosphate and p-nitrophenol from nitrophenyl phosphate. Thereafter, a hydroxide is added, to react

with the p-nitrophenol to remove the phenolic proton and form p-nitrophenolate, which is yellow in colour (Figure 3.5).

For the assay, 0.3 ml 0.1M sodium acetate buffer (pH 5.5), 0.3ml 3.8mM p-nitrophenyl phosphate and 0.3 ml sample are mixed and incubated for 15 minutes at 37°C. The reaction is terminated by the addition of 3 ml 0.2M NaOH. The absorbance of p-nitrophenol is measured at 410 nm. The calibration curve is presented in Appendix A. The enzyme activity is defined as the amount of enzyme that releases 1 micromole of p-nitrophenol in one minute. The coefficient of variance was calculated to be 12.0 %.

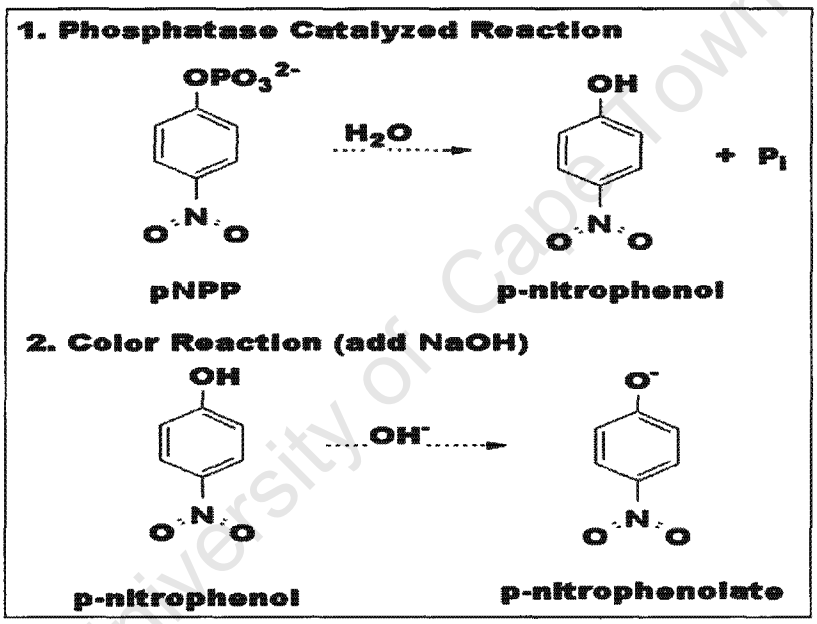


Figure 3.5 **Reactions occurring in acid phosphatase assay (Campbell, Available Online http://www.bio.mtu.edu/campbell/bl482/lectures/lec2/482ex2a.htm)**

3.5.6 β-galactosidase

The enzyme β-galactosidase catalyses the breakdown of lactose (the substrate) into galactose and glucose. These compounds readily enter the glycolytic pathway. The reaction is shown below:

$$Lactose \xrightarrow{\beta-galactosidase} Galactosidase + Glucose \quad \textbf{Equation 3.5}$$

An artificial substrate, ortho-nitrophenyl-β-galactoside (ONPG) is substrate for the enzyme assay. In the presence of β-galactosidase, ONPG is converted to galactose and ortho-nitrophenyl (ONP). ONP is colourless at a neutral or acidic pH, but if alkaline conditions are present, it turns yellow.

For the assay reaction, 0.05 ml of sample is mixed with 2 ml of ONPG prepared in a PPB-Mn buffer at pH 6.6. The mixture is incubated for 5 minutes at 37°C and terminated by the addition of 0.5 ml of 1M Na_2CO_3. The absorbance is read at 420 nm to quantify the ONP released. The unit of enzyme activity is defined as the number of moles of o-nitro phenol produced by the enzyme per minute. The extinction coefficient of ONP is 3.1 $mM^{-1}cm^{-1}$. The coefficient of variance of triplicate samples was calculated to be 4.0 %.

3.5.7 Microscopic Observation

Micrographs of the cells were taken to determine the physical damage to the cell structure caused by the separate pretreatment and mechanical processes. Micrographs were taken using an optical light microscope (Olympus Bx40), with phase contrast optics at 1000 x magnification. The cells were stained with methylene blue and viewed under a light microscope (Olympus Bx40) at 1000 x magnification and photographed. Micrographs were taken before pretreatment, after pretreatment and at stages through the mechanical disruption. These showed the gradual degradation and breakdown of the cell. This allowed a qualitative observation of disruption.

3.6 Mixing Studies

To determine the degree of mixing and mixing time in the holding tanks for the high pressure homogeniser and hydrodynamic cavitation, mixing studies were performed. The mixing studies provide data for the distribution of the cell suspension in the disruption equipment. These were conducted using 2M and 3M KCl tracer solutions. A 1ml aliquot of this tracer was pipetted into the centre of holding tank of each piece of equipment and the conductivity probe was almost completely immersed in the

solution, also at the centre of the tank to measure the conductivity as the salt solution is spread through the system. The conductivity was measured from the time the tracer was added to the point where the conductivity became stable. The mixing time is defined as the time between the injection of the disturbance and the time at which the measured concentration is within 0.1% of the average concentration (Kramers *et al.*, 1953). Conductivity is dependent on temperature and therefore, the temperature had to be maintained in order to evaluate the mixing in the tanks accurately. The expected concentration value was determined by rigorously agitating a system, injecting the same amount of salt and recording the steady state value.

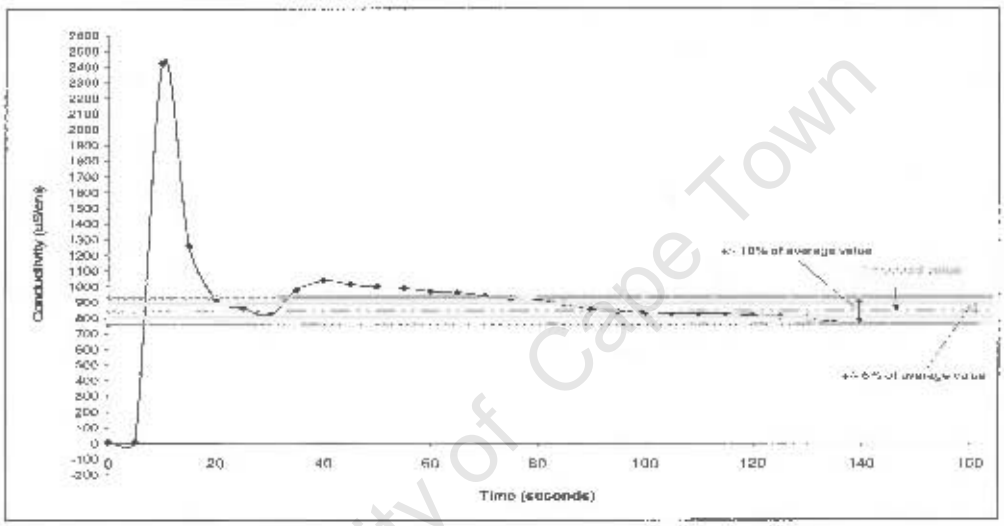


Figure 3.6 Conductivity profile for high pressure homogeniser (300 ml sample with 1 ml of 2M KCl injection)

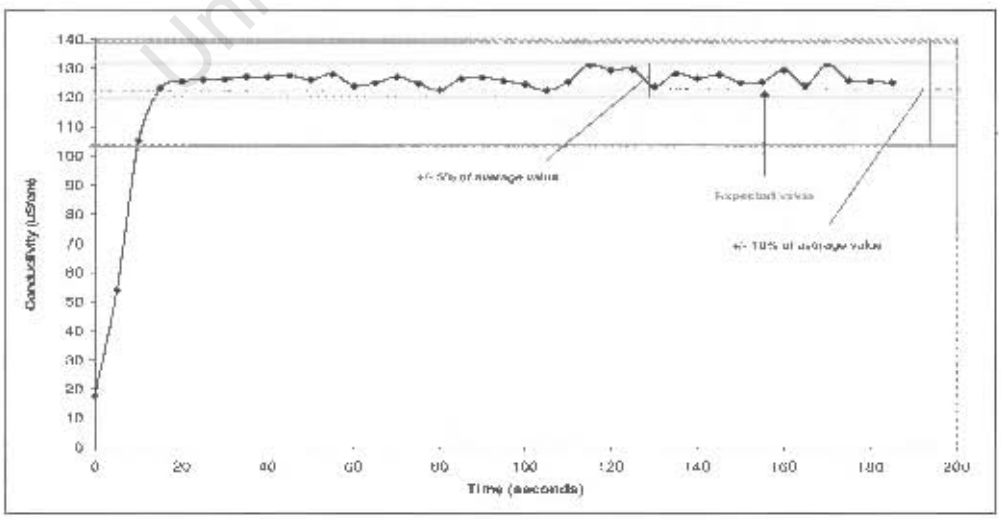


Figure 3.7: Conductivity profile for hydrodynamic cavitation (18 l sample with 5ml of 3M KCl injection)

Figures 3.6 and 3.7 show typical data to determine the mixing time in the high pressure homogeniser and hydrodynamic cavitation rig. The conductivity values measured are very close to the expected conductivity values for a well mixed vessel. This indicates that both vessels are well mixed and there is minimal potential for channelling in each system due to time spent in the circulation lines. The expected value falls within 5 to 10% of the average steady state measured value (Figure 3.6 and 3.7), further supporting the absence of channelling. If channelling had occurred, much larger deviations from the average value, further in the system, would be observed in the conductivity profiles. The time required for the high pressure homogeniser to reach the steady state average value is approximately 45 seconds, and the hydrodynamic cavitation requires 20 seconds.

3.7 Conclusions

The objective of this study is to determine the effect of pretreatments, used in combination with mechanical disruption techniques, on different microorganisms. The pretreatments with potential were chosen from literature and the mechanisms by which these pretreatments worked were specific to the organism's cell structure. A high pressure homogeniser and hydrodynamic cavitation rig were used as the mechanical disruption methods. The methods used to carry out the pretreatments and mechanical breakage of cells were outlined in detail with all reaction conditions. The effect of the pretreatments combined with mechanical methods was measured in terms of release rates of total soluble protein and enzymes from specific locations within the organisms. The rates at which these enzymes and protein were released were also reviewed and calculated using the kinetics of the specific equipment used. Enzyme assays were used to detect the amount of enzyme released after pretreatment and disruption and the enzyme activity was calculated for a quantitative measurement of the disruption and effect of pretreatment. An optical light microscope was used to view the physical cell damage caused by the pretreatment procedure and stages through the mechanical disruption process to observe the gradual cell degradation. Mixing studies were also performed to verify that adequate mixing was taking place in the holding tanks and no dead zones were formed. Good mixing is necessary to ensure that the distribution of cell disruption is even and affects all cells.

Chapter 4

COMBINED PRETREATMENT AND MECHANICAL DISRUPTION

4.1 Introduction

High pressure homogenisation and hydrodynamic cavitation as microbial cell disruption techniques have been introduced and reviewed in detail in Section 2.3. The use of pretreatments in the form of non-mechanical methods that cause permeabilisation or lysis of the microbial cell, have also been reviewed in Section 2.4. The potential of the combination of these two techniques to increase the extent of disruption and amount of intracellular component release, while decreasing the energy consumption for the process has been reviewed in Section 2.6. In Chapter 3, the methodology used to assess this potential is presented. The results of the implementation of these combinations on an experimental level are detailed in this chapter.

The microorganisms used for this study were *Saccharomyces cerevisiae* (Baker's yeast) and *Escherichia coli*. Each microorganism has different cell wall components that are resistant to disruption. These specific components have been identified in Section 2.2. The main structural strength of *Saccharomyces cerevisiae* is given by the outer protein-mannan layer and the inner layer of glucan (Walker, 1998). In bacterial cells, the peptidoglycan

layer provides most resistance to disruption (Engler, 1985). Effective non-mechanical methods of cell permeabilisation or lysis with potential as pretreatment methods were identified from the literature review. The pretreatment methods used were selected based on their ability to cause permeabilisation or weaken the cell envelope. The selected pretreatment methods were studied over a range of concentrations and conditions to ascertain the optimum conditions required for cell envelope weakening or permeabilisation. These pretreatments were assessed in terms of the release of intracellular protein, the release of indicator intracellular enzymes and microscopic observation. A subset of pretreatment conditions was selected to assess the potential of a combination of pretreatment with mechanical disruption through high pressure homogenisation or hydrodynamic cavitation to effect intracellular product release.

Section 4.2 presents the results of the pretreatment and justification for the concentrations of pretreatments used in combination with mechanical disruption equipment. Further, the effect of pretreatments combined with high pressure homogenisation or hydrodynamic cavitation on different microorganisms obtained from experimental studies performed are presented. The extent of release of intracellular components, release kinetics and rates of release of the indicator enzymes and soluble protein are discussed with respect to each pretreatment. Interference of the pretreatment with the assays performed is also discussed. The effect of the combination is also discussed qualitatively by means of phase contrast light microscopy.

4.2 Permeabilisation or Lysis of Microbial Cells Following Chemical Pretreatment

Following selection of the pretreatments from the review of the literature, all pretreatments chosen were tested to confirm permeabilisation of model organisms. Pretreatments were selected to cause chemical permeabilisation or cell envelope weakening. The release of proteins was measured after pretreatment. The chemicals used in the pretreatments were chosen according to their region of attack. In all cases, a combination of chemicals were used, one to attack the cell wall and one to attack the cell membrane. The chemicals used, their position of attack, the range of concentrations tested

and reaction conditions are shown in Table 4.1 for yeast and in Table 4.7 for bacteria. Following treatment, the supernatant was analysed for soluble protein release and release of indicator enzymes. For the pretreatments performed on Baker's yeast, soluble protein and either α-glucosidase or invertase or both were analysed. For pretreatments performed on *E. coli*, soluble protein release and the release of intracellular enzymes were measured. For all the assays, the supernatant contained the pretreatment chemicals. To correct for potential contribution of the chemical to the absorbance reading, the same concentration of chemical was added to the blank. Further potential interference of the chemical present with the protein and enzyme assays is presented in each pretreatment.

Permeabilisation is a procedure which causes small perforations in the cell wall of a microorganism. These perforations may be very small in size and therefore may not allow the passage of proteins into the extracellular medium. The cell envelope is damaged and allows low-molecular weight substances to enter and leave the cell (Galabova *et al.*, 1996). A substrate that is smaller than the perforation could move through the perforation and react to form a product which is released from the cell. Further, the pretreatments may permeabilise or weaken the cell wall, making it easier to disrupt mechanically. The disruption resulting on exposure to mechanical treatment can therefore be improved, by a decrease in energy consumption through lower pressure and reduced exposure to the disruption region. Further, less micronisation may occur with an increase in the release of intracellular compounds.

4.2.1 Chemical Pretreatment of Baker's Yeast

Based on Section 2.5 of the literature review, effective chemical and enzymic treatments for the permeabilisation of Baker's yeast were identified. To determine the appropriate concentration of the chemicals used in combined cell disruption methods, each pretreatment was studied across a range of concentrations, detailed in Table 4.1. Where possible, the lowest chemical concentration was chosen to minimise the amount of chemical requiring removal. The successful pretreatments at the optimum concentrations were used in combination with the high pressure homogeniser and hydrodynamic cavitation.

Table 4.1 Pretreatment Conditions for Baker's Yeast (1% wet weight)

Cell Wall	Concentrations Tested	Cell Membrane	Concentrations Tested	Time (min)	Temp (°C)
Ethanol	20, 40, 60, 80%	Toluene	1, 5%	15	30
EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	Triton X-100	0.10%	60	30
EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	CTAB	0.1, 0.2%	15	30
Lyticase	0.01, 0.1, 1 mg/g yeast (wet wt)	-	-	120	37

4.2.1.1 Ethanol and Toluene

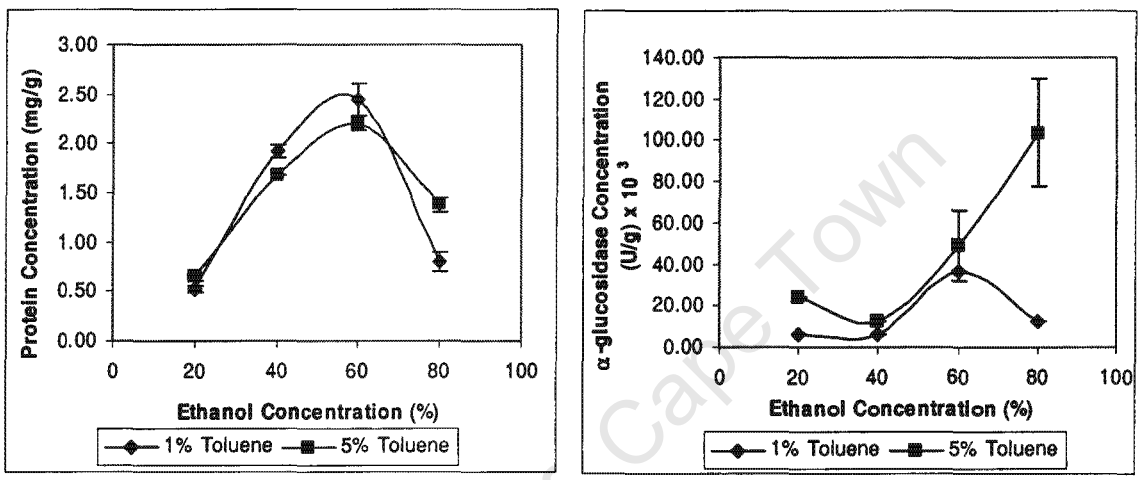
Pretreatment with ethanol and toluene was studied over a wide range of ethanol concentrations, between 20% and 80%, and two toluene concentrations: 1% and 5% (De Smet *et al.*, 1978; Murakami *et al.*, 1980; Fenton, 1982; Decleire *et al.*, 1987; Flores *et al.*, 1994; Novella *et al.*, 1994; Kondo *et al.*, 2000; Chow and Palecek, 2004). For the purposes of analysis, total soluble protein release and the release of α-glucosidase were assayed. The release results are compiled in Table 4.2 and Figure 4.1.

Table 4.2 Release of total soluble protein and α-glucosidase from Baker's yeast (1% wet weight) following pretreatment with ethanol and toluene at 30°C for 15 minutes with agitation at 120 rpm in a shake flask

Ethanol Concentration	1% Toluene		5% Toluene	
	Total Soluble	Periplasmic	Total Soluble	Periplasmic
(%)	Protein (mg/g)	α-glucosidase (U/g) x 10^3	Protein (mg/g)	α-glucosidase (U/g) x 10^3
20	0.52	6.10	0.64	24.4
40	1.92	6.10	1.67	12.2
60	2.45	36.6	2.21	48.8
80	0.80	12.2	1.38	104

Figure 4.1a shows that the release of total soluble protein increases with increasing ethanol concentration to a maximum at 60% ethanol. Thereafter, it decreases as the ethanol concentration is increased further to 80%. The same trend with ethanol concentration is found at toluene concentrations of 1% and 5%. Further, the magnitude of release is very similar, showing that an increase in the toluene concentration had no enhanced effect on protein release. The release of α-glucosidase at a 1% toluene concentration increased with increasing ethanol concentration to a maximum release at 60% ethanol and then release decreased as the ethanol concentration was increased to

80%, in much the same way as protein release occurred (Figure 4.1b). When the toluene concentration was increased to 5%, the α-glucosidase release showed a maximum at 80% and good release at 60%. In summary, increasing ethanol concentration increased the release of cytoplasmic proteins, reaching a maximum at 60%. Increase in toluene concentration beyond 1% had no effect on protein release, but showed increased release of α-glucosidase at 80% ethanol concentration. The concentrations chosen for the ethanol and toluene pretreatment method were 60% and 1% respectively.



(a) (b)

Figure 4.1 Release of total soluble protein and α-glucosidase from Baker's yeast (1% wet weight) during pretreatment with ethanol and toluene (1%) as a function of ethanol concentration at 30°C for 15 minutes with agitation at 120 rpm

4.2.1.2 EDTA and Triton X-100

The EDTA concentrations were varied over the range 0.025M to 0.1M (De Smet *et al.*, 1978; Novella *et al.*, 1994; Bansal-Mutalik and Gaikar, 2003). Triton X-100 was used at a single concentration of 0.1% based on the literature (King *et al.*, 1991; Galabova *et al.*, 1996; Laouar *et al.*, 1996). For the purposes of analysis, total soluble protein, α-glucosidase and invertase release were measured. These data are presented in Table 4.3 and Figure 4.2.

Table 4.3 **Protein and enzyme release from Baker's yeast (1% wet weight) following pretreatment with EDTA and Triton X-100 (0.1%) at 30°C for 1 hour with agitation at 120 rpm in a shake flask**

	Total Soluble	Periplasmic	Cell wall
EDTA Concentration (M)	Protein (mg/g)	α-glucosidase (U/g) x 10^3	Invertase (U/g)
0.025	3.07	12.2	1.24
0.050	2.61	12.2	0.94
0.075	3.05	17.1	0.42
0.100	3.20	6.10	0.20

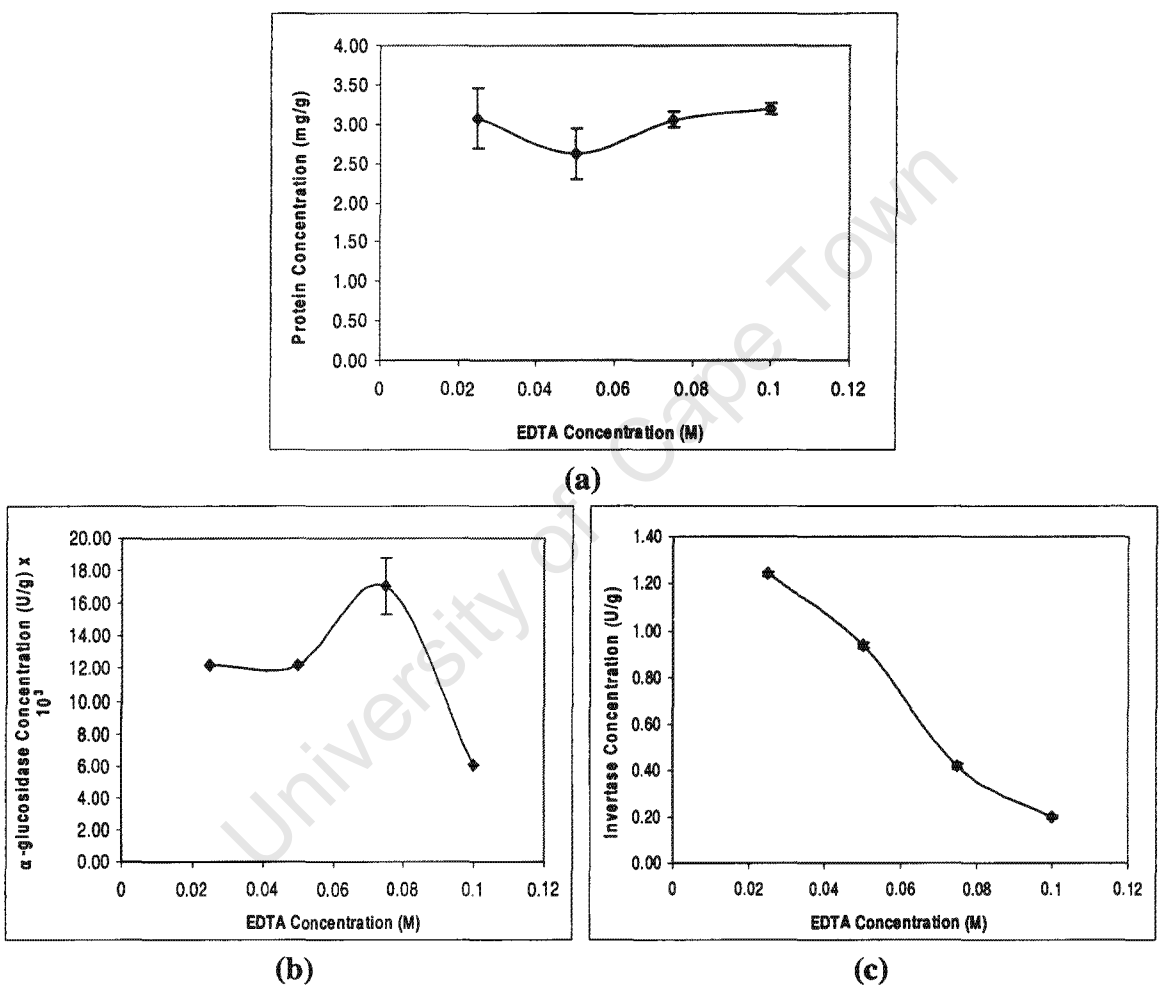


Figure 4.2 **Soluble protein, α-glucosidase and invertase release from Baker's yeast (1% wet weight) during pretreatment with EDTA and Triton X-100 (0.1%) as a function of concentration at 30°C for 1 hour with agitation at 120 rpm**

Figure 4.2a shows a slight decrease in soluble protein release when the EDTA concentration is increased from 0.025M to 0.050M, however, the error bars on these data indicate that this may not be the case. Further increases in the EDTA show little

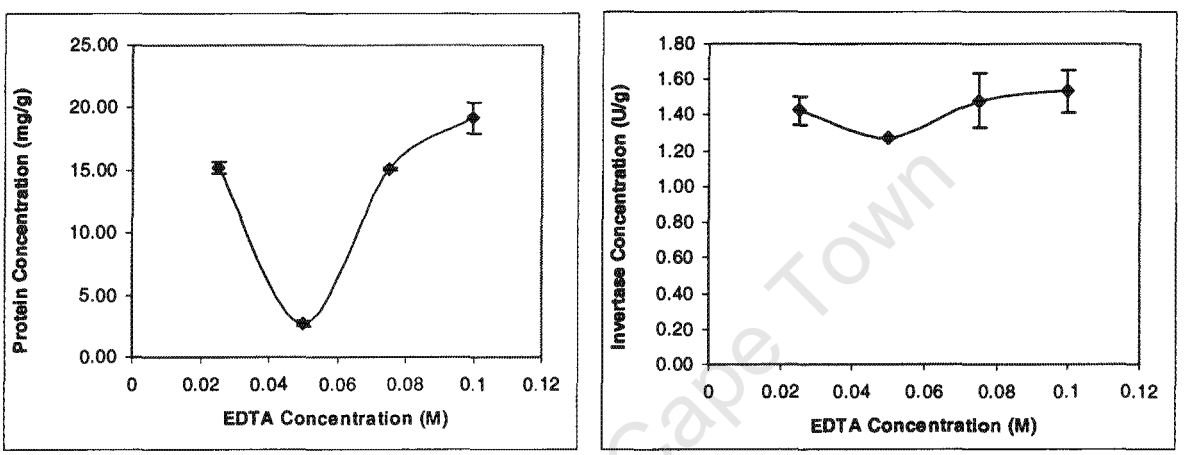
improvement in the release of total soluble protein. This indicates that the effect of increasing EDTA concentration on soluble protein release is not significant. The release of α-glucosidase remained the same when EDTA concentration was increased from 0.025M to 0.050M. Extracellular enzyme activity increased at 0.075M, but decreased as EDTA concentration was further increased to 0.1M (Figure 4.2b). The release of invertase showed an inverse relationship with EDTA concentration (Figure 4.2c). As EDTA concentration was increased, a steady decrease in invertase concentration was observed. In summary, the results showed a maximum invertase release at 0.025M EDTA and a maximum α-glucosidase release at an EDTA concentration of 0.075M. Significant enzyme inhibition of extracellular activity was observed at 0.1M EDTA suggesting the lower concentration would be preferred. Little significant trend is observed for total soluble protein release with increasing EDTA concentration. Owing to the more marked effect on the enzymes studied, at 0.025M EDTA and 0.1% Triton X-100 concentrations were selected for further studies. Selection of 0.075M EDTA was shown to increase α-glucosidase release by 42% while reducing invertase release to 0.33% of the value determined, with little effect on total soluble protein release.

4.2.1.3 EDTA and CTAB

The EDTA concentration was varied over the range 0.025M to 0.1M (De Smet *et al.*, 1978; Novella *et al.*, 1994; Bansal-Mutalik and Gaikar, 2003). The CTAB concentration was tested at two levels, 0.1% and 0.2% (Gowda *et al.*, 1991; Alamae and Jarviste, 1995; Sekhar *et al.*, 1999). Total soluble protein release, α-glucosidase and invertase were measured. No extracellular α-glucosidase was detected, indicating that significant enzyme inhibition has occurred or the enzyme was not released using this method. Testing at 0.2% CTAB revealed negative absorbance values across all assays, indicating that the increased CTAB concentration interfered with the assays such that the release could not be measured. The results of the pretreatment test at 0.1% CTAB are presented in Table 4.4 and Figure 4.3.

Table 4.4 **Protein and invertase release from Baker's yeast (1% wet weight) following pretreatment with EDTA and CTAB (0.1%) as a function of concentration at 30°C for 15 minutes with agitation at 120 rpm**

	Total Soluble	Cell wall
EDTA Concentration (M)	Protein (mg/g)	Invertase (U/g)
0.025	15.3	1.43
0.050	2.72	1.27
0.075	15.1	1.48
0.100	19.2	1.53



(a) (b)

Figure 4.3 **Total soluble protein and invertase release from Baker's yeast (1% wet weight) during pretreatment with EDTA and CTAB (0.1%) as a function of concentration at 30°C for 15 minutes with agitation at 120 rpm**

The release of total soluble protein and invertase followed the same trend with a decrease in release from 0.025M to 0.050M EDTA concentration and then steady increases as EDTA concentration was increased further. A small increase in protein release of 33% was observed on increasing EDTA concentration to 0.1M. The release of invertase showed a slight decrease at 0.050M EDTA concentration and no statistically significant difference in release at 0.025, 0.075 and 0.1M. In summary, to ease further downstream processing in removal of the chemical at a later stage, an EDTA concentration of 0.025M was used since the increased release seen at higher concentrations was limited. The CTAB concentration used was 0.1%.

4.2.1.4 Lyticase

The lyticase pretreatment used enzyme concentrations in the range 0.01 mg enzyme/g yeast to 1.0 mg/g (Knorr *et al.*, 1979, Baldwin and Robinson, 1990, 1993). Total soluble protein was measured to determine the release due to the pretreatment. Two methods were used for enzyme treatment. In the first method, based on Knorr *et al* (1979), the yeast was incubated at various enzyme concentrations in the range 16 to 4000 μg enzyme/g yeast or using an enzyme: yeast ratio ranging from 1:250 to 1:64000 for 2 hours at 37°C. In the second method, taken from Baldwin and Robinson (1990), a 3ml yeast cell suspension (2 mg dry weight/ml) was added to 1 ml of a 0.1mg/ml enzyme solution, 5 ml of a phosphate buffer, pH 7.5 and 1 ml deionised water were added and incubated for 2 hours at 25°C. To minimise the amount of enzyme used, a maximum enzyme concentration of 1.0 mg/g yeast was used and range of concentrations below this was used. The results of these data are presented in Table 4.5 and Figure 4.4.

Table 4.5 Protein release from Baker's yeast (1% wet weight) following pretreatment with lyticase at various concentrations at 37°C for 2 hours with agitation at 100 rpm for Method 1 and at 25°C with gentle shaking for Method 2 in shake flasks

	Method 1	Method 2
Lyticase Concentration (mg/g)	Total Soluble Protein (mg/g)	Total Soluble Protein (mg/g)
0.01	0.59	-
0.10	3.56	-
0.17	-	0.45
0.33	-	2.64
1.00	1.35	3.18

The results of the lyticase testing with method 1 showed that lower enzyme concentrations resulted in better soluble protein release. Further increases in the enzyme concentration decreased total soluble protein release. Maximum release was observed at a concentration of 0.1 mg/g. Protein release with method two showed increased release initially, which eventually slowed down as the enzyme concentration was further increased to 1 mg/g. In summary, the maximum release was observed at 0.1mg/g with method 1 and therefore this concentration and method were used. Due to the expense of such small quantities of the enzyme, lower concentrations were preferred.

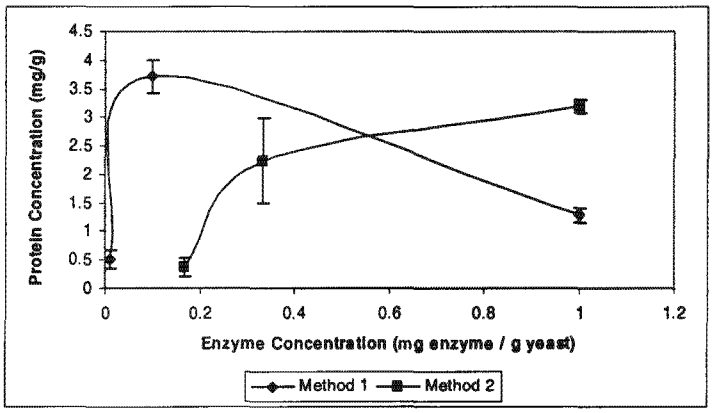


Figure 4.4 Protein release from Baker's yeast (1% wet weight) during pretreatment with lyticase as a function of concentration at 37°C for 2 hours with agitation at 100 rpm

4.2.1.5 Summary of Pretreatment of Baker's yeast

Table 4.6 presents a summary of protein and enzyme release following chemical pretreatment of Baker's yeast. For the pretreatments: ethanol + toluene, EDTA + Triton X-100 and lyticase approximately 3% of the total soluble protein available in the cell was released, whereas, EDTA + CTAB released 15% of the soluble protein. These results indicate that some release has occurred. α-glucosidase release with ethanol + toluene and EDTA + Triton X-100 accounted for approximately 0.1 and 0.03% respectively of the total available, while invertase release using EDTA + Triton X-100 and EDTA + CTAB accounted for 0.01% of the maximum available. Literature results showed higher release, however different enzymes were measured and therefore a comparison cannot be made.

Enzymes from different location within a cell have been released using differential product release (Huang *et al.*, 1991). The cell wall is first removed by enzyme digestion to release the cell wall bound enzymes, followed by disruption of the cytoplasmic membrane to release cytoplasmic components. Huang *et al.* (1991) reported that approximately 11% protein available in *Saccharomyces cerevisiae* is periplasmic. If permeabilisation of the cells has occurred during the testing, most of the periplasmic contents of the cell could be expected to be released under these pretreatment testing conditions. However, the results showed that complete permeabilisation had not occurred since 11% of the periplasmic protein available from permeabilisation has not been released, except EDTA + CTAB.

Table 4.6 Summary of permeabilisation of Baker's yeast following chemical pretreatment

Yeast Pretreatment	Total Soluble Protein (mg/g)	Periplasmic α-glucosidase (U/g) x 10^3	Cell wall Invertase (U/g)	Comments
Ethanol 60%, Toluene 1%	2.45	36.6	-	Similar protein release to EDTA/Triton X-100 and to Lyticase pretreatments, good α-glucosidase release compared to EDTA/Triton X-100
EDTA 0.025M, Triton X-100 0.1%	3.07	12.2	1.24	Protein release comparable to Ethanol/Toluene and lyticase pretreatments, invertase release is similar to EDTA/CTAB pretreatment
EDTA 0.025M, CTAB 0.1%	15.3		1.43	Significant protein release and good invertase release
Lyticase 0.1mg/g	3.56			Similar protein release to Ethanol/Toluene and EDTA/Triton X-100 pretreatments

4.2.2 Chemical Pretreatment of *Escherichia coli*

A review of the literature in Section 2.5 revealed effective chemical and enzymic treatments for the permeabilisation of *Escherichia coli.* Pretreatments were selected and studied across a range of concentrations to determine the optimum condition required for permeabilisation or cell wall weakening. Total soluble protein and marker enzymes (acid phosphatase: periplasm and β-galactosidase: cytoplasm) were measured to determine the effect of the pretreatment. The pretreatments selected and the concentrations tested are presented in Table 4.7.

Table 4.7 Pretreatment Conditions for *Escherichia coli* (1% wet weight)

Cell Wall	Concentrations Tested	Cell Membrane	Concentrations Tested	Time (min)	Temp (°C)
EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	-	-	10	37
EDTA	0.020, 0.040, 0.060, 0.080, 0.1M	Triton X-100	2%	60	4
G-HCl	0.1, 0.5, 1.0, 1.5, 2.5M	Triton X-100	2%	60	4

4.2.2.1 EDTA and EDTA Triton X-100

EDTA was used alone and in combination with Triton X-100, to enhance the permeabilisation or cell envelope weakening. The experimental approach is detailed in Section 3.4.6, based on the literature. EDTA concentrations were varied over the range 0.020M to 0.1M. Where appropriate, 2% Triton X-100 was added (Schnaitman, 1971a,b; Felix 1982; Bansal-Mutalik and Gaikar, 2003). Total soluble protein, acid phosphatase and β-galactosidase were measured to determine the release of proteins. These data are presented in Table 4.8 and Figure 4.5.

Table 4.8 Protein and enzyme release from *Escherichia coli* (1% wet weight) following permeabilisation with EDTA (37°C for 10 minutes with agitation at 120 rpm) and EDTA + Triton X-100 (4°C for 1 hour with intermittent shaking)

	Total Soluble	Periplasmic	Cytoplasmic
EDTA Concentration (M)	Protein (mg/g)	Acid Phosphatase (U/g)	β-galactosidase (U/g)
0.02	2.25	14.2	18.4
0.04	3.58	14.9	28.0
0.04 (+ 2% Triton X-100)	0.77	5.42	0.39
0.06	2.43	4.07	16.6
0.08	1.86	4.07	21.6
0.10	1.00	4.74	5.03

The release of soluble protein and enzymes showed a maximum release at an EDTA concentration of 0.040M. The amounts of soluble protein and β-galactosidase released at EDTA concentrations of 0.020, 0.060 and 0.080 achieved similar values of 60 to 65% of the maximum observed. On increasing EDTA to a concentration of 0.1M, the release of both soluble protein and β-galactosidase is decreased to 28% and 18% of the maximum release respectively. The release of acid phosphatase showed a small increase when EDTA concentration was increased from 0.020M to 0.040M, but further increase in EDTA concentration was accompanied by a much reduced extracellular activity. Hence, 0.040M was chosen as the EDTA concentration.

When the EDTA concentration of 0.040M was used in combination with 2% Triton X-100, the released proteins were all lower than the amounts released by EDTA alone, indicating the interference of Triton X-100 with the protein and enzyme assays,

which caused incorrect measurement of protein release. It is difficult to compare the two methods as they have different conditions, and therefore further experimentation is required to determine if the low release is due to the lower temperature for the combined method or due to the deactivation of proteins by Triton X-100.

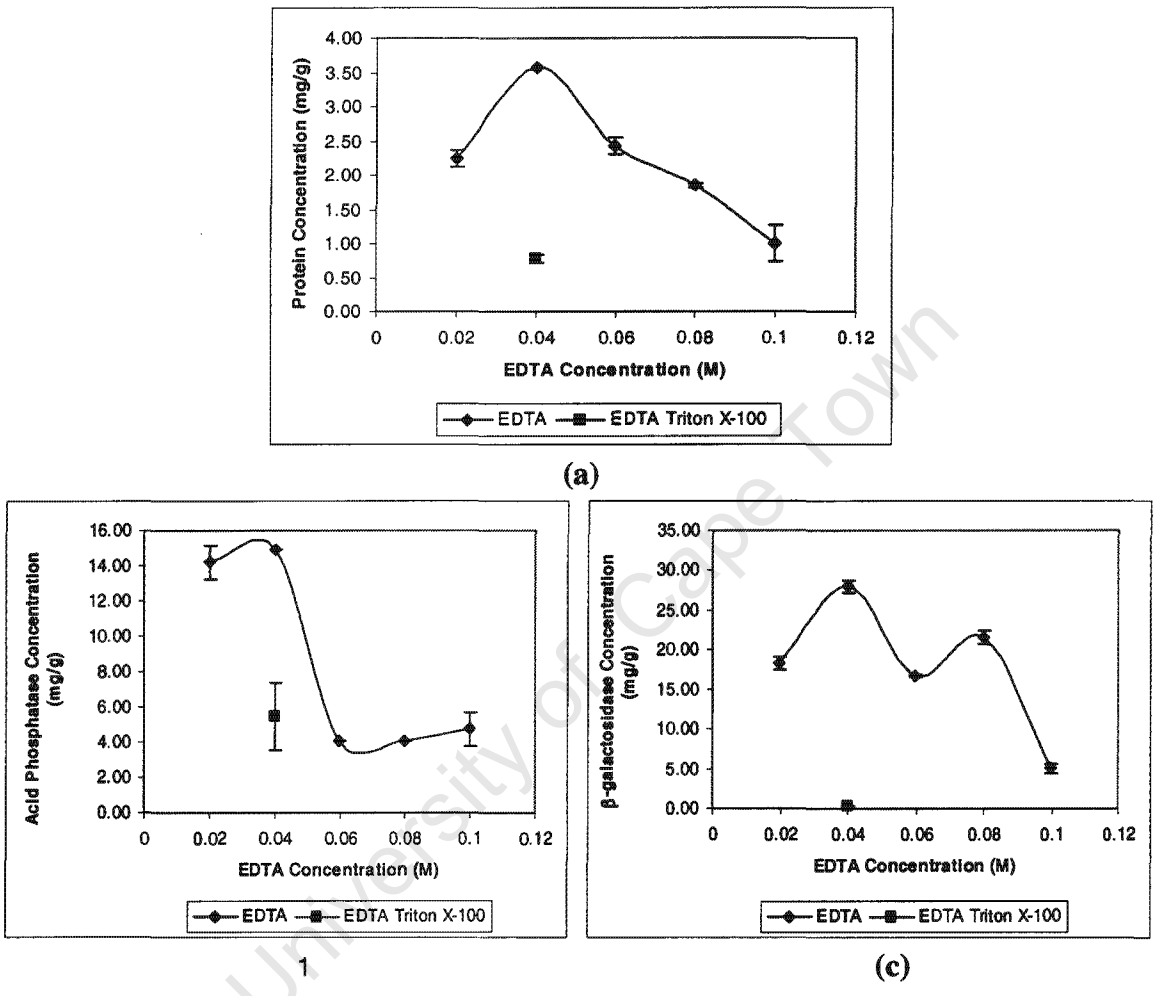


Figure 4.5 **Protein release, acid phosphatase and β-galactosidase from *Escherichia coli* (1% wet weight) during pretreatment with EDTA as a function of concentration (37°C for 10 minutes, agitation at 120 rpm) and EDTA with Triton X-100 (2%) (4°C for 1 hour, intermittent shaking)**

4.2.2.2 G-HCl and Triton X-100

Literature studies report that the main cause of permeabilisation in bacterial cells in the presence of G-HCl and Triton X-100 is due to G-HCl, while Triton X-100 merely acts to slightly enhance the release (Hettwer and Wang, 1989; Naglak and Wang, 1990). The

guanidium hydrochloride concentration was varied in the range 0.1 to 2.5M according to literature recommendations. The Triton X-100 concentration was only used at the optimum reported concentration of 2% (Hettwer and Wang, 1989; Naglak and Wang, 1990). Treatment times of 1 and 2 hours were used. The release of total soluble protein, acid phosphatase and β-galactosidase were measured. These results in presented in Table 4.9 and Figure 4.6.

Table 4.9 Protein and enzyme release from *Escherichia coli* (1% wet weight) following permeabilisation with G-HCl and Triton X-100 (2%) at 4°C for 1 and 2 hours with intermittent shaking

1 hour incubation			
	Total Soluble	Periplasmic	Cytoplasmic
G-HCl Concentration (M)	Protein (mg/g)	Acid Phosphatase (U/g)	β-galactosidase (U/g)
0.10	0.43	395	35.9
0.50	1.53	268	22.3
1.00	2.25	89.0	0.26
1.50	0.00	116	1.03
2.50	12.3	48.8	1.94

2 hour incubation			
G-HCl Concentration (M)	Protein (mg/g)	Acid Phosphatase (U/g)	β-galactosidase (U/g)
0.10	0.68	304	35.0
0.50	1.14	322	21.6
1.00	1.88	88.1	0.13
1.50	0.00	167	1.03
2.50	12.2	49.5	1.48

As the G-HCl concentration is increased, the soluble protein release increased gently from 0.1M to 1.0M, before decreasing to 0 at 1.5M. A sharp increase in release is found at 2.5M (Figure 4.6a). Similar trends were observed by Hettwer and Wang (1989), where a peak in the protein release was achieved with 0.12M G-HCl and 2% Triton X-100, correlating to 45% of the maximum attainable protein release. As G-HCl concentration was increased, a wave like release was noted, where the protein release increased, then decreased and increased again. Contrary to the protein release, the acid phosphatase release decreased with increasing G-HCl concentration from 0.1M to 2.5M (Figure 4.6b). The release of β-galactosidase also showed a steady decline from 0.1M G-HCl to 1.0M G-HCl with little or no release at higher G-HCl concentrations (Figure 4.6c). Protein release following a 2 hour treatment was similar to 1 hour for all proteins measured, therefore the lower treatment time was selected for further use. Since maximum release of acid phosphatase

and β-galactosidase observed at a G-HCl concentration of 0.1M suggested inhibition or denaturation of these enzymes at increased G-HCl concentrations, 0.1M G-HCl was chosen, even though protein release at this concentration was lower. This could be attributed to interference of the Triton X-100 on the soluble protein assay. Literature has shown G-HCl on its own to be effective in permeabilisation, where 10% of the maximum protein available is released at a G-HCl concentration of 0.12M (Hettwer and Wang, 1989).

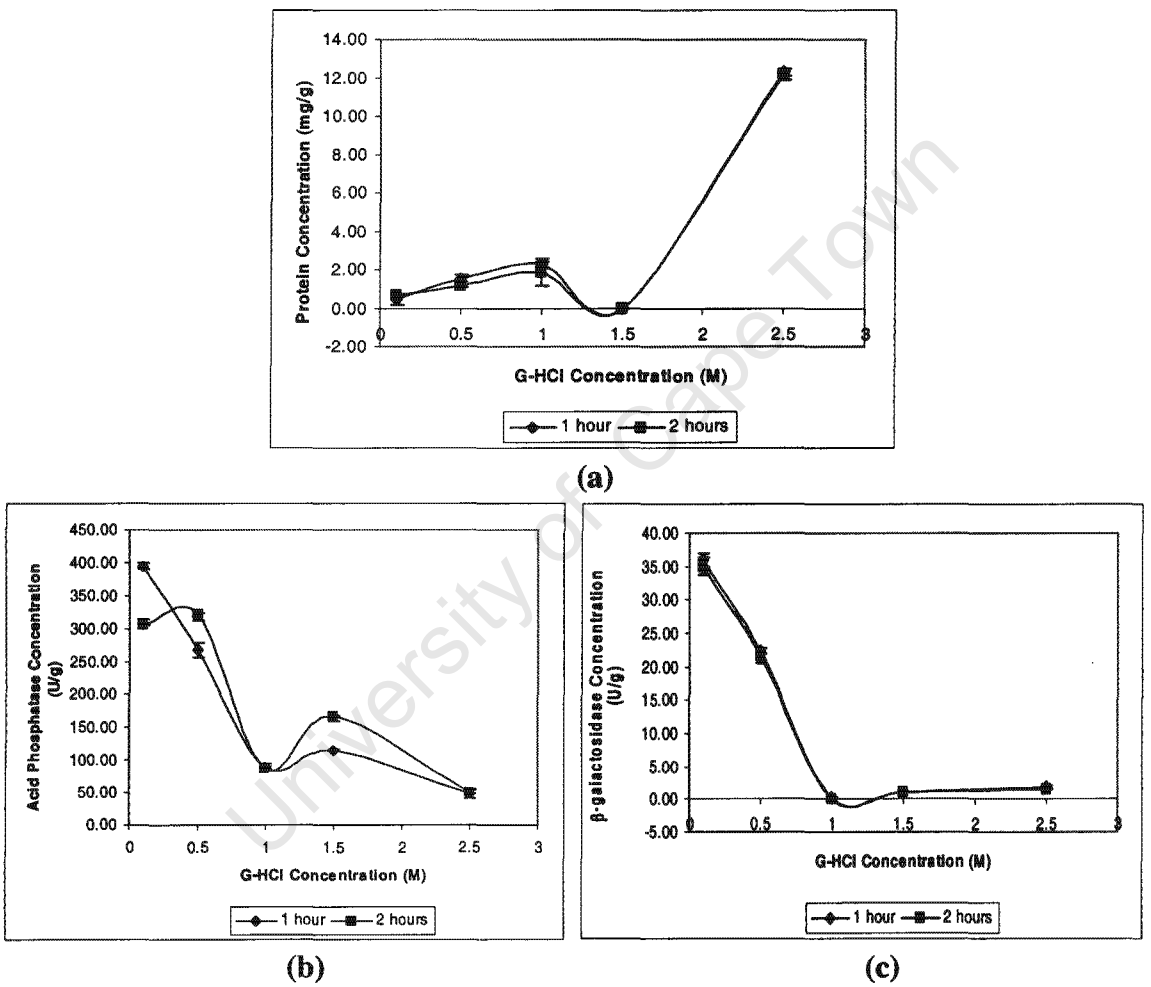


Figure 4.6 **Protein release, acid phosphatase and β-galactosidase from *Escherichia coli* (1% wet weight) following permeabilisation with G-HCl and Triton X-100 (2%) as a function of G-HCl concentration at 4°C for 1 and 2 hours with intermittent shaking**

4.2.2.3 Summary of Pretreatment of bacteria

Table 4.10 presents a summary of protein and enzyme release following chemical pretreatment of bacteria. The total soluble protein released using the pretreatments, was approximately 2.3, 0.5 and 0.3% of the maximum available for EDTA, EDTA + Triton X-100 and G-HCl + Triton X-100 respectively. Similar findings were observed with β-galactosidase at 1.4, 0.02 and 1.8% released. Acid phosphatase release with EDTA and EDTA + Triton X-100 was low at 1.7 and 0.6% respectively, however, 45% of the maximum available was released on treatment with G-HCl and Triton X-100, indicating that cell breakage had occurred. The permeabilisation was expected to release the majority of the periplasmic contents, which accounts for a percentage of the maximum protein available.

Table 4.10 Summary of permeabilisation of *Escherichia coli* following chemical pretreatment

	Total Soluble	Periplasmic	Cytoplasmic	
Bacteria Pretreatment	**Protein (mg/g)**	**Acid Phosphatase (U/g)**	**β-galactosidase (U/g)**	**Comments**
EDTA 0.040M	3.58	14.9	28.0	Good soluble protein, periplasmic and cytoplasmic enzyme release
EDTA 0.040M, Triton X-100 2%	0.77	5.42	0.39	Reduced effect with addition of Triton X-100, possible enzyme inhibition
G-HCl 0.1M, Triton X-100 2%	0.43	395	36.0	Significant acid phosphatase release with good cytoplasmic release, while protein release was lowest of all three pretreatments tested

4.3 Mechanical Microbial Cell Disruption

In order to provide a benchmark for the comparison of combined microbial cell disruption protocol, results obtained on the mechanical disruption of yeast and bacteria by homogenisation in the absence of pretreatment are presented here. Further, the framework of analysis used in further studies is presented.

4.3.1 Disruption of Yeast using the High Pressure Homogeniser

The conditions used in the homogenisation of yeast, including the protein and enzymes assayed to quantify release are listed in Table 4.11. All experiments were performed using a 1% cell concentration (wet weight).

Table 4.11 Experiments performed with 1% cell concentration (wet weight) of Baker's yeast using the High Pressure Homogeniser

Microorganism	Pressure (MPa)	Max. No. of Passes	Proteins Analysed
Baker's yeast (1% wet weight)	13.8	24	Total soluble protein, Invertase, α-glucosidase, G6PDH
	34.5	24	Total soluble protein, Invertase, α-glucosidase, G6PDH
	69.0	4	Total soluble protein, Invertase, α-glucosidase, G6PDH

For comparative purposes, the high pressure homogeniser was operated at three separate pressures: 13.8 MPa, 34.5 MPa and 69.0 MPa. The recirculation of the cell suspension was necessary to produce the number of passes required. Chilled cooling water was used to maintain the temperature of the yeast suspension below 37°C to minimise protein denaturation. The results for yeast disruption prior to chemical treatment in the high pressure homogeniser are presented in Figure 4.8 and Table 4.12.

At a pressure of 13.8 MPa, the soluble protein release was significant reaching a maximum of 112 mg/g wet weight. The maximum soluble protein release of 155 mg/g was not obtained following 24 passes at 13.8 MPa. As the pressure was increased to 34.5 MPa, the soluble protein release increased, reaching 124 mg/g wet weight within 4 passes and a maximum value of 154 mg/g wet weight after 15 passes, by which stage, significant micronisation had occurred.

Further increase in the operating pressure to 69.0 MPa did not result in an increased extent of soluble protein release. It is expected that denaturation of the proteins resulted from increased temperature and shear. Temperature control at higher pressures becomes difficult and temperature spikes above 37°C can occur very quickly, before the cooling can be increased and the temperature reduced. The release rate of protein at a pressure of 69.0 MPa was higher than at 34.5 MPa, with a release of 120 mg/g wet weight being

achieved in 3 passes. Pressure reduces the number of passes required to achieve maximum release, and therefore effective disruption is achieved under these conditions. A maximum release was obtained at a pressure of 34.5 MPa, after approximately 12 passes, where the concentration released at the 12th pass lies within the standard deviation of the average of the concentrations released at the 12th, 16th, 20th and 24th passes.

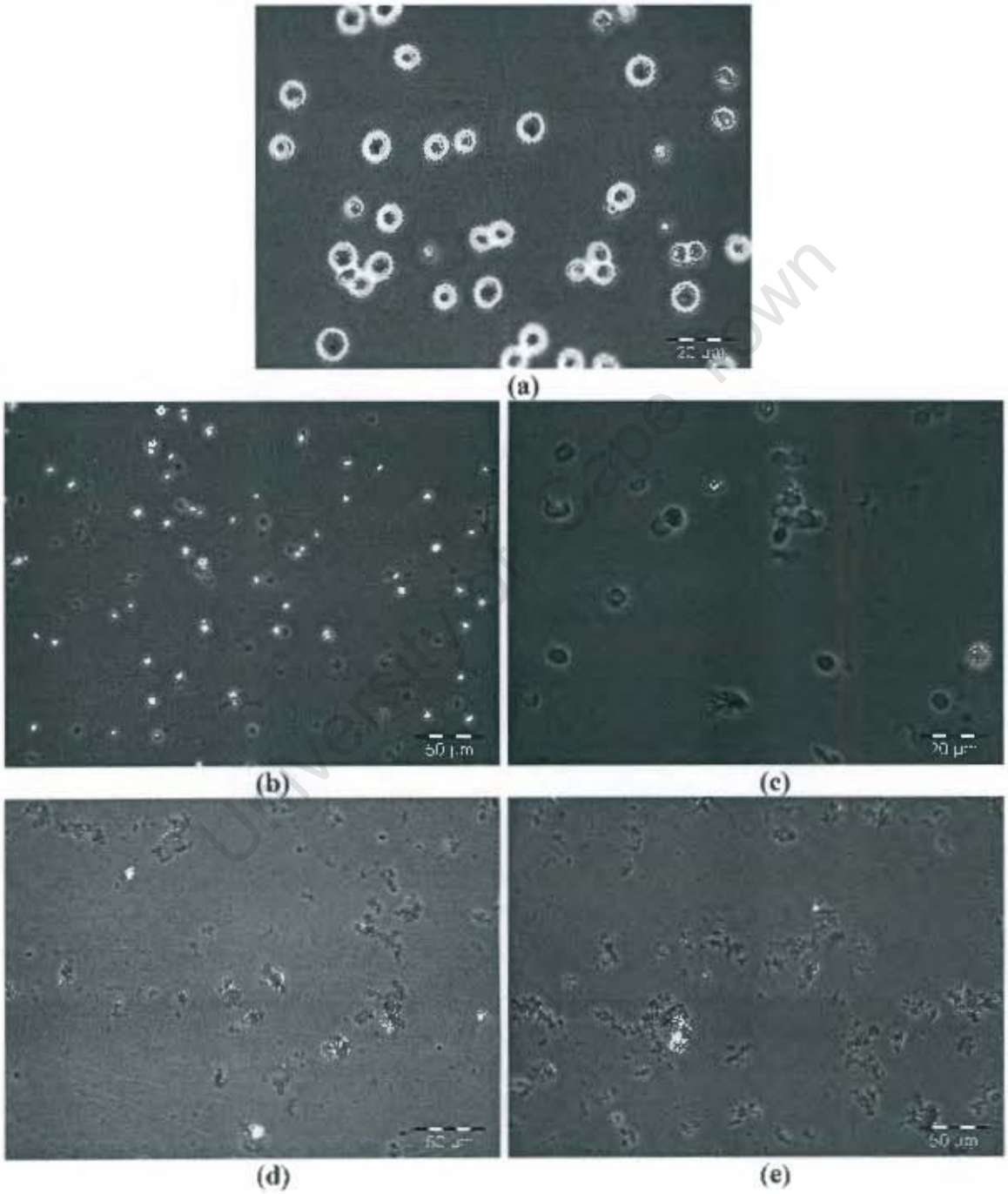


Figure 4.7 **Gradual degradation of Baker's yeast with HPH at 13.8 MPa (a: undisrupted cells, b: cells at 12th pass, c: cells at 24th pass) and 34.5 MPa (d: cells at 12th pass, e: cells at 24th pass showing micronisation)**

Figure 4.7 shows the disruption of Baker's yeast under a pressure of 13.8 MPa and 34.5 MPa observed by light microscopy. Clear intact cells are shown in Figure 4.7a using phase contrast microscopy under 100 x magnification. Cell breakage has occurred by the 12th at 13.8 MPa pass through the homogeniser (Figure 4.7b), but the breakage is partial as intact cells are still present. Figure 4.7c shows broken cells and some cell debris under 40 x magnification. Figure 4.7d and 4.7e shows the disruption of Baker's yeast under 34.5 MPa of pressure. In Figure 4.7d, cell breakage has occurred with a significant amount of cell debris, some micronisation and random intact cells. At the 24th pass, complete cell breakage has occurred with micronisation of cell debris. Micronisation occurs when the cell debris becomes extensively fragmented and membrane bound proteins as well as cytoplasmic proteins are released.

The R_{max} (maximum release available) was determined from the asymptote of release for soluble protein and each enzyme measured. The release profiles of total soluble proteins and enzymes from different locations in the cell were studied. Maximum release of invertase, a cell wall associated enzyme of 1.61 x 10^4 U/g was obtained on 16 passes through the homogeniser at a pressure of 34.5 MPa. At a pressure of 13.8 MPa, 80% of this value had been released after 24 passes. Release at a higher pressure, 69.0 MPa, did not achieve the maximum release, due to denaturation as a result of insufficient temperature control. Cell wall and periplasmic enzymes are expected to be released faster than cytoplasmic enzymes due to their close proximity to the wall, where disruption begins to take place. Maximum release of the periplasmic enzyme, α-glucosidase of 4.38 x 10^7 U/g was achieved in 12 passes through the homogeniser at a pressure of 34.5 MPa. Release achieved at 13.8 MPa was found to be 37% of this maximum value at 1.61 x 10^7 U/g. α-glucosidase release at 69.0 MPa, did not result in maximum release due to denaturation and release was found to be 1.46 x 10^7 U/g. The cytoplasmic enzyme also required 12 passes to achieve maximum release at 34.5 MPa of 7.1 U/g G6PDH. The release achieved at both 13.8 MPa and 69.0 MPa did not achieve this maximum after 24 and 4 passes respectively and the amounts released were 63 and 73% of the R_{max} found for G6PDH respectively.

The extent of release of soluble proteins and enzymes obtained under each pressure after 24 passes for 13.8 and 34.5 MPa and 4 passes for 69.0 MPa were studied and compared to

the R_{max}. These results are presented as percentages of the maximum in Table 4.12 and release profiles are presented in Figure 4.8.

Table 4.12 The release of total soluble protein and enzymes released from Baker's yeast using a 1% cell concentration (wet weight), at various pressures, with cooling to maintain the temperature below 37°C

	Total Soluble		Cell wall		Periplasmic		Cytoplasmic	
HPH Conditions	Protein		Invertase		α-glucosidase		G6PDH	
	mg/g	%	(U/g) x 10^4	%	(U/g) x 10^7	%	(U/g)	%
13.8 MPa, 24 passes	112	72.1	1.29	80.3	1.61	36.7	4.49	63.4
34.5 MPa, 24 passes	151	97.8	1.45	90.2	4.27	97.3	6.97	98.3
69.0 MPa, 4 passes	120	77.7	1.14	70.7	1.46	33.2	5.14	72.6
R_{max}	155		1.61		4.38		7.08	

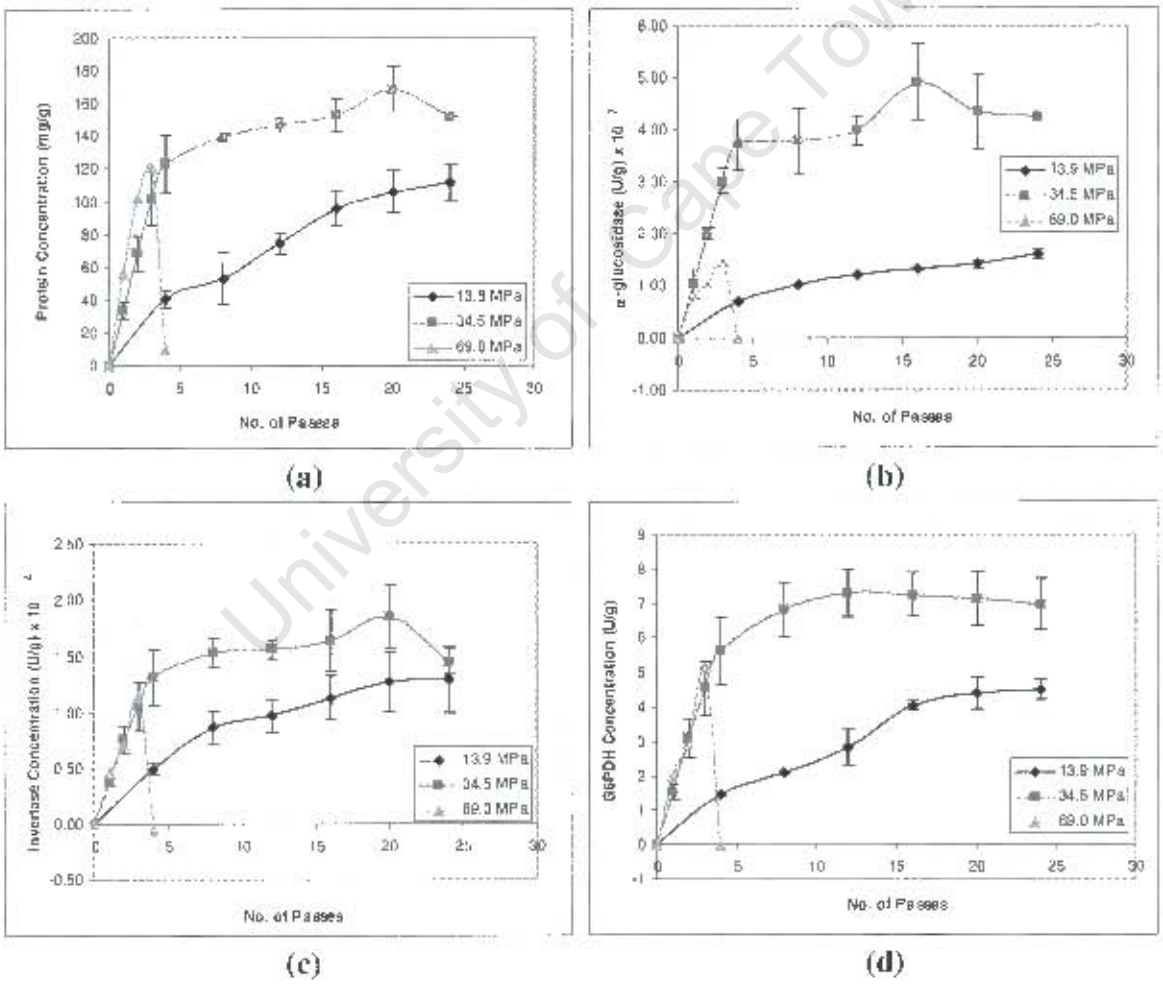


Figure 4.8 The release profile of total soluble protein, α-glucosidase, invertase and G6PDH from a Baker's yeast suspension with a 1% cell concentration in the high pressure homogeniser as a function of pressure

The effects of high pressure homogenisation on a cell occur in two stages, first the cell is ruptured by point forces acting on the cell with release of soluble material. Further shear, turbulent eddies and attrition cause the cell to be disintegrated to release further intracellular contents and the cell debris is fragmented (Harrison, 1990a). Various mechanisms have been proposed for the disruption of cells by HPH, including turbulence, cavitation, impact on solid surfaces and viscous shear (Harrison, 1990a; Keshavarz-Moore *et al.*, 1990a; Middelberg, 1995). Further studies have revealed cavitation, inertial forces and viscous shear to be important mechanisms, however, the clear mechanism remain unproved (Kleinig and Middelberg, 1998). Optimum cell disruption can be achieved at a pressure that releases the maximum amount available in the fewest number of passes possible, without extensive fragmentation of the cell debris or denaturation of the proteins. Temperature control is also necessary to prevent denaturation. Hence, the increase in the soluble protein release rate with increasing temperature can only be exploited in the absence of a labile protein product (Harrison, 1990a).

4.3.2 Disruption of Bacteria using the High Pressure Homogeniser

The experiments performed on bacteria in the high pressure homogeniser are defined in Table 4.13. The experiments were all performed using a 1% (wet weight) cell concentration and two operating pressures: 13.8 MPa and 34.5 MPa. Release of total soluble protein, the periplasmic enzyme acid phosphatase and the cytoplasmic enzyme β-galactosidase from the cell were measured. Results are presented in Table 4.14 and Figure 4.10.

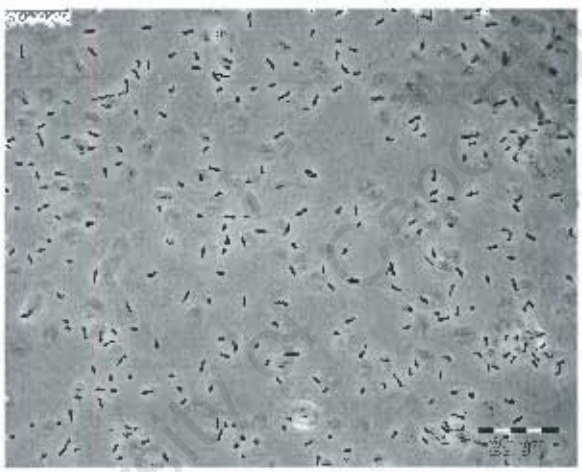
Table 4.13 Experiments performed with 1% cell concentration (wet weight) of *Escherichia coli* using the High Pressure Homogeniser

Microorganism	Pressure (MPa)	No. of Passes	Proteins Analysed
Escherichia coli (1% wet weight)	13.8	20	Total soluble protein, Acid Phosphatase, β-galactosidase
	34.5	20	Total soluble protein, Acid Phosphatase, β-galactosidase

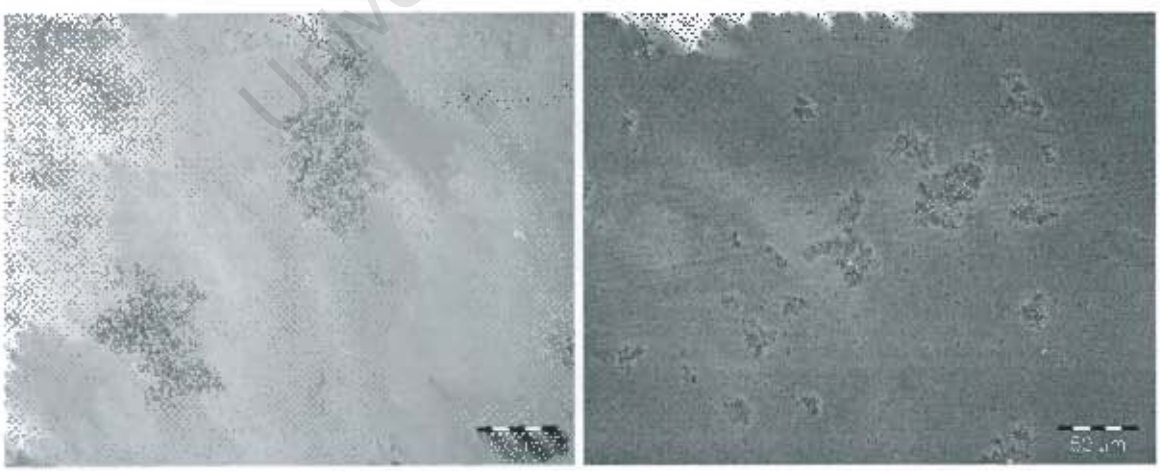
The homogenisation of bacteria required fewer passes than yeast to obtain the maximum protein release to under each condition, as expected owing to less robust cell wall present

in Gram-negative bacteria. At 34.5 MPa, a maximum protein release of 157 mg/g was achieved in approximately 5 passes, whereas at 13.8 MPa, the R_{max} was achieved at 20 passes (Figure 4.10). The number of passes required increased for a lower pressure. The amounts of protein and enzyme released at each pressure are presented in Table 4.14 as a percentage of the maximum available (R_{max}) for release.

Figure 4.9a shows intact bacterial cells viewed under 100 x magnification using phase contrast. Figure 4.9b shows clear cell breakage and cell debris and no intact cells on disruption by 12 passes at 13.8 MPa. Complete cell breakage at 34.5 MPa is seen in Figure 4.9c with no intact cells remaining, with micronisation of cell debris. Therefore, a pressure of 13.8 MPa is sufficient for complete cell breakage of *Escherichia coli*.



(a)



(b) (c)

Figure 4.9 Gradual degradation of bacteria with HPH at 13.8 MPa (a: undisrupted sample, b: cells at 20th pass) and at 34.5 MPa (c: cells at 20th pass)

Table 4.14 **The release of total soluble protein and enzymes released from *Escherichia coli* using a 1% cell concentration (wet weight), at various pressures, with cooling to maintain the temperature below 37°C**

HPH Conditions	Total Soluble Protein		Periplasmic Acid Phosphatase		Cytoplasmic β-galactosidase	
	mg/g	%	(U/g)	%	(U/g)	%
13.8 MPa, 20 passes	156	99.4	437	49.6	1203	60.1
34.5 MPa, 20 passes	157	99.5	888	100	2016	100
R_{max}	157		882		2002	

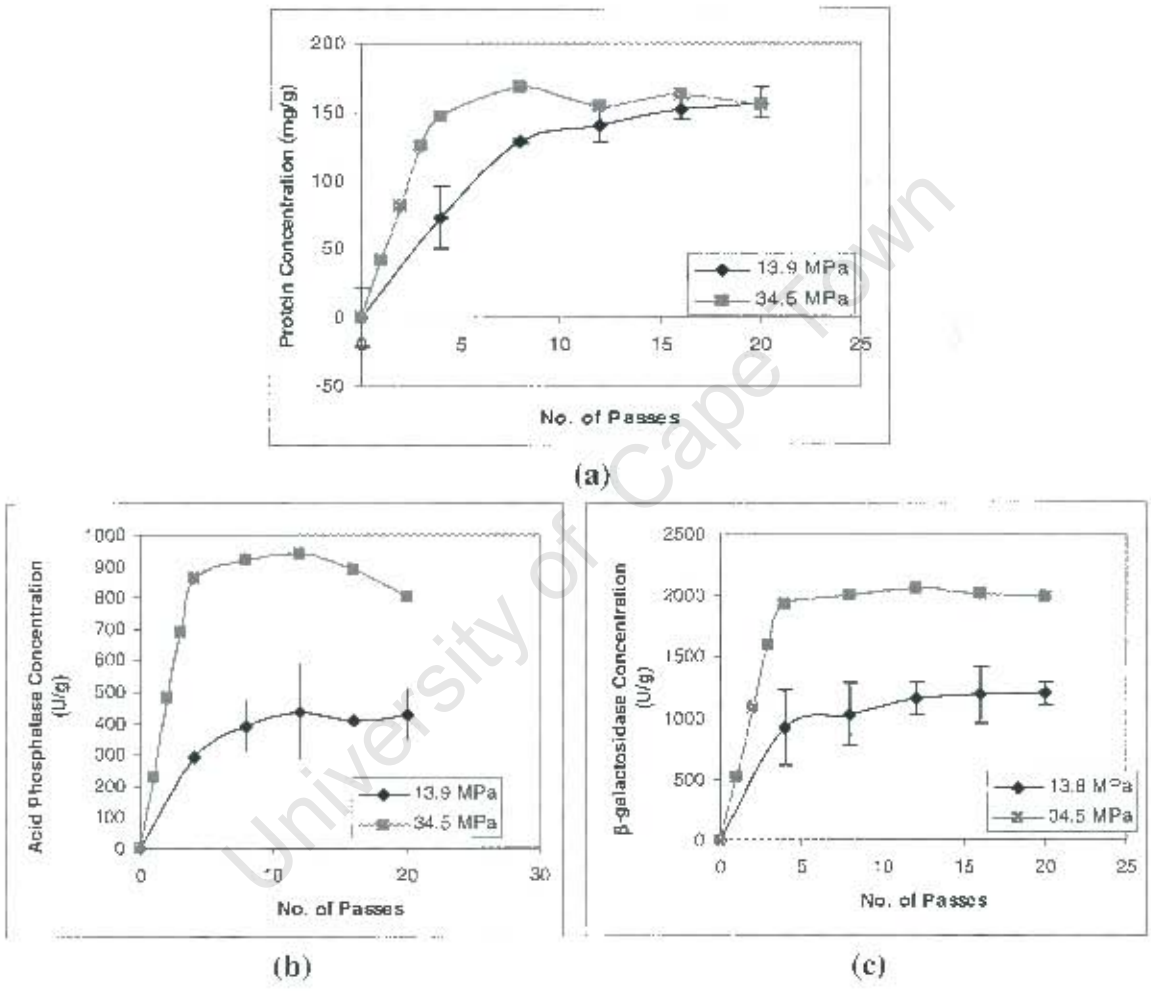


Figure 4.10 **The release profile of total soluble protein, acid phosphatase and β-galactosidase from *Escherichia coli* in the high pressure homogeniser at pressures of 13.8 MPa and 34.5 MPa**

At a pressure of 13.8 MPa, the release of total soluble protein reached 156 mg/g bacteria i.e. R_{max}, while 50.3% of the acid phosphatase and 60.2% of the β-galactosidase was released. Increase in the pressure to 34.5 MPa resulted in the attainment of R_{max} for soluble protein, acid phosphatase and β-galactosidase. Maximum acid phosphatase and β-

release was obtained at approximately 5 passes at 34.5 MPa, while release of these enzymes at 13.8 MPa did not reach the maximum, release of intracellular contents stabilised after 20 passes.

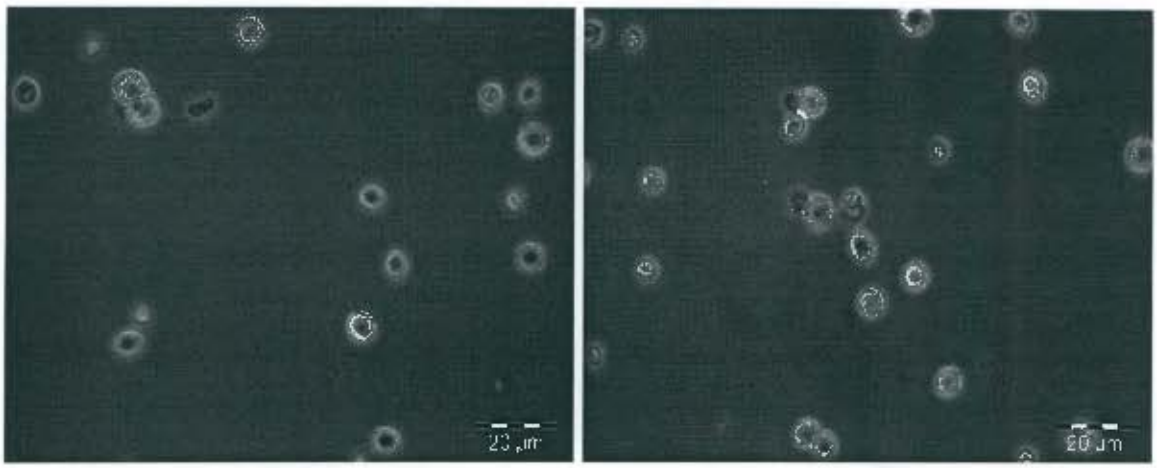
4.3.3 Disruption of Yeast using Hydrodynamic Cavitation

Hydrodynamic cavitation has been used for microbial disruption is reviewed in Section 2.3.2. The intensity of cavitation is measured in terms of a dimensionless number, the cavitation number, C_v and the intensity of cavitation can be varied to cause cell disruption. Each orifice plate corresponds to different cavitation number at a specific flow rate and therefore causes different intensities of cavitation. Hydrodynamic cavitation was tested with the two orifice plates that gave the best disruption results (Balasundaram, 2004). In general, increased cavitation is observed with decreasing cavitation number but at very low cavitation numbers, a maximum is observed. The two plates used fall within a very narrow region, including the maximum ($C_v = 0.13$) and a point just before the maximum ($C_v = 0.09$). The experiments performed on yeast are listed in Table 4.15. The maximum release, R_i achieved under the specific experimental conditions is compared with the maximum available for release from the cell, R_{max}, determined by high pressure homogenisation. These release results are presented in Table 4.16 and the release profiles in Figure 4.13. Figures 4.11 and 4.12 show the breakage of yeast with hydrodynamic cavitation ($C_v = 0.13$).

Table 4.15 Experiments performed with 1% cell concentration (wet weight) of Baker's yeast using the Hydrodynamic Cavitation

Microorganism	Cavitation Number	No. of Passes	Proteins Analysed
Baker's yeast (1% wet weight)	0.09	1000	Total soluble protein, Invertase, α-glucosidase, G6PDH
	0.13	1000	Total soluble protein, Invertase, α-glucosidase, G6PDH

Cell breakage in hydrodynamic cavitation is difficult to observe by phase contrast light microscopy in Figure 4.11. Transmission electron microscopy has revealed that cell damage does occur, which is mainly observed as wall damage rather than rupture (Figure 4.12).



(a) (b)

Figure 4.11 Degradation of Baker's yeast with hydrodynamic cavitation (a: cells at 500th pass, b: cells at 1000th pass)

Figure 4.12 Transmission electron micrograph of yeast (1% cell concentration) disrupted using hydrodynamic cavitation after 1000th passes (Balasundaram, 2004)

The cavitation number is the ratio of forces collapsing cavities to those forces that form cavities. Total collapse pressure is greater of the cavities is greater than the cavity formation forces at low cavitation numbers, resulting in increased intensity of cavitation. Cavitating conditions increase as the cavitation number is decreased, however, the extent of release passes through a maximum is at a cavitation number of 0.13 (Balasundaram, 2004). The increase in release observed as cavitation number is decreased is due to the increase in intensity of cavitation. This increased intensity is a result of reduced flow area

and increased orifice velocity (Balasundaram, 2004). It has been found that the collapse pressure decreases when cavitation intensity increases, and therefore the number of cavities (Gogate and Pandit, 2000).

Table 4.16 The release of total soluble protein and enzymes from Baker's yeast at a 1% cell concentration (wet weight), on hydrodynamic cavitation with cooling to maintain the temperature below 32°C

	Protein (mg/g)		Invertase (U/g) x 10^2		α-glucosidase (U/g) x 10^5		G6PDH (U/g)	
	R_i	%	R_i	%	R_i	%	R_i	%
C_v = 0.09	5.61	3.62	2.57	0.59	0.15	0.09	0.11	1.61
C_v =0.13	6.23	4.03	11.6	2.65	6.68	4.16	0.35	4.93
HPH R_{max}	155		438		161		7.08	

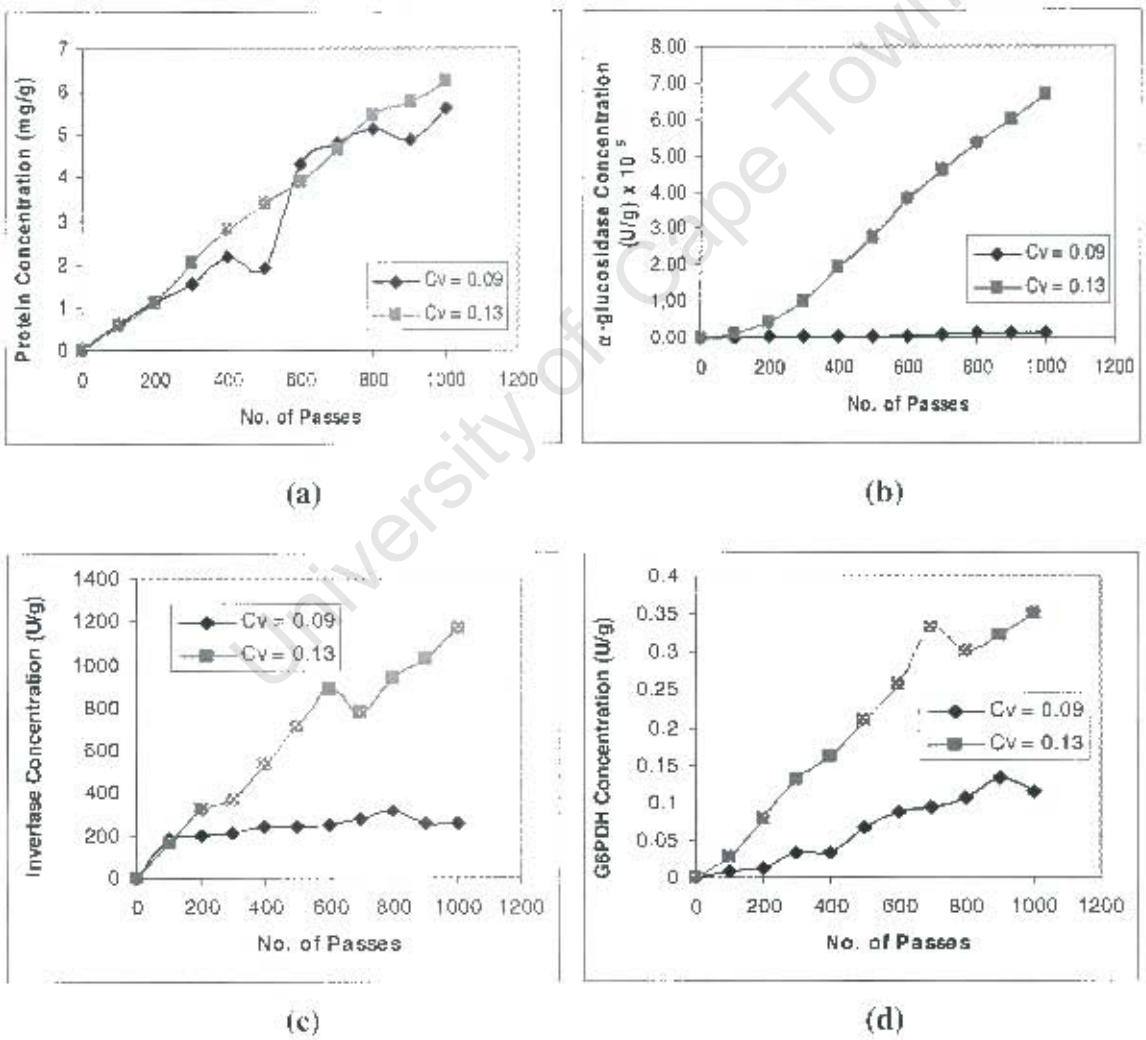


Figure 4.13 The release profile of total soluble protein, α-glucosidase, invertase and G6PDH from hydrodynamic cavitation at cavitation numbers of 0.09 and 0.13

The extent of release of total soluble protein increased from 3.6% to 4.0% of the maximum available as the cavitation number was increased from 0.09 to 0.13. An increase of 2% was noted with invertase release with an increase in the cavitation number, while a 4% increase in α-glucosidase release is seen. The release of G6PDH increased from 1.6% to 4.9% with an increase in the cavitation numbers. The results indicate that a cavitation number of 0.13 releases the intracellular contents to a greater extent than a cavitation number of 0.09.

4.3.4 Energy Efficiency

The energy input for a high pressure homogeniser is calculated using Equation 4.1:

$$E = PN \qquad \textbf{Equation 4.1}$$

where energy, E is measured in terms of MJ/m^3, N is the number of passes used and P is the operating pressure in MPa. Therefore, the number of passes and pressure are direct contributions to the energy input.

Table 4.17 Energy consumption of HPH

	Pressure (MPa)	No. of Passes	Energy (MJ/m^3)
High Pressure Homogenisation	13.8	24	331
	34.5	24	828
	69.0	4	276
Hydrodynamic Cavitation	1.50	1000	1500

4.4 Release Rate Kinetics

The protein release kinetics for different methods of mechanical disruption has been outlined in Section 2.3.1. The use of bead mills, ultrasonication and high pressure homogenisation are common mechanical disruption processes. The release kinetics for high pressure homogenisation was originally studied by Hetherington *et al.* (1971) who described the release kinetics by Equation 4.3. Protein release by high pressure homogenisation is first order with respect to the protein available for release. The equation used to model the protein release through high pressure homogenisation was developed in the following manner:

$$\frac{dR}{dN} = k'R \qquad \textbf{Equation 4.2}$$

Rearranging this equation gives,

$$\frac{dR}{R} = k'dN \qquad \textbf{Equation 4.3}$$

Integrating this equation, one obtains

$$\ln\left(\frac{R_m}{R_m - R}\right) = kP^a N = k'N \qquad \textbf{Equation 4.4}$$

This approach of Hetherington *et al.* in 1971 has been supported through numerous subsequent studies of yeast and bacterial cell disruption (Gray *et al.*, 1972; Limon-Lason *et al.*, 1979; Engler and Robinson, 1981; Keshavarz-Moore *et al.*, 1990a; Harrison *et al.*, 1991c). Equation 4.4 describes the ratio of the protein initially available for release to the amount of protein still available for release at time *t*. R_m is the maximum protein available for release, R is the protein release at time *t*. The variable N refers to the number of passes through the homogeniser, P is the operating pressure and k' (kP^a) is the effective disruption release rate constant, a function of temperature, with units of $pass^{-1}$, which is essentially regarded as unitless unless the passes are converted to time. The exponent a is dependent on the type of organism used for disruption and its conditions of growth.

The release rate kinetics for high pressure homogenisation can be modified and applied to hydrodynamic cavitation. In accordance with the analysis of other mechanical cell disruption methods hydrodynamic cavitation has also shown to follow first order kinetics (Balasundaram, 2004). However, the use of hydrodynamic cavitation for disruption does not attain R_m and therefore, new conditions must be defined for a first order process. An effective maximum, dependent on the method of disruption and the operating conditions, can be defined as R_i (Scholtz-Brown *et al.*, 1997; Scholtz-Brown, 1998). The release rate constants calculated from hydrodynamic cavitation are calculated based on a first order approach to this effective maximum according to Equation 4.5:

$$\ln\left(\frac{R_i}{R_i - R}\right) = k'N \qquad \textbf{Equation 4.5}$$

With the use of a pretreatment, the equation needs to be modified, to incorporate the release of protein during permeabilisation and before passing the suspension through the

high pressure homogeniser. Baldwin and Robinson (1993) modified Hetherington's (1971) equation for enzymatic pretreatment of the cell suspension to the form:

$$\ln\left(\frac{R_m - R_o}{R_m - R}\right) = kP^a N = k'N \qquad \textbf{Equation 4.6}$$

Where R_m is the maximum protein available in the cell for release, R_o is the amount of protein released during the pretreatment, R is the amount of protein released, k the disruption constant, P the operating pressure, N is the number of passes and a is the exponent of pressure. This equation is used when the cells are subjected to mechanical disruption in the same suspension in which they were enzymatically pretreated.

For the use of pretreatments where the pretreatment solution is removed prior to mechanical rupture, the equation needs to be further modified. The protein content of the cell and its release can be described by the following components:

R_o is the protein released during the pretreatment process alone,
R_B is the amount of protein released by mechanical rupture,
R_C is the protein remaining in the cell that has not been released and
R_m is the maximum protein available in a cell for release.

Expressed as fractions, the first three categories sum to unity:

$$\frac{R_O}{R_m} + \frac{R_B}{R_m} + \frac{R_C}{R_m} = 1 \qquad \textbf{Equation 4.7}$$

In the case of the pretreatment procedures used for this study, the cells were pretreated in the chemical medium and the supernatant from this procedure was analysed for the release of proteins. The chemicals were then removed by centrifugation and then the cells were washed to remove any remaining chemicals. Therefore, when the cells were passed through the homogeniser, the amount available for release was no longer R_m, but R_m - R_o. Using the same basis for the development of the equation with the ratio of protein initially available in the cell to the protein still remaining in the cell after partial rupture, the equation describing first order cell disruption becomes modified as follows:

$$\ln\left(\frac{R_m - R_O}{R_m - R_O - R}\right) = kP^a N = k'N \qquad \textbf{Equation 4.8}$$

All experiments in which pretreatments were used were analysed according to Equation 4.8 to determine the release rate of soluble protein as well as individual enzymes. This modified model satisfactorily predicts the release for use with a pretreatment.

Table 4.18 The effective disruption rate (k') and regression coefficients R^2 for protein release by HPH of Baker's yeast

	Protein		Invertase		α-glucosidase		G6PDH	
Yeast – Pressure (MPa)	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**
13.8	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
34.5	252	0.98	394	0.99	207	0.98	341	0.98
69.0	512	1.00	414	1.00	128	0.97	438	0.99

Table 4.18 presents the effective disruption rate constants k' ($pass^{-1}$) found on high pressure homogenisation of yeast according to Equation 4.4, where the linear plot of ln ($R_m/(R_m-R)$) was plotted as a function of number of passes to give a line with a slope of k' in Figure C.1 in Appendix C. As the operating pressure was increased, the release rates of soluble protein, invertase and G6PDH increased. The release rate of α-glucosidase increases when the pressure was increased from 13.8 MPa to 34.5 MPa, but decreased on further increases in the pressure to 69.0 MPa. From Figure 4.8, it is seen that the initial disruption rate constant at 69.0 MPa approaches that at 34.5 MPa. The decrease in the rate of release can be attributed to the denaturation experienced at 69.0 MPa as a result of insufficient temperature control. At 13.8 MPa, the release rate of invertase was the fastest of the proteins studied, as expected due to its location in the cell wall, followed by soluble protein, G6PDH and α-glucosidase. α-glucosidase was released the slowest in all cases, which was unexpected since it is a periplasmic protein and closest to the initial impact of disruption. These release trends do not follow those observed by Follows *et al.* (1971), where cell wall and periplasmic enzymes are release first followed by soluble protein and lastly, cytoplasmic enzymes. However, faster release of specific enzymes is only expected under partial release conditions, which is not possible under high pressures. Further increase in pressure to 34.5 MPa, revealed invertase as the enzyme released fastest, followed by cytoplasmic release, soluble protein and α-glucosidase. At 69.0 MPa, soluble protein, G6PDH, invertase and α-glucosidase was the order in which the proteins and enzymes were released. Clearly, the release of soluble protein and enzymes is not only dependent on their location in the cell but the conditions of disruption such as pressure.

Table 4.19 shows the release rates of soluble protein and enzymes from *Escherichia coli*, analysed according to Equation 4.4, where the linear plot of ln $(R_m/(R_m-R))$ was plotted as a function of number of passes to give a line with a slope of k' in Figure C.4 in Appendix C. The higher pressure of 34.5 MPa revealed higher release rates than 13.8 MPa. At a pressure of 13.8 MPa, soluble protein was released fastest, followed by cytoplasmic enzyme β-galactosidase and the periplasmic enzyme acid phosphatase. At 34.5 MPa, soluble protein was released fastest, followed by acid phosphatase and then β-galactosidase. These trends followed those observed by Follows *et al.* (1971).

Table 4.19 The release rate constant (k') and regression coefficients R^2 for protein release by HPH of *Escherichia coli*

	Protein		Acid Phosphatase		β-galactosidase	
Bacteria – Pressure (MPa)	**k' x 10^{-3}**	**R^2**	**k' x 10^{-3}**	**R^2**	**k' x 10^{-3}**	**R^2**
13.8	244	0.96	73.1	0.96	90.7	0.86
34.5	516	0.94	506	0.96	393	0.98

The release rates for hydrodynamic cavitation were modelled using Equation 4.5 where the linear plot of ln $(R_m/(R_m-R))$ was plotted as a function of number of passes to give a line with a slope of k' in Figure C.5 in Appendix C. Since the R_i is specific to the conditions of each experiment, appropriate R_i values for each treatment method were used for the calculation of the release rate constants. These results are presented in Table 4.20.

Table 4.20 The release rate constant (k') and regression coefficients R^2 for protein release by hydrodynamic cavitation of Baker's yeast

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
Yeast	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**
C_v = 0.09	2.90	0.98	3.70	0.97	1.30	0.95	3.80	0.97
C_v =0.13	1.90	0.97	2.30	0.97	3.30	0.95	2.80	0.96

The release rate constants with cavitation number 0.09 are higher than at a cavitation number of 0.13 for all proteins, with the exception of α-glucosidase. This should be higher due to location in the periplasm which is the region most susceptible to cell disruption, allowing the enzyme to be released. For a cavitation number of 0.09, the invertase and G6PDH are released fastest, followed by soluble protein and α-glucosidase. At a C_v of 0.13, α-glucosidase is released fastest, followed by G6PDH, then invertase and soluble protein. The order of release in both cases does not follow trends observed by Follows *et al.* (1971).

The exponent *a*, is a measure of the relationship between the release rate constant and pressure in a high pressure homogeniser (Hetherington *et al.*, 1971). It was found that the pressure exponent was equal to 2.9 for Baker's yeast. Various different values of *a* reported by are presented in Table 4.21. In these studies, contradicting trends are reported for *a* as a function of the specific growth rate. Therefore, the specific growth rate may influence the resistance to disruption by both *k* and *a*. The resistance to disruption is best seen on comparison of the effective disruption constant, k', (which is a function of growth rate) at a fixed pressure. The exponent relationship can be determined from the slope of the natural log of k' as a function of Ln (P), as presented in Figure 4.14.

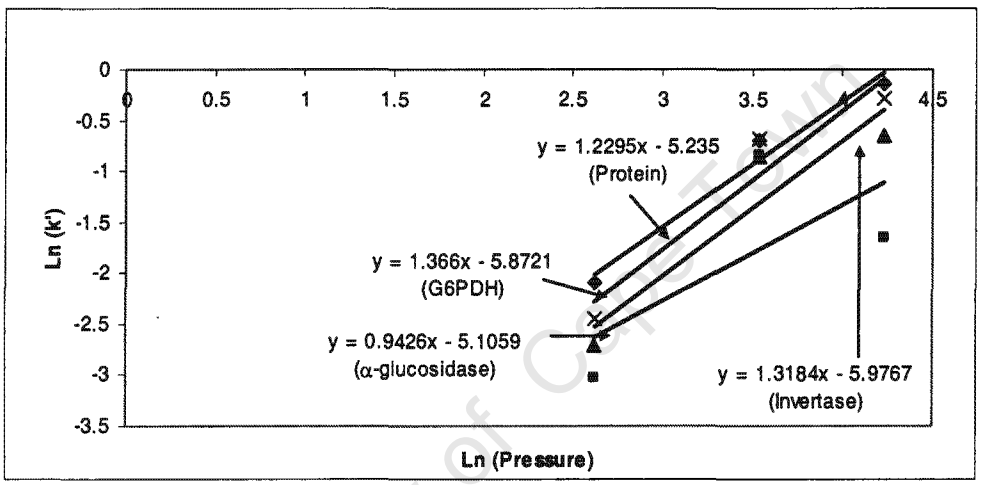


Figure 4.14 The relationship between Ln (k') and Ln (Pressure) to determine the pressure exponent *a* for homogenisation of Baker's yeast

Table 4.21 Pressure exponent *a* values found in literature

Microorganism	Exponent	Pressure (MPa)	k' (MPa^{-a})	Reference
Saccharomyces cerevisiae	2.90			Hetherington *et al.*, 1971
Saccharomyces cerevisiae	1.87	60.0	4.76×10^{-9}	Engler and Robinson, 1981
Ralstonia eutrophus				Harrison *et al.*, 1991c
- exponential growth	3.08	60.0	3.08×10^{-5}	
- stationary phase	1.60 to 1.70	60.0	2.16 to 1.18×10^{-3}	
Escherichia coli	2.20	39.2	0.22	Gray *et al.*, 1972
Escherichia coli	1.43		1.4×10^{-3}	Sauer *et al.*, 1989
Recombinant Escherichia coli	1.41		1.8×10^{-3}	Sauer *et al.*, 1989

Table 4.22 presents the pressure exponent obtained for the release of total soluble protein and individual enzymes from Baker's yeast on homogenisation in the absence of pretreatment. Through the R^2 value, the quality of the fit is assessed. The values for the

exponent found for α-glucosidase and invertase were fairly inaccurate with regression coefficients of 0.47 and 0.83. The exponents found for invertase, G6PDH and total soluble protein are similar: 1.23 for soluble protein, 1.37 for G6PDH and 1.32 for invertase. These values differ from those found for yeast by Hetherington *et al.* (1971) and Engler and Robinson (1981) of 2.90 and 1.87 respectively (Table 4.21). The variation in the exponent reported in literature may be due to different operating conditions, which affect the rate and extent of disruption. Therefore, the value of the exponent *a* requires further investigation.

Table 4.22 The determination of exponent *a* and the regression coefficients (R^2) on homogenisation of Baker's yeast

	Protein	Invertase	α-glucosidase	G6PDH
a	1.23	1.32	0.94	1.37
R^2	0.97	0.88	0.47	0.93

4.5 Effect of Pretreatment Combined with High Pressure Homogenisation on Disruption of Baker's Yeast

Following the selection of chemical pretreatments to permeabilise and weaken the cell envelope and the assessment of appropriate chemical concentrations to be used, cell disruption was studies through the combination of pretreatments with high pressure homogenisation. Table 4.6 provides a summary of the selected pretreatments, the concentrations chosen and the release of proteins achieved at those concentrations for Baker's yeast. The objectives of these studies were to increase the extent of disruption and the rate of intracellular release of proteins while decreasing the energy requirements and micronisation of cell debris.

The release of proteins was measured after pretreatment and subsequently at every fourth pass during homogenisation for a pressure of 13.8 MPa. On using a pressure of 34.5 MPa, measurements were made every pass for the first four passes and thereafter every fourth pass. A low operating pressure of 13.8 MPa was selected to study combined cell disruption using high pressure homogenisation, as it was expected that the permeabilisation or weakening of the cell envelope would result in increased release of intracellular protein at a low pressure in comparison to untreated yeast at the same

pressure. Use of the lower pressure would therefore ensure sensitivity to observe differences between experimental conditions. It was expected that the use of pretreatment in combination with high pressure homogenisation would enable similar disruption at a lower pressure when compared with homogenisation of untreated yeast. The lower pressure would result in lower energy consumption and less micronisation of cell debris.

A mass balance can be performed for all experiments using pretreatments combined with high pressure homogenisation in the following manner:

$$R_H = R_o + R_B + R_D \qquad \textbf{Equation 4.9}$$

R_H is the total amount of protein released from the yeast cells by the combined cell disruption process, R_o is the amount of protein released during pretreatment process, R_B is the amount of protein released by high pressure homogenisation after pretreatment and R_D is the amount of protein denatured.

4.5.1 Yeast Cell Disruption by HPH Following Pretreatment with Ethanol and Toluene

4.5.1.1 Extent of Release

Ethanol is known to inhibit the cross linking in the structural components of the cell wall while toluene causes disorganisation of the cell membrane. The concentrations selected for pretreatment were 60% and 1% respectively (Section 4.2.1.1). Proteins were released into the supernatant during the pretreatment procedure, with small amounts of α-glucosidase and invertase, indicating that permeabilisation had occurred since periplasmic and cell wall enzymes were released. Table 4.23 presents the results of the ethanol and toluene pretreatment used in combination with high pressure homogenisation at 13.8 MPa. The data presented above has been averaged from triplicate experiments, with a standard deviation of 0.03 mg/g for protein and coefficient of variance of 1.1%. The extent of release measured with the combination is compared to the maximum available in the cell for release from untreated yeast, R_{max}. Figure 4.20 presents the release profiles achieved for this combination, where it can be compared with untreated yeast and

all other pretreatment combinations used on Baker's yeast homogenised at 13.8 MPa. The raw data generated can be found in Appendix B.

The total protein release measured following the combination was 5.4 mg/g whereas 112 mg/g was released on homogenisation of untreated yeast at 13.8 MPa, indicating a significant protein loss. A similar proportion of all enzymes tested were lost on pretreatment, with a 72 and 99% invertase and α-glucosidase loss respectively. The maximum amount of invertase released by homogenisation following pretreatment was 2790 U/g yeast. Prior to this, 660 U/g yeast was released during the permeabilisation. The combined release accounts for 21.5% of the amount available for release. The total amount of α-glucosidase released was 1.31 x 10^5 U/g yeast, which is 0.3% of the maximum available. It is postulated that the rest of the proteins were denatured by chemicals. Glucose-6-phosphate dehydrogenase is a cytoplasmic enzyme, which was not released during the pretreatment procedure. Further, no G6PDH release was observed after pretreatment and homogenisation. This suggests that denaturation of the enzymes by the chemicals had occurred.

Table 4.23 Protein release following ethanol (60%) and toluene (1%) pretreatment and homogenisation of Baker's yeast with a comparison of the release to R_{max}

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	mg/g	%	(U/g) x 10^3	%	(U/g) x 10^5	%	(U/g)	%
Pretreatment	1.97		0.66		0.46		0.00	
HPH (13.8 MPa, 24 pass) of pretreated cells	3.47		2.79		0.85		0.00	
TOTAL	5.44	3.51	3.45	21.47	1.31	0.30	0.00	0.00
R_i at 13.8 MPa	**112**	**72.1**	**11.3**	**70.2**	**141**	**32.2**	**2.81**	**40.0**
R_{max}	**155**		**16.1**		**438**		**7.08**	

While a thorough washing protocol was used after the pretreatment to remove all solvents, it is possible that the chemicals penetrated and remained within the cell, adhering to portions of the cell during the homogenisation and after the washing steps, causing the denaturation of these proteins. The adverse effect of ethanol and toluene on proteins was confirmed by testing their effect on the enzymes, with results presented in Section 4.5.1.2.

4.5.1.2 Interference of the Chemicals

The interference of ethanol and toluene in determining release of total soluble protein and specific enzymes was determined by treating the homogenate prepared by homogenisation at 13.8 MPa at the chemical concentrations used in the pretreatments for their specific treatment times. Samples of the homogenate were then centrifuged to remove the cell debris and the supernatant was analysed for protein content and enzyme activity.

The difference in the amount of protein measured in the homogenate prior to and post exposure to chemicals gives an indication of interference protein denaturation by the chemicals. Table 4.24 presents the relative soluble protein and enzymes detected using this method.

During pretreatment testing, the assays performed on the supernatant contained the chemicals used. To correct for potential contribution of the chemical to the absorbance reading, the same concentration of chemical was added to the blank. This method of analysis does not eliminate the deactivation or precipitation of the protein, but does correct for any contribution of the chemical to the absorbance reading.

Table 4.24 Interference of ethanol and toluene on protein measurement

	Untreated Yeast (R_i) 13.8 MPa	Ethanol and Toluene	% Denatured
Protein (mg/g)	112	5.51	95.1
Invertase (U/g) x 10^4	1.29	0.07	94.8
α-glucosidase (U/g) x 10^5	161	0.06	99.9
G6PDH (U/g)	7.08	0.00	100

Table 4.24 shows that the addition of ethanol and toluene to a cell homogenate resulted in significant denaturation or inactivation of the enzymes. The resultant measurements in the presence of the chemicals correlate well with the amounts released during the high pressure homogenisation of the pretreated cells presented in Table 4.23. It can therefore be concluded that the use of ethanol and toluene as a pretreatment to enhance intracellular release in subsequent high pressure homogenisation results in denaturation, in excess of 90% of the proteins and is therefore not a viable pretreatment method.

The Bradford protein assay is often used for protein determination because it is quick and reliable. Interference of the Bradford assay is mostly caused by detergents. Solvent interference has not been reported in literature. A concentration of 95% ethanol has been reported to cause no interference with the assay (Bradford, 1976). It is possible that these results differ from those obtained on measurement of protein in the presence of 60% ethanol due to differing reaction conditions such as temperature and pH. The Bradford assay measures bovine serum albumin (BSA) as the protein while protein measurements in the experiment were performed to determine the soluble protein produced by Baker's yeast.

4.5.1.3 Release Rate Kinetics

The release rate kinetics for the high pressure homogenisation of treated cells has been discussed in Section 4.4 and Equation 4.8 was used to model these kinetics. The release rates of all proteins and enzymes from the treated cells were compared to those of the untreated yeast at the same pressure. Table 4.25 presents the release rates calculated.

The release rate kinetics for the pretreated cells homogenised at 13.8 MPa do not exceed those of untreated yeast at 13.8 MPa due to the large amounts of protein deactivation. These release rates are therefore provided as further confirmation of the significant deactivation caused by ethanol and toluene as a combination pretreatment method. The release rates will therefore not be discussed further in terms of order of release or rate.

Table 4.25 Release rate constants (k') and regression coefficients R^2 for protein release by HPH prior to and post pretreatment with ethanol and toluene

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Yeast (HPH at 13.8 MPa)	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
Pretreatment with Ethanol and Toluene + HPH at 13.8 MPa	1.10	0.98	9.40	0.98	0.08	1.00		

4.5.1.4 Discussion

The use of solvents to release enzymes from yeast has been studied previously and results showed 80 to 90% release of β-galactosidase with 95% ethanol and isopropanol treatments. The use of concentrated solvents for intracellular enzyme recovery kills the majority of the yeast cells. The mechanism has not been fully studied but it is believed that the solvent extracts a lipid component from the yeast cell envelope, allowing leakage of intracellular or periplasmic protein (Fenton, 1982). Fenton's data were presented as percentage release defined as the total units released divided by the total units measured intracellularly, however, these amounts are not presented. A small amount of toluene in ethanol has been used to achieve good permeabilisation of yeast, with β-galactosidase activity of 1.53 U/mg dry weight, while toluene alone was found to be ineffective. A minimum concentration of 40% ethanol was required for good permeabilisation (Decleire *et al.*, 1987). The synergistic effect of ethanol and toluene resulted in a quick permeabilisation method which could be associated with a change in membrane fluidity, rendering it more accessible (Flores *et al.*, 1994). Ethanol-toluene treatment of *Saccharomyces cerevisiae* gave incomplete permeabilisation unless heat was applied at a temperature of 40°C. Low activities were observed in some cases suggesting incomplete permeabilisation or severe changes in environmental conditions. Morphological changes observed were possibly due to the extraction of lipid from the membrane by toluene (Murakami *et al.*, 1980). The use of alcohol to permeabilise yeast and the viability of the cells as a result of the treatment have been studied and results show that increases in alcohol concentrations above 40% resulted in poor growth, indicating that most of the cells died during the permeabilisation treatment (Kondo *et al.*, 2000). The literature on permeabilisation with ethanol and toluene has not discussed, or in general, considered potential for denaturation by the chemicals. However, Flores *et al.* (1994) indicated that the measurement of cytoplasmic constituents used appropriate blanks with solvents. The results obtained through permeabilisation have not explicitly presented the data with reference to the total amount of enzyme or protein available for release, and therefore no overall comparison can be drawn between the methods in literature and the results presented in Table 4.23.

The above results and discussion of the use of ethanol and toluene as a pretreatment combined with HPH has proved this unsuccessful and infeasible due to the extensive

deactivation of protein by the chemicals. The washing procedure to remove the chemicals was expected to minimise the interference of the chemicals, however it is evident that the washing did not reduce the interference, suggesting permeation of the chemicals into the cell. The deactivation of the proteins by the chemicals, confirmed by the interference test, has resulted in a reduced release of active protein in comparison to untreated yeast at the same pressure of 13.8 MPa. The deactivation and subsequent reduced release of proteins renders the method ineffective as a pretreatment method for the disruption of Baker's yeast.

4.5.2 Yeast Cell Disruption by HPH following pretreatment with EDTA and Triton X-100

4.5.2.1 Extent o f Release

EDTA was chosen for its ability to increase permeability of the cell wall while Triton X-100 removes fragments of the cell membrane and solubilises protein. By attacking both the cell wall and membrane, the mechanical cell disruption process is expected to be easier. The concentrations selected for pretreatment were 0.020M and 0.1% respectively. Proteins and small amounts of α-glucosidase and invertase were released into the supernatant during pretreatment, indicating that permeabilisation had occurred. Table 4.26 presents the results of the EDTA and Triton X-100 pretreatment used in combination with high pressure homogenisation at 13.8 MPa and 34.5 MPa. The extent of release achieved with the combination is compared to the maximum released from untreated yeast, R_{max}. Figures 4.20 and 4.21 present the release profiles achieved for this combination at pressures of 13.8 MPa and 34.5 MPa respectively, in comparison with untreated yeast and all other pretreatment combinations used on Baker's yeast. The raw data generated can be found in Appendix B.

Table 4.26 **Protein release following EDTA (0.020M) and Triton X-100 (0.1%) pretreatment and homogenisation of Baker's yeast with a comparison of the release to R_{max}**

Treatment	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	mg/g	%	(U/g) x 10^3	%	(U/g) x 10^7	%	(U/g)	%
Pretreatment	3.90		1.30		0.01		0.00	
Pretreated Yeast								
HPH (13.8 MPa, 24 pass)	33.8		7.33		0.12		3.17	
HPH (34.5 MPa, 24 pass)	152		14.9		4.17		6.31	
TOTAL (13.8 MPa)	37.7	24.3	8.63	53.7	0.13	3.08	3.17	44.7
TOTAL (34.5 MPa)	156	100	16.2	100	4.18	95.3	6.31	89.1
Untreated Yeast								
HPH (13.8 MPa, 24 pass)	112	72.1	1.29	80.3	1.61	36.7	4.49	63.4
HPH (34.5 MPa, 24 pass)	151	97.8	14.5	90.2	427	97.3	6.97	98.3
R_{max}	155		16.1		4.38		7.08	

The amount of protein released at 13.8 MPa did not achieve that using untreated yeast at the same pressure. The soluble protein release was 24% of the maximum available in the cell. Invertase and G6PDH release accounted for approximately 50% of the R_{max}, while only 3% of the maximum α-glucosidase available was released. It appears that enzyme denaturation occurred.

The treated cells were also homogenised at a higher pressure of 34.5 MPa, where the release equalled or slightly exceeded that achieved with no pretreatment, indicating complete cell breakage had occurred. R_{max} was achieved using this pretreatment combination at 34.5 MPa within 12 passes through the homogeniser. In this case, 100% of the available soluble protein and invertase were released while 95% of the α-glucosidase and 89% of the G6PDH were released.

The reduced levels of active protein measured following HPH at 13.8 MPa, suggest that insufficient washing to remove the chemicals may have resulted. The interference data presented in Table 4.27 indicated that deactivation does occur in the presence of EDTA and Triton X-100. The increased release at 34.5 MPa indicates that complete cell breakage has occurred, confirmed by micrographs, presented in Figure 4.15 and that deactivation is not evident.

4.5.2.2 Interference of Chemicals

The interference of the chemicals used was determined as described in Section 3.4.7. Table 4.27 presents the interference determined and the percentage denaturation of the enzymes due to EDTA and Triton X-100. The addition of the chemicals to a cell homogenate generated by high pressure homogenisation of the untreated cells at 13.8 MPa resulted in denaturation or inactivation of the enzymes, ranging from 68% for total soluble protein to 99% for α-glucosidase. The interference data presented here can be compared with interference data obtained for the use of EDTA and Triton X-100 as a pretreatment method on bacteria (Table 4.40). The latter data found shows a 30% denaturation of the total soluble protein, while Table 4.27 shows 67% denaturation of protein. The interference data was not performed in triplicate and therefore the correct amount is expected to lie within this range.

Table 4.27 Interference of EDTA and Triton X-100 on proteins measured with the percentage denatured in relation to release achieved from untreated yeast at the same pressure

	Untreated Yeast (R_i) 13.8 MPa	EDTA and Triton X-100	% Denatured
Protein (mg/g)	112	36.2	67.6
Invertase (U/g) x 10^4	1.29	0.31	75.6
α-glucosidase (U/g) x 10^5	161	2.08	98.7
G6PDH (U/g)	7.08	1.26	82.2

Detergents such as Triton X-100 have been found to interfere with the Bradford protein assay, and a concentration of 0.1% Triton X-100, causes a change in the absorbance at 595 nm of 0.013 (Bradford, 1976). This change in absorbance was managed by treatment of the blank with the chemicals at the appropriate concentrations. This method of managing potential interference of the chemical was indicated in literature, however, no denaturation due to the chemical was mentioned. Interference of the assay has primarily been reported by detergents causing an over or under estimation of the response and a reduction of the linear response range. To improve the linearity and sensitivity of the assay, it is suggested that the detergent is removed before performing the assay (Compton and Jones, 1985). It has also been found that Triton X-100 appears to facilitate non-ionic interactions of the dye with proteins of limited capacity for ionic binding. Results have shown that the incorporation of low concentrations of a non-ionic detergent may be useful

in improving sensitivity and variability of the Bradford assay (Friedenauer and Berlet, 1989). The variability refers to the inconsistent response of Coomassie Brilliant Blue to different proteins. The addition of Triton X-100 compensated the specificity of the dye response to arginine residues, the content of which accounts for most of the variability of the assay with individual proteins (Compton and Jones, 1985).

4.5.2.3 Release Rate Kinetics

In Section 4.4, Equation 4.8 was proposed to model the release rate kinetics for the high pressure homogenisation of treated cells. The release rates of all proteins and enzymes from the treated cells are compared to those of the untreated yeast at the same pressure in Table 4.28 for the EDTA and Triton X-100 pretreatment method. The release profiles can be seen in Figure 4.20 and 4.21, where they are compared against the other pretreatment method combinations used.

Table 4.28 Release rate constants (k') and regression coefficients R^2 for protein release by HPH prior to and post pretreatment with EDTA and Triton X-100

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Treatment (13.8 MPa, 24 pass)								
Untreated Yeast	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
EDTA and Triton X-100	11.0	0.97	42.2	0.98	2.70	0.98	28.1	0.98
Treatment (34.5 MPa, 24 pass)								
Untreated Yeast	252	0.98	394	0.99	207	0.98	414	0.99
EDTA and Triton X-100	378	0.99	193	0.97	182	0.99	183	0.97

From Table 4.28, it is seen that the release rates of all proteins from treated yeast are lower than those of untreated yeast, with the exception of soluble protein at 34.5 MPa, where the soluble protein from the treated cells was released faster than the soluble protein from untreated yeast cells. For experiments performed at 13.8 MPa, the pretreated yeast released total soluble protein at a rate of 11.0 x 10^{-3}, whereas the release rate of protein from untreated yeast was 54.1 x 10^{-3}. The same trend followed for all enzymes measured. The release rates of invertase in both cases were fastest, followed by G6PDH and then total soluble protein. The release rate of α-glucosidase was slowest at 2.70 x 10^{-3}.

At 34.5 MPa, the release rate of protein from treated cells (378 x 10^{-3}) exceeded protein release from untreated cells at 252 x 10^{-3}. This increased release rate is possibly due to minimal deactivation of the proteins as a result of adequate removal of the chemicals. All other enzymes measured were released at rates slower than those of untreated yeast. The release rate of invertase was fastest at 193 x 10^{-3}, i.e. half the rate at which invertase was released from untreated yeast (394 x 10^{-3}). G6PDH was released at 183 x 10^{-3} compared to 414 x 10^{-3} of untreated yeast, while α-glucosidase was released at a rate that was equal to 90% of the rate of α-glucosidase from untreated yeast, revealing the same release trend seen at 13.8 MPa. In the above cases, the cell wall bound enzyme, invertase was released fastest, except for soluble protein at 34.5 MPa, which has already been accounted for, and therefore followed the trends found by Follows *et al.* (1971).

4.5.2.4 Discussion

Permeabilisation using Triton X-100 has been studied and shown to be successful with growth of yeast in the presence of Triton X-100 increasing the permeability of the cell. A three-stage process occurs including membrane-interaction by incorporation into the lipid bilayer, disruption of the bilayer and then separation of the lipid from the protein components. The first two processes occur rapidly (within 1 minute), while the last can take up to 20 hours. Triton X-100 is effective in facilitating the uptake of alcohol substrates and the cofactor NADH for the enzyme ADH, with 350 U ADH/g wet weight measured compared to 80 U/g from untreated cells (Laouar *et al.*, 1996). Permeabilisation is only possible if the surfactant can penetrate the cell wall and reach the membrane and therefore Triton X-100 alone cannot permeabilise *Saccharomyces cerevisiae* (King *et al.*, 1991). EDTA was used to assist the Triton X-100 by increasing permeability of the cell wall. The success of the permeabilisation depends on the composition of the cell wall and membrane as well as the location of the proteins assayed. The permeability depended more on the detergent concentration than the ratio of detergent to cells. Triton X-100 appears to cause slight ultra structural changes in the cell wall and membrane suggesting a possible weakening of the cohesion between proteins and lipids in the zones attacked by the detergent. *Yarrowia lipolytica* cells treated with 0.1% Triton X-100 showed maximum acid phosphatase activity and 82% alkaline phosphate activity. The maximum activity was determined in a disrupted cell suspension (Galabova *et al.*, 1996). The literature on

permeabilisation with EDTA and Triton X-100 has not discussed or in general considered potential for denaturation by the chemicals, despite the vast amount of literature available on Triton X-100. However, Zhao and Yu (2001) accounted for Triton X-100 interference by ensuring that all samples assayed contained the same amount of Triton X-100 as the standard protein solution.

The above results and discussion of the use of EDTA and Triton X-100 as a pretreatment combined with HPH has proved to be successful if adequate washing and thorough removal of the chemicals is achieved. If the proteins are exposed to the chemicals, deactivation does occur. The lower extent of release achieved from treated cells homogenised at 13.8 MPa confirms the deactivation of the proteins due to insufficient removal of the chemicals. This is further confirmed by the treatment of Baker's yeast with EDTA and Triton X-100, followed by homogenisation at 34.5 MPa where the maximum soluble protein and invertase release was obtained and approximately 95% of the maximum available of α-glucosidase and G6PDH was released. The deactivation of the released proteins at 34.5 MPa is not apparent but, it is evident from micrographs (Figure 4.15) that complete cell breakage had occurred and therefore if deactivation had occurred, the extent of release achieved would have been lower. The method therefore requires careful and complete removal of the chemicals to minimise the interference of the chemicals.

4.5.3 Yeast Cell Disruption by HPH following pretreatment with EDTA and CTAB

4.5.3.1 Extent of Release

EDTA increases permeability of the cell wall, while CTAB, is postulated to interact with the hydrophobic moieties with the membranes. Their concentrations, selected through the screening process (Section 4.2.1.3), were 0.020M and 0.1% respectively. The pretreatment procedure released small amounts of soluble protein, α-glucosidase and invertase, indicating that permeabilisation had occurred. Table 4.29 presents the results of the EDTA and CTAB pretreatment used in combination with high pressure homogenisation at 13.8 MPa and 34.5 MPa. These are compared to the maximum protein available in the cell

for release from untreated yeast, R_{max}. Figures 4.20 and 4.21 present the release profiles achieved on homogenisation following pretreatment with EDTA and CTAB. These can be compared with untreated yeast and all other pretreatment combinations used on Baker's yeast. The raw data generated through these experiments are found in Appendix B.

Table 4.29 Protein release following EDTA (0.020M) and CTAB (0.1%) pretreatment and homogenisation of Bakers yeast with a comparison of the release to R_{max}

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	mg/g	%	(U/g) x 10^3	%	(U/g) x 10^5	%	(U/g)	%
Pretreatment	18.1		3.79		0.23		0.00	
Pretreated Yeast								
HPH (13.8 MPa, 24 pass)	23.6		5.41		2.37		1.65	
HPH (34.5 MPa, 24 pass)	21.5		7.04		32.9		1.72	
TOTAL (13.8 MPa)	44.7	27.0	9.20	57.3	2.60	2.37	1.65	23.2
TOTAL (34.5 MPa)	39.6	25.6	10.8	67.4	33.2	33.0	1.72	24.2
Untreated Yeast								
HPH (13.8 MPa, 24 pass)	112	72.1	12.9	80.3	161	36.7	4.49	63.4
HPH (34.5 MPa, 24 pass)	151	97.8	14.5	90.2	427	97.3	6.97	98.3
R_{max}	155		16.1		438		7.08	

Total soluble protein release on permeabilisation was less than 15% R_{max}. On homogenisation, an increase in pressure did not increase the apparent extent of disruption. At the two pressures investigated, the total soluble protein release attained through pretreatment and HPH was 25 % of the maximum available. Negligible α-glucosidase was released using 13.8 MPa in comparison to the R_{max} and 33% was released at 34.5 MPa. About 60% of the wall associated invertase available was released and almost 25% of the cytoplasmic G6PDH at both pressures.

Micrographs (Figures 4.16 and 4.17), taken under 100 x magnification using phase contrast microscopy, revealed that the cells were not broken throughout the homogenisation process at either pressure. It is postulated that the pretreatment procedure resulted in strengthening of the cells and increased their resistance to disruption. The micrographs are presented and discussed with the other pretreatments in Section 4.5.5.

4.5.3.2 Interference of Chemicals

The interference of the chemicals used was determined as described in Section 3.4.7. Table 4.30 presents the percentage denaturation of the enzymes due to EDTA and CTAB. The addition of the chemicals to a cell homogenate resulted in significant denaturation or inactivation of the enzymes. Deactivation of soluble protein, invertase, α-glucosidase and G6PDH measured during the interference test were 82%, 36%, 100% and 53% respectively. These values can be compared to those of Sekhar *et al.* (1999), where 0.4% CTAB treatment resulted in 36% decrease in ADH activity and a 30% decrease in catalase activity with a treatment time of 2 hours.

Ionic and non-ionic detergents have been reported to interfere with the Bradford assay. In addition to stabilising the neutral dye species, some detergents are known to decrease assay response to a number of enzymes, most likely due to competition with the dye for protein. These competitive effects lead to protein underestimation and may be overcome through inclusion in the standard calibration (Compton and Jones, 1985). In all samples where the chemicals were still present, the chemicals were added to the blank in the same concentrations used for the pretreatment procedure.

Table 4.30 Interference of EDTA and CTAB on proteins measured with the percentage denatured in relation to release achieved from untreated yeast at 13.8 MPa

	Untreated Yeast (R_I) 13.8 MPa	EDTA and CTAB	% Denatured
Protein (mg/g)	112	19.7	82.3
Invertase (U/g) x 10^4	1.29	0.83	35.9
α-glucosidase (U/g) x 10^5	161	0.37	99.8
G6PDH (U/g)	7.08	3.37	52.4

4.5.3.3 Release Rate Kinetics

The release rate kinetics during high pressure homogenisation of pretreated cells were calculated using Equation 4.8 (Section 4.4). Table 4.31 presents the release rates calculated using the EDTA and CTAB pretreatment method. These are compared to those of untreated yeast at the same pressure. The release profiles for the method can be seen in

Figure 4.20 and 4.21, where they are compared against the other pretreatment method combinations used.

Table 4.31 Release rate constants (k') and regression coefficients R^2 for protein release by HPH prior to and post pretreatment with EDTA and CTAB

	Soluble Protein		Invertase		α-glucosidase		G6PDH	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Treatment HPH (13.8 MPa, 24 pass)								
Untreated Yeast	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
EDTA and CTAB	7.50	0.98	22.6	0.97	0.20	0.99	11.1	1.00
Treatment HPH (34.5 MPa, 24 pass)								
Untreated Yeast	252	0.98	394	0.99	207	0.98	414	0.99
EDTA and CTAB	9.10	0.96	38.4	0.98	4.40	0.96	11.8	0.97

The release rates of all proteins from pretreated yeast are lower than those of untreated yeast. For experiments performed at 13.8 MPa, the pretreated yeast released total soluble protein at a rate of 7.5 x 10^{-3}, whereas the release rate of protein from untreated yeast was 54.1 x 10^{-3}, eight times faster than the treated yeast. The same trend followed for all enzymes measured. The release rates of invertase in both cases were fastest but still three times slower than untreated yeast, followed by G6PDH (four times slower than untreated yeast), and then soluble protein. The release rate of α-glucosidase was slowest at 0.2 x 10^{3}, this amount differing 65 fold from the rate released by untreated yeast.

At 34.5 MPa, the release rate of all proteins followed the same trends as at 13.8 MPa. All release rates slower than those of untreated yeast at the same pressure. At this pressure, the release rate of invertase was fastest at 38.4 x 10^{-3}, followed by G6PDH at 11.8 x 10^{-3} and then soluble protein at 9.10 x 10^{-3} and lastly α-glucosidase at 4.40 x 10^{-3}. For both pressures, the cell wall bound enzyme, invertase was released faster that intracellular material following the trends found by Follows *et al.* (1971). There is very little change in release rate at the different pressures following pretreatment, further supporting that HPH had little impact on the cells which were protected or strengthened by the treatment.

4.5.3.4 Discussion

The use of the detergent CTAB has been widely used to permeabilise cells and cause increased effective activity of intracellular enzymes in the cell suspension (Giovenco and Verheggen, 1987; Gowda *et al.*, 1991; Alamae and Jarviste, 1995; Sekhar *et al.*, 1999; Bansal-Mutalik and Gaikar, 2003). The use of 0.2% CTAB at 24°C for 15 minutes has shown maximum permeabilisation of *Saccharomyces cerevisiae*, with an alcohol dehydrogenase (ADH) activity of 347.2 U/g cells (measured in suspension) compared to 0.6 U/g cells activity from cells that were not treated (Gowda *et al.*, 1991). Detergent molecules are believed to interact through their hydrophobic moieties with the cell membranes. Results have shown that permeabilisation is dependent on the ratio of cell to detergent more than the detergent concentration (Gowda *et al.*, 1991). The permeabilisation of *Saccharomyces cerevisiae* by CTAB is governed by a number of parameters: concentration, pH, contact time and temperature. Maximum activity of catalase was observed at 15 minutes contact time, a pH between 4.0 and 9.0 and at a concentration of 0.4% (Sekhar *et al.*, 1999). Catalase activity decreased at CTAB concentrations beyond 0.4%, and decreased ADH activity was observed on increase in the CTAB concentration beyond 0.2%. These decreases have been attributed to inactivation of the enzymes by the detergent (Sekhar *et al.*, 1999). The treatment of cells with CTAB has reduced cell viability quite rapidly, with only 10% of cells remaining viable after a 5 minute treatment with 0.1% CTAB and 50% remain viable after treatment with 0.01% for 15 minutes (Alamae and Jarviste, 1995).

The above results and discussion of the use of EDTA and CTAB as a pretreatment combined with HPH has suggested that the yeast cells strengthened or protected by the presence of CTAB. The extent of disruption achieved with the treatment and homogenisation of the cells was much lower than expected, as were the release rates. Micrographs (Figures 4.16 and 4.17) confirmed that disruption of the cells did not take place at both pressures of 13.8 and 34.5 MPa. This physical evidence of the absence of cell breakage by homogenisation confirms that the low extent of disruption is not due to inactivation of the proteins by the chemicals, but due to the increased strength of the cells or their protection from shear forces Therefore, the use of EDTA and CTAB as a pretreatment method for Baker's yeast results in the strengthening of the cells and increased resistance to disruption by HPH.

4.5.4 Yeast Cell Disruption by HPH following pretreatment with Lyticase (Zymolase)

4.5.4.1 Extent of Release

Lyticase was selected for the enzymatic lysis of Baker's yeast owing to its ability to hydrolyse 1, 3 β glucosidic linkages in 1,3 β glucans. The enzymatic lysis was followed by the homogenisation of the cells at two selected pressures: 13.8 MPa and 34.5 MPa. The optimum enzyme concentration, determined during the pretreatment testing phase of the research, was found to be 0.1 mg enzyme/g yeast. The pretreatment procedure released small amounts of soluble protein and invertase indicating that permeabilisation had occurred. Table 4.32 presents the extent of protein release following pretreatment used in combination with high pressure homogenisation at 13.8 MPa and 34.5 MPa. Figures 4.20 and 4.21 present the release profiles achieved for this combination, compared with untreated yeast and all other pretreatment combinations used on Baker's yeast. The raw data generated in these experiments can be found in Appendix B.

Table 4.32 Protein release following lyticase (0.1 mg/g yeast) pretreatment and homogenisation of Baker's yeast with a comparison of the release to R_{max}

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	mg/g	%	(U/g) x 10^3	%	(U/g) x 10^7	%	(U/g)	%
Pretreatment	1.30		0.41		0.00		0.00	
Pretreated Yeast								
HPH (13.8 MPa, 24 pass)	109		11.9		1.83		4.16	
HPH (34.5 MPa, 24 pass)	146		15.6		4.39		6.54	
TOTAL (13.8 MPa)	111	71.4	12.3	76.5	1.83	41.7	4.16	58.8
TOTAL (34.5 MPa)	147	94.9	16.0	99.7	4.39	100	6.54	92.3
Untreated Yeast								
HPH (13.8 MPa, 24 pass)	112	72.1	12.9	80.3	1.61	36.7	4.49	63.4
HPH (34.5 MPa, 24 pass)	151	97.8	14.5	90.2	4.27	97.3	6.97	98.3
R_{max}	155		16.1		4.38		7.08	

Protein release in the homogeniser at 13.8 MPa following lyticase pretreatment followed a release pattern similar to that of untreated yeast. Figure 4.20 presents the release profiles achieved on homogenisation following pretreatment with lyticase. These can be compared with untreated yeast and all other pretreatment combinations used on Baker's yeast. The release of α-glucosidase was enhanced with the release achieved (41.7% of R_{max}) exceeding the amount released by homogenisation of untreated cells at 13.8 MPa (36.7% of R_{max}) but not exceeding the maximum available for release. The release of invertase, soluble protein and G6PDH from pretreated cells at 13.8 MPa was similar to release from untreated yeast. At a pressure of 13.8 MPa, 70 to 80% of the maximum soluble protein and invertase, some 60% of the G6PDH available and 40% α-glucosidase were released.

On homogenisation at 34.5 MPa following lyticase pretreatment, maximum release of invertase and α-glucosidase was achieved while 93% and 95% of the maximum G6PDH and soluble protein available was released respectively. The pretreatment method did not appear to alter the extent of disruption significantly as enhanced release in comparison to untreated yeast at the same pressures was not observed (Figure 4.21).

Micrographs (Figures 4.18 and 4.19), taken using phase contrast, revealed that the cells were completely disrupted with the homogenisation process at both pressures: 13.8 and 34.5 MPa. The micrographs are presented and discussed relative to the other pretreatments in Section 4.5.5.

4.5.4.2 Interference of Chemicals

The potential interference of lyticase was determined as described in Section 3.4.7. Table 4.33 presents the results in terms of the percentage denaturation due to the enzyme. Minimal interference of lyticase with the assays performed was observed. The small differences of 5.6% soluble protein and 3.8% invertase observed may be due to experimental error. However, 45% G6PDH appears to have been deactivated on addition of the lyticase to the homogenate. Denaturation of G6PDH is not apparent in homogenisation of the pretreated cells, presented in Table 4.32. The release of α-glucosidase was not deactivated by lyticase, and release increased on addition of the enzyme. The enzyme lyticase has not been noted in literature as a possible interfering

substance. Baldwin and Robinson (1994) have indicated that much of the Zymolase may remain bound to the cell wall debris and hence be removed from the total soluble protein during centrifugation following disruption and that removal of the Zymolase is only necessary if the fraction remaining on the wall is significant.

Table 4.33 Interference of lyticase on proteins measured with the percentage denatured in relation to release achieved from untreated yeast at the same pressure

	Untreated Yeast (R_i) 13.8 MPa	Lyticase	% Denatured
Protein (mg/g)	112	105	5.60
Invertase (U/g) x 10^4	1.29	1.24	3.82
α-glucosidase (U/g) x 10^5	161	192	excess by 20%
G6PDH (U/g)	7.08	3.86	45.5

4.5.4.3 Release Rate Kinetics

The release rate kinetics on high pressure homogenisation of treated cells were modelled according to Equation 4.8, (Section 4.4). The release rates from the treated cells were compared to those of the untreated yeast at the same pressure in Table 4.34.

The release profiles for the method can be seen in Figure 4.20 and 4.21, where they are compared against the other pretreatment method combinations used. The release rate of invertase from treated yeast is some 67% that of untreated yeast at 13.8 MPa, while that of G6PDH is some 87%. The release rate of α-glucosidase doubled for pretreated cells when compared with untreated cells, while a small increase in the rate of release of total soluble protein was found (57.1 x 10^{-3}, compared with 54.1 x 10^{-3}). The release rate of soluble protein was fastest, followed by invertase. The release rate of α-glucosidase was slowest at 22.3 x 10^{-3}.

At 34.5 MPa, higher release rates for soluble protein and invertase for treated cells compared to untreated at the same pressure were observed. The release rate of soluble protein was again fastest at 598 x 10^{-3}, doubling the release rate of untreated yeast. Invertase was released next fastest, at a rate 1.4 times faster than the untreated yeast at 537 x 10^{-3}. G6PDH was released at a rate slower than the untreated yeast, at approximately

the same rate of the untreated yeast as that observed at 13.8 MPa. The rate release of α-glucosidase from the treated cells was equal to the release rate of treated cells.

Table 4.34 Release rate constants (k') and regression coefficients R^2 for protein release by HPH prior to and post pretreatment with lyticase

	Total Soluble Protein		Invertase		α-glucosidase		G6PDH	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Treatment (13.8 MPa, 24 pass)								
Untreated Yeast	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
Lyticase	57.1	0.97	46.3	0.98	22.3	0.98	37.7	1.00
Treatment (34.5 MPa, 24 pass)								
Untreated Yeast	252	0.98	394	0.99	207	0.98	414	0.99
Lyticase	598	0.94	537	0.96	202	0.97	302	1.00

4.5.4.4 Discussion

Studies have shown that the partial softening and weakening of the cell wall by enzyme treatment can facilitate mechanical rupture. Zymolase hydrolyses the glucan and mannan components of the carbohydrate-protein complex of *Saccharomyces cerevisiae* cell wall. The pretreatment method resulted in release of more than 80% of the total nitrogen available, an amount comparable to release obtained by mechanical methods. Protein denaturation with this method is negligible (Knorr *et al.*, 1979). Zymolase has been effective in the lysis of Baker's yeast cell walls, with a 100% disruption in 4 passes at 95 MPa achieved in combination with HPH, in comparison to 32% disruption within 4 passes at 95 MPa on HPH in the absence of Zymolase (Baldwin and Robinson, 1990). Partial enzymatic lysis of *Candida utilis* by Zymolase followed by mechanical disruption has shown increased release under the same operating conditions, with 95% disruption at 95 MPa with the treatment compared to 65% disruption of untreated cells (Baldwin and Robinson, 1993).

The results and discussion presented have showed the use of lyticase as a pretreatment method, however, the increased extent of disruption observed by Baldwin and Robinson (1993) was not found. Increased release of invertase and α-glucosidase with the pretreatment was observed when compared with the untreated yeast at 34.5 MPa.

Micrographs (Figures 4.18 and 4.19) confirmed that complete disruption took place at both pressures of 13.8 and 34.5 MPa.

4.5.5 Microscopic Observation of Cell Damage on HPH with Pretreatment of Baker's yeast

Micrographs of the pretreated and untreated cells are presented to determine the physical damage to the cell structure caused by the separate pretreatment and mechanical processes. The micrographs were taken before and after pretreatment and at stages through the mechanical disruption, allowing a qualitative measurement of the effect of pretreatment on Baker's yeast. The effect of the combination of pretreatment and mechanical disruption methods can be compared with untreated yeast micrographs presented in Section 4.3.1. For each pretreatment, micrographs are presented of a pretreated sample prior to homogenisation (a), followed by a sample taken half way through disruption (b) and a final sample taken at 24 passes through the homogeniser (c).

EDTA and Triton X-100

Figure 4.15 provides micrographs of Baker's yeast following pretreatment with EDTA and Triton X-100 and following subsequent homogenisation at 34.5 MPa. In Figure 4.15a, the cells were intact and there were no signs of breakage in the cells, due to the chemical treatment. Figure 4.15b shows the yeast at the 12^{th} pass through the homogeniser at a pressure of 34.5 MPa, with clear cell breakage and cell debris present. In Figure 4.15c, following 24 passes in the homogeniser, it can be seen that complete cell breakage had occurred and micronisation of the cell debris. The evidence of complete cell breakage supports the discussion in Section 4.5.2.4 that maximum release obtained with homogenisation of treated cells at 34.5 MPa. Therefore, disruption at 13.8 MPa showed low release of proteins from treated cells homogenised due to denaturation of the proteins as a result of insufficient washing off of the chemicals.

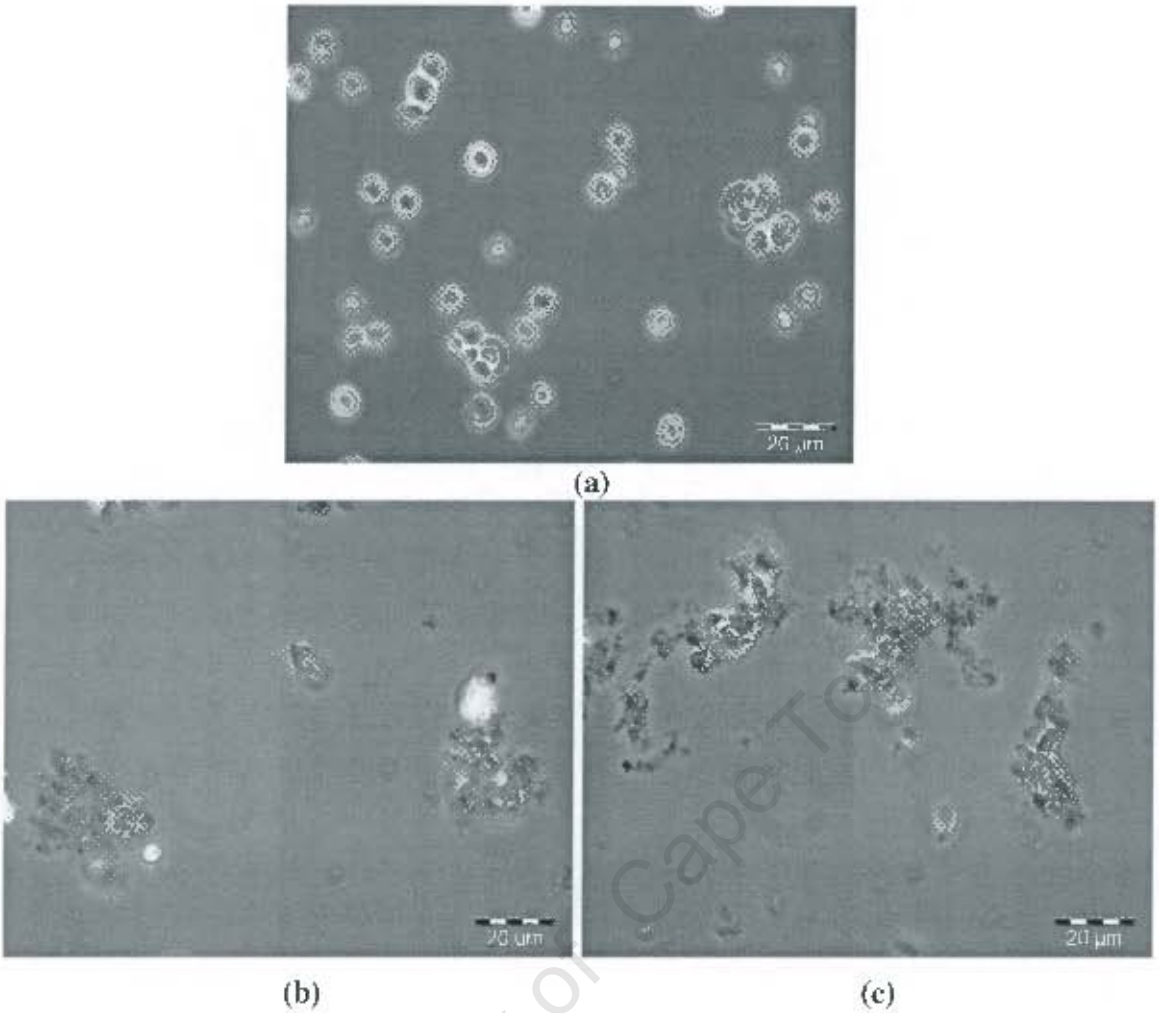


Figure 4.15 Degradation of Baker's yeast by HPH at 34.5 MPa following pretreatment with EDTA and Triton X-100 (a: pretreated cells prior to homogenisation, b: cells at 12th pass, c: cells at 24th pass)

EDTA and CTAB

Figures 4.16 and 4.17 provide micrographs of Baker's yeast following pretreatment with EDTA and CTAB and following subsequent homogenisation at 13.8 MPa and 34.5 MPa respectively. Pretreated Baker's yeast cells were observed prior to disruption in Figure 4.16a, where the cells were clearly intact. Following passage through he homogeniser at 13.8 MPa, it is evident that the cells remained unbroken (Figures 4.16b and c), even though they were subjected to conditions typically resulting in mechanical breakage.

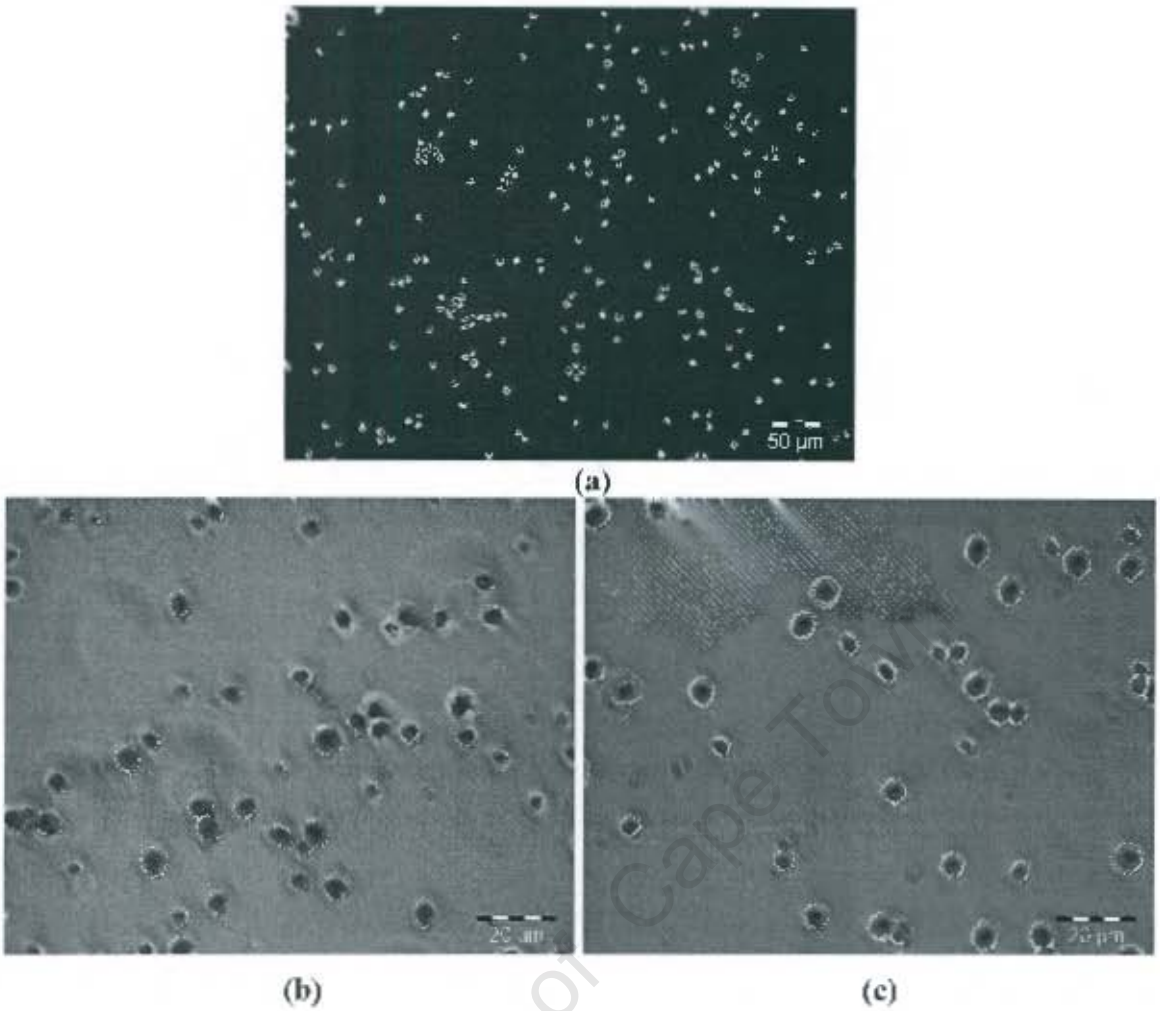
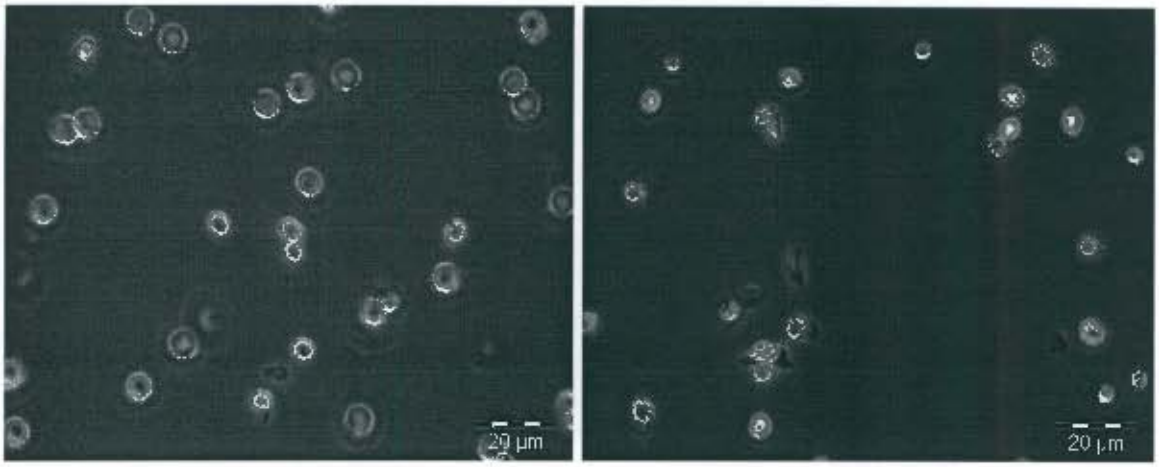


Figure 4.16 Degradation of Baker's yeast by HPH at 13.8 MPa following pretreatment with EDTA and CTAB (a: pretreated cells prior to homogenisation, b: cells at 12th pass, c: cells at 24th pass)

Figure 4.17 illustrates exposure of pretreated yeast to homogenisation at 34.5 MPa. In Figure 4.17a and b, a small amount of cell breakage was observed but not complete cell breakage and no cell debris was present. It is apparent that strengthening of the cells has occurred and this supports the results obtained for the decreased extent of disruption and protein release rates compared with untreated yeast. Therefore, it can be concluded that the pretreatment of Baker's yeast with EDTA and CTAB resulted in the protection of the cells, thereby increasing their resistance to disruption and contradicting the expected weakening of the cell envelope by EDTA and CTAB associated with permeabilisation.



(a) (b)

Figure 4.17 Degradation of Baker's yeast by HPH at 34.5 MPa following pretreatment with EDTA and CTAB (a: cells at 12th pass, b: cells at 24th pass)

Lyticase

Figures 4.18 and 4.19 provide micrographs of Baker's yeast following pretreatment with lyticase and following subsequent homogenisation. In Figure 4.18a, Baker's yeast, treated with lyticase was observed prior to mechanical disruption. Figure 4.18b shows clear cell breakage at the 12th pass through the homogeniser, with some cells still unbroken. In After the 24th pass (Figure 4.18c), cell breakage and cell debris, but some cells remain unbroken. Figure 4.19a shows complete cell breakage had occurred by the 12th pass at 34.5 MPa and at the 24th pass, (Figure 4.19b), cell debris was present and micronisation of debris occurred. The extent of release at 13.8 MPa did not achieve maximum release due to the incomplete breakage of all cells by the final pass. The increased release in invertase and α-glucosidase observed at 34.5 MPa is supported by the physical evidence of the nature of the cells under these conditions, where complete cell breakage and micronisation have occurred.

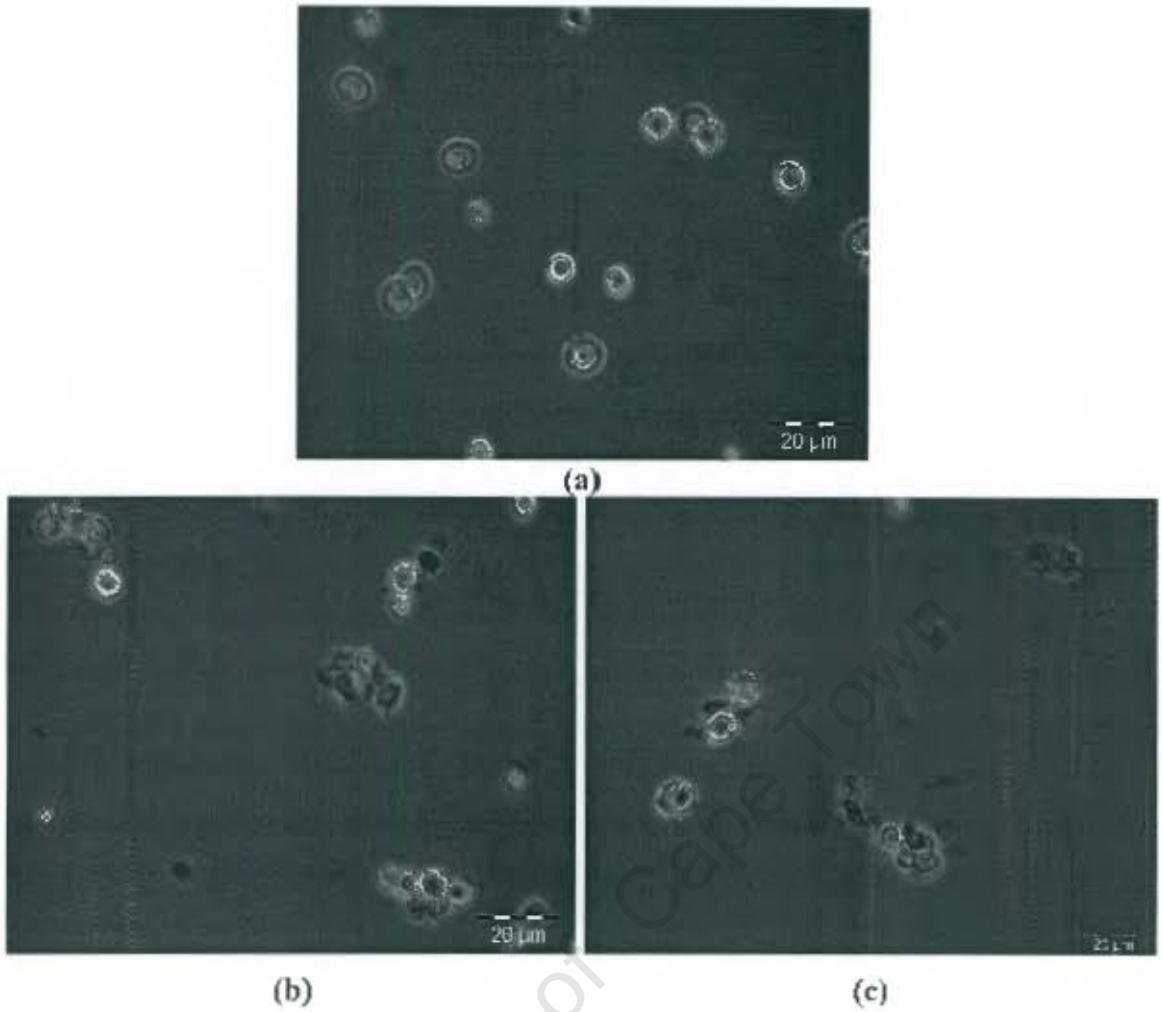


Figure 4.18 **Degradation of Baker's yeast by HPH at 13.8 MPa following pretreatment with lyticase (a: pretreated cells prior to homogenisation, b: cells at 12th pass, c: cells at 24th pass)**

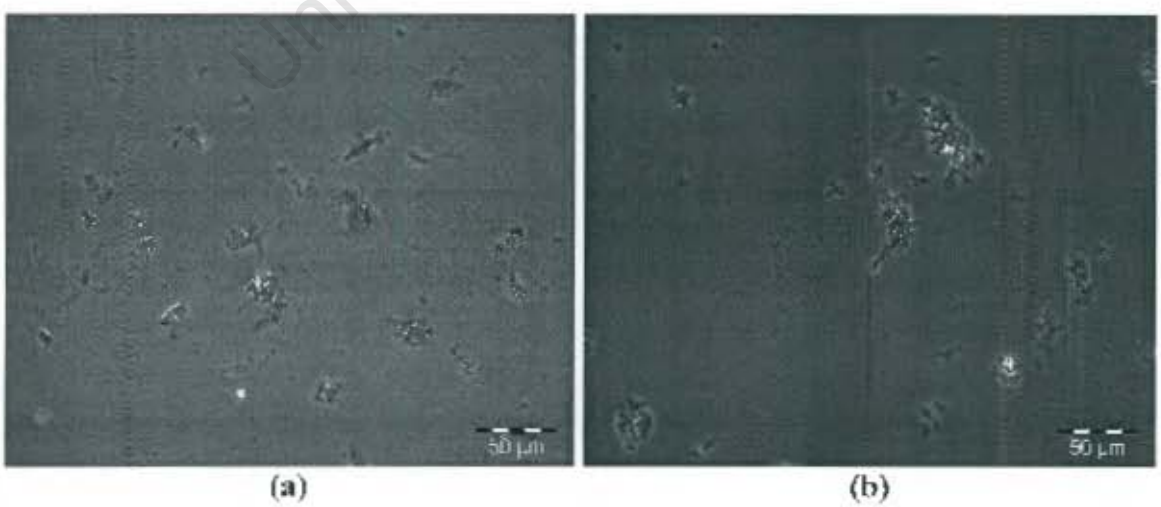


Figure 4.19 **Degradation of Baker's yeast by HPH at 34.5 MPa following pretreatment with Lyticase (a: cells at 12th pass, b: cells at 24th pass)**

4.5.6 Summary of Combined Pretreatment and High Pressure Homogenisation Methods with Baker's Yeast

The release profiles for all pretreatment methods combined with homogenisation at 13.8 MPa and 34.5 MPa are shown in Figure 4.20 and 4.21 respectively. These profiles are compared to release of protein from untreated yeast by HPH at the same pressure. The release of total soluble protein and all enzymes as a function of the number of passes did not achieve maximum release following 24 passes at 13.8 MPa, at 1% cell concentration for all pretreatments. Following all pretreatments, with the exception of lyticase, the enzymes and protein release tended to an asymptote even though the maximum release was not achieved. Under some pretreatments, it appeared that the cell released the majority of the cytoplasmic contents, but the maximum extracellular protein concentration was not determined due to denaturation of the proteins. Following pretreatment with EDTA and CTAB method, complete disruption did not result. The untreated yeast was also found to asymptote even though R_{max} was not achieved.

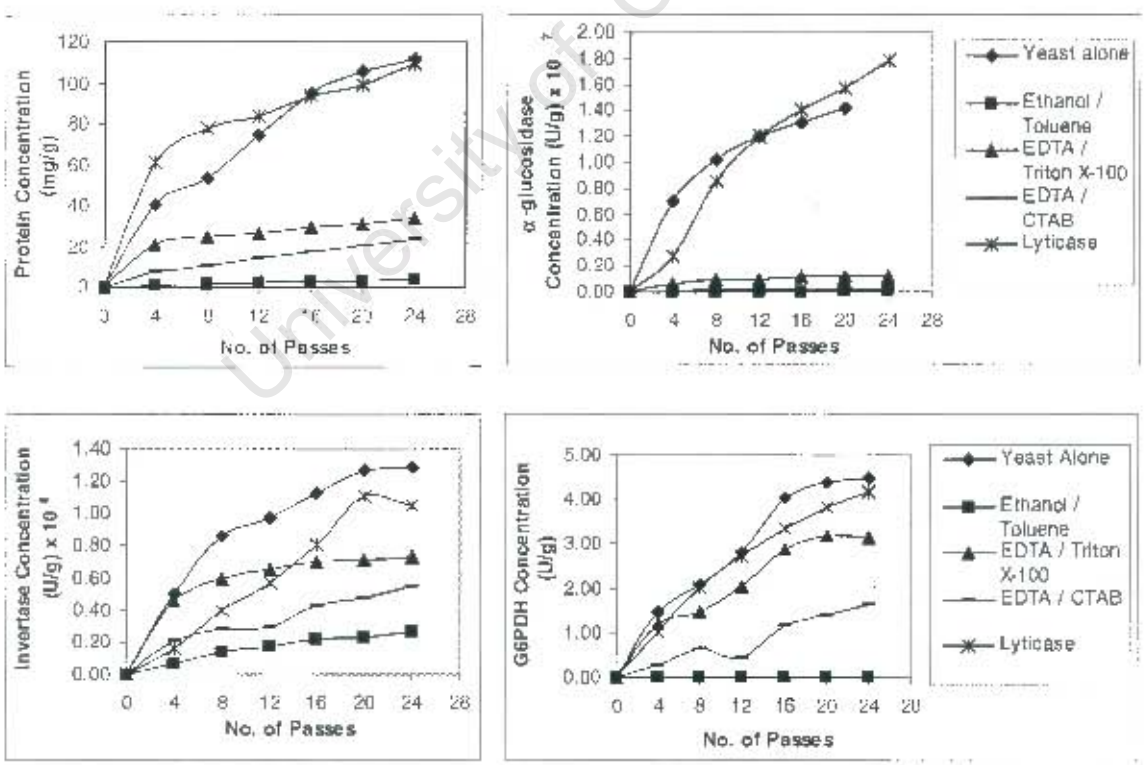


Figure 4.20 **Release of total soluble protein, invertase, α-glucosidase and G6PDH as a function of the number of passes for all pretreatments combined with homogenisation (13.8 MPa) and untreated yeast**

It is evident from the interference of the chemicals that significant protein and enzyme denaturation occurred following pretreatment with the ethanol and toluene. This suggests that the chemicals permeated the cell and caused significant denaturation. On direct exposure of the proteins to EDTA + Triton X-100 and EDTA + CTAB, protein deactivation occurred. Therefore, the chemicals have adverse effects on the proteins, however, if a thorough washing procedure is followed after the pretreatments and prior to homogenisation to remove the chemicals, the effect can be minimised, unless the chemicals permeate the cell during pretreatment.

The pretreatments with lyticase and EDTA and Triton X-100, followed by HPH at 34.5 MPa showed maximum release. The use of EDTA and Triton X-100 at this pressure showed higher release rates of total soluble protein and invertase when compared to the untreated yeast The EDTA and CTAB pretreatment, once again did not reach R_{max}. Micrographs showed that the cells were not broken during HPH and it is therefore suggested that they were protected due to the pretreatment chemicals.

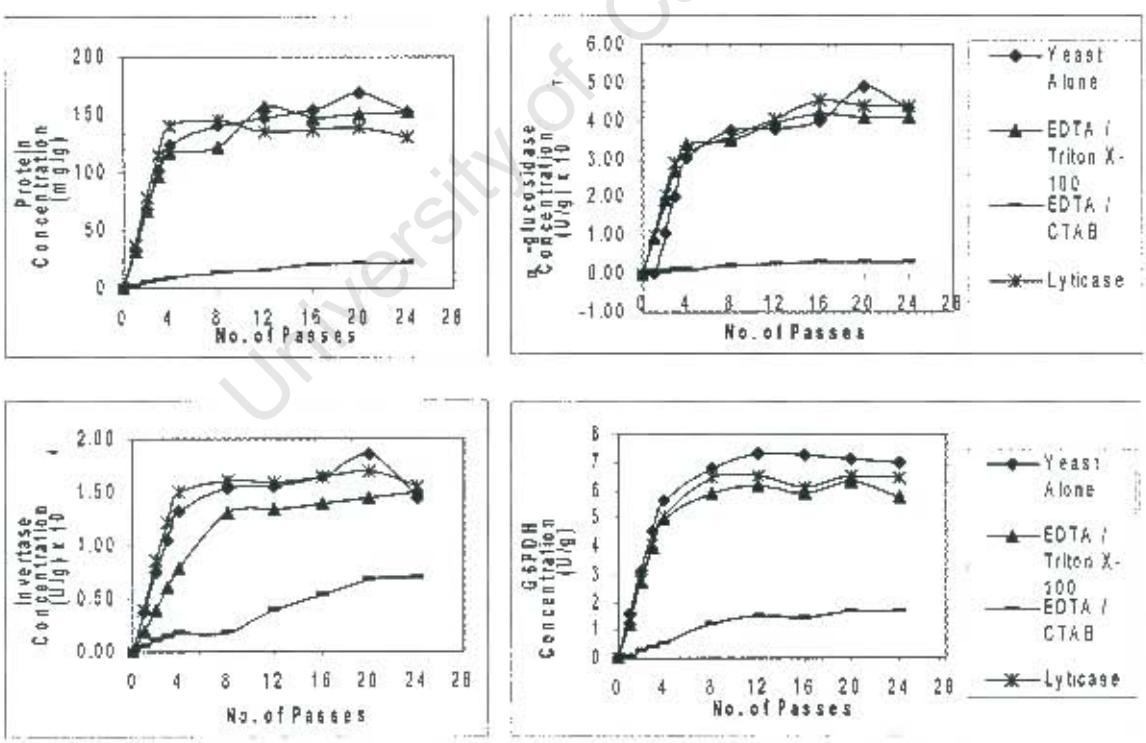


Figure 4.21 Release of total soluble protein, invertase, α-glucosidase and G6PDH as a function of the number of passes for all pretreatments combined with homogenisation (34.5 MPa) and untreated yeast

The use of ethanol and toluene as a pretreatment method resulted in reduced extent of disruption compared to untreated yeast at the same pressure. The chemicals caused significant protein deactivation as the chemicals permeated the cell during the pretreatment procedure, and therefore were not removed during the washing procedure. The deactivation, confirmed by interference testing of the chemicals, and subsequent reduced release of proteins in the presence of micronisation of the cell renders the method ineffective as a pretreatment method for the disruption of Baker's yeast for protein recovery.

The use of EDTA and Triton X-100 as a pretreatment method followed by HPH showed increased protein release at 34.5 MPa compared to 13.8 MPa. Interference testing revealed that the chemicals do cause significant protein deactivation. Since the micrographs showed that complete cell breakage had occurred at 34.5 MPa, the decreased extent of disruption observed at 13.8 MPa is due to insufficient washing off of the chemicals and subsequently deactivation occurred.

Cells treated with EDTA and CTAB showed decreased disruption compared to untreated yeast at pressures of 13.8 and 34.5 MPa. The release of proteins from treated cells was observed to be similar for both pressures, indicating that pressure had little effect. Micrographs revealed that the cell had not broken during the high pressure homogenisation as expected and that the EDTA and CTAB protected the cells, increasing their resistance to disruption.

The use of lyticase to hydrolyse 1, 3 β glucosidic linkages in 1,3 β glucans and weaken the yeast cell wall proved effective with respect to release of invertase and α-glucosidase at 34.5 MPa, where maximum release was attained. Micrographs showed that cell breakage had occurred at 13.8 MPa but some cells still remained intact, while 34.5 MPa resulted in complete cell breakage.

4.6 Effect of Pretreatment Combined with High Pressure Homogenisation on Disruption of *Escherichia coli*

4.6.1 Bacteria Cell Disruption by HPH following Pretreatment with EDTA

4.6.1.1 Extent of Disruption

EDTA destabilises the outer membrane, forming complexes with divalent cations. The changes in the outer membrane cause weakness in the inner membrane, resulting in higher permeability of the cell after EDTA treatment. The concentration selected for pretreatment (Section 4.2.2.1) was 0.040M. Release of small amounts of soluble protein, acid phosphatase and β-galactosidase during pretreatment indicated that permeabilisation had occurred since periplasmic and intracellular enzymes were released. A low pressure was used for the homogenisation of the pretreated cells, because complete disruption was achieved with 34.5 MPa and 20 passes and the pretreatment was expected to result in a reduced pressure to achieve the same extent of disruption. Table 4.35 presents the results of the EDTA pretreatment used in combination with high pressure homogenisation at 13.8 MPa. The extent of release measured with the combination is compared to the maximum available in the cell for release from untreated bacteria, R_{max}. Figure 4.25 presents the release profiles achieved for this combination, in comparison with untreated bacteria and all other pretreatment combinations used on *Escherichia coli*. The raw data generated can be found in Appendix B.

Table 4.35 Protein release following EDTA (0.040M) pretreatment and homogenisation of *E. coli* at 13.8 MPa

	Total Soluble Protein		Acid Phosphatase		β-galactosidase	
	mg/g	%	(U/g)	%	(U/g)	%
Pretreatment	0.85		13.6		5.16	
HPH (13.8 MPa, 20 pass)	176		1050		2278	
TOTAL (13.8 MPa)	176	112	1063	121	2283	114
Untreated Bacteria						
HPH (13.8 MPa, 20 pass)	156	99.4	437	49.6	1203	60.1
HPH (34.5 MPa, 20 pass)	157	99.5	888	100	2017	100
R_{max}	**157**		**882**		**2002**	

At a pressure of 13.8 MPa and after 20 passes, the amounts of total soluble protein, acid phosphatase and β-galactosidase released were 176 mg/g, 1050 U/g and 2278 U/g respectively. The release observed exceeded release of proteins from untreated bacteria at the same pressure (Table 4.35). Due to the success and maximum release attained from treated cells at a pressure of 13.8 MPa, it was not necessary to conduct the pretreatment experiments using the higher pressure of 34.5 MPa.

4.6.1.2 Interference of Chemicals

The amount of enzyme denatured by EDTA was assessed by homogenizing a 1% (wet weight) cell concentration sample of *Escherichia coli* at 13.8 MPa and treating the homogenate with 0.040M EDTA at the temperature and duration used in the pretreatment procedure. The sample was analysed for soluble protein and enzyme release. This allowed a direct attack of the chemical on the released proteins to evaluate denaturation. The results are presented in Table 4.36. The method revealed that EDTA showed little interference with soluble protein and acid phosphatase. However, 40% β-galactosidase denaturation occurred.

Table 4.36 Interference of EDTA on proteins measured with the percentage denatured in relation to release achieved from untreated bacteria at the same pressure

	Untreated Bacteria (R_I)	EDTA	% Denatured
Protein (mg/g)	126	122	3.02
Acid Phosphatase (U/g)	427	369	13.5
β-galactosidase (U/g)	1203	712	40.8

The literature has in general not considered the potential for denaturation by the chemicals used (De Smet et *al.*, 1978; Felix, 1982). However, concentration of 0.1M EDTA has been reported to cause a 0.004 change in the optical density at 595 nm with the assay (Bradford, 1976). The experiments performed for the pretreatment used a concentration of 0.040M that was less than half of the concentration reported to interfere with the assay. This change in absorbance was corrected for by adding the chemical at the selected concentration to the blank for samples taken that contained the permeabilising solution. Therefore, interference with the protein assay by EDTA was minimal with 3% soluble protein denatured on exposure to the chemical. This result confirms that the significant

denaturation observed with the use of EDTA and Triton X-100 on both yeast and bacteria (Sections 4.5.2.2 and 4.6.2.2), in the range of 30 to 60% denaturation is mostly due to Triton X-100.

4.6.1.3 Release Rate Kinetics

The release rate constants of total soluble protein and the specific enzymes from the treated cells were calculated using Equation 4.8 and are compared to those of the untreated bacteria at the same pressure in Table 4.37.

The release rate of soluble protein following pretreatment was greater than that of untreated bacteria at 13.8 MPa, and equal to the release rate of untreated bacteria at 34.5 MPa. The release rates of acid phosphatase and β-galactosidase were considerably greater at 401 x 10^{-3} and 705 x 10^{-3} respectively with the pretreatment in comparison to untreated bacteria at 13.8 MPa with 73.1 x 10^{-3} and 91.0 x 10^{-3} at 13.8 MPa and were approximately 80% of the release rate of untreated bacteria at 34.5 MPa. For homogenisation of pretreated cells at 13.8 MPa, the cytoplasmic enzyme was released fastest, followed by soluble protein and the periplasmic enzyme, acid phosphatase. These trends do not follow the order of release for untreated bacteria, where at 13.8 MPa the soluble protein from the bacteria was released fastest, followed by β-galactosidase and acid phosphatase, while untreated bacteria homogenised at 34.5 MPa were released in the following order: β-galactosidase fastest followed by both acid phosphatase and total soluble protein. It appears that release from untreated cells may not only be dependent on the location of the enzyme in the cell, but also on pressure. At a lower pressure, the cell is gradually degraded, releasing cell wall enzymes and soluble protein and lastly intracellular enzymes. As the pressure is increased, the degradation is not as gradual and the impact greater, causing non-selective release of all intracellular material. When EDTA is used to decrease the resistance to disruption, the order of release mimics that of untreated bacteria at 34.5 MPa and the soluble protein release for both cases is equal.

Table 4.37 Release rate constants (k') and regression coefficients R^2 calculated for protein release by HPH prior to and post pretreatment with EDTA

	Protein		Acid Phosphatase		β-galactosidase	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Treatment HPH (13.8 MPa, 20 pass)						
EDTA	521	1.00	401	0.89	705	1.00
Untreated Bacteria	244	0.96	73.1	0.96	91.0	0.86
Treatment HPH (34.5 MPa, 20 pass)						
Untreated Bacteria	516	0.94	506	0.96	867	0.97

4.6.1.4 Energy Efficiency

Energy required was calculated using Equation 4.1 as discussed in Section 4.3.1. The use of EDTA as a pretreatment method in combination with high pressure homogenisation has shown significant decrease in energy consumption. Table 4.38 shows an energy reduction of 60% with the use of the combination on release of soluble protein. Maximum release of soluble protein was obtained in 4 passes at 13.8 MPa with the pretreatment compared to 4 passes at 34.5 MPa for untreated bacteria. Maximum acid phosphatase release was obtained with the pretreatment in 8 passes at 13.8 MPa resulting in a 20% reduction in energy usage. The release of β-galactosidase reached a maximum in 4 passes at 13.8 MPa resulted in a 60% reduction in energy usage. Clearly, this method is advantageous for its increased release and decreased energy consumption.

Table 4.38 Energy efficiency calculated for maximum intracellular protein release with EDTA pretreatment combined with HPH

	HPH Conditions		EDTA + HPH Conditions		% Energy Reduction
		Energy (MJ/m^3)		Energy (MJ/m^3)	
Total Soluble Protein	4 passes, 34.5 MPa	138	4 passes, 13.8 MPa	55.2	60
Acid Phosphatase	4 passes, 34.5 MPa	138	8 passes, 13.8 MPa	110	20
β-galactosidase	4 passes, 34.5 MPa	138	4 passes, 13.8 MPa	55.2	60

4.6.1.5 Discussion

EDTA has been successful in decreasing the resistance of bacteria to disruption. The EDTA forms complexes with divalent cations resulting in destabilisation of the outer

membrane. The changes in the outer membrane cause weaknesses in the inner membrane resulting in a high permeability of the cell after EDTA treatment (Felix, 1982). EDTA has been known to destabilise the outer membrane of *E. coli* cells and increase the release of intracellular dehydrogenases by 20%, compared to the absence of EDTA (De Smet *et al.*, 1978).

The above results and discussion of the use of EDTA as a pretreatment method combined with HPH has proved to be successful with maximum release achieved with disruption at 13.8 MPa, whereas untreated bacteria required a pressure of 34.5 MPa to achieve release of proteins. The enhanced intracellular release at a lower pressure with minimal denaturation of proteins on exposure to the chemical renders this method effective as a pretreatment method combined with HPH for increased release. Sufficient removal of the chemicals is necessary to minimise any potential interference of the chemical. The method is advantageous with increased release at a lower pressure, resulting in decreased energy requirement of 60% on release of soluble protein and decreased micronisation of cell debris.

4.6.2 Bacteria Cell Disruption by HPH following Pretreatment with EDTA and Triton X-100

4.6.2.1 Extent of Disruption

Triton X-100 is known for its ability to permeabilise the cell membrane. Its action is augmented on membrane destabilisation with EDTA. The concentrations selected were 0.040M EDTA and 2% Triton X-100. Table 4.39 presents the results of the pretreatment in combination with high pressure homogenisation at 13.8 MPa. The extent of release measured with the combination is compared to the maximum available in the cell for release from untreated bacteria, R_{max}. Figure 4.25 presents the release profiles achieved for this combination, in comparison with untreated bacteria and all other pretreatment combinations used on *Escherichia coli*. The raw data generated can be found in Appendix B.

The amount of total soluble protein released by HPH following pretreatment was 1.61 mg/g bacteria, accounting for only 1.04% of the maximum available. Similarly, the release of 24.0 U/g bacteria β-galactosidase equated to 1.20% of the R_{max}. The acid phosphatase release was 0.47% of the total available at a value of 4.07 U/g bacteria.

After the pretreatment, the cells formed a gelatinous substance in comparison to the normal paste-like material of untreated *Escherichia coli* after the incubation. This gelatinous nature is typical of DNA release without shear.

Table 4.39 Protein release following EDTA (0.040M) and Triton X-100 (2%) pretreatment and homogenisation of *E. coli* at 13.8 MPa with comparison to R_{max}

	Total Soluble Protein		Acid Phosphatase		β-galactosidase	
	mg/g	%	(U/g)	%	(U/g)	%
Pretreatment	0.45		16.3		0.26	
HPH (13.8 MPa, 20 pass)	1.61		4.07		24.0	
TOTAL (13.8 MPa)	2.06	1.31	20.3	2.30	24.3	1.21
Untreated Bacteria						
HPH (13.8 MPa, 20 pass)	156	99.4	437	49.6	1203	60.1
HPH (34.5 MPa, 20 pass)	157	99.5	888	100	2017	101
R_{max}	**157**		**882**		**2002**	

4.6.2.2 Interference of Chemicals

Table 4.40 presents the percentage denaturation of the enzymes due to the chemicals. Significant enzyme denaturation of acid phosphatase and β-galactosidase and 30% denaturation of soluble protein occurred. The latter does not correspond to the 1.3% protein release achieved during the treatment combination. A 67% protein denaturation was observed on yeast homogenate (Section 4.5.2.2). Due to the data not being performed in triplicate and no error data being available, the amount of denaturation could lie within the two values found. The presence of the gelatinous material, typical of DNA release suggests that the low protein concentrations determined was due to interference with the enzyme assay, rather than release. Micrographs taken of the cells after pretreatment and prior to homogenisation show that amalgamated mass of cells, together and no individual

cell is clearly visible. The micrographs, presented in Section 4.6.4 show that disruption has taken place, as cell debris is present in Figures 4.23b and c. Since the micrographs show disruption, the reduced protein concentration detected after homogenisation is due to denaturation. These results are consistent with those found with EDTA and Triton X-100 as a pretreatment method used on Baker's yeast, where significant deactivation of enzymes was detected if the chemicals were not removed sufficiently.

Table 4.40 Interference of EDTA and Triton X-100 on proteins measured with the percentage denatured in relation to release achieved from untreated bacteria at the same pressure

	Untreated Bacteria (R_i)	EDTA and Triton X-100	% Denaturation
Protein (mg/g)	126	87.3	30.8
Acid Phosphatase (U/g)	427	6.78	98.4
β-galactosidase (U/g)	1203	67.0	94.4

4.6.2.3 Release Rate Kinetics

Due to considerable reduction in the protein concentrations determined following disruption of the treated cells, owing to protein denaturation, the release rates calculated were not meaningful.

4.6.2.4 Discussion

Previously, Schnaitman (1976b) reported that the treatment of *Escherichia coli* cell wall by Triton X-100 resulted in 15 to 25% solubilisation of the protein, whereas treatment on the cell membrane resulted in 60 to 80% protein solubilisation. The combination treatment was expected to enhance the release of intracellular material with EDTA destabilising the outer membrane and allowing the Triton X-100 to attack the cell membrane. This combination was used for extraction of protein and resulted in solubilisation of half of the cell wall protein but drastic changes in the morphology of the EDTA-Triton insoluble material. The EDTA is believed to be able to destabilise the outer membrane and cause a weakness in the cell wall to a point where it is susceptible to mechanical damage of the LPS and protein components by detergents (Schnaitman, 1971a). The severe changes in

morphology of the cells are analogous with the findings of this thesis with the EDTA and Triton X-100 pretreatment procedure producing gelatinous cells.

The above results and discussion indicate that use of EDTA and Triton X-100 as a pretreatment method combined with HPH has proved to be unsuccessful with minimal release achieved with disruption at 13.8 MPa. Since the use of EDTA as a pretreatment method resulted in maximum release, the addition of the Triton X-100 appears to result in severe adverse effects including a changed morphology, as well as significant protein denaturation.

4.6.3 Bacteria Cell Disruption by HPH following Pretreatment with G-HCl and Triton X-100

4.6.3.1 Extent of Disruption

Guanidine hydrochloride is known to inhibit the cross linking of peptidoglycan and therefore cell wall synthesis. It also solubilises protein from membrane fragments and alters the hydrophobic interactions. Triton X-100 was used to permeabilise the cell membrane. From study of pretreatment conditions (Section 4.2.2.2), the optimum concentrations determined were 0.1M G-HCl and 2% Triton X-100. Protein release following homogenisation of the pretreated bacteria is compared to the maximum available, R_{max} in Table 4.41. Release from the untreated bacteria has been presented as a percentage of the R_{max} to provide comparison Figure 4.25 presents the release profiles achieved for this combination, in comparison with untreated bacteria and all other pretreatment combinations used on *E. coli*. The raw data generated can be found in Appendix B.

Table 4.41 shows that the use of the pretreatment with homogenisation at 13.8 MPa increases the release of proteins when compared with untreated bacteria homogenised at the same pressure. The pretreatment resulted in maximum release achieved at a pressure of 13.8 MPa in terms of soluble protein and acid phosphatase. The amount of β-galactosidase released was 1.4 times greater than the release from untreated bacteria at 13.8 MPa and

89% of the maximum available (R_{max}). Only a very small increase in pressure would be required to cause the maximum release of cytoplasmic enzymes and therefore, the pressure of 34.5 MPa was not investigated.

Table 4.41 Protein release following G-HCl (0.1M) and Triton X-100 (2%) pretreatment and untreated homogenisation of *E. coli* at 13.8 MPa with a comparison of the release to R_{max}

	Total Soluble Protein		Acid Phosphatase		β-galactosidase	
	mg/g	%	(U/g)	%	(U/g)	%
Pretreatment	31.6		40.7		19.4	0.97
HPH (13.8 MPa, 20 pass)	138		915		1771	88.5
TOTAL (13.8 MPa)	170	108	955	108	1790	89.5
Untreated Bacteria						
HPH (13.8 MPa, 20 pass)	156	99.4	437	49.6	1203	60.1
HPH (34.5 MPa, 20 pass)	157	99.5	888	101	2017	101
R_{max}	**157**		**882**		**2002**	

4.6.3.2 Interference of Chemicals

Table 4.42 presents the percentage denaturation of the enzymes due to the chemical. The data show a small amount of denaturation of soluble protein, and slightly larger amounts of acid phosphatase and β-galactosidase. Underestimation of protein may result from reduced dye binding in the Bradford assay. Competition with the dye for the protein has been observed with guanidine hydrochloride leading to protein underestimation. These competitive effects may be compensated through their inclusion in the standard calibration (Compton and Jones, 1985). Introduction of the chemicals into the blank at the appropriate concentration was carried out where samples were taken and included the permeabilising solution. After the washing procedure, it was expected that all chemicals had been removed and therefore no inclusion of the chemicals in the blanks of these samples was necessary.

Table 4.42 Interference of G-HCl and Triton X-100 on proteins measured with the percentage denatured in relation to release achieved from untreated bacteria at the same pressure

	Untreated Bacteria (R_i)	G-HCl and Triton X-100	% Denatured
Protein (mg/g)	126	116	7.55
Acid Phosphatase (U/g)	427	357	16.5
β-galactosidase (U/g)	1203	923	23.3

4.6.3.3 Release Rate Kinetics

The kinetics of protein on the high pressure homogenisation of treated cells was modelled according to Equation 4.8, as discussed in Section 4.4. The release rates of the total soluble protein and specific enzymes from the treated cells were compared to those of the untreated bacteria at the same pressure in Table 4.43. The release rates of all proteins were higher then untreated bacteria at the same pressure of 13.8 MPa, and lower than the release rates of untreated bacteria at 34.5 MPa. Pretreatment with G-HCl and Triton X-100 has shown enhanced release in comparison to untreated bacteria at the same pressure. The order of release showed that soluble protein was released fastest at 268 x 10^{-3}, followed by 129 x 10^{-3} for β-galactosidase and lastly acid phosphatase. The trends observed with this method do not follow those observed by Follows *et al.* (1971), but due to the breakage of the cells after pretreatment with the chemicals, confirmed by micrographs (Section 4.6.4), the release of soluble protein is expected to be fastest and since breakage has already occurred, there is no selective release of periplasmic or cytoplasmic enzymes on further homogenisation.

Table 4.43 Release rate constants (k') and regression coefficients R^2 calculated protein release by HPH prior to and post pretreatment with G-HCl and Triton X-100

	Protein		Acid Phosphatase		β-galactosidase	
	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2	k' x 10^{-3}	R^2
Treated Bacteria, HPH (13.8 MPa, 20 pass)						
G-HCl / Triton X-100	268	1.00	111	0.96	129	0.97
Untreated Bacteria						
HPH (13.8 MPa, 20 pass)	244	0.96	73.1	0.96	90.7	0.86
HPH (34.5 MPa, 20 pass)	516	0.94	506	0.96	867	0.97

4.6.3.4 Energy Efficiency

The energy calculations were performed using Equation 4.1. The use of G-HCl and Triton X-100 as a pretreatment method in combination with high pressure homogenisation resulted in increased release at 13.8 MPa in comparison to untreated bacteria at the same pressure. An energy reduction of 30% was noted on 50% release of β-galactosidase, 20% reduction for release of 50% total soluble protein and acid phosphatase with the use of the combination when compared with the same amount of protein release from untreated bacteria at the same pressure (Table 4.44). The method is advantageous for increased release and decreased energy consumption.

Table 4.44 Energy efficiency for 50% intracellular release from untreated bacteria and with G-HCl and Triton X-100 combined with HPH

	HPH Conditions		G-HCl and Triton X-100 + HPH Conditions		% Energy Reduction
		Energy (MJ/m^3)		Energy (MJ/m^3)	
Total Soluble Protein	2 passes, 34.5 MPa	69.0	4 passes, 13.8 MPa	55.2	20
Acid Phosphatase	2 passes, 34.5 MPa	69.0	4 passes, 13.8 MPa	55.2	20
β-galactosidase	2 passes, 34.5 MPa	69.0	3.5 passes, 13.8 MPa	48.3	30

4.6.3.5 Discussion

The use of these chemicals for bacterial permeabilisation has shown enhanced release of periplasmic proteins with 50% extraction of intracellular protein (Naglak and Wang, 1990). A pronounced synergistic effect of the two chemicals has shown dramatic protein release yields. The permeabilisation involves a solubilisation of the inner membrane and an outer wall alteration occurring on a molecular level, not detectable by microscopy. The process is dominated by G-HCl since the use of the chemical on its own results in 20% protein release and the use of Triton X-100 enhances the protein release such that protein release increases to 35% (Hettwer and Wang, 1989).

The permeabilisation of *Escherichia coli* by guanidium hydrochloride has been investigated and in some cases, the permeabilisation has been used in combination with high pressure homogenisation to produce a combined effect that increases the release of

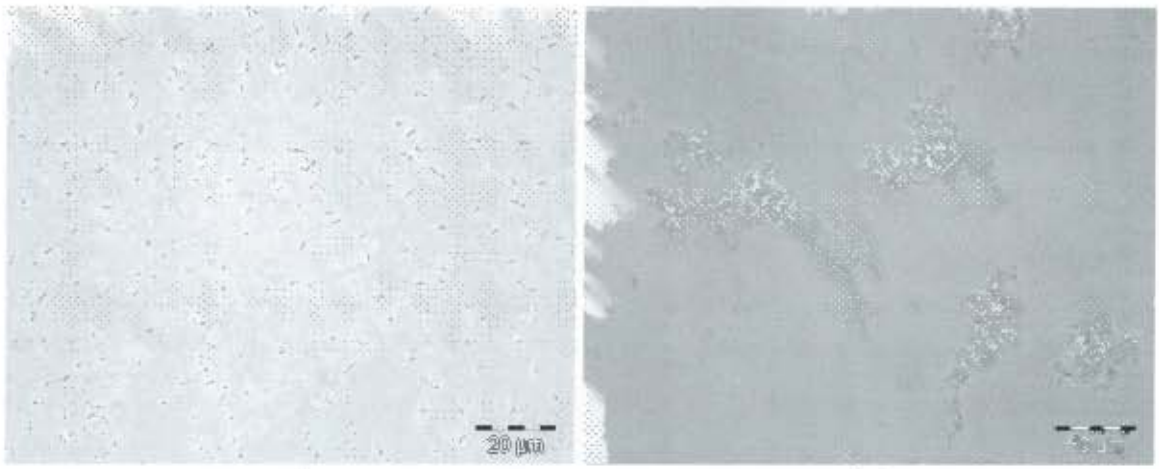
intracellular proteins. The G-HCl and Triton X-100 method has resulted in increased intracellular release compared to untreated bacteria at the same pressure, with maximum release of soluble protein and acid phosphatase achieved. This increase in release at a lower pressure has resulted in reduced energy requirements of 20% on the release of soluble protein and acid phosphatase and 30% on release of β-galactosidase. Micrographs of the disruption of the cells by HPH after treatment with G-HCl and Triton X-100 are presented in Section 4.6.4. These micrographs show that the bacterial cells are disrupted during the pretreatment process, and no intact cells are viable. On homogenisation of the cells for further breakage, cell debris is present.

4.6.4 Microscopic Observation of Cell Damage on HPH with Pretreatment of *Escherichia coli*

Micrographs of the pretreated and untreated cells were taken to determine the physical damage to the cell structure caused by the separate pretreatment and mechanical processes. The effect of the combination of pretreatment and mechanical disruption can be compared with micrographs of untreated bacteria presented in Section 4.3.2.

EDTA

Figure 4.22 provides micrographs of *Escherichia coli* following pretreatment with EDTA and following subsequent homogenisation at 13.8 MPa. In Figure 4.22a, the cells were intact and there were no signs of breakage in the cells, due to the treatment. Figure 4.22b shows the bacteria at the 12^{th} pass through the homogeniser at a pressure of 13.8 MPa, with clear cell breakage and cell debris is present, there are no intact cell remaining. In The evidence of complete cell breakage supports the discussion in Section 4.6.1.1 that increased release from treated cells at 13.8 MPa is due to complete cell breakage and release of all available intracellular material.



(a) (b)

Figure 4.22 Degradation of *Escherichia coli* by HPH at 13.8 MPa following pretreatment with EDTA (a: pretreated cells prior to homogenisation, b: cells at 12th pass)

EDTA and Triton X-100

Figure 4.23 provides micrographs of *Escherichia coli* following treatment with EDTA and Triton X-100 and following subsequent homogenisation at 13.8 MPa. Figure 4.23a shows the disruption of bacteria after treatment with EDTA and Triton X-100, where the cells have formed an amalgamated mass, of a gelatinous nature. No intact, individual cells are visible. Figures 4.23b and c show the disruption at the 12th and 20th pass through the homogeniser, where cell debris is present indicating that disruption had occurred and therefore the reduced extent of protein from treated cells to untreated cells is due to denaturation of the proteins by the chemicals. This has been confirmed for both yeast and bacteria pretreatment with EDTA and Triton X-100.

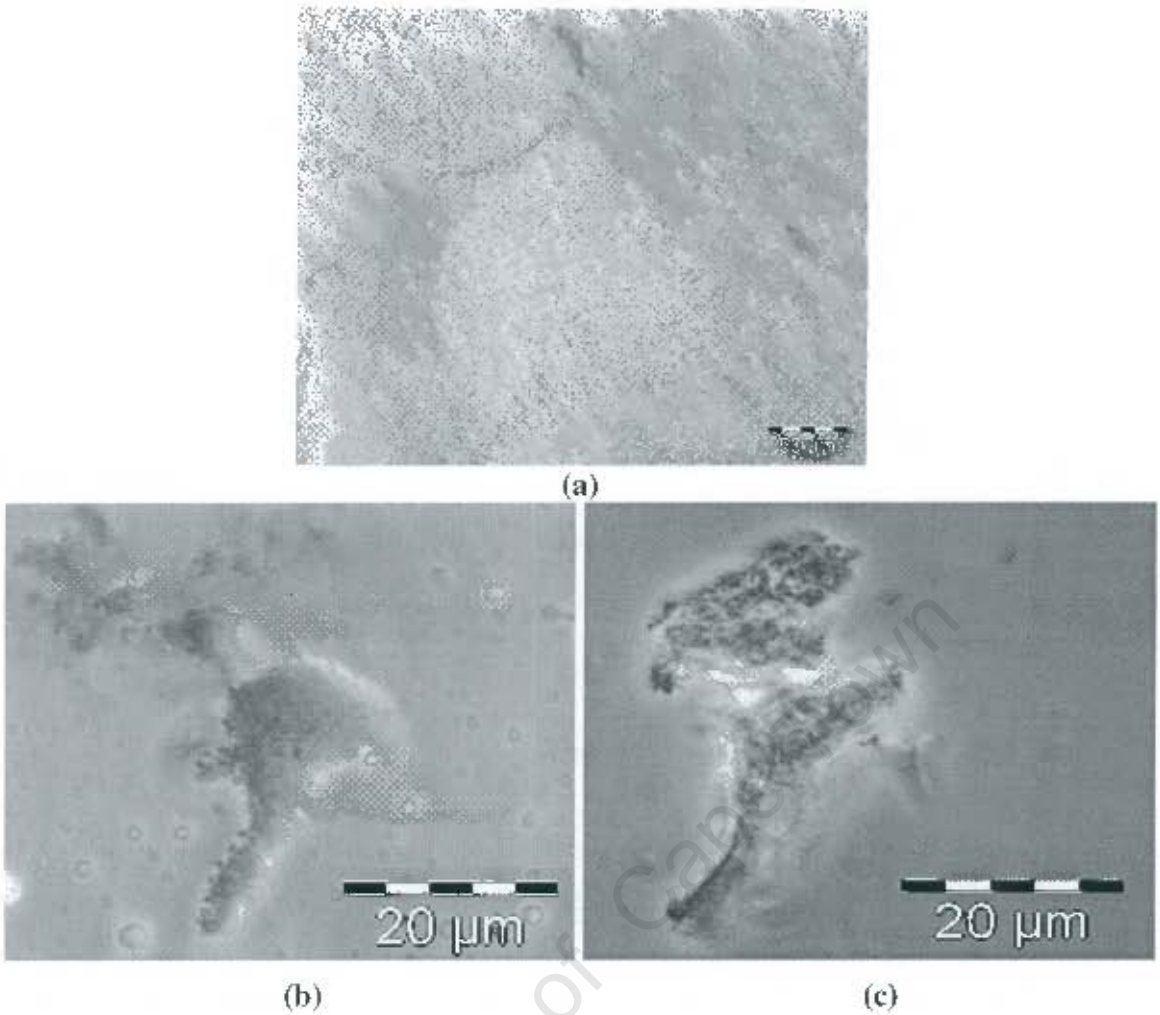
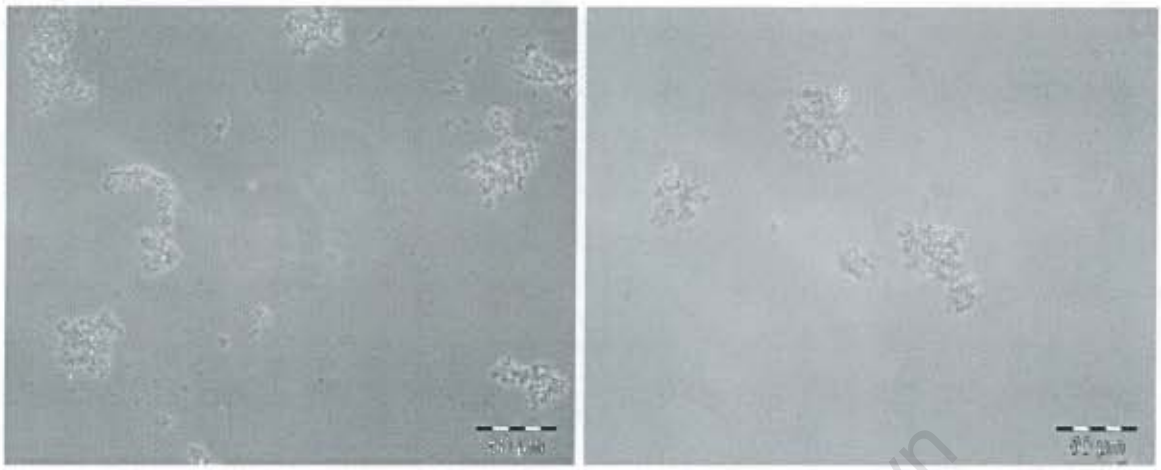


Figure 4.23 Degradation of *Escherichia coli* by HPH at 13.8 MPa following pretreatment with EDTA and Triton X-100 (a: pretreated cells prior to homogenisation, b: cells at 12th pass, c: cells at 20th pass)

G-HCl and Triton X-100

Figure 4.24 provides the micrographs of *Escherichia coli* following pretreatment with G-HCl and Triton X-100 and following subsequent homogenisation at 13.8 MPa. Figure 4.24a shows the cells after pretreatment and prior to homogenisation, where cell breakage by the pretreatment was evident and no intact cells are present. Figure 4.24b shows the bacteria at the 12th pass through the homogeniser at a pressure of 13.8 MPa, with micronisation of cell debris. The evidence of complete cell breakage supports the discussion in Section 4.6.3.1 that increased release from treated cells at 13.8 MPa is due to complete cell breakage and release of the maximum available soluble protein and acid phosphatase. The breakage of cells during the pretreatment process results in a reduction

in the pressure required for complete breakage and subsequent reduced energy requirement.



(a) (b)

Figure 4.24 Degradation of *Escherichia coli* by HPH at 13.8 MPa following pretreatment with G-HCl and Triton X-100 (a: pretreated cells prior to homogenisation, b: cells at 12th pass)

4.6.5 Summary of Combined Pretreatment and High Pressure Homogenisation Methods with Bacteria

The release of total soluble protein with number of passes followed the same trend for untreated bacteria and bacteria subjected to all pretreatments shown in Figure 4.25. Maximum release of untreated bacteria was obtained in four passes through the homogeniser at 34.5 MPa, however, the R_{max} was not achieved at 13.8 MPa, even though the release stabilised such that an increase in the number of passes did not further release. The release of total soluble protein using the EDTA pretreatment at 13.8 MPa achieved the maximum available, mimicking the release profile of 34.5 MPa. Increased release at 13.8 MPa was found with the G-HCl and Triton X-100 treatment in comparison to untreated bacteria homogenized at the same pressure. The results of these two pretreatments have shown that the use of pretreatments in combination with homogenisation can result in the increased release of intracellular material, requiring fewer passes in the homogeniser or a lower operating pressure, thereby decreasing the

energy consumption of the high pressure homogeniser. The EDTA and Triton X-100 pretreatment did not result in increased release due to extensive protein denaturation. The release of acid phosphatase and β-galactosidase followed the same trends for all pretreatments tested, indicating that the increased release is extended to periplasmic and cytoplasmic enzymes. Energy efficiency was observed with the use of EDTA and G-HCl and Triton X-100.

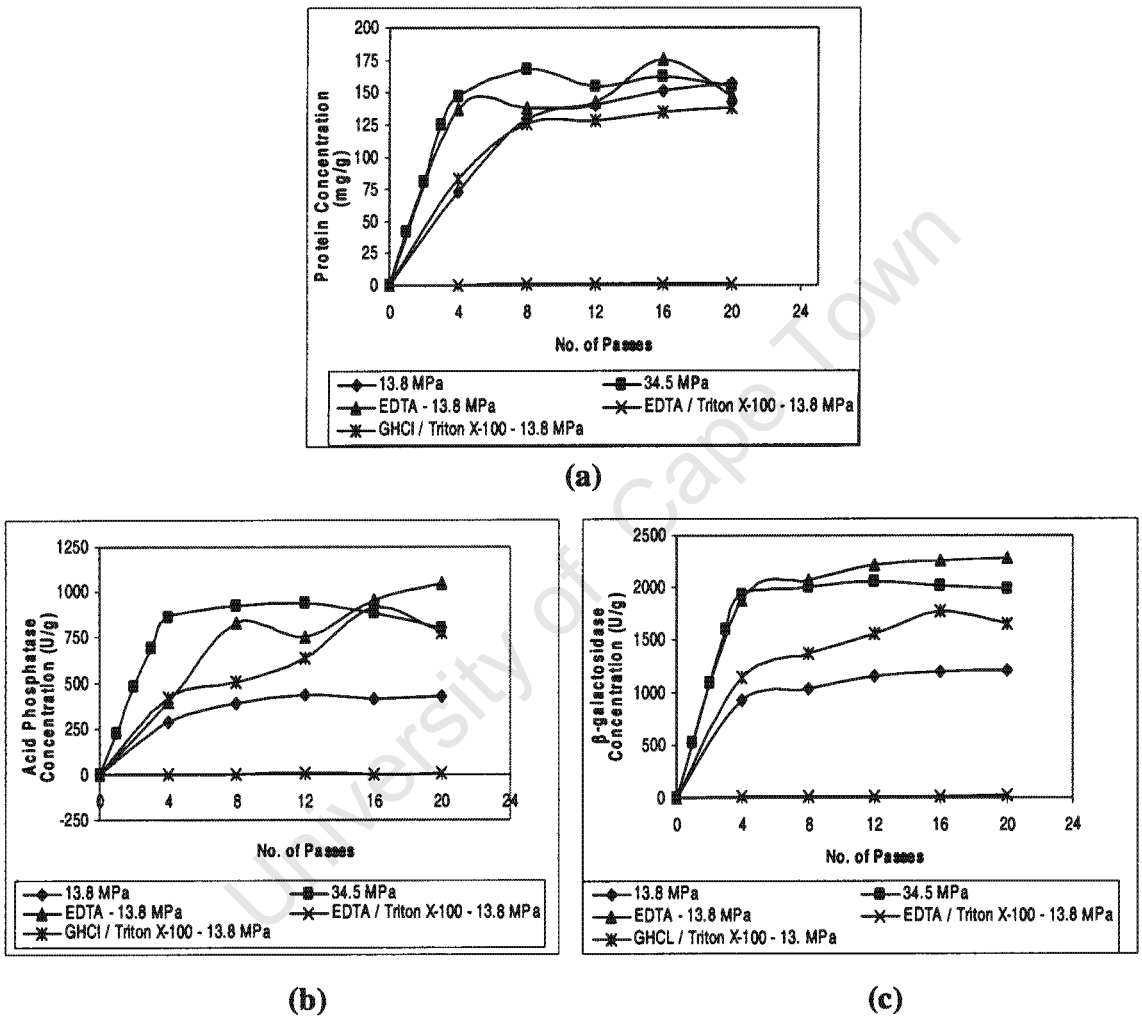


Figure 4.25 Release of total soluble protein, acid phosphatase and β-galactosidase as a function of the number of passes for all pretreatments combined with homogenisation at 13.8 MPa and untreated bacteria at 13.8 MPa and 34.5 MPa

The use of EDTA as a pretreatment method in combination with HPH has proved to be successful with maximum release of proteins achieved on homogenisation of treated cells at 13.8 MPa, whereas maximum release was obtained at 34.5 MPa for untreated bacteria.

The increased release at a reduced pressure resulted in decreased energy consumption up to 60% for the release of soluble protein. Clearly, this method is advantageous due to increased release, decreased energy and micronisation of cell debris. The interference of EDTA with the proteins was also found to be minimal, with the exception of β-galactosidase.

On treatment of *Escherichia coli* with EDTA and Triton X-100, the expected increased release trends were not observed. A gelatinous cell mixture was obtained after treatment of the cells with the chemicals and significant protein denaturation was observed. Similar results were also found on treatment of Baker's yeast with EDTA and Triton X-100, however, the yeast cell morphology was not altered. EDTA and Triton X-100 is therefore not a feasible pretreatment method due to the complications arising from interference of the chemicals and denaturation of proteins.

G-HCl and Triton X-100 treatment on bacterial cells was successful with increased intracellular release during subsequent homogenisation of the cells at 13.8 MPa. Cell breakage after pretreatment and maximum release of soluble protein and acid phosphatase after homogenisation was observed, resulting in a reduced energy requirement of up to 40% on release of β-galactosidase. The chemicals do cause denaturation of the proteins, but if sufficient washing to remove the chemicals is achieved, the denaturation is minimised.

4.7 Effect of Pretreatment on Baker's yeast with Hydrodynamic Cavitation

Following experimentation with Baker's yeast and *Escherichia coli* using the high pressure homogeniser, two pretreatments were selected for use in combination with another mechanical disruption method, hydrodynamic cavitation. Bacteria were not used for disruption by hydrodynamic cavitation due to the large amounts of cells required for disruption and the ready availability of yeast. The use of lyticase as a pretreatment for use in combination with hydrodynamic cavitation was not a feasible option due to the expense of the enzyme and large amounts required in hydrodynamic cavitation. The ethanol and toluene method was not successful in increasing intracellular release due to significant

protein denaturation and therefore was not studied further. The use of EDTA and CTAB resulted in strengthening of the cells and increased resistance to disruption, confirmed by micrographs of the treated cells during homogenisation and therefore this method was also not a good pretreatment choice to increase intracellular release. Hence, the remaining pretreatment, EDTA and Triton X-100 was chosen to be used in combination with hydrodynamic cavitation. The same conditions were used as in the previous experiments, however due to the large scale of the hydrodynamic cavitation apparatus, the pretreatment was conducted using a more concentrated cell suspension and diluted thereafter to the required cell concentration. These alterations to the method have been outlined in Section 3.4.4.

Hydrodynamic cavitation was tested with the two orifice plates previously shown to give the best protein release results (Balasundaram, 2004). In general, increased cavitation is observed with decreasing cavitation number but at very low cavitation numbers, a maximum is observed. The two plates used fall within a very narrow region, including the maximum ($C_v = 0.13$) and a point just before the maximum ($C_v = 0.09$). The pretreatment of EDTA and Triton X-100 was tested on each orifice plate. The release of proteins was measured after permeabilisation and subsequently during hydrodynamic cavitation at every 100^{th} pass for 1000 passes.

4.7.1 Yeast Cell Disruption by Hydrodynamic Cavitation following Pretreatment with EDTA and Triton X-100

4.7.1.1 Extent of Release

Table 4.45 presents the extent of protein release with pretreatment used in combination with hydrodynamic cavitation with cavitation numbers of 0.13 and 0.09. Figure 4.27 presents the release profiles achieved for this combination, compared with untreated yeast at both cavitation numbers. The raw data generated in these experiments can be found in Appendix B.

The amounts of total soluble protein, α-glucosidase and G6PDH released by hydrodynamic cavitation following pretreatment are greater at both cavitation numbers than in the absence of pretreatment. The total soluble protein increased from 4.0% to 8.8% with a cavitation number of 0.13. The release of G6PDH at a cavitation number of 0.09, increased from 1.6% to 13.5%. At a cavitation number of 0.13, the increase was from 4.9% to 13.6%. The release of α-glucosidase increased from 0.2% to 3.6% at a cavitation number of 0.09 and from 0.04% to 4.8% at a cavitation number of 0.13. Increased release of invertase was not apparent by the use of pretreatments. At both cavitation numbers, the amount released was lower than the release of invertase from untreated yeast.

Table 4.45 Protein release following EDTA and Triton X-100 pretreatment and hydrodynamic cavitation of Baker's yeast with a comparison of the release to R_{max}

	Total Soluble Protein (mg/g)		Invertase (U/g) x 10^2		α-glucosidase (U/g) x 10^5		G6PDH (U/g)	
	R_I	%	R_I	%	R_I	%	R_I	%
Pretreatment	5.08		0.85		1.52		0.39	
Treated Yeast								
EDTA / Triton: Cv =0.09	7.86		1.41		3.57		0.57	
EDTA / Triton: Cv =0.13	8.51		4.18		6.10		0.58	
TOTAL (Cv = 0.09)	12.9	8.36	2.26	0.51	5.09	3.17	0.96	13.48
TOTAL (Cv = 0.13)	13.6	8.77	5.03	1.15	7.62	4.75	0.97	13.59
Untreated Yeast								
Cv = 0.09	5.61	3.62	2.57	0.59	0.15	0.09	0.11	1.61
Cv =0.13	6.23	4.03	11.6	2.65	6.68	4.16	0.35	4.93
HPH R_{max}	**155**		**438**		**161**		**7.08**	

4.7.1.2 Interference of Chemicals

Denaturation of proteins with EDTA and Triton X-100 has been observed. The denaturation can be avoided or minimised by effective removal of the chemicals.

4.7.1.3 Discussion

The cavitation number is the ratio of forces collapsing cavities to those forces that form cavities. Total collapse pressure of the cavities is greater than the cavity formation forces

at low cavitation numbers, resulting in increased intensity of cavitation. Cavitating conditions increase as the cavitation number is decreased. This increased intensity is a result of reduced flow area and increased orifice velocity (Balasundaram, 2004). Optimum cell disruption is expected at maximum collapse pressure and will pass through a maximum with cavitation number due to the opposing effects of cavitation number on collapse pressure and the number of cavities (Balasundaram, 2004). Since maximum protein release was observed at a cavitation number of 0.13, the orifice plate corresponding to this cavitation number was used. The cavitation number and corresponding orifice plate which gave the next best release was 0.09 (Balasundaram, 2004). Therefore, the cavitation numbers used in hydrodynamic cavitation for this study correspond to cavitation numbers in a very narrow region, which resulted in maximum release. It is due to this region that the opposing trends of increased disruption at a higher cavitation number are observed.

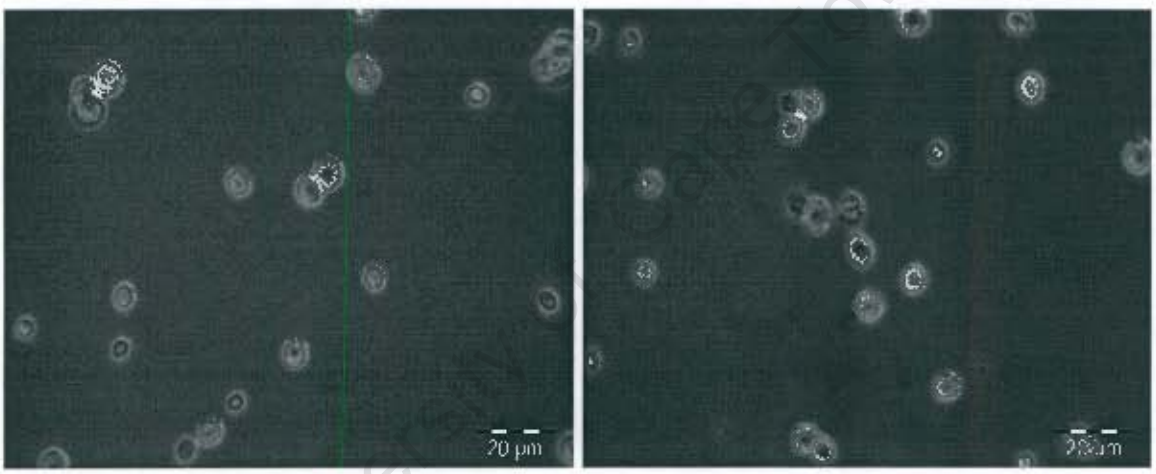
The expected increase in release of proteins is apparent in the release of total soluble protein, α-glucosidase and G6PDH on treatment of the cells with EDTA and Triton X-100 and subsequent hydrodynamic cavitation. The decreased release of invertase with pretreatment in comparison to untreated yeast is possibly due to enzyme denaturation. The wall location of invertase results in it experiencing extended contact with the pretreatment chemicals prior to washing and mechanical disruption.

The use of EDTA and Triton X-100 as a pretreatment method in combination with hydrodynamic cavitation to increase intracellular release involves a number of considerations. The method has previously shown significant protein denaturation on exposure of the chemicals to proteins. The denaturation can be minimised if there is complete removal of the chemicals by washing the cells prior to mechanical disruption. Due to the large volume treated for hydrodynamic cavitation, complete washing off of the chemicals is difficult and tedious to ensure.

The following shows the micrographs of treated cells, including descriptions of the effect of the process on the cells. The effect of the combination of pretreatment and mechanical disruption methods can be compared with untreated yeast micrographs presented in Section 4.3.3.

EDTA and Triton X-100 (C_v = 0.13)

Figure 4.26 provides the micrographs of Baker's yeast following pretreatment with EDTA and Triton X-100 and following subsequent hydrodynamic cavitation, with a cavitation number of 0.13. Figure 4.26a and b show the yeast at the 500th and 1000th pass respectively and breakage is not clearly visible by light microscopy under phase contrast, with cell debris is present. Optical light microscopy does not explicitly show the breakage of cells by hydrodynamic cavitation as already shown in Figure 4.11, which does not appear to be different from Figure 4.26. Figure 4.12, presented in Section 4.3.3, showed a transmission electron micrograph of Baker's yeast at the 1000th pass disrupted at a cavitation number of 0.13. Clear breakage of the cell wall can be seen, indicating that disruption by hydrodynamic cavitation occurs.



(a) (b)

Figure 4.26 Degradation of Baker's yeast by hydrodynamic cavitation (Cv = 0.13) following pretreatment with EDTA and Triton X-100 (a: pretreated cells at 500th pass, b: cells at 1000th pass)

4.7.2 Summary of Combined Pretreatment and Hydrodynamic Cavitation Pretreatment Methods with Baker's Yeast

The release profiles in Figure 4.27 shows the enhanced release of total soluble protein and G6PDH with both cavitation numbers. The release of invertase was not increased with the pretreatment used. The rate of α-glucosidase release was enhanced following pretreatment

at a cavitation number of 0.13. However, the extent of release at 600 to 1000 passes is equal to that of untreated yeast. At a cavitation number of 0.09, an increase in the release of α-glucosidase is evident across 1000 passes. The use of EDTA and Triton X-100 is complicated with interference of the chemicals and subsequent denaturation of proteins and the difficult removal of the chemicals after the pretreatment due to the large volumes used in hydrodynamic cavitation. The pretreatment procedure requires careful selection to minimise protein denaturation.

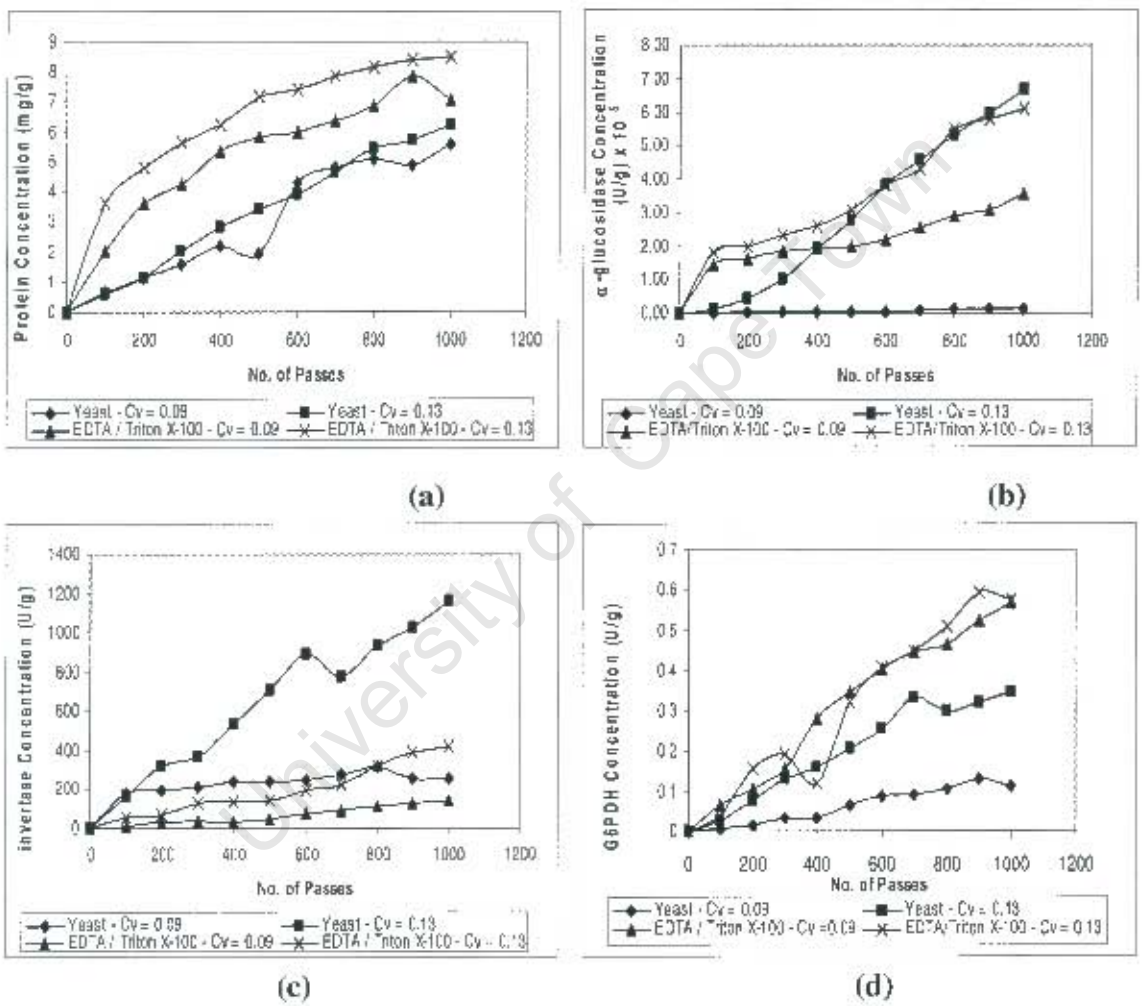


Figure 4.27 Release of total soluble protein, α-glucosidase, invertase and G6PDH as a function of the number of passes for all pretreatments combined hydrodynamic cavitation with cavitation numbers of 0.09 and 0.13

4.8 Summary and Conclusions

The use of pretreatment needs to be a careful selection process in which all effects of the chemicals on the proteins and cell morphology must be investigated. The use of pretreatment can be useful to enhance the mechanical disruption process with clear advantages in terms of reducing extensive fragmentation, decreased energy requirements for the process with increased release in some instances. Optimisation of the permeabilisation process is necessary to avoid the denaturation of proteins. A number of variables can be altered to investigate the optimum conditions, pH, temperature, treatment time and concentration of the chemicals. The removal of the chemicals is an important consideration for the downstream processing and for maximising the desired product. The removal of the chemicals is necessary to avoid irreversible loss of product activity and suboptimum yields that may result. The optimum pretreatment conditions must be defined in order to balance the advantages and disadvantages depending on the desired product.

The disruption of yeast and bacterial cells by high pressure homogenisation is a common technique for the release of intracellular material. The method is energy intensive and results in micronisation of the cell debris at high pressures. The use of pretreatments to weaken or permeabilise the cell envelope is expected to decrease the pressure required to achieve maximum release, thereby decreasing the energy required and micronisation of the cell debris.

Four pretreatments were selected for use on Baker's yeast in combination with HPH. All pretreatments were expected to result in increased intracellular release in comparison to untreated cells at the same pressure. The ethanol and toluene pretreatment method combined with HPH at 13.8 MPa resulted in a considerable reduction in the release of intracellular material compared to untreated yeast at 13.8 MPa. A thorough washing procedure was carried out after pretreatment and prior to homogenisation to ensure removal of the chemicals. However, despite the removal of the chemicals, the amount of intracellular material released was significantly lower than that of untreated yeast. It is believed that during the pretreatment process, the chemicals permeated the cell causing considerable protein denaturation leading to a reduced extent of disruption and release rates. The interference of the chemicals with the assays proved that the chemicals had an

adverse effect on the proteins with up to 90% or more denaturation of the proteins on exposure to the chemicals.

EDTA and Triton X-100 were used to pretreat yeast and bacteria in combination with HPH and hydrodynamic cavitation. Release on homogenisation of Baker's yeast at 13.8 MPa was much lower than that of untreated cells, while homogenisation of the treated cells at 34.5 MPa resulted in maximum release of the proteins. The difference has been attributed to insufficient washing off of the chemicals, which caused denaturation of the proteins, especially since complete breakage of the cells was confirmed by micrographs. On treatment of the bacteria, the cells formed an amalgamated mass of a gelatinous nature. The gelatinous behaviour is typical of DNA release and therefore the reduced extent of disruption observed on homogenisation of treated bacterial cells compared to untreated bacteria is due to the denaturation of proteins by the chemicals. The interference of the chemicals was confirmed by directly exposing the proteins to the chemicals. The treated yeast cells used in combination with hydrodynamic cavitation showed increased release of soluble protein and G6PDH, but decreased invertase release. Once again, evidence of protein denaturation was apparent.

Interesting results were seen with treatment of Baker's yeast with EDTA and CTAB, where the treatment procedure strengthened the cells and increased their resistance to disruption. The extent of disruption achieved for treated cells homogenised at both 13.8 and 34.5 MPa was much lower than release from untreated cells, and no increase in release was observed on increase in pressure during homogenisation of the treated cells. The micrographs confirmed the absence of disruption and breakage of cells by homogenisation at both pressures.

The enzyme lyticase was used as a pretreatment method in combination with HPH on Baker's yeast. The release achieved at 13.8 and 34.5 MPa did not significantly increase the extent of disruption.

The use of EDTA to reduce the resistance of bacteria to disruption was successfully achieved with maximum release of proteins on homogenisation of the treated cells at 13.8 MPa, whereas maximum release from untreated cells required a pressure of 34.5 MPa. The increased release at the reduced pressure resulted in decreased energy requirements up

to 60% and reduction in the micronisation of the cell debris. The chemical was found to cause minimal interference with soluble protein and acid phosphatase but approximately 40% denaturation of β-galactosidase, further confirming that the significant denaturation of all chemicals with EDTA and Triton X-100 is mostly due to the Triton X-100.

G-HCl and Triton X-100 as a pretreatment method for bacteria resulted in cell breakage after the pretreatment method prior to homogenisation, observed in micrographs. The treated cells resulted in increased released of proteins compared to untreated cells at 13.8 MPa. A 50% release of soluble protein from treated cells compared to untreated cells resulted in a decrease in energy requirement of approximately 20%.

Therefore, the two most successful pretreatment methods that achieved the objectives of the use of pretreatment in combination with HPH were EDTA and G-HCl + Triton X-100 on bacteria. Increased release observed at reduced pressures, with reduced energy requirements and micronisation of cell debris have shown that it is possible to use the correct pretreatment procedure combined with HPH to increase intracellular release.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The combination of pretreatments, in the form of non-mechanical disruption methods, with mechanical disruption methods to increase the extent of disruption and subsequent release of intracellular material was investigated in this study. The pretreatments used were methods chosen from literature that proved effective in permeabilisation and cell wall weakening. The mechanical methods used included the high pressure homogeniser and hydrodynamic cavitation. Four pretreatment methods were studied on *Saccharomyces cerevisiae* and three on *Escherichia coli*. The extent of disruption was measured by total soluble protein and intracellular enzymes released. Microscope studies were also performed to determine the physical damage of the cells caused by the pretreatments and mechanical breakage.

A thorough literature review of cell disruption was performed to determine the most effective pretreatment methods to use. An analysis of the cell wall compositions and structures allowed the main locations of resistance to cell disruption to be identified. This knowledge provided the basis for the choice of pretreatment due to the specificity of the pretreatment methods, which maximised the weakening of the cell wall during permeabilisation. The release of intracellular contents may be specific and the process of permeabilisation does not result in fine debris. The pretreatments were

used as combinations of chemicals to attack the outer and inner membranes and cell wall components of the cell envelope. The pretreatments chosen for *Saccharomyces cerevisiae* were ethanol and toluene, EDTA and Triton X-100, EDTA and CTAB and lyticase. The pretreatments used on *Escherichia coli* were EDTA, EDTA and Triton X-100 and lastly G-HCl and Triton X-100. The mechanical methods are energy intensive, non-selective and cause micronisation of cell debris. The reduction in the energy usage is desirable, but not at the cost of reduced product release. The combination of the methods to develop a new technique was applied for increased release and decreased energy consumption.

The experimental design (Chapter 3) was implemented where the pretreatments were tested on *Saccharomyces cerevisiae* and *Escherichia coli.* These pretreatments were then used in combination with mechanical methods of disruption to determine the effect of the pretreatments on the extent of cell disruption and subsequent release of intracellular protein. The aims of the project were achieved and the extent to which these aims were achieved was outlined in the results and discussion section (Chapter 4) of this dissertation.

The use of the ethanol and toluene pretreatment on *Saccharomyces cerevisiae* resulted in large enzyme deactivation during the pretreatment process, which led to very little active protein release during the subsequent homogenisation steps. The EDTA and Triton X-100 pretreatment of yeast resulted in increased release at a pressure of 34.5 MPa. The EDTA and CTAB pretreatment gave unexpected results with the pretreatment resulting in the protection of the cells, preventing their disruption in the high pressure homogeniser. The intracellular release was very low. It is postulated that the cationic detergent CTAB interacted with the cell envelope, protecting it from shear forces, providing resistance to disruption. This was confirmed by light microscope studies performed at stages through the disruption process, indicating that little or no breakage has occurred at pressures of 13.8 and 34.5 MPa. The lyticase pretreatment did not appear to enhance the extent of disruption or release. Significant interference of the chemicals with the assays was evident in all cases with the exception of lyticase.

The use of the EDTA pretreatment on *Escherichia coli* showed that the pretreatment enhanced the release of intracellular material, with the release with pretreatment at 13.8 MPa exceeding the release with no pretreatment at 13.8 MPa and equalling the release with no pretreatment at 34.5 MPa. This pretreatment case, showed a decrease in the operating pressure required and therefore a reduction in energy requirement to the value of 60% for soluble protein, 20% for acid phosphatase and 60% for β-galactosidase. The use of EDTA and Triton X-100 caused protein denaturation and possible DNA release. The G-HCl and Triton X-100 pretreatment resulted in increased release in comparison to no pretreatment at the same pressure of 13.8 MPa, but the release did not exceed the maximum available. Once again, increased release was detected and decreased energy usage with a 20% reduction in energy required with the pretreatment at 13.8 MPa compared to untreated bacteria for the release of 50% soluble protein and acid phosphatase and a 30% reduction for the release of β-galactosidase.

The use of EDTA and Triton X-100 in combination with hydrodynamic cavitation showed increased release in comparison to no pretreatment for both cavitation numbers investigated, C_v = 0.09 and 0.13. Hydrodynamic cavitation has proved to be much less energy intensive than high pressure homogenisation and therefore provides an alternative method for microbial cell disruption.

The following conclusions can be drawn from the outcome of the project:

- It is possible to pretreat cells with various non-mechanical methods to cause permeabilisation or weaken the cell wall of microorganisms.
- In some instances, the pretreatment combined with the mechanical method resulted in an increase in the release of intracellular soluble protein and enzymes in comparison to the mechanical method alone with no pretreatment.
- The successful combined methods showed increased intracellular release with decreased operating pressure and number of passes during HPH. The decrease in pressure and passes results in decreased energy consumption.
- Complications do arise with regard to the type of pretreatment used. Since all pretreatments used in this study were chemical and enzymatic in nature, there was a need for the removal of these chemicals and enzyme prior to mechanical

breakage to prevent deactivation of the proteins and interference of the chemicals in the enzyme and protein assays performed.

- The use of pretreatment needs to be a careful selection process in which all effects of the chemicals on the proteins and cell morphology must be investigated.
- Pretreatment can be useful to enhance the mechanical disruption process with clear advantages in terms of reducing extensive fragmentation, decreased energy requirements for the process with increased release in some instances. The optimum pretreatment conditions must be defined in order to balance the advantages and disadvantages depending on the desired product.
- Product damage was difficult to minimise due the nature of the chemicals used. Some of the pretreatments resulted in large amounts of enzyme deactivation by the chemicals.

5.2 Recommendations

The ability to increase intracellular release and decrease energy consumption by the use of pretreatments combined with mechanical methods on a laboratory scale has proven the system to be effective in specific instances. Some of the aims of the project were not achieved.

The following is an attempt to discuss some of the unsuccessful components of our project and to recommend further developments of the process:

- The pretreatment testing stage of the process must be thoroughly investigated to determine how the pretreatment affects the cell and proteins associated with the microorganism and assays to be used. This stage is extremely important and an in depth analysis of how the chemical attacks the cell must be performed before moving to the mechanical stage of disruption.
- There are a wide range of possible pretreatment methods that can be investigated for use in combination with mechanical methods. If selected correctly, these methods would result in less complicated downstream processing.

- Once a comprehensive study of the pretreatments has been conducted, the use of these methods may have potential for combination with hydrodynamic cavitation.

Chapter 6

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Appendix A
ANALYTICAL METHODS

A.1 Total Soluble Protein – Bradford Method

Reagents used: Bradford
- 100 mg of Coomassie Brilliant Blue G-250 in 95% ethanol
- add 100 ml of 85% (v/v) phosphoric acid
- add distilled water up to 1 litre

Bovine Serum Albumin (BSA) standard
- prepare BSA standard solution in concentration range: 0 µg/ml to 100 µg/ml.

Procedure:
1. Pipette 0.1 ml of sample into a plastic cuvette
2. Add 1 ml of Bradford reagent (store in fridge at 4°C and in dark, light sensitive) to sample
3. Let reaction occur in cuvette for 2 minutes at room temperature
4. Don not allow to stand for more than an hour
5. Measure absorbance at 595 nm against a blank (NaH_2PO_4 buffer, pH 7.0)

Table 0.1 Reproducibility of the Bradford method for protein analysis

Sample	Protein (mg/g)
1	65.2
2	56.9
3	67.5
Average	63.2
Standard Deviation	5.56
Coefficient of Variance (%)	**8.80**

A.1.1 Calibration Curve for Bradford method for protein analysis

Procedure:
1. Prepare standard solutions of BSA in the concentration range 0 µg/ml to 100 µg/ml

2. Pipette 0.1 ml of each concentration of standard BSA solution into a plastic cuvette
3. Add 1 ml of Bradford reagent and wait for 2 minutes
4. Measure absorbance at 595 nm against a blank of distilled water

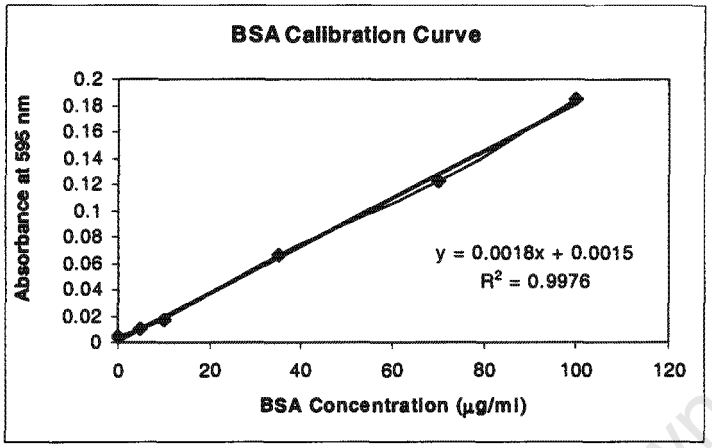

Figure A.1 Calibration curve for Bradford method of protein analysis

A.2 Invertase

Reagents used:

DNSA (dinitrosalicylic acid)
- dissolve 5 g of 3, 5 dinitrosalicyclic acid in 100 ml of 2M NaOH
- dissolve 150 g of sodium potassium tartrate (NaKtartrate) in 250 ml of distilled water
- Mix the two solutions together and make up with distilled water to 500 ml

0.5M sucrose
0.2M potassium di-hydrogen phosphate (KH_2PO_4)
0.1M sodium acetate buffer (pH 5.5)

Procedure:

1. Measure out 1 ml of sample
2. Add 1.5 ml of 0.1M acetate buffer (pH 5.5)
3. Add 0.5 ml of 0.5M sucrose
4. Mix together and incubate in water bath at 55°C for 10 minutes
5. Add 3 ml of 0.2M KH_2PO_4 and place in boiling water for 3 minutes to stop the reaction
6. Remove 1 ml of this mixture and add 1ml of DNSA reagent to new test tube
7. Incubate in boiling water for 10 minutes
8. Measure absorbance at 540 nm against a blank (NaH_2PO_4 buffer, pH 7.0)
9. Dilute samples accordingly

Table A.2 Reproducibility of Invertase analysis

Sample	Invertase (U/g)
1	716
2	704
3	735
Average	718
Standard Deviation	15.2
Coefficient of Variance (%)	**2.12**

A.2.1 Calibration Curve for DNS assay for reducing sugars

Procedure:

1. Prepare standard solutions of glucose in the concentration range of 0 to 0.05 m moles
2. Pipette 1 ml of each concentration of standard glucose solution
3. Add 1 ml of DNSA reagent to the sample
4. Incubate in boiling water for 10 minutes
5. Measure absorbance at 540 nm against a blank of distilled water
6. Dilute samples accordingly

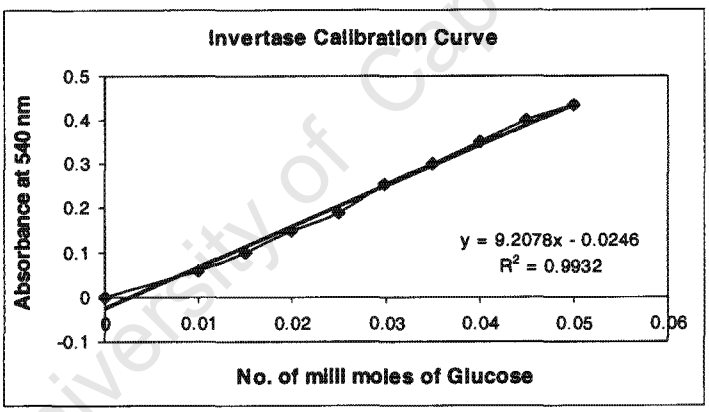

Figure A.2 Calibration curve for glucose analysis

A.3 α-glucosidase

Reagents used: 5.0 mM p-nitrophenol -α-glucosidase (PNPG) in 0.05M phosphate buffer (NaH_2PO_4, pH 7.0)
0.1M sodium carbonate (Na_2CO_3)

Procedure:

1. Pipette 0.1 ml of sample out into a test tube
2. Add 2 ml of 5.0 mM PNPG solution to sample
3. Incubate in water bath at 30° for 10 minutes
4. Add 2 ml of 0.1M of Na_2CO_3 to stop reaction
5. Measure absorbance at 410 nm against a blank (NaH_2PO_4 buffer, pH 7.0)
6. Dilute samples accordingly

Table A.3 Reproducibility of α-glucosidase analysis

Sample	α-glucosidase (U/g)
1	3.66×10^4
2	3.96×10^4
3	3.66×10^4
Average	3.76×10^4
Standard Deviation	1.76×10^3
Coefficient of Variance (%)	**4.68**

A.3.1 Calibration Curve for α-glucosidase

Procedure:

1. Prepare standard solutions of p-nitrophenol in the concentration range of 0 to 10 moles
2. Dissolve solutions in 0.1M Na_2CO_3
3. Measure absorbance at 410 nm against a blank of distilled water
4. Dilute samples accordingly

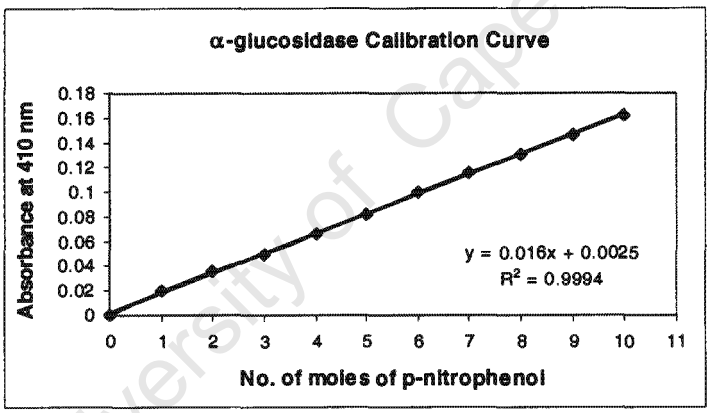

Figure A.3 Calibration curve for α-glucosidase analysis

A.4 Glucose-6-Phosphate Dehydrogenase

Reagents used:
249 mM Tris-HCl buffer (pH 7.6)
10 mM glucose-6-phosphate
10 mM β-nicotinamide adenine dinucleotide phosphate (βNADP)
0.1 M magnesium chloride ($MgCl_2$)

Procedure:

1. Place 1 ml of Tris-HCl buffer (pH 7.6) into a quartz cuvette
2. Add 0.3 ml of glucose-6-phosphate
3. Add 0.2 ml of $MgCl_2$
4. Add 0.12 ml of β-NADP
5. Add 1.38 ml of sample and shake gently, but quickly

6. Measure absorbance at 340 nm every 15 seconds for 2 minutes against a blank (NaH_2PO_4 buffer, pH 7.0)
7. Dilute samples accordingly

Table A.4 Reproducibility of Glucose-6-Phosphate Dehydrogenase analysis

Sample	Glucose-6-Phosphate Dehydrogenase (U/g)
1	0.25
2	0.36
3	0.33
Average	0.31
Standard Deviation	0.05
Coefficient of Variance (%)	**14.6**

A.5 β-galactosidase

Reagents used: PPB-Mn Buffer
- Measure out 50 mM potassium dihydrogen phosphate (KH_2PO_4)
- Add 10M potassium hydroxide (KOH) to adjust pH to 6.6
- Add 0.1mM $MgCl_2$
- 12.0 mM o-nitrophenyl-β-galactosidase (ONPG) in PPB-Mn buffer

1 M sodium carbonate (Na_2CO_3)

Procedure:
1. Pipette 0.05 ml sample into a test tube
2. Add 2 ml of ONPG in buffer to sample
3. Incubate in water bath at 37°C for 5 minutes
4. Add 0.5 ml of 1 M Na_2CO_3 to stop reaction
5. Measure absorbance at 420 nm against a blank (NaH_2PO_4 buffer, pH 7.0)
6. Dilute samples accordingly

Table A.5 Reproducibility of β-galactosidase analysis

Sample	β-galactosidase (U/g)
1	1052
2	955
3	968
Average	992
Standard Deviation	53.3
Coefficient of Variance (%)	**5.37**

A.6 Acid Phosphatase

Reagents used:
0.1 M sodium acetate buffer (pH 5.0)
3.8 mM p-nitrophenol phosphate (PNP) in sodium acetate buffer
0.2 M sodium hydroxide (NaOH)

Procedure:

1. Pipette 0.3 ml of sample into a test tube
2. Add 0.3 ml of PNP solution
3. Add 0.3 ml of sodium acetate buffer
4. Incubate in water bath at 37°C for 15 minutes
5. Add 3 ml of 0.2 M NaOH to stop reaction
6. Measure absorbance at 410 nm against blank (NaH_2PO_4 buffer, pH 7.0)
7. Dilute samples accordingly

Table A.6 Reproducibility of Acid Phosphatase analysis

Sample	Acid Phosphatase (U/g)
1	283
2	278
3	251
Average	270
Standard Deviation	17.3
Coefficient of Variance (%)	**6.41**

A.6.1 Calibration Curve for Acid Phosphatase

Procedure:

1. Prepare standard solutions of p-nitrophenol in the concentration range of 0 to 5 moles
2. Dissolve in 0.2 M NaOH
3. Measure absorbance at 410 nm against a blank of distilled water
4. Dilute sample accordingly

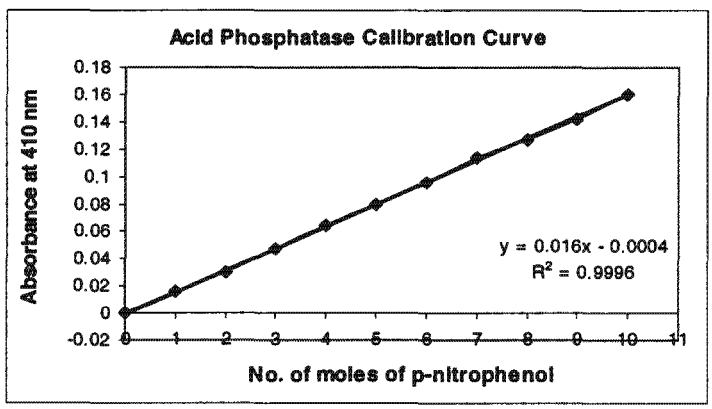

Figure A.4 Calibration curve for Acid Phosphatase

Appendix B
RAW DATA FOR EXPERIMENTS PERFORMED ON YEAST AND BACTERIA

B.1 Pretreatment Testing on Baker's Yeast

Pretreatment was performed on a 1% Baker's Yeast suspension (wet weight). Soluble protein and indicator enzyme release were tested to determine the optimum conditions for the pretreatment procedure. The tables below present the triplicate raw data for the release of soluble protein, invertase or α-glucosidase during pretreatment of Baker's yeast. Italicised values are those which are clear anomalies and have been omitted to reduce the coefficient of variance. Average, standard deviation and coefficient of variance are based on all data. For calculation of Corrected Average, Corrected Standard deviation and Corrected Coefficient of Variance, the anomalies data is omitted.

Table B.1 **Raw data for ethanol and toluene pretreatment testing with error data**

Toluene = 1%	Protein (mg/g)								
Ethanol Concentration (%)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
20	0.50	*0.41*	0.54	0.48	0.52	0.07	0.03	14.5	6.23
40	1.97	*1.74*	1.88	1.86	1.92	0.12	0.07	6.21	3.38
60	2.57	2.34	*1.60*	2.17	2.45	0.50	0.16	23.3	6.63
80	0.73	0.87	*1.37*	0.99	0.80	0.34	0.10	34.3	12.2
	α-glucosidase (U/g) x 10^3								
Ethanol Concentration (%)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
20	6.10	*0.00*	6.10	4.07	6.10	3.52	0.00	86.6	0.00
40	6.10	6.10	*12.2*	8.13	6.10	3.52	0.00	43.3	0.00
60	18.3	*0.00*	18.3	12.2	36.6	10.6	0.00	86.6	0.00
80	6.10	*12.2*	6.10	8.13	12.2	3.52	0.00	43.3	0.00
Toluene = 5%	Protein (mg/g)								
Ethanol Concentration (%)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
20	0.68	0.61	0.64	0.64	0.64	0.14	0.04	22.0	5.45
40	1.67	*2.38*	1.67	1.91	1.67	0.46	0.00	23.9	0.00
60	2.13	2.27	2.22	2.21	2.21	0.14	0.07	6.36	3.18
80	1.44	1.30	1.40	1.38	1.38	0.33	0.07	23.6	5.09
	α-glucosidase (U/g) x 10^4								
Ethanol Concentration (%)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
20	2.44	2.44	*1.22*	2.03	2.44	0.70	0.00	34.64	0.00
40	1.22	1.22	1.22	1.22	1.22	1.41	0.00	115	0.00
60	6.10	3.66	*11.0*	6.91	4.88	3.93	1.72	56.9	35.4
80	*0.00*	12.2	8.54	6.91	10.4	6.30	2.59	91.1	25.0

Table B.2 **Raw data for EDTA and 0.1% Triton X-100 pretreatment testing with error data**

	Protein (mg/g)								
EDTA Concentration (M)	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Corrected Average**	**Standard Deviation**	**Corrected Standard Deviation**	**Coefficient Of Variance**	**Corrected Coefficient Of Variance**
0.025	3.35	*1.83*	2.80	2.66	3.07	0.77	0.39	28.9	12.7
0.050	*1.74*	2.84	2.38	2.32	2.61	0.55	0.33	23.9	12.4
0.075	3.12	*2.38*	2.98	2.83	3.05	0.39	0.10	13.8	3.20
0.100	3.12	3.26	3.21	3.20	3.20	0.07	0.07	2.20	2.20
	α-glucosidase (U/g) x 10^4								
EDTA Concentration (M)	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Corrected Average**	**Standard Deviation**	**Corrected Standard Deviation**	**Coefficient Of Variance**	**Corrected Coefficient Of Variance**
0.025	*1.83*	1.22	1.22	1.42	1.22	0.35	0.00	24.7	0.00
0.050	1.22	1.22	1.22	1.22	1.22	0.00	0.00	0.00	0.00
0.075	1.83	*1.22*	1.59	1.54	1.71	0.31	0.17	19.9	10.1
0.100	0.61	0.61	0.61	0.61	0.61	0.00	0.00	0.00	0.00
	Invertase (U/g)								
EDTA Concentration (M)	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Corrected Average**	**Standard Deviation**	**Corrected Standard Deviation**	**Coefficient Of Variance**	**Corrected Coefficient Of Variance**
0.025	1.25	*1.08*	1.23	1.19	1.24	0.09	0.01	7.94	0.81
0.050	0.92	0.94	0.95	0.94	0.94	0.01	0.01	1.33	1.33
0.075	0.41	0.44	0.42	0.42	0.42	0.01	0.01	2.96	2.96
0.100	0.21	*0.10*	0.20	0.17	0.20	0.06	0.01	36.2	3.33

Table B.3 Raw data for EDTA and 0.1% CTAB pretreatment testing with error data

	Protein (mg/g)								
EDTA Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.025	14.9	15.6	*18.0*	16.2	15.3	1.00	0.47	6.21	3.09
0.050	2.84	2.43	2.89	2.72	2.72	0.25	0.25	9.31	9.31
0.075	*17.2*	15.0	15.2	15.8	15.1	1.22	0.13	7.76	0.86
0.100	*13.1*	20.1	18.3	17.2	19.2	3.61	1.24	21.0	6.43
	Invertase (U/g)								
EDTA Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.025	1.51	1.41	1.36	1.43	1.43	0.09	0.08	6.53	5.42
0.050	1.27	*1.41*	1.27	1.32	1.27	0.08	0.00	5.79	0.00
0.075	1.33	1.63	1.48	1.48	1.48	0.15	0.15	10.2	10.2
0.100	1.41	1.65	1.54	1.53	1.53	0.12	0.12	8.02	8.02

Table B.4 Raw data for Lyticase pretreatment testing with error data

Method 1 – Protein (mg/g)									
Enzyme Concentration (mg/g)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.01	*0.36*	0.50	0.68	0.51	0.59	0.16	0.13	31.4	22.0
0.10	*4.04*	3.63	3.49	3.72	3.56	0.29	0.10	7.72	2.74
1.00	1.33	*1.14*	1.37	1.28	1.35	0.12	0.03	9.50	2.41
Method 2 – Protein (mg/g)									
Enzyme Concentration (mg/g)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.17	*0.22*	0.54	0.36	0.38	0.45	0.16	0.13	42.9	28.7
0.33	*1.42*	2.89	2.38	2.23	2.64	0.75	0.36	33.5	13.6
1.00	3.17	3.07	3.30	3.18	3.18	0.12	0.12	3.64	3.64

B.2 Pretreatment Testing on *Escherichia coli*

Pretreatment testing was performed on 1% *Escherichia coli* suspension (wet weight). Soluble protein and indicator enzyme release were tested to determine the optimum conditions for the pretreatment procedure. The tables below present the triplicate raw data for the release of soluble protein, acid phosphatase and β-galactosidase during pretreatment of bacteria with pretreatments selected as a function of concentration. Italicised values are those which are clear anomalies and have been omitted to reduce the coefficient of variance. Average, standard deviation and coefficient of variance are based on all data. For calculation of Corrected Average, Corrected Standard deviation and Corrected Coefficient of Variance, the anomalies data is omitted.

Table B.5 Raw data for EDTA pretreatment testing with error data

	Protein (mg/g)								
EDTA Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.02	2.15	*1.60*	2.34	2.03	2.25	0.38	0.13	18.9	5.79
0.04	3.58	*3.17*	3.58	3.44	3.58	0.24	0.00	6.94	0.00
0.06	2.34	*1.83*	2.52	2.23	2.43	0.36	0.13	16.0	5.35
0.08	1.83	*2.66*	1.88	2.12	1.86	0.47	0.03	21.9	1.75
0.10	1.19	*1.65*	0.82	1.22	1.00	0.41	0.26	34.0	25.9
	Acid Phosphatase (U/g)								
EDTA Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.02	*10.8*	14.9	13.6	13.1	14.2	2.07	0.96	15.8	6.73
0.04	*16.3*	14.9	14.9	15.4	14.9	0.78	0.00	5.09	0.00
0.06	4.07	4.07	4.07	4.07	4.07	0.00	0.00	0.00	0.00
0.08	4.07	4.07	*5.42*	4.52	4.07	0.78	0.00	17.3	0.00
0.10	*2.71*	4.07	5.42	4.07	4.74	1.36	0.96	33.3	20.2

	β-galactosidase (U/g)								
EDTA Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.02	17.6	18.3	19.2	18.4	18.4	0.84	0.84	4.57	4.57
0.04	28.8	27.6	27.5	28.0	28.0	0.71	0.71	2.54	2.54
0.06	16.8	16.5	16.5	16.6	16.6	0.15	0.15	0.90	0.90
0.08	22.2	22.1	20.7	21.6	21.6	0.86	0.86	3.97	3.97
0.10	4.65	5.42	*3.61*	4.56	5.03	0.91	0.55	19.9	10.9

Table B.6 **Raw data for 0.040M EDTA and 2% Triton X-100 pretreatment testing with error data**

EDTA and Triton X-100									
	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
Protein (mg/g)	0.73	0.82	*1.00*	0.85	0.77	0.14	0.07	16.5	8.39
Acid Phosphatase (U/g)	*1.36*	4.07	6.78	4.07	5.42	2.71	1.92	66.7	35.4
β-galactosidase (U/g)	0.39	*0.65*	0.39	0.47	0.39	0.15	0.00	31.5	0.00

Table B.7 **Raw data for G-HCl and 2% Triton X-100 pretreatment testing with error data**

1 hour Incubation	Protein (mg/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	0.27	0.59	*0.96*	0.61	0.43	0.35	0.23	56.9	52.9
0.5	1.42	*1.00*	1.65	1.36	1.53	0.33	0.16	24.0	10.6
1.0	*3.26*	2.11	2.38	2.58	2.25	0.60	0.20	23.2	8.69
1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5	12.4	*11.6*	12.2	12.1	12.3	0.43	0.16	3.54	1.32

	Acid Phosphatase (U/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	392	398	*427*	406	395	18.7	4.79	4.61	1.21
0.5	260	276	*304*	280	268	21.9	11.5	7.82	4.29
1.0	86.7	90.8	89.4	89.0	89.0	2.07	2.07	2.33	2.33
1.5	117	*130*	115	121	116	8.24	0.96	6.83	0.83
2.5	43.4	54.2	*78.6*	58.7	48.8	18.0	7.67	30.7	15.7
	β-galactosidase (U/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	35.2	36.7	*44.1*	38.7	36.0	4.78	1.00	12.4	2.79
0.5	22.1	22.6	22.5	22.4	22.3	0.27	0.36	1.20	1.63
1.0	*0.52*	0.26	0.26	0.34	0.26	0.15	0.00	43.3	0.00
1.5	1.03	1.03	*0.77*	0.95	1.03	0.15	0.00	15.8	0.00
2.5	*1.42*	1.81	2.06	1.76	1.94	0.32	0.18	18.4	9.43

2 hours incubation	Protein (mg/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	*1.00*	0.64	0.73	0.79	0.68	0.19	0.07	24.2	9.52
0.5	*0.82*	1.19	1.10	1.04	1.14	0.19	0.07	18.5	5.69
1.0	1.37	2.38	*3.26*	2.34	1.88	0.94	0.72	40.4	38.1
1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5	12.3	12.4	11.9	12.2	12.2	0.27	0.27	2.22	2.22

	Acid Phosphatase (U/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	306	294	313	304	304	9.61	9.61	3.16	3.16
0.5	322	321	*272*	305	322	28.6	0.96	9.35	0.30
1.0	*51.5*	82.7	93.5	75.9	88.1	21.8	7.67	28.7	8.70
1.5	*137*	165	168	157	167	17.3	1.92	11.0	1.15
2.5	52.9	*35.2*	46.1	44.7	49.4	8.89	4.79	19.9	9.69
	β-galactosidase (U/g)								
G-HCl Concentration (M)	Sample 1	Sample 2	Sample 3	Average	Corrected Average	Standard Deviation	Corrected Standard Deviation	Coefficient Of Variance	Corrected Coefficient Of Variance
0.1	33.7	36.3	35.0	35.0	35.0	1.29	1.29	3.69	3.69
0.5	20.8	22.8	21.2	21.6	22.0	1.10	1.10	5.08	5.08
1.0	0.13	*0.65*	0.13	0.30	0.13	0.30	0.00	99.0	0.00
1.5	1.03	*1.29*	1.03	1.12	1.03	0.15	0.00	13.3	0.00
2.5	1.42	1.55	*2.32*	1.76	1.48	0.49	0.09	27.7	6.15

B.3 High Pressure Homogenisation of Baker's Yeast

The homogenisation of Baker's yeast (1%, wet weight) was performed at three pressures: 13.8 MPa, 34.5 MPa and 69.0 MPa. The release of soluble protein and indicator enzymes were tested. The tables below present the raw data obtained for the release of soluble protein, invertase, α-glucosidase and glucose-6-phosphtae dehydrogenase (G6PDH).

Table B.8 **Raw data for homogenisation of Baker's yeast at 13.8 MPa**

	Protein (mg/g)					
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00
4	37.0	22.6	44.1	40.6	5.07	12.5
8	94.9	41.9	64.6	53.2	16.0	30.0
12	97.1	70.1	78.9	74.5	6.24	8.38
16	106	85.0	94.9	95.0	10.8	11.3
20	120	96.6	100	106	12.7	12.0
24	122	100	112	112	11.0	9.89
	α- glucosidase (U/g) x 10^7					
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00
4	0.70	0.15	0.70	0.70	0.00	0.49
8	1.02	0.33	1.03	1.03	0.00	0.34
12	1.20	0.58	1.21	1.20	0.00	0.29
16	1.31	0.94	1.32	1.31	0.00	0.26
20	1.46	1.32	1.46	1.41	0.08	5.67
24	1.54	1.16	1.67	1.61	0.09	5.58
	Invertase (U/g) x 10^4					
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00
4	0.45	0.54	0.51	0.50	0.05	9.17
8	0.76	0.97	0.48	0.86	0.15	17.2
12	0.88	1.06	0.68	0.97	0.14	14.6
16	0.98	1.28	0.70	1.13	0.20	17.4
20	1.08	1.46	0.82	1.27	0.27	21.0
24	1.08	1.50	0.90	1.29	0.30	23.0

	G6PDH (U/g)					
Pass	Sample 1	Sample 2	Sample 3	Average	Standard Deviation	Coefficient of Variance
0	0.00	0.00	0.00	0.00	0.00	0.00
4	1.53	1.21	1.42	1.47	0.08	5.15
8	3.25	2.06	2.09	2.07	0.02	1.11
12	4.13	3.17	2.45	2.81	0.51	18.3
16	4.13	3.95	2.79	4.04	0.13	3.27
20	4.71	4.06	2.87	4.39	0.46	10.5
24	4.70	4.28	3.12	4.49	0.29	6.54

Table B.9 **Raw data for homogenisation of Baker's yeast at 34.5 MPa**

	Protein (mg/g)				
Pass	Sample 1	Sample 2	Average	Standard Deviation	Coefficient of Variance
0	0.00	0.00	0.00	0.00	0.00
1	37.0	30.0	33.5	5.00	14.8
2	76.0	61.0	68.5	10.6	15.5
3	113	90.0	102	15.9	15.7
4	136	111	123	17.6	14.2
8	141	137	139	2.73	1.96
12	144	150	147	4.68	3.19
16	160	146	153	9.75	6.39
20	178	159	169	13.7	8.10
24	152	151	152	0.39	0.26

	α-glucosidase (U/g) x 10^7				
Pass	**Sample 1**	**Sample 2**	**Average**	**Standard Deviation**	**Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00
1	1.10	1.00	1.05	0.07	6.73
2	2.20	1.80	2.00	0.28	14.1
3	3.10	2.92	3.01	0.13	4.23
4	3.90	3.55	3.73	0.25	6.69
8	4.15	3.45	3.80	0.49	13.0
12	4.44	3.55	4.00	0.63	15.7
16	5.12	4.71	4.92	0.29	5.93
20	4.88	3.83	4.35	0.74	17.0
24	4.78	3.75	4.27	0.73	17.0
	Invertase (U/g) x 10^4				
Pass	**Sample 1**	**Sample 2**	**Average**	**Standard Deviation**	**Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00
1	0.40	0.35	0.38	0.04	9.43
2	0.84	0.67	0.76	0.12	15.9
3	1.20	0.90	1.05	0.21	20.2
4	1.49	1.14	1.31	0.25	18.8
8	1.44	1.62	1.53	0.12	8.14
12	1.50	1.62	1.56	0.08	5.34
16	1.83	1.45	1.64	0.27	16.4
20	2.05	1.65	1.85	0.28	15.2
24	1.37	1.52	1.45	0.11	7.38

	G6PDH (U/g)				
Pass	Sample 1	Sample 2	Average	Standard Deviation	Coefficient of Variance
0	0.00	0.00	0.00	0.00	0.00
1	1.40	1.70	1.55	0.21	13.7
2	2.70	3.50	3.10	0.57	18.3
3	4.00	5.10	4.55	0.78	17.1
4	4.95	6.33	5.64	0.98	17.3
8	6.23	7.36	6.80	0.80	11.7
12	6.81	7.79	7.30	0.69	9.45
16	6.79	7.69	7.24	0.64	8.80
20	6.56	7.69	7.12	0.80	11.2
24	6.45	7.49	6.97	0.73	10.5

Table B.10 Raw data for homogenisation of Baker's yeast at 69.0 MPa

Pass	Protein (mg/g)	α-glucosidase (U/g) x 10^7	Invertase (U/g) x 10^4	G6PDH (U/g)
0	0.00	0.00	0.00	0.00
1	55.7	0.78	0.46	2.01
2	102	1.04	0.72	2.91
3	120	1.46	1.14	5.14
4	9.93	0.00	-0.06	-0.04

B.4 High Pressure Homogenisation of *Escherichia coli*

The homogenisation of bacteria (1%, wet weight) was performed at two pressures: 13.8 MPa and 34.5 MPa. The release of soluble protein and indicator enzymes were tested. The tables below present the raw data obtained for the release of soluble protein, acid phosphatase and β-galactosidase. . Italicised values are those which are clear anomalies and have been omitted to reduce the coefficient of variance.

Table B.11 Raw data for homogenisation of bacteria at 13.8 MPa

	Protein (mg/g)								
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**	**Corrected Average**	**Corrected Standard Deviation**	**Corrected Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	58.4	*121*	88.3	89.2	31.3	35.1	73.3	21.1	28.8
8	*66.1*	145	112	108	39.8	37.0	129	23.3	18.0
12	*65.4*	142	139	116	43.3	37.5	140	1.63	1.16
16	*67.9*	161	143	124	49.1	39.7	152	12.5	8.26
20	*65.1*	162	151	126	53.0	42.1	156	7.64	4.88
	Acid Phosphatase (U/g)								
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**	**Corrected Average**	**Corrected Standard Deviation**	**Corrected Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	218	*454*	196	289	143	49.4	207	15.2	7.31
8	*257*	542	373	390	143	36.7	457	120	26.2
12	275	*623*	413	437	176	40.1	344	97.9	28.5
16	323	312	*589*	408	157	38.6	317	7.76	2.45
20	270	379	*630*	427	184	43.2	325	77.0	23.7
	β-galactosidase (U/g)								
Pass	**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**Standard Deviation**	**Coefficient of Variance**	**Corrected Average**	**Corrected Standard Deviation**	**Corrected Coefficient of Variance**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	941	1375	*447*	921	465	50.4	1158	307	26.6
8	964	*1533*	602	1033	469	45.4	783	256	32.7
12	994	*1680*	813	1162	457	39.4	903	128	14.2
16	1102	*1697*	774	1191	468	39.3	938	232	24.7
20	976	*1785*	850	1203	507	42.2	913	89.3	9.78

Table B.12 Raw data for homogenisation of bacteria at 34.5 MPa

Pass	Protein (mg/g)	Acid Phosphatase (U/g)	β-galactosidase (U/g)
0	0.00	0.00	0.00
1	42.0	225	520
2	81.0	480	1090
3	125	690	1600
4	147	860	1935
8	168	921	1999
12	154	942	2057
16	163	888	2017
20	155	799	1990

B.5 Combined Pretreatment and High Pressure Homogenisation

The following is a compilation of the raw data for experiments performed on Baker's yeast using pretreatment followed by high pressure homogenisation. Combined experiments were performed in triplicate on yeast in the high pressure homogeniser but not with hydrodynamic cavitation or bacteria and high pressure homogenisation.

Table B.13 Combined pretreatment of Baker's yeast with ethanol and toluene and high pressure homogenisation at 13.8 MPa

HPH Extent of Disruption at 24th Pass	Sample 1	Sample 2	Sample 3	Average	Standard Deviation	Coefficient of variance	Corrected Average	Corrected Standard Deviation	Corrected Coefficient of Variance
Protein (mg/g)	3.49	*5.23*	3.44	4.06	1.02	25.2	3.47	0.04	1.08
α-glucosidase (U/g)	7.93×10^4	9.15×10^4	*6.16×10^5*	2.62×10^5	3.06×10^5	117	8.54×10^4	8.62×10^3	10.1
Invertase (U/g)	2.65×10^3	2.93×10^3	*1.94×10^3*	2.51×10^3	510	20.4	2.79×10^3	196	7.04
G6PDH (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Pretreatment (R_o)									
Protein (mg/g)	*1.42*	2.02	1.92	1.79	0.32	18.0	1.97	0.07	3.30
α-glucosidase (U/g)	4.88×10^4	4.27×10^4	*3.05×10^4*	4.07×10^4	931.00	22.9	4.57×10^4	4.31×10^3	9.43
Invertase (U/g)x 10^2	682	636	*459*	592	118	19.9	659	32.7	4.96
G6PDH (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table B.14 Combined pretreatment of Baker's yeast with EDTA and Triton X-100 and high pressure homogenisation at 13.7 MPa

HPH Extent of Disruption at 24th Pass	Sample 1	Sample 2	Sample 3	Average	Standard Deviation	Coefficient of variance	Corrected Average	Corrected Standard Deviation	Corrected Coefficient of Variance
Protein (mg/g)	31.9	*49.4*	35.6	39.0	9.20	23.6	33.8	2.62	7.75
α-glucosidase (U/g)	1.22×10^6	1.24×10^6	*1.33×10^4*	8.26×10^5	7.04×10^5	85.2	1.23×10^6	1.72×10^4	1.40
Invertase (U/g)	7.28×10^3	*7.56×10^3*	7.38×10^3	7.41×10^3	139	1.88	7.33×10^3	70.2	0.96
G6PDH (U/g)	*4.05*	3.03	3.30	3.46	0.52	15.2	3.17	0.19	5.93

Pretreatment (R_o)									
Protein (mg/g)	*9.85*	2.29	5.50	5.88	3.79	64.5	3.90	2.27	58.2
α-glucosidase (U/g)	1.22×10^5	*9.76×10^4*	11.1×10^5	1.10×10^5	1.22×10^4	11.1	1.16×10^5	7.74×10^3	6.65
Invertase (U/g)	1.29×10^3	*11.33×10^3*	1.31×10^3	1.31×10^3	22.9	1.75	1.30×10^3	11.8	0.91
G6PDH (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table B.15 Combined pretreatment of Baker's yeast with EDTA and CTAB and high pressure homogenisation at 13.7 MPa

HPH Extent of Disruption at 24th Pass	Sample 1	Sample 2	Sample 3	Average	Standard Deviation	Coefficient of variance	Corrected Average	Corrected Standard Deviation	Corrected Coefficient of Variance
Protein (mg/g)	22.1	25.2	*32.2*	26.5	5.19	19.6	23.6	2.21	9.37
α-glucosidase (U/g)	2.20×10^5	*1.90×10^4*	2.55×10^5	1.65×10^5	1.27×10^5	77.4	2.37×10^5	2.51×10^4	10.6
Invertase (U/g)	5.49×10^3	5.32×10^3	5.43×10^3	5.41×10^3	85.1	1.57	5.41×10^3	85.1	1.57
G6PDH (U/g)	1.61	*1.55*	1.68	1.61	0.07	4.03	1.65	0.05	2.86

Pretreatment (R_o)									
Protein (mg/g)	18.7	17.6	*15.0*	17.1	1.88	11.0	18.1	0.80	4.40
α-glucosidase (U/g)	2.44×10^4	2.12×10^4	*2.88×10^4*	2.48×10^4	3.82×10^3	15.39	2.28×10^4	2.26×10^3	9.90
Invertase (U/g)	3.62×10^3	*3.33×10^3*	3.95×10^3	3.63×10^3	310	8.54	3.79×10^3	232	6.14
G6PDH (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table B.16 Combined pretreatment of Baker's yeast with lyticase and high pressure homogenisation at 13.7 MPa

HPH Extent of Disruption at 24th Pass	Sample 1	Sample 2	Sample 3	Average	Standard Deviation	Coefficient of variance	Corrected Average	Corrected Standard Deviation	Corrected Coefficient of Variance
Protein (mg/g)	105	110	113	109	3.62	3.31	109	3.62	3.31
α-glucosidase (U/g)	1.92×10^7	1.74×10^7	1.83×10^7	1.83×10^7	8.78×10^5	4.80	1.83×10^7	8.78×10^5	4.80
Invertase (U/g)	1.24×10^4	1.14×10^4	*7.63×10^3*	1.05×10^4	2.51×10^3	24.0	1.19×10^4	744	6.27
G6PDH (U/g)	3.86	4.38	4.25	4.16	0.27	6.45	4.16	0.27	6.45

Pretreatment (R_o)									
Protein (mg/g)	1.02	1.57	*0.47*	1.02	0.55	54.1	1.30	0.39	30.1
α-glucosidase (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Invertase (U/g)	360	*1.66×10^3*	462	828	724	87.5	411	72.1	17.6
G6PDH (U/g)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table B.17 Raw data for experiments performed on bacteria with pretreatment combined with high pressure homogenisation at 13.8 MPa

EDTA	Pretreatment (R_o)	High Pressure Homogenisation at 20th pass	Total
Protein (mg/g)	0.85	176	176
Acid Phosphatase (U/g)	13.6	1.05×10^3	1.06×10^3
β-galactosidase (U/g)	5.16	2.28×10^3	2.28×10^3

EDTA and Triton X-100	Pretreatment (R_o)	High Pressure Homogenisation at 20th pass	Total
Protein (mg/g)	0.45	1.61	2.06
Acid Phosphatase (U/g)	16.3	4.07	20.3
β-galactosidase (U/g)	0.26	24.0	24.3
G-HCl and Triton X-100			
Protein (mg/g)	31.6	138	170
Acid Phosphatase (U/g)	40.7	915	955
β-galactosidase (U/g)	19.4	1.77×10^3	1.79×10^3

Table B.18 Raw data for experiments performed on Baker's yeast with pretreatment combined with hydrodynamic cavitation

EDTA / Triton X-100: Cv = 0.09	Pretreatment (R_o)	Hydrodynamic Cavitation at 1000th pass	Total
Protein (mg/g)	5.79	7.86	13.7
Invertase (U/g)	91.3	1.41	92.7
α-glucosidase (U/g)	1.83×10^5	3.57	1.83×10^5
G6PDH (U/g)	0.25	0.57	0.82
EDTA / Triton X-100: Cv = 0.13			
Protein (mg/g)	4.37	8.51	12.9
Invertase (U/g)	78.1	4.18	82.3
α-glucosidase (U/g)	1.22×10^5	6.10	1.22×10^5
G6PDH (U/g)	0.53	0.58	1.10

Appendix C
RELEASE RATE KINETICS FOR EXPERIMENTS PERFORMED ON YEAST AND BACTERIA

C.1 Release Rate Kinetics for Untreated Baker's Yeast

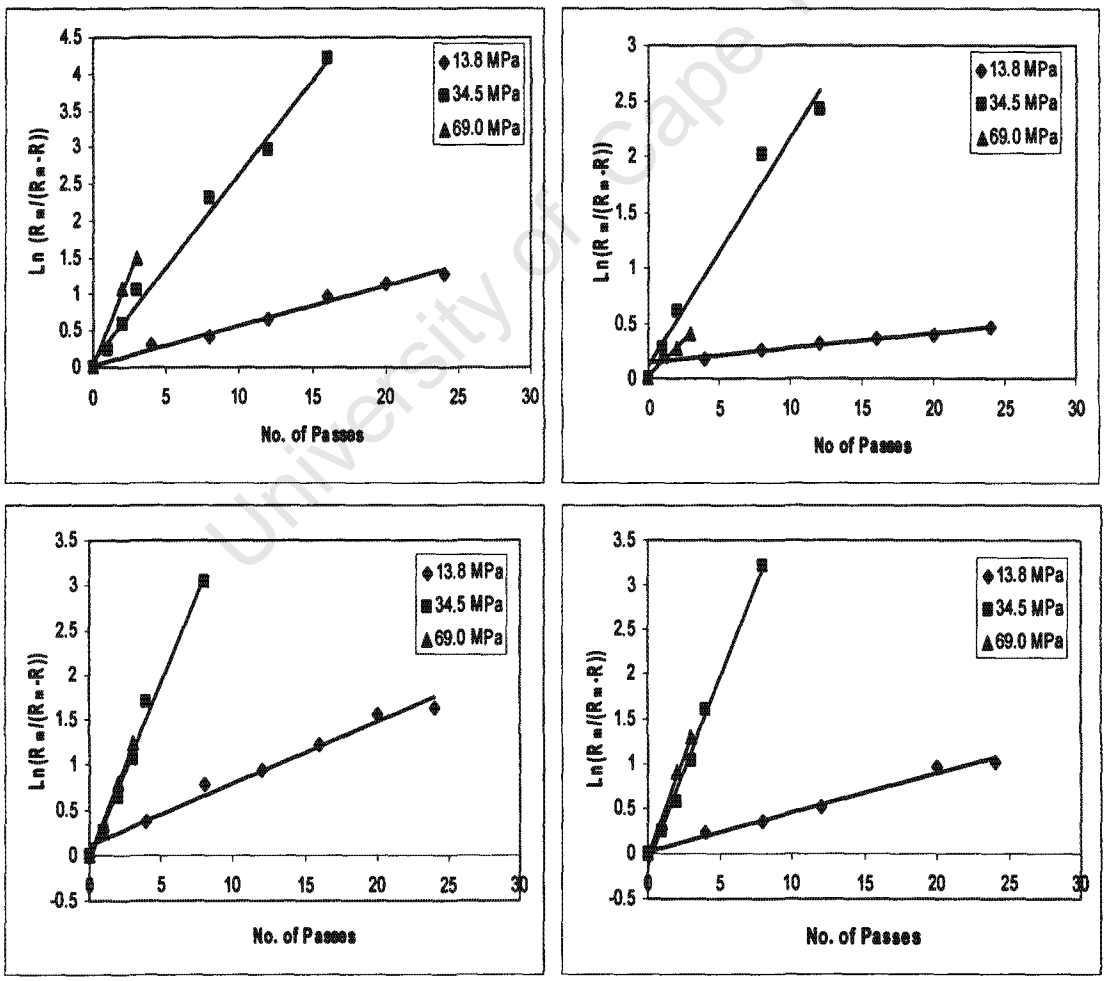

Figure 0.1 **Soluble protein, α-glucosidase, invertase and G6PDH release rate kinetics from Baker's yeast at three pressures in the high pressure homogeniser**

Table C.1 Release rate constants (k') and regression coefficients for above graphs

Yeast	Protein		Invertase		α-glucosidase		G6PDH	
Pressure (MPa)	k x 10^{-3}	R^2	k x 10^{-3}	R^2	k x 10^{-3}	R^2	k x 10^{-3}	R^2
13.8	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
34.5	252	0.98	394	0.99	207	0.98	341	0.98
69.0	512	1.00	414	1.00	128	0.97	438	0.99

C.2 Release Rate Kinetics for Combined Pretreatment and High pressure Homogenisation of Baker's Yeast

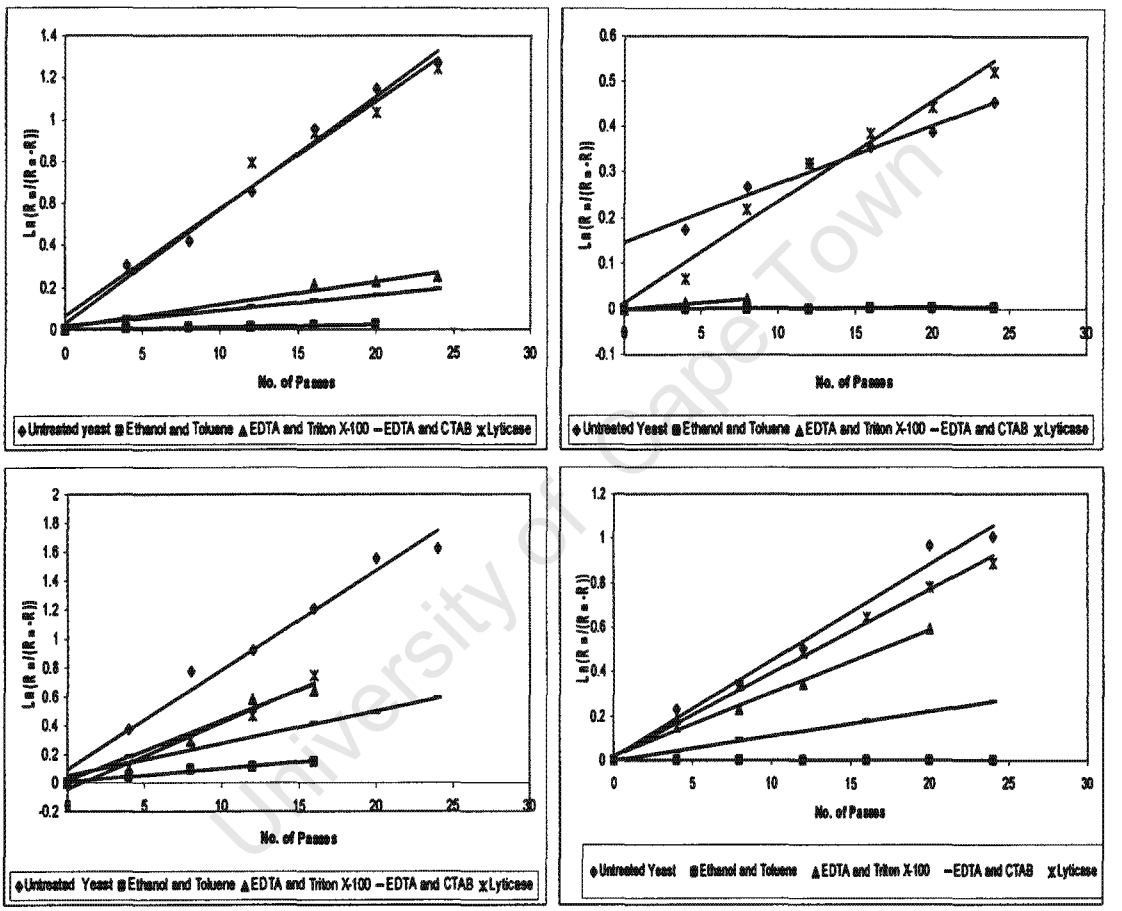

Figure C.2 Soluble protein, α-glucosidase, invertase and G6PDH release rate kinetics from Baker's yeast for all pretreatments used in combination with HPH at 13.8 MPa

Table C.2 Release rate constants (k') and regression coefficients for graphs

	Protein		Invertase		α-glucosidase		G6PDH	
Yeast – 13.8 MPa	k x 10^{-3}	R^2	k x 10^{-3}	R^2	k x 10^{-3}	R^2	k x 10^{-3}	R^2
Untreated	54.1	0.99	68.7	0.98	13.0	0.97	43.3	0.98
Ethanol / Toluene	1.10	0.98	9.40	0.98	0.08	1.00		
EDTA / Triton X-100	11.0	0.97	42.2	0.98	2.70	0.98	28.1	0.98
EDTA / CTAB	7.50	0.98	22.6	0.97	0.20	0.99	11.1	1.00
Lyticase	57.1	0.96	46.3	0.98	22.3	0.98	37.7	1.00

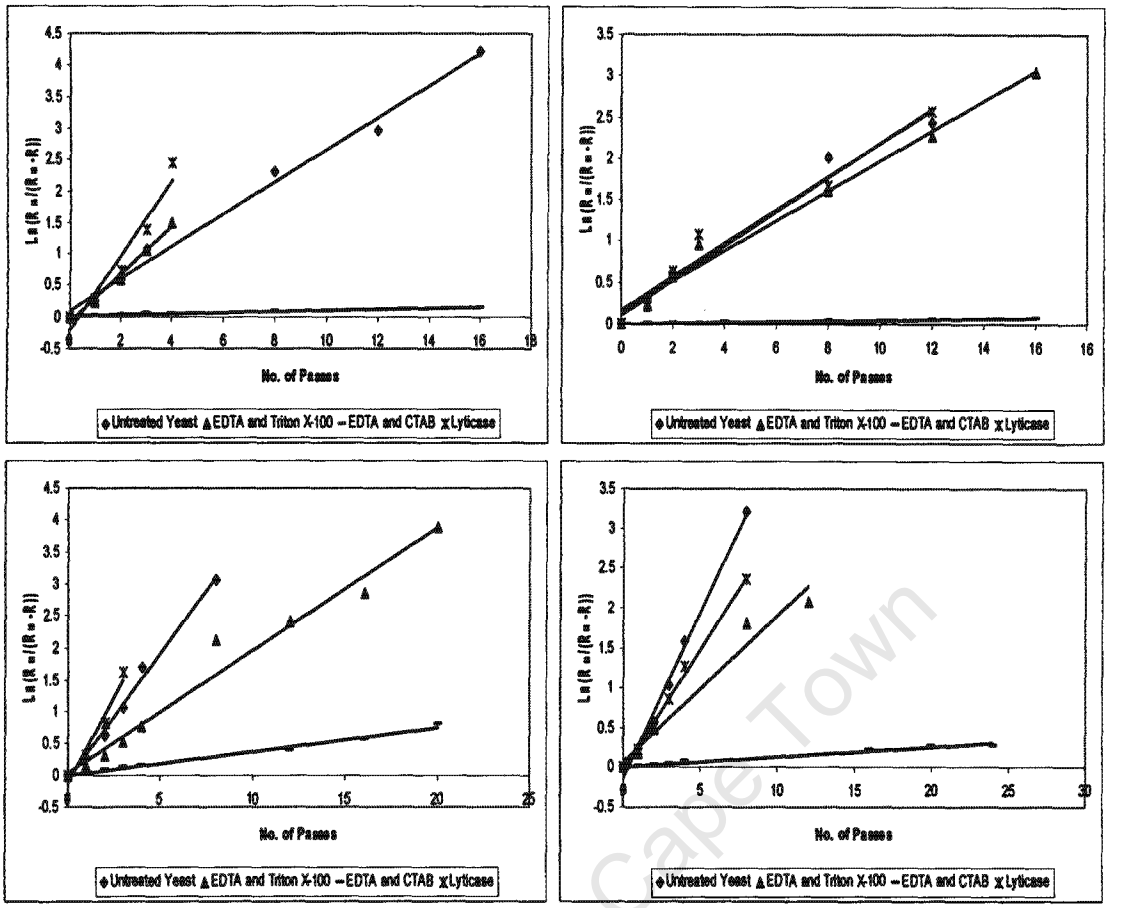

Figure C.3 **Soluble protein, α-glucosidase, invertase and G6PDH release rate kinetics from Baker's yeast for all pretreatments used in combination with HPH at 34.5 MPa**

Table C.3 **Release rate constants (k') and regression coefficients for above graphs**

	Protein		Invertase		α-glucosidase		G6PDH	
Yeast – 34.5 MPa	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**
Untreated	252	0.98	394	0.99	207	0.98	341	0.98
EDTA / Triton X-100	378	0.99	193	0.97	182	0.99	183	0.97
EDTA / CTAB	9.10	0.96	38.4	0.98	4.40	0.96	11.8	0.97
Lyticase	598	0.94	537	0.96	202	0.97	302	1.00

C.3 Release Rate Kinetics for Combined Pretreatment and High pressure Homogenisation of *Escherichia coli*

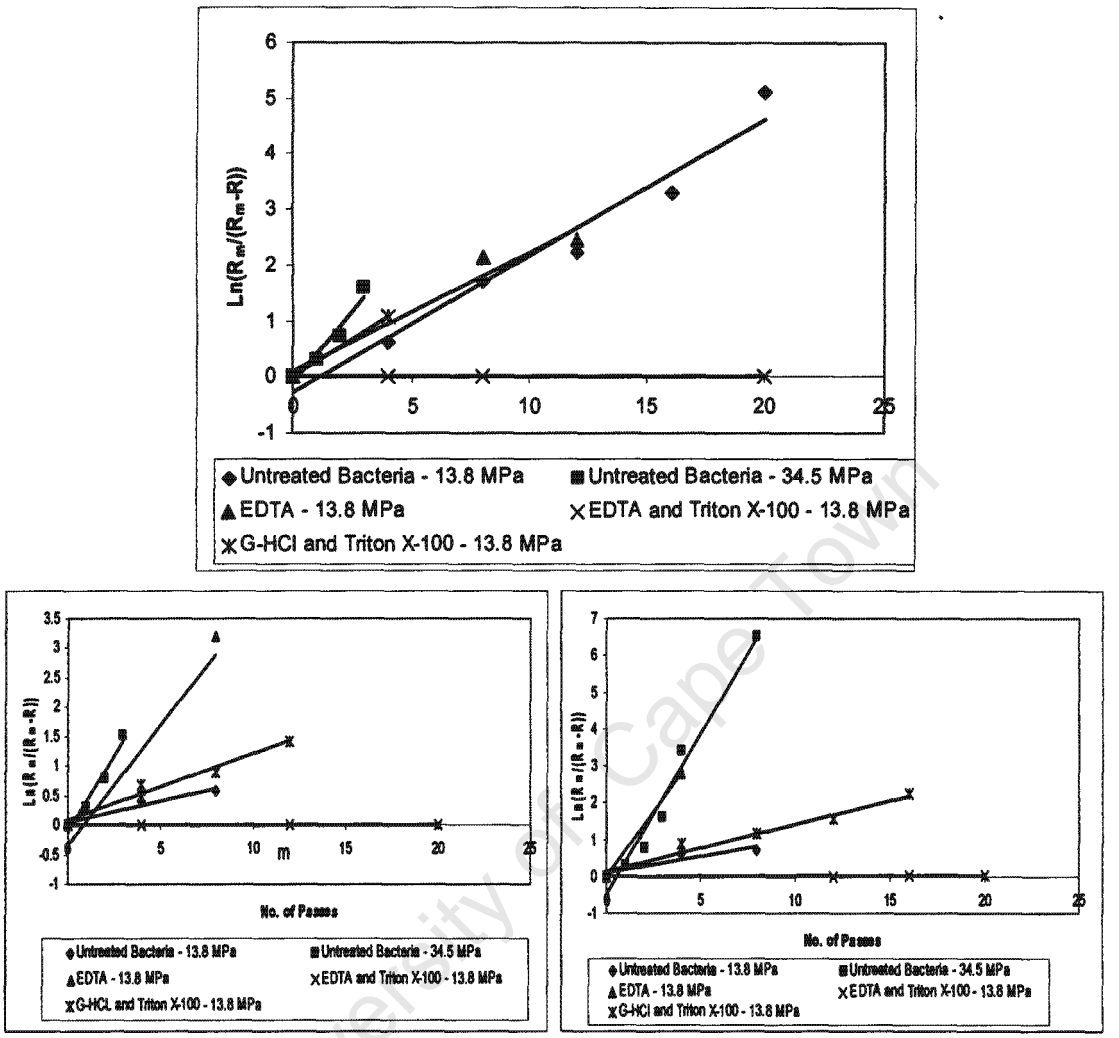

Figure C.4 **Soluble protein, acid phosphatase and β-galactosidase release rate kinetics from bacteria for all pretreatments used in combination with HPH**

Table C.4 **Release rate constants (k') and regression coefficients for above graphs**

	Protein		Acid Phosphatase		β-galactosidase	
Bacteria	**k' x 10^{-3}**	**R^2**	**k' x 10^{-3}**	**R^2**	**k' x 10^{-3}**	**R^2**
13.8 MPa	244	0.96	73.1	0.96	90.7	0.86
34.5 MPa	516	0.94	506	0.96	393	0.98
EDTA - 13.8 MPa	521	1.00	401	0.89	705	1.00
EDTA / Triton X-100 - 13.8 MPa	0.50	0.99	0.20	0.98	0.60	0.98
G-HCl / Triton X-100 - 13.8 MPa	268	1.00	111	0.96	129	0.97

C.4 Release Rate Kinetics for Combined Pretreatment and Hydrodynamic Cavitation of Baker's Yeast

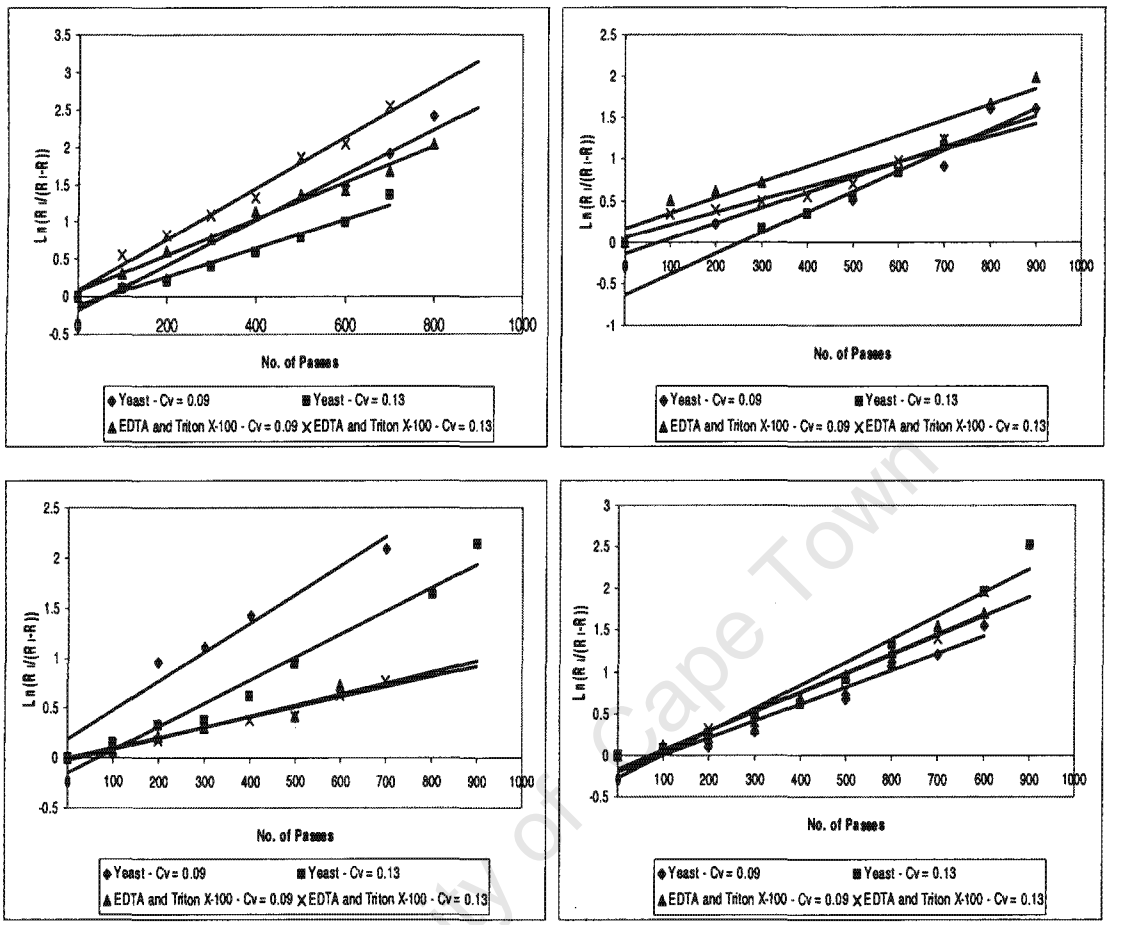

Figure C.5 Soluble protein, α-glucosidase, invertase and G6PDH release rate kinetics from Baker's yeast for pretreatments used in combination with hydrodynamic cavitation

Table C.5 Release rate constants (k') and regression coefficients for above graphs

	Protein		Invertase		α-glucosidase		G6PDH	
Yeast	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**	**k x 10^{-3}**	**R^2**
Cv = 0.09	2.90	0.98	3.70	0.97	1.30	0.95	3.80	0.97
Cv =0.13	1.90	0.97	2.30	0.97	3.30	0.95	2.80	0.96
EDTA / Triton: Cv =0.09	2.40	0.99	4.60	0.97	2.20	1.00	2.30	0.98
EDTA / Triton: Cv =0.13	3.70	0.98	3.80	0.98	4.40	0.97	2.80	0.98