

Effects of recombinant precursor pathway variations on poly[(*R*)-3-hydroxybutyrate] synthesis in *Saccharomyces cerevisiae*

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Abstract

Different recombinant *R*-3-hydroxybutyryl-CoA (3-HB) synthesis pathways strongly influenced the rate and accumulation of the biopolymer poly[(*R*)-3-hydroxybutyrate] (PHB) in *Saccharomyces cerevisiae*. It has been previously shown that expression of the *Cupriavidus necator* PHB synthase gene leads to PHB accumulation in *S. cerevisiae* [Leaf, T., Peterson, M., Stoup, S., Somers, D., Srienc, F., 1996. *Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. *Microbiology* 142, 1169–1180]. This finding indicates that native *S. cerevisiae* expresses genes capable of synthesizing the correct stereochemical substrate for the synthase enzyme. The effects of variations of 3-HB precursor pathways on PHB accumulation were investigated by expressing combinations of *C. necator* PHB pathway genes. When only the PHB synthase gene was expressed, the cells accumulated biopolymer to approximately 0.2% of their cell dry weight. When the PHB synthase and reductase gene were co-expressed, the PHB levels increased approximately 18 fold to about 3.5% of the cell dry weight. When the beta-ketothiolase, reductase and synthase genes were all expressed, the strain accumulated PHB to approximately 9% of the cell dry weight which is 45 fold higher than in the strain with only the synthase gene. Fluorescent microscopic analysis revealed significant cell-to-cell heterogeneity in biopolymer accumulation. While the population average for the strain expressing three PHB genes was approximately 9% of the cell dry weight, some cells accumulated PHB in excess of 50% of their cell volume. Other cells accumulated no biopolymer. In addition, the recombinant strain was shown to co-produce ethanol and PHB under anaerobic conditions. These results demonstrate that the technologically important organism *S. cerevisiae* is capable of accumulating PHB aerobically and anaerobically at levels similar to some bacterial systems. The easily assayed

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PHB system also creates a convenient means of probing in vivo the presence of intracellular metabolites which could be useful for studying the intermediary metabolism of *S. cerevisiae*.

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1. Introduction

PHB is a member of the polyhydroxyalkanoate (PHA) family of biodegradable polyesters. PHAs are widely studied as potential alternatives to some petroleum-based thermoplastics (for a recent review see Sudesh et al., 2000) and have been isolated from more than 240 strains of bacteria and have been found in more than 100 different structural variations (Steinbüchel and Valentin, 1995; Kessler and Witholt, 2001). Genetic engineering techniques have made it possible to express the PHA biosynthetic genes in foreign hosts. This ability is valuable for taking advantage of the unique metabolic capabilities found in different organisms. A short list of recombinant hosts includes such prokaryotic and eukaryotic organisms as *Escherichia coli* (Slater et al., 1988; Schubert et al., 1988; Peoples and Sinskey, 1989), *Arabidopsis thaliana* (Poirier et al., 1992), cotton (John and Keller, 1996), maize (Hahn et al., 1997), tobacco (Nakashita et al., 1999), *Saccharomyces cerevisiae* (Leaf et al., 1996; Poirier et al., 2001; Carlson et al., 2002; Breuer et al., 2002), *Pichia pastoris* (Poirier et al., 2002; Vijayasanakaran et al., 2005), *Arxula adenivorans* (Terentiev et al., 2004), and insect culture cells (Williams et al., 1996). This continually expanding list demonstrates that this polymer can be synthesized probably in any cell environment providing that the pathway enzymes are present and that precursors and cofactors are present at favorable concentrations for the synthesis reaction to occur. However, many of the listed eukaryotic cell systems are reported to accumulate only relatively small amounts of PHB. From the point of view of possible technological applications it is therefore of increasing interest to investigate conditions that result in faster synthesis rates, in increased final accumulation levels of this polymer and how the diversion of intermediates from the host metabolism affects host cell physiology. While the conditions for enhanced PHB synthesis

are in principle known and predictable, they are difficult to realize experimentally particularly in eukaryotic cell systems because of the inherent limitations of expressing multiple genes. High level expression can be quickly realized with a strong promoter and with multicopy plasmids. Such constructs can be relatively quickly made for single genes, but they suffer from the lack of a uniform copy number and may be inherently unstable. Chromosomal integration can alleviate the stability problem but it reduces the gene copy number and the level of expressed gene products unless multiple gene integrations have been made. We have realized here the high level expression of three genes through a combination of two multicopy plasmids and expression from a bivalent promoter that enables expression of two genes from a single promoter. Although the system is not yet sufficiently developed to be technologically useful, the studies have resulted in interesting data that demonstrate how the reaction flux through this relatively simple pathway is affected if the ratios of the expressed enzymes are altered. Furthermore, the estimation of PHB accumulation in some of the cells in a pronounced heterogeneous cell population demonstrated the capacity of PHB accumulation that cells can achieve.

The intracellular availability of the correct stereochemical precursors can limit flux through recombinant pathways. In the current study, we demonstrate that the native *Saccharomyces cerevisiae* R-3-hydroxybutyryl-CoA (3-HB) synthesis route can be augmented with foreign genes to improve both the poly[(R)-3-hydroxybutyrate] (PHB) synthesis rates and accumulation levels. While population averaged biopolymer levels are modest, microscopic analysis reveals this commercially important organism can accumulate PHB in excess of 50% of the cell volume. In addition, the anaerobic co-production of PHB with the industrially important chemical ethanol is demonstrated.

2. Materials and methods

2.1. Strains and media

Saccharomyces cerevisiae strain D603 (MAT α *ura3-52 lys2-801 met his3 ade2-101 reg1-501* [cir $+$]) (Srienc et al., 1986) was used in all described yeast studies. The *reg1-501* mutation partially releases catabolite repression of the GAL1-10 promoter (Matsumoto et al., 1983; Hovland et al., 1989). This mutation permits the partial induction of the GAL1-10 promoter in the presence of glucose with galactose concentrations as low as 0.2 g/L (Piper and Kirk, 1991). Common maintenance and manipulation of DNA plasmids were done in *E. coli* strain DH5 α (Life Technologies). When a restriction site was sensitive to methylation, DAM methylase negative *E. coli* strain GM34 was used.

Non-recombinant *S. cerevisiae* D603 cultures were grown at 30 °C in YPD media (Sherman, 1991). Transgenic yeast strains were grown at 30 °C on minimal media (1.6 g/L Bacto Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco, Detroit, MI), 5 g/L ammonium sulfate, 10 g/L galactose, and 10 g/L glucose). Adenine (100 mg/L), histidine (80 mg/L), lysine (150 mg/L), methionine (100 mg/L), and uracil (80 mg/L) were added as needed to complement auxotrophic mutations. The minimal media components were filter sterilized (Supor-200, 0.2 μ m, Gelman Sciences, Ann Arbor, MI). The use of two carbon sources, namely galactose and glucose, permitted rapid growth and good expression levels from the GAL1-10 promoter (Da Silva and Bailey, 1989). *E. coli* strains were typically grown in Luria-Bertanini (LB) media (Sambrook et al., 1989). When appropriate, either ampicillin (100 μ g/mL) or kanamycin (100 μ g/mL) was added.

The fraction of plasmid containing cells was determined by plating equal numbers of yeast cells on selective, minimal medium agar plates and non-selective YPD agar plates.

2.2. Cell growth

Shake flask experiments were done in 250 mL Erlenmeyer flasks with 50 mL of minimal media. The flasks were incubated at 30 °C in a Lab-line incubator-shaker operated at 200 RPM.

A 51 B. Braun Biostat B (Bethlehem, PA) was used for all bioreactor experiments. The reactors were batched with 3 l of filter sterilized media. The pH was maintained at 4.5 with the addition of either 2% H₃PO₄ or 1 M NaOH and the temperature was set at 30 °C. The reactor was agitated at 300 RPM and sparged with air at 1 vessel volume per minute (1VVM).

2.3. DNA transformation

Yeast cells were transformed using the technique outlined by Soni et al. (1993).

2.4. Metabolite analysis

Glucose concentrations were determined using Sigma Diagnostics Glucose kit (No. 510-A, St. Louis, MO). Galactose concentrations were determined using Boehringer-Mannheim Lactose/D-Galactose assay kit (Darmstadt, Germany). Ethanol concentrations were determined by mixing 0.5 mL of culture supernatant with 0.1 mL of internal standard solution (3 g/L 1-propanol). Samples were run on a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) with a DB-WAX 30W capillary column using a flame ionization detector. PHB content was analyzed as previously described (Leaf et al., 1996). Residual biomass is defined as the total biomass minus the PHB mass.

2.5. Nile Red staining

The hydrophobic stain Nile Red (Sigma, St. Louis, MO) was used to visualize the PHB with UV microscopy (Ostle and Holt, 1982). To stain the hydrophobic PHB inclusions, 10 μ L of a 5 g/L solution of Nile Red was added to 1 mL of cell suspension. The solution was incubated at room temperature for approximately 5 min before being analyzed using UV microscopy.

2.6. Plasmid construction

A modified divergent GAL1-10 promoter was constructed from plasmid pRS169 (gift from Dr. P. Hieter) using the following PCR primers and *pfu* polymerase (Stratagene, La Jolla, CA): GAAGTGAATTCACCTTTGTAAACATCGATCTCATTTTTAT-TGAATT and CCGGTACAATTCGGGTCTCGACGTT-

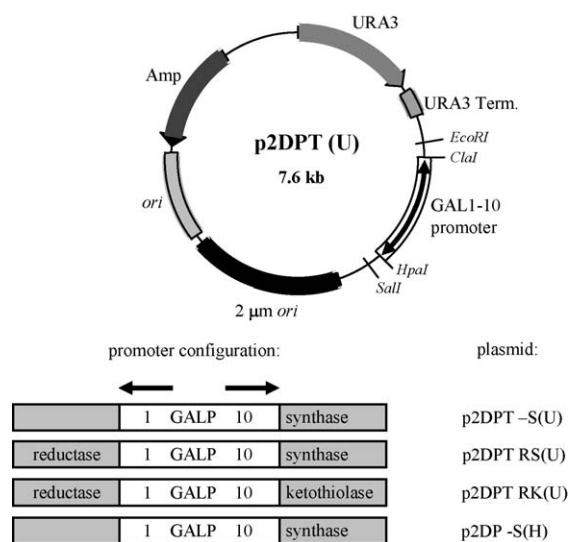


Fig. 1. High copy number plasmid system based on the divergent GAL1-10 promoter. p2DPT (U) is the backbone for all of the PHB plasmids except p2DP-S(H) which utilizes a HIS3 *S. cerevisiae* selection marker. The bar diagram illustrates the GAL1-10 promoter configuration for the listed plasmids.

AACTCTCCTT. The first primer introduces an *EcoRI* (GAATTC) and a *ClaI* (ATCGAT) restriction site while the second primer introduces a *SalI* (GTCGAC) and an *HpaI* (GTTAAC) restriction site (Fig. 1). The first primer introduces an ATG start site for the GAL10 promoter which results in the addition of three amino acids (Met-Arg-Ser) to the N-terminus of the protein. The GAL1 site does not contain an ATG site. The PCR product was digested with *SalI* and *EcoRI* and ligated into a similarly cut pRS306 (Sikorski and Hieter, 1989) yielding plasmid pDP306. The 'DP' designation refers to the 'divergent promoter.'

The 2.2 kb *EcoRI* fragment of the 2 μ m origin of replication was isolated from pLGSD5 (Guarente et al., 1982) and rendered blunt with Klenow DNA polymerase (Life Technologies, Rockville, MD). The origin was ligated into pRS303 (Sikorski and Hieter, 1989) which had been digested with *XhoI* and rendered blunt with Klenow DNA polymerase (Life Technologies, Rockville, MD). The 2 μ m origin was then cut out of the multicloning site using a *KpnI* and *EcoRI*. This fragment was ligated into pRS306 which was cut with the same restriction enzymes creating plasmid p2RS306. The URA3 transcription termination sequence was cut out of pRS169 using *SstI* and *EcoRI* and introduced

into p2RS306 using the same restriction enzymes creating p2RS306T. The '2' and 'T' refers to the '2 μ m origin of replication' and the 'URA3 transcription termination sequence.' The 2 μ m origin of replication has been reported to maintain plasmids in the range of 9–50+ copies per cell (Christianson et al., 1992; Rose and Broach, 1990).

The *Cupriavidus necator* (formerly known as *Wautersia eutropha*, *Ralstonia eutropha*, and *Alcaligenes eutrophus*) PHB reductase gene was isolated from pAeT41 (Peoples and Sinskey, 1989) using *pfu* polymerase (Stratagene, La Jolla, CA) and the following primers ATTATCGATGACTCAGCGCATTGCGTATGTG and ATTGAATTCAGCCCATATGCAGGCCGCCG which introduced a 5'-*ClaI* and a 3'-*EcoRI* restriction site, respectively. The PCR product was digested with *ClaI* and *EcoRI* and rendered blunt with Klenow DNA polymerase. The blunt insert was ligated into pDP306 which had been digested with *HpaI* in the presence of calf intestinal alkaline phosphatase (CIAP) (Life Technologies, Rockville, MD) creating plasmid pDP306 R. The reductase gene was cloned into p2RS306T with an *EcoRI/SalI* digest creating p2DPT R. The 2 μ m sequence also provides a termination sequence for the gene in the GAL1 cloning slot (Hitzeman et al., 1983).

The *C. necator* synthase gene was isolated from pAeT41 using *pfu* polymerase with the following primers ATTATCGATGGCGACCGGCAAAGGCGCGGC and GCCGAATTCATGCCTTGGCTTTGACGTATC which introduced a 5'-*ClaI* and a 3'-*EcoRI* restriction site. The PCR product was digested with *ClaI* and *EcoRI* and ligated into a *ClaI/EcoRI* digested pDP306 creating pDP306-S. Because of the methylation sensitive *ClaI* site, the synthase gene was isolated from pDP306-S with an *EcoRI* digest and a partial *AgeI* digest. The appropriate *AgeI/EcoRI* fragment was ligated into the GAL10 gene slot of p2DPT R which was prepared with an *AgeI/EcoRI* restriction digest. This construct was named p2DPT RS(U) (Fig. 1). The reductase gene was under regulation of the GAL1 promoter and is listed first, the synthase gene was under control of the GAL10 promoter and is listed second. 'U' refers to the RS306 uracil selection marker.

To construct a plasmid containing only the PHB synthase gene, pDP306-S was partially digested with *SalI* and *EcoRI* and the fragment harboring the synthase gene and the GAL1-10 promoter was

isolated and ligated into p2RS306T which was prepared with a *Sall*/*EcoRI* digest. The plasmid was named p2DPT – S(U). With this construct, the GAL1 gene slot was empty.

The *C. necator* beta-ketothiolase gene was isolated from plasmid pAet41 using the following PCR primers ATTATCGATGACTGACGTTGTCATCGTATC and TAAGAATTCTTATTTGCGCTCGACTGCCAG. The PCR primers introduced a 5'-*ClaI* and a 3'-*EcoRI* restriction site. The gene fragment was ligated into plasmid pDP306 using the *ClaI* and *EcoRI* restriction sites. The plasmid was named pDP306 K. The ketothiolase gene was isolated from pDP306 K using an *AgeI*/*EcoRI* digest and was ligated into a similarly digested p2DPT RS(U). The resulting plasmid was named p2DPT RK(U).

In order to express all three PHB genes in a single host, a plasmid containing the PHB synthase gene on a *HIS3* selection marker was constructed. Plasmid p2DPT – S(U) was digested with *ApaI* and *EcoRI*. The cassette containing the 2 μ m *ori*, the GAL1-10 promoter, and the PHB synthase gene, was ligated into a similarly digested pRS303 (Sikorski and Hieter, 1989). The resulting plasmid contains a *HIS3* selection marker and was named p2DP – S(H).

The functionality of the bi-directional promoter system was confirmed by expressing the reporter enzyme green fluorescence protein (GFP) either individually from the GAL1 or the GAL10 promoter or by co-currently expressing GFP from both sides of the promoter (data not shown).

3. Results

3.1. Recombinant 3-HB pathway effects

Recombinant *S. cerevisiae* accumulates low levels of PHB when the *C. necator* synthase gene is expressed indicating that the organism natively expresses genes involved in the synthesis of the PHB precursor R-3-hydroxybutyryl-CoA (3-HB) (Leaf et al., 1996). To address the effect of different levels of 3-HB synthesis pathway enzymes on PHB accumulation, different combinations of three *C. necator* PHB pathway genes were expressed from the divergent GAL1-10 promoter system. The inducible bi-directional promoter simplified cloning and co-expression of multiple genes in a

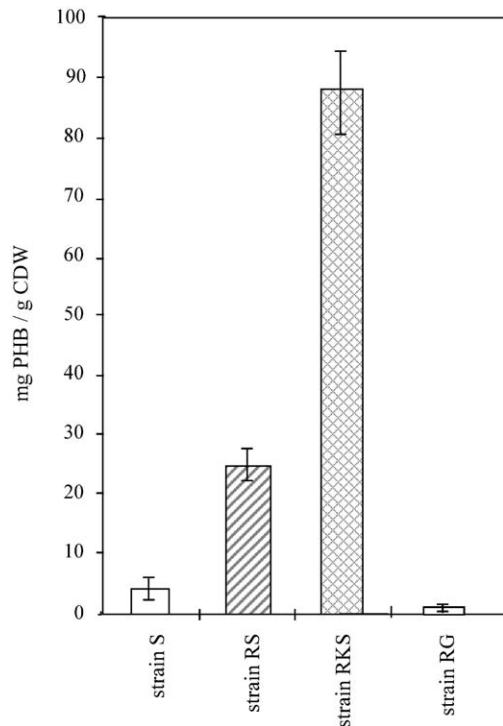


Fig. 2. 3-Hydroxybutyryl-CoA pathway gene dosage effect on specific PHB content in recombinant *S. cerevisiae* D603 cultures. Strain S: synthase only, strain RS: reductase and synthase, strain RKS: beta-ketothiolase, reductase, and synthase genes. Strain RG is a negative control strain expressing the reductase gene and the reporter gene GFP. Cultures were grown for approximately 4 days.

eukaryotic host from a single promoter sequence. The promoter system also permitted recombinant pathway expression independent of the native 3-HB pathway regulation.

The strain containing only the synthase gene, 'S' (plasmid p2DPT – S(U)), accumulated biopolymer to approximately 0.2% of its cell dry weight. When the PHB synthase and reductase genes were co-expressed, strain 'RS' (plasmid p2DPT RS(U)), PHB levels increased approximately 18 fold to about 3.5% of the cell dry weight. When all three PHB genes were expressed, strain 'RKS' (plasmids p2DPT RK(U) and p2DP – S(H)), PHB accumulation increased approximately 45 fold over strain S to about 9% of the cell dry weight (Fig. 2).

A control strain which co-expressed the reductase gene along with the reporter gene GFP from the divergent promoter system indicated the increase in

observed PHB levels was due to polymer and not accumulated precursor (Fig. 2). This experimental control was included because the propanolysis analytical method is not capable of distinguishing between polymer and intracellular 3-HB pools. Either metabolite would produce the same 3-HB-propyl ester although the monomer presumably can be eliminated by careful washing of the sample of permeabilized cells before esterification.

3.2. Kinetic studies

In bioreactor experiments the PHB synthesis kinetics for the three PHB accumulating strains was characterized. Strain S and strain RS were grown under uracil selection while strain RKS was grown under both uracil and histidine selection.

All three strains demonstrated some similar culturing trends as described in detail below. The profiles for strain RS are shown as a representative case (Fig. 3). Profiles for strain RKS can be found in Carlson et al. (2002). The specific growth rates, specific PHB production rates, volumetric PHB production rates, final specific PHB content, and final biomass concentrations for each of the three strains are summarized in Table 1.

Strain RS grew with a maximum specific growth rate of 0.24 h^{-1} for approximately 20 h before entering a slow growth phase (Fig. 3). Glucose was preferentially consumed. While strain D603 contains the *reg1-501* mutation (see Section 2), galactose was not significantly metabolized until after the glucose was exhausted. The ethanol levels increased until both sugars were depleted. At this point, the ethanol level decreased likely due to a combination of diauxic growth and evaporation in the sparge gas (Nissen et al., 1997).

PHB production seems to occur with the culture entering the slow growth phase which coincides with glucose exhaustion. During this phase, the residual biomass increased by approximately 1.5 g/L. The specific PHB levels increased with a nearly linear trend for about 20 h with a maximum specific production rate of 1.18 mg PHB/g residual biomass/h. During this time, galactose is exhausted but the nearly linear specific accumulation of PHB continues for approximately another 10 h. The linear PHB accumulation after the exhaustion of both sugars suggests PHB is synthesized from metabolic by-products like ethanol or acetate (Oura, 1977). This is similar to the accumulation patterns seen in a recombinant PHB producing *P. pastoris* strain (Vijayasanakaran et al., 2005). The GAL1-10 promoter is tightly regulated by galactose (Johnston,

Table 1
Summary of bioreactor rates, final specific PHB content, final biomass concentration and product yields

Strain	Rates			Final PHB content (% CDW)	Final cell mass (g/L)
	Specific growth rate (h^{-1})	Specific PHB rate (mg PHB/g residual biomass/h)	Volumetric PHB rate (mg PHB/L/h)		
S	0.23	0.06	0.16	0.2	3.4
RS	0.24	1.18	2.79	3.8	3.6
RKS	0.19	2.45	4.27	7.5	3.6
RKS (anaerobic)	0.11	0.37	0.19	3.5	0.6
Strain	Yields				
	g biomass/g glc	g EtOH/g glc	g EtOH/g gal	g PHB/g EtOH	
S	0.17	N.D.	N.D.	N.D.	
RS	0.16	0.32	0.3	0.033	
RKS	0.15	0.33	0.25	0.051	
RKS (anaerobic)	0.05	0.35	0.32	0.002 ^a	

The various parameters were calculated from three different recombinant *S. cerevisiae* cultures containing either the PHB synthase gene (strain S), the PHB reductase and synthase genes (strain RS) or the PHB beta-ketothiolase, reductase, and synthase genes (strain RKS). The strains were grown at pH 4.5, 30 °C, and 300 RPM on defined medium with 1% glucose and 1% galactose used as the carbon sources.

^a PHB yield based on galactose.

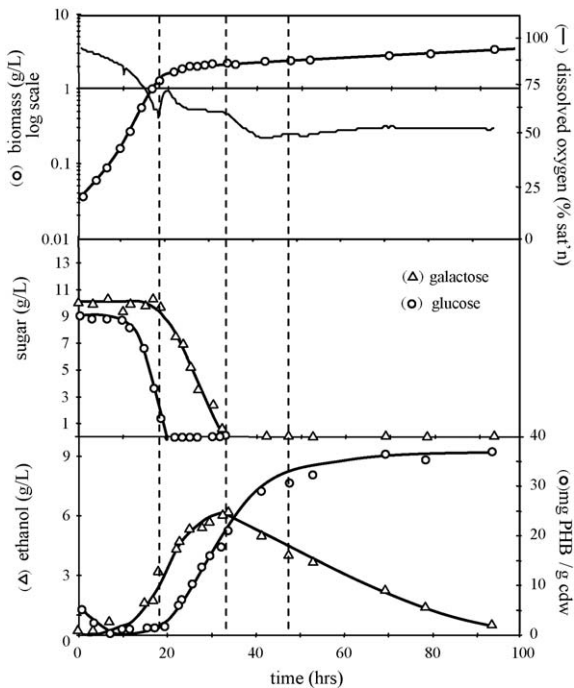


Fig. 3. Bioreactor time profiles from recombinant *S. cerevisiae* D603 strain RS expressing PHB reductase and synthase from a high copy number vector (p2DPT RS(U)). The culture was sparged with air (1VVM) and the pH was controlled at 4.5. The vertical dotted lines high light the boundaries between the four different phases (see text for details).

1987) and likely stopped expressing the PHB genes after the depletion of the galactose. The linear accumulation of PHB for 10 h after the exhaustion of galactose suggests either the recombinant mRNA or, more likely, the recombinant enzymes are quite stable in *S. cerevisiae*. If these products were unstable, PHB accumulation would be expected to cease immediately after the exhaustion of galactose.

The culture seems to go through four distinct phases. These four distinct phases were seen with all three tested strains S, RS, and RKS. During the first phase, the culture grew exponentially on glucose. When glucose is exhausted, the culture enters a second phase characterized by utilization of galactose, slowed growth and the start of PHB production. The third stage starts after galactose is exhausted. This phase involves decreasing levels of ethanol, little growth, and continued PHB accumulation. The fourth phase begins with the slowing of PHB accumulation and lasts until

the end of the experiment. Events during these phases like the depletion of glucose seem to correspond to trends in the dissolved oxygen profiles. The yields from Table 1 were calculated from different phases. Biomass yields from glucose and ethanol yields from glucose were determined during phase one. Ethanol yields from galactose were determined during phase two and PHB yields from ethanol were determined from phase three.

While all three PHB accumulating cultures go through the same four phases described above, the PHB synthesis kinetics and the overall PHB accumulation are quite different. The specific PHB production rates for *S. cerevisiae* expressing three *C. necator* PHB genes from two high copy number plasmids is about 2.45 mg PHB/g residual biomass/h compared to 1.18 mg PHB/g residual biomass/h for strain RS and 0.06 mg PHB/g residual biomass/h for strain S (see Table 1). The fraction of plasmid containing cells for strains S and RS was approximately 80% at the beginning of the PHB accumulation phase and fell to approximately 60% at the end of the experiment. Approximately 70% of strain RKS cells contained both plasmids at the beginning of the PHB accumulation phase. This number fell to about 45% at the end of the experiments. During aerobic studies in shake flasks and reactors, the final, population averaged, PHB content for strain RKS ranged from approximately 1 to 12% of the cell dry weight (average = 4.9%, S.D. = 2%, $n = 58$). Possible explanations for the observed range of PHB levels are given in Section 4.

3.3. Anaerobic PHB synthesis

The feasibility of anaerobic co-production of PHB and ethanol in a recombinant *S. cerevisiae* was predicted using a biochemical network model (Carlson et al., 2002). Anaerobic PHB production in *S. cerevisiae* offers a number of potentially interesting advantages like process simplification by removing the need for aeration. Co-producing other potentially valuable products could be an interesting strategy for both fuel ethanol and PHB production.

Prior to inoculation, the reactor contents were sparged with nitrogen gas to remove the dissolved oxygen. During cultivation, the reactor was sparged with 50 mL/min of nitrogen gas to maintain anaerobic conditions and positive reactor pressure. The cells grew exponentially with a maximum growth rate of

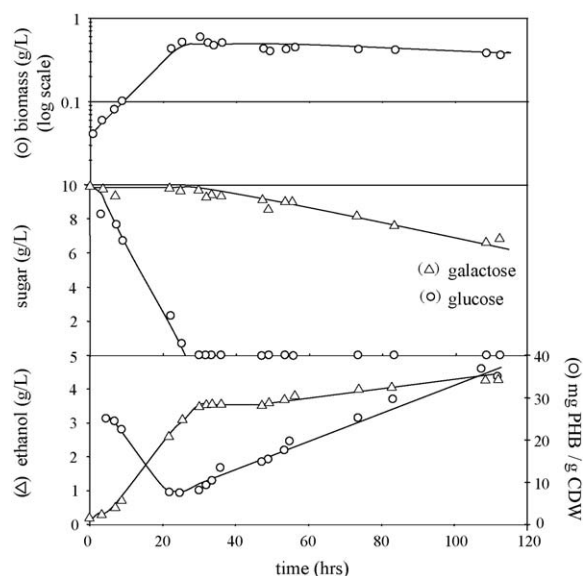


Fig. 4. Bioreactor time profiles for anaerobically grown recombinant *S. cerevisiae* D603 strain RKS expressing PHB synthase, reductase, and beta-ketothiolase (plasmids p2DPT RK(U) and p2DP – S(H)). The pH was controlled at 4.5.

approximately 0.11 h^{-1} . The yeast culture entered stationary phase after the depletion of glucose. This point also corresponds to the initial cessation of ethanol production (Fig. 4). Galactose was slowly consumed after the depletion of glucose. Ethanol is again produced during stationary phase as PHB is synthesized. Final ethanol concentrations reached about 4.5 g/L. The population averaged specific PHB production rate was $0.37 \text{ mg PHB/g CDW/h}$. The average specific PHB content reached roughly 3.5% of the cell dry weight. Experiment-to-experiment variation in final specific PHB content for anaerobic conditions in shake flasks and reactor experiments ranged from approximately 1–7% of the cell dry weight (average = 2.4%, S.D. = 1.5%, $n = 28$). Possible explanations for this heterogeneity are proposed in Section 4. Anaerobic PHB synthesis has also been recently described in recombinant *E. coli* (Carlson et al., 2005).

3.4. Microscopic analysis

Cells from strain RKS were stained with Nile Red and studied with fluorescence microscopy (Fig. 5). The

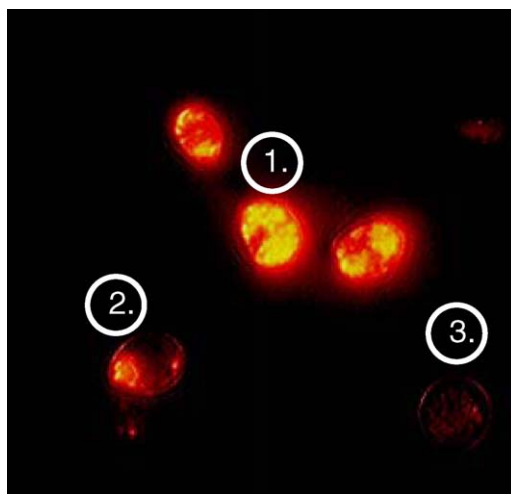


Fig. 5. Nile Red stained *S. cerevisiae* D603 strain RKS expressing the PHB synthase, reductase, and beta-ketothiolase. Nile Red is a hydrophobic dye that localizes to hydrophobic bodies like PHB. The picture illustrates the population heterogeneity. Some cells appear to have accumulated PHB in excess of 50% of their volume while other cells did not synthesize any PHB. Cell 1: high PHB content; Cell 2: low PHB content; Cell 3: no PHB. Control cultures, which do not express the PHB pathway genes, grown under the same conditions appear very homogeneous with all of the cells appearing like Cell 3 in the above micrograph.

staining showed significant heterogeneity in the PHB content under both aerobic and anaerobic conditions. As estimated from the volume occupied by PHB inclusions some cells appeared to contain in excess of 50% PHB while a significant fraction of the cells had no PHB. These results demonstrate that *S. cerevisiae* is capable of accumulating PHB to levels similar to some bacterial systems both aerobically and anaerobically. The heterogeneity is likely due to a number of factors including the cell-to-cell variation in plasmid copy number, cell age, and plasmid recombination. Quantitative single cell PHB data have been recently obtained in a study utilizing an automated staining and flow cytometry system to examine population heterogeneity in native and recombinant PHB production systems (Kacmar et al., 2005). Section 4 below includes additional comments on the observed heterogeneity. Due to the pronounced population heterogeneity and the anticipated small size of the intracellular metabolite pools, much smaller than the measured PHB levels, we have not attempted to determine the population-averaged,

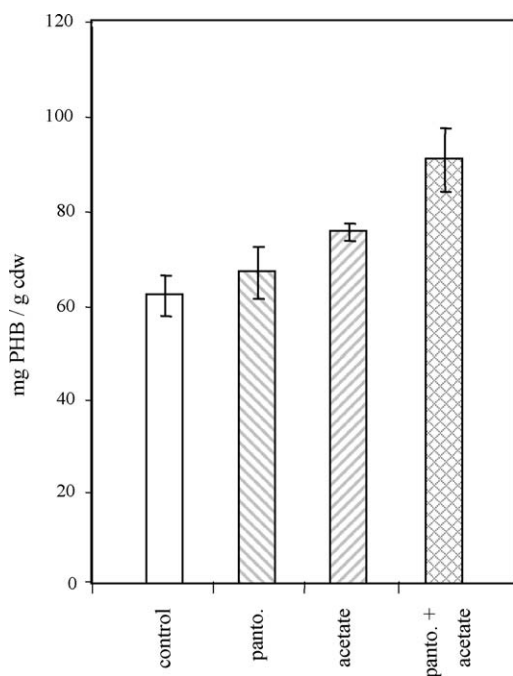


Fig. 6. Acetate and pantothenate shake flask experiment with *S. cerevisiae* D603 strain RKS. Acetate (0.5 g/L) and coenzyme A precursor pantothenate (1000 mg/L) were added either separately or together to glucose and galactose containing medium. The pH of the acetate solution was adjusted to 4.5 before adding to the medium. Cultures were grown for approximately 4 days.

intracellular concentrations of the PHB precursor concentrations.

3.5. Medium supplements

The feeding of acetyl-CoA precursors was tested as a means of increasing polymer accumulation. Acetate (0.5 g/L, pH adjusted to 4.5) and the coenzyme A precursor pantothenate (1000 µg/L) individually and together improved PHB production when added to the minimal medium containing glucose and galactose (Fig. 6). The supplements were added at the beginning of the experiment. Feeding a combination of acetate and pantothenate improved PHB production by approximately 45% over the control strain. Acetate concentrations higher than 0.5 g/L were inhibitory to cell growth.

Similarly, the medium pH was examined for its effect on PHB accumulation. The use of a citrate buffer (50 mM) had a strong negative effect on poly-

mer accumulation. The citrate buffer, regardless of the pH, reduced accumulated PHB levels to approximately 50–60% of the non-buffered medium levels. While *S. cerevisiae* is not able to grow on externally supplied citrate (Barrett, 1997), it appears citrate still exerts a strong effect on the cellular metabolism. This is potentially due to tight regulation of intracellular citrate concentrations. The condensation of acetyl-CoA and oxaloacetate via citrate synthase is a major citrate synthesis route in yeast. If the intracellular levels of citrate are high from the buffer, the cells may downregulate acetyl-CoA levels which could adversely affect PHB accumulation. Bioreactor experiments carried out without pH control had specific PHB levels comparable to cultures grown with pH control even though the culture pH dropped to approximately 2.6.

Unlike many native PHB accumulating organisms (Dawes and Senior, 1973), limiting nitrogen availability did not lead to elevated levels of polymer accumulation. Limiting nitrogen reduced PHB accumulation likely because of reduced recombinant protein production. It has been suggested that growth on nitrate as a nitrogen source would require a highly active NADPH production system (Bruinenberg et al., 1983). Since elevated NADPH levels have been implicated in enhanced PHB accumulation (Dawes and Senior, 1973), nitrate was analyzed as a possible nitrogen source for growth and PHB synthesis. However, the recombinant strains showed little to no growth on nitrate and under these conditions, PHB synthesis was severely inhibited.

4. Discussion

S. cerevisiae expressing the *C. necator* PHB synthase gene accumulates low levels of the biopolymer PHB (Leaf et al., 1996). In the current study, a bidirectional GAL1-10 gene expression system was used to augment the native genes which produce the PHB synthase enzyme substrate R-3-hydroxybutyryl-CoA. Augmenting the 3-HB pathway improved both the PHB synthesis rate and total accumulated levels of biopolymer by approximately 40 fold. In addition, the anaerobic co-production of PHB and ethanol was established. Microscopic analysis of the recombinant strain expressing three PHB genes demonstrated that *S. cerevisiae* is capable of accumulating biopolymer to levels

similar to some bacterial systems. The existence of relatively simple gas chromatography analytical techniques (Riis and Mai, 1988) and of fluorescent single cell assays also makes this acetyl-CoA derived biopolymer an attractive tool for studying host metabolisms by permitting the probing of in vivo intracellular metabolite pools.

Cells typically have a variety of regulatory mechanisms that work to maintain intracellular homeostasis (Fell, 1998). Critical biochemical intermediates from the central metabolism are often supported by flexible regulatory networks that work to maintain desirable intracellular pools. The recombinant pathways in the three PHB producing strains each tapped a different combination of metabolite pools for biopolymer production. For instance, the precursor supplied by the native *S. cerevisiae* gene(s) for strain S is R-3-hydroxybutyryl-CoA. The exact role of this compound in *S. cerevisiae*'s metabolism has not been determined, however, a role in fatty acid synthesis or β -oxidation has been hypothesized (Leaf et al., 1996). The intracellular pool of this metabolite is likely small and the regulatory mechanisms which maintain this pool have probably not evolved to handle large perturbations. The PHB synthase enzyme in strain RS, is capable of handling a flux at least 18 times greater than what is supplied by the 3-HB metabolite pool in strain S (see Table 1). If Michaelis–Menten kinetics are assumed for the synthase enzyme (Leaf and Srienc, 1998), the 18 fold increase in flux could be explained as a 18 fold increase in precursor concentration (first-order region of saturation kinetics), an 18 fold increase in synthase enzyme concentration (zeroth order region of saturation kinetics), or a combination of the two. Since the synthase enzyme is expressed from the same promoter and same selection marker in both strain S and RS, any increase in enzyme levels is unlikely much less an 18 fold increase. This suggests the different fluxes are based on different metabolite pool sizes and that PHB synthesis in strain S is limited by 3-HB levels. This proposed explanation is consistent with the theoretical predictions of recombinant system behavior made by Fell (2005). Strain RS draws on both the 3-HB and the acetoacetyl-CoA metabolite pools. Examination of the data in Table 1 suggests PHB synthesis in strain RS is limited by acetoacetyl-CoA levels because expression of the ketothiolase gene further increases PHB accumulation by approximately twofold. When all three

PHB genes are expressed, three different metabolite pools are drawn on for flux through the recombinant pathway; the R-3-hydroxybutyryl-CoA pool, the acetoacetyl-CoA pool and the cytosolic acetyl-CoA pool.

Because of its central metabolic role, the regulatory mechanisms that maintain the cytosolic acetyl-CoA concentration are probably better capable of handling perturbations like the flux of carbon toward PHB than the other PHB precursor pools. Although the fact that acetate and pantothenate feeding increased PHB accumulation (Fig. 5) suggests that in strain RKS, acetyl-CoA levels may play a role in limiting biopolymer synthesis.

Population heterogeneity is a well known biological phenomenon and can have several different origins (for instance: Spudich and Koshland, 1976; Elowitz et al., 2002). This is especially true in engineered systems that often suffer from genetic instabilities. Because eukaryotes do not typically express polycistronic messages, this heterogeneity is further compounded when multiple recombinant genes are expressed from multiple plasmids. Potential sources of heterogeneity in the current study include varying copy number/stability of the two plasmids, recombination of the two plasmids both of which are based on pBLUESCRIPT backbones (Sikorski and Hieter, 1989), cell-to-cell differences in promoter expression levels, differences in cell age, cross feeding of auxotrophic compounds between plasmid containing and plasmid free cells, and numerous other stochastic events (McAdams and Arkin, 1997). It is the view of the authors that this heterogeneity is not often reported nor appreciated in many genetically engineered systems. Knowledge of this data highlights the limitations of population-averaged data that is often reported and that is difficult to interpret (Kacmar et al., 2004).

When the fraction of plasmid containing cells is factored into the specific PHB production rates, the difference between strain RKS and the other strains is magnified. When the specific rate for strain RKS is adjusted for plasmid stability, the rate increases to 3.50 mg PHB/g residual biomass/h. Adjusting the specific rates for plasmid stability increases strain RS's rate to 1.45 mg PHB/g residual biomass/h and strain S's rate to 0.08 mg PHB/g residual biomass/h. The plasmid stability adjusted specific PHB production rate for strain RKS is approximately 2.4 times faster than strain RS

and approximately 44 times faster than strain S. A typical PHB production rate for *C. necator* metabolizing fructose is 33 mg PHB/g residual biomass/h (Kelley and Srienc, 1999). The plasmid stability adjusted specific PHB accumulation rate for *S. cerevisiae* strain RKS is therefore about 10% of the *C. necator* rate.

We acknowledge that the population heterogeneity presents a challenge in the data interpretation. However, certain conclusions are clear from the presented results. These conclusions are that biopolymer levels can be increased by augmenting the native 3-HB synthesis pathway(s), that the organism is capable of accumulating significant amounts of polymer, that biopolymer levels can be increased with substrate feeding schemes, and that the biopolymer can be accumulated under anaerobic conditions with the co-production of ethanol. The requirement of galactose for promoter induction and the population heterogeneity complicate the analysis however this strain can be interpreted as a 'proof of concept' strain. A number of additional expression systems were explored which increased gene stability by using more stable centromeric/autonomous replicating sequence (CEN/ARS) origins of replication or by using integrative plasmids. However, these systems all accumulated less PHB than the presented expression system likely due to reduced expression levels from the low gene copy number (data not shown). Expression systems based on the PGK, TEF1 and TEF2 promoter were tested because they would theoretically provide strong expression levels from integrated gene constructs and because they did not require galactose. However, the systems also produced significantly less PHB than the presented GAL1-10 system (data not shown). Although, the TEF based systems have been successfully utilized to study GFP expression and to produce medium chain polyhydroxyalkanoate utilizing the *Pseudomonas oleovorans* polymerase (Kacmar et al., 2004; Zhang et al., 2005).

A recombinant strain of the industrially important organism *S. cerevisiae* was capable of accumulating high amounts of the biopolymer PHB under both aerobic and anaerobic conditions. Stabilizing the recombinant genes and developing feeding schemes with metabolites like acetate and pantothenate are expected to significantly improve the population averaged PHB content in this promising biotechnological platform.

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