Novel Thermal Route to an Amorphous, Film-Forming Polymer Latex

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ABSTRACT: The bacterial storage polymer, poly(3-hydroxybutyrate) (PHB), is remarkable in that while the isolated polymer is crystalline, the polymer is produced and stored in cells in the form of amorphous granules. The amorphous physical state in vivo has been attributed to the slow nucleation kinetics that are operative for small, isolated particles. On isolation, the polymer granules are usually found to crystallize. We report here a new thermal method that returns the purified crystalline particles to a fully amorphous state. In the new method, a suspension of crystalline particles is heated briefly under pressure to a temperature above the polymer T_m and then rapidly cooled. The thermally treated particles have a median size of 0.83 μm and, unlike their crystalline counterparts, form coherent films under ambient conditions. At temperatures where bulk amorphous PHB crystallizes within a few minutes, the suspension of thermally treated particles remains in a metastable amorphous state for at least several weeks.

Introduction

Poly(3-hydroxyalkanoate)s (PHAs, **1**) are linear, stereoregular polyesters that are produced naturally by bacteria and other organisms.[1,2] The polymers are of high molecular weight, typically from 5×10^4 to 2×10^6. Although in nature the polymers function primarily as carbon and energy storage materials, PHAs have found use commercially as biodegradable thermoplastics. The most abundant of the polymers is poly(3-hydroxybutyrate) (PHB, **2**), first identified in 1925. The related copolymer poly[(3-hydroxybutyrate)-*co*-3-(hydroxyvalerate)] (PHBV or Biopol, **3**) has been produced on an industrial scale for several years by fermentation of the soil bacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). *Pseudomonas* bacteria are known to produce other PHAs having longer alkyl side chains, generally from C_3 to C_{11}. The latter PHAs are thermoplastic elastomers or amorphous polymers.

Naturally occurring PHAs are hydrophobic and water-insoluble and for this reason form inclusion bodies or "granules" within bacterial cells. The granules are typically 0.1−1.0 μm in size and contain essentially pure PHA with a surface coating of protein and/or lipid, which comprise about 2% of the total mass.[3] The nature of the surface coating has been studied by biochemical, immunochemical, and ultrastructural techniques, and it is well established that the PHA synthase is attached at the granule surface and is a major surface constituent.[4] The presence of other proteins, known as phasins, has also been reported.[5] Incubation of the purified PHA synthase together with its natural substrate, 3-hydroxybutyryl-CoA, results in the formation of granules in vitro;[6] the particle size of these granules can be controlled by varying the protein concentration during synthesis.[7] PHA granule biosynthesis has thus been compared with an emulsion or precipitation polymerization, in which the synthase serves as both catalyst and emulsifier.[8]

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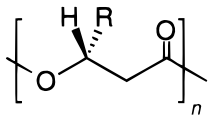
Scheme 1



1: PHA, R = various alkyl, alkenyl, and aryl groups ($C_{1\text{-}11}$)
2: PHB, R = CH_3
3: PHBV (Biopol), R = CH_3, CH_2CH_3

A striking feature of the PHB storage granules is that they remain amorphous in vivo, even at temperatures where the isolated bulk polymer crystallizes rapidly.[9] This surprising stability in the amorphous state has been explained on the basis of nucleation kinetics.[10] The theoretical model predicts that the observed rate of crystallization for an ensemble of polymer particles varies inversely with the particle volume (i.e., the third power of particle diameter). The half-time for the crystallization process can be expressed as $(\log_e 2)/Iv$, where I is the nucleation rate constant and v is the volume of an individual particle. Using known nucleation rate constant data,[11] it has been predicted that a suspension of 0.25 μm amorphous PHB particles will crystallize with a half-time of >1000 years at 30 °C.[12] Particles of 2.5 μm size would crystallize with a half-time of about a year, in this model.

Horowitz and Sanders provided experimental confirmation for the model by showing that purified, crystalline PHB or PHBV could be reconstituted into a suspension of amorphous, submicron particles.[12] These artificial granules were prepared by dissolving the polymer in chloroform, emulsifying with an excess of an aqueous surfactant solution, and then evaporating away the organic solvent. No crystallization of the artificial granule suspension was observed even after 2 years of storage, when an appropriate surfactant was present. However, when the suspension was applied to a surface and dried, rapid crystallization ensued with the formation of a polymer film. Similar amorphous, artificial granules of medium chain length PHAs (e.g., poly(3-hydroxyoctanoate), PHO) were reported by Marchessault et al.[13] As in the previous work, the method

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Table 1. Purification of PHB Particle Suspension

purification stage[a]	% solids	particle size (μm)[b]	% CI	% purity[c]
bacterial cells	10.4	0.72	0	42
protease	5.8	0.48	0	63
ozone	4.9	0.49	20	69
hydrogen peroxide	7.5	0.61	50	96

[a] Values obtained after complete diafiltration in each case. [b] Median particle size on a volume basis. [c] Determined by GC.

employed a dilute polymer solution which was then dispersed in water.

Suspensions of artificial PHA granules can be compared to conventional polymer latices, with the additional feature that films derived from PHA latices ultimately crystallize. Crystalline films are expected to have significant advantages in toughness and barrier properties, for example. PHA latices are thus of interest in a variety of coating applications, including paints, thermolyzable lacquers, and paper and food coatings. However, native PHA storage granules are frequently observed to crystallize during isolation, for reasons discussed below. It is accordingly difficult to prepare useful amorphous latices by direct extraction of PHA-containing cells. Likewise, the solvent-based routes reported by Horowitz and Marchessault are only useful for small samples. Moreover, solvent emulsification is only applicable to polymers for which there exist good, volatile solvents; insoluble polymers cannot be studied.

It was of interest therefore to develop a general, scalable method for preparing amorphous suspensions of crystalline polymers. We report here a new method for producing an amorphous, aqueous suspension of PHB particles. In this method, native PHB storage granules are isolated from cells in a manner that yields a suspension of submicron, crystalline particles. The crystalline suspension is processed through a novel apparatus designed to rapidly heat and cool the suspension. The apparatus is designed so that the peak temperature exceeds the polymer T_m.

Results and Discussion

Preparation of Polymer Suspension. It was important for this work to develop a method that yielded preparative quantities of crystalline PHB particles of low particle size, ideally less than 1 μm. Fermentation of *Ralstonia eutropha* on glucose yielded a cell slurry containing 10.4% dry solids, of which 42% was PHB. PHB granules were isolated from bacterial cells by an aqueous process similar to the published commercial processes[14] (see Table 1). The current process differed in some respects, such as (a) the avoidance of a heat shock, (b) the use of ozone as a bleaching agent or oxidant, and (c) the use of microfiltration to effect the separation of the polymer particles from solubilized biomass. In addition, a nonionic emulsifier (stearyl-21-ethoxylate, Brij 721) was added during the purification process. The surfactant helped prevent any significant aggregation of the particles, which otherwise occurs quite readily. Thus, the final product was an aqueous PHB suspension having a median particle size of 0.61 μm, just slightly larger than intact native storage granules. GC analysis of the dried polymer suspension showed it to contain 96% pure PHB; the remainder was primarily residual surfactant (3.4% by ^{1}H NMR). The dried polymer had $T_m = 174$ °C, $T_g = -1$ °C, $M_w = 4.5 \times 10^5$, and $M_n = 1.2 \times 10^5$.

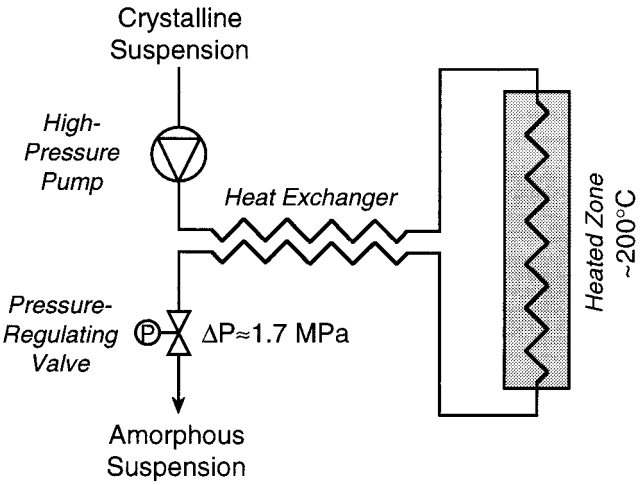

Figure 1. Schematic diagram for thermal apparatus.

Crystallization of the polymer particles occurred progressively during the purification process, despite the presence of surfactant and the maintenance of a small particle size. At the end of the process a crystallinity index (CI) of 50% was reached, as described below. The observed rate of crystallization during purification thus clearly exceeds that predicted by homogeneous nucleation kinetics and probably reflects heterogeneous nucleation.[10] Although the exact mechanism remains to be determined, it is likely that the presence during the purification process of surfaces, foreign particles, and impurities coupled with high agitation and shear are responsible for the observed nucleation and crystallization.

Thermal Treatment of Polymer Suspensions. The principle of the new process is that submicron, crystalline particles of PHB can be melted by heating to above the polymer T_m and then undercooled without inducing crystallization. The process is conceptually similar to that used in early studies of polymer nucleation,[15] where suspensions of highly diluted polymer particles in oil were heated above T_m, cooled, and then observed microscopically. The new method differs in that (a) the suspending medium is water, (b) the polymer particles are present in a high concentration, and (c) the process is continuous. Unlike oil, water boils below the T_m of PHB and other polymers of potential interest, and thus it was necessary to carry out the thermal process under elevated pressure.

The colloidal stability of concentrated PHB suspensions in water at high temperatures (≥180 °C) was very limited. In preliminary experiments, crystalline PHB suspensions were heated at 180−200 °C for 4−6 min in sealed test tubes and then cooled (data not shown). Suspensions of metastable, amorphous particles could be obtained in this way. However, a significant fraction of the polymer was found to have coalesced into one or more large masses, which in accord with the nucleation theory crystallized rapidly upon cooling. No surfactants could be found that prevented coalescence under these harsh temperature conditions.

Accordingly, a novel apparatus was developed for performing a continuous flow thermal treatment, shown schematically in Figure 1. The apparatus has three main components: (a) a high-pressure liquid pump, (b) a loop wherein rapid heating and cooling treatments can be applied to the polymer suspension, and (c) a pressure-regulating valve. Heating is applied by means of a

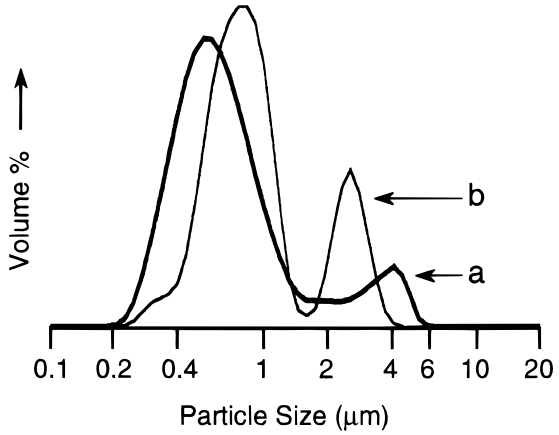

Figure 2. Particle size distribution of purified PHB suspension (a) before thermal treatment and (b) after thermal treatment.

silicone oil bath, into which is immersed a thin coil of copper tubing. Within the loop, pressure is maintained at a sufficient level to prevent the boiling of the suspending medium (e.g., 1.6−1.7 MPa for water at 180 °C). While the pump is operating, the residence time of the polymer suspension in the oil bath is less than 10 s. A heat exchanger serves the dual purpose of prewarming the suspension entering the oil bath and, more importantly, rapidly cooling the superheated product before particle aggregation can occur. After cooling in the heat exchanger, the product is discharged through the pressure-regulating valve at a temperature below the atmospheric pressure boiling point.

The apparatus was operated on a suspension of crystalline PHB from above (CI = 50%, solids content 7.5%) using a bath temperature of 197 °C and a flow rate of 16 mL/min. The temperature of the suspension leaving the oil bath was 180 °C; leaving the outlet side of the heat exchanger, the temperature had been reduced to 61 °C. The product was then cooled to room temperature and analyzed. The total dry matter content of the suspension was unchanged from before treatment. The particle size distribution was shifted only slightly by the thermal treatment (Figure 2). Median particle size was determined to be 0.83 μm compared to 0.61 μm prior to treatment. It is likely that the presence of residual surfactant (Brij 721) as well as trace cellular contaminants (e.g., amphipathic lipids) contribute to the colloidal stability of the PHB suspension. Owing to the brevity of thermal treatment, degradation of the polymer by thermolysis or hydrolysis was minimal. After thermal treatment the polymer showed $M_w = 4.8 \times 10^5$ and $M_n = 1.4 \times 10^5$, essentially the same as the starting material.

Determination of Crystallinity. The product of thermal treatment was found to be completely amorphous. PHB crystallinity indices were determined by a modification of the method of Bloembergen et al.[16] FTIR spectra were collected directly on aqueous suspensions of PHB particles by attenuated total reflectance (Figure 3). Amorphous particles showed characteristic IR bands at 1185 and 1303 cm^{-1}, while crystalline particles showed characteristic bands at 1228, 1280, and 1288 cm^{-1}. Percent crystallinity index was determined by comparison with standards of known crystallinity. A crystallinity index of 100% represents the maximum crystallinity that the polymer is normally capable of achieving; for PHB this corresponds to an X-ray crystallinity of approximately 70%. Density analyses have generally shown that PHA suspensions with intermediate crystallinity indices represent mixtures of two distinct populations: fully amorphous and fully crystalline particles.[17] The PHB suspension purified from cells of *R. eutropha* had a crystallinity index of 50% (Figure 3c). After thermal treatment, the PHB suspension had an FTIR spectrum that was indistinguishable from the amorphous polymer standard (see Figure 3). The suspension remained amorphous for at least 2 weeks at 32 °C or for several months at 4 °C.

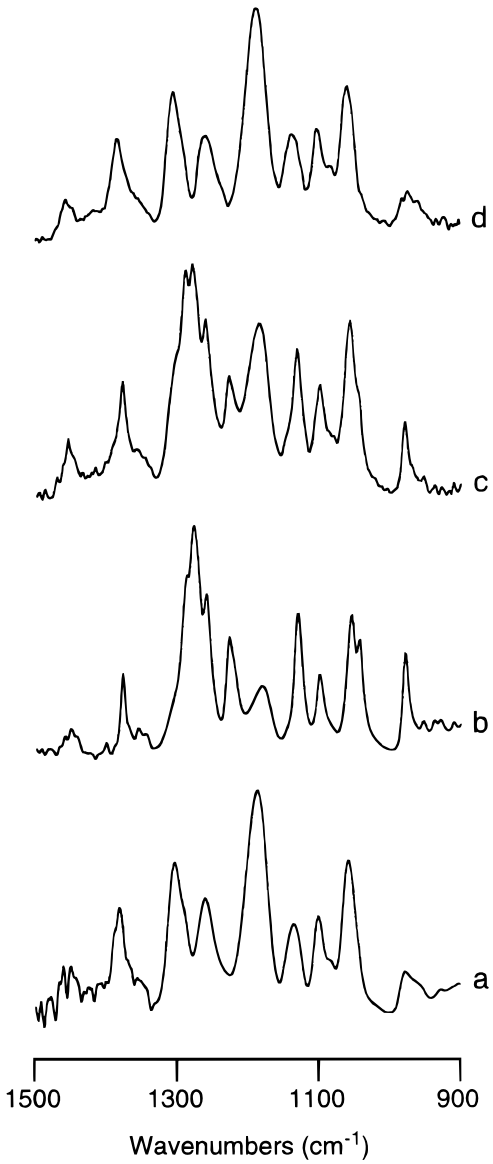

Figure 3. FTIR spectra of aqueous suspensions of PHB particles. Spectra were obtained from (a) artificial amorphous granules,[12] (b) a dispersion of dried, crystalline PHB particles in water, (c) PHB suspension after extraction and purification from cells of *R. eutropha*, and (d) PHB suspension after thermal treatment.

The crystalline PHB suspension could be supplemented with additional surfactant (e.g., potassium oleate, 0−1% w/v, with 5 mM EDTA). Addition of the surfactant did not markedly alter the properties of the suspension produced by thermal treatment. However, higher surfactant concentrations were associated with higher stabilities of the amorphous particles toward crystallization. After 4 weeks storage at 32 °C, the suspension containing the highest concentration of

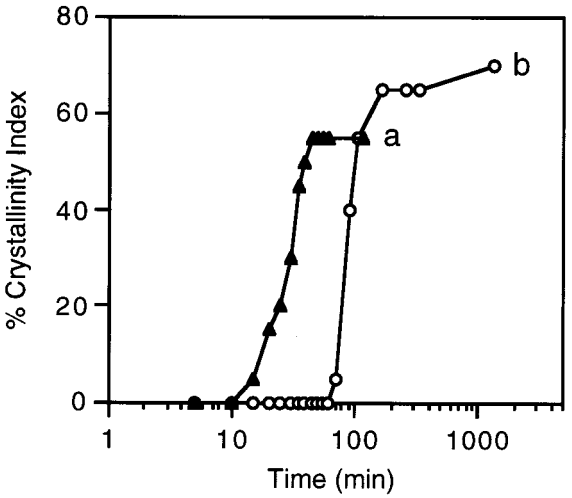

Figure 4. Crystallization of amorphous PHB particles applied to a surface and allowed to dry under ambient conditions. Two curves represent initial solids concentrations of (a) 27% (w/v) and (b) 7.5% (w/v).

potassium oleate showed a crystallinity index of 15%, whereas in the absence of any added surfactant the value was 60%. It is likely that other surfactants could be identified which would further extend the lifetime of the particles in the amorphous state. In any case the stability is less than that reported for solvent-derived artificial amorphous granules, which showed no detectable crystallization over a period of 2 years.[12] The solvent-prepared granules were up to a 1000-fold smaller in volume than the particles reported here and thus may differ in colloidal stability, sedimentation behavior, and nucleation rate.

Crystallization and Film Formation. Upon application of the thermally treated, amorphous PHB particles to a surface and evaporation of the liquid phase, the particles crystallize with concomitant formation of a polymer film. The crystallization process was followed in situ by FTIR spectroscopy. The observed rate of crystallization was dependent upon particle concentration. At a high solids concentration (27%), crystallinity was detectable within 15 min of application and was essentially complete within 1 h; at a lower concentration (7.5%) the bulk of crystallization occurred between 1 and 3 h of application (Figure 4). The data are thus consistent with the evaporation of water (and hence loss of surfactant stabilization) being the triggering event for crystallization.

PHA suspensions or latices are unique in that the polymer can exist in both amorphous and crystalline states. Current latices used for film formation are made from amorphous polymers; often, a volatile organic solvent is added to temporarily depress the minimum filming temperature (MFT) below the temperature of application. PHA latices are of practical interest, since slow nucleation obviates the need for a solvent and also since crystalline films may have superior properties. Film formation by the suspensions in the current work was examined by atomic force microscopy (AFM). A thin layer of PHB suspension was coated on glass, either before or after thermal treatment (Figure 5), under defined temperature conditions. The micrograph of the film produced prior to thermal treatment (Figure 5a) shows a total lack of particle coalescence; discrete spherical particles are visible with minimal interparticle contacts. These films were macroscopically opaque and

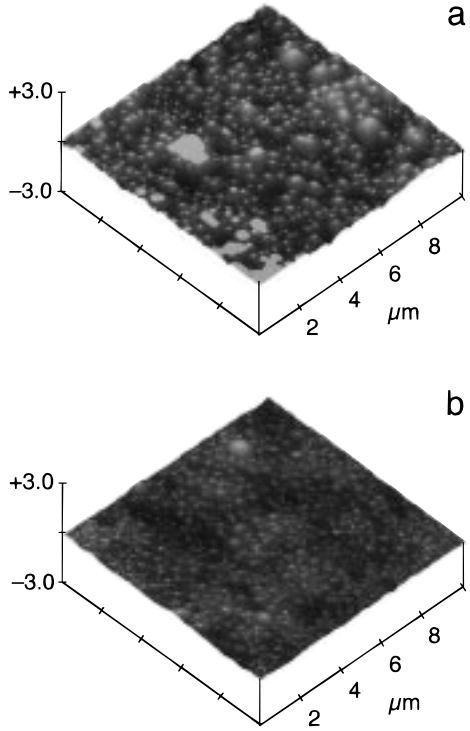

Figure 5. Atomic force micrographs of polymer films derived from (a) PHB suspension prior to thermal treatment and (b) PHB suspension after thermal treatment. Both films were prepared at room temperature.

incoherent. It has been reported previously that crystalline PHA particles are incapable of ambient temperature film formation and must be heated and/or exposed to high pressure or solvent vapors in order to undergo coalescence.[18]

By contrast, the amorphous suspension produced by thermal treatment gave a clear film, which under the microscope showed a comparatively high degree of particle coalescence (Figure 5b). Although these two films were formed at room temperature, similar results were obtained at temperatures from 20 to 100 °C (data not shown). At 4 °C (near T_g), the thermally treated, amorphous suspension also failed to coalesce. It is likely that film formation by amorphous PHA latices is dependent upon a competition between the two processes, coalescence and crystallization. Maximum film coherence is achieved when coalescence is rapid relative to crystallization. Thus, slow crystallizing copolymers such as PHBV give ideal behavior. Of course, the rates of both crystallization and coalescence will be strongly influenced by temperature, surfactant concentration, and possibly additional factors.

Conclusions

The results reported here show that purified, crystalline PHB can be reconstituted into an aqueous, amorphous latex by a brief thermal treatment at $T \geq T_m$. The thermal treatment may conveniently be performed using a continuous-flow heating and cooling device. The latex, which has a particle size under 1 μm and shows no detectable crystallinity, forms coherent, crystalline films upon drying under ambient conditions. In contrast to previous methods for preparing amorphous PHA latices, the new method is straightforward and solvent-free and can be carried out at any scale.

The remarkable stability of PHA particles in the amorphous state can be attributed to two factors: (a)

the small size of the particles and (b) the rather low nucleation rate constant of PHB.[11] Whether the latter is an effect of purity or reflects an intrinsic property of the polymer is not known. However, it seems likely that other crystalline polymers could be identified that would exhibit similar behavior and form amorphous latices, which depending upon the particle size would be metastable with respect to crystallization. The solvent-free, thermal method described here should provide a means to prepare such amorphous latices, either for the study of nucleation behavior or for practical use.

Experimental Section

General. *Ralstonia eutropha* strain NCIMB 40124 was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. PHB analyses were performed by gas chromatography using acidic butanolysis.[19,20] Solids concentrations were determined using a Mettler HG53 halogen moisture analyzer. Gel-permeation chromatography (GPC) determinations of molecular weight were performed in chloroform using a Waters Styrogel HT6E column with polystyrene standards (Polysciences, Inc.). Thermal properties were measured using a Perkin-Elmer Pyris I DSC at a heating rate of 10 °C/min. Particle size distributions were obtained by laser light scattering (Coulter LS230 particle analyzer) using an optical model derived for PHB particles. Atomic force microscopy (AFM) was performed using a Digital Instruments NanoScope IIIa operating in contact mode. Microscope samples were prepared by spreading a thin film of PHB suspension (100 μL) on a temperature-equilibrated glass pane (7.5 cm square) using a 10 μm drawdown rod (Leneta Co., Mahwah, NJ) and then drying in air at the appropriate temperature. Infrared spectra were recorded at room temperature using a Nicolet Impact 410 spectrometer equipped with a SpectraTech Gemini horizontal attenuated total reflectance (HATR) accessory; spectra of aqueous samples were corrected using a background spectrum for water. Crystallinity indices were determined by comparison with standards, which were mixtures of fully amorphous and fully crystalline particles in aqueous suspension. The fully amorphous particles were prepared by the method of Horowitz et al.,[12] while the fully crystalline particles were prepared by resuspension and microfluidization of spray-dried PHB powder.

Preparation of Crystalline PHB Suspension. Microfiltrations were performed using a pilot-scale tangential flow filtration apparatus (Niro Filtration). The system was equipped with a tubular ceramic filtration element (U.S. Filter Membralox, 0.1 μm nominal cutoff), a 60 L holding tank, a variable-speed centrifugal feed pump, and a heat exchanger. Operating conditions were as follows: temperature, 20 °C; filter inlet pressure, 0.41 MPa; filter outlet pressure, 0.28 MPa; cross-flow rate, 68−75 L/min. For constant volume diafiltration, water or surfactant solution was added continuously to the recirculating suspension by means of a proportional valve which maintained constant tank volume.

Ralstonia eutropha strain NCIMB 40124 was fermented on glucose under conditions of nutrient limitation as described previously.[21] The cell broth (20 L) containing 163 g/L dry solids was then washed by constant volume diafiltration as described above using distilled water (5 vol). The suspension was then adjusted to pH 10 using 30% ammonium hydroxide, 10 μL/L commercial nuclease was added (Benzonase, American International Chemical), and the suspension was passed twice through a Microfluidizer M110EH homogenizer (Microfluidics International Corp.) at an operating pressure of 124 MPa. Stearyl-21-ethoxylate (Brij 721, ICI Surfactants) was added to a concentration of 0.25%, and the suspension was heated to 45 °C. The pH of the suspension was adjusted to 7.0 with ammonium hydroxide and maintained there by means of a pH controller. Chicken egg-white lysozyme (0.2 g/L, Schweizerhall, Inc.) was added, and the suspension was digested at 45 °C for 1 h. The suspension was then adjusted to 50 °C, pH 8.0, and a commercial protease (Alcalase, 1 mL/L, Novo Nordisk) was added. After 8 h incubation at 50 °C, the suspension was diafiltered at constant volume using distilled water (5 vol).

The washed suspension was supplemented with 0.1% (w/v) Brij 721 and Antifoam A (2 mL, Sigma), cooled to 4 °C, and then sparged with ozone (ca. 2% stream in oxygen, 4 L/min) for 12 h with vigorous stirring. The suspension was then diafiltered at constant volume using distilled water (5 vol). The suspension was then transferred to a reactor, Antifoam A (2 mL) was added, and the suspension was heated to 80 °C. Hydrogen peroxide (30 wt %) was pumped in gradually to a concentration of 1.5% (w/v). Reaction pH was maintained at 7.0 using a pH controller (30% ammonium hydroxide for pH control). After 5 h at 80 °C, the reaction was cooled to 20 °C and diafiltered at constant volume using distilled water (10 vol). The washed suspension was then concentrated by further microfiltration to a solids content of 7.5% (10 L). Analysis of a dried sample of the suspension gave a residual nitrogen content of 0.76%.

Thermal Treatment of Crystalline PHB Suspension. The experimental apparatus was assembled from an electronic diaphragm metering pump (Pulsatron E series, 1.7 MPa rated, maximum flow rate 0.79 L/h), a heat exchanger (inlet 9.5 mm o.d. × 305 mm stainless pipe; outlet 3.2 mm o.d. × 305 mm copper pipe), a submersible coil (3.2 mm o.d. × 1.6 mm i.d. copper pipe, four turns of ca. 100 mm diameter), a thermostatically controlled oil bath (Dow Corning 550 silicone), and a pressure-regulating valve (Swagelok, 0.34−2.4 MPa adjustable). In addition, the apparatus was equipped with a pressure gauge, two electronic thermocouple thermometers, two in-line stainless mesh filters, and two purge valves for eliminating gas bubbles. All components were connected by means of 1.6 mm i.d. copper pipe. The apparatus was insulated to minimize thermal losses.

The silicone oil bath was preheated to 197 °C, and the apparatus was operated on a pure water feed stream for 15 min to purge air bubbles and approach thermal equilibrium. Internal system pressure was maintained at 1.6−1.7 MPa using the pressure-regulating valve. After 15 min the feed was changed to a crystalline PHB suspension (7.5% w/v) prepared as above. At a flow rate of 16 mL/min, the residence time of the suspension within the heated zone was approximately 9 s. Samples of crystalline PHB suspension (200 mL) were processed sequentially through the apparatus. After thermal processing the samples were stored at 4 °C. If necessary, samples were concentrated to a high solids (25−30%) by dialysis against 20% (w/v) aqueous poly(ethylene glycol) (MW 20 000).

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