

Disruption of *Alcaligenes latus* for Recovery of Poly(β -hydroxybutyric acid): Comparison of High-Pressure Homogenization, Bead Milling, and Chemically Induced Lysis

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Alcaligenes latus, a producer of the intracellular bioplastic poly(β -hydroxybutyric acid), was disrupted by chemical and mechanical means. Chemical lysis employed sequential treatment with sodium dodecyl sulfate (SDS) and sodium hypochlorite. The SDS treatment alone never released more than 60% of the cellular protein (a measure of disruption), but more than 90% of the protein could be released by the two-stage chemical process. Mechanical disruption used a continuous flow bead mill, or a high-pressure homogenizer. Bead mill disruption obeyed first-order kinetics. The diameter of grinding beads did not affect the disruption rate, but protein release improved with increasing bead loading. At low biomass concentrations the homogenizer was ineffective for disruption, but the bead mill could release almost all available protein. Disruption in the mill was independent of the slurry biomass concentration, but the performance of the homogenizer improved with increasing concentration until concentration-related blockages made operation impractical.

Introduction

Poly(β -hydroxybutyric acid), PHB, is an intracellular cytoplasmic storage polymer of the poly(hydroxy alkanate) (PHA) type that is produced by many bacteria (Poirier et al., 1995; Lee and Chang, 1995) as granular inclusions. PHAs are biodegradable and, at sufficiently large molecular weights, they are thermoplastics. Because of those features, PHA bioplastics are attracting considerable attention as potential limited replacements of traditional thermoplastics that constitute a persistent postconsumer waste. Furthermore, because of biocompatibility, PHAs are potentially useful in medical applications such as suture filaments (Poirier et al., 1995) and prolonged release drug formulations. Commercial production of PHAs is proven, but only as expensive specialty plastics. Important contributors to cost of production are the fermentation substrate (Poirier et al., 1995) and downstream processing (Berger et al., 1989; Lee and Chang, 1995). Inexpensive and scaleable PHA recovery schemes need to be developed to achieve low-cost production that is competitive with traditional thermoplastics.

This study compares mechanical disruption—high-pressure homogenization and bead milling—and chemically induced lysis of PHB producing *Alcaligenes latus* for potential use in product recovery. No prior information exists on bead mill disruption of this organism and because disruption depends strongly on the microorganism and culture conditions (Chisti and Moo-Young, 1986, 1991), data on the other disruption methods are essential for a reasonable comparison.

Literature Review

A PHA copolymer (hydroxy butyrate/hydroxy valerate) was commercially produced by Imperial Chemical Industries (ICI) in the United Kingdom. A 100–120-h-fed batch culture of *Alcaligenes eutrophus* was employed, and the product was marketed under the trade name Biopol (Byrom, 1990). Zeneca, a former subsidiary of ICI, inherited the production process which was later acquired by Monsanto. An outline of ICI's production scheme has been published (Marchessault et al., 1990). A different product, the PHB homopolymer, was produced by the Austrian company Chemie Linz GmbH via continuous culture of *A. latus* on sucrose (Hänggi, 1990; Hrabak, 1992; Poirier et al., 1995). Unlike in *A. eutrophus*, PHB production in *A. latus* is growth associated; hence, a one-stage fermentation with a shorter overall culture period can be employed. A single-stage fermentation is also more suited to continuous culture.

Chemie Linz employed solvent extraction for PHB recovery. Harvested and washed biomass was extracted with methylene chloride (Hänggi, 1990; Hrabak, 1992). The polymer-laden solvent was recovered by decanter centrifugation, and the PHB was precipitated by adding water (Hrabak, 1992). Other suitable extraction solvents have been reported, including dichloroethane, 1,1,2-trichloroethane, chloroform, acetic anhydride, propylene carbonate, and ethylene carbonate (Berger et al., 1989). Solvent extraction is expensive, creates disposal problems and other hazards, and destroys the unique natural morphology of PHA granules that is useful in certain applications (Barham, 1990; Ramsay et al., 1990). For example, the native noncrystalline amorphous state of the PHB granules could be especially useful in producing strong fibers (Barham, 1990).

Like Chemie Linz, ICI initially used solvent extraction for PHA recovery, but that process was considered

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too expensive (Byrom, 1990; Poirier et al., 1995). Instead, a series of aqueous enzyme and detergent washes were developed to solubilize the heat-shocked cells to leave behind the PHA granules (Byrom, 1990). Yet other recovery processes have employed treatment of biomass with hypochlorite. Hypochlorite treatment is effective in solubilizing cellular components, but hypochlorite is known to severely degrade the PHA polymer (Hahn et al., 1994; Ramsay et al., 1990). Optimizing treatment regimen may limit the molecular weight loss (Berger et al., 1989). Less severe hypochlorite treatments in combination with other methods have been used to obtain a purer product while limiting hypochlorite-associated degradation. Examples of such procedures are the various surfactant-hypochlorite treatments noted by Ramsay et al. (1990). Surfactant treatment alone is also effective, but the purity of the recovered pellet is lower than that with combination treatments (Ramsay et al., 1990). Surfactants such as sodium dodecyl sulfate (SDS) and the nonionic Triton X-100 do not degrade the polymer. Among other surfactants, synthetic palmitoyl carnitine—a widespread natural surfactant—has been shown to lyse *A. eutrophus* and *A. latus*, with the latter being significantly more susceptible (Lee et al., 1993). Lysis of *A. eutrophus* exceeded 70%, whereas that of *A. latus* was over 85% when treated with 1 mM palmitoyl carnitine in 0.1 M Tris-HCl buffer (pH 7.0, 30 °C, 60 min) with gentle shaking (Lee et al., 1993).

A combination of chloroform and thioglycolic acid has also been used (Ramsay et al., 1990). Another novel approach combined sodium hypochlorite digestion with chloroform extraction to substantially lower PHB losses (Hahn et al., 1994). A dispersion of chloroform in aqueous hypochlorite solution was used to treat the biomass. As the cells were digested and released the PHB granules, the polymer dissolved into the organic phase; hence, its exposure to hypochlorite was minimized. This mode of recovery reduced degradation relative to treatment with hypochlorite alone, but degradation could not be entirely eliminated. Under optimal conditions—30% w/v hypochlorite, 1:1 volume ratio of chloroform-to-aqueous phase, 4% w/v cells in dispersion, 30 °C, 90-min treatment—91% PHB could be recovered from *A. eutrophus* (Hahn et al., 1994). The purity of the product exceeded 97% (Hahn et al., 1994).

Mechanical cell disruption is widely used for recovering intracellular proteins (Chisti and Moo-Young, 1986, 1991, 1994; Harrison, 1991; Kula and Schütte, 1987; Middelberg, 1995), but PHA recovery by mechanical means has not received much attention. Large-scale cell disruption devices include high-pressure homogenizers and bead mills (Chisti and Moo-Young, 1986). Combinations of chemical and mechanical methods seem particularly promising (Harrison et al., 1991b). High-pressure homogenization with and without chemical pretreatment was examined for PHB recovery from *A. eutrophus* (Harrison et al., 1991b). An APV-Gaulin homogenizer (Chisti and Moo-Young, 1986) fitted with a ceramic valve seat (Chisti and Moo-Young, 1991) was employed. Complete disruption required three passes at 60–69 MPa (Harrison et al., 1991b). To limit total energy input to below 70 MJ·m⁻³, a single-pass disruption was necessary; thus, Harrison et al. (1991b) investigated various pretreatments to achieve that objective. Alkaline pretreatment of biomass (pH 10.5, 7 °C, ≤1 min) substantially improved single-pass disruption

performance, but a minimum of two passes were still needed for complete protein release (Harrison et al., 1991b). Cells pretreated with 0.1% w/v SDS (70 °C, 20 min) could be completely ruptured in a single homogenizer pass at a relatively low operating pressure of 62 MPa (Harrison et al., 1991b). Single-pass operating pressure could be reduced yet further to only 34.5 MPa when the cells were pretreated with a larger amount of SDS (1% w/v, 70 °C, 20 min). Pretreatments with sodium chloride or potassium chloride (≈0.14 M, 60 °C, 60 min) also enhanced disruption relative to salt-free thermal treatment (60 °C, 60 min) presumably because of the known thermal injury enhancing effect of monovalent metal ions (Harrison et al., 1991b). Pretreatments with lysozyme, EDTA, and a combination of the two were seen to improve single-pass disruption relative to untreated material (Harrison et al., 1991b). In further work with enzymes, Harrison et al. (1991a) reported essentially complete lysis of *A. eutrophus* cells upon treatment (37.5 °C, pH 7.3, 60 min) with lytic enzymes of *Cytophaga* sp. without any mechanical processing. But lytic enzymes (e.g., hen lysozyme) are generally too expensive for large-scale applications.

Unlike solvents, surfactants, and other chemicals, mechanical disruption, possibly in combination with a benign treatment such as heat shock, is likely to be less expensive and less damaging to the product. Furthermore, mechanical treatment should not contaminate the product, and it should be environmentally less burdensome. This work reports on the mechanical disruption of *A. latus*. Release of intracellular protein is used as a measure of disruption in keeping with other work on the recovery of intracellular products (Chisti and Moo-Young, 1986; Harrison, 1991; Middelberg, 1995) including PHB (Harrison et al., 1991a,b; Tamer et al., 1997). Intracellular protein release has been shown to correlate with the release of PHB irrespective of the specific method used for disrupting the cells (Tamer et al., 1997).

Materials and Methods

Microorganism and Culture Conditions. An intracellular PHB producer, *A. latus*, ATCC 29713, was used throughout. The culture was maintained at 4 °C on slants of Bacto nutrient agar (Difco Laboratories, Detroit, MI). Two-staged inocula were grown in shake flasks held at 33 °C, 200 rpm, for 2–3 days on a rotary shaker (Innova 4330; New Brunswick Scientific Co., Inc., New Brunswick, NJ). For the first stage, a loopful of slant culture was inoculated into a 250-mL flask containing 50 mL of Bacto nutrient broth (Difco Laboratories, Detroit, MI) supplemented with 10 g·L⁻¹ of sucrose. A portion of this preculture (5 mL) was inoculated into a 2-L flask that contained 500 mL of the following medium (per liter): 20 g of sucrose, 1.4 g of (NH₄)₂SO₄, 1.5 g of KH₂PO₄, 1.8 g of Na₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.3 g of H₃BO₃, 0.2 g of CoCl₂·6H₂O, 0.1 g of ZnSO₄·7H₂O, 30 mg of MnCl₂·4H₂O, 30 mg of Na₂MoO₄·2H₂O, 28 mg of NiSO₄·7H₂O, and 10 mg of CuSO₄·5H₂O, as previously employed by Yamane et al. (1996), and 60 mg of ammonium iron(III) citrate. Fermentations were carried out in a 15-L (nominal) stirred tank fermenter (MBR Bioreactor AG, Switzerland) equipped with pH, temperature, and dissolved oxygen controllers. A presterilized medium (4 L) at 33 °C, pH 7.0, was inoculated with 500 mL of the inoculum described earlier. The fermentation medium was identical to that of the second stage of preculture.

The cells were grown in batch and fed-batch modes. Approximately 1.5 L of 50% w/v sucrose solution and 160 mL of 20% w/v ammonia solution were fed from separate reservoirs. The ratio of the two feeds (C:N) was 7–10, and the feeding was controlled in response to a pH control signal as in a pH-stat previously used also by Yamane et al. (1996). The culture lasted about 50 h. The dissolved oxygen concentration was maintained above 20% of air saturation by constant aeration at 1 vvm; the agitation speed (400–900 rpm) varied in response to the dissolved oxygen level. The pH was controlled at 6.5 by control of aqueous ammonia feed as previously noted.

Just prior to harvest, the temperature was raised to 80 °C and cooled to ambient temperature over a total period of 20 min. At harvest the biomass concentration was typically 35 kg·m⁻³, and the PHB content of the biomass was 49–53% w/w. The harvested broth was kept refrigerated (4 °C) until needed. The storage period did not exceed 1 month. The cells were recovered at 5500g, 15 min, in a laboratory centrifuge (IEC Centra-HN, International Equipment Company, Needham Heights, MA); for larger quantities a Sorvall centrifuge (RC 5B Plus, Dupont, Wilmington, DE) running at 16 250g, 15 min, was employed. The cells were then washed by resuspending in deionized water, centrifuged, and made into slurry in a phosphate buffer (0.1 M, pH 7.0) as needed.

Chemically Induced Lysis. Sodium dodecyl sulfate, SDS, and commercial sodium hypochlorite solution were employed for cell lysis. For treatment with SDS, equal amounts of the detergent solution and biomass slurry were mixed (shake flask, 120 rpm; Innova 4330 reciprocating shaker, New Brunswick Scientific Co., Inc., New Brunswick, NJ) and incubated at 35 °C for 1 h. The resulting suspension was centrifuged (5500g, 15 min; IEC Centra-HN centrifuge, International Equipment Company, Needham Heights, MA) to recover the supernatant for protein assays. Concentrations of the SDS and the biomass were varied to obtain different treatment combinations. The SDS-treated pellet was further treated with sodium hypochlorite solution (Javex-5, Colgate-Palmolive Canada Inc., Toronto). The treatment procedure was identical to that used with SDS, except that the incubation period was 24 h. The recovered pellet was gravimetrically analyzed for PHB.

Bead Mill Disruption. A continuous flow high-speed bead mill (Annu Mill 01, Sulzer Brothers Limited, Winterthur, Switzerland) as previously described for disintegration of *Saccharomyces cerevisiae* (Garrido et al., 1994) was used. Briefly, the equipment consisted of a vertical cylindrical grinding chamber having a concentric cylinder variable speed rotor for agitation of glass beads. The rotor and the outer cylinder (stator) were 50 and 62 mm in diameter, respectively; hence, the annular gap had a width of 6 mm. The length of the rotor was 150 mm. The cell slurry entered the mill at its base, flowed up the annular gap between the rotor and stator, and exited near the top through a perforated stainless steel screen which retained the beads. A mechanical seal prevented leakage where the rotor shaft penetrated the grinding chamber. Operation of the mill generated heat that was removed by circulating cooling water in the jacket that surrounded the grinding chamber. The disintegration media were lead-free soda lime silica glass beads of diameter 425–600 μm (Sigma Chemical Co., St. Louis, MO; catalog no. G9268) and

710–1180 μm (Sigma; catalog no. G9393). Bead loading was either 75% or 85% of chamber volume (210 mL). Those loading figures were based on the packed volume measured prior to loading. The density of the beads was 2740 kg·m⁻³. In view of the results of earlier work (Garrido et al., 1994), the agitation rotor speed (2800 rpm) and the slurry flow rate (90 mL·min⁻¹) remained constant. The selected slurry flow rate was too low to cause fluidization of grinding media and a consequent loss in disruption performance. Cooling of the grinding chamber ensured that the slurry remained at 22–24 °C, an acceptable range in view of the thermal stability of the product. At 2800 rpm the agitation rotor speed was such that any further increase would not have improved disruption performance (Garrido et al., 1994). Before each run, the grinding chamber was filled with deionized water and the rotation speed was set. The cell slurry was introduced once the system had stabilized. Dilution of the slurry was avoided by discarding at least 150 mL of the initial flow from the grinding chamber. Typically, 750–1000 mL of broth was used in disruption experiments. Samples (4 mL) were collected at the exit port of the grinding chamber for each pass. The samples were diluted 2–3-fold with deionized water to facilitate debris removal by centrifugation at approximately 10 000g (Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA) for 15 min.

High-Pressure Homogenization. A Microfluidics high-pressure homogenizer, the “microfluidizer”, (model M110T with extra heavy duty pump; Microfluidics Corp., Newton, MA) was used. This device consisted of an air-driven positive displacement pump which forced the cell slurry through two parallel slots (2 × 100 μm) under high pressure (90–95 MPa in this work). The resulting parallel fluid streams impinged on a vertical plate, flowed toward each other, recombined, and were forced out. Disruption occurred at ambient temperature (25 °C) which was maintained by immersing the disruption chamber and the exit lines in ice throughout operation.

Protein Assay. Protein released by chemical disruption was followed by the micro-Kjeldahl method (Lang, 1958). The absorbance was read at 420 nm (Pye Unicam SP6–550 UV/vis spectrophotometer, Philips Scientific and Analytical Equipment) after nesslerization. Mechanically disrupted samples were analyzed with the Lowry method (Lowry et al., 1951). Measurements were made directly on the supernatant. A protein standard (Sigma Chemical Co., St. Louis, MO; catalog no. P7656) prepared from bovine serum albumin was used for calibration. Absorbance was read at 750 nm (Pye Unicam SP6–550 UV/vis spectrophotometer, Philips Scientific and Analytical Equipment) after treatment with Folin-Ciocalteu phenol reagent (Sigma Chemical Co., St. Louis, MO; catalog no. F9252). The maximum amount of releasable protein was measured when further passes through the mill yielded no additional protein. Maximum protein release amounted to 90 g of protein/kg of total dry biomass.

The two assay procedures gave equivalent results. Compared to Kjeldahl, the Lowry method was simpler, but it could be used only with mechanically disrupted samples because chemicals such as SDS interfered with the Lowry assay.

Poly(β-hydroxybutyric acid) Assay. A gravimetric method similar to those employed previously by

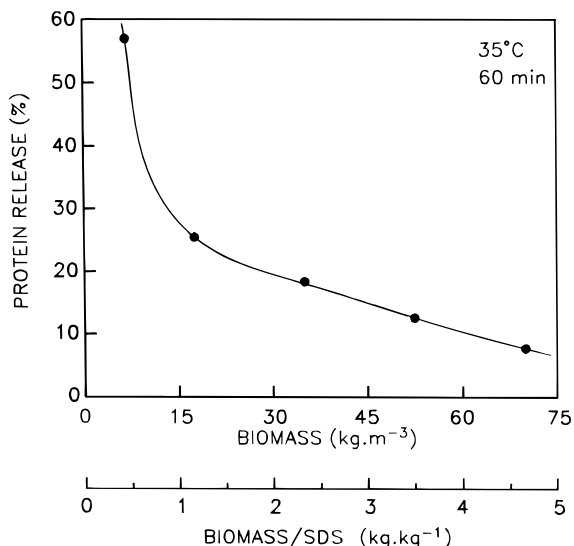


Figure 1. Effect of relative amounts of cells and sodium dodecyl sulfate (SDS) on protein release with SDS treatment (1.5% w/v SDS solution, pH 10, 1:1 volume ratio of SDS-to-biomass slurry).

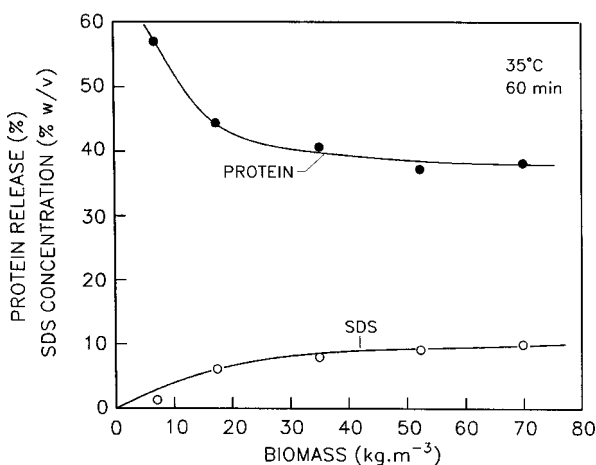


Figure 2. Effect of relative amounts of cells and SDS on protein release by high-level SDS treatment (pH 10, 1:1 volume ratio of SDS-to-biomass slurry, mass ratio of biomass-to-SDS varied over 0.47–0.7).

Marchessault et al. (1990) and Ramsay et al. (1990) was used. The pellet portion of the mechanically disrupted, centrifuged (10 000g, 15 min; Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA; room temperature) samples (4 mL) was used to estimate the PHB content. The pellet was mixed with 1.5% w/v sodium dodecyl sulfate (4 mL, pH 10, 35 °C, 1 h). The mixture was centrifuged and the remaining solids were washed with deionized water, recovered, and washed with alkaline (pH 13) commercial sodium hypochlorite solution (Javex-5, Colgate-Palmolive Canada Inc., Toronto; 4 mL, 5.64% w/v sodium hypochlorite). The remaining solids were recovered by centrifugation and thoroughly washed with deionized water. The final pellet was dried (55 °C, 24 h) to constant weight in preweighed aluminum dishes.

Results and Discussion

Chemical Disruption. Release of available protein upon treatment with different amounts of SDS is shown in Figures 1 and 2. The data in Figure 1 were obtained by mixing equal volumes of a dilute SDS solution (3%

w/v SDS) and a biomass slurry having different amounts of suspended biomass. The final SDS concentration in the mixture was 1.5% w/v. Almost 60% of the available protein was released when the slurry biomass concentration was low (7 kg DW·m⁻³) corresponding to a biomass:SDS ratio of about 0.5 (Figure 2). As this ratio increased to one or higher, protein release declined rapidly to less than 30% levels. The sharp rise in protein release at a biomass:SDS ratio of about 0.5 suggested that at least that value was necessary to achieve saturation of the cell membrane with SDS and consequent rupture. The data in Figure 2 were obtained at relatively high levels of SDS. The biomass and the SDS concentrations increased in a range of 7–70 kg DW·m⁻³ and 1.5–10% w/v, respectively. The mass ratio of biomass-to-SDS varied over 0.47–0.7. The results were consistent with those in Figure 1. From Figure 2, a near constant amount (~40%) of the protein was released for a fixed proportion of SDS (~10% w/v) despite an increasing concentration of biomass (30–70 kg·m⁻³), suggesting good contact between SDS and the cells.

Surfactants such as the anionic SDS disrupt cells by incorporating into the lipid bilayer membrane. As more surfactant is added, more of it enters the membrane to increase the volume of the cell envelope (Ramsay et al., 1990) until the envelope is saturated. Further addition ruptures the membrane to produce micelles of surfactant and membrane phospholipids (Ramsay et al., 1990). The polymer granules are left enclosed in a matrix of peptidoglycan and cell debris (Ramsay et al., 1990).

The observed inability of SDS to fully release all the protein was consistent with other findings. For example, in studies with a recombinant *S. cerevisiae*, Garrido et al. (1994) observed that SDS–chloroform-induced lysis was ineffective in quantitatively releasing all the cellular protein, whereas mechanical disruption in a bead mill attained complete release. Furthermore, SDS–chloroform combination was shown to denature β -galactosidase even though small amounts of SDS were used and the concentration of chloroform did not exceed 5% v/v (Garrido et al., 1994). SDS is a well-known protein denaturant; hence, it is contraindicated particularly when bioactive proteins are the desired product.

Because SDS could not release all of the available protein, further treatment became necessary. Hypochlorite treatment was used. Figure 3 depicts protein release obtained by sodium hypochlorite treatment of the pelleted solids of the SDS treatment shown in Figure 1. The data points corresponding to the same biomass concentration in the two figures represent the same initial sample. Thus, by difference, the additional protein released by hypochlorite treatment of the five samples with 7–70 kg DW·m⁻³ of biomass levels corresponded to, respectively, 42%, 71%, 76%, 80%, and 83%. Based on the undisrupted biomass remaining after the first stage, the second-stage hypochlorite treatment always released much more protein than did the SDS treatment. Note, however, that hypochlorite treatment required 24 h versus 60 min for the SDS treatment. If the additional protein release is assumed to depend linearly on the exposure period, then hypochlorite treatment appears to be quite poor relative to SDS treatment. The kinetics of hypochlorite-induced release were not examined, and much of the protein release may well have occurred during a fraction of the

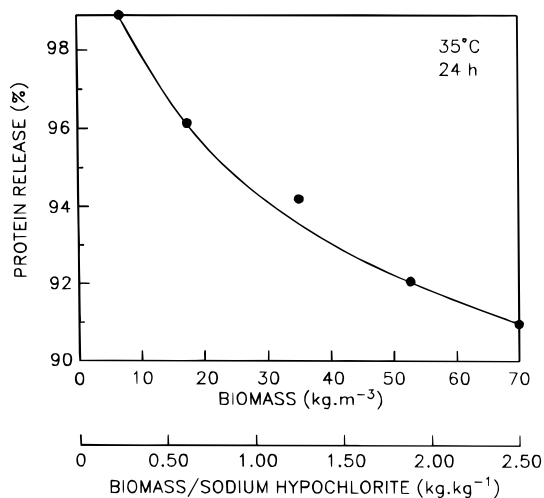


Figure 3. Effect of relative amounts of cells and sodium hypochlorite on protein release with hypochlorite treatment (5.64% w/v sodium hypochlorite, pH 13, 1:1 volume ratio of hypochlorite solution-to-biomass slurry).

total exposure time. Note, however, the fact that for most of the data shown in Figure 3 complete release of protein had not yet been attained even after 24 h.

Despite the high levels of protein release achieved with the two-stage SDS/hypochlorite treatment, this procedure is unlikely to be commercially useful. If a total protein release of 95% is deemed acceptable, then, based in Figures 3 and 1, a rather dilute biomass slurry of 23 kg DW·m⁻³ would need to be sequentially treated with 16.4 kg·m⁻³ of SDS followed by 28 kg·m⁻³ of sodium hypochlorite, and the total treatment time would exceed 25 h. Such large chemical inputs and long processing periods do not make economic sense for recovering at most 10–15 kg·m⁻³ of PHB. Furthermore, this scenario does not yet include the expense of treating the SDS/hypochlorite-containing waste. Clearly, chemical-free mechanical processing is to be preferred if it can be shown to be effective.

Mechanical Disruption. Cell disruption in bead mills and high-pressure homogenizers typically obeys first-order kinetics (Chisti and Moo-Young, 1986, 1991; Kula and Schütte, 1987). For the mill employed in this work, Garrido et al. (1994) had established the equation

$$\ln \frac{R_m}{R_m - R} = \frac{k(1 - \phi)V}{F} N \quad (1)$$

which applied to protein release from a recombinant *S. cerevisiae*. In eq 1 R is the amount of protein released per unit cell mass after N passes through the mill, R_m is the maximum amount of releasable protein per unit cell mass, k is the first-order disruption rate constant, V is the volume of the milling chamber, F is the flow rate of the cell suspension through the mill, and ϕ is the volume fraction of the beads in the mill. As shown for one set of data in Figure 4, plots of $\ln[R_m/(R_m - R)]$ versus the number of passes were linear for a given bead loading, flow rate, and mill volume; hence, confirming eq 1 for disruption of *A. latus* which has a much smaller cell size—only about $1/10$ —than the yeast *S. cerevisiae*. From eq 1, the first-order disruption rate constant was

$$k = \frac{SF}{(1 - \phi)V} \quad (2)$$

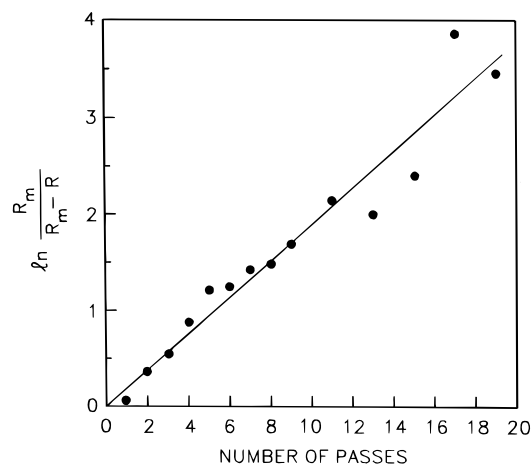


Figure 4. Plot of $\ln[R_m/(R_m - R)]$ versus number of passes in the bead mill (8 kg DW·m⁻³ cell concentration, 75% bead loading, 710–1180- μ m bead diameter).

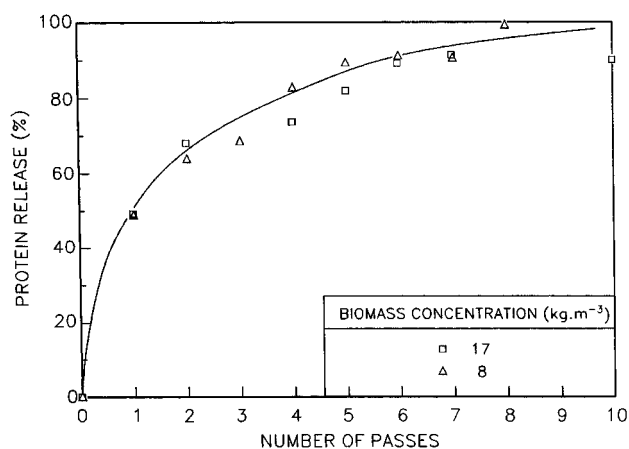


Figure 5. Effect of biomass concentration on protein release by bead mill disruption (85% bead loading, 425–600- μ m bead diameter).

where S was the slope of the line in Figure 4. The data shown in Figure 4 produced a k value of 0.321 min⁻¹.

For disruption of a recombinant *S. cerevisiae* in the same mill, Garrido et al. (1994) achieved complete disruption within three passes while operating at 2000 rpm rotor speed, 5 kg DW·m⁻³ of cell concentration, 100 mL·min⁻¹ slurry flow rate, and 85% loading of 500- μ m beads of 2500 kg·m⁻³ density. Under similar conditions (8 kg DW·m⁻³ of biomass concentration, 90 mL·min⁻¹ flow rate, 2800 rpm, 85% loading of somewhat denser beads, and 512- μ m mean bead diameter), complete disruption of *A. latus* required eight passes (Figure 5). The significantly higher resistance to disruption was associated with the much smaller size of the bacterium relative to that of the yeast. The slight difference in cell concentrations between the yeast slurry of Garrido et al. (1994) and this work cannot account for differences in disruption behavior, because numerous studies have shown bead mill disruption to be independent of the cell concentration (Chisti and Moo-Young, 1986, 1991; Kula and Schütte, 1987). Indeed, Garrido et al. (1994) observed no differences in disruption performance when the yeast cell concentration was varied over 5–20 kg DW·m⁻³. Similarly, using the same machine, Mao and Moo-Young (1990) had shown that yet higher concentrations of yeast (250–600 kg·m⁻³ packed weight of *S. cerevisiae*) did not affect the disruption rate. In this work, too, changes in bacterial biomass concentration

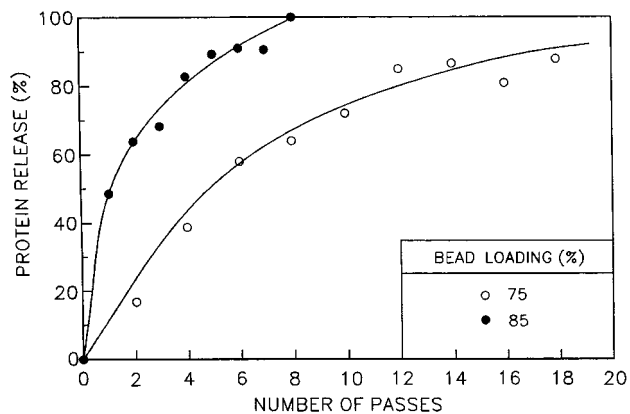


Figure 6. Effect of number of passes and bead load on protein release by bead mill disruption (8 kg DW·m⁻³ cell concentration, 425–600- μ m bead diameter).

(8–45 kg·m⁻³) in the slurry did not affect disruption (Figure 5 and others). In all cases, 75%–80% of the soluble protein could be released by the fifth pass. The noted differences in the disruption behavior of the two microorganisms under similar conditions reflect the influence of the different cell sizes and cell wall strengths.

Fewer passes are generally necessary for the disruption of yeasts in bead mills than those needed for disintegration of bacteria (Chisti and Moo-Young, 1986). Under optimal conditions, three or four passes are generally sufficient for complete disruption of bacteria (Chisti and Moo-Young, 1986; Kula and Schütte, 1987). As noted, *A. latus* required a significantly larger number of passes, suggesting a relatively robust cell even among bacteria.

Note that the comparison of the disruption of *A. latus* and *S. cerevisiae* is based on slightly different slurry flow rates: 90 mL·min⁻¹ in this work and 100 mL·min⁻¹ used by Garrido et al. (1994). However, this difference does not explain the vastly different disruption rates. A lower flow rate (i.e., longer residence time in the grinding chamber) actually improves disruption as clearly documented for yeast disruption (Garrido et al., 1994). Although the *A. latus* slurry was disrupted at 2800 rpm agitation speed versus 2000 rpm used with the yeast, earlier work in the same mill had shown that changes over 2000–4000 rpm did not affect disruption behavior (Garrido et al., 1994). Clearly, therefore, differences in intrinsic physical characteristics of the two microorganisms were the cause of the observed differences in disruption performance.

In keeping with prior experience (Garrido et al., 1994; Kula and Schütte, 1987; Chisti and Moo-Young, 1986), disruption was quite sensitive to the loading of glass beads. With 512- μ m beads, complete disruption was achieved by the eighth pass when the loading was 85%, whereas a reduced loading of 75% did not release all the cellular protein even after 16 passes (Figure 6). The bead-loading effect on disruption was far more pronounced than generally observed in earlier studies, again suggesting significant differences in bacterial disruption relative to the more commonly used yeasts. Thus, in the same mill, Garrido et al. (1994) noted a significantly weaker improvement in disruption of *S. cerevisiae* when the bead loading was increased from 75% to 85%.

For the small *A. latus* cells, the disruption rate was little affected by changes in the mean diameter of the glass beads (Figure 7) even though the diameter varied

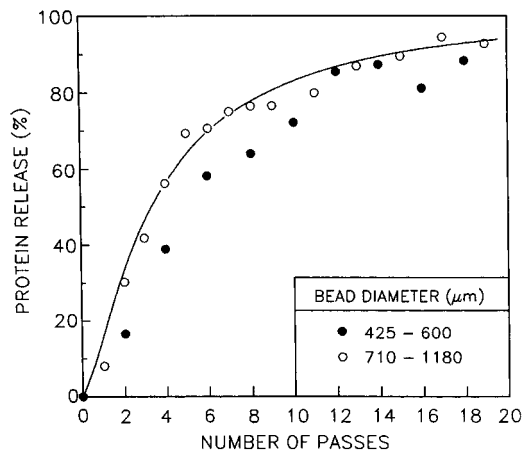


Figure 7. Effect of number of passes and mean bead diameter on protein release by bead mill disruption (8 kg DW·m⁻³ cell concentration, 75% bead loading).

by almost 2-fold from 512 to 945 μ m. Apparently, both those sizes were outside the range in which a bead size optimum may exist for the small cells. Earlier, using larger yeast cells, Garrido et al. (1994) had identified an optimal disruption bead diameter of 500 μ m that produced better disruption relative to either 250- or 750- μ m beads. This work did not test beads smaller than 512 μ m because of a concern for the integrity of PHB granules. Evidence for micronization of PHB with increasing exposure to severe disruption conditions is discussed later in this section.

Bead mills with vertical disruption chambers are prone to the loss of performance if an upflow of the cell slurry causes fluidization of the grinding media. Although the peculiar geometry of the mill used provided only a small cross-sectional area (the annulus) for flow and it was susceptible to easy fluidization, a low flow rate (90 mL·min⁻¹) assured absence of fluidization. The calculated sedimentation velocity of the smallest beads exceeded the superficial slurry velocity in the annulus by a factor of several hundred. Moreover, with the same mill and similar but lighter beads (500- μ m diameter, 2500 kg·m⁻³ density), an earlier work also demonstrated lack of fluidization even at flow rates that were almost 3-fold greater than the one used here (Garrido et al., 1994).

Biomass concentration was the only parameter varied during disruption by the homogenizer. At a relatively low biomass concentration of 17 kg·m⁻³ the homogenizer was far less effective than the bead mill (Figure 8). Thus, less than 50% of the protein was released at the ninth pass, whereas in the mill more than 80% had been released by the sixth pass. The performance of the homogenizer improved during processing of a higher biomass (45 kg·m⁻³) slurry (Figure 9), but the disruption in the mill was insensitive to the biomass level. However, even for that biomass level, the homogenizer released only about 15% more protein than did the mill at the fifth pass (Figure 9). Note that the decline in bead mill protein release seen between passes seven and eight (Figure 9) was due to leakage of some lubricating/cooling water from the mechanical seal (where the rotor entered the grinding chamber) into the homogenate; thus, the protein solution was diluted. For 45 kg·m⁻³ of slurry biomass level, a cumulative residence time of less than 10 min in the bead mill was sufficient to release all the soluble protein. Residence time data for the homogenizer are largely meaningless as disruption

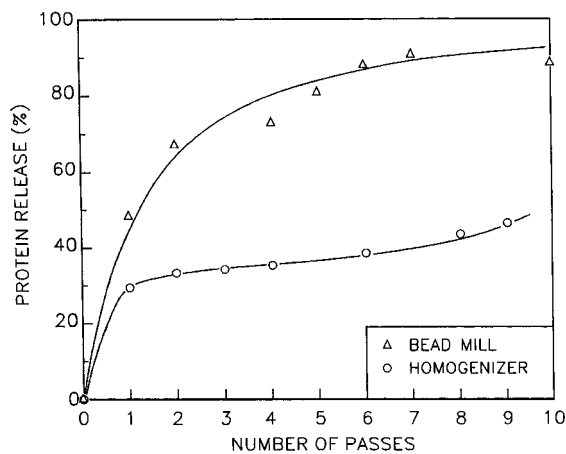


Figure 8. Comparison of the bead mill (85% bead loading, 425–600- μm bead diameter) and the homogenizer (90–95 MPa). Biomass concentration in slurry was 17 kg DW $\cdot\text{m}^{-3}$.

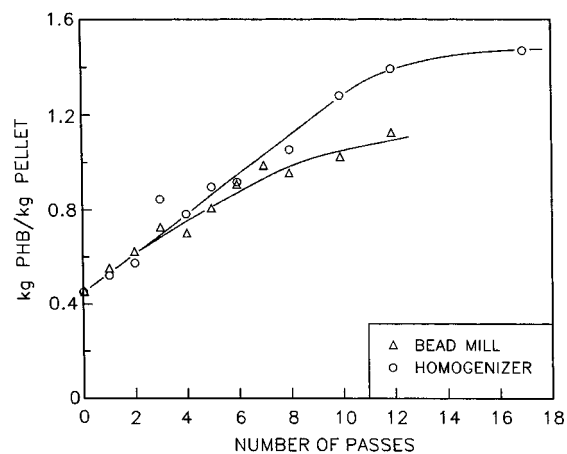


Figure 10. Comparison of the bead mill (85% bead loading, 425–600- μm bead diameter) and the homogenizer (90–95 MPa) for PHB recovery. Biomass concentration in slurry was 45 kg DW $\cdot\text{m}^{-3}$.

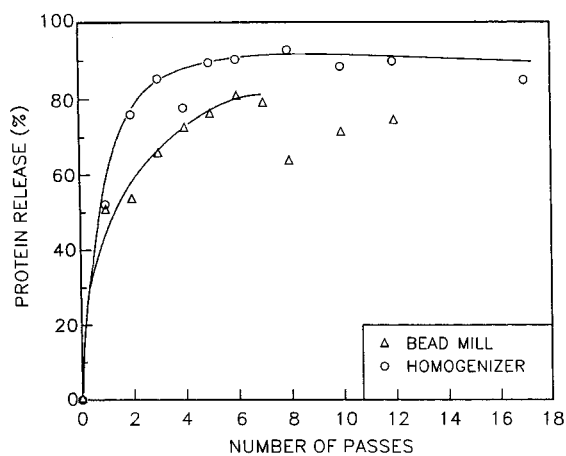


Figure 9. Comparison of the bead mill (85% bead loading, 425–600- μm bead diameter) and the homogenizer (90–95 MPa). Biomass concentration in slurry was 45 kg DW $\cdot\text{m}^{-3}$.

occurs mainly during passage through the slit and possibly when the fluid stream impacts upon the wall or other stationary surface (Chisti and Moo-Young, 1986). In any case, an accurate determination of residence time for the homogenizer proved impossible because of frequent blockages.

At a slurry biomass level of 66 kg $\cdot\text{m}^{-3}$, the homogenizer was entirely ineffective due to continual plugging, but the bead mill could process this concentration. The viscosity of the 66 kg $\cdot\text{m}^{-3}$ of *A. latus* slurry was 0.3 Pa $\cdot\text{s}$. In contrast to the machine used here, other designs of commercial high-pressure homogenizers are known to effectively process nonfilamentous bacterial slurries without suspended solids (Chisti and Moo-Young, 1986; Harrison et al., 1991b). Those other devices have a higher upper limit on viscosity at 1 Pa $\cdot\text{s}$ (Chisti and Moo-Young, 1991), and they typically operate at lower pressures, usually less than 60 MPa (Chisti and Moo-Young, 1986). Some such industrially useful machines have been discussed by Chisti and Moo-Young (1986, 1991). Because power consumption is a linear function of the operating pressure (Chisti and Moo-Young, 1986), excessively high operating pressures are not wanted unless the number of passes can be reduced sufficiently that the total power input can be kept small for a given level of disruption. In high-pressure homogenizers, design of the discharge valve seat has a major impact on disruption performance (Chisti and Moo-Young,

1986, 1991; Kula and Schütte, 1987); therefore, the relatively poor performance of the Microfluidics machine was most likely due to the peculiarities of its discharge slits. A further explanation for the blockages may be the nature of the product: unlike most intracellular products, PHB is a dense solid which could deposit around the slit because of high-speed impaction and gradually block the opening.

The actual amount of PHB in the cells was determined by chemical digestion and gravimetry. The mass ratio of this pure PHB to that of the crude pellet from various passes of the homogenizer and the mill are plotted in Figure 10 as functions of the number of passes. The PHB-to-pellet ratio is an indicator of the purity of the crude pellet. A ratio of unity suggests a pure product. The value of this ratio at zeroth pass (Figure 10) corresponds to the mass fraction of PHB in undisrupted cells. For both modes of mechanical disruption, that ratio increased with an increasing number of passes: as more of the cellular material was solubilized and micronized, the total mass of the pellet declined. In both machines, the ratio approached unity around the seventh pass (Figure 10) during the processing of 45 kg DW $\cdot\text{m}^{-3}$ of cell slurry. (See Figure 9 for the corresponding protein release data.) An increase in the PHB-to-pellet ratio to greater than 1.0 after the seventh pass (Figure 10) suggested a loss of PHB by micronization to levels that could not be sedimented. As shown in Figure 10, micronization was more severe in the homogenizer than in the bead mill. PHB loss by micronization suggests the need to achieve complete disruption under relatively less severe conditions and to limit the exposure to such conditions. An eight-pass disruption corresponded to cumulative exposure of 8.75 min in the mill. Note that increasing PHB loss by micronization occurs even in an aqueous slurry (no other chemicals) as the severity of mechanical treatment increases. Data in Figure 10 suggest that in relative terms the bead mill is better than the homogenizer in reducing PHB losses.

Conclusions

Chemically induced lysis of *A. latus* with sodium dodecyl sulfate alone was unsatisfactory for disruption even though quite high SDS levels were tested (0.2–2 kg of SDS/kg of biomass). Sequential treatment with SDS-to-biomass ratio of at least 0.2 (pH 10, 35 °C, 60

min) followed by a 24-h sodium hypochlorite wash (at least 1.3 kg of hypochlorite/kg of biomass, pH 13, 35 °C) was necessary to remove 95% of the cellular protein. In contrast, similar disruption levels were achieved within minutes by best mechanical treatments in the bead mill.

Bead mill disruption was independent of the biomass concentration (8–66 kg DW·m⁻³); disruption performance was consistent and predictable, thus ensuring ease of scale-up. First-order disruption kinetics were observed. The mill was not susceptible to blockages, and complete disruption was achieved within eight passes (2800 rpm, 85% loading of 512- μ m beads, 90 mL·min⁻¹ slurry flow rate). Diameter of the grinding beads did not affect the disruption rate, but the rate depended strongly on bead loading. Compared to the homogenizer, data indicated little micronization of the mill-processed PHB during eight passes.

The performance of the homogenizer depended on biomass concentration. Relative to the mill, the homogenizer performed quite poorly at low biomass levels, but at 45 kg DW·m⁻³ of cell concentration the homogenizer was a somewhat better disrupter. Nonetheless, frequent blockages made processing difficult.

In view of the performance, relatively low-power consumption, and the fact that bead mills are robust and relatively inexpensive devices that are widely used commercially (Chisti and Moo-Young, 1986; Kula and Schütte, 1987), bead mill disruption is recommended as the preferred method for recovering PHB from heat-shocked *A. latus*. Mechanical disruption recovered PHB without solubilizing it; hence, the native amorphous morphology of the polymer was retained.

Acknowledgment

Enrico Grothe assisted with the fermentations. This research was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

Nomenclature

DW: dry weight
 EDTA: ethylenediaminetetraacetic acid
 F: flow rate of the cell slurry, mL·min⁻¹
 k: disruption rate constant, min⁻¹
 N: number of passes through bead mill or homogenizer
 PHA: poly(hydroxyalkanoate)
 PHB: poly(β -hydroxybutyric acid)
 R: specific protein release after N passes, m⁻³
 R_m: maximum amount of releasable protein per unit biomass, m⁻³
 S: slope of ln[R_m/(R_m - R)] versus N plots
 SDS: sodium dodecyl sulfate
 V: volume of the milling chamber, mL
 ϕ : volume fraction of the beads in the milling chamber

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Received for review October 24, 1997

Revised manuscript received February 6, 1998

Accepted February 7, 1998

IE9707432