A Detailed Investigation of Microbial Cell Disruption by Hydrodynamic Cavitation for Selective Product Release

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Hydrodynamic cavitation is a novel method for microbial cell disruption, mediated by intense pressure fluctuations caused by cavity oscillation and collapse. Selective release of intracellular microbial products is desirable to reduce the cost involved in their downstream processing. A study of the process variables that affect microbial cell disruption by hydrodynamic cavitation is presented in order to ascertain the conditions required for a selective release. Two model systems were considered (yeast and *E. coli*). Enzymes from different locations of the cell were studied and the release compared with other methods of disruption.

On varying the cavitation number over the range 0.09 to 0.92, an optimum cavitation number of 0.13 was found where the extent of release of extracytoplasmic enzymes (α-glucosidase and invertase) from Brewers' yeast was highest. An optimum cell-to-cavity ratio leads to an effective interaction between the cells and cavities resulting in a maximum release at 0.5 % w/v (wet wt). An increasing release of invertase on increasing the number of passes to 5000 passes indicated the predominant effect of cavitation on the cell wall. A point rupture of yeast cell wall was observed by transmission electron microscopy at less intense conditions. Little micronization resulting in cell debris was seen. Gel electrophoresis indicated a reduced amount of contaminating proteins released by hydrodynamic cavitation in comparison with French Press.

The extent of release of acid phosphatase and β-galactosidase from *E. coli* was maximum at a cavitation number of 0.17. The release of periplasmic enzyme increased with number of passes even after 600 passes while the release of soluble protein and cytoplasmic β-galactosidase asymptoted at 600 passes, indicating the predominant effect of cavitation on the cell wall. Higher specific activity can be achieved at less intense cavitation conditions; however, a compromise between maximising specific activity and recovery results. Transmission electron micrographs indicated fragments of cell and cell debris along with few intact cells at intense cavitation conditions. Gel electrophoresis showed reduced number of protein bands with hydrodynamic cavitation.

Integration of cell disruption by hydrodynamic cavitation with solid-liquid separation and protein recovery and purification using aqueous two-phase extraction resulted in an increase in the partition coefficient of β-galactosidase, while maintaining equivalent recovery on comparison with the separate unit operations. The yeast cells retained viability to a significant extent without compromising metabolic activity on their subjection to hydrodynamic cavitation, indicating potential for integrated product formation and extraction with cell recycle.

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Bala

Summary

The recovery of an intracellular product requires the disruption or damage of the protective cell envelope of the microbial cell. A wide variety of techniques can be found in the literature. The methods available can be broadly classified into mechanical and non-mechanical methods. Some of the mechanical methods include bead mill, high pressure homogenization, sonication and hydrodynamic cavitation. Non-mechanical methods include chemical, enzymatic, physical stress, osmotic shock etc. The cost of a protein product of biotechnological derivation is strongly influenced by the unit operations involved in its purification. A selective release of an intracellular product will have a beneficial impact in this regard. Selective release of intracellular enzymes has been reported for non-mechanical methods more often than mechanical methods. Mechanical methods are often preferred in the industry due to the operational and economic reasons. Hence selective release of intracellular product by a mechanical method would be rewarded.

Harrison and Pandit (1992) reported the use of hydrodynamic cavitation for cell disruption. Hydrodynamic cavitation is the phenomenon of formation, growth and collapse of vapour cavities induced by flow restriction in a closed system. It is a versatile technique because the intensity of cavitation can be varied over a wide range by the operating conditions. It is hypothesized that less intense conditions can permeabilize the cell releasing periplasmic or cell-wall associated products and complete disruption can be achieved at conditions of higher intensity. To investigate this, the influence of the various process variables that affect the cell disruption by cavitation was studied. Two model microbial systems were chosen: Brewers' yeast and *E. coli*.

The release of total soluble protein, α-glucosidase (periplasmic), invertase (cell-wall associated), alcohol dehyrogenase (ADH; cytoplasmic) and glucose-6-phosphate dehyrogenase (G6PDH; cytoplasmic) from Brewers' yeast were studied. The process variables investigated include: cavitation number, initial concentration of cell suspension

and the number of passes. The disruption by hydrodynamic cavitation was compared with French Press and high pressure homogenization. The maximum extent of release of the enzymes, α -glucosidase and invertase was observed at an optimum cavitation number of 0.13. A higher number of cavities are formed on increasing the intensity of cavitation (corresponding to a decrease in the cavitation number) but the collapse pressure of the individual cavity decreased. The total collapse pressure (number of cavities x collapse pressure of single cavity) is maximum at an optimum cavitation number resulting in the higher extent of disruption.

The cell-to-cavity ratio was optimum at a particular concentration of cell suspension (0.5 % w/v, wet wt) and the maximum interaction that occurred between the cells and cavities at this concentration resulted in the higher release of enzymes and soluble protein. The release of soluble protein and α-glucosidase was shown to asymptote to a maximum, R_i, on extending the number of passes through the cavitation zone. The increase in the release of invertase was sustained for up to 5000 passes through the cavitation zone, at which stage 85 % of total available enzyme was released. The formation of liquid micro-jet during cavity collapse is postulated to result in a water-hammer effect concentrating the bulk of energy on the small surface on the cell wall of yeast loosening the cell wall structure to release invertase. This mechanical effect is augmented by the chemical effects: reduction of the disulphide bridges that trap the invertase molecule by the free radicals generated during cavitation.

The release kinetics was found to follow first-order kinetics. The release rate constant was highest at an optimum cavitation number. Maximum release rate of soluble protein was observed at a cavitation number of 0.17 (1 % w/v, wet wt). The release rate constant of invertase was found to increase with decrease in cavitation number and reached a maximum value between C_v of 0.17 and 0.13 for 0.1 % and 2.5 % cell concentration. But for 1 % cell concentration the release rate constant of invertase was increasing on decreasing the cavitation number until C_v 0.13. Transmission electron micrographs showed a point rupture of the yeast cell that was treated at C_v of 0.17 (1 % w/v, wet wt, 1000 passes) indicating a selective release of periplasmic proteins. Ghosts of yeast cell

along with fragments of cell wall were observed from transmission electron micrographs of yeast cells subjected to C_v of 0.13 (0.1 % w/v, wet wt, 1000 passes) indicating severe damage at intense cavitation conditions.

E. coli was disrupted by hydrodynamic cavitation for the release of soluble protein, acid phosphatase (periplasmic) and β-galactosidase (cytoplasmic). The disruption was compared with French Press, high pressure homogenization, osmotic shock and EDTA treatment. The effect of cavitation number, number of passes and growth rate of *E. coli* on disruption by hydrodynamic cavitation was studied.

The release of soluble protein and enzymes were found to increase on increasing the intensity of cavitation (decreasing the cavitation number). The release of acid phosphatase and β-galactosidase reached a maximum extent of 87 % and 67 % respectively at a C_v of 0.17. The increase in the release of protein decreased when cavitation number was decreased below 0.17.

The release of soluble protein and β-galactosidase resembled a saturation function with respect to the number of passes through the cavitation zone and approached a maximum, R_i, around approximately 600 passes. For acid phosphatase, the release increased through 800 passes approached an asymptote around approximately 1500 passes. Increased damage of the cell wall after 600 passes resulted in a higher damage of the cell wall, resulting in the sustained release of periplasmic enzyme up to 1500 passes.

The release of the soluble protein and enzymes from *E. coli* follows first-order kinetics. The release rates pass through a maximum with respect to cavitation number. The maximum release rate was observed at a cavitation number of 0.17 for acid phosphatase and β-galactosidase. Specific activity of acid phosphatase was 4 times higher following release by osmotic shock when compared to disruption at C_v 0.13, but the recovery was only 59 % with osmotic shock compared to 87 % by hydrodynamic cavitation. The specific activity of β-galactosidase was higher on disruption by hydrodynamic cavitation than other methods at all different cavitation numbers studied. Cell fragments along with

a few intact cells were observed by the transmission electron micrographs of *E. coli* disrupted at C_v of 0.13. SDS-PAGE analysis indicated a reduced number of protein bands from the supernatant of hydrodynamic cavitation on comparing with French Press.

Integration of the steps involved in the production and purification of biological molecules has potential to increase the total recovery of the product and reduce the cost involved in the manufacture while maintaining or improving product specification. This can be achieved by the integration of the production phase in the bioreactor with the unit operations of the downstream processing or two or more steps of the downstream processing can be integrated for practical and economic interest.

On integration of cell disruption by hydrodynamic cavitation (C_v - 0.4, 5 % w/v wet wt, 500 passes) with aqueous two-phase extraction using PEG 4600 directly from the cell lysates, the partition coefficient of β-galactosidase increased to 11.6 from 2.3 compared to the discrete unit operations while maintaining equivalent recovery. The viability of yeast cells following cavitation was maintained at 73 % for yeast cells treated at C_v of 0.17 and 1000 passes. The percentage viability increases on decreasing the intensity of cavitation. The maximum specific growth rate of yeast cells subjected to cavitation was not affected significantly (0.236 hr^{-1} to 0.231 hr^{-1}). The yeast cells retain the viability to a significant extent without compromising the metabolic activity on subjecting to hydrodynamic cavitation, indicating potential for integrated product formation and extraction with cell recycle.

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Abbreviations

2,4-D	2,4 Dicholor phenoxy acetic acid
2-HEDS	Hydroxy Ethyl Di-Sulfide
6PGDH	6-Phospho Gluconate Dehydrogenase
ADH	Alcohol Dehydrogenase
AOT	Sodium bis(2-ethylhexyl) sulfosuccinate
APS	Ammonium Per Sulphate
ASME	American Society of Mechanical Engineers
ATP	Adenosine Tri Phosphate
CA	Contrast Agent
CTAB	Cetyl Trimethyl Ammonium Bromide
DTT	Dithiothreitol
EBA	Expanded Bed Adsorption
EDTA	Ethylene Diamine Tetra Acetic acid
FDA	Fluorescein Diacetate
G6PDH	Glucose-6-Phosphate Dehydrogenase
GDP	Guanosine Di-Phosphate
GTP	Guanosine Tri-Phosphate
HPH	High Pressure Homogenization
IgG	Immunoglobulin
IMAC	Immobilized Metal Ion Chromatography
IPTG	Iso Propyl Thio Galactoside
LB	Luria Broth
MPYG	Malt extract, Peptone, Yeast extract, Glucose
NAD	Nicotine Adenine Di-nucleotide (oxidized form)
NADH	Nicotine Adenine Di-nucleotide hydrogen (reduced form)
NADP	Nicotine Adenine Di-nucleotide phosphate (oxidized form)
NADPH	Nicotine adenine Di-nucleotide phosphate hydrogen (reduced form)
NPSH	Net Positive Suction Head

$NPSH_a$	Net positive Suction Head available
$NPSH_r$	Net Positive Suction Head required
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PFHb	Plasma Free Haemoglobin
PHB	Polyhydroxybutyrate
R_b	Maximum radius of bubble
R_{BR}	Blast radius
R_g	Maximum radius of globule
R_k	Killing radius
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SLS	Sodium Lauryl Sulphate
TYE	Tryptone Yeast Extract

Nomenclature

μ	Growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
ρ	Density of the liquid media (Kg/m^{-3})
C_v	Cavitation number
D	$R_m/(R_m-R)$
ID	Internal diameter of the pipe (m)
K	Effective disruption rate constant ($K = kP^a$), MPa^{-1}
k	First order release rate constant (s^{-1} – bead mill, MPa^{-1} – high pressure homogenization, s^{-1} – sonication, dimensionless – hydrodynamic cavitation)
K_c	Generalised rate constant for cell disruption (k (V/P)), s^{-1}
k_i	Enzyme release rate constant (s^{-1})
K_i	Generalised rate constant for cell disruption (k_i (V/P)), s^{-1}
k_t	Protein release rate constant, (s^{-1})
K_t	Generalised rate constant for cell disruption (k_t (V/P)), s^{-1}
N	Number of passes

P_3	Pressure downstream of the orifice plate (MPa)
P_v	Vapour pressure of the liquid (MPa)
R	Amount of soluble protein or enzymes released (mg/g)
R_i	Maximum amount of soluble proteins or enzyme released under the specified conditions (mg/g)
R_m	Maximum amount of soluble protein or enzymes available for release from cell (mg/g)
t	Time (s)

Chapter 1: Introduction

Microbial cells can be used as a source of a large variety of biological products of commercial interest. The ability to manipulate the genetic information of the microorganisms has increased the potential usage of microbial cells in medical, diagnostic, therapeutic, food and chemical industry. *E. coli* and *S. cerevisiae* will continue to be the hosts for many first generation large scale processes, but these hosts do not excrete high level of proteins to the medium (Middelberg, 1995). The intracellular accumulation of the product of interest by these organisms is considered a bottleneck in the production and purification of the biotechnological products. The genetic manipulation of the microbial cell to make them leaky or manipulation of the microorganism to secrete the product into the culture medium has found limited commercial application to date.

Besides giving shape and strength to the microorganism, the cell wall envelope of bacteria and yeast also forms the barrier for the excretion of intracellular products. The network components of the cell wall have to be disrupted for the release of the products of intracellular derivation.

The methods of disruption can be broadly classified into mechanical and non-mechanical methods. The mechanical methods include the use of the bead mill, high pressure homogenization and sonication. The non-mechanical methods include physical (osmotic), chemical, enzymatic and novel techniques. Mechanical methods enable a higher recovery of the product and they are preferred for microbial cell disruption over non-mechanical methods from an industrial application perspective. However, these methods suffer from several drawbacks. Due to the cells being broken completely, the product of interest must be separated from host of other contaminant proteins. The release of DNA increases the viscosity of the crude mixture posing severe limitations for the application of chromatography in the purification of the products. The particle size of the cell debris decreases when the cell suspension is passed through the disruption equipment several times, limiting its quantitative elimination due to the formation of finer cell debris. Most

of the energy supplied to the disruption equipment is dissipated as heat; hence temperature control of the suspension during disruption is necessary. A fine control of the cell disruption for a selective release of the product from a particular location (i.e. cell wall or periplasm) in the absence of release of cytoplasmic enzymes is challenging with mechanical methods. While enzymes from different locations are released at different release rates (Follows *et al.*, 1974), the difference in release rates, is not considered sufficient to fractionate the product groups.

Non-mechanical methods are often limited to laboratory scale because of the practical and economic limitations. Selective release of periplasmic enzyme while maintaining the spheroplasts intact has been reported by osmotic shock (Anraku and Heppel, 1967; Dvorak *et al*, 1967; Nossal and Heppel, 1967).

The use of hydrodynamic cavitation for microbial cell disruption was investigated by Harrison and Pandit, (1992). Cavitation is the formation, growth and collapse of vapour cavities in a liquid due to the reduction in the static pressure below the liquid vapour pressure. Cavitation can be induced in the flow system by restricting the flow using the orifice plate. The size and geometry of the orifice plate can be manipulated to vary the intensity of cavitation. These cavities oscillate through collapse and rebound cycles until they are destroyed by the pressure recovery. Damage of the microbial cell occurs during the collapse of the cavities during pressure recovery, downstream of the orifice plate.

The various process variables that can be manipulated to vary the intensity of cavitation include number and size of the orifice plates, initial concentration of the cell suspension, operating temperature, number of passes through the cavitation zone. Hydrodynamic cavitation offers the potential to vary the intensity of damage to a cell. Hydrodynamic cavitation is reported to be more energy efficient than other disruption methods (Harrison and Pandit, 1992; Jyoti and Pandit, 2001; Kalumuck and Chahine, 2003; Save *et al.*, 1994, 1997). The ease of construction of the apparatus and operation also makes it attractive.

This dissertation presents an investigation of the effect of the process variables on the release of enzymes from different locations within the microbial cell by hydrodynamic cavitation. Further, its potential for selective release of intracellular products is presented. The potential of the hydrodynamic cavitation for integration with upstream and downstream processing was also investigated. The methods of cell disruption available and the research performed to date using these methods are reviewed in Chapter 2. A brief description of the cell wall structure of yeast and bacteria is also included. Special emphasis is laid on the influence of location of the enzymes on the selective release potential of each disruption technique. A review of the phenomenon of cavitation and the literature available on cell disruption by hydrodynamic cavitation is presented in Chapter 3. A detailed description of the various cavitation rigs used for the current research is presented in Chapter 4. The procedure for cell disruption using high pressure homogenization, the French Press and hydrodynamic cavitation; the analytical procedures for determining protein release and the activity of various enzymes, along with the sodium dodecyl polyacrylamide electrophoresis (SDS-PAGE) and Transmission Electron Microscopy (TEM) procedures employed for the current study are also described in Chapter 4.

The results of the study of disruption of Brewers' yeast (*Saccharomyces cerevisiae)* by hydrodynamic cavitation is presented in Chapter 5. The effect of varying the cavitation number, initial cell concentration of the suspension and number of passes through the cavitation zone on the release of enzymes from various locations of the yeast cell is presented in this chapter. Release kinetics of the total soluble protein and enzymes and the selective release of the enzymes from yeast by hydrodynamic cavitation are also discussed in this chapter.

The results of the disruption of *E. coli* by hydrodynamic cavitation are presented in Chapter 6. The effect of varying the cavitation number, number of passes through the cavitation zone, and growth rate of the *E. coli* cells on the release of the enzymes from various locations of the cell is presented in this chapter. Release kinetics of total soluble protein and representative enzymes from various locations of *E. coli* and selective release

of the enzymes from *E. coli* by hydrodynamic cavitation is compared with the other methods of disruption such as osmotic shock, the French Press and high pressure homogenization in this chapter.

The integration of cell disruption by hydrodynamic cavitation with the aqueous two-phase separations for the extraction of β-galactosidase from *E. coli* was investigated and the results are presented in Chapter 7. A brief review of the literature on aqueous two-phase separation is included. The potential of hydrodynamic cavitation for integration with an upstream process is investigated and discussed in this chapter.

In Chapter 8, the conclusions drawn from the study are presented and recommendations are made of areas in which future work may yield fruitful results on integration of cell disruption by hydrodynamic cavitation with both the upstream product formation and downstream product purification processes.

Chapter 2: Literature Review on Cell Disruption

2.1. Introduction

2.1.1. The need for microbial cell disruption

The need for microbial cell disruption has limited the large-scale production of commercial biotechnological products of intracellular derivation. Microorganisms produce a variety of useful enzymes and protein that are not excreted into the culture medium. Hence disruption of the cells is essential for the recovery of such products. The product range has been extended greatly by the production of recombinant products by genetic engineering techniques. With the increase in the use of genetic engineering techniques to use microbial cells as hosts for the production of variety of proteins such as insulin, growth hormone, interferon, β-galactosidase, glucose oxidase, asparaginase, etc., the importance of cell disruption is also increasing. The first step in the downstream processing of intracellular proteins or other intracellular molecules is the disruption of the microbial cell.

Saccharomyces cerevisiae and *E. coli* are the preferred hosts for the production of a variety of biological products (Middelberg, 1995). These hosts frequently do not secrete the product of interest into the culture medium. A signal sequence is necessary for the proteins to be transported from cytoplasm across the cytoplasmic membrane to periplasm. The cytoplasmic membrane forms the barrier and prevents the secretion of protein molecules which lack an appropriate signal sequence. The cell wall forms the barrier for bulkier molecules which cannot permeate through the pores in the outer cell wall. Hence the heterologous proteins that are expressed in yeast and *E. coli* can be sequestered into the periplasm (if the molecule is bulky) or in the cytoplasm if they lack the appropriate N-terminal signal sequence. The signal sequence of host microbe, and sometimes the signal sequence associated with the gene cloned, can be genetically engineered along with the gene of interest for the product to be secreted into the periplasm. The signal sequence is cleaved either during translation (co-translational translocation mechanism) or post translationally (post-translational translocation). Because of the bulky nature of the enzyme it may not cross the cell wall barrier and hence is retained in periplasmic or extracytoplasmic region of yeast

and bacteria. Hence cell disruption or partial cell disruption becomes inevitable in the release of these heterologous products. The selection of the cell disruption technique requires the knowledge of the composition and structure of the cell envelope as well as the principle and performace of the technique.

2.1.2. Cell structure

2.1.2.1. Yeast

Saccharomyces cerevisiae cells are generally ellipsoidal in shape with a large diameter ranging from 5 to 10 µm. Brewing strains of *S. cerevisiae* are generally bigger than the laboratory strains. For example ale yeast (NCYC 1006) has a typical mean diameter of 13.4 µm (Walker, 1998) while the lager yeast SAB has a typical mean diameter of 6.1 – 6.8 µm (Robinson, 2001). Yeast represents a typical eurkaryotic cell with the following structures present: nucleus, mitochondria, Golgi apparatus, secretory vesicles, endoplasmic reticulum, vacuoles, ribosomes and microbodies. These cellular contents are encased by an envelope comprising plasma membrane, periplasm and cell wall. The yeast cell envelope surrounds and protects the cytoplasm, and it is comprised of (from inside looking out); the plasma membrane, the periplasmic space and the cell wall. The yeast cell envelope occupies about 15 % of the total cell volume (Walker, 1998). A typical structure of the yeast cell envelope is shown in Figure 2.1.

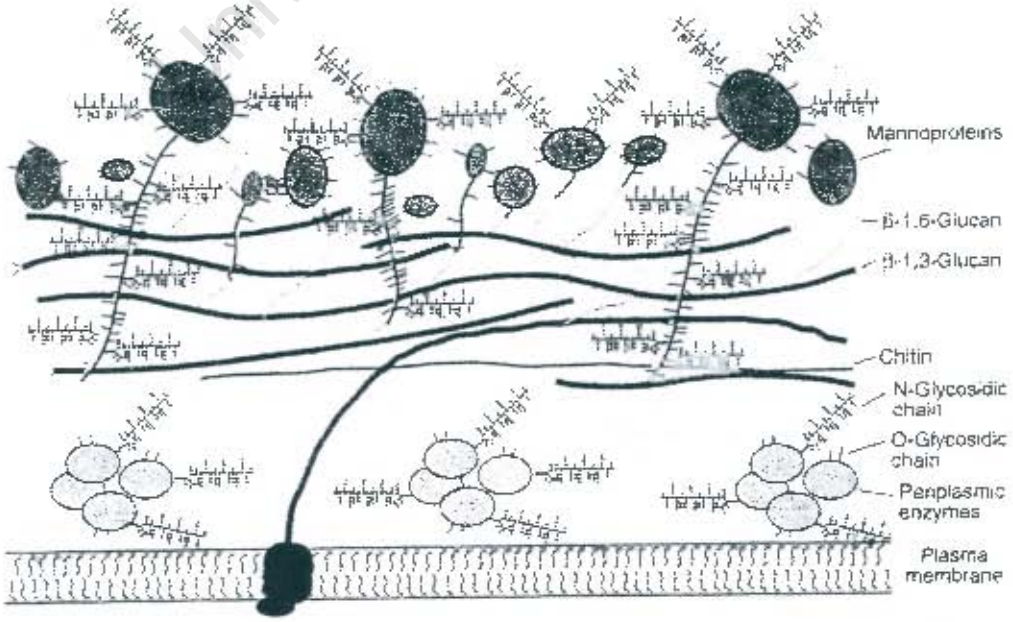

Figure 2.1 Cell wall envelope of *Saccharomyces cerevisiae* (Walker, 1998)

Plasma membrane: The plasma membrane is about 7.5 nm thick and and forms the biological barrier of the cell. It is a lipid bilayer interspersed with globular proteins which form a fluid mosaic. The lipid components comprise mainly of phospholipids and sterols (ergosterol and zymosterol). The phospholipids confer fluidity and sterols provide rigidity. The protein components include those involved in the solute transport (ATPase, permeases), biosynthesis of the cell wall components such as glucan and chitin, transmembrane signal transduction and cytoskeletal anchoring. The plasma membrane offers selective permeability for the uptake of sugars, nitrogenous sources, ions etc. Other functions of the plasma membrane include signal transduction, exocytosis and endocytosis.

Periplasm: This is a thin (3.5 – 4.5 nm) cell wall-associated region external to the plasma membrane and internal to the cell wall. The periplasm comprises mainly secreted proteins (eg. mannoproteins) which are unable to permeate the cell wall.

Cell wall: The wall of yeast cells represents quite a thick (generally 100 - 200 nm) structure comprising between 15 - 25 % of the total dry mass of the cell. The thickness of the cell wall increases with age. The main structural constituent of the yeast cell wall is polysaccharide, which accounts for 80 – 90 % of the cell wall components. These are principally glucans and mannans with a minor proportion of chitin. The strength of the cell wall is mainly due to its glucan component. The glucan components are linked together by both β-1,6 and β-1,3 linkages. Mannans are present as an α-1,6-linked inner core with α-1,2 and α-1,3 side chains. Chitin, a polymer of N-acetylglucosamine, is present in small quantity in *S. cerevisiae* (2 - 4 %) and is associated with bud scars. The cell wall is a layered structure, the outermost section of which comprises cross-linked mannoproteins. These are linked by hydrophobic interaction or by disulphide bonds. They are also linked to the inner fibrillar glucan network by covalent bonds. The mannoproteins are considered to be important in determining the porosity of the yeast cell wall which is known to exclude molecules greater than about 600 Da. Some β-glucan of the inner layer is cross-linked to chitin which itself is a major constituent of the bud scars in budding yeasts. The functions of the yeast cell wall include mechanical strength, cell protection, shape maintenance, cellular interactions, and specialised enzymatic activities.

2.1.2.2. Bacteria

Nearly all bacterial cell walls contain a basic peptidoglycan network. There are two classes of bacteria defined on the basis of their staining characteristics of the cell wall, *Gram-negative* and *Gram-positive*. The difference between the cell envelope of *Gram-postitive* and *Gram-negative* bacteria is shown in Figure 2.2. The walls of *Gram-positive* bacteria are relatively thick (15 - 50 nm) and contain 40 - 90 % peptidoglycan, the remainder being polysaccharides and teichoic acids. In *Gram-negative* bacteria, the cell wall consists of a thinner peptidoglycan layer (1.5 - 2.0 nm) and an outer membrane enclosing the periplasmic space (Engler, 1985).

Cytoplasmic membrane: The cytoplasmic membrane is approximately 4 nm wide and is comprised mainly of phospholipids and protein. It contains about half of the phospholipids of the cell envelope and is readily dissociable by Triton X-100 and other detergents (Schnaitman, 1971a). It has a complex protein composition, typical of other biological membranes. It maintains the concentration gradients, houses transport systems and is involved in ATP generation.

Periplasm: The cytoplasmic membrane is separated from the outer cell wall by an electron-transparent region with a minimum dimension of about 10 nm (Schnaitman, 1971b). This is defined as periplasmic space.

Cell wall: The cell wall is made up of outer membrane (in *Gram-negative* bacteria only) and the inner peptidoglycan layer. The peptidoglycan structure is similar in all bacteria and is comprised of linear polysaccharide chains of alternating N-acetyl-D-glucosamine (NAG) and N-acetyl-muramic acid (NAM) residues joined by β-(1-4) glycosidic bonds. The chains are cross-linked by a tetra peptide of basic structure L-alanyl-D-glutamyl-L-R_3-D-alanine attached to the C_3 lactic acid side chains of the NAM residue. The L-R_3 amino acid residue may be one of the several di-aminoacids, frequently diaminopimelic acid. The peptide branches of the parallel chains are further cross-linked (Holtje and Glauner, 1990). The resulting rigid structure acts as a single macromolecular network to provide the shape, tensile strength and osmotically protective nature of the cell envelope. The peptidoglycan layer provides the mechanical strength of the cell.

The cell envelope of *Gram-positive* bacteria is composed of 50 - 80 % peptidoglycan, associated with teichoic acids, presenting a greater structural resistance to breakage. In *Gram-positive* cells, the peptidoglycan layer is not protected from the external environment by an outer membrane, but provides greater structural strength.

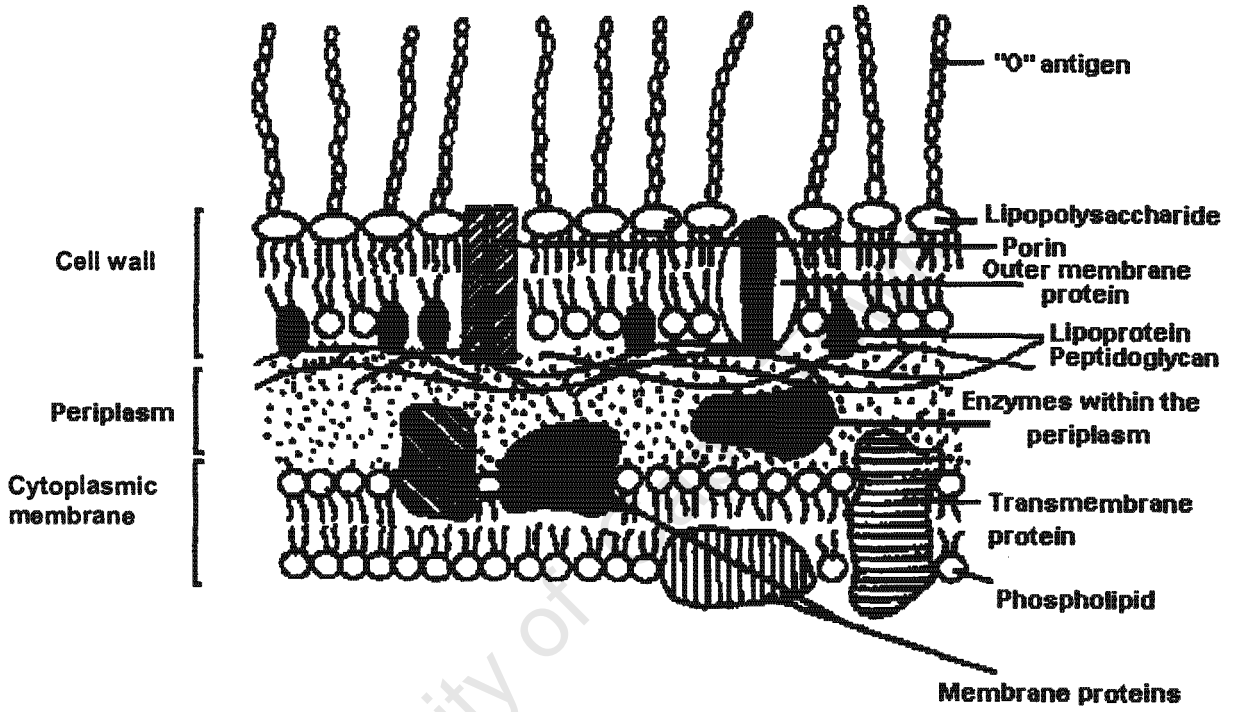

Figure 2.2 Cell wall envelope of *Gram-negative* bacteria (Schnaitman, 1971b)

2.1.3. Selective product release

The microbial cell contains a variety of enzymes and proteins. Hence on a non-selective disruption of the microbial cell, the target enzyme is liberated in a complex mixture of proteins and other biomolecules. The downstream processing of the target enzyme becomes increasingly challenging with complete cell disruption because of

the release of a host of contaminant proteins and other compounds. Further, micronization of cells by the mechanical disruption complicates solid-liquid separation for removal of cell debris for the product recovery (Agerkvsit and Enfors, 1990; Quirk and Woodrow, 1984). This in turn compromises the yield achievable. Hence a selective release of the desired enzyme relative to release of other intracellular proteins without the formation of fine cell debris would be rewarded.

Enzymes are confined to a particular compartment of the cell. Different enzymes are located in different compartments of the cell such as the cell wall, periplasm, cytoplasm, and cytoplasmic organelles. This segregation of the enzyme to a particular compartment of the cell can be exploited for the selective release of enzymes. A disruption strategy in which the outer wall of the yeast or *Gram-negative* bacteria is permeabilized or broken open in the first step to release the extracytoplasmic enzymes while maintaining the spheroplasts intact, and the rupture of the cytoplasmic membrane in the second step for the recovery of the cytoplasmic enzymes would be ideal. A similar approach using enzymes and chemicals for release of enzymes from various locations of the yeast cell was proposed and demonstrated by Huang *et al.* (1991).

In the heterologous production of proteins, the product of interest can be cloned along with a signal sequence to transport the product from the cytoplasm to the periplasm. The periplasmic location of bacteria has several advantages. The periplasmic space contains 7 out of 25 known cellular proteases and comprises only 4 - 8 % of total cell protein (French *et al.*, 1996). The mature secreted protein does not incorporate N-formyl methionine and the oxidative environment of the periplasm facilitates correct disulfide bonding and protein folding. A complete disruption of the cell or intense disruption conditions is not required for a product which is located in the periplasm. A selective rupture of the outer cell wall or permeabilization of the outer cell wall to make it leaky could result in the release of the periplasmic product without releasing the constituents of the cytoplasm. The principle advantage of this selective product strategy would be the higher purity of the product and avoidance of the micronization of cells, which will ease the solid-liquid separation process subsequent to the cell disruption.

2.1.4. Cell disruption

Cell disruption can be achieved by variety of means. The methods available for disruption can be broadly classified into mechanical and non-mechanical methods (Figure 3). Mechanical methods can be combined with non-mechanical methods to increase the efficiency of cell disruption. Mechanical methods include the bead mill, high-pressure homogenizer, and use of cavitation generated through either sonication or by fluid flow. Non-mechanical methods include chemical treatment, enzymatic lysis, osmotic shock, freeze/thaw cycles and novel methods.

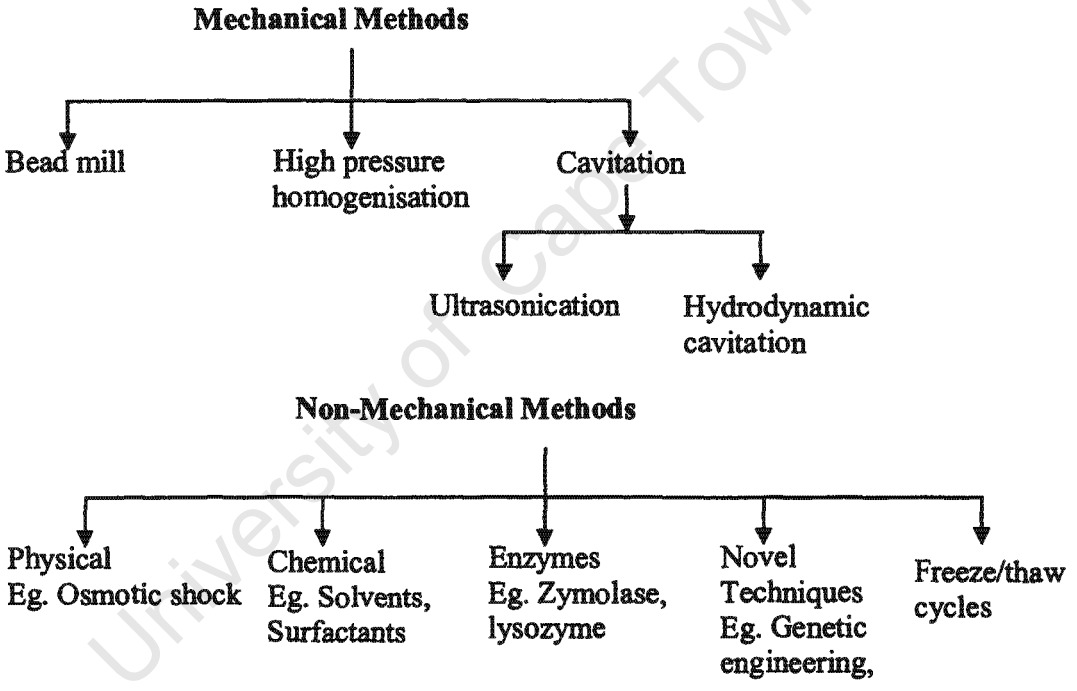

Figure 2.3 Classification of the techniques available for microbial cell disruption

Although a variety of techniques are available for intracellular enzyme release, the mechanical methods have found greater commercial application than the non-mechanical methods because of the operational and economic limitations of the latter at process scale. The bead mill and the high-pressure homogenizer are used in the large-scale microbial cell disruption. The other mechanical and all the non-mechanical methods are predominantly used at the laboratory scale or in the high value low volume processes such as in the release of plasmid DNA. The bead mill and

the high-pressure homogenizer offer a complete disruption of the cells and are easier to scale up for industrial process. Ultrasonication is not used in the large-scale cell disruption because of the difficulty in transmitting sufficient power to large volumes of cell material (Kalumuck and Chahine, 2000). Most of the energy absorbed by the suspension during ultrasonication appears as heat, making it challenging to provide good temperature control. Chemical and enzymatic methods are limited to laboratory scale because of the difficulties in removing the exogenous chemicals or enzymes from product and also due to the cost of the chemicals or enzymes required for disruption at large scale (Baldwin and Robinson, 1994).

Cell disruption has been reviewed previously (Chisti and Moo-Young, 1986; Geciova *et al.*, 2002; Harrison, 1991; Kula and Schutte 1987; Middelberg, 1995; Schutte and Kula, 1990). The literature review that follows provides an overview of the principle of product release and its operation for the selective product release and the significance of the location of the enzyme in the microbial cell for achieving selective product release with each unit operation for disruption.

2.2. Mechanical methods

The mechanical methods used routinely for cell disruption include the bead mill, high pressure homogenization and ultrasonication. These methods offer a near complete liberation of the intracellular products from the microbial cells. The bead mill and high pressure homogenization are the preferred methods of cell disruption for large scale and industrial production processes owing to the ease of scale-up (Middelberg, 1995). Because of the harsh conditions during the cell disruption, mechanical methods are unsuitable for shear sensitive products like plasmid DNA (Ciccolini *et al.*, 1998). A significant amount of energy used during these processes is converted to heat and hence temperature control of the suspension during disruption is necessary (Middelberg, 1995). Micronization of the cells is common and hence removal of the cell debris from the supernatant in the subsequent solid-liquid separation is a challenge.

2.2.1. Bead mill

A bead mill is characterised by a hollow cylinder rotated on a horizontal axis loaded with a certain quantity of the grinding element and a central agitator to keep the grinding elements moving. The advantage of the horizontal as compared to the vertical position of the grinding chamber is the almost complete elimination of gravity forces as a parameter of the milling process (Engler, 1985). On the agitator shaft, different impellers are fixed in the form of discs, rings or pins. The impellers can be mounted centrically or eccentrically on the shaft. The construction of the agitator and impellers is directed towards an optimal transfer of kinetic energy from the rotating parts to the grinding elements, typically glass beads (Kula and Shutte, 1987). For microbial cell disruption, lead-free glass beads of 2.5 g/cm^3 are generally used (Engler, 1985). This technique is widely used in industry. The importance of location of the enzymes on their release rates has been demonstrated (Melendres *et al.*, 1993; Ricci-Silva *et al.*, 2000; Schutte *et al.*, 1983; Torner and Asenjo, 1991; van Gaver and Huyghebaert, 1990).

2.2.1.1. Mechanism of disruption

Kinetic energy from the agitator is transported to the grinding elements. The differential velocity profile created generates high shear forces depending on the peripheral velocity of the agitator and the size of the grinding elements. Shear forces generated along with the frequency and strength of collision between the beads are responsible for the disintegration of microbial cells. Scholtz-Brown *et al.* (1998) has shown that breakage kinetics in the bead mill and slurry reactor can be modelled by the same basic equations. Further, the dominant mechanism is solid-cell-solid collision.

2.2.1.2. Kinetics of disruption

Disintegration of cells in a high-speed agitator bead mill is a first order process (Currie *et al.*, 1972; Garrido *et al.*, 1994; Limon-Lason *et al.*, 1979; Marffy and Kula, 1974; Melendres *et al.*, 1993; Schutte and Kula, 1988; Torner and Asenjo, 1991, Woodrow and Quirk, 1982) and can be described for the solubilization of proteins by Equation 2.1.

$$\ln\left(R_m / (R_m - R)\right) = kt \qquad \textbf{2.1}$$

where 'R_m' is the maximum protein available for release, 'R' is the protein released at a time 't' seconds and 'K' is the first order release constant.

2.2.1.3. Factors affecting disruption

Various factors affecting disruption by bead mill include agitator speed, feed rate, ratio of the cell to bead size and density of the beads, cell concentration, temperature and bead loading. A summary of the studies presented in the literature on cell disruption by the bead mill as a function of process variables is presented in Table 2.1

2.2.1.3.1. Agitation speed

Increased speed of the agitator leads to a higher frequency of contact between the attritive elements and a greater shear stress as their differential velocities are increased. These result in an increase in the disruption. Table 2.2 summarizes the effect of agitation speed on the cell disruption studied by various researchers. The extent of release and the release rate of G6PDH from Bakers' yeast was found to increase with increase in the agitation speed up to 1800 min^{-1} (Shutte and Kula, 1988). Increasing the agitation speed from 1100 to 3100 rpm increased the extent of disruption of Bakers' yeast from 60.9 to 88.5 % (Ricci-Silva *et al.*, 2000). The release rate of total soluble protein, maltase, acid phosphatase, malate dehydrogenase, G6PDH and 6-phosphogluconate dehydrogenase from Brewers' yeast was found to increase with increasing the agitation speed in the range 2000 - 4500 rpm. Increasing the agitation speed increased the release rate of β-galactosidase from recombinant *Saccharomyces cerevisiase* up to 2000 rpm. Further increase in agitation did not increase the release rate. Agitation higher than 2000 rpm was said to cause increased slip between the rotor and the suspension of the grinding elements and hence the effectiveness of the momentum transfer to the beads did not improve with an increase in the agitation speed beyond 2000 rpm (Garrido *et al.*, 1994). The release rate of total soluble protein from *Enterobacter cloacae* increased from 0.015 to 0.023 s^{-1} on increasing the agitation speed from 3000 to 4500 rpm while the release rate of β-lactamase increased from 0.02 to 0.037 s^{-1} (Woodrow and Quirk, 1982).

Table 2.1 Summary of the literature on the process variables affecting cell disruption in the bead mill

Microorganism	Agitation speed (rpm)	Initial cell concentration	Bead size (mm)	Bead loading (v/v)	Miscellaneous	Enzymes	Reference
Bakers' yeast	1100, 1700, 2300, 3100	170, 250 and 325 g/l	0.5	20, 30, 40 %		Protein, G6PDH	Ricci-Silva *et al.*, 2000
Bakers' yeast	600 - 1800	0 – 600 g/l	0.5 - 3	3-10 kg (4.2 litre tank)	Temperature 5 - 40 °C	Proteins	Currie *et al.*, 1972
Recombinant S. cerevisiae	1000 - 4000	0 - 20 g/l	0.25 - 0.75	70 – 85 %		β-galactosidase, protein	Garrido *et al.*, 1994
Bakers' yeast, *E. coli, L. casei, L. confusus, B. cerus, C. boidinii*	150 - 1800	50 – 600 g/l	0.1 - 1.5	40 – 95 %		Proteins	Schutte & Kula, 1988
Enterobacter cloacae, E. coli, Pseudomonas sp.	2000, 3000, 4500	250 and 400 g/l	0.1, 0.25, 0.45	80 – 85 %		β-lactamase, carboxy peptidase, protein	Woodrow and Quirk, 1982
Brewers' yeast	2000 - 4500		0 - 0.75	87 %	Flow rate, Cell density	Maltase, phosphatase, malate dehydrogenase, G6PDH, 6PGDH, protein	Marffy and Kula, 1974

Microorganism	Agitation speed (rpm)	Initial cell concentration	Bead size (mm)	Bead loading (v/v)	Miscellaneous	Enzymes	Reference
S. cerevisiae	2000	300 g/l (dry wt)	0.375, 0.625, 0.875	0.3 -0.85		Invertase, acid phosphatase, ADH, alkaline phosphatase, protein	Melendres *et al.*, 1993
S. cerevisiae, C. boidinii	Data not available	50 g/l	0.5	70 %		Invertase, α-glucosidase, fumarase, ADH	Torner and Asenjo 1991
Bakers' yeast, Brewers' yeast, *Candida utilis*	1640, 2340, 2930	110 g/l (dry wt)	0.5 - 0.75	50 %		Acid phosphatase, alkaline phosphatase, fumarase, ADH G6PDH, protein 6PGDH	Mogren *et al.*, 1974
Pichia pastoris	10 ms^{-1}	350-450 g/l	0.5 - 0.75	85 %		Protein	Canales *et al.*, 1998
Bakers' yeast	1900	0 - 30 % w/w	0.75 - 1.00	10 – 80 %		Invertase, G6PDH, nitrogen	van Gaver and Huyghebaert, 1990

Table 2.2 Summary of the literature on the effect of agitation speed on cell disruption in the bead mill

Agitation speed (rpm)	Results/comments	Reference
600 – 1800	Release rate of G6PDH increases with increase in agitation speed up to 1800 rpm	Schutte and Kula, 1988
1100 – 3100	Increase in agitation speed increases the extent of disruption from 60.9 to 88.5%	Ricci-Silva *et al.*, 2000
2000 – 4500	Release rate of proteins and enzymes increases with agitation speed up to 4500 rpm	Marffy *et al.*, 1974
1000 – 4000	Release of β-galactosidase increases with agitation speed up to 2000 rpm and higher speed did not improve cell disruption	Garrido *et al.*, 1994
3000 and 4500	$k_{proteins}$ was 0.015 and 0.023 s^{-1} while $k_{\beta\text{-lactamase}}$ was 0.2 and 0.037 s^{-1} respectively	Woodrow and Quirk, 1982

2.2.1.3.2. Initial concentration of cell suspension

The effect of initial cell concentration on cell disruption by bead mill was studied by Garrido *et al.* (1994); Ricci-Silva *et al.* (2000); Schutte and Kula (1988); van Gaver and Huyghebaert (1990); Woodrow and Quirk (1982). The results are summarized in Table 2.3. In the concentration range of 0.5 to 2 % (5 - 20 g/l, dry wt), the cell concentration had no influence on the release of soluble proteins and β-galactosidase from recombinant *Saccharomyces cerevisiae* (Garrido *et al.*, 1994). Concentrations of up to 30 % (300 g/l, packed yeast) had no influence on the protein release from Bakers' yeast (van Gaver and Huyghebaert, 1990). On the study with *Enterobacter cloacae*, increasing the cell concentration from 25 % (250 g/l, cell paste) to 40 % (400 g/l, cell paste) had little effect on the disruption (Woodrow and Quirk, 1982). Schutte and Kula (1988) reported that faster disintegration could be achieved when slurries with cell concentration below 40 % w/v (packed Bakers' yeast) were used. Increasing the cell concentration of yeast from 17 % to 32.5 % (170 g/l to 325 g/l, packed yeast) was found to decrease the percentage disruption of Bakers' yeast from 97.3 to 86.6 by Ricci-Silva *et al.* (2000). One gram of packed yeast has been reported to be equivalent to 0.28 dry weight of yeast (Hetherington *et al.*, 1971). This value was used to normalize all the data available from the literature for comparison (Table 2.3). van Gaver and Huyghebaert (1990), reported no influence of cell concentration over the range 14 – 84 % w/v (dry wt). However, Ricci-Silva *et al.* (2000) reported a decrease in the extent of release of proteins on increasing the cell concentration from 48 to 91 % w/v (dry wt).

Table 2.3 Summary of literature on the effect of initial cell concentration of the suspension on cell disruption in the bead mill

Initial cell concentration (%, w/v)	Calculated dry wt (g/l)	Results/comments		Microorganism	Reference
0.5 – 2 (dry wt)	5 – 20	No influence on the extent of release of protein and β-galactosidase		recombinant *S. cerevisiae*	Garrido *et al.*, 1994
5-30 (packed yeast)	14 – 84	No significant effect on the extent of release of proteins		Bakers' yeast	van Gaver and Huyghebaert, 1990
Cell paste		$k_{proteins}$ s^{-1}	$k_{\beta\text{-lactamase}}$ s^{-1}	*Enterobacter cloacae*	Woodrow and Quirk, 1982
25		0.018	0.02		
40		0.021	0.021		
0-60 (packed yeast)	0 – 168	Complete disintegration achieved faster when the slurry conc. is less than 112 % (dry wt)		Bakers' yeast	Schutte and Kula, 1988
17, 24.9, 32.5 (packed yeast)	48, 70, 91	Extent of disruption decreased from 83.8 to 61.8 % on increasing the cell concentration from 48 to 91 g/l (dry wt)		Bakers' yeast	Ricci-Silva *et al.*, 2000

2.2.1.3.3. Bead size

The effect of bead size on the disruption of microbial cells, reported in the literature (Currie *et al.*, 1972; Garrido *et al.*, 1994; Melendres *et al.*, 1993; Schutte and Kula, 1988; Woodrow and Quirk, 1982) is summarized in Table 2.4. Increasing the bead size was found to decrease the protein release rate constant from Bakers' yeast in the size range of 0.5 mm to 3 mm by Currie *et al.* (1972), to reduce the disruption rate constant of *Enterobacter cloacae* using beads of 0.1, 0.25 and 0.45 mm diameter by Woodrow and Quirk (1982) and decrease the release rate constant of alkaline phosphatase from Bakers' yeast when using the beads of sizes 0.325 (0.032 s^{-1}), 0.625 (0.018 s^{-1}) and 0.875 mm (0.009 s^{-1}) by Melendres *et al.* (1993). In the disruption of recombinant yeast for the release of β-galactosidase, 0.5 mm diameter beads were more effective than 0.25 mm or 0.75 mm beads. The optimum bead size also depends on the type of micro organism used. The following bead size fractions were reported for optimal release: 0.25 - 0.5 mm maximum protein release rate from *S. carlbergensis* (Marffy *et al.*, 1974), approximately 1 mm was effective for Bakers' yeast (*S. cerevisiae*), 0.25 - 0.50 mm for *Bacillus* and *Brevibacterium sp.* and 0.5 - 0.75 mm fraction for *Lactobacillus* and *E. coli* (Schutte and Kula, 1988).

2.2.1.3.4. Bead loading

The bead loading used for disruption also affects the disruption of cells. Increased bead loading provides a better transfer of the disruption energy resulting in higher disruption efficiency. Increasing the bead loading up to 70 % v/v was found to increase the extent of disruption (van Gaver and Huyghebaert, 1990; Ricci-Silva *et al.*, 2000; Curie *et al.*, 1972). The release rate was found to increase on increasing the bead loading from 75 to 85 % by Garrido *et al.* (1994). A 90 % bead loading for yeast and 80 - 85 % bead loading for *E. coli* was recommended by Schutte and Kula (1988) for a higher extent of release.

Table 2.4 Summary of the literature on the effect of size of beads on microbial cell disruption in the bead mill

Bead size (mm)	**Results/comments**		**Microorganism**	**Reference**
0.5 – 2.8	Protein release rate constant decreases with increase in bead size		Bakers' yeast	Currie *et al.*, 1972
0.1, 0.25 and 0.45	Rate of breakage decreased with increasing bead size		*Enterobacter cloacae*	Woodrow and Quirk, 1982
0.325, 0.625, 0.875	$k_{alkaline\ phosphtase}$ decreases with increase in sizes 0.032, 0.018, 0.009 s^{-1} respectively		Bakers' yeast	Melendres *et al.*, 1993
0.25, 0.5 and 0.75	Extent of release of β-galactosidase was best with 0.5 mm beads. Fluidization effect with 0.25 size beads. Decline in collision frequency with 0.75 mm size beads		recombinant *Saccharomyces cerevisiae*	Garrido *et al.*, 1994
0.1-0.25, 0.25-0.5, 0.5-0.75	Protein release rate was highest with 0.25-0.5 mm fraction		*S. carlbergensis* (Brewers' yeast)	Marffy and Kula, 1974
0.1-0.25, 0.25-0.5, 0.5-0.75, 0.75-1.0, 1.0-1.5	Optimum size fraction depends on the microorganism		Bakers' yeast, *Candida boidinii*, *L. casei*, *L. confusus*, *B. cereus*, *Brevibacterium ammoniagenes*	Schutte and Kula, 1988
	1.0-1.5	*Candida*, Bakers' yeast		
	0.5-0.75	*Lactobacillus sp.*, *E. coli*		
	0.25-0.50	*Bacillus* and *Brevibacterium*		

2.2.1.4. Significance of location and selectivity

The location of the enzyme or biomolecule of interest in the microbial cell influences its release from the cell and subsequent recovery. The enzyme can be located in the cell wall, periplasm, cytoplasm, cytoplasmic membrane or cell organelles etc. The release rate of the enzyme will therefore depend on the location of the enzyme and the mechanism of disruption. The maximum release of invertase (cell wall bound), α-D-glucosidase (periplasmic), alcohol dehydrogenase (ADH, cytoplasmic) and fumarase (mitochondria) from *Saccharomyces cerevisiae* were obtained following 2, 5, 10 and 15 minutes of disruption in the bead mill respectively (0.5 mm beads diameter, 70 % bead loading, 5 % w/v cell concentration). The first order release rate constant was highest for total soluble protein release (0.029 s^{-1}) followed by invertase (0.026 s^{-1}) α-glucosidase (0.024 s^{-1}), ADH (0.023 s^{-1}) and fumarase (0.011 s^{-1}) (Torner and Asenjo, 1991). Similar results were obtained by Melendres *et al.* (1993) during the disruption of *Saccharomyces cerevisiae* under the following conditions: 0.325 mm bead diameter, 85 % bead loading, 2000 rpm, 30 % w/v, dry wt. The enzyme invertase (3.10 s^{-1}) was released faster than the others because of its location in cell wall, followed by the release of periplasmic acid phosphatase (2.61 s^{-1}), cytoplasmic ADH (2.22 s^{-1}) and cytoplasmic membrane bound alkaline phosphatase (0.032 s^{-1}) at decreasing rates.

The release rates of maltase and acid phosphatase (periplasmic) were higher than the cytoplasmic enzymes, malate dehydrogenase, G6PDH and 6PGDH in the disruption of Brewers' yeast *S. carlbergensis* in the bead mill at 2000 rpm, 0.25 - 0.5 mm bead diameter and 87 %, bead loading (Marffy and Kula, 1974).

A CoBall-Mill MS-12, similar in principle to the conventional bead mill, was used to disrupt the yeast cells (30 % w/w cell concentration, 80 % bead loading, 1900 rpm, 0.75 - 1.00 mm beads). The ball mill consisted of a conical rotor fitting into a conical stator. The ratio of surface area to volume of the disruption chamber is much higher than the conventional bead mill providing good temperature control in the chamber through heat exchange. The location of the enzyme was found to influence the extent of release. The release after one pass was higher for invertase (cell wall bound) than G6PDH (cytoplasmic). The release of G6PDH follows the same trend as protein

(maximum amount was released after 4 passes) while maximum amount of invertase was released in 2 passes (van Gaver and Huyghebaert, 1990). The cytoplasmic enzyme G6PDH was released from Bakers' yeast almost parallel to protein, whereas the release of enzyme fumarase was retarded due to its location in the mitochondria (Schutte and Kula, 1988).

The release rates of enzyme based on its location in the microbial cell in the decreasing order as reported by various workers is summarized in Table 2.5. The cell wall bound enzyme would be expected to be released faster than the periplasmic enzyme followed by the total soluble protein, cytoplasmic enzymes, cytoplasmic membrane bound enzymes and enzymes enclosed in the organelles such as mitochondria. The bulk of the proteins are present in the cytoplasm and hence the total soluble protein release could be expected to be faster than or at the same rate as a cytoplasmic enzyme. This was seen with all cases except that reported by Torner and Asenjo (1991), where the release of total soluble proteins exceeds that of cell wall bound and periplasmic enzyme.

Table 2.5 Sequence of locations of enzymes in the microbial cell based on their release rates of enzymes by various researchers

Sequence of locations of enzymes based on their release rates	Reference
Total soluble protein > cell wall bound > periplasmic > cytoplasmic > mitochondrial	Torner and Asenjo, 1991
Cell wall bound > periplasmic > cytoplasmic > cytoplasmic membrane	Melendres *et al.*, 1993
Periplasmic > total soluble protein > cytoplasmic	Marffy and Kula, 1974
Periplasmic > protein ~ cytoplasmic	van Gaver and Huyghebaert, 1990
Protein ~ cytoplasmic > mitochondria	Schutte and Kula, 1988

The various operating parameters, such as the size of beads and the agitation speed can be manipulated to achieve a differential release rate of the enzymes from various locations of the cell. When the size of the beads used in bead mill is reduced, there is an increase in the shear force due to increase in the number of beads for the same volume, increased contact between the beads and microorganism, and hence an increase in the number of stream layers producing shear forces. A higher shear force

is necessary for the release of enzymes from cytoplasm while a low shear force is sufficient for the release of enzymes from periplasm (Schutte *et al.*, 1983). It is found that in the disruption of *S. cerevisiae*, the location of the desired enzyme in the cell is of importance for the choice of glass bead size. The extent of the cytoplasmic enzyme G6PDH released was at its maximum with glass beads of 0.55 – 0.85 mm while the periplasmic enzyme α-D-glucosidase was released to its maximum extent with the bead diameter of 1 mm or even larger. This was because complete disintegration, which occurs with the lower bead diameter, was not necessary for the release of periplasmic enzyme α-D-glucosidase (Schutte *et al.*, 1983).

The agitation rate in a bead mill can be controlled to obtain an enzyme with higher specific activity (units/mg of protein released) as demonstrated by G6PDH release from Bakers' yeast by Ricci-Silva *et al.*, (2000). At a low agitation speed (2300 rpm), the cell wall and cell membrane were largely destroyed whereas the cell organelles (mitochondria, lysosomes, golgi complex etc.) also rich in proteins, were less damaged. As G6PDH is located in the cytoplasmic, it was readily liberated representing a higher proportion of the bulk protein at 2300 rpm than at 3100 rpm. Thus cytoplasmic G6PDH released at 2100 rpm was of higher specific activity (0.15 U/mg of protein as opposed to 0.05 U/mg of protein after 1 minute of disruption using 32.3 % w/v, packed yeast cell concentration) than that was obtained with 3100 rpm agitation speed.

2.2.2. High pressure homogenisation

In the high pressure homogenizer, disruption is accomplished by passing a pressurized cell suspension with concomitant pressure release through an adjustable, restricted orifice discharge valve. The operating variables of the homogenizer include discharge pressure, rate of pressure release, design of the valves and the impact system. Further, the number of passes through the discharge valve and suspension concentration also affects separation. The French Press is an apparatus used in the laboratory for small scale experiments. It operates on the same general principle as high pressure homogeniser, particularly disrupting cells on response to sudden changes in the operating pressure.

2.2.2.1. Mechanism of disruption

The cells are subjected to shear, turbulence, rapid pressure drop and cavitation in a high-pressure homogenizer (Engler, 1990). However the predominant mechanism of disruption is debated rigorously (Middelberg, 1995, Keshavarz-Moore *et al.*, 1990b, Harrison, 1990). The significant role of the magnitude of the pressure drop (Brookman, 1974), rate of pressure drop, turbulence (Doulah and Hammond, 1975), impingement (Keshavarz-Moore *et al.*, 1990b, Engler and Robinson, 1981a) and the cavitation (Harrison, 1991, Shirgoankar *et al.*, 1997) has been demonstrated. Their relative dominance remains unresolved.

2.2.2.2. Kinetics of disruption

The kinetics of disruption in an industrial high pressure homogenizer has been reported to follow first-order kinetics for Bakers' yeast (Hetherington *et al.*, 1971), *E. coli* (Gray *et al.*, 1972; Sauer *et al.*, 1989), *Alcaligenes eutropus* (Harrison *et al.*, 1991a), *Candida utilis* and *Bacillus subtilis* (Engler and Robinson, 1981a). The release kinetics for yeast slurries not exceeding 600 g/l (packed yeast) was described by Hetherington *et al.* (1971), as:

$$\ln\left(R_m / (R_m - R)\right) = kNP^a \qquad 2.2$$

where 'R' is the protein released, 'R_m' is the maximum protein available for release and the dimensional constant 'k' is a function of the temperature, 'N' is the number of passes 'P' is the operating pressure and 'a' is the pressure exponent. The value of the exponent 'a' represents the resistance of the microorganism to disruption (Sauer *et al.*, 1989).

The value of the exponent 'a' in the disruption of various microorganisms reported by researchers is summarised in Table 2.6. No particular trend in the pressure exponent between various microorganisms or for the same microorganism is observed. For instance, the value of the exponent for the disruption of *Saccharomyces cerevisiae* was reported as 2.9 (Bakers' yeast) by Hetherington *et al.* (1971), 1.72 (Bakers' yeast) by Doulah and Hammond (1975), 1.87 (Brewers' yeast) and 0.86 for *S. cerevisiae* cultivated by continuous fermentation of a μ of 0.1 hr^{-1} by Engler and Robinson (1981b). This variation in the pressure exponent may result from the data having

been collected over a range of operating conditions affecting the rate and extent of disruption

Table 2.6 Summary of the values of the pressure exponent in the release kinetics equation for various micro-organisms from literature

Microorganism	Batch	Continuous	Reference
Saccharomyces cerevisiae (Bakers' yeast)	2.9		Hetherington *et al.*, 1971
Saccharomyces cerevisiae (Brewers' yeast)	1.87		Engler and Robinson, 1981b
Saccharomyces cerevisiae		0.86 (μ - 0.1 hr^{-1})	Engler and Robinson, 1981b
Saccharomyces cerevisiae (Bakers' yeast)	1.72		Doulah and Hammond, 1975
Candida utilis		1.77 (μ - 0.1 hr^{-1}) 1.17 (μ - 0.5 hr^{-1})	Engler and Robinson, 1981b
Escherichia coli	2.2		Gray *et al.*, 1972
Escherichia coli	1.8±0.2		Fonseca and Cabral, 2002
rEscherichia coli (NM989)	1.407 ($\mu = \mu_{max} = 0.35$)	0.662 (μ-0.35 hr^{-1}) 0.633 (μ-0.17 hr^{-1})	Sauer *et al.*, 1989
Escherichia coli	1.427 ($\mu = \mu_{max} = 0.35$)	1.771 (μ - 0.17hr^{-1}) 1.545 (μ - 0.24hr^{-1}) 1.378 (μ - 0.33hr^{-1})	Sauer *et al.*, 1989
Alcaligenes eutropus	3.08 (exponential) 2.80 (late exponential) 1.69 (early stationary), 1.59 (late stationary)	Average value of the exponent 2.1 (μ 0.07 - 0.12 hr^{-1})	Harrison *et al.*, 1991a,b
Bacillus subtilis		1.07 (μ - 0.2 hr^{-1})	Engler and Robinson, 1981b

The effect of specific growth rate on cell disruption has been studied for Candida utilis, E. coli and Alcaligenes eutropus. In these studies contradicting trends are reported for the pressure exponent 'a' as a function of specific growth rate. In the disruption of *Candida utilis*, growth rate of 0.1 hr^{-1} gave a value of 1.77 while the exponent was 1.17 for cells cultivated at 0.5 hr^{-1}. Similar results were observed in the disruption of non-recombinant *E. coli* by Sauer *et al.* (1989): 'a' was 1.771 for 0.17 hr^{-1} and 1.378 for 0.33 hr^{-1}. The pressure exponent was found to be higher for faster

grown cells (exponential) by Harrison *et al.* (1991a, b). As the specific growth rate may influence the resilience to disruption through both 'k' and 'a'. It is more appropriate to characterise the resilience to disruption as the function of growth rate through the disruption rate constant (K), given by $K = kP^a$ from the Equation 2.3

$$\ln\left(R_m/(R_m - R)\right) = LnD^{-1} = kNP^a \qquad \textbf{2.3}$$

'K' was calculated ('a' and 'k' values from Table 2.6 and 2.9 respectively) across the typical operating pressure range according to Equation 2.3. The results are presented in Figure 2.4. It could be seen that the disruption rate constant 'K' is higher for faster growing cells across all the literature data compared.

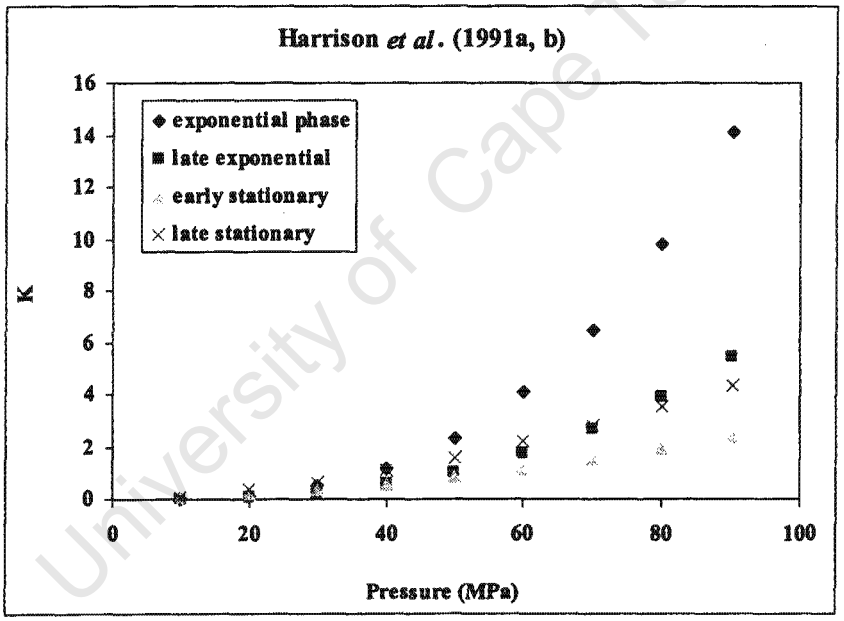

Figure 2.4 Effective 'K' ($K = kP^a$) as a function of pressure calculated from Harrison *et al.* (1991a, b)

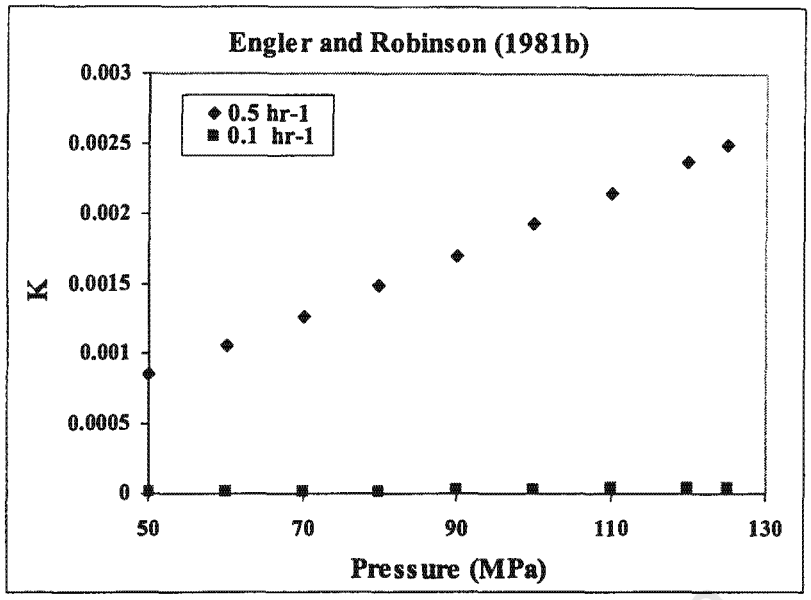

Figure 2.5 Effecive 'K' (K = kP^a) as a function of pressure calculated from Engler and Robinson (1989b)

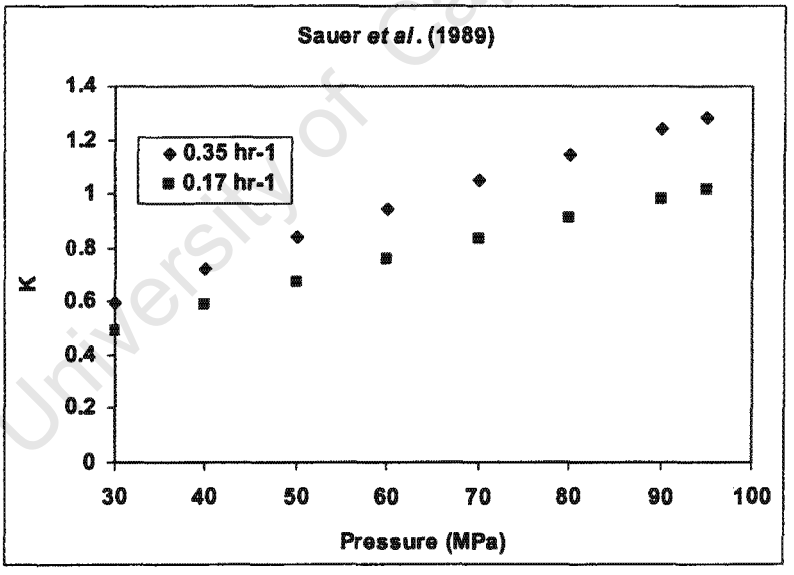

Figure 2.6 Plot of effecive 'K' (K = kP^a) as a function of pressure calculated from Sauer *et al.* (1989)

2.2.2.3. Factors affecting disruption

The various factors that affect the microbial cell disruption process by high pressure homogenization include the operating pressure, design of the valve unit, cell density, physiological factors and the temperature of the cell suspension. A summary of the literature on high pressure homogenization, along with the process variables studied, is presented in the Table 2.7.

2.2.2.3.1. Effect of operating pressure

A summary of the effect of operating pressure is presented in Table 2.8. An Increase in the operating pressure results in an increase in the cell disruption by high pressure homogenization. The release rate of total soluble protein from Bakers' yeast was found to increase with an increase in the pressure up to 49 MPa i.e. across the range studied (Hetherington *et al.*, 1971). The extent of total soluble protein release from Bakers' yeast was found to increase with the increase in the operating pressure of up to 138 MPa, while the release of the cytoplasmic enzyme, G6PDH increased only up to 100 MPa. The particle size distribution of the cell debris broadened with increasing the pressure. This effect of micronization is postulated to be responsible for the sustained release of proteins with increasing operating pressure (Whitworth, 1974). The extent and rate of soluble protein release from *C. lipolytica* increased with an increase in the operating pressure over the range 29.42 - 54.91 MPa. The maximum soluble protein release (R_m) was found to be dependent on the operating pressure and hence the process was not described by the first-order expression (Whitworth, 1974). The release rate of soluble protein and β-galactosidase from *E. coli* was found to increase with an increase in the pressure over the range 19.61 to 49.03 MPa. The fraction of the cells disrupted was found to increase with the pressure up to 95 MPa for both recombinant and the non-recombinant *E. coli* (Sauer *et al.*, 1989). Cell disruption was found to be a sigmoid function of the operating pressure in the study with *Alcaligenes eutropus* over the pressure range 0 - 62 MPa. The release of total soluble protein from *Alcaligenes eutropus* increases with the increase in pressure and maximum protein release was obtained after 2 passes at 62 MPa, while at 15 MPa maximum available protein from the cell was not released regardless of the number of passes. The maximum soluble DNA release was dependent on the operating pressure over the range 17.4 MPa to 62 MPa and independent over the pressure range 62 – 122.7 MPa (Harrison *et al.*, 1991a).

Table 2.7 Summary of the literature on the effect of process variables on microbial cell disruption by high pressure homogenization

Microorganism	Operating Pressure (MPa)	Initial cell concentration	Other variables	Analysis	Reference
E. coli	19.61 – 49.03 (200-500 kgf/cm^2)		No. of passes	Proteins, β-galactosidase	Gray *et al.*, 1972
E. coli, S. cerevisiae S. aureous, L. casei, B .subtilis, S. faecalis, C. perfringens, S. zoepidermicus, A. fumigatus, Chlorella sp. Fusarium sp	0 – 0.28 (0 - 40 lb/in^2)			A_{280nm}	Kelemen and Sharpe, 1979
Bakers' yeast	50 - 275	0 – 90 g/l		Protein, G6PDH	Lovitt *et al.* 2000
Bakers' yeast	20.68 – 172.37 (3 - 25 x 10^3 psi)	35 - 240 g/l (dry wt)		Total soluble protein	Brookman, 1974
S. cerevisiae		37 - 158 g/l (dry wt)		Total soluble protein	Doulah, 1977
Candida utilis	50 - 125	93 - 148 g/l (dry wt)		Total soluble protein, Invertase	Engler and Robinson 1981a
Bakers' yeast	46	450 gl/ (wet wt)	No. of passes, geometry of valve seat, impact ring dimensions	Total soluble protein	Keshavarz-Moore *et al.*, 1990a
Bakers' yeast	0 – 49.03 (0 – 500 kgf/cm^2)	0.30-0.75 g/ml	Valve (flat vs knife edge)	Total soluble protein	Hetherington *et al.*, 1971

Microorganism	Operating Pressure (MPa)	Initial cell concentration	Miscellaneous	Enzymes	Reference
Alcaligenes eutropus	0 - 122.7	96-257 g/l (dry wt)	Temperature, valve design, shape, size and growth phase of cells	Total soluble protein, PHB, DNA	Harrison *et al.*, 1991a
Alcaligenes eutropus	0 – 62	96.6-307 g/l (dry wt)	Energy input, cell size, cell shape, growth phase,	Total soluble protein, PHB	Harrison *et al.*, 1990
E. coli	30 – 95	2-174 g/l (dry wt)	Growth rate (0.12, 0.17, 0.33 hr^{-1}), recombinant *E. coli* vs non-recombinant	Total soluble protein	Sauer *et al.*, 1988
Candida utilis, S. cerevisiae, B. subtilis	50 – 100		Growth rate	Nitrogen	Engler and Robinson, 1981b
E. coli	0 - 65	5 - 150 g/l (wet wt.)	viscosity	Cell size distribution	Kleinig *et al.*, 1995
Rhizopus nigricans	10 – 50	8 - 30 g/l (dry wt)		Protein, ADH	Keshavarz-Moore *et al.*, 1990b
Candida lipolytica	29.42 – 54.91 (300-560 kgf/cm^2)	300 g/l (wet wt)		G6PDH, ADH, Total soluble protein	Whitworth, 1974
Bakers' yeast	9.81 – 45.11 (100-460 kgf/cm^2)	450 & 750 g/l	Temp. (5 & 30 °C), valve seat design	ADH, 6PGDH, protein, acid & alkaline phosphatase	Follows *et al.*, 1971
E. coli	30 – 75	23 g/l (dry wt)		Penicillin acylase, proteins	Fonseca and Cabral, 2002

the fermentor are subjected to increased shear during fermentation leading to enhanced wall toughness (Keshavarz *et al.*, 1990a). Pressure required for cell disruption was found to be related to the cell shape and composition of the cell wall by Kelemen and Sharpe (1979).

2.2.2.3.2. Initial concentration of the cell suspension

Disruption was found to be independent of the yeast cell concentration over the range 300 - 600 g/l of packed yeast (Hetherington *et al.*, 1971). The disruption was independent of the cell concentration over the range 96 to 257 g/l (dry wt) for *Alcaligenes eutropus* disruption for the recovery of total soluble protein and PHB (Harrison *et al.*, 1991a).

Table 2.9 Summary of the literature on the effect of growth rate/culture history on the disruption rate constant by high pressure homogenization

Microorganism	Results/comments		Reference
	Growth rate (hr^{-1})	Disruption rate constant (MPa^{-a})	
Candida utilis	0.5	8.78×10^{-6}	Engler and Robinson, 1981b
	0.1	8.53×10^{-9}	
Escherichia coli	batch ($\mu=\mu_{max}=0.35$)	1.4×10^{-3}	Sauer *et al.*, 1989
	0.33	1.6×10^{-3}	
	0.24	0.60×10^{-3}	
	0.17	0.27×10^{-3}	
Escherichia coli (recombinant)	batch ($\mu=\mu_{max}=0.35$)	1.8×10^{-3}	Sauer *et al.*, 1989
	0.35	62.9×10^{-3}	
	0.17	56.7×10^{-3}	
Alcaligenes eutropus	Exponential phase	1.35×10^{-5}	Harrison *et al.*, 1991a,b
	Late exponential phase	1.83×10^{-5}	
	Early stationary phase	1.18×10^{-3}	
	Late stationary phase	2.16×10^{-3}	

2.2.2.3.3. Effect of culture history

The disruption rate constant obtained by researchers with different microorganism and different growth phase/rate is presented in Table 2.9. Disruption characteristics of a given organism can be altered significantly by changing the growth conditions. High growth rate produces cells having weaker cell walls. The faster growing cells do not direct metabolic energy to produce material for reinforcing the cell wall structure

unless essential. Hence these cells can be disrupted easily. At a pressure of 88 MPa, *Candida utilis* cells produced at growth rate of 0.5 hr^{-1} gave 87 % disruption while only 53 % disruption was observed for cells which were grown at 0.1 hr^{-1}. The disruption rate constant of higher growth rate cells (0.5 hr^{-1}) was 8.78 x 10^{-6} and the slower grown cell (0.1 hr^{-1}) was 8.53 x 10^{-9} MPa^{-a} (Engler and Robinson, 1981b). *E. coli* (both recombinant and non-recombinant) grown at a higher specific growth rate was disrupted more readily than cells cultivated at a slower growth rate. Increasing the growth rate from 0.17 to 0.33 hr^{-1} was found to increase the disruption constant of *E. coli* cells from 0.27 to 1.6 x 10^{-3} MPa^{-a} (Sauer *et al.*, 1989). In contrast to the above results, in the disruption of *Alcaligenes eutropus* the disruption rate constant of faster growing exponential phase cells (1.35 x 10^{-5} MPa^{-a}) was lower than the slower growing stationary phase cells (2.16 x 10^{-3} MPa^{-a}, Harrison *et al.*, 1990). Though these results appear contradictory, the effective 'K' calculated as in Section 1.2.2.2 and presented in Figure 2.4, 2.5 and 2.6 clearly demonstrates that the faster grown cells are disrupted more easily.

2.2.2.4. Significance of location and selectivity

The significance of location of the protein and enzymes within the cell on the release rate has been reported. In the disruption of Bakers' yeast by high-pressure homogenisation Follows *et al.* (1971) reported that the release rate of the enzymes invertase, acid phosphatase, G6PDH, 6PGDH, alkaline phosphatase and fumarase from Bakers' yeast correlated with the location of the enzymes in the cell (Figure 4). Release of the enzymes acid phosphatase and invertase, which are predominantly outside the cell membrane (extracytoplasmic), was faster than that of the overall soluble protein. The cytoplasmic enzymes ADH, G6DPH and 6PDDH were released slightly faster or at the same rate as the overall soluble protein and the plasma membrane-bound alkaline phosphatase and mitochondrial fumarase were released more slowly than the total soluble protein. The difference between release rates was not sufficient to allow fractionation of the enzymes into groups (Follows *et al.*, 1971).

2.2.3. Ultrasonication

Ultrasound, sound of frequency higher than 15 - 20 kHz which is inaudible to the human ear, is known to cause inactivation and, at higher power input, disruption of the microbial cells in suspension.

2.2.3.1. Mechanism of disruption

In ultrasonication, cavities are generated by the sound waves. The formation, growth and collapse of vapour bubbles is termed cavitation. When sounds waves are transmitted through a liquid medium the molecules of the medium compress and stretch alternatively. When the intramolecular forces are exceeded a cavity is formed. During the collapse of the cavities, large quantities of the sonic energy are converted to the mechanical energy in the form of elastic waves. When the kinetic energy content of the cell exceeds the wall strength, the microbial cell disintegrates. The mechanism for disintegration of yeast cells is proposed as due to the shear stress developed during the cavitation by ultrasonication (Doulah, 1977).

2.2.3.2. Kinetics of disruption

The release of total soluble protein from *E. coli* by ultrasonication at 200 W and 26 g/l (dry wt) was found to follow first order release kinetics. The release constant was $0.0075 \pm 0.0008\ s^{-1}$ (Fonseca and Cabral, 2002). First order release kinetics was confirmed by Kuboi *et al.* (1995) in the disruption of *E. coli* by ultrasonication.

2.2.3.3. Factors affecting disruption

The factors that affect cell disruption using ultrasonication are acoustic power input per unit volume, temperature of the suspension and initial cell concentration. Literature on the effect of process variables on the microbial cell disruption by ultrasonication is summarised in Table 2.10.

2.2.3.3.1. Acoustic power

The protein release constant was found to increase linearly with the increase in the acoustic power over the range of 67 - 187 W during the disruption of Brewers' yeast (James *et al.*, 1972). The percentage survival of *Acetobacter peroxydans* decreased

from 50 to 36 % on increasing the power from 30 to 100 W (Kapucu, *et al.*, 2000). The disruption rate was found to increase linearly for *E. coli* in the range of 20 to 80 W by Kuboi *et al.* (1995) and in the range 100 to 200 W by Fonseca and Cabral (2002).

Table 2.10 Summary of the literature on the effect of process variables on microbial cell disruption by ultrasonication

Micro organism	Acoustic power (W)	Initial cell concentration	Working volume (ml)	Miscellaneous	Analysis	Reference
Brewers' yeast	67 – 195 (20 kHz)	20 – 60 % w/v (wet wt)	75 – 450	Temperature 17 – 30 °C	Soluble protein	James *et al.*, 1972
Bakers' yeast	5 – 20 (20kHz)		5 – 40	Pressure 0 – 0.59 MPa (85 psi)	Nitrogen	Neppiras and Hughes, 1964
Acetobacter peroxydans	30 – 100 (20kHz)	60 % w/v (wet wt)		pH 3 - 10	Soluble protein	Kapucu, 2000
E. coli, S. cerevisiae, B. subtilis	20 – 30 (20kHz)	35 - 200 % w/v (wet wt)	2.5 - 10		Soluble protein , acid phosphatase, G6PDH, fumarase	Kuboi *et al.*, 1995
E. coli	100 – 250 (20kHz)	130 % w/v (dry wt)			Soluble protein , penicillin acylase	Fonseca & Cabral, 2002

2.2.3.3.2. Initial concentration of the cell suspension

No detectable dependence of cell concentration on the disruption rate of Brewers' yeast was observed over the range 20 - 60 % w/v (wet wt) by James *et al.* (1972). Cell concentration in the range 3 - 20 g/l of *E. coli* cells also showed no effect on the disruption rate constant by ultrasonication (Kuboi *et al.*, 1995).

2.2.3.3.3. Working volume

The disruption rate of *E. coli* cells was found to decrease linearly on increasing the volume from 2.5 to 10 ml at a power input of 40 W (20 kHz, 2.5 g/l wet wt, Kuboi *et al.*, 1995). In the disruption of Bakers' yeast (20 % w/w, dry cake, 20 kHz, 30 W) the amount of protein (measured as total nitrogen) released decreased from 50 % to 25 % on increasing the volume of suspension used for disruption from 10 ml to 20 ml and

the release increased to 35 % on increasing the volume further to 40 ml (Neppiras and Hughes, 1964). Four different volumes were studied by James *et al.* (1972), in the disruption of Brewers' yeast: 75, 100, 300 and 450 ml (20 % w/v wet wt, 140 W, 20 kHz). The protein release (expressed as the product of the release rate constant and volume of the suspension used, k x V, to account for the difference in volume) was 53.4, 55.6, 53.2, 53.5 ml min^{-1} showing that the protein release was independent of the volume of the flow chamber within the range of 75 - 400 ml.

2.2.3.3.4. Temperature

Total soluble protein release increased from 52 % to 63 % when the temperature was increased from 17 to 30 °C (James *et al.*, 1972). However, while increased temperature is known to enhance cell disruption, it increases protein denaturation and should generally be avoided.

2.2.3.3.5. Ambient pressure

The amount of nitrogen released increased regularly with increase in ambient pressure to a peak at about 0.24 MPa (35 psi) and decreased thereafter in the disruption of Bakers' yeast by Neppiras and Hughes (1964). Cavitation inception is reached more easily with increase in pressure, but extent of cavitation decreases and hence the competing influence is responsible for the optimum.

2.2.3.4. Significance of location and selectivity

Kuboi *et al.* (1995) determined the optimum conditions for the selective recovery of β-galactosidase from the recombinant *E. coli ML308*, disrupted using ultrasonic disruptor UD-200 (Tomy seiko co. Ltd, Tokyo 20 kHz). First order kinetics (Equation 2.1) were used and the disruption rate constant (k_c) (determined from the changes in absorbance at 660 nm), the protein release rate constant (k_t) and enzyme release rate constant (k_i) determined. The rate constants were generalised by multiplying the respective release constant with the ratio of volume to power used for sonication (K = k x V/P)). The generalised rate constant for cell disruption (K_c) total protein release (K_t) and enzyme release (K_t) were used to identify the release efficiency and release selectivity. The ratio K_t/K_c was defined as the release efficiency while K_i/K_t was

defined as the release selectivity. The enzymes acid phosphatase and β-galactosidase were used as periplasmic markers and G6PDH was used as cytoplasmic marker. The release selectivity values were found to increase in the order of G6PDH (cytoplasmic), β-galactosidase (periplasmic), acid phosphatase (periplasmic). The release selectivity was found to indicate the location of the enzyme in the cell. The enzyme β-galactosidase is overproduced and found in periplasm of the recombinant *E. coli ML308*. Addition of 5 % PEG 1540 was found to improve the release selectivity. The release selectivity for the periplasmic acid phosphatase and β-galactosidase was found to increase when increasing the concentration of PEG to 5 % and reduced thereafter (Kuboi *et al.*, 1995).

2.3. Non-mechanical methods

The non-mechanical methods of microbial cell disruption reported include chemical treatment, enzymatic lysis, osmotic shock and some novel techniques. The extent of recovery of the intracellular products depends on the location of the product, the method used and its operating conditions. There is a higher potential for the selective release of products from the cell wall and periplasm with these methods because they can cause selective damage on the cell wall or cytoplasmic membrane of the microbial cell. Non-mechanical methods are suitable for shear sensitive products like plasmid DNA. Micronization of the cells can be minimised and heat generation owing to conversion of mechanial energy to heat energy during the cell disruption process is avoided. The need for the removal of the exogenous chemicals or enzymes may complicate subsequent product purification. The chemicals and the enzymes needed for disruption may be expensive. These methods are are not preferred in industry where product purification is required on a large scale but may find application in the production of high value, low volume products.

2.3.1. Chemical methods

A variety of chemical methods are available that act selectively on the outer cell wall resulting in the selective leakage of the periplasmic constituents. These chemicals are not generally selected to disrupt the cell completely, unlike chemicals such as chloroform and sodium hydroxide. The cell morphology is not affected by most chemicals selected and the particle size of cell debris is large. Hence the cell debris can be readily separated from the crude suspension by centrifugation or microfiltration.

The use of chemical methods can be categorised based on whether the chemical agent released the product extracellularly or the cells were just permeabilized by the chemical agent, allowing the substrate molecule to diffuse into the cell for conversion into product. In permeabilized cells, the outer cell wall is 'leaky' allowing the smaller molecular weight molecules to diffuse in and out of the cell, while trapping the bulkier enzyme molecules within the cell. The permeabilized cells can allow the substrate molecule to diffuse into the cells to access the catalytic enzyme and the

resultant product to diffuse out. These permeabilized cells can be immobilized on a support providing a source of the enzyme within a functional unit in the place of a purified enzyme for the biocatalysis industry (eg. *E. coli* cells producing penicillin acylase were permeabilized and immobilized, (Prabhune *et al.*, 1992). This cell environment provides benefit through the generation of cofactors such as NAD and ATP, while removing the transport limitations hampering typical whole cell systems.

Table 2.11 Summary of literature on release of the intracellular products from microbial cells by chemical treatment

Chemical agent	Micro organism	Analysis	Location	Reference
Solvents				
Ethanol, Methanol, Isopropanol, Butanol	*Yeast*	β-galactosidase		Fenton, 1982
Chloroform and Sod. hypochlorite	*Alcalingenes eutropus*	PHB	Cytoplasmic	Hahn *et al.*, 1993, 1994
Glycine	*E. coli*	α-amylase	Cell wall	Ariga *et al.*, 1989
Chaotropic Agent				
Urea + EDTA	*E. coli*	Long-R^3-IGF-I	Cytoplasmic	Falconer *et al.*, 1999
Surfactants				
SDS, SLS (cationic)	*E. coli*	-	-	Woldringh, 1970
SDS (cationic)	*E. coli*	Intracellular material	Cytoplasmic	Ciccolini *et al.*, 1998
AOT (cationic)	*E. coli*	Penicillin acylase	Periplasmic	Gaikar and Kulkarni, 2001
CTAB (anionic)	*Acetobacter vinelandii*	G6PDH, βhydroxybutyrate, Isocitrate dehydrogenase	Cytoplasmic	Giovenco *et al.*, 1987
Tween 80 (Non-ionic)	*Neurospora crassa*	Invertase	Cell wall associated	Buzzi *et al.*, 1993
Triton X-100 (Non-ionic) + KH_2PO_4	*E. coli*	Asparaginase	Periplasmic	Zhao and Yu, 2001
Reducing Agents				
Cysteine	*K. fragilis*	invertase	Cell wall associated	Lam and Grootwassink, 1985
Mercapto ethanol	*S. fragilis*	Invertase	Cell wall associated	Kidby and Davies, 1970
DTT	*S.carlbergensis*	Invertase	Cell wall associated	Sommer and Lewis, 1971

2.3.1.1. Extracellular release

The release of the intracellular product of interest by chemical treatment can be discussed under various classes of chemicals. A summary of the chemical methods reported to release enzymes from various locations of the cell is presented in the Table 2.11

2.3.1.1.1. Solvents

Solvents like ethanol, methanol, isopropanol and butanol extract the lipid component of the cell and, at a sufficient concentration, can cause disruption of the membrane structure sufficiently to allow for the diffusion of the protein molecule from the cell. The solvent ethanol released 90 % of β-galactosidase from *Kluyveromyces fragilis* while isopropanol released 85 % of the same enzyme when the cells were treated with the solvents for 90 minutes at a concentration of 80 % w/v followed by the extraction of the product into a phosphate buffer for 20 hours (Fenton, 1982).

Sodium hypochlorite was used for the isolation of PHB granules from the cytoplasm of *Alcaligenes eutropus*. Exposure to a 30 % v/v sodium hypochlorite for 90 minutes gave 97 % recovery of PHB with a purity of 91 %. Sodium hypochlorite was used in conjunction with chloroform (1:1 v/v) to extract the product into the chloroform phase as soon as the PHB molecules were released by sodium hypochlorite to prevent the degradation of PHB by sodium hypochlorite (Hahn *et al.* 1993, 1994). The enzyme α-amylase was released from the periplasm of recombinant *E. coli* cells when incubated for 3 hours with 1 % glycine (Ariga *et al.* 1989, 1991).

2.3.1.1.2. Chaotropic agents

The weakening of hydrophobic interactions is proposed as a general mechanism by which chaotropic agents act on the *E. coli* cell wall. The potency of chaotropic anions with regard to the inhibition of cross-linking of the peptidoglycan layer of the cell envelope and the causing of cell lysis has been reported to follow the expected chaotropic series. The potency of the compounds is as follows: trichloroacetate > perchlorate ≃ thiocyanate > nitrate > urea. The lytic effects of chaotropic agents were roughly additive. Chaotropic salts that weaken hydrophobic associations promote lysis and inhibit cell wall assembly (Ingram, 1981).

A two stage selective extraction process was used for the isolation of the recombinant protein Long-R^3-IGF-1 from inclusion bodies located in the cytoplasm of *E. coli*. In the first stage, cells were permeabilised with a buffer containing 0.1 M Tris at pH 9.0, 6 M urea and 3 mM EDTA. Co-solubilisation of the recombinant protein was prevented using 15 mM 2-HEDS such that the contaminating host protein can be separated from the insoluble recombinant protein by centrifugation. Thereafter, the recombinant protein was resolubilised with DTT and buffer, yielding a soluble denatured product of relatively high purity. This is then renatured and recovered. This selective extraction process was compared with the conventional disruption using the high-pressure homogenizer. The conventional process gave 88 % w/w recovery while the selective extraction process gave 81 % recovery. The purities resulting from the conventional and selective extraction process were 41 % and 46 % respectively (Falconer *et al.*, 1999).

2.3.1.1.3. Anionic surfactants

When *E. coli* cells were treated with 0.05 % SDS for two minutes, the plasma membrane was dissolved and the nucleoplasm contracted to a smoothly contoured structure in the centre of the cell. The other anionic surfactants like sodium lauryl sarcosinate and sodium deoxycholate showed similar effects (Woldringh, 1970). The release of intracellular materials occurred rapidly over a time of 30 - 40 seconds when SDS was used to disrupt the *E. coli* cells depending on the cell strain (Ciccolini *et al.*, 1998).

The anionic surfactant AOT was used to extract periplasmic penicillin acylase selectively from *E. coli* cells. Reverse micellar permeabilization was performed using an AOT-isooctane reverse micellar solution. The surfactant assisted in permeabilizing the bacterial cells and accommodated the liberated enzyme in the water pool of reverse micelles. On comparison with sonication, the purification was 8 times higher with this selective extraction technique while the recovery was reduced to 60 % of that obtained by sonication (Gaikar and Kulkarni, 2001).

2.3.1.1.4. Cationic surfactants

The cationic surfactant, CTAB was used for a selective reverse micellar extraction of isocitrate dehydogenase and β-hydroxybutyrate dehydrogenase. *Acetobacter vinelandii* was permeabilized with CTAB in a hexanol-octane reverse micellar system. A 6.2 fold purification for isocitrate dehydrogenase and 7.6 fold purification for β-hydroxybutyrate dehydrogenase were achieved compared to sonication (Giovenco *et al.*, 1987).

2.3.1.1.5. Non-ionic surfactants

Non-ionic surfactant Triton X-100 was used for the release of periplasmic asparaginase from *E. coli* cells. The outer membrane of *E. coli* cells shows an asymmetric structure i.e. glycerophospholipids in the inner layer and lipopolysaccharide layer on the outer layer. High concentration of salt (K_2HPO_4) weakens some interactions in the lipopolysaccharide layer after which Triton X-100 is able to act on the lipids in the inner layer and the cell envelope is permeabilized releasing the periplasmic enzyme asparaginase selectively. A suspension containing 12.5 % w/v K_2HPO_4, 2 % w/v Triton X-100 and 3 x 10^8 cell ml^{-1} released 70 % of the enzyme compared to the release by sonication. Electron microscopy indicated that the chemical treatment altered the surface structure of *E. coli* but did not disrupt the cell physically (Zhao and Yu, 2001).

2.3.1.1.6. Reducing agents

Reducing agents like cysteine, dithiothreitol (DTT) and mercaptoethanol were found to create pores in the cell wall by reducing the disulphide links. β-D-fructofuranosidase (an exo-inulinase) from *K. fragilis* was extracted using 0.1 M acetate buffer (pH 5) and cysteine. The combined action of high salt (ionic effect) and cysteine (reducing effect) expands the cell wall thereby increasing the porosity and permitting inulase molecules that were trapped in the cell wall matrix or in the periplasmic space to diffuse out (Lam and Grootwassink, 1985). Mercaptoethanol was used for the release of the enzyme invertase from the cell wall of *Saccharomyces fragilis* (Kidby and Davies, 1970) and DTT was used for the release of the enzyme invertase from *Saccharomyces cerevisiae* (Sommer and Lewis, 1971).

Table 2.12 Summary of the permeabilization of the microbial cells by chemical methods

Chemical agent	Micro organism	Enzyme	Location	Reference
Solvents				
Toluene + EDTA	*E. coli*	Dehyogenases and oxidases	Cytoplasmic	De Smet *et al.*, 1978
Toluene + Heat	Bakers' yeast			Murakami, *et al.*, 1980
Chloform + Toluene	*Kluyveromyces lactis*	β-galactosidase	Cytoplasmic	Flores *et al.*, 1994
Surfactants				
SLS (anionic)	*Yeast*	β-galactosidase	Cytoplasmic	Kippert, 1995
CTAB (cationic)	Bakers' yeast	ADH, G6PDH, hexokinase	Cytoplasmic	Gowda *et al.*, 1991
CTAB , Digitonin	Pichia pinus	Alcohol oxidase, formaldehyde dehydrdogenase, formate dehydrogenase	Cytoplasmic	Alamae and Jarviste, 1995
CTAB	Bakers' yeast	Catalase	Cytoplasmic	Sekhar *et al.*, 1999
CTAB	*E. coli*	Penicillin acylase	Periplasmic	Prabhune *et al.*, 1992
Triton X-100 (Non-ionic)	*E. coli*	Cytoplasmic membrane bound proteins		Schnaitman, 1971a
Triton X-100	*Yarrowia lipolytica*	Acid phosphatase alkaline phosphatase	Periplasmic	Galabova *et al.*, 1996
PF – 68 (Non-ionic)	*Yeast*	Uptake of FDA	-	King *et al.*, 1991
PF – 68	*Saccharomyces cerevisae*	ADH	Cytoplasmic	Laouar *et al.*, 1996
Triton X-100, oxgall	*Streptococcus thermophilus*	β-galactosidase	Cytoplasmic	Somkuti and Steinberg, 1994
Triton X-100	*Nocardia rhodochrous*	Cholersterol oxidase	Cell membrane bound	Cheetham *et al.*, 1980

2.3.1.2. Permeabilization

A summary of the chemicals used to permeabilize various microorganisms to access enzymes from various locations of the microbial cell is presented in Table 2.12.

2.3.1.2.1. Solvents

Toluene can damage the cytoplasmic membrane of yeast or *E. coli*, hence it has been used in conjunction with EDTA successively for *E. coli* permeabilization (De Smet *et al.*, 1978), and in conjunction with heat treatment for yeast permeabilization (Murakami *et al.*, 1980). It is postulated that toluene disorganises the bilayer by removing phospholipids from the cytoplasmic membrane when used for permeabilization. In the presence of magnesium which stabilises the outer membrane, there was no increase in permeability, while in the presence of EDTA which destabilised the outer membrane, *E. coli* cells were permeabilized and 40 to 60 % of intracellular dehydrogenases and oxidase activities were detected. There were no observable changes in the cross sections of the outer membrane and it did not disintegrate upon treatment with 1 % or 10 % toluene (De Smet *et al.*, 1978). When *Saccharomyces cerevisiae* was heated to 40 – 45 °C in the presence of toluene, 100 % of the pyruvate kinase, AMP deaminase, phosphofructokinase, and 54 % of ADH were released (Murakami *et al.*, 1980). The cells of *Kluyveromyces lactis* were permeabilized with chloroform, toluene and ethanol for β-galactosidase activity (Flores *et al.*, 1994). *Saccharomyces cerevisiae* was permeabilized by isopropyl alcohol for the enzyme glyoxalase I (Kondo *et al.*, 2000). *Lactobacillus plantarun* was permeabilized by toluene, chloroform and diethyl ether to access the LDH activity (Krishnan *et al.*, 2000).

2.3.1.2.2. Anionic surfactants

Yeast cells (*Saccharomyces cerevisiae* and *Saccharomyces pombe*) were permeabilised with 0.1 % sodium lauryl sarcosinate for the quantification of β-galactosidase activity (Kippert, 1995).

2.3.1.2.3. Cationic surfactants

The surfactant CTAB (0.2 %) was used for the permeabilization of Bakers' yeast for the release of the enzymes ADH, G6PDH, hexokinase (Gowda *et al.*, 1991) and catalase (Sekhar and Bhat, 1999). The detergent interacts with the hydrophobic moieties with the membranes. Proteins of molecular weight less than 60,000 were released following CTAB treatment. When compared with digitonin, 75 % more alcohol oxidase from the yeast *Pichia pinus* could be detected following CTAB pre-

treatment (Alamae and Jarviste, 1995). *E. coli* cells with penicillin acylase activity were permeabilised with CTAB (0.1 %) and immobilized for the production of 6-APA from benzyl penicillin (Prabhune *et al.*, 1992).

2.3.1.2.4. Non-ionic surfactants

When the cell envelope of *E. coli* was treated with Triton X-100, the cytoplasmic membrane proteins were solubilised but the cell wall morphology was not affected (Schnaitman, 1971b) About 60 to 80 % of cytoplasmic membrane proteins were released by Triton X-100 (Schnaitman, 1971a). The action of Triton X-100 is improved by the use of EDTA in conjunction. *Yarrowia lipolytica* cells were permeabilised above the critical micellar concentration of Triton X-100 (0.1 to 0.2 %) and the periplasmic enzymes acid phosphatase and alkaline phosphatase were analysed to measure the effect of permeabilization. 100 % of acid phosphatase and 82 % of alkaline phosphatase activity could be detected. Triton X-100 caused slight ultrastructural alterations in the cell wall and membrane (Galabova *et al.*, 1996). Triton X-100 in the concentration range of 0.025 - 0.5 % was found to be more effective in the permeabilization of *Streptococcus thermophilus* for β-galactosidase than Tween 80, Brij 35, deoxycholate-sodium and bile salts [Somkuti and Steinberg, 1994].

2.3.2. Enzymatic methods

Lytic enzymes, which are secreted by certain microorganisms, can be used to attack the cell wall of other species for the release of intracellular products. These enzymes can selectively attack the cell wall without affecting the inner cytoplasmic membrane and hence these can be used for the release of periplasmic products and for production of protoplasts (Kuo and Yamamoto, 1975). Since the cell wall components of yeast and bacteria are different, separate lytic enzymes are available for each group of microorganisms. The scope for the selective release of periplasmic products is higher with this disruption technique. The subject of enzymatic disruption for controlled lysis and selective product release has been reviewed by Andrews and Asenjo (1987).

2.3.2.1. Yeast

The yeast cell wall can be digested with suitable enzymes without damaging the cytoplasmic membrane resulting in the formation of protoplast which will allow the release of periplasmic enzymes selectively. A number of enzymes have been reported for the preparation of protoplasts from yeast cells. Isolation of protoplasts from yeasts using snail gut enzyme was reported by Eddy and Williamson (1957), Indge (1968), Kuo and Yamamoto (1975). When these protoplasts were transferred into the growth media, glucan and chitin were formed and retained by the protoplast, while most of the mannan was secreted into the medium. Glycoprotein enzymes (extracytoplasmic) such as invertase and acid phosphatase were synthesized and liberated into the medium, whereas the non-glycoprotein enzymes, α-glucosidase and alkaline phosphatase were retained by the protoplasts (Kuo and Yamamoto, 1975)

Brewers' yeast, on treatment with the enzyme Zymolase, released 2.5 times more carbohydrates than control cells in the absence of enzyme. The enzyme lysozyme did not have any effect on the yeast cells on its own. Combination of these two enzymes released 44 % more carbohydrates than zymolase alone. Zymolase caused partial hydrolysis of the glucan and mannan components of the carbohydrate protein complex of the cell wall. Lysozyme hydrolysed the glucosidic bonds of mannoprotein and glucan complexes of the cell wall after partial disorganisation of the cell wall by zymolase (Knorr *et al.*, 1979).

Lytic enzymes for yeast cell wall were produced from other microorganisms at several instances. A lytic enzyme for the yeast cells was isolated from *Cytophaga* species and tested on the Bakers' yeast and the Brewers' yeast (Asenjo and Dunnill, 1981). The use of the enzyme 1-3, β-glucanase from *Aspergillus niger* on yeast cell wall glucan preparation has been reported (Kery *et al.*, 1991). Glucanase enzyme, which lyses the Bakers' yeast cell wall, was isolated from *Bacillus circulans WL-12,* and was studied on isolated cell walls of yeast (Fleet and Phaff, 1974).

2.3.2.2. Gram-negative bacteria

Lysozyme and EDTA can cause the dissolution of the outer cell wall of *Gram-negative* bacteria. *E. coli* cells were treated with EDTA to destabilize the outer membrane and Lysozyme used to attack the peptidoglycan cell wall for spheroplast formation. It was found that 93 % of the periplasmic enzyme, alkaline phosphatase was released during this process while the activity of cytoplasmic enzymes G6PDH and glutamic dehydrogenase remained almost completely associated with the spheroplasts (Malamy and Horecker 1964). In a separate study 99 % of alkaline phosphatase was released while cytoplasmic enzyme β-galactosidase remained associated with the spheroplasts (Malamy and Horecker 1964). This is recognized as a standard laboratory procedure for the extraction of intracellular proteins from *Gram-negative* bacteria as the spheroplasts are easily disrupted by osmotic shock. Further Cytophaga lysing enzymes have been shown to disrupt the cell wall of *Gram-negative* bacteria, using the release of PHB from *Alcaligenes eutropus* as a case study (Harrison *et al.*, 1991b)

2.3.2.3. Fungi

Fungal protoplasts were produced by using commercial enzymes. The protoplasts were identified by the absence of cell wall and osmotic sensitivity. The enzymes used were Cellulase CP and Cellulase CT (E. Sturge Ltd., Selby, UK), Novozyme 234 and Cereflo 200L (Novo Enzyme Products Ltd., Windsor) and β-d-Glucoronidase (Sigma). The effect of these enzymes separately and in combination was examined on the species of *Aspergillus, Penicillium chrysogenum, Volvariella volvacea* and *S. cerevisiae* (Hamlyn *et al.*, 1981). Protoplasts were prepared from *Aspergillus niger* using a commercial enzyme caylase C3. A 24 hour grown culture was found to yield protoplasts within 30 minutes application of the enzyme in 0.7 M KCl. Vital staining of protoplasts showed that approximately 90% of the protoplasts were alive and intact (Sorensen *et al.*, 1996).

2.3.3. Osmotic shock

E. coli, on sudden changes in osmotic pressure, can release periplasmic enzymes selectively. *E. coli* cells from the stationary phase were subjected to osmotic shock

using EDTA and sucrose in the first stage and magnesium chloride in the second stage. A set of extracytoplasmic enzymes were released, which include cyclic phosphodiesterase (100 %), 5 nucleotidase (100 %), acid hexose phosphatase (90 %) and insignificant amount of cytoplasmic enzyme β-galactosidase (0.4 %). In this osmotic shock procedure EDTA helps in two ways. It greatly improves the selective release of extracytoplasmic enzymes and it also enables bacteria to withstand osmotic shock more successfully because the amount of cytoplasmic β-galactosidase released increased from 0.4 % to 3.4 % in the absence of EDTA and the protein concentration increased from 3.2 mg/g to 10 mg/g in the absence of EDTA (Anraku and Heppel, 1967).

Osmotic shock procedure was used for the selective release of periplasmic enzyme acid phosphatase from *E. coli* cells (Dvorak *et al.*, 1967). The specific activity of the enzyme increased from 9.9 U/mg protein to 70 U/mg protein and 90 % of the enzyme could be recovered compared to sonication.

Osmotic shock was used for the selective release of the periplasmic enzyme penicillin acylase from *E. coli* cells. Under optimum conditions for the osmotic shock procedure (pH 8.0, 5 °C), a 94 % release could be achieved with a specific activity of 3.9 U/mg protein, while the mechanical methods sonication and high pressure homogenization gave 100 % recovery with a specific activity of only 0.1-0.3 U/mg protein (Fonseca and Cabral, 2002).

The selective release of enzyme by osmotic shock takes place under milder conditions with exponentially growing *E. coli* cells than with the cells in stationary phase, as indicated by the reduced requirement for both EDTA and sucrose (Nossal and Hepel, 1966).

2.3.4. Novel techniques

A variety of novel techniques have been proposed in the literature for the release of intracellular enzymes. Many of the novel techniques are centred on selective release of enzymes owing to the significance of its successful implementation on the design and yield of product recovery trains. The genes responsible for the outer cell wall

proteins can be manipulated by genetic engineering, mutation etc., to make the microbial cells leaky and hence selective release of periplasmic proteins can be achieved.

2.3.4.1. Deficient outer wall

In several colicin-encoding plasmids, an operon has been identified that, in addition to the structural gene for colicin, contains a gene that is responsible for colicin release and cell death. This gene is called 'kil' gene. The plasmid *pEAP1* and *pEAP2* has a dormant 'kil' gene which was activated by the promoter in the inserted DNA fragment for the enzyme penicillinase from alkalophilic *Bacillus*. The activation of this 'kil' gene makes the outer cell wall permeable (Kobayashi *et al.*, 1986). When plasmids (*pEAP1* and *pEAP2*) bearing the penicillinase gene of alkalophilic *Bacillus* were cloned into *E. coli*, the enzyme penicillinase was excreted through the outer membrane of *E. coli*. It is found that 58 % of alkaline phosphatase, 21 % of total protein, and 83 % of penicillinase were excreted into the culture broth of *E. coli* bearing the plasmid *pEAP2*. These results suggest that the outer membrane of *E. coli* was changed by the introduction of *pEAP2* into the cells because a periplasmic enzyme (penicillinase) was secreted extracellularly while the cytoplasmic enzyme β-galactosidase was not (Kudo *et al.*, 1983)

E. coli cells containing plasmid *ptac11* could be induced with IPTG to produce increased β-lactamase. This high level production of β-lactamase resulted in the secretion of the periplasmic enzyme owing to interference with the synthesis of cell envelope proteins which altered the outer membrane permeability (Gerogiou and Shuler, 1988). The outer wall of *E. coli* becomes defective because the production of outer membrane proteins *ompA* and *ompC* (which form part of the cell envelope) are present in lower quantities.

2.3.4.2. Mutation with nitrosoguanidine

E. coli and *Salmonella typhimurium* cells with abnormalities in the cell envelope were prepared by mutation with nitrosoguanidine. The leaky mutants selected were found to leak only the periplasmic enzyme ribonuclease I while the cytoplasmic enzymes G6PDH and phophoglucoisomerase remained inside the cell (Lopes *et al.*, 1972).

Cellulase producing genes from *Cellulomonas fimi* were cloned into *Escherichia coli* and the enzyme was excreted into the periplasm of *E. coli*. On mutation with nitrosoguanidine, the enzyme was found to be released extracellularly along with the other periplasmic enzymes β-lactamase and alkaline phosphatase. In these leaky mutants the function of the outer membrane is lost due to the changes in its composition (Gilkes *et al.*, 1984).

2.3.4.3. Temperature sensitive/regulated expression

A plasmid *pPLc28K1* containing the '*kil*' gene (gene responsible for the release of colicin and cell death) was constructed. This '*kil*' gene was used under the control of a thermo sensitive CI repressor. On increasing the temperature of the culture from 28 to 42 °C, *E. coli* cells bearing this plasmid were found to release 80 % periplasmic proteins due to the induction of the '*kil*' gene. All the β-lactamase diffused out readily from the periplasmic space while there was no release of the cytoplasmic enzyme β-galactosidase. The protein synthesis capacity of the cells was found to be arrested on the induction of the gene (Steidler *et al.*, 1994).

A plasmid (*pRD21*) capable of triggering lysis in *E. coli* cells when the temperature is increased from 30 °C to 42 °C was constructed and the release of cytoplasmic β-galactosidase was studied. This plasmid carries the ΦX174 lysis gene 'E' downstream from the λ pL promoter. Temperature induction alone was found to be unsatisfactory because only 24 % of total β-galactosidase was found in the supernatant while the rest were found associated with the biomass. Hence sonication of these induced cells was performed for 60 seconds to release the remaining periplasmic enzyme from the cell. Temperature induction followed by a short sonication period is recommended for effective release of β-galactosidase from *E. coli 15224* (*pRD21;* Dabora *et al.*, 1989).

The genes *PDE2, SRB1/PSA1* and *PKC1* involved in the cell wall biogenesis of *S. cerevisiae* were controlled by a tightly regulated promoter *pMET3*. The repression of the genes individually did not give good lysis when compared to a double mutant where both the genes *SRB1/PSA1* and *PKC1* were suppressed simultaneously. Such a

system was found to secrete both homologous and heterologous proteins efficiently (Zhang *et al.*, 1999).

2.3.4.4. Heat Stress

It has been demonstrated that the translocation of β-galactosidase from the cytoplasm to periplasm can be induced in *E. coli* by exposing the cells to a heat stress at 42 to 47 °C for 10 minutes (Umakoshi *et al.*, 1998a). The translocation is attributed to changes in surface hydrophobicity of the enzyme and inner cytoplasmic membrane of the cells. The local hydrophobicity of the β-galactosidase increased along with that of the inner membrane of *E. coli* cells during the heat stress. The enhanced hydrophobic interaction between the membrane and enzyme aids its potential passage across the membrane.

2.3.4.5. Differential product release (DPR)

Enzymes from different locations in the cell were released successively using the strategy of differential product release (Huang *et al.*, 1991). In the first step the outer cell wall of yeast was dissolved by cell wall digesting enzyme from *Oerskovia xanthincolytica* to release the wall associated enzyme, invertase, while maintaining the spheroplasts using an osmotic stabiliser. In the second step the cytoplasmic membrane was disrupted to release the cytoplasmic enzyme using the polybase compound DEAE-Dextran and glucose incubation, while maintaining the inner organelles like mitochondria intact by using membrane stabilisers. Osmotic imbalance caused the rupture of the cytoplasmic membrane and the cytoplasmic enzyme ADH was released. In the third step, mitochondria was permeabilized with the detergent Triton X-100 to release the enzyme fumarase. By this successive disruption of various cell compartments, proteins from differential locations of the microbial cell can be released with higher specific activity. This approach was defined as differential product release and has been used by Huang *et al.* (1991) to release enzymes from different locations of the yeast *Saccharomyces cerevisiae.*

2.3.4.6. Immobilization of permeabilized cells

A plasmid *pKK* having a *tac* promoter upstream from the normal β-lactamase gene was constructed. This promoter can be induced by iso-propyl thio galactosidase (IPTG). Because of the overproduction of β-lactamase, the *E. coli* cells became leaky on induction with IPTG. These cells were immobilized on a matrix for the continuous production of β-lactamase. Cell growth and the formation of the intact cell wall in their immobilized state was controlled by reducing or eliminating phosphate in the medium and thus the production of target protein of β-lactamase was sustained for longer periods. Immobilisation offered protection to the leaky cells. The overproduction of β-lactamase saturated the potential secretion sites on the inner membrane partially blocking the transport of normal outer membrane proteins. Their reduced level was hypothesized to have resulted in the construction of an incomplete outer membrane allowing leakage of the periplasmic β-lactamase molecules. Production levels of 390 U/hr were sustained for at least 120 days. β-lactamase was found to constitute 40 % of the protein released (Gerogiou *et al.*, 1985).

2.4. Combined methods

Cells can be pretreated with chemical agents or lytic enzymes to improve subsequent mechanical disruption. This has potential to reduce the treatment time and energy requirement for the mechanical disruption and to reduce the damage of the product during the mechanical disruption. Micronization of the cells can be reduced, easing the purification of the product. However, this pre-treatment implies an additional cost in downstream processing which may or may not offset against energy savings.

2.4.1. Chemical pre-treatment

E. coli cells harbouring human growth hormone inclusion bodies were pre-treated with guanidine hydrochloride and Triton X-100 before high-pressure homogenization. The addition of 1.5 % Triton X-100 reduced the concentration of guanidine required from 4 M to 1.5 M. The pre-treatment was found to reduce the number of passes required for disruption and allowed for operation at the lower operating pressure. Some 82 % of the protein was released from pretreated cells at 41 MPa after 1 pass, while the untreated cells required 2 passes to release 93 % of proteins at the same

pressure. The average particle size of the cell debris after homogenization of pretreated cells was 0.18 µm while it was 0.16 µm for untreated cells. One pass through the homogenizer at a low operating pressure allows for shorter process times, lower energy consumption and less reduction in the size of the cell debris compared to multiple passes at higher operating pressure (Bailey *et al.*, 1995).

Lysis of cold adapted bacteria is difficult to achieve by normal disruption processes because of the high amount of mucous present. Pre-treatment with Tween was found to enhance the release of proteins, postulated to be due to the removal of mucous adhering to the cell wall or the combined action of mucous removal and cell permeabilisation (Nandakumar *et al.*, 2000).

The use of chemical pre-treatment to decrease cell wall strength prior to mechanical breakage by homogenization has been studied using the *Gram-negative* bacterium *Alcaligenes eutropus*. The release of protein and DNA were studied. The yield of total soluble protein was increased by 37.5 % when the cells were subjected to alkaline pre-treatment (pH 10.5). Protein release on a single pass at 62.8 MPa following pre-treatment with 1 % sarkosyl (an anionic detergent) approached that found on 2 passes of an untreated suspension. Effect of pre-treatment with cations was studied using Na and K. Over the range of operating pressure considered an average increase of 30 % in soluble protein release was obtained in the presence of 0.137 M salt (NaCl or KCl) with respect to a similarly treated culture in its absence. The addition of EDTA or both EDTA and lysozyme increased the extent of protein release observed on a single pass through the homogenizer valve at a pressure less than 70 MPa (Harrison *et al.*, 1991b).

2.4.2. Enzymatic pre-treatment

Candida utilis cells were pre-treated with the wall lysing enzyme, Zymolase, and homogenized using a microfluidiser design of high-pressure homogenizer. Without pre-treatment at 95 MPa, the overall disruption was only 27.5 %. Following pre-treatment, the overall fraction disrupted increased to 65 %, a 2 - 4 fold improvement (Baldwin and Robinson, 1994). The yeast *Saccharomyces cerevisiae* was pretreated with Zymolase for disruption in the high pressure homogenizer (microfluidizer). The

fraction disrupted at 60 MPa increased from 0.38 to 0.7 by pre-treatment (Baldwin and Robisnon, 1990).

The number of passes required to get 90 % cell disintegration in a high pressure homogenizer was reduced from 5 passes to 1 passes when *Bacillus cereus* cells were pretreated with 0.5 g cellulosyl per g of cells (Cellulosyl, a partially purified extracellular enzyme from *Sptreptomyces coelicolor* was purchased from Hoeck AG, Frankfurt, Germany). Pre-treatment is also found to improve cell disruption with a bead mill. Pre-heating of *Bacillus cereus* at 55 °C for 30 minutes before disruption with high-pressure homogenizer or bead mill resulted in an increase in the enzyme and protein release rate. The specific activity of leucine dehydrogenase released was two fold higher following heat pre-treatment after mechanical disruption by the bead mill or high-pressure homogenization (Vogels and Kula, 1992).

A filamentous fungus *N. sitophila* was pre-treated with Zymolyase before mechanical disruption in the bead mill to facilitate the recovery of constitutive cellulases. Incubation with the enzyme Zymolyase caused approximately 25 % disruption. Subsequent bead milling of the pre-treated fungi achieves 100 % disruption with 1 pass at 1000 rpm and 80 L/min flow rate compared to only 45 % without pre-treatment (Baldwin and Moo-Young, 1991).

2.4.3. Combined stress

E. coli cells were subjected to chemical stress and heat stress to obtain a selective recovery of cytoplasmic β-galactosidase (Umakoshi *et al.,* 1998a). Triton X-100 and EDTA were used for chemical stress and heating at 45 °C for 0 - 60 minutes was used to apply heat stress to the bacteria. The effect of the sequence of these two stresses on the selective release of cytoplasmic β-galactosidase was studied. Chemical stress followed by heat stress was favourable in terms of high specific activity of the enzyme (26.5 U/mg of protein compared to only 2.62 U/mg of protein when the cells were heat stressed first followed by chemical stress).

Plasmid DNA (cytoplasmic) from *E. coli* was recovered by a combination of heat and chemical treatment by Wang *et al.* (2002). Heat treatment for 5 min at 95 °C and a

combination of 5 % Triton X-100 and 100 mM EDTA was found to yield results similar to the traditional alkaline-lysis methods for plasmid release.

The recombinant protein α-amylase was recovered from *E. coli* by chemical, osmotic and enzymatic methods. Osmotic shock, chloroform treatment or glycine treatment separately did not give a good yield compared to sonication. Lysozyme/EDTA treatment greatly increased the yield of α-amylase from the periplasm. The enzyme is entrapped in the peptidoglycan gel matrix and hence methods lacking lysozyme did not give a good recovery. When lysozyme and EDTA treatment were combined with osmotic shock, 90 % of enzyme could be recovered (French *et al.*, 1996).

A variety of chemical methods were tested for the release of penicillin acylase from the periplasm of *E. coli* cells separately. The methods include Triton X-100, guanidine, guanidine and EDTA, lysozyme and EDTA, ethanol/toluene, freezing/thawing and sonication. It was found that the combined use of guanidine and EDTA released 90 % of the enzyme with a specific activity 25 times greater than that obtained from sonication (Novella *et al.*, 1994).

2.4.4. Effect of Freeze and thawing

Recombinant cells of *Saccharomyces cerevisiae* producing virus-like particles (Ty-VLP) were used to study the effect of freezing and thawing on cell disruption with high pressure homogenization and bead mill. The effect of freeze thawing alone was not found to release the Ty-VLP's. Cells subjected to freeze-thaw cycle were four times more resistant to high-pressure homogenization and twice as resistant to disruption in the bead mill. The reduction in the rate of disruption caused by freezing and thawing was 68 % to 90 % for homogenizer compared with only 43 % to 54 % in the bead mill. When cells were freeze thawed, the cells were more 'flaccid' than cells that were not frozen. This is postulated to make the cells more resilient to disruption by impingement, a major mechanism for cell disruption by high pressure homogenization. The effect of freeze thawing was found to be independent of the time for which the cells are held in the frozen state and it was rather dependent on the number of freeze thaw cycles (Milburn and Dunnill, 1994).

Table 2.13 Summary of the literature on the selective release of enzymes or proteins by mechanical disruption techniques

Microorganism	Enzyme (Location)	Results and Comments	Reference
Bead Mill			
Saccharomyces cerevisiae	Invertase (cell wall bound), α-glucosidase (Periplasmic), ADH (cytoplasmic), fumarase (mitochondria)	$k_{proteins}(0.029\ s^{-1}) > k_{invertase}(0.026\ s^{-1}) > k_{\alpha\text{-glucosidase}}(0.023\ s^{-1}) > k_{ADH}(0.023\ s^{-1}) > k_{fumarase}(0.011\ s^{-1})$	Torner and Asenjo, 1991
Saccharomyces carlbergensis	Maltase (periplasmic), acid phosphatase (periplasmic), G6PDH (cytoplasmic), 6PDGH (cytoplasmic)	Release rate of maltase and acid phosphatase higher than the others	Marffy and Kula, 1974
Saccharomyces cerevisiae	Invertase (cell wall bound), Acid phosphatase (periplasmic), ADH (cytoplasmic), alkaline phosphatase (cytoplasmic membrane)	$k_{invertase} > k_{acid\ phosphatase} > k_{ADH} > k_{alkaline\ phosphatase}$	Melendres *et al.*, 1993
Bakers' yeast	Invertase (cell wall bound), G6PDH (cytoplasmic membrane)	Maximum amount of the soluble protein and G6PDH released in 4 passes, maximum invertase was released in one pass	van Gaver and Huyghebaert, 1990
Bakers' yeast	G6PDH (cytoplasmic), fumarase (mitochondria)	G6PDH released parallel to the soluble protein while fumarase release was retarded due to its location in the mitochondria	Schutte and Kula, 1988
Bakers' yeast	α-glucosidase(periplasmic), G6PDH (cytoplasmic)	0.55-0.85 mm beads required for cytoplasmic enzyme, 1 mm beads for periplasmic enzyme	Schutte *et al.*, 1983
Bakers' yeast	G6PDH	High specific activity of G6PDH obtained at 2300 rpm agitation speed than at 3100 rpm	Ricci-Silva *et al.*, 2000
High Pressure Homogenization			
Saccharomyces cerevisiae	Invertase (cell wall bound), acid phosphatase(periplasmic), G6PDH , 6PGDH (cytoplasmic), alkaline phosphatase, (cytoplasmic membrane bound) fumarase (mitochondrial)	Release rates were in the order of acid phosphatase = invertase >G6PDH = 6PGDH>fumarase & alkaline phosphatase	Follows *et al.*, 1971
Ultrasonication			
E. coli	Acid phosphatase (periplasmic), β β-galactosidase (periplasmic), G6PDH (cytoplasmic)	Release selectivity of acid phosphatase > β-galactosidase > G6PDH	Kuboi *et al.*, 1995

Microorganism	Enzyme (Location)	Chemicals/Enzymes	Resuls and comments	Reference
Enzymatic methods				
E. coli	Alkaline phosphatase (periplasmic), G6PDH (cytoplasmic), glutamic dehydrogenase (cytoplasmic)	Lysozyme/EDTA	93 % of alkaline phosphatase released on the formation of spheroplast	Malamy and Horecker, 1964
E. coli	Alkaline Phosphatase (periplasmic), β-galactosidase (cytoplasmic)	Lysozyme/EDTA	99 % of alkaline phosphatase was released	Malamy and Horecker, 1964
Osmotic shock				
E. coli	Phosphodiesterase, 5-nucleotidase, acid hexose phosphatase (periplasmic), B-galactosidase (cytoplasmic)	Osmotic shock	Phosphtodiesterase 100 %, 5-nucleotidase 100 %, acid hexose phosphatase 90 % released while only 0.4 % of β-galatosidase was released	Anraku and Heppel, 1967
E. coli	Acid phosphatase (periplasmic)	Osmotic shock	Specific activity increased from 9.9 U/mg to 70 U/mg of protein compared to sonication and a recovery of 90 %	Dvorak *et al.*, 1967
E. coli	Penicillin acylase	Osmotic shock	94 % recovery with a specific activity of 3.9 U/mg protein while only 0.1 - 0.3 U/mg specific activity was obtained with sonication and high pressure homogenization	Fonseca and Cabral, 2000
Novel Techniques				
E. coli	Alkaline phosphatase (periplasmic), penicllinase (periplasmic)	Manipulation of the genetic information	58 % of Alkaline phosphatase, 21% of soluble protein and 83 % of penicillinase were released	Kudo *et al.*, 1983
E. coli	Ribonuclease I (periplasmic), G6PDH (cytoplasmic), Phosphogluoisomerase (cytoplasmic)	Mutation with nitrosoguanidine	Ribonuclease I excreted into medium while other two remained inside the cell	Lopes *et al.*, 1972
E. coli	B-lactamase (periplasmic)	Temperature induction of '*kil*' gene	80 % of periplasmic proteins and 100 % of β-lactamase released	Steidler *et al.*, 1994

2.5. Conclusions

The summary of the literature on selective release by mechanical and non-mechanical methods is presented in Table 2.13 and 2.14. The detailed analysis of the structure and composition of cell envelope of yeast and *Gram-negative* bacteria indicates that a highly selective release of a cell wall bound or periplasmic enzyme is possible by a selective product release strategy. The mechanical methods differentiate the cell wall bound and periplasmic enzymes from enzymes which are located in the other regions of the cell by the difference in their release rates. This difference in the release rates has not allowed the enzyme to be fractionated (Follows *et al.*, 1974). A highly selective protein release strategy is not reported for a mechanical method. Relatively, a greater amount of literature is available for selective release by non-mechanical methods. With the exception of release of enzymes by osmotic shock, the recovery of the product by non-mechanical methods was always lower than mechanical methods. The non-mechanical methods can give a purer product at an early stage in the downstream processing of an enzyme. The reduced recovery may compromise the benefit obtained with selective product release if the process conditions are not optimised carefully, taking into account both the selectivity and yield. The additional cost of the chemical or enzyme is currently expected to make these methods uneconomical. Isolated reports of novel techniques appear but none considered beyond the research interest. Most of the non-mechanical methods were not demonstrated in pilot scale. Thus a technique for microbial cell disruption with high recovery, selectivity and ease of scale-up would be rewarded.

Chapter 3: Literature Review on Hydrodynamic Cavitation

3.1. The phenomenon of cavitation

The phenomenon of formation, growth and collapse of vapour cavities or bubbles in a liquid is known as cavitation. Each cavitation occurs over a very small time interval (milliseconds) and is associated with the release of large amounts of energy over a very small region. Cavities can be formed by reduction in the local pressure by static or dynamic means or a pre-existing bubble can expand. During the collapse of cavities, pressures as high as 120 MPa and temperatures as high as 9700 °C can be generated locally (Young, 1989). The physical effects of the cavitation phenomenon result in erosion, damage or disintegration of solid particles in a solid-liquid system, emulsification or homogenization in a liquid-liquid system. Degassing of liquid and atomisation of liquid in air occur in a gas-liquid system. The energy dissipated by cavitation leads to the rupture of the bonds of the water molecule resulting in the formation of free radicals. The chemical effects of cavitation are mainly due to the oxidation reactions involving these free radicals. Some of the reactions involved in the formation of free radicals in an aqueous system can be written as follows (Suslick, 1989),

$$H_2O \rightarrow H^{\bullet} + OH^{\bullet} \qquad 3.1$$

$$H^{\bullet} + H^{\bullet} \rightarrow H_2 \qquad 3.2$$

$$OH^{\bullet} + OH^{\bullet} \rightarrow H_2O_2 \qquad 3.3$$

The oxidation of potassium iodide, commonly known as Weissler's reaction (Equation 3.4) is widely used in sonochemistry to quantify the formation of free radicals and hence cavitation. The free radicals oxidise potassium iodide to liberate iodine which can be measured at 355 nm (Suslick *et al.*, 1997). This reaction was used by Senthil Kumar *et al.* (2000) to study the phenomenon of cavitation induced hydrodynamically.

$$H_2O_2 + 2\,KI + 2H\,(+) \rightarrow I_2 + 2\,H_2O \qquad 3.4$$

Some of the chemical effects of cavitation include ligand-metal bond cleavage in transition-metal complexes, disruption of solvent structure (formation of free radicals such as OH*, H*, and H_2O_2 in an aqueous system), rupture of polymers, etc. The effects of cavitation can be both physical and mechanical effects such as the erosion of metals.

3.2. Nature and cause of cavitation

There are four different types of cavitation, based on its source, (Young, 1989):

- **Acoustic cavitation** is caused by sound waves in a liquid
- **Hydrodynamic cavitation** is caused by the pressure variation in a flowing system induced by change in the system geometry
- **Optic cavitation** is caused by high intensity (laser) light rupture in a liquid
- **Particle cavitation** is caused by elementary particle (a proton, rupturing a liquid, as in a bubble chamber)

Of these types of cavitation, acoustic cavitation and hydrodynamic cavitation have been studied extensively and they are discussed in this section along with the bubble dynamics.

3.2.1. Acoustic cavitation

The sonic spectrum ranges from 20 kHz to 10 MHz and it can be subdivided into three main regions: low frequency, high power ultrasound (20 -100 kHz); high frequency, medium power ultrasound (100 kHz – 1 MHz); and high frequency, low power ultrasound (1-10 MHz). The biological and chemical applications use ultrasound in the range 20 kHz to 1 MHz while the low power, high frequency ultrasound is used for the medical and diagnostic purposes.

When ultrasound is transmitted through a medium, the vibrational motion of the molecules induces a pressure wave and the molecules compress and stretch alternatively.

The molecules oscillate around a mean position. When the power of ultrasound is increased sufficiently, the intramolecular forces cannot hold the molecules intact, leading to the formation of a cavity or a bubble. In the absence of dissolved gas in the medium, vapour filled cavities form. Bubbles, containing vapour and gas, form in the presence of dissolved gases in the medium, on cavitation. Millions of cavities and bubbles are produced any instant. These cavities and bubbles respond to the pressure waves and oscillate through expansion and contraction cycles alternatively, resulting in the dissipation of energy.

Two different forms of cavitation can be distinguished: stable and transient. During stable cavitation the cavity oscillates around a mean position and undergoes several refraction and compression cycles. During transient cavitation the cavities undergo only few oscillation cycles then increase in size before collapsing violently. This occurs rapidly, of the order of milliseconds. The transient cavitation was found to be responsible for the ultrasound-mediated membrane permeabilization of 3T3 mouse cells by Sundaram *et al.* (2003). The size, life time and the fate of the vapour cavities and bubbles will depend on the sound frequency, intensity, solvent and the presence of any dissolved gases.

3.2.2. Hydrodynamic cavitation

Cavitation induced in a flow system by reduction in the pressure or pressure variation is termed hydrodynamic cavitation. Vapour filled cavities are produced when constriction of flow causes a reduction in pressure to approach the vapour pressure of the liquid. Cavitation bubbles may form at pressures above the vapour pressures of the liquid. The cavitation bubbles and cavities travel along with the fluid to the region of elevated pressure where they collapse. The pressure variations required to induce cavitation can be achieved by flow restriction and thereby increased linear velocity through the presence of a venturi, orifice plate or jet nozzles. The pressure and velocity profile in the presence of an orifice plate can be represented by Figure 3.1. The size, life time and the fate of the vapour cavities and bubbles will depend on the size of orifice, inlet pressure, temperature and presence of any dissolved gases.

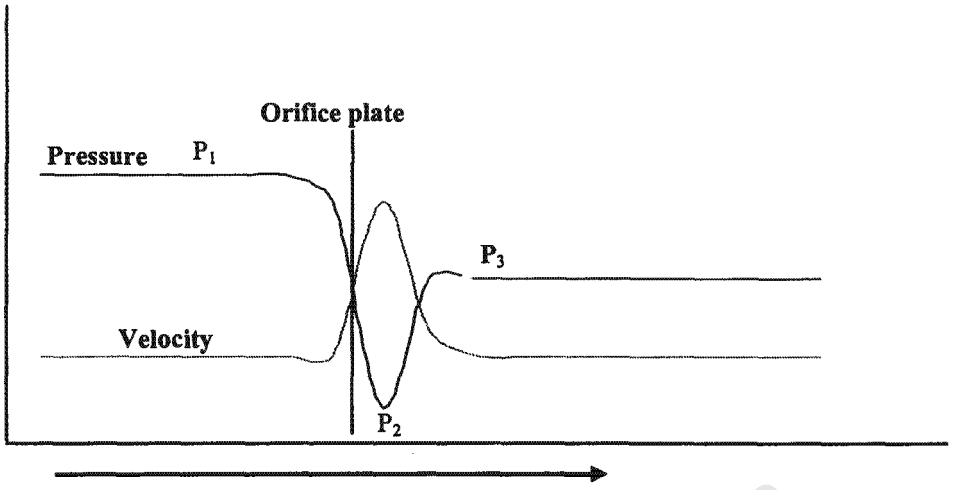

Figure 3.1 Representation of the pressure and velocity profile across an orifice plate

3.2.2.1. Bubble dynamics

From the pressure profile (Figure 3.1), it can be seen that the pressure reaches a minimum value at the vena contracta (P_2) before it recovers. Some amount of static pressure is lost due to the friction in the pipes. The liquid jet formed at the orifice plate is fully spread at the point where the pressure is recovered. The formation growth and collapse of the cavities and bubbles have been discussed in detail by Yan (1989). The vapour cavities and bubbles behave similarly except during collapse. The formation and growth of cavities and bubbles begins at the vena contracta where the static pressure reduces to a mimimum. The growth rate and stability of cavities and bubbles were found to depend on the extent of reduction in static pressure (Yan, 1989). The onset and growth of cavities and bubbles were not confined to the region between orifice plate and vena contracta, but can extend beyond the vena contracta (Yan, 1989).

3.2.2.1.1. Cavitation inception

The term cavitation inception can be defined as the conditions at which the formation of cavities is initiated. In general, for cavitation inception to occur, the static pressure must be reduced below the vapour pressure of the liquid. However, cavitation was observed at

conditions where the minimum pressure at the vena contracta (P_2) did not reach the vapour pressure, owing to the presence of dissolved gases producing nuclei for cavitation (Yan, 1989). The presence of dissolved gases was found to provide the nuclei for cavitation. Chahine (1994) found the presence of nuclei or weak spots in the liquid to be essential for the cavitation inception using the bubble static equilibrium model. Bubble formation by cavitation in the presence of dissolved gases was found to occur at one-tenth of the energy required when cavitation nuclei were absent (Dean, 1944). Senthil Kumar *et al.* (2000) reported that the degradation rate of potassium iodide was higher at the beginning of the experiment and decreased as the deaeration of the system occurred. The presence of dissolved gases increases the number of cavities generated (Gogate and Pandit, 2001). In deaerated water, the pressure at the vena contracta has to be reduced to below its vapour pressure for cavitation inception. The presence and stability of cavitation nuclei in various liquids was studied by Hayward (1970). Water (tap and distilled) was the only liquid in their study that contained stabilized cavitation nuclei. The organic liquids tested contained no nuclei (benzene, heptane, ethyl alcohol, methyl ethyl ketone, kerosene, mineral oil, water-in-oil emulsion hydraulic fluid). The stability of the cavitation nuclei in distilled water in the presence of organic contaminants was also studied and the stability was found to be only little affected.

3.2.2.1.2. Cavity collapse

The cavities formed can be transient or stable. Transient cavities contract and rebound alternatively with decreasing amplitude until the cavities collapse completely. Yan (1989) explains this phenomenon using the analogy of a pendulum, which can be reproduced as follows. When a pendulum is lifted from its equilibrium position it possesses maximum potential energy. This corresponds to a bubble that is about to collapse (when the external pressure of the flow system is higher than the pressure inside the bubble after P_3). When the pendulum is released it gains maximum kinetic energy at the bottom and it rises in the opposite direction. This bottom position of the pendulum corresponds to the maximum wall velocity and maximum pressure energy inside the bubble (the pressure inside the bubble at this position is higher than the external pressure) and hence it rebounds. A pressure disturbance is generated in the vicinity of the bubble

and it is propagated spherically. A cavity in the medium seldom collapses as an isolated event. The cavitation collapse occurs as a cloud at intense cavitation conditions due to the interaction between the neighbouring cavities. The interaction between bubbles leads to a collapse pressure that is orders of magnitude higher than that of the isolated bubble collapse (Chahine, 1984). The characteristic noise of cavitation is due to the collapse of the cavities (Chahine, 1984; Yan, 1989). The mechanisms of bubble collapse in the presence of a solid wall, between two solid walls, in the vortex, near deformable bodies and near simulated cells were discussed in detailed by Chahine (1994). Collapse of the cavities near a solid wall, near deformable bodies and simulated cells is of significance for the current study and are discussed in Section 3.3.1 on cavitation for microbial cell disruption.

3.2.3. Applications of cavitation

The phenomenon of cavitation is considered an unwanted phenomenon in the design of pumps and marine propellers due to the erosion of the metal surfaces that it causes. However, cavitation induced effects by means of ultrasound is used extensively (or has potential for use) in the chemical industry (for performing chemical reactions), biotechnology (cell disruption, waste water treatment) and medical purposes (such as dissolving renal calculi). Due to the enormous number of applications of cavitation by ultrasound, the current discussion is restricted to the applications of cavitation induced hydrodynamically. Hydrodynamic cavitation has potential for use mainly in the chemical and biotechnology industry. The energy efficiency of this technique bas been reported and it is discussed in Section 3.6.

3.2.3.1. Chemical applications

This phenomenon of hydrodynamic cavitation has been used in the control flow cavitation™ (CFC) technology by Five Star Technologies (Ohio, USA) for homogenization, deagglomeration, particle reduction and encapsulations to name a few applications. Dynaflow Inc., (USA) has patented the fluid jet cavitation method and

system for efficient decontamination of liquids (Chahine and Kalumuck, 2001; United States Patent No. 6,200,486). Hydrodynamic cavitation is used to oxidise organic compounds such as *p*-nitrophenol, methyl orange (Kalumuck and Chahine, 2000), pesticides, volatile organic compounds, arsenic, the herbicide 2,4-D (2,4 – dicholorophenoxy acetic acid) and Malathion (Kalumuck *et al.*, 2003). The hydrolysis of castor oil by hydrodynamic cavitation has also been reported (Pandit and Joshi, 1993). The use of hydrodynamic cavitation for fine particle floatation was demonstrated by Zhou *et al.* (1997).

3.2.3.2. Biological applications

The destructive power of cavitation in a solid-liquid system can be used for the removal of microorganism in drinking water (Kalumuck *et al.*, 2003), recovery of intracellular enzymes and soluble protein from microorganisms (Balasundaram and Pandit, 2001a; Harrison and Pandit, 1992; Save *et al.*, 1994; Save *et al.*, 1997) and treatment of silt waste of sewage treatment plants (Ivanenko, 2001). The physical destruction and decomposition of microorganisms (such as *E. coli* and *Salmonella*) and larvae (such as Zebra mussel larvae) effectively and remediating polluted waters by hydrodynamic cavitation was demonstrated by Kalumuck and Chahine (2000).

3.2.4. Comparison of acoustic and hydrodynamic cavitation

While the phenomenon of cavitation is responsible for the effects observed with both acoustic and hydrodynamic cavitation, these differ in the mechanism of generation of cavities. Ultrasound is used for the generation of cavities in acoustic cavitation while a flow restriction is used for the generation of cavities for hydrodynamic cavitation. Suslick *et al.* (1997) reported a higher rate for the Weissler reaction compared to hydrodynamic cavitation. The collapse pressure generated in the case of acoustic cavitation is an order of magnitude higher than that for hydrodynamic cavitation (Gogate *et al.*, 2001). However, the hydrodynamic cavitation converts the fluid pressure energy into

cavitational collapse pressures much more efficiently than the acoustic cavitation (Gogate and Pandit, 2000).

Constraints exist in the large scale commercial application of ultrasound in the process industry. The erosion of the sonicator probe occurs over a period of usage and hence it has to be replaced frequently. Also the difficulty in transmitting the power to higher volumes makes scale-up of ultrasound processes more challenging. The energy efficiency of the hydrodynamic cavitation compared to acoustic cavitation is orders of magnitude higher (discussed in Section 3.5). Also the intensity of cavitation can be varied across a wide range easily using different orifice plates by hydrodynamic cavitation (Senthil Kumar *et al.*, 2000). Hence the use of hydrodynamic cavitation as a preferred unit operation for microbial cell disruption is considered in this dissertation.

3.3. Hydrodynamic cavitation for microbial cell disruption

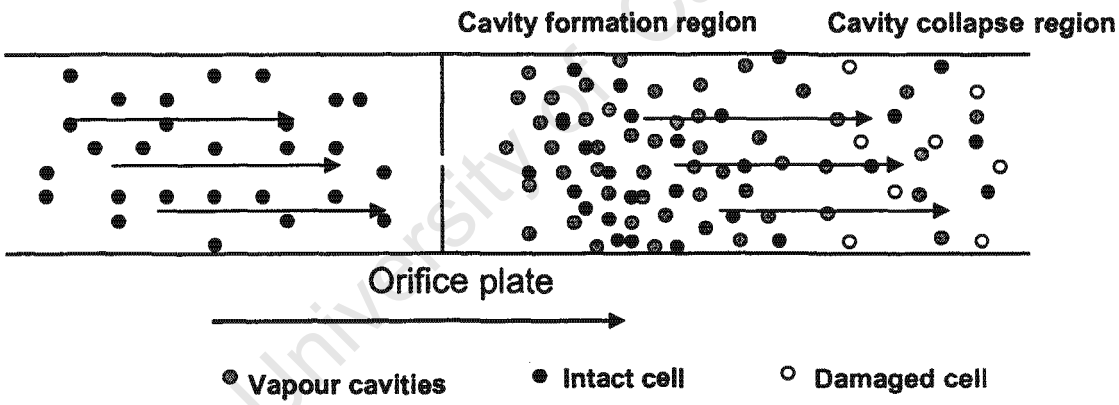

Figure 3.2 Schematic representation of hydrodynamic cavitation occurring in a cell suspension across an orifice plate

Harrison and Pandit (1992) first demonstrated the use of hydrodynamic cavitation for the disruption of *S. cerevisiae* and *Alcaligenes eutropus*. The use of hydrodynamic cavitation for the release of proteins and release of invertase from Bakers' yeast was studied by

Save *et al.* (1994, 1997). Hydrodynamic cavitation was used for the disinfection of water by Jyoti and Pandit (2001) and Kalumuck *et al* (2003). The disruption of microbial cells using hydrodynamic cavitation can be represented schematically as shown in the Figure 3.2

3.3.1. Mechanism of disruption

The phenomenon of cavitation has been introduced in the Section 3.2. The mechanism of disruption of microbial cells by cavitation will be considered in this section. The precise mechanism of disruption of cells by hydrodynamic cavitation is not yet understood. The effects of cavitation can be distinguished as mechanical and chemical. The mechanical effect of cavitation is due to the collapse of the cavities and the chemical effects of cavitation are due to the generation of free radicals (Equations 3.1 to 3.3).

3.3.1.1. Mechanical effects

The mechanism of the cavity collapse has been discussed in detail by Chahine (1994) and Yan (1989). The collapse of the cavity in the presence of a solid wall, sphere or simulated cell can be represented as shown in the Figure 3.3. Three different events are reported in the literature during cavity collapse, namely formation of the liquid micro-jet, shock wave and radial bubble motion.

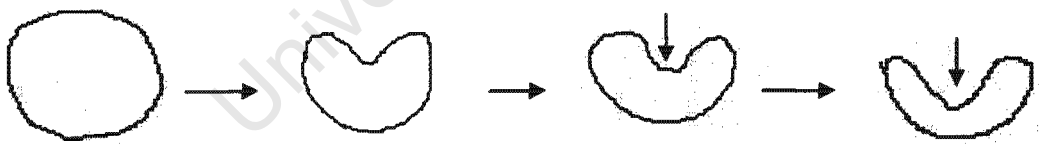

Figure 3.3 Schematic representation of the collapse of a cavity (Gogate and Pandit, 2001)

Liquid micro-jet: When the cavity collapses, its spherical shape is disturbed and involution, as shown in the Figure 3.3, occurs. A liquid micro-jet is formed, threading the cavity. The resulting liquid jet concentrates the large amount of bubble energy in a small site. The resulting water hammer effect was claimed to be responsible for the mechanical

damage observed by cavitation (Brujan, 2004). From a theoretical analysis of this water jet, the water hammer pressure is an order of magnitude less than that required for the erosion of metals. But it is sufficient to disintegrate renal calculi, dental tartar or intraocular lenses. Numerical simulation by Chahine *et al.* (1994) revealed that the collapse of the bubbles in the vicinity of a solid wall generates a micro-jet. A re-entering region was formed initiating a micro-jet that pierced the bubble and impinged on the wall. Numerical simulation of the collapse of a cavity in the presence of a sphere was also considered. Three different cases were studied: (i) an immovable, rigid sphere (II) a movable, rigid sphere (III) a deformable, movable sphere. The bubble collapse proceeded with the formation of a re-entering jet perpendicular to the sphere in the first case. In the second case constriction of the bubble top was observed prior to the formation of the liquid micro-jet. In the third case a re-entering jet was formed but it was wider and slower than that achieved in the presence of a rigid sphere.

Radial bubble motion: Transient cavities exposed to ultrasound at 26.5 kHz were shown to grow from a initial radius of ~5 µm to a radius of 37 µm in ~16 µseconds and collapse in ~ 3 µseconds (Wu and Roberts, 1993). This radial motion of the bubbles creates a bubble wall velocity sufficient to cause some deformation of any cell located at a sufficient distance from the bubble.

Shock wave: The collapse pressure generated by the cavity collapse induces a shock wave. The shock waves propagate spherically around the centre of bubble collapse.

However, these effects do not appear to occur in isolation. Sundaram *et al.* (2003) reported that the shock wave induced by the collapse pressure along with the radial bubble motion was responsible for the membrane permeabilization of 3T3 mouse cells by low frequency ultrasound. The shock waves generated are sufficient for membrane permeabilization but they last only of the order of nanoseconds. The stresses encountered during radial bubble motion last in the order of microseconds. Thus the collective effect due to the shock waves and the radial bubble motion is said to be responsible for the permeabilization. Ohl *et al.* (2003) hypothesized that the liquid jet induced the shear

stress that was responsible for the membrane permeabilization of female uterus cancer cells for the uptake of fluorescein isothiocyanate dextran (FITC-dextran). The shock wave and micro-jet of fluid are known to be associated with the collapse of the cavities (Leighton, 1994).

3.3.1.2. Chemical effects

The chemical effects of cavitation are mediated through the oxidation reactions by the free radicals generated during cavitation (equations 3.1 to 3.3). The generation of these free radicals during hydrodynamic cavitation has been by reported by Kalumuck *et al.* (2003), Senthil Kumar *et al.* (2000) and Suslick *et al.* (1997).

The chemical effects of cavitation induced hydrodynamically due to the free radicals such as $OH^{\bullet}$, $H^{\bullet}$ and $^{\bullet}O_2$ was also observed by Suhr *et al.* (1994). Tocopherol (Vitamin E) is a well known scavenger of free radicals. When rats were supplemented in the diet with tocopherol, the erythrocytes in the blood were seemed to be protected against cavitation. The percentage of destroyed erthrocytes was 30.6 ± 5.3 and the same was 17.5 ± 1.4 for the tocopherol-depleted and tocopherol supplemented rats respectively. The membrane permeability of erythrocytes from tocopherol-depleted rats was increased, implying that the permeabilization is mediated by the chemical effects of cavitation through the generation of free radicals.

The cavitation events were reported to rupture the cell wall or outer membrane of *E. coli* and *Salmonella* and larvae (such as Zebra mussel) and to generate bactericidal compounds such as peroxides and hydroxy radicals that assist in the destruction of these organisms (Chahine and Kalumuck, 2001). Following the disruption of the cell wall or outer membrane, the inner components are susceptible to oxidation. No further results were available from the literature supporting this claim. Furuta *et al.* (2004) reported that the ultrasonic shock wave (produced during cavity collapse) was more important than the indirect effect of free radicals during the inactivation of *E. coli* by ultrasound.

3.3.2. Bubble behaviour near a globule

The behaviour of a cavitating bubble near a simulated blood cell (a globule) was studied by Chahine (1994). The bubble behaviour was dependent on the size ratio between the bubble and globule. When the maximum radius of the bubble (R_b) of the bubble was equal to the maximum radius (R_g) of globule, a sharp intrusion of fluid into the globule and a rupture of the globule interface were observed. When the maximum radius of the bubble (R_b) was half R_g, no rupture was observed and there was only a stretch of the globule.

3.4. Factors affecting hydrodynamic cavitation

A dimensionless number known as the cavitation number (C_v) has generally been used to relate the flow conditions to cavitation intensity. It is the ratio between the force that tends to collapse the cavities and the force that initialises cavity formation:

$$C_v = \frac{(P_3 - P_v)}{\frac{1}{2}\rho v^2} \qquad 3.5$$

where, 'P_3' is the fully recovered downstream pressure (kPa), 'P_v' is the vapor pressure of the medium (kPa), 'ρ' is the density of the medium and 'v' is the velocity at the orifice (m/s). The cavitation number at which the inception of cavitation occurs is known as cavitation inception number (C_{vi}). Cavitation inception typically occurs at the value of C_v of one and there are significant cavitational effects at C_v less than 1 (Gogate and Pandit, 2001; Kalumuck and Chahine, 2000). However, cavitation has been observed at cavitation numbers greater than one due to the presence of cavitation nuclei provided by the presence of dissolved gases (Gogate and Pandit, 2000). The geometry of the flow constriction and the operating conditions such as flow rate and temperature of the suspension can be manipulated to vary the intensity of cavitation. The various parameters affecting the cavitation phenomenon include operating pressure, magnitude of flow constriction (β), number of passes through the cavitation zone, operating temperature, biomass concentration, nature of suspending medium, dissolved solute, concentration and viscosity of the suspending medium.

3.4.1. Effect of operating pressure

Increase in the operating pressure (inlet pressure) was found to result in an increase in the orifice velocity and a decrease in the cavitation number (Table 3.1; Harrison and Pandit, 1992). The inlet pressure also increases with the decrease in the flow area (or the orifice size), resulting in an increase in the orifice velocity and hence lower cavitation number (Senthil Kumar *et al.*, 2000). Thus intense cavitation conditions (low cavitation number) are achieved when the inlet pressure is increased and when the flow area is reduced (i.e. by using smaller orifice).

Table 3.1 Relation between operating pressure and cavitation number (Harrison and Pandit, 1992)

operating pressure (kPa)	orifice velocity (v) (m/s)	cavitation number (C_v)
90	14.2	0.99
60	12.0	1.37
35	8.4	2.79

The collapse pressure of the cavities and the number of cavities generated should be considered when analyzing the effect of inlet pressure and cavitation number

3.4.1.1. Collapse pressure

When the inlet pressure is increased, the pressure drop across the orifice plate increases and hence energy dissipation is higher. Using numerical simulations, Gogate and Pandit (2000) found that at higher energy dissipation the collapse pressure of any single cavity decreases. Chahine (1984) found that the cumulative effect of the bubble collapse leads to a collapse pressure, orders of magnitude higher than that expected from the simple addition of single bubble effects.

3.4.1.2. Number of the cavities

Increasing the inlet pressure, thereby increasing the orifice velocity and reducing the cavitation number leads to an increase in the number of cavities generated (Oba *et al.*, 1986). However, on increasing the number of cavities, the neighboring cavities influence each other and the cavity efficiency decreases. Guzman *et al.* (2003) used the term 'blast radius' to explain the effects of increasing the concentration of cavities. The cavities introduced were called contrast agents, CA (albumin-stabilized gas cavities of diameter of 2.0 to 4.5 μm termed 'Optison' and obtained commercially were used). The permeabilization of human prostrate cancer cells by ultrasound and uptake of calcein, a green fluorescent molecule by in the presence of these contrast agents was studied.

The concept of blast radii can be explained using Figure 3.4. The collapsing cavity was located in the centre. The cells that were present within the killing radius (R_k) became non-viable, the cells that were present within the blast radius (R_{BR}) were reversibly permeabilized and the cells that were present outside this blast radius are unaffected by the cavity collapse. On increasing the concentration of CA, the number of cells permeabilized or killed per cavity present decreased. The neighboring collapsing bubbles influenced each other, resulting in the decrease of the bubble efficiency with increasing CA. The bubbles ability to permeabilize cells was found to be more affected than its ability to kill owing to the relative size of the R_k and R_{BR}. The CA bubbles present at low concentration was found to be more efficient for permeabilization because of the effective blast radii. However when the cell concentration in the suspension was varied, effective permeabilization was found to occur at an optimum cell to bubble ratio. On a similar note Tsukamoto *et al.* (2004), found that the *Saccharomyces cerevisiae* cells that were located within the zone of cavitation were killed by ultrasound (bacteriocidal effects) irrespective of the time of exposure.

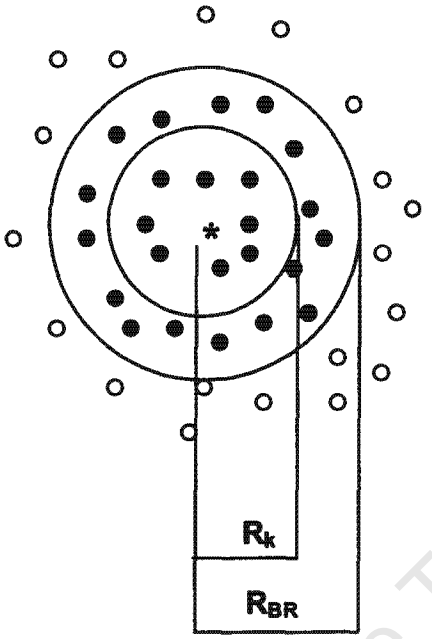

Figure 3.4 Illustration of the concept of blast radius where R_k is the killing radius, R_{BR} is the blast radius. * represents the collapsing bubble, o the intact cell, ● permeabilized cell, ● dead cell (redrawn from Guzman *et al.*, 2003)

The total amount of collapse pressure generated depends on the number of cavities generated and the collapse pressure of each cavity. The collapse pressure generated is maximum at an optimum inlet pressure and cavitation number. This optimum was observed during the conversion of potassium iodide to iodine by Senthil Kumar *et al.* (2000). Inlet pressure was studied over the range 0.14 to 0.34 MPa (20 - 50 psi). The conversion was found to increase with pressure up to 0.28 MPa (40 psi) and decrease thereafter. The percentage of cells disrupted was also reported to increase with an increase in the inlet pressure up to a point and then decrease (Isaacs and Coetzee, 1996), however this was not related to cavitation intensity or collapse pressure. The effect of inlet pressure and cavitation number on various cavitation applications reported in the literature is summarized in Table 3.2

Decrease in the cavitation number indicates an increase in the intensity of cavitation (Kalumuck and Chahine, 2000; Gogate and Pandit, 2001). The rate of formation of I_3^- increased with the increase in liquid pressure over the pressure range studied (0.17 kbar to 1.30 kbar) and concomitant decrease in cavitation number. Its rate of formation was

0.02 μmoles/min at 17 MPa (0.17 kbar) and 0.35 μmoles/min at 130 MPa (1.30 kbar, Susslick *et al.*, 1997). The conversion of potassium iodide to iodine by hydrodynamic cavitation was studied over the pressure range 0.14 – 0.34 MPa (20 - 50 psi). The conversion was found to increase with pressure up to 0.28 MPa (40 psi) and decrease thereafter (Senthil Kumar *et al.*, 2000). The reduction in the concentration of herbicide 2,4-D was faster at a pressure of 0.45 MPa (65 psi) than at 0.34 MPa (50 psi). The degradation of methyl orange was studied over the pressure range of 0.15 – 0.55 MPa (15 – 80 psi). The degradation was found to increase with pressure up to 0.41 MPa (60 psi) and then decrease with increase in pressure above 0.41 MPa.

Table 3.2 Effect of inlet pressure or cavitation number (C_v) on hydrodynamic cavitation reported in the literature

Range of pressure, MPa/cavitation no.	Results/comments	Reference
17 -130	Increase in the rate of formation of I_3^- with inlet pressure	Suslick *et al.*, 1997
0.45 and 0.34 (65 and 50 psi)	Degradation of 2,4-D was faster at 0.45 MPa (65 psi)	Kalumuck *et al.*, 2003
0.15 – 0.55 (15 – 80 psi)	Degradation of methyl orange increased with pressure up to 0.41 MPa (60 psi) and decreased thereafter	Kalumuck *et al.*, 2003
0.14 – 0.34 (20 -50 psi)	Oxidation of potassium iodide to iodine was optimum at 0.28 MPa (40 psi)	Senthil kumar *et al.*, 2000
0.14 – 0.21 (20 – 30 psig)	The amount of proteins released (measured as A_{280nm}) from Bakers' yeast increased from 0.069 to 0.077	Save *et al*, 1994
0.301 and 0.315	Extent of breakage of *Alcaligenes eutropus* increased from 64 to 77 %	Harrison, 1990
Cavitation number 0.99 – 1.35	Rate of hemolysis increased with decrease in cavitation number	Chambers *et al.*, 2000
0.41 – 0.52 and 1.03 (60-75 psi and 150)	Reduction in the concentration of viable bacteria was higher at 150 psi	Kalumuck *et al.*, 2003
0.172 and 0.517 (1.72 and 5.17 bar)	Reduction in CFU increased from 35 % to 43 - 54 % on increasing the inlet pressure	Jyoti and Pandit, 2001

The extent of breakage of *Alcaligenes eutropus* increased from 64 % to 77 % on increasing the pressure from 0.301 MPa to 0.315 MPa (Harrison, 1990). The release of total soluble proteins measured as the absorbance of the supernatant at 280 nm (A_{280nm}), was found to increase from 0.069 to 0.077 on increasing the operating pressure from 0.14 MPa (20 psig) to 0.21 MPa (30 psig) when Bakers' yeast (0.01 % w/w) was subjected to cavitation (Save *et al.*, 1994). The effect of cavitation number on the hemolysis was studied by Chambers *et al.*, (2000). The measure of plasma-free haemoglobin (PFHb) was used as an indicator of haemolysis. The rate of PFHb generation increased with decrease in cavitation number over the range studied (0.99 to 1.35). The decrease in viable bacterial concentration was found to be greater at an operating pressure of 1.03 MPa (150 psi) than at 0.41 – 0.52 MPa (60 - 75 psi; Kalumuck *et al.*, 2003). Hydrodynamic cavitation was used for the disinfection of water and the reduction in colony forming units increased from 35 % to 43 - 54 % on increasing the discharge pressure from 0.172 MPa (1.72 bar) to 0.517 MPa (5.17 bar; Jyoti and Pandit, 2001).

3.4.2. Geometry of orifice plate

The size and number of orifices or the geometry of the jet in a nozzle influences the intensity of cavitation. Gogate and Pandit (2000) studied the effect of varying the orifice size for a constant flow area by numerical simulation. It was found that at a constant inlet pressure, cavitation number and flow area, when the orifice size was increased the collapse pressure of any single cavity increases, but the number of cavities generated decreases. It is postulated that the effects of cavitation are maximum at an optimum orifice size, when the total amount of collapse pressure generated is maximum (number of cavities x collapse pressure of each cavity). To date no such optimum has yet been reported in the literature. Increase in the number of orifices by reducing the size of orifice for same flow area results in an increase in the overall free surface area exposed to the intense shear and turbulence zone leading to intense cavitation conditions. The rate of iodine liberation in the Weissler reaction decreases with an increase in the size of the orifice (Vichare *et al.*, 2000).

The effect of the size and the number of jets in the nozzle was investigated by Chahine and Kalumuck (2001). Four different configurations were used: 12 nozzles of 0.381 x 10^{-2} m diameter, 36 nozzles of 0.221 x 10^{-2} m diameter, 72 nozzles of 0.156 x 10^{-2} m diameter and 216 nozzles of 0.102 x 10^{-2} m diameter were used. The flow area for the first 3 nozzles were the same (1.37 x 10^{-4} m^2) while the flow area for the last nozzle was 25 % greater than the others. The flow rate for the first three configurations was 3.49 l/s while it was 4.32 l/s for the fourth nozzle. The extent of *p*-nitrophenol removed in 2 hrs was determined and the results reported are presented in Table 3.3. The operating conditions were maintained at 42 °C, pH 3.8 and the ambient pressure at 0.14 MPa (20 psia). The oxidation efficiency increased with a decrease in the nozzle size and increase in the number of nozzles. Thus a large number of smaller diameter nozzles are desirable to achieve intense cavitation.

Table 3.3 Effect of the number and size of nozzles on the degradation of *p*-nitrophenol by hydrodynamic cavitation (Chahine and Kalumuck, 2001)

Number of nozzles	Diameter of nozzles (x 10^{-2} m)	Flow area (x 10^{-4} m^2)	Flow rate (dm^3/s)	% of *p*-nitrophenol removed in 2 hrs
12	0.381	1.367	3.49	37
36	0.221	1.380	3.49	26
72	0.156	1.375	3.49	57
216	0.102	1.764	4.32	64

3.4.3. Effect of cell concentration

Cell disruption was found to decrease with increase in the initial concentration of cell suspension (*Sacchormyces cerevisiae* over the range studied 50 - 250 g/l (dry wt; Harrison and Pandit, 1992). Save *et al.* (1997) studied the influence of cell concentration on the disruption by hydrodynamic cavitation using three different concentrations of initial suspension of Bakers' yeast (9 %, 6 % and 3.5 % w/v packed yeast). One gram of

packed yeast is shown to be equivalent to 0.28 g dry wt yeast (Hetherington *et al.*, 1971). Using this correlation, 9 %, 6 % and 3.5 % w/v packed yeast is calculated to be equivalent to 25.2, 16.8, 9.8 g/l, dry wt. When the discharge pressure was 0.21 or 0.28 MPa (30 or 40 psig), the extent of cell disruption was directly proportional to cell concentration (i.e. with an increase in the concentration of cells there was an equivalent increase in the amount of protein released per gram of yeast). When the discharge pressure was 0.34 MPa the extent of proteins released per gram of yeast was almost equivalent for both 3.5 % and 6 % cell suspension. Thus when the cavitation was severe the effect of cell concentration was substantially reduced (Save *et al.*, 1997). Thus from the above two reports, increase in the cell concentration over the range 9.8 to 25.2 g/l dry wt, increases the extent of cell disruption of yeast (Save *et al.*, 1997), whereas over the range 50 to 250 g/l dry wt the extent of disruption decreases (Harrison and Pandit, 1992).

The effects of cavitation were maximum when the ratio of cell to cavity is at its optimum (Guzman, *et al.*, 2003). The blast radius and the killing radius (explained under the Section 3.4.1) was found to be unaffected by increasing the number of cells during the permeabilization of human prostrate cells for the uptake of calcein, by ultrasound in the presence of contrast agents (CA). On increasing the cell density, the number of cells permeabilized was found to increase, illustrating the effective interaction of the bubbles with the cells at higher concentration. On varying both the number of cells and the cavities (CA), an optimum cell-to-cavity ratio was observed. The cell concentrations studied by Save *et al.* (1997) and Harrison and Pandit (1992) were at different ranges and hence an optimum cell concentration has not been reported for the disruption of yeast by hydrodynamic cavitation.

3.4.4. Effect of number of passes

Disruption of *A. eutropus* and *Saccharomyces cerevisiae* was found to increase with the increase in the number of passes through the cavitation zone (Harrison and Pandit, 1992). The effect of number of passes for Bakers' yeast (1 %, w/v) was studied at 0.24 MPa for 25 passes. The amount of soluble protein released was found to increase with the increase

in the number of passes, shown in Table 3.4 (Save *et al.*, 1997). The effect of number of passes on the disruption of Brewers' yeast (2 % w/v) was studied at various discharge pressures (0.17, 0.24 and 0.31 MPa). The effect of number of passes was only marginal at low discharge pressure but increased at higher discharge pressures (Save *et al.*, 1997).

Table 3.4 Effect of the number of passes through the cavitation zone on the release of protein from Bakers' yeast at a 1 % w/v concentration and operating pressure of 0.24 MPa. (Save *et al.*, 1997)

No. of passes	Amount of protein (mg/ml)
1	0.58
5	0.67
15	0.67
25	0.86

3.4.5. Effect of temperature

Effect of cell breakage is a function of the operating temperature. Increase in the temperature increases the vapour pressure thereby increasing the pressure at which cavitation onset occurs, and reduces the viscosity and surface tension. A rise in temperature reduces the gas solubility the chief source of cavity nuclei. This reduces the rate of occurrence of cavitation events (Gogate and Pandit, 2001). The influence of bulk solution temperature on the rate of formation of I_3^- was investigated by Suslick *et al.* (1997). The I_3^- production rate decreases sharply with increase in the temperature. Increase in the temperature increases the vapour pressure of the solvent allowing the easier onset of cavitation. However, the vapour inside the bubble before collapse increases and the cushioning effect was responsible for the reduced effects of cavitation at higher temperatures. But the rate of disruption of Bakers' yeast was found to be higher when the operating temperature was increased from 10 °C to 20 °C at 90 kPa (Harrison and Pandit, 1992). The increase in temperature reduced the viscosity and hence reduced dampening of the cavitation. Increase in the temperature also leads to increase in the

vapour pressure and hence cavitation occurs more easily. The variation of vapour pressure with temperature is greater than the variation due to the other properties.

Thus the effect of temperature is dependent on various factors such as vapour pressure, dissolved gases, collapse pressure generated, viscosity etc. Hence the overall effect depends on the predominant factor at any particular temperature. Further, from the current literature available, it is apparent that while free radical induced reaction may decrease with increase in temperature, mechanical effects may be enhanced depening on operating conditions.

3.5. Significance of location and selectivity

The location factor is defined as the ratio of the release rate of the enzyme of interest to the release rate of total soluble protein. First order kinetic relationship has been used for the calculation of the release rate of protein and enzymes in mechanical disruption cell processes (Limon-Lason *et al.*, 1979; Hetherington *et al.*, 1971; Fonseca and Cabral, 2000), according to Equation 3.6,

$$\ln\left(R_m / (R_m - R)\right) = kt \qquad 3.6$$

where 'R_m' is the maximum protein available for release, 'R' is the protein released at a time 't' seconds and 'k' is the first order release constant (s^{-1}). Periplasmic enzymes maybe expected to be released faster than total soluble protein and hence the location factor was expected to be greater than one for periplasmic enzymes and less than one for cytoplasmic enzymes. The use of the location factor to deduce the selectivity obtained with a particular method of disruption has been demonstrated by Balasundaram and Pandit (2001a). Different values of location factor for the same enzyme liberated by different disruption techniques were obtained. For the release of invertase from Bakers' yeast, the location factor was 1.54 by hydrodynamic cavitation (1 % cells, 0.52 MPa, 50 minutes), 1.10 by high pressure homogenization (1 % cells, 34.47 MPa, 15 passes) and

1.16 by sonication (1 % cells, 20 minutes, 600 W, 20 kHz, 15% amplitude). This is due to the difference in the mechanism of disruption by each technique. Cytoplasmic products were not released to a significant level by hydrodynamic cavitation of 1 % Bakers' yeast at 0.52 MPa for 50 minutes and hence total soluble protein, most of which is located in the cytoplasm, was released at a very slow rate ($k = 0.00065\ s^{-1}$) compared to invertase ($k = 0.0160\ s^{-1}$). Thus the higher value of the location factor for hydrodynamic cavitation indicated the selective release of invertase from Bakers' yeast by hydrodynamic cavitation under the specified operating conditions (1 % cells, 0.52 MPa, 50 minutes; Balasundaram and Pandit, 2001a).

Invertase from Bakers' yeast was released more selectively by hydrodynamic cavitation than by high pressure homogenization or sonication. The selectivity was defined as the ratio between concentration of invertase released and that of total soluble protein released (milligram of invertase per milligram of protein released) The selectivities for invertase were found to be 0.50 mg invertase/mg protein by hydrodynamic cavitation (1 % cells, 0.52 MPa, 10 minutes) 0.0177 mg invertase/mg protein by sonication (1 % cells, 20 minutes, 600 W, 15 % amplitude, 20 kHz) and 0.207 mg invertase/mg protein by high pressure homogenization (1 % cells, 20.68 MPa, 15 passes). However, the yield of invertase, defined as the amount of invertase released per unit biomass, of 0.462 mg/gm of yeast on hydrodynamic cavitation was lower, when compared to the yield of 0.53 mg/gm by sonication and 5.10 mg/gm by high pressure homogenization (Balasundaram and Pandit, 2001b).

3.6. Energy efficiency of hydrodynamic cavitation

Hydrodynamic cavitation has been claimed to be energy efficient by several researchers (Harrison and Pandit; 1992, Jyoti and Pandit, 2001; Kalumuck and Chahine, 2000; Kalumuck *et al.*, 2003; Save *et al.*, 1994; Save *et al.*, 1997; Senthil Kumar *et al.*, 2000). The efficiency of transfer of electric energy into the reaction mixture using ultrasonication is in the order of only 15 % (Chahine and Kalumuck, 2001). The amount of energy required for jet cavitation was compared with ultrasonication for the process of

degradation of *p*-nitrophenol. The cavitation chamber with a 216 nozzle configuration was compared with a 20 kHz magnetostrictive ultrasonic horn under the same conditions (pH 3.8 and 42 °C). The oxidation efficiency was 2.30 mg/MJ using hydrodynamic cavitation while it was only 0.05 mg/MJ by ultrasonication. Thus jet cavitation is more energy efficient than ultrasonication in mediating the oxidation reaction. Similarly in the oxidation of arsenic, DYNAJET, hydrodynamic cavitation system was two orders of magnitude more efficient than ultrasonication in terms of the energy required for oxidation (Kalumuck *et al.*, 2003). The amount of iodine liberated per unit energy input was found to be orders of magnitude higher than acoustic cavitation (Senthil kumar *et al.*, 2000).

The energy requirement for equivalent protein release from microbial cells (*Saccharomyces cerevisiae*) by hydrodynamic cavitation was compared with high pressure homogenization and ultrasonication. Hydrodynamic cavitation was found to be more efficient than high pressure homogenization by an order of magnitude and 3 times more efficient than ultrasonication (Harrison and Pandit, 1992). In the study with Brewers' yeast, the energy efficiency of hydrodynamic cavitation was compared with ultrasonication. For the release of same amount of protein, ultrasonication was found to use 600 J/(ml of suspension), while only 13.5 J/(ml of suspension) was required for hydrodynamic cavitation (Save *et al.*, 1997). In the same study, freshly grown Bakers' yeast was investigated and the energy required for equivalent protein release, was 360 J/ml for ultrasonication while it was 5.4 J/ml for hydrodynamic cavitation. The energy efficiency of hydrodynamic cavitation was compared with sonication, high pressure homogenization and ultrasonication for the disinfection of water. The extent of disinfection based on the energy efficiency of the equipment and energy dissipated in each system is presented in Table 3.5. Based on the energy consumed, high speed homogenization (a blender) was found to be more efficient. However, in terms of the actual energy dissipated hydrodynamic cavitation was found to be more efficient than the other mechanical methods of disruption (Jyoti and Pandit, 2001).

Table 3.5 Energy efficiency during disinfection of contaminated water with various cell disruption equipment (Jyoti and Pandit, 2001)

Equipment and scale	Electrical energy consumption (W)	Energy dissipated (W)	Energy efficiency (%)	Extent of disinfection based on energy consumption (CFU killed/J)	Extent of disinfection based on actual energy dissipated (CFU killed/J)
Ultrasonication horn (50 ml)	240	7	3	1	45
High speed homogenization (1000 ml)	105	45	43	23	55
High pressure homogenization (1000ml)	2090	1137	54	3	5
Hydrodynamic cavitation (75 litres)					
1.72 bar	5500	1915	34	21	60
3.44 bar	5500	2497	45	7	16
5.17 bar	5500	247	4	13	310

3.7. Conclusions

The mechanical and chemical effects of cavitation have wide range of applications including mediating oxidation reactions, reducing microbial viability and releasing intracellular microbial products. The presence of dissolved gas in the suspending medium provides the nuclei for cavitation inception (Yan, 1989; Gogate and Pandit, 2000) for cavitation inception and the amount of energy required is reduced (Dean, 1944). Water (tap and distilled) contains stabilised cavitation nuclei (Hayward, 1970).

Hydrodynamic cavitation was reported to be energy efficient and have higher potential for large scale operation than ultrasonication (Harrison and Pandit; 1992, Jyoti and Pandit, 2001; Kalumuck and Chahine, 2000; Kalumuck *et al.*, 2003; Save *et al.*, 1994; Save *et al.*, 1997; Senthil Kumar *et al.*, 2000). It also allows the cavitation intensity to be varied across a wide range through manipulation of operating variables (Senthil Kumar *et*

al., 2000). The use of hydrodynamic cavitation for cell disruption has been demonstrated (Harrison and Pandit, 1992; Harrison, 1990; Isaacs and Coetzee, 1996; Save *et al.* 1994, 1997).

The effects of cavitation can be distinguished as mechanical and chemical. Three different events, namely liquid micro-jet, radial bubble motion and the generation of shock waves are responsible for the mechanical effects of cavitation. The collective effect of shock waves and radial bubble motion were reported to be responsible for the permeabilization of 3T3 mouse cells (Sundaram *et al.*, 2003). Shear stress induced by the liquid micro-jet was reported to be responsible for the permeabilization of female uterus cancer cells. (Ohl *et al.*, 2003). The chemical effects of cavitation mediated through the generation of free radicals were confirmed by Kalumuck *et al.* (2003), Suslick *et al.* (1997) and Suhr *et al.* (1994). However, Furura *et al.* (2004), reported that shock waves were more important than free radicals for the inactivation of *E. coli* by ultrasound.

The effect of inlet pressure depends on the collapse pressure of the cavities and the number of cavities generated. At intense cavitation conditions (high inlet pressure or low cavitation number) the collapse pressure of each cavity decreases (Gogate and Pandit, 2000) but the number of cavities generated increases (Oba *et al.*, 1986). Thus an optimum cavitation number is expected where the effect of cavitation is maximum because the overall collapse pressure generated is maximum (number of cavities x collapse pressure of each cavity). The existence of this optimum was observed during the degeneration of potassium iodide by Senthil Kumar *et al.* (2000) and during cell disruption by Isaacs and Coetzee (1996).

The effect of the initial concentration of the cell suspension used for disruption depends on the ratio of number of cavity to number of cell. The existence of this optimum was explained using the concept of blast radii by Guzman *et al.* (2003). For the same flow area, cavitation intensity was greater with higher number of orifices of smaller size (Chahine and Kalumuck, 2001; Sethil Kumar *et al.*, 2000). Cell disruption increases with the increase in the number of passes through the cavitation zone (Harrison and Pandit,

1992; Save *et al.*, 1997). The effect of temperature depends on the predominant factor affected such as vapour pressure, viscosity, dissolved gases, collapse pressure etc., at any particular temperature. Hydrodynamic cavitation was found to be useful for the selective release of invertase (Balasundaram and Pandit, 2001a; Balasundaram and Pandit, 2001b).

Chapter 4: Materials and Methods

4.1 Introduction

The cavitation apparatus used for the experiments is described in this chapter. The microorganisms used, their source and the cultivation of *E. coli*, along with the experimental protocol followed are discussed. The principle and methods of analysis used are also defined.

4.2 Cell disruption

4.2.1 Hydrodynamic cavitation

When cavitation is induced in a flow system by restricting the flow using orifice plates or jet nozzle or venturi, it is termed as hydrodynamic cavitation. In this study an orifice plates is introduced into the flow system, downstream of the pump using flanges. The geometry of the orifice plates can be varied to achieve cavitation of different intensities. The ratio of the size of the orifice that can be drilled into the plate to the inside diameter of the pipe and the flow rate generated by the pump are factors that need to be considered in the design of an experimental apparatus for hydrodynamic cavitation.

The cavitation unit I described below did not allow the intensity of cavitation to be varied over a wide range owing to the internal diameter of the flow system. The minimum C_v established were 0.17 using a single orifice of 4 mm diameter and 0.22 using 25 orifices of 1 mm each. Hence cavitation unit II was constructed to allow the cavitation intensity, measured as cavitation numbers to vary across a wide range (C_v 0.99 to 0.09). Due to the large capacity of the tank, the pipe work and the pump in unit II, a minimum of 16 -18 litres of suspension was required for each experiment. For the study on integrated extraction of proteins using aqueous two-phase polymers (Chapter 7), a third rig (unit III) was used because it could be operated with smaller quantities of suspension, typically

100 ml and above. The orifice plates had flat edged orifices, the sides of which were beveled.

4.2.1.1 Cavitation unit 1

The hydrodynamic cavitation apparatus essentially consisted of a closed loop circuit comprised of a holding tank, a centrifugal pump and a flange to accommodate the orifice plate (Figure 4.1). The suction side of the centrifugal pump is connected to the bottom of the tank. The orifice plate is flanged to the discharge line and the discharge line terminates well inside the tank to avoid any induction of air into the liquid due to the plunging liquid jet. The inside diameter of the delivery line of centrifugal pump is 26.64 mm (cross sectional area, 557.11 x 10^{-6} m^2). The tank and the entire flow line are constructed of stainless steel (SS316). A tank of 10 litre capacity was used. Pressure gauges are provided to measure the inlet pressure (P_1) the pressure drop across orifice plate (P_1 - P_2) and fully recovered downstream pressure (P_3). The flanges for the orifice plate were at a distance of 50 pipe diameters from the outlet of the pump and P_3 was measured 10 pipe diameters downstream of the orifice plate. Four different orifice plates were used. The hydraulic characteristics and the geometry of the orifice plates are given in Table 4.1.

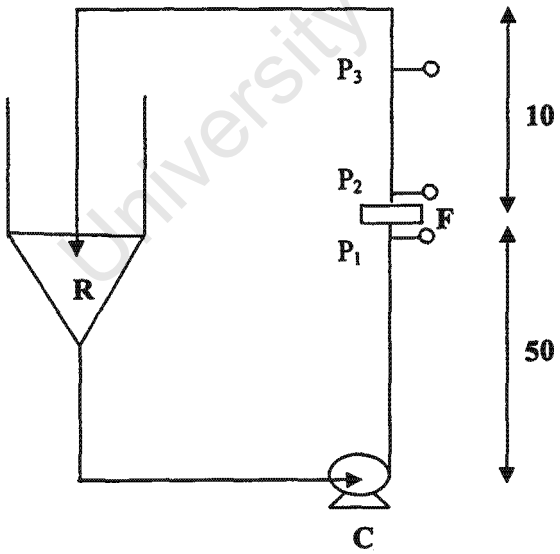

Figure 4.1 Schematic representation of the hydrodynamic cavitation unit I (C- centrifugal pump, F- Flanges for orifice plate, P – Pressure gauges, R- Reservoir for cell suspension)

Table 4.1 Orifice plate geometry and hydraulic characteristics of cavitation unit 1

No.	Orifice diameter (mm)	No. of orifices	Flow rate (dm^3/s)	Orifice velocity (m/s)	Cavitation number	Inlet pressure (kPa)	Reynolds number x 10^{-4}	Time for 100 passes of 6 litres of suspension (min:sec)
Ia	4	1	0.43	34.3	0.17	1080	14	23:30
Ib	1	25	0.59	30.0	0.22	800	15	17:30
Ic	1.8	17	0.92	21.3	0.43	260	16	11:30
Id	2	25	1.11	14.1	0.99	50	14	9:00

4.2.1.2 Cavitation unit II

The centrifugal multistage high pressure pump (model MCH 14BX7, Southern Pumps S.A. (PTY) Ltd., Cape Town, South Africa) made of cast iron coupled to a 15 kW 2 pole Ip55 380/3/50 TFC electric motor was used in the construction of this unit (Figure 4.2). This unit contained a special cylindroconical tank with a capacity of 20 liters. The inside diameter of the pipe work used was 26.67 x 10^{-3} m (cross sectional area of 557.11 x 10^{-6} m^2) except for the pipe of 50 x 10^{-3} m ID that connected the base of the tank to the inlet of the pump to prevent cavitation in the pump due to the net positive suction head (NPSH). The net positive suction head required ($NPSH_r$) by the pump was 2 m (obtained from the pump curve). The net positive suction head available ($NPSH_a$) was calculated to be 5.4 m. Thus the $NPSH_a$ is greater than $NPSH_r$ to prevent the formation of cavities in the pump. All the components of this rig (the pipe, flanges, tank etc.,) were made of SS316 stainless steel. The pressure tappings, the valves and the orifice plates were located according to the ASME (Bean, 1971) standards. Flanges for the orifice plate were at a distance of 25 pipe diameters from the outlet of the pump. A 'T' piece was used to introduce the by-pass line at a distance of 10 pipe diameters from the outlet of the pump. A needle valve was used in the by-pass line at a distance of 4 pipe diameters from the 'T' junction. An 'L' bend was placed at 10 pipe diameters downstream of the orifice plate. The pressure gauges P_1, P_5, and P_2, P_3 were at a distance of one pipe diameter away from the needle valve and the orifice plate respectively and P_4 at a distance of 8 pipe diameters

from the orifice plate. Orifice plates of different geometry were designed to give a range of cavitation numbers ranging from 0.99 to 0.09. These are described in Table 4.2.

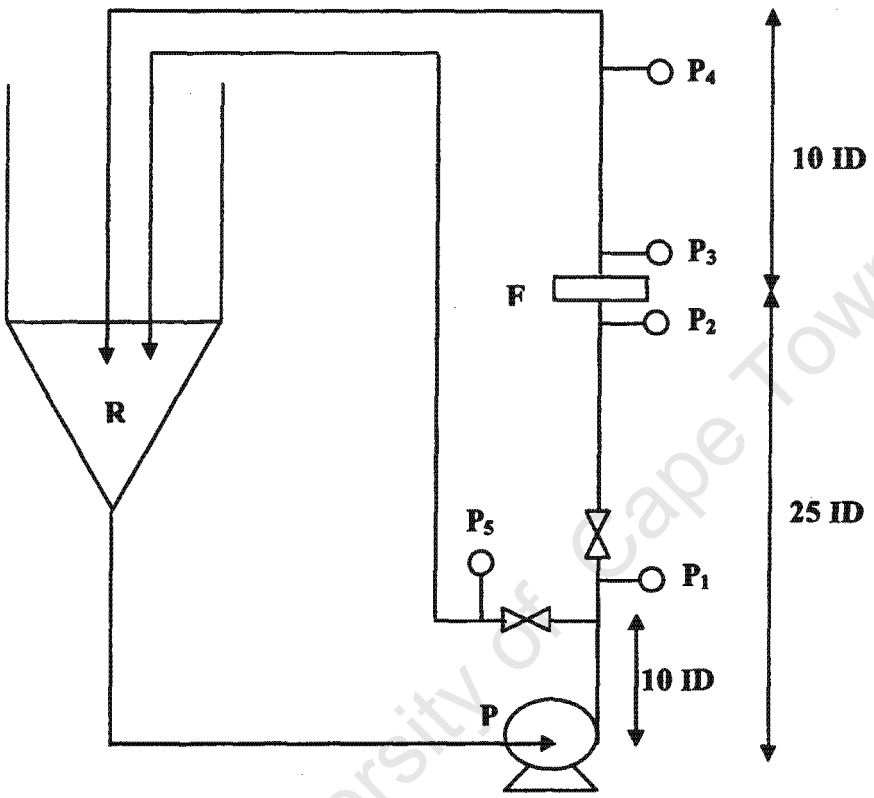

Figure 4.2 Schematic representation of the hydrodynamic cavitaiton unit II (C-centrifugal pump, F- Flanges for orifice plate, P – Pressure gauges, R-Reservoir for cell suspension, ID – Internal diameter of the pipe, 26.64 mm)

4.2.1.3. Cavitation unit III

The cavitation unit III was similar to the unit I consisting of a centrifugal pump, a tank and a flange for the orifice plate. The inside diameter of the pipe work used was 5 mm and the orifice plate had a single orifice of 1.2 mm diameter. The flow rate was 0.025 l/sec, orifice velocity 22.1 m/s, Reynolds number 59 x 10^4 and cavitation number 0.4.

Table 4.2 Orifice plate geometry and hydraulic characteristics of cavitation unit II

Serial No	Orifice diameter (mm)	No. of orifices	Flow rate $m^3 s^{-1} \times 10^3$	Orifice velocity (m/s)	Reynolds number $\times 10^{-4}$	Cavitation number	Inlet pressure (kPa)	Time for 100 passes of 18 litres of suspension (min:sec)
IIa	3	4	1.32	38.0	124	0.09	1550	23:10
IIb	2	25	2.99	46.7	101	0.13	1500	10:00
IIc	12	1	3.86	34.2	910	0.17	1380	8:20
IId	14	1	4.89	31.8	85	0.19	1170	6:10
IIe	15	1	5.28	29.9	80	0.22	1000	6:10
IIf	16	1	5.50	27.4	73	0.26	710	5:50
IIg	17	1	5.57	24.6	65	0.32	400	5:40
IIh	18	1	5.47	21.5	57	0.42	400	5:50
III	19	1	5.61	19.8	53	0.49	310	5:30
IIj	22	1	5.51	14.5	39	0.92	0	5:40

4.3 Microorganism

4.3.1 Yeast

Brewers' yeast (*Saccharomyces cerevisiae*) was obtained as a flocculated cropped yeast slurry at a concentration of approximately 600 g wet weight per litre, at the end of Castle Lager fermentations, from South African Breweries, Newlands, Cape Town, South Africa. Bakers yeast was obtained as a 'sales' yeast suspension following its concentration to approximately 250 g wet weight per litre from Anchor Yeast (Cape Town, South Africa). The yeast cells were washed twice with distilled water and the final washing was performed with a phosphate buffer of pH 7.0 (25 mM) using Beckman cooling centrifuge (Avanti J-25) at 7741 g for 10 minutes at 15 °C. The cells were stored at 4 °C and used within one week of collection. A specified amount of yeast was weighed (wet wt) and suspended in phosphate buffer of pH 7.0 (25 mM) to achieve the required concentration for each experiment.

4.3.2. E. coli

Escherichia coli (*E. coli CSH 36*) culture was obtained from Dr. Val Abratt's laboratory at the Department of Molecular and Cellular Biology, UCT. *E. coli* was sub-cultured every week and stored at 4 °C on agar slants. The Tryptone Yeast Extract (TYE) growth medium used contained: Tryptone 10 g/L, Yeast extract 5 g/L, Sodium chloride 5 g/L. For solid growth media, 1.5 % agar was added to the above medium.

4.3.2.1. Cultivation of *E. coli*

A pre-inoculum was prepared on TYE media and grown in a shake flask at 150 rpm and 37 °C for 12 hours. For cultivation of the inoculum, a 5 % v/v aliquot of the pre-inoculum was added to fresh TYE media and cultivated in shake flasks at 150 rpm and at 37 °C for 12 hrs. This inoculum was used to inoculate a 14 liter New Brunswick BioFlo reactor to obtain both the growth curve of *E. coli* and to provide culture for further experiments. Some 9.5 litres of TYE media containing antifoam (0.01 % v/v) was sterilized. The temperature was set at 37 °C, the agitation at 400 rpm (flat blade turbine impellers) and the pH was maintained at pH 7.0 using 4 N NaOH and 4 N acetic acid. Samples were withdrawn at 2 hour intervals starting from time zero and continuing for 24 hours. Bacterial growth was monitored by absorbance at 660 nm to obtain the growth curve. The cells were found to enter the stationary phase at 12 hours. The maximum specific growth rate was calculated from the growth curve to be 0.36 hr^{-1}.

For the hydrodynamic cavitation experiments, a large quantity of biomass was needed and hence continuous culture of *E. coli* was performed at a growth rate of 0.36 hr^{-1} and 0.11 hr^{-1}. The feed (TYE growth media) was started after 8 hours of batch culture at a 10 liter volume. Approximately 80 litres of media was fed at the rate of 3.6 liters/hr while a reactor volume of 10 liters was maintained. The cells were separated from the spent

media by centrifugation at 6371 g for 8 minutes. The cells were washed with distilled water and re-suspended in phosphate buffer of pH 7.0 (25 mM) for disruption.

4.4. Experimental procedure

4.4.1. Experiments with yeast

The disruption of yeast by hydrodynamic cavitation is compared with the disruption by well established techniques such as the French Press and the high pressure homogenization. Further the effect of the following operating conditions on cell disruption and selective protein release was investigated: cavitation number, initial concentration of the cell suspension and the number of passes through the cavitation zone.

4.4.1.1. Hydrodynamic cavitation of yeast

4.4.1.1.1. Cavitation unit I

The effect of cavitation number, initial cell concentration of suspension and number of passes were studied using cavitation unit I. A Brewers' yeast suspension of concentrations 0.1 %, 1 % and 2.5 % (wet wt) in phosphate buffer of pH 7.0 (25 mM) was subjected to disruption for 1000 passes (re-circulation) using each of the four different orifice plates separately. A 6 litre aliquot of cell suspension was used for each experiment. The cell suspension was maintained at 25 °C during the cavitation process using cooling coils through which the coolant ethylene glycol was circulated at 4 °C from the chiller unit. Samples of 5 ml were withdrawn after every 100 passes to estimate the amount of total soluble protein and specific enzymes released. The samples were centrifuged at 7741 g for 10 minutes to remove cells and cell debris before the supernatant was analysed for the total soluble protein (Folin-Lowry method), invertase and ADH. The release kinetics of proteins and enzymes were studied using orifice plate

Ia, Ib, Ic and Id using 0.1 % , 1 % and 2.5 % cell concentration (wet wt, w/v) and a total exposure of 1000 passes. TEM and SDS-PAGE analysis was also performed.

4.4.1.1.2 Cavitation unit II

The effect of varying the cavitation number for cell disruption was studied using each of orifice plates available with cavitation unit II (IIa to IIj) and a 1 % (wet wt, w/v) suspension of Brewers' yeast was re-circulated across the orifice plates for 1000 passes. Orifice plate IIb was used to study the effect of varying cell concentration (0.1, 0.5, 1, 2.5, 5 % w/v, wet wt). The release kinetics of soluble protein and enzymes were studied using orifice plate IIb using 1 % cell concentration (wet wt, w/v) and a total exposure of 2500 passes and the number of passes was extended to 5000 passes to obtain the release profile of invertase on prolonged exposure to cavitation. An 18 litre aliquot of yeast suspension was used in each experiment. In all the cases, 5 ml samples were withdrawn at intervals of 200 passes. The temperature of the suspension was maintained at 32 °C during the exposure to cavitation using cooling coils through which cold water (5 °C) from the chiller unit was recirculated. Owing to the increased energy dissipation and operational limitations on the heat removal the temperature could not be maintained at 25 °C as done with the cavitation unit I. The samples were centrifuged at 7741 g for 10 minutes and the supernatant was analysed for the total soluble proteins (Bradford's method) and the enzymes, invertase, α-glucosidase, glucose-6-phosphate dehydrogenase (G6PDH) and alcohol dehydrogenase (ADH). TEM and SDS-PAGE analysis and yeast cell viability following cavitation was also studied.

4.4.1.2 French Press disruption

A suspension of Brewers' yeast (1 % w/v wet wt, 40 ml) in phosphate buffer of pH 7.0 (25 mM) was subjected to disruption in the French Press (40 ml cell, model number FA 073) at 20 MPa for 5 passes. Preliminary studies revealed that no further protein release occurs on exposing yeast to 5 passes or more at 20 MPa. Further the maximum release of protein observed was consistent with literature findings. After 5 passes, samples were

centrifuged in the Beckman cooling centrifuge (Avanti J-25) at 12096 g for 10 minutes at 15 °C. The supernatant was analysed for total soluble protein and the enzymes invertase and ADH. The total soluble protein release was estimated by the method of Folin-Lowry.

4.4.1.3 High pressure homogenization

A cell suspension of Brewers' yeast (1 % w/v wet wt, 300 ml) in phosphate buffer of pH 7.0 (25 mM) was subjected to high pressure homogenization for 51.7 MPa (7500 psi) for 10 passes using APV Rannie AS high pressure homogenizer. These conditions were chosen for the maximum release of protein and enzymes in a similar manner to French Press disruption. After every 2nd pass, 1 ml sample was withdrawn and centrifuged at 12,096 g for 10 minutes. The supernatant was analysed for total soluble protein (Folin-Lowry method) and the enzymes α-glucosidase, invertase, G6PDH and ADH.

4.4.1.4 Stability of enzymes during hydrodynamic cavitation

To investigate the stability of enzymes during hydrodynamic cavitation, Brewers' yeast was disrupted by high pressure homogenization (10 % w/v, wet wt, 10 passes, 51.7 MPa) and the cell debris separated by centrifugation at 12,093 g for 10 minutes. The supernatant (crude mixture) was subjected to hydrodynamic cavitation using orifice plate IIb for 500 passes (32 °C). The amount of α-glucosidase and invertase present at the beginning and at the end was determined. The results presented in Table 4.3. It can be seen that the stability of the enzymes was not affected by cavitation. No significant decrease in the soluble protein indicated the absence of protease activity in the disrupted cell suspension.

Table 4.3 Stability of enzymes during hydrodynamic cavitation (C_v 0.13, 32 °C)

No. of passes	α-glucosidase (U/ml)	Invertase (U/ml)	Soluble protein (µg/ml)
0	192	14.60	266
500	188	14.57	254

4.4.2 Experiments with *E. coli*

The disruption of E. coli cells by hydrodynamic cavitation was compared with the disruption by French Press, high pressure homogenization, osmotic shock and EDTA treatment.

4.4.2.1 Hydrodynamic cavitation of *E. coli*

Effect of cavitation number was studied by subjecting 0.5 % cell suspension (wet wt, w/v) to 2500 passes through cavitation unit II using orifice plates IIb, IIc, IIe, IIg, IIi, and IIj. The effect of duration of exposure across the orifice plate and the effect of culture history (growth rate of 0.36 and 0.11 hr^{-1}) were also studied, with 0.5 % w/v (wet wt) suspension of *E. coli* using orifice plate IIb. Samples were withdrawn after every 200 pass and centrifuged at 7741 g for 10 minutes. The supernatant was used to quantify the total soluble protein and enzymes acid phosphatase and β-galactosidase released. The total soluble protein release was estimated by Bradford's method.

4.4.2.2 French Press disruption

A suspension of *E.coli* (1 % w/v wet wt, 40 ml) of growth rate 0.36 hr^{-1} and 0.11 hr^{-1} in phosphate buffer of pH 7.0 (25 mM) was subjected to French Press disruption at 20 MPa for 5 passes. After 5 passes, samples were centrifuged in the Beckman cooling centrifuge (Avanti J-25) at 12,096 g for 10 minutes at 15 °C and the supernatant analysed for total soluble protein released and the enzymes acid phosphatase and β-galactosidase. The total soluble protein release was estimated by Bradford's method.

4.4.2.3 Osmotic shock

E. coli cells, cultivated by a batch culture in a shake flask (1 g, wet wt) were re-suspended in 72 ml of Tris-Cl buffer (0.03 M, pH 8.0) and 8 ml of 0.01 M EDTA. An amount of 16 g of sucrose was added and mixed in a 500 ml shake flask for 10 minutes (120 rpm, 37 °C). Thereafter the mixture was centrifuged at 13000 g for 10 minutes at 4

°C. The supernatant was removed and the well drained pellet was rapidly re-suspended in cold water to the original volume of suspension and incubated in a rotary shaker for 10 minutes (120 rpm, 37 °C). After 10 minutes, the suspension was centrifuged at 7741 g and the cells and cell debris was separated. The supernatant, called the osmotic shock fluid, was analysed for total soluble protein (Bradford's method), acid phosphatase and β-galactosidase.

4.4.2.4 EDTA treatment

A suspension of *E. coli* cells (1 gm, wet wt) in 72 ml of Tris-Cl buffer (0.03 M, pH 8.0) and 8 ml of 0.01 M EDTA was prepared in a 500 ml shake flask and incubated for 10 minutes (120 rpm, 37 °C). After 10 minutes the suspension was centrifuged at 7741 g and the amount of total soluble protein, acid phosphatase and β-galactosidase released were estimated from the supernatant. The total soluble protein release was estimated by Bradford's method.

4.5. Analytical methods

4.5.1. Enzyme analysis

In order to assess the extent of disruption by various disruption techniques used the release of enzymes from different location of yeast and bacteria were studied.

4.5.1.1. α-glucosidase (periplasmic)

This enzyme catalyses the hydrolysis of terminal non-reducing 1,4-linked α-D-glucosides to form α-D-glucose. Its estimation is based on the principle of release of *p*-nitrophenol from *p*-nitrophenol-α-D-glucoside by the enzyme (Oliveira *et al.*, 1981). The reaction was terminated by the addition of 0.1 M Na_2CO_3 and the absorbance measured at 410 nm. A calibration curve with *p*-nitrophenol (Appendix A.1.1) was obtained to calculate the enzyme activity. Enzyme activity is defined as that amount which produces 1 mole of *p*-nitrophenol in one minute. The detailed method of estimation is presented in Appendix A.1. The coefficient of variance was calculated to be 12.41 %.

4.5.1.2. β-galactosidase (cytoplasmic)

This is an enzyme which catalyses the hydrolysis of lactose to β-D-galactoside and D-glucose.

$$\text{Lactose} + H_2O \longleftrightarrow \beta\text{-D-galactosidase} + \text{D-glucose}$$

Its analysis is based on the principle of release of *o*-nitrophenol from *o*-nitrophenyl-β-galactosidase at 37 °C in PPB-Mn buffer (described in Appendix A.5) of pH 6.6 (Flores *et al.*, 1994). The extinction coefficient of *o*-nitrophenol (3.1 M^{-1} cm^{-1}) is used for the calculation of number of moles of *o*-nitrophenol produced. Enzyme activity is defined as the number of moles of *o*-nitrophenol produced per minute at the pH of 6.6 and 37 °C.

The full method of analysis is provided in Appendix A.2. The coefficient of variance for replicate samples was 4.7 %.

4.5.1.3. Acid phosphatase (periplasmic)

This enzyme acts as a catalyst for the hydrolysis of the distal phosphoryl residues of GTP and of the regulatory nucleotide guanosine 5'3'-biphosphate under acidic conditions.

$$H_20 + GTP \longleftrightarrow GDP + Phosphate$$

Its estimation is based on the hydrolysis of *4*-nitrophenyl phosphate to *4*-nitrophenol. The *4*-nitrophenol released undergoes dissociation and rearrangement under alkaline conditions to form *4*-nitrophenolate ion which has a characteristic absorption maximum at 410 nm (Galabova *et al.*, 1996).

$$\text{4-nitrophenyl phosphate} + H_2O \longrightarrow \text{4-nitrophenol} + \text{Phosphate}$$

$$\text{4-nitrophenol} \longrightarrow \text{4-nitrophenolate ion (at pH 11)}$$

A calibration curve with *p*-nitrophenol is used to estimate the number of moles of *p*-nitrophenol formed (Appendix A.3.1). The enzyme unit was defined as the amount of enzyme releasing 1 micromole of *p*-nitrophenol in one minute at the pH of 5.5 and temperature 25 °C. The complete method of analysis is presented in Appendix A.3. The coefficient of variance was found to be 5.87 %.

4.5.1.4. Alcohol dehydrogenase (ADH, cytoplasmic)

Alcohol dehydrogenase catalyses the conversion of acetaldehyde to ethanol *in vivo* in yeast. During alcoholic fermentations, yeast re-oxidise NADH to NAD using pyruvate decarboxylase and alcohol dehydrogenase. Its estimation is based on the principle of conversion of ethanol to acetaldehyde with stoichiometric consumption of NAD^+.

$$R\text{-}CH_2OH + NAD^+ \longleftrightarrow R\text{-}CHO + NADH + H^+$$

In this equilibrium redox reaction, there is simultaneous production of the reduced form of the cofactor i.e., NADH from NAD^+, which gives a characteristic absorbance band at 340 nm ($\varepsilon_{340} = 6.2$ mM cm^{-1}). The rate of formation of NADH is used for the calculation of enzyme activity (Racker, 1950). One unit of enzyme activity is defined as that amount which causes a change in optical density of 0.001 per minute in 0.06 M sodium pyrophosphate at pH 8.5. The comprehensive method of estimation is presented in Appendix A.4. The coefficient of variance was calculated to be 15 %.

4.5.1.5. Glucose-6-phosphate dehydrogenase (G6PDH, cytoplasmic)

Glucose-6-phosphate dehydrogenase is an enzyme which catalyses the conversion of D-glucose-6-phosphate to D-gluconate-6-phosphate.

$$\text{D-Glucose-6-phosphate} + NADP^+ \longleftrightarrow \text{D-Gluconate-6-phosphate} + NADPH + H^+$$

Its estimation is based on the conversion of glucose-6-phosphate to D-gluconate-6-phosphate in the presence of NADP. The simultaneous reduction of β-NADP to β-NADPH is monitored by measuring the increase in the absorbance at 340 nm (Schutte et al., 1983). One unit of enzyme is defined as the conversion of number of millimoles of substrate (glucose-6-phosphate) per minute under the assay conditions (pH 7.6). The detailed method of analysis is included in Appendix A.5. The coefficient of variance was calculated to be 19 %.

4.5.1.6. Invertase (cell wall associated)

This enzyme catalyses the conversion of sucrose to glucose and fructose. Its estimation is based on the principle of hydrolysis of sucrose, a non-reducing disaccharide to glucose and fructose, which are reducing monosaccharides (Gascon and Lampen, 1968).

Sucrose ⟶ Glucose + Fructose

The amount of invertase can be estimated from the amount of glucose and fructose formed. The reducing property of the sugars produced forms the basis for their estimation by DNSA method. The free carbonyl groups, i.e. aldehyde in glucose and ketone functional group in fructose are reduced by 3,5-dinitrosalicyclic acid with its concomitant reduction to 3-amino 5-nitrosalicylic acid during the process. This gives an orange colour which can be measured at 540 nm using the UV-visible spectrophotometer. One mole of glucose and one of fructose is formed from one mole of sucrose. One molecule of 3,5-dinitrosalicyclic acid reacts with one molecule of glucose and fructose each. Hence two molecule of 3,5-dinitrosalicylic acid is used by one mole of sucrose. The number of moles of sucrose hydrolysed can be estimated from the calibration curve and hence the invertase activity. A calibration curve with glucose was obtained to calculate the amount of glucose and fructose produced from sucrose (Appendix A.6.1). One unit of invertase is defined as the amount of enzyme, which hydrolyses 1 micromole of sucrose in one minute at 55 °C in sodium acetate buffer of pH 5.5. The exhaustive method of estimation is presented in Appendix A.6. The coefficient of variance was calculated to be 7.5 %.

4.5.2. Total soluble protein

4.5.2.1. Folin-Lowry Method

This estimation is based on the reduction of phophomolybdic-tungstic mixed acid (Folin & Ciocalteu reagent) by proteins and the consequent loss of 1, 2 or 3 oxygen atoms from the mixed acids leading to the formation of reduced species with a characteristic blue colour which can be measured at 600 nm (Peterson, 1979). Copper chelates with the peptide structure of the protein molecule and facilitates electron transfer to the mixed acid chromogen, increasing the sensitivity to protein. The comprehensive method of estimation is included in Appendix A.7. The coefficient of variance for replicate samples was calculated to be 8 %.

4.5.2.2. Bradford Method

This method is based on the principle of binding of the protein molecule to the dye, Coomassie Brilliant Blue G-250 (Bradford, 1976). The dye exists in two different forms. When the protein molecule binds to the dye the red, form is converted to the blue form which has a characteristic absorption maximum at 595 nm. The method of analysis is provided in Appendix A.8. The coefficient of variance was found to be 9.8 %.

4.5.3. Microscopy

4.5.3.1. Viability

Yeast viability subsequent to cavitation was determined by the slide culture method (Robinson, 2001). Yeast cells which are metabolically functional will undergo reproduction forming buds when transferred to a growth media. The budding cells can be distinguished from those which do not produce buds, under a microscope. In the slide culture technique, a thin layer of solid media on a glass slide is used. The yeast sample is spread over this and incubated at 37 °C for 3-4 hours. The enumeration of the budding yeasts forms the basis of this method of estimation of viability. The experimental protocol followed is described in Appendix A.9.

4.5.3.2. Optical microscopy

The damage of yeast cells by hydrodynamic dynamic cavitation was estimated qualitatively by observing the samples under the light microscope. Mercurochrome (2 %) is used to stain the yeast cells which were viewed under microscope at 1000 X magnification (Olympus BX40 microscope) and photographed.

4.5.3.3. Electron microscopy

The effect of cavitation on the cell wall integrity of yeast and *E. coli* was estimated qualitatively under higher magnification using the electron microscope. Samples were fixed with 2 % formaldehyde, 2 % glutaraldehyde in phosphate buffered saline (PBS).

Fixed cells were pelleted and enrobed in 2 % agarose. Small pellets 1 x 1 x1mm (1 mm cubed) were infiltrated with 2.3 M sucrose (in PBS) for at least 16 hours. Thereafter the cells were rapidly plunge-frozen at -170 degrees using the Leica KF80. Ultrathin cryosections were cut on the Reichert Ultracut S at -100 degrees. The sections were collected on Formvar coated grids, then stained and embedded in methyl cellulose containing 0.2 % uranyl acetate. The samples were viewed on JEM 1200EX (JEOL) Transmission Electron Microscope operating at 120 kV and photographed.

4.5.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis is the technique of separation of macromolecules in an electric field using acrylamide as the solid support. When sodium dodecyl sulphate (SDS) is used for denaturing the protein molecule in the samples, it is called as SDS-PAGE analysis. This technique is widely used for qualitative analysis of protein mixtures and for monitoring protein purification. The separation of proteins is based on the difference in the molecular weight of the polypeptides and hence its size. In order to assess the purity of the soluble protein released by cavitation, SDS-PAGE analysis was performed and the results compared with the other techniques of disruption such as the French Press, the high pressure homogenization, EDTA treatment and the osmotic shock. The procedure followed for this analysis is described in the Appendix A.10.

4.6 Reproducibility of hydrodynamic cavitation experiments

The reproducibility of the hydrodynamic cavitation experiments were studied by repeating the experiments using orifice plate IIb at 1 % w/v (wet wt) cell concentration for 1000 passes (for both yeast and *E.coli*). The experiments were repeated four times at the same operating conditions and the release of soluble protein and enzymes released were determined. The repeated data standard deviation and the coefficient of variance calculated for soluble protein and the enzymes are presented in the Appendix A, below the respective method of analysis. Furthermore the coefficient of variance presented for each analytical procedure in Section 4.5 represent the reproducibility of the complete experiment, not merely the analytical method.

4.7 Concluding remarks

In this chapter the design of the cavitation units and their hydraulic characteristics have been discussed. The procedure used for the cultivation and the preparation of micro organisms for experiments have been detailed. The various disruption techniques performed with yeast and *E. coli* are described. The principle of assay for the quantification of the each enzyme and total soluble protein along with the accuracy of each method has been quantified in terms of a coefficient of variance of the measurements. Finally the qualitative techniques used to asses the effect of cavitation on yeast and *E. coli* and the purification of the proteins released have also been explained.

Chapter 5: Hydrodynamic Cavitation of Yeast

5.1. Introduction

The phenomenon of cavitation and the use of hydrodynamic cavitation for microbial cell disruption have been introduced in Chapter 3. Yeast cells are known are to possess thick cell wall envelope, about 100 – 200 nm and the glucan component of the cell wall envelope provides the mechanical strength. Yeast cell envelope is made up of an outer cell wall and an inner cytoplasmic membrane. The region between these is known as the periplasmic region. Thus the yeast cell can be considered as made up of three primary compartments or locations: outer compartment or periplasm, an inner compartment or cytoplasm and membrane bound cytoplasmic organelles, one enclosed within the other. A selective damage or a point breakage of the outer cell wall could result in a selective release of the cell wall associated and periplasmic proteins.

The effect of cavitation number on the intensity of cavitation has been reported by Senthil Kumar *et al.* (2000). On varying the intensity of cavitation, the extent of cell disruption or damage can be varied. It is postulated that advantage can be taken of the mechanism of cavity collapse to cause a puncture or a point breakage in the cell wall at less intense cavitation conditions, leaving the cell largely intact, while a significant extent of disruption can be achieved at intense cavitation conditions (low cavitation number) or low concentration of the cells in the suspension. The importance of cell to cavity ratio reported by Guzman *et al.*, (2003) indicates that the extent of damage can also be varied by changing the initial concentration of cell suspension. In accordance with the above postulation, by manipulating the operating variables, variable disruption from point breakage to total disruption of yeast cells can be achieved. Thus a detailed understanding of the effect of the various process parameters on cavitation is required to ascertain the conditions for a selective release of the cell wall associated and periplasmic enzymes.

In this chapter the experimental study of the effect of varying intensities of hydrodynamic cavitation generated by manipulating operating conditions on the disruption of Brewers' by hydrodynamic cavitation is presented. The experiments that were performed are listed in Table 5.1. Yeast samples from four different batches were used for the experiments. In order to eliminate the batch variation, the total amount of protein and enzymes available from yeast cells for each batch was quantified separately using French Press or high pressure homogenization (HPH). Batch I and II were used for the experiments with cavitation unit I. Batch III (for the study of effect of cavitation number) and batch IV (for the study of effect of cell concentration) were used for the experiments with cavitation unit II. The results obtained with the experiments using French Press and HPH are presented in the Table 5.2. The raw data for the experiments with hydrodynamic cavitation are presented in the Appendix D.

5.2. Effect of cavitation number on the disruption of Brewers' yeast

The intensity of cavitation occurring in a closed system can be defined using a dimensionless parameter called the cavitation number, C_v:

$$C_v = \frac{(P_3 - P_v)}{\frac{1}{2}\rho v^2} \qquad 5.1$$

where, 'P_3' is the recovered downstream pressure (kPa), 'P_v' is the vapor pressure of medium (kPa), and 'v' is the orifice velocity (m/s). Typically cavitation is said to occur when the cavitation number lies below 1.0 (Gogate and Pandit, 1992). The cavitation number is the ratio of forces collapsing cavities to those initiating their formation. Low cavitation number implies the total collapse pressure of all the cavities is greater than the initiating forces i.e. greater cavitational activity. Different intensities of cavitation can be achieved in the system by changing the flow rate across the orifice plate or by changing the number and size of the orifices in the orifice plate.

Table 5.1 Experiments that were performed with Brewers' yeast at different operating conditions with cavitation unit I and II

Cavitation number	Cell concentration (w/v, wet wt)	No. of passes	Yeast batch	Proteins/Enzymes analysed
Cavitation unit I				
0.17	0.1 , 1 , 2.5	1000	I & II	Total soluble protein, invertase
0.22	0.1 , 1 , 2.5	1000	I & II	Total soluble protein, invertase
0.43	0.1 , 1 , 2.5	1000	I & II	Total soluble protein, invertase
0.99	0.1 , 1 , 2.5	1000	I & II	Total soluble protein, invertase
Cavitation unit II				
0.09, 0.13, 0.17, 0.19, 0.22, 0.26, 0.32, 0.42, 0.49, 0.92	1	1000	III	Total soluble protein, invertase, α-glucosidase, ADH, G6PDH
0.13	0.1, 0.5 , 1, 2.5, 5	1000	IV	Total soluble protein, invertase, α-glucosidase, ADH, G6PDH
0.13	1 & 0.1 %	2500	IV	Total soluble protein, invertase,
	1 %	5000	IV	Invertase

Table 5.2 Results of the experiments with French Press and high pressure homogenizer (1 % w/v, wet wt) for the determination of total amount of soluble protein and enzymes available in the cell (n/a – not analaysed)

Batch/Method	Inlet pressure, MPa	No. of passes	Total soluble protein (mg/g)	Invertase (U/g)	α-glucosidase (U/g)	ADH (U/g)	G6PDH (U/g)
I / French Press	20	5	88.1	1075	n/a	n/a	n/a
II / French Press	20	5	79.7	1183	n/a	n/a	n/a
III / HPH	51.7 (7500 psi)	10	62.4	772	54,401	1.70	40,000
IV / HPH	51.7 (7500 psi)	10	51.6	1723	55,991	1.53	24.800

In the current study, the number and size of orifices were varied by using different orifice plates for each cavitation number in the range 0.09 - 0.99 across two different cavitation units. Four different orifice plates were used with the cavitation unit I. The geometry and the hydraulic characteristics of the unit with these plates are given in Table 4.1. The effects of cavitation numbers of 0.17, 0.22, 0.43, and 0.99 on cell damage at different cell concentrations (0.1 %, 1 % and 2.5 % w/v, wet wt) were studied by re-circulating the cell suspension across the orifice plate for 1000 passes. The amount of total soluble protein and invertase released from yeast cells was determined. The results are presented in the Figure 5.1. The total amount of proteins and enzymes available for release was estimated by using French Press method of disruption (1 % w/v, 20 MPa, 5 passes) and is presented in Table 5.2.

The effect of cavitation number on cell damage in terms of protein and invertase release is seen clearly from the experiments with unit I at 0.1 % cell concentration (Figure 5.1). At a cavitation number greater than 0.4, cavitation number has little effect on release of invertase or total soluble protein. As the cavitation number decreased below 0.4, the amount of the protein and invertase released was found to increase. Invertase and soluble protein release appeared to reach a maximum at C_v around 0.17 to 0.22. While similar trends were observed at 1 and 2.5 % yeast concentrations, these were less marked and the conditions for maximum release were not attained.

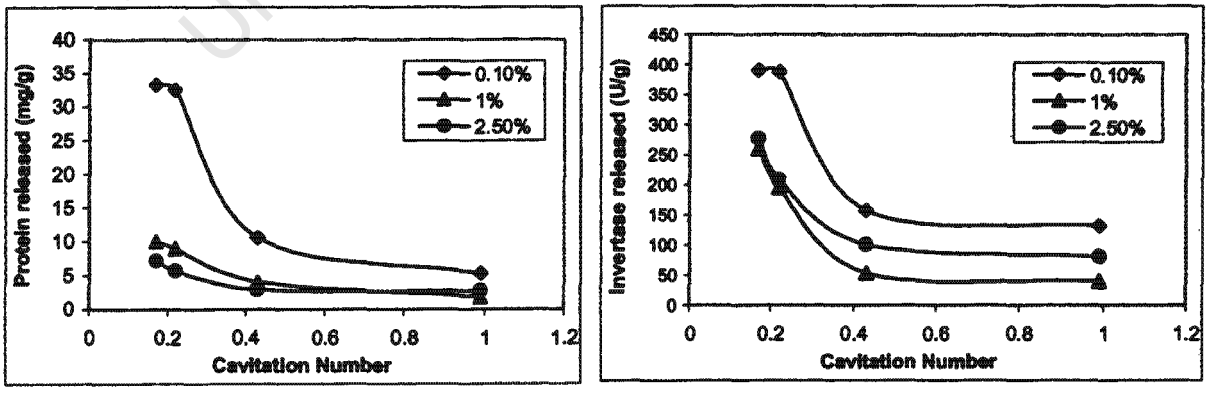

Figure 5.1 Protein and invertase release as a function of cavitation number at different cell concentrations using cavitation unit II (1000 passes)

With cavitation unit II, 10 different orifice plates for cavitation numbers within the range 0.99 to 0.09 were studied at a 1 % cell concentration. Each experiment was carried out for 1000 passes. The geometry and the hydraulic characteristics for this system are given in Table 4.2. The relation between flow area, cavitation number and inlet pressure are given in Figure 5.2. The cavitation number increases with increase in flow area and decreases with increasing inlet pressure. A sigmoid curve was obtained for the relationship between inlet pressure and cavitation number. The effect of cavitation number on the release of proteins and enzymes from various locations of the yeast cell were studied. The release profile of the enzymes that were studied with unit II include invertase (cell wall associated), α-glucosidase (periplasmic), G6PDH, (cytoplasmic) and ADH (cytoplasmic). The total amount of soluble protein and enzymes available for release from the cell was determined by high pressure homogenization (51.7 MPa, 1 % w/v, wet wt) of yeast cells for 10 passes and the results are presented in Table 5.2. The extent of release of the enzymes and protein by hydrodynamic cavitation compared to the high pressure homogenization is presented in the Table 5.3 and Figure 5.4.

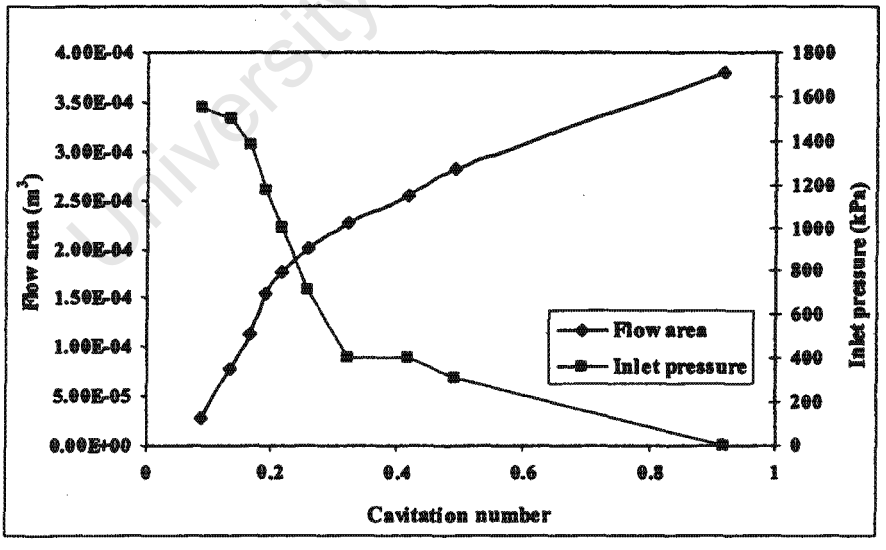

Figure 5.2 Relation between the cavitation number, flow area and inlet pressure using the various orifice plates with cavitation unit II (using water at 25 °C)

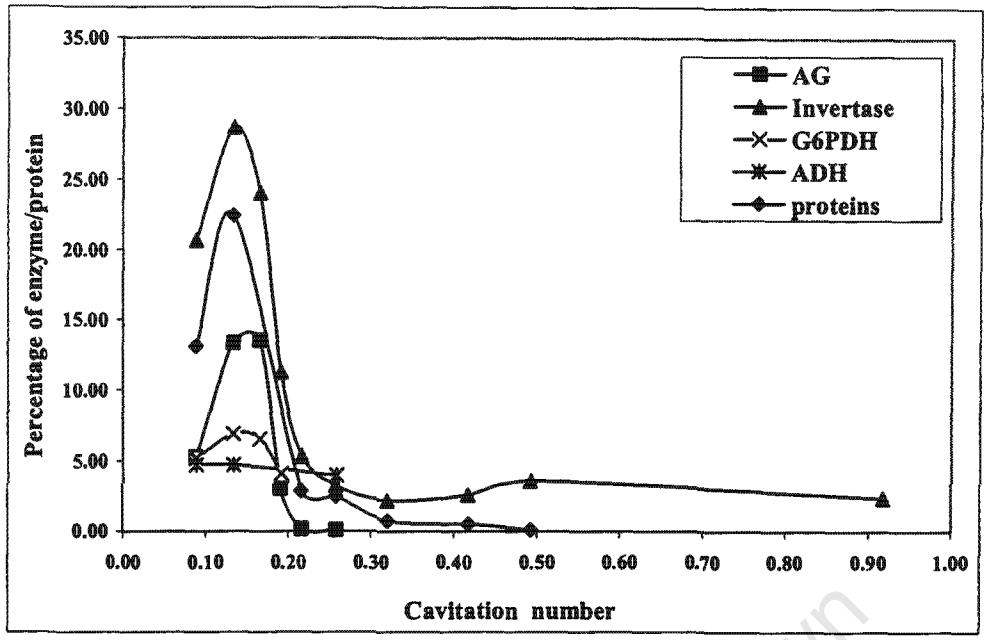

Figure 5.3 **Extent of release of total soluble protein and marker enzymes by hydrodynamic cavitation unit II as a function of the cavitation number (1000 passes, 1 % w/v, wet wt)**

Table 5.3 **The release of total soluble protein and marker enzymes released from Brewers' yeast at 1 % w/v (wet wt) cell concentration following 1000 passes through hydrodynamic cavitation unit II as a function of cavitation number. Comparison is made with disruption by high pressure homogenization (1 % w/v, wet wt, 51.7 MPa psi, 10 passes)**

Cavitation number	Total soluble protein		α-glucosidase (periplasmic)		Invertase (cell-wall associated)		G6PDH (cytoplasmic)		ADH (cytoplasmic)	
	mg/g	%	U/g	%	U/g	%	U/g	%	U/g	%
0.09	8.2	13.1	2870	5.3	160	20.7	0.09	5.2	1900	4.8
0.13	14.0	22.4	7280	13.4	221	28.7	0.12	6.9	1900	4.8
0.17			7380	13.6	185	24.0	0.11	6.6		
0.19			1670	3.1	87	11.3	0.07	4.1	1600	4.0
0.22	1.8	2.9	94	0.2	41	5.5				
0.26	1.6	2.5	57	0.1	25	3.3				
0.32	0.5	0.7			17	2.2				
0.42	0.3	0.5			20	2.6				
0.49	0.1	0.1			28	3.6				
0.92					18	2.4				
HPH	62.4		54400		772		1.70		40000	

The extent of release of the extracytoplasmic enzymes (invertase and α-glucosidase) was found to increase as a weak function of C_v at C_v above 0.25 and as a strong function of C_v when the cavitation number is reduced below 0.25. The extent of release passes through a maximum with respect to cavitation number in the region around a C_v of 0.13. A similar effect was observed for the release of total soluble protein.

The effect of the cavitation number on the release of cytoplasmic enzyme ADH was not significant. The extent of release remained below 5 % across all cavitation numbers used. The extent of release of cytoplasmic G6PDH increased from 4.1 % at cavitation number 0.19 to 6.9 % at cavitation number 0.13 and decreased to 5.2 % at cavitation number 0.09. Hence limited release of cytoplasmic enzymes from yeast was observed under cavitation conditions studied. The extent of release of periplasmic α-glucosidase increased from 3.1 % at cavitation number 0.19 to 13.4 % at cavitation number 0.13 and decreased to 5.3 % at cavitation number 0.09.

The increase in the release of extracytoplasmic enzymes and total soluble proteins on decreasing the cavitation number is due to the concomitant increase in the intensity of cavitation. The intensity of cavitation increases on decreasing the cavitation number because the flow area is reduced and orifice velocity increases and hence the energy dissipation rate increases. Gogate and Pandit (2000), by numerical simulations, found that the collapse pressure of the cavities decrease on increasing the intensity of cavitation. The number of cavities increases on increasing the intensity of cavitation (Oba *et al.*, 1984). The overall effect of cavitation is a function of the total collapse pressure (Senthil Kumar *et al.*, 2000) i.e. the product of the number of cavities and the collapse pressure of single cavity. Hence optimum cell disruption can be expected at maximum total collapse pressure and the latter will pass through a maximum with cavitation number owing to the opposing influence of cavitation number on its components, the collapse pressure and the number of cavities. This is consistent with the disruption maximum at cavitation number around 0.13. When the number of cavities increases the interaction between the neighboring cavities decreases the cavitation efficiency (Guzman *et al.*, 2003). Thus there is an optimum intensity at which the effects of cavitation is highest. Such an optimum was reported by Senthil Kumar *et al.* (2001), on the decomposition of potassium iodide to iodine by

hydrodynamic cavitation (Equation 3.4). The amount of iodine liberated was found to be optimum at a particular cavitation number. Over the range of cavitation number studied (0.17 to 0.30) iodine liberation from potassium iodide was found to be maximum at cavitation number of 0.20 (Senthil Kumar *et al.*, 2000). A similar effect was observed in the current study. From the Figure 5.3, when the cavitation number was 0.13, the release of protein, α-glucosidase and invertase was at its maximum.

The amount of enzyme and protein released decreases when cavitation number was reduced (i.e. when the intensity of cavitation was increased) from 0.13 (orifice plate Ia) to 0.09 (orifice plate IIa). The decrease in the release observed could also be due to the reduction in the number of orifices. The orifice plate used for the cavitation number 0.13 had 25 holes of 2 mm diameter each (flow area = 7.85×10^{-5} m^2) while for the cavitation number 0.09 the orifice plate had 4 orifices of 3 mm each (flow area = 2.83×10^{-5} m^2). Though the flow area for the latter is less than the orifice plate for cavitation number 0.13, intensity of cavitation was not greater because the number of orifices and hence the number of jets arising from 2mm 25 orifice plate is higher and therefore overall surface area of the jet that is exposed to this intense shear and turbulence zone is much higher. The higher density of cavities at cavitation number of 0.13 leads to intense cavitation. Similar effects of increase in intensity of cavitation with increase in the number of orifice for same flow area have also been reported by Chahine and Kalumuck (2001) and Vichare *et al.* (2000).

The trend in enzyme and protein release with cavitation number was found to be similar across cavitation units I and II (Figure 5.4). The absolute amount of protein and invertase activity released with the cavitation unit I was higher at cavitation number above 0.2 than that released with the cavitation unit II. This is due to the temperature of operation. Experiments with unit I were performed at 25 °C while experiments with unit II were performed at 32 °C.

Increase in the temperature increases the vapor pressure thereby allowing the more ready onset of cavitation, but the collapse pressure reduces and hence the intensity of cavitation decreases. The rise in temperature also reduces the gas solubility which is the source of cavity nucleation and hence reduces the rate of occurrence of cavitational events. Thus the overall number of the cavities formed decreases, thereby

reducing the effect of the cavitation phenomenon (Gogate and Pandit, 2001). Suslick *et al.* (1997) reported a decrease in the rate of production of I_3^- with increase in the temperature. Out study is consistent with the above reports. The amount of protein and invertase released decreased because of the increase in operating temperature from 25 °C to 32 °C.

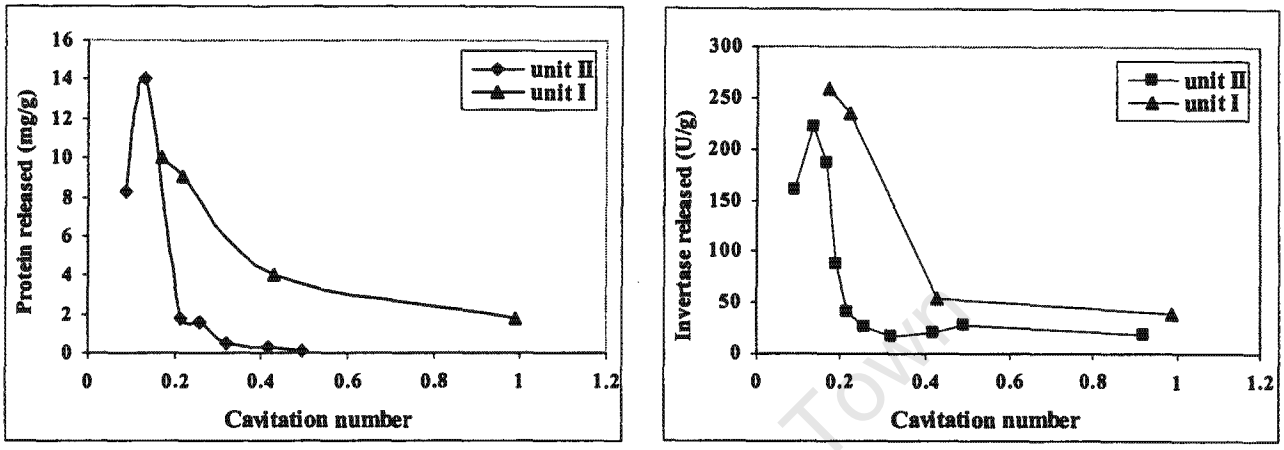

Figure 5.4 Comparison of the release trend of protein and invertase as a function of cavitation numbers across different two cavitation units (I & II)

5.3. Effect of cell concentration

While maintaining all other parameters for cell disruption constant, the cell concentration was varied to study its effect on the release of enzymes and proteins with both the units (I and II). Cell concentrations of 0.1 %, 1 % and 2.5 % were used for experiments with unit I with each of the four orifice plates (cavitation numbers 0.17, 0.22, 0.49 and 0.99). The results are given in Figure 5.5. It can be seen that the amounts of protein and invertase released per unit mass of cells were found to increase on decreasing the cell concentration. This increase was more pronounced for proteins When cell concentration was decreased from 1 % to 0.1 % the protein released increased by 3.2 times while invertase released increased by 1.5 to 2.0 fold in the C_v range of 0.17 to 0.22. It is postulated that at 0.1 % cell concentration the same cell undergoes repeated interaction with oscillating cavities and this is more frequent than with 1 % cell concentration and hence damage was aggravated with each

subsequent pass resulting in an increase in the release of protein than the cell wall associated invertase.

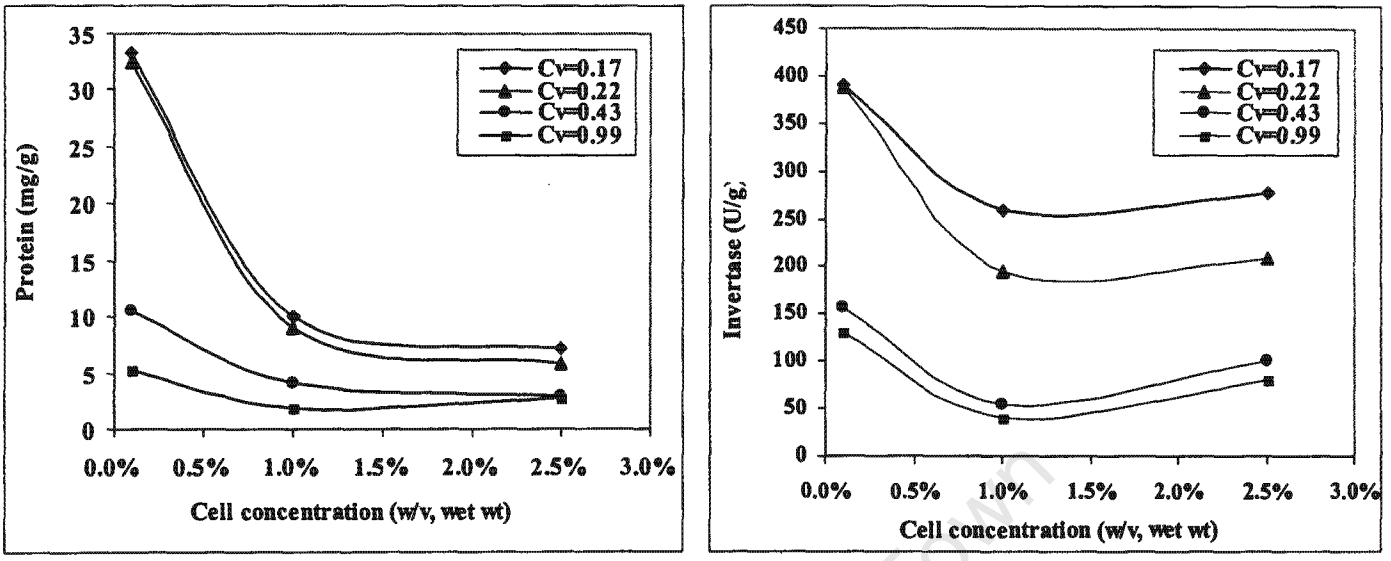

Figure 5.5 Release of soluble protein and invertase as a function of cell concentration using cavitation unit I (1000 passes)

The effect of cell concentration (0.1 %, 0.5 %, 1 %, 2.5 % & 5 % w/v, wet wt.) was studied using the orifice plate IIb (cavitation number 0.13) with cavitation unit II. The release of total soluble protein, invertase, α-glucosidase, G6PDH and ADH released was quantified. The results are shown in Figure 5.6. The total amount of soluble protein and amount of each enzyme available in the cell for release was estimated by disruption with the high pressure homogenizer (1 % cell concentration, 7500 psi, 10 passes). From Figure 5.6 it can be seen that there was an optimum cell concentration (0.5 %) at which the percentage release of proteins, invertase and α-glucosidase were maximum. The protein release increased from 15 % to 28.5 %, α-glucosidase release increased from 16.5 % to 27 % and invertase release increased from 23 % to 39 % on decreasing the cell concentration from 1 % to 0.5 %. On further decreasing the cell concentration from 0.5 % to 0.1 % the amount of total soluble protein decreased from 29 % to 21 %, α-glucosidase release decreased from 27 % to 6.6 % and invertase released was found to decrease from 39.4 to 39.0 %.

The existence of this optimum cell concentration is due to the optimum ratio of number of cavities to number of cells. Since the cavitation conditions were

maintained constant, the number of cavities generated was the same while the density of cells was varied. At lower cell concentration the cavities interact among themselves and the cavitation efficiency decreases while at higher cell concentrations the number of cavities available is saturated. At an optimum ratio, the interaction between the cavities generated and the cells present is postulated to be highest resulting in a maximum release of soluble protein and enzymes (0.5 %). An optimum cell-to-cavity ratio was noted by Guzman *et al.* (2003) during the permeabilization of human prostrate cells by ultrasound for the uptake of calcein.

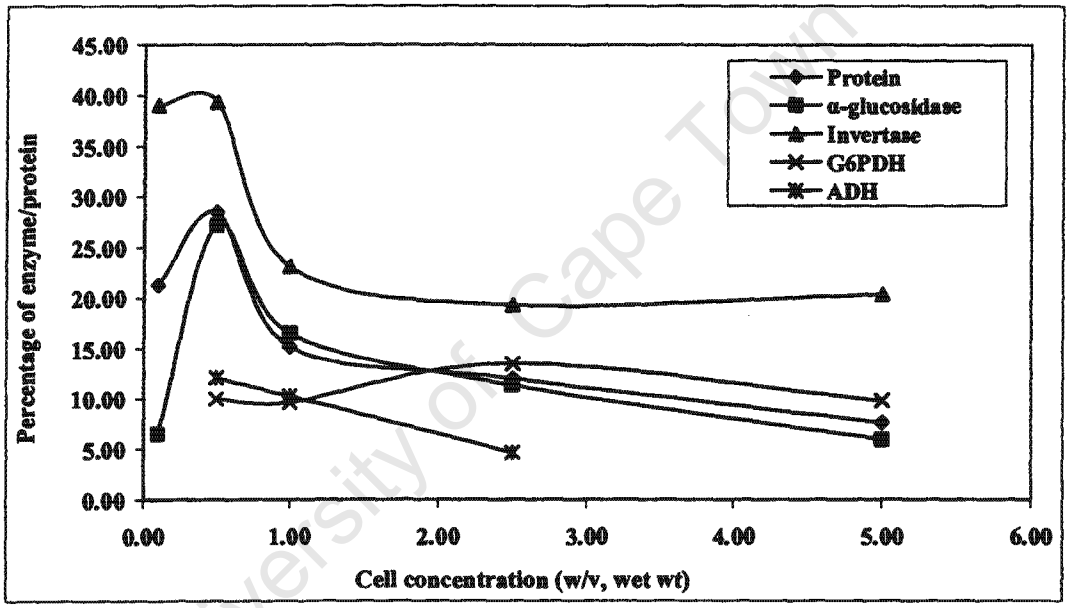

Figure 5.6 Extent of release of total soluble protein and markers enzymes as a function of cell concentration using cavitation unit II (cavitation number 0.13, 1000 passes)

5.4. Effect of number of passes

The effect of number of passes of yeast suspension across the orifice plate on the release of total soluble proteins, α-glucosidase and invertase was studied at cavitation number 0.13 (cavitation unit II) and a temperature of 32 °C for two different cell concentrations 1 % and 0.1 % for 2500 passes. The results are presented in the Figure 5.7, 5.8 and 5.9 and in Table 5.4. The total amount of protein and enzymes available

in the cell for release was estimated by disruption of 1 % yeast suspension (wet wt) at 51.7 MPa (7500 psi) on 10 passes through the high pressure homogenizer. The increase in the amount of protein released as a function of the number of passes took the form of a saturation function, asymptoting to a maximum value at approximately 2000 passes. At a 1 % cell concentration and a cavitation number 0.13, in cavitation unit II, the maximum amount of protein released by cavitation was 12.9 mg/g wet yeast (Figure 5.7). This represents 25 % of protein available for release by high pressure homogenizer. At a 0.1 % cell concentration, the maximum amount of protein released under the same conditions was 15.80 mg/gm representing 31 % of total soluble protein available for release.

Table 5.4 Maximum amount of total soluble protein, invertase and α-glucosidase released using hydrodynamic cavitation (cavitation number 0.13, cavitation unit II) subjected to 2500 passes

Cell disruption	Cell conc.	Proteins		α-glucosidase		Invertase	
		mg/g	%	U/g	%	U/g	%
Hyd. cavitation	1 %	12.9	25	9600	17	1060	62
Hyd. cavitation	0.1 %	15.8	31	3900	7	1110	64
HPH	1 %	51.6		56,000		1720	

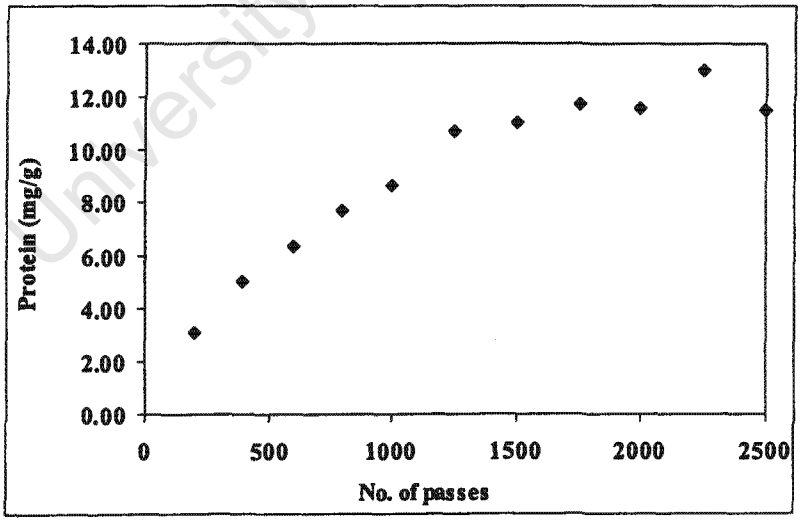

Figure 5.7 Release of total soluble protein as a function of the number of passes using cavitation unit II (1 % w/v, wet wt, cavitation number 0.13)

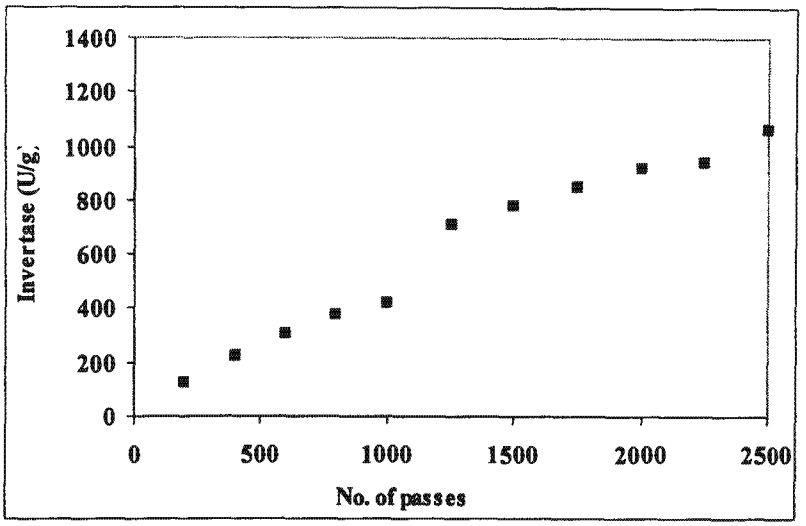

Figure 5.8 Release of invertase as a function of the number of passes using cavitation unit II (1 % w/v, wet wt, cavitation number 0.13)

The release of invertase was found to increase until 2500 passes (Figure 5.8). It did not asymptote under the conditions of the experiment (1 % w/v wet wt, cavitation number 0.13 and 2500 passes). The maximum amount of invertase released after 2500 passes was 1160 U/g. This corresponds to 62 % of the invertase available for release by high pressure homogenization. When a 0.1 % cell concentration was used, the maximum amount of invertase released after 2500 passes was 1110 U/g, corresponding to 64 % of the invertase available for release by high pressure homogenization.

The release of α-glucosidase as a function of the number of passes was found to be linear up to 1000 passes and reached an approximately constant value after 1000 passes with both 1 % and 0.1 % cell concentration separately. The maximum release of α-glucosidase with 1 % cell concentration was 9600 U/g yeast which is 17 % of the amount released by high pressure homogenization. The maximum amount of enzyme released with 0.1 % cell concentration was 3900 U/g yeast which corresponds to 7 % of the enzyme available for release.

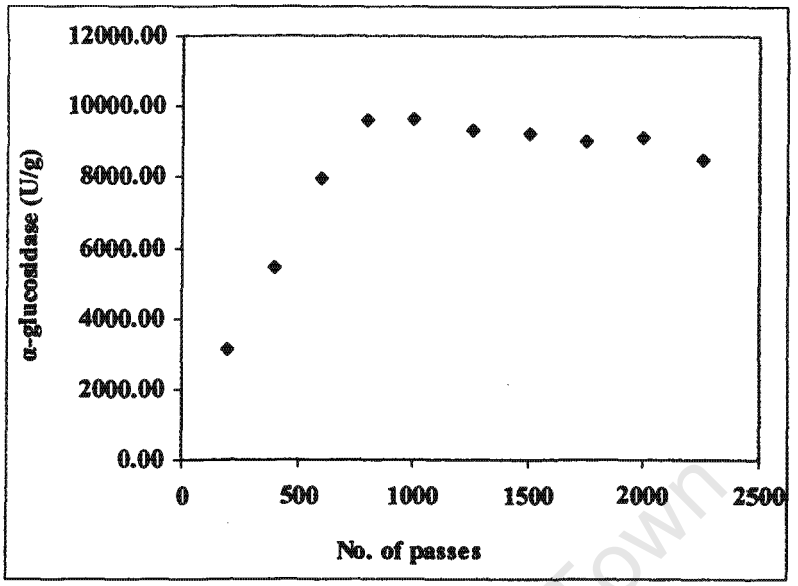

Figure 5.9 Release of α-glucosidase as a function of the number of passes using cavitation unit II (1 % w/v, wet wt, cavitation number 0.13)

The release of the total soluble protein and of the periplasmic enzyme α-glucosidase as a function of number of passes was found to asymptote, though complete release of the available proteins or periplasmic enzymes was not achieved under the conditions of the experiment (1 % w/v wet wt, 2500 passes, and cavitation number 0.13). Conversely, the release of invertase (cell wall associated) was sustained up to 2500 passes. When the disulphide bonds of the outer cell wall of yeast were reduced by dithiothreitol, invertase was found to be released (Sommer and Lewis, 1971). The formation of short lived reactive species such as OH*, H* and H_2O_2 during hydrodynamic cavitation that are capable of secondary oxidation and reduction reactions has been reported at several occasions. (Kalumuck *et al.*, 2003; Senthil Kumar *et al.*, 2000; Suslick *et al.*, 1997). On continued exposure of the cells to cavitation, the impact of the collapsing cavities weakens the cell wall by loosening the cell wall matrix. Thus the combined effects of cavitation both chemical and mechanical were postulated to result in the sustained release of invertase.

5.5. Release kinetics

The release kinetics of protein by various disruption techniques has been discussed in detail in the Sections 1.2.1.2, 1.2.2.2 and 1.2.3.2 in Chapter 2. Disintegration of cells in high-speed agitator bead mills (Currie *et al.*, 1972, Garrido *et al.*, 1994, Schutte and Kula, 1988, Woodrow and Quirk, 1982, Marffy and Kula, 1974, Melendres *et al.*, 1993, Torner and Asenjo, 1991, Limon-Lason *et al.*, 1979) is a first order process and the solubilization of proteins can be described by Equation 5.2:

$$\ln\left(R_m/(R_m - R)\right) = kt \qquad 5.2$$

where 'R_m' is the maximum protein available for release, 'R' is the protein released at a time 't' (seconds) and 'k' is the first order release constant (s^{-1}).

The kinetics of disruption of Bakers yeast in an industrial high pressure homogenizer was studied and reported as follow first-order process by Engler and Robinson, 1981; Gray *et al.*, 1972; Harrison, 1990; Hetherington *et al.* (1971), Sauer *et al.*, 1989. The release kinetics for yeast slurries not exceeding 600 g/l (packed yeast) was described by Equation 5.3 (Hetherington *et al.*, 1971):

$$\ln\left(R_m/(R_m - R)\right) = kNP^{2.9} \qquad 5.3$$

where 'R' amount is the protein released, 'R_m' is the maximum protein available for release, the dimensional release rate constant 'k' (MPa^{-1}) is a function of temperature, 'N' is the number of passes and 'P' is the operating pressure.

The release of total soluble proteins by ultrasonication at 200 W and 26 g/l (dry weight) was found to follow first order release kinetics (Fonseca and Cabral, 2002, Kuboi *et al.*, 1995). Thus microbial cell breakage has been shown to follow first-order kinetics for several different mechanical methods of disruption.

First order kinetics is based on the assumption that the rate of release of soluble protein or of a specific enzyme is directly proportional to the amount of unreleased protein or enzyme, as shown in Equation 5.4:

$$dR/dN = k(R_m - R) \qquad 5.4$$

where 'R' is the amount of intracellular protein or enzyme released after 'N' passes, 'R_m' is the maximum amount of protein or enzyme available for release and 'k' is the first order rate release constant (dimensionless). Integrating Equation 5.4 between the limits of R = 0 at N = 0 and R = R at N = N results in Equation 5.5:

$$\ln\left(R_m/(R_m - R)\right) = LnD^{-1} = kN \qquad 5.5$$

It can be argued that for each disruption mechanism, a certain fraction of the protein present in the cells is available for release. Where micronisation occurs, this will be greater than in its absence. This maximum, dependent on method of disruption and operating conditions, is termed 'R_i' (Scholtz-Brown, 1998). First order kinetics requires the use of 'R_i' to conform to the assumptions of the Equation 5.5. For the calculation of release rate of protein (using 1 % w/v, wet wt) at different cavitation numbers, the 'R_i' value calculated as the asymptote value was used, instead of 'R_m' since maximum amount of protein or enzyme released as estimated by French Press or high pressure homogenization was not achieved under the current operating conditions of hydrodynamic cavitation. For the cavitation number of 0.13 the release of soluble protein asymptotes after approximately 2000 passes through the cavitation zone (Figure 5.7). However, in order to compare the release kinetics across different cavitation numbers, the maximum amount of soluble protein released during 1000 passes through the cavitation zone were used as R_i (Figure 5.10). The value of $\ln\left(R_i/(R_i - R)\right) = \ln D^{-1}$ is plotted against N according to the form of Equation 5.5 to estimate the release rate constant. Straight line fits were obtained with the regression coefficients typically greater than 0.98 or 0.99. These fits given in Appendix B (B.1) illustrate that release kinetics fit the data well. The R_i values used for the determination of 'k', the regression coefficients (R^2) obtained and the first order constants are presented in Table 5.5. The release rate constant as a function of cavitation number is presented in Figure 5.11.

Table 5.5 R_i values used for calculation of protein release rate constant (k) and the corresponding regression co-efficient (R^2) obtained with hydrodynamic cavitation (1 %, w/v, wet wt)

C_v	R_i (mg/g)	k (x 10^{-3})	R^2
0.09	8.2	1.45	0.98
0.13	14	2.03	0.92
0.17	10	3.76	0.98
0.22	8.3	2.48	0.99
0.26	1.6	1.67	0.84

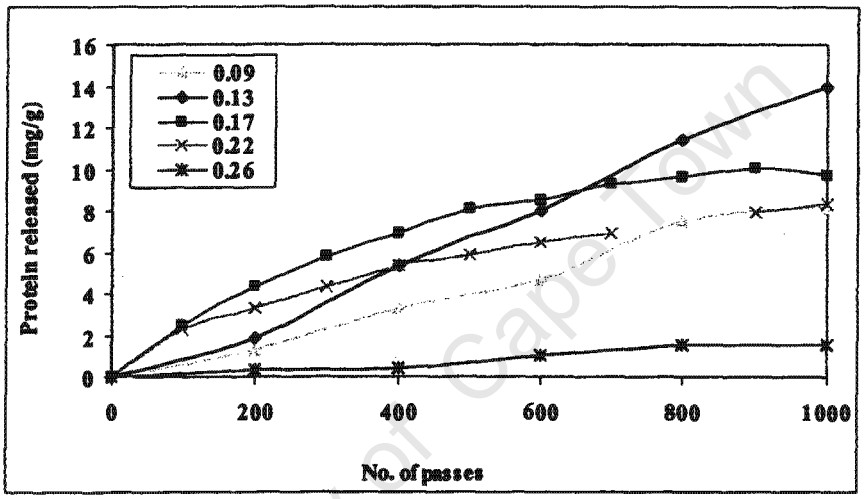

Figure 5.10 Release of total soluble protein as a function of number of passes at different cavitation numbers (1 % w/v, wet wt)

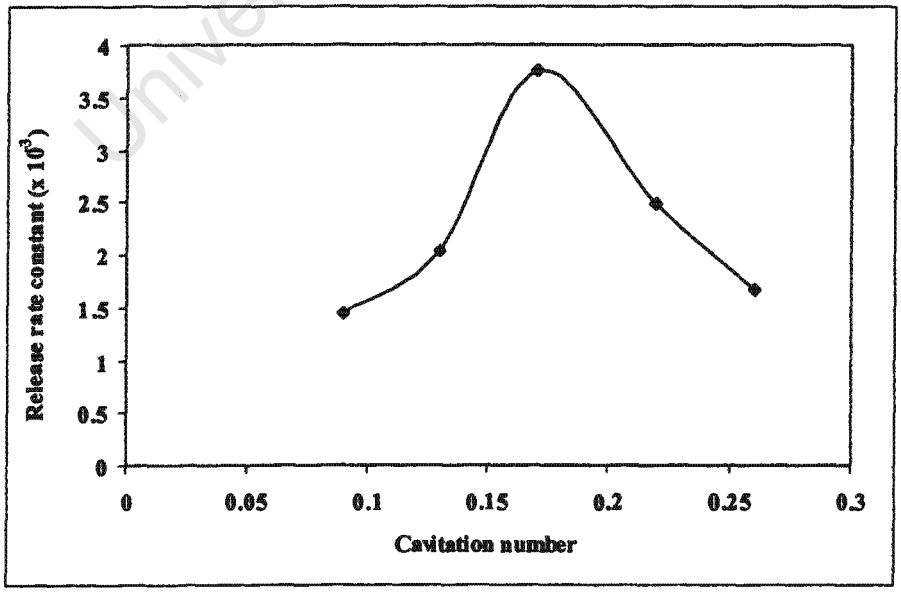

Figure 5.11 Release rate constant of soluble protein as a function of cavitation number using cavitation unit II (1000 passes, 1 % w/v, wet wt)

While using 1 % cell concentration for disruption the release rate of protein was found to be maximum at cavitation number of 0.17 and the release rates decreases with increase or decrease in cavitation number from 0.17 (Figure 5.11). The cell-to-cavity ratio (discussed under section 5.3) was optimum at this conditions (cavitation number 0.17, 1 % w/v, wet wt, 1000 passes) resulting in a higher release rate.

There was a sustained increase in the release of invertase on extending the number of passes through the cavitation zone for up to 5000 passes (1 % w/v, wet wt; Figure 5.12). Some 1474 units of invertase were released and this corresponds to 86 % of the enzyme available from the cell. Similarly at 0.1 % cell concentration (w/v, wet wt) the number of passes through the cavitation zone was extended up to 3000 passes, again the invertase release was found to increase until 3000 passes, reaching 1256 units, which corresponds to 73 % of the total enzyme available (Figure 5.12). The release of invertase did not asymptote for the range of cavitation numbers studied (Figure 5.12). Since a significant amount of invertase was released by hydrodynamic cavitation and the release did not asymptote the total amount of the enzyme available could be expected to be released under the conditions of hydrodynamic cavitation studied. Hence, the maximum amount of invertase obtained by high pressure homogenization (R_m) was used for the calculation of release rate constant. The values of $\ln\left(R_m/(R_m - R)\right)$ = Ln D^{-1} was plotted against the number of passes to calculate the release rate constant. The R_m values used for the determination of 'k', the regression coefficients (R^2) obtained and the first order constants are presented in Table 5.6. Straight line fits were obtained with the regression coefficients typically greater than 0.98 or 0.99. These fits given in Appendix B (B.2. 3) illustrate that release kinetics fit the data well.

Table 5.6 R_m values used for calculation of invertase release rate constant (k) and the corresponding regression co-efficient (R^2) obtained with hydrodynamic cavitation

Cell conc.	0.10 %			1 %			2.5 %		
C_v no.	k (x 10^{-4})	R_m	R^2	k (x 10^{-4})	R_m	R^2	k (x 10^{-4})	R_m	R^2
0.13	4.27	1720	0.98	3.88	1720	0.98	2.41	1720	0.98
0.17	4.42	1180	0.98	2.58	1080	0.98	2.86	1180	0.99
0.22	3.38	1180	0.99	1.97	1080	0.96	2.14	1180	0.98
0.43	1.64	1180	0.99	0.46	1080	0.97	0.65	1180	0.99
0.99	1.69	1180	0.98	0.35	1076	0.99	0.49	1180	0.99

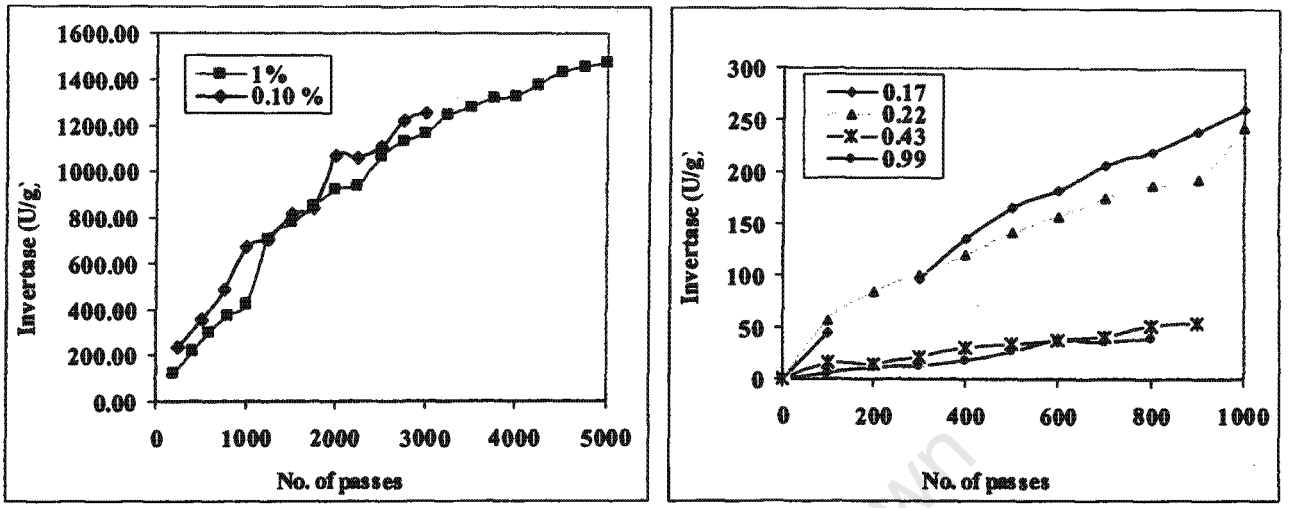

Figure 5.12 Release of invertase as a function of number of passes (A) at different cell conc. (cavitation number 0.13) (B) at different cavitation numbers

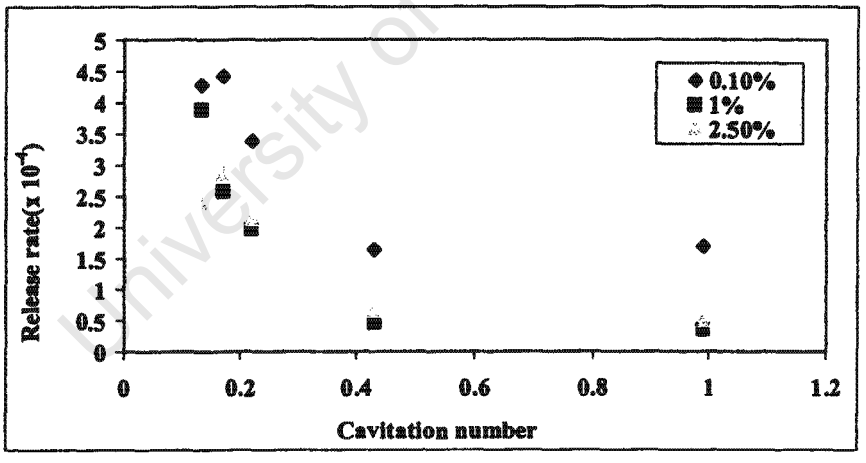

Figure 5.13 Release rate constant of invertase as a function of cavitation number at different cell concentrations using cavitation unit II (cavitation number 0.13)

The release rate of invertase as a function of cavitation number using different cell concentrations is presented in Figure 5.13. The release rate decreases when the cavitation number is less than 0.17 for 0.1 % and 2.5 % cell concentration. The release rate constant was found to be optimum at cavitation number of 0.17 and the

value decreases on either increasing or decreasing the cavitation number. The release rate of invertase was found to increase with decrease in cavitation number for 1 % w/v (wet wt) cell concentration.

Hetherington *et al.* (1971) reported the relation between the release rate and pressure when using high pressure homogenizer for disruption of bakers yeast (Equation 5.3). The pressure exponent was found to be 2.9. No such linear relation was found between the release rates and cavitation number in our study (Figure 5.11 and 5.13). The R_m value for proteins when using the fungus *Rhizopus nigricans* for disruption by high pressure homogenizer were found to be different at different discharge pressure (Keshavarz *et. al.*, 1990) and it was concluded that the soluble protein release is a weak function of pressure. In our system the release rate was found to have an optimum cavitation number for each cell concentration.

5.6. Selectivity

The mechanism of disruption by cavitation has been discussed in the Section 3.3.1. The disruption of yeast can be due to the mechanical or chemical effects or both. Three different mechanisms have been reported in the literature for the mechanical effects of cavitation. During the collapse of the cavity the liquid micro-jet induces a water hammer effect and the resultant water hammer pressure has been estimated by various researchers for different cavitation conditions by numerical simulations (Brujan, 2004; Philipp and Lauterborn, 1998). When this pressure exceeds the yield point of the metal, corrosion occurs. The pressure necessary to disrupt half the population of *Saccharomyces cerevisiae* was reported as 150 MPa by Kelemen and Sharpe (1979). The calculation of this water hammer pressure is complicated due to the limited knowledge of the initial size of the cavity and the probable distance of the cavity from the yeast at any point of time. The importance of these two factors on the water hammer pressure has been demonstrated by Philipp and Lauterborn (1998). Such a water hammer effect in the case of yeast cell disruption could give rise to a point breakage on the cell wall of yeast, because the energy dissipation is focused on a small region by this water jet.

The transmission electron micrographs of yeast subjected to cavitation at a cavitation number of 0.17 (cavitation unit II, 1 % w/v, wet wt, 1000 passes), presented in Figure 5.14 shows point breakage clearly. From a numerical analysis, the water hammer effect was found to be useful for the disintegration of renal calculi, dental tartar or intraocular lens (Brujan, 2004).

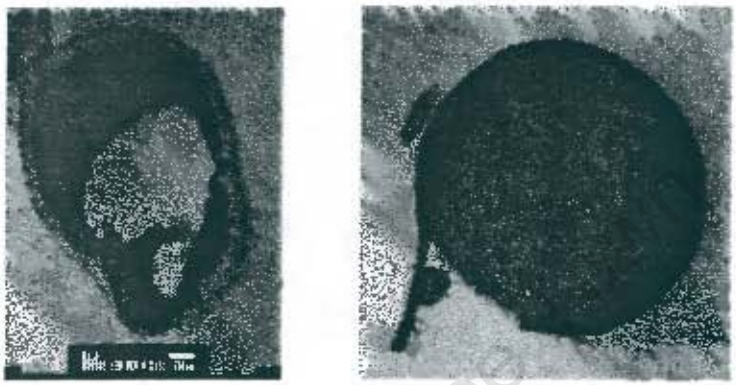

Figure 5.14 Transmission electron micrograph of yeast cells subjected to cavitation (cavitation unit I, cavitation number 0.17, 1 % w/v, wet wt, 1000 passes)

However, the disruption may occur by other mechanisms too, such as the shock wave generated during cavity collapse and the radial wall motion. The shock wave generated by cavity collapse was found to be more important than the water hammer effect in the case of erosion of metal by Philipp and Lauterborn (1998). Hartman and Delgado (2004) used numerical simulations to propose that the mechanical damage to the yeast cell occurs due to the pressure oscillations of greater than 700 MHz and not due to the transient pressure applied. Thus the oscillation of the cavities will lead to a mechanical damage of yeast cell wall. The oscillation of the cavities is responsible for the shock wave generation and radial wall motion. The combination of shock waves and radial wall motion was found to be responsible for the permeabilization of 3T3 mouse cells by Sundaram *et al.* (2003). Shock waves generated by cavitation bubbles were found to be responsible for the permeabilization of female uterus cells for the uptake of fluorescein isothiocyanate (Ohl *et al.*, 2003).

It is postulated that the intensity of these forces generated will depend on the collapsing cavities which in turn depend on the conditions of cavitation. The extent of damage will depend on the strength of the cell and also the size of the cell. The importance of cell to cavity ratio on the extent of damage has been demonstrated by the numerical simulations of a bubble and a simulated blood cell by Chahine (1994, discussed in Section 3.3.2.)

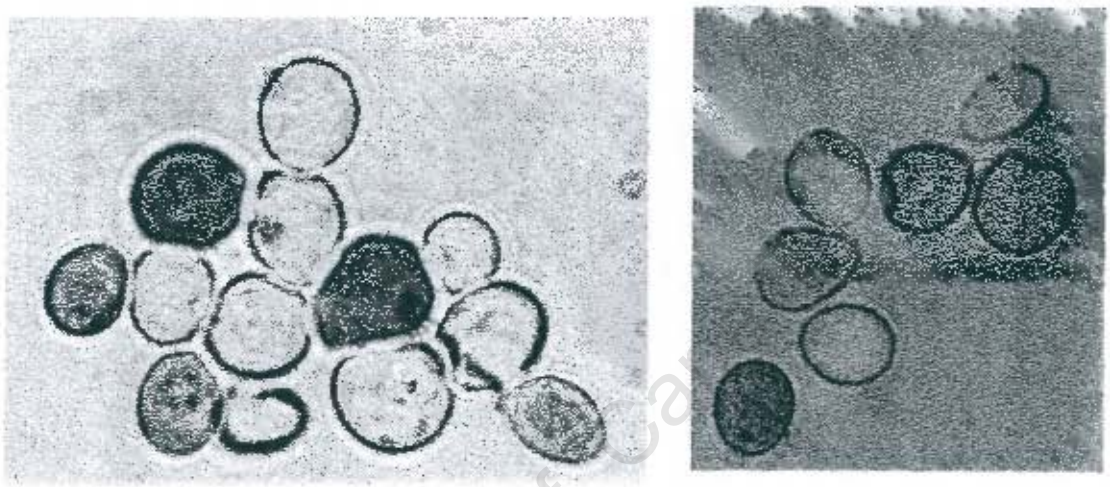

Figure 5.15 Optical microscope images of yeast cells subjected to cavitation (cavitation unit I, cavitation number 0.17, 1 % w/v, wet wt, 1000 passes)

In Table 5.3 and Figure 5.3, the percentage of enzymes released by hydrodynamic cavitation at various cavitation numbers (1 % w/v wet wt, 1000 passes) is compared to the amount of enzyme released using high pressure homogenization (51.7 MPa, 10 passes, 1 % w/v, wet wt). Some 22 % of the release of total soluble protein was achieved at a cavitation number of 0.13 and cell concentration of 0.5%. Since only 22 % of total soluble protein was released the yeast cells were not disrupted completely. The amount of periplasmic protein in *S. cerevisiae* was reported to be 11% (Huang *et al.*, 1991). If permeabilization of the cell wall occurred due to the various forces of cavitation as observed by Ohl *et al.* (2003) and Sundaram *et al.* (2003), then most of the periplasmic constituents could be expected to be released at these disruption conditions from *S. cerevisiae*. However, only 13 % of the periplasmic enzyme α-glucosidase (AG) was released. When yeast cells were permeabilized using EDTA and osmotic shock little protein was released yet the cells were permeabilized to

permit the *in situ* determination of isocitrate dehydrogenase and G6PDH (Crotti *et al.*, 2001). In studying metal corrosion, Philipp and Lauterborn (1998) showed that, instead of one big pit, multiple number of pits were formed on the metal (due to corrosion) when a large number of smaller bubbles collapsed. Hence it can be postulated that a large number of smaller holes might have developed in the yeast cell wall resulting in the release of periplasmic protein but the pore size created might restrict the leaching of macromoleules like α-glucosidase.

The bud scar on the yeast indicates the age of the cell. These bud scars were found to be resistant to digestion by zymolase because of the presence of chitin (Pringle *et al.*, 1979). Hence a distribution of yeast cell strength, related to cell age, may be expected. The damage of cells with relatively weaker cell wall is postulated to have contributed to the smaller amount of cytoplasmic enzymes released (cytoplasmic enzymes G6PDH and ADH released were low: 7 & 5 % of that available respectively at a C_v of 0.13, and 0.5 % w/v, wet wt). Absence of a significant release of cytoplasmic enzyme and absence of the micronization of the yeast cells as seen from electron micrographs presented in Figure 5.14 and optical microscopic images, presented in Figure 5.15, indicate that the cells were not ruptured completely.

Sommer and Lewis (1971) used dithiothreitol (DTT) to release invertase from the cell wall of yeast. DTT has two thiol groups which reduce the disulphide bonds in proteins. The formation of the reactive species such as OH*, H* and H_2O_2 (responsible for oxidation and reduction reactions) during hydrodynamic cavitation can be expected to cause some similar damage to the cell wall. Cavitation was found to be more effective in releasing the cell wall bound enzyme than enzyme from other locations of the cell (Table 5.7). The amount of total soluble proteins and periplasmic enzyme released were lower.

Table 5.7 Release of extracytoplasmic proteins and total soluble protein using orifice plate IIb (cavitation number 0.13)

No. of passes	Cell conc. (% w/v, wet wt)	Soluble protein (mg/g)	α-glucosidase (U/g)	Invertase (U/g)
1000	0.1	21	7	39
2500	0.1	25	7	68
1000	1	15	17	23
2500	1	31	17	73
1000	5	8	6	20

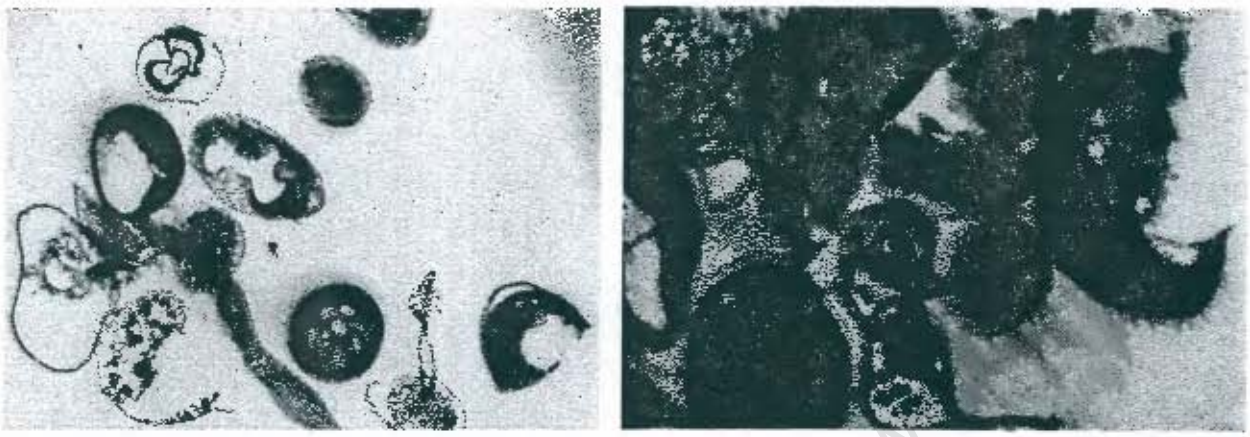

Figure 5.16 Transmission electron micrograph of yeast cells subjected to hydrodynamic cavitation (cavitation unit II, cavitation number 0.13, 1000 passes, 0.1 % w/v, wet wt)

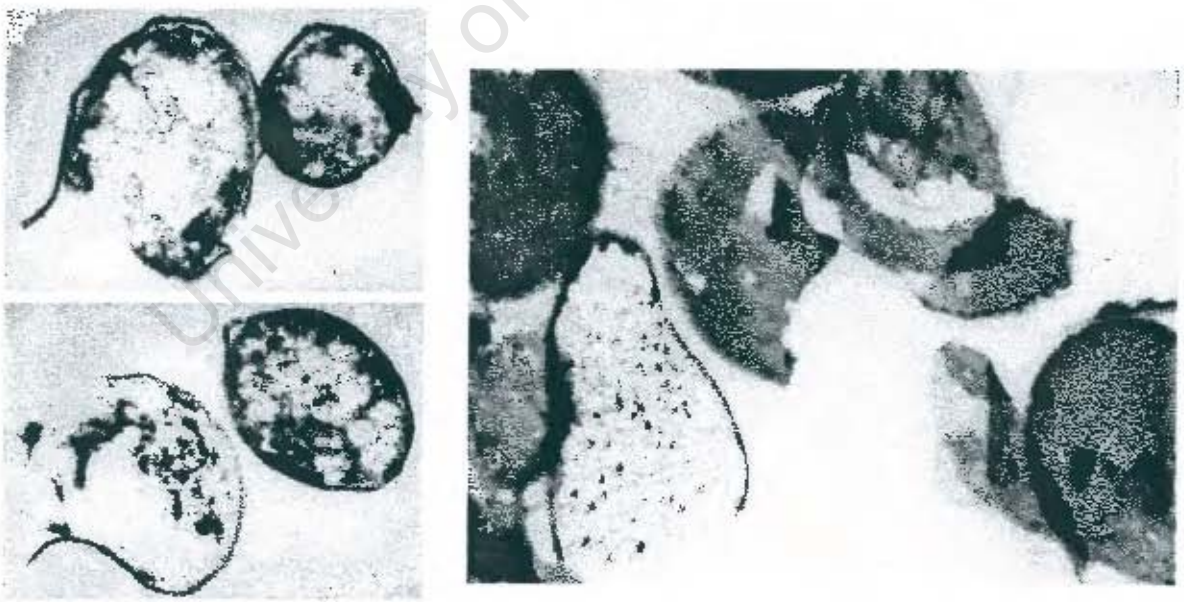

Figure 5.17 Transmission electron micrograph of yeast cells subjected to hydrodynamic cavitation (cavitation unit II, cavitation number 0.13, 1000 passes, 1 % w/v, wet wt)

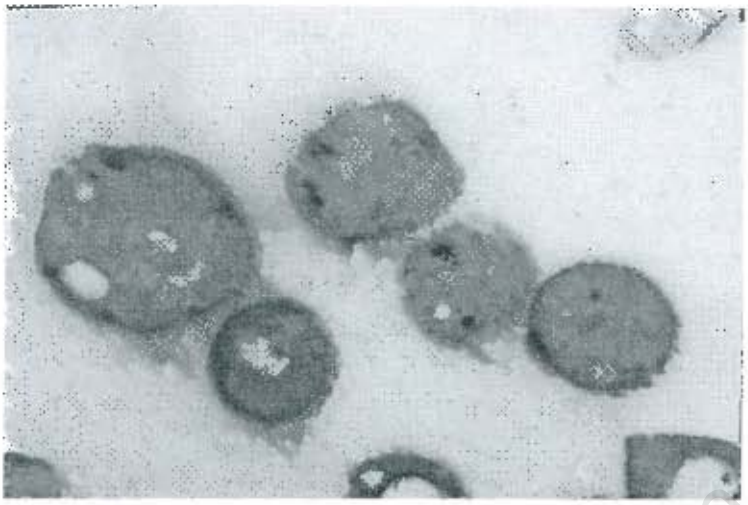

Figure 5.18 Transmission electron micrograph of yeast cells subjected to hydrodynamic cavitation (cavitation unit II, cavitation number 0.13, 1000 passes, 5 % w/v, wet wt)

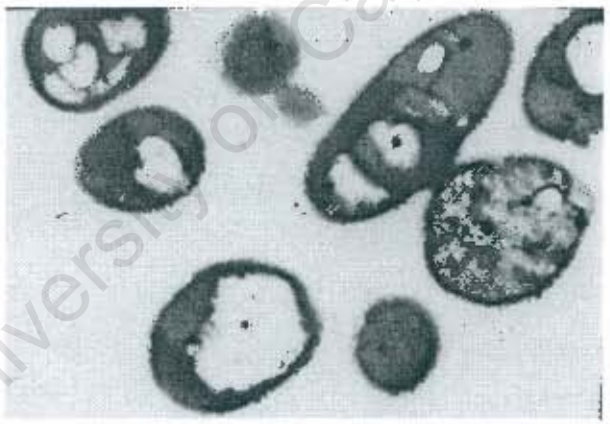

Figure 5.19 Transmission electron micrograph of yeast cells (untreated)

Microscopic studies have also suggested that hydrodynamic cavitation attacks the cell wall at isolated regions leading to a point breakage of the cell (Figures 5.14 & 5.15). The extent of damage on yeast as observed from TEM studies correlates well with the soluble protein and enzyme release. At 0.1 % cell concentration and C_v of 0.13 (1000 passes, cavitation unit II), the extent of breakage of yeast cell appeared higher resulting in the formation few fragments of cell envelope materials. At a 1 % cell

concentration breakage was observed but fragmentation was not obvious. These observations were based on the study of 5 to 10 micrographs at each operating conditions, examples of which are presented in Figures 5.16 and 5.17. The transmission electron micrograph of yeast subjected to hydrodynamic cavitation at cavitation number of 0.13 and 5 % cell concentration appeared intact (1000 passes, cavitation unit II; Figure 5.18). No significant damage of the cell wall could be observed under these conditions correlating well with the poor release of total soluble protein and enzyme fraction (Table 5.7). In Figure 5.19, transmission electron micrographs of untreated yeast cells are presented for comparison.

Further, SDS-PAGE analysis was performed to compare the range of protein molecules that are released by hydrodynamic cavitation to those released by French Press. The supernatant of the cell suspension disrupted by hydrodynamic cavitation was concentrated 5 fold (approximately) prior to its analysis by SDS-PAGE electrophoresis. The samples were run adjacent to the supernatant from cell suspension disrupted in the French Press (20 MPa, 5 passes, 1 % w/v, wet wt). The resultant gel banding pattern is presented in Figure 5.20. The release of proteins as a function of cavitation number may be seen by comparing lane 2 and lane 3 to 8. In particular comparison of lane 7 and 8, in which supernatants prepared following cavitation at C_v of 0.22 and 0.13 respectively are compared, shows that at cavitation number 0.13 more proteins corresponding to 44, 24 and 14.2 kDa were released. No protein bands were observed for the samples from other cavitation numbers (0.92, 0.49, 0.32 and 0.26) owing to limited sensitivity.

Owing to the higher number of proteins of different sizes in the French Press sample, a smear was seen in the region between 36 and 24 and again below 20 kDa. The number of protein bands separated following cavitation of the yeast is less than those obtained following disruption in the French Press. This supports the observation of a reduced release of cytoplasmic compounds. Further, it illustrates that a higher specific activity may be expected for those proteins released by hydrodynamic cavitation allowing easier purification of a specific periplasmic protein or enzyme molecule following disruption by hydrodynamic cavitation.

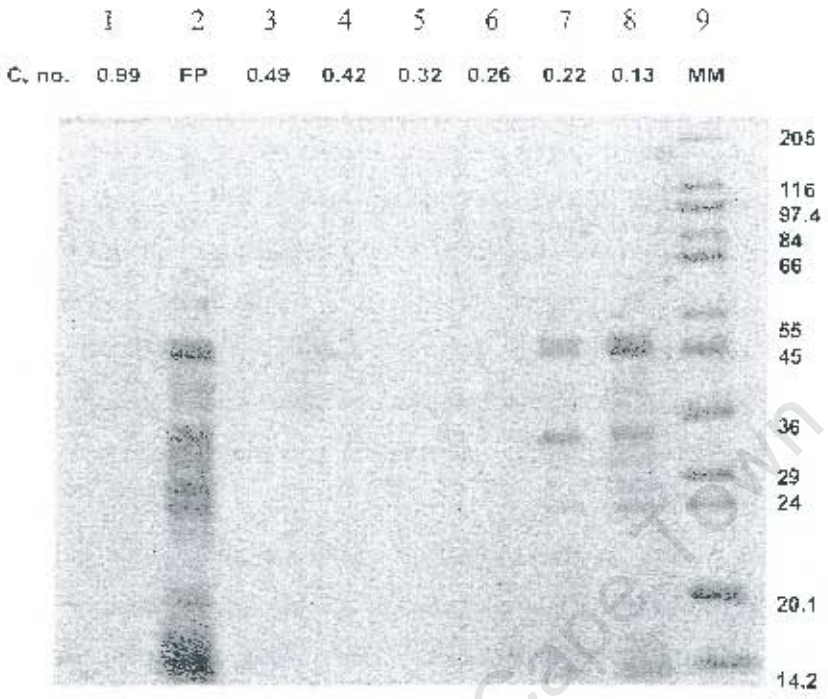

Figure 5.20 SDS-PAGE analysis of supernatants following yeast cell disruption by hydrodynamic cavitation (1 % w/v, 1000 passes) and HPH (51.7 MPa, 1 % w/v, 10 passes) MM- Molecular marker, FP – French Press (20 MPa, 5 passes, 1 % w/v, wet wt)

5.7. Conclusions

The release of total soluble protein and enzymes was found to increase on increasing the intensity of cavitation (at cavitation numbers less than 0.4). Over the range of cavitation number studied an optimum release was observed at cavitation number 0.13 for the release of total soluble protein, α-glucosidase and invertase. The number of cavities generated increases on increasing the intensity of cavitation, but the collapse pressure passes through a maximum with respect to cavitation number due to the opposing influence of cavitation number on its components: number of cavities and collapse pressure of each cavity. Thus the maximum damage occurring at an optimum cavitation number is postulated to be due to the maximum collapse pressure generated at these conditions. The total soluble protein and invertase release trend were found to be similar across the cavitation units I and II.

Of the various yeast cell concentrations studied (0.1 % to 5 % w/v, wet wt), the maximum extent of release of total soluble proteins, α-glucosidase and invertase was found at 0.5 % w/v (wet wt). There exists an optimum cell concentration at which the ratio of number of cavities generated to the number of cells present are optimal. Study of the effect of the number of passes indicated that there was a sustained release of invertase up to 5000 passes through the cavitation zone while the release of proteins and α-glucosidase reached a maximum after 1500 and 800 passes respectively (C_v of 0.13, 1 % w/v, wet wt). The invertase molecule is entrapped in the outer cell wall by the disulphide bonds. The generation of reactive species such as OH* H* and H_2O_2 during cavitation assists to release the entrapped invertase. Thus the combined effects of cavitation both chemical and mechanical result in the sustained release of invertase.

The release of protein and enzymes by hydrodynamic cavitation was found to follow first-order kinetics. The release rate constants of soluble protein and invertase were found to reach a maximum at an optimum cavitation number. Higher quantities of cell wall entrapped invertase were released than the enzymes from the other locations of the cell due to the combined effects of cavitation (chemical and mechanical) by hydrodynamic cavitation. At intense cavitation a large number of cavities were formed but the collapse pressure was lower and hence the cavities collapse less violently. This led to a large number of less intense cavities damaging the cell wall or just loosening the cell wall matrix and releasing the cell wall entrapped invertase enzyme (combined with the chemical effects due to the reactive species generated) to a greater extent than the enzymes from the other locations.

A lower number of protein bands with SDS-PAGE analysis of hydrodynamic cavitation samples compared to samples from French press indicated that a certain degree of preliminary purification can be achieved on cell disruption by hydrodynamic cavitation over disruption with French Press. The extent of damage of the yeast cells as observed by the TEM studies was found to coordinate well with the protein and enzymes released at various cavitation numbers. TEM studies indicated that cavitation damages the yeast cell wall causing discrete openings in the absence of micronisation.

The versatile nature of the hydrodynamic allows a wide range of damage of yeast cell by varying the conditions of cavitation. Higher extent of damage can be achieved by using intense cavitation conditions. From the above results it can be seen that hydrodynamic cavitation can be used for a selective release of cell wall associated proteins. Absence of micronization could ease the subsequent solid-liquid separation following disruption and higher selectivity aids the purification of the protein.

Chapter 6: Hydrodynamic Cavitation of *E. coli*

6.1. Introduction

In this chapter the results of the study of hydrodynamic cavitation for the release of intracellular and periplasmic products from the *Gram-negative* bacteria *E. coli* is presented. The concept of hydrodynamic cavitation and its role in microbial cell disruption is reviewed in Chapter 3. Its effect on the yeast *Saccharomyces cerevisiae* has been discussed in Chapter 5. *E. coli* was selected as a model organism to represent *Gram-negative* bacteria. The cell wall of *Gram-negative* bacteria is about 15-30 nm in thickness. The peptidoglycan network in the cell wall provides the mechanical rigidity to the cell. The cell envelope of *Gram-negative* bacteria is made up of outer cell wall and inner cytoplasmic membrane separated by the periplasm. Thus the cytoplasm can be considered as the inner compartment and periplasm the outer. A selective removal of the outer cell wall or a point breakage on the cell wall could lead to selective leakage of enzymes from the outer compartment (periplasmic enzymes).

The destructive forces generated by hydrodynamic cavitation can be varied by manipulating the operating conditions (Senthil Kumar *et al.*, 2000). It is postulated that milder condition of cavitation can be used to inflict a point breakage on the cell wall and intense conditions can be employed to rupture the cell. The knowledge of the effect of the various operating conditions on hydrodynamic cavitation is therefore essential. The effect of cavitation number, number of passes and physiological state of the biomass varied here through specific growth rate of the bacteria on the disruption by hydrodynamic cavitation, were investigated through quantification of the extent and rate of release of the total soluble protein, acid phosphatase and β-galactosidase.

This chapter is organized first to define the growth conditions for *E. coli* and characterize the biomass produced (Section 6.2). Thereafter results of the effect of cavitation number (Section 6.3) and number of passes (Section 6.4) on cell disruption are presented and

discussed. The kinetics of cell disruption are analysed in Section 6.5. The effect of physiological state of biomass is investigated in Section 6.6 and potential for selective release considered in Section 6.7.

6.2. Production and characterization of *E. coli*

6.2.1. Growth curve

The growth profile of *E. coli,* determined in batch culture as described in Section 4.3.2.1, is shown in Figure 6.1. The maximum specific growth rate was found to be 0.36 hr^{-1}. Exponential growth at μ_{max} 0.36 hr^{-1} was found from 5 to 10 hours in the batch culture.

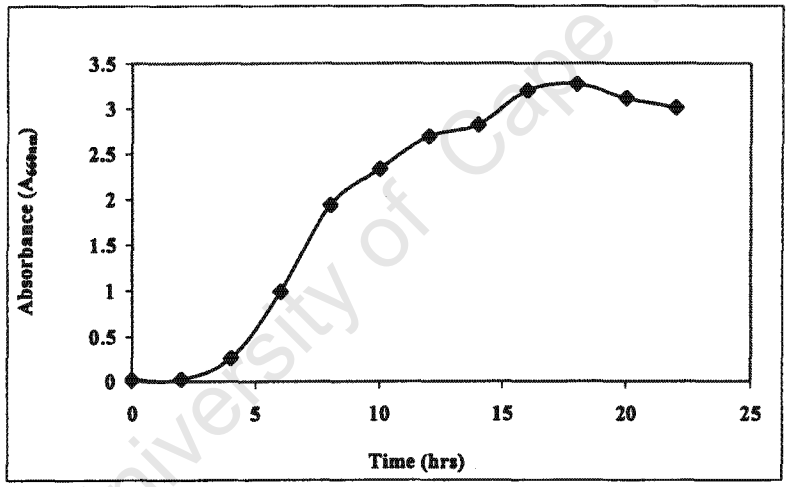

Figure 6.1 Growth curve of *E. coli* in LB medium (37 °C, pH 7.0, 400 rpm)

6.2.2. Continuous culture

A volume of *E. coli* suspension of 18 litres was required for each experiment using hydrodynamic cavitation. For a 0.5 % w/v (wet wt) concentration of suspension, 90 g of *E. coli* cells was required for each experiment. Thus *E. coli* was required in abundant quantities. Hence *E. coli* cells were produced by continuous culture. To study the effect

of growth rate, continuous culture was performed at two different growth rates (0.36 and 0.11 hr^{-1})

For continuous fermentation, 9.5 litres of media was inoculated with a 500 ml inoculum grown on a shake flask (as described in the Section 4.3.2.1). The feed pump was started after 8 hours (when the cells were multiplying at a specific growth rate of 0.36 hr^{-1}) at the rate of 3.6 l/hr and harvest pump was set at the same flow rate, corresponding to a dilution rate of 0.36 hr^{-1}, to obtain *E. coli* culture at a growth rate 0.36 hr^{-1}. To obtain *E. coli* culture of growth rate 0.11 hr^{-1}, the feed was started after 10 hours at which the specific growth rate has decreased to 0.11 hr^{-1}. The feed pump and the harvest pump were set at the speed of 1.1 l/hr, corresponding to a dilution rate of D (= F/V) of 0.11 hr^{-1}. The biomass collected in the fermentor eluent was centrifuged at 6371 g for 8 minutes and re-suspended in phosphate buffer of pH 7.0 (25 mM) at the specified biomass concentration for disruption.

Table 6.1 Amount of soluble protein and enzymes release from *E. coli* by various disruption techniques (0.5 % w/v, wet wt)

Specific growth rate (hr^{-1})	Cell disruption technique	Operating conditions	Total soluble Protein (mg/g)	Acid phosphatase (U/g)	β-galactosidase (U/g)
0.36	French Press	20 MPa, 5 passes	90.7	597	1502
0.11	French Press	20 MPa, 5 passes	97.2	462	1505
Batch (stationary phase)	Osmotic shock	10 minutes	15.5	723	16
Batch (stationary phase)	High pressure homogenization	51.7 MPa, 5 passes	107	1235	1039

Table 6.2 Experiments performed with hydrodynamic cavitation (cavitation unit II, 0.5 % w/v, wet wt, 32 °C, 2500 passes)

Cavitation number	Specific growth rate (hr^{-1})	Enzymes analysed
0.13, 0.17, 0.22, 0.32, 0.49, 0.99	0.36	Total soluble protein, acid phosphatase, β-galactosidase
0.13	0.11	Total soluble protein, acid phosphatase, β-galactosidase

6.2.3. Characterization of biomass

A sample of *E. coli* from each batch was disrupted using French Press or high pressure homogenization to determine the total amount of soluble protein and enzyme available from the cell. The results are presented in Table 6.1. *E. coli* biomass grown at the specific growth rate of 0.36 hr^{-1} was used for the study of the effect of cavitation number and the number of passes across the orifice plate. For osmotic shock release, stationary phase cells cultivated by batch culture were used. The cavitation experiments performed are listed in Table 6.2.

6.3. Effect of cavitation number

The intensity of cavitation defined by the dimensionless parameter called cavitation number has been defined in Section 5.2. Lower cavitation number implies greater cavitational activity. The intensity of cavitation number was varied by using different orifice plates for each cavitation number. Cavitation unit II was used for all the experiments with *E. coli*. The cavitation numbers studied include 0.13, 0.17, 0.22, 0.32, 0.49 and 0.99. Each experiment was carried out for 2500 passes at a cell concentration of 0.5 % w/v (wet wt). The total amount of soluble protein and activity of specific enzymes released from *E. coli* was determined by disruption of a 1 % w/v (wet wt) suspension of *E. coli* cells using the French Press at 20 MPa for 5 passes. The release of total soluble protein, acid phosphatase and β-galactosidase were determined. The amount of the

enzyme released (as percentage compared to the amount released by French press disruption) as a function of the cavitation number is presented in the Figure 6.2. The maximum amount of total soluble protein and activity of enzyme released at each cavitation number is presented in Table 6.3. The detailed data is presented in Appendix E.

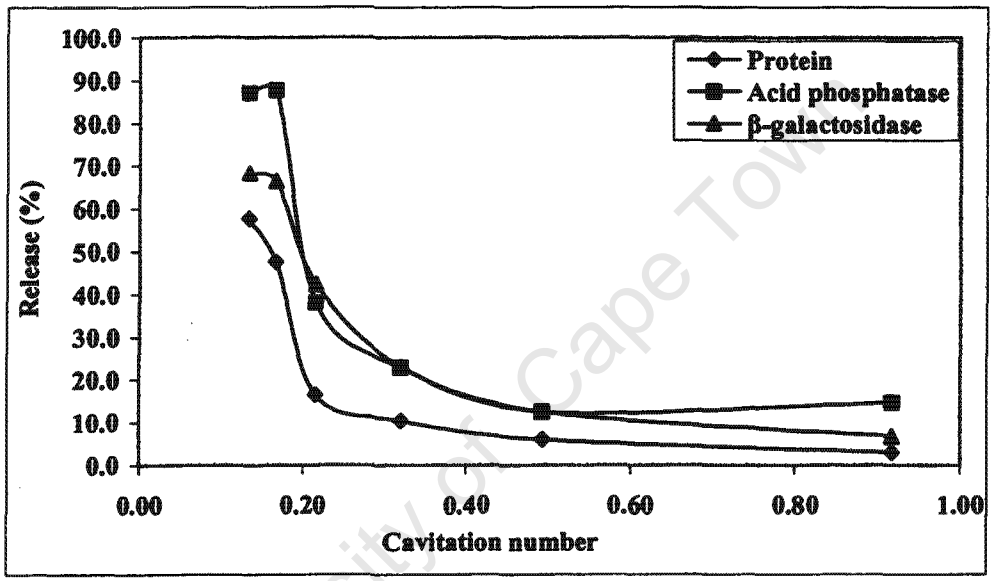

Figure 6.2 Release of total soluble protein and enzymes from *E. coli* by hydrodynamic cavitation (0.5 % w/v wet wt, 2500 passes) as a function of cavitation number expressed as a fraction of the release by French Press (20 MPa, 5 passes, 1 % w/v, wet wt)

It can be seen that the activity of enzymes and total soluble protein released at cavitation numbers greater than 0.4 were less than 15 % of that released by French Press. The release of total soluble protein, acid phosphatase and β-galactosidase increased significantly on decreasing the cavitation number below 0.4. On increasing the intensity of cavitation from 0.22 to 0.17 the release of total soluble protein increased from 17 to 48 % and β-galactosidase activity released increased from 43 to 68 % while acid phosphatase activity in the supernatant increased from 38 to 88 %. The increase in the extent of release was found to be highest for the periplasmic enzyme. Multiple numbers

of pits were seen to form instead of one when a higher number of smaller bubbles collapsed near a metal surface (Philipp and Lauterborn, 1998). By comparison it can be postulated that the formation of many number of smaller punctures on the outer cell wall of bacteria facilitates periplasmic enzymes, such as acid phosphatase leaking out of the cell.

Cavitation number	Total soluble proteins		Acid phosphatase		β-galactosidase	
	(mg/g)	%	(U/g)	%	(U/g)	%
0.13	52.5	58	520	87	1026	68
0.17	43.2	48	524	88	999	67
0.22	15.0	17	227	38	638	42
0.32	9.47	10	137	23	348	23
0.49	5.48	6	75	13	188	13
0.92	2.79	3	88	15	102	6.8
French press	90.7		597		1502	

Table 6.3 Release of total soluble protein and enzymes from *E. coli* by hydrodynamic cavitation (0.5 % w/v wet wt, 2500 passes) as a function of cavitation number expressed as a fraction of the release by French Press (20 MPa, 5 passes, 1 % w/v, wet wt)

When changing the cavitation number from 0.17 to 0.13 the release of acid phosphatase and β-galactosidase reached a constant value of 88 and 67 % respectively, while the release of total soluble protein was found to be approaching a constant value, as illustrated by its decreased dependence on cavitation number (Figure 6.2). Although the extent of protein release did not reach a constant value, the dependence of the release on increasing the intensity of cavitation (decreasing cavitation number from 0.17 to 0.13) decreases, indicating approach towards the maximum. On increasing the intensity of cavitation, the number of cavities formed increases (Oba *et al.*, 1986), but the collapse pressure of the cavities decreases (Gogate and Pandit, 2000). Thus the total collapse pressure (product of the number of cavities and collapse pressure of each cavity) is optimum at a particular cavitation number. Further, the cavitation efficiency decreases

above a critical cavity density owing to the decreased interaction between cavity and cell. At an optimum cavity to cell ratio, the permeabilization of human prostrate cells for the uptake of calcein by ultrasound was at its maximum (Guzman *et al.*, 2003). Hence at an optimal cavitation number the effects of cavitation are highest. Thus the maximum release seen between the cavitation numbers of 0.13 and 0.17 represents the optimum cavitation number for the release of acid phosphatase and β-galactosidase. Such an optimum cavitation number for the decomposition of potassium iodide was noted by Senthil Kumar *et al.* (2000). When varying cavitation number over the range 0.17 to 0.30, maximum decomposition was found to be at cavitation number of 0.20. The optimum cavitation number of soluble protein release for *E. coli* is predicted to be found slightly below C_v of 0.13.

6.4. Effect of number of passes

The effect of number of passes through the cavitation zone on the disruption of *E. coli* was investigated at the cavitation number of 0.13 and a 0.5 % w/v (wet wt) cell concentration using cavitation unit II. Samples were taken at equal intervals and the amounts of the total soluble protein as well as the activity of acid phosphatase and β-galactosidase released were determined. The percentage of the total soluble protein and enzymes released (compared to the release by French Press disruption) as a function of the number of passes is presented in the Figure 6.3.

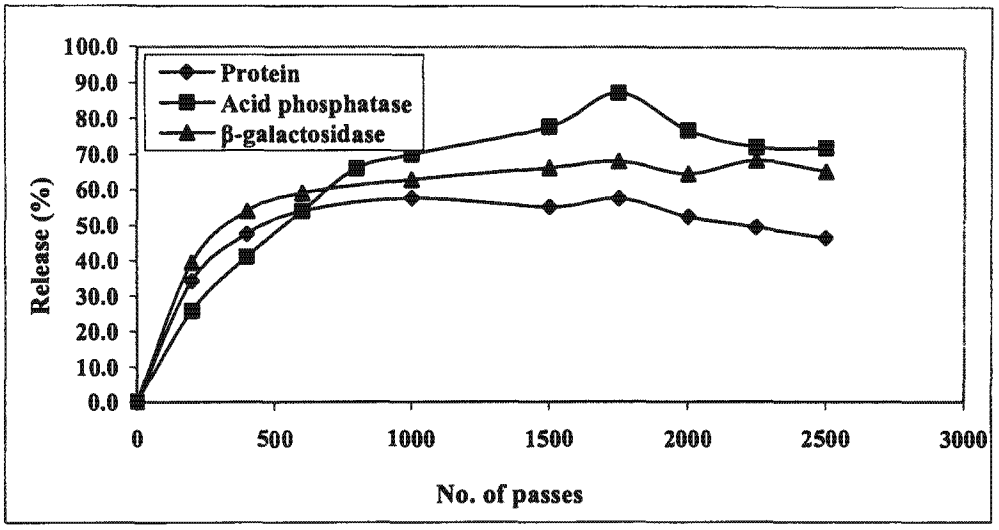

Figure 6.3 Release of total soluble protein and enzymes from *E. coli* by hydrodynamic cavitation (cavitation number 0.13, 0.5 % w/v, wet wt) as a function of number of passes compared to the release by French Press (20 MPa, 5 passes, 1 % w/v, wet wt)

It is clearly seen that the release of total soluble protein and activity of β-galactosidase released increase up to approximately 600 passes and asymptote on further increase in the number of passes. A maximum release of 54 % for total soluble protein and 59 % for β-galactosidase activity were observed. The release of the periplasmic enzyme acid phosphatase was found to increase rapidly with an increase in the number of passes, going up to 800 passes. Further increase in the number of passes resulted in a reduced dependence on the number of passes.

The effect of cavitation with increasing number of passes on *E. coli* disruption is postulated to occur through three stages of cell disruption represented schematically in the Figure 6.4. In the first stage, the mechanical effects of cavitation inflict pores on the outer cell wall releasing a portion of the periplasmic enzymes and proteins. In the second stage the mechanical effects combined with chemical effects (described in Section 3.3.1.1) can now reach the inner cytoplasmic membrane releasing some cytoplasmic products. In the third stage, on sustaining the exposure of the cells to cavitation more effects can be observed on the outer cell wall due of its greater accessibility to the cavitation than the inner membrane. Increased exposure of the *E. coli* cells to the

cavitation zone weakens the cell wall due to fatigue resulting from repeated exposure to oscillating cavities and the collapse of the cavities closer to the cell wall. Hence the third stage of disruption occurring after 600 passes resulted in the sustained increase in the release of periplasmic acid phosphatase.

The effect of number of passes on the release of proteins and enzymes was also observed from the results of SDS-PAGE analysis. The result of the SDS-PAGE analysis is shown in the Figure 6.6. Comparing lanes 3 (2500 passes), 9 (1000 passes) and 10 (100 passes) at cavitation conditions of C_v of 0.13, 0.5 % w/v (wet wt) cell concentration, three prominent protein bands could be observed between the region 24 kDa and 20.1 kDa and one between the 14.2 and 20.1 kDa for the 2500 passes sample (0.13 cavitation number, 0.5 % w/v, wet wt). The corresponding protein bands were not prominent with 100 passes and 1000 passes samples (0.13 cavitation number, 0.5 % w/v, wet wt). The same protein bands were not seen for the samples that were collected at the less intense cavitation conditions (cavitation numbers 0.92, 0.49, 0.32 and 0.22; 0.5 % w/v, wet wt, 2500 passes). Thus intense cavitations and higher number of passes releases greater number of smaller molecular weight proteins.

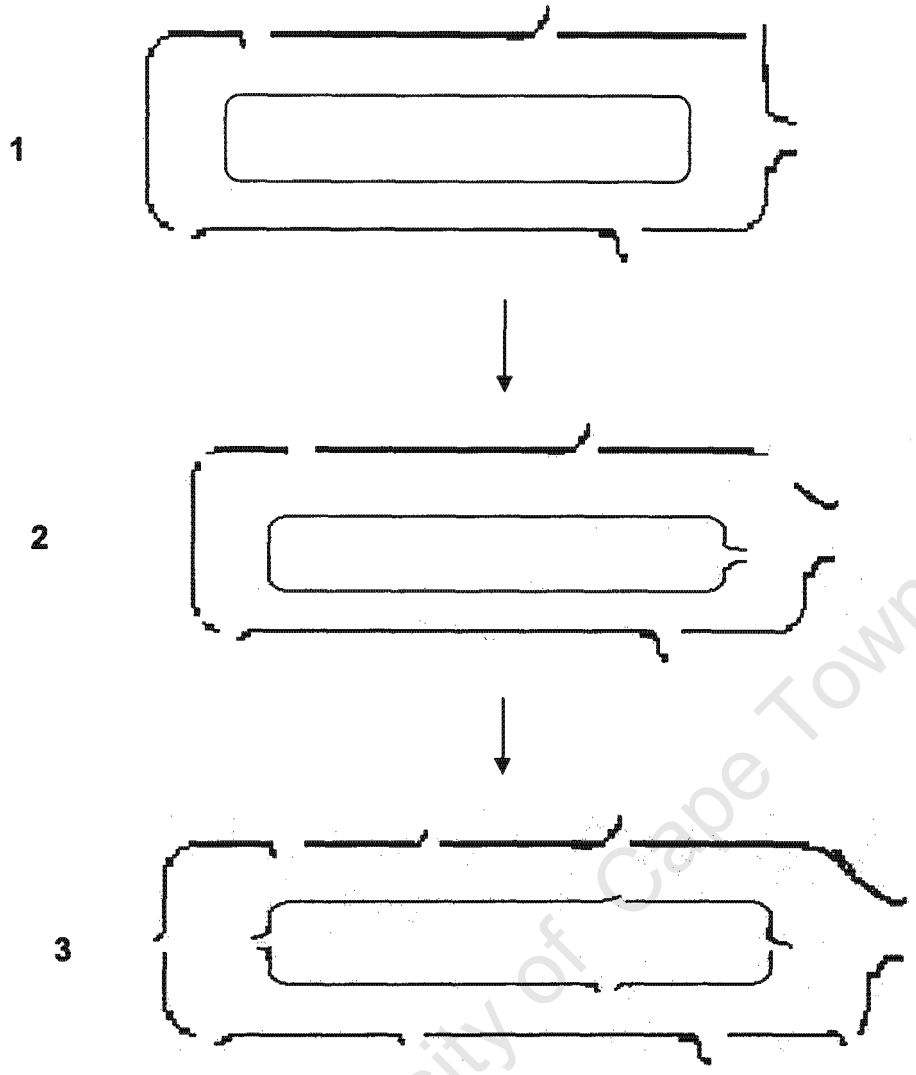

Figure 6.4 Schematic representation of the postulated stages of cell disruption by hydrodynamic cavitation

6.5. Release kinetics

Cell disruption was found to follow first order kinetics during the disruption of *E. coli* using the high speed bead mill (Woodrow and Quirk, 1982), the high pressure homogenizer (Gray *et al*, 1972) and on ultrasonication [Kuboi *et al*., 1995]. The release profile of total soluble protein and enzymes with respect to number of passes through the cavitation zone given in Figure 6.3 suggests that the release follows first-order kinetics. To assess the ability of the first order disruption rate law to describe cell breakage,

Equation 6.1 was used to calculate the release rate constant for the release of total soluble protein, acid phosphatase and β-galactosidase:

$$\ln\left(R_i/(R_i - R)\right) = LnD^{-1} = kN \qquad 6.1$$

where 'R' is the amount of protein or enzyme released after 'N' passes and 'R_i' is the maximum amount of proteins or enzymes available for release using the method of disruption and operating conditions of interest (Scholtz-Brown, 1989). 'R_i' was used instead of 'R_m' the maximum amount of protein or enzymes as estimated by French Press or high pressure homogenization, as complete release of all soluble intracellular proteins was not achieved under the current conditions of hydrodynamic cavitation. The maximum amount of proteins and enzymes released with each experiment (R_i) was used for the calculation for the release rate. The value of Ln ($R_i/(R_i-R)$) represented as Ln D^{-1} was plotted against the number of passes through the cavitation zone. The kinetic analysis is shown in Appendix C (C.1 – C.3) for the disruption of cells cultivated under the growth rate of 0.36 hr^{-1} at the cavitation number of 0.13, 0.17, 0.22 and 0.32 using 0.5 % cell concentrations. The data were well described by first order release kinetics and, in general, a regression coefficient greater than 0.95 resulted. The R_i values, release rate constant (k) and the regression coefficients (R^2) obtained are given in the Table 6.4. The effect of cavitation number on both the extent and the release rate is presented in Figure 6.5.

Table 6.4 Release rate constant (k), regression coefficient (R^2), maximum enzyme or protein released (R_i) for various cavitation numbers, obtained by the kinetic analysis of the cell disruption by hydrodynamic cavitation (μ_{max} 0.36 h^{-1}, cavitation unit II, 2500 passes, 0.5 % w/v, wet wt)

	Total soluble proteins			Acid phosphatase			β-galactosidase		
Cavitation number	R_i (mg/g)	k x 10^{-3}	R^2	R_i (U/g)	k x 10^{-3}	R^2	R_i (U/g)	k x 10^{-3}	R^2
0.13	52.4	4.6	0.99	520	1.7	0.99	1026	2.2	0.95
0.17	-	-	-	524	2.0	0.97	999	3.0	0.98
0.22	15	3.8	0.97	227	1.3	0.98	638	1.3	0.94
0.32	9.47	1.3	0.99	137	1.0	0.94	348	1.1	0.90

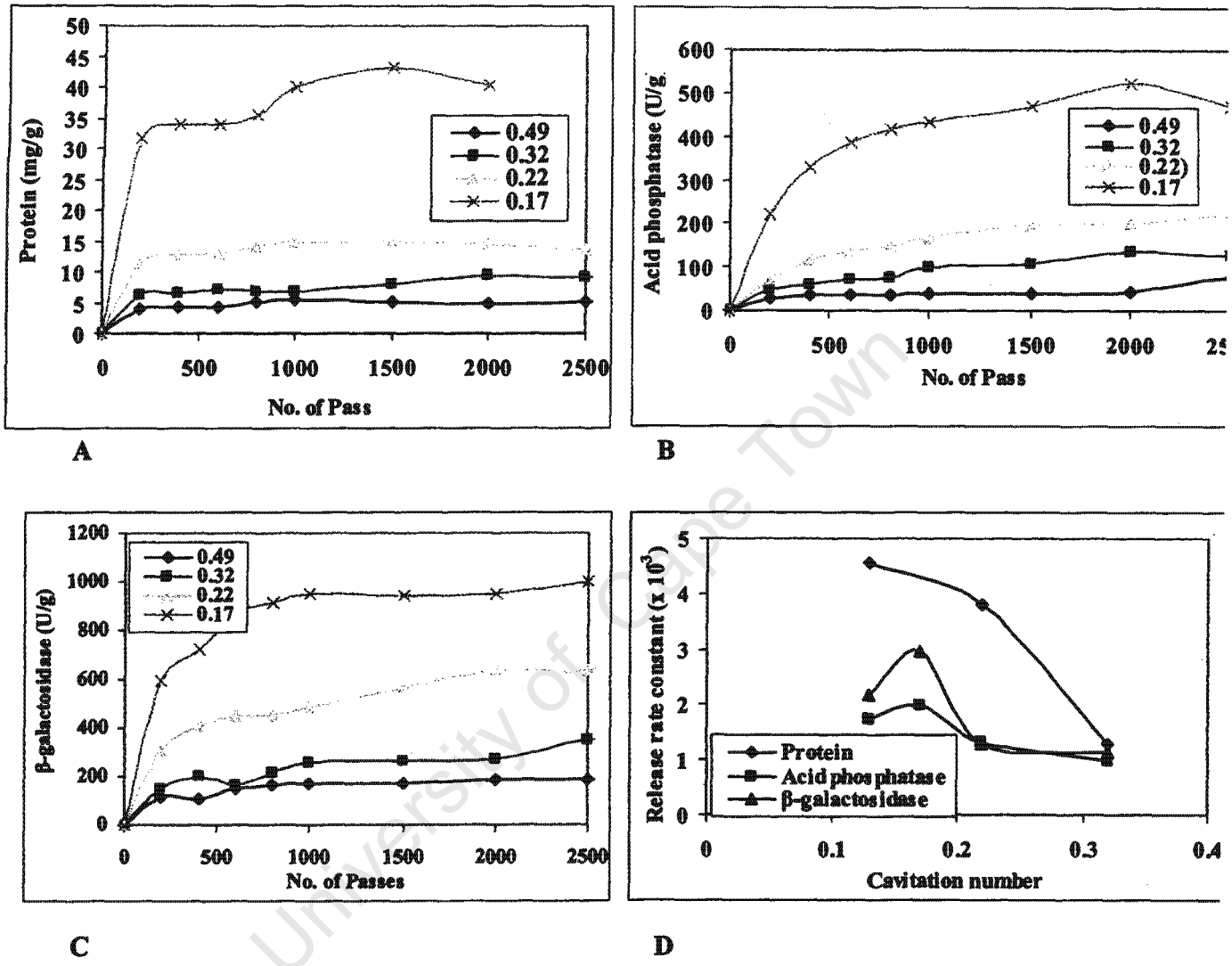

Figure 6.5 Release of soluble protein and marker enzymes as a function of number of passes (A, B, C) at various cavitation numbers, (D) effect of cavitation number on the release rates of soluble protein and enzymes by hydrodynamic cavitation (unit II, 2500 passes, 0.5 % w/v, wet wt)

As observed with yeast disruption for protein release, an optimum cavitation number at which the release rate constant of an enzyme is at its maximum was observed for the release of acid phosphatase and β-galactosidase from *E. coli*. The highest release rate constant was obtained with cavitation number 0.17. Here the release rate constants were 2.0×10^{-3} and 3.0×10^{-3} for acid phosphatase and β-galactosidase respectively. Thus at a

cavitation number of 0.17 the number of cavities generated and the number of *E. coli* cells present was at an optimum proportion resulting in the highest release rate of acid phosphatase and β-galactosidase. Such an optimum cell-to-cavity ratio needed for the effective permeabilization of human prostrate cells for the uptake of calcein by ultrasound was noted by Guzman *et al.* (2003). The release rate constant of total soluble protein was found to increase with decreasing cavitation number (i.e. on increasing the intensity of cavitation). Though it did not reach a maximum the decreasing dependence of the release rate constant on cavitation number on approaching a C_v of 0.13 suggested approach toward an optimum (similar to that noted in the release trend; Figure 6.3).

Over a large number of passes, the rate of release was found to be higher in the initial stages of cell disruption (Figure C1 – C5: Appendix C). The reduction in the release rates on increasing the number of passes is postulated to be due to the degassing effect (Senthil Kumar *et al.*, 2000). The presence of dissolves gases reduces the threshold pressure necessary for cavitation resulting in the increase in the number of cavities generated. On increasing the number of passes, the dissolved gases from the system is released and its effect on cavitation reduces resulting in the reduced rate of cell disruption.

6.6. Effect of growth rate

Microbial cells in the rapidly growing phase i.e. that are cultivated at a higher growth rate have been reported to disrupt more easily than stationary phase or slow growing cells (Baldwin and Robinson, 1993; Engler and Robinson, 1981; Gray *et al.*, 1972; Harrison *et al.*, 1990; Nossal and Heppel, 1966; Sauer *et al.*, 1989). In this current study, the effect of two different growth rates (0.36 hr^{-1} and 0.11 hr^{-1}) of *E. coli* on the disruption by hydrodynamic cavitation at a cavitation number of 0.13 and a cell concentration of 0.5 % w/v (wet wt) over 2500 passes was investigated. Samples were taken for the determination of total soluble protein, acid phosphatase and β-galactosidase. The release rate constants were calculated as described in Section 6.4. The straight lines fitted to calculate the release rates are shown in the Appendix C (C.4 – C.6). The 'R_i' values, the release rate constants (k) and the regression coefficient (R^2) obtained, are presented in Table 6.5.

It can be seen that the release rate constant for soluble proteins was higher (4.6×10^{-3}) on subjecting the rapidly growing *E. coli* (μ_{max} of 0.36 hr^{-1}) than that obtained for *E. coli* μ_{max} of 0.11 hr^{-1} (1.8×10^{-3}) by a factor of 2.5. The release rate constant of β-galactosidase was slightly higher for the rapidly growing cells (μ_{max} of 0.36; $k = 2.2 \times 10^{-3}$) than slower growing cells (μ_{max} of 0.11; $k = 1.96 \times 10^{-3}$).

Table 6.5 Release rate constant (k), maximum enzyme or protein released (R_i) and regression coefficient values (R^2) for various cavitation numbers, obtained by the kinetic analysis of the cell disruption by hydrodynamic cavitation as a function of specific growth rate (μ_{max} 0.36 h^{-1}, Unit II, 2500 passes, 0.5 % w/v, wet wt)

Growth rate (hr^{-1})	Total soluble protein			Acid phosphatase			β-galactosidase		
	R_i	k x 10^{-3}	R^2	R_i	k x 10^{-3}	R^2	R_i	k x 10^{-3}	R^2
0.36	52.4	4.6	0.99	520	1.7	0.99	1026	2.2	0.95
0.11	65.8	1.9	0.96	280	1.7	0.99	1101	2.0	0.96

The slower growing cells reinforce the cell wall by adding more and more of peptidoglycan, unlike the rapidly growing cells and hence these cell walls are stronger (Engler and Robinson, 1981; Sauer *et al.*, 1989; Harrison *et al.*, 1990; Nossal and Heppel, 1961; Baldwin and Robinson, 1993; Keshavarz *et al.*, 1990). The disruption rate constant reported by Harrison *et al.* (1990) for exponential phase cells was lower than cells from stationary phase. Though these results appear contradictory, the effective 'K' calculated as in Section 1.2.2.2 and presented in Figure 2.4, 2.5 and 2.6 clearly demonstrates that the faster grown cells are disrupted easily.

The results from the current study were compared with that from literature in Table 6.6. The cells that were grown rapidly (higher growth rate) can be disrupted easily and hence the release rate of protein obtained with 0.36 hr^{-1} cells (4.6×10^{-3}) was higher than obtained with cells grown at 0.11 hr^{-1} (1.8×10^{-3}). The results from the current study is in accordance with the literature.

Table 6.6 Comparison of the effect of growth rate on cell disruption by hydrodynamic cavitation with literature

Microorganism/ Disruption method	Growth rate/Culture history	Results/Comments		Reference
Alcaligenes eutropus/HPH		Disruption rate constant, k MPa^{-a}	Pressure exponent, 'a'	Harrison *et al.*, 1990
	Exponential	1.35×10^{-5}	3.08	
	Late exponential	1.83×10^{-5}	2.80	
	Early stationary	1.18×10^{-3}	1.69	
	Late stationary	2.16×10^{-3}	1.59	
E .coli / HPH	Higher growth rate vs slower growth rate of recombinant and non-recombinant cells	Higher specific growth rate cells disrupt readily than the slower growing cells		Sauer *et al.*, 1989
C. utilis/HPH	Growth rate (hr^{-1})	Disruption rate constant, k (MPa^{-a})	Pressure exponent, 'a'	Engler and Robinson, 1981
	0.5	8.78×10^{-4}	1.17	
	0.1	8.52×10^{-9}	1.77	
E. coli / HPH	Cells grown on complex media (yeast extract, glycerol and salts) vs defined media (glycerol and salts)	Release rate of β-galactosidase was lower for cells grown on complex media compared to cells grown on defined media		Gray *et al.*, 1972
E.coli / Ultrasonication	Stationary phase cells vs early exponential growth phase cells	$1/K_c$ values for *E. coli* disrupted by ultrasonication was about 70% greater for sationary phase cells than that of early exponential growth phase cells		Kuboi *et al.*, 1995
E.coli / Hydrodynamic cavitation	Growth rate	Release rate constant of total soluble protein		Our study
	0.36 hr^{-1}	4.6×10^{-3}		
	0.11 hr^{-1}	1.8×10^{-3}		

$1/K_c$ value is the inverse of the generalized disruption rate constant, which indicates the amount of energy needed per unit decrease in cell concentration and is considered to depend on the cell strength of the individual cell and the magnitude of the disruptive stress

Table 6.7 Extent of disruption at different growth rates achieved using hydrodynamic cavitation (cavitation unit II, cavitation number 0.13, 0.5 % w/v, wet wt, 2500 passes) compared to the release by French Press (20 MPa, 1 % w/v, wet wt, 5 passes)

Growth rate (hr^{-1})	Cell disruption technique	Proteins		Acid phosphatase		β-galactosidase	
		mg/g	%	U/g	%	U/g	%
0.36	Hydrodynamic cavitation	52.4	58	520	87	1026	68
0.36	French Press	90.7		597		1502	
0.11	Hydrodynamic cavitation	65.8	68	280	61	1101	73
0.11	French Press	97.2		462		1505	

Comparing the percentage of total soluble proteins and enzymes released at different growth rates to the total amount available for release (determined by French press disruption, 1% w/v, wet wt, 20 MPa, 5 passes; Table 6.7) a significantly higher amount of acid phosphatase could be released from the faster grown cells grown (μ_{max} of 0.36 hr^{-1}) than slower grown cells (μ_{max} of 0.11 hr^{-1}; 87 % as opposed to 61 %). This is because of the weaker cell walls of *E. coli* (as explained above) that was grown at higher specific growth rate (0.36 hr^{-1}) than the cells that were grown at the specific growth rate of 0.11 hr^{-1}.

6.7. Selectivity

The selectivity that can be achieved with any particular disruption technique of cell disruption can be assessed by studying the release profile of enzymes from various locations of the cell. The enzyme acid phosphatase was selected as periplasmic marker and β-galactosidase was selected as cytoplasmic marker to study selective protein release from *E. coli* using hydrodynamic cavitation. The damage of the cell by hydrodynamic cavitation postulated in Figure 6.4 could be expected to release periplasmic enzymes at high purity. Osmotic shock method of release has been reported to be highly selective for the release of periplasmic enzymes in several studies (Anraku and Heppel, 1967; Dvorak

et al., 1967; Fonseca and Cabral, 2000; Malamy and Horecker, 1964). The literature available on osmotic shock of *E. coli* is presented in Table 6.8. In all cases, the release of periplasmic enzyme was 60 % or greater while the release of total soluble protein did not exceed 25 %. Hence the ratios of extent of periplasmic protein to total soluble protein released were in the range of 2.7 to 7.0 fold.

Table 6.8 Comparison of the results of the osmotic shock release of total soluble protein and enzymes from *E. coli* with the literature

Total soluble protein released	Enzymes studied	Extent of enzyme released	Reference
12.87 %	Acid phosphatase (periplasmic)	90 %	Dvorak *et al*, 1967[a]
-	Acid phosphatase (periplasmic)	90 %	Anraku and Heppel, 1967[a]
	β-galactosidase (cytoplasmic)	< 2 %	
25 %	Penicillin acylase (periplasmic)	67 %	Fonseca and Cabral 2000[a]
14.5 %	Acid phosphatase (periplasmic)	59 %	Our study[b]

[a] Total proteins and enzyme release by sonication
[b] Total proteins and enzyme release by high pressure homogenization

In the current research the disruption of *E. coli* cells by hydrodynamic cavitation, French Press, high pressure homogenization, osmotic shock and EDTA treatment were studied to compare the selectivity obtained with various cell disruption techniques. *E. coli* cells (stationary phase) cultivated by batch culture were subjected to osmotic shock and EDTA treatment as described in the Materials and Methods Sections 4.4.2.3 and 4.4.2.4. The total amount of these enzymes available for release was determined by high pressure homogenization of 1 % w/v, (wet wt) *E. coli* cells at 51.7 MPa for 5 passes. The experimental results are presented in Table 6.9. It was found that EDTA treatment released only 10 % of periplasmic acid phosphatase, while osmotic shock released 59 % of acid phosphatase. Some 2.7 % of the total soluble protein was released by EDTA treatment while 15 % was released by osmotic shock. The acid phosphatase release from

the periplasm of *E. coli* by osmotic shock was reported to be 90 % by Dvorak *et al.* (1967) and Anraku and Heppel (1967). Similarly 67 % of periplasmic penicillin ayclase (Fonseca and Cabral, 2000) were released from *E. coli* by osmotic shock.

Table 6.9 Soluble protein and enzyme release by osmotic shock release (method described in section 4.4.2.3) compared with the high pressure homogenization (51.7 MPa, 5 passes, 1 % w/v, wet wt)

Cell disruption Technique	Total soluble protein		Acid phosphatase		β-galactosidase	
	mg/g	%	U/g	%	U/g	%
EDTA treatment	2.9	2.7	39	3	11	1.1
Osmotic shock	15.4	14.5	723	59	16	1.5
High pressure homogenization	106	100	1235	100	1038	100

The specific activity (defined as the activities of enzyme released per milligram of protein released) of acid phosphatase and β-galactosidase released by hydrodynamic cavitation, EDTA treatment and osmotic shock is compared with that obtained by high pressure homogenization and French Press disruption in Table 6.10. The extent of release is also included. The optimum release of acid phosphatase by hydrodynamic cavitation occurred at 0.17 (0.5 % w/v, wet wt). The specific activity of acid phosphatase obtained under these hydrodynamic cavitations was approximately 4 times lower than obtained with osmotic shock. The extent of release of acid phosphatase was 88 % with hydrodynamic cavitation at a cavitation number of 0.17 compared to only 59 % with osmotic shock. On comparing the release of acid phosphatase under these cavitation conditions with high pressure homogenization and French Press method of disruption a similar specific activity was obtained. The purity of acid phosphatase was higher by hydrodynamic cavitation at cavitation numbers of 0.17 and above. A compromise in extent of release with increasing purity was observed with increasing cavitation number.

Table 6.10 Comparison of the specific activity of acid phosphatase and β-galactosidase released by various disruption techniques (Hydrodynamic

cavitation: cavitation unit II, μ_{max} of 0.36 hr^{-1}, 0.5 % w/v, wet wt, 2500 passes; French Press: 20 MPa, 1 % w/v, wet wt, 5 passes; high pressure homogenization: 51.7 MPa, 1 % w/v, wet wt, 5 passes, osmotic shock and EDTA treatment described in the Section 4.4.2.3

Cavitation number	Specific activity of acid phosphatase (U/mg)	Extent of release (%)	Specific activity of β-galactosidase (U/mg)	Extent of release (%)
0.13	10	87	20	68
0.17	12	88	23	67
0.22	15	38	43	42
0.32	14	23	37	23
0.49	14	13	34	13
0.92	31	15	36	7
French Press	7		17	
EDTA treatment	13	3	4	1.1
Osmotic shock	47	59	1	1.5
High pressure homogenization	12		10	

The specific activity of β-galactosidase was found to be higher with hydrodynamic cavitation than with any of the other methods of disruption studied. The maximum release of β-galactosidase of 67 % corresponded with a specific activity of 23 U/mg at the cavitation number of 0.17. The maximum specific activity obtained of 43 U/mg corresponded to an extent of release of 42 % at a cavitation number of 0.22.

Higher recovery of enzymes using hydrodynamic cavitation coupled with higher selectivity can be illustrated with the results from the experiments with cavitation number 0.17, when 88 % of acid phosphatase and 67 % of β-galactosidase could be released while maintaining the release of total soluble proteins to a lower level (only 48 % was released). The absence of total cell disruption is indicated by the reduced amount of total soluble protein release. To further validate the selectivity obtained with hydrodynamic cavitation SDS-PAGE analysis was performed on supernatants following disruption to provide the profile of the proteins released by various disruption methods.

For SDS-PAGE analysis samples from hydrodynamic cavitation were concentrated approximately 5 times while osmotic shock samples were concentrated 7 fold prior to loading on the gel. Samples from disruption in the French Press and molecular markers (6.5 to 205 KDa range) were prepared as described in the Section 4.5.4 prior to loading onto the gel. The operating parameters for the electrophoresis separation are detailed in Section 4.5.4. The protein banding patterns obtained are presented in Figure 6.6. It can be observed that there is no significant difference in the size of the proteins molecules released by hydrodynamic cavitation between various cavitation numbers 0.13 (2500 passes sample, Lane 3) and 0.17 (Lane 2). But these differ significantly from isolate obtained at a cavitation number of 0.32 (Lane 8) between the region 55 and 45 kDa, and the region between 20 and 24 kDa, indicating the higher number of proteins of different sizes being released at these intense cavitation conditions. Only a single band corresponding to 116 kDa was observed with the samples from cavitation number 0.92 and 0.49. French Press isolates yielded a higher number of bands than samples of cavitation indicating release of a larger number of proteins. Comparing the isolate prepared following 2500 passes (Lane 3) through the cavitation unit II at cavitation number of 0.13 with the French Press isolate (Lane 7) a larger number of protein bands were observed (two in the regions between 205 and 116 kDa, 84 and 66 kDa, 20.1 and 24 kDa and one in the region between 66 and 55 kDa).

The samples from osmotic shock release and EDTA treatment are compared with the sample from the high pressure homogenizer (Figure 6.7). It can be seen that the EDTA treatment does not release proteins to any significant extent and osmotic shock released fewer proteins than high pressure homogenization. Similar results during the SDS-PAGE analysis of osmotically shocked *E. coli* cells for the release of periplasmic penicillin acylase was observed by Fonseca and Cabral (2002). The higher number of protein bands seen with the French Press and high pressure homogenizer samples compared to hydrodynamic cavitation indicates the selectivity obtained with hydrodynamic cavitation.

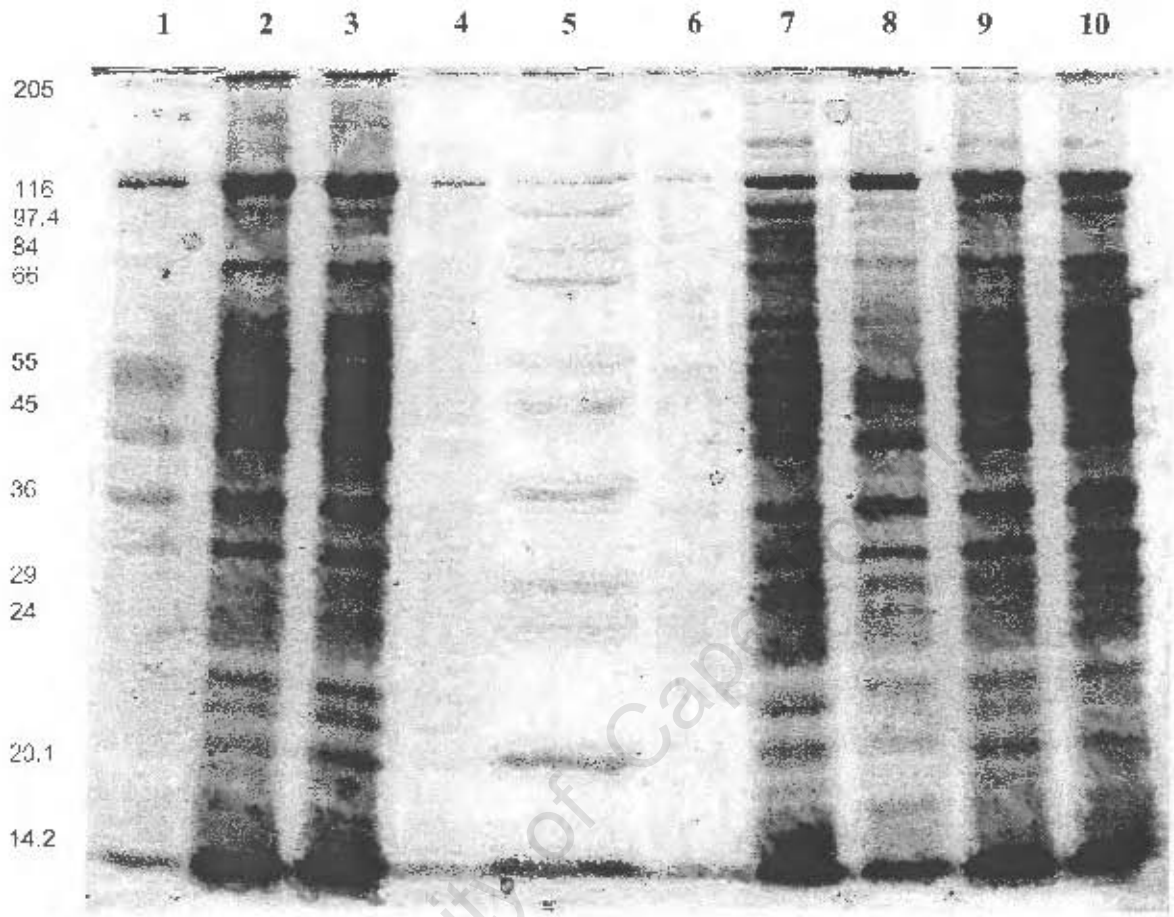

Figure 6.6 SDS-PAGE analysis of samples from hydrodynamic cavitation (0.5 % w/v, 2500 passes unless specified) and French press (20 MPa, 5 passes, 1 % w/v, wet wt)

1 Cavitation number 0.22
2 Cavitation number 0.17
3 Cavitation number 0.13 (2500 passes)
4 Cavitation number 0.92
5 Molecular Marker (wide range, 6.5 to 205 kDa)
6 Cavitation number 0.49
7 French Press
8 Cavitation number 0.32
9 Cavitation number 0.13 (1000 passes)
10 Cavitation number 0.13 (100 passes)

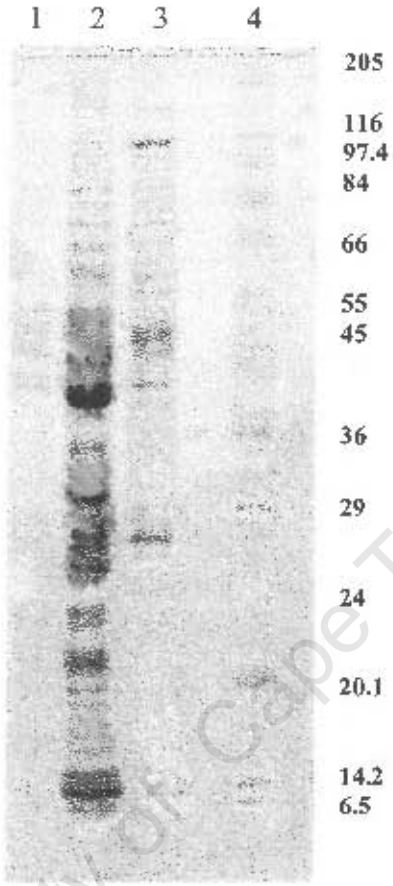

Figure 6.7 SDS-PAGE analysis of samples from *E. coli* cells disrupted by high pressure homogenization (1 % w/v, 5 passes, 51.7 MPa), osmotic shock and EDTA treatment (method described in the Section 4.4.2.3: 1. EDTA treatment 2. high pressure homogenization 3. osmotic shock 4. molecular marker)

E. coli cells subjected to cavitation when observed under electron microscope could provide some information to understand the mechanism of damage and extent of damage. Transmission electron microscopy (TEM) of *E. coli* cells subjected to cavitation was performed as described in Section 4.5.3.3. The results are presented in Figures 6.8 to 6.10. Compared to untreated cells (Figure 6.8) transmission electron micrograph of cells which were subjected to cavitation number 0.49 (μ_{max} 0.36 hr^{-1}, 2500 passes, 0.5 % w/v, wet wt) given in Figure 6.9 showed discontinuities on the outer cell wall. Parts of the outer cell wall appeared to have been damaged. The cells which were subjected to

cavitation at a C_v of 0.13 (μ_{max} 0.36 hr^{-1}, 2500 passes, 0.5 % w/v, wet wt), shown in Figure 6.10 appeared to be damaged to a greater extent. Cell debris could be observed along with a few partly damaged ghost cells. Fragments of cell wall debris and clumps of electron-dense cytoplasmic material were found. Thus the damage observed in the Figure 6.9 could be correlated with stage 1 of disruption (Figure 6.4) and Figure 6.10 could be correlated with stage 3 of cell disruption by hydrodynamic cavitation as proposed in the Figure 6.4. TEM analysis of a similar Gram-negative (*Alcaligenes eutropus*, renamed *Ralstonia eutropus*) bacterium disrupted by high pressure homogenization was performed by Harrison *et al.* (1990). On subjecting the cells to single pass at 67 MPa, the cells were found to be almost intact with only discreet breaks in the cell envelope. On subjection to 4 passes, the cells were micronized and only fragments of cell debris were seen, with no intact cell. In the current study even at intense cavitation conditions intact cells, could be seen along with fragments of cell debris. Thus the micronization observed with high pressure homogenization is reduced by hydrodynamic cavitation.

Figure 6.8 TEM of untreated *E. coli* cell (growth rate 0.36 hr^{-1})

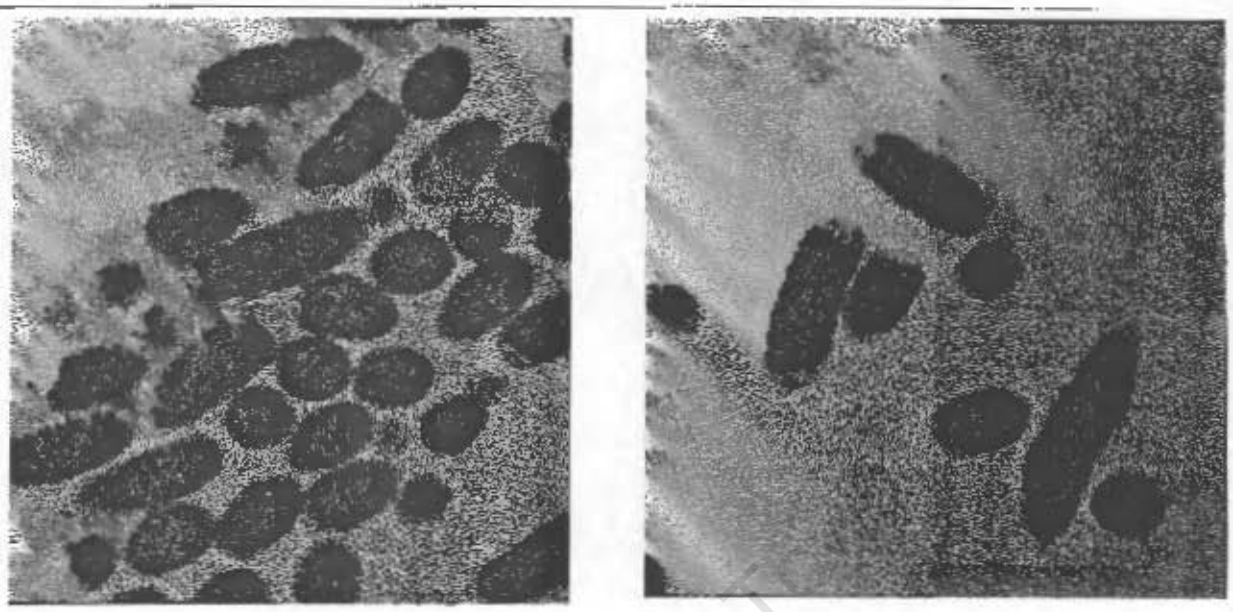

Figure 6.9 Transmission electron micrographs of samples from *E. coli* cells disrupted by hydrodynamic cavitation at a cavitation number of 0.49 (cavitation unit II, μ_{max} 0.36 hr^{-1}, 2500 passes, 0.5 % w/v, wet wt)

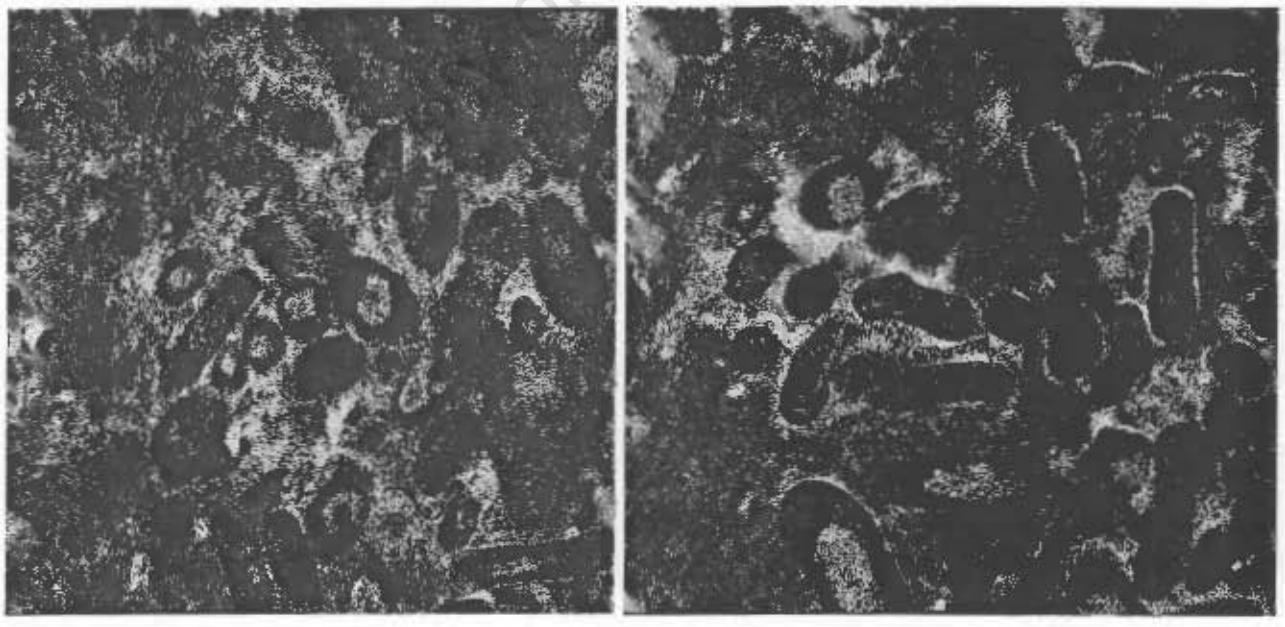

Figure 6.10 Transmission electron micrographs of samples from *E. coli* cells disrupted by hydrodynamic cavitation at a cavitation number of 0.13 (cavitation unit II, μ_{max} 0.36 hr^{-1}, 2500 passes, 0.5 % w/v, wet wt)

6.8. Conclusions

The release of total soluble protein and the enzymes acid phosphatase and β-galactosidase from *E. coli* were found to increase with a decrease in the cavitation number less than 0.5. The maximum extent of acid phosphatase and β-galactosidase were observed at cavitation number 0.17. Here 87 % of acid phosphatase and 68 % of β-galactosidase present in the bacteria were released. The number of cavities formed increases with increase in the intensity of cavitation but the collapse pressure of the cavities decreases. At an optimum cavitation number, the total collapse pressure which is a product of the number of cavities and collapse pressure of each cavity is at a maximum. The maximum extent of release indicates the optimum cavitation number postulated to occur where the total collapse pressure was at its maximum leading to the highest recovery.

The effect of the number of passes on cell disruption indicated the occurrence of stage 3 of cell disruption as represented in the Figure 6.4. The increase in the damage of the cell wall on the sustained exposure of *E. coli* to cavitation zone led to a sustained increase of periplasmic acid phosphatase up to 1500 passes though at a reduced rate after 800 passes while the release of the cytoplasmic enzyme β-galactosidase and total soluble protein asymptoted after 600 passes.

Disruption of *E. coli* cells by hydrodynamic cavitation was found to follow first-order kinetics showing a saturating profile as a function of number of passes. The release rate constant of acid phosphatase, β-galactosidase and total soluble protein increased with decrease in cavitation number over range of 0.17 - 0.49. An optimum cavitation number at which the enzyme release rates were maximum was observed and was attributed to the ratio of the number of cavities generated to the number of cells present being optimum at these conditions.

E. coli cells grown at a higher specific growth rate were found to be disrupted more easily. The release rate constant of proteins from cells cultivated at a μ_{max} of 0.36 hr^{-1} was 4.6×10^{-3} compared with 1.87×10^{-3} for the slower grown cells (μ_{max} 0.11 hr^{-1}). Also the

percentage of periplasmic enzyme acid phosphatase that was released from cells which were grown at the higher specific growth rate (0.36 hr^{-1}) was higher than for the slower grown cells 0.11 hr^{-1} (87 % as opposed to 61 %). It is postulated that hydrodynamic cavitation damages the cell wall to a predominantly than cytoplasmic membrane. In the weaker cell walls of faster grown cells this effect is pronounced resulting in the higher amount of release of periplasmic enzyme (acid phosphatase).

To investigate the selectivity obtained with hydrodynamic cavitation the specific activity of acid phosphatase and β-galactosidase was compared with other disruption techniques. Osmotic shock released the enzyme at higher purity than the French press, hydrodynamic cavitation or high pressure homogenization. Compared to the enzyme released at a cavitation number of 0.17, specific activity of acid phosphatase was four times higher on release by osmotic shock, but the recovery was only 59 % while the recovery was 87 % with hydrodynamic cavitation. Compared to the other mechanical methods, the specific activity of β-galactosidase was always higher with hydrodynamic cavitation. Some 87 % of acid phosphatase and 68 % of β-galactosidase could be released at cavitation number 0.17. At this cavitation number, the specific activity of acid phosphatase released was 1.8 times higher and specific activity of β-galactosidase was 1.4 times higher than the French Press method of disruption. Although osmotic shock gave a higher purity, the extent of release was less. Thus disruption of *E. coli* cells by hydrodynamic cavitation can yield a more pure enzyme than French Press while compromising the recovery slightly. The higher selectivity obtained with hydrodynamic cavitation was also evident from the SDS-PAGE analysis. A higher number of protein bands was obtained for the isolates from French Press and high pressure homogenization than from hydrodynamic cavitation. This indicates that a certain degree of preliminary purification can be achieved by disruption with hydrodynamic cavitation. Further evidence on the selectivity was also seen from the TEM results. Intact cells were seen at less intense cavitation conditions and the absence of micronization characteristic of high pressure homogenization, confirms the relative selectivity that can be obtained by hydrodynamic cavitation

Chapter 7: Process Integration

7.1. Introduction

The simplified generic unit operations involved in the production and purification of an intracellular microbial product can be schematically represented in Figure 7.1. The microbial cells produced in the bioreactor are separated from the broth or concentrated before being disrupted to release their intracellular contents. The soluble protein is then separated from the solids by a solid-fluid separation such as centrifugation or microfiltration. The crude mixture contains the desired protein among a host of contaminants. The product of interest is separated from the contaminants by chromatography or aqueous two-phase extraction or precipitation, usually through a train of recovery and purification steps. This scheme of purification is generic and it varies for any particular protein depending on the purity requirements and properties of the protein. For instance a protein produced in the form of inclusion bodies is readily recovered from the cell debris after cell disruption by a differential centrifugation but also requires renaturation besides the usual purification operations.

Integration of the steps involved in the production and purification of biological molecules is one of the potential means of increasing the total recovery of the product and reducing the cost involved in the manufacture while maintaining or improving product specification. This can be achieved by the integration of the operations production phase in the bioreactor with the unit operations of the downstream processing or two or more steps of the downstream process can be integrated for practical and economic interest. In this chapter integration of the cell disruption by hydrodynamic cavitation with aqueous two-phase extraction to circumvent the solid-liquid separation is discussed in Section 7.2. Also the potential for integration of hydrodynamic cavitation with the bioreactor for the continuous production and extraction of extracytoplasmic proteins is analysed in Section 7.3.

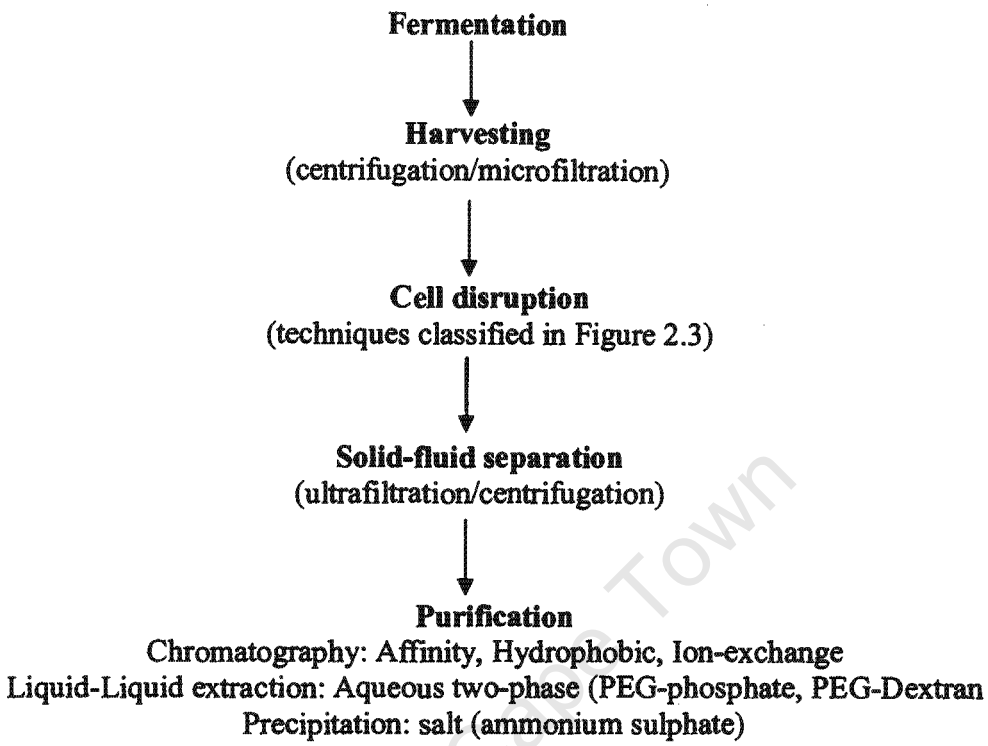

Figure 7.1 Simplified schematic representation of the generic unit operations involved in the purification of an intracellular protein

7.2. Integration of downstream processing steps

7.2.1. The value of integration

The recovery of an intracellular enzyme requires cell disruption as an early step in downstream processing train followed by separation of the cell debris either by microfiltration or centrifugation. Thereafter, the product of interest is purified and concentrated. Unit operations used may include a selection of the following: aqueous two-phase separation: adsorption, affinity or hydrophobic chromatography; and precipitation, depending on the specific product requirement. The number of steps involved in the concentration and purification of the intracellular product depends on the disruption technique used, the nature of the contaminant compounds and the purity

requirements of the product. The cost of the product is often dictated by the number of the downstream processing steps involved in the purification of the product. During the purification of a protein a considerable amount of product is lost with unit operations involved in the process. For instance, in the purification of acid phosphatase from *E. coli* cells, the recovery was only 33 %, as shown in the Table 7.1 based on the total amount available from the cell determined by sonication (Dvorak *et al.*, 1967). Hence integration of the downstream processing steps while maintaining or enhancing the product quality could help to formulate an economical purification strategy while achieving a higher recovery. Expanded bed chromatography and aqueous two-phase systems are employed for the direct extraction of product of interest from microbial cell lysates, thus avoiding the solid-liquid separation step and also achieving a higher degree of purification in one step.

Unit operation	Acid phosphatase (U)	Recovery (%)
Sonication	4500	100
Osmotic shock lysis	4000	90
Dialysis	3900	88
Chromatography (DEAE-cellulose)	2700	61
Chromatography (Hydroxyapatite)	1455	33

Table 7.1 Percentage recovery of acid phosphates from *E. coli* cells during each unit operation in its purification (Dvorak *et al.*, 1967)

7.2.2. Literature review

7.2.2.1. Aqueous two-phase separations

Aqueous two-phase systems have been investigated by several workers for the purification of enzyme molecules. Many biomolecules are denatured by organic solvents, hence PEG is used for the liquid-liquid extraction of such molecules. The high water

content in the PEG system provides a protective environment for biological materials (Albertson, 1986). The extraction is based on the principle of the affinity of the product of interest to one of the phases. The protein of interest partition's selectively to one of the phases from the crude mixture, thus enabling the separation

A clear supernatant or the disrupted suspension (containing cell debris) can be used for aqueous two-phase extraction. The solid-fluid separation step can be eliminated when extracted directly from the cell lysates. The product is extracted simultaneously into a protective environment from the proteases, which can damage the product. Also a high purification of the enzyme early in the downstream process is achieved with the integrated aqueous two-phase extraction. This approach of integration has been demonstrated by many authors (Rito-Palomares and Lyddiatt, 2002; Su and Feng, 1999).

A two-phase aqueous system can be obtained by mixing two or more polymers or a mixture of polymers and salt at high concentrations. For a PEG-salt system, when the polymers and salt are combined in appropriate proportions, mixed thoroughly and allowed to settle, a two-phase system occurs. Separation of the mixture into two-phases can be hastened by its centrifugation. Polyethylene glycol (PEG) in combination with high concentration of salts or dextran is commonly used in the preparation of aqueous two-phase systems. In a PEG-salt system, the PEG rich phase is less dense and forms the top phase while the salt rich phase settles at the bottom. The composition and the volume of the phases depend on the molecular weight of the polymer, polymer concentration and the concentration of the salt. Aqueous two-phase systems are always prepared on a mass basis (w/w) for the sake of practical convenience. The use of aqueous two-phase extraction for the purification of enzymes has been reported (Kroner *et al.*, 1978; Veide *et al.*, 1983). When an enzyme molecule is added to the two-phase system it migrates to either top or bottom phase depending on the partition coefficient of the molecule.

7.2.2.1.1. Effect of the cell disruption technique on the extraction by aqueous two-phase systems

The impact of the different cell disruption techniques employed for the recovery of the product on the extraction by the aqueous two-phase systems has been reported (Rito-Palomares and Lyddiatt, 1996, 2000). The effect of different disruption equipment on the recovery of bulk protein, pyruvate kinase (cytoplasmic) and fumarase (mitochondrial) from Bakers' yeast by aqueous two-phase extraction with a 27 % PEG 1000 and 14 % phosphate system at pH 9.0 was reported by Rito-Palomares and Lyddiatt (2000). Three different disruption methods were used: the high speed bead mill, APV Gaulin high pressure homogenizer and Constant Systems high pressure homogenizer. In the Constant System homogenizer the impingement effect is less than APG Gaulin high pressure homogenizer since the outlet from the jet orifice ends in the disruption chamber directly without the presence of the impact ring. The fractionation of cell debris and proteins by aqueous two-phase systems did not seem to be dependent on the origin of the crude extract. The difference between the partition coefficients of the total soluble protein that was extracted from the cell lysates produced from three different types of disruption equipment was less than 5 %. Pyruvate kinase (4 %) and fumarase (9 %) were found to behave similarly. Kroner *et al.* (1978) reported the effect of disruption equipment employed (high pressure homogenization and bead mill) in the disruption of *E. coli* cells for the extraction of the enzyme aminoacyl tRNA sythetase by aqueous two-phase system with a PEG 6000 (10 %) potassium phosphate (18 %) system at pH 7.8. The amount of total soluble protein extracted depended only a small degree on the source of origin of the lysates. Some 49 % of protein was extracted from the lysates that originated from high pressure homogenizer while 43 % of protein was extracted from that obtained by high speed bead mill. However, the above studies compare only two different techniques of cell disruption. The influence of other disruption techniques such as sonication, hydrodynamic cavitation, osmotic shock etc., may not behave similarly since the particle size of the cell debris varies with cell disruption technique as does the amount of contaminant protein present in the lysate.

7.2.2.1.2. Choice of the molecular weight of the polymer on the extraction

Existence of an optimum molecular weight of the polymer for an efficient partitioning of the protein to the PEG phase has been reported. The partition coefficient of the protein and desired enzyme is influenced by the molecular weight of the polymer. The amount of hydrophilic end groups present on the polymer chains influences the specific hydrophocity of the polymer and hence the partition coefficient of proteins. The low molecular weight polymers such as PEG 400 and PEG 1000 promote the partition of yeast proteins to top phase while the same was partitioned to the bottom phase on increasing the molecular weight of PEG to 3350 (Huddleston *et al.*, 1991).

The recovery of aminoacyl tRNA synthetase from the *E. coli* homogenate was found to be higher (96 %) with PEG 6000 (10 % w/w) whereas the yield was only 21 % with PEG 1550 (17 % w/w; Kroner *et al.*, 1978). The yield on the top phase of the PEG-phosphate system for β-galactosidse from *E. coli* was reported to be higher with PEG 6000 than PEG 4000 and 10,000 (Szoke *et al.*, 1988). Their system comprised 10 % (w/w) PEG, 8 % (w/w) phosphate and 20 % (w/w) biomass at pH 8.0. The recovery was found to be 88 % for PEG 4000, 95 % for PEG 6000 and 79 % for PEG 10,000. Thus for the efficient extraction of β-galactosidse an optimum molecular weight of the polymer was noticed.

7.2.2.1.3. Selective extraction with aqueous two-phase systems

Purification of smaller molecular weight proteins can be enhanced by genetic fusion to the enzyme β-galactosidase, since the latter separates well into the PEG-rich top phase in a PEG-phosphate aqueous two-phase system. This approach was reported in two separate instances. Veide *et al.*, (1983) demonstrated this principle using the fused protein called SpA-βgal (*Staphylococcal* protein A was fused with *E. coli* β-galactosidase). Strandberg *et al.*, (1991) purified the fused protein AG-βgal (IgG binding region of protein A from *Staphylococcus aureus* and two IgG binding regions of protein G from *Streptococcus* again were fused with the β-galactosidase from *E. coli*) from *E. coli* disrupted by bead mill. The aqueous two-phase extraction system comprised PEG 4000 (7.4 %, w/w), phosphate (10.4 %, w/w) and cell lysate (12.5 %, w/w) at pH 7.0. However, this new

strategy was not compared with conventional purification to compare the additional benefits.

For a selective extraction of a biomolecule, the PEG can be derivatized for a selective interaction of the target protein with the modified PEG. This principle was demonstrated by Gavasane and Gaikar (2003). A range of PEG derivatives were synthesized including PEG benzoate (PEG-Bz), trimethylamine (PEG-tma), palmitate (PEG-pal) and phenylacetamide (PEG-paa) and compared with the underivatized PEG-phosphate system ($PEG\text{-}PO_4$). The selective extraction of penicillin acylase from *E. coli* disrupted by sonication was investigated using these derivatives. The affinity interaction of penicillin acylase was found to be higher with PEG-phenylacetamide (PEG-paa) than other derivatives. The enrichment factor was found to be 9.4 for PEG-paa, 8.5 for PEG-Bz, 6.3 for PEG-tma, 3 for $PEG\text{-}PO_4$ and 2 for PEG-pal and. Molecular simulation confirmed the specific interaction of phenyl acetamide group with the active site of the enzyme through π-π interactions and through hydrogen bonding. Hence potential exists to design polymer derivatives through molecular simulation to achieve high recovery and separation of specific proteins.

7.2.2.1.4. Integrated extraction with aqueous two-phase systems

The aqueous two-phase system can be employed to extract the desired protein from the crude cell lysate into one of the phases leaving the cell debris and contaminating proteins in another phase because of the higher partition coefficient of the biomolecule of interest to the PEG phase. The phase forming constituents, when added to the cell suspension for disruption, extract the protein efficiently as and when it is released by cell disruption. Thus cell disruption, solid-liquid separation and aqueous two-phase extraction can be integrated to reduce the number of steps involved in the purification, to remove the protein liberated from a hostile environment rapidly and to concentrate the desired product in a single step.

Integration of the aqueous two-phase extraction with microbial cell disruption has been reported. Ariga *et al.* (1994) studied the release of β-galactosidase from a recombinant

E. coli. β-galactosidase was released by glycine and simultaneously extracted. The cells were suspended in the aqueous two-phase system containing glycine and incubated at 37 °C for 3 hrs (PEG 6000 13 % w/w, potassium phosphate 10 %, w/w). The effect of PEG concentration and phosphate concentrations on the release and extraction was examined. Higher phosphate concentration was found to repress the release of the enzyme by glycine. The glycine concentration was optimized as 1 %. However the extent of the release of the product was low (approximately 10% compared to sonication) in this system and the release rate was slow due to the repression by the high phosphate concentration. The mechanism of action of glycine for the release of β-galactoasidase was not reported.

The bead mill was used both as cell disrupter and product extractor by Rito-Palomares and Lydiatt (2002) in the recovery of G3PDH and proteins from Bakers' yeast. This integrated process was compared with the conventional process (cell disruption and aqueous two-phase extraction as discrete operations). In the integrated process, the required amounts of PEG and phosphate were added to the cell suspension and fed to the Dyno-mill. The disrupted effluent was centrifuged for phase separation and analysed for soluble protein and G3PDH. Of the various concentrations of PEG and phosphate used, 22.2 % (w/w) polymer and 19.0 % (w/w) phosphate gave 66.5 % recovery of G3PDH by integrated process while the conventional process gave a 55.9 % yield. The total protein recovery on the top phase was 80.8 % with conventional process while it was 79.8 % for the integrated process. At this concentration of PEG and phosphate, the cell debris was found to be in the top phase too. Thus the advantage of the integrated extraction is compromised by the presence of the cell debris in the top phase. The PEG and phosphate salt concentration were thus varied for the integrated process. The product recovery was improved to 73 % (G3PDH) with a 41 % recovery of total protein (12 % (w/w) PEG 1000 and 28 % w/w, phosphate). The cell debris was found to be in the bottom phase.

In another study by Su and Feng (1999), the integration of cell disruption by the bead mill and aqueous two-phase extraction was compared with the conventional process where cell disruption and aqueous two-phase extraction were performed separately.

Bakers' yeast was disrupted and the extraction of ADH by an aqueous two-phase system comprising PEG 600 and ammonium sulphate was studied. The partition coefficient of ADH for the conventional process was a little higher than the integrated process (19.5 compared with 16.0) while the purification factor was slightly improved with the integrated process (1.46 compared with 2.57). In other respects, the integrated process performed similarly to the conventional process. The enzyme activity in the top phase was higher with the integrated process (173 U/ml compared to 100 U/ml with conventional process), postulated to be due to the protection afforded to the enzyme during cell disruption owing to its immediate removal from other enzymes.

7.2.2.2. Expanded bed adsorption (EBA)

Chromatography is a well recognized unit operation developed for commercial protein purification some 50 years ago. Separation may be based on affinity, ion-exchange or hydrophobic interactions. Typically the chromatography system is operated as a packed bed, purifying proteins from clarified solutions.

In expanded bed chromatography the fluid phase (crude mixture of proteins or the suspension after cell disruption) is pumped up through the bed of adsorbent beads fluidizing this. The column is fitted with flow adapter to prevent the escape of the beads, while permitting the passage of debris and other contaminants in the crude mixture. The expansion of the bed increases the distances between the adsorbent particles and hence the cell debris and other suspended solids can pass through the bed and the column unhindered. Thus a product of interest can be captured on to the surface of the adsorbent from the disrupted cell suspension directly without the need for the solid-fluid separation following cell disruption. An optimum bed expansion is needed for an effective adsorption of the product. The various factors that needed to be considered include flow velocity of the suspension, size of the particles in the suspension, viscosity, pH, ionic strength etc. The disrupted cell suspension can be used directly with EBA technology thereby avoiding the solid-fluid separation step following the cell disruption. The product of interest can be concentrated significantly in this single step. The selective adsorption

of the protein of interest can be improved by using affinity ligands on the beads that are used.

Expanded bed adsorption was performed using an anion exchanger as the adsorbent (DEAE) and the process was compared with the traditional process of disruption followed by centrifugation and packed bed adsorption for the purification of *Pseudomonas aeruginosa* exotoxin expressed in *E. coli* (Johansson *et al.*, 1996). The total processing time was three times less for EBA compared to conventional bed and also the product was concentrated three times more.

A highly selective affinity system using EBA where the product of interest can be purified to a greater extent was reported by Chase and Draeger (1992). Human polyclonal immunoglobulin G was adsorbed onto the protein A sepharose using this principle. The principle of hydrophobic interaction expanded bed chromatography was used by Ghose and Chase (2000) in the purification of alcohol dehydrogenase and α-glucosidase from unclarified yeast homogenates.

The interaction between the adsorbent and protein (product) can also be increased by means of a metal ion. Certain metal ions like Ni^{2+} and Cu^{2+} have higher affinity for the amino acid histidine. A sequence of six histidine is fused to the C or N terminus of the protein of interest and the hexahistidine tag is used for the selective adsorption by immobilized metal ion chromatography (IMAC). The metal ions are immobilized on the adsorbent by means of ligands such as iminodiacetic acid and nitriloacetic acid. The protein which contains the histidine is adsorbed to the metal ions on the surface of the adsorbent and retained, while the solids and other contaminant proteins goes out of the column without getting adsorbed. This approach has been used several times (Arvidsson *et al.*, 2003; Choe *et al.*, 2002; Clemmitt and Chase, 1999; Glynou *et al.*, 2003; Mukhija *et al.*, 1995).

7.2.2.3. Conclusions

The possibility of integration of the unit operations involved in the purification of a protein is seen from the above literature review. Solid-liquid separation can be integrated with the purification step by using expanded bed adsorption (EBA). But in the case of aqueous two-phase extraction three different steps involved in the purification can be integrated: cell disruption, solid-liquid separation and purification. Such integration can help to reduce the loss of product in the train of downstream processing events, concentrate the product and also to achieve a certain degree of preliminary purification in the early stages of the product isolation process. In the current study β-galactosidase was released from *E. coli* by hydrodynamic cavitation and extracted by aqueous two-phase extraction. This process was integrated and compared with the discrete operations.

7.2.3. Integrated extraction of β-galactosidase using hydrodynamic cavitation and aqueous two-phase extraction

7.2.3.1. Materials and Methods

7.2.3.1.1. Preparation of stock solutions

PEG of three different molecular weights 2000, 4600 and 8000 were used. 50 % w/w of PEG was dissolved in deionised water separately. A 30 % w/w potassium phosphate (K_2HPO_4/KH_2PO_4 in the molar ratio of 1:1.42 was prepared in deionised water and stored at 4 °C.

7.2.3.1.2. Conventional process

Cell disruption: 12.5 g of *E. coli* (wet wt) was suspended in 250 ml of phosphate buffer pH 7.0 (25mM) and disrupted using cavitation unit III (described in the Section 4.2.3) for 500 passes (cavitation number 0.4, 5 % w/v, wet wt, 25 °C).

Aqueous two-phase extraction: 12 g of PEG from stock, 13.33 g of phosphate from stock and 14.67 g of disrupted crude suspension (from the above) were

mixed together and shaken at 25 °C for 30 minutes. The final concentrations in the mixture were 15 % w/w PEG and 10 % w/w potassium phosphate. The mixture was centrifuged at 3000 rpm for 20 minutes for a complete phase separation. Samples were withdrawn from the top phase and bottom phase for the determination of total soluble protein and activity of β-galactosidase. The volume of the phases and location of the cell debris was also recorded.

7.2.3.1.3. Integrated process

For the integrated process, PEG 4600 was chosen. Some 75 g of PEG 4600 from the stock solution, 83.33 g of phosphate solution from the stock solution, 12.5 g (wet wt) of *E. coli* cells and 79.42 g of phosphate buffer of pH 7.0 (25 mM) were mixed together. The final concentration in the mixture was 5 % w/w (wet wt) biomass, 15 % w/w PEG 4600 and 10 % w/w potassium phosphate. The mixture was subjected to hydrodynamic cavitation using the cavitation unit III (cavitation number of 0.4) for 500 passes and the temperature was maintained at 25 °C. After 500 passes through the cavitation zone, the mixture was centrifuged at 3000 rpm for 20 minutes for phase separation. Samples were withdrawn from the top phase and bottom phase for the determination of total soluble protein and β-galactosidase. The volume of the each phase and the location of the cell debris were also recorded.

7.2.3.2. Results and Discussion

7.2.3.2.1. Choice of molecular weight of the polymer

The results of the extraction of β-galactosidase using PEG of three different molecular weights: 2000, 4600 and 8000 are presented in Table 7.2. By visual observation a clear top phase was seen and the cell debris was found to be in the bottom phase with all 3 molecular weights of PEG studied. The partition coefficients ($k_{proteins}$, $k_{\beta\text{-galactosidase}}$) are defined as the ratio of total amount of protein or enzyme extracted in the top phase to that extracted into the bottom phase.

Table 7.2 Extraction of β-galactosidase in PEG-phosphate aqueous two-phase system (PEG 15 % w/w, phosphate 10 % w/w and pH 7.0) as a function of molecular mass of the polymer

PEG	Total enzyme extracted (U)	Top phase (U)	Bottom phase (U)	Recovery (%)	$k_{\beta\text{-galactosidase}}$
2000	14	13	0.6	1.2	22.6
4600	29	20	9	1.8	2.3
8000	22	16	6	1.4	2.7

The partition coefficient for β-galactosidase was found to be 22.6 for PEG 2000 while it was only 2.3 with PEG 4500 and 2.7 for PEG 8000 (Table 7.2). But the total amount of enzymes extracted (i.e. the sum of both top and bottom phase) is 14 U for PEG 2000, 29 U for PEG 4600 and 22 U for PEG 8000. The percentage recovery of the enzyme to the PEG phase (top phase) were 1.2 % for PEG 2000, 1.8 % for PEG 4600 and 1.4 % for PEG 8000. Thus PEG 4600 extracts more enzyme into the top phase than the others. The cells were not disrupted sufficiently by hydrodynamic cavitation as to release all the β-galactosidase available and hence the low recovery by any polymer but the relative difference indicates that the PEG 4600 is the preferred polymer for the extraction of β-galactosidase to maximize recovery. This is consistent with the reports by other authors (Strandberg *et al.*, 1991; Szoke *et al.*, 1988). Also the specific activity of β-galactosidase (U/mg of protein) in the top phase was higher for PEG 4600 (19.8 U/mg) compared with other PEG molecular weights (13.4 U/mg for PEG 8000 and 5.4 U/mg for PEG 2000). Both the recovery and purity of β-galactosidase were highest with PEG 4600. Thus PEG 4600 was chosen for the integrated process.

7.2.3.2.2. Comparison of the conventional process with the integrated process

The results of the experiments of the integrated process for β -galactosidase extraction for *E. coli* are presented in the Table 7.3. On comparing the conventional process with the integrated process, it is found that the amount of enzyme extracted into the top phase was marginally higher (21 units compared to 20) for integrated process. Similar results in

which the integrated process was only slightly better than the conventional process have been reported by Su and Feng (1999). The percentage recovered from the cell was 1.9 % compared to the total amount of enzyme available from the cells (recovery was 1.8 % with the conventional process).

PEG	Volume of top phase (ml)	Proteins (mg)	$k_{proteins}$	β - galactosidase (U)	Specific activity of β - galactosidase (U/mg of proteins)	Recovery (%)	$k_{\beta\text{-}galactosidase}$
Conventional process (hydrodynamic cavitation followed by aqueous two-phase extraction							
2000	36.3	2.4	1.9	13	5.4	1.2	22.6
4600	26.5	1.0	0.2	20	19.8	1.8	2.3
8000	26.5	1.2	0.2	16	13.4	1.4	2.7
Integrated extraction							
4600	26.5	2.31	0.4	21	9.1	1.9	11.6

Table 7.3 Comparison of extraction of protein and β-galactosidase with conventional process (cavitation followed by extraction) and integrated process (cavitation – cavitation unit III, cavitation number 0.4, 5 % w/v, wet wt, 500 passes, 25 °C; Recovery - compared to the total amount of enzyme present in the cell determined by French Press disruption of 1 % w/v, wet wt, *E. coli* cells at 20 MPa for 5 passes)

Total amount of enzyme available was determined by French press disruption of 1 % w/v (wet wt) *E. coli* cells at 20 MPa for 5 passes. The partition coefficient of β-galactosidase increased from 2.3 to 11.6 by the integrated process though the specific activity decreased from 19.8 to 9.1 U/mg. From Table 6.3 it is seen that at cavitation numbers over the range 0.32 to 0.49, some 13 % to 23 % release of β –galactosidase is expected, whereas this was increased to 68 % release under more extreme cavitation conditions (C_v 0.13 to 0.17). Further, enzyme release has been shown to decrease with increasing cell concentration over the range 0.5 to 5 % (w/v, wet wt). During disruption by hydrodynamic cavitation in the presence of polymer only 23 U was released (cavitation number 0.4, cavitation unit III, 5 % w/v, wet wt, 500 passes). The higher concentration of

cell suspension together with the lower intensity of cavitation employed resulted in the poor release of β-galactosidase during the integrated process. The intensity of damage on the cells was lower and hence the cells might have been punctured at this less intense cavitation conditions (Further evidence reported in Section 6.6). Hence smaller molecular weight proteins could have leached out and got extracted to a higher extent because of the turbulence and presence of PEG during the integrated process reducing the specific activity of β-galactosidase in the top phase.

Also during the conventional process the two-phase system was in contact with the disrupted suspension for 30 minutes, while during the integrated process the contact was maintained over the whole operation with duration of some 85 minutes. Hence during the integrated process the PEG was in contact with the cells for longer time. Further the enzyme extraction away from the cell debris could occur as soon as it is released during disruption. This is postulated to be responsible for the reduction in the specific activity and increase in the partition co-efficient of β-galactosidase by the integrated process.

The cell debris was found to be in the bottom phase and hence the additional step of microfiltration or centrifugation needed during the conventional process can be eliminated by this integrated process.

7.3. Integration of upstream processing with downstream processing

7.3.1. Introduction

In the manufacture of an intracellular product if the viability and metabolic activity of the microbial cells can be maintained after the release of the desired product the microbial cells can be recycled to the bioreactor following product extraction to enable the use of the biomass to synthesize more of the desired product again. It is proposed that if cell damage during product extraction is minimized, this cycle can be sustained for a few life

cycles of the microorganism. The ideal requirements for the feasibility of this process are, among others (i) the location of the product in the periplasm or associated with the cell-wall so that the release of product does not require permeabilization of the cytoplasmic membrane, the biological barrier controlling many metabolic functions of the microorganism; (ii) an ideal cell 'disruption' technique that can release the product without disrupting the cell functionally or viability; (iii) the absence of any exogenous chemicals or enzymes in the disrupted suspension; (iv) ease of scale up for large scale production; and (v) aseptic operation of the process.

7.3.2. Continuous extraction of extracytoplasmic proteins

Exposure of yeast to hydrodynamic cavitation at appropriate conditions released the extracytoplasmic enzymes like invertase (cell wall associated) or α-glucosidase (periplasmic) without micronization. No release of cytoplasmic enzymes was detected. Absence of a significant damage of the yeast cell indicates that the metabolic function of the yeast cell may not be compromised. The yeast cells may be expected to function metabolically and physiologically and also reproduce when transferred to a growth media. Hence the viability of the yeast cells and their potential for continued growth and metabolism was investigated experimentally following subjection to hydrodynamic cavitation. Yeast cells (1% w/v, wet wt) were subjected to cavitation for 1000 passes using different orifice plates and their viability following 1000 passes through the cavitation zone was determined. The viability was estimated by slide-count method as described in Section 4.5.3.1. The results are presented in the Table 7.4 and the amount of total soluble protein, α-glucosidse and invertase released at the corresponding cavitation conditions is presented in Table 7.5

It can be seen from Table 7.4 that the viable cell number after subjecting the yeast to hydrodynamic cavitation decreases on increasing the intensity of cavitation (decreasing cavitation number). The maximum release was obtained with orifice plate for cavitation number 0.13. Some 22.4 % of protein, 28.7 % of invertase (cell-wall associated) and 13.4

% of α-glucosidase (periplasmic) were released at this cavitation number and 72.3 % of cells were viable.

Table 7.4 Results of the viability of the yeast cells following disruption hydrodynamic cavitation (cavitation unit II, 1000 passes, 1 % w/v, wet wt) estimated by slide-count method described in section 4.5.3.1

Cavitation number	Total cells counted	Viable cell count	Viability (%)	Non-viable cell count	% Non-viable
0.92	552	508	92.0	44	8.0
0.49	637	617	95.4	30	4.6
0.42	641	578	90.2	63	9.8
0.32	486	439	90.3	47	9.7
0.22	662	483	73.0	170	27.0
0.13	827	598	72.3	229	27.7

Table 7.5 Release of total soluble protein, α-glucosidase and invertase by hydrodynamic cavitation (cavitation unit II, 1000 passes, 1 % w/v, wet wt) compared with release by high pressure homogenization (1 % w/v, wet 25, 7500 psi, 10 passes)

Cavitation number	Protein mg/gm	%	α-glucosidase (U/gm)	%	Invertase (U/gm)	%
0.92					18	2.4
0.49	0.1	0.1			28	3.6
0.42	0.3	0.5			20	2.6
0.32	0.5	0.7			17	2.2
0.22	1.8	2.9	94	0.2	41	5.4
0.13	14.0	22.4	7276	13.4	221	28.7
HPH	62.4		54401		772	

The changes in the metabolic and physiological activity of yeast subjected to hydrodynamic cavitation was investigated by transferring the yeast cells following

disruption by hydrodynamic cavitation to a growth media. The growth profile of yeast was observed before and after cavitation.

Yeast cells were cultivated in a shake flask (MPYG medium, 250 rpm, 37 °C) and subjected to cavitation using the cavitation unit III (5 % w/v, wet wt, cavitation number 0.4, 500 passes). Yeast cells (*S. cerevisiae W330*) were grown in 25 ml of MPYG medium as a pre-inoculum. The pre-inoculum culture was transferred to 475 ml of medium and the cells were grown for 30 hours. Samples were withdrawn every 2 hours and increase in the biomass concentration observed by absorbance at 660 nm. The growth curves are presented in Figure 7.2. After 26 hours the yeast cells were harvested aseptically and suspended in phosphate buffer of pH 7.0 (25mM) before being subjected to cavitation using cavitation unit III for 500 passes (5% w/w, wet wt, cavitation number 0.4). After 500 passes the cell suspension was centrifuged aseptically and inoculated into fresh medium to compare the growth curve of the yeast cells. The growth curves obtained are plotted in Figure 7.2. No significant difference in the growth rate of the yeast cells was observed between the growth curve of fresh cells and cells which were subjected to hydrodynamic cavitation. The growth rate (μ_{max}) of yeast was not altered siginificantly 0.236 hr^{-1} to 0.231 hr^{-1}.

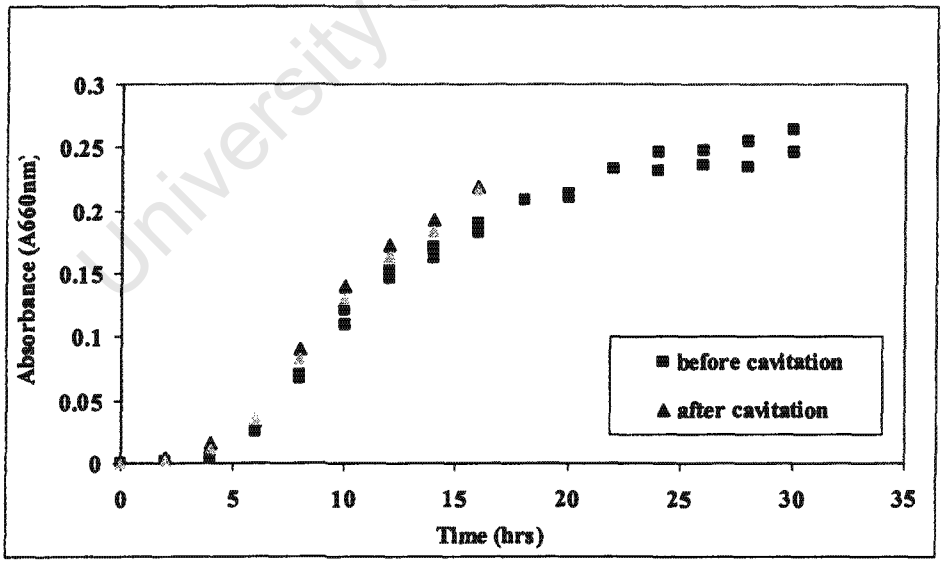

Figure 7.2 Growth curve of yeast (*Saccharomyces cerevisiae*) in MPYG medium (250 rpm, 37 °C) before and after hydrodynamic cavitation (cavitation unit III, cavitation number 0.4, 5 % w/v, wet wt, 500 passes)

Based on these findings, it is proposed that continuous extraction of periplasmic or wall-associated proteins may be achieved from Bakers' yeast using an integrated circuit of the nature presented in Figure 7.3. Implementation of such a system will form the subject of further study. In particular the impact of the process yield will be considered owing to the reduced requirements to produce fresh biomass.

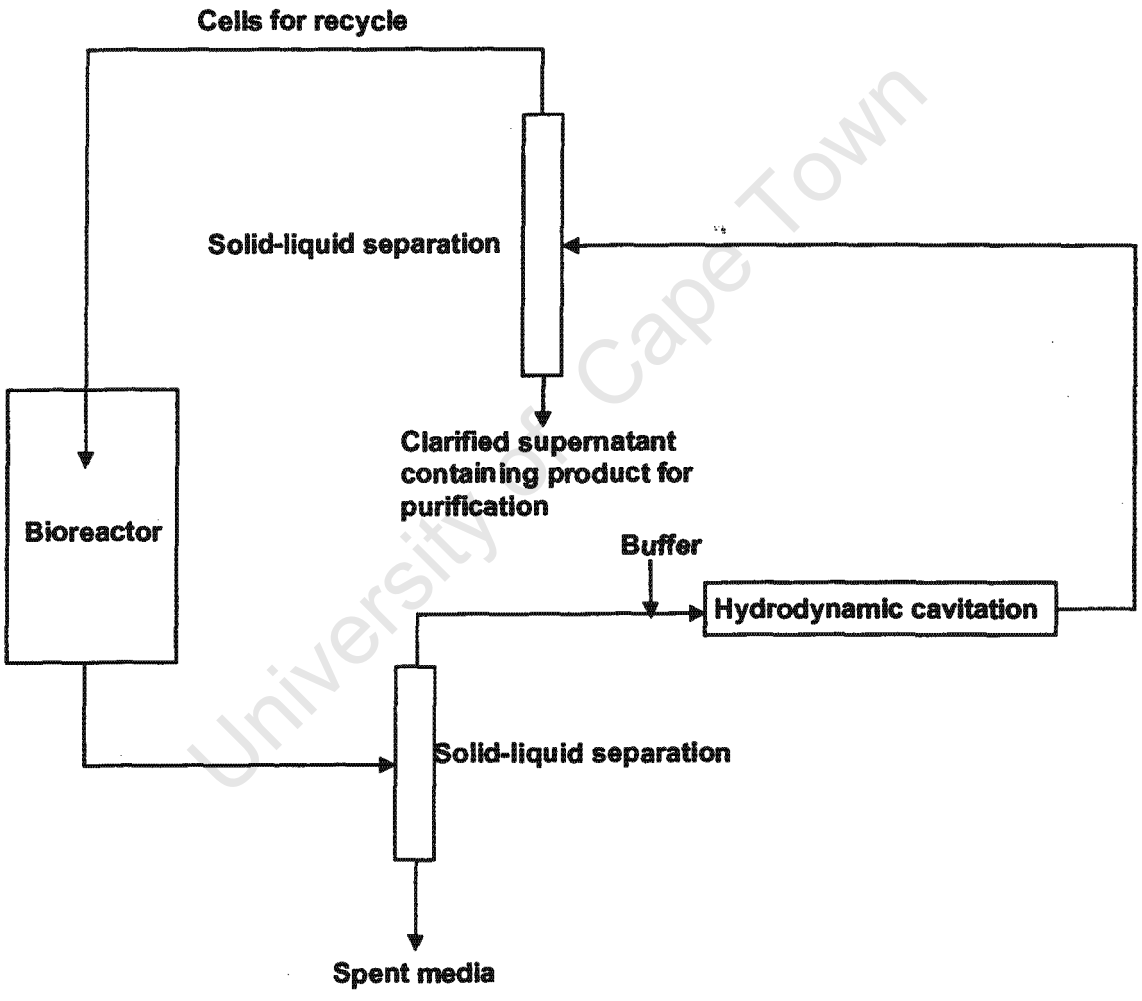

Figure 7.3 Schematic representation of the proposed, continuous extraction of extracytoplasmic proteins using hydrodynamic cavitation

7.4. Conclusions

The integration of the aqueous two-phase extraction with hydrodynamic cavitation resulted in an increase in the partition coefficient of β -galactosidase. However the total recovery improved only marginally. The cell debris was found in the bottom phase. Thus cell disruption, solid-fluid separation and extraction can be integrated with some potential benefits. However, the cavitation conditions employed were not of sufficient intensity to release a significant amount of the enzyme. The benefits of the integration can be expected to improve on employing intense cavitation conditions.

Hydrodynamic cavitation was found to maintain the viability of the yeast cells and the yeast cells were also found to be functional. There was no significant difference in the growth rate of fresh yeast cells and yeast subjected to hydrodynamic cavitation (C_v of 0.4, cavitation unit III, 5 % w/v, 500 passes). Thus hydrodynamic cavitation has potential to sustain the viability of yeast cell while extracting the extra-cytoplasmic and cell-wall associated proteins for continuous production.

Chapter 8: Conclusions

The disruption of microbial cells for selective release of intracellular products using hydrodynamic cavitation was investigated in the current study. Two model systems were used: Brewers' yeast and *E. coli*. The techniques of disruption employed for yeast disruption include hydrodynamic cavitation, high pressure homogenization and French Press. The release of α-glucosidase (periplasmic), invertase (cell wall bound), alcohol dehydrogenase (cytoplasmic), glucose-6-phosphate dehydrogenase (cytoplasmic) and total soluble protein were determined. Two different cavitation units were used namely: unit I and unit II. The effects of the process variables such as the cavitation number, the initial concentration of cell suspension and the number of passes through the cavitation zone on the release of the above enzymes were studied. The extent of disruption was compared with the disruption by French Press and high pressure homogenization. The selective release was also analysed by microscopy (optical and transmission electron microscopy) and SDS-PAGE.

The effect of cavitation number was studied over the range 0.09 to 0.99. There was a significant amount of protein and enzyme release at cavitation number less than 0.4. The release of extracytoplasmic enzymes (α-glucosidase and invertase) and total soluble protein was found to reach a maximum extent at a cavitation number of 0.13. Some 22 % of total soluble protein, 13 % of α-glucosidase and 29 % of invertase were released (C_v - 0.13, 1 % w/v, wet wt, 1000 passes). The number of cavities generated increases when increasing the intensity of cavitation (lower cavitation number) but the collapse pressure of the individual cavity decreases (Gogate and Pandit, 2001). Thus the maximum collapse pressure (the product of the number of cavities and collapse pressure of each cavity) generated at the optimum cavitation number is postulated to have resulted in a maximum extent of disruption. The trend in the release of the enzymes and soluble protein as a function of cavitation number was found to be similar with both the cavitation units (unit I and unit II) although the absolute amounts released were different because of the different temperatures at which they were operated.

When the cell concentration was varied over the range 0.1 % to 5 % (w/v, wet wt) the release of extracytoplasmic enzymes and total soluble protein were highest at 0.5 %. Some 28.5 % of soluble protein, 27 % of α-glucosidase and 39 % of invertase were released at 0.5 % w/v (wet wt, 1000 passes, and cavitation number 0.13). At higher cell concentration, the number of cavities available to interact with microbial cells was saturated and at lower cell concentration the cavities interact among themselves. The maximum interaction that occurs, when the cell-to-cavity ratio was optimum was postulated to be responsible for the highest release at 0.5 % cell concentration.

On subjecting the yeast cells to an extended number of passes, the release of total soluble protein and α-glucosidase reached an approximately constant value after 1500 and 1000 passes respectively. Some 25 % of protein and 17 % of α-glucosidase were released. The release of invertase increased up to 5000 passes at which stage 86 % of total enzyme available was released. This is postulated to be due to the combined effect of mechanical and chemical effects of cavitation on the cell wall of yeast. Invertase, trapped in the cell wall matrix by disulphide bridges was released by the reduction reactions of the free radicals generated during hydrodynamic cavitation. The higher impact of hydrodynamic cavitation on the yeast cell wall relative to the cytoplasmic membrane was also seen by optical microscopy.

The release of soluble protein was found to follow first-order kinetics. An optimum cavitation number at which the release rate constant was maximum was observed. On analyzing the release kinetics of total soluble protein over the ranges of cavitation numbers from 0.09 to 0.26, the highest release rate was observed at a cavitation number of 0.17 (1000 passes, 1 % w/v, wet wt). First order release kinetics was used to calculate the release rate constant of invertase over the range of cavitation numbers from 0.13 to 0.99. The release rate constant was found to increase on decreasing the cavitation number, reaching a highest value between cavitation number of 0.17 and 0.13 for 0.1 % and 2.5 % w/v cell concentration. From the data collected the maximum release rate constant for 1 % cell concentration was postulated to occur at a cavitation number below 0.13.

On subjecting the yeast cells to a cavitation number of 0.17 and 1000 passes at 1 % w/v cell concentration, 11.5 % of total soluble protein was released. The periplasmic protein in yeast has been quantified as 11 %. Absence of micronization seen from the optical microscopic images and absence of the cytoplasmic enzyme ADH indicated the selective release of periplasmic proteins. Transmission electron micrographs showed point rupture on the yeast cell wall. At a cavitation number of 0.13, 1 % w/v cell concentration and 1000 passes, 22 % of total soluble protein, 13 % of α-glucosidase and 39 % of invertase was released. This amount of soluble protein exceeds that present in the periplasmic space. However, only 13 % of periplasmic enzyme α-glucosidase was released. It is postulated that the yeast cells were permeabilized to release the periplasmic protein but retained the bulkier periplasmic enzyme molecule within (such as α-glucosidase). Higher amount of release of the cell wall associated invertase indicated the predominant effect of cavitation on the cell wall of yeast.

Transmission electron micrographs of cells exposed to hydrodynamic cavitation at a cavitation number of 0.13 and cell concentration of 0.1 % (w/v, wet wt) showed that the cells were ruptured and ghosts of yeast cell and fragments of cell wall were observed. At 1 % cell concentration and cavitation number of 0.13 the cell wall was damaged but the cells were largely intact. At 5 % cell concentration no significant difference was observed compared to an untreated yeast cell. SDS-PAGE analysis of supernatants from hydrodynamic cavitation was compared with French Press. Fewer protein bands were observed for samples from hydrodynamic cavitation illustrating higher specific activity may be expected for those proteins released by hydrodynamic cavitation, allowing easier purification of the proteins released by hydrodynamic cavitation.

The disruption of *E. coli* was studied using cavitation unit II. The release of acid phosphatase (periplasmic), β-galactosidase and total soluble protein was studied. The disruption was compared with the release by French Press, high pressure homogenization, osmotic shock and EDTA treatment. The effect of cavitation number, number of passes and the growth rate of *E. coli* on its disruption by hydrodynamic cavitation were studied.

The release of soluble protein, acid phosphatase and β-galactosidase was found to increase with a decrease in cavitation number (hence increasing the intensity of cavitation). The extent of release of acid phosphatase and β-galactosidase reached a maximum of 88 % and 67 % respectively at a cavitation number of 0.17. The release of soluble protein appeared to approach a maximum extent at a cavitation number below 0.13. On studying the effect of number of passes through the cavitation zone the extent of release of soluble protein and β-galactosidase was found to approach a constant value after 600 passes. The release of acid phosphatase was increasing until 800 passes and the increase in the release decreased beyond 800 passes. The predominant effect of cavitation on the cell wall caused discrete openings on the cell wall, resulting in the sustained release of periplasmic enzyme up to 800 passes. The disruption of *E. coli* was found to follow first-order kinetics. The release rate constants of soluble protein, acid phosphatase and β-galactosidase were calculated. The release rate constants were maximum at a cavitation number of 0.17 for acid phosphatase and β-galactosidase. The release rate constant of total soluble protein increased with decrease in cavitation number across the range studied.

E. coli cells that were cultivated at the maximum specific growth rate of 0.36 hr^{-1} were found to disrupt more readily than cells cultivated at a specific growth rate of 0.11 hr^{-1}. The release rate constant of soluble protein was found to be 4.6×10^{-3} for μ of 0.36 hr^{-1} while it was 1.9×10^{-3} for μ of 0.11 hr^{-1} at a cavitation number of 0.13 and cell concentration of 0.5 % w/v (wet wt). This is in agreement with the general literature on cell disruption which illustrates that the weaker cell walls of faster grown cells are disrupted more easily and hence yield a higher release rate of intracellular proteins. *E. coli* grown at a μ of 0.36 hr^{-1} released 87 % periplasmic acid phosphatase compared to 61 % from cells grown at a μ of 0.11 hr^{-1}. Thus the predominant effect of cavitation on the cell wall combined with the weaker cell walls of faster grown cells resulted in the higher extent of release of periplasmic enzyme acid phosphatase than total soluble protein and cytoplasmic β-galactosidase.

The specific activity of acid phosphatase and β-galactosidase were compared with other methods of disruption. The specific activity of acid phosphatase was found to be 4 times higher by osmotic shock than at a cavitation number of 0.13, however the recovery was 59 % with osmotic shock, while 88 % recovery was obtained with hydrodynamic cavitation. On comparison with high pressure homogenization the specific activity of 12 U/mg (same as high pressure homogenization) and 87 % recovery obtained at a cavitation number of 0.13 compared favourably with high pressure homogenization. A higher specific activity of acid phosphatase could be obtained at a higher cavitation number (31 U/mg at C_v of 0.92), however recovery was compromised (15 %). The specific activity of β-galactosidase was always higher with hydrodynamic cavitation than other methods of disruption. High pressure homogenization gave a specific activity of 10 U/mg, while hydrodynamic cavitation at a C_v of 0.13 resulted in a specific activity of 20 U/mg and 68 % recovery.

The absence of micronization was indicated by the reduced amount of protein released at any cavitation number. The reduced number of contaminating proteins was also seen from the SDS-PAGE analysis. The French Press sample had additional protein bands (two between regions of 205 and 116 kDa, 84 and 66 kDa, 20.1 and 24 kDa and one in the region between 66 an 55 kDa) compared to cavitation number 0.13. The higher number of soluble proteins released under intense cavitation conditions was also seen on comparing the protein bands obtained at a of C_v of 0.32 and 0.13. Transmission electron micrographs of *E. coli* subjected to cavitation at a cavitation number of 0.92 showed largely intact cells without much damage under intense cavitation (C_v 0.13), cells were ruptured, fragments of cell wall debris and clumps of electron-dense cytoplasmic material could be seen along with few intact cells.

One or more of the steps involved in the production and purification of biological molecules can be integrated to increase the recovery, reduce the cost involved in its manufacture and to improve the product specification. On integration of the cell disruption by hydrodynamic cavitation (C_v - 0.4, 5 % w/v wet wt, 500 passes) with aqueous two-phase extraction using PEG 4600 the partition coefficient of β-galactosidase

increased to 11.6 from 2.3 compared to the discrete unit operations while maintaining the recovery. Thus cell disruption by hydrodynamic cavitation, solid-fluid separation and purification by aqueous two-phase extraction can be integrated with some potential benefits.

The viability of yeast cells following cavitation was maintained at 73 % for yeast cells treated at C_v of 0.17 and 1000 passes. The percentage viability increases on decreasing the intensity of cavitation. The maximum specific growth rate of yeast cells subjected to cavitation was not affected significantly (0.236 hr^{-1} to 0.231 hr^{-1}). Thus yeast cells can retain the viability to a significant extent without compromising the metabolic activity indicating potential for integrated product formation and extraction with cell recycle.

The yeast cell is known to possess a rigid cell wall. The cell wall envelope of yeast is relatively thicker and stronger compared to *Gram-negative* bacteria. Under the same conditions of cavitation (same intensity) the extent of disruption of a microbial cell will depend on its strength. At cavitation number of 0.13 and cell concentration of 0.5 % w/v (wet wt) 28.5 % of total soluble protein was released from yeast during 1000 passes through the cavitation zone. Under similar conditions 58 % of total soluble protein was released from *E. coli* after 600 passes through the cavitation zone. Thus *E. coli* was disrupted more readily than yeast.

On extending the number of passes through the cavitation zone the extent of release of invertase increased from 23 % at 1000 passes to 73 % after 2500 passes while the extent of release of α-glucosidase remained constant at 17 % (cavitation unit II, cavitation number 0.13, 1 % w/v, wet wt). Thus cell wall bound enzyme from yeast can be released to a higher extent and also selectively. In the disruption of *E. coli* for the release of periplasmic enzyme, acid-phosphatase from *E. coli*, hydrodynamic cavitation was found to perform similar to the high pressure homogenizer (87 % recovery at cavitation number 0.13, 0.5 % w/v, wet wt and 1750 passes). The cytoplasmic enzyme β-galactosidase can be released with higher specific activity compared to French Press and high pressure homogenization, with a compromise on the extent of release.

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Appendix A: Analytical methods

A.1. α-glucosidase

Reagents

5.0 mM *p*-nitrophenol-α-D-glucoside in 0.05 M phosphate buffer (pH 6.8)

0.05 M Phosphate buffer (pH 6.8)

0.1 M Sodium carbonate solution in distilled water

Method

1. Pipette out 100 µl of enzyme supernatant into a clean test tube
2. Add 2 ml solution of 5.0 mM *p*-nitrophenol-α-D-glucoside dissolved in the phosphate buffer of pH 6.8 (0.05 M)
3. Incubate at 30 °C for 10 minutes in a water bath
4. Remove the test tubes from the water bath and add 2 ml of 0.1 M Na_2CO_3 as stop reagent
5. Measure the absorbance at 410 nm against a blank (buffer pH 7.0) treated similar to the sample

Table A.1 Reproducibility of α-glucosidase analysis

Sample no.	α-glucosidase (U/gm)
1	7311
2	9263
3	8091
4	9618
Average	8571
Standard deviation	1064
Coefficient of variance (%)	12.41

A.1.1. Calibration curve for *p*-nitrophenol

1. Prepare standard solutions of *p*-nitrophenol (1 – 5 M) dissolved in 0.1 M Na_2CO_3
2. Measure the absorbance at 410 nm against blank (distilled water)

The calibration curve obtained is shown in Figure A.1

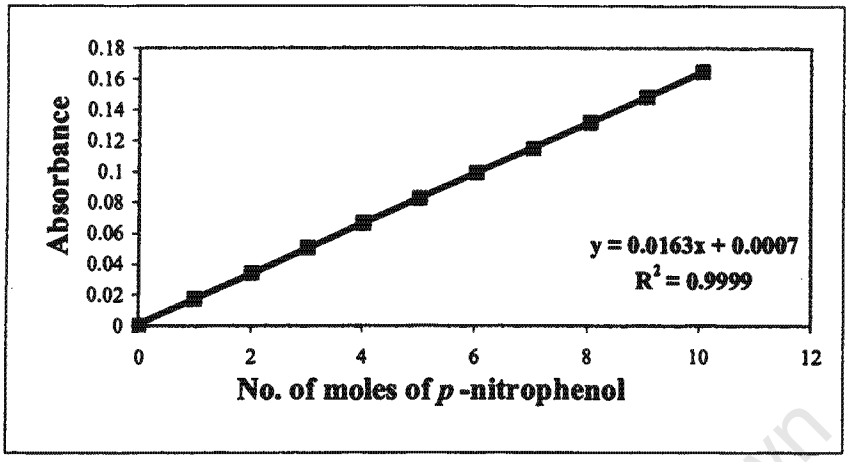

Figure A.1 Calibration curve for *p*-nitrophenol

A.2. β-galactosidase

Reagents

PPB-Mn buffer: Add 10 M KOH to 50 mM KH_2PO_4 to obtain a final pH 6.6 and add 0.1 mM $MnCl_2$

?mM *o*-nitrophenyl-β-D-galactoside dissolved in PPB-Mn buffer

1 M Na_2CO_3 dissolved in distilled water

Method

1. Pipette 50 micro litres of enzyme supernatant into a clean test tube
2. Add 2 ml solution of *o*-nitrophenyl-β-D-galactoside dissolved in PPB-Mn buffer
3. Incubate the reaction mixture for 5 minutes at 37 °C in a water bath
4. Add 0.5 ml of 1 M Na_2CO_3 to terminate the reaction
5. Read the absorbance at 420 nm against a blank (buffer pH 7.0) treated similar to the sample

Table A.2 Reproducibility of β-galactosidase analysis

Sample no.	β - galactosidase (U/gm)
1	1502
2	1492
3	1624
Average	1540
Standard deviation	73.53
Coefficient of variance (%)	4.77

A.3. Acid phosphatase

Reagents

0.1 M Sodium acetate buffer pH 5.0

3.8 mM p-nitrophenyl phosphate in sodium acetate buffer (0.1 M, pH 5.0)

0.2 M sodium hydroxide

Method

1. Pipette out 300 µl of sample into a clean test tube
2. Add 300 µl of p-nitrophenylphosphate dissolved in sodium acetate buffer (pH 5.0, 0.1 M)
3. Add 100 µl of sodium acetate buffer (pH 5.5, 0.1 M)
4. Incubate at 37 °C for 15 minutes in a water bath
5. After 15 minutes add 3 ml of 0.2 M NaOH to terminate the reaction
6. Measure the absorbance at 410 nm against a blank (phosphate buffer pH 7.0) treated similar to the sample

Table A.3 Reproducibility of acid phosphatase analysis

Sample no.	Acid phosphatase (U/gm)
1	597
2	547
3	612
Average	585
Standard deviation	34.33
Coefficient of variance	5.87

A.3.1. Calibration curve for *p*-nitrophenol

3. Prepare standard solutions of *p*-nitrophenol (1 – 10 M) dissolved in 0.2 M NaOH
4. Measure the absorbance at 410 nm against blank (distilled water)

The calibration curve obtained is shown in Figure A.2

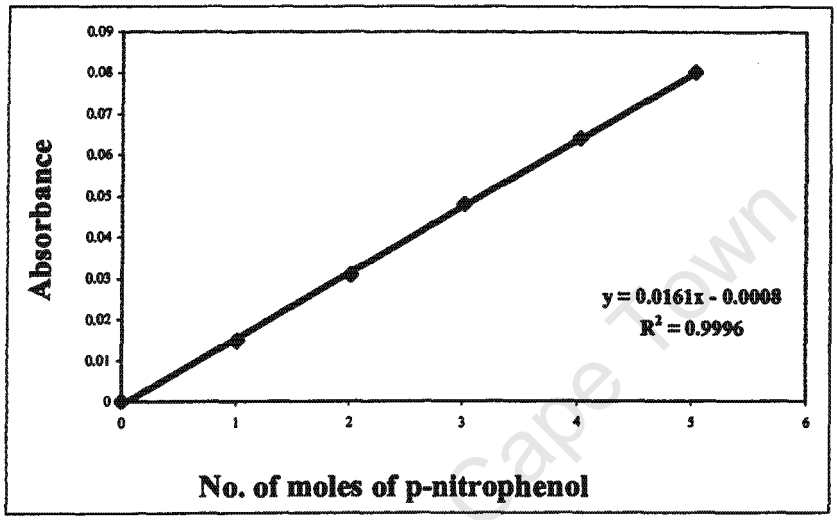

Figure A.2 Calibration curve for *p*-nitrophenol

A.4. Alcohol dehydrogenase

Reagents

0.06 M Sodium pyrophosphate buffer (pH 8.5)

0.1 M Nicotine adenine di-nucleotide (NAD) in distilled water

0.1 M Ethanol in distilled water

Method

1. Pipette out 2.2 ml of distilled water into a quartz cuvette
2. Add 0.5 ml of 0.06 M pyrophosphate buffer
3. Add 0.1ml of 0.1 M ethanol followed by 0.1 ml of 0.1 M NAD
4. Add 0.1 ml of the appropriately diluted enzyme supernatant
5. Place the cuvette immediately in the spectrophotmeter and record the absorbance at intervals of 15 seconds for two minutes at 340 nm against a blank (buffer pH 7.0) treated similar to the sample

Table A.4 Reproducibility of alcohol dehydrogenase analysis

Sample no.	Alcohol dehydrogenase (U/gm)
1	1900
2	2550
3	2600
4	2700
Average	2437
Standard deviation	363
Coefficient of variance (%)	15

A.5. Glucose-6-phosphate dehydrogenase

Reagents

249 mM Tris-HCl buffer (pH 7.6)

10 mM Glucose-6-phosphate in distilled water

10 mM β-Nicotine adenine di-nucleotide phosphate (β-NADP) in distilled water

0.1 M Magnesium chloride in distilled water

Method

1. Pipette out 1 ml of Tris-HCl buffer (0.249 mM, pH 7.6) into a quartz cuvette
2. Add 0.3 ml of 10 mM glucose-6-phosphate followed by 0.12 ml of 10 mM NADP and 0.20 ml of 0.1 M $MgCl_2$
3. Add 1.38 ml of appropriately diluted enzyme sample.
4. Place the cuvette immediately in the spectrophotmeter and record the absorbance at intervals of 15 seconds for two minutes at 340 nm against a blank (buffer pH 7.0) treated similar to the sample

Table A.5 Reproducibility of glucose-6-phosphate dehydrogenase analysis

Sample no.	Glucose-6-phosphate dehydrogenase (U/gm)
1	0.118
2	0.148
3	0.141
4	0.096
Average	0.126
Standard deviation	0.024
Coefficient of variance (%)	19

A.6. Invertase

Reagents

0.1 M Sodium acetate buffer of pH 5.5

0.5 M Sucrose in distilled water

0.2 M Potassium di-hydrogen phosphate (KH_2PO_4) in water

DNSA reagent: Dissolve 150 grams of sodium potassium tartrate in 250 ml distilled water. Dissolve 5 gms of 3, 5 di-nitrosalicylic acid in 100 ml of 2 N NaOH. Mix the above two solutions and make up to 500 ml with distilled water

Method

1. Pipette 1 ml of sample from the supernatant into a clean test tube
2. Add 1ml of acetate buffer of pH 5.5 (0.1 M)
3. Add 0.5 ml of 0.5 M sucrose
4. Incubated at 55 °C for 10 minutes in a water bath.
5. After 10 minutes add 3 ml of 0.2 M KH_2PO_4 to terminate the reaction
6. Place the reaction mixture in boiling water bath for 3 minutes.
7. Pipette out 1 ml of this reaction mixture into another clean test tube
8. Add 1 ml of DNSA reagent and place in a boiling water bath for 10 minutes
9. Add 10 ml of distilled water to the above reaction mixture
10. Read the absorbance at 540 nm against blank (buffer pH 7.0) treated similar to the sample

Table A.6 Reproducibility of invertase analysis

Sample no.	Invertase (U/gm)
1	443
2	399
3	475
4	419
Average	434
Standard deviation	32.63
Coefficient of variance (%)	7.5

A.6.1. Calibration curve for glucose estimation by DNSA

1. Prepare glucose standard solutions over the range (0.01 – 0.05 mM)
2. Pipette out 1 ml of standard solution into a clean test tube
3. Add one ml of DNSA reagent (described in A.6)
4. Place the test tubes in a boiling water bath for 10 minutes
5. Add 10 ml of distilled water
6. Read the absorbance at 540 nm against blank (distilled water) treated similar to the sample

The calibration curve obtained is presented in the Figure A.3

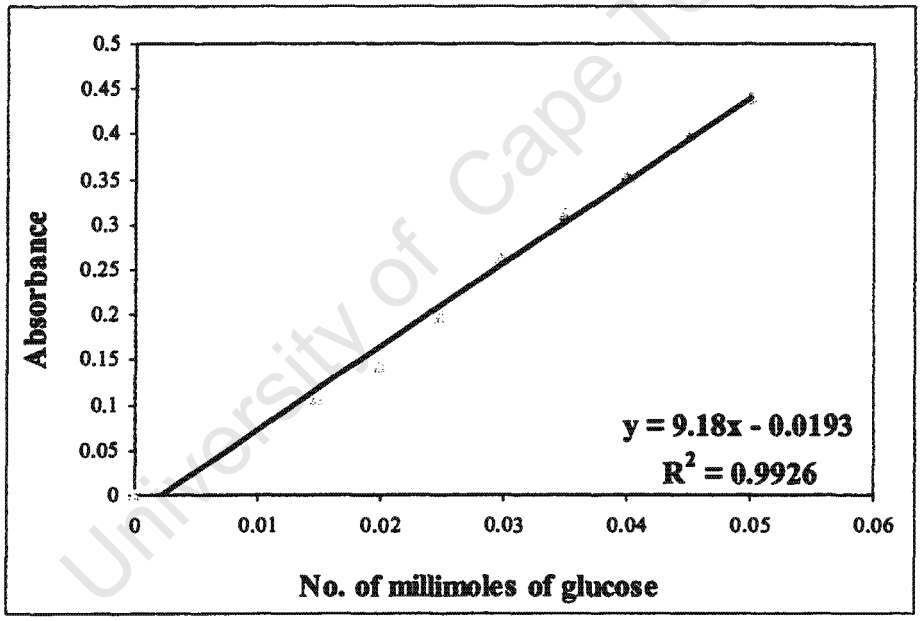

Figure A.3 Calibration curve for glucose by DNSA method of analysis

A.7. Folin-Lowry method of estimation of total soluble protein

Reagents

Reagent A: Dissolve 5 g of NaOH in 1 litre to which 20 g of Na_2CO_3 is added

Reagent B: Dissolve 0.5 g of $CuSO_4$ in a 100 ml distilled water containing 1g of sodium potassium tartrate.

Reagent C: Mix 2 ml of Reagent B with 100 ml of reagent A

Reagent D: 2 N Folin-Ciocalteu reagent is diluted 1:1

Bovine serum albumin standard (BSA): Prepare standard solutions of BSA in distilled water over the range 0.01 to 0.1 mg/ml

Method

1. Pipette 1 ml of sample into a clean test tube
2. Add 2 ml of reagent C and incubate at room temperature for 10 minutes
3. Add 0.1 ml of reagent D to the reaction mixture and incubate for 30 minutes
4. Dilute the reactions mixture with 3 ml of distilled water
5. Read the absorbance at 600 nm against a blank

Table A.7 Reproducibility of Folin-Lowry method of analysis of soluble protein

Sample no.	Souble protein (mg/ml)
1	0.062
2	0.067
3	0.057
Average	0.062
Standard deviation	0.0049
Coefficient of variance	8

A.8. Bradford's method of estimation of total soluble protein

Bradford reagent: Dissolve 100 mg of Coomassie Brilliant Blue G-250 in 95 % ethanol. Add 100 ml of 85 % (w/v) phosphoric acid to this solution. Dilute the resulting solution to a final volume of 1 litre.

Bovine serum albumin standard (BSA): Prepare standard solutions of BSA in distilled water over the range 100 to 10 µg/ml

Method

1. Pipette 0.1 ml of sample into a 2 ml plastic cuvette
2. Add 1 ml of Bradford reagent to the above and incubate for 2 - 5 minutes at room temperature

3. Read the absorbance after 2 minutes and before one hour at 595 nm against a blank (buffer pH 7.0) treated similar to the sample

Table A.8 Reproducibility of Bradford method of analysis for protein

Sample no.	Protein (mg/gm)
1	280
2	314
3	290
4	348
Average	308
Standard deviation	30.243
Coefficient of variance (%)	9.8

A.9. Slide-culture method for viability estimation

Yeast growth media (MPYG media)

Malt extract	3 g/l
Yeast extract	3 g/l
Peptone	5 g/l
Glucose	10 g/l
Agar	15 g/l

1. Dissolve the constituents in distilled water and sterilize by autoclaving and store at 4 °C
2. Melt the media using a microwave when needed and spread a thin layer on a glass slide using a pipette, aseptically and allow to cool down and solidify
3. Dilute the yeast samples 1:100 with 0.9 % saline
4. Spread a small volume on the surface of the solidified media and place a cover slip over it and incubate at 30 °C for 4 hours (Yeast cells when metabolically functional starts budding)
5. After 4 hours examine the slide under a microscope using 100X magnification

6. Count approximately 300-500 cells for each sample. (The cells with buds were considered as viable cells and cells with no buds were considered non-viable cells)
7. Calculate the percent viability based on the total number of cells counted

A.10. SDS-PAGE

A vertical polyacrylamide gel apparatus VG/01 purchased from Omeg scientific, Cape Town, South Africa was used. It consisted of 120 (h) x 100 (w) mm glass plates, 2 plastic spacers, a gel holder, a plastic comb, 2 combs, a upper and lower buffer chamber and a powerpack unit to cast and run the gel. The composition of the solutions and buffers used are presented in A.10.1

Preparation of gel

- Prepare a resolving gel by mixing the gel components (composition given in Table A.9) and mix them and cast to a height of 5 mm below the level of combs (Figure A.4)
- Level the resolving gel with distilled water and set it aside to polymerise for 1 hour
- After the separating gel had set, decant the distilled water and the unpolymersied material and wash with distilled water.
- Remove the excess distilled water on the gel surface using filter paper.
- Prepare the stacking gel according to Table A.10 and cast over the resolving gel
- Place the comb in the stacking gel and leave it to polymerize for 30 minutes.

Table A.9 Concentrations of the resolving gels (15 % acryl amide)

Acrylamide /bisacrylamide (ml)	16
4 X Tris-HCl / SDS pH 8.8 (ml)	8
Distilled Water (ml)	8
10% APS (µl)	108
TEMED (µl)	20

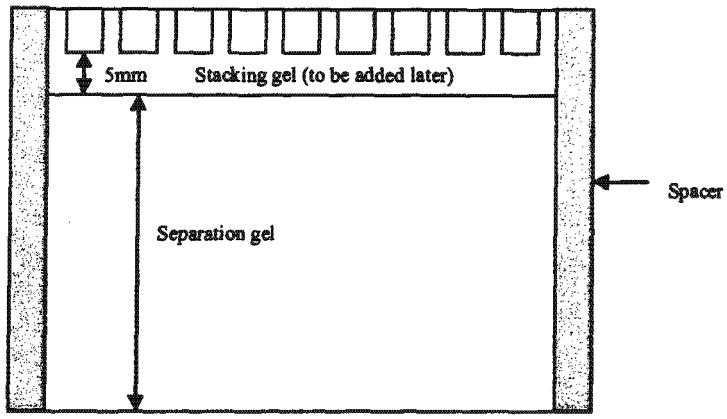

Figure A.4: Schematic representation of the glass plate setup used to cast the SDS PAGE gels

Table A.10 Volumes of various components of the stacking gel

Acrylamide/bisacrylamide (ml)	1.6
4 X Tris-HCl / SDS pH 6.8 (ml)	3
Distilled Water (ml)	7.32
10% APS (µl)	120
TEMED (µl)	12

Sample preparation and loading

- Concentrate approximately 50 ml of the supernatant from disrupted cell suspension from hydrodynamic cavitation to 10 ml using a pellicon Biomax 5 (5 kDa) membrane filter (5 times concentration)
- Concentrate 10 ml samples from high pressure homogenizer to 5 ml (2 times concentration)
- To 100 µl of samples add 200 micro litres of sample buffer and incubate at 60 °C for 20 minutes
- To 10 µl of molecular weight marker (purchased from Sigma, wide range molecular marker, 6.5 - 205 kDa) add 20 µl of sample buffer and incubate at 60 °C for 20 minutes

- Load approximately 50 micro litres of the treated samples and markers onto the well in the gel.
- Fit the gel to the apparatus and fill the tank with electrophoresis running buffer
- Run the gel at 15 mA for 6h and then remove and stain for 2 hours on a gel shaker with Coomassie blue stain
- After 2 hours destained the gel by removing the stain solution and adding destain I for 1 hour. Repeat this process with destain 1 for 30 minutes and then with destain 2 until the background of the gel is totally destained. Then transfer the gel to a clean transparent sheet and scan the gel

A.10.1. Composition of the solutions and buffers used for SDS-PAGE

Acrylamide/Bisacrylamide: 30 g Acrylamide mixed with 0.8 g Bisacrylamide and made up to 100 ml with distilled water and covered with foil

4X Tris-HCl/SDS pH 6.8: 12.08 g Tris mixed with 0.8 g SDS and made up to 170 ml with distilled water and adjusted to pH 6.8 with HCl and made up to 200 ml finally with distilled water

4X Tris-HCl/SDS pH 8.8: 36.4 g Tris mixed with 0.8 g SDS and made up to 170 ml with distilled water and adjusted to pH 8.8 with HCl and made up to 200 ml finally with distilled water

10X Electrophoresis buffer: 30.25 g Tris, 144 g Glycine and 10 g SDS were mixed and made up to 950 ml with distilled water and pH adjusted to 8.3 with HCl and made up to 1 litre finally with distilled water

2X SDS Sample buffer: 3.04 g Tris, 8 g SDS, 20 ml mercaptoethanol, 40 ml glycerol and 0.02 g bromophenol blue were mixed and made up to 170 ml with distilled water and pH adjusted to 6.8 with HCl and the volume made up to 200 ml with distilled water

Ammonium Per Sulphate (APS): 0.1 g in 1 ml of distilled water

Stain: 1.2 g Coomassie Blue R250 is dissolved in a mixture of 500 ml methanol, 400 ml distilled water and 100 ml Acetic acid

Destain I: 150 ml Acetic acid + 250 ml Methanol + 1600 ml distilled water

Destain II: 140 ml Acetic acid + 800 ml Methanol + 1060 ml distilled water

Appendix B: Analysis of protein and invertase release kinetics from yeast by hydrodynamic cavitation

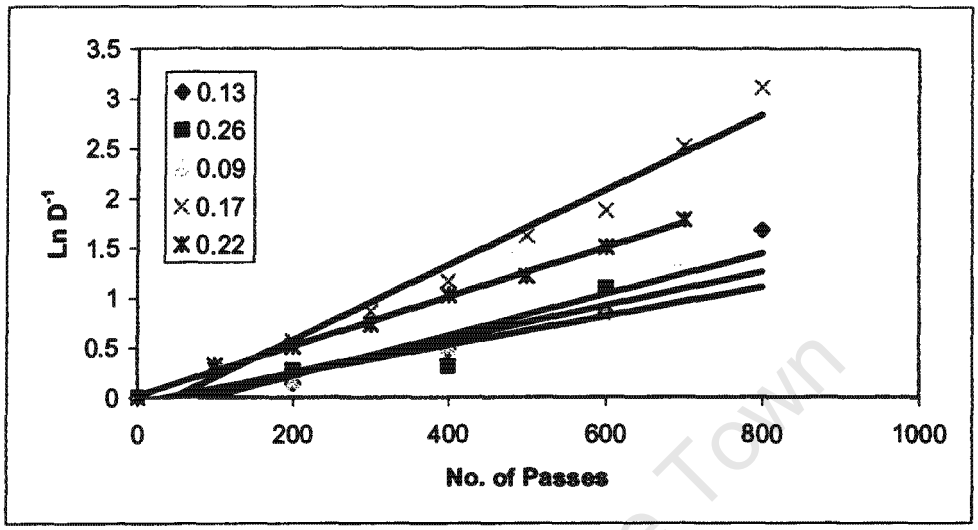

Figure B.1 **Analysis of soluble protein release kinetics from yeast by hydrodynamic cavitation at various cavitation numbers (1 % w/v, wet wt)**

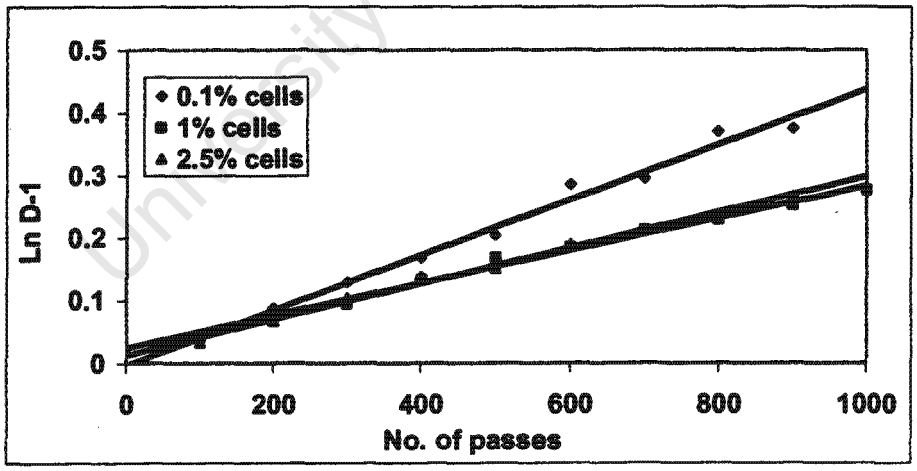

Figure B.2 **Analysis of invertase release kinetics from yeast by hydrodynamic cavitation at various cell concentrations (cavitation number 0.17)**

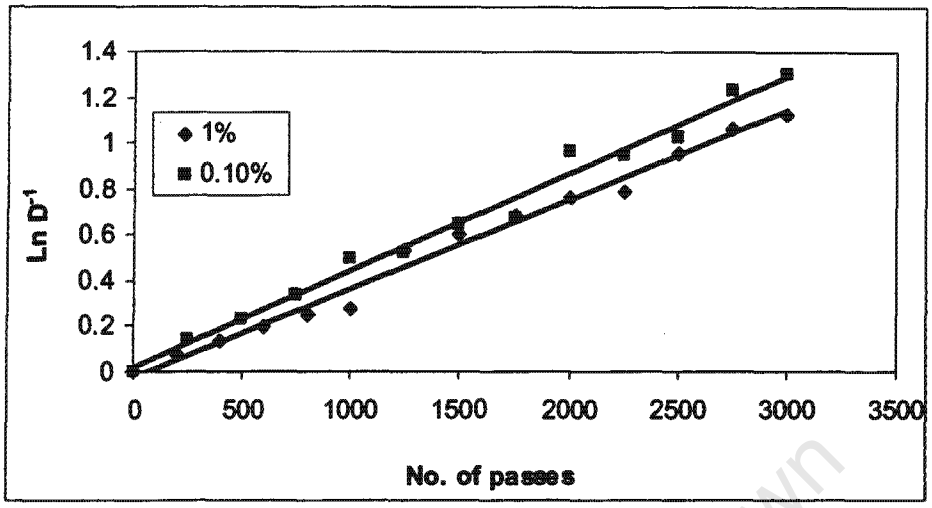

Figure B.3 Analysis of release kinetics of invertase from yeast by hydrodynamic cavitation at various cell concentrations (cavitation number 0.13)

Appendix C: Analysis of release kinetics of protein and enzyme from *E. coli* by hydrodynamic cavitation

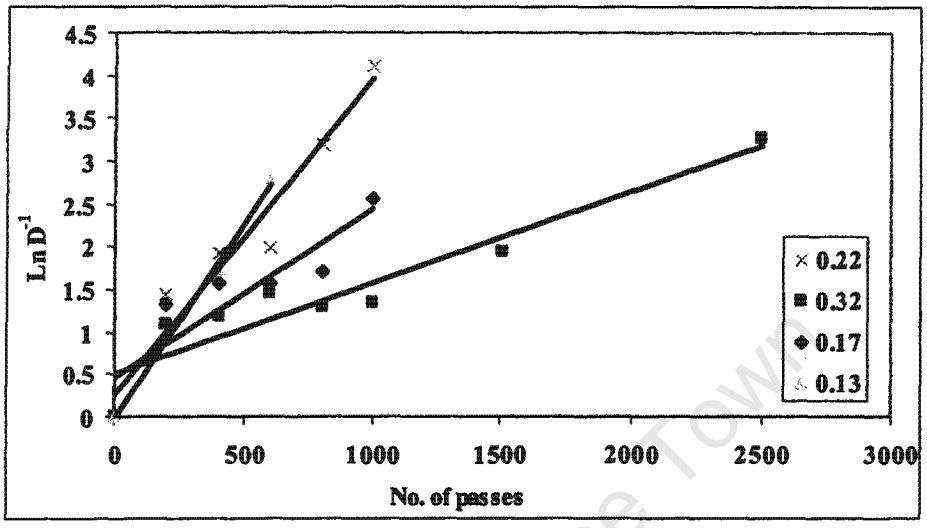

Figure C.1 Release kinetics of total soluble protein using hydrodynamic cavitation at different cavitation numbers (0.5 % w/v wet wt, μ_{max} 0.36, cavitation unit II)

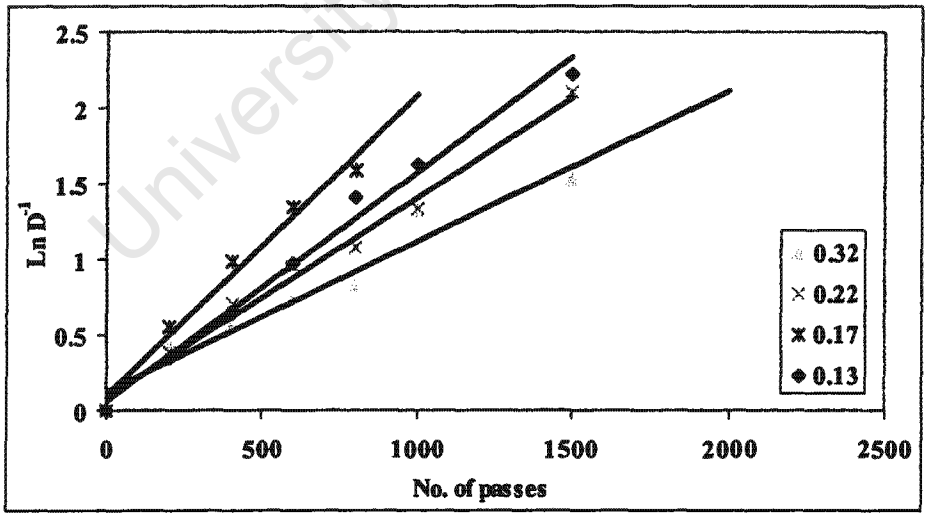

Figure C.2 Release kinetics of acid phosphatase using hydrodynamic cavitation at different cavitation numbers (0.5 % w/v wet wt, μ_{max} 0.36, cavitation unit II)

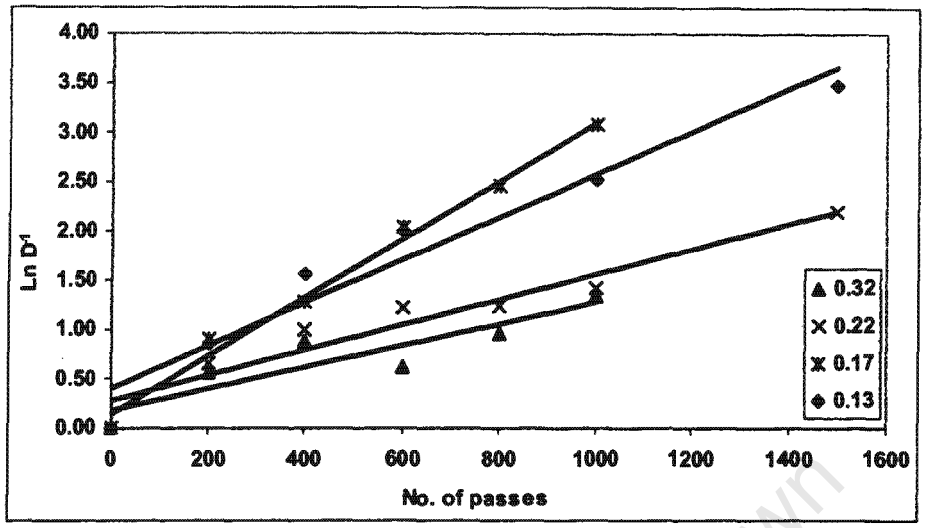

Figure C.3 Release kinetics of β-galactosidase from *E. coli* using hydrodynamic cavitation at different cavitation numbers (0.5 % w/v wet wt, μ_{max} 0.36, cavitation unit II)

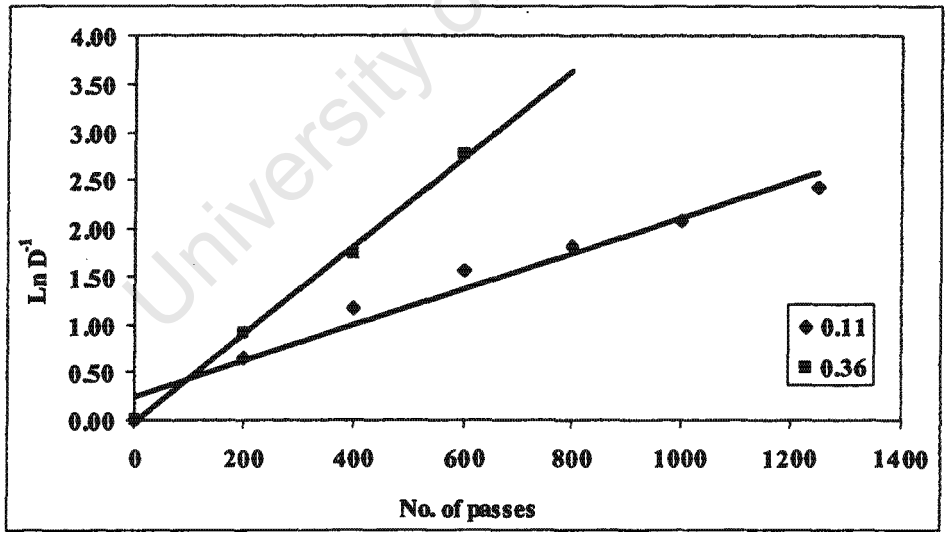

Figure C.4 Release kinetics of total soluble protein from *E. coli* using hydrodynamic cavitation at different growth rates (0.5 % w/v wet wt, cavitation number 0.13, cavitation unit II)

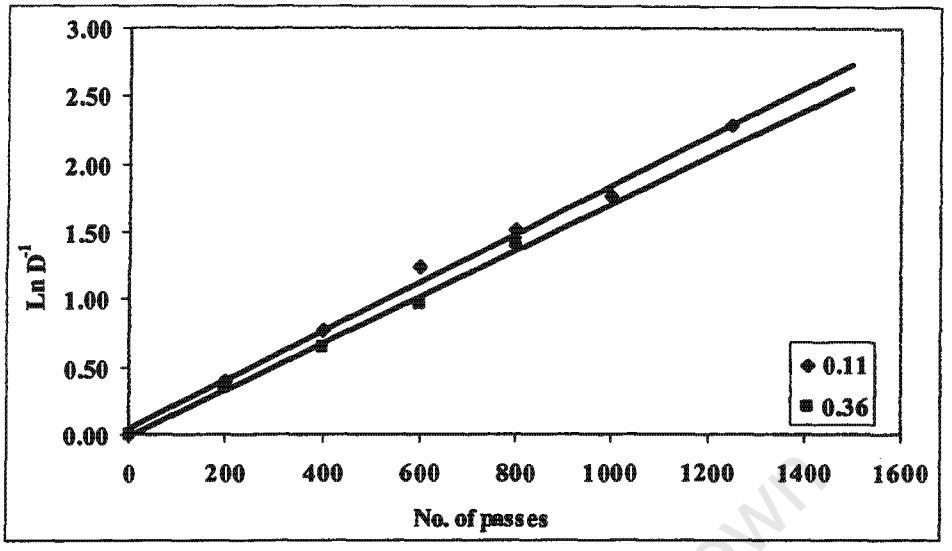

Figure C.5 Release kinetics of acid phosphatase from *E. coli* using hydrodynamic cavitation at different growth rates (0.5 % w/v wet wt, cavitation number 0.13, cavitation unit II)

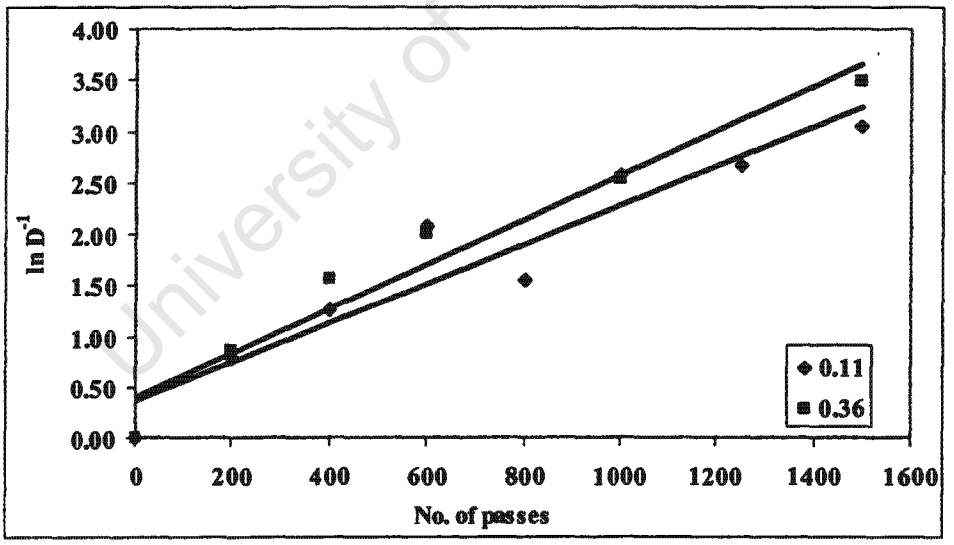

Figure C.6 Release kinetics of β-galactosidase from *E. coli* using hydrodynamic cavitation at different growth rates (0.5 % w/v wet wt, cavitation number 0.13, cavitation unit II)

Appendix D: Raw data from the yeast experiments

Release of proteins from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.17 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	0.1 % cells		1 % cells		2.5 % cells	
	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}
0	0	0	0	0	0	0
100	11.2	0.15	2.5	0.29	1.8	0.28
200	14.5	0.20	4.4	0.58	2.7	0.46
300	19.2	0.28	5.8	0.87	4.7	1.03
400	21.2	0.31	6.9	1.18	4.9	1.11
500	23.1	0.34	8.1	1.65		
600	24.2	0.36	8.5	1.91	6.3	2.04
700	28.7	0.45	9.3	2.59	6.6	2.40
800	30.1	0.47	9.6	3.24	7.3	
900	30.5	0.48	10.1		7.0	
1000	33.3	0.54	9.7			
French press	79.7		88.1		79.7	

Release of invertase from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.17 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	0.1 % cells		1 % cells		2.5 %cells	
	U/g	Ln D^{-1}	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}
0	0	0	0	0	0	0
100	41	0.04	44.0	0.19	38	
200	100	0.09			78	0.15
300	144	0.13	97.3	0.47	117	0.33
400	184	0.17	135	0.73	153	0.55
500	218	0.20	167	1.03	166	0.80
600	294	0.29	182		205	0.91
700	302	0.30	207	1.58	228	1.34
800	366	0.37	220	1.87	250	1.72
900	370	0.38	239	2.54	277	2.32
1000	390	0.40	260			
French press	118		1075		1183	

Release of proteins from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.22 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	0.1 %		1 %		2.5 %	
	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}
0	0	0	0	0	0	0
100	4.9	0.16	2.3	0.30	1.2	0.22
200	10.9	0.41	3.3	0.46	2.6	0.61
300	14.1	0.57	4.4	0.66	3.7	1.06
400	18.2	0.82	5.3	0.90	4.2	1.33
500	25.2		5.8	1.06	5.0	2.09
600	24.1	1.36	6.5	1.28	5.1	2.29
700	24.5	1.40	6.9	1.47	5.5	3.15
800	27.1	1.80			5.5	3.16
900	29.3	2.33	7.9	2.11	5.7	
1000	32.5		8.3	2.58	5.3	
French press	79.7		88.1		79.7	

Release of invertase from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.22 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	0.1 %		1 %		2.5 %	
	U/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0	0	0	0	0	0	0
100	113	0.34	57	0.35		
200	134	0.42	84	0.56	26	0.14
300	187	0.66	100	0.73	55	0.31
400	207	0.76	120	0.97	91	0.57
500	244	1.00	142	1.31	102	0.67
600			156	1.64	134	1.04
700	325	1.83	175	2.35	136	1.06
800	333	1.96	188	3.52	178	1.93
900	358	2.59	194	6.43	197	2.89
1000	387		194		208	
French press	1183		1075		1183	

Release of proteins from brewers yeast at 1% w/v (wet wt) cell concentration using cavitation unit I and using orifice plate for cavitation number 0.43 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	1 %	
	mg/g	Ln D^{-1}
0	0	0
100	3.3	0.38
200	4.7	0.58
300	6.2	0.88
400	5.2	
500	7.6	1.27
600	5.9	0.81
700	5.6	0.75
800	7.6	1.24
900	7.5	1.21
1000	10.6	
French press	79.7	

Release of invertase from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.43 for 1000 passes, French press disruption was performed using 1 % w/v (wet wt) concentration of yeast suspension at 20 MPa for 5 passes

No of passes	0.1%		1%		2.5 %	
	U/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0	0	0	0	0	0	0
100	41	0.30	16	0.36	43	0.55
200	58	0.46	14	0.29	56	0.82
300	75	0.64	22	0.52	74	1.34
400	97	0.95			83	1.72
500	101	1.02	30	0.84	88	2.12
600	111	1.21	33	0.98	90	2.26
700	117	1.36	37	1.19	92	2.50
800	145	2.54	40	1.42	93	2.68
900			51	3.06	99	4.26
1000	158		53		101	
French press	1183		1076		1183	

Release of invertase from brewers yeast at different cell concentrations (w/v, wet wt) using cavitation unit I and using orifice plate for cavitation number 0.99 for 1000 passes, French press disruption was performed with 1 % w/v (wet wt) concentration of suspension at 20 MPa for 5 passes

No of passes	0.1% cells		1% cells		2.5 % cells	
	U/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0				0		0.00
100	17	0.39	5	0.13	23	0.33
200	42	0.62	10	0.29	45	0.81
300	60	0.85	12	0.38	52	1.04
400	75				56	1.21
500	94	0.99	18	0.60	62	
600	82	1.23	27	1.10	62	1.46
700	93	1.25			65	1.67
800	94	1.79	38	2.90	75	2.65
900	109		36	2.23	80	
1000	131		40		80	
French press	1183		1076		1183	

Release of proteins from brewers yeast 1 % w/v (wet wt) cell concentration using cavitation unit II and using orifice plate for cavitation number 0.26 and 0.09 for 1000 passes each

No. of passes	Cavitation no. 0.26		Cavitation no. 0.09	
	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}
0	0	0	0	0
200	0.3	0.28	1.3	0.17
400	0.4	0.32	3.3	0.51
600	1.1	1.10	4.7	0.85
800	1.6	4.36	7.5	2.46
1000	1.6		8.2	

Release of proteins, invertase and alpha-glucosidase using cavitation unit II, 1 % w/v (wet wt) cell concentration and using orifice plate for cavitation number 0.13 for 3000 passes

No of passes	Proteins		Invertase		α-glucosidase	
	mg/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0		0.00		0.00		0.00
200	3.1	0.27	121	0.11	3154	0.40
400	5.0	0.49	216	0.21	5471	0.84
600	6.4	0.67	301	0.30	7951	1.75
800	7.7	0.90	373	0.39	9594	5.97
1000	8.7	1.11	419	0.45	9618	
1250	10.7	1.75	707	0.94	9305	
1500	11.0	1.87	776	1.10	9244	
1750	11.7	2.34	851	1.32	9020	
2000	11.6	2.25	919	1.56	9122	
2250	13.0		941	1.66	8512	
2500	11.5	2.16	1063	2.46		
2750	12.5	3.26	1130	3.58		
3000	12.2	2.85	1163			

Release of proteins, invertase and alpha-glucosidase using cavitation unit II, 0.1 % w/v (wet wt) cell concentration and using orifice plate for cavitation number 0.13 for 3000 passes

No of passes	Proteins		Invertase		α-glucosidase	
	mg/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0		0.00		0.00		0.00
250	4.5	0.33	234	0.21	1179	0.36
500	6.0	0.47	354	0.33	1667	0.55
750	8.8	0.79	489	0.49	3333	1.87
1000	11.0	1.16	673	0.77	3943	
1250	11.8	1.33	703	0.82	3618	
1500	12.3	1.45	818	1.05	3374	
1750	13.0	1.67	843	1.11	3455	
2000	13.5	1.86	1067	1.89	3780	
2250	12.3	1.45	1057	1.84	3618	
2500	15.8	4.16	1107	2.13	2927	
2750	16.0		1221	3.58	3780	
3000	16.0		1256		2602	

Release of proteins and invertase using cavitation unit II, 2.5 % w/v (wet wt) cell concentration and using orifice plate for cavitation number 0.13 for 1000 passe

No of passes	Proteins		Invertase	
	mg/g	Ln D^{-1}	U/g	Ln D^{-1}
0	0	0	0	0
200	1.8	0.33	110	0.40
400	2.9	0.62	161	0.66
600	4.7	1.39	222	1.10
800	4.7	1.39	319	3.17
1000	6.2		333	

Maximum amount of proteins and enzymes released from brewers yeast at various cell concentrations using hydrodynamic cavitation unit II and using orifice plate for cavitation number 0.13, for 1000 passes compared with disruption by high pressure homogenization of 1 % w/v (wet wt) cell concentration at 7500 psi for 10 passes

Cell conc.	Protein mg/g	%	α-glucosidase (U/g)	%	Invertase (U/g)	%	G6PDH (U/g)	%	ADH (U/g)	%
0.10	11.0	21.32	3670	6.55	673	39.06				
0.50	14.7	28.49	15218	27.18	678	39.35	0.15	10.00	3000	12.10
1.00	7.9	15.21	9263	16.54	399	23.15	0.15	9.67	2550	10.28
2.50	6.2	12.02	6356	11.35	333	19.33	0.21	13.50	1160	4.68
5.00	4.0	7.66	3341	5.97	351	20.37	0.15	9.84		
HPH	51.6		55991		1723		1.53		24800	

Percentage of total soluble proteins and invertase released at various cavitation numbers and at various cell concentrations (wet wt) for 1000 passes using cavitation unit I

Total soluble proteins				Invertase			
Cavitation no.	Cell concentration			Cavitation no.	Cell concentration		
	0.10 %	1 %	2.50 %		0.10 %	1 %	2.50 %
0.17	33.3	10.1	7.2	0.17	390	260	277
0.22	32.5	9.0	5.7	0.22	387	194	208
0.43	10.6	4.1	2.9	0.43	158	53	101
0.99	5.3	1.8	2.7	0.99	131	40	80

Appendix E: Raw data from the *E. coli* experiments

Raw data for disruption of *E. coli* by hydrodynamic cavitation (cavitation unit II 0.5 % w/v, wet wt, cavitation number 0.13, 2500 passes, μ - 0.36 hr^{-1}) and French Press (1 % w/v, wet wt, 20 MPa, 5 passes, μ - 0.36 hr^{-1})

No of passes	Protein		No of passes	Acid phosphatase		No of passes	β-galactosdiase	
	mg/g	%		U/g	%		U/g	%
0	0	0	0	0	0	0	0	0
200	31	34	200	154	26	200	594	40
400	43	48	400	245	41	400	812	54
600	49	54	600	322	54	600	888	59
1000	52	58	800	395	66	1000	945	63
1500	50	55	1000	418	70	1500	995	66
1750	52	58	1500	464	78	1750	1022	68
2000	48	53	1750	520	87	2000	970	65
2250	45	50	2000	458	77	2250	1026	68
2500	42	47	2250	430	72	2500	981	65
			2500	429	72			
French press	91			597			1502	

Release kinetics data for the calculation of the release rate of total soluble protein by hydrodynamic cavitation of *E. coli* cells at different cavitation numbers (μ_{max} 0.36, 2500 passes, 0.5 % w/v, wet wt, cavitation unit II)

No of passes	C_v no. 0.32		C_v no. 0.22		C_v no. 0.17		No of passes	C_v no 0.13	
	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}	mg/g	Ln D^{-1}		mg/g	Ln D^{-1}
0	0	0	0	0	0	0	0	0	0.00
200	6.	1.08	11.4	1.44	31.6	1.31	200	31.1	0.90
400	6.5	1.17	12.8	1.91	34.1	1.56	400	43.1	1.74
600	7.2	1.47	12.9	1.99	34.1	1.56	600	49.1	2.76
800	6.9	1.29	14.4	3.19	35.4	1.71	1000	52.4	
1000	7.0	1.34	14.8	4.11	39.9	2.56	1500	50.1	
1500	8.1	1.95	15.0		43.2		1750	52.3	
2000	9.5		14.6	3.65	40.2	2.67	2000	47.6	
2500	9.1	3.27	13.7	2.42	28.7		2250	45.0	
							2500	42.2	

Release kinetics data for the calculation of the release rate of acid phosphatase by hydrodynamic cavitation of *E. coli* cells at different cavitation numbers (μ_{max} 0.36, 2500 passes, 0.5 % w/v, wet wt, cavitation unit II)

No of passes	C_v no. 0.32		C_v no. 0.22		C_v no. 0.17		No of passes	C_v no 0.13	
	U/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}		U/g	Ln D^{-1}
0	0	0	0	0	0	0	0	0	0.00
200	48	0.44	73	0.39	222.2	0.55	200	154	0.35
400	59	0.56	115	0.70	328.8	0.99	400	246	0.64
600	71	0.73	139	0.94	387.3	1.34	600	323	0.97
800	76	0.82	150	1.08	417.4	1.59	800	394	1.41
1000	100	1.32	167	1.33	435.0	1.77	1000	418	1.62
1500	107	1.52	199	2.10	472.6	2.32	1500	464	2.22
2000	137		203	2.25	523.9		1750	520	
2500	127		227		470.1		2000	458	
							2250	430	
							2500	429	

Release kinetics data for the determination of release rate constant of β-galactosidase by hydrodynamic cavitation of *E. coli* cells at different cavitation numbers (μ_{max} 0.36, 2500 passes, 0.5 % w/v, wet wt, cavitation unit II)

No of passes	C_v no. 0.32		C_v no. 0.22		C_v no. 0.17		No of passes	C_v no 0.13	
	U/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}		U/g	Ln D^{-1}
0	0	0	0	0	0	0	0	0	0.00
200	149	0.56	307	0.66	593	0.90	200	594	0.86
400	203	0.88	403	1.00	719	1.27	400	812	1.57
600	161	0.62	449	1.22	868	2.03	600	888	2.01
800	214	0.95	452	1.23	914	2.46	1000	945	2.53
1000	258	1.35	485	1.43	953	3.07	1500	995	3.49
1500	262	1.40	567	2.20	943	2.87	1750	1022	5.52
2000	273	1.53	638		953	3.08	2000	970	2.91
2500	348		636		999		2250	1026	
							2500	981	

Release kinetics data for the determination of release rate constants of soluble protein and enzymes by hydrodynamic cavitation of *E. coli* cells (cavitation number 0.13, 2500 passes, 0.5 % w/v, wet wt, μ_{max} 0.11, cavitation unit II)

No. of passes	Total soluble proteins		Acid phosphatase		β-galactosidase	
	mg/g	Ln D^{-1}	U/g	Ln D^{-1}	U/g	Ln D^{-1}
0		0	0	0	0	0
200	31.6	0.65	91	0.39	594	0.78
400	45.2	1.16	150	0.77	788	1.26
600	51.9	1.55	199	1.23	964	2.08
800	55.0	1.81	219	1.52	864	1.53
1000	57.6	2.08	232	1.76	1016	2.56
1250	60.0	2.42	252	2.28	1025	2.67
1500	55.7	1.87	269	3.21	1049	3.04
1750	55.3	1.83	280	5.83	1101	
2000			280		1090	
2250	65.8		277			