Improving product release from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* by pretreatment for cell wall weakening and selective product release

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Abstract

Efficient intracellular product release from yeast is required for the recovery of many bioproducts, recombinant or other. Traditionally such product release is achieved by non-selective, energy demanding mechanical disruption. The fine debris resulting from mechanical disruption is also challenging in the solid-liquid separation in downstream process. This study investigates the effect of the pretreatment on the energy efficiency of cell disruption, the extent of product release and its selective product release. *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were used as the model microorganisms while disruption following pretreatment was achieved on exposure to ultrasound or passing through the high pressure homogenisation (HPH).

Pretreatments were selected for their ability to weaken the yeast cell wall, rather than to permeabilise the cell. This allowed product release to be concentrated into the disruption step only, not distributed between the disruption and pretreatments steps. Rapid temperature treatment at 40 to 60℃, pH shock across the range pH 9 to 11 and osmotic pressure between 0.5 MPa and 5 MPa were used as single pretreatment. Combined pretreatments were also considered. These were affected by diluting the yeast suspension into a pre-warmed pH or high osmolarity buffer. On dilution, the temperature was increased rapidly to 40℃, while the pH or osmotic pressure was increased to pH 10 or 1 MPa.

Increase in total soluble protein release and decrease in energy requirement of equivalent cell disruption were obtained after a rapid shift in temperature, pH or osmotic pressure. The optimum ranges for pretreatment found were temperatures between 40 and 50℃, pH 10 and 1 MPa, respectively. Combined pretreatment resulted more protein released than each single pretreatment, however, the impact of combination compared to the heat pretreatment at 40℃ was small. Improvement in both the rate and extent of protein release was illustrated. In all cases, the pretreatment by short time exposure was most efficient in ensuring cell weakening. Extended duration of pretreatment offered no advantage in overall protein release and frequently was disadvantageous owing to denaturation of protein. The selectivity of enzyme release achieved by pretreatment was investigated by enzyme analysis. The condition of pretreatment for marker enzymes release depended on the location of the enzyme. Harsh conditions, such as higher temperature, pH and osmotic

pressure and longer holding time were preferred by cytoplasmic enzymes. Longer sonication time and a greater number of passes were preferred by intracellular enzymes. The cell debris obtained on HPH following a combined pretreatment was analysed by the Malvern size analyser and microscopy. The micronisation of debris for equivalent product release could be solved by pretreatment. To sum up, the energy efficiency of microbial cell disruption, the ability to achieve selective product release and the degree of micronisation of cell debris can be manipulated by use of pretreatment prior to mechanical cell disruption. Heat pretreatment was more efficient than other single and combined pretreatments.

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Abbreviations and Nomenclature

Abbreviations

ADH	alcohol dehydrogenase
CTAB	cetyltrimethylammonium bromide
EDTA	ethylenediaminetetraacetic acid
G6PDH	glucose-6-phosphate dehydrogenase
G-HCl	guanidium hydrochloride
HPH	high pressure homogenisation
NAD	nicotine adenine di-nucleotide (oxidized form)
NADH	nicotine adenine di-nucleotide hydrogen (reduced form)
NADP	nicotine adenine di-nucleotide phosphate (oxidized form)
NADP	nicotine adenine di-nucleotide phosphate hydrogen (reducedform)
PHB	polyhydroxybutyrate
SDS	sodium dodecyl sulphate

Nomenclature

ρ	density of the suspension	kg/m^3
a	pressure exponent of soluble protein release	
C_v	cavitation number	
E	energy per unit volume	J/m^3
E_n	normalised enzyme release	
k	kinetic rate constant (HPH)	$pass^{-1}$
N	number of passes	
P	operating pressure	MPa
P_3	pressure downstream of the orifice plate	MPa
P_v	vapour pressure of the liquid	MPa
R	soluble protein release	mg/g
R_i	maximum soluble protein release under specific conditions	mg/g
R_m	maximum soluble protein available for release	mg/g
R_i/R_m	extent of protein release	
T	temperature	°C
t	time	min
v	orifice velocity	m/s

Chapter 1
Introduction

Microorganisms provide a large variety of biological products of commercial interest. The genetic manipulation has increased the potential usage of microorganisms in the medical, food and chemical industry. Some microbial products can be found extracellularly due to the natural transport of the products from the cell to the environment. However, microorganisms do not excrete the majority of biological products into the medium. For the exploitation of intracellular products of interest, such as intracellular enzymes and products of recombinant DNA, cell disruption is typically required.

The intracellular products are separated from the external medium by the cell wall and membrane, which also give shape and strength to the microorganism. The network components of the cell wall have to be disrupted to release the intracellular products. Hence, to understand the composition and structure of cell wall is important for analysis of cell disruption.

Cell disruption can be broadly classified in mechanical and non-mechanical methods. Mechanical methods enable high recovery of the products and are preferred by the industry. High pressure homogenisation and bead mill are widely used on an industrial scale. However, several drawbacks are associated with mechanical methods. These techniques provide no selective release of intracellular product, hence, the product of interest needs to be separated from other contaminant products. These methods are energy-intensive. Heat removal processes are required, due to the dissipation of the energy expended as heat. The cells are passed repeatedly through the disruption equipment to increase the extent of disruption causing a considerable decrease in the particle size of the debris, resulting in a more complicated solid-liquid separation.

Non-mechanical methods include chemical, physical and biological methods. These methods are less energy intensive and some have been demonstrated to achieve

selective product release. However, owing to restriction of process economics or efficiency, they are generally limited to a small scale. Consequently, they are used in primarily at laboratory scale. The combination of a mechanical method and a non-mechanical pretreatment has potential for reduction in the energy requirement.

This study investigates the extent and selectivity of product release and minimisation of the energy required for mechanical cell disruption by using pretreatments. Moreover, it seeks to maximise the size of cell debris resulting to aid solid-liquid separation while still ensuring efficient product release. To achieve this, it is necessary to identify pretreatments to weaken cell structure while not denaturing product. *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were chosen as the model microorganism, while high pressure homogenisation and ultrasonication were used to achieve mechanical disruption. Pretreatments include heat, pH, osmotic shock and combined pretreatment. These pretreatments were implemented for the permeabilisation or weakening of the cell envelope prior to mechanical disruption. The effect of pretreatment on micronisation was studied using the Malvern size analyser and microscope.

A detailed description of the cell envelop of the microorganisms and a literature review of cell disruption are provided in Chapter 2. The experimental procedure and methods are described in Chapter 3. The rate and extent of total protein release and enzyme release from Baker's yeast on different mechanical methods in the absence of pretreatment are presented in Chapter 4 to provide a benchmark for the comparison of combined microbial cell disruption. The effect of single pretreatment on the energy efficiency of cell disruption and selective product release on Baker's using ultrasonication is discussed in Chapter 5. The comparison of each single and combined pretreatment on protein and enzyme release from *S. cerevisiae* and *K. lactis* using high pressure homogenisation are demonstrated in Chapter 6 and 7, respectively. In Chapter 8, the conclusions drawn from this project and recommendations made for further work are presented.

Chapter 2
Literature Review

2.1 INTRODUTION

The requirement of microbial cell disruption has restricted large-scale production of commercial biotechnological products of intracellular derivation to value added products, owing to both the energy intensity of the disruption step and the increased purification challenge on release of the many intracellular constituents. Not only do the cell wall and membrane give shape and strength to the microorganism, but also form a barrier to the release of the intracellular products. Cell disruption is the most prominent unit for the isolation of intracellular products that are not secreted by the microorganism. A selective release of the desired enzyme relative to release of other intracellular proteins without the formation of fine cell debris would be rewarded.

2.2 THE STRUCTURE OF YEAST

Yeasts are typical eukarytotic microorganisms, comprised of nucleus, mitochondria, Golgi apparatus secretory vesicles, endoplasmic reticulum, vacuoles, ribosomes and microbodies. These cellular contents are encased by a cell envelop, which occupies about 15% of the total cell volume (Walker, 1998). The cell envelope consists of a rigid cell wall, a plasma membrane and a periplasmic space. The cell wall provides a rigid outer support while the concentration gradients between the interior and exterior of the cell are established across cytoplasmic membrane which provides the biological barrier to the cell. The cytoplasmic membrane generally is not concerned in disruption processes, since the primary resistance of disruption is provided by the cell wall. Hence, it is important to understand the composition and structure of cell wall for analysis of cell disruption (Harrison, 1991; Middelberg, 1995).

2.2.1 Cell wall

The yeast cell wall is generally thick, approximately 100 to 200 nm, comprising 15 to 25 % of total dry mass of the cell (Walker, 1998). For example, Moor and Muhlethaler (1963) reported that the cell wall of the baker's yeast strain they studied was some 70 nm thick during growth, with the thickness increasing with the age. The basic structural components are glucan, mannan and proteins (Phaff, 1971). The glucan and mannans account for roughly 80 to 90% of the cell wall. There are two types of glucan linkages, namely β-1,6 and β-1,3 linkages. Mannans are characterised by a backbone of mannose residues in α-1,6 linkage having a short oligosaccharide side chains composed of α-1,2 and α-1,3 lingakage. The majority of proteins complexed with mannans are functional enzymes rather than structural components. Chitin, a polymer of N-acetylglucosamine, is present in small amounts and is associated with bud scars.

The cell wall is a layered structure consisting of an inner layer of glucan microfibrils and an outer surface of mannan-protein. The outer surface comprises cross-linked mannoproteins, which are linked together by hydrophobic interaction or by disulphide bonds. The porosity of the yeast cell wall is determined by the mannoproteins which are selectively permeable to solutes larger than 600 Da. The linkage between the outer surface and inner fibrillar glucan network is covalent. Figure 2.1 depicts the structure of the yeast cell wall. The physicological functions of the yeast cell wall include mechanical strength, cell protection and shape maintenance. Hence, the resistance of yeast cell walls to disruption appears to be a function of the degree of cross-linking and the thickness of the structural components (Walker, 1998).

2.2.2 Periplasm

This cell wall-associated region between the cell wall and cytoplasmic membrane is 3.5 to 4.5 nm thick. Mainly secreted proteins are found in the periplasmic space (Hunter and Asenjo, 1988; Bailey and Ollis, 1986). Invertase, a glycoprotein enzyme, is associated with the periplasmic space (Tuite and Oliver, 1991; Walker, 1998). Since invertase is a cell wall associated protein, it can be used as an indicator of damage of the yeast cell wall.

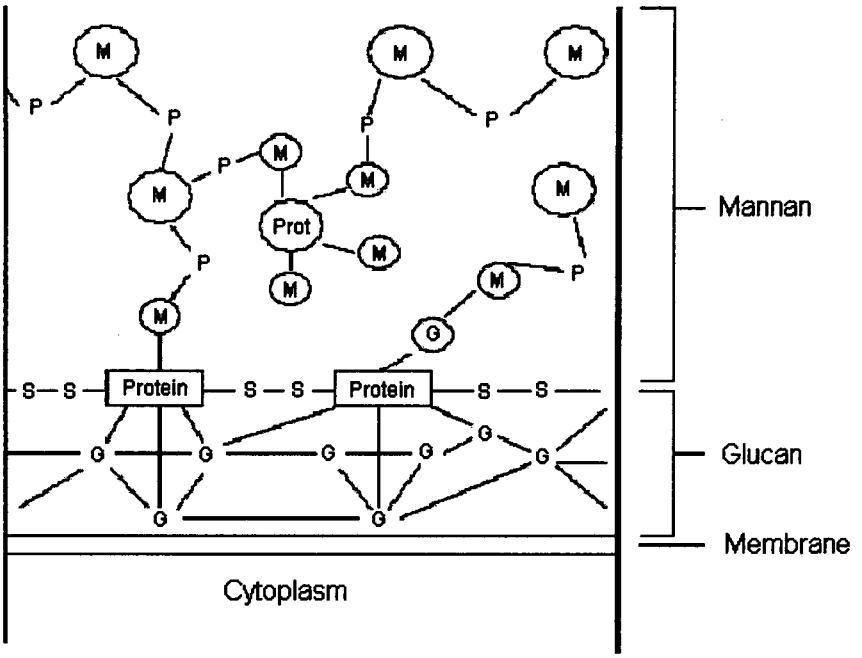


Figure 2.1 Schematic structure of yeast cell wall: the outer portion contains mannans (M) which may be linked by phosphodiester bridges (P); the inner portion contains cross-linked glucans (G); proteins are linked to mannans and glucans and are themselves linked by disulphide bonds. (Lampen, 1968).

2.2.3 Cell membrane

The plasma membrane forms the biological barrier of the cell and is approximately 7.5 nm thick. It is a lipid bilayer scattered with globular proteins representing a fluid mosaic. The lipid components are comprised mainly of phospholipids and sterols (Tuite and Oliver, 1991). The phospholipids confer fluidity and sterols provide rigidity of the membrane. The protein components present in the plasma membrane are involved in solute transport (ATPase and permeases), the cell wall biosynthesis, transmembrane signal induction and cytoskeletal anchoring.

The main function of the membrane is to maintain concentration gradients between the interior and exterior of the cell. Sugars, nitrogenous sources, ions and solutes are selectively transported across the membrane. Other functions of the membrane include signal transduction, exocytosis and endocytosis (Walker, 1998).

2.2.4 Cell wall structure and disruption

For liberation of intracellular products, the cell wall and cytoplasmic membrane must be damaged such that they no longer to separate the intracellular components from the environment (Engler, 1985; Harrison, 1991). The major resistance to disruption is provided by the cell wall. The shape and strength of the cell depends on the degree of the cross-linking of the structural cell wall polymers. The covalent bond of the structural network must be overcome during the disruption.

The nature of the yeast cell wall is greatly dependent on the type of the microorganism, the growth conditions including temperature of cultivation and nutrients available, the composition of the growth medium, growth phase and specific growth rate (Suterlan, 1975; Engler and Robinson, 1981; Engler, 1985; Harrison, 1991; Collis *et al.*, 1995, Middelberg, 1995).

Based on the understanding of the cell wall, altering growth conditions may affect thee strength of the cell wall and make the cell easier to disrupt. Similarly, certain pretreatments can attack the component of the cell wall specifically to weaken it, and therefore make the subsequent disruption easier.

The microorganism contains a variety of proteins and enzymes. A non-selective disruption results the target protein liberated in a complex mixture of proteins and other biomolecules. Moreover, the micronisation of cell debris by the mechanical disruption complicates solid-liquid separation. Enzymes are located in different compartments of the cell such as the cell wall, periplasm, cytoplasm and cytoplasmic organelles. The release rate of the enzyme depends on the location of the enzyme and the mechanism of disruption. The cell wall bound enzyme would be expected to be released faster than the periplasmic membrane followed by the total soluble protein, cytoplasmic enzymes, cytoplasmic membrane bound enzymes and enzymes enclosed in the organelles. The cell wall of microorganism broken or permeabilised in the first step to release the periplasmic enzymes, while the rupture of the cytoplasmic membrane in the second step to release the cytoplasmic enzymes would be a ideal disruption strategy. Huang et al. (1991) had reported using chemicals and enzymes for enzyme release from different location of the cell; however, the methodology proposed was not readily scaleable to large scale disruption. 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.

2.3 CELL DISRUPTION

Cell disruption is the most prominent unit for the isolation of intracellular products that are not secreted by the microorganism. There are two major categories of cell disruption, namely mechanical and non-mechanical. Several mechanical methods have been used to release intracellular products. These include bead mills (Schutte *et al.*, 1983; Melendres *et al.*, 1993; Garrido *et al.* 1994), high pressure homogenisation (Follows *et al.*, 1971; Kula and Schutte, 1987; Sauer *et al.*, 1989) and cavitation (Neppiras and Hughes, 1964; Balasundaram and Pandit, 2001). Non-mechanical methods include physical, chemical and enzymatic methods. The methods used for cell disruption are shown in Figure 2.2.

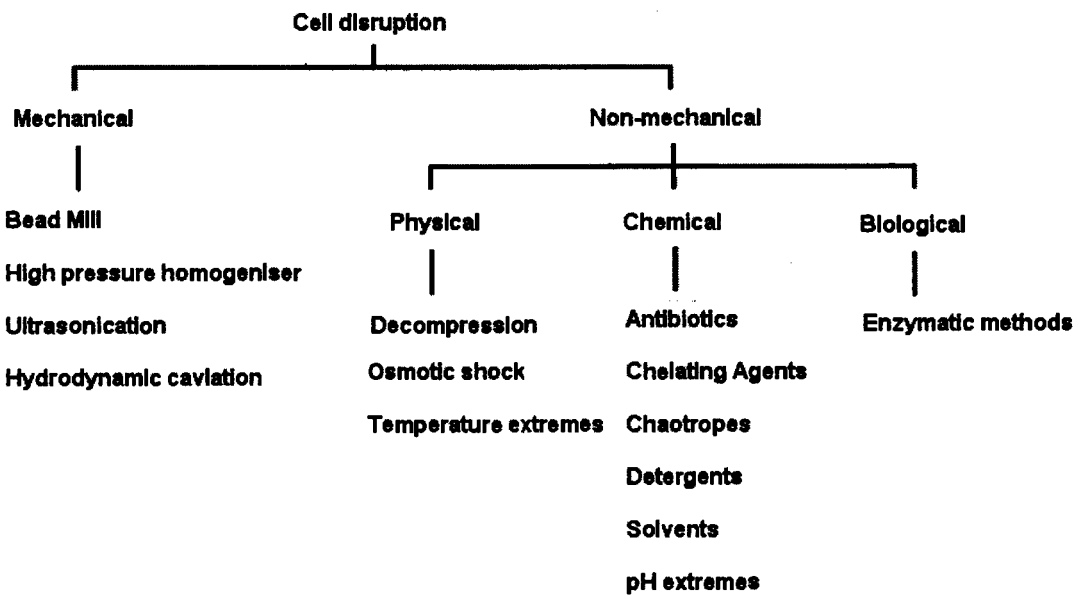


Figure 2.2 Methods of microbial cell disruption (adapted from Chisti and Moo-Young, 1986; Middelberg, 1995)

The mechanical methods are more commonly used in commercial application than the non-mechanical methods, because the extent of breakage by the latter is limited. Further there are difficulties in removing of the exogenous chemicals or enzymes from the product and enzymatic lysis may be cost prohibitive. Typically, the bead mill and the high-pressure homogeniser are used in the large-scale cell disruption. Non-mechanical methods can be used in high value, low volume processes as for the release shear sensitive products, such as plasmid DNA.

Mechanical cell disruption typically results in the complete disruption of cells, making it necessary for the target products to be separated from many other contaminants. Hence a selective release of the desired intracellular product relative to total release

of cytoplasmic material would be rewarded. Although the non-mechanical methods are not preferred in industry as sole disruption methods, they can be used as pretreatment prior to mechanical methods for weakening the cell wall to allow product release at lower energy disruption (Harrison *et al.*, 1991a), and potentially to enhance selective product release.

2.4 MECHANICAL METHODS

The mechanical methods used for cell disruption are depicted in Figure 2.3. Bead mill and high pressure homogenisation have been most frequently used in industry. Common disadvantages of mechanical disruption include high capital investment and energy costs (Harrison, 1991; Chisti and Moo-Young, 1986). These methods offer a near complete liberation of the intracellular products, which complicates the subsequent purification of the target proteins. They are not suitable for the shear sensitive products due to the harsh conditions during the disruption. A significant amount of energy is converted to heat, hence excellent heat exchange to control the temperature is required (Middelberg, 1995). Micronisation of the cell debris is another challenge of the mechanical methods.

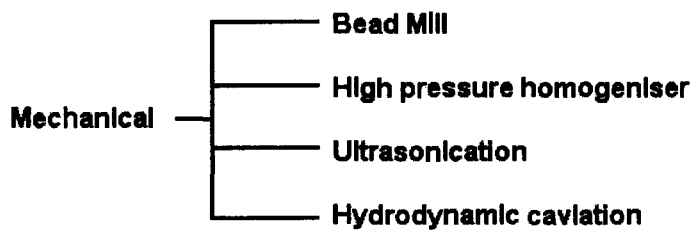


Figure 2.3 Mechanical methods of microbial cell disruption

2.4.1 Ultrasonication

Ultrasonication is one of the most used laboratory disruption methods (Engler, 1985). Ultrasound, sound waves of frequency higher than 15 to 20 kHz which is not audible to the human ear, can cause both inactivation and, at higher power acoustic power input, disruption of microbial cells in suspension. In principle, ultrasonic devices can be scaled up and operated continuously (James *et al.*, 1972). However, ultrasonication is typically ineffective for the large scale disruption because of the excessive heating. Most of the ultrasound energy absorbed into the cell suspension is converted to heat, hence good temperature control is necessary. Sonication may

cause significant degradation of enzymes due to heat denaturation because of insufficient cooling in close proximity of the sonication probe (Chisti and Moo-Young, 1985; Engler, 1985). It is also very sensitive to operating volume used. Finally, ultrasonic cavitation produces very fine cell debris, which may complicate subsequent processing, specifically solid-liquid separation (Chisti and Moo-Young, 1986).

In ultrasonication, the mechanism of the cell disruption is associated with the cavitation phenomena. The molecules of the medium compress and stretch alternatively when sounds waves are transmitted through a liquid medium. A cavity is formed when the intramolecular forces are exceeded. The formation of cavities by ultrasound differs from hydrodynamic cavitation with the former being assymmetrical leading to free radical formation. Disruption is caused by shear stresses developed by viscous dissipative eddies arising from shock waves produced by oscillating and imploding cavities (Doulah, 1977). When the cavities collapse, the sonic energy release is converted to mechanical energy. The cell disintegrates when the kinetic energy content of the cell exceeds the strength of the cell wall.

The release kinetics of ultrasonication was found to follow first order release kinetics and confirmed by Kuboi *et al.* (1995) in the disruption of *Escherichia coli*. The first order kinetics is discussed in Section 2.4.3.The factors that affect cell disruption using ultrasonication include acoustic power input, temperature of the suspension and working volume. The disruption rate was found to increase linearly for *E. coli* with acoustic power increasing in the range of 20 to 80 W (Kuboi *et al.*, 1995) and in the range 100 to 200 W (Fonseca and Cabral, 2002). Similarly, James *et al.* (1972) reported that the disruption rate was found to increase linearly with the acoustic power increase over the rate of 67 to 187 W when sonicating 200 ml of a 20 % brewer's yeast suspension. They also showed that the amount of total soluble protein release increased from 52 % to 63 % with the temperature increased from 17 to 30°C. However, increase in temperature may result in protein denaturation, which should generally be avoided. Therefore good temperature control is necessary (Chisti and Moo-Young, 1986) and the upper limit of temperature of cell suspension needs to be considered. The summary of the effects of working volume on ultrasonication is shown in Table 2.1. The disruption rate decreased linearly with an increase in working volume.

Table 2.1 Summary of the effect of working volume on disruption using ultrasonication

Microorganism	Power, cell concentration	Working volume	Results	Reference
Baker's yeast	140 W (20 kHz), 20 % w/v (wet wt)	75-450 ml	The protein release reported independent of the volume	James *et al.*, 1972
Brewer's yeast	30 W (20 kHz), 20 % w/v (wet wt)	10-20 ml	The protein release decreased from 50 % to 25 % with volume increase	Neppiras and Hughes, 1964
Escherichia coli	40 W (20 kHz), 2.5 g/l (wet wt)	2.5-10 ml	The disruption rate decreased with increase in volume	Kuboi *et al.*, 1995

2.4.2 High pressure homogenisation

High pressure homogenisation is a widely known disruption method, and remains the method of choice in the bioprocess industry (Middlberg, 1995) for disruption of non-filamentous organisms on a large scale disruption. Low pressure homogenisation, used for milk industry to break large fat globules to smaller ones (<1 μm) to prevent the separation of cream from the milk, was introduced in the 1950's (Loo *et al.*, 1950). High pressure homogenisation is commonly used in food and pharmaceutical industries (Shutte and Kula, 1987).

The disruption of *Saccharomyces cerevisiae* on exposure to high pressure homogenisation is well documented (Hetherington *et al.*, 1971; Follows *et al.*, 1971; Brookman, 1974; Doulah and Hammond, 1975, Limon-Lason *et al.*, 1979; Engler and Robinson, 1981; Keshavarz-Moore *et al.*, 1990). Further studies of bacterial cell disruption by high pressure homogenisation include Gray *et al.* (1972), Engler and Robinson (1981), Sauer *et al.* (1989), Harrison *et al.* (1991a,b), and Fonseca and Cabal (2002). The homogeniser consists of a high pressure positive displacement piston pump and a homogeniser valve. The cell suspension is delivered by the positive piston pump, and forced through a spring-loaded or hydraulically-controlled orifice to adjust the pressure (Middelberg, 1995). The fluid flows radially across the valve and strikes an impact ring. The suspension exits the valve assembly and flows to either a second valve or to discharge. Different valve units can be used, examples of which are presented in Figure 2.4. The knife edged units release more proteins than the standard units due to the more rapid pressure drop determined by the narrowness of the valve opening (Chisti and Moo-Young, 1986).

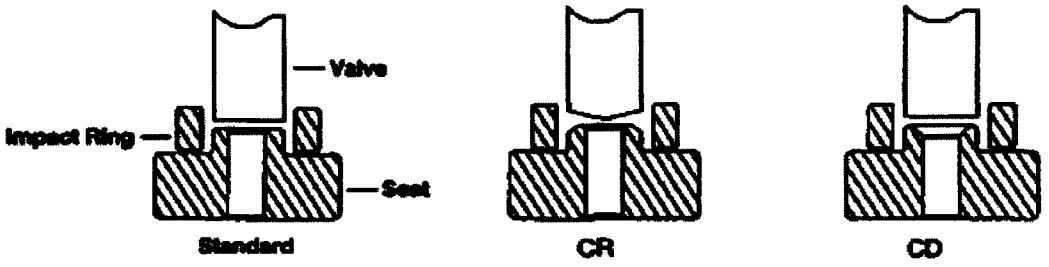


Figure 2.4 **Various homogeniser valve designs (Middelberg, 1995).**
'Standard': flat-edge 'Standard' unit (which is typically employed for emulsion and dispersion application);
'CR': knife-edge 'Cell rupture' unit;
'CD': knife-edge 'Cell Disruption unit.

The most widely used homogeniser is Manton-Gaulin APV design (Middelberg, 1995). Increase of the temperature of cell suspension is common, due to adiabatic compression in the homogeniser of about 2°C per 10 MPa of pressure (Chisti and Moo-Young, 1986). With increase in pressure, the number of passes required for equivalent disruption decreased, but the increase in temperature may result in increased protein denaturation, unless sufficient cooling during the disruption is required.

Disruption of microbial cells in the high pressure homogeniser is accomplished by passing the cell suspension through an adjustable restricted orifice discharge valve. There are fewer operational parameters to consider than with high speed bead mills (Moo-Young, 1995). These parameters include operating pressure, number of passes of cell suspension through the valve (Engler, 1985), homogeniser design (Keshavarz-Moore et al., 1990a) and suspension temperature (Hetherington et al., 1971). The French Press operates on the same principle as the high pressure homogeniser and is a batch system used for small scale operation in the laboratory. Here the valve is replaced by a capillary orifice and the impact ring absent. The kinetics of Baker's yeast cell disruption in a high pressure homogeniser was first studied by Hetherington et al. (1971). The process was independent of the cell concentration across the range 300 to 600 g/l (wet weight). The release kinetics for yeast and the pressure exponent described by Hetherington *et al.* (1971) is calculated using Equation 2.1 and Equation 2.2:

$$kN = \ln\frac{R_m}{R_m - R} \qquad \textbf{Equation 2.1}$$

$$k = k'P^a \qquad \textbf{Equation 2.2}$$

where 'R_m' is the maximum soluble protein available for release; 'R' is the soluble protein released; 'k' is the rate constant; 'N' is the number of passes through homogeniser; 'k'' is the dimensional constant; 'P' is the operating pressure; 'a' is pressure exponent. The pressure exponents of different microorganisms are illustrated in Table 2.2.

Table 2.2 Summary of the a (pressure exponent) with different microorganisms

Microorganism	Phase	a	Reference
Saccharomyces cerevisiae	Stationary	2.9	Hetherington *et al.*, 1971
Saccharomyces cerevisiae	Stationary	1.87	Engler and Robinson, 1981
Escherichia coli *Escherichia coli* (Recombinant)	Stationary Stationary	1.43 1.41	Sauer et al., 1989
Escherichia coli	Stationary	2.2	Gray *et al.*, 1972
Cupriavidus necator (*Alcaligenes eutrophus*)	Exponential Stationary	3.08 1.59 to 1.69	Harrison *et al.*, 1991c

The protein release is dependent on pressure, the number of passes and temperature. To achieve the same protein release, more passes are required at a lower pressure than at high pressure. A single pass is required for 75 % of total protein release at a pressure of 69 MPa, while the same level of disruption was achieved at 27.6 MPa on three passes, when disrupting *Cupriavidus necator* (Harrison *et al.*, 1991c). Table 2.3 illustrates the effect of operating pressure.

Table 2.3 Summary of the effect of operating pressure on cell disruption

Microorganism	Pressure (Pa)	Results	Reference
Baker's yeast	0-49.03	The release rate of total protein increased with increase in the pressure	Hetherington *et al.*, 1971
Escherichia coli	15-95	The fraction of the cells disrupted increased with increase in the pressure	Sauer *et al.*, 1989
Escherichia coli	19.61-49.03	The release rate of total protein and β-galactosidase increased with increase in the pressure	Gray *et al.*, 1972
Cupriavidus necator	0-62	Disruption was a sigmoidal function of the pressure	Harrison *et al.*, 1991c

Micronisation of cell debris results in complex downstream processing (Harrison, 1991). It occurs even at low operating pressure, and is aggravated by an increase in the number of passes. The ease of disruption using high pressure homogenisation is related to the composition of the cell wall, cell size and shape (Engler, 1985). Yeasts are more difficulty to disrupt in the homogeniser than Gram-negative bacteria because of their cell wall structure and thickness. Disruption characteristics of a microoganism can be altered by changing the growth condition. Table 2.4 summarises the effects of the growth rate on the disruption rate constant (k). Microbial cells with higher specific growth rate can be disrupted rapidly (Engler and

Robinson, 1981). Growth at a fast growth rate produced cells with weaker cell walls, as the cells would not have enough time to produce material for reinforcing the cell wall structure. Hence these cells can be disrupted easily.

Table 2.4 Summary of the literature on the effect of growth rate (μ) on the disruption rate constant (k)

Microorganisms	μ (hr^{-1})	k (MPa^{-1})	Reference
Escherichia coli	0.35 (μ_{max})	1.4×10^{-3}	Sauer *et al.*, 1989
	0.33	1.6×10^{-3}	
	0.24	0.6×10^{-3}	
	0.17	0.27×10^{-3}	
Escherichia coli (Recombinant)	0.35 (μ_{max})	62.9×10^{-3}	
	0.17	56.7×10^{-3}	
Cupriavidus necator	Exponential phase	1.35×10^{-3}	Harrison *et al.*, 1991c
	Later exponential phase	1.85×10^{-3}	
	Stationary phase	1.18×10^{-3}	
	Later stationary phase	2.16×10^{-3}	

2.4.3. Bead mill

The bead mill provides a simple and effective means for disrupting different types of microorganisms, and is widely used in large-scale cell disruption. Bead mills were developed in industry for fine grinding and dispersion of dyestuffs and pigments and their use extended to bioprocesses. Lead-free glass beads of 2.5 g/cm^3 are generally used for microbial cell disruption (Engler, 1985). Collisions and grinding between stream layers of solid particles of different velocity results in disruption of the microbial cells (Engler, 1985; Harrison *et al.*, 1991a). Complete disintegration of mycelial microbial cells is best be achieved by bead mill, because they may block, the valve of the high pressure homogeniser owing to their mycelial or pellet morphology (Chisti and Moo-Young, 1986).

Various designs of bead mill are available depending on the size of the unit and the manufacturer. Mills consist of either a vertical or a horizontal grinding chamber containing rotating discs or impellers mounted, concentrically or off-centred, on a motor driven shaft. The grinding action is provided by beads typically occupying 80 to 85 % of the free working volume of the chamber (Chisti and Moo-Young, 1986). The units must be equipped with high capacity cooling systems for processing temperature sensitive materials. The volume of bead mill ranges between 0.6 and 200 l. The maximum volume is limited by the heat removal required (Chriti and Moo-Young, 1986). Horizontal units are generally preferred for cell disruption as the grinding action in vertical mills is reduced due to fluidising effects of the upward fluid flow on the beads (Engler, 1985). Figure 2.5 shows the structure of bead mill.

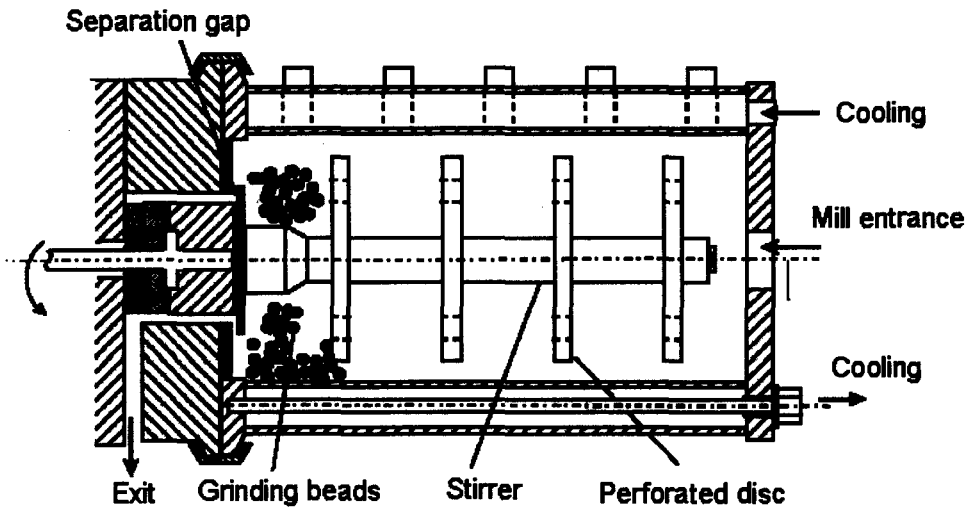


Figure 2.5 Bead mill with a horizontal grinding chamber (Frances, 2004)

Currie *et al.* (1972) first studied the kinetics of the cell disruption process. Cell disruption in the bead mill is a first order process with respect to time (Currie *et al.*, 1972; Schutte and Kula, 1988; Garrido *et al.*, 1994) and can be described by Equation 2.3:

$$kt = \ln \frac{R_m}{R_m - R} \qquad \textbf{Equation 2.3}$$

where 'R_m' is the maximum soluble protein available for release; 'R' is the soluble protein released; 'k' is the disruption rate constant and 't' is the treatment time. The bead disrupts yeast cells more efficiently than bacteria (Middelberg, 1995), owing to the smaller size of bacterial cells. The factors that influence cell disruption in the bead mill include the bead size and loading, impeller design and speed, initial concentration of cell suspension, the type of microorganism, the nature and location of the product and temperature.

In general, the release rate decreases with increasing bead size. The optimal bead size depends on the type of microorganism. Typically for yeast cells, approximately 1 mm is effective for Baker's yeast, while 0.5-0.75 mm is preferred by *E. coli* (Schutte and Kula, 1988). By using larger beads, enzymes located in the periplasmic space can be released preferentially whereas smaller beads are required for the release of cytoplasmic enzymes (Schutte *et al.*, 1983).

Bead loading used also affects the disruption of cells due to the bead-bead interaction. Increased bead loading provides a better transfer of the disruption energy resulting in higher disruption efficiency. Schutte and Kula (1988) recommended that a

80-85% bead loading for *E. coli* for a high extent of release and a 90 % bead loading for yeast.

Increased rotational speed of the agitator leads to a higher frequency of contact between the attractive elements and a greater shear stress as their differential velocities are increased. These result in an increase in the disruption.

The influence of the initial concentration on the release of protein was studied by Morgren *et al.* (1974). The concentration of yeasts in 4 to 20 % w/v (wet weight) had no influence on the disruption rate. In contrast, the disruption rate decreased with the yeast concentration increase between 30 and 60 % w/v (wet weight).

2.4.4 Hydrodynamic caviation

The application of hydrodynamic cavitation for disruption of *S. cerevisiae* and *C. necator* were first investigated by Harrison and Pandit (1992), and positive results was shown (Harrison, 1990; Harrison and Pandit, 1992; Save *et al.*, 1994; Save *et al.*, 1997; Kumar *et al.*, 2000; Balasundaram and Pandit, 2001; Balasundaram and Harrison, 2006). Moreover, its application on the disinfection of water also be demonstrated (Jyoti and Pandit, 2001; Kalumuck et al., 2003). It is more energy efficient than the high pressure homogenisation and ultrasonication (Harrison and Pandit, 1992) with reduced energy input required for equivalent breakage and a decrease in energy dissipated (Save *et al.*, 1994). It provides a cheap and simple approach to cell disruption.

A typical hydrodynamic cavitation set-up is presented in Figure 2.6. It mainly consists of a tank, a centrifugal pump and an orifice plate. The tank is used to hold the cell suspension with large volume. The pump is connected to the bottom of the tank and used to separate the stream into two flows. One stream passes through the orifice plate where the cavitation occurs, while the other one serves as a bypass to control the flow. To avoid air induction, both the streams are returned below the liquid level of the reservoir tank. Cooling coils are placed within the tank to maintain the temperature of the suspension, thereby preventing the denaturation of the protein.

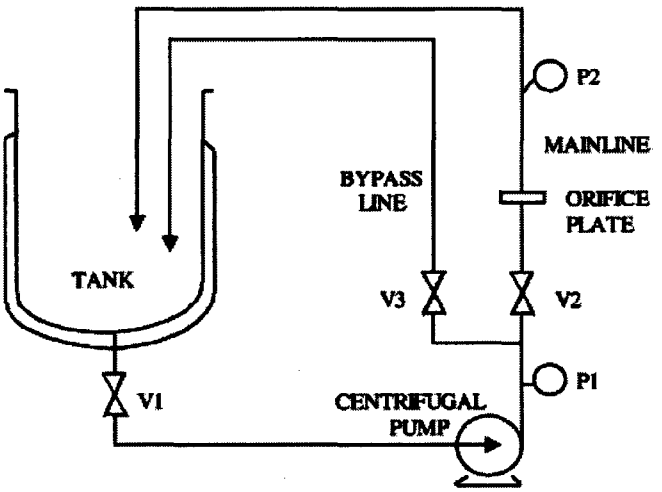


Figure 2.6 The hydrodynamic cavitation set up using orifice plates at pilot plant scale (Balasundaram and Pandit, 2001)

The type of cavitation generated is based on its source (Young, 1989). Hydrodynamic cavitation is caused by the pressure variation in a flowing system induced by change in the system geometry. However, the exact mechanism of hydrodynamic cavitation for disruption has not been understood. The effects of cavitation can be distinguished as physical and chemical. The physical effects include the generation of shock waves (Sundaram *et al.*, 2003), the water hammer effect (Brujan, 2004), and radial bubble motion.

A dimensionless parameter known as the cavitation number (C_v) is used to define the intensity of cavitation. C_v is the ratio of forces collapsing cavities to those initiating their formation.

$$C_v = \frac{P_3 - P_v}{0.5\rho v^2} \qquad \textbf{Equation 2.4}$$

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 suspension medium and 'v' is the orifice velocity (m/s). Typically, cavitation occurs when the cavitation number is below 1.0 (Kalumuchk and Chahine, 2000; Gogate and Pandit, 2001; Gogate and Pandit, 2002). The intensity of cavitation can be varied over a wide range by manipulation of the operating pressure, initial concentration of the cell suspension, number of passes through the cavitation zone, temperature, and viscosity (Gogate and Pandit, 2001).

Harrison and Pandit (1992) showed that as the operating pressure increased from 35 to 90 kPa, the orifice velocity increased from 8.4 to 14.2 m/s while the cavitation number decreased from 2.79 to 0.99. The total soluble protein released decreased with increase in the initial concentration of cell suspension over a range between 50 and 250 g/l (dry weight), when *S. cerevisiae* was disrupted by hydrodynamic cavitation. They also showed that increase in the number of passes through the orifice increased the total soluble protein released.

Gogate and Pandit (2000) found that, at a constant operating pressure, cavitation number and flow area, an increase in the orifice size resulted in the number of cavities generated decreasing and the collapse pressure of any single cavity increased.

First order disruption kinetics was used to predict total soluble protein release during the mechanical cell disruption process is described by Equation 2.5.

$$kt = \ln\frac{R_m}{R_m - R} \qquad \textbf{Equation 2.5}$$

where 'R_m' is the maximum soluble protein available for release; 'R' is the soluble protein released; 'k' is the disruption rate constant and 't' is the treatment time. However, hydrodynamic cavitation can not disrupt a cell completely, hence the R_m value must be replaced by R_i, the maximum soluble protein released under a specific conditions (Pearce, 1993). The amount of protein released from a cell under the specific conditions can be represented by R_i/R_m fraction of the total maximum R_m available to quantify the extent of disruption.

2.5 NON-MECHANICAL METHODS

The non-mechanical methods of microbial cell disruption include chemical, biological and physical processes. They are suitable for shear sensitive products like plasmid DNA. The micronisation can be minimised. Similarly, heat generation owing to conversion of mechanical energy to heat during disruption can be avoided. Non-mechanical disruption has a higher potential to release protein selectively because selective damage of the cell wall or cytoplasmic membrane of the cells can be targeted. However, non-mechanical disruption is not preferred in industry, due to the practical and commercial limitations. Further, the removal of the exogenous chemicals and enzymes may complicate subsequent product purification. However,

these methods may be used for the production of high value, low volume products. Figure 2.7 summarises the non-mechanical methods used for cell disruption.

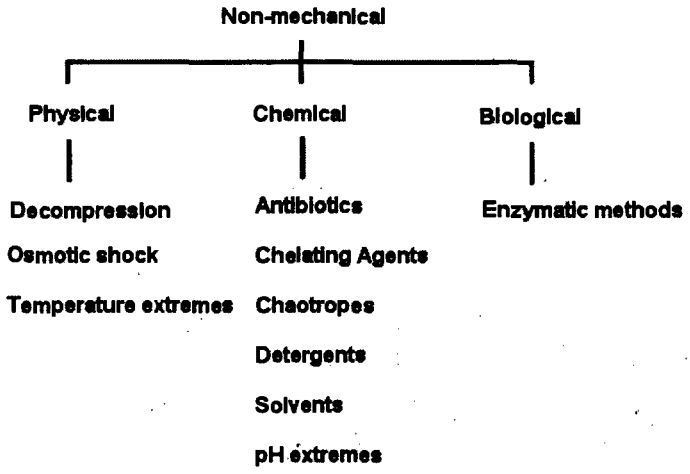


Figure 2.7 Non-mechanical methods of microbial cell disruption

2.5.1 Physical methods

A limited number of physical methods exist with potential process-scale application. These include decompression, one of the oldest techniques of cell rupture, osmotic shock and temperature extremes. These methods result in large cell debris, which is an advantage for solid-liquid separations prior to recovery and purification of proteins, enzymes or other bioproducts.

2.5.1.1 Decompression

Decompression is based on introducing a pressurised subcritical or supercritical gas into the cell suspension causing cell disruption after release of the applied pressure by expansion. Fraser (1951) reported that *E. coli* disrupted at laboratory scale using decompression. Decompression is extremely gentle and reported to result in large debris which may ease consequent separation. However, it generally results in less disruption than high pressure homogenisation or other mechanical methods (Engler, 1985).

2.5.1.2 Temperature extremes

Temperature extremes include heating and freezing. Freezing and thawing involves the formation and subsequent melting of ice crystals. It is commonly used on a small scale and gives low yields with a loss in enzyme activity. Moreover, denaturation of protein may result when using this technique (Engler, 1985).

The technique has several advantages for specific products for pilot scale use. The elevated temperature may kill or deactivate cells reducing or eliminating the possible release of recombinant organisms. If the products are not temperature sensitive, the preferential protease deactivation may occur, according to the Arrhenius law. This technique results in large cell debris which eases subsequent separation. A number of drawbacks exist. First, due to the denaturation of protein at high temperature, the temperature range and duration time of exposure to high temperature must be considered for temperature sensitive products. Second, killing the cells may lead to smaller, toughened cells, which makes consequent mechanical disruption less efficient (Collis *et al.*, 1995). Third, a significant increase in viscosity owing to intact nucleic acid and the subsequent appearance on non-Newtonian viscoelastic behavior may be observed (Watson and Cumming, 1987).

The specific temperature range used for disruption is dependent on the type of microorganism, growth phase and the location of the enzymes. The periplasmic proteins from *E. coli* are released when the cells are heated to 50-55°C (Katsui *et al.*, 1981). The cytoplasmic protein was released within 10 min at 90°C (Watson *et al.*, 1987). Watson *et al.* also showed the Gram-positive bacteria *Bacillus megaterium* gave only low levels of protein release even at high temperature. Unlike mechanical disruption, the stationary cells are more easily disrupted than the exponential cells. Temperature stress may be induced by step changes (heat shock) and gradual linear changes (heat slope). A cell volume reduction has been observed in cells submitted to a sub-lytic heat shock but not for cells, submitted to a sub-lytic heat slope (Gervais and de Marañon, 1995).

Thermolysis has been increasingly used in large scale disruption. A production of poly-β-hydroxybutyrate (PHB) employed a heat shock technique for the disruption of the Gram negative bacterium *C. necator* at a pilot scale (Holmes and Lim, 1985; Harrison, 1990). Beck *et al.* (1978) reported a similar process for the production of yeast protein extracts for the food industry.

2.5.1.3 Osmotic shock

The intracellular medium always has a slightly higher osmotic pressure than the external medium, and the difference is balanced by the cell wall. A low increase rate of osmotic pressure maintains an important viability of the cells even across a high change in osmotic pressure. Gervais *et al.* (1992) demonstrated that the viablility of

yeast can be maintained at about 90 to 100 % under a slow variation in osmotic pressure across 10^8 Pa, due to modification of cytoplasmic membrane to survive during gradual dehydration. The same phenomenon applies on slow rehydration. Mill *et al.* (2002) showed slow rehydration harmless to the viability of cells, since the cells were allowed to recovery their initial area-to-volume ratio. The drastic decrease in cell volume during a rapid shift in osmotic pressure may induce a mechanical effect on the membrane with possible disruption.

Osmotic shock is a gentle cell lysis method, and results in large cell debris. In osmotic shock, cells are first equilibrated briefly at a high osmotic pressure such as 1 M sucrose or salt solution. Thereafter the medium is diluted rapidly. Water enters the cells, increasing the hydrostatic pressure and thereby causing the disruption of the cells and limited efficiency. However, it is usually restricted to small scale application, due to the high cost of the additives and limited efficiency. Moreover, the contamination of product may result because of high salt concentrations (Engler, 1985).

2.5.2 Biological methods

Autolysis, phage lysis and lysis by addition of foreign lytic enzymes are the primary biological methods (Middelberg, 1995). Autolysis is easy to scale-up, The host cells produce lytic enzymes that increase the porosity of the cells or cause cell lysis. It is sometimes classified as a chemical or physical treatment, as it may be induced by solvent shock, pH shock and thermal shock. The phage lysis is not preferred on an industrial scale, due to the possibility of the premature lysis in subsequent batches owing to the residual phage, as well as the cellular contents being altered (Engler, 1985). An 80 % fractional release of β-galactosidase from *E. coli* was obtained after expression of the cloned phage proteins was induced for 2.3 hr.

Alternatively, cell lysis can result from adding lytic enzymes (Schutte and Kula, 1990). The advantages of enzymatic cell lysis are biological specificity, mild operating conditions, low capital investment, low energy requirements and the avoidance of harsh physical conditions such as high shear stress or temperature (Engler, 1995; Harrison, 1991) which may denature products. To date enzymic lysis is a slow and costly process (Baldwin and Robinson, 1990). If the enzyme or enzyme cocktail can be immobilised or is readily produced by microorganisms, this technique would be

considered for large scale use (Engler, 1985). The selection of the lytic enzymes is dependent on the specific structure of bacterial and yeast cell walls. Three types of bacteriolytic enzymes have been identified: glycosidase, acetylmuramy-L-alanine amidase and endopeptidases (Andrew and Asenjo, 1987). Most bacteriolytic enzymes are not active on viable cells. Hence, the biomass requires sensitisation by heat inactivation, chemical pretreatment, freezing or lyophilisation (Andrew and Asenjo, 1987, Golovina *et al.*, 1973).

Currently, lysozyme is common bacteriolytic enzyme available commercially for large scale application (Harrison, 1991). It attacks β-1,4 glucosidic linkages of polysaccharide chains of peptidoglycan. Gram-positive bacterial cell walls are more susceptible to be attacked by lysozyme, since peptidoglycan forms the outer surface of the cell, unlike Gram negative bacteria which have an outer membrane. A chelating agent EDTA or a non-ionic detergent, Triton X-100 may be employed to destabilise or remove the outer membrane of the Gram-negative bacteria. The morphological changes of the cell wall were confirmed by Andrews and Asenjo (1987) using light microscopy. The lysis of *E. coli* using the lytic enzyme system of *Cytophaga* sp. requires the removal of the outer membrane (Andrews and Asenjo, 1987), while lysis in the absence of its removal was found for *C. necator* (Harrison *et al.*, 1991c).

The application of biological methods is limited by the cost of the enzymes. The operating costs may be reduced by the enzyme immobilisation. However, this reduces contact between the enzyme and cell wall. Hence, biological disruption is only used on a small scale.

2.5.3 Chemical methods

There are many chemical methods available. They act selectively on the outer cell wall resulting in the selective leakage of the periplasmic constituents. The chemical agent may cause cell lysis releasing the product extracellularly or cells permeabilisation, allowing the substrate molecule to diffuse into the cell for conversion into product.

2.5.3.1 pH extremes

The alkaline lysis is an inexpensive method and can be applied in almost any scale

of operation. Alkaline treatment at a pH of 11.5-12.5 for 20 to 30 minutes causes cell lysis (Stanbury and Whitaker, 1984). Alkaline conditions are reported in the preparation of protein concentrates for feeds from yeasts and bacteria. The pH range of 11.0-11.5 is preferable (Hedenskog *et al.*, 1970). A 50 % release of total soluble protein on disruption of *C. necator* for the recovery of PHB was obtained by alkaline lysis at pH 10 and 45°C (Harrison, 1990). The acid lysis using 6 N HCl of *Candida lipolytica* has been reported by Engler (1985). This process resulted in the concomitant hydrolysis of protein to amino acids and required 6 to 12 hr. These processes are relevant where active protein molecules are not required.

2.5.3.2 Antibiotics

Antibiotics for Gram-negative bacteria lysis can be applied on a laboratory scale. The cell lysis using antibiotics is caused by a particular mechanism: inhibition of the synthesis of cell wall and disorganisation or distortion of the cell membrane (Middelberg, 1995). To inhibit cell wall synthesis, the penicillin was added during the growth phase to initiate cell lysis (Engler, 1985). The effect of antibiotics on the changing of *E. coli* peptidoglycan structure determined by a new chromatographic technique has been reported by Kohlrausch and Hőltje (1991). The cell is unable to maintain the osmotic pressure without an intact peptidoglycan structure, and intracellular products are released. The cost of antibiotics is generally high and the effectiveness of antibiotic lysis is dependent on the state of the culture hence the use of antibiotics for large scale work has not been reported.

2.5.3.3 Chelating agents - EDTA

Ethlenediamine tetra-acetic acid (EDTA) cause the chelation of divalent cations, Mg^{2+} and Ca^{2+}, which are necessary for the stabilisation of the outer membrane of the Gram-negative cell envelop. This causes removal or destabilisation of the outer membrane. A 33 to 50 % loss in lipopolysaccharide content and small amount of protein and phospholipid resulted (Felix, 1982). Although EDTA disrupted the outer membrane and therefore released periplasmic proteins, it is not useful for recovery of cytoplasmic enzymes. The release of alkaline phosphatase from periplasm by EDTA has been reported by Neu and Heppel (1964). Anand *et al.* (2007) confirmed very low release on treatment with EDTA. Some 2 % total soluble protein and approximately 2% of both the cytoplasmic and periplasmic enzymes were released from *E. coli* by 0.04 M EDTA at 37°C for 10 min with agitation at 120 rpm in a shake flask. The low cytoplasmic enzyme release was contrary previous report.

2.5.3.4 Chaotropes

Chaotropic agents are describe as cell lysis mediators (Becker *et al.*, 1983; Hettwer and wang, 1989). The potency degree is: trichoroacetate > perchlorate ≈ hiocyanate > nitrate > urea. These agents are known to solubilise protein from membrane fragments of *E. coli* by altering the hydrophobic interactions. Ingram (1981) reported that the chaotropic salts weakened hydrophobic associations to promote lysis and inhibited the cross-linking of the cell wall assembly. The cell structure of *E. coli* was altered by guanidine hydrochloride (G-HCl) at a concentration over 2 M, resulting in a substantial amount of protein release (Hettwer and Wang, 1989). They also reported some protein release at 0.1 M G-HCl. The chaotropes have only been used on a small scale, due to the cost of high concentration of chaotropic agents, dependence on the state of the microorganism, as well as the waste disposal of chaotropic agents.

2.5.3.5 Solvents

Microorganisms can be permeabilised by non-polar solvents to release intracellular products. Solvents such as ethanol, methanol, isopropanol and butanol can cause disruption of the cytoplasmic membrane. When *K. fragilis* was treated with ethanol or isopropanol at a concentration over a range of 80 to 90 % w/v followed by the extraction of the product into a phosphate buffer for 20 hr, 90 % of β-galactosidase was released by ethanol and 85 % on using isopropanol (Fenton, 1982). Removal of the solvent for prevent enzyme denaturation was required when the solvent concentration exceeded 80 to 90 %. Toluene is frequently employed. The results are dependent on the concentration of the toluene and the working temperature. When *S. cerevisiae* was treated with 2 % toluene at 40 to 45°C, the maximum release of intracellular enzymes pyruvate kinase, AMP deaminase and phosphofructokinase was obtained, while 54 % of ADH was released (Murakami *et al.*, 1980). Sodium hypochlorite at a concentration of 30 % v/v for 90 min resulted 97 % recovery of the PHB from *A. eutropus* (Hahn *et al*, 1993). The purity of the PHB was 91 %.

2.5.3.6 Detergents

Detergents are amphipathic molecules with a hydrophilic portion, which is ionic and a hydrophobic region. They are able to interact with water and lipid (Helenius and Simons, 1975). Detergents include anionic detergents (sodium dodecyl sulphate), cationic detergents (cetyltrimethylammonium bromide) and non-ionic detergents (Triton X-100).

Sodium dodecyl sulphate (SDS) induces disorganisation of the phospholipids in the plasma membrane, causing release of intracellular compounds. In an alkaline environment, the cytoplasmic membrane of *E. coli* was dissolved by SDS and the intracellular compounds released rapidly. The release time is about 30 to 40 s and dependent on the type of the cells (Woldringh and van Iterson, 1972).

Cetyltrimethylammonium bromide (CTAB) causes permeabilisation of the cells, but the mechanism of the surfactant and the exact site of attachment is debated. CTAB has been used for the permeabilisation of both yeast and bacteria. The optimal conditions are: concentration over a range of 0.1 to 0.4 %, the pH between 4 and 10, the temperature from 24 to 37°C, with a 15 min treatment time. On permeabilisation of *Acetobacter vinelandii* using CTAB in a hexanol-octane reverse micellar system, a 6.2 fold purification for isocitrate dehydrogenase and 7.6 fold purification of β-hydroxybutyrate dehydrogenease were obtained, when compared to cells exposure to ultrasound (Giovenco *et al.*, 1987). About 10 % total soluble protein was released from Baker's yeast on exposure to EDTA and CTAB (0.1 %) at 30°C for 15 min with agitation at 120 rpm in a shake flask (Anand, 2004).

Triton X-100 acts on the cytoplasmic membrane, and results in protein release from both bacterial and yeast cells. Buckland *et al.* (1976) reported a 2 % protein and 70 % cholesterol oxidase release from *Nocardia rhodococcus* by Triton X-100. A maximum activity of β-galactosidase of 40 % resulted on permeabilisation of *S. cerevisiae* by Triton X-100 at 0.3 to 1.0 % for 30 min at 30°C (Chow and Palecek, 2004). Some 2 % protein was released from Baker's yeast by EDTA and Triton X-100 (0.1%) at 30°C for 1 hr with agitation at 120 rpm in a shake flask (Anand, 2004).

2.6 COMBINED METHODS

The various methods of disruption use different modes of action. Hence, combined methods to result in a higher extent of release may be beneficial. There are two ways: mechanical disruption with non-mechanical pretreatment and combined non-mechanical methods. A selective product release strategy by breakage of the outer membrane prior to lysis of cytoplasmic membrane may be obtained by using different non-mechanical methods. EDTA, polymyxin and chaotropes typically affect destabilisation of the outer membrane. Solvent can be used to attack the cytoplasmic membrane. However, the combined non-mechanical methods are limited to small

scale due to the expensive cost of chemicals or enzymes, the removal and recovery of the exogenous additives, and low efficiency.

The mechanical disruption has been used in industry. However, these processes have several drawbacks, including non-selective product release, high energy consumption and micronisation of the cell debris. An approach using non-mechanical pretreatment prior to mechanical disruption for improving product release, reducing energy requirements and enhancing selective product release is desirable. For example, complete cell disruption of *S. cerevisiae* was obtained on microfluidizer at 95 MPa for 4 passes when the cells were pretreated by Zymolyase for 2 hr, while only 32 % disruption was obtained by the untreated cells (Baldwin and Robinson, 1993). A 82 % protein release was obtained using 1.5 G-HCl and 1.5 % Triton X-100 from *E. coli* on high pressure homogenisation at 41 MPa for 1 pass, while a 62 % protein was released from untreated cells on high pressure homogenisation at the same pressure for 2 passes (Bailey *et al.*, 1995). The pH pretreatment release for β-galactosidase release from *Kluveromyces lactis* on ultrasonication was studied by Farkade *et al.* (2006). The maximum activity of β-galactosidase (95±3 U/ml, wet weight) was obtained on pretreating the cells at pH 4.4 for 6 h with subsequent exposure to ultrasound. The enzyme activity on ultrasonication without pretreatment was 7.2±0.6 U/ml. The pretreatment using EDTA for permeabilisation of *E. coli* resulted the maximum release on high pressure homogenisation at 13.8 MPa, while the maximum release of untreated cells was achieved at 34.5 MPa (Anand *et al.*, 2007). They also reported that the G-HCl and Triton X-100 increased intracellular protein release and decreased energy requirement.

High-pressure homogenisation with and without chemical pretreatment has been examined for PHB recovery from *C. necator* (Harrison et al., 1991a). To achieve complete disruption, 3 passes were required on high pressure homogenisation at 60 to 69 MPa. Improved single pass disruption at same operating pressure was achieved by using alkaline pretreatment with pH 10.5 with duration less than 1 min at 7°C, but a minimum of two passes were necessary for complete protein release. Completely rupture on high pressure homogenisation by a single pass at 62 MPa was obtained when *C. necator* was pretreated with 0.1% w/v SDS for 20 min at 70°C. Osmotic pretreatment using sodium chloride or potassium chloride with concentration of about 0.14 M for 60 min at 60°C was less effective than SDS pretreatment. But cell disruption was improved relative to salt-free thermal treatment, presumably because of the thermal injury enhancing effect of monovalent metal ions.

2.7 UPSTREAM AND DOWNSTREAM PROCESSES

Upstream and downstream processes must be considered in selection of the cell disruption protocol. The upstream processes involve all factors prior to disruption. As the major resistance to cell breakage is the strength of the cell wall, the microorganism type markedly influences the cell disruption (Keleman and Sharpe, 1979). Cell disruption is more readily accomplished following the cells growing on a defined medium than on a complex medium (Gray *et al.*, 1972). The resistance to cell disruption is decreased with increasing growth rate and dependent on the growth phase. The exponentially growing cells show less resistance than the stationary phase cells (Engler and Robinson, 1981; Sauer *et al.*, 1988; Harrison *et al.*, 1991b).

When the intracellular components are the desired products, the cell disruption is required in the first place in the downstream processes. The considerations for the selection of a cell disruption approach include the tolerance of the desired product to conditions used, the extent of product release and the process economics (Johnstone-Robertson *et al.*, 2008; Balasundaram and Harrison, 2008). The downstream processes, including the separation and purification of the product of interest, must be best balanced through the cost of the process, the extent of product release, the solubility of the desired product and debris, as well as the removal and recovery of the exogenous chemicals and enzymes.

2.8 CONCLUSIONS

The detail of the structure and the composition of cell envelop indicates that a selective product release strategy is possible for some products. Mechanical methods have found greater commercial application than the non-mechanical methods because of practical and economic limitation. However, mechanical processes are very non-specific and energy-intensive. The dissipation of the energy expended as heat to prevent product deterioration increases the energy requirement. The micronisation resulting in more difficult solid-liquid separation is another challenge of mechanical methods. Non-mechanical methods are less energy intensive and have a high potential for the selective product release. Moreover, micronisation of the cells can be minimised and heat generation is reduced. However, owing to the restrictions of process efficiency and economics, they are limited to a small scale. The need for the removal of the exogenous chemicals or enzymes may

complicate subsequent product purification. Hence, the combined use of non-mechanical methods as pretreatment prior to mechanical disruption can be applied for decreased energy consumption and micronisation as well as increased selective product release.

The primary objective to be investigated through this thesis, in the light of current literature understanding are:

- minimise the energy required while achieving increased efficiency of product release through pretreatment
- enhance selectivity of product release by mechanical methods using pretreatments
- maximise the size of cell debris resulting, while still ensuring efficient product release
- identify pretreatments to weaken cell structure and improve product release on subsequent mechanical disruption while not denaturing product

The hypotheses developed for examination in addressing these objectives are:

- By using the appropriate pretreatments, the energy required for product release by mechanical cell disruption can be minimised and micronisation can be avoided.
- Selective enzyme release on mechanical disruption can be achieved by using pretreatments.

Chapter 3
Methodology

3.1 INTRODUCTION

The methodologies used in this project are presented in this chapter. The microorganisms and their culture conditions are described in Section 3.2. The experimental apparatus is described in Section 3.3. Procedures of pretreatment are described in Section 3.4. In Section 3.5, protein and enzymes assays and their calibration curves, size analysis, dry weight analysis and optical microscopy are detailed.

3.2 MICROORGANISM

3.2.1 Baker's yeast (*Saccharomyces cerevisiae*)

Bakers yeast (*Saccharomyces cerevisiae*) was obtained as a stationary phase yeast cream from Anchor Yeast (Cape Town, South Africa). Residual media components were removed from yeast suspension by centrifugation at 18,000 g for 10 min at room temperature in the Beckman centrifuge (Avanti-J25) with a JA-10 rotor and washing twice with sodium phosphate buffer (NaH_2PO_4, 0.5 M, pH 7). The yeast was stored at 4°C for a maximum of one week.

3.2.2 *Kluyveromyces lactis*

Kluyveromyces lactis CBS 2359 was obtained from the University of Free State (Bloemfontein, South Africa). The stock culture was maintained on an agar slant with YMPD medium at 4°C. YMPD medium contained malt extract (3 g/l), yeast extract (3 g/l), peptone (5 g/l) and glucose (10 g/l). An inoculum was prepared on the medium inoculated from the agar slant and grown in a shake flask at 175 rpm for 24 hr. A 5 % (v/v) inoculum was transferred to 4 l culture medium in the New

Brunswick Scientific Bioflo 110 bioreactor of 7 l. The culture medium was composed of lactose (25g/l), yeast extract (5g/l), KH_2PO_4 (2.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.6 g/l), $CaCl_2 \cdot 2H_2O$ (0.1 g/l), $FeSO_4 \cdot 7H_2O$ (2.5 mg/l), $ZnSO_4 \cdot 7H_2O$ (1 mg/l), $MnSO_4 \cdot H_2O$ (0.5 mg/l); $CuSO_4 \cdot 5H_2O$ (0.25 mg/l). Following batch operation for 24 h, fed-batch culture using a lactose feed solution (50 g/l) enhanced biomass formation over a further 24 hr. The pH of the culture medium was maintained at pH 5.5 by 1 N H_3PO_4 or 1 N NaOH. The aeration and agitation rates were 1 vvm and 600 rpm, respectively. All cultures were carried out at 30°C. Final dry biomass concentration reached 33 g /l. The yeast was separated from the media by centrifugation at 18,000 g for 10 min at room temperature and re-suspended in sodium phosphate buffer (pH 7). The concentration of the yeast suspension was adjusted to 1.5 % by volume on a dry weight basis before mechanical cell disruption.

3.3 MECHANICAL CELL DISRUPTION

3.3.1 High pressure homogeniser

The Rannie high pressure laboratory homogeniser (Model MINI-LAB, type 8.30 H), manufactured by APV, is shown in Figure 3.1. The working pressure of the homogeniser ranged between 13.8 and 69.0 MPa. A 300 ml working volume was used. The temperature during the disruption was maintained at 20°C by heat transfer through a cooling coil. An overhead stirrer was used to maintain the homogeneity of the yeast suspension in the reservoir during the disruption. A 1 ml sample was taken after each pass and centrifuged at 16,060 g for 10 min. The supernatant was subjected to protein and enzyme analysis. The homogenisation conditions used with on each microorganism, the homogenisation pressure selected, and the pretreatments with which HPH was used are summarised in Table 3.1. The number of passes required to achieve the maximum protein release at different operating pressures are different, hence the method of calculation of the energy requirement for disruption and the specific heating is reported in Chapter 4 and Chapter 5.

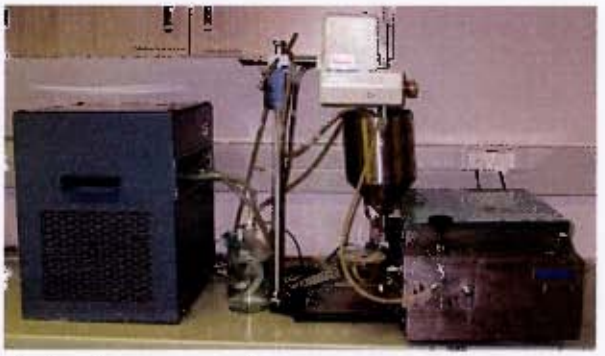


Figure 3.1 Rannie high pressure homogeniser in the laboratory

Table 3.1 Operating conditions of pretreatment and high pressure homogenisation for each microorganism

Microorganism	Pressure (MPa)	No. of Passes	Pretreatments used				
			No	Heat	pH	Osmotic	Combined
Baker's yeast (*S. cerevisiae*)	13.8	10	√				
	27.6		√	√	√	√	√
	41.4		√	√			
	55.2	5	√				
	69.0		√				
K. lactis	27.6	10	√	√			
	41.4		√	√	√	√	√
	69.0		√				

3.3.2 Ultrasonication

The VirSonic 100 ultrasonic cell disruptor, shown in Figure 3.2, operated at 22.5 kHz and power settings of 40, 60 or 80 W was used. A 15 ml yeast suspension was sonicated on ice. A 3 or 5 min pause was given after every 3 or 5 min sonication period to minimise the temperature increase of cell suspension during sonication. Typically, the temperature increased to 37°C after sonication and decreased to 10°C after each pause. To ensure that significant denaturation did not occur under the ultrasound regime used (T_{max} = 37°C), an equivalent experiment compared protein release following sonication for the same total time using 1 min sonication and cooling cycles. This resulted in a T_{max} less than 20°C. Comparison of the resultant soluble protein concentration indicated the denaturation of protein was less than 4 %. The disrupted cells were harvested by centrifugation at 16,060 g for 10 min. The supernatant was subjected to protein and enzyme analysis. Use of sonication centred on finding the optimum condition of each single pretreatment, and was only applied to disrupt Baker's yeast (*Saccharomyces cerevisiae*). Table 3.2 summarises the experiments performed using ultrasonication to select conditions for single pretreatments.

Figure 3.2 Virsonic 100 Ultrasonication in the laboratory

Table 3.2 conditions of ultrasonication for Baker's yeast

Power (W)	Length of sonication cycle	Length of cooling cycle	Number of sonication cycle	Pretreatments used			
				No	Heat	pH	Osmotic
40	5 min	5 min	5	√	√		
60	3 min	3 min	7	√			
80	3 min	3 min	7	√		√	√

3.4 PROCEDURE OF PRETREATMENT

Three pretreatments were used to decrease the energy requirement of mechanical cell disruption and increase potential product release: heat, pH and osmotic pretreatment. Following determination of optimum conditions for each pretreatment, combinations were investigated.

3.4.1 Heat pretreatment

The heat pretreatment was carried out by either dilution or heat exchange. The maximum temperature used, heating rate and holding time at maximum temperature were also considered.

3.4.1.1 Heat pretreatment using dilution

To introduce a rapid temperature shock, the Baker's yeast suspension was diluted into pre-warmed buffer to achieve the desired maximum temperature: 40, 50 and 60°C. The heating rate was over 20°C/s. A similar dilution into cooled buffer was used to return the temperature of suspension back to room temperature. The holding time at maximum temperature was minimised. Volumes of starting yeast buffer solutions were calculated to achieve the desired temperatures and a final yeast concentration of 1.5 % (w/v, dry weight).

3.4.1.2 Heat pretreatment using heat exchange

To control the rate and extent of heat pretreatment, the Baker's yeast suspension was pumped through a heating coil with an internal diameter of 6 mm and total length of 250 cm. The coil was immersed in a water bath, controlled to the specified temperature for heating of the suspension to 40, 45, 50, 52, 55 and 60°C at a flow-rate of 0.02 l/s.

Heating rate was varied using both the heating coil and STR. The Baker's yeast suspension was heated from room temperature to 40°C by varying the flow rate through the coil from 0.003 l/s through 0.01 l/s to 0.02 l/s, corresponding to a heating rate of 0.5°C/s, 1.7°C/s and 3.5°C/s. The jacketed STR, agitated at 750 rpm, was used to provide a slow heating rate of 0.1°C/s.

To study the influence of holding time, the yeast suspension was preheated to 40°C at a heating rate of 3.5°C/s. It was then maintained at 40°C for a holding period of 5, 10, 20 and 60 min.

After a given heat pretreatment, the solution was pumped through the same coil, immersed in an ice water bath at the maximum pump speed. The temperature of the suspension was decreased to room temperature. In Table 3.3a, the procedure of heat pretreatment prior to high pressure homogenisation of both Baker's yeast and *K. lactis* was based on the optimised conditions determined by ultrasonication. Heat pretreatment prior to ultrasonication is summarised in Table 3.3b. These conditions were confirmed by varying maximum temperature and holding time across the range 40 to 50°C, and 0 to 5 min prior to HPH. After heat pretreatment, cell suspension was disrupted at either 27.6 or 41.4 MPa.

Table 3.3a The heat pretreatment procedure prior to ultrasonication

Microorganism	T_{max}	Heating rate	Holding time	Cooling	Disruption
Baker's yeast (*S. cerevisiae*)	40,50 and 60°C	Dilution > 20°C/s	Minimal	Dilution > 20°C/s	Suspension was disrupted at 40 W power input for 25 min, after given a heat pretreatment,
	40, 45, 50, 52, 55 and 60°C	3.5, 3.9, 4.3. 4.5, 4.8 and 5.2°C/s	Minimal	Cooling coil	
	40°C	0.1 (STR), 0.5, 1.7 and 3.5°C/s	Minimal		
	40°C	3.5°C/s	5, 10, 20 and 60 min		

Table 3.3b The heat pretreatment procedure prior to HPH

Microorganism	T_{max}	Heating rate	Holding time	Cooling	Disruption pressure	
					27.6 MPa	41.4 MPa
Baker's yeast (*S. cerevisiae*)	40°C	3.5°C/s	Minimal	Cooling coil	√	
	50°C	4.3°C/s	Minimal		√	√
	50°C	4.3°C/s	5 min			√
K. lactis	40°C	3.5°C/s	Minimal			√
	50°C	4.3°C/s	Minimal		√	√

3.4.1.3 Protein denaturation

Typically, the energy dissipated per pass results in a increase in suspension temperature of 15°C. In the absence of sufficient cooling, this heat dissipation may result in a temperature increase of the suspension. Here, potential for protein denaturation following this temperature increase was investigated. The denaturation of enzymes from different locations was also considered. Following yeast disruption at 69.0 MPa for 4 passes using high pressure homogenisation, the supernatant of the lysate was prepared. This supernatant was exposed to heat pretreatment conditions at 40 and 50°C. A 1 ml sample was taken at 0, 10, 20, 60, 180 and 300 s, and its soluble protein content analysed for comparison with the sample obtained before heating.

3.4.2 pH pretreatment

The pH pretreatment was investigated in terms of extent and duration. The pH of yeast suspension was elevated to a maximum pH for the pre-determined holding time. Further the effect of holding time of the yeast suspension at pH 10 was investigated over 30 s to 5 min. Through use of rigorous mixing, the duration of the pH increase was limited to less than 15 s. To introduce a rapid change in pH, a concentrated Baker's yeast suspension was diluted using a carbonate buffer (0.5 M) of specified pH, to achieve final suspension of pH 9, 9.5, 10, 10.5 and 11. Carbonate buffer of 0.05 M and 0.5 M were used to investigate the effect of osmotic pressure of pH buffer and its buffering capacity on subsequent mechanical disruption.

The holding time of pH pretreatment at pH 10 using both carbonate buffers was studied across 5 durations: 0, 0.5, 1, 2 and 5 min. The protein denaturation introduced by higher pH was not considered. Following pH pretreatment, phosphate buffer (0.05 or 0.5 M) was added to adjust the pH of yeast suspension back to pH 7. The decrease in pH was achieved in less than 15 s. The final concentration of yeast

suspension was 1.5 % (dry weight, w/v). The procedures of pH pretreatment related to this study are summarised in Table 3.4.

Table 3.4a The pH pretreatment procedure prior to ultrasonication

Microorganism	pH_{max}	pH increase $NaHCO_3$		Holding time (min)	pH decrease NaH_2PO_4 (pH7)		Conditions of disruption
		0.5 M	0.05M		0.5 M	0.05M	
Baker's yeast (*S. cerevisiae*)	9, 9.5, 10, 10.5 and 11	√		Minimal	√		Cells were sonicated at 80 W for 21 min
	10	√		0, 0.5, 1, 2 and 5	√		
	10		√			√	

Table 3.4b The pH pretreatment procedure prior to HPH

Microorganism	pH_{max}	pH increase	Holding time	pH decrease	Disruption pressure	
					27.6 MPa	41.4 MPa
Baker's yeast	10	$NaHCO_3$ (0.5 M)	2 min	NaH_2PO_4 (0.5 M, pH7)	√	
K. lactis						√

3.4.3 Osmotic pretreatment

A concentrated Baker's yeast suspension was introduced into a glycerol or NaCl solution of increased osmotic pressure (0.25, 0.5, 1 and 5 MPa), and subsequently rehydrated by phosphate buffer (NaH_2PO_4, 0.5 M, pH 7) to reduce the osmotic pressure to the starting range. The duration of both the dehydration and rehydration steps was less than 15 s. The ratio of the yeast and buffers was calculated to achieve the final concentration of the yeast suspension of 1.5 % (dry weight, w/v). Table 3.5 shows the procedure of osmotic pretreatment.

Table 3.5 The osmotic pretreatment procedure

Mechanical disruption	Microorganism	Osmotic buffer	Osmotic pressure $_{(max)}$	Conditions of disruption
Sonication	Baker's yeast (*S. cerevisiae*)	Glycerol	0.25, 0.5, 1 and 5 MPa	Cells were disrupted at 80 W for 21 min
		NaCl		
HPH	Baker's yeast (*S. cerevisiae*)	NaCl	1 MPa	Cells were disrupted at 27.6 MPa
	K. lactis			Cells were disrupted at 41.4 MPa

3.4.4 Combined pretreatment

Yeast suspension diluted by a pre-warmed pH or osmotic buffer was used to introduce a combined pretreatment. On dilution, the temperature was shifted to 40°C, while the pH or osmotic pressure was shifted to pH 10 or 1 MPa. To eliminate the

protein denaturation at these extreme conditions, cooled phosphate buffer was introduced to return the cells to the neutralised condition. Procedures of combined pretreatment are listed in Table 3.6, and were used with both Baker's yeast and *K. lactis*. After exposure to the combined pretreatment, the yeast suspension was disrupted in the high pressure homogeniser. The operating pressure for HPH of Baker's yeast and *K.* lactis are 27.6 MPa and 41.4 MPa, respectively.

Table 3.6 Combined pretreatment procedures of *S. cerevisiae* and *K. lactis*

Combined	T_{max}	Heating rate	Extreme condition	Holding time	Neutralised buffer
Heat and pH	40°C	Dilution > 20°C	pH_{max} 10; using carbonate buffer (0.5M)	5 min	Cooled phosphate buffer 0.5 M
Heat and osmotic			Osmotic pressure $_{(max)}$ 1MPa; using NaCl solution (1MPa)		Cooled phosphate buffer 0.05 M

3.5 ANALYTICAL METHODS

In order to assess the extent of cell disruption, total soluble protein release was measured, while the release of enzymes from different locations within the yeast cell was used to quantify selective product release. The total protein and enzymes analysed for both Baker's yeast and *K. lactis* are defined in Table 3.7.

Table 3.7 Protein and enzymes analysed for each yeast

Microorganism	Total soluble protein	Cell wall associated enzyme	Periplasmic enzyme	Cytoplasmic enzyme		
		Invertase	α-glu.	ADH	G6PDH	β -gal.
Baker's yeast	√	√	√	√	√	
K. lactis	√	√		√	√	√

3.5.1 Total soluble protein

Total soluble protein was assayed according to the Bradford assay spectrophotometrically at 595 nm (Bradford, 1976). This method is based on the principle of binding of the protein molecule to the dye, Coomassie Brilliant Blue G-250. The dye exists in two different forms. Unbound, it absorbs at 465 nm. When the dye binds to the protein molecule, the protein-dye complex has a strong absorption at 595 nm. The process requires only 2 min, and the protein-dye complex remains stable for approximately 1 hr. The calibration curve and the exhaustive method of estimation are presented in Appendix A.1. The coefficient of variance for triplicate samples was 4.8 %.

3.5.2 Enzyme analysis

3.5.2.1 Invertase (cell wall associated)

Invertase activity was estimated by hydrolysis of sucrose, a non-reducing disaccharide to glucose and fructose, which are reducing monosaccharides (Gascon and Lampen, 1968):

$$\text{Sucrose} + H_2O \rightarrow \text{Glucose} + \text{Fructose} \qquad \text{Equation 3.1}$$

The activity of invertase can be measured from the amount of glucose and fructose formed. The dinitrosalicyclic acid (DNS) assay is performed to quantify the reducing sugars, glucose and fructose. The DNS assay is based on the free carbonyl groups, aldehyde in glucose and ketone functional group in fructose, reducing 3,5-dinitrosalicyclic acid to 3-amino 5-nitrosalicylic acid, an orange compound. This is measured spectrophotometrically at 540 nm. One mole of sucrose can form two mole reductions of 3,5-dinitrosalicylic acid. A standard calibration curve using glucose concentrations was used. The unit of enzyme activity is defined as that which catalyses the hydrolysis of one micromole of sucrose per min at 55°C in sodium acetate buffer of pH 5.5. The procedure for this analysis is described in Appendix A.2. The coefficient of variance for triplicate samples was 6.1 %.

3.5.2.2 α-glucosidase (periplasmic)

The principle of the α-glucosidase assay is based on the release of *p*-nitrophenol from *p*-nitrophenol-α-D-glucosides according to Oliveira *et al.* (1981):

$$p\text{-nitrophenol-}\alpha\text{-D-glucosides} \rightarrow p\text{-nitrophenol} + \alpha\text{-D-glucose} \qquad \text{Equation 3.2}$$

The reaction was terminated by the addition of 0.1 M Na_2CO_3. The absorbance, read at 410 nm, was converted to *p*-nitrophenol concentration using a calibration curve. The unit of enzyme activity is defined as the amount of enzyme that produces 1 mole of *p*-nitrophenol in one minute at 30°C. The experimental protocol is described in Appendix A.3. The coefficient of variance for triplicate samples was 6.7 %.

3.5.2.3 Alcohol dehydrogenase (ADH, cytoplasmic)

Alcohol dehydrogenase (ADH) activity was determined from the conversion of ethanol to acetaldehyde with stoichiometric consumption of NAD^+ (Racker, 1950):

$$R\text{-}CH_2OH + NAD^+ \longleftrightarrow R\text{-}CHO + NADH + H^+ \qquad \text{Equation 3.3}$$

NADH has a characteristic absorbance band at 340 nm. The enzyme activity is calculated by the rate of formation of NADH. One unit of enzyme activity is defined as

the conversion of 100 moles of substrate (NAD) per minute (equivalent to ΔA of 0.01) in 0.06 M sodium pyrophosphate at pH 8.5. The detailed method of analysis is included in Appendix A.4. The coefficient of variance for triplicate samples was 8.5 %.

3.5.2.4 Glucose-6-phosphate dehydrogenase (G6PDH, cytoplasmic)

The analysis of G6PDH was based on the conversion of glucose-6-phosphate to D-gluconate-6-phosphate in the presence of NADP (Schutte *et al.*, 1983):

D-Glucose-6-phosphate + $NADP^+$ ⟷ D-Gluconate-6-phosphate + NADPH + H^+ **Equation 3.4**

The simultaneous reduction of β-NADP to β-NADPH is monitored by measuring the increase in the absorbance at 340 nm. The unit of enzyme is defined as that catalysing the conversion of one millimole of substrate (glucose-6-phosphate) in one minute under the assay conditions at pH 7.6. The comprehensive method of estimation is presented in Appendix A.5. The coefficient of variance for triplicate samples was 5.5 %.

3.5.2.5 β-galactosidase (cytoplasmic)

The analysis of β-galactosidase is based on the principle of release of *o*-nitrophenol (ONP) and galactose from *o*-nitrophenyl-β-galactoside (ONPG) at 37°C in PPB-Mn buffer of pH 6.6 (Flores *et al.*,1994):

o-nitrophenyl-β-galactoside ⟷ o-nitrophenol + galactose **Equation 3.5**

The extinction coefficient of *o*-nitrophenol (3.1 $mM^{-1}cm^{-1}$) is used for the calculation of number of moles of *o*-nitrophenol produced. The unit of enzyme activity is defined that which catalyses production of one moles of *o*-nitrophenol in 1 min at the pH 6.6 and 37°C. The comprehensive method of estimation is included in Appendix A.6. The coefficient of variance for triplicate samples was 10.2 %.

3.5.3 Dry weight analysis

The dry weight of yeast in suspension was measured to enable the results of total soluble protein or enzyme release to be presented on a specific basis i.e. 'per g dry weight yeast'. The cell pellet was collected from 1 cm^3 suspension by centrifugation in an Eppendorf microfuge tube and dried at 80°C for 48 h. The analysis was carried out in triplicate.

3.5.4 The maximum total soluble protein and enzyme release

High pressure homogenisation (HPH) was performed to establish the maximum total soluble protein and enzymes available for release (R_m) for both Baker's yeast and *Kluyveromyces lactis*, as described in Section 3.3.1. To determine R_m, the yeast suspension was passed through the high pressure homogeniser at 69.0 MPa for an increasing number of passes until no further protein release resulted. The number of passes required depended on the species of microorganism and the location of the enzyme.

3.5.5 Malvern size analysis

The Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK; serial number: 33265-44), used to determine the size of the cell, is shown in Figure 3.3. The Malvern Mastersizer is based on the principle of laser light scattering. Samples taken after each pass using high pressure homogenisation with or without pretreatment (single) were agitated using a vortex mixer to avoid debris agglomerating. A 1 ml aliquot of sample was injected into the reservoir of the analyser containing particle-free water. The resultant suspension was mixed by agitation and sonication prior to measurement of the size of the cell debris. The control sample, not subjected to disruption, was also measured to provide a benchmark for other samples. As the size of the cell or cell debris is not uniform, the Malvern size analyser generates the particle size distribution and gives five indicator values, D[v, 0.1], D[v, 0.5], D[v, 0.9], D[4,3] and D[3,2]. D[v, 0.1] and D[v, 0.9] show that the diameters below which 10 % and above which 90 % of the particle lie, while D[4,3], D[v,0.5] and D[3,2] are the volume or mass moment mean, the volume median diameter and the surface area moment mean, respectively. The values of D[4,3], D[v, 0.1] and D[v, 0.9] were used to determine the effect of combined pretreatment on the micronisation caused by mechanical cell disruption. Table 3.8 illustrates the setting of parameters for the Marlven analyser.

Figure 3.3 Malvern Mastersizer 2000 in the laboratory

Table 3.8 The parameters of the system setting in the laboratory

Distribution type	Range lens	Beam length	Presentation	Sample's density
Volume	300RFmm	3.40 mm	3THD	1.000 g/l

3.5.6 Optical microscopy

The physical damage of yeast cell structure caused by high pressure homogenisation was estimated qualitatively by observing the samples under the light microscope. Samples were diluted with phosphate buffer, stained with methylene blue, and subsequently viewed under a light microscope (Olympus Bx40) at 1000 X magnification. Micrographs were taken for yeast disrupted at 27.6, 41.4 and 69.0 MPa at each pass with or without pretreatment (combined), and compared to the control sample not subjected to disruption.

3.6 CONCLUSIONS

The aim of this study is to determine the effect of physicochemical pretreatments on the microbial cell. Specifically, reduction in energy consumption of the mechanical disruption step, reduction in micronisation of cell debris and enhancement of selective enzyme release were desired. The procedure for each of *S. cerevisiae* and *K. lactis* has been detailed. The two approaches to mechanical cell disruption and their application have been defined. The effect of different pretreatments on the energy consumption and selective enzyme release were detected by total soluble protein assay and specific enzyme assays. The physical cell damage was detected by observation by optical microscopy. This was used to observe the gradual cell degradation.

Chapter 4
Cell Disruption of Baker's yeast by Different Mechanical Methods

4.1 INTRODUCTION

The results of Baker's yeast (*Saccharomyces cerevisiae*) disrupted by different mechanical methods in the absence of pretreatment are presented in this chapter to provide a benchmark for the comparison of combined microbial cell disruption. The release kinetics of each mechanical cell disruption method under different conditions are demonstrated. The framework of analysis is presented for use in the further studies.

4.2 DISRUPTION USING HIGH PRESSURE HOMOGENISATION

The experiments using high pressure homogenisation (HPH) for mechanical cell disruption on Baker's yeast are defined in Table 4.1. All experiments were performed using 300 ml yeast suspension with a 1.5 % (dry weight) cell concentration corresponding to 5 % in wet weight. The effect of operating pressure on protein release was investigated by disrupting the yeast suspension at five different pressure differences: 13.8, 27.6, 41.4, 55.2 and 69.0 MPa. A cooling coil was used to maintain the recirculated suspension at 20℃ to avoid the protein denaturation. The total protein release as a function of number of passes under different operating pressures is presented in Figure 4.1. Data for the total soluble protein release on HPH at different pressures are shown in Table B.1 of Appendix B.

Table 4.1 Experimental conditions for high pressure homogenisation using 1.5 % cell concentration (dry weight) of Baker's yeast

Microorganism	Pressure (MPa)	No. of Passes	Protein and enzymes measured
Baker's yeast (*Saccharomyces cerevisiae*)	13.8	10	Total soluble protein Invertase α-glucosidase ADH G6PDH
	27.6		
	41.4		
	55.2	5	
	69.0		

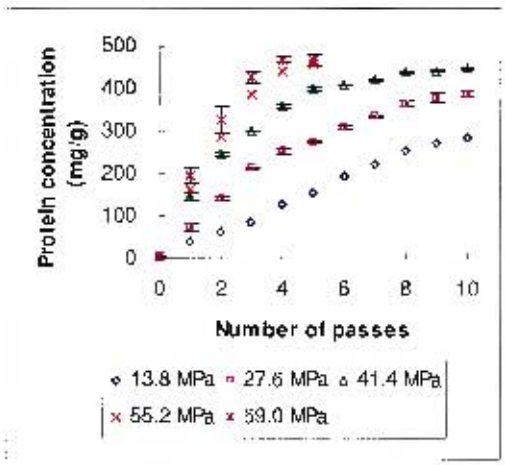


Figure 4.1 Total soluble protein release from Baker's yeast (1.5 % dry weight) by high pressure homogenisation as a function of number of pass and operating pressure

From Figure 4.1, it is seen that increased total soluble protein release resulted on an increase in the operating pressure. The maximum soluble protein release was obtained for 4 passes at 69.0 MPa. This release of 470 mg/g was used as the maximum soluble protein release (R_m) for kinetic calculations. The maximum protein release (R_i) obtained at 13.8 MPa was 288 mg/g following 10 passes. As the pressure increased to 27.6 MPa, the soluble protein release at 10 passes (R_i) increased to 388 mg/g. A maximum protein release of 440 mg/g at 41.4 MPa was achieved on 8 passes. A similar protein release of 442 mg/g was obtained on 4 passes at 55.2 MPa. Therefore, the total protein release was influenced by both operating pressure and number of passes, synergistically. The number of passes required to achieve maximum protein release is reduced by increasing pressure. The energy requirement per gram protein release on HPH at each operating pressure is shown in Table 4.2. The energy input per pass at 13.8, 27.6, 41.4, 55.2 and 69.0 MPa can be estimated as 13.8, 27.6, 41.4, 55.2 and 69.0 kJ/kg (Anand *et al.*, 2007). The energy requirement per protein release increased from 0.48 kJ/g (energy/protein) at 13.8 MPa through 0.66 kJ/g at 27.6 MPa to 0.75 kJ/g, and decreased to 0.60 and 0.59 kJ/g when the operating pressure increased to 55.2 and 69.0 MPa, respectively, owing to the reduced number of passes used. This suggests that partial release of periplasmic enzymes is less energy intensive."

Table 4.2 Energy requirement of HPH (*S. cerevisiae*, control)

Pressure	Passes	R_i (mg/g)	Energy (kJ/kg)	Energy/protein (kJ/g)
13.8 MPa	10	288	138	0.48
27.6 MPa	9	379	248	0.66
41.4 MPa	8	440	331	0.75
55.2 MPa	5	458	276	0.60
69.0 MPa	4	470	276	0.59

The effect of operating pressure and number of passes on release of specific enzymes is shown in Figure 4.2. Data are presented in Tables B.2 of Appendix B. Increasing operating pressure reduced the number of passes required to reach maximum enzyme release while increasing the extent of release. This is similar to the trends observed for total soluble protein release. The operating pressure of 69.0 MPa was preferred by each enzyme to achieve maximum release. The number of passes required for maximum release at 69 MPa was different for each enzyme. Maximum invertase release was achieved by pumping yeast suspension through homogeniser for 3 passes, α-glucosidase and G6PDH required 4 passes, while ADH required 5 passes to reach maximum enzyme release. The ease of enzyme release is a function of the location of enzyme.

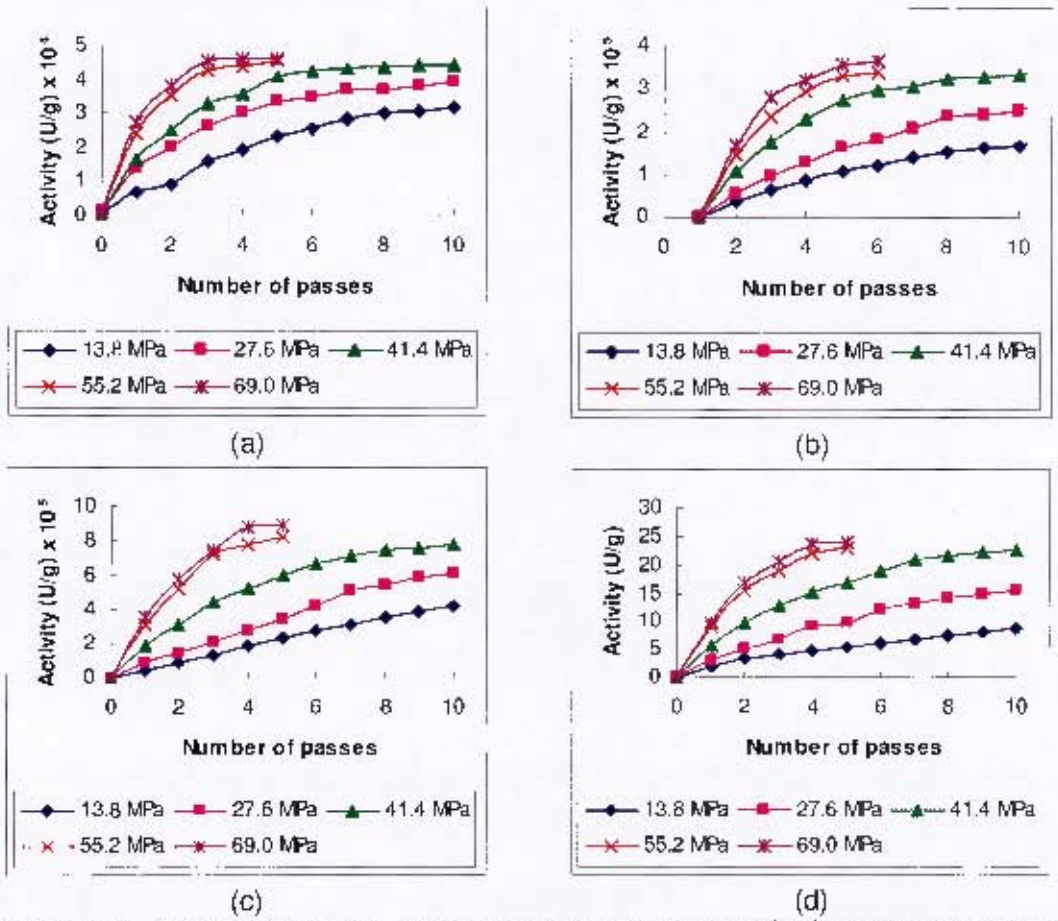


Figure 4.2 **(a) Invertase, (b) α-glucosidase, (c) ADH and (d) G6PDH released from Baker's yeast (1.5 % dry weight) by high pressure homogeniser as a function of no. of passes and operating pressure**

Table 4.3 **The maximum protein (R_m) or enzyme activity available for release on disruption**

	Protein (mg/g)	Invertase (U/g)	α-glucosidase (U/g)	ADH (U/g)	G6PDH (U/g)
passes	4	3	4	4	5
R_m	470	4.57×10^4	3.53×10^5	8.73×10^5	24.0

The kinetic rate constant (k) was determined using Equation 4.1, where 'R' is the protein or enzyme release obtained following 'N' passes, 'R_m' is maximum soluble protein available for release.

$$kN = \ln \frac{R_m}{R_m - R} \qquad \text{Equation 4.1}$$

The kinetic rate constant (k) for total soluble protein release determined from the slope of $\ln(R_m/(R_m-R))$ as a function of number of passes as shown in Figure 4.3, and listed in Table 4.4. The k of total soluble protein increased with the increase in operating pressure. Through the correlation coefficient R^2 the quality of the fit is assessed.

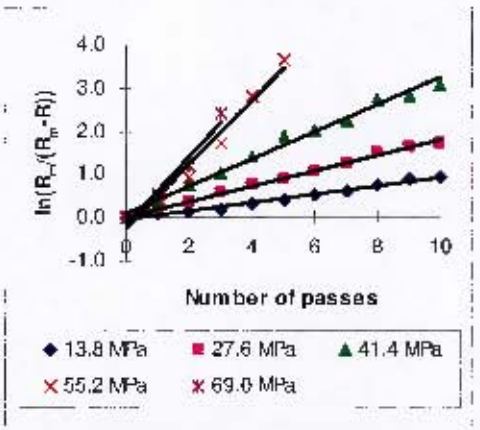


Figure 4.3 **Kinetic rate constant (k) of total soluble protein released from Baker's yeast by high pressure homogenisation is determined as the slope of the relationship between $\ln(R_m/(R_m-R))$ and number of passes**

Table 4.4 **Kinetic rate constant (k) of total soluble protein released obtained by high pressure homogenisation at different operating pressure**

Pressure (MPa)	13.8	27.6	41.4	55.2	69.0
k (pass^{-1})	0.10	0.18	0.35	0.75	0.78
R^2	0.9887	0.9954	0.9917	0.9750	0.9620

The extent of release (R_i/R_m) was determined using Equation 4.2, where 'R_i' is the maximum protein or enzyme release obtained at each operating pressure.

$$\text{Extent of release} = \frac{R_i}{R_m} \qquad \text{Equation 4.2}$$

The R_i/R_m of total soluble protein release as a function of operation pressure is shown in Figure 4.4. The R_i/R_m of total soluble protein increased with increasing operating pressure from 0.61 at 13.8 MPa through 0.81 at 27.6 MPa and 0.93 at 41.4 MPa to 0.97 at 55.2 MPa. Little further increase occurred on increase of operating pressure in the range 41.4 to 69.0 MPa. However, the number of passes for maximum protein release decreased with increasing operating pressure from

8 passes at 41.4 MPa through 5 passes at 55.2 MPa to 4 passes at 69.0 MPa. Hence, with increasing operating pressure, the kinetic rate constant and the extent of release of total soluble protein increases, while the number of passes for maximum protein release decreases.

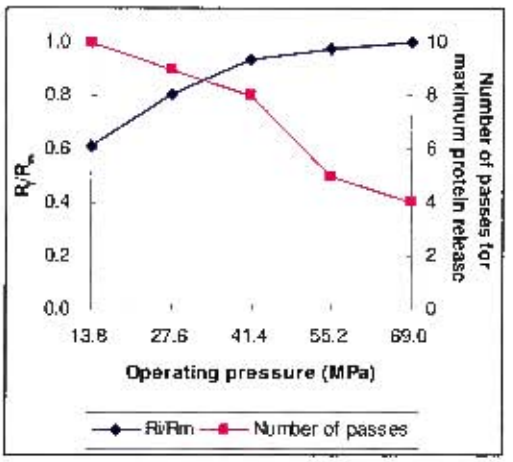


Figure 4.4 **The extent of release of total soluble protein release from Baker's yeast by high pressure homogenisation as a function of operating pressure**

Determination of the kinetic rate constants (k) for enzyme release determined by the relationship between number of passes and $\ln(R_m/(R_m-R))$ are listed in Table 4.5. The values of k of wall-associated and periplasmic enzymes are higher than the values of k of cytoplasmic enzymes at same operating pressure. The quality of the fit is assessed by the correlation coefficient R^2. The kinetic rate constant and the extent of release for protein and enzymes assayed released are shown in Figure 4.5. The rate constants increased with the increasing operating pressure. Figure 4.5a presents the release sequence of enzymes: cell wall associated enzyme > periplasmic enzyme > cytoplasmic enzyme. This is in agreement with Torner and Asenjo (1991), Melendres *et al.* (1993) and Balasundaram and Pandit (2001). From Figure 4.5b, it is seen that the extent of release for total soluble protein and cell-associated enzyme increased with increasing operating pressure. The values of the R_i/R_m of invertase and α-glucosidase increased with the operating pressure from 13.8 to 41.4 MPa. With further increase in the pressure above 41.4 MPa, no significant increase was obtained. The values of the R_i/R_m of ADH and G6PDH depended more strongly on the operating pressure, increasing with pressure from 13.8 MPa to 69.0 MPa. Therefore, the kinetic rate constant and the extent of release of total soluble protein and enzymes depend on the operating pressure, number of passes and enzyme location.

Table 4.5 **Kinetic rate constant (k) of protein and enzyme released obtained by high pressure homogenisation at different disruption pressure**

Pressure (MPa)	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
13.8	0.10	0.9887	0.14	0.9946	0.08	0.9951	0.07	0.9982	0.04	0.9843
27.6	0.18	0.9954	0.22	0.9885	0.15	0.9981	0.13	0.9875	0.11	0.9946
41.4	0.35	0.9917	0.40	0.9883	0.31	0.9976	0.23	0.9979	0.29	0.9930
55.2	0.75	0.9750	0.82	0.9893	0.65	0.9896	0.58	0.9923	0.62	0.9870
69.0	0.78	0.9620	0.88	1	0.70	0.9886	0.64	0.9863	0.65	0.9953

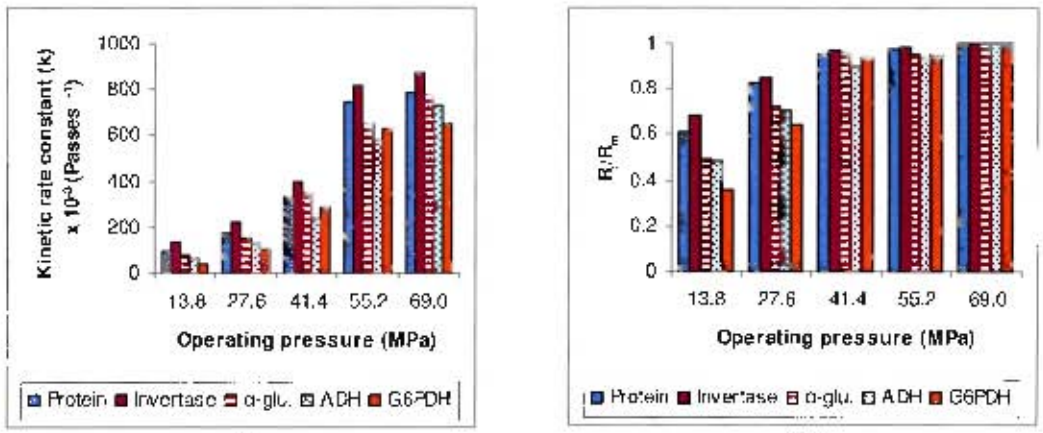


Figure 4.5 **(a) The kinetic rate constant (k) and (b) the extent of release for protein and enzymes released from Baker's yeast by high pressure homogenisation as a function of operating pressure**

The nature of dependence of k on operating pressure is described by the power law where the pressure exponent (a) is determined using Equation 4.3, shown by Hetherington *et al.* (1971) and demonstrated in Figure 4.6

$$k = k'P^a \qquad \text{Equation 4.3}$$

The pressure exponent of protein and enzymes studied are listed in Table 4.6. R^2 demonstrates the goodness of fit. The pressure exponent of total soluble protein determined in Figure 4.6 was 1.39. A similar value of 1.41 was reported by Anand (2004) for the same cell disruption. These are compared with literature values in Table 4.7. The difference of pressure exponents may result from use of different homogeniser designs, operating conditions and microbial species. The pressure exponent increased with increasing intracellular location as follows: cell wall associated enzyme > total soluble protein > periplasmic enzyme > cytoplasmic enzyme.

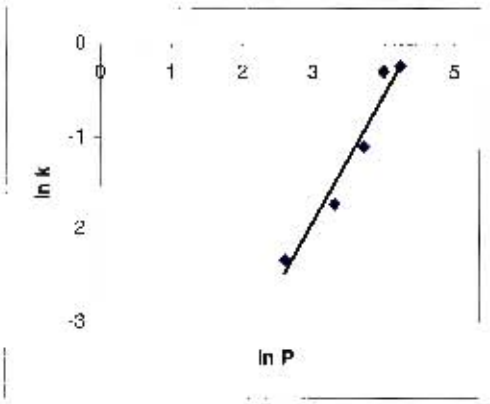


Figure 4.6 **Pressure exponent of total soluble protein from Baker's yeast by high pressure homogenisation determined by the releationship between ln(k) and ln(P)**

Table 4.6 **Pressure exponent of protein and different enzymes from Baker's yeast by high pressure homogenisation**

Enzymes	Protein	Invertase	α-glucosidase	ADH	G6PDH
a	1.39	1.24	1.46	1.48	1.85
R^2	0.9532	0.9462	0.9593	0.9460	0.9742

Table 4.7 **Summary of the pressure exponent reported for different microorganisms**

Microorganisms	Operating pressure (MPa)	Initial cell concentration (g/l)	a	Reference
S. cerevisiae	0-49.03	300-750	2.9	Hetherington *et al.*, 1971
S. cerevisiae	50-125	93-148	1.87	Engler and Robinson, 1981
S. cerevisiae	13.8-69.0	10	1.41	Anand, 2004
E. coli *E.coli* (Recombinant)	30-95	2-174 (dry weight)	1.43 1.41	Sauer et al., 1989
Alcaligenes eutrophus (*Cupriavidus necator*) (Exponential phase) (Stationary phase)	0-122.7	96-257 (dry weight)	3.08 1.59- 1.69	Harrison *et al.*, 1991b

4.3 DISRUPTION OF YEAST USING ULTRASONICATION

The ultrasonication conditions used were varied across power input, continuous disruption time, cooling time, total disruption time and protein and enzymes assayed. These are listed in Table 4.8. The effect of power input on total protein release was investigated by sonication at three different power inputs: 40, 60 and 80 W. All experiments were performed using a 15 ml yeast suspension at a 1.5 % cell concentration (dry weight). The yeast suspension was disrupted on ice to avoid protein denaturation.

Table 4.8 Experimental conditions for sonication using 1.5 % cell concentration (dry weight) of Baker's yeast

Power	Length of sonication cycle	Length of cooling cycle	Number of sonication cycle	Protein and enzymes measured				
				Protein	Invertase	α-glu.	ADH	G6PDH
40 w	5 min	5 min	5	√	√	√	√	√
60 w	3 min	3 min	7	√				
80 w	3 min	3 min	7	√	√	√	√	√

The total protein release is shown as a function of sonication time and power input in Figure 4.7. Data for the total soluble protein released on ultrasonication at different power inputs are presented in Table B.3 of Appendix B. The total soluble protein release increased with increasing sonication time. The protein concentration reached a maximum of 304 mg/g when the yeast suspension was sonicated at 40 W for 25 min. To release this amount protein at 60 or 80 W, 15 min sonication was required. As the sonication time was increased to 21 min, the concentration of total soluble protein released at 60 and 80 W increased to 319 and 353 mg/g, respectively. Hence, the total protein release was influenced by both the sonication power and sonication time, synergistically.

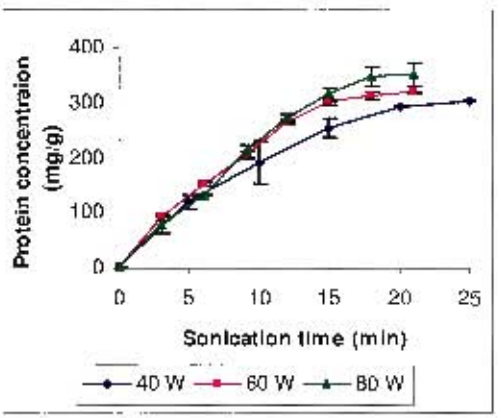


Figure 4.7 Total soluble protein release from Baker's yeast (1.5% dry weight) by ultrasound as a function of time and power input

The energy requirement per gram protein release on ultrasonication at each power input is shown in Table 4.9. The energy requirement per protein release increased from 0.88 kJ/g at 40 W through 1.06 kJ/g at 60 W to 1.27 kJ/g at 80 W. This may relate to the partial release of periplasmic enzymes is less energy intensive, which has been shown on protein release using high pressure homogenisation.

Table 4.9 Energy requirement of ultrasonication (*S. cerevisiae*, control)

Power	Time	R_i (mg/g)	Energy (kJ/kg)	Energy/protein (kJ/g)
40 W	25 min	304	267	0.88
60 W	21 min	319	338	1.06
80 W	21 min	353	449	1.27

The effect of power input on enzyme release was investigated by sonication of the yeast suspension (1.5 %, dry weight) at 40 and 80 W. The release profiles of invertase, α-glucosidase, ADH and G6PDH as a function of power input and sonication time are shown in Figure 4.8. Raw data for enzyme release by ultrasonication are listed in Tables B.4 of Appendix B. Similar to total soluble protein release, enzyme release increased with increasing power or sonication time or both. At both 40 and 80 W, to achieve a significant amount of invertase (cell wall associated enzyme) release, 15 min sonication was required. Further increase in the sonication time did not result in an increased extent of invertase release. The required duration of sonication for maximum enzyme release was a function of the location of enzyme. To result in significant α-glucosidase (periplasmic enzyme) release, 25 min sonication was required when yeast suspension was sonicated at 40 W. The maximum α-glucosidase release was achieved after 18 min sonication at 80 W. The maximum release of cytoplasmic enzyme ADH and G6PDH were obtained at 80 W and 25 min sonication. In summary, sonication time required for maximum enzyme release increased with increasing intracellular location and with decreasing power input.

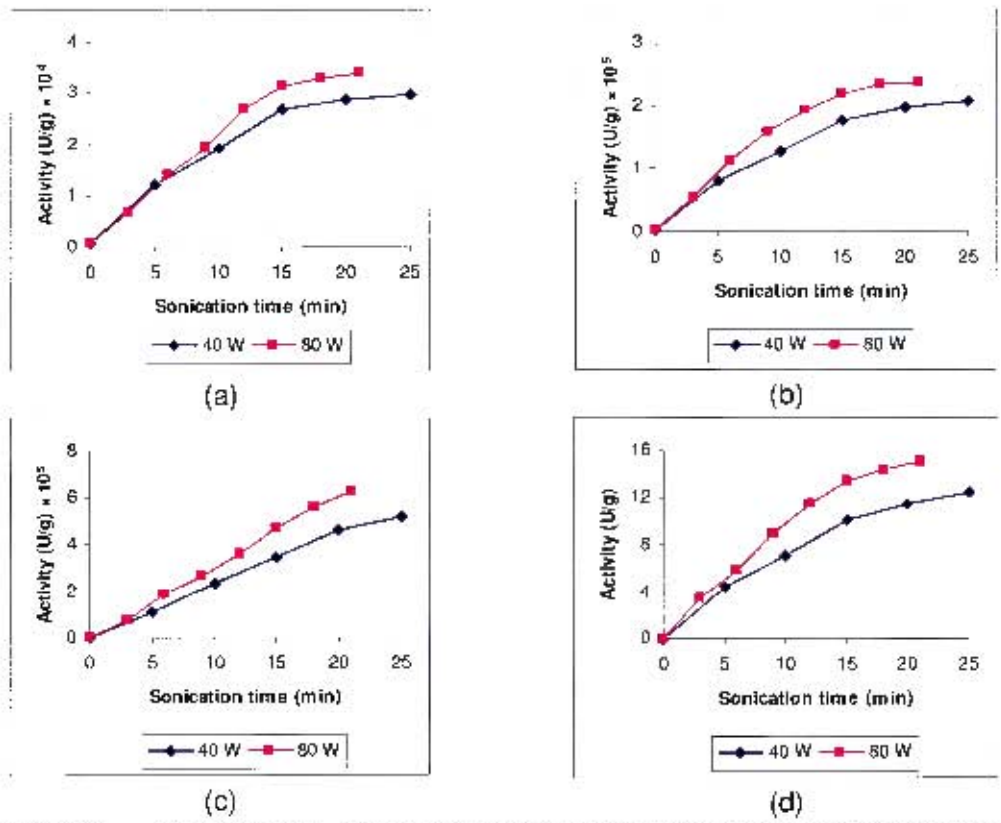


Figure 4.8 **(a) Invertase, (b) α-glucosidase, (c) ADH and (d) G6PDH released from Baker's yeast (1.5 % dry weight) by ultrasound as a function of power**

The maximum soluble protein release (R_m) and enzyme activities were determined by passing the yeast suspension through the high pressure homogeniser at 69.0 MPa. The maximum total soluble protein and enzymes available from disruption (R_m) are illustrated in Table 4.2. The first order kinetic rate constant for protein and enzyme release (k) was determined using Equation 4.4, where 'R' is the protein or enzyme release obtained in each sample at time 't':

$$kt = \ln \frac{R_m}{R_m - R} \qquad \text{Equation 4.4}$$

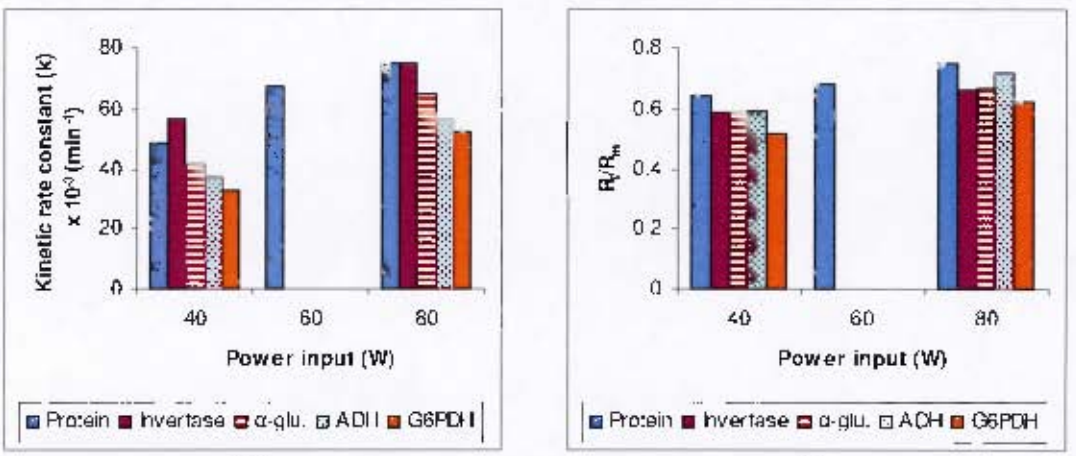
These kinetic rate constants for total soluble protein and different enzymes, are listed in Table 4.10 and Table 4.11, and shown in Figure 4.9a. Figure 4.9b presents the extent of release for protein and enzymes on sonication as a function of power input.

Table 4.10 Kinetic rate constant (k) of total soluble protein released obtained by ultrasonication at different power input

Power input (w)	k (min^{-1})	R^2
40	0.05	0.9957
60	0.07	0.9975
80	0.08	0.9882

Table 4.11 Kinetic rate constant (k) of different enzymes released obtained by ultrasonication at different power input

Power input (w)	Invertase		α-glucosidae		ADH		G6PDH	
	k (min^{-1})	R^2	k (min^{-1})	R^2	k (min^{-1})	R^2	k (min^{-1})	R^2
40	0.06	0.9954	0.04	0.9907	0.04	0.9942	0.03	0.9909
80	0.08	0.9818	0.06	0.9983	0.06	0.9725	0.05	0.9956



(a) (b)

Figure 4.9 (a) The kinetic rate constant (k) and (b) the extent of release for protein and enzymes released from Baker's yeast by ultrasonication as a function of power input

In Table 4.10, the k of total soluble protein release obtained using sonication at 40 W is reported as 0.05 min^{-1}. On further increasing in the power input to 60 and 80 W, the

k increased by 40 and 55 %, illustrating an increasing rate of protein release with increasing power input. Tables 4.10 and 4.11 and Figure 4.9a show the k of release for protein and enzymes was increased with the increasing power. The sequence of enzymes released by ultrasonication was similar to using high pressure homogenisation: cell wall associated enzyme > total soluble protein > periplasmic enzyme > cytoplasmic enzyme. In Figure 4.9b, the extent of release for total soluble protein and enzymes is seen to increase with increasing power. When the power increased from 40 to 80 W, the R_i/R_m of protein increased by 16 %, the R_i/R_m of invertase and α-glucosidase by 30 %, and the R_i/R_m of ADH and G6PDH was obtained. Hence, the extent of release depended on the location of the enzyme, and increased with increasing intracellular location.

4.4 CONCLUSIONS

The total soluble protein and enzyme release using high pressure homogenisation increased with increasing operating pressure and number of passes, synergistically, and thereby with increasing power input. Similarly, the total soluble protein and enzyme release using sonication increased with increasing power input and sonication time, synergistically, and thereby with increasing energy input. The ease of enzyme release was a function of the location of enzyme. The number of passes or the sonication time required for maximum enzyme release increased with increasing intracellular location. The values of the maximum release (R_m) for total soluble protein and enzymes were defined on HPH at 69.0 MPa. These values were used to determine the kinetic rate constant and the extent of release. The kinetic rate constant and the extent of release increased with increasing power input. Compared to other enzymes, the power input had more influence on the values of k and R_i/R_m of the cytoplasmic enzymes. The release sequence for the enzymes was determined: cell wall associated enzyme > periplasmic enzyme > cytoplasmic enzyme. This result is in agreement with those of Torner and Asenjo (1991), Melendres *et al.* (1993) and Balasundaram and Pandit (2001). The pressure exponent (a) of total soluble protein release by HPH was 1.39. A similar result of 1.41 has been reported by Anand (2004). The pressure exponent was dependent on the location of the enzyme: cell wall associated enzyme > periplasmic enzyme > cytoplasmic enzyme.

Chapter 5
Effect of Pretreatment on Ultrasonication

5.1 INTRODUCTION

The effect of single pretreatment on the energy efficiency of cell disruption and selective product release using ultrasonication is presented in this chapter. Baker's yeast (*Saccharomyces cerevisiae*) was used as model organism. The optimised condition of each single pretreatment was determined. Ultrasound was used to mediate disruption as it can be conducted at small scale compared to high pressure homogenisation, allowing efficient optimisation studies.

Three single pretreatment methods were used for cell weakening before sonication: heat pretreatment, pH shock and osmotic shock. All experiments were performed using a 15 ml yeast suspension containing 1.5 % yeast (dry weight). Total soluble protein release was used to measure the disruption of yeast cultures on sonication following a single pretreatment, while the selective product release was determined by release of a suite of enzymes. The release kinetics following each pretreatment method were quantified across a range of conditions.

5.2 HEAT PRETREATMENT

To assess the impact of heat pretreatment, the magnitude and rate of temperature change and duration of exposure to elevated temperature are considered. The maximum temperature of treatment, rate of heating and holding time were varied over the ranges from 40 to 60°C, 0.5°C/s to >20°C/s and 0 to 1 hr, respectively. The conditions of heat pretreatment, heating methods, maximum temperature of pretreatment, heating rate and holding time are listed in Table 3.3. The yeast suspension was sonicated at 40 W following a heat pretreatment. In Section 5.2.1, the effect of heat pretreatment on extent of disruption is considered. In Section 5.2.2, the effect of temperature pretreatment on protein denaturation is considered. Section

5.2.3 considered the impact on selective release, while in Section 5.2.4, this is extended to evaluate the effect of heat pretreatment on the rate of subsequent disruption.

5.2.1 Effect of heat pretreatment on protein release

5.2.1.1 Comparing the effect of heat pretreatment on protein release using different heating methods

Two methods were used to introduce heat pre-treatment: dilution into a pre-warmed buffer and heat exchange in a heating coil to achieve temperature in the range 40 to 60°C. The fastest flow rate (0.02 l/s) delivered by the pump was employed for coil experiments. This resulted in heating rates of 3.5, 4.3 and 5.2°C/s for pretreatments at 40, 50 and 60°C, respectively. The heating rates for the dilution experiments exceeded 20°C/s. Total soluble protein release results obtained on sonication following these pretreatments are presented in Table C.1 to C.2 of Appendix C and Figure 5.1. The rate and extent of protein release is given as a function of sonication time across a range of temperature pretreatments.

When the pretreatment was carried out at the same maximum temperature, the total protein released on disruption following heat exchange is similar to that using temperature shock by dilution as shown in Figure 5.1a-c. In Figure 5.1.d, it is seen that the maximum total protein release increased with heat pretreatment compared to the control as an increasing function of temperature from 365 mg/g at 40°C to 390 mg/g at 50°C, and decreased to about 90 mg/g at a temperature of 60°C. The equivalent protein release obtained following a 25 min sonication of the control of 304 mg/g was obtained on 12 min sonication following heat pretreatment at maximum temperature varying between 40 and 50°C, illustrating that heat pretreatment decreases the energy requirement of cell disruption.

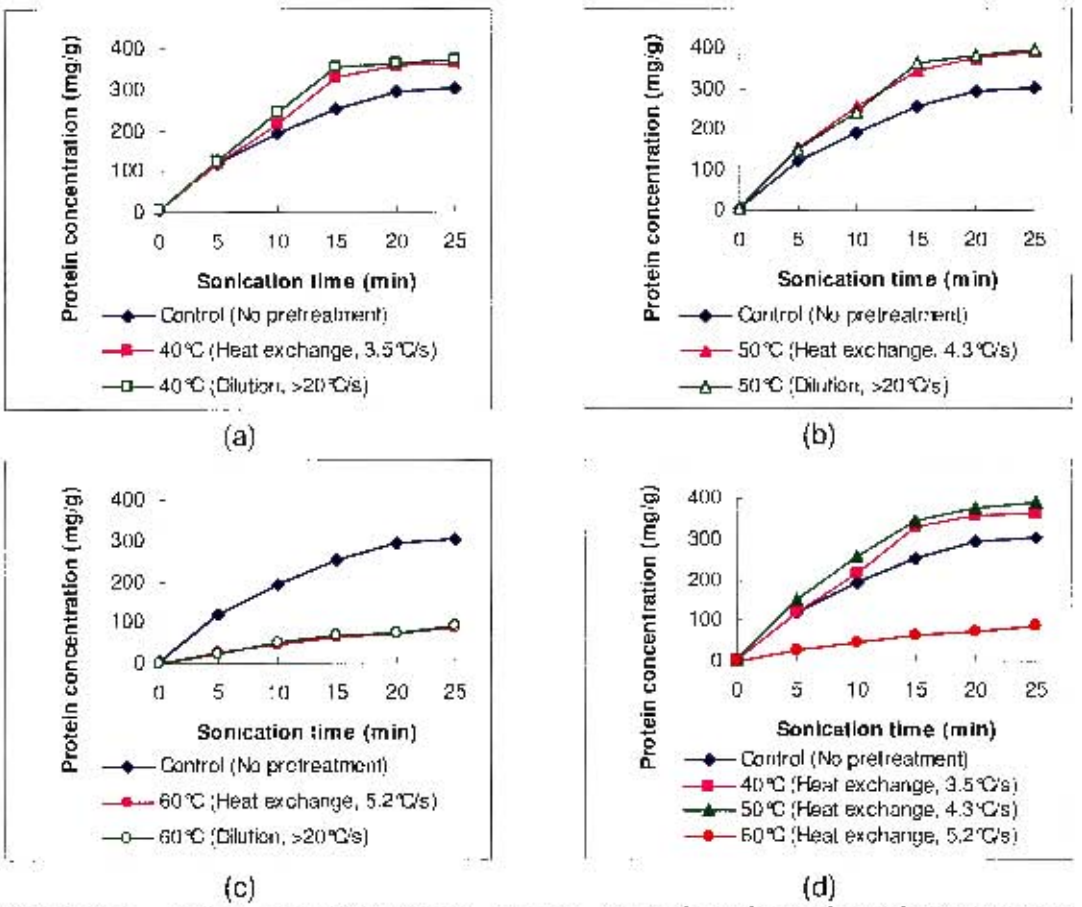


Figure 5.1 **Total soluble protein release as a function of sonication time following heat pretreatment at a maximum temperature of (a) 40°C, (b) 50°C and (c) 60°C using heat exchange or dilution. Holding time was minimised, and subsequent sonication was at 40 W. (d) Total soluble protein release on sonication of 40 W following heat pretreatment at different maximum temperatures using heat exchange.**

The energy requirement per gram protein release on ultrasonication at 40 W following heat pretreatment at 40 and 50°C is shown in Table 5.1. The energy input required for heating was determined using Equation 5.1, where 'E' is the specific energy input required for heating in terms of kJ/kg, 'c' is the specific heat of water (4.2 kJ/kg°C), 'ΔT' is the temperature difference.

$$E=c\Delta T \qquad \text{Equation 5.1}$$

Hence, the energy input required for heat pretreatment at 40 and 50°C are 75.6 and 118 kJ/kg, respectively. Following heat pretreatment at 40 and 50°C, the energy requirement per protein release decreased to 0.71 and 0.81 kJ/g, which represents 21 % and 6 % energy saving compared to the control (0.86 kJ/g). This suggests that heat pretreatment could improve the energy efficiency of ultrasonication.

Table 5.1 **Energy requirement of ultrasonication following heat pretreatment at 40 and 50°C (*S. cerevisiae*)**

Power	Pretreatment conditions	Time	Protein conc.	Energy (kJ/kg)	Energy/protein (kJ/g)
40 W	No pretreatment	25	304	267	0.86
40 W	Heat pretreatment at 40°C, minimal holding	15	329	236	0.71
40 W	Heat pretreatment at 50°C, minimal holding	15	344	278	0.81

5.2.1.2 Effect of temperature of heat treatment

To investigate the effect of the maximum temperature of the pretreatment on protein release, six temperatures were used: 40, 45, 50, 52, 55 and 60°C. Total soluble protein release following sonication at 40 W for 25 min after heat pretreatment is shown in Figure 5.2. Data are presented in Table C.2 of Appendix C. The total soluble protein release increased with the increase in temperature of heat pretreatment from 362 mg/g at 40°C through 379 mg/g at 45°C to 389 mg/g at 50°C, compared with the control at 304 mg/g indicated by the dashed line in Figure 5.2. These data show good agreement with replicate data presented in Figure 5.1. On heat pretreatment at 52°C an 8 % increase in total soluble protein concentration with respect to the control was obtained, compared with the 19 % obtained at 40°C release over the control and 28 % at 50°C heat pretreatment. The total soluble protein concentration following disruption decreased dramatically as the temperature of heat pretreatment exceeded 52°C, assumed to be due to protein denaturation at higher temperature. The soluble protein in the supernatant decreased to 114 mg/g and 88mg/g on heat pretreatment at 55 and 60°C, respectively. Hence, for energy efficient heat pretreatment, the optimal temperature range is between 40 and 50°C.

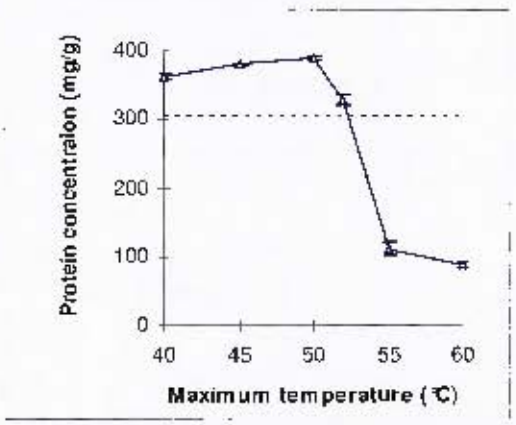


Figure 5.2 **Maximum soluble protein release as a function of the maximum temperature of heat pretreatment by heat exchange.** Sonication was conducted for 25 min at 40 W. The dashed line represents protein release in the control in the absence of pretreatment.

5.2.1.3 Effect of heat treatment at different heating rate

The effect of rate of heating was investigated using the maximum temperature of 40°C. The rates of temperature increase from room temperature to 40°C were varied from 3.5°C/s through 1.7°C/s and 0.5°C/s (heating coil) to 0.1°C/s (STR). Figure 5.3 illustrates that the concentration of protein released increased on subsequent ultrasonication with increasing rate of heating in heat pretreatment. The data are listed in Table C.3 of Appendix C.This is in accordance with the influence of the rate of increase of the temperature increase on the viability of the yeast reported by Gervais and Maranon (1995). The yeasts may adapt to survive during the slow temperature shift. At a gradual heating rate of 0.1°C/s achieved in the STR, little improvement (2 %) in protein release was observed with respect to the control, whereas at a heating rate of 3.5°C/s, the protein release increased by 19 %. On comparison of heating by exchange at 3.5°C/s and the dilution experiment in which the heating rate exceeded 20°C/s, shown in Figure 5.1a, further increase in heating rate beyond 3.5°C/s has little effect on disruption. Hence, the fast heating rate (3.5°C/s) is preferred.

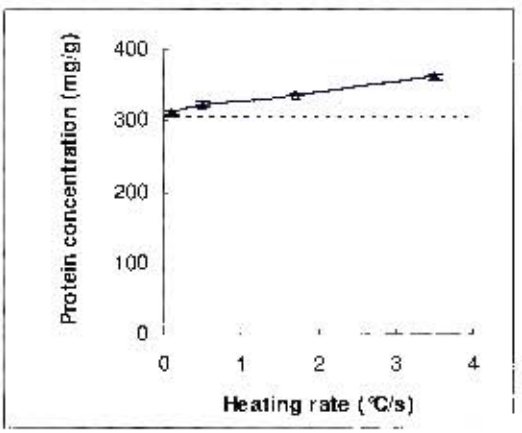


Figure 5.3 **Maximum soluble protein release as a function of heating rate during heat pretreatment.** Heat pretreatment was conducted at 40°C with minimal holding time. Sonication was conducted for 25 min at 40 W. The dashed line represents protein release in the control in the absence of pretreatment.

5.2.1.4 Effect of holding time of heat pretreatment

The effect of holding time of pre-treatment at 40°C on subsequent protein release by sonication was studied. Total soluble protein release on sonication at 40 W for 25 min following a temperature pretreatment with increasing holding time is shown in Figure 5.4. The data are presented in Table C.4 of Appendix C.

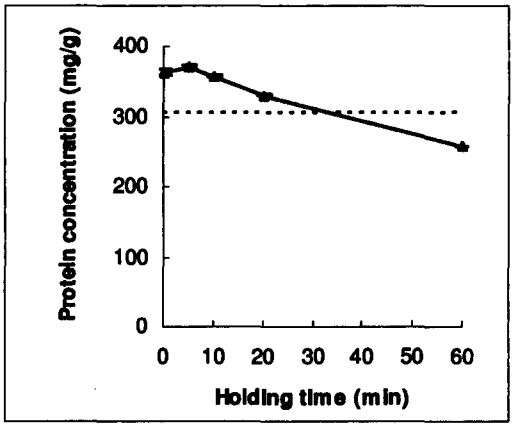


Figure 5.4 **Maximum soluble protein release as a function of holding time during heat pretreatment.** Heat pretreatment was conducted at 40°C with heat rate of 3.5°C/s. Sonication was conducted for 25 min at 40 W. The dashed line represents protein release in the control in the absence of pretreatment.

In Figure 5.4, on varying the holding time from 0 to 10 min, no significant influence on the protein release was observed. On extending the holding time to 20 min and greater, the resultant protein concentration decreased, suggesting protein denaturation occurred. In Figure 5.11a of Section 5.2.4, the maximum kinetic rate constant of total soluble protein was obtained using heat pretreatment with 5 min holding. Hence, heat pretreatment with a holding time of 5 min or less is preferred for efficient cell disruption.

5.2.2 Effect of temperature on denaturation of protein and enzymes

The denaturation of protein and enzymes resulting from temperature pretreatment was investigated by exposing the supernatant following disruption to a temperature of 40 or 50°C. Samples were taken at 0, 10, 20, 60, 180 and 300 s. The fractional denaturation was calculated using Equation 5.2, where 'R' is the protein concentration or enzyme activity of each sample, 'R_0' is the protein concentration or enzyme activity obtained before heating.

$$\text{Denaturation} = 1 - \frac{R}{R_0} \qquad \text{Equation 5.2}$$

The fractional denaturation as a function of holding time at 40 and 50°C is presented in Figure 5.5. From Figure 5.5, the denaturation for protein and enzymes increased with increasing holding time and temperature. Compared to other enzymes, ADH showed the greatest denaturation at both 40 and 50°C. G6PDH was more stable at 40°C than other enzymes, but showed similar denaturation to the remainder of suite

of enzymes at 50°C. It is clear that to minimise denaturation of protein and enzymes released, holding time must be minimised to less than 1 min at 40°C, while less than 20 s holding time is preferred at 50°C. As illustrated by the disruption studies, denaturation is expected to be retarded on exposure of intracellular proteins as compared with proteins in solution.

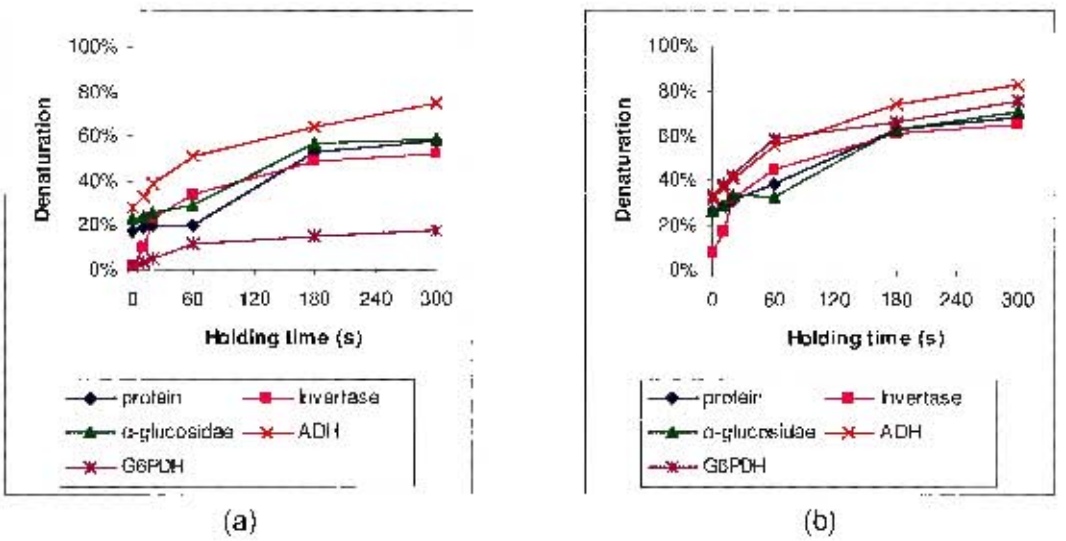


Figure 5.5 Fractional denaturation of protein and enzymes released into the supernatant of the cell suspension as a function of holding time at (a) 40°C and (b) 50°C

5.2.3 Effect of heat pretreatment on selective product release

The selective product release was investigated by release of enzymes from varying locations. Based on location of the intracellular enzymes, its ease of release from the cell is different. Hence, selective product release manipulated by heat pretreatment has been considered. Following the experimental approach described in Section 3.5, data for enzyme release by ultrasonication following a heat pretreatment are listed in Tables C.5 to C.8 of Appendix C. To enable comparison across enzymes, selective product release is analysed in terms of normalised enzyme release (E_N) relative to the maximum available for release in control. E_N is calculated using Equation 5.3

$$E_N = \frac{\text{Maximum enzyme release under operating conditions}}{\text{Maximum enzyme release in control}} \qquad \text{Equation 5.3}$$

As with total protein release, the enzyme released by ultrasonication following heat exchange is similar to that following heat pretreatment using dilution.

5.2.3.1 Effect of temperature of heat pretreatment on enzyme release

The normalised enzyme activity release for each enzyme is compared with that of total soluble protein as a function of the temperature of heat pretreatment in Figure 5.6. From Figure 5.6, it is seen that varying the maximum temperature of pretreatment between 40 and 50°C had strong effect on selective release of α-glucosidase. The E_N of α-glucosidase released was constant at 130 % of that obtained in the control experiment, and decreased as the temperature increased over 50°C. This demonstrates some preferential release of α-glucosidase over other proteins when compared to the increase in soluble protein release of 110 to 120%. The optimal temperature of heat pretreatment for α-glucosidase was 40 to 50°C. The values of E_N of ADH and G6PDH released obtained following heat pretreatment at maximum temperature between 40 and 50°C were quite similar, and approached to the E_N of protein released. This illustrated that the heat pretreatment did not assist selective release of ADH and G6PDH. The E_N of G6PDH lay in the range 110 % to 120 % on temperature of pretreatment from 40 to 52°C, and decreased as the temperature of pretreatment exceeded 52°C. The E_N of ADH reached a maximum of 120 % on pretreatment at 45°C, decreasing slightly at 50°C and losing activity rapidly as the temperature exceeded 50°C. The maximum temperatures of pretreatment of 45 and 52°C were preferred for ADH and G6PDH release, respectively. However, heat pretreatment at temperatures between 40 and 50°C was also sufficient for G6PDH release. Hence, the optimal temperature of heat pretreatment for G6PDH release was in the range from 40 to 52°C. The E_N of invertase obtained on heat pretreatment temperatures between 40 and 50°C was lower than the E_N of protein. While a 5 % increase in invertase release was obtained, this was at the expense of selectivity. Hence, the optimal temperature of heat pretreatment for invertase release was between 40 and 50°C. The optimal condition of pretreatment for selective product release must be chosen on a product by product basis. For the marker enzymes used, it appears heat pretreatment only favours selective release of α-glucosidase.

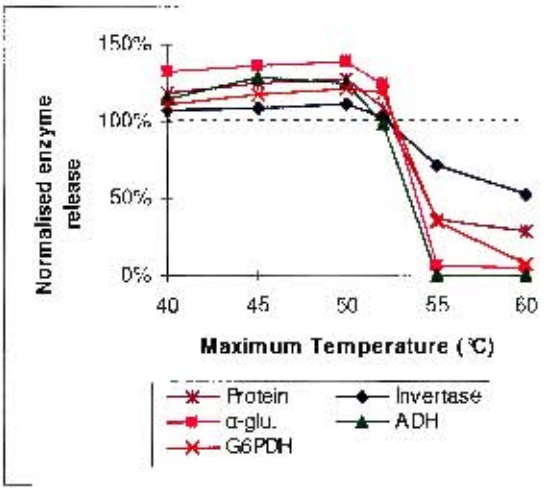
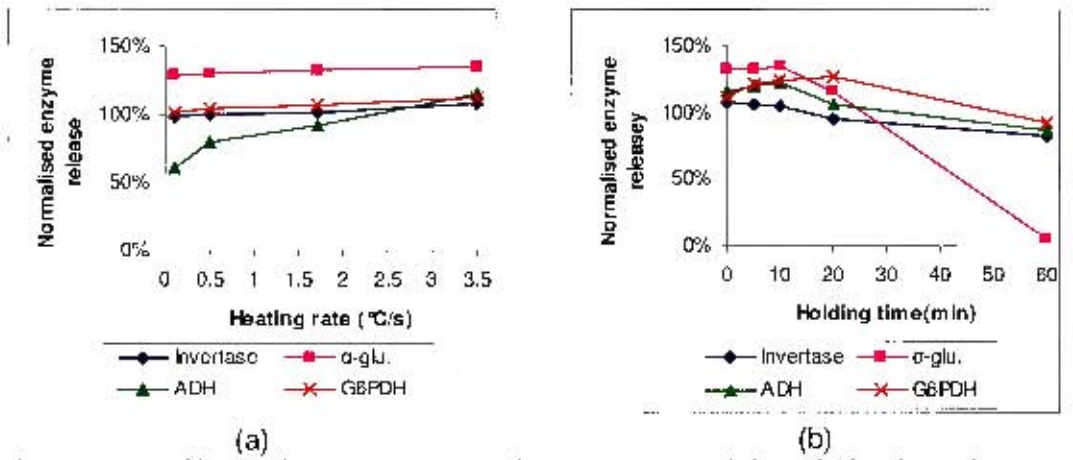


Figure 5.6 Normalised release of enzyme activity following heat pretreatment and sonication at 40 W as a function of pretreatment temperature

5.2.3.2 Effect of heating rate and holding time of heat pretreatment

Figure 5.7a presents the enzyme activity in the supernatant, normalised relative to the control following the 40°C heat pretreatment at different heating rates and subsequent sonication at 40 W. A sonication time of 12 minutes was used here, compared to a sonication time of 25 minutes required for maximum enzyme release in the absence of pretreatment. The normalised activity of the target enzymes released as a function of increasing holding time is presented in Figure 5.7b. On varying the heating rate, no significant change in the values of E_r of invertase, α-glucosidase and G6PDH were obtained, while the E_n of ADH increased with increasing heating rate. Hence, the fast heating rate (3.5°C/s) is preferred. The E_N of all enzymes studied was maintained with holding time from 0 to 10 min. E_N decreased when the holding time exceeded 10 min in all cases except G6PDH. Compared with the enzymes obtained on the control, more than 90 % of α-glucosidase was denatured on the heat pretreatment with 1 hr holding. Hence, the optimal range of holding times of heat pretreatment for invertase, α-glucosidase and ADH are less than 10 min, while less than 20 min holding is preferred by G6PDH. The holding time of heat pretreatment is not only dependent on the stability of protein and enzyme at higher temperature, but the location of the enzymes. The values of E_N of the range of enzymes following a 40°C heat pretreatment at heating rate of 3.5°C/s and minimal holding time is presented in Figure 5.8 as a function of sonication time. The resultant sonication times required for maximum enzyme release are different depending on the location of each enzyme. For invertase (cell wall associated enzyme), a 15 min sonication was required to reach the maximum release, while

greater than 25 min sonication was preferred for release of the other enzymes (periplasmic or cytoplasmic enzymes).



(a) (b)

Figure 5.7 Normalised release of enzyme activity following heating pretreatment at 40°C and sonication at 40 W as a function of: (a) heating rate where holding time was kept minimal, (b) holding time where the heating rate was 3.5°C/s

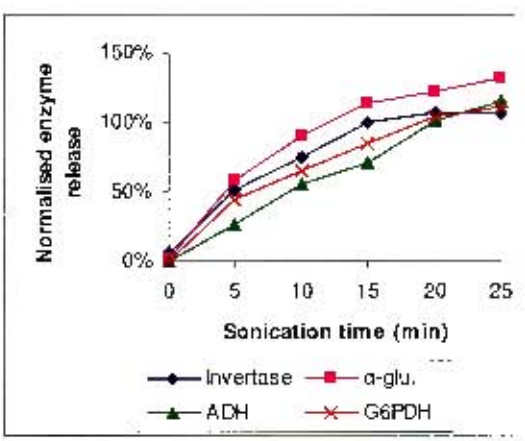


Figure 5.8 Normalised release of enzyme activity following heating pretreatment at 40°C and sonication at 40 W as a function of sonication time where holding time was kept minimal

5.2.4 The kinetic rate constant for protein release following heat pretreatment

The kinetic rate constant for protein release on sonication following heat pretreatment was calculated using Equation 4.4. Data are listed in Tables C.9 to C.11 of Appendix C. To compare the effect of pretreatment on the kinetic rate constant across total soluble protein and enzyme release, the ratio of the kinetic rate constant following pretreatment (k) to that in the control (k_c) was employed (k/k_c).

5.2.4.1 The effect of maximum temperature of heat pretreatment

The ratios of kinetic rate constants (k/k_c) and the extent of release (R_i/R_m) from Baker's yeast following sonication at 40 W for 25 min as a function of maximum temperature of heat pretreatment are presented in Figure 5.9. When the temperature was varied between 40 and 50℃, the heat pretreatment had a stronger effect on the kinetic rate constant of total soluble protein and ADH than other enzymes. The k/k_c of total soluble protein increased with the increase in temperature of heat pretreatment from 1.62 at 40℃ to 1.78 at 50℃, and decreased when the temperature exceeded 50℃. Owing to the effect of temperature influencing both protein release and protein denaturation, the data are insufficient to be further analysed to demonstrate the Arrhenius dependence of each effect. The k/k_c of invertase and α-glucosidase released were constant at 1.00 and 1.60 on heat pretreatment temperatures between 40 and 50℃, and decreased as the temperature over 50℃. The maximum k/k_c of ADH was achieved at the temperature of heat pretreatment of 45℃ and presented about 50 % increase in kinetic rate constant compared to the control. The k/k_c of G6PDH released was constant at 1.10 as the temperature of heat pretreatment varied between 40 and 52℃, and decreased when the temperature exceeded 52℃. When the maximum temperature was varied between 40 and 52℃, the maximum release (R_i) of total soluble protein and enzymes were higher than the controls. The R_i/R_m of total soluble protein, invertase, α-glucosidase and G6PDH release were largely constant at 0.75, 0.65, 0.80 and 0.60, as temperature varied between 40 and 50℃, and decreased as temperature over 50℃. The maximum R_i/R_m of ADH was obtained at the temperature of heat pretreatment of 45℃ and was 76 %. The R_i/R_m of G6PDH was constant at 0.60 when the temperature of heat pretreatment varied between 40 and 52℃. Hence, the optimal range temperatures of heat pretreatment for protein, invertase, α-glucosidase, ADH and G6PDH release were 40 to 50℃, 40 to 50℃, 40 to 50℃, 45℃ and 40 to 52℃, respectively.

greater than 25 min sonication was preferred for release of the other enzymes (periplasmic or cytoplasmic enzymes).

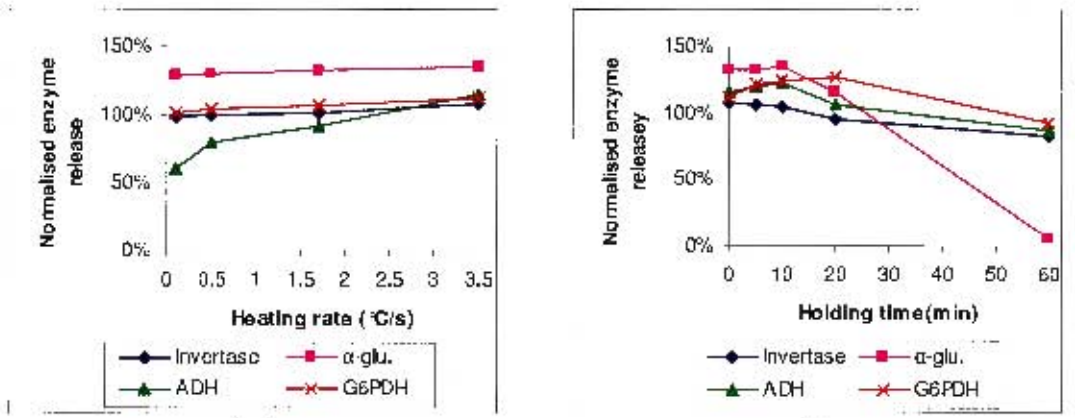


Figure 5.7 **Normalised release of enzyme activity following heating pretreatment at 40°C and sonication at 40 W as a function of: (a) heating rate where holding time was kept minimal, (b) holding time where the heating rate was 3.5°C/s**

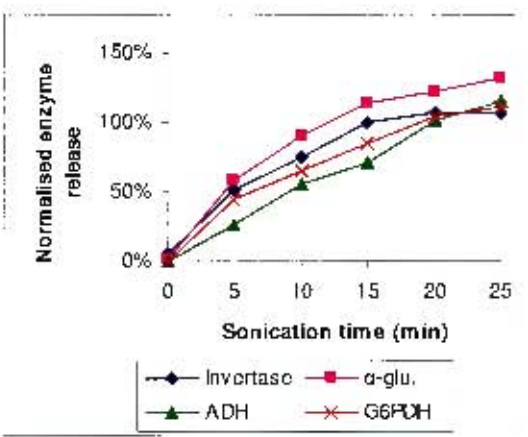
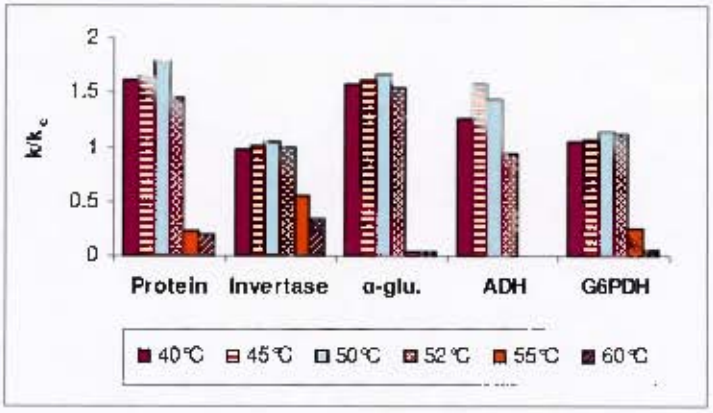


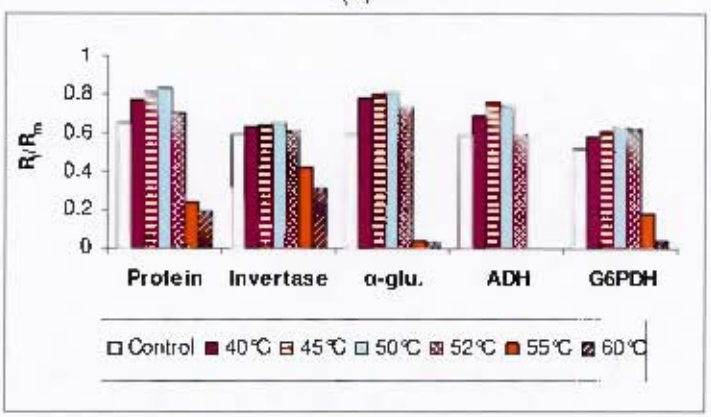
Figure 5.8 **Normalised release of enzyme activity following heating pretreatment at 40°C and sonication at 40 W as a function of sonication time where holding time was kept minimal**

5.2.4 The kinetic rate constant for protein release following heat pretreatment

The kinetic rate constant for protein release on sonication following heat pretreatment was calculated using Equation 4.4. Data are listed in Tables C.9 to C.11 of Appendix C. To compare the effect of pretreatment on the kinetic rate constant across total soluble protein and enzyme release, the ratio of the kinetic rate constant following pretreatment (k) to that in the control (k_c) was employed (k/k_c).



(a)



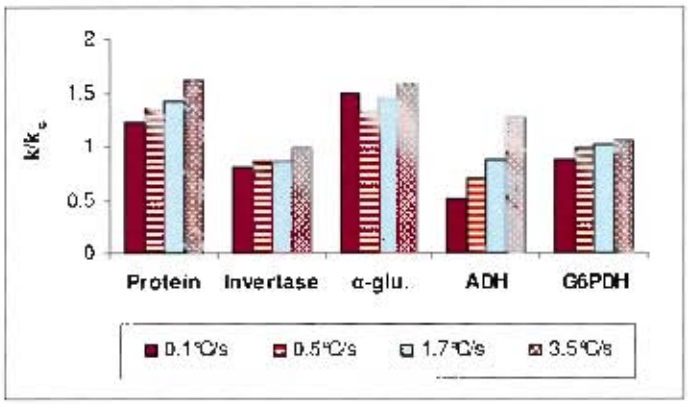
(b)

Figure 5.9 Comparison of (a) rate of release k/k_c and (b) extent of release R_i/R_m of total soluble protein and enzyme release from Baker's yeast as a function of pretreatment temperature following heat pretreatment and sonication. Holding time was minimised. Sonication was conducted at 40 W for 25 min.

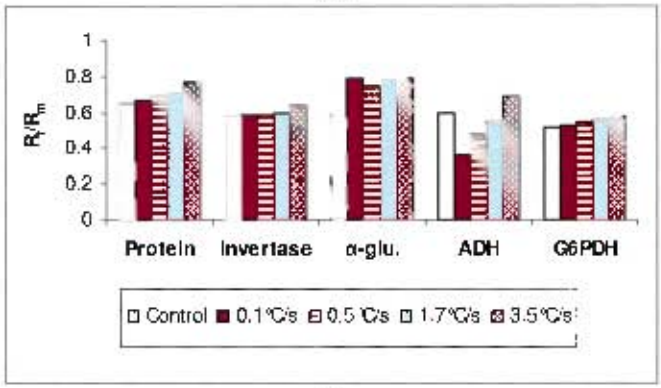
5.2.4.2 The effect of heating rate on heat pretreatment at 40℃

The ratio of the kinetic rate constants (k/k_c) and the extent of release (R_i/R_m) from Baker's yeast following sonication at 40 W as a function of heating rate for pretreatment at 40℃ are presented in Figure 5.10. From Figure 5.10, the k/k_c for total soluble protein and enzyme release increased with increasing heating rate. The maximum k/k_c for total soluble protein, invertase, α-glucosidase, ADH and G6PDH were obtained following 40℃ heat pretreatment with heating rate of 3.5℃/s and were 1.62, 0.98, 1.87, 1.25 and 1.05, respectively. Similarly, the maximum R_i/R_m of total soluble protein and enzyme release were obtained at a heating rate of 3.5℃/s. The maximum R_i/R_m of total soluble protein, invertase, α-glucosidase, ADH and G6PDH release were 0.77, 0.63, 0.79, 0.69 and 0.58, respectively, representing 4 % to 17 % increase in protein or enzyme release compared to the control. Therefore, across these experimental conditions, the optimal heating rate was 3.5℃/s. Further, comparison of protein and enzyme release following pretreatment by heat exchange

at 3.5°C/s and by dilution at 20°C/s, presented in Figure 5.1, illustrated equivalent release. Hence it is concluded that the optimal heating rate for cel weakening to enhance protein release is 3.5°C/s or greater.



(a)



(b)

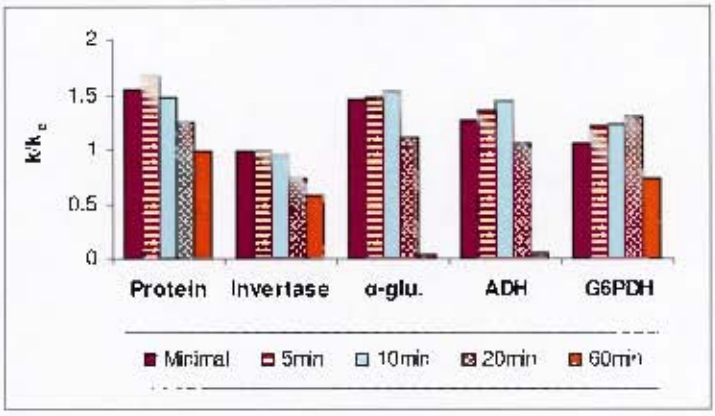
Figure 5.10 Comparison of (a) rate of release k/k_c and (b) extent of release R_i/R_m of total soluble protein and enzyme release from Baker's yeast as a function of heating rate during heat pretreatment. Heat pretreatment was conducted at 40°C with minimal holding time, followed by sonication at 40 W for 25 min.

5.2.4.3 The effect of holding time of 40°C heat pretreatment

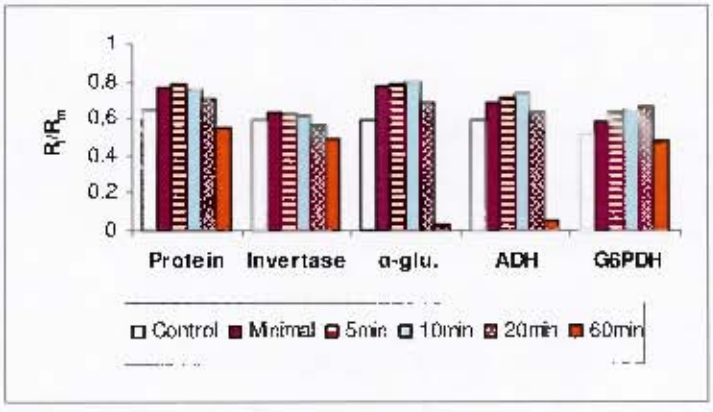
The ratio of the kinetic rate constants (k/k_c) and the extent of release (R_i/R_m) from Baker's yeast following sonication at 40 W as a function of holding time of 40°C heat pretreatment are presented in Figure 5.11. From Figure 5.11a, the maximum k of total soluble protein release was obtained on a holding time of 5 min, increasing to 1.66 fold that of the control. No significant effect of heat pretreatment on the k/k_c of invertase was observed on varying the holding time from 0 to 10 min. An extended holding time of 20 min and greater resulted in denaturation in accordance with Figure 5.5. The k/k_c ratio of α-glucosidase and ADH increased with increasing holding time of 40°C heat pretreatment between 0 and 10 min, and decreased when the holding

time exceeded 10 min. The maximum k/k_c of α-glucosidase and ADH were 1.53 and 1.43, respectively. The maximum k/k_c of G6PDH of 1.29 was obtained when the holding time of heat pretreatment at 40°C was 20 min.

In Figure 5.11b, it is seen that the R_i/R_m of total soluble protein, invertase, α-glucosidase and ADH were constant when the holding time was varied between minimal and 10 min, while the range of holding time from minimal to 20 min was preferred on G6PDH release. The maximum R_i/R_m of total soluble protein, invertase, α-glucosidase, ADH and G6PDH obtained at the holding time used to obtain the maximum kinetic rate constant, were 0.79, 0.63, 0.80, 0.73 and 0.66, respectively, representing a 4 % to 20 % increase in protein or enzyme release compared to the control.



(a)



(b)

Figure 5.11 Comparison of (a) rate of release k/k_c and (b) extent of release R_i/R_m of total soluble protein and enzyme release from Baker's yeast as a function of holding time during heat pretreatment. Heat pretreatment was conducted at 40°C with heat rate of 3.5°C/s. Sonication was conducted for 25 min at 40 W.

5.2.5 Optimal conditions of heat pretreatment

The optimal conditions for cell disruption and selective product release following heat pretreatment and sonication release are concluded in Table 5.2. The maximum temperature of 50°C is preferred by most enzymes, except ADH (45°C) which denaturates at a lower temperature than other enzymes. Harsh conditions, maximum temperature at 52°C and 20 min holding at 40°C heat pretreatment, were preferred by G6PDH which showed less rapid denaturation. Longer sonication time (25 min) is preferred by intracellular enzymes.

Table 5.2 The optimal condition of heat pretreatment for protein and enzymes selective release

Protein/Enzyme	Maximum temperature	40°C heat pretreatment (heating rate: 3.5°C/s)	
		Holding time	Sonication time
Total soluble protein	40 to 50°C	Minimal to 5 min	15 min
Invertase	40 to 50°C	Minimal	15 min
α-glucosidase	40 to 50°C	Minimal to 10 min	25 min
ADH	40 to 45°C	Minimal to 10 min	25 min
G6PDH	40 to 52°C	Minimal to 20 min	25 min

5.3 pH PRETREATMENT

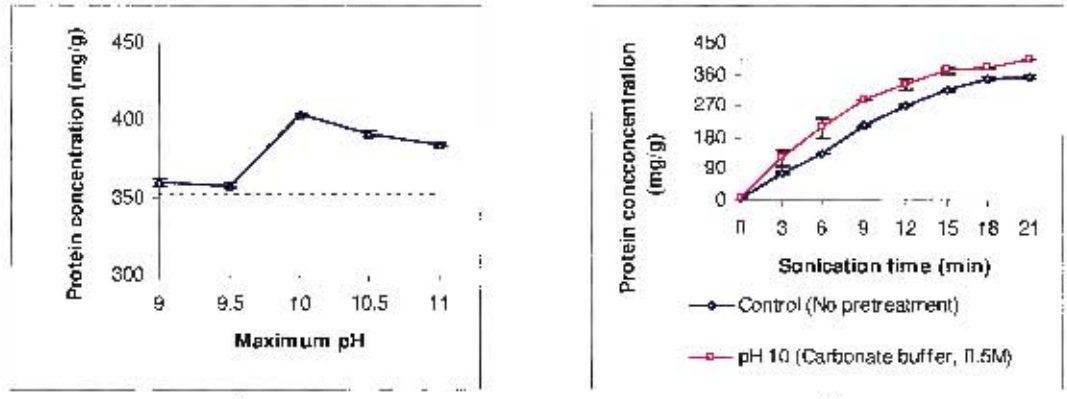
To evaluate the influence of pH pretreatment on mechanical cell disruption and selective product release maximum pH used and the holding time at pH 10 were considered. These were varied over pH 9 to pH 11 and 0 to 5 min, respectively. The conditions of pH pretreatment are listed in Table 3.4. As with heat pretreatment, focus was on cell weakening rather than cell permeabilisation; hence the proteins released were not exposed directly to the extreme pH conditions which may be expected to effect their activity. In this section where the effect of pretreatment was less marked, sonication power was increased from 40 W to 80 W to ensure maximum disruption within an appropriate time. The yeast suspension was sonicated at 80 W for 21 min following pH pretreatment.

5.3.1 Effect of pH pretreatment on protein release

5.3.1.1 Effect of maximum pH of pretreatment

The pH pretreatment was affected by diluting the Baker's yeast suspension (1.5 %, dry weight in 0.05 M phosphate buffer) in a carbonate buffer (0.5 M). The results are shown in Section 5.3.1.2. On dilution, the pH of yeast suspension was shifted from neutral to between pH 9 and pH 11 with a pH interval of 0.5. Following the holding time, the suspension was neutralised on further dilution. Total soluble protein release

on sonication at 80 W for 21 min following pH pretreatment is shown in Figure 5.12a. The protein release as a function of sonication time at 80 W power input following pH 10 pretreatment is presented in Figure 5.12b. Data are presented in Table C.12 of Appendix C. Pretreatment at pH 9 to pH 9.5 did not have a significant effect on total soluble protein release. The maximum protein release of 409 mg/g was obtained on pretreatment at pH 10, representing a 16 % increase over the control. To obtain the equivalent protein release seen in the control, 12 min sonication was required following pH 10 pretreatment. The total soluble protein release decreased when pH pretreatment exceeded pH 10 through 392 mg/g at pH 10.5 to 384 mg/g at pH 11, representing 11 % and 9 % increase in protein release relative to the control. Hence, pH pretreatment decreases the energy requirement of cell disruption through decreasing the sonication time required at the same sonication power, while the maximum pH 10 is preferred.



(a) (b)

Figure 5.12 (a) Maximum soluble protein released following sonication for 21 min at 80 W as a function of the maximum pH of pretreatment. Holding time was minimised. The dashed line represents protein release in the control in the absence of pretreatment. (b) Total soluble protein release on ultrasonication at 80 W as a function of time under different conditions (with or without pretreatment at pH 10).

5.3.1.2 Effect of holding time of pH pretreatment

To investigate the holding time of pH 10 pretreatment on total soluble protein release, 5 durations were used: 0, 0.5, 1, 2 and 5 min. The sodium carbonate buffers of different concentrations (0.05 M and 0.5 M) were used to investigate the effect of buffering capacity on total soluble protein release during the pH pretreatment. The total soluble protein release on sonication at 80 W for 25 min following a pH 10 pretreatment with increasing holding time is presented in Figure 5.13. Data are presented in Table C.13 of Appendix C. On varying the holding time from minimal to 2 min using 0.5 M carbonate buffer, no significant change in total soluble protein

release was obtained. The maximum total soluble protein release of 416 mg/g was recorded following pretreatment at pH 10 with 2 min holding using 0.5 M carbonate buffer. A protein release of 345 mg/g was recorded on pretreatment at pH 10 with 5 min holding using 0.5 M carbonate buffer. This was lower than the total soluble protein release obtained in the control (353 mg/g), due the protein denaturation. The total soluble protein release increased slightly with increasing holding time of pH 10 pretreatment using 0.05 M carbonate buffer from 395 mg/g at no holding through 401 mg/g at 1 min holding to 408 mg/g at 2 min holding. The protein release decreased to 352 mg/g with 5 min holding. The difference in buffering capacity caused by sodium carbonate buffer had slight influence on total soluble protein release. The optimal holding time of the pH 10 pretreatment is 2 min or less.

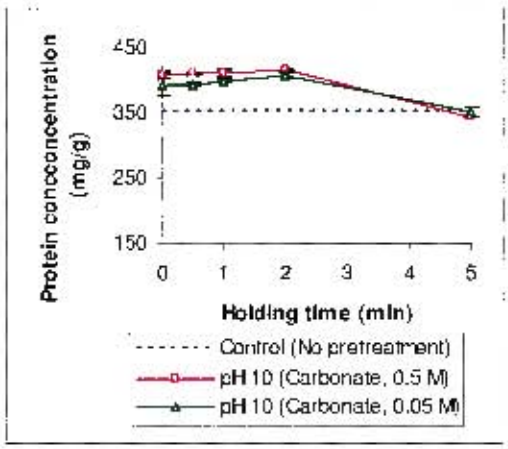


Figure 5.13 Maximum soluble protein released as a function of holding time of pH 10 pretreatment using carbonate buffer following sonication for 21 min at 80 W. The dashed line represents protein release in the control in the absence of pretreatment.

5.3.2 Effect of pH pretreatment on selective product release

The enzyme release was used to investigate the selective product release influenced by pH pretreatment. To enable comparison across enzymes, normalised enzyme release (E_N) was employed, which is calculated using Equation 5.3. Data are presented in Tables C.13 to C.16 of Appendix C.

5.3.2.1 Effect of maximum pH of pretreatment on product release

The normalised release of enzyme activity as a function of maximum pH of pretreatment is shown for each enzyme in Figure 5.14. The maximum E_N of invertase, α-glucosidase and G6PDH, obtained at pH 10, were 118 %, 121 % and 114 %, respectively. Decrease in E_n on further increase in pH may result from protein denaturation. The E_N of ADH increased with increasing pretreatment pH from 9 to

10.5, and decreased as pH exceeded 10.5. The maximum E_N of ADH was 114 %. Hence the optimal pHs of pH pretreatment for invertase, α-glucosidase, ADH and G6PDH release are 10, 10, 10.5 and 10, respectively.

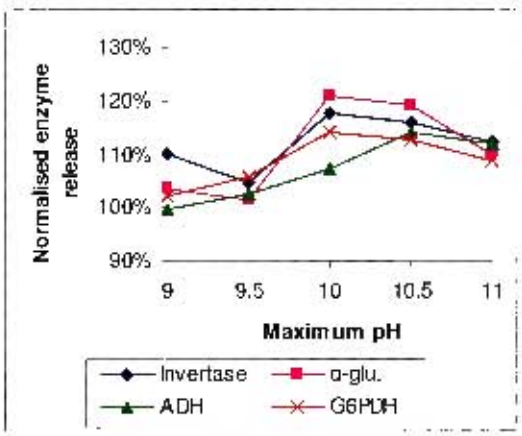


Figure 5.14 Normalised enzyme release as a function of the maximum pH of pretreatment with minimal holding time following sonication for 21 min at 80 W.

The E_N of different enzymes following pretreatment at pH 10 for minimal holding time is presented in Figure 5.15 as a function of sonication time. For invertase, 15 min sonication was required to reach the maximum release. To obtain maximum α-glucosidase release, 18 min sonication was required. A 25 min sonication was preferred by ADH and G6PDH. Hence, longer sonication time is preferred for the intracellular enzyme release. The pH pretreatment is expected to weaken the cell wall by changing protonation and thereby Ionic bonding. The heat pretreatment may affect on both cell wall and cell membrane, while translocation of cytoplasmic enzymes to periplasm may also result by heat pretreatment. Hence the pH pretreatment was less selective than heat pretreatment.

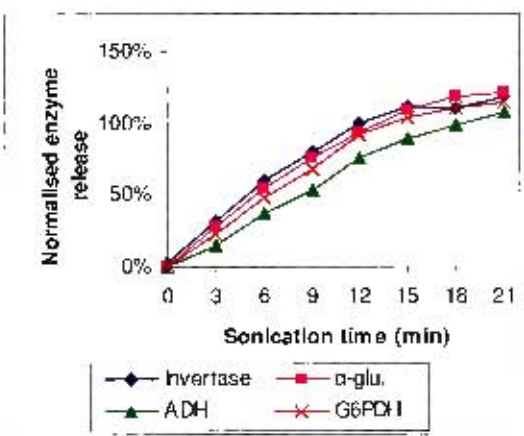


Figure 5.15 Normalised enzyme release following pretreatment at pH 10 as a function of sonication time with 80 W power input. Holding time was minimised.

5.3.2.2 Effect of holding time of pH pretreatment

The E_N for each enzyme as a function of holding time of pH 10 pretreatment using sodium carbonate buffer is shown in Figure 5.16. In Figure 5.16a, the E_N of invertase was constant at 118 % on holding time between 0 and 1 min, and decreased as the holding time exceeded 1 min. The E_N of α-glucosidase increased with holding time from 0 (121 %) to 2 min (127 %), and decreased as holding time exceeded 2 min. To achieve the maximum ADH and G6PDH release, 5 min holding time of pH 10 pretreatment was preferred. The maximum E_N of invertase, α-glucosidase, ADH and G6PDH were 119 %, 127 %, 114 % and 119 %, respectively. Using a lower buffering capacity of 0.05 M illustrated in Figure 5.16b, optimal holding times of pH 10 pretreatment for invertase, α-glucosidase, ADH and G6PDH release were 2, 1, 2 to 5 and 2 to 5 min, respectively. The maximum E_N of invertase, α-glucosidase, ADH and G6PDH under these optimal conditions were 119 %, 123 %, 106 % and 112 %, respectively.

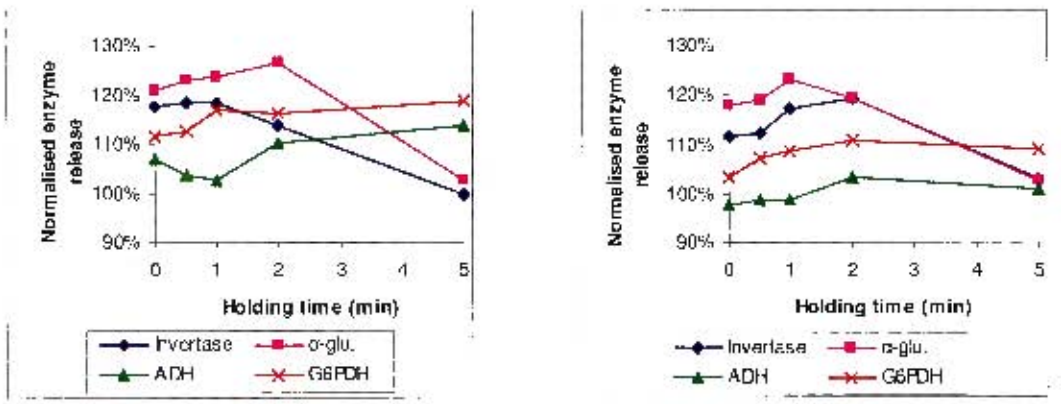


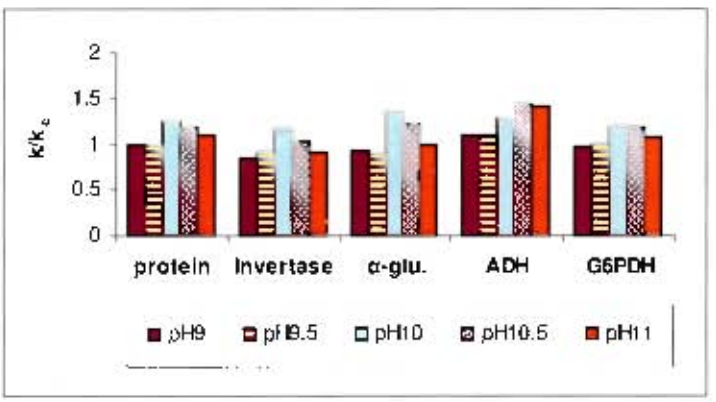
Figure 5.16 Normalised enzyme release following sonication for 21 min at 80 W is given as a function of holding time of pretreatment pH 10 (a) using 0.5 M sodium carbonate buffer (b) using 0.05 M sodium carbonate buffer

5.3.3 The kinetic rate constant of pH pretreatment

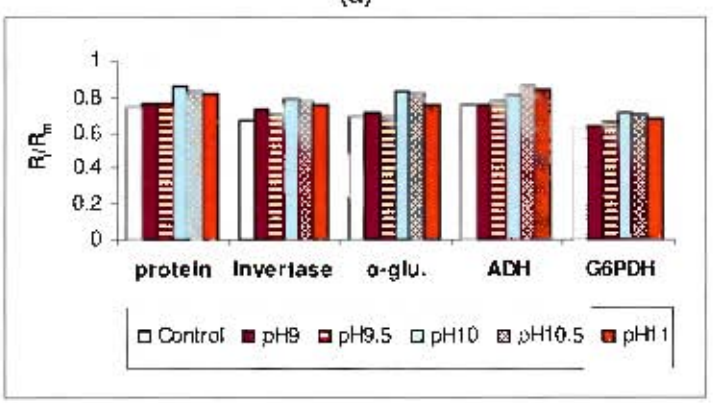
5.3.3.1 Effect of maximum pH of pretreatment on product release kinetics

The kinetic rate constant of pH pretreatment was calculated using Equation 4.4. Data are listed in Tables C.17 to C.18 of Appendix C. The ratio of the kinetic rate constants (k/k_c) was employed to quantify the pH pretreatment on the kinetic rate constant across protein and enzymes. The ratios k/k_c and R_i/R_{in} from Baker's yeast following sonication at 80 W as a function of maximum pH of pretreatment are presented in Figure 5.17. On varying the pH of pretreatment from 9 to 9.5, no significant change of

k was observed. The maximum k/k_c of total soluble protein, invertase, α-glucosidase and G6PDH obtained at the pretreatment pH of 10, were 1.39, 1.16, 1.36 and 1.22, respectively. The maximum k/k_c of ADH of 1.43 was obtained at a pH of pretreatment of 10.5. As the maximum pH of pretreatment was varied between 10 and 11, the R_i/R_m of total soluble protein and enzymes were higher than the control. The maximum R_i/R_m of total soluble protein, invertase, α-glucosidase and G6PDH, obtained at the pretreatment pH used to obtain the maximum kinetic rate constant, were 0.86, 0.78, 0.81, 0.82 and 0.71, respectively. Therefore, to achieve both maximum k/k_c and maximum R_i/R_m of total soluble protein, invertase, α-glucosidase, ADH and G6PDH, the optimal pH of pretreatment are 10, 10, 10, 10.5 and 10, respectively. The maximum pH of 10.5 was preferred by ADH, this may related to the inner location of the enzyme.



(a)



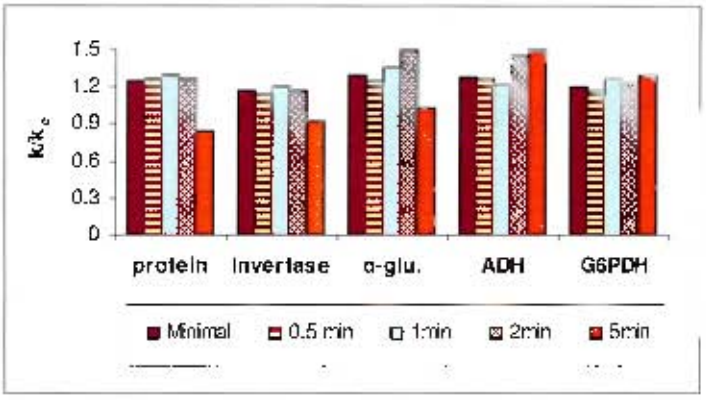
(b)

Figure 5.17 **(a) k/k_c and (b) R_i/R_m of total soluble protein and enzyme release from Baker's yeast as a function of maximum pH of pretreatment using 0.5 M carbonate buffer and minimal holding time, followed by sonication at 80 W for 21 min.**

5.3.3.2 Effect of holding time of pH pretreatment

The ratio of kinetic rate constants (k/k_c) from Baker's yeast following sonication at 80 W as a function of holding time at pH 10 pretreatment using 0.5 and 0.05 M

carbonate buffer is presented in Figure 5.18.



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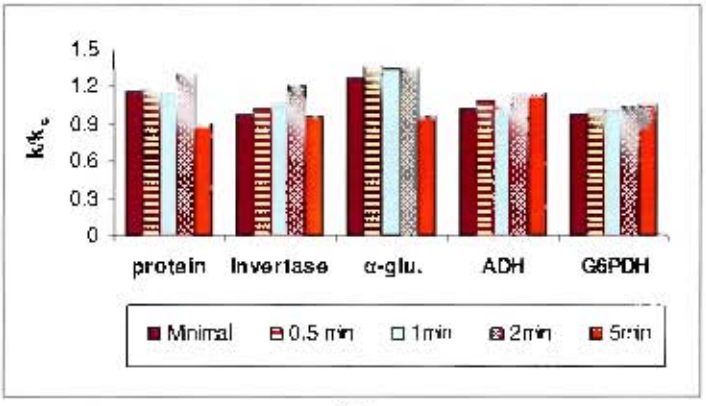


(b)

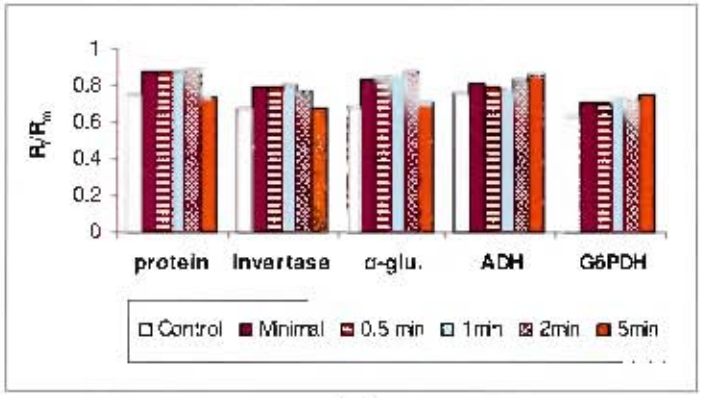
Figure 5.18 k/k_c of total soluble protein and enzyme release from Baker's yeast as a function of holding time at pH 10 pretreatment using (a) 0.5 M carbonate buffer or (b) 0.05 M carbonate buffer and minimal holding time, followed by sonication at 80 W for 21 min

In Figure 5.18a, it is seen that on varying the holding time at pH 10 pretreatment from 0 to 2 min using 0.5 M carbonate buffer, the k/k_c of total soluble and invertase were constant at 1.25 and 1.15, respectively. These decreased on holding time over 2 min. The maximum k/k_c of α-glucosidase, obtained at a holding time of 2 min, represented a 50 % increase over the control. The maximum k/k_c of ADH, obtained on holding times between 2 and 5 min, represented a 45 % increase over the control. Holding time showed little effect on the k/k_c of G6PDH, which increased from1.20 at the minimal holding time to a maximum of 1.29 at a holding time of 5 min. From Figure 5.18b, the maximum k/k_c of total soluble protein and invertase were 1.31 and 1.21 obtained on pH 10 pretreatment at a holding time of 2 min using 0.05 M carbonate buffer. The k/k_c of α-glucosidase increased from 1.26 at 0.5 min holding to 1.36 at 1 min holding. The k/k_c of α-glucosidase was constant at 1.35 when the holding time of pretreatment varied from 0.5 and 2 min, and decreased when the holding time exceed 2 min. The k/k_c of ADH was constant 1.05 as the holding time of pretreatment

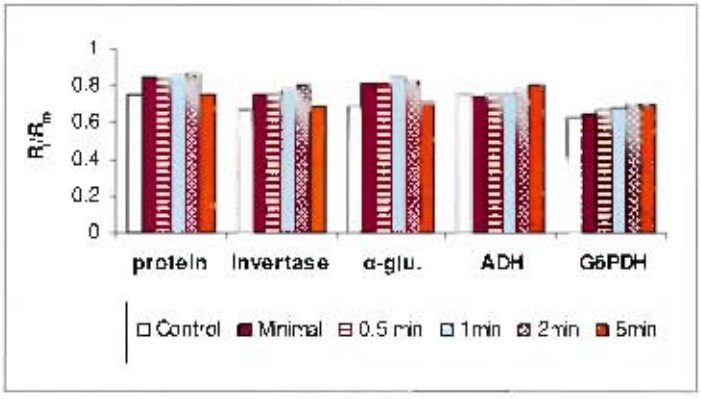
between minimal and 1 min, and step increased to 1.15 when the holding time varied in the range of 2 to 5 min.

The extent of release (R_i/R_m) is presented in Figure 5.19. In Figure 5.19a, it is seen that on varying the holding time at pH 10 pretreatment from minimal to 2 min using 0.5 M carbonate buffer, the R_i/R_m of total soluble protein and invertase, α-glucosidase was constant at 0.9, 0.8 and 0.85, respectively, and decreased when the holding time of pretreatment exceeded 2 min. The R_i/R_m of ADH was constant at 0.8 when at holding time of pretreatment between minimal and 1 min, and increased through 0.83 at 2 min holding, to 0.86 at 5 min holding. The R_i/R_m of G6PDH increased from 0.70 at holding time between minimal and 0.5 min to 0.73-0.75 at holding times between 1 and 5 min. The maximum R_i/R_m of G6PDH obtained on pretreatment at 5 min holding represented 19 % increase over the control of 0.63. From Figure 5.19b, the R_i/R_m of total soluble protein, invertase and α-glucosidase were constant at 0.85, 0.78 and 0.82 when the holding time at pH 10 pretreatment was varied between minimal to 2 min, and decreased as the holding time over 2 min. On varying the holding time between minimal to 1 min, no change on ADH release resulted. The R_i/R_m of ADH increased with increasing holding time from 2 min to 5 min. The maximum R_i/R_m of ADH was 0.80, presenting 5 % increase over the control. The R_i/R_m of G6PDH increased with increasing holding time of pretreatment from 0.63 at minimal to 0.70 between 2 and 5 min holding.

The R_i/R_m of total soluble protein and each enzyme resulting on 0.05 and 0.5 M buffers were quite similar. In all cases, the release using 0.5 M buffer was greater; however the release using the 0.05 M buffer was 93 to 98 % of this. Greater k/k_c ratios were obtained for α-glucosidase, ADH and G6PDH using 0.5 M carbonate buffer. This may be relative to the different osmotic pressure of the buffers, since an osmotic shock may be induced on pretreatment using 0.5 M carbonate buffer, which results cell membrane weakening during the pretreatment. The optimal holding time at pH 10 pretreatment using 0.5 M carbonate buffer for total soluble protein, invertase, α-glucosidase release are between minimal to 2 min, while ADH and G6PDH preferred the holding time of pretreatment between 2 to 5 min.



(a)



(b)

Figure 5.19 **R_i/R_m of total soluble protein and enzyme release from Baker's yeast as a function of holding time of pH 10 pretreatment using (a) 0.5 carbonate buffer or (b) 0.05 M carbonate buffer and minimal holding time, followed by sonication at 80 W for 21 min**

5.3.4 Optimal conditions of pH pretreatment

The optimal conditions of pH pretreatment for protein and enzymes selective release using 0.5 M sodium carbonate buffer are concluded in Table 5.3. The maximum pH of 10 is preferred by most enzymes, except ADH (pH 10.5). Similar as heat pretreatment, extreme conditions such as longer holding time (2 to 5 min) and sonication time (18 to 25 min) are preferred by intracellular enzymes, ADH and G6PDH.

Table 5.3 **The optimal condition of pH pretreatment using 0.5 M carbonate buffer for protein and enzymes selective release**

Protein/Enzyme	Maximum pH	pH 10 pretreatment	
		Holding time	Sonication time
Total soluble protein	10	Minimal to 2 min	15 min
Invertase	10	Minimal to 2 min	15 min
α-glucosidase	10	Minimal to 2 min	18 min
ADH	10.5	2 to 5 min	21 min
G6PDH	10	2 to 5 min	21 min

5.4 OSMOTIC PRETREATMENT

To introduce the osmotic pretreatment, two solutions were used: sodium chloride and glycerol. These allowed differing influence on conductivity. The osmotic pressure of these solutions was varied over the range 0.25 MPa to 5 MPa. The conditions of osmotic pretreatment are listed in Table 3.5. The yeast suspension was subsequently disrupted by ultrasonication at power input of 80 W. As the impact of osmotic pressure on the cell results from its inability to adjust its shape and size sufficiently rapidly to mitigate the osmotic pressure effect, the holding time of osmotic pretreatment was minimised, and not varied.

5.4.1 Effect of osmotic pretreatment on protein release

Total soluble protein release as a function of osmotic pressure of pretreatment using both solutions on sonication at 80 W for 21 min is shown in Figure 5.20a. The total protein release as a function of sonication time at a power input of 80 W following a pretreatment at 1 MPa using sodium chloride is presented in Figure 5.20b. Data are presented in Table C.19 of Appendix C.

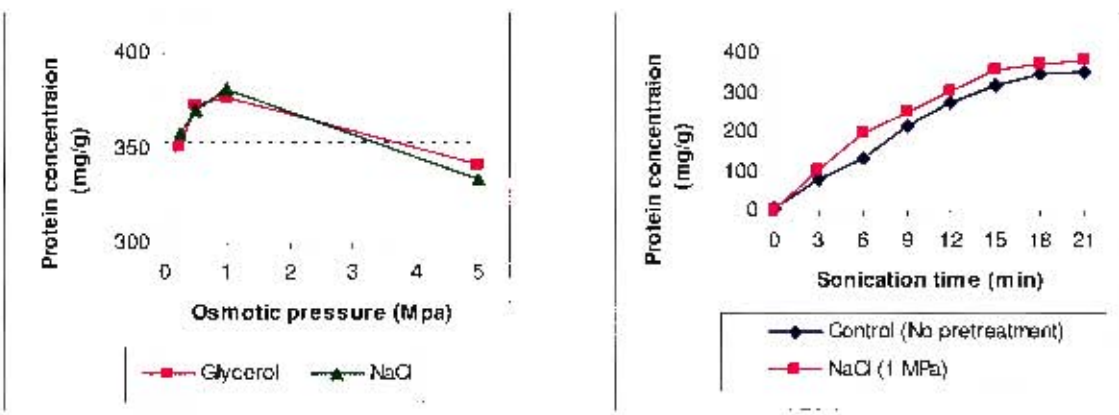


Figure 5.20 (a) Total soluble protein release as a function of osmotic pressure of pretreatment using NaCl or glycerol solution. Sonication for 21 min at 80 W The dashed line represents protein release in the control in the absence of pretreatment. (b) Total soluble protein release as a function of sonication time at 80 W power input following a 1 MPa osmotic pretreatment using sodium chloride.

In Figure 5.20a, it is seen that the profiles of total soluble protein release on osmotic pretreatment using both solutions were similar, indicating osmolarity as the dominant mediator over conductivity. The protein concentration increased with increasing

osmotic pressure of pretreatment from 0.25 MPa to 1MPa, but decreased as the osmotic pressure reached 5 MPa. The maximum total soluble protein release was 380 mg/g, representing 8 % increase over the control. From Figure 5.13.b, to achieve equivalent maximum total soluble protein release obtained in the control, sonication time was reduced from 21 to 15 min on pretreatment at 1 MPa using sodium chloride solution, illustrating that inclusion of osmotic pretreatment decreased the energy requirement of cell disruption proportionally (direct dependence of sonication energy on sonication time).

5.4.2 Effect of osmotic pretreatment on selective product release

To evaluate the effect of osmotic pretreatment on the selective product release, enzyme analysis was employed. The normalised enzyme release, calculated using Equation 5.3, was used for comparison. The E_N of each enzyme as a function of osmotic pressure of pretreatment is presented in Figure 5.21. Data are presented in Tables C.20 of Appendix C. The normalised enzyme release of each enzyme as a function of sonication time at 80 W power input on pretreatment at 1 MPa using sodium chloride is presented in Figure 5.22.

From Figure 5.21, the profiles of invertase and α-glucosidase release on osmotic pretreatment using NaCl and glycerol solutions were similar. The maximum E_N of invertase and α-glucosidase were 113 % and 110 % relative to the enzyme release obtained in the control, at the osmotic pressure of 1 MPa. On varying the osmotic pressure of pretreatment from 0.25 to 5 MPa, osmotic pretreatment using either glycerol or NaCl solution had no effect on ADH release. The maximum E_N of ADH obtained on pretreatment at 5 MPa using both solutions were 101 %. The osmotic pretreatment using NaCl solution had greater effect on G6PDH release than using glycerol solution. The maximum E_N of G6PDH obtained on pretreatment at 1 MPa using glycerol solution was 106 %, compared with 116 % at 1 to 5 MPa using NaCl solution. Hence, osmotic pretreatment using NaCl solution was preferred for G6PDH release. The optimal osmotic pressure of pretreatment for total soluble protein, α-glucosidase, and G6PDH were 1, 1, 1, and 5, respectively. While mechanistic studies on the effect of osmotic pretreatment were not conducted as it is not the pretreatment of choice, the more extreme requirement for G6PDH may be consistent with its intracellular location.

In Figure 5.22, it is seen that longer sonication time was preferred for intracellular enzyme release. The sonication time for maximum invertase, α-glucosidase, ADH and G6PDH release were 15, 18, 21, and 21 min, respectively.

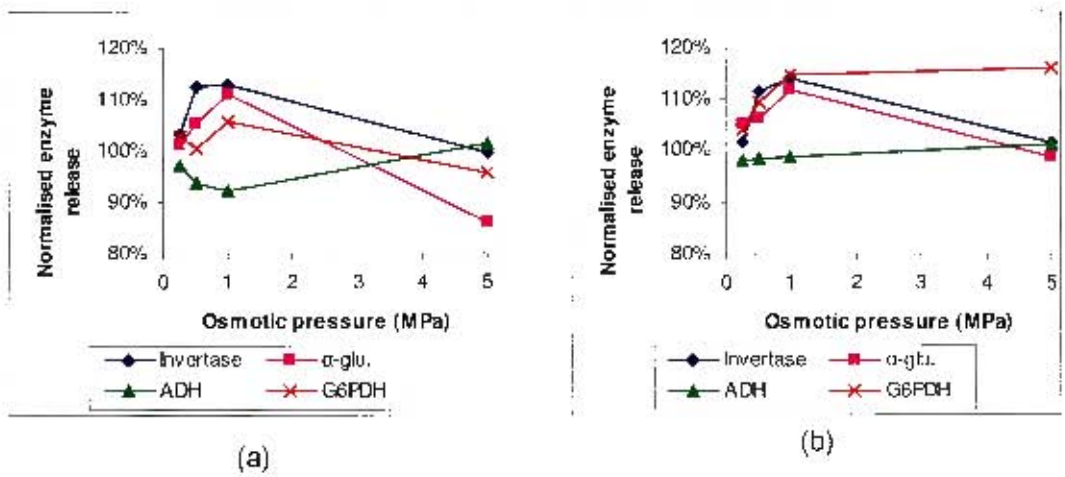


Figure 5.21 Normalised enzyme release following sonication (21 min at 80 W) is given as a function osmotic pressure of pretreatment (a) using glycerol solution (b) using sodium chloride solution

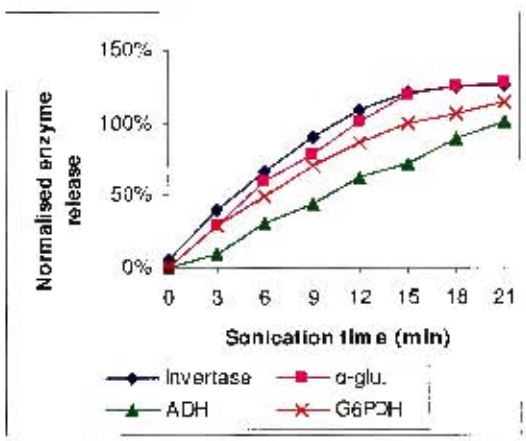
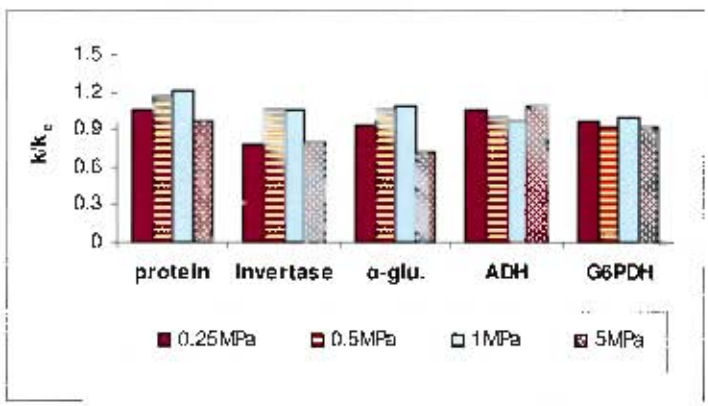


Figure 5.22 Normalised enzyme release following a 1 MPa osmotic pretreatment as a function of sonication time with 80 W power input.

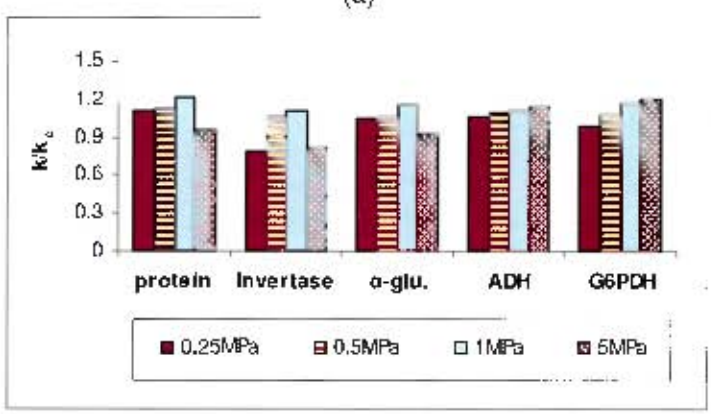
5.4.3 The kinetic rate constant of osmotic pretreatment

The kinetic rate constants of osmotic pretreatment were calculated using Equation 4.3. Data are listed in Tables C.21 of Appendix C. The ratio of the kinetic rate constant (k/k_c) following sonication at 80 W power input as a function osmotic pressure of pretreatment are presented in Figure 5.23.

On pretreatment using glycerol (Figure 5.23a), k/k_c of total soluble protein, invertase and α-glucosidase increased with increasing osmotic pressure between 0.25 and 0.5 MPa, remained constant at osmotic pressures of pretreatment between 0.5 and 1 MPa, and decreased when the osmotic pressure exceeded 1 MPa. The maximum k/k_c of total soluble protein, invertase and α-glucosidase were 120 %, 105 %, 110 %, respectively. The maximum k/k_c of ADH was obtained on the pretreatment at 5 MPa, presenting a 9 % increase over the control. The k/k_c of G6PDH varied between 90 % to 100 % illustrating the osmotic pressure of pretreatment between 0.25 to 5 MPa did not affect the kinetic rate constant of G6PDH. In Figure 5.23b, the k/k_c of total soluble protein, invertase and α-glucosidase increased with increasing osmotic pressure from 0.25 to 1 MPa, and decreased as osmotic pressure over 1 MPa. The k/k_c of ADH and G6PDH increased with increasing osmotic pressure in a range of 0.25 to 5 MPa. The maximum k/k_c of total soluble protein, invertase, α-glucosidase, ADH and G6PDH were 122 %, 110 %, 116 %, 113 % and 120 %, respectively.



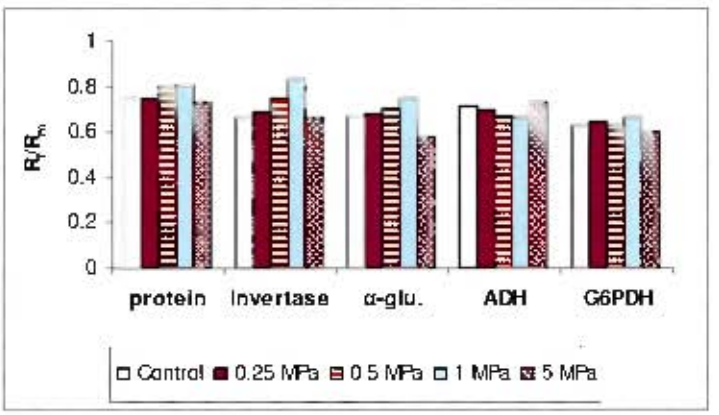
(a)



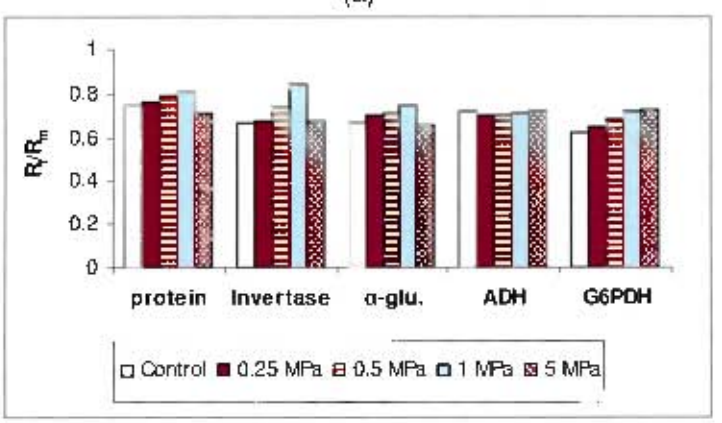
(b)

Figure 5.23 **k/k_c of total soluble protein and enzyme release from following sonication (21 min at 80 W) as a function of osmotic pressure of osmotic pretreatment using (a) glycerol solution or (b) NaCl solution.**

The ratio of the extent of release (R_i/R_m) following sonication at 80 W power input as a function osmotic pressure of pretreatment are presented in Figure 5.24. In Figure 5.24, it is seen that the osmotic pretreatment using both solutions had specific effect on the R_i/R_m of total soluble protein, invertase and α-glucosidase. The maximum R_i/R_m of total soluble protein, invertase and α-glucosidase obtained on pretreatment at osmotic pressure of 1 MPa were 0.80, 0.85 and 0.75, respectively. The osmotic pretreatment using either solution did not affect the R_i/R_m of ADH, while only the osmotic pretreatment using NaCl solution had effect on the R_i/R_m of G6PDH. The maximum R_i/R_m of G6PDH obtained on pretreatment using NaCl was 0.73, presented 16 % increase compared to the control.



(a)



(b)

Figure 5.24 **R_i/R_m of total soluble protein and enzyme release from Baker's yeast following sonication (21 min at 80 W) as a function of osmotic pressure of osmotic pretreatment using (a) glycerol solution or (b) NaCl solution.**

Hence, the optimal osmotic pressure of pretreatment to achieve the maximum k/k_c and the maximum R_i/R_m of total soluble protein invertase and α-glucosidase using either solutions was 1 MPa. The pretreatment at osmotic pressure of 5 MPa using NaCl solution was preferred by G6PDH. Although osmotic pretreatment using either

protein release lay between 40 and 50℃ using the fast heating rates of 3.5 and 4.3℃/s. A 20 % increase over the control resulted. The maximum temperature of 50℃ was preferred by most enzymes, except ADH, whose maximum release was obtained on the pretreatment at 45℃. The optimal temperature for invertase, α-glucosidase, ADH and G6PDH were 40 to 50℃, 40 to 50℃, 40 to 50℃, 45℃ and 40 to 52℃, respectively. In all cases the fast heating rate was preferred and gave equivalent results to heating by dilution. The maximum kinetic rate constants for release found for each optimal condition for protein and enzyme release represented an increase of 5 % to 87 % compared to the control.

The pH pretreatment was induced using carbonate buffers at concentrations of 0.05 and 0.5 M. The maximum total soluble protein, invertase, α-glucosidase and G6PDH release were achieved as the pretreatment at pH 10, while pH 10.5 was preferred for ADH release. The duration of pretreatment at pH 10 was considered, and depended on the location of enzymes. The optimal holding time for total soluble protein, invertase, α-glucosidase lay in a range of minimal to 2 min, while longer holding time of 2 to 5 min was required to achieve the maximum release of the cytoplasmic enzymes ADH and G6PDH. While in all cases greater release resulted with 0.25 M buffer than 0.05 M buffer, the difference in extent of release was small. The rate of release was improved by pH pretreatment.

The osmotic pretreatment was affected using glycerol and NaCl solutions with osmotic pressures of 0.25 to 5 MPa. The profiles of total soluble protein and enzyme release were quite similar using these solutions, except G6PDH. The optimal osmotic pressure of pretreatment to achieve the maximum rate (k/k_c) and extent (R_i/R_m) of total soluble protein, invertase and α-glucosidase release were obtained on pretreatment at 1 MPa. Although osmotic pretreatment using both solutions did not affect the extent of release of ADH, a 13 % increase in its rate over the control was obtained on pretreatment at an osmotic pressure of 5 MPa using both solutions. The pretreatment at an osmotic pressure of 5 MPa using NaCl solution was preferred by G6PDH.

Chapter 6
Effect of Pretreatment on High Pressure Homogenisation

6.1 INTRODUCTION

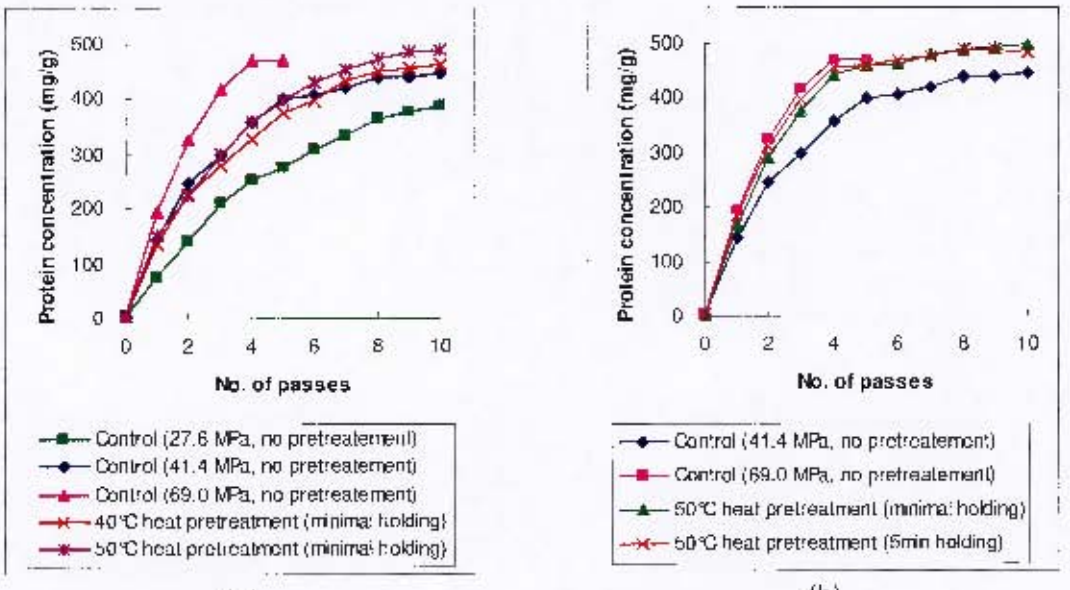
The influence of the single and combined pretreatments on energy efficiency and selective product release from *Saccharomyces cerevisiae* using high pressure homogenisation is demonstrated in this chapter. The optimal conditions of heat, pH and osmotic pretreatment have been determined in Chapter 5 from those listed in Table 3.3, Table 3.4 and Table 3.5, respectively. The condition of each combined pretreatment is shown in Table 3.6. The protein and enzyme analyses were used to measure the disruption and selective product release. The micronisation of cell debris on disruption, determined by size analysis, was also considered.

6.2 EFFECT OF PRETREATMENT ON PROTEIN RELEASE

6.2.1 Effect of heat pretreatment on protein release

The effect of heat pretreatment at maximum temperatures of 40 and 50°C and holding times of minimal duration and 5 min on cell disruption of Baker's yeast using high pressure homogenisation was investigated. The results are shown in Figure 6.1. Data are listed in Table D.1 of Appendix D. In Figure 6.1a, protein release on HPH at 27.6 MPa following heat pretreatment is compared to homogenisation of untreated *S. cerevisiae* at 27.6, 41.4 and 69.0 MPa as a function of the number of passes. To achieve the equivalent protein release to the control of HPH following 10 passes at 27.6 MPa (388 mg/g), 6 and 5 passes were required for the disruption at 27.6 MPa following heat pretreatment at 40 and 50°C. The maximum soluble protein release obtained on HPH at 27.6 MPa for 8 passes following 40°C heat pretreatment represented a 20 % increase over the maximum protein release obtained for the

control at the same pressure, while a 26 % increase was found on protein release following 50°C heat pretreatment at same operating pressure for 8 passes. These amounts of protein release approached to the maximum protein release obtained of HPH at 69.0 MPa without pretreatment. The profiles of total protein release on HPH at 27.6 MPa following 40 or 50°C heat pretreatment were similar to those of the control of HPH at 41.4 MPa. In Figure 6.1b, on varying the holding time of 50°C heat pretreatment from 0 to 5 min, no significant influence on the subsequent protein release was obtained. Approximate 10 % increase in the maximum protein release was obtained on HPH at 41.4 MPa following 50°C heat pretreatment compared to the maximum protein release on the control of HPH at 41.4 MPa. The profiles of total protein release on HPH at 41.4 MPa following 50°C heat pretreatment with minimal and 5 min holding were similar to the control of HPH at 69.0 MPa. The number of passes required to achieve the maximum protein release obtained on HPH of control at 41.4 MPa was decreased from 8 to 10 to 4 when HPH operated at 41.4 MPa following 50°C heat pretreatment with minimal or 5 min holding. Hence, the energy requirement of high pressure homogenisation decreases following heat pretreatment at the maximum temperature between 40 and 50°C. As shown in Chapter 5, increasing the holding time of heat pretreatment from 0 to 5 min does not increase total soluble protein release. The optimal condition of heat pretreatment is the maximum temperature at 50°C without holding.



(a) (b)

Figure 6.1 **(a) Total soluble protein release from Baker's yeast on high pressure homogeniser at 27.6 MPa following heat pretreatment at 40 and 50°C, with minimal holding time. (b) Total soluble protein release from Baker's yeast on high pressure homogeniser at 41.4 MPa following 50°C heat pretreatment at 4.3°C/s heating rate and holding times of minimal duration and 5 min.**

The energy input per pass at 27.6, 41.4 and 69.0 MPa can be estimated as 27.6, 41.4 and 69.0 kJ/kg (Anand *et al.*, 2007). The energy input required for heat pretreatment at 40 and 50°C are 75.6 and 118 kJ/kg, respectively. The effect of each pretreatment on energy saving was considered. The energy calculation for heating has been related through Equation 5.1. The comparison of the energy input required for each pretreatment and the controls are illustrated in Table 6.1. It is seen that, more energy was required on HPH at 27.6 MPa following 50°C heat pretreatment than the control. The energy saving was obtained on the HPH at 41.4 MPa following a single or a combined pretreatment. Osmotic and pH pretreatments were less efficient than heat pretreatment, owing to the slight increase in energy requirement and the extra cost of the buffer. The combined pretreatments had no advantage on total protein release and energy saving to single heat pretreatment at same maximum temperature. The maximum total soluble protein release obtained on HPH at 41.4 MPa following 50°C heat pretreatment approached to the R_m obtained on HPH at 69.0 MPa for 8 passes, while the energy requirement for them were similar. Hence, each pretreatment decreases the energy requirement of *K. lactis* disruption. The heat pretreatment is more efficient than pH or osmotic pretreatment.

Table 6.1 Effect of heat pretreatment on energy efficiency of total soluble protein release from Baker's yeast on HPH

Effect of heat pretreatment on energy efficiency of HPH at 27.6 MPa					
Pressure	**Pretreatment conditions**	**Passes**	**Protein conc. (mg/g)**	**Energy (kJ/kg)**	**Energy/protein (kJ/g)**
27.6 MPa	No pretreatment	10	226	276	1.22
27.6 MPa	Heat pretreatment at 50°C, minimal holding	7	213	311	1.46
Effect of heat pretreatment on energy efficiency of HPH at 41.4 MPa					
Pressure	**Pretreatment conditions**	**Passes**	**Protein conc. (mg/g)**	**Energy (kJ/kg)**	**Energy/protein (kJ/g)**
41.4 MPa	No pretreatment	9	365	373	1.02
41.4 MPa	Heat pretreatment at 40°C, minimal holding	6	361	324	0.90
41.4 MPa	pH 10 pretreatment	8	379	331	0.87
41.4 MPa	Osmotic pretreatment at 1 MPa	8	366	331	0.90
41.4 MPa	Combined heat-pH pretreatment	6	373	324	0.87
41.4 MPa	Combined heat-osmotic pretreatment	6	349	324	0.93
41.4 MPa	Heat pretreatment at 50°C, minimal holding	10	433	532	1.23
69.0 MPa	No pretreatment	8	447	552	1.23

6.2.2 Effect of pH pretreatment on protein release

The condition of pH pretreatment was determined in Chapter 5. To introduce the pH pretreatment, 0.5 M sodium carbonate buffer was used. The pH of Baker's yeast suspension was shifted to pH 10, and held at this pH for 2 min, subsequently the pH was brought back to pH 7 by sodium phosphate buffer. The total protein release as a function of number of passes on HPH at 27.6 MPa following a pH 10 pretreatment with 2 min holding is shown in Figure 6.2. Data is presented in Table D.1 of Appendix D. To achieve the maximum protein release on HPH of the control at 27.6 MPa, 7 passes were required using pH 10 pretreatment with 2 min holding. The maximum protein release obtained at pH 10 pretreatment was 443 mg/g. This approached to the maximum protein release from the control on HPH at 41.4 MPa for 9 passes (449 mg/g), and presented a 14 % increase compared to the control, homogenised at the same operating pressure of 27.6 MPa. Hence, energy saving on high pressure homogenisation can be achieved following pH 10 pretreatment with 2 min holding.

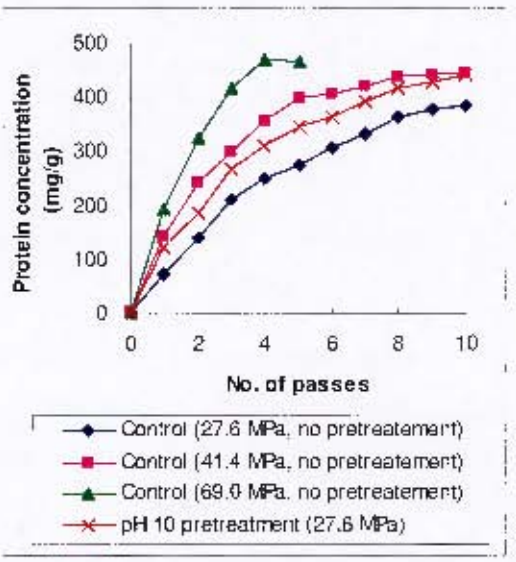


Figure 6.2 Total soluble protein release from Baker's yeast as a function of number of passes on HPH at 27.6 MPa following a pH 10 pretreatment with 2 min holding.

6.2.3 Effect of osmotic pretreatment on protein release

Based on the osmotic pretreatment study presented in Chapter 5, a sodium chloride solution with an osmotic pressure of 1 MPa was used. The holding time of osmotic stress was minimised. The total soluble protein release on HPH at 27.6 MPa as a function of number of passes following osmotic pretreatment is presented in Figure 6.3. Data is listed in Table D.1 of Appendix D. The maximum protein release achieved

on HPH at 27.6 MPa following osmotic pretreatment using 1 MPa sodium chloride solution was 403 mg/g. It represented a 4 % increase in total protein release compared to the control of HPH at 27.6 MPa, illustrating that osmotic pretreatment has the lowest effect on protein release of pretreatments used.

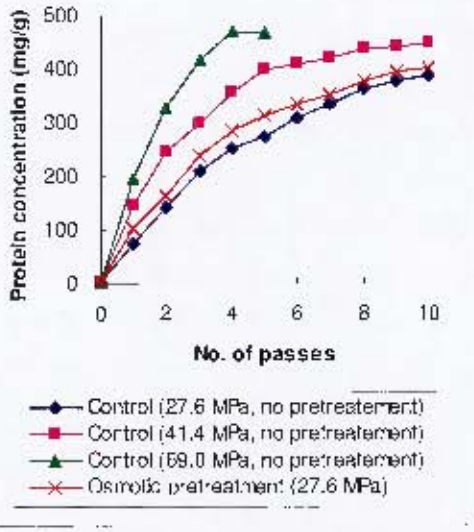


Figure 6.3 Total soluble protein release from Baker's yeast as a function of number of passes on HPH at 27.6 MPa following an osmotic pretreatment using 1 MPa NaCl solution.

6.2.4 Comparison of heat, pH and osmotic pretreatment on subsequent protein release by HPH

To compare the different single pretreatments on the protein release using HPH, the HPH was operated at 27.6 MPa, and the optimal condition of each single pretreatment used is listed in Table 6.2. The comparison of effect of single pretreatment on total soluble protein release from Baker's yeast as a function of number of passes is presented in Figure 6.4. As the high pressure homogeniser was operated at same pressure, the change in the total protein release depended only on the single pretreatment used. Figure 6.4 presents the increase in total protein release following heat, pH and osmotic pretreatment are 26 %, 14 % and 4 %, respectively. Heat pretreatment is recognised in cell wall weakening and translocation of enzymes from cytoplasm to periplasm. Pretreatment at extreme pH and osmotic pressure have been reported to influence the cell wall or cell membrane, respectively. It is clearly seen that short term pretreatment at elevated temperature is the most efficient of these investigated.

Table 6.2 The condition of each single pretreatment on Baker's yeast using high pressure homogenisation

Pretreatment	Conditions	Pressure
Heat	Heat exchanger, maximum temperature: 50 °C, minimal holding	27.6 MPa
pH	Na_2CO_3 (0.5M), maximum pH: 10 with 2 min holding	
Osmotic	NaCl (1 MPa), minimal holding	

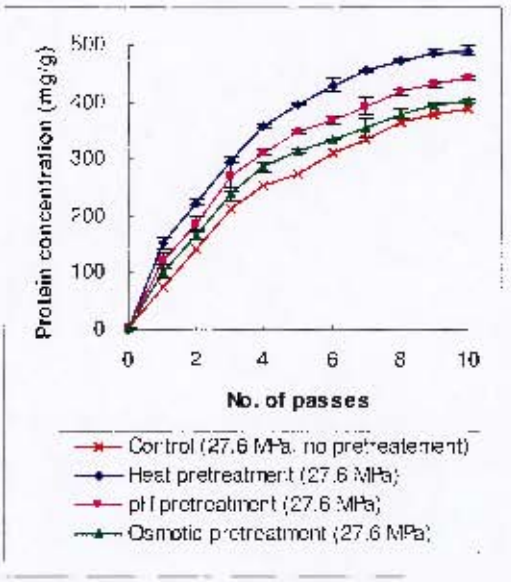
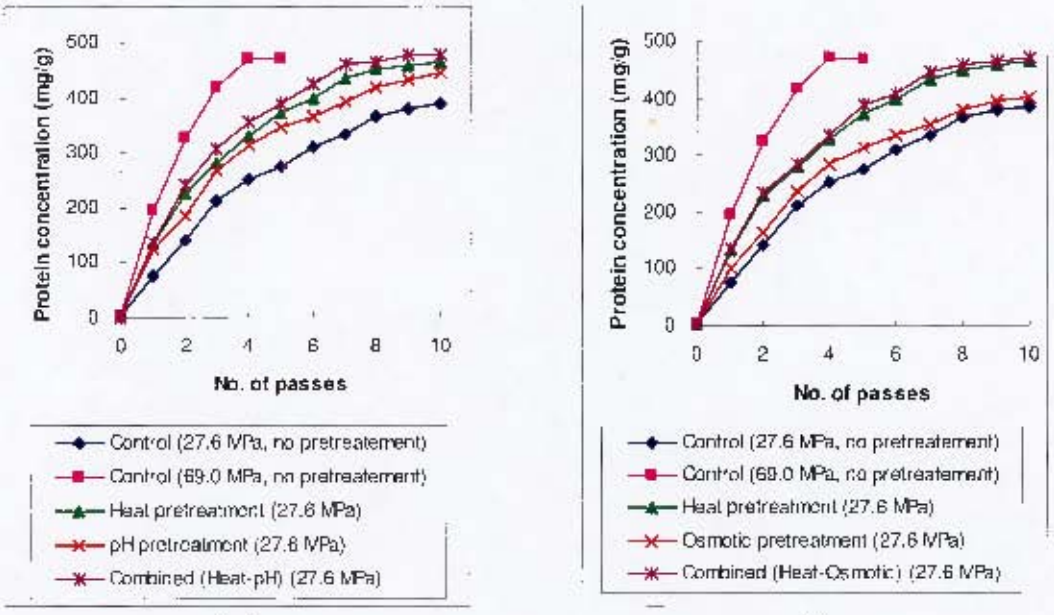


Figure 6.4 Total soluble protein release from Baker's yeast as a function of number of passes on HPH at 27.6 MPa following a heat, pH or osmotic pretreatment.

6.2.5 Effect of combined pretreatment on protein release

The condition of each combined pretreatment was listed in Table 3.6 of Chapter 3. To introduce the combined pretreatment, the Baker's yeast suspension was diluted into a pre-warmed pH or osmotic buffer. Sodium carbonate buffer with concentration of 0.5 M and 1 MPa sodium chloride solution were used. On dilution, the temperature was shifted to 40 °C while the pH or osmotic pressure was shifted to pH 10 or 1 MPa. The total soluble protein release as a function of number of passes on HPH at 27.6 MPa following a combined pretreatment is shown in Figure 6.5. Data is presented in Table D.1 of Appendix D.



(a) (b)

Figure 6.5 Total soluble protein release from Baker's yeast by high pressure homogeniser at 27.6 MPa as a function of number of passes without or following pH or osmotic or combined pretreatment ((a) Heat-pH, (b) Heat-osmotic)).

In Figure 6.5, it is seen that the maximum protein release achieved on HPH at 27.6 MPa following heat-pH pretreatment was 478 mg/g, a 26 % increase in total soluble protein release compared to the control at the same pressure. A 21 % increasing in total soluble protein release was obtained on HPH at 27.6 MPa following heat-osmotic pretreatment. When compared with single effect heat and pH pretreatments, heat-pH pretreatment presented 4 % and 8 % increase in total soluble protein release, respectively. A 2 % and a 16 % increasing in total soluble protein release were obtained using heat-osmotic pretreatment compared with single effect heat and osmotic pretreatments, respectively. Hence, combined pretreatment decreased the energy requirement of mechanical cell disruption, and resulted in higher protein release than all single pretreatments. However, the increase over the heat pretreatment is small. There are some associated disadvantages on using combined pretreatment compared to single heat pretreatment. These include the cost of chemicals, handling of the process fluid and the increased cost of product recovery.

6.3 Effect of pretreatment on selective product release

The selective product release is analysed in terms of enzyme activity released normalised against that release in the control (10 passes at 27.6 MPa) calculated to

enable comparison across enzymes. The E_N is calculated using Equation 5.3. Data for enzyme release by high pressure homogeniser following a single or combined pretreatment are listed in Tables D.2 of Appendix D.

6.3.1 Effect of heat pretreatment on selective product release

The normalised enzyme release from Baker's yeast on HPH following 50°C heat pretreatment is presented at different operating pressure and as function of number of passes in Figure 6.6. To achieve maximum enzyme release of invertase, α-glucosidase, ADH, G6PDH on HPH at 27.6 MPa, the number of passes were 6, 7, 10 and 10, while 4, 6, 9 and 8 passes was required on HPH at 41.4 MPa. Hence, the number of passes to achieve maximum enzyme release depends on the operating pressure

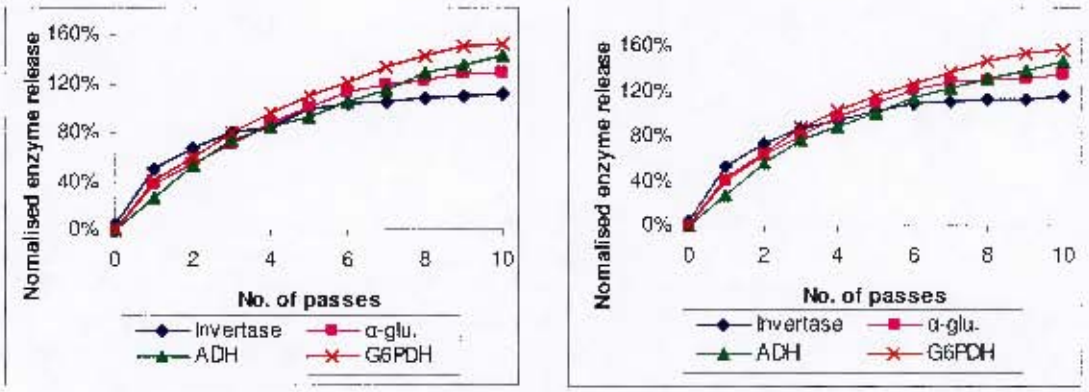


Figure 6.6 **E_N of different enzyme from Baker's yeast following 50°C heat pretreatment on HPH at (a) 27.6 MPa and (b) 41.4 MPa as a function of number of passes. Holding time was minimised.**

The E_N of each enzyme from Baker's yeast on HPH at different operating pressure following heat pretreatment at varying maximum temperature and holding time is presented in Figure 6.7. Data for each enzyme release is listed in Table D.2 of Appendix D. In Figure 6.7a, the E_N of different enzyme increased between 3 and 5 %, on varying the maximum temperature of heat pretreatment between 40 and 50°C. The E_N of ADH and G6PDH were higher than α-glucosidase and invertase. In Figure 6.7b, varying the holding time of 50°C heat pretreatment between minimal and 5 min did not have influence on the E_N of different enzymes. In Figure 6.7c, varying the operating pressure between 27.6 MPa and 41.4 MPa following 50°C heat pretreatment without holding, no significant increase in E_N was obtained. While the

effect of varying temperature and holding time of pretreatment on enzyme release was small, a significant increase in E_N on pretreatment was obtained relative to the control. For invertase, α-glucosidase, ADH and G6PDH lay in the range 110 % to 120 %, 130 to 135 %, 140 to 145 % and 150 % to 160 %, respectively. Heat pretreatment has more influence on cytoplasmic enzyme release than periplasmic and cell wall associated enzyme release. Cell wall weakening by heat pretreatment resulted in more cytoplasmic enzyme release at lower operating pressure. This may related to the transportation of cytoplasmic enzyme from cytoplasm to periplasm during the heat pretreatment.

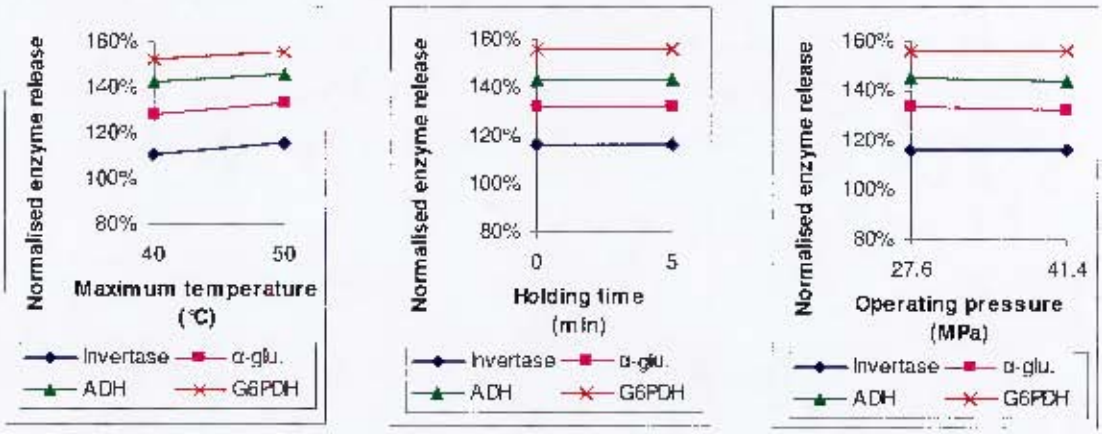


Figure 6.7 **Enzyme release, expressed as E_N, from Baker's yeast following HPH for 10 passes: (a) as a function of the maximum temperature of heat pretreatment with 5 min holding and HPH at 27.6 MPa. (b) as a function of the holding time of 50°C heat pretreatment with HPH at 41.4 MPa. (c) as a function of operating pressure following 50°C heat pretreatment with 5 min holding.**

6.3.2 Effect of pH and osmotic pretreatment on selective product release

The E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa following a pH or an osmotic pretreatment as a function of number of passes is shown in Figure 6.8. The number of passes required to obtained the maximum E_N of invertase, α-glucosidase, ADH and G6PDH following pH 10 pretreatment with 2 min holding were, 6, 8, 10 and 10, while 8, 9, 10 and 10 passes were required for the E_N following osmotic pretreatment using 1 MPa NaCl solution. The enzyme activities obtained following pretreatment at pH 10 were increased by 10 % to 50 %, while 5 % to 20 % increase in enzyme activities were obtained following osmotic pretreatment using 1 MPa NaCl solution. When the high pressure homogeniser operating at the same pressure, the number of passes to obtain specific enzyme maximum release depends on the location of the enzyme and the pretreatment been used.

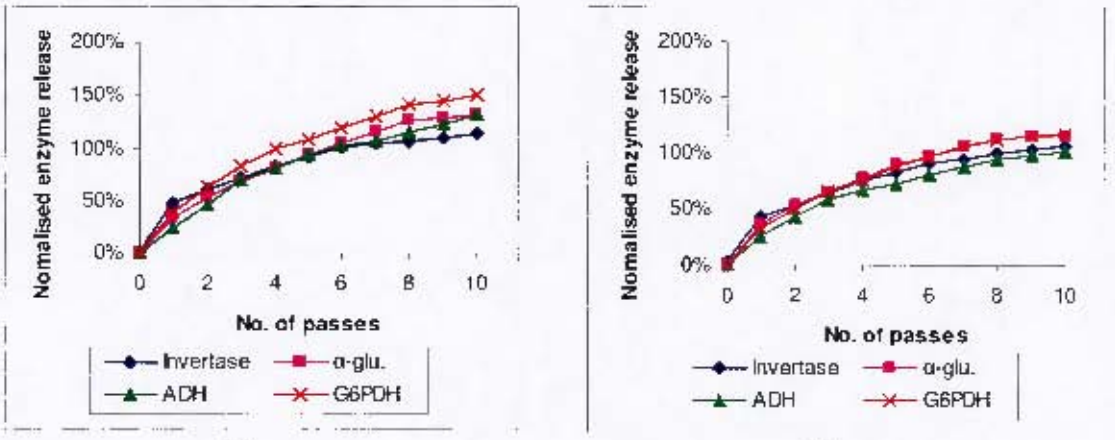


Figure 6.8 **(a) E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa following pH 10 pretreatment with 2 min holding as a function of number of passes. (b) E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa following osmotic pretreatment using 1 MPa NaCl solution as a function of number of passes.**

6.3.3 Comparison of single and combined pretreatment on selective product release

The maximum E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa following different single or combined pretreatments is presented in Figure 6.9. The E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa following a combined pretreatment is presented as a function of number of passes in Figure 6.10. The condition of each combined pretreatment is listed in Table 6.3. The highest E_N of each enzyme was obtained following heat pretreatment at 50°C, combined pretreatment, heat-pH and heat-osmotic, which were similar to heat pretreatment with the maximum temperature at 50°C. Following each combined pretreatment, the maximum E_N of G6PDH and AHD were about 155 % and 140 %, while 115 % and 135 % were obtained for invertase and α-glucosidase activity. Hence, combined pretreatment improves intracellular enzyme release and has more influence on the cytoplasmic enzyme release. Each enzyme released following 50°C heat pretreatment was higher than other single pretreatments. Hence, the best single pretreatment for intracellular enzyme release is heat pretreatment at 50°C. In Figure 6.10, the profile of E_N following combined heat-pH pretreatment was similar to the E_N following combined heat-osmotic pretreatment. The number of passes to achieve maximum release of invertase, α-glucosidase, ADH and G6PDH following each combined pretreatment were 5, 7 10 and 10, respectively.

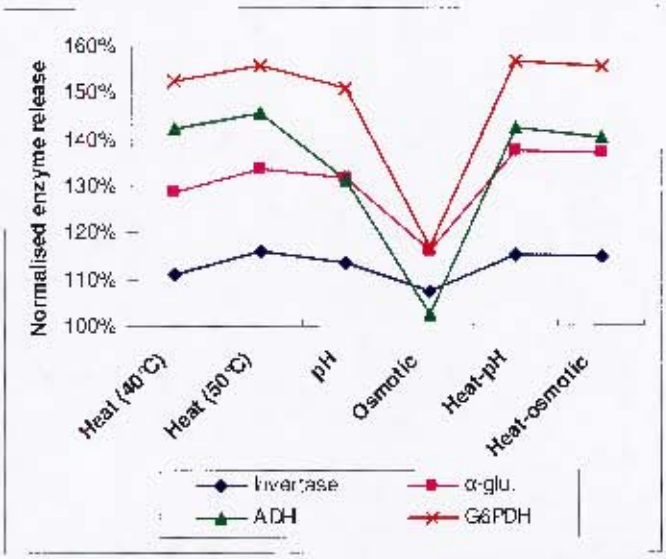


Figure 6.9 E_N of each enzyme from Baker's yeast on HPH at 27.6 MPa for 10 passes following single or combined pretreatment

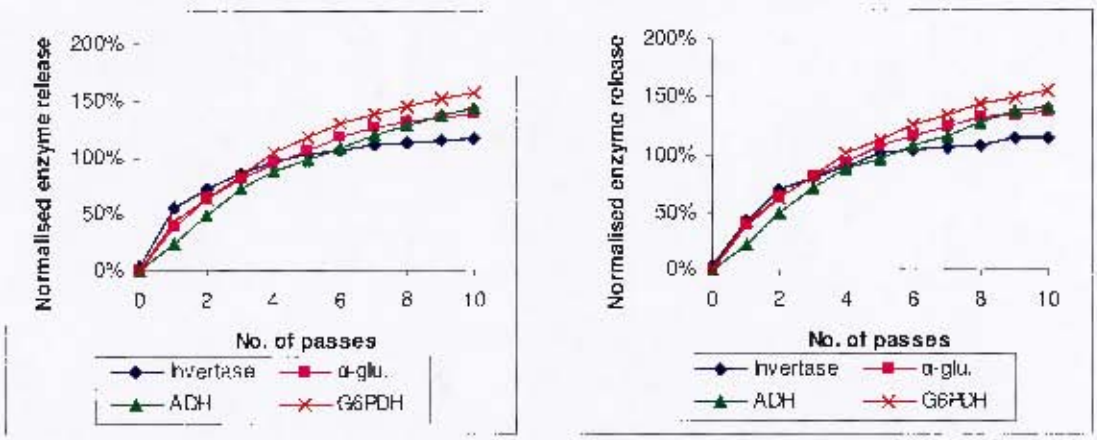


Figure 6.10 E_N of each enzyme from Baker's yeast on HPH at 27.6 following a combined pretreatment ((a) heat-pH (b) heat-osmotic)) as a function of number of passes

Table 6.3 The condition of each combined pretreatment on Baker's yeast using high pressure homogenisation

Combined pretreatment	Conditions
Heat-pH	Warmed Na_2CO_3 (0.5M) buffer; pH_{max}:10, T_{max}: 40°C
Heat-osmotic	Warmed NaCl (1 MPa) solution; T_{max}: 40°C

6.3.4 The optimal condition of each single and combined pretreatment for protein release and selective enzyme release

The optimal condition of each single and combined pretreatment on protein and enzyme release is summarised in Table 6.4. Following a single or combined pretreatment, more passes were required for maximum release of cytoplasmic

enzymes release over other enzymes.

Table 6.4 The optimal condition of each single and combined pretreatment on Baker's yeast using high pressure homogenisation at 27.6 MPa

Pretreatment	Pretreatment conditions	Passes				
		P	I	α	A	G
Control	No pretreatment	10	8	9	10	10
Heat	Heat exchanger, T_{max}: 50°C, no holding	8	6	7	10	10
pH	Na_2CO_3 (0.5M), pH_{max}: 10, 2 min holding	9	6	8	10	10
Osmotic	NaCl (1 MPa), minimal holding	9	8	9	10	10
Combined Heat-pH	Warmed Na_2CO_3 (0.5M) buffer; pH_{max}:10, T_{max}: 40°C	7	5	7	10	10
Combined Heat-osmotic	Warmed NaCl (1 MPa) solution; T_{max}: 40°C	7	5	7	10	10

P: protein; I: invertase; α: α-glucosidase; A: ADH; G: G6PDH.

6.4 RELEASE RATE KINETICS

The kinetic rate constant (k) of total soluble protein and each enzyme following single or combined pretreatment were calculated using Equation 4.1. Data are listed in Tables D.3 of Appendix D.

6.4.1 Comparison of single pretreatment on release rate kinetics

The release rate constant k for total soluble protein and the marker enzymes obtained on HPH at different operating pressures following heat pretreatment at 40 or 50°C is shown in Figure 6.11. The rate constants following heat pretreatment at 40 and 50°C were higher than the k obtained for the control at 27.6 MPa. The k of protein and marker enzymes following 40°C heat pretreatment with minimal holding on HPH at 27.6 MPa approached the k obtained for the control disrupted at 41.4 MPa while the k values following 50°C heat pretreatment with minimal holding on HPH at 27.6 MPa exceeded the control. On comparing soluble protein release following heat pretreatment with the control, the k increased by 1.9 and 1.8 on HPH at 27.6 and 41.4 MPa, respectively. Varying the holding time of 50°C pretreatment between minimal and 5 min on HPH at 41.4 MPa did not have significant influence on k of the indicator enzymes.

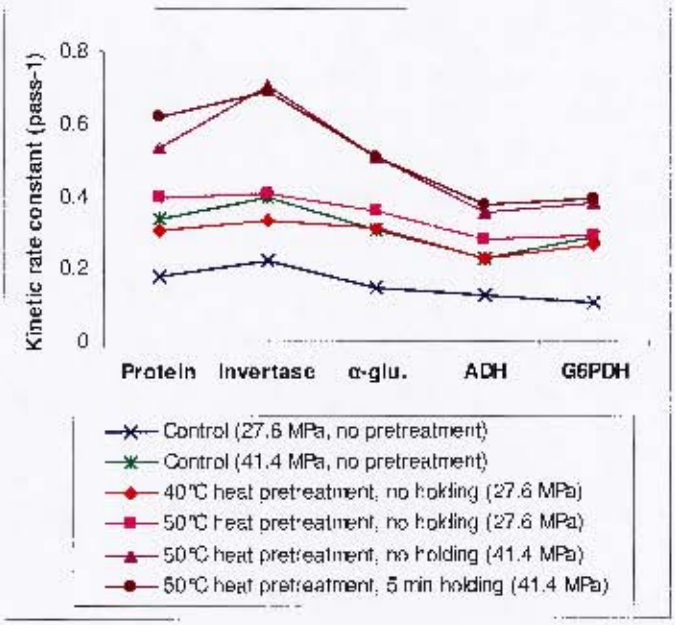


Figure 6.11 Release rate constant, k, of total soluble protein and each enzyme from Baker's yeast on HPH at 27.6 or 41.4 MPa following 40 or 50°C heat pretreatment with minimal or 5 min holding

The k of total soluble protein and marker enzymes on HPH at 27.6 MPa are compared across pretreatments in Figure 6.12. The k of total soluble protein and each enzyme following heat pretreatment at 40 and 50°C were higher than the k obtained following other pretreatments. No significant increase in the k of total soluble protein, invertase and ADH following osmotic pretreatment was obtained compared to the k on control on HPH at 27.6 MPa, while the k of α-glucosidase and G6PDH were increased about 30 % and 50 %. The k for total soluble protein and marker enzymes following pH pretreatment on HPH at 27.6 MPa increased by 35 % to 65 % over the control at 27.6 MPa, but remained lower than the k values for the control at 41.4 MPa. Hence, pH pretreatment showed an enhancement of product release rate, however it did not perform as well as heat pretreatment.

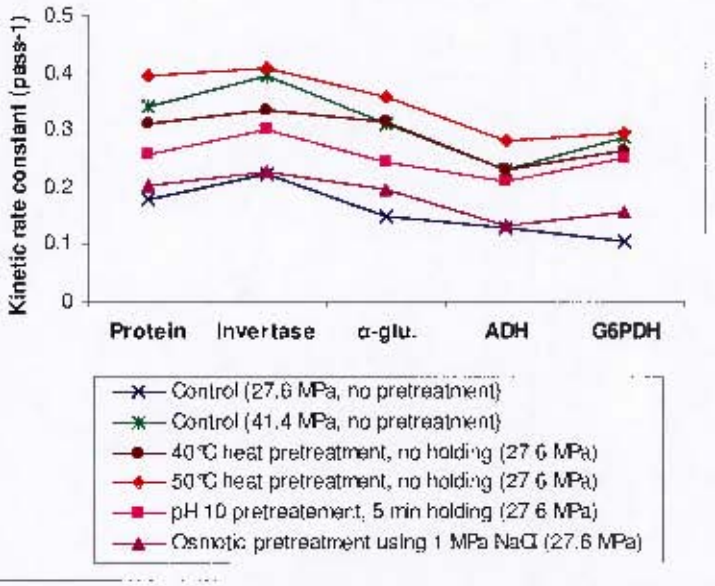


Figure 6.12 Comparison of release rate constant, k, of total soluble protein and indicator enzyme from Baker's yeast across single pretreatments following HPH at 27.6 MPa

6.4.2 Comparison of combined pretreatment on release rate kinetics

Comparison of the rate constants of single heat pretreatment or combined pretreatment HPH at 27.6 MPa is shown in Figure 6.13. The k of total soluble protein and marker enzymes following each combined pretreatment were higher than the single 40°C heat pretreatment. Following combined heat-pH pretreatment on HPH at 27.6 MPa, the k of total soluble protein (0.37 pass^{-1}), invertase (0.39 pass^{-1}) and G6PDH (0.31 pass^{-1}) were higher than the k following combined heat-osmotic pretreatment. The k of α-glucosidase (0.31 pass^{-1}) and ADH (0.28 pass^{-1}) were similar to the k following combined heat-osmotic pretreatment. The k of total soluble protein, invertase, α-glucosidase, ADH and G6PDH following combined heat-osmotic pretreatment were 0.34, 0.35, 0.32, 0.27 and 0.28 pass^{-1} respectively. However, k of the combined pretreatment at 40°C remained lower than the single 50°C heat pretreatment. The number of passes required for maximum protein and enzyme release following heat and combined pretreatment are shown in Table 6.4.

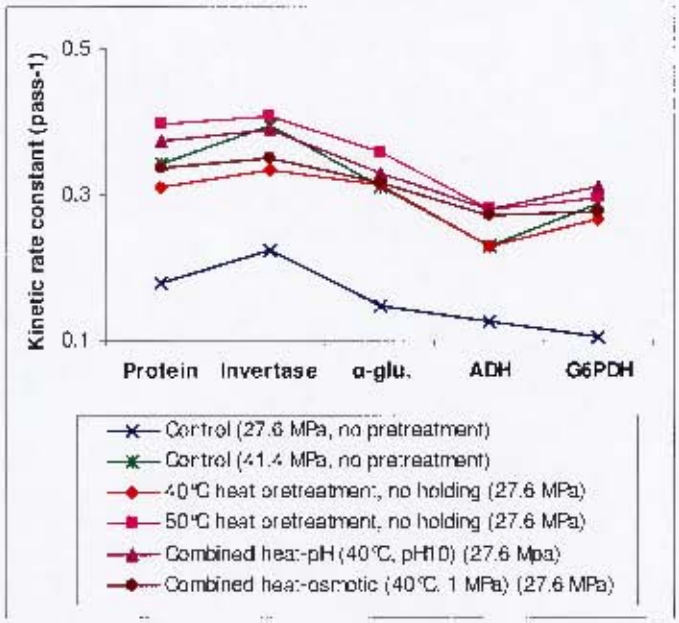


Figure 6.13 Release rate constant, k, of total soluble protein and each enzyme from Baker's yeast on HPH at 27.6 following single heat pretreatment or combined pretreatment

6.4.3 Comparison of single and combined pretreatment on extent of release

The extent of release (R_i/R_m) of total soluble protein and marker enzymes from Baker's yeast on HPH at 27.6 MPa following single or combined pretreatment is presented in Figure 6.14. The R_i/R_m of total soluble protein and marker enzymes following osmotic pretreatment varied between 0.7 and 0.9, while the R_i/R_m following other pretreatments varied between from 0.95 and 1.05. The maximum total soluble protein and enzyme release following both combined pretreatments and 50°C were approached or higher than the R_m obtained on HPH at 69.0 MPa without pretreatment. Hence, it is noted that while the efficiency of heat pretreatment approaches that of combined pretreatments in terms of extent of release, the rate of release was enhanced by the combined pretreatments relative to the single pretreatments. This in turn implies that the number of passes through the homogeniser is reduced.

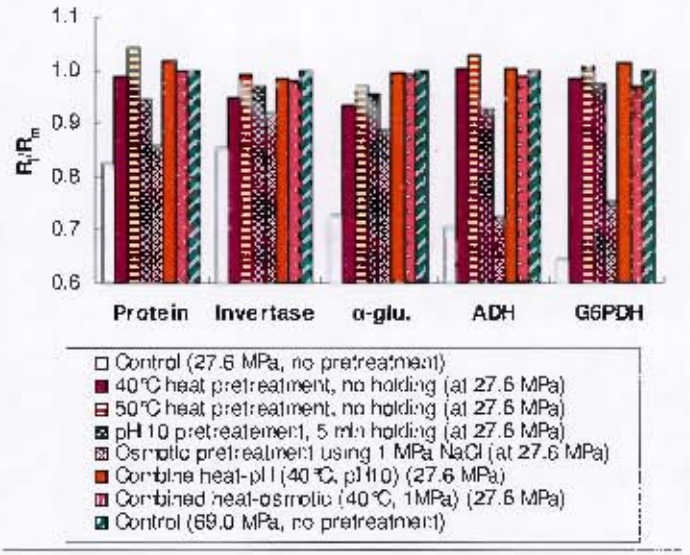


Figure 6.14 R_i/R_m of total soluble protein and each enzyme from Baker's yeast on HPH at 27.6 following single or combined pretreatment

6.5 SIZE ANALYSIS

Due to the relationship between cell size and the ease of solid/liquid separation, the size of Baker's yeast before and after disruption was considered. The size of Baker's yeast was measured by light microscopy and the Malvern size analyser. The data are presented in Tables D.4 of Appendix D. Light micrographs of disrupted Baker's yeast suspensions on HPH as a function of operating pressure and number of passes are presented in Figure 6.15. These were taken by phase contrast microscopy under 100 x objective magnification. The intact cells are shown in Figure 6.15a. The cell breakage presented in Figure 6.15b was obtained on HPH at 69.0 MPa for 5 passes, under which conditions R approaches R_m and micronisation occurred. In Figure 6.15c and 6.15d, it is seen that the extent of breakage increased with increased number of passes, however intact cells were still present in the control following HPH at 27.6 MPa for 10 passes. The micronisation increased with number of passes and increasing pressure. Under the conditions of Figure 6.15e and 6.15f, protein release approached that of complete breakage was obtained, while micronisation was less than the complete breakage conditions of 69.0 MPa for 5 passes, shown in Figure 6.15, illustrating the micronisation induced by mechanical cell disruption could be minimised by following a pretreatment.

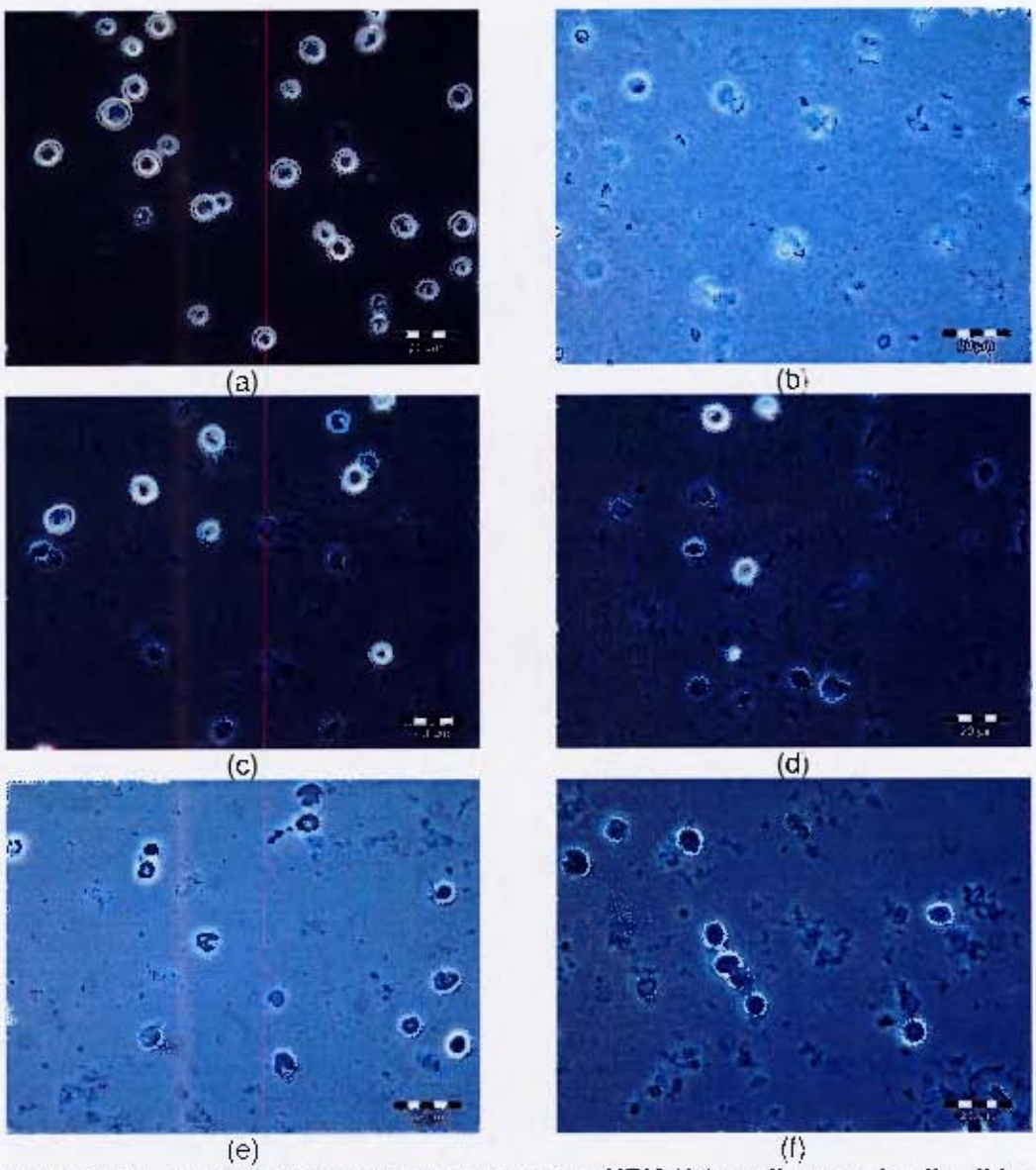


Figure 6.15 **Micrograph of Baker's yeast on HPH ((a) undisrupted cells, (b) at 69.0 MPa for 5 passes, (c) at 27.6 MPa for 6 Passes, (d) at 27.6 MPa for 10 Passes (e) at 27.6 MPa for 10 passes following heat-pH pretreatment, (f) at 27.6 MPa for 10 passes following heat-osmotic pretreatment).**

Figure 6.16 shows the particle diameter distribution of the undisrupted Baker's yeast suspension. The diameters below which obtained under 10 % and 90 % of the full distribution are shown as D[v, 0.1] and D[v, 0.9], with values of 3.9 µm and 8.8 µm, respectively. At the mode diameter of 6.1 µm, more than 19 % was obtained. This value was close to the volume mean diameter D[4,3] of 6.06 µm.

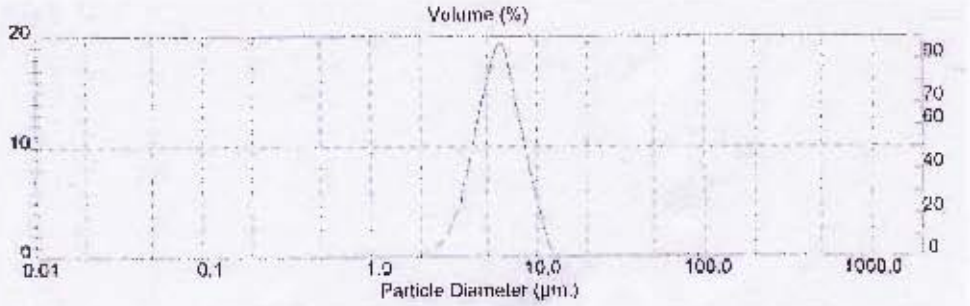


Figure 6.15 The particle diameter distribution of undisrupted Baker's yeast, measured by laser light scattering

The volume mean diameter (D[4,3]) was used to compare cell size following combined pretreatment on HPH as function of number of passes with disruption in the absence of pretreatment in Figure 6.17. Prior to pretreatment and disruption, the yeast D[4,3] was 6.06 µm. After pretreatment and before disruption, the D[4,3] of combined heat-pH pretreatment and combined heat-osmotic pretreatment were 5.69 and 5.67 µm, respectively. The D[4,3] decreased with increasing number of passes for the controls. The D[4,3] of the cell decreased with increasing operating pressure of HPH, from 5.22 µm at 27.6 MPa through 5.11 µm at 41.4 MPa to 4.89 µm at 69.0 MPa. The D[4,3] on combined heat-pH pretreatment on HPH at 27.6 MPa varied between 5.48 and 5.69 µm, while the D[4,3] following combined heat-osmotic pretreatment decreased from 5.67 µm to about 5.3 µm. The minimum D[4,3] obtained following combined pretreatments were greater than the size obtained on the control at 27.6 MPa. The mean diameters of the cell debris were also measured by the light microscopy; 10 random cell debris in different sizes were measured. The light microscopy shows 3 diameters, mean diameter, minimum circular diameter and maximum circular diameter. Data are illustrated in Table 6.5 and compared with the results obtained on the Malvern size analyser. The mean diameters obtained by both methods were similar. The diameters of small debris (D[v,0.1]) obtained on HPH at 27.6 MPa following combined pretreatments were bigger than the control (27.6 MPa). The mean diameter of cell debris decreased with increasing operating pressure. The ease of solid/liquid separation can be improved by combined pretreatment. The micronisation induced by mechanical cell disruption could be minimised by following a pretreatment.

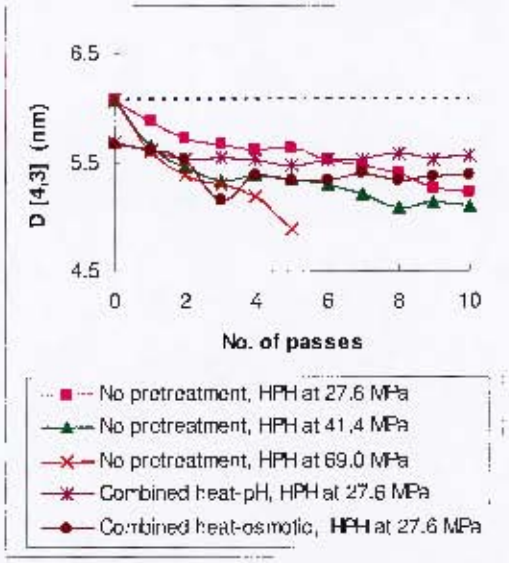


Figure 6.16 Volume mean diameter (D[4,3]) of Bakers' yeast using Malvern size analyser on HPH at 27.6 MPa following each combined pretreatment compared with the controls as function of number of passes. The dash line shows the size of undisrupted cells.

Table 6.5 The particle size distribution obtained on HPH using microscopy and the Malvern size analyser

	Microscopy (μm)			Malvern (μm)		
	Mean	Min	Max	D[4,3]	D[v,0.1]	D[v,0.9]
Undisrupted	6.29	4.54	8.52	6.06	3.78	8.70
Control (27.6MPa, 10passes)	4.76	2.81	7.50	5.26	3.16	7.68
Control (69.0MPa, 10passes)	4.34	2.11	5.69	4.89	2.75	5.30
Heat-pH (27.6MPa, 10passes)	5.26	2.21	6.97	5.48	3.36	7.76
Heat-Osmotic (27.6MPa, 10passes)	4.99	2.38	7.66	5.39	3.46	7.63

6.6 CONCLUSIONS

The total soluble protein release from Baker's yeast increased with increasing maximum temperature of pretreatment from 40 to 50°C. No significant change in total soluble protein release was obtained on varying the holding time of the 50°C heat pretreatment between minimal and 5 min. The profiles of total soluble protein release obtained on HPH at 27.6 MPa following heat pretreatment at 40 and 50°C were similar to the control at 41.4 MPa, while the profiles of total soluble protein release obtained on HPH at 41.4 MPa following heat pretreatment at 50°C with minimal or 5 min holding approached the control at 69.0 MPa. To achieve the same amount of total soluble protein release at same operating pressure, the energy requirement was decreased following heat pretreatment at 40 and 50°C. The R_m obtained at 69 MPa could be obtained on HPH at a low operating pressure following heat pretreatment at

40 and 50℃, but not in the absence of pretreatment. However, the lowest energy requirement for disruption using HPH was reported at the highest pressure of 69.0 MPa used owing to the reduced number of passes. The ability to further improve energy efficiency by combining heat pretreatment with HPH at this pressure remains to be investigated.

An energy saving on HPH at a constant pressure operation can be achieved following pH 10 pretreatment with 2 min holding, however the energy saving was less than that on heat pretreatment, while the osmotic pretreatment had the lowest effect on protein release compared to the other single pretreatment. The influence of combined pretreatments on energy saving were better than all single pretreatments at same maximum temperature, maximum pH or maximum osmotic pressure; however, the impact of the combination was small compared to the heat pretreatment at 40℃, and was less than heat pretreatment at 50℃.

The heat pretreatment enhanced the relative release of cytoplasmic enzymes more than periplasmic and cell wall associated enzyme release. The E_N of the marker enzymes did not change when the maximum temperature and the holding time of heat pretreatment varied from 40 to 50℃ and minimal to 5 min. The pH and osmotic pretreatment had less effect on the E_N than the heat pretreatment. The E_N obtained on combined pretreatments (pH 10, 40℃ or 1 MPa, 40℃) approached that of heat pretreatment at 50℃ and exceeded any single pretreatment.

The optimal condition of each single and combined pretreatment for protein and enzyme release was concluded in Table 6.5. The number of passes required for maximum release of protein and marker enzymes decreased following single or combined pretreatments, while the best results were obtained on HPH following combined pretreatments.

The highest release rate constants were obtained on HPH following heat pretreatment at 50℃. The k for combined pretreatments were higher than all single pretreatment at same maximum temperature, maximum pH or maximum osmotic pressure, while no significant increase in the k of total soluble protein, invertase and ADH following osmotic pretreatment was obtained compared to the control at same operating pressure. The R_i/R_m obtained following single and combined pretreatment were quite similar except the R_i/R_m obtained following osmotic pretreatment, which was unchanged from the control.

The sizes of Baker's yeast and cell debris were measured by light microscopy and the Malvern size analyzer. The indicators of the distribution obtained using both methods were quite similar. These decreased with increasing in the number of passes. Complete breakage was obtained on the control at 69.0 MPa for 5 passes and on HPH at 27.6 MPa for 10 passes following combined heat-pH pretreatment and combined heat-osmotic pretreatment. The diameters of small debris obtained on HPH at 27.6 MPa following combined pretreatments were bigger than the control at same operating pressure. The micronisation induced by mechanical cell disruption was minimised.

Chapter 7
Effect of Pretreatment on *Kluyveromyces lactis* Disruption

7.1 INTRODUCTION

The influence of the single and combined pretreatment on energy efficiency and selective product release from *Kluyveromyces lactis* using high pressure homogenisation (HPH) is demonstrated in this chapter. The conditions of single and combined pretreatments are listed in Tables 3.3 to 3.6, respectively. The ease of disruption and selective product release were measured by release of total soluble protein and marker enzymes. The micronisation of disruption were also considered and determined by size analysis.

7.2 DISRUPTION OF *KLUYVEROMYCES LACTIS* USING HPH WITHOUT PRETREATMENT

The experiments using the high pressure homogenisation for mechanical cell disruption of *Kluyveromyces lactis* are defined in Table 7.1. All experiments were performed using a 300 ml yeast suspension with a 1.5 % (dry weight) cell concentration. The effect of operating pressure on total soluble protein release was investigated by disrupting the yeast suspension at three operating pressures: 27.6, 41.4 and 69.0 MPa. A cooling coil was used to maintain the suspension at 20°C to avoid the protein denaturation.

Table 7.1 Experimental conditions for HPH using 1.5 % cell concentration (dry weight) of *K. lactis*

Microorganism	Pressure (MPa)	No. of Passes	Protein and enzymes measured
Kluyveromyces lactis	27.6	10	Total soluble protein
	41.4		Total soluble protein, Invertase, ADH, G6PDH and β-galactosidase
	69.0		

The total protein release as a function of number of passes under different operating pressures without pretreatment is presented in Figure 7.1. Data are shown in Table E.1 of Appendix E. Increasing operating pressure reduced the number of passes required to reach maximum total soluble protein release while increasing the extent of release. The maximum protein release (R_i) obtained at 27.6 MPa was 226 mg/g following 10 passes. A similar total soluble protein release was obtained on 5 passes at 41.4 MPa and 2 passes at 69.0 MPa. The R_i increased to 365 mg/g on 9 passes at 41.4 MPa. The total soluble protein release obtained for 7 passes at 69.0 MPa was 431 mg/g. This was used as the maximum protein release (R_m) for the calculation of the kinetic rate constant. Therefore, the total protein release was influenced by both operating pressure and number of passes, synergistically.

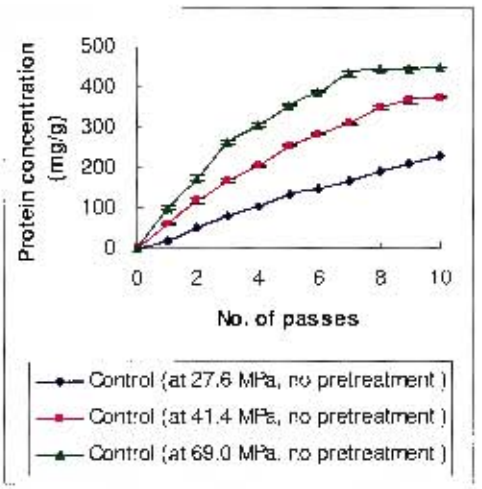


Figure 7.1 Total soluble protein release from *K. lactis* by HPH at different operating pressure without pretreatment as a function of number of passes.

The marker enzyme release by HPH at different operating pressure as a function of number of passes is shown in Figure 7.2. Data are presented in Tables E.2 of Appendix E. Increasing operating pressure reduced the number of passes required to reach the maximum enzyme release while increasing the extent of release. The maximum invertase, ADH, G6PDH and β -galactosidase obtained on HPH at 41.4 MPa for 8, 10, 10, 10 passes were 3.14 $X10^4$, 6.21 $X10^5$, 17.1 and 4.59 $X10^3$ U/g, respectively. The maximum invertase (cell wall associated enzyme) release was achieved by HPH at 69.0 MPa for 6 passes, while cytoplasmic enzymes required 9 passes to reach the maximum enzyme release. The maximum enzyme release (R_m) of invertase, ADH, G6PDH and β -galactosidase were 3.74 $X10^4$, 7.70 $X10^5$, 20.9 and 5.82 $X10^3$ U/g, respectively. Hence, the ease of enzyme release is a function of location of enzyme.

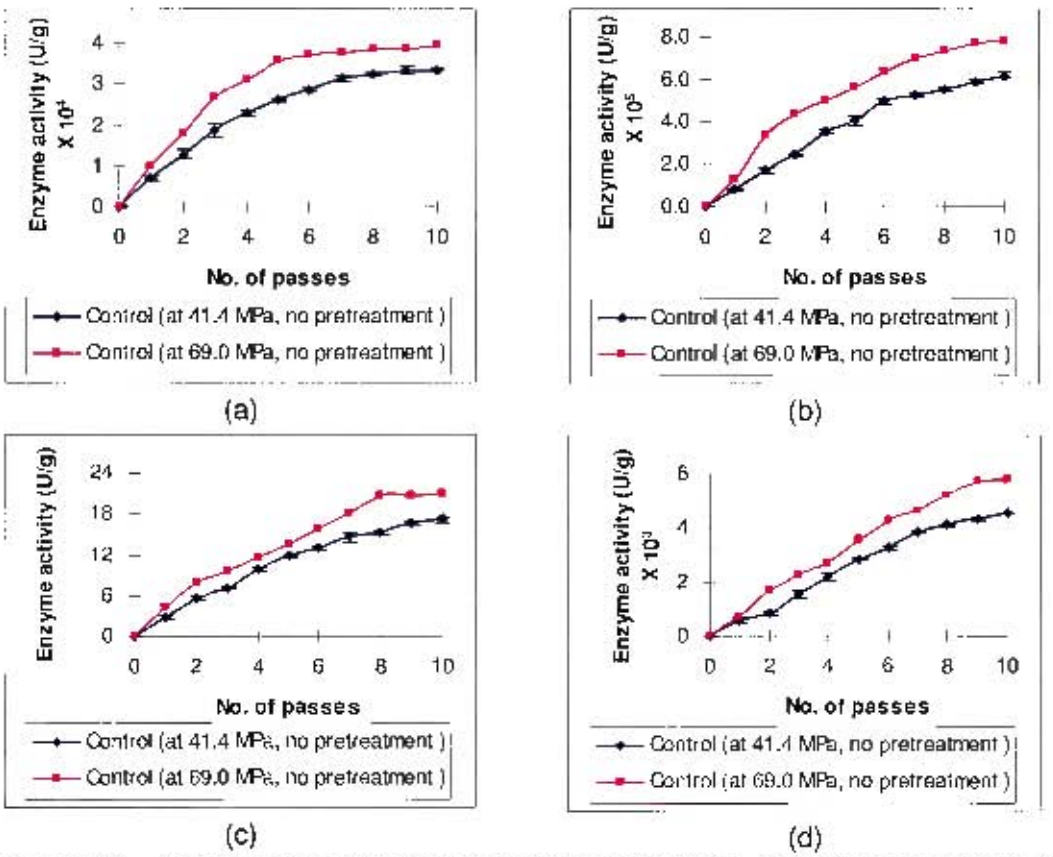


Figure 7.2 **(a) Invertase, (b) ADH, (c) G6PDH and (d) β -galactosidase from *K. lactis* by HPH at different operating pressure without pretreatment as a function of number of passes.**

The extent of disruption (R_i/R_m) for total soluble protein and different enzymes from *K. lactis* by HPH as a function of operating pressure is presented in Figure 7.3. Increasing the operating pressure increased the R_i/R_m. The R_i/R_m of invertase obtained at 41.4 MPa was 0.86, while the R_i/R_m of total soluble protein, ADH, G6PDH and β -galactosidase were 0.90, 0.81, 0.83 and 0.80, respectively. Hence, the ease of enzyme release depended on the location of the enzyme: cell wall associated (invertase) > total soluble protein > cytoplasmic enzyme (ADH, G6PDH and β-galactosidase).

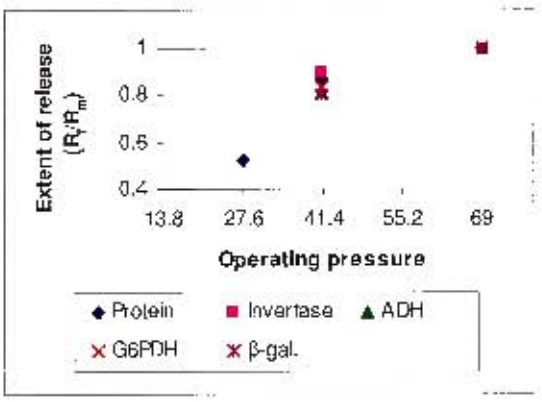


Figure 7.3 R_i/R_m of total soluble protein and different enzymes from *K. lactis* by HPH as a function of operating pressure

The first order kinetic rate constants (k) of release of total soluble protein and marker enzymes were determined using Equation 4.1. Table 7.2 illustrates the k obtained from *K. lactis* by high pressure homogenisation at different operating pressure. The kinetic rate constant (k) of total soluble protein obtained by high pressure homogenisation increased from 0.08 pass^{-1}at 27.6 MPa through 0.21 pass^{-1} at 41.4 MPa to 0.37 pass^{-1}at 69.0 MPa. The k of each enzyme listed in Table 7.2 demonstrates increased k with the increasing pressure. The value of k for cell wall associated enzyme (Invertase) is higher than for cytoplasmic enzymes (ADH, G6PDH and β -galactosidase), illustrating the cell wall associated enzymes released more rapidly than the cytoplasmic enzymes. Through the correlation, R^2, the quality of the fit of first order disruption kinetics is assessed. The release sequence of total soluble protein and different enzymes are: cell wall associated enzyme > total soluble protein > cytoplasmic enzyme. This is in agreement with Torner and Asenjo (1991), Melendres *et al.* (1993) and Balasundaram and Pandit (2001). The dependence of k on number of passes and operating pressure has been related through Equation 4.2. The pressure exponent (a) of total soluble protein from *K. lactis* was determined by linearising Equation 4.2. On plotting ln k as a function of ln P, as shown in Figure 7.4, the slope of (a) was determined as 1.71. The goodness of fit is quantified by the correlation coefficient R^2 of 0.9542.

Table 7.2a Kinetic rate constant (k) of total soluble protein release from *K. lactis* by HPH at different operating pressure

Pressure(MPa)	k (pass^{-1})	R^2
27.6	0.08	0.9977
41.4	0.21	0.9824
69.0	0.37	0.9792

Table 7.2b **Kinetic rate constant (k) of different enzymes release from *K. lactis* by HPH at different operating pressure**

Pressure (MPa)	Invertase		ADH		G6PDH		β-galactosidase	
	K ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
41.4	0.24	0.9916	0.17	0.9935	0.18	0.9953	0.17	0.9873
69.0	0.45	0.9755	0.29	0.9933	0.23	0.9832	0.24	0.9628

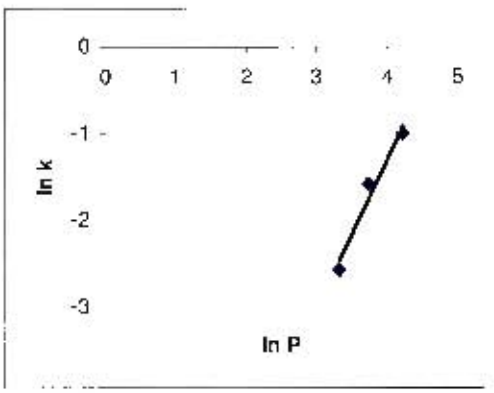


Figure 7.4 **Pressure exponent of total soluble protein from K. *lactis* by HPH determined as the slope of the releationship between ln(k) and ln(P)**

7.3 EFFECT OF SINGLE AND COMBINED PRETREATMENT ON PROTEIN RELEASE FROM *K. LACTIS*

To assess the impact of heat pretreatment, the temperature of *K. lactis* was increased to 40 or 50°C by heat exchange before returning to room temperature and subsequent disruption by HPH at 27.6 and 41.4 MPa. The total protein from *K. lactis* as a function of number of passes on HPH following a heat pretreatment is shown in Figure 7.5. Data are presented in Table E.3 of Appendix E. The maximum total soluble protein release increased with the increase in temperature of heat pretreatment from 365 mg/g in the absence of pretreatment to 415 mg/g at 40°C and 422 mg/g at 50°C, following HPH at 41.4 MPa. This approached the R_m of 431 mg/g and presented about a 14 % increase compared with the control at 41.4 MPa. To reach the maximum protein release following heat pretreatment with different maximum temperatures, 8 passes were required. The maximum total soluble protein release on HPH at 27.6 MPa following 50°C heat pretreatment for 10 passes (287 mg/g) represented presented a 27 % increasing compared with the control at 27.6 MPa. However, it remained lower than the maximum protein release on HPH at 41.4 MPa without pretreatment (349 mg/g). Hence, heat pretreatment with maximum temperature between 40 and 50°C decreases the energy requirement of *K. lactis* disruption at the same pressure.

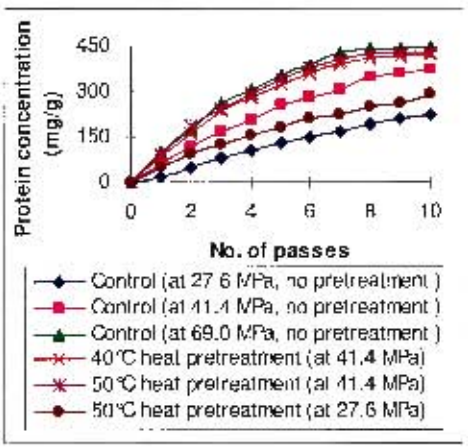


Figure 7.5 Total soluble protein release form *K. lactis* on HPH at 27.6 and 41.4 MPa following 40 and 50°C heat pretreatment as a function of number of passes. Holding time was minimised.

The total soluble protein release from *K. lactis* on HPH following a single pretreatment as a function of number of passes is presented in Figure 7.6. The conditions of each single pretreatment are listed in Table 7.3. To reach the maximum protein release following pH and osmotic pretreatment, 9 passes were required. The maximum protein release following pH and osmotic pretreatment were 393 and 380 mg/g at 41.4 MPa, presenting 8 % and 4 % increase compared to the control, while a 14 % increase in maximum protein release was obtained following heat pretreatment. Hence, energy saving on disruption of *K. lactis* using high pressure homogenisation can be achieved following pretreatment through heat, pH or osmotic shock. The total protein release following heat pretreatment was higher and more rapid than the protein release following pH and osmotic pretreatment.

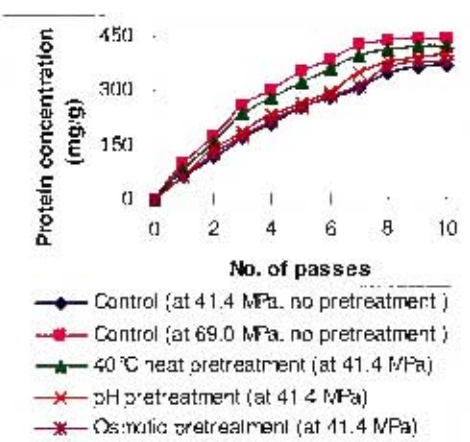


Figure 7.6 Total soluble protein release form *K. lactis* on HPH at 41.4 MPa following heat, pH and osmotic pretreatment as a function of number of passes. Holding time was minimised.

Table 7.3 The condition of each single pretreatment on *K. lactis* using high pressure homogenisation

Pretreatment	Conditions	Pressure
Heat	Heat exchanger, maximum temperature: 40°C without holding	41.4 MPa
pH	Na_2CO_3 (0.5M), maximum pH: 10 with 2 min holding	
Osmotic	NaCl (1 MPa), minimal holding	

The combined pretreatment was induced by diluting the *K. lactis* suspension using a pre-warmed pH or osmotic buffer. On dilution, the temperature was shifted to 40°C while the pH or osmotic pressure was shifted to pH 10 or 1 MPa. The holding time of combined pretreatment was minimised. The total soluble protein release from *K. lactis* as a function of number of passes on HPH at 41.4 MPa following a combined pretreatment is shown in Figure 7.7. The maximum protein release achieved on HPH at 41.4 MPa following combined heat-pH and combined heat-osmotic pretreatments were 419 and 417 mg/g, representing a 15 % increase compared to the control. Only 1 % increase was obtained on total soluble protein release following combined pretreatments compared with single heat pretreatment. This may relate to the major impact of combined pretreatment on protein release was induced by heat. Hence, combined pretreatments have more effect on decreasing the energy requirement of mechanical cell disruption than pH and osmotic pretreatments. However, the increase over the heat pretreatment only s small.

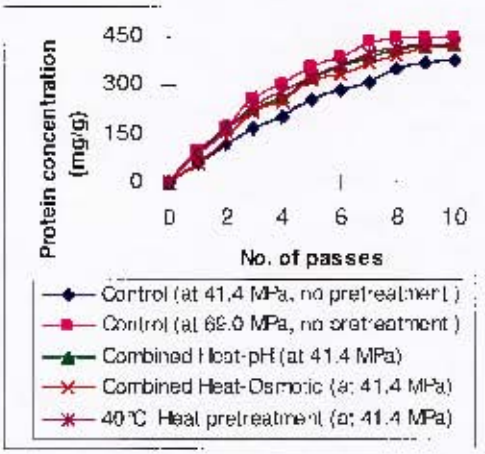


Figure 7.7 Total soluble protein release from *K. lactis* by high pressure homogeniser at 41.4 MPa following 40°C heat pretreatment or combined pretreatment as a function of number of passes.

The effect of each pretreatment on energy saving was considered. The energy calculation for heating has been related through Equation 5.1. The comparison of the energy input required for each pretreatment and the controls are illustrated in Table 7.4. It is seen that, more energy was required on HPH at 27.6 MPa following 50°C heat pretreatment than the control. The energy saving was obtained on the HPH at

41.4 MPa following a single or a combined pretreatment. Osmotic and pH pretreatments were less efficient than heat pretreatment, owing to the slight increase in energy requirement and the extra cost of the buffer. The combined pretreatments had no advantage on total protein release and energy saving to single heat pretreatment at same maximum temperature. The maximum total soluble protein release obtained on HPH at 41.4 MPa following 50°C heat pretreatment approached to the R_m obtained on HPH at 69.0 MPa for 8 passes, while the energy requirement for them were similar. Hence, each pretreatment decreases the energy requirement of *K. lactis* disruption. The heat pretreatment is more efficient than pH or osmotic pretreatment.

Table 7.4 Effect of heat pretreatment on energy efficiency of total soluble protein release from *K. lactis* on HPH

Effect of heat pretreatment on energy efficiency of HPH at 27.6 MPa					
Pressure	**Pretreatment conditions**	**Passes**	**Protein conc. (mg/g)**	**Energy (kJ/kg)**	**Energy/protein (kJ/g)**
27.6 MPa	No pretreatment	10	226	276	1.22
27.6 MPa	Heat pretreatment at 50°C, minimal holding	7	213	311	1.46
Effect of heat pretreatment on energy efficiency of HPH at 41.4 MPa					
Pressure	**Pretreatment conditions**	**Passes**	**Protein conc. (mg/g)**	**Energy (kJ/kg)**	**Energy/protein (kJ/g)**
41.4 MPa	No pretreatment	9	365	373	1.02
41.4 MPa	Heat pretreatment at 40°C, minimal holding	6	361	324	0.90
41.4 MPa	pH 10 pretreatment	8	379	331	0.87
41.4 MPa	Osmotic pretreatment at 1 MPa	8	366	331	0.90
41.4 MPa	Combined heat-pH pretreatment	6	373	324	0.87
41.4 MPa	Combined heat-osmotic pretreatment	6	349	324	0.93
41.4 MPa	Heat pretreatment at 50°C, minimal holding	10	433	532	1.23
69.0 MPa	No pretreatment	8	447	552	1.23

7.4 EFFECT OF SINGLE AND COMBINED PRETREATMENT ON SELECTIVE PRODUCT RELEASE

Selective product release was analysed by relative enzyme release. The normalised enzyme release (E_N) related to the maximum enzyme release was calculated by Equation 7.1.

$$E_N = \frac{\text{Enzyme release}}{\text{Enzyme release in control (at 41.4 MPa)}} \qquad \text{Equation 7.1}$$

The E_N of each marker enzyme from *K. lactis* on HPH following pretreatment at 40 and 50°C and 10 passes through the homogeniser at 41.4 MPa is presented in

Figure 7.8. The E_N of different enzymes from *K. lactis* on HPH at 41.4 MPa following different single pretreatments as function of number of passes is presented in Figure 7.9. Data of each enzyme release are listed in Table E.4 of Appendix E.

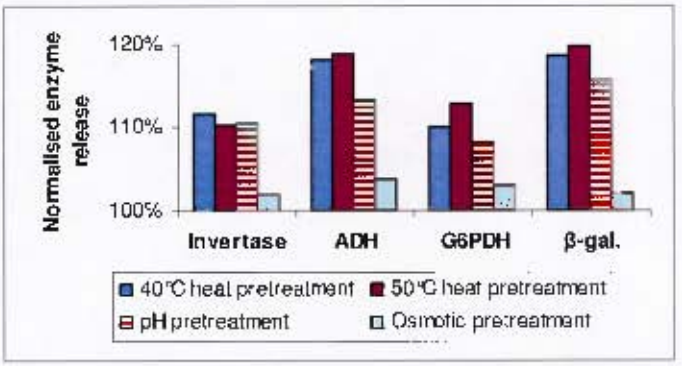


Figure 7.8 **Maximum E_N of different enzymes from *K. lactis* by high pressure homogeniser for 10 passes at 41.4 MPa following each single pretreatment.**

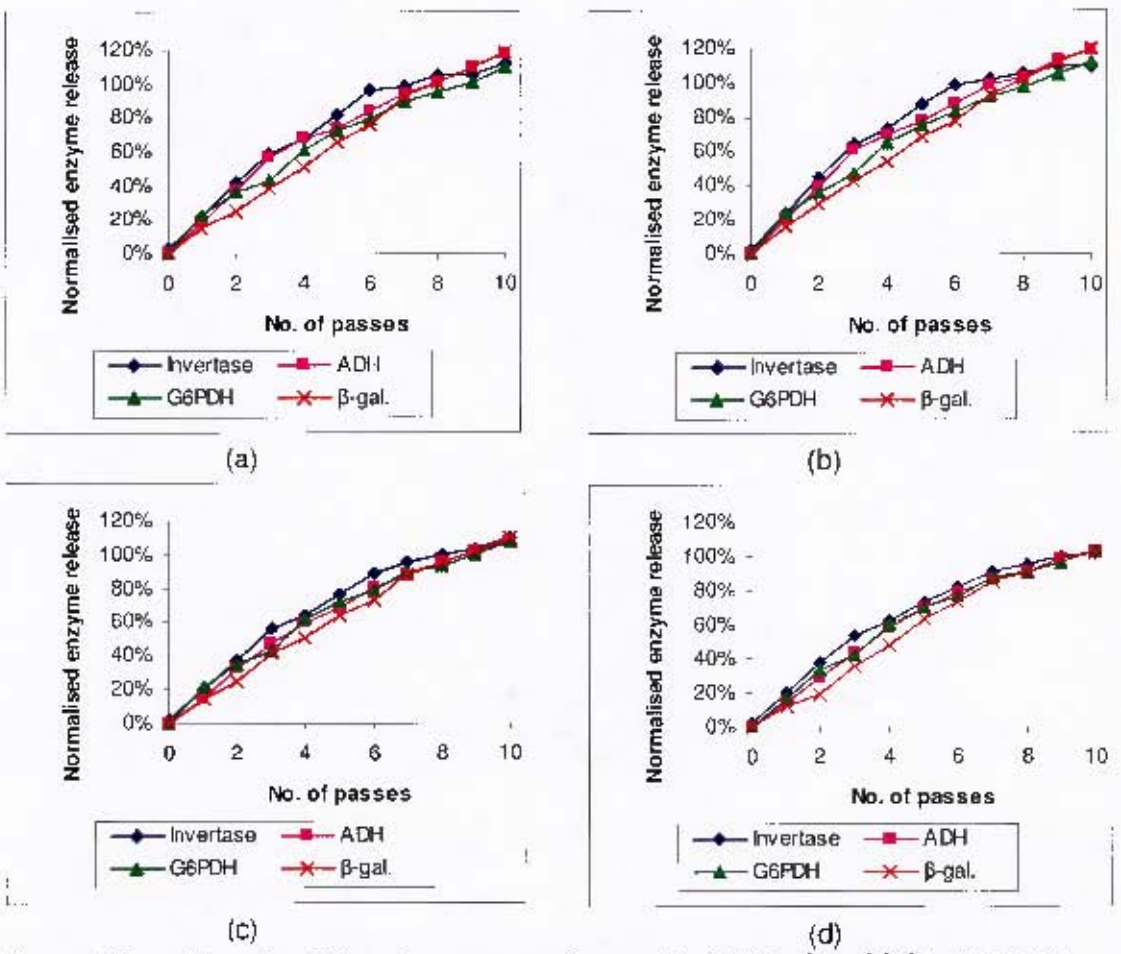


Figure 7.9 **E_N of different enzyme from *K. lactis* by high pressure homogeniser at 41.4 MPa following (a) 40°C heat pretreatment, (b) 50°C pretreatment, (c) pH pretreatment and (d) osmotic pretreatment as function of number of passes.**

The E_N of each marker enzyme obtained on HPH following heat pretreatment at 40 or 50°C were similar. An 18 % increase of the E_N of ADH and β -galactosidase was obtained following heat pretreatment, while a 12% increase was demonstrated for invertase and G6PDH. To reach the maximum E_N of invertase, ADH and G6PDH and β -galactosidase on HPH at 41.4 MPa following heat pretreatment at either 40 or 50°C, 8, 10, 10 and 10 passes were required, respectively. An increase of some 10 % in E_N of invertase and G6PDH was obtained following pH pretreatment, while that of ADH and β -galactosidase release increased by 15 % increase. Osmotic pretreatment did not affect the E_N of marker enzymes significantly. To reach the maximum E_N of invertase, ADH and G6PDH and β -galactosidase following pH or osmotic pretreatment, 8, 10, 10 and 10 passes were required.

The E_N of different enzymes from *K. lactis* on HPH at 41.4 MPa following different combined pretreatments as function of number of passes are presented in Figure 7.10. To achieve the maximum E_N of invertase, ADH, G6PDH and β -galactosidase following combined pretreatments with heat, 8, 10, 10 and 10 passes were required respectively.

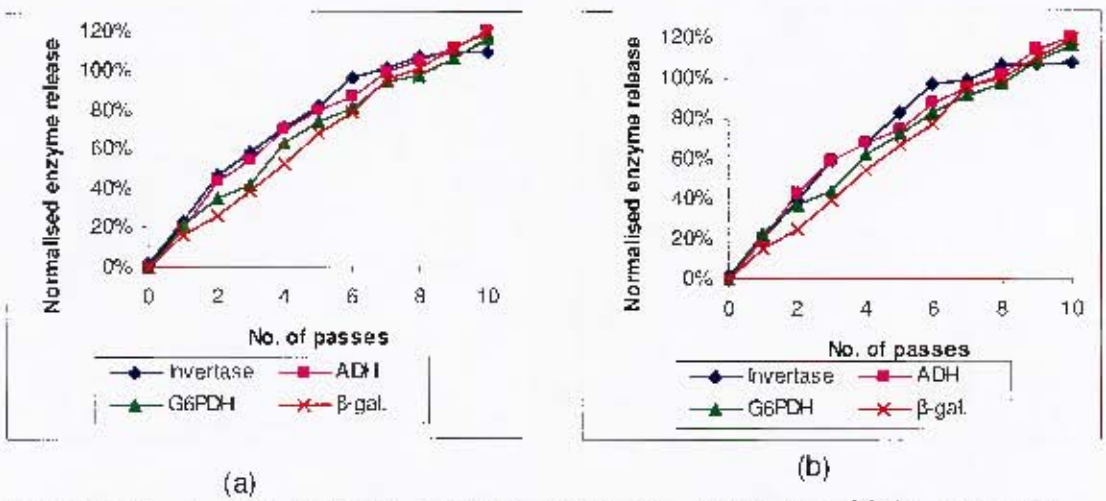


Figure 7.10 **E_N of enzyme release from *K. lactis* by high pressure homogeniser at 41.4 MPa as function of number of passes following (a) combined heat-pH pretreatment and (b) combined heat-osmotic pretreatment. Holding time was minimised.**

To compare the influence of enzyme release across different single and combined pretreatments, the maximum E_N of the marker enzymes following pretreatment are shown in Figure 7.11.

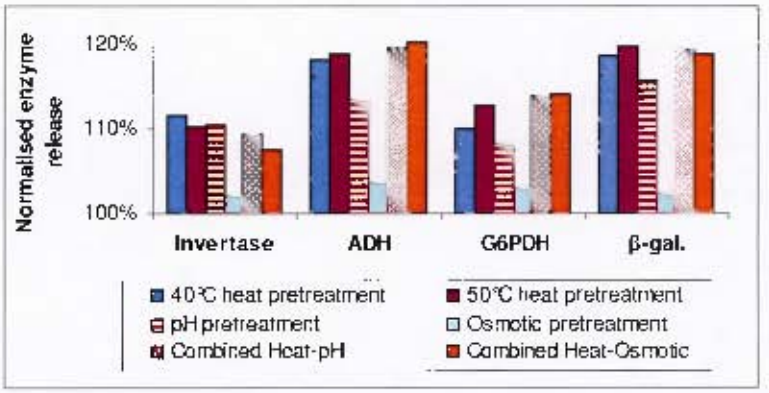


Figure 7.11 Maximum E_N of enzyme release from *K. lactis* on high pressure homogenisation at 41.4 MPa following single or combined pretreatments

From Figure 7.11, it is seen that a 2 % increase on invertase release was obtained following osmotic pretreatment, while about 10 % increase of invertase activity was obtained following other single or combined pretreatments. The maximum E_N of ADH (120 %), G6PDH (116 %) and β -galactosidase (119 %) were obtained following 40°C heat pretreatment and combined pretreatments. These had similar effect on enzymes release. Hence, combined pretreatment was not advantageous over heat pretreatment with respect to E_N.

7.5 RELEASE RATE KINETICS

The first order kinetic rate constant (k) of total soluble protein following single or combined pretreatment were calculated using Equation 4.1. The k of total soluble protein and marker enzymes release from *K. lactis* on HPH at 41.4 MPa following pretreatment is presented in Figure 7.12. Data are listed in Table E.5 of Appendix E. The single and combined pretreatment did not affect the sequence of release on HPH at 41.4 MPa: cell wall associate enzyme (invertase) > total soluble protein > cytoplasmic enzyme (ADH, G6PDH and β -galactosidase).On HPH at 41.4 MPa, the maximum k of total soluble protein was obtained following heat pretreatment at 50°C (0.29 $pass^{-1}$), representing a 40 % increase over the k of total soluble protein obtained by the control. When the maximum temperature decreased to 40°C, the k of total soluble protein decreased to 0.26 $pass^{-1}$. The k following combined heat-pH and combined heat-osmotic pretreatment at 40°C were both 0.28 $pass^{-1}$. The k of total soluble protein release following pH and osmotic pretreatment were 0.25 and 0.23 $pass^{-1}$, which representing a 20 % and 10 % increase over the control, respectively. For invertase, the largest k of 0.36 $pass^{-1}$ was obtained following 50°C

heat pretreatment, compared to 0.31 $pass^{-1}$ on pretreatment at 40°C. The k of invertase was 0.28 $pass^{-1}$ on pH pretreatment and 0.25 $pass^{-1}$ on osmotic pretreatment. On combined heat-pH and combined heat-osmotic pretreatments, the rate constant was 0.34 $pass^{-1}$ and 0.33 $pass^{-1}$, respectively. The heat pretreatments and combined pretreatments had similar influence on the k of cytoplasmic enzymes varying between 0.20 to 0.23 $pass^{-1}$, while a k of 0.18 to 0.20 $pass^{-1}$ was obtained on pH and osmotic pretreatment.

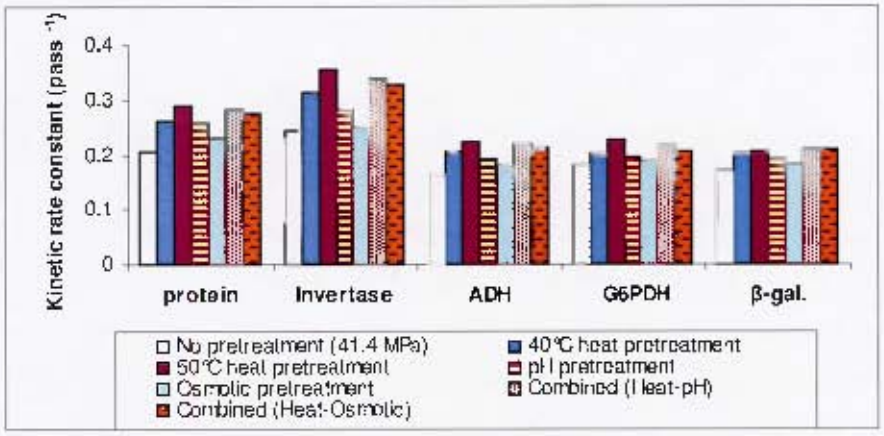


Figure 7.12 Kinetic rate constant (k) of total soluble protein and enzyme release from *K. lactis* by high pressure homogeniser at 41.4 MPa following pretreatment.

The extent of release (R_i/R_{ri}) of total soluble protein and marker enzymes on HPH at 41.4 MPa following different pretreatments is shown in Figure 7.13. The R_i of total soluble protein and marker enzymes on HPH at 41.4 MPa following combined pretreatments and heat pretreatment with maximum temperature between 40 and 50°C were approached to the maximum release (R_m). These represented 12 % increase in total soluble protein release compared to the control at 41.4 MPa. The R_i/R_m of total soluble protein on HPH at 41.4 MPa following pH and osmotic pretreatments were 0.93 and 0.89, respectively. The R_i/R_m of invertase on HPH at 41.4 MPa following pH pretreatment was 0.99, while 91 % invertase was released following osmotic pretreatment. The R_i/R_m of invertase on HPH at 41.4 MPa following heat and combined pretreatment were approached to 1. About 95 % of ADH and β–galactosidase were released oh HPH at 41.4 MPa following heat and combined pretreatments, while the R_i/R_{ri} of G6PDH on HPH at 41.4 MPa following pH and osmotic pretreatment were 0.90 and 0.86, respectively. The R_i/R_m of ADH, G6PDH and β–galactosidase on HPH at 41.4 MPa following pH and osmitc pretreatment were varying between 0.8 and 0.9.

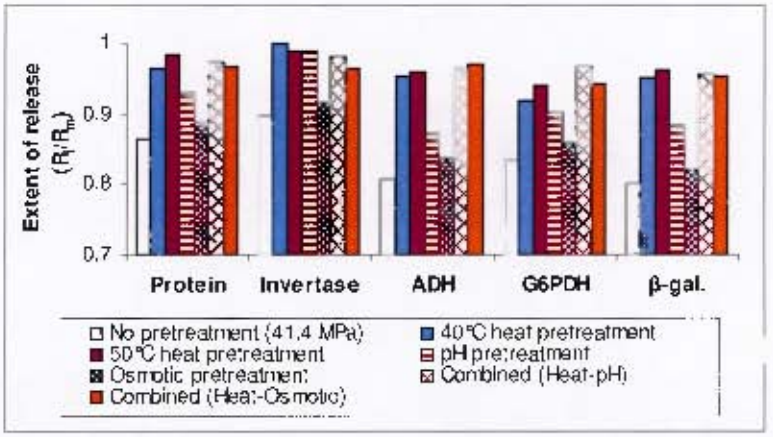


Figure 7.13 Extent of release (R_i/R_m) of total soluble protein and marker enzymes from *K. lactis* on HPH at 41.4 MPa following pretreatment.

7.6 SIZE ANALYSIS

Micrographs taken under the light microscope with phase contrast optics at 100 x objective magnification are presented in Figure 7.14 for the disruption of *K. lactis* on HPH. In Figure 7.15, it is seen that most of the cells were disrupted on HPH at 41.4 MPa for 10 passes following a combined pretreatment, while micronisation was less than the control at 41.4 MPa for 10 passes.

The volume mean diameter (D[4.3]) determined by the Malvern size analyser following combined pretreatment on HPH as function of number of passes compared with the controls is presented in Figure 7.15. Data are shown in Table E.6 of Appendix E. The D[4,3] of cells before disruption was 3.60 nm, and decreased with the number of passes increasing. The D[4,3] obtained on HPH for 10 passes decreased with increasing operating pressure from 3.32 µm at 27.6 MPa through 2.91 µm at 41.4 MPa to 2.90 µm at 69.0 MPa. The D[4,3] obtained on HPH at 41.4 MPa for 10 passes following combined heat-pH pretreatment and combined heat-osmotic pretreatment were 3.38 and 3.29 nm, respectively, which were similar to the control at 27.6 MPa. The mean diameters of *K. lactis* measured the light microscopy and the Malvern size analyser are shown in Table 7.5. The results obtained on both methods were quite similar. The diameters of small debris (D[v,0.1]) obtained following combined pretreatments were bigger than the controls at 41.4 MPa. Hence, the combined pretreatment can improve the solid/liquid separation, while the micronisation induced by HPH is minimised.

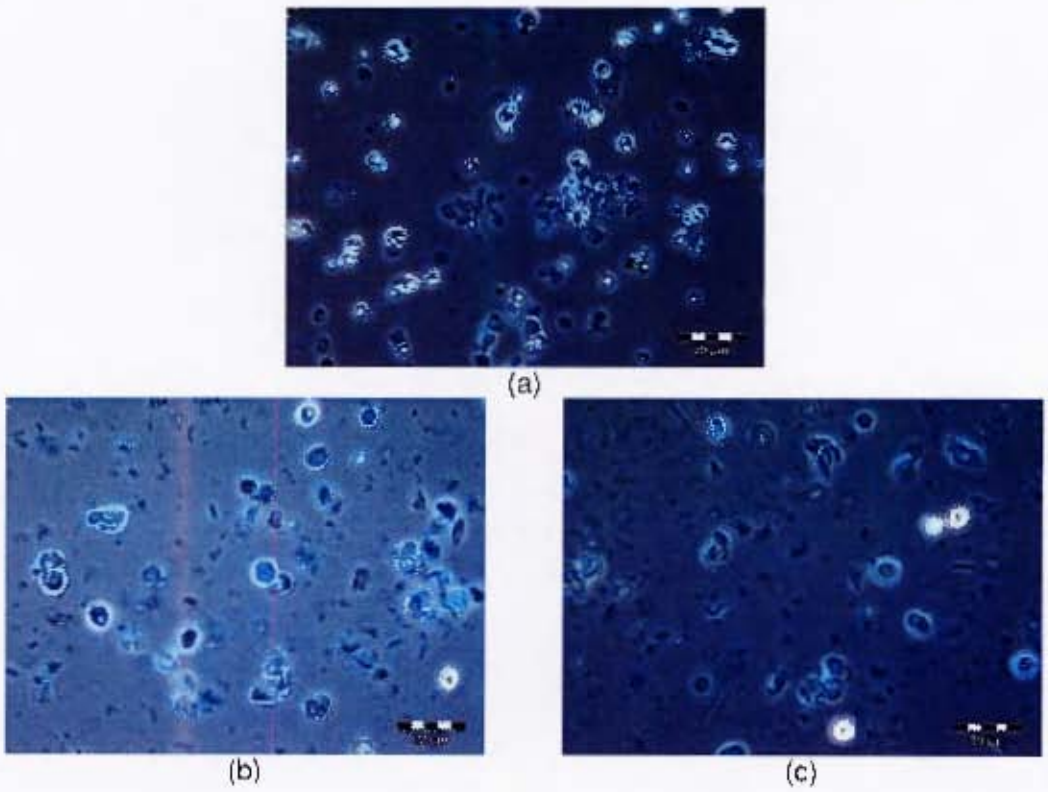


Figure 7.14 Micrograph of *K. lactis* on HPH (a) at 41.4 MPa for 10 passes (b) at 41.4 MPa for 10 passes following heat-pH pretreatment, (c) at 41.4 MPa for 10 passes following heat-osmotic pretreatment).

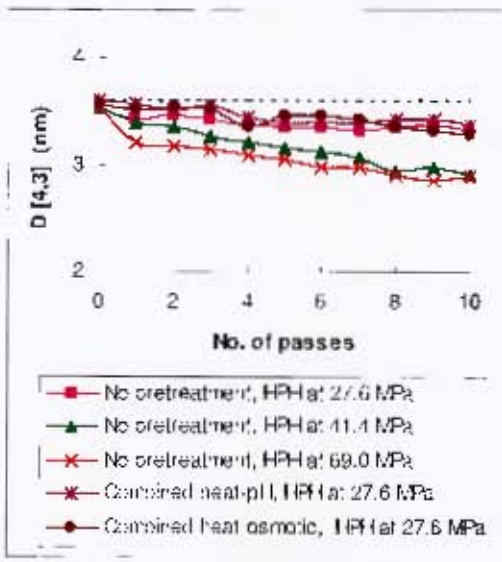


Figure 7.15 Volume mean diameter (D[4,3]) of *K. lactis* using Malvern size analyser on HPH at 41.4 MPa following each combined pretreatment compared with the controls as function of number of passes. The dash line shows the size of undisrupted cells.

Table 7.5 The mean diameters of *K. lactis* measured by the microscopy and the Malvern size analyser

	Microscopy (μm)			Malvern (μm)		
	Mean	Min	Max	D[4,3]	D[v,0.1]	D[v,0.9]
Undisrupted	4.33	3.65	7.76	3.60	3.78	8.70
Control (41.4MPa, 10passes)	3.46	1.44	4.71	2.91	2.05	3.82
Control (69.0MPa, 10passes)	2.79	1.06	3.22	2.71	1.93	3.51
Heat-pH (41.4MPa, 10passes)	4.26	2.15	5.48	3.38	2.25	4.76
Heat-Osmotic (41.4MPa, 10passes)	4.29	2.04	5.29	3.29	2.12	5.16

7.7 CONCLUSIONS

The maximum extent of total soluble protein and enzyme release and the first order kinetic rate constant obtained on high pressure homogenisation of *Kluyveromyces lactis* increased with increase in operating pressure and number of passes, synergistically, The release sequence of total soluble protein and marker enzymes was: cell wall associated enzyme > total soluble protein > cytoplasmic enzyme. To achieve equivalent total soluble protein release from *Kluyveromyces lactis* using high pressure homogenisation, the number of passes was decreased following a single or combined pretreatment compared to the control at same operating pressure. As with disruption of Baker's yeast, more passes were required for equivalent release of cytoplasmic enzymes.

The heat pretreatment at 40 or 50℃ and combined pretreatment with heating to 40℃ had similar effect on the disruption of *Kluyveromyces lactis*. The maximum total soluble protein and enzyme release obtained on HPH at 41.4 MPa following one of these pretreatments approached the R_m obtained at 69.0 MPa in the absence of pretreatment. The pH pretreatment was less effective than heat and combined pretreatment. No significant increase in total soluble protein and enzyme release was obtained on HPH following osmotic pretreatment compared to the control. Less energy input was required for equivalent release on HPH at the same pressure following heat pretreatment or combined pretreatment compared to other pretreatment. However, the chemical cost implies heat pretreatment alone is preferred. The decrease in energy requirement was obtained on HPH following the pH and osmotic pretreatment, although they were less efficient than other pretreatments.

Improvement in both the rate and extent of protein release on HPH following single and combined pretreatment was obtained. The maximum first order kinetic rate

constant (k) and extent of release (R_i/R_m) were obtained on HPH following heat pretreatment at 50°C. The k and (R_i/R_m) for combined pretreatments were higher single pretreatments at same temperature, pH and osmotic pressure, however, the advantage compared to single heat pretreatment at 40°C was small. The degree of micronisation was reduced following a combined pretreatment. This was confirmed by Malvern size analysis and microscopy. This further confirmed that micronisation is an effect of cell disruption and not a requirement to achieve adequate disruption.

Chapter 8
Conclusions and Recommendations

8.1 CONCLUSIONS

The effect of pretreatment on energy efficiency of microbial cell disruption and selective product release was investigated in this study. Two model systems were used: *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The pretreatments were chosen, using knowledge from literature, for their ability to weaken the yeast cell wall rather than to permeabilise the cell. Subsequent to pretreatment, cell disruption was carried out by ultrasonication or high pressure homogenisation. The protein and enzyme analyses were used to measure cell disruption and selective product release. The degree of micronisation of cell debris on high pressure homogenisation following a combined pretreatment was analysed by the Malvern size analyzer and light microscopy.

The key findings of the effect of single pretreatments on protein and enzyme release from Baker's yeast were studied on a small scale using ultrasound. These are detailed below.

Heat pretreatment:
No significant difference in total soluble protein release following ultrasonication was obtained when comparing heat pretreatment in the heat exchanger at a maximum heating rate with heat pretreatment by dilution for the same maximum temperature. The total soluble protein release increased with increasing temperature of pretreatment from 40 to 50°C, and decreased as the temperature exceeded 50°C. The latter is assumed to be due to protein denaturation. The optimal pretreatment temperatures for invertase, α-glucosidase, ADH and G6PDH release on ultrasonication were 40 to 50°C, 40 to 50°C, 40 to 50°C, 45°C and 40 to 52°C, respectively. In all cases, the fastest heating rate of 3.5°C/s of 40°C heat pretreatment was preferred. Longer sonication times and longer holding time were required to maximize release of intracellular enzymes. The maximum kinetic rate

In summary, efficient cell disruption and selective product release could be achieved on mechanical disruption following pretreatment. Pretreatment could be used to improve protein and enzyme release from different microorganisms. The micronisation induced by mechanical disruption was minimised by pretreatment. Heat pretreatment was the most efficient compared to other single pretreatments. Combined pretreatment did not have advantage over single heat pretreatment at same maximum temperature; however the rate of protein release was enhanced.

8.2 RECOMMENDATIONS

Following on from the study reported, the following recommendations are put forward for further study:

While disruption at the same operating pressure following pretreatment was more energy efficient than in the absence of pretreatment, the most energy efficient disruption occurred at the highest operating pressure of HPH. It is proposed that by combining heat pretreatment with high pressure homogenisation at this most extreme operating pressure, further improvement of energy efficiency may result and complete disruption on a single pass at 69.0 MPa may be achieved.

Further understanding of the effect of pretreatment on the cell wall structure may have potential for optimization of pretreatment and facilitate selection of operating conditions for disruption of other microorganisms.

It is noticed that the extent of soluble protein release is increased by the pretreatments. Further study would enable characterisation of the proteins released to ascertain whether this is caused by more effective release of soluble proteins or by the release of proteins from the wall and membrane fractions. Should the latter occur, this may enable improved recovery of such proteins.

References

1. Anand H. (2004). The effect of pretreatment used in combination with mechanical methods on the extent of cell disruption and subsequent release of intracellular protein. Msc Thesis. University of Cape Town.
2. Anand H., Balasundaram B., Pandit A.B., Harrison S.T.L. (2006). The effect of chemical pretreatment combined with mechanical disruption on the extent of disruption and release of intracellular protein from *E. coli*. *Biochemical Engineering Journal*, **35**, 166-173.
3. Andrews B.A. and Asenjo J.A. (1987). Enzymatic lysis and disruption of microbial cell. *Tibtech*, **5**, 273-277.
4. Bailey J.E. and Ollis D.F. (1986) Biochemical Engineering Fundamentals. McGraw Hill Pub. Co. New York, USA, 208-218.
5. Bailey S.M., Blum P.H. and Meagher M.M. (1995). Improved homogenization of recombinant *Eschrichia coli* following pre-treatment with guanidine hydrochloride. *Biotechnol. Prog.*, **11**, 533-539.
6. Balasundaram, B. and Harrison, S. T. L. (2008). Influence of method and extent of disruption of Bakers' yeast on protein adsorption in expanded beds. *Journal of Biotechnology*, **133**, 360-369.
7. Balasundaram B. and Pandit A.B. (2001). Selective release of invertase by hydrodynamic cavitation. *Biochemical Engineering Journal*, **8** (3), 251-256.
8. Balasundaram B. (2004). A detailed investigation of microbial cell disruption by hydrodynamic cavitation for selective product release. PhD thesis. University of Cape Town.
9. Balasundaram B. and Harrison S.T.L. (2006). Disruption of Baker's yeast by hydrodynamic cavitation: process variables and their influence on selective release. *Biotechnology and Bioengineering*, **94** (2), 303-311.
10. Baldwin C. and Robinson C.W. (1990). Disruption of *Saccharomyces cerevisiae* using enzymatic pretreatment and high-pressure homogenization. *Biotechnology Techniques*, **4**, 329-334.
11. Baldwin C.V. and Robinson C.W. (1993). Enhanced disruption of *Candida utilis* using enzymatic pretreatment and high–pressure homogenization. *Biotechnology and Bioengineering*, **43**, 46-56.

12. Beck D., Kreuter T., Sonnenkalb W. and Pauli G. (1978). Verfahren zur Gewinnung von Rotein aus Mikroorganismen. DDR Patentschrift 129270.
13. Becker T., Ogez J.R. and Builder S.E. (1983). Downstream processing of proteins. *Biotechnol. Advs.*, **1**, 247-261.
14. Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-265.
15. Brookman J.S.G. (1974). Mechanism of cell disintegration in a high pressure homogenizer. *Biotechnol. Bioeng.*, **16**, 371-383.
16. Brujan E.A. (2004). The role of cavitation micro-jets in the therapeutic applications of ultrasound. *Ultrasound in Med. & Biol.*, **3**, 381-387.
17. Buckland B.C., Lilly M.D. and Dunnill P. (1976). The kinetics of cholesterol oxidase synthesis by *Nocardia rhodocrous*. *Biotechnology and Bioengineering*, **18**, 601-621.
18. Chisti Y., and Moo-Young M. (1986). Disruption of microbial cells for intracellular products. *Enzyme Microbiology and Technology*, **8**, 194-204.
19. Chow C.-K. and Palecek S.P. (2004). Enzyme encapsulation in permeabilized *Saccharomyces cerevisiae* cells. *Biotechnology Progress*, **20**, 449-456.
20. Collis M.A., O'Neill B.K. and Middelberg A.P.J. (1995) Thermal deactivation affects disruption *of Escherichia coli. Biotechnol. Tech.*, **9**, 91-94.
21. Currie J.A., Dunnill P and Lilly M.D. (1972). Release of protein from Baker's yeast (Saccharomyces cerevisiae) by disruption in an industrial agitator mill. *Biotechnology and Bioenginnering*, **14**, 725-736.
22. de Maranon I.M. Marechal P.-A. and Gervais P. (1996). Passive response of *Saccharomyces cerevisiae* on osmotic shifts: cell volume variations depending on the physiological state. *Biochemical and Biophysical Research Communications*, **227**, 519-523.
23. de Smet M.J., Kingma J. and Witholt B. (1978). The effect of toluene on the *Escherichia coli. Biochimica et Biophysica Acta*, **506**, 64-80.
24. Doulah M.S. (1977). The formation of bubbles. *J. Appl. Sci.*, **15**, 445-451.
25. Doulah M.S. and Hammond T.H. (1975). A hydrodynamic mechanism for the disintegration of *Saccharomyces cerevisiae* in an industrial homogenizer. *Biotechnol. Bioeng.*, **17**, 845-858.
26. Engler C.R. (1985). Disruption of microbial cells. *Comprehensive Biotechnology*, **2**, Ed. M. Moo-Young, Ch. 20, 305-324. Pergamon Press, Oxford.
27. Engler C.R. and Robinson C.W. (1981). Disruption of *Candida utilis* cells in high pressure flow devices. *Biotechnol. Bioeng.*, **23**, 765-780.

28. Farkade V.D., Harrison S.T.L. and Pandit A.B. (2006). Improve cavitational cell disruption following pH pretreatment for the extraction of β-galactosidase from *Kluveromyces lactis*. *Biochemical Engineering Journal*, **31**, 25-30.
29. Felix H. (1982). Permeabilized cells. *Analyt. Biochem.*, **120**, 211-234.
30. Fenton D.M. (1982). Solvent treatment for β-D-galactosidase release from yeast cells. *Enzyme and Microbial Technology*, **4**, 229-232.
31. Flores M.V., Voget C.E. and Ertola R.J.J. (1994). Permeabilisation of yeast cells (*Kluyveromyces lactis*) with organic solvents. *Enzyme and Microbial Technology*, **16**, 340-346.
32. Follows M., Hetherington P.J., Dunnill P. and Lilly M.D. (1971). Release of enzymes from Baker's yeast by disruption in an industrial homogeniser. *Biotechnol Bioeng.*, **8**, 549-560.
33. Fonseca L.P. and Cabral J.M.S. (2002). Penicillin acylase release from Escherichia coli cells by mechanical cell disruption and permeabilization. *J. Chem. Tech. Biotechnol.*, **77**, 159-167.
34. Frances C. (2004). On modelling of submicronic wet milling processes in bead mills. *Powder Technology*, **143–144**, 253– 263.
35. Fraser D. (1951). Bursting bacteria by release of gas pressure. *Nature*, **167**, 33-34.
36. Garrido F., Banerjee U.C., Chisti Y. and Moo-Young M. (1994), Disruption of a recombinant yeast for the release of β-galactosidase. *Bioseparation*, **4**, 319-328.
37. Gascon, S. and Lampen, J.O. (1968). Purification of the internal invertase of yeast. *J. Biol. Chem.*, **243**, 1567-1572.
38. Geciova J., Bury D. and Jelen P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry. *International Dairy J.*, **12**, 541-553.
39. Gervais P. and de Maranon I. M. (1995). Effect of the kinetics of temperature variation on *Saccharomyces cerevisiae* viability and permeability. *Biochimica et Biophysica Acta*, **1235**, 52-56.
40. Gervais P., Marechal P. A., and Molin P. (1992). Effects of the kinetics of osmotic pressure variation on yeast viability. *Biotechnol. Bioeng.* **40**, 1435–1439.
41. Giovenco S. and Verheggen F. (1987). Purification of intracellular enzymes from whole bacterial cells using reversed miscelles. *Enzyme and Microbial Technology*, **9**, 470-473.
42. Gogate P.R. and Pandit A.B. (2000). Engineering design methods for cavitation reactors II: Hydrodynamic cavitation. *AIChE Journal*, **46**, 1641-1649.

43. Golovina I.G., Saveleva N.D., Loginova L.G. and Zavarzin G.A. (1973). Lysis of *Hydrogenomonas eutropha* Z-1 cells by the enzymes of *Micromonospora vulgaris* PA II-4. *Mikrobiologiya*, **42** (5), 899-903.
44. Gray P.P, Dunnill P. and Lilly M. (1972). The continuous-flow isolation of enzymes. *Fermentation Technology Today*, 347-351.
45. Grogate R.R., and Pandit A.B. (2001). Hydrodynamic cavitation reactors: A state of the art review. *Rev. in Chem. Eng.*, **17**, 1-85.
46. Hahn S.K., Chang Y.K., Kim B.S., Lee K.M. and Chang H.N. (1993). The recovery of poly (3-hydroxybutyrate) by using dispersions of sodium hypochlorite solution and chloroform. *Biotechnol. Tech.*, **7**, 209-212.
47. Harrison S.T.L. (1990). The extraction and purification of PHB from *Alcaligenes eutrophus*. PhD dissertation, University of Cambridge.
48. Harrison S.T.L. (1991). Bacteria cell disruption: A key unit operation in the recovery of intracellular products. *Biotechnology Advances*, **9**, 217-240.
49. Harrison S.T.L., Chase H.A. and Dennis J.S. (1991a). Combined chemical and mechanical processes for the disruption of bacteria. *Bioseparation*, **2**, 95-105
50. Harrison S.T.L., Chase H.A. and Dennis J.S. (1991b). The disruption of *Alcaligenes eutrophus* by high pressure homogenisation: key factors involved in the process. *Bioseparation*, **2**, 155-166.
51. Harrison S.T.L., Chase, H.A. and Dennis J.S. (1991c). The lysis of Gram negative *Alcaligenes eutrophus* by enzymes from *Cytophaga*. *Biotechnol. Techn.*, **5**, 115-120.
52. Harrison S.T.L. and Pandit A.B. (1992). The disruption of microbial cells by hydrodynamic cavitation. *9th International Biotechnology Symposium*, Washington D.C. August 17-21.
53. Hedenskog G., Morgren H. and Enebo L. (1970). A method for obtaining protein concentrates from microorganisms. *Biotechnol. Bioeng.*, **12**, 947-959.
54. Helenius A. and Simons K. (1975). Solubilization of membranes by detergents. *Biochimica et Biophys. Acta.*, **415**, 29-79.
55. Hetherington P.J., Follows M. Dunnill P. and Lilly M.D. (1971). Release of protein from Bakers yeast (*Saccharomyces cerevisiae*) by disruption in an industrial homogenizer. *Inst. Pasteur Res. Microbiol.*, **141**, 75-103.
56. Hettwer D. And Wang H. (1989). Protein release from *Escherichia coli* cells permeabilised with guanidine-HCl and Triton X-100. *Biotechnol. Bioeng.*, **33**, 886-895.
57. Holmes P.A. and Lim G.B. (1985). Separation processes. European Patent Application, 145, 223.

58. Hopkins T.R. (1991). Physical and chemical cell disruption for the recovery of intracellular proteins. *Purification and Analysis of Recombinant Proteins.* (editors Seetharam, R. and Sharma, S.K.,), Chap. 3, Marcel Dekker (New York), 57-83.

59. Huang R.B., Andrews B.A. and Asenjo J.A. (1991), Differential product release (DPR) of proteins from yeast: a new technique for selective product recovery from microbial cells. *Biotechnol. Bioeng.*, **38**, 977-985.

60. Hunter J.B. and Asenjo J.A. (1988). A structured mechanistic model of the kinetics of enzymatic lysis and disruption of yeast cells. *Biotechnol. Bioeng.*, **31**, 929-943.

61. Ingram L.O. (1981). Mechanism of lysis of *Escherichia coli* by ethanol and other chaotrophic agents. *Journal of Bacteriology*, **146**, 331-336.

62. James C.J., Coakley W.T. and Hughes D.E. (1972). Kinetics of protein release from yeast sonicated in batch and flow systems at 20 kHz. *Biotechnol. Bioeng.*, **14**, 33-42.

63. Johnstone-Robertson, M, Clarke, K.G. and Harrison S.T.L. (2008) Comparison of the distribution of glucose oxidase in Penicillium sp. CBS 120262 and *Aspergillus niger* NRRL-3 cultures. *Biotechnology and Bioengineering*, **99** (4) 910 - 918.

64. Jyoti K.K. and Pandit A.B. (2000). Water disinfection by acoustic and hydrodynamic cavitation *Biochemical Engineering Journal*, **7**, 201-202.

65. Kalumuck K.M. and Chahine G.L. (2000). The use of cavitating jets to oxidize organic compounds in water. *ASME J. Fluids Engr.*, **22**, 465-470.

66. Kalumuck K.M., Chahine G.L., Hsiao C.-T. and Choi J.-K. (2003). Remediation and disinfection of water using jet gernerated cavitation. *Fifth International Symposium on Cavitation (CAV, 2003)*, Osaka Japan, November 1-4.

67. Katsui N., Tsuchido T., Takano M. and Shibasaki I. (1981). Effect of preincubation on the heat resistance of *Escherichia coli* having different fatty acid composistions. *J. Gen. Microbiol.*, **122** preincubation, 357-361.

68. Keleman M.V. and Sharpe J.E.E. (1979). Controlled cell disruption: a comparison of the forces required to disrupt different micro-organisms. *J. Cell Sci.*, **35**,431-441.

69. Keshavarz-Moore E., Hoare M. and Dunnill P. (1990a). Disruption of bakers yeast in a high-pressure homogenizer: New evidence on mechanism. Enzyme Microb. Technol., **12**, 764-770.

70. Keshavarz-Moore E., Bonnerjea J., Hoare M. and Dunnill P. (1990b). Disruption of fungal organism, Rhizopus nigricans in a high-pressure homogenizer. *Enzyme Microb. Technol.*, **12**, 494-498.

71. Kohlrausch U. and Holtje J.-V. (1991). Analysis of murein and murein precursors during antibiotic-induced lysis of *Escherichia coli. J. Bacteriol.*, **173**, 3425-3431.

72. Kuboi R., Umakoshi H., Takagi N., and Komasawa I. (1995). Optimal disruption methods for the selective recovery of β-galactosidase from E. coli. *Journal of Fermentation and Bioengineering*, **79**(4), 335–341.

73. Kula M.-R. and Schutte H. (1987). Purification of proteins and the disruption of microbial cells. *Biotechnol. Prog.*, **3**, 31-42.

74. Kumar P.S. Kumar, M.S. and Pandit A.B. (2000). Experimental quantification of chemical effects of hydrodynamic caviation. *Chemical Engineering Science*, **55**, 1633-1639.

75. Limon-Lason J., Hoare M., Orsborn C.B., Doyle J. and Dunnill P. (1979). Reactor properties of high-speed bead bill for microbial cell rupture. *Biotechnol. Bioeng.*, **21**, 745-774.

76. Loo C.C, Slatter W.L. and Powell R.W. (1950). A study of the cavitation effect in the homogenization of dairy products. *Journal of Diary Science*, **33**, 692-702.

77. Melendres A.V., Honda H., Shiragami N. and Unno H. (1993). A concept of critical velocity for cell disruption by bead mill. *J. Chemical Eng. Japan*, **25**, 354-356.

78. Middelberg A.P.J. (1995). Process-Scale disruption of microorganisms. *Biotechnology Advances*, **13** (3), 491-551.

79. Mille Y., Beney L. and Gervais P. (2002). Viability of Escherichia coli after combined osmotic and thermal treatment: a plasma membrane implication. *Biochimica et Biophysica Acta*, **1567**, 41– 48.

80. Mogren H., Lindblom M. and Hedenskog G. (1974). Mechanical disintegration of microorganisms in an industrial homogenizer. *Biotechnol. Bioeng.*, **16**, 261-274.

81. Moor H. and Mühlethaler K. (1963). Fine structure in frozen-etched yeast cell. *Journal of Cell Biology*, **17**, 609-628.

82. Murakami K., Nagura H. and Yoshino M. (1980). Permeabilization of yeast cells: Application to study on the regulation of AMP deaminase activity *in situ. Gene*, **105**, 407-413.

83. Neppiras E.A. and Hughes D.E. (1964). Some experiments on the disintegration of yeast by high intensity ultrasound. *Biotechnol. Bioeng.* **6**, 247–270.

84. Neu H.C. and Heppe L.A. (1964). On the surface localization of enzymes in *E. coli*. *Biochem. Biophys. Res. Comm.*, **17**, 215-219.

85. Norrish R.S. (1966). An equation for the activity coefficients and equilibrium relative humidities of water in confectionary syrups. *J. Food Technol.* **1**, 25-39.

86. Oliveira, D.E., Santos, Neta A. L. C. and Panek, A. D. (1981). Permeabilization of yeast for *in situ* determination of α-glucosidase. *Anal. Biochem.*, **113**, 188-192.

87. Pearce S.A.J. (1993). The disruption of microorganisms due to agitation in slurries of fine particles. MSc Thesis. University of Cape Town.

88. Phaff H.J. (1971). Structure and biosythesis of the yeast cell envelop. In *The Yeasts*. Ed. A.H. Rose and J.S. Harrison. Vol. 2, Chap. 5, 135-210. Academic New York.

89. Racker, E. (1955). Alcohol dehydrogenase from bakers yeast. Chapter 79, In: *Methods in Enzymeology*, Colowick, S.P. and Kaplan, N.O., (ed.) Vol. I, 500-503.

90. Record M.T., Courtenay D.S., Cayley D.S. and Guttman H.J. (1998). Response of *Escherichia coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem. Sci.*, **23**, 143–148.

91. Sauer T., Robinson C.W. and Glick B.R. (1989). Disruption of native and recombinant *Escherichia coli* in a high-pressure homogenizer. *Biotechnol. Bioeng.*, **33**, 1330-1342.

92. Saver S.S., Pandit A.B. and Joshi J.B. (1994). Microbial cell disruption : role of cavitation. *The Chemical Engineering Journal*, **55**, 67-72.

93. Save S.S., Pandit A.B. and Joshi J.B. (1997). Use of hydrodynamic cavitation for large-scale microbial cell disruption. *Trans. Inst. Chem. Eng. 71C*, **75**, 41-48.

94. Schutte H., Kroner K.H., Hustedt H., and Kula M.-R. (1983). Experience with a 20 litre industrial bead mill for the disruption of microorganism. *Enzyme and Microbial Technology*, **5**, 143–148.

95. Schutte H. and Kula M.-R. (1987). Purification of proteins and the disruption of microbial cells. *Biotechnology Progress*, **3**, 31-42.

96. Schutte H., Kroner, K.H., Hustedt H. and Kula M.-R. (1983). Experiences with a 20 litre industrial bead mill for the disruption of microorganisms. *Enzyme and Microbial Technology*, **5**, 143-148.

97. Schutte H. and Kula M.-R. (1988). Analytical disruption of microorganisms in a mixer mill. *Enzyme Microb. Technol.*, **10**,552-558.

98. Schutte H. and Kula M.-R. (1990). Pilot and process-scale techniques for cell disruption. *Biotechnology and Applied Biochemistry*, **12**, 599-620.

99. Stanbury P.F. and Whitaker A. (1984). *Principles in Fermentation Technology*, 206-208. Pergamon Press.

100. Sundaram J., Mallein B.R. and Mitragotri S. (2003). An experimental and theoretical analysis of ultrasound-induced permeabilization of cell membrane. *Biophys. J.*, **84**, 3087-3101.

101. Sutherland I.W. (1975). The bacterial wall and surface. *Process Biochemistry*, **10** (3), 4-8.

102. Tuite M.F. and Oliver S.G. (1991). Biotechnology Handbook, New York, Ed. Plenum Press, **4**, 1-3.

103. Walker G.M. (1998). Yeast Physiology and Biotechnology: Chapter 2, John Wiley and Sons, New York.

104. Watson J.S., Cumming R.H., Street G. and Tuffnell J.M. (1987). Release of intracellular protein by thermolysis. *Separations for Biotechnology*, (editors Neijssel O.M, van der Meer R.R. and Luyben K.), Vol. 2, 526-529. Elsevier (Amsterdam).

105. Woldringh C.L. and van Iterson W. (1972) Effects of treatment with sodium dodecyl sulphate on the ultrastructure of *Escherichia coli*. *Journal of Bacteriology*, **11**, 801-813.

106. Young F.R. (1989). Cavitation, McGraw Hill Book Co. New York.

Appendix A
Analytical methods

A.1 Total soluble protein - Bradford's method

Bradford reagent:

Dissolve 100 mg of Coomassie Brilliant Blue G-250 in 50ml absolute ethanol. Add 100 ml of phosphoric acid to this solution. Dilute the resulting solution to a final volume of 1 l with distilled water.

Procedure:

1. Pipette out 0.1 ml of sample (diluted) into a plastic cuvette (2 ml)
2. Add 1 ml of Bradford reagent
3. Incubate for 2-5 min at room temperature (do not stand for longer than 1 hr)
4. Read the absorbance at 595 nm against a blank (phosphate buffer pH 7) treated similar to the sample

Table A.1 Reproducibility of Bradford method for protein analysis

Sample 1 (mg/g)	Sample 2 (mg/g)	Sample 3 (mg/g)	Average (mg/g)	Standard deviation	Coefficient of variance (%)
340	369	330	346	16.60	4.80

Calibration curve for Bradford method for protein analysis

1. Prepare standard solutions of Bovine Serum Albumin (BSA) in the concentration range from 0 mg/ml to 0.1 mg/ml.
2. Pipette out 0.1 ml of standard BSA solution into a plastic cuvette (2ml)
3. Add 1 ml of Bradford reagent and wait for 2-5 min
4. Read the absorbance at 595 nm against a blank

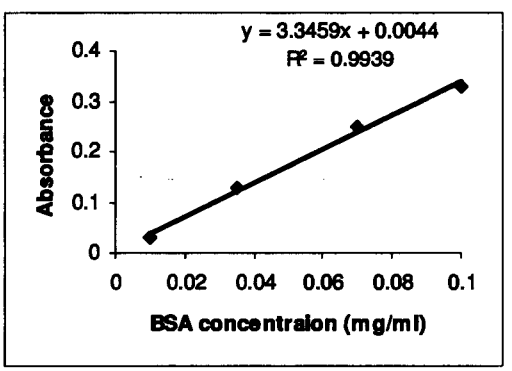


Figure A.1 Calibration curve for Bradford method of protein release

A.2 Invertase

DNS reagent:

Dissolve 150 g of sodium potassium tartrate in 250 ml distilled water. Dissolve 5 g of 3,5 di-nitrosalicylic acid in 100 ml of 2 M NaOH. Mix the above two solutions and dilute to a final volume of 500 ml with distilled water.

Other 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 distilled water

Procedure:

1. Pipette out 1 ml of sample (diluted) into a clean test tube
2. Add 1 ml of 0.1 M acetate buffer of pH 5.5 (0.1 M)
3. Add 0.5ml of 0.5 M sucrose
4. Incubate at 55°C for 10 min in a water bath
5. 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 min
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 min
9. Add 10ml of distilled water to the above reaction mixture
10. Read the absorbance at 540 nm against a blank treated similar to the sample

Table A.2 Reproducibility of Invertase analysis

Sample 1 (U/g)	Sample 2 (U/g)	Sample 3 (U/g)	Average (U/g)	Standard deviation	Coefficient of variance (%)
3.90×10^4	3.73×10^4	4.20×10^4	3.94×10^4	0.24	6.08

Calibration curve for glucose estimation by DNS

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

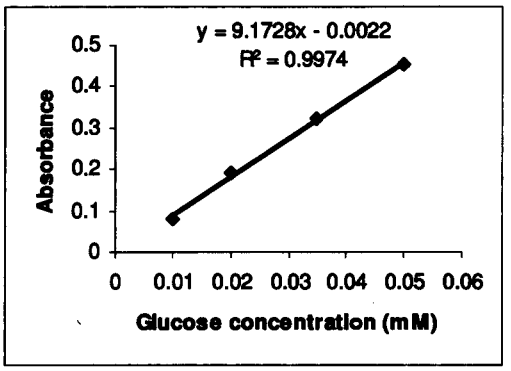


Figure A.2 Calibration curve for glucose analysis

A.3 α-glucosidase

Reagents:

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

0.05 M sodium phosphate buffer (pH 6.8)

0.1 M sodium carbonate (Na_2CO_3) solution in distilled water

Procedure:

1. Pipette out 100 µl of sample (diluted) into a clean test tube
2. Add 2 ml solution of 5 mM *p*-nitrophenol-α-D-glucoside dissolved in phosphate buffer of pH 6.8 (0.05 M)
3. Incubate at 30°C for 10 min 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. Read the absorbance at 410 nm against a blank treated similar to the sample

Table A.3 Reproducibility of α-glucosidase analysis

Sample 1 (U/g)	Sample 2 (U/g)	Sample 3 (U/g)	Average (U/g)	Standard deviation	Coefficient of variance (%)
2.59×10^5	2.67×10^5	2.34×10^5	2.53×10^5	0.17	6.72

Calibration curve for *p*-nitrophenol

1. Prepare standard solution of *p*-nitrophenol (1-5 M) dissolved in 0.1 M Na_2CO_3
2. Read the absorbance at 410 nm against a blank

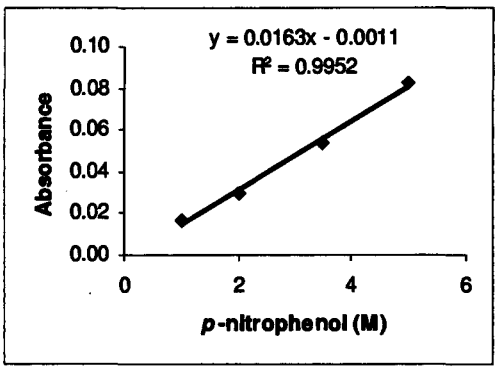


Figure A.3 Calibration curve for *p*-nitrophenol

A.4 Alcohol dehydrogenase (ADH)

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

Procedure:

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

Table A.4 Reproducibility of ADH analysis

Sample 1 (U/g)	Sample 2 (U/g)	Sample 3 (U/g)	Average (U/g)	Standard deviation	Coefficient of variance (%)
6.08×10^5	5.30×10^5	5.22×10^5	5.53×10^5	0.48	8.53

A.5 Glucose-6-phosphate dehydrogenase (G6PDH)

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 (NADP) in distilled water

0.1 Magnesium chloride in distilled water

Procedure:

1. Pipette out 1ml of Tris-HCl buffer (0.249 mM, pH 7.6) into a quartz cuvette (4ml)
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 sample (diluted)
4. Place the cuvette immediately in the spectrophotmeter and record the absorbance at intervals of 15 seconds for 2 minutes at 340nm against a blank treated similar to the sample

Table A.5 Reproducibility of G6PDH analysis

Sample 1 (U/g)	Sample 2 (U/g)	Sample 3 (U/g)	Average (U/g)	Standard deviation	Coefficient of variance (%)
14.30	14.01	15.51	14.61	0.80	5.54

A.6 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 $MnCl_2$

12 mM *o*-nitrophenyl-β-D-galactoside (ONPG) dissolved in PPB-Mn buffer

1 M Na_2CO_3 dissolved in distilled water

Procedure:

1. Pipette 50 ml of sample (diluted) a clean test tube
2. Add 2 ml of ONPG dissolved in PPB-Mn buffer
3. Incubate at 37°C for 5 minutes in a water bath
4. Add 0.5 ml of 1M Na_2CO_3 to terminate the reaction
5. Read the absorbance at 420 nm against a blank treated similar to the sample

Table A.6 Reproducibility of β-galactosidase analysis

Sample 1 (U/g)	Sample 2 (U/g)	Sample 3 (U/g)	Average (U/g)	Standard deviation	Coefficient of variance (%)
$1.49 X10^3$	$1.38 X10^3$	$1.51 X10^3$	$1.46 X10^3$	70.94	4.84

Appendix B
Cell disruption of Baker's yeast by different mechanical methods

Raw data for homogenisation of Baker's yeast without pretreatment

The homogenisation of Baker's yeast (1.5%, dry weight, w/v) was performed at five pressure: 13.8, 27.6, 41.4, 55.2 and 69.0 MPa. The tables below present the data obtained for the release of total soluble protein and enzyme release.

Table B.1 Data for total soluble protein release on homogenisation of untreated yeast

Protein	13.8 MPa	Protein (mg/g) release at 27.6 MPa						Protein (mg/g) release at 41.4 MPa				
Passes	mg/g	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.71	2.71	2.93	2.63	2.76	0.16	5.67	2.71	2.86	2.78	0.11	3.91
1	39.4	82.7	76.0	64.7	74.5	9.09	12.2	141	151	146	7.42	5.08
2	63.4	147	138	142	142	4.59	3.23	242	250	246	5.40	2.20
3	85.8	212	215	208	211	3.26	1.54	298	302	300	2.19	0.73
4	129	254	247	258	253	5.22	2.06	360	354	357	4.30	1.20
5	152	279	271	274	275	3.93	1.43	398	403	401	3.57	0.89
6	194	305	314	308	309	4.94	1.60	411	408	409	1.72	0.42
7	222	335	331	338	335	3.15	0.94	424	418	421	3.77	0.89
8	254	366	372	360	366	6.25	1.71	438	441	440	2.07	0.47
9	273	381	391	366	379	12.5	3.29	441	444	442	2.04	0.46
10	288	387	394	381	388	6.47	1.67	449	448	448	1.12	0.25

Protein	55.2 MPa	Protein (mg/g) release at 69.0 MPa				
Passes	mg/g	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.71	2.71	3.06	2.88	0.25	8.59
1	164	183	208	195	17.3	8.84
2	287	305	349	327	30.8	9.41
3	387	438	417	428	15.07	3.52
4	441	465	476	470	7.66	1.63
5	458	460	478	469	12.8	2.73

Table B.2a Data for invertase release on homogenisation of untreated yeast

Invertase	13.8 MPa	Invertase (U/g x 10^4) release at 27.6 MPa						41.4 MPa	55.2 MPa	69.0 MPa
Passes	U/g x 10^4	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	U/g x 10^4	U/g x 10^4	U/g x 10^4
0	0.07	0.07	0.08	0.13	0.09	0.03	33.5	0.07	0.07	0.07
1	0.66	1.29	1.38	1.41	1.36	0.06	4.73	1.67	2.36	2.72
2	0.93	1.87	1.93	2.17	1.99	0.16	8.09	2.51	3.53	3.83
3	1.62	2.56	2.63	2.68	2.62	0.06	2.21	3.29	4.24	4.62
4	1.94	2.94	2.98	3.05	2.99	0.05	1.82	3.59	4.38	4.59
5	2.32	3.30	3.33	3.42	3.35	0.06	1.80	4.10	4.53	4.62
6	2.54	3.52	3.46	3.47	3.49	0.03	0.98	4.24		
7	2.87	3.66	3.68	3.73	3.69	0.03	0.93	4.29		
8	2.98	3.69	3.67	3.76	3.71	0.05	1.33	4.36		
9	3.05	3.80	3.84	3.85	3.83	0.03	0.68	4.41		
10	3.16	3.88	3.95	3.98	3.94	0.05	1.32	4.46		

Table B.2b Data for α-glucosidase release on homogenisation of untreated yeast

α-glucosidase	13.8 MPa	α-glucosidase release (U/g x 10^5) at 27.6 MPa						41.4 MPa	55.2 MPa	69.0 MPa
Passes	U/g x 10^5	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	U/g x 10^5	U/g x 10^5	U/g x 10^5
0	0.01	0.01	0.01	0.01	0.01	0.00	0.00	1.76	0.01	0.01
1	0.37	0.56	0.55	0.55	0.58	0.56	0.02	3.02	1.44	1.67
2	0.62	0.95	0.93	0.97	0.95	0.95	0.02	2.05	2.33	2.78
3	0.85	1.27	1.26	1.27	1.28	1.27	0.01	0.75	2.92	3.07
4	1.08	1.61	1.60	1.63	1.59	1.61	0.02	1.05	3.30	3.53
5	1.23	1.81	1.82	1.79	1.83	1.81	0.02	1.10	3.38	3.62
6	1.38	2.08	2.08	2.03	2.13	2.08	0.05	2.30		
7	1.54	2.32	2.32	2.30	2.33	2.32	0.02	0.69		
8	1.61	2.36	2.40	2.33	2.37	2.36	0.04	1.49		
9	1.67	2.49	2.48	2.48	2.51	2.49	0.02	0.67		
10	1.73	2.56	2.53	2.59	2.56	2.56	0.03	1.19		

Table B.2c Data for ADH release on homogenisation of untreated yeast

ADH	13.8 MPa	ADH (U/g x 10^5) release at 27.6 MPa						41.4 MPa	55.2 MPa	69.0 MPa
Passes	U/g x 10^5	exp. 1	exp. 2	exp. 3	ave.	Passes	U/g x 10^5	exp. 1	exp. 2	exp. 3
0	0	0	0	0	0	0	0	0	0	0
1	0.40	0.85	1.00	0.87	0.90	1	0.40	0.85	1.00	0.87
2	0.87	1.53	1.33	1.47	1.44	2	0.87	1.53	1.33	1.47
3	1.33	2.13	2.00	2.20	2.11	3	1.33	2.13	2.00	2.20
4	1.87	2.73	2.73	2.87	2.78	4	1.87	2.73	2.73	2.87
5	2.33	3.47	3.40	3.33	3.40	5	2.33	3.47	3.40	3.33
6	2.73	4.20	4.20	4.40	4.27	6	2.73	4.20		
7	3.13	4.80	5.13	5.27	5.07	7	3.13	4.80		
8	3.53	5.33	5.40	5.47	5.40	8	3.53	5.33		
9	3.87	5.80	5.87	6.00	5.89	9	3.87	5.80		
10	4.20	6.20	6.07	6.20	6.16	10	4.20	6.20		

Table B.2d Data for G6PDH release on homogenisation of untreated yeast

G6PDH	13.8 MPa	G6PDH (U/g) release at 27.6 MPa						41.4 MPa	55.2 MPa	69.0 MPa
Passes	U/g	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	U/g	U/g	U/g
0	0	0	0	0	0	0	0	0	0	0
1	2.03	3.04	2.85	2.90	2.93	0.10	3.43	5.85	9.03	9.86
2	3.24	4.83	5.12	4.98	4.98	0.14	2.91	9.71	15.4	16.9
3	4.01	6.91	6.96	6.76	6.88	0.10	1.46	12.7	18.9	20.5
4	4.78	8.74	9.23	9.08	9.02	0.25	2.75	15.3	22.1	23.6
5	5.46	10.10	9.95	9.66	9.90	0.22	2.24	16.9	22.9	24.0
6	6.09	11.6	12.6	12.1	12.1	0.48	4.00	18.9		
7	6.76	12.9	13.4	13.0	13.1	0.26	1.95	20.8		
8	7.44	14.0	14.5	14.1	14.2	0.28	2.00	21.6		
9	8.02	14.8	14.8	14.9	14.8	0.03	0.19	22.2		
10	8.60	15.5	15.4	15.6	15.5	0.07	0.48	22.6		

Raw data for ultrasonication of Baker's yeast without pretreatment

The ultrasonication of Baker's yeast (1.5%, dry weight, w/v) was performed at three different power inputs: 40, 60 and 80 W. The tables below present the data obtained for the release of total soluble protein and enzyme release.

Table B.3 Data for total soluble protein release on ultrasonication of untreated yeast

Protein	Protein release (mg/g) at 40 W					
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	2.73	2.93	2.63	2.77	0.15	5.52
5	135	111	118	121	12.1	9.98
10	229	153	196	193	38.1	19.8
15	265	235	265	255	17.3	6.77
20	294	291	294	293	1.90	0.65
25	304	302	304	304	1.43	0.47

Protein	Protein release (mg/g) at 60 W						Protein release (mg/g) at 80 W					
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	2.73	2.77	2.63	2.71	0.07	2.56	3.21	2.90	2.71	2.94	0.25	8.63
3	89.7	91.0	98.2	93.0	4.60	4.95	62.8	92.0	80.4	78.4	14.7	18.7
6	153	152	156	154	2.36	1.53	133	132	131	132	1.36	1.03
9	207	211	198	205	6.28	3.06	225	208	205	213	10.6	5.01
12	266	263	261	263	2.49	0.95	267	279	273	273	6.19	2.27
15	301	306	296	301	5.22	1.73	303	326	319	316	12.2	3.87
18	318	313	304	312	6.73	2.16	368	341	333	348	18.4	5.29
21	322	316	318	319	2.91	0.91	375	350	333	353	21.2	6.02

Table B.4a Data for invertase release on ultrasonication of untreated yeast

Invertase (U/g X 10^{-4}) release at 40 W							Invertase (U/g X 10^{-4}) release at 80 W						
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	0.05	0.06	0.06	0.06	0.00	5.45	0	0.07	0.07	0.07	0.07	0.00	4.86
5	1.17	1.14	0.98	1.10	0.09	7.83	3	0.66	0.60	0.56	0.60	0.04	6.85
10	1.82	1.73	1.70	1.75	0.05	2.91	6	1.33	1.24	1.22	1.26	0.05	3.79
15	2.56	2.32	2.49	2.46	0.10	4.10	9	1.76	1.74	1.73	1.74	0.01	0.72
20	2.69	2.59	2.56	2.61	0.06	2.13	12	2.47	2.39	2.42	2.43	0.03	1.36
25	2.74	2.69	2.72	2.72	0.02	0.76	15	2.87	2.82	2.75	2.81	0.05	1.75
							18	3.02	2.97	2.95	2.98	0.03	0.99
							21	3.05	3.04	3.09	3.06	0.02	0.71

Table B.4b Data for α-glucosidase release on ultrasonication of untreated yeast

α-glucosidase (U/g X 10^5) release at 40 W							α-glucosidase (U/g X 10^5) release at 80 W						
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	0.02	0.02	0.02	0.02	0.00	1.90	0	0.01	0.01	0.01	0.01	0.00	3.25
5	0.68	0.93	0.77	0.79	0.11	13.2	3	0.62	0.50	0.53	0.55	0.05	9.84
10	1.21	1.33	1.24	1.26	0.05	4.05	6	1.24	1.07	1.05	1.12	0.09	7.61
15	1.80	1.68	1.80	1.76	0.06	3.21	9	1.62	1.54	1.56	1.57	0.03	2.16
20	2.02	1.99	1.91	1.97	0.05	2.35	12	1.88	1.96	1.94	1.93	0.03	1.76
25	2.09	2.07	2.07	2.08	0.01	0.45	15	2.13	2.20	2.18	2.17	0.03	1.36
							18	2.23	2.39	2.36	2.33	0.07	2.98
							21	2.24	2.41	2.43	2.36	0.09	3.61

Table B.4c Data for ADH release on ultrasonication of untreated yeast

ADH (U/g X 10^5) release at 40 W							ADH (U/g X 10^5) release at 80 W						
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.93	1.27	1.20	1.13	0.15	12.8	3	0.80	0.87	0.70	0.79	0.07	8.70
10	2.40	2.27	2.40	2.36	0.06	2.60	6	1.87	1.80	1.82	1.83	0.03	1.61
15	3.27	3.47	3.65	3.46	0.16	4.48	9	2.67	2.73	2.40	2.60	0.14	5.52
20	4.47	4.52	4.79	4.59	0.14	3.06	12	3.70	3.67	3.47	3.61	0.10	2.83
25	5.20	5.13	5.23	5.19	0.04	0.81	15	4.53	4.67	4.93	4.71	0.17	3.52
							18	5.47	5.60	5.60	5.56	0.06	1.10
							21	6.23	6.13	6.37	6.24	0.10	1.58

Table B.4d Data for G6PDH release on ultrasonication of untreated yeast

G6PDH (U/g) release at 40 W							G6PDH (U/g) release at 80 W						
Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.	Time(min)	exp. 1	exp. 2	exp. 3	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	4.06	4.60	4.75	4.47	0.36	8.14	3	3.35	3.29	3.72	3.45	0.23	6.75
10	7.34	6.57	7.17	7.03	0.41	5.78	6	6.14	6.18	5.22	5.85	0.54	9.32
15	8.99	10.7	10.6	10.1	0.94	9.38	9	9.34	9.23	8.49	9.02	0.46	5.10
20	10.7	11.6	12.2	11.5	0.74	6.41	12	11.4	11.5	11.4	11.4	0.02	0.22
25	11.9	12.5	12.9	12.5	0.53	4.27	15	13.5	13.6	13.0	13.3	0.31	2.31
							18	14.6	14.7	13.8	14.4	0.51	3.52
							21	15.2	15.5	14.4	15.0	0.56	3.74

Appendix C
Effect of Pretreatment on Ultrasonication

Raw data for homogenisation of Baker's yeast following heat pretreatment

The total soluble protein and enzyme release from Baker's yeast (1.5%, dry weight, w/v) on ultrasonication at 40 W following heat pretreatment. The tables below present the data obtained for the release of total soluble protein and enzyme release and kinetic rate constant k.

Table C.1 Data for protein release on ultrasonication following heat pretreatment at different temperature using dilution

Protein	Maximum T: 40℃; (mg/g)					Maximum T: 50℃; (mg/g)					Maximum T: 60℃; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.47	2.30	2.38	0.12	4.94	2.60	2.73	2.67	0.09	3.54	0.97	1.10	1.03	0.09	9.12
5	119	130	125	8.01	6.42	143	158	150	10.3	6.84	22.9	24.2	23.6	0.94	4.00
10	240	245	243	3.94	1.62	233	250	241	11.7	4.84	52.3	48.6	50.5	2.62	5.19
15	354	358	356	2.40	0.68	361	364	362	2.62	0.72	68.1	71.5	69.8	2.38	3.41
20	363	364	364	1.23	0.34	384	383	383	0.66	0.17	69.3	77.5	73.4	5.77	7.87
25	370	372	371	1.56	0.42	399	393	396	4.01	1.01	91.6	93.7	92.7	1.48	1.60

Table C.2 Data for protein release on ultrasonication following heat pretreatment at different temperature using heat exchanger

Protein	Maximum T: 40℃; (mg/g)					Maximum T: 45℃; (mg/g)					Maximum T: 50℃; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.67	2.88	2.77	0.11	3.90	2.27	2.43	2.35	0.08	3.55	2.30	2.49	2.39	2.30	3.88
5	137	107	122	15.0	12.3	137	125	131	6.10	4.64	148	160	154	5.86	3.81
10	222	212	217	4.99	2.30	226	221	223	2.33	1.04	252	261	256	4.77	1.86
15	342	316	329	13.3	4.05	350	345	347	2.65	0.76	355	332	344	11.67	3.40
20	360	353	356	3.18	0.89	361	366	364	2.43	0.67	380	376	378	1.99	0.53
25	366	358	362	4.06	1.12	377	382	379	2.50	0.66	386	392	389	3.05	0.78
Protein	Maximum T: 52℃; (mg/g)					Maximum T: 55℃; (mg/g)					Maximum T: 60℃; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	1.67	1.73	1.70	0.03	1.96	0.90	0.70	0.80	0.10	12.5	0.73	0.83	0.78	0.05	6.38
5	131	122	126	4.47	3.54	30.8	26.6	28.7	2.10	7.32	27.6	29.3	28.5	0.85	2.99
10	233	223	228	4.67	2.05	51.1	60.8	56.0	4.83	8.64	41.7	49.6	45.6	3.93	8.62
15	300	312	306	5.75	1.88	70.7	76.2	73.5	2.75	3.74	59.9	67.7	63.8	3.90	6.11
20	304	324	314	9.92	3.16	105	116	110	5.30	4.80	71.1	77.3	74.2	3.08	4.16
25	319	338	328	9.28	2.83	102	121	112	9.30	8.34	84.0	91.6	87.8	3.78	4.31

Table C.3 Data for protein release on ultrasonication following 40℃ heat pretreatment with different heating rate

Protein	Heat rate: 0.1℃/s; (mg/g)					Heat rate: 0.5℃/s; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	1.97	2.88	2.42	0.46	18.8	3.00	2.01	2.50	0.49	20.0
5	122	126	124	1.98	1.60	112	109	110	1.45	1.31
10	206	222	214	7.85	3.66	213	215	214	1.16	0.54
15	278	279	279	0.34	0.12	294	295	294	0.58	0.20
20	305	310	307	2.20	0.72	319	311	315	4.06	1.29
25	310	315	313	2.80	0.90	327	319	323	3.77	1.17

Protein	Heat rate: 1.7℃/s; (mg/g)					Heat rate: 3.5℃/s; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	3.17	2.22	2.70	0.47	17.6	2.67	2.88	2.77	0.11	3.90
5	121	115	118	2.90	2.46	137	107	122	15.00	12.3
10	225	213	219	6.09	2.78	222	212	217	4.99	2.30
15	305	301	303	2.03	0.67	342	316	329	13.33	4.05
20	326	330	328	2.03	0.62	360	353	356	3.18	0.89
25	337	336	337	0.58	0.17	366	358	362	4.06	1.12

Table C.4 Data for protein release on ultrasonication following heat 40℃ heat pretreatment with different holding time

Protein	Holding: minimal; (mg/g)					Holding: 5 min; (mg/g)					Holding: 10 min; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.67	2.88	2.77	0.11	3.90	2.30	3.23	2.77	0.47	16.9	2.53	2.10	2.32	0.22	9.35
5	137	107	122	15.00	12.3	138	153	145	7.37	5.07	143	133	138	4.92	3.56
10	222	212	217	4.99	2.30	252	244	248	4.05	1.63	240	232	236	4.28	1.82
15	342	316	329	13.33	4.05	349	362	355	6.47	1.82	342	335	338	3.43	1.02
20	360	353	356	3.18	0.89	361	370	365	4.47	1.22	347	346	346	0.45	0.13
25	366	358	362	4.06	1.12	367	372	369	2.52	0.68	356	358	357	1.40	0.39
Protein	Holding: 20 min; (mg/g)					Holding: 60 min; (mg/g)									
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.					
0	2.93	2.23	2.58	0.35	13.6	2.27	1.80	2.03	0.23	11.5					
5	131	119	125	5.83	4.66	147	159	153	5.58	3.65					
10	233	242	237	4.13	1.74	192	228	210	18.30	8.72					
15	304	313	308	4.15	1.35	241	245	243	2.12	0.87					
20	318	321	320	1.58	0.50	256	248	252	3.70	1.47					
25	328	331	329	1.67	0.51	257	259	258	1.07	0.41					

Table C.5a Data for invertase release on ultrasonication following heat pretreatment at different temperature using dilution

Invertase	Maximum T: 40 °C; (U/g X 10^{-4})					Maximum T: 50 °C; (U/g X 10^{-4})					Maximum T: 60 °C; (U/g X 10^{-4})				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.16	0.16	0.16	0.00	0.79	0.16	0.16	0.16	0.00	0.83	0.18	0.16	0.17	0.01	3.46
5	1.33	1.40	1.37	0.03	2.56	1.43	1.40	1.41	0.02	1.10	0.63	0.68	0.66	0.02	3.51
10	2.03	2.00	2.01	0.02	0.94	2.03	2.02	2.03	0.00	0.19	0.96	1.13	1.05	0.09	8.13
15	2.73	2.75	2.74	0.01	0.35	2.81	2.77	2.79	0.02	0.64	1.28	1.29	1.29	0.00	0.33
20	2.90	2.89	2.90	0.00	0.05	2.97	2.93	2.95	0.02	0.61	1.42	1.40	1.41	0.01	0.55
25	2.90	2.94	2.92	0.02	0.64	2.98	2.95	2.96	0.01	0.48	1.41	1.41	1.41	0.00	0.24

Table C.5b Data for α-glucosidase release on ultrasonication following heat pretreatment at different temperature using dilution

α-glucosidase	Maximum T: 40 °C; (U/g X 10^{-5})					Maximum T: 50 °C; (U/g X 10^{-5})					Maximum T: 60 °C; (U/g X 10^{-5})				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	7.41	0.01	0.01	0.01	0.00	9.50	0.01	0.01	0.01	0.00	13.4
5	1.04	1.13	1.09	0.05	4.31	1.31	1.35	1.33	0.02	1.48	0.04	0.04	0.04	0.00	6.10
10	1.72	1.74	1.73	0.01	0.50	1.79	1.91	1.85	0.06	3.38	0.05	0.04	0.04	0.00	7.53
15	2.34	2.19	2.27	0.08	3.46	2.36	2.28	2.32	0.04	1.68	0.08	0.07	0.07	0.00	3.63
20	2.63	2.55	2.59	0.04	1.52	2.66	2.64	2.65	0.01	0.50	0.10	0.12	0.11	0.01	6.54
25	2.68	2.73	2.71	0.03	0.94	2.79	2.85	2.82	0.03	0.99	0.12	0.13	0.12	0.01	4.76

Table C.5c Data for ADH release on ultrasonication following heat pretreatment at different temperature using dilution

ADH	Maximum T: 40 °C; (U/g X 10^{-5})				Maximum T: 50 °C; (U/g X 10^{-5})				Maximum T: 60 °C; (U/g X 10^{-5})						
Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1.27	1.30	1.28	5	1.27	1.30	1.28	5	1.27	1.30	1.28	5	1.27	1.30	1.28
10	2.60	2.77	2.68	10	2.60	2.77	2.68	10	2.60	2.77	2.68	10	2.60	2.77	2.68
15	3.83	4.03	3.93	15	3.83	4.03	3.93	15	3.83	4.03	3.93	15	3.83	4.03	3.93
20	5.18	5.33	5.26	20	5.18	5.33	5.26	20	5.18	5.33	5.26	20	5.18	5.33	5.26
25	6.00	5.93	5.97	25	6.00	5.93	5.97	25	6.00	5.93	5.97	25	6.00	5.93	5.97

Table C.5d Data for G6PDH release on ultrasonication following heat pretreatment at different temperature using dilution

G6PDH	Maximum T: 40℃; (U/g)					Maximum T: 50℃; (U/g)					Maximum T: 60℃; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	6.44	6.12	6.28	0.16	2.51	5.71	6.22	5.97	0.26	4.31	0.42	0.52	0.47	0.05	10.7
10	8.93	9.64	9.29	0.36	3.85	8.66	9.61	9.14	0.48	5.21	0.45	0.54	0.49	0.05	9.50
15	11.1	11.4	11.3	0.17	1.48	11.0	11.6	11.3	0.33	2.91	0.59	0.59	0.59	0.00	0.65
20	12.8	12.3	12.6	0.26	2.04	12.8	12.2	12.5	0.32	2.56	0.75	0.73	0.74	0.01	1.52
25	14.6	14.4	14.5	0.07	0.50	14.0	14.3	14.1	0.11	0.80	0.94	0.95	0.95	0.00	0.50

Table C.6a Data for invertase release on ultrasonication following heat pretreatment at different temperature using heating exchanger

Invertase	Maximum T: 40℃; (U/g X 10^4)					Maximum T: 45℃; (U/g X 10^4)					Maximum T: 50℃; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.
0	0.16	0.16	0.16	0	0.16	0.16	0.16	0	0.16	0.16	0.16	0	0.16	0.16	0.16
5	1.38	1.44	1.41	5	1.38	1.44	1.41	5	1.38	1.44	1.41	5	1.38	1.44	1.41
10	2.03	2.05	2.04	10	2.03	2.05	2.04	10	2.03	2.05	2.04	10	2.03	2.05	2.04
15	2.70	2.73	2.71	15	2.70	2.73	2.71	15	2.70	2.73	2.71	15	2.70	2.73	2.71
20	2.87	2.93	2.90	20	2.87	2.93	2.90	20	2.87	2.93	2.90	20	2.87	2.93	2.90
25	2.88	2.96	2.92	25	2.88	2.96	2.92	25	2.88	2.96	2.92	25	2.88	2.96	2.92
Invertase	Maximum T: 52℃; (U/g X 10^4)					Maximum T: 55℃; (U/g X 10^4)					Maximum T: 60℃; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.	Time(min)	exp. 1	exp. 2	ave.
0	0.13	0.12	0.12	0	0.13	0.12	0.12	0	0.13	0.12	0.12	0	0.13	0.12	0.12
5	1.28	1.38	1.33	5	1.28	1.38	1.33	5	1.28	1.38	1.33	5	1.28	1.38	1.33
10	2.07	2.24	2.16	10	2.07	2.24	2.16	10	2.07	2.24	2.16	10	2.07	2.24	2.16
15	2.71	2.65	2.68	15	2.71	2.65	2.68	15	2.71	2.65	2.68	15	2.71	2.65	2.68
20	2.83	2.79	2.81	20	2.83	2.79	2.81	20	2.83	2.79	2.81	20	2.83	2.79	2.81
25	2.80	2.78	2.79	25	2.80	2.78	2.79	25	2.80	2.78	2.79	25	2.80	2.78	2.79

Table C.6b Data for α-glucosidase release on ultrasonication following heat pretreatment at different temperature using heating exchanger

α-glucosidase	Maximum T: 40℃; (U/g X 10^5)					Maximum T: 45℃; (U/g X 10^5)					Maximum T: 50℃; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.02	0.01	0.00	11.9	0.01	0.01	0.01	0.00	9.09	0.00	0.01	0.01	0.00	10.3
5	1.15	1.26	1.21	0.05	4.31	1.31	1.17	1.24	0.07	5.82	1.17	1.12	1.15	0.02	2.04
10	1.99	1.74	1.86	0.12	6.66	1.73	1.79	1.76	0.03	1.96	1.75	1.98	1.87	0.11	6.10
15	2.40	2.35	2.37	0.02	1.01	2.40	2.32	2.36	0.04	1.52	2.35	2.30	2.33	0.02	1.01
20	2.51	2.58	2.54	0.04	1.48	2.55	2.65	2.60	0.05	1.96	2.59	2.67	2.63	0.04	1.48
25	2.70	2.79	2.75	0.04	1.62	2.80	2.85	2.82	0.03	0.99	2.79	2.95	2.87	0.08	2.91
α-glucosidase	Maximum T: 52℃; (U/g X 10^5)					Maximum T: 55℃; (U/g X 10^5)					Maximum T: 60℃; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	6.98	0.01	0.01	0.01	0.00	6.98	0.01	0.01	0.01	0.00	10.3
5	1.11	0.95	1.03	0.08	7.53	0.07	0.06	0.07	0.01	11.7	0.05	0.06	0.05	0.00	7.83
10	1.62	1.49	1.56	0.06	4.17	0.08	0.09	0.08	0.00	4.76	0.07	0.07	0.07	0.00	6.10
15	2.17	2.26	2.22	0.04	1.96	0.12	0.12	0.12	0.00	0.50	0.09	0.10	0.09	0.00	1.96
20	2.30	2.47	2.38	0.08	3.45	0.12	0.13	0.13	0.00	3.38	0.11	0.10	0.11	0.00	1.01
25	2.49	2.66	2.57	0.09	3.38	0.13	0.14	0.13	0.01	5.66	0.11	0.12	0.11	0.00	2.44

Table C.6c Data for ADH release on ultrasonication following heat pretreatment at different temperature using heating exchanger

ADH	Maximum T: 40℃; (U/g X 10^5)					Maximum T: 45℃; (U/g X 10^5)					Maximum T: 50℃; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1.27	1.50	1.38	0.12	8.43	1.27	1.37	1.32	0.05	3.80	1.23	1.37	1.30	0.07	5.13
10	2.73	2.97	2.85	0.12	4.09	2.67	3.00	2.83	0.17	5.88	2.60	3.00	2.80	0.20	7.14
15	3.63	3.77	3.70	0.07	1.80	4.00	4.10	4.05	0.05	1.23	3.80	4.00	3.90	0.10	2.56
20	5.20	5.33	5.27	0.07	1.27	5.67	6.10	5.88	0.22	3.68	5.13	5.50	5.32	0.18	3.45
25	5.87	6.10	5.98	0.12	1.95	6.73	6.57	6.65	0.08	1.25	6.43	6.57	6.50	0.07	1.03

ADH	Maximum T: 52℃; (U/g X 10^5)					Maximum T: 55℃; (U/g X 10^5)					Maximum T: 60℃; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1.27	1.47	1.37	0.10	7.32	0	0	0	0	0	0	0	0	0	0
10	2.07	2.03	2.05	0.02	0.81	0	0	0	0	0	0	0	0	0	0
15	3.03	3.23	3.13	0.10	3.19	0	0	0	0	0	0	0	0	0	0
20	4.23	4.43	4.33	0.10	2.31	0	0	0	0	0	0	0	0	0	0
25	5.10	5.20	5.15	0.05	0.97	0	0	0	0	0	0	0	0	0	0

Table C.6d Data for G6PDH release on ultrasonication following heat pretreatment at different temperature using heating exchanger

G6PDH	Maximum T: 40℃; (U/g)					Maximum T: 45℃; (U/g)					Maximum T: 50℃; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	5.25	5.98	5.62	0.37	6.54	5.32	5.74	5.53	0.21	3.85	5.61	6.62	6.11	0.50	8.26
10	7.79	8.42	8.10	0.31	3.85	7.96	8.44	8.20	0.24	2.91	7.82	8.22	8.02	0.20	2.44
15	11.3	9.9	10.6	0.74	6.95	10.5	10.6	10.5	0.05	0.50	10.9	11.23	11.1	0.2	1.48
20	13.0	12.8	12.9	0.13	1.01	12.6	11.0	11.8	0.82	6.95	12.9	12.52	12.7	0.2	1.52
25	14.1	13.7	13.9	0.21	1.52	14.3	15.0	14.7	0.36	2.44	14.8	15.39	15.1	0.3	1.96
G6PDH	Maximum T: 52℃; (U/g)					Maximum T: 55℃; (U/g)					Maximum T: 60℃; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	6.06	6.54	6.30	0.24	3.85	0.96	1.18	1.07	0.11	10.3	0.31	0.38	0.34	0.03	9.50
10	8.92	9.55	9.23	0.31	3.38	2.19	2.57	2.38	0.19	7.96	0.40	0.43	0.41	0.02	3.85
15	11.2	12.2	11.7	0.50	4.31	2.84	3.06	2.95	0.11	3.85	0.63	0.64	0.64	0.01	0.99
20	13.0	12.9	12.9	0.07	0.50	3.76	3.87	3.81	0.06	1.48	0.67	0.69	0.68	0.01	1.48
25	14.6	15.0	14.8	0.22	1.48	4.40	4.36	4.38	0.02	0.50	0.95	1.03	0.99	0.04	3.85

Table C.7a Data for invertase release on ultrasonication following 40℃ heat pretreatment with different heating rate

Invertase	Heat rate: 0.1℃/s; (U/g X 10^4)					Heat rate: 0.5℃/s; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.15	0.20	0.18	0.03	15.3	0.13	0.14	0.13	0.00	3.77
5	1.07	1.10	1.08	0.02	1.48	1.08	1.10	1.09	0.01	0.99
10	1.78	2.05	1.91	0.13	6.98	1.84	1.87	1.85	0.01	0.79
15	2.44	2.24	2.34	0.10	4.17	2.49	2.44	2.47	0.02	0.94
20	2.66	2.61	2.63	0.03	1.01	2.62	2.70	2.66	0.04	1.48
25	2.67	2.75	2.71	0.04	1.48	2.67	2.71	2.69	0.02	0.73
Invertase	Heat rate: 1.7℃/s; (U/g X 10^4)					Heat rate: 3.5℃/s; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.15	0.15	0.15	0.00	1.25	0.16	0.16	0.16	0.00	2.25
5	1.31	1.44	1.38	0.06	4.51	1.38	1.44	1.41	0.03	2.18
10	1.91	1.87	1.89	0.02	1.08	2.03	2.05	2.04	0.01	0.45
15	2.61	2.44	2.52	0.09	3.47	2.70	2.73	2.71	0.02	0.66
20	2.72	2.65	2.69	0.04	1.45	2.87	2.93	2.90	0.03	1.05
25	2.75	2.75	2.75	0.00	0.08	2.88	2.96	2.92	0.04	1.24

Table C.7b Data for α-glucosidase release on ultrasonication following 40℃ heat pretreatment with different heating rate

α-glucosidase	Heat rate: 0.1℃/s; (U/g X 10^5)					Heat rate: 0.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.02	0.01	0.01	9.09	0.01	0.01	0.01	0	6.08
5	1.15	1.25	1.20	0.46	3.85	1.31	1.35	1.33	0.18	1.35
10	1.65	1.70	1.68	0.25	1.48	1.73	1.67	1.70	0.3	1.78
15	2.40	2.33	2.36	0.36	1.52	2.40	2.39	2.39	0.04	0.18
20	2.51	2.48	2.49	0.13	0.50	2.42	2.47	2.44	0.24	0.97
25	2.70	2.89	2.80	0.95	3.38	2.70	2.61	2.66	0.47	1.78

α-glucosidase	Heat rate: 1.7℃/s; (U/g X 10^5)					Heat rate: 3.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.02	11.9	0.01	0.02	0.02	0.02	11.9
5	1.17	1.26	1.21	0.42	3.50	1.15	1.26	1.21	0.52	4.31
10	1.75	1.73	1.74	0.12	0.67	1.99	1.74	1.86	1.24	6.66
15	2.35	2.33	2.34	0.10	0.45	2.40	2.35	2.37	0.24	1.01
20	2.43	2.44	2.43	0.07	0.28	2.51	2.58	2.54	0.38	1.48
25	2.79	2.79	2.79	0.03	0.10	2.80	2.79	2.80	0.05	0.19

Table C.7c Data for ADH release on ultrasonication following 40℃ heat pretreatment with different heating rate

ADH	Heat rate: 0.1℃/s; (U/g X 10^5)					Heat rate: 0.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
5	0.37	0.43	0.40	0.03	8.33	0.80	0.90	0.85	0.05	5.88
10	0.97	1.03	1.00	0.03	3.33	1.43	1.57	1.50	0.07	4.44
15	1.60	1.73	1.67	0.07	4.00	2.33	2.07	2.20	0.13	6.06
20	2.73	2.87	2.80	0.07	2.38	3.43	3.63	3.53	0.10	2.83
25	3.13	3.23	3.18	0.05	1.57	4.10	4.20	4.15	0.05	1.20
ADH	Heat rate: 1.7℃/s; (U/g X 10^5)					Heat rate: 3.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
5	1.30	1.47	1.38	0.08	6.02	1.27	1.50	1.38	0.12	8.43
10	2.00	2.03	2.02	0.02	0.83	2.73	2.97	2.85	0.12	4.09
15	2.97	3.30	3.13	0.17	5.32	3.63	3.77	3.70	0.07	1.80
20	4.23	4.70	4.47	0.23	5.22	5.20	5.33	5.27	0.07	1.27
25	4.70	4.83	4.77	0.07	1.40	5.87	6.10	5.98	0.12	1.95

Table C.7d Data for G6PDH release on ultrasonication following 40℃ heat pretreatment with different heating rate

G6PDH	Heat rate: 0.1℃/s; (U/g X 10^5)					Heat rate: 0.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
5	4.60	5.47	5.04	0.44	8.68	4.72	4.52	4.62	0.10	2.24
10	7.17	7.60	7.39	0.22	2.91	7.29	7.14	7.21	0.08	1.08
15	10.1	10.2	10.2	0.05	0.50	11.0	11.1	11.1	0.05	0.46
20	11.9	10.4	11.1	0.71	6.38	12.2	12.1	12.1	0.06	0.50
25	12.7	12.6	12.7	0.06	0.50	13.0	12.9	13.0	0.06	0.44
G6PDH	Heat rate: 1.7℃/s; (U/g X 10^5)					Heat rate: 3.5℃/s; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
5	5.10	5.14	5.12	0.02	0.37	5.25	5.98	5.62	0.37	6.54
10	7.60	7.45	7.53	0.07	0.98	7.79	8.42	8.10	0.31	3.85
15	11.2	11.3	11.3	0.05	0.45	11.3	9.9	10.6	0.74	6.95
20	12.8	12.7	12.7	0.01	0.07	13.0	12.8	12.9	0.13	1.01
25	13.4	13.3	13.3	0.04	0.32	14.1	13.7	13.9	0.21	1.52

Table C.8a Data for invertase release on ultrasonication following heat 40℃ heat pretreatment with different holding time

Invertase	Holding: minimal; (U/g X 10^4)					Holding: 5 min; (U/g X 10^4)					Holding: 10 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.16	0.16	0.16	0.00	2.25	0.14	0.16	0.15	0.01	9.09	0.12	0.15	0.14	0.01	8.68
5	1.38	1.44	1.41	0.03	2.18	1.26	1.42	1.34	0.08	5.66	1.23	1.32	1.27	0.05	3.85
10	2.03	2.05	2.04	0.01	0.45	1.94	2.07	2.01	0.07	3.38	1.89	2.02	1.95	0.07	3.38
15	2.70	2.73	2.71	0.02	0.66	2.70	2.76	2.73	0.03	0.99	2.60	2.68	2.64	0.04	1.48
20	2.87	2.93	2.90	0.03	1.05	2.79	2.65	2.72	0.07	2.56	2.76	2.67	2.71	0.04	1.52
25	2.88	2.96	2.92	0.04	1.24	2.82	2.92	2.87	0.05	1.70	2.79	2.88	2.83	0.04	1.48

Invertase	Holding: 20 min; (U/g X 10^4)					Holding: 60 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.08	0.09	0.09	0.00	2.91	0.07	0.07	0.07	0.00	1.96
5	0.93	0.96	0.94	0.01	1.48	0.93	1.05	0.99	0.06	6.10
10	1.67	1.60	1.63	0.03	2.04	1.36	1.39	1.38	0.01	0.99
15	2.27	2.11	2.19	0.08	3.63	2.04	1.76	1.90	0.14	7.53
20	2.44	2.49	2.46	0.02	0.99	2.25	2.14	2.19	0.06	2.56
25	2.59	2.62	2.60	0.01	0.50	2.24	2.31	2.27	0.03	1.48

Table C.8b Data for α-glucosidase release on ultrasonication following heat 40℃ heat pretreatment with different holding time

α-glucosidase	Holding: minimal; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)					Holding: 10 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.02	0.02	11.9	0.01	0.01	0.01	0.01	8.68	0.01	0.01	0.01	0.01	8.68
5	1.15	1.26	1.21	0.52	4.31	1.31	1.42	1.36	0.52	3.85	1.17	1.25	1.21	0.41	3.38
10	1.99	1.74	1.86	1.24	6.66	1.73	1.78	1.75	0.26	1.48	1.75	1.91	1.83	0.79	4.31
15	2.40	2.35	2.37	0.24	1.01	2.40	2.52	2.46	0.60	2.44	2.35	2.42	2.38	0.35	1.48
20	2.51	2.58	2.54	0.38	1.48	2.48	2.74	2.61	1.29	4.95	2.56	2.61	2.59	0.26	1.00
25	2.70	2.79	2.75	0.45	1.62	2.70	2.81	2.76	0.54	1.96	2.79	2.84	2.82	0.28	1.00
α-glucosidase	Holding: 20 min; (U/g X 10^5)					Holding: 60 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.01	8.26	0.01	0.01	0.01	0.01	10.3
5	1.11	1.19	1.15	0.39	3.38	0.07	0.06	0.07	0.05	6.95
10	1.63	1.45	1.54	0.89	5.82	0.08	0.08	0.08	0.01	1.01
15	2.17	2.01	2.09	0.80	3.81	0.12	0.14	0.13	0.1	7.41
20	2.30	2.26	2.28	0.23	1.01	0.12	0.13	0.13	0.05	3.85
25	2.42	2.39	2.40	0.17	0.70	0.13	0.12	0.12	0.02	1.52

Table C.8c Data for ADH release on ultrasonication following heat 40°C heat pretreatment with different holding time

ADH	Holding: minimal; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)					Holding: 10 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1.27	1.50	1.38	0.12	8.43	1.13	1.33	1.23	0.10	8.11	1.33	1.17	1.25	0.08	6.67
10	2.73	2.97	2.85	0.12	4.09	2.10	2.17	2.13	0.03	1.56	2.57	3.13	2.85	0.28	9.94
15	3.63	3.77	3.70	0.07	1.80	3.57	3.87	3.72	0.15	4.04	4.30	4.63	4.47	0.17	3.73
20	5.20	5.33	5.27	0.07	1.27	5.43	5.07	5.25	0.18	3.49	5.67	5.13	5.40	0.27	4.94
25	5.87	6.10	5.98	0.12	1.95	6.00	6.40	6.20	0.20	3.23	6.17	6.63	6.40	0.23	3.65
ADH	Holding: 20 min; (U/g X 10^5)					Holding: 60 min; (U/g X 10^5)									
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.					
0	0	0	0	0	0	0	0	0	0	0					
5	1.33	1.03	1.18	0.15	12.7	0.15	0.17	0.16	0.01	7.37					
10	2.20	2.27	2.23	0.03	1.49	0.20	0.24	0.22	0.02	8.96					
15	3.20	3.63	3.42	0.22	6.34	0.30	0.28	0.29	0.01	2.30					
20	4.37	4.60	4.48	0.12	2.60	0.40	0.43	0.42	0.01	2.81					
25	5.27	5.73	5.50	0.23	4.24	0.45	0.46	0.46	0.00	0.37					

Table C.8d Data for G6PDH release on ultrasonication following heat 40°C heat pretreatment with different holding time

G6PDH	Holding: minimal; (U/g)					Holding: 5 min; (U/g)					Holding: 10 min; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	5.25	5.98	5.62	0.37	6.54	5.73	6.36	6.04	0.32	5.21	6.06	7.03	6.54	0.48	7.41
10	7.79	8.42	8.10	0.31	3.85	8.80	9.59	9.20	0.40	4.31	8.92	9.46	9.19	0.27	2.91
15	11.3	9.9	10.6	0.74	6.95	11.4	11.9	11.7	0.28	2.44	12.1	11.7	11.9	0.18	1.52
20	13.0	12.8	12.9	0.13	1.01	14.0	14.3	14.1	0.14	0.99	14.2	14.5	14.3	0.14	0.99
25	14.1	13.7	13.9	0.21	1.52	15.2	15.0	15.1	0.08	0.50	15.6	15.3	15.4	0.16	1.01

G6PDH	Holding: 20 min; (U/g)					Holding: 60 min; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
5	5.95	6.42	6.18	0.24	3.85	4.77	5.68	5.22	0.45	8.68
10	8.81	8.90	8.85	0.04	0.50	6.18	6.61	6.39	0.22	3.38
15	11.5	10.7	11.1	0.40	3.63	8.43	9.10	8.77	0.34	3.85
20	14.3	14.9	14.6	0.29	1.96	9.97	9.58	9.77	0.20	2.04
25	15.3	16.4	15.8	0.53	3.38	11.3	11.9	11.6	0.34	2.91

Table C.9 **Kinetic rate constant (k) of protein and enzyme release on ultrasonication following heat pretreatment at different temperature with maximum heating rate and no holding time using heat exchanger**

Maximum T	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
40℃	0.08	0.9699	0.06	0.9945	0.06	0.9819	0.05	0.9835	0.03	0.9845
45℃	0.08	0.9661	0.06	0.9958	0.07	0.9908	0.06	0.9677	0.04	0.9825
50℃	0.09	0.9943	0.06	0.9835	0.07	0.9967	0.05	0.9679	0.04	0.9875
52℃	0.07	0.9974	0.06	0.9966	0.06	0.9909	0.04	0.9835	0.04	0.9775
55℃	0.01	0.9934	0.03	0.9853	0.0014	0.8764	-	-	0.01	0.9860
60℃	0.01	0.9908	0.02	0.9649	0.0011	0.9258	-	-	0.0018	0.9545

Table C.10 Kinetic rate constant (k) of protein and enzyme release on ultrasonication following 40℃ heat pretreatment with different heat rate with no holding time using heat exchanger

Heating rate	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
0.1℃/s	0.06	0.9999	0.05	0.9934	0.06	0.9843	0.02	0.9654	0.03	0.9744
0.5℃/s	0.07	0.9937	0.05	0.9999	0.05	0.9515	0.03	0.9714	0.03	0.9658
1.7℃/s	0.07	0.9934	0.05	0.9879	0.06	0.9804	0.03	0.9788	0.03	0.9665
3.5℃/s	0.08	0.9699	0.06	0.9945	0.06	0.9819	0.05	0.9835	0.03	0.9845

Table C.11 Kinetic rate constant (k) of protein and enzyme release on ultrasonication following 40℃ heat pretreatment with heating rate of 3.5℃/s with different holding time using heat exchanger

Holding time	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
Minimal	0.07	0.9809	0.06	0.9945	0.06	0.9783	0.05	0.9835	0.03	0.9845
5 min	0.08	0.9663	0.06	0.9957	0.06	0.9650	0.05	0.9635	0.04	0.9853
10 min	0.07	0.9643	0.05	0.9974	0.06	0.9881	0.05	0.9876	0.04	0.9853
20 min	0.06	0.9661	0.04	1	0.05	0.9605	0.04	0.9846	0.04	0.9883
60 min	0.05	0.9435	0.03	0.0367	0.00	0.8870	0.03	0.9830	0.02	0.9662

Raw data for homogenisation of Baker's yeast following pH pretreatment

The total soluble protein and enzyme release from Baker's yeast (1.5%, dry weight, w/v) on ultrasonication at 80 W following pH pretreatment. The tables below present the data obtained for the release of total soluble protein, enzyme release and kinetic rate constant k.

Table C.12 Data for protein release on ultrasonication following pH pretreatment at different maximum pH using 0.5 carbonate buffer

Protein	Maximum pH 9; (mg/g)					Maximum pH 9.5; (mg/g)					Maximum pH 10; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	3.52	3.44	3.48	0.04	1.08	4.35	3.87	4.11	0.24	5.87	3.54	3.14	3.34	0.20	5.97
3	78.5	88.1	83.3	4.77	5.73	75.0	102	88.3	13.3	15.1	98.8	144	122	22.71	18.7
6	127	134	131	3.74	2.86	120	135	127	7.76	6.09	179	236	207	28.56	13.8
9	173	184	179	5.71	3.20	185	206	199	10.3	5.25	287	288	287	0.36	0.12
12	242	248	245	3.04	1.24	256	279	267	11.5	4.30	350	317	333	16.52	4.96
15	313	336	325	11.9	3.65	328	342	335	6.56	1.96	362	380	371	8.87	2.39
18	350	355	353	2.39	0.68	340	349	344	4.83	1.40	382	379	381	1.53	0.40
21	362	358	360	1.98	0.55	356	360	358	1.85	0.52	403	404	404	0.52	0.13

Protein	Maximum pH 10.5; (mg/g)					Maximum pH 10; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	4.27	3.97	4.12	0.15	3.70	2.53	2.88	2.70	0.17	6.35
3	129	110	120	9.58	7.99	59.0	78.3	68.6	9.69	14.1
6	196	179	188	8.67	4.62	122	152	137	15.1	11.0
9	252	275	263	11.6	4.40	155	177	166	11.4	6.86
12	322	340	331	8.86	2.68	233	216	225	8.34	3.71
15	362	345	354	8.41	2.38	304	314	309	4.83	1.56
18	376	382	379	3.11	0.82	356	361	358	2.52	0.70
21	393	390	392	1.67	0.43	383	386	384	1.61	0.42

Table C.13a Data for protein release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

Protein	Holding: minimal; (mg/g)					Holding: 30 s; (mg/g)					Holding: 1 min; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	3.54	3.14	3.34	0.20	5.97	3.23	4.10	3.66	0.44	11.9	3.07	3.69	3.38	0.31	9.12
3	98.8	144	121	22.7	18.7	157	129	143	13.84	9.66	167	125	146	20.77	14.2
6	179	236	207	28.6	13.8	244	179	211	32.61	15.4	242	177	210	32.58	15.5
9	287	288	287	0.36	0.12	287	277	282	5.02	1.78	293	268	280	12.33	4.40
12	350	317	333	16.5	4.96	319	346	333	13.59	4.08	332	350	341	9.00	2.64
15	362	330	346	16.1	4.66	336	371	354	17.83	5.04	341	379	360	18.92	5.26
18	382	379	381	1.53	0.40	385	385	385	0.17	0.04	388	391	390	1.44	0.37
21	413	404	409	4.48	1.10	410	412	411	1.11	0.27	407	417	412	5.39	1.31

Protein	Holding: 2 min; (mg/g)					Holding: 5 min; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	3.42	3.17	3.30	0.13	3.83	2.53	2.87	2.70	0.17	6.20
3	104	106	105	0.89	0.85	104	115	110	5.33	4.87
6	205	218	211	6.42	3.04	156	146	151	4.78	3.17
9	275	298	286	11.71	4.09	240	254	247	6.94	2.81
12	349	323	336	13.15	3.91	283	288	286	2.85	1.00
15	383	366	375	8.32	2.22	296	301	299	2.64	0.88
18	386	385	386	0.11	0.03	332	321	327	5.54	1.70
21	415	418	416	1.70	0.41	345	344	345	0.67	0.19

Table C.13b Data for protein release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

Protein	Holding: minimal; (mg/g)					Holding: 30 s; (mg/g)					Holding: 1 min; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.40	3.25	2.82	0.42	15.1	3.40	4.03	3.72	0.32	8.52	3.63	3.07	3.35	0.28	8.46
3	91.2	97.9	94.5	3.37	3.56	114	146	130	15.9	12.2	139	122	130	8.47	6.49
6	165	184	174	9.88	5.67	165	212	188	23.7	12.6	231	196	213	17.82	8.35
9	265	239	252	12.9	5.11	261	284	273	11.7	4.29	293	271	282	10.87	3.86
12	290	301	295	5.50	1.86	303	311	307	4.22	1.37	315	312	313	1.33	0.43
15	332	342	337	4.97	1.47	328	326	327	1.00	0.31	344	333	339	5.60	1.65
18	348	406	377	29.3	7.78	364	375	369	5.18	1.40	386	372	379	6.87	1.81
21	378	411	394	17.0	4.30	391	397	394	2.83	0.72	403	398	401	2.57	0.64

Protein	Holding: 2 min; (mg/g)					Holding: 5 min; (mg/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	2.90	3.10	3.00	0.10	3.33	2.63	2.87	2.75	0.12	4.24
3	133	125	129	3.79	2.94	108	105	106	1.33	1.25
6	207	228	217	10.3	4.72	179	154	166	12.2	7.32
9	307	291	299	7.90	2.64	240	270	255	15.0	5.87
12	355	330	342	12.1	3.52	287	295	291	4.33	1.49
15	375	365	370	5.00	1.35	306	314	310	4.02	1.29
18	396	392	394	1.62	0.41	341	333	337	4.17	1.24
21	412	404	408	3.88	0.95	360	345	352	7.22	2.05

Table C.14a Data for invertase release on ultrasonication following pH pretreatment at different maximum pH using 0.5M carbonate buffer

Invertase	Maximum pH 9; (U/g X10^{-4})					Maximum pH 9.5; (U/g X10^{-4})					Maximum pH 10; (U/g X10^{-4})				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.08	0.09	0.08	0.00	2.24	0.10	0.10	0.10	0.00	4.46	0.08	0.09	0.08	0.00	5.19
3	0.64	0.73	0.68	0.06	8.71	0.68	0.72	0.70	0.03	4.20	0.94	0.99	0.96	0.04	3.86
6	1.25	1.33	1.29	0.05	4.20	1.22	1.30	1.26	0.06	4.46	1.65	2.00	1.82	0.25	13.6
9	1.46	1.42	1.44	0.03	1.93	1.60	1.82	1.71	0.15	8.69	2.36	2.50	2.43	0.10	4.20
12	2.17	2.05	2.11	0.09	4.14	2.37	2.76	2.57	0.27	10.55	2.96	3.12	3.04	0.11	3.64
15	2.89	2.73	2.81	0.12	4.13	2.98	2.96	2.97	0.02	0.55	3.30	3.49	3.40	0.13	3.86
18	3.02	3.21	3.12	0.13	4.33	2.95	3.04	3.00	0.06	2.16	3.26	3.48	3.37	0.15	4.46
21	3.12	3.63	3.38	0.36	10.6	3.11	3.30	3.20	0.13	4.21	3.55	3.66	3.60	0.08	2.18

Invertase	Maximum pH 10.5; (U/g X10^{-4})					Maximum pH 11; (U/g X10^{-4})				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.10	0.10	0.10	0.00	3.13	0.06	0.08	0.07	0.01	13.6
3	0.92	0.98	0.95	0.04	3.85	0.53	0.56	0.55	0.02	4.46
6	1.63	1.89	1.76	0.19	10.6	0.98	1.00	0.99	0.02	1.61
9	2.16	2.29	2.23	0.09	4.21	1.36	1.77	1.57	0.29	18.5
12	2.94	2.76	2.85	0.12	4.28	1.99	1.88	1.94	0.08	4.28
15	3.15	3.08	3.12	0.05	1.57	2.75	3.02	2.89	0.19	6.45
18	3.25	3.43	3.34	0.13	3.87	3.07	3.26	3.17	0.14	4.33
21	3.40	3.70	3.55	0.21	5.92	3.34	3.54	3.44	0.14	4.21

Table C.14b Data for α-glucosidase release on ultrasonication following pH pretreatment at different maximum pH using 0.5M carbonate buffer

α-glucosidase	Maximum pH 9; (U/g X10^5)					Maximum pH 9.5; (U/g X10^5)					Maximum pH 10; (U/g X10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	3.86	0.01	0.02	0.01	0.00	3.67	0.01	0.01	0.01	0.00	1.56
3	0.52	0.57	0.55	0.03	6.37	0.66	0.70	0.68	0.03	4.42	0.67	0.63	0.65	0.03	4.15
6	1.06	1.23	1.14	0.12	10.7	1.19	1.26	1.23	0.05	3.88	1.32	1.24	1.28	0.06	4.36
9	1.39	1.52	1.45	0.09	6.36	1.37	1.54	1.45	0.12	8.15	1.77	1.80	1.79	0.02	1.19
12	1.80	1.97	1.89	0.12	6.49	1.78	1.90	1.84	0.08	4.39	2.10	2.27	2.18	0.12	5.40
15	2.12	2.08	2.10	0.03	1.26	2.10	2.08	2.09	0.01	0.62	2.63	2.50	2.57	0.10	3.71
18	2.24	2.38	2.31	0.10	4.22	2.28	2.31	2.30	0.02	0.92	2.78	2.82	2.80	0.03	0.92
21	2.37	2.52	2.45	0.11	4.53	2.33	2.46	2.39	0.09	3.87	2.83	2.88	2.86	0.03	1.08

α-glucosidase	Maximum pH 10.5; (U/g X10^5)					Maximum pH 11; (U/g X10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	6.64	0.01	0.01	0.01	0.00	6.36
3	0.82	0.87	0.84	0.03	3.86	0.48	0.52	0.50	0.03	5.86
6	1.42	1.50	1.46	0.06	3.86	0.87	0.94	0.90	0.05	5.77
9	1.91	2.21	2.06	0.21	10.2	1.23	1.31	1.27	0.05	4.23
12	2.29	2.40	2.34	0.08	3.53	1.78	1.96	1.87	0.12	6.51
15	2.50	2.66	2.58	0.11	4.33	2.12	2.26	2.19	0.10	4.39
18	2.61	2.59	2.60	0.01	0.56	2.40	2.34	2.37	0.04	1.71
21	2.70	2.92	2.81	0.16	5.51	2.57	2.61	2.59	0.02	0.93

Table C.14c Data for ADH release on ultrasonication following pH pretreatment at different maximum pH using 0.5M carbonate buffer

ADH	Maximum pH 9; (U/g X10^5)					Maximum pH 9.5; (U/g X10^5)					Maximum pH 10; (U/g X10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0.80	0.83	0.82	0.02	2.89	0.88	0.96	0.92	0.05	5.90	0.83	0.93	0.88	0.07	8.00
6	1.83	1.67	1.75	0.12	6.73	2.29	2.23	2.26	0.04	1.82	2.13	2.47	2.30	0.24	10.3
9	2.62	2.53	2.58	0.06	2.29	2.98	2.77	2.87	0.15	5.32	3.17	3.50	3.33	0.24	7.07
12	3.53	3.63	3.58	0.07	1.97	3.49	4.30	3.89	0.57	14.8	4.60	4.90	4.75	0.21	4.47
15	4.83	4.83	4.83	0.00	0.00	4.25	5.30	4.78	0.74	15.5	5.80	5.30	5.55	0.35	6.37
18	5.67	5.70	5.68	0.02	0.41	5.04	6.00	5.52	0.68	12.3	6.27	5.97	6.12	0.21	3.47
21	6.27	6.17	6.22	0.07	1.14	6.16	6.67	6.42	0.36	5.54	6.87	6.53	6.70	0.24	3.52

ADH	Maximum pH 10.5; (U/g X10^5)					Maximum pH 11; (U/g X10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	0.83	0.87	0.85	0.02	2.77	1.10	1.23	1.17	0.09	8.08
6	2.53	2.60	2.57	0.05	1.84	2.40	2.53	2.47	0.09	3.82
9	3.60	3.73	3.67	0.09	2.57	3.50	3.60	3.55	0.07	1.99
12	4.73	5.00	4.87	0.19	3.87	4.53	4.43	4.48	0.07	1.58
15	5.87	6.20	6.03	0.24	3.91	5.73	6.07	5.90	0.24	3.99
18	6.20	6.07	6.13	0.09	1.54	6.27	6.60	6.43	0.24	3.66
21	6.93	7.33	7.13	0.28	3.97	6.90	7.07	6.98	0.12	1.69

Table C.14d Data for G6PDH release on ultrasonication following pH pretreatment at different maximum pH using 0.5M carbonate buffer

G6PDH	Maximum pH 9; (U/g)					Maximum pH 9.5; (U/g)					Maximum pH 10; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	3.19	3.29	3.24	0.07	2.09	3.26	3.42	3.34	0.12	3.45	3.36	3.49	3.42	0.09	2.77
6	5.64	5.86	5.75	0.16	2.77	6.04	6.82	6.43	0.55	8.63	6.93	7.41	7.17	0.34	4.78
9	8.83	10.2	9.49	0.94	9.87	10.0	10.7	10.4	0.50	4.78	10.9	9.47	10.2	1.00	9.83
12	11.3	10.1	10.7	0.88	8.23	12.3	12.1	12.2	0.17	1.43	13.6	13.9	13.8	0.19	1.40
15	13.9	13.5	13.7	0.29	2.15	13.7	12.0	12.8	1.16	9.03	15.7	15.2	15.5	0.37	2.41
18	14.5	14.7	14.6	0.20	1.40	14.7	14.8	14.8	0.10	0.70	16.6	16.4	16.5	0.10	0.60
21	15.0	15.8	15.4	0.53	3.45	15.7	16.2	15.9	0.33	2.09	17.1	17.2	17.2	0.11	0.64

G6PDH	Maximum pH 10.5; (U/g)					Maximum pH 11; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	3.42	3.53	3.47	0.07	2.09	3.32	3.49	3.41	0.12	3.45
6	6.87	7.42	7.15	0.39	5.44	6.77	7.92	7.35	0.81	11.1
9	10.5	10.2	10.4	0.22	2.15	10.4	10.8	10.6	0.22	2.09
12	13.7	14.5	14.1	0.58	4.12	12.8	12.5	12.6	0.18	1.43
15	15.2	15.4	15.3	0.11	0.70	14.6	14.0	14.3	0.41	2.89
18	16.1	16.2	16.2	0.11	0.70	15.4	15.8	15.6	0.33	2.09
21	17.6	16.3	17.0	0.90	5.29	16.2	16.5	16.4	0.23	1.40

Table C.15a Data for invertase release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

Invertase	Holding: minimal; (U/g X 10^4)					Holding: 30 s; (U/g X 10^4)					Holding: 1 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.08	0.09	0.08	0.00	5.19	0.09	0.10	0.09	0.01	10.6	0.08	0.11	0.09	0.02	19.1
3	0.94	0.99	0.96	0.04	3.86	1.10	1.17	1.14	0.05	4.20	1.13	1.19	1.16	0.04	3.86
6	1.65	2.00	1.82	0.25	13.6	1.69	1.91	1.80	0.16	8.71	1.67	1.78	1.73	0.07	4.21
9	2.36	2.50	2.43	0.10	4.20	2.31	2.52	2.42	0.15	6.18	2.30	2.67	2.48	0.26	10.5
12	2.96	3.12	3.04	0.11	3.64	2.96	3.14	3.05	0.13	4.20	3.03	3.21	3.12	0.13	4.20
15	3.08	3.49	3.28	0.29	8.79	3.15	3.31	3.23	0.11	3.54	3.21	3.41	3.31	0.14	4.33
18	3.26	3.48	3.37	0.15	4.46	3.30	3.37	3.34	0.05	1.61	3.34	3.45	3.39	0.08	2.25
21	3.55	3.66	3.60	0.08	2.18	3.57	3.68	3.62	0.08	2.18	3.58	3.69	3.63	0.08	2.17

Invertase	Holding: 2 min; (U/g X 10^4)					Holding: 5 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.08	0.07	0.08	0.00	3.95	0.06	0.07	0.07	0.00	4.21
3	0.81	0.94	0.87	0.09	10.5	0.84	1.57	1.21	0.51	42.4
6	1.69	1.91	1.80	0.16	8.69	1.44	1.68	1.56	0.17	10.7
9	2.35	2.49	2.42	0.10	4.21	2.03	2.30	2.16	0.19	8.76
12	2.98	3.08	3.03	0.07	2.16	2.54	2.87	2.70	0.24	8.76
15	3.34	3.49	3.41	0.11	3.22	2.66	2.92	2.79	0.18	6.51
18	3.30	3.41	3.36	0.07	2.17	2.80	2.97	2.89	0.12	4.19
21	3.50	3.47	3.49	0.02	0.62	2.99	3.12	3.06	0.09	2.92

Table C.15b Data for α-glucosidase release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

α-glucosidase	Holding: minimal; (U/g X 10^5)					Holding: 30 s; (U/g X 10^5)					Holding: 1 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	1.56	0.02	0.02	0.02	0.00	13.6	0.02	0.02	0.02	0.00	9.95
3	0.67	0.63	0.65	0.03	4.15	0.81	0.91	0.86	0.07	8.08	0.81	0.88	0.85	0.05	5.77
6	1.32	1.24	1.28	0.06	4.36	1.42	1.45	1.44	0.02	1.62	1.41	1.43	1.42	0.01	0.92
9	1.77	1.80	1.79	0.02	1.19	1.80	1.94	1.87	0.10	5.53	1.86	1.81	1.83	0.04	1.93
12	2.10	2.27	2.18	0.12	5.40	2.12	2.23	2.18	0.08	3.66	2.17	2.13	2.15	0.03	1.26
15	2.63	2.50	2.57	0.10	3.71	2.49	2.42	2.45	0.05	1.99	2.58	2.71	2.65	0.09	3.53
18	2.78	2.82	2.80	0.03	0.92	2.70	2.76	2.73	0.04	1.62	2.76	2.86	2.81	0.07	2.51
21	2.83	2.88	2.86	0.03	1.08	2.87	2.94	2.91	0.05	1.62	2.92	2.93	2.93	0.01	0.23

α-glucosidase	Holding: 2 min; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.02	0.02	0.00	4.20	0.01	0.02	0.01	0.00	10.2
3	0.80	0.93	0.86	0.09	10.1	0.74	0.76	0.75	0.01	1.62
6	1.44	1.53	1.49	0.06	4.21	1.41	1.50	1.46	0.06	4.20
9	1.97	2.01	1.99	0.03	1.49	1.89	2.02	1.95	0.09	4.71
12	2.37	2.26	2.31	0.08	3.25	2.05	2.12	2.09	0.05	2.25
15	2.72	2.75	2.73	0.03	0.93	2.13	2.26	2.20	0.10	4.33
18	2.88	2.96	2.92	0.06	2.09	2.26	2.29	2.27	0.02	0.92
21	2.98	3.00	2.99	0.02	0.62	2.36	2.49	2.43	0.09	3.86

Table C.15c Data for ADH enzyme release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

ADH	Holding: minimal; (U/g X 10^5)					Holding: 30 s; (U/g X 10^5)					Holding: 1 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0.83	0.93	0.88	0.07	8.00	0.87	0.90	0.88	0.02	2.67	0.87	0.93	0.90	0.05	5.24
6	2.13	2.47	2.30	0.24	10.3	2.05	2.10	2.08	0.03	1.59	2.24	2.37	2.30	0.09	3.89
9	3.17	3.50	3.33	0.24	7.07	3.12	3.30	3.21	0.13	3.97	3.36	3.47	3.41	0.08	2.21
12	4.60	4.90	4.75	0.21	4.47	4.40	4.43	4.42	0.02	0.53	4.53	4.83	4.68	0.21	4.53
15	5.80	5.30	5.55	0.35	6.37	5.60	5.93	5.77	0.24	4.09	5.47	5.13	5.30	0.24	4.45
18	6.27	5.97	6.12	0.21	3.47	6.27	6.07	6.17	0.14	2.29	6.13	6.30	6.22	0.12	1.90
21	6.87	6.53	6.70	0.24	3.52	6.40	6.60	6.50	0.14	2.18	6.27	6.57	6.42	0.21	3.31

ADH	Holding: 2 min; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	1.00	1.13	1.07	0.09	8.84	1.13	1.27	1.20	0.09	7.86
6	2.33	2.40	2.37	0.05	1.99	2.71	2.87	2.79	0.11	4.06
9	3.60	3.50	3.55	0.07	1.99	4.20	4.13	4.17	0.05	1.13
12	4.67	4.60	4.63	0.05	1.02	5.33	5.27	5.30	0.05	0.89
15	6.00	6.17	6.08	0.12	1.94	6.17	6.33	6.25	0.12	1.89
18	6.53	6.93	6.73	0.28	4.20	6.53	6.73	6.63	0.14	2.13
21	6.67	7.10	6.88	0.31	4.45	6.97	7.27	7.12	0.21	2.98

Table C.15d Data for G6PDH release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

G6PDH	Holding: minimal; (U/g X 10^5)					Holding: 30 s; (U/g X 10^5)					Holding: 1 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	3.36	3.49	3.42	0.09	2.77	3.54	3.69	3.61	0.10	2.77	3.17	3.36	3.27	0.13	4.12
6	6.93	7.41	7.17	0.34	4.78	6.71	6.91	6.81	0.14	2.09	6.28	6.72	6.50	0.31	4.78
9	10.9	9.5	10.2	1.00	9.83	10.7	12.0	11.4	0.91	8.00	10.2	11.0	10.6	0.58	5.44
12	13.6	13.9	13.8	0.19	1.40	13.0	13.7	13.3	0.46	3.45	12.7	14.6	13.6	1.35	9.87
15	15.7	16.2	16.0	0.33	2.09	15.2	14.8	15.0	0.32	2.15	15.7	16.2	16.0	0.33	2.09
18	16.6	17.7	17.2	0.82	4.78	16.6	16.2	16.4	0.23	1.43	16.5	16.2	16.4	0.23	1.43
21	17.1	16.6	16.8	0.36	2.15	16.8	17.3	17.0	0.36	2.09	16.9	18.3	17.6	0.96	5.44

G6PDH	Holding: 2 min; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	3.92	3.99	3.96	0.06	1.40	4.11	4.21	4.16	0.08	1.85
6	6.28	6.72	6.50	0.31	4.78	7.61	8.04	7.82	0.30	3.89
9	10.5	10.8	10.6	0.22	2.09	11.9	12.9	12.4	0.67	5.37
12	12.5	13.7	13.1	0.80	6.09	14.1	13.9	14.0	0.15	1.10
15	14.5	14.3	14.4	0.21	1.43	16.2	15.3	15.7	0.62	3.92
18	16.8	17.4	17.1	0.47	2.77	17.3	17.7	17.5	0.28	1.58
21	17.3	17.8	17.5	0.37	2.09	18.0	17.8	17.9	0.09	0.51

Table C.16a Data for invertase release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

Invertase	Holding: minimal; (U/g X 10^4)					Holding: 30 s; (U/g X 10^4)					Holding: 1 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.07	0.01	0.04	0.09	0.09	0.09	0.02	0.06	0.08	0.10	0.09	0.01	0.05	0.07	0.01
3	0.75	0.03	3.86	1.11	1.00	1.06	0.08	7.56	1.00	1.14	1.07	0.09	8.76	0.75	0.03
6	1.75	0.11	6.17	1.91	1.80	1.86	0.08	4.21	1.71	1.93	1.82	0.15	8.41	1.75	0.11
9	2.12	0.08	3.66	2.37	2.24	2.30	0.10	4.20	2.31	2.51	2.41	0.14	5.74	2.12	0.08
12	2.71	0.12	4.46	2.96	2.72	2.84	0.16	5.79	2.78	2.95	2.87	0.12	4.21	2.71	0.12
15	3.09	0.12	3.86	2.97	2.91	2.94	0.04	1.49	3.02	3.12	3.07	0.07	2.44	3.09	0.12
18	3.20	0.04	1.26	3.31	3.17	3.24	0.10	3.12	3.25	3.28	3.26	0.03	0.78	3.20	0.04
21	3.41	0.02	0.62	3.46	3.42	3.44	0.03	0.92	3.48	3.70	3.59	0.16	4.33	3.41	0.02

Invertase	Holding: 2 min; (U/g X 10^4)					Holding: 5 min; (U/g X 10^4)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.07	0.07	0.07	0.00	3.13	0.07	0.07	0.07	0.00	4.46
3	0.99	1.04	1.02	0.03	3.13	0.82	0.90	0.86	0.05	6.38
6	1.75	1.84	1.79	0.07	3.67	1.59	1.73	1.66	0.10	6.05
9	2.45	2.67	2.56	0.16	6.17	2.09	2.42	2.25	0.23	10.25
12	3.04	3.11	3.08	0.05	1.55	2.58	2.74	2.66	0.11	4.20
15	3.29	3.26	3.28	0.02	0.62	2.76	2.85	2.81	0.06	2.17
18	3.38	3.58	3.48	0.15	4.22	2.89	2.95	2.92	0.04	1.49
21	3.54	3.76	3.65	0.15	4.22	3.06	3.25	3.15	0.13	4.19

Table C.16b Data for α-glucosidase release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

α-glucosidase	Holding: minimal; (U/g X 10^5)					Holding: 30 s; (U/g X 10^5)					Holding: 1 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	2.85	0.02	0.02	0.02	0.00	1.06	0.01	0.01	0.01	0.00	10.9
3	0.65	0.62	0.64	0.03	4.15	0.65	0.64	0.65	0.01	1.13	0.66	0.66	0.66	0.00	0.06
6	1.28	1.21	1.25	0.05	4.36	1.22	1.12	1.17	0.07	6.31	1.24	1.25	1.25	0.01	0.90
9	1.73	1.76	1.74	0.02	1.19	1.71	1.62	1.67	0.06	3.57	1.80	1.82	1.81	0.02	0.94
12	2.05	1.88	1.97	0.11	5.83	2.09	2.24	2.17	0.10	4.82	2.19	2.24	2.21	0.03	1.45
15	2.57	2.44	2.50	0.09	3.71	2.58	2.65	2.62	0.05	1.79	2.60	2.51	2.56	0.07	2.57
18	2.71	2.75	2.73	0.03	0.92	2.72	2.81	2.77	0.06	2.21	2.70	2.86	2.78	0.11	4.02
21	2.76	2.80	2.78	0.03	1.08	2.77	2.86	2.81	0.06	2.28	2.76	3.06	2.91	0.21	7.14

α-glucosidase	Holding: 2 min; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0.01	0.01	0.01	0.00	4.40	0.01	0.02	0.01	0.00	10.5
3	0.86	0.65	0.76	0.15	19.2	0.66	0.70	0.68	0.03	4.48
6	1.48	1.34	1.41	0.10	6.80	1.26	1.45	1.35	0.13	9.94
9	1.99	1.78	1.88	0.15	7.70	1.68	1.73	1.70	0.03	1.82
12	2.33	2.30	2.31	0.02	0.73	2.01	2.00	2.01	0.01	0.32
15	2.55	2.71	2.63	0.11	4.34	2.19	2.17	2.18	0.01	0.62
18	2.73	2.78	2.75	0.03	1.20	2.32	2.45	2.39	0.09	3.67
21	2.79	2.85	2.82	0.04	1.48	2.40	2.45	2.42	0.04	1.56

Table C.16c Data for ADH release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

ADH	Holding: minimal; (U/g X 10^5)					Holding: 30 s; (U/g X 10^5)					Holding: 1 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1.00	1.07	1.03	0.05	4.56	0.97	1.17	1.07	0.14	13.26	1.10	1.23	1.17	0.09	8.08
6	2.00	2.10	2.05	0.07	3.45	2.03	2.17	2.10	0.09	4.49	2.40	2.47	2.43	0.05	1.94
9	2.80	3.33	3.07	0.38	12.3	2.83	3.30	3.07	0.33	10.8	3.27	3.53	3.40	0.19	5.55
12	3.67	4.23	3.95	0.40	10.1	4.07	4.57	4.32	0.35	8.19	4.47	4.57	4.52	0.07	1.57
15	4.67	4.70	4.68	0.02	0.50	5.13	5.10	5.12	0.02	0.46	4.90	4.73	4.82	0.12	2.45
18	5.40	5.50	5.45	0.07	1.30	5.50	5.97	5.73	0.33	5.76	5.43	5.53	5.48	0.07	1.29
21	6.00	6.20	6.10	0.14	2.32	6.07	6.27	6.17	0.14	2.29	6.00	6.37	6.18	0.26	4.19

ADH	Holding: 2 min; (U/g X 10^5)					Holding: 5 min; (U/g X 10^5)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	1.03	1.17	1.10	0.09	8.57	1.10	1.17	1.13	0.05	4.16
6	2.50	2.60	2.55	0.07	2.77	2.40	2.60	2.50	0.14	5.66
9	3.43	3.63	3.53	0.14	4.00	3.50	3.97	3.73	0.33	8.84
12	4.43	4.50	4.47	0.05	1.06	4.53	4.83	4.68	0.21	4.53
15	5.13	5.20	5.17	0.05	0.91	5.37	5.87	5.62	0.35	6.29
18	5.77	5.63	5.70	0.09	1.65	5.70	6.07	5.88	0.26	4.41
21	6.30	6.60	6.45	0.21	3.29	6.23	6.37	6.30	0.09	1.50

Table C.16d Data for G6PDH release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

G6PDH	Holding: minimal; (U/g)					Holding: 30 s; (U/g)					Holding: 1 min; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	3.92	3.99	3.96	0.05	1.16	3.98	4.15	4.07	0.12	2.98	4.06	5.12	4.59	0.75	16.4
6	6.76	7.22	6.99	0.33	4.68	6.74	7.16	6.95	0.30	4.27	6.82	7.61	7.21	0.56	7.73
9	9.79	10.5	10.1	0.47	4.68	9.84	10.25	10.04	0.29	2.92	9.89	10.4	10.1	0.36	3.52
12	12.4	12.2	12.3	0.14	1.10	12.0	11.7	11.9	0.24	1.99	12.6	12.4	12.5	0.08	0.62
15	14.0	13.6	13.8	0.28	2.06	14.0	13.9	13.9	0.09	0.62	14.1	15.0	14.6	0.64	4.42
18	14.6	15.4	15.0	0.60	3.98	14.6	15.2	14.9	0.38	2.54	14.7	15.1	14.9	0.33	2.24
21	15.5	15.7	15.6	0.15	0.93	15.9	16.3	16.1	0.29	1.79	16.2	16.4	16.3	0.15	0.92

G6PDH	Holding: 2 min; (U/g)					Holding: 5 min; (U/g)				
Time(min)	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.
0	0	0	0	0	0	0	0	0	0	0
3	4.10	4.36	4.23	0.19	4.48	4.08	4.30	4.19	0.16	3.76
6	6.73	7.51	7.12	0.55	7.73	6.84	7.94	7.39	0.78	10.6
9	10.0	10.6	10.3	0.40	3.88	9.97	10.2	10.1	0.19	1.85
12	11.9	13.0	12.5	0.77	6.18	12.1	13.6	12.8	1.07	8.36
15	14.1	13.8	13.9	0.17	1.24	14.1	14.0	14.0	0.08	0.55
18	14.9	14.5	14.7	0.28	1.92	15.1	16.1	15.6	0.67	4.27
21	16.3	17.1	16.7	0.53	3.20	16.1	16.7	16.4	0.41	2.48

Table C.17 Kinetic rate constant (k) of protein and enzyme release on ultrasonication following pH pretreatment at different maximum pH using 0.5M carbonate buffer

Maxim pH	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
9	0.07	0.9733	0.06	0.9746	0.06	0.9966	0.06	0.9727	0.05	0.9882
9.5	0.07	0.9704	0.07	0.9703	0.06	0.9951	0.06	0.9771	0.05	0.9874
10	0.09	0.9916	0.09	0.9951	0.09	0.9952	0.07	0.9911	0.06	0.9848
10.5	0.09	0.9900	0.08	0.9942	0.08	0.9708	0.08	0.9775	0.06	0.9789
11	0.08	0.9910	0.07	0.9644	0.06	0.9853	0.08	0.9832	0.06	0.9900

Table C.18a Kinetic rate constant (k) of protein and enzyme release on ultrasonication following pH 10 pretreatment using 0.5 M carbonate buffer with different holding time

Holding time	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
minimal	0.09	0.9916	0.09	0.9951	0.09	0.9952	0.07	0.9911	0.06	0.9848
30 s	0.09	0.9959	0.09	0.9932	0.08	0.9980	0.07	0.9805	0.06	0.9859
1 min	0.10	0.9962	0.09	0.9872	0.09	0.9895	0.07	0.9895	0.07	0.9842
2 min	0.09	0.9940	0.09	0.9935	0.10	0.9954	0.08	0.9759	0.07	0.9902
5 min	0.06	0.9962	0.07	0.9852	0.07	0.9505	0.08	0.9944	0.07	0.9874

Table C.18b Kinetic rate constant (k) of protein and enzyme release on ultrasonication following pH 10 pretreatment using 0.05 M carbonate buffer with different holding time

Holding time	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2	k ($pass^{-1}$)	R^2
minimal	0.09	0.9983	0.07	0.9896	0.08	0.9863	0.06	0.9926	0.05	0.9856
30 s	0.09	0.9911	0.08	0.9971	0.09	0.9862	0.06	0.9935	0.05	0.9950
1 min	0.09	0.9941	0.08	0.9999	0.09	0.9969	0.06	0.9937	0.05	0.9876
2 min	0.10	0.9938	0.09	0.9959	0.09	0.9962	0.06	0.9956	0.05	0.9911
5 min	0.07	0.9962	0.07	0.9984	0.06	0.9873	0.06	0.9917	0.06	0.9935

Raw data for homogenisation of Baker's yeast with osmotic pretreatment

The total soluble protein and enzyme release from Baker's yeast (1.5%, dry weight, w/v) on ultrasonication at 80 W following osmotic pretreatment. The tables below present the data obtained for the release of total soluble protein, enzyme release and kinetic rate constant k.

Table C.19 Data for protein release on ultrasonication following osmotic pretreatment at different osmotic pressure using glycerol or NaCl solution

Protein	Glycerol solution; (mg/g)				NaCl solution; (mg/g)			
Time(min)	0.25 MPa	0.5 MPa	1MPa	5MPa	0.25 MPa	0.5 MPa	1MPa	5MPa
0	2.94	1.86	2.18	1.57	2.94	1.70	2.34	1.53
3	78.4	83.5	80.4	91.2	78.4	90.5	88.1	101.3
6	132	126	150	187	132	134	155	194
9	213	213	212	237	213	217	217	249
12	273	284	275	291	273	288	283	302
15	316	325	353	356	316	335	342	355
18	347	341	365	370	347	349	358	369
21	352	350	373	377	352	358	370	381

Table C.20a Data for invertase release on ultrasonication following osmotic pretreatment at different osmotic pressure using glycerol or NaCl solution

Invertase	Glycerol solution; (U/g X 10^4)				NaCl solution; (U/g X 10^4)			
Time(min)	0.25 MPa	0.5 MPa	1MPa	5MPa	0.25 MPa	0.5 MPa	1MPa	5MPa
0	0.19	0.14	0.10	0.19	0.14	0.12	0.14	0.16
3	0.69	1.03	1.16	0.77	0.76	1.00	1.22	0.86
6	1.57	1.52	1.84	1.45	1.51	1.54	2.03	1.45
9	1.93	2.17	2.61	1.70	1.96	2.23	2.75	2.09
12	2.37	2.98	3.03	2.56	2.38	2.81	3.34	2.60
15	2.78	3.19	3.53	2.74	2.77	3.26	3.69	2.76
18	3.09	3.43	3.77	3.00	3.04	3.39	3.84	3.05
21	3.16	3.45	3.83	3.05	3.11	3.42	3.87	3.11

Table C.20b Data for α-glucosidase release on ultrasonication following osmotic pretreatment at different osmotic pressure using glycerol or NaCl solution

α-glucosidase	Glycerol solution; (U/g X 10^5)				NaCl solution; (U/g X 10^5)			
Time(min)	0.25 MPa	0.5 MPa	1MPa	5MPa	0.25 MPa	0.5 MPa	1MPa	5MPa
0	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02
3	0.53	0.57	0.72	0.24	0.57	0.58	0.70	0.66
6	1.08	1.33	1.39	0.86	1.33	1.35	1.41	1.24
9	1.58	1.72	1.81	1.04	1.72	1.82	1.85	1.69
12	2.01	2.28	2.33	1.40	2.28	2.32	2.37	2.07
15	2.44	2.63	2.78	1.66	2.63	2.62	2.82	2.17
18	2.63	2.80	2.96	2.04	2.80	2.83	2.93	2.26
21	2.71	2.82	3.06	2.03	2.82	2.85	3.00	2.33

Table C.20c Data for ADH release on ultrasonication following osmotic pretreatment at different osmotic pressure using glycerol or NaCl solution

ADH	Glycerol solution; (U/g X 10^5)				NaCl solution; (U/g X 10^5)			
Time(min)	0.25 MPa	0.5 MPa	1MPa	5MPa	0.25 MPa	0.5 MPa	1MPa	5MPa
0	0	0	0	0	0	0	0	0
3	0.60	0.53	0.60	0.64	0.93	1.13	1.53	1.73
6	2.00	1.92	1.92	1.92	1.87	2.00	2.53	2.47
9	2.60	2.77	2.73	2.67	2.67	2.93	3.40	3.53
12	4.00	3.63	3.93	3.60	3.53	4.07	4.60	4.67
15	4.67	4.59	4.47	4.32	4.73	5.20	5.67	5.73
18	5.60	5.44	5.53	5.44	5.67	6.07	6.33	6.27
21	6.07	5.84	6.33	5.76	6.33	7.00	7.40	7.47

Table C.20d Data for G6PDH release on ultrasonication following osmotic pretreatment at different osmotic pressure using glycerol or NaCl solution

G6PDH	Glycerol solution; (U/g)				NaCl solution; (U/g)			
Time(min)	0.25 MPa	0.5 MPa	1MPa	5MPa	0.25 MPa	0.5 MPa	1MPa	5MPa
0	0	0	0	0	0	0	0	0
3	3.45	2.85	3.61	3.72	3.45	3.43	3.91	4.44
6	5.85	6.33	6.73	6.86	5.85	6.28	6.81	7.39
9	9.02	9.03	8.86	9.42	9.02	9.66	10.2	10.6
12	11.4	11.6	10.8	11.5	11.4	11.2	12.1	12.9
15	13.3	13.1	13.1	13.3	13.3	13.9	14.4	15.0
18	14.4	14.5	14.5	14.7	14.4	14.7	15.5	16.0
21	15.0	15.5	15.1	15.9	15.0	15.7	16.5	17.2

Table C.21a Kinetic rate constant (k) of protein and enzyme release on ultrasonication following osmotic pretreatment using glycerol solution

Glycerol	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2
0.25 MPa	0.08	0.9793	0.06	0.9928	0.06	0.9946	0.06	0.9842	0.05	0.9944
0.5 MPa	0.09	0.9571	0.08	0.9807	0.07	0.9930	0.06	0.9878	0.05	0.9938
1 MPa	0.09	0.9812	0.08	0.9850	0.07	0.9940	0.05	0.9830	0.05	0.9985
5 MPa	0.07	0.9587	0.06	0.9729	0.05	0.9826	0.06	0.9729	0.05	0.9724

Table C.21b Kinetic rate constant (k) of protein and enzyme release on ultrasonication following osmotic pretreatment using NaCl solution

NaCl	Protein		Invertase		α-glucosidase		ADH		G6PDH	
	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2	k (pass^{-1})	R^2
0.25 MPa	0.08	0.9787	0.06	0.9984	0.07	0.9983	0.06	0.9754	0.05	0.9910
0.5 MPa	0.08	0.9804	0.08	0.9909	0.07	0.9922	0.06	0.9872	0.06	0.9945
1 MPa	0.09	0.9938	0.08	0.9979	0.08	0.9893	0.06	0.9834	0.06	0.9963
5 MPa	0.07	0.9943	0.06	0.9896	0.06	0.9908	0.06	0.9856	0.06	0.9982

Appendix D
Effect of Pretreatment on High Pressure Homogenisation

Raw data for homogenisation of Baker's yeast following single and combined pretreatment

The total soluble protein and enzyme release from Baker's yeast (1.5%, dry weight, w/v) on high pressure homogenisation (HPH) at 27.6 and 41.4 MPa following pretreatment. The tables below present the data obtained for the release of total soluble protein and enzyme release and kinetic rate constant k.

Table D.1 Data for protein release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

Protein release (mg/g) on HPH at 27.6 and 41.4 MPa following heat pretreatment								
T_{max}	40℃	50℃					50℃	50℃
Holding	Minimal	Minimal					Minimal	5 min
Pressure	27.6 MPa	27.6 MPa					41.4 MPa	41.4 MPa
Passes	-	exp. 1	exp. 2	ave.	S.D.	C.V.	-	-
0	2.66	1.57	1.77	1.67	0.14	8.49	1.83	1.73
1	134	160	145	152	10.8	7.05	168	185
2	227	227	220	224	4.83	2.16	292	312
3	280	302	295	299	4.86	1.63	377	398
4	329	354	359	357	3.23	0.91	444	456
5	373	398	395	397	2.22	0.56	461	459
6	399	438	422	430	11.9	2.76	464	471
7	433	456	454	455	1.18	0.26	479	476
8	450	473	475	474	1.30	0.27	490	491
9	458	483	490	487	5.19	1.07	492	490
10	465	484	496	490	8.67	1.77	499	485

Protein release (mg/g) on HPH at 27.6 MPa following pH, osmotic and combined pretreatment												
Passes	pH pretreatment					Osmotic pretreatment					Heat and pH (T_{max}: 40 °C; pH_{max} 10)	Heat and osmotic (T_{max}: 40 °C, 1 MPa)
	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.		
0	3.54	3.22	3.38	0.23	6.67	2.37	2.73	2.55	0.26	10.2	1.63	2.93
1	132	113	123	13.3	10.9	95	111	103	11.4	11.1	138	138
2	195	179	187	11.4	6.11	161	170	166	6.25	3.77	243	233
3	287	251	269	25.4	9.44	247	230	239	12.5	5.24	307	284
4	316	308	312	6.08	1.95	281	291	286	7.38	2.58	355	335
5	350	345	348	3.02	0.87	311	317	314	4.55	1.45	389	388
6	362	372	367	7.02	1.91	334	335	335	0.49	0.15	425	409
7	382	404	393	15.2	3.87	343	366	355	15.8	4.47	460	445
8	413	422	417	5.76	1.38	373	386	379	9.24	2.44	465	460
9	428	434	431	4.67	1.08	397	395	396	1.96	0.49	477	465
10	445	442	443	2.40	0.54	401	405	403	2.64	0.66	478	470

Table D.2a Data for Invertase release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

Invertase release (U/g) X 10^4 on HPH at 27.6 and 41.4 MPa following heat pretreatment								
T_{max}	40°C	50°C					50°C	50°C
Holding	Minimal	Minimal					Minimal	5 min
Pressure	27.6 MPa	27.6 MPa					41.4 MPa	41.4 MPa
Passes	-	exp. 1	exp. 2	ave.	S.D.	C.V.	-	-
0	0.16	0.15	0.17	0.16	0.01	6.43	0.16	0.16
1	1.95	2.13	2.04	2.09	0.06	2.94	2.40	2.35
2	2.65	2.92	2.90	2.91	0.02	0.70	3.22	3.13
3	3.17	3.46	3.36	3.41	0.07	2.10	3.87	3.86
4	3.36	3.72	3.67	3.70	0.04	0.97	4.41	4.34
5	3.87	4.03	4.01	4.02	0.02	0.38	4.51	4.50
6	4.08	4.22	4.37	4.30	0.10	2.38	4.52	4.53
7	4.16	4.32	4.35	4.34	0.03	0.61	4.56	4.55
8	4.24	4.44	4.34	4.39	0.07	1.59	4.55	4.50
9	4.31	4.46	4.43	4.44	0.02	0.46	4.62	4.57
10	4.37	4.60	4.54	4.57	0.04	0.89	4.58	4.53

Invertase release (U/g) X 10^4 on HPH at 27.6 MPa following pH, osmotic and combined pretreatment												
Passes	pH pretreatment					Osmotic pretreatment					Heat and pH (T_{max}: 40°C; pH_{max} 10)	Heat and osmotic (T_{max}: 40°C, 1 MPa)
	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.		
0	0.09	0.12	0.10	0.03	24.4	0.14	0.15	0.14	0.01	7.07	0.12	0.16
1	1.87	1.87	1.87	0.00	0.00	1.66	1.74	1.70	0.06	3.30	2.17	1.72
2	2.46	2.36	2.41	0.07	2.96	2.15	2.03	2.09	0.08	3.91	2.85	2.71
3	2.88	2.82	2.85	0.04	1.43	2.54	2.44	2.49	0.07	2.67	3.39	3.09
4	3.26	3.24	3.25	0.01	0.31	3.03	2.94	2.98	0.06	2.05	3.75	3.50
5	3.47	3.81	3.64	0.23	6.45	3.30	3.24	3.27	0.04	1.25	4.04	3.97
6	3.79	4.17	3.98	0.27	6.67	3.62	3.58	3.60	0.03	0.71	4.20	4.07
7	3.95	4.25	4.10	0.21	5.23	3.78	3.74	3.76	0.03	0.68	4.37	4.14
8	4.10	4.34	4.22	0.17	4.12	3.98	3.95	3.97	0.02	0.52	4.41	4.26
9	4.27	4.43	4.35	0.11	2.58	4.11	4.09	4.10	0.02	0.37	4.49	4.47
10	4.40	4.54	4.47	0.10	2.28	4.21	4.25	4.23	0.03	0.72	4.54	4.52

Table D.2b Data for α-glucosidase release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

α-glucosidase release (U/g) X 10^5 on HPH at 27.6 and 41.4 MPa following heat pretreatment								
T_{max}	40°C	50°C					50°C	50°C
Holding	Minimal	Minimal					Minimal	5 min
Pressure	27.6 MPa	27.6 MPa					41.4 MPa	41.4 MPa
Passes	-	exp. 1	exp. 2	ave.	S.D.	C.V.	-	-
0	0.02	0.02	0.02	0.02	0.00	0.92	0.02	0.02
1	0.98	1.03	0.95	0.99	0.06	5.99	1.11	1.14
2	1.42	1.69	1.57	1.63	0.08	5.03	2.03	2.07
3	1.82	2.09	2.01	2.05	0.06	2.90	2.46	2.47
4	2.23	2.50	2.43	2.47	0.05	1.95	2.99	2.97
5	2.56	2.86	2.73	2.79	0.09	3.18	3.11	3.09
6	2.89	3.17	3.04	3.10	0.09	3.01	3.39	3.40
7	3.06	3.28	3.25	3.26	0.03	0.82	3.39	3.41
8	3.12	3.37	3.28	3.33	0.07	1.97	3.40	3.42
9	3.28	3.39	3.29	3.34	0.07	2.17	3.42	3.41
10	3.29	3.46	3.39	3.42	0.05	1.42	3.39	3.40

α-glucosidase release (U/g) X 10^5 on HPH at 27.6 MPa following pH, osmotic and combined pretreatment												
Passes	pH pretreatment					Osmotic pretreatment					Heat and pH (T_{max}: 40°C; pH_{max} 10)	Heat and osmotic (T_{max}: 40°C, 1 MPa)
	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.		
0	0.02	0.02	0.02	0.00	1.00	0.01	0.01	0.01	0.00	2.10	0.02	0.01
1	0.85	0.83	0.84	0.01	1.61	0.95	0.88	0.92	0.05	5.55	0.99	0.99
2	1.33	1.39	1.36	0.04	2.99	1.34	1.38	1.36	0.03	1.91	1.62	1.60
3	1.74	1.74	1.74	0.00	0.26	1.61	1.73	1.67	0.09	5.12	2.06	2.08
4	1.97	2.13	2.05	0.11	5.44	2.06	1.90	1.98	0.11	5.62	2.41	2.37
5	2.33	2.47	2.40	0.10	4.17	2.26	2.30	2.28	0.03	1.31	2.72	2.74
6	2.59	2.73	2.66	0.10	3.76	2.43	2.49	2.46	0.04	1.68	3.01	2.94
7	2.92	3.00	2.96	0.06	2.06	2.75	2.67	2.71	0.06	2.04	3.21	3.19
8	3.21	3.26	3.23	0.03	1.07	2.86	2.90	2.88	0.03	1.01	3.37	3.39
9	3.32	3.27	3.30	0.03	0.89	2.98	2.92	2.95	0.04	1.39	3.46	3.43
10	3.43	3.30	3.36	0.09	2.60	3.02	2.93	2.97	0.06	2.16	3.51	3.50

Table D.2c Data for ADH release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

ADH release (U/g) X 10^5 on HPH at 27.6 and 41.4 MPa following heat pretreatment								
T_{max}	40°C	50°C					50°C	50°C
Holding	Minimal	Minimal					Minimal	5 min
Pressure	27.6 MPa	27.6 MPa					41.4 MPa	41.4 MPa
Passes	-	exp. 1	exp. 2	ave.	S.D.	C.V.	-	-
0	0	0	0	0	0	0	0	0
1	1.60	1.67	1.60	1.63	0.05	2.89	1.80	1.83
2	3.27	3.60	3.40	3.50	0.14	4.04	4.50	4.57
3	4.50	4.73	4.73	4.73	0.00	0.00	5.67	5.50
4	5.17	5.53	5.47	5.50	0.05	0.86	6.47	6.43
5	5.67	6.07	6.40	6.23	0.24	3.78	6.97	7.17
6	6.50	6.60	7.47	7.03	0.61	8.71	7.67	7.77
7	7.03	7.20	7.87	7.53	0.47	6.26	8.03	8.10
8	7.90	7.93	8.27	8.10	0.24	2.91	8.43	8.57
9	8.33	8.33	8.67	8.50	0.24	2.77	8.80	8.67
10	8.77	9.00	8.93	8.97	0.05	0.53	8.83	8.77

ADH release (U/g) X 10^5 on HPH at 27.6 MPa following pH, osmotic and combined pretreatment												
Passes	pH pretreatment					Osmotic pretreatment					Heat and pH (T_{max}: 40°C; pH_{max} 10)	Heat and osmotic (T_{max}: 40°C, 1 MPa)
	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.		
0	0	0	0	0	0	0	0	0	0	0	0	0
1	1.53	1.37	1.45	0.12	8.13	1.57	1.53	1.55	0.03	1.94	1.40	1.33
2	2.97	2.77	2.87	0.14	4.93	2.73	2.63	2.68	0.07	2.64	3.00	3.07
3	4.27	4.40	4.33	0.09	2.18	3.73	3.50	3.61	0.16	4.40	4.50	4.40
4	4.83	5.03	4.93	0.14	2.87	4.05	4.13	4.09	0.05	1.30	5.40	5.37
5	5.83	5.77	5.80	0.05	0.81	4.38	4.45	4.41	0.05	1.20	6.00	5.95
6	6.33	6.27	6.30	0.05	0.75	5.00	4.88	4.94	0.09	1.79	6.63	6.57
7	6.50	6.60	6.55	0.07	1.08	5.60	5.25	5.43	0.25	4.56	7.33	7.16
8	7.00	7.27	7.13	0.19	2.64	5.75	5.95	5.85	0.14	2.42	7.90	7.87
9	7.50	7.57	7.53	0.05	0.63	6.05	5.98	6.01	0.05	0.88	8.37	8.43
10	8.17	8.00	8.08	0.12	1.46	6.38	6.25	6.31	0.09	1.40	8.77	8.63

Table D.2d Data for G6DH release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

G6PDH release (U/g) on HPH at 27.6 and 41.4 MPa following heat pretreatment								
T_{max}	40°C	50°C					50°C	50°C
Holding	Minimal	Minimal					Minimal	5 min
Pressure	27.6 MPa	27.6 MPa					41.4 MPa	41.4 MPa
Passes	-	exp. 1	exp. 2	ave.	S.D.	C.V.	-	-
0	0	0	0	0.0	0	0	0	0
1	6.4	6.6	6.4	6.5	0.14	2.10	7.25	7.45
2	9.2	10.2	10.0	10.1	0.10	1.01	13.0	13.3
3	12.3	13.5	13.3	13.4	0.14	1.02	15.4	15.0
4	14.8	16.0	15.8	15.9	0.14	0.86	18.7	18.9
5	17.0	17.8	18.3	18.0	0.38	2.08	20.5	20.4
6	18.6	19.4	19.7	19.5	0.24	1.22	21.2	21.2
7	20.7	21.1	21.3	21.2	0.14	0.64	22.5	22.7
8	22.1	22.7	22.6	22.7	0.07	0.30	23.5	23.6
9	23.3	23.7	23.9	23.8	0.14	0.57	23.9	23.8
10	23.6	24.2	24.2	24.2	0.03	0.14	24.2	24.3

G6PDH release (U/g) on HPH at 27.6 MPa following pH, osmotic and combined pretreatment												
Passes	pH pretreatment					Osmotic pretreatment					Heat and pH (T_{max}: 40°C; pH_{max} 10)	Heat and osmotic (T_{max}: 40°C, 1 MPa)
	exp. 1	exp. 2	ave.	S.D.	C.V.	exp. 1	exp. 2	ave.	S.D.	C.V.		
0	0	0	0	0	0	0	0	0	0	0	0	0
1	6.3	5.7	6.0	0.38	6.25	5.1	5.0	5.1	0.08	1.52	6.71	6.37
2	10.1	9.0	9.6	0.79	8.19	7.8	7.6	7.7	0.18	2.33	9.91	9.80
3	12.9	12.4	12.7	0.34	2.70	10.4	10.1	10.3	0.26	2.50	12.9	12.6
4	15.3	15.5	15.4	0.14	0.89	11.9	11.8	11.9	0.05	0.43	16.3	15.6
5	17.2	16.5	16.9	0.55	3.24	13.4	13.6	13.5	0.13	0.95	18.4	17.4
6	18.8	18.3	18.6	0.34	1.84	15.0	14.8	14.9	0.13	0.86	20.0	19.4
7	20.8	19.4	20.1	1.02	5.10	16.4	16.5	16.4	0.10	0.62	21.4	20.8
8	22.1	21.5	21.8	0.38	1.72	17.3	17.3	17.3	0.03	0.20	22.4	22.2
9	22.8	22.3	22.5	0.34	1.52	17.9	17.8	17.8	0.10	0.57	23.5	23.1
10	23.4	23.3	23.4	0.06	0.25	18.2	18.0	18.1	0.08	0.42	24.3	24.1

Table D.3 Kinetic rate constant (k) of protein and enzyme release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

Pretreatment	Pressure	Conditions	Holding	Protein		Invertase		α-glucosidase		ADH		G6PDH	
				k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2
Heat	41.4	T_{max}: 50℃	minimal	0.54	0.9924	0.70	0.9734	0.51	0.9671	0.36	0.993	0.38	0.9938
	41.4	T_{max}: 50℃	5 min	0.62	0.9890	0.69	0.9853	0.51	0.9595	0.37	0.9936	0.39	0.9834
	27.6	T_{max}: 40℃	minimal	0.31	0.9980	0.33	0.9853	0.31	0.9854	0.23	0.9942	0.26	0.9857
	27.6	T_{max}: 50℃	minimal	0.40	0.9863	0.41	0.9880	0.36	0.9826	0.28	0.9928	0.29	0.9922
pH	27.6	pH_{max}: 10	2 min	0.26	0.9965	0.30	0.9899	0.24	0.9858	0.21	0.9948	0.25	0.9972
Osmotic	27.6	Osmotic: 1 MPa	minimal	0.20	0.9902	0.23	0.9889	0.20	0.9944	0.13	0.9868	0.16	0.9943
Combined	27.6	T_{max}: 40℃; pH_{max}: 10	minimal	0.37	0.9926	0.39	0.9944	0.33	0.9871	0.28	0.979	0.31	0.9902
	27.6	T_{max}: 40℃; 1 MPa	minimal	0.34	0.9937	0.35	0.9898	0.32	0.9880	0.27	0.9764	0.28	0.9934

Table D.4 The volume mean diameter (D[4,3]) determined by Malvern size analyser

D[4,3] (μm) determined by Malvern size analyser					
Passes	Control (No pretreatment)			Heat and pH pretreatment	Heat and osmotic pretreatment
	27.6 MPa	41.4 MPa	69.0 MPa	27.6 MPa	27.6 MPa
0	6.08	6.08	6.08	5.69	5.67
1	5.88	5.65	5.59	5.61	5.62
2	5.73	5.46	5.39	5.53	5.52
3	5.66	5.34	5.30	5.54	5.16
4	5.62	5.39	5.19	5.52	5.38
5	5.63	5.35	4.89	5.48	5.33
6	5.53	5.30	-	5.53	5.33
7	5.48	5.20	-	5.53	5.40
8	5.4	5.08	-	5.58	5.33
9	5.26	5.13	-	5.53	5.37
10	5.22	5.11	-	5.57	5.39

Appendix E
Effect of pretreatment on *Kluyveromyces lactis* disruption

Raw data for homogenisation of *K. lactis* without pretreatment

The homogenisation of *K. lactis* (1.5%, dry weight, w/v) was performed at three pressures: 27.6, 41.4 and 69.0 MPa. The tables below present the data obtained for the release of total soluble protein and enzyme release.

Table E.1 Data for total soluble protein release on HPH of untreated *K. lactis*

Passes	27.6 MPa (mg/g)	41.4 MPa (mg/g)	69.0 MPa (mg/g)
0	1.22	1.15	1.13
1	17.6	61.1	99.3
2	52.7	118	173
3	79.0	167	261
4	105	204	304
5	132	255	353
6	149	282	387
7	169	308	431
8	191	349	441
9	211	365	445
10	226	373	447

Table E.2 Data for enzyme release on HPH of untreated *K. lactis*

Passes	41.4 MPa				69.0 MPa			
	Invertase (U/g) X 10^4	ADH (U/g) X10^5	G6PDH (U/g)	β-galactosidase (U/g) X10^3	Invertase (U/g) X 10^4	ADH (U/g) X10^5	G6PDH (U/g)	β-galactosidase (U/g) X10^3
0	0.02	0	0	0	0.02	0	0	0
1	0.71	0.79	2.87	0.59	0.97	1.31	4.10	0.74
2	1.29	1.71	5.63	0.85	1.77	3.33	7.83	1.71
3	1.86	2.43	7.08	1.59	2.70	4.37	9.50	2.29
4	2.28	3.53	9.81	2.22	3.11	5.04	11.65	2.73
5	2.63	4.05	11.81	2.88	3.58	5.67	13.60	3.54
6	2.86	5.00	13.05	3.29	3.74	6.35	15.90	4.26
7	3.13	5.29	14.54	3.89	3.77	7.02	18.08	4.67
8	3.23	5.54	15.26	4.11	3.86	7.34	20.58	5.23
9	3.34	5.92	16.61	4.37	3.88	7.70	20.53	5.72
10	3.35	6.21	17.11	4.59	3.93	7.79	20.85	5.82

Raw data for homogenisation of *K. lactis* following single and combined pretreatment

The total soluble protein and enzyme release from *K. lactis* (1.5%, dry weight, w/v) on high pressure homogenisation at 27.6 and 41.4 MPa following pretreatment. The tables below present the data obtained for the release of total soluble protein and enzyme release and kinetic rate constant k.

Table E.3 Data for protein release on HPH at 27.6 and 41.4 MPa following single and combined pretreatment

Passes	Single pretreatment					Combined pretreatment	
	50°C heat pretreatment	40°C heat pretreatment	50°C heat pretreatment	pH pretreatment	Osmotic pretreatment	heat and pH pretreatment	heat and osmotic pretreatment
	27.6 MPa	41.4 MPa	41.4 MPa	41.4 MPa	41.4 MPa	41.4 MPa	41.4 MPa
0	0.63	0.70	0.59	1.03	1.21	0.76	0.72
1	50.2	86.3	96.1	61.6	63.5	90.7	59.1
2	94.7	161	184	141	128	171	140
3	127	237	247	188	173	235	228
4	158	281	292	231	215	276	259
5	183	324	346	263	256	337	330
6	215	361	381	299	286	373	349
7	228	396	409	351	314	390	377
8	251	415	422	379	366	419	397
9	263	422	432	393	380	425	420
10	293	424	433	400	383	429	426

Table E.4a Data for invertase release on HPH at 41.4 MPa following single and combined pretreatment

Invertase (U/g) X10^4	40°C heat	50°C heat	pH	Osmotic	heat and pH	heat and osmotic
0	0.01	0.01	0.01	0.02	0.01	0.01
1	0.74	0.79	0.66	0.67	0.77	0.67
2	1.39	1.49	1.24	1.26	1.56	1.30
3	1.99	2.13	1.86	1.80	1.97	1.99
4	2.28	2.45	2.13	2.07	2.39	2.27
5	2.71	2.92	2.54	2.46	2.75	2.76
6	3.22	3.29	2.98	2.77	3.22	3.26
7	3.30	3.42	3.20	3.07	3.41	3.33
8	3.52	3.55	3.33	3.21	3.60	3.57
9	3.54	3.68	3.46	3.34	3.67	3.59
10	7.34	7.38	7.04	6.76	7.43	7.47

Table E.4b Data for ADH release on HPH at 41.4 MPa following single and combined pretreatment

ADH (U/g) X10^5	40°C heat	50°C heat	pH	Osmotic	heat and pH	heat and osmotic
0	0	0	0	0	0	0
1	1.13	1.26	0.95	0.95	1.22	1.26
2	2.34	2.43	2.13	1.89	2.70	2.66
3	3.51	3.74	3.07	2.84	3.42	3.65
4	4.19	4.32	3.87	3.78	4.37	4.19
5	4.55	4.82	4.49	4.54	4.95	4.64
6	5.22	5.40	5.29	5.15	5.40	5.45
7	5.85	6.12	5.72	5.62	6.17	5.90
8	6.26	6.44	6.19	5.91	6.57	6.35
9	6.84	7.07	6.66	6.43	6.89	7.11
10	7.34	7.38	7.04	6.76	7.43	7.47

Table E.4c Data for G6PDH release on HPH at 41.4 MPa following single and combined pretreatment

G6PDH (U/g)	40°C heat	50°C heat	pH	Osmotic	heat and pH	heat and osmotic
0	0	0	0	0	0	0
1	3.95	4.12	3.72	2.91	3.68	3.85
2	6.18	6.05	5.93	5.71	5.93	6.27
3	7.35	8.01	7.31	7.18	7.00	7.41
4	10.44	11.12	10.54	10.22	10.63	10.44
5	12.48	12.88	12.30	11.88	12.48	12.18
6	13.62	14.25	13.36	13.05	13.50	13.86
7	15.30	15.81	15.27	15.11	15.80	15.26
8	16.33	16.78	15.98	15.65	16.42	16.36
9	17.32	18.09	17.03	16.57	17.80	18.20
10	18.83	19.28	18.51	17.61	19.48	19.50

Table E.4d Data for β-galactosidase release on HPH at 41.4 MPa following single and combined pretreatment

β-galactosidase (U/g) $X10^3$	40°C heat	50°C heat	pH	Osmotic	heat and pH	heat and osmotic
0	0	0	0	0	0	0
1	0.69	0.70	0.68	0.61	0.76	0.72
2	1.12	1.32	1.21	0.94	1.21	1.15
3	1.74	1.97	1.98	1.70	1.81	1.78
4	2.36	2.48	2.44	2.29	2.41	2.47
5	3.02	3.13	3.08	3.07	3.15	3.07
6	3.50	3.55	3.49	3.51	3.61	3.55
7	4.25	4.30	4.26	4.10	4.38	4.41
8	4.60	4.67	4.60	4.37	4.64	4.60
9	5.03	5.14	4.92	4.75	5.12	5.08
10	5.44	5.50	5.31	4.92	5.47	5.46

Table E.5 Kinetic rate constant (k) of protein and enzyme release on HPH following single and combined pretreatment

Pretreatment	Pressure (MPa)	Conditions	Holding	Protein		Invertase		α-glucosidase		ADH		G6PDH	
				k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2	k $pass^{-1}$	R^2
Heat	27.6	T_{mas}: 50℃	Minimal	0.26	0.9673	0.28	0.9929	0.19	0.9822	0.20	0.9919	0.19	0.9718
	41.4	T_{mas}: 40℃	Minimal	0.26	0.9756	0.31	0.9917	0.21	0.9674	0.20	0.9898	0.20	0.9547
	41.4	T_{mas}: 50℃	Minimal	0.29	0.9643	0.36	0.9905	0.22	0.9756	0.23	0.9834	0.21	0.9592
pH	41.4	pH_{max}: 10	2 min	0.25	0.9673	0.28	0.9929	0.19	0.9822	0.20	0.9919	0.19	0.9718
Osmotic	41.4	Osmotic: 1 MPa	Minimal	0.23	0.9609	0.25	0.9951	0.18	0.9931	0.19	0.9934	0.18	0.9842
Combined	41.4	T_{mas}: 40℃; pH_{max}: 10	Minimal	0.28	0.985	0.34	0.9891	0.22	0.9629	0.21	0.9800	0.21	0.9569
	41.4	T_{mas}: 40℃; 1 MPa	Minimal	0.28	0.981	0.33	0.9921	0.21	0.9633	0.21	0.9887	0.21	0.9533

Table E.6 The volume mean diameter (D[4,3]) determined by Malvern size analyser

Passes	D[4,3] (μm) determined by Malvern size analyser				
	Control (No pretreatment)			Heat and pH pretreatment	Heat and osmotic pretreatment
	27.6 MPa	41.4 MPa	69.0 MPa	41.4 MPa	41.4 MPa
0	3.54	3.54	3.54	3.61	3.57
1	3.43	3.39	3.22	3.58	3.51
2	3.46	3.35	3.17	3.51	3.53
3	3.43	3.27	3.14	3.55	3.51
4	3.37	3.22	3.09	3.44	3.35
5	3.35	3.16	3.06	3.39	3.46
6	3.35	3.13	2.99	3.41	3.47
7	3.32	3.08	2.98	3.40	3.43
8	3.36	2.94	2.91	3.42	3.36
9	3.38	2.99	2.85	3.43	3.33
10	3.32	2.91	2.90	3.38	3.29