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

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1 Biotechnology and Bioengineering

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3 Title: Production of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by *Ralstonia eutropha*

4 in High Cell Density Palm Oil Fermentations

5 Running title: P(HB-*co*-HHx) Production by *R. eutropha* from Palm Oil

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21 Keywords: *Ralstonia eutropha*, Polyhydroxyalkanoate, Poly(hydroxybutyrate-*co*-

22 hydroxyhexanoate), Plant oil, high cell density fermentation, Palm oil

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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 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).

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39 **INTRODUCTION**

40 Today, the majority of industrial plastic production is dependent on fossil–based petroleum.
41 To reduce this dependency, new polymer production processes based on alternative substrates
42 must be developed. Polyhydroxyalkanoate (PHA) is a microbially produced polyester used for
43 carbon and energy storage. PHA can be used effectively as a biodegradable and biocompatible
44 alternative to petroleum-based plastic (Haywood et al. 1990; Steinbuchel et al. 1992; Sudesh
45 et al. 2000). One key motivation for the migration to biodegradable plastic is the increasing
46 accumulation of non-biodegradable waste in the environment, which has recently been
47 reviewed (Barnes et al. 2009; Ryan et al. 2009). PHA already has a wide variety of
48 applications. Traditionally, PHA has been used to produce everyday items such as packing
49 material or containers for storage of household products (Philip et al. 2007). Because of its
50 biocompatibility and ability to degrade to non-toxic compounds in the human body, PHA is
51 used in tissue engineering as a scaffold material (Chen and Wu 2005; Williams et al. 1999;
52 Zhao et al. 2002). As a biofuel, methyl-esters derived from PHA monomers have been shown
53 to have potential as transportation fuels (Zhang et al. 2009).

54 A summary of 24 PHA-producing companies offered by (Chen 2009) demonstrates the global
55 interest in production of these polymers. It is expected that lower production costs will help
56 accelerate further commercialization of PHA. For low-cost industrial PHA production, high
57 space-time yields are required. Optimal fermentation processes would be based on either
58 renewable carbon substrates with a high yield per hectare or inexpensive secondary products.
59 Tian *et al.* (Tian et al. 2009), review the utilization of waste water, whey, molasses and
60 various plant oils as carbon substrates for PHA production. The advantage of utilizing plant
61 oils is their high carbon content as well as high conversion rate to PHA (Akiyama et al. 2003;
62 Fukui and Doi 1998; Kahar et al. 2004; Loo et al. 2005; Ng et al. 2010). Because of their high
63 carbon content, low flow rate feed streams can be applied, reducing the dilution of the

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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 β-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 Steinbuchel 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 6 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 (Hanisch 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.

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91 **MATERIALS AND METHODS**

92 **Bacterial strain**

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

96

97 **Growth media and preculture cultivation conditions**

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

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

110 Re2058/pCB113 was initially grown overnight in 4 mL TSB from a single colony. Cells were
111 centrifuged at 16,100 x *g* and the pellet was resuspended in 0.85% saline and used to inoculate
112 50 or 100 mL minimal medium flask cultures containing 2% fructose and 0.1% NH_4Cl or
113 0.06% urea to an initial OD_{600} of 0.05. After approximately 24 h of incubation, cells were
114 centrifuged at 6,500 x *g* and the pellet was resuspended in 0.85% saline for inoculation of the
115 fermenter culture to an initial OD_{600} of 0.1.

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117 **General fermentation conditions**

118 A Bioengineering multiple fermenter system (R'ALF PLUS TRIO) consisting of 2 L double
119 jacketed glass vessels with a working volume of 1.2 L (Wald ZH/Switzerland) was used for
120 fermentation studies. The temperature of the cultures was kept constant at 30°C, and the pH
121 was maintained at 6.8 ± 0.1, through controlled addition of 2 M NaOH (NH_4OH was used for
122 pH controlled nitrogen feeding) and 0.67 M H_3PO_4. Cultures were stirred using two six-blade
123 Rushton impellers at speeds ranging from 300-1,500 rpm. Air was supplied through a ring
124 sparger at 0.5 vvm unless noted otherwise. The dissolved oxygen concentration was
125 maintained at levels above 40% by addition of pure oxygen and kept at a constant flow rate by
126 a mass flow controlled pO2 cascade. Foam in the cultures was broken mechanically with pairs
127 of cable ties attached to the shaft of the impellor.

128

129 **Extended batch fermentation**

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

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135 **Fed batch fermentation (NH_4OH), with pH controlled nitrogen feeding**

136 Fermenter cultures containing 1 L of minimal medium with 20 g/L PO and 4 g/L NH_4Cl (75
137 mM nitrogen) were inoculated with precultures, as described above. During cultivation, PO
138 was fed between 24-84 h in approximately 5 g/L steps, pulse-wise with a pump in 2 h
139 intervals, to a total concentration of approximately 170 g/L, based on initial volume. For
140 initial pH control and nitrogen feeding, a calculated volume of a stock solution of 14% (w/v)
141 NH_4OH was provided to the culture, from the base reservoir of the fermenter, to reach a total

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nitrogen concentration of 480 mM nitrogen, based on initial volume. After the NH_4OH 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 6,500 x *g* 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 resuspended in 2 mL cold water, frozen at -80°C, lyophilized and then the cell dry weight (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.

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3 167 For molecular weight determinations of P(HB-*co*-HHx), the polymer was extracted from
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6 168 freeze-dried cells with chloroform. Equal masses of freeze-dried cells were weighed in screw
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8 169 capped glass tubes to form a 3 mg/mL PHA solution with a total volume of 2 mL. Samples
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10 170 were kept at 50°C for 4 h and briefly shaken by hand every 30 min. After PHA extraction, the
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13 171 samples were filtered through a 0.2 µm polytetrafluoroethylene (PTFE) membrane and the
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15 172 molecular weight was measured via gel permeation chromatography (GPC) relative to
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18 173 polystyrene standards as described previously (Budde et al. 2010).
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20 174 For testing the nitrogen content of cell supernatants, frozen culture supernatants were thawed
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23 175 at room temperature, centrifuged for 5 min at 16,100 x *g,* and filtered through a 0.2 µm PTFE
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25 176 membrane (if necessary). Ammonium and urea concentrations were measured from clarified
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27 177 supernatants with an ammonium assay kit (Sigma-Aldrich, Cat. No. AA0100) or a urea assay
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30 178 kit (BioVision, Cat. No. #K375-100) respectively, as defined in the text.
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32 179 Lipids were extracted from the culture by using a mixture of chloroform/methanol (2:1, v/v)
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35 180 as previously described (Budde et al. 2011a). Samples for lipid recovery were taken prior to
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37 181 any PO feeding steps at a given time point. The distribution of fatty acids in the recovered
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39 182 lipids was determined by the same methanolysis assay described above, which leads to
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42 183 formation of fatty acid methyl esters. The species and proportion of fatty acids in the lipids
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44 184 recovered from the chloroform/methanol extraction were identified by thin layer
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47 185 chromatography (TLC). The extracted lipids were dissolved in chloroform to a final
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49 186 concentration of 3 mg/mL. Aliquots of 10 µL (30 µg lipids) were spotted on a silica gel TLC
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51 187 plate (250 µm thickness; EMD Chemicals, Gibbstown, NJ). A mixture of defined standards
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54 188 was also spotted: triacylglycerol (TAG, 1,2-distearoyl-3-oleoyl-rac-glycerol; 10 µg),
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56 189 diacylglycerol (DAG, 1,2-dipalmitoyl-rac-glycerol; 20 µg), monoacylglycerol (MAG, 1-
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59 190 palmitoyl-rac-glycerol; 20 µg) (Nu-check Prep, Inc., Elysian MN) and free fatty acid (FFA,
60 191 palmitic acid; 10 µg). TLC was then performed as described previously (Budde et al. 2011a).

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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 (≥15mol%) 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 17mol% HHx after 96 h of cultivation in minimal medium containing 40 g/L PO as the carbon source and 4 g/L NH_4Cl (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 (2.1 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 NH_4Cl, 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

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was already 45% of CDW at 24 h (Figure 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 17mol% (Figure 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 (NH_4OH), 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.

In the first fed batch cultivation (Figure 2), NH_4Cl was the initial nitrogen source and NH_4OH was fed to the culture 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 NH_4Cl (75 mM nitrogen). As the initial NH_4^+ from NH_4Cl was consumed, the decrease in culture pH resulted in the addition of NH_4OH by the pH controller, resulting in nitrogen levels returning to their initial concentration during NH_4OH feeding causing the nitrogen concentration to remain approximately constant during NH_4OH feeding. It was observed, during NH_4OH 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 24mol% HHx.

Fed batch fermentation (urea)

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In the second fed batch culture (Figure 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% of CDW) was produced containing 19mol% 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 (Figure 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 5 is that the proportion of residual fatty acids stayed almost constant 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 5 times greater than it had been at 48 h. Linoleic acid had decreased almost 4 fold 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 (Figure 6) 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 h to 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 48-91 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 NH4+ (Table I), along with the adjustment of PO concentration. These increases in the concentrations of

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growth substrates allowed for the production of larger quantities of biomass, resulting in larger amounts of PHA per culture. The key observation of 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 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_4OH) where NH_4Cl 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 space time yield (STY) for all fermentations was calculated. Figure 4 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, Figures 1-3). At early time points, a high level of HHx monomer was seen in cultures, reaching above 40mol% 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

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polymer produced over the course of the fed batch fermentation (urea) (Figure 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.

DISCUSSION

Production of P(HB-*co*-HHx) was increased by using different fermentation strategies to attain a maximum concentration of 102 g/L PHA (Figure 4). 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 content of CDW was always over 70% with high HHx level (>17mol%) at the conclusion of all fermentations (Table 2), 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 (~100 g/L CDW) of *R. eutropha* (Srinivasan et al. 2003).

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*., (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 impellor. 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 (Figure 6). 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 NH_4OH feeding and NH_4Cl as the initial nitrogen source. Previous studies have described urea as the best nitrogen

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3 375 source for PHA production by *R. eutropha* (Khanna and Srivastava 2004; Ng et al. 2010).
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6 376 CO_2 remains after the consumption of nitrogen from urea (data not shown), which could
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8 377 potentially have a positive effect on cell growth (*i.e.* as a secondary carbon source). With a
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10 378 STY of 1.1 g/L/h PHA in fed batch fermentation with urea as the nitrogen source, we
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13 379 establish a high productivity process. However, a further increase of the STY would result in
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15 380 an increase of the total amount of PHA produced and also shorten the fermentation process. A
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17 381 higher biomass could be reached with higher total carbon and nitrogen concentrations along
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20 382 with an adjusted feeding strategy. The lag phase could potentially be shortened by first using a
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22 383 soluble carbon source (e.g. sodium butyrate) in the growth media, so that the carbon would be
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24 384 immediately available for consumption by the cells. Such a procedure would allow for faster
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27 385 growth. A second feeding of oil could then follow, which would be quickly emulsified due to
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29 386 a high cell concentration generating more lipase activity. Another potential method for
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31 387 shortening the lag phase in growth would be to emulsify the initial PO added to the culture
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34 388 while also decreasing the initial PO concentration (decreasing the amount of emulsifying
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36 389 agent needed), and then feeding unemulsified PO.
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39 390 PHA production is normally triggered through a nutrient limitation (Haywood et al. 1990),
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41 391 which in our fermentations was nitrogen. However, it was observed at early time points in the
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43 392 fermentations (where no nutrient limitation yet occurred) a surprisingly high PHA
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46 393 accumulation of over 40% CDW (Figures 1-3). This early high PHA production is possibly
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48 394 due to the PHA production genes being located on a plasmid that results in higher gene
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50 395 dosage and consequently higher gene expression.
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53 396 Moving forward, the results of high density PHA production on different oil palm products
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55 397 will be examined and their effects on copolymer content (e.g. HHx) and on molecular weight
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58 398 will be determined. The presented fed batch fermentation strategy using urea as the nitrogen
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60 399 source offers the possibility for production of P(HB-*co*-HHx) with a high HHx concentration

400 (19 mol%), which will allow high cell density fermentation processes with a yield of 102 g/L

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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.

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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[a,c]	75	NH_4Cl	70.9 ± 1.9	17.5 ± 1.1
Extended batch[b]	150	Urea	72.3 ± 0.5	32.6 ± 1.9
Fed batch (NH_4OH)[b]	480	NH_4Cl/NH_4OH	70.1 ± 0.3	68.9 ± 1.3
Fed batch (urea)[a]	480	Urea	73.5 ± 2.3	102.1 ± 8.1

[a] Results obtained from triplicate cultures with error bars indicating ± SD

[b] Results obtained from duplicate cultures with error bars indicating maximum and minimum values

[c] From Budde et al., 2011b

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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[a,c]	25	26.2 ± 3.3	43.7 ± 2.8	3.0 ± 1.2	96	70.9 ± 1.9	17.3 ± 1.1	24.7 ± 1.0
Extended batch[b]	24	45.0 ± 2.5	28.1 ± 1.4	3.1 ± 2.2	96	72.2 ± 0.5	17.5 ± 1.4	45.1 ± 2.4
Fed batch (NH_4OH)[b]	24	35.2 ±3.9	42.2 ± 0.9	5.9 ± 1.8	109	70.1± 0.3	23.7 ± 1.0	98.3 ± 1.4
Fed batch (urea)[a]	24	41.6 ± 4.0	27.7 ± 4.5	4.7 ± 0.7	96	73.5 ± 2.3	19.1 ± 0.1	138.8 ± 7.5

[a] Results obtained from triplicate cultures with error bars indicating ± SD

[b] Results obtained from duplicate cultures with error bars indicating maximum and minimum values

[c] From Budde et al., 2011b

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FIGURE LEGENDS

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 (%, top), HHx content of PHA (mol%, bottom, filled triangles), residual cell dry weight (g/L, bottom, filled circles) and PHA produced (g/L, filled boxes) are shown. Data points are mean values from duplicate fermentations. Error bars indicate maximum and minimum values.

FIGURE 2

Fed batch fermentations for P(HB-*co*-HHx) production by *R. eutropha* Re2058/pCB113 from palm oil (PO), using NH_4^+ as nitrogen source. Initial concentrations of 20 g/L PO and 4 g/L NH_4Cl (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 (pH 6.8 ± 0.1) using a 14% NH_4OH 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.

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

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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 ± SD. Values for 15 h and 91 h data points are means of duplicate samples.

FIGURE 4

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 ± 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_4OH) in duplicate with error bars indicating maximum and minimum values).

FIGURE 5

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 ± SD. Fatty acid content was determined by quantification of fatty acid methyl esters using known quantities of standard compounds.

FIGURE 6

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 µg 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.

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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 ± SD.

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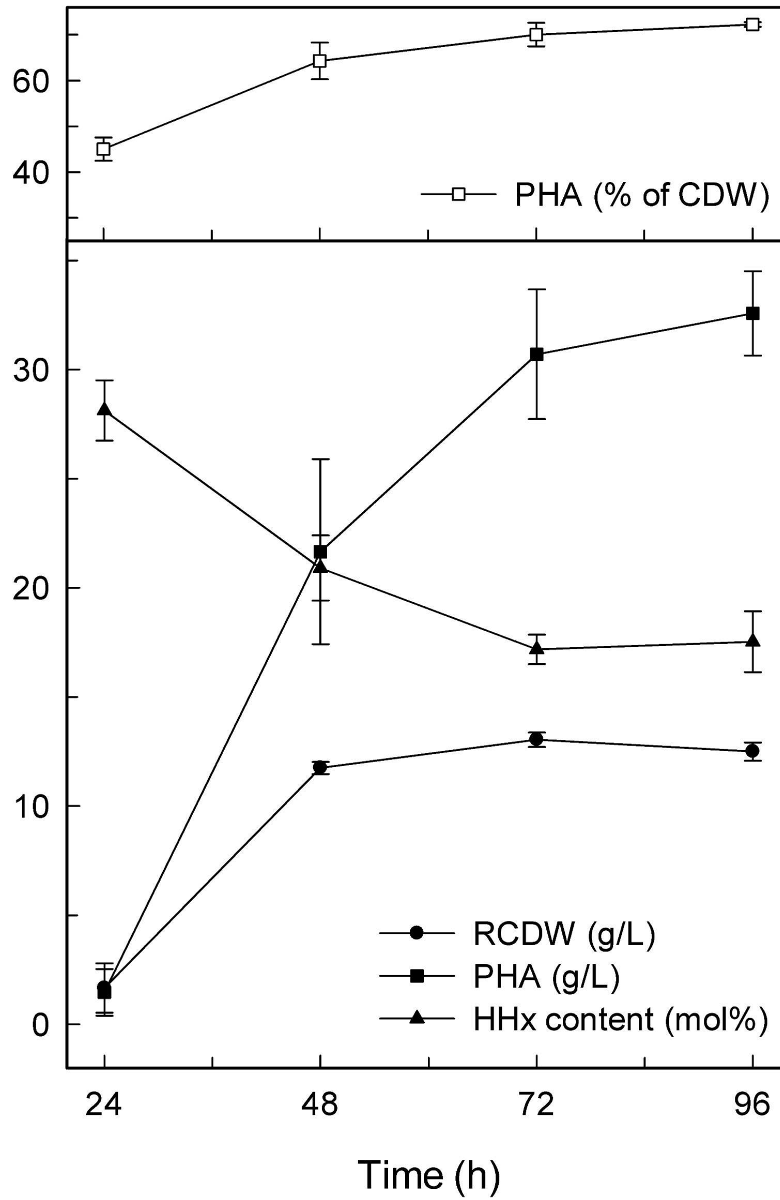

Figure 1
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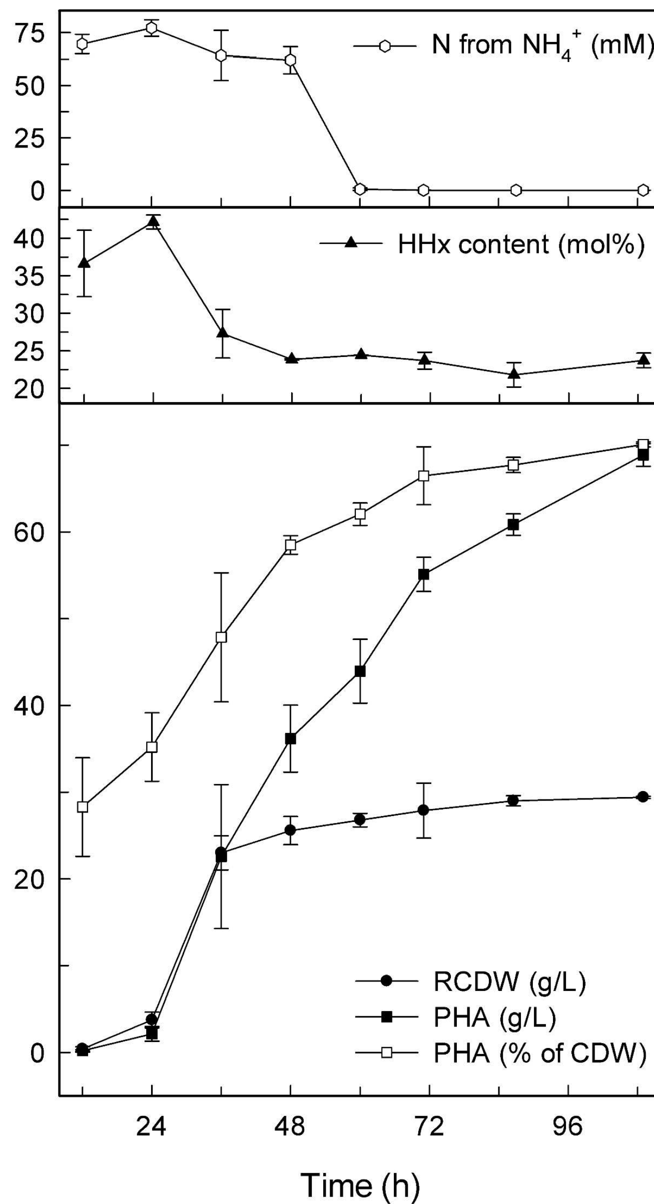

Figure 2
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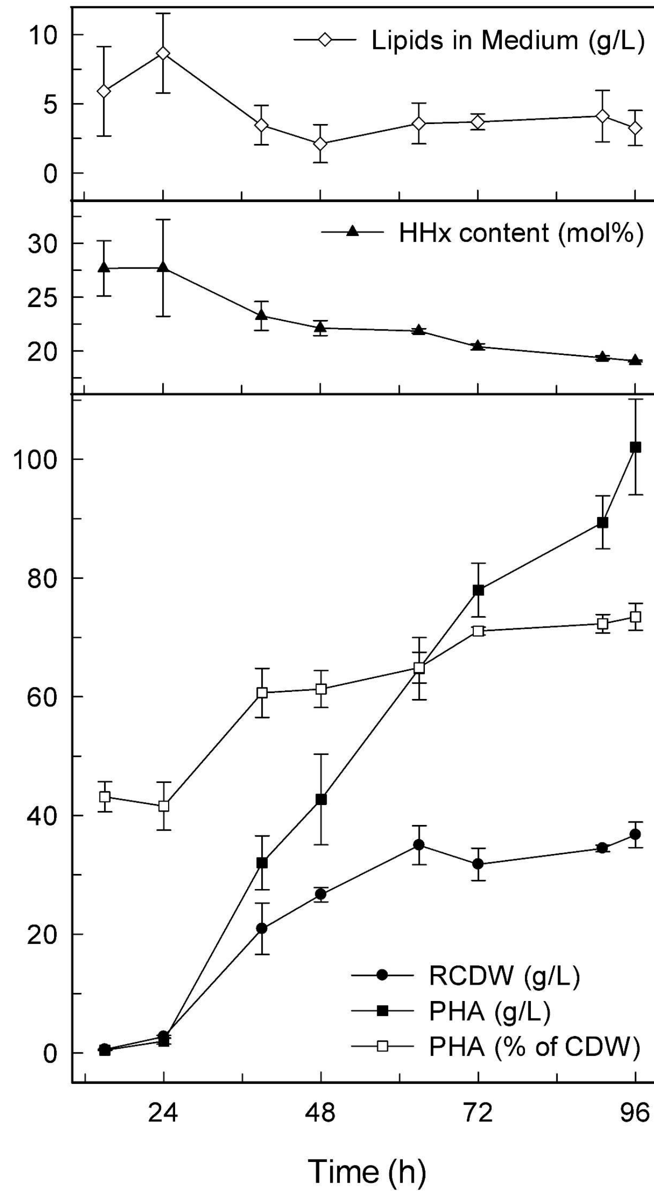

Figure 3
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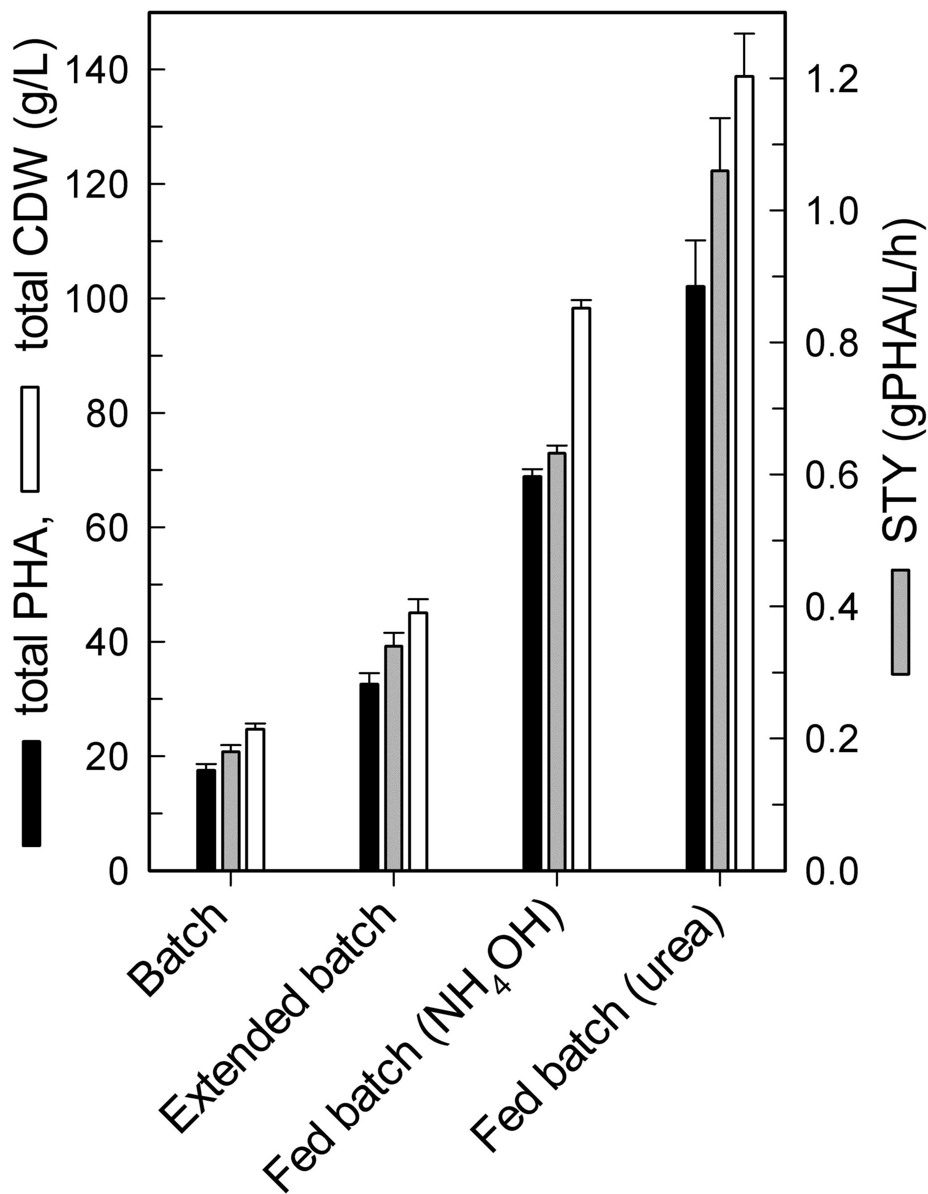

Figure 4
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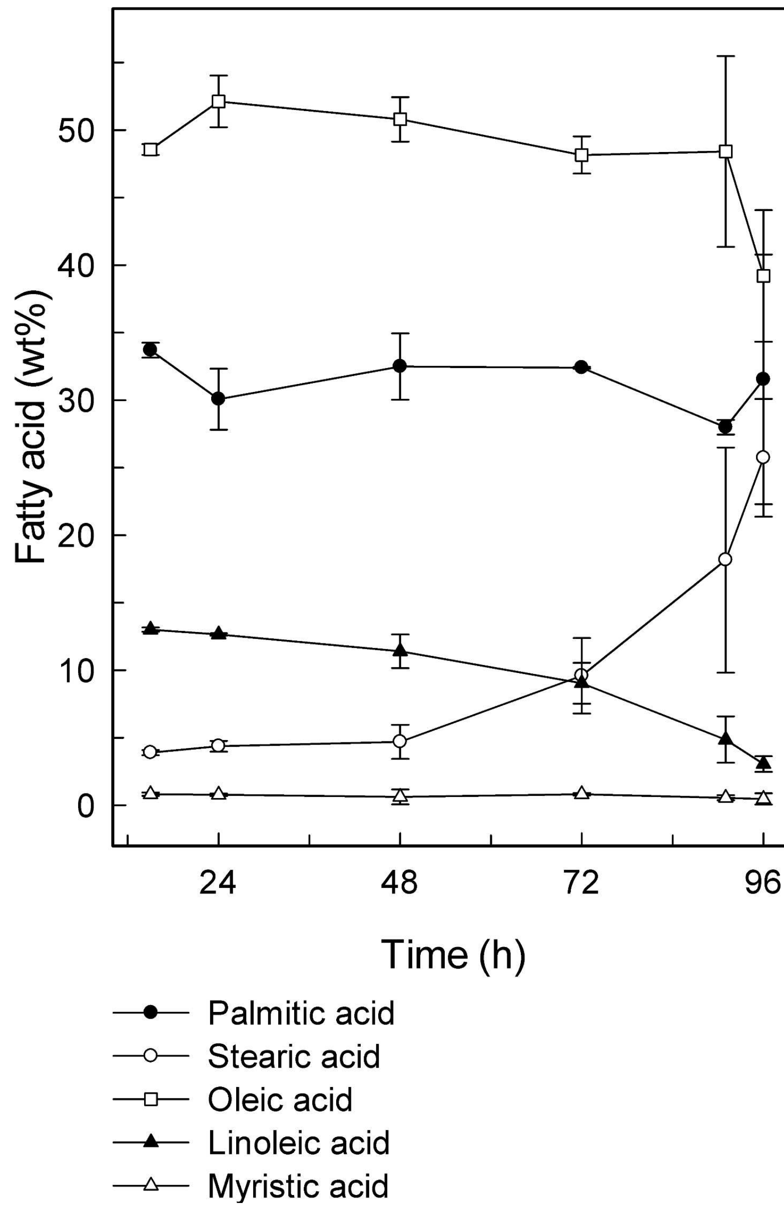

Figure 5
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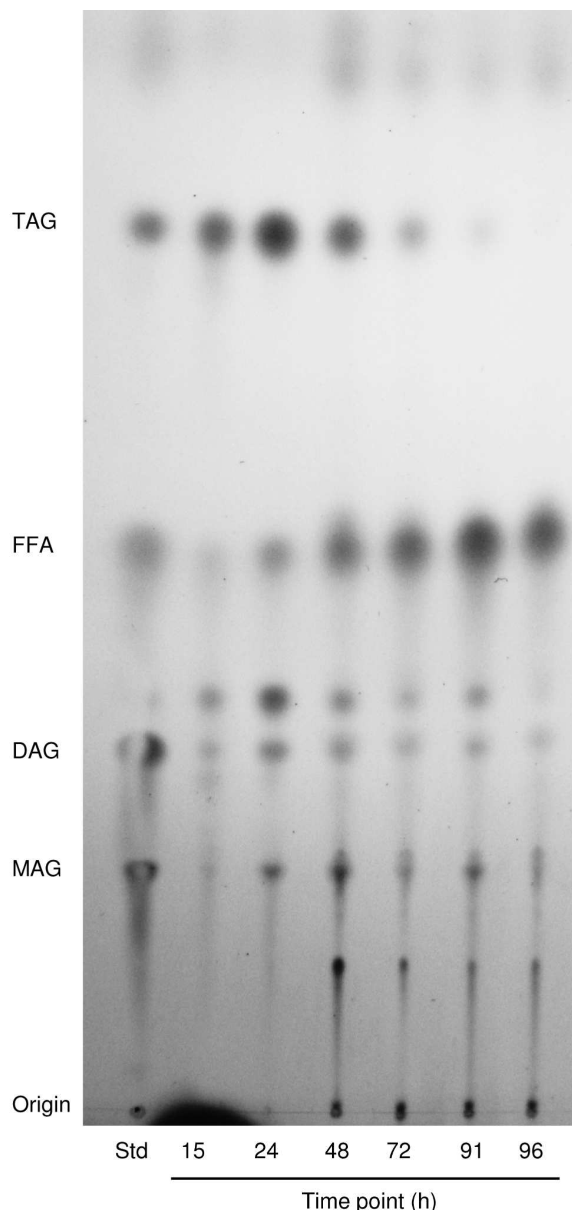

Figure 6
84x172mm (300 x 300 DPI)

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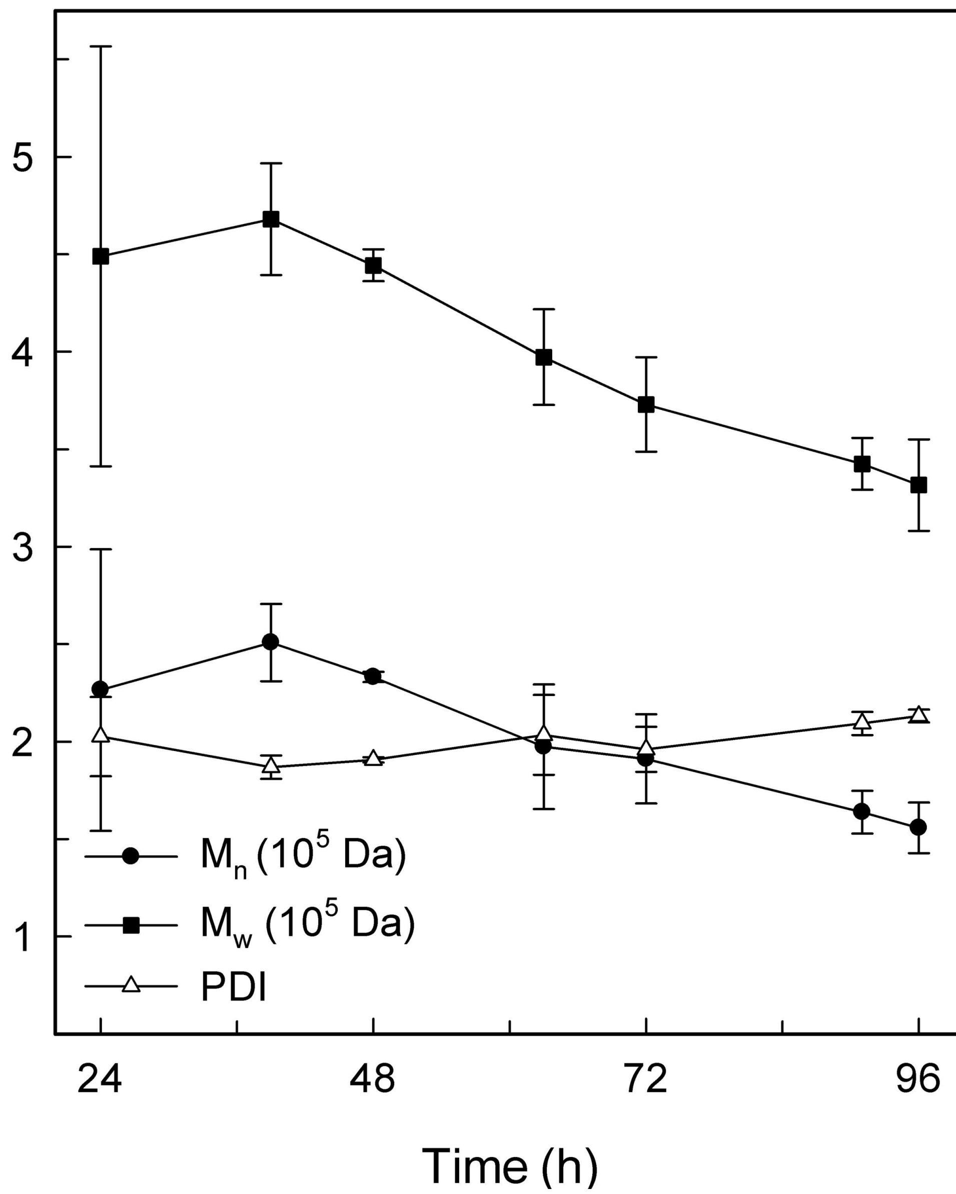

Figure 7
104x128mm (600 x 600 DPI)