

# Production of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by *Ralstonia eutropha* in High Cell Density Palm Oil Fermentations

Sebastian L. Riedel,<sup>1</sup> Johannes Bader,<sup>1,6</sup> Christopher J. Brigham,<sup>1</sup> Charles F. Budde,<sup>2</sup> Zainal Abidin Mohd Yusof,<sup>7</sup> ChoKyun Rha,<sup>3</sup> Anthony J. Sinskey<sup>1,4,5</sup>

<sup>1</sup>Bldg. 68-370, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139; telephone: (617) 253-6721; fax: (617) 253-8550; e-mail: asinskey@mit.edu

<sup>2</sup>Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

<sup>3</sup>Biomaterials Science & Engineering Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts

<sup>4</sup>Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts

<sup>5</sup>Engineering Systems Division, Massachusetts Institute of Technology, Cambridge, Massachusetts

<sup>6</sup>Department of Applied and Molecular Microbiology, Technische Universität Berlin, Berlin, Germany

<sup>7</sup>Research and Technology Division, SIRIM Berhad, Shah Alam, Malaysia.

Received 29 May 2011; revision received 6 July 2011; accepted 22 July 2011

Published online 1 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23283

**ABSTRACT:** Improved production costs will accelerate commercialization of polyhydroxyalkanoate (PHA) polymer and PHA-based products. Plant oils are considered favorable feedstocks, due to their high carbon content and relatively low price compared to sugars and other refined carbon feedstocks. Different PHA production strategies were compared using a recombinant strain of *Ralstonia eutropha* that produces high amounts of P(HB-co-HHx) when grown on plant oils. This *R. eutropha* strain was grown to high cell densities using batch, extended batch, and fed batch fermentation strategies, in which PHA accumulation was triggered by nitrogen limitation. While extended batch culture produced more biomass and PHA than batch culture, fed batch cultivation was shown to produce the highest levels of biomass and PHA. The highest titer achieved was over 139 g/L cell dry weight (CDW) of biomass with 74% of CDW as PHA containing 19mol% HHx. Our data suggest that the fermentation process is scalable with a space time yield (STY) better than 1 g PHA/L/h. The achieved biomass concentration and PHA yield are among the highest reported for the fermentation of recombinant *R. eutropha* strains producing P(HB-co-HHx).

Biotechnol. Bioeng. 2012;109: 74–83.

© 2011 Wiley Periodicals, Inc.

**KEYWORDS:** *Ralstonia eutropha*; Polyhydroxyalkanoate; Poly(hydroxybutyrate-co-hydroxyhexanoate); plant oil; high cell density fermentation; palm oil

## Introduction

Today, the majority of industrial plastic production is dependent on fossil-based petroleum. To reduce this dependency, new polymer production processes based on alternative substrates must be developed. Polyhydroxyalkanoate (PHA) is a microbially produced polyester used for carbon and energy storage. PHA can be used effectively as a biodegradable and biocompatible alternative to petroleum-based plastic (Haywood et al., 1990; Steinbüchel et al., 1992; Sudesh et al., 2000). One key motivation for the migration to biodegradable plastic is the increasing accumulation of non-biodegradable waste in the environment, which has recently been reviewed (Barnes et al., 2009; Ryan et al., 2009). PHA already has a wide variety of applications. Traditionally, PHA has been used to produce everyday items such as packing material or containers for storage of household

Correspondence to: A.J. Sinskey

products (Philip et al., 2007). Because of its biocompatibility and ability to degrade to non-toxic compounds in the human body, PHA is used in tissue engineering as a scaffold material (Chen and Wu, 2005; Williams et al., 1999; Zhao et al., 2002). As a biofuel, methyl-esters derived from PHA monomers have been shown to have potential as transportation fuels (Zhang et al., 2009).

A summary of 24 PHA-producing companies offered by (Chen, 2009) demonstrates the global interest in production of these polymers. It is expected that lower production costs will help accelerate further commercialization of PHA. For low-cost industrial PHA production, high space time yields (STY) are required. Optimal fermentation processes would be based on either renewable carbon substrates with a high yield per hectare or inexpensive secondary products. Tian et al. (2009), review the utilization of waste water, whey, molasses, and various plant oils as carbon substrates for PHA production. The advantage of utilizing plant oils is their high carbon content as well as high conversion rate to PHA (Akiyama et al., 2003; Fukui and Doi, 1998; Kahar et al., 2004; Loo et al., 2005; Ng et al., 2010). Because of their high carbon content, low flow rate feed streams can be applied, reducing the dilution of the fermentation broth and optimizing product concentration. Compared with all other oilseed plants, the production of palm oil offers the highest yield per area with an average oil production of 4 metric tons per hectare (Basiron, 2007).

The  $\beta$ -proteobacterium *Ralstonia eutropha* is known as the model organism for PHA production, mainly because it can store high amounts of PHA under nutrient limitation in the presence of ample carbon source (Haywood et al., 1990; Reinecke and Steinbüchel, 2009). The components of PHA are mainly divided in short chain length (SCL) monomers with 3–5 carbon atoms and medium chain length (MCL) monomers with six or more carbon atoms (Rehm, 2003). *R. eutropha* strain H16 can store up to 90% of its cell dry weight (CDW) as polyhydroxybutyrate (PHB), a polymer consisting of only SCL monomers (Hänisch et al., 2006; Uchino and Saito, 2006). However, PHA copolymers consisting of both SCL and MCL monomers are better suited for replacement of petroleum-based plastics than PHB due to certain enhanced properties, such as flexibility and ease of processing (Noda et al., 2005b). These enhanced properties are partially dependent on the MCL monomer concentration of the copolymer, which regulates variables such as melting temperature and crystallinity (Noda et al., 2005a). It has been shown that the copolymer poly(hydroxybutyrate-co-hydroxyhexanoate) (P(HB-co-HHx)) with a high HHx level of 17 mol% has similar properties to low-density polyethylene (LDPE) (Doi et al., 1995).

We recently described the recombinant *R. eutropha* strain Re2058/pCB113, which was engineered from *R. eutropha* H16 (ATCC 17699) and which produces P(HB-co-HHx) when grown on palm oil as the sole carbon source (Budde et al., 2011b). In the work presented here, the optimization of the fermentation conditions of Re2058/pCB113 with palm oil as the sole carbon substrate is discussed.

## Materials and Methods

### Bacterial Strain

Experiments were performed with the recombinant *R. eutropha* strain Re2058/pCB113, which was engineered from strain H16 (ATCC 17699) and produces P(HB-co-HHx) when grown on plant oils (Budde et al., 2011b).

### Growth Media and Preculture Cultivation Conditions

Dextrose-free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD) was used for overnight cultivations. To ensure maintenance of the plasmid pCB113 in rich media, TSB medium was always supplemented with 200  $\mu$ g/mL kanamycin sulfate. The addition of kanamycin for plasmid maintenance in minimal media cultures was unnecessary, due to the presence of an addiction system based on proline auxotrophy (Budde et al., 2011b). All growth media contained 10  $\mu$ g/mL gentamicin sulfate. Phosphate buffered minimal medium used for precultures and fermentations was described previously (Budde et al., 2010). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

The carbon sources fructose or palm oil (PO, Wilderness Family Naturals, Silver Bay, MN) and nitrogen sources (ammonium chloride, ammonium hydroxide, or urea) were all used as described in the text. Cultures were always grown aerobically at 30°C, and nitrogen limitation was used to trigger maximum PHA production.

Re2058/pCB113 was initially grown overnight in 4 mL TSB from a single colony. Cells were centrifuged at 16,100g and the pellet was resuspended in 0.85% saline and used to inoculate 50 or 100 mL minimal medium flask cultures containing 2% fructose and 0.1% NH<sub>4</sub>Cl or 0.06% urea to an initial OD<sub>600</sub> of 0.05. After approximately 24 h of incubation, cells were centrifuged at 6,500g and the pellet was resuspended in 0.85% saline for inoculation of the fermenter culture to an initial OD<sub>600</sub> of 0.1.

### General Fermentation Conditions

A Bioengineering multiple fermenter system (R'ALF PLUS TRIO) consisting of 2 L double jacketed glass vessels with a working volume of 1.2 L (Wald ZH/Switzerland) was used for fermentation studies. The temperature of the cultures was kept constant at 30°C, and the pH was maintained at 6.8  $\pm$  0.1, through controlled addition of 2 M NaOH (NH<sub>4</sub>OH was used for pH controlled nitrogen feeding) and 0.67 M H<sub>3</sub>PO<sub>4</sub>. Cultures were stirred using two six-blade Rushton impellers at speeds ranging from 300–1,500 rpm. Air was supplied through a ring sparger at 0.5 vvm unless noted otherwise. The dissolved oxygen concentration was maintained at levels above 40% by addition of pure oxygen and kept at a constant flow rate by a mass flow controlled pO<sub>2</sub> cascade. Foam in the cultures was broken

mechanically with pairs of cable ties attached to the shaft of the impeller.

### Extended Batch Fermentation

Cultures consisting of 1 L minimal medium with 40 g/L PO and 4.5 g/L urea (150 mM nitrogen) were inoculated from precultures as described above. A bolus of 20 g/L PO, based on initial culture volume, was fed to the fermenter after 32 h of cultivation. Air was supplied at 0.2–1 vvm during fermentation.

### Fed Batch Fermentation (NH<sub>4</sub>OH), With pH Controlled Nitrogen Feeding

Fermenter cultures containing 1 L of minimal medium with 20 g/L PO and 4 g/L NH<sub>4</sub>Cl (75 mM nitrogen) were inoculated with precultures, as described above. During cultivation, PO was fed between 24–84 h in approximately 5 g/L steps, pulse-wise with a pump in 2 h intervals, to a total concentration of approximately 170 g/L, based on initial volume. For initial pH control and nitrogen feeding, a calculated volume of a stock solution of 14% (w/v) NH<sub>4</sub>OH was provided to the culture, from the base reservoir of the fermenter, to reach a total nitrogen concentration of 480 mM nitrogen, based on initial volume. After the NH<sub>4</sub>OH solution was depleted, the pH control was switched to 2 M NaOH in order to initiate nitrogen limitation. After 48 h cultivation, a solution consisting of trace metal salts (Budde et al., 2010), magnesium sulfate, calcium chloride, and potassium sulfate was added in initial concentrations to prevent undesired nutrient limitation.

### Fed Batch Fermentation (urea)

Cultures consisting of 1 L minimal medium with initial concentrations of 20 g/L PO and 2.2 g/L urea (75 mM nitrogen) were inoculated with precultures as described above. PO was fed to the fermenter culture as described above. Urea was fed in approximately 0.2 g/L steps (7 mM nitrogen) in 30 min intervals, starting at 18 h, until a total concentration of 14.4 g/L urea (480 mM nitrogen) was reached based on initial volume. After 48 h cultivation, a solution consisting of trace metal salts (Budde et al., 2010), magnesium sulfate, calcium chloride, and potassium sulfate was added in initial concentrations to prevent undesired nutrient limitation.

### Analytical Methods

Aliquots of 3–14 mL from fermenter cultures were sampled in pre-weighed polypropylene test tubes. The samples were centrifuged for 10–15 min at 6,500g and 1 mL of the supernatant was frozen at –20°C. The pellets were washed with a mixture of 5 mL cold water and 2 mL cold hexane to

remove residual oil. The washed cell pellet was then re-suspended in 2 mL cold water, frozen at –80°C, lyophilized and then the CDW was determined. The content and composition of PHA from dried cells were determined using a methanolysis protocol described previously (Budde et al., 2011b). In this procedure, pure standards of methyl 3-hydroxybutyrate and methyl 3-hydroxyhexanoate were used to generate calibration curves. Residual cell dry weight (RCDW) was defined as CDW minus the mass of the PHA.

For molecular weight determinations of P(HB-*co*-HHx), the polymer was extracted from freeze-dried cells with chloroform. Equal masses of freeze-dried cells were weighed in screw capped glass tubes to form a 3 mg/mL PHA solution with a total volume of 2 mL. Samples were kept at 50°C for 4 h and briefly shaken by hand every 30 min. After PHA extraction, the samples were filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane and the molecular weight was measured via gel permeation chromatography (GPC) relative to polystyrene standards as described previously (Budde et al., 2010).

For testing the nitrogen content of cell supernatants, frozen culture supernatants were thawed at room temperature, centrifuged for 5 min at 16,100g, and filtered through a 0.2 μm PTFE membrane (if necessary). Ammonium and urea concentrations were measured from clarified supernatants with an ammonium assay kit (Sigma-Aldrich, Cat. No. AA0100) or a urea assay kit (BioVision, Cat. No. #K375-100), respectively, as defined in the text.

Lipids were extracted from the culture by using a mixture of chloroform/methanol (2:1, v/v) as previously described (Budde et al., 2011a). Samples for lipid recovery were taken prior to any PO feeding steps at a given time point. The distribution of fatty acids in the recovered lipids was determined by the same methanolysis assay described above, which leads to formation of fatty acid methyl esters. The species and proportion of fatty acids in the lipids recovered from the chloroform/methanol extraction were identified by thin layer chromatography (TLC). The extracted lipids were dissolved in chloroform to a final concentration of 3 mg/mL. Aliquots of 10 μL (30 μg lipids) were spotted on a silica gel TLC plate (250 μm thickness; EMD Chemicals, Gibbstown, NJ). A mixture of defined standards was also spotted: Triacylglycerol (TAG, 1,2-distearoyl-3-oleoyl-*rac*-glycerol; 10 μg), diacylglycerol (DAG, 1,2-dipalmitoyl-*rac*-glycerol; 20 μg), monoacylglycerol (MAG, 1-palmitoyl-*rac*-glycerol; 20 μg) (Nu-check Prep, Inc., Elysian MN), and free fatty acid (FFA, palmitic acid; 10 μg). TLC was then performed as described previously (Budde et al., 2011a).

### Results

The polymer P(HB-*co*-HHx) has been shown to exhibit thermal and mechanical properties that rival those of petroleum-based polymers (Doi et al., 1995; Noda et al., 2005b). Our group is interested in the production of high concentrations of P(HB-*co*-HHx) in fermentation cultures

using plant oils as the sole carbon source, with high yields of PHA from oil. The production of P(HB-co-HHx) containing a high concentration ( $\geq 15\text{mol}\%$ ) of HHx monomers is a novel undertaking, as such polymers have not been produced in high quantities thus far by microorganisms grown on raw carbon sources. We have recently described the engineered *R. eutropha* strain Re2058/pCB113, which accumulates high amounts of PHA per CDW with a high HHx level when grown on plant oils. In that work, we demonstrated the strain's performance in a batch fermentation in which it produced 25 g/L CDW with a PHA content of 71% with 17 mol% HHx after 96 h of cultivation in minimal medium containing 40 g/L PO as the carbon source and 4 g/L  $\text{NH}_4\text{Cl}$  (75 mM nitrogen) as the nitrogen source (Budde et al., 2011b).

In this study, to further enhance PHA production, we evaluated different fermentation strategies with Re2058/pCB113 to increase biomass concentrations, which allows for higher PHA titers.

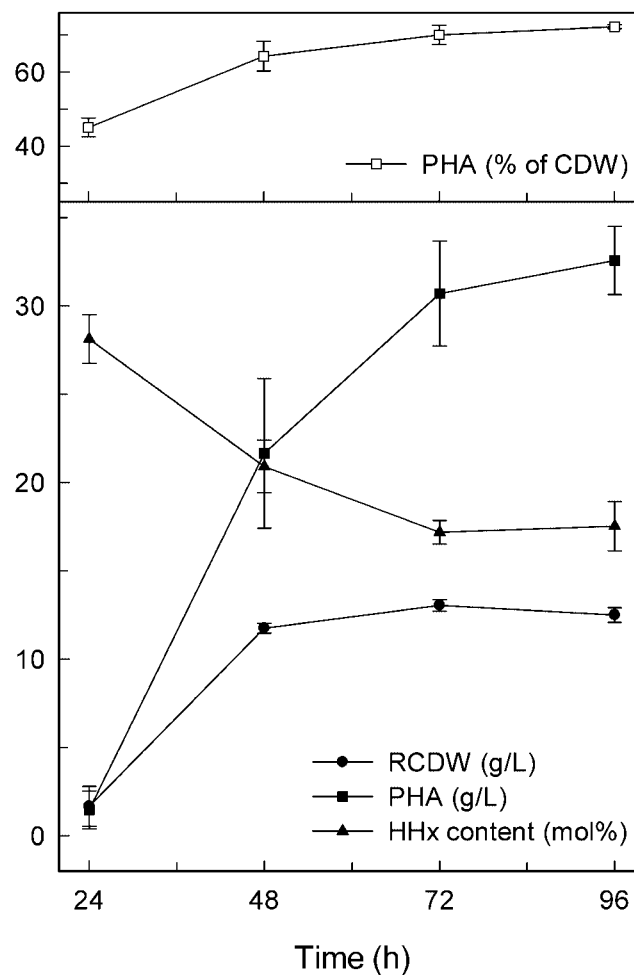
### Extended Batch Fermentation

To begin to optimize the yield of PHA produced by *R. eutropha* Re2058/pCB113, an extended batch fermentation was performed with a two fold increase in the culture's total nitrogen concentration (4.5 g/L urea, 150 mM nitrogen) compared to the batch fermentation described previously (Budde et al., 2011b). Urea was used as nitrogen source instead of  $\text{NH}_4\text{Cl}$ , as urea is a less costly nutrient and allows for better growth of *R. eutropha* (Khanna and Srivastava, 2004; Ng et al., 2010). The total PO concentration of the culture was increased by 1.5-fold to 60 g/L. In the extended batch fermentation, it was observed that the PHA content was already 45% of CDW at 24 h (Fig. 1), before the culture had reached nitrogen limitation. After 48 h, nitrogen became limiting, resulting in a constant residual biomass and allowing for maximum PHA production. After 96 h, cultures had produced 32.5 g/L PHA (72% of CDW) with an HHx level of 17 mol% (Fig. 1). Over the entire fermentation, polymer was formed at a yield of 0.52 g PHA/g PO. During 48–96 h the PHA yield increased to 0.77 g PHA/g PO.

### Fed Batch Fermentation ( $\text{NH}_4\text{OH}$ ), With pH Controlled Nitrogen Feeding

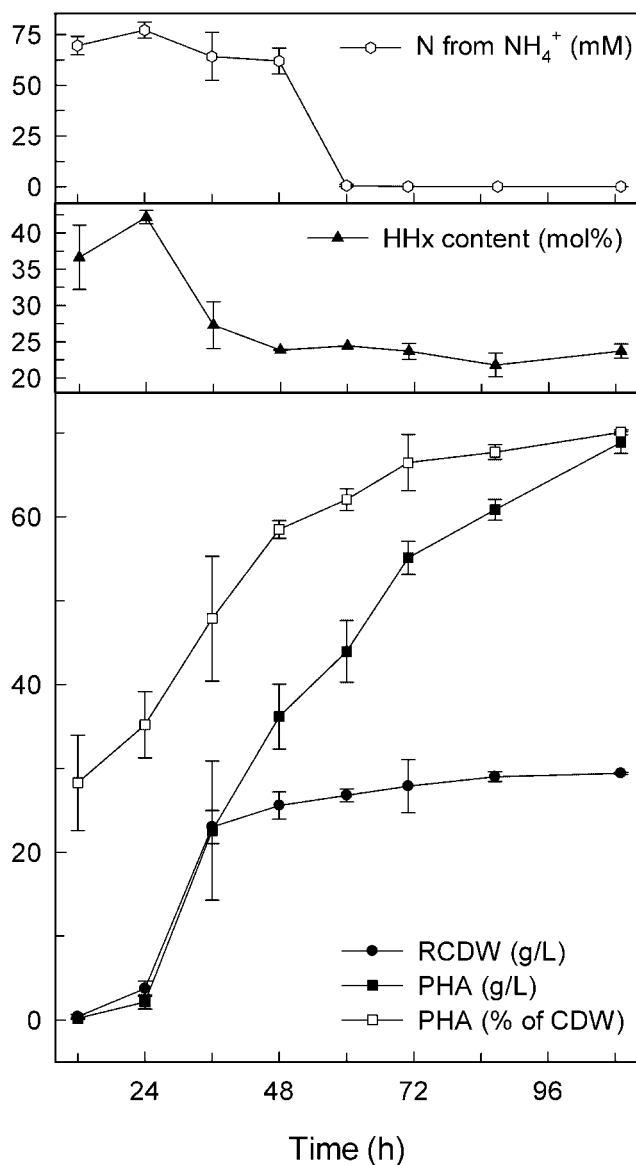
While the results of the extended batch fermentation represented an improvement over the initial batch fermentation, we continued to improve fermentation performance using fed batch strategies. Thus, we implemented two fed batch strategies with two different kinds of nitrogen feeding. In both strategies, the initial nitrogen concentration of the culture was 75 mM, and nitrogen was fed to a final concentration of 480 mM as described in Materials and Methods section.

In the first fed batch cultivation (Fig. 2),  $\text{NH}_4\text{Cl}$  was the initial nitrogen source and  $\text{NH}_4\text{OH}$  was fed to the culture



**Figure 1.** Extended batch fermentations for P(HB-co-HHx) production by *R. eutropha* Re2058/pCB113 using palm oil (PO) as the sole carbon source. PO (initial concentration = 40 g/L) and urea (initial concentration = 4.5 g/L; 150 mM nitrogen) were added as carbon and nitrogen sources, respectively to 1 L minimal medium. A bolus of 20 g/L PO was added to the culture after 32 h based on initial volume. PHA content of cell dry weight (%), HHx content of PHA (mol%), residual cell dry weight (g/L), and PHA produced (g/L) are shown. Data points are mean values from duplicate fermentations. Error bars indicate maximum and minimum values.

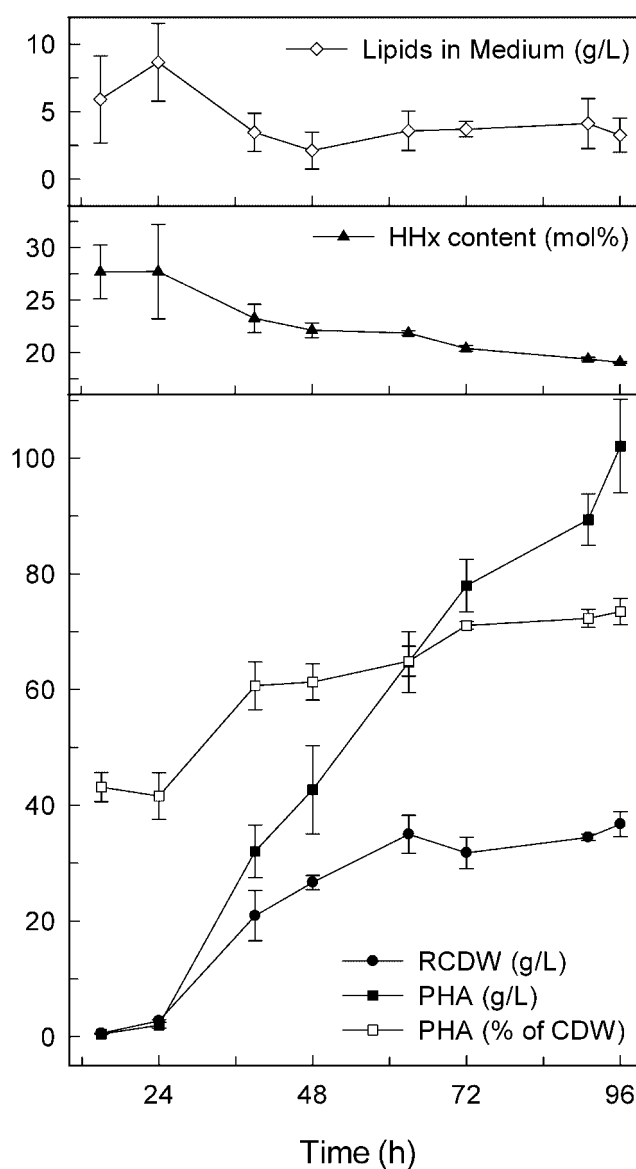
for pH control. During the nitrogen feeding stage of the culture, the nitrogen level remained constant, equivalent to the initial level of 4 g/L  $\text{NH}_4\text{Cl}$  (75 mM nitrogen). As the initial  $\text{NH}_4^+$  from  $\text{NH}_4\text{Cl}$  was consumed, the decrease in culture pH resulted in the addition of  $\text{NH}_4\text{OH}$  by the pH controller, resulting in nitrogen levels returning to their initial concentration during  $\text{NH}_4\text{OH}$  feeding causing the nitrogen concentration to remain approximately constant during  $\text{NH}_4\text{OH}$  feeding. It was observed, during  $\text{NH}_4\text{OH}$  feeding (between 12–48 h), that the PHA content of CDW increased from 28 to 59%, even though nitrogen was still present in the culture. After 60 h of cultivation, nitrogen limitation was observed and by the end of the fermentation the culture reached 98 g/L CDW with a PHA content of 70% PHA containing 24 mol% HHx.



**Figure 2.** Fed batch fermentations for P(HB-co-HHx) production by *R. eutropha* Re2058/pCB113 from palm oil (PO), using  $\text{NH}_4^+$  as nitrogen source. Initial concentrations of 20 g/L PO and 4 g/L  $\text{NH}_4\text{Cl}$  (75 mM nitrogen) were used in 1 L media. PO was fed between 24–84 h to a total concentration of 170 g/L based on initial volume. Nitrogen was fed over pH control ( $\text{pH } 6.8 \pm 0.1$ ) using a 14%  $\text{NH}_4\text{OH}$  stock solution to a total nitrogen concentration of 480 mM nitrogen. Concentration of nitrogen from ammonium (g/L, top), HHx content of PHA (mol%, middle), residual cell dry weight (g/L, bottom, filled circles), PHA produced (g/L, bottom, filled boxes), and PHA content of CDW (%) (bottom, open boxes) are shown. Data points are mean values from duplicate fermentations. Error bars indicate maximum and minimum values.

### Fed Batch Fermentation (urea)

In the second fed batch culture (Fig. 3), urea was used as the sole nitrogen source with an initial concentration of 2.2 g/L (75 mM nitrogen). After 15 h, the PHA content of CDW was already at 43%. The PHA content increased to 61% after 48 h, and nitrogen limitation was observed after 63 h of cultivation. At the end of fermentation, 102 g/L PHA (73%



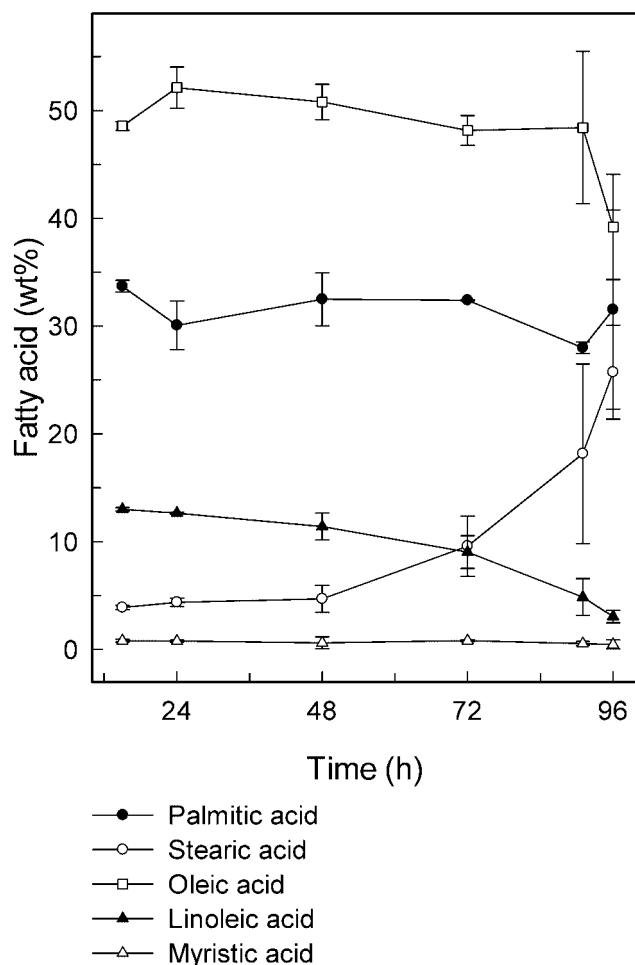
**Figure 3.** Fed batch fermentations for P(HB-co-HHx) production by *R. eutropha* Re2058/pCB113 using palm oil (PO) as carbon source and urea as nitrogen source. Initial concentrations of 20 g/L PO and 2.2 g/L urea (75 mM nitrogen) were added to 1 L media. PO and urea were fed between 24–84 h and 18–48 h, respectively, to total concentrations of 170 g/L PO and 480 mM nitrogen, based on initial volume. Concentration of lipids in the medium (g/L, top), HHx content of PHA (mol%, middle), residual cell dry weight (g/L, bottom, filled circles), PHA produced (g/L, bottom, filled boxes), and PHA content of cell dry weight (%) (open boxes) are shown. Data points are means from triplicate fermentations and error bars indicate  $\pm$  SD. Values for 15 h and 91 h data points are means of duplicate samples.

of CDW) was produced containing 19 mol% HHx. Residual lipids from culture supernatants were isolated to follow the utilization of PO during the course of the experiment and also to calculate the PHA yield from PO (g PHA/ g PO). The concentration of recovered lipids stayed almost constant around 5 g/L during the entire fermentation, which indicated a balanced PO feed (Fig. 3). Over the course of the entire cultivation, polymer was accumulated at a yield

of 0.63 g PHA/g PO. During 63–96 h, the PHA yield was 0.78 g/g PO.

### Recovery and Analysis of Residual Lipids From Culture Supernatants

Recovery of residual lipids in fermenter cultures is useful for determination of many different process parameters, such as PHA production yield (g PHA/g oil), fatty acid composition of residual lipids, and lipid consumption profiles. Methanolysis was used to identify the residual fatty acids and quantify their distribution in the lipids extracted from culture supernatants from the fed batch fermentation in which urea was the nitrogen source. One of the key observations that can be seen in Figure 4 is that the proportion of residual fatty acids stayed almost constant



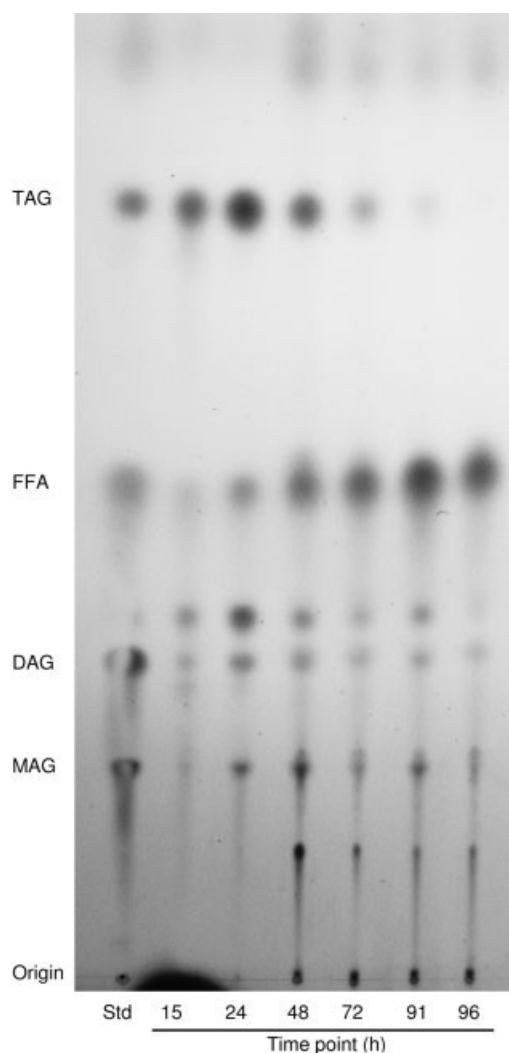
**Figure 4.** Fatty acid distributions in lipid samples extracted from medium over the course of the fed batch fermentations (urea), were determined. Data points are means from triplicate fermentations (with the exception of the 91 h data point, which represents the mean of duplicate) and error bars indicate  $\pm$  SD. Fatty acid content was determined by quantification of fatty acid methyl esters using known quantities of standard compounds.

during PO feeding (24–84 h). Approximately, 12 h after the last PO feeding step, at 96 h, the proportion of stearic acid was over five times greater than it had been at 48 h. Linoleic acid had decreased almost fourfold by that time, and the proportion of oleic acid decreased from 51% (w/w) to 39% (w/w). The proportion of palmitic acid stayed constant over the 96 h course of the fermentation, however the standard deviation (SD) for these values was large at 96 h (over 9%). Myristic acid was present throughout the fermentation in a proportion of less than 1% (w/w) of the total fatty acids. An increase of certain fatty acids in the supernatant over the course of the fermentation could be due to the cells preferring some fatty acids as carbon sources, thus certain fatty acids are not readily utilized and build up in the culture media. On the other hand, fatty acids that decrease in concentration might be more readily consumed by the cells. A biohydrogenation of the unsaturated fatty acids linoleic acid (C18:2) and oleic acid (C18:1) to produce stearic acid (C18:0), has been shown to occur using a consortium of rumen bacteria (Jenkins et al., 2008). This phenomenon could potentially occur in PO cultures, and could account for a decrease in linoleic and oleic acids and a concomitant increase of stearic acid. However, homologs of genes and enzymes responsible for this process have not yet been identified in *R. eutropha*.

TLC analysis of the same extracted lipids (Fig. 5) showed that the concentration of TAGs in the lipid extracts decreased continuously from 24 h until all TAGs had been broken down by the end of the fermentation (96 h). DAGs also decreased proportionally until the end of fermentation. At the same time, the concentration of FFAs in the extracted lipids continuously increased up until 91 h. The concentration of MAGs fluctuated over the course of fermentation. The PO feeding between 24 and 84 h did not result in an increase in the proportion of TAGs, indicating balanced PO feeding throughout the experiment. At 48 h, a new species was observed below the spot corresponding to MAG migration. This new species appears in the TLC area thought to be for polar lipid separation (King et al., 1977). We hypothesize that this spot represents a polar lipid (or polar lipids) extracted from culture supernatants. From 48 to 91 h, the intensity of the spot representing the unknown species decreased.

### Comparison of PHA Production From the Different Fermentations

The amount of biomass produced in Re2058/pCB113 fermentations was increased using different strategies, as described above. The amount of total PHA produced increased due to the increase in the total nitrogen supplied to the cultures from either urea or  $\text{NH}_4^+$  (Table I), along with the adjustment of PO concentration. These increases in the concentrations of growth substrates allowed for the production of larger quantities of biomass, resulting in larger amounts of PHA per culture. The key observation of



**Figure 5.** Thin layer chromatography indicating the time course of residual lipids and fatty acid present in the medium of fed batch fermentations (urea) with palm oil as the sole carbon source. In all sample lanes, 30  $\mu\text{g}$  of extracted lipids were loaded. Proportions of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and free fatty acids (FFA) are shown. Std = lipid standards as described in Materials and Methods section.

these experiments was the comparison of the PHA production of the batch culture and of the fed batch culture using urea as the nitrogen source. In this case, the fold increase of the total nitrogen (6.4-fold, Table I) added

**Table I.** Improvement of PHA Production from palm oil by Re2058/pCB113 with different fermentation strategies using several nitrogen sources. All values represent means from duplicate or triplicate cultivations.

Fermentation	Total N (mM)	N source	PHA (% of CDW)	PHA (g/L)
Batch <sup>a,c</sup>	75	NH <sub>4</sub> Cl	70.9 ± 1.9	17.5 ± 1.1
Extended batch <sup>b</sup>	150	Urea	72.3 ± 0.5	32.6 ± 1.9
Fed batch (NH <sub>4</sub> OH) <sup>b</sup>	480	NH <sub>4</sub> Cl/NH <sub>4</sub> OH	70.1 ± 0.3	68.9 ± 1.3
Fed batch (urea) <sup>a</sup>	480	Urea	73.5 ± 2.3	102.1 ± 8.1

<sup>a</sup>Results obtained from triplicate cultures with error bars indicating ± SD.

<sup>b</sup>Results obtained from duplicate cultures with error bars indicating maximum and minimum values.

<sup>c</sup>From Budde et al., 2011b.

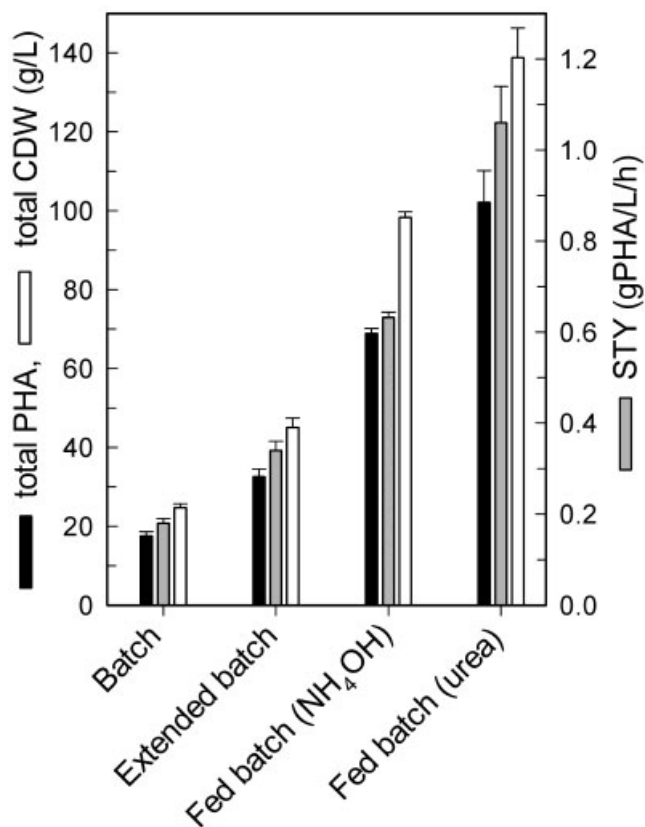
to the culture correlates with the increase in PHA produced by the cultures (5.8-fold, Table I). However, when the output of the batch culture is compared to that of the pH controlled fed batch fermentation (NH<sub>4</sub>OH) where NH<sub>4</sub>Cl was used as the initial nitrogen source, the increase in PHA production (3.9-fold, Table I) was significantly smaller than the increase of total nitrogen input (6.4 fold, Table I). These results suggest that the fed batch strategy with urea as the nitrogen source is the superior PHA production strategy.

For efficient industrial scale PHA production, it is important for a production strain to produce large amounts of PHA in a relatively short time. Thus, the STY for all fermentations was calculated. Figure 6 shows that we were able to increase the STY of PHA production from 0.2 g/L/h from the initially described batch fermentation (Budde et al., 2011b) to 1.1 g/L/h from the fed batch fermentation (urea) with a total PHA production of 102 g/L.

In all fermentations discussed here, the HHx content of PHA was observed to decrease over time (Table II, Figs. 1–3). At early time points, a high level of HHx monomer was seen in cultures, reaching above 40 mol% in some cases. Over the course of the fermentation, the HHx monomer percentage decreased and eventually leveled off. This phenomenon was also observed in the batch fermentation published previously (Budde et al., 2011b).

### Molecular Weight Analysis of PHA

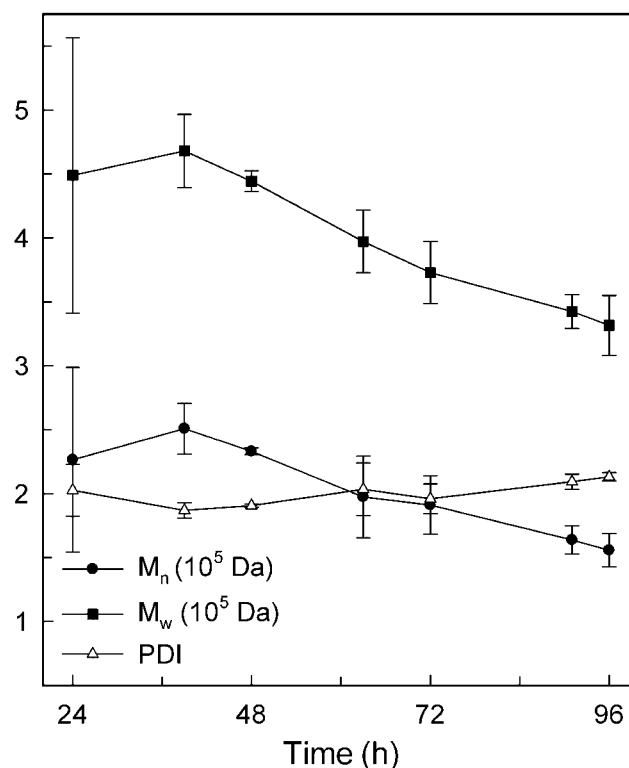
Molecular weight of PHA polymer chains plays an important role in determining whether the polymer can be processed (Sim et al., 1997; Tsuge et al., 2007). Using GPC analysis, the weight average ( $M_w$ ) and number average ( $M_n$ ) molecular weights was determined of the PHA polymer produced over the course of the fed batch fermentation (urea) (Fig. 7). The average molecular weight of the PHA decreased from 500,000 Da after 39 h to 300,000 Da after 96 h. A similar decrease over time in molecular weight of PHA was also seen in our previous study (Budde et al., 2011b) where the molecular weight decreased from 400,000 Da after 48 h to 300,000 Da after 96 h. The polydispersity index (PDI) of PHA from the fed batch culture increased from 1.9 to 2.1 during 39–96 h, which indicates narrow molecular weight distributions.



**Figure 6.** Improvements to PHA production (g/L), the space time yield of PHA production (g/L/h), and cell dry weight production (g/L) comparing batch culture (Data points are means from triplicate and error bars indicate  $\pm$  SD, Budde et al., 2011b), extended batch culture (Data points are means from duplicate with error bars indicating maximum and minimum values), and fed batch cultures (fed batch (urea) in triplicate with error bars indicating SD. Fed batch (NH<sub>4</sub>OH) in duplicate with error bars indicating maximum and minimum values).

## Discussion

Production of P(HB-co-HHx) was increased by using different fermentation strategies to attain a maximum concentration of 102 g/L PHA (Fig. 6). It was shown that the addiction system present in Re2058/pCB113 for plasmid maintenance (Budde et al., 2011b) was robust in high cell density fermentations of up to 140 g/L CDW. The PHA



**Figure 7.** GPC analyses of P(HB-co-HHx) during the fed batch fermentations (urea). PHA was extracted from freeze-dried cells with chloroform and molecular weights determined relative to polystyrene standards. The number average molecular weight ( $M_n \times 10^5$  Da, filled circles), weight average molecular weight ( $M_w \times 10^5$  Da, filled boxes), and polydispersity index (PDI, open triangles) are shown. Data points are means from triplicate fermentations (with the exception of the 91 h data point, which represents the mean of duplicate) and error bars indicate  $\pm$  SD.

content of CDW was always over 70% with high HHx level ( $>17$  mol%) at the conclusion of all fermentations (Table II), indicating that the plasmid-borne PHA production genes were still present at the conclusion of fermentation. Therefore, this system overcomes previously reported issues of plasmid instability in high cell density fermentations ( $\sim 100$  g/L CDW) of *R. eutropha* (Srinivasan et al., 2003).

**Table II.** HHx monomer content of PHA produced by Re2058/pCB113 at early and late stages in fermentations using palm oil as the sole carbon source. All values represent means from duplicate or triplicate cultivations.

Fermentation	Early time point				End of cultivation			
	t (h)	PHA (% of CDW)	HHx (mol%)	CDW (g/L)	t (h)	PHA (% of CDW)	HHx (mol%)	CDW (g/L)
Batch <sup>a,c</sup>	25	26.2 $\pm$ 3.3	43.7 $\pm$ 2.8	3.0 $\pm$ 1.2	96	70.9 $\pm$ 1.9	17.3 $\pm$ 1.1	24.7 $\pm$ 1.0
Extended batch <sup>b</sup>	24	45.0 $\pm$ 2.5	28.1 $\pm$ 1.4	3.1 $\pm$ 2.2	96	72.2 $\pm$ 0.5	17.5 $\pm$ 1.4	45.1 $\pm$ 2.4
Fed batch (NH <sub>4</sub> OH) <sup>b</sup>	24	35.2 $\pm$ 3.9	42.2 $\pm$ 0.9	5.9 $\pm$ 1.8	109	70.1 $\pm$ 0.3	23.7 $\pm$ 1.0	98.3 $\pm$ 1.4
Fed batch (urea) <sup>a</sup>	24	41.6 $\pm$ 4.0	27.7 $\pm$ 4.5	4.7 $\pm$ 0.7	96	73.5 $\pm$ 2.3	19.1 $\pm$ 0.1	138.8 $\pm$ 7.5

<sup>a</sup>Results obtained from triplicate cultures with error bars indicating  $\pm$  SD.

<sup>b</sup>Results obtained from duplicate cultures with error bars indicating maximum and minimum values.

<sup>c</sup>From Budde et al., 2011b.



Plant oils such as PO are favorable feedstocks because of their lower price per mass and higher carbon content compared to sugars. Furthermore, plant oils are shown to be an excellent carbon source for PHA production in *R. eutropha* cultures (Akiyama et al., 2003; Fukui and Doi, 1998). They can also be utilized from pure stocks, which minimize added volume during carbon substrate feeding. Brigham et al. (2010) showed that *R. eutropha* expresses lipases which are essential for growth on unemulsified plant oils. In a previous study, we developed an emulsification process to allow for immediate availability of PO in growth media, thus shortening the lag phase in growth (Budde et al., 2011a). We did not use this method in the current study in order to avoid the costs of external emulsification agents. After 24 h in fed batch fermentations, the PO added initially appeared to be emulsified, thus feeding was initiated after that time. Additional PO was emulsified shortly after addition to the cultures. An excess of PO during cultivation (e.g., during feeding) resulted in excessive foaming and thickening of the culture broth (data not shown), which likely inhibited the oxygen transfer, and thus resulted in inefficient production of PHA. It is largely for this reason that proper dosage of PO during feeding is crucial for maximum PHA productivity. Strong foam formation was anticipated, so we kept the aeration rate at a constant low level of 0.5 vvm. Any foam that did occur was broken mechanically with pairs of cable ties attached to the shaft of the impeller. Foam centrifuges, a more powerful tool, could be used in larger fermenters, which would allow for a higher aeration rate to minimize the amount of pure oxygen needed. Chemical antifoams cannot be used for several reasons. First, they interfere with our oil extraction method. Polyethylene glycol also decreases the molecular weight of PHA (Shi et al., 1996). Finally, upon addition to the culture, silicone oil seems to get co-emulsified, which minimizes silicon oil's antifoam effect and thus results in high usage. During cell growth, MAGs, DAGs, and FFAs were produced from the breakdown of TAGs (Fig. 4). These lipids may act as emulsifiers when interacting with unemulsified PO. A 2% (w/v) initial PO concentration and a linear feeding strategy after 24 h in small steps every 2 h was identified as an efficient process for oil addition. PHA yield in the storage phase of the fed batch fermentation urea was 0.78 g/g PO, which is similar to PHB production yields described in previous studies (Budde et al., 2011b; Kahar et al., 2004; Ng et al., 2010) in which plant oils were used as the sole carbon source.

The maximum cell growth, and consequently maximum PHA production, was highly dependent on the nitrogen source used. In the fed batch fermentation with urea, we could produce 48% more PHA compared to the fed batch fermentation with  $\text{NH}_4\text{OH}$  feeding and  $\text{NH}_4\text{Cl}$  as the initial nitrogen source. Previous studies have described urea as the best nitrogen source for PHA production by *R. eutropha* (Khanna and Srivastava, 2004; Ng et al., 2010).  $\text{CO}_2$  remains after the consumption of nitrogen from urea (data not shown), which could potentially have a positive effect on cell

growth (i.e., as a secondary carbon source). With a STY of 1.1 g/L/h PHA in fed batch fermentation with urea as the nitrogen source, we establish a high productivity process. However, a further increase of the STY would result in an increase of the total amount of PHA produced and also shorten the fermentation process. A higher biomass could be reached with higher total carbon and nitrogen concentrations along with an adjusted feeding strategy. The lag phase could potentially be shortened by first using a soluble carbon source (e.g., sodium butyrate) in the growth media, so that the carbon would be immediately available for consumption by the cells. Such a procedure would allow for faster growth. A second feeding of oil could then follow, which would be quickly emulsified due to a high cell concentration generating more lipase activity. Another potential method for shortening the lag phase in growth would be to emulsify the initial PO added to the culture while also decreasing the initial PO concentration (decreasing the amount of emulsifying agent needed), and then feeding unemulsified PO.

PHA production is normally triggered through a nutrient limitation (Haywood et al., 1990), which in our fermentations was nitrogen. However, it was observed at early time points in the fermentations (where no nutrient limitation yet occurred) a surprisingly high PHA accumulation of over 40% CDW (Figs. 1–3). This early high PHA production is possibly due to the PHA production genes being located on a plasmid that results in higher gene dosage and consequently higher gene expression.

Moving forward, the results of high density PHA production on different oil palm products will be examined and their effects on copolymer content (e.g., HHx) and on molecular weight will be determined. The presented fed batch fermentation strategy using urea as the nitrogen source offers the possibility for production of P(HB-co-HHx) with a high HHx concentration (19 mol%), which will allow high cell density fermentation processes with a yield of 102 g/L PHA. A scale-up of the developed process to industrial scale is being planned. The design and implementation of an integrated biorefinery for PHA production is imaginable. Palm oil mills usually produce excess heat and electricity by burning waste material (e.g., fruit fiber) (Basiron, 2007), which could be also used to power a PHA plant. Hence, the results presented in this study may contribute to commercialization of biodegradable bioplastics made from palm oil products, and reduce the dependency of the plastics industry on fossil fuels.

## Acknowledgements

The authors thank Mr. John W. Quimby for his helpful ideas, discussions, and editing of the manuscript. They also thank Ms. Karen Pepper for critical reviewing of the manuscript. This work was funded by the Malaysia MIT Biotechnology Partnership Programme (MMBPP). Authors thank their MMBPP collaborators for their helpful discussions and support throughout the course of this study.

## References

- Akiyama M, Tsuge T, Doi Y. 2003. Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon sources by bacterial fermentation. *Polym Degrad Stab* 80:183–194.
- Barnes DKA, Galgani F, Thompson RC, Barlaz M. 2009. Accumulation and fragmentation of plastic debris in global environments. *Phil Trans R Soc B* 364:1985–1998.
- Basiron Y. 2007. Palm oil production through sustainable plantations. *Eur J Lipid Sci Technol* 109:289–295.
- Brigham CJ, Budde CF, Holder JW, Zeng Q, Mahan AE, Rha C, Sinskey AJ. 2010. Elucidation of beta-oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. *J Bacteriol* 192(20): 5454–5464.
- Budde CF, Mahan AE, Lu J, Rha C, Sinskey AJ. 2010. Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J Bacteriol* 192(20):5319–5328.
- Budde CF, Riedel SL, Hubner F, Risch S, Popovic MK, Rha C, Sinskey AJ. 2011a. Growth and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium. *Appl Microbiol Biotechnol* 89(5):1611–1619.
- Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ. 2011b. Production of Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate) from Plant Oil by Engineered *Ralstonia eutropha* Strains. *Appl Environ Microbiol* 77(9):2847–2854.
- Chen GQ. 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem Soc Rev* 38(8):2434–2446.
- Chen GQ, Wu Q. 2005. The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* 26(33):6565–6578.
- Doi Y, Kitamura S, Abe H. 1995. Microbial Synthesis and Characterization of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromolecules* 28:4822–4828.
- Fukui T, Doi Y. 1998. Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. *Appl Microbiol Biotechnol* 49(3):333–336.
- Hänisch J, Wältermann M, Robenek H, Steinbüchel A. 2006. The *Ralstonia eutropha* H16 phasin PhaP1 is targeted to intracellular triacylglycerol inclusions in *Rhodococcus opacus* PD630 and *Mycobacterium smegmatis* mc2155, and provides an anchor to target other proteins. *Microbiology* 152(Pt 11):3271–3280.
- Haywood GW, Anderson AJ, Ewing DF, Dawes EA. 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. *Appl Environ Microbiol* 56(11):3354–3359.
- Jenkins TC, Wallace RJ, Moate PJ, Mosley EE. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J Anim Sci* 86(2):397–412.
- Kahar P, Tsuge T, Taguchi K, Doi Y. 2004. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. *Polym Degrad Stab* 83:79–86.
- Khanna S, Srivastava AK. 2004. Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Process Biochem* 40:2173–2182.
- King RJ, Martin H, Mitts D, Holmstrom FM. 1977. Metabolism of the apoproteins in pulmonary surfactant. *J Appl Physiol* 42(4):483–491.
- Loo CY, Lee WH, Tsuge T, Doi Y, Sudesh K. 2005. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. *Biotechnol Lett* 27:1405–1410.
- Ng K-S, Ooi W-Y, Goh L-K, Shenbagarathai R, Sudesh K. 2010. Evaluation of jatropha oil to produce poly(3-hydroxybutyrate) by *Cupriavidus necator* H16. *Polym Degrad Stab* 95:1365–1369.
- Noda I, Bond EB, Green PR, Melik DH, Narasimhan K, Schechtman LA, Satkowski MM. 2005a. Preparation, properties, and utilization of biobased biodegradable nodax copolymers. *Polym Biocatal Biomater* 900:280–291.
- Noda I, Green PR, Satkowski MM, Schechtman LA. 2005b. Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. *Biomacromolecules* 6(2):580–586.
- Philip S, Keshavarz T, Roy I. 2007. Polyhydroxyalkanoates: Biodegradable polymers with a range of applications. *J Chem Technol Biotechnol* 82:233–247.
- Rehm BH. 2003. Polyester synthases: Natural catalysts for plastics. *Biochem J* 376(Pt 1):15–33.
- Reinecke F, Steinbüchel A. 2009. *Ralstonia eutropha* strain H16 as a model organism for PHA metabolism and for biotechnological production of technically interesting polymers. *J Mol Microbiol Biotechnol* 16(1–2): 91–108.
- Ryan PG, Moore CJ, Franeker JA, Moloney CL. 2009. Monitoring the abundance of plastic debris in the marine environment. *Phil Trans R Soc B* 364:1999–2012.
- Shi F, Ashby R, Gross RA. 1996. Use of poly(ethylene glycol)s to regulate poly(3-hydroxybutyrate) molecular weight during *Alcaligenes eutrophus* cultivations. *Macromolecules* 29(24):7753–7758.
- Sim SJ, Snell KD, Hogan SA, Stubbe J, Rha C, Sinskey AJ. 1997. PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate in vivo. *Nat Biotechnol* 15(1):63–67.
- Srinivasan S, Barnard GC, Gerngross TU. 2003. Production of recombinant proteins using multiple-copy gene integration in high-cell-density fermentations of *Ralstonia eutropha*. *Biotechnol Bioeng* 84(1):114–120.
- Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A, Valentin H. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiol Rev* 9(2–4):217–230.
- Sudesh K, Abe H, Doi Y. 2000. Synthesis, structure, and properties of polyhydroxyalkanoates: Biological polyesters. *Prog Polym Sci* 25:1503–1555.
- Tian PY, Shang L, Ren H, Mi Y, Fan DD, Jiang M. 2009. Biosynthesis of polyhydroxyalkanoates: Current research and development. *Afr J Biotechnol* 8(5):709–714.
- Tsuge T, Watanabe S, Sato S, Hiraishi T, Abe H, Doi Y, Taguchi S. 2007. Variation in copolymer composition and molecular weight of polyhydroxyalkanoate generated by saturation mutagenesis of *Aeromonas caviae* PHA synthase. *Macromol Biosci* 7(6):846–854.
- Uchino K, Saito T. 2006. Thiolysis of poly(3-hydroxybutyrate) with polyhydroxyalkanoate synthase from *Ralstonia eutropha*. *J Biochem* 139(3):615–621.
- Williams SF, Martin DP, Horowitz DM, Peoples OP. 1999. PHA applications: Addressing the price performance issue: I. Tissue engineering. *Int J Biol Macromol* 25(1–3):111–121.
- Zhang X, Luo R, Wang Z, Deng Y, Chen GQ. 2009. Application of (R)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. *Biomacromolecules* 10(4):707–711.
- Zhao K, Deng Y, Chen JC, Chen GQ. 2002. Polyhydroxyalkanoate (PHA) scaffolds with good mechanical properties and biocompatibility. *Biomaterials* 24:1041–1045.