Preface

Considerable effort and time is allocated to introducing cell culture and fermentation technology to undergraduate students in academia, generally through a range of courses in industrial biotechnology and related disciplines. Similarly, a large number of textbooks are available to describe the applications of these technologies in industry. However, there has been a general lack of appreciation of the significant developments in downstream processing and isolation technology, the need for which is largely driven by the stringent regulatory requirements for purity and quality of injectable biopharmaceuticals. This is particularly reflected by the general absence of coverage of this subject in many biotechnology and related courses in educational institutions.

For a considerable while I have felt that there is increasing need for an introductory text to various aspects of downstream processing, particularly with respect to the needs of the biopharmaceutical and biotechnology industry. Although there are numerous texts that cover various aspects of protein purification techniques in isolation, there is a need for a work that covers the broad range of isolation technology in an industrial setting. It is anticipated that *Downstream Processing of Proteins: Methods and Protocols* will play a small part in filling this gap and thus prove a useful contribution to the field. It is also designed to encourage educational strategists to broaden the coverage of these topics in industrial biotechnology courses by including accounts of this important and rapidly developing element of the industrial process. The hope is that this will result in graduates having a reasonable understanding of downstream processing principles and techniques, and thus be better prepared to fulfill the ever-increasing demand for competent isolation scientists in industries.

This is, of course, achieved with the help of the dedicated contributing authors of *Downstream Processing of Proteins: Methods and Protocols*, without whose willingness to contribute and patience it would not have been possible. I would also like to thank the Humana Press and Prof. John Walker (the series editor) for their encouragement and prompt feedback. My thanks are also due to the Medeva Pharma Development management for providing me with the time and opportunity to fulfill this task, and without whose support it would have been impossible. Finally, I wish to thank my wife, children, and family members for allowing me to persevere with my editing activities in perhaps what should have been their time.

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Microbial Cell Disruption by High-Pressure Homogenization

Anton P. J. Middelberg

1. Introduction

The disruption of a cell's wall is often a primary step in product isolation, particularly when hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*, which generally do not excrete product, are employed. Of the available methods, high-pressure homogenization is dominant at moderate or large process volumes.

The high-pressure homogenizer is essentially a positive-displacement pump that forces cell suspension through a valve, before impacting the stream at high velocity on an impact ring. Operating pressures range up to 1500 bar. Several valve designs are available, but cell-disruption applications (as opposed to fat-globule dispersion) generally utilize a tapered cell-disruption design (*see* **Fig. 1**). The mechanism of disruption is still a matter of some debate ***(1,2)*** and of little concern in the current context. Disruption performance is, however, optimized by maintaining small valve gaps and hence high-velocity jets, with short impact-ring diameters. As complete disruption is rarely achieved with a single homogenizer pass, multiple passes are often employed.

This chapter describes some practical issues surrounding microbial cell disruption, and highlights issues not discussed extensively in the general scientific literature. It will, therefore, be of most use to those inexperienced with homogenization, or those with a practical focus. For more detailed information, the reader is referred to reviews, which provide pointers to the literature and information on other methods of cell disruption ***(2–4)***.

The structure addresses four major themes. Equipment layout is discussed in some detail. A simple method for cell disruption is then provided, and issues that affect performance are discussed in the Notes section. Some time is also

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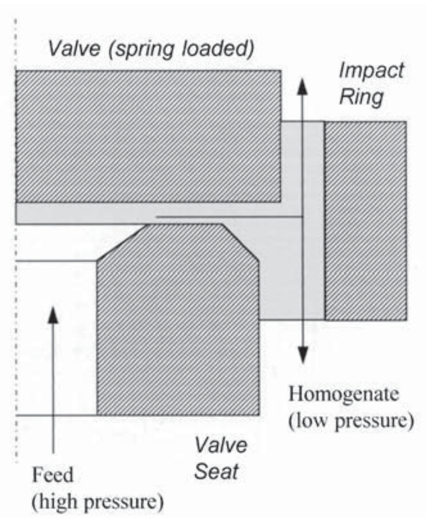

Fig. 1. Cross-section of a typical high-pressure homogenizer valve for use in cell-disruption applications.

spent discussing the analysis of both disruption and debris size. The latter is a particularly difficult problem, and often not of concern in laboratory settings. At process scale, however, a reasonable quantification of debris size is critical for optimal process design and operation.

2. Materials

2.1. Solutions and Reagents

Buffer may be required to dilute the cell suspension prior to homogenization. Buffer choice depends on product stability. Redox reagents may be required in the buffer to prevent the oxidation of certain products. Similarly, improved yields of soluble protease-sensitive proteins can be obtained by the addition of appropriate inhibitors. For stable products such as inclusion bodies, the use of a simple buffer such as 50 m*M* phosphate pH 7.4 is often acceptable. Where the fermentation broth is not concentrated prior to homogenization, simple dilution with water may prove adequate. For analysis of disruption, reagents for soluble protein quantitation are required. The Bradford dye-binding assay *(5)* is widely employed. This is now available as a commercial kit (Bio-Rad Laboratories, Hercules, CA).

2.2. Equipment and Layout

It should be stated at the outset that there is no universally optimal homogenizer system design. The final design depends very much on the scale of

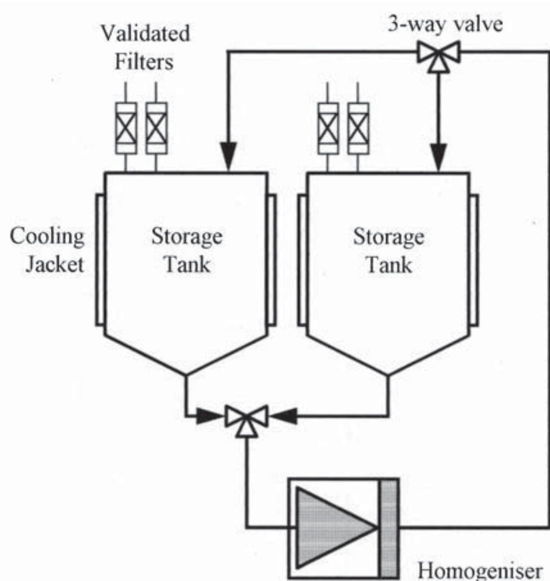

Fig. 2. Example of a high-pressure homogenization system.

application, the need for process cleanability with or without equipment disassembly, containment requirements, and process validation considerations (*see* **Notes 1** and **2**).

Several manufacturers such as APV-Rannie (Copenhagen, Denmark), Niro-Soavi (Parma, Italy), and APV-Gaulin (Wilmington, MA) offer competing homogenizer designs. Features include double-seal arrangements to prevent accidental release to cooling water, steam-sterilization of the high-pressure side, and simplified high-pressure delivery systems to facilitate cleaning. For laboratory-scale work, small homogenizers such as the APV-Gaulin 15 MR are well suited to processing typical fermentation volumes (e.g., 2–10 L) rapidly and efficiently. These often require disassembly after use for thorough cleaning of the valve assembly (*see* **Note 2**).

Figure 2 shows a typical layout for a cell-disruption system based on high-pressure homogenization. Two storage tanks are employed as improved disruption efficiency is achieved by operating a discrete-pass strategy, where the homogenizer feed is drawn from one tank, whereas the homogenate is passed to the other. The location of the three-way valves enables feed to be drawn from, and fed to, either tank. The feed and homogenate tanks, therefore, alternate for multiple-pass strategies (the normal operational mode). As homogenization generates considerable heating of the suspension (typically 2.5°C per 10 MPa of operating pressure), the tanks are jacketed and cooled at 5°C. Additional heat-transfer capacity can be obtained by including internal cooling

coils or an in-line heat exchanger, but with considerably more difficulty in final cleaning. For most laboratory or pilot-scale applications, the external jacket should suffice. Suspension of the tank contents is important, particularly where storage before subsequent processing is required. Tanks fitted with stirrers are ideal but expensive, and cleanability is again an issue. In laboratory settings, effective suspension can be obtained using a recirculation loop on each tank. This can be conveniently implemented using, for example, a double-headed peristaltic pump to simplify cleanability.

2.3. Cell Disruption, Analysis of Disruption, and Analysis of Debris Size

The procedures described here are defined as simply as possible, to rely on standard equipment available in most biological laboratories. Specifically, cell-disruption analysis will require access to a microscope (preferably with phase-contrast optics) and a spectrophotometer. The spectrophotometer is used in conjunction with a dye-binding assay such as the Bradford assay ***(5)*** to estimate the released protein concentration, and hence the extent of cell disruption. This assay is now available as a commercial kit (Bio-Rad).

3. Method

3.1. Cell Disruption

1. With reference to **Fig. 2**, load the cells to be disrupted into one tank. The cell suspension can be the fermentation broth without pretreatment, or may be preconcentrated and resuspended (e.g., by filtration or centrifugation) if removal of the fermentation media or volume-reduction is required.
2. Adjust the cell broth to an appropriate concentration by dilution with a suitable buffer. Cell concentration can vary considerably as disruption efficiency is essentially independent of this parameter although analysis is complicated at higher concentrations (*see* **Notes 3** and **4**). Dilution may be unnecessary if the fermentation broth is not concentrated prior to disruption. Buffer choice is dictated by the stability of the product being released, as homogenization efficiency is relatively insensitive unless specific pretreatments such as EDTA-containing buffers are employed (*see* **Notes 5–7**).
3. Take a small sample of the feed cells for microscopy (*see* **Subheading 3.2.**) and for protein estimation. Sediment the cells and determine the supernatant protein concentration using the protein estimation kit (full instructions are provided in the kit). Alternatively determine the concentration of the specific product of interest. For concentrated feed suspensions, correct the protein concentration for volume fraction (*see* **Note 4**). This value is the feed protein concentration and is a measure of the initial cell disruption (e.g., by upstream units or pretreatments).
4. Switch the three-way valves to feed material from the tank containing the material to be homogenized, with homogenizer discharge set to the other tank.

5. Connect the cooling-water supply to the homogenizer and ensure it is switched on. Connect and switch on other utilities as required for the specific homogenizer design (e.g., steam).
6. Commence homogenization with the operating pressure set to zero. Watch the pressure rise on the instrument gauge to ensure a flow path is available, especially if the homogenizer is not fitted with a high-pressure cutout.
7. Cautiously adjust the operating pressure to the desired value, watching for system problems (e.g., seal leaks, etc.).
8. Allow disruption to proceed while monitoring the system. Ensure an adequate supply of feed by monitoring the tank level.
9. When the feed supply runs low, release the homogenizer pressure back to zero and shut off the system (a system of tank-level detectors coupled to an alarm or a homogenizer shutoff system is advisable).
10. Determine the extent of cell disruption (*see* **Subheading 3.2.**).
11. Allow the homogenate to cool to the desired temperature, and then repeat the above procedure as necessary until the desired performance criterion is met (adequate cell disruption, maximum product release, or acceptable debris size).
12. Thoroughly clean and disinfect the system, using installed clean-in-place systems and adequate flushing. Dismantle and clean, if necessary, after chemical sterilization.

3.2. Analysis of Disruption

1. Analysis of disruption is desirable as soon as possible after cell disruption. Viable cells will remain in the broth after disruption, and these may multiply using substrate available from the disrupted cells (sample fixation using, e.g., 0.2% formaldehyde can inhibit this growth without compromising disruption estimation).
2. Observe the homogenate sample using a phase-contrast or bright-field microscope. Compare with the feed sample to qualitatively estimate the extent of cell disruption. Phase-contrast optics facilitate cell-debris observation, also providing qualitative information on debris size and its impact on subsequent processing.
3. Sediment a sample of the homogenate and determine the protein concentration in the supernatant using the protein estimation kit again. Alternatively, measure the specific product of interest (*see* **Notes 8** and **9**). For concentrated feed suspensions, correct the protein concentration for volume fraction (*see* **Note 4**).
4. Compare the supernatant protein concentration (C_n), with that for the previous homogenizer pass (C_{n-1}), and decide whether further homogenizer passes are required. When protein concentration reaches a plateau or begins to decline, then homogenization should be terminated. Note that a decrease in protein concentration indicates product loss through inactivation ***(6)***. The final plateau value estimates the maximum protein concentration achievable (C_{max}). This may be used to estimate the fractional release of protein after each homogenizer pass (C_n/C_{max}). This ratio, expressed as a percentage, is the simplest measure of cell disruption, although in the strictest sense disruption can only be quantitated using a direct method (*see* **Notes 8** and **9**).

3.3. Analysis of Debris Size

1. Obtain a qualitative assessment of debris size using the phase-contrast microscope. Latex standards of defined size may be incorporated into the sample if calibration is required.
2. Decide whether a quantitative assessment of debris is required (e.g., for process optimization). Several methods are available, but all are either tedious or prone to error (*see* **Note 10**). Cumulative sedimentation analysis (CSA) is a recently developed method that overcomes the limitations of other methods and requires only equipment available in a standard laboratory (e.g., centrifuge with swing-out rotor, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), scanning densitometry) (*see* **Note 11**). It involves centrifuging the homogenate sample in a swing-out rotor of known dimensions for various times, thus sedimenting different fractions of the particulate cell debris. The supernatant and concentrate samples are then analyzed by SDS-PAGE with quantitation of outer-membrane proteins by scanning densitometry. A comparison with the initial homogenate provides an estimate of the fraction of debris sedimented at each centrifugation time. This can then be used to construct a cumulative size distribution using standard mathematical techniques.

4. Notes

4.1. Equipment and Layout

1. As indicated in the Introduction, a homogenizer is essentially a high-pressure positive-displacement pump that forces the cell suspension through a homogenizer valve. In designing the equipment layout, it is important to ensure that an unconstricted flow path is provided while the homogenizer operates. The three-way valves in **Fig. 2** should not be capable of positive shutoff. Furthermore, the storage tanks must be sealed to prevent aerosol release, usually by validated absolute filters (e.g., 0.22 μm). It is important that the filters be designed and selected to minimize blocking potential (e.g., hydrophilic filters mounted on an adaptor (e.g., elbow), with an integrated condenser for rigorous applications). Pressure-relief systems or connection to a validated air removal system at regulated pressure can also be employed to prevent tank overpressure. It is also important that the feed to the homogenizer be maintained without interruption. Manually monitoring tank levels to ensure feed does not exhaust is tedious; level alarms on the tanks are strongly recommended for moderate-scale laboratory operation. At higher automation levels, these can be tied to a cut-out system for the homogenizer. A pressurized feed system can also enhance delivery to the homogenizer, but is generally not required as the core of the homogenizer consists of a positive-displacement pump with no net-positive-suction-head requirement (provided the tanks are above the intake plane of the homogenizer).
2. Cleanability in these systems is a major concern. Spray balls or heads on the tanks are highly recommended, as is chemical disinfection by recirculating appropriate cleaning solutions through the homogenizer. Given the extreme pres-

sure that these systems operate under, regular maintenance is most definitely required. It is strongly recommended that the manufacturer's suggested maintenance schedules be followed, and that a sufficient stock of spare parts be maintained if operational downtime at critical junctures is undesirable.

4.2. Cell Disruption

3. Kleinig et al. ***(7)*** examined the effect of cell concentration for *E. coli* in a Gaulin 15 MR high-pressure homogenizer. In the range of 5–150 g/L wet weight, a small decrease in homogenization efficiency was observed at higher concentrations. **Eq. 1** described the effect of wet cell concentration, X (g/L), on disruption efficiency

$$\ln (1/1 - D) = (0.0149 - 2.75 \times 10^{-5} X)\, N^{0.71}\, P^{1.165} \qquad (1)$$

   where D is the fractional release of protein (the protein release at a specific point divided by the maximum release attainable), N is the number of discrete homogenizer passes, and P is the homogenizer operational pressure in MPa. It was concluded that the decrease in homogenization efficiency at high concentrations was not sufficient to warrant dilution of the suspension before homogenization. The decrease in homogenization efficiency could be easily compensated by additional homogenizer passes. This approach proves more cost effective than diluting the broth and homogenizing the larger volume. However, viscosity increases significantly at the higher concentrations, and it therefore appears that the maximum homogenization concentration is limited by practical constraints related to high viscosity.
4. Protein analysis of highly concentrated samples is prone to error because of the excluded-volume of the cell mass. As disruption proceeds, the volume-fraction of packed material can change significantly. This in turn affects the supernatant volume in a given sample, and hence the protein concentration (when comparing samples throughout the disruption procedure, and calculating D in the above equation). A dilution method of correcting for this increase in aqueous volume fraction has been developed ***(8)***. For samples containing partially denatured protein, dilution during protein estimation can lead to variable results. A method using Kjeldahl nitrogen analysis is available that overcomes this problem ***(9)***, but is considered to be less accurate than the dilution procedure because of several assumptions in the analysis.
5. Homogenization efficiency can be improved, with consequent reduction in the need for homogenization, using chemical pretreatments. Strategies for weakening the cell wall focus largely on enzymatic attack of the strength-conferring elements. For example, treatment of *Bacillus cereus* with the lytic enzyme *cellosyl* prior to homogenization increased disruption efficiency to 98% from 40% after a single homogenizer pass at 70 MPa ***(10)***. For *E. coli*, pretreatment with a combination of ethylenediamine tetra-acetic acid (EDTA) and lysozyme has been used to marginally increase the efficiency of mechanical disruption ***(11)***. Yeast, such as *S. cerevisiae* and *Candida utilis*, may be effectively weakened by pretreatment with zymolyase preparation, available commercially from

Seikagaku America (Rockville, MD) ***(12,13)***. In general, however, the cost of these enzyme preparations can be quite high and recovery and recycle is difficult and costly to implement. Significantly enhanced disruption is required to justify this cost, and results of pretreatment will be very organism- and condition-specific. Often, a simpler and more practical strategy is simply to increase the number of homogenizer passes.

6. It is often desirable to inactivate the broth prior to release from the fermenter for downstream processing. An attractive method for cell inactivation is thermal treatment, as chemical addition to the broth is unnecessary. However, thermal deactivation can significantly reduce the efficiency of cell disruption during homogenization ***(14)***. Results are very procedure-specific, reflecting changes in cell wall composition and cell size. Collis et al. ***(14)*** were able to show that by charging stationary-phase cells with glucose prior to thermal deactivation, an increase in disruption efficiency was actually obtained. Furthermore, product release can actually be enhanced through thermolysis at higher temperatures, provided the product is thermally stable. For example, incubation of *E. coli* at 90°C is reported to release cytoplasmic contents within 10 min. The effects are clearly dependent on the state of the microorganism, and the regime of heat treatment (specifically the temperature and the rate of deactivation).
7. Some products may be degraded during homogenization. For example, Augenstein et al. ***(6)*** have clearly demonstrated product degradation when homogenizing *B. brevis* for the release of a shear-sensitive enzyme. Perhaps the greatest problem arises because of heat generation, which can usually be mitigated by precooling the feed to 5°C (and rapidly cooling the homogenate). The literature also suggests that protein denaturation is intimately tied to the existence of interfaces. Consequently, degassing before homogenization may provide benefits.

4.3. Analysis of Disruption

8. Methods for quantifying disruption may be broadly classed as direct or indirect ***(2)***. Direct methods measure the number or volume-fraction of cells destroyed during the homogenization process. Indirect methods infer the volume or number fraction of cells by measuring, for example, the release of total protein during homogenization. In the procedure described earlier, microscopy provides a direct qualitative measure of disruption, whereas the measurement of total protein release provides an indirect quantitation of the volume fraction of cells destroyed. In this case, the indirect method allows definition of a fractional protein release. Several other methods for quantifying disruption are also available ***(2)***. Microscopy can be conducted in a quantitative manner by cell counting. This can be automated using a hemocytometer with methylene-blue dye exclusion ***(16)*** or an Elzone particle-size analyzer (Coulter Electronics, Fullerton, CA) ***(10)***. The Elzone method provides difficult quantitation, however, because of overlap with the debris resulting from cell disruption. Centrifugal disk photosedimentation (CDS) also provides a rapid and direct measure of cell disruption for *E. coli* ***(17)***.

9. The most appropriate method for monitoring cell disruption in a practical sense is to follow the release of the specific product of interest. If the product is an enzyme, then monitoring the release of specific activity using a standard test will be most appropriate. If the product is nonenzymatic, then immunofluorescent methods offer a rapid and relatively simple means of monitoring the rate of product release. Under this approach, maximizing the fraction of cells destroyed is of secondary importance to maximizing the release of product. It is particularly appropriate if the product degrades during homogenization, as the point of maximum product recovery is unlikely to occur at complete cell disruption.

4.4. Analysis of Debris Size

10. Several techniques are available to analyze debris size, but each has limitations. Consequently, the only method provided above is a qualitative assessment of debris size by light microscopy. Methods previously employed to characterize debris include photon correlation spectroscopy (PCS), CDS, electrical sensing zone measurement (ESZ), and CSA. PCS is an inherently low-resolution technique, so sample preparation including the removal of undisrupted cells is required. This may be achieved by filtration ***(18)***. Mild centrifugation has also been used to separate debris from inclusion bodies before sizing ***(19)***. However, fractionation will selectively remove larger debris and distort the measured distribution toward lower sizes. For example, Jin ***(20)*** has shown that up to 47% of the cell debris is removed from the supernatant (the "debris" fraction) using Olbrich's ***(19)*** fractionation scheme. ESZ has the disadvantage of low sensitivity at smaller debris sizes. Sensitivity can be improved by reducing orifice size, but at the risk of continual blocking. It is typically unsuitable for analyzing *E. coli* debris. It has been used with some success for yeast debris sizing ***(21)***, although sensitivity is lost below 1 μm (where a significant amount of debris should be detected). CDS also suffers from low sensitivity below approximately 0.2 μm ***(17)***, where a considerable fraction of *E. coli* debris lies after homogenization. Resolution is limited by baseline problems and uncertainties associated with light extinction as particles approach the wavelength of light ***(17)***.
11. CSA, developed by Wong et al. ***(22)*** for sizing *E. coli* debris in the presence or absence of inclusion bodies, suffers none of the limitations of PCS, CDS, and ESZ. Its key limitation is that full determination of debris-size distributions is labor intensive. For downstream operations, such as the centrifugal fractionation of inclusion bodies and cell debris, however, information on debris size is important for optimal results. In such cases, CSA is the method of choice as it provides a Stokes sedimentation diameter for direct use in the relevant centrifuge performance equations (*see* **Chapter 5**).

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