Relation Between Cell Disruption Conditions, Cell Debris Particle Size, and Inclusion Body Release

Pim van Hee,1 Anton P.J. Middelberg,2 Rob G.J.M. van der Lans,1 Luuk A.M. van der Wielen1

1Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands; telephone: +31-15-278-2361; fax: +31-15-278-2355; e-mail: L.a.m.vdwielen@tnw.tudelft.nl
2Division of Chemical Engineering, University of Queensland, St. Lucia, Australia

Received 20 January 2004; accepted 4 June 2004
Published online 10 September 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20343

Abstract: The efficiency of physical separation of inclusion bodies from cell debris is related to cell debris size and inclusion body release and both factors should be taken into account when designing a process. In this work, cell disruption by enzymatic treatment with lysozyme and cellulase, by homogenization, and by homogenization with ammonia pretreatment is discussed. These disruption methods are compared on the basis of inclusion body release, operating costs, and cell debris particle size. The latter was measured with cumulative sedimentation analysis in combination with membrane-associated protein quantification by SDS-PAGE and a spectrophotometric peptidoglycan quantification method. Comparison of the results obtained with these two cell debris quantification methods shows that enzymatic treatment yields cell debris particles with varying chemical composition, while this is not the case with the other disruption methods that were investigated. Furthermore, the experiments show that ammonia pretreatment with homogenization increases inclusion body release compared to homogenization without pretreatment and that this pretreatment may be used to control the cell debris size to some extent. The enzymatic disruption process gives a higher product release than homogenization with or without ammonia pretreatment at lower operating costs, but it also yields a much smaller cell debris size than the other disruption process. This is unfavorable for centrifugal inclusion body purification in this case, where cell debris is the component going to the sediment and the inclusion body is the floating component. Nevertheless, calculations show that centrifugal separation of inclusion bodies from the enzymatically treated cells gives a high inclusion body yield and purity. © 2004 Wiley Periodicals, Inc.

Keywords: cell debris particle size; Pseudomonas putida; chemical lysis; homogenization; polyhydroxyalkanoate; inclusion body

INTRODUCTION

Inclusion bodies are formed when products accumulate in certain compartments of cells or when products aggregate spontaneously inside cells. Some examples of inclusion bodies (IBs) are polyhydroxyalkanoate (PHA) produced by Pseudomonas putida (Fuller, 1999), β-carotene produced by Blakeslea trispora (Roukas and Mantzouridou, 2001), and recombinant proteins, such as insulin-like growth factor (Wangsa-Wirawan et al., 2001) and α-glucosidase (Walker and Lyddiatt, 1999), produced in E. coli. The product inside IBs can often be recovered by dissolution after permeabilization of the cell wall (Page and Cornish, 1993). In this process, the properties of the product depend strongly on the extraction conditions and in some cases the product loses its valuable properties during the process. For instance, the extraction of polyhydroxyalkanoates with organic solvents causes a decrease in molecular weight of the polymer and thus changes the properties of the material irreversibly (Luzier, 1992).

An alternative for product recovery by extraction is cell disruption followed by particle-particle separation with filtration (Souza et al., 2002), centrifugation (Hoare and Dunnill, 1989), interfacial partitioning (Jauregi et al., 2001), or selective flotation (Wangsa-Wirawan et al., 2001). This recovery strategy prevents product degradation and may reduce the number of downstream processing operations due to the high product purity inside the IBs.

When combining cell disruption with physical separation of IBs, the influence of cell disruption conditions on product release, cell debris particle size, and operating costs should be investigated. This has been done by Kula et al. (1990), Siddiqi et al. (1996), Wong et al. (1997a), and others for mechanical cell disruption, but the effects of chemicals on cell debris particle size has not been studied thus far. In this work, the effect of chemicals on Pseudomonas putida cell debris particle size, PHA IB release, and operating costs is investigated for enzymatic cell disruption and
homogenization with and without ammonia pretreatment. In addition, the influence of cell disruption on IB purification with centrifugation is investigated.

The particle size of cell debris can be measured with methods such as dynamic light scattering and electrical sensing zone techniques. However, these methods cannot discriminate between cell debris and IBs and therefore require the purification of cell debris prior to particle size determination. This may affect the particle size distribution due to aggregation or losses during the purification process. Therefore, cumulative sedimentation analysis (CSA), which was developed by Wong et al. (1997b), is used for cell debris particle size determination in this work. This method requires the quantification of cell debris particles, which is done with SDS-PAGE analysis of membrane-associated proteins and spectrophotometric peptidoglycan quantification (Van Hee et al., 2004). By combining CSA with these two analysis methods, information is obtained on the chemical composition of the cell debris particles and the compatibility of CSA with the various cell disruption methods.

**MATERIALS AND METHODS**

**Theoretical Background**

**Homogenization**

Hetherington et al. (1971) described protein release as a function of homogenization pressure and the number of homogenizer passes. Sauer et al. (1989) modified the relation by including an exponent for the number of passes, as is shown in Equation 1,

\[
\ln \left( \frac{R_{\text{max}}}{R_{\text{max}} - R} \right) = K \cdot N^a \cdot \Delta P^b
\]

where \( R \) is the released soluble protein (mg protein/g dry mass), \( R_{\text{max}} \) is the maximal protein concentration available for release (mg protein/g dry mass), \( K \) is the rate constant, \( N \) is the number of homogenizer passes, \( \Delta P \) is the pressure drop (Pa), \( a \) is the pressure exponent, and \( b \) is the exponent for the number of homogenizer passes. In this work, \( R \) and \( R_{\text{max}} \) will be used as the released IB fraction and the total IB fraction in the mixture, respectively.

Chemical pretreatment can very efficiently lower the energy requirement for homogenization. For example, Harrison et al. (1991) performed an osmotic/pH shock with sodium hydroxide and showed that this pretreatment very efficiently increases soluble protein release in homogenization. In this work, pretreatment is performed with ammonia instead of sodium hydroxide, because ammonia can be applied as a gas under pressure. When ammonia is dissolved, ammonium hydroxide is formed and the pH of the liquid phase is increased. Reduction of pressure after cell disruption results in the evaporation of ammonia, which can then be recovered with a stripping column and reused. This method potentially produces less wastewater compared to the use of sodium hydroxide or other nonvolatile bases.

**Cumulative Sedimentation Analysis**

Cumulative sedimentation analysis (Wong et al., 1997b) is a method that makes use of Stokes’ law to measure the particle size distribution. During sedimentation of a dilute homogeneous mixture of particles over a height \( H \), the particle mass fraction that has settled to form the concentrate (\( F \)) will increase with increasing effective settling time (\( t_e \)). \( F \) consists of particles that have a settling velocity larger than or equal to \( H/t_e \) and particles with a settling velocity smaller than \( H/t_e \). By discriminating between these two classes of particles with Allen’s relation (Allen, 1990), a cumulative curve for the oversized settled particle mass fraction (\( W \)) can be obtained (Equation 2).

\[
W(t_e) = F - t_e \cdot \frac{dF}{dt_e} = F - \frac{dF}{d \ln t_e}
\]

**IB Purification With Centrifugation**

Centrifugal separation can be modeled with the sigma theory, which is shown in Equation 3 (Ambler, 1961). The sigma theory makes use of a critical particle diameter (\( d_c \)), which results from the balance between Stokes’ drag and the centrifugal force, to calculate the particle-liquid separation efficiency in a centrifuge as follows:

\[
d_c = \sqrt{\frac{18 \cdot \mu \cdot Q}{\sum \Delta \rho \cdot g}}
\]

where \( \mu \) is the liquid viscosity (Pa s), \( Q \) is the feed rate (m³/s), \( \Sigma \) is the equivalent clarification area (m²), \( \Delta \rho \) is the density difference between the particle and liquid phase (kg/m³), and \( g \) is the gravitational acceleration (m/s²).

Particle-particle separation requires particles to move in opposite direction in the centrifuge. This requires the liquid-phase density to be in between the densities of the particles, which in this case have densities of 1,000 kg/m³ (PHA) and 1,085 kg/m³ (cell debris). The choice of liquid-phase density influences both the separation efficiency and the operating costs due to the addition of chemicals for obtaining the desired liquid-phase density. When the liquid-phase density is known, the critical particle diameters for both particle types can be calculated. This critical diameter in combination with the particle size distribution (PSD) of cell debris and the IBs can be used to calculate the IB yield and purity that can be obtained with centrifugation.

**Experimental Procedures**

**Fermentation**

Pseudomonas putida KT2442 was produced in a 10 L fermentor that was run in fed-batch mode at 30°C as described
by Weusthuis et al. (2001). The pH was maintained at 7.0 with a 25% v/v ammonia solution that served as the main nitrogen source for the microorganism. Coconut oil-free fatty acids (Vereenigde Oliefabrieken, Rotterdam, The Netherlands) were used as carbon source, forcing the organism to produce inclusion bodies of medium-chain-length polyhydroxyalkanoate (mcl-PHA), which is a biodegradable polymer with carbon chains of C-6 to C-16. After fermentation, the broth was stored at 4–8°C for a maximum of 3 months.

Storage of fermentation broth affects the cell wall strength, which is an important parameter in cell disruption as was reported by Chisti and Moo-Young (1986), Sauer et al. (1989), Middelberg and O’Neill (1993), and others. It generally weakens the cell wall, causing an increase in cell disruption efficiency as compared to working with fresh cells. For instance, Rees et al. (1995) showed that storage of cells at a temperature below 10°C significantly increased protein release during cell rupture. In this work, fermentation broth was provided by Agrotechnology and Food Innovations (Wageningen, The Netherlands) and storage of the broth could not be avoided. The cells were thus weakened before cell disruption was performed. However, as this work focuses on cell disruption methodology rather than industrial application, storage is not an issue. Evaluation of the effect of the investigated disruption processes on IB purification, on the other hand, is very much affected by storage and the data presented in this work should only be used as a guide for designing an industrial process.

Homogenization

The fermentation broth was set to the desired pH ranging from 9 to 11 with 25% v/v ammonium hydroxide. After 30 min, the pH was neutralized with 2 M HCl and a homogenizer (Constant Cell Disruption Systems, Low March, Daventry, Northants, I.K.S. BV) was used for cell rupture. The homogenizer was operated at 10°C and various pressures and the broth was cooled between passes to avoid large temperature fluctuations. The manufacturer reported a pressure drop of 1,600 bar as the required pressure for IB release in P. putida.

Enzymatic Lysis

The enzymatic disruption procedure started with the addition of 0.3 g lysozyme per kg dry cell weight (DCW) at 40°C. After 1 hr, the pH was increased from 7.0 to 8.5 with sodium hydroxide. Then 4.8 mL Novozyme 342 concentrate at a concentration of 1 g enzyme per mL (Novo Nordisk Industri), which is a commercially available cellulase mixture, was added per kg DCW and the mixture was again stirred for 1 hr. After enzymatic treatment, the broth was stored at −20°C until further use.

Cumulative Sedimentation Analysis

Immediately after cell rupture, the broth was diluted with a 100 mM phosphate buffer to approximately 2.5 g DCW/L. Samples were centrifuged for the desired effective settling times with a Beckman L-70 ultracentrifuge equipped with a swing-out rotor. After centrifugation, the supernatant and concentrate were separated and samples were taken for peptidoglycan and membrane-associated protein quantification. The density and viscosity of the liquid phase were measured with a DMA 48 density meter (Anton Paar, Graz, Austria) and a Haake VT 550 viscosity meter (Thermo Scientific, Breda, The Netherlands).

SDS-PAGE

Samples were taken of the concentrate and the supernatant. The samples were centrifuged in an eppendorf centrifuge at 17,000 g for 60 min to settle all protein-marked cell debris particles with a size above 0.07 μm. The membrane-associated proteins of Pseudomonas putida have a size of 19 and 38.5 kDa (Rodriguez-Herva and Ramos, 1996). After centrifugation, the pellet was mixed with sample buffer (NuPAGE LDS, Invitrogen, Breda, The Netherlands) and heated to 70°C for 10 min. A 12% Bis Tris gel (Invitrogen) was used in an Invitrogen Xcell surelock minicell with MOPS running buffer and a broad-range protein marker (Invitrogen Benchmark Protein Ladder: 220, 160, 120, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kD). The gel was stained with SimplyBlue stain (Invitrogen). The linearity of SDS-PAGE quantification was confirmed with a calibration curve with the protein marker.

Muramic Acid Analysis

A calibration curve was made with lactic acid (Acros Organics) solutions of concentrations in the range of 0 and 1,000 µg lactic acid/L milli-Q water: 1 mL samples of the concentrate and the calibration curve were mixed with 2 mL 5M sulfuric acid and heated for 2 hr at 90–100°C. After cooling to room temperature, 2 mL 10 M sodium hydroxide in water was added to neutralize the pH; 1 mL of 0.30 mg trichloroacetic acid/mL water was then added to precipitate all proteins from the solution (wash 1). The mixture was centrifuged for 15 min at 2,500 g and 2.5 mL of the supernatant was mixed with 0.5 mL 20% (w/w) copper sulfate pentahydrate in water. The volume was increased to 5 mL with milli-Q water and the pH neutralized by adding 100 µl 25% (v/v) ammonium hydroxide. Approximately 0.5 gram of calcium hydroxide was added and the mixture was shaken vigorously (wash 2). After at least 30 min with occasional shaking, the tubes were centrifuged for 15 min at 2,500 g. The supernatant, now free of proteins and other interfering substances, was collected for the colorimetric muramic acid analysis as described by Taylor (1996). Samples of the supernatant were diluted with 96% sulfuric acid at a volume ratio of 1:6. The samples were heated at
90–100°C for at least 30 min in screw-capped borosilicate tubes. After cooling to room temperature, 50 μL of a 4% (w/w) copper sulfate pentahydrate solution in water was added to 3.5 mL of the samples. Then 100 μL of a 1.5% (w/w) p-phenylphenol solution in 95% ethanol was added and the liquid was immediately mixed well. After at least 30 min, the absorbance was measured at 570 nm.

Inclusion Body Release

Samples of ruptured broth were centrifuged for 3 hr at 30,000 g. The resulting pellet and top phase were separated, dried in a freeze dryer, and analyzed for PHA content with a gas chromatography method described by De Rijk et al. (2001). Due to the difference in IB (~1,000 kg/m³) and cell debris density (~1,085 kg/m³), the released IBs were expected to be collected in the supernatant, while the unreleased IBs were collected in the pellet.

Inclusion Body Particle Size Distribution

The PSD of the PHA inclusion bodies was measured with a Coulter Multisizer II equipped with a 15 μm orifice (Beckman Coulter, Mijdrecht, The Netherlands) after purification of the IB with multiple centrifugation and washing steps.

RESULTS AND DISCUSSION

Inclusion Body Release

Influence of Pressure

Figure 1 shows the influence of homogenization pressure and ammonia pretreatment on the IB release efficiency after one homogenizer pass. R was taken from the analysis of the supernatant after centrifugation (mass of released IBs) and \( R_{\text{max}} \) was taken from the analysis of the whole mixture (total IB mass). The data show that homogenization with ammonia pretreatment causes more efficient IB release than homogenization without pretreatment and that it increases the pressure dependence of the disruption process for pressures up to 1.6 kbar. When the data for homogenization without pretreatment are correlated to Equation 1, \( K \) and the pressure exponent, \( a \), yield values of 0.38 (standard error, 0.032) and 1.2 (standard error, 0.17), respectively. The data for homogenization with ammonia pretreatment do not seem to correlate with Hetherington’s equation because IB release does not increase when the pressure is increased from 1.6 to 2.0 kbar. However, the data point at 2.0 kbar for homogenization with ammonia pretreatment is inaccurate because the total IB mass in the mixture was lower than in the other samples, while the cell debris mass was the same. This loss of IBs was caused by foam formation during homogenization, which led to inaccurate sampling. The foam probably contained a large fraction of the released IBs that were excluded from the measurement as the foam was not included in the samples. In all other samples, less foam was created during homogenization, indicating that there might be a correlation between foaming, homogenization pressure, and ammonia pretreatment. When the data point at 2.0 kbar is corrected for IB losses in the analysis procedure, the value of \( \ln(R_{\text{max}}/(R_{\text{max}} - R)) \) is approximately 1, which indicates that at pressures equal or higher than 1.6 kbar, IB release does not increase with pressure. The mechanism of cell disruption by homogenization with ammonia pretreatment thus deviates from the mechanism that is described by Hetherington. Regardless of this different disruption mechanism, correlation of the data with Hetherington’s relation for pressures up to 1.6 kbar yields values for \( K \) and \( a \) of 0.38 (standard error, 0.071) and 2.3 (standard error, 0.45), respectively.

Influence of Number of Homogenizer Passes

Since parameters \( K \) and \( a \) were obtained from the dependence of IB release on homogenization pressure, exponent \( b \) can be obtained by varying the number of homogenizer passes. Figure 2 shows the relation between the number of homogenizer passes and IB release at 1.6 kbar. For homogenization without ammonia pretreatment, \( R \) and \( R_{\text{max}} \) were again obtained by analysis of the top phase after centrifugation and the whole mixture, respectively. Sampling of the broth after homogenization with ammonia pretreatment, on the other hand, was inaccurate due to foam formation. Sampling of the broth at \( N = 0 \) was accurate since no homogenization was performed and thus
The relation between the number of homogenization passes at 1.6 kbar, pretreatment conditions, and IB release for PHA inclusion bodies from *Pseudomonas putida* cells that were stored for about 9 weeks at 4°C. The curve for homogenization without pretreatment is calculated with Equation 1 for $K = 0.34$, $a = 1.2$, and $b = 0.33$. The curve for homogenization with ammonia pretreatment is calculated with Equation 1 for $K = 0.32$, $a = 2.3$, and $b = 0.28$.

An exponential fit was made with the values for exponent $a$ from the previous section to estimate the values of $K$ and $b$. The $K$ values for pH 11 pretreatment and no pretreatment are 0.32 (standard error, 0.073) and 0.34 (standard error, 0.073), respectively, which is in the same range as the values obtained in the previous section, and the values of exponent $b$ are 0.28 (standard error, 0.057) and 0.33 (standard error, 0.058), respectively. These values of $b$ deviate from the value for soluble protein release in Bakers’ yeast that was reported by Hetherington et al. (1971), indicating that the release mechanism for soluble proteins is different from the release mechanism for IBs. This difference is probably related to the different locations of the products inside the cells as described by Marr (1960) and Follows et al. (1971).

**Influence of Biomass Concentration**

In the above-described experiments, ammonia was added to give the cells a pH/osmotic shock. This addition causes dilution of the broth, which may have an effect on cell rupture efficiency. Experiments showed that biomass concentration does not have a significant effect on cell rupture in the concentration range of 0.06–115 g DW/L, which is in accordance with values reported by Brookman (1974). Since the experiments from the previous sections were performed at concentrations within this range, we may conclude that there is no concentration effect that has to be accounted for.

**Comparison of Cell Debris Detection Methods**

Cell rupture yields cell debris particles and causes IB release. In the rupture process, the PSD of the cell debris particles, which is important for subsequent separation steps, is influenced by the rupture conditions. In this section, the relation between the rupture conditions and the cell debris particle size determination with CSA is presented for homogenization with ammonia pretreatment, homogenization without ammonia pretreatment, and enzymatic treatment.

In Figure 3, the mass fraction of settled particles ($F$) is plotted against the effective settling time ($t_e$) for the two analysis methods. As a reference, optical density measurements are shown. The SDS-PAGE data for homogenized cells with and without pretreatment are based on the 19 kDa membrane-associated protein of *P. putida* (Rodriguez-Herva and Ramos, 1996). A typical result of an SDS-PAGE gel is presented in Figure 4. For enzymatically treated broth, the 38.5 kDa membrane-associated protein was used for densiometry because the bands of the 19 kDa protein were very broad and unclear (Fig. 5). In the analysis, $F$ is set to unity when the effective settling times represent a cut size of 0.07 μm. For all samples, this limiting cut size is much smaller than the median cell debris size, which justifies the use of this cell debris mass as the total mass in the sample. For enzymatic treatment, this is not the case, thus the median particle size may be smaller than the median that is implied by the graph.
The results show that homogenized broth after two passes without pretreatment gives similar results for the peptidoglycan-based assay (MA) and the membrane-associated protein assay (SDS-PAGE). This is in accordance with our previous result for whole cells of P. putida and E. coli (Van Hee et al., 2004). Conversely, the SDS-PAGE analysis for enzymatically ruptured cells and homogenized cells after pH 9.5 pretreatment shows a different

**Figure 3.** Results from cumulative sedimentation analysis for *Pseudomonas putida* with the muramic acid assay (MA) and membrane-associated proteins by SDS-PAGE for enzymatic rupture, two homogenizer passes at 1.6 kbar, and homogenization at 1.6 kbar with pH 9.5 and 11 pretreatment. $t_e$ is given in seconds.

**Figure 4.** A typical result for SDS-PAGE analysis of cumulative sedimentation analysis of *Pseudomonas putida*. This photograph shows analysis of samples after two passes through a homogenizer without pretreatment. Lanes 1 – 6 and 8 – 10 show the protein content in supernatant samples with decreasing effective settling time. Lane 7 shows the Invitrogen Benchmark Protein Ladder 220, 160, 120, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kD.

**Figure 5.** SDS-PAGE analysis of cumulative sedimentation analysis of *Pseudomonas putida* for enzymatically ruptured broth. Lanes 1 – 4 and 6 – 10 show the protein content in supernatant samples with decreasing effective settling time. Lane 5 shows the Invitrogen Benchmark Protein Ladder 220, 160, 120, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kD. The 19 kD membrane-associated proteins show vague bands while the 38.5 kD bands are clear enough for densiometry.
Position. On the other hand, the variation in chemical com-
velocities are affected by this variability in chemical com-
determines relative settling velocities, while particle size
may affect their densities and thus their physical separation.
debris particles of interest.
detection method in CSA that is representative for the cell
We may thus conclude that it is very important to choose a
for the protein-based assay and the peptidoglycan-based assay.
glycan-based assay, which also causes differences between
a representative amount of peptidoglycan for the peptido-
and/or without peptidoglycan. These particles will not carry
associated proteins and components of the outer wall with
leads to further degradation of the cell wall. This process
which makes peptidoglycan susceptible for lysozyme and
since two enzymes are used that both play a different role
ference between the two analysis methods is more difficult
values in the SDS-PAGE analysis and thus makes the
difference between the two analysis methods smaller.
Because of these inaccuracies in the SDS-PAGE data, the
results of the peptidoglycan-based assay will be used for
debris size analysis in the next section.
For enzymatically treated cells, interpretation of the dif-
ference between the two analysis methods is more difficult
since two enzymes are used that both play a different role
in cell disruption. Lysozyme breaks the N-acetylmuramic
acid and N-acetylglucosamine bonds in peptidoglycan
(Carson and Eagon, 1966). This may cause the release of
proteins from the cell wall leading to the formation of cell
debris particles with a membrane-associated protein con-
tent that is not representative for the particle. Even the
existence of cell debris particles without any membrane-
associated proteins becomes very likely due to the small
cell debris size that was measured with the peptidoglycan-
based assay. These particles cause differences between the
two analysis methods. Cell rupture by cellulase (Novozyme
342) may be related to the breakage of sugar bonds in
extracellular polysaccharides (EPSs) in the outer wall of the
cells, as was reported by Loiselle and Anderson (2003),
which makes peptidoglycan susceptible for lysozyme and
leads to further degradation of the cell wall. This process
may cause the formation of particles containing membrane-
associated proteins and components of the outer wall with
and/or without peptidoglycan. These particles will not carry
a representative amount of peptidoglycan for the peptido-
glycan-based assay, which also causes differences between
the protein-based assay and the peptidoglycan-based assay.
We may thus conclude that it is very important to choose a
detection method in CSA that is representative for the cell
debris particles of interest.
The differences in chemical composition of the particles
may affect their densities and thus their physical separation.
This effect is accounted for in the CSA measurement as it
determines relative settling velocities, while particle size
analysis techniques that do not measure relative settling
velocities are affected by this variability in chemical com-
position. On the other hand, the variation in chemical com-
position makes interpretation of the PSD that is obtained
with CSA very difficult because $F$ is the settled mass
fraction of the marker component, which does not by
definition correspond to the settled particle mass frac-
tion when the chemical composition of the particles in
the mixture varies. In this work, the results of the pepti-
doglycan-based assay will be used for analysis of the par-
ticle size distribution.

### Particle Size Distribution of Cell Debris

The particle size distribution of ruptured cells obtained
from the muramic acid detection method of CSA samples is
shown in Figure 6. $W$ was calculated from the best fit of
$F$ against $\ln(t_e)$ with Equation 2 and the particle diameter
was calculated from the effective settling time ($t_e$) by
assuming a particle density of $1,085 \text{ kg/m}^3$ (Wong et al.,
1997b). This value is accurate enough for this work, be-
cause it is concerned with relative settling velocities of
IB versus cell debris. On the other hand, the density could
be critical for particle separation with other separation
methods, as the apparent size strongly depends on the
assumed densities.

The results show that homogenized broth after pH 11
pretreatment has a smaller median cell debris diameter than
homogenized broth after pH 9.5 pretreatment. The reason
for this decrease in cell debris size with an increase in am-

![Figure 6](image-url)

**Figure 6.** Comparison of the PSD of cell debris obtained from several
cell disruption methods: enzymatic treatment (enzymatic), two homoge-
nizer passes at 1.6 kbar (2 passes), pH 9.5 ammonia pretreatment followed
by one homogenizer pass at 1.6 kbar (pH 9.5 pretreatment), and pH 11
ammonia pretreatment followed by one homogenizer pass at 1.6 kbar
(pH 11 pretreatment). $W$ is the mass fraction of particles with a diameter
larger than the cut size. The cut size was calculated with Stokes’ law by
assuming a cell debris density of $1,085 \text{ kg/m}^3$ (Wong et al., 1997b). For
enzymatic disruption, the limiting cut size is larger than the median cell
debris size, which makes the PSD presented in this graph inaccurate. The
actual PSD will have an even smaller median particle size. The curve
for whole cells is obtained from literature data of Wong et al. (1997b) for
*E. coli* since it has great similarity to *P. putida*. 

106 BIOTECHNOLOGY AND BIOENGINEERING, VOL. 88, NO. 1, OCTOBER 5, 2004
monia concentration could be the destruction of muramic acid with ammonium hydroxide, as reported by Tipper (1968), and/or the osmotic shock caused by the addition of this chemical. Furthermore, enzymatic disruption yields the smallest cell debris size when peptidoglycan is used as a marker. Cell debris particle size in mechanical cell disruption is dependent on the cell disruption system that is used (Agerkvist and Enfors, 1990), indicating that other homogenizers may give a different result. Therefore, a general comparison between enzymatic treatment and mechanical cell disruption based on these results is impossible.

In Figure 7, IB release is plotted against the median particle diameter. Comparison between two homogenizer passes and one pass after pH 11 pretreatment shows that ammonia pretreatment gives an increase in IB release, while the median cell debris diameter remains approximately the same. In addition, pH 9.5 pretreatment with one homogenizer pass gives the same release as two homogenizer passes without pretreatment, while the median cell debris diameter is larger. The cell debris size can thus be tuned to some extent without affecting the IB release by either using homogenization with an ammonia pretreatment or homogenization without ammonia pretreatment. Besides the influence of ammonia on IB release and cell debris size, the graph shows a correlation between the median cell debris particle diameter and IB release. As the cell debris particle size decreases, an increase in IB release is observed. This indicates that IBs are only released when the cell debris is too small to retain the IBs.

**Particle Size Distribution of IBs**

The IB PSD that was measured with a Coulter Counter is presented in Figure 8. The graph does not show the full range of inclusion bodies because particle sizes below 0.36 μm cannot be measured with the Coulter Counter. This will have a small effect on the separation efficiency, however, since the volumetric contribution of these particles is expected to be very small. A much larger inaccuracy is caused by losses during purification of the IBs prior to particle size analysis. Therefore, these data will only be used as an indication of the IB particle size.

**Inclusion Body Purification**

The purification of IBs from cell debris by centrifugation is dependent on the particle size of cell debris and IBs, as well as the released IB fraction. This makes the separation efficiency strongly dependent on the cell disruption process that is used. Since the cell strength was affected by storage, the cell debris PSD and IB release presented in this work are not representative for an industrial process. Nevertheless, evaluation of IB purification based on these data will give insight of the influence of the various cell disruption strategies on IB purification. In the following sections, the operating costs for cell disruption are calculated and the effect of cell disruption on IB purification is investigated.

**Operating Costs**

The operating costs of the rupture process per kg released product can be roughly estimated with Equation 4, which accounts for the influence of energy consumption, addition of chemicals, and temperature change:

\[
OC_d = \frac{PE \cdot N}{C_{IB} \cdot X_{IB}} \cdot (\Delta P + \rho \cdot C_p \cdot \Delta T \cdot \eta) + \sum C_{c,i} \cdot PC_i \cdot \frac{1}{C_{IB} \cdot X_{IB}}
\]

where \(OC_d\) are the operating costs for disruption per kg of released product ($/kg), \(PE\) is the energy price ($/J), \(N\) is the number of homogenizer passes, \(C_{IB}\) is the IB concentration...
(kg/m$^3$), $X_{IB}$ is the fraction of IBs released after homogenization, $\Delta P$ is the operating pressure for homogenization (Pa), $\rho$ is the density of the liquid phase (kg/m$^3$), $C_p$ is the heat capacity of the mixture (J/kg/K), $\Delta T$ is the sum of all temperature changes in the disruption process (K), $\eta$ is the cooling/heating efficiency, $C_{c,j}$ is the concentration of added chemicals (kg/m$^3$), and $PC_j$ is the price of these chemicals ($/kg$).

Table I shows the result of the calculation of the disruption costs for homogenization without pretreatment, homogenization with ammonia pretreatment, and enzymatic treatment. The prices for energy, lysozyme, Novozyme 342, and ammonia are assumed to be 0.009 S/MJ and 0.5, 10, and 0.4 $/kg, respectively. These prices are based on the SuperPro Designer database (Intelligen). In the calculations, the efficiency for cooling and heating is 0.80 and all energy input in homogenization is assumed to be dissipated as heat. The heat capacity and density of the mixture were assumed to be equal to that of water.

Comparison of the disruption costs for the two homogenization processes shows that ammonia does not have a significant effect on the cost of the disruption process, while it gives a significant increase in product release and therefore lowers the costs per kg released product. A recycle of ammonia in the disruption process thus has a negligibly small effect on the operating costs of the disruption process. Ammonia recycling may, however, become worthwhile when the costs for wastewater treatment are taken into account since it can be recycled.

The enzymatic process gives a much higher IB release in comparison to homogenization with ammonia pretreatment at the same operating costs for cell disruption. Therefore, the enzymatic treatment seems to be favorable. However, for decent comparison of the disruption processes, their effect on IB purification and equipment capacity has to be taken into account. In the production of mcl-PHA, the substrate costs are most likely to be the cost-determining factor since the IB yield on the substrate is 0.40 kg product/kg substrate (Weusthuis et al., 2001) and the substrate price is approximately 0.9 $/kg coconut oil-free fatty acids (estimated on the basis of fatty acid prices reported at www.chemspy.com). This indicates that an increase in IB release, which reduces the fermentation capacity, has a larger impact on the overall operating costs than reduction of the operating cost for cell disruption. The enzymatic rupture process is thus favorable unless other disruption processes enable more efficient separation.

### Centrifugation

The result of this calculation is shown in Table II. The yield and purity obtained with centrifugal separation of the IBs from the cell debris can be calculated with the sigma theory discussed above. The parameters and assumptions that were used in this calculation are: $Q/\Sigma$ is $2.0 \times 10^{-9}$ m/sec, which is a reasonable value for centrifugation at high accelerations (Wong et al., 1997a); the fermentation broth contains 50 kg non-IB biomass/m$^3$ and 50 kg IBs/m$^3$ (Weusthuis et al., 2001); nonreleased IBs are assumed to go to the bottom outlet since the complex of cell debris and IBs will very likely have a reasonable size and a density near that of cell debris, and cell debris with a size below the critical particle diameter is assumed to be distributed homogeneously throughout the centrifuge as the centrifugal force exerted onto these particles will be too small to give the particles a significant velocity in the centrifuge. In addition, the top phase is assumed to contain 40% v/v of the inclusion bodies. This value is estimated on the basis of results obtained by Van Wegen et al. (1998), who showed that PHA can be concentrated approximately 2.4 times from a diluted mixture at a $Q/\Sigma$ ratio of $1.0 \times 10^{-8}$ m/sec. Based on these data, a concentration factor of 12 (60% v/v IBs in the top phase) may be expected in our process. However, for the higher particle concentration that is used in this case, the concentration factor is expected to be approximately 30% lower.

The results are shown for the optimal liquid-phase density that is defined here as the lowest density that yields the highest top phase purity. The calculations show that the enzymatic rupture process is the only process that reaches acceptable yields for industry. This is mainly due to the high IB release that is obtained with this method compared to two homogenizer passes at 1.6 kbar and one homogenizer pass at 1.6 kbar with ammonia pretreatment. Homogenization with ammonia pretreatment may give higher IB release with multiple homogenizer passes but the costs for disruption will go up as well (Table I). Therefore, this disruption

### Table I. Costs for homogenization at 160 MPa with and without pretreatment at pH 11.*

<table>
<thead>
<tr>
<th>Number of passes</th>
<th>Costs ($/kg released product)</th>
<th>Without pretreatment</th>
<th>pH 11 pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.097 (0.39)</td>
<td>0.063 (0.61)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.165 (0.46)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.237 (0.48)</td>
<td>0.163 (0.70)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.249 (0.61)</td>
<td>0.211 (0.72)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.316 (0.60)</td>
<td>0.235 (0.81)</td>
</tr>
<tr>
<td>Enzymatic disruption</td>
<td></td>
<td>0.149 (0.90)</td>
<td></td>
</tr>
</tbody>
</table>

*In between parentheses the product release fraction is shown.

*Not calculated because data point was an outlier in the measurement.

### Table II. Yield and purity in the top outlet for centrifugation at the optimal liquid phase density.

<table>
<thead>
<tr>
<th>Disruption method</th>
<th>Liquid density (kg/m$^3$)</th>
<th>Fraction IB released (w/w)</th>
<th>IB purity top yield (g/g DW)</th>
<th>IB yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two passes</td>
<td>1.050</td>
<td>0.46</td>
<td>0.80</td>
<td>0.46</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>1.055</td>
<td>0.90</td>
<td>0.88</td>
<td>0.90</td>
</tr>
<tr>
<td>pH 11 + one pass</td>
<td>1.045</td>
<td>0.61</td>
<td>0.87</td>
<td>0.60</td>
</tr>
<tr>
<td>pH 9.5 + one pass</td>
<td>1.050</td>
<td>0.50</td>
<td>0.86</td>
<td>0.50</td>
</tr>
</tbody>
</table>
process will only be favorable if the cell debris PSD that is obtained during cell disruption significantly increases the yield and/or purity of the product in comparison to the enzymatic rupture process. This is very unlikely as the median cell debris diameter for homogenization with pH 11 ammonia pretreatment is near the separation limit of the centrifuge and will only decrease with an increase in the number of homogenizer passes.

CONCLUSIONS

The comparison of disruption methods that was presented in this work shows that ammonia pretreatment in homogenization can be used to increase IB release and/or manipulate the cell debris particle size. Furthermore, enzymatic cell rupture produces cell debris particles with a varying chemical composition, while this is not the case with homogenization with and without ammonia pretreatment. This variation gives valuable information on the chemical composition of the cell debris particles, but it makes interpretation of the CSA data for enzymatic cell rupture difficult.

Centrifugal IB purification after enzymatic cell rupture, which yields small cell debris particles, is favorable in comparison to the other disruption processes that were investigated mainly because the enzymatic rupture process gives the highest IB release. However, since in this process cell debris is collected in the sediment and the IBs in the top phase, the separation efficiency in centrifugation decreases with a decrease in cell debris size. Therefore, other purification strategies, such as direct chemical extraction of the product from the cells, may be more efficient. Chemical extraction causes degradation of mcl-PHA and physical separation is thus preferable in this process. In other processes, where solvents do not affect the product, chemical extraction may be more attractive than physical separation when the product has to be separated from the small cell debris that is obtained with the enzymatic disruption process.

The authors thank Gert van der Steen for his collaboration with the GC analysis.

References


Van Hee P, Middelberg APJ, van der Lans RGJM, van der Wielen LAM.