Synthesis of Poly[(*R*)-3-hydroxybutyric acid) in the Cytoplasm of *Pichia pastoris* under Oxygen Limitation†

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We have constructed a tandem gene expression cassette containing three *Ralstonia eutropha* poly[(*R*)-3-hydroxybutyrate] (PHB) synthesis genes under the control of the *Pichia pastoris* glyceraldehyde-3-phosphate promoter and the green fluorescent protein (Gfp) under the control of the *P. pastoris* alcohol oxidase promoter. The inducible Gfp reporter protein has been used to rapidly isolate transformed strains with two copies of the entire expression cassette. The isolated strain exhibits Gfp induction kinetics that is twice as fast as that of the strains isolated without cell sorting. In addition, the sorted strains exhibited higher PHB contents in preliminary screening experiments. PHB synthesis was characterized in more detail in the sorted strain and was found to be dependent on culture conditions. It was observed that the specific PHB synthesis rate was dependent on the carbon source utilized and that the conditions of oxygen stress lead to increased fractional PHB content. When this strain is cultivated on glucose under oxygen-limited conditions, the cultures accumulated ethanol during the initial growth phase and then consumed the ethanol for the accumulation of PHB and biomass. While PHB was not synthesized during initial growth on glucose, significant levels of PHB were synthesized when ethanol was subsequently consumed. PHB was also synthesized under aerobic conditions when ethanol was the only carbon source. During growth on ethanol, the specific growth rate of the culture was reduced under oxygen-limited conditions but the specific PHB synthesis rate was relatively unaffected. Thus, the high accumulation of PHB which exceeded 30% of the cell dry weight appears to be the consequence of the decreased biomass growth rate under severe oxygen limitation.

Introduction

Poly[(*R*)-3-hydroxybutyrate] (PHB) belongs to a class of structurally simple macromolecules of poly(hydroxyalkanoates) (PHAs) that are synthesized in a wide variety of bacteria.[1] This natural, biodegradable thermoplastic has been widely studied for its properties and as an alternative to traditional petroleum-based polymers.[2] Synthesis of PHAs has been investigated in several recombinant organisms such as *Escherichia coli*,[3] insect cells,[4] plants,[5] and yeast.[6−9] In *S. cerevisiae* the expression of only the synthase enzyme leads to synthesis of small but detectable quantities of PHB indicating that the correct stereoisomeric monomer can be natively made in the cytoplasm. Coexpression of bacterial ketothiolase and reductase results in higher PHB levels.[7] Yeasts are of specific interest because they represent a model for eukaryotic cells that can be easily grown in the laboratory and, from a more applied view, because of the possibility of coproducing fuel ethanol and PHAs.

Synthesis of PHB in foreign organisms usually involves the expression of several genes that complement absent reactions of the entire PHB pathway. Expression of multiple genes in eukaryotic cells is more difficult than in prokaryotes because normally each gene has to be equipped with its own promoter and termination sequences. For example, PHB can be made in *S. cerevisiae* with combinations of multi-copy plasmids each encoding individual PHB synthesis genes, but the system is genetically unstable. Hence, PHB production is very heterogeneous and typical population-averaged PHB contents in such cultures are low. Physiological effects caused by the introduction of this pathway are, therefore, small and may not become apparent. *Pichia pastoris* is known for the efficient expression of proteins from single gene copies, and systems for high-level expression of heterologous proteins have been well-developed.[10] Two strong promoters, the constitutive glyceraldehyde-3-phosphate (GAP) promoter and the methanol inducible alcohol oxidase (AOX1) promoter, have been frequently used to obtain high cellular expression of various proteins.[11,12] Methods for the expression of multiple genes in *P. pastoris* have also been developed.[13] This is particularly useful for metabolic engineering of entire pathways consisting of several genes such as in the case of PHB synthesis. Moreover, the genes are integrated into the chromosome of *P. pastoris* resulting in recombinant strains that are very stable and that do not require selective pressure to keep the genes of interest during culture conditions. Furthermore, it has been already demonstrated that *P. pastoris* is capable of synthesizing medium-chain-length

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Table 1. Plasmids Used in This Study[a]

	arrangement of expression cassettes	references
pGAPZ	GAP	16
pGAPZ^m	GAP^m	18
pPICZ	AOX1	16
pSL-301-FM2	lac-Gfp	21
pPT500		19
pCR-KT		19
pGAP−phaC	GAP−phaC	this work
pGAP^m−phaA	GAP^m−phaA	this work
pGAP^m−phaB	GAP^m−phaB	this work
pGAP−phaC−phaA	GAP−phaC:GAP^m−phaA	this work
pPHB	GAP−phaC:GAP^m−phaA:phbB−GAP^m	this work
pPICZ−Gfp	AOX1−Gfp	this work
pPHB−AOX1−Gfp	GAP−phaC:GAP^m−phaA:AOX1−Gfp:phaB−GAP^m	this work
pGAP^m−Gfp	GAP^m−Gfp	this work
pPHB−Gfp	GAP−phaC:GAP^m−phaA:GAP^m−Gfp:phaB−GAP^m	this work

[a] GAP^m refers to the GAP promoter with the *AvrII* site removed.[18] Plasmids pPHB, pPHB−AOX1−Gfp, and pPHB−Gfp contained only one copy of the unmutated GAP promoter to ensure that a unique *AvrII* site was available for linearization of the plasmid for chromosomal integration. phaA, phaB, and phaC refer to the ketothiolase, reductase, and the synthase genes, respectively.

PHAs in peroxisomes when a polymerase is targeted into this organelle.[14] Methods for cultivating *P. pastoris* to high cell densities have been reported.[15] Therefore, this organism could be an attractive host for the production of PHAs.

To test whether a PHB pathway leading to high PHB accumulation levels can be assembled in the cytoplasm of *P. pastoris*, we have constructed an integrative plasmid with a tandem gene expression cassette including the *Ralstonia eutropha* synthase, reductase, and ketothiolase genes each under the control of the *P. pastoris* GAP promoter. We have integrated this cassette into the genome of *P. pastoris* resulting in cytoplasmic PHB accumulation that is significantly higher than that observed in *S. cerevisiae* cultures. We show that in addition to stable and efficient protein expression, specific cultivation conditions are needed to favor PHB accumulation at significantly elevated levels.

Materials and Methods

Strain and Plasmid. The wild-type *P. pastoris* strain X33 (Invitrogen, Carlsbad, CA) was used in this work. Plasmids were derived from the pGAPZ B vector[16] (*P. pastoris* expression system manual, Invitrogen, Carlsbad, CA) that uses the constitutive GAP promoter for high-level expression of recombinant proteins.[11] This plasmid can be linearized at the unique *AvrII* site in the GAP promoter region for integration into the *P. pastoris* genome. Downstream of the GAP promoter, it contains a multicloning site for convenient insertion of genes of interest and the AOX1 transcription terminator sequence. The gene expression cassette consisting of the GAP promoter, multicloning site, and transcription terminator is flanked by the complementary restriction sites *BglII* and *BamHI*, which can be used to construct vectors containing multiple copies of a gene of interest or to insert multiple genes such as genes of a pathway. This vector also contains the *Streptoalloteichus hindustanus* bleomycin gene that confers resistance to the antibiotic zeocin to prokaryotic and eukaryotic cells containing the vector.[17]

Construction of Plasmids. To produce PHB, a plasmid containing the three *Ralstonia eutropha* PHB pathway genes encoding for synthase, reductase, and β-ketothiolase was constructed. The methodology for constructing such a multigene *P. pastoris* vector has been previously described.[13] Plasmids constructed and used in this study are listed in Table 1. The pGAPZ^m vector (gift from Dr. Claudia Schmidt-Dannert at the University of Minnesota, Saint Paul) contains a single base pair mutation on the GAP promoter sequence that deletes the *AvrII* site but that does not abolish promoter function.[18] This promoter is used for expression of all individual genes in the expression cassette except the synthase gene that uses the original GAP promoter with the functional *AvrII* site. This site is used for linearizing the plasmid before integration. The synthase gene was polymerase chain reaction (PCR)-amplified from plasmid pPT500[18] using the primers 5′-TAT TAG ATT TCA TGG CGA CCG GCA AAG GC-3′ and 5′-ATT ACT CGA GTC ATG CCT TGG CTT TGA CG-3′. The PCR product was digested with *EcoRI* and ligated with the *EcoRI* digested fragment of plasmid pGAPZ to result in plasmid pGAP−phaC. The reductase gene was also PCR-amplified from plasmid pPT500 using the primers 5′-ATT GAA TTC ATG ACT CAG CCG CAT TGC GTA TGT G-3′ and 5′-ATT GCG GCC GCT CAG CCC ATA TGC AGG CCG CCG-3′. The PCR product was digested with *EcoRI* and cloned into the *EcoRI* site in the multicloning sequence in pGAPZ^m to result in plasmid pGAP^m−phaB. The ketothiolase gene was excised from the plasmid pCR-KT[19] by digesting with *EcoRI*. The ketothiolase fragment was ligated with the *EcoRI* digested fragment of plasmid pGAPZ^m to result in plasmid pGAP^m−phaA. The ketothiolase expression cassette from pGAP^m−phaA was excised by digesting with *BamHI* and *BglII* and was ligated with the *BamHI* digested fragment of plasmid pGAP−phaC, which gave plasmid pGAP−phaC−phaA. The reductase expression cassette was excised from pGAP^m−phaB by digesting with *BamHI* and *BglII* and was ligated with the *BamHI* digested fragment of plasmid pGAP−phaC−phaA to yield the plasmid pPHB (Figure 1a). The sequence of the FM2 mutant of green fluorescent protein (Gfp)[20] was PCR-amplified from pSL-301-FM2[21] using the primers 5′-GCG GGA GAA TTC ATG AGT AAA GGA GAA-3′ and

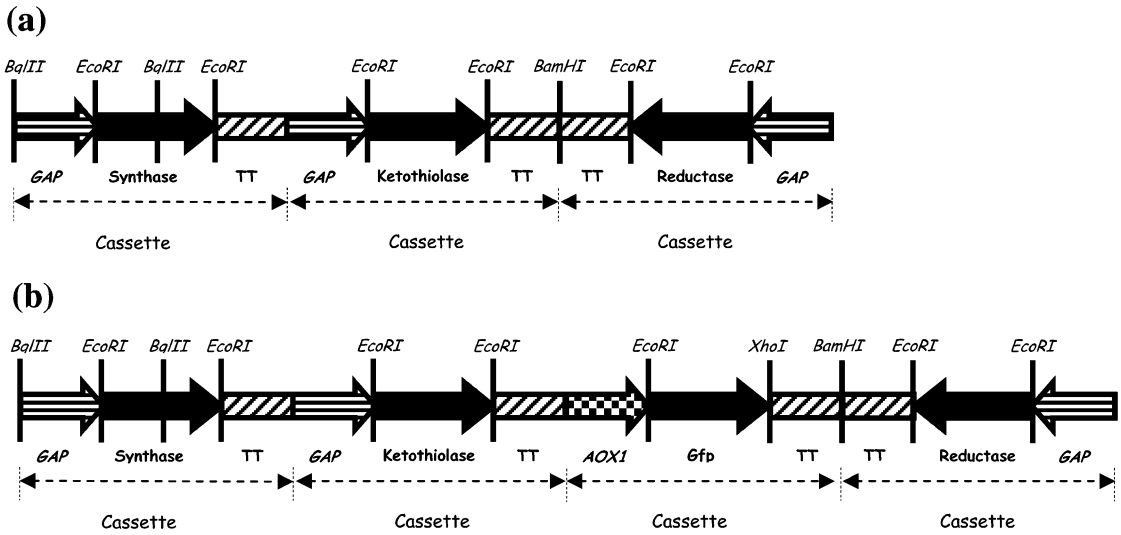

Figure 1. Schematic illustration of the constructed multigene cassettes. Integrative plasmid (a) pPHB encoding the three *R. eutropha* PHB genes was constructed. Complementary restriction sites, *BglII* and *BamHI*, were used to concatenate individual gene expression cassettes, each containing one of the three PHB genes with its own GAP promoter and transcription termination sequence (TT). An additional gene expression cassette containing the gene for the Gfp under the control of the AOX1 promoter was added to pPHB to construct (b) pPHB−AOX1−Gfp. This plasmid was used for flow cytometric strain improvement.

5′-GAG GCA CTC GAG TTA TTT GTA TAG-3′. The Gfp fragment was digested with *EcoRI* and *XhoI* and ligated with the pPICZ (catalog no. V190-20, Invitrogen, Carlsbad, CA) fragment digested with *EcoRI* and *XhoI* to result in vector pPICZ−Gfp. The gene expression cassette containing the Gfp gene was excised from this plasmid by digesting with *BamHI* and *BglII* sites and cloned into the unique *BamHI* site in the pPHB vector, to create pPHB−AOX1−Gfp (Figure 1b). The plasmid pPHB−Gfp containing the Gfp gene under the control of the GAP promoter in addition to the three PHB synthesis genes was similarly constructed. All plasmids contain a unique *AvrII* site which was used for linearization and integration into the *P. pastoris* genome. PHB-producing strains X33:PHB and X33:APHB were created by transforming wild-type *P. pastoris* X33 with plasmids pPHB and pPHB−AOX1−Gfp using electroporation according to established protocols (*P. pastoris* expression system manual, Invitrogen, Carlsbad, CA).

Flow Cytometry and Cell Sorting. A FACSCalibur flow cytometer was used for flow cytometric analysis and sorting experiments of PHB- and Gfp-producing cultures. The strain X33 was transformed with the plasmid pPHB−AOX1−Gfp or pPHB−Gfp by electroporation. After electroporation, the cells were resuspended and grown in YPD liquid media (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) containing 50 μg/mL of zeocin for approximately 2 days until the turbidity of the culture visibly increased. When the plasmid pPHB−AOX1−Gfp was used, the cells were centrifuged and resuspended in BMMY medium (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L yeast nitrogen base, 5 g/L methanol, and 100 mM potassium phosphate, pH 6.0) to induce the AOX1 promoter. These cells were grown in BMMY for 24 h. Cellquest software (Becton Dickinson, San Jose, CA) was used for the analysis and sorting of these cells using the flow cytometer. A gate corresponding to the 0.01% of cells with the highest green fluorescence was used as a basis for sorting cells that were analyzed at a rate of 2000 cells s^{-1}. When the plasmid pPHB−Gfp was used, flow cytometric sorting was performed without regrowing the cells in the BMMY medium. In both cases, the sorted cells were directly plated onto YPD plates containing 50 μg/mL zeocin.

Induction of Gfp Expression. The induction kinetics of strains obtained by cell sorting was determined as follows. A 100-μL aliquot of the frozen stock was inoculated in 25 mL of the glycerol-based medium BMGY (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L yeast nitrogen base, 10 g/L glycerol, and 100 mM potassium phosphate, pH 6.0) in 250-mL Erlenmeyer shake flasks and incubated in a shaker at 30 °C at 220 rpm. After 24 h, the cells were centrifuged and resuspended in BMMY to induce the AOX1 promoter, and the Gfp fluorescence was monitored using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Media and Growth Conditions. PHB-producing strains were cultivated on the complex YPD medium. For the induction of the AOX1 promoter for sorting purposes, the BMMY medium was used. For shake flask experiments, single colonies were inoculated into 25 mL of YPD medium in 250-mL Erlenmeyer shake flasks and cultured for 72 h with an agitation of 250 rpm in a rotary incubator at 30 °C. A 2.5-L BIOSTAT B bioreactor (B Braun Biotech., Bethlehem, PA) with a 1.5-L working volume was used for controlled growth experiments. The temperature was controlled at 30 °C. The pH was not controlled. The dissolved oxygen concentration was controlled above 20% air saturation using agitation in the aerobic experiments. In hypoxic experiments, the oxygen supply was controlled by maintaining constant aeration and agitation rates. However, agitation was not used to control the dissolved oxygen concentration. The applied aeration and agitation rates for the various reactor experiments are summarized in Table 2.

Analysis of Bioreactor Samples. Cell dry weights were measured gravimetrically. The PHB content was measured as previously described.[22] Glucose concentrations were determined using a Sigma Diagnostics D-glucose kit (Sigma, St. Louis, MO). Ethanol was determined by mixing 1 mL

Table 2. Maximal Concentrations of Ethanol, Residual Biomass, PHB, and % PHB of *P. pastoris* Cultures in Controlled Bioreactor Experiments[a]

	aeration [L min^{-1}]	agitation [rpm]	ethanol [g/L]	residual biomass [g/L]	PHB [g/L]	% PHB
aerobic experiment	4.0	DO control	not detected	18	0.33	1.76
hypoxic experiment I	3.0	100	5.58	10.1	2.22	19
hypoxic experiment II	1.33	50	11	8.6	3	27
aerobic ethanol experiment	4.0	DO control	7.31	11.5	0.66	5.47

[a] The agitation was either constant as listed or varied automatically to control the dissolved oxygen (DO) concentration.

of culture supernatant with 200 μL of internal standard solution (3 g/L propanol) and analyzing on a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) with a DB-WAX 30 W capillary column with a flame ionization detector. Lactate and succinate were assayed as described elsewhere[23] using the same gas chromatography equipment. Acetate was assayed using an enzymatic acetic acid kit (R-BIOPHARM, Darmstadt, Germany).

Dipyrromethene boron Difluoride (BODIPY) Staining and Fluorescence Microscopy. A 1-mL sample of the culture was diluted with YPD medium to an optical density (600 nm) of approximately 10. This diluted cell suspension (1 mL) was stained with 10 μL of BODIPY stock solution containing 100 μg/mL of BODIPY 493/503 (Molecular Probes, Eugene, OR) for 5 min at room temperature. The cells were photographed using a fluorescence microscope (model E800, Nikon, Melville, NY) using an excitation wavelength of 488 nm.

Growth of *P. pastoris* under Nitrogen-Limited Conditions. A 100-μL aliquot of the frozen stock of *P. pastoris* X33:APHB-S3 was inoculated in 25 mL of YPD medium in 250-mL Erlenmeyer shake flasks and incubated in a shaker at 30 °C at 220 rpm. After 24 h, the cells were centrifuged and resuspended in minimal medium containing trace mineral salts and 2% glucose but lacking a nitrogen source (8 g/L sodium chloride, 0.2 g/L potassium chloride, 2.72 g/L sodium phosphate, 0.24 g/L potassium phosphate, 0.0261 g/L cupric sulfate, 3.48×10^{-4} g/L sodium iodide, 0.01305 g/L manganese sulfate, 8.7×10^{-4} g/L sodium molybdate, 8.7×10^{-5} g/L boric acid, 0.002175 g/L cobalt chloride, 0.087 g/L zinc chloride, 0.28275 g/L ferrous sulfate, 8.7×10^{-4} g/L biotin, 0.02175 g/L sulfuric acid, and 20 g/L glucose). These cultures were sampled after 48 and 72 h, and their PHB contents were analyzed.

Specific Synthesis Rates. The specific rates of production of PHB, biomass, and Gfp were calculated using the following equations.

$$\frac{\mathrm{dPHB}}{\mathrm{d}t} = q_{\mathrm{PHB}} R_x \tag{1}$$

$$\frac{\mathrm{d}R_x}{\mathrm{d}t} = \mu R_x \tag{2}$$

$$\frac{\mathrm{dGfp^*}}{\mathrm{d}t} = q_{\mathrm{Gfp}} N \tag{3}$$

PHB and R_x are the PHB and residual biomass concentrations (g L^{-1}), respectively. q_{PHB} and μ are the specific PHB (g PHB (g residual biomass)$^{-1}$ h^{-1}) and residual biomass synthesis (h^{-1}) rates, respectively. Gfp* is the total amount of Gfp per mL expressed as the product of mean green fluorescence intensity and the cell concentration N (cells mL^{-1}). Cellular fluorescence was measured using a FACSCalibur flow cytometer in arbitrary fluorescence units. Cells were excited with a laser at 488 nm, and green fluorescence was measured using a band-pass filter (530/30 nm). q_{Gfp} is the specific Gfp synthesis rate in (fluorescence units) h^{-1}. Assuming a constant specific synthesis rate, integration of eq 1 yields

$$\mathrm{PHB}(t) = q_{\mathrm{PHB}} \int_0^t R_x \, \mathrm{d}t \tag{4}$$

The residual biomass was integrated graphically. The specific PHB synthesis rate was then calculated as the slope of the line when the PHB concentration is plotted versus the time-dependent integrated residual biomass. The specific growth rate and the Gfp synthesis rate were similarly calculated.

Results

Production of PHB in *P. pastoris.* To produce PHB in the cytoplasm of *P. pastoris*, we transformed the wild-type strain X33 with plasmid pPHB. The average PHB content was about 3% of the cell dry weight after growing in shake flasks for 72 h. Cells transformed with a plasmid containing only the polymerase gene did not result in detectable amounts of PHB. When cells were stained with the hydrophobic stain BODIPY,[24,25] PHB inclusions could be visualized using fluorescence microscopy (Figure 2b). PHB was clearly visible as bright fluorescent inclusions in the cytosol. These inclusions were absent in wild-type strain X33 cultivated and stained under conditions comparable to those of the control (Figure 2a).

Enhancement of PHB Production through Cell Sorting. High PHB accumulation requires (i) high expression levels of the PHB synthesis enzymes and (ii) the cultivation of cultures under physiological conditions that favor PHB synthesis. It was expected that an increase in the gene copy number of the three PHB-producing enzymes would lead to increased expression levels of the PHB enzymes, resulting in increased PHB production. To be able to detect and to isolate cells with high protein expression levels, we included the gene for the Gfp as a fourth protein in the expression cassette resulting in plasmid pPHB−AOX1−Gfp. The Gfp gene was placed under the control of the methanol-inducible AOX1 promoter[12] enabling the expression of the reporter protein only when it was desired. The AOX1 promoter is only induced in the presence of methanol, and, hence, it could be induced for the selection of high protein-producing cells and repressed during PHB production. This plasmid was

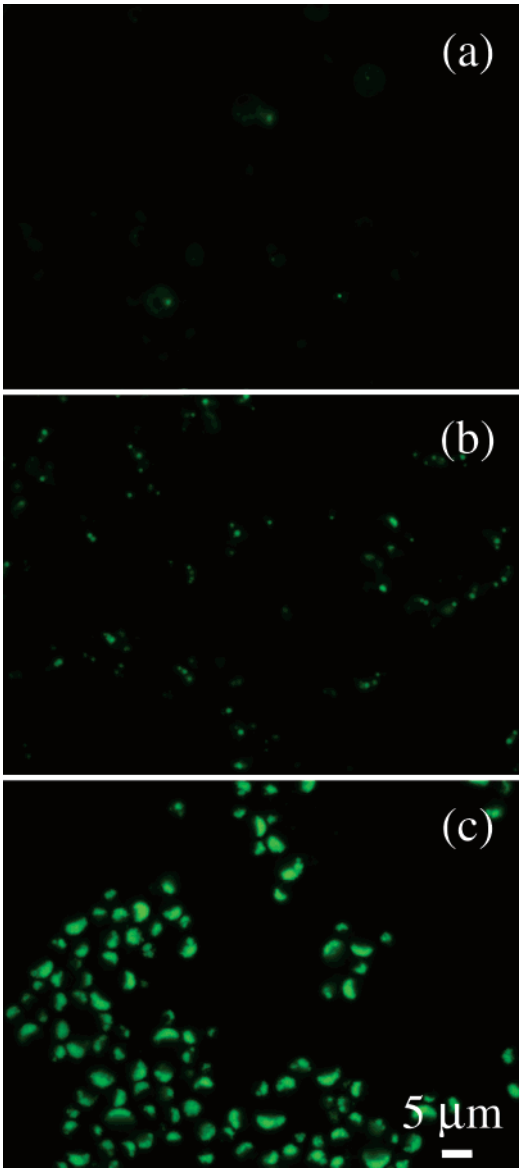

Figure 2. Fluorescent micrographs of *P. pastoris*. The strains X33 and X33:PHB were cultivated in the complex YPD medium at 30 °C for 72 h. X33:APHB-S3 was cultivated in a bioreactor under hypoxic conditions. They were subsequently stained with the hydrophobic stain BODIPY and photographed using fluorescence microscopy. (a) Strain X33 not producing PHB. (b) Strain X33:PHB producing 3% PHB. (c) Strain X33:APHB-S3 producing 19% PHB.

transformed into strain X33, and high Gfp-producing cells in the transformation mixture were sorted with flow cytometry as described in the methods section. The sorted cells were plated on YPD plates containing 50 μg/mL zeocin.

Ten colonies from this plate were picked and screened for PHB content. The highest PHB production level observed was 6.9% while the highest PHB production among unsorted colonies was 4.3%. Starting from individual colonies of these cultures the screening assay was repeated in triplicate. The results confirmed the initially observed difference in PHB content. The higher producing strain obtained from cell sorting, strain X33:APHB-S3, had a PHB content of 7.1 ± 1.5% while the unsorted strain, X33:APHB, had a PHB level of 3.1 ± 2.0%.

We compared Gfp induction kinetics of the sorted and unsorted strains. The cells were initially grown on BMGY medium and then shifted to the methanol-based complex medium to induce the AOX1 promoter. The Gfp fluorescence of the cells was followed using flow cytometry. The integrated form of eq 3 was used to compare the specific Gfp synthesis rate of the two strains (see Figure 3). The abscissa of this plot is the integrated cell number, while the ordinate is the Gfp fluorescence. The slope of this graph represents the specific Gfp production rate. It can be seen that the Gfp production rate of X33:APHB-S3 is approximately twice that of X33:APHB, suggesting that the sorted strain contains duplicate copies of the Gfp gene. This also suggests that the improved PHB production was likely because of the increased levels of protein expression due to the integration of duplicate copies of the gene expression cassette into the chromosome of *P. pastoris*.

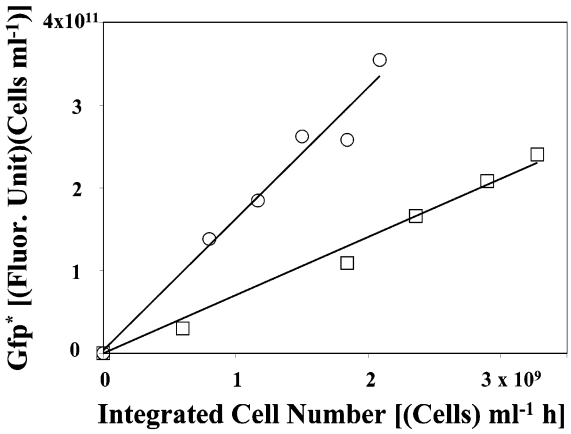

Figure 3. Gfp induction kinetics of strains with chromosomally integrated expression cassettes. The Gfp synthesis rates of strains X33:APHB-S3 (○) and X33:APHB (□) were compared. The strains were initially grown in BMGY medium and then shifted to methanol-based BMMY medium to induce the AOX1 promoter. The fluorescence intensity was measured in arbitrary units. The specific Gfp synthesis rate was 70.3 (fluorescence) h^{-1} for X33:APHB while it was 158 (fluorescence) h^{-1} for X33:APHB-S3.

Enhancing the PHB Production Level through Cultivation Conditions. Controlled batch bioreactor experiments in which the dissolved oxygen concentration was not allowed to drop below 20% resulted in cultures with maximum PHB content up to 1.8% of the cell dry weight (Figure 4). This is significantly lower than the PHB levels observed in shake flasks. In comparison to bioreactor growth conditions, conditions in the shake flask likely become oxygen-mass-transfer-limited (hypoxic) as a result of poor mixing.

To approximate shake flask conditions in the bioreactor the rate of oxygen supply was reduced by lowering the aeration and agitation rates (Table 2). Two different bioreactor experiments were conducted under hypoxic growth conditions created by lowered agitation and aeration rates. The aeration and agitation rates were lower for reactor II than they were for reactor I. Hence, the cells in hypoxic reactor II were under a more severe oxygen stress. The results from these experiments are shown in Figure 5 and are summarized in Table 2. In both experiments, the dissolved oxygen concentration rapidly decreased as the cells started growing and dropped to 0% of the air saturation levels. The growth rate was reduced, and the cells continued to grow linearly as a result of oxygen limitation. The final biomass

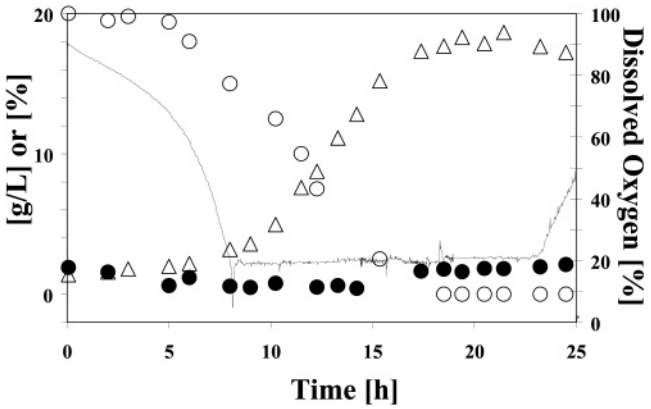

Figure 4. Aerobic cultivation of *P. pastoris* containing PHB genes. The strain X33-APHB-S3 was grown in a controlled bioreactor where the dissolved oxygen concentration was maintained at least at 20% of the air saturation by feeding air at the rate of 4 L min^{-1} and by controlling the agitation rate. (△) Residual biomass (g/L), (●) % PHB, (○) glucose (g/L), (−) dissolved oxygen concentration (% air saturation).

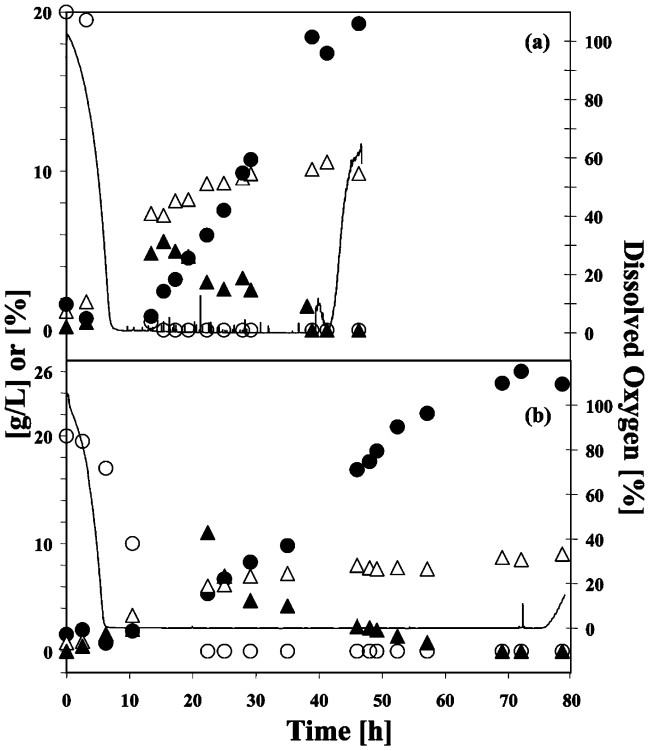

Figure 5. Cultivation of X33:APHB-S3 under oxygen-limiting conditions. (a) Hypoxic reactor I: dissolved oxygen concentration was not controlled. The aeration was set at 3 L min^{-1}, and the agitation was set at 100 rpm. (b) Hypoxic reactor II: The aeration was set at 1.33 L min^{-1}, and the agitation was set at 50 rpm. (△) Residual biomass (g/L), (●) % PHB, (▲) ethanol (g/L), (○) glucose concentration (g/L), (−) dissolved oxygen (% air saturation).

concentration decreased as a function of the oxygen limitation. The biomass concentration was lower in experiment II, which had the greater oxygen limitation. The PHB concentration increased linearly with culture time in both hypoxic reactors and reached a final concentration of 2.22 g/L in reactor I and 3 g/L in reactor II. This corresponded to a PHB content of 19% of the cell dry weight in reactor I and 27% of the cell dry weight in reactor II. Cells from reactor I containing up to 19% PHB were stained and photographed using fluorescence microscopy (Figure 2c).

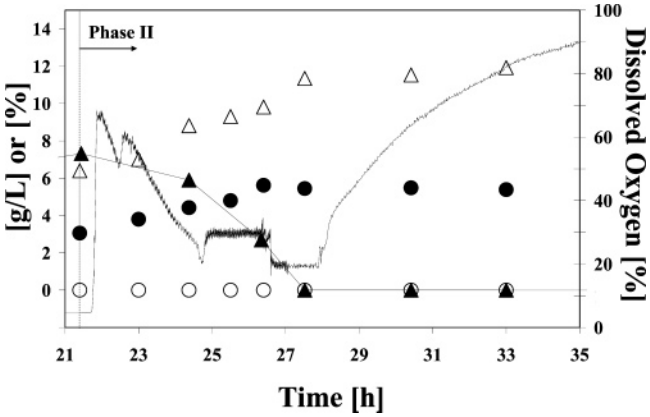

Figure 6. Strain X33:APHB-S3 was initially cultivated under hypoxic conditions (aeration, 1.33 L min^{-1}; agitation, 50 rpm) to force them to produce ethanol (phase I). The aeration was increased to 4 L min^{-1}, and the agitation was used to control the dissolved oxygen level at least at 20% of the air saturation (phase II). Data shown only for phase II (see text). (△) Residual biomass (g/L), (●) % PHB, (▲) ethanol (g/L), (○) glucose (g/L), (−) dissolved oxygen (% air saturation).

Large clumps of polymer can be seen inside the cells instead of individual granules. This granule morphology was likely caused by the absence of expressed phasins. For instance, mutant *R. eutropha* strains not containing phasin PhaP accumulate PHB in the form of a few very large granules.[26] One can also visually see that the PHB content is much higher than in the cells from the shake flask cultures (Figure 2b).

The medium was analyzed for concentrations of glucose and for metabolic byproducts such as ethanol, acetate, succinate, and lactate. None of the cultures produced any acetate, succinate, or lactate. In the hypoxic reactor experiments, glucose was exhausted many hours before the cells stopped growing. While the aerobic reactor did not produce any metabolic byproducts, the hypoxic reactors I and II produced ethanol after the culture was limited by the availability of oxygen. Hypoxic reactor I accumulated up to 5.58 g/L of ethanol after 15.45 h while hypoxic reactor II accumulated up to 11 g/L of ethanol after 22 h. This ethanol was subsequently consumed for biomass and PHB production. If glucose was repeatedly added to the reactor during PHB accumulation the sugar was immediately converted into ethanol and ethanol was subsequently consumed for PHB synthesis to reach intracellular levels up to 31% (data not shown).

The reduced oxygen supply evidently results in increased PHB formation. Moreover, significant PHB synthesis occurred when glucose was exhausted and ethanol served as the carbon and energy source. To determine the PHB and biomass synthesis rates under completely aerobic conditions when ethanol was the carbon source, a culture was grown under identical growth conditions as in the hypoxic experiment II for the initial 15 h. At this point, when glucose was exhausted and the highest ethanol concentration was obtained, the aeration and agitation rates were increased to create aerobic conditions. The dissolved oxygen concentration was controlled subsequently at a level greater than 20% of air saturation (Figure 6). One can see from the data that, at the end of the hypoxic growth phase, glucose was

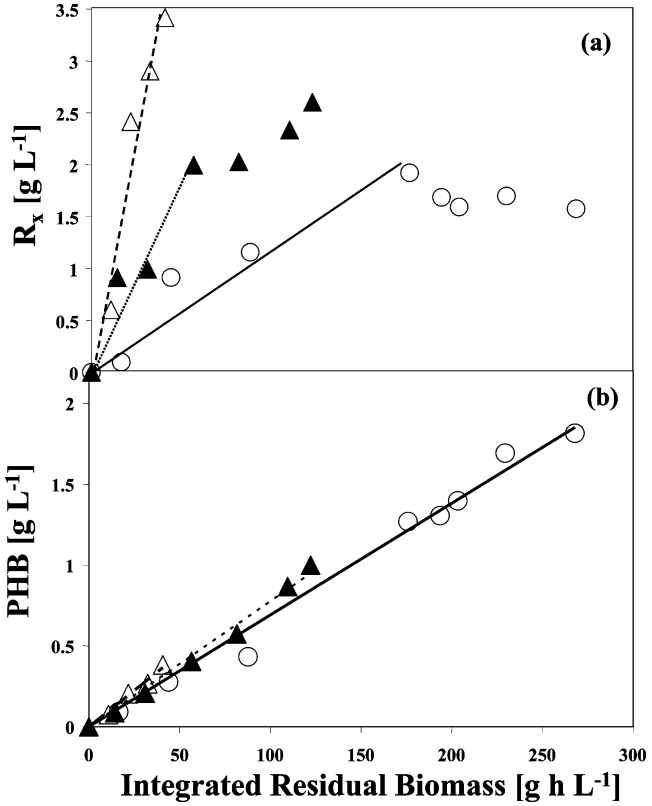

Figure 7. Effect of oxygen availability on residual biomass and PHB production rates. The abscissa is the integrated residual biomass and the ordinates are the (a) residual biomass and (b) PHB concentration. The slopes of this plot give the specific rates of (a) residual biomass and (b) PHB synthesis. Only points where the ethanol concentration was detected in the medium are shown. The points on the biomass plot do not fall on a straight line, indicating that the residual biomass growth rate changes with time. The specific growth rate was 0.09 h^{-1} under aerobic conditions (△) while it was 0.04 and 0.01 h^{-1} when the oxygen supply was reduced in hypoxic experiments I (▲) and II (○), respectively. The specific PHB synthesis rate was 0.009 g g^{-1} h^{-1} under aerobic conditions, while it was 0.008 and 0.007 g g^{-1} h^{-1} in hypoxic experiments I and II, respectively.

exhausted and ethanol reached a concentration of 7.31 g/L. Once the air supply was increased, the available ethanol was quickly consumed in 6 h. In that time, residual biomass increased from 6.4 g L^{-1} to 11.35 g L^{-1} and fractional PHB content increased from 3 to 5.6% of the cell dry weight. There was no detectable increase in the concentration of the residual biomass or in the PHB content after ethanol was exhausted.

Integrated forms of eqs 1 and 2 were used to compare the specific PHB and biomass synthesis rates for the different reactors. Figure 7a,b shows the residual biomass and PHB synthesis rates for the different cases of oxygen limitation. The slope of the curves in the plot represents the specific PHB and residual biomass production rates. It is clear from this graph that the growth rates of the hypoxic reactors was significantly lower than that of the aerobic ethanol reactor indicating that the growth rate is significantly affected by oxygen supply. The biomass profiles do not represent straight lines, indicating changing growth rates during the course of the bioreactor culture time due to oxygen limitation. The specific PHB production rate appears to be less affected than the biomass growth rate because of hypoxic conditions. It is clear from these data that in comparison to the specific residual biomass growth rate, the specific PHB accumulation rate was relatively less affected by the extent of oxygen limitation.

Discussion

We have shown that fractional PHB content can be enhanced by increasing the expression level of the three PHB pathway enzymes through gene dosage. The applied strain isolation technique has been designed to minimally interfere with the cellular metabolism during PHB synthesis. The reporter protein, Gfp, is expressed under the control of the tightly regulated inducible AOX1 promoter which is only induced when methanol is present in the medium. Because this gene is part of the expression cassette that has been integrated into the chromosome, it served as a reporter for the copy number of the entire cassette. The reporter function could be induced only for the cell-sorting purpose and is repressed during PHB synthesis when it is desired to avoid diversion of cellular resources toward production of proteins that are not needed. This strategy has been developed after initial attempts to isolate cells with multiple integrations using Gfp, expressed with the constitutive GAP promoter, were not successful.

Limitation of nutrients is known to increase the cellular PHB content in several other organisms.[27] Our experiments show that *P. pastoris* does not accumulate significant levels of PHB under conditions of nitrogen limitation (data not shown). The controlled growth experiments demonstrate that high PHB accumulation in *P. pastoris* is favored under oxygen-limited hypoxic conditions.

Under completely aerobic conditions with glucose as the carbon source, cells appear not to accumulate PHB and they do not secrete significant levels of metabolites. This indicates that a balanced redox state can be attained under aerobic conditions and that intracellular metabolic concentrations do nor favor PHB accumulation. When the cells grow aerobically on glucose, the cellular PHB content decreases to very low levels and cells do not accumulate PHB even if growth is limited by nitrogen. When oxygen becomes limiting, a balanced redox state can be attained only when significant amounts of ethanol are excreted. While little information about the metabolism of *P. pastoris* is available, it is known that, for instance, *Pichia stipitus* is a respiratory yeast and the regulation of its aerobic and fermentative metabolism is different from that of *S. cerevisiae*.[28] The alcohol dehydrogenase gene in *P. stipitus* is induced under oxygen-limiting conditions leading to the production of ethanol. Thus, respirofermentative metabolism is used to support cell growth under oxygen-limiting conditions. In the case of *P. pastoris*, significant PHB synthesis occurs when glucose is exhausted and ethanol serves as the carbon and energy source. Synthesis of PHB in *S. cerevisiae* was previously analyzed using elementary mode analysis which also suggested that this yeast could synthesize PHB more efficiently when ethanol or acetate is used as the carbon source.[7]

We further show that oxygen-limited conditions lead to increased fractional PHB content. Oxygen limitation is known to affect specific PHB formation rates in certain

nitrogen fixing bacteria through a complex regulatory mechanism.[27,29] In *P. pastoris*, the specific PHB synthesis rate is little affected by the extent of oxygen limitation during growth on ethanol. In fact, the specific PHB synthesis rate is little altered when cells grow either under aerobic or under hypoxic conditions. In contrast, the residual biomass growth rate is significantly reduced under hypoxic conditions. Although the specific PHB synthesis rate remains unaffected under aerobic growth conditions, the cellular PHB content is very low because biomass grows much faster. When the oxygen becomes limiting, the biomass growth rate is reduced and PHB accumulates to greater levels in the cells. The level of cellular accumulation of PHB is, thus, not subject to a specific regulation mechanism of the direct PHB pathway but appears to be the result of the modulation of the specific biomass growth rate of cells. We have observed a fractional PHB content in excess of 30% of the cell dry weight and a maximal specific PHB synthesis rate of 0.009 g g^{-1} h^{-1}. It is plausible that *P. pastoris* can accumulate PHB to much higher levels in a fed-batch reactor under optimized conditions.

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References and Notes

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