Cell Disruption: Breaking the Mould: An Overview of Yeast and Bacteria High-Pressure Cell Disruption

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Cell Disruption plays a key part in the isolation and preparation of intra-cellular products for biopharma-ceutical technology. Many biotech-nology fields, especially recombinant technology, demand efficient, temperature-controlled, repeatable cell disruption systems. The prepara-tion of many biological products requires cell disruption to release intracellular material. *Saccharomyces cerevisiae, Pichia pastoris* and *Escherichia coli* have been tested to show percentage soluble protein release and to offer an overview of high-pressure cell disruption.

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Introduction

The disruption of cells is an important stage in the isolation and preparation of intracellular products for biopharmaceutical technology. From research levels through to production, many areas of biotechnology, particularly recombinant technology, necessitate the use of efficient, temperature controlled, and repeatable cell disruption systems.

This is especially true for commercial operations whereby product recovery yields and scalability are vital to the successful development and manufacture of a product.

Although some biological products are secreted from the cell or released during autolysis, the preparation of many others requires cell disruption to release intracellular material[1].

Yeasts, gram-positive bacteria and gram-negative bacteria to a lesser extent, have considerably harder cell walls in comparison to animal cells and quite extreme conditions are required at the cell disruption stage. The use of extremely high pressure has been successfully developed to achieve the optimum conditions for cell lysis to take place.

Testing *Saccharomyces cerevisiae, Pichia pastoris* and *Escherichia coli* has been performed at the University of Wales, Swansea and another confidential source to illustrate percentage soluble protein release and to give an overview of high-pressure cell disruption.

Principles and Background

Cell disruption focuses on obtaining the desired product from within the cell, and it is the cell wall that must be disrupted to allow the contents of the cell out. The cell wall conveys its strength to the cell and can be formed from differing kinds of complex polysaccharides which are generally cross-linked by peptides to a degree and give different organisms varying levels of strength[2].

In essence the objectives of cell disruption are as follows:

1. To solubilise the maximum amount of the product present in the cell whilst still maintaining maximum biological activity.
2. To avoid secondary alteration of the product that will render it useless e.g denaturisation and oxidation
3. To limit the detrimental effects of the disruption stage on the following separation steps

A wide range of techniques have been developed in trying to achieve the above three objectives. These can be grouped into two categories 'mechanical' and 'non-mechanical' as in figure 1.0.

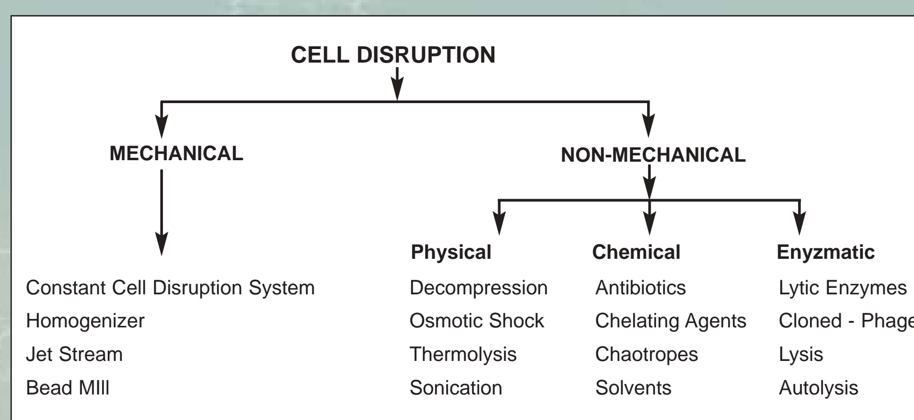

Figure 1.0

The article will focus on mechanical methods specifically high-pressure means of cell disruption. High-pressure cell disruption is often confused as the same process as homogenisation, however it is not, due to the difference in mechanical design, principle and final outcome.

This is also emphasised in the pure definition of disruption and homogenisation: 'Disruption' – to break apart or bring disorder to. 'Homogenise' - to make the sample consist of parts all of the same kind; to produce a homogeneous fluid'[1].

Homogenisers, and arguably high-pressure cell disrupters, originate from the dairy industry. The dairy industry still use these today to decrease and disperse the size of lipid globules in milk. If steps are not used lipid globules rise to the top of the milk as cream[3]. Based on processing for the food industry applications have been found in biopharmaceuticals.

High-pressure cell disrupters and homogenisers are both positive displacement pumps however each differs in the way they create pressure and transfer the sample from one pressurised chamber to another chamber at lower pressure.

High-pressure cell disruption systems developed by Constant Systems Ltd (Daventry, UK) use a hydraulic mechanism that acts on a piston seal within a cylinder to force the sample through a fixed orifice to a chamber of lower pressure. The sample is not released, but accelerated and forced through an orifice up to speeds of 550 m/sec and achieving pressures up to 40 kpsi or 2,700 Bar.

The electrically controlled hydraulic system and fixed orifice guarantee the disruption environment is repeatable between operating intervals. The precision high pressures ensure greater yielding breakage of the hardest micro-organisms.

Homogenisers, on the other hand, pressurise the sample in a chamber (via a crank shaft mechanism or compressed gas) and then release it through a manually or automatically controlled valve (homogenising valve) into another chamber. Traditionally these cannot go to extremely high pressures (40 kpsi or 2,700 Bar) necessary to disrupt hard cell walled micro organisms and due to the homogenising valve and pressure creation mechanism, variability over the pressure can have implications on percentage breakage and repeatability between operating intervals.

Cell disruption

Saccharomyces cerevisiae, Pichia pastoris and *Escherichia coli* have long been used in the biopharmaceutical process as each has reasonably well known genetic systems and act as a good host to produce desired intracellular material such as proteins.

This process relies on the ability to extract the valuable contents of the cells in a swift and efficient way. Yeast cells are regarded as particularly hard to break, often needing multiple passes to achieve the high disruption rates required. The Constant Systems method of breakage has been used widely with yeast and *Escherichia coli*. Below are results obtained from a proportion of this work.

Measuring cell disruption

To have a definite base line for evaluation, measurement of cell disruption is imperative. Measuring the efficiency of disruption can be done in several ways. A visual count of disruption can be seen physically under a microscope although this is not accurate and does not guarantee a thorough observation. For this series of investigations a protein assay was used, this is widely recognised as a good measurement of cell disruption. The method measures the amount of protein released after disruption. The mechanically disrupted cells are then tested and checked against this number for percentage breakage.

There are several types of protein assay but for these tests the Folin Reaction (Lowry Assay) method is often used which is comparatively simple and consistent through out results. This is a colorimetric method and has a sensitivity to protein of around 8_g/ml in the assay solution. The assay turns blue in the presence

of proteins due to the reaction of copper ions in the alkaline solution with protein and the reduction of phosphomolybdate-phosphotungstic acid in the Folin reagent by aromatic amino acids in the treated protein[2].

Fractional protein release, Rp, is calculated using the following equation and multiplying the result by 100:

$$R_p = \frac{C_f - C_b}{C_t - C_b}$$

C_f = Free protein
C_t = Total protein
C_b = Background protein

This gives the actual disruption percentage taking into account the background levels of protein before disruption.

Controlling temperature during cell disruption

Another important factor in cell disruption is the inactivation or denaturisation of the contents of the cells due to temperature rise. Due to the extreme conditions present at high pressures, various equipment have design issues with temperature control.

In other homogenisers such as the French Press, energy used to produce the high pressure is released as heat due to compression and frictional forces as the fluid passes through the valve. The fluid temperature rises by 1-2 ˚C for every 1,000 psi to which the sample is subjected[4].

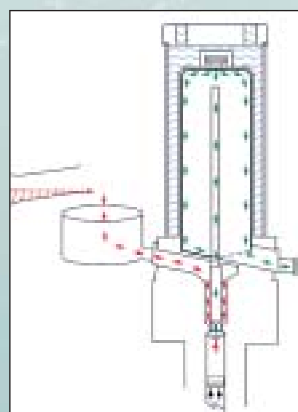

Figure 1.1: Cell disrupter and built-in cooling jacket

Tests have been made using the Constant Systems cell disrupter and standard built-in cooling jacket (Figure 1.1). A cooling chamber whereby coolant is circulated through surrounds the entire disruption head, giving a large surface area for cooling exchange to take place. Although the machine reaches high pressures up to 40 kpsi or 2,700 Bar, the energy is imparted in the sample and retained as kinetic energy in a 'jet'. The energy is then dissipated as the product slows down on the cooled surfaces of the chamber. Temperature is measured by a thermocouple positioned at the outlet and is digitally displayed on the control panel so the operator has visibility throughout the process.

The following results in figure 1.2 were obtained when testing this principle with a Z Plus Series 1.1 kW model. This shows a model of the temperature throughout the disruption cycle.

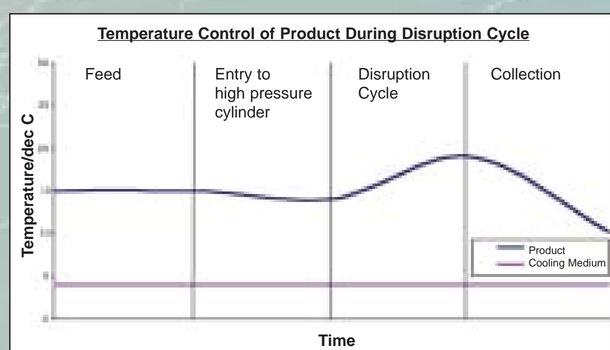

Figure 1.2: Graph of sample temperature during one disruption cycle on a continuous processing model

Yeast cell disruption

Two yeast species were investigated in this example; these being bakers' yeast (*Saccharomyces cerevisiae*), and *Pichia pastoris*. The bakers yeast was obtained from a commercial supplier as a block of fresh yeast, and *Pichia pastoris* was grown on standard YPD medium (Yeast extract 1%, Mycological peptone 2%, Glucose 2%, PH5.5).

Production of cells

The batches were grown for 48 hours at 27 ˚C in a 10 litre airlift fermenter. Both species were harvested and suspended in 25 mM phosphate buffer, (PH 7.0). Bakers' yeast also underwent a diafiltration stage to wash the cells before suspension. This was to free the cells of the majority of peptides present to concentrate the volumes. The cells (concentration 15-20 g/l) were then disrupted.

Procedure

Disruption was conducted using the Constant Systems Z-plus 1.1 kW model cell disrupter.

The cells were cooled to 4 ˚C prior to disruption and 100 ml of each cell suspension was passed through the machine at selected pressures. The first 50 ml of each cycle was discarded to avoid any risk of contamination or dilution from washing cycles with distilled water. The second 50 ml was collected in a bottle and placed on ice immediately.

Results

Table 1.0 and figure 1.3 show disruption percentages for *Pichia pastoris* with one pass to be 76% at 30 kpsi and increasing to 87% at 40 kpsi. If the sample is passed through a second pass disruption percentages further increase to 100% at 30 kpsi.

Bakers yeast has complete disruption (100%) at 35 kpsi and 40 kpsi. Due to high percentage disruption of Bakers yeast, more than one pass was not necessary.

Pressure Bar & kpsi	Pichia pastoris (1 pass)	Pichia pastoris (2 passes)	Bakers Yeast S. cerevisiae
2700 (40kpsi)	87	N/A	100
2400 (35kpsi)	N/A	N/A	100
2050 (30kpsi)	76	100	89
1700 (25kpsi)	N/A	N/A	85
1400 (20kpsi)	53	78	81
1000 (15kpsi)	N/A	N/A	62
700 (10kpsi)	22	39	43
350 (5kpsi)	7	15	19
170 (2.5kpsi)	5	9	6

Table 1.0

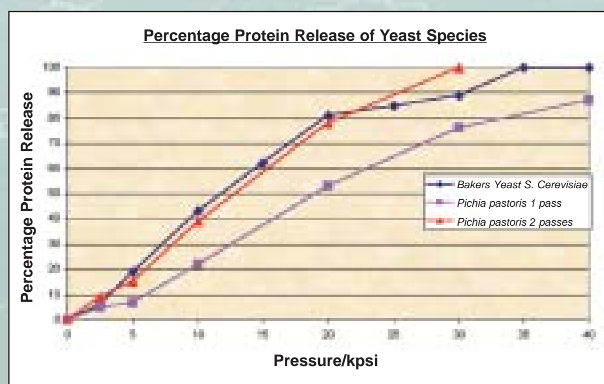

Figure 1.3

Conclusion

The disruption profiles indicate a high percentage disruption rate for both species of yeast tested. *Pichia pastoris* is a significantly harder organism to break in comparison to *Saccharomyces cerevisiae*.

Although a high level of breakage was recorded with *Pichia pastoris* in one pass (87%), it is evident that for 100% two passes are required. It can be concluded that with a second pass of *Pichia pastoris* pressures above 30 kpsi are not required due to 100% being attained.

Saccharomyces cerevisiae disruption profile illustrates a high protein release at lower pressures reaching 100% percentage protein release at 35 kpsi.

It can be concluded that *Saccharomyces cerevisiae* and *Pichia pastoris* are effectively disrupted giving high yields of protein release using pressures between 35 kpsi and 40 kpsi.

Escherichia coli cell disruption

Production of cells

The disrupter was cleaned prior to use with a 2% Virkon solution in order to prevent contamination from previous use. 250 ml of 2% Virkon solution was passed through the machine at 40 kpsi. The system was then flushed with 250 ml distilled water.

The sample was prepared from a 24-hour culture in Nutrient Broth (CM 67). This was then separated into 7 x 50 ml aliquots in order to test at each pressure setting.

Procedure

The pressure settings used were 15, 20, 25, 30, 35 and 40 kpsi. The samples were passed through the machine separately starting with 40kpsi and working downwards through the pressures.

Results/conclusion

The disruption profile for *Escherichia coli* indicated low protein release percentage up to a 20 kpsi pressure setting, however a dramatic rise up to over 99% was realised with the higher pressures (35-40 kpsi). The difference in protein release between 35 kpsi and 40 kpsi is minimal therefore it is concluded that 35 kpsi be the maximum pressure used for *Escherichia coli*.

Pressure/kpsi	Protein Yield/%
2700 (40kpsi)	99.99
2400 (35kpsi)	99.93
2050 (30kpsi)	83
1700 (25kpsi)	50
1400 (20kpsi)	40
1000 (15kpsi)	16.6
700 (10kpsi)	10.7

Table 1.0

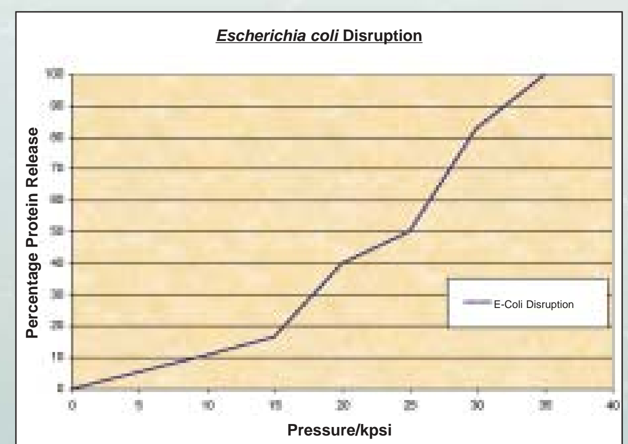

Figure 1.4

Discussion

For optimal cell lysis conditions, a high yielding and temperature controlled cell disruption stage needs to take place.

The high-pressure cell disrupter demonstrated superior proportions of soluble protein release from three species of yeast and bacteria respectively. Importance here is placed on the ability to disrupt the tough organism *Pichia pastoris* and receive 100% percentage soluble protein release with two passes and 100% with *Saccharomyces cerevisiae* and *Escherichia coli* with one pass. Optimise Testing has demonstrated pressure versatility of the cell disrupter. Being able to disrupt at specific pressure settings with a high degree of control has a significant impact on characterisation of pressure vs. soluble protein release. As cell wall strengths differ (if only slightly) between growth mediums and length of growth cycles, having the ability to control different pressures accurately gives the operator an improved disruption tool when optimising the process.

However, having high percentage breakage and prominent yields alone is not enough; for being able to operate in continuous processing mode whilst maintaining temperature control is fundamental. It can be seen that a high surface area for cooling exchange is important in maintaining a controlled temperature throughout the disruption cycle.

Constant Systems (Daventry, UK) offer modern high-pressure cell disruption solutions to traditional methods with efficiency, automation and control.

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