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Optimization of poly(β-hydroxybutyric acid) recovery from *Alcaligenes latus*: combined mechanical and chemical treatments

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Abstract Recovery of the intracellular bioplastic poly(β-hydroxybutyric acid) or PHB from fed-batch cultured *Alcaligenes latus*, ATCC 29713, was examined using combinations of chemical and mechanical treatments to disrupt the cells. Chemical pretreatments used sodium chloride and sodium hydroxide. For salt pretreatment the cells were exposed to NaCl (8 kg m^{-3}) and heat (60 °C, 1 h), cooled to 4 °C, and mechanically disrupted. For alkaline treatments, the cells were exposed to sodium hydroxide (0.025–0.8 kg NaOH per kg biomass) and mechanically disrupted at ambient temperature. A combined treatment with sodium chloride (8 kg m^{-3}), heat (60 °C, 1 h), and alkaline pH shock (pH 11.5, 1 min) was also tested. Mechanical disruption employed a continuous flow bead mill (2,800 rpm agitation speed, 90 ml min^{-1} slurry flow rate, 512 μm mean bead diameter, bead loadings of 80% or 85% of chamber volume). Disruption was quantified by protein release. Over most of the disruption period, the release of PHB was approximately proportional to protein release. Regardless of the pretreatment or bead load, the disruption obeyed first order kinetics; hence, the rate of protein release was directly proportional to the amount of unreleased protein. Relative to untreated biomass, pretreatment always produced earlier protein release during milling. Pretreatment with a minimum of 0.12 kg NaOH per kg biomass was necessary to enable complete disruption within three passes (85% bead load). Untreated biomass required more than twice as many passes. Irrespective of the chemical pretreatment, the bead loading strongly influenced the disruption rate which was higher at the higher loading. Alkaline hydrolysis associated PHB loss was observed, but it could be limited to insignificant levels by immediate neutralization of disrupted homogenates.

List of symbols

C_{NaOH}	mass ratio of alkali-to-biomass
EDTA	ethylenediaminetetraacetic acid
F	flow rate of the cell slurry, ml min^{-1} or m^3 s^{-1}
f	efficiency factor
I	electric current, A
k	disruption rate constant, min^{-1}
N	number of passes through bead mill
NTU	nephelometric turbidity units
PHA	poly(hydroxyalkanoic acid)
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}
R_p	mass ratio of PHB-to-pellet
R_s	specific protein release, g protein kg^{-1} biomass
S	slope of $\ln[R_m/(R_m - R)]$ versus N plots
SDS	sodium dodecyl sulfate
V	volume of the milling chamber, ml

Greek symbols

α	constant in Eq. (5)
β	constant in Eq. (5), kg g^{-1}
ϕ	volume fraction of the beads in the milling chamber
v	voltage, V
ξ	energy input, J m^{-3}

1 Introduction

The intracellular bioplastic poly(β-hydroxybutyric acid) (PHB) is produced by many bacteria (Poirier et al., 1995; Lee and Chang, 1995) as granular inclusions. Like other poly(hydroxyalkanoic acids) (PHAs), PHB is biodegradable and biocompatible. Physical properties of PHB are similar to those of the commonly used, non-biodegradable, petrochemical-derived thermoplastic polypropylene; hence, PHB is widely regarded as a potential replacement of certain traditional thermoplastics that constitute a persistent post consumer waste (Tamer et al., 1998).

Microbial production of all PHAs is expensive, thus those polymers are used at present only as specialty plastics. Significant contributors to cost of production are

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the fermentation carbon source (Poirier et al., 1995) and downstream processing (Berger et al., 1989; Lee and Chang, 1995). Inexpensive and scaleable recovery schemes need to be devised to achieve low-cost production that is competitive with traditional thermoplastics (Tamer et al., 1998). This work attempts to develop such simple, rapid, and economic strategies for PHB recovery from *Alcaligenes latus*, a microorganism that has in the past been used to commercially produce PHB (Hänggi, 1990; Hrabak, 1992; Poirier et al., 1995).

Another microorganism, *Alcaligenes eutrophus*, has also been used in industrial production of PHA biopolymers (Byrom, 1990; Marchessault et al., 1990), but, unlike *A. latus*, *A. eutrophus* requires a less desirable two-stage culture: the polymer is produced only after the growth has ceased. PHB synthesis in *A. latus* is growth associated, hence, a shorter overall culture period can be employed. Moreover, a one-stage fermentation is also more amenable to continuous culture. Whereas the fermentation methodology and the choice of microorganism can reduce costs, low-cost product recovery remains essential. The PHB recovery methods reported here focus on chemical pretreatment of the biomass prior to mechanical disruption in a high-speed bead or colloid mill.

2 Review

PHB recovery processes have been reviewed by Tamer et al. (1998). Most such processes have relied upon solvent extraction, surfactants such as sodium dodecyl sulfate (SDS) and aggressive chemicals such as sodium hypochlorite. Based on experimental studies, treatments with SDS or sequential treatment with SDS followed by hypochlorite have been found too expensive for commercial recovery of PHB (Tamer et al., 1998). Solvent extraction is also expensive, creates disposal problems, other hazards, and it destroys the unique natural morphology of PHA granules that is useful in certain applications (Barham, 1990; Ramsay et al., 1990). Indeed, because of the expense the British chemical company Imperial Chemical Industries (ICI) did abandon solvent extraction as a viable recovery scheme (Byrom, 1990; Poirier et al., 1995). Instead, ICI employed a series of aqueous enzyme and detergent washes to solubilize the heat shocked cells (*A. eutrophus*) to leave behind the PHA granules (Byrom, 1990). Yet, the cost of production remained high.

Mechanical disruption of producing cells is another recovery option. Mechanical cell disruption in bead mills and high-pressure homogenizers is widely used for recovering intracellular proteins (Chisti and Moo-Young, 1986, 1991, 1994; Harrison, 1991; Kula and Schütte, 1987; Middelberg, 1995), but PHB recovery by mechanical means has received little attention (Harrison, 1991; Tamer et al., 1998). High-pressure homogenization with and without chemical pretreatment has been examined for PHB recovery from *A. eutrophus* (Harrison et al., 1991c). An APV-Gaulin homogenizer (Chisti and Moo-Young, 1986) fitted with a ceramic valve seat (Chisti and Moo-Young, 1991) was used. Complete disruption required three passes at 60–69 MPa (Harrison et al., 1991c). Alkaline pretreatment of biomass (pH 10.5, 7 °C, ≤1 min) substantially improved single pass disruption performance, but a minimum of two passes were necessary for complete protein release (Harrison et al., 1991c).

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., 1991c). 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) were less effective, but disruption improved relative to salt-free thermal treatment (60 °C, 60 min) presumably because of the thermal injury enhancing effect of monovalent metal ions (Harrison et al., 1991c). Pretreatments with lysozyme, EDTA, and combination of the two, were seen to improve single pass disruption relative to untreated material (Harrison et al., 1991c). 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 commercial scale processing.

Whereas homogenization after a suitable pretreatment is a proven recovery method, homogenizers are susceptible to blockages (Tamer et al., 1998), and only relatively dilute biomass slurries can be satisfactorily processed because of viscosity constraints: an upper limit on viscosity is 1 Pa s (Chisti and Moo-Young, 1991), although significantly lower values are preferred. In one study with *A. latus*, Tamer et al. (1998) reported an upper limit of less than 0.3 Pa s that corresponded to a biomass concentration of only 66 kg DW m^{-3}. Using a different homogenizer, Harrison et al. (1991b) were able to disrupt 96–300 kg DW m^{-3} cell slurries of *A. eutrophus*, but disruption rate declined beyond a concentration of 257 kg DW m^{-3}. Bead mills can process much higher concentrations (Chisti and Moo-Young, 1986). Typical energy consumption reported for complete disintegration of various yeasts in bead mills has ranged over 0.20–0.33 kW h kg^{-1} (Rehacek and Schaefer, 1977). Power consumption depends strongly on bead loading, agitation speed, and the type of agitator (Chisti and Moo-Young, 1986). In a typical setup, with 88% bead load, 0.15 m^3 h^{-1} flow rate, and 15 m s^{-1} agitator tip speed, Rehacek and Schaefer (1977) reported a power consumption of about 8 kW for complete disruption of *S. cerevisiae*. Based on those values, disintegration in their mill required ~53 kW h m^{-3}.

Unlike solvents, surfactants, and other hazardous and environmentally burdensome chemicals, mechanical bead mill disruption alone or in combination with more benign treatments such as heat shock, salt or easily neutralized hydroxide is likely to be less expensive overall. For disruption of *A. latus*, an earlier study (Tamer et al., 1998) compared the performance of high-pressure homogenization in a ‘microfluidizer’ (Microfluidics® model M110T with extra heavy duty pump) and the same bead mill as used in this work. No chemical pretreatment was employed. The authors found bead mill disruption to substantially superior to homogenization. Furthermore, in

view of the relatively low power consumption and widespread use of bead mills in commercial processing (Chisti and Moo-Young, 1986; Kula and Schütte, 1987), that method was recommended for recovering PHB from heat-shocked *A. latus*. Nonetheless, complete disruption of bacteria in the mill required at least 8 passes through the machine (2,800 rpm agitation speed, 85% loading of 512 μm beads) despite a relatively low slurry flow rate of 90 ml min^{-1}. Too many passes through disruption devices reduce throughput and the total energy input can be relatively large. Furthermore, overexposure to severe disruption conditions has been implicated in unwanted reduction of the size of the PHB particles (Tamer et al., 1998). Therefore, exposure must be minimized. This work reports on thermal and relatively innocuous chemical pretreatments that substantially improve bead mill disruption of *Alcaligenes latus*.

3 Materials and methods

3.1 Microorganism and culture conditions

An intracellular PHB producer, *Alcaligenes latus*, ATCC 29713, was used throughout. The culture was maintained at 4 °C on slants of Bacto® nutrient agar (Difco Laboratories, Detroit, MI, USA). 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, USA). For the first stage, a loopful of slant culture was inoculated into a 250 ml flask containing 50 ml Bacto® nutrient broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10 gl^{-1} 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 sucrose, 1.4 g $(NH_4)_2\ SO_4$, 1.5 g KH_2PO_4, 1.8 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 30 mg $Na_2MoO_4 \cdot 2H_2O$, 28 mg $NiSO_4 \cdot 7H_2O$, and 10 mg $CuSO_4 \cdot 5H_2O$, as previously employed by Yamane et al. (1996), and 60 mg 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. Presterilized medium (4 l) at 33 °C, pH 7.0, was inoculated with 500 ml 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 hours. 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 over a total period of twenty minutes. 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 one month. The cells were recovered at 5,500-*g*, 15 min, in a laboratory centrifuge (IEC Centra-HN, International Equipment Company, Needham Heights, MA, USA); for larger quantities a Sorvall centrifuge (RC 5B Plus, Dupont, Wilmington, DE, USA) running at 16,250-*g*, 15 min, was employed. The cells were then washed by resuspending in deionized water, centrifugation, and made into slurry in phosphate buffer (0.1 M, pH 7.0) as needed.

3.2 Chemical pretreatments

3.2.1 Alkaline pretreatment

Sodium hydroxide (3 M) was added to the cell slurry to obtain predetermined ratios of alkali-to-biomass (0.025–0.8 kg NaOH kg^{-1} biomass). The resulting suspension was processed immediately in the bead mill. The entire process took about 45 min. Samples collected at each pass were separated into supernatant and pellet by centrifugation (10,000-*g*, 15 min; Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA, USA; room temperature). The supernatant and the pellet were analyzed for protein and PHB, respectively.

3.2.2 Sodium chloride and heat pretreatment

Sodium chloride was added to the cell slurry to give a salt concentration of 8 kg m^{-3}. The resulting suspension was incubated on a water bath (60 °C, 1 h). The suspension was then cooled to 4 °C and mechanically disrupted in the bead mill. The milling was completed within about 45 minutes, and the samples were analyzed as described previously.

3.2.3 Combined salt, alkali and thermal pretreatment

The cell slurry was supplemented with sodium chloride (8 kg m^{-3}), the resulting suspension was held on a water bath (60 °C) for one hour, and cooled to 4 °C. The pH was adjusted to 11.5 using saturated sodium hydroxide. After about a minute, the suspension was neutralized to pH 7 with hydrochloric acid. The suspension was now disrupted in the mill. Milling was complete within 45 minutes. The samples were collected for each pass and analyzed as noted.

3.3 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

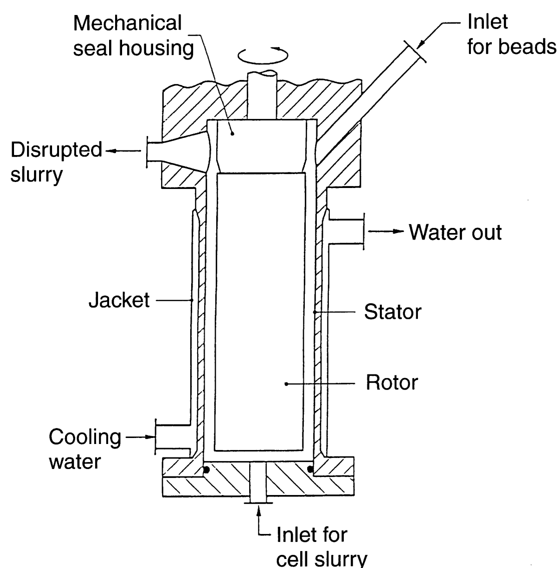

Fig. 1. Schematic of the bead mill disruption chamber. Dimensions (mm): diameter of stator, 62; diameter of rotor, 50; length of rotor, 150; annular gap, 6. Based on Garrido et al. (1994)

for agitation of glass beads (Fig. 1). 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. 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, USA; catalog no. G9268). Bead loading was either 80% or 85% of chamber volume (210 ml). Those loading figures were based on packed volume. The density of the beads was 2,740 kg m^{-3}. In view of the results of earlier work (Garrido et al., 1994), the agitation rotor speed (2,800 rpm, or 7.3 m s^{-1} tip speed) 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 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 2,800 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, 750–1,000 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 10,000-*g* (Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA, USA) for 15 minutes.

3.4
Protein assay

Protein released by disruption was quantified with the Lowry method (Lowry et al., 1951). A protein standard (Sigma Chemical Co, St Louis, MO, USA; 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, USA; 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 protein kg^{-1} total dry biomass.

3.5
Poly(β-hydroxybutyric acid) assay

A gravimetric method similar to those employed previously by Marchessault et al. (1990) and Ramsay et al. (1990) was used. The pellet portion of the mechanically disrupted, centrifuged (10,000-*g*, 15 min; Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA, USA; 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, 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 pre-weighed aluminum dishes.

3.6
Turbidity measurements

In attempts to quantify the possible impact of mechanical disruption conditions on the released PHB particles, purchased standard microbial PHB (ICN Biomedicals Inc., OH, USA; *Alcaligenes* sp 26063-00-3, catalog no. 156325) was suspended in deionized water to a concentration of 1 kg m^{-3}, and fed to the bead mill under conditions specified earlier. Samples (4 ml) collected at each pass were analyzed for turbidity (HACH 2100 P portable turbidimeter, Hach Co., Loveland, CO, USA). Care was taken to thoroughly mix the sample just prior to the measurement. Formazin polymer supplied as a kit with the turbidimeter was used as the reference turbidity standard.

This nephelometric method of assessing particle size is based on a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under identical conditions (Greenberg et al., 1992). For a given concentration of solids, suspensions of smaller particles scatter more light. Turbidity as a measure of particle disintegration has been employed also by others (Chang et al., 1994).

4
Results and discussion

As shown in Fig. 2, for alkaline pretreatment alone, increasing proportion of the sodium hydroxide relative to biomass improved disruption. At a hydroxide concentration of 0.4 kg kg^{-1}, most of the protein could be released within three passes, whereas untreated biomass required at least ten passes for the same level of disruption. Thus, alkali pretreatment improved performance so much so that the power requirements were reduced to about a third relative to untreated biomass. All data in Fig. 2 were obtained at 4.85 kg DW m^{-3} biomass concentration and a relatively low bead loading of 80%. At a higher, more realistic bead loading of 85%, pretreatment with a yet lower amount of alkali (0.12 kg kg^{-1} biomass) was sufficient to ensure complete protein release by the third pass as shown in Fig. 3. Again, at any fixed bead loading, increasing alkali concentration during pretreatment increased disruption rate. Although the results in Fig. 3 were obtained with

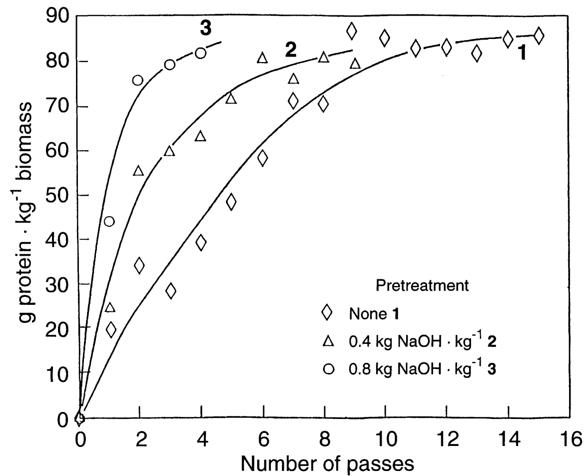

Fig. 2. Effect of alkaline pretreatments and number of passes in the mill on specific protein release (80% bead loading; biomass concentration was 4.85 kg DW m^{-3})

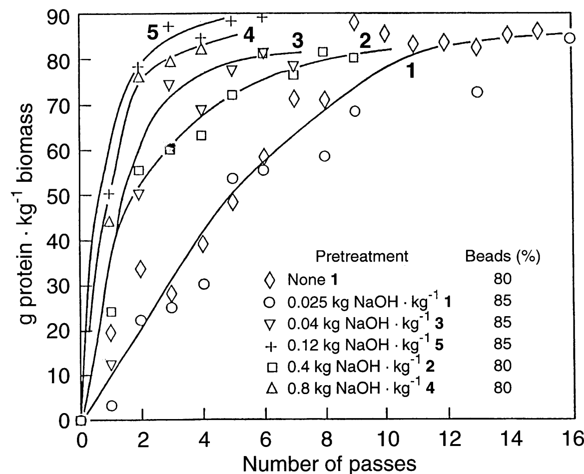

Fig. 3. Effect of alkaline pretreatments, bead loading, and number of passes on specific protein release. Biomass concentrations were: (□, △) 4.85 kg DW m^{-3} ; (▽, ○) 22 kg DW m^{-3}; and (+) 33 kg DW m^{-3}

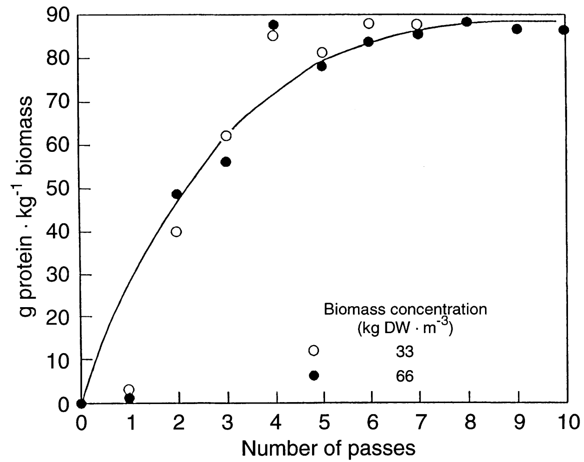

Fig. 4. Effect of biomass concentration on specific protein release from cells pretreated with 0.05 kg NaOH kg^{-1} biomass (85% bead loading)

slurries having different amounts of biomass (4.85–33 kg DW m^{-3}), the absolute amount of cells did not influence the disruption rate as long as the alkali-to-biomass ratio remained constant as confirmed in Fig. 4. The quantity of cells in Fig. 4 varied 2-fold. Similarly, for a fixed ratio of alkali-to-biomass, the ratio of PHB-to-pelleted solids was insensitive to the slurry biomass concentration, thus suggesting unchanged kinetics of solubilization of cellular solids.

Using the higher bead loading (85%), alkaline pretreatment with 0.12 kg sodium hydroxide kg^{-1} biomass was better than all other alkaline treatments tested (Fig. 3). In comparison, the two sodium chloride treatments produced disruption performances that equated with the results obtained by pretreating at a much reduced alkali level of 0.04 kg kg^{-1} biomass. Biomass pretreated with those three methods required 6–8 passes for complete disrup-

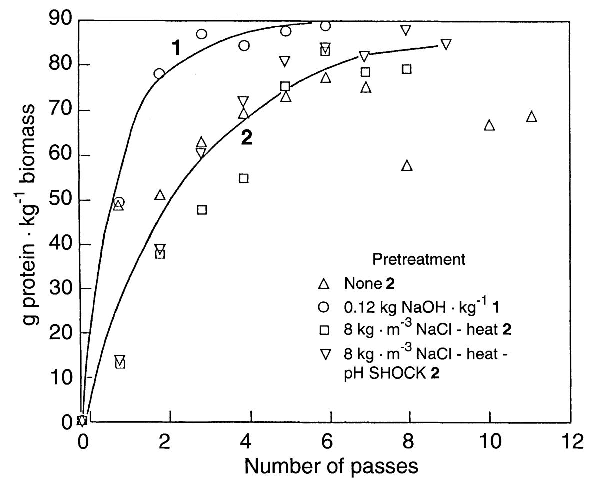

Fig. 5. Comparison of specific protein release from alkali treated cells ((○) 0.12 kg NaOH kg^{-1} biomass, 33 kg DW m^{-3} biomass concentration), salt treated material ((□) 8 kg DW m^{-3} biomass, 8 kg m^{-3} NaCl, 60 °C, 1 h), salt-pH shock treated slurry ((▽) 4.85 kg DW m^{-3} biomass, 8 kg m^{-3} NaCl, 60 °C, 1 h), and untreated biomass ((△) 45 kg DW m^{-3}). The bead loading was 85% in all cases

tion. Note further that combing the sodium chloride treatment with a short-lived pH shock had little added impact on disruption relative to material treated only with the salt (Fig. 5). Note further that the three low protein release values for untreated cells at pass ⩾8 were caused by leakage of some lubricating/cooling water from the mechanical seal (Fig. 1) into the grinding chamber.

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

$$\ln \frac{R_m}{R_m - R} = \frac{k(1-\phi)V}{F} \,.\, N \;, \tag{1}$$

which applied to disruption of a recombinant *Saccharomyces 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. Equation (1) has also been confirmed for disruption of *A. latus* (Tamer et al., 1998). From Eq. (1), the slope S of a plot of $\ln[R_m/(R_m - R)]$ versus the number of passes can be used to determine the rate constant k:

$$k = \frac{SF}{(1-\phi)V} \;. \tag{2}$$

Irrespective of the pretreatment used, bead mill disruption followed first order kinetics as revealed by linear plots in Figs. 6 and 7. Values of the disruption rate constant are summarized in Table 1. All pretreatments improved disruption rate relative to untreated cells at the same bead loading (Table 1).

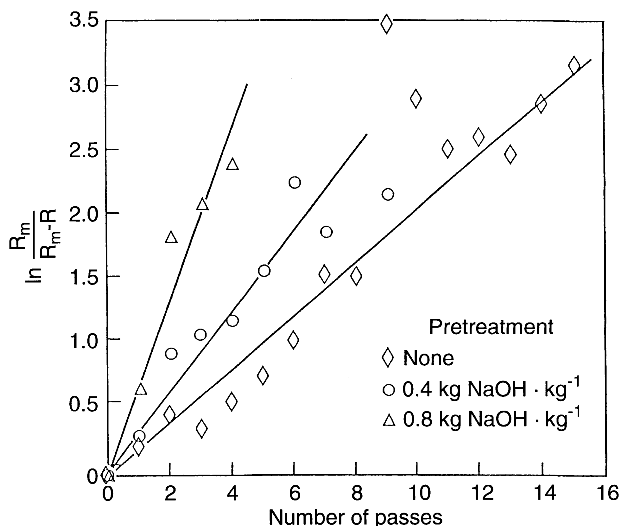

Fig. 6. Plots of $\ln[R_m/(R_m - R)]$ versus number of passes in the bead mill for alkali treated cells. Effects of pretreatment conditions (80% bead loading; biomass concentration was 4.85 kg DW m^{-3})

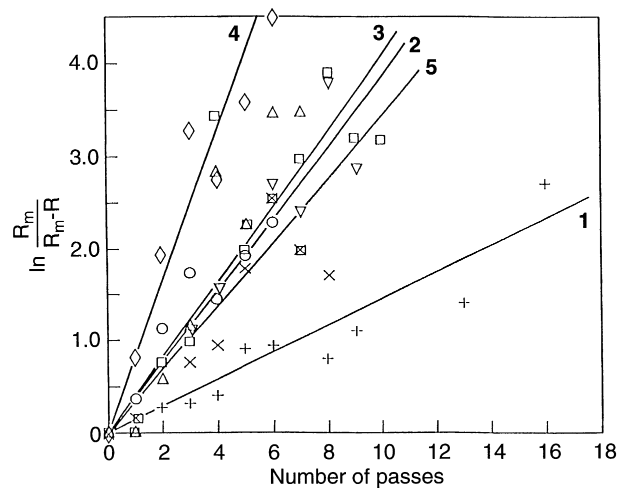

Fig. 7. Plots of $\ln[R_m/(R_m - R)]$ versus number of passes in the bead mill for variously treated cells (85% bead loading): (1) 0.025 kg NaOH kg^{-1} biomass, 22 kg DW m^{-3} biomass concentration (+); (2) 0.04 kg NaOH kg^{-1} biomass, 22 kg DW m^{-3} biomass concentration (○); and salt-pH shock treatment (8 kg m^{-3} NaCl 60 °C, 1 h, 1-min pH shock (▽)); (3) 0.05 kg NaOH kg^{-1} biomass, biomass concentration was either 33 kg DW m^{-3} (△) or 66 kg DW m^{-3} (□); (4) 0.12 kg NaOH kg^{-1} biomass, 33 kg DW m^{-3} biomass concentration (◇); (5) salt treatment without pH shock (8 kg m^{-3} NaCl, 60 °C, 1 h, (×))

The combined effects of alkaline pretreatment and bead loading on disruption rate constant are clearly revealed in Fig. 8 where the rate constant is plotted against the alkali-to-biomass ratio used during pretreatment. As shown in the figure, the impact of alkali concentration on enhancing disruption rate depends strongly on the value of the bead loading. Alkali-induced weakening of the cell wall is insufficient unless the cells are also exposed to a sufficiently mechanically disruptive environment. At a loading of 85%, a milder alkali treatment with only 0.12 kg NaOH kg^{-1} biomass yields a rate constant that is almost twice as large as when much more severely treated cells (0.8 kg NaOH

Table 1. Observed first order disruption rate constants for variously pretreated biomass samples

Pretreatment	Bead loading (%)	Disruption rate constant (min^{-1})
Alkaline treatments		
0.025 kg NaOH kg^{-1} biomass	85	0.42
0.04 kg NaOH kg^{-1} biomass	85	1.11
0.05 kg NaOH kg^{-1} biomass	85	1.17
0.12 kg NaOH kg^{-1} biomass	85	2.72
0.4 kg NaOH kg^{-1} biomass	80	0.66
0.8 kg NaOH kg^{-1} biomass	80	1.43
Sodium chloride treatments		
8 kg m^{-3} NaCl (60 °C, 1 h)	85	0.99
8 kg m^{-3} NaCl (60 °C, 1 h, pH shock)	85	1.11
No pretreatment		
	85	0.69
	80	0.44

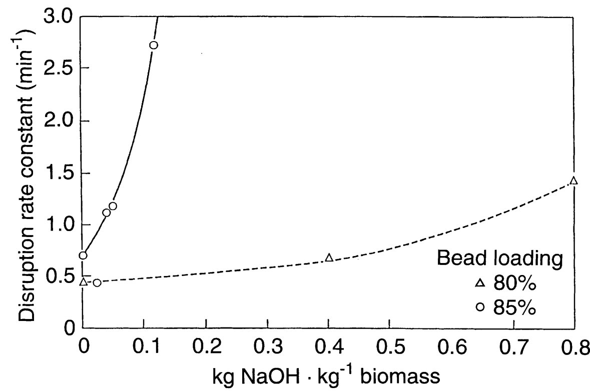

Fig. 8. Effect of alkali-to-biomass ratio and bead loading on the disruption rate constant

kg^{-1} biomass) are disrupted with 80% bead loading (Fig. 8). Whereas, relative to untreated cells, the milder alkaline treatment (85% bead load) improves disruption rate by over 5-fold. Also noteworthy (Fig. 8) is the relatively small effect of bead loading on disruption of untreated cells (zero alkali concentration). Thus, clearly, a suitable combination of alkaline pretreatment *and* bead loading is essential for rapid disruption. For *A. latus*, the optimal combination is pretreatment with 0.12 kg NaOH kg^{-1} biomass followed by disruption at 85% bead loading. The data of Fig. 8 were correlated with the empirical equation:

$$k = 2.15 \cdot \phi^7 \cdot e^{1.475(0.8/\phi)^{-33.866}} \cdot C_{\mathrm{NaOH}} \quad , \qquad (3)$$

where C_{NaOH} was the ratio of alkali-to-biomass. Good agreement of the data with Eq. (3) is shown in Fig. 9. The pronounced effect of bead load on disruption rate is obvious in Eq. (3), and it is consistent with other similar observations (Chisti and Moo-Young, 1986; Garrido et al., 1994; Kula and Schütte, 1987; Tamer et al., 1998).

The energy input ξ to the cell slurry was calculated from the voltage v and the measured current I drawn by the motor; thus

$$\xi = \frac{f \cdot v \cdot I \cdot N}{F} \quad , \qquad (4)$$

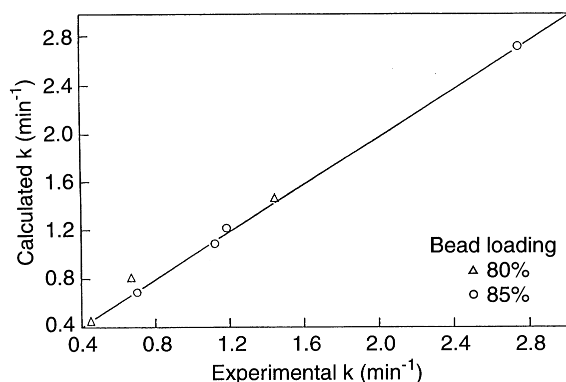

Fig. 9. Comparison of the calculated, Eq. (3), and the observed values of the disruption rate constant k. The diagonal represents exact agreement

where the efficiency factor f was 0.64. This value was based on a very typical 80% motor efficiency, and 80% efficiency of the transmission system which consisted of pulley-and-belt drive, two bearings, and a mechanical seal. The motor operated at 220 V, and it drew a 6 A current under the processing conditions. For three-pass disruption, the energy input was 1,689.6 MJ m^{-3}. In practice, a more suitable basis of assessing operational efficiency is energy input per unit mass of the cells disrupted. On that basis, for a 66 kg DW m^{-3} slurry, the energy input was 25.6 MJ kg^{-1}.

Yet better energy efficiency may be easily attained because highly concentrated slurries can be processed in bead mills. For example, in the same mill Mao and Moo-Young (1990) processed up to 600 kg m^{-3} (packed weight) of *S. cerevisiae* without seeing any concentration effect on disruption rate. Furthermore, Garrido et al. (1994) observed no differences in disruption performance when the yeast cell concentration was varied over 5–20 kg DW m^{-3}, and Rehacek and Schaefer (1977) achieved complete single-pass disruption of yeast slurries that contained up to 170 kg DW m^{-3} biomass. Yet higher yeast biomass slurries at 300 kg DW m^{-3} were completely disrupted within two minutes in another study (Melendres et al., 1992), and Schütte et al. (1983) processed even more concentrated slurries at 500 kg DW m^{-3}. Disruption rate was unaffected even at that high biomass level so long as a high agitation rate ($\sim$ 8 m s^{-1} mean tip speed) was used (Schütte et al., 1983) as in the present work. Thus, if the mill is used to disrupt a slurry with 500 kg DW m^{-3} biomass, then the energy demand works out to only 3.38 MJ kg^{-1}. These energy values are fairly small. For a better perspective, a 60 W light bulb consumes 5.184 MJ over a 24-hour period. If the disruption efficiency is measured in terms of the mass of protein released per joule (Limon-Lason et al., 1979), then for three-pass disruption of an alkali pretreated (0.12 kg NaOH kg^{-1} biomass) slurry with 66 kg DW m^{-3}, the efficiency was 3.5 g J^{-1}. In comparison, an eight-pass disruption of untreated material had an efficiency of 1.3 g J^{-1}. Thus, pretreatment improved efficiency by 2.7-fold.

The specific throughput of the mill amounted to 9.5 m^3 h^{-1} per m^3 of gross mill volume for complete disruption within three passes. This compared well with a value of 13 m^3 · h^{-1} per m^3 of gross mill volume observed by Rehacek and Schaefer (1977) for 94–96% disintegration of *S. cerevisiae* in a 0.02 m^3 (nominal) horizontal mill operated with 80% bead load and 0.26 m^3 · h^{-1} flow rate. Our throughput value is especially reasonable considering that small bacterial cells are generally harder to break than yeast cells (Chisti and Moo-Young, 1986; Tamer et al., 1998).

Unlike bead mills, high-pressure homogenizers are unable to handle high viscosities, hence the acceptable biomass concentration in the broth is restricted. In one case, a 66 kg DW m^{-3} slurry of *A. latus* (viscosity = 0.3 Pa · s) could not be processed, and a 45 kg DW m^{-3} slurry could be barely handled (Tamer et al., 1998) despite a high operating pressure (90–95 MPa). In contrast, we have successfully processed up to 66 kg DW m^{-3} *A. latus* in the same mill (Tamer et al., 1998).

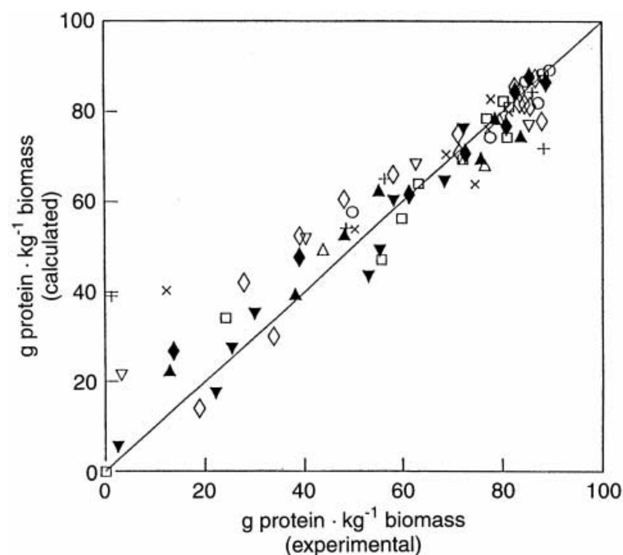

Fig. 10. Comparison of the observed values of the specific protein release with those calculated using Eq. (5) for the various pretreatment regimens: (◇) no pretreatment, 4.85 kg DW m^{-3} biomass concentration, 80% bead loading; (▼) 0.025 kg NaOH kg^{-1} biomass, 22 kg DW m^{-3} biomass concentration, 85% bead loading; (×) 0.04 kg NaOH kg^{-1} biomass, 22 kg DW m^{-3} biomass concentration, 85% bead loading; (▽) 0.05 kg NaOH kg^{-1} biomass, 33 kg DW m^{-3} biomass, concentration, 85% bead loading; (+) 0.05 kg NaOH kg^{-1} biomass, 66 kg DW m^{-3} biomass concentration, 85% bead loading; (○) 0.12 kg NaOH kg^{-1} biomass, 33 kg DW m^{-3} biomass concentration, 85% bead loading; (□) 0.4 kg NaOH kg^{-1} biomass, 4.85 kg DW m^{-3} biomass concentration, 80% bead loading; (△) 0.8 kg NaOH kg^{-1} biomass, 4.85 kg DW m^{-3} biomass concentration, 80% bead loading; (▲) 8 kg m^{-3} NaCl, 60 °C, 1 h, 8 kg DW m^{-3} biomass concentration, 85% bead loading; (◆) 8 kg m^{-3} NaCl, 60°C 1 h, 1-min pH shock, 4.85 kg DW m^{-3} biomass concentration, 85% bead loading. The diagonal represents exact agreement

Regardless of the type of pretreatment practiced, the mass ratio of PHB-to-pellet (R_p) correlated with specific protein release (R_s g protein kg^{-1} biomass) during disruption. This relationship was found to be:

$$R_p = \alpha + \beta R_s \ , \quad (5)$$

where the α and β values were respectively 0.47 ± 0.09 and 0.0082 ± 0.0028 kg g^{-1}. Figure 10 compares the specific protein release values determined with Eq (5) and the experimental data. The comparison shown spans 80% and 85% bead loadings, biomass concentrations of 4.85–66 kg DW m^{-3}, all three pretreatment protocols, and alkali concentrations of 0.025–0.8 kg NaOH kg^{-1} biomass.

The actual amount of PHB in cells was determined by chemical digestion and gravimetry as described by Tamer et al. (1998). The mass ratio of this pure PHB to that of the crude pellet from various passes of the mill are plotted in Fig. 11–13 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 corresponds to the mass fraction of PHB in undisrupted cells. The value of that ratio increased with increasing number of passes: as more of the cellular material was solubilized and micronized, the total mass of the pellet declined. The ratio approached unity around the second pass (Fig. 11) during processing of alkali pretreated cells. Untreated cells attained a unit ratio around the seventh pass (Fig. 11). As shown in Fig. 12, the absolute amount of biomass in the slurry did not affect the number of passes required to attain a given value of PHB-to-pellet mass ratio so long as the ratio of alkali-to-biomass used in pretreatment remained unchanged.

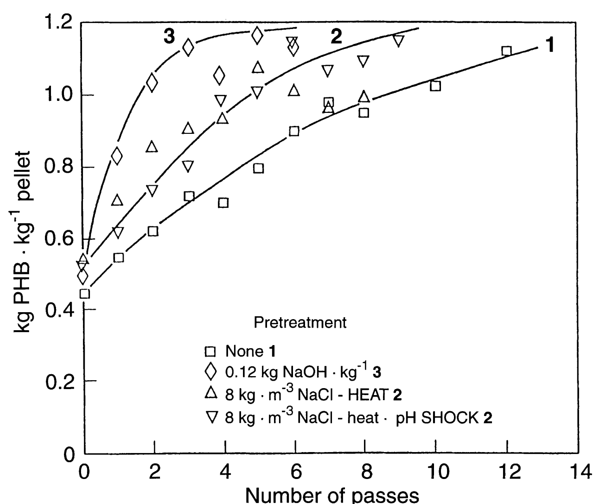

Fig. 11. PHB-to-biomass ratio versus the number of passes: comparison of various pretreatments. The bead loading was 85%. Biomass concentrations were: (▽) 4.85 kg DW m^{-3}; (△) 8 kg DW m^{-3}; (◇) 33 kg DW m^{-3}; and (□) 45 kg DW m^{-3}

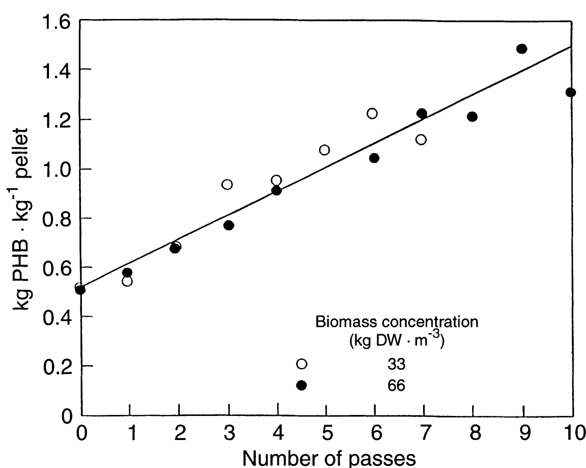

Fig. 12. PHB-to-biomass ratio versus the number of passes: effect of biomass concentration at a constant alkali-to-biomass ratio of 0.05 kg kg^{-1}. The bead loading was 85%

Increase in the PHB-to-pellet ratio to greater than one (Figs. 11–13) suggested loss of PHB by micronization to levels that could not be sedimented. Alkaline hydrolysis related PHB loss apparently also contributed as shown in Fig. 13 where the rate of increase in PHB-to-pellet mass ratio beyond a value of unity increased with increasing amounts of alkali. However, so long as the number of passes was limited to two, little loss of PHB occurred even

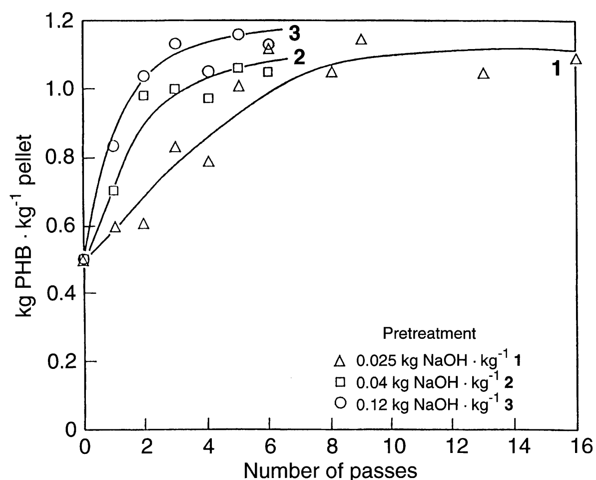

Fig. 13. PHB-to-biomass ratio versus the number of passes: effect of alkali concentration. The bead loading was 85%. Biomass concentrations were: (□, △) 22 kg DW m^{-3}; and (○) 33 kg DW m^{-3}

at the high alkali concentration (0.12 kg kg^{-1}) that was recommended for optimal recovery (Fig. 13).

The hypothesis of PHB loss by micronization was examined by processing a standard PHB suspension through the mill. Turbidity of the suspension was used to quantify the extent of size reduction (Greenberg et al., 1992; Chang et al., 1994). The initial decline in turbidity during the first pass was attributed to sedimentation of some granules in the reservoir and the inlet lines (Fig. 14). However, as the operation proceeded, the turbidity increased, almost doubling by the twelfth pass (Fig. 14). Because micronization is not wanted, processing should be restricted to four and fewer passes. Turbidity increased little beyond the sixth pass, presumably because the particles had been so much reduced in size that further milling was ineffective.

5 Conclusions

All pretreatments examined improved bead mill disruption to various degrees relative to untreated biomass;

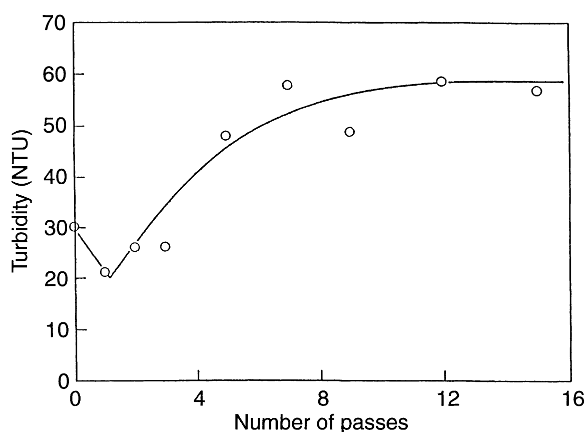

Fig. 14. Effect of number of passes on turbidity of a PHB slurry (1 kg m^{-3}) upon milling (2,800 rpm agitation speed, 90 ml min^{-1} slurry flow rate, 512 µm mean bead diameter, 85% bead loading)

however, the two sodium chloride pretreatments (8 kg NaCl m^{-3}, 60 °C, 1 h; and the same combined with a 1-min pH 11.5 shock) were less effective. The biomass concentration in the slurry (4.85–66 kg DW m^{-3}) did not affect disruption rate, but the rate strongly depended on the bead loading; higher loadings produced faster protein release. For otherwise fixed conditions, increases in alkali-to-biomass ratio (0.025–0.8 kg NaOH kg^{-1}) during pretreatment enhanced mechanical disruption rates. Regardless of the bead load and the type of pretreatment, first-order disruption kinetics prevailed, and the rate of protein release depended on the amount of unreleased protein. Equation (3) correlated the combined effects of alkali concentration and bead loading on the disruption rate constant. For all cases, the release of PHB was linearly related to protein release in accordance with Eq. (5).

Of all pretreatments examined, alkaline treatment with 0.12 kg NaOH kg^{-1} biomass (60 °C, 1 h) followed by disruption at 85% bead loading (2,800 rpm agitation speed, 90 ml min^{-1} slurry flow rate, and 512 µm mean diameter of grinding beads) was the most effective. That recovery method is recommended; however, especial care is necessary to ensure that the disrupted homogenate is neutralized immediately after processing to protect against PHB loss by hydrolysis. Excessive mechanical processing was implicated in unwanted micronization of PHB to difficult-to-sediment particles; however, the yield loss was minimal if milling was restricted to four and fewer passes (85% bead loading). The recommended disruption procedure released nearly all cellular protein within two passes – a cumulative residence time of 2.2 minutes – through the grinding chamber. This represented a substantial improvement over the 6+ passes required to fully disrupt the untreated *Alcaligenes latus*. The advocated pretreatment enhanced disruption energy efficiency by 2.7-fold to 3.5 kg DW cells kJ^{-1}.

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